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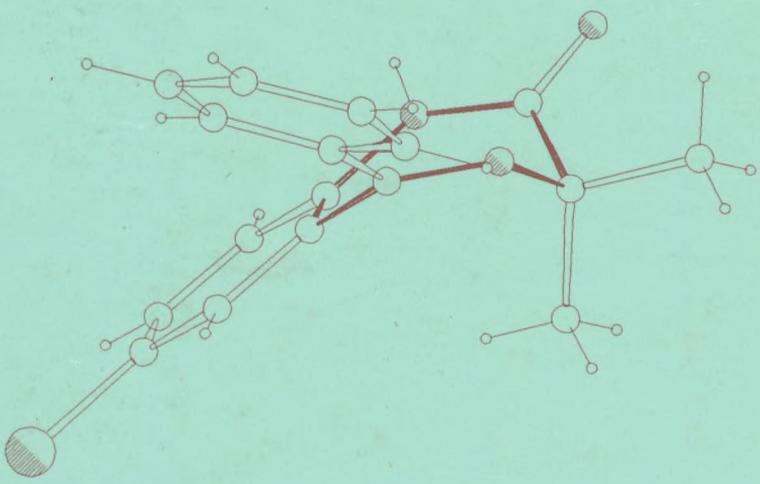
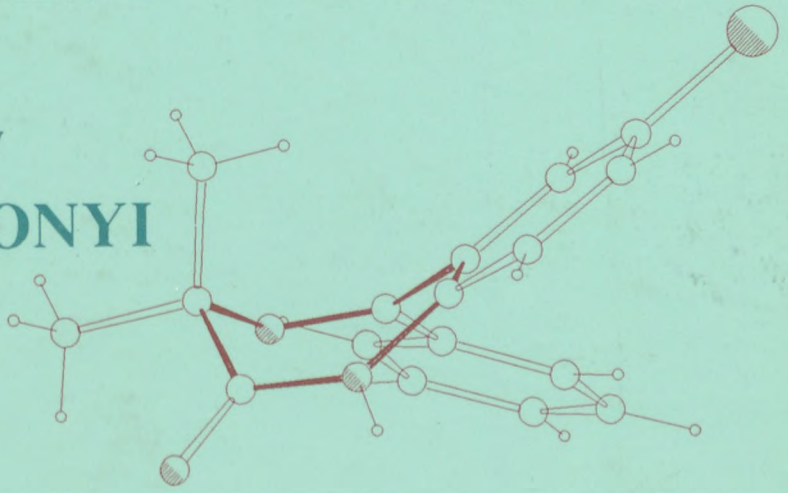


CHIRAL MOLECULES



Edited by

M. SIMONYI

AKADÉMIAI KIADÓ, BUDAPEST

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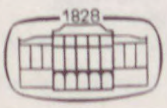

Akadémiai Kiadó, Budapest 1990

ISBN 963 05 5881 5

Printed in Hungary
by Akadémiai Kiadó és Nyomda Vállalat

EDITOR'S FOREWORD

Establishing our system for stereochemical description, Cahn, Ingold and Prelog carefully defined priority rules by which the arrangement of three of the centers around a tetravalent element of chirality is found either clockwise or counterclockwise, thereby unequivocally defining the stereochemical class, R or S, which the given structure falls into. One thing they found obvious, and so did not define, was the idea: clockwise.

Of course! Any literate person is familiar with the clock.

Or certainly had been before the digital watch became dominant. Could not clockwise be defined by a natural phenomenon?

But it is! Just push a stick into the ground and make a sundial. The shadow of the stick will rotate clockwise.

The 10th International Congress of Pharmacology successfully held in Sydney, Australia in 1987 comprised 40 symposia, one of which was specifically devoted to enantioselective effects. Besides, participants of the Congress could observe the northern sun, a phenomenon remembered from school age, and might notice its immediate consequence: the sun moves on its orbit from right to left, hence the sundial on the ground in Sydney should be numbered counterclockwise.

Hm ..... this probably proves that the clock was invented in the north. But the idea of clockwise can still be related to sundials used in the Northern hemisphere.

Actually, sundials are useless between the tropics where the sun's orbit passes the zenith back and forth during the

year. While Sydney receives northern sunshine throughout the year (it lies on 33° 52′ S), Jerusalem (31° 47′ N) is always lit from the south. Nevertheless, the sundial built before the Dome of the Rock is numbered counterclockwise, simply because the plane of the dial is vertical.

Even more unexpected is the product of a Chinese craftsman in the Imperial Palace of Beijing. It is a double dial with

sloping parallel planes. The upper dial lit around noon and seen by the Emperor is numbered clockwise. The lower dial lit during the early and late hours and seen by the court is numbered counterclockwise. Geographic location (39° 54′ N), the Emperor's timetable and the seasonal variation together had

to be taken into consideration for the meticulous calculation of the angle of the slope.

Well, it would need a sophisticated definition to relate "clockwise" to a sundial, indeed.

Instead, look at your hands with fingers outstretched. The order of your nails from thumb through forefinger to the little finger of the right hand does define clockwise, while the same order is counterclockwise on the left hand. These orders are independent of position, standing or lying, with your hand being above your head or below. Thus, clockwise is identical with right-hand-wise.

And what if you forget which is your right hand.

Just find your heart. It should certainly be on your left.

***

This book is the result of a joint venture offering competent treatment of a range of subjects in which the structures of chiral molecules serve to give the basis of further considerations. Finding a problem is the beginning of a scientific study, solving the problem is the aim. Between the two, usually there is much work to be done leading to a pivotal point where the problematic turns to the obvious; where arguments which have appeared controversial, become now reconciled. This turning point is a marvelous moment for the human mind. Since biblical times, from the construction of the Tower of Babel, the ordinary state of human affairs is confusion. Understanding is a real achievement, an intellectual wonder, the reward of scientific struggles.

This book can promise no less than wonders of understanding related to chiral molecules. The authors, many of them being the best in their subjects, offer a hand to the reader and help him/her ascend high peaks in the area, opening new vistas over as yet unexplored fields.

Just hold on! I wish you a pleasant climb.

Budapest, September 1989

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CHEMISTRY

BIOCHEMISTRY

Akadémiai Kiadó
Budapest, 1990

STEREOSPECIFIC REACTIONS ON NON-CHIRAL SUBSTRATES IN BIOLOGY

DOUGLAS W. YOUNG

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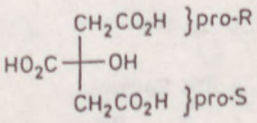
It has been known for a very long time that enzymes may distinguish between enantiomeric compounds and so enantiomers can have very different biological properties. This may have presented conceptual problems some years ago but stereochemical ideas are now so generally understood that we have little difficulty in accepting the fact. Although enantiomers have identical chemical properties, when they combine with a chiral reagent, the new compounds or complexes are diastereoisomers and so have different properties. Enzymes are almost perfect chiral reagents and so extremely high degrees of enantioselectivity are expected in enzyme-catalysed reactions.



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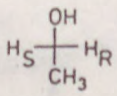

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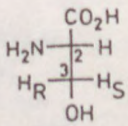

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The idea that an enzyme might distinguish between two apparently identical groups 'a', such as the $-CH_2CO_2H$ groups in citric acid (1) or the hydrogens of the methylene group of ethanol (2), in a prochiral* centre, Ca_2bc, was not apparent until 1948 when Ogston[1] pointed out that

*The term prochiral and the R/S; pro-R/pro-S; and *re/si* nomenclature for defining the stereochemistry of atoms and faces are described in the appropriate references[2] for those readers who are unfamiliar with them.

this could be the case. He used a theory involving three points of attachment between enzyme and substrate but, in fact, this theory is not necessary. If we first consider the $-CH_2-$ centre in the amino acid serine (3), the presence of the chiral centre at C-2 causes the C-3 hydrogens to be diastereotopic. They will thus be in intrinsically different environments as can be seen from the ^1H-nmr spectrum (Figure 1).

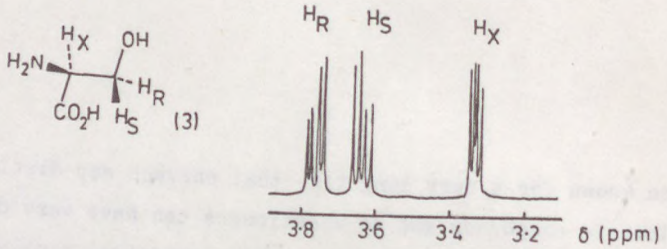


Figure 1: ^1H-nmr spectrum of L-serine in 10% $NaO^2H/^2H_2O$

The hydrogen atoms of the methylene group of ethanol (2) are enantiotopic and so they are in identical environments, having identical shifts in the ^1H-nmr spectrum. Even in the camphanoyl ester where the hydrogen atoms are now diastereotopic, these appear at the same chemical shifts (Figure 2a). Addition of a shift reagent, however, causes these hydrogens to absorb at different chemical shifts (Figures 2b and 2c).

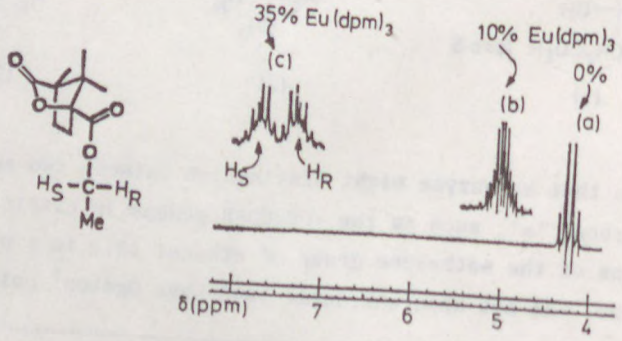
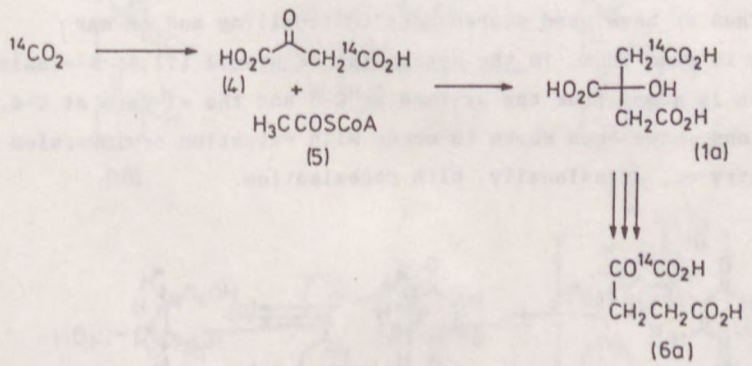


Figure 2: ^1H-nmr spectrum in CCl_4 of the methylene protons of the (1S,4R) camphanoate of ethanol - (a) without shift reagent; (b) with 10% of shift reagent; (c) with 35% of shift reagent.

Since enzymes are almost perfect chiral reagents, enantiotopic hydrogens of substrates will behave very differently in an enzyme complex where they will be diastereotopic. Thus alcohol dehydrogenases specifically remove the pro-R hydrogen (H_R) of ethanol (2) when it is oxidised to acetaldehyde. To prove this fact, samples of ethanol (2) labelled specifically in the 1-pro-R or 1-pro-S positions with tritium or deuterium were used. A stereospecific synthesis of labelled ethanol from compounds of known chirality was required to verify the absolute stereochemistry of the labelled ethanols.[3,4]

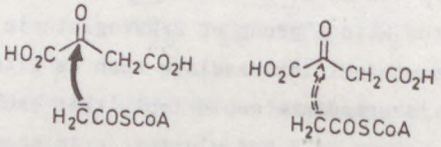
The quick acceptance of Ogston's concept allowed some isotopic experiments to be reinterpreted in their true light. Thus, although citric acid (1) had been the cornerstone of Krebs' tricarboxylic acid cycle, the fact that $^{14}CO_2$ was shown to be incorporated specifically into the carboxyl group α to the ketone group of 2-ketoglutaric acid (6)[5] was thought to rule out a symmetrical intermediate such as citric acid (1). It was felt that such an intermediate would imply that each of the terminal carboxylic acid groups in 2-ketoglutaric acid should have half of the original label. After Ogston's postulate, however, it was realised that enzymes could treat the pro-R-CH_2CO_2H group of citric acid (1) differently from the pro-S-CH_2CO_2H group and so asymmetric labelling in the reaction products was allowed.



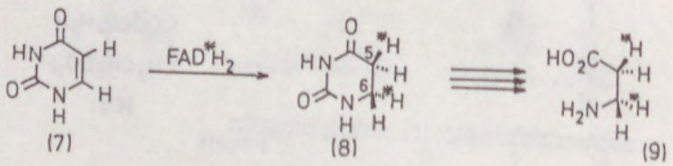
Scheme 1

Experimental evidence was soon found to back up this interpretation[6] and citric acid was reinstated in the tricarboxylic acid cycle. It was

later shown that, although mammalian enzymes incorporate $^{14}CO_2$ *via* oxaloacetate (4) into the pro-R-CH_2CO_2H group of citric acid (1a), there are bacterial enzymes which incorporate this label *via* oxaloacetate into the pro-S-CH_2CO_2H group.[7] This implies that, in the aldol condensation mediated by the mammalian enzyme, acetyl-CoA (5) reacts at the *si** face of oxaloacetate (4) whilst in the bacterial enzyme acetyl-CoA (5) reacts at the *re** face. By now it will not be surprising that enzymes can distinguish between the enantiotopic faces of the carbonyl group in oxaloacetate (4) since the faces are diastereotopic in the enzyme complex. The mammalian and bacterial enzymes are termed *si*-citrate synthase and *re*-citrate synthase, respectively.



Hydrogen atoms at prochiral centres, Ca_2bc, are intrinsically different in the presence of an enzyme and enzymic reactions may create and destroy prochiral centres in a stereospecific manner. The overall course of such reactions may be determined using isotopically labelled hydrogen. Thus we have used stereospecific labelling and ^{1}H-nmr spectroscopy to show that, in the metabolism of uracil (7) to β-alanine (9), hydrogen is added from the *si*-face at C-5 and the *si*-face at C-6.[8] Other reactions have been shown to occur with retention or inversion of stereochemistry or, occasionally, with racemisation.

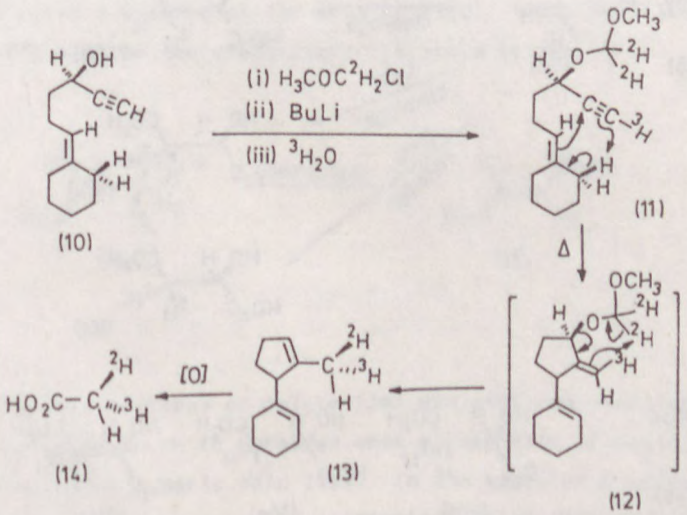


Scheme 2

Prochiral groups Ca_2bc require substitution of one of the identical

groups 'a' to become chiral and so allow the stereochemistry of their enzymic reactions to be deduced. Groups Ca_3b or Ca_2b_2 which require two substitutions to achieve chirality are termed *proprochiral*. Methyl groups and phosphate groups are proprochiral groups of the 'Ca_3b' type and malonic acid is a proprochiral molecule of the 'Ca_2b_2' type. Although isotopic substitution allows us to synthesise chirally labelled methyl, phosphate and malonate using fairly normal stereospecific synthesis, the atoms or groups are not intrinsically different from each other in an enzyme complex. Assay is therefore a more difficult problem than in the case of prochiral atoms where the 'identical' atoms are intrinsically different in an enzyme complex.

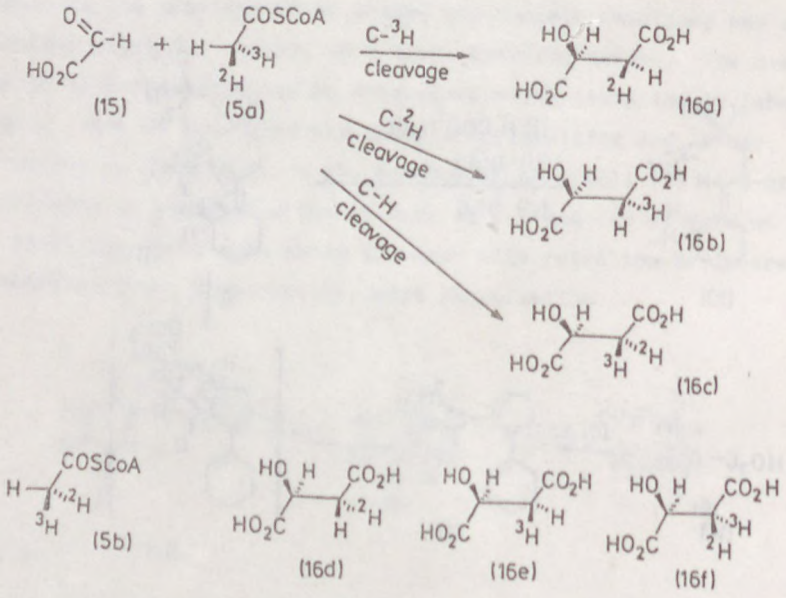
Considering first the methyl group, stereospecific replacement of two protium atoms by deuterium and tritium will yield a stereospecifically labelled methyl group. Synthesis therefore presents no different problems from synthesis of compounds containing prochiral atoms which are stereospecifically labelled. Several syntheses have been developed and it will be of interest to consider the most elegant of these. In this synthesis,[9] the starting material for (2R)[2-$^3H_1$,2-$^2H_1$]-acetate (14) is the optically pure alcohol (10) whilst (2S)[2-$^3H_1$,2-$^2H_1$]-acetate may be obtained from the enantiomer of this alcohol.



Scheme 3

Conversion of alcohol (**10**) to the ether (**11**) was achieved by alkylaton followed by exchange of the acetylenic hydrogen by tritium. Compound (**11**) is chiral and contains the three isotopes protium, deuterium and tritium. These were now assembled on the same atom in a stereospecific manner by two consecutive ene-reactions which occurred on thermolysis of the ether (**11**). In the first ene-reaction, protium was stereospecifically transferred to the triple bond to yield the ring-closed intermediate (**12**) with the exocyclic (Z)-olefinic bond. A second ene-reaction then ensued with transfer of deuterium to the *re*-face of the $=CH^3H$ atom in intermediate (**12**), yielding the product (**13**). This had a chirally labelled methyl group whose stereochemistry was defined by the method of synthesis. Kuhn-Roth oxidation then yielded (2R)[2-$^3H_1$,2$^2H_1$] acetate (**14**).

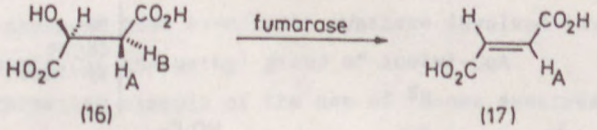
Since the hydrogens of the methyl group are not intrinsically different to the enzyme, assay of the chirality of such groups presents problems. Enzymic reactions at such groups or which create such groups still occur with inversion or retention of stereochemistry or with racemisation and it is necessary to discover the overall reaction stereochemistry if we are to understand the mechanisms of such reactions.



Scheme 4

A method of assay was discovered independently by two groups[10,11] and it is still the most popular method in use today. It relies on the use of an enzyme which forms a bond at a chiral methyl group and exhibits a primary kinetic isotope effect. The method relies on measurement of the isotope tritium which is never present in more than trace amounts and so it is important that levels of deuterium in the chiral acetate are high so that all tritium originates from chiral methyl and not from C^3HH_2.

The enzyme chosen for the assay, malate synthase, catalyses a stereospecific aldol reaction between glyoxylate (**15**) and acetyl-CoA (**5**). (R)[2-$^3H_1$,2-$^2H_1$] Acetate (**14**), stereospecifically synthesised to be of known chirality, was converted to (2R)[2-$^3H_1$,2-$^2H_1$] acetyl-CoA (5a) *in situ* using a mixture of enzymes. Since malate synthase cannot differentiate between the hydrogens of acetyl-CoA, each of the C-H bonds may be cleaved in the enzymic reaction. Thus, if this reaction involves inversion of stereochemistry, (2R)[2-$^3H_1$,2-$^2H_1$] acetyl-CoA (5a) will yield the malates (**16a**), (**16b**) and (**16c**), respectively. Since the assay relies on measurement of tritium, malate (**16a**) will be "invisible" and so we need only consider compounds (**16b**) and (**16c**). Given a normal isotope effect, $k_{2_H} < k_{1_H}$, C-H cleavage will be preferred to C-^2H cleavage and so there will be more of the malate (**16c**) in the final mixture. A similar argument may be used to show that (2S)[2-$^3H_1$,2-$^2H_1$] acetyl-CoA (5b) will yield a mixture of the malates (16d), (16e) and (16f) and that malate (16f) will be the predominant tritiated malate.



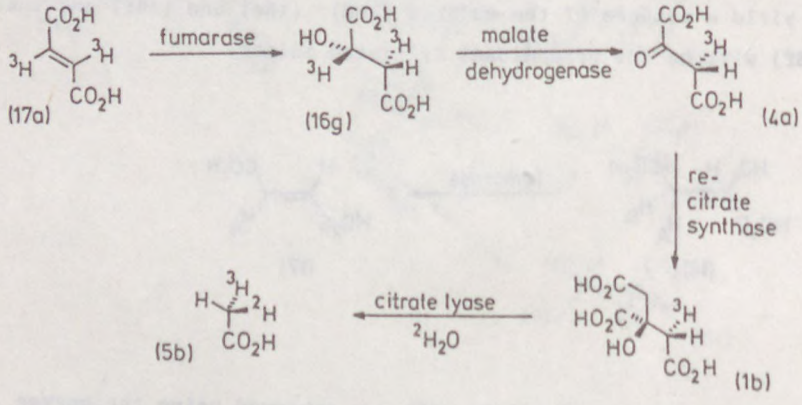
(16) (17)

Scheme 5

Assay of the mixtures of malate (**16**) was achieved using the enzyme fumarase which is known to catalyse *anti* elimination of water from malic acid (**16**) to yield fumaric acid (**17**). In the assay of R-acetate (5a), this enzyme would be expected to eliminate tritium from the minor isomer (**16b**) as tritiated water but to produce tritiated fumaric acid (**17**, H_A = ^3H) from the major isomer (**16c**). The percentage of tritium retained in

the organic fraction after removal of water is known as the F-value. The F-value from the action of fumarase on the malates obtained from reaction of malate synthase on (2R)[2-$^{3}H_1$,2-$^{2}H_1$] acetyl-CoA (5a) was F=79 whilst that from the corresponding (S)-isomer (**5b**) was F=21. This was in keeping with the expected product ratios if the malate synthase reaction proceeded with inversion of stereochemistry. The stereochemistry of the malate synthase reaction had thus been proven and the assay could now be used to test the chirality of acetates of unknown stereochemistry.

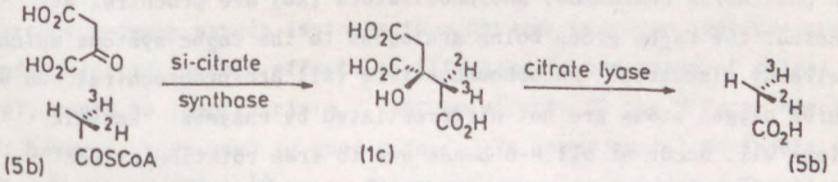
This assay was used to further the understanding of the biosynthesis of citric acid (1). As we have seen, this synthesis may be catalysed either by *si*- or by *re*-citrate synthase. These allow addition of acetyl-CoA (5) from the *si*-face or the *re*-face, respectively of oxaloacetic acid (**4**). The chirality of the reaction at the carbonyl group of oxaloacetic acid (**4**), involving development of prochiral $-CH_2CO_2H$ groups which are intrinsically different to the enzyme, has been worked out as shown in Scheme 1. Assessment of the chirality of the reaction at the methyl group of acetyl-CoA (5), however, was quite another problem and an interesting use was made of the fact that there are two synthases, *si*- and *re*-, in its solution.



Scheme 6

Eggerer and his colleagues[12] prepared (3S)[3-$^{3}H_1$] oxaloacetic acid (**4a**) by first incubating [2,3-$^{3}H_2$] fumaric acid (**17a**) with fumarase and then oxidising the resultant (2S,3S)[2,3-$^{3}H_2$] malic acid (**16g**) using

malate dehydrogenase. The labelled oxaloactate was incubated with *re*-citrate synthase to obtain the labelled citrate (1b). Since the chirally labelled centre was not created in this reaction, there was no doubt about the stereochemistry of the citrate (**1b**), which was labelled in the pro-S-CH_2CO_2H group with tritium substituting for the pro-S-hydrogen of the CH_2 group. When the citrate (**1b**) was used as a substrate for citrate lyase in the presence of ^2H_2O, labelled acetate was obtained for which an F-value of F=43 was found using the malate synthase/fumarase assay. The compound was therefore mainly (2S)[2-$^3H_1$,2-$^2H_1$] acetate (**5b**) and the fission of citrate with citrate lyase had involved inversion of stereochemistry.

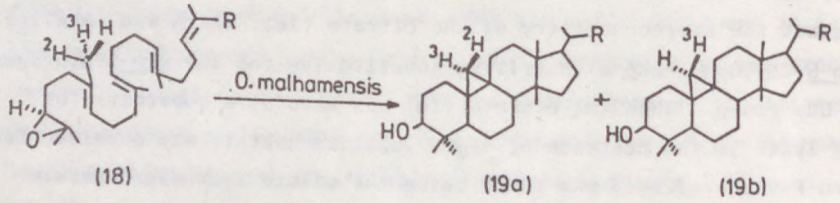


Scheme 7

si-Citrate synthase was now used to convert (2S)[2$^3H_1$,2-$^2H_1$] acetyl-CoA (**5b**) to citrate (**1c**). Change from the *re* to the *si* synthase allowed the label to be in the pro-S CH_2CO_2H group as before. The citrate (**1c**) was now incubated with citrate lyase and H_2O to yield acetate of F=41. The acetate was therefore (S) and, since citrate lyase involves inversion, the citrate (**1c**) must have had the stereochemistry indicated. It was therefore apparent that *si*-citrate synthase involved inversion of stereochemistry at the methyl group of acetyl-CoA.

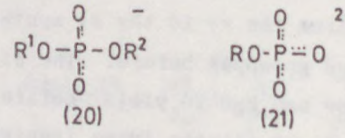
An interesting example of the use of ^3H-nmr spectroscopy in assessing the stereochemistry of a reaction occurring at a methyl group is to be found in the work of Altman.[13] He synthesised 2,3-oxidosqualene (**18**) bearing predominantly an R-methyl group at C-6. This was incubated with a microsomal preparation of the alga *Ochromonas malhamensis* to yield a sample of cycloartenol (**19**). The ^3H-nmr spectrum of this compound showed a major species (**19a**) where the tritium was in the *exo* (19-pro-R) position and was coupled to deuterium, and a minor species (**19b**) with tritium in the *endo* (19-pro-S) position coupled to a protium in the 19-pro-R position. These results indicated that, in the formation of the cyclopropane ring,

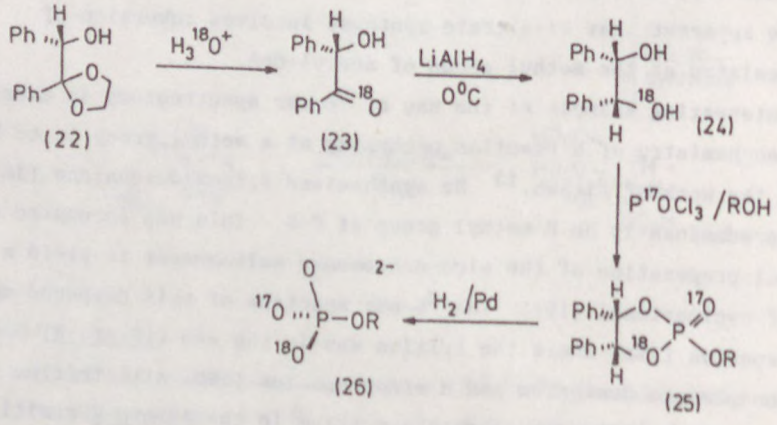
the methyl hydrogen was substituted with retention of stereochemistry.



Scheme 8

In phosphorus compounds, phosphodiesters (**20**) are prochiral at phosphorus, the Pa_2bc group being analogous to the Ca_2bc systems which we have already discussed. Phosphomonoesters (21) are proprochiral and so the three oxygen atoms are not differentiated by enzymes. Enzymic reactions will occur at all P-O bonds due to free rotation at the phosphate. Synthesis of chirally labelled phosphomonoesters presents no special problems and an elegant and straightforward synthesis[14] is shown in Scheme 9.





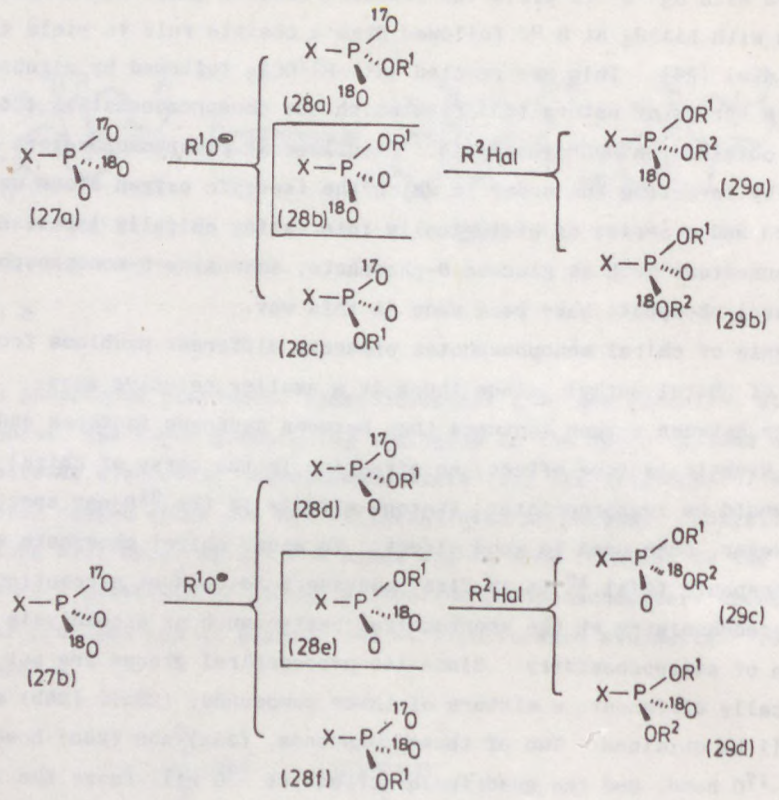
Scheme 9

Here, the chiral alcohol (**22**), prepared from S-mandelic acid, was hydrolysed with $H_3{}^{18}O^+$ to yield the labelled benzoin (**23**). Stereospecific reduction with $LiAlH_4$ at 0 ^oC followed Cram's chelate rule to yield the labelled diol (**24**). This was reacted with $P^{17}OCl_3$ followed by alcoholysis to yield a series of esters (**25**) from which the phosphomonoesters (**26**) could be obtained on hydrogenolysis. Enantiomeric phosphomonoesters were prepared by inverting the order in which the isotopic oxygen atoms were introduced and a series of biologically interesting chirally labelled phosphomonoesters such as glucose-6-phosphate, adenosine-5-monophosphate and glycerol-phosphate have been made in this way.

Analysis of chiral monophosphates presents different problems from analysis of chiral methyl, since there is a smaller relative mass difference between oxygen isotopes than between hydrogen isotopes and so use of a kinetic isotope effect, so effective in the assay of chiral methyl, would be inappropriate. Isotope effects in the ^{31}P-nmr spectra have, however, been used to good effect. To assay chiral phosphate such as the phosphate (**27a**),[15] it is first necessary to conduct a reaction of known stereochemistry at the proprochiral centre such as alcoholysis with inversion of stereochemistry. Since the proprochiral groups are not intrinsically different, a mixture of three compounds, (**28a**), (**28b**) and (28c) will be obtained. Two of these compounds, (28a) and (28c) however have a $P-{}^{17}O$ bond, and the quadripolar effect of ^{17}O will cause the lines in the ^{31}P-nmr spectrum due to these compounds to broaden. At best these lines will be broad and, at worst, 'invisible'. Hence only compound (**28b**), which contains no ^{17}O, will have a sharp spectrum. Alkylation of the mixture now produces a mixture containing only two non-^{17}O-containing compounds (**29a**) and (**29b**).

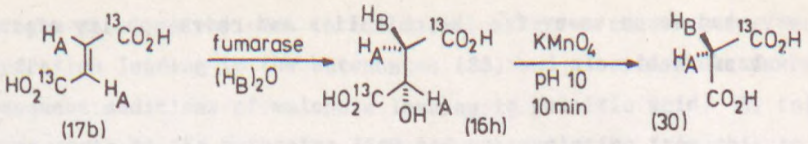
Compound (**29a**) is identical with (**29c**) derived from the enantiomeric phosphate (27b), except for the fact that the ^{18}O label is in a non-bridging, P=O, group in (**29a**) and in a bridging P-O group in (**29c**). The former causes an isotope shift of *ca.* 0.4 ppm in the ^{31}P absorption whilst the latter causes a shift of only *ca.* 0.2 ppm. Assuming that either X or R^1 is chiral, compounds (**29b**) and (**29d**) will be diastereoisomeric with (**29a**) and so the phosphorus absorption will be quite different. Using the Scheme it is therefore possible to differentiate the phosphate (27a) from its enantiomer (27b) using ^{31}P nmr spectroscopy and without having to separate the mixture of labelled

compounds produced in the sequence.



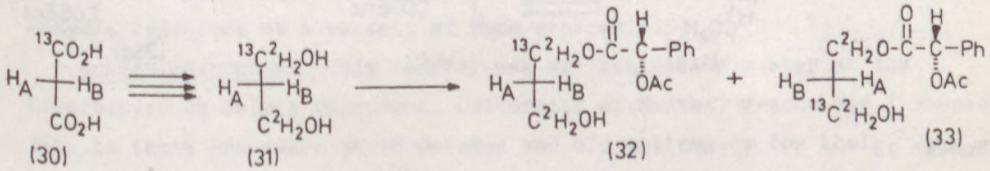
Scheme 10

Labelled malonic acids (30, H_A *or* H_B=^{2}H) have recently been prepared as the first examples of labelled proprochiral compounds of the type Ca_2b_2 which are optically active. The same synthesis was independently devised by two groups[16,17] and it is shown in Scheme 11. Using fumarase and [1,4-$^{13}C_2$] fumarates (**17b**, H_A=^{2}H) and (**17b**, H_A=H), *anti*-addition of water or deuterium oxide gave respectively the labelled malates (**16h**, H_A=^{2}H) and (**16h**, H_B=^{2}H). Kuhn-Roth oxidation under mild conditions for a very short time then gave (2S)[1-$^{13}C_1$,2-$^2H_1$] malonate (30, H_A=^{2}H) and (2R)[1-$^{13}C_1$,2-$^2H_1$] malonate (30, H_B=^{2}H), respectively.



Scheme 11

As always, the synthesis proved the easier part of the work, the fact that proprochiral groups could not be differentiated chemically or enzymatically making the assay the more novel aspect of the problem. Floss[16] solved this problem using nmr spectroscopic techniques. Taking undeuteriated and (R) and (S) malates (**30**), he converted them to the diesters which, on reduction with $LiAl^2H_4$ gave the dialcohols (**31**). These were reacted with (S)(+)-O-acetylmandelyl chloride and, since the two alcohol groups in (**31**) could not be differentiated, a mixture of esters (**32**) + (**33**) was obtained in each case. Although the results are complicated by further mixtures, the protons H_A and H_B in (**32**) and (**33**) are diastereotopic and occur at different chemical shifts. Further, they show a small coupling to the ^{13}C atoms.

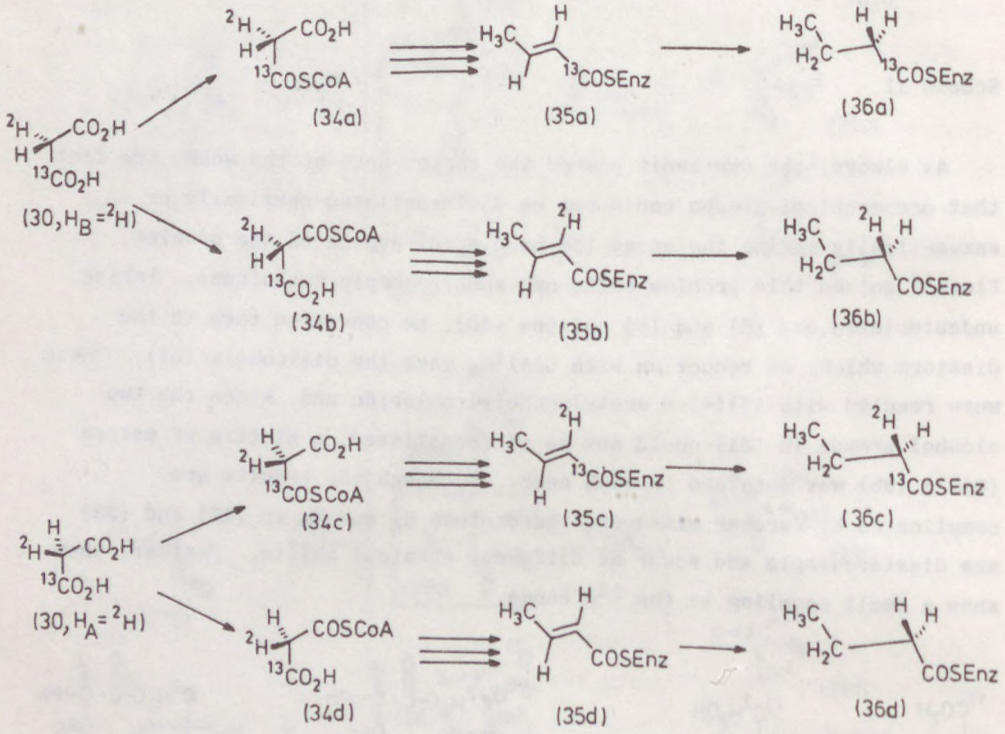


Scheme 12

When the downfield ($^{13}C^2H_2$-O-ester) ^{13}C-enriched-carbon was irradiated in the mixture (**32**, $H_B=^2H$) + (**33**, $H_B = ^2H$) derived from (R)[1-$^{13}C_1$, 2-$^2H_1$]-malonate (**30**, $H_B=^2H$), then the proton H_A of ester (**32**, $H_B=^2H$) collapsed to a singlet while H_A of (**33**, $H_B=^2H$) remained a doublet. The opposite pattern was observed when the upfield ($^{13}C^2H_2OH$) ^{13}C-enriched carbon in the spectrum of the mixture was irradiated.

Different decoupling was observed when the mixture (**32**, $H_A=^2H$) + (**33**, $H_A=^2H$) derived from (S)[1-$^{13}C_1$, 2-$^2H_1$] malonate (**30**, $H_A=^2H$) was assayed in

the same way, and so an assay for the chirality and chiral purity of the malonates was available.



Scheme 13

The assay for chiral malonate developed by Jordan[17] used mass spectroscopic methods and relied on an enzyme, fatty acid synthase, whose stereochemistry had previously been worked-out by Cornforth[18] using enantiomerically tritiated samples of malonyl-CoA. Jordan used succinyl-CoA transferase to convert the (R) and (S) malonates (30) to malonyl-CoA (34) which, in the presence of fatty acid synthase, yielded palmitic ester. This was methylated and the ester examined by mass spectroscopy.

Since succinyl-CoA transferase cannot distinguish between the two carboxyl groups, each of (R)[1-$^{13}C_1$,2-$^2H_1$] malonate (**30**, $H_B = {}^2H$) and (S)[1-$^{13}C_1$,2-$^2H_1$] malonate (**30**, $H_A = {}^2H$) will yield a mixture, (**34a**) + (**34b**) and (34c) + (34d), respectively. Fatty acid synthetase will remove the

hydrogen H_R of malonyl-CoA in the decarboxylative condensation/reduction/dehydration leading to the butenoates (**35**) and also in each of the six subsequent additions of malonate leading to palmitic acid. By following the sequence to the butyrates (**36**) and extrapolating from this to the palmitates, we can see that there is an equal chance of incorporating ^2H *or* ^{13}C at each stage when (R)[1-$^{13}C_1$,2-$^2H_1$] malonate (**30**, H_B=$^2H_1$) is used, but *both* isotopes cannot be incorporated. Conversely, palmitate derived from (S) malonate (**30**, H_A=^2H) will either incorporate both isotopes at each stage or no isotopes at each stage.

Assuming no isotope dilution, we can see that we would expect *only* an M+7 parent ion for palmitate biosynthesised from (R)[1-$^{13}C_1$,2-$^2H_1$] malonate (**30**, H_B=^2H). Palmitate derived from (S)[1-$^{13}C_1$,2-$^2H_1$] malonate (**30**, H_B=^2H) would be expected to have two peaks M+0 and M+14 for the parent ion. Although this ideal is not fully met in practice, the results are sufficiently clear-cut to allow the method to be used as an assay.

We have seen how enzymes can differentiate between enantiomers and enantiomeric groups and faces and that the enzymic reactions are stereospecific. Although differentiation of proprochiral groups by enzymes is not possible, the ingenuity of several groups of workers has allowed the development of methods to follow the stereochemistry of enzymic reactions at a variety of such centres.

Acknowledgements: This chapter was written during a stay at the Laboratoire de Chimie Organique, University of Nantes, France and I should like to thank Professor Hervé Quiniou and his colleagues for their hospitality to me during my visit.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

LEFT-HANDED RNA OLIGOMERS

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INTRODUCTION

Since the discovery of the right-handed double-helical DNA structure by Watson and Crick[1], DNA is known to adopt various right-handed conformations such as A-, B- and C-DNA, and the fundamental concept of a regular DNA structure has become one of the foundations of molecular biology.

However, this situation has changed dramatically since 1972, with the discovery by Pohl and Jovin[2] of the two distinct forms of poly(dG-dC):poly(dG-dC) structure in solution, with a cooperative transition occurring between them at high salt concentration. In fact, the circular dichroism spectrum of the high salt form is nearly an inversion of that of the low salt form. This suggests that the transition to the high salt form involves a rearrangement of the helical sense, i.e. more precisely, each helical structure of the two is different in its own handedness, and the transition occurs between the right-handed and the left-handed helical conformations. Simply speaking, we have now two chiral molecules composed of identical structural components, namely the purine and pyrimidine bases, 1-β-D-deoxyriboses and phosphates. The left-handed Z-DNA structure was first solved by Rich and co-workers[3] in 1979.

On the other hand, it has been shown that the A-RNA double-helical structure[4,5] is less flexible than that of B-DNA due to the rigidity of the sugar conformation in the former, adapting 3'-endo conformation owing to the introduction

of a 2'-hydroxyl group. Poly(rG-rC) is more difficult to be converted from the A-RNA conformation to Z-RNA, as this transition requires a high concentration (4M) of $NaClO_4$, and temperature above 35°C[6].

The most unusual structural feature of Z-DNA[3] and the main cause for its adoption of a left-handed helix is the syn conformation around the glycosyl bond in the deoxyguanosine residue. The easiest way to make the purine nucleotide take on the syn conformation is a covalent modification at the C8 position with a bulky group, for instance, methyl group or bromine atom. In fact, the purine nucleotide or nucleoside chemically substituted at the C8 position adopts the syn conformation around the glycosyl bond as has been found in the crystal of 8-methyladenosine-3'-monophosphate[7].

In this paper, we describe the molecular structure in the Z-conformation of two G-C alternating ribonucleotide oligomers containing 8-bromo- and 8-methylguanine residues.

MATERIALS AND METHODS

Two G-C alternating ribonucleotide oligomers containing 8-bromo- or 8-methylguanine, $r(C\text{-}br^8G)_2$ or $r(C\text{-}m^8G)_3$, were synthesized via the phosphotriester method by Ikehara and co-workers[8,9] and given to us for X-ray structure analysis.

The oligomers were crystallized with 2-methyl-2,4-pentanediol (2-MPD) as precipitant at room temperature by the sitting drop method of vapor diffusion. The hexagonal crystals of the methylated hexamer, $r(C\text{-}m^8G)_3$, were obtained in solution containing the oligomer, sodium cacodylate buffer (pH 7.0), magnesium chloride, spermine and 2-MPD, and equilibrated against 30% 2-MPD as shown in Table 1. The crystals of the brominated tetramer, $r(C\text{-}br^8G)_2$, were obtained after two weeks under similar conditions as the methylated hexamer, except for spermine which was not added because of precipitation[10].

Nickel-filtered Cu-K radiation from a rotating anode (Rigaku RU-300) was used at 50 KV and 250 mA to record the X-ray diffraction pattern at 7°C. The crystallographic data for the two oligoribonucleotides are shown in Table 2 together with

Table 1. Crystallization of Z-RNA oligomers

Droplet	$r(C\text{-}m^8G)_3$	$r(C\text{-}br^8G)_2$
cacodylate buffer (pH=7.0)	27 mM	27 mM
$MgCl_2$	13 mM	27 mM
RNA (oligonucleotide)	2.4mM	3.6mM
spermine 4HCl	9.3mM	none
2-MPD	1.3 %	3 %
total volume	70 µl	37.5µl
Reservoir		
2-MPD	30 %	30 %
(volume)	25 ml	25 ml

at room temperature

related DNA oligomers. A total of 791 unique reflections within 5 Å to 1.5 Å resolution for the $r(C\text{-}m^8G)_3$ hexamer were collected at a 4.0σ(F) level above background and used in the refinement. For $r(C\text{-}br^8G)_2$, 1154 unique reflections within 1.5 Å resolution were used in the refinement. The two oligomer crystal structures have been solved by the molecular replacement method using the atomic coordinates of Z-DNA oligomer, and then refined by using the Konnert-Hendrickson constraint procedures. After several cycles of refinement, 44 and 37 solvent

Table 2. Crystallographic data of Z-RNA and Z-DNA oligomers

Compound	Space group	Cell constants a(Å)	b(Å)	c(Å)	Nucleotides in asymmetric unit	Resolution (Å)
$r(Cm^8GCm^8GCm^8G)$	$P6_5$	18.59	18.59	42.73	4	1.5
$r(Cbr^8GCbr^8G)$	$P3_212$	32.49	32.49	42.56	12	1.7
d(CGCG)	$P6_5$	31.25	31.25	44.06	12	1.5
d(CGCGCGCG)	$P6_5$	31.27	31.27	43.56	12	1.6
d(CGCATGCG)	$P6_5$	30.90	30.90	43.14	12	2.5
d(CGCGCG)	$P2_12_12_1$	17.88	31.55	44.58	12	0.9

water molecules per asymmetric unit were found in the crystal structures of $r(C\text{-}br^{8}G)_2$ and $r(C\text{-}m^{8}G)_3$ oligomers, respectively. The final R-factor was 26.4% and 25.6%, respectively. The two structures have final root mean square deviations of bond lengths from the ideal value of 0.028 Å and 0.040 Å, respectively.

RESULTS AND DISCUSSION

The atomic coordinates of two left-handed RNA oligomers together with those of the solvent water molecules are listed in the Appendix.

Figure 1 indicates the electron density map found in the $r(C\text{-}m^{8}G)_3$ crystal structure, in which the density contour of three sections were superimposed. One G-C base pair region can be clearly seen in this map. One of the solvent molecules (W-A)

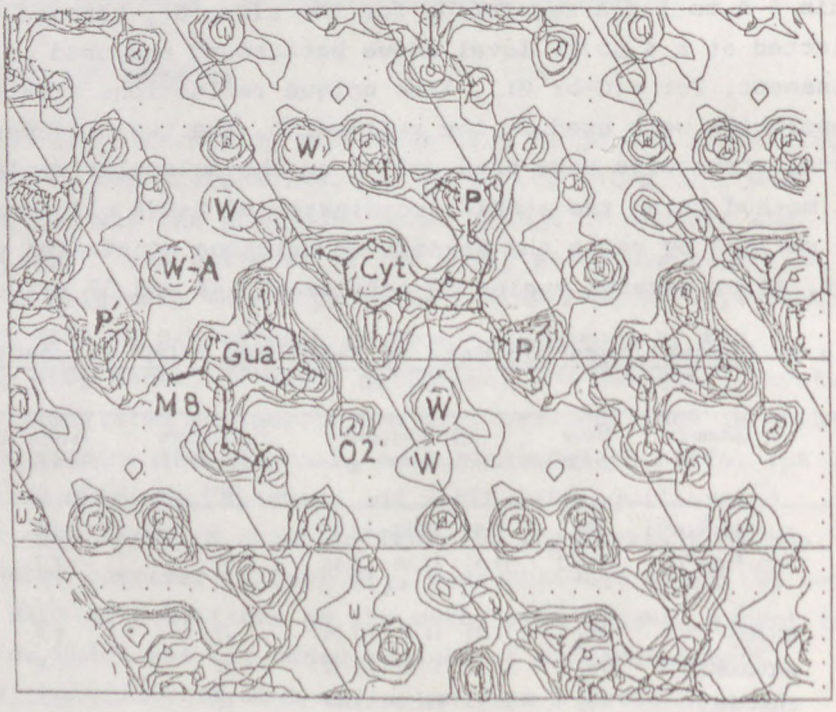


Figure 1. Electron density map of $r(C\text{-}m^{8}G)_3$; ranges from Z = 3.8 Å to Z = 6.8 Å.

probably binds to nitrogen N7 of the guanine base and also to the oxygen of the neighboring phosphate group (P'). The oxygen O2' of the sugar moiety in the cytidine residue at one residue below 8-methylguanosine in this electron density map binds to nitrogen N2 of the 8-methylguanine base, and this intramolecular hydrogen bond may stabilize the left-handed Z-RNA structure, similarly to the hydrogen bond between N2 of the guanine base and the solvent molecule (water) found in the Z-DNA oligomer, $d(CG)_3$ [3]. The left-handed molecular conformations of the two RNA oligomers are shown in Figure 2 (a, the ball-and-stick model; b, the space-filling model). The N2...O2' hydrogen bond formation (distance 2.8 Å) is clearly shown in Fig. 2; the bromine atoms (the shaded spheres in Fig. 3) stick out from the helical surface. The alternation of anti-syn conformations in the glycosyl angles is associated with the alternating pyrimidine-purine sequences of the molecule.

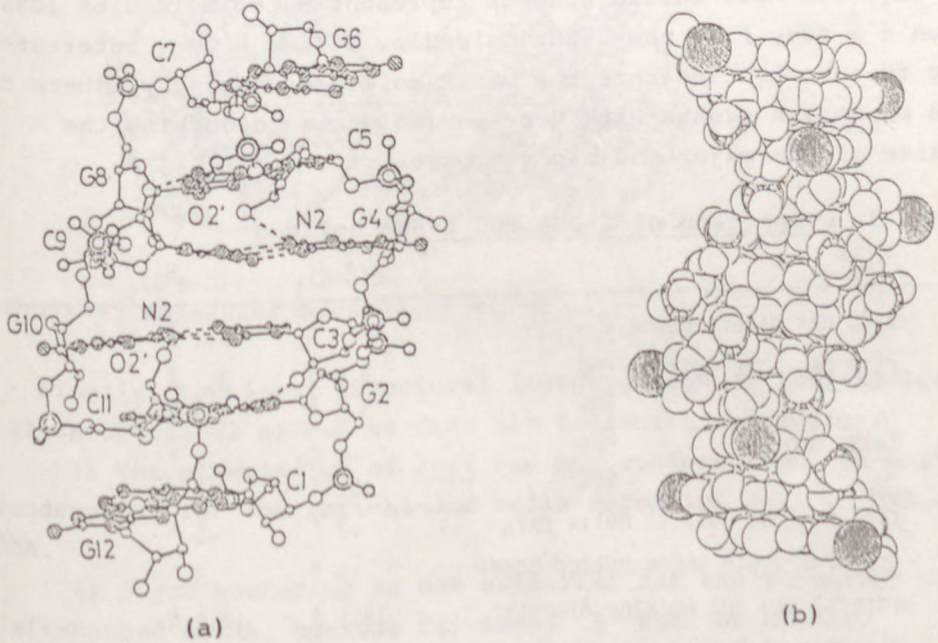
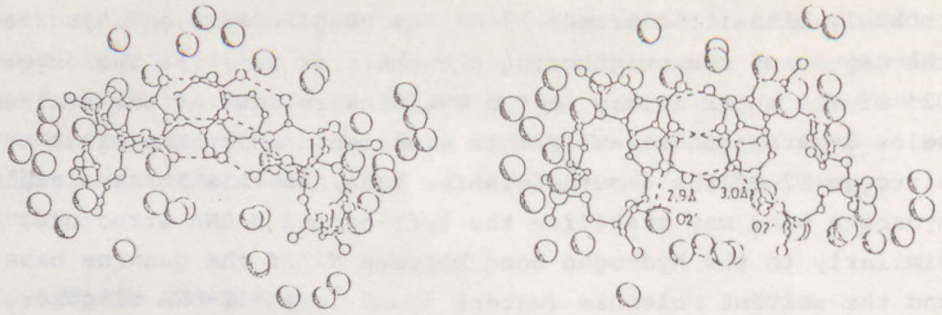


Figure 2. Molecular conformation of (a) $r(C\text{-}m^8G)_3$ and (b) $r(C\text{-}br^8G)_2$; for sake of clarity the intertwinement of the duplex is not shown for (a).



(a) (GpC duplex) (b) (CpG duplex)

Figure 3. Hydration of $r(C\text{-}m^8G)_3$.

As already mentioned , the two Z-RNA oligomers are surrounded by solvent water molecules and hence these water molecules were added during X-ray refinement, by the inspection of the electron density and difference density maps. The hydration shells around GpC and CpG double-helical regions for the $r(C\text{-}m^8G)_3$ hexamer are shown in Figure 3 (a) and (b), respectively. The half-shaded spheres represent water molecules less than 4 Å away from the Z-RNA molecule. It was a very interesting feature to find that the water molecules strongly adhere to the phosphate groups with very few molecules occupying the inside of the major and minor groove.

Table 3. Comparison of Z-DNA and Z-RNA helices

		$d(C\text{-}G)_3$	$r(C\text{-}m^8G)_3$
rise per dinucleoside		7.4 Å	7.1 Å
rise per base pair	CpG	3.9 Å	3.7 Å
	GpC	3.5 Å	3.4 Å
twist angle	CpG	-9 °	-12 °
	GpC	-51 °	-48 °
tilt of base pair to helix axis		-7 °	-5 °
dihedral angle among paired bases		5 °	3 °
lateral crystal packing diameter		18.1 Å	18.6 Å
distance of P from axis	pG	6.3 Å	6.5 Å
	pC	7.4 Å	8.1 Å

The molecular packing of $r(C\text{-}m^8G)_3$ in the hexagonal cell is shown in Figure 4. The space between the left-handed Z-RNA oligomer molecules is occupied by solvent water molecules. As mentioned before, the two Z-RNA oligomers showed left-handed conformations very similar to that of Z-DNA oligomers. Table 3 compares Z-RNA helical parameters with those of Z-DNA. As expected, the two sets of parameters are almost identical indicating a very similar molecular conformation for Z-RNA and Z-DNA.

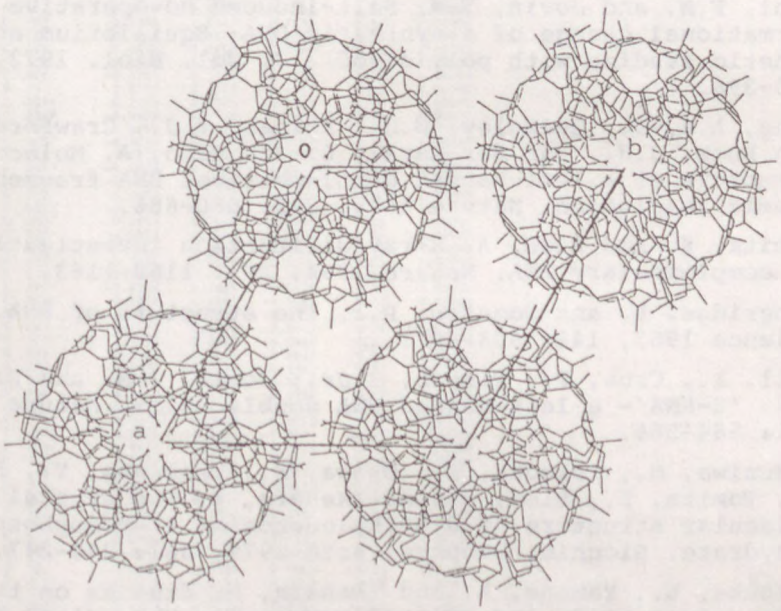


Figure 4. Molecular packing of $r(C\text{-}m^8G)_3$.

Finally, the X-ray structural investigation of left-handed oligonucleotides allows to draw the following conclusions.

1) The alternation of anti and syn conformations is most fundamental for the left-handed helix formation both in DNA and RNA.

2) Sugar puckering is not essential for the formation of left-handed helix, because C2'-endo, C3'-endo or C4'-exo puckerings are possible for purine or pyrimidine nucleosides in the left-handed helix.

3) Since the circular dichroism pattern of Z-DNA is nearly an inversion of that of B-DNA, the predominant factor to induce

CD peaks is the transition moment vector of the purine or pyramidine bases. Therefore,the signs of the CD peaks are changeable with the helical sense. However,the CD pattern of Z-RNA is not so simple[11], and probably the 2'-OH group may affect both the sign and magnitude of the CD pattern in both A-RNA and Z-RNA.

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APPENDIX

$r(C\text{-}br^{8}G)_2$ atomic coordinates

ATOM			X	Y	Z	B	OCC
1	C	1O5'	-0.2124	-0.0203	-0.1288	10.28	1.00000
2	C	1C5'	-0.1722	0.0106	-0.1493	9.70	1.00000
3	C	1C4'	-0.1311	0.0167	-0.1284	9.65	1.00000
4	C	1O1'	-0.1414	-0.0264	-0.1132	9.06	1.00000
5	C	1C1'	-0.1005	-0.0330	-0.1143	9.39	1.00000
6	C	1C2'	-0.0711	-0.0055	-0.1438	9.50	1.00000
7	C	1O2'	-0.0225	0.0085	-0.1363	9.46	1.00000
8	C	1C3'	-0.0839	0.0331	-0.1473	9.78	1.00000
9	C	1O3'	-0.0496	0.0773	-0.1370	9.84	1.00000
10	C	1N1	-0.1217	-0.0850	-0.1175	9.26	1.00000
11	C	1C2	-0.0943	-0.1057	-0.1205	9.55	1.00000
12	C	1O2	-0.0510	-0.0845	-0.1192	9.62	1.00000
13	C	1N3	-0.1180	-0.1547	-0.1252	9.96	1.00000
14	C	1C4	-0.1671	-0.1821	-0.1267	9.61	1.00000
15	C	1N4	-0.1877	-0.2296	-0.1307	10.11	1.00000
16	C	1C5	-0.1946	-0.1593	-0.1245	9.60	1.00000
17	C	1C6	-0.1709	-0.1120	-0.1204	9.56	1.00000
18	G	2P	-0.0383	0.1310	-0.1369	9.90	1.00000
19	G	2O1P	0.0133	0.1571	-0.1433	9.55	1.00000
20	G	2O2P	-0.0671	0.1450	-0.1551	9.24	1.00000
21	G	2O5'	-0.0465	0.1321	-0.0995	9.74	1.00000
22	G	2C5'	-0.0237	0.1702	-0.0786	9.76	1.00000
23	G	2C4'	-0.0640	0.1801	-0.0704	9.95	1.00000
24	G	2O1'	-0.1094	0.1394	-0.0782	9.69	1.00000
25	G	2C1'	-0.1396	0.1267	-0.0521	9.81	1.00000
26	G	2C2'	-0.1103	0.1479	-0.0220	9.82	1.00000
27	G	2O2'	-0.1362	0.1641	-0.0029	9.86	1.00000
28	G	2C3'	-0.0657	0.1888	-0.0354	9.81	1.00000
29	G	2O3'	-0.0600	0.2355	-0.0323	9.66	1.00000
30	G	2N9	-0.1686	0.0762	-0.0535	9.87	1.00000
31	G	2C8	-0.2161	0.0536	-0.0570	9.81	1.00000
32	G	2B8	-0.2467	0.0871	-0.0601	10.82	1.00000
33	G	2N7	-0.2356	0.0069	-0.0578	9.64	1.00000
34	G	2C5	-0.1976	0.0001	-0.0544	9.86	1.00000
35	G	2C4	-0.1553	0.0420	-0.0516	9.68	1.00000
36	G	2N3	-0.1111	0.0490	-0.0477	9.65	1.00000
37	G	2C2	-0.1132	0.0065	-0.0468	9.52	1.00000
38	G	2N2	-0.0748	0.0082	-0.0437	9.31	1.00000
39	G	2N1	-0.1515	-0.0377	-0.0493	9.51	1.00000
40	G	2C6	-0.1946	-0.0424	-0.0532	9.73	1.00000
41	G	2O6	-0.2270	-0.0846	-0.0557	9.54	1.00000
42	C	3P	-0.0256	0.2632	-0.0030	10.26	1.00000
43	C	3O1P	-0.0326	0.3020	0.0078	9.90	1.00000
44	C	3O2P	0.0219	0.2725	-0.0124	10.14	1.00000
45	C	3O5'	-0.0458	0.2212	0.0229	9.39	1.00000
46	C	3C5'	-0.0411	0.2175	0.0546	9.01	1.00000
47	C	3C4'	-0.0241	0.1826	0.0617	8.57	1.00000
48	C	3O1'	-0.0623	0.1359	0.0669	8.53	1.00000
49	C	3C1'	-0.0557	0.1014	0.0490	8.17	1.00000
50	C	3C2'	-0.0283	0.1296	0.0194	8.51	1.00000
51	C	3O2'	-0.0024	0.1085	0.0069	8.50	1.00000
52	C	3C3'	0.0064	0.1767	0.0355	8.44	1.00000
53	C	3O3'	0.0441	0.1735	0.0468	8.21	1.00000
54	C	3N1	-0.1021	0.0588	0.0469	8.05	1.00000
55	C	3C2	-0.1049	0.0160	0.0395	7.96	1.00000
56	C	3O2	-0.0721	0.0093	0.0340	7.70	1.00000
57	C	3N3	-0.1493	-0.0230	0.0383	7.87	1.00000
58	C	3C4	-0.1908	-0.0218	0.0441	8.05	1.00000
59	C	3N4	-0.2331	-0.0642	0.0416	8.44	1.00000
60	C	3C5	-0.1872	0.0221	0.0518	7.88	1.00000
61	C	3C6	-0.1435	0.0602	0.0532	7.99	1.00000
62	G	4P	0.0778	0.1835	0.0755	8.38	1.00000
63	G	4O1P	0.0599	0.1393	0.0932	7.81	1.00000
64	G	4O2P	0.1277	0.2052	0.0628	7.90	1.00000
65	G	4O5'	0.0780	0.2258	0.0945	8.51	1.00000
66	G	4C5'	0.1187	0.2721	0.0926	10.24	1.00000
67	G	4C4'	0.1245	0.3059	0.1190	11.36	1.00000
68	G	4O1'	0.0888	0.3198	0.1157	11.79	1.00000
69	G	4C1'	0.0640	0.3146	0.1448	12.51	1.00000
70	G	4C2'	0.0895	0.3025	0.1714	11.95	1.00000
71	G	4O2'	0.1265	0.3430	0.1879	11.77	1.00000
72	G	4C3'	0.1205	0.2878	0.1535	11.62	1.00000
73	G	4O3'	0.1660	0.3085	0.1679	11.53	1.00000
74	G	4N9	0.0144	0.2812	0.1398	12.96	1.00000
75	G	4C8	-0.0212	0.2910	0.1382	13.47	1.00000
76	G	4B8	-0.0091	0.3517	0.1423	15.07	1.00000
77	G	4N7	-0.0647	0.2531	0.1344	13.04	1.00000
78	G	4C5	-0.0554	0.2167	0.1333	12.87	1.00000
79	G	4C4	-0.0080	0.2319	0.1363	12.87	1.00000
80	G	4N3	0.0138	0.2060	0.1365	12.61	1.00000
81	G	4C2	-0.0181	0.1604	0.1326	12.50	1.00000
82	G	4N2	0.0013	0.1363	0.1328	12.53	1.00000
83	G	4N1	-0.0652	0.1396	0.1287	12.35	1.00000
84	G	4C6	-0.0857	0.1669	0.1294	12.75	1.00000
85	G	4O6	-0.1308	0.1445	0.1265	12.42	1.00000
86	C	5P	0.4838	0.3854	-0.1667	13.13	0.50000
87	C	5O1P	0.4432	0.3900	-0.1567	13.10	0.50000
88	C	5O2P	0.5276	0.4275	-0.1777	12.94	0.50000
89	C	5O5'	0.4944	0.3651	-0.1351	12.92	1.00000
90	C	5C5'	0.5137	0.3964	-0.1082	12.57	1.00000
91	C	5C4'	0.5649	0.4094	-0.1029	12.21	1.00000
92	C	5O1'	0.5725	0.3704	-0.0956	11.91	1.00000
93	C	5C1'	0.6056	0.3861	-0.1161	11.56	1.00000
94	C	5C2'	0.5963	0.3869	-0.1467	11.75	1.00000
95	C	5O2'	0.6318	0.3963	-0.1687	11.83	1.00000
96	C	5C3'	0.5987	0.4307	-0.1323	12.21	1.00000
97	C	5O3'	0.6439	0.4662	-0.1216	12.44	1.00000
98	C	5N1	0.5935	0.3156	-0.1186	11.27	1.00000
99	C	5C2	0.6280	0.3048	-0.1230	11.08	1.00000

ATOM			X	Y	Z	B	OCC
100	C	5O2	0.6692	0.3331	-0.1285	11.21	1.00000
101	C	5N3	0.6151	0.2569	-0.1238	11.38	1.00000
102	C	5C4	0.5695	0.2205	-0.1177	10.85	1.00000
103	C	5N4	0.5642	0.1766	-0.1191	10.91	1.00000
104	C	5C5	0.5341	0.2326	-0.1109	10.90	1.00000
105	C	5C6	0.5469	0.2786	-0.1109	11.08	1.00000
106	G	6P	0.6532	0.5018	-0.0938	12.16	1.00000
107	G	6O1P	0.7042	0.5373	-0.0928	13.11	1.00000
108	G	6O2P	0.6200	0.5191	-0.1002	12.37	1.00000
109	G	6O5'	0.6454	0.4757	-0.0607	12.26	1.00000
110	G	6C5'	0.6521	0.4986	-0.0311	12.67	1.00000
111	G	6C4'	0.6143	0.5099	-0.0190	12.83	1.00000
112	G	6O1'	0.5738	0.4895	-0.0391	12.62	1.00000
113	G	6C1'	0.5312	0.4637	-0.0225	13.17	1.00000
114	G	6C2'	0.5414	0.4722	0.0136	13.01	1.00000
115	G	6O2'	0.5292	0.5074	0.0209	13.14	1.00000
116	G	6C3'	0.5944	0.4925	0.0150	12.82	1.00000
117	G	6O3'	0.6126	0.5289	0.0386	12.45	1.00000
118	G	6N9	0.5077	0.4136	-0.0294	13.43	1.00000
119	G	6C8	0.4612	0.3856	-0.0347	13.41	1.00000
120	G	6N8	0.4206	0.4074	-0.0342	13.71	1.00000
121	G	6N7	0.4492	0.3406	-0.0398	13.60	1.00000
122	G	6C5	0.4913	0.3410	-0.0375	13.34	1.00000
123	G	6C4	0.5278	0.3849	-0.0308	13.25	1.00000
124	G	6N3	0.5736	0.3985	-0.0264	13.52	1.00000
125	G	6C2	0.5811	0.3618	-0.0292	13.44	1.00000
126	G	6N2	0.6236	0.3763	-0.0260	13.58	1.00000
127	G	6N1	0.5489	0.3167	-0.0355	13.46	1.00000
128	G	6C6	0.5028	0.3040	-0.0397	13.41	1.00000
129	G	6O6	0.4733	0.2616	-0.0460	12.90	1.00000
130	C	7P	0.6135	0.5235	0.0758	12.16	0.50000
131	C	7O1P	0.5737	0.4766	0.0843	11.99	0.50000
132	C	7O2P	0.6143	0.5654	0.0895	11.60	0.50000
133	C	7O5'	0.6605	0.5221	0.0840	11.98	1.00000
134	C	7C5'	0.7077	0.5473	0.0737	12.43	1.00000
135	C	7C4'	0.7328	0.5188	0.0718	12.45	1.00000
136	C	7O1'	0.6949	0.4694	0.0749	12.57	1.00000
137	C	7C1'	0.6971	0.4415	0.0495	12.56	1.00000
138	C	7C2'	0.7199	0.4787	0.0226	12.50	1.00000
139	C	7O2'	0.7353	0.4587	-0.0015	12.54	1.00000
140	C	7C3'	0.7590	0.5183	0.0415	12.22	1.00000
141	C	7O3'	0.7944	0.5070	0.0491	12.33	1.00000
142	C	7N1	0.6461	0.4057	0.0466	12.72	1.00000
143	C	7C2	0.6326	0.3594	0.0408	12.74	1.00000
144	C	7O2	0.6609	0.3458	0.0372	12.69	1.00000
145	C	7N3	0.5845	0.3285	0.0386	12.90	1.00000
146	C	7C4	0.5499	0.3414	0.0428	12.93	1.00000
147	C	7N4	0.5028	0.3089	0.0404	12.75	1.00000
148	C	7C5	0.5656	0.3898	0.0495	12.72	1.00000
149	C	7C6	0.6124	0.4193	0.0516	12.77	1.00000
150	G	8P	0.8498	0.5375	0.0413	12.46	1.00000
151	G	8O1P	0.8703	0.5122	0.0264	12.17	1.00000
152	G	8O2P	0.8489	0.5781	0.0255	12.50	1.00000
153	G	8O5'	0.8775	0.5541	0.0740	12.51	1.00000
154	G	8C5'	0.9020	0.6044	0.0806	12.46	1.00000
155	G	8C4'	0.8903	0.6097	0.1141	12.81	1.00000
156	G	8O1'	0.8559	0.6273	0.1157	12.85	1.00000
157	G	8C1'	0.8352	0.6176	0.1467	12.88	1.00000
158	G	8C2'	0.8567	0.5898	0.1642	12.71	1.00000
159	G	8O2'	0.9018	0.6222	0.1752	12.45	1.00000
160	G	8C3'	0.8637	0.5657	0.1355	12.71	1.00000
161	G	8O3'	0.8806	0.5350	0.1404	13.08	1.00000
162	G	8N9	0.7839	0.5935	0.1469	12.80	1.00000
163	G	8C8	0.7570	0.6138	0.1479	12.74	1.00000
164	G	8N8	0.7839	0.6776	0.1483	14.17	1.00000
165	G	8N7	0.7106	0.5835	0.1475	12.63	1.00000
166	G	8C5	0.7078	0.5405	0.1464	12.66	1.00000
167	G	8C4	0.7521	0.5448	0.1455	12.67	1.00000
168	G	8N3	0.7630	0.5099	0.1437	12.25	1.00000
169	G	8C2	0.7236	0.4676	0.1430	12.33	1.00000
170	G	8N2	0.7339	0.4360	0.1415	12.28	1.00000
171	G	8N1	0.6780	0.4572	0.1440	12.36	1.00000
172	G	8C6	0.6683	0.4931	0.1458	12.48	1.00000
173	G	8O6	0.6243	0.4811	0.1460	12.56	1.00000
174	C	9O5'	1.0973	0.6903	-0.0878	7.99	1.00000
175	C	9C5'	1.1330	0.7126	-0.1113	8.04	1.00000
176	C	9C4'	1.1754	0.7081	-0.1075	8.26	1.00000
177	C	9O1'	1.1671	0.6591	-0.1101	8.55	1.00000
178	C	9C1'	1.2142	0.6666	-0.1172	8.91	1.00000
179	C	9C2'	1.2240	0.6979	-0.1469	8.69	1.00000
180	C	9O2'	1.2730	0.7154	-0.1553	9.08	1.00000
181	C	9C3'	1.2152	0.7351	-0.1322	8.09	1.00000
182	C	9O3'	1.2556	0.7669	-0.1159	7.32	1.00000
183	C	9N1	1.2136	0.6218	-0.1229	9.17	1.00000
184	C	9C2	1.2546	0.6203	-0.1205	9.45	1.00000
185	C	9O2	1.2940	0.6534	-0.1137	9.50	1.00000
186	C	9N3	1.2523	0.5777	-0.1268	9.66	1.00000
187	C	9C4	1.2112	0.5364	-0.1348	9.54	1.00000
188	C	9N4	1.2125	0.4959	-0.1401	9.76	1.00000
189	C	9C5	1.1690	0.5390	-0.1371	9.68	1.00000
190	C	9C6	1.1722	0.5810	-0.1311	9.72	1.00000
191	G	10P	1.2948	0.8209	-0.1203	5.97	1.00000
192	G	10O1P	1.3399	0.8235	-0.1268	6.72	1.00000
193	G	10O2P	1.2771	0.8451	-0.1408	6.13	1.00000
194	G	10O5'	1.2956	0.8378	-0.0848	7.06	1.00000
195	G	10C5'	1.3285	0.8771	-0.0670	7.75	1.00000
196	G	10C4'	1.2980	0.8840	-0.0423	8.57	1.00000
197	G	10O1'	1.2487	0.8489	-0.0480	8.29	1.00000
198	G	10C1'	1.2239	0.8312	-0.0189	8.29	1.00000
199	G	10C2'	1.2560	0.8645	0.0069	8.08	1.00000
200	G	10O2'	1.2524	0.9060	0.0080	7.84	1.00000
201	G	10C3'	1.3015	0.8733	-0.0076	8.36	1.00000
202	G	10O3'	1.3422	0.9071	0.0093	8.97	1.00000
203	G	10N9	1.2103	0.7819	-0.0168	8.35	1.00000
204	G	10C8	1.1672	0.7463	-0.0090	8.32	1.00000
205	G	10N8	1.1191	0.7556	0.0029	10.04	1.00000
206	G	10N7	1.1646	0.7042	-0.0100	8.32	1.00000
207	G	10C5	1.2084	0.7141	-0.0198	8.12	1.00000
208	G	10C4	1.2182	0.7617	-0.0245	8.18	1.00000
209	G	10N3	1.2838	0.7831	-0.0344	7.85	1.00000

ATOM			X	Y	Z	B	OCC
210	G	10C2	1.2991	0.7528	-0.0386	7.52	1.00000
211	G	10N2	1.3403	0.7702	-0.0472	7.70	1.00000
212	G	10N1	1.2743	0.7056	-0.0346	8.00	1.00000
213	G	10C6	1.2289	0.6851	-0.0252	8.04	1.00000
214	G	10O6	1.2093	0.6413	-0.0213	8.12	1.00000
215	C	11P	1.3576	0.8818	0.0351	9.44	1.00000
216	C	11O1P	1.3197	0.8337	0.0430	9.45	1.00000
217	C	11O2P	1.3748	0.9176	0.0606	9.61	1.00000
218	C	11O5'	1.4018	0.8776	0.0228	9.17	1.00000
219	C	11C5'	1.4346	0.8861	0.0465	9.06	1.00000
220	C	11C4'	1.4582	0.8585	0.0502	8.80	1.00000
221	C	11O1'	1.4197	0.8115	0.0581	8.61	1.00000
222	C	11C1'	1.4261	0.7777	0.0381	8.33	1.00000
223	C	11C2'	1.4492	0.8064	0.0079	8.68	1.00000
224	C	11O2'	1.4714	0.7864	-0.0097	8.13	1.00000
225	C	11C3'	1.4873	0.8527	0.0238	8.84	1.00000
226	C	11O3'	1.5215	0.8425	0.0341	9.27	1.00000
227	C	11N1	1.3757	0.7423	0.0417	8.21	1.00000
228	C	11C2	1.3603	0.6949	0.0422	7.95	1.00000
229	C	11O2	1.3856	0.6776	0.0390	7.72	1.00000
230	C	11N3	1.3127	0.6662	0.0466	7.88	1.00000
231	C	11C4	1.2799	0.6813	0.0497	7.95	1.00000
232	C	11N4	1.2325	0.6491	0.0539	8.09	1.00000
233	C	11C5	1.2967	0.7308	0.0490	7.92	1.00000
234	C	11C6	1.3439	0.7586	0.0455	8.18	1.00000
235	G	12P	1.5763	0.8743	0.0407	10.01	1.00000
236	G	12O1P	1.5998	0.8572	0.0189	10.11	1.00000
237	G	12O2P	1.5848	0.9237	0.0388	10.49	1.00000
238	G	12O5'	1.5851	0.8673	0.0764	10.37	1.00000
239	G	12C5'	1.5425	0.8508	0.0954	11.04	1.00000
240	G	12C4'	1.5487	0.8945	0.1126	11.10	1.00000
241	G	12O1'	1.5162	0.9093	0.1022	11.22	1.00000
242	G	12C1'	1.4952	0.9189	0.1280	11.35	1.00000
243	G	12C2'	1.5105	0.9056	0.1599	10.96	1.00000
244	G	12O2'	1.5370	0.9472	0.1784	10.28	1.00000
245	G	12C3'	1.5436	0.8883	0.1487	11.10	1.00000
246	G	12O3'	1.5903	0.9142	0.1624	11.20	1.00000
247	G	12N9	1.4447	0.8956	0.1247	11.37	1.00000
248	G	12C8	1.4204	0.9185	0.1219	11.47	1.00000
249	G	12B8	1.4458	0.9819	0.1193	13.04	1.00000
250	G	12N7	1.3738	0.8905	0.1204	11.17	1.00000
251	G	12C5	1.3693	0.8467	0.1232	11.03	1.00000
252	G	12C4	1.4116	0.8474	0.1261	11.14	1.00000
253	G	12N3	1.4190	0.8103	0.1295	10.81	1.00000
254	G	12C2	1.3777	0.7698	0.1295	10.67	1.00000
255	G	12N2	1.3789	0.7325	0.1323	10.81	1.00000
256	G	12N1	1.3343	0.7647	0.1268	10.86	1.00000
257	G	12C6	1.3273	0.8013	0.1236	10.89	1.00000
258	G	12O6	1.2868	0.7959	0.1205	10.68	1.00000
259	W	13W	0.1951	0.1945	0.0264	13.48	1.00000
260	W	14W	0.5236	0.9689	0.0295	23.84	1.00000
261	W	15W	0.3266	0.2810	0.1250	17.92	1.00000
262	W	16W	0.8009	0.2854	0.0531	12.69	1.00000
263	W	17W	0.1446	0.9540	0.0784	18.60	1.00000
264	W	18W	0.0630	0.8816	0.0483	23.79	1.00000
265	W	19W	0.0030	0.3960	0.0594	19.46	1.00000
266	W	20W	0.4038	0.5496	0.0592	21.91	1.00000
267	W	21W	0.6807	0.8258	0.0052	19.22	1.00000
268	W	22W	0.7888	0.1555	0.0235	21.24	1.00000
269	W	23W	0.2875	0.3308	0.1468	18.00	1.00000
270	W	24W	0.7165	0.7616	0.1063	24.54	1.00000
271	W	25W	0.0128	0.6540	0.0536	12.37	1.00000
272	W	26W	0.4874	0.3891	0.0621	9.35	1.00000
273	W	27W	0.8464	0.4421	0.0761	13.48	1.00000
274	W	28W	0.4671	0.4879	0.1262	17.89	1.00000
275	W	29W	0.3981	0.4796	0.1657	24.49	1.00000
276	W	30W	0.5761	0.6252	0.1412	13.90	1.00000
277	W	31W	0.0944	0.8177	0.0980	18.74	1.00000
278	W	32W	1.0098	0.5469	0.1398	22.50	1.00000
279	W	33W	0.9130	0.4140	0.0962	18.56	1.00000
280	W	34W	0.0621	0.8413	0.1767	15.61	1.00000
281	W	35W	0.1683	0.6790	0.0573	8.55	1.00000
282	W	36W	0.6501	0.6416	0.0553	19.81	1.00000
283	W	37W	0.6648	0.7553	0.0512	17.27	1.00000
284	W	38W	0.3198	0.1808	0.1158	8.85	1.00000
285	W	39W	0.4655	0.5150	0.0487	6.30	1.00000
286	W	40W	0.6524	0.9472	0.1054	17.63	1.00000
287	W	41W	0.9333	0.5041	0.1015	24.72	1.00000
288	W	42W	0.4725	0.3772	0.1219	23.67	1.00000
289	W	43W	0.5422	0.0099	0.0955	16.03	1.00000
290	W	44W	0.2303	0.8225	0.1278	25.56	1.00000
291	W	45W	0.9795	0.6055	0.1297	23.05	1.00000
292	W	46W	0.4132	0.2430	0.0069	19.39	1.00000
293	W	47W	0.3209	0.2966	0.0650	12.75	1.00000
294	W	48W	0.2384	0.7872	0.0668	22.75	1.00000
295	W	49W	0.1163	0.5096	0.0065	20.58	1.00000
296	W	50W	0.0283	0.3158	0.0558	25.43	1.00000
297	W	51W	0.5447	0.6027	0.0645	22.02	1.00000
298	W	52W	0.0980	0.5734	0.0673	21.64	1.00000
299	W	53W	0.3673	0.1176	0.0903	18.77	1.00000
300	W	54W	0.7623	0.2271	0.1372	23.29	1.00000
301	W	55W	0.7760	0.1580	0.1222	18.21	1.00000
302	W	56W	0.9765	0.7014	0.1482	21.18	1.00000

$r(C\text{-}m^8G)_3$ atomic coordinates

1	C	1P	0.4769	0.3947	0.1245	14.81	0.66667
2	C	1O2P	0.5026	0.4365	0.1548	14.60	0.66667
3	C	1O1P	0.5180	0.3590	0.1078	16.70	0.66667
4	C	1O5'	0.3793	0.3159	0.1313	14.58	1.00000
5	C	1C5'	0.3944	0.2521	0.1431	14.86	1.00000
6	C	1C4'	0.3174	0.1741	0.1454	14.45	1.00000
7	C	1O1'	0.2525	0.1927	0.1507	14.41	1.00000
8	C	1C1'	0.1788	0.1299	0.1380	12.02	1.00000
9	C	1C2'	0.2048	0.1083	0.1067	14.68	1.00000
10	C	1O2'	0.1409	0.0200	0.1014	18.03	1.00000
11	C	1C3'	0.2854	0.1166	0.1161	16.81	1.00000
12	C	1O3'	0.2700	0.0378	0.1229	19.55	1.00000
13	C	1N1	0.1291	0.1748	0.1358	10.87	1.00000

ATOM			X	Y	Z	B	OCC
14	C	1C2	0.0440	0.1231	0.1359	10.21	1.00000
15	C	1O2	0.0133	0.0477	0.1366	10.80	1.00000
16	C	1N3	-0.0001	0.1647	0.1349	9.76	1.00000
17	C	1C4	0.0340	0.2487	0.1338	7.92	1.00000
18	C	1N4	-0.0180	0.2818	0.1342	8.79	1.00000
19	C	1C5	0.1220	0.3008	0.1352	9.23	1.00000
20	C	1C6	0.1645	0.2587	0.1359	9.68	1.00000
21	G	2P	0.2502	-0.0227	0.1524	20.86	1.00000
22	G	202P	0.1766	-0.0288	0.1682	19.34	1.00000
23	G	201P	0.2378	-0.1027	0.1406	20.79	1.00000
24	G	205'	0.3284	0.0183	0.1756	18.33	1.00000
25	G	2C5'	0.3691	-0.0224	0.1886	16.24	1.00000
26	G	2C4'	0.4295	0.0319	0.2126	12.95	1.00000
27	G	201'	0.4482	0.1122	0.2045	13.29	1.00000
28	G	2C1'	0.4653	0.1606	0.2317	11.07	1.00000
29	G	2C2'	0.4216	0.1063	0.2603	12.00	1.00000
30	G	202'	0.4641	0.1047	0.2886	14.93	1.00000
31	G	2C3'	0.4026	0.0242	0.2471	14.09	1.00000
32	G	203'	0.4404	-0.0104	0.2665	14.11	1.00000
33	G	2N9	0.4443	0.2236	0.2286	8.51	1.00000
34	G	2C8	0.4938	0.3065	0.2281	7.79	1.00000
35	G	2N7	0.4514	0.3465	0.2260	5.56	1.00000
36	G	2C5	0.3707	0.2865	0.2233	6.94	1.00000
37	G	2C4	0.3637	0.2117	0.2248	7.21	1.00000
38	G	2N3	0.2978	0.1355	0.2251	6.02	1.00000
39	G	2C2	0.2288	0.1428	0.2217	5.88	1.00000
40	G	2N2	0.1699	0.0685	0.2209	5.00	1.00000
41	G	2N1	0.2234	0.2128	0.2196	7.04	1.00000
42	G	2C6	0.2937	0.2855	0.2195	7.08	1.00000
43	G	206	0.2878	0.3510	0.2177	8.15	1.00000
44	G	2N8	0.5834	0.3451	0.2301	6.26	1.00000
45	C	3P	-0.3944	0.0587	0.2459	14.50	0.66667
46	C	301P	-0.4204	0.0855	0.2199	14.80	0.66667
47	C	302P	-0.4534	-0.0276	0.2586	14.23	0.66667
48	C	305'	-0.3141	0.0587	0.2315	15.09	1.00000
49	C	3C5'	-0.3318	0.0074	0.2050	11.21	1.00000
50	C	3C4'	-0.2776	-0.0272	0.2026	12.80	1.00000
51	C	301'	-0.1924	0.0396	0.1986	12.12	1.00000
52	C	3C1'	-0.1386	0.0156	0.2121	10.79	1.00000
53	C	3C2'	-0.1878	-0.0051	0.2446	11.83	1.00000
54	C	302'	-0.1475	-0.0333	0.2635	13.35	1.00000
55	C	3C3'	-0.2752	-0.0665	0.2353	12.99	1.00000
56	C	303'	-0.2819	-0.1440	0.2330	15.84	1.00000
57	C	3N1	-0.0608	0.1021	0.2135	8.81	1.00000
58	C	3C2	0.0146	0.1099	0.2140	8.62	1.00000
59	C	302	0.0269	0.0513	0.2130	9.43	1.00000
60	C	3N3	0.0799	0.1908	0.2154	8.18	1.00000
61	C	3C4	0.0693	0.2593	0.2168	5.39	1.00000
62	C	3N4	0.1367	0.3383	0.2178	6.22	1.00000
63	C	3C5	-0.0088	0.2481	0.2163	7.76	1.00000
64	C	3C6	-0.0719	0.1710	0.2142	8.68	1.00000
65	G	4P	-0.3390	-0.2379	0.2421	15.41	1.00000
66	G	401P	-0.3228	-0.3047	0.2373	16.17	1.00000
67	G	402P	-0.3640	-0.2380	0.2745	16.21	1.00000
68	G	405'	-0.4194	-0.2590	0.2196	16.50	1.00000
69	G	4C5'	-0.3955	-0.2488	0.1868	16.21	1.00000
70	G	4C4'	-0.4566	-0.2764	0.1613	13.19	1.00000
71	G	401'	-0.4766	-0.2126	0.1608	12.31	1.00000
72	G	4C1'	-0.4753	-0.1733	0.1343	9.10	1.00000
73	G	4C2'	-0.4593	-0.2225	0.1088	10.31	1.00000
74	G	402'	-0.5349	-0.2790	0.0930	10.21	1.00000
75	G	4C3'	-0.4325	-0.2762	0.1254	12.58	1.00000
76	G	403'	-0.4651	-0.3542	0.1116	13.41	1.00000
77	G	4N9	-0.4155	-0.0873	0.1331	9.02	1.00000
78	G	4C8	-0.4357	-0.0283	0.1320	6.11	1.00000
79	G	4N7	-0.3670	0.0489	0.1308	8.75	1.00000
80	G	4C5	-0.3029	0.0330	0.1306	7.36	1.00000
81	G	4C4	-0.3288	-0.0471	0.1338	6.99	1.00000
82	G	4N3	-0.2802	-0.0817	0.1322	7.59	1.00000
83	G	4C2	-0.2004	-0.0233	0.1316	7.38	1.00000
84	G	4N2	-0.1615	-0.0622	0.1311	7.02	1.00000
85	G	4N1	-0.1667	0.0593	0.1301	5.30	1.00000
86	G	4C6	-0.2159	0.0913	0.1291	5.32	1.00000
87	G	406	-0.1870	0.1691	0.1304	8.19	1.00000
88	G	4N8	-0.5209	-0.0386	0.1327	6.05	1.00000
89	W	5W	0.0632	-0.3784	0.0250	5.14	1.00000
90	W	6W	0.4396	-0.0988	0.0198	14.15	1.00000
91	W	7W	0.5430	-0.0947	0.0475	5.04	1.00000
92	W	8W	0.5019	0.0236	0.0526	23.23	1.00000
93	W	9W	0.4358	0.1101	0.0533	22.99	1.00000
94	W	10W	0.3634	-0.1666	0.0984	6.92	1.00000
95	W	11W	-0.4434	0.2611	0.1112	15.14	1.00000
96	W	12W	0.3227	-0.4118	0.1413	6.97	1.00000
97	W	13W	-0.3376	0.2091	0.1451	16.06	1.00000
98	W	14W	-0.3249	-0.5132	0.1532	12.73	1.00000
99	W	15W	-0.0016	0.5172	0.1490	22.45	1.00000
100	W	16W	-0.3215	0.3570	0.1579	5.14	1.00000
101	W	17W	0.1973	-0.3890	0.1480	21.62	1.00000
102	W	18W	-0.3030	-0.3782	0.1443	21.64	1.00000
103	W	19W	-0.1664	-0.1618	0.1752	5.25	1.00000
104	W	20W	0.1391	0.4715	0.1462	14.65	1.00000
105	W	21W	0.2647	-0.2600	0.0701	16.02	1.00000
106	W	22W	0.4275	-0.3478	0.0456	10.16	1.00000
107	W	23W	-0.3559	0.3195	0.0652	10.61	1.00000
108	W	24W	-0.1343	0.3352	0.0412	5.18	1.00000
109	W	25W	-0.4321	0.4414	0.0280	12.03	1.00000
110	W	26W	-0.4809	-0.2323	0.0448	18.48	1.00000
111	W	27W	0.0885	-0.4882	0.1126	29.30	1.00000
112	W	28W	0.1587	-0.1177	0.0501	23.76	1.00000
113	W	29W	0.0456	-0.3414	0.1691	5.00	1.00000
114	W	30W	0.1894	-0.1732	0.1882	23.79	1.00000
115	W	31W	0.3210	-0.2131	0.1509	23.19	1.00000
116	W	32W	0.1883	-0.2499	0.1415	17.79	1.00000
117	W	33W	-0.0024	-0.0867	0.1238	12.38	1.00000
118	W	34W	0.0632	-0.2553	0.1276	10.72	1.00000
119	W	35W	-0.1286	0.4002	0.0990	8.17	1.00000
120	W	36W	0.1325	-0.1077	0.1115	31.64	1.00000
121	W	37W	-0.4168	0.1848	0.0601	10.89	1.00000
122	W	38W	0.2274	0.0322	0.0335	21.73	1.00000
123	W	39W	0.3022	-0.0697	-0.0053	5.06	1.00000
124	W	40W	-0.0002	-0.0756	0.1807	20.07	1.00000
125	W	41W	0.4137	0.0596	0.1211	51.96	1.00000

Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

THE ROLE OF 11-BETA-HYDROXYL IN GLUCOCORTICOID HORMONE ACTION

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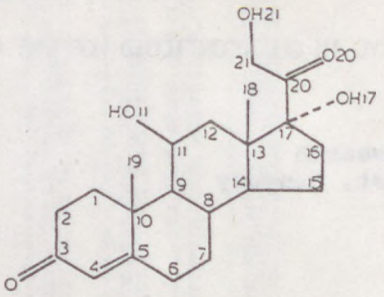
Significant physiological and pharmacological effects of adrenal steroids prompted a long lasting search for synthetic analogs and led to the discovery of the potent glucocorticoid agonists prednisolone, triamcinolone acetonide, etc. Literally thousands of steroids have been prepared and screened for receptor binding and pharmacologic effects since the early sixties. Owing to this unrivalled effort, we got quite far in understanding how the steroid structure affects affinity for the glucocorticoid receptor protein (GR). Intensive studies on the properties of GR, at the same time, shed light on the chain of molecular events that are triggered by ligand binding and are the prerequisite of hormone action. An excellent presentation of the major results of the research on glucocorticoid hormone action up to the end of the seventies is given in a monograph dedicated to Gordon Tomkins[1]. This chapter reviews chiefly more recent data with reference to earlier results where necessary for understanding. I will concentrate on the steroid - GR interaction with special emphasis on the role of the substituent at the chiral C-11 atom in the mechanism of hormone action.

