A Thesis

entitled

"An Investigation of Enantiomer Resolution by Gas Chromatography. Application in Drug Metabolism Studies."

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Certain sections of the work described in this thesis have been published :

1. New derivatives for gas-phase analytical resolution of enantiomeric alcohols and amines.
   C.J.W. Brooks, Mary T. Gilbert and J.D. Gilbert.
   Anal. Chem., 1973, 45(6), 896-902.
2. Determination of configurations by gas chromatography.
   C.J.W. Brooks, J.D. Gilbert and M.T. Gilbert,
   Mass Spectrometry in Biochemistry and Medicine,
   ed. A. Frigerio and N. Castagnoli, Raven Press,
   New York, 1974, pp. 379-384.
3. Studies of urinary metabolites of (2-(4-isobutyl-phenyl)propionic acid by gas chromatography-mass spectrometry.
   C.J.W. Brooks and M.T. Gilbert,
   J. Chromatog., 1974, 99, 541-551.
4. Gas phase analytical resolution of enantiomeric amines as diastereomeric amides. Gas chromatography-mass spectrometry of α-phenylbutyramides, α-phenylpropionamides and α-chlorophenylacetamides.
   M.T. Gilbert, J.D. Gilbert and C.J.W. Brooks,
   Biomedical Mass Spectrometry, 1974, 1, 274-280.

CONTENTS

SUMMARY

The use of gas chromatography for the analytical resolution of optical enantiomers provides a means of assigning configurations, on a correlative basis, to small, and often impure, samples of biological origin. This problem has previously been approached in two ways, viz : direct resolution of optical enantiomers on chiral stationary phases, or the employment of chiral reagents to form diastereomeric derivatives which have been separated on conventional columns. In the present work, the latter approach was employed.

It was considered that conferment of sufficiently distinctive chromatographic properties would depend on the enhancement of conformational differences between diastereomers. Accordingly, it seemed likely that reagents in which the chirality and the functional group were embodied in a rigid molecular skeleton would be useful as chromatographic resolving agents. On the basis of this hypothesis, a study of the suitability of terpenoid acids for the resolution of enantiomeric alcohols and amines was initiated. The two acids examined were the sesquiterpenoid drimanoic acid and the monoterpenoid chrysanthemic acid. Many good separations were in fact observed for a wide range of enantiomeric alcohols, amines and amino acid methyl esters. The results achieved with these derivatives on columns packed with a non-selective stationary phase (SE-30) are comparable with, or in many cases superior to, those reported

with other chiral reagents on capillary columns.

An observation that the α-phenylbutyryl derivatives of (R)-α-phenylethylamine were well separated on conventional packed columns prompted an investigation into the use of α-substituted phenylacetic acids as gas chromatographic resolving agents for amines and amino acid methyl esters. Within the series studied, a resolution was achieved for every enantiomeric pair as their amides derived from at least one, and in many cases all of the chiral phenylacetic acids.

The mechanisms involved in the resolution are still open to speculation, but examination of the separations obtained within a series of related diastereomeric amides indicated some trends. In general, the degree of resolution achieved increased with the size of the substituent on the chiral acid centre (i.e. R = Me < Et < iPr). Introduction of a more polar substituent (as in α-methoxyphenylacetic acid) produced resolutions comparable to those obtained with the isopropylphenylacetic acid but some effects which could be attributed to the increased polarity of the substituent were noted. The derivatives of α-chlorophenylacetic acid showed satisfactory resolutions, but this reagent was judged unsuitable because of racemisation during acylation.

All the derivatives were easily prepared, in good yield, without racemisation (except for the α-chlorophenylacetamides). The diastereomers were thermally and chromatographically stable, as shown by analysis using

combined gas chromatography-mass spectrometry (GC-MS). The considerable degree of regularity observed in the elution order of these derivatives rendered the correlative assignment of configuration a feasible proposition.

The techniques developed during the examination of enantiomer resolution were subsequently applied to a study of the *in vivo* metabolism of the oral anti-rheumatic drug (RS)-2-(4-isobutylphenyl) propionic acid ("ibuprofen") in humans. Four metabolites, resulting from oxidation of the isobutyl side chain, were identified by GLC and GC-MS.

Gas chromatographic resolution of diastereomeric amides formed with (R)-(+)-α-phenylethylamine showed that the excreted drug was enriched in the (S)-(+)-enantiomer. Similarly, two of the hydroxylated metabolites were shown to be enriched in the (S)-form. Synthesis of one of the minor hydroxylated metabolites, 2,4'-(1-hydroxy-2-methylpropyl) phenylpropionic acid, allowed complete characterisation of the metabolite. Separation of the enantiomeric alcohols as esters of (R)-(-)-α-phenylbutyric acid indicated some stereoselectivity in the metabolic hydroxylation.

A brief examination of the metabolism of the aldosterone antagonist, spironolactone was also undertaken. Several drug-related components were excreted as conjugates in the urine of patients receiving treatment. Structures were proposed for nine of these on the basis of the data obtained on examination by TLC, GLC, and GC-MS. Some of the metabolites contained oxidised sulphur functions at C-7 which

proved thermally labile, rendering complete structural identification of small samples difficult. Isolation of more material under mild conditions should enable confirmation of the structures which have been tentatively proposed.

Section 1. Introduction

1.1. Stereochemical aspects of metabolism

1.1.1. Introduction

Metabolic processes are controlled by the stereochemical requirements of the enzyme systems which have evolved through processes of natural selection.[1,2] Most foreign compounds are oxidised or reduced by the "drug-metabolising" enzymes, associated with the smooth endoplasmic reticulum of liver cells,[3-6] although small amounts of the enzymes are also located in those organelles of the gastro-intestinal tract, kidneys, lung,[7] and placenta.[8] The most common foreign compound biotransformations are probably oxidations catalysed by "mixed function oxidases"[9] or "mono-oxygenases"[10], which require a reducing agent (NADPH), atmospheric oxygen and cytochrome P-450.[11] (A phrase which encompasses several enzyme systems.) These enzymic reactions include aromatic hydroxylation, oxidation of aliphatics, and alkyl side-chains, deamination, N-oxidation, sulphoxide formation, N-dealkylation, and O-dealkylation[12]. Stereochemical aspects of these metabolic processes can be divided into three types.[13] When the two enantiomers of an asymmetric substrate are metabolised at different rates, without the introduction of a subsequent asymmetric centre, the reaction shows substrate stereoselectivity; when an asymmetric centre is created in an achiral molecule, and the two stereoisomeric products are formed at different rates, product stereoselectivity is displayed and when an asymmetric substrate is transformed into diastereomeric

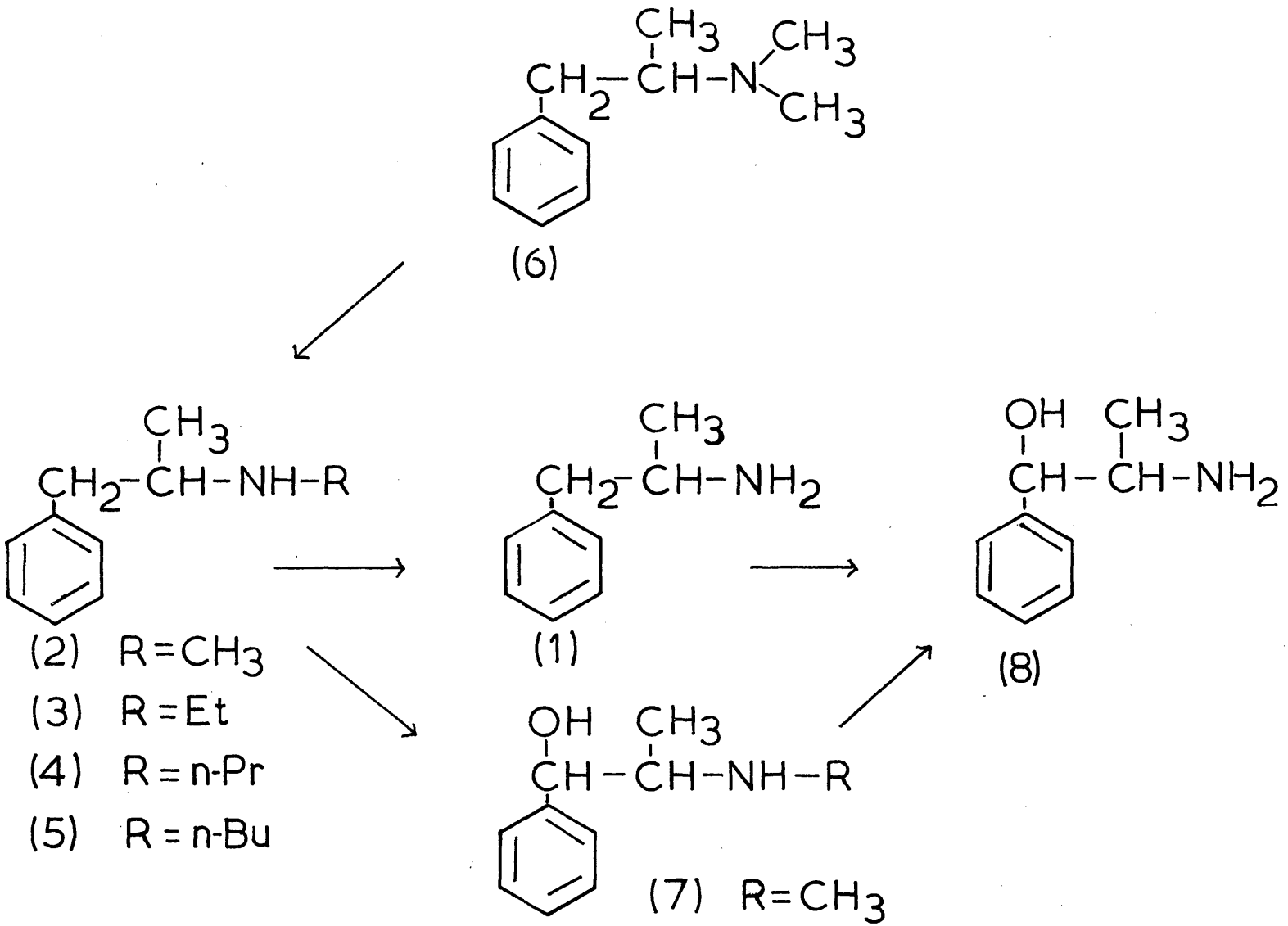


Fig. 1. Metabolic relationship between amphetamines and ephedrines.

products at different rates both substrate and product stereoselectivity are involved.

1.1.2. Substrate stereoselectivity

Amphetamines (Fig. 1)

The metabolism of amphetamine (1) has been extensively studied in man[14-21] and various animals[14,15,22-25] both in vivo and in vitro.

The major route of metabolism in man is deamination,[15] but α-C-oxidation,[14,15,20] and hydroxylation of the aromatic ring also occur. Recovery of the unmetabolised amphetamine indicated excretion of an excess of the (R)-(-)-enantiomer over the (S)-(+)-form when either the racemic drug,[16] or individual enantiomers[17,18] were ingested. Gunne,[19] by gas chromatographic analysis of the excreted amphetamines as N-trifluoroacetyl-(S)-(-)-prolyl derivatives, found that approximately equal amounts of each enantiomer were excreted during the first twelve hours after ingestion of racemic drug, but subsequent samples contained decreasing amounts of (S)-(+)-amphetamine. These results indicated some stereoselective metabolism of the (S)-(+)-enantiomer. The stereoselectivity was shown to be in the deamination process,[15] since the benzoic acid production from (-)-amphetamine was less than from either (+)-amphetamine or the racemic mixture, with little difference being observed in the other metabolism routes.

Considerable species variation has been observed[26] both

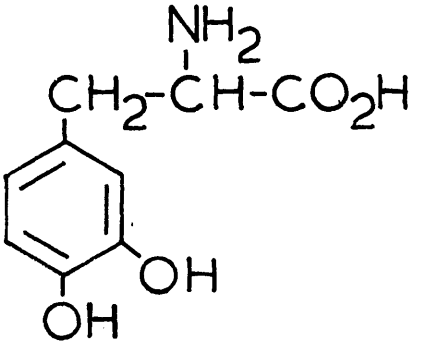
in mode of metabolism and in the stereoselectivity encountered. The results obtained in several species are summarised in Table 1.

The *in vivo* metabolism of *N*-methylamphetamine (2) showed stereoselective demethylation of the (*S*)-(+)-enantiomer.[19,27,28,30] The *N*-alkyl chain length, however, affected the metabolism since the (+)-isomers of methyl- and ethyl-amphetamine (3) were *N*-dealkylated more rapidly than their (-)-enantiomers[29] but (-)-n-propyl-amphetamine (4) was *N*-dealkylated more rapidly than the (+)-isomer.[31] Also, the total metabolism of (+)-methyl-, ethyl-, and n-propyl-amphetamine was greater than that of the corresponding (-)-isomers but there was no difference in the total metabolism of the (+)- and (-)-n-butyl-amphetamine (5). Similarly, under conditions of acidic urinary pH, (+)-dimethylamphetamine (6) was *N*-dealkylated to (+)-methylamphetamine to a greater extent than the (-)-isomer.[32] The (-)-enantiomer produced larger quantities of the *N*-oxide than the (+)-dimethylamphetamine, but this was probably due to the faster removal of the (+)-isomer by dealkylation.

Studies on the *in vitro* demethylation of the four diastereomeric ephedrines (7) using male rabbit 78,000 x *g* hepatic preparations, showed that (1*R*, 2*S*)-(-)-ephedrine and (1*S*, 2*S*)-(+)-pseudo-(ψ)-ephedrine were *N*-demethylated at rates 15-31% greater than their respective enantiomers.[33] These results suggest that the rate of demethylation depends on the configuration at the 2-position. (-)-Ephedrine was

Table 1. Species variation in stereoselectivity of amphetamine metabolism

Species	Stereoselectivity (Rate of metabolism)	Route	Ref.
Rabbit	(R)-(-)- > (S)-(+)-	Deamination	15,23,24
Guinea pig	(S)-(+)- > (R)-(-)-	Deamination	14
Rat	(S)-(+)- ≡ (R)-(-)-	-	14,15
Man	(S)-(+)- > (R)-(-)	Deamination	14,15,16,17,18,19
Swine	(S)-(+)- ≡ (R)-(-)-	-	25
Cattle	(S)-(+)- ≡ (R)-(-)-	-	
Horse	(S)-(+)- > (R)-(-)-	Unknown	
Sheep	(S)-(+)- > (R)-(-)-	"	
Rhesus monkey	(S)-(+)- ≡ (R)-(-)-	-	15
Mouse	(S)-(+)- ≡ (R)-(-)	-	



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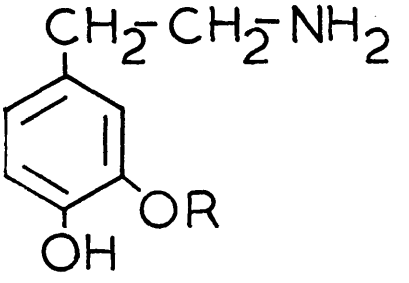
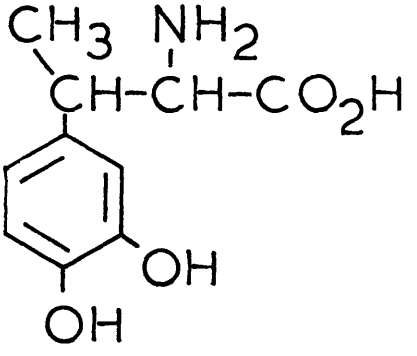


(10) R=H

(11) R=Me



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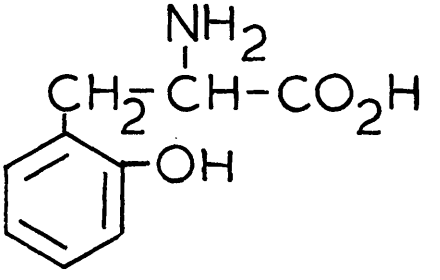

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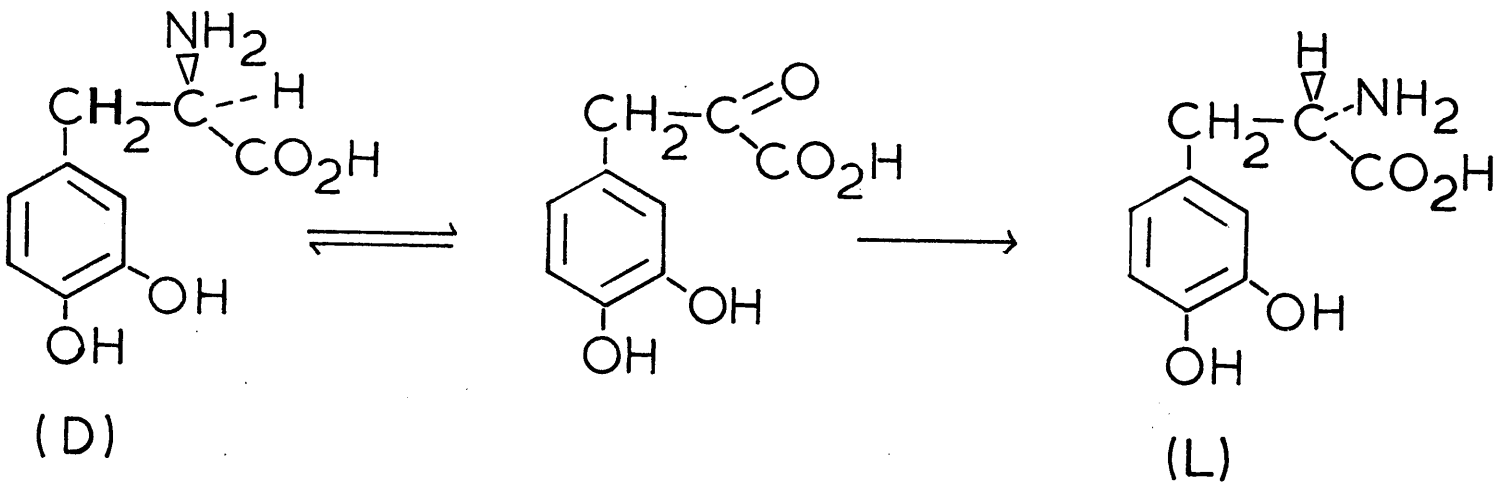


Fig. 2. Proposed metabolic interconversion of (D)- and (L)-Dopa.

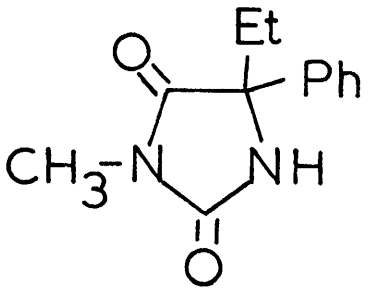
also found to be deaminated faster than the (+)-enantiomer,[34] and since (-)-ephedrine was deaminated to a greater extent than (-)-nor-ephedrine (8), the stereoselectivity was probably not due to different rates of N-dealkylation.

Dopa and related compounds

The metabolism of (D)-dopa (3,4-dihydroxy-phenylalanine) (9) by isolated perfused rat liver was slower than that of (L)-dopa in all respects except in the formation of the 3-methyl ether.[35] After administration of ^{14}C-(D)-dopa radioactivity was detected in the bile due to the presence of dopamine (10) and 3-O-methyl dopamine (11). Since (D)-dopa is not a substrate for dopa decarboxylase,[36] these results were explained by deamination of (D)-dopa by (D)-amino acid oxidase to give 3,4-dihydroxyphenylpyruvic acid which was then transaminated to (L)-dopa (Fig. 2). These reactions, were, however, minor compared to the decarboxylation of (L)-dopa.

The metabolism of α-methyldopa (12) also showed stereoselectivity for the (L)-form,[37] the metabolites being free and conjugated α-methyl dopa, free and conjugated 3-O-methyl-α-methyldopa, α-methyldopamine, 3-O-methyl-α-methyldopamine and 3,4-dihydroxyphenylacetone.

The metabolism of o-hydroxy-phenylalanine-2-^{14}C (13) was studied in rats.[38] Both (D) and (L) forms gave the same metabolites, mainly o-hydroxyphenylacetic acid and o-tyramine, but injection of the (D)-isomer produced more unchanged material due to slower metabolism. In vitro



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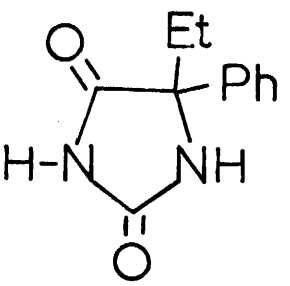

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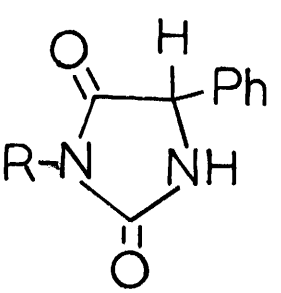
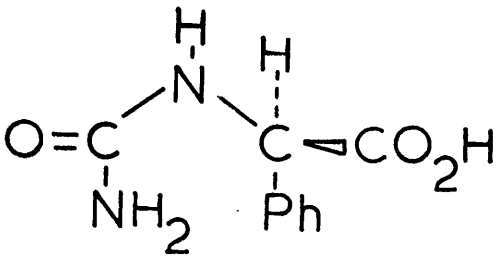


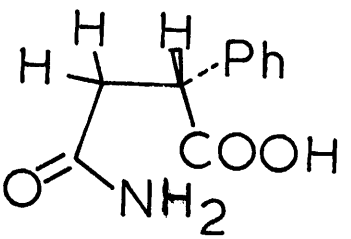
(16) R=H

(17) R=Me

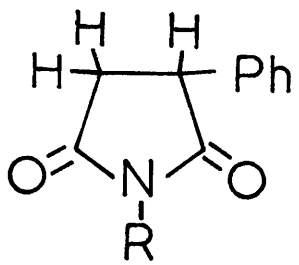
(18) R=Et



(R)-(-)-(19)

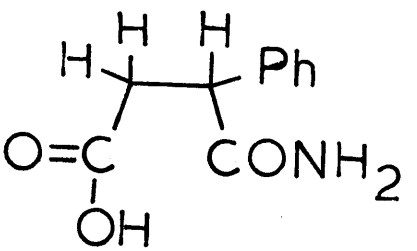


(R)-(-)-(20)



(21) R=Me

(22) R=H



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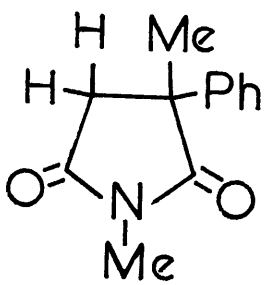

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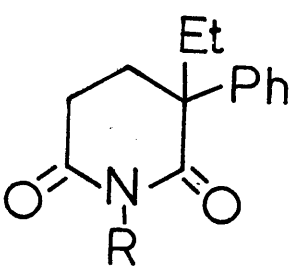


(25) R=H

(26) R=Me

evidence was obtained for the deamination of the (D)-isomer and subsequent transamination to the (L)-form.

Anti-convulsants

Mesantoin ((±)-5-ethyl-3-methyl-5-phenylhydantoin) (14) is N-demethylated to give Nirvanol (5-ethyl-5-phenylhydantoin) (15). In dogs and humans the excretion of (-)-Nirvanol in the urine is greater than that of the (+)-isomer, whether (±)-Mesantoin or (±)-Nirvanol is administered.[39] This suggests initial complete demethylation of Mesantoin followed by stereoselective metabolism of Nirvanol.

In dogs, administration of the (RS)-forms of 5-phenylhydantoin (16), its 3-methyl derivative (17), or its 3-ethyl derivative, ethotoin (18), resulted in the excretion of (RS)-5-phenylhydantoin in the urine.[40] (R)-(-)-Phenylhydantoic acid (19) was the major metabolite of all three drugs. The yields of (R)-(-)-(19), and the racemic nature of the initial urinary metabolite (16) indicated that the residual (S)-form of (16) underwent epimerisation in the body.

2-Phenylsuccinamic acid (20), isolated from the urine of dogs treated either with (RS)-N-methyl-2-phenylsuccinimide (21) or (RS)-α-phenylsuccinimde (22) was the optically pure (-)-form shown to have the (R)-configuration.[41] The absence of 3-phenylsuccinamic acid (23) was demonstrated indicating the high regiospecificity of the enzyme system. Administration of (RS)-(20) showed that the stereoselectivity was not due to removal of the (S)-form and metabolic studies

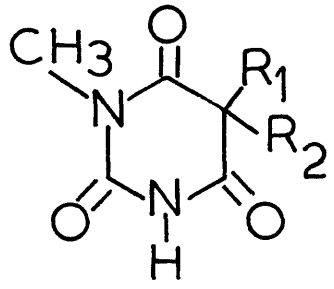
on (S)-(22) showed that no ring opening of the (S)-form occurred. Once again epimerisation of the (S)-enantiomer to the (R)-enantiomer which then undergoes ring opening, was proposed.

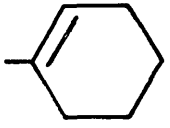
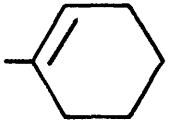
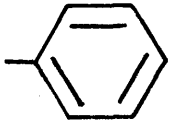
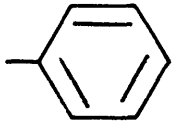
5,5-disubstituted hydantoins undergo little ring cleavage.[42,43] (RS)-2-methyl-2-phenylsuccinimide (24) undergoes significant demethylation and ring cleavage[44] but the stereochemistry of the products has not been determined. Ring cleavage of 2-phenyl-2-ethylglutarimide (25) did not occur in dogs.[45] but the metabolism was stereospecific. The (-)-isomer underwent hydroxylation of the 2-ethyl group while the (+)-isomer was hydroxylated in the glutarimide ring. N-methylglutethimide (26) showed partially stereospecific metabolism after demethylation,[46] and both isomers were excreted as glucuronides of hydroxymethylglutethimide.

Barbiturates

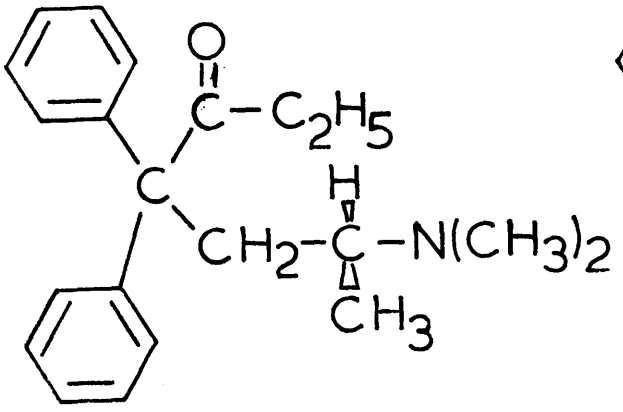
The enantiomers of hexobarbital (27) showed sex differences in rats both in anaesthetic activity and metabolic elimination.[47-49] The increased potency of (+)-hexobarbital appeared to be related to the decreased ability of the (-)-isomer to cross the blood-brain barrier.[50] The sex difference could partially be ascribed to the poorer abilityof female rats to transform the (+)-isomer.[47,50] Male rats metabolised the (+)-form faster than the (-)-isomer. The isomers were also affected differently by enzyme-inducing agents, and both *in vivo*[49] and *in vitro*[51] studies showed that phenobarbital (29)

Table 2. Effect of C-5 substituents on potency and metabolism of barbiturates



	N-methyl-5-cyclohexenyl-5-methylbarbital (27)	N-methyl-5-cyclohexenyl-5-ethylbarbital (28)	N-methyl-5-phenyl-5-methylbarbital (29)	N-methyl-5-phenyl-5-ethylbarbital (30)
R_1				
R_2	$-CH_3$	$-C_2H_5$	$-CH_3$	$-C_2H_5$
Anaesthetic activity	(+) > (-)	(-) > (+)	(+) > (-)	(-) > (+)
Metabolic elimination	(+) > (-)	(+) > (-)	(+) > (-)	(+) > (-)
Albumin binding	-	(-) > (+)	-	(-) > (+)

From Buch et al (52,53)



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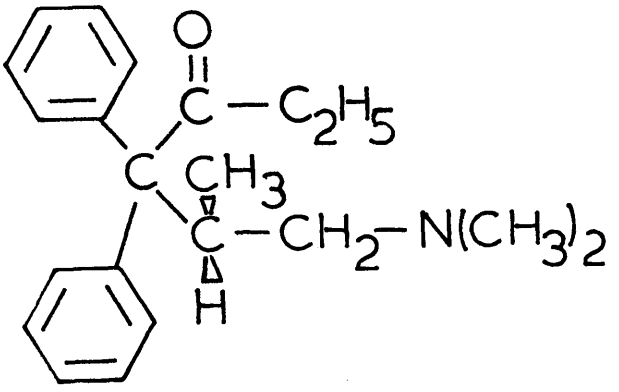

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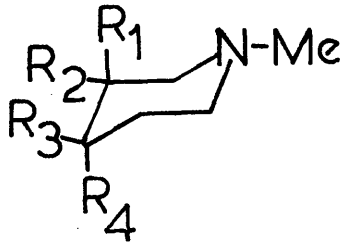
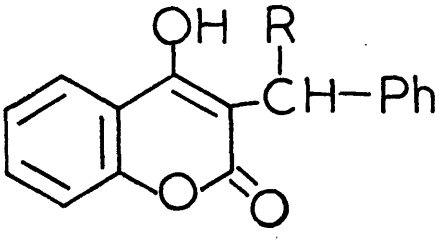


	R_1	R_2	R_3	R_4
(33)	H	Me	Ph	OCOEt
(34)	Me	H	OCOEt	Ph
(35)	Me	H	Ph	OCOEt
(36)	H	Me	OCOEt	Ph



(37) R = CH_2–$COCH_3$

(38) R = CH_2–CH_3

increased the total amount of (+)-hexobarbital oxidase, while the content of (-)-hexobarbital oxidase was unchanged although the substrate affinity was increased. The effect of varying the substituents at C-5 on the anaesthetic activity and metabolism has also been examined[52,53] (Table 2).

Analgesics

The in vitro metabolism of methadone (31) and its isomer (32) by rat liver microsomes showed[54] that (S)-(+)-(31) and (R)-(+)-(32) were metabolised to a greater extent than their (-)-enantiomers. Using guinea pig microsomes, however, (S)-(+)-(31) and (S)-(-)-(32) were metabolised faster.[54] Also, using the guinea pig preparation more N-oxide was formed from (S)-(-)-(32) than from the (+)-isomer. N-oxidation of (31), was minor. In vivo studies in rats using methadone-1-^{3}H-confirmed the stereoselectivity.[55,56]

The metabolism of (+)- and (-)-α-prodine (33,34) and (+)- and (-)-β-prodine (35,36 respectively) has been studied in mice.[57] In vitro the (+)-isomers were demethylated at a slower rate than the (-)-isomers. In vivo only the β-prodine isomers showed any significant difference in rates of decline of blood and brain levels. No relationship between metabolism and analgesic activity could be proposed.

Warfarin

After administration of warfarin-4-^{14}C (37) to female

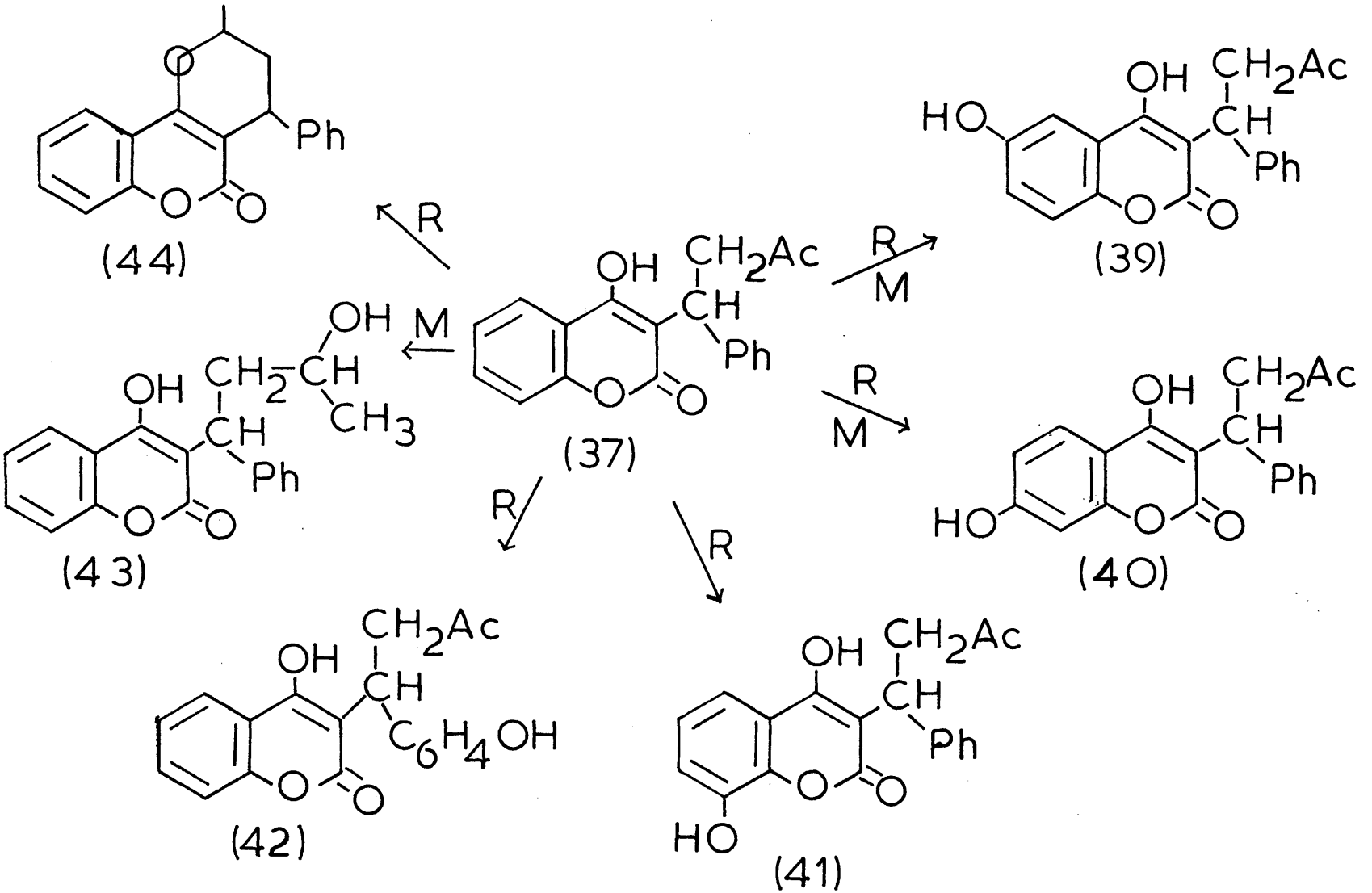


Fig. 3. The <u>in vivo</u> metabolites of warfarin identified in rats (R) and man(M).

rats the less active (R)-(+)-isomer was more rapidly excreted.[58] The same results were obtained with phenoprocoumon (38). Both warfarin-resistant and non-resistant rats excreted the (R)-(+)-form faster than the (S)-(-)-form.[59] The potency of the (S)-(-)-form can be explained partly by its longer plasma half-life [60,61] and partly by a difference in inhibition of prothrombin complex synthesis. The metabolism of warfarin in rats[62] and man[63] has been elucidated with the aid of synthetic compounds, mass spectra, and fragmentation patterns (Fig. 3). The stereochemical aspects of the metabolism in man have also been examined and stereoselectivity observed.[64] (R)-(+)-warfarin was regioselectively oxidised to 6-hydroxy warfarin (39) and reduced to the (R,S)-diastereomeric alcohol (43). In contrast (S)-(-) warfarin gave 7-hydroxy warfarin (40) as a major metabolite and some 6-hydroxy warfarin (39). Reduction gave the (S,S)-alcohol (43). Warfarin, therefore, shows both substrate and product stereoselectivity. Detection of a new metabolite of warfarin, produced by hydroxylation in the benzylic position, was achieved using an ion doublet chemical ionisation mass spectrometry technique.[65] Larger amounts of the metabolite were obtained from (R)-warfarin than from (S)-warfarin during *in vitro* experiments with rat liver microsomal preparations.

1.1.3. Product stereoselectivity

a) Hydroxylation

Initial studies[66] suggested that ethylbenzene was hydroxylated in the rabbit without any stereoselectivity, but a reinvestigation subsequently showed that the (R)-(+)-isomer always predominated.[67] The methylphenylcarbinol produced showed substrate stereoselectivity in rats, since the (R)-(+)-isomer was excreted as a glucuronide, while the (S)-(-)-form was further metabolised to (S)-(-)-mandelic acid.[68] The oxidation mechanism was studied using $^{18}O_2$ and D_2O media and was shown to involve molecular oxygen.[68] The oxidation of (S)-(+)-α-[$^2H_2$]-ethylbenzene showed that microsomal hydroxylation proceeded with retention of configuration i.e. by an "insertion" mechanism.[68] The use of microsomal inducing agents reduced the stereoselectivity of metabolism.[67,68]

Indane (45) was hydroxylated by rat-liver microsomes to (S)-(+)-indanol (70%).[69] The stereoselectivity of hydroxylation would be even higher than it appeared since the (S)-(+)-indanol was selectively oxidised to indanone. The formation of indanone was catalysed by an $NADP^+$-dependent dehydrogenase which was subsequently isolated.[70] Similar systems have been found which dehydrogenate trans-acenaphthene-1,2-diol[71] and methylphenylcarbinol.[72]

The in vivo metabolism of cyclohexylamine (46) in man and animals was examined using GLC to determine the trans/cis

Table 3. Products of in vivo hydroxylation of cyclohexylamine

	Rabbit		Rat		Guinea pig		man	
	% dose	t/c ratio	%	t/c		t/c	%	t/c
3-amino-cyclohexanol	11.9	19	2.3	22	1.4	6	0	-
4-amino-cyclohexanol	0.6	2	2.2	0.3	0.4	1	0	-

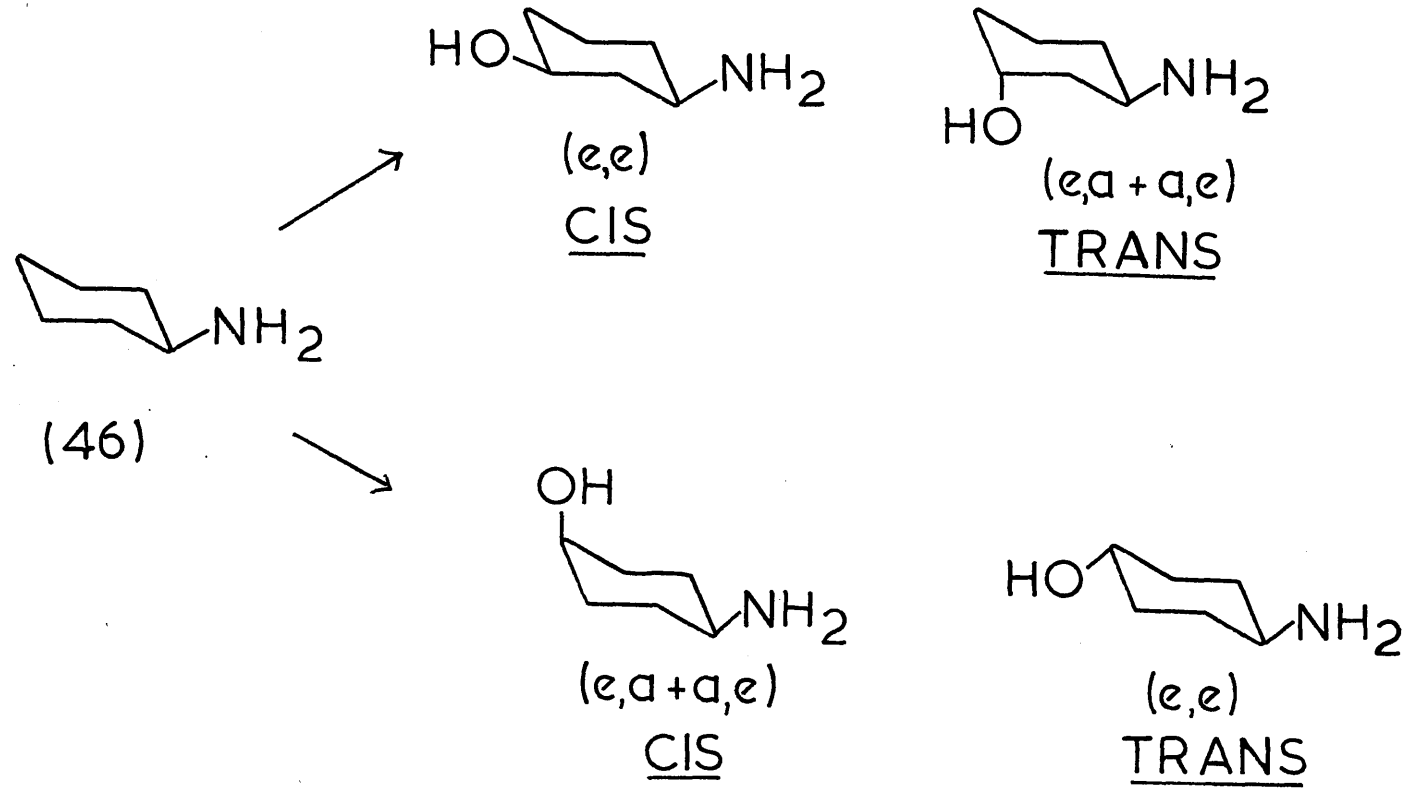


Fig. 4. The products obtained by metabolic hydroxylation of cyclohexylamine.

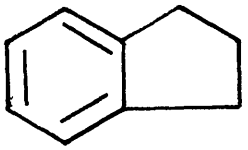
ratio of the products[73] (Table 3). If it is assumed that the cyclohexylamine is in the preferred conformation, with the amino-group equatorial, during hydroxylation then "the addition of the hydroxyl group is mainly axial",[73] (Fig. 4).

Hydroxylation of butamoxane (47) both *in vivo* and *in vitro* by rat liver yielded 6-hydroxy (47) and 7-hydroxy-(47) in a ratio of 2:1.[74] In addition the microsomal oxidation of (47) yielded the catechol, 6,7-dihydroxy-(47). This same catechol was produced by hydroxylation of either 6-hydroxy-(47) or 7-hydroxy-(47).

The use of $^{18}O_2$ supported the concept that 6,7-dihydroxy (47) was formed by two consecutive hydroxylations, rather than *via* a dihydrodiol or an endoperoxide.[74]

b) The hydration of epoxides

Since the first suggestion[75] that epoxides might be intermediates in the metabolism of aromatic hydrocarbons there have been indications that arene oxides are concerned in the biological effects produced by these compounds.[76] The oxidative metabolism of naphthalene in rabbit and rat liver slices and kidney homogenates yielded three main products : 1-naphthol, *S*-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione, and 1,2-dihydro-1,2-dihydroxy-naphthalene.[77] Indene was similarly converted to the dihydro-*trans*-diol derivative.[78] These results supported the suggestion of arene oxides as metabolic intermediates. The system catalysing the hydrolysis of epoxides to *trans*-



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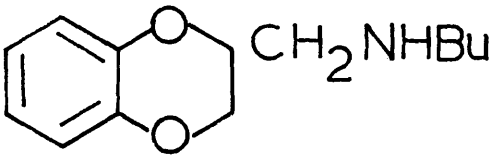

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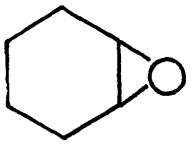

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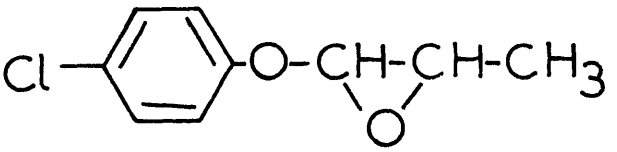

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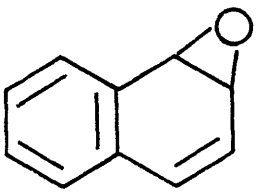

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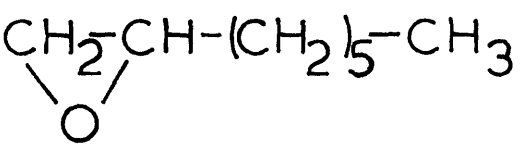

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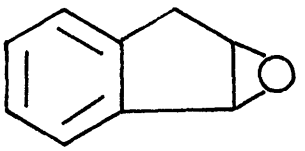

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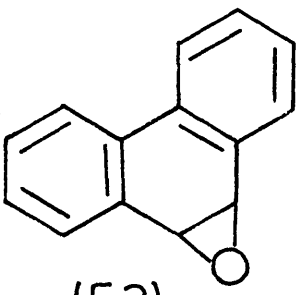

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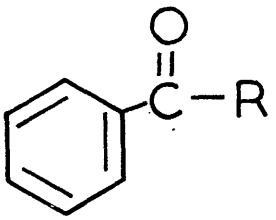
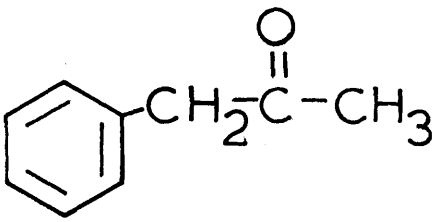




(54) R = CH_3

(55) R = CH_2CH_3

(56) R = $CH_2CH_2CH_3$

(57)

diols is commonly known as epoxide hydrase. The purification of styrene epoxide hydrase from guinea pig microsomes has been reported.[79] The enzyme, which requires no co-factors, catalyses the hydration of a variety of epoxides, including cyclohexene oxide (48), p-chlorophenyl-2,3-epoxypropyl ether (49), naphthalene-1,2-oxide (50), octene-1,2-oxide (51), indene-1,2-oxide (52), and phenanthrene-9,10-oxide[80] (53).

c) Ketone reduction

Acetophenone (54) was stereoselectively reduced in the rabbit to (S)-(-)-methylphenylcarbinol.[66,81] Examination of higher homologues showed that ethylphenyl (55) and phenyl n-propyl ketones (56) were selectively reduced to the corresponding (-)-carbinols and benzylmethyl ketone (57) to the (+)-carbinol.[82] These carbinols all had the (S)-configuration.

One of the most important and well-studied enzymatic reactions is the oxidation-reduction reaction catalysed by nicotinamide-nucleotide-linked dehydrogenases. The subject has been extensively reviewed.[83-85]

The coenzyme nicotinamide adenine dinucleotide (NAD^+) (58, R = adenosinediphosphoribosyl) in conjunction with the enzyme alcohol dehydrogenase (ADH) oxidises ethanol to acetaldehyde with the formation of the reduced coenzyme NAD-H (59) (Fig. 5). The reduction of acetaldehyde is the reverse of this reaction.

It has been shown[83,86,87] that the hydrogen transfer is direct, reversible, and stereospecific. ADH catalyses

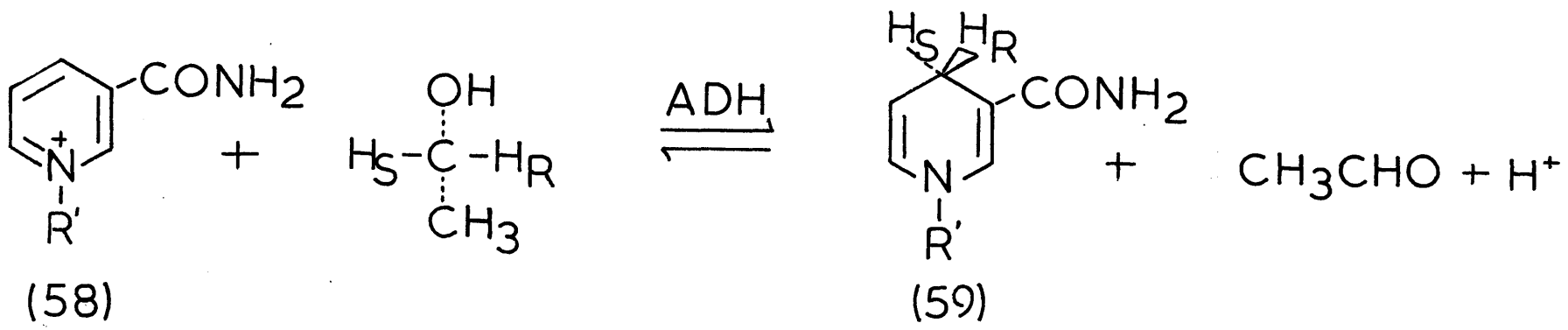


Fig. 5. The stereospecific interconversion of ethanol and acetaldehyde by alcohol dehydrogenase (ADH) using nicotinamide adenine dinucleotide as coenzyme (R' = adenosine diphosphoribosyl).

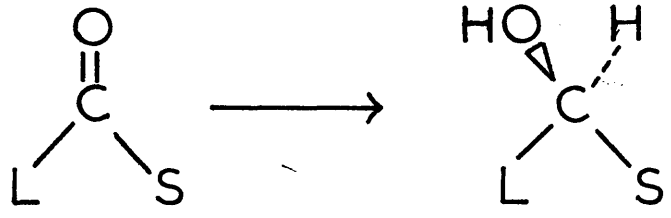
the reversible addition of hydrogen to only one side of the plane of the nicotinamide ring of NAD^+. The pro-(R) hydrogen[88] of ethanol is always transferred in the presence of the enzyme and becomes the pro-(R) hydrogen at C-4 of reduced NAD (Fig. 5). Isotopic substitution does not alter the stereospecificity, i.e. reduction of acetaldehyde-1-^{2}H with NAD-H in the presence of ADH produces ethanol-1-^{2}H which, upon enzymatic reoxidation yields only acetaldehyde-1-^{2}H with no loss of deuterium.[86]

In order to furnish proof of the stereospecificity of carbonyl group reduction by actively fermenting yeast, Mosher and co-workers[89] investigated the yeast reduction of a series of ketones which included all combinations of the substituents methyl, ethyl, n-propyl, n-butyl, and phenyl. Eight of the nine secondary alcohols produced possessed the (S)-configuration (the configuration of the ninth compound, (-)-3-heptanol, is in doubt).

These results, together with the observations that the reduction of acetaldehyde-1-^{2}H by purified yeast ADH-NADH[87,90] and trimethylacetaldehyde-1-^{2}H by actively fermenting yeast[91] give alcohols of the (S)-configuration agree with the assumption that the same enzyme system is responsible for the in vitro and the in vivo reductions.

Prelog has studied[92] the rates and products of reduction of a large number of alicyclic ketones using horse liver alcohol dehydrogenase and an extensively purified oxido-reductase from Curvularia falcata. He found that

the product stereospecificity of the reduction could be represented by the very simple scheme :



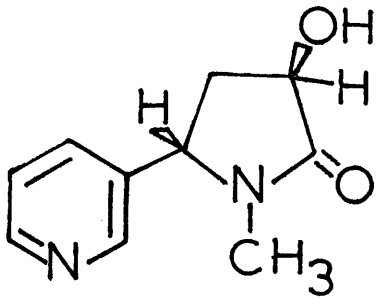
Where L is a large group and S a smaller one.

1.1.4. Substrate produced stereoselectivity

a) Hydroxylation reactions

Dopamine β-hydroxylase catalyses the hydroxylation of dopamine (10) to noradrenaline, the (1R) isomer being preferentially formed.[93] α-Methyl-dopa (12) is metabolised *in vivo* to (1R, 2S)-(-)-α-methylnoradrenaline *via* the intermediate (2S)-(+)-α-methyldopamine.[94] (+)-, (-)-, or (±)-α-methyldopamine administration to mice showed conversion of the (2S)-(+)-isomer to (1R, 2S)-α-methyl-noradrenaline, with hardly any (1S, 2S)-isomer being produced. (2R)-(-)-α-methyl-dopamine underwent only negligible β-hydroxylation.[94,95]

trans-1-Methyl-3(R)-hydroxy-5(S)-(3-pyridyl)-2-pyrrolidinone [(3R, 5S)-hydroxy cotinine)] (60) was



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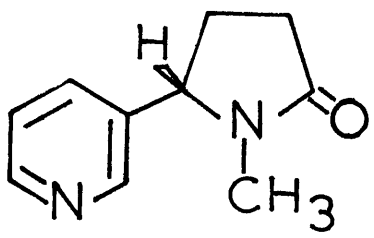

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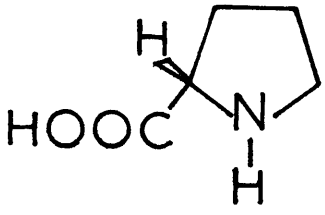

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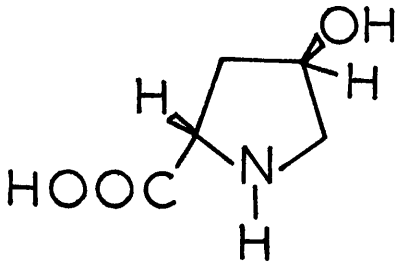

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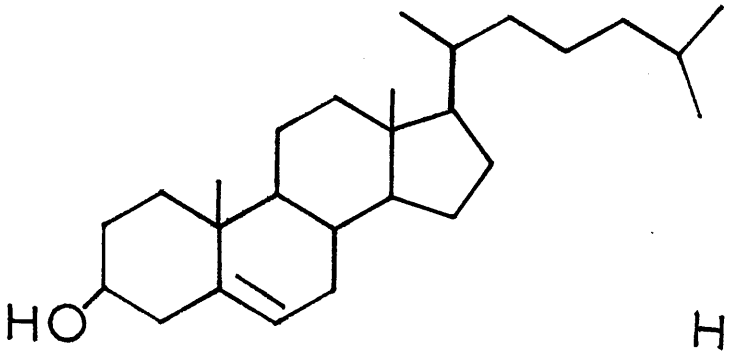

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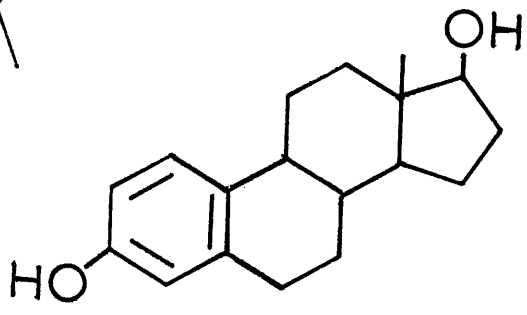

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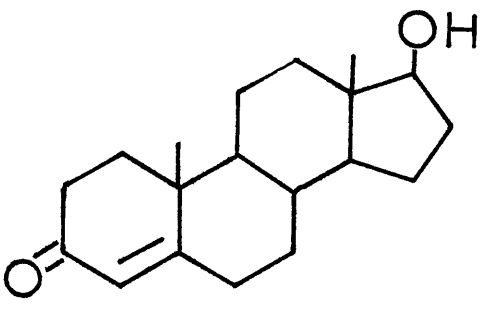

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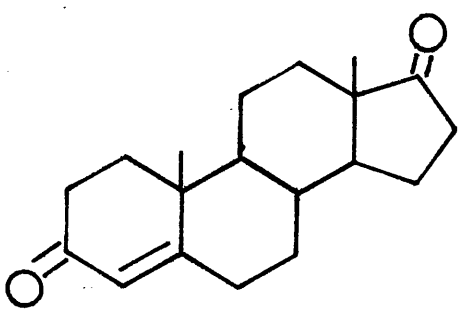

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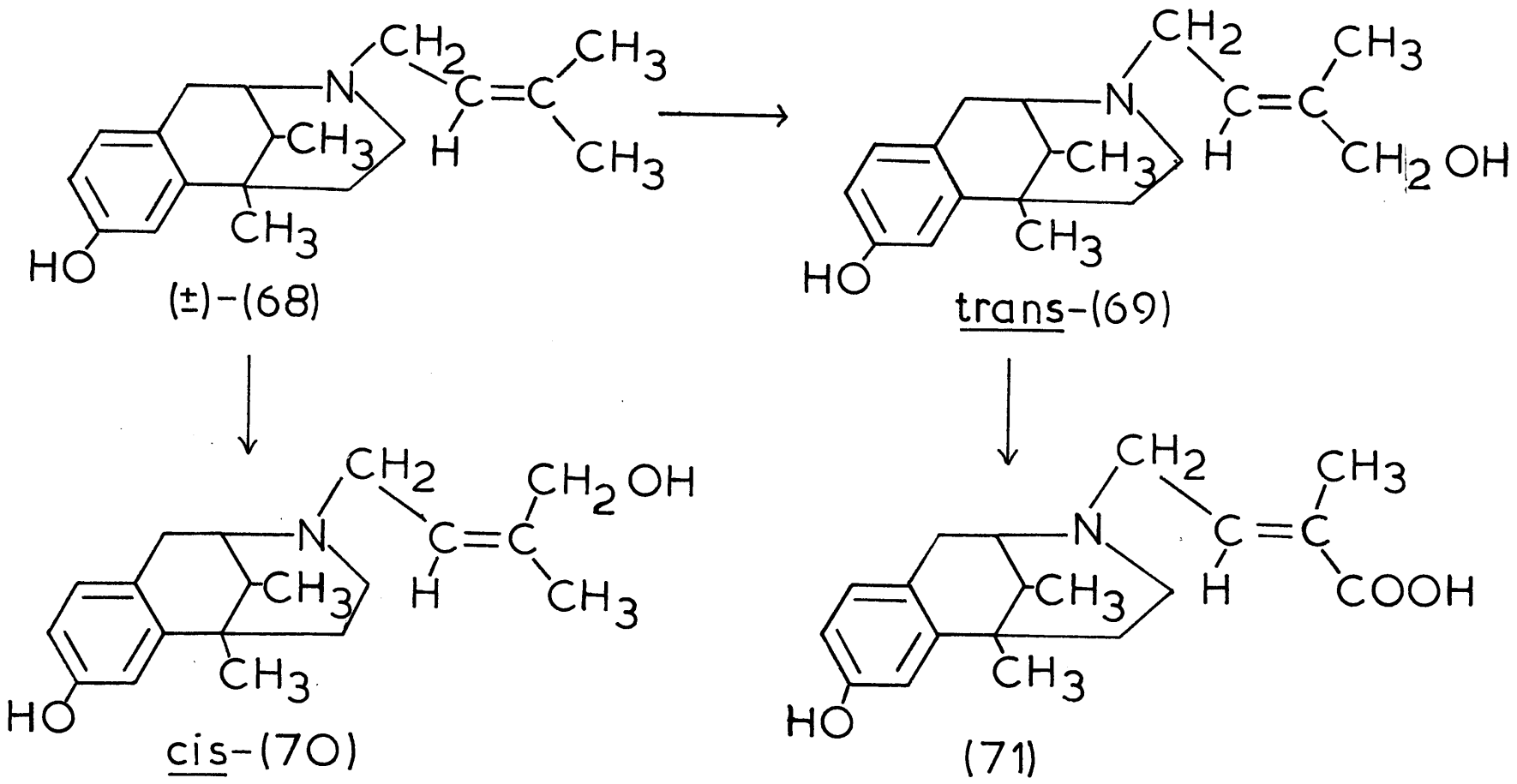


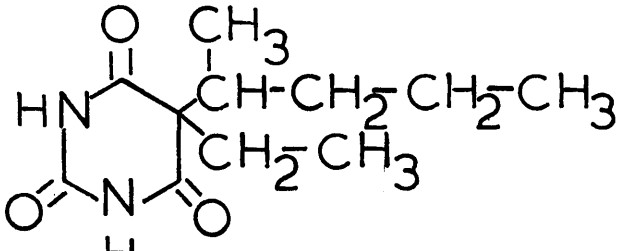
Fig. 6. Metabolism of pentazocine.

identified by NMR and MS in the urine of monkeys[96] treated with (S)-(-)-cotinine (61), a metabolite of nicotine. No cis-product was detected in the NMR. (S)-(-)-proline (62) was also stereoselectively metabolised to (2S, 4R)-hydroxyproline (63).[97]

The use of cis-4-^{3}H-(S)-proline and trans-4-^{3}H-(S)-proline showed that the mechanism involved front side displacement with complete retention of configuration.[97] The similarity between the proline and cotinine metabolism suggests an identical mechanism.

Hydroxylation of steroids proceeds stereoselectively. Double-labelling experiments with cholesterol (64) showed that the hydroxylation mechanism occurred by front-side displacement.[98] Some examples of stereoselective steroid hydroxylations are the preferential formation of the 6-α-hydroxylated metabolite of 17-β-estradiol (65) by rat liver microsomes[99] and the hydroxylation of testosterone (66) and Δ^{4}-androstene-3,17-dione (67),[100] to mainly 6-β- and 7-α-products.

Substrate stereoselectivity was also observed in the hydroxylation of (±)-pentazocine[101] (68) (Fig. 6). Both isomeric hydroxylated products were observed using rat or mouse hepatic preparations but the stereoselectivity was variable. Using mouse microsomes, the trans-isomer (69) predominated over the cis-form (70), while the rat preparation produced an excess of the cis-over the trans. In vitro and in vivo experiments performed with the rhesus monkey using ^{3}H-pentazocine (68) showed the presence of



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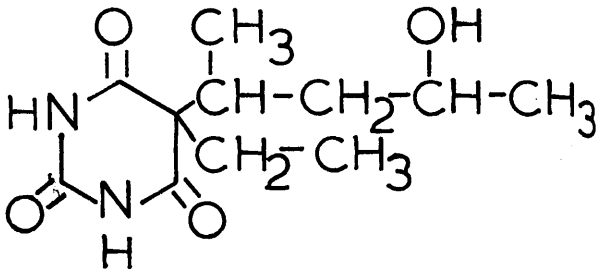

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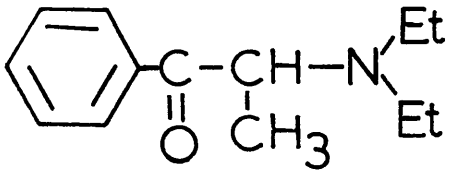

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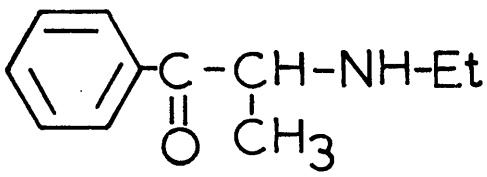

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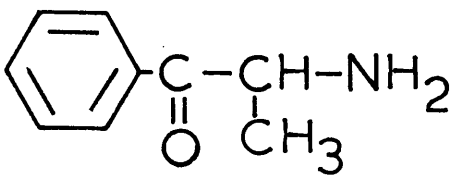

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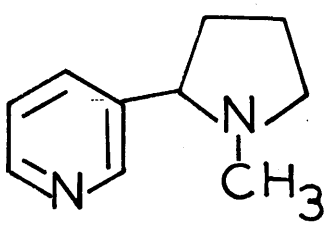


(77)

the acid (71), obtained by further oxidation of the trans-isomer. In this case the amount of the trans-forms (69) and (71) exceeded that of the cis-isomer (70).

Pentobarbital (72) is hydroxylated in the (ω-1) position to 5-ethyl-5-(3'-hydroxy-1'-methylbutyl)barbituric acid (73).

In dogs, two diastereomers of (73) were produced.[102,103] Administration of (R)-(+)-pentobarbital gave (1'R, 3'R) and (1'R, 3'S) (73) in a 1:1 ratio, while (S)-(-)-pentobarbital was metabolised into (1'S, 3'S) and (1'S, 3'R) (73) in a 1:5 ratio. These results showed that there was no substrate stereoselectivity but that product stereoselectivity varied with the substrate. (R)-(+)-pentobarbital showed no product stereoselectivity but the metabolism of (S)-(-)-pentobarbital showed a high degree of stereoselectivity.

b) Ketone reduction

The metabolism of N-diethyl-amino-propiophenone (74) has been extensively studied.[104,105] The major mode of metabolism was N-de-ethylation[104] but the stereochemistry of the β-hydroxylated metabolites has been examined[105] from the point of view of substrate and product stereoselectivity. The major hydroxylated metabolites of racemic N-ethylaminopropiophenone (75) and aminopropiophenone (76) have also been stereochemically examined.[106] The results are summarised in Table 4. The tertiary and secondary amines showed substrate stereo-

Table 4. Stereochemical composition of amino alcohols produced by reduction in man of the corresponding racemic ketones

	(1S, 2R) (+)-erythro, %	(1R, 2S) (-)-erythro, %	(1S, 2S) (+)-threo, %	(1R, 2R) (-)-threo, %
N-diethyl-norephedrine	——————— 2 ———————		80	18
N-ethyl-norephedrine	6	20	60	14
Norephedrine	2	37	0	61

B. Testa (106)

selectivity since 80% of the reduced ketone was the (2S)-enantiomer. The major products had the (1S, 2S) configuration and the small proportion of (2R)-amino ketone which was reduced preferentially gave the (1R, 2R)-amino alcohol. Product stereoselectivity was, therefore, also important. The primary amino-ketone, however, showed only slight substrate stereoselectivity (63% (2R) reduced) but very high product stereoselectivity since the products had almost exclusively the (1R) configuration.

c) N-oxidation

Nicotine (77) is metabolised to nicotine-1' N-oxide and cotinine by 10,000 x *g* hepatic preparations from rats, mice, rabbits, guinea pigs and hamsters. Little evidence was obtained for the stereoselective disappearance of nicotine but some evidence was obtained for stereoselectivity of total metabolite formation.[107,108] There are four possible diastereomeric N-oxides.[109] (S)-(-)-nicotine was transformed by rats, mice, hamsters and guinea pig into more (R,S)-cis than (SS)-trans-N-oxide. However, (R)-(+)-nicotine produced more (S,R)-trans than (R,R)-cis. Using rabbit microsomes more cis diastereomer was produced from both enantiomers. Similar results obtained previously[110,111] had been explained on the basis of two distinct receptors for N-oxidation.[111] Jenner *et al*, however, proposed[108] a single receptor site which could account for the formation of both diastereomers in terms of orientation of the

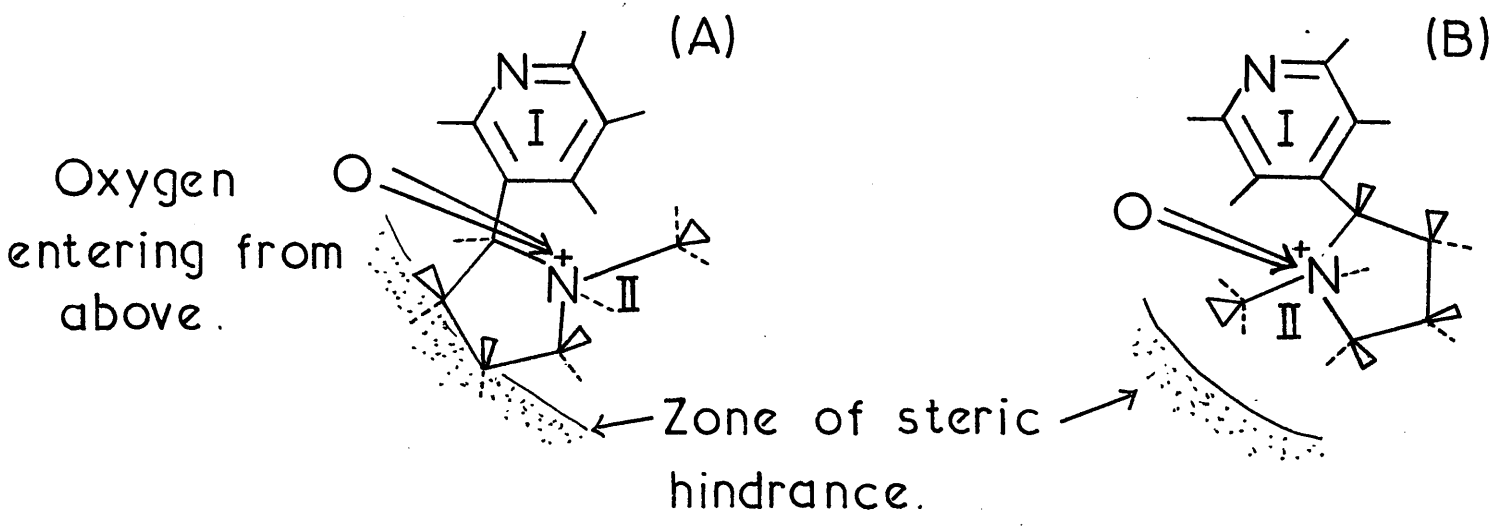


Fig. 7. Orientation of (A), (RS)-cis and (B), (SS)-trans-protonated nicotine at the N-oxidation surface. (Preferential attachment of the (SS)-trans-form due to the unhindered orientation, followed by oxygen attack from above leads to a greater amount of (RS)-cis-N-oxide due to an S_N2 inversion of configuration.)

