6-n-PROPYLCHROMONE-2-CARBOXYLIC ACID (6-n-PCCA) : A PROBE FOR THE STEREOSELECTIVITY OF ALKYL HYDROXYLATION

by

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A Thesis Submitted
for the Degree of
Doctor of Philosophy
in the University of London

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November 1990

Abstract

6-n-Alkylchromone-2-carboxylic acids are metabolised only by aliphatic hydroxylation. Early studies (Winter 1987) using female Dutch rabbits, showed the 6-n-propyl congener underwent regio- and stereo-selective metabolism resulting in a single metabolite 6-(2'-hydroxypropyl)-chromone-2-carboxylic acid (6-2'-HPCCA), and that this oxidation formed the S-enantiomer preferentially (R/S 24:76). This compound was therefore considered an ideal probe with which to investigate the influences of species and enzyme induction upon the regio- and stereo-selectivity of metabolism.

All chromone compounds to be used in the experiments were synthesised *de novo* including dose compounds, suspected metabolites, and all derivatives of the aforementioned compounds. A new methodology was established for the purification of these compounds from urine using solid phase extraction, and TLC. Final identification was achieved using MS and NMR. Enantiomeric composition was determined by derivatisation with Mosher's reagent ((+)-(S)-α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPA-Cl)) and HPLC analysis.

Initially, [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (6-n-PCCA) was administered to male Wistar rats (500μmol/kg, 20μCi/kg, i.p.) and was found to be oxidised at all 3 positions along the side chain giving metabolites which were excreted unconjugated. These metabolites were :-

6-(1'-hydroxypropyl)chromone-2-carboxylic acid (6-1'-HPCCA)

6-(2'-hydroxypropyl)chromone-2-carboxylic acid (6-2'-HPCCA)
6-(3'-carboxyethyl)chromone-2-carboxylic acid (6-3'-CECCA).
This latter presumably arising from the precursor 6-(3'-hydroxypropyl)chromone-2-carboxylic acid (6-3'-HPCCA).

After further investigation, the enantiomeric composition of the 6-2'-HPCCA metabolite was found to be 90:10 (R/S). An authentic standard of 6-1'-HPCCA was synthesised, purified, its Mosher's derivatives separated by preparative HPLC and its absolute configuration assigned by ^{1}H and ^{19}F NMR. Using these data, analysis of the enantiomeric composition of the 6-1'-HPCCA was found to be 7:93 (R/S) in male rats.

Pretreatment of male rats with phenobarbitone, clofibrate, isosafrole and ß-naphthoflavone (using dose regimes well known to cause well defined patterns of induction of cytochrome P450) altered the excretion of unchanged 6-n-PCCA in all cases, with marked changes to regioselectivity. However the stereoselectivity of the hydroxylation was essentially unaltered with the enantiomeric composition remaining R/S 89:11 and R/S 8:92 for 6-2'-HPCCA and 6-1'-HPCCA respectively, the only notable exception being that of 6-1'-HPCCA formed after pretreatment with ß-naphthoflavone, resulting in an enantiomeric composition of R/S 2:98.

Administration of [2,2'-^{14}C]-6-n-PCCA (500µmol/kg, 20µCi/kg, i.p.) to male guinea pigs revealed that this species was unable to metabolise this compound, whilst administration of 6-n-PCCA (500µmol/kg, i.p.) to a female Dutch rabbit confirmed the excretion of only one metabolite i.e. 6-2'-HPCCA and its near reverse enantiomeric ratio R/S 20:80.

In the rat, the stereoselectivity of this oxidation, considered to be due to the orientation of the hydroxylation, as the racemic 1'-hydroxy and 2'-hydroxy alcohols, when dosed, were excreted as racemates, with no observable stereoselective metabolism or conversion of either to their respective ketones.

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Acknowledgements

I would like to express my sincere thanks to Prof. Robert Smith for allowing me the opportunity to work at St Mary's and to my supervisor, Prof. John Caldwell, for his invaluable guidance and encouragement over the past four years. I would also like to thank all my friends and colleagues in the department, for making this studentship rewarding both intellectually and socially.

I am greatly indebted to Mr David Wilkinson of Fisons plc, Pharmaceutical Division, Loughborough, for his help throughout the past four years, and for the use of the company's NMR and mass spectrometry facilities.

I am also grateful to Dr Peter Grice of the Department of Chemistry, Imperial College, University of London, for his helpful discussions and computing expertise.

Finally, I include special thanks to Debbie for her support and to my family for their never ending patience and love.

This work was supported by a Science and Engineering Research Council CASE award in collaboration with Fisons plc, Pharmaceutical Division.