GLUCOCORTICOID AGONISTS AND ANTAGONISTS

The naturally occurring steroids with glucocorticoid activity belong to the pregnane series. All have rings B and C in the chair form with the B:C and C:D fusions in the trans configuration. X-ray crystallographic studies showed that steroid configurations are conditioned by a few basic structural elements. As to the structure of the glucocorticoid agonist cortisol (Fig. 1), it may assume two conformations that can be observed in pyridine and methanol complexes, respectively. The difference between those

a



b

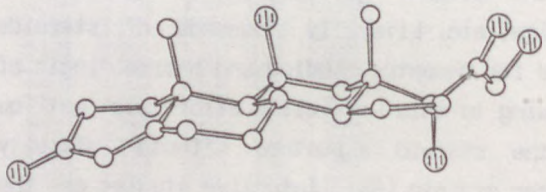


Figure 1. Structure of hydrocortisone (cortisol). Numbering of atoms (a) and ball-and-stick representation (b) of hydrocortisone in methanol complex.

is that ring A is bent toward the alpha face to a higher degree in the pyridine complex[2], whereas the C(17) side chain orientation is largely invariant[3]. Conformations of 9α-fluoro derivatives, which are generally more active glucocorticoids than the parent compounds, are characterized by a pronounced inclination of the A-ring toward the α-face[4].

It is interesting in this respect that several Δ4,9 and Δ4,9,11 unsaturated steroids have far more flexible structures than Δ4 steroids; the former easily adopt slightly bent or highly bent conformations. This may be the reason why many of them display indiscriminately high affinity for receptors of different classes[5].

The 18 and 19 methyl groups both point to the β-side of the ring system. The isomers which differ from hydrocortisone in the configuration of any of the chiral carbon atoms at the ring fusions would consequently

exhibit a rather different shape. This gives a satisfactory explanation why chirality of those centers cannot be changed while preserving receptor binding and/or pharmacological activity.

It was early recognized that glucocorticoid agonist activity lies with the 11β,21-dihydroxy-4-ene-3,20-dione structure [6,7], since natural and synthetic agonists almost invariably had those functions. Systematic studies[8] and fortuitous discoveries[9,10], however, revealed that other A-ring substituents could replace the 3-oxo group without loss of activity. Moreover, very strong binding to the GR was seen in case of some synthetic steroids with unusual structures (Fig. 2), e.g. RU 38486, or RU 25055[11]. Nonpolar substituents on the steroid core form numerous contacts with GR contributing to the van der Waals interactions that are the major source of the free energy change which drives complex formation[12,13]. It should be noted, however, that lack of 11β-hydroxyl substituent[14,15] or its replacement with a bulky apolar group[11], as well as changing the configuration of the C-11 atom[14,15], would all result in glucocorticoid antagonists or inactive molecules in different systems. The affinity of GR is not necessarily lower for these steroids than for their 11β-hydroxyl counterparts.

However, 11-deoxycortisol, an effective antiglucocorticoid in vitro[16], exhibited glucocorticoid agonist activity in intact rats in vivo. This was attributed to its conversion to cortisol by 11-hydroxylation[17]. Analogs that were less susceptible to metabolic activation, or could not be 11-hydroxylated at all, such as Δ1,9(11)-deoxycortisol[18] or 11-oxa-11-deoxycortisol[19] retained in vivo antiglucocorticoid activity.

The antagonist character of 11β-hydroxy-17β-carboxamidosteroids[20] in some in vivo systems (e.g. rat liver) is difficult to explain, all the more, since in other systems (human peripheral blood lymphocytes) these same steroids behaved like glucocorticoid agonists[21].

The covalent antagonist dexamethasone 21-mesylate (Fig. 2) will be discussed later.

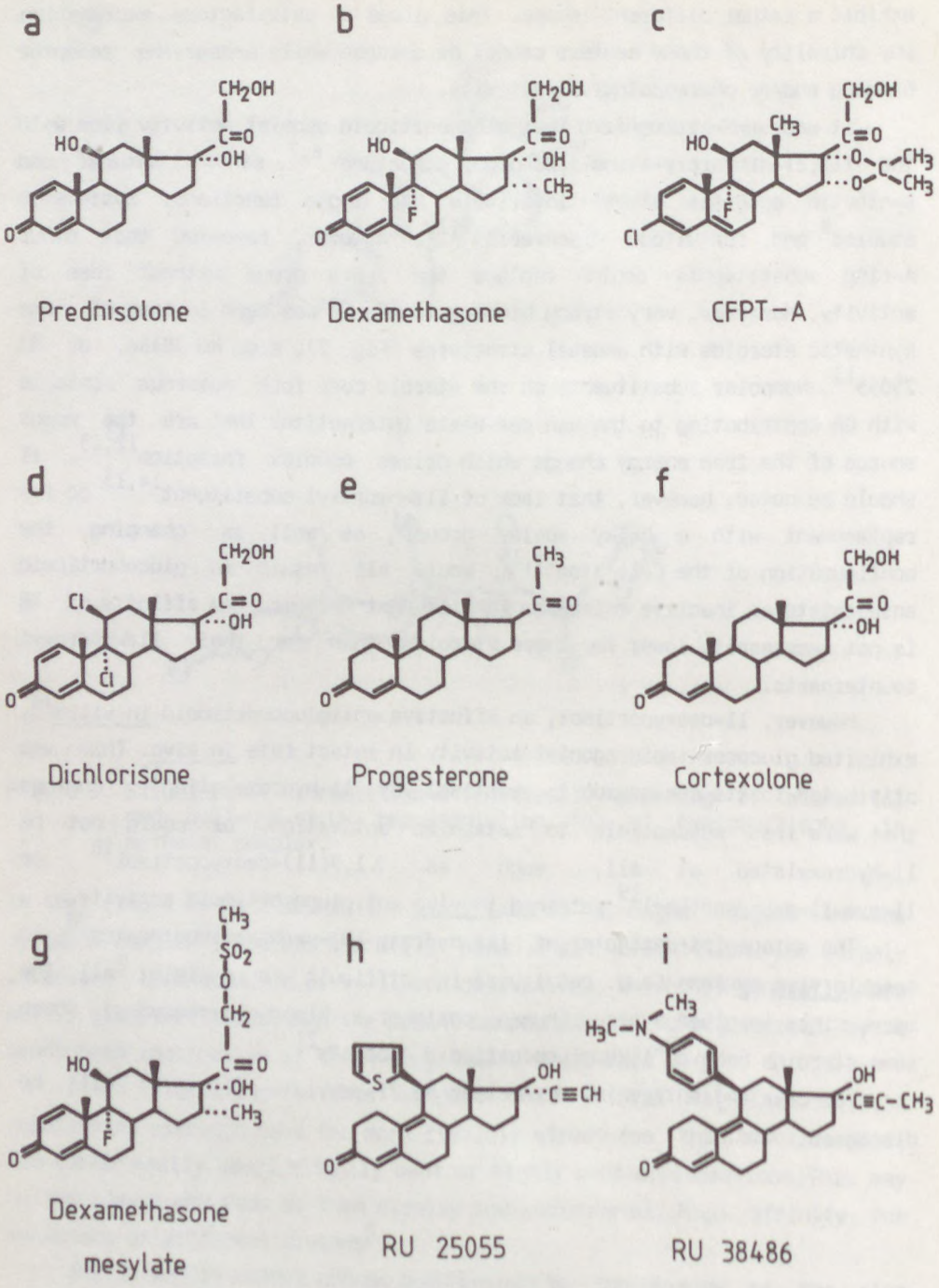


Figure 2. Structures of glucocorticoid agonists (a-d) and antagonists (e-i).

Many different tissues respond to glucocorticoid hormones. The type of response varies with the target cell, ranging from mainly anabolic effects like tyrosine aminotransferase induction[22,23] or stimulation of gluconeogenesis[24] in liver or cultured hepatoma cells, to catabolic effects demonstrated in lymphoid tissues[25]. Still, structure and properties of GR from different sources appear to be essentially identical.

To date, human[26], rat[27] and murine[28] GR have been cloned and sequenced. Primary sequences of these proteins are strongly homologous. Discovery of extensive homologies with other steroid receptors and apparently unrelated proteins led to the recognition of the steroid/thyroid receptor gene family[29]. Examination of insertion and deletion mutants and site directed mutagenesis helped in elucidating the roles of several structural elements in the mechanism of GR action[30,31]. Human GR, derived from the lymphoid cell line M9, consists of 777 amino acids and rodent GR's are a few amino acids longer. The carboxy terminal part forms the steroid binding pocket. A stretch of 86 amino acids in the middle region[27], which is the most conserved among steroid receptors, confers DNA-binding properties on GR. This DNA-binding domain seems to determine specificity of function, since replacement of this domain of estrogen receptor (ER) with that of GR gave a hybrid ER that behaved like GR in the presence of estradiol[32].

Cytosolic GR, which is used in general in steroid binding experiments, is but a small fraction of the cytosolic protein mass, usually less than 0.01 per cent. In buffers of low ionic strength and at low temperature, it is a trimer or perhaps a tetramer[33]. This trimer is called the nontransformed GR and it consists of one steroid binding subunit discussed above, and two other subunits unable to bind steroids. The latter are identical with the hsp 90 heat shock protein[34] and are constituents of other nontransformed steroid receptors as well[35]. The nontransformed GR has a molecular weight of about 300,000 with a sedimentation coefficient (S_{20}) of 9-10 S and a Stokes radius (R_S) of about 8 nm[36,37]. The complex is stabilized by molybdate ions.

Some authors advocate for the involvement of various other constituents, "factors", within the nontransformed GR. The strongest evidence supports perhaps the participation of a 50-60 kD protein[33,38].

Different laboratories became engaged in studying structure-activity relationships for glucocorticoids in the seventies, using receptor binding as a tool[8,39]. In these experiments, crude cytosol of rat hepatoma or liver was used as a source of GR and tritiated dexamethasone was selected as radioligand. Dexamethasone (Fig.2) offered the following advantages over the natural glucocorticoids cortisol or corticosterone: it did not bind to rat serum proteins, it was not metabolized in the assay conditions and it was even more selective and displayed higher affinity (K_D=6nM for GR) than those[39].

Analysis of data was simplified by the fact that a single binding site was detected with no sign of cooperativity. The steroids available for study were included in the incubation mixture and their binding affinity for the cytosol GR was determined. Affinity constants were not really required to draw - mostly qualitative - conclusions concerning the influence of certain elements of the steroid structure on biding affinity. Therefore, relative binding affinity, RBA, was mostly used with non-radioactive dexamethasone as the reference compound[8,40]. It was learned through pairwise comparisons of dissociation constants or RBA's of steroids differing in a single substituent that the introduction into the pregnenedione structure of a double bond in position 1, a 9α-fluoro group, an 11β-hydroxyl group, or a 16α-methyl group consistently increased binding affinity for GR[8,39]. On the other hand, presence of 1-methyl or 11α-hydroxyl group or reduction of the 20-ketone strongly diminished receptor binding.

It was also realized that incubation conditions influenced the apparent RBA values considerably, namely, compounds that dissociate fast from the receptor would have RBA's that decrease as incubation proceeds. On the contrary, RBA's increase with time if the dissociation rate of the test steroid is slow[41]. The reason of this finding is probably the systematic error due to the lack of true equilibrium between free and receptor-bound steroid[42]. It was suggested that kinetic, rather than equilibrium constants should be determined to avoid arteficial results[43]. Reexamination of the influence of structural elements on GR binding using kinetic methods[44] supported the earlier conclusions and it permitted a more detailed

understanding of the steroid-GR interaction in the vicinity of the C-11 atom.

Notably, steroids with a Δ1 double bond, especially in conjunction with a 9α-F atom, showed exceedingly slow dissociation rates from the GR. Association rate constants were not affected by these functions[44].

On the other hand, the presence of a 17α-hydroxyl substituent decreased association rate constants (k_a) and increased dissociation rate constants (k_d) severalfold, resulting in a substantial increase in equilibrium dissociation constant (K_D) of the steroid - GR complex, as compared with the parent compound. For example, k_a, k_d, and K_D for progesterone (cf. Fig. 2) were $2.6X10^{6}M^{-1}min^{-1}$, $4.6X10^{-3}min^{-1}$ and 1.8 nM, respectively, whereas the values for 17α-hydroxyprogesterone were $3.7X10^{5}M^{-1}min^{-1}$, $1.0X10^{-2}min^{-1}$ and 27 nM.

As to the 11β-hydroxyl group, its oxidation or epimerization resulted in decreased association and increased dissociation rates, the association rate constant being more severely changed (e.g. in case of the prednisolone - epiprednisolone couple, k_a was reduced sixfold with a twofold increase in k_d value). Interestingly, 11-deoxycorticosterone had about the same rate constants as its parent compound, whereas cortexolone (Fig. 2) dissociated significantly faster and associated more slowly with GR than hydrocortisone[44].

When dissociation kinetics of 11β-hydroxysteroids in deuterated water were studied, significant (about twofold) decrease in k_d values was observed. No kinetic deuterium isotope effect was seen with any other steroids lacking the 11β-hydroxyl substituent (Table 1). For instance, in case of prednisolone (Fig. 2), the ratio of k_d's observed in H_2O and D_2O was 1.82, while a ratio of 1.08 was found for 11-epiprednisolone. These observations strongly suggested that the 11β-hydroxyl group formed a kinetically relevant H-bond with the steroid binding pocket of GR[45].

Recently, a novel series of 11β-chloro-corticosteroids were shown to be potent glucocorticoid agonists[46]. Observation of a kinetic deuterium isotope effect of similar magnitude in case of the 11β-chloro-steroids dichlorisone and 16α-methyldichlorisone[47] (Fig. 2), and demonstration of their glucocorticoid agonist character by the tyrosine aminotransferase induction assay allowed to conclude that the 11β-substituent of high electronegativity is an acceptor in the hydrogen bond between GR and the glucocorticoid agonist. This finding harmonizes with the statement of Duax

et al.[4]: "the 11β-hydroxyl group appears to be more important for activity than for binding".

Table 1

Deuterium isotope effect on steroid-GR complex dissociation[a]

Steroid	k_H/k_D[b] (Mean±SEM)	n
Hydrocortisone	2.05±0.18[c]	8
Corticosterone	1.82±0.11[c]	5
Prednisolone	1.82±0.25[c]	5
Epiprednisolone	1.08±0.11	6
11-Deoxycorticosterone	1.10±0.07	4
Progesterone	1.02±0.04	3

[a] Data taken from reference 45.

[b] k_H: Dissociation rate constant in H_2O; k_D: dissociation rate constant in D_2O.

[c] Different from 1.00 by the t test at $p<0.01$

It is of interest that no deuterium isotope effect was seen in the association reaction between GR and the 11β-hydroxysteroids. It can be shown by assuming an intramolecular rearrangement within the steroid-GR complex that lack of deuterium isotope effect in the association reaction (H-bond making) along with a significant deuterium isotope effect in the dissociation reaction (H-bond breaking) does not contradict the principle of microscopic reversibility (M. Simonyi, personal communication).

As to the amino acid side chains constituting the steroid binding site, not much is known as yet. It is suspected that two cysteinyl groups are near the opening of the binding cavity. One of those in the rat GR is Cys-656, which is covalently labeled by dexamethasone mesylate[48]. Met-622 and Cys-754 are probably in close contact with ring A of the ligand, since these side chains are covalently labeled after photoactivation of the triamcinolone acetonide-GR complex[49]. We have no idea at present of which amino acid would participate in the hydrogen bond with glucocorticoid agonists.

The specific interaction between glucocorticoid agonists and GR

manifests itself in a stabilizing effect: agonists protect GR against heat inactivation[50]. Antagonists, on the other hand, do not influence denaturation of GR[51].

CONSEQUENCES OF STEROID BINDING - TRANSFORMATION

Steroid-bound cytosolic GR undergoes transformation if incubated at moderately elevated temperature or in solutions of high ionic strength. Transformation is a complex conformational change and involves dissociation of the oligomeric structure[52]. The molecular weight will drop to 94,000 with an R_S of about 6.0 nm and an S_{20} of 4 S.

Dissociation rates of glucocorticoid agonist but not of antagonist steroids from the GR become slower after transformation[51]. Apparently, the conformation of the binding site changes so that the steroid is more strongly bound than before transformation.

The transformed, steroid-bound GR subunit will elute at lower salt concentration from DEAE cellulose than the nontransformed complex.

Last but not least, the DNA-binding domain of the steroid binding subunit becomes exposed and it will display considerable affinity for DNA-cellulose or isolated nuclei. Specific GR-binding sequences, termed glucocorticoid responsive elements, have been identified in the 5' flanking regions of hormone-inducible genes[53]. This is probably the essence of the mechanism of GR action: specific interaction of transformed GR with glucocorticoid responsive elements provides for an enhanced transcriptional activity of the regulated gene.

Transformation of GR may follow different routes depending on how it is triggered[54]. Transformation by salt can proceed also in the absence of hormone, whereas temperature mediated transformation is a hormone dependent event[55]. High salt media may induce dissociation of the subunits of GR without perhaps much involvement of the steroid. However, the important role of the glucocorticoid agonist in heat induced transformation has been emphasized by different authors[54,56,57]. Dexamethasone promotes separation of the steroid binding subunit from hsp 90 also within lymphoma cells[58].

The mechanism of agonist-induced GR transformation can be modeled as shown in Figure 3. The 11β-hydroxysteroid forms a primary complex with the nontransformed GR whose stability mostly depends on van der Waals interactions, but at least one hydrogen bond adds some extra strength and

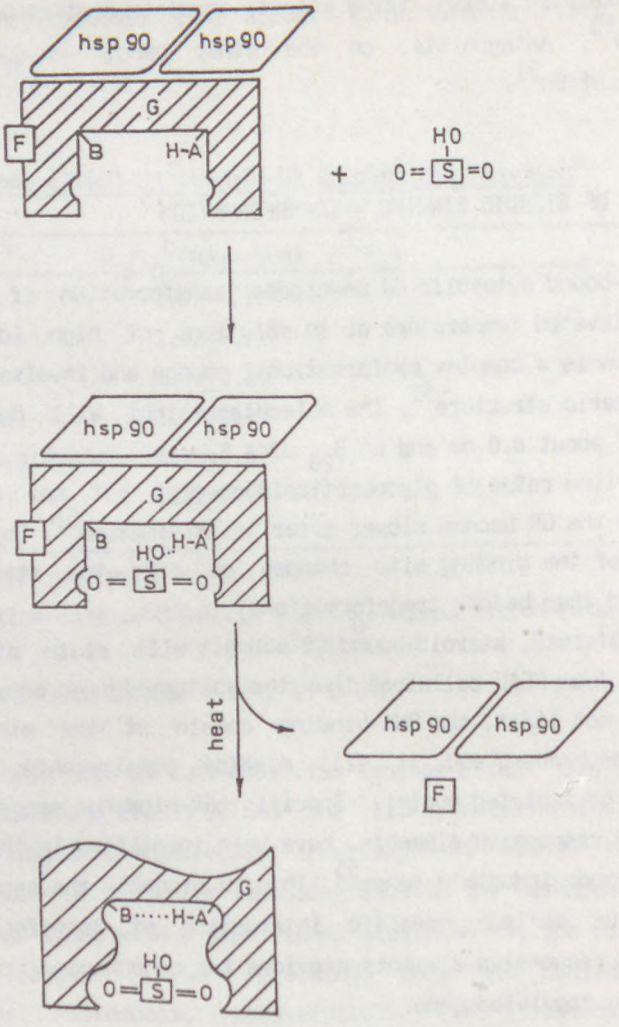


Figure 3. Formation of the transformed GR-agonist complex. The 11β-hydroxyl substituent is explicitly shown on the steroid ligand.

specificity to it. Upon incubation at around room temperature, rearrangement of the bonds holding the steroid takes place. As a result, dissociation rate of the steroid decreases. Furthermore, this

conformational change spreads over the structure and makes GR to shed the two hsp 90 subunits. It is important in this respect, that hsp 90 has recently been shown to interact with the steroid binding domain[59], more precisely, with a subregion near its amino terminal part[60]. Transformation is already detectable at this point. However, for the full development of transformation a further change in the structure of GR (not shown in the Figure) is probably needed in order for the monomeric GR to obtain increased DNA-binding capacity[61]. Factor F has not yet been unequivocally identified. It is possible, however, that factor F is the first to leave the complex when incubated in high salt medium. GR complexes of the noncovalent antagonist RU 38486 and of the covalent antagonist dexamethasone mesylate (cf. Fig. 2) can undergo transformation, too, i.e. the subunits of the nontransformed complex get separated upon prolonged incubation at elevated temperature. This is, however, much slower in both cases than in case of GR-agonist complexes[62]. RU 38486 obviously cannot form H-bond near the C-11 atom. The covalent bond between Cys-656 and the 21-mesylate group may prevent correct positioning of the steroid within the binding cavity, preventing, in turn, the formation of the pivotal H-bond.

CONCLUSIONS

Concentrated efforts of synthetic chemists, X-ray crystallographers, biochemists and pharmacologists have shown many details of the mechanism of glucocorticoid hormone action. A hydrogen bond forming substituent on the chiral C-11 atom of the steroid appears to play a pivotal role. The other pillar of the bridge, the one that belongs to the receptor protein, is still unknown. Similarly, the interactions of the amino acid side chains constituting the steroid binding cavity with the rest of the GR molecule, as well as dynamic changes in the network of noncovalent forces stabilizing the nontransformed GR complex remain to be identified.

Acknowledgment

This work has been supported by OTKA (Grant No. 180/1986).

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

STERIC COURSE AND MECHANISM OF ENZYMIC REACTIONS

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INTRODUCTION

All biopolymers and most of their building blocks are chiral. Furthermore they are produced by living cells in high enantiomeric purity. If we want to understand and artificially influence living systems (e.g. by applying drugs), we must first learn how such a high enantioselectivity is achieved. It has been known for a long time that in non-enzymic reactions the stereochemical integrity of molecules may be either lost or retained. The latter is true for concerted reactions, whereas the occurrence of metastable intermediates may lead to more than one stereoisomer. The ground for the loss of stereochemical integrity lies in the geometrical instability or in the symmetry of intermediates. Although most enzymic reactions are non-concerted, their intermediates are controlled by specific binding at the active site. In addition to geometrical or conformational control, symmetrical intermediates are subject to chiral recognition by the enzyme. Chiral recognition involves not only enantiomers but also enantiotopic groups or faces in an achiral molecule.

Paradoxically, the almost perfect stereospecificity of enzymic reactions makes the linkage between steric course and mechanism more difficult. Whereas the steric course of non-enzymic reactions gives a clue to the geometry of the transition state, e.g. Walden inversion points to a pentacoordinate transition state, no such simple conclusion can be drawn from

the steric course alone of enzymic substitution reactions. Here additional evidence for concertedness is also required. In this article I shall discuss a few principles and selected examples, in which enzymic stereospecificity and mechanism of action could be linked.

CHIRAL RECOGNITION OF ENANTIOTOPIC GROUPS AND FACES

Many achiral molecules, among them enzyme substrates have enantiotopic atoms, groups of atoms or faces of trigonal centres. Examples are shown in Figure 1.

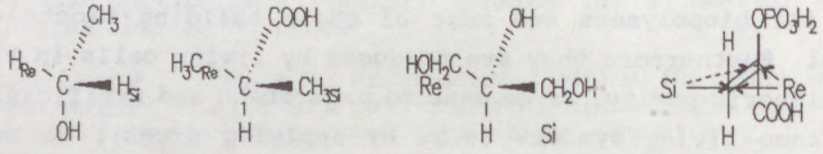

Fig. 1

For the specification of reflection-related as well as of diastereotopic groups or faces the Re/Si system suggested by Prelog and Helmchen[1] will be used throughout this article. We already discussed the properties and advantages of this system at an earlier occasion[2] and compared it with the one suggested by Hanson[3] . The essential feature of the Prelog-Helmchen system is the equal treatment of groups and faces. The molecule will be divided by the reflection symmetry element (e.g. plane of symmetry) into two enantiotopic halves. The three ligands in each half will be ordered according to the Cahn-Ingold-Prelog (CIP) priority rules and their orientation (clockwise = Re or anti-clockwise = Si) determined from the site remote to the symmetry element (Fig. 2).

If the alphabetic order corresponds to the CIP rank order, the two chemically identical ligands b and b' will be specified as indicated in Fig. 2.

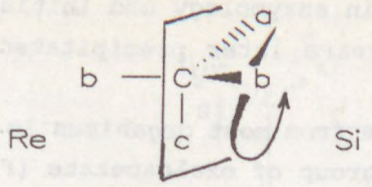

Fig. 2

Of course, enzymes also differentiate between diastereotopic groups and faces within a molecule but this property is shared also by achiral reagents. In some cases, however, enzymes can treat diastereotopic groups and faces as if they were enantiotopic, i.e. the influence of near or remote chiral centres is either ignored or it is restricted to the reaction velocity. Thus many oxido-reductases reduce enantiomeric ketones or aldehydes to a separable mixture of enantiomerically pure diastereomers[4-7].

STERIC COURSE OF ENZYMIC ALDOL-TYPE CONDENSATIONS

The best-known and most remarkable enzyme catalysing an aldol-type condensation is the citrate synthase. It catalyses the irreversible formation of citrate from acetyl-CoA and oxaloacetate (Eq. 1).

$$CH_3COSCoA + HO_2CCOCH_2CO_2H \longrightarrow HO_2CCH_2-\underset{\displaystyle COOH}{\overset{\displaystyle OH}{\overset{|}{\underset{|}{C}}}}-CH_2COOH + CoA \quad (1)$$

Although, except for the CoA portion, neither the educts nor the product are chiral, the reaction is very interesting from the stereochemical point of view. Citrate was the compound for which chiral recognition of enantiotopic groups was postulated for the first time. In 1948 Ogston suggested that aconitase is able to differentiate between the two enantiotopic carboxymethyl groups of citrate and this type of enantioselectivity is shared by enzymes in general[8] . The so-called Ogston

rule is a milestone in enzymology and initiated an extensive research that many years later precipitated in a number of monographs[2,9,10].

Citrate synthase from most organisms is specific for the Si-face of the keto group of oxaloacetate (Fig. 3).

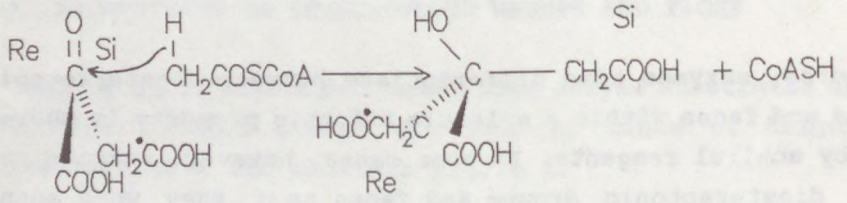

Fig. 3

Labelling the β-carboxyl group of oxaloacetate leads then to an enantiospecifically labelled citrate (Fig. 3, C = ^{14}C). When the latter is further reacted with aconitase, elimination of water takes place in such a way that the label is exclusively in the carboxyl group conjugated to the double bond. Thus aconitase is specific for the $CH_2CO_2H_{Re}$ branch. It is noteworthy that citrate synthase from certain microorganisms, e.g. *Clostridium kluyveri* [11], is specific for the Re face of the keto group of oxaloacetate. Consequently the two condensing enzymes are called Si- and Re-citrate synthases, respectively.

Another question concerns the steric course of the substitution at the methyl group of acetyl-CoA. Will one of the H-atoms be replaced by the carbonyl-C-atom of oxaloacetate with retention or inversion? The answer to this question required the synthesis of chiral, enantiomerically pure [^2H,^3H]acetyl-CoA samples of known absolute configuration. Their first syntheses were described some 20 years ago[12,13]. Enzymic condensation thereof with oxaloacetate furnished citrate samples, in which a portion of the molecules were labelled both with deuterium and tritium in the $CH_2CO_2H_{Si}$-branch (Fig. 4).

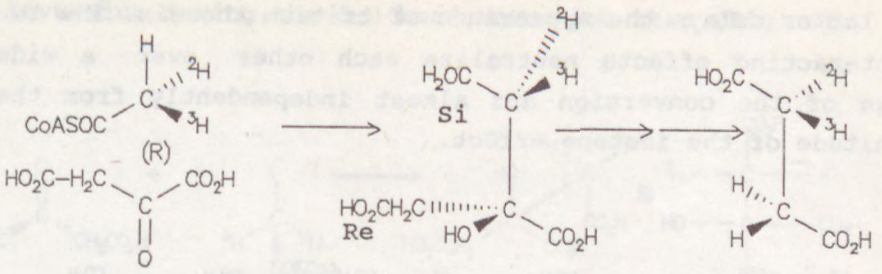

Fig. 4

Determination of the absolute configuration of the doubly labelled citrate molecules required considerable sophistication, but the problem could be solved in three different ways[14-16]. Only one of these will be described here[15]. The citrate samples obtained from (R)- and (S)-[^{2}H,^{3}H]acetate, respectively, were reacted further with enzymes of the citrate cycle, like aconitase and isocitrate dehydrogenase. The resulting α-ketoglutarate was oxidised with H_2O_2 to yield succinate still containing the intact doubly labelled methylene group. Former research revealed that the next enzyme of the cycle, succinate dehydrogenase, oxidises succinate to fumarate by abstracting an H_{Re}- and an H_{Si}-atom from adjacent positions[17]. Moreover the kinetic deuterium isotope effects (k_H/k_{2_H}) differ considerably for the abstraction of the two enantiotopic H atoms ($k_{H_{Re}}/k_{2_H} = 5.3$; $k_{H_{Si}}/k_{2_H} = 1.35$)[18]. Application of the Swain equation [$k_H/k_{3_H} = (k_H/k_{2_H})^{1.44}$][19] allows also calculation of the kinetic tritium isotope effects ($k_{H_{Re}}/k_{3_H} \approx 12$; $k_{H_{Si}}/k_{3_H} \approx 1.5$). When samples of (R)- and (S)-acetates were processed through the reaction sequence leading to fumarate, 31 % and 49 % of the original radioactivity was retained, respectively. Succinate samples containing only tritium lose approximately half of their radioactivity upon enzymic oxidation to fumarate, irrespective of their absolute configuration[15]. This results from the interplay of the intramolecular competition between the two abstractable hydrogen pairs and of the intermolecular competition between tritiated and unlabelled molecules. The former effect tends to retain more than half of the tritium in fumarate (isotope effect),

the latter delays the appearance of tritium therein. The two counteracting effects neutralize each other over a wide range of the conversion and almost independently from the magnitude of the isotope effect.

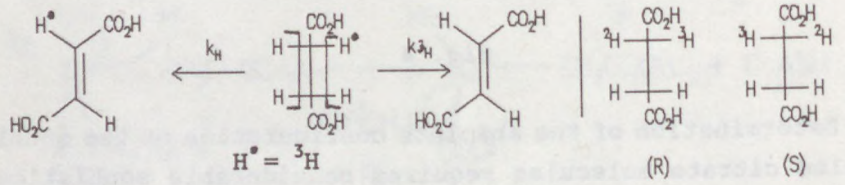

Fig. 5

The situation is quite different, however, in the case of doubly labelled [^{2}H,^{3}H]succinate species (Fig. 5). In the (R)-enantiomer deuterium is in the pro-S position and its abstraction is associated only with a small isotope effect ($k_{H_{Si}}/k_{2_H}$ = 1.35). The behaviour of this species will differ only insignificantly from that of the tritiated ones. In contrast, in (S)-[^{2}H,^{3}H]succinate deuterium abstraction is difficult ($k_{H_{Re}}/k_{2_H}$ = 5.3) while tritium abstraction is easy ($k_{H_{Si}}/k_{3_H} \approx$ 1.5). Here intra- and intermolecular isotope effects are synergetic and lead to an impoverishment of tritium in fumarate. Thus the experimentally found 31 % tritium retention identifies (S)-[^{2}H,^{3}H]succinate as the major tritiated species in the sample derived from (R)-[^{2}H,^{3}H]acetate (Fig. 5). This means that the protium is replaced by the carbonyl-C-atom of oxaloacetate with inversion at the labelled carbon center. This rules out a concerted mechanism for the substitution.

Since fluoroacetyl-CoA is a good substrate of citrate synthase, the steric course of the substitution could be investigated also in a different way. Only one isotope substitution is required to render the fluoromethyl group chiral. (2R)-[2-$^2H_1$]fluoroacetyl-CoA was prepared and treated with citrate synthase to form (2R,3R)-[2-^{2}H]fluorocitrate (Fig. 6). Almost complete deuterium retention in the product, shown both by ^{1}H- and ^{19}F-NMR spectroscopy[20,21] provided further evidence

for inversion during the C-C bond formation step (Fig. 6).

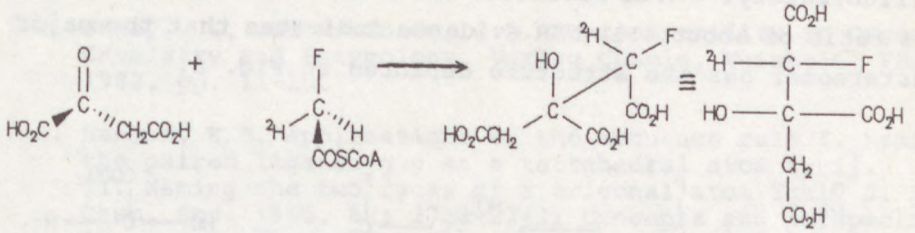

Fig. 6

Since the condensation yields (2R,3R)fluorocitrate in 97-98 % diastereomeric purity[22], citrate synthase stereoselectively abstracts the 2-H_{si}-atom of fluoroacetyl-CoA. In other words, the conformation of the fluoromethyl group is fixed at the active site so that only H_{si} is in the reach of the proton abstracting basic group. In the case of the normal substrate, acetyl-CoA, a slow rotation of the methyl group must exist at the active site as revealed by an intramolecular isotope discrimination ($k_H/k_{^2H} \approx 2$). A comparison with the related malate synthase reaction is very instructive. Instead of oxaloacetate the carbonyl component is here glyoxylate (Fig.7).

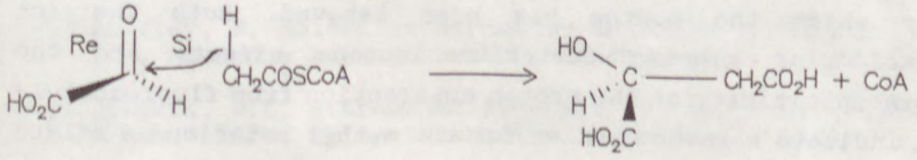

Fig. 7

Malate synthase is also Si-face specific for glyoxylate, and the formal substitution at the methyl group occurs also with inversion. The rotational barrier for the methyl group at the active site seems to be lower, since the intramolecular discrimination of deuterium is higher ($k_H/k_{^2H} = 4$)[23].

This is also supported by the lack of stereoselectivity for the diastereotopic H-atoms of fluoroacetyl-CoA. Starting

from unlabelled substrates two diastereomeric 3-fluoromalates are formed in an approximate ratio of 1:1. With (2R)-[2-$^2H_1$]fluoroacetyl-CoA as substrate one diastereomer predominates in a ratio of about 4:1. NMR evidence indicates that the major diastereomer has the structure depicted in Fig. 8.

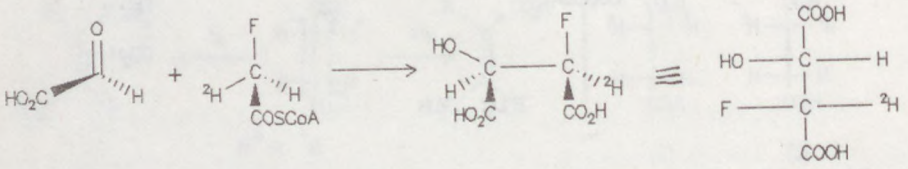

Fig. 8

CONCLUSION

Investigation of the citrate and malate synthase reactions with stereospecifically labelled substrates led to a detailed steric picture of the events at the enzymes' active site. After binding of both substrates, a basic group of the enzyme removes a proton from the methyl group of acetyl-CoA. The resulting enolate adds to the Si-face of the electrophilic carbonyl group of the other substrate on the opposite site from which the proton has been removed. Both the intramolecular kinetic deuterium isotope effects and the stereospecificity of the proton abstraction from fluoroacetyl-CoA indicate a lower barrier for the methyl rotation on malate synthase and a higher one on citrate synthase.

ACKNOWLEDGMENT

It is a pleasure to thank my colleagues who contributed to the work carried out in collaboration with the author. The work in the Karlsruhe laboratory was generously supported by the *Deutsche Forschungsgemeinschaft* and the *Fonds der Chemischen Industrie*.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders of Chiral Molecules (ed. M.Simonyi)

QUANTITATIVE INTERPRETATION OF STEREOSELECTIVITY IN BIOCHEMICAL REACTIONS

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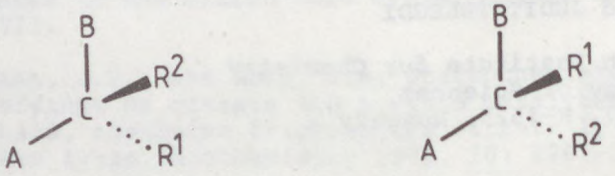
Linear free energy relationships for quantitative characterization of the reactivity of chemically congeneric series of compounds in organic, bio-organic and biochemical transformations have become generally accepted during the last four decades. Such a description of biochemical and pharmacological processes is equivalent to the quantitative representation of substrate or pharmacon specificity in given series of compounds. The method is generally known as quantitative structure-activity relationship (QSAR). Among the different parameters of QSAR the steric effect has an important, in many cases decisive role. The influence of steric effects on the transformations of congeneric series of compounds has been expressed by different forms of steric substituent constants (E_S, E'_S, E^C_S, M_W, M_R, μ, Verloop parameters [1,2]). These constants proved useful to describe the relative reactivity (biological activity) of pairs of compounds having similar structure, with the exception of enantiomers. The present paper is to demonstrate that the problem of enantioselective reactivity (substrate stereospecificity) can be approached by an analysis of the steric properties of chiral substituents.

Theoretical basis of the concept

The idea of "chiral substituent" is defined in centrally chiral molecules as a set of different atoms or groups and the attached center of asymmetry. The type of substituent $-CR^1R^2A$

exists in enantiomeric forms: $(-CR^1R^2A)_r$ and $(-CR^1R^2A)_s$. When these chiral groups are bound to the same molecular moiety B (different from any of R^1, R^2 and A), we get enantiomeric compounds:



As all structural elements are identical in these two molecules, the different reactivity of the enantiomers arises from different steric effects of the chiral substituents under the given (asymmetric) reaction conditions. At this point the steric constants summarized above become quite useless, because the nature of these constants does not allow us to make any distinction between the steric effects of $(-CR^1R^2A)_s$ and $(-CR^1R^2A)_r$ substituents.

As follows, we have to search for other parameters to describe enantioselectivity quantitatively. An empirical approach is given by Ruch and Ugi[3]. Our starting point was the analysis of the shape of the substituent determined by its localization in the course of chemical or biochemical transformation.

The effect of steric requirement on reactivity depends on the shape of the substituent in the rate-determining step of the process. Konwledge on that shape gives a possibility for the explanation of the steric effect. The main problem in the reaction of small molecules in solution is the unknown shape of substituents. The analysis of these factors is much simpler for reactions of biopolymers, since both the direction of the attack and the position of the moieties of the reacting molecules are fixed by the macromolecule. This idea served as the starting point of our consideration.

In Fig. 1 the plane of the paper represents the surface of the macromolecule or the edge of its cavity termed in enzyme chemistry as "pocket". This part of the macromolecule contains A' and B' groups capable of binding the groups A and

B of the compound, as shown in the Figure. In this way a cross-linkage is formed; A...A′ and B...B′ attachments may be covalent or hydrogen bonds, ionic or hydrophobic interactions. Consequently, R^1 and R^2 substituents of the chiral carbon atom are necessarily oriented. These orientations are denoted by μ and τ, respectively. Since μ does not exert substantial steric effect, the reactivity should mainly depend on the steric hindrance caused by the substituent which occupies the τ position[4,5].

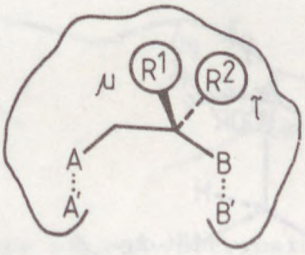


Figure 1. Localization of a small molecule on the surface of a macromolecule. Position τ is inside of the cavity.

In case of enzyme-catalyzed reactions

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

both the complex formation between the biopolymer and substrate (ES-complex) and the subsequent chemical transformations are influenced by the "orientational steric effect" of the τ substituent.

Orientational steric substituent effect in enzyme-catalyzed reactions

One of the most widely studied enzymatic reactions is the ester hydrolysis catalyzed by α-chymotrypsin. The rate of hydrolysis of esters containing an aryl group in β-position is by 1-2 orders of magnitude higher than that of those containing aliphatic substituents. The reaction is highly stereospecific. The presence of an N-acyl group in α-position increases the rate of hydrolysis and the stereoselectivity.

In their fundamental publication Hein and Niemann[6] pointed out three oriented positions for the substrate in the enzyme-substrate complex. Accordingly, ρ^3 is the reaction center, ρ^2 is the most essential binding site for the β-aryl group, and ρ^1 is the binding site of the N-acyl group. Compounds of L-configuration are very good substrates, while enantiomeric compounds of D-configuration are inhibitors, in support of the Niemann assumption. Binding constants of pairs of L- and D-compounds are similar.

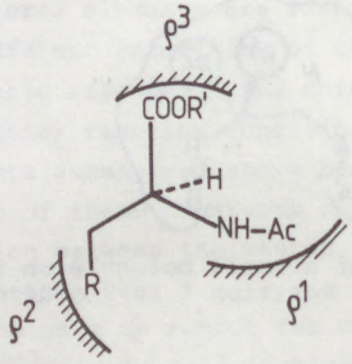


Figure 2. Three oriented positions of a substrate in the enzyme-substrate complex of α-chymotrypsin.

The reaction mechanism was first elucidated by Sturtevant[7] and subsequently described in detail by several authors[8,9], as illustrated below.

$$E + R\text{-}COOR' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.....R\text{-}COOR' \xrightarrow{k_2} E\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}R + R'\text{-}OH \xrightarrow{k_3} E + R\text{-}COOH$$

If $R = p\text{-}C_6H_4\text{-}NO_2$, then $k_2 >> k_3$.

In case of active esters, e.g. p-nitrophenyl ester, the k_2 value is much higher then k_3, hence the rate-determining step is the decomposition of the acyl-enzyme. Both steps take place with the participation of a heterocyclic nitrogen atom of histidine-57. The imidazole ring activates the OH group of

serine in position-195 in the second step, and promotes the attack of water molecule in the third step. The catalytic effect of imidazole has been studied in separate investigations. The steric sturcture of the active site of α-chymotrypsin was determined by X-ray diffraction[10].

The active site of the enzyme is shown schematically in Fig. 3 illustrating the positions of the Niemann binding site ρ^2, serine-195, and the participating histidine-57.

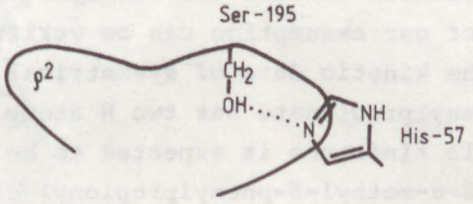


Figure 3. The cavity of α-chymotrypsin.

Figure 4 shows α-chymotrypsin acylated by an α-substituted β-phenylpropionic acid. The β-phenyl group is bound to the ρ^2 site and serine-195 is acylated. Owing to the cross-linkage formed, the R^2 substituent faces the inside of the biopolymer, while the R^1 substituent is oriented towards the solvent.

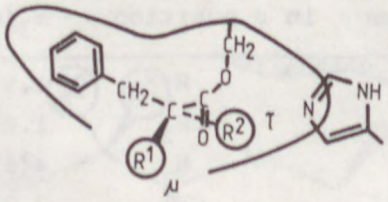


Figure 4. Acylated α-chymotrypsin.

The μ position exerts no substantial steric effect, while the R^2 group, depending on its size, hinders the attack of water molecule assisted by the heterocyclic base. The substituents R^1 and R^2 are in interchanged positions in substrates of R and S configurations, localized by cross-linkage. In chiral substrates R^1 and R^2 groups exert different degrees

of steric hindrance, and stereospecificity is due to this difference.

In the acyl-enzyme involving S-α-methyl-β-phenylpropionate, the H atom is oriented towards the catalytic site of the enzyme, while in the case of R-α-methyl-β-phenylpropionyl-chymotrypsin the methyl group is located in this position. The difference in hydrolytic rates of the enantiomers is in accord with the difference in steric hindrance between the two substituents oriented towards the catalytic site (Table 1).

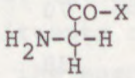
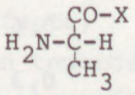
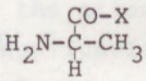
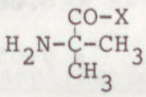
The validity of our assumption can be verified by consideration of the kinetic data of symmetrical substrates. Unsubstituted β-phenylpropionate has two H atoms in α-position, consequently, steric hindrance is expected to be the same as in case of the S-α-methyl-β-phenylpropionyl derivative. In the acyl-enzyme formed by the α,α-dimethyl-β-phenylpropionyl analog, the nucleophilic attack on the ester carbonyl group is hindered by a methyl group as in the case of R-α-methyl-β-phenylpropionyl chymotrypsin, hence their reactivities are expected to be similar. These assumptions are fairly well supported by experimental data in Table 1.

Table 1. Deacylation constant (k_3) of acylated chymotrypsin

Substituent in τ position	in μ position	$k_3(s^{-1})$
H	H	$1.9 \cdot 10^{-1}$
H	CH_3	$1.2 \cdot 10^{-1}$
CH_3	H	$4.1 \cdot 10^{-3}$
CH_3	CH_3	$3.2 \cdot 10^{-3}$

Another example is the dipeptide hydrolysis catalyzed by aminoacylase. According to Greenstein[11], this enzymatic transformation is highly stereospecific. The rate-determining step is the decomposition of the ES complex[12], therefore k_{cat}/K_M values characterize the reactivities of the compounds. Table 2 presents data on alanyl-L-norvaline derivatives.

Table 2. Reactivity constants of alanyl-L-norvaline and related dipeptides in aminoacylase-catalyzed hydrolysis

Substrate (X = L-norvaline)	Configuration	k_{cat}/K_M ($mM^{-1}s^{-1}$)
	-	55
	L	70
	D	0.13
	-	0.09

The ratio of the rate constants of L-L and D-L dipeptides is higher than 500. The k_{cat}/K_M value of L-alanyl-L-norvaline is similar to that of glycyl-L-norvaline, while the rate of hydrolysis of the D-alanyl derivative corresponds to that of the α-amino-isobutyryl compound. In the first two cases steric hindrance is due to the hydrogen atom, and in the latter two cases it may be attributed to the methyl group.

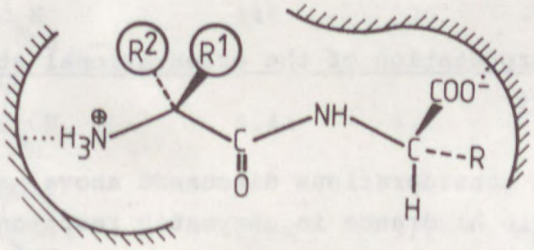


Figure 5. Dipeptide bound to the active site of aminoacylase.

The conformation of the substrate in the enzyme-substrate complex is mainly fixed by interactions with the C-terminal carboxylate moiety and the N-terminal amino group[13] (Fig. 5).

Table 3. Reactivity constants of aminoacyl-L-norvaline dipeptides in aminoacylase-catalyzed hydrolysis

R^1	R^2	Configuration of substrate	k_{cat}/K_M ($mM^{-1}s^{-1}$)
CH_3	H	LL	70
$n\text{-}C_3H_7$	H	LL	38
$i\text{-}C_3H_7$	H	LL	0.6
C_6H_5	H	LL	0.3
H	CH_3	DL	0.13
H	$n\text{-}C_3H_7$	DL	0.06
H	$i\text{-}C_3H_7$	DL	< 0.05
H	C_6H_5	DL	< 0.05

Kinetic data of the hydrolysis of further stereoisomer dipeptides are collected in Table 3. As seen from the data in Table 3, if the R^1 substituent branches in α position, the reactivity decreases significantly, indicating that a bulky group - even in the favorable position - can inhibit the enzymatic reaction as a consequence of its overlap with the τ position.

Quantitative interpretation of the orientational steric substituent effect

Based on the considerations discussed above, our conclusion is that steric hindrance in enzymatic reactions is caused by the substituents which occupy the τ position. For single atoms or groups of spherical symmetry, the steric requirement can be expressed by the van der Waals radii[14] of the substituents, $r_v(\tau)$[15]. This relationship is described by eq. (1):

$$\log k = \sigma^* \rho^* + \kappa\, r_v(\tau) + c \qquad (1)$$

where κ is the orientation constant, and the $\sigma^*\rho^*$ term is the same as in the Taft-Ingold formula for general steric effects (eq. 2):

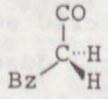
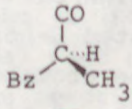
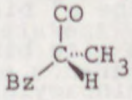
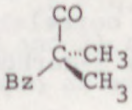
$$\log \frac{k}{k_{CH_3}} = \sigma^*\rho^* + \delta E_S \qquad (2)$$

As obvious, the E_S constant in eq. (2) cannot be distinguished for substituents of opposite chirality. While δ in eq. (2) expresses the susceptibility of the reaction series towards steric effects, κ depends mainly on the orientation of the τ position towards the catalytic site of the enzyme, thereby hindering the attack on the reaction center of the substrate.

The application of eq. (1) for the hydrolysis of acyl-α-chymotrypsin is presented in Table 4.

Table 4. Application of eq. (1) for substrates of chymotrypsin

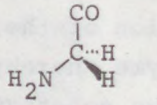
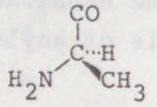
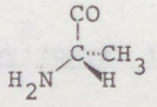
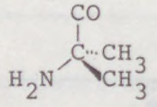
$$\log k = \sigma^*\rho^* \underset{(\pm 0,08)}{- 1.98}\, r_v(\tau) \underset{(\pm 0.09)}{+ 1.59}$$

Acyl group Bz = $C_6H_5-CH_2$	$10^3 \cdot k_{cat}$ (s^{-1}) calculated	observed	σ^*	$r_v(\tau)$
	245	190	+0.08	1.20
	148	120	-0.02	1.20
	4.44	4.1	-0.02	1.97
	2.68	3.2	-0.12	1.97

For the hydrolysis of dipeptides catalyzed by aminoacylase (Table 5) the κ value was -3.46, which shows that in this reaction the τ position exerts a higher steric effect than in the case of chymotrypsin.

Table 5. Application of eq. (1) for dipeptide substrates (acyl-L-norvalines) in aminoacylase-catalyzed hydrolysis

$$\log k = \sigma^*\rho^* - 3.46 \cdot r_v(\tau) + 1.35$$
$$(\pm 0.15) \qquad (\pm 0.10)$$

Acyl group	k_{cat}/K_M ($mM^{-1}s^{-1}$) calculated	observed	σ^*	$r_v(\tau)$
	68	55	+0.08	1.20
	56	70	-0.02	1.20
	0.12	0.13	-0.02	1.97
	0.10	0.09	-0.12	1.97

Tables 4 and 5 indicate that stereoselectivity can be expressed quantitatively by the orientational steric substituent effect, and eq. (1) is suitable for a general description of substrate specificity. For further application, the enzymatic hydrolysis of aliphatic acid esters and acylated L-norvaline derivatives were studied. In the absence of the aromatic ring it is uncertain which group is localized near the ρ^2 binding site of α-chymotrypsin (cf. Fig. 3), or at the site binding the amino terminal of dipeptides in case of aminoacylase (cf. Fig. 5). Nevertheless, the reactivities can be correlated with one of the van der Waals radii attached to the α-carbon of the acyl moiety.

Since the acetyl group (No. 1) can only orient a hydrogen, and the pivaloyl group (No. 9) only a methyl towards the τ position, these two reactivities essentially determine the straight line in Figure 6. The stereoselectivity of enantiomers (Nos. 3 vs. 8 and Nos. 4 vs. 7) is in accord with the sugges-

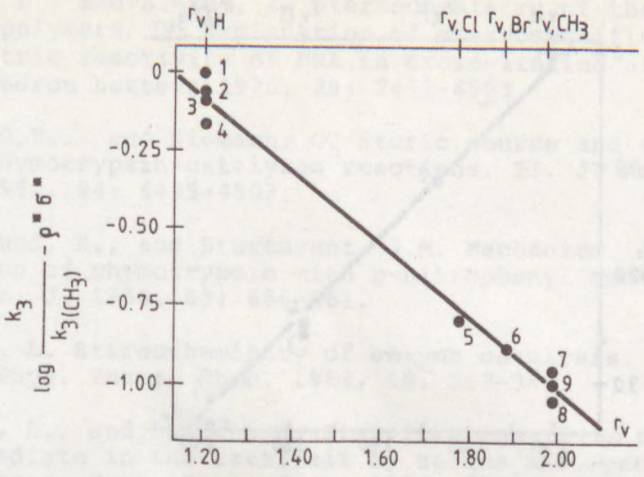


Figure 6. Relationship between the reactivity of acylated chymotrypsin and the van der Waals radius of one of the substituents attached to the α-carbon of the acylating group: 1. CH_3CO; 2. $(CH_3)_2CHCO$; 3. R-$CH_3CH(Cl)CO$; 4. R-$CH_3CH(Br)CO$; 5. Cl_2CHCO; 6. Br_2CHCO; 7. S-$CH_3CH(Br)CO$; 8. S-$CH_3CH(Cl)CO$; 9. $(CH_3)_3CCO$.

tion that R enantiomers place the hydrogen, and S enantiomers direct the methyl group to position τ, while the halogen atom occupies the ρ^2 site. It is, however, less comforting that the reactivities of dihaloacetyl derivatives can be correlated with the radii of the halogens (Nos. 5 and 6), while the isobutyl (No. 2) fits best the radius of hydrogen, an empirical fact as yet not explained.

The reactivities in the aminoacylase-catalysed hydrolysis can consistently be interpreted (Fig. 7) by the assumption that the non-hindering μ position is occupied by a hydrogen atom (R^1 in Fig.5). Accordingly, dihaloacetyl derivatives (Nos. 2 and 3) are correlated with the van der Waals radii of halogen atoms, and the hydrolysis rate of isobutyryl-L-norvaline fits the radius of methyl group (No. 6). The pair of enantiomers, Nos. 4 and 5, then orient the appropriate group towards position τ in accord with the correlation in Figure 7.

Although the numerical values of κ are different in Figures 6 and 7 from those of Tables 4 and 5, respectively,

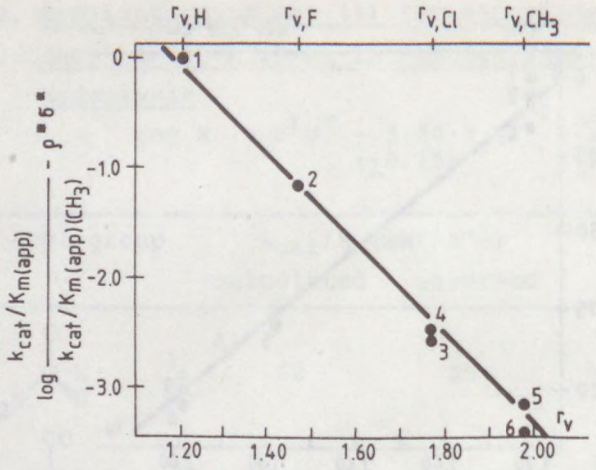


Figure 7. Connection between the reactivity of acylated L-norvaline derivatives and one of the van der Waals radii in the aminoacylase-catalyzed reaction. Acylating groups: 1. CH_3CO; 2. F_2CHCO; 3. Cl_2CHCO; 4. S-$CH_3CH(Cl)CO$; 5.R-$CH_3CH(Cl)CO$; 6. $(CH_3)_2CHCO$.

the difference between the substrate series gives adequate reason for it. However, the aminoacylase-catalysed reactions indicate definitely higher sensitivity to the orientational steric effect in either case.

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Akadémiai Kiadó
Budapest, 1990

OPTICALLY ACTIVE AZAHETEROCYCLES. A CD STUDY

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Some basic principles

Nitrogen-containing heteroaromatic rings briefly called "azaheterocycles" are found in many natural products occurring, in general, as optically active compounds (e.g. nucleosides, alkaloids involving structural building blocks of pyrimidine, pyridine, or indole). Since these heteroaromatic moieties are also characteristic chromophores, this group of compounds can be conveniently investigated by optical spectroscopy. In case of optically active compounds, this structure elucidating technique can be usefully completed by the application of circular dichroism (CD) spectroscopy.

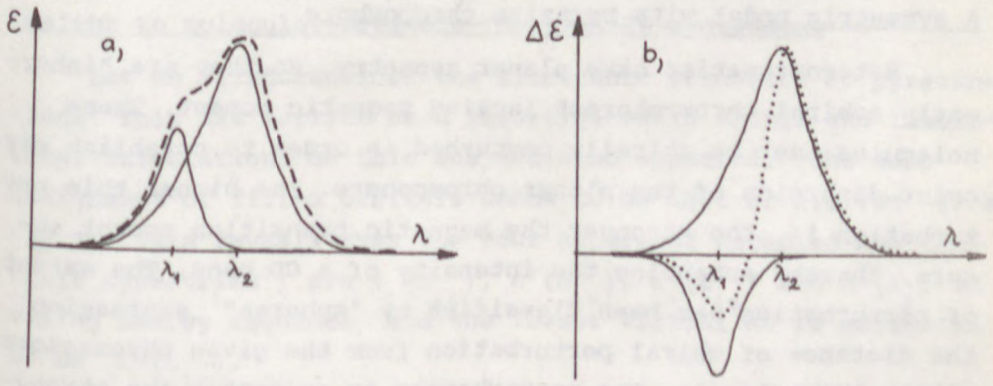


Figure 1. Comparison of detectabilities of two absorptions and Cotton effects at close wavelengths (λ_1 ; λ_2) by UV (a; -----) and CD (b; .....).

The advantage of CD spectra over UV ones is that absorptions very near to each other - overlapping sometimes in the UV or appearing as a hardly detectable "shoulder" - may have opposite signs in the CD and thereby appear well separated. Such an example is shown in Fig. 1: the detectable UV curve appears as a sum of two peaks deriving from absorptions at λ_1 and λ_2 wavelengths and, because of poor resolution, these peaks are not separated. If, however, these two absorptions have opposite signs in CD, the two maxima can distinctly be registered.

Another important point of the joint application of UV and CD spectroscopy is that the conditions of the appearance of a band in the two spectra are different. While in UV, the size of an absorption peak is determined by the dipole strength ($D = \mu^2$), the corresponding band in CD[1] is related to the rotation strength (R) which is the the scalar product of the electric (μ) and magnetic transition moments:

$$R = \vec{\mu}\ \vec{m}\ \cos(\vec{\mu},\vec{m})$$

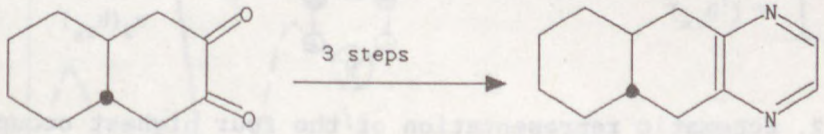
This means that certain transitions giving intense bands in CD ("magnetically allowed transitions") might become "electrically forbidden" (fairly observable) in the UV. In other cases the reverse can be true.

A symmetric model with pyrazine chromophore

Heteroaromatics have planar geometry, so they are "inherently achiral chromophores" lacking magnetic moment. These molecules must be chirally perturbed in order to establish circular dichroism of the planar chromophore. The higher this perturbation is, the stronger the magnetic transition moment occurs, thereby enhancing the intensity of a CD band. The extent of perturbation has been classified by "spheres"[2] expressing the distance of chiral perturbation from the given chromophore within the molecule. The perturbation is evidently the strongest when the chromophore itself becomes chiral by certain geometrical distortions ("first chiral sphere"). Inherently achiral chromophores such as heteroaromatics must, however, be perturbed by a chiral surrounding. One of the most effective modes

of such perturbation appears when the heteroaromatic ring is attached to a chiral skeleton (called the "second chiral sphere"). Further, the magnetic moment of the heteroaromatic system can also be induced by introduction of a chiral substituent (called "third chiral sphere"); in this case, however, the induced moment will be relatively small, and the corresponding CD bands are generally very weak.

Here we deal with the second possibility described above and discuss the spectral properties of octahydronaphto[g]pyrazine (**2**) as model compound constructed from a six-membered heteroaromatic chromophore (pyrazine) fused to the rigid chiral *trans*-decalin skeleton.



1 2

This model compound (**2**) has recently been synthesized[3] by starting from *trans*-decalin-2,3-dione (**1**) *via* a three-step route including also the tedious resolution of one of the racemic intermediates.

Insight to molecular orbitals (MOs). A UV assignment

Let us first consider the electronic structure of pyrazine alone. This has earlier been investigated in detail and theoretical calculations on this subject also appeared.[4] The best assignment of filled orbitals seems to be that of Gleiter[5] (see Fig 2). This reveals that the four uppermost occupied MOs (and their symmetries[6]) are $\pi_2(b_{2g})$, $n^-(b_{1u})$, $\pi_3(b_{1g})$ and $n^+(a_g)$ in rising energy sequence, and the lowest virtual MO is estimated to be $\pi^*(b_{3u})$.

The transition energies expected for pyrazines have also been calculated by several authors[7]. There is agreement with the assignments of the electrically allowed transitions (i.e. I,II, and IV in Fig.2). Evidence for the ($n^- \rightarrow \pi_4^*$) transition (III in Fig.2), on the other hand, is controversial and apart

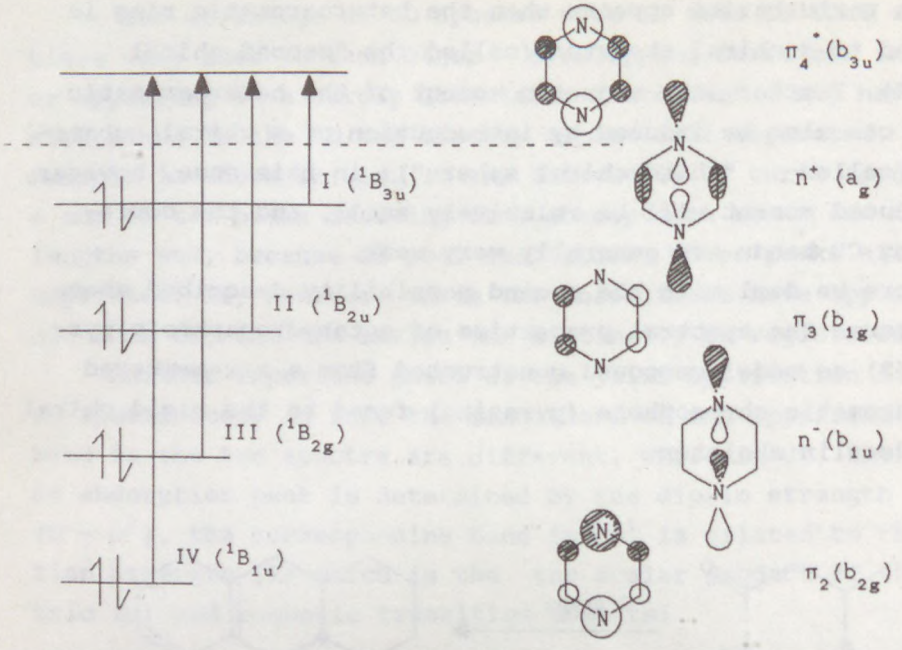


Figure 2. Schematic representation of the four highest occupied MOs and the LUMO of pyrazine. Arrows indicate the possible transitions denoted by Roman numerals. Orbital mixing can be neglected since all one-electron configurations have different irreducible representations. Hatched and open areas stand for the two opposite signs of the electronic functions.

from some ambiguous statements about it in respect of gas or matrix spectra[8], no experimental identification has so far appeared.

In the UV spectrum of $2^{1,3}$ (Fig 3), three main bands can be observed. At the highest wavelength - around 320 nm - a band with a clearly visible fine structure appears which is ascribed to the $n^+ \rightarrow \pi^*$ transition (I in Fig.2). This is allowed[6] and the finding that the fine structure of this band disappears in more polar solvents verifies its $n \rightarrow \pi^*$ nature.

The second band system around 260 nm shows a less pronounced fine structure (called "diffuse system") and is assigned to the $\pi_3 \rightarrow \pi^*$ transition (II in Fig.2).

The third band at still shorter wavelengths (around 195 nm) has been assigned the $\pi_2 \rightarrow \pi_4^*$ transition (IV in Fig.2).

A new finding in the CD spectrum

From the MO diagram shown in Fig. 2 a fourth transition, *viz.* $n^- \rightarrow \pi_4^*$ (III) can also be anticipated. According to its energy[7], this transition would appear at a wavelength between 200 and 300 nm. It is electrically forbidden[7], however, and can not be localized in the UV spectrum in solution. Since it is, however, magnetically allowed, its appearance in the CD spectrum can be expected.

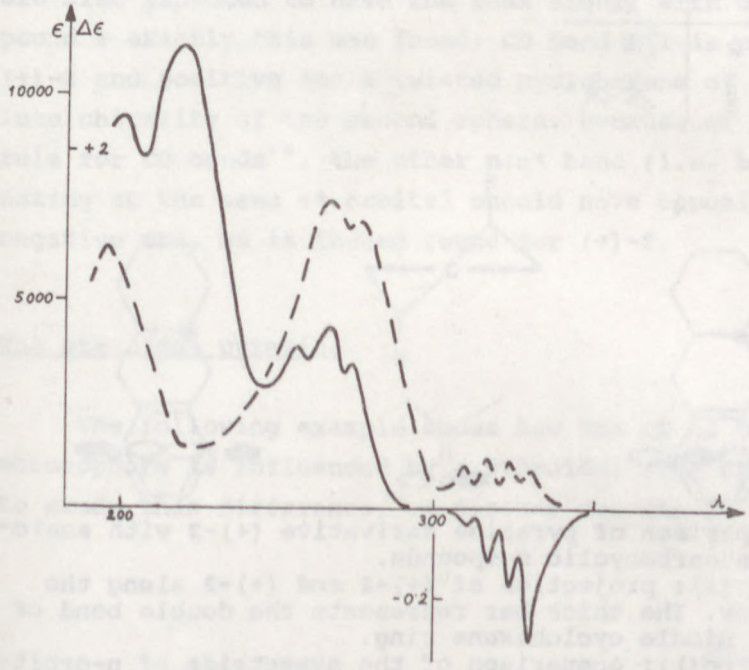


Figure 3. UV (- - - - - -) and CD (————) spectra of (+)-2 (in isooctane, 25°).

In Fig. 3 the CD spectrum of (+)-2[1,3] is also depicted together with the UV curve. Interestingly enough, this comparison shows that the CD curve has four bands: three of them correspond to the analoguous UV bands, whereas a fourth one in the spectrum appears at a wavelength (230 nm) where the UV spectrum has just a minimum. Obviously, this band corresponds to an electrically dipole forbidden but magnetically allowed transition and can, therefore, be assigned to the above mentioned

and by UV not yet detected $n^- \rightarrow \pi_4^*$ transition (III in Fig.2).

As Fig. 3 shows, CD band I has negative sign, whereas the other bands are positive. This difference in sign can be interpreted on the basis of the following symmetry arguments.

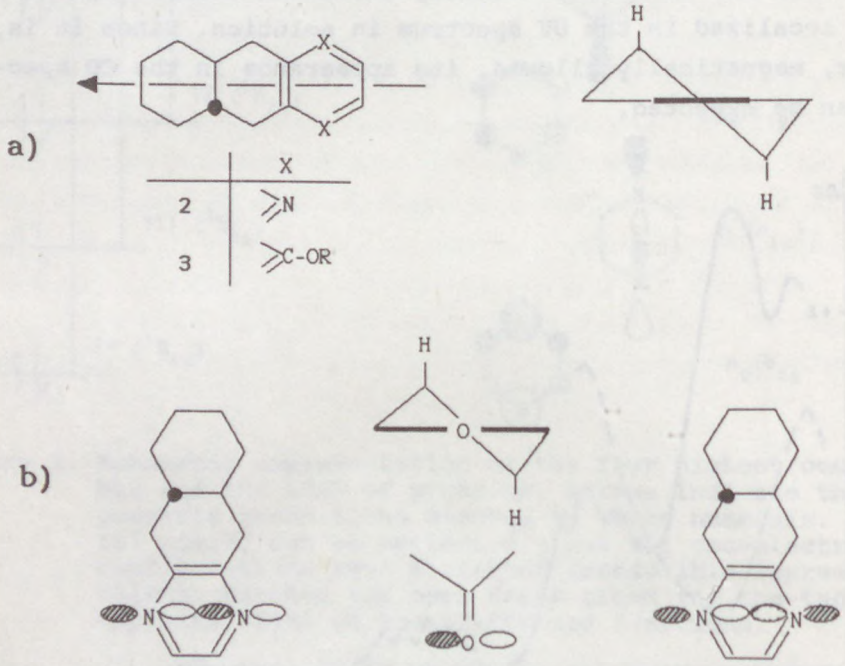


Figure 4. Comparison of pyrazine derivative (+)-**2** with analogous carbocyclic compounds.
Top (a): projection of (+)-**2** and (+)-**3** along the arrow. The thick bar represents the double bond of the middle cyclohexene ring.
Bottom(b): comparison of the symmetries of n-orbitals of (+)-**2** with those of cyclohexanone. Left: $n^-(b_2)$-orbital of (+)-**2**. Middle: $n(b_2)$-orbital of cyclohexanone (projection along the oxygen towards C for a twisted alkanone (above)). Right: $n^+(a_1)$ orbital of (+)-**2**.

Our model pyrazine compound **2** with C_2 symmetry is comparable to a p-disubstituted octahydroantracene (**3**)[9] as shown in Fig. 4. Bands II and IV of a pyrazine correspond to $^1B_{2u}$ and $^1B_{1u}$ bands of the carbocyclic reference compound **3** (benzene chromophore notation used). With the same absolute configuration, these two CD bands are indeed positive in both compounds.

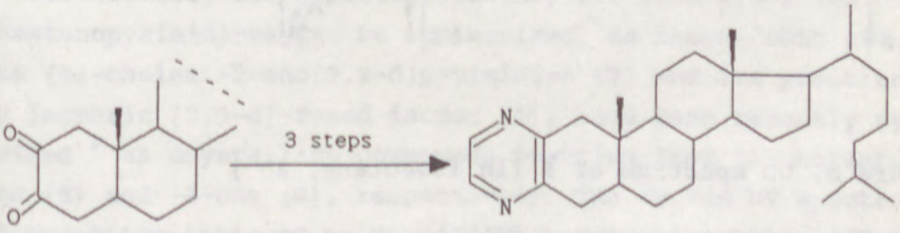
Bands I and III of **2** can, on the other hand, be compared with the $n \rightarrow \pi^*$ transition of a carbonyl chromophore. Actually

the point group of **2** is C_2, so one has to compare its CD with that of a cyclohexanone in the twist configuration with the C=O in the "center" of the twist (Fig. 4).

Since the n orbital of (+)-**2** has the same symmetry as the n-orbital of the carbonyl, and also the π^* orbitals of the two compounds have the same symmetry properties[3], the symmetry of the $n \rightarrow \pi^*$ transitions of the two compounds will obviously be identical. This means that the CDs of $n \rightarrow \pi^*$ transitions of compounds of identical absolute configuration in the second sphere are also expected to have the same signs. With our model compound **2** exactly this was found: CD band III is positive for (+)-**2** and positive for a twisted cyclohexane of identical absolute chirality of the second sphere. Because of the local sum rule for CD bands[10], the other $n \rightarrow \pi^*$ band (i.e. band I) terminating at the same π^* orbital should have opposite sign, i.e. a negative one, as is indeed found for (+)-**2**.

The steroidal pyrazine

The following example shows how the CD of the pyrazine chromophore is influenced by a steroidal ring system. In order to study this difference, we discuss now the CD of 5α-cholest-



4 **5**

2-eno-[2,3-b]-pyrazine[11] (**5**) which is available through a synthesis similar to that shown for **2**, starting from 5α-cholestan-2,3-dione (**4**). The choice of the model compound (**5**) compared to the previous one (**2**) is obviously more favourable from preparative point of view, since the starting material is optically active and no resolution is therefore necessary. There are two

major changes in this structure compared to **2** of C_2 symmetry: the presence of rings C and D with the long alkyl chain attached to C-17, and the presence of an axial methyl group in angular position (C-10).

As shown in Fig. 5, the CD spectrum of **5** also shows four Cotton effects similarly to the tricyclic analogue: the first and third bands correspond to the $n^+ \rightarrow \pi^*$ and $n^- \rightarrow \pi^*$ transitions, respectively, while the second and fourth ones have been assigned to the $\pi_3 \rightarrow \pi^*$ the $\pi_2 \rightarrow \pi^*$ transitions, respectively (cf. Fig. 2). Comparison of this CD spectrum with that of **2** (Fig.3) reveals that the signs and magnitudes of those two CD bands corresponding to $n \rightarrow \pi^*$ transitions remained unchanged, whereas the $\pi_2 \rightarrow \pi^*$ band inverted its sign.

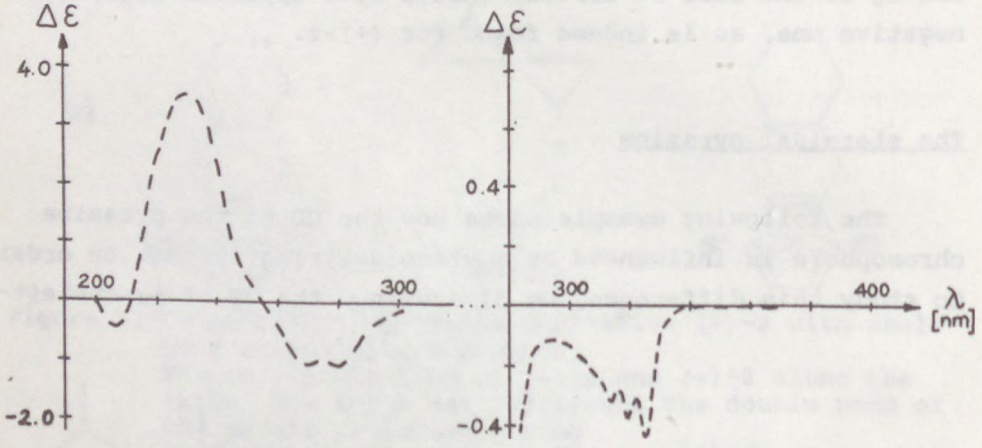
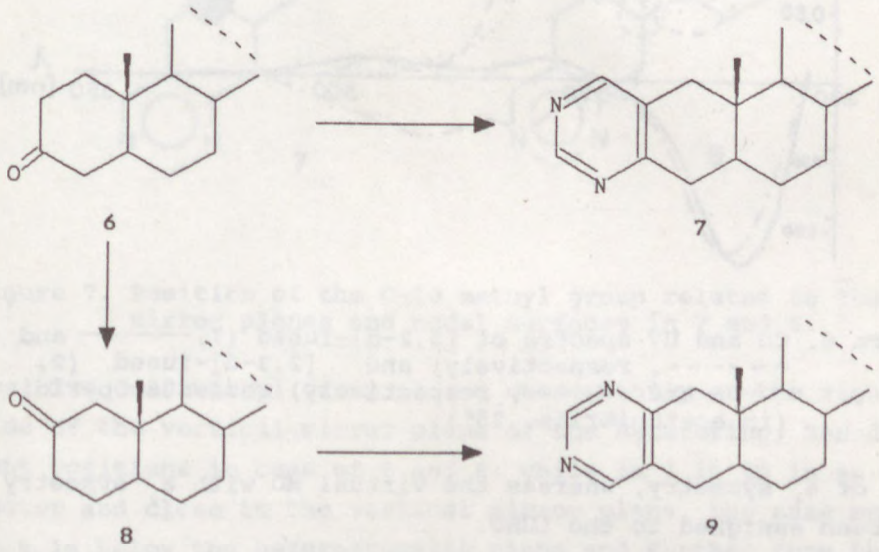


Figure 5. CD spectrum of **5** (in isooctane, 25°)

This difference found with the $\pi \rightarrow \pi^*$ transition is very probably due to the presence of the angular methyl group as has also been found in spectra of analogous furan and thiophene derivatives[12,13]. Thus, we also observe here that, similarly to the much better investigated ketones, $n \rightarrow \pi^*$ transitions can safer be correlated with the absolute configuration of the molecules than $\pi \rightarrow \pi^*$ transitions.

Further step towards lower symmetry: the pyrimidine chromophore

Owing to the relative positions of the two nitrogens to the bond of fusion, the whole cholestenopyrimidine ring system cannot retain the original symmetry even if we neglect the presence of the angular methyl group and rings C and D. Nevertheless, the local symmetry of the pyrimidine chromophore C_{2v} should still be taken into consideration.



6 7

8 9

Furthermore, for symmetry reasons, two such 2,3-fused cholestenopyrimidines can be synthesized, as shown. Both of these (5α-cholest-2-eno[3,2-d]pyrimidine (**7**) and its positionally isomeric [2,3-d]-fused isomer (**9**)) have been recently synthesized[14] as crystalline compounds starting from 5α-cholestan-3-one (**6**) and -2-one (**8**), respectively. The CD and UV spectra of **7** and **9** are depicted in Fig. 6. This comparison shows that in both cases three Cotton effects are detectable around 290, 260 and 220 nm. The first CD band corresponds to a shoulder, the second to a maximum, and the third to a minimum in the UV spectra.