I. Attachment of pyridine ring involving van der Waals forces.

II. Anionic site reinforced by H-bonding. (According to Jenner et al, 108.)

protonated enantiomers (Fig. 7).

Significant stereoselective reduction of the N-oxide occurs under anaerobic but not aerobic conditions.[111] Guinea pig hepatic microsomes reduced the (R,S)-cis-N-oxide six times faster than the (SS)-trans-diastereomer. An excess of (S,S)-trans-N-oxide was isolated from smokers' urine,[110] contrary to the in vitro findings, but this could be the result of overall stereoselective oxidation and reduction.

1.2. The gas chromatographic resolution of enantiomers

1.2.1. Introduction

The analytical resolution of optical enantiomers is of considerable importance in the examination of biological or biochemical systems such as are involved in the study of drug metabolism or various enzymic reactions. Since such systems often involve working with small and frequently impure samples a chromatographic method of resolution is desirable.

There have been many investigations of the chromatographic resolution of racemates on optically active adsorbents.[112] Some success has been achieved with D-lactose,[113] starch,[114,115] cross-linked chitosan ,[116] or synthetic polymers containing asymmetric centres,[117-119] as adsorbent. Multi-functional amino acids have been resolved by paper chromatography[120,121] through interaction with the asymmetric centres of cellulose, and ligand-exchange chromatography of racemic α-amino acids on asymmetric resins containing optically active bi- or tridentate α-amino acids as fixed ligands co-ordinated with Cu^{2+}, Ni^{2+} or other transition metal ions has been effective.[122-125]

Recently, complete optical resolution of α-amino acids has been described by differential complexation in solution between a chiral cyclic polyether and the amino acid.[126] Diastereomeric di- and tri-peptides have been separated on paper and thin layer chromatography[127-130]

and Sephadex gel chromatography.[131] A modified gel containing L-arginine residues was reported[132] to resolve (RS)-3,4-dihydroxyphenylalanine.

Partial separation of some asymmetric aromatic compounds has been reported[133] on a column of silicic acid impregnated with optically active (2,4,5,7-tetranitro-9-fluoroenylideneaminooxy)propionic acid. Mandelic acid can be resolved partially on either polyacrylic esters or amides of optically active ephedrine derivatives[134] or by liquid liquid partition chromatography on Sephadex gels.[135]

The most successful area of chromatographic resolution has, however, been in the field of gas liquid chromatography (GLC).[136] Gas chromatographic resolution of enantiomers may be effected by one of two methods : a) preparation of diastereomers by reaction of the enantiomers with a suitable asymmetric reagent and chromatography on an inactive phase and b) direct resolution of the enantiomers on an optically active stationary phase. Both approaches have been applied successfully as outlined below.

1.2.2. Gas chromatographic resolution of enantiomers by formation of diastereomeric derivatives

The separation of diastereomers by gas chromatography can be described in thermodynamic terms using the relative volatility, α, obtained from the retention times of the two components under isothermal conditions.[137]

$$\alpha = \frac{t_{R_2} - t_a}{t_{R_1} - t_a} = \frac{K_2}{K_1}$$

Where t_{R_1} and t_{R_2} are the retention times for the first and second components respectively, t_a is the inert gas retention time, and K_1 and K_2 are the respective gas-liquid partition coefficients.

The isothermal determination of α allows the computation of the standard free energy differences between the diastereomeric pairs with respect to their gas-liquid partition equilibria. Thus :

$$\Delta G^o = -RT\ln K$$

and $$\Delta G^o_2 - \Delta G^o_1 = \Delta(\Delta G^o) = -RT\ln\alpha$$

Where R is the gas constant = 8.31432×10^7 erg $(g\ mole)^{-1}\ deg^{-1}$ and T the temperature in ^{o}Kelvin.

a) Alcohols

The resolution of alcohols by formation of diastereomeric esters has received considerable attention since work started in this field in the early 1960's.[138] The preferred reagent for derivatising 2-n-alcohols has been α-acetoxypropionic acid.[139,140] A series of 2-n-alcohols containing 4-19 C atoms were well separated[139] as their α-alkanoyloxypropionates on capillary columns coated with both non-polar (squalane) and polar (polypropyleneglycol and FS-1265) stationary phases. The polar phases produced better resolutions and polypropyleneglycol gave

the best results for alkanols containing 4-10 carbon atoms while the higher alcohols were better resolved on FS-1265. Separation factors (α-values) of 1.03-1.04 were reported for the lower homologues and they increased with increasing chain length to a maximum of 1.10. A plot of the relative retention volume versus the number of carbon atoms in the alkanol, within the series studied, resulted in pairs of almost linear and parallel curves. In all cases the lower curve corresponded to the (RS) or (SR) diastereomer. Series of straight- and branched-chain alkan-2-ols (4-8 C atoms) were examined[140] both as the α-acetoxypropionates and α-hydroxypropionates on packed columns (1,2,3-tris-(2-cyanoethoxy)-propane or DC710 silicone oil). In general the straight-chain methylcarbinyl α-acetoxy esters yielded better separations than the corresponding α-hydroxy esters. The branched-chain methylcarbinyl α-hydroxy esters were separable on both columns but the $\Delta(\Delta G^{o})$ values for the diastereomers were greater on the non-polar column, contrary to the results obtained with the α-acetoxy esters. The authors also examined the derivatives of four alkan-3-ols and found them to be less well resolved than the analogous esters at the 2-position.

Westley et al.[141] resolved a series of straight- and branched-chain alcohols as their N-trifluoroacetyl(TFA)-(S)-phenylalanine esters on a polyester phase. They also observed the best resolutions when the alcohol was in the 2-position and an increase in branching of the chain,

particularly close to the asymmetric centre, improved the value of $\Delta(\Delta G^{o})$.

Good resolutions were obtained on capillary columns for both straight and branched chain alcohols containing 4-8 carbon atoms as their (R)-(+)-1-phenylethylurethanes,[142] derivatives originally applied to the resolution of alkan-2-ols by thin layer chromatography.[143] In the majority of cases studied by gas chromatography the alcohol was in the 2-position but four examples of alkan-3-ols are given. This reagent has also been found suitable[144] for the resolution of long chain hydroxy fatty acid methyl esters where the alcohol function is in the ω-2 position in the chain. When the alcohol function was in the 3-,ω-4-, andω-3-positions, better derivatives were[145] the 2(S)-phenylpropionates. Preparation of esters by reaction with menthylchloroformate[146] also produced derivatives suitable for the optical analysis of α-hydroxy fatty acid methyl esters. These derivatives have proved useful in biochemical situations in allowing the determination of the configuration of the 2-hydroxy acids from brain cerebrosides,[147] of the 2-hydroxy-palmitic acid produced in the peanut oxidation system,[148] and of the hydroperoxides produced by lipoxygenase oxygenation of linoleic acid.[149] The use of this reagent, however, for the optical analysis of hydroxy fatty acid methyl esters where the hydroxyl group was further removed from the carboxyl end of the chain proved unsuccessful.[150]

Resolution factors of the order of 1.1 were obtained[146]

for the (R)-(-)-menthylcarbonates of a series of phenylalkylcarbinols. Since these derivatives were formed in weakly basic medium at room temperature they were suitable for heat- and acid-labile compounds. Comparable resolution factors have been reported[151] for these carbinols as the derivatives of 3β-acetoxy-Δ^5-etienic acid.

Few examples of alicyclic alcohol resolutions have been reported. Three asymmetric cyclohexanol derivatives were separated[141] as their N-trifluoroacetyl-(S)-phenylalanine esters, and the resolution of the (R)-(+)-1-phenylethylurethane of menthol has been reported.[142] The resolution factors obtained when the asymmetric centre is within a rigid ring system are, as expected, fairly large (1.10-1.17 cf. ref. 141).

b) Amines

On the basis of preliminary experiments using amphetamine with several chiral acylating reagents Halpern and Westley chose[152] N-trifluoroacetyl-(S)-(-)-prolyl chloride[153] as the resolving agent for amines. This has been the preferred reagent since that time. Halpern and Westley have successfully resolved aliphatic, arylaliphatic,[152,154] and heterocyclic amines[154,155] as amides of this reagent. The observed elution order of the aliphatic or arylaliphatic amides was always (RS) < (SS),[154] but a reversal of this order was noted for the heterocyclic amides whose absolute configuration was known. During a

study of the N-trifluoroacetyl-(S)-prolyl derivatives of various ring and N-alkyl substituted amphetamines, seventeen enantiomeric pairs were examined.[156] In all cases the derivatives of the (2R) enantiomers had shorter retention times than the derivatives of the (2S) enantiomers. The introduction of a 1-OH group to give ephedrines and ψ-ephedrines did not alter the control of the relative retention times by the geometry of the carbon-2. Steric analysis of a series of homologous amines possessing two asymmetric centres (i.e. 2-amino-3-methylpentane— 2-amino-4-methylhexane) as their N-TFA-(S)-prolyl derivatives showed four peaks on GLC.[157] The order of emergence of the peaks, with respect to the asymmetric carbon atom carrying the amino function, was in agreement with the elution pattern observed for diastereomeric amides derived from aliphatic amines containing only one asymmetric centre, i.e. (SR) < (SS).

The N-TFA-(S)-prolylamides of ring-substituted 1-phenyl or naphthylalkylamines were better resolved[158] on a relatively polar column (DEGS) than on a non-polar column (SE-30). On the contrary, for a series of ring-substituted 1,2-diphenylethylamines, the diastereomeric amides were better resolved on the non-polar column.

The N-TFA-(S)-prolylamides of amphetamine were sufficiently resolved to allow quantitative enantiomeric determinations. Metabolism studies conducted after ingestion of (RS)-amphetamine showed that the amount of (+)-amphetamine excreted in the urine was less than that

of the (-)-enantiomer.[19]

An excess of (+)-amphetamine, however, was found in the urine of subjects receiving doses of methamphetamine. The same derivatives were used to show that no in vivo racemisation occurred when either (+)- or (-)-amphetamine was ingested.[159] The use of N-TFA-(S)-prolyl derivatives has been recommended as the standard method for the quantitative determination of the ratio of (+)- and (-)-amphetamine in commercial preparations.[160] Nanogram quantities of amphetamine and related substituted arylalkylamines can be analysed by using electron capture detection of the derivatives formed with N-pentafluoro-benzoyl-(S)-prolyl-1-imidazolide[161] or α-methyl-α-methoxy-pentafluorophenylacetyl-imidazolide.[162] The latter reagent can also be used for the correlation of the absolute configuration of certain substituted β-arylethylamines by NMR.

c) Aliphatic and alicyclic acids

As already discussed, enantiomeric alcohols or amines may be resolved on gas chromatography by the formation of diastereomers with a suitable chiral acylating agent. Conversely, the optical analysis of asymmetric acids can be performed with the aid of chiral alcohols or amines. Examples of both approaches can be found in the literature.

A series of α-chloro-alkanoic acids were examined[163] as the amides formed with valine methyl ester in an

investigation of steric effects about the amide bond. The results showed that the differences between the diastereomers were enhanced by increased crowding about the amide bond. The resolution also improved on going from a non-polar phase (SE-30) to a more polar polyester phase, suggesting secondary bonding between the amide carbonyl and the polyester phase.

As an extension of their method for determining the absolute configuration of amino compounds using the partial kinetic resolution of (±)-α-phenylpropionic acid,[164] Cervinka et al developed[165] a method for the determination of the absolute configuration of asymmetric carboxylic acids by reaction with (S)-(+)-methamphetamine. Using this approach they claimed that (+)-α-alkylphenylacetic acids have the absolute configuration (S) when the alkyl group is Me, Et, n-Pr, but are of the (R) configuration when the α-group is iso-Pr or iso-Bu. Gas chromatographic analysis of these amides, however, showed that the (S)-(+)-methamphetamine amides of all the (+)-acids, normal and branched chain, were eluted earliest.[166] The order of emergence of diastereomers was shown to be a reliable criterion for the assignment of configuration in this case when the absolute configuration of (-)-α-isopropylphenylacetic acid was reassigned as (R).[167-169]

With the increasing use of insecticides based on pyrethroids containing chrysanthemic acid it became necessary to develop analytical methods to determine the optical purity of synthetic material. (+)-Trans- and

(-)-trans-Chrysanthemic acid were separated[170] as the esters of (-)-menthol or (-)-borneol, but no resolution could be obtained for the cis-acids. The separation of the bornyl esters was poorer than that of the menthyl derivatives. Subsequently both the (±)-trans and (±)-cis-chrysanthemic acids were resolved as their (+) or (-)-2-octyl esters on packed columns,[171] or as (+)-α-phenylethylamine amides on capillary columns,[172] and these methods were applied to the determination of the enantiomeric purity of synthetic pyrethroids.

Methods for the resolution of acyclic isoprenoid acids as their (-)-menthyl esters on capillary columns have been developed and applied to the determination of the stereochemistry of the major acids present in geologically ancient sediments.[173-175]

The gas chromatographic method has also been extended to the optical analysis of carbohydrates. Complete acetylation of the alcohol function followed by oxidation of the aldoses to aldonic acids produced compounds which could be resolved as esters of 2-butanol, 3-methyl-2-butanol or 3,3-dimethyl-2-butanol.[176,177] The order of elution of the peaks was determined by the configuration at the carbon adjacent to the carboxyl group.

d) Amino acids

Diastereomers can be formed from amino acids in two ways : the acid group may be treated with an asymmetric alcohol or amine to form an ester or amide, or the amino

function can be derivatised with an optically active acid. The remaining polar group can then be derivatised with a non-asymmetric reagent prior to gas chromatography.

The former approach has been applied by many workers who have resolved N-trifluoroacetyl (N-TFA) amino acid alkanol esters. The most frequently used asymmetric alkanols have been 2-butanol,[139,178-183] 2-octanol[139,178] and menthol.[184-187] Many amino acids have been resolved by this method on both packed and capillary columns. Ayers *et al*[188] examined the resolution of amino acid diastereomers by packed column chromatography, using various alcohols for derivatisation and various columns for the resolution. N-trifluoroacetyl-derivatives were generally found to be superior to N-acetyl-, N-trichloroacetyl- or N-propyl-derivatives. The effect of the alcohol structure was quite pronounced. No resolution was achieved with 2-methyl-1-butyl esters and in the alkan-2-ol series the degree of resolution attainable increased with the size of the alcohol. 3-Methyl-2-butanol and 3,3-dimethyl-2-butanol were significantly superior in effecting a resolution to any other alcohols tested, and 3,3-dimethyl-2-butanol was the best. Using the latter derivatives fourteen protein amino acid diastereomers could be resolved to 93% or better. Aspartic acid and proline derivatives could be resolved to the extent of 70% and 82% respectively.

A problem is presented by the trifunctional amino acids which would form di-TFA derivatives under normal

conditions and are poorly resolved.[179] A superior method was found to be preparation of O- (or S-) acetyl-N-TFA-2-butyl esters.[180]

N-TFA-2-butyl esters, have been successfully employed in the identification, and determination of enantiomeric composition of the amino acids occurring in meteorites,[189-192] soils[183] and sediments.[193-195] This method has found application in the estimation of the age of fossils or bones by determination of the amino acid enantiomeric composition.[196] Several non-protein amino acids occurring in the Murchison meteorite were poorly resolved as their 2-butyl esters.[190] However examination of the use of various acyl groups and alcohols[197] led to the choice of the 2-pentyl- or 2-hexyl-N-pentafluoropropionyl derivatives in this case.

The alternative approach to amino acid resolution employs the nitrogen function to form a diastereomeric amide. In some of the earliest examples examined[198,199] the second asymmetric centre was already present in the diastereomeric dipeptide. Subsequently many amino acids have been resolved as their N-TFA-prolyl- amino acid methyl esters.[154,200,201] Polyfunctional amino acids have been successfully examined[201] by trimethylsilylating any free hydroxyl or sulphydryl groups before reaction with N-TFA-(S)-prolyl chloride. Both (R)-[202] and (S)-[202,203] isomers of N-TFA-prolyl chloride have been synthesised to 99% optical purity and used to determine the relative amounts of (R)- and (S)-threonine and (R)- and (S)-allothreonine

in a mixture,[202] and in the quantitative determination of (R)- and (S)-leucine enantiomers.[203]

Iwase has studied[204] the effect of changing the N-perfluoroacyl group, and the ester function, on the separation of four racemic amino acids (alanine, valine, leucine, and proline) and found an increase in the separation factors, except for proline, as the number of CF_2 groups in the perfluoroacyl group increased : he also found that esters of secondary alcohols based on n-alkanes were more favourable than those of branched alcohols. He also compared[205] the use of proline, hydroxyproline, 4-thiazolidine carboxylic acid and pyroglutamic acid as resolving agents and concluded that proline gave the best results. An examination of the relationship between separation factors and structure was undertaken for fifteen amino acids as their N-TFA-(S)-prolyl amino acid methyl esters,[206] and for twenty-five racemic amino acids as their N-TFA-(S)-prolyl amino acid n-butyl esters.[207]

α-Halogeno-acyl chlorides were shown[208] to be good reagents for the gas chromatographic analysis of amino acid methyl esters on SE-30, FFAP or DEGS phases. In particular the use of α-chloroisovaleryl chloride was recommended. Both α-chloropropionic[209,210] acid chloride and α-bromopropionic acid chloride[211] have been found suitable reagents for resolving amino acids but the use of α-chloropropionic acid is preferred [209] since it is readily prepared in optically pure form.

Recently three camphor-related compounds, (+)-isoketopinyl chloride, (-)-dihydroteresantalinyl chloride

and (-)-teresantalinyl chloride, have been applied[212] to the gas chromatographic resolution of amino acid methyl esters. Initial studies with DL-alanine using various stationary phases led to the choice of (-)-teresantalinyl chloride as resolving agent and twelve pairs of enantiomeric neutral and acidic amino acid derivatives were run on PEGA. Separation factors of the order 1.01-1.07 were achieved, for the n-butyl esters, and 1.03-1.10 for the methyl esters. The high resolution factors obtained reflect the utility of the rigid carbon skeleton directly linked to the amino acid *via* an amide bond, as suggested previously by ourselves[213] and other workers.[214]

1.2.3. Mechanism of separation

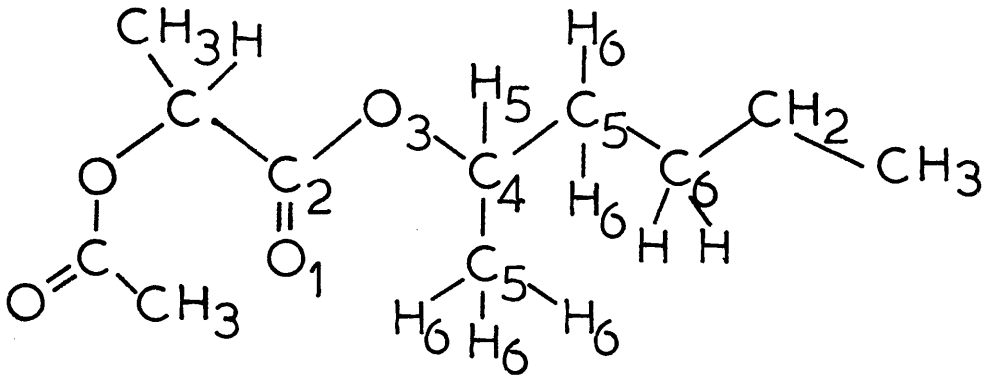
Various mechanisms have been suggested to account for the order of elution of diastereomers. In the case of unsaturated alcohols or diols, the diastereomer having the lower population of molecules in the hydrogen-bonded form has the higher retention volume on a polar phase.[215] Nurok *et al.*, when studying the separation of diastereomeric dialkyl esters of butane-2,3-diol, suggested[216] that a polar solvent stabilises the most polar conformer of a molecule with polar groups on adjacent atoms (*i.e.* the molecule with *syn* dipolar groups). The proportion of conformers existing with aligned dipoles will differ for each diastereomer, and hence the stabilisation will be

different for each diastereomer.

Differential interaction of functional groups with the stationary phase, due to different steric environments, can result in a separation of diastereomers. This reasoning has been applied to the study of the resolution of diastereomeric esters and amides, where the "key functional group" is the ester or amide grouping flanked by asymmetric centres.

It has been suggested[217] that the degree of steric crowding around the central polar group may be computed empirically by Newman's "Rule of Six".[218]

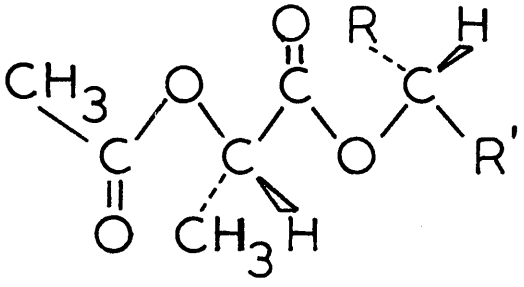
The calculation of the "six number" is illustrated below for 2-hexyl-α-acetoxypropionate.



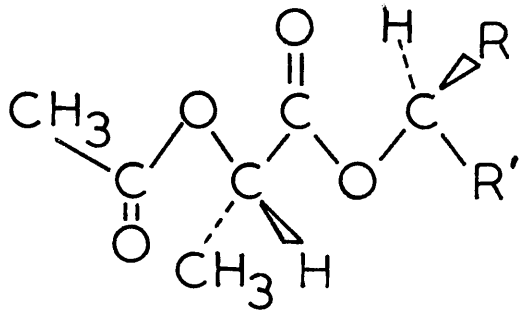
Commencing the count with the carbonyl oxygen, the number of atoms in position six is called the "six number". The rule is based on the assumption that a high "six number" is directly associated with increased steric hindrance at the group in position 1-2. The magnitude of the

effect will be dependent on absolute configuration and thus will be different for each diastereomer, leading to a separation. Correlations between resolution factors and the "six number" have been observed in the separation of α-acetoxypropionates of secondary alcohols[217] and of α-chloroalkanoyl valine methyl esters.[163] Data, however, have been cited[214,219] in disagreement with the empirical rule and it should only be used with caution.

Several workers have systematically studied[140,141,214,220,221] the influence of structural and polar variations on the separation factors and $\Delta(\Delta G^{o})$ values for series of diastereomeric esters and amides. Examination of the gas chromatographic behaviour of α-acetoxypropionates of secondary alcohols showed[140,220] that the $\Delta(\Delta G^{o})$ values for, and hence the separation of diastereomeric pairs increased as the size differential of the groups on the alcoholic asymmetric centre increased. The esters were assumed to exist in "preferred" conformations in solution which allowed differential accessibility to the central functional group in diastereomeric pairs.



a) (SS)-diastereomer



b) (SR)-diastereomer

As the size differential between R and R' increased the conformational mobility about the carbon-oxygen bond would decrease, resulting in an increased population of the preferred conformers. The (SS)-diastereomer would be more accessible for interaction with the stationary phase since the two bulkier groups, $-CH_3$ and R, are on the same side of the plane passing through the ester linkage, and the two hydrogen atoms are on the other side. As R increased in size the difference in accessibility to the diastereomers would also increase, with the resultant increase in $\Delta(\Delta G^{o})$. This proposal also accounted for the observed order of elution since the (SS)-diastereomer would be preferentially retained because of the increased interaction with the stationary phase.

Similar trends were noted in the separation of diastereomeric N-TFA-prolyl derivatives of racemic amines and amino-acids.[141,155] Incorporation of the alcoholic asymmetric centre into a ring system resulted in fairly large $\Delta(\Delta G^{o})$ values[141] due to the increased conformational immobility about the carbon-oxygen bond. A similar increase in $\Delta(\Delta G^{o})$ was observed for the N-TFA-(S)-prolyl derivatives of racemic cyclic amines.[155] The effect of the increased rigidity of the ring system in imposing "asymmetric preferred conformations" on the molecule was most noticeable in the case of the diastereomeric N-TFA-(S)-prolyl derivatives of 3-methylpiperidine, where a $\Delta(\Delta G^{o})$ value of -78 cal/mole was obtained. Previously introduction of a methylene group between the asymmetric centre and the

ester group had resulted in indistinguishable diastereomers.[220]

Examination of a series of structurally related diastereomers in which the groups attached to either side of the polar linkage were the same, showed[221] that the diastereomeric amide (R'CONHR') was better separated than the corresponding ester ($R'CO_2R'$) (R' = 1,2-dimethylpropyl). The reason for this difference in $\Delta(\Delta G^o)$ was attributed to the ability of the amide to hydrogen-bond to the solvent via the proton attached to the nitrogen. This reasoning was substantiated by a lowering of the $\Delta(\Delta G^o)$ value when this proton was replaced by a methyl group. (R'CO.NMe.R'). Introduction of an ethereal oxygen (R'-O-CO-OR', R^2-O-CO-OR^2), (R^2 = 1,2,2-trimethylpropyl) did not markedly affect the $\Delta(\Delta G^o)$ value although the separation of the asymmetric centres was increased. Ethereal oxygens can themselves contribute to the separation since R'O.CH_2.OR' and R^2O.CH_2.OR^2 were still resolved. However, further separation of the chiral centres in the di-ester of oxalic acid ($R^2O_2CCO_2R^2$) produced indistinguishable diastereomers. Diastereomeric symmetrically substituted hydrocarbons showed better resolution in compounds having a methylene group between the chiral centres than those without it.[222] Replacement of the methylene group by an oxygen atom produced even higher resolution factors.[223] When one of the chiral carbons, however, was replaced by silicon the resolution factors decreased appreciably, and when both

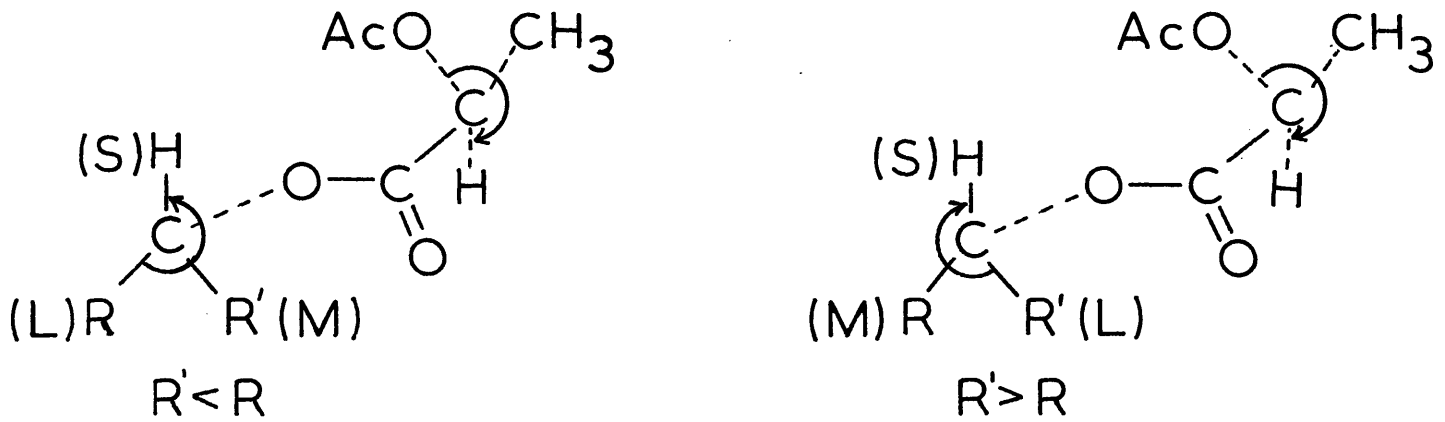


Fig. 8. Space projection of an (S)-alkanol-(S)-α-acetoxypropionate.

asymmetric centres were asymmetric silicon atoms no resolution was observed.[223]

Stern and coworkers[214] showed that steric effects are not the only factors involved in the separation mechanism, and electronic effects must also be considered. A series of methylalkyl α-alkylpropionates, where there is no polar group on the acidic centre, gave much poorer separations than the corresponding α-acetoxypropionates. These results, and those obtained on the separation of methyl 2(DL),4(L)-dimethylhexanoate[214] indicate that the polar group on the acidic centre may interact differently with the solvent for each member of the diastereomeric pair. Thus the total polar linkage, and not just the central ester linkage, may be thought of as contributing to the separation in terms of differential accessibility for the two diastereomeric forms.

Using experience gained during work on chiral stationary phases, Feibush proposed[225] a mechanism of resolution which explained both the observed order of elution of diastereomers and the separation factors obtained. If the diastereomer is viewed along its long axis, from the alcohol to the acid moiety, (Fig. 8) the substituents on the asymmetric carbons have either a clockwise or anti-clockwise arrangement going from the large (L) to the medium (M) to the small (S) group. The rule states that the diastereomer emerging last from the column is the one in which both asymmetric carbons have their substituents arranged in the same direction. If

the effective size of the two groups on the alcohol asymmetric centre is equal no separation will be observed. This was the case in the series of unsaturated alkylcarbinyl α-acetoxypropionates when R was methyl and R' vinyl ($CH=CH_2$).[214] As the difference in bulk between the R and R' groups increases the separation of the diastereomers also increases. If R' is bigger than R, the (SS) diastereomer will emerge last (Fig. 8) but if R becomes larger than R' the order of elution will reverse. Examination of the data obtained for the series of diastereomeric saturated and unsaturated alkylcarbinyl α-acetoxypropionates by Stern et al,[214] showed that a given structural change produced a constant difference in the free energy of solvation of the isomers.[225]

When considering the separation of α-hydroxy-propionates of 2-n-alkanols the formation of an intra-molecular hydrogen-bond between the free hydroxyl group and the carbonyl group was thought to contribute to the separation.[140,214] Disruption of this hydrogen-bond was given as a reason[214] for the decrease of $\Delta(\Delta G^{o})$ obtained as the alkyl chain attached to the alcoholic asymmetric centre was lengthened. A similar reasoning was used to explain the observed order of elution of the 2-alkanol esters of (S)-(+)-mandelic acid.[226] The diastereomers were believed to assume preferred conformations which resembled cis-trans isomers. The "cis"-form (SS) involved more intramolecular hydrogen bonding and was, therefore, eluted before the "trans"

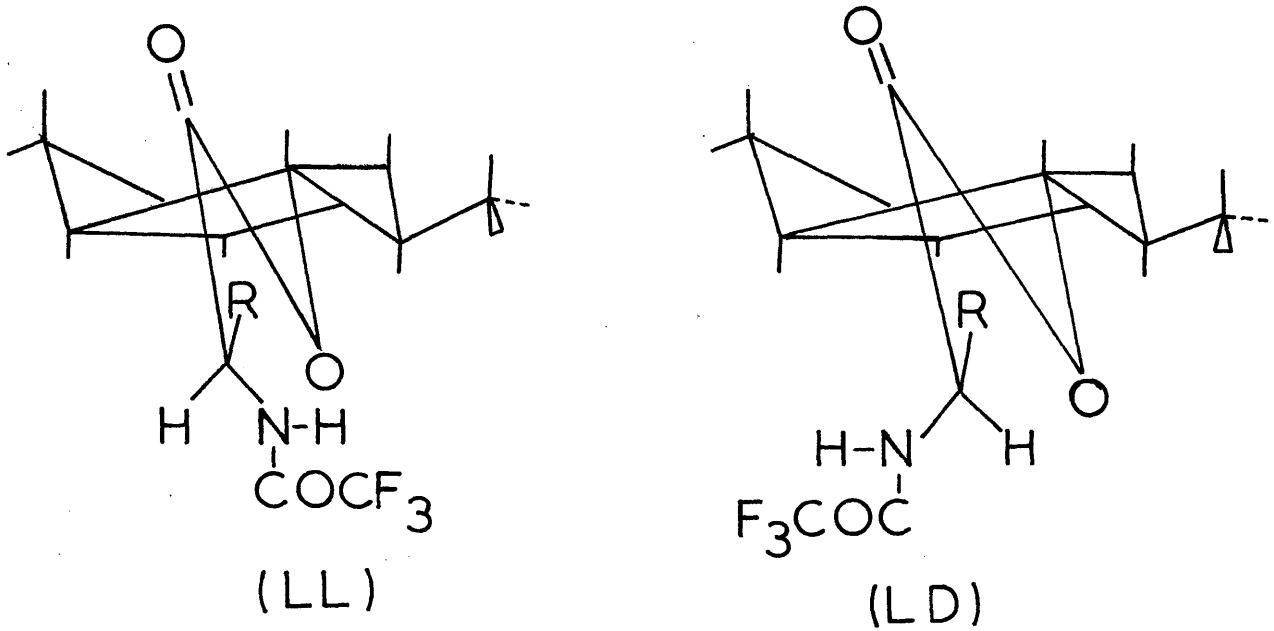


Fig. 9. Space projection of the diastereomeric derivatives of an N-trifluoroacetyl-α-amino acid with (L)-menthol.

(SR) which experienced more association with the phase.

Differential accessibility of the polar groups has recently been used to explain[187] the observed order of elution of the (L)-menthyl esters of N-TFA-amino acids. The steric repulsion between the isopropyl group in the menthyl moiety and the R-group in the amino acid fixes the "preferred" conformations as shown in Figure 9. In the ester of the (D)-amino acid the ester carbonyl and the amide group lie on the same side, where the steric hindrance of the isopropyl group is weak. In the derivative of the (L)-enantiomer the amide group is at the same side as the isopropyl group and is, therefore, shielded from the environment by this bulky group. This will result in stronger interactions between the (LD) diastereomer and the phase and hence its preferential retention. Infra-red evidence indicates that there is increased hydrogen-bonding between the (LD) diastereomer and polar solvents.

1.2.4. Resolution of enantiomers on chiral stationary phases

The first direct separation of enantiomers by gas chromatography was reported in 1966 by Gil-Av and co-workers.[227] Since that time a number of studies have evaluated various optically-active phases.[228-238] Two general classes of chiral solvent have been used :

1) derivatives of α-amino acids,[227,228] and di- and

tri-peptides.[229-235]

2) N,N'-disubstituted urea derivatives.[236,238]

In the former approach, promising results were obtained for the resolution of N-TFA-α-amino acid esters on capillaries coated with N-TFA-(L)-isoleucine lauryl ester or N-TFA-(L)-phenylalanine cyclohexyl ester.[227,228] Much higher resolution factors were obtained, however, when dipeptide phases were examined,[230] and N-TFA-(L)-valyl-(L)-valine cyclohexyl ester produced sufficient resolution to be applicable to packed column chromatography.[229] Examination of various solute structures showed that the separations improved with increasing bulk of the alcohol used to esterify the amino acid, (i.e. prim. < sec. < tert.)[230,231,233] and isopropyl esters were recommended[231] since they combined reasonable retention times with good resolution factors. Comparison of various perfluoroacyl derivatives indicated[239] that N-pentafluoropropionyl (PFP) derivatives were more volatile than N-TFA derivatives without adversely affecting the resolution. N-TFA-(L)-valyl-(L)-valine cyclohexyl ester was found to be a very useful phase for resolving the more volatile amino acids,[231,240] although some overlap of different amino acids did occur (e.g. serine and leucine). Operation of this phase above 100-115°C, however, was not feasible, and N-TFA-(L)-phenylalanyl-(L)-leucine cyclohexyl ester,[232] which could be operated up to 140°C, was more useful for resolving the less volatile amino acids.[246] Subsequent introduction of N-TFA-(L)-α-aminobutyryl-(L)-α-aminobutyric acid cyclohexyl

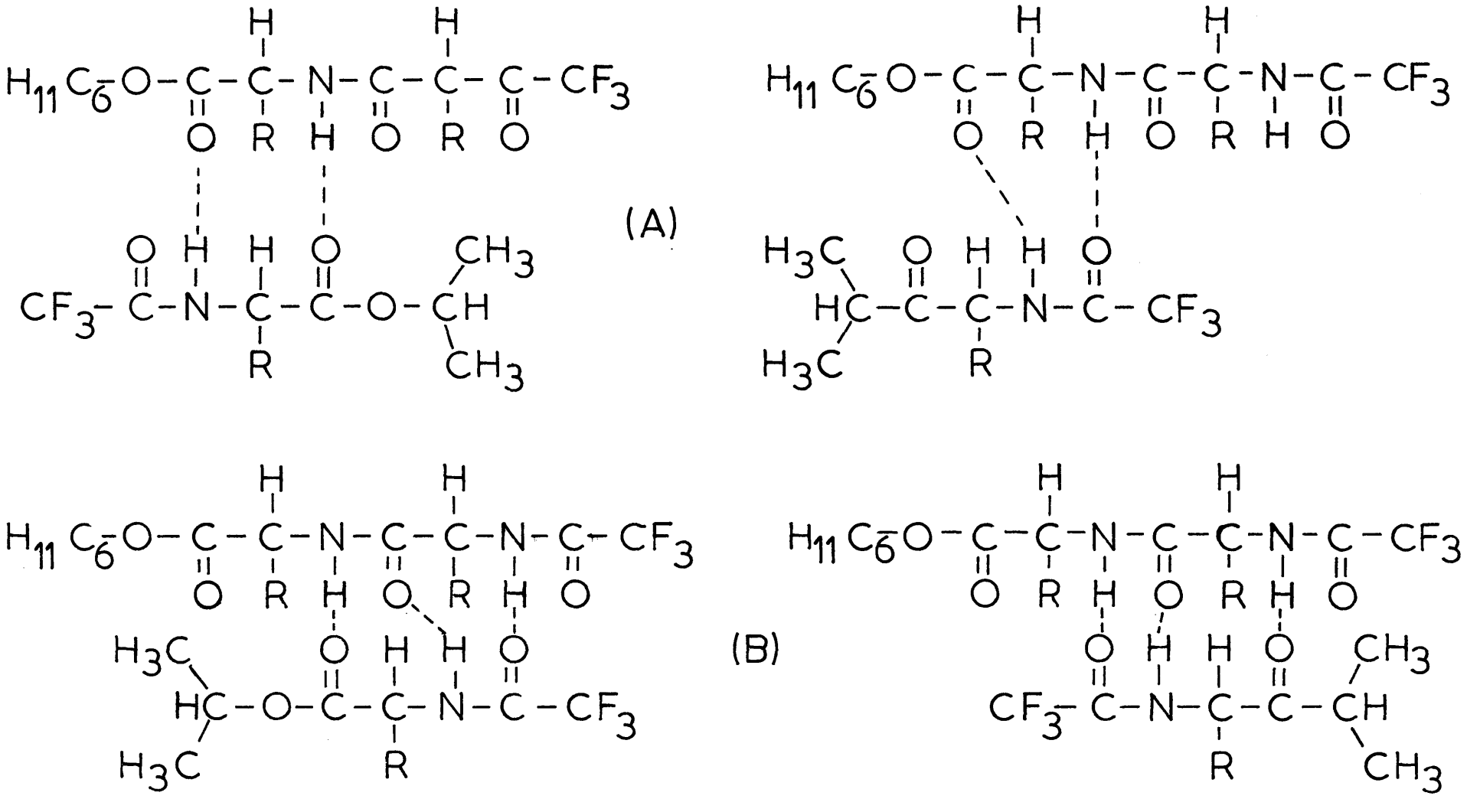
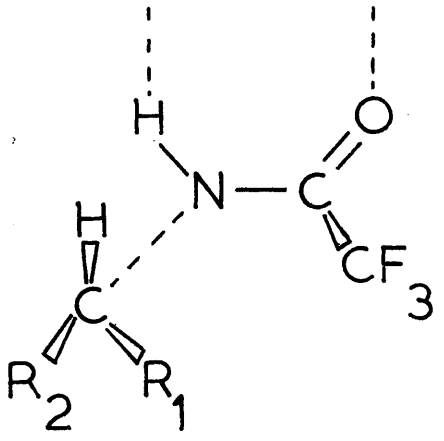


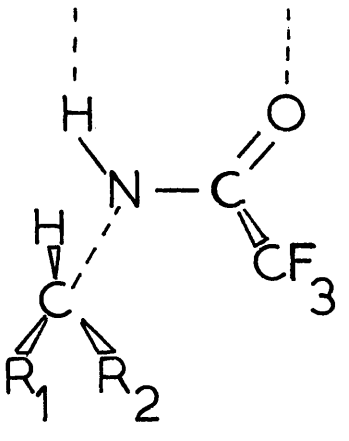
Fig. 10. Possible hydrogen-bonded complexes formed between a chiral solute and a chiral stationary phase involving either two (A) or three (B) points of interaction.

ester[235] allowed unambiguous separation of the more volatile amino acids. The separation of N-TFA-(DL)-amino acid isopropyl esters has been examined on several dipeptide phases[241] with respect to separation factors (α) and thermodynamic properties of interaction. An increase in size of the alkyl group on the asymmetric centre of the dipeptide stationary phase was found to produce greater solvent-solute interaction. The same modification applied to the side chain on the α-carbon of the solute, however, caused a decrease in interaction. Studies of this nature can lead to the prediction of the stationary phase necessary for particular separations.

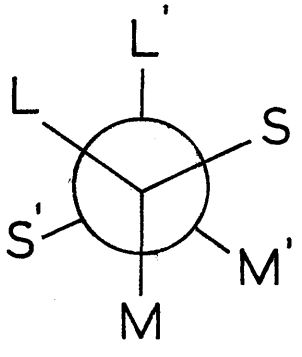
On all the (L)-peptide phases examined the (D)-amino acids were eluted before the (L). It was also noteworthy that only α-amino acids could be resolved on these phases. Both the resolution and the observed regularity of elution order were explained by the formation of "diastereomeric" association complexes between the solute and solvent[230,234] (Fig. 10). A three-point interaction complex[121] was favoured and subsequent experiments confirmed that it was the amide portion of the peptide (complex B) which was involved in the interaction.[242,243] Consideration of the association complex showed that the [-CO-NH-$\overset{H}{\underset{R}{C}}$-CO-] group was necessary for the formation of three hydrogen-bonds. This explained why only α-amino acid esters could be resolved. Examination of various solutes on N-TFA-(L)-α-amino-n-butyryl-(L)-α-amino-n-butyric acid cyclohexyl ester, however, showed[244] that the important structural feature for the resolution was the two carbonyl groups



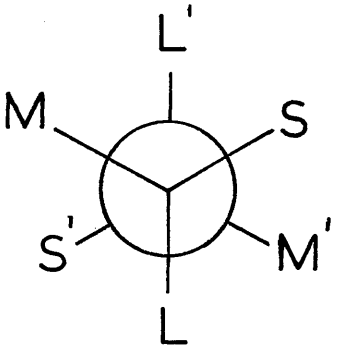
(1)



(2)



(3)



(4)

Fig. 11. Suggested association of enantiomers with a chiral phase to give either a clockwise (1) or an anti-clockwise (2) arrangement of R_1, R_2, and H. The order of elution of the enantiomers from the phase can be predicted if it is assumed that the two 'diastereomeric' association complexes can be described as if the two asymmetric centres were on vicinal carbons. [(3) and (4).]

separated by two atoms. Since N-TFA-prolyl esters could be resolved, although with poorer resolution factors, the N-H group could not be essential and a two-point association complex might be adequate in some instances.

The direct resolution of enantiomeric amines was eventually achieved on the optically active ureide, carbonyl-bis-(N-(L)-valine isopropyl ester).[236] The enantiomers were found to emerge in a consistent manner when the bulk of the substituents on the chiral carbon was considered. These observations led to the proposal of a mechanism of resolution[245] which was successfully applied to the assignment of the absolute configuration of optical isomers.[246] The ureide was considered to be linked through two hydrogen-bonds to the amide group (Fig. 11). Viewing the asymmetric carbon from the side remote from the nitrogen atom, projection formulae were obtained with clockwise (1) or anticlockwise (2) arrangements of the substituents, (R^1, R^2 and H). The order of emergence of the enantiomers and the magnitude of the resolution factors would depend on the relative stability of these "diastereomeric" association complexes. It was assumed that differences in stability could be interpreted in terms of "preferred" conformations and that the non-bonded interactions between the chiral solvent and the enantiomers could be described as if the two asymmetric centres were on vicinal carbons ((3) and (4)). One of the combinations of the enantiomers with the chiral solvent

would be less stable than the other (cf. Fig. 11. (3) less stable than (4) because of the interaction between L and L'). The larger the difference in size between the large and medium groups, the larger would be the effect on the relative stability and hence on the resolution factors. For a given configuration of solvent the order of emergence would depend on the clockwise ($_S$  $_M$) or anticlockwise ($_M$  $_S$) arrangement according to the size of the substituents, R^1, R^2 and H. On the (L)-ureide phase the enantiomer with the anti-clockwise arrangment emerged first. The configuration of an unknown could, therefore, be determined from its order of emergence if the relative sizes of R^1 and R^2 were known, e.g. for an amino acid R-CH-(NH)-CO_2R', the
$COCF_3$
first peak would have the D-configuration if $CO_2R' > R$ and the (L)-configuration if $CO_2R' < R$. Studies of the selectivity of hydrogen-bond formation in chiral ureide-amide systems have been performed with the aid of ^1H and ^{13}C NMR,[247] and the only significant hydrogen-bond interaction appeared to be between the ester carbonyl of the stationary phase and the amide amido proton.

Corbin and Rogers[248,249] significantly extended this work when they showed that separations could easily be achieved on the solid ureide of (L)-valine isopropyl ester as well as on the liquid phase. The resolution factors obtained on the solid ureide phase were larger than those obtained on the liquid phase, with a significantly shorter analysis time.

Further examination revealed that the ureide of (L)-valine isopropyl ester existed in two forms below its melting point[249] and that it was the high temperature form which effected the good resolutions. Examination of several ureide phases in the smectic liquid crystalline state and the isotropic liquid state has shown that enantiomers can be resolved with very large α values on optically active mesophases.[237,238]

1.3. Applications of gas chromatography-mass spectrometry in metabolism studies

1.3.1. Introduction

Mass spectrometry has become a powerful tool for metabolism studies since a large amount of structural information can be obtained with little expenditure of sample. The combination of gas chromatography with mass spectrometry allows the simultaneous analysis of multi-component mixtures, thus eliminating the necessity for extensive purification prior to analysis which considerably shortens analysis time. The extensive use of gas chromatography-mass spectrometry (GC-MS) in the metabolism field is exemplified by the vast amount of literature available on the subject and several pertinent reviews have been published.[250-253] The technique has application both in the detection and identification of metabolites and in their quantification in biological material.

1.3.2. The detection and structural identification of metabolites

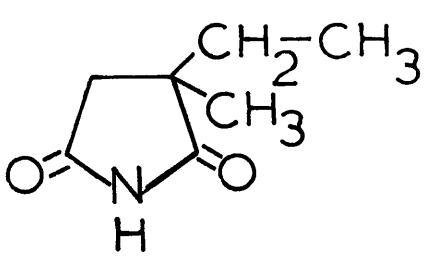
Direct analysis by GC-MS, of suitably derivatised extracts from body fluids or incubation media after the administration of a drug can produce valuable information about metabolite structures. Comparison of the spectra obtained with those of authentic material or closely related analogues can lead to complete structural identification.

Permethylation of barbiturates produces compounds amenable to GC-MS analysis.[254-256] This method has been applied to the metabolic study of several barbiturates in man.[257-260]

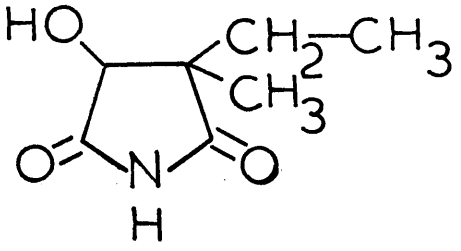
A previously unreported major urinary metabolite of ethosuximide (78) was isolated from a patient suffering from petit mal epilepsy and identified as the ring hydroxylated ethosuximide (79) by GC-MS and NMR of the N-methyl derivative.[261]

Hydroxylamines are now recognised as important metabolites of phenylalkylamines.[14,262] The hydroxylamines formed from primary amines are readily characterised by GC-MS as the corresponding oximes resulting from oxidation during GLC,[263] or as their more stable trimethylsilyl (TMS) ethers.[262,264] Further oxidation to C-nitroso- and nitro-compounds has been observed.[265] Secondary amines are metabolised to hydroxylamines, oximes and nitrones,[263] which are amenable to GC-MS analysis, although some decomposition of the secondary hydroxylamine may occur. The hydroxylamines derived from N-demethylchlorpromazine and its sulphoxide were identified as metabolites of chlorpromazine[266] and the mass spectra obtained by direct inlet techniques were compared with those of the main thermolytic products obtained during GC-MS analysis.[267] A knowledge of any thermal breakdown which occurs during GC-MS analysis permits the use of this technique even when labile compounds are being analysed.

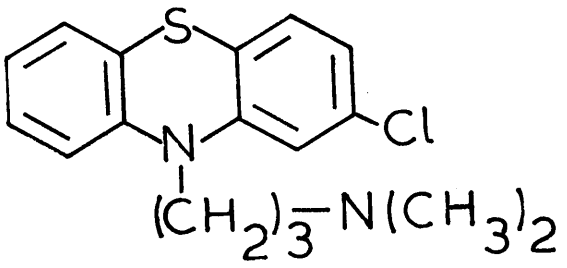
The metabolites of compounds which contain an atom



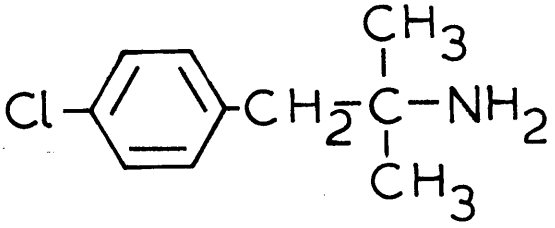
(78)



(79)



(80)



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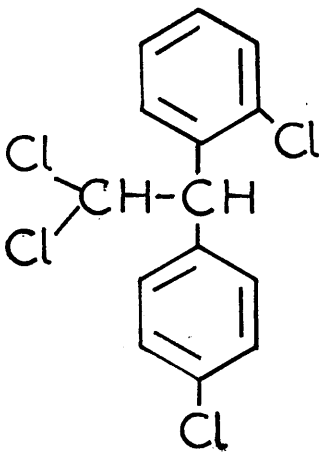


(82)

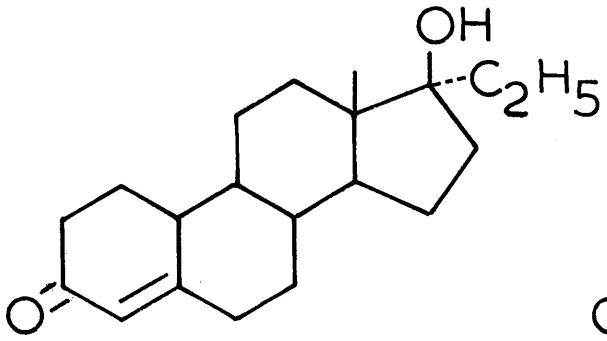
with an abundant second isotope e.g. chlorine (^{35}Cl : ^{37}Cl, 3:1) are easily recognised by the appearance of ion clusters of known relative abundance. The metabolism of many chlorinated compounds has been studied using this approach e.g. chlorpromazine,[268] (80) chlorophentermine,[265] (81) and mitotane (o,p'-DDD)[269] (82). When no such naturally occurring stable isotopes are present in the molecule use has been made of synthetic materials. Drugs or other precursors have been partially labelled with ^{15}N,[270] ^{13}C,[271,272] or ^{2}H[273] in such a way that the $[M]^{+}$ and $[M+1]^{+}$ ions occur in a 1:1 ratio. The metabolites are then easily recognised by the presence of the 'twin ions'. Other ion clusters can be easily created by the choice of suitable isotope combinations.[273]

The analysis of more complex mixtures of biological origin has been achieved with the increasing application of capillary column GC-MS[274-278] and the use of data handling systems.[279-285] When the computer is programmed with retention index data as well as mass spectra the number of authentic spectra which require searching during the identification of an unknown is greatly reduced.[286,287]

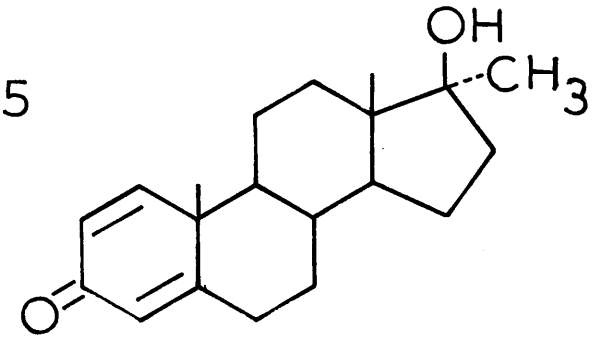
An alternative method of GC-MS analysis uses the mass spectrometer as a highly specific gas-chromatographic detector. This is achieved by fixing the applied accelerating voltage to focus one particular ion - single ion monitoring (SIM). Quickly switching the applied voltage to a number of pre-selected values, while keeping the magnetic field constant, brings a series of ions

into focus within a short time - multiple ion detection (MID). MID was achieved by application of an accelerating voltage alternator (AVA) which could monitor up to three characteristic ions by automatically altering the applied accelerating voltage so swiftly that the different ions were all apparently recorded simultaneously.[288] With the latest magnetic sector instruments up to four mass fragments within a 10-30% mass range can be monitored using the AVA.[289] Recent modifications employing computer systems have improved the stability of the system.[290,291] (Many more mass fragments, however, over the whole mass range can be monitored using a quadrupole mass spectrometer.[292]) This technique has been termed "mass fragmentography" by Hammar et al[268] and its application in biological research has been recently reviewed.[293]

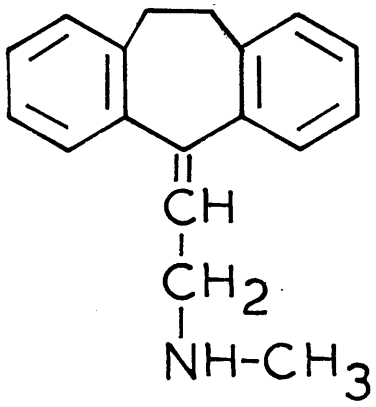
Mass fragmentography allows very specific metabolite searches to be carried out. A knowledge of the precursor structure and metabolism of related compounds permits the prediction of metabolite structures and the choice of characteristic fragment ions. Monitoring of one or more predicted ions can establish the presence or absence of the metabolites postulated to be present in the biological material. TMS ethers of steroid drug metabolites containing 17α-alkyl-, 17α-alkenyl-, and 17α-alkynyl-17β-ols may be detected selectively by the abundant ions at m/e 128+R which in general do not occur in the mass spectra of natural steroids.[294] The fragmentation mechanism has been


OH
C2H5
O

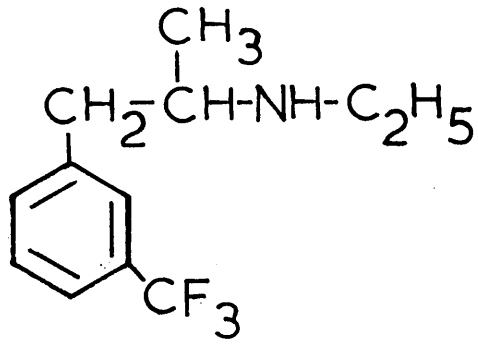
(83)


OH
CH3
O

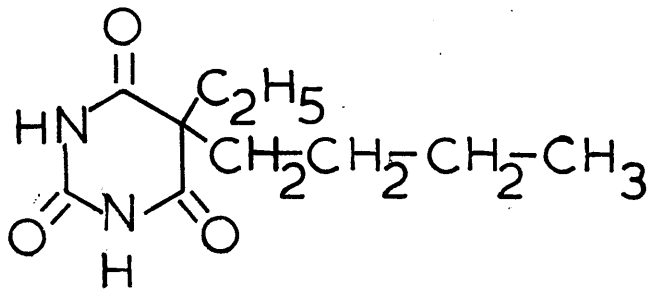
(84)


CH
CH2
NH-CH3

(85)

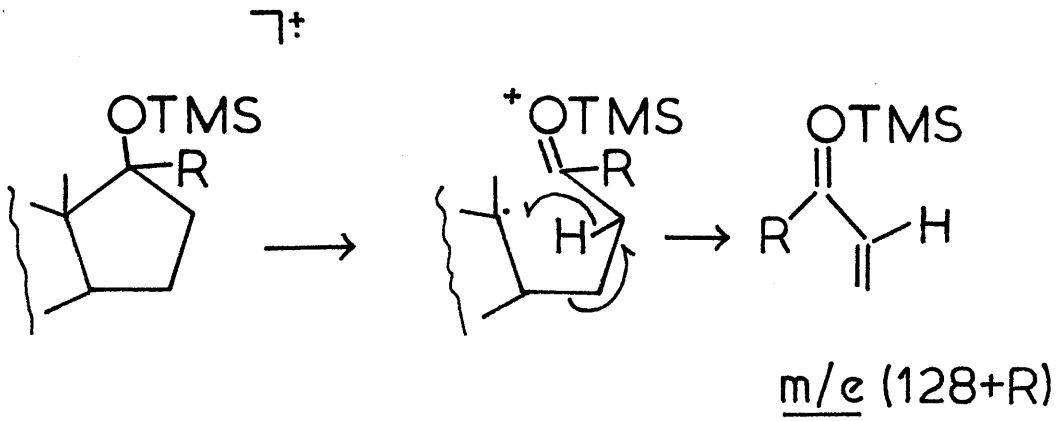

CH3
CH2-CH-NH-C2H5
CF3

(86)


O
C2H5
HN
CH2CH2CH2CH3
O
N
O
H

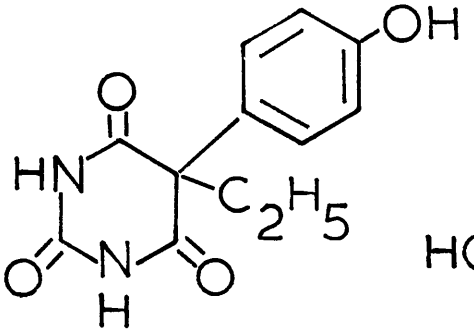
(87)

elucidated by deuterium labelling :[295]

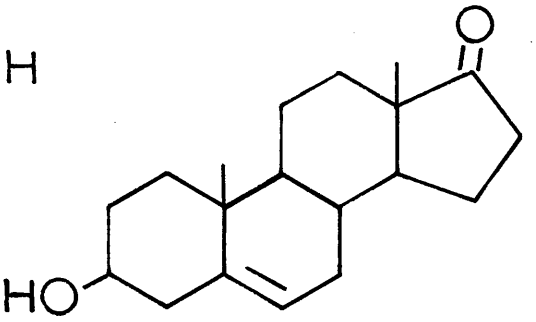


Preliminary investigations of the metabolism of the anabolic steroids, 17α-ethyl-17β-hydroxyestr-4-en-3-one (83) and 17β-hydroxy-17α-methylandrostan-1,4-dien-3-one (84) used these techniques.[294] Mass fragmentography has been successfully applied to the identification of 10-hydroxynortriptyline, desmethylnortriptyline and demethyl-10-hydroxynortriptyline, metabolites of nortriptyline (85).[296,297] The technique also facilitated the detection of the N-dealkylated metabolites of methamphetamine (2) and fenfluramine (86) in rat tissue after i.p. administration.[30] A new metabolite of butobarbitone (87) 3'-ketobutobarbitone, was detected in urine by mass fragmentography.[259]

The use of precursors labelled with stable isotopes is readily amenable to mass fragmentographic analysis. Simultaneous monitoring of fragment ions at the isotopic masses allows identification of metabolites. An excess of



(88)



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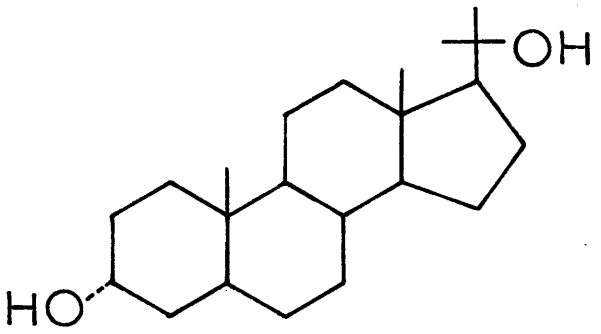
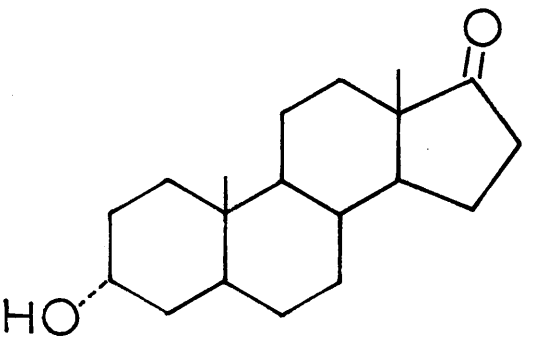
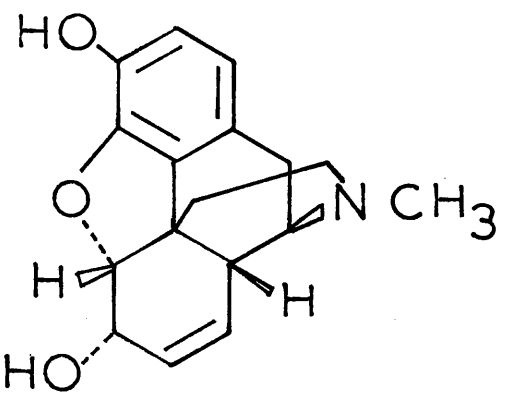


(90)



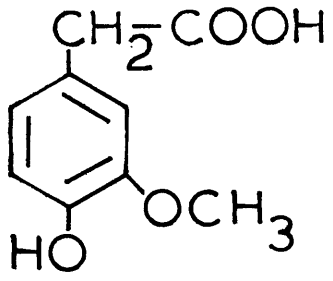
(91)



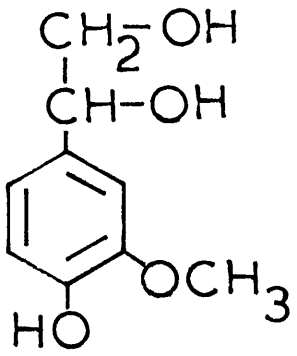
(92)

added labelled compound may also act as a carrier for endogenous material and reduce losses during work up and GLC. This has been applied to prostaglandin analysis.[298,299] The use of stable isotopes may lead to the discovery of previously unknown metabolites as in the case of the study of the metabolism of nortriptyline labelled with ^{2}H and ^{15}N.[300]

Chemical ionisation mass spectrometry employs a milder form of ionisation produced by ion molecule reactions between the sample and ionised reagent gas. This reaction normally results in the formation of the $[M+H]^{+}$ ion or quasi-molecular ion. (The abundance of this ion is lower when methane is used as reagent gas.) By selection of an appropriate reagent gas the degree of fragmentation obtained can also be varied, although in general little fragmentation is observed. This technique should have considerable application in drug metabolism work because of the production of abundant quasi-molecular ions, however, few studies have been reported as yet. Using methane as reagent gas the major metabolites of sodium phenobarbital (29), p-hydroxyphenobarbital (88), dehydroepiandrosterone (89), pregnanediol (90) and androsterone (91) were easily detected by their quasi-molecular ions.[301] It was not necessary to separate all the metabolites as individual peaks in the gas chromatograph to obtain identification. Normorphine and norcodeine have been identified by their chemical ionisation spectra as being metabolites of morphine (92)



(93)



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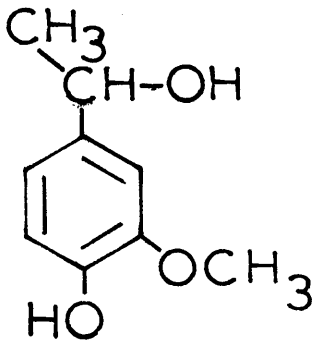

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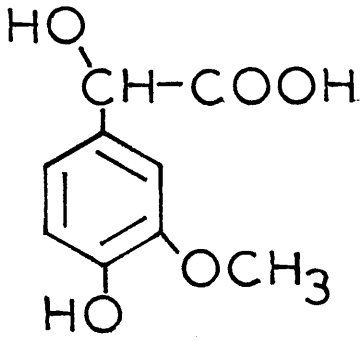

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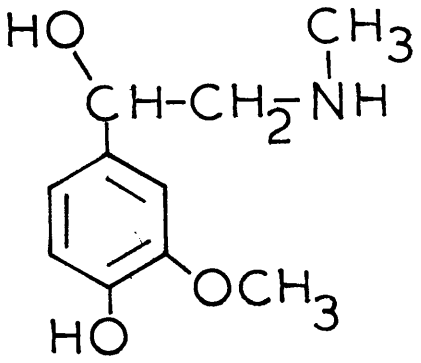

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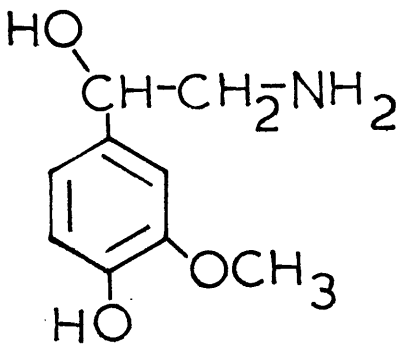

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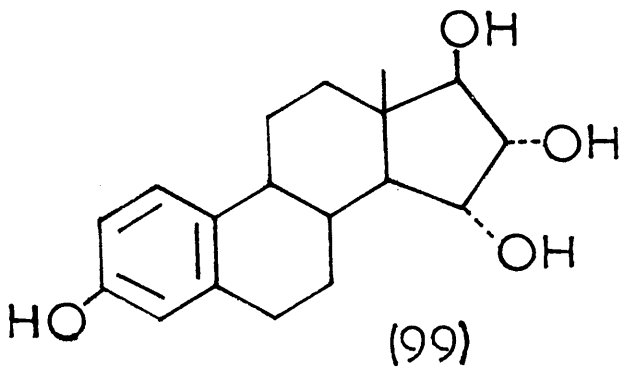


(99)

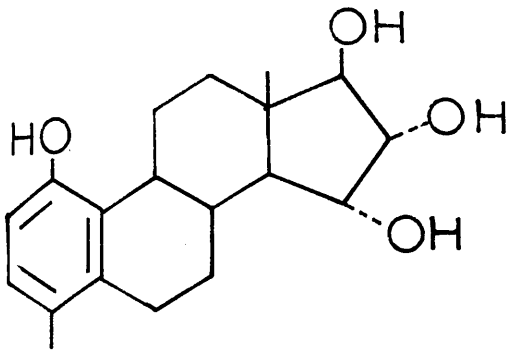
in man.[302]

1.3.3. The quantification of drugs and drug metabolites in biological material

Quantification is most suitably effected by the techniques of mass fragmentography already described. Addition of an internal standard to the biological material prior to extraction accounts for any losses which may occur during work-up. Two types of internal standard have been employed *viz* : a closely related analogue with suitable gas chromatographic properties or the drug itself labelled with a suitable stable isotope.