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Chapter 1

General Introduction

1.1 Introduction

The metabolism and disposition of endogenous and exogenous substrates is governed by interactions between the biological system and the stereochemical and physicochemical properties of the substrate molecule. This implies that isomeric substrates may react differently in a given metabolic process, resulting in observed substrate selectivity, be it substrate regio- or stereo-selectivity, or that the same substrate may give rise to two or more isomeric products at different rates and/or in different amounts, resulting in observed product selectivity, be it product regio- or stereo-selectivity.

These additional complexities in the metabolic processes of isomeric compound may lead to increased experimental difficulties, but often prove to be more informative. In particular they are powerful probes in gaining specific insight into such areas as, enzyme-substrate interactions, enzyme nature and multiplicity, enzymatic mechanisms and active site topology. At the same time they are often relevant to pharmacological and toxicological effects (Testa and Trager 1983).

Structural isomerism

When structural isomers have identical atomic composition and identical atomic connectivity (i.e. the pattern of bonding connections of atoms in a molecule) but differ in the spatial arrangement of their atoms, they are designated stereoisomers (Testa 1990). Any two stereoisomers are enantiomers or diastereomers, depending upon whether or not they are mirror images of each other. Either two stereoisomers are related to each other as objects and

nonsuperimposable mirror images or they are not. In the former case, they are enantiomers, arising from the presence of a chiral centre. Chirality is the necessary and sufficient condition for the existance of enantiomers, i.e. a compound containing a chiral centre exists as enantiomers; achiral compounds cannot exist as enantiomers. Stereoisomers which are not enantiomers are diastereomers. While a molecule can have two and only two enantiomers it may have several diastereomers, but two stereoisomers cannot, at the same time be both enantiomers and diastereomers of each other, and thus the relationships are mutually exclusive.

Substrate stereo- and regio-selectivity

Substrate stereoselectivity involves the differential metabolism of enantiomeric or diastereomeric substrates under identical conditions. This discrimination arises from the chirality of the enzyme, with which diastereomeric complexes are formed on binding. These diastereomeric substrate-enzyme complexes will differ in stability and transition energies, which will ultimately result in differential kinetics of metabolism. It is noteworthy that enantiomers have identical physical and chemical properties, whereas diastereomers differ in every chemical and physical property, and as such resemble regioisomers more than enantiomers. This is particularly relevant in drug disposition (absorption, distribution and excretion) where interactions with chiral environments are not necessarily involved.

When structural isomers have identical atomic composition and differ in atomic connectivity, i.e. their functional groups are adjacent to different atoms, they are designated regioisomers (e.g. 1-propanol and 2-

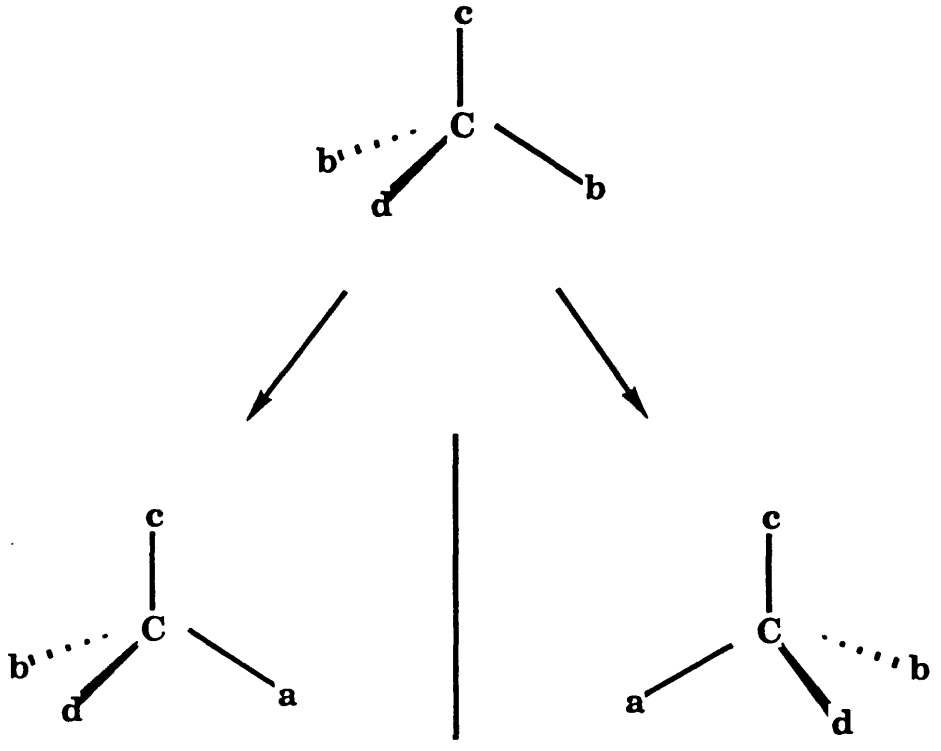
propanol). Thus substrate regioselectivity is defined as the preferential reaction of one regioisomer as compared to the other(s). This discrimination may or may not be of enzymic origin, as regioisomers differ in their physical and chemical properties which may account for selectivity. For example, differences in lipophilicity may result in unequal quantities of individual regioisomers entering an enzyme phase.