As known from photoelectron spectroscopy (PS) studies[15], the highest MOs of pyrimidine are placed as follows: HOMO is the n^- combination (b_2) of the two lone pairs of the nitrogen

atoms, followed downwards in MO-energy by the highest π-MO of a_2 symmetry (corresponding to π_2 of benzene), the next is the n combination (a_1) of lone pairs and the fourth is another

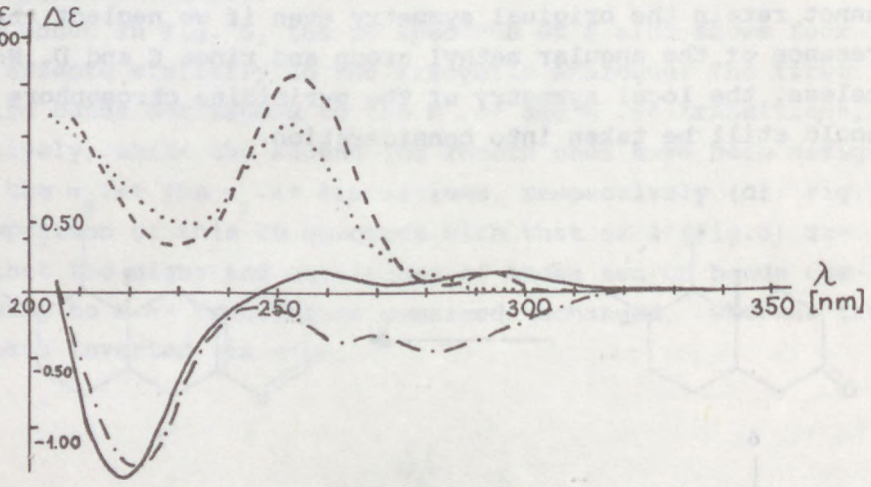


Figure 6. CD and UV spectra of [3,2-d]-fused (**7**, ——— and - - - - - -, respectively) and [2,3-d]-fused (**9**, —·—·— and ·····, respectively) cholestenopyridines (in acetonitrile, 25°).

π-MO of a_2 symmetry, whereas the virtual MO with a_2 symmetry had been assigned to the LUMO.

If we neglect again configurational interaction, this assignment fits nicely the experimental CD and UV bands. The first band at highest wavelength corresponds to the n^--π* transition which has B_1 symmetry and is weakly allowed (because of small overlap). The second is of π-π* parentage with B symmetry and is allowed too, whereas the third is either of n^+-π* or n^--π* parentage which both lead to an A_2 state and will probably be mixed. Such transitions are electrically forbidden but magnetically allowed and one expects therefore a stronger CD band for the 220 nm Cotton effect which is indeed the case. The third Cotton effect of both compounds (**7** and **9**) is negative, whereas the first two are positive for the [3,2-d] fused compound (**7**), but negative for the [2,3-d] isomer (**9**).

The most important difference in the geometry of **7** and **9** in the vicinity of the chromophore is the position of the 10-

methyl group. This can be well demonstrated by an appropriate projection perpendicular to the plain of the heterocyclic ring, as shown in Fig. 7.

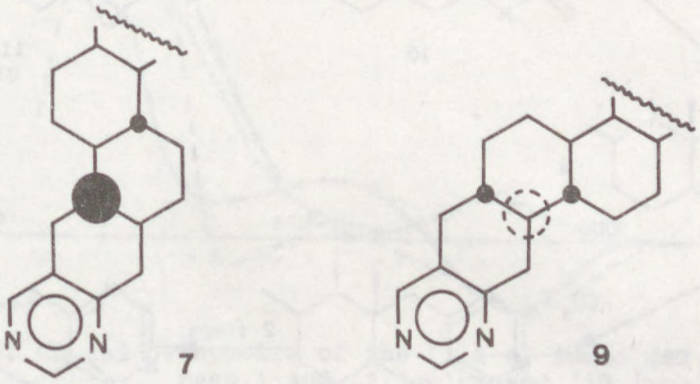
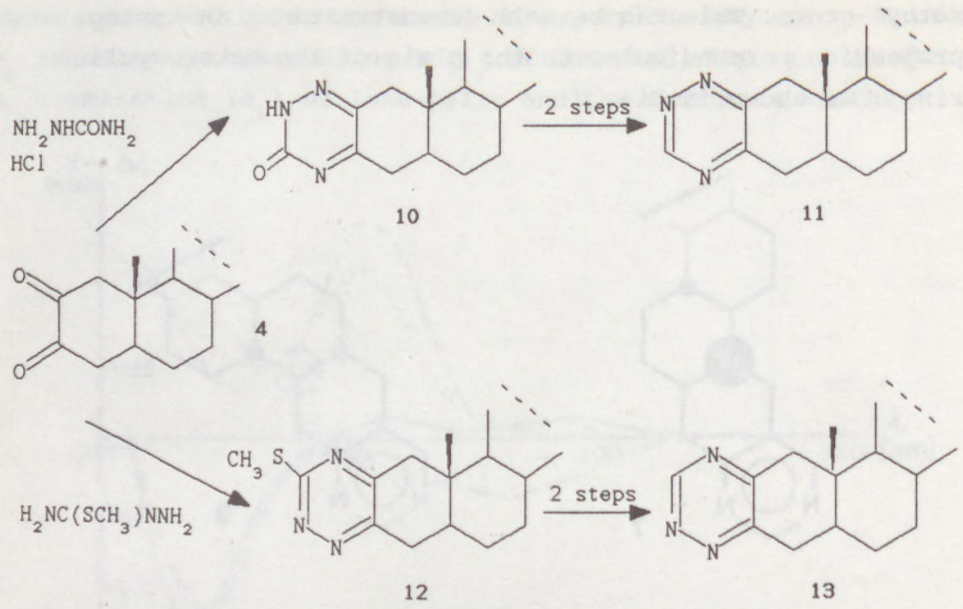


Figure 7. Position of the C-10 methyl group related to the mirror planes and nodal surfaces in **7** and **9**.

The C-10 methyl group placed consequently on the right side of the vertical mirror plane of the heteroring, has different positions in case of **7** and **9**: while in **7** it is in an upper sector and close to the vertical mirror plane, the same methyl in **9** is below the heteroaromatic plane and farther from the vertical one. The position of the C-10 methyl group in compounds **7** and **9** is found, therefore, in different sectors of opposite sign.

A more sophisticated case: 1,2,4-triazine

As a last example for analysis of CDs of azaheteroaromatics, we are going now to interpret the chiroptical properties of 1,2,4-triazines. In this case, the number of nitrogens is increased and the symmetry has even more been destroyed except for the mirror plane of the planar aromatic chromophore. The recently published preparative tool[16] by which two differently fused cholesteno-1,2,4-triazines can be prepared from the same starting compound allows the interesting comparison of these two chiral triazine systems.



As shown above, cholestan-2,3-dione (**4**) when reacted with semicarbazide hydrochloride (acidic medium) affords the oxo derivative of the [3,2-e]-fused cholesteno-1,2,4-triazine **10**, whereas reaction of the same starting compound with S-methyl-isothiosemicarbazide (under basic conditions) gives rise to the derivative of the other possible [2,3-e]-fused system as a methylthio compound (**12**). The functional groups can be removed by routine procedures in both cases to give the two isomeric unsubstituted cholestenotriazines **11** and **13**.

UV and PS studies[15,17] on 1,2,4-triazines reveal that the first absorption at 347 nm is of $n \rightarrow \pi^*$ nature, and the next UV band around 247 nm can be assigned to a $\pi \rightarrow \pi^*$ transition. This is in agreement with the assignment of the HOMO to a nonbonding MO and the next lower one to a π-MO, if configurational interaction would not be important. Below these, two other MOs are placed by PS, so one can expect that at least two $n \rightarrow \pi^*$ bands will appear in the absorption and CD spectra.

CD and UV spectra of **11** and **13** are depicted in Fig. 8. This shows for both isomers at longest wavelengths a negative Cotton effect at 380 to 384 nm with practically the same mag-

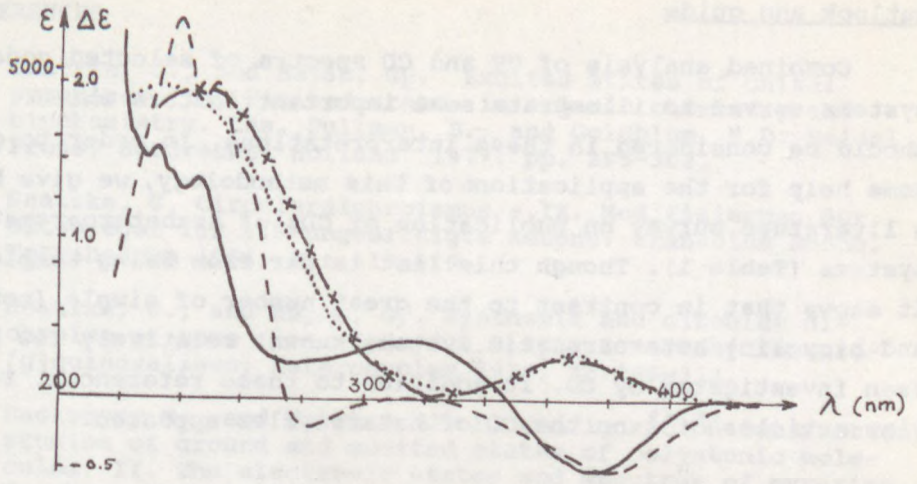


Figure 8. CD and UV spectra of the [3,2-e]-fused (**11**, ——— and ····· , resp.) and [2,3-e]-fused (**13**, – – – – and —×—×— , resp.) cholesteno-1,2,4-triazines (in acetonitrile, 25°).

nitude of -0.35. This band must be associated with the first $n \rightarrow \pi^*$ transition. Between this and the position of the first $\pi \rightarrow \pi^*$ band, however, two more Cotton effects appear: one of these (at about 320 nm) is detectable in the CD spectrum of **11** as a positive, and the other (at 280 nm) in the spectrum of **13** as a negative maximum. Since neither of these Cotton effects coincide with UV maxima, they are probably associated with $n \rightarrow \pi^*$ transitions. In the range of the intense π-π^* UV maximum, both isomers (**11** and **13**) give distinct positive Cotton effects between 220 to 260 nm with fine structure. In case of **11**, this is located on the slope of another, even stronger positive CD, which is not any more fully detectable.

We would like to note that in spite of the relatively low symmetry of these model compounds, CD spectroscopy has effectively complemented the UV and allowed the experimental observation of two additional electronic transitions.[16] Because of the great number of n orbitals and therefore the fairly complicated spectra, no assignments of additional transitions are possible without tedious calculations.

Outlook and guide

Combined analysis of UV and CD spectra of selected model systems served to illustrate some important factors which should be considered in these interpretations. In order to give some help for the applications of this methodology, we give here a literature survey on publication of CDs of azaheteroaromatic systems (Table 1). Though this list is far from being complete, it shows that in contrast to the great number of simple (mono- and bicyclic) heteroaromatic systems known, relatively few have been investigated by CD. In addition to these references, review articles[18,19] on the CD of heterocycles appeared.

Table 1. Collection of some representative literature data on CDs of compounds with heteroaromatic chromophores

Heteroaromatic chromophore	Reference
Benzthiazole	20
Oxadiazole	21
Pyrazine	1, 3, 11
Pyridine	22, 23, 24
Pyridine in alkaloids	25
Pyridine-N-oxide	26
Pyridinium	27
Pyrimidine	14, 28, 29, 30, 31, 32
Pyrrole	33
Tetrazine	21
Triazine	15
Triazole	21

We would like, however, to recall the important fact that the CD is actually determined by the absolute conformation of a molecule and therefore only indirectly by its absolute configuration. Nevertheless, selection of an appropriate model compound and comparison of its CD and UV to those of a related unknown derivative can conveniently lead to recognition of the absolute configuration of the latter, and can thereby serve as an effective aid to structure elucidation.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

THE HOMOCHIRAL VERSUS HETEROCHIRAL PACKING DILEMMA

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One of the simplest and most efficient methods for preparing optically active materials rests on the *spontaneous resolution* which takes place on crystallization of certain racemates. This phenomenon, discovered by Pasteur in 1848, has been, for half a century, the basis of several important industrial optical resolution processes, applied for example to the preparation of compounds such as *L*-glutamic acid, *L*-α-methyldopa, *L*-carbidopa, chloramphenicol and thiamphenicol, *l*-menthol, etc.[1-3]

In recent improvements of the technique, the racemization of the substrate is induced in solution during the course of crystallization, so as to convert all of the material into a single enantiomer. This method may be applied to substances with a single asymmetric center, which can be racemized in the presence of appropriate catalysts,such as certain amino acids (lysine, *p*-hydroxyphenylglycine) or the antiinflamatory drug Naproxen.

However, the vast majority of racemates crystallize as racemic compounds (heterochiral packing), and hence cannot be resolved by a direct crystallization method (Fig. 1). Only a few of them, 10% or less, spontaneously resolve into a mixture of homochiral *d* and *l* crystals; 10%, however, implies that thousands of conglomerates must exist among the huge number of known substances, even though only a few hundred cases of spontaneous resolution have been identified as yet.[1-3]

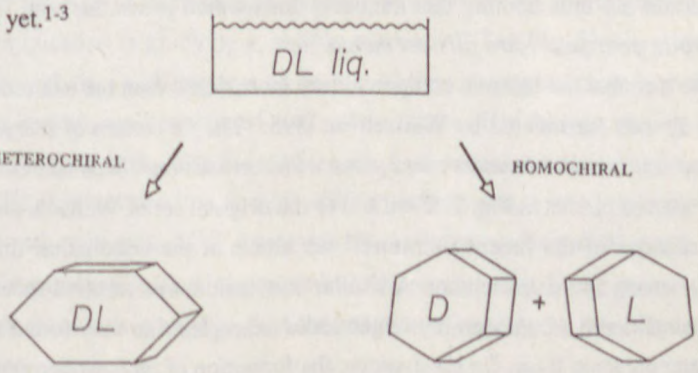


Fig. 1. *The homochiral vs. heterochiral packing dilemma*

What factors govern the alternative depicted in Fig. 1? Can we understand the highly unequal distribution between the two racemate types? Can we predict the occurrence of a conglomerate from the structure of a molecule?

At this stage, I would like to make a remark. As chemists, we generally investigate systems which are very complex, and, paradoxically, we can in many instances give appropriate answers to problems which we are not able to comprehend fully. For instance we can predict the direction of addition of a hydride to a carbonyl group by using different *models*. The models change from time to time, and they probably have little to do with the actual reaction mechanism. Yet they generally work fairly well.

Here, on the contrary, we are facing a problem which is well identified and experimentally perfectly well defined as a kind of dilemma: yes or no, black or white, racemic compound or conglomerate. Yet, we are still largely unable to understand what is going on, or even to build an empirical model which would at least allow some predictions to be made.

For a long time, the origins of chiral discrimination phenomena have been the object of theoretical studies. In this respect, I should mention here the work of Craig and his school in Australia, Mason in England, and others.[4-6] These theoretical approaches are interesting in the sense that they may inform us about the possible origins of the energy differences between homochiral and heterochiral interactions. However, they are generally so far removed from real systems that their predictive value is extemely weak, and their conclusions are almost impossible to check experimentally. No theory has been able as yet to predict or even to explain the occurrence of a spontaneous resolution. The aim of this paper is to present the opinion or, more exactly, the feeling of an experimentalist on the question.

Before proceeding farther, it may be interesting to comment on some of the main ideas that have generally prevailed among chemists since the end of the last century. To tell the truth, the most common opinion was that there wasn't any real problem. Racemic compounds dominate because they are normally more stable, and they are more stable because they are more *efficiently packed* in the crystalline state than are the pure enantiomers. The conglomerates are thus nothing that the exceptions which prove the rule, as we sometimes say. *"Et voila pourquoi votre fille est muette"*...

The idea that the racemic compounds are more dense than the enantiomers in the crystal state was already formulated by Wallach[7] in 1895: *"The formation of a crystalline racemate from the optically active isomers takes place with contraction"*. This rule actually rests on ten cases, the starred circles in Fig. 2. If we add to the original set of Wallach some new examples taken at random in the recent literature,[1] we arrive at the conclusion that there is clearly something wrong in this statement. A similar conclusion was reached recently by Dunitz *et al.*,[8] who considered a considerably larger set of examples that they found in the Cambridge Crystallographic Data Base. *On the average*, the formation of racemic compounds does not take

place with contraction, and therefore there should exist other reasons to explain their wide occurrence.

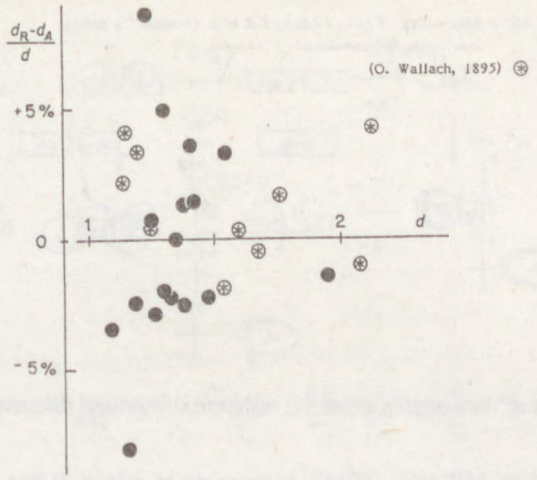


Fig. 2. *Density difference between homochiral and heterochiral packings;* d_A *and* d_R *are the densities of enantiomer and racemic compound crystals, respectively, and* $d=(d_R+d_A)/2$.

Another interesting view on the question can be found in the famous book by Professor Eliel, *"Stereochemistry of Carbon Compounds"*, which has influenced thousands of chemists since its release in the sixties.[9] And thus every chemist must be convinced that *"unlike enantiomers have greater affinity for one another than like enantiomers"*, and that *"racemic compounds have lower enthalpies than pure enantiomers"*.

Let me suggest that these statements might perhaps be taken with a grain of salt.

In effect, if we want to discuss racemate stability in terms of thermodynamics, we have to consider the sign of the free energy change ΔG^ϕ corresponding to the solid state conversion of a conglomerate into a racemic compound[10] (Fig. 3). The formation of a racemic compound will take place if the sign of ΔG^ϕ for this reaction is negative. At a temperature *T*, the free energy change in question is given by a simple expression (See Fig. 3) where $\Delta H_{A \text{ or } R}$ and $\Delta S_{A \text{ or } R}$ are the enthalpy and entropy of fusion of the enantiomers and of the racemic compound. This expression consists of two main terms. The former, which I call ΔG^i, represents the intrinsic stability difference between homochiral and heterochiral packing. In other words, ΔG^i expresses the free energy difference between a *pure enantiomer* and the racemic compound, whereas ΔG^ϕ expresses the corresponding difference between a *conglomerate of d and l crystals* and the racemic compound. In Fig. 3, the racemic compound is arbitrarily represented as more stable than the enantiomer crystal, but this is by no means the rule, as we shall see later.

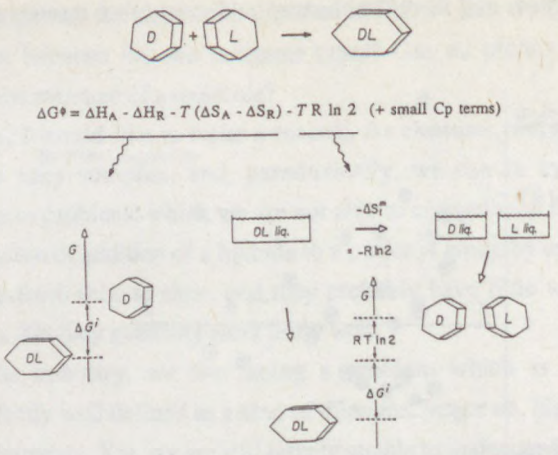


Fig. 3. *Thermodynamics of the conglomerate vs. racemic compound dilemma*

The second term of ΔG^{ϕ}, i.e., TRln2, is entropic in origin. It has nothing to do with chirality nor with crystal stability. It comes from the fact that, if the racemic melt or solution wants to crystallize in the form of a conglomerate, the system must split into two phases containing the pure enantiomers, and from a thermodynamic point of view the process can be formally described in the way shown in Fig. 3. The phase separation decreases the entropy of the system by Rln2, the inverse of the entropy of mixing ΔS_l^m of the pure enantiomers in the liquid state. The cost of this phase separation is equivalent to giving to the conglomerate a free energy debit of TRln2 with respect to the racemic compound, for which no separation of the system is necessary. Since TRln2 at room temperature is about 0.5 kcal/mol, we arrive at the conclusion that the formation of a conglomerate implies that the enantiomer crystals must be more stable than the racemic compound by at least half a kcal/mol, in order to overcome the unfavorable entropy term. At this step, it may be useful to make a short digression, in order to show how the existence of this entropy term can be justified experimentally.

The thermodynamic cycle in Fig. 4 describes two different ways to make a conglomerate, starting from the pure crystalline enantiomers. On one hand, the enantiomer crystals are melted separately at their melting point T_A, then the two liquids are mixed, the resulting liquid racemate is cooled to T_R, and crystallized to the conglomerate at this temperature. On the other hand, the enantiomer crystals are simply cooled down to T_R and then mechanically mixed to produce the conglomerate. The point is that in this cycle the only unknown parameter is the entropy of mixing ΔS_l^m of the enantiomers in the liquid state, all the others being accessible experimentally. Without going to details, one can arrive at the entropy of mixing by using the equation shown in Fig. 4. Table 1 shows the results of these

calculations for a dozen conglomerates.[10] The experimental values of ΔS_l^m (1.33 cal/mol/K on the average) are very close indeed to the actual value of Rln2, 1.38 cal/mol/K.

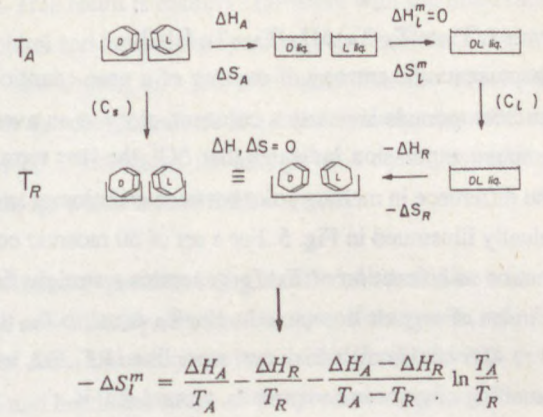


Fig. 4. *Entropy of mixing of enantiomers in the liquid state (theory).*

Table 1. *Entropy of mixing of enantiomers in the liquid state (experiments)* [10]

	"Rln2"
o-Chloromandelic acid	1.29
p-Bromophenylhydracrylic acid	1.40
p-Chlorophenylhydracrylic acid	1.40
m-Fluorophenylhydracrylic acid	1.24
Phenylhydracrylic acid	1.30
3-Hydroxy-3-phenylpivalic acid	1.26
1,2-Dichloroacenaphthene	1.41
2-Naphthoxypropionamide	1.28
Hydrobenzoin	1.30
α-Methyl-4-methoxydesoxybenzoin	1.35
Methylphenylnaphthylfluorosilane	1.47
Anisylidenecamphor	1.27
	<1.33>

We are now ready to draw some important information from the expression of the free energy of formation of racemic compounds that we have just seen. If we calculate the value of ΔG^{ϕ} at the melting point of the racemate T_R, we arrive after appropriate rearranging at a new equation,

$$\Delta G^{\phi} = (T_A - T_R)\Delta H_A/T_A - T_R \, R\ln 2$$

In this equation, $\Delta H_A/T_A$ represents the entropy of melting of a pure enantiomer. In fact, the entropy of fusion of organic compounds is nearly a constant, close to an average value of 17 cal/mol/K. Therefore, the above expression indicates that ΔG^i, the first term of ΔG^{ϕ}, should be proportional to T_A-T_R, the difference in melting point between enantiomer and racemate.

This result is graphically illustrated in Fig. 5. For a set of 60 racemic compounds, a plot of the free energy of formation as a function of T_A-T_R generates a straight line. The slope of this line is the entropy of fusion of organic compounds. For T_A equal to T_R, the line intersects the *y* axis in a region (-0.5 to -0.6 kcal/mol) which corresponds to $RT_m\ln 2$, when T_m is a kind of average temperature of melting of organic compounds, around 400 K.

Therefore, there is a direct correspondence between ΔG^{ϕ} and the shape of the phase diagram. The most stable racemic compounds have ΔG^{ϕ} over -2 kcal/mol, and for a majority of them it is smaller than -1 kcal/mol. All compounds above the line corresponding to $\Delta G^{\phi}=RT_m\ln 2$ have negative ΔG^i, hence correspond to racemic compounds which are more stable than the pure enantiomers. Below this line, on the contrary, ΔG^i is positive and therefore the racemic compounds in this region are less stable than are the pure enantiomers. They are, however, more stable than is the conglomerate, because of the TRln2 term. Conglomerates themselves would be below the line corresponding to $\Delta G^{\phi}=0$, but for these compounds it is not possible to calculate the magnitude of ΔG^{ϕ}, as the data corresponding to the racemic compound do not exist.

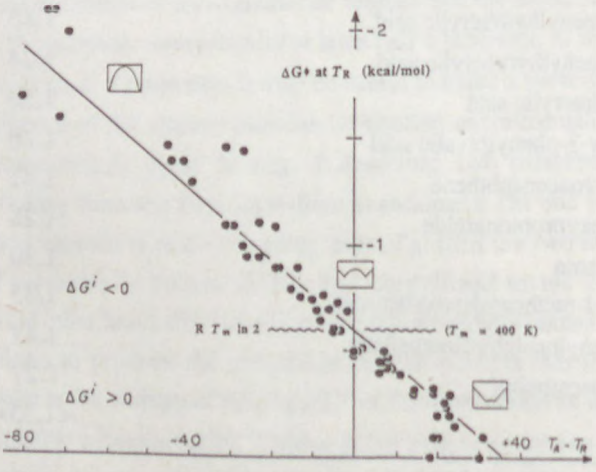


Fig. 5. *Free energy of formation* (ΔG^{ϕ}) *of racemic compounds as a function of* T_A-T_R

Now, the point is that in this set of 60 compounds, there are about the same number of racemates having positive and negative ΔG^i. Hence, the idea that the heterochiral packing would be generally more stable than the homochiral packing does not seem to be verified experimentally. This result is entirely consistent with the observation made above that, on the average, homochiral and heterochiral packings are equivalent in terms of compactness.

In fact, one should now be convinced that the observed 9:1 ratio of the racemic compounds over the conglomerates is a mere consequence of the $RT\ln 2$ contribution, rather than the effect of a structural advantage of a racemic packing over the corresponding homochiral packing, which indeed does not exist.

This preliminary question being clarified, I would like to move to the second part of the story. Could we now identify structural factors, if they exist, which would determine the order of magnitude of ΔG^i, and which in turn would determine the position of a racemate on the graph of Fig. 5 and hence the shape of the *dl* phase diagram? The answer to this question does not only have theoretical interest. In effect, the ease with which a pure enantiomer can be separated from a partially resolved mixture does not depend on the skill of the chemist, but rather on the location of the eutectics in the phase diagrams of the *dl* system, as shown (Fig. 6).

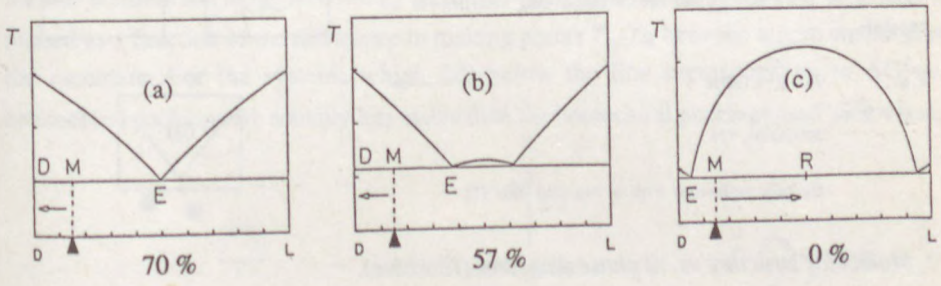


Fig. 6. *Yield* (ME/DE) *of optically pure material that can be obtained by recrystallization of a 70% ee sample.*

For instance, the yield of a pure enantiomer that can be obtained from a sample of 70% enantiomeric excess (ee), is given by the ratio ME/DE in the two diagrams on the left. In a conglomerate (a), the yield is equal to the ee, whereas it is lower in racemic compound (b), because some enantiomer is lost in the mother liquors. In the last case (c), crystallization will only afford the racemate.

The tentative ideas I would like to discuss now are summarized in Fig. 7. I do not pretend that they are true in an absolute sense, but I suggest that they may reflect a real trend in

the behaviour of racemic systems. In fact, I have simply divided the set of the chiral molecules into two sub-sets.

The first sub-set encompasses molecules which can pair up about a center of symmetry. I propose that such molecules will always have racemic packings that are intrinsically more stable than homochiral packings (in other words, they will display negative ΔG^i). Accordingly, this class of molecules will exhibit *dl* phase diagrams of type (a) in Fig. 7 and will never form conglomerates. The only clear example of such a behaviour found thus far is the carboxylic acids R*COOH in which the chiral group R* does not contain strong H-bonding substituents such as OH, SH etc.

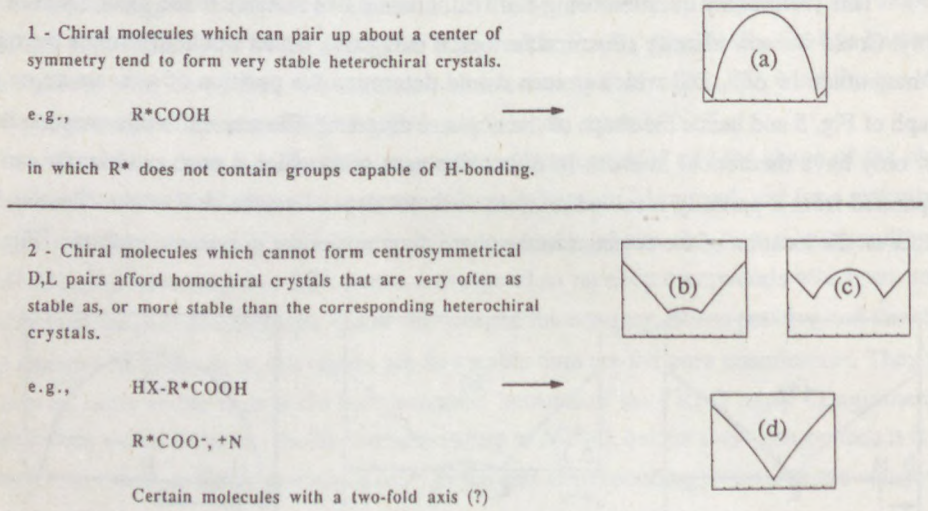


Fig. 7. *Molecular structure vs. dl phase diagrams (tentative).*

The second sub-set comprises molecules which, on the contrary, cannot pair up about a center of symmetry, for reasons I shall discuss later. Then, on the average, homochiral or racemic packings will have about the same efficiency. Hence, the phase diagrams for this sub-set will, in general, be of the types (b)-(d), and the conglomerates, for which ΔG^i is positive and greater than $RT\ln 2$, will be relatively frequent. It seems that at least three classes of chiral molecules fall in this sub-set: (i) carboxylic acids containing additional OH groups; (ii) carboxylic acid salts; (iii) possibly, certain molecules which belong to the C2 point group.

Some arguments which support these ideas are discussed below.

Fig. 8 depicts in a simple way the general trend that one observes for chiral carboxylic acids. Naphthyl and naphthoxypropionic acids are representatives of a category in which the chiral center is close to the acid function, and the substituents are not capable of H bonding.

Such acids always form very stable racemic compounds. On the contrary, hydroxy acids such as mandelic acid and analogues usually form unstable racemic compounds, and occasionally afford conglomerates.

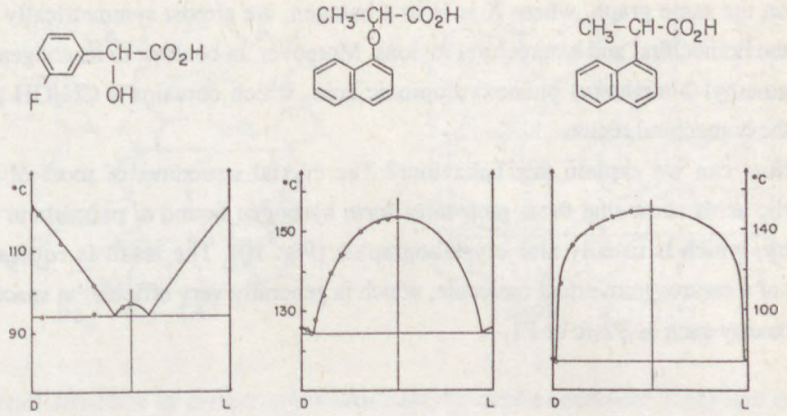


Fig. 8. *Typical dl phase diagrams for simple carboxylic acids.*

This behaviour is illustrated in a more quantitative way in the graph of Fig. 9. Here only the ΔG^i contribution to the free energy of formation of racemic compounds, in a set of acids, is plotted as a function of the difference in melting points T_A-T_R between a pure enantiomer and the racemate. For the systems which fall below the line corresponding to ΔG^i=0, the heterochiral packings are actually less stable than the homochiral packings, and vice versa.

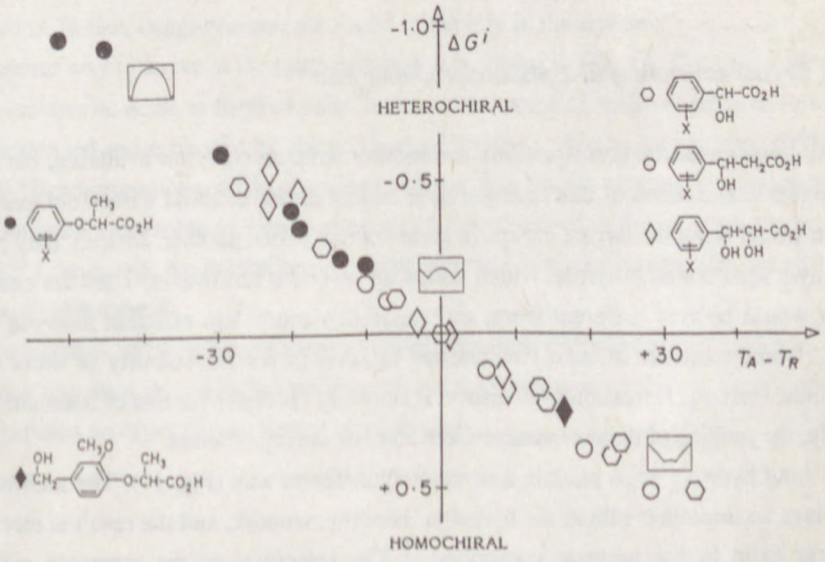


Fig.9. *Plot of* ΔG^i *vs.* T_A-T_R *for carboxylic acids.*

The black circles represent a series of substituted 2-phenoxypropionic acids, with X being halogen or nitro groups. They all display negative ΔG^i, and indeed form very stable racemic compounds. On the contrary, the racemic compounds of the various hydroxy acids shown on the same graph, where X is H or a halogen, are almost symmetrically distributed among the homochiral and heterochiral regions. Moreover, in contrast to its congeners, a 2-(4-hydroxymethyl-2-methoxy) phenoxypropionic acid, which contains a CH_2OH group, also falls in the homochiral region.

How can we explain this behaviour? The crystal structures of most of the simple carboxylic acids show that these molecules form hydrogen bound *dl* pairs about a center of symmetry, which is usually also crystallographic (Fig. 10). The result is equivalent to the packing of a centrosymmetrical molecule, which is generally very efficient in space groups of low symmetry such as $P2_1/c$ or $P\bar{1}$.

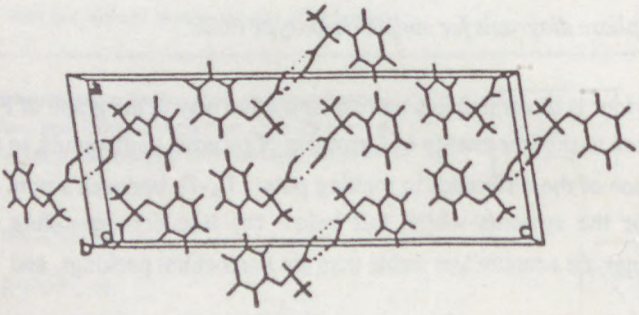
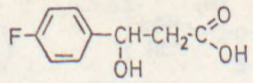


Fig. 10. *Crystal structure of dl-2-phenoxypropionic acid.*[11]

In these cases the corresponding enantiomer structures are not available, but we may suppose that enantiomers of like configuration cannot dimerize about a two-fold axis, as this situation would bring the largest groups in close contact to one another. Instead, they probably form a hydrogen bound polymer, which winds up around a screw axis. Then the enantiomer packing would be very different from, and apparently much less efficient than the racemic packing. Thus, there are at least two reasons to account for the stability of these racemic compounds. First, the formation of *dl* dimers is sterically favored over that of homochiral pairs. Secondly, the packing of these centrosymmetrical pairs is very efficient.

Chiral hydroxy acids pack in a completely different way (Fig. 11). The additional OH group plays an important role in the hydrogen bonding network, and the result is that *dl* pairs no longer exist in the racemic compound.[12] The structures of the racemate and of the enantiomer have in common the existence of layers of molecules of like configuration.



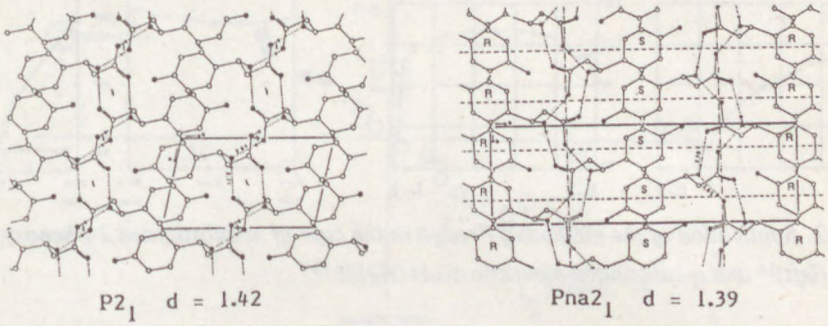


Fig. 11. *Crystal structure of p-fluorophenylhydracrylic acid enantiomer (left) and racemic compound (right).*[12]

These layers are linked to one another on each side by hydrogen bonds involving the carboxyl groups and the OH groups. In the racemate, homochiral layers alternate. In this case, the enantiomer packing is more dense than the racemic packing. We have made similar observations for mandelic acids and other hydroxy acids. In these compounds, the homochiral and the heterochiral arrangements have almost the same structure, and depending on each particular case, the one or the other may be more stable, and very often the homochiral structure wins. In fact, conglomerates are found frequently in these series.

Another way to arrive at the same conclusion is shown in Fig. 12. The strong tendency of simple carboxylic acids to form *dl* pairs, and the reluctance of hydroxy acids to form such pairs is evidenced quite clearly by the method of Fredga.[13] For instance, two differently substituted phenoxypropionic acids of unlike configuration form a very stable quasi-racemate, in which molecules assemble as dimers similar to those observed in the ordinary racemates of this series.[14] Conversely, the corresponding enantiomers of like configuration do not mix at all, and form a simple eutectic.

The *o*-chloro and *o*-bromomandelic acids behave quite differently.[15] The enantiomers of opposite configuration do not form a compound, but a pseudo-conglomerate, whereas those of like configuration co-crystallize to form a solid solution.

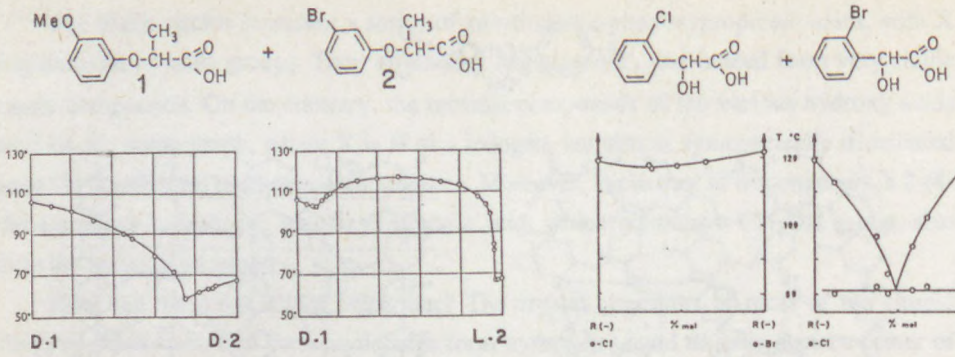


Fig. 12. *Application of the method of Fredga in the case of m-substituded 2-phenoxypropionic acids (left)*[14] *and o-substituted mandelic acids (right).*[15]

A second class of compounds for which we may suspect that the enantiomer packing may be often more stable than the racemic packing is the carboxylic acid salts. The first indication of this trend comes from a statistical study of Jacques and coworkers.[16] In a set of 1308 neutral racemates taken from Beilstein, they found 83 conglomerates, a frequency between 5 and 7.6% at a 95% confidence level. This set was compared with a set of 94 crystalline salts, which were prepared for this purpose, and in which they found 14 conglomerates. This ratio corresponds to a frequency between 7 and 23%, a very wide range due to the small size of the set. For that reason, the statistical argument is not very strong. Nevertheless, the same conclusion may be drawn from a different approach. Instead of counting the number of conglomerates in a given set of molecules, which is always very small, one can calculate the stability of the racemic compounds that are present in the set.

This approach[17] is summarized in the graph of Fig. 13, which shows the effect of salt formation on the magnitude of ΔG^i, for the same set of substituted 2-phenoxypropionic acids we have seen above. The open circles represent the ΔG^i of racemic salts of these acids with achiral amines such as propylamine, cyclohexylamine or dicyclohexylamine. It is quite clear that, in contrast to the acids themselves, most of the salts fall in the homochiral region. In other words, in most of these salts, the homochiral packing is intrinsically more stable than the racemic packing. How can we explain this result?

There seems to exist a common way of packing for the salts of carboxylic acids and amines. The acid and the base form a hydrogen bound polymer which winds up around a screw axis in the way shown (Fig.14). The point is that the chiral ions, either the acid or the base, must have the same handedness throughout a column. Then, the racemic compound is made by assembling homochiral columns of the two handedness, and it is perhaps less efficient to pack together right and left handed screws than to pack homochiral screws.

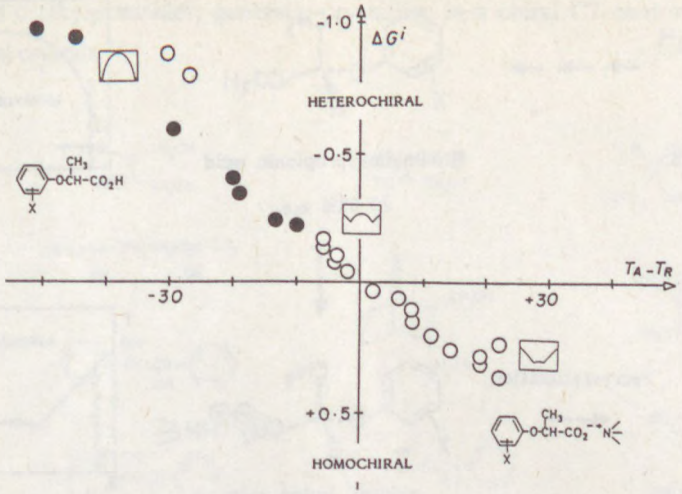


Fig.13. *Plot of* ΔG^i *vs.* T_A-T_R *for a series of substituted 2-phenoxypropionic acids (black circles) and their salts with n-propyl-, cyclohexyl- and dicyclohexylamine (open circles).*

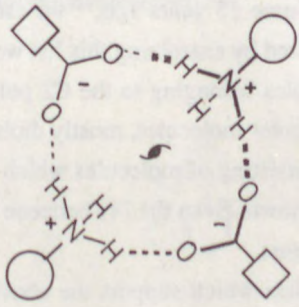


Fig. 14. *Packing of carboxylic acid/amine salts.*

Whatever the reason for this behaviour is, it may have important practical applications. Fig. 15 shows a straightforward preparation of optically pure substituted *R*-2-phenoxypropionic acids. Some of these compounds are important herbicides. An SN_2 reaction on an *S*-lactic acid derivative gives access to the *R* acids with 75-88% ee. These acids cannot be purified, however, due to their unfavorable *dl* phase diagrams. Nevertheless, purification can be easily done after conversion of the partially resolved acids to appropriate salts with achiral bases, which generally have much more favorable *dl* phase diagrams.

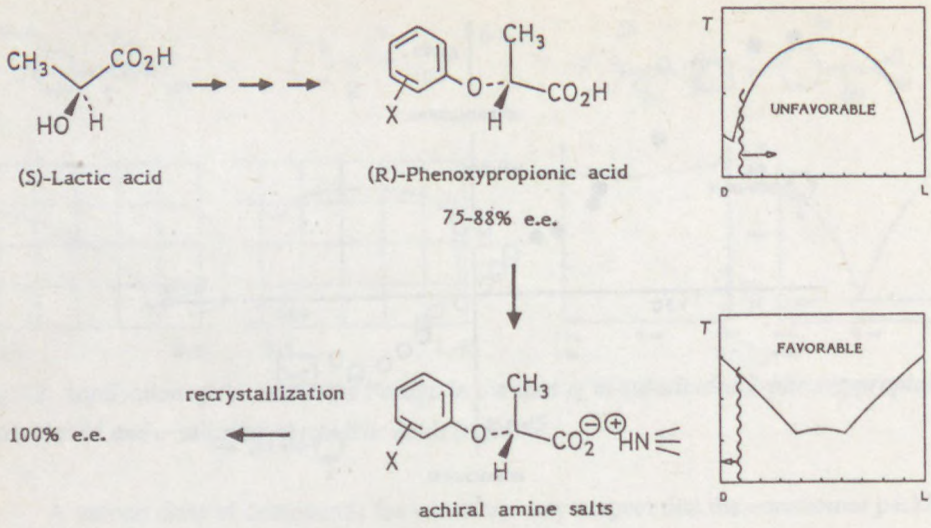


Fig. 15. *Straightforward preparation of R-2-phenoxypropionic acids.*[17]

Finally, we arrive at a last category of molecules which, perhaps, might prefer to crystallize in a homochiral way. Some 15 years ago,[18] we established a list of 170 neutral molecules forming conglomerates, and by examining this list we were rather impressed by the apparently large number of molecules belonging to the C2 point group, 24% of the set. For instance, Fig. 16 shows a series of polar molecules, mostly diols, which form conglomerates, and Fig. 17 displays another set consisting of molecules which do not form hydrogen bonds. The case of the helicenes is well known. Even the [4]-helicene forms chiral crystals, but the molecule racemizes instantly in solution.

There are some statistical data which support the above idea. In a set of 752 chiral molecules taken from Beilstein, we found only 13% of molecules which belong to the C2 point group. This is to be compared with the presence of 42 C2 molecules, 24%, among the 170 conglomerates. This ratio is even larger, 35%, if we only take into account a sub-set of 57 non-polar molecules forming conglomerates, of which 20 belong to the C2 point group. Another interesting clue in this context is the remark that achiral molecules such as hydrogen peroxide,

diaryl and dialkylperoxides, generally crystallize in a chiral C2 conformation, and form homochiral crystals.[19]

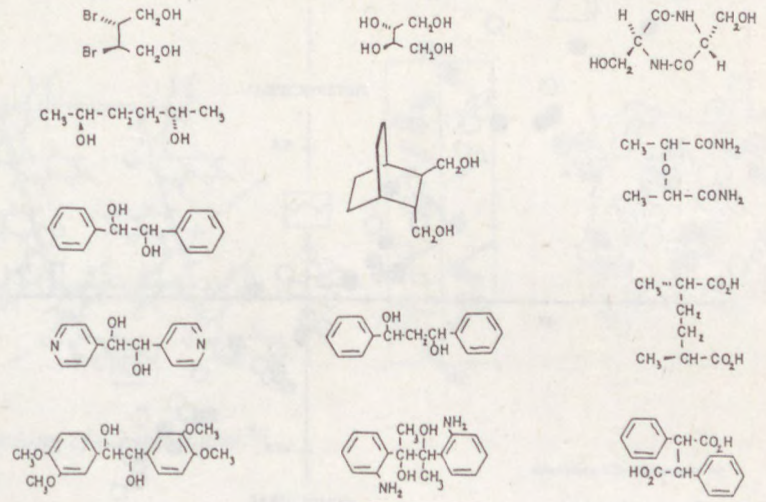


Fig. 16. *Polar C2 molecules forming conglomerates.*

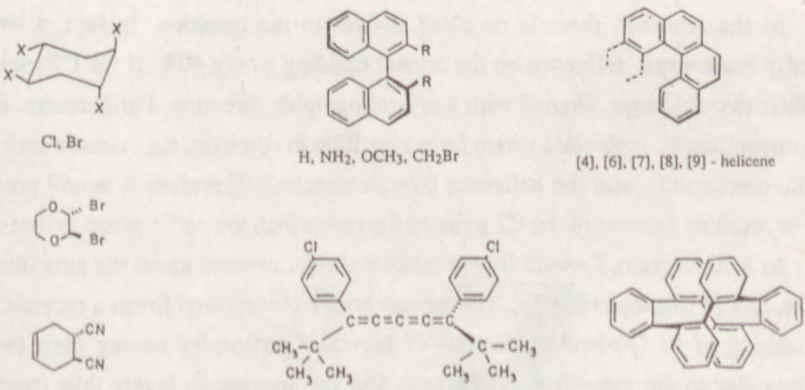


Fig. 17. *Non-H-bonding C2 molecules forming conglomerates.*

However, thermodynamics does not seem to confirm the importance of the above statistical data. In a set of 55 non-acidic C2 molecules forming racemic compounds, we observe an almost equal distribution between homochiral and heterochiral preferences, and

furthermore some of the C2 molecules of the set form very stable racemic compounds (Fig. 18). What does that mean?

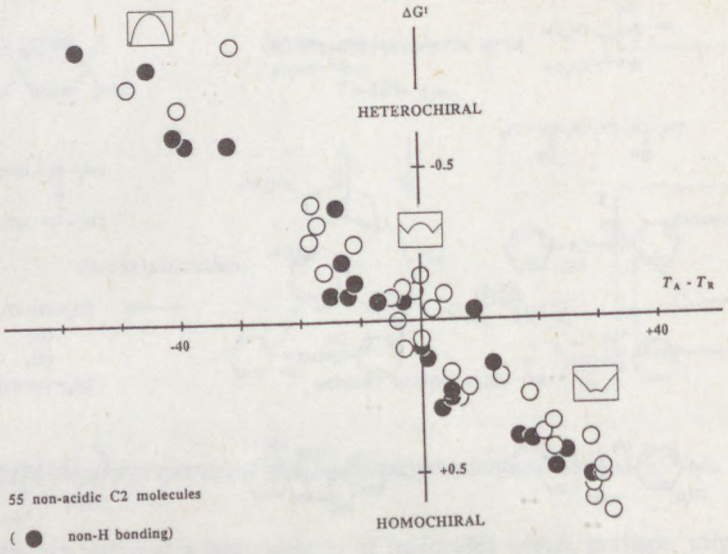


Fig. 18. *Plot of* ΔG^i *vs.* T_A-T_R *in a set of 55 non-acidic C2 molecules forming racemic compounds.*

At the moment, there is no clear answer to the question. In fact, a two-fold axis generally has a weak influence on the crystal building - only 40% of the C2 molecules pack with their two-fold axis aligned with a crystallographic direction. Furthermore, the C2 point group encompasses molecules which have very little in common, e.g., tartaric acid (very stable racemic compound) and the helicenes (conglomerates). Therefore it would probably make sense to consider sub-sets of the C2 point group rather than the entire group in that context.

As a conclusion, I would like to make a short comment about the structure of the two C2 molecules[20] displayed (Fig.19). The ethano bridged binaphthyl forms a racemic compound. The packing of its (+)-isomer consists of layers of molecules having their two-fold axis perpendicular to the three-fold screw axis, and the successive layers thus rotate from one another by 120° around that axis. One thus obtains a kind of super-twisted structure with a short pitch of three molecular layers along the screw axis direction. The structure of the racemate is even more unusual. One observes the formation of homochiral helical columns in which molecules of the same handedness wind up around a four-fold screw axis, and here again, the racemate is made of the juxtaposition of right and left helices. There are no *dl* pairs in this structure.

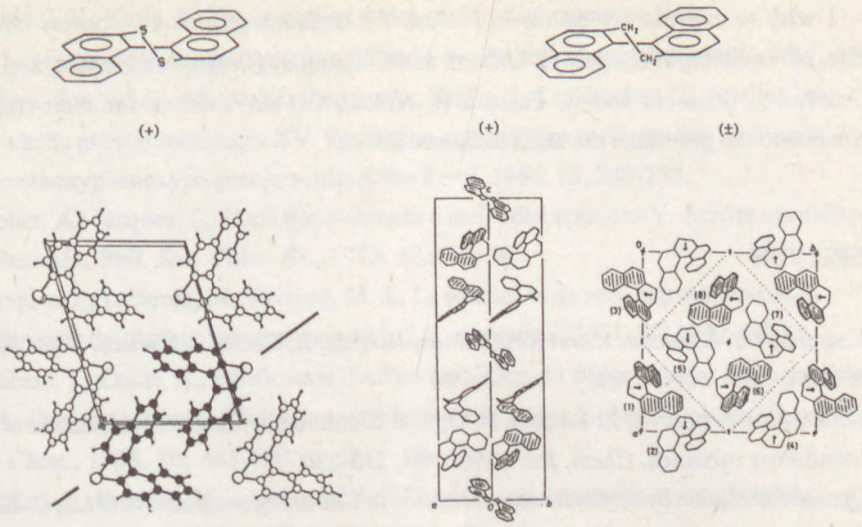


Fig. 19. *Packing of C2 molecules.*[20]

Finally, in the disulfide analogue, which forms a conglomerate, if one looks along the direction indicated in Fig. 19, the molecules pack in a way which is exactly that of the wood models of Professor Jacques,[21] a crystallographic equivalent to Horeau's coupe du Roi (Fig. 20). These simple models nicely account for the homochiral packing preference of certain classes of C2 molecules.

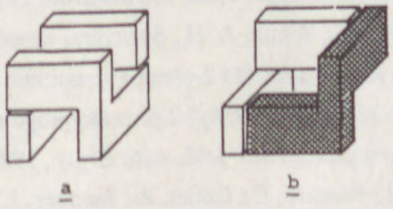


Fig. 20. *The wood models of J. Jacques : a pair of models of like handedness can pack with their C2 axes aligned to form a close-packed dimer (a cube); models of opposite handedness cannot pack as efficiently.*[21]

I wish to express my thanks to Professor J. Jacques and his co-workers Drs M.-J. Brienne, M. Leclercq, J. Canceill, J. Gabard, and C. Fouquey (Collège de France), and to Drs. M. Cesario, J. Guilhem and C. Pascard (C.N.R.S., Gif-sur-Yvette), for their important contribution to the genesis of the ideas discussed above.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

CHIRAL RECOGNITION IN THE LIGHT OF MOLECULAR ASSOCIATIONS

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> "A crystal and each constituent space-filling molecule are 'images of each other' in overall shape."

Scientists have always sought simple and general axioms for exploring and visualizing the most complicated problems of Nature. As Mason[1] pointed out, Haüy's above postulation, dating from 1809 and based on crystal cleavage observation, had a great influence on crystallography and stereochemistry in the 19th century. It gained an extension to the chemical and physical properties of substances in Mitscherlich's law on isomorphism, which linked the similarity of crystal shape to an equivalence in stoichiometric chemical composition[2] and helped Herschel to establish the connection between the morphological handedness of quartz crystals and the sign of the crystal optical activity.[3]

Crystal packing, as well as chemical and biochemical reaction mechanisms are frequently symbolized by trivial models taken from everyday life. Attempts to demonstrate packing facilities may involve children's building blocks; for stereochemical considerations different types of wire or hemisphere models are used; for enzymatic reactions the classical "key and lock" model of Emil Fischer[4] is often applied. The mechanistic picture of our macroscopic surroundings determines and restricts the way of our thinking about molecular processes.

As any type of reaction requires properly positioned electronically and sterically complementary interacting groups, the two (or more) components of the reaction should fit to-

gether in the same way as the pieces of a jig-saw puzzle, and this complementarity is not fulfilled for the mirror image of molecules (Figure 1).

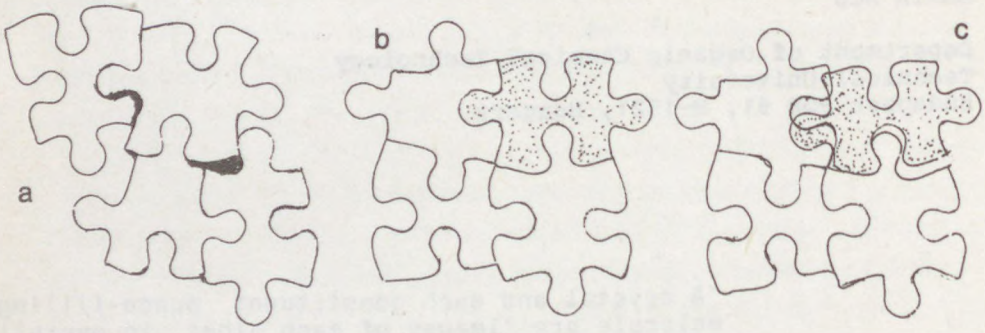


Figure 1. The problem of multipoint steric fit as illustrated by pieces of a jig-saw puzzle: a) "wrong" random packing; b) perfect tessellation of the same pieces; c) wrong fit due to the mirror image of one of the components.

For chemical reactions not only the surroundings (e.g. the solvent), but also the larger part of the molecule is often disregarded when attempts are being made to elucidate the reaction mechanism. For instance, an isolated double bond is expected to exhibit normal addition reactions with electrophilic reagents, but changes in the electronic properties of the molecule can inhibit the reactivity of the double bond (e.g. vinyl halogens). In addition, steric hindrance can entirely suppress reactivity when merely a stereochemical "wall" hinders the addition of the reagents (Figure 2).

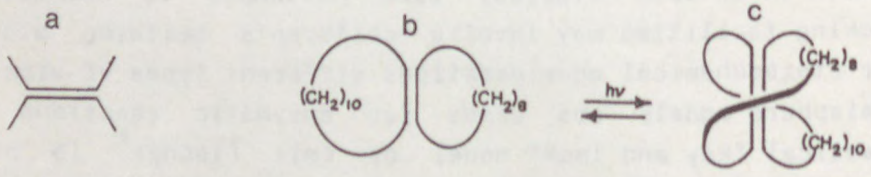


Figure 2. Reactivity of differently substituted alkenes with the same agents: dichlorocarbene and catalytic hydrogenation afford the saturated addition products for a) and b), whereas [10,8]betweenanene (c) does not react under conditions when the cis isomer (b) undergoes transformation (ref.5 and ref.6)

An increasing number of new types of molecular systems display confusing behaviour under the usual reaction conditions. Therefore interest starts being focused on the weak second order interactions possibly taking place between substrates and reagents. Without assuming the existence of specific associations no plausible explanation for biochemical reactions, chromatographic separations, asymmetric synthesis or even optical resolution can be provided.

It is currently considered that

1) most experimental data can be explained on the basis of the generalized three-point interaction model[7] of molecular recognition and discrimination;

2) as a consequence of discriminating weak second order interactions, different types of homo- and heterochiral associations can be detected even in solution;[8,9]

3) in the case of chiral species, the influence of associations is manifested in different products of the chemical reactions of enantiomerically pure, impure and racemic substances, even with achiral reaction partners.

THE THREE-POINT INTERACTION MODEL OF MOLECULAR RECOGNITION

As a result of the discriminating reactivity of a given molecule, new kinds of molecular formations can be generated. They may differ from each other in chemical composition (stoichiometry), molecular symmetry, and consequently in macroscopic physico-chemical properties. These differences provide the basis for their separability. The origin of these differences in macroscopic properties lies in the possibilities of distinct second-order interactions.

Molecular recognition among "small" molecules can be clearly observed when using chiral species. During covalent bond-forming reactions the transition state geometry largely determines the configuration of the resulting stereoisomers. In these kinetically controlled cases second order interactions cannot easily be studied. Reversible reactions to be investigated here include optical resolutions via diastereoisomeric salt formation, where one of the optical

isomers of a given chiral molecule reacts with the enantiomers of a substance of opposite chemical character.

In the reaction of a chiral molecule (R) with a mixture of enantiomers (ℱ and F), the formation of two diastereoisomeric associations (ℱR + FR: compounds, salts, complexes, etc.) is always possible. Similarly to this association forming ability, the existence of diastereoisomeric homo- (FF; ℱℱ) and heterochiral (ℱF) dimers can be assumed even in solutions of chemically homogeneous enantiomer mixtures.[8] The diastereoisomeric dimers (ℱF- FF or FR-ℱR) produced as a result of homo- and heterochiral interactions can react in different ways. The question arises as to what kinds of interactions and of what strengths of these are needed to make diastereoisomers macroscopically distinguishable. In the overall behaviour of systems built up from chiral components significant differences were found in the interactions of corresponding molecular pairs.[8]

For reasons of clarity asymmetrically substituted tetrahedra have been chosen to symbolize the chiral species. A free one-point vertex interconnection between two tetrahedra can result in the formation of 10 homo- and 10 heterochiral diastereoisomeric dimer pairs, while 16-16 diastereoisomeric salt pairs may be formed between the enantiomers and resolving agent. If at least two vertices are involved in the interactions, and one of the possible contacts is fixed (e.g. an ionic bond, or hydrogen bond), the number of possible dimers along one edge are 6 in the case of enantiomer associations, and 9 for diastereoisomeric salt pairs. Selection and fixation of one of the edges causes a further reduction to two in the number of possible contacts along the faces of the tetrahedra (Figure 3). The first two contacts are equally probable in any diastereoisomeric pair, but the third will be different, because of the mirror image relationship between the enantiomeric components of the diastereoisomers. Therefore, the different properties leading to separability are due to the difference in the third contact both in diastereoisomers and also in non-racemic mixtures of enantiomers. The "third", differential interaction, is rarely so well-defined

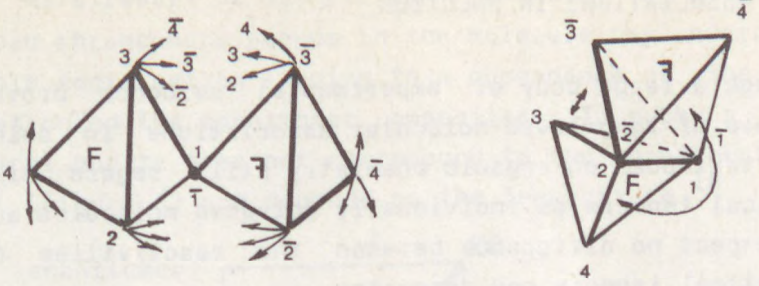


Figure 3. Interaction possibilities between two mirror image molecules (F and 7); a) one vertex-pair is fixed (1-$\bar{1}$), b) an edge is fixed (1-$\bar{1}$ and 2-$\bar{2}$).

that it could be identified by traditional spectroscopic methods. A detectable diastereoisomeric dimer interconnection illustrating the three-point interaction model was reported in 1987. Pirkle and Pochapsky trying to analyze the chiral recognition mechanisms of 3,5-dinitrobenzoylamino acids (used as chiral modifiers for stationary phase in HPLC enantiomer separation of amino acids) were able to identify different interactions between the R-S and S-S dimeric association (Figure 4).

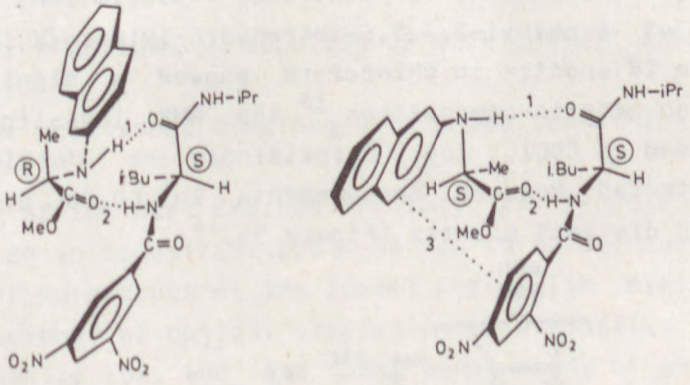
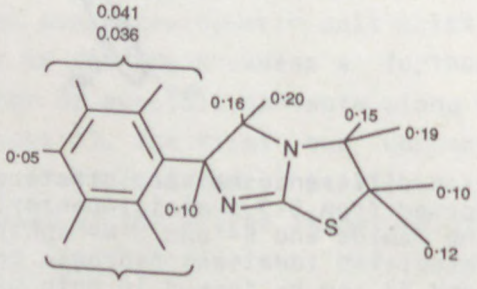


Figure 4. Interaction difference between diastereoisomeric dimers formed from S-3,5-dinitrobenzoyl-N-propyl-isoleucine amide and R- and S-N-naphtylalanine methyl ester. Two identical hydrogen bonds (dotted lines 1 and 2) can be formed in both diastereoisomers. The charge transfer complex formation (dotted line 3 in the right-hand dimer) characterizes only the dimer with SS-configuration (assigned by ^{1}H-NMR and UV-spectroscopy).(refs10, 11.).

Although a large body of experimental evidence proves the existence of long-lived molecular associations in solution, most textbooks on organic chemistry still regard dissolved optical isomers as individually solvated molecules and therefore expect no difference between the reactivities of solvated optical isomers and racemates.

The problem is that the difference between these types of molecular associations (sometimes simple van der Waals interactions) can scarcely be assigned. Only a few examples have been described when ^{1}H-NMR spectra reveal significant differences between the enantiomeric and racemic forms, indicating that at least in the third contacts the FF and ⅁F dimers may assumed to be different. For dihydroquinine[12] and different α-alkyl (Et, i-Pr) substituted succinic acids[13] and some amino acids[14] these differences resulted in signal splitting in the spectra of non-racemic mixtures.

The detection of such interactions can fail because of the wrong choice of the solvent. On the basis of preparative experiments, the existence of enantiomer associations could be assumed for 6-phenyl-2,3,5,6-tetrahydro-imidazo[2,1b]thiazol. As the IR spectra in chloroform showed a significant dependence on enantio-composition,[15] the NMR investigations were performed in $CDCl_3$, too. Surprisingly no distinction could be detected. However, measurements in CD_3OD produced the expected distinct spectra (Figure 5).[16]



$\sigma_{⅁F} - \sigma_{FF}$ in ^{1}H-NMR

Figure 5. Chemical shift differences in the spectra of racemic and optically active 6-phenyl-2,3,5,6-tetrahydro-imidazo[2,1b]thiazol (^{1}H-NMR spectra recorded in CD_3OD, ref.16).

As a result of association-formation, the chirally perturbed chromophore groups in the molecule may undergo considerable modification leading to a dependence of the specific rotation on the enantiomer composition. In such a case the optical purity does not correspond to the enantiomeric purity[17] (Figure 6.) and depends on the temperature.[18]

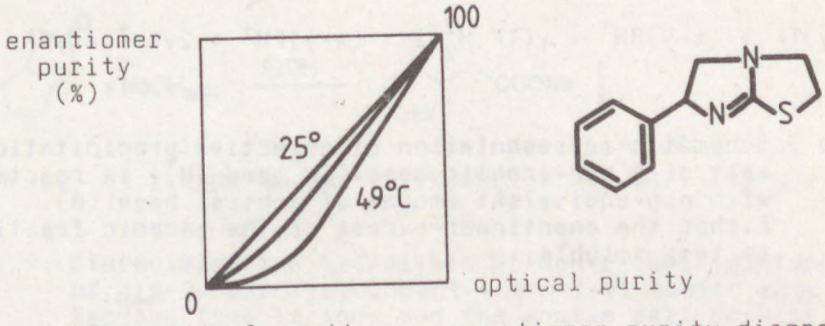


Figure 6. The optical purity vs. enantiomer purity diagram of the model compound deviates from the diagonal valid only in the absence of enantiospecific interactions (ref. 18). The actual difference is magnified.

Making use of the association-formation one can perform selective reactions under achiral conditions.

SELECTIVE REACTIONS IN SOLUTION OF ENANTIOMERIC ASSOCIATIONS

Acid-base reactions, combined with phase transition

One of the most serious problems in producing optical isomers on an industrial scale is how to manufacture enantiomerically pure drugs at the lowest cost with minimum loss. The efficiency of optical resolution or asymmetric synthesis is frequently low, and the traditional means of purification (such as fractional crystallization) sometimes also prove to be ineffective.

When a non-racemic mixture is reacted with less than equivalent amount of achiral substance of opposite chemical character, the optical purities of the precipitating fraction and the portion remaining in the mother liquor may be different. Then by changing the amount of the achiral reagent, a very efficient separation of the dissolved eutectic composi-

tion can be achieved in one step (Figure 7).[19,20,21]

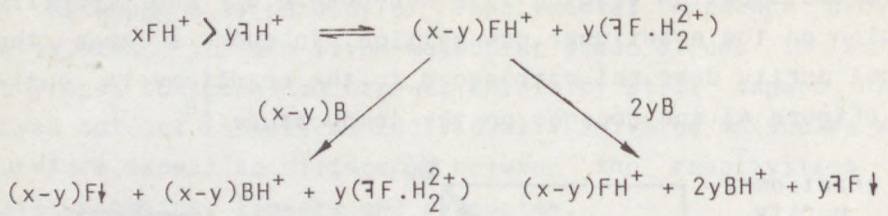
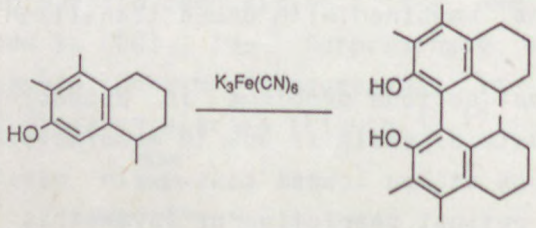


Figure 7. Schematic representation of selective precipitation; salt of a non-racemic base (FH^+ and $\overline{F}H^+$) is reacted with non-equivalent amount of achiral base (B). Either the enantiomer-excess or the racemic fraction is less soluble.

Reactions with covalent bond formation/breaking

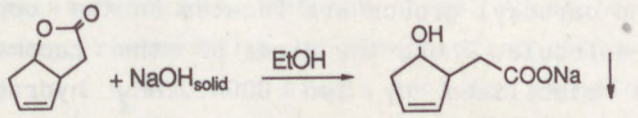
To the best of our knowledge, the first paper reporting different results for a chemical reaction using optically pure and racemic starting materials was published by Wynberg and Feringa.[22] In the course of an oxidative dimerization reaction they obtained different stereoisomers as a function of the optical purity of the starting material (Figure 8).



		products		
		trans	cis	meso
sub-	optically pure	97.5	-	-
strate:	racemate	66.0	7.9	26.1

Figure 8. Chemical yield depending on the enantio-composition of the substrate.

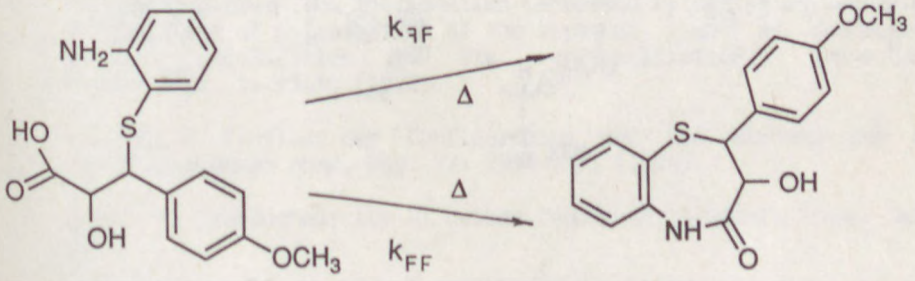
Similarly, when hydrolyzing cis-2-hydroxy-cyclopent-4-enyl acetic acid lactone with a non-equivalent amount of solid sodium hydroxide, the compositons of the precipitated sodium salt and the unreacted lactone in the mother liquor were different from each other (Figure 9).[23]



$$yF\ x\overline{7} + 2xNaOH \longrightarrow x(\overline{7}F)Na_2 + (y-x)F \qquad y > x$$

Figure 9. Stereoselective hydrolysis of non-racemic mixture of cis-2-hydroxycyclopent-4-ene-1-yl acetic acid lactone (the lactone and the sodium salt coexist).

A further example is the intramolecular ring closure reaction of erythro- and threo-2-hydroxy-3-(2-aminophenylthio)-3-(4-methoxyphenyl)-propionic acid (Figure 10).[25] The rate of reaction is almost twice as high for the optically pure compounds as for the racemic species.[24]



$$k_{FF}/k_{\overline{7}F} > 2$$

Figure 10. An enantio-composition dependent ring closure.

The solid-state IR spectra reveal that all carboxyl and amino groups in the racemate are ionized and the alcoholic hydroxyl group takes part in a single type of hydrogen bond. In the optically active substance the amino and carboxyl groups are in part free and at least three types of hydrogen

bonds can be assigned, including the alcoholic hydroxyl group. The ^{1}H-NMR spectra did not show the difference, probably because DMSO had to be used as solvent owing to the poor solubility of the racemate.

MM2 calculations indicate that one of the most stable conformers for the monomer has an elongated structure in which the amino and carboxyl groups are located on the opposite sides of the molecule. Thus, the dimer of the racemate (Figure 11) can be stabilized by two COOH...NH_2 hydrogen bonds and by the formation of two intramolecular C=O...HO hydrogen bonds, in accordance with the IR spectra.

The solid state structure of the enantiomers can be characterized by complicated associations in which some of the amino and carboxyl groups are free and can therefore take part more easily in the ring closure reaction. As a result of

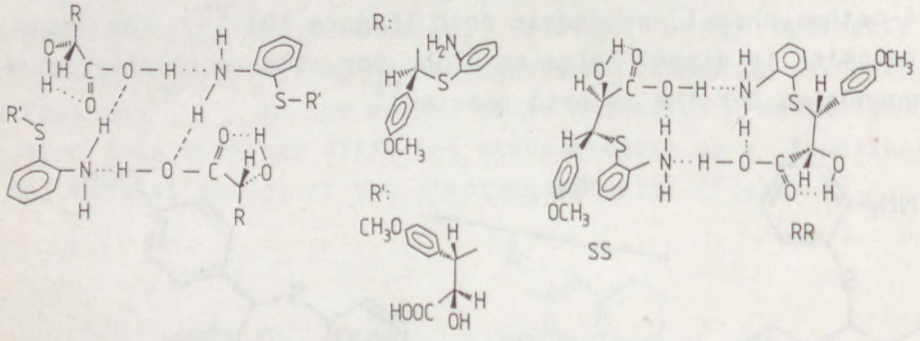


Figure 11. Hypothetical associations based on IR spectra and reactivity differences for the optically active (left) and racemic (right) species.

these differences, the optically active isomer can produce quantitatively the product in melt within 5 minutes (the racemate only reacts at a temperature 40° higher and gives a lower yield, with much by-product). The optically active benzothiazepinone ring system is formed even in solid state within 90 minutes, at a temperature 15° lower than its melting point. (At the same time the racemate does not undergo such solid state reaction).[25]

CONCLUSIONS AND PROSPECTS

With the accumulating experimental evidence and the rapidly developing experimental techniques, the existence of molecular associations in solution can no longer be neglected. Apt consideration can lead to a deeper understanding of chemical reactions, and the chiral molecules with their appearing, reappearing and disappearing optical activity provide us a useful and simple tool for explaining "miraculous" reaction outcome. At the same time the final target of these studies is development of new technologies based upon the better understanding.

Acknowledgement: The author thanks Professor E. Fogassy for his valuable comments.

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DRUGS

MEDICINAL CHEMISTRY

PHARMACOLOGY

Akadémiai Kiadó
Budapest, 1990

SIGNS: THE CODE OF CLARIFICATION

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A young man in a fairy tale reached a branching path in the middle of a thick forest. One way led safely to town, the other to a swamp. A shack stood nearby where two dwarfs lived; one, a deceiver that always lied and the other always truthful. The man found only one of the dwarfs at home and didn't know which of the two it was. Besides, he could only ask a single question, and he did: "Which is the safe path according to your mate?" The reply unconditionally indicated the wrong path, hence he took the other one and safely reached home. Note that our man was an ingenious pragmatist because he obtained the essential information without learning which dwarf he was talking to.

In real life the practising physician often plays the role of this young man, trying to find his way through the jungle of drugs. Since no dwarf, true or liar, stands by, the medical doctor has to rely on the information available for marketed drugs. His primary concerns include establishing the right diagnosis, finding adequate therapy and keeping an eye open for the eventuality of side-effects or drug interactions. Stereochemical identification of pharmaceutical agents is not the physician's responsibility, however. Still, world-wide neglect of this issue leaves him unassisted. Just listen to Drayer's advice[1]:

"To my knowledge, no source of information exists that lists all drugs that are administered as racemic mixtures. This situation should be changed in the future. Until proper

published references are available, perhaps contacting the drug company is the best way to identify a drug that can have stereoisomers".

Alternatively, it is reasonably required that non-proprietary (generic) drug names should carry the information[2,3] necessary to the medical practitioner. To this aim a new nomenclature, a stereochemically informative generic-name system (abbreviated: SIGNS) has recently been worked out[4].

This new system introduces simple prefixes to established generic names of most drugs for which stereoisomers exist. The prefix indicates whether the drug is of single or composite character and gives further information on the kind of stereoisomer or on the type of stereoisomeric mixture implied by the given drug name. Table 1 summarizes and specifies these prefixes.

Table 1. SIGNS prefixes for stereoisomeric drugs

Single drugs		Mixtures	
prefix	meaning	prefix	meaning
dextro-	dextrorotatory enantiomer	*rac-*	racemate
levo-	levorotatory enantiomer	*mep-*	mixture of epimers
cis-	diastereomer (geometrical isomer) generally having Z descriptor in chemical nomenclature[5]	*diam-*	diastereomeric mixture (involving achiral diastereomers and the mixture of racemates)
trans-	diastereomer (geometrical isomer) generally having E descriptor in chemical nomenclature[5]		

The philosophy of SIGNS

Since thousands of drug names exist, SIGNS aims to achieve an informative nomenclature while causing the least disturbance of the present usage of established names. Besides enlightening practising physicians SIGNS also conveys the minimum of essential information on drug identity to pharmacologists thereby saving them from ignoring stereoselectivity in clinical pharmacological studies involving racemates[6]. At the same time SIGNS does not pretend to be an alternative of the stereochemical nomenclature established by Cahn, Ingold and Prelog[5], for two reasons. First, because SIGNS deals with drug names, not with chemical names, and serves primarily medical people, not chemists. Second, because SIGNS has to deal with mixtures, a pharmaceutical concept.

The racemate, an optically inactive equimolar mixture of dextro- and levorotatory enantiomers, is a natural consequence of non-stereoselective synthesis leading to each form with identical probability. Resolution of a racemate into individual enantiomers of identical chemical properties usually requires special techniques based on the use of a chiral handle. Therefore, racemates are mixtures whose ingredients are most intimately related. Diastereomeric mixtures are composed of components different in their physicochemical properties, thus, these represent "cruder" kinds of mixtures which could be separated by simpler methods. By this practical criterion the mixture of two racemates is regarded by SIGNS as a diastereomeric mixture rather than a racemic one. While these two kinds of mixtures are optically inactive, mixtures of epimers possess optical activity, a property in itself insufficient as evidence for optical purity.

By defining mixtures SIGNS considers also the problem of chemical stability. Special attention should be paid to compounds showing the tendency of racemization or epimerization, since these agents are transformed into mixtures themselves. If such a transformation under physiological conditions (37 ^{o}C, aqueous solution, pH 7.4) has a half-life of less then 24 h, the substance is regarded as a mixture by its nature and SIGNS

prefixes are not applied to its name. Consequently, *rac-*, *mep-*, and *diam-* designate drugs which could be applied in the pure, i.e. single form.

The enantiomeric prefixes *dextro-*, and *levo-* refer to the direction of optical rotation which may well depend on experimental conditions. Since the subject is drugs, SIGNS gives preference to physiological conditions (37 °, aqueous solution, pH 7.4) or among organic solvents to a more polar one, when the direction of optical rotation is defined.

Last but not least, about 40 % of all drugs are substances that have no stereoisomers. These drugs will retain their names without any prefix, i.e. in unchanged form, in SIGNS. Hence, the lack of prefix will have the meaning that stereochemistry has no bearing on the purity of the drug, and the drug could not be applied in any purer form.

Table 2 lists SIGNS names of some established drugs.

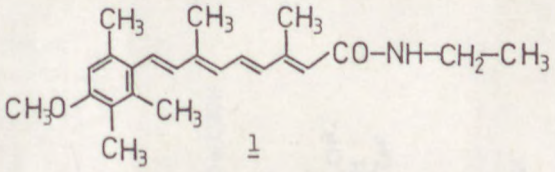
Possible objections to SIGNS

Few experts would deny the need for greater stereochemical awareness in contemporary medical, pharmacological, even chemical literature. Most scientists will also agree that the present confusion arises mainly from the indiscriminate application of drug names to both single stereoisomers and mixtures. Hence, most possible objections could fall into the category of asking the question: "Why so, and not otherwise?"

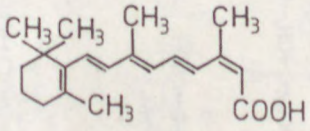
The *dextro-* and *levo-* prefixes were chosen because these are in current use incorporated into numerous names of single-enantiomer drugs and so they would sound familiar as SIGNS prefixes. To some extent *cis-* and *trans-* have also been applied. For the designation of racemates Ariens first suggested R,S- or d,l-prefixes[7], but later became a supporter of *rac-*[8]. Only *mep-* and *diam-* are unfamiliar since they were invented for SIGNS[4]. Inevitably, any endeavour to bring about radical improvement could meet opponents and SIGNS is no exception. We try to address here some of the conceptual difficulties.

Consider a case when the molecule has several double bonds in defined configurations (Figure 1). The generic name

motretinide (1) refers to all-*trans*-N-ethyl-9-(4-methoxy--2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenamide, an exact chemical name clearly unsuitable for naming a drug. Since we are dealing with a single agent, SIGNS will call it *trans*-motretinide. The situation is more complex if one of the double bonds accomodates *cis* (Z) configuration, as in the case of 13-*cis*-retinoic acid (2). This denomination implies that the all-*trans* isomer of 2 is simply called retinoic acid, while it is more informative to name it all-*trans*-retinoic acid[9]. However, the name in SIGNS will be *trans*-retinoic acid, while 2 will be called 13-*cis*-retinoic acid. Analogously, if the double bond neighboring the amide group in 1 changes configuration, the isomer will be called 13-*cis*-motretinide.



1

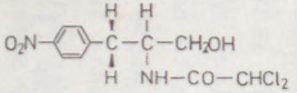
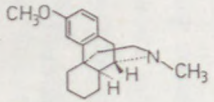
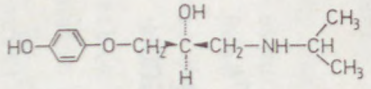
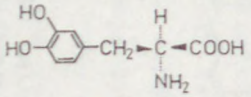
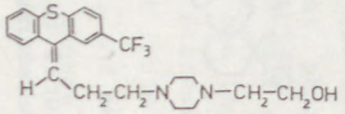
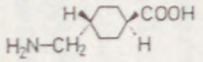
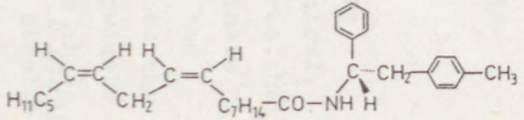


2

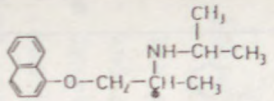
Figure 1. Structural formulae of *trans*-motretinide (1) and 13-*cis*-retinoic acid (2).