Biogenic amines occurring at low concentrations have been analysed by SIM of various derivatives, often also suitable for electron capture analysis *e.g.* pentafluoropropionates,[303,304] heptafluorobutyrates,[304] TMS ethers,[305] and pentafluorobenzylimine - TMS derivatives.[306] Homovanillic acid (93) has been estimated in body fluids using deuterated[307] or isomeric[308] compounds as internal standards. Similar methods were used for 4-hydroxy-3-methoxyphenylglycol (94),[309] 4-hydroxy-3-methoxyphenylethanol (95)[310] and 4-hydroxy-3-methoxymandelic acid[311] (96). Alternatively, metanephrine (97) and normetanephrine (98) could be quantified as the *O*-ethyl ethers produced by derivatisation with EtOH-HCl.[312]

It was found possible to measure estra-1,3,5,-triene-3,15α,16α,17β-tetrol (estetrol) (99) in pregnancy urine



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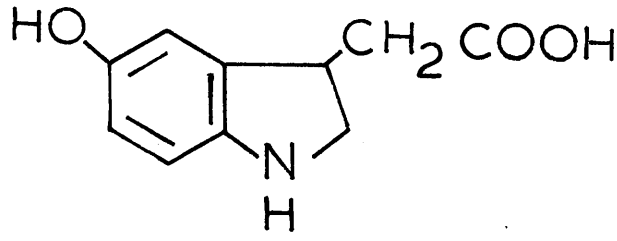

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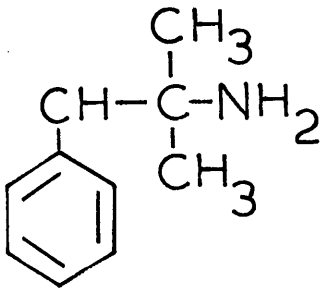

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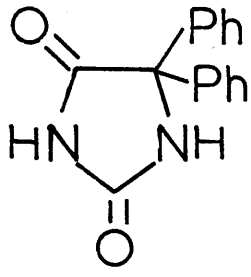

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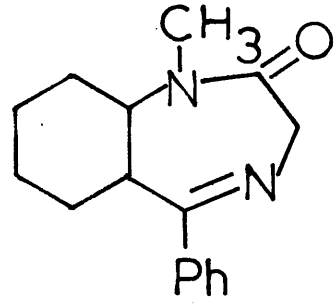

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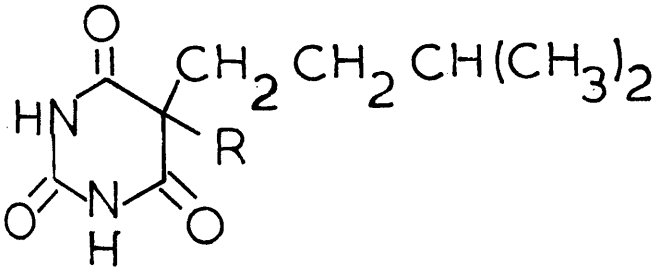
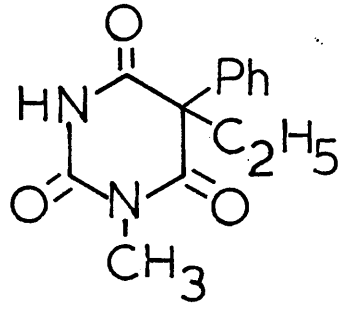
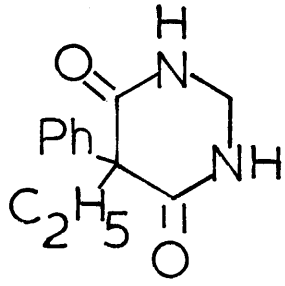


(105) R = C_2H_5

(106) R = CH_2–CH=CH_2



(107)



(108)

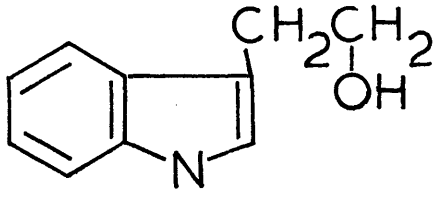
from the tenth week of pregnancy by monitoring the ion at m/e 191 and using 4-methyl-estra-1,3,5(10)-triene-1,15α,16α,17β-tetrol (100) as internal standard.[313]

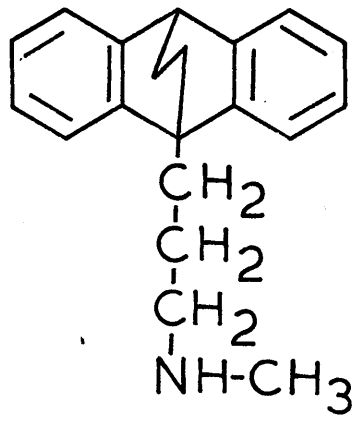
There have been many reports published on the determination of drugs and drug metabolites at the nanogram (ng) level using closely related analogues as internal standards.[314-317] Some examples of such studies are included in Table 5.

The use of deuterium labelled derivatives of the studied substance as an internal standard allows quantitative determination of biological metabolites occurring at low levels. Examples of analyses include 5-hydroxyindole-3-acetic acid (101) in cerebrospinal fluid,[318] amphetamine (1)[320,321] and phentermine (102)[321] in plasma and brain, rare amino acids in heart cell culture, endogenous corticosteroids in rat adrenals and blood, catecholamines in rat adrenals, and estrogens in non-pregnant women.[322]

^{13}C-labelled drugs have also been used as internal standards to quantify drugs and drug metabolites in urine, plasma, and breast milk.[323-325] [2,4,5,-$^{13}C_3$]-diphenylhydantoin, [2,4,5-$^{13}C_3$]-phenobarbital, and [1-C^2H_3] valium were used to quantify diphenylhydantoin (103),[323-325] phenobarbital (29),[324,325] and valium (104)[325] respectively. [2,4,5-$^{13}C_3$]-phenobarbital has been used to quantify amobarbital (105), secobarbital (106), butobarbital (87) and pentobarbital (72),[323,325] and mephobarbital (107) and primidone (108).[324] Analyses

Table 5. The quantitative estimation of drugs and drug metabolites in biological media using mass fragmentography.

Compound	Standard	Sensitivity	Ref.
Homovanillic acid (HVA)	HVA-[$^2H_3$]-methyl ester	9 ng/ml	307
	Isohomovanillic acid or 4-methoxy mandelic acid	cerebro-spinal fluid (CSF)	308
4-hydroxy-3-methoxy-phenyl glycol (HMPG)	a) 1-(4-hydroxy-3-methoxyphenyl)-2-[$^2H_2$]-glycol	1 ng/ml	
	b) 4-hydroxy-3-methoxy mandelic acid methyl ester		309
	c) HMPG-[$^2H_3$]		
4-hydroxy-3-methoxy-phenyl ethanol	tryptophol 	20 ng	310
4-hydroxy-3-methoxy mandelic acid	4-hydroxy-3-[$^2H_3$]-methoxy mandelic acid		311
5-hydroxyindole-3-acetic acid (HIAA)	[$^2H_2$]-HIAA	2-50 ng/ml CSF	318, 367

Compound	Standard	Sensitivity	Ref.
Nortriptyline and metabolites	CIBA 34276 	10 ng/ml plasma	319
Piribedil	2-N-benzylamino-5-chloro-benzophenone	10 ng/g or ng/ml brain tissue or plasma	317
Lidocaine and metabolites	Trimecaine	0.5-10 µg/ml	314
Oestetrol	4-methyl-oestra-1,3,5(10)-triene-1,15α,16α,17β-tetrol	5 ng	313
Amphetamine	[$^{2}H_{3}$]-amphetamine	0.6-1 ng/ml	320
Phentermine	[$^{2}H_{3}$]-Phentermine	1 ng/ml	321
Diphenylhydantoin	[2,4,5-$^{13}C_{3}$]-diphenylhydantoin	pg	323-325
Phenobarbital	[2,4,5-$^{13}C_{3}$]-phenobarbital	pg	324-325
Secobarbital	"	pg	323, 325

Compound	Standard	Sensitivity	Ref.
Pentobarbital	[2,4,5-$^{13}C_3$]-phenobarbital	pg	323, 325
Mephobarbital	"	pg	324
Primidone	"	pg	324
Amylobarbital	"	pg	323, 325
	butobarbitone	0.01 μg/ml	315
Butobarbitone	pentobarbitone		316
	[2,4,5-$^{3}C_3$]-phenobarbital	pg	324, 325
Valium	1-C[$^{2}H_3$]-valium		325
Prostaglandin E_1	[$^{2}H_3$]-methoxime of PGE_1	3 ng	299
Prostaglandins E_2 and $F_{2\alpha}$	[3,3,4,4-$^{2}H_4$]-PGE_2 [3,3,4,4-$^{2}H_4$]-$PGF_{2\alpha}$	250 pg	298
Indole alkylamines	α-methylserotonin N-acetyltryptamine	10^{-12}-10^{-13} moles	303a
Biogenic amines	S.I.M.	10^{-15} moles	303c 304 305

were carried out in the picogram - nanogram range using both electron ionisation and chemical ionisation techniques with the aid of data handling systems.

Section 2. Experimental

Table 6

Source of Reagents

Supplier	Reagent/Substrate
Dr. S.W. Head Pyrethrum Marketing Board, Nakuru, Kenya	(R)-(+)-trans-chrysanthemic acid
Norse Laboratories Inc., Santa Barbara, Ca., U.S.A.	(R)-(-)-Phenylbutyric acid (S)-(+)-Phenylbutyric acid (S)-(+)-α-Chlorophenylacetyl chloride
Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.	(RS)-Phenylpropionic acid
PCR Inc., Gainesville, Florida, U.S.A.	(RS)-3-Methyl-2-phenylbutyric acid
Burdick and Jackson, Laboratories Inc., Muskegon, Michigan, U.S.A.	(RS)-α-Methoxyphenylacetic acid
Dr. A.A. Manian, NIMH, Bethesda, Rockville, Md., U.S.A.	para-chloroamphetamine

Fluka A.G.,	(R)-(-)-Menthol
Buchs,	(R)-(-)-Borneol
Switzerland	(RS)-Borneol
	(S)-(+)-2-Octanol
	(R)-(-)-2-Octanol
	(R)-(-)-Mandelic acid
	(S)-(+)-Mandelic acid
	(R)-(+)-N-methyl-α-phenylethylamine
	(S)-(-)-N-methyl-α-phenylethylamine
	(R)-(-)-Valine
	(RS)-Proline
	(RS)-Phenylglycine
	(R)-(-)-Phenylglycine
	(R)-(+)-Phenylalanine
	(S)-(-)-Phenylalanine
	(1S,2R)-(+)-Ephedrine HCl
Koch-Light Laboratories Ltd	(RS)-Pantolactone
Colnbrook,	(RS)-3-Methoxy-4,5-
Bucks.,	methylenedioxyamphetamine
England	(R)-(-)-Norvaline
L. Light & Co. Ltd.,	(1R,2R)-(-)-ψ-Ephedrine HCl
Colnbrook,	(1S,2R)-(+)-Nor-ephedrine Sulphate
England	(1R, 2R)-(-)-Nor-ψ-ephedrine HCl

Sigma,	(R)-(-)-Pantolactone
St. Louis,	(S)-(+)-Amphetamine sulphate
Mo., U.S.A.	(S)-(+)-Methamphetamine HCl
	(RS)-Methamphetamine HCl
	(S)-(-)-Proline
R.N. Emanuel Ltd.,	(R)-(+)-α-Phenylethylamine
Wembley,	(S)-(-)-α-Phenylethylamine
Middlesex	(R)-(-)-Amphetamine
BDH Ltd.,	(RS)-Norvaline
Poole,	(S)-(+)-Valine Methyl ester HCl
England	(1R,2S)-(-)-Ephedrine HCl
Prof. J.I.G. Cadogan,	(S)-(+)-Menthol
University of Edinburgh	(S)-(+)-Isomenthol
	(RS)-Isomenthol
	(S)-(+)-Neomenthol
	(RS)-Neomenthol
Dr. J.D. Gilbert,	Methyl (RS)-12-hydroxystearate
University of Glasgow	Methyl (R)-(-)-12-hydroxystearate
	Methyl (RS)-13-hydroxystearate
	Methyl (S)-(+)-13-hydroxystearate
Drs. G. Amiard and	(±)-Estradiol 3-methyl ether
R. Bucourt,	(+)-Estradiol 3-methyl ether
Roussel-UCLAF,	
Romainville, France	

2.1. Gas chromatographic resolution of enantiomers

2.1.1. Reagents and substrates

Most reagents and substrates were obtained commercially or as gifts, as shown in Table 6.

Drimanoic acid was synthesized by the oxidation of drimanol, obtained by catalytic hydrogenation of drimenol.[326] To drimanol (250 mg) in "AnalaR" acetic acid (3 ml) was added dropwise a solution of chromium trioxide (87 mg) in 80% acetic acid (3 ml) containing potassium hydrogen sulphate (83 mg). After 1 hour, the solution was diluted with water and extracted with diethyl ether. After washing with water to remove acetic acid, drying and removing the solvent, the extract yielded a pale green oil. This oil in ethanol (10 ml) was added dropwise to a freshly prepared suspension of silver oxide (prepared from 360 mg $AgNO_3$ by treatment with aqueous potassium hydroxide) and the resulting solution adjusted to pH 11. After stirring and refluxing at 95°C for 1 h, the solution was cooled, diluted with water, and filtered. Ether extraction removed neutral organic material. Acidification and ether extraction yielded a pale yellow oil which crystallized from aqueous methanol to give white prisms of drimanoic acid (60 mg), m.p. 133-136°C.

(S)-(+)-α-Phenylpropionic acid was obtained from the racemic acid by fractional crystallisation of the salts formed with (R)-(+)-α-phenylethylamine, from acetone. After four recrystallisations the free acid was liberated with HCl and extracted into ether. The liberated acid had $[\alpha]_D$ +45°

(C, 1 g/100 ml in chloroform). Gas chromatographic analysis of the R-(+)-α-phenylethylamide indicated an optical purity of approximately 90%.

Isoborneol

The lithium aluminium hydride reduction of (+)- and (-)-camphor produced (-)- and (+)-isoborneol, respectively as the major product. The minor product was (+)- or (-)-borneol.

Fenchol

Similarly the major product from the reduction of (+)-fenchone was (-)-fenchol, and (+)-fenchol was obtained from (-)-fenchone.

(±)-17β-hydroxy-4-estren-3-one was obtained by the selective borohydride reduction[327] of 4-estrene-3,17-dione.

2.1.2. Methods of acylation

(a) Acid chlorides

Acid chlorides were prepared in toluene by the action of freshly redistilled thionyl chloride on the acid, at 40-60°C for 1 h. In the small scale preparations, excess thionyl chloride was removed in a stream of nitrogen and the acid chloride was used immediately without purification.

Drimanoyl and chrysanthemoyl derivatives were prepared with 1 mg of alcohol or amine and an excess of acid chloride (3 molar ratio), in toluene at 60°C or 40°C respectively, for 1-2 h.

Amides of the (2)-phenylalkanoic acids were prepared on a 10 μ molar scale using a molar equivalent of acid chloride, in toluene at room temperature for 10 min.

The products of these acylations were analysed by gas liquid chromatography (GLC) without purification. Synthesis of some reference samples of esters and amides allowed standardisation of the analytical method.

Drimanoic acid (30 mg) in dry toluene (3 ml) was treated with redistilled thionyl chloride (300 μl) under reflux for 1 h. Removal of excess thionyl chloride and solvent yielded a yellow oil which was purified by vacuum distillation ($60^{o}C/0.2$ mm Hg) to give a colourless oil, which showed $\nu_{C=O}$ 1805 cm^{-1} (liquid film). The purified drimanoyl chloride was added to a solution of l-menthol (30 mg) in toluene and refluxed for 2 h. The toluene was removed by distillation leaving a dark yellow oil which was purified by preparative thin layer chromatography (TLC) using benzene:EtOAc, 20:1 v/v as mobile phase, yielding a pale yellow oil (25 mg), $\nu_{C=O}$ = 1725 cm^{-1} (liquid film). After purification by vacuum sublimation (block temperature $80^{o}C/0.5$ mm Hg), analysis gave C, 80.0; H, 11.9% $C_{25}H_{44}O_{2}$ requires C, 79.7; H, 11.8%.

Similarly l-menthol chrysanthemate was prepared, Found : C, 78.2; H, 11.2; Calc. for $C_{20}H_{34}O_{2}$: C, 78.4; H, 11.2%.

The chrysanthemoyl derivative of l-amphetamine was purified by vacuum sublimation ($80^{o}C/0.2$ mm Hg) to give white

crystals, m.p. 97-101°C; ν_{N-H} = 3430, $\nu_{C=O}$ = 1675 cm^{-1} (CCl_4 solution). Found : C, 79.9; H, 9.6; N, 4.9% $C_{19}H_{27}ON$ requires C, 80.0; H, 9.5; N, 4.9%.

(b) Acid anhydrides

Acid anhydrides were prepared by the reaction of the acid chloride with a slight excess of the sodium salt, in toluene solution, under reflux for 1 h. After filtration to remove sodium chloride, and removal of solvent in vacuo, traces of unreacted acid were removed by vacuum sublimation using a cold finger apparatus. The purity of the anhydride was checked by infra red spectroscopy.

Phenylbutyric : acid $\nu_{C=O}$ = 1700; acid-Cl $\nu_{C=O}$ = 1799; anhydride $\nu_{C=O}$ = 1810 and 1742 cm^{-1}.

Phenylpropionic : acid $\nu_{C=O}$ = 1705; acid-Cl $\nu_{C=O}$ = 1800; anhydride $\nu_{C=O}$ = 1815 and 1745 cm^{-1}.

Preparation of amides : The amine or amino acid methyl ester (10 μmol) was treated with an equimolar quantity of acid anhydride in toluene (50 μl) at room temperature, for 10 min. After dilution with ethyl acetate (1 ml), the products were analysed by GLC and gas chromatography-mass spectrometry (GC-MS) without further purification.

A reference standard of amide was prepared from (+)-α-phenylethylamine and (±)-α-phenylbutyric anhydride. After purification by vacuum sublimation (block temperature 105°C, 4 mm) this had m.p. 70-73°C. Found : C, 80.9; H, 7.8; N, 5.4%. $C_{18}H_{21}NO$ requires C, 81.2; H, 7.9; N, 5.3%. Infra-red spectroscopy showed ν_{NH} = 3280 and $\nu_{C=O}$ = 1640 cm^{-1}.

(c) Imidazolides

The preparation of imidazolides is outlined below for phenylbutyric acid.

N,N'-carbonyldiimidazole (180 mg) was stirred at room temperature with (RS)-α-phenylbutyric acid (180 mg) in dry, redistilled chloroform for 30 min. Evolution of CO_2 was observed. After shaking with water to remove imidazole the chloroform layer was dried over Na_2SO_4 and evaporated to dryness to yield a pale yellow oil which crystallised on standing. Recrystallisation was effected from benzene yielding white crystals, m.p. 75-80°C, $\nu_{C=O}$ = 1740 cm^{-1} (CCl_4 soln.).

Found : C, 71.3; H, 6.8; N, 12.8%. $C_{13}H_{14}ON_2$ requires C, 71.9; H, 6.6; N, 13.1%.

The ^1H NMR spectrum showed δ, 8.15 (1H, s)[-H on C-2 of imidazole ring]; 7.4 (1H, d, J = 2 Hz) [-H on C-5 of imidazole ring]; 7.2 (5H, s) [aromatic-H]; 6.94 (1H, d, J = 2 Hz) [-H on C-4 of imidazole ring]; 4.0 (1H, t, J = 7 Hz) [benzylic-CH-]; 2.0 (2H, m, J = 7 Hz) [$-CH_2-$]; 0.92 (3H, t, J = 6 Hz) [$-CH_3$].

The resonances were assigned to the protons in the imidazole ring on the basis of the ^1H NMR spectrum of N-acetyl imidazole.[328]

The mass spectrum (22.5 eV) showed M^+ 214, 12%; 91, 100% [tropylium cation]; 119, 92% $[Ph-CH-Et]^+$; 146, 47% [M^+-imidazole].

For small scale reactions the imidazolides were

prepared using 10 μmol of acid in dry chloroform (50 ul) with an equimolar amount of N,N'-carbonyldiimidazole. After 10-15 min at room temperature the amine or amino acid methyl ester (10 μmol) was added and the reaction mixture left at room temperature for 2½ h. The solution was shaken with 50 μl water for 1 min, then centrifuged for 5 min. The chloroform layer was removed, passed through a small column of Na_2SO_4, and blown to dryness in a stream of nitrogen. The sample was then dissolved in ethyl acetate for analysis by GLC.

2.2. Drug metabolism

2.2.1. Isolation of urinary metabolites

(a) Spironolactone metabolites

Steroids and steroid conjugates were selectively adsorbed from urine onto the surface of Amberlite XAD-2 (Rohm and Haas, Ltd.), a synthetic insoluble crosslinked polystyrene polymer, supplied in bead form. 1 Kg resin was washed with distilled water until free from chloride and washed with methanol until no further elution of styrene was observed. A column was packed using a slurry of the resin in distilled water and prepared for use according to the method of Bradlow.[329] 2-4 Litres of urine were percolated through the column under gravity flow, followed by 4-6 litres of water. The steroids were then eluted with 4-5 litres of methanol.

After removal of the solvent under vacuum the residue was partitioned between chloroform and water, yielding free steroids (chloroform layer) and conjugated steroids (aqueous layer).

(b) Ibuprofen metabolites

Urine was diluted with saturated brine and extracted with ether and ethyl acetate. The pH was then adjusted to ~ 1 with 12M HCl and the urine was re-extracted with ether-ethyl-acetate. The combined extracts were back-washed with aqueous NaOH solution and the organic phase was discarded. After re-acidification the aqueous layer was extracted with

ether and ethyl acetate and the combined extracts dried over Na_2SO_4 and evaporated at reduced pressure to yield a crude acidic extract. The aqueous layer was retained since it contained conjugated metabolites.

2.2.2. Hydrolysis of conjugated metabolites

Conjugates were hydrolysed by incubation with "Glusulase" (Endo Lab. Inc. ; 179,000 units/ml β-glucuronidase, 47,000 units/ml sulphatase) in 0.1M acetate buffer at pH 4.6 and 37°C for 24 h. 2.5 ml enzyme was used per litre of urine. After adjustment of the pH to 5.5 and addition of another 1.5 ml enzyme per litre urine, the incubation was continued for a further 24 h. The solution was then diluted with saturated brine and solvent extracted as before.

2.2.3. Rat liver preparations

A rat was killed by a blow to the neck, the liver removed and immediately minced and homogenised in phosphate buffer (0,1M, pH 7.6). The homogenate was spun at 2,000 g for 10 min and the supernatant removed. NADP, NADPH, GSH, Glc-6-phosphate, $MgCl_2$ and EDTA were added to the supernatant as co-factors, then ibuprofen and Glc-6-phosphate-dehydrogenase were added and the mixture incubated at 37°C for 3 h. Solvent extraction as previously described yielded a crude acidic extract.

2.3. Analytical techniques

2.3.1. Column chromatography

Silicic acid columns were prepared using silica gel for "dry column chromatography" (Woelm, Ltd., Eschwege, G.F.R.). The silica was packed dry in glass columns fitted with sintered glass discs and stopcocks. A loading of approximately 10 mg of crude material per g of silica was used and the sample was applied to the top of the column in a small portion of solvent. The stopcock was left open during application of the sample and addition of the solvent to avoid the introduction of gaps into the column.

A suitable solvent was chosen by examining the separations achieved on TLC. R_F values obtained on the columns were in general slightly higher than those found for the same compounds on TLC using the same mobile phase. An elution technique was employed, and the size of the fractions collected was estimated from the thin layer chromatographic separation. Progress was monitored by micro-plate TLC.

Liquid-gel chromatography was performed on the lipophilic gel, Sephadex LH-20 (Pharmacia, Uppsala, Sweden) with 1,2-dichloroethane/methanol, 7:3 v/v as mobile phase or on the substituted gel N1518 LH20[330,331] using benzene (straight phase) or methanol/heptane 9:1 v/v (reverse phase) as solvent. All solvents were dried and redistilled prior to use, and glassware was silanised with a 5% solution of

dimethyldichlorosilane in toluene. The gel was pre-swollen in the elution solvent and packed by sedimentation under gravity flow in glass columns (100 x 0.9 cm), fitted with Glenco No. 3020 Teflon Connectors at the bottom.[332] Samples were applied to the top of the column in a small portion of eluting solvent using a drawn-out Pasteur pipette or a Hamilton syringe. The eluate was monitored with a Pye-Unicam System 2 liquid chromatograph and fractions were collected with an automatic fraction collector (BTL Chromafrac).

2.3.2. Thin layer chromatography (TLC)

Glass plates (20 x 20 cm or 5 x 20 cm) were coated to a thickness of 0.25 mm or 0.5 mm with Kieselgel HF_{254} (E. Merck, Darmstadt, Germany) using a Quickfit (Quickfit and Quartz Ltd., Stone, Staffordshire) spreader. The adsorbent was applied as a slurry in distilled water. Plates were air dried for 20 min after spreading, then activated at 120°C for 30 min-1 h in a clean oven.

Chromatograms were developed in Shandon Chromatanks (Shandon Scientific Co. Ltd., London) (20 x 20 cm) or gas-jars (5 x 20 cm) lined with filter paper to aid atmosphere saturation, through a distance of 15 cm, from origin to solvent front.

For analytical TLC samples (10-30 µg in a volatile solvent) were applied to the 0.25 mm. layers using a 10 µl Hamilton syringe or drawn-out capillary tubes. Spots on the developed chromatograms were generally

detected by spraying with a solution of 5% (w/v) ceric sulphate in 10% sulphuric acid followed by heating at 150°C for a few min. In many cases, particularly steroids, characteristic colours developed after heating for 1-2 min. The ibuprofen metabolites were not well detected with ceric sulphate, and a superior spray reagent for their detection was found to be 5% (w/v) phosphomolybdic acid in ethanol. After heating for 2-3 min, blue spots appeared. Ketonic material was detected by spraying with a solution of 0.5% (w/v) 2,4-dinitrophenylhydrazine in ethanol (1 litre) plus 10 ml concentrated hydrochloric acid. Yellow or orange spots appeared without heating.

A load of approximately 30-40 mg material could be applied to a 20 x 20 cm, 0.5 mm layer for preparative TLC without considerable deterioration in separation. Samples were applied in a band across the plate using a 50 µl Hamilton syringe. Bands on developed chromatograms were located by the quenching of the fluorescence of the modified layer at 254 nm. On the few occasions where no material was visible under u.v. light, brief exposure to iodine vapour was employed. The silica was scraped from the plate and the material eluted in chloroform:methanol 9:1 (v/v). In the case of aromatic and/or very polar compounds the silica was first deactivated with water and then extracted with ethyl acetate.

2.3.3. Gas liquid chromatography (GLC)

GLC was performed on either a Perkin-Elmer 881 or a Pye Unicam Series 104-dual column chromatograph, both fitted with flame ionisation detectors. Glass columns of length 1.5 m, 3 m or 5 m and 3 mm i.d. were used. Before packing, the columns were treated with a 5% solution of dimethyldichlorosilane in toluene. Column packings were obtained from Applied Science Laboratories, Inc., State College, Pa., and the following were used :

1% SE-30 (methyl siloxane polymer) on 100-120 mesh Gas-Chrom Q.

1% OV-1 (methyl siloxane polymer) on 100-120 mesh Gas-Chrom Q.

1% OV-17 (phenyl methyl siloxane polymer, 50% phenyl) on 100-120 mesh Gas-Chrom Q.

1% QF-1 (fluoroalkyl siloxane polymer) on 100-120 mesh Gas-Chrom Q.

Oxygen-free nitrogen was used as carrier gas with a flow rate of 40 ml/min. Both instruments were fitted with injection port heaters which were kept approximately 50° above the temperature of the column.

Samples for GLC were dissolved in ethyl acetate (1-2 mg/ml) and aliquots of 1-2 µl were injected using a 10 µl Hamilton syringe. Retention indices were measured with respect to n-alkanes.

2.3.4. Gas chromatography-mass spectrometry (GC-MS)

Mass spectra were recorded at electron energy 22.5 or 70 eV using an LKB 9000 gas chromatograph-mass spectrometer. A glass column (3 m x 3 mm i.d.) packed with 1% OV-1 was used to effect GC separations with helium as carrier gas. The trap current was 60 μA; accelerating voltage 3.5 kV; electron multiplier voltage, 2.3-2.7 kV; molecule separator temperature, 250-270°C; ion source temperature, 250-270°C; entrance slit, 0.1 mm; exit slit, 0.1 mm. Spectra were obtained using an oscillographic recorder. Scans taken in the absence of sample allowed correction of the spectra for background ions.

Accurate mass measurements were carried out by Mr. A. Ritchie using an A.E.I. MS-902S instrument at 70 eV.

2.3.5. Infra-red spectroscopy (IR)

IR spectra were recorded as carbon tetrachloride solutions, KBr discs, or liquid films using a Perkin-Elmer 257 Spectrophotometer.

2.3.6. Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra were recorded at 100MHz on a Varian HA 100 instrument, using $CDCl_3$ as solvent. Interpretation of the spectrum of hydroxy-fenoprofen was facilitated by the use of a lanthanide shift reagent - europium 1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionate[$Eu(fod)_3$]. Spectra were recorded at 0.2 M-1M $Eu(fod)_3$ concentrations.

2.3.7. Ultra-violet spectroscopy (UV)

UV spectra were recorded in ethanol solution using a Pye-Unicam SP 8000 spectrophotometer.

2.4. Chemical techniques

2.4.1. Preparation of methyl esters

Carboxylic acids were methylated with an ethereal solution of diazomethane (freshly prepared from bis-(N-methyl-N-nitroso)terephthalamide)[333] at room temperature. When the yellow colour of diazomethane persisted the reaction was complete and excess reagent was removed in a stream of nitrogen.

Amino acids were converted to their methyl esters by refluxing in methanolic HCl solution (prepared from methanol and acetyl chloride) for 1 h. After removal of methanol the free bases were liberated by aqueous bicarbonate and the amino acid methyl ester extracted into ethyl acetate.

2.4.2. Trimethylsilylation of alcohols

Unhindered alcohols were trimethylsilylated in dry pyridine with hexamethyldisilazane-trimethylchlorosilane (5:1), or bis(trimethylsilyl)acetamide without catalyst, at 60°C for 15-30 min.

More hindered tertiary alcohols were completely trimethylsilylated with bis(trimethylsilyl)acetamide-trimethylchlorosilane at 60° after 30 min.

Perdeuterated trimethylsilyl ethers were formed with [d_{18}]-bis(trimethylsilyl)acetamide, (Merck Sharp & Dohme, Montreal, Canada) under the same conditions.

2.4.3. Preparation of chloromethyldimethylsilyl (CMDMS) ethers

CMDMS ethers were prepared with 1,3-bis-(chloromethyl) -1,1,3,3-tetramethyldisilazane-chloromethyldimethylchlorosilane (2:1) in pyridine for 1 h at 60°C.

2.4.4. Reduction of esters with lithium aluminium hydride

The ester sample (100-500 µg) was dissolved in sodium-dried diethyl ether and $LiAlH_4$ added. The mixture was warmed gently for 10 min, then excess reagent was removed by cautious addition of ethyl acetate. When effervescence had ceased water and more ether was added. The products were recovered from the dried ethereal layer.

Deuterated products were obtained by reducing the ester as above with lithium aluminium deuteride (Ciba, Basle, Switzerland).

2.4.5. Alkaline hydrolysis of esters

The ester was treated with an ethanolic solution of potassium hydroxide (6 ml 33% KOH in 50 ml ethanol) at 40°C for 1 h. Ether extraction removed any neutral material; subsequent acidification followed by ether extraction yielded the acidic components.

2.4.6. Oxidation

Oxidations with CrO_3/H_2SO_4 were carried out in acetone at 5-10° for 15 min.

2.5. Synthesis of 2,4'-(1-hydroxy-2-methylpropyl) phenylpropionic acid

Ibuprofen methyl ester (200 mg) was dissolved in carbon tetrachloride and N-bromosuccinimide (150 mg) in carbon tetrachloride added. The reaction mixture was refluxed for 5 h, then left at room temperature overnight. The crystallised succinimide was removed by filtration and after removal of solvent *in vacuo* a pale yellow oil (267 mg) was obtained. The oil was dissolved in methanol (10 ml) and 10% aqueous $NaHCO_3$ solution (10 ml) was added. After refluxing for 2 h the solution was acidified with dilute HCl and extracted with ether. The ether extract was dried over Na_2SO_4 and evaporated to dryness yielding a pale yellow oil (217 mg).

The purity of the product was assessed by TLC and GLC. Analytical TLC showed three spots,

R_F = 0.49, 0.63, 0.74 ($CHCl_3$/EtOAc, 3:1 v/v)

GLC on 10' 1% OV-1 at 150°C indicated that the product was 40% 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester (I = 1700) and 25% unreacted starting material (I = 1510). The third peak (I = 1605) was shown by GC-MS to be the methyl ether of the product, presumably produced on hydrolysis of the bromide.

Purification was effected by preparative TLC with chloroform/ethyl acetate 3:1 v/v as mobile phase, and the product characterised as described in Sec. 3.3.2.

Section 3. Results

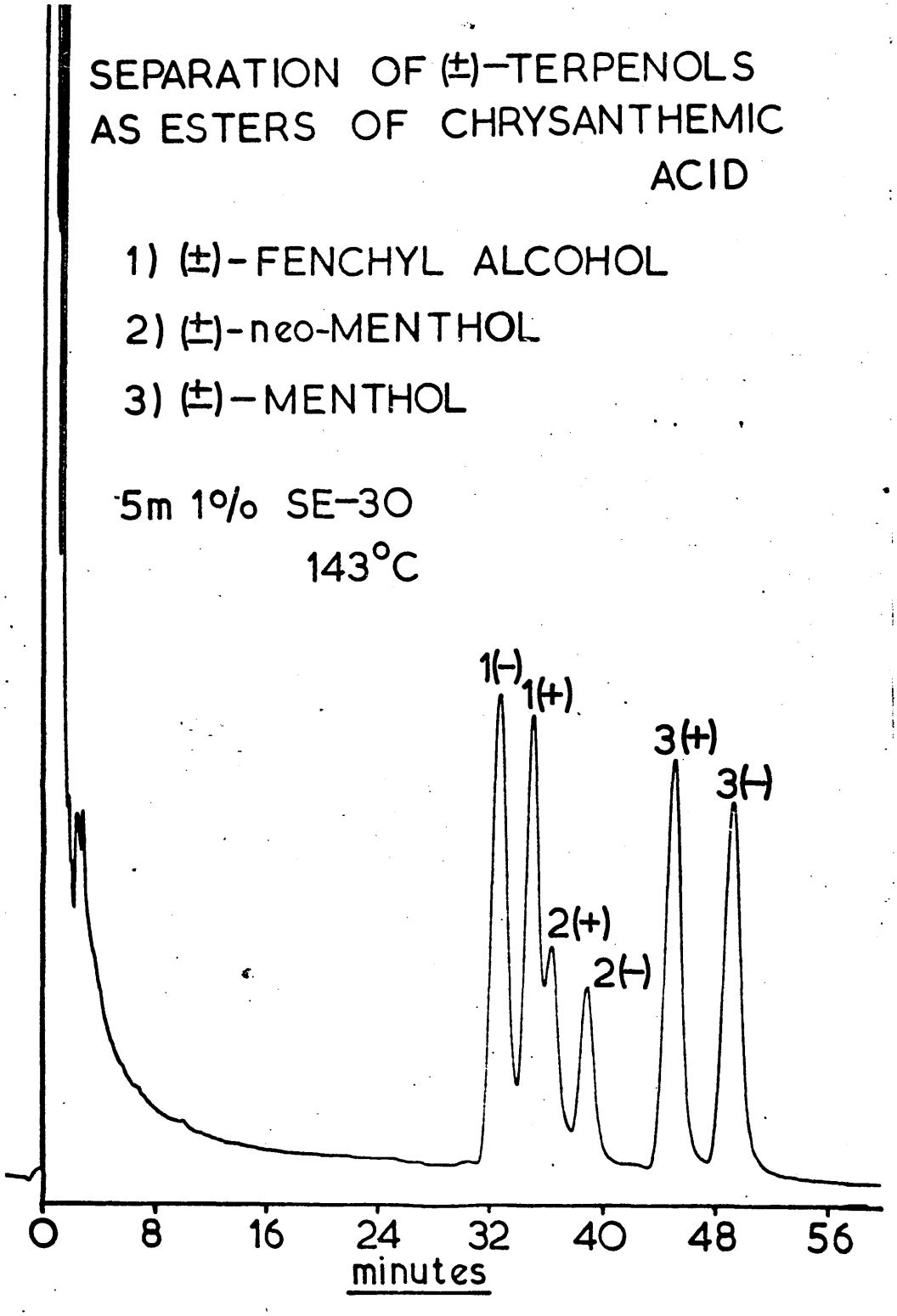


Fig. 12. Gas chromatographic separation of (±)-fenchol, (±)-neomenthol and (±)-menthol as esters of chrysanthemic acid.

3.1. Resolution of enantiomeric alcohols and amines with terpenoid reagents

3.1.1. Drimanoates and chrysanthemates of chiral secondary alcohols

The degree of resolution achieved varied greatly (Table 7), although in general chrysanthemic acid proved a more suitable resolving agent for the alcohols studied. The isomeric menthols illustrate this variation. Although the drimanoates of (+)- and (-)-menthol were well separated those of the enantiomeric neomenthols showed no resolution while the isomenthols showed only a partial separation ($\Delta I = 5$). The chrysanthemates of all three diastereomeric pairs gave a retention index difference of 15, which gives complete peak resolution (e.g. Fig.12. and Table 7). A better measure of diastereomeric differences is the relative volatility, α :[137]

$$\alpha = \frac{t_{R_2} - t_a}{t_{R_1} - t_a} = \frac{K_2}{K_1}$$

Where t_{R_1} and t_{R_2} are the retention times of the first and second components respectively, t_a is the inert gas retention time, and K_1 and K_2 are the respective gas-liquid partition coefficients.

Using the retention time of the solvent as t_a, α values were calculated. On this basis the enantiomeric neomenthols ($\alpha = 1.13$) showed a better resolution than the menthols ($\alpha = 1.11$) and the isomenthols ($\alpha = 1.07$). These

Table 7. Retention index values for drimanoates and chrysanthemates

Alcohols		Drimanoates $I^{1\%\ SE\text{-}30}_{190 \pm 2^{o}}$	ΔI	Chrysanthemates $I^{1\%\ SE\text{-}30}_{143 \pm 1^{o}}$	ΔI
(+)-2-Octanol	(S)	2280	20	1725	15
(-)-2-Octanol	(R)	2300		1740	
(+)-Menthol	(S)	2435	15	1880	15
(-)-Menthol	(R)	2450		1895	
(+)-Isomenthol	(S)	2430	5	1880	15
(-)-Isomenthol	(R)	2435		1895	
(+)-Neomenthol	(S)	2395	0	1835	15
(-)-Neomenthol	(R)	2395		1850	
(+)-Borneol	(S)	2475	0	1900	5
(-)-Borneol	(R)	2475		1905	
(+)-Isoborneol	(S)	2475	0	1895	10
(-)-Isoborneol	(R)	2475		1905	
(+)-Fenchol	(R)	2410	5	1825	15
(-)-Fenchol	(S)	2405		1810	
(+)-Pantolactone	(S)	2405	15	1820	10
(-)-Pantolactone	(R)	2390		1810	
Methyl (R)-(-)-12-hydroxystearate		3580[a]	15	2920[e]	10
Methyl (S)-(+)-12-hydroxystearate		3565[a]		2910[e]	
Methyl (R)-(-)-13-hydroxystearate		3570[b]	15	-	-
Methyl (S)-(+)-13-hydroxystearate		3555[b]		-	
Methyl (R)-(-)-mandelate		2580	5	2030[c]	10
Methyl (S)-(+)-mandelate		2575		2020[c]	
(±)-17β-Hydroxy-4-estren-3-one		4110[d]	-	3310[e]	-
(±)Estradiol 3-methyl ether		-		3355[f,g]	

[a] 262°C; [b] 272°C; [c] 160°C; [d] 285°C; [e] 225°C; [f] 250°C; [g] partial resolution of peak.

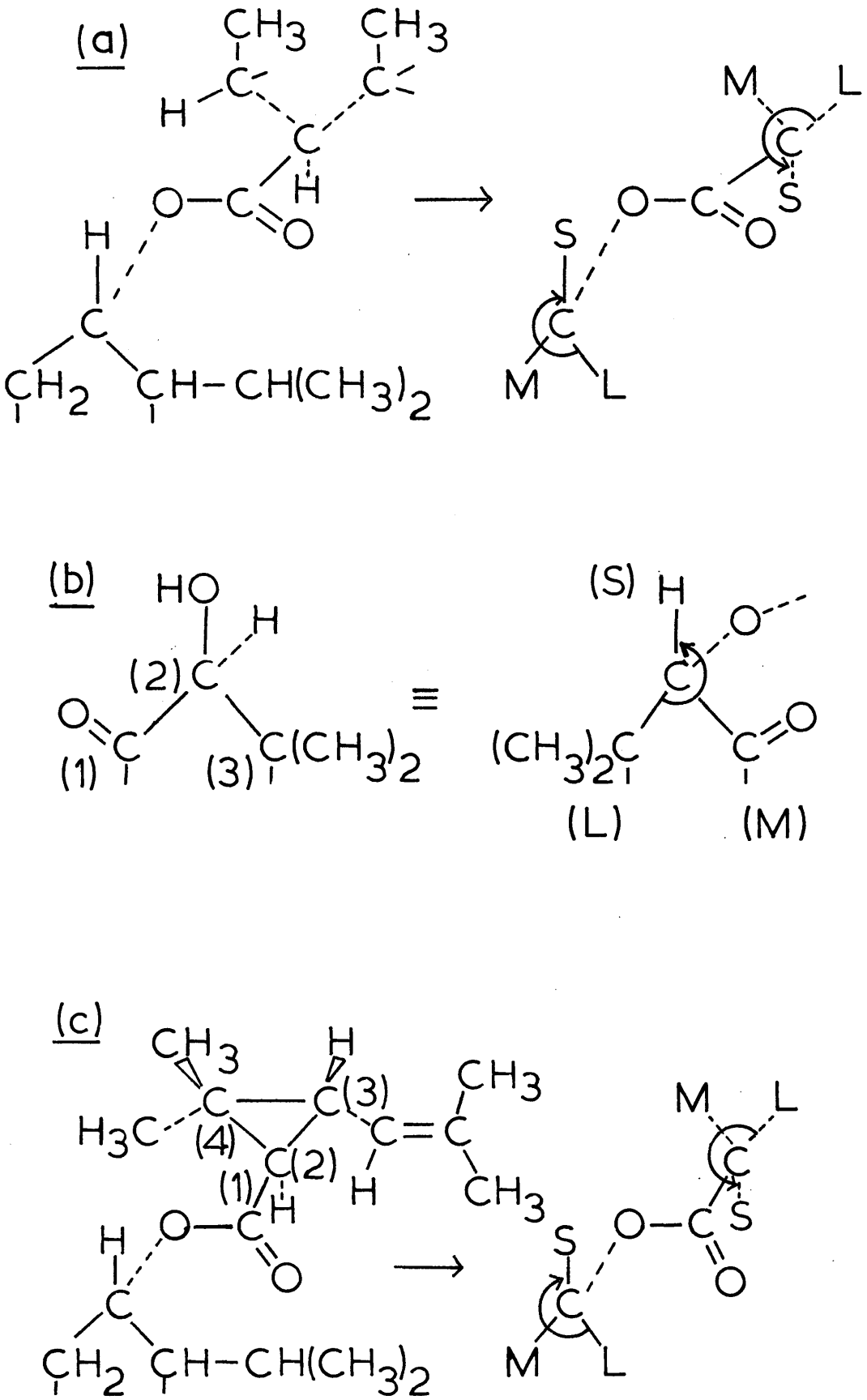


Fig. 13. Space projection of (a) (S)-menthol -(S)-drimanoate, (b) (S)-pantolactone, and (c) (S)-menthol-(R)-chrysanthemate.

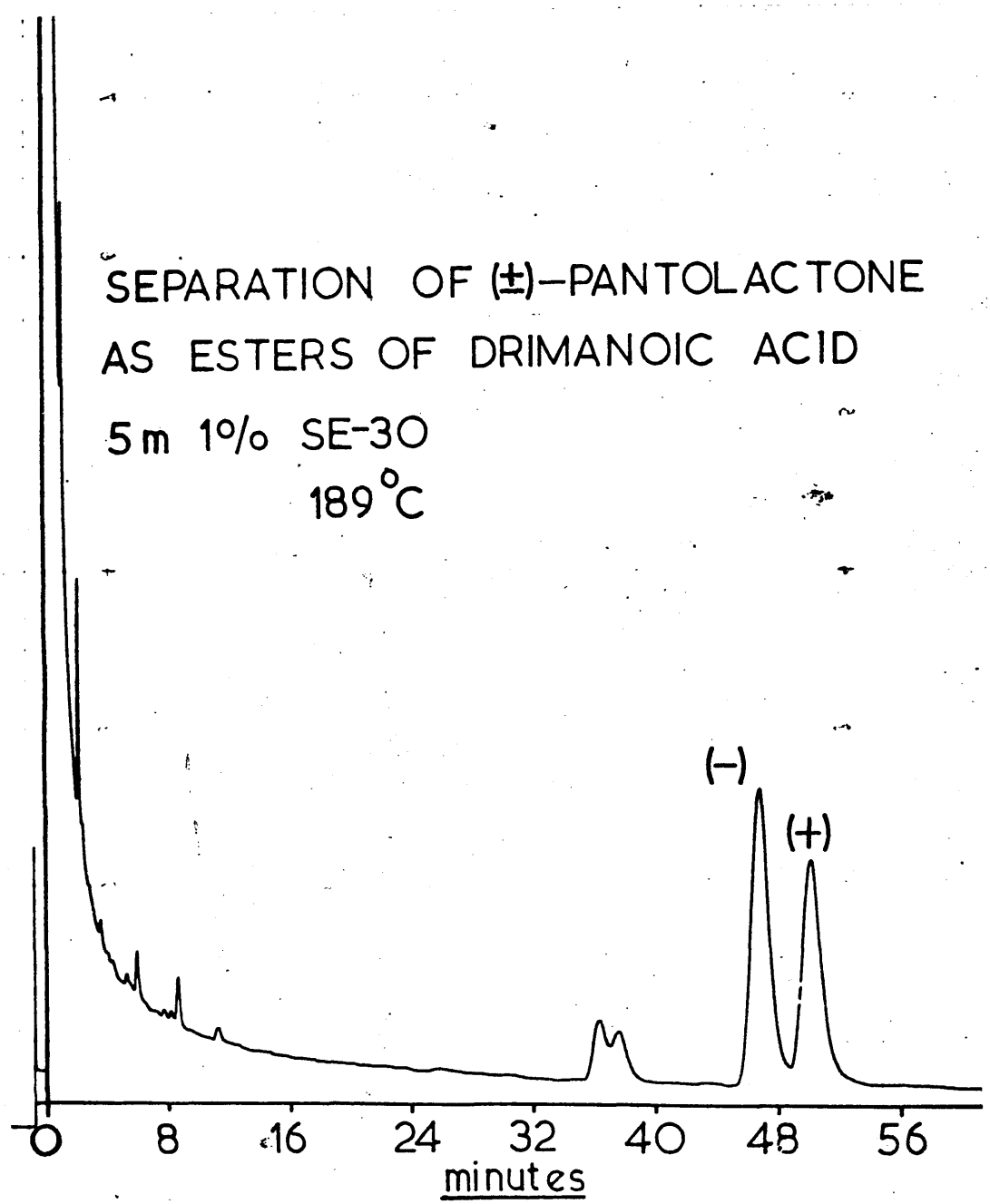


Fig. 14. Gas chromatographic separation of (±)-pantolactone as esters of drimanoic acid.

were some of the best separation factors achieved. Trends could also be observed in the order of elution of the diastereomers. With the exception of pantolactone, the diastereomeric drimanoates were always eluted in the order (SS) before (SR) while the chrysanthemates showed the reverse order, (RS) before (RR). On examination of molecular models these elution orders could be correlated with the mechanism of "bulkiness chirality" proposed by Feibush [225]: the diastereomer emerging last from the column, when viewed along its long axis, has the same direction of bulkiness chirality (L → M → S) at both asymmetric carbons. Figure 13 illustrates the situation for menthol. Viewing the (SS)-drimanoate diastereomer it is seen that the two asymmetric centres have the substituents arranged in opposite directions (L → M → S) and this should be the diastereomer eluted first. This is also the situation for all the other alcohols except pantolactone. In this case (Fig.13) the carbonyl carbon (C-1) takes precedence over C-3 in the sequence rule but the dimethyl group is bulkier than the carbonyl and the (SS) diastereomer is eluted later than (SR) (Fig.14).

A similar correlation may be observed in the case of the chrysanthemates. Although, according to the sequence rule C-4 takes precedence over C-3, the isobutenyl group is bulkier than the dimethyl group so the chrysanthemoyl moiety shows an anticlockwise arrangement in the bulkiness diagram (Fig.13) and consequently the (RS) diastereomers are eluted first.

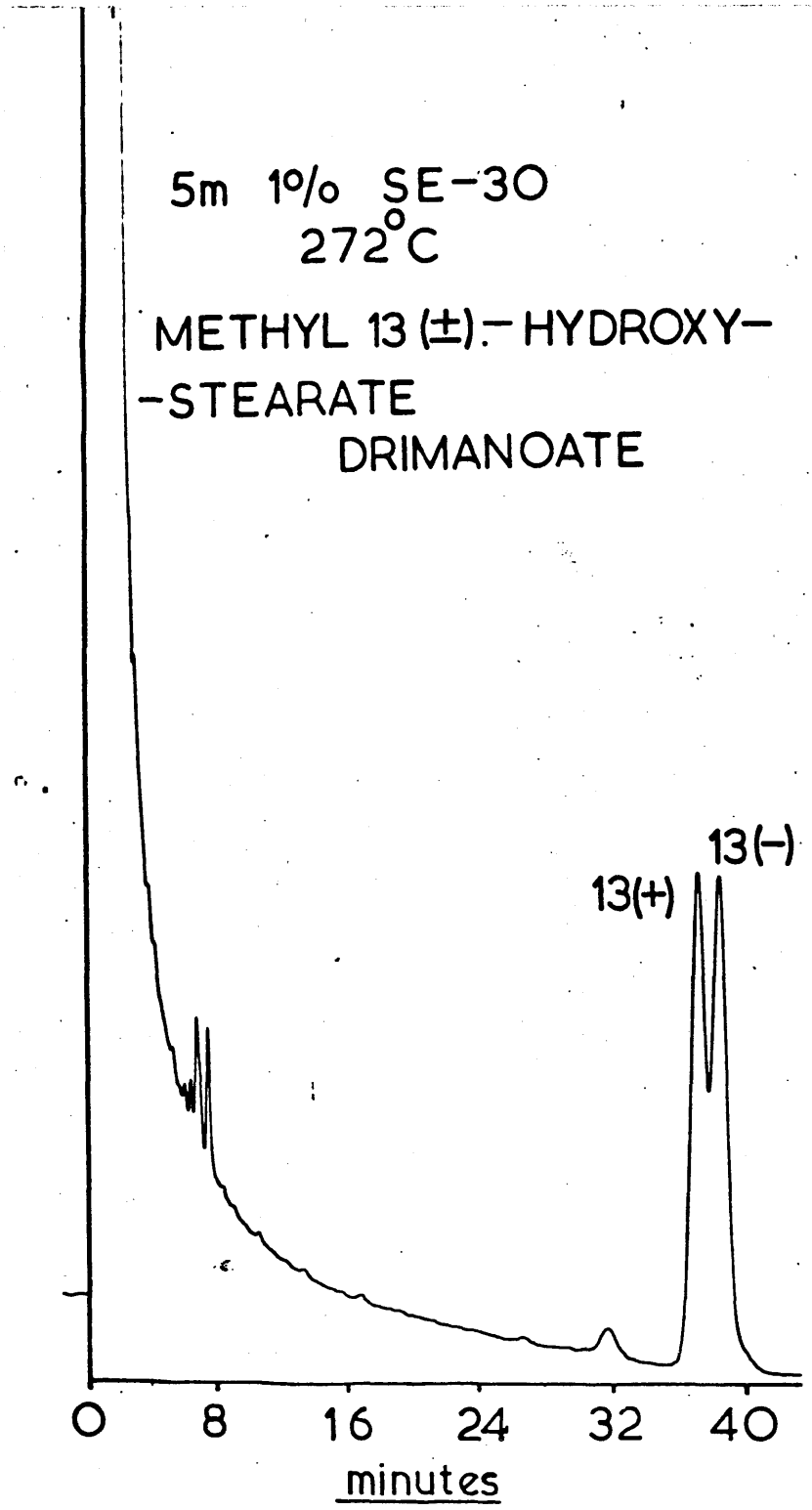


Fig. 15. Gas chromatographic separation of (±)-methyl-13-hydroxystearate as esters of drimanoic acid.

Enantiomeric alcohols in which the chiral carbinol group is followed by methylene chains are difficult to resolve as diastereomeric derivatives.[150] Several methyl hydroxystearates have been analysed as diastereomeric 2(D)-phenylpropionate derivatives using QF-1 as stationary phase.[145] Good separations were achieved for methyl-3-hydroxy-, 16-hydroxy-, and 17-hydroxy stearates and an adequate separation of methyl 15-hydroxystearates was observed. However no visible separation of the phenyl-propionates of methyl 13-hydroxystearates could be obtained. Attempts to resolve the enantiomeric methyl 13-hydroxy-stearates as (R)-(+)-1-phenylethylurethanes or (R)-(-)-menthylcarbonates, or (R)-(-)-menthoxyacetates proved unsuccessful, but the corresponding drimanoates were satisfactorily distinguished,[213,334] (Fig. 15). Partial separation of methyl 12-hydroxystearates, both as drimanoyl and chrysanthemoyl derivatives, was also achieved.

The minor peaks of shorter retention time, observed in the traces of all drimanoyl derivatives, were shown by GC-MS to be isomeric with the major derivatives. The shorter retention time and the reversal of the order of elution of diastereomers suggested that these might be 9-epidrimanoyl derivatives. Methylation of a sample of drimanoic acid with diazomethane produced one peak on GLC, $I^{1\%\ OV-1}_{130^oC}$ = 1700, but methylation *via* the acid chloride with methanol gave two peaks, $I^{1\%\ OV-1}_{130^oC}$ = 1665, 1700 the minor peak being eluted first. GC-MS confirmed that these peaks were isomeric methyl drimanoates. However,

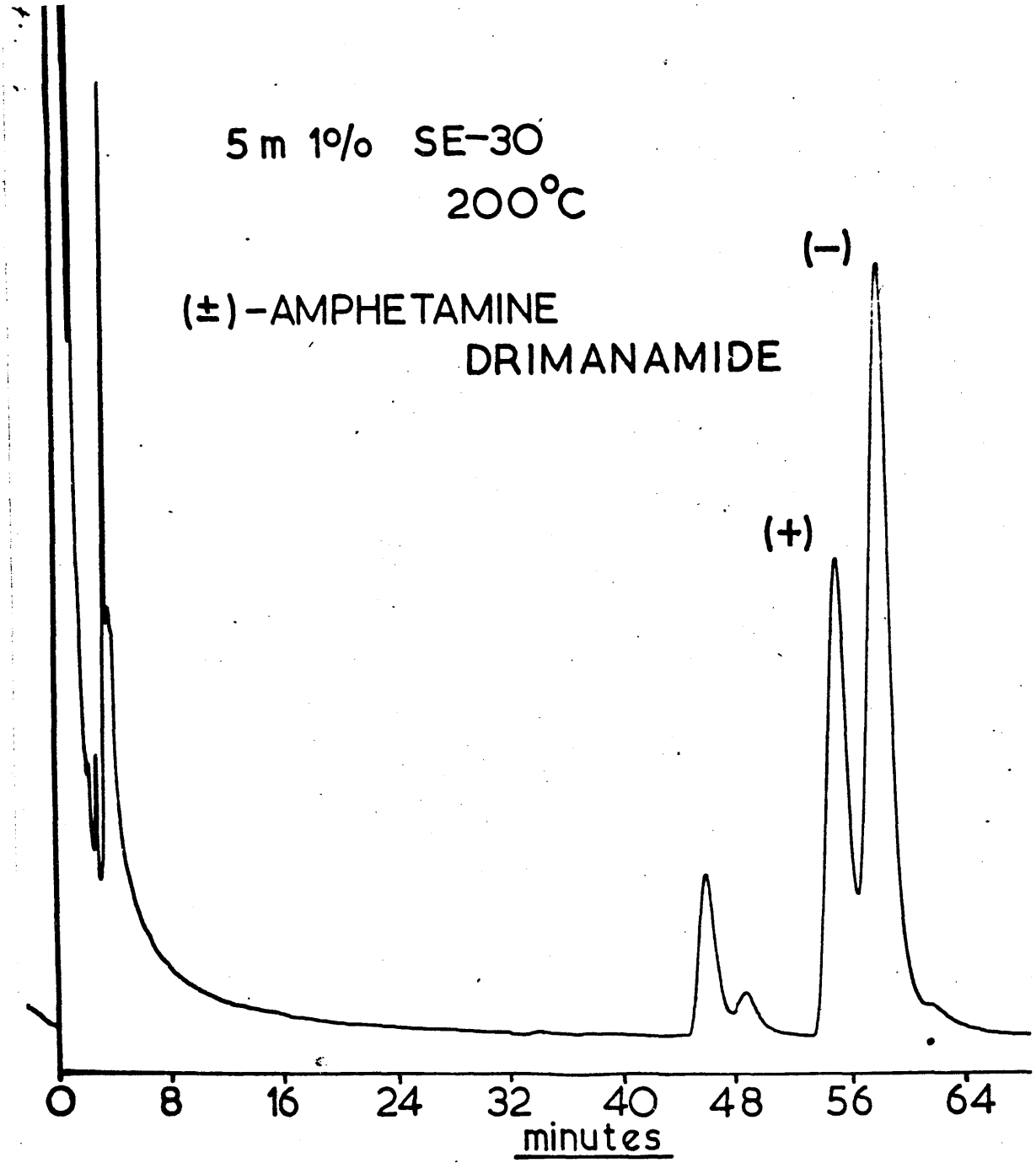


Fig. 16. Gas chromatographic separation of the N-drimanoyl derivatives of (+)- and (-)-amphetamine.

attempts to separate and fully characterise these minor products formed during the acylation process were unsuccessful. No similar complications were observed with chrysanthemoyl chloride.

3.1.2. Drimanoyl and chrysanthemoyl derivatives of chiral amines and amino acids

Considerable variations were observed in the degree of resolution achieved within the series of amines and amino acids studied (Table 8). The primary, amines were adequately resolved by drimanoic acid, as illustrated by the N-drimanoylamphetamines in Figure 16. However the secondary amine, N-methyl α-phenylethylamine showed no separation. It has been suggested[141] that the amide hydrogen is involved in hydrogen bonding which enhances the differential between diastereomers. This could explain the loss in resolution on replacement of the amide hydrogen by a methyl group.

The results obtained with chrysanthemic acid did not directly match those of the drimanoyl derivatives. Since no separation was observed for either α-phenylethylamine or N-methyl α-phenylethylamine, while both amphetamine and N-methylamphetamine were well resolved, a critical factor might appear to be the distance of the amino function from the aromatic ring. A similar result was noted with the amino acid methyl esters : phenylglycine methyl ester was not separated as the chrysanthemamide, whereas the N-chrysanthemoyl-phenylalanine methyl esters were well

Table 8. Retention index values for drimanamides and chrysanthemamides

	Drimanamides			Chrysanthemamides		
	$I^{1\%\ SE-30}$	ΔI	Temp.	$I^{1\%\ SE-30}$	ΔI	Temp.
(R)-(+)-α-Phenyl-ethylamine	2535	20	187	1990	0	171
(S)-(-)-α-Phenyl-ethylamine	2515		187	1990		171
(S)-(+)-Amphetamine	2600	20	200	2025	10	160
(R)-(-)-Amphetamine	2620		200	2035		160
(R)-(+)-N-methyl-α-phenylethylamine	2630	0	200	2035	0	171
(S)-(-)-N-methyl-α-phenylethylamine	2630		200	2035		171
(S)-(+)-Methamphetamine				2095	15	186
(R)-(-)-Methamphetamine				2110		186
(R)-(-)-Valine methyl ester	2335	5	190	1800	15	143
(S)-(+)-Valine methyl ester	2340		190	1815		143
(R)-(+)-Proline methyl ester	2465	5	220	1925	5	143
(S)-(-)-Proline methyl ester	2470		220	1930		143
(R)-(-)-Phenylglycine methyl ester	2750	0	220	2170	0	189
(S)-(+)-Phenylglycine methyl ester	2750		220	2170		189
(R)-(+)-Phenylalanine methyl ester	2810	10	218	2240	15	189
(S)-(-)-Phenylalanine methyl ester	2820		218	2255		189

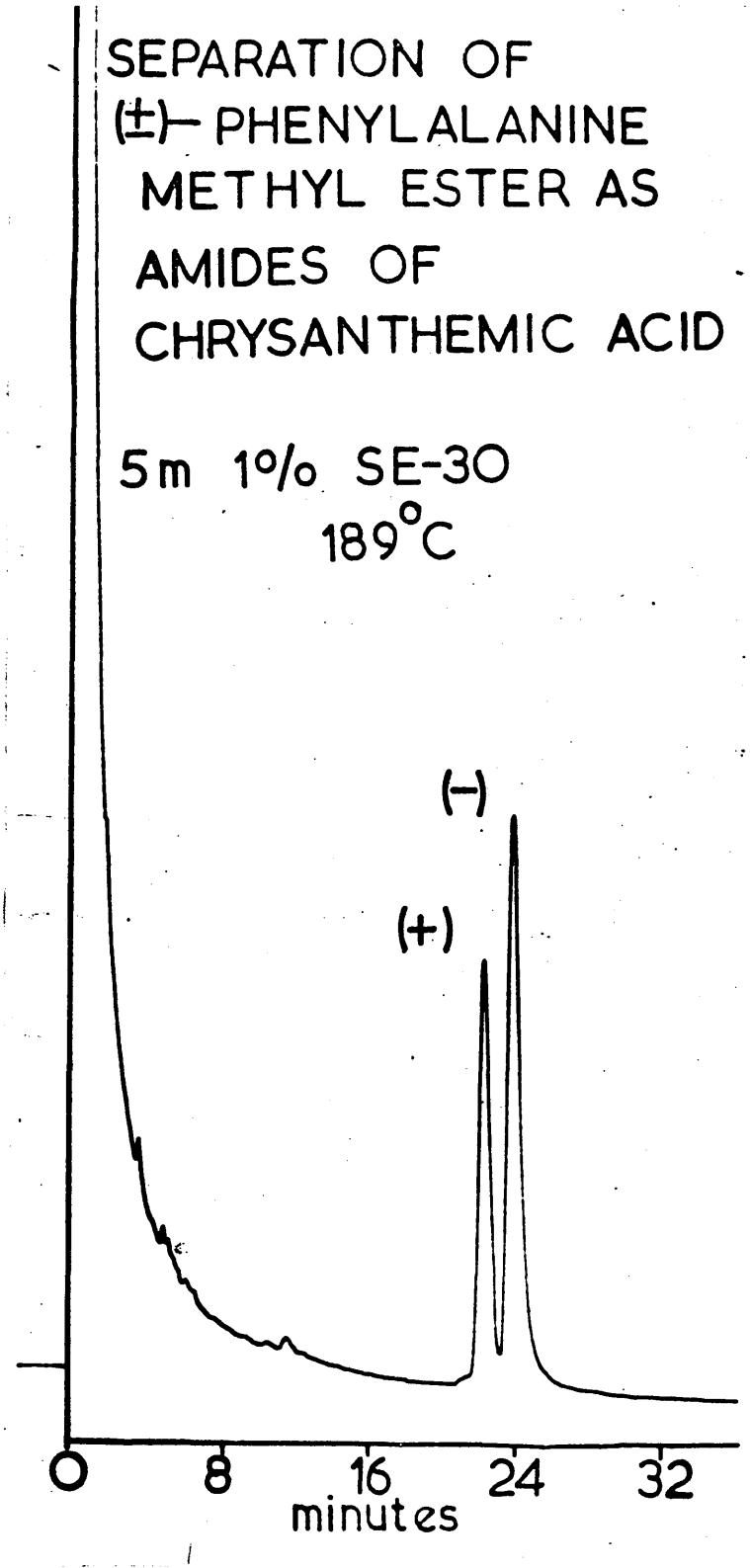


Fig. 17. Gas chromatographic separation of the N-chrysanthemoyl derivatives of (+)- and (-)-phenylalanine methyl ester.

distinguished (Fig.17).

The order of elution of the diastereomers predicted on the basis of the bulkiness of the groups around the chiral centres agreed with that observed in all cases. In the case of the four amines studied the order of groups in the sequence rule was the same as the order of bulkiness and the observed elution pattern was (SS) before (SR) for the drimanoyl derivatives and (RS) before (RR) for the chrysanthemamides. When considering the amino acids the carbomethoxyl group took precedence in the sequence rule but the phenyl group (phenylalanine) or the isopropyl group (valine) was seen to be larger on examination of molecular models and the order of elution of the diastereomers was reversed. In the case of proline methyl ester the rigid ring system must take preference over the carbomethoxyl group to explain the observed elution order, but this was not obvious from an examination of molecular models. Hence the N-drimanoyl amino acid methyl esters followed the elution order proposed by Westley and Halpern,[154] based on the analysis of N-trifluoroacetyl-(S)-(-)-prolyl derivatives, viz. an (RS) or (SR) derivative generally has a shorter retention time than its (RR) or (SS) diastereomer. The N-chrysanthemoyl amino acid methyl esters, however, showed the reverse order.

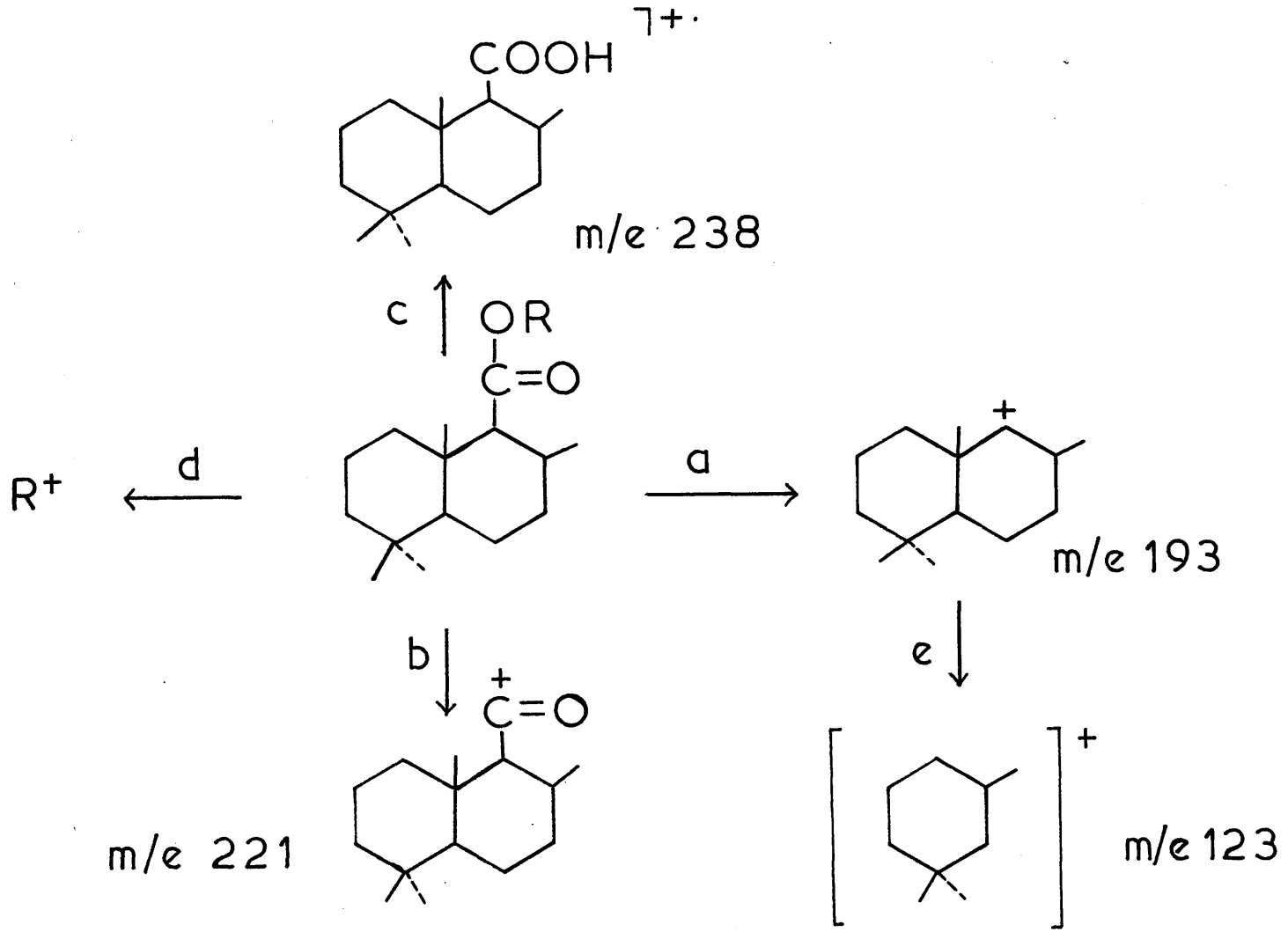


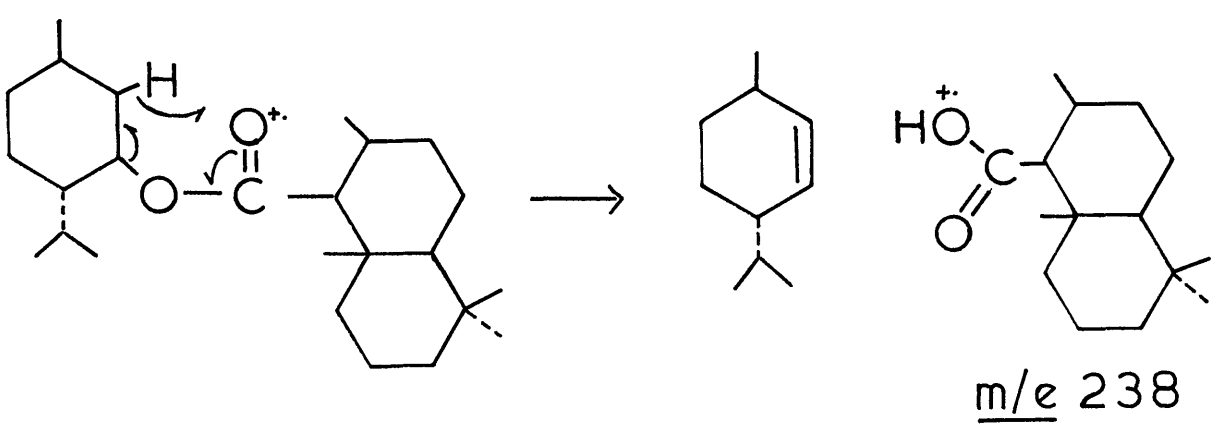
Fig. 18. Mass spectral fragmentation scheme for derivatives of drimanoic acid.