Another issue is the large number of single-enantiomeric drugs, biosynthesized or prepared carefully through stereoselective synthetic steps, such as penicillins, cephalosporins, alkaloids, steroids,etc. According to SIGNS,enantiomeric prefixes should be attached to all these names. When the drug also exists in racemic form as in case of *rac*-norgestrel, the SIGNS requirement is - beyond doubt - justified. Some may feel it an unnecessary formality to use names like *dextro*-estradiol, or *dextro*-penicillin V. However, we believe that omitting the

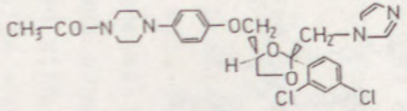
Table 2. The application of SIGNS to established drugs

Name	Formula	Remark
dextro-chloramphenicol		dextrorotatory in ethanol, levorotatory in ethyl acetate; prefix is defined by the more polar solvent
dextromethorphan		remains unchanged in SIGNS
levo-prenalterol		
levodopa		remains unchanged in SIGNS
cis-flupenthixol		Lundbeck markets the pure Z stereoisomer under the trade name Fluanxol Depot
trans-tranexamic acid		
levo-moctamide		enantiomeric purity is preferred over diastereomeric purity in defining prefix

rac-propranolol

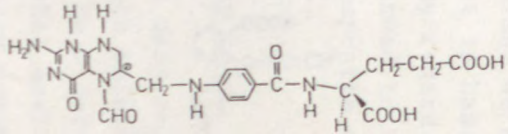


rac-ketoconazole



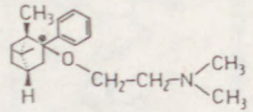
Z configuration with respect to the dioxolane ring cannot be used to define *cis*-prefix for the racemate; open wedges indicate only relative configuration

mep-leucovorin



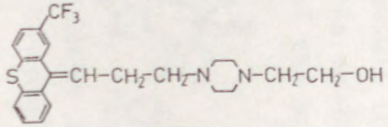
mixture of epimers due to a non-stereoselective synthetic step

mep-EGYT 3886



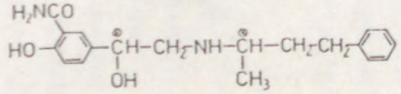
mixture of epimers due to a non-stereoselective synthetic step

diam-flupenthixol



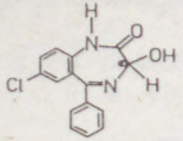
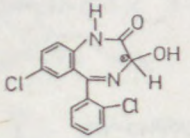
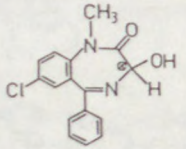
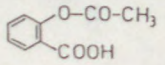
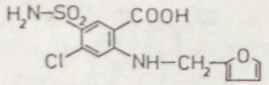
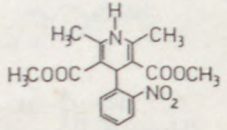
mixture of Z and E isomers marketed by Lundbeck under the trade name Fluanxol

diam-labetalol



mixture of two racemates

Table 2 (continued)

oxazepam		subject to fast racemization in aqueous solution; the compound is racemic by its nature under physiological conditions, hence cannot be applied in any purer form
lorazepam		cf. oxazepam
temazepam		cf. oxazepam
acetylsalicylic acid		compound having no stereoisomer
furosemide		compound having no stereoisomer
nifedipine		compound having no stereoisomer

prefixes in such cases may serve as an invitation to further neglect, thereby weakening the hope of ever eliminating the confusion. Once SIGNS is uniformly accepted and applied, *dextro-* and *levo-* will not sound clumsy any more but will be taken as a sign of quality indicating the purity of the pharmaceutical agent.

Finally consider those names that will have no prefix in SIGNS, since they have no stereoisomers, or racemize fast and cannot be applied in purer form.

How will such a name formally differ from mixtures denominated in the pre-SIGNS era? In other words, will the introduction of SIGNS not cause additional confusion arising from the non-conformity of earlier and new drug manuals? Well, it certainly may, although setting a certain reasonable date after which only the SIGNS denomination will be legal could help a great deal.

In more general terms, we should not be a afraid of improving something that is basically wrong. In ten years time students of pharmacy may regard it unbelievable that pharmaceutical firms were allowed to sell mixtures under names which did not indicate the composite character of the products. It is unbelievable even today.

The temporary end of the tale

The seven prefixes *dextro-*, *levo-*, *cis-*, *trans-*, *rac-*, *mep-* and *diam-* will stand before many drug names like seven true dwarfs. None will be a deceiver, and the medical doctor may find his task somewhat easier by their help. But only if bodies concerned with drug denomination undertake to play the role of Snow White and take proper care of the seven dwarfs.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

CHROMATOGRAPHIC ANALYSIS OF DRUG ENANTIOMERS: APPLICATIONS OF 2,3,4,6-TETRA-O-ACETYL-ß-D-GLUCOPYRANOSYL ISOTHIOCYANATE

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Methods for the simultaneous analysis of enantiomeric forms of chiral drugs are needed in a variety of situations. These include, for example, the analysis of the enantiomeric composition of drugs or synthetic intermediates during resolution procedures or stereospecific syntheses in manufacturing or in research, the determination of the optical purity of a pharmacological agent before pharmacological testing, the analysis of the enantiomeric composition of drugs or their metabolites isolated from biological fluids in studies of the stereoselectivity of drug-metabolism and pharmacokinetics, etc. A variety of methods are available for the determination of the enantiomeric composition of chiral compounds, including chiroptical methods, nmr, mass spectrometry, immuno- and receptor assays, and chromatography. It has become evident in recent years, however, that among these chromatography is unrivalled for its powerful combination of resolving ability, specificity, sensitivity, and versatility.

To separate two enantiomerically related substances by chromatographic means, a homochiral (single-enantiomeric) environment or "handle" must be present in some form in the chromatographic system. This is because in the absence of a homochiral environment the two enantiomeric analytes will have identical physicochemical properties (except for optical rotation, of course), and will therefore also have identical chromatographic behavior, i.e., will be unresolvable. If a homochiral handle is present, on the other hand, its interaction with the two enantiomers may, in principle, be different. The result of such differences in the interaction of the two enantiomers will be a difference in their energy content, and a concomitant difference in their chromatographic behavior, i.e., chromatographic resolution.

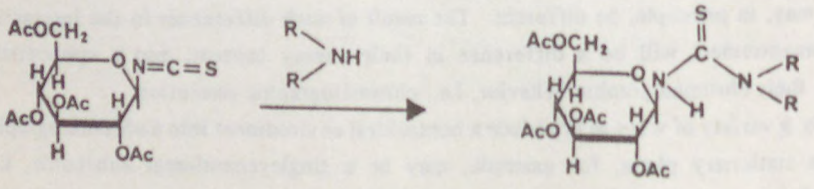
There is a variety of ways to introduce a homochiral environment into a chromatographic system. The stationary phase, for example, may be a single-enantiomer substance, i.e., homochiral. This approach is widely used today in high-performance liquid chromatography (HPLC), with dozens of such homochiral columns, often referred to as "chiral columns", offered by commercial suppliers, and many others described in the research literature. Furthermore, several homochiral gas-liquid-chromatographic (GLC) stationary phases are also commercially available. In another approach, the mobile phase in HPLC is rendered chiral by the addition of a homochiral substance. This approach has the theoretical advantage that the choice of homochiral additive may be made from a large number and a large variety of resolved

compounds but in practice at the present time only a few homochiral mobile-phase systems enjoy popularity.

The third widely used approach to the chromatographic separation of enantiomeric antipodes is based on the use of a homochiral reagent that is made to react with the two enantiomers to be separated. In this situation, i.e., when two enantiomeric substances react with a single enantiomer of another chiral compound, the two products of the reaction are no longer enantiomerically related, but are diastereomers. This means that, unlike enantiomers, they are not mirror images of each other, but are stereoisomers that have, in principle, different physicochemical properties. This, in turn, means that they may be separable in non-chiral chromatographic systems, i.e., using "ordinary" (non-chiral) chromatographic columns and mobile phases. This approach to the separation of enantiomers is often referred to as the "indirect" approach or method, because the enantiomers are not separated per se, but are first converted to diastereomeric derivatives.

The choice of homochiral derivatizing agent (HDA) in the indirect method depends, naturally, on the available functional group(s) in the analyte molecules and on the requirements of the chromatographic system used. A variety of HDAs are available from commercial sources[1] and many others have been described in the literature. While the use of an HDA for the separation of enantiomers requires the observation of certain precautions, this approach has been highly successful in a variety of stereospecific drug analysis problems.

Among the HDAs available from commercial suppliers is a rather remarkable reagent, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT, Figure 1). By virtue of being an isothiocyanate, TAGIT readily reacts with a primary or secondary amine to yield the corresponding thiourea (Figure 1). This reaction is facile and rapid, and is selective for the amino function, inasmuch as hydroxyl and carboxyl groups do not react under the mild conditions that suffice for the formation of thioureas. Since many drugs, synthetic intermediates of drugs, and drug metabolites are amines, TAGIT has proven in recent years of considerable value in HPLC for the stereospecific analysis and chromatographic resolution of chiral amines of pharmacological interest.



TAGIT thiourea

Figure 1. The chemical structure of TAGIT and its reaction with amines.

The first use of TAGIT in the chromatographic resolution of enantiomeric substances was described by Nimura et al., who demonstrated that the enantiomers of amino acid ethyl esters are separable on reversed-phase C_{18} columns as their TAGIT derivatives[2]. It was shown that the thiourea derivatives formed had a high absorption at 250 nm, while the absorption by TAGIT was ca. 1/12 of that of the derivatives[2]. Subsequently, the procedure was extended to the resolution of unesterified amino acids[3]. It is interesting to note that in this case the derivatization reaction was carried out in a mixture of water and acetonitrile to ensure the dissolution of the amino acids, and that the presence of water was not detrimental to TAGIT. The stability of TAGIT in aqueous solutions is an important advantage when the derivatization of polar, water-soluble compounds is considered, and in this regard TAGIT is superior to many other HDAs, such as acid chlorides and isocyanates, that do not tolerate water in the reaction medium.

The ability of TAGIT to separate amino acid stereoisomers proved useful in a study of penicillin biosynthesis[4]. In this work the biosynthetic cell-free cyclization of α-L-aminoadipyl-L-cysteinyl-D-valine (1, Figure 2) to penicillin in extracts of Cephalosporium acremonium was examined[4]. Isolation of the reaction product was followed by derivatization with TAGIT and analysis by HPLC to identify it as isopenicillin N (2, i.e. with the R configuration at the α-amino acid chiral center designated with an asterisk in Figure 2), rather than penicillin N (3, Figure 2). The TAGIT derivatives of 2 and 3 were separated on a C_{18} column, an interesting illustration of the power of the reagent, since the two stereoisomers differ in the configuration of one asymmetric center out of four. It should be pointed out that this example illustrates the separation of epimers, rather than enantiomers, via derivatization with TAGIT.

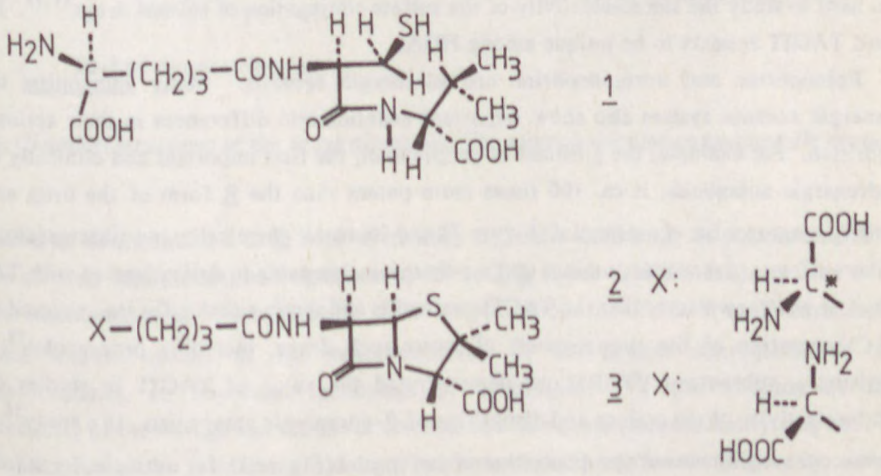


Figure 2. The chemical structures of the penicillins and precursor studied by Neuss et al[4].

The utility of TAGIT was subsequently extended to a variety of other compounds. Nimura et al. reported that the enantiomers of epinephrine and of norepinephrine (Figure 3) can be readily resolved on reversed-phase columns as their TAGIT derivatives[5]. Excellent separation of the diastereomeric derivatives of epinephrine was achieved, while those of norepinephrine were less-well resolved. Epinephrine and norepinephrine are polar and easily oxidizable compounds, whose HPLC resolution is indeed best performed via derivatization with TAGIT, and this was recognized by chemists of the United States Food and Drug Administration, who applied the method to the determination of the enantiomeric purity of epinephrine in various pharmaceutical dosage forms[6,7]. (R)-(-)-Epinephrine is considerably more potent as an adrenergic (vasoconstrictor) agent than its antipode, and is the active ingredient of a variety of pharmaceutical preparations. It is important, therefore, to monitor its enantiomeric purity and stability in drug dosage forms. Using the TAGIT method, enantiomeric impurities of less than 1% could be determined, and it was found in a survey of a variety of intravenous, cardiac, and dental preparations that contamination by (+)-epinephrine was small (<5%) even after the expiration date had passed[6,7]. However, in a similar study by another laboratory also using TAGIT it was found that during long-term storage of (-)-epinephrine injection preparations the proportion of the (+)-isomer increased considerably[8]. After 10-12 years, for example, the range of (+)-epinephrine was 23-34%[8].

It is also worth noting that derivatization with TAGIT followed by reversed-phase HPLC resolution is also suitable for the stereospecific analysis of many other adrenergic agents[9,10]. Particularly valuable is the procedure for drugs that contain a phenolic or a catechol moiety[10], because few other approaches to the chromatographic resolution of such compounds are available. It is also of interest in this context that the phenolic hydroxyl groups of such drugs often undergo metabolic conjugation with sulfate, and this biotransformation may be stereoselective. Derivatization of the amino group of these sulfate metabolites with TAGIT has been used to study the stereoselectivity of the sulfate conjugation of several drugs[11,12]. In this regard TAGIT appears to be unique among HDAs.

Epinephrine and norepinephrine are adrenergic agonists. Many antagonists of the adrenergic nervous system also show important enantiomeric differences in their actions and disposition. For example, the S isomer of propranolol, the first important and clinically useful β-adrenergic antagonist, is ca. 100 times more potent than the R form of the drug as a β-adrenergic antagonist. Propranolol (Figure 3) and its many chemically and pharmacologically similar analogs are secondary amines and are therefore amenable to derivatization with TAGIT. Indeed, it was shown early that the TAGIT method is eminently suitable for the reversed-phase HPLC separation of the stereoisomers of many such drugs, including propranolol[13]. Not surprisingly, subsequent publications demonstrated the value of TAGIT in studies of the enantioselectivity of the actions and disposition of β-adrenergic antagonists. In a study[14] of the pharmacogenetic aspects of the disposition of metoprolol (Figure 3), for example, Lennard et al. used TAGIT to determine the S/R ratio of the drug in the urine of subjects who ingested a single oral dose. Significant differences in the S/R ratios between Caucasians and Nigerians were found; furthermore, within the Caucasian population those subjects that were poor

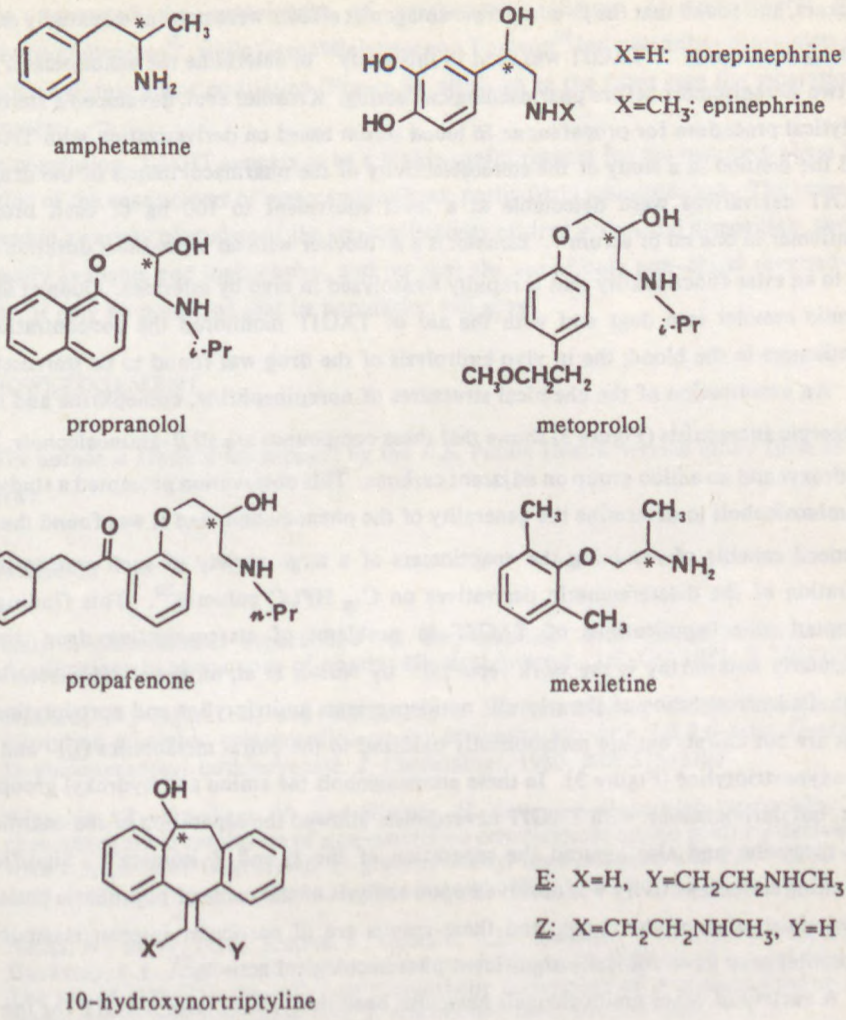


Figure 3. Chemical structures of the drugs discussed. The asterisk denotes an asymmetric center.

metabolizers of debrisoquin, a drug used to identify a genetic deficiency in certain oxidative pathways of drug metabolism, had significantly lower S/R ratios than the subjects who were extensive metabolizers of debrisoquin[14]. Other applications of TAGIT to β-adrenergic antagonists include studies of the stereoselectivity of the sulfate conjugation of 4-hydroxypropranolol, an important metabolite of propranolol[11,15], and a study of the stereoselectivity of the storage and release of atenolol in a line of rat pheochromocytoma cells[16]. Burnett et al. studied the β-adrenergic antagonist properties of propafenone (Figure 3), a new antiarrhythmic drug with a chemical structure similar to those of the above-discussed β-

blockers, and found that the β-adrenergic antagonist effects were present essentially exclusively in the S enantiomer[17]. TAGIT was used in this study[17] to determine the enantiomeric purity of the two stereoisomers before pharmacological testing. Kroemer et al. developed a stereospecific analytical procedure for propafenone in blood serum based on derivatization with TAGIT, and used the method in a study of the stereoselectivity of the pharmacokinetics of the drug[18]. The TAGIT derivatives were detectable at a level equivalent to 100 ng of each propafenone enantiomer in one ml of serum[18]. Esmolol is a β-blocker with an ultra-short duration of action due to an ester functionality that is rapidly hydrolyzed in vivo by esterases. Quon et al. infused racemic esmolol into dogs and with the aid of TAGIT monitored the concentration of the enantiomers in the blood; the in vivo hydrolysis of the drug was found to be stereoselective[19].

An examination of the chemical structures of norepinephrine, epinephrine and of the β-adrenergic antagonists (Figure 3) shows that these compounds are all β-aminoalcohols, i.e., have a hydroxyl and an amino group on adjacent carbons. This observation prompted a study of other β-aminoalcohols to determine the generality of the phenomenon, and it was found that TAGIT is indeed capable of resolving the enantiomers of a large variety of such aminoalcohols via separation of the diastereomeric derivatives on C_{18} HPLC columns[20]. This finding in turn prompted other applications of TAGIT to problems of stereoselective drug disposition. Particularly noteworthy is the work reported[21] by Nusser et al. on the stereoselectivity of the metabolic hydroxylation of the tricyclic antidepressants amitriptyline and nortriptyline. These drugs are not chiral, but are metabolically oxidized to the chiral metabolites (E)- and (Z)-10-hydroxynortriptyline (Figure 3). In these aminoalcohols the amino and hydroxyl groups are far apart, but derivatization with TAGIT nevertheless allowed the separation of the enantiomers of each racemate, and also assured the separation of the E and Z isomers[21]. Significant and interesting stereoselectivity was observed upon analysis of the urine of psychiatric patients after the administration of the drugs, and these results are of particular interest inasmuch as the metabolites may have clinically significant pharmacological activity[21].

A variety of other aminoalcohols have also been derivatized with TAGIT for the purpose of HPLC separation of epimeric and enantiomeric forms[22,23]. In one of these studies[22] the enantiomers of epoxides were resolved via an initial ring-opening reaction with a simple amine to produce enantiomeric aminoalcohols that were, in turn, derivatized with TAGIT.

The reasons for the particularly facile resolution of the TAGIT derivatives of enantiomeric aminoalcohols are not clearly understood, but it is likely that an intramolecular hydrogen bond between the hydroxyl group and the thiourea moiety may be involved. Such bonding will change the overall polarity of the molecule and will result in a reduction of conformational mobility, and it has been suggested that such changes in molecular structure enhance the physicochemical differences between diastereomers which in turn facilitate chromatographic resolution[24].

It should be emphasized, however, that the utility of TAGIT is not restricted to aminoalcohols. For example, as discussed earlier, the enantiomers of amino acids and their esters are separable with TAGIT[2,3]; furthermore, other types of racemates that are not aminoalcohols have also been resolved via HPLC separation of their TAGIT derivatives. Miller et al., for

example, separated the enantiomers of amphetamine (Figure 3) and several related phenylisopropylamines[25], while Grech-Bélanger and Turgeon[26] separated the enantiomers of the new antiarrhythmic agent mexiletine (Figure 3), although in the latter case the separation was not complete.

In conclusion, TAGIT appears to be a highly useful reagent for the reversed-phase HPLC separation of the enantiomers of many amine drugs, particularly aminoalcohols. The reagent has been used in a variety of studies of the stereoselectivity of drug action and disposition, and since it is readily available and inexpensive, and requires the use of only non-chiral reversed-phase columns, it may be predicted that its popularity will grow.

ACKNOWLEDGEMENT

The author is grateful for support by the U.S. Public Health Service in the form of Grant HL 34789.

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Akadémiai Kiadó
Budapest, 1990

GOSSYPOL, THE NATURAL MALE CONTRACEPTIVE: FROM DISCOVERY TO CLINICAL STUDIES

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Discovery of the antifertility effect

Excerpts from the earliest report by Liu Bao-Shan,[1] a traditional herb doctor, on infertility caused by the consumption of cottonseed oil which contained gossypol:

"In the spring of 1929 I accompanied my schoolmate Wang Yin-Min to pay a visit to Wang Village situated at the juncture of the Wu-xi, Jiang-yin and Chang-shu counties in the Jiang-su province. I discovered that the some 30 families in that village were quite well to do owing to their diligence and thriftiness. All the people there ate and clad economically. They chose to consume cottonseed oil as their cooking oil, because it was much cheaper than other kinds of cooking oil. As a result, within the 10-15 years while using cottonseed oil not a single child, be it a boy or a girl, was born to any of the 30 families. For quite a period of time nobody knew what was the reason, why all the families that were quite healthy had had no childbirths. Many farmers tried to take a concubine, but still had no children. Some farmers even tried to marry women who had given multiple births in the past, but when these women came to Wang Village, they immediately became unable to conceive. After some time some farmers became impatient and sent their concubines away to some neighboring villages. To their surprise, these women promptly got pregnant when married to men in other villages. This puzzling phenomenon certainly made the farmers of Wang Village furious. They thought the Almighty was trying to exterminate the people in Wang Village on purpose.

So, for at least 10 years, people in Wang Village were horrified because they thought they were going to be exterminated and did not know what to do. But the tragedy did not last indefinitely. By the early forties, the mass production of soybean in the northeastern provinces made soybean oil much cheaper than cottonseed oil. So the farmers in Wang Village quickly shifted from cottonseed oil to soybean oil for daily cooking. Quite unexpectedly, many of the wives in the thirty families began to conceive and have children.

I have not done any scientific research .... I hope our scientists will strive to do .... because based on the experience in Wang Village, cottonseed oil is definitely effective in preventing conception. And whenever childbirth is desired again, just cease to take cottonseed oil .... This appears to me a very simple and convenient way of contraception."

Unfortunately, Liu Bao-Shan's paper did not arouse appropriate attention until the late 1960's, when many parts of rural China also began to recognize effects of cottonseed oil. Farmers from areas such as the Hubei and Hebei provinces complained of fatigue and of burning in the face, extremities, and other exposed parts of their bodies. The farms raised cotton, but the afflicted people found work in the fields impossible, preferring to hide in the shade and lie on rocks in order to remain cool. Local doctors remained puzzled while the disease reached epidemic proportions. The peasants referred to their disease as "the burning fever".[2]

Burning fever was expecially prevalent in Xingtai, a county in the Hebei province. The local doctors discovered that these peasants consumed raw, homemade cottonseed oil. Commercially produced cottonseed oil had been used in cooking for many years. Only in the 1960's, however, did the peasants begin to make the oil from uncooked seeds, using their own pressing machines. Raw cotton seeds contain the substance gossypol which is destroyed by heat. Unlike the commercial process, preparation of homemade oil does not include heating. Consequently, gossypol remains dissolved in homemade oil. This substance was discovered to be the cause of the burning fever.

As soon as crude cottonseed oil was found to be the source of burning fever, Xingtai doctors advised their patients to stop pressing their own raw oil. The burning and fatigue stopped, but several years later many couples were experiencing infertility problems. A large number of women had amenorrhea. Very few men, however, recovered from their infertility and impotency. Examination of these men revealed azoospermia or oligospermia, and, in addition, some men noted a decrease in testicular size.

Because of the difficult situation, medical and scientific research workers from universities and hospitals were sent to the area to investigate. They confirmed the findings of the local doctors. Infertility was prevalent, and women seemed to recover at a much higher rate than men did. Those men who did recover from infertility were found to have had a lower total intake of cottonseed oil; both time and quantity played a part. This information naturally led people to speculate as to whether measured doses of purified gossypol could be effectively used as a male contraceptive. Observational studies in the country had shown that burning fever, fatigue and infertility were the most common adverse effects of gossypol. Mortality was not observed as a result of burning fever. Because the rate of recovery from male infertility was dependent on the individual amount of cottonseed oil a man had consumed, scientists conjectured that infertility would most likely be reversible if the gossypol dosage could be adjusted.

Pharmacology and toxicology of gossypol: animal experiments

Gossypol occurs in the genus Gossypium. It was first isolated by Marchlewski who coined its name.[3] For many decades, scientists have spent much time and effort trying to develop cotton seeds as food material for humans and animals, since the seeds contain vegetable protein and fat, and occur abundantly in nature. However, owing to gossypol's toxicity, cotton seeds, especially uncooked, proved harmful to most animals as well as poultry.[4] For instance, gossypol had been fed for long periods to cats and rabbits. The animals eventually developed weakness, loss of appetite, and, in severe cases, paralysis, shortness

of breath, cardiac hypertrophy, edema of lungs, etc. Other animals such as rats, dogs and cattle exhibited similar symptoms and signs of poisoning.

The toxicity of gossypol attracted much attention, and research on gossypol was conducted for nearly a century. Although voluminous papers had been published in connection with the pharmacology and toxicology, until 1970 nothing had been reported on the toxic effects of gossypol on the genital systems of the animals.

Chinese scientists devoted more attention to the reproductive aspects of male animals. Among other findings, gossypol's action was found to be focused directly on the testis, specifically to the development of spermatids. Scientists also found that the toxicity of gossypol is dose-related. Thus, if the dosage is minimized, gossypol can exert an antifertility effect without producing pronounced toxicity. Studies using male rats, mice, rabbits, hamsters, dogs and monkeys yielded almost identical infertility results.[5,6] The absorption, distribution and excretion of gossypol were also found to be similar in these animals.[5,6] The biological half-life of (racemic) gossypol in the gastrointestinal tract of the rat is 9.6 hours. Elimination of gossypol takes place mainly through the bile-fecal pathway, while excretion through the kidney is minimal.[7] Elimination of gossypol from the body is slow. It takes a rat 19 days to eliminate 97 % of the dose from its body.[7] Continued administration, therefore, could lead to accumulation.

The order of gossypol distribution throughout the body in all animals is liver, gastrointestinal tract, spleen, lymph nodes, kidneys, heart, lungs, pancreas, salivary glands, muscle, adipose tissue, testes, blood, bladder, brain and spinal cord.[7] Although the testes do not retain much gossypol, sperm cells are vulnerable to the substance. Because gossypol concentration is high in the liver and kidneys, there should be special concern about toxic effects on these organs. Fortunately, since the liver and kidneys can rely on their high regeneration and compensation activity, relatively less harm is done to these organs.

Studies with gossypol enantiomers*

Enantiomers of gossypol are atropisomers; rotation about the single bond in the bis-β-naphthyl derivative is hindered by neighboring methyl and hydroxyl groups. Both enantiomers occur in nature. (-)-Gossypol of 10-25 % enantiomeric excess was isolated from Gossypium barbadense,[8] while apparently pure (+)-gossypol was obtained from the bark Thespesia populnea.[9] Resolution of racemic gossypol has been achieved,[10-11] apparently without establishing the absolute configuration of the enantiomers. The X-ray structure has so far been determined for racemic gossypol only.[12] The two naphthalene rings are almost perpendicular to each other (cf. Figure 1).

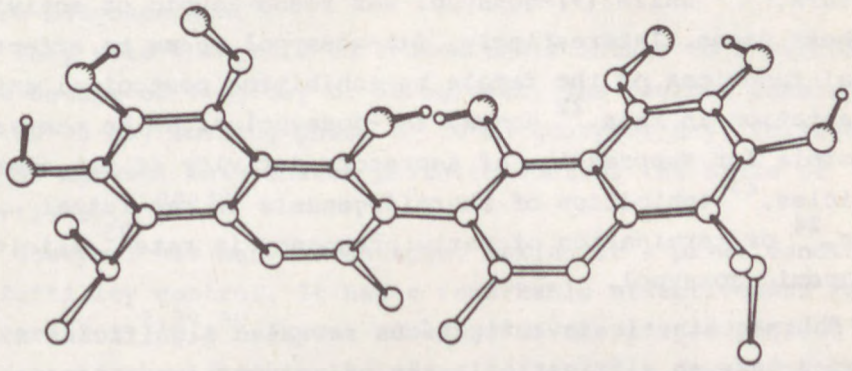

Figure 1. Molecular structure of racemic gossypol crystallized from ligroin (from atomic coordinates published in ref. 12).

The chemical properties of gossypol are determined by its aromatic hydroxyl and formyl groups. Its chemical stability depends on the solvent. HPLC analysis of gossypol revealed extra peaks appearing in ethanolic solution within 15 min, and acetone has been indicated as a better solvent for extraction of gossypol from plant material.[13]

Schiff bases of gossypol are readily formed with primary amines, a reaction investigated for the resolution of racemic gossypol with optically active amines.[10,11] It has been noted that gossypol Schiff bases racemise, while Schiff bases of the

* This section was added by the Editor

hexaacetate of gossypol are enantiomerically stable.[14] However, Schiff base formation in vivo seems to be absent, and the enantiomers appear to have remarkable stability. Gossypol binds strongly to albumin,[15] while gossypol derivatives in which the CHO groups have been converted into CN groups bind even more tightly.[16] Moreover, modification of the ε-amino groups of lysine has no effect on the interaction.[17] Nevertheless, both isopropyl and formyl groups have been found essential for the biological activity of gossypol.[18]

It seems now well established that the antifertility activity of gossypol in males resides in the (-) enantiomer. (-)-Gossypol has stereoselective antispermatogenic effect,[19] it is the fully active enantiomer in male rats,[20] and male hamsters,[9,21] while (+)-gossypol was found devoid of activity in these cases. Interestingly, (+)-gossypol seems to effect sexual functions of the female by inhibiting postcoital antiimplantation in rats.[22] Hence, (+)-gossypol might be the active principle for suppression of secretory activity of rat ovarian follicles,[23] inhibition of steroidogenesis in rat luteal cells,[24] or termination of early pregnancy in rats[25] elicited by racemic gossypol.

Pharmacokinetic investigations revealed significant differences between elimination rates of gossypol enantiomers, with the (+) enantiomer having a slower elimination rate in rats,[26] dogs,[27] and men.[27] This difference is especially large in humans with elimination $t_{1/2}$ of the (+) enantiomer being about 30 times higher than that of (-)-gossypol. The finding that the enantiomer active in males does not accumulate in man provides a good prospect for the clinical application of (-)-gossypol.

Clinical studies testing gossypol as a male contraceptive

In 1972, scientists in Nanjing thought it would be safe to start a phase-one clinical study, using the smallest possible gossypol dosage.[28] Volunteers were carefully monitored with periodic interviews and physical and laboratory check-ups including blood electrolyte estimations, liver and kidney

function tests, and ECG. Ordinary gossypol was used at a dosage of 60 mg/day. At the end of 40 days volunteers showed either azoospermia or severe oligospermia. No side effects were apparent.

In 1973 more hospitals and research institutions from all over China joined the clinical study. More than 10,000 volunteers took gossypol in the form of ordinary gossypol or gossypol acetate. Both forms produced similar antifertility effects. For safety's sake, attempts were made to reduce the dosage even further. It was first reduced to 30 mg/day; then a further reduction was made to 20 mg/day. In 1975 this dose was considered by most researchers to be the optimal dose. At the end of 60-75 days, most patients showed azoospermia or severe oligospermia.[2]

They were then told to reduce their intake to a maintenance dosage of 7 mg/day or 50 mg/week. The regimen consisted of a 60-75 day loading phase of 20 mg gossypol/day, followed by a 50 mg/week maintenance phase to control the state of infertility.[2,29,30]

Gossypol has many advantages, making it a prime candidate for fertility control. It has a remarkable effectiveness rate of over 99 %.[2,29,30] Anyone adhering to the proper regimen is bound to become temporarily infertile. As long as the maintenance dosage is taken, sperm will not appear in the semen. Side effects are not prominent, and there are no alarming symptoms in the loading phase. The cost of gossypol is quite low as cotton is an abundant natural resource.

A paper published in 1978 reported on a period of about 5 years during which more than 4,000 participants from 14 provinces were participating in the clinical trial. Some side effects were present. For example, it was found that 12 % complained of fatigue, 5 % of decreased libido and 5 % of a decrease in appetite.[29,30] The symptoms usually occurred 2-3 weeks after the beginning of the loading phase. They tended to become less apparent, however, toward the end of the loading phase. The symptoms were thus attributed to physiological adjustment to the new substance and usually disappeared after the loading phase. All liver and kidney functions, ECG results,

and blood and urine analyses were normal. In most men the side effects did not recur during the maintenance phase. But there were occasional cases of a condition known as hypokalemia.

Gossypol induced clinical hypokalemia in about 1 % of all cases. The main manifestation of gossypol-induced hypokalemia is a weakening of the voluntary muscles. Afflicted patients are sometimes unable to stand up, to dress themselves, or to get onto a bicycle. In these instances serum potassium can be as low as 2 mEq/liter. There are sometimes bradycardia and typical ECG changes associated with hypokalemia. Gossypol-induced hypokalemia occurred most often in the southern part of China, in areas like the Yangtze Valley. This was considered to be a dietary related phenomenon.[31] People in the south eat a great deal of rice, while in the northern areas, wheat (higher in potassium) is the main grain in the diet.

The mechanism of gossypol-induced hypokalemia is not understood. Potassium is known to be lost through the kidneys, probably the result of gossypol enhancing prostaglandin biosynthesis. This, in turn, leads to renal potassium loss or an inhibitory effect of gossypol on sodium/potassium ATPase.[31] Hypokalemia occurs almost exclusively in the maintenance phase. This may be due to the fact that, in the loading phase, gossypol has not reached the crucial level of toxicity. As a recent study has shown, potassium salt supplementation did not reverse gossypol-related hypokalemia.[32]

Another problem with gossypol arose when a small percentage of men failed to regain their fertility after ending treatment. In a study of 2,067 cases, when patients were administered the standard dose for 1/2 to 4 1/2 years, semen analyses showed a recovery rate of 90.1 %, while 9.9 % remained azoospermic years after gossypol withdrawal.[33]

Biopsies of gossypol-treated animal and human testes revealed similar damage to the seminiferous epithelia.[7] This phenomenon helps to explain the basis of gossypol's antifertility action. It also explains the reason for irreversibility. Extensive gossypol exposure and subsequent severe damage to the spermatogenic epithelium appear to reduce the chance of fertility recovery following cessation of treatment. Clinical obser-

vations disclosed that more irreversible cases occur in men approximately 35 years and older.[33] The germinal epithelium of older men cannot withstand the same amount of gossypol given to younger men. In order to avoid irreversibility, researchers advise that gossypol exposure should not exceed 2 years. Moreover, it is advisable that patients do not take more than a total of 6 g gossypol.[33]

Gossypol's effects, if any, on offspring warrant consideration and further investigation. In the areas of epidemic sterility due to cottonseed oil, the children born after recovery from azoospermia all appeared quite normal.[33] Moreover, 53 women gave birth to normal babies after their husbands ceased taking gossypol.

No clinical studies applying gossypol enantiomers have been disclosed so far.

Conclusions and prognosis

Why do so many researchers consider gossypol the most promising male antifertility agent? Gossypol has been found to have very effective antifertility properties. One advantage, already mentioned, is its availability and low cost. Of even more interest is its mechanism of action. Gossypol acts locally, at the level of the reproductive organs themselves.[34] It does not act indirectly, interfering with hormone production, as do gonadotropin hormone releasing factor and steroid hormones. During the first few weeks of treatment, gossypol acts on maturing sperm stored in the epididymis and renders them immotile. Later, after man has taken gossypol for a period of time, the drug also acts in the testes to check sperm production. These characteristics, the direct action on the motility of mature spermatozoa and on the growth of immature sperm cells differentiate gossypol from other potential chemical fertility regulating agents. Gossypol acts without interfering with the Leydig cells or with the pituitary-gonadal system. It, therefore, should not affect libido and should not exert profound disturbances on the general hormonal regulatory system.

Gossypol is not without its disadvantages. The slow onset

of the antifertility effect is merely inconvenient, while the risks of sterility and hypokalemia are much graver problems. Toxicity is still the main issue of concern. Gossypol has been considered poisonous ever since its discovery almost a hundred years ago. The toxic effects in certain animal species have further alarmed those who already view gossypol's toxicity as an insurmountable barrier. One should remain aware of, though not be alarmed by, the results of animal experiments using dogs and other species which are apparently more sensitive to the toxic effects of gossypol than man. Other animal species, especially primates, exhibit a much lower toxic reaction and require a lower antifertility dose. This is not to say that one ought to be completely optimistic. One cannot deem gossypol the ideal male antifertility agent, ignoring the occurrence of hypokalemia, possible toxic manifestations in the heart and muscle, and permanent sterility.

More research is needed in this area, especially with gossypol enantiomers. Although antifertility action in males is associated with the (-) enantiomer probably also responsible for the risk of sterility, it is absolutely unknown how far does (+)-gossypol contribute to hypokalemia. Besides, the (+) isomer may potentiate the effect of its antipode by competing for the same albumin binding site.[15] Hence, (-)-gossypol alone may well have milder effects than a double dose of the racemate.

Developing a contraceptive requires decades of painstaking research. We are dealing with a drug to be taken by many healthy young and middle-aged people. The world population is increasing steadily, however, and always at a faster pace, especially in recent years. The projected world population for the year 2000 is 6 billion. We cannot rely on the contraceptives already in use. They are not sufficient, and some methods have proved unsatisfactory. In a time when there are not enough male fertility regulating methods available, gossypol appears to be an agent of promise. One cannot expect women only to continue taking the contraceptive measures. Men should share the responsibility. Research must proceed carefully and efficiently. No time should be lost, as population growth will not stop to wait for new scientific findings.

This chapter is based on the following paper:
Liu, G.-Z., and Frick, J. Cottonseed oil for birth control. In Human Fertility, Health and Food., Puett, D. ed. United Nations Fund for Population Activities, New York, 1984, pp. 81-89.

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Akadémiai Kiadó
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Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

COUMARIN ANTICOAGULANTS: THE INFLUENCE OF STEREO-STRUCTURE OF WARFARIN ON METABOLIC BEHAVIOR AND AS A PROBE OF CYTOCHROME P-450

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Some 50 years ago, the toxic principle present in spoiled sweet clover hay that was responsible for the hemorrhagic disorder that afflicted cattle throughout the northern plains of North America in the 1920's, was identified as 3,3'-methylene-bis-4-hydroxycoumarin (dicumarol). Its potential therapeutic usefulness as an orally effective anticoagulant agent was rapidly recognized but because of its poor absorption characteristics analogs having more desirable dispositional properties were sought. Since the initial discovery a number of synthetic analogs have been introduced into clinical medicine but the most well studied and certainly the most popular oral anticoagulant, at least in the United States, is warfarin (Figure 1). It was first

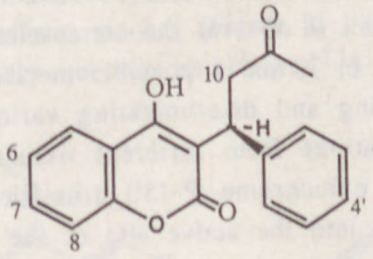


Figure1.
The structure of (S)-warfarin. The numbers indicate the sites of cytochrome P-450 catalyzed hydroxylation.

synthesized at the University of Wisconsin by K. P. Link and was given the name warfarin in appreciation of the financial support provided by the Wisconsin Alumni Research Foundation to do the initial

research. At first warfarin was thought to be potentially too toxic for use in man and as a consequence was initially marketed as a rodenticide. To this day it is still the world's major rat poison and is the active ingredient present in D-CON, a rodenticide that is available in most hardware stores in North America. It was finally introduced into clinical medicine in 1952 and is the mainstay of oral anticoagulant therapy in the United States.

Although warfarin is chiral by virtue of having an asymmetric benzylic carbon, it like many drugs is only available for clinical use as the racemic mixture. Nevertheless, the compound has been resolved and (-)-warfarin has been shown to be approximately 5 times more potent than (+)-warfarin in inhibiting the synthesis of the vitamin K dependent clotting factors in both the rat[1] and humans[2]. The absolute S configuration has been assigned to the (-) isomer by relating it to (-)-(R)-β-phenylcaproic acid through a series of reactions not involving the asymmetric center[3]. This assignment was subsequently confirmed by X-ray crystallographic techniques[4].

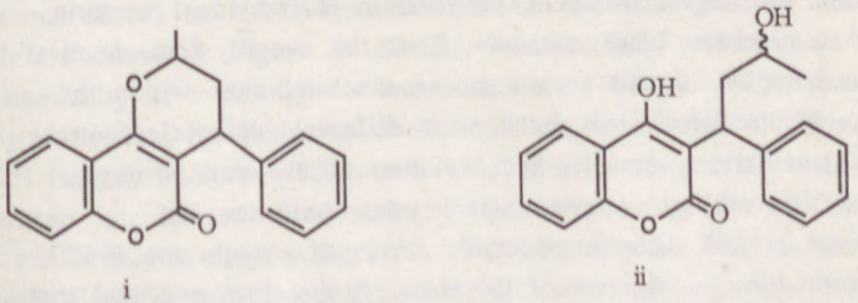
Once the basic stereochemical properties of the molecule had been established, the biological impact of stereoselective processes in its metabolism could begin to be assessed. Such considerations have not only demonstrated considerable value in accounting for a number of seemingly paradoxical drug interactions in man[5] but have offered a powerful, yet subtle probe of the cytochrome P-450 complex in both animal and man. For example, in the rat the stereoselective oxidative profile of warfarin to the 4', 6, 7, and 8 phenolic metabolites has provided a means of assessing and discriminating various induction states in microsomal preparations from different tissues[6]; provided mechanistic information on cytochrome P-450 aromatic hydroxylation[7] and provided insight into the active site of the 3-methylcholanthrene inducible form of the enzyme[8]. Analogous studies have been and are currently being used with human liver preparations to assess enzyme purity, develop a primary catalytic standard for isozyme identification and a standard for correlating the metabolism of diverse drug entities to specific isozymes[9]. Thus, consideration of stereochemical principles has moved warfarin stereoisomerism from the status of a simple phenomenological observation to that of a powerful tool for probing various aspects of drug metabolism. In the

discussion to follow a number of examples will be presented that highlight the kinds of information that can be obtained using this approach.

METABOLISM IN THE RAT

a. *In Vivo*

Despite the fact that warfarin had been introduced into clinical medicine in the early 1950's, the first metabolic study to appear in the literature was a study by Barker et al.[10]. These workers administered ^{14}C labeled warfarin, i.p., to male Holtzman rats and collected and pooled urine and feces for a one-week period. Scintillation counting established that 2/3 of the dose was excreted in the urine while 1/3 of the dose was excreted in the feces. Chromatographic analysis of the excreta and comparison to authentic standards established 4'-, 6-, 7-, and 8-hydroxywarfarin as primary urinary metabolites of the drug in relative amounts of 21%, 15.4%, 35% and 8.9% of the administered dose, respectively. In addition, they identified a reduced ring-closed derivative (Figure 2, i) as a metabolite, 6.6%, together with unchanged drug, 6.6%, and a minor amount, 3.9%, of the glucuronide of 7-hydroxywarfarin. In total, the identified materials accounted for 93.5% of the radioactivity present in urine. It now appears that the ring-closed compound is probably an artifact generated from cyclization of warfarin alcohol (Figure 2, ii) (a metabolite whose structure was subsequently established[11]) in the workup procedure.



i ii

Figure 2.

The structure of i) the reduced ring-closed metabolite of warfarin and ii) warfarin alcohol.

While Barker et al's study did not probe any stereoselective effects that might be associated with the metabolism of warfarin, it did provide the foundation for major metabolic processes involved in the disposition of the drug in both the rat and man. Subsequently, the stereochemistry involved in the metabolism of warfarin was determined by i.p. administration of the ^{14}C labeled individual enantiomers of the drug to male Sprague-Dawley rats[12]. Analysis of the excreta gave a metabolic profile in good general agreement with the study of Barker et al. except for the formation of compound i and the degree of conjugation. In the Pohl et al[12] study compound i was also found but it was presumed to arise from chemical cyclization of the warfarin alcohols which were also present in an amount about equal to that of i. In addition, large amounts of polar metabolites were found to be present in urine which could be liberated by treatment with β–glucuronidase.

In terms of stereochemistry the major metabolic process, 7-hydroxylation, was found to favor (R)-warfarin over (S)-warfarin by a ratio of approximately 2/1. Conversely, formation of 4'-hydroxy-warfarin, the next most abundant metabolite, favored (S)-warfarin as substrate by a ratio of about 4/1. The formation of the 6- and 8-hydroxy metabolites occurred with little apparent stereoselectivity. Formation of the four possible warfarin alcohols appeared to be both substrate- and product-selective for (S)-warfarin. However, this apparent stereochemical preference for warfarin alcohol formation must be viewed with caution, since possible stereochemical differences in the rate and degree of cyclic dehydration of individual warfarin alcohols to generate i are unknown. Thus the overall stereochemical profile is complex in that even adjacent structural sites within the parent molecule are hydroxylated with different stereoselectivities, e.g. 6-hydroxylation versus 7-hydroxylation. These data strongly suggested that multiple enzymes (reductases, oxidases and glucuronidases) and different isozymic forms of cytochrome P-450 were involved in metabolism of the drug. It therefore appeared that the stereoselective biotransformation of warfarin might prove to be a useful probe for identifying and categorizing various enzymes and, in particular, various isozymic forms of cytochrome P-450. It is also interesting to note that the major metabolite produced by the rat is

the biologically inactive (R)-7-hydroxywarfarin from the biologically less potent (approximately 1/5 the activity) (R)-warfarin enantiomer. Just the opposite situation is seen in man, as will be discussed below, in that the major metabolite formed, and at the fastest rate, is the inactive 7-hydroxy product from the biologically most active (S)-warfarin enantiomer. This simple observation would seem to offer a ready explanation for why the rat appears to be particularly sensitive to warfarin poisoning.

b. *In Vitro*

Since cytochrome P-450 is a complex of an unknown number of closely related enzymes whose composition is a function both quantitatively and qualitatively of numerous variables, such as induction state, sex, species, age and nutrition, considerable effort has gone into defining the system in terms of the specific cytochrome P-450's present for any given set of circumstances. One approach to studying the system is to take advantage of the various catalytic profiles exhibited by these enzymes in producing multiple products from a single substrate. As mentioned above, warfarin is such a substrate and has been used by ourselves and others to study the P-450 complex isolated from different sources, in various states of purity and induction. For example, the regioselectivity and stereoselectivity for the aromatic ring-hydroxylated metabolites of the enantiomers of warfarin from microsomes obtained from untreated rats (Sprague-Dawley) and phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), β-naphthoflavone (BNF) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced rats are shown[13] (Figure 3). Clearly, the metabolic profile can change dramatically as a function of induction state. While 7-hydroxylation of (R)-warfarin is the major metabolic pathway in uninduced, PB and PCN induced microsomes, it is only a minor pathway in BNF and TCDD induced microsomes. Conversely, 8-hydroxylation of (R)-warfarin which is only a minor pathway in uninduced, PB and PCN induced preparations is now the major pathway, along with 6-hydroxylation of (R)-warfarin, for the later two induction states.

Closer examination of the quantitative data reveals that whereas the overall metabolism of warfarin was induced in the PB microsomes by about 2-fold on a per mg protein basis, it was essentially unchanged

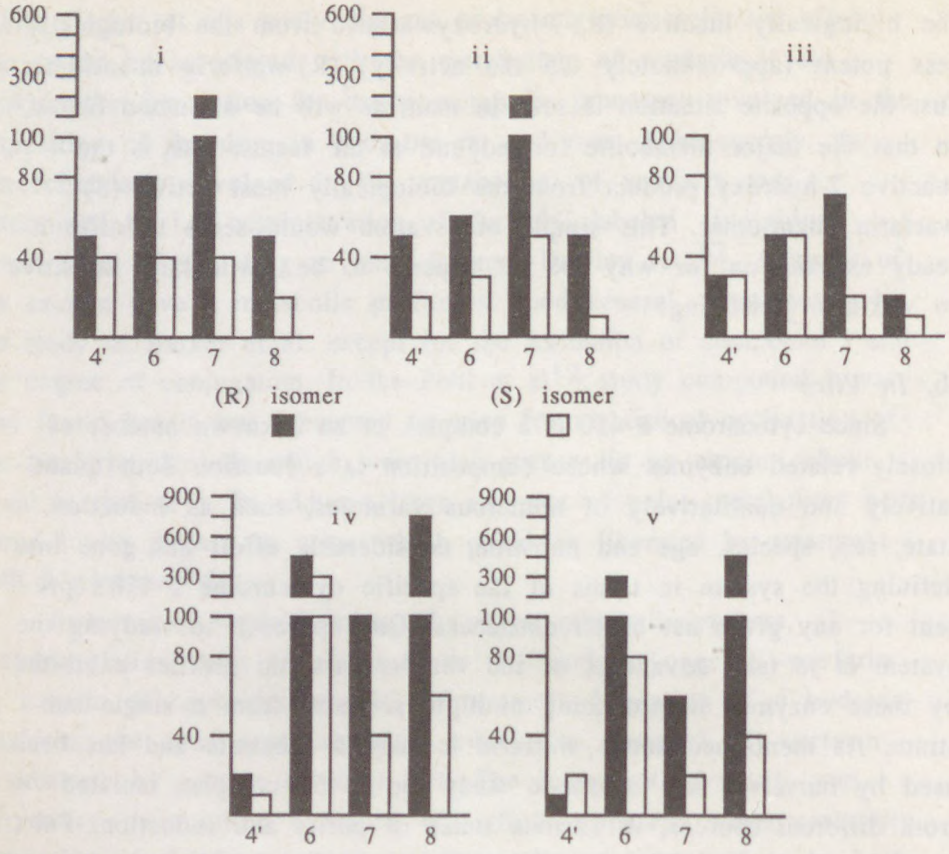


Figure 3.

The stereoselective oxidative metabolism of warfarin in pmol product/nmol P-450/min catalyzed by liver microsomes from rats pretreated with various inducing agents: i) control, ii) PB, iii) PCN, iv) BNF, v) TCCD. The numbers indicate sites of hydroxylation.

relative to the uninduced state on a per nanomole P-450 basis. That is, when the data were normalized with respect to P-450 content, the pattern of metabolism from the two states was essentially indistinguishable, indicating that PB induces all the different isozymes of P-450 involved in the oxidative metabolism of (R)- and (S)-warfarin to equal extents, within the limits of the experimental determination. In contrast, PCN induction resulted in about a 50% decrease in overall metabolism relative to the uninduced state on a P-450 content basis, and an even greater reduction on a per mg protein basis. These data seem to imply that while PCN induces P-450, it induces an isozyme or

isozymes that either do not accept warfarin as a substrate and/or metabolize it at some site within the molecule, other than the aromatic rings, that was not measured, e.g. the aliphatic side chain. Recent data (unpublished results) support this interpretation and indicate that the major site of oxidation with PCN induced microsomes is the methylene position in the side chain (10-hydroxywarfarin). Finally, the quantitative data indicate that with the polycyclic aromatic hydrocarbon (PAH) inducing agents, BNF and TCDD, the overall metabolism is increased as much as 5-fold on a cytochrome P-450 content basis relative to the uninduced state. Similar results had also been found earlier with 3-methylcholanthrene (3-MC) induction, indicating that high levels of 6- and 8-hydroxylation of (R)-warfarin may provide a sensitive marker for the presence of the specific P-450 isozyme involved in the formation of the carcinogenic diol epoxide metabolite of benzo[a]pyrene.

Given the results of these experiments, it was interesting to speculate that while there was no compelling reason to presuppose that an analogous PAH inducible enzyme existed in the human, yet if it did, it might have the same activity towards warfarin. If true, such a relatively non-toxic marker substance could be of considerable use in the *in vivo* detection of such activity in the human being in various industrial and environmental settings. To explore this possibility, we initiated a study in which the stereoselective oxidative metabolism of warfarin by microsomes from human placenta from smoking and non-smoking mothers was compared[14]. The placenta from smoking mothers were characterized by their ability to hydroxylate benzo[a]-pyrene (high PAH activity) and those from non-smoking mothers by the lack of this catalytic activity (low PAH activity). The results of the study are shown (Figure 4). The major metabolite formed by microsomes from the placenta displaying low PAH activity is (R)-7-hydroxy-warfarin followed by (S)-6-hydroxy, (R)-8-hydroxy and finally (R)-4'-hydroxywarfarin. Overall, the degree of stereoselectivity is relatively low. By contrast, the major metabolite formed from placenta displaying high PAH activity is (R)-8-hydroxywarfarin followed by (R)-6-hydroxy, (R)-7-hydroxy and then (R)-4'-hydroxywarfarin. Overall, the system is induced about 7-fold and is highly stereoselective for the (R) enantiomer. The data obtained from placenta with

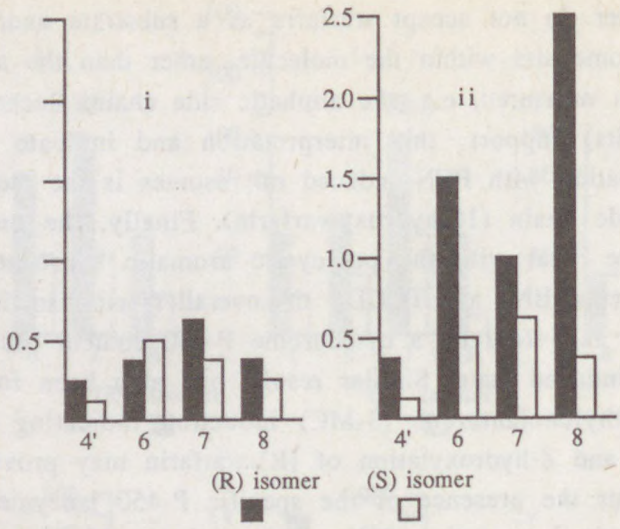


Figure 4.

The stereoselective oxidative metabolism of warfarin by microsomes from the placenta of mothers in pmol product/ mg protein/30 min with i) low PAH activity and ii) high PAH activity. The numbers indicate the sites of hydroxylation.

the high PAH activity are clearly consistent with the results obtained earlier from PAH induced rat liver microsomes and suggests that indeed the human responds to exposure by these toxins to produce a cytochrome P-450(s) that is catalytically similar to that produced by the rat. It is also interesting to note that the metabolic profile obtained from the placenta with low PAH activity is substantially different from that obtained from human liver microsomes (see later). For example, with human liver microsomes the major metabolite found at similar concentrations of substrate (650 μM) is (R)-6-hydroxywarfarin, while (R)-7-hydroxywarfarin is a relatively minor metabolite. These data would indicate that the constitutive forms of cytochrome P-450 in human liver differ markedly, at least quantitatively if not qualitatively, from those constitutive forms present in human placenta.

While the stereoselective metabolism of warfarin was being explored using microsomes from rats pretreated with various inducing agents, analogous studies were also conducted with phenprocoumon (Figure 5), an oral anticoagulant, used extensively in clinical medicine

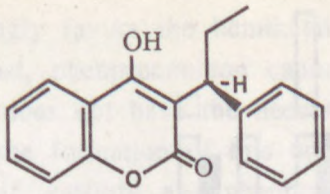


Figure 5.
The structure of (S)-phenprocoumon.

in continental Europe. The chemical structures of the two drugs, warfarin and phenprocoumon, only differ in that phenprocoumon lacks a carbonyl group in the side chain. Thus it is not surprising that the mechanism of action of phenprocoumon, competitive inhibition of the synthesis of the vitamin K dependent clotting factors, is identical to that of warfarin. Nor is it surprising that the biologically most active enantiomer has the S absolute configuration, since this configuration is spatially related to (S)-warfarin, the biologically most active enantiomer of warfarin. What was surprising was the profound differences in the microsomal metabolism of the two drugs. For example, with uninduced rat liver microsomes the rate of metabolism of phenprocoumon is only about 1/5 that of warfarin, while with animals pretreated with PAH inducers the general level of metabolism of phenprocoumon is at least as great as that of warfarin and perhaps even greater[13]. Even more unexpected was the reversal in the stereoselectivity for the major metabolic pathways of the two drugs seen with the PAH induced preparations[13] (Figure 6). For example, after induction with BNF, 6-hydroxylation of (S)-phenprocoumon was increased 45-fold and 8-hydroxylation was increased 62-fold. The formation of both metabolites was highly selective for the (S)-enantiomer with S/R ratios of approximately 6.0 and 2.5, respectively. When the same microsomal preparation was used to catalyze the hydroxylation of warfarin, 6- and 8-hydroxylation were again the major pathways induced (approximately 10-fold and 16-fold, respectively) but with reversed stereoselectivity (Figure 4). That is, (R)-warfarin was preferentially metabolized to form the 6- and 8-hydroxy metabolites by R/S ratios of approximately 2.5 and 12, respectively. Thus the metabolism of the two drugs with regard to 6- and 8-hydroxylation bears an essentially mirror image relationship. The obvious question is: why?

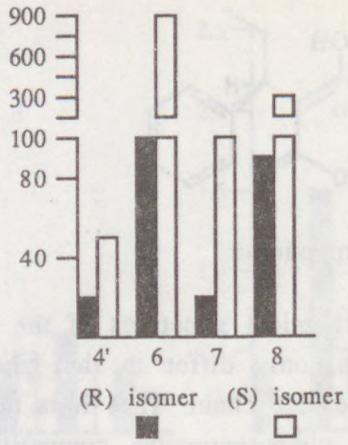


Figure 6.

The stereoselective oxidative metabolism of phenprocoumon by liver microsomes in pmol product/ nmol P-450/min from rats pretreated with BNF. The numbers indicate the site of hydroxylation.

One possible explanation for the phenomenon was provided by studies of the preferred solution conformations of the two drugs[15]. Warfarin is known to exist in solution as an equilibrium mixture of a ring-opened keto tautomer (Figure 1) (as it is normally depicted in most papers and textbooks) and as the diastereomeric ring-closed cyclic hemiketal tautomers[16] (Figure 7). Moreover, it is known that

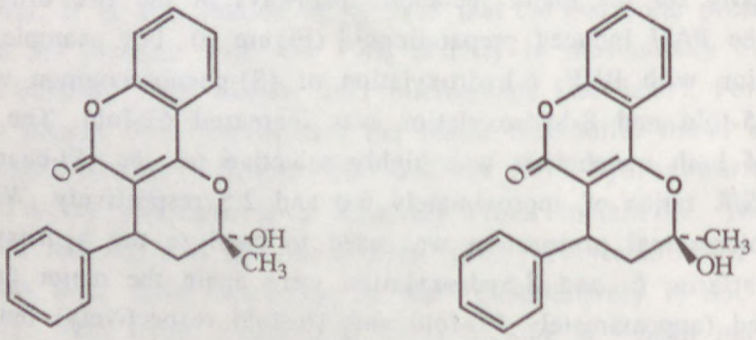


Figure 7.

The structure of the two diastereomeric warfarin cyclic hemiketal tautomers.

the equilibrium strongly favors the hemiketal forms in all solvents examined. By contrast, phenprocoumon cannot adopt an analogous cyclic form since it does not have the necessary side chain carbonyl group that leads to the formation of this tautomeric species. This would suggest that, if warfarin and phenprocoumon are metabolized by a set of isozymes (PAH inducible) at comparable rates with similar regioselectivity, then some conformational similarity between the cyclic hemiketal form of warfarin and the open-chain form of phenprocoumon must exist. That is, one might expect spatially related three-dimensional species to be presented to the enzymes in order to obtain similar rates of metabolism at corresponding sites within the two molecules. NMR studies had indicated that the preferred conformation of phenprocoumon in solution maintains an intramolecular spatial relationship between the coumarin and phenyl rings of the molecule that is identical to that found in the preferred conformation of either of the cyclic tautomeric hemiketal forms of warfarin[15]. In addition, circular dichroism (CD) studies on the resolved compounds revealed that opposite enantiomers of the two drugs gave essentially identical CD spectra above 220 nm, while the corresponding enantiomers gave mirror image spectra[8]. Construction of molecular models of the two compounds in their preferred conformations then revealed that when the relative spatial orientations of the coumarin and phenyl rings were taken as a point of reference, the spatial orientations of (R)-warfarin and (S)-phenprocoumon were essentially superimposable (Figure 8). Thus, if it is assumed that the cyclic hemiketal form of

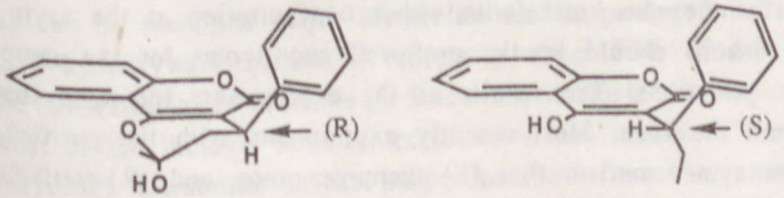
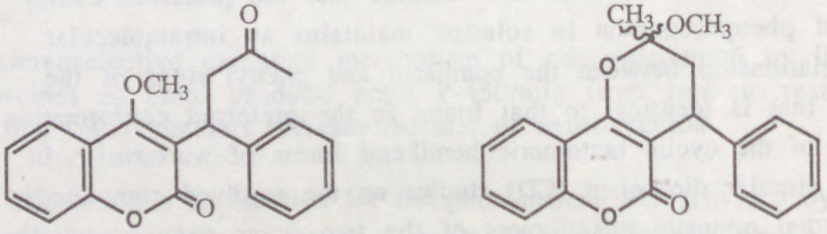


Figure 8.

Comparison of the preferred conformations of (R)-warfarin and (S)-phenprocoumon.

(R)-warfarin is the preferred substrate for PAH inducible enzyme(s), then (S)-phenprocoumon in its preferred conformation would be

expected to be the preferred substrate for the same enzyme(s). To test the hypothesis studies were carried out that demonstrate that when warfarin is extracted from aqueous buffer at physiological pH into a lipophylic environment that is presumed to be closer to the environment of the active site of the enzyme, it rearranges from the open chain form to the cyclic hemiketal form[17]. Model analogs of warfarin, conformationally fixed in either a ring-opened form by methylation of the 4-hydroxyl group to generate warfarin-4-methyl ether (Figure 9) or a cyclic ring-closed form by formation of the cyclic methyl ketal, warfarin methyl ketal (Figure 9), were then synthesized and incubated



i ii

Figure 9.
Structure of i) warfarin-4-methyl ether and ii) warfarin methyl ketal.

with BNF induced rat liver microsomes[18]. If the hypothesis was correct, then analogous to (S)-phenprocoumon, (S)-warfarin-4-methyl ether should be the preferred enantiomer for 6- and 8-hydroxylation, while (R)-warfarin cyclic ketal (with either configuration at the asymmetric ketal carbon) should be the preferred enantiomer for the same regioselective oxidations. The results of the experiments indicated that this was indeed the case. Most recently, experiments with the purified form of the enzyme confirm that (S)-phenprocoumon and (R)-warfarin are the preferred substrates, that they yield the 6- and 8-hydroxy metabolites and that they are mutual inhibitors of each other (unpublished results).

METABOLISM IN THE HUMAN

a. *In Vivo*

Just as in the rat, the metabolism of warfarin in the human is highly stereoselective. A typical excretory profile is presented in Table 1. The data are based on total 15 day recoveries of parent drug

Table 1

A 15 day total urinary excretion of warfarin and its metabolites after a single oral dose of the drug, 1.5 mg/kg, expressed as a percent of each enantiomer

	(R)	(S)	(S)/(R)
Warfarin	3.3	2.1	0.64
6-hydroxy	31.0	18.9	0.61
7-hydroxy	15.4	75.6	4.9
8-hydroxy	23.4	<0.1	<0.01
Alcohol-1	27.1	-	<0.01
Alcohol-2	<0.1	3.0	>30

and metabolites from urine and stool after oral administration of a single 1.5 mg/kg dose of pseudoracemic warfarin[19] (a racemic dose of warfarin in which one of the enantiomers has been selectively labeled with a stable isotope so that the two enantiomers can be quantitatively determined in the presence of each other using mass spectrometry). As can be seen, the major metabolite that is produced is (S)-7-hydroxywarfarin and it represents about 70% of the dose of the (S)-warfarin administered. Since (S)-warfarin is responsible for most of the biologic activity, its selective conversion to the inactive 7-hydroxy metabolite is the major metabolic event involved in modulating and terminating the anticoagulant response attained by administration of the drug. The remaining metabolites of (S)-warfarin in decreasing order of quantitative importance are (S)-6-hydroxywarfarin, (S,S)-warfarin alcohol-2 and (S)-8-hydroxywarfarin. In addition, a total of about 11% of unchanged (S)-warfarin was recovered from urine and stool. In the case of (R)-warfarin, 6-hydroxylation was the major pathway (about 31%). This was followed by

(R,S)-warfarin alcohol-1, (R)-7-, (R)-8-hydroxywarfarin and a trace of (R,R)-warfarin alcohol-2. Unchanged (R)-warfarin from both urine and stool accounted for about 16% of the administered dose. If a simple S/R ratio is computed for each metabolic pathway, Table 1, it is clear that like the rat, multiple isozymes of cytochrome P-450 are probably involved in the metabolism of warfarin. For example, 7-hydroxylation has an S/R ratio of 4.9, while that of 8-hydroxylation, an adjacent site in the molecule, is about 0.01. While these data alone do not exclude the possibility that a single isozyme accounts for the difference in ratios, recent studies with human microsomal[9] and purified P-450's[20] unambiguously establish that multiple isozymic forms of the enzyme are responsible.

The oral anticoagulants, in general, are susceptible to the phenomenon of drug interactions, i.e. the presence of a second drug will often perturb the biologic response elicited by a given dose of anticoagulant. A likely mechanism whereby a second drug could alter the intensity of the biologic response due to the primary medication is by decreasing its rate of elimination through inhibition of the enzymes responsible for its metabolism. In the case of warfarin, since it is metabolized by a number of different isozymes with differing regio-selectivity and stereoselectivity, there is little reason to presuppose that a second drug would inhibit all the isozymes involved in the metabolism of warfarin to the same degree. Indeed, just the opposite outcome would be expected. This can raise serious problems in drug interaction studies that involve a drug that is administered as a racemic mixture. In such a study one attempts to correlate the pharmacologic response to the plasma concentration of a given dose of the primary drug in the presence and absence of the interacting drug. If the secondary drug is exerting its effect by inhibiting the metabolism of the primary drug, then one would expect an increased plasma level and an increased biologic response. However, as indicated above, there is no reason to presuppose that an inhibitor would effect all the metabolic pathways to the same degree. As a consequence, it is possible that the major effect of the second drug is to inhibit the metabolism of the least potent enantiomer, in which case one would observe an increased plasma level of total drug relative to control with little increase in biologic effect. Thus, unless some means is

introduced into the experiment by which the individual pharmacokinetic behavior of the two enantiomers can be monitored in the presence of each other, a meaningful correlation between the plasma concentration of the drug and its pharmacologic effect is essentially impossible to achieve. What is critical in such studies is not the plasma concentration of total drug, but the fractional contribution of each of the enantiomers to the total concentration. This assumes, of course, that the two enantiomers have inherently different degrees of activity.

An example of a drug interaction study that illustrates this point is the interaction between the uricosuric agent sulphinpyrazone and warfarin[19]. In this study six male volunteers were given 200 mg of sulphinpyrazone, twice a day, for 3 days prior to receiving a single oral dose, 1.5 mg/kg, of pseudoracemic warfarin (a mixture containing 50% (S)-warfarin-2-^{13}C and 50% (R)-warfarin). The daily sulphinpyrazone regimen was continued throughout the course of the study (15 days) in which serial plasma, stool, and urine samples were collected for analysis of parent drug and metabolites. In addition, prothrombin times were determined daily to provide a measure of biologic activity. In every subject studied, administration of warfarin and sulphinpyrazone together always resulted in an enhanced anticoagulant response. In Table 2 are listed the elimination $t_{1/2}$'s

Table 2

The plasma elimination $t_{1/2}$'s for subjects D. C. and J. A. in the presence and absence of sulphinpyrazone

Subject	Warfarin $t_{1/2}$ hr	Warfarin + Sulphinpyrazone $t_{1/2}$ hr
J. A.	32	44
D.C.	65	41

and difference in the anticoagulant response seen for subjects D. C. and J. A. in the presence and absence of sulphinpyrazone. What is seen is that while the rate of elimination of warfarin decreases in J. A. in the presence of sulphinpyrazone it increases in D. C. despite the fact

that the anticoagulant response increases in both individuals. On first analysis this result is perplexing because one would normally expect to find an increased biologic response accompanied by an increased plasma concentration of the drug eliciting that response, as is the case with J. A. The question then arises is, how can one rationally account for a decreased plasma concentration of warfarin in the presence of sulphinpyrazone in D. C. being associated with an increased biologic effect? Clearly, there does not appear to be any direct relationship between the plasma concentration of the drug and the observed biologic response. However, if the $t_{1/2}$'s for the individual warfarin enantiomers are determined as shown in Table 3, the data begin

Table 3

The plasma elimination $t_{1/2}$'s for (R)- and (S)-warfarin from subjects J. A. and D. C. in the presence and absence of sulphinpyrazone

Subject	Warfarin $t_{1/2}$ hr		Warfarin + Sulphinpyrazone $t_{1/2}$ hr	
	(R)	(S)	(R)	(S)
J. A.	46.2	18.2	38.5	49.5
D. C.	86.8	49.5	25.4	57.7

to make sense. In both individuals the elimination $t_{1/2}$ for (R)-warfarin is decreased, while that for (S)-warfarin is increased in the presence of sulphinpyrazone. Since (S)-warfarin is at least 5 times as potent as (R)-warfarin, it is responsible for most of the biologic activity that is observed. Thus the effect of sulphinpyrazone on (S)-warfarin is entirely consistent with the biologic response, while its effect on (R)-warfarin has little biologic consequence. Since the total warfarin plasma concentration being measured is the sum of the individual contributions from (R) and (S)-warfarin, it is possible, depending on their relative contributions, that a lower total warfarin plasma concentration will be accompanied with an enhanced biologic response. This is exactly what happens with D. C. The decrease in the $t_{1/2}$ of (R)-warfarin in the presence of sulphinpyrazone is so great

(87 to 25 hrs.) as to completely overwhelm the increase in the $t_{1/2}$ of (S)-warfarin (49 to 58 hrs). Thus, while the total warfarin plasma data are confusing and reveal nothing,consideration of the enantiomeric data indicates that the overall interaction mechanism is probably a consequence of competitive inhibition of the biotransformation of the biologically more potent (S)-warfarin enantiomer to the inactive (S)-7-hydroxywarfarin metabolite. Analysis of the metabolites, in the same study, indicated that this was indeed the case and that inhibition of the formation of the other metabolites also occurred but to varying degrees. Finally, the increase in elimination (decrease in the plasma $t_{1/2}$) of (R)-warfarin in the presence of sulfinpyrazone was found to be due to a stereoselective displacement of this warfarin enantiomer from plasma protein binding sites[21].

b. *In Vitro*

The importance of the status of the drug metabolizing complex to drug response has long been recognized, but a means to effectively assess inherent metabolic activity and to correlate *in vivo* and *in vitro* results is still lacking. The relatively recent discovery of genetically determined variants of cytochrome P-450 and the correlation of specific isozymes to specific metabolic transformations has stimulated new work in this direction. If subjects are available that lack a specific cytochrome P-450 enzyme activity, e.g. the isozyme responsible for the benzylic hydroxylation of debrisoquine or the 4'-hydroxylation of (S)-mephenytion, then correlating the activities of either of these specific isozymes to specific metabolic pathways seen in other drugs is a relatively straightforward process. However, the power of the technique is limited to the enzymatic activity of the few isozymes presently known to exhibit polymorphism and to the small number of individuals (less than 10% of the Caucasian population) known to have a deficiency.

An alternate but complementary approach is to use a substrate that is metabolized to different products by a number of different isozymes. One can then use changes in the quantitative production of the different products under different conditions (presence of an inhibitor) to monitor and identify specific enzyme activities. If the substrate is also chiral, then one has the added advantage of using

stereoselective differences in the various metabolic pathways as a further means of segregating different enzyme activities. Warfarin is such a substrate. Wang et. al.[20] have demonstrated that at least 5 purified forms of human cytochrome P-450 contribute to the oxidative metabolism of (R)- and (S)-warfarin. However, only a single isozyme, P-450$_7$, catalyzes formation of (R)-6-hydroxywarfarin. Cytochrome P-450$_7$ also catalyzes formation of some (S)-7-hydroxywarfarin as does P-450$_8$. Since these two products were the major metabolites in a majority of 33 human liver preparations investigated by Kaminsky et al.[9], it would appear that quantitative stereoselective warfarin metabolism could be an effective tool to monitor and characterize the catalytic properties of at least these isozymes.

We have begun a program to explore the possibility of correlating the *in vivo* and *in vitro* metabolism of warfarin. The intent of these studies is to elucidate and establish the parameters necessary for such a correlation and then to attempt to predict *in vivo* drug interaction based on *in vitro* data. The approach we are taking is to first reproduce *in vitro* a known, well characterized, *in vivo* drug interaction. If this can be achieved, the reverse experiment will then be tried. Prior to examining the effects of an interacting drug on the metabolism of warfarin *in vitro*, it was first necessary, as mentioned above, to construct an *in vitro* model that would reflect the oxidative metabolic profile of warfarin *in vivo*. To this end the kinetics of the oxidative metabolism of (R)- and (S)-warfarin by human liver microsomes were studied in detail[22] and it was found that the quantitative pattern of oxidized products changed dramatically as a function of substrate concentration. Apparent K_m values for the formation of 4', 6, 7 and 8-hydroxywarfarin indicated the presence of two easily distinguishable subsets of human liver cytochrome P-450; a high affinity subset (K_m 3-15μM) and a low affinity subset of isozymes (K_m > 200μM), Table 4. The high affinity subset is primarily responsible for the metabolism of (S)-warfarin, while the low affinity subset is largely responsible for metabolism of (R)-warfarin. Thus the stereoselective profile that is obtained for the metabolism of pseudoracemic warfarin either *in vivo* or *in vitro* can be expected to depend heavily on the concentration of substrate to which the enzymes are

Table 4

Representative apparent V_{max} (pmol product/nmol P-450/min) and K_m (μM) parameters for the formation of phenolic warfarin metabolites by human liver microsomes

Metabolite	V_{max}	K_m
(R)-4'-hydroxy	22	180
(R)-6-hydroxy	593	738
(R)-7-hydroxy	85	498
(R)-8-hydroxy	95	405
(S)-4'-hydroxy	55	455
(S)-6-hydroxy$_1$	31	388
(S)-6-hydroxy$_2$	5	4.3
(S)-7-hydroxy	12	5.6
(S)-8-hydroxy	7.4	360

exposed. The problem then becomes, what *in vitro* values are to be compared to what *in vivo* values. Clearly, one would want to use some parameter that is independent of substrate concentration. A parameter that meets this requirement *in vitro* is a V_{max}/K_m value, since it is a measure of the intrinsic ability of an enzyme to process a substrate. The corresponding parameter *in vivo* is the intrinsic formation clearance for metabolite formation. The intrinsic formation clearances for the various warfarin metabolites that were determined in an earlier drug interaction study[21] were totaled and each value is presented as a percent of the total, Table 5, together with percent of total V_{max}/K_m values (averaged values from three different human liver microsomal preparations) obtained in the recent study. The data in Table 5 were normalized in this way (as a percent of total) to provide a common base for comparison, since units of V_{max}/K_m values are in ml/min/μmol P-450, whereas intrinsic formation clearances are in L/hr. Inspection of Table 5 reveals that the *in vitro* kinetic parameters accurately predict the rank order of formation clearances obtained from the *in vivo* experiment for each of the phenolic warfarin metabolites with respect to both regio- and stereoselectivity. What remains to be demonstrated, is that addition of the

Table 5

Comparison of the percent V_{max}/K_m values (*in vitro*) to the percent intrinsic formation clearances (*in vivo*) for the phenolic metabolites of warfarin

Metabolite	% V_{max}/K_m	% intrinsic formation clearance
(R)-4'-hydroxy	1.3	0
(R)-6-hydroxy	12.0	9.2
(R)-7-hydroxy	2.4	4.7
(R)-8-hydroxy	4.0	6.4
(S)-4'-hydroxy	3.1	0
(S)-6-hydroxy	25.0	16.3
(S)-7-hydroxy	52.3	63.1
(S)-8-hydroxy	0.2	0.3

interacting drug to the microsomal preparations accurately reflects what occurs *in vivo*. Based on the data that have already been obtained with warfarin alone, even though limited, it appears that the approach offers an excellent chance for success.

In conclusion, it is hoped that the reader will leave this chapter not only with an appreciation for the importance of stereochemical discrimination in biological systems, but with an awareness of the power that stereochemical considerations can provide to the investigator as a research tool.

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Akadémiai Kiadó
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Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

METABOLIC CHIRAL INVERSION: IBUPROFEN

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1. INTRODUCTION

Manaton Murderer Hanged
The Morning Express, November 30, 1930.

In 1929, George Harrison, aged 56 years, Head of the Accounts Department of Messrs. Frobisher, Wiley and Teddington, Electrical Engineers, was found dead in his little cottage, called 'The Shack'. George was fond of eating natural foods, particularly mushrooms, collected from the English woods.

At the inquest of December 5, 1929, Sir James Lubbock, the Home Analyst, gave evidence that he had identified a considerable quantity of muscarine, the poisonous principle of the fungus, *Amanita muscaria*, in the stomach and vomitus and in an unconsumed portion of the fungus. Under questioning from the Coroner, Sir James stated that he had not the slightest doubt that Harrison had died of poisoning by muscarine, ingested in the preparation of fungus, submitted to him for analysis. The jury, after a brief consultation, brought in a verdict of Accidental Death, due to poisoning by *Amanita muscaria*.

This would have been the end of events except for John Bunting, an artist, novelist and friend of the family who did not believe that Harrison had mistaken the mushroom for an edible variety. In the course of his sleuthing activities, a scientist recounts to Bunting how it is that "life is a kind of bias - a lopsidedness so to speak" and that it was possible to differentiate a substance of natural origin, one formed by living tissue, from that produced synthetically in the laboratory. The means of distinguishing the two forms, the racemate from the

natural product, was to place solutions of the muscarines in a polarimeter, where 'so long as the artificial substance remains.....in the racemic form...this would be optically inactive, while that from the living tissues would rotate the beam of polarised light.'

With this startling information, Bunting is off to Sir Lubbock's laboratory. The sceptical Sir Lubbock is persuaded to view a solution of the muscarine isolated from the fungal preparation under the polarimeter, whereupon they discover that the Laws of Nature have been suspended and that this muscarine preparation is racemic.

The source of the racemic synthetic muscarine was linked to Mrs Harrison's lover and so it ensued that the Morning Express carried the headline on November 30, 1930, "Manaton Murderer Hanged."

The Documents in the Case
Dorothy L. Sayers & Robert Eustace[1]

There has been some criticism of the scientific validity of this murder mystery[2], but there was no flaw in the concept which makes the clear and important distinction between synthetic drugs and those produced by nature. So it is today that many synthetic drugs are racemic while the natural products are usually enantiomerically pure.

If, however, muscarine readily racemised during cooking, as would have been the case for hyoscyamine, the link between Mrs Harrison's lover and George's death might not have been so readily established. Under these circumstances there would have been no way to differentiate the natural from the synthetic alkaloid.

It should not be too surprising to discover that other drugs, but not muscarine to my knowledge, racemise in aqueous solution under physiological conditions (Table 1). Rates of racemisation vary from very rapid for hyoscyamine, oxazepam and oxyphenbutazone, to a more leisurely, but still biologically significant rate for the phthalidomide anaolgue, EM-12.

Interestingly, the susceptibility of arbaprostil, an inactive R-15-methylprostaglandin analogue, to racemise to produce the active S-enantiomer, has been used to deliver the S-enantiomer at a rate controlled by the pH of the gastric contents. This elegant approach

helps to minimise the amount of active enantiomer available for systemic absorption with consequent potential for adverse effects. It should be stated, however, that more often than not, the enanantiomeric forms of drugs are relatively stable and interconversion of enantiomers is the exception rather than the rule.

Table 1.
Examples of Drugs which Racemise 'Spontaneously' in Solution

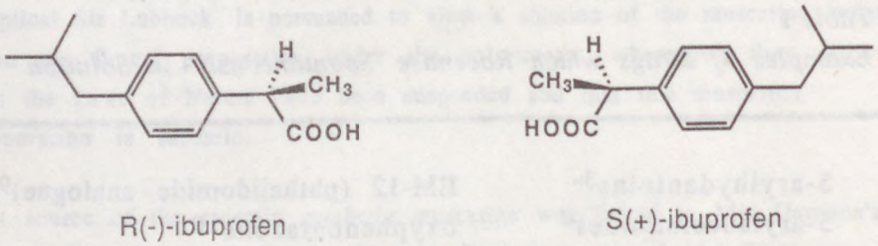
5-arylhydantoins[3]	EM-12 (phthalidomide analogue)[9]
5-arylsuccinimides[4]	oxyphenbutazone[10]
oxazepam[5,6]	hyoscyamine[11]
15R-15-methyl-PGE_2[7,8]	

While racemisation may occur spontaneously as in the above examples, it also may be mediated by enzymes specific for this purpose. Thus alanine racemase for example, provides D-alanine for the D-alanyl-D-alanine dipeptide used by bacteria for the synthesis of the peptidoglycan layer of their cell walls.

In contrast to racemisation which thus can occur either enzymatically or non-enzymatically, unidirectional inversion of one enantiomer to the other needs chiral intervention. Consequently, it was very interesting to discover that there was unidirectional inversion of a commonly prescribed non-steroidal anti-inflammatory drug, ibuprofen (Fig. 1) *in vivo*. It is this novel biotransformation and the consequences of the mechanism by which this transformation takes place which is the subject of this chapter. The apparent association with intermediary metabolism of lipids is one of particular import as is the role of coenzyme A (CoA) thioesters in the metabolism of not only these, but other carboxylic acid xenobiotics.

The interconversion of one enantiomer to the other might, at first glance, appear to be a trivial metabolic transformation in comparison to other oxidative and conjugative modes of metabolism. Inversion results in a product which has the same molecular formula, interatomic distances, polarity and solubility as the parent. But as

illustrated by the contents of this chapter, biochemically and pharmacologically, inversion of configuration is in many instances of greater import than any other possible metabolic modification.



R(-)-ibuprofen S(+)-ibuprofen

Figure 1. The enantiomers of ibuprofen. S-ibuprofen is an inhibitor of prostaglandin synthetase. The R-enantiomer is inactive in this respect.

2. CHIRAL INVERSION

Ibuprofen belongs to the chemical class of 2-arylpropionates which represents the largest single group of drugs used in the amelioration of the pain and inflammation associated with the arthropathies such as rheumatoid arthritis (Table 2). They have also found wider application as analgesics for the treatment of pain associated with dysmenorrhoea and metastatic bone cancer, and more recently in over-the-counter formulations for the self-medication of headache and minor pain.

Perhaps the most impressive feature of this array of drugs, however, is the diversity of the aromatic functionalities which although modifying or fine tuning some of the general characteristics of the drugs, do not change their mechanism of action ie. inhibition of prostaglandin synthesis. In contrast, a change in the chirality of these drugs from the S to the R-configuration results in a total loss of inhibitory activity. Thus the most interesting features of drugs related to ibuprofen are that: (i) they are asymmetric, (ii) with the exception of naproxen they are administered as their racemates, (iii) only the S-

enantiomers inhibit prostaglandin synthesis, and (iv) there is *in vivo* inversion of the inactive R-enantiomer to the active S-enantiomer.

Table 2.
Typical 2-Arylpropionic Acid Anti-Inflammatory Drugs

benoxaprofen	indoprofen
carprofen	ketoprofen
fenoprofen	naproxen*
flurbiprofen	pirprofen
ibuprofen	tiaprofenic acid

*The S-enantiomer; all other drugs racemic.

These pharmacokinetic and pharmacodynamic characteristics of the enantiomers are a clear reminder of the importance of chirality in drug disposition[12,13]. The focus of this chapter is on the chiral inversion of ibuprofen[14] and, in particular, emphasises that to understand a drug, one must understand the chirality of its action and disposition. Additionally, there is the continual reminder that the best way to probe a chiral system is with a chiral agent.