3.1.3. Mass spectral characteristics

Salient features of the mass spectra of O- and N-drimanoyl derivatives are cited in Table 9. Simple fragmentation modes represented in Figure 18 are indicated by superscripts in the table. Ions below m/e 80 have been disregarded since they are generally of limited value in structural diagnosis. Molecular ions were observed in all instances and were particularly abundant in the spectra of the N-drimanoyl derivatives.

Drimanoates

Major fragments characteristic of the drimanoyl moiety were observed in all the spectra . Alkyl-oxygen cleavage produced an ion at m/e 237 from the drimahoyl portion or $[M-237]^+$ characteristic of the alcohol. The abundance of the $[M-237]^+$ ion varied considerably : this fragment constituted the base peak in the spectra of the esters of borneol, fenchol and methyl mandelate, but was absent in those of 2-octyl drimanoate and pantolactone drimanoate. Alkyl-oxygen cleavage also occurred with rearrangement of one hydrogen atom to produce the drimanoic acid ion, m/e 238 (Scheme 1).



Scheme 1

Table 9. Principal mass spectrometric data for O- and N-drimanoyl derivatives

	PRINCIPAL IONS IN MASS SPECTRUM (Relative abundance in parentheses)							
	$M^{+}\cdot$	Other Significant Ions						
DRIMANOATE ESTERS								
2-Octanol	350(9)	123(100)[e]	223(74)	87(70)	182(58)	237(40)	238(35)[c]	95(21)
Menthol	376(2)	123(100)[e]	238(95)[c]	83(80)	223(68)	139(52)[d]	237(40)	138(36)
Borneol	374(2)	137(100)	81(38)	193(28)[a]	123(16)	221(10)[b]	109(9)	237(6)
Fenchol	374(2)	137(100)[a]	193(42)	81(30)	221(17)[b]	123(17)[e]	97(12)	95(10)
Pantolactone	350(10)	123(100)[e]	335(45)	122(32)	109(22)	95(21)	199(18)	294(17)
Methyl 13-hydroxystearate	534(0.5)	238(100)[c]	123(64)[e]	223(58)	182(34)	193(31)[a]	237(22)	297(20)
Methyl mandelate	386(5)	149(100)	237(80)	123(56)[e]	193(41)[a]	93(40)	137(28)	95(27)
AMIDES OF DRIMANOIC ACID								
α-Phenylethylamine	341(48)	163(100)	105(51)[d]	190(28)	85(18)	123(16)[e]	164(14)	326(13)
N-methyl-α-phenylethylamine*	355(45)	177(100)	105(30)[d]	204(26)	100(18)	120(15)	218(15)	230(10)
Amphetamine	355(28)	193(100)[a]	264(92)	221(90)[b]	86(74)	118(64)	97(62)	123(52)[e]
Valine methyl ester*	351(48)	132(100)	173(64)	123(40)[e]	200(31)	114(26)	336(20)	295(14)
Phenylglycine methyl ester*	385(56)	166(100)	106(99)	207(65)	123(52)[e]	149(30)	234(28)	370(17)
Phenylalanine methyl ester	399(57)	180(100)	162(76)	221(52)[b]	123(48)[e]	120(38)	248(24)	193(23)[a]

For superscripts see Fig.18 * 70 eV (others 22.5 eV)

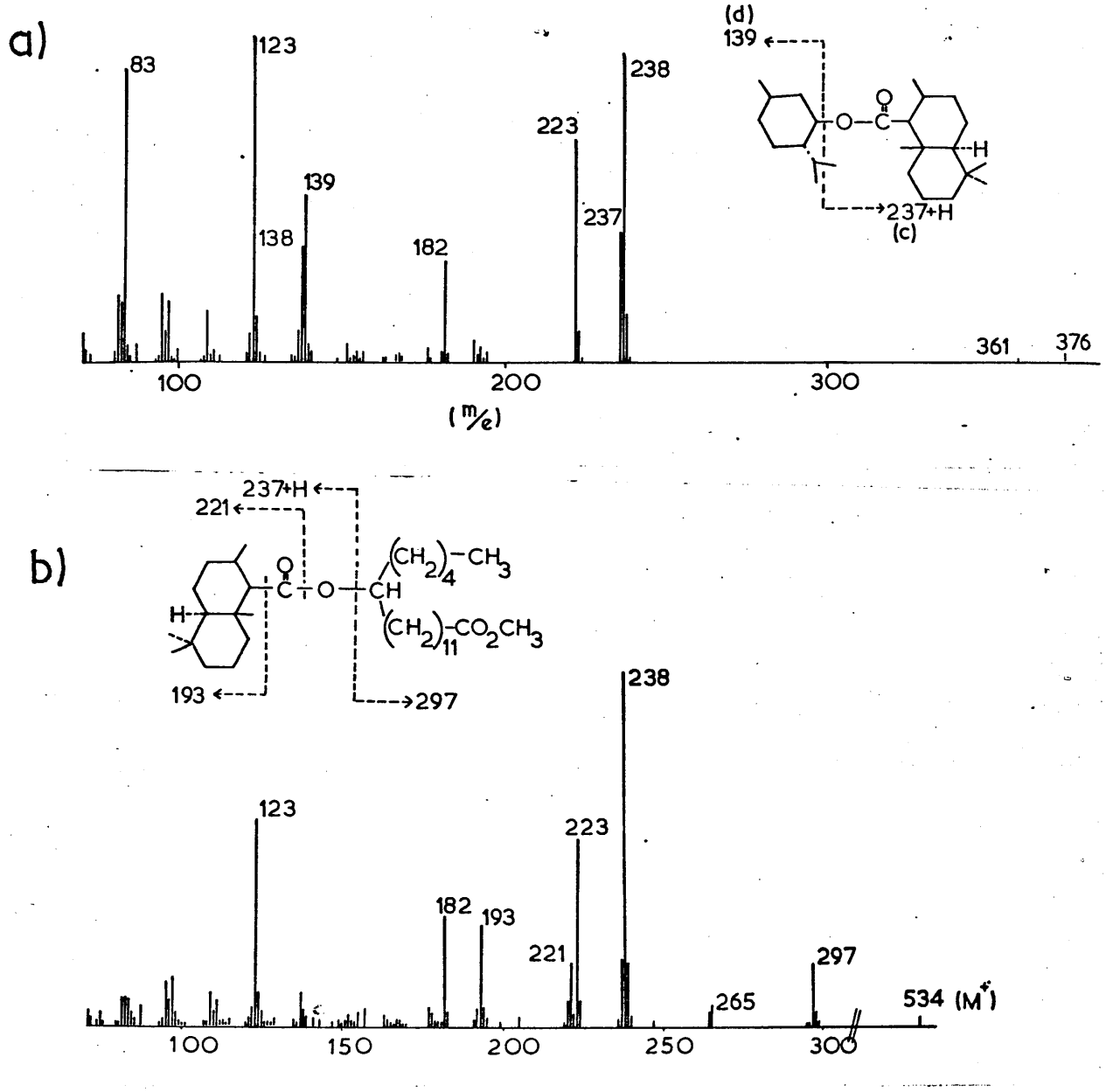


Fig. 19. Illustrative mass spectra of **O**-drimanoyl derivatives.

a) Mass spectrum of menthyl drimanoate.

b) Mass spectrum of methyl-13-hydroxystearate drimanoate.

Acyl-oxygen cleavage produced the drimanoyl ion $[C_{15}H_{25}O]^+$ at m/e 221. No evidence was obtained in the present work for the occurrence of acyl-oxygen cleavage with the charge residing on the alcohol moiety. Other characteristic ions of high abundance were the nordrimanyl ion $[C_{14}H_{25}]^+$, m/e 193, and the fragment from ring A of the drimane nucleus at m/e 123.

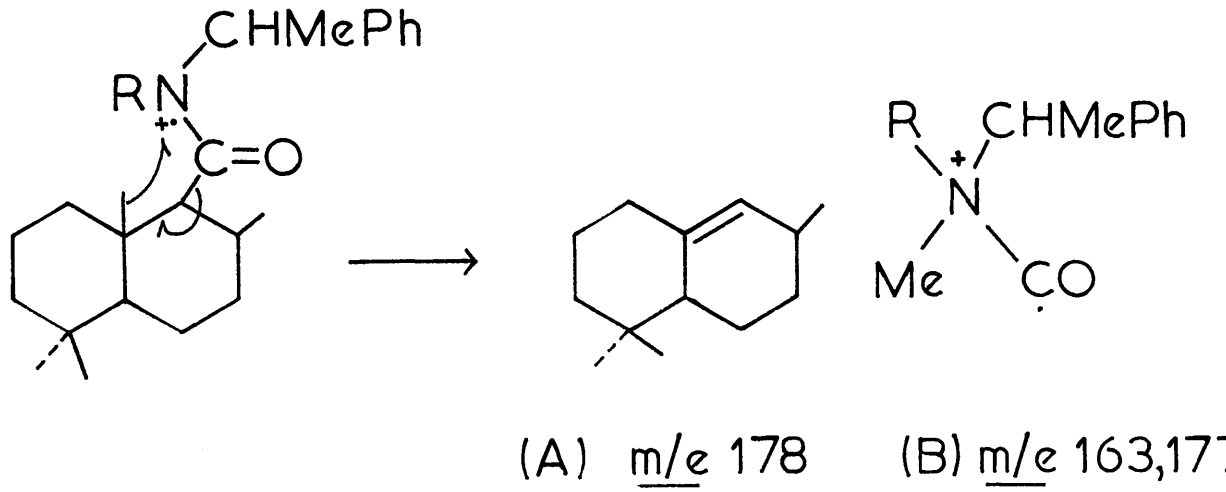
Typical ester spectra are presented in Figure 19 for menthyl drimanoate and methyl 13-hydroxystearate drimanoate.

N-drimanoyl derivatives

In addition to the ions already mentioned from the drimanoyl portion, the amide spectra showed more ions characteristic of the amine or amino acid under consideration. Salient features of these are further discussed below.

a) α-Phenylethylamine and N-methyl-α-phenylethylamine

The base peaks in these spectra at m/e 163 and 177, respectively were ascribed to species formed by methyl group migration, (Scheme 2).



(A) m/e 178 (B) m/e 163,177

Scheme 2

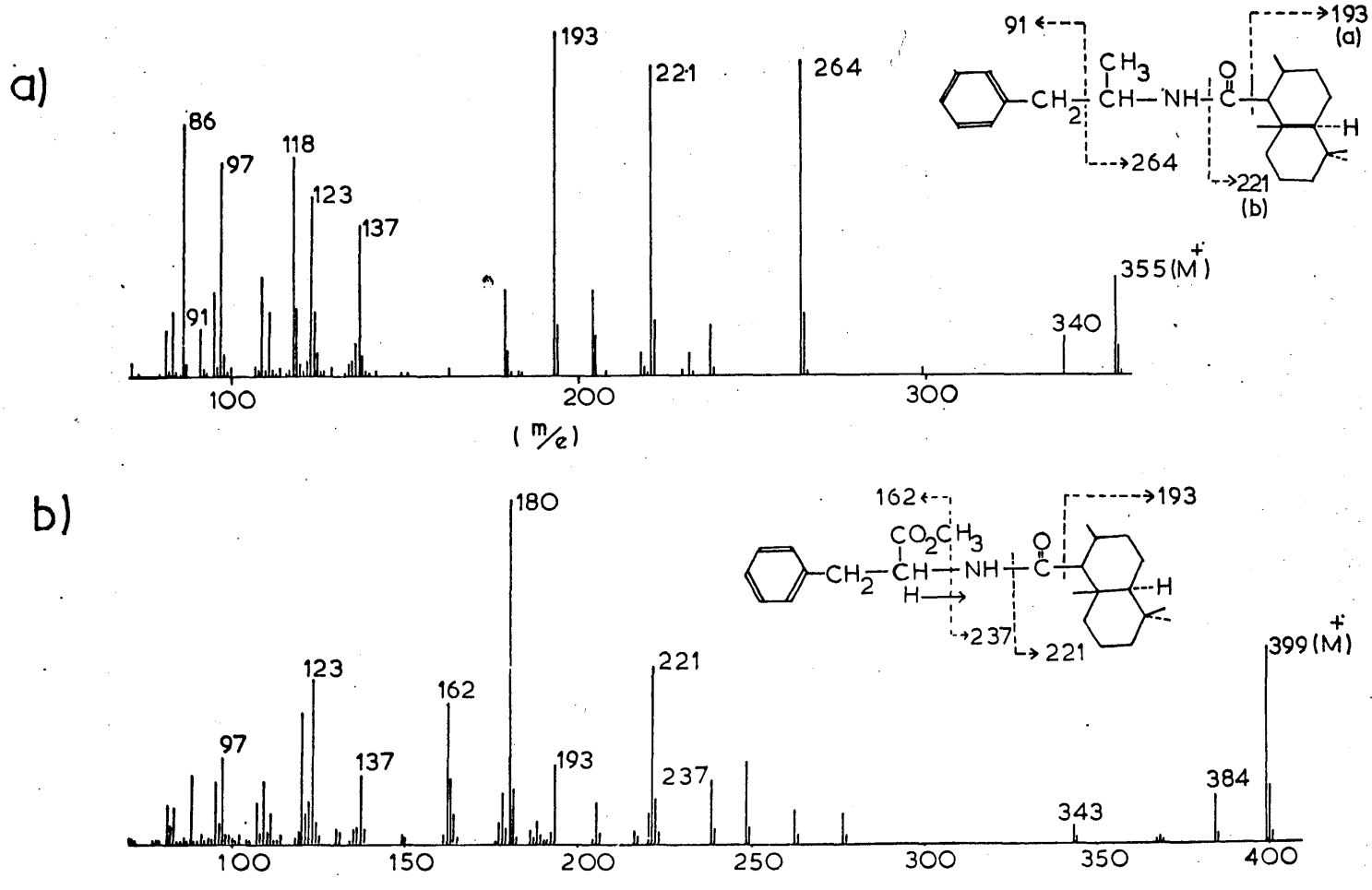


Fig. 20. Illustrative mass spectra of N-drimanoyl derivatives.

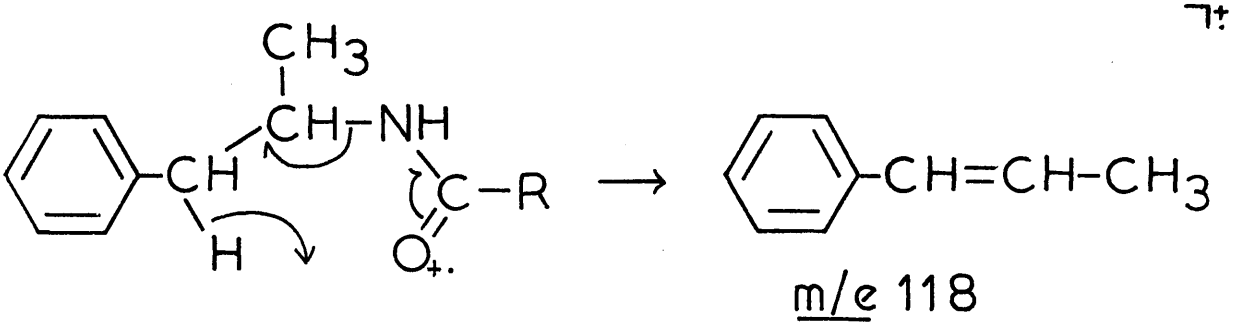
a) Mass spectrum of amphetamine drimanamide.

b) Mass spectrum of N-drimanoyl phenylalanine methyl ester.

High resolution mass spectrometry of the N-drimanoyl derivative of N-methyl-α-phenylethylamine showed the peak at m/e 177 to comprise two ions in the ratio 4:1, viz $C_{11}H_{15}NO$ ((B) as above) : calc. 177.1154; found, 177.1158; and $C_{13}H_{21}$, corresponding to $[(A)-H]^+$: calc. 177.1643; found, 177 1647.

b) Amphetamine (Fig. 20a)

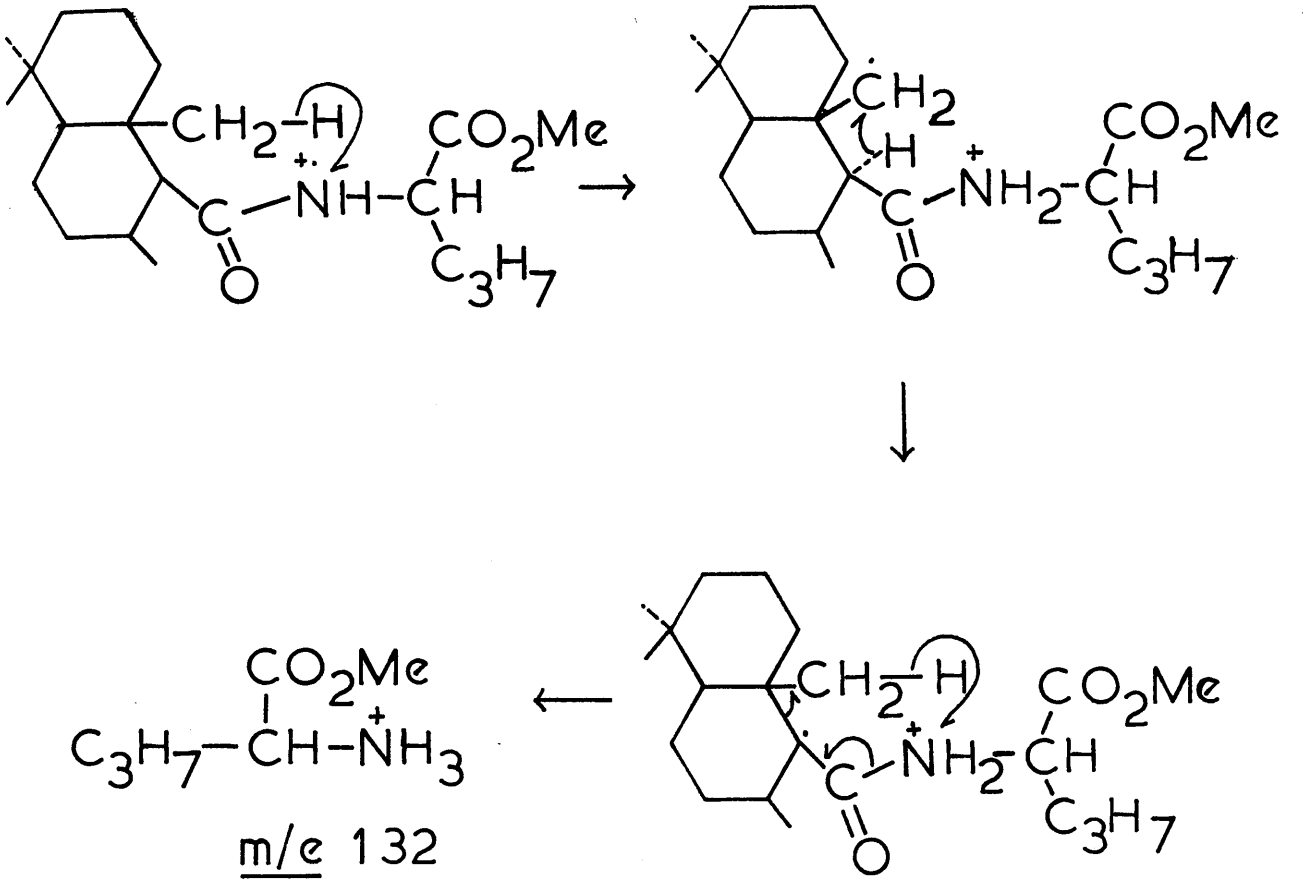
The major ions in the mass spectrum of amphetamine drimanamide were produced by simple fissions as shown in Figure 20a. Evidence for formation of the base peak, the 11-nordrimanyl ion (m/e 193) via the ion of m/e 221. was obtained from a "metastable" ion at m/e 168.6. The abundant ion at m/e 118 was formed by alkyl-nitrogen cleavage with transfer of one hydrogen atom as shown in Scheme 3.



Scheme 3

c) Valine methyl ester

The base peak (m/e 132) in this spectrum was formed by cleavage of the amide bond together with the transfer of two hydrogen atoms on to the nitrogen (Scheme 4).



Scheme 4

The abundant ion at m/e 173 was formed by cleavage adjacent to the C=O group with migration of a methyl group, according to Scheme 2, while that at m/e 114 was due to alkyl-nitrogen cleavage with hydrogen transfer as shown in Scheme 3.

d) Phenylglycine methyl ester and Phenylalanine methyl ester.

Again the base peaks (m/e 385 and 399 respectively) were formed by cleavage of the amide bond with rearrangement of two hydrogen atoms (cf. Scheme 4). Major ions at m/e 106 and 120 respectively, could be attributed to the imonium fragment,[335] (Scheme 5).

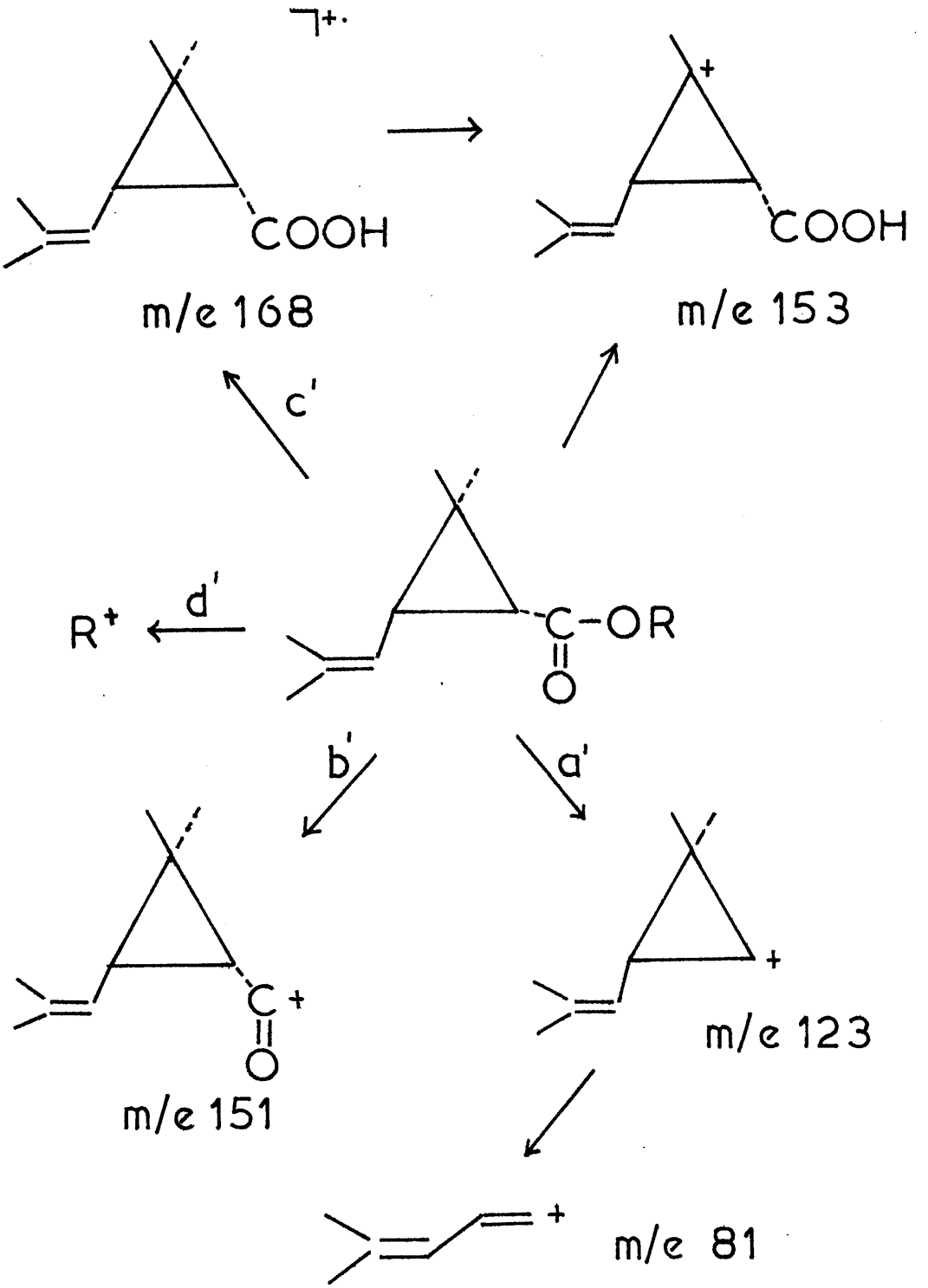
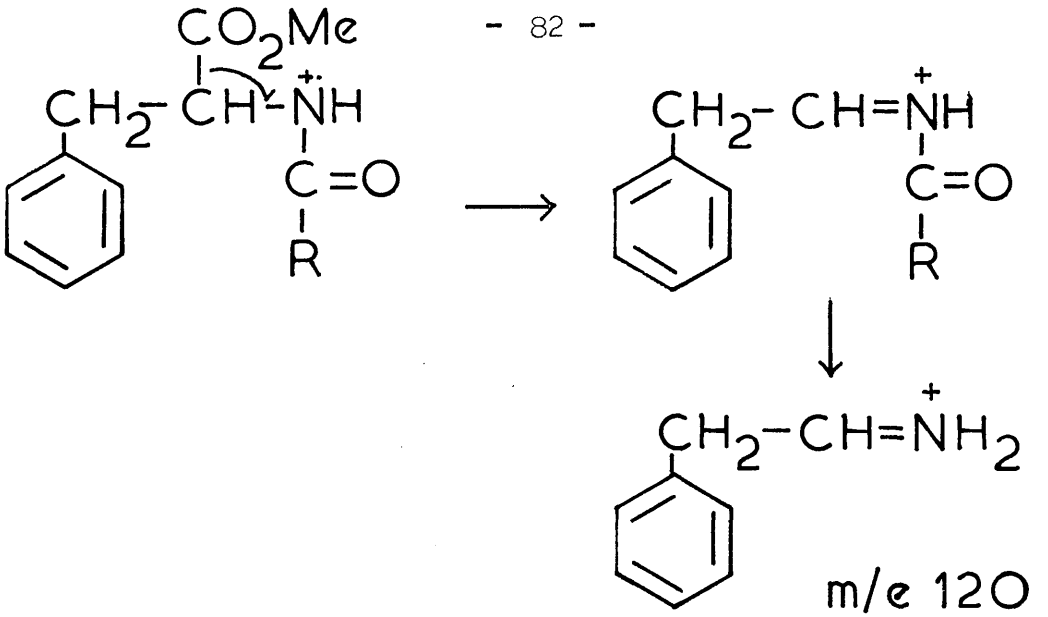


Fig. 21. Mass spectral fragmentation scheme for derivatives of chrysanthemic acid.



Scheme 5

Fragmentation according to Scheme 2 was also observed producing ions at m/e 207 and 221. The ion at m/e 221 in the spectrum of phenylalanine methyl ester drimanamide (Fig. 20b) could also be formulated as the drimanoyl ion $[C_{15}H_{25}O]^+$. Alkyl-nitrogen cleavage produced the ion at m/e 149 in the spectrum of phenylglycine methyl ester drimanamide but this fragmentation was accompanied by hydrogen transfer in the case of N-drimanoyl phenylalanine methyl ester to give the ion at m/e 162 (cf. Scheme 3).

The mass spectral fragmentation of chrysanthemate esters is already known for the naturally occurring pyrethrins.[336] The principal ions previously observed are presented in Figure 21 and indicated by superscripts in Table10.

Chrysanthemates

The synthetic diastereomeric esters showed a similar fragmentation pattern to that noted earlier.[336] The base peak in the majority of the spectra was represented by the norchrysanthemyl cation $[C_9H_{15}]^+$ (m/e 123), and other major ions were produced from the chrysanthemoyl moiety.

Table 10. Principal mass spectrometric data for O- and N-chrysanthemoyl derivatives

	PRINCIPAL IONS IN MASS SPECTRUM (Relative abundance in parentheses)							
	M^+.	Other Significant Ions						
CHRYSANTHEMATE ESTERS								
2-Octanol	280(2)	123(100)[a']	81(19)	151 (9)[b']	153(6)	107(6)	168(5)[c']	107(5)
Menthol	306(1)	123(100)[a']	83(93)	168(16)[c']	139(15)[d']	97(15)	81(13)	153(10)
Isomenthol	306(1)	83(100)	123(98)[a']	81(29)	168(15)[c']	97(15)	153(12)	139(12)[d']
Neomenthol	306(1)	123(100)[a']	83(100)	81(26)	168(17)[c']	97(16)	139(10)[d']	153(10)
Borneol	304(2)	81(100)	123(65)[a']	137(62)[d']	95(15)	151(15)[b']	168(10)[c']	118(8)
Isoborneol	304(2)	81(100)	137(99)[d']	123(43)[a']	151(20)[b']	168(20)[c']	95(15)	109(7)
Fenchol	304(7)	123(100)[a']	81(95)	137(80)[d']	151(24)[b']	149(20)	95(15)	168(13)[c']
Pantolactone	280(8)	123(100)[a']	149(57)	107(17)	81(15)	135(11)	167(7)	223(7)
Methyl 12-hydroxystearate	464(0.5)	123(100)[a']	168(25)[c']	151(14)	153(8)	297(4)	265(3)	97(3)
Methyl mandelate	316(8)	123(100)[a']	149(15)[d']	121(15)	81(14)	150(10)	167(10)	151(9)
AMIDES OF CHRYSANTHEMIC ACID								
α-Phenylethylamine*	271(5)	123(100)[a']	105(76)[d']	124(45)	81(40)	109(15)	91(8)	107(8)
N-methyl-α-phenylethylamine*	285(15)	105(100)[d']	123(52)[a']	122(30)	81(28)	96(21)	134(17)	166(11)
Amphetamine	285(12)	123(100)[a']	124(85)	91(35)	109(30)	119(27)[d']	151(20)[b']	194(15)
Methamphetamine*	299(17)	91(100)	123(60)[a']	119(52)[d']	151(38)[b']	81(32)	284(17)	208(16)
Valine methyl ester*	281(6)	123(100)[a']	130(41)	124(40)	81(38)	109(32)	107(28)	98(16)
Proline methyl ester*	279(22)	128(100)	123(88)[a']	81(48)	107(47)	264(22)	109(20)	130(16)
Phenylglycine methyl ester*	315(8)	123(100)[a']	124(55)	149(47)[d']	81(34)	109(26)	123(24)	107(22)
Phenylglycine methyl ester*	329(5)	123(100)[a']	124(45)	81(34)	109(25)	91(24)	206(24)	146(20)

For superscripts see Fig. 21. * 70 eV (others 22.5 eV)

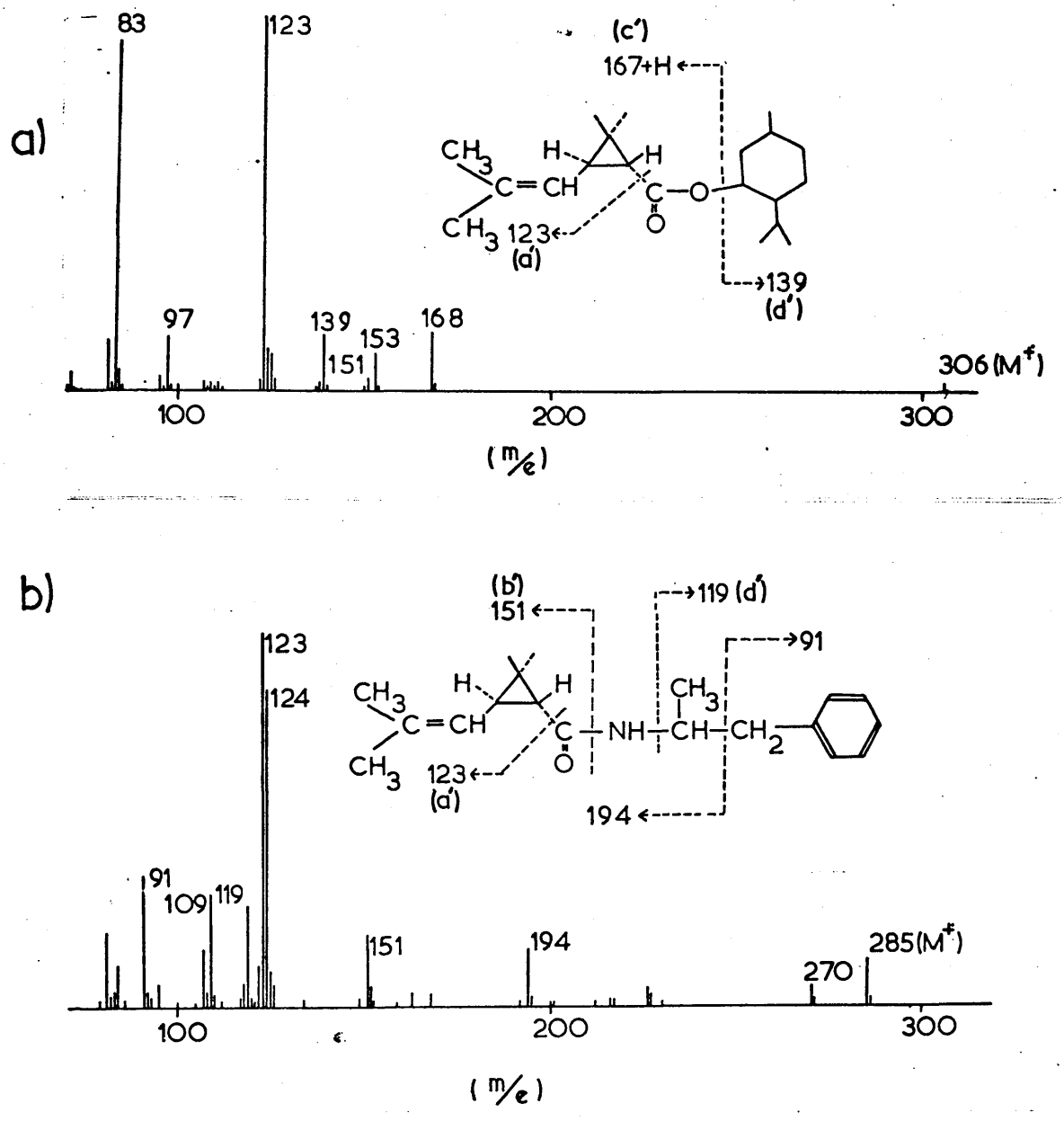


Fig. 22. Illustrative mass spectra of O- and N-chrysanthemoyl derivatives.

a) Mass spectrum of menthyl chrysanthemate.

b) Mass spectrum of amphetamine chrysanthemamide.

Ions characteristic of the alcohol were produced by alkyl-oxygen cleavage in all cases except those of 2-octyl chrysanthemate and pantolactone chrysanthemate. The terpenol esters (cf. Fig. 22a for menthol chrysanthemate) showed ions at m/e 83 $[C_6H_{11}]^+$ or m/e 81 $[C_6H_9]^+$ due to further fragmentation of the alcohol portion.

N-Chrysanthemoyl derivatives

Major fragments were again observed from the chrysanthemoyl moiety and β-cleavage produced ions characteristic of the amine or amino acid. The base peaks in the spectra of derivatives of N-methyl-α-phenylethylamine (m/e 105) and methamphetamine (m/e 91) were the methyl-benzyl and benzyl ions respectively. The spectrum of amphetamine chrysanthemamide is represented in Figure 22b and the formation of the major ions is indicated. Ions produced by N-acyl cleavage with the charge residing on the amine fragment were not observed to any great extent except in the case of chrysanthemoylproline methyl ester, where the methyl pyrrolidine-2-carboxylate ion (m/e 128) formed the base peak. The rearrangement ions which characterised the spectra of the N-drimanoyl derivatives were not observed from the chrysanthemamides.

3.1.4. Comparison of other chiral reagents

As a means of assessing the usefulness of the terpenoid reagents a comparison was made with a few gas chromatographic resolving agents which had previously been

Table 11. Retention index values for diastereomeric derivatives of (±)-Amphetamine and (±)-Menthol (1% SE-30 column)

Derivative	Δ M[a]	Amphetamine			Menthol		
		$I_{(+)}$	$I_{(-)}$	Δ I	$I_{(+)}$	$I_{(-)}$	Δ I
(+)-Chrysanthemoyl[b]	150	2025	2035	10	1880	1895	15
(-)-Menthoxycarbonyl[c]	168	2205	2205	0	2080	2080	0
(+)-α-Phenylethyl-carbamoyl[c]	161	-	-	-	2100	2110	10
(-)-Menthoxyacetyl[c]	182	2355	2350	5	2200	2200	0
Drimanoyl[b]	220	2600	2620	20	2435	2450	15
3β-Acetoxy-5-etienyl[d]	360	3840	3840	0	3570	3570	0

[a] Molecular weight increment accompanying derivative formation

[b] Column temperatures as in Tables 7 and 8.

[c] 185°

[d] 280°

used, viz : (R)-(-)-menthylchloroformate,[146] (R)-(-)-menthoxyacetic acid,[337] (R)-(+)-α-phenylethylisocyanate,[142, 144] and 3β-acetoxy-5-etienic acid.[151]

The results obtained for a representative alcohol, menthol, and amine, amphetamine, are presented in Table 11.

The only separations observed were for the (R)-1-phenylethylcarbamates of (±)-menthol and (R)-(-)-menthoxyacetyl derivatives of (±)-amphetamine but the retention index differences were considerably inferior to those obtained with the terpenoid acids.

Table 12. Retention indices of amides of (1) (R)-(-)-phenylbutyric (2) (S)-(+)-phenylpropionic (3) (RS)-3-methyl-2-phenylbutyric, (4) (RS)-α-methoxy-phenylacetic acid, and (5) (S)-(+)-α-Cl phenylacetic acid.

	$I^{1\%\ SE-30}_{190°C}$ * 180°C					$I^{1\%\ OV-17}_{220°C}$ * 205°C.				
	(1)	(2)	(3)	(4)	(5)	(1)	(2)	(3)	(4)	
(R)-α-phenylethylamine	2030	1995	2040	2050	2090	2370	2355	2410	2445	
(S)-α-phenylethylamine	2060	1965	2070	2085	2120	2400	2330	2450	2475	
(R)-amphetamine	2115	2065	2135	2150	2180	2455	2420	2495	2525	
(S)-amphetamine	2125	2065	2155	2175	2195	2470	2410	2520	2550	
(RS)-p-chloro-amphetamine	2305	2235	2320	2315		2685			2710	
	2320	2220	2345	2345		2705			2750	
(RS)-3-methoxy-4, 5-methylenedioxyamphetamine	2615	2570	2660	2640						
	2635	2550	2700	2690						
(R)-N-methyl-α-phenylethylamine	2105	2060	2110	2150	2230	2420	2415	2450	2520	
(S)-N-methyl-α-phenylethylamine	2120	2050	2120	2150	2230	2445	2400	2465	2520	
(R)-methamphetamine	2160	2130	2190	2210	2310	2490	2485	2520	2585	
(S)-methamphetamine	2195	2110	2215	2215	2310	2515	2460	2550	2600	
(R)-norvaline methyl ester*	1900	1830	1920	1910	1970	2190	2140	2235	2260	
(S)-norvaline methyl ester*	1900	1830	1920	1925	1970	2190	2140	2235	2280	
(R)-valine methyl ester*	1870	1805	1900	1875	1920	2145	2100	2190	2225	
(S)-valine methyl ester*	1880	1790	1900	1875	1940	2155	2080	2190	2225	
(R)-proline methyl ester	1990	1955	2015	2010	2120	2360	2360	2385	2455	
(S)-proline methyl ester	2015	1940	2045	2010	2130	2390	2335	2430	2465	
(R)-phenylglycine methyl ester	2230	2155	2225	2235	2305	2640	2580	2665	2700	
(S)-phenylglycine methyl ester	2215	2165	2250	2270	2285	2620	2600	2690	2740	
(R)-phenylalanine methyl ester	2300	2245	2330	2325	2375	2700	2650	2740	2775	
(S)-phenylalanine methyl ester	2300	2245	2330	2340	2375	2700	2650	2760	2795	

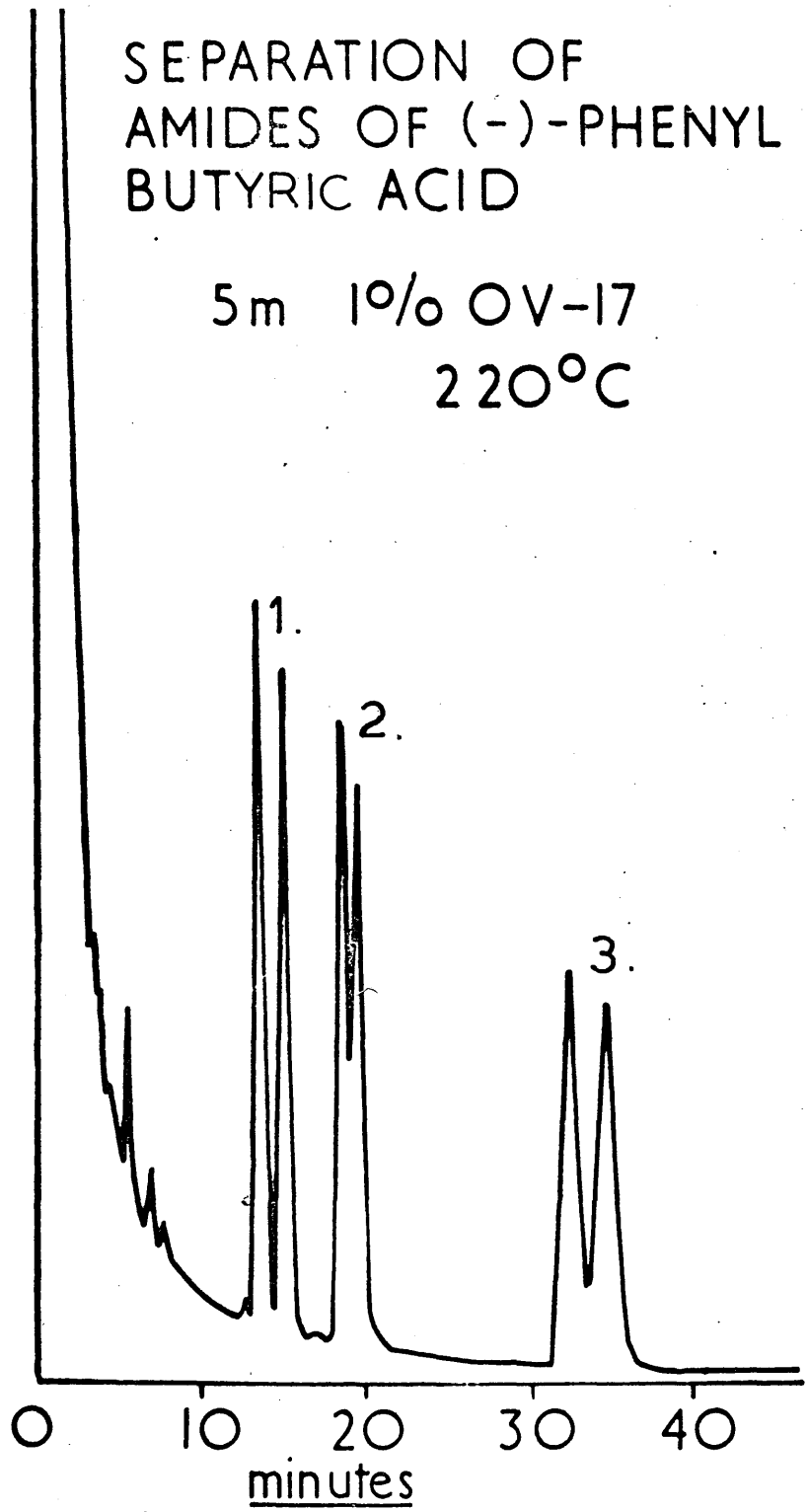


Fig. 23. Gas chromatographic separation of (1) (R,S)-proline methyl ester; (2) (R,S)-amphetamine; (3) (R,S)-phenylglycine methyl ester as amides of (R)-(-)-α-phenylbutyric acid.

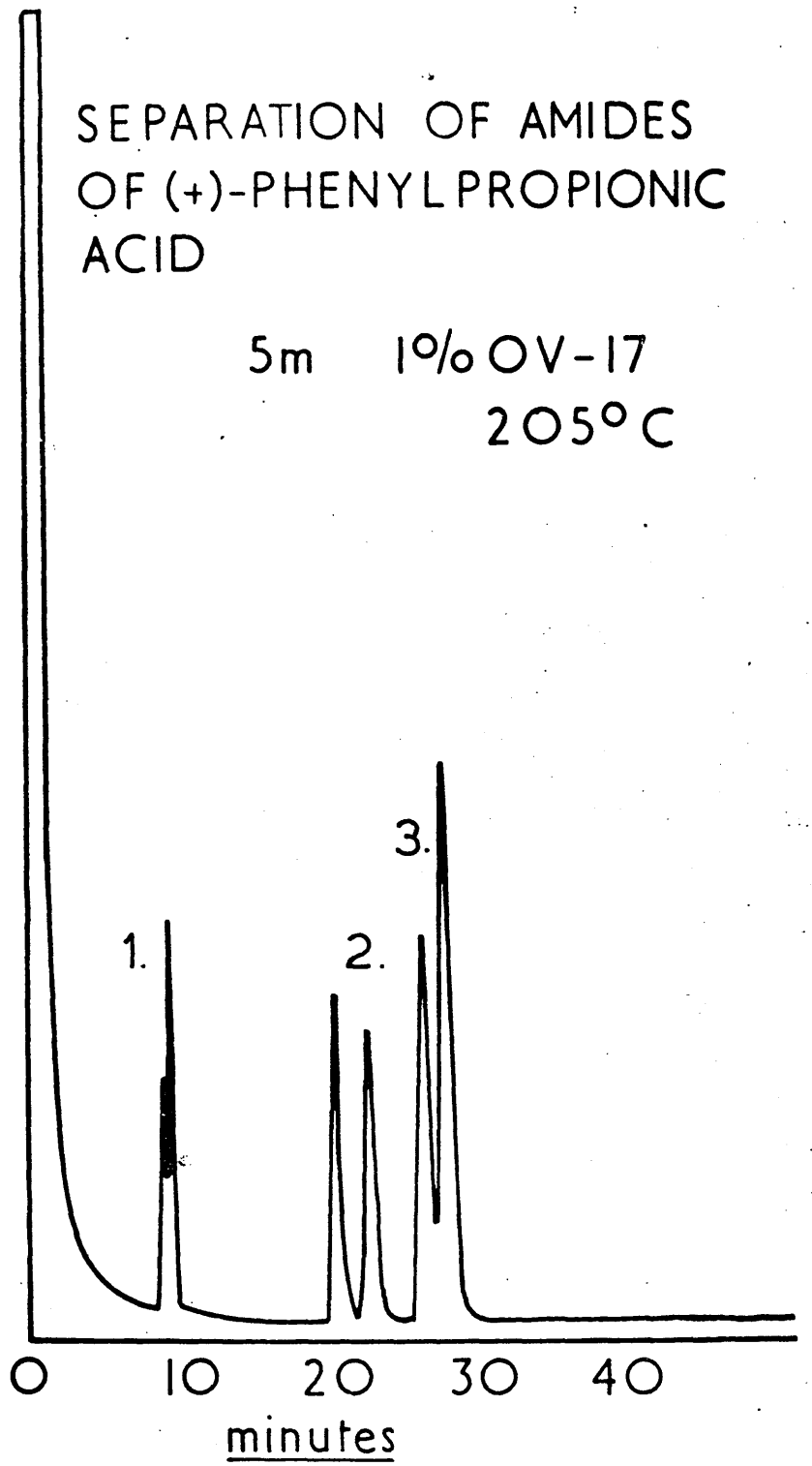


Fig. 24. Gas chromatographic separation of (1) (R,S)-valine methyl ester; (2) (R,S)-α-phenylethylamine (3) (R,S)-N-methyl-α-phenylethylamine as amides of (S)-(+)-α-phenylpropionic acid.

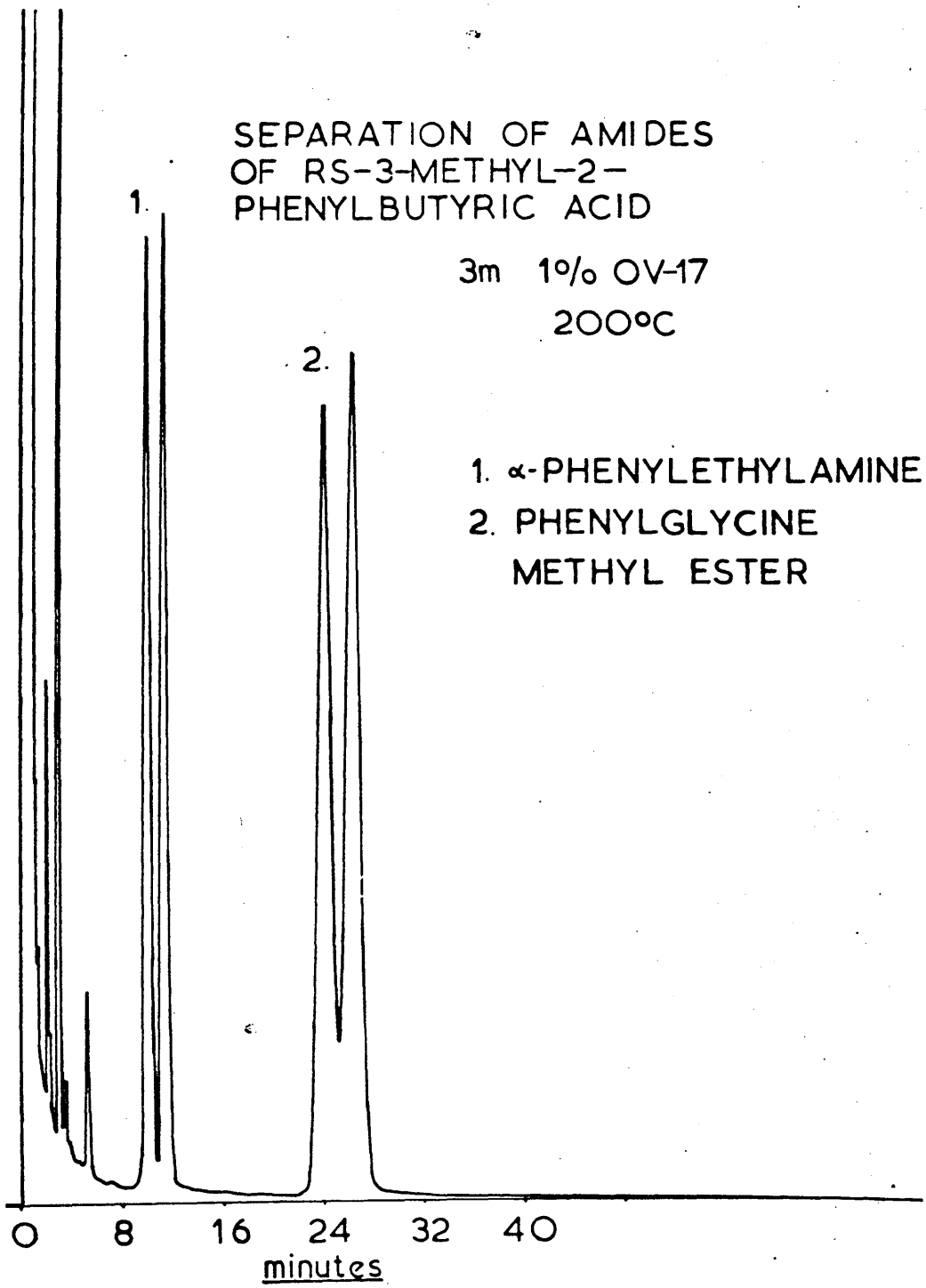


Fig. 25. Gas chromatographic separation of (R,S)-3-methyl-2-phenylbutyric acid as amides of (1) (R)-(+)-α-phenylethylamine and (2) (R)-(-)-phenylglycine methyl ester.

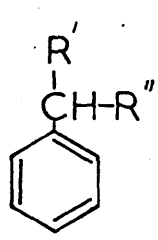
3.2. Gas chromatographic resolution of amines and amino acids by formation of diastereomeric amides with substituted phenylacetic acids

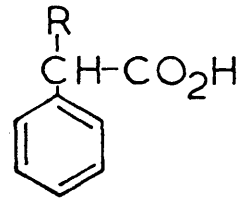
3.2.1. Gas chromatography

The acids used as resolving agents all had the general formula R-CH(Ph)-CO_2H. Considerable differences could be noted in the degree of separation achieved as R varied (Table 12.).

a) R = methyl, ethyl, isopropyl

Examples of the resolutions achievable with these acids are shown in Figures 23-25. In general, as R increased in size from methyl to ethyl to isopropyl, the retention index difference between any two diastereomers increased. This is illustrated in Figure 26. No significant differences were observed between the resolutions achieved on SE-30 and OV-17 phases, except in the case of phenylalanine methyl ester 3-methyl-2-phenylbutyramide, suggesting that hydrogen bonding with the phase is not a dominant factor involved in the separation mechanism. However there was some evidence for intramolecular hydrogen bonding between the amide hydrogen and the phenyl ring (cf. Fig. 26) : (1) an increase in alkyl chain length between the chiral centre and the aromatic centre decreased the separation (e.g. α-phenylethylamine and amphetamine or phenylglycine methyl ester and phenylalanine methyl ester); (2) substitition of the amide hydrogen by a methyl group in the α-phenylethylamine derivatives, resulted in a decrease in resolution of the





R'	R''	R: CH_3	CH_2CH_3	$CH(CH_3)_2$	OCH_3
CH_3	NH_2	25	30	40	30
CH_3	$NH\text{-}CH_3$	15	25	15	0
H	CH_3 $CH\text{-}NH_2$	10	15	25	25
H	CH_3 CH_3 $CH-NH$	25	25	30	15
CO_2CH_3	NH_2	20	20	25	40
H	CO_2CH_3 $CH\text{-}NH_2$	0	0	20	20

Comparison of ΔI values (OV-17) for a series of structurally related diastereomeric amides.

Fig. 26. The variation in gas chromatographic separation achieved for a series of structurally related amides as the side chain substituents are changed.

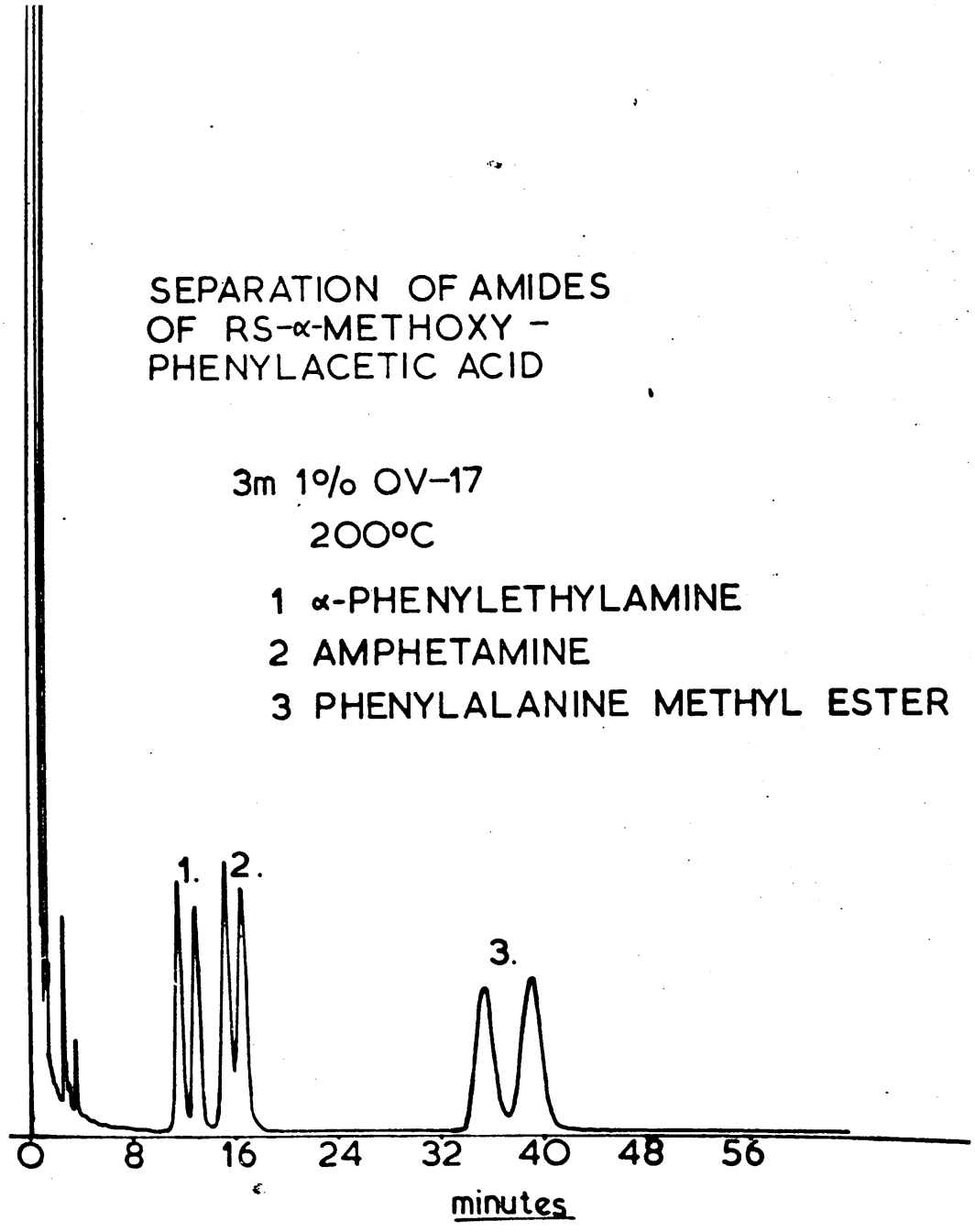


Fig. 27. Gas chromatographic separation of (R,S)-α-methoxy-phenylacetic acid as amides of (1) (R)-(+)-α-phenylethylamine, (2) (R)-(-)-amphetamine and (3) (R)-(+)-phenylalanine methyl ester.

diastereomers. When such bonded conformations could not be achieved the influence of bulky groups around the chiral centre probably assumed greater importance, hence the increased resolution for methamphetamine compared to amphetamine.

Norvaline methyl ester proved very difficult to resolve but the isomeric valine methyl esters, containing the branched alkyl chain, were well distinguished as amides of α-phenylpropionic acid. In this example the degree of resolution achieved decreased as R increased in size and no difference was observed for the 3-methyl-2-phenylbutyramides.

b) R = methoxyl

Substitution of the alkyl group in the resolving agent by a methoxyl group caused an enhancement of the separations with the primary amines but sharply decreased those of the secondary amines. This suggests that hydrogen bonding involving the amide hydrogen is an important factor in the resolution. The most noticeable differences were observed with the amino acid methyl esters. The previously unobserved resolution of norvaline methyl ester was achieved, but no separation could be obtained for the diastereomeric valine methyl ester derivatives. Phenylalanine methyl ester had also presented problems with the α-alkylphenylacetic acids but a satisfactory separation was achieved of the α-methoxy-phenylacetamides (Fig. 27). The increased polarity of the methoxyl group, with respect to the previous alkyl groups,

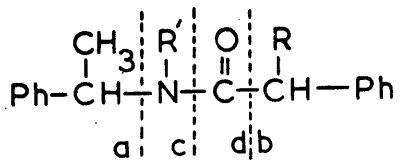
produced a slight enhancement of the resolutions on the more polar OV-17 phase. The most striking example of this was observed with proline methyl ester : no separation was observed on SE-30 but a retention index difference of ten units was obtained on OV-17.

c) R = chloro

α-Chlorophenylacetic acid proved to be a less useful resolving agent for two reasons. Fewer resolutions were achieved throughout the series and secondly racemisation of the reagent was observed during the acylation.

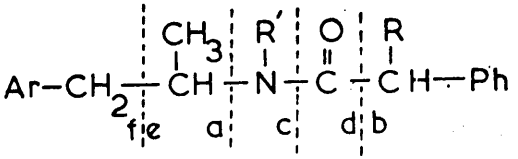
The elution order of the α-phenylpropionamides and α-phenylbutyramides was (RR)/(SS) before (RS)/(SR) in all cases except that of phenylglycine methyl ester. This is the reverse of the sequence established for N-trifluoroacetyl prolyl amides[154,156,202] but is in agreement with the order predicted by Feibush's rule.[225] The anomalous behaviour of phenylglycine has been noted previously as the N-trifluoroacetyl-2-octyl ester.[338]

Since the 3-methyl-2-phenylbutyric acid and α-methoxyphenylacetic acid employed were racemic it is not possible to state the elution order in these cases. The absolute configuration of 3-methyl-2-phenylbutyric acid was at one time assigned[164] as (R)-(+), (S)-(-), while α-phenylpropionic and α-phenylbutyric acids were known to be (S)-(+), (R)-(-). The amides of the (+)-acids with (S)-(+)-methamphetamine were, however, found to elute before those of the (-)-acids on GLC, in every



I: R′ = H

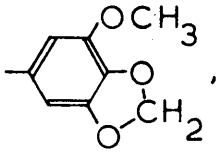
II: R′ = CH_3

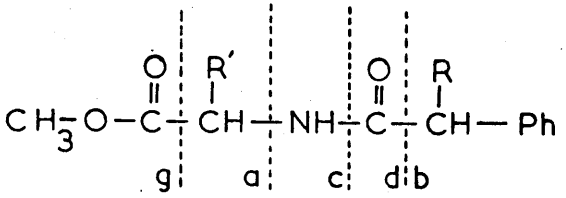


III: Ar = C_6H_5, R′ = H

IV: Ar = C_6H_5, R′ = CH_3

V: Ar = p-Cl C_6H_4, R′ = H.

VI: Ar =  , R′ = H

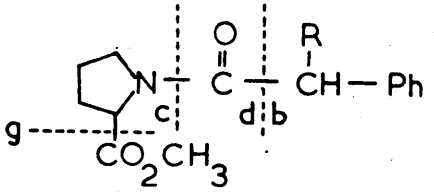


VII: R′ = n-C_3H_7

VIII: R′ = $CH(CH_3)_2$

X: R′ = $CH_2C_6H_5$

XI: R′ = C_6H_5



IX

Fig. 28. Mass spectral fragmentation modes for amides of substituted phenylacetic acids.

case.[166] On re-examination[168,169] the assignment of absolute configuration on the basis of the gas chromatographic data was shown to be correct.

It, therefore, seems likely that the 3-methyl-2-phenylbutyramides would follow the same elution order as that observed for the derivatives of the other two 2-phenylalkanoic acids. It is not possible, however to predict the effect of substituting a more polar substituent, and in fact a reversal of the elution order was observed for the α-chlorophenylacetamides.

3.2.2. Mass spectra

The principal ions in the 22.5 eV mass spectra of the amides are presented in Table 13. Ions below m/e 80 have been excluded (except for proline, valine and norvaline derivatives) since they have limited diagnostic value. Molecular ions were observed in all instances, although they were generally of low abundance in the α-methoxyphenylacetamides and the α-chlorophenylacetamides. The superscripts in Table 13 refer to the fragmentation modes shown in Figure 28. Salient features of the spectra are discussed below :

a) α-Phenylethylamine and N-methyl-α-phenylethylamine

The base peak in all the spectra, except those of the α-methoxyphenylacetamides was at m/e 105 due to benzylic cleavage at the NH group. The spectra of the N-α-methoxyphenylacetyl derivatives were dominated by a base

Amine/Amino acid		[M]$^{+\cdot}$	Other ions (% abundance)					
α-Phenylethylamine	(1)	267(25)	105[a](100)	119[b](55)	91 (40)	120[c](19)	118 (18)	106 (15)
	(2)	253(18)	105[a,b](100)	106 (19)	104 (17)	132 (10)	120[c](0.9)	
	(3)	281(24)	105[a](100)	133[b](70)	91 (61)	134 (18)	106 (15)	239 (11)
	(4)	269(0.1)	121[b](100)	122 (30)	105[a](15)	77 (5)	91 (4)	123 (3)
	(5)	273(0.4)	105[a](100)	106 (12)	120[c](8)	104 (4)	237 (4)	125[b](1)
N-Methyl-α-phenylethylamine	(1)	281(25)	105[a](100)	119[b](30)	91 (25)	106 (11)	120 (11)	118 (7)
	(2)	267(28)	105[a,b](100)	106 (10)	104 (7)	163 (6)	120 (4)	162[d](3)
	(3)	295(26)	105[a](100)	133[b](39)	91 (32)	136 (19)	106 (10)	118 (9)
	(4)	283(0.9)	121[b](100)	105[a](25)	122 (8)	77 (2)	118 (2)	134 (2)
	(5)	287(3)	105[a](100)	134[c](40)	118 (12)	251 (11)	132 (10)	106 (9)
Amphetamine	(1)	281(2)	190[e](100)	119[a,b](69)	91[f](56)	118(215)	191 (14)	120 (12)
	(2)	267(2)	176[e](100)	105[b](42)	118 (17)	91[f](15)	167 (13)	119[a](10)
	(3)	295(2)	204[e](100)	133[b](88)	91[f](63)	118 (33)	119[a](18)	178 (10)
	(4)	283(0.9)	121[b](100)	122 (30)	192[e](26)	91[f](7)	118 (3)	119[a](3)
	(5)	287(3)	196[e](100)	118 (77)	119[a](45)	91[f](40)	198[e](35)	125[b](18)
Methamphetamine	(1)	295(0.3)	204[e](100)	119[a,b](32)	91[f](18)	205 (15)	118 (7)	178 (3)
	(2)	281(0.3)	190[e](100)	105[b](27)	191 (14)	91[f](5)	119[a](5)	118 (3)
	(3)	309(0.2)	218[e](100)	133[b](68)	91[f](68)	136 (29)	118 (23)	219 (18)
	(4)	297(0.2)	121[b](100)	206[e](21)	119[a](16)	91[f](12)	122 (9)	176 (8)
	(5)	301(0.2)	210[e](100)	212[e](32)	91[f](15)	118 (15)	125[b](14)	119[a](11)
p-Chloroamphetamine	(1)	315(1)	190[e](100)	119[b](43)	152 (23)	91 (21)	191 (13)	164 (9)
	(2)	301(1)	176[e](100)	105[b](70)	152 (26)	177 (12)	154 (10)	125[f](9)
	(3)	329(1)	204[e](100)	133[b](94)	91 (50)	152 (32)	205 (15)	134 (14)
	(4)	317(0.4)	121[b](100)	192[e](24)	122 (19)	152 (3)	193 (3)	91 (2)
3-Methoxy-4,5-methylene-dioxyamphetamine	(1)	355(3)	192 (100)	119[b](12)	193[a](12)	91 (4)	165[f](3)	190[e](3)
	(2)	341(3)	192 (100)	105[b](17)	193[a](14)	165[f](5)	176[e](4)	166 (3)
	(3)	369(3)	192 (100)	133[b](19)	193[a](13)	91 (10)	165[f](5)	204[e](3)
	(4)	357(2)	192[e](100)	121[b](70)	193[a](15)	122 (8)	173 (6)	190 (4)
Norvaline methyl ester	(1)	277(3)	130[c](100)	72 (95)	91 (50)	120 (46)	158[d](29)	235 (25)
	(2)	263(2)	130[c](100)	72 (70)	106 (54)	158[d](46)	105[b](32)	221 (30)
	(3)	291(6)	130[c](100)	72 (97)	118 (60)	249 (50)	91 (38)	133[b](27)
Norvaline methyl ester	(4)	279(0.1)	121[b](100)	122 (33)	130[c](7)	158[d](5)	91 (2)	123 (2)
	(5)	283(0.06)	130[c](100)	158[d](45)	88 (16)	72 (15)	98 (8)	224[g](8)
Valine methyl ester	(1)	277(7)	130[c](100)	72 (90)	91 (46)	120 (40)	119[b](35)	115[a](30)
	(2)	263(9)	130[c](100)	72 (72)	106 (60)	105[b](53)	115[a](37)	158[d](33)
	(3)	291(6)	72 (100)	130[c](86)	118 (48)	91 (35)	133[b](33)	249 (25)
	(4)	279(0.1)	121[b](100)	122 (34)	130[c](9)	158[d](7)	123 (3)	91 (2)
	(5)	283(0.1)	130[c](100)	158[d](40)	98(17)	224[g](9)	72 (8)	131 (8)
Proline methyl ester	(1)	275(9)	70 (100)	128[c](43)	91 (34)	119[b](18)	216[g](15)	156[d](7)
	(2)	261(13)	70 (100)	128[c](51)	202[g](32)	156[d](20)	105[b](15)	203 (5)
	(3)	289(5)	70 (100)	128[c](34)	91 (14)	247 (14)	133[b](10)	118 (9)
	(4)	277(2)	121[b](100)	128[c](20)	156[d](12)	122 (9)	218[g](2)	247 (2)
	(5)	281(3)	128[c](100)	70 (53)	156[d](35)	222[g](17)	118 (8)	129 (8)
Phenylglycine methyl ester	(1)	311(4)	106 (100)	149[a](54)	252[g](44)	91 (41)	164[c](39)	119[b](23)
	(2)	297(5)	106 (100)	238[g](65)	149[a](53)	105[b](41)	164[c](36)	239 (14)
	(3)	325(3)	106 (100)	149[a](49)	164[c](35)	91 (33)	266[g](26)	118 (25)
	(4)	313(0.01)	121[b](100)	122 (46)	149[a](10)	192[d](6)	164[c](2)	254[g](2)
	(5)	317(0.8)	149[a](100)	192[d](45)	118 (32)	164[c](30)	106 (30)	258[g](28)
Phenylalanine methyl ester	(1)	325(4)	162 (100)	119[b](65)	120 (51)	163[a](45)	91 (45)	146 (14)
	(2)	311(4)	162 (100)	105[b](45)	163[a](30)	106 (18)	120 (17)	88 (11)
	(3)	339(8)	162 (100)	133[b](70)	135 (50)	163[a](46)	91 (36)	120 (31)
	(4)	327(0.6)	121[b](100)	122 (37)	206[d](10)	178[c](4)	91 (3)	146 (3)
	(5)	331(0.5)	162 (100)	178[c](18)	206[d](18)	146 (15)	163[a](13)	88 (10)

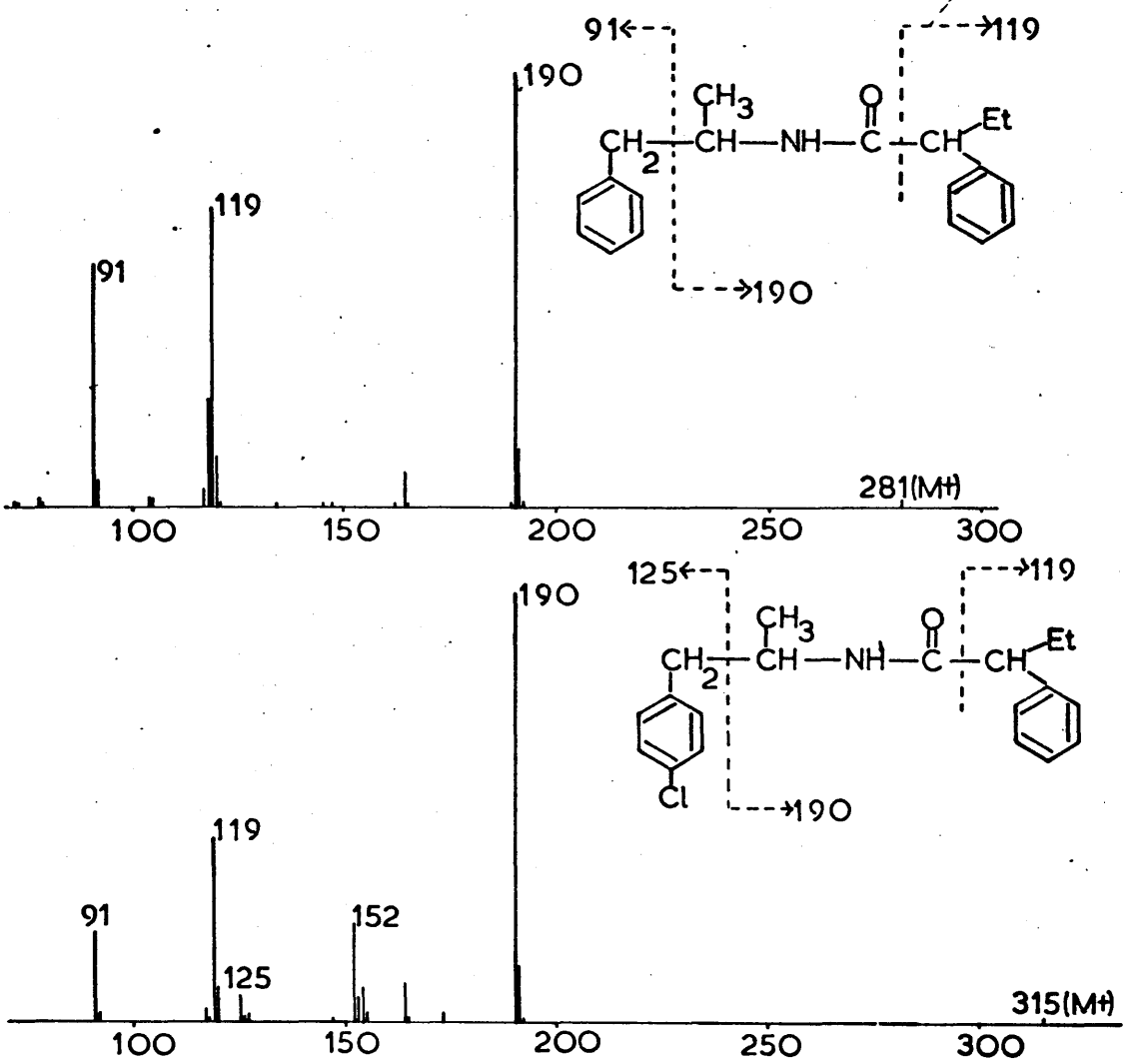


Fig. 29. Illustrative mass spectra of α-phenylbutyramides.

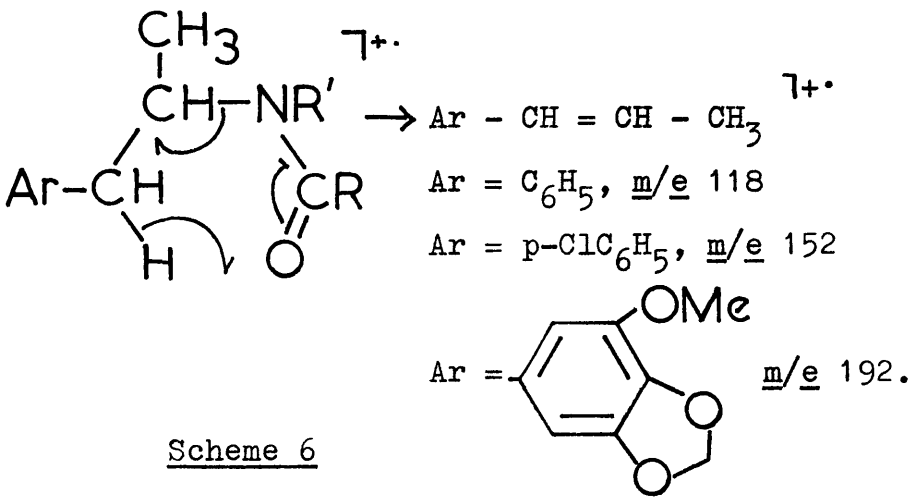
a) Mass spectrum of amphetamine α-phenylbutyramide.

b) Mass spectrum of p-chloroamphetamine α-phenylbutyramide.

peak at m/e 121 produced by cleavage adjacent to the carbonyl group. This fragmentation was also important in the phenylalkanoic acid derivatives (m/e 105, 119, 133 respectively) but was largely suppressed in the α-chlorophenylacetamides. Ions produced by cleavage of the amide bond with elimination of PhCHR-$\dot{C}$=O were also present but they were generally of fairly low abundance. However, this fragmentation appeared to be enhanced in the case of N-methyl-α-phenylethylamine α-chlorophenylacetamide producing an ion at m/e 134 of 40% abundance. The structure of this ion was confirmed by high resolution mass spectrometry : found, m/e 134.0972; calc. for $C_9H_{12}N$, 134.0972. A characteristic ion, although of fairly low abundance, was produced from the 3-methyl-2-phenylbutyramides by elimination of $C_3H_6^{\cdot}$ from the side chain.

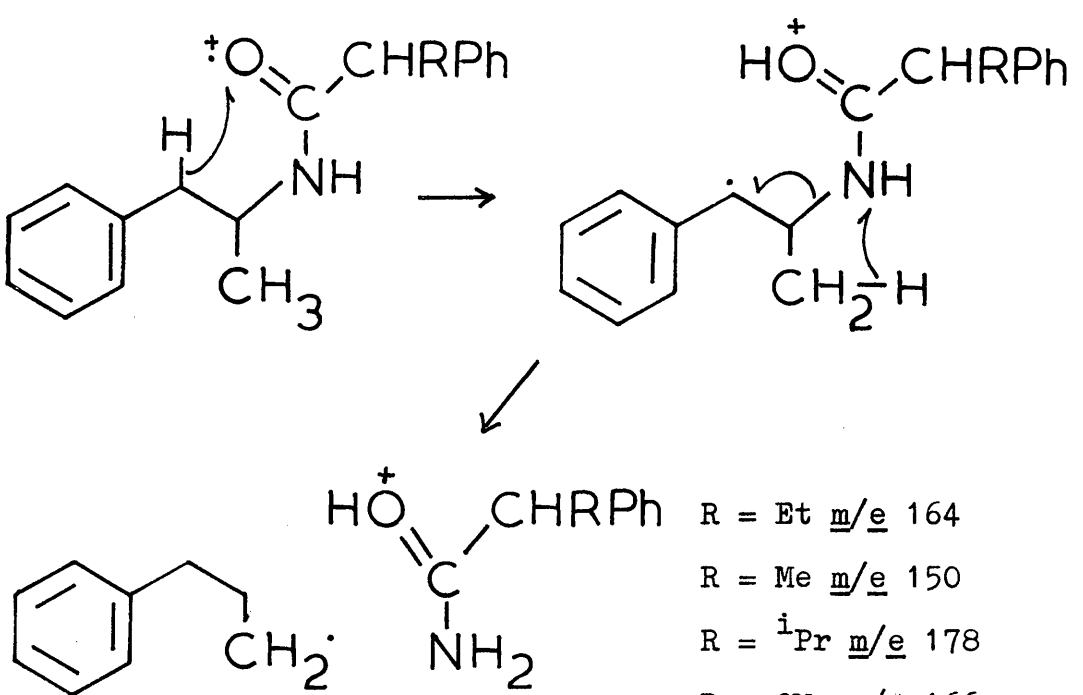
b) Amphetamine, methamphetamine, p-chloroamphetamine, and 3-methoxy-4,5-methylenedioxyamphetamine.

The base peak in most of the spectra arose from benzylic cleavage with the charge remaining on the amide fragment. The ions obtained from the aralkyl portion were also present but to a lesser extent. These fragmentations are illustrated for the α-phenylbutyramides in Figure 29. In addition, abundant ions were produced by benzylic fission at the acyl group; the base peaks in the spectra of the α-methoxyphenylacetamides were formed in this way. Other major fragments were due to alkyl-nitrogen fission, or alkyl-nitrogen cleavage accompanied by hydrogen transfer (Scheme 6).



Scheme 6

The latter process produced the base peak in all the spectra of 3-methoxy-4,5-methylenedioxyamphetamine derivatives. High resolution mass spectrometry confirmed the assignment of the ion at m/e 192 to this fragment in the case of the α-phenylbutyramide : found, m/e 192.0778; calc. for $C_{11}H_{12}O_3$, 192.0786. Rearrangement ions with double hydrogen transfer were also present, although to a lesser extent (Scheme 7).

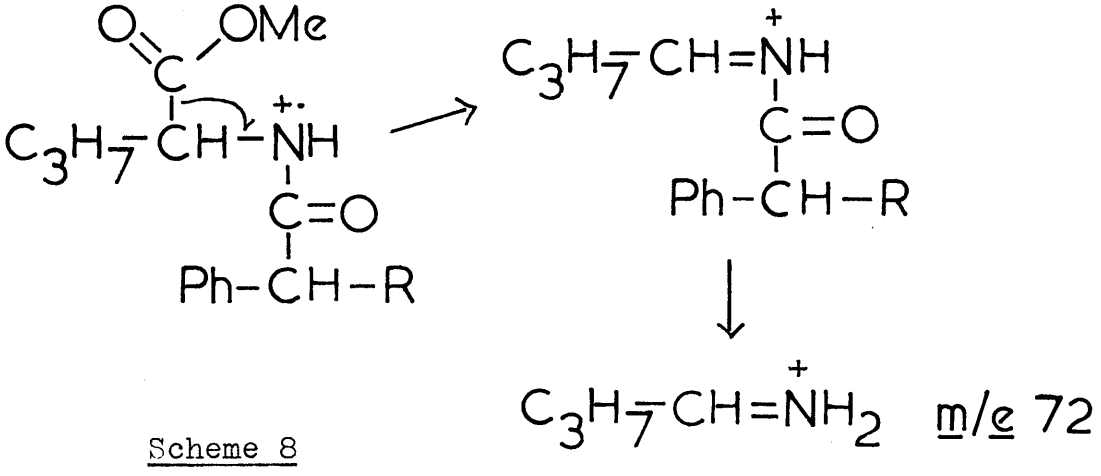


Scheme 7

For amphetamine α-chlorophenylacetamide accurate mass measurement at m/e 170 gave m/e 170.0374; calc. for $C_8H_9NO^{35}Cl$, 170.0373.

c) Norvaline methyl ester and valine methyl ester.

The spectra of the isomeric amides were very similar with the base peaks in most cases being produced by cleavage of the amide bond. The exceptions were the α-methoxyphenylacetamides where benzylic cleavage was the major process. Very abundant ions at m/e 72 in the spectra of the phenylalkanoic acid amides were shown to be due to the imonium fragment[335] (Scheme 8) by accurate mass measurement in the case of the N-phenylbutyramide. (Found : m/e 72.0808; calc. for $C_4H_{10}N$, 72.0813).



Scheme 8

This ion was of lower abundance in the spectra of the α-chlorophenylacetamides and was not observed from the α-methoxyphenylacetamides. In the spectra of the amides of the phenylalkanoic acids ions produced by benzylic fission with hydrogen transfer were observed (Scheme 9).

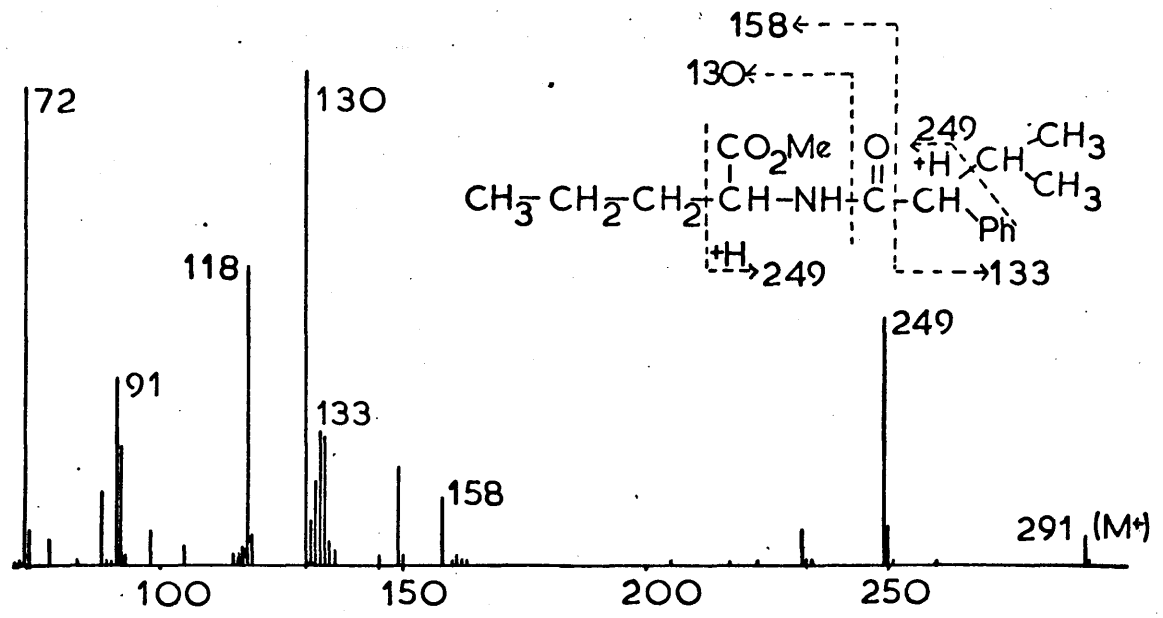


Fig. 30. Mass spectrum (22.5 eV) of norvaline methyl ester 3-methyl-2-phenylbutyramide.

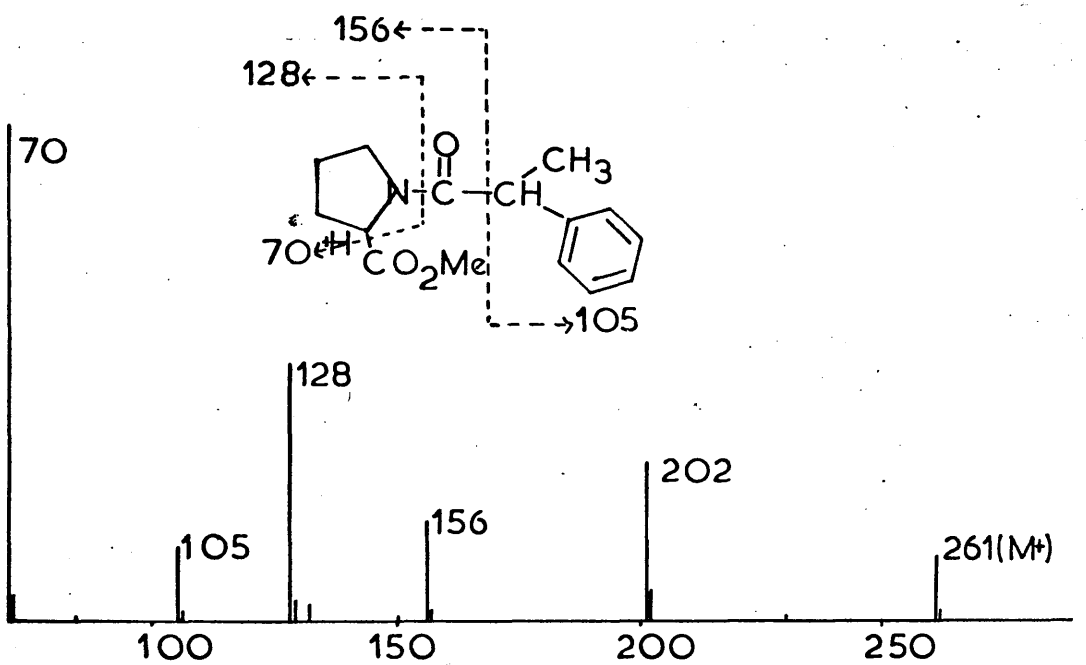
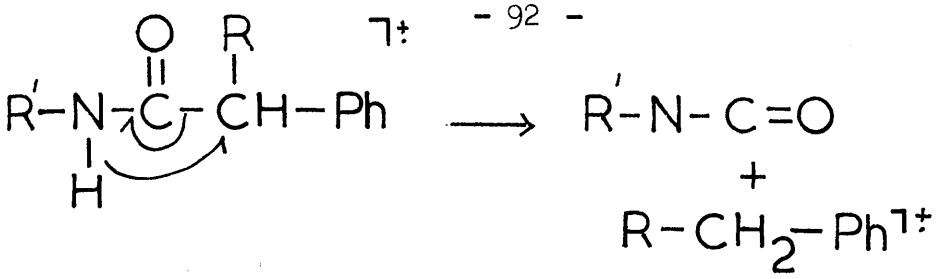


Fig. 31. Mass spectrum (22.5 eV) of proline methyl ester α-phenylpropionamide.



R = Et, m/e 120

R = Me, m/e 106

R = ^{i}Pr, m/e 134

Scheme 9

When R = Et and Me the ions produced by benzylic cleavage accompanied by hydrogen transfer were more prominent than those produced by cleavage without hydrogen transfer, but the reverse was true when R = ^{i}Pr (Fig. 30). Both ions were also observed in the spectra of the α-chlorophenylacetamides but they were of considerably lower abundance. In all the spectra ions due to loss of the carbomethoxyl group were observed.

d) Proline methyl ester

The major fragments in all the spectra, except that of the α-methoxyphenylacetamide, were the pyrrolidinium ion (m/e 70) and the ion produced by amide cleavage (m/e 128) (Fig. 31). Benzylic cleavages were also observed in all the spectra with the base peak (m/e 121) being produced by this process in the α-methoxyphenylacetamide. Ions produced by loss of $-CO_2CH_3$ were present in all cases.

e) Phenylglycine methyl ester and phenylalanine methyl ester

As noted in the spectra of the drimanamides major ions due to the imonium fragment (m/e 106, 120 respectively) were produced (cf. Scheme 8). This fragment produced the base peak in the spectra of the phenylalkanoic acid amides

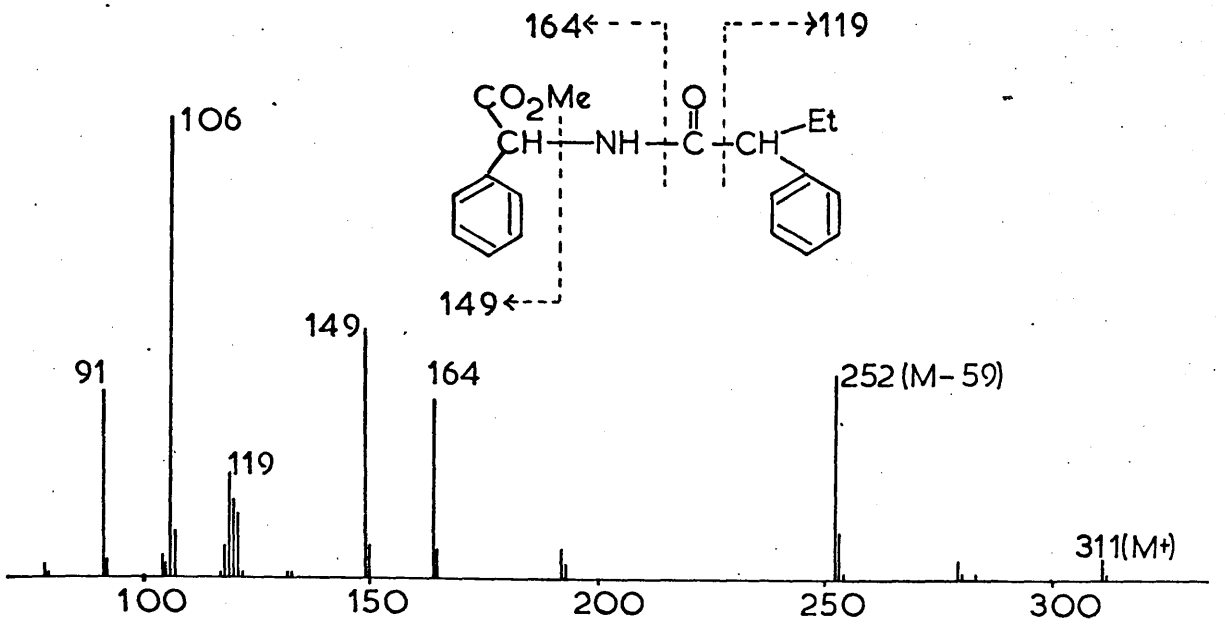
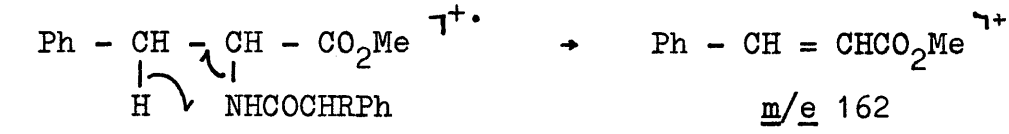


Fig. 32. Mass spectrum (22.5 eV) of phenylglycine methyl ester α-phenylbutyramide.

of phenylglycine methyl ester. Abundant ions also arose by simple benzylic cleavages as illustrated in Figure 32. The base peak in the spectra of the amides of phenylalanine methyl ester (except the α-methoxy phenylacetamide) arose by alkyl-nitrogen fission with hydrogen transfer resulting in a methyl cinnamate ion[339] (Scheme 10).



Scheme 10

The composition of this ion was verified (for the N-phenylbutyramide) by accurate mass measurement. (Found, m/e 162.0680; calc. for $C_{10}H_{10}O_2$; 162.0681).

3.2.3. Application to amino-alcohols : The ephedrines

By making use of the greater reactivity of the amino function it was found possible to derivatise the amine in the presence of the alcohol without producing any bis-derivative. Using an equimolar quantity of phenylbutyryl chloride or phenylbutyric anhydride the best yields were obtained with a ten-minute reaction time at room temperature. Use of phenylbutyric imidazolide provided more selectivity since imidazolides react only very slowly with alcohols at room temperature.[340] When the imidazolide was used a 30 min reaction time was employed. Under these conditions no bisphenylbutyryl derivatives were detected on GLC.

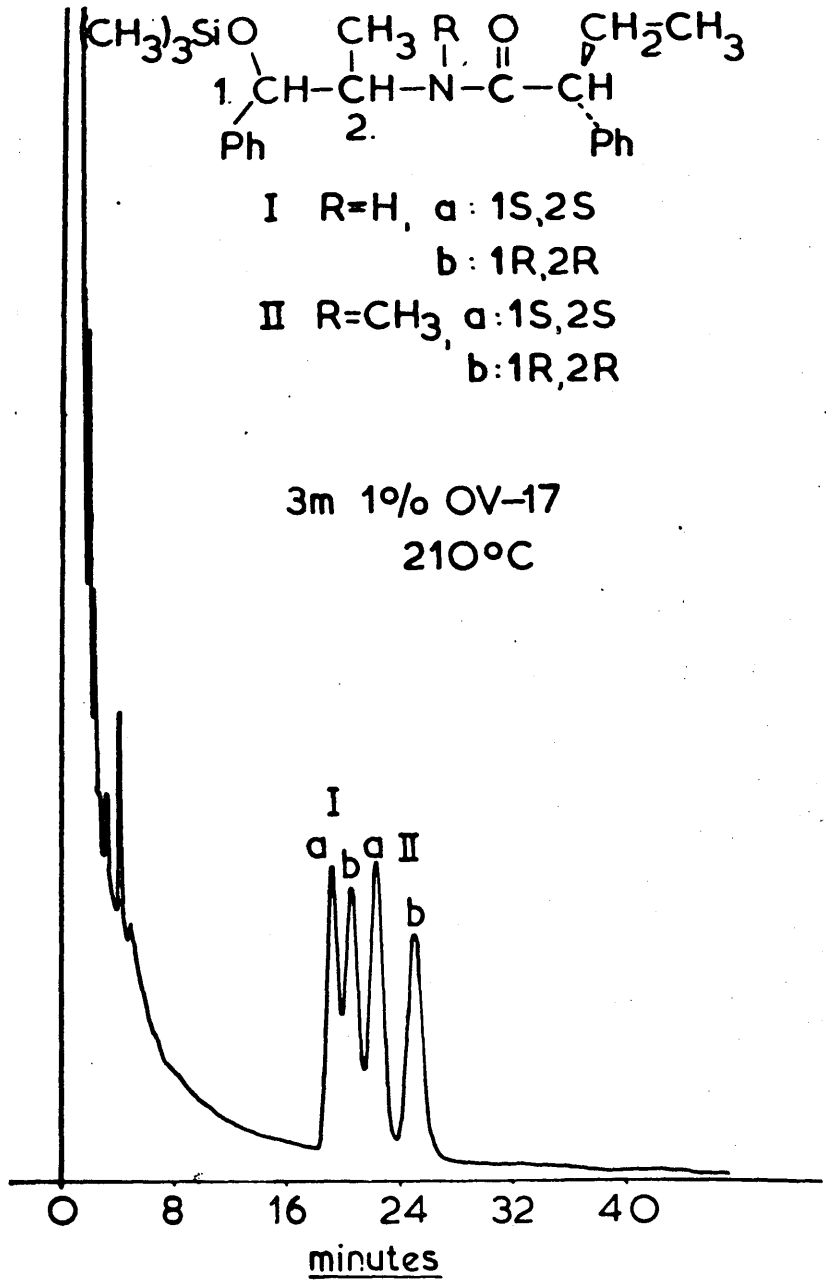


Fig. 33. Gas chromatographic separation of (1) (±)-nor-ψ-ephedrine-O-TMS and (2) (±)-ψ-ephedrine-O-TMS as (R)-N-phenylbutyryl derivatives.

Only ephedrine itself gave any indication of a resolution as the N-phenylbutyryl derivative. However, when the free hydroxyl group was trimethylsilylated, good separations were also observed for the ψ-ephedrines and nor-ψ-ephedrines (Table 14, Fig. 33). This change was presumably due to the steric requirements of the large trimethylsilyloxy group forcing "preferred" conformations on the molecule which enhanced the differences between the diastereomers. The free hydroxyl group would participate in hydrogen bonding, and removal of this effect would also change the preferred conformations. The order of elution of the diastereomers, with respect to the chiral centre carrying the amine, was (RR) < (RS) for the ephedrine derivative but (RS) < (RR) for the derivatives of the ψ-ephedrines.

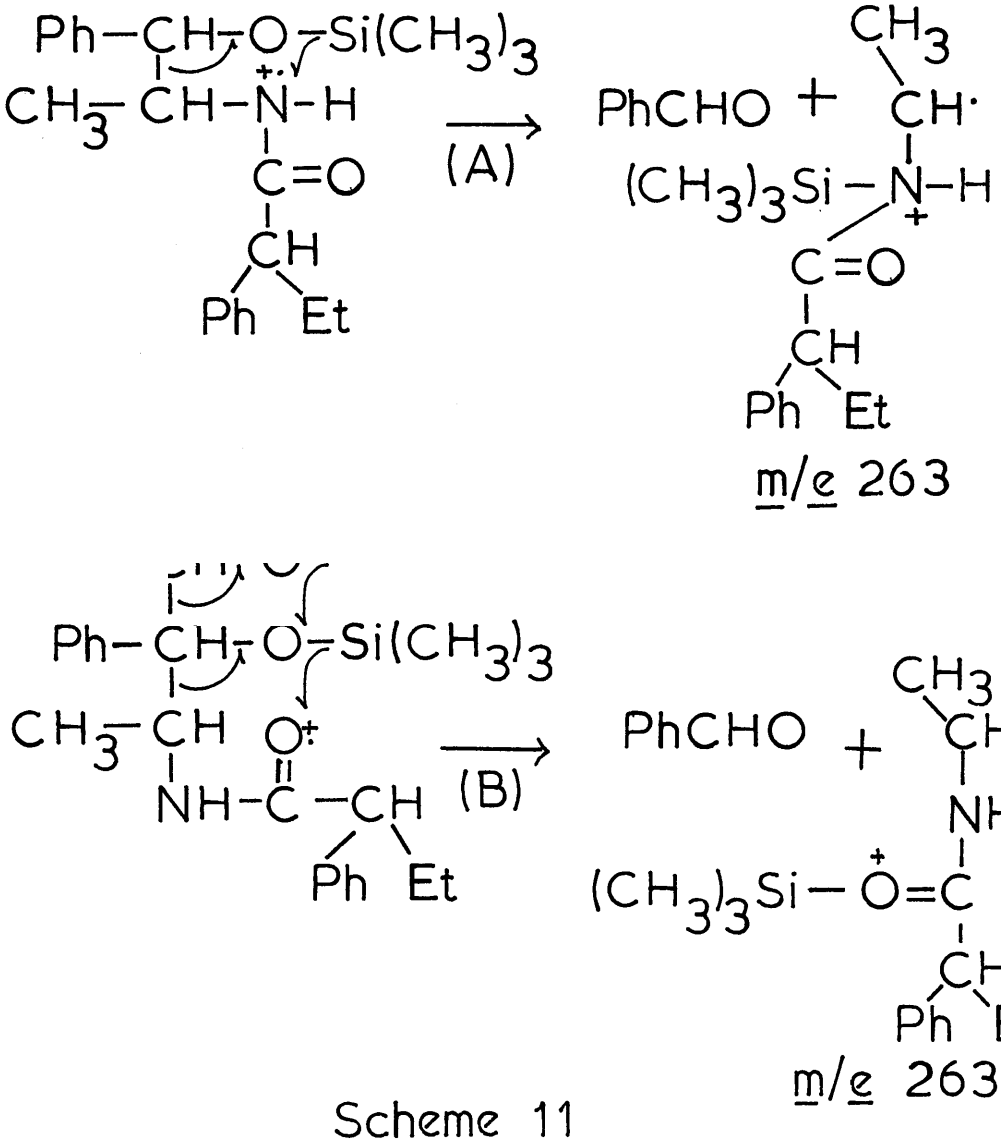
Under electron ionisation (E.I.) conditions (22.5 eV) the N-phenylbutyryl, O-TMS-ephedrine derivatives were completely fragmented. The molecular ions were of very low abundance (0.1-0.2%). The base peak in all cases were produced by benzylic cleavage. The fragment containing the amine moiety was the base peak in the spectra of the ephedrine derivatives (m/e 204), but the charge was preferentially retained on the fragment containing the trimethylsilyl ether group in the spectra of the nor-ephedrine derivatives (m/e 179). Cleavage adjacent to the carbonyl produced the ion at m/e 119 characteristic of the phenylbutyryl moiety.

An abundant ion at m/e 263 was observed in the spectra

Table 14. Gas chromatographic and mass spectral characteristics of (R)(-)-phenylbutyryl derivatives of the ephedrines, E.I.-electron ionisation; C.I.-isobutane chemical ionisation

	$I^{1\%\ OV\text{-}1}_{190^{\circ}C}$	$I^{1\%\ OV\text{-}17}_{210^{\circ}C}$	Mass spectral characteristics		
				$M^{+\cdot}$	Other ions (% abundance)
(1S,2R)-(+)-Ephedrine-N-phenyl-butyramide	2350				
(1R,2S)-(-)-Ephedrine-N-phenyl-butyramide	2360				
(1S,2R)-(+)-Ephedrine-O-TMS-N-phenylbutyramide	2300	2555	E.I.	383(0.1)	204(100); 119(30); 205(18); 91(14); 179(9)
(1R,2S)-(-)-Ephedrine-O-TMS-N-phenylbutyramide	2310	2570	C.I.	384(97)	294(100); 204(71); 91(50); 119(25)
(1S,2S)-(+)-ψ-Ephedrine-O-TMS-N-phenylbutyramide	2290	2570	E.I.	383(0.1)	204(100); 119(25); 205(15); 91(12); 179(8)
(1R,2R)-(-)-ψ-Ephedrine-O-TMS-N-phenylbutyramide	2325	2600	C.I.	384(100)	204(87); 294(64); 91(33); 119(20)
(±)-nor-Ephedrine-O-TMS-N-phenylbutryamide	2280	2550	E.I.	369(0.2)	179(100); 263(42); 190(36); 119(32); 180(18); 206(14); 91(14)
(1S,2S)-(+)-nor-ψ-Ephedrine-O-TMS-N-phenylbutyramide	2240	2520	E.I.	369(0.1)	179(100); 263(43); 190(36); 119(35); 180(18); 91(14); 206(12)
(1R,2R)-(-)-nor-ψ-Ephedrine-O-TMS-N-phenylbutyramide	2260	2545	C.I.	370(68)	280(100); 179(31); 91(23); 263(21)

of the nor-ephedrine derivatives. This rose by 9 a.m.u. to m/e 272 when perdeuterated TMS ethers were prepared, indicating that the fragment contained the trimethylsilyl group. This could be ascribed to a rearrangement ion produced by transfer of the trimethylsilyl group and elimination of benzaldehyde. The silyl group could possibly be transferred either to the nitrogen (A) or to the carbonyl oxygen (B) as represented in Scheme 11.



Scheme 11

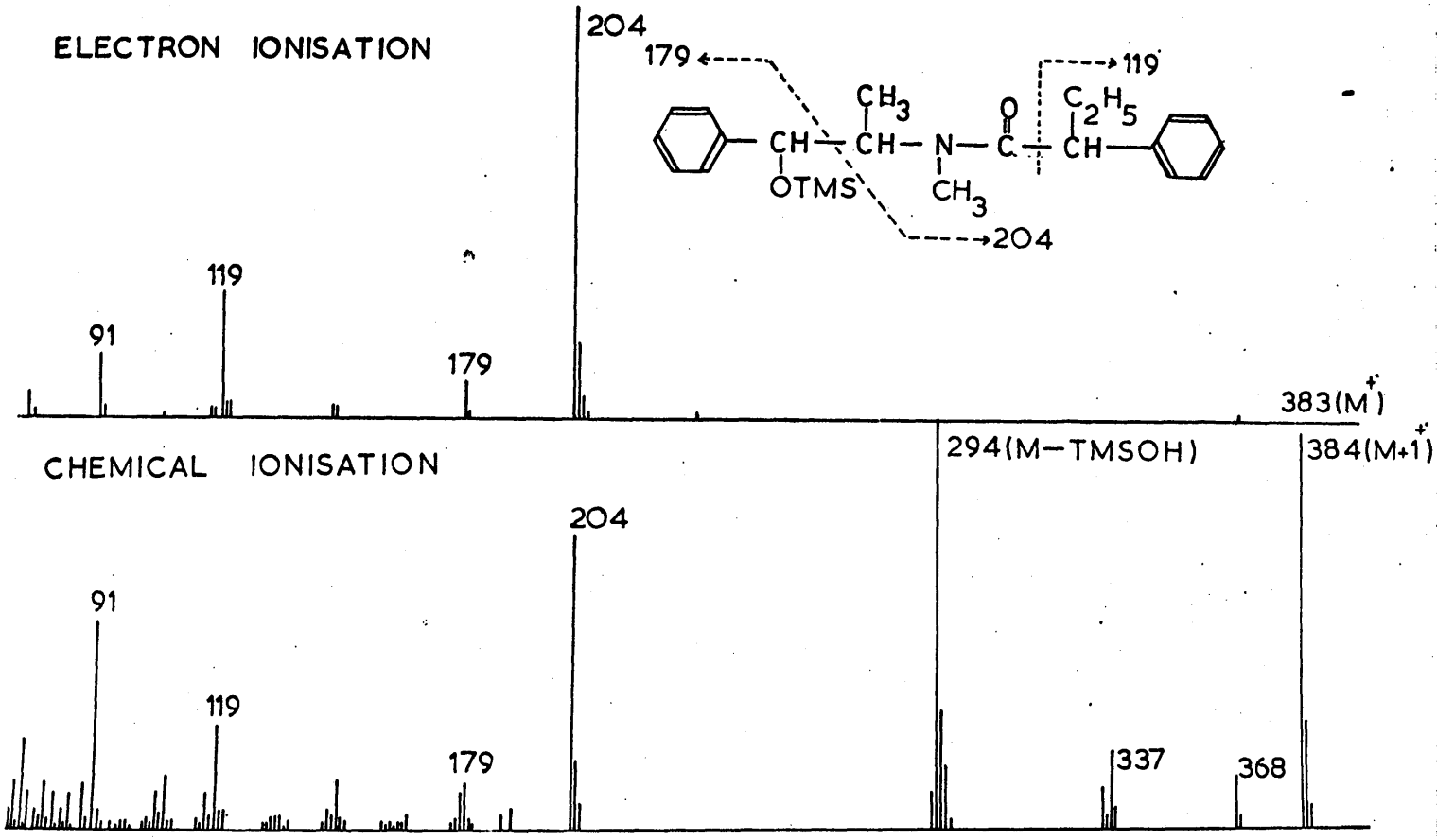


Fig. 34. Comparison of the mass spectra of N-phenylbutyryl-O-TMS-ephedrine under electron ionisation (22.5 eV) and chemical ionisation (isobutane) conditions.

The chemical ionisation (C.I.) spectra using isobutane as reagent gas, showed considerable enhancement of the ions in the higher mass range. Abundant $[M+1]^{+\cdot}$ ions were observed in all cases (this constituted the base peak in the C.I. spectrum of the ↳-ephedrine derivative), but fragment ions were also present. A major fragmentation under C.I. conditions was loss of trimethylsilanol $[[M+1]^{+\cdot} - 90]$, and otherwise the molecules fragmented in a manner similar to that observed under E.I. conditions.

A comparison of the E.I. and C.I. spectra of N-phenylbutyryl, O-TMS-ephedrine is shown in Fig. 34.

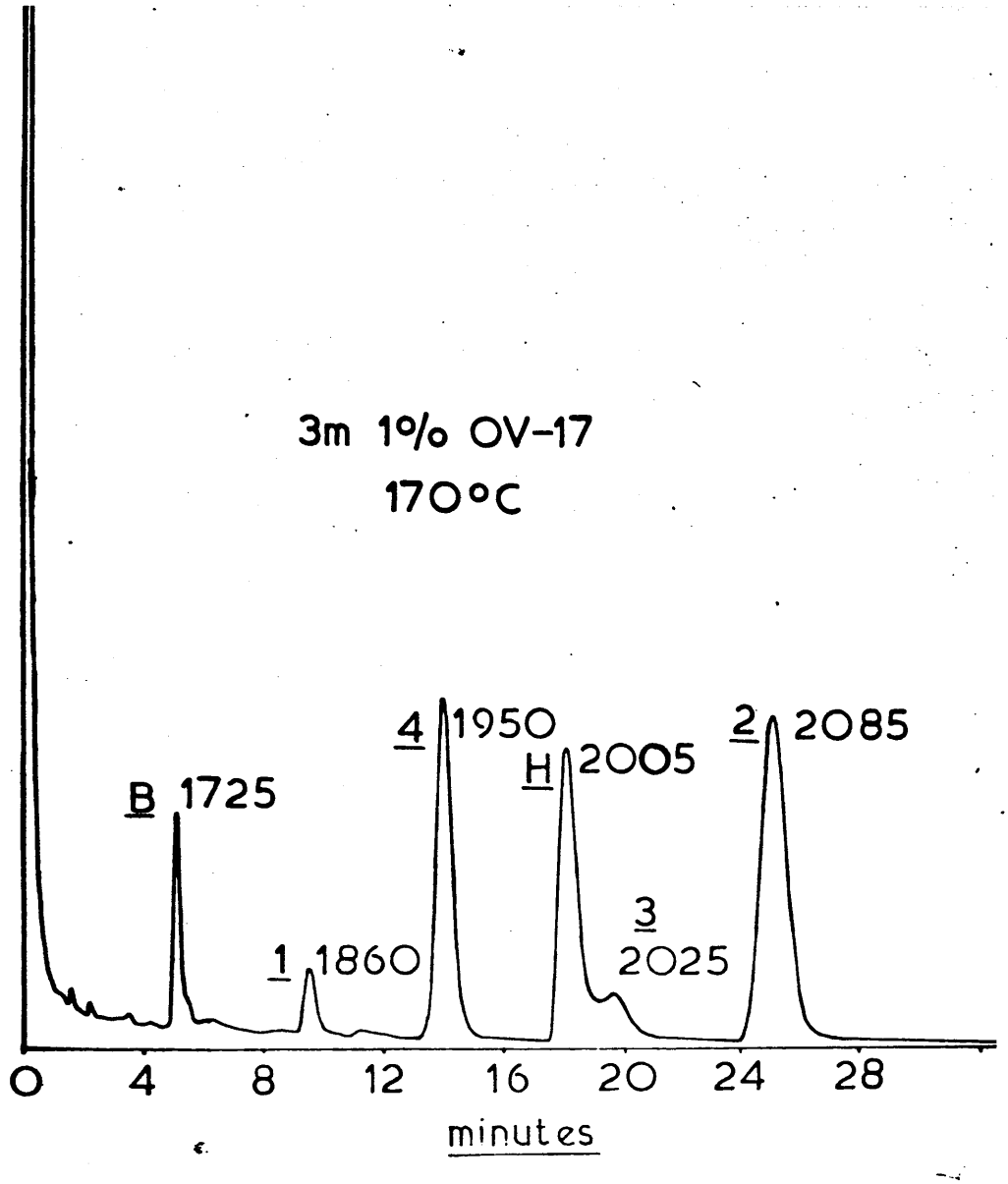


Fig. 35. Gas liquid chromatogram of the urinary metabolites of ibuprofen as their methyl ester trimethylsilyl ethers. (B = ibuprofen; H = hippuric acid; the metabolites are numbered 1 to 4 as in the text.)

3.3. The urinary metabolism of ibuprofen, 2-(4-isobutylphenyl)propionic acid

3.3.1. Isolation and identification of metabolites

Urine samples were initially obtained from a male volunteer after ingestion of 3 x 200 mg. tablets of "Brufen" and subsequently from patients receiving daily treatment with the drug.

Purification of the extract, obtained as described in section 2.2.1., was at first attempted by chromatography of the free acidic components on a column of silica gel, using chloroform-methanol-acetic acid (80:20:5 v/v) as eluting solvent. Examination of aliquots from each fraction, after methylation, on GLC showed that the major components had been eluted in the first three fractions. Further purification of these samples was achieved by preparative TLC (mobile phase : chloroform-methanol-acetic acid, 20:1:0.5). After methylation and trimethylsilylation the samples were examined by GLC and GC-MS. The major component of fractions 1 and 2 was shown to be unmetabolised 2-(4-isobutylphenyl)propionic acid by comparison of its GLC and GC-MS behaviour with that of the authentic acid isolated from "Brufen" tablets. Fraction 3 contained four components which were identified as metabolites of ibuprofen (Fig. 35).

Subsequently extracts were first methylated and then purified by preparative TLC using chloroform-ethyl acetate (3:1 v/v) as mobile phase. Typical R_F values and gas liquid chromatographic data for ibuprofen and its metabolites are

Table 15. Chromatographic data for derivatives of 2-(4-isobutylphenyl)propionic acid and its metabolites. Solvent system for TLC : chloroform-ethylacetate (3:1)

	R_F value	$I^{1\%\ OV-1}_{150^oC}$	$I^{1\%\ OV-17}_{170^oC}$	$I^{1\%\ QF-1}_{150^oC}$
2-(4-Isobutylphenyl)-propionic acid methyl ester	0.70	1515	1725	1800
Metabolite 1 methyl ester	0.50	1705	1905	2225
Metabolite 1 methyl ester trimethylsilyl ether	-	1700	1860	2135
Metabolite 2 dimethyl ester	0.56	1775	2080	2260
Metabolite 3 methyl ester	-	1780	2090	-
Metabolite 3 methyl ester trimethylsilyl ether	-	1840	2025	-
Metabolite 4 methyl ester	0.34	1660	1935	2090
Metabolite 4 methyl ester trimethylsilyl ether	-	1780	1950	2080

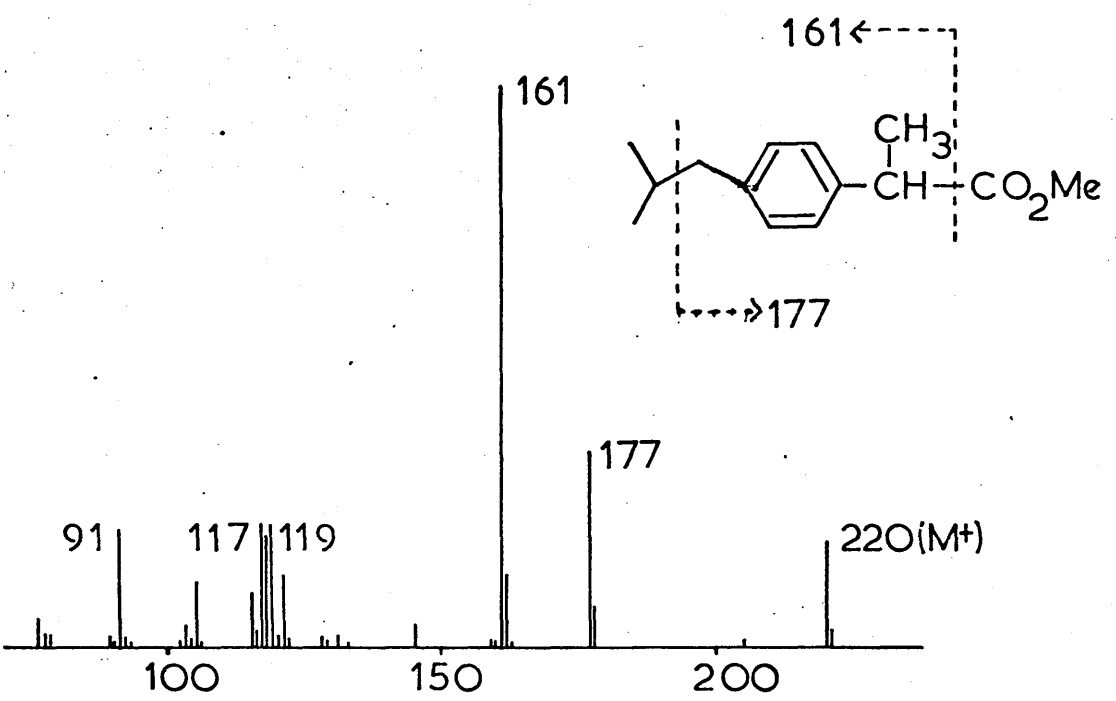


Fig. 36. Mass spectrum of 2-(4-isobutylphenyl)-propionic acid methyl ester.

Table 16. Gas liquid chromatographic and mass spectrometric data for derivatives of 2-(4-isobutylphenyl)propionic acid

Derivative	$I^{1\% \text{ OV-1}}_{150^{o}C}$	Mass spectral characteristics	
		$M^{+}\cdot$	Other ions (% abundance)
Methyl ester	1515	220(19)	161(100); 177(35); 117(22); 119(22); 91(21)
$LiAlH_4$ reduction product as TMS ether	1575	264(7)	73(100); 161(98); 103(95); 75(38); 117(18); 249(18)
$LiAl^2H_4$ reduction product as TMS ether	1565	266(6)	161(100); 73(100); 105(95); 75(31); 251(18); 119(17)

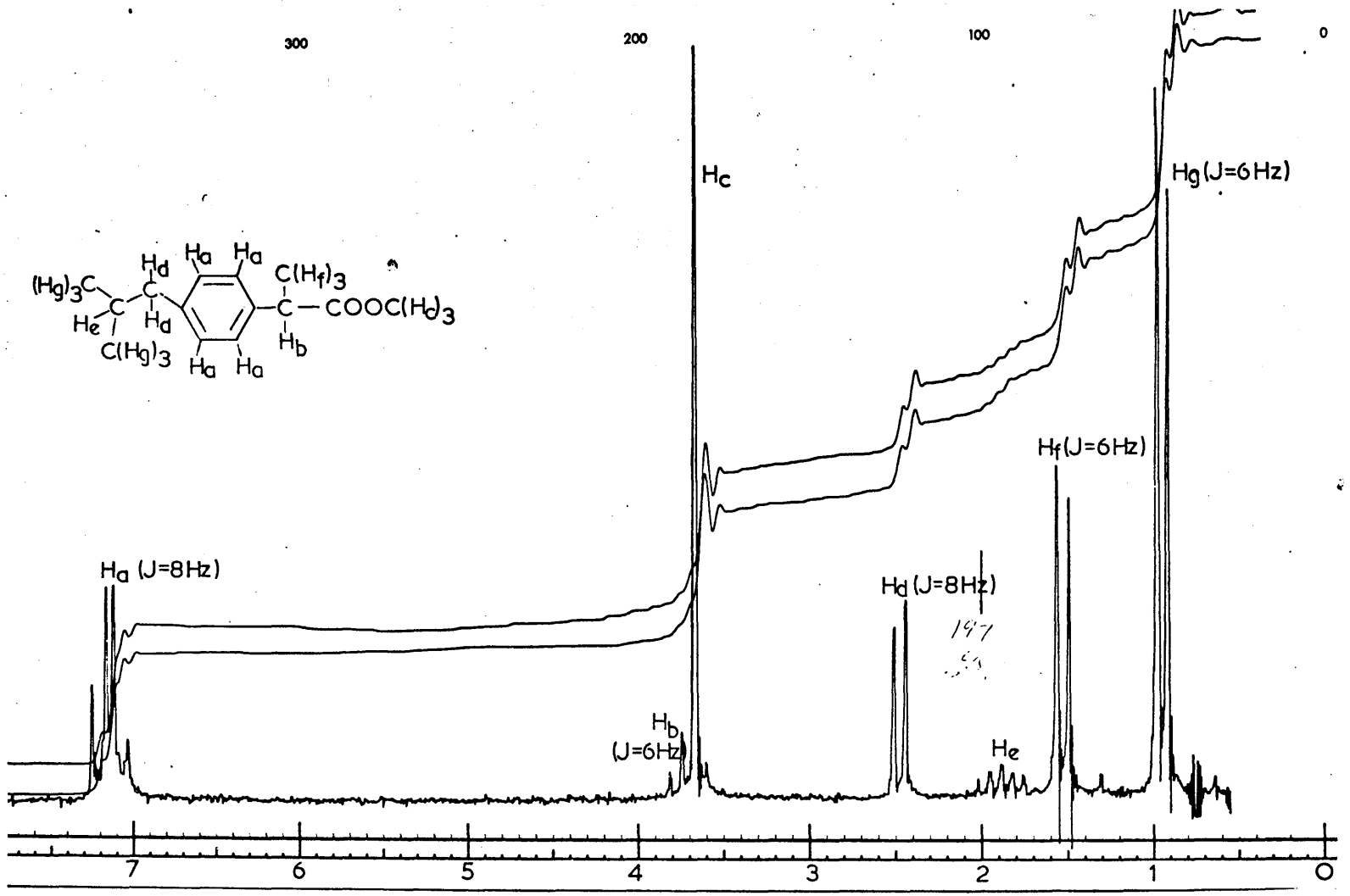


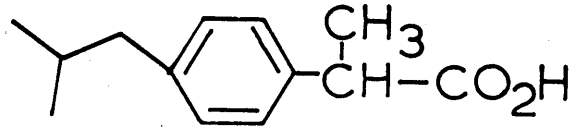
Fig. 37. Proton NMR spectrum of 2-(4-isobutylphenyl)propionic acid methyl ester.

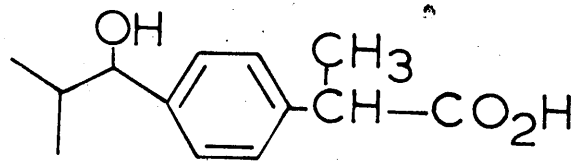
summarised in Table 15.

The mass spectrum of ibuprofen methyl ester is shown in Figure 36. The base peak in this spectrum was due to loss of the carbomethoxyl group and a major fragment was observed due to cleavage of the side chain at the tertiary centre. Ibuprofen was reduced with $LiAlH_4$ and $LiAl^2H_4$ and the diols characterised as their trimethylsilyl ethers by GLC and GC-MS (Table 16). The base peak in the spectrum of the diol-TMS was again at m/e 161, produced by cleavage adjacent to the primary trimethylsilyl ether group. Abundant ions were observed from the trimethylsilyloxy group at m/e 73, $[Si(CH_3)_3]^+$ and m/e 75, $[\overset{+}{H}O = Si(CH_3)_2]$ and the ion characteristic of the primary trimethylsilyloxy function was present at m/e 103(105), $[CH_2 = \overset{+}{O} - Si(CH_3)_3]$, $[CD_2 = \overset{+}{O} - Si(CH_3)_3]$.

Methyl ibuprofen was further characterised by ^1H NMR spectrometry (Fig. 37) : δ7.2 (4H, q, J = 8 Hz) [aromatic-H]; 3.7 (3H, S) [$-COOCH_3$]; 3.6 (1H, q, J = 6 Hz) [benzylic CH-]; 2.5 (2H, d, J = 8 Hz) [benzylic-CH_2-]; 1.9 (1H, m) [side-chain CH-]; 1.5 (3H, d, J = 6 Hz) [$-CH_3$ coupled to benzylic CH-]; 1.0 (6H, d, J = 6 Hz) [side chain-CH_3].

I.R. spectroscopy showed an ester carbonyl, $\nu_{C=O}$ = 1745 cm^{-1} (CCl_4) and the U.V. spectrum of the free acid gave λ_{max}^{EtOH} = 222 nm. (ε = 4,120) and λ_{max}^{EtOH} = 263 nm (ε = 129).





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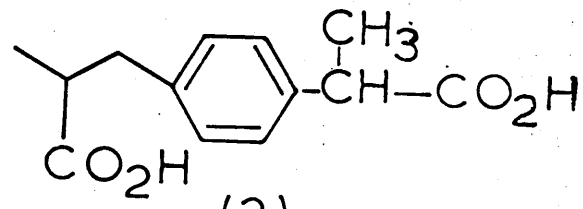

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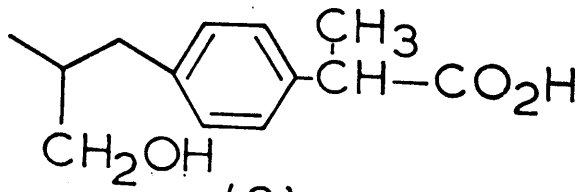

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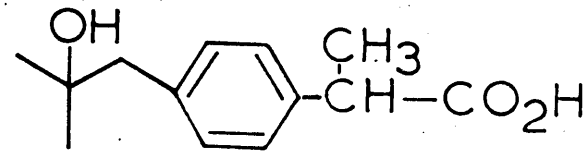


(4)

Fig. 38. Structures of ibuprofen and four of its urinary metabolites.

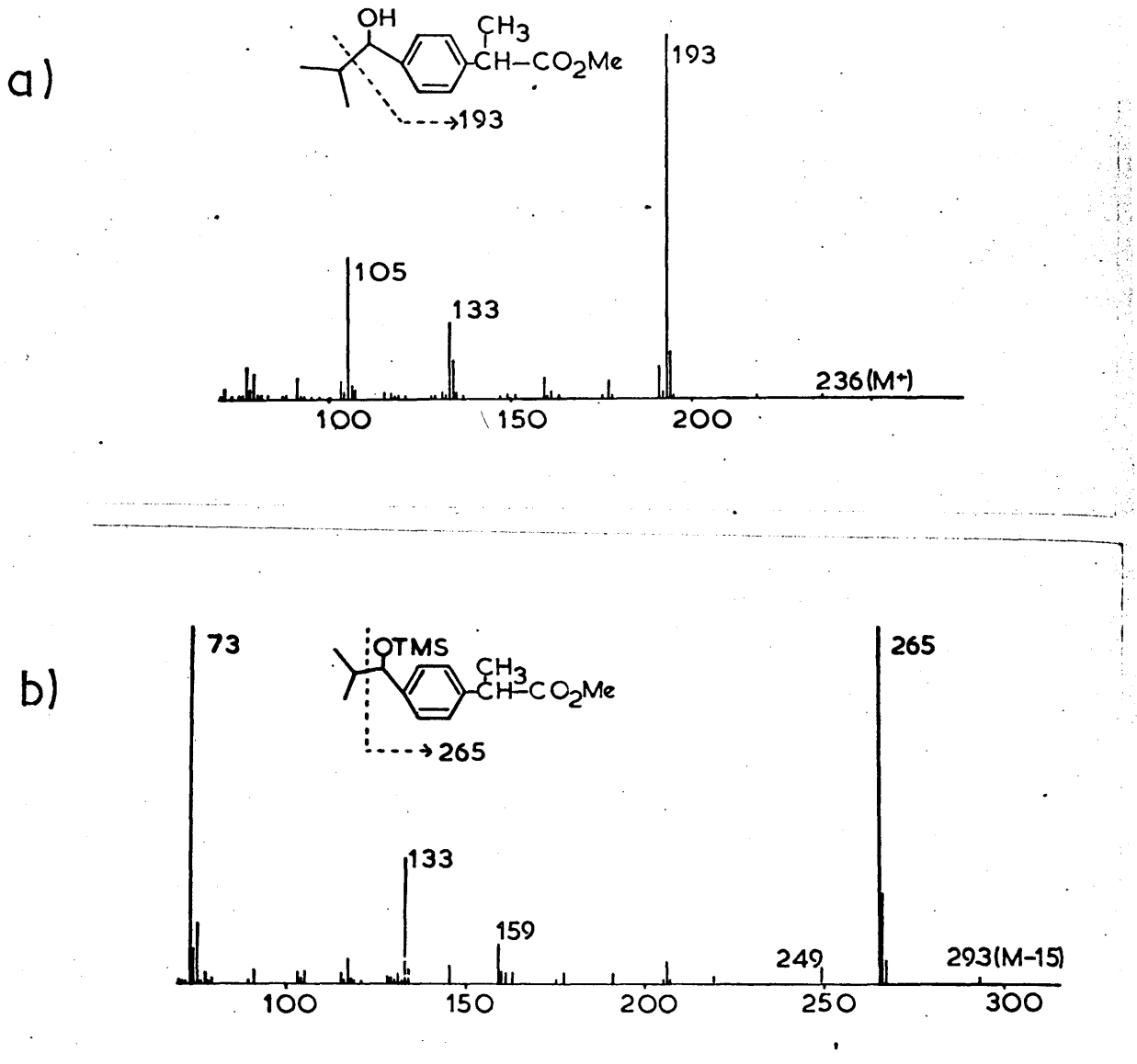


Fig. 39. Mass spectral characterisation of metabolite 1.

a) Mass spectrum of metabolite 1 methyl ester.

b) Mass spectrum of metabolite 1 methyl ester trimethylsilyl ether.

Table 17. Gas liquid chromatographic and mass spectrometric data for metabolite 1

Derivative	$I^{1\%\ OV-1}_{150^{\circ}C}$	Mass spectral characteristics	
		$M^{+\cdot}$	Other ions (% abundance)
Methyl ester	1705	236(1)	193(100); 105(39); 133(21); 194(13); 134(11)
Methyl ester TMS ether	1700	308(0)	265(100); 73(99); 133(35); 266(25); 75(16); 159(11)
Methyl ester $\underline{d}_9$-TMS ether	1700	317(0)	82(100); 274(95); 133(32); 275(23); 83(9); 159(7); 215(6)
Methyl ester CMDMS ether	1930	342(0.1)	299(100); 79(42); 301(40); 107(24); 300(23); 133(22)
$LiAlH_4$ reduction product as TMS ether	1760	352(0.1)	73(100); 309(67); 75(31); 310(19); 74(6); 103(6); 311(6)
$LiAl^2H_4$ reduction product as TMS ether	1755	354(0.1)	73(100); 311(68); 75(25); 312(21); 74(7); 105(7); 313(7)
Ketone formed on oxidation of methyl ester	1705	234(1)	191(100); 103(20); 104(13); 192(11); 77(8); 78(6); 132(5); 175(4)

Characterisation of metabolites

The structures of the three hydroxylated metabolites and the dicarboxylic acid (Fig. 38) were proposed on the basis of their GLC and GC-MS characteristics. Confirmation of the structures was aided by micro-chemical transformations, monitored by GLC and GC-MS. Metabolites 2 and 4 had previously been identified.[341,342]

Metabolite 1 : 2,4'-(1-hydroxy-2-methylpropyl)phenyl-propionic acid

The mass spectrum of metabolite 1 methyl ester (Fig. 39) showed a very small molecular ion at m/e 236. This molecular weight corresponded to a mono-hydroxylated metabolite. Confirmation of this structure was obtained by formation of the trimethylsilyl ether. No molecular ion was observed after trimethylsilylation (Fig. 39) but an ion at m/e 293 corresponded to loss of a methyl radical from a molecule of weight 308. The base peaks in these spectra (Table 17) corresponded to a loss of 43 a.m.u. from the molecular ion. This cleavage at the tertiary centre was observed in the spectrum of the unhydroxylated acid methyl ester but its high abundance in this instance suggested that hydroxylation had occurred at the benzylic position in the side chain. Loss of the carbomethoxyl group produced the fragments at m/e 177 and 249 in the spectra of the free and trimethylsilylated derivatives respectively. These fragments together with loss of water or trimethylsilanol produced the ions at m/e 159. Elimination of trimethylsilanol from the molecular ion

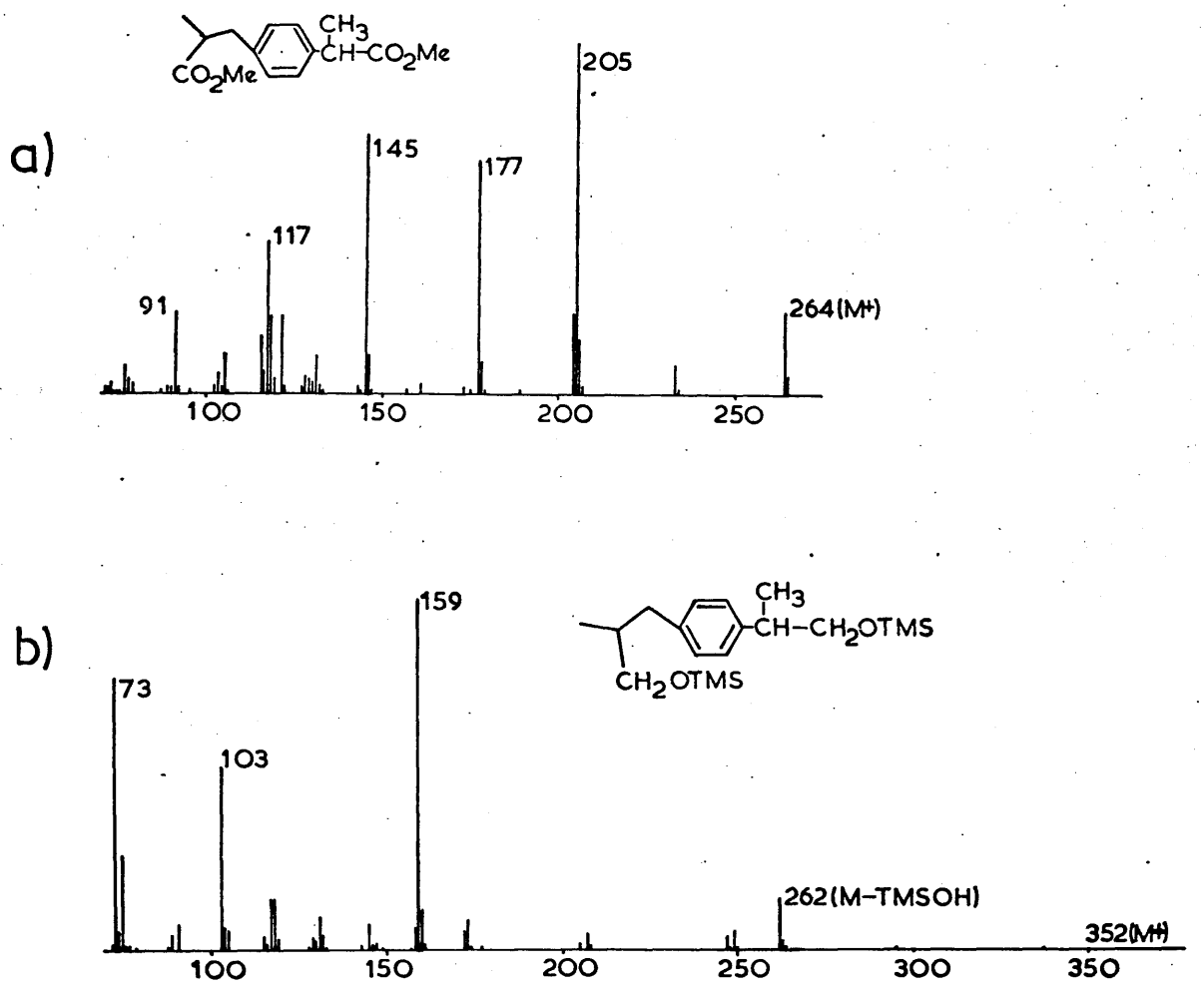


Fig. 40. Mass spectral characterisation of metabolite 2.

a) Mass spectrum of metabolite 2 methyl ester.

b) Mass spectrum of the $LiAlH_4$ reduction product of metabolite 2 as the trimethylsilyl ether.

itself was not observed, but an ion of low abundance at m/e 219 indicated the loss of a trimethylsilyloxy radical as expected from a benzylic trimethylsilyl ether. The assignment of ions to silyl ether fragments was aided by formation of the perdeuterated[343] trimethylsilyl ether or the chloromethyldimethylsilyl ether.