2.1 Inversion in man

The first evidence of this interesting phenomenon came from data for ibuprofen in man. Urinary metabolites were determined to be dextrorotatory after administration of racemic drug[15] but more telling were the data showing that metabolites were dextrorotatory regardless of whether the R or the S-enantiomer was administered[16]. Plasma concentrations after separate administration of the enantiomers to a volunteer clearly illustrate the unidirectional nature of the inversion process (Fig. 2)[17,18]. However, inversion of configuration has only been demonstrated unequivocally in man for two other arylpropionates, fenoprofen[19] and benoxaprofen[20]. No significant inversion was detected in man for indoprofen[21] or flurbiprofen[22] while the data are also suggestive that tiaprofenic acid[23], ketoprofen[24,25,26] and carprofen[27] are not inverted in man.

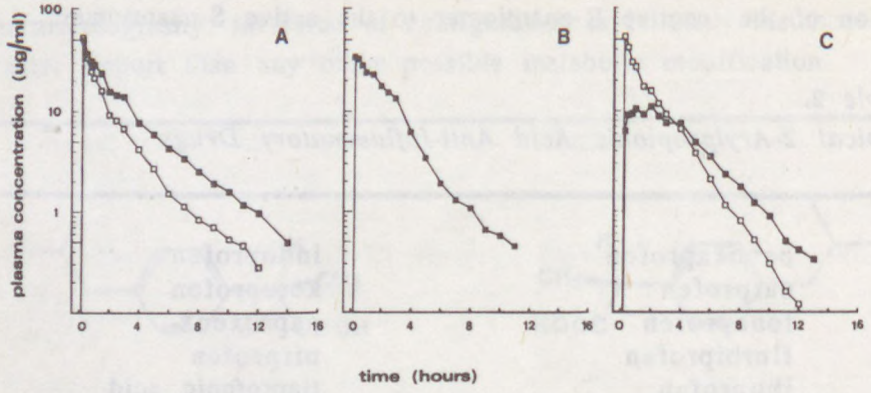


Figure 2. Plasma concentration-time profiles of the R (□) and S-enantiomers (■) of ibuprofen in a healthy volunteer after oral administration of solutions of: A. 800mg R,S-ibuprofen; B. 400mg S-ibuprofen; C. 400mg R-ibuprofen[17]. (Reproduced with permission of the copyright owner, the American Pharmaceutical Association).

2.2 Inversion in animals

Complementary data came from studies in animals where it was found that while R-ibuprofen did not inhibit prostaglandin synthetase *in vitro*, it was active *in vivo* in animal models of inflammation. This was reflected by a change in the potency ratio (S/R) of the enantiomers from 160 *in vitro* to 1.3 *in vivo*[28]. Similarly, it was found that clidanac for example had a potency ratio of 1000 *in vitro* (guinea pig skin microsomes[29]) but *in vivo* the ratio was 1 (ultraviolet erythema model, guinea pigs[30]). However, *in vitro* and *in vivo* potencies were the same for other 2-arylpropionates such as carprofen (>16 *in vitro*, 14 *in vivo*[31]) suggesting that this drug was not significantly inverted. The animal data suggest that there is more diversity in the inversion process across animal species than there is in man. Future comparative studies between species will give valuable insight into the mechanism and potential consequences of the inversion process.

3. MECHANISM OF INVERSION

The mechanism by which the novel inversion process occurs has already become the focus of some very interesting studies which have given insights into areas not previously considered. It is this increased insight obtained by investigating biochemical and pharmacological systems with asymmetric probes which is the great reward of such studies.

Based on the observation that metabolites were dextrorotatory regardless of whether R or S-ibuprofen was administered, it was concluded that inversion occurred during oxidation of the isobutyl side chain[16]. Thus it was also suggested that binding must occur both near the asymmetric carbon atom and to the isobutyl group. Later studies using deuterium labelled analogues of ibuprofen led to the belief that there was loss of a methyl hydrogen from ibuprofen via the action of a dehydrogenase on the CoA thioester to form the methylene intermediate (Fig. 3)[32].

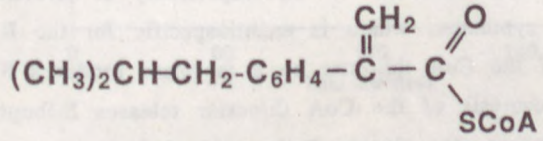


Figure 3. Proposed methylene intermediate formed by the action of a dehydrogenase on R-ibuprofen CoA thioester. A stereospecific enoyl reductase reforms the CoA thioester, but of the S-configuration[32].

Further studies showing retention of the 2-methyl hydrogens, have recently discounted these data[33]. It is now reasonably established that the key intermediate is the ibuprofen-CoA thioester itself. In summary, the stereospecificity of inversion is believed to be controlled by the CoA synthetase. Only the R-enantiomer is a substrate for this enzyme. The racemase then acts on R-ibuprofen-CoA thioester to produce the S-CoA thioester. Finally hydrolases of unknown specificity cleave the R and S-CoA thioesters (Fig. 4)[34].

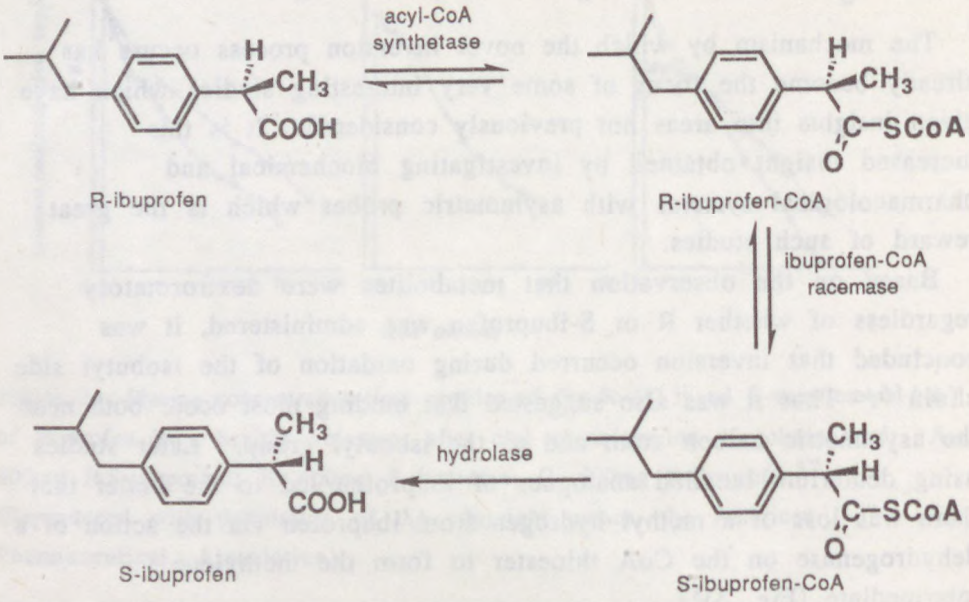


Figure 4. Mechanism of inversion of ibuprofen and related anti-inflammatory drugs as first proposed by Nakamura *et al*[34]. Stereospecificity of inversion is controlled by the acyl-CoA synthetase which is enantiospecific for the R-enantiomer. Racemisation of the CoA thioester by a racemase produces S-ibuprofen-CoA. Finally, hydrolysis of the CoA thioester releases S-ibuprofen. The same or a similar hydrolase also cleaves R-ibuprofen-CoA.

3.1 Role of coenzyme A thioester formation.

Nakamura and coworkers determined that inversion of R-ibuprofen by rat liver homogenate required coenzyme A and ATP as cofactors. Additionally, they reported that while S-ibuprofen was not inverted, S-ibuprofen-CoA was inverted. Further data supporting the enantiospecific control dictated by the acyl-CoA synthetase come from two sources. The first is indirect evidence from studies of the specificity of incorporation of ibuprofen[35] and fenoprofen[36] into lipids (See 2.4.2). The second is a study of the specificity of acyl-CoA synthetase isolated from rat liver microsomes[37]. In this latter elegant

study, the acyl-CoA synthetase was adsorbed to an agarose affinity chromatography column. Solutions of the enantiomers of fenoprofen were recirculated through the column. There was disappearance of R-fenoprofen from the solution with formation of the CoA thioester. S-fenoprofen was not a substrate (Fig. 5). Similarly, we found that R-ibuprofen, but not S-ibuprofen, was a substrate for the synthetase[38].

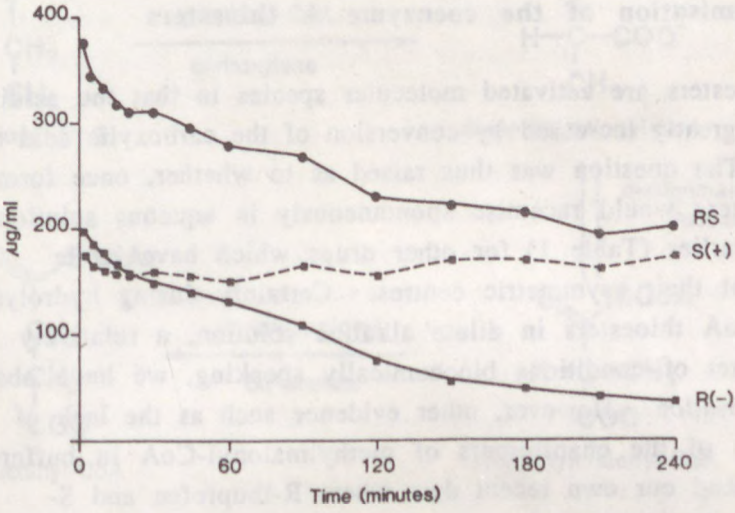


Figure 5. Long chain acyl-CoA synthetase was adsorbed to a Matrex gel Red A column. A solution of R,S-fenoprofen was recirculated through the column over 4 hours. The concentration of R-fenoprofen decreased by 82% over 4 hours, while the S-enantiomer was unchanged[37]. (With permission of the authors and Pergamon Press).

It is of interest that tetradecylglycidic acid, which is an epoxide analogue of the 2-arylpropionates and a hypolipidaemic agent, had previously been shown to form the CoA thioesters in an enantioselective, but not enantiospecific, manner[39]. There are several other recently identified CoA synthetases, such arachidonyl-CoA synthetase[40], which have been shown to be distinct from the long

chain acyl-CoA synthetase. It will be of interest to determine the relative enantiomeric specificities of such enzymes.

Perhaps the most important observation from these data is that, as for the determinants of inhibitory activity of prostaglandin synthetase, chirality is a much more critical factor determining whether the 2-arylpropionate is a substrate, than apparently more significant structural changes, such as the chain length or the degree of unsaturation of the fatty acid.

3.2 Racemisation of the coenzyme A thioesters

The thioesters are activated molecular species in that the acidity of the 2-H is greatly increased by conversion of the carboxylic acid to its thioester. The question was thus raised as to whether, once formed, CoA thioesters would racemise spontaneously in aqueous solution[41] as illustrated earlier (Table 1) for other drugs which have labile hydrogens at their asymmetric centres. Certainly during hydrolysis of ibuprofen-CoA thioesters in dilute alkaline solution, a relatively aggressive set of conditions biochemically speaking, we have observed some racemisation. However, other evidence such as the lack of racemisation of the enantiomers of methylmalonyl-CoA in buffered solution[42] and our own recent data where R-ibuprofen and S-ibuprofen CoA thioesters were stable at physiological pH[38] are reasonable evidence that this is an enzyme-mediated process. (In fact the CoA thioesters are diastereomerically related and the enzyme, strictly speaking, is an epimerase).

It is interesting to speculate on the endogenous function of the 2-arylpropionate-CoA racemase. One analogous enzyme which comes to mind is methylmalonyl-CoA racemase, an enzyme with similar catalytic activities as the 2-arylpropionate-CoA racemase. Conversion of propionyl-CoA by intermediary metabolism to methylmalonyl-CoA occurs enantiospecifically with formation of the S-enantiomer (Fig. 6). For unknown reasons, the subsequent mutase which forms succinyl-CoA, requires the R-enantiomer. The intermediate step, racemisation, provides the substrate in the appropriate configuration. In fact, it was the observation that synthetic methylmalonyl-CoA, but not enzymatically synthesised thioester, was a substrate for the mutase

which led to the characterisation of methylmalonyl-CoA racemase[43]. Again we see the contrast between the synthetic racemic drug and that produced biologically. Paradoxically, it was the synthetic methylmalonyl-CoA (or at least the 50% which is of the R-configuration) which was a substrate, while the enzymically synthesised drug, being of the S-configuration, was not a substrate.

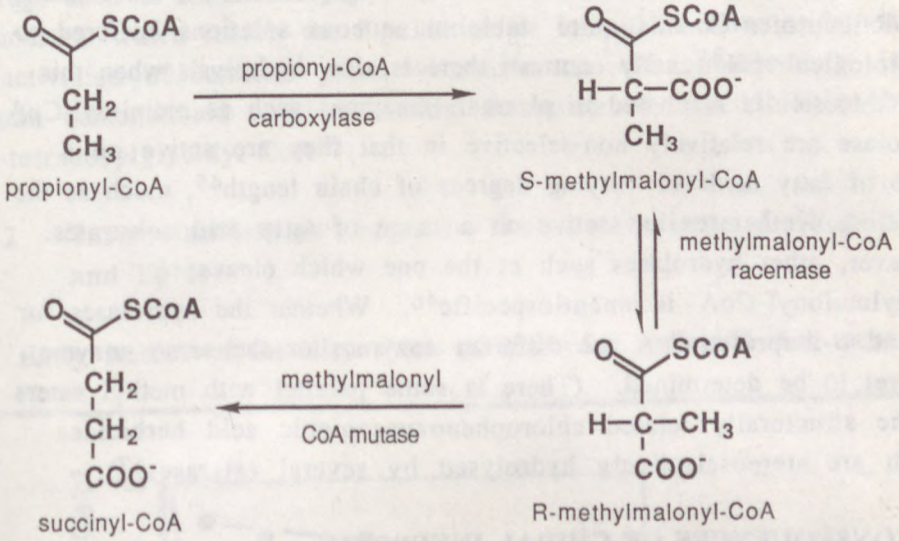


Figure 6. Propionyl-CoA is produced by catabolism of the amino acids valine, isoleucine, methionine and threonine, by degradation of cholesterol and by ß-oxidation of odd-chain fatty acids. The methylmalonyl-CoA produced is of the S-configuration. However, the mutase which subsequently converts it to succinyl-CoA only acts on the R-CoA thioester. A racemase (strictly, an epimerase) provides the appropriate conformation for the mutase.

The diversity of inherited diseases suggests that there might be cases of methylmalonyl aciduria attributable to a defect in the racemase as well as the mutase. One such case was reported[44]. However, it was subsequently found that this was due to a defect in the mutase. It is particularly interesting to speculate that there may

be individuals who lack the enzymes capable of racemising 2-arylpropionate-CoA thioesters, or for that matter, of forming the CoA thioesters.

3.3 Hydrolysis of coenzyme A thioesters

There are no data on the characteristics of the enzymes responsible for the hydrolysis of the CoA thioesters of ibuprofen. We have found that R-ibuprofen-CoA is quite stable in aqueous solutions buffered to physiological pH[38]. By contrast, there is slow hydrolysis when this CoA thioester is incubated in plasma. Enzymes such as palmitoyl-CoA hydrolase are relatively non-selective in that they are active on a range of fatty acids of varying degrees of chain length[45], much as the acyl-CoA synthetases are active on a range of fatty acid substrates. However, other hydrolases such as the one which cleaves S-methylmalonyl-CoA is enantiospecific[46]. Whether the hydrolases for R- and S-ibuprofen-CoA are different enzymes or the same enzyme, has yet to be determined. (There is some parallel with methyl esters of the structurally related chlorophenoxypropionic acid herbicides which are stereoselectively hydrolysed by several esterases[47]).

4. CONSEQUENCES OF CHIRAL INVERSION

4.1 Coenzyme A thioesters - Toxic metabolites?

CoA thioesters are not the first metabolites which come to mind when one thinks of the disposition of drugs, but they may in fact be very important in the understanding of the disposition and toxicities of drugs of the carboxylic acid class. The toxicities of the inherited acidurias, such as methylmalonic aciduria, are in part a consequence of the severe disruption of metabolic function due to accumulation, not so much of the free acids, but of the CoA thioesters. Jamaican vomiting sickness, an often fatal disease, is an example of a CoA thioester-mediated toxicity[48,49]. Toxicity results from eating the unripe ackee fruit. Before maturation, the fruit contains hypoglycin, an amino acid which is metabolised to the CoA thioester of methylenecyclopropyl-acetic acid. This metabolite disrupts fatty acid oxidation causing

secondary inhibition of gluconeogenesis. Severe hypoglycaemia and vomiting are sequelae and are associated with significant mortality. These examples raise the question of the potential direct toxicity of 2-arylpropionate-CoA thioesters. This is an unexplored area, but one whose greatest single determinant is the chirality of the propionate.

On the other hand, CoA thioesters may also be therapeutically beneficial because of their hypoglycaemic and antiketogenic actions. Drugs such as RS-tetradecylglycidic acid owe their activity to the CoA thioesters which inhibit the ß-oxidation of long-chain fatty acids via inactivation of carnitine palmitoyl-transferase. Enantioselectivity is again demonstrated with preferential formation of, and inhibition by, R-tetradecylglycidyl-CoA[50].

4.2 Interrelationships between the metabolism of ibuprofen and lipids

Early studies of the disposition of ibuprofen indicated that there

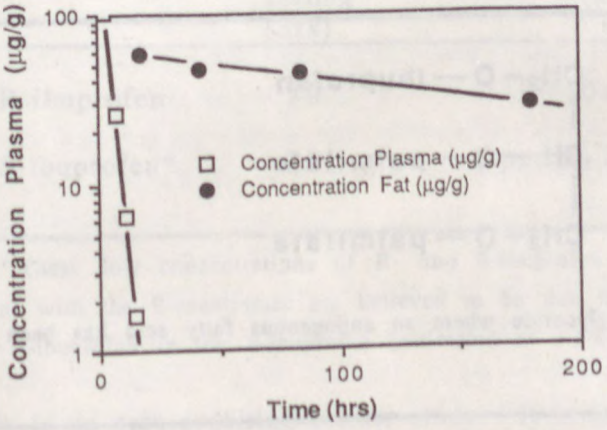


Figure 7. Time course of fat concentrations of R,S-ibuprofen after repeated oral doses of ^{14}C-labelled drug (20mg/kg, twice daily, 55 doses). Plasma concentrations are extrapolated from the data (plasma concentration 0.8µg/ml at 17hr) and assuming a half-life estimated from single dose data of approx. 2hr)[51].

was some accumulation of ibuprofen in lipid-rich tissues[51]. What was rather surprising and an aspect overlooked in the original report was the very slow elimination of drug from fat tissues (Fig. 7). The estimated elimination half-life was of the order of 7 days from this tissue in contrast to the very rapid elimination from blood where the half-life was of the order of 2 hours. This could not be explained by simple aqueous-lipid partition coefficients. The long half-life corresponded more with the turnover time of the tissue.

The interesting studies of Fears *et al.* some 9 years later were the solution to the slow accumulation and release of ibuprofen from lipids[52,53]. These workers were studying hypolipidaemic agents which were derivatives of phenylacetic acid. The initial work was with 4-benzyloxybenzoic acid. They found that a proportion of this drug, rather than being metabolised to more readily excreted hydrophilic products, was incorporated into triglycerides. This unusual product, where one or more of the fatty acids was replaced by the drug are known as 'hybrid' triglycerides[54] (Fig. 8). Of particular interest, was that they also reported that ibuprofen and fenoprofen were similarly metabolised.

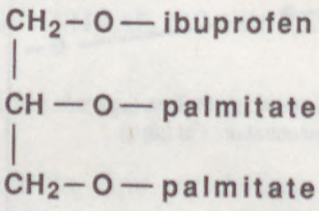


Figure 8. A 'hybrid' triglyceride where an endogenous fatty acid has been replaced by ibuprofen.

The convergence of this interest in the formation of hybrid lipids containing the 2-arylpropionates and the stereochemistry of the inversion process came with the hypothesis that, since both processes involved metabolic activation to the CoA thioesters, itself a stereospecific step, uptake into lipids should also be stereospecific[55]. Moreover the data were suggestive that the reason for the

accumulation of ibuprofen in lipid-rich tissues following chronic dosing to rats was due to formation of hybrid triglycerides.

We thus repeated the chronic dosing study to rats, but in this instance the enantiomers were also administered separately. The results confirmed the hypothesis[35]. Administered S-ibuprofen did not accumulate in fat, but there was accumulation following treatment with the R-enantiomer (Table 3). Subsequently, similar data have also been obtained for the incorporation of fenoprofen into triglycerides *in vitro* in adipocytes[36]. Moreover, we have also found that qualitatively similar accumulation occurs in man after chronic treatment with racemic ibuprofen (unpublished data).

Table 3.
Concentrations of Ibuprofen Enantiomers Isolated from Lipids of Rats Treated Chronically with R- or S-Ibuprofen (20mg/kg b.d., 7 days, n=4)[35]

Treatment	Time after Dosing (hrs)	Concentration (µg/g) (mean ± s.e.m.) R	S
R-ibuprofen	20	40±12	24±4
S-ibuprofen*	20	(approx) 1	1

* These low concentrations of R- and S-ibuprofen present after treatment with the S-enantiomer are believed to be due to the low contamination of the R-ibuprofen in the S-ibuprofen (estimated at 4-5%).

This is an area requiring further study. It is not known whether such hybrid lipids can be considered to be inert or whether they could contribute to adverse effects. The broader implications of these observations are very interesting. One cannot help but wonder as to the potential for toxicity by the phenoxypropionic acid herbicides such as mecoprop. Herbicidal activity is mediated by the R-enantiomers (same configuration as the S-arylpropionates). Inversion of the S-

enantiomer to the R-enantiomer by fresh soils, but not by soils which have been sterilised, has been demonstrated for fluazifop (a phenoxypropionate)[11]. Presumably a similar selectivity of the CoA synthetases for the S-enantiomers will be found for these xenobiotics. The ability to avoid the potential accumulation of long lasting lipid stores of the herbicides in man by use of the R-enantiomers is a question of significant toxicological interest. The production of enantiomerically pure herbicides may be a significant improvement in the toxicological profile of this group of herbicides. One wonders as to similar implications for other carboxylate drugs and xenobiotics.

4.3 Implications for variability in response

One of the dilemmas in the management of the rheumatic diseases is the variability with which different patients respond to the same dose of anti-inflammatory drug[56]. An interesting question for those 2-arylpropionates which are inverted is, whether there is likely to be significant inter-subject variability in the degree of inversion. In respect to inversion, the R-enantiomer can be considered to be a prodrug with the prospect that patients may range from no inversion to total inversion to the active S-enantiomer. The consequence is that two patients ostensibly being treated with the same dose of drug may in fact be receiving a dose of drug which differs by a factor of two. This is the extreme situation of course, but it is a contribution to variability which could be avoided by use of the pure S-enantiomer[55,57].

4.4 R-2-arylpropionates - Prodrugs or toxins?

In the general controversy as to whether drugs should be used as racemates or pure enantiomers, those 2-arylpropionates which are inverted, are not as easily categorised. The question begs asking as to whether those R-enantiomers which are inverted should be considered to be prodrugs or toxins. Evidence has been given to suggest either may be the case. Thus one may take the view that the drugs should be administered as the S-enantiomers to avoid "toxicity" associated with formation and consequent disposition of the CoA thioesters. This

assumes that there are significant adverse events associated with these CoA thioesters or the hybrid lipids which may be formed, and for this there is as yet no evidence. Clinically, however, there is as yet no clear distinction between the adverse effects associated with S-naproxen and those observed for the other racemic non-steroidal anti-inflammatory agents. After all, the primary toxicity of this group of drugs is gastrointestinal and apparently is an extension of the therapeutic action viz. the inhibition of gastroprotective prostaglandins.

Alternatively, the R-enantiomers may be viewed as prodrugs thus protecting the gastric mucosa from the effects of local high concentrations of active drug (assuming little inversion in the gut) and the consequent damage secondary to inhibition of prostaglandin synthetase. One might even design drugs of the R-configuration which are more readily inverted, approaching 100% formation of the active S-enantiomers after absorption. This advantage might be offset, however, by resulting in high concentrations of the active enantiomer at the sites of inversion with the possibility of associated adverse effects and the increased potential for toxicities mediated either directly or indirectly (eg. hybrid lipids) by the CoA intermediates.

It is apparent also that there is the potential that the R-enantiomers are hypolipidaemic agents, and this activity might be separated from effects on prostaglandin synthetase if it is that drugs can be designed which form CoA thioesters but which are not subsequently racemised. This type of activity appears, however, to be more clearly associated with the related, but achiral 2,2-dimethyl-propionates, such as clofibrate.

Another question of general interest is, if it is that one enantiomer should be developed as the clinical formulation, how much enantiomeric impurity is acceptable? This is rather a difficult question and will depend on the relative pharmacodynamic and pharmacokinetic characteristics of the enantiomers.

5. CONCLUSIONS

It perhaps should be restated in conclusion that inversion of enantiomers is not a common phenomenon in man, even apparently for

the 2-arylpropionates. However, for drugs such as ibuprofen where it does occur, it is a very important consideration with potential implications for drug formulation, drug design and clinical use. There are broader implications for the disposition and toxicities of some of the structurally related herbicides, as well as for understanding the disposition of other carboxylic acids and their potential for long term storage in lipids.

Ibuprofen is inverted via an enantiospecific CoA synthetase (long-chain acyl-CoA synthetase); a non-enantioselective racemase and an hydrolase or hydrolases of unknown specificity. Sequestration of 2-arylpropionates into lipid products occurs enantiospecifically consistent with the enantiospecific formation of the CoA thioesters.

Pragmatically, it is still not clear that the cost-benefit ratio is sufficient to justify the resolution of presently used racemic 2-arylpropionates and administration of the active S-enantiomers alone. However, future design of new anti-inflammatory agents must consider the stereochemistry of the final preparation, with enantiomerically pure drugs the rule rather than the exception. The chirality of this class of drugs dictates that enantiomers frequently are sufficiently different pharmacologically to be considered to be different drugs.

In general, racemic drugs will ultimately be the leftovers of a time when one could be executed for making such a basic error as to ignore nature's chiral character.

Poisoning: a natural product is best, but otherwise use one which is readily racemised or inverted.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

STRUCTURAL, STEREOCHEMICAL AND CONFORMATIONAL REQUIREMENTS FOR ACTIVATION AND BLOCKADE OF CENTRAL GLUTAMIC ACID RECEPTORS

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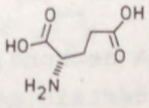
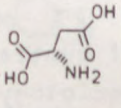
The acidic amino acids (S)-aspartic acid (ASP) and, in particular, (S)-glutamic acid (GLU) are generally accepted to be the major excitatory neurotransmitters in the mammalian central nervous system (CNS). Most, if not all, CNS neurones are sensitive to these excitatory amino acids (EAAs), reflecting that a large majority of the central neurones may be under EAA control[1-4].



GLU



ASP

In addition to GLU and ASP, which are found at high concentrations in the CNS, other less abundant EAAs are also found in the CNS. These include the sulphur-containing amino acids (S)-homocysteic acid[5] and (S)-serine-O-sulphate[6], the dipeptide N-acetyl-(S)-aspartyl-(S)-glutamic acid (NAAG) and related dipeptides, all of which may serve neurotransmitter or neuromodulator functions[7]. The tryptophan metabolite quinolinic acid (QUIN) (see Figure 4) is unlikely to be a neurotransmitter but may play an as yet unclarified role in excitotoxic processes in the CNS[2].

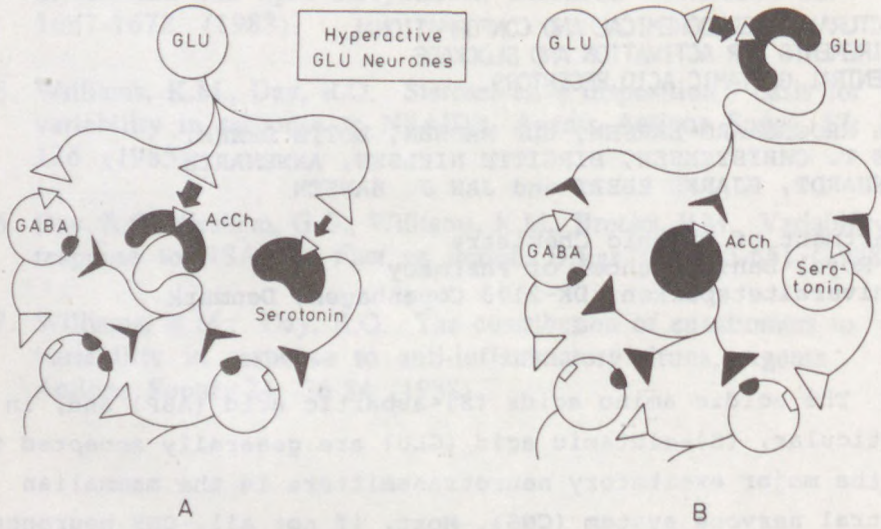


Figure 1. Schematic illustration of (A) the neurodegeneration of acetylcholine (AcCh) and serotonin neurones caused by hyperactive (S)-glutamic acid (GLU) neurones; (B) the neurodegeneration of GLU neurones caused by hyperactive GLU neurones. GABA: γ-aminobutyric acid.

Hyperactivity of central EAA neuronal pathways has been associated with the etiology of certain neurodegenerative diseases, such as status epilepticus, Huntington's chorea and dementia of the Alzheimer type[3,8-11]. In Alzheimer patients a regional degeneration of neurones, notably cholinergic and serotonergic neurones, is observed[12]. In addition, loss of glutamatergic neurones is seen in the progression of Alzheimer's disease[10] (Figure 1). Thus, hyperactive as well as hypoactive neuronal mechanisms may be operative in Alzheimer's disease, and this hypoactivity has recently been proposed to be associated with learning and memory deficits[10] (see subsequent section). The neuronal degeneration observed after ischemia, including stroke, hypoxia, and hypoglycemia may also be due to prolonged and excessive stimulation of EAA receptors[8]. There is some evidence that hypoactivity of the central EAA system(s) may be a key factor in schizophrenia[13].

Due to the accumulating evidence implicating hyperactivi-

ty as well as hypoactivity of the EAA neuronal systems, in for example, Alzheimer's disease, drugs capable of protecting as well as activating EAA receptors may be of therapeutic interest. An obvious challenge is to design partial agonists with appropriately balanced agonist/antagonist profiles.

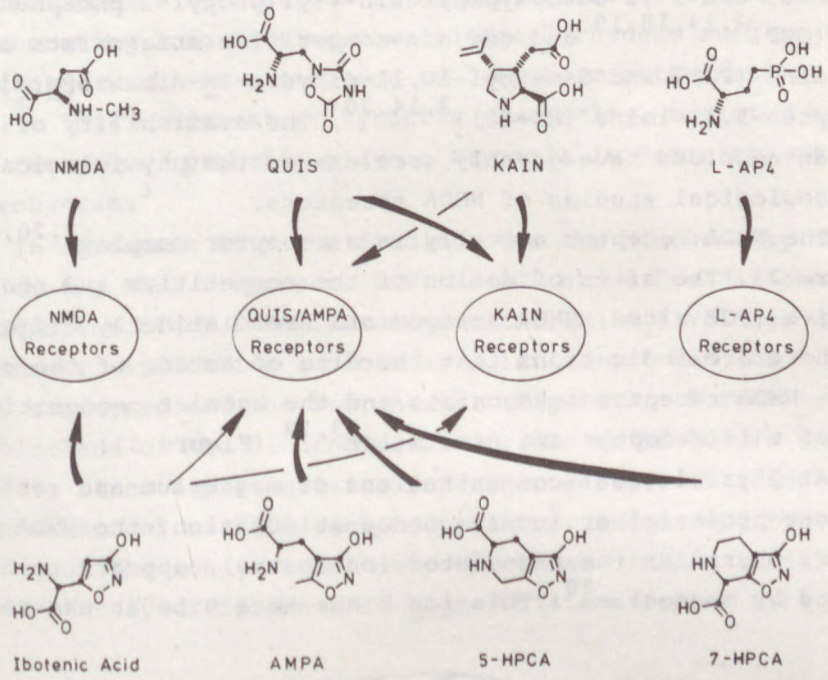


Figure 2. Schematic illustration of the classification of excitatory amino acid receptors. Structures of some agonists and L-AP4 with indications of sites of action.

MULTIPLICITY OF EXCITATORY AMINO ACID RECEPTORS

In analogy with other neurotransmitter systems, GLU and ASP operate through multiple receptors. These receptors are at present subdivided into four main classes, some of which probably are heterogeneous[1-3,7,14,15]: (1) N-Methyl-D-aspartic acid (NMDA) receptors at which NMDA and ibotenic acid are selective agonists (Figure 2); (2) QUIS/AMPA receptors at which quisqualic acid (QUIS) is a non-selective and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) a highly selective agonist[16]; (3) kainic acid (KAIN) receptors, which are selectively activated by KAIN[4,17]; (4) L-AP4 receptors, at which L-2-a-

mino-4-phosphonobutyric acid (L-AP4) inhibits synaptic excitations[15].

A variety of compounds have been shown to possess the ability to block NMDA-induced neuronal excitation, including the competitive antagonists (R)-2-amino-5-phosphonovaleric acid ((R)-AP5) and 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP)[2,14,18,19] and the non-competitive antagonists phencyclidine (PCP) and 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801)[3,14,20]. The availability of these NMDA antagonists have greatly accelerated the physiological and pharmacological studies of NMDA receptors.

The NMDA receptor actually is a receptor complex[2,20,21] (Figure 3). The sites of action of the competitive and non-competitive (PCP sites) NMDA antagonists are distinctly different, and there are indications that the site of action of the competitive NMDA receptor antagonists and the agonist recognition site of this receptor are dissimilar[3,19] (Figure 3).

At physiological concentrations of magnesium and resting membrane potential or low-frequency stimulation, the NMDA receptor, or rather the associated ion channel, appears to be blocked by magnesium[20]. This ion binds to a site at the recep-

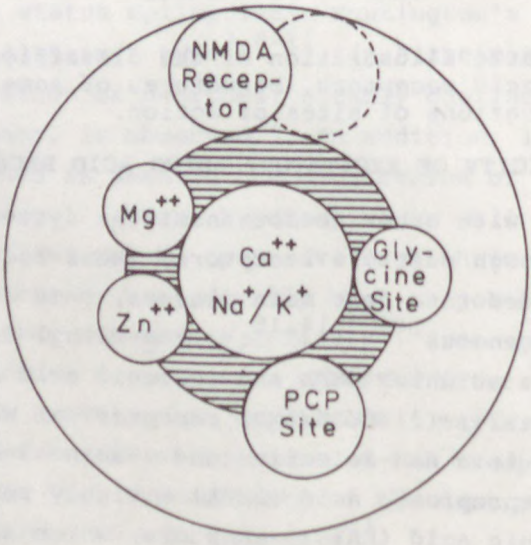


Figure 3. A schematic illustration of the NMDA receptor complex.

tor complex, which is different from those, at which zinc and glycine interact[3,20,21] (Figure 3). Following membrane depolarization, for example via activation of QUIS/AMPA or KAIN receptors in the cell membrane, the magnesium blockade is released, causing further depolarization. Thus, the NMDA receptor complex appears to function as an amplification system capable of generating long lasting physiological changes such as those associated with the phenomenon of long term potentiation (LTP)[3]. This component of synaptic plasticity, mediated by NMDA receptors, probably plays a key role in learning and memory mechanisms[3].

In agreement with the findings for the blockade by magnesium of the NMDA responses, PCP, MK-801 and other dissociative anaesthetics block NMDA-induced excitation in a voltage-dependent manner, indicating a close association between the binding sites concerned and the ion channel (Figure 3). Furthermore, the blockade of the binding sites for PCP or MK-801 is use-dependent, since a prerequisite for their antagonist effects is the presence of an NMDA receptor agonist, suggesting that the opening of the ion channel uncovers these binding sites, which are distinctly different from sigma opiate receptor sites[20].

STEREOCHEMICAL AND CONFORMATIONAL REQUIREMENTS FOR ACTIVATION OF EXCITATORY AMINO ACID RECEPTORS

Chirality and a high degree of conformational flexibility are traits of the molecule of GLU (see Figure 5). Furthermore, the enantiotopic hydrogen atoms at each carbon atom of the backbone of GLU become mutually distinct upon the interaction of GLU with the chiral macromolecules concerned with the function of GLU in synaptic transmission. These molecular characteristics probably are essential for the complex synaptic activities of GLU, but inherently make GLU itself unsuitable for studies of its molecular mechanism of action at different synaptic sites. A rational approach to these problems involves design and structure-activity studies of GLU analogues, in which stereochemical, conformational, and electronic parameters are systematically modified.

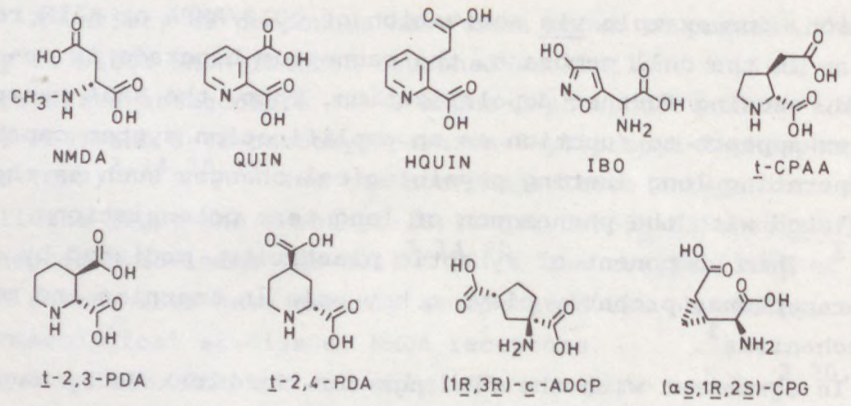


Figure 4. Structures of some NMDA receptor agonists. The amino acids t-CPAA, t-2,3-PDA and t-2,4-PDA are arbitrarily shown as single enantiomers.

Among the conformationally restricted, cyclic analogues of GLU, particularly the cis but also the trans forms of the cyclopentyl derivative 1-amino-1,3-dicarboxycyclopentane (ADCP) show potent excitatory actions and are considerably more potent than the corresponding rac-cis- and rac-trans-1,3-cyclohexane derivatives[1]. The four stereoisomers of ADCP have recently been prepared[22], and the (1R,3R)-cis isomer, shown in Figure 4, mimics the effects of NMDA, including induction of burst firing and antagonism by (R)-AP5, whereas the other cis isomer and the two trans isomers cause excitations comparable to those elicited by KAIN[22]. Similarly, the cis forms of racemic 2-carboxycyclopropylglycine (CPG) are agonists at NMDA receptors, albeit weaker than cis-ADCP, while "trans-CPG" is reported to show weak excitatory activity with mixed agonist and antagonist properties[1]. CPG contains three asymmetric centres, and of the eight possible stereoisomers the four isomers with (S)-configuration at the α-carbon have recently been obtained[23]. Among these four stereoisomeric compounds containing GLU, conformationally restricted by a cyclopropyl moiety, the highest activity at EAA receptors was observed for the two cis isomers[23], in agreement with the results for the racemic mixture[1]. The (αS,1R,2S)-isomer, depicted in Figure 4, shows the highest ac-

tivity, especially at NMDA-sensitive GLU receptor sites, but its agonist activity is not blocked by antagonists selective for NMDA receptors, indicating that at least part of its activity is mediated by non-NMDA receptors[23]. Like the pyrrolidine amino acid rac-trans-2-carboxy-3-pyrrolidineacetic acid (t-CPAA)[24], the two piperidine analogues of ASP and GLU, respectively, rac-trans-piperidine-2,3-dicarboxylic acid (t-2,3-PDA), rac-trans-piperidine-2,4-dicarboxylic acid (t-2,4-PDA) (Figure 4) show agonist properties at NMDA receptors[2], and both show considerable conformational mobility[25].

Structure-activity studies on the PDAs using high-resolution ^{1}H NMR techniques supported by molecular mechanics calculations have shed light on the conformational requirements for activation and blockade of NMDA receptors[25,26]. Whereas rac-cis-2,3-PDA is a partial agonist/antagonist at NMDA receptors, rac-trans-2,3-PDA is an NMDA agonist and, similarly, rac-cis-2,4-PDA and rac-trans-2,4-PDA show NMDA antagonist and agonist profiles, respectively[2,25]. In aqueous solution rac-trans-2,3-PDA shows a high degree of conformational flexibility, existing as a mixture of the conformers with diequatorial and diaxial orientations of the carboxylate groups, whereas rac-cis-2,3-PDA exists exclusively in the depicted conformation (Figure 5). Similarly, rac-trans-2,4-PDA exists as an approximately 1:1 mixture of the two conformers with axial-equatorial orientations of the carboxylate groups, whereas only the conformer of rac-cis-2,4-PDA having the carboxylate groups in equatorial orientations could be detected[25] (Figure 5). Finally, the conformationally mobile rac-cis-2,5-PDA is a weak but full NMDA agonist, whereas the rigid isomer, rac-trans-2,5-PDA, does not interact with NMDA receptors[25] (not illustrated).

These results are consistent with the view that a certain degree of conformational flexibility of the agonists is a prerequisite for activation of NMDA receptors. This structure-activity relationship does not comprise QUIN, homoquinolinic acid (HQUIN) (Figure 4), and related dicarboxylic acids capable of activating NMDA receptors via as yet not fully understood mechanisms[2,18,25].

Ibotenic acid, which is a relatively flexible 3-isoxazolol

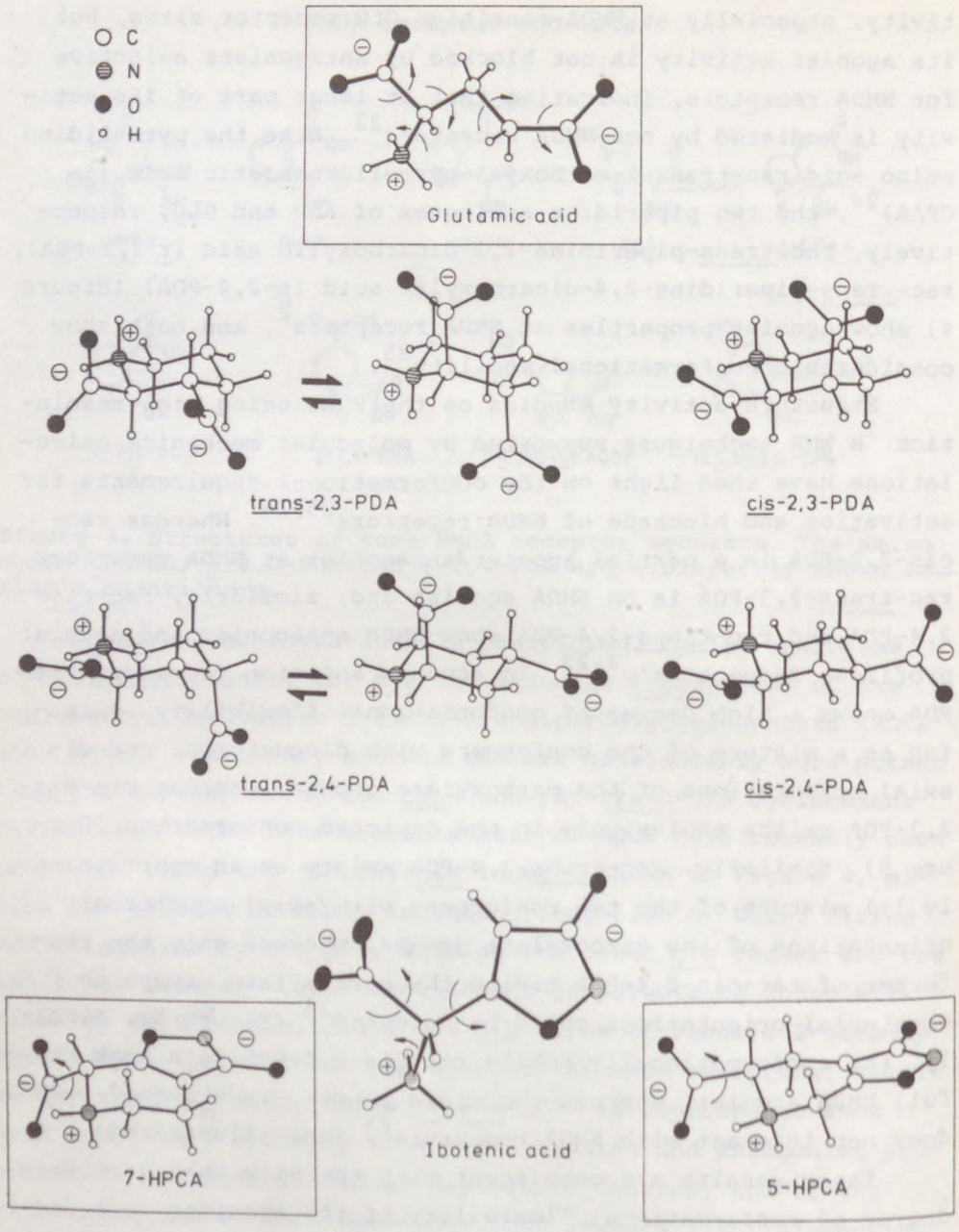


Figure 5. The structures of GLU and a number of mono- and bicyclic analogues. The approximate conformational flexibility of the compounds and the conformations in aqueous solution of trans- and cis-2,3- and trans- and cis-2,4-PDAs, as determined by NMR spectroscopy, are illustrated.

bioisostere of GLU (Figure 5), is a potent and selective NMDA agonist[2,16]. In the crystalline state, ibotenic acid adopts the conformation illustrated in Figure 5 as determined by an X-ray analysis[26]. It is likely that this conformation of ibotenic acid is recognized by the NMDA receptor site and that the agonist-receptor complex subsequently undergoes conformational changes during the receptor activation[27].

Interestingly, the conformationally restricted analogue of ibotenic acid, 7-HPCA (Figures 5 and 6), does not interact with NMDA receptors, but 7-HPCA is a specific agonist at QUIS/AMPA receptors[28,29]. The orientation of the carboxylate group of 7-HPCA in the crystalline state, as established by X-ray crystallography[26,29] (Figure 5) is different from that of ibotenic acid. Thus, 7-HPCA may be unable to adopt conformations recog-

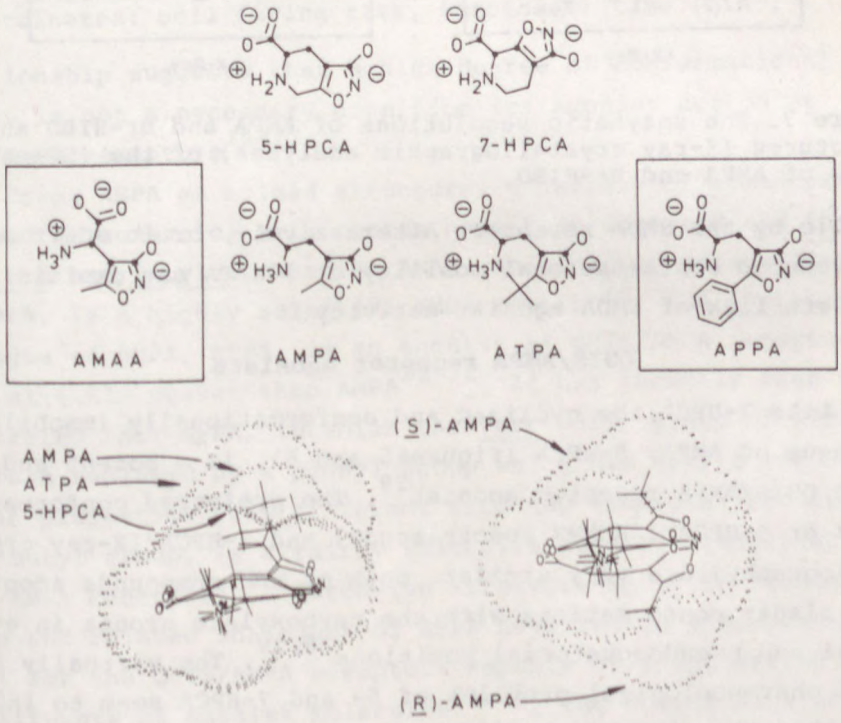


Figure 6. The structures of a number of 3-isoxazolol bioisosteres of glutamic acid and aspartic acid and a comparison of the relative volumes (solvent accessible surfaces) of some of these analogues.

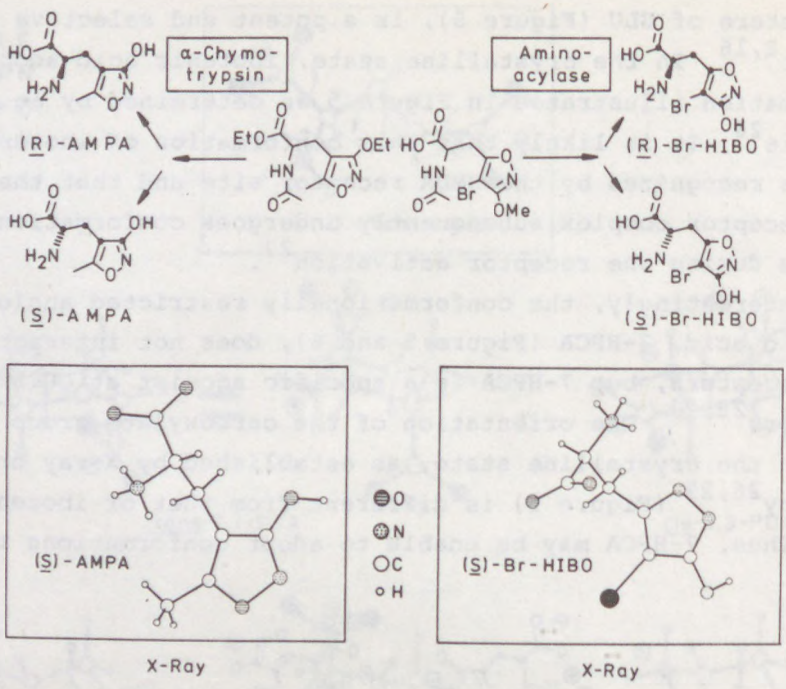


Figure 7. The enzymatic resolutions of AMPA and Br-HIBO and the structures (X-ray crystallographic analyses) of the (S)-enantiomers of AMPA and Br-HIBO.

nizable by the NMDA receptor. Alternatively, or in addition, the reduced conformational mobility of 7-HPCA may explain its complete lack of NMDA agonist activity.

QUIS/AMPA receptor agonists

Like 7-HPCA, the cyclized and conformationally immobilized analogue of AMPA, 5-HPCA (Figures 5 and 6), is a potent and specific QUIS/AMPA receptor agonist[29]. The preferred conformations of 5-HPCA (^{1}H NMR spectroscopy) and 7-HPCA (X-ray crystallography) are very similar: both of the compounds adopt almost planar conformations with the carboxylate groups in equatorial and pseudoequatorial positions[26,29]. The virtually identical pharmacological profiles of 5- and 7-HPCA seem to indicate that the structural difference between them is irrelevant to their agonist actions; hence these rigid model compounds essentially reflect the active conformations of AMPA and GLU at QUIS/AMPA receptors. Furthermore, this structure-activity

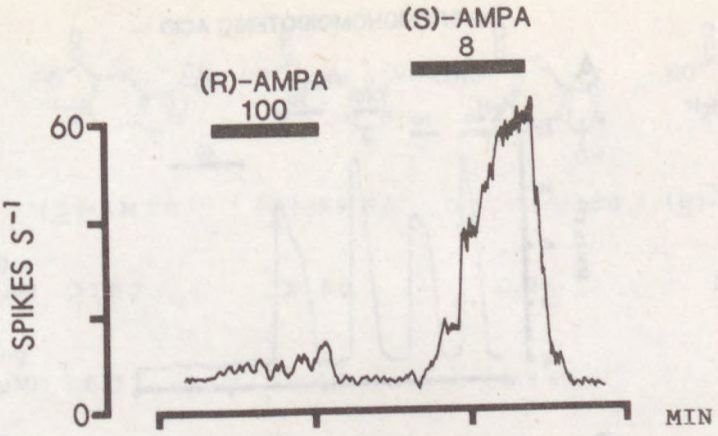


Figure 8. Comparison of the excitation of a spinal interneurone by (S)- and (R)-AMPA, both ejected microelectrophoretically from 0.1 M solutions (pH 8) within different barrels of a seven-barrel micropipette using currents (nA) and ejection times indicated by the numbers and horizontal bars, respectively. Ordinates: cell firing rate. Abscissae: time (min).

relationship suggests that a high degree of conformational mobility is not a necessary condition for agonist action at QUIS/AMPA receptors[25-27].

Using AMPA as a lead structure, a variety of structurally related 3-isoxazolol amino acids have been designed and tested *in vitro* and *in vivo*. Whereas AMAA, an aspartic acid analogue of AMPA, is a highly selective NMDA agonist[16], the *tert*-butyl analogue of AMPA, ATPA, is an agonist at QUIS/AMPA receptors only slightly weaker than AMPA[29-32]. It has recently been demonstrated that APPA, in which the *tert*-butyl group of ATPA has been replaced by a phenyl group which has steric and electronic properties very different from the spherical and bulky *tert*-butyl group, is a fairly selective partial agonist at QUIS/AMPA receptors[32]. Extensive structure-activity studies on these and related analogues of AMPA have led to a hypothetical model for the QUIS/AMPA receptors capable of accomodating bulky substituents of agonist molecules[21,26]. The steric bulk of the methyl group of (R)-AMPA may, to some extent, be responsible for the lack of interaction of this compound with the QUIS/AMPA receptor (Figure 6).

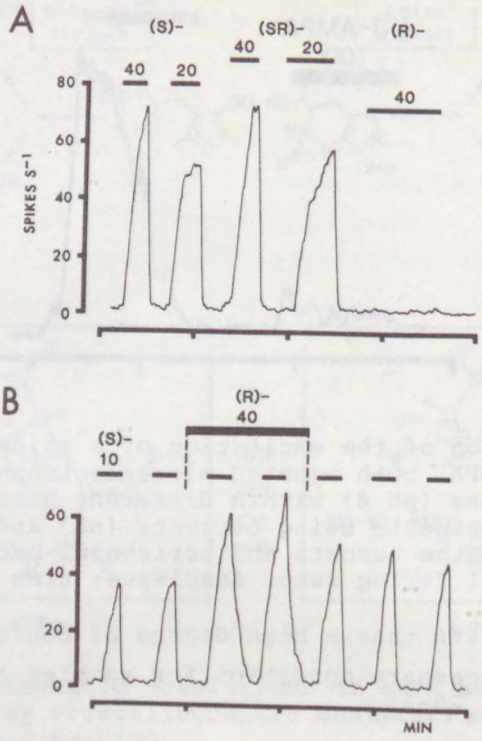
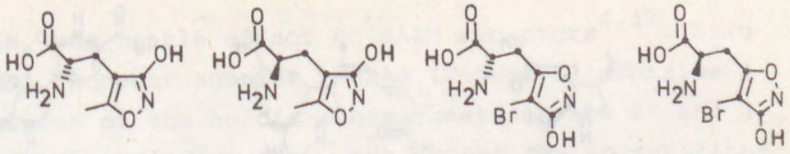


Figure 9. Comparison of the excitation of a spinal interneurone by (R)-, (S)-, and (RS)-4-bromohomoibotenic acid (Br-HIBO), all ejected microelectrophoretically from a seven-barrel micropipette (see legend for Figure 8) (Reproduced with permission from ref. 34).

The stereochemical requirements for activation of QUIS/AMPA receptors are at present being mapped out[27,30,33-35]. Like AMPA, 4-bromohomoibotenic acid (Br-HIBO) (Figure 7) is a very potent agonist at QUIS/AMPA receptors[16,34]. The (S)- and (R)-isomers of AMPA and Br-HIBO have been synthesized using enzymatic procedures[33,34] (Figure 7). Similar procedures have been used for the production of the enantiomers of other QUIS/AMPA receptor agonists, including 5-HPCA and 4-methylhomoibotenic acid (Me-HIBO)[27].

These enantiomers have been studied electrophysiologically (Figures 8 and 9) and as inhibitors of the binding of various radioactive ligands for EAA receptor sites, including ^{3}H-AMPA and ^{3}H-GLU (in the presence of calcium chloride) (Figure 10).



	(S)-AMPA	(R)-AMPA	(S)-Br-HIBO	(R)-Br-HIBO
GLU Binding ($CaCl_2$)(IC_{50},μM)	>100	>100	0.24	0.14
AMPA Binding (IC_{50},μM)	0.02	76	0.34	32
Neuroexcitation (Rel. potency)	+++++	(+)	++++	(+)

Figure 10. Neuroexcitatory actions of the enantiomers of AMPA and Br-HIBO on cat spinal neurones and their effects in two EAA receptor binding assays.

None of these compounds interact significantly with NMDA and kainic acid receptor sites or with GLU uptake mechanisms. There generally is a positive correlation between affinity for ^{3}H-AMPA binding sites and potency as neuronal excitants at QUIS/AMPA receptors of the enantiomers studied[26,27,30,34]. In all cases, the neuroexcitatory effects reside in the (S)-enantiomers of the compounds (Figure 10).

In contrast to (S)- and (R)-AMPA, the enantiomers of Br-HIBO are inhibitors of calcium chloride-dependent ^{3}H-GLU binding[34] (Figure 10). Whereas the neuroexcitatory action of Br-HIBO resides exclusively in the (S)-enantiomer, (R)-Br-HIBO is somewhat more active in this particular ^{3}H-GLU binding assay, and this receptor or, perhaps, transport affinity may underlie the potentiation of the excitatory effect of (S)-Br-HIBO by its (R)-enantiomer[34]. This observation may indicate that calcium chloride-dependent ^{3}H-GLU binding reflects a functionally relevant mechanism at EAA synapses, which may have pharmacological interest.

The physiological relevance of this binding is largely unknown, but it has been suggested that it may represent binding to glial transport sites[36]. If this is the case, then both en-

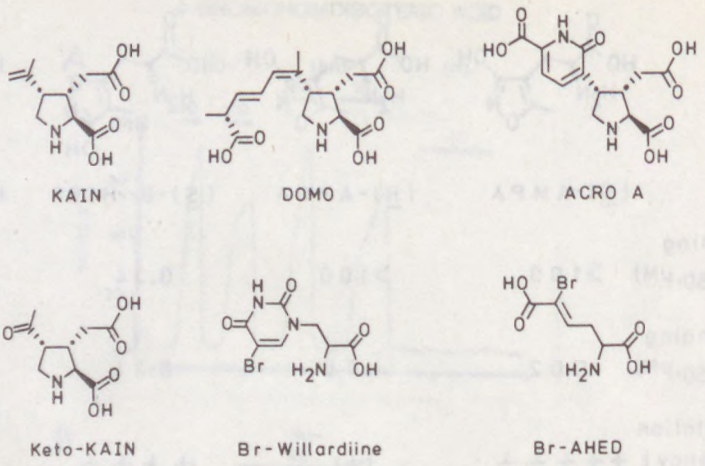


Figure 11. Structures of some KAIN receptor agonists.

antiomers of Br-HIBO are likely to be substrates for a glial uptake mechanism. This, in turn, could provide an explanation for the apparent paradox, that the presence of the "inactive" (R)-form increases the excitatory potency of the (S)-enantiomer, because (R)-Br-HIBO would then be expected to inhibit glial uptake of the excitatory (S)-Br-HIBO, thus prolonging its presence near receptors and resulting in an increased excitatory response. The rapid offset of excitation, especially observed after application of (S)-Br-HIBO alone, may support the presence of an uptake mechanism, but the above interpretation is still quite speculative, and other explanations are possible.

KAIN receptor agonists

A common structural feature of KAIN receptor agonists is the presence of a π-electron system in the molecule (Figure 11). This unsaturated moiety appears to be advantageous for interactions with KAIN receptors, and the extended conjugation in domoic acid (DOMO) and acromelic acid A (ACRO-A) gives even more potent excitatory effects than observed in KAIN[4]. The KAIN derivative keto-KAIN also contains π-electrons in the 4-substituent, and keto-KAIN shows considerable activity both as an excitant and as an inhibitor of ^{3}H-KAIN binding[4,17,27]. In contrast, dihydro-KAIN, obtained by reduction of the unsaturated side-

chain of KAIN, has little effect on KAIN receptors[4,17]. Like KAIN, the NMDA receptor agonist t-CPAA (Figure 4) contains a trans arrangement of the acidic substituents in the 2- and 3-position of the pyrrolidine ring, but t-CPAA has no substituent in the 4-position and predominantly interacts with NMDA receptors besides showing a weak affinity for KAIN receptors[24]. Three of the four stereoisomers of ADCP (see Figure 4) have been stated to be "KAIN-like"[22], indicating that an unsaturated moiety is not an absolute requirement for activity at KAIN receptors, but all three isomers are considerably less potent than KAIN[22]. The bromo-containing amino acids (RS)-Br-willardiine and (RS)-2-bromo-5-amino-trans-hex-2-enedioic acid (Br-AHED) (Figure 11) are EAA receptor agonists interacting primarily with KAIN receptors[4,27].

CONCLUSION

During the past 10-15 years a large number of GLU analogues have been developed as model compounds for molecular pharmacological studies of EAA synaptic mechanisms, for which GLU is the major, if not the only, ligand/substrate. These model compounds have been designed by systematic variation of the structural parameters of the molecule of GLU. Structure-activity studies on conformationally restricted or immobilized GLU analogues and chiral analogues with established stereochemistry have provided detailed information about the structural requirements of the different EAA synaptic mechanisms. These studies have demonstrated that the stereochemical and conformational constraints imposed on agonists for different subtypes of EAA receptors are distinctly different, and, furthermore, that the structural requirements of the EAA transport (uptake) systems are different from those of the EAA receptors.

In continuation of these studies we are in the process of developing compounds with specific actions at EAA synaptic mechanisms and with pharmacological properties acceptable for animal behavioural and clinical studies. Hopefully, these studies will lead to therapeutically useful drugs.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

BICUCULLINE SALTS: CONFUSION OF THE SIGN

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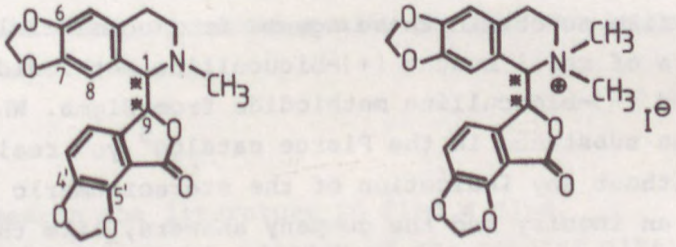
Suppose that you are a newcomer in the field of GABAergic neurotransmission. And for a newcomer every joke is new.

1st Stage

You come across a paper by Fernández-Guasti et al.[1] reporting a comparison of the effects of different isomers of bicuculline (cf. formula) on the male sexual behavior of rats.



You learn that little attention has been paid to the possible role of amino acids in the mediation of copulation behavior, hence an investigation on the effect of γ-aminobutyric acid (GABA) holds promise in this respect. The authors refer to the effective use of bicuculline methiodide as a GABA antagonist[2], and point out that of the two enantiomers the (+)-form has the GABA antagonistic effect, while the (-)-form does not antagonize GABAergic neurotransmission[3,4]. The new findings of Fernández-Guasti et al.[1] are cited below.

"*Intracerebral infusion of the (-)-form of bicuculline methiodide did not produce any deviations in the mating pattern*

compared to that displayed by saline-treated controls. However, in its (+)-form, bicuculline methiodide drastically changed the mating pattern."
"Results clearly show that infusion in the medial preoptic area, of (+)-bicuculline methiodide stimulates masculine sexual behavior in the rat, while (-)-bicuculline methiodide has no such effect." ... "Therefore, the action of (+)-bicuculline methiodide on masculine sexual behavior seems to be specifically associated with its property of antagonizing a GABAergic inhibitory input on the neural substrates of this behavior."

The above results are new and unexpected, and you may find them interesting. Apart from the small oversight that the title talks about different isomers of bicuculline while the paper[1] itself refers to the enantiomers of bicuculline methiodide, you regard it as a nice piece of work.

2nd Stage

In view of the valuable application of bicuculline methiodide enantiomers displaying stereoselective antagonism, you would like to obtain these agents from commercial source. The authors of ref.1 bought (+)-bicuculline methiodide from Pierce, and (-)-bicuculline methiodide from Sigma. When you look up the substance in the Pierce catalog[5] you really find it, but without any indication of the stereoisomeric form. You make then an inquiry and the company answers, like this:

21676 hipec nl

october 18, 1988

this product is no longer available and we have not got any information about the optical rotation, so we do not know which enantiomer of bicuculline methiodide was sold.

regards
pierce europe bv

21676 hipec nl

which is a bit disappointing. Then you look up the stuff at Sigma and by chance pick a 1982 catalog[6] in which you find both (+)-bicuculline and (+)-bicuculline methiodide, i.e. the enantiomer the above authors purchased from Pierce[1]. Somewhat confused, you turn to later catalogs. In 1984 Sigma sold (-)-bicuculline methiodide of approx. 95 % purity while the (+)-methiodide salt disappeared[7]. In 1985 Sigma marketed crystalline (-)-bicuculline methiodide[8], and from 1986 and on their catalog[9] lists crystalline (-)-bicuculline methiodide "produced from (+)-bicuculline". You realize then that the stereochemistry of bicuculline methiodide is unusually complicated and turn to other sources for information on its absolute configuration.

Fluka[10] lists (+)-bicuculline and gives its formula displaying (1S,9R) configuration. Aldrich[11] sells (-)-bicuculline methiodide and the formula attached shows (1R,9S) configuration. Finally, at Research Biochemicals Inc.[12] you learn that both (+)-bicuculline and its levorotatory quaternary salts (methiodide, methyl bromide, and methyl chloride) are assigned by the same (1S,9R) configuration.

By this time you become really puzzled who is telling you the truth.

3rd Stage

You search the literature to find a clue.

The GABA antagonist potency of the natural alkaloid bicuculline was established in 1970 by Curtis and coworkers[13]. Two years later the same group reported advantage of the quaternary salt bicuculline methochloride over the alkaloid[14] owing to the low solubility of the latter in water. The stereostructure of bicuculline was unequivocally determined by X-ray diffraction by Gilardi[15], who obtained the alkaloid from Pierce. The configuration of bicuculline was found to be (1S,9R) at variance with earlier incorrect models of the molecule[16,17] depicting diastereoisomers of bicuculline.

The first report on the stereoselective biological action of bicuculline quaternary salts was published by Collins and

Hill [3]. As they pointed out [3], all pharmacological experiments performed earlier had used commercially available (+)-bicuculline and so they prepared the (-)-enantiomer from (-)-β-hydrastine (cf. Table 1). The configuration of the asymmetric centers are not affected by methylation at nitrogen, and the methochloride salts were prepared from (+)- and (-)-bicuculline [3]. As Collins and Hill found, only one of the two methochloride salts had the potency to antagonize the biological effect of GABA, and it was the one prepared from (+)-bicuculline [3]. The authors argued, as follows [3]:

"(+)-Bicuculline methochloride possesses the 1S,9R configuration, whereas (-)-bicuculline methochloride has the 1R,9S configuration, these configurations of course, being shared by the parent compounds (+)- and (-)-bicuculline."

Soon afterwards radiolabeled quaternary salts of bicuculline were found valuable pharmacological agents and applied as such, adhering to the above assignation [18,19].

Valuable information on the optical rotation of bicuculline-related alkaloids and their hydrochloride salts were

Table 1. The structure and sign of optical rotation for phthalideisoquinoline alkaloids and their hydrochloride salts at 586 nm (ref. 20)

Alkaloid	Configuration*	Substituents*	Sign of rotation in $CHCl_3$	in 0.1n HCl
(+)-bicuculline	1S,9R	6,7-OCH_2O, 8-H, 4',5'-OCH_2O	(+)	(-)
(+)-adlumidine	1S,9S	6,7-OCH_2O, 8-H, 4',5'-OCH_2O	(+)	(+)
(-)-capnoidine	1R,9R	6,7-OCH_2O, 8-H, 4',5'-OCH_2O	(-)	(-)
(+)-corlumine	1S,9R	6,7-$(OCH_3)_2$, 8-H, 4',5'-OCH_2O	(+)	(-)
(+)-corlumidine	1S,9R	6-OCH_3, 7-OH, 8-H, 4',5'-OCH_2O	(+)	(-)
(+)-adlumine	1S,9S	6,7-$(OCH_3)_2$, 8-H, 4',5'-OCH_2O	(+)	(+)
(-)-β-hydrastine	1R,9S	6,7-OCH_2O, 8-H, 4',5'-$(OCH_3)_2$	(-)	(+)
(-)-α-narcotine	1R,9S	6,7-OCH_2O, 8,4',5'-$(OCH_3)_3$	(-)	(+)
(-)-β-narcotine	1R,9R	6,7-OCH_2O, 8,4',5'-$(OCH_3)_3$	(-)	(-)

*According to ref. 21.

published[20] as early as 1964 indicating for some bases that the sign of optical rotation may be reversed upon the formation of the salt. From data collected in Table 1 you may reach two conclusions:

i/ for alkaloid bases the sign of optical rotation (in $CHCl_3$) is empirically associated with the configuration of C-1: 1S is positive, 1R is negative;

ii/ the signs of rotation for base and salt are different if the stereochemical descriptors for C-1 and C-9 differ (1S,9R and 1R,9S : erythro series), while the signs are the same for identical descriptors (1R,9R and 1S,9S : threo series).

Although the narcotines contain an extra OCH_3 group at C-8, both rules apply to them in accordance with the observation[22] that the additional 8-OCH_3 substituent makes little difference to the chiroptical properties.

But you did not find the sign of the methiodide enantiomer prepared from (+)-bicuculline.

4th Stage

You venture to gain the information yourself by transforming authentic alkaloid bases into methiodide salts and by taking measurements on the optical rotation. Table 2 collects the results obtained[23].

Finally, you become convinced that quaternary (methiodide) salts of bicuculline, α-narcotine and β-hydrastine (representatives of the erythro series of phthalideisoquinolines) possess reversed sign of optical rotation compared to their parent bases, in accordance with hydrochloride salts shown in Table 1. The reason for the reversed rotation can be seen on the CD spectra of (+)-bicuculline and its salts (Fig.1). Hydrochloride and methiodide salts give almost identical spectra. At the same time the positive charge at nitrogen in the salts increases the intensity of the aromatic transition between 200 and 250 nm, which results in a remarkable shift of the spectrum of the base without changing its overall character

Table 2. Optical rotation of erythro phthalideisoquinoline methiodide salts in $CHCl_3$ at 589 nm (ref.23)

Alkaloid*	[α] of methiodide salt at c = 1	c = 0.4
(+)-bicuculline $[\alpha]_D$ + 137.2 ° $(CHCl_3)^{24}$	-102.5 °	-92.0 °
(-)-α-narcotine $[\alpha]_D$ -200 ° $(CHCl_3)^{21}$	+96.0 °	+89.2 °
(-)-β-hydrastine $[\alpha]_D$ -68 ° $(CHCl_3)^{21}$	+157 °	+153 °

* For structural information refer to Table 1.

at higher wavelengths. This conceivably indicates similar conformations for the salts and the parent base[22], a prerequisite for the biological activity to be associated with both salt and base of identical configuration[25].

Conclusions

1. (+)-Bicuculline and (-)-bicuculline methiodide both share identical (1S,9R) configuration and possess GABA antagonist potency. They are commercially available pharmacological agents.

2. How ever did Fernández-Guasti et al. find[1] difference between experiments applying apparently the same agent is a mystery you are no more interested in.

3. By now you are not a newcomer any more, but hold newcomers in high esteem. Their mind is fresh, they note everything. You set the highest standards before yourself when

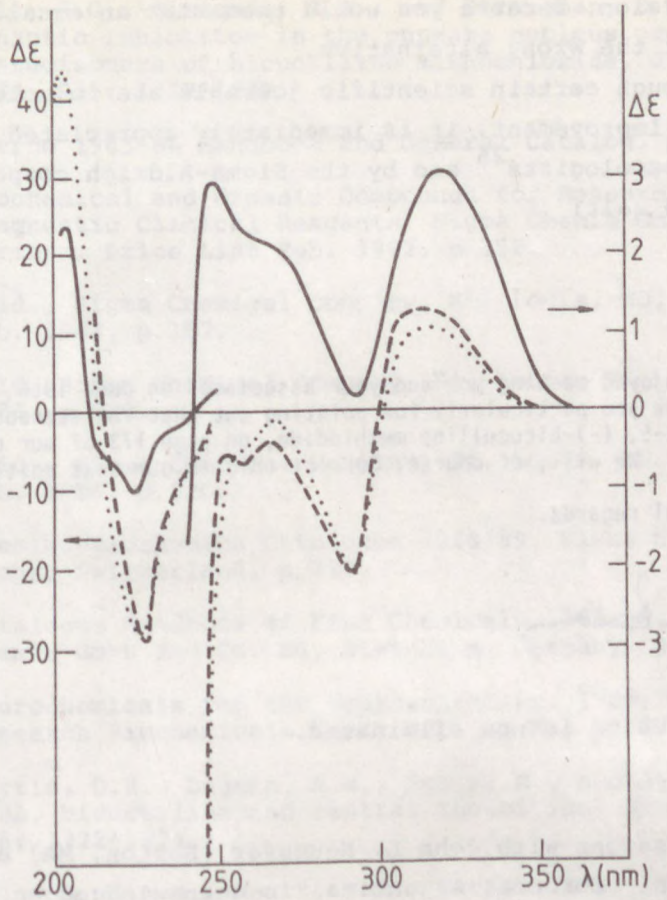


Figure 1. Circular dichroism spectra of (+)-(1S,9R)-bicuculline (2.0 mg/10 ml, ——), (-)-(1S,9R)-bicuculline hydrochloride (2.2 mg/10 ml, ...), and (-)-(1S,9R)-bicuculline methiodide (2.35 mg/10 ml, ----) in ethanol. Pathlength: 0.05 cm (200-250 nm, left-hand axis) and 0.5 cm (above 245 nm, right-hand axis). Taken from ref. 23.

try to address the newcomer, who certainly deserves true information.

4. You do not try to blame any individual for all the mess. To be blamed is rather the inherent human defect called carelessness especially dangerous in the field of chirality. You will never rely on the high, 50 % probability to find

the right version, because you would then risk an equally high chance to hit the wrong alternative.

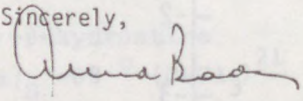
5. Although certain scientific journals are reluctant to publish your improvement, it is immediately appreciated by careful pharmacologists[26] and by the Sigma-Aldrich corporation marketing the salt:

Dr. Alfred Bader
Chairman

We really enjoyed meeting you and your associates on June 19th, and I want to thank you particularly for pointing out that the stereochemistry of our 28464-5, (-)-bicuculline methiodide, on page 173 of our catalog, is in error. We will, of course, correct this in our next edition.

Best personal regards.

Sincerely,



Alfred Bader

A bit of confusion is now eliminated.

Helpful discussions with John L. Neumeyer (Boston, MA) and Jorge D. Brioni (Cordoba, Argentina) is acknowledged.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

CHIRAL DISCRIMINATION BY DOPAMINE RECEPTORS

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The catecholamines dopamine (DA), norepinephrine (NE) and epinephrine (EPI) are well recognized neurotransmitters in several species [1,2]. Their biosynthesis occurs in specific tissues via a series of enzymatic reactions. The biosynthesis of DA is depicted in Fig.1. The step-wise sequence begins with the essential amino acid L-tyrosine which possesses chirality at the α -carbon. This chirality is retained during the initial rate-limiting aromatic hydroxylation of L-tyrosine to L-DOPA by the enzyme tyrosine hydroxylase. However, in the next step, the chirality is lost when L-DOPA is decarboxylated by the enzyme aromatic L-amino acid decarboxylase to DA.

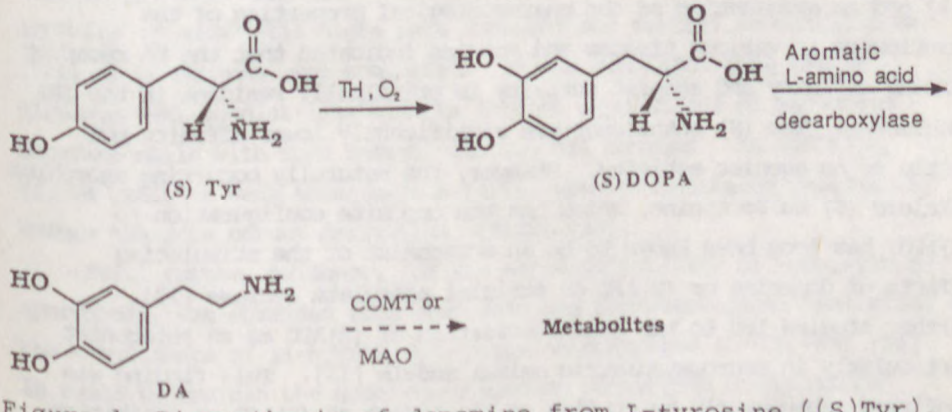


Figure 1. Biosynthesis of dopamine from L-tyrosine ((S)Tyr).

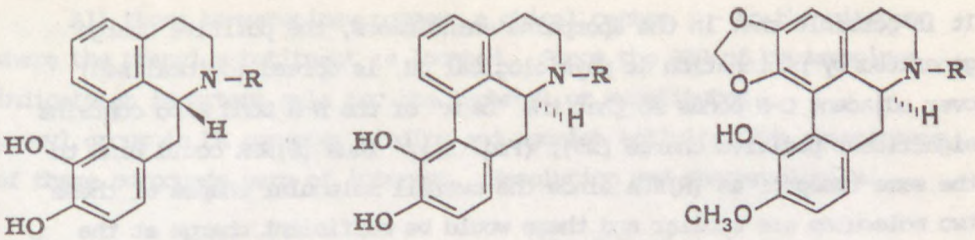
Dopamine, as a neurotransmitter, exerts its actions by binding to specific DA receptors located at presynaptic as well as postsynaptic neuronal sites. This binding triggers a series of events resulting

ultimately in a characteristic physiological/biochemical response. The action of DA can be duplicated by DA receptor agonists and blocked by DA receptor antagonists belonging to a wide variety of chemical classes [3,4]. Postsynaptic DA receptors have been classified on the basis of extensive pharmacological and biochemical studies, into D-1 and D-2 receptors [5,6]. While DA itself is achiral, both the D-1 and D-2 receptors exhibit a remarkable degree of stereoselectivity in agonist and antagonist binding and agonist activation of the transducer-effector systems. Amazingly enough, this stereoselectivity appears to be omnipresent with almost all chemical classes of DA receptor agonists and antagonists exhibiting it to a greater or lesser extent.

In order to illustrate the chiral properties of DA receptors, some important DA receptor agonists and antagonists belonging to distinct chemical classes will be examined.

APORPHINES

Apomorphine (APO), derived from morphine via an acid catalysed rearrangement, is the prototypical DA agonist and has a chiral center at C-6a position. Its absolute configuration was determined to be (R), by various chemical [7,8] and physical (ORD [9] and X-ray crystallographic [10]) methods. The synthesis of its (S) enantiomer was later carried out [11] and an examination of the pharmacological properties of the enantiomers in various tissues and species indicated that the DA receptor binding affinity and agonist activity is principally resident in the (R) enantiomer. The (S) enantiomer has significantly lower affinity but little or no agonist activity. However, the naturally occurring aporphine alkaloid (S) bulbocapnine, which has the opposite configuration to (R)APO, has long been known to be an antagonist of the stimulating effects of dopamine or (R)APO on striatal adenylate cyclase [12]. Further studies led to the characterisation of (S)APO as an antagonist particularly in neuropsychiatric animal models [13]. This finding was confirmed subsequently by studies on the effect of (S)APO on inhibition of DA and ACh uptake by (R)APO [14] and from competition binding experiments with various radioligands selective for D-1 and D-2 receptors [15]. Among the N-alkylated norapomorphine derivatives, (R)N-n-propylnorapomorphine (NPA) was found to be the most potent analogue [16,17], (Fig. 2.), as a centrally acting emetic, an indication



$R=CH_3$ R(-) APO
$R=C_3H_7$ R(-) NPA

S(+) APO
S(+) NPA

(+)BULBOCAPNINE

Figure 2. Structures of (R)APO, (R)NPA, their corresponding antipodes and (S) Bulbocapnine.

of DA agonist activity.

Synthesis of the corresponding (S) enantiomer of NPA and comparative evaluation of the two enantiomers in various biochemical and pharmacological tests provided further evidence for the predominant activity of the (R) enantiomer in the aporphine series of DA receptor agonists and antagonists [18,19]. Recent reports have suggested that the (S) enantiomer of NPA and its p.o. active prodrug derivative, (S)-10,11-methylenedioxy-N-n-propylnoraporphine ((S)MDO-NPA), exhibit limbic-selective dopamine antagonist actions in behavioral paradigms in rodents [20-22]. Recently, the effects of NPA enantiomers on single unit activity of substantia nigra pars compacta and ventral tegmental area (VTA) of DA neurons were evaluated. It was concluded that (S)NPA displays weak agonist-like effects in both populations of mid-brain dopamine cells with some selectivity for VTA neurons. In addition, (S)NPA exhibits weak antagonist activity when administered immediately before the more potent derivative, (R)NPA [23].

Still further evidence, for the stereoselectivity of hydroxylated aporphines, was obtained from synthesis and pharmacological evaluation of the enantiomers of 11-hydroxy-N-n-propylnoraporphine (11-OH NPa) [24]. In order to explain the agonist/antagonist properties of aporphine stereoisomers, a computer assisted stereochemical study was carried out with stereoisomeric pairs [25]. This study led to the proposition that the orientation of the ammonium hydrogen (or the lone pair of electrons on the nitrogen) is the most important factor in determining agonist/antagonist activity of the aporphines at DA receptors [25,26].

It is possible that in the aporphine enantiomers, the positive charge generated by protonation at physiological pH, is spread (delocalised) over adjacent C-N bonds so that the "back" of the N-H bond also contains significant positive charge [25], (Fig. 3.). Thus (S)NPA could bind to the same receptor as (R)NPA since the overall molecular shapes of these two molecules are similar and there would be sufficient charge at the

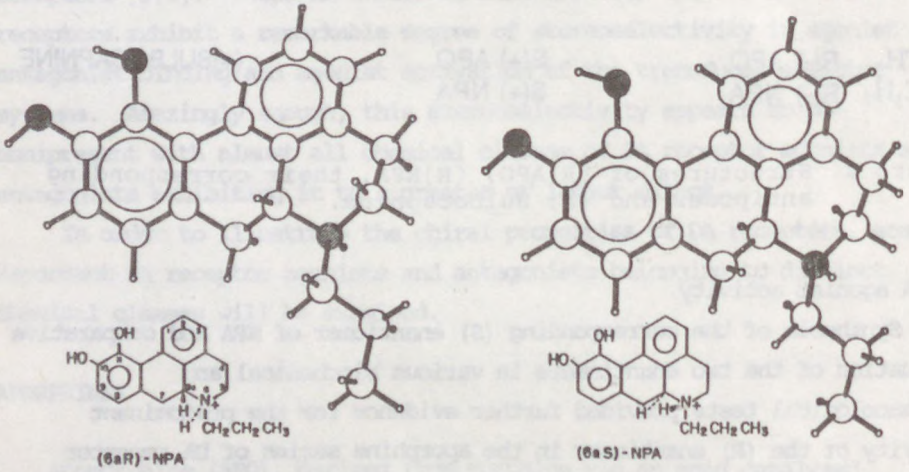


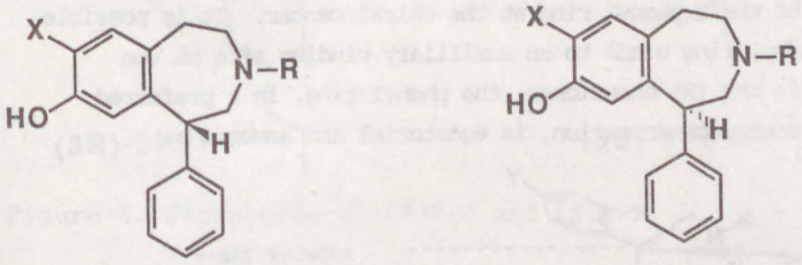
Figure 3. Computer generated structures of (R)- and (S)NPA (taken from ref. 25).

"back" of the N-H bond, in (S)NPA, to allow binding to a complementary electrostatic site on the receptor [25]. Antagonists could thus bind to the same receptor site as agonists, but it is probable that agonist activity would require a proper orientation of the N-H bond [25].

BENZAZEPINES

For the D-1 DA receptor, the currently available agonists and antagonists belong, almost exclusively, to the benzazepine class. The 7,8-dihydroxy-1-phenyl-1H-3-benzazepine (SKF 38393) was the first selective agonist at D-1 receptors [27]. Subsequently, the 6-chloro analogue was found to be more potent and selective [28]. A 4'-hydroxy group in the 1-phenyl ring provides SKF 82526 (Fenoldopam) which is a potent renal vasodilator antihypertensive devoid of CNS effects [29]. On the other hand, the 7-halogenated (Cl, Br, I)-8-hydroxy-3-methyl analogues lack agonist activity but are highly potent and selective D-1 antagonists [30], (Fig. 4.).