The fact that the metabolite contained a secondary hydroxyl group was confirmed by its oxidation to a ketone with CrO_3/acetone. The mass spectrum of the ketone was very similar to that of the hydroxylated metabolite with the major ion being produced by cleavage adjacent to the carbonyl group (m/e 191).

The metabolite was further characterised by reduction and analysis of the trimethylsilyl ether of the resulting diol. The major mode of fragmentation was still cleavage α to the secondary trimethylsilyloxy group.

Metabolite 2 : 2,4'-(2-carboxypropyl)phenylpropionic acid

The mass spectrum of metabolite 2 as the methyl ester gave a molecular ion of 264, corresponding to a di-carboxylic acid dimethyl ester (Fig. 40). The absence of hydroxyl groups agreed with the polarity observed for this compound on TLC and was confirmed by the unchanged retention index and mass spectrum, after treatment with trimethylsilylating reagent (Table 18). The position of the second carboxyl group was deduced by consideration of the mass spectrum. The ion at m/e 177 was observed in the spectrum of 'ibuprofen' methyl ester due to cleavage of the side chain at the tertiary centre. The occurrence

Table 18. Gas liquid chromatographic and mass spectrometric data for derivatives of Metabolite 2

Derivative	$I^{1\% \text{ OV-1}}_{150^{\circ}C}$	Mass spectral characteristics	
		$M^{+\cdot}$	Other ions (% abundance)
Methyl ester	1775	264(23)	205(100); 145(74); 177(66); 117(43); 91(23); 118(22); 121(22); 204(22)
$LiAlH_4$ reduction product as TMS ether	1890	352(0.1)	159(100); 73(78); 103(53); 75(28); 117(15); 118(15); 262(15)
$LiAlH_4$ reduction product as d_9-TMS ether	1890	370(0.1)	159(100); 82(80); 112(44); 81(23); 271(18); 118(14); 117(13)
$LiAl^2H_4$ reduction product as TMS ether	1890	356(0.1)	73(100); 161(95); 105(70); 75(35); 159(32); 266(16); 118(16)

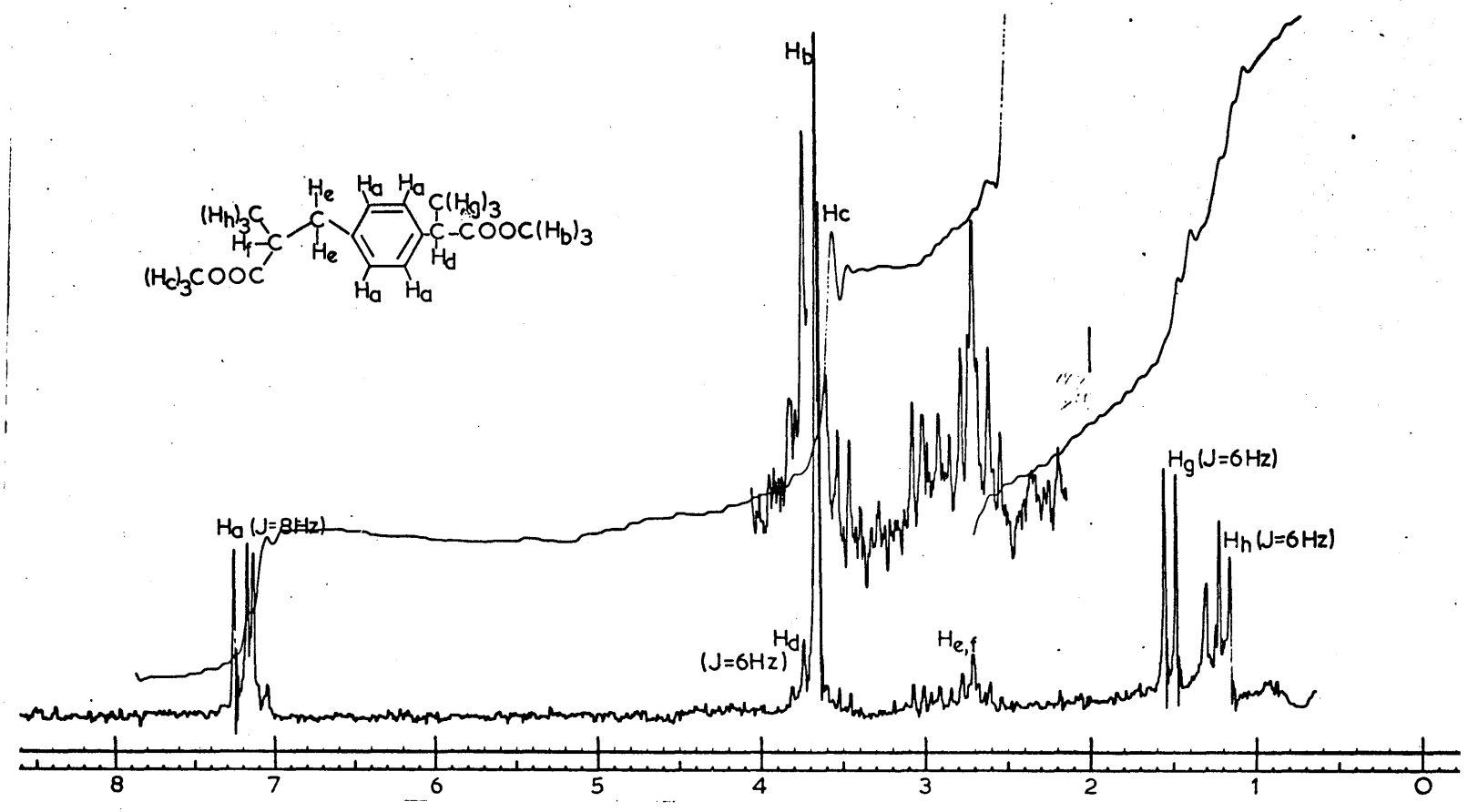


Fig. 41. Proton NMR spectrum of metabolite 2 methyl ester.

of this ion in the spectrum of metabolite 2 methyl ester suggested that it was one of the methyl groups in the isobutyl side chain that had been oxidised. The major fragments could be ascribed to the loss of a carbomethoxy group (m/e 205) and to the combined loss of carbomethoxy and $C_2H_4O_2$. Reduction with $LiAlH_4$ (or $LiAl^2H_4$) followed by trimethylsilylation yielded a product of molecular weight 352 (or 356) thus confirming the presence of two acid moieties. The base peak (m/e 159) in the mass spectrum of this product (Fig. 40) was produced by a combination of two processes, viz. loss of trimethylsilanol (m/e 262) and elimination of the primary $[CH_2\text{-}OTMS]^{\bullet}$ radical. The ion characteristic of the primary trimethylsilyl ether group, $[CH_2=\overset{+}{O}\text{-}Si(CH_3)_3]$ was observed at m/e 103. Its identity was confirmed by a shift of 9 a.m.u. to m/e 112 when perdeuterated TMS ethers were formed and by the change to m/e 105 when the reduction was performed with $LiAl^2H_4$.

Metabolite 2 was the major metabolite of ibuprofen and isolation of 4-5 mg of pure material was achieved by a combination of lipophilic gel chromatography on Sephadex LH-20 (mobile phase : 1,2-dichloroethane-methanol, 7:3) and preparative TLC (mobile phase : chloroform-ethyl acetate, 3:1 v/v) of the methyl ester. This allowed confirmation of the metabolite structure by ^1H NMR spectrometry (Fig. 41) : δ, 7.15 (4H, q, J = 8 Hz) [aromatic-H]; 3.65 (3H, s) [original $-COOC\underline{H}_3$]; 3.6 (3H, s) [new $-COOC\underline{H}_3$]; 3.6 (1H, q, J = 6 Hz) [benzylic C<u>H</u>-]; 2.8 (3H, m) [benzylic $-C\underline{H}_3$- and side chain C<u>H</u>-]; 1.5 (3H, d,

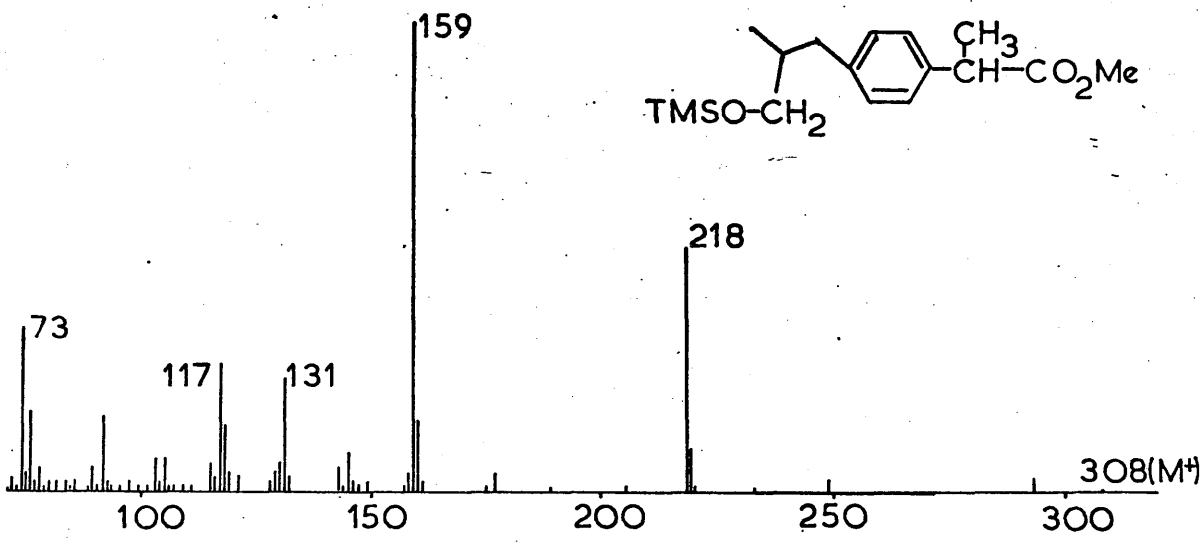


Fig. 42. Mass spectrum of metabolite 3 as its methyl ester trimethylsilyl ether.

J = 6 Hz) [-$\underline{CH}_3$ coupled to benzylic CH-]; 1.25 (3H, d, J = 6 Hz) [side-chain -$\underline{CH}_3$].

The IR spectrum showed $\nu_{C=O}$ 1745 cm^{-1} (CCl_4 solution) and confirmed the absence of hydroxyl groups. U.V. spectrometry gave λ_{max}^{EtOH} = 221 nm (ε = 5,200).

Subsequent isolation of more material as the free acid by preparative TLC using toluene-acetic acid, 9:1 v/v (R_F = 0.31) and recrystallisation from benzene-hexane gave white crystals, m.p. 96-103°C.

Metabolite 3: 2,4'-(2-hydroxymethylpropyl)phenylpropionic acid

The mass spectrum of the methyl ester trimethylsilyl ether of metabolite 3 (Table 19, Fig. 42) was characterised by the fact that none of the major ions contained the trimethylsilyloxy function, as shown by comparison with the spectrum of the perdeuterated trimethylsilyl ether or the chloromethyldimethylsilyl ether. The base peak (m/e 159) was produced by loss of the carbomethoxy group together with elimination of trimethylsilanol. The latter process alone produced a major ion at m/e 218 (52%). The ion at m/e 103, though of relatively low abundance (7%), was significant since it was shifted to m/e 112 in the spectrum of the perdeuterated trimethylsilyl ether; it thus corresponded to $CH_2=\overset{+}{O}-Si(CH_3)_3$, consistent with a primary hydroxyl function. The proposed position of hydroxylation was confirmed by $LiAlH_4$ reduction of the ester function since the trimethylsilyl ether of the resulting diol was identical to that obtained from

Table 19. Gas liquid chromatographic and mass spectrometric data for derivatives of metabolite 3

Derivative	$I^{1\%\ OV-1}_{150^oC}$	Mass spectral characteristics	
		$M^{+\cdot}$	Other ions (% abundance)
Methyl ester TMS ether	1840	308(0.1)	159(100); 218(52); 73(35); 117(27); 131(24)
Methyl ester $\underline{d}_9$-TMS ether	1840	317(0.1)	159(100); 218(50); 82(42); 131(23); 117(19)
Methyl ester CMDMS ether	2075	342(0)	159(100); 218(54); 117(35); 131(20); 91(17)
$LiAlH_4$ reduction product as TMS ether	1890	352(0.2)	73(100); 159(95); 103(46); 75(32); 117(18); 262(16); 118(13)
$LiAl^2H_4$ reduction product as TMS ether	1890	354(0.2)	73(100); 159(89); 105(62); 75(36); 264(18); 117(17); 118(15); 160(15)

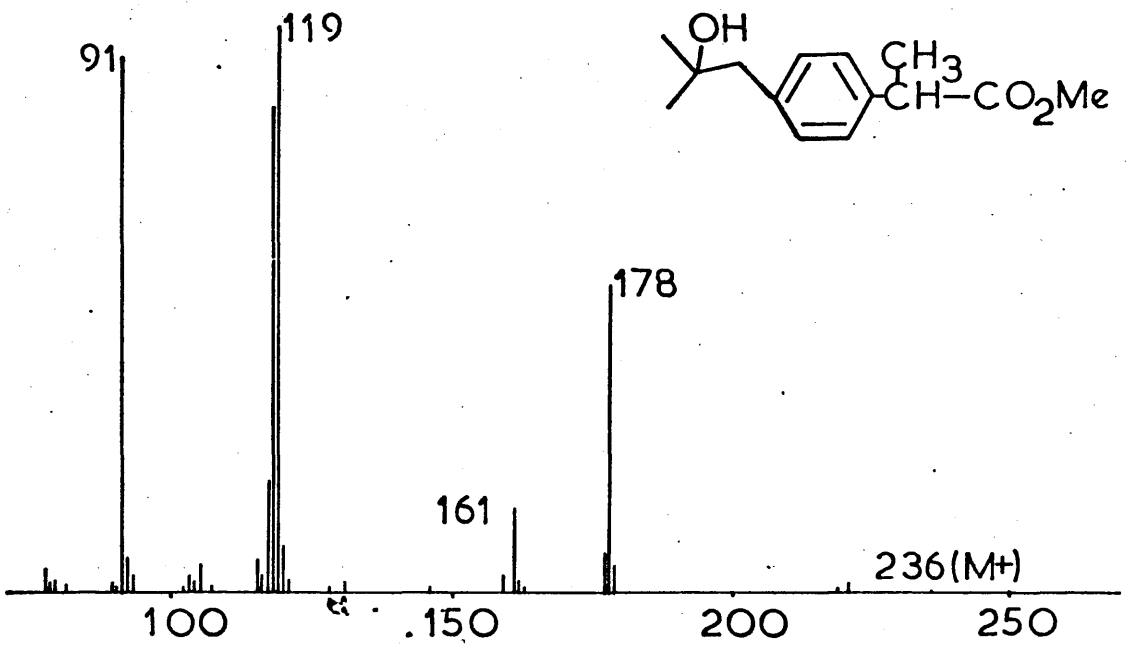


Fig. 43. Mass spectrum of metabolite 4 as its methyl ester.

metabolite 2 in both GLC and GC-MS behaviour.

Metabolite 4 : 2,4'-(2-hydroxy-2-methylpropyl)phenyl-propionic acid

The mass spectral characteristics of derivatives of metabolite 4 are presented in Table 20 . The small molecular ion at m/e 236 in the mass spectrum of the methyl ester (Fig. 43) suggested that this was a hydroxylated metabolite. However, difficulty was experienced in forming the trimethylsilyl ether, suggesting that the hydroxyl group was at the tertiary position in the isobutyl side-chain. In accordance with this, the mass spectrum of the methyl ester showed intense peaks at m/e 178 ($[M-58]^{+\cdot}$) due to cleavage adjacent to the tertiary centre with hydrogen transfer, and m/e 119, resulting from additional loss of a carbomethoxyl radical. In the mass spectrum of the methyl ester trimethylsilyl ether, the base peak occurred at m/e 131, corresponding to $(CH_3)_2C=\overset{+}{O}-SiMe_3$. As expected, this changed to m/e 140 on formation of the perdeuterated trimethylsilyl ether, and to m/e 165 in the chloromethyldimethylsilyl ether. The ion at m/e 250 in the methyl ester trimethylsilyl ether spectrum corresponded to a rearrangement of the trimethylsilyl group accompanied by elimination of acetone (M-58). This was shifted to m/e 259 and m/e 284 in the perdeuterated trimethylsilyl ether and chloromethyl dimethylsilyl ether spectra respectively. After reduction of metabolite 4 with $LiAlH_4$, and trimethylsilylation, the fragment at m/e 131 was still the base peak. Isolation

Table 20. Gas liquid chromatographic and mass spectrometric data for derivatives of metabolite 4

Derivative	$I^{1\% \text{ OV-1}}_{150^{\circ}C}$	Mass spectral characteristics	
		$M^{+\cdot}$	Other ions (% abundance)
Methyl ester	1660	236(0.1)	119(100); 91(95); 118(86); 178(55); 117(20); 161(15)
Methyl ester TMS ether	1780	308(0)	131(100); 73(70); 75(17); 132(11); 159(5); 74(4); 250(4)
Methyl ester d_9-TMS ether	1780	317(0)	82(100); 140(63); 161(27); 141(20); 142(10); 299(7); 159(6); 259(4)
Methyl ester CMDMS ether	2010	342(0)	165(100); 107(46); 167(36); 79(30); 75(25); 109(17); 159(16); 81(13); 166(13); 284(10)
$LiAlH_4$ reduction product as TMS ether	1835	352(0.02)	131(100); 73(59); 75(22); 72(14); 132(13); 247(8); 103(7)
$LiAl^2H_4$ reduction product as TMS ether	1830	354(0)	131(100); 73(58); 75(20); 132(14); 105(6); 74(5); 133(5)

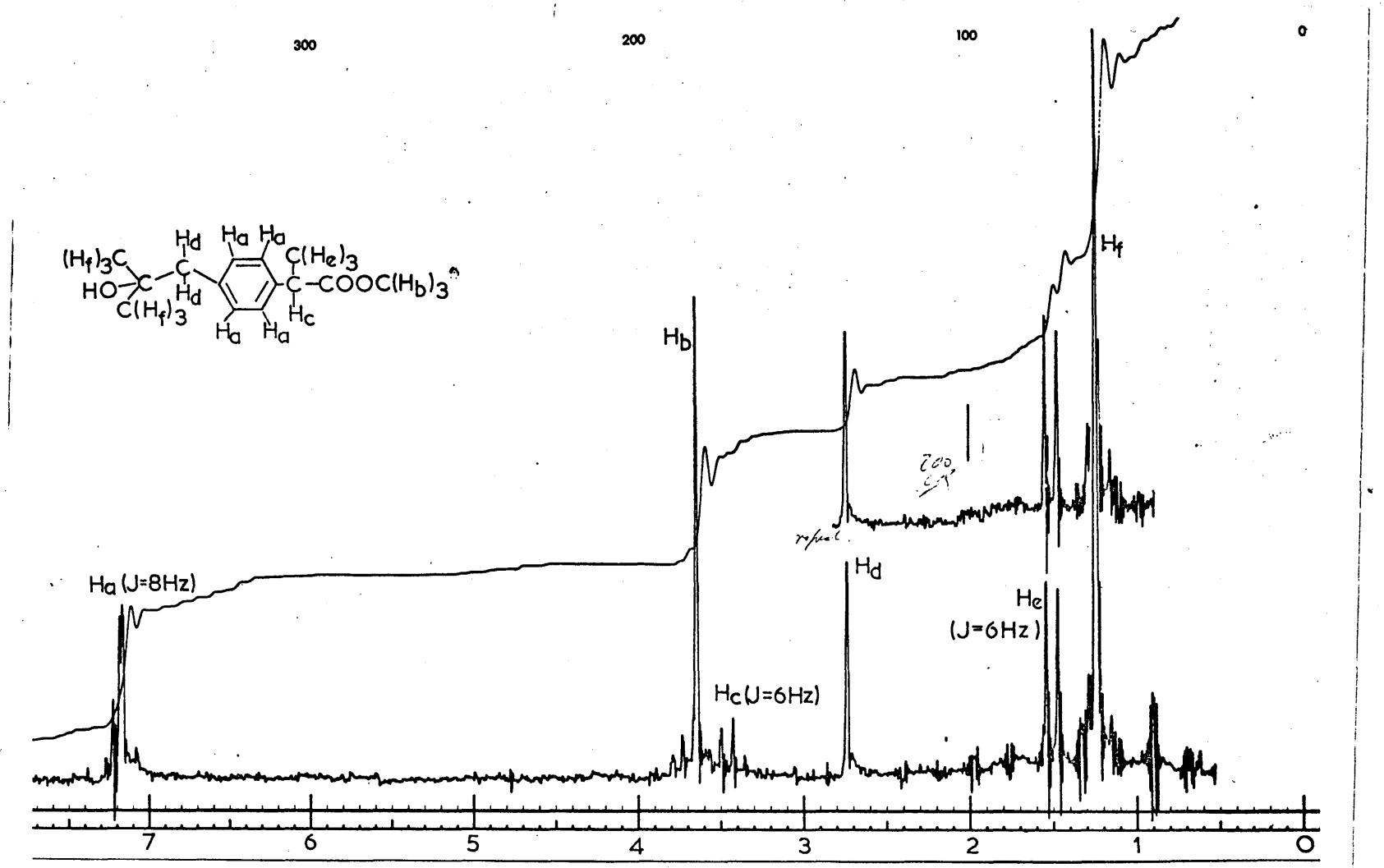


Fig. 44. Proton NMR spectrum of metabolite 4 methyl ester.

Table 21. Gas liquid chromatographic and mass spectral data for derivatives of 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid

Derivative	$I^{1\%\ OV-1}_{150^oC}$	Mass spectral characteristics (70 eV) $M^{+\cdot}$	Other ions (% abundance)
Methyl ester	1700	236(2)	193(100); 105(33); 133(20); 194(13); 134(9); 77(5); 79(4); 177(4);
Methyl ester TMS ether	1705	308(0)	265(100); 73(64); 133(25); 266(20); 75(9); 159(7); 74(6); 206(5)
Methyl ester methyl ether	1605	250(0.2)	207(100); 148(16); 208(13); 133(10); 105(7); 77(5); 191(4); 91(3)
Methyl ester α-phenylbutyrate*	2430 2465	382(0.1)	119(100); 219(76); 91(74); 159(30); 177(21); 117(15); 220(12); 120(9)

* 230°C

of a few mg of purified metabolite 4 methyl ester allowed the verification of the proposed structure by other spectroscopic techniques.

I.R. spectrum (CCl_4) solution : ν_{OH} = 3600 cm^{-1}

$\nu_{C=O}$ = 1745 cm^{-1}

^1H NMR spectrum (Fig. 44) : δ, 7.2 (4H, q, J = 8 Hz) [aromatic -H]; 3.7 (3H, s) [$-COOCH_3$]; 3.6 (1H, q, J = 6 Hz) [benzylic CH-]; 2.75 (2H, s) [benzylic $-CH_2$]; 1.5 (3H, d, J = 6 Hz) [$-CH_3$ coupled to benzylic CH-]; 1.25 (6H, s) [side-chain $-(CH_3)_2C(OH)-$].

3.3.2. Synthesis and characterisation of 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid

The structure of metabolite 1 was confirmed by the synthesis of 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester by the action of N-bromosuccinimide on 2-(4-isobutylphenyl)propionic acid methyl ester, and subsequent hydrolysis of the bromide (cf. Sec. 2.5.). The GLC and GC-MS characteristics (Table 21) of the synthetic material as the methyl ester and the methyl ester trimethylsilyl ether were identical to those found for metabolite 1 (Table 17).

The methyl ester was a pale yellow oil which was purified by vacuum sublimation using a cold-finger apparatus (0.1 mm Hg, block temp. 60°C). Found : C, 62.26 H, 8.40% *; Calc. for $C_{14}H_{20}O_3$. C, 71.16; H, 8.53%.

The IR spectrum (CCl_4 solution) showed ν_{OH} = 3610 and $\nu_{C=O}$ = 1745 cm^{-1}. The UV spectrum showed the predicted

* This result indicates the presence of water of crystallisation.

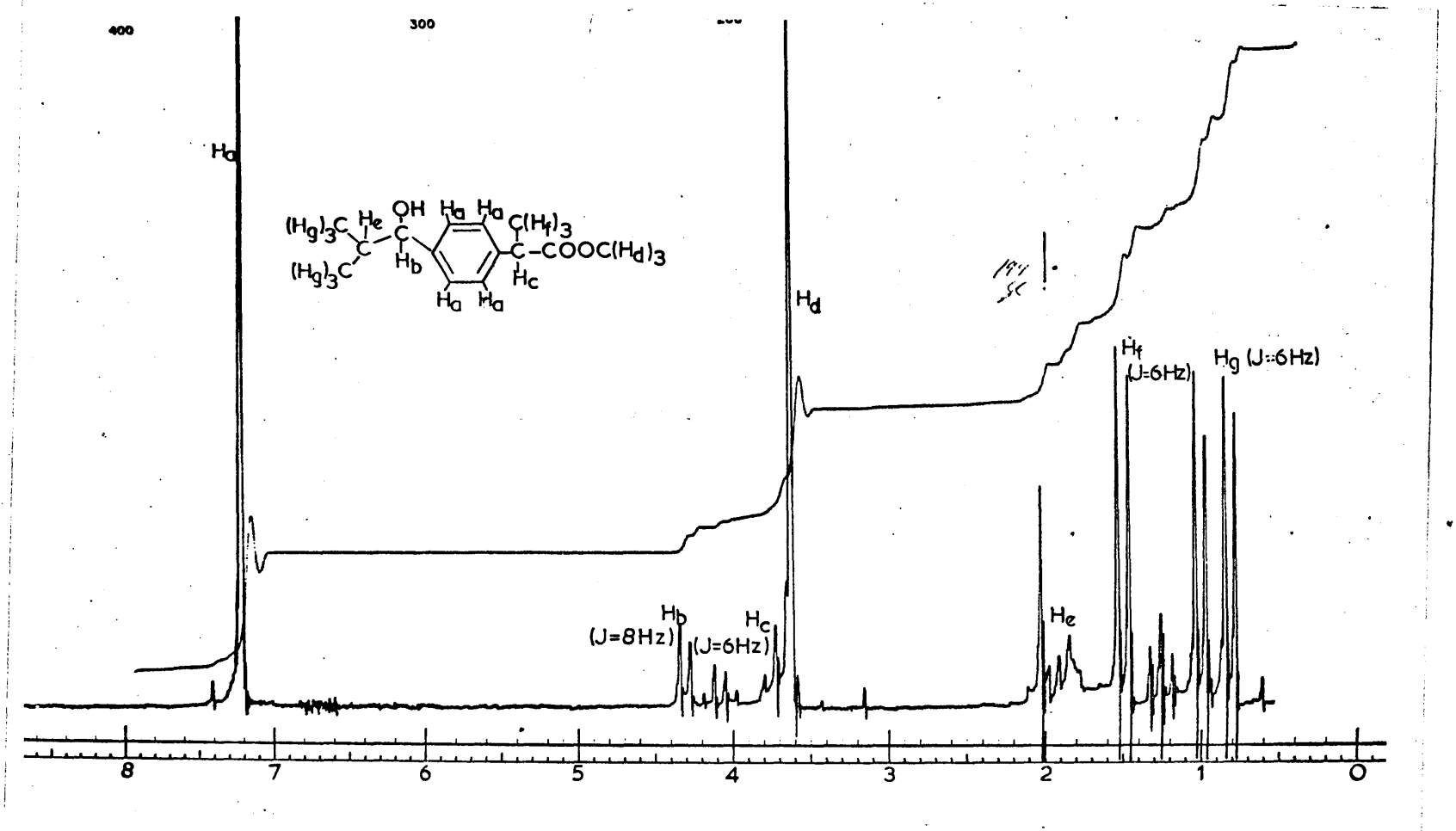


Fig. 45. Proton NMR spectrum of 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester.

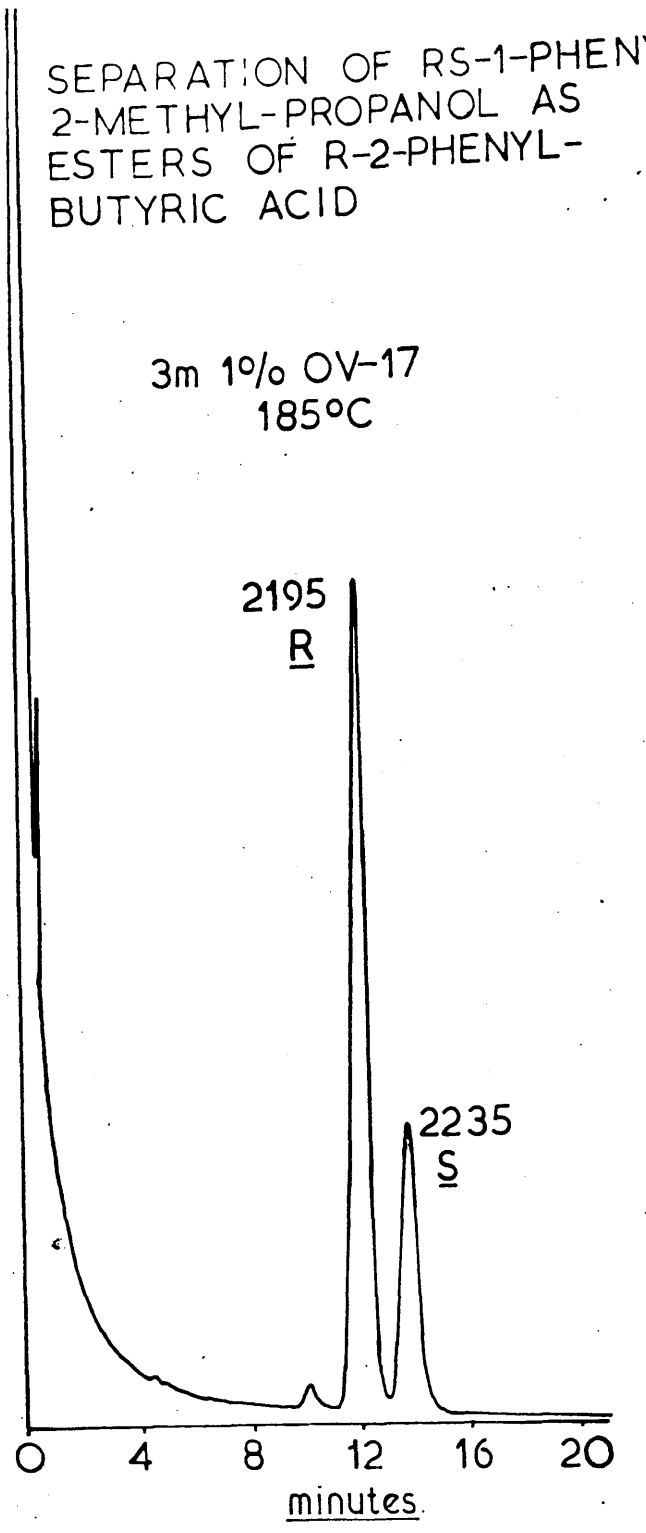
aromatic absorptions : λ_{max} 215 nm, (ε = 3,330); λ_{max} 250 nm, (ε = 1,550).

Full characterisation was achieved by ^{1}H NMR spectrometry (Fig. 45) : δ, 7.2 (4H, s) [aromatic -H]; 4.3 (1H, d, J = 8 Hz) [benzylic -CH(OH)-]; 3.7 (1H, q, J = 6 Hz) [benzylic CH-]; 3.6 (3H, s) [-COOCH$_3$]; 2.0 (1H, m) [side-chain CH-]; 1.5 (3H, d, J = 6 Hz) [-CH$_3$ coupled to benzylic CH-]; 1.0 (3H, d, J = 6 Hz) [side-chain -CH$_3$]; 0.80 (3H, d, J = 6 Hz) [side-chain -CH$_3$].

An interesting feature of the NMR spectrum was the non-equivalence of the methyl groups in the side-chain. This verified the position of the hydroxyl group at the benzylic carbon since the introduction of an asymmetric centre produced non-equivalent environments for the two methyl groups on the adjacent carbon atom and such diastereotopic groups are distinguishable by NMR spectrometry.

When the bromide was hydrolysed in methanolic solution a less polar by-product was observed (R_F $CHCl_3$/EtOAc 3:1 = 0.63, free hydroxy compound R_F = 0.49). Analysis of this by-product by GC-MS showed a compound with a molecular ion at m/e 250 and a fragmentation pattern similar to that of the major product. The molecular weight suggested that this was a methyl ether produced by displacement of bromide ion by $^-$OMe instead of $^-$OH. Isolation by preparative TLC ($CHCl_3$:EtOAc, 3:1) and ^{1}H NMR spectrometry confirmed this postulation : δ, 7.2 (4H, q, J = 2Hz) [aromatic -H]; 3.7 (1H, q, J = 6Hz) [benzylic CH-]; 3.6 (3H, s) [-COOCH$_3$]; 3.2 (1H, d, J = 2 Hz) [benzylic -CH(OMe)-]

a) SEPARATION OF RS-1-PHENYL-2-METHYL-PROPANOL AS ESTERS OF R-2-PHENYL-BUTYRIC ACID



b)

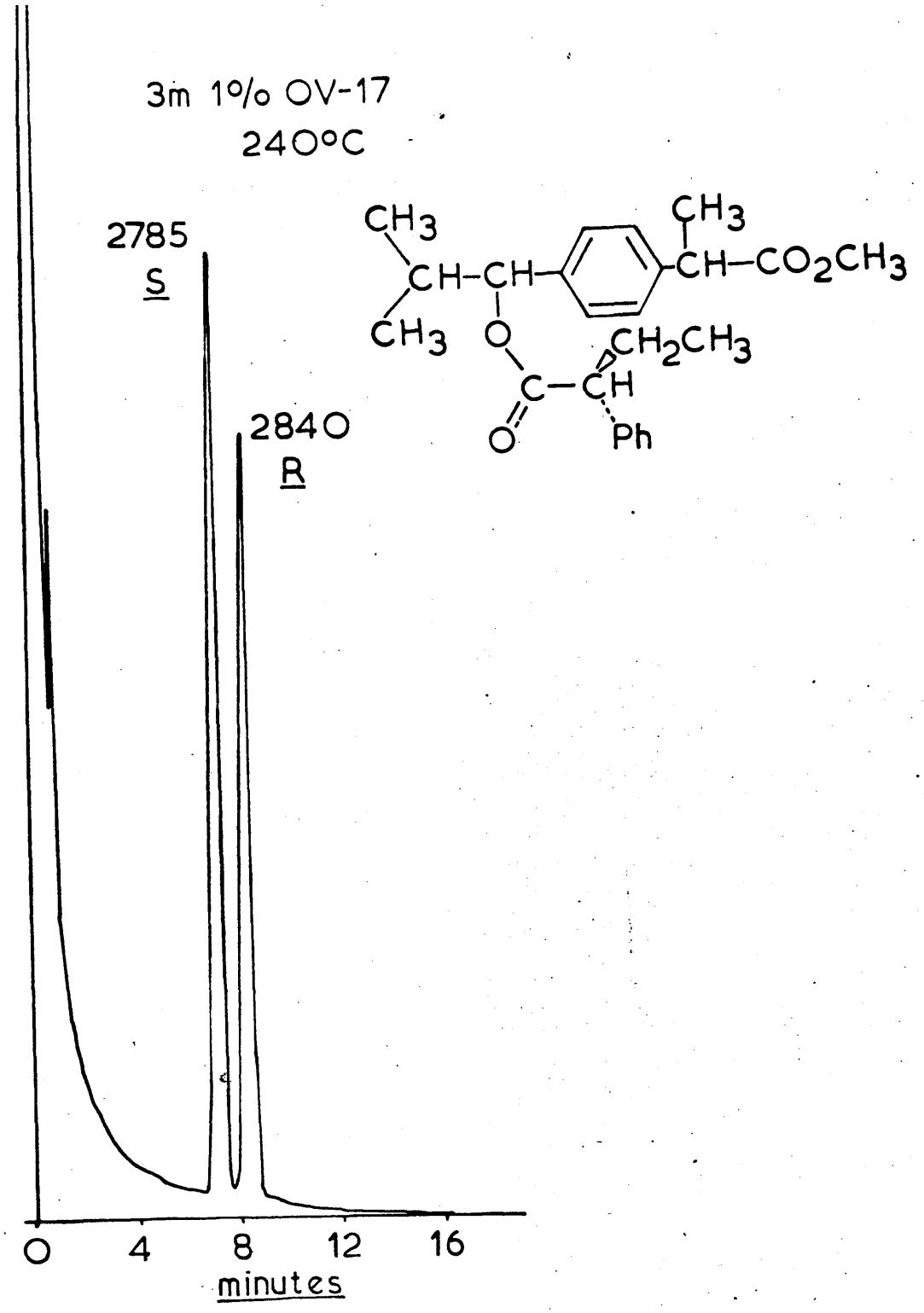


Fig. 46. Gas chromatographic separation of the (R)-(-)-phenylbutyryl derivatives of (a) (RS)-1-phenyl-2-methylpropanol and (b) (RS)-2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester.

3.15 (3H, s) [-O-C$\underline{H}_3$]; 1.9 (1H, m) [side-chain C$\underline{H}$-]; 1.0 (3H, d, J = 6 Hz) [side-chain -C$\underline{H}_3$]; 0.75 (3H, d, J = 6 Hz) [side-chain -C$\underline{H}_3$].

Since hydroxylation at the benzylic position in the side-chain introduced a new asymmetric centre into the 'ibuprofen' structure it was of interest to be able to characterise the configuration at this centre. The gas chromatographic separation of enantiomeric alcohols was first studied using a model compound viz 1-phenyl-2-methylpropanol obtained by reduction of isobutyrophenone with $LiAlH_4$. Since good separations were achieved with enantiomeric alcohols using chrysanthemic acid to form diastereomers (cf. Section 3.1.) this was the procedure employed. However, no resolution was observed for the diastereomeric 1-phenyl-2-methylpropanol chrysanthemates (Table 22) on a 5 m 1% SE-30 column. Subsequent preparation of the (R)-(-) α-phenylbutyrates showed a separation of 30 index units between the two enantiomers on 1% SE-30 phase and 40 index units on OV-17 phase (Fig. 46a). Both peaks were shown to correspond to esters of phenylbutyric acid by GC-MS.

When the phenylbutyrates were prepared from the synthetic 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester, complete chromatographic resolution was achieved (Fig. 46b). To avoid any possible interference due to the second chiral centre (the original centre) another sample of 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester was synthesised starting with (R)-(-) 2-(4-isobutylphenyl)propionic acid methyl ester (obtained

Table 22. Gas chromatographic data for diastereomeric derivatives of benzylic secondary alcohols

Ester	$I^{5m1\%SE-30}$	Temp.°C	$I^{3m1\%OV-17}$	Temp.°C
1-phenyl-2-methyl propanol chrysanthemate	1905	190		
1-phenyl-2-methyl propanol α-phenylbutyrate	1960 1990	190	2195 2235	185
2,4'-(1-hydroxy-2-methylpropyl)phenyl propionic acid methyl ester α-phenylbutyrate	2430 2465	230	2785 2840	240

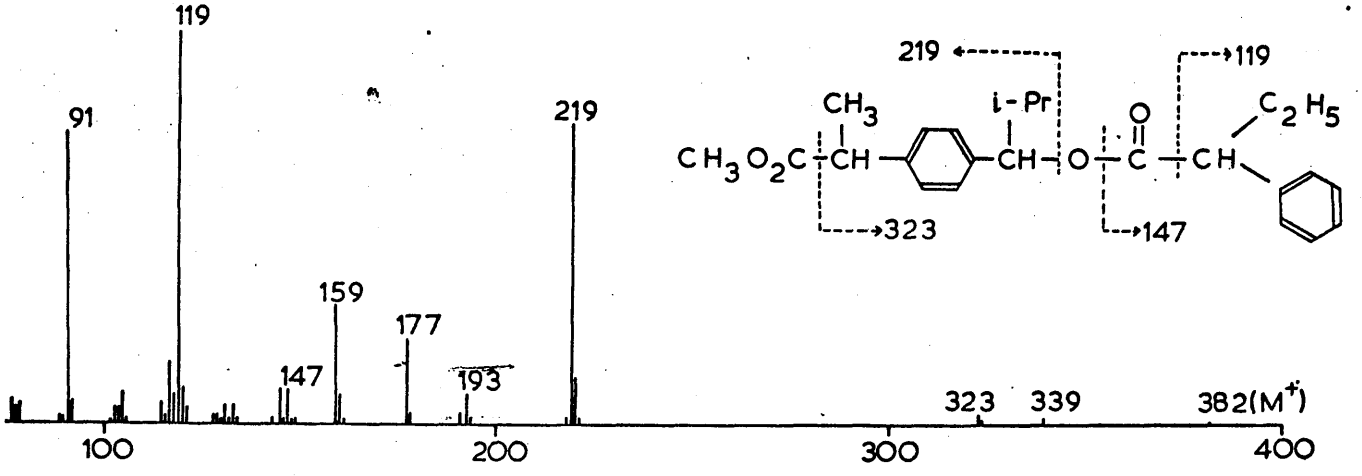


Fig. 47. Mass spectrum of the (R)-(-)-α-phenylbutyryl derivative of (RS)-2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester.

by resolution as salts of (R)-(+) α-phenylethylamine). Preparation of the phenylbutyrates and analysis by GLC gave the same two peaks as previously observed confirming that it was the pair of enantiomeric alcohols which was being resolved. The structure of the esters was confirmed by GC-MS analysis. The mass spectrum (Fig. 47) was similar to those observed for amides of phenylbutyric acid (Sec. 3.2.). The base peak (m/e 119) was produced by cleavage adjacent to the carbonyl group, and O-alkyl cleavage gave the abundant ion at m/e 219. Subsequent loss of the carbomethoxy group gave the ion at m/e 159, or cleavage adjacent to the tertiary centre the ion at m/e 177.

In order to determine the order of elution of the phenylbutyrates on GLC it was necessary to resolve the alcohols and determine the absolute configuration of one of them. The resolution of the enantiomeric alcohols was achieved by TLC of the diastereomeric (R)-(-)-α-phenylbutyrates. After preparative TLC (two developments with mobile phase benzene : EtOAc, 20:1 v/v) analysis of the lower band by GLC showed it to consist of 67% the ester of shorter retention time. (The approximation of peak height x retention time was used to measure peak areas). Further TLC of the material from the lower band using a continuous elution method, in a Desaga chamber, for 2½ h (mobile phase : benzene : EtOAc, 100:1 v/v) enhanced the separation so that material of 88% enantiomeric purity was obtained. Mild alkaline hydrolysis, acidification and extraction yielded the alcohol.

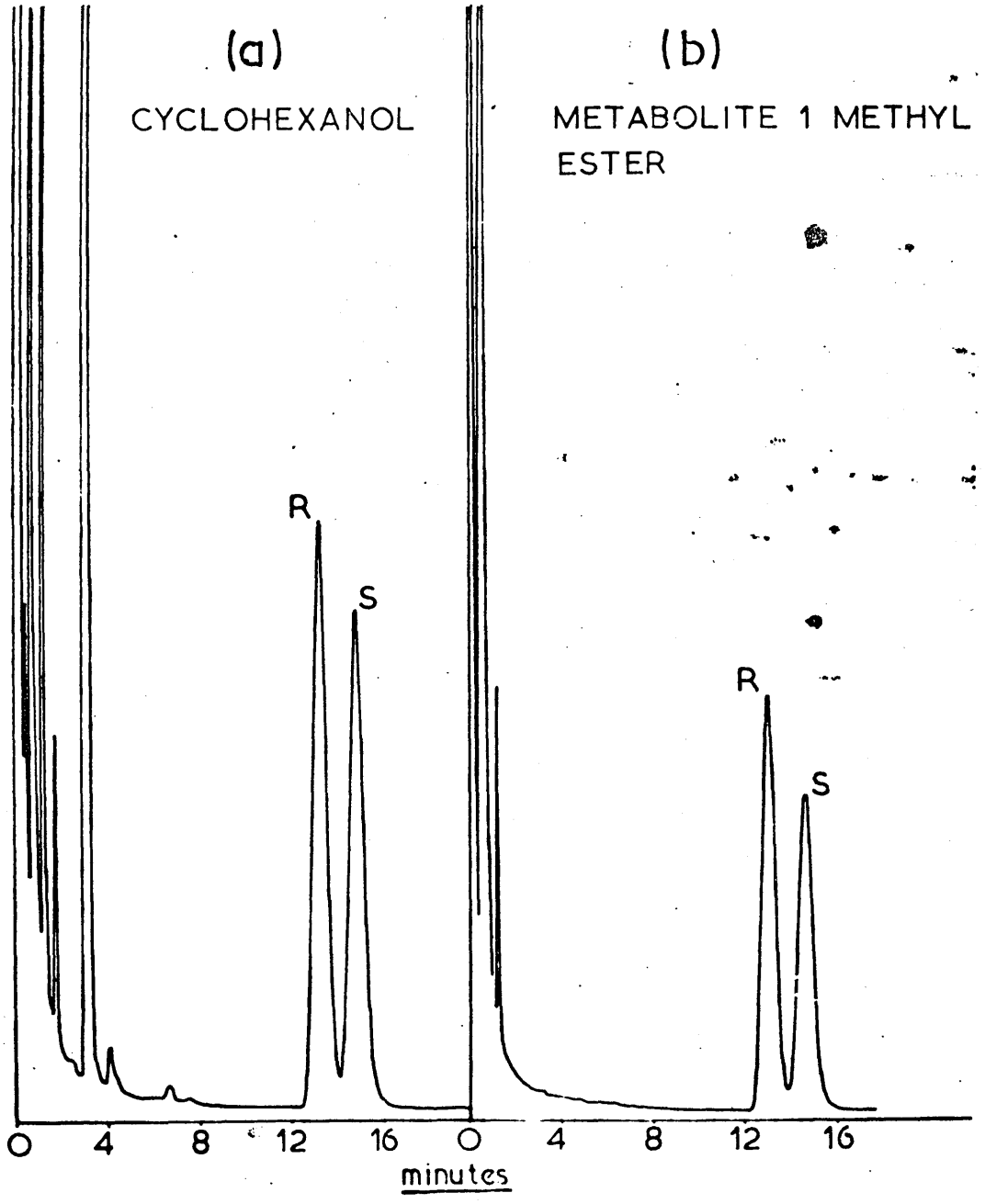


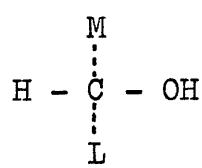
Fig. 48. Gas chromatogram of the N-phenylbutyryl derivatives of α-phenylethylamine obtained by reaction of the amine with excess α-phenylbutyric anhydride left after the reaction of (RS)-α-phenylbutyric anhydride with (a) cyclohexanol and (b) 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester.

Determination of absolute configuration using the Micro-Horeau Method

The absolute configuration of the secondary alcohol could be determined on a micro-scale using the recently published[344,345] modification of Horeau's method.

After methylation of the acid function with diazomethane the alcohol (10 μmole) was dissolved in pyridine (7 μl) and treated with (RS)-α-phenylbutyric anhydride (6.4 μl) at 50°C for 1½ h. A parallel blank reaction was carried out with cyclohexanol. (R)-(+)-α-phenylethylamine was added and mixed thoroughly. After 15 min the mixture was diluted with dry ethyl acetate (400 μl) and a sample analysed by GLC (Fig. 48).

Peak area ratios were measured using the approximation of peak height x retention time. The percentage area representing the amide of the (-)-(R)-acid was assessed. Subtraction of the corresponding value for the reaction with cyclohexanol gave an increment of +2.54 representing excess of (-)-(R)-acid. This suggested that the alcohol was of Horeau's "Type II".



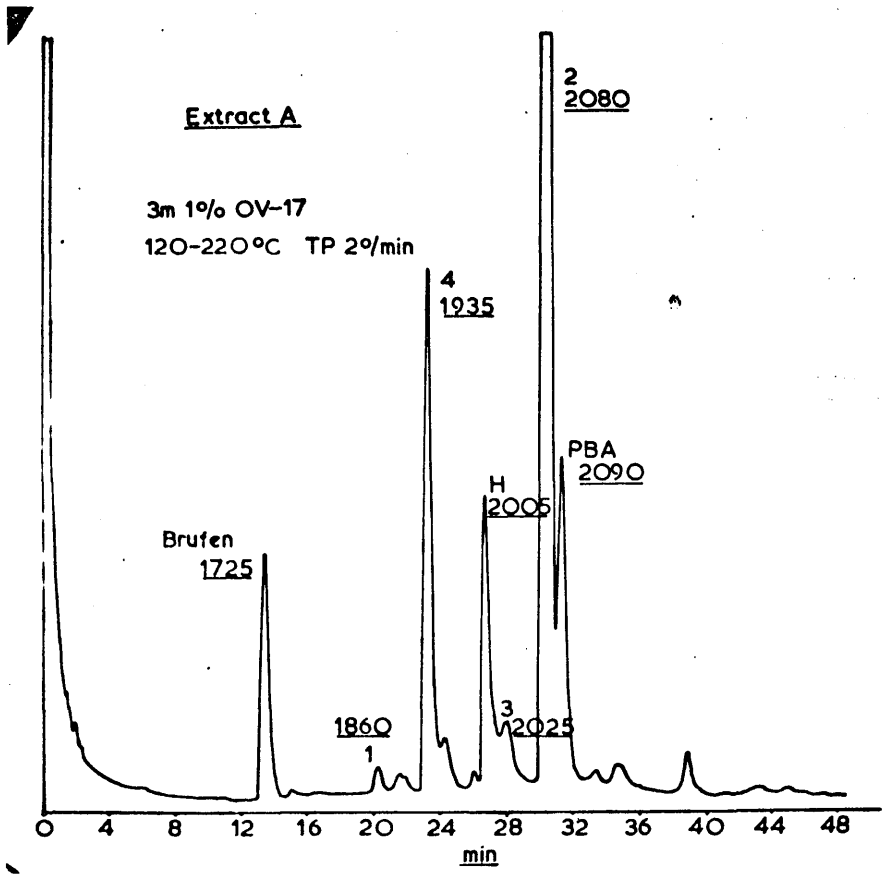
Type II

In most cases type II alcohols have the (S)-configuration however, the previous work on 1,1-diphenyl-alkan-2-ols using the classical Horeau procedure showed[347] that in the case where the side chain was an isopropyl group the isopropyl substituent took preference over the diphenyl-

methyl group. In that situation the (S)-(-)-alcohol behaved as a type I alcohol. In order to clarify this situation 1-phenyl-2-methylpropanol was used. Asymmetric reduction of isobutyrophenone using the complex hydride prepared in situ by reaction of $LiAlH_4$ with (-) quinine produced[348] an excess of the (R)-(+)-carbinol. Optical produced[348] rotation confirmed that the product contained an excess of the dextroratory enantiomer ($[\alpha]_D$ = +3.3). GLC analysis of the derived phenylbutyrates showed only a slight enhancement (64%) of the first ester peak, but the optical purity was increased to 86% by repeated TLC of the diastereomeric phenylbutyrates (mobile phase : benzene : EtOAc, 100:1 v/v). After hydrolysis of the ester with $LiAlH_4$ a sample of the alcohol was treated with (RS)-α-phenylbutyric anhydride as previously described. A blank reaction was also performed with cyclohexanol. (R)-(+)-α-Phenylethylamides were prepared with the excess reagent and analysed as before. The peak increment for the amide of the (R)-acid was -1.52. (+)-1-Phenyl-2-methylpropanol therefore behaved as a type I alcohol, and its absolute configuration was known to be (R). On this basis the order of elution of the enantiomeric 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester as esters of (R)-(-)-α-phenylbutyric acid was (S) < (R), and the resolved alcohol had the (S)-configuration.

3.3.3. Quantification of metabolites

A 24 h urine was obtained from a female patient receiving daily 'Brufen' treatment. Extraction and work



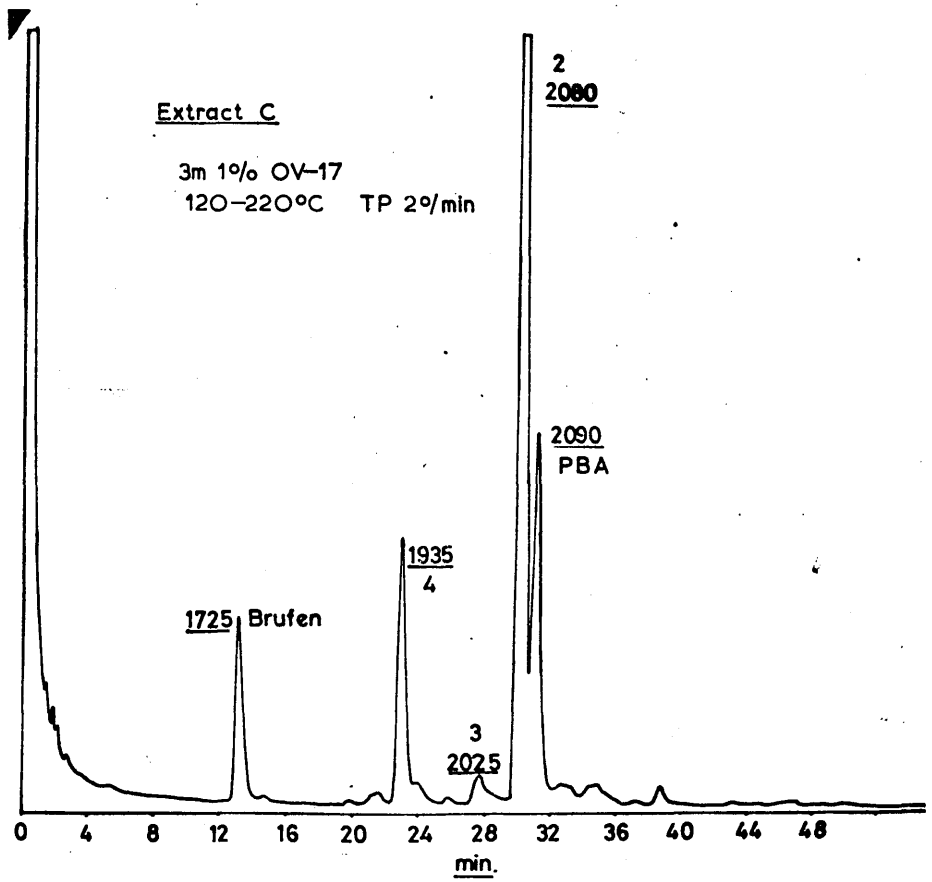


Fig. 49. Gas chromatographic profiles of acidic urine extracts obtained from a patient receiving treatment with ibuprofen. Extract A : free components as their methyl ester trimethylsilyl ethers. Extract C : conjugated components after enzymic hydrolysis as methyl ester trimethylsilyl ethers. (H = hippuric acid; PBA = 4-phenylbenzoic acid added as internal standard; metabolites are numbered 1 to 4.)

up as described in section 2.2.1. yielded extract A. Hydrolysis of the aqueous acidic urine with "Glusulase" and work-up yielded extract C.

$\frac{1}{7}$ of the total of extract A was methylated with ethereal diazomethane and trimethylsilylated with BSA at 60°C for 10 min : the product was dissolved in 1.5 ml ethyl acetate. A known aliquot was removed and a solution of 4-phenylbenzoic acid methyl ester (2 mg/ml) was added to give a final concentration of 1 mg/ml with respect to the standard. Similarly $\frac{1}{10}$ of extract C was methylated and trimethylsilylated and a sample containing 1 mg/ml internal standard was prepared. Aliquots from these samples were analysed by GLC on a 3 m 1% OV-17 column using temperature programming at 2°/min from 120 to 220°C (Fig. 49).

A standard solution of 2 mg/ml concentration of methylibuprofen in ethyl acetate was prepared. 100 µl of this was added to 100 µl of a solution of 2 mg/ml 4-phenylbenzoic acid methyl ester. This was the reference standard for the quantification.

The quantity of ibuprofen methyl ester could be calculated :

$$Q = 2 \times \frac{F_s}{F_{ref}} \times V$$

Where F_s = $\frac{\text{peak height ibuprofen methyl ester}}{\text{peak height standard}}$ in extract

F_{ref} = $\frac{\text{peak height ibuprofen methyl ester}}{\text{peak height standard}}$ in reference standard

Table 23. Quantities of ibuprofen and its metabolites present in a 24 h urine from a patient receiving daily treatment.

Extract A - free acid and metabolites

Metabolite	Quantity(mg)	Concentration(µg/ml)
Ibuprofen	4	5.7
1	0.7	1
2	88	125.7
3	1.4	2
4	15	21.4

Extract C - conjugated acid and metabolites

Metabolite	Quantity(mg)	Concentration(µg/ml)
Ibuprofen	5.7	8.1
1	1	1.4
2	100	142.8
3	1	1.4
4	14.8	21.1

V = volume of extract (i.e. 1.5 ml for extract A)
This equation holds if 100 µl of extract from V is added to 100 µl of the 2 mg/ml solution of the standard. If this is not the case a suitable correction factor must be included.

Using this method the quantity of unmetabolised acid present as the free acid (extract A) or as a conjugate (extract C) was estimated (Table 23). Assuming that the response factors on GLC for the metabolites would be the same as that of ibuprofen methyl ester, and by applying a correction for the differences in retention time, values were also calculated for the amounts of metabolites present.

From Table 23 it can be seen that ibuprofen and metabolite 2 are excreted to a slightly greater extent as conjugates whereas more of metabolite 3 is present in the free extract. Metabolites 1 and 4 appear to be present to the same extent in both extracts.

This method does not take account of any losses which may occur during work-up and therefore too much reliance cannot be placed on these values. However, it gives an indication of the relative amounts of the various metabolites present.

3.3.4. Stereochemical aspects of metabolism

The amides of (RS)-2-(4- isobutylphenyl)propionic acid with (R)-(+)-α-phenylethylamine were completely resolved ($\Delta I = 35$) on a 5 m 1% OV-17 column (Table 24)

Table 24 . Gas liquid chromatographic and mass spectrometric data for the amides of 2-(4-isobutylphenyl)propionic acid with R-(+)-α-phenylethylamine

	$I^{1\%\ SE-30}_{230^oC}$	$I^{1\%\ OV-17}_{220^oC}$	Mass spectral characteristics	
			M^+.	Other ions (% abundance)
Amide of (+)-2-(4-isobutyl-phenyl)propionic acid	2340	2660	309(12)	161(100); 105(72); 162(36); 119(27); 117(14)
Amide of (-)-2-(4-isobutyl-phenyl)propionic acid	2310	2625	309(12)	161(100); 105(80); 162(35); 119(28); 117(14)

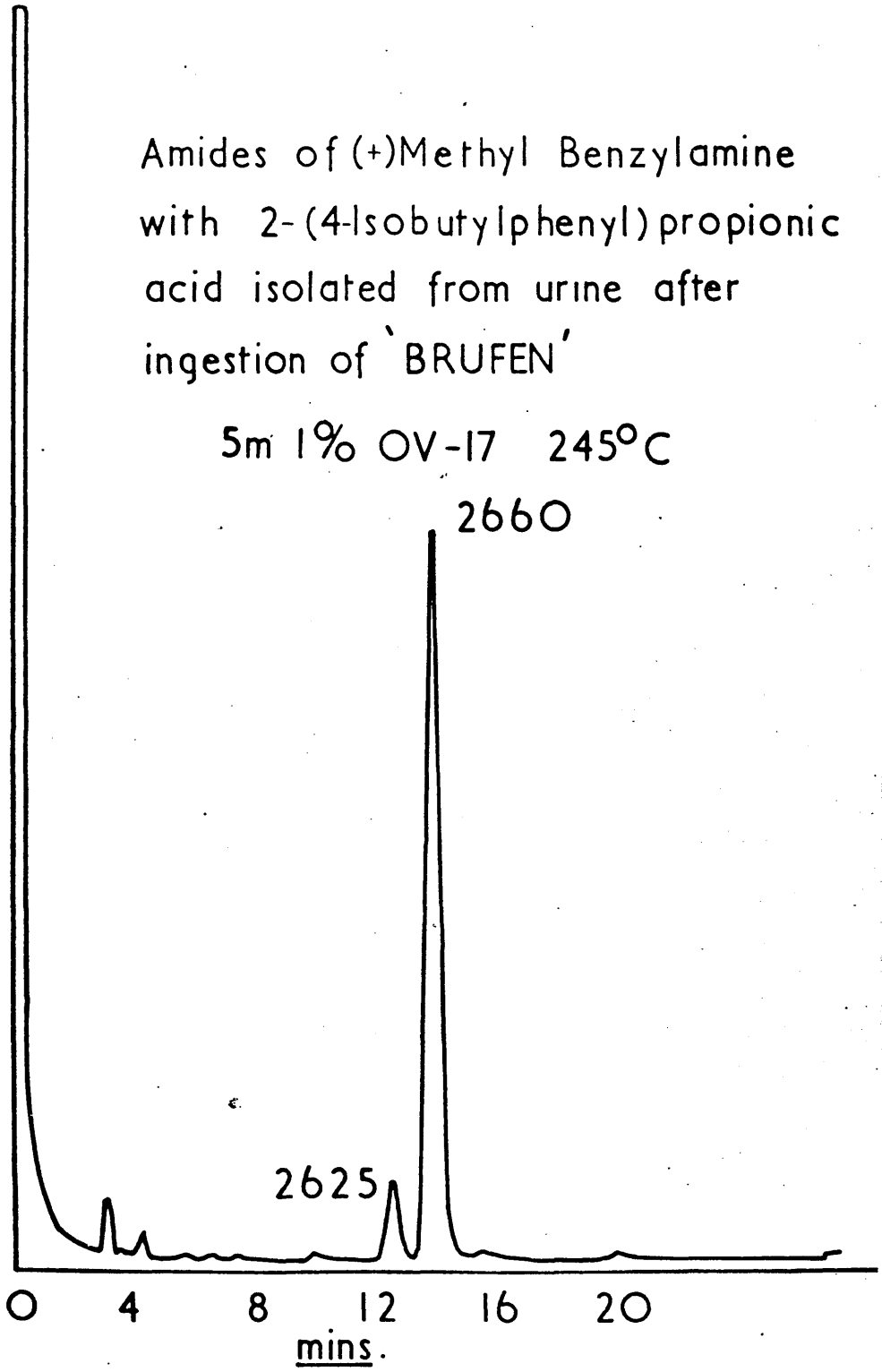


Fig. 50. Gas chromatographic resolution of 2-(4-isobutyl-phenyl)propionic acid, isolated from urine, as amides with (R)-(+)-α-phenylethylamine.

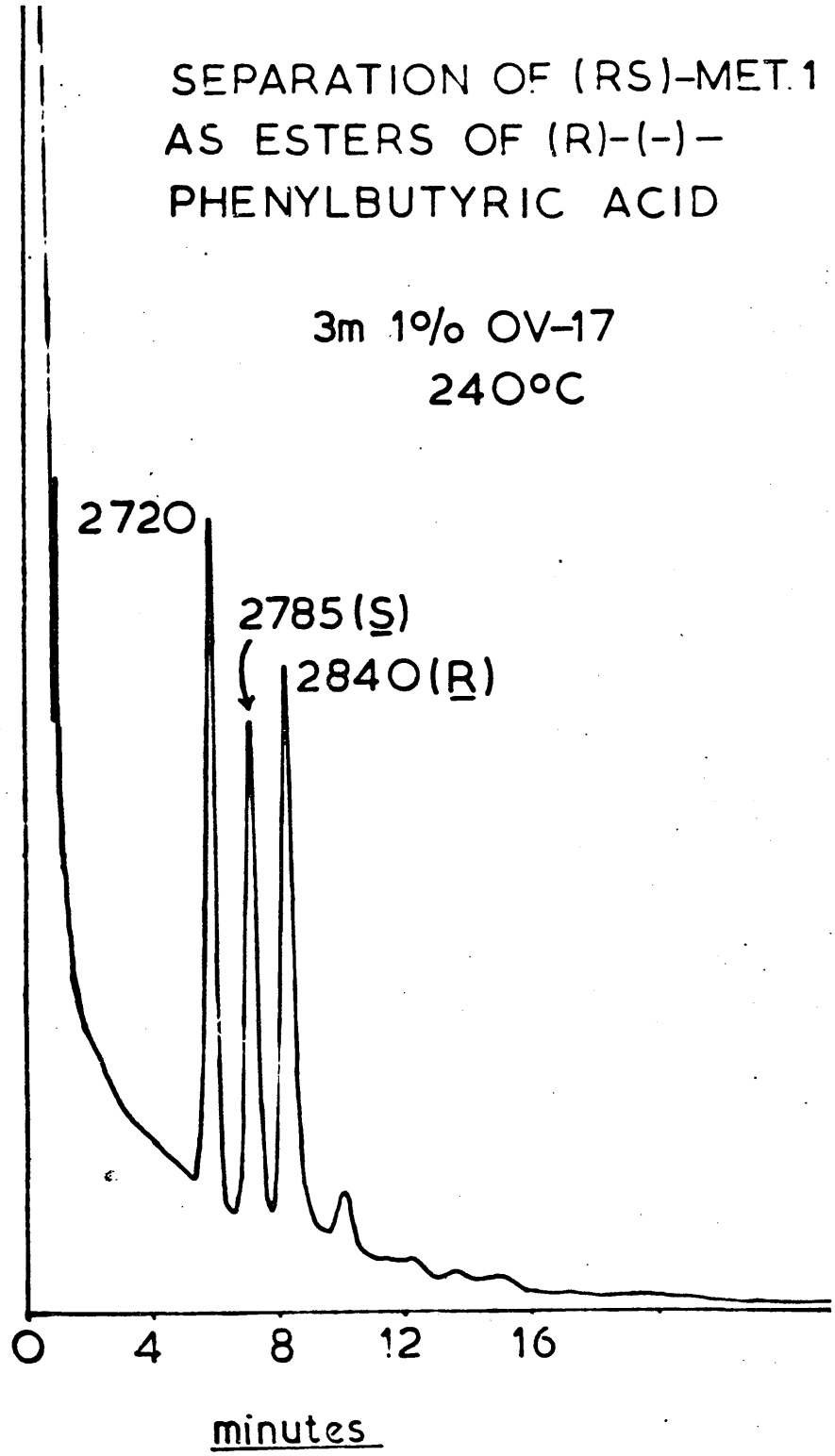


Fig. 51. Gas chromatographic resolution of 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester, isolated from urine, as esters with (R)-(-)-α-phenylbutyric acid.