All these benzazepines possess a chiral center β - to the nitrogen where the phenyl substituent is located. Since the SAR of benzazepines indicate an important role for the 1-phenyl or substituted phenyl group in DA receptor binding and agonist activity, the enantiomers of these compounds were of interest. Resolution and pharmacological



X = OH, R = H (R)SKF38393
X = Cl, R = CH_3 (R)SCH23390

X = OH, R = H (S)SKF38393
X = Cl, R = CH_3 (S)SCH23390

Figure 4. Structures of substituted benzazepine enantiomeric pairs.

characterisation of the enantiomers revealed that dopaminergic activity resides almost exclusively in the (R) enantiomer [31], (Table 1.).

A comparison of the 8-hydroxy-1-phenyl-3-benzazepines with other DA agonists and antagonists suggests that the same structural features,

Table 1. Dopaminergic activity of (RS), (R) and (S) SKF 38393 (taken from ref. 31)

test system	(RS)	(R)	(S)
DA sensitive adenylate cyclase stimulation: EC_{50}, mol	7.1×10^{-8}	3.2×10^{-8}	-
inhibition of spiperone binding to rat caudal tissue, IC_{50}, µmol	34.43	33.86	197.4
contralateral rotation in lesioned rat: RD_{500}, mg/kg, ip	0.7	0.5	2.0
renal vasodilator activity, dogs: ED_{15}, µg/kg (iv)	31	25	550

postulated to be essential for dopaminergic activity, are present in this class of compounds viz a meta-OH group and a phenethylamine moiety. The mode of binding of the benzazepines to the DA receptor must, therefore, be similar to that of other chemical classes of ligands. Enantioselectivity, then persumably arises from the differences in orientations of the 1-phenyl ring at the chiral center. It is possible that the 1-phenyl ring binds to an auxilliary binding site at the receptor and in the (R) enantiomer, the phenyl ring, in a preferred (reasonable) energy conformation, is equatorial and assumes a

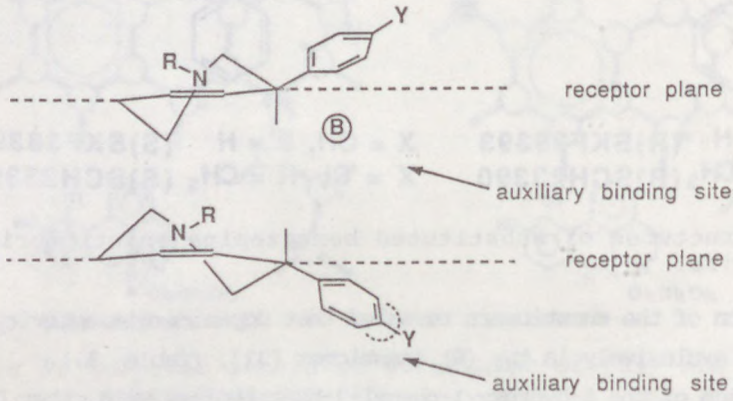


Figure 5. Postulated model to explain the activity/inactivity of benzazepine enantiomeric pairs (taken from ref. 32).

location slightly above the plane of the auxilliary binding site, whereas in the (S) enantiomer the same phenyl ring takes the place slightly below the site [32], (Fig. 5.). These differences in the position of the phenyl ring with respect to the auxilliary binding site may serve to explain the activity/inactivity of various substituted dopaminergic benzazepine agonists and antagonists [32,33].

PHENYLPIPERIDINES

Most DA receptor agonists, belonging to a wide variety of chemical classes, act at postsynaptic sites. The phenylpiperidines provided the first selective ligands for the DA autoreceptors (presynaptic receptor sites) [34]. 3-PPP was the most potent and selective compound in a series of N-alkylated analogues [35]. 3-PPP is chiral at the 3-position of the piperidine ring, β to the nitrogen and at the same position at which the

m-OH phenyl group is located (Fig. 6.).

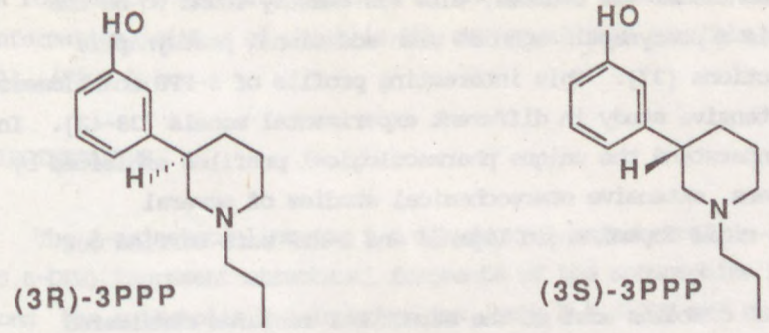


Figure 6. Structures of (R)PPP and (S)PPP.

This structural feature is similarly encountered in the 1-phenyl-3-benzazepines as discussed before. Resolution of 3-PPP and examination of the activity of its enantiomers provided interesting results [36] (Table 2).

Table 2. Presynaptic and postsynaptic activity of PPP enantiomers (taken from ref. 36)

compound	presynaptic agonism ED_{50}, nmol/kg		postsynaptic agonism motor activity	
	limbic	striatum	dose µmol/kg	accumulated counts/30 min
(R) PPP	1000	1300	13	78 ± 14
(S) PPP	800	1700	213	12 ± 2

The (R) enantiomer behaves as a pre- as well as postsynaptic agonist. Earlier, the racemate had been shown to be devoid of postsynaptic agonist actions. Taking these two observations into consideration together, it would thus logically appear that, in the racemate, the (S) enantiomer must have an antagonist effect on the

postsynaptic stimulation of the DA receptor by the (R) enantiomer. When the pure (S) enantiomer was studied, this was exactly found to be the case : (S)PPP is a presynaptic agonist with additional postsynaptic antagonistic actions [37]. This interesting profile of 3-PPP enantiomers prompted an extensive study in different experimental models [38-42]. In an effort to understand the unique pharmacological profiles exhibited by 3-PPP enantiomers, extensive stereochemical studies of several rigid and semi-rigid dopaminergic ligands and 3-PPP were carried out [39].

Since 3-PPP contains most of the structural features considered essential for ligand binding to DA receptors, it can be concluded that, in general, the structural, spatial requirements for binding to and activation of central pre and postsynaptic DA receptors are similar if not identical [43]. The presynaptic selectivity of PPP cannot be explained on the basis of geometrical fit of the molecule alone. Rather, specific properties of the molecule such as the size of the N-alkyl

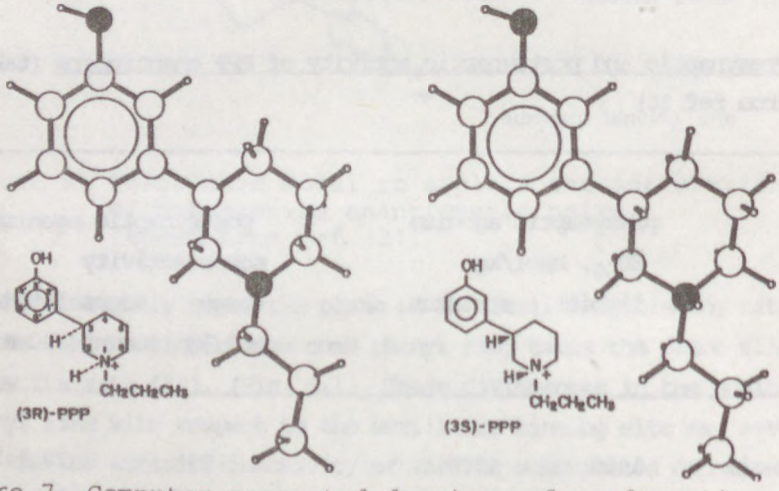


Figure 7. Computer generated drawings of conformations of (R)PPP and (S)PPP which serve to explain their different pharmacologic activity (taken from ref. 25).

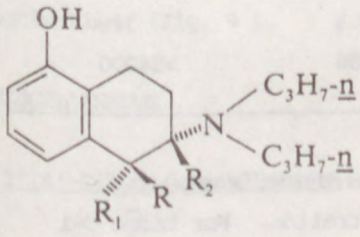
substituent, lipophilicity, conformational probability, directionality and conformational energy required for the molecule to acquire a correct geometry, have to be taken into consideration [43].

The presynaptic selectivity of (S)PPP and the nonselectivity of (R)PPP probably arise from different N-alkyl directionalities in their active (agonist) conformation and the conformational energy necessary to

acquire these conformations [43]. (S)PPP probably acts as an agonist in one rotameric conformation and as an antagonist in another rotameric conformation neither of which is the energy minimum for this molecule [43] (Fig. 7.).

AMINOTETRALINS

The 2-aminotetralins (eg. 5,6-dihydroxy-2-aminotetralins or A-5,6-DTN) represent structural fragments of the apomorphine molecule. Among the catecholic 2-aminotetralins, both A-5,6-DTN and the isomeric A-6,7-DTN possess considerable dopaminergic activity. Among the monohydroxy aminotetralins, the 5-hydroxy analogue is the most potent with a degree of selectivity for the D-2 subtype [44]. All the dopaminergic 2-aminotetralins have a chiral center, located α to the amino nitrogen analogous to the aporphines, which remains part of the pharmacophore of the aminotetralin skeleton. A-6,7-DTN stereoisomers were resolved and their absolute configurations determined by both chemical degradation methods and X-ray crystallography [45,46]. Other aminotetralins including A-5,6-DTN and 5-hydroxy analogues were also resolved and extensive pharmacological studies provided interesting and unexpected results, (Fig. 8).



R	R_1	R_2
CH_3	H	H (trans)
H	CH_3	H (cis)
H	H	H

Figure 8. Structures of aminotetralin enantiomers.

In the A-5,6-DTN series, the more active enantiomer is (R) [44,46] while in the A-6,7-DTN and 5-hydroxy series, the more active enantiomer is (S) [45, 47-52] (Table 3).

McDermed et al. proposed a conformational DA receptor model to explain these unusual findings. They proposed that for agonist binding to DA receptors, steric orientation of the amino group of the agonist is controlled by the position of the hydroxy group and hence in order to

achieve a complementary agonist-receptor binding site interaction, a proper orientation of the amino and hydroxyl groups maybe attained by rotation of the molecule. Thus while (R)APO and (S)5-OH-DPAT have the similar orientation, absolute configuration and hence bind similarly, (R)A-6,7-DTN having the opposite configuration, possibly undergoes a rotation to fit to the same complementary binding sites on the receptor [45,53,54].

Table 3. Pharmacological activity of various aminotetralin enantiomers (taken from ref. 58)

compound	^{3}H-spiperone pIC_{50}	^{3}H-NPA pIC_{50}	ED_{50} nmol/kg
2S-5-OH DPAT	6.24	8.24	3.7
2R-5-OH DPAT	4.15	6.80	530
1S,2S-1-Me-5-OH DPAT	<1	5.79	8700
1R,2R-1-Me-5-OH DPAT	<1	4.28	>54000
1R,2S-1-Me-5-OH DPAT	5.42	7.53	340
1S,2R-1-Me-5-OH DPAT	5.15	6.56	>54000

Further interesting observations came from the work on C-1 methylated analogues of the 5-hydroxy-2-aminotetralins. For these C-1 methylated analogues, the introduction of the second chiral center provides four possible diastereomers with considerably different pharmacological profiles [55-58]. The two cis enantiomers have a pronounced selectivity for DA autoreceptors [56]. However, while the (1R, 2S) enantiomer is a centrally acting DA agonist, the corresponding (1S, 2R) enantiomer is a centrally acting DA antagonist [56,57]. In contrast, the two trans enantiomers (1R, 2R) and (1S, 2S) are either inactive (former) or considerably less potent (latter) [57]. The low potency/inactivity of the trans enantiomers has been proposed to be due to unfavourable steric interaction of the C-1 methyl group with the

ammonium hydrogen atom which persumably leads to improper orientations of the N-H bond or unfavourable energies of the conformation required for optimum DA receptor binding and activation [58]. On the other hand, among the cis enantiomeric pair, the agonist/antagonist activity of

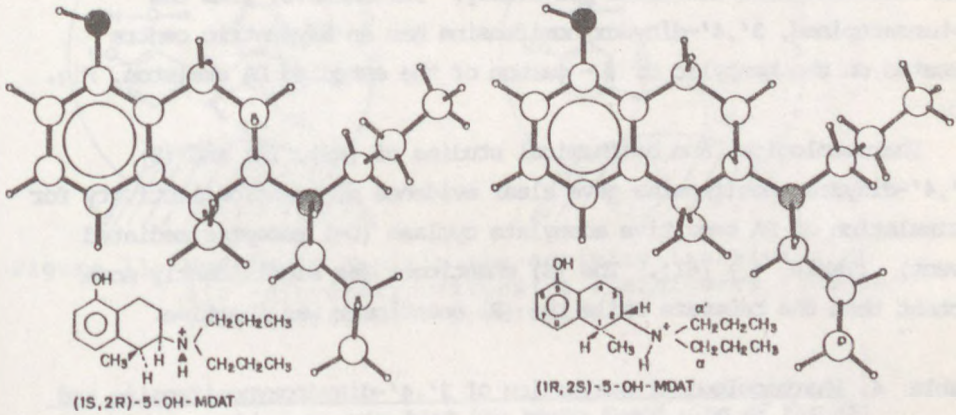
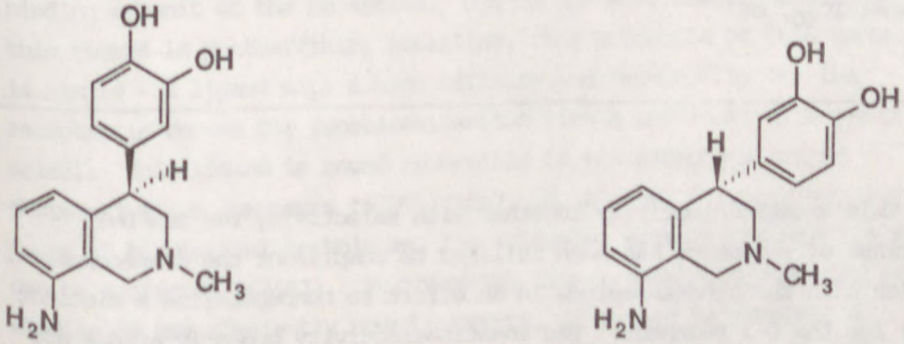


Figure 9. Conformations of cis-1-methyl aminotetralin enantiomers (taken from ref 25).

the pair probably arises due to energetically unfavourable 1,2-diaxial interactions between the ammonium hydrogen atom (or the nitrogen electron pair) and the C-2 hydrogen in the conformations required for DA receptor activation (Fig. 9).

MISCELLANEOUS

3',4'-Dihydroxynomifensine:



(S) 3',4'-dihydroxynomifesine (R) 3',4'-dihydroxynomifesine

Figure 10. Structures of 3',4'-dihydroxynomifensine enantiomers.

3',4'-Dihydroxynomifensine, a tetrahydroisoquinoline, is a well characterised central as well as peripheral DA receptor agonist [59,60]. It was a particularly valuable tool owing to its selectivity for the D-1 sub-class of DA receptors, a property which it possesses in common with the 3-benzazepines discussed previously. Furthermore, like the 3-benzazepines, 3',4'-dihydoxynomifensine has an asymmetric centre located on the benzylic or β - carbon of the embodied DA skeleton, Fig. 10.

Pharmacological and biochemical studies of (RS), (R) and (S) 3',4'-dihydroxynomifensine gave clear evidence of enantioselectivity for stimulation of DA sensitive adenylate cyclase (D-1 receptor mediated event) (Table 4) [61]. The (S) enantiomer was significantly more potent than the racemate while the (R) enantiomer was inactive.

Table 4. Pharmacological evaluation of 3',4'-dihydroxynomifensine and its enantiomers (taken from ref. 61)

test system	(RS)	(R)	(S)
DA-sensitive adenylate cyclase stimulation: EC_{50}, μM	2.88	inactive	1.87
Displacement of spiperone binding to rat caudate tissue: IC_{50}, μM	92	99.1	85.8

This enantioselectivity together with selectivity for the D-1 sub-class of receptors has been utilised to complement the stereochemical studies with the 3-benzazepines in an effort to conceptualize a distinct model for the D-1 receptor. The enantioselectivity probably arises due to the fact that in the (S) enantiomer of 3',4'-dihydroxynomifensine, the bulk of the tetrahydroisoquinoline ring system is directed away from a

postulated site of steric occlusion, whereas in the (R) enantiomer, this bulk is directed precisely toward the site of intolerance.

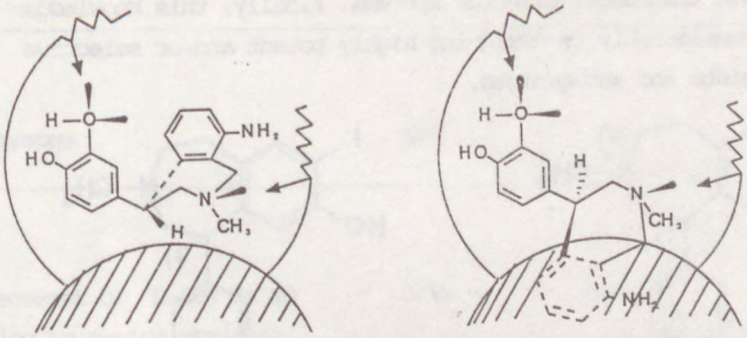


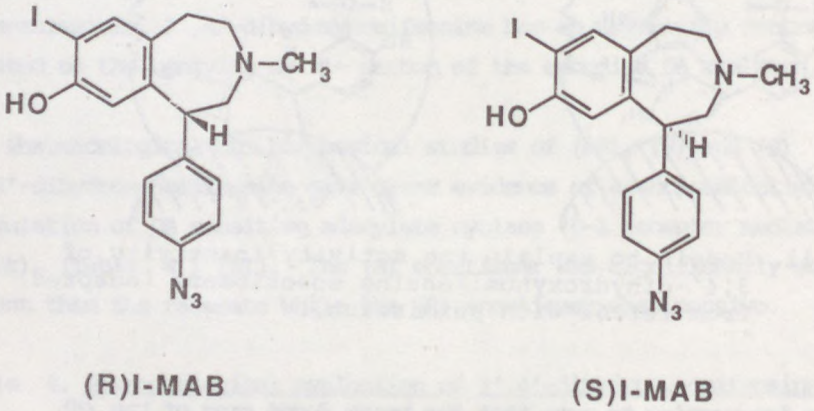
Figure 11. Models to explain the activity/inactivity of 3',4'-dihydroxynomifensine enantiomers (adapted from ref.61 with permission).

It is interesting to note that the benzo fused ring of the (S) enantiomer occupies a position above the plane of the postulated site of steric occlusion on the receptor, that is virtually identical with the site occupied by the 1-aryl ring of the benzazepine D-1 DA receptor agonists. This similarly implies the existence of an accessory binding site on the D-1 receptor in the vicinity of the site of steric hindrance (Fig. 11).

I-MAB

Development of more potent and/or selective DA receptor agonists could be achieved by understanding the molecular nature of the ligand binding subunit of the receptors. One of the most useful techniques in this regard is photoaffinity labelling. The principle of this technique is simple - a ligand with a high affinity and selectivity for the receptor is chosen for functionalisation with a photoactivable group (eg. azide). This ligand is bound reversibly to the receptors but on photoactivation (exposure to UV light), it is bound irreversibly and by means of biochemical techniques, the molecular size of the bound receptor can be estimated [62,63]. Furthermore, from proteolytic degradation studies of the covalently bound receptor, it should be possible to identify the peptide sequence forming part of the ligand binding site of the receptor. A 3-D mapping of the ligand binding site should then be

possible and this in turn should lead to identification of the actual amino acids involved in ligand-receptor binding and agonist activation of the appropriate transducer-effector systems. Finally, this knowledge should help considerably in designing highly potent and/or selective receptor agonists and antagonists.



(R)I-MAB (S)I-MAB

Figure 12. Structures of I-MAB enantiomers.

The development of I-MAB enabled the preliminary characterisation of the binding subunits of the D-1 receptor [64-66]. Like other benzazepines, I-MAB possesses a chiral centre at the C-1 position β - to the nitrogen (Fig. 12).

Resolution of I-MAB and studies with the enantiomers indicated that the (R) enantiomer is considerably more potent than the (S) enantiomer a finding (Table 5) which consolidates the earlier discussed proposition that, in the benzazepine series of DA receptor agonists and antagonists, the D-1 receptor binding and activation ability resides principally in the (R) enantiomer.

Further biochemical studies with (R)I-MAB led to the observation that (R)I-MAB is bound covalently and stereoselectively to the D-1 receptor [67]. This provided a confirmation of the previous estimate of the molecular size of the receptor obtained from studies with (RS)I-MAB [64,65]. Thus (R)I-MAB provides an example of a stereoselective photoaffinity label for the further molecular studies of the D-1 receptor. In particular the stereoselectivity of I-MAB could serve as a useful tool to explore the chiral nature of the D-1 receptor binding site.

Table 5. Affinity of I-MAB and its enantiomers for D-1 receptor sites (taken from ref. 67)

test system	(RS)	(R)	(S)
Displacement of ^{3}H-SCH 23390 binding to canine striatal membranes: K_I, nM	0.38	0.28	28

ACKNOWLEDGEMENTS

This work was supported in part by USPHS grants NS 15439 and MH 47370 and a graduate research award to NB from Research Biochemicals Inc.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

THE ENANTIOSELECTIVE PHARMACOLOGICAL SPECTRUM OF *levo*-DEPRENYL

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Amazing as it is, hundreds of preparations containing a chiral molecule and its mirror image are described and still treated in national lists of drugs as well as in pharmacological handbooks as pharmacologically homogeneous entities. It is of course common knowledge that in a variety of pharmacological tests considerable quantitative differences between the enantiomers are usually detectable and even tests in which only one of them is active may exist. Such quantitative differences between the enantiomers are obviously not incompatible with the handling of the racemic mixture in therapy as a single drug. The deceptive nature of this practice, however, becomes immediately clear if the almost always existing qualitative differences in the pharmacological spectrum of the enantiomers are carefully detected. It seems to be a warmly recommendable good practice for the future to use in therapy a clinical molecule without "mixing" it, for evidently financial reasons, with its mirror image. This paper, summarizing the *levo*-deprenyl story, is devoted to demonstrate the practical importance of this recommendation by an experimental example.

The "cheese effect"; the physiolgically inherent danger accompanying monoamine oxidase (MAO) inhibition

Potentiation of the pressor effect of tyramine is likely to be the main cause of the dangerous hypertensive reaction which supervenes after the intake of certain foodstuffs containing high amounts of the free amines (e.g. cheese, yeast

products, beans, Chianti wines, pickled herring, chicken liver, etc.) in patients treated with MAO inhibitors. The "cheese reaction" seriously discredited the MAO inhibitors and restricted their therapeutic use, which needs careful medical control. The "cheese effect", first described by Blackwell in 1963 was thought to be primarily a consequence of inhibition of the intestinal enzyme[1].

There is now general agreement that two main forms of mitochondrial MAO exist. The discovery of this situation and the development of our present knowledge concerning the dual nature of MAO is inseparable from the introduction of two substrate-selective highly potent irreversible inhibitors, deprenyl and clorgyline.

rac-Deprenyl (N-phenylisopropyl-N-methyl-propargylamine) was developed by Knoll et al. in 1964 and noted to be a compound with a new spectrum of MAO inhibitory action. Detailed analysis of its pharmacological and biochemical effects revealed that (-)deprenyl is a selective inhibitor of a particular type of MAO which deaminates benzylamine, metaiodobenzylamine and phenylethylamine[2,3].

Clorgyline (N-2,4-dichlorophenoxypropyl-N-methyl-propargylamine), a compound similar in structure to deprenyl, was developed in 1968 by Johnston, who found it to be a selective inhibitor of that type of MAO which deaminates 5-HT. To distinguish the two forms of MAO, one highly sensitive to clorgyline and one relatively insensitive to it, he introduced the terms 'type A' and 'type B'. This nomenclature has become widely accepted. MAO-A is selectively inhibited by clorgyline, MAO-B by (-)deprenyl.

As mentioned above, the inhibition of intestinal MAO was thought to play the main role in the "cheese effect". Squires demonstrated[4] that MAO-A is the predominant activity in the gut of many different species. It is in good agreement with the finding that *levo*-deprenyl is much less potent[2] than clorgyline in inhibiting intestinal MAO.

The practical importance of an MAO inhibitor free of the "cheese effect" is obvious. Is it enough to use a selective inhibitor of MAO-B type to this aim? This was the question

raised by the findings with deprenyl.

The introduction of *levo*-deprenyl, the first and still the only safe MAO inhibitor in therapy free of the "cheese effect"

In 1960, we were attracted by the finding that *rac*-amphetamine improved (in lower doses) or disturbed (in higher doses) the performance of the rat in the one-way avoidance test. Aiming at the development of new derivatives with more selective actions on the CNS than the parent compound, we synthesized and analysed a series of new methamphetamine derivatives and learned that proper substitution at the para position leads to structures with remarkable selectivity for catecholaminergic or serotonergic systems[5].

In 1963, the "cheese effect" focussed our attention on MAO inhibitors and we decided to extend our structure-activity relationship study with methamphetamine derivatives in an MAO inhibitor direction. *rac*-Methamphetamine itself is a reversible inhibitor of MAO. We knew from our unpublished observations with newly synthesized *rac*-methamphetamine derivatives that compounds with substitutions at the nitrogen moiety might, by comparison with the parent compound, practically lose the ability to release biogenic amines from the cytoplasmic pools of the nerve endings. We learned from the structure of pargyline, which had been introduced at that time into clinical practice, that the attachment of a propargyl group to the nitrogen in benzylamine inhibits MAO irreversibly; we therefore synthesized a number of new *rac*-metamphetamine derivatives containing this group and analysed their MAO inhibitory ability and the changes in their pharmacological spectrum. As *rac*-methamphetamine was the parent compound, we naturally attached the propargyl group to it in our first series of experiments. *rac*-N-phenylisopropyl-N-methyl-propargylamine HCl (E-250) was soon selected as a highly potent irreversible MAO inhibitor with a peculiar spectrum of pharmacological activity. The most remarkable finding was that, in striking contrast to *rac*-methamphetamine, its propargyl derivatives, especially the levorotatory isomer, inhibited the release of biogenic amines from nerve terminals

and acted in vivo and in vitro as a potent antagonist against tyramine. We expressed the hope "that this tyramine inhibiting property of E-250 may be highly valuable for human therapy"[6] and argued that (-)-R-N-phenylisopropyl-N-methyl-propargylamine HCl, later named *levo*-deprenyl, is the first highly potent MAO inhibitor to be found which does not have the "cheese effect".

Our claim that *levo*-deprenyl is an MAO inhibitor without the "cheese effect" was substantially supported by the experience of clinicians, who never observed hypertensive reactions[2].

Clinical advantage of the peculiar pharmacological spectrum of *levo*-deprenyl was first taken in connection with the levodopa treatment of parkinsonian patients. The possibility of potentiating the levodopa effect by concurrent administration of an MAO inhibitor had always been apparent, and was checked by Birkmayer and Hornykiewicz in 1962. MAO inhibitors, however, potentiated the unwanted effects of levodopa and the danger of hypertensive crises supervening made such a combination impossible.

levo-Deprenyl, an MAO inhibitor without the "cheese effect", lent itself particularly well to this purpose because it could be safely combined with levodopa. Birkmayer et al. demonstrated between 1975 and 1977 the clinical benefit of concurrent administration of levodopa plus a peripheral decarboxylase inhibitor plus *levo*-deprenyl. Their finding was corroborated by many authors[2]. *levo*-Deprenyl was proved conclusively to potentiate the therapeutic effect of levodopa, without increasing its side-effects.

The example of *levo*-deprenyl led in a confusing manner to the view that selective inhibitors of MAO-B type, in general, are free of the "cheese effect".

According to our experiences the pulmonal artery strip of the rabbit is the most sensitive preparation to test the tyramine-potentiating ability of a compound. Cumulative doses (1, 3 and 8 µg/ml) of tyramine are first given to check the noradrenaline releasing effect of this indirectly acting amine. There is a dose-related cumulative increase in the tone of the vascular smooth muscle preparation due to the tyramine-induced

release of noradrenaline. The contraction of the smooth muscle is measured quantitatively. Normal tone returns readily after washing out tyramine from the tissue. The tyramine-potentiating effect of an MAO inhibitor is easily detectable in this sensitive test by checking the tyramine-induced contractions of the smooth muscle before and 20 minutes after the administration of the MAO inhibitor into the bathing fluid in which the pulmonal artery strip of the rabbit is suspended.

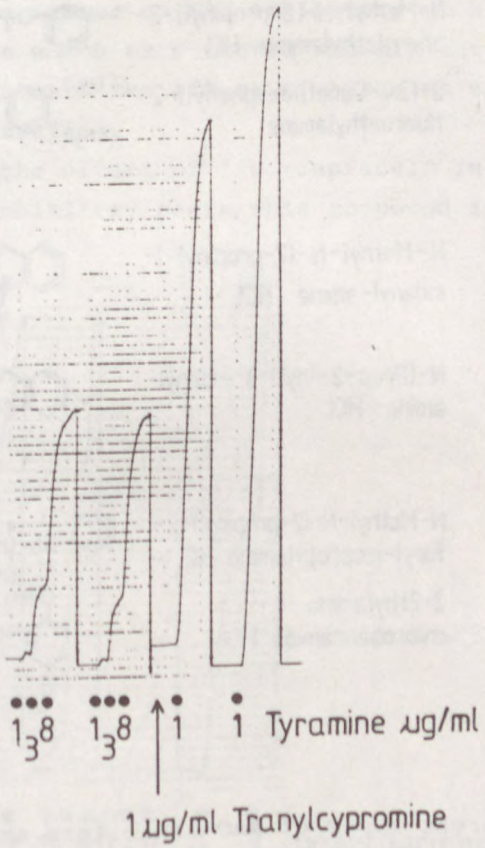
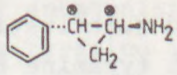
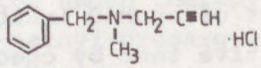
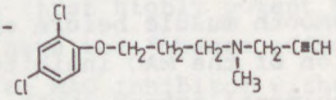
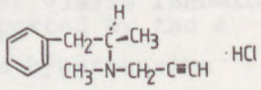
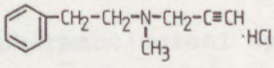
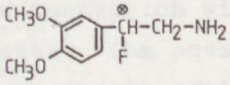
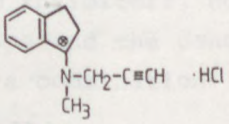
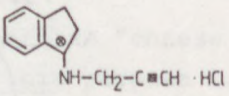


Figure 1. The effect of *rac*-tranylcypromine on the tyramine-induced contractions of the pulmonal artery strip of the rabbit.

Phenylalkylamine derivatives

rac-Tranylcypromine	*trans*-(±)-2-Phenylcyclo-propylamine		ref. 8
Pargyline	N-Methyl-N-(2-propinyl)-benzylamine · HCl		ref. 9
Clorgyline	N-[3-(2.4-Dichlorophenoxy)-propyl]-N-methyl-prop-2-inylamine		ref. 10
levo-Deprenyl	(-)-R-N-Methyl-N-(2-propinyl)-1-phenylisopropylamine · HCl		ref. 11
TZ-650	N-Methyl-N-(2-propinyl)-2-phenylethylamine · HCl		ref. 12
rac-MDL-72145	2-(3.4-Dimethoxyphenyl)-2-fluoroethylamine		ref. 13

Indane derivatives

rac-J-508	N-Methyl-N-(2-propiny)-1-indanyl-amine · HCl		ref. 12
rac-AGN-1135	N-(Prop-2-inyl)-1-indanyl-amine · HCl		ref. 14

Miscellaneous

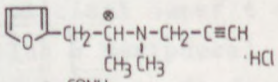
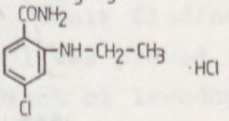
rac-U-1424	N-Methyl-N-(2-propinyl)-2-furyl-isopropylamine · HCl		ref. 12
RO-16-6491	2-Ethylamino-4-chlorobenzamide · HCl		ref. 15

Figure 2. Different types of MAO inhibitors analysed on the pulmonal artery strip of the rabbit.

Fig. 1 shows a typical experiment demonstrating the tyramine-potentiating effect of *rac*-tranylcypromine, one of the few MAO inhibitors still in medicinal use. *rac*-Tranylcypromine is a highly potent, non-selective MAO inhibitor, i.e. it inhibits both forms of MAO equally. Clorgyline, which is the prototype of the selective inhibitors of MAO-A type, potentiates the effect of tyramine on the pulmonal artery strip of the rabbit as effectively as *rac*-tranylcypromine. Using this sensitive test we found, however, that even selective inhibitors of MAO-B type potentiate the effect of tyramine.

Fig. 2 shows a number of MAO inhibitors with different chemical structures which were tested and all but *levo*-deprenyl were found to potentiate the effect of tyramine on the vascular smooth muscle preparation.

Fig. 3 shows the effect of *levo*-deprenyl. In striking contrast to its MAO-inhibitory peers, this compound is a potent in-

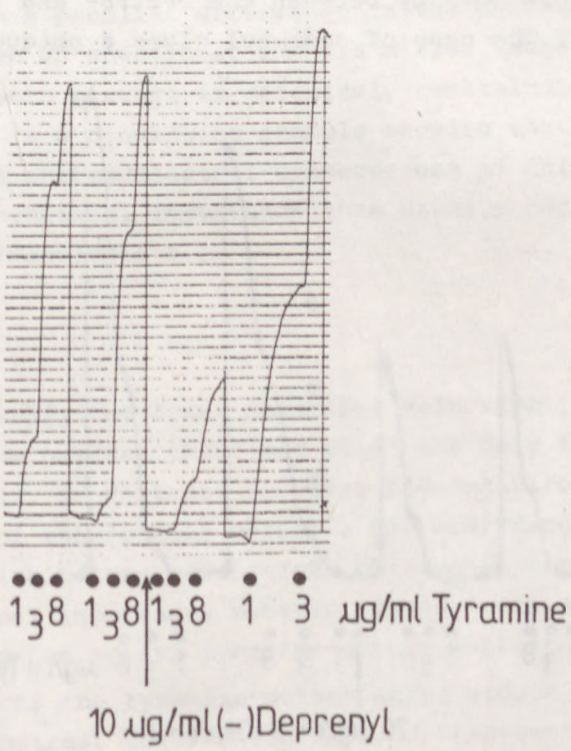


Figure 3. The effect of *levo*-deprenyl on the tyramine-induced contractions of the pulmonal artery strip of the rabbit.

hibitor of the uptake of tyramine in the test. It is evident that this effect is independent from its MAO inhibitory potency. The safety of *levo*-deprenyl is due to its tyramine-uptake inhibitory potency which counteracts the tyramine potentiating effect of MAO inhibition. This was found to be a very rare combination of pharmacological effects. During the 25 years of deprenyl research we synthesized over 300 analogues, but 5 compounds only shared with *levo*-deprenyl the peculiar pharmacological spectrum to inhibit B-type MAO and tyramine-uptake simultaneously.

Enantioselectivity in the safety of deprenyl

As it was mentioned in the introduction of this paper, an enantiomer possesses usually the same pharmacological spectrum as its mirror image, though, depending on the test, remarkable differences in potency between the *dextro*- and *levo*-isomers might exist. The case of deprenyl gives a unique example that a

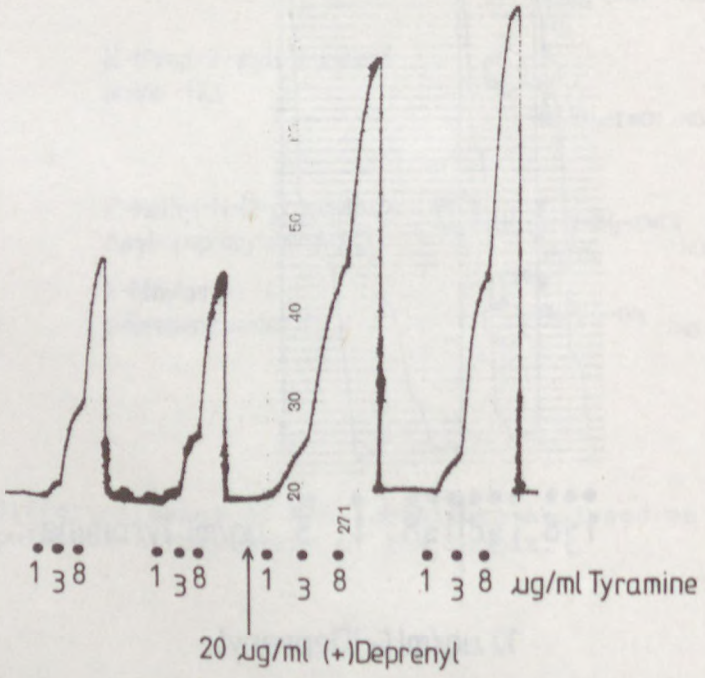


Figure 4. The effect of *dextro*-deprenyl on the tyramine-induced contractions of the pulmonal artery strip of the rabbit.

certain lucky combination of potencies in different pharmacological tests may have the final result that one enantiomer is from the practical point of view a qualitatively differently acting drug than the other. The MAO inhibitory nature of both dextro- and levorotatory deprenyl results in the potentiation of the tyramine effect and the tyramine-uptake inhibitory potency works against this effect. The result will finally determine the safety of the compounds.

Fig. 4 shows that *dextro*-deprenyl potentiates the effect of tyramine as all other MAO inhibitors do. Thus, *dextro*-deprenyl does not share with *levo*-deprenyl the exceptional ability to be a safe MAO inhibitor. *levo*-Deprenyl has to be given uninterruptedly for decades[7], thus, its safety is of peculiar practical importance. Because of the tyramine-potentiating effect of the *dextro*-isomer, even the racemic mixture is unsafe.

The case of *levo*-deprenyl is up to the present a unique example of such a peculiar difference in the pharmacological spectrum between an enantiomer and its mirror image that the use of the racemic mixture is definitely contraindicated. The deprenyl-story is a convincing example showing that the detailed analysis of the pharmacological consequences of chirality might be of greater practical importance than usually realized at present by pharmacologists.

Summary

levo-Deprenyl, the first described selective inhibitor of B-type monoamine oxidase (MAO) and still the only one in clinical practice, is, in contrast to other MAO inhibitors used in therapy, free of the "cheese effect", the most dangerous side-effect of MAO inhibitors. The safety of *levo*-deprenyl, which is of high practical importance because it has to be administered for decades, is due to its tyramine-uptake inhibitory potency which counteracts the tyramine-potentiating effect of MAO inhibition. In contrast to *levo*-deprenyl, *dextro*-deprenyl shares with other MAO inhibitors the tyramine-potentiating effect.

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Akadémiai Kiadó
Budapest, 1990

THE CHIRAL PROPERTY OF 1,4-BENZODIAZEPINES RECOGNIZED BY CENTRAL RECEPTORS

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The problem

1977 was the year of discovery for the benzodiazepine (BZ) receptor in mammalian brain.[1,2] The receptor's ability of chiral discrimination was shown[2] by applying 3-methyl-flunitrazepam; the (+)-(3S)-methyl enantiomer displaced ^{3}H-diazepam at a concentration about 200 times lower than that effective for the (-)-(3R)-methyl antipode. The center of asymmetry is, however, not the only element of chirality for 3-Me-1,4-BZs, since the diazepine ring exists in two kinds of boat conformation, P and M, chiral themselves regardless of the substitution pattern at position 3 (Figure 1).

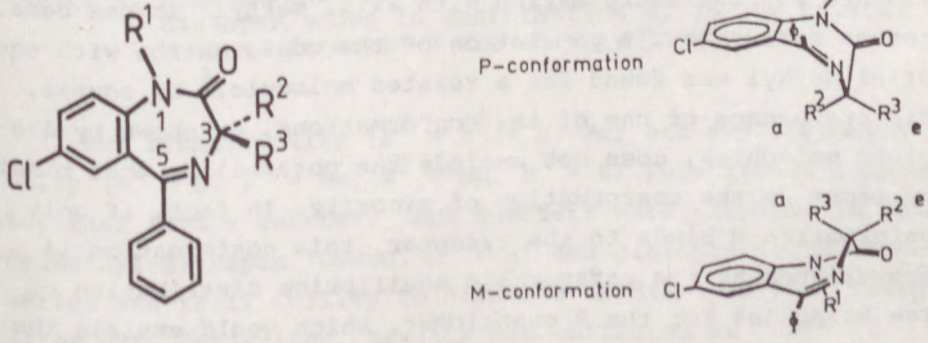

Figure 1. Conformations are assigned P (plus), or M (minus) according to the sign of the torsion angle C2-C3-N4-C5. Axial (a) and equatorial (e) positions are indicated for substituents attached to C3.

Stereoselective binding to BZ receptors was also demonstrated by applying conformationally defined anthramycine-type analogs of 1,4-BZs;[3] the (3S) enantiomer restricted to conformation M was at least 160 times more active than the (3R) stereoisomer closed into conformation P. It appeared, therefore, that chiral discrimination by central BZ receptors is confined to selecting the conformation M of 1,4-BZs.

There is, however, a problem inherent in any approach applying conformationally defined analogs of flexible molecules. Conformational restriction is always associated with additional steric requirements of the extra group of atoms which force the molecule into the restricted conformation. Hence, we can only detect experimentally the conformational preference superimposed by some extra steric effect. 3-Methyl-substituted 1,4-BZs offer a way leading to the separation of these two factors.[4]

The quartet principle

In fact, a 3-methyl substituent brings about a kind of preference between the conformations depicted in Figure 1; the molecule is more stable in solution if the methyl group occupies equatorial position. Consequently, if R^2 is methyl and R^3 is hydrogen (S configuration), the molecule prefers conformation M (cf. Fig.1). The reverse is true for the R enantiomer. In more quantitative terms, NMR measurements indicated no evidence for the conformation with axial methyl[5] in one case, whereas more than 97% population of the conformation with equatorial methyl was found for a related molecule.[6] Of course, this preference of one of the conformations, as shown by dissolved molecules, does not exclude the possibility that binding may occur in the conformation of minority. In fact, if only conformation M binds to the receptor, this conformation is impoverished by the unfavorable equilibrium distribution of free molecules for the R enantiomer, which would explain the stereoselectivity giving preference for the S enantiomer. In that case, however, the R enantiomer binds to the receptor with an axial methyl group (conformation M!), and the question arises as to whether the axial methyl group can be tolerated

by the receptor. It is possible to obtain an unequivocal answer to this question by testing the binding ability of 3,3-dimethyl-1,4-BZs that identically populate both conformations and have an axial methyl group in either case. Such compounds can be synthesized as proved by the X-ray structure shown in Figure 2. Data of structure determination are given in the Appendix.

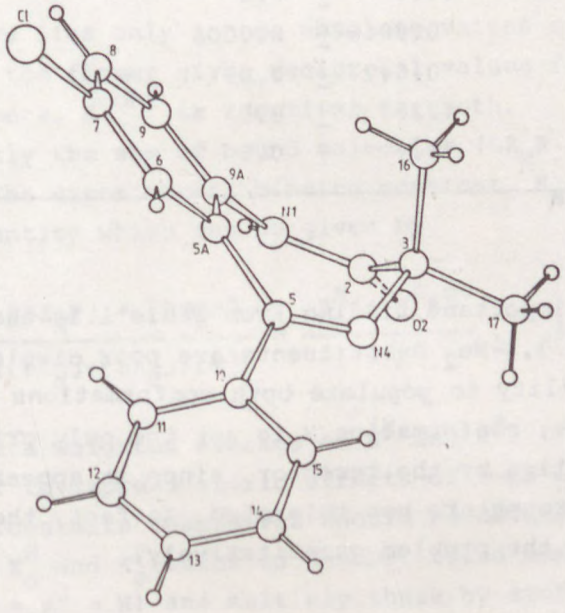

Figure 2. Molecular structure of 3,3-dimethyl-desmethyl-diazepam shown in conformation M. In the crystal both conformations are equally populated.

Two symmetrically (R^2 = R^3 = H, Me) and two asymmetrically (R^2 = H, R^3 = Me; R^2 = Me, R^3 = H) substituted compounds together form a quartet. Such quartets were prepared in both the desmethyl-diazepam (DeMeD, R^1 = H) and diazepam (D, R^1 = Me) series and their ability to displace ^{3}H-diazepam from receptor sites was determined. Results are collected in Table 1.

Table 1. Receptor binding of the methyl quartets

Compound	IC_{50} (μM)	$IC_{50}(R)/IC_{50}(S)$
DeMeD	0.010 ± 0.002	
(3S)-Me-DeMeD	0.061 ± 0.004	
(3R)-Me-DeMeD	68 ± 11	1117
3,3-Me_2-DeMeD	14.3 ± 2.4	
D	0.0046 ± 0.0005	
(3S)-Me-D	0.047 ± 0.011	
(3R)-Me-D	12.8 ± 3.3	274
3,3-Me_2-D	4.7 ± 0.5	

The most important finding from Table 1 is that both compounds with 3,3-Me_2 substituents are poor displacers despite their ability to populate both conformations equally in solution. Hence, conformation M is not the only criterion of chiral recognition by the receptor, since it appears that axial methyl groups are not tolerated. In fact, the quartets allow to treat the problem quantitatively.

Mathematical description of the quartets

Suppose that both conformations participate in receptor binding and they differ in affinity:

$$BZ_M + R \overset{K^M}{\rightleftharpoons} BZ_MR \qquad (1)$$

$$BZ_P + R \overset{K^P}{\rightleftharpoons} BZ_PR \qquad (2)$$

where BZ_M and BZ_P are free molecules accomodating the conformations indicated, R is a vacant receptor site, K^M and K^P are binding constants for the individual conformations. Symmetri-

cally substituted members of the quartets ($R^2 = R^3 = H$, Me) populate the two conformations is solution identically. For 3-Me enantiomers the population is determined by an equilibrium constant that can be defined in two ways:

$$K^c = \frac{[BZ_p]}{[BZ_M]} \; ; \quad \text{or} \quad K^{conf} = \frac{[Me^e]}{[Me^a]} \; . \qquad (3)$$

The difference lies only in the absolute values of K^c and K^{conf}; while the former gives reciprocal values for (3S) and (3R) enantiomers, K^{conf} is identical for both.

Since only the sum of bound molecules ($BZ_MR + BZ_PR$) is measurable, the experimental binding constant, K_{app} is a composite quantity which can be given by

$$K_{app} = \frac{[BZ_MR] + [BZ_PR]}{\{[BZ_M]+[BZ_p]\}[R]} = \frac{K^M + K^PK^c}{1 + K^c} \; , \qquad (4)$$

hence K_{app} is a weighted average of K^M and K^P.

In order to express steric effects of 3-Me substituents, the binding constants themselves should be defined more exactly. Let K_o^M and K_o^P refer to unsubstituted members of the quartets ($R^2 = R^3 = H$) and multiply these by steric factors f^e and f^a, in order to describe the effects of equatorial and axial methyl groups, respectively. This treatment gives the following expressions for members of the quartets:

$$R^2 = R^3 = H \qquad K_{app} = \frac{K_o^M + K_o^P}{2} \qquad (5)$$
$$K^c = 1$$

$$\text{(3S)-Me} \qquad K_{app} = \frac{f^eK_o^M + f^aK_o^PK^c}{1 + K^c} =$$
$$R^2 = Me$$
$$R^3 = H$$
$$K^c = \frac{1}{K^{conf}} \qquad = \frac{f^eK_o^MK^{conf} + f^aK_o^P}{K^{conf} + 1} \qquad (6)$$

(3R)-Me

R^2 = H, $K_{app} = \frac{f^a K_o^M + f^e K_o^P K^c}{1 + K^c} =$

R^3 = Me

$K^c = K^{conf}$

$$= \frac{f^a K_o^M + f^e K_o^P K^{conf}}{1 + K^{conf}} \qquad (7)$$

$R^2 = R^3$ = Me $\qquad K_{app} = f^e f^a \frac{K_o^M + K_o^P}{2} \qquad (8)$

$K^c = 1$

Four independent equations involving four unknown quantities allow a mathematical solution to be obtained. A technical problem is that K^{conf} is not known exactly for the enantiomers, so the lower limit of K^{conf} = 32 was used expressing 97% population for the conformation with equatorial methyl group.[6] For K_{app} values the reciprocal of IC_{50} data were used.

Table 2. Conformational preference (K^{conf}) of 3-Me-BZ enantiomers, conformational recognition (K_o^M/K_o^P) by the receptor and steric factors (f^a, f^e) computed from IC_{50} values (Table 1) by eqs. (5)-(8)

Parameter	DeMeD series	D series	Condition
K^{conf} for 3-Me-BZ enantiomers	114	109	$K_o^P < 0$
K_o^M/K_o^P	4641	1001	K^{conf}=150
K_o^M/K_o^P	1450	351	K^{conf}=500
f^a	0.0084	0.0197	K^{conf}=150
f^a	0.0085	0.0198	K^{conf}=500
f^e	0.083	0.050	K^{conf}=150
f^e	0.083	0.050	K^{conf}=500

The first, unexpected result of the computation was that K^{conf} should exceed 100, otherwise $K_o^P < 0$ which is, of course, nonsense. Moreover, the exact value of K^{conf}, as input parameter, has a strong influence on the K_o^M/K_o^P ratio. Interestingly, f^a and f^e values are independent of K^{conf}. The results are collected in Table 2.

In spite of the uncertainty concerning exact values for K_o^M/K_o^P, these are unconditionally high. Their lowest possible values, obtained as a ratio of eqs. (6) and (7) for the limiting case of $K^{conf} \to \infty$, are the experimental stereoselectivity ratios given in Table 1. In other words, conformational recognition is high for unsubstituted compounds (DeMeD and D) which bind exclusively in conformation M. This is in agreement with the prediction of Blount et al.[3]

As seen from Table 2, f^a values are very small, expressing a high extent of steric strain hindering perfect fit for molecules with axial methyl groups. It is in line with a recent supposition.[7] On the other hand, the f^e values indicating significant steric hindrance to receptor binding, which is associated with equatorial methyl substituents, are entirely unexpected. Earlier receptor models concluded to a flat receptor cavity[8] in which the space occupied by equatorial 3-substituents appeared to be an open entrance to the binding site.

Since the presence of axial and equatorial methyl groups sterically interferes with the binding of 3,3-Me_2-1,4-BZs in either conformation, chiral recognition of these compounds by the receptor is given by the K_o^M/K_o^P values of Table 2. Hence 3,3-Me_2-1,4-BZs also bind to the receptor exclusively in conformation M. Direct experimental proof for that cannot be obtained owing to the low concentration of the receptor. For an analogous case, binding-induced CD spectra have been observed (Figure 3).

Further characterization of 3-alkyl-1,4-BZs

In order to confirm the reality of f^e values, the receptor binding of 1,4-BZs with 3-substituents larger than methyl were also studied. Results given in Table 3 indicate that increasing

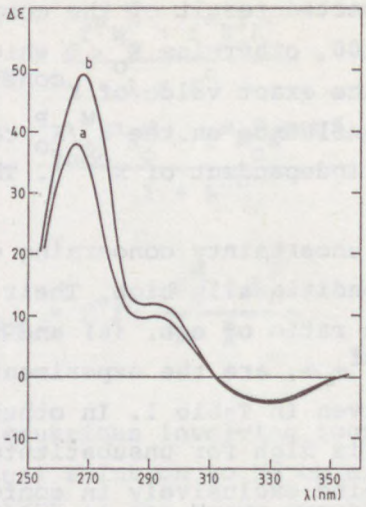

Figure 3. CD spectra induced by interaction, when 3,3-Me_2-DeMeD (a) and 3,3-Me_2-D (b) bind to human serum albumin, proving that only conformation M is recognized by the binding site (taken from ref.9).

Table 3. Displacing potency of 3-Et- and 3-Pr^i-1,4-BZ enantiomers

Compound	IC_{50} (μM)	$IC_{50}(R)/IC_{50}(S)$
(3S)-Et-DeMeD	0.61 ± 0.05	
(3R)-Et-DeMeD	73 ± 16	119
(3S)-Pr^i-DeMeD	57 ± 12	
(3R)-Pr^i-DeMeD	267 ± 176	4.7[a]
(3S)-Et-D	0.48 ± 0.03	
(3R)-Et-D	46 ± 18	96
(3S)-Pr^i-D	73 ± 36	
(3R)-Pr^i-D	101 ± 45	1,4[a]

[a] These values are not significantly different from each other owing to the high standard deviation indicated.

size of the 3-substituent is associated with both decreasing binding potency and lower enantioselectivity of 1,4-BZs. Hence data of Table 3 corroborate the reality of f^e values computed from the mathematical treatment of methyl quartets. In other words, there is steric hindrance to binding, which arises from 3-alkyl substituents occupying equatorial position.

Further experiments revealed that, despite its low potency, 3,3-Me_2-DeMeD is an agonist pharmacologically[4] (not shown), and its binding causes ligand-induced changes in receptor conformation which are similar to those elicited by the highly potent DeMeD. We think this ligand-induced change offers explanation for the f^e values. Upon agonist binding, central BZ receptors may close around the molecule in such a way that steric constraints become more stringent around C3 than they are for the binding of antagonists.

Binding conformation for 3-Me-1,4-BZ enantiomers

Having unraveled conformational and steric effects, we can now address the problem outlined in the first part of this chapter. For (3S)-Me enantiomers eq. (6) clearly expresses the answer. Since $K_o^M \gg K_o^P$, and $f^e K^{conf} \gg f^a$, only conformation M takes part in the binding process. The case is not so simple for (3R)-Me enantiomers, as indicated by eq. (7): here conformational recognition ($K_o^M \gg K_o^P$) appears to be compensated to some extent by both the unfavorable equilibrium and the different steric effects of axial and equatorial substituents ($f^a \ll f^e K^{conf}$). Furthermore, the relative contribution of the conformations depends on the actual value of K^{conf}. For illustration let us take $K^{conf} = 150$, which defines the contribution of conformation P to overall binding as 25% and 27% for (3R)-Me-DeMeD and (3R)-Me-D, respectively. Hence, about a quarter of these molecules bind to the receptor in conformation P favored by these molecules in solution. This conformational preference is reflected by the fact that (3R)-Me enantiomers in both DeMeD and D series are even poorer displacers of the radiolabel than their 3,3-Me_2 analogs (cf. Table 1). It is, therefore, conceivable that conforma-

tional recognition by the receptor is still a dominant factor in the binding of (3R)-Me-1,4-BZ enantiomers.

Conclusion

Close analogy in both the structure and the pharmacological efficacy of our model compounds allowed a quantitative treatment of binding potencies in terms of methyl quartets leading to the separation of conformational and steric effects operative in BZ receptor binding. Central BZ receptors selectively recognize conformation M in accordance with the earlier suggestion of Blount et al.[3] Conformational preference of free 3-Me-1,4-BZ enantiomers are so high that less than 1% of all molecules accomodates the conformation with axial methyl, in agreement with NMR measurements of Sunjic et al.[5] Compounds with axial methyl groups experience strong steric hindrance on binding to BZ receptors, which confirms a recent suggestion by Wermuth et al.[7] Equatorial methyl groups are subject to lower, but still substantial steric hindrance, a feature that seems to be characteristic of agonists. Owing to the differential steric effects exerted by axial and equatorial methyl groups, chiral recognition by central BZ receptors implies both conformational and configurational selection of 3-Me-1,4-BZs. Of the two components, conformational recognition is dominant.

Since ligands are not inflexible casts, and receptors are not rigid molds, it seems to be an advisable strategy to deal with steric properties of receptor binding separately for agonists and antagonists. Since agonist binding is accompained by ligand-induced changes in receptor conformation, while this is not the case for antagonists, attempts to define a common cavity for both agonist and antagonist binding implies the oversight of the pharmacological profile. Such treatments unavoidably lend a static character to the receptor models derived.[7,8,10]

This chapter is a shortened and modified version of ref.4, to be published in Bioorganic Chemistry. With permission

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APPENDIX

Crystal data of 3,3-dimethyl-1,4-BZ derivatives

	3,3-Me_2-DeMeD	3,3-Me_2D
Formula	$C_{17}H_{15}ON_2Cl$	$C_{18}H_{17}ON_2Cl$
M.w.	298.77	312.80
Cryst. sym.	monoclinic	
Space group	$P2_1/n$	$P2_1/c$
a(A)	9.271(1)	18.641(3)
b(A)	13.818(3)	9.012(1)
c(A)	12.894(2)	25.445(3)
β(deg.)	92.44(2)	131.12(1)
U(A^3)	1554.78(8)	3220.2(1.8)
D_c(g cm^{-3})	1.276	1.290
Z	4	8 §
μ(cm^{-1})	2.43(Mo-K$\bar{\alpha}$)	21.32(Cu-K$\bar{\alpha}$)
F(000)	628	1312
N(F_{tot})	3199	4247
N(F_{obs})	2028 [$F^2 > 2\sigma(F^2)$]	3937 [$F^2 > 3\sigma(F^2)$]
Weight	$w = 4F_o^2/\sigma(F_o^2)^2$	
R_{obs}	0.045	0.042
R_w	0.051	0.063
R_{tot}	0.045	0.068
ρ_{max}(eA^{-3})	0.185	0.224
Approx. cryst. size (mm^3)	0.10 x 0.12 x 0.30	0.12 x 0.15 x 0.25

§ Two molecules in the asymmetric unit.

Selected torsion angles

	3,3-Me_2-DeMeD[a]	3,3-Me_2D[b]
N1-C2-C3-N4	65.9(3)	61.2(4)
C2-C3-N4-C5[c]	-67.2(4)	-66.2(5)
C3-N4-C5-C5A	2.2(4)	0.6(4)
N4-C5-C5A-C9A	38.3(4)	40.3(5)
C5-C5A-C9A-N1	0.3(4)	2.4(4)
C5A-C9A-N1-C2	-38.0(4)	-46.3(4)
C9-C9A-N1-C2	144.8(5)	134.2(5)
C9A-N1-C2-C3	-1.4(4)	8.6(4)
C9A-N1-C2-O2	179.1(5)	-172.2(5)
O2-C2-C3-N4	-114.7(4)	-118.0(4)
N1-C2-C3-C17	179.1(4)	175.8(4)
C17-C3-N4-C5	176.7(4)	177.3(5)
O2-C2-C3-C17	-1.5(4)	-3.4(4)
C11-C10-C5-C5A	26.5(4)	36.4(5)
C11-C10-C5-N4	-152.9(5)	-146.1(5)
C15-C10-C5-C5A	-157.4(4)	-143.4(5)
C15-C10-C5-N4	23.2(4)	34.2(5)

Standard deviations are given in parenthesis.

[a]Data of a single molecule accommodating conformation *N*; conformation *P* is equally populated.

[b]Values are means of two molecules both accommodating conformation *N*; conformation *P* is equally populated.

[c]Torsion angle defining conformation *N*.

Fractional coordinates of nonhydrogen atoms for 3,3-Me_2-DeMeD

Atom	x/a	y/b	z/c
Cl	0.56717(8)	0.83636(6)	0.65139(6)
O2	1.1587(2)	0.4363(1)	0.4588(1)
N1	0.9585(2)	0.3607(1)	0.5114(1)
N4	1.1845(2)	0.1878(1)	0.4113(1)
C2	1.0983(2)	0.3570(1)	0.4853(1)
C3	1.1758(2)	0.2532(1)	0.4988(1)
C5	0.9739(2)	0.1562(1)	0.4166(1)
C5A	0.8727(2)	0.1817(1)	0.4989(1)
C6	0.7778(2)	0.1072(1)	0.5348(1)
C7	0.6823(2)	0.1389(2)	0.6085(1)
C8	0.6767(3)	0.2295(2)	0.6498(2)
C9	0.7696(3)	0.3023(2)	0.6158(2)
C9A	0.8698(2)	0.2802(1)	0.5423(1)
C10	0.9232(2)	0.0888(1)	0.3208(1)
C11	0.7792(2)	0.0797(2)	0.2957(1)
C12	0.7481(3)	0.0179(2)	0.2185(2)
C13	0.8436(3)	-0.0319(2)	0.1591(2)
C14	0.9862(3)	-0.0237(2)	0.1896(2)
C15	1.0255(2)	0.0356(2)	0.2745(1)
C16	1.1088(3)	0.2078(2)	0.6085(2)
C17	1.3303(3)	0.2662(2)	0.4566(2)

Fractional coordinates of nonhydrogen atoms for 3,3-Me_2D

Atom	x/a	y/b	z/c
Cl	0.66967(4)	0.11103(9)	0.64284(3)
O2	0.4096(1)	0.7991(2)	0.65411(7)
N1	0.4668(1)	0.5699(2)	0.66870(8)
N4	0.3886(1)	0.6453(2)	0.52491(8)
C2	0.4407(1)	0.7091(2)	0.63839(9)
C3	0.4513(1)	0.7459(2)	0.5851(1)
C5	0.4041(1)	0.5858(2)	0.53888(9)
C5A	0.4033(1)	0.4258(2)	0.59447(9)
C6	0.3526(1)	0.3175(2)	0.59812(9)
C7	0.4081(1)	0.2425(2)	0.6491(1)
C8	0.4345(1)	0.2717(3)	0.7129(1)
C9	0.5065(1)	0.3779(3)	0.7177(1)
C9A	0.5121(1)	0.4596(2)	0.65988(9)
C10	0.3370(1)	0.4159(2)	0.44497(9)
C11	0.3151(1)	0.2694(2)	0.4478(1)
C12	0.2531(1)	0.1888(3)	0.4868(1)
C13	0.2138(1)	0.2517(3)	0.5429(1)
C14	0.2327(1)	0.3967(3)	0.5399(1)
C15	0.2940(1)	0.4789(2)	0.4884(1)
C16	0.5562(1)	0.7442(3)	0.6179(1)
C17	0.4126(1)	0.9820(3)	0.5573(1)
C18	0.4599(1)	0.5470(3)	0.7222(1)
C1'	-0.15932(4)	1.37454(8)	-0.01257(3)
O2'	0.8991(1)	0.6771(2)	0.24336(8)
N1'	0.8442(1)	0.9888(2)	0.20520(8)
N4'	0.1271(1)	0.8473(2)	0.13914(8)
C2'	0.8711(1)	0.7715(2)	0.1995(1)
C3'	0.8647(1)	0.7410(2)	0.1378(1)
C5'	0.1180(1)	0.9873(2)	0.13644(9)
C5A'	0.8302(1)	1.0610(2)	0.11884(9)
C6'	-0.0187(1)	1.1743(2)	0.0687(1)
C7'	-0.0787(1)	1.2395(2)	0.0516(1)
C8'	-0.1312(1)	1.1968(3)	0.0846(1)
C9'	-0.0827(1)	1.0878(3)	0.1354(1)
C9A'	-0.0824(1)	1.0194(2)	0.15208(9)
C10'	0.1744(1)	1.0881(2)	0.1279(1)
C11'	0.2016(1)	1.2225(3)	0.1557(1)
C12'	0.2569(1)	1.3076(3)	0.1494(1)
C13'	0.2842(1)	1.2550(4)	0.1145(1)
C14'	0.2581(1)	1.1153(3)	0.0872(1)
C15'	0.2058(1)	1.0273(3)	0.0945(1)
C16'	-0.0394(1)	0.7432(3)	0.0675(1)
C17'	0.1059(1)	0.5887(3)	0.1448(1)
C18'	0.0477(1)	0.9255(3)	0.2645(1)

Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

AFFINITY AND STEREOSELECTIVITY IN BINDING TO THE SUBSITES OF THE $GABA_A$ RECEPTOR COMPLEX

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Attempts to find a general correlation between drug potency and stereoselectivity have resulted in limited success. As Pfeiffer concluded in 1956, the lower the effective dose of a drug the greater the difference in the pharmacological effect of the optical isomers[1]. Pfeiffer's rule was elaborated later and formulated as eudismic-affinity correlations[2]. The more and less active isomers were called eutomer and distomer, respectively. In many systems the logarithm of the ratio of their activities, called the eudismic index (EI), correlated linearly with the logarithm of the potencies of the eutomer[2]. In most cases the slope of the correlation (the eudismic affinity quotient, EAQ) was found to be positive according to Pfeiffer's rule. With an analogy of Lehmann[3], if you have a pair of shoes fitting perfectly, they are "stereoselective" as well. But you can easily put on loose shoes in an exchanged manner too.

This chapter will illustrate Pfeiffer's rule by receptor binding in the field of the $GABA_A$ receptor complex, and that this rule is violated in certain cases. Originally, in Pfeiffer's rule the ligands are variable and the biological target is constant. However, living organisms often apply different macromolecular entities to perform similar enzymatic or receptive tasks. These are called isoenzymes or isoreceptors, respectively. Furthermore, allosteric regulation also contributes to the variability of a binding site. Therefore variable receptors will also be examined here with a fixed pair of enantiomers.

Gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter, binds to its postsynaptic receptors and opens the coupled chloride ionophores. This $GABA_A$ receptor complex contains several binding sites which regulate each other allosterically (i.e. by modification of the receptor conformation). These are binding sites for GABA, 1,4-benzodiazepines (BZ), cage convulsants and barbiturates, etc[4]. Three binding sites can be labeled with specific radioligands and chiral displacers will be applied in kinetic and equilibrium binding studies.

3 - Substituted 1,4-benzodiazepines

3-Me-BZs have been of crucial importance for the demonstration of chiral recognition by the BZ binding site[5]. It is interesting to compare stereoselective displacing potencies of 3-acyloxy and 3-alkyl derivatives of diazepam and 1-demethyl-diazepam described in the previous chapter. The eudismic indices (EI) are plotted against the affinities ($-\log IC_{50}$) of the eutomeric S enantiomers in Figure 1.

The positive slopes for two 3-alkyl-substituted series mean that higher affinity is accompanied by greater stereoselectivity, in agreement with Pfeiffer's rule. In order to understand the molecular nature of this correlation, let us summarize first the relevant conclusions of the previous chapter: 1/ The binding site prefers the boat conformation M of the diazepine ring. 2/ Calculations for successive 3-methylated BZs established that 3-axial (a) methyl substituents are less tolerated for receptor binding than 3-equatorial (e) ones. But how does further hindrance develop for greater alkyl groups? Since the 3-alkyl groups vary mainly in bulk and branching, Hancock's steric parameter E_S^c can be used as an informative structural parameter of the substituents. Let us consider $\log IC_{50}$ values - related to the reciprocal of displacing potency, i.e. affinity - to characterize steric hindrance. Figure 2 shows that steric hindrance develops steeply for the R stereoisomers as a function of the steric

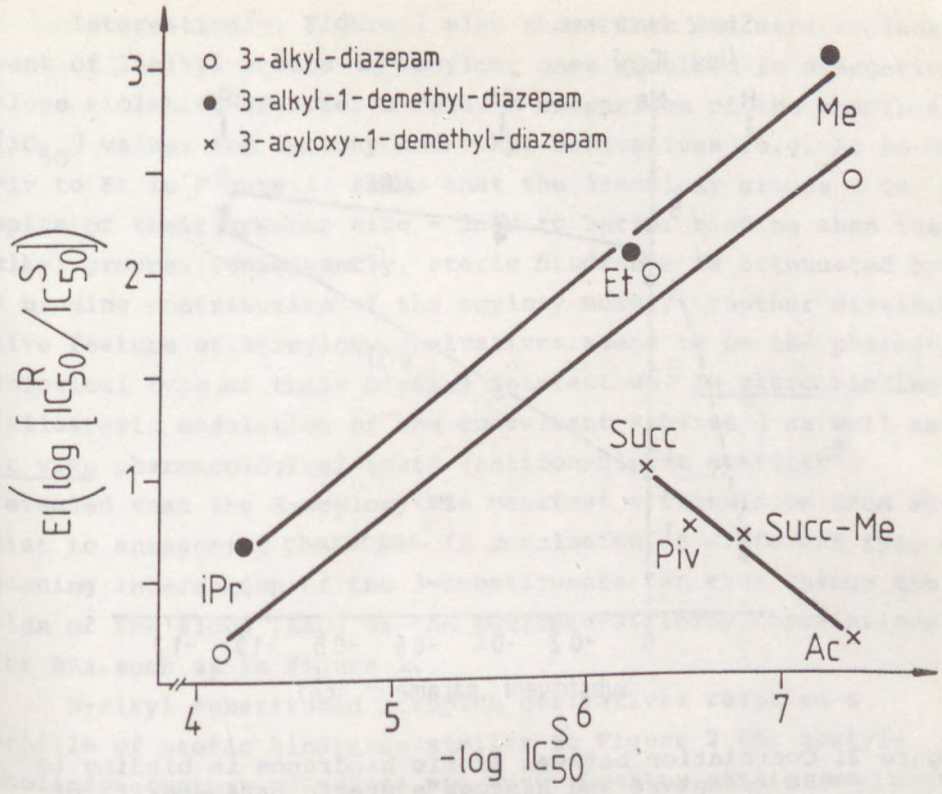


Figure 1. Eudismic-affinity correlations for 3-substituted 1,4-benzodiazepines. Displacing potencies (IC_{50}) of BZs were determined for ^{3}H-diazepam binding in the presence of 10 µM GABA. Data on 3-alkyl-BZs were taken from ref. 5 and oxazepam hemisuccinate (Succ) from ref. 6. Data on oxazepam acetate (Ac), pivaloate (Piv) and succinate methyl esters (Succ-Me) are unpublished.

parameter and reaches saturation. For the S enantiomers steric hindrance approaches to the same plateau but more gradually. Since the origo of the steric parameter was set to the methyl group, unfortunately we cannot attribute a numerical abscissa value to the unsubstituted compounds (no E_S^c value is given for H). Therefore the starting point of the curves may move horizontally and the curves are tentative (dashed) up to the value for methyl. But this failure does not essentially influence the saturating type of the curves.

If the preference of the binding site for conformation M is also valid for greater 3-substituents, we receive two

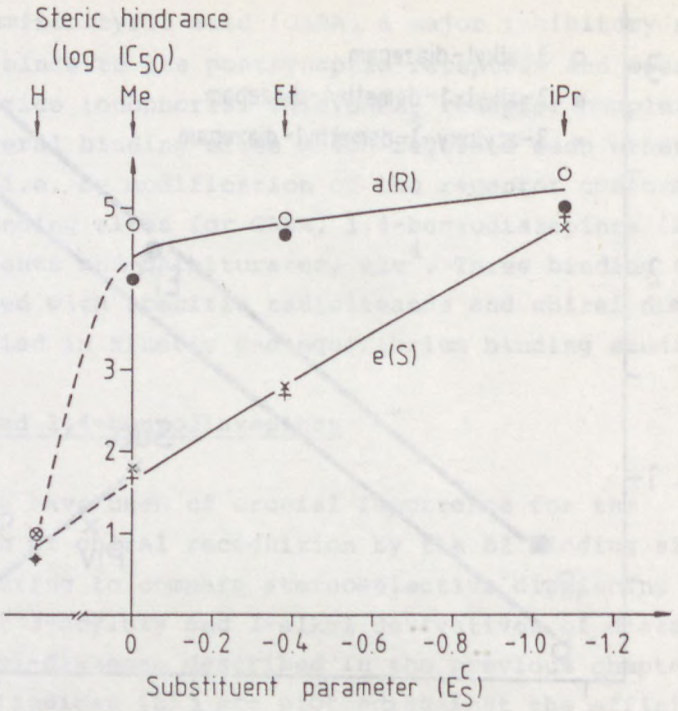


Figure 2. Correlation between steric hindrance in binding to BZ receptors and Hancock's steric parameter E_S^c of 3-alkyl substituents in a and e positions (corresponding to R and S enantiomers, respectively). Enantiomers of 3-alkyl diazepam (R:●, S:+) and 1-demethyl-diazepam (R:o, S:x) derivatives. IC_{50} values expressed in nM are taken from ref.5.

separate curves for the 3-axial and 3-equatorial directions to distinguish their susceptibilities for steric hindrance (Figure 2). Due to differential steric susceptibilities of increasing a and e substituents, stereoselectivity reaches maximum for 3-methyl groups and then decreases. Thus, steric hindrance seems to be a predominant factor which diminishes displacing potency for 3-alkyl-BZs. In spite of the great variations in potency, the type of binding interaction seems to be analogous. It was supported by the finding that GABA exerted the same allosteric enhancement of displacing potency for a potent 3-unsubstituted BZ and its 3,3-dimethyl derivative of low activity[5]. The same value of this in vitro indicator suggested that these 3-alkyl-BZs belong to the same type of agonists.

Interestingly, Figure 1 also shows that the mere replacement of 3-alkyl groups by acyloxy ones resulted in a negative slope violating Pfeiffer's rule. A comparison of the abscissa (IC_{50}) values for acyloxy and alkyl derivatives (e.g. Ac to Me, Piv to Et in Figure 1) shows that the 3-acyloxy groups - in spite of their greater size - lead to better binding than the alkyl groups. Consequently, steric hindrance is attenuated by a binding contribution of the acyloxy moiety. Another distinctive feature of 3-acyloxy derivatives seems to be the pharmacological type of their binding interaction. In vitro binding (allosteric modulation of the convulsant subsite[7]) as well as in vivo pharmacological tests (anticonvulsant activity[8]) revealed that the 3-acyloxy-BZs manifest a transition from agonist to antagonist character. In conclusion, a different type of binding interaction of the 3-substituents can even change the sign of the slope (EAQ) of the eudismic-affinity correlations for BZs, such as in Figure 1.

N-alkyl substituted atropine derivatives revealed a profile of steric hindrance similar to Figure 2 for acetylcholine receptors of guinea pig gut[9]. Greater steric sensitivity for the N-axial direction is unequivocal there, since the "chair" conformation of the tropane ring is conformationally constrained. For increasing N-alkyl derivatives stereoselectivity increases first, then decreases such as for 3-alkyl BZs in Figure 2. It suggests a broader validity of this kind of correlation between stereochemical structure and receptor binding, if steric hindrance is the predominant factor. For steric hindrance in the binding of saturated ring systems, the stereoselectivity profile may be determined by differential steric susceptibilities towards axial and equatorial substituents.

Heterogeneity of the GABA binding site

$GABA_A$ binding sites are heterogeneous having high, low and "super-low" affinity populations[10]. Conformational restriction of GABA by an isoxazole ring led to the development of ^3H-muscimol, the high affinity radioligand of the $GABA_A$ binding

sites. Interestingly, chiral dihydromuscimol (DHM) displayed opposite stereoselectivities for receptor binding and transport: its S(+) enantiomer (Figure 3) binds preferentially to the high affinity $GABA_A$ sites while synaptosomal GABA uptake sites are selective for R(-)-DHM[11].

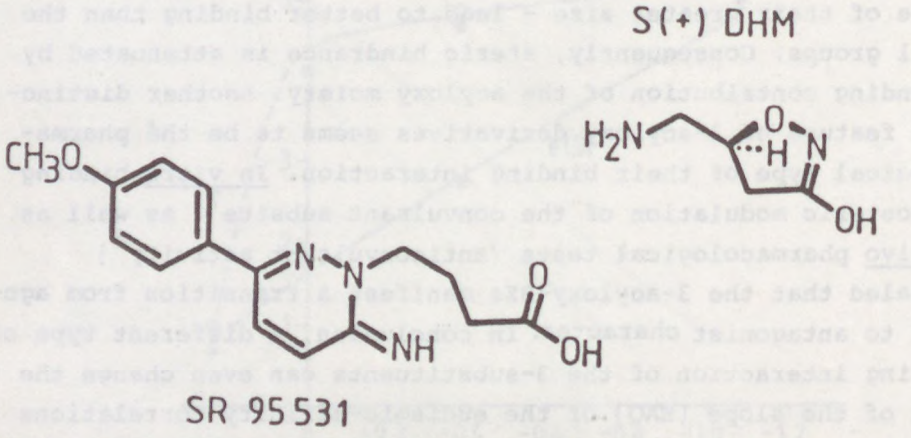


Figure 3. Structure of the $GABA_A$ agonist S(+)-dihydromuscimol and the antagonist SR 95531.

Direct $GABA_A$ antagonists all have hydrophobic ring systems attached to the GABA mimetic polar part: e.g. the arylpyridazinyl derivatives such as SR 95531 (Figure 3). SR 95531 is the highest affinity radioligand of antagonist type for the $GABA_A$ binding sites[12]. The high affinity component of SR 95531 binding corresponded to the "super-low" affinity population of $GABA_A$ sites[10]. This opposite affinity-relationship was found to be valid for all $GABA_A$ antagonists tested[13]. The preferential binding of antagonists to the lower affinity $GABA_A$ isoreceptors was attributed to the additional binding of their rings to hydrophobic accessory sites[9] accessible only around the lower affinity isoreceptors[10,13].

^{3}H-Muscimol and ^{3}H-SR 95531 bindings were used here to characterize the high and "super-low" affinity populations, respectively, of $GABA_A$ sites. The enantiomers of DHM were applied as enantioselective probes of binding. Table 1 shows that DHM displaced ^{3}H-muscimol binding with high affinity and substantial stereoselectivity. This is similar to previous reports on other agonist radioligands[11]. DHM, like all other

GABA agonists displaced ^{3}H-SR 95531 binding with much less potency (Table 1) since SR 95531 preferentially labels the lower affinity GABA sites. The enantioselectivity of displacement was decreased simultaneously.

Table 1. Displacing potencies of DHM enantiomers on GABA agonist and antagonist binding*

Radioligand	IC_{50} (nM)		R/S
	S(+)-DHM	R(-)-DHM	
^{3}H-Muscimol	7.8±1.3	64±33	8.2
^{3}H-SR 95531	210±55	819±204	3.9

*Freeze-thawed membrane preparations of whole rat brain were incubated in 50 mM Tris citrate (pH=7.1) with 1.8 nM ^{3}H-muscimol or 3.5 nM ^{3}H-SR 95531 for 20 min at 4°C and filtered. Data are mean (±S.D.) of five experiments. DHM enantiomers were kindly donated by Prof. P. Krogsgaard-Larsen (Denmark).

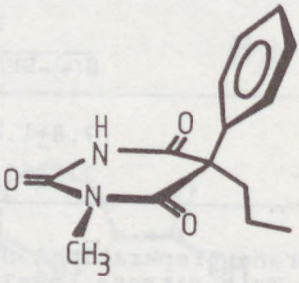
How can we explain the different stereoselectivities? Muscimol analogues bind to the polar center of the high affinity $GABA_A$ sites where the steric orientation of the isoxazole ring is of great importance. In contrast, SR 95531 predominantly binds to the hydrophobic accessory sites around lower affinity GABA, sites and in its displacement by DHM the orientation of the isoxazole ring is of secondary importance. Chiral indicator ligands have been similarly used for mapping critical and non-critical regions of receptor binding[9].

In conclusion, the lower affinity $GABA_A$ isoreceptors bind the enantiomers of DHM with less stereoselectivity, thus they obey Pfeiffer's rule. A similar correlation was reported for methacholine stereoisomers at "super-high", high and low affinity muscarinic isoreceptors[14].

Barbiturate interaction with the convulsant binding site

The $GABA_A$ receptor complex also contains a binding site for cage convulsants which can be labeled with ^{35}S-t-butyl-bicyclo-phosphorothionate (TBPS)[4]. Such cage-structured bicyclic

compounds block the chloride channel and cause convulsions. In contrast to these GABAergic inhibitory agents, there are GABAergic facilitatory agents called central depressants. Barbiturates, a well-known group of such agents exert dual activities. These activities are separated most clearly in the enantiomers of 1-methyl-5-phenyl-5-propyl-barbituric acid (MPPB): its S(+) enantiomer is convulsant while the R(-) antipode is depressant.



R(-) MPPB

MPPB displaces specific ^{35}S-TBPS binding in a stereoselective manner (Table 2). The affinity of MPPB to the convulsant (TBPS) sites can be modulated allosterically by other subsites. Table 2 shows the effect of the GABA binding site. A steroidal GABA antagonist, R 5135 was applied to block the GABA binding site. In contrast, GABA enhanced the displacing potencies of MPPB enantiomers and increased their stereoselectivity[15]. Thus, this interaction obeys Pfeiffer's rule.

Table 2. Displacing potencies of MPPB enantiomers at the convulsant (TBPS) site in the presence of GABA and its antagonist R 5135*

In presence of	IC_{50} (μM)		S/R
	R	S	
10^{-8}M R 5135	335	1600	4.8
10^{-6}M GABA	142	1200	8.5

*Taken from ref. 15.

The IC_{50} values of Table 2 refer to binding competition of equilibrium nature when the GABA site modulated the affinities of both barbiturates and the convulsant TBPS. Let us examine now the elementary kinetic steps of this binding competition. The dissociation of TBPS binding was slow when elicited by a completely displacing concentration of convulsants such as TBPS or S(+)MPPB (■ in Figure 4)[16]. In contrast, the depressant R(-)MPPB greatly accelerated the dissociation of ^{35}S-TBPS (o in Figure 4) contradicting to its simple competition with TBPS for the same binding sites[16]. An allosteric interaction can account for the accelerated dissociation according to the following scheme:

$$B + CL \longleftrightarrow BCL \longleftrightarrow BC^{x}L \longleftrightarrow BC^{x} + L$$

Barbiturates (B) bind to the receptor complex (C) previously equilibrated with ^{35}S-TBPS (L) and elicit a conformational change (C^{x}) so that the radioligand dissociates. The stereoselectivity of MPPB can be manifested at different steps.

From the stereoselectivity data of Table 2 we can conclude about the first step of the scheme. Since IC_{50} values are related to the dissociation constant K_D:

$$K_D = \left(\frac{k_d}{k_a}\right)_R < \left(\frac{k_d}{k_a}\right)_S .$$

That is, the dissociation rate constant (k_d) of R(-)-MPPB is smaller and/or its association rate constant (k_a) is greater than the corresponding ones for S(+)-MPPB. The effect of MPPB on the rate of ^{35}S-TBPS dissociation was studied in two ways.

Case 1

The first step of the scheme was excluded by the addition of a partially displacing concentration (0.3 mM) of the barbiturates to the receptor before ^{35}S-TBPS was added. Thereafter dissociation was elicited by an excess of convulsants (e.g. picrotoxin, ■ and □ in Figure 4). Table 3 shows the computed half-lives of dissociation following the separation of a minor first phase. Stereoselectivity is rather small (2.1).

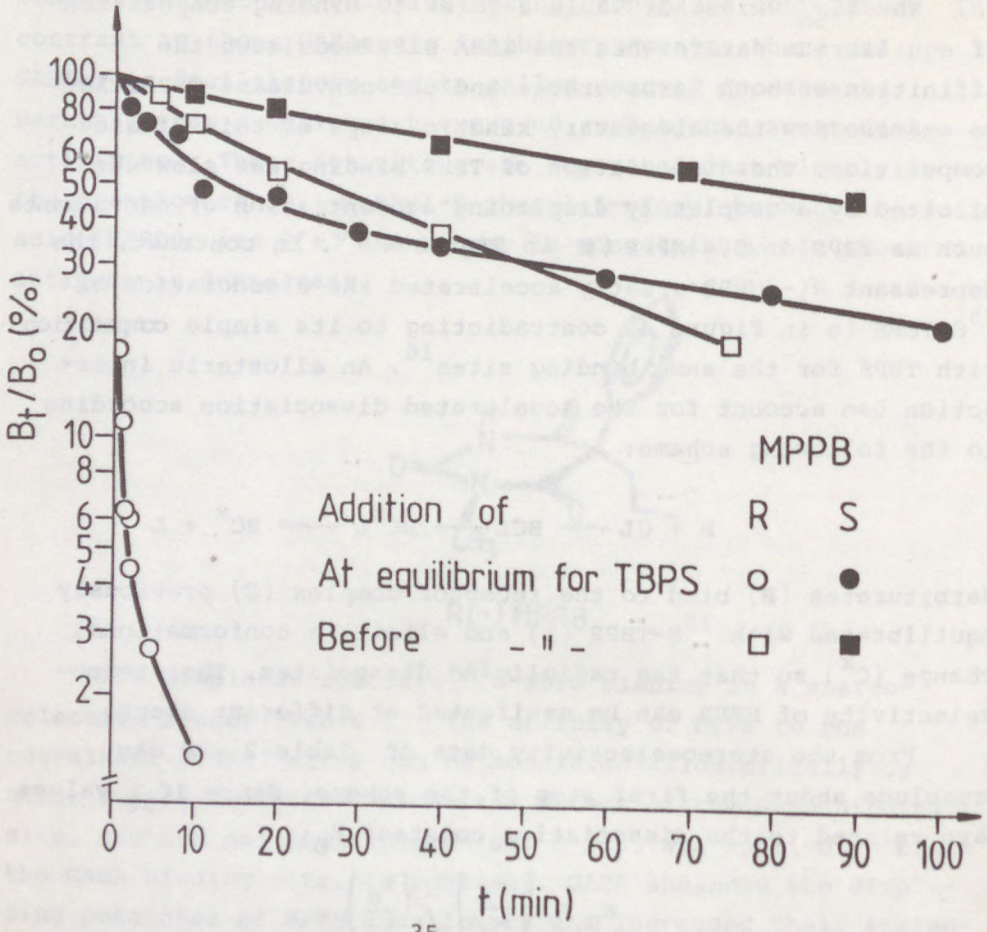


Figure 4. Dissociation of ^{35}S-TBPS binding in the presence of MPPB enantiomers. Freeze-thawed cortical membranes were incubated with 3 nM ^{35}S-TBPS at 25°C for 130 min in 5 mM Tris HCl buffer containing 200 mM KCl. Case 1: 0.3 mM of MPPB enantiomers (R: □, S: ■) were added before ^{35}S-TBPS and dissociation was initiated with 20 µM picrotoxin[17]. Case 2: dissociation was initiated with 2 mM of MPPB enantiomers (R:o, S:●)[16].