The peak with I=2720 was not investigated.

This allowed the determination of the enantiomeric composition of the "unmetabolised" acid recovered from urine. After formation of the amides with (R)-(+)-α-phenylethylamine, GLC showed that the excreted acid consisted of 80% (+)-2-(4-isobutylphenyl)propionic acid (Fig. 50). The principal MS characteristics of the amides are also contained in Table 24. Identical mass spectra were obtained for the amides of the authentic and isolated acid. The fragmentations were similar to those observed for corresponding amides of α-phenylbutyric and α-phenylpropionic acids (Section 3.2.), C-acyl cleavage yielding m/e 161 characteristic of the acid and N-alkyl cleavage giving m/e 105, characteristic of the amine moiety.

Metabolite 1

Hydroxylation at the benzylic position in the side chain introduces a new chiral centre and hence there are four possible diastereomers. A sample of metabolite 1, isolated from a urine extract was methylated and treated with excess (R)-(-)-phenylbutyryl chloride. Analysis of the resulting phenylbutyrates on GLC showed two peaks (Fig. 51) which corresponded to those previously observed with the synthetic material (Table 22). GC-MS confirmed that these were the phenylbutyryl esters. The mass spectra of the esters obtained from the isolated material were identical to those of the synthetic alcohol (Fig. 47). Hydroxylation at the benzylic position appeared not to have been stereospecific since both ester peaks were present. However, the alcohol was not completely racemic.

Table 25. Gas liquid chromatographic and mass spectrometric data for the amides of metabolites of 2-(4-isobutylphenyl)propionic acid with (R)-(+)-α-phenyl ethylamine

	$I^{1\%\ OV-1}_{225^oC}$	$I^{1\%\ OV-17}_{220^oC}$	Mass spectral characteristics	
			M^+.	Other ions (% abundance)
Amides of oxidised Met. 1	2475 2515		323(4)	105(100); 176(36); 106(12); 77(10); 79(10); 104(10)
Amide of (+)-Met. 4	2485		325(0.1)	105(100); 119(50); 163(29);
Amide of (-)-Met. 4	2465			267(24); 91(19); 106(15)
Amide of (+)-Met. 4-TMS	2590	2890	397(0)	131(100); 73(80); 105(33); 75(18); 339(13)
Amide of (-)-Met. 4-TMS	2565	2855		

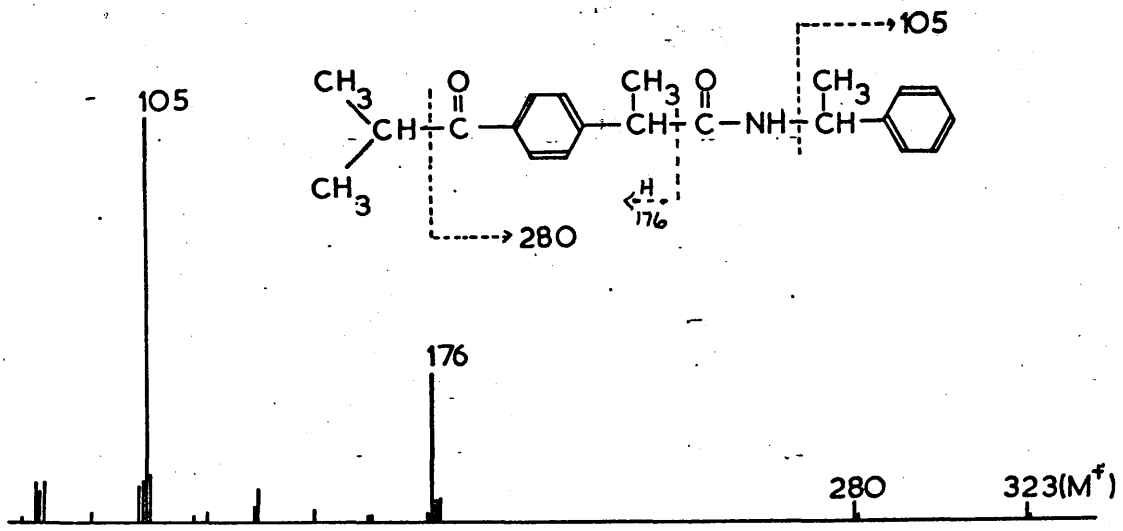


Fig. 52. Mass spectrum of the amide of oxidised metabolite 1 with (R)-α-phenylethylamine.

Measurement of peak areas showed that it contained 57% of the (R)-form. Since the extraction procedure involved the use of basic solutions it was possible that some racemisation might have occurred. A sample of the resolved alcohol (88% -(S)) (cf. Sec. 3.3.2.) was taken through the normal work-up procedure, methylated and esterified with (R)-(-)-α-phenylbutyryl chloride. Analysis of the resulting phenylbutyrates showed that the enantiomeric composition was 87% of (S)-form to 13% of (R). This confirmed that no racemisation had taken place during work-up, and that the excreted metabolite was 57% the (R)-form.

In order to determine the enantiomeric composition at the second chiral centre without any possible interference from the asymmetric centre in the side chain, the alcohol was first oxidised to the ketone with CrO_3/H_2SO_4. After formation of (R)-(+)-α-phenylethylamides, GLC showed that the metabolite comprised 58% of the (S)-form and 42% of the (R)-form. The principal MS characteristics of the amides are presented in Table 25. The base peak was produced by N-alkyl cleavage and the abundant ion at m/e 176, characteristic of the acid moiety, by C-acyl cleavage with hydrogen transfer, (Fig. 52).

Metabolite 4

(R)-(+)-α-phenylethylamides were prepared for metabolite 4 via the imidazolide to prevent any interaction with the hydroxyl group. GLC and MS data for the amides of the metabolite, both as the free hydroxy-

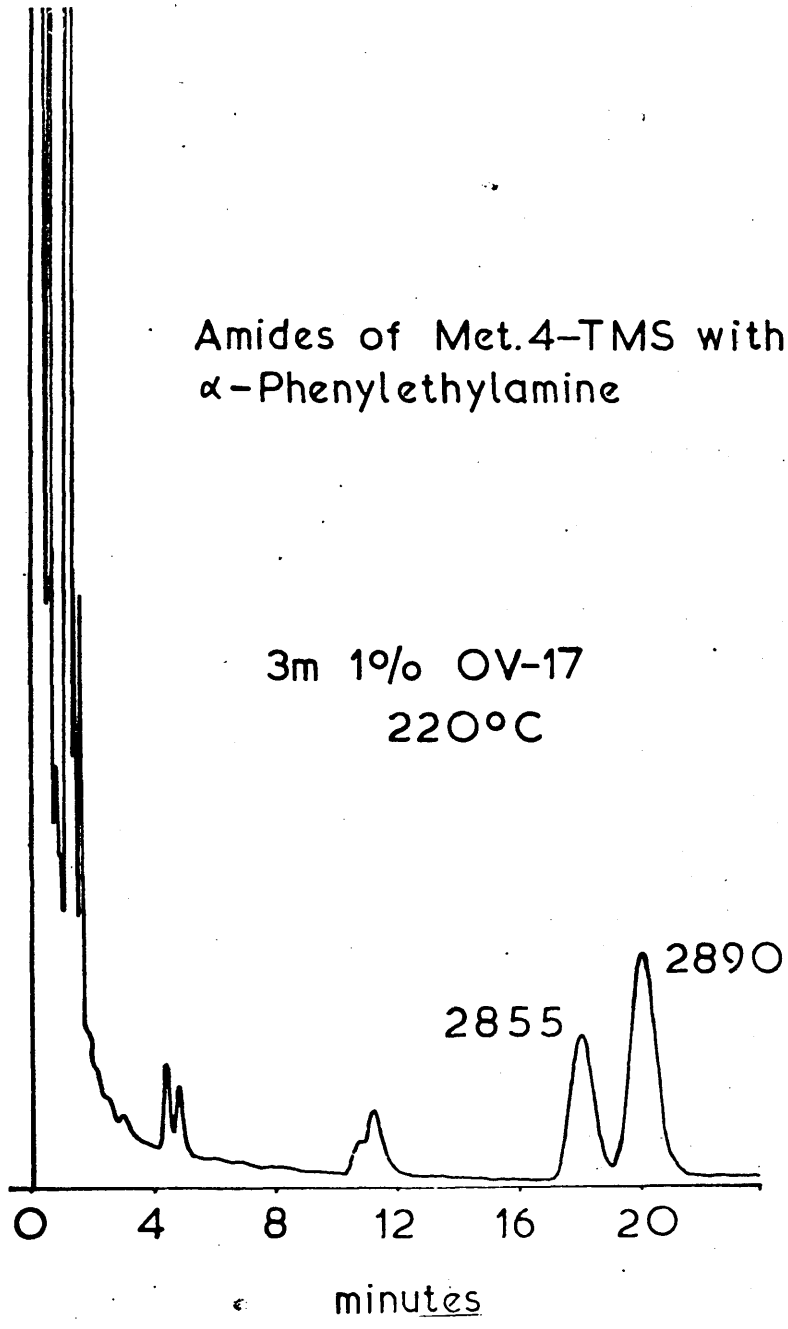


Fig. 53. Gas chromatographic resolution of metabolite 4 - trimethylsilyl ether as the amides formed with (R)-(+)-α-phenylethylamine.

compound and as the TMS ether are presented in Table 25. The excreted metabolite was shown to be 64% the (S)-2,4'-(2-hydroxy-2-methylpropyl)phenylpropionic acid, (Fig. 53) assuming the order of elution of the amides is the same as that for the derivatives of ibuprofen methyl ester.

The base peak in the mass spectrum of the amide of metabolite 4 was produced by N-alkyl cleavage (m/e 105) and N-acyl cleavage gave the abundant ion at m/e 119. Loss of acetone formed the ion at m/e 267, and (combined with N-alkyl cleavage) the ion at m/e 163. After trimethylsilylation, the base peak (m/e 131) was produced by cleavage at the tertiary centre. The ethylbenzyl ion was still abundant (33%). The ion at m/e 339 could be ascribed to loss of acetone from the molecular ion and required rearrangement of the trimethylsilyl-group.

Table 26. Details of metabolites obtained from incubation of ibuprofen with a rat liver homogenate

Zone from TLC plate	$I^{1\%\ OV-1}_{150^oC}$	$I^{1\%\ OV-17}_{160^oC}$	GC-MS identification
R_F 0.70 - 0.84	1505	1725	Ibuprofen
0.55 - 0.64	1660	1935	Met. 4
	1710	1950	Met. 1
0.4 - 0.55	1660	1935	Met. 4
	1780	2090	Met. 3

3.3.5. In vitro metabolism using rat liver preparation

(RS)-ibuprofen (10 mg) was incubated with an enzymatic preparation obtained from a rat liver homogenate, for 3 h. After extraction of an acid fraction the total sample was methylated and applied to a preparative plate which was eluted in chloroform:ethyl acetate, 3:1 v/v. The material isolated from the plate was examined on GLC and GC-MS and three metabolites of ibuprofen were identified, as well as unmetabolised ibuprofen (Table 26). All three hydroxylated metabolites were present, but no evidence was observed for the presence of the dicarboxylic acid, metabolite 2. The primary hydroxylated metabolite, metabolite 3, was only a minor constituent of the urine extracts but was present to a much larger extent in this case. It seemed likely that this was an intermediate on the pathway to the dicarboxylic acid and under the present conditions further oxidation had not occurred.

After mild alkaline hydrolysis, the α-phenylethylamides of the unmetabolised ibuprofen and metabolite 4 were prepared. GLC analysis showed that both these acids were racemic and no evidence for stereospecific metabolism was obtained.

Table 27. Gas chromatographic and mass spectral characteristics of fenoprofen and its metabolites as methyl ester and trimethylsilyl derivatives

Peak	$I^{1\%\ OV-1}$	Mass spectral characteristics $M^{+\cdot}$	Other ions (% abundance)	Structural assignment
I	1840	256(40)	197(100); 77(38); 91(32); 104(24)	Fenoprofen
II	2100	286(100)	227(69); 77(43); 123(38); 103(32); 105(25); 91(24)	Methoxy-fenoprofen
III(a)	2175	272(85)	213(100); 109(47); 91(21); 77(19); 103(18)	Hydroxy-feno-profen
III(b)	2215	344(100)	73(84); 89(53); 135(25); 285(20); 91(18); 75(15); 269(14); 77(13); 151(11)	Hydroxy-fenoprofen-TMS ether

3.4. The urinary metabolism of fenoprofen, (RS)-2-(3-phenoxyphenyl)propionic acid

During the study of ibuprofen metabolism a urine sample was obtained from a patient who had also received treatment with fenoprofen, 2-(3-phenoxyphenyl)propionic acid. Previous work[349] on the metabolism of this drug had identified the main urinary constituents as the drug itself and a hydroxylated derivative of the drug as well as the glucuronide conjugates of both of these.

3.4.1. Gas chromatographic and mass spectral characteristics of metabolites

A 20 ml sample of urine was extracted with ether and ethyl acetate in the same manner as described for the isolation of metabolites of ibuprofen, to yield extract A. The acidified urine (pH ~ 1) was heated at 80°C for 1 h to hydrolyse any acid-labile conjugates and re-extracted with ether and ethyl acetate to yield extract B. Another 20 ml sample of urine was treated with "Glusulase" in acetate buffer for 48 h at 37°C. After extraction this yielded extract C. Each sample was then methylated and trimethylsilylated and analysed by GLC on a 1% OV-1 column, programming from 120-220°C at 2°/min (Fig. 54). Extracts A and B showed three peaks of longer retention time than the ibuprofen metabolites. GC-MS analysis of these peaks (Table 27) showed that peak I corresponded to fenoprofen methyl ester. Peak II had M^+ 286 corresponding to a methoxy-derivative of fenoprofen methyl ester and

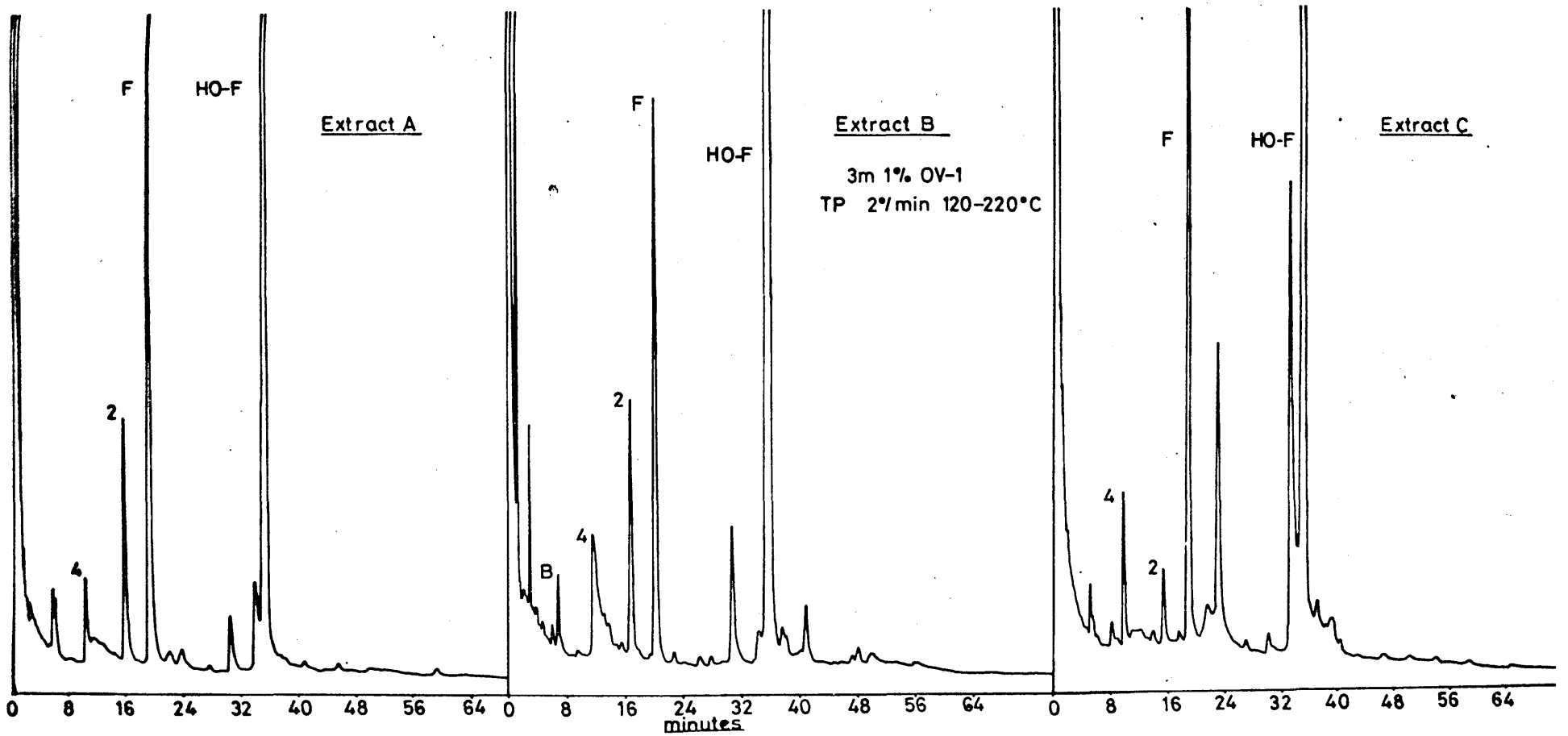


Fig. 54. Gas chromatographic profiles of urinary acidic extracts obtained from a patient receiving treatment with ibuprofen and fenoprofen. Extract A : free components; Extract B : HCl labile components; Extract C : free and conjugated components after enzymic hydrolysis, all as methyl ester trimethylsilyl ethers. (B = ibuprofen; F = fenoprofen; HO-F = hydroxy-fenoprofen; 2 and 4 are ibuprofen metabolites.)

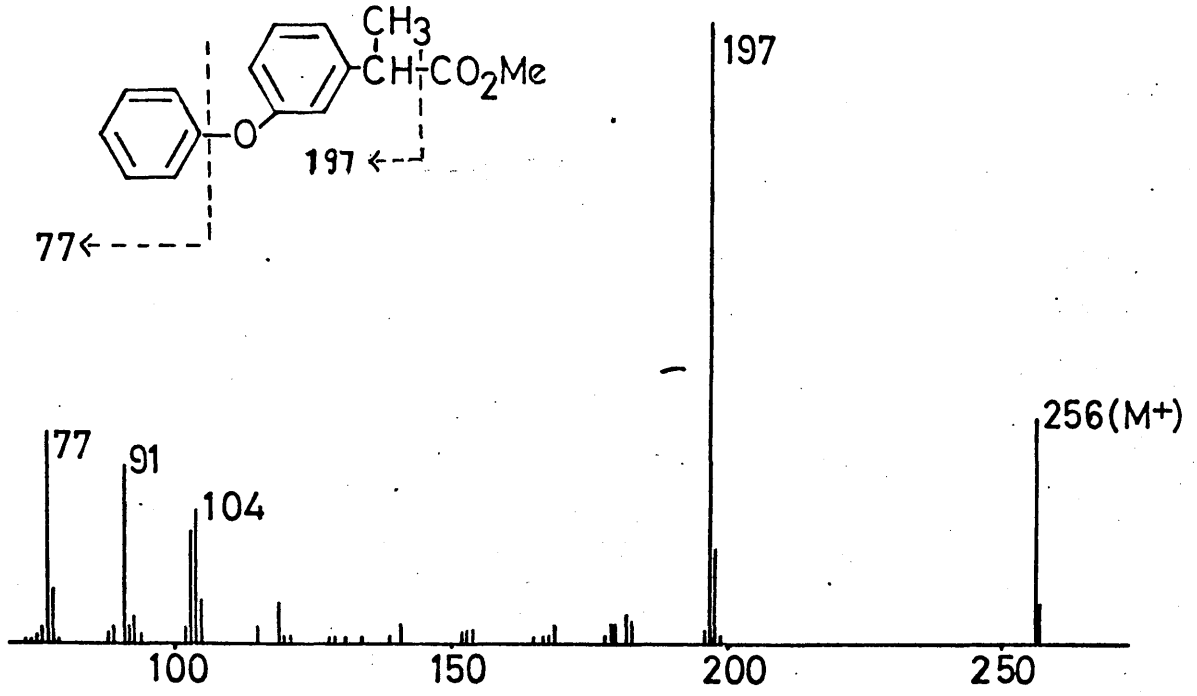


Fig. 55. Mass spectrum of fenoprofen methyl ester.

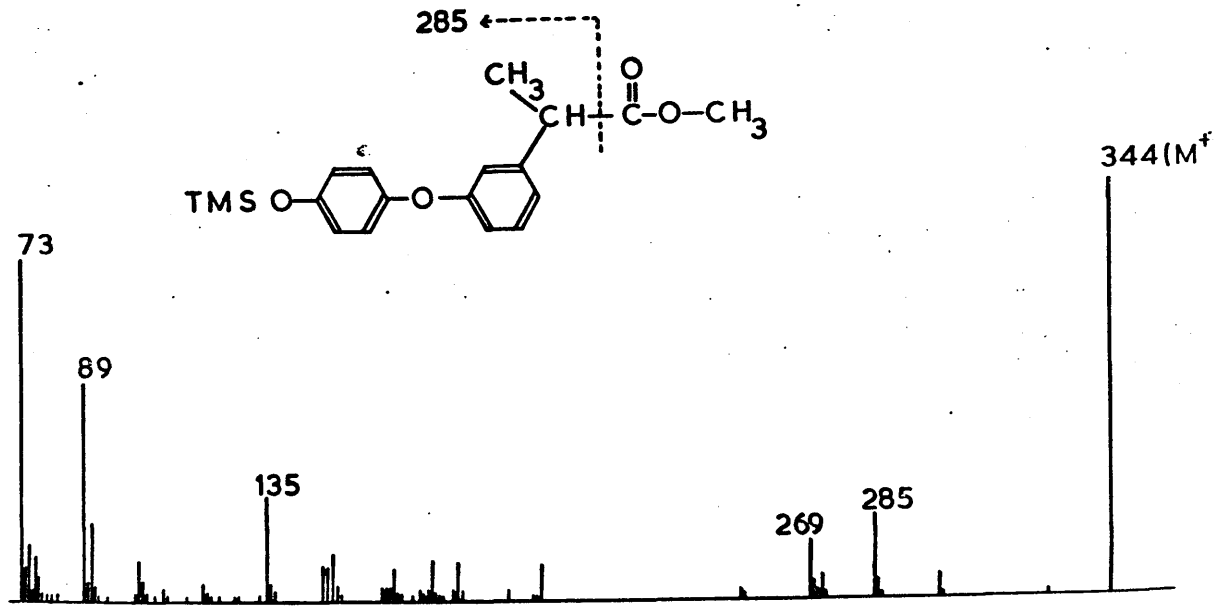


Fig. 56. Mass spectrum of hydroxy-fenoprofen as its methyl ester trimethylsilyl ether.

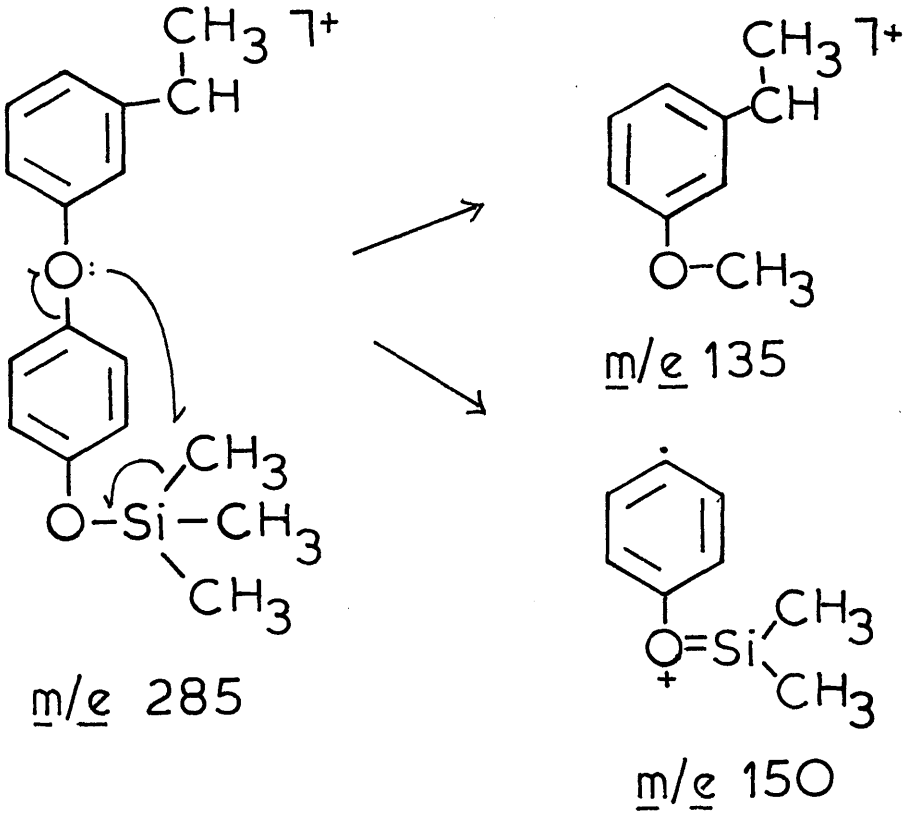
peak III had M^+ 344, corresponding to a trimethylsiloxy derivative.

The major fragmentation in the mass spectrum of fenoprofen methyl ester (Fig. 55) was loss of the carbomethoxy group (M-59). Other large fragments were the benzyl (m/e 77) and tropylium (m/e 91) cations produced by cleavage adjacent to the ethereal oxygen, and loss of the carbomethoxy and methyl radicals from the phenyl-propionic acid moiety.

Peak II may have arisen by methylation of the phenolic hydroxyl group on treatment with diazomethane and may not in fact be a genuine metabolite. The base peak in the spectrum of this methyl ester was the molecular ion. A large ion was observed due to the loss of the carbomethoxy group (m/e 227) and cleavage adjacent to the phenyl ether oxygen with the charge being retained on the ethereal fragment produced the ion at m/e 123.

Hydroxy-fenoprofen (peak III) The base peak in the spectrum of the methyl ester was produced by loss of the carbomethoxy group, while cleavage at the ethereal oxygen produced the abundant ion at m/e 109. After trimethylsilylation of the hydroxyl group the molecular ion became the base peak. Loss of the carbomethoxy group produced an ion of 20% abundance (m/e 285) (Fig. 56). The ion at m/e 151 was shown to contain two methyl groups from the trimethylsilyl ether group by preparation of the per-deuterated ether. It could be seen to arise by loss of a methyl radical and cleavage adjacent to the phenyl-ether with hydrogen transfer (Scheme 12). Transfer of a methyl

radical from the trimethylsilyl ether group on to the phenolic ether produced the ion at m/e 135. This changed to m/e 138 on formation of the perdeuterated trimethylsilyl ether.



Scheme 12

3.4.2. Isolation and identification of hydroxy-fenoprofen

According to previous work[349] the position of hydroxylation in fenoprofen is para to the ether, in the second ring. Since no evidence was presented for this structure it was decided to isolate purified material and use spectroscopic evidence to verify the structure.

The methylated extract was applied to a silicic

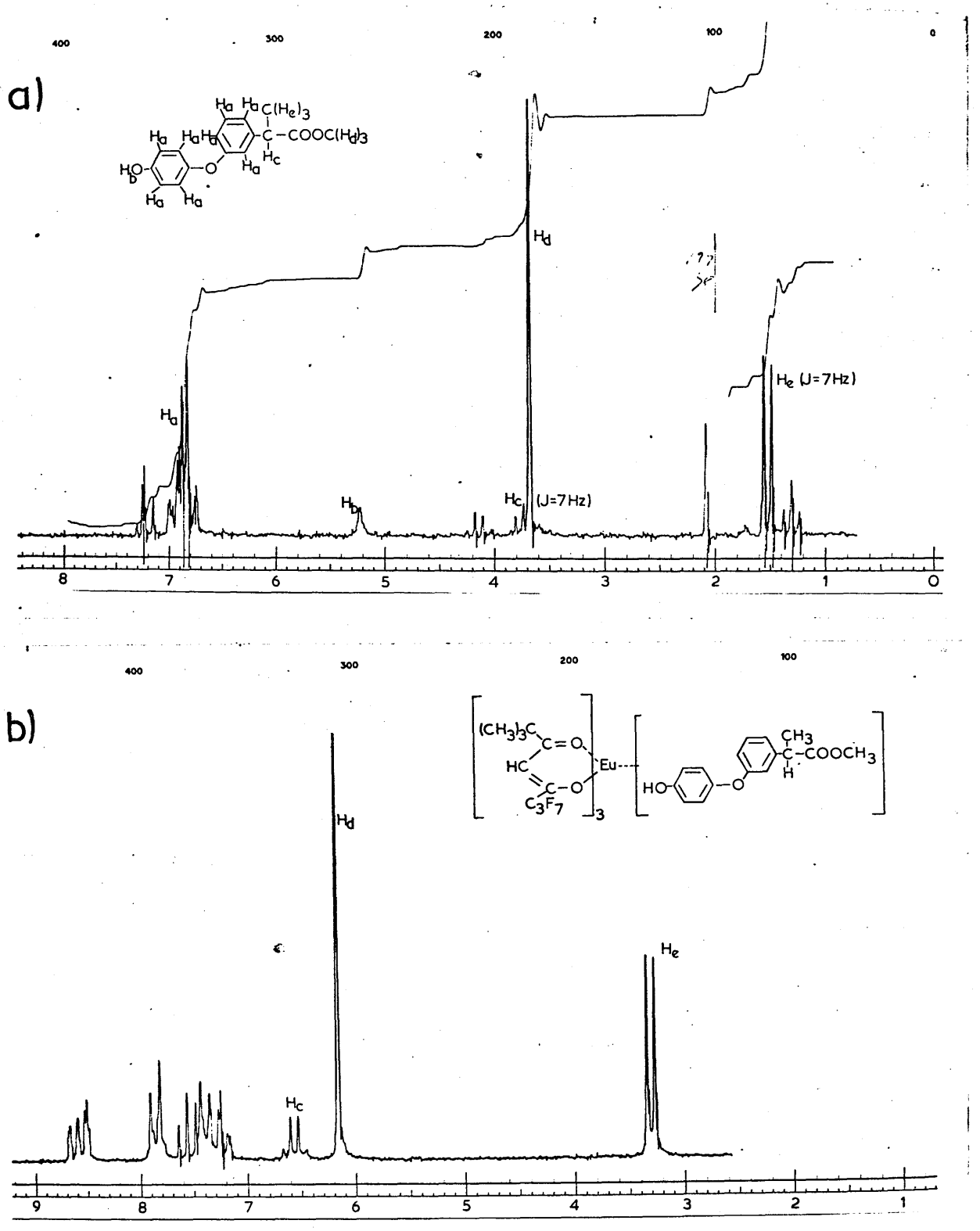


Fig. 57. Proton NMR spectrum of hydroxy-fenoprofen methyl ester.

a) Normal spectrum.

b) Spectrum obtained with added paramagnetic shift reagent, $Eu(fod)_3$ (0.4 M concentration).

acid column which was eluted with $CHCl_3$/EtOAc, 3:1 v/v. The first compounds to appear in the eluate were fenoprofen, ibuprofen and dicarboxy ibuprofen (Metabolite 2). The next fraction contained hydroxy-fenoprofen (R_F = 0.46). Removal of solvent yielded crystalline material which was recrystallised from benzene to give white needles, m.p. 118-120°C. Found : C, 70.05; H, 5.70%. Calc. for $C_{16}H_{16}O_4$: C, 70.57; H, 5.92%. Analysis by IR spectroscopy gave ν_{OH} = 3600 and $\nu_{C=O}$ = 1740 cm^{-1} (CCl_4 solution).

The ^1H NMR spectrum showed : δ, 7.3-6.7 (8H) [aromatic -H]; 5.3 (1H) [-OH]; 3.7 (1H, q, J = 7 Hz) (benzylic CH-]; 3.7 (3H, s) [-COOCH$_3$]; 1.5 (3H, d, J = 7 Hz) [-CH$_3$ coupled to benzylic CH-].

The aromatic region of the spectrum was too complicated to determine any substitution pattern. However, simplification was effected by the use of a paramagnetic shift reagent - $Eu(fod)_3$. Several reagent concentrations were used but the best spectrum was obtained with a 0.4 M concentration (Fig. 57). This showed the presence of two doublets (δ = 8.65 and 7.9 J = 8 Hz) which could be assigned to the protons in the hydroxylated phenyl ring. This could only arise from an A_2B_2 system present in a para-substituted ring. The signal at δ 8.5 was assigned to the proton at C-2 in the first phenyl ring and the multiplet at δ 7.4 to the ABX system at C-4,5 and 6 . This evidence confirmed that the hydroxyl group was in the 4'-position.

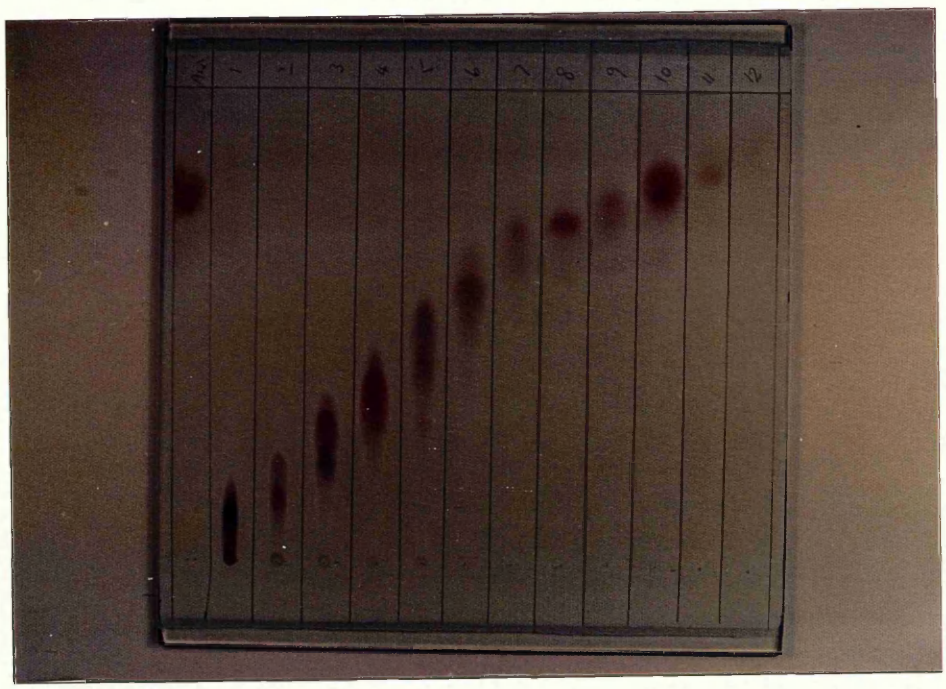


Fig. 58. Analytical thin layer chromatogram of material isolated by preparative TLC from a urinary extract, after enzymic hydrolysis, obtained from a patient receiving spironolactone treatment.

Table 28. Chromatographic data for urinary metabolites of spironolactone

Metabolite	R_F $CHCl_3$:MeOH, 9:1	Colour with $Ce(SO_4)$	1% OV-1 $I_{250°C}$ Free	1% OV-1 $I_{250°C}$ TMS	$M^{+\cdot}$ Free	$M^{+\cdot}$ TMS	$M^{+\cdot}$ d_9-TMS
1	0.26	Pink	3335	3350	356	428	437
2	0.21	Pink	3335	3350	356	428	437
3	0.35	Yellow	3335	3350	356	428	437
4	0.44	Yellow	3305	3360	358(M-48)	550	568
5	0.52	Yellow	3170	-	340	-	-
6	0.64	Yellow	3175	-	340	-	-
7	0.69	Yellow		3350	356	428	437
				3410		476	485
8	0.77	Yellow	3275	-	356	-	-
9	0.80	Yellow	3170	-	340	-	-

3.5. The urinary metabolism of spironolactone

Urine, from patients receiving spironolactone treatment, was obtained *via* the Blood Pressure Unit at the Western Infirmary. After extraction of the steroids and steroid conjugates from the urine, by adsorption onto the neutral resin, Amberlite XAD-2, the free steroids were separated from the conjugates by solvent extraction and the conjugates were hydrolysed enzymically. Analysis of the steroid fractions by analytical TLC (mobile phase $CHCl_3$:MeOH, 9:1 v/v) showed several components. Purification was effected by preparative TLC using chloroform:methanol, 9:1 v/v as mobile phase. Since previous workers had identified[350] several components of the free steroid extract, it was decided to concentrate on the hydrolysate, using the free extract for comparison. After purification of the hydrolysate by preparative TLC, the material recovered from each band was re-examined by analytical TLC (Fig. 58) and R_F values were recorded for the major components (Table 28). The purified fractions were analysed by GLC and GC-MS before and after trimethylsilylation. The use of perdeuterated trimethylsilyl ethers aided identification by indicating the number of hydroxyl groups present.

Several metabolite structures could be postulated by examination of their mass spectral fragmentations. Unfortunately most of the sulphur functions proved thermally labile so that introduction into the mass

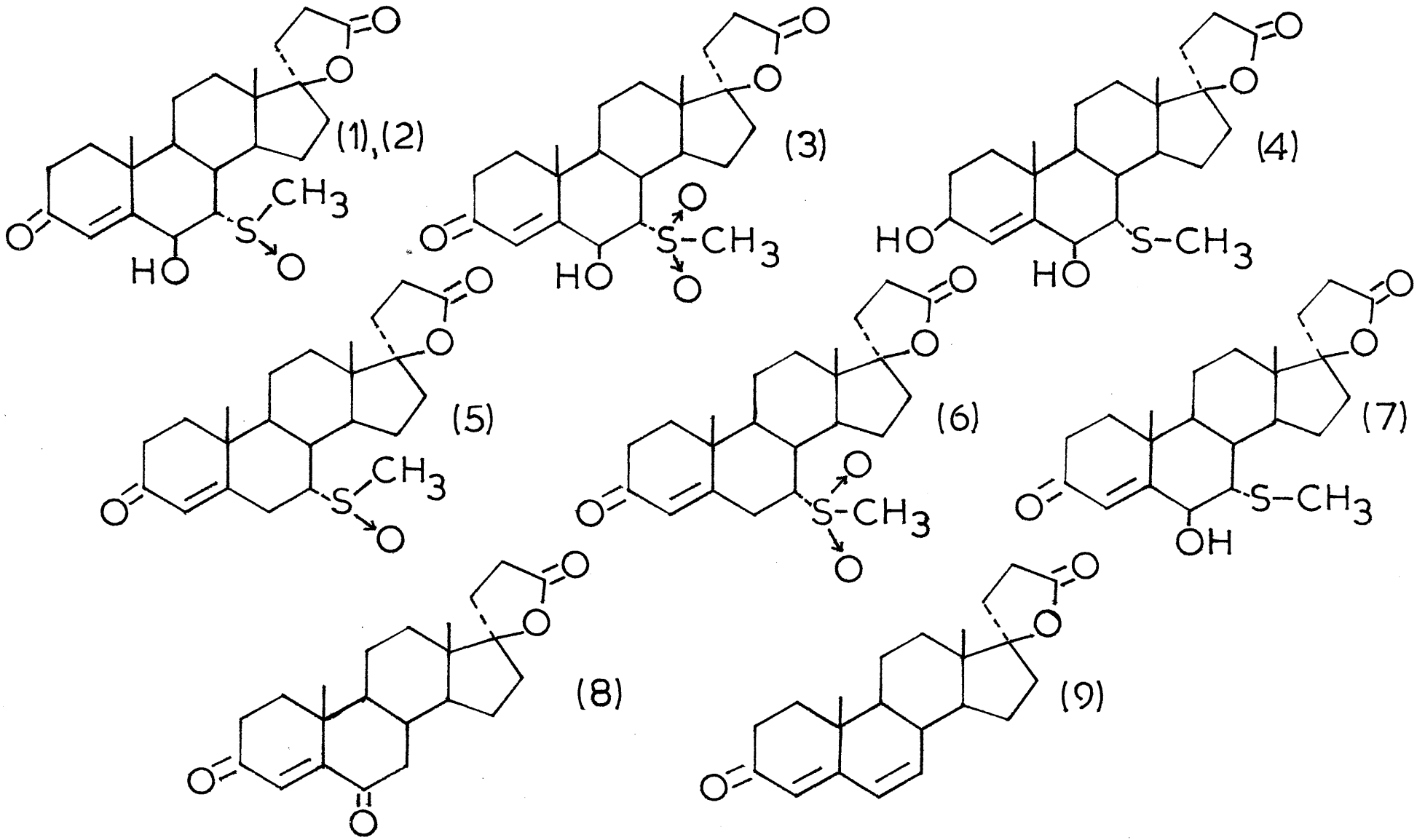


Fig. 59. Proposed structures of metabolites of spironolactone.

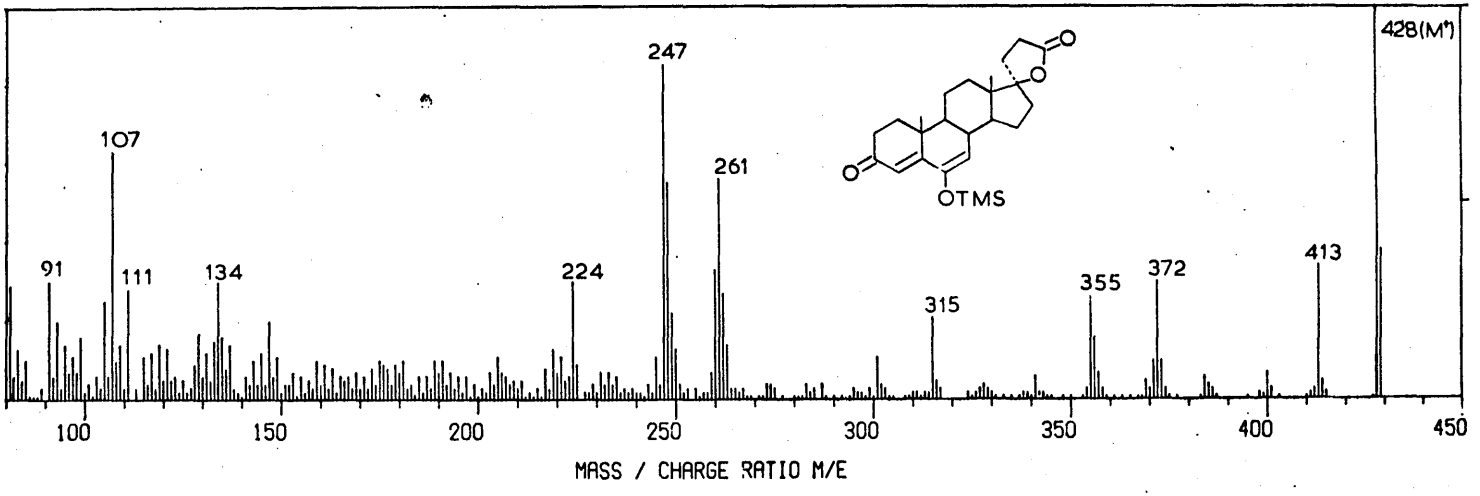
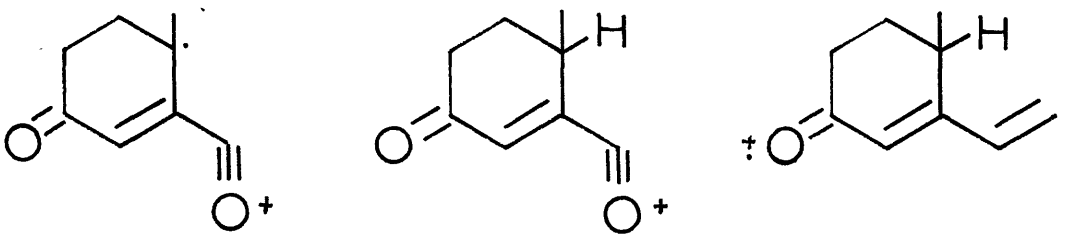


Fig. 60. Mass spectrum of metabolite 1 as its trimethylsilyl ether.

spectrometer *via* the GLC system resulted in the spectra of the thermal degradation products containing a Δ6-7 double bond. However, knowledge of the mobility on TLC allowed reasonable speculation as to the oxidation state of the sulphur function present (Fig. 59).

Band 4 showed a major component at R_F 0.26 (metabolite 1) and a minor component, R_F 0.21 (metabolite 2). Metabolite 2 was also present in band 3. These metabolites had similar gas chromatographic and mass spectral properties. Mass spectral analysis indicated a molecular weight of 356. Trimethylsilylation gave a molecular ion at m/e 428 (Fig. 60) rising to 437 on treatment with d_{18}-BSA, demonstrating that the molecule contained one free hydroxyl group. The presence of abundant ions at m/e 136 and 137 in the spectrum of the underivatised sample was consistent with a 6-ketone,[351] or $\Delta^{4,6}$-3-one[352] structure.



m/e 136 137 136

Ions of this type disappeared after trimethylsilylation, perhaps suggesting that the 6-keto or

$\Delta^{4,6}$-3-one structure arose from thermal degradation. If the metabolite contained a 6-hydroxyl group and a 7-sulphoxide or sulphone, thermal elimination of the sulphur function followed by tautomerism would produce the required structure. Trimethylsilylation of the hydroxyl function prior to gas chromatographic analysis would prevent the latter process, and the spectrum obtained implied the possible formation of an enol ether, since no loss of trimethylsilanol was observed from the molecular ion. It seemed likely from the polarity of the metabolites (indicated by mobility on TLC) that the function at C-7 should be a sulphoxide. The two metabolites could then be assigned as the two sulphoxide epimers, since such epimeric compounds are known to show different chromatographic properties.[353] This structure of a methyl sulphoxide would agree with that of a major metabolite which had been identified[350] in a free steroid extract.

Band 5 contained two drug related compounds : metabolite 3 (R_F 0.35) and metabolite 4 (R_F 0.44). Metabolite 3 also showed the same gas chromatographic and mass spectral properties as metabolites 1 and 2. This metabolite must, therefore, have the same basic structure as metabolites 1 and 2 but have a different sulphur function as C-7. It seemed probable that the function at C-7 was a sulphone, which would give metabolite 3 the structure shown in Fig. 59 .

Metabolite 4 had a molecular weight of 550 after trimethylsilylation which rose to 568 on formation of the

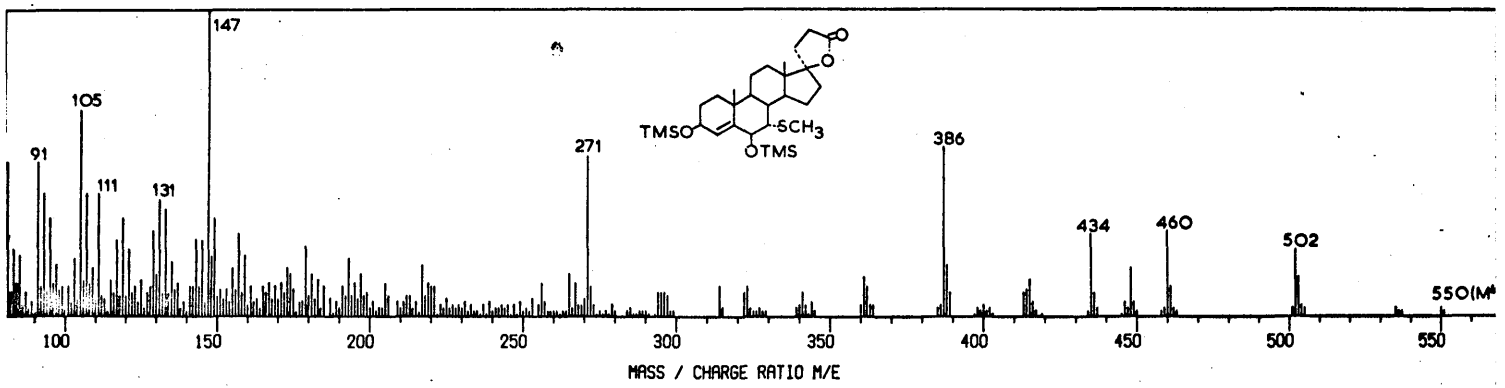
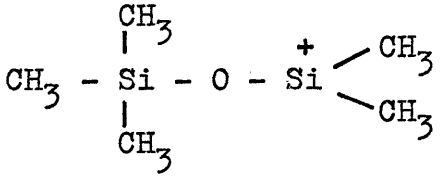


Fig. 61. Mass spectrum of metabolite 4 as its trimethylsilyl ether.

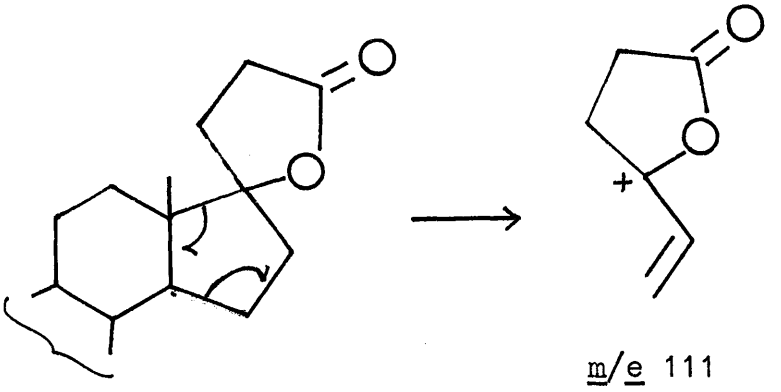
d_9-TMS ether indicating the presence of two hydroxyl groups. A loss of 48 (methylthiol) suggested the possibility of an intact methylsulphide at C-7. It seemed feasible that this function might be less prone to elimination. No molecular ion was observed in the spectrum of the untrimethylsilylated sample, but an ion at m/e 358 due to loss of methylthiol was noted. Once again fairly abundant ions were observed at m/e 136 and 137, suggesting that one hydroxyl group was at C-6. Since the original drug contained a keto - group at C-3 this was the most likely site for the second hydroxyl group.

The base peak in the spectrum of the trimethylsilyl ether (Fig. 61) arose by rearrangement and combination of the two trimethylsilyoxy functions. This was confirmed by a



m/e 147

rise of 15 a.m.u. to m/e 162 when perdeuterated TMS ethers were formed. An abundant ion at m/e 111 could be ascribed to the lactone ring by cleavage at ring D (Scheme 13)



m/e 111

Scheme 13

In this spectrum loss of trimethylsilanol from the molecular ion was observed (m/e 460) and loss of both silyl groups was also seen (m/e 370).

Band 6 contained a major metabolite with R_F 0.52. The retention index and mass spectrum of this metabolite was identical to that obtained from a reference standard of aldadiene, a previously identified[354] metabolite of spironolactone. However this compound was more polar than aldadiene on TLC. The metabolite was therefore presumed to have contained a C-7 sulphur function which was eliminated in the flash heater. Comparison of these results with the metabolites which had been identified from the free extract[350] suggested that this was a methyl sulphoxide.

The sulphur function was found to be labile and the metabolite was partially converted to aldadiene on work-up of the TLC band.

Band 8 contained two metabolites; a minor one at R_F 0.64 (Metabolite 6) and a major one at R_F 0.69 (metabolite 7).

Metabolite 6 corresponded to aldadiene on GLC and gave an identical mass spectrum. Once again this metabolite was more polar than aldadiene but was readily converted to aldadiene, indicating the presence of a labile sulphur function at C-7. This metabolite was assigned the sulphone structure on consideration of data from TLC.

Metabolite 7 gave two peaks on GLC as the TMS ether,

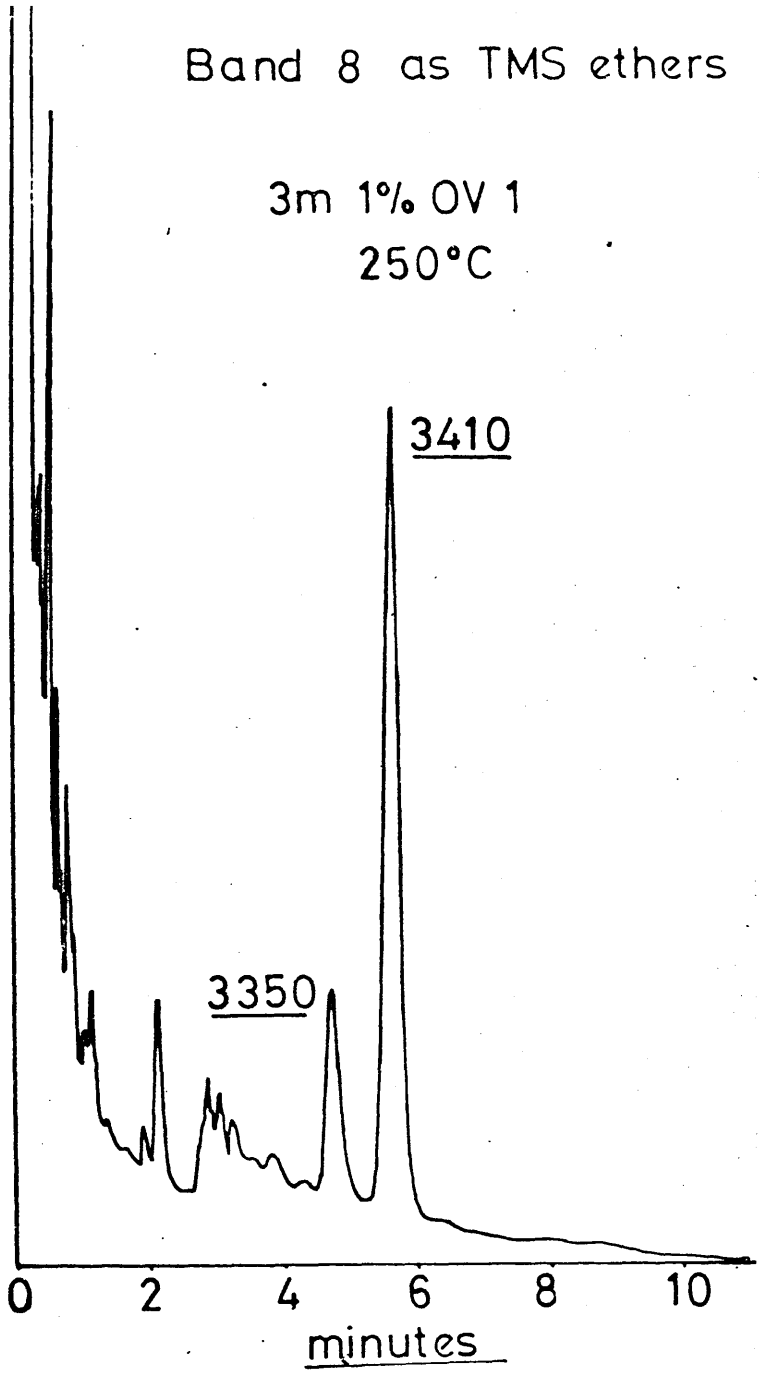


Fig. 62. Gas chromatogram of the material eluted in band 8 as the trimethylsilyl ether.

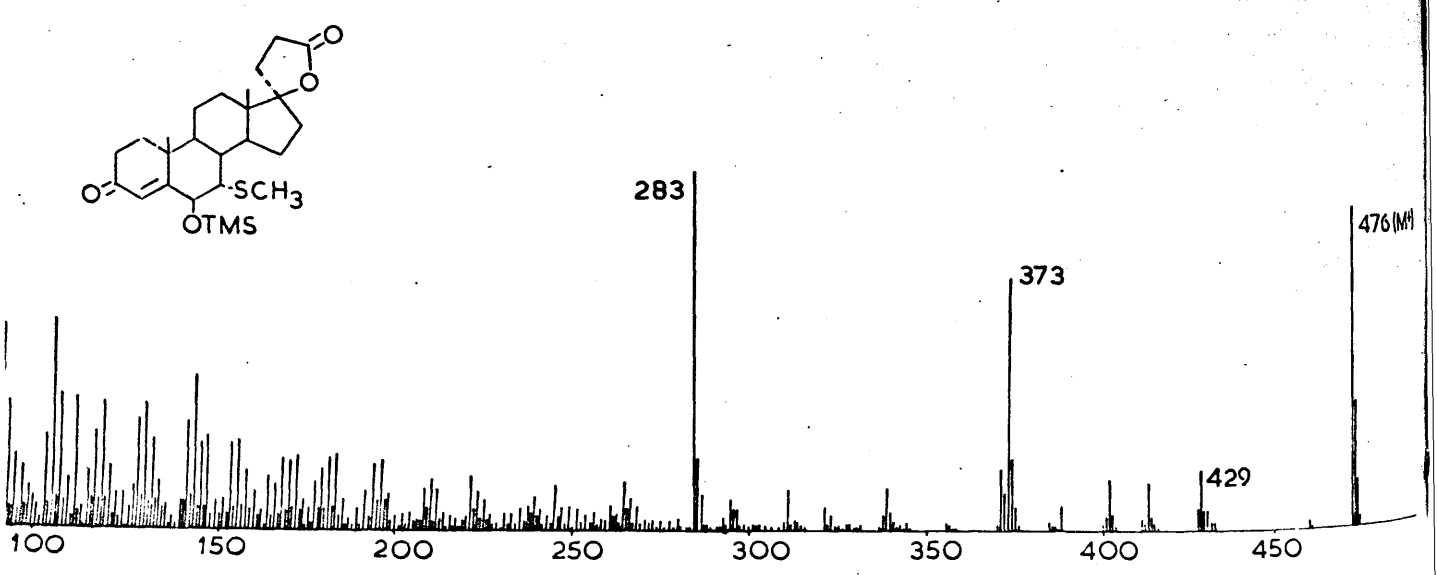


Fig. 63. Mass spectrum of metabolite 7(a) as its trimethylsilyl ether.

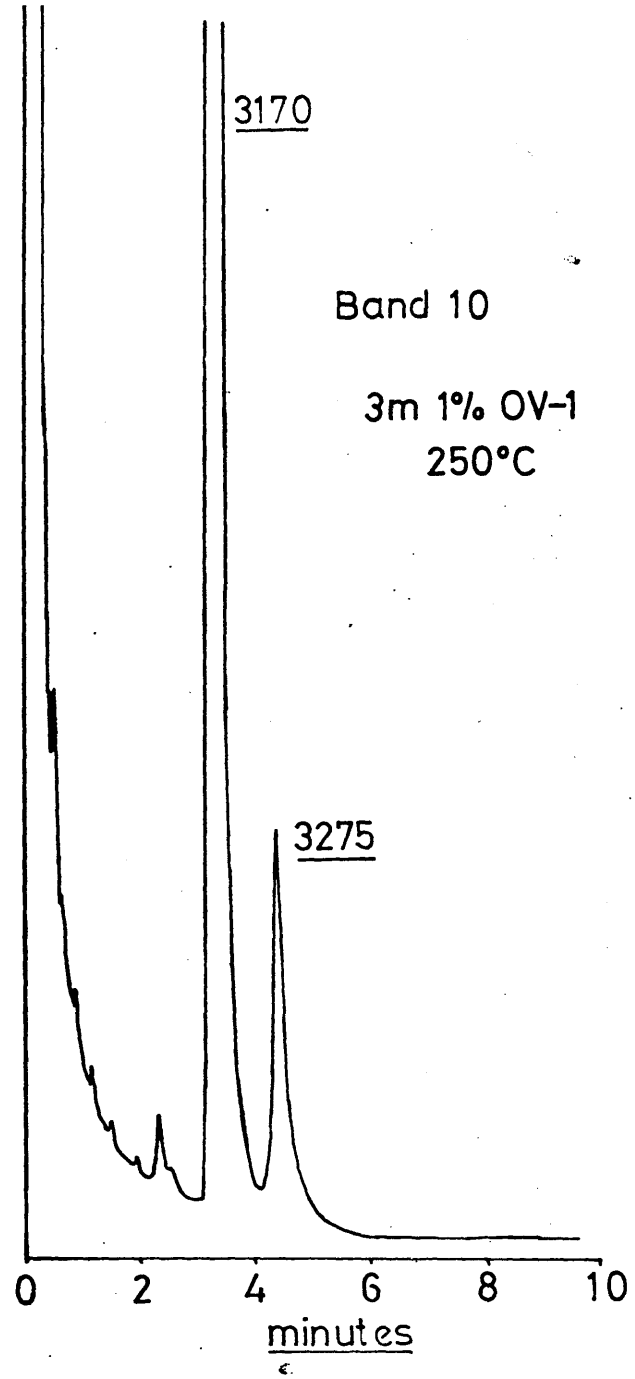


Fig. 64. Gas chromatogram of the material eluted in band 10.

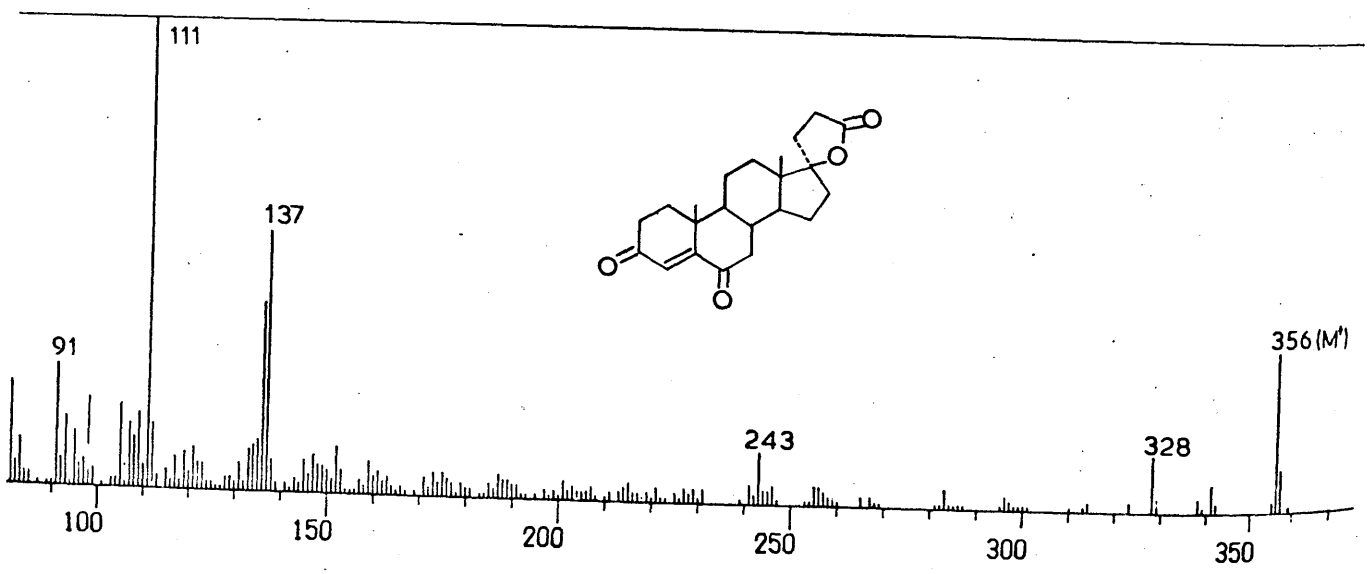


Fig. 65. Mass spectrum of metabolite 8.

I = 3350 and I = 3410 (Fig. 62). The peak with lower retention index gave a mass spectrum similar to that of metabolite 1. This suggested that metabolite 7 contained a C-6-hydroxyl group. The second peak had a molecular ion at m/e 476 which rose by 9 a.m.u. to m/e 485 on formation of the perdeuterated TMS ether. A loss of 47 a.m.u. from the molecular ion suggested that the function at C-7 might be a methyl sulphide. Either both these peaks arise from the same metabolite - the methyl sulphide, by partial elimination of methylthiol on GLC or the spot observed on TLC was a mixture of two metabolites, one of which (the methyl sulphide), was thermally stable : and the other (a thiol) labile.

The mass spectrum of metabolite 7(a) as the TMS ether is shown in Fig. 63.

Band 10 also contained two metabolites, metabolite 8, (R_F 0.77) and metabolite 9 (R_F 0.80) (Fig. 64). Neither of these metabolites showed a change in retention index or mass spectrum when treated with trimethylsilylating reagent, indicating that no hydroxyl groups were present. The mass spectrum of metabolite 8 (Fig. 65) showed a molecular weight of 356. The spectrum was very similar to that obtained for metabolite 1 before trimethylsilylation and the Δ^4-3,6-dione structure was proposed on the basis of its polarity on TLC. The base peak in the spectrum (m/e 111) could be ascribed to the lactone ring produced by cleavage across ring D. Abundant ions were also observed at m/e 136 and 137 confirming the presence of a

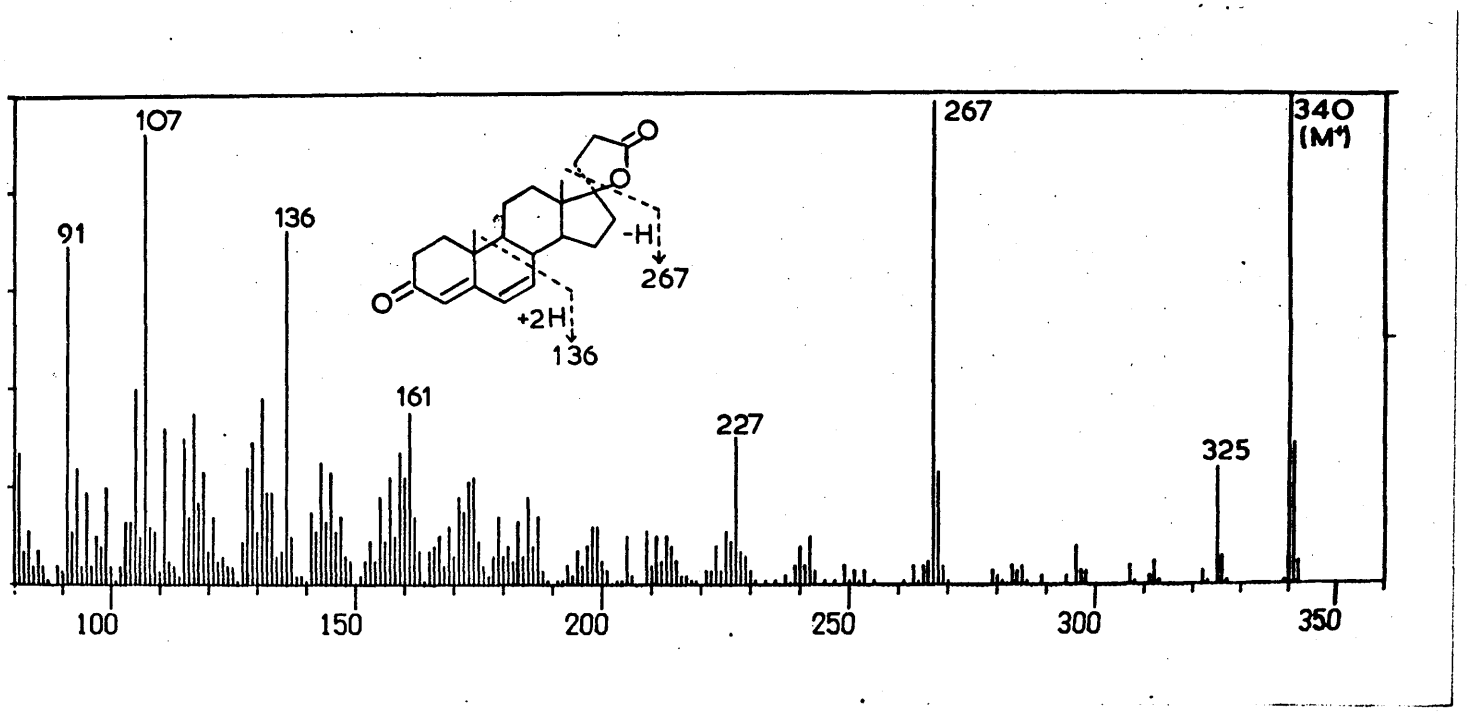


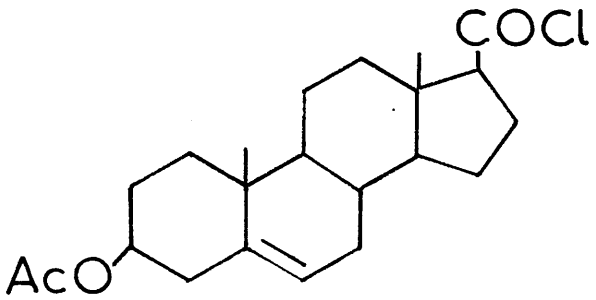
Fig. 66. Mass spectrum of metabolite 9.