Association of ^{35}S-TBPS was also found earlier to be accelerated by R(-)-MPPB[18] so that the reversal of the last step of the scheme is also stereoselective.

Case 2

Dissociation of ^{35}S-TBPS was also elicited by higher concentrations (2 mM) of MPPB enantiomers (● and o in Figure 4). Table 3 summarizes the computed half-lives for two separated phases of dissociation. Stereoselectivity is much greater this

way but declines somewhat for the second phase.

How can we explain that kinetic stereoselectivity observed in TBPS dissociation depends on time and on the experimental conditions? In case 1, smaller barbiturate concentrations are equilibrated with the receptor, resulting in comparable BC^xL concentrations,[17] thus the small kinetic stereoselectivity mainly reflects the last step of the scheme. In case 2, high barbiturate concentrations trigger a sequence of stereoselective processes that amplifies the stereoselectivity observed. However, this kinetic stereoselectivity decreases for the second phase, while the system reaches steady-state conditions for the intermediates of the scheme.

Table 3. Kinetic parameters of ^{35}S-TBPS dissociation in the presence of MPPB enantiomers

	Dissociation phases			
	Case 1		Case 2	
	First	Second	First	Second
MPPB	Contribution* (%)	Half-life (min)	Half-lives (min)	
R	23	43	< 0.4	10
S	6	90	6.8	76
S/R		2.1	>17	7.6

Data were fitted to the curves in Figure 4.

*Due to the minority of the first phase, only % contribution can be determined.

Beyond this kinetic explanation a functional interpretation may also arise. A correlation has been observed between the kinetics of TBPS binding and the kinetic states of the chloride ionophore[18]. The rapid and slow phases of TBPS dissociation were supposed to represent open and closed ion channels, respectively[18]. In the presence of the convulsants S(+)-MPPB and ^{35}S-TBPS the ionophores are mainly closed and a slow phase of TBPS dissociation can be observed. The depressant R(-) barbiturate opens the ionophores and results in rapid dissociation of TBPS (o in Figure 4). However, longer

occupation of the receptors with depressant barbiturates is known to desensitize the ion channels and reclose them, therefore the contribution of the first phase of dissociation corresponding to open channels is very suppressed after pre-incubation with R(-)-MPPB (□ versus o in Figure 4).

In conclusion, for the convulsant(TBPS)sites the displacing potency of MPPB enantiomers can be allosterically increased by the GABA site which enhances their equilibrium stereoselectivity. In kinetic studies of a sequence of stereoselective processes (allosteric displacement of TBPS binding by barbiturates) the kinetic stereoselectivity observed in TBPS dissociation is transient and can be amplified (case 2) and attenuated (case 1) by the experimental conditions. The dissociation data and stereoselectivity for MPPB enantiomers are in agreement with their effects on the kinetic states of the chloride ionophore and their pharmacological properties, respectively.

In general conclusion, most eudismic-affinity correlations for binding to the subsites of the $GABA_A$ receptor complex obey Pfeiffer's rule for both structural variation of the ligands and binding site modification. However, the 3-acyloxy-BZs illustrate that deviations from Pfeiffer's rule may occur if the character of the binding interaction is altered.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

ULTRAFILTRATION OF RACEMATES: HOW TO DETECT THE ENANTIOMER?

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In order to elucidate molecular properties, the application of racemates to experiments proceeding in chiral environment does not seem to be a problem for many investigators. At one extreme, some are not aware of the ambiguity arising from the simultaneous presence of two enantiomers that have different effects. Others are simply misled by the careless drug nomenclature accepted as the current practice[1]. Since the racemic character of various substances is often hidden behind names we are accustomed to, failure to consult the structural formula in the mistaken belief that *one* name necessarily refers to a *single* molecular species could easily become a trap for the unsuspecting pharmacologist.

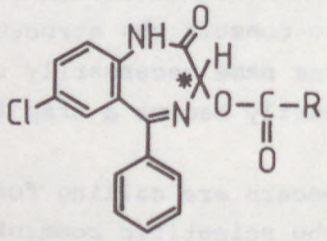
This is why voices of growing concern are calling for increased awareness on the part of the scientific community[2-4]. Perhaps the most eloquent among them is Ariens, who dismisses non-stereospecific pharmacokinetic studies applying racemates because they lead to "pseudoscientific nonsense"[5]. Deriving a kinetic constant, or the potency of a racemate is "like talking about the age and body weight of a married couple"[6].

As the critical voices intensify, some are apparently pushed to the other extreme, like the medicinal chemist who was heard to declare at the end of a conference: "I shall now ban the synthesis of all chiral compounds in my laboratory for the future!"[7]. Since about 50 % of all drugs currently on the market are chiral[2], such a decision does not seem reasonable. The real problem arises from the fact that one half of the chiral drugs can only be found in racemic form[2].

Should the experimenter then abandon the idea to detect enantiomeric effects from experiments applying racemates? This chapter gives a few examples of how the problem can be circumvented in studies of protein binding when only racemic drugs are available.

The principle of ultrafiltration in protein binding

A paper by Müller and Wollert in 1975 became a milestone demonstrating high stereoselectivity in the binding of a drug to human serum albumin[8]. This drug was oxazepam hemisuccinate (I) and the authors applied resolved enantiomers. We had in our laboratory a series of oxazepam esters - all in the racemic form (II-VII, cf. formula) - and wanted to know how the stereoselectivity varies with the modification of the esterifying group. Ultrafiltration was selected as the method for the experiments.



Code	R
I	$-CH_2-CH_2-COOH$
II	$-CH_3$
III	$-CH_2-CH_3$
IV	$-CH_2-CH_2-CH_3$
V	$-CH(CH_3)_2$
VI	$-C(CH_3)_3$
VII	$-CH_2-CH_2-COOCH_3$

Semipermeable membranes retain macromolecular species, i.e. protein molecules and protein-bound drugs, while they let unbound drug molecules filter through. If the membrane does not adsorb the small molecules - a prerequisite for binding studies - then the concentration of the free drug (c_f) determined by the binding equilibrium can be measured in the ultrafiltrate. The overall binding equilibrium may be quite complex; let P_i denote the i-th kind of protein binding site the number of which is n_i per molecule; each P_i is saturated upon complexation with a single drug molecule (L) when it forms a 1:1 complex

(P_iL),

$$P_i + L \underset{}{\overset{K_i}{\rightleftharpoons}} P_iL \qquad (1)$$

$$n_i c_p^o - c_b^i \qquad\qquad c_b^i$$

and the overall equilibrium constant (K) is given by:

$$K = \frac{\sum_i c_b^i}{\sum_i (n_i c_p^o - c_b^i)\, c_f} = \frac{c_b}{(nc_p^o - c_b)\, c_f} \qquad (2)$$

where c_b is the concentration of bound drug, and c_p^o is the total concentration of the protein. Note that the free drug concentration, c_f is a common denominator if several elementary equilibria are operative.

When ultrafiltration is done in a stepwise manner, the collection of ultrafiltrate is associated with concentrating the macromolecular retentate (Fig.1) and it may appear that this procedure can influence the binding equilibrium. In other words, the question arises how c_f depends on x, the volume of filtrate collected.

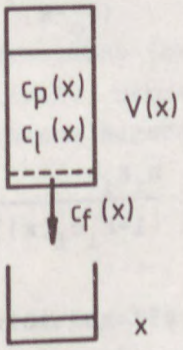


Figure 1. Free fraction of the drug is separated in the ultrafiltrate of volume x, while the volume of the sample, V(x) decreases and the concentrations of protein, $c_p(x)$ and ligand, $c_l(x)$ in the cell increase.

Let V_o denote the original volume of solution characterized by initial concentrations of $c_l(o)$ and $c_p(o)$ for drug (ligand) and protein, respectively. During ultrafiltration these quantities vary as given by:

$$V(x) = V_o - x \tag{3}$$

$$c_p(x) = \frac{V_o}{V_o - (x)} c_p(o) \tag{4}$$

$$c_l(x) = c_f(x) + c_b(x) = \frac{V_o c_l(o) - \int_o^x c_f(x)dx}{V_o - x} \tag{5}$$

Since $c_b(x)$ can be expressed from the equilibrium, we also have an independent equation for the ligand concentration:

$$c_l(x) = c_f(x)\{1 + c_p(x) \sum_i \frac{n_i K_i}{1 + K_i c_f(x)}\} \tag{6}$$

The first derivative of the ligand concentration can be obtained both from eq. (5):

$$\frac{dc_l(x)}{dx} = \frac{V_o c_l(o) - \int_o^x c_f(x)dx - c_f(x)(V_o - x)}{(V_o - x)^2} = \frac{c_b(x)}{c_o - x} \tag{7}$$

and from eq. (6):

$$\frac{dc_l(x)}{dx} = \{1 + c_p(x) \sum_i \frac{n_i K_i}{[1 + K_i c_f(x)]^2}\} \frac{dc_f(x)}{dx} + \frac{c_b(x)}{V_o - x} \tag{8}$$

As obvious from eqs. (7) and (8):

$$\frac{dc_f(x)}{dx} = 0 \tag{9}$$

i.e. the drug concentration in the ultrafiltrate is independent of the volume of the filtrate. In simpler words, ultrafiltration equally concentrates occupied and vacant binding sites of the

protein molecule, hence the equilibrium constant predicts c_f to be independent of filtrate volume (cf. eq. (2)). Since the position of the equilibrium is monitored by measuring the free drug concentration, a more appropriate form of eq. (2) is used for practical purposes:

$$K = \frac{1-\alpha}{\alpha\,[nc_p^o-(1-\alpha)c_o]} \qquad (10)$$

where c_o is the total drug concentration and α is its free fraction, i.e.

$$\alpha = \frac{c_f}{c_o} . \qquad (11)$$

If the drug is a racemate, enantiomeric equilibria exist simultaneously and independently, provided that the saturation is not too high. Then α is a composite quantity, α_{rac}:

$$\alpha_{rac} = \frac{c_f^d + c_f^l}{\frac{c_o}{2} + \frac{c_o}{2}} = \frac{\alpha_d + \alpha_l}{2} \qquad (12)$$

the arithmetic mean of free fractions for *d*extro- and *l*evo-rotatory enantiomers, respectively. Hence, unraveling enantiomeric effects is equivalent to the separate determination of α_d and α_l.

Stepwise ultrafiltration

A direct consequence of stereoselective binding (i.e. $\alpha_d \neq \alpha_l$) is that a simple ultrafiltration step leads to partial resolution of the initially racemic ligand. If the drug is available both as radiolabeled and nonlabeled racemates, stereoselectively labeled quasi-racemic mixtures can be prepared[9].

Consider two ultrafiltration cells which contain racemic

drug and protein solution of identical composition in equal volumes (V_o) with the only difference that the drug is labeled in one cell while nonlabeled in the other (Fig.2a). Filter both samples so as to collect identical volumes of the filtrates (V_f, Fig.2b). This step results in an excess of one enantiomer in the filtrate with an identical excess for the other enantiomer left in the retentate. Then restore the quasi-racemic composition by quantitative cross-mixing of radioactive filtrate with inactive retentate, and nonlabeled filtrate with radiolabeled retentate (Fig.2c). The result is enantioselective labeling; the enantiomer with lower binding affinity that appeared in an excess in the filtrate has now a relatively higher specific activity in the cell containing the radioactive filtrate, and the reverse is true in the other cell.

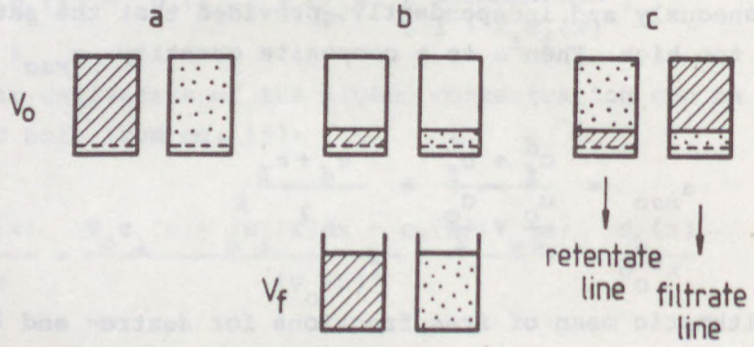


Figure 2. The principle of stereoselective labeling[9]. Hatched and dotted areas represent radioactive and radioinactive solutions, respectively. Identical volumes of filtrates (b) are cross-mixed quantitatively (c), to yield filtrate and retentate lines starting from the cells containing the corresponding labeled material from the first step.

While in the filtration of the original samples (Fig.2b) both UV spectrophotometric determination and liquid scintillation counting provided identical filtrate concentrations (a_{rad}), this is no longer true if filtration of the cross-mixed samples are performed. When overall (radiolabeled + nonlabeled) concentrations are measured, the second filtrates still reflect the

racemic α value, but the extent of radioactivity is obviously different from it if drug binding is enantioselective. The cell containing radioactive filtrate from the first step should produce in the second filtrate a relative excess of radioactivity for the enantiomer bound less tightly to the protein (Fig.2c, filtrate line), hence the α value increases for liquid scintillation counting as expressed by

$$\alpha_{2,f} = \frac{\alpha_d^2 + \alpha_l^2}{\alpha_d + \alpha_l} \qquad (13)$$

On the other hand, the cell containing the labeled retentate from the first filtration step gives a decreased α value for radioactivity (retentate line) that is given by

$$\alpha_{2,r} = \frac{\alpha_d q_d + \alpha_l q_l}{q_d + q_l} \qquad (14)$$

where

$$q_d = 1-\alpha_d \frac{V_f}{V_o} \; ; \; q_l = 1 - \alpha_l \frac{V_f}{V_o} \qquad (15)$$

In principle, eqs. (13) - (15) are sufficient to calculate the enantiomeric α values. In practice, it is advisable to increase the extent of stereoselective labeling in further subsequent ultrafiltration steps. Since radio-inactive retentate efficiently removes the labeled enantiomer of higher binding affinity, while nonlabeled filtrate selectively washes away the weakly bound radioactive isomer from labeled retentate, multi-step ultrafiltration should follow the outline shown in Figure 3.

If the volume of filtrates is kept constant throughout the procedure (note that $\alpha_{2,r}$ depends on V_f), we can express the radioactive α values after the i-th step of the two lines, as follows:

$$\alpha_{i,f} = \frac{\alpha_d^i + \alpha_l^i}{\alpha_d^{i-1} + \alpha_l^{i-1}} \qquad (16)$$

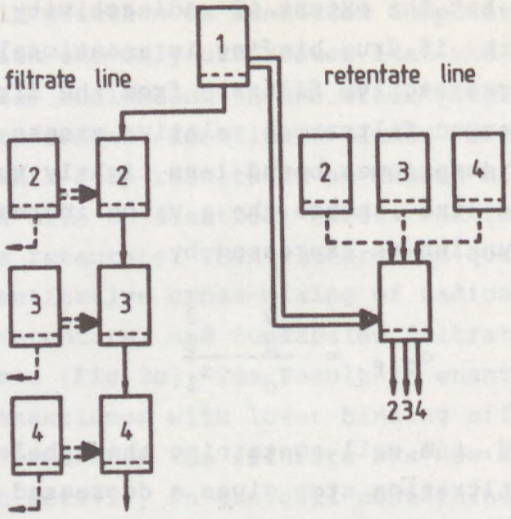


Figure 3. The strategy of stepwise ultrafiltration[9]. Single and double lines indicate filtrate and retentate, respectively. Dashed lines represent nonlabeled material. Step numbers (i) are indicated.

$$\alpha_{i,r} = \frac{\alpha_d \, q_d^{i-1} + \alpha_l \, q_l^{i-1}}{q_d^{i-1} + q_l^{i-1}} . \qquad (17)$$

Suppose that in a given system

$$\alpha_l > \alpha_d \qquad (18)$$

Then we can obtain limiting values from eqs. (16) and (17) for an infinitely large number of steps:

$$\lim_{i \to \infty} \alpha_{i,f} = \alpha_l \qquad (19)$$

$$\lim_{i \to \infty} \alpha_{i,r} = \alpha_d \qquad (20)$$

yielding finally the enantiomeric α values.

The method was applied to study the binding of racemic II to human serum albumin[9]. Results are given in Table 1.

Table 1. Four-step ultrafiltration of *rac*-oxazepam acetate (II) and human serum albumin

No.	i	$\alpha_{i,f}$	$\alpha_{i,r}$
1*	1	0.35	
	2	0.50	0.24
	3	0.59	0.21
	4	0.57	0.17
	$\rightarrow \infty$	0.59	0.12
2**	1	0.55	
	2	0.62	0.49
	3	0.70	0.46
	4	0.73	0.42
	$\rightarrow \infty$	0.76	0.34

*c_o = 73 µM, c_p^o = 105 µM; **c_o = 53 µM, c_p^o = 46 µM.

It must be admitted that stepwise ultrafiltration is a tedious procedure and the conditions (identical filtrate volumes, quantitative cross-mixing) place a heavy burden on the experimenter. The retentate line is technically easier because the radioactive material could remain in the original cell. Hence, it allows a simplification of the method.

Stationary ultrafiltration

The name refers to a continuous operation in which the nonlabeled protein-free feed solution entering the cell and the protein-free filtrate leaving the cell have an identical total of ligand concentrations (radioactive + radio-inactive). Then the overall concentration of ligand in the cell remains constant leaving the binding equilibrium in a stationary state while the isotopic replacement of the ligand takes place continuously[10] (Fig.4).

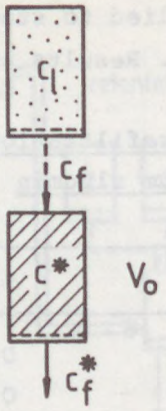


Figure 4. Stationary ultrafiltration does not affect the position of the equilibrium during isotopic replacement of the ligand; $c_l(o) = c^*(o)$.

Since the amount of radioactive ligand lost by the cell ($-dc^*V_o$) appears in the filtrate ($\alpha c^* dx$), the radioactive concentration of the filtrate (c_f^*) depends on the filtrate volume (x), viz.,

$$c_f^* = \alpha c_o \exp\left(-\frac{\alpha x}{V_o}\right) \qquad (21)$$

yielding an expression valid if only one ligand is involved (cf. Fig.5).

For a racemic ligand simultaneous enantiomeric equilibria are reflected by eq. (22) containing a sum of exponentials:

$$c_f^* = \frac{c_o}{2}\left[\alpha_d \exp\left(-\frac{\alpha_d x}{V_o}\right) + \alpha_l \exp\left(-\frac{\alpha_l x}{V_o}\right)\right] \qquad (22)$$

Eq. (22) expresses the idea of the retentate line: if x is sufficiently high, the less tightly bound radioactive enantiomer that has the higher enantiomeric α value is practically washed out of the cell leaving behind the other optical isomer labeled selectively. Since eq. (22) equals eq. (12) for $x = o$, the free fraction of radioactivity, α^* depends on the volume of the filtrate, as given by:

$$\alpha^*(x) = \frac{V_o c_f^*(x)}{V_o c_o - \int_o^x c_f^*(x)dx} = \frac{\alpha_d \exp(-\frac{\alpha_d x}{V_o}) + \alpha_l \exp(-\frac{\alpha_l x}{V_o})}{\exp(-\frac{\alpha_d x}{V_o}) + \exp(-\frac{\alpha_l x}{V_o})} \quad (23)$$

Limiting cases of eq. (23) are eq. (12) for x=o, and eq. (20) if x becomes large.

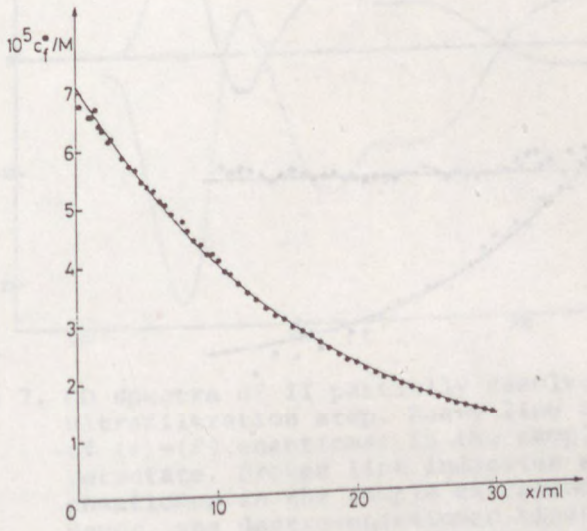


Figure 5. Experimental data (closed circles) satisfy eq. (21) (curve drawn) for the isotopic replacement of 5-[^{3}H]salicylic acid in a solution of human serum albumin; c_o = 210 µM; V_o = 5.9 ml; α = 0.34.

Eq. (23) agrees well with the experimental data as shown in Fig.6, in contrast to an achiral ligand for which α^* is constant as predicted by eq. (23) if $\alpha_d = \alpha_l = \alpha$.

While the methods described above can unequivocally indicate when α_d differs from α_l, the relative magnitude of the two has only been assumed so far by the inequality (18). Since even a single ultrafiltration step could partially resolve racemic ligands if binding is stereoselective, extraction of the ligand

from both filtrate and retentate and subsequent measurements of the optical activity will determine experimentally the relative magnitude of the enantiomeric α values. Figure 7 demonstrates the mirror image relation of circular dichroism spectra of the recovered samples and proves the inequality (18) for oxazepam acetate (II).

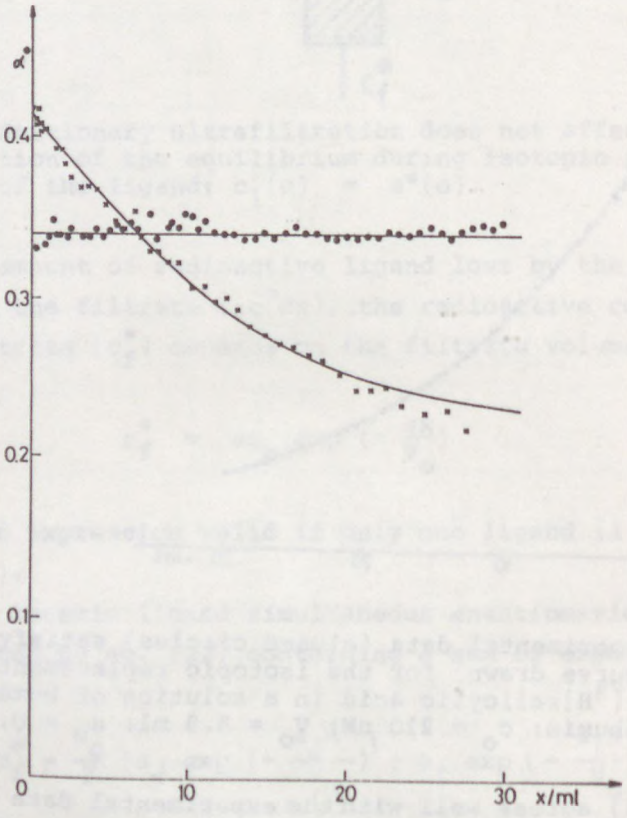


Figure 6. Experimental data satisfy eq. (23) (curve drawn) for stationary ultrafiltration of *rac*-oxazepam acetate (II) and human serum albumin; c_o = 49 μM; V_o = 32 ml; α_{rac} = 0.40; α_d = 0.19; α_l = 0.61. Data of salicylic acid (closed circles taken from the experiment shown in Fig.5) define α^* = 0.34. From ref. 10, with permission.

Since not all racemic drugs are available in radioactive form, optical purity measurements combined with ultrafiltration

provide a technique[11] applicable to studies on stereoselective binding of nonlabeled racemates.

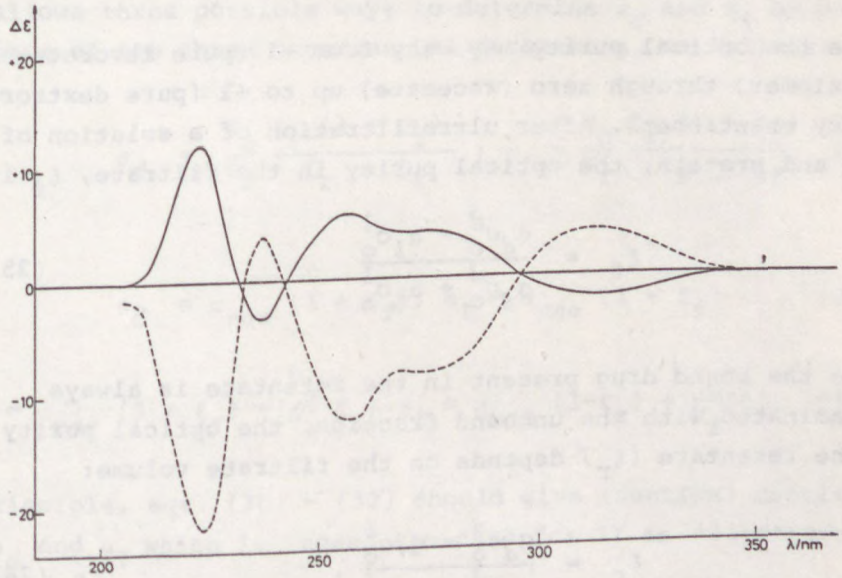


Figure 7. CD spectra of II partially resolved in a single ultrafiltration step. Heavy line represents excess of (+)-(S) enantiomer in the sample extracted from retentate. Broken line indicates excess of (-)-(R) enantiomer in the sample extracted from filtrate. Hence, the dextro-enantiomer binds more strongly to human serum albumin, thus, $\alpha_l > \alpha_d$.

Optical purity of filtrate and retentate

In order to determine the optical purity of a compound, CD spectral data of the enantiomers are required. These are either available from literature, or the resolution of the racemate is unavoidable[11]. The resolution of oxazepam esters can be performed conveniently by chromatography on immobilized human serum albumin. This technique is the subject of a separate chapter.

The optical purity (ξ) of a mixture of enantiomers with concentrations c_o^d and c_o^l is defined, as

$$\xi = \frac{c_o^d - c_o^l}{c_o^d + c_o^l} , \qquad (24)$$

hence the optical purity may vary from -1 (pure levorotatory enantiomer) through zero (racemate) up to +1 (pure dextrorotatory enantiomer). After ultrafiltration of a solution of drug and protein, the optical purity in the filtrate, ξ_f is

$$\xi_f = \frac{\alpha_d c_o^d - \alpha_l c_o^l}{\alpha_d c_o^d + \alpha_l c_o^l} . \qquad (25)$$

Since the bound drug present in the retentate is always contaminated with the unbound fraction, the optical purity of the retentate (ξ_r) depends on the filtrate volume:

$$\xi_r = \frac{q_d c_o^d - q_l c_o^l}{q_d c_o^d + q_l c_o^l} , \qquad (26)$$

where q_d and q_l have the meaning, as before (cf. eq. (15)). Starting with a racemic ligand, $c_o^d = c_o^l$, thus eqs. (25) and (26) take simpler forms, viz.,

$$\xi_f = \frac{\alpha_d - \alpha_l}{\alpha_d + \alpha_l} \qquad (27)$$

and

$$\xi_r = \frac{q_d - q_l}{q_d + q_l} = \frac{\alpha_d - \alpha_l}{\alpha_d + \alpha_l - 2\,\frac{V_o}{V_f}} , \qquad (28)$$

respectively. From eqs. (12), (27) and (28) the basic equation of the method can be found; it relates experimental parameters (α_{rac}, ξ_f and ξ_r) to enantiomeric α values:

$$\alpha_{rac} = \frac{\alpha_d + \alpha_l}{2} = \frac{V_o}{V_f} \frac{\xi_r}{\xi_r - \xi_f} \qquad (29)$$

and allows three possible ways to determine α_d and α_l by using any pair of the three experimental parameters, as follows:

$$\alpha_d = \frac{V_o}{V_f} \frac{\xi_r (1 + \xi_f)}{\xi_r - \xi_f} \; ; \; \alpha_l = \frac{V_o}{V_f} \frac{\xi_r (1 - \xi_f)}{\xi_r - \xi_f} \qquad (30)$$

$$\alpha_d = \alpha_{rac} (1 + \xi_f); \; \alpha_l = \alpha_{rac} (1 - \xi_f) \qquad (31)$$

$$\alpha_d = \alpha_{rac} (1 + \xi_r) - \frac{V_o}{V_f} \xi_r; \; \alpha_l = \alpha_{rac} (1-\xi_r) + \frac{V_o}{V_f} \xi_r. \qquad (32)$$

In principle, eqs. (30) - (32) should give identical results for α_d and α_l which is indeed the case for II as illustrated in Table 2.

Table 2. Determination of stereoselective binding of *rac*-oxazepam acetate (II) to human serum albumin by optical purity measurements[11]

Experimental parameters	equation applied	α_d	α_l
c_o = 81 µM			
c_p^o = 150 µM	(30)	0.15	0.52
V_o = 400 ml	(31)	0.14	0.50
V_f = 382 ml			
α_{rac} = 0.321	(32)	0.13	0.52
ξ_f = -0.565			
ξ_r = +0.265			

If, however, adsorption of the drug to the ultrafiltration membrane occurs, all calculations based on the value of α_{rac} will be false. In such a case eq. (30) still can be used if the preparative losses are not stereoselective[11]. This method was also applied to study the binding of compounds III-VII to human serum albumin[12]. Results are collected in Table 3.

Table 3. Stereoselective binding of *rac*-oxazepam esters to human serum albumin as evaluated by eq. (30)[12]

Drug	c_o μM	c_p^o μM	ξ_f	ξ_r	α_d	α_l	K^d/K^l
II	81	150	-0.565±0.01	+0.265±0.01	0.15	0.52	6.5±1
III	30	45	-0.41 ±0.02	+0.58 ±0.03	0.35	0.85	10.0±3
IV	32	30	-0.31 ±0.01	+0.67 ±0.03	0.48	0.92	12.0±3
V	29	38	-0.35 ±0.01	+0.56 ±0.03	0.41	0.85	8.0±3
VI	11	15	-0.39 ±0.02	+0.44 ±0.01	0.33	0.74	6.0±2
VII	51	45	-0.26 ±0.02	+0.37 ±0.02	0.45	0.76	4.0±2

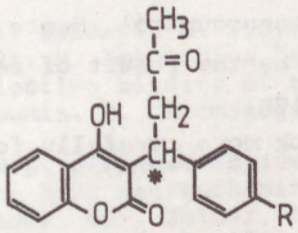
The extent of stereoselectivity (last column of Table 3) is calculated from the approximate eq. (33) implying common binding sites for the enantiomers and low saturation, i.e. $nc_p^o >> (1-\alpha)c_o$ in eq. (10):

$$\frac{K^d}{K^l} = \frac{(1-\alpha_d)\,\alpha_l}{\alpha_d(1-\alpha_l)} \qquad (33)$$

Although the experimental uncertainty is rather high, and the stereoselectivity does not seem to vary too much with the structure of II-VII, *none* of our compounds showed nearly as high a stereoselectivity as the hemisuccinate ester studied by Müller and Wollert[8].

Application to the binding of *rac*-acenocoumarol

Pharmacokinetics, pharmacodynamics, and metabolism are known to be stereoselective for coumarin derivatives like acenocoumarol and warfarin.



R=NO_2 Acenocoumarol

R=H Warfarin

In the binding of warfarin enantiomers to both protein components of blood, human serum albumin[13] (HSA) and α_1-acid glycoprotein[14] (AGP), a slight preference for *levo*-warfarin has been indicated, but no information has been available for the binding of acenocoumarol enantiomers. Optical purity measurements were made on the ultrafiltrate of racemic coumarin derivatives[15]. Measurable optical activity was not found for the filtrates of *rac*-warfarin with either of HSA and AGP. Results for *rac*-acenocoumarol are collected in Table 4.

Table 4. Stereoselective binding of *rac*-acenocoumarol to human serum albumin and to α_1-acid glycoprotein[15] as evaluated by eq. (31)

Protein	c_o	c_p^o	α_{rac}	ξ_f	α_d	α_l	$\frac{K^d}{K^l}$
HSA	25	25	0.32	-0.21	0.25	0.39	1.9
	50	50	0.21	-0.30	0.15	0.27	2.1
	57	30	0.39	-0.24	0.30	0.48	2.2
AGP	50	50	0.50	+0.26	0.63	0.37	0.34
	57	58	0.52	+0.33	0.69	0.35	0.24
	100	100	0.45	+0.28	0.58	0.32	0.34

Interestingly, inverse stereoselectivity is demonstrated for the two proteins, similarly to the binding of propranolol enantiomers[16]. The extent of stereoselectivity is higher for AGP than for HSA; in fact, aconocoumarol exhibits the highest degree of enantioselective binding reported for AGP so far. Our method was not sensitive enough to detect optical activity in the serum filtrate of *rac*-acenocoumarol. Hence, an overall lack of enantioselectivity can be the result of selective molecular events of opposite sign.

Just another reason to look more carefully for enantioselective interactions.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

RESOLUTION BY IMMOBILIZED HUMAN SERUM ALBUMIN

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INTRODUCTION

Stereoselective binding of chiral molecules to serum proteins can be an initial step in enantioselective drug action[1,2]. Following the administration of a racemate, protein binding modifies the equimolar ratio of the enantiomers in the pharmacologically active "free" (not protein-bound) phase. The serum contains several components among which albumin is the most abundant (about 4 %) and the most studied protein[3] even from the point of view of binding stereoselectivity. Human serum albumin (HSA) is supposed to have two main specific ligand binding sites which were found to be stereoselective as well[4]. These sites can be characterized by the binding of the drugs diazepam and *rac*-warfarin or of the endogenous ligands L-tryptophan and bilirubin, respectively.

DEFINITION OF BINDING STEREOSELECTIVITY

The binding process is a reversible association between the ligand (L) and the protein (P) and can be characterized by the equilibrium constant (K) and the number of binding sites (n).

$$P + nL \rightleftharpoons PL_n \qquad (1)$$

In case of stereoselective binding the enantiomeric equilibrium constants (K_R and K_S) are different and their ratio is considered

a measure of stereoselectivity. The binding processes however, are usually much more complex than the scheme above and the determination of K values from the binding isotherms is not unambiguous. There are two main theoretical approaches to describe multiple equilibria[5]. The Scatchard model gives the "site" binding constants which are different from the stoichiometric "stepwise" binding constants. Therefore numerical values reported for stereoselectivity should be compared carefully.

AFFINITY CHROMATOGRAPHY AND CHIRAL RECOGNITION

In the chromatographic technique,which involves a series of consecutive equilibria between free and bound ligands, even small differences in the binding of the enantiomers can bring about resolution of racemates on immobilized protein. It is known that proteins immobilized in hydrophilic gels usually maintain their specific binding affinity towards small molecules[6]. The pioneer work with albumin was done by Stewart and Doherty[7], who resolved DL-tryptophan on bovine serum albumin (BSA) coupled to succinoethyl-agarose gel. They suggested to extend the term of affinity chromatography to all chromatographic separations based on specific biological interactions. Ever since only a few laboratories have contributed to the development and application of this chiral separation technique, probably because of the rapid appearance of more effective and convenient synthetic chiral stationary phases. The main reason of the utilization of protein columns is to obtain relevant pharmacological information for binding stereoselectivity. Besides, these columns can be succesfully applied for the analysis of certain enantiomeric mixtures.

Evaluation of binding equilibrium constants

Several chromatographic techniques have been developed for quantitative studies of drug-protein binding interactions[8], but special attention was seldom given to stereoselectivity. The work of Lagercrantz and co-workers[9] is of basic importance.

They immobilized BSA by coupling it to BrCN-activated Sepharose 4B ($c_{BSA} = 1.4 \times 10^{-4}$ M) and the elution volumes of radioactive ligands (less than 10 nmol) were measured using buffer eluent. They worked out how the association constants can be evaluated from the elution volumes if certain column parameters are known. The validity of eq.(2) was proved,

$$V_e - V_o = V_g c_p \sum_{j=1}^{m} n_j K_j = V_g c_p K_1 \qquad (2)$$

where V_e: elution volume of the ligand studied,

V_o: elution volume of ligands having no special interaction with the protein,

V_g: volume of the gel (total volume minus void volume),

c_p: concentration of protein in the gel.

Since eq.(2) is derived for very low ligand concentration when the saturation of the binding sites on the protein is practically negligible, the binding affinity is characterized either by the Scatchard type $\sum_{j=1}^{m} n_j K_j$ expression (vertical intercept) where site binding constants and number of binding sites cannot be separated, or by the first stoichiometric binding constant K_1 which gives no information on the presence of multiple binding sites. According to eq.(2) the binding stereoselectivity can be obtained as a ratio of the V_e-V_o values of the enantiomers. Besides the above described "zonal" chromatography, Lagercrantz et al. also discussed the use of "frontal" chromatography, where instead of pure buffer the eluent is a ligand solution. They studied[10] the binding enantioselectivity of tryptophan and warfarin on immobilized albumins from a number of different biological species including man, and revealed considerable variations in sign and magnitude.

Analytical application of protein columns

Allenmark and co-workers successfully utilized BSA-Sepharose stationary phase for the resolution of widely different molecules like aromatic amino acids[11] and compounds having chirality with respect to sulphur atom[12]. Being interested in the analysis of enantiomeric mixtures produced by biochemical reactions, they developed[13,14] a much more efficient separation method combining albumin as chiral stationary phase with HPLC methodology. BSA immobilized on silica is commercially available as "Resolvosil" column from Macherey, Nagel and Co. (Düren, F.R.G.). Variations in mobile phase composition(e.g. pH, buffer and organic additives) can optimize the separations.

Another serum protein with stereoselective binding ability, α_1-acid glycoprotein (α_1-AGP, orosomucoid) was also developed as a highly selective chiral stationary phase for HPLC by Hermansson[15]. Commercial α_1-AGP columns are "EnantioPac" from LKB and "Chiral-AGP" from ChromTech AB as an improved version.

SELECTIVE BINDING ON HSA-SEPHAROSE COLUMN

A few examples are being selected - mainly from our laboratory - providing useful information for the stereoselective binding of chiral drugs with special attention to the specific binding sites. We used the technique of Lagercrantz et al.[9] Results are compared with those obtained by other methods.

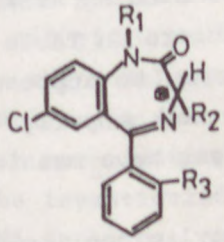
Binding to the benzodiazepine-indole binding site

In 1958 McMenamy and Oncley reported[16] about a hundred-fold selectivity for the binding of tryptophan enantiomers to albumin favoring the naturally occurring L-amino acid. This impressive number is still cited as the classical example for stereoselective binding on albumin. The resolution of *rac*-tryptophan on immobilized albumin has been mentioned already[7,10], however factors of only 6 and 8 were obtained for BSA and HSA, respectively. Being interested in the reliability of the affinity chromatographic method we could not avoid interfering

with this discrepancy. We came to the conclusion[17] that the large effect was connected with the author's definition of stereoselectivity[16]. The high K_L/K_D ratio was obtained[16] on the assumption that while one specific binding site would exist for L-tryptophan, the binding of the D-antipode was not specific with the number of binding sites being at least by an order of magnitude greater. It means that the factor of 100 reflects the ratio of slopes of the Scatchard plots of the enantiomers (the smaller slope being close to zero), while the chromatographic technique is characteristic of the vertical intercepts, i.e. ΣnK values (cf. eq. 2). We think that the latter concept is less misleading.

Among drugs, oxazepam hemisuccinate is the classic example. Müller and Wollert reported[18] $n_S K_S / n_R K_R = 35$ according to the Scatchard plots obtained for the binding of the enantiomers to HSA. The high stereoselectivity preferring the (S)-enantiomer was explained[19] by a special structural feature of the 3-substituted 1,4-benzodiazepine molecule; due to the inversion of the diazepine ring the enantiomers differ not only in configuration of the chiral center but also in chiral conformation. By chromatography on HSA-Sepharose columns we studied the binding stereoselectivity of several 3-acyloxy- and 3-hydroxy-[20,21] (Table 1) as well as 3-alkyl-1,4-benzodiazepines[22].

Table 1. Structure of 3-hydroxy-1,4-benzodiazepines investigated



R_1	R_2	R_3	
H	OH	H	oxazepam
H	OH	Cl	lorazepam
CH_3	OH	Cl	Me-lorazepam
CH_3	OH	H	temazepam
CH_3	H	H	diazepam

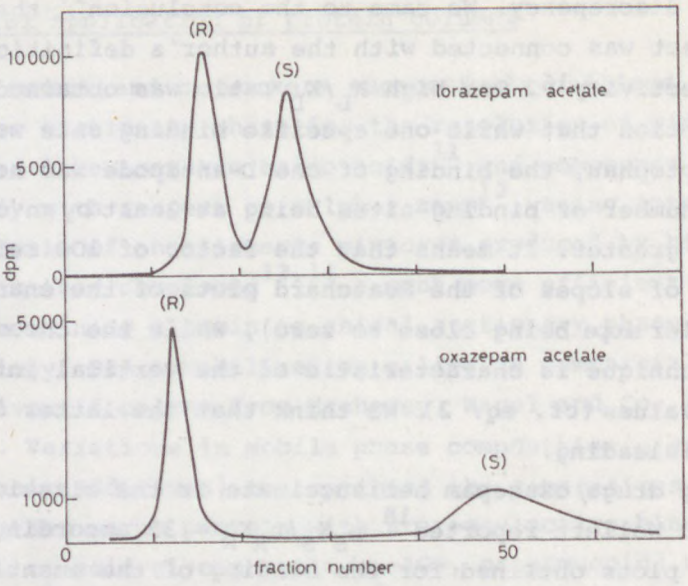


Figure 1. Radiochromatogram of *rac*-[^{14}C]lorazepam acetate and *rac*-[^{14}C]oxazepam acetate on a HSA-Sepharose column. V_o = 15.3 ml, $V_{fraction}$ = 3.2. ml; taken from ref.21.

Figure 1 shows the chromatograms of racemic acetates of lorazepam and oxazepam. The stereoselectivity values of 2 and 6, respectively, were in good agreement with those obtained by other methods. It can be seen that the decreased stereoselectivity due to the ortho-chlorine substituent is the result of increased binding of the (R)- and decreased binding of the (S)-enantiomer. In Table 2 the elution volumes of a series of oxazepam esters are collected.

These data were measured on a column of rather low binding capacity allowing to detect a wide range of binding affinities. It should be noted that the selectivity factors in Table 2 are smaller than those obtained on better columns. We suppose that the prerequisite conditions of eq. (2) were not properly fulfilled. Nevertheless, the relative changes have meaning. The following tendencies could be observed:

1. Increasing hydrophobicity of the acyl group increases the binding of both enantiomers without significant

Table 2. Resolution of *rac*-oxazepam esters on HSA-Sepharose column (V_o = 11 ml). V_R and V_S are the elution volumes of the enantiomers (from ref. 20)

Oxazepam ester	V_R (ml)	V_S (ml)	$\frac{V_S-11}{V_R-11}$
acetate	19	46	4.4
propionate	23	53	3.5
n-butyrate	28	85	4.4
i-butyrate	28	85	4.4
pivaloate	37	96	3.3
α-ethylbutyrate	65	> 150	
phenylacetate	73	> 150	
methylsuccinate	20	34	2.6
hemisuccinate	16	70	11.8

variation in stereoselectivity. A similar study on 3-alkyl derivatives[22] supported this conclusion.

2. The presence of polar atoms (cf. methylsuccinate) decreases both the binding affinities of the enantiomers and the stereoselectivity.

3. The negative charge on the acyl moiety (cf. hemisuccinate) selectively increases the binding of the (S)-enantiomer.

The information obtained by the chromatographic method for the "benzodiazepine" binding site on HSA is in agreement with the conclusion achieved by other approaches[19,23]: it is a hydrophobic pocket in the protein structure with a cationic charge, and gives preference to that conformation of the molecule which belongs to the (S)-configuration.

3-Hydroxy-1,4-benzodiazepines in solution are subject to very fast racemization, thus their binding stereoselectivity cannot be investigated by conventional binding methods. On short HSA-Sepharose and on BSA-silica HPLC[24] columns very good resolution of oxazepam occurred. With increasing time of chromatog-

raphy (slow rate or long column) an irregular elution profile shown in Fig.2 could be observed.

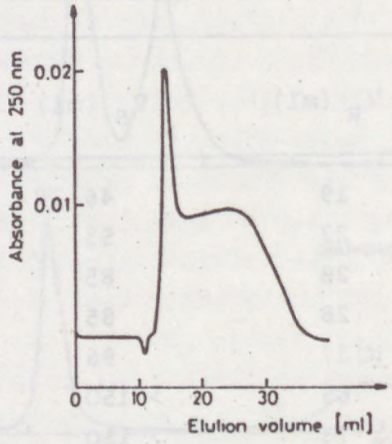


Figure 2. Elution profile of oxazepam on an HSA-Sepharose column. V_o = 11 ml, flow rate = 22 ml/hour. Taken from ref.20.

Binding to the warfarin binding site

The stereoselectivity of this binding site of HSA has been found to be less pronounced than that of the benzodiazepine site[4]. By chromatographic technique Lagercrantz et al.[10] determined $K_S = 4.4 \times 10^5\ M^{-1}$ and $K_R = 3.3 \times 10^5\ M^{-1}$ values for the binding of warfarin enantiomers to HSA. These parameters for albumins of other animal species showed large differences. Micropreparative resolution of [^{14}C]*rac*-warfarin could be performed on immobilized BSA ($K_R > K_S$) and pig serum albumin ($K_S > K_R$).

On a short HSA-Sepharose column where (S)-warfarin showed slightly stronger binding than the (R)-enantiomer, a structurally related compound, acenocoumarol, indicated a factor of two in favor of the (R)-enantiomer[25]. This unexpected result could be confirmed by a more tedious method combining ultrafiltration and CD-spectroscopy (cf. preceding chapter).

Interaction between binding sites: improved resolution

The application of frontal chromatography has been suggested for competition studies[9,26]. When the eluent contained salicylic acid, an improved resolution of *rac*-warfarin was achieved on HSA-Sepharose column; it was due to a differential decrease in the elution volumes of the enantiomers[9]. Displacement owing to competition can be expected between ligands which bind to the same site on the protein. The marker compounds diazepam and *rac*-warfarin do not change the binding of each other to HSA[27]. By chromatography we investigated whether the presence of (R)- or (S)-warfarin in the eluent influences the stereoselective binding of 3-substituted 1,4-benzodiazepines[21].

Table 3. The effect of warfarin enantiomers on the elution volumes (V_1, V_2 in ml) of racemic [^{14}C]3-hydroxy- and [^{14}C]3-acyloxy-1,4-benzodiazepin-2-ones from HSA-Sepharose column (V_o = 6.4 ml). From ref.21

Compound	Buffer		(R)-warfarin		(S)-warfarin	
	V_1	V_2	V_1	V_2	V_1	V_2
oxazepam	11	35	11	23	11	23
temazepam	10	48	10	35	10	35
lorazepam	13		12		12	28
Me-lorazepam	18*		18*		18*	
rac-oxazepam acetate	12	31	12	28	12	67
rac-oxazepam methyl-succinate	13	39	12	31	12	48
rac-temazepam acetate	9	41	9	34	9	35
rac-lorazepam acetate	13	20	12	45	13	130§
rac-Me-lorazepam acetate	11	28	11	26	11	24

* Broad peak of irregular shape.

§ Half of the total radioactivity could only be eluted with 1 % HSA solution.

Data collected in Table 3 indicate surprisingly selective changes for the binding of (S)-benzodiazepines (V_2 values). While (R)-warfarin either did not change, or decreased the elution

volumes (with the only exception of (S)-lorazepam acetate), in the presence of (S)-warfarin the binding of certain (S)-benzodiazepines was enhanced resulting in increased resolutions. The most remarkable effect was observed for (S)-lorazepam acetate which in the presence of (S)-warfarin was practically stuck to HSA. The effect of (S)-warfarin on the chromatograms of lorazepam and lorazepam methylether[28] was also very spectacular. While the binding of these compounds is weak and not stereoselective, in the presence of (S)-warfarin good resolution occurs due to increased binding of the (S)-enantiomer (cf. Fig.3).

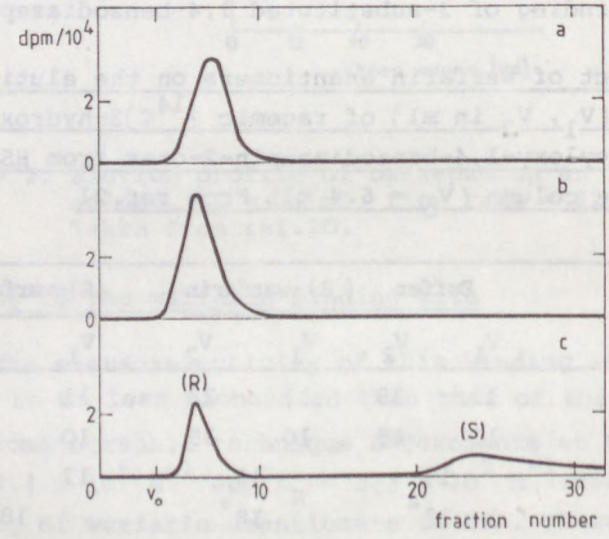


Figure 3. Radiochromatogram of *rac*-[^{14}C]lorazepam methylether on an HSA-Sepharose column (V_o = 6.4 ml, $V_{fraction}$ = 1.9 ml). Elution was made by (a) buffer, (b) 10^{-4} M (R)-warfarin, (c) 10^{-4} M (S)-warfarin. Taken from ref. 28.

The inverse experiment revealed[29] that this binding interaction is mutual: the chromatogram of *rac*-warfarin with (S)-lorazepam acetate in the eluent indicates the elution of the (R)-enantiomer only; (S)-warfarin could be removed from the column by albumin solution. Practically it means that the column provides a completely stereospecific affinity

chromatography and the whole binding capacity is available for resolution. The phenomenon itself proves that the "benzodiazepine" and *rac*-warfarin binding sites on HSA are not independent. There is a stereoselective allosteric interaction between the bound ligands, which is manifested by either mutually increased or decreased binding, depending on the structure of the interacting molecules. In the enhancement the ortho-chlorine substituent on the detached phenyl ring of the benzodiazepine molecule seems to play an important role, while the N(1)-methyl substituent prevents the phenomenon. Optically inactive clonazepam being an equimolar mixture of two conformers of opposite chirality is also able to provoke an improved resolution of warfarin[30] as seen in Fig.4.

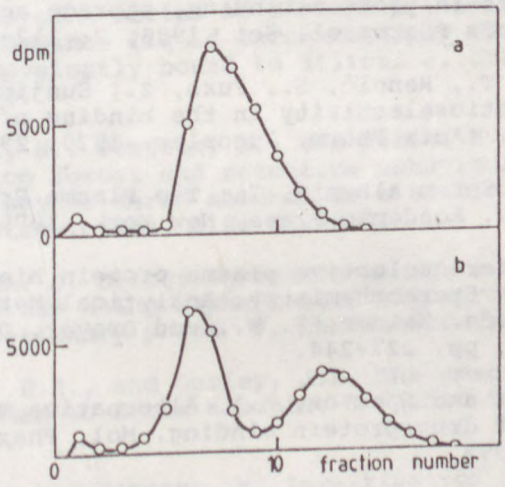


Figure 4. Radiochromatogram of *rac*-[^{14}C]warfarin on an HSA-Sepharose column (V_o = 3 ml, $V_{fraction}$ = 8 ml). Elution was made by (a) buffer, (b) 10^{-4} M clonazepam. Taken from ref.30.

CONCLUSION

Resolution of chiral ligands on immobilized HSA may have, in principle, analytical, preparative and pharmacological applications. In practice, the first two do not have much importance, either if combined with HPLC methodology, since plenty of more effective and convenient chiral stationary phases are available for these purposes. However, as far as the

pharmacological relevance is concerned, affinity chromatography preserving the original conformational flexibility of the protein gives fast and useful information for the stereoselectivity which may occur in physiological binding and binding interaction processes. This chromatographic technique makes small changes very spectacular even in those cases when by conventional techniques the significance of the effect is not convincing.

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Akadémiai Kiadó
Budapest, 1990

Problems and Wonders
of Chiral Molecules (ed. M.Simonyi)

BETA-ADRENERGIC BLOCKERS: PROPERTIES OF THE ENANTIOMERS

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The adrenergic nervous system plays a major part in controlling the function of the body. It is activated by the two adrenergic hormones noradrenaline (NA), which is released locally upon stimulation of the adrenergic nerves, and adrenaline (ADR), a circulating agent released from adrenal glands. Activation of the system causes profound effects on many body functions, including heart rate and force, blood pressure, kidney function, skeletal, intestinal and uterine smooth muscle tone, and many aspects of the central nervous system.[1]

These hormones act at so-called beta-adrenergic receptors (BAR) on cell membranes causing, via the 'second messenger' cyclic AMP, a biochemical cascade of events leading to the observed effect. In 1967 Lands classified these receptors into two sub-classes which he called beta-1 and beta-2[2]; activation of beta-1 receptors causes an increase in heart rate and force, releases renin from the kidney, and stimulates lipolysis, while activation of beta-2 receptors relaxes blood vessels, bronchi, and uteri, and causes a skeletal muscle tremor.

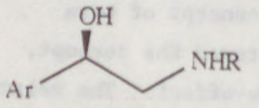
In the early 1960's J.W.Black proposed that, as anginal attacks occurred when the oxygen demand of heart muscle exceeded the oxygen supply through diseased coronary arteries, prevention of heart rate increases by blockade of beta receptors in the heart would reduce oxygen demand and prevent anginal attacks.[3] This led to the concept of beta blockers, and the first of these, pronethalol, proved the concept, though it failed subsequently due to a toxic side-effect. The related compound, propranolol, was not toxic, and proved to be an effective antianginal agent.

Subsequent clinical studies with propranolol showed that it was effective, also in the treatment of hypertension, glaucoma (a disease of the eye), migraine, tremor, anxiety, and a number of other indications, and opened the way for a large number of competitive products. These differ in potency, beta-1/beta-2 selectivity (propranolol is non-selective), lipid solubility, and residual degree of beta partial agonism, and are used in the many clinical areas.
Early work showed that R propranolol was more than a hundred times less potent than S propranolol at blocking cardiac beta receptors,[4] and the drug is sold as the racemate, the R isomer simply acting as an inert diluent. This pattern has been observed with subsequent drugs, with the result that only timolol, penbutolol, levobunolol and pacrinolol are sold as enantiomers.

In this chapter the differences between the properties of the enantiomers of beta blockers will be discussed in terms of pharmacology, clinical studies, distribution, and metabolism, and the chemical aspects of their synthesis and purity determination will be covered briefly.

The hormones NA and ADR are examples of arylethanolamines (I), and the activity resides in the natural R(-)enantiomer shown, the distomer being 100-1000 times less potent.[5] Many synthetic agonists, and the antagonists pronethalol and sotalol, have this configuration also, while most of the antagonists, such as propranolol, have the aryloxypropanol-amine structure II, the eutomer having the S(-) configuration illustrated.

Beta blockers have very small optical rotations, and so until the advent of sophisticated HPLC/GLC/NMR methods optical purity was very hard to define accurately. One should use, therefore, great caution when evaluating reports of eudismic ratios in various systems where accurate enantiomer ratios have not been defined.



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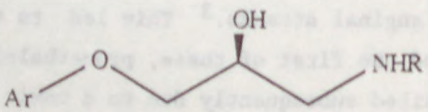


II

As one would expect, the isomers of propranolol, the first commercially successful beta blocker, have been investigated more than all the other cases. Being a lipophilic amine some of the properties of propranolol are due to non-specific anaesthetic effects in cell membranes, often termed 'membrane-stabilising activity' or MSA; and, unlike the beta blocking effects, MSA is exhibited equally by the two enantiomers. A number of studies have been carried out to determine the significance of the two effects (beta blockade vs. MSA) in various biological situations.

Studies in both rats[6] and man[7,8] have shown that the antianginal and antihypertensive effects of propranolol are due to beta blockade caused by the S isomer, the R isomer being completely ineffective. Propranolol has anti-arrhythmic properties, and there is evidence that both beta blockade and MSA contribute to this property. S propranolol has a far greater effect than R propranolol on the ventricular fibrillation threshold in dogs,[9] while the enantiomers are equivalent in their effect on both refractory period,[10] and vulnerability to fibrillation in the rat heart.[11] In several models in cat and dog both propranolol enantiomers are anti-arrhythmic, though S propranolol is more potent.[4]

MSA appears to be the cause of propranolol's effect in mania,[12] as an anticonvulsant,[13] and as an inhibitor of sperm motility;[14] the cardiovascular effects of R,S propranolol obviously preclude its widespread use as a vaginal contraceptive, though it is effective. At clinical oral doses the drug has no effect on sperm motility.[15]

Propranolol inhibits skeletal muscle tremor of the type induced by stress or anxiety. The activity resides with the S isomer,[16] as it is a consequence of stimulation of beta receptors in skeletal muscles.

A number of other beta blockers have ancillary properties which may reside with one or both enantiomer; it is even possible that it is the opposite enantiomer. Prizidolol possesses vasodilating and beta blocking properties; the enantiomers have equal potencies as vasodilators but, as usual, the S isomer is more potent, by 25-50 times, as a beta blocker.[17] Marketing the S isomer would, therefore, have

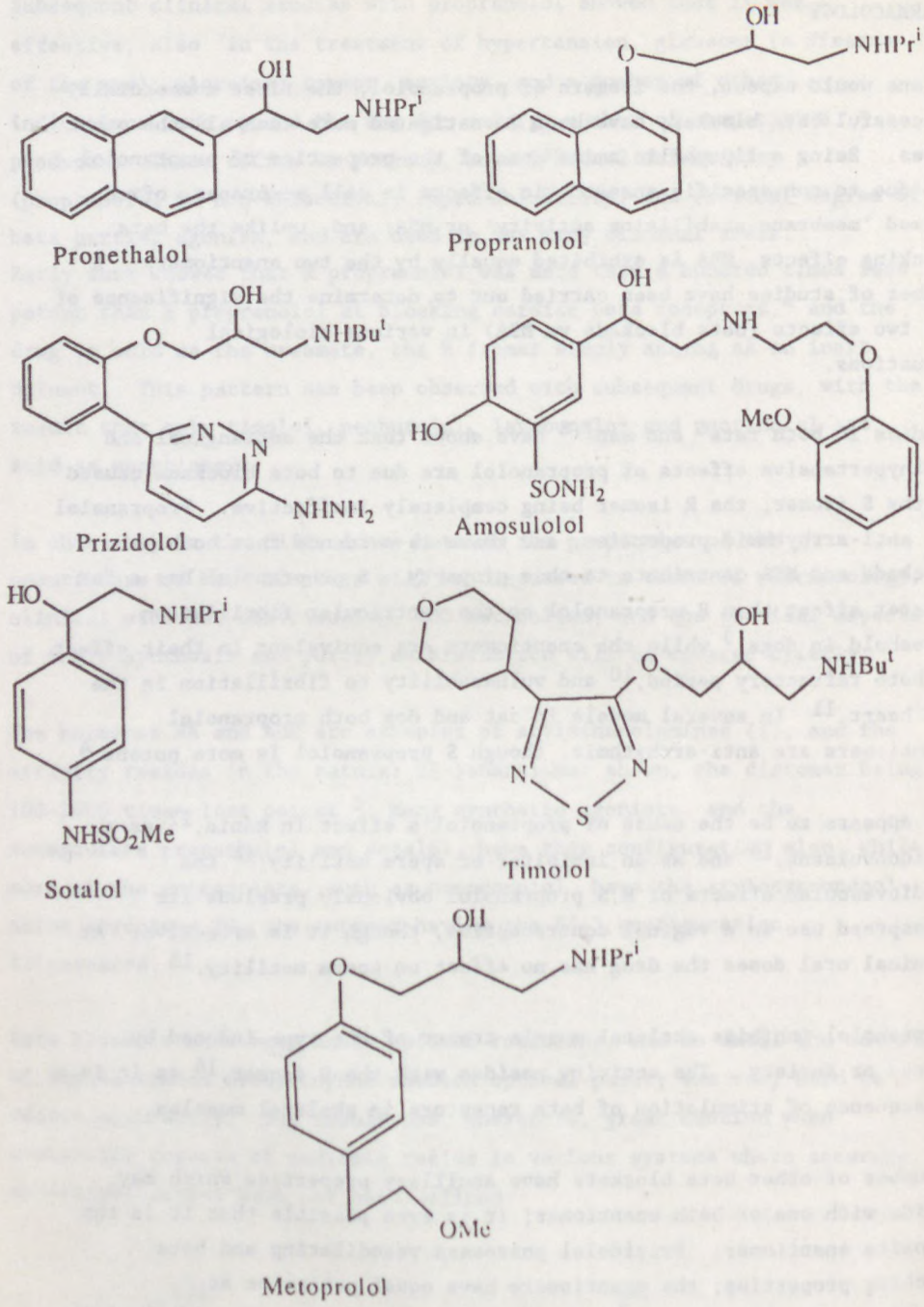


Figure 1. Structure of Selected Beta Blockers.

produced a slightly different cardiovascular pharmacology than the racemate.

Amosulalol also possesses beta blocking and anti-hypertensive properties, these latter being due to alpha adrenoceptor blockade; unlike prizidolol, however, these properties are possessed by different isomers. The R(-) isomer is less potent, by a factor of 11:1, than the S(+) isomer as an alpha blocker, while it is more potent, by a factor of 60:1 as a beta blocker.[18] Obviously, to act as an alpha + beta blocker the drug must be dosed as a racemate, as neither enantiomer possesses both properties.

The situation with two other alpha/beta blockers, labetalol (III) and medroxalol (IV), is even more complex, as both contain, in addition to the asymmetric carbon atom bearing the benzylic alcohol function, an additional centre of assymetry in the side chain. Four isomers, comprising two enantiomeric pairs of diastereoisomers, are possible and, in the case of labetalol the mixture of these isomers which is produced by the chemical synthesis is the product marketed; fortunately the ratio of isomers produced is reproducible. The synthesis of the four labetalol isomers has been described,[19,20] and the properties are listed in Table 1. The picture is complex, and supports the concept that dosing a fixed-ratio mixture is reasonable.

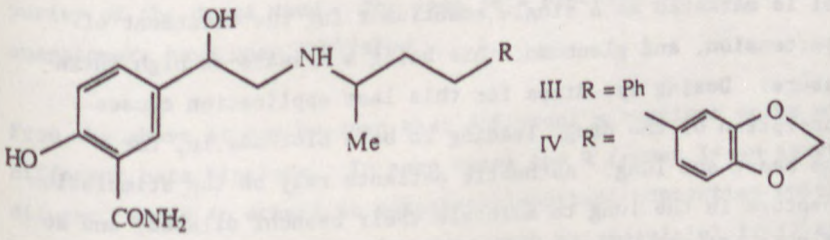


Table 1. Alpha and Beta Blocking Properties of the Isomers of Labetalol in Anaesthetised Rats

Isomer	Beta block. ED_{50}	Alpha block. ED_{50}	Ratio $\frac{\text{Alpha}}{\text{Beta}}$
R,R	0.07 mg/kg	35.0 mg/kg	500
R,S	4.1	inactive	-
S,R	5.0	1.3	0.26
S,S	inactive	4.8	0
labetalol	0.25	7.1	28.4

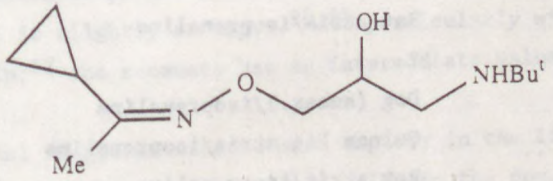
One isomer of labetalol, the R,R enantiomer, has been selected by the Schering-Plough company for development as an antihypertensive agent. Known as SCH 19927, it is about three times more potent than labetalol as a beta blocker, and five times less potent as an alpha blocker.[21] The alpha:beta ratio is therefore 500 as compared to 28 for labetalol. Both of these drugs must be considered as separate entities.

A very similar situtation is seen with the structurally related drug medroxalol.[22] Although the four isomers could not be obtained completely pure, they show beta:alpha potency ratios of 5, 1500, 0.5 and 400 compared with 55 for medroxalol (i.e. more potent at beta than alpha receptors). Medroxalol is marketed as the (constant) mixture.

Racemic sotalol possesses beta blocking and anti-arrhythmic properties; as sotalol is a hydrophilic drug, these latter are not due to MSA. The beta receptor effects reside with the R(-) isomer (this corresponds to the same configuration as, for example, S(-) propranolol, as sotalol has an aryl ethanolamine structure), but the S(+) isomer has potent antiarrythmic effects in dogs[23] and in man,[24,25] this activity being comparable to that exhibited by the R(-) isomer. R(-) sotalol (usually referred to as D-Sotalol) is hence a much safer antiarrhythmic drug in patients where beta blockade is undesirable. The racemic drug is marketed for the treatment of angina and hypertension.

S(-) timolol is marketed as a single enantiomer for the treatment of angina, hypertension, and glaucoma; this being a disease of high intra-ocular pressure. Dosing eye drops for this last application causes systemic absorption of the drug, leading to beta blockade in, for example, the heart and lung. Asthmatic patients rely on the stimulation of beta receptors in the lung to maintain their bronchi dilated, and so blockade of this effect has serious repercussions. Fatalities have occurred from the side effects of timolol,[26] and so it is very significant that the R isomer has also been found to have a beneficial effect on intraocular pressure.[27,28] This isomer should be a far safer drug for glaucoma due to its relatively poor beta blocking potency (50-90 times less than timolol[29]).

The ketoxime derived beta blocker falintolol (V) presents a very interesting case. The syn- and anti- forms of both enantiomers were prepared and the biological properties compared.[30] It is evident that stereochemical discrimination in this area is very poor, with R and S isomers having very similar properties. A model of 'pseudo-symmetry' is proposed in this case to explain the data.



V

In many cases the only data published which relate the biological properties of pairs of enantiomers in a particular system (the eudismic ratio) is a simple comparison of the affinities for beta receptors, measured by radioligand binding, or the ability to displace a standard agonist (usually isoprenaline) in an in vitro or in vivo system; the most common system is the heart rate response in an isolated atrium or an anaesthetised animal. The eudismic ratio for a number of drugs is shown in Table 2, remembering the cautionary note regarding optical purity of the drugs used. For some beta blockers no data on the enantiomers have been published.

From the above it may be seen that different situations occur with different beta blockers. In some cases the R isomer is an inert diluent, while in others it possesses important properties which may be beneficial or deleterious. In cases such as amosulalol it is essential for both isomers to be present, while for timolol and sotalol it may be desirable to omit the active beta blocking isomer in order to benefit from another effect. It is desirable that the enantiomers of any new drug should be studied in detail before a final choice of racemate or enantiomer is chosen for the market.

Table 2. Eudismic Ratios for Beta Blocking Drugs

Drug	Ratio	Test System	Reference
Alprenolol	100,426	Rat atria/isoprenaline	31, 33
	300	Adenylate cyclase/isoprenaline	32
Atenolol	12	Receptor binding	33
Befunolol	300	Guinea Pig atria/isoprenaline	34
Betaxolol	25	Rat atria/isoprenaline	35
Bucindolol	250	?	36
Bucumulol	40	Dog (anaes.)/isoprenaline	37
	270	Guinea Pig atria/isoprenaline	
Bupranolol	65	Rat atria/isoprenaline	33
Indenolol (YB2)	50-100	Guinea Pig atria and anaes. Dog	38
Metoprolol	270-380	Rabbit atria/isoprenaline	39
Moprolol	Qualitative	Human volunteers	40
Oxprenolol	10-35	Anaes. cats/isoprenaline	41
Penbutolol	50	Guinea Pig papillary muscle	42
Pindolol	200	Guinea Pig atria	43
Practolol	12	Receptor binding	33
Propranolol	160	Receptor binding	33
	60-100	Anaes. cat/isoprenaline	44
Sotalol	22	Anaes. dog/isoprenaline	45
Talinolol	20	Anaes. dog/isoprenaline	46
Timolol	50-90	Guinea Pig atria/isoprenaline	29
Tolamolol	30	Guinea Pig atria/isoprenaline	47

PHARMACOKINETICS

As the enantiomers of beta blockers have different absolute chemical structures but identical physical properties, one would expect that they should be absorbed at identical rates, assuming no active transport, and providing that they are adequately soluble. As enzymes are chiral, one would also expect the enantiomers to be metabolised differently, and this is the case.

In some cases the R isomer affects the rate of metabolism of the S isomer of a drug, and this may be for a physicochemical reason, or because of competition for a common enzyme. For example, dosing S propranolol on its own produces lower blood levels in man than when co-dosed with the same weight of R propranolol (i.e. as racemate).[48,49] The R isomer could be displacing the S isomer from plasma protein, or it could be competing for the same metabolising enzyme. Both enantiomers undergo comparable plasma protein binding, although binding with S propranolol is slightly stronger,[50,51] particularly with alpha-1-acid glycoprotein;[52] the racemate has an intermediate value.

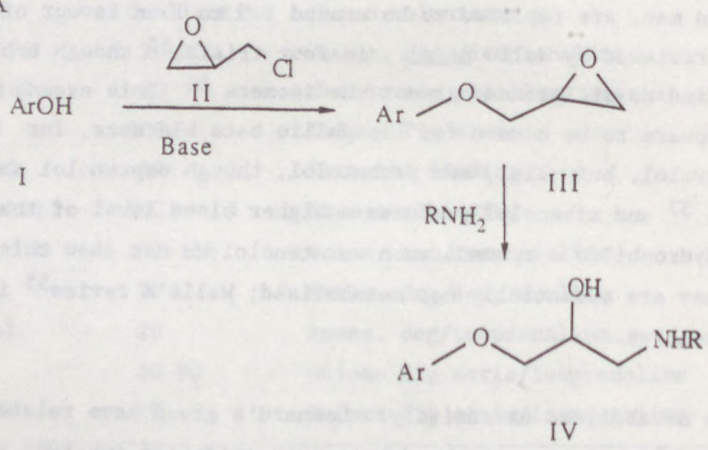
S propranolol is glucuronidated more rapidly in the liver (dog) than R propranolol by three- to fourfold.[53] Also in the dog, oxidation to 4-hydroxy-propranolol is more rapid with the S isomer,[54] though in man the order is reversed. Overall plasma ratios of S and R propranolol, after oral dosing in man, are reported to be around 1.5 to 1 in favour of the S isomer, as reviewed by Walle et al., in four trials,[55] though Bobik's group could find no difference between the isomers.[56] This excess of S enantiomer appears to be common for lipophilic beta blockers, for example metoprolol, bufuralol, and penbutolol, though oxprenolol shows no difference,[57] and xibenolol produces a higher blood level of the R enantiomer. Hydrophilic compounds such as atenolol do not show this effect, as they are essentially not metabolised; Walle's review[55] is quite comprehensive.

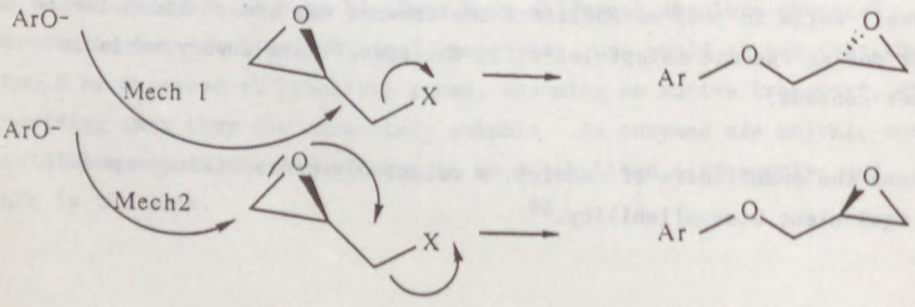
Metoprolol is metabolised extensively. Lennard's group have related the degree of metabolism in man to the debrisoquin oxidation sub-type and shown that the oxidation is stereoselective.[58] For good metabolisers S metoprolol levels were 35% higher than for the R isomer (areas under the curves), while in poor metabolisers the reverse was true. Blood levels after dosing racemic metoprolol will, therefore, have a very variable isomer content.

In rats the enantiomers of nadolol, a relatively hydrophilic compound, had equivalent bioavailability.[59]

The majority of racemic beta blockers have been prepared by reaction of a phenol (I) with epichlorhydrin (II) and a basic catalyst to give an epoxide (III) which reacts with amines to give the final products (IV), as depicted in Scheme A. It would seem logical to use enantiomerically pure epichlorhydrin to prepare a pure enantiomer of IV, but this is not as simple as it seems. Epichlorhydrin is attacked by nucleophiles in two modes; via direct S_N2 displacement of halide (mechanism 1), or by epoxide ring opening followed by ring closure (mechanism 2). These different mechanisms give rise to opposite enantiomers of the epoxide, hence unless the reaction can be limited to one pathway a mixture will ensue.[60]

Scheme A

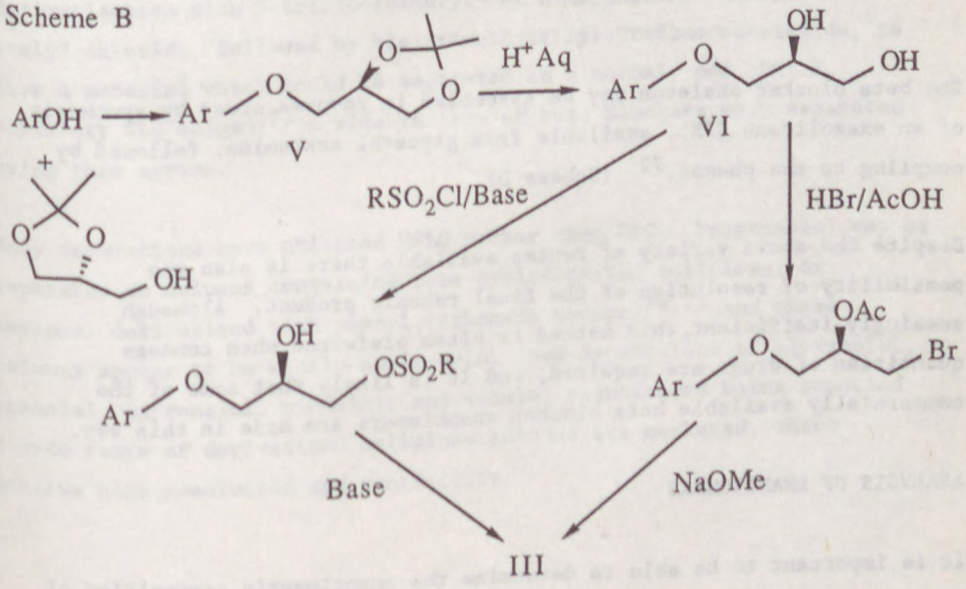




Replacement of the chlorine atom by better leaving groups such as trifluoromethanesulphonyl[61] or 3-nitrobenzenesulphonyloxy[62] gives virtually 100% direct S_N2 displacement, the 4-toluene sulphonate derivative being almost specific enough for mechanism 2 to be synthetically useful.[62] The 'Sharpless oxidation' technique allows preparation of both enantiomers of glycidol in good yield, though the optical purity is poor, and crystalline derivatives have to be made to afford pure products.[63]

Glycerol acetonide (isopropylidene glycerol, 'solketal') has been prepared from several sources; cleavage of mannitol derivatives gives the S isomer,[64] and the R isomer is obtained from ascorbic acid.[65] Recently the R isomer has become readily available via microbiological kinetic resolution,[66] bringing its cost down to a reasonable level. Reaction of the R isomer with phenols gives acetonides (V), which are easily cleaved to diols (VI) using acidic hydrolysis. These diols may be converted to epoxides using either sulphonyl chlorides/base,[67] or hydrogen bromide in acetic acid, followed by base.[68] The former may possibly cause some racemisation via the secondary sulphonate, whereas the latter route is reported to be stereospecific. It is presumed that reaction of III with amines to give IV is stereospecific (Scheme B).

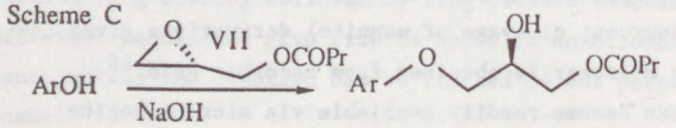
Scheme B

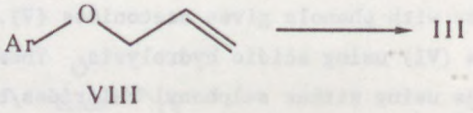


The same intermediate diol (VI) is produced by the reaction of glycidyl butyrate (VII) with phenols, followed by basic hydrolysis.[69] (Scheme C). VII is now available at moderate cost from the kinetic resolution of racemic VII using porcine pancreatic lipase.[70]

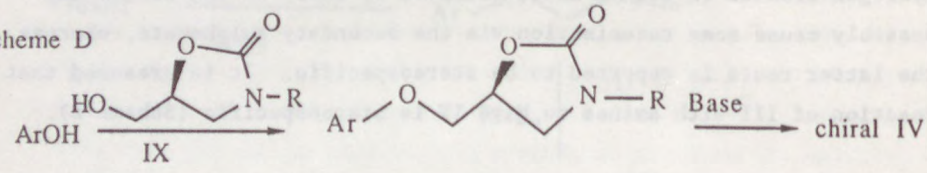
Aryl alkyl ethers (VIII) have been converted directly to epoxides (III) using another microbiological system. The best organism was Ps. Oleovorans, which gave a good reaction rate and high optical purity.[71]

Scheme C





Scheme D



The beta blocker skeleton may be assembled in reverse order by synthesis of an oxazolidone (IX), available from glycerol acetonide, followed by coupling to the phenol.[72] (Scheme D).

Despite the above variety of routes available there is also the possibility of resolution of the final racemic product. Although seemingly inefficient, this method is often preferred when tonnage quantities of drugs are required, and it is likely that some of the commercially available beta blocker enantiomers are made in this way.

ANALYSIS OF ENANTIOMERS

It is important to be able to determine the enantiomeric composition of beta blocking agents in both bulk drugs and in samples of biological

fluids. These two applications have different problems; for the former, determination of low-level contamination of one isomer by the other is required, while with biological fluids such as plasma or urine the major problem is sensitivity. For pure drugs resolution needs to be better, probably much better, than 1%; a beta blocker which has a true eudismic ratio of 100 would exhibit a ratio of 20 if only 5% of the eutomer was present in the distomer. The converse is not true, as 5% of the distomer in the eutomer will affect its potency very little.

Until the last few years, measurement of optical rotation was the only method available and, due to the low rotation of most beta blockers, this was very imprecise. More recently, a number of analytical methods have been developed which have the resolution required; these may be direct, or may involve derivatisation with either a chiral or non-chiral reagent.

Using capillary GLC all three types have been employed: betaxolol enantiomers are separated directly on a tris(3,5-dimethylphenyl-carbamate) cellulose column, and most other beta blockers available commercially may be separated similarly.[73] Derivatisation with phosgene, giving the less polar oxazolidone, has enabled the separation of metoprolol isomers to be achieved,[74] while an Italian group used derivatisation with N-trifluoroacetyl- or N-heptafluorobutyryl-L-prolyl chloride, followed by bis(trimethylsilyl)trifluoroacetamide, to give a material which could be separated on a normal, non chiral, capillary GLC column;[75] a wide variety of beta blockers were separated using this system.

Many separations have utilised HPLC rather than GLC. Propranolol may be separated on columns containing beta cyclodextrin, cellulose, or amylose, derivatised with phenyl carbamate groups,[76,77] and these columns appear to be widely applicable, good separations of alprenolol, atenolol, oxprenolol, pindolol, and sotalol enantiomers being reported. A wide range of derivatised cellulose columns are marketed, which achieve high resolution and sensitivity.

'Pirkle' HPLC columns[78] contain R-N(3,5 dinitrobenzoyl)phenylglycine bonded onto silica, and the phosgene derivative of propranolol separates on this; the system is sensitive enough to allow the estimation of propranolol enantiomer levels in human serum.[79] Propranolol may also be resolved using ion-pair chromatography with camphor-10-sulphonic acid.[80]

A number of chiral derivatising agents have been used to prepare beta blockers for non-chiral HPLC assay. These include S-α-phenylethyl isocyanate, the derivatives being separable on either normal[81] or reversed-phase,[82] S-benoxaprofen chloride (TLC separation),[83] and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate or 2,3,4,6-tetra-O-acetyl-β-D-glycopyranosyl isothiocyanate (reverse-phase HPLC).[84] This last paper describes the separation of ten beta blocker racemates. Atenolol has been resolved by derivatisation with tartaric acid anhydride (reverse-phase HPLC).[85]

With biological samples, such as plasma or urine, the major problem is obtaining an assay sensitive to nanogram quantities of drug. Normally one wishes to either estimate the levels of an enantiomer, or compare enantiomer ratios roughly. The determination of, say, 1% of one enantiomer in the presence of 99% of the other, accompanied by large numbers of other chemicals, is unrealistic, and is not usually required. This subject has been reviewed by Testa.[86]

In addition to the method described previously[79] for the determination of propranolol enantiomers in plasma, derivatisation by N-trifluoroacetyl-L-prolyl chloride enables plasma concentrations of 10 ng/ml to be measured.[87] Antibodies have been raised to S-propranolol which do not cross-react with R-propranolol, enabling a radio-immune assay to be developed.[88]

Betaxolol enantiomers have been separated by HPLC after derivatisation with R-α-naphthylethyl isocyanate;[89] fluorimetric detection allowed quantification down to 0.5 ng/ml, and enabled the authors to show equivalent pharmacokinetics for the two enantiomers in human volunteers.

One must conclude that enantiomeric analysis of both pure drugs and body fluids is, though difficult, possible for a number of beta blockers, and these methods are getting better with time as new techniques and instruments are developed. The determination of enantiomers in plasma is rapidly becoming routine.

CONCLUSIONS

Great changes have occurred in the general perception of beta blocker isomers. Initially regarded as a mixture of active drug and inert diluent, it is now accepted that the isomers are two distinct drugs which should be treated as such, and not dosed as racemates unless there is good reason.

Differences may be seen in both pharmacology and in pharmacokinetics where recent analytical developments have enabled plasma and urine levels of enantiomers to be measured. Improved synthetic methods have been developed giving good yields of pure products, whereas when these drugs were first discovered only traditional resolution of racemates was possible.

These changes in both ideology and technology should mean that, in the future, beta blockers should be developed in a logical and efficient manner.

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Akadémiai Kiadó
Budapest, 1990

THE INDUSTRIAL SYNTHESIS OF OPTICALLY ACTIVE COMPOUNDS

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1. INTRODUCTION - CHIRALITY & BIOLOGICAL ACTIVITY

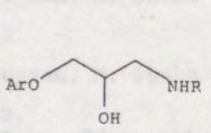
In the last two decades there has been growing public awareness and concern with regard to the exposure of man and his environment to an ever increasing number of chemicals. The benefits arising from the use of therapeutic agents, pesticides, food and feed additives, etc., are manifold. But in addition to the desired biological effects these products can exhibit undesirable side-effects. Consequently, a proper assessment of their potential benefits and risks requires a thorough knowledge of their interactions with biological organisms. Only then can their efficacy be optimized to provide maximum benefits with minimum undesirable side-effects.

This is particularly relevant where the use of chiral compounds is involved. Many biologically active substances contain one or more chiral centres and consist, therefore, of two or more optical isomers which should be considered as different substances. Thus, in general, only one of the enantiomers (the eutomer) of a racemic mixture is reponsible for the desired biological activity towards the target organism[1]. At best, the other isomer (the distomer) constitutes unnecessary ballast the removal of which would significantly reduce the chemical burden on the environment.

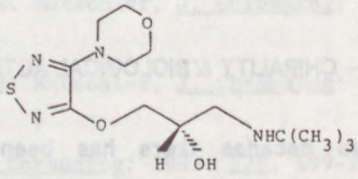
However, more often than not the distomer inhibits the desired effect of the eutomer and/or exhibits adverse side-effects. A much-quoted example is the drug thalidomide which was marketed in the sixties as a sedative and administered as a racemic mixture. Unfortunately, it was not

known at that time that although the R-enantiomer is an effective sedative the S-enantiomer is one of the most potent teratogens known.*

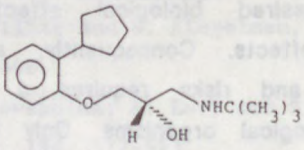
Many other less dramatic but nevertheless significant examples are known. For example, β-adrenergic blocking agents (β-blockers) characteristically have the aryloxypropanolamine structure (1) containing one chiral centre. In general, β-blocking activity resides virtually completely in the S-enantiomer. Some of these drugs have been marketed as the single, biologically active S-enantiomer, e.g. timolol (Merck, Sharp & Dohme), penbutolol (Hoechst) and levobunolol (Warner-Lambert).



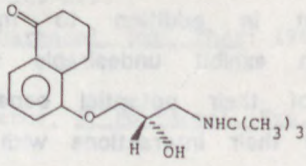
(1)



(S)-TIMOLOL



(S)-PENBUTOLOL



(S)-LEVOBUNOLOL

Similarly, it is the S-enantiomer of the α-arylpropionic acid (2) of anti-inflammatory drugs which is responsible for the desired therapeutic effect.