6-ketone. An ion at m/e 328 could be assigned to loss of carbon monoxide from the molecular ion.

Metabolite 9 was identical in chromatographic and mass spectral properties to aldadiene.

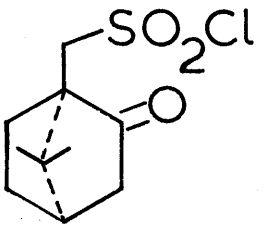
The base peak in the mass spectrum (Fig. 66) was the molecular ion. Major ions could be assigned to simple cleavages : loss of the lactone by cleavage at C-17 produced an ion at m/e 227 and with the charge remaining on the lactone fragment, m/e 111; fragmentation between rings B and C produced a strong ion at m/e 161 and further fragmentation of ring B produced the ion characteristic of the $\Delta^{4,6}$dien-3-one structure at m/e 136.

Section 4. Discussion



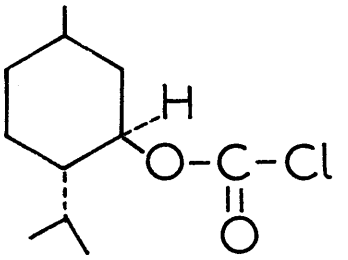
3β-Acetoxy-5-etienyl chloride

Anders & Cooper, 1971[151]



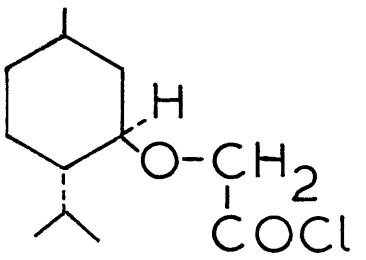
(+)-Camphor-10-sulphonyl chloride

Hoyer et al, 1972[355]

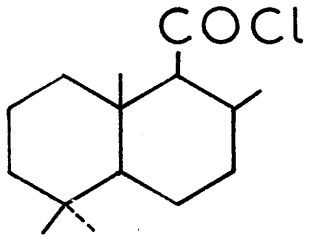


(R)-Menthyl chloroformate

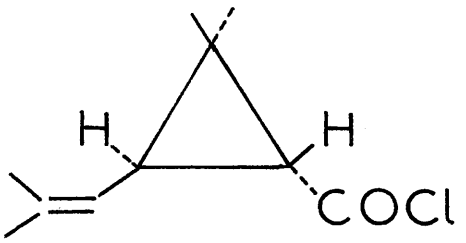
Westley & Halpern, 1968[146]



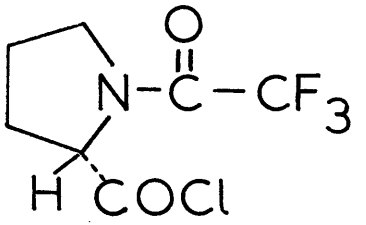
(R)-Menthoxyacetyl chloride



Drimanoyl chloride



(R)-(+)-trans-Chrysanthemoyl chloride



N-TFA-(S)-(-)-prolyl chloride

Westley & Halpern

Fig. 67. Chiral acid chlorides containing rigid carbon skeletons rendering them suitable reagents for the gas chromatographic resolution of enantiomers.

4.1. Gas chromatographic resolution of enantiomers

4.1.1. Terpenoid reagents

The choice of the terpenoid acids as gas chromatographic resolving agents was based on the hypothesis that an increase in the conformational immobility of the diastereomers, due to the incorporation of one of the chiral centres into a ring structure, would enhance the population of the "preferred" conformations of the diastereomers and hence the differences in their chromatographic mobility. This property is displayed by some of the reagents which have previously been applied to gas chromatographic resolutions (Fig. 67). In several cases, however, the rigid reagent skeleton is linked to the substrate *via* a flexible chain which will reduce the degree of conformational immobility. The increase in rigidity which would be conferred on the system by directly linking the chiral reagent to the substrate *via* an ester or amide bond was predicted to lead to better separations.

Drimanoic acid proved particularly effective for the resolution of straight-chain alcohols. When the alcohol is near the end of the chain, as in octan-2-ol, there is considerable size differential between the two alkyl groups on the asymmetric carbon and the achievement of a resolution is relatively straight-forward. Previous work on the resolution of alkan-2-ols, however, had mainly been based[139,220] on capillary columns or columns packed

with very polar phases. The octan-2-ol drimanoates showed very good separation on a packed column coated with non-selective SE-30 phase.

When the alcohol function is near the middle of a long alkyl chain the size differential no longer applies and considerable difficulty had been experienced[145,150] with the resolution of long-chain hydroxy fatty acids of this type. During the present study no separation was observed for the (R)-(+)-1-phenylethylurethanes, (R)-(-)-menthylcarbonates, or the (R)-(-)-menthoxy acetates of the enantiomeric methyl 13-hydroxystearates, but the corresponding drimanoates were well resolved. A comparable separation was also achieved for the drimanoyl derivatives of the methyl-12-hydroxystearates. These results reflect the enhancement of diastereomeric differences produced when two chiral centres are directly linked *via* an ester bond.

From the preceding discussion it would seem likely that when both chiral centres are constrained within ring systems even better resolutions should result. This, however, was not always observed, indicating the complexity of the separation mechanism.

Of the cyclic asymmetric alcohols studied the best resolutions achieved with the O-drimanoyl derivatives were for the enantiomeric pantolactones (2-hydroxy-3,3-dimethyl-4-butyrolactones) and the menthols. No noteworthy results were obtained with the other terpenols. However, (R)-(+)-*trans*-chrysanthemic acid proved a very useful

resolving agent for the series of enantiomeric monoterpenols. Very good resolutions were achieved for all the enantiomeric pairs of isomeric menthols and also the fenchyl alcohols. The results were not quite so good for the borneols or isoborneols, but separations were observed. This last result indicates that the increased rigidity of the borneol ring compared to menthol does not improve the separation, and other opposing factors must be involved in this situation. This decrease in resolution had also been noted by Murano[170] when he used (-)-menthol and (-)-borneol as resolving agents for (±)-*trans*-chrysanthemic acid.

An important criterion in the choice of a resolving agent is that it be enantiomerically pure. Both (*S*)-(+) drimanoic acid and (*R*)-(+)-*trans*-chrysanthemic acid comply with this requirement, being obtained in pure forms from naturally occurring materials.[326,356] The formation of derivatives with enantiomerically pure alcohols (*e.g.* (+)-octanol or (-)-menthol) gave only one ester peak on GLC, verifying that no racemisation occurred during acylation *via* the acid chloride. This confirmed the suitability of these reagents for determining the enantiomeric composition of an unknown mixture. The use of drimanoyl derivatives showed[334] that the 13-hydroxy-9,11-octadecadienoic acid isolated from human arterial cholesteryl ester fractions was racemic, and allowed postulation of a mechanism for its formation. The minor products formed during the preparation of drimanoyl derivatives (tentatively

assigned to 9-epidrimanoyl derivatives) were always eluted much earlier on gas chromatography and hence did not interfere with any correlations.

In the present work a comparison was made of the effectiveness of several previously used resolving agents for the resolution of (±)-menthol (Table11). Of the derivatives formed only the (R)-1-phenylethylcarbamates of (±)-menthol showed any resolution on 1% SE-30, and the separation was considerably inferior to those obtained with the terpenoid acids.

This present study is limited by the number of alcohols examined, but it differs from most previous ones in that a wide range of types of secondary alcohol have been considered. Since there is no simple structural relationship between most of the alcohols studied it is very difficult to draw any conclusions about the mechanism involved in the separation. However, the few comparisons which have been made between these derivatives and those formed with previously used gas chromatographic resolving agents indicate, in many instances, a considerable improvement in resolution with the new reagents. This effect is probably due to the presence of a rigid cyclic system, incorporating a chiral centre directly linked to the asymmetric carbinol *via* an ester bond. Further improvements in the resolution observed with the chrysanthemoyl derivatives could be ascribed either to the involvement of the two centres of electron density in the isobutenylcyclopropane system or simply to the lower molecular weight of the derivatives

allowing the use of lower chromatographic temperatures.

Six distinct types of secondary alcohol have been examined and resolutions were achieved for every enantiomeric pair with at least one, and in many cases both derivatives, with the exception of the steroidal alcohols. These present special problems because the high molecular weights which are involved require the use of temperatures above the optimum for gas chromatographic resolution.

In conclusion (S)-(+)-drimanoic acid and (R)-(+)-trans-chrysanthemic acid appear to be useful and versatile reagents for the gas chromatographic resolution of secondary alcohols.

Amines and amino acids

Excellent resolutions were obtained with drimanoic acid for the primary amines α-phenylethylamine and amphetamine. There has been considerable interest in determining the configuration of amphetamine and various analogues by gas chromatography. The most frequently used derivatives have been the N-trifluoroacetyl-(S)-(-)-prolylamides[156,160] which have been found suitable for the quantitative determination of (+)- and (-)-amphetamine. The separation achieved with drimanoic acid would be suitable for semi-quantitative analysis and it could, presumably, be improved by the use of high efficiency capillary columns. A very good resolution was also obtained for the amphetamine N-chrysanthemoyl derivatives. However, under these conditions no separation was observed

for the α-phenylethylamine chrysanthemamides nor either of the terpenoid acyl derivatives of N-methyl-α-phenylethylamine. Improved resolution can, however be achieved by using longer, more efficient chromatographic columns and complete resolution of both (±)-trans and (±)-cis-chrysanthemic acids has been obtained when they are analysed as amides of (+)-α-phenylethylamine on capillary columns.[172] The application of chrysanthemic acid to the resolution of several 1-phenyl or 1-naphthylalkylamines or 1,2-diphenylethylamines has been reported.[357] The derivatives of trans-chrysanthemic acid were separated better than those of cis-chrysanthemic acid, but in general poor resolutions have been noted for the 1-phenyl or 1-naphthylalkylamine derivatives. The proximity of the phenyl ring to the amide centre seems, therefore, to have an adverse effect on the resolution. This is contrary to previous results for other diastereomeric derivatives.[141] Extensive studies with α-phenylethylamine on optically active stationary phases have indicated[237,238,247] that the presence of the bulky phenyl group at the asymmetric centre in close proximity to hydrogen bonding involving the amino group gave excellent resolution. The resolution of N-TFA-(S)-prolylamides[141] and more recently N-perfluoroacyl-(L)-leucyl-, -(L)-alanyl- and -(L)-valyl-derivatives[358] have all indicated a decrease in resolution when the asymmetric centre and phenyl ring are separated by a methylene group. Similar results were obtained with the phenylalkylamides of substituted phenyl-

acetic acids (Sec. 3.2.1.). The excellent result obtained for (±)-methamphetamine-N-chrysanthemamide also indicates that hydrogen bonding involving an amide proton is not an important factor in this case. It would seem likely that in the case of the chrysanthemoyl derivatives electronic effects involving the isobutenylcyclopropane system are important, and interaction of this moiety with another electron dense region at close proximity can adversely affect the resolution.

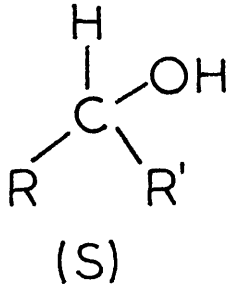
Only four amino acid methyl esters were studied and the best resolutions were obtained for the chrysanthemoyl derivatives of valine methyl ester and phenylalanine methyl ester ($\Delta I = 15$).

Proline methyl ester was expected to be resolved well since the chirality is embodied in a ring system. This is the basis of the choice of N-trifluoroacetyl-(S)-(-)-prolyl chloride as a gas chromatographic resolving agent. Separations were observed with both drimanoic and chrysanthemic acids.

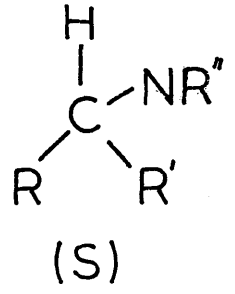
Phenylglycine methyl ester presents an interesting case. No separation was observed for either the N-drimanoyl or N-chrysanthemoyl derivatives. The analogous diastereomeric O-drimanoyl and O-chrysanthemoyl derivatives of mandelic acid methyl ester, however, were easily distinguished. This result is contrary to previous findings that diastereomeric amides are better separated by GLC than analogous esters (e.g. ref. 221). Again this suggests that hydrogen bonding of an amido-proton is not

an important factor for the resolution of derivatives of these terpenoid acids. Since the homologous phenylalanine methyl ester showed good separations with both the drimanoyl and chrysanthemoyl derivatives the distance of the phenyl ring from the asymmetric centre is again seen to be critical.

Although the mechanism involved in these resolutions appears to be complicated the order of elution of diastereomers could be easily predicted on the basis of bulkiness chirality.[225] For a representative alcohol or amine :



(S)



(S)

the order of elution of the (S*)-drimanoyl derivatives was (S*S) before (S*R), and of the (R*)-chrysanthemoyl derivatives, (R*S) before (R*R), as long as R' was bulkier than R. When R became bulkier than R' a reversal of elution order was noted.

The degree of success achieved with these reagents confirms the expectations of the value of chiral reagents containing conformationally immobile ring systems. The use of drimanoic acid is to some extent restricted by the large molecular weight increment produced on derivatisation.

The high temperatures which are required to elute high molecular weight compounds e.g. derivatives of steroid alcohols, are above the optimum for good column efficiency and hence resolution of these diastereomers is more difficult.

The efficiency of a packed column can be given by[359]

$$H = 4\lambda r + 2\gamma Dg/u + \frac{8Kd^2}{\pi^2(1+K)^2 D_l} u$$

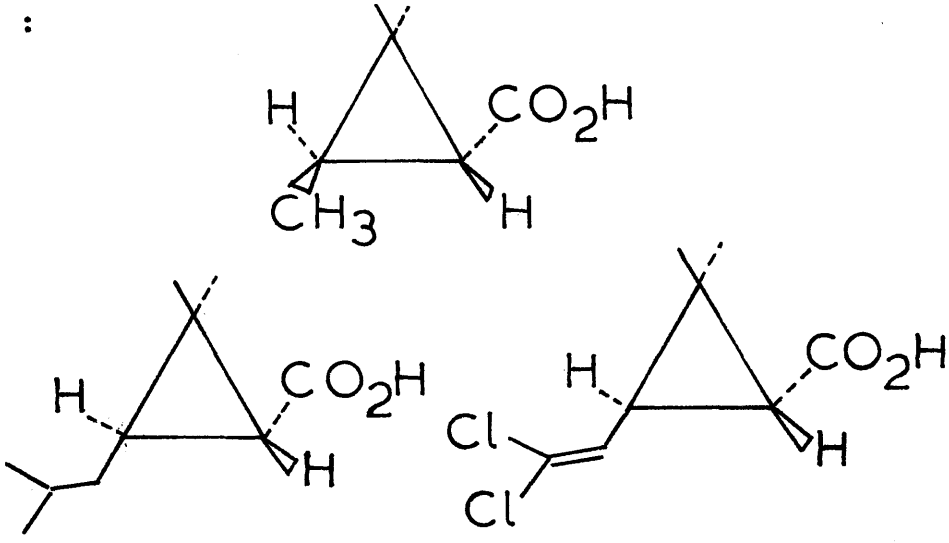
Where H is the height equivalent to a theoretical plate; λ and γ are constants of the order of unity; r = average particle radius; d = average liquid film thickness; Dg and D_l = diffusion coefficients of vapour being chromatographed in the gas and liquid phases; u = true linear gas velocity in the column; K is the column capacity coefficient.

This equation can be simplified to :

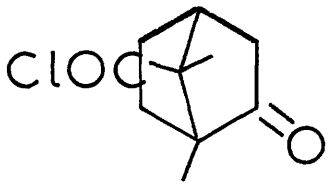
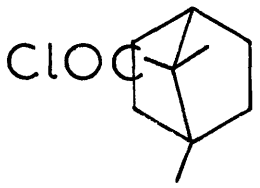
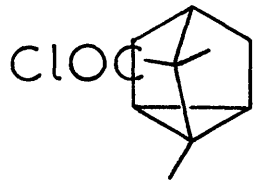
$$H = A + B/u + Cu$$

Temperature is the most complex of the variables affecting column efficiency.[360] Increase of temperature increases D_l and Dg and decreases K. The variation of D_l and Dg with temperature are not great provided that the liquid phase is not highly viscous. Their effects on column efficiency will tend to cancel out since one appears in the numerator of B and the other in the denominator of C. The major effect of temperature is through K. Any decrease in K tends to increase C and therefore increase of temperature will lower column efficiency.

On this basis resolving agents analogous to chrysanthemic acid probably hold more promise for the resolution of high molecular weight compounds. A partial peak resolution was in fact noted for (±)-estradiol 3-methyl ether as its chrysanthemoyl derivative. Natural chrysanthemic, pyrethric, and chrysanthemum-dicarboxylic acid[356,361] are readily accessible sources of a variety of related acids which would retain chirality. The examination of chrysanthemic acid analogues of lower molecular weight or higher polarity is being undertaken at present by a colleague. Examples of some of the acids are :



Other monoterpenoid reagents may also be readily obtained e.g. (-)-cis-myrtanic acid, camphanecarboxylic acids. Three camphor related compounds : (+)-isoketopinyl chloride(I), (-)-dihydroteresantalinyl chloride(II) and (-)-teresantalinyl chloride(III), have recently been successfully

(I) (II) (III)

applied to the gas chromatographic resolution of racemic amino acids.[212] The more rigid structure of the (-)-teresantalinyl moiety, possessing a cyclopropane ring, was reflected in superior resolutions with this reagent.

4.1.2. Substituted phenylacetic acids

The best amine resolutions observed in this series were for the derivatives of α-phenylethylamine, where a retention index difference of 30 or more was achieved with every derivative. The lower resolution factors obtained with N-methyl-α-phenylethylamine and amphetamine derivatives indicate that the resolution mechanism involves hydrogen bonding of the proton on the amine and that the distance of the aromatic ring from the amide bond is important. In this case, unlike the terpenoid derivatives, the derivatives in which the aromatic ring is nearer to the amide bond were better separated.

Although thermodynamic parameters were not calculated during this study the retention index differences which were measured are related to the separation factors, α, as shown below.

In isothermal gas chromatography the retention index of any substance is found by logarithmic interpolation between the two relevant n-paraffins.[362]

$$I = 100 \frac{\log r_{(subst)} - \log r_{(n-Cz)}}{\log r_{(n-C_{z+1})} - \log r_{(n-C_z)}} + 100\,z$$

$$[r_{(n-C_z)} \leq r_{(subst)} \leq r_{(n-C_{z+1})}]$$

Where r = adjusted retention time, net retention volume; $n\text{-}C_z$ = n-paraffin with z carbon atoms, and $n\text{-}C_{z+1}$ = n-paraffin with (z+1) carbon atoms.

Therefore, for two compounds A and B,

$$\Delta I = 100 \frac{\log r_B - \log r_A}{\log r_{(n-C_{z+1})} - \log r_{(n-C_z)}}$$

$$= 100 \frac{\log r_B/r_A}{\log r_{(n-C_{z+1})} - \log r_{(n-C_z)}}$$

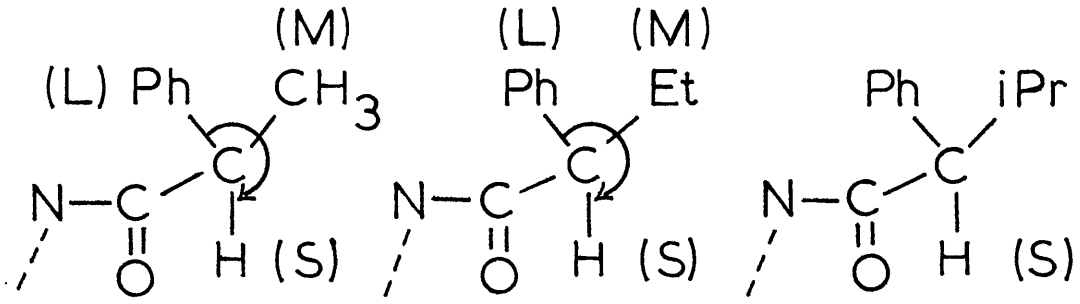
$$= 100 \frac{\log \alpha}{\log r_{(n-C_{z+1})} - \log r_{(n-C_z)}}$$

Since α can be directly related to the free energy differences for the diastereomers,

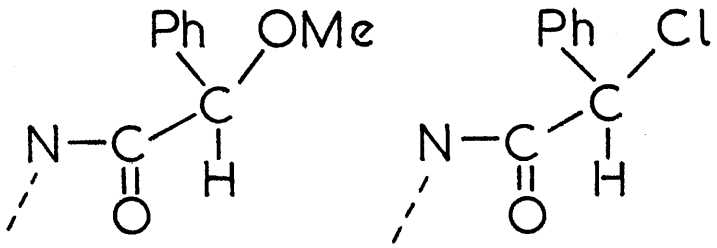
$$-\Delta(\Delta G^o) = RT\ln\alpha$$

(see Sec. 1.2. p. 24)

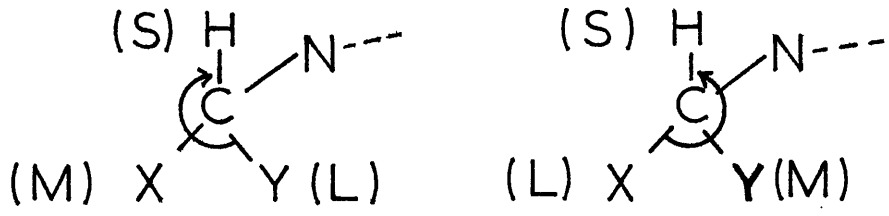
the trends observed in the retention index differences quantitatively reflect the variation of free energy differences for the pairs of diastereomers. $-\Delta(\Delta G^o)$ can be expected to increase as the substituent on the α-carbon of the resolving agent increases in size (Me < Et < ^{i}Pr), since the separations increase in



(R)-Phenylpropionyl- (R)-Phenylbutyryl- (R)-3-Methyl-2-phenylbutyryl-



(R)-α-Methoxyphenylacetyl- (R)-α-Chlorophenylacetyl-



(S)-amine

Fig. 68. Space projection of the (R)-substituted phenylacetic acids and an appropriate (S)-amine. When Y was bulkier than X the phenylpropionamides and phenylbutyramides were eluted in the order (RR) < (RS); the reverse order was observed when X was bulkier than Y. The α-chlorophenylacetamides behaved in the opposite manner.

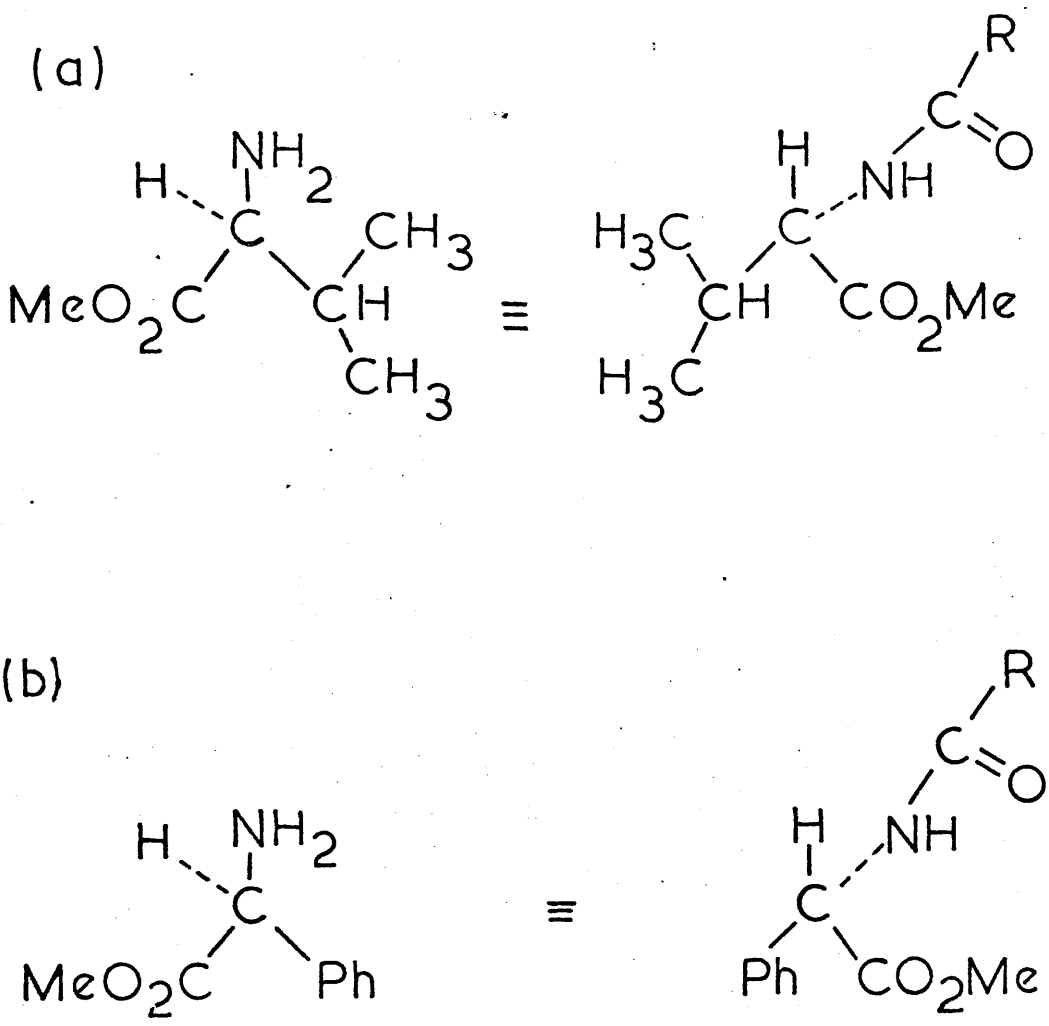


Fig. 69. The apparently anomolous behaviour of the amino acids.

a) Space projection of (S)-valine methyl ester. The observed order of elution of these amides suggests that the carbomethoxyl group is bulkier than the isopropyl group when R is phenylpropionyl or phenylbutyryl, isosteric with the isopropropyl group when R is 3-methyl-2-phenylbutyryl, and smaller than the isopropyl group when R is drimanoyl or chrysanthemoyl.

b) Space projection for (S)-phenylglycine methyl ester. The observed elution order of the phenylpropionyl- or phenylbutyryl-derivatives of phenylglycine methyl ester can be explained by the phenyl group being bulkier than the carbomethoxyl group.

this order. The introduction of a more polar substituent, however, (-OMe or -Cl) produces electronic effects which makes interpretation of the results more difficult. Introduction of polar substituents into the aromatic ring in p-chloroamphetamine and 3-methoxy-4,5-methylene-dioxyamphetamine increased the resolution (relative to amphetamine). This could either be an electronic effect or simply a result of the increased bulkiness.

The elution order of these diastereomers can also be predicted by Feibush's rule.[225] When R' is bulkier than R (Fig. 68) the (RR)/(SS) diastereomer is eluted before the (RS)/(SR) diastereomer as observed for the series of phenylbutyramides and phenylpropionamides. This order should remain the same for the isopropylphenyl-acetamides provided that the isopropyl group is not bulkier than the phenyl substituent. The same elution order has been observed for all three α-alkyl substituted phenylacetyl derivatives of (±)-methyl amphetamine[166] which would suggest that the isopropyl group does not appear bulkier than the phenyl ring.

When the amino acid derivatives are considered the reversal of elution order observed with the terpenoid derivatives and explained on the basis of R having a greater effective bulk than R' was not apparent, except in the case of phenylglycine methyl ester. In these derivatives the carbomethoxyl group is effectively larger than the isopropyl group in valine and the ring in proline, but not the phenyl group in phenylglycine (Fig. 69). Since the relative effective bulk of two groups is not

expected to change when considered under similar conditions, this interpretation of the results may be oversimplified. It is interesting to note that in the case of valine methyl ester the resolution achieved decreases as the bulk of the α-group in the reagent increases (i.e. Me > Et > ^{i}Pr). This might suggest that there are two opposing resolution mechanisms in operation which effectively cancel each other out when the α-isopropylphenylacetyl derivative is used, since no separation is obtained. It would be interesting to examine the situation with α-t-butylphenylacetyl derivatives to ascertain whether or not a reversal of elution order is observed.

The α-methoxyphenylacetyl derivatives should follow the same elution order as the other derivatives as long as the phenyl group is bulkier than the methoxyl group and no opposing polarity effects occur. This elution order has still to be ascertained. The α-chlorophenylacetamides were expected to follow the same elution order since the phenyl ring was thought to be bulkier than the chlorine atom; however, examination of the experimental results showed the opposite order. If these observed elution orders are to agree with Feibush's rule then the chlorine must appear bulkier than the phenyl ring, but the polarity of this group may result in a different resolution mechanism.

Assignments of configuration from gas chromatographic data may be made within a series of related compounds. On the basis of the known order of elution of amphetamine

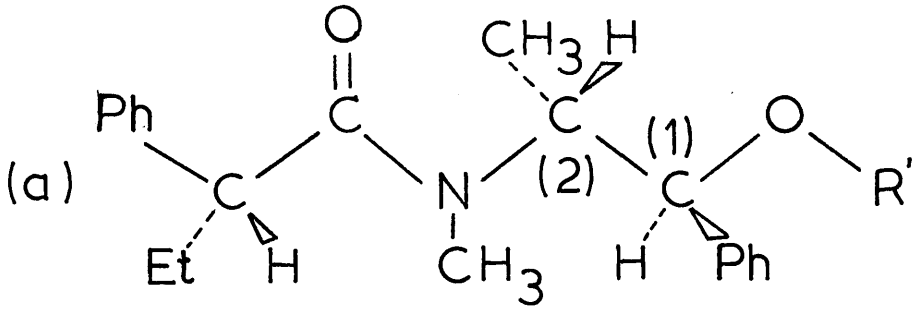
and methamphetamine phenylbutyramides as (RR) before (RS), the peak of shorter retention time for the diastereomeric (R)-phenylbutyryl derivatives of p-chloroamphetamine or 3-methoxy-4,5-methylenedioxyamphetamine can be assigned to the (R)-enantiomer.

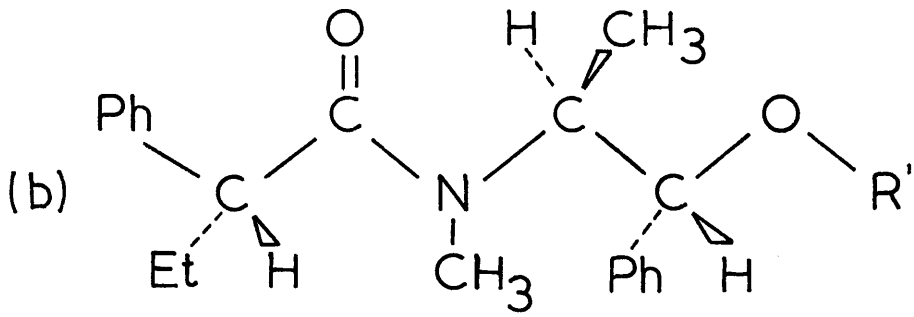
Contrary to the results obtained with the terpenoid reagents, good resolutions were achieved for phenylglycine methyl ester with the arylalkanoic acids, but phenylalanine methyl ester proved to be unresolved in most cases. A good resolution was obtained, however, for the 3-methyl-2-phenylbutyryl derivative of phenylalanine methyl ester on OV-17 phase, reflecting the enhanced diastereomeric differences achieved when the ethyl group in phenylbutyryl derivatives is replaced by an isopropyl group. Since no separation of these diastereomers was observed on SE-30 phase the increased polarity of the stationary phase must also play a role in the separation mechanism. Replacement of the α-alkyl group by an α-methoxyl group produced a reagent which adequately resolved both arylamino acid methyl esters on SE-30 ($\Delta I = 35$ for phenylglycine methyl ester and $\Delta I = 15$ for phenylalanine methyl ester) and OV-17 phase ($\Delta I = 40$ and $\Delta I = 20$ respectively).

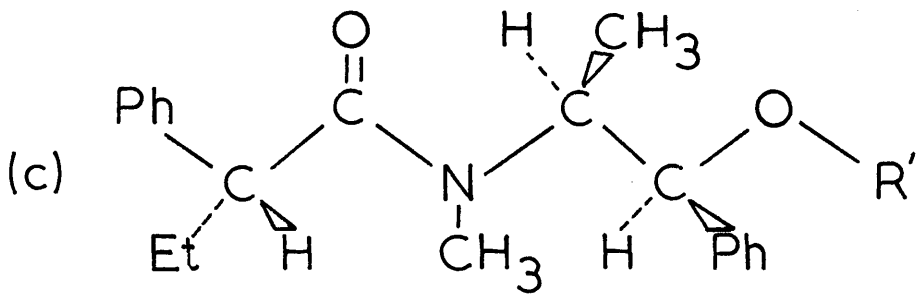
Norvaline methyl ester presented problems with all the reagents, although the (branched) isomeric valine methyl ester was sufficiently differentiated with α-phenylpropionic, α-phenylbutyric, and α-chlorophenylacetic acids. Once again the α-methoxyphenylacetic acid

proved a useful reagent in a difficult situation and a separation of 20 index units was obtained on OV-17 for the N-acyl norvaline methyl esters.

Compounds containing more than one functional group present particular problems. The simplest case is the amino acid where the acid function can be methylated followed by derivatisation of the amino function with a chiral reagent. Alternatively a diastereomeric derivative can be obtained by esterification of the acid function with a chiral alcohol and the polar amino group may be trifluoroacetylated or trimethylsilylated. The situation becomes more difficult when both an amino function and an hydroxyl function are present since they react with the same reagents. The selective derivatisation of multifunctional amino acids has been accomplished in several ways :[180,201,363] O-TMS, N-(N-TFA-prolyl)-amino acid esters have been prepared by two approaches.[201,363] β-Hydroxy-α-amino acid methyl esters have been examined by trimethylsilylation with hexamethyldisilazane (HMDS) before reaction with N-TFA-(S)-prolylchloride.[201] Alternatively the greater reactivity of the amino function has been used to form the N-TFA-(S)-prolyl derivative before trimethylsilylation of the phenolic amino acid n-butyl esters.[363] The diastereomeric derivatives prepared by esterification of the amino acid with 2-butanol and trifluoroacetylation of the hydroxyl and amino functions have been observed not to yield good chromatographic peaks.[180] It has been found, however,







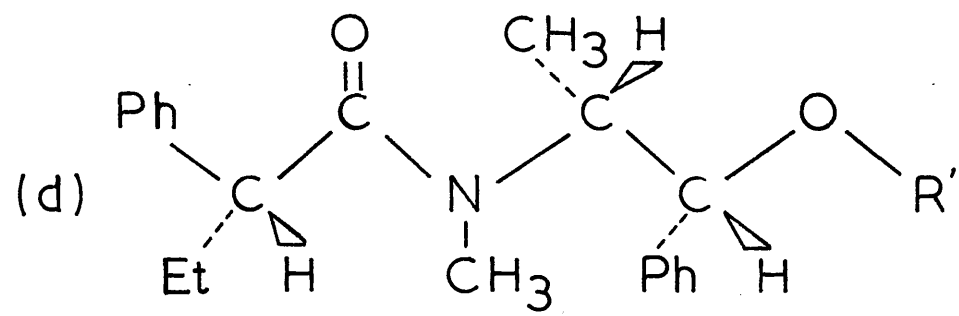


Fig. 70. Suggested "preferred" conformation for the N-(R)-phenylbutyryl derivative of ephedrine O-TMS :
a) (2R, 1S)-(+)-erythro; b) (2S, 1R)-(-)-erythro;
c) (2S, 1S)-(+)-threo; d) (2R, 1R)-(-)-threo
[R' = $Si(CH_3)_3$].

that leaving the sample in methanol for 24h at room temperature quantitatively removed the trifluoroacetate group from the hydroxyl function which could then be acetylated, resulting in a considerable improvement in chromatographic performance.

In the present work the amino alcohol ephedrine was examined. Previous workers have prepared the N-TFA-(S)-prolylamides by reacting the amino alcohol with one equivalent of N-TFA-(S)-prolylchloride.[368] A similar approach was successfully employed in the present study using (R)-(-)-phenylbutyryl chloride or (R)-(-)-phenylbutyric anhydride with a short reaction time. Increased selectivity was expected with the imidazolide of phenylbutyric acid since carboxylic acid imidazolides had been reported[340] to react readily with amines, but only very slowly with alcohols, at room temperature. Derivatisation was complete after ½h at room temperature and no O-phenylbutyryl derivative could be detected by TLC or GLC.

Only ephedrine (Fig. 70 -erythro, R=H) was resolved as its (R)-(-)-phenylbutyramide on GLC. The observed elution order was (+)-erythro (1S, 2R) before (-)-erythro (1R, 2S). Considering the amine chiral centre (2) alone this shows the same elution order as that observed with the (R)-(-)-phenylbutyramides of the phenylalkylamines, i.e. (RR) before (RS). A similar result has been found for the diastereomeric ephedrines as the N-TFA-(S)-prolyl derivatives.[156]

After trimethylsilylation of the free hydroxyl group

resolutions were also observed for the (R)-(-)-phenylbutyryl derivatives of ψ-ephedrine (Fig. 70 -threo, R = TMS) and nor-ψ-ephedrine. No separation was observed for the derivatives of nor-ephedrine.

The bulkiness of the trimethylsilyl ether group, and the removal of the hydroxylic proton for hydrogen bonding would change the preferred conformations of the diastereomers, producing different associations with the stationary phase and hence the observed resolutions. The (R)-(-)-phenylbutyryl derivatives of the ψ-ephedrines (O-TMS) were eluted in the order (+)-threo (1S, 2S) before (-)-threo (1R, 2R), which is the opposite to that found for the N-TFA-(S)-prolyl derivatives.[156] These results can, however, be explained on the basis of differential accessibility to the central functional group. If the preferred conformation is assumed to be one in which the bulky trimethylsilyl ether group is as far removed from the amide centre as possible (Fig. 70) and the molecule is observed along the plane through the amide bond, the (-)-threo derivative has all the bulky groups, (Et, CH_3 Ph) on one side of the plane and all the hydrogen atoms on the other side. The (+)-threo derivative will be less accessible to the stationary phase since there are bulky groups on both sides. The (-)-threo-(R)-(-)-phenylbutyramide would, therefore, be expected to interact more strongly with the stationary phase and be retained relative to the (+)-threo-derivative. This is the observed elution order. A similar argument applies to the ephedrine case (erythro) although in this situation

neither diastereomer has all bulky groups on the one side of the plane. In this case the diastereomer which has the two bulkiest groups (Et and Ph) on the same side of the plane i.e. (-)-erythro, allows the greatest accessibility to the phase and is retained relative to the (+)-erythro-derivative. The elution order cannot, therefore, be predicted on the basis of the configuration at the amino-chiral centre[156] alone, and the spatial arrangement of the whole molecule must be considered.

This series of substituted phenylacetic acids are suitable for the analytical gas chromatographic resolution of many enantiomeric amino-compounds. All the compounds studied during this survey were effectively resolved by at least one of the reagents, and in many instances complete resolution was obtained. This renders these derivatives useful for the determination of the enantiomeric composition of amines derived from biological extracts. Only the α-chlorophenylacetyl chloride proved unsatisfactory because of its ready racemisation during acylation.

Several extensions of this project can be proposed. Mandelic acid would be a suitable substrate for the preparation of chiral acids incorporating various bulky or polar substituents in the α-position e.g. α-acetoxy-phenylacetic acid or α-trimethylsilyloxy-phenylacetic acid. It would also be interesting to examine the resolutions obtained using the analogous amino acid-phenylglycine - as resolving agent. In this way an armoury of reagents can be built up, thus increasing the versatility of the

method.

As a corollary, several substituted phenylpropionic acids were successfully resolved on GLC as the amides with (R)-(+)-α-phenylethylamine (Appendix). Use of N,N'-carbonyldiimidazole allowed the formation of the (R)-(+)-α-phenylethylamide of a hydroxy-acid without any interference of the hydroxyl group, during work on the hydroxylated metabolites of 'Brufen' (Sec. 3.3.4.). Work is also in progress on the selective derivatisation of one carboxyl group in diastereomeric dicarboxylic acids. It is hoped to extend this work to preparative scale diastereomer resolution either by TLC or liquid chromatography.

4.2. Mass spectral characteristics of diastereomeric esters and amides

The major mass spectral fragmentations of the diastereomeric esters and amides are discussed fully in Section 3. The main features of interest are the high abundance of molecular ions and characteristic fragmentations which would be of use in the identification of unknown compounds, particularly in the amide series.

No significant differences were noted between the spectra of diastereomeric compounds.

The use of chiral derivatives such as these in association with mass fragmentography could lead to the determination of the enantiomeric composition of chiral compounds occurring at very low levels in biological fluids.

4.3. The metabolism of ibuprofen

Ibuprofen [(±)-2-(4-isobutylphenyl)propionic acid] has been shown[341,364] to be a potent orally active anti-inflammatory antipyretic and analgesic agent. It is in widespread use for long term maintenance therapy at a dosage of about 500 mg/day. When drugs are administered in relatively high dosage over a long term it is important to establish the contribution of any metabolites to the pharmacological effects. It is of particular importance when racemic drugs are administered, to determine whether or not both enantiomeric forms have equal biological activity.

With these aims in view, gas chromatographic methods for determining the urinary metabolites of ibuprofen were devised.

4.3.1. General aspects of metabolism

Four urinary metabolites were observed and identified as the oxidation products of the isobutyl side-chain. The three possible isomeric side-chain hydroxylated metabolites were identified and the fourth metabolite was a dicarboxylic acid obtained by further oxidation of the primary alcohol. This pattern is qualitatively similar to the one which had been observed[365] in the metabolism of the hypoglycaemic agent, 2-*p*-methoxybenzene sulphonamido-5-isobutyl-1,3,4-thiadiazole. Further metabolism consisted of conjugation of ibuprofen

and its metabolites.

The three isomeric hydroxy derivatives were easily distinguished by GLC as their methyl esters or methyl ester TMS ethers. The primary hydroxylated metabolite (metabolite 3), however, was indistinguishable from the dicarboxylic acid (metabolite 2) as their methyl esters, but trimethylsilylation of the alcohol function produced two well separated peaks.

The mass spectra obtained from the derivatives of ibuprofen and its metabolites were characterised by simple cleavages which aided structural elucidation. Unfortunately, the molecular ions obtained from the hydroxylated metabolites were of relatively low abundance but this did not hinder identification. The preparation of perdeuterated derivatives and the use of simple chemical transformations helped to confirm proposed structures.

Isolation of a few mg of each of the two major metabolites (2 and 4) allowed the use of infrared and NMR spectroscopy to substantiate further the suggested structures. These two metabolites had previously been identified[341,342] in the serum and urine of experimental animals and humans after treatment with ibuprofen.

The two minor metabolites (1 and 3) were previously unidentified. The structure of metabolite 1 was confirmed by synthesis of authentic material from ibuprofen, and complete characterisation was achieved by GLC, MS, IR and NMR spectroscopy. The isolated metabolite was

identical to the synthetic material in all chromatographic and mass spectral properties.

Confirmation of the structure of metabolite 3 was obtained by reduction of the acid function to give a product identical to that obtained by reduction of metabolite 2.

The initial identification of these metabolites was obtained from the analysis of a single 20h urine sample. This indicates the advantage of using GLC and GC-MS for metabolism studies since a great deal of structural information can be obtained without requiring the extraction of large batches of fluid.

4.3.2. Stereochemical aspects of metabolism

The (R)-(+)-α-phenylethylamides of (±)-ibuprofen were completely separated on OV-17 phase. This allowed the determination of the enantiomeric composition of the "unmetabolised" ibuprofen excreted in urine. The excreted acid was found by GLC to be 80% (S)-(+)-2-(4-isobutylphenyl)-propionic acid. A similar enhancement of the (S)-enantiomer was observed with the ibuprofen excreted as conjugated material. This result could indicate stereoselective metabolism of the (R)-(-)-acid or enhanced excretion of the (S)-(+)-acid. Both forms, however, had been reported[341] to have equal biological activity. Preparation of the (R)-(+)-α-phenylethylamides of the metabolised acid and GLC analysis, also showed an excess of the (S)-(+)-form. Metabolite 4 was shown

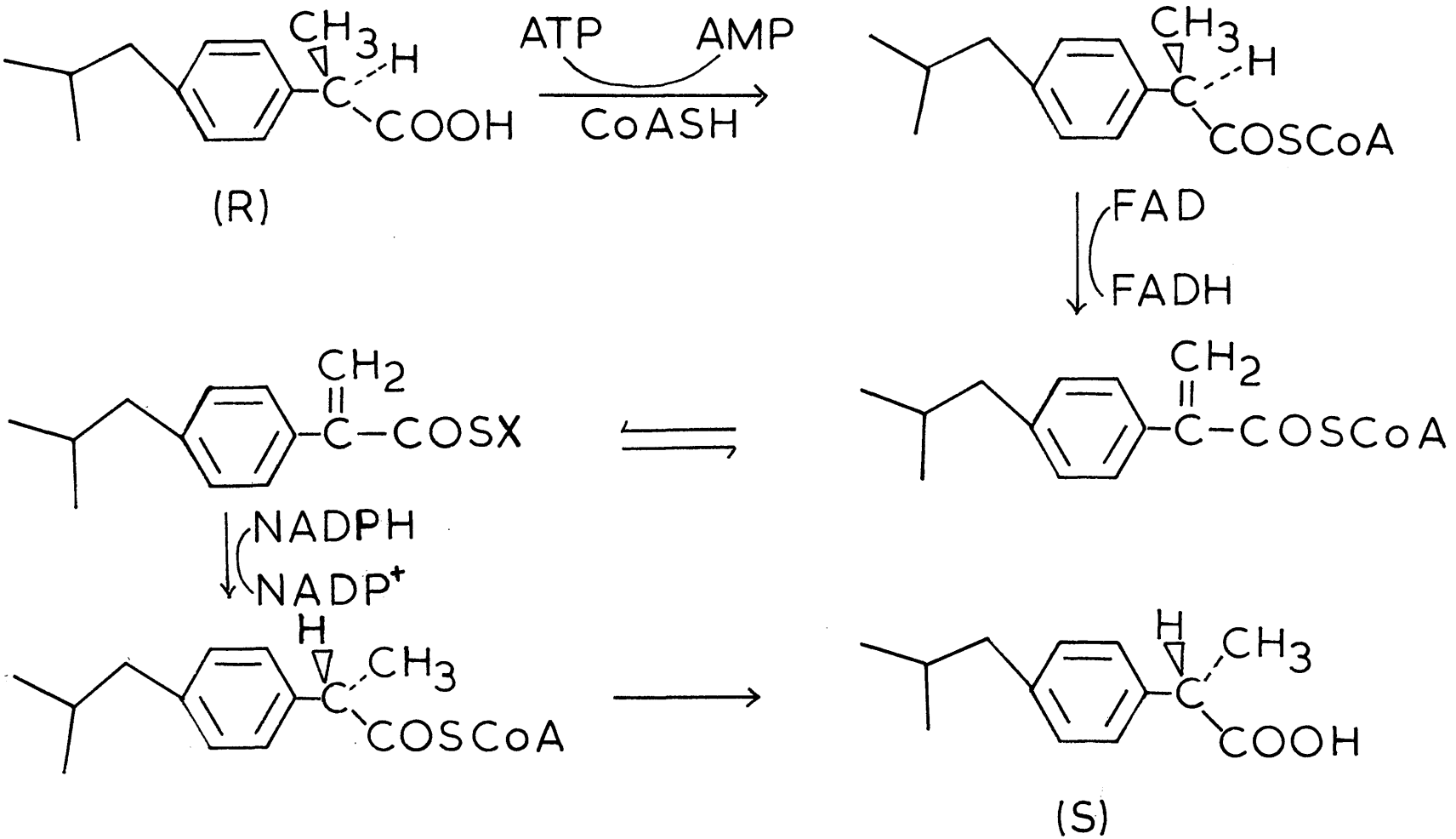


Fig. 71. Mechanism of *in vivo* epimerisation of (R)-ibuprofen proposed by Wechter *et al* (366).

by GLC of the derived amides to contain 64% of the (S)-(+)-enantiomer, while metabolite 1, after oxidation of the alcohol to a ketone to remove the second chiral centre, was found to contain 58% of the (S)-form. In accordance with these results, it had previously been found[341,342] that the two major metabolites of ibuprofen, isolated from human urine, were dextrorotatory, even when (R)-(-)-acid was administered.[342] Metabolite 2 contains a second chiral centre which could have accounted for the change in rotation observed for this metabolite, No such explanation however, could be proposed for metabolite 4, and an inversion of configuration must have occurred. In the present work the isolation of "unmetabolised" acid consisting mainly of the (S)-(+)-enantiomer also suggests that the (R)-(-)-acid is epimerized. Determination of the enantiomeric composition of the metabolites indicates that both forms of the ibuprofen are metabolised, and the enhancement of the (S)-(+)-form could be produced, either by epimerization of the (R)-(-)-acid before metabolism, or by the occurrence of a similar inversion at the chiral centre in the metabolite. Recent work by the Upjohn Co., involving the administration of the resolved forms of ibuprofen and deuterium labelled, (R)-(-)-[$^2H_4$]-ibuprofen to male volunteers, has led to the proposition[366] of the existence of an (R)-aryl-propionic acid isomerase system in man (Fig. 71).

An initial *in vitro* experiment performed with the racemic acid using an enzyme preparation from rat liver

gave no indication of any isomerase activity. Both the isolated "unmetabolised" ibuprofen and metabolite 4 were racemic. Further work is required however, particularly with the resolved forms of the drug, before any conclusions can be drawn about this system.

Metabolite 2 presented a special problem since two chiral centres are involved. The selective derivatisation of one centre at a time in order to determine the enantiomeric composition at both, is under investigation. It does, however, seem likely from the results obtained with metabolites 1 and 4, that the configuration at the original centre will be enhanced in the (S)-form.

Metabolite 1 also contains a second chiral centre but the enantiomeric composition at the alcohol centre was readily determined by GLC of the diastereomeric esters prepared with (R)-(-)-phenylbutyric acid. The second ester peak was larger than the first (57%) indicating that the hydroxylation showed some stereoselectivity. The absolute configuration of the alcohol was determined, using the resolved synthetic sample, by the modified micro-Horeau method,[344,345] and the excreted metabolite was found to comprise 57% the (R)-enantiomer.

This metabolite could be produced by either of two metabolic processes : either direct enzymic hydroxylation, or benzylic addition of oxygen to form a ketone followed by reduction to the alcohol. Studies of the metabolic hydroxylation of alkylbenzenes have shown[68] a preponderance of the (R)-enantiomer in the product, whereas metabolic

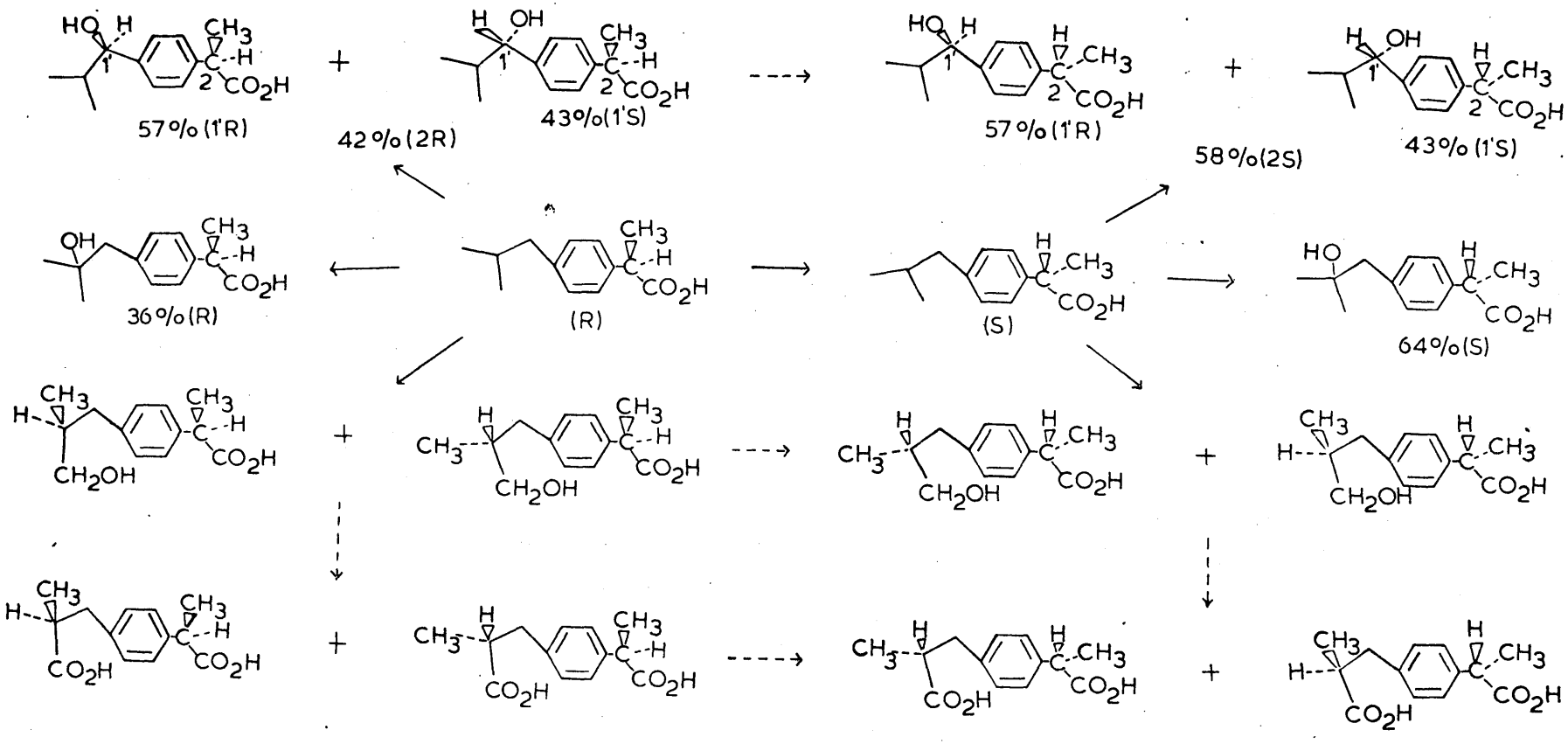


Fig. 72. Proposed metabolic profile for ibuprofen.

reduction of benzylic ketones has been seen to produce[82] a greater proportion of the (S)-enantiomer. It would, therefore, seem likely that metabolite 1 is produced by a direct hydroxylation process which shows some product stereoselectivity.

These results (Fig. 72) further illustrate the convenience of gas-phase methods for the micro-analytical characterisation of enantiomers and present a practical application of the techniques described in sections 3.1. and 3.2.

4.4. The metabolism of fenoprofen

During the work on ibuprofen metabolism one urine sample was obtained from a patient who had received treatment (unknown to us at the time) with fenoprofen, (±)-2-(3-phenoxyphenyl)propionic acid, as well as ibuprofen. Analysis of the acidic urine extract by GLC showed three peaks of longer retention time than the ibuprofen metabolites. The conjugated fraction showed the same peaks. These were identified by GC-MS as fenoprofen methyl ester, a hydroxylated metabolite and a methoxylated metabolite. Only the 4'-hydroxyfenoprofen had been previously identified[349] as a metabolite, as well as the conjugates of fenoprofen and 4'-hydroxyfenoprofen. Since the hydroxyl group is phenolic it is possible that it was partially methylated when the acid was treated with diazomethane. This would explain the presence of the methoxylated derivative as an artefact and not a metabolite. The mass spectrum was consistent with this being the methylated derivative of the hydroxy-compound.

Fenoprofen was fully characterised by GLC and GC-MS as its methyl ester. The structure of the hydroxylated metabolite was determined by GC-MS and confirmed by isolation of purified material followed by NMR spectroscopy. The position of hydroxylation was proved by NMR spectroscopy with the help of a lanthanide shift reagent, $Eu(fod)_3$, to spread out the aromatic region of the spectrum and simplify interpretation.

The pattern of metabolism was in agreement with the one which had already been described.[349] However, although the relative amounts of conjugated and unconjugated material excreted were not accurately measured, the urinary profiles (Fig. 54) indicated a larger portion of unconjugated fenoprofen and 4'-hydroxy-fenoprofen than had previously been suggested (2-5% free; 45% conjugated). Perhaps this difference could be attributed to an effect of the long term treatment with ibuprofen before administration of fenoprofen.

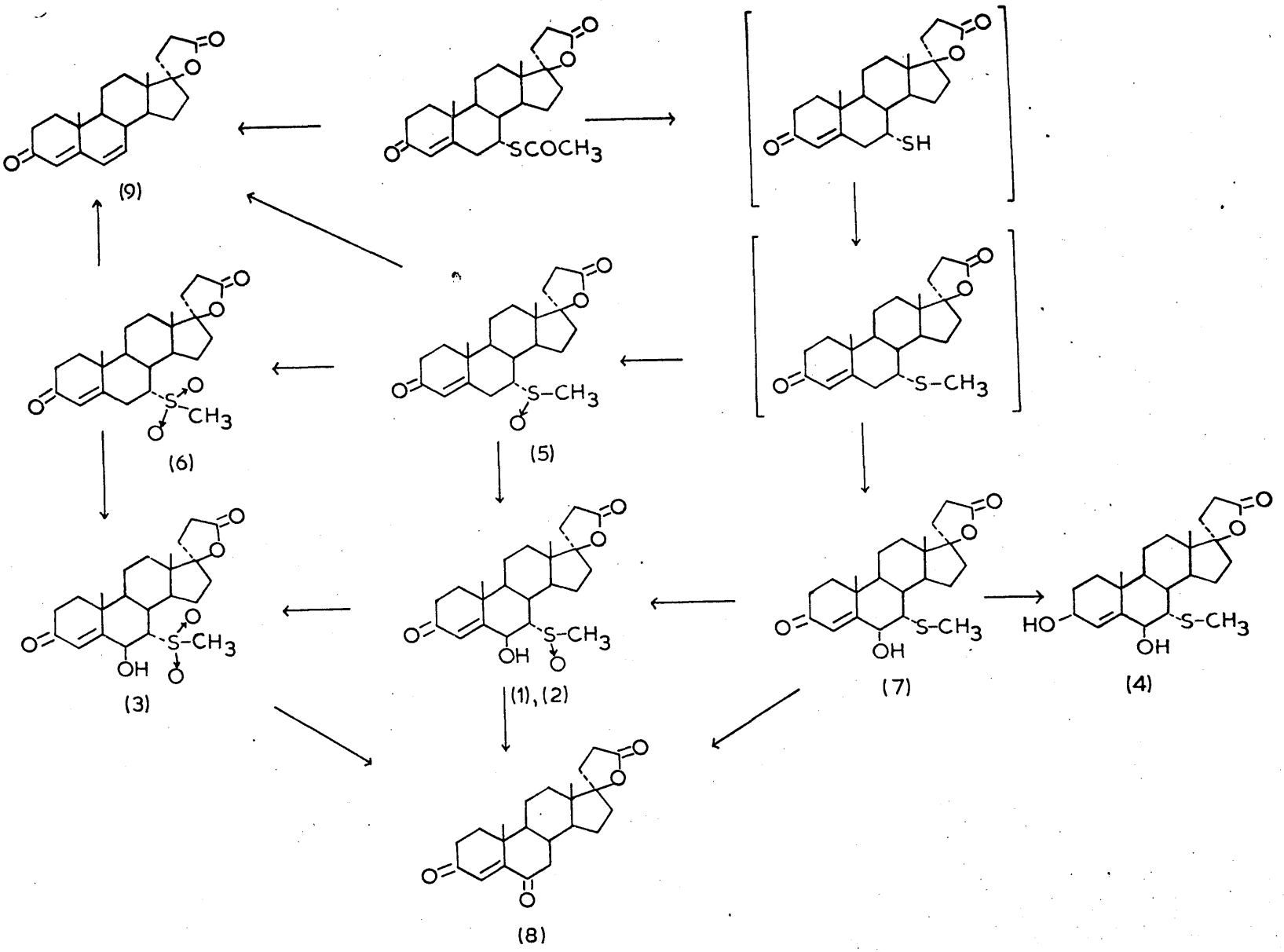


Fig. 73. Proposed metabolic profile for spironolactone.

4.5. Spironolactone metabolism

In previous metabolism studies of spironolactone [3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl)propionic acid γ-lactone], two major urinary metabolites have been identified in the free steroid extracts, viz : aldadiene,[354] [3-(3-oxo-17β-hydroxy-4,6-androstadiene-17α-yl)propionic acid γ-lactone] and 3-(3-oxo-7α-methylsulphinyl-6β,17β-dihydroxy-4-androsten-17α-yl)-propionic acid γ-lactone.[350] The structures of three other minor sulphur-containing metabolites have also been proposed.[350] The present study confirmed the presence of these metabolites in the conjugated fraction of urine extracts and provided additional mass spectral data in accordance with the proposed structures. Four other minor metabolites were tentatively identified on the basis of their chromatographic mobilities (TLC and GLC) and mass spectral characteristics.

A possible metabolic pathway for spironolactone is represented in Fig. 73. The thiol resulting from hydrolysis of the thioacetyl group and the product of its S-methylation were not observed during this study but may presumably be intermediates in the metabolism.

The major sulphur containing metabolite (1) could arise from the proposed methylthiol intermediate either via metabolite (5) by oxidation of the methyl sulphide followed by hydroxylation, or by initial hydroxylation to metabolite (7) followed by sulphoxidation. Evidence was obtained on TLC for the presence of both sulphoxide

epimers suggesting that this oxidation was not stereospecific. Further oxidation of the sulphoxide would produce the methylsulphone (3). Similarly the sulphone (6) could arise by oxidation of the sulphoxide (5).

These latter metabolites were present only in minor amounts and the lability of the sulphur functions rendered isolation of material difficult. The proposed structures are, therefore, only tentative assignments.

The major non-sulphur-containing metabolite was the de-thioacetylated compound, aldadiene (9). This metabolite might also arise from metabolites (5) and (6) by elimination of the sulphur function. These metabolites were easily converted to aldadiene under mild conditions and it seems feasible that a portion of the observed aldadiene would arise as an artefact during work-up.

Similarly metabolite (8) could arise by the elimination of the sulphur functions from metabolites (1), (2), (3) and (7). This elimination occurred thermally and the former three metabolites were chromatographed completely as the Δ^4-3,6-dioxo steroid (8) on GLC. The methylthiol function appeared less labile and this metabolite (7) underwent chromatography intact. Whether any of metabolite (8) occurred metabolically or whether it was present merely as an artefact produced during work-up has not been ascertained.

The minor products of the metabolism of spirono-lactone have been tentatively identified and the proposed

structures fit into a logical metabolic pathway. However, further work using milder isolation procedures is required to obtain full structural identification. Initial observations using high pressure liquid chromatography of spironolactone indicate that it chromatographs with the thioacetate function intact. This could provide a means of differentiating and isolating the various sulphur containing metabolites for further spectroscopic examination.

APPENDIX

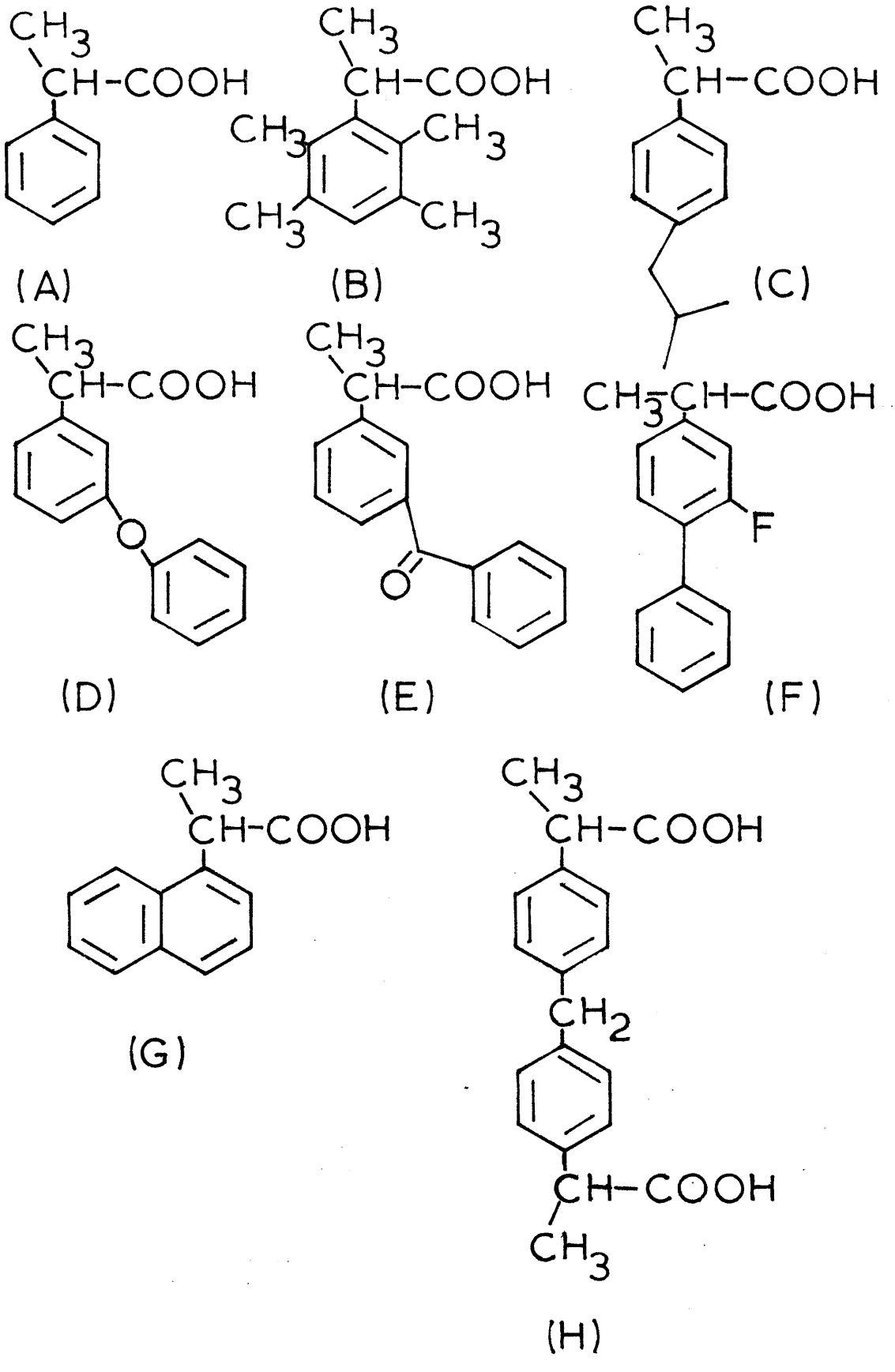


Fig. 74.

Table 29. Gas chromatographic data for the amides of substituted phenylpropionic acids with (R)-(+)-α-phenylethylamine

Acid (see Fig. 74)		1% OV-1 I	1% OV-1 T°C	1% OV-17 I	1% OV-17 T°C
2-Phenylpropionic (A)	(R)	1965	190	2330	220
	(S)	1995		2355	
2-(2,3,5,6-tetramethyl-phenyl)propionic (B)*		2305	210	2690	220
		2350		2740	
2-(4-isobutylphenyl) propionic (C)†	(R)	2310	230	2625	220
	(S)	2340		2660	
2-(3-phenoxyphenyl) propionic (D)‡		2640	230	3185	245
		2675		3215	
2-(3-benzoylphenyl) propionic (E)**		2850	230	3475	245
		2890		3525	
2-([3-fluoro-4-phenyl] phenyl)propionic (F)+		2655	230	3155	255
		2695		3200	
α-(naphthyl) propionic (G)*		2440	210	2900	220
		2455		2920	
2-(4-[4'-(1-carboxy ethyl)-benzyl]phenyl)propionic (H) as mono-methyl ester-mono-amide*		3260	250	3865	255
		3275		3900	

Sources of acids : * Organon Ltd., New House, Lanarkshire

† Boots Ltd., Nottingham

‡ E. Lilly & Co. Ltd., Basingstoke

** May and Baker Ltd., Dagenham, Essex

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