When one considers that biological activity is generally a result of selective binding of the substance in question to a receptor site or to the active site of an enzyme in the target organism, it is not so surprising that the two enantiomers of a racemate display completely different properties. In fact it was suggested[2] as long ago as 1956 that the potency of a drug is directly proportional to the so-called eudismic

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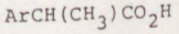
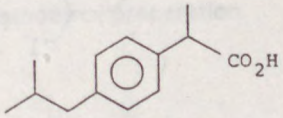
*Editor's footnote. For a comprehensive view on thalidomide cf. De Camp, W.H. The FDA perspective on the development of stereoisomers. Chirality 1989, 1: 2-6.

ratio (the activity ratio of the eutomer compared to the distomer). Simply stated, Pfeiffer's rule says that the potency of the eutomer is determined by how well it fits into the active site and if both enantiomers are able to bind it is unlikely to be an exclusive fit.

Moreover, in comparing the biological properties of two enantiomers it is not just a question of comparing their respective activities per se. For a proper comparison it is essential to study the effect of enantioselectivity on the rates of absorption, transport, accumulation and especially biotransformation (metabolism) of the two enantiomers in vivo.



(2)



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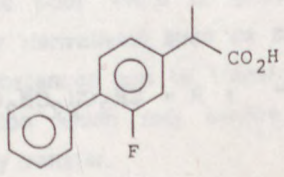
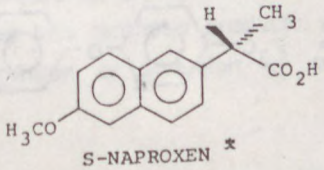
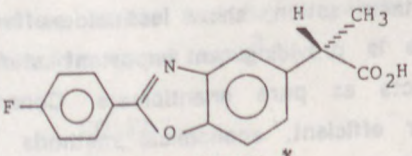


FLURBIPROFEN



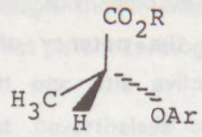
S-NAPROXEN *



S-FLUNOXAPROFEN *

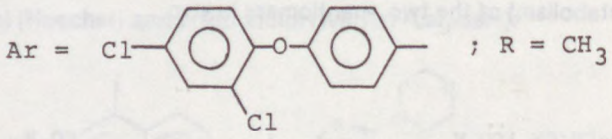
* Marketed as the single enantiomer

As noted above, this phenomenon of enantioselectivity is not restricted to pharmaceuticals but is characteristic of all biologically active agents. Another market segment which is being subjected to increasing scrutiny in this context is agrochemicals. For example, it is well-known that the herbicidal activity of the α-phenoxypropionic acid (3) group of plant protection agents resides predominantly in the R-enantiomer. Consequently, there is an increasing trend to market these compounds as single enantiomers, thereby reducing significantly their ecological impact.

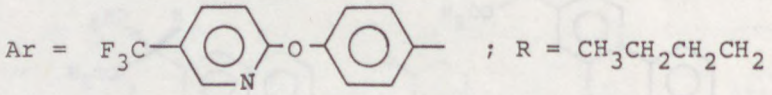


(3)

R-Diclofop-methyl (Hoechst) :



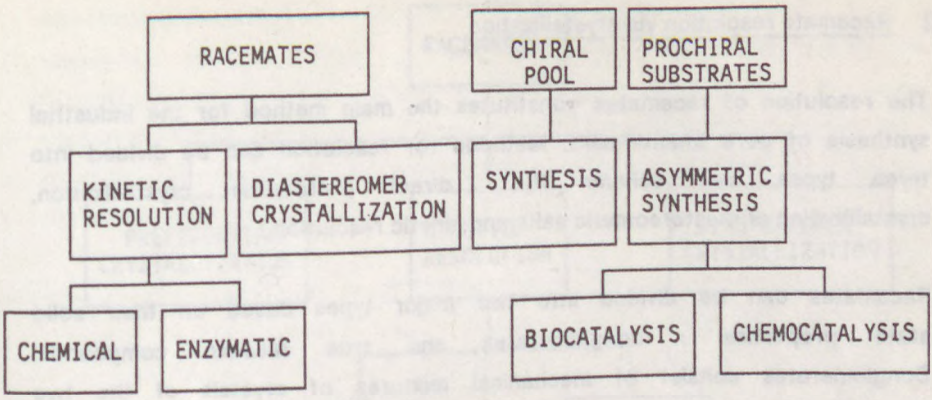
R-Fluazifop-butyl (ICI) :



In short, the ever-increasing demand for more selective drugs, pesticides, etc., which are more targeted in their action, show less side-effects and are more environmentally acceptable is providing an important stimulus to companies to market these products as pure enantiomers. Consequently, there is an increasing demand for efficient, economical methods for the industrial-scale synthesis of optically active compounds.

2. SYNTHETIC METHODOLOGY

Synthetic routes to optically active compounds can be conveniently divided into three groups, on the basis of the type of raw material used (see scheme 1).

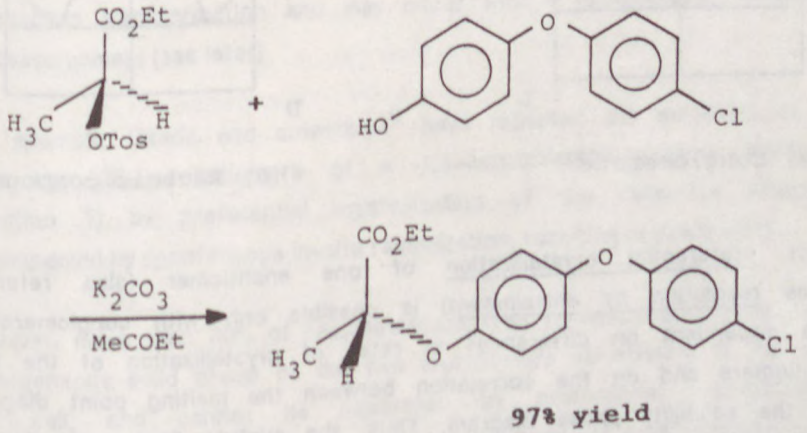


SCHEME 1. Optically active compounds : methods of preparation.

2.1 The Chiral Pool

The chiral pool refers to inexpensive, readily available natural products (and their derivatives) such as carbohydrates, amino acids and lactic acid. These substances can be transformed into synthetic products via chemical manipulation which may involve retention or inversion of configuration, or chirality transfer.

For example, the optically active α-phenoxypropionic acid herbicides referred to earlier can be prepared from the appropriate enantiomer of lactic acid as shown below[3].

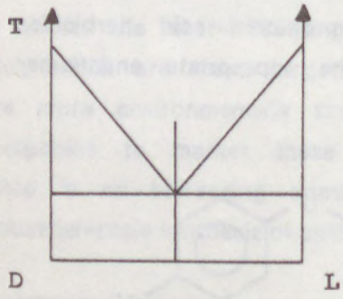


SCHEME 2. Use of the chiral pool.

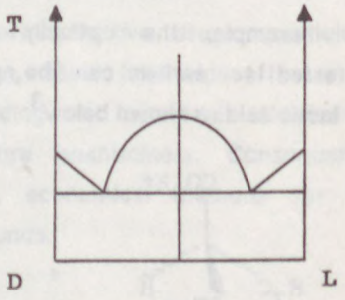
2.2 Racemate resolution via crystallization

The resolution of racemates constitutes the main method for the industrial synthesis of pure enantiomers. Methods for resolution can be divided into three types (see scheme 3) : direct preferential crystallization, crystallization of diastereomeric salts, and kinetic resolution.

Racemates can be divided into two major types based on their solid state properties : conglomerates and true racemic compounds. Conglomerates consist of mechanical mixtures of crystals of the two enantiomers. True racemic compounds, on the other hand consist of a homogeneous solid phase of the two enantiomers co-existing in the same unit cell. To which of the two classes a particular racemate belongs is readily seen by reference to its melting point diagram (a graph of melting point vs. composition for various ratios of enantiomers) which is readily determined using a differential scanning calorimeter. A conglomerate has a minimum melting point for the racemic mixture, whilst a racemic compound shows a maximum melting point for the 1:1 mixture of enantiomers (see below).

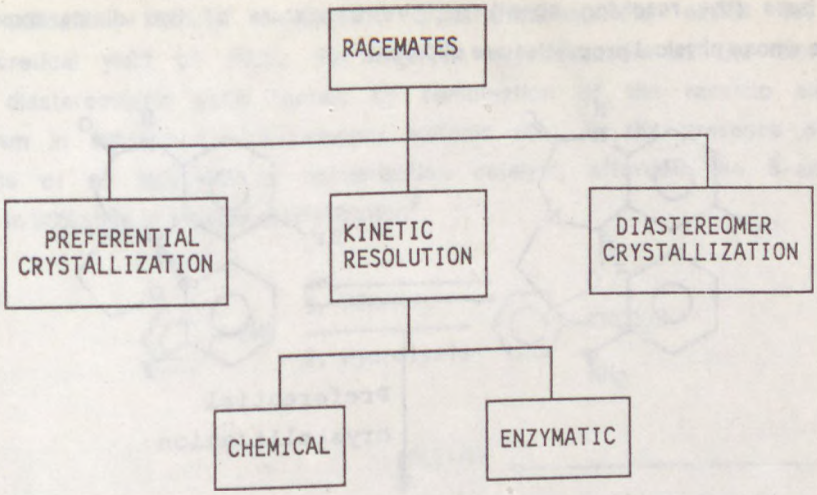


(a) Conglomerate



(b) Racemic compound

Direct preferential crystallization of one enantiomer (also referred to as resolution by entrainment) is possible only with conglomerates. It is dependent on differences in rates of crystallization of the two enantiomers and on the correlation between the melting point diagram and the solubility phase diagram. Thus, the mixture having the lowest melting point is the most soluble and for a conglomerate this is the racemic mixture.



SCHEME 3. Resolution of racemates.

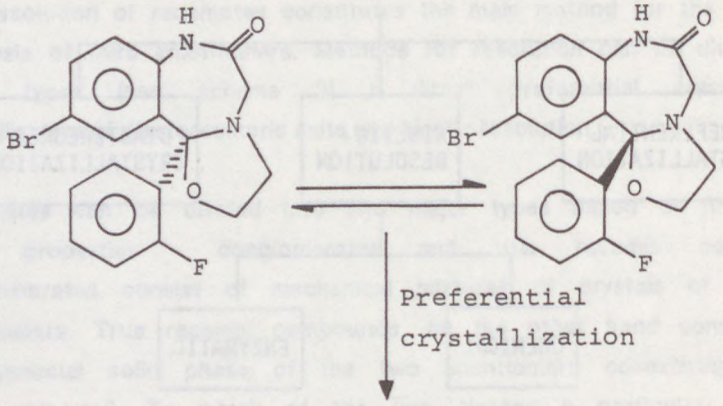
Hence, by seeding a supersaturated solution of the racemate with crystals of one enantiomer, it is possible to achieve preferential crystallization.

Resolution by entrainment is widely used on an industrial scale, for example in the manufacture of α-methyl-L-dopa[4] and chloramphenicol[5]. It is a particularly attractive method when it is accompanied by spontaneous in-situ racemization which allows for a theoretical yield of 100%. Such a process is referred to as a crystallization-induced asymmetric transformation and may occur with a racemate or a mixture of diastereomers (see later).

For example, Okada and coworkers[6] have reported the synthesis of the (R)- and (S)- enantiomers of a 1,4-benzodiazepinooxazole derivative (reaction 1) by preferential crystallization of the racemate which is accompanied by spontaneous in-situ racemization, resulting in yields >50%.

However, more than 80% of racemates are true racemic compounds, i.e. a homogeneous solid phase of the two enantiomers co-existing in the same unit cell, and cannot be separated by preferential crystallization. Racemic compounds may be separated by diastereomer crystallization which generally involves reaction of the racemate with an optically pure acid

or base (the resolving agent) to give a mixture of two diastereomeric salts whose physical properties are different.



(1)

(R)- or (S)- enantiomer

Thus, when a racemic acid A is combined with an optically pure base B, a mixture of two diastereomeric salts is formed (reaction 2) which is separated by crystallization.

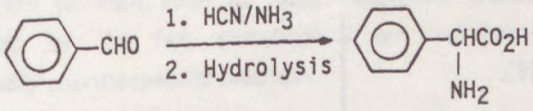
$$\underset{\text{Racemate}}{(DL)\text{-}A} + \underset{\text{Resolving agent}}{(L)\text{-}B} \longrightarrow \underset{\text{Diastereomers}}{(D)A(L)B + (L)A(L)B} \quad (2)$$

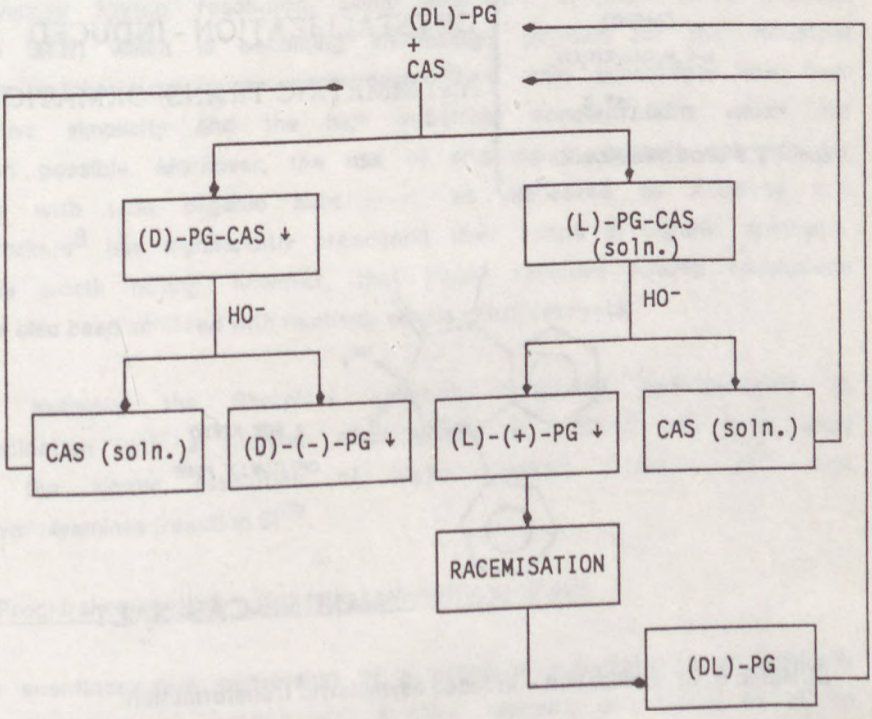
Diastereomer crystallization is widely used for the industrial manufacture of pure enantiomers. A typical example is the Andeno process (see scheme 4) for the manufacture of D-(-)-phenylglycine, an antibiotic intermediate, using optically pure camphor sulfonic acid as the resolving agent. This process is used for the production of more than a thousand tons per annum of D(-)-phenylglycine.

The theoretical, once-through yield of a resolution via diastereomer crystallization is 50%. In practice, chemical yields of >40% of material of high (>95%) optical purity, in a single crystallization, are considered to be good resolutions. In general, industrial processes involve racemisation of the unwanted enantiomer, for obvious economic reasons.

It is possible to obtain once-through yields >50% when the diastereomer remaining in solution undergoes spontaneous epimerization, often referred to as diastereomer interconversion. Such a process is referred to as a

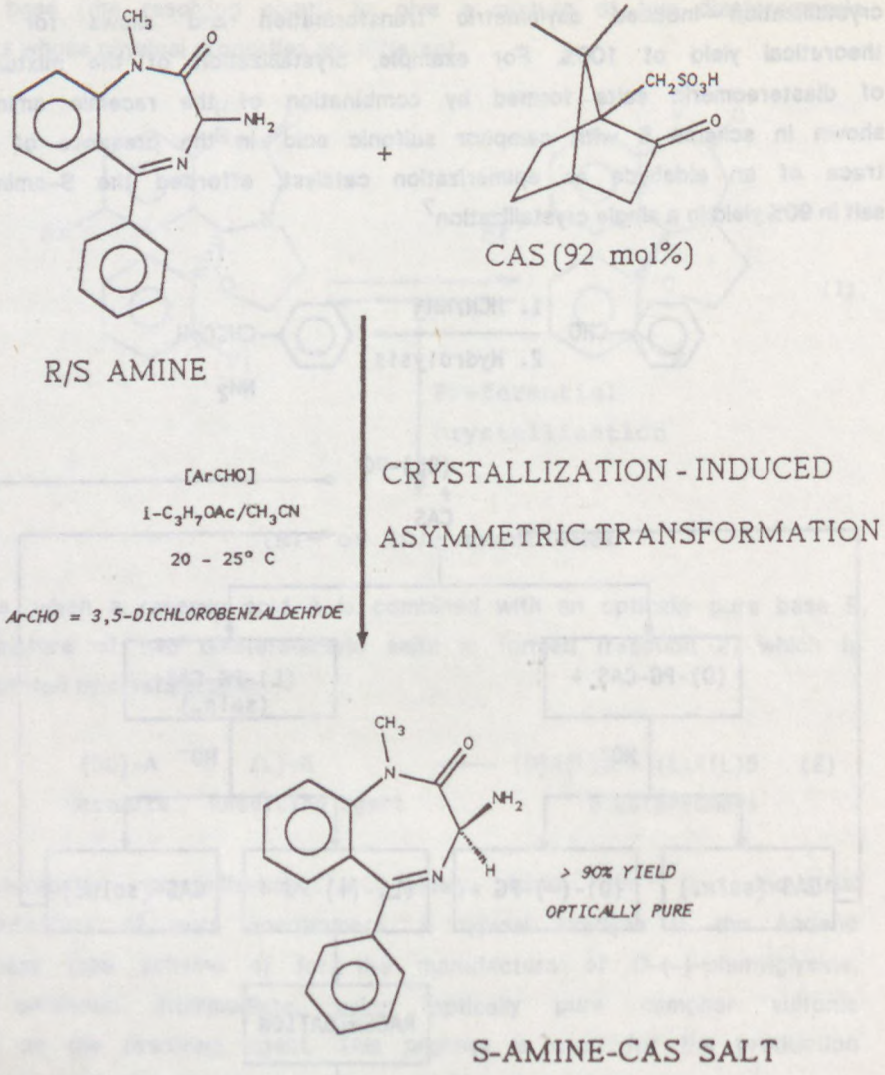
crystallization – induced asymmetric transformation and allows for a theoretical yield of 100%. For example, crystallization of the mixture of diastereomeric salts formed by combination of the racemic amine shown in scheme 5 with camphor sulfonic acid, in the presence of a trace of an aldehyde as epimerization catalyst, afforded the S-amine salt in 90% yield in a single crystallization[7].





CAS = (+)-Camphorsulfonic acid

SCHEME 4. Manufacture of D-phenylglycine DSM/Andeno.



SCHEME 5. Crystallization – induced asymmetric transformation.

2.3 Kinetic resolution of racemates

Kinetic resolution depends on the fact that the rates of reaction of two enantiomers with an optically active reagent are different. Preferably, the optically active reagent should function in catalytic quantities and may be an enzyme or a simple chemical catalyst. The so-called enantiomeric ratio E (see eq. 3) is a measure of the efficiency of a particular kinetic resolution (in this case the required enantiomer is the unreacted substrate).

$$\frac{\ln ([1-c]\,[1-ee_s])}{\ln ([1-c]\,[1+ee_s])} = E \qquad (3)$$

where c = degree of conversion of substrate

ee_s = enantiomeric excess of remaining substrate

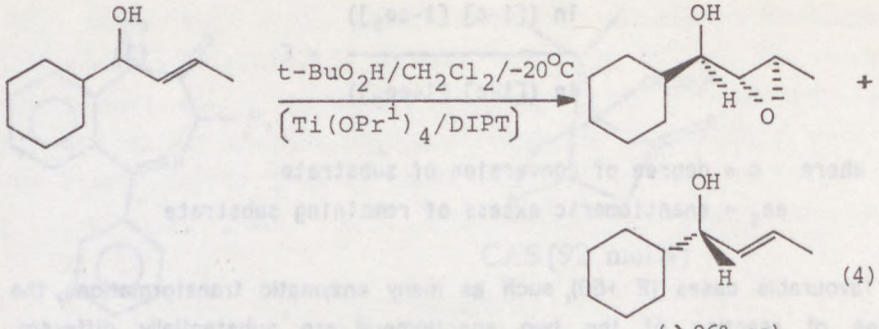
In favourable cases (E >50), such as many enzymatic transformations, the rates of reaction of the two enantiomers are substantially different, resulting in a virtually enantiospecific reaction.

Enzymatic kinetic resolution using hydrolytic enzymes is a method (see later) which is becoming increasingly popular for the industrial scale synthesis of pure enantiomers. Their main advantages are their relative simplicity and the high substrate concentrations which are often possible. Moreover, the use of enzymes in organic solvents (or even with neat organic substrates), as pioneered by Klibanov and coworkers[8] has significantly broadened their scope in organic synthesis. It is worth noting, however, that highly selective kinetic resolutions have also been achieved with relatively simple chiral catalysts.

For example, the Sharpless reagent, tert-butyl hydroperoxide in combination with a titanium alkoxide/tartrate catalyst, has been used for the kinetic resolution of allylic alcohols (reaction 4)[9a] and β-hydroxyamines (reaction 5)[9b].

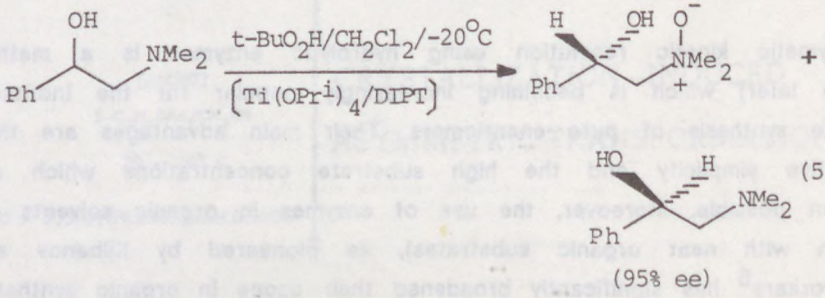
2.4 Prochiral compounds – Catalytic asymmetric synthesis

The enantioselective conversion of a prochiral substrate to an optically active product, by reaction with a chiral reagent, is referred to as an asymmetric synthesis. From an economic viewpoint the optically active reagent should function in catalytic quantities. Catalytic asymmetric syntheses can be divided into two groups based on whether they involve a simple chemical catalyst or a biocatalyst. An example of the former is the well-known Monsanto process for the manufacture of L-dopa[10] by catalytic asymmetric hydrogenation (reaction 6). An example of the latter is the Genex process[11] for the synthesis of L-phenylalanine by L-phenylalanine ammonia lyase (PAL)-catalyzed addition of ammonia to trans-cinnamic acid (reaction 7).



(4)

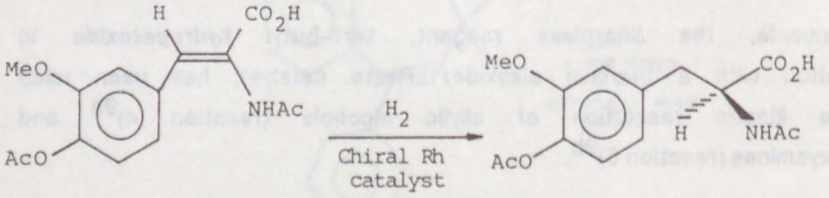
(> 96% ee)



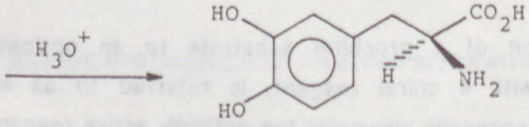
(5)

(95% ee)

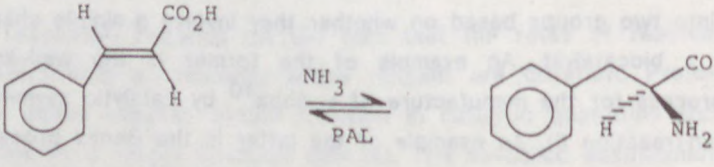
DIPT = L-(+)-Di-isopropyl tartrate



(6)



L-DOPA



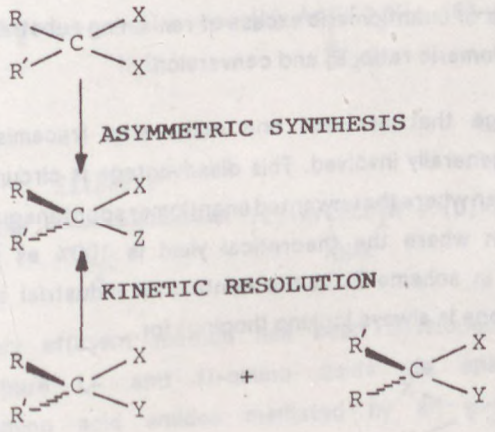
(7)

L-PHENYLALANINE

2.5 Asymmetric synthesis vs. kinetic resolution

In considering the relative merits (economics) of different methods for the synthesis of pure enantiomers the question often arises : which is more attractive, asymmetric synthesis or kinetic resolution? This is a difficult question to answer. In the first place different raw materials are involved and the economics are obviously dependent on their relative price and availability.

Superficially it would seem that asymmetric synthesis is more attractive since it has a theoretical yield of 100% compared to 50% for kinetic resolution as shown in the hypothetical example (scheme 6).



SCHEME 6. Asymmetric synthesis vs kinetic resolution.

However, further inspection often reveals that kinetic resolutions have several advantages compared to asymmetric synthesis. One important advantage is the fact that the optical purity can, in principle, be tuned to any required value by adjusting the degree of conversion as illustrated in figure 1. This is a consequence of the fact that a kinetic resolution involves, by definition, reaction of a mixture (of enantiomers), the molar ratio of which varies with conversion, whilst asymmetric synthesis pertains to the reaction of a single (prochiral) compound and enantioselectivity is independent of conversion.

Moreover, kinetic resolutions tend, in general, to be simpler chemical processes than asymmetric syntheses. On the other hand, they suffer

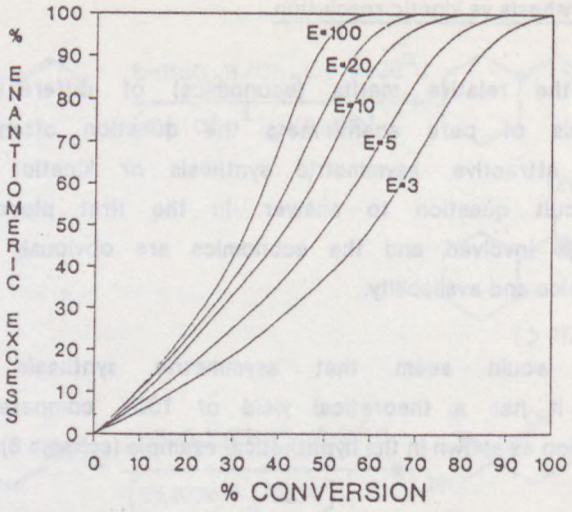
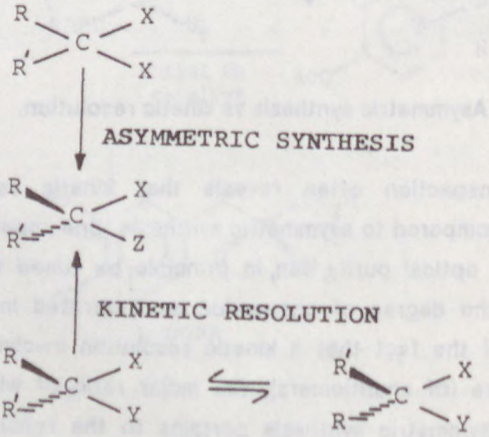


FIGURE 1. Dependence of enantiomeric excess of remaining substrate on relative rate (Enantiomeric ratio, E) and conversion.

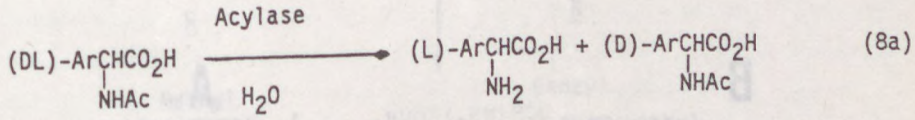
from the disadvantage that at least one extra step (racemisation of the unwanted isomer) is generally involved. This disadvantage is circumvented when conditions can be chosen where the unwanted enantiomer spontaneously racemises, leading to a situation where the theoretical yield is 100% as shown in the hypothetical example in scheme 7. In the context of industrial syntheses this is the situation which one is always looking (hoping) for.



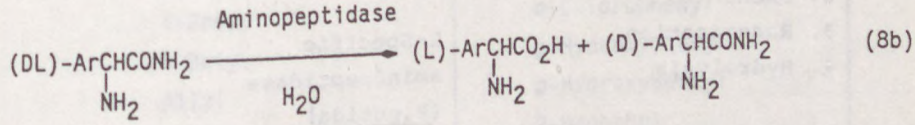
SCHEME 7. Asymmetric synthesis vs. kinetic resolution + spontaneous racemisation.

3. ENZYMATIC KINETIC RESOLUTION OF AMINO ACIDS

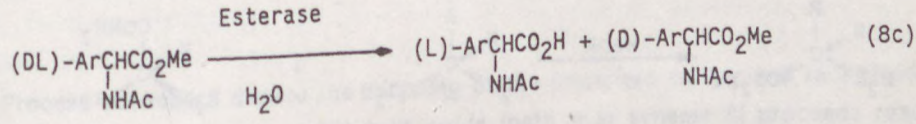
Various methods (see reactions 8a–c) have been reported in the literature[12] for the enzymatic resolution of amino acids. For example, several L–amino acids are produced on a commercial scale by acylase–catalyzed enantioselective hydrolysis of N–acetyl–D,L–amino acids (reaction 8a)[13].



(8a)



(8b)



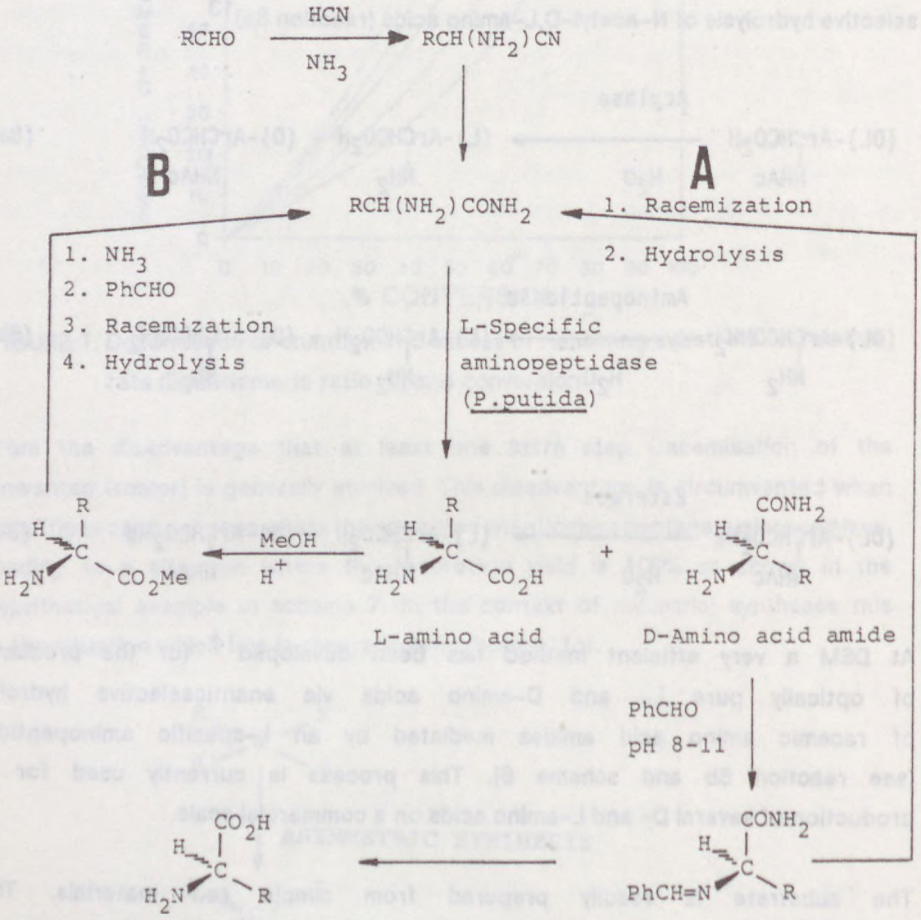
(8c)

At DSM a very efficient method has been developed[14] for the production of optically pure L– and D–amino acids via enantioselective hydrolysis of racemic amino acid amides mediated by an L–specific aminopeptidase (see reaction 8b and scheme 8). This process is currently used for the production of several D– and L–amino acids on a commercial scale.

The substrate is readily prepared from simple raw materials. Thus, Strecker reaction of the appropriate aldehyde with hydrogen cyanide and ammonia affords the corresponding amino nitrile. The latter is converted in high yield to the corresponding racemic amino amide by alkaline hydrolysis in the presence of a catalytic amount of acetone[15].

The resolution step[16] is carried out with permeabilized whole cells of *Pseudomonas putida* ATCC 12633 resulting in a virtually enantiospecific hydrolysis of the L–amino acid amide. A significant advantage of this process is its broad substrate specificity which allows for the resolution of a wide range of amino acids (see table 1). Another advantage is the

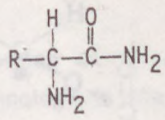
fact that the substrate is a precursor of the amino acid rather than a derivative. This means, in principle, that fewer steps are needed compared to processes which use a derivative as the substrate and involve the initial preparation of the racemic amino acid followed by derivatization.



SCHEME 8. DSM-process for the production of optically pure L- and D-amino acids.

The biocatalyst is used in soluble form in a batchwise process, thus allowing for the resolution of even poorly soluble amino acids. Recycling of the biocatalyst is in principle possible. A very simple and elegant method has been developed for the separation of the L-amino acid and D-amino acid amide products. Thus, addition of one equivalent of benzaldehyde (with respect to D-amino amide) results, surprisingly, in the formation of a water-insoluble Schiff base of the D-amino amide which is readily separated[17]. Subsequent acid hydrolysis affords the optically pure D-amino acid.

TABLE 1. Substrate specificity of the amino peptidase from *Pseudomonas putida* ATCC 12633 (selected examples).



R	R
Methyl	Benzyl
Ethyl	2-Phenylethyl
n-Propyl	Phenyl
i-Propyl	o-Chlorophenyl
i-Butyl	p-Hydroxyphenyl
Allyl	p-Hydroxybenzyl
2-Methylthioethyl	2-Naphthyl
3-Methylthiopropyl	2-Thienyl

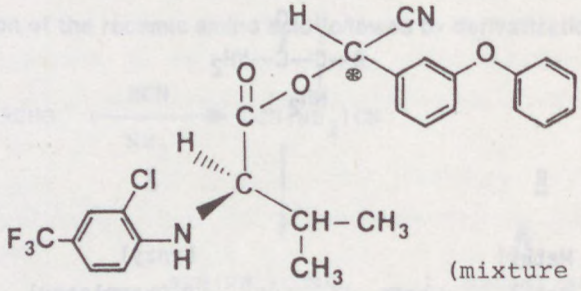
Process economics dictate the recycling of the unwanted isomer, and racemisation of the D-N-benzylidene amino acid amide (path A in scheme 8) proceeds readily under mild conditions[18]. After removal of the benzaldehyde, the racemic amino acid amide can be recycled to the enzymatic hydrolysis step.

A suitable method for the racemisation and recycling of the L-amino acid (path B, scheme 8) comprises conversion to the methyl ester followed by reaction with ammonia affording the amino amide. Addition of benzaldehyde and racemisation at pH 13 gives the DL-amino amide. Although the synthesis of D-amino acid involves overall more steps than for the corresponding L-amino acid (see following section), the operations are relatively simple and virtually quantitative yields of D-amino acids are obtained.

Both D- and L-amino acids are widely used as pharmaceutical intermediates. For example, the earlier mentioned D-enantiomer of phenylglycine is a key intermediate in the manufacture of several semi-synthetic penicillins and cefalosporins.

Interest in optically pure amino acids as agrochemical intermediates

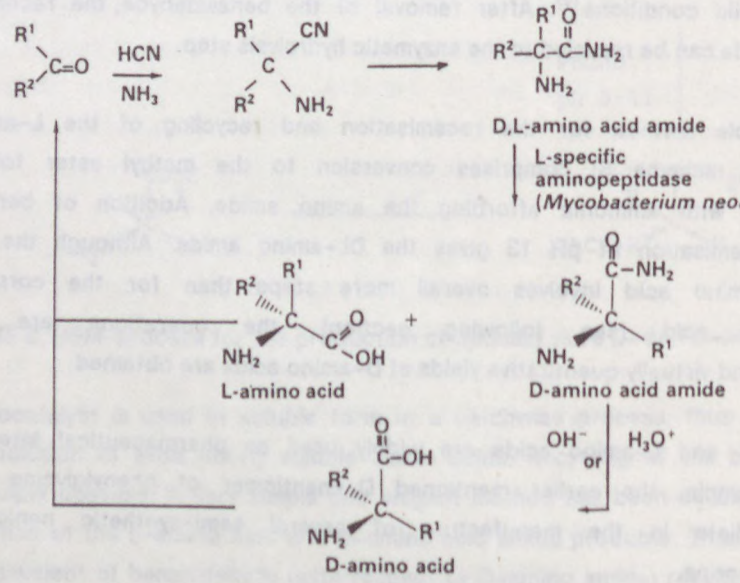
is steadily growing. For example, D-valine is used in the synthesis of fluvalinate (4), a broad spectrum pyrethroid insecticide developed by Zoecon.



(mixture of epimers)

(4)

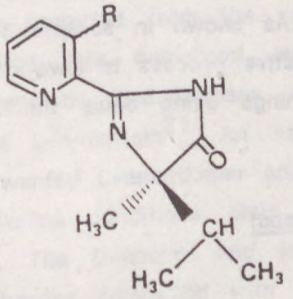
An essential structural feature for the *Pseudomonas putida* – derived aminopeptidase is the presence of an α-hydrogen in the substrate. On the other hand, optically pure α-disubstituted amino acids are also of commercial interest. To provide access to this class of amino acids a new biocatalyst derived from *Mycobacterium neoaurum* has been developed by DSM[19]. This L-specific aminopeptidase mediates the enantioselective hydrolysis of a range of α-disubstituted amino acid amides (see scheme 9). They are readily prepared by a Strecker reaction of hydrogen cyanide and ammonia with appropriate ketone.



SCHEME 9. Enzymatic method for the optical resolution of racemic α-disubstituted amino acids.

It is impossible to racemise α-disubstituted amino acids directly due to the absence of the acidic α-C-H bond. Recycling of the unwanted isomer is achieved, therefore, by conversion back to the starting ketone (see scheme 9).

A possible commercial application of an optically pure α-disubstituted amino acid accessible via this technology is the use of D-α-methylvaline amide as an intermediate for the synthesis of the biologically active form of the broad spectrum herbicide Arsenal (5) developed by American Cyanamide[20]. It can be inferred from the patent literature that the D-enantiomer is significantly more active than the racemate in certain applications.



(5)

4. FACTORS EFFECTING THE ECONOMICS OF RESOLUTION PROCESSES

Numerous factors are involved in determining the economics of resolution processes : the price and ease of recycling of the resolving agent or enzyme, the chemical, optical and volume yield of the resolution, etc. However, three general points are worthy of further explanation :

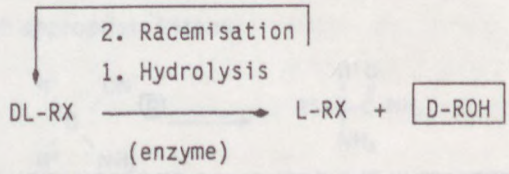
1. The ease of racemisation of the unwanted isomer.
2. Total number of steps (attractive vs.subtractive processes).
3. The position of the resolution step in the overall synthesis.

Although the ease of racemisation is obviously of paramount importance, especially with expensive raw materials, it is not generally recognized that the racemisation step is often the most difficult one in an

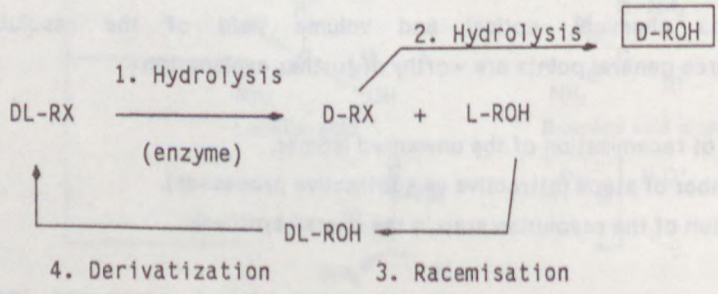
industrial resolution process. For example, racemisation of simple amines is often difficult and can be achieved only under forcing conditions, where it is accompanied by extensive decomposition.

The total number of steps is important for the economics of any process. More steps generally involve longer overall reaction times and lower volume yields (kilos per unit reactor volume per unit time). In resolution processes the total number of steps is determined by whether or not the required product is formed directly. For example, in an enzymatic kinetic resolution (see scheme 10) a derivative RX is enantioselectively hydrolysed to ROH. If the D-enantiomer is the required product and it is formed directly, we speak of an attractive process. If the L-enantiomer of ROH is formed, then we speak of a subtractive process. As shown in scheme 10, the difference between an attractive and a subtractive process is always two extra chemical steps in the latter. All other things being equal the subtractive process is always less economical.

Attractive process (two steps)



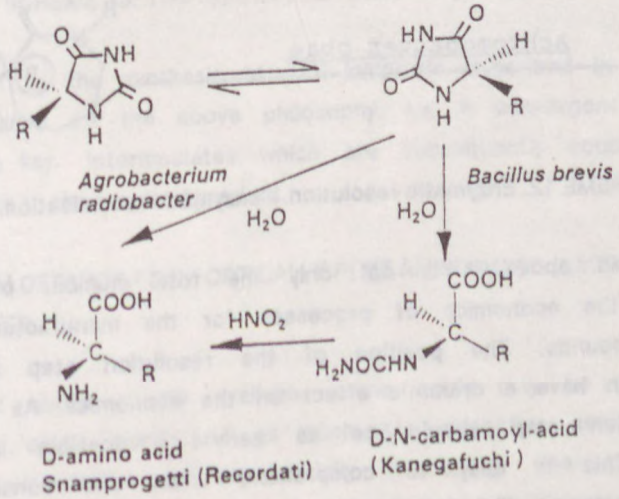
Subtractive process (four steps)



SCHEME 10. Attractive vs. subtractive resolutions.

Examination of the enzymatic resolutions (reactions 8a–c) of amino acids discussed earlier reveals that they are all attractive processes for the manufacture of L-amino acids, but subtractive for D-amino acids. Another factor which has a bearing on the economics is whether or not the substrate for the resolution step is a precursor or a derivative of the racemate. As mentioned in the previous section in connection with the DSM process fewer steps are involved when the substrate is a precursor of the racemic compound.

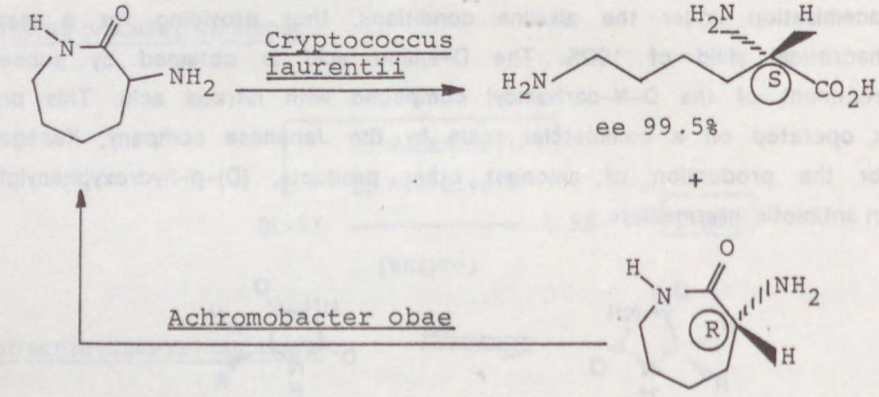
An example of an enzymatic resolution process which is "attractive" for D-amino acids, and applies what can be considered a precursor as substrate, is the hydantoinase-mediated, D-specific hydrolysis of hydantoins. Thus, racemic hydantoins, readily prepared from the corresponding aldehydes via the Bucherer – Berg reaction, are converted by microbial cells (*Bacillus brevis*), containing a D-specific hydantoinase, to a mixture of D-N-carbamoyl amino acid and L-hydantoin[21]. An additional advantage derives from the fact that the unwanted L-enantiomer undergoes rapid, spontaneous racemization under the alkaline conditions, thus providing for a maximum theoretical yield of 100%. The D-amino acid is obtained by subsequent treatment of the D-N-carbamoyl compound with nitrous acid. This process is operated on a commercial scale by the Japanese company, Kanegafuchi, for the production of, amongst other products, (D)-p-hydroxyphenylglycine, an antibiotic intermediate.



SCHEME 11. Enzymatic kinetic resolution of hydantoins.

Recordati (Italy) uses an even more elegant approach for the production of D-p-hydroxyphenylglycine on an industrial scale. The microorganism *Agrobacterium radiobacter* is able to produce both D-hydantoinase and a second enzyme, N-carbamoyl-D-amino acid amidohydrolase, which catalyzes the hydrolysis of the N-carbamoyl-D-amino acid, thus allowing for a one-step conversion in a theoretical yield of 100%.[22]

In the above examples, spontaneous racemization occurs under the alkaline reaction conditions, due to the acidic character of the C-H bond in the hydantoin ring. If such a spontaneous racemization is not feasible, one may be able to effect in-situ racemization by employing a racemase. For example, the microorganism *Cryptococcus laurentii* contains an enzyme which mediates the enantioselective hydrolysis of α-amino-ε-caprolactam to L-lysine (see scheme 12). In the presence of a second microorganism, *Achromobacter obae*, the remaining D-α-amino-ε-caprolactam undergoes a racemase-mediated racemization.[23]

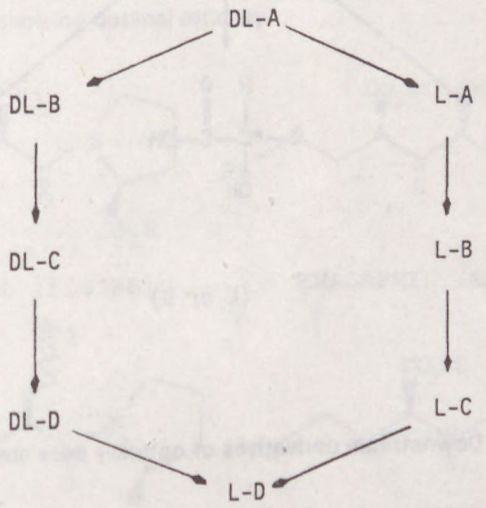


SCHEME 12. Enzymatic resolution + enzymatic racemisation.

As mentioned above, it is not only the total number of steps which determines the economics of processes for the manufacture of optically active compounds. The position of the resolution step in the overall synthesis can have a dramatic effect on the economics. As a general rule, the resolution step should be as early as possible in the overall synthesis. This is easy to comprehend when one considers that all subsequent steps will require half of the amount of reagents, solvents, reactor volume, etc., per kilo endproduct compared with the corresponding

steps performed with the racemate. In other words, in scheme 13, other things being equal, the right-hand route will be economically more attractive than the left-hand route, even though the total number of steps in both routes is the same.

Obviously, the difference in cost price will be more pronounced when the steps which are carrying 'isomeric ballast' involve the use of expensive, difficult to recycle reagents.



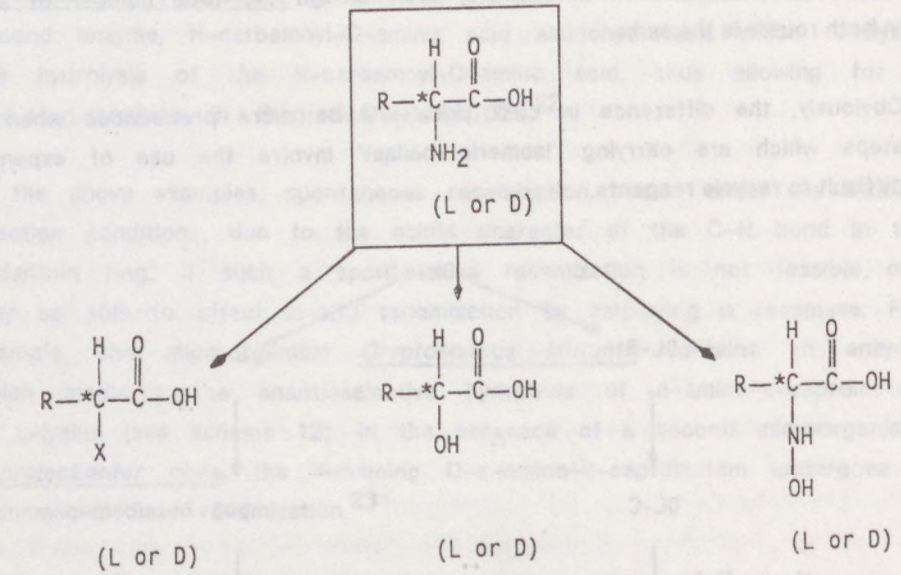
SCHEME 13. Two hypothetical resolution processes.

Our approach to the synthesis of ACE-Inhibitors described in the following section is based on the above philosophy, i.e. a convergent synthesis of optically pure key intermediates which are subsequently coupled in clean, high-yield steps to the required product.

5. DOWNSTREAM DERIVATIVES OF OPTICALLY PURE AMINO ACIDS – ACE INHIBITOR INTERMEDIATES

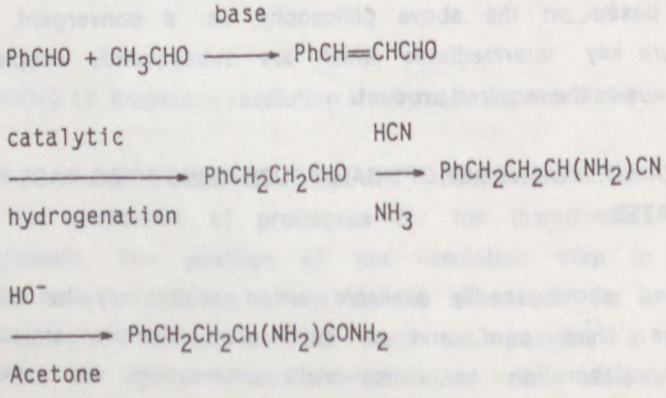
As mentioned above, readily available amino acids may be considered as part of the 'chiral pool' and as such are attractive starting materials for further elaboration to commercially interesting fine chemicals. For example, efficient methods have been developed by DSM/Andeno for the conversion of D- or L-amino acids, available from the above described

enzymatic resolution technology, to the corresponding optically pure α-hydroxy acids, α-haloacids and α-hydroxyamino acids (see scheme 14).



SCHEME 14. Downstream derivatives of optically pure amino acids.

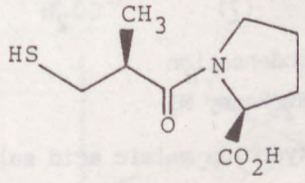
An example of an amino acid which has been successfully resolved on a commercial scale using the DSM technology is homophenylalanine. The substrate is readily prepared starting from benzaldehyde as shown in scheme 15.



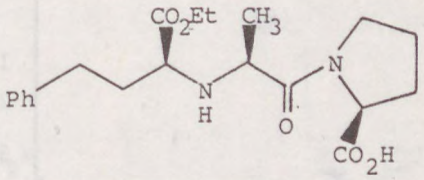
SCHEME 15. Synthesis of DL-homophenylalanine amide.

Both L- and D-homophenylalanine constitute commercially interesting starting materials for the production of a variety of Angiotensin Converting Enzyme (ACE) Inhibitors, a relatively new class of antihypertensive agents.

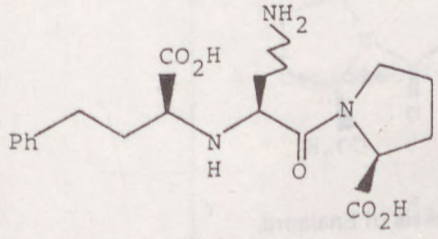
The ACE-inhibitors[24] have found wide application in the treatment of hypertension since the market introduction of the first example, Captopril by Squibb in 1981 (see scheme 16 for structure). A remarkable feature of all of these drugs is that they are all chiral and they are all marketed as a single optical isomer, i.e. they are a good example of a modern class of therapeutic agents showing optimal efficacy.



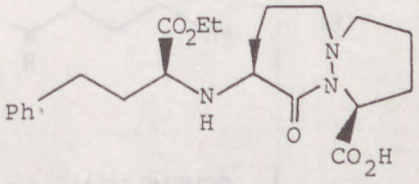
CAPTOPRIL (SQUIBB)



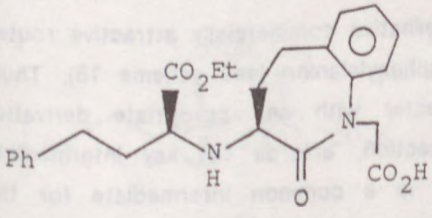
ENALAPRIL (MERCK)



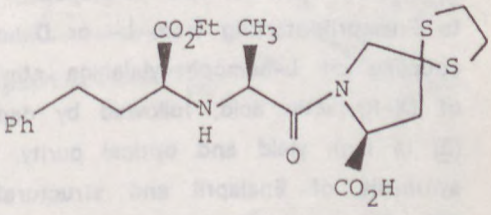
LISINOPRIL (MERCK)



CILAZAPRIL (HOFFMANN-LA ROCHE)



BENAZAPRIL (CIBA-GEIGY)

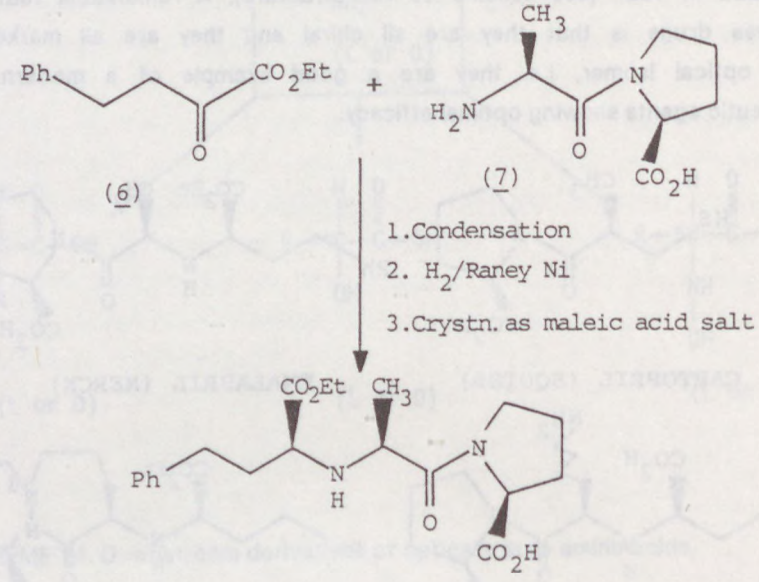


SPIRAPRIL (SCHERING-PLOUGH SANDOZ)

SCHEME 16. Structures of various ACE Inhibitors.

Subsequent to the introduction of Captopril a variety of ACE Inhibitors have been developed many of which contain the homophenylalanine moiety as a common structural feature. The prime example of this group is Enalapril developed by Merck, Sharp & Dohme and introduced in 1984. Enalapril is

currently manufactured by Merck via reductive amination of 4-phenyl-2-ketobutyric acid ethyl ester (6) with L-alanyl-L-proline (7) as shown in scheme 17. The best results were obtained using a Raney nickel catalyst which gave an 80-90% yield of a product containing 87% of the required (SSS)-diastereomer together with 13% of the (RSS)-isomer[25]. The crude enalapril was purified via crystallization of its maleic acid salt.

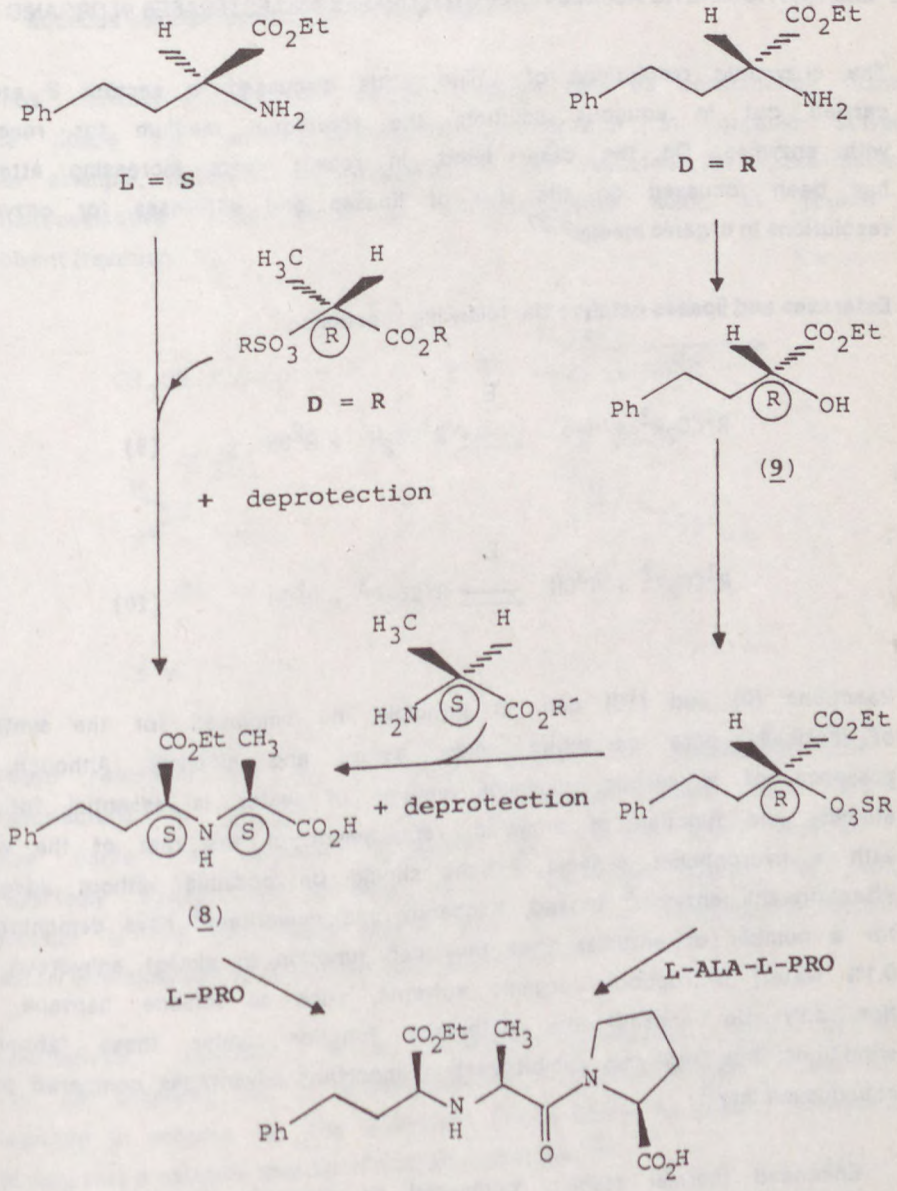


SCHEME 17. Merck synthesis of Enalapril.

At Andeno/DSM we have developed[26] alternative commercially attractive routes to Enalapril starting from L- or D-homophenylalanine (see scheme 18). Thus, coupling of L-homophenylalanine ethyl ester with an appropriate derivative of D(=R)-lactic acid, followed by deprotection, affords the key intermediate (8) in high yield and optical purity. (8) is a common intermediate for the synthesis of Enalapril and structurally related ACE-inhibitors (see scheme 16). For example, reaction of the acid chloride hydrochloride salt of (8) with L-proline affords Enalapril in excellent yield.

Alternatively, D-homophenylalanine ethyl ester can be converted selectively, without significant loss of optical activity, to the corresponding α-hydroxy compound (9). Treatment of the appropriate derivative of (9) with L(=S)-alanine ester followed by deprotection affords the key intermediate (8).

An alternative, shorter route to Enalapril, starting from D-homophenylalanine involves reaction of the appropriate derivative of (9) with L-alanyl-L-proline. This route has the advantage that it is also applicable to the synthesis of other ACE Inhibitors, such as Lisinopril, Cilazapril and Benazapril (see scheme 16 for structures) which possess the homophenylalanine moiety but are not based on the key intermediate (8).



SCHEME 18. Synthesis of Enalapril from L- or D-homophenylalanine.

All of the above mentioned routes to ACE inhibitors starting from L- or D-homophenylalanine have one common advantage : the key resolution step is early in the overall synthesis. As discussed in the preceding section this is an important factor in determining the economics of processes for the synthesis of optically active compounds in general.

6. ENZYMATIC KINETIC RESOLUTIONS WITH LIPASES AND ESTERASES IN ORGANIC MEDIA

The enzymatic resolutions of amino acids discussed in section 3 are all carried out in aqueous solution, the traditional medium for reactions with enzymes. On the other hand, in recent years increasing attention has been focussed on the use of lipases and esterases for enzymatic resolutions in organic media.[8,27]

Esterases and lipases catalyze the following reactions :

$$R^1CO_2R^2 + H_2O \xrightleftharpoons{E} R^1CO_2H + R^2OH \qquad (9)$$

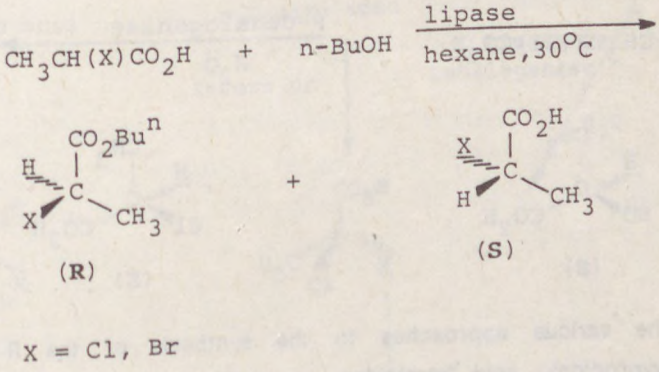
$$R^1CO_2R^2 + R^3OH \xrightleftharpoons{E} R^1CO_2R^3 + R^2OH \qquad (10)$$

Reactions (9) and (10) can, in principle, be employed for the synthesis of optically pure carboxylic acids, esters and alcohols. Although the presence of a certain minimum amount of water is essential for the stability and function of enzymes, replacement of the rest of the water with a hydrophobic organic solvent should be possible without adversely effecting the enzyme.[8] Indeed, Klinbanov and coworkers[8] have demonstrated for a number of enzymes that they can function in almost anhydrous (ca. 0.1% water) hydrophobic organic solvents, such as toluene, heptane, etc. Not only do lipases and esterases function under these 'abnormal' conditions, but they also exhibit certain important advantages compared to an aqueous medium[8] :

- Enhanced thermal stability compared to aqueous media. Reactions can often be carried out at 100°C in toluene, for example.

- The enzymes often exhibit increased substrate specificity presumably because the 'dry' enzyme has a more rigid structure.
- Reactions in organic solvents are more compatible with organic syntheses which generally involve water-insoluble substrates.
- Reactions become possible, e.g. esterification (the reverse of reaction 9) and transesterification (reaction 10) which are not feasible in aqueous solution due to unfavorable equilibria.

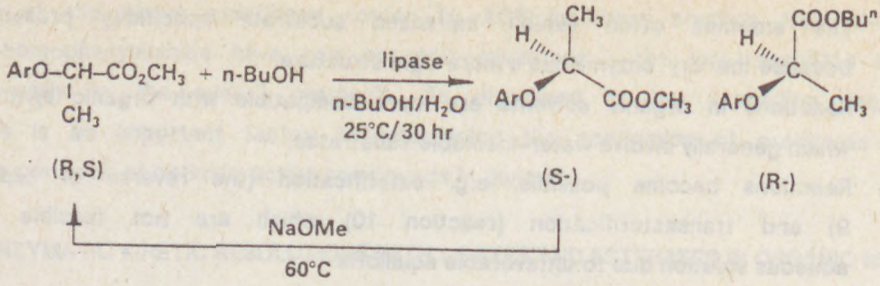
Thus, racemic mixtures of carboxylic acids can be conveniently resolved via lipase (or esterase)-catalyzed esterification in organic solvents. For example, Klibanov and coworkers[28] have reported the lipase-mediated enantioselective esterification of α-halopropionic acids in hexane as solvent (reaction 11).



(11)

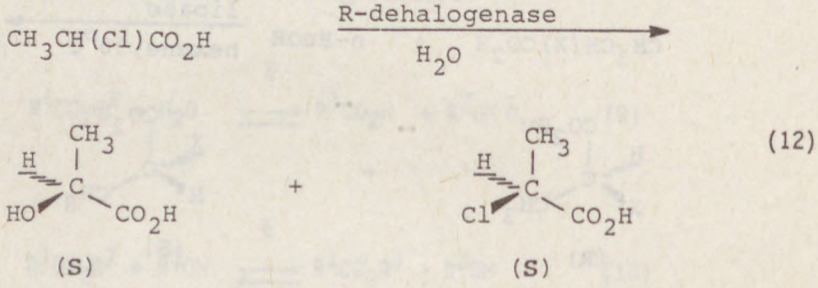
These reactions are of commercial interest in connection with the manufacture of optically active (R)-α-phenoxypropionic acid herbicides (see earlier) of general structure (3). Chemie Linz (Austria) has reportedly scaled this process up to pilot-plant scale. The required product is the (S)-α-halopropionic acid which is subsequently converted (via an inversion) to the (R)-α-phenoxypropionic acid.

Alternatively, optically active R-α-phenoxypropionic acid derivatives can be prepared by a lipase-catalyzed transesterification[30] reaction as depicted in scheme 19. The unwanted S-enantiomer is readily racemized by heating with a catalytic amount of sodium methoxide.[30]



SCHEME 19. Enantioselective transesterification.

Yet another approach involves the synthesis of (S)–α–chloropropionic acid via enantioselective, dehalogenase–catalyzed hydrolysis of the C–Cl bond as reported by ICI workers.[31] The co–product of the reaction is (S)–lactic acid since the enzyme–mediated hydrolysis occurs with inversion of configuration.



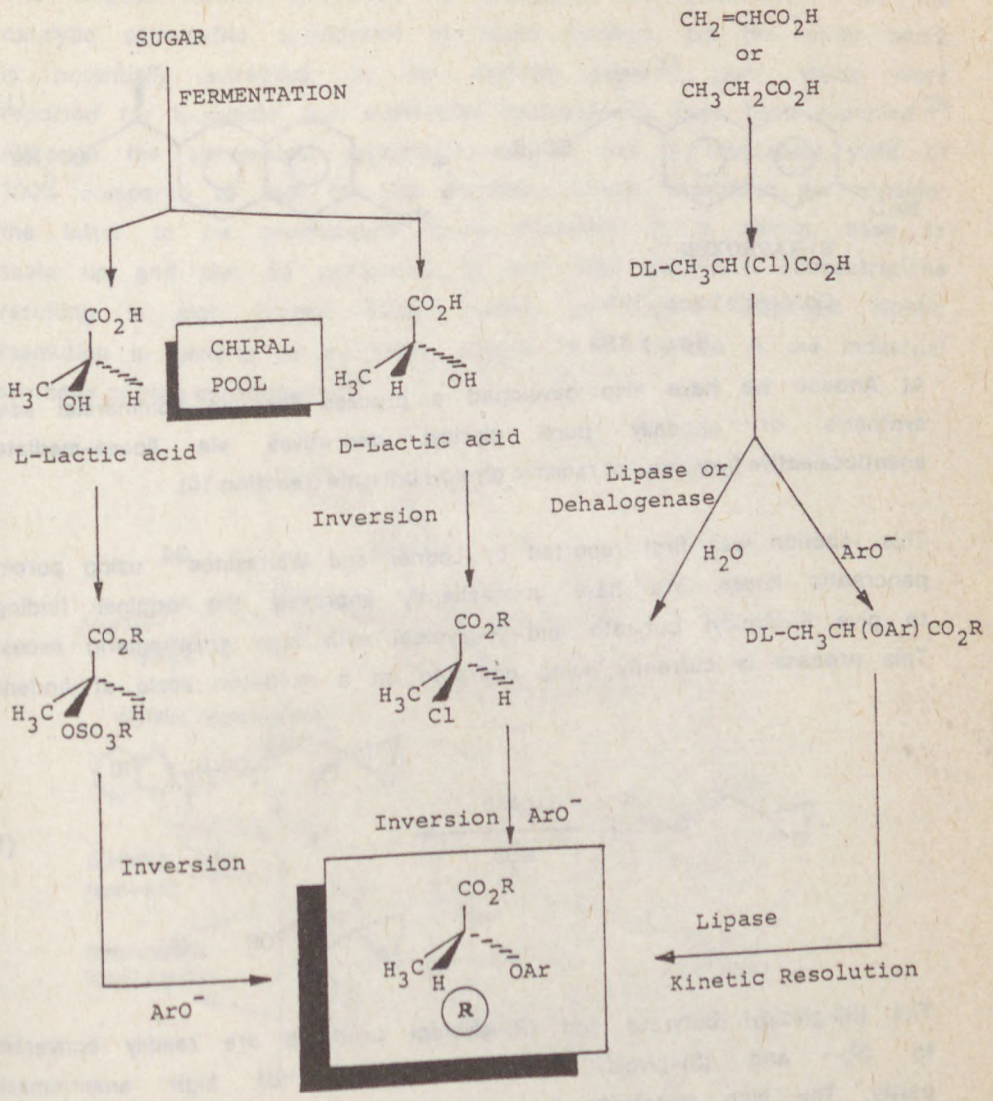
(12)

In short, the various approaches to the synthesis of the R–enantiomers of α–phenoxypropionic acid herbicides, as outlined in scheme 20, represent a good illustration of the sort of choices one is faced with in choosing an industrial synthesis of an optically active product.

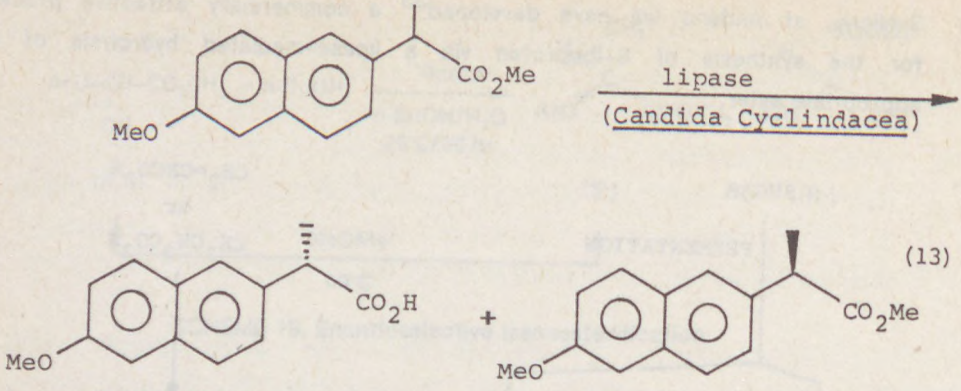
Thus, one can make use of the chiral pool, in this case represented by (D)– or (L)–lactic acid, or carry out a (kinetic) resolution at a particular stage in the synthesis.

The therapeutically active isomers of the structurally related α–aryl–propionic acid class of anti–inflammatory drugs (2) can similarly be prepared by lipase–mediated hydrolysis or transesterification of an appropriate ester. The preparation of S–naproxen by lipase–catalyzed hydrolysis of the methyl ester (reaction 13), for example has been reported by Sih and coworkers.[32]

Similarly, at Andeno we have developed[33] a commercially attractive process for the synthesis of S-Ibuprofen via a lipase-mediated hydrolysis of an appropriate ester.



SCHEME 20. Alternative routes to (R)-α-phenoxypropionic acids.



(13)

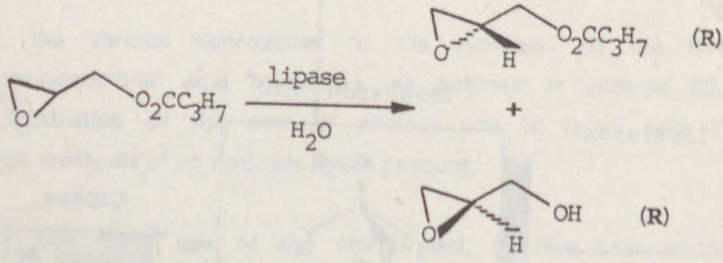
S-NAPROXEN

Conversion: 39%

ee: > 98%

At Andeno we have also developed a process for the commercial scale synthesis of optically pure glycidyl derivatives via lipase-mediated enantioselective hydrolysis of racemic glycidyl butyrate (reaction 14).

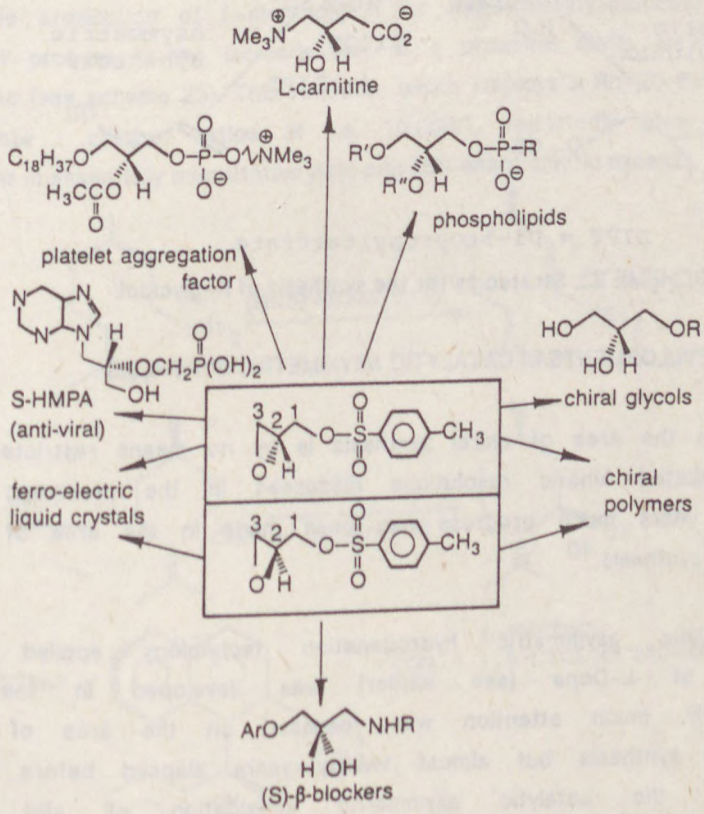
This reaction was first reported by Ladner and Whitesides[34] using porcine pancreatic lipase. We have subsequently improved the original findings to give R-glycidyl butyrate and R-glycidol with high enantiomeric excess. This process is currently being operated on a multi-ton scale at Andeno.



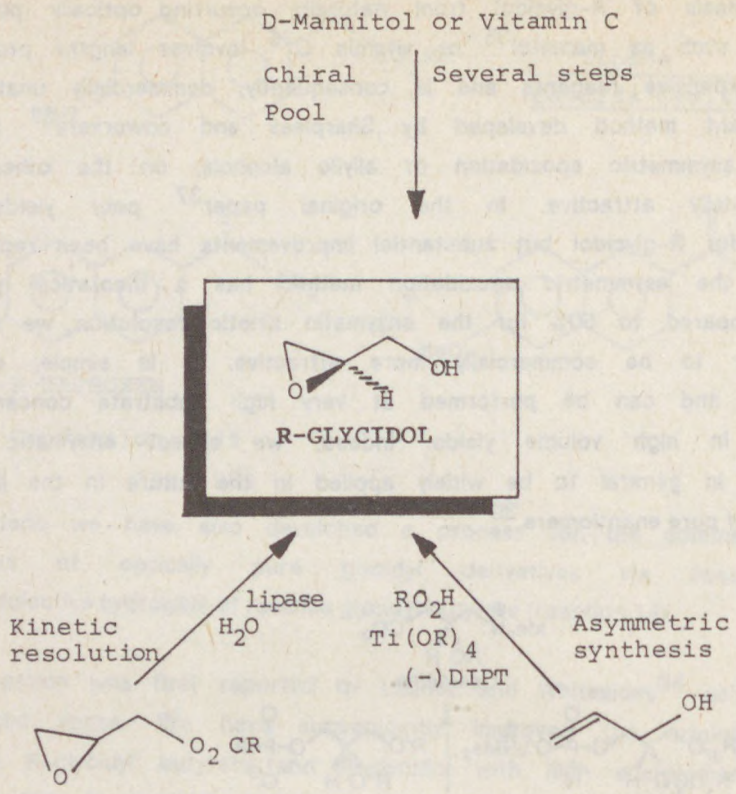
(14)

The (R)-glycidyl butyrate and (R)-glycidol products are readily converted to (R)- and (S)-glycidyl tosylate, respectively, of high enantiomeric purity. The high selectivity for nucleophilic attack at C_1 makes these glycidyl tosylates highly attractive synthons for a wide range of commercially interesting products, such as the optically active β-blockers discussed earlier, phospholipids and many more (see scheme 21). Interestingly, the key intermediate for the synthesis of S-β-blockers, R-glycidol, can be prepared using the three different strategies referred to earlier, i.e. the chiral pool, kinetic resolution or asymmetric synthesis (see scheme 22).

The synthesis of R-glycidol from naturally occurring optically pure raw materials such as mannitol[35] or vitamin C[36] involves lengthy procedures and/or expensive reagents and is, consequently, commercially unattractive. The elegant method developed by Sharpless and coworkers[37] for the catalytic asymmetric epoxidation of allylic alcohols, on the other hand, is potentially attractive. In the original paper[37] poor yields were reported for R-glycidol but substantial improvements have been reported.[38] Although the asymmetric epoxidation method has a theoretical yield of 100% compared to 50% for the enzymatic kinetic resolution, we consider the latter to be commercially more attractive. It is simple, easy to scale up and can be performed at very high substrate concentrations resulting in high volume yields. Indeed, we expect enzymatic kinetic resolution in general to be widely applied in the future in the industrial synthesis of pure enantiomers.[39]



SCHEME 21. Applications for (R)- and (S)-Glycidyl tosylates.



DIPT = Di-isopropyltartrate

SCHEME 22. Strategies for the synthesis of R-glycidol.

7. RECENT DEVELOPMENTS IN CATALYTIC ASYMMETRIC SYNTHESIS

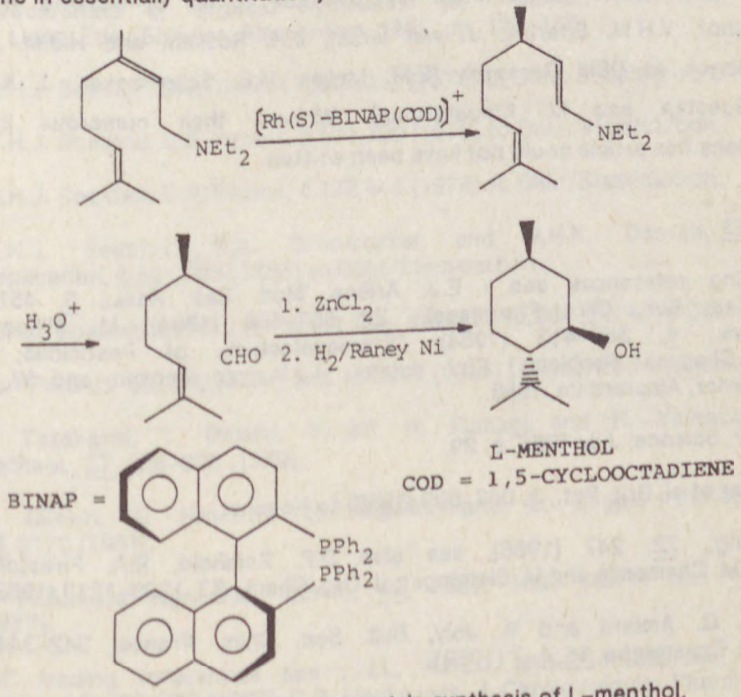
Progress in the area of chiral synthesis is by no means restricted to the enzyme-mediated kinetic resolutions discussed in the preceding section. In recent years much progress has been made in the area of catalytic asymmetric synthesis.[40]

The catalytic asymmetric hydrogenation technology applied in the synthesis of L-Dopa (see earlier) was developed in the sixties. Subsequently, much attention was focussed on the area of catalytic asymmetric synthesis but almost twenty years elapsed before Sharpless reported[37] the catalytic asymmetric epoxidation of allyl alcohols, the only example where the success of the asymmetric hydrogenation was really emulated.

Nevertheless, both the asymmetric hydrogenation and epoxidation reactions suffer from the same drawback : their scope (substrate specificity) is limited (to unsaturated-α-acylamino carboxylic acids and allylic alcohols, respectively). There is still a definite need, therefore, for chiral catalyst systems with a broader substrate specificity, which are able to mediate the asymmetric hydrogenation, oxidation, carbonylation and isomerisation of relatively simple molecules.

Recently, very promising results have been reported by Noyori and co-workers[40,41] who have employed chiral metal-Binap complexes in catalytic isomerisations and hydrogenations. These systems exhibit very high activities (substrate catalyst ratios often >1000) and enantioselectivities (>95%). Moreover, they appear to have a broad scope and are effective with relatively simple, largely unfunctionalized substrates.

This technology has reportedly been commercialised by Takasago in a new process for the production of L-menthol[42]. The key, chirality-inducing step in this elegant process is the isomerisation of a prochiral allylic amine to a chiral enamine (see scheme 23). This reaction, which employs a Rh-(S)-Binap catalyst at substrate : catalyst ratios of ca. 10.000:1, reportedly gives the required enamine in essentially quantitative yield and 98% enantiomeric excess.



SCHEME 23. Catalytic asymmetric synthesis of L-menthol.

8. CONCLUDING REMARKS

Revolutionary advances are currently being made in the industrial synthesis of optically active compounds. These developments are largely stimulated by the growing awareness of the importance of chirality in conjunction with biological activity.

With regard to the question of which method is the most economically attractive, e.g. enzymatic kinetic resolution, catalytic asymmetric synthesis or classical resolution via crystallization, there is no simple, all-encompassing answer. Numerous factors are involved[43] in determining the economics of different routes to a particular product and the method of choice will obviously vary from one product to another. One generalisation, however, usually holds true and that is : the earlier in the synthesis the chirality is introduced the better. Finally, another general conclusion can be drawn : with the steadily growing arsenal of effective methods at our disposal there is no longer any excuse for marketing such products as racemic mixtures.

9. ACKNOWLEDGEMENTS

The author wishes to acknowledge his gratitude to his co-workers at Andeno (L.A. Hulshof, V.H.M. Elferink, J. van Iersel, J.H. Roskam and H.J.M. Zeegers) and colleagues at DSM Research (E.M. Meijer, H.E. Schoemaker, J. Kamphuis, W.H.J. Boesten and M. Kloosterman). Without their numerous innovative contributions this article could not have been written.

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Close by the imposing Dôme des Invalides and modestly hiding behind the walls of the surrounding garden, Musée Rodin opens from a quiet street in Paris. You may even miss the entrance if you are not determined to find it. Once inside, however, you are fascinated by a different world.

One of my visits there coincided with a rare event. A blind visitor was led around by a lady. In order to perceive the forms, he was allowed to touch the sculptures. He did this

with each one for a long time in careful concentration. Being a witness by chance, my first impression was to appreciate French tolerance, but soon I became quite moved by the sight of his fumbling hands struggling for perception. I left him with the selfish feeling of being rich and cast exultant glances around to test my sight.

In another room I stayed before one of my favorite works of Rodin, the Cathedral. I had always liked it for its simplicity: for the church's nave, pillars and arches, and all the

grandeur expressed by human hands. Still being affected by seeing the blind man, I thought that he would have an easier job in perceiving these hands than more complex figures such as a bust. No sooner had I recalled the blind man's hands than I became astounded, staring at the statue and ignoring everything around. Realizing that the blind man would recognize something overlooked by myself, I felt as if a secret message were reaching me; a message openly encoded in the statue by the artist himself: "Man, don't you see that the sculpture is composed of two right hands? They belong to two people who hold them together with a single will. Don't you see that the supporting strength of the gothic arch comes from human collaboration?"

I was slow to recover and then felt grateful to the blind man who had unintentionally taught me a lesson. Handedness, though obvious to the touch, may easily pass unnoticed by the eye.

Years later I ran across an attractive album of Rodin's works which gave the following description of the Cathedral: "... the idea came to him one day in 1908 of representing the high pointed naves by two tapering hands joined in a gesture of prayer." Well, not quite that. But the author of the text had excuses. Certainly, he had never seen a blind man interested in sculpture. And, of course, blind people are not employed as referees.

The Editor

AUTHOR INDEX

820.-

This book is the result of a joint venture offering competent treatment of a range of subjects in which the structures of chiral molecules serve to give the basis of further considerations. Finding a problem is the beginning of a scientific study, solving the problem is the aim. Between the two, usually there is much work to be done leading to a pivotal point where the problematic turns to the obvious; where arguments which have appeared controversial, become now reconciled. This turning point is a marvelous moment for the human mind. Since biblical times, from the construction of the Tower of Babel, the ordinary state of human affairs is confusion. Understanding is a real achievement, an intellectual wonder, the reward of scientific struggles.

This book can promise no less than wonders of understanding related to chiral molecules. The authors, many of them being the best in their subjects, offer a hand to the reader and help him/her ascend high peaks in the area, opening new vistas over as yet unexplored fields.

The book will be of interest to students, as well as to both academic and industrial staff, who work in chemistry, biochemistry, pharmacy or pharmacology, and are interested in drug R&D.

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