Product enantioselectivity

Product stereoselectivity implies the preferential formation of one of a number of possible stereoisomeric metabolites from a given substrate. This in turn means that an element of stereoisomerism is generated during the metabolic reaction. Thus the generation of an element of stereoisomerism results from the transformation of an element of prostereoisomerism, i.e. the substrate contains a prochiral centre or an element of prochirality. For example the prochiral centre (C), when present in an achiral molecule, containing two identical ligands (b) can be represented as Cbbcd (Fig 1.1). When one of these enantiotopic ligands (b) is replaced by another ligand (a), a chiral centre is generated, Cabcd and there are two enantiomeric products. If this prochiral centre is present in a molecule already containing one chiral centre, replacement of one of the diastereotopic ligands (b) with another ligand (a) would generate two chiral diastereoisomeric products. At first sight enantiotopic and diastereotopic groups do not appear to be different but discrimination is fundamental.

Figure 1.1

Formation of a chiral centre from a prochiral molecule.



Enantiotopic groups require a chiral environment for discrimination to occur, which is provided by the enzyme itself and often results from equivocal modes of binding of the substrate to the enzyme. In contrast, diastereotopic groups differ in their properties and reactivities just like regiotopic groups and it is these differences which influence enzyme discrimination.

Product stereoselectivity may occur via two types of prochiral-chiral transformations (Caldwell *et al* 1988); (a) those in which the prochiral centre is itself the site of metabolism, e.g. the carbonyl reduction of

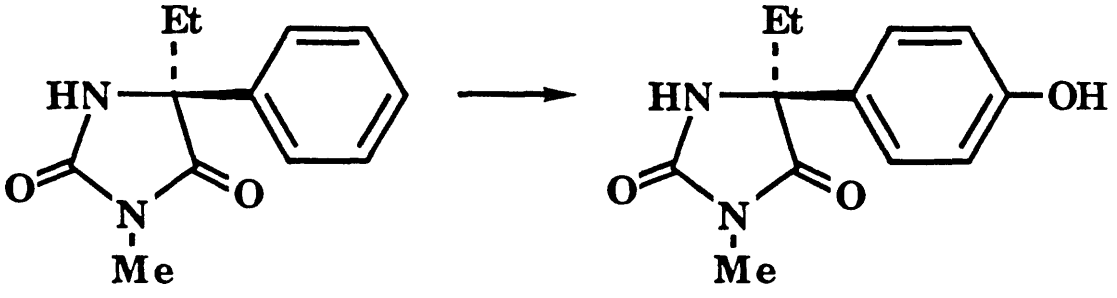
fenofibric acid (Weil *et al* 1986) and the aliphatic hydroxylation of ethylbenzene to 1-phenylethanol (McMahon 1982), (b) where metabolism remote from a prochiral centre renders it chiral e.g. the hydroxylation of diphenylhydantoin.

The metabolism of diphenylhydantoin gives a good example of product enantioselectivity. This is a prochiral compound having two enantiotopic phenyl rings and in man displays highly selective para-hydroxylation of the pro-S ring (Poupaert *et al* 1975). In comparison mephenytoin, a closely related chiral compound, undergoes para-hydroxylation which is highly selective for the (+)-(S)-enantiomer (Kupfer *et al* 1982). Clearly the predominately hydroxylated phenyl groups are topographically equivalent in mephenytoin and diphenylhydantoin (Fig 1.2), which may suggest these compounds share the same mode of binding to a common P450 isozyme, mediating the reactions. However, recent evidence has suggested that the pro-S para-hydroxylation of diphenylhydantoin and the para-hydroxylation of (S) mephenytoin are mediated by distinct isozymes (Kalow 1986 and Fritz *et al* 1987). Interestingly, these results indicate that it is the (S)-mephenytoin and pro-R-diphenylhydantoin which are mediated by the same P450 isozyme, and that the pro-R and pro-S-hydroxylation of diphenylhydantoin is mediated by at least two isozymes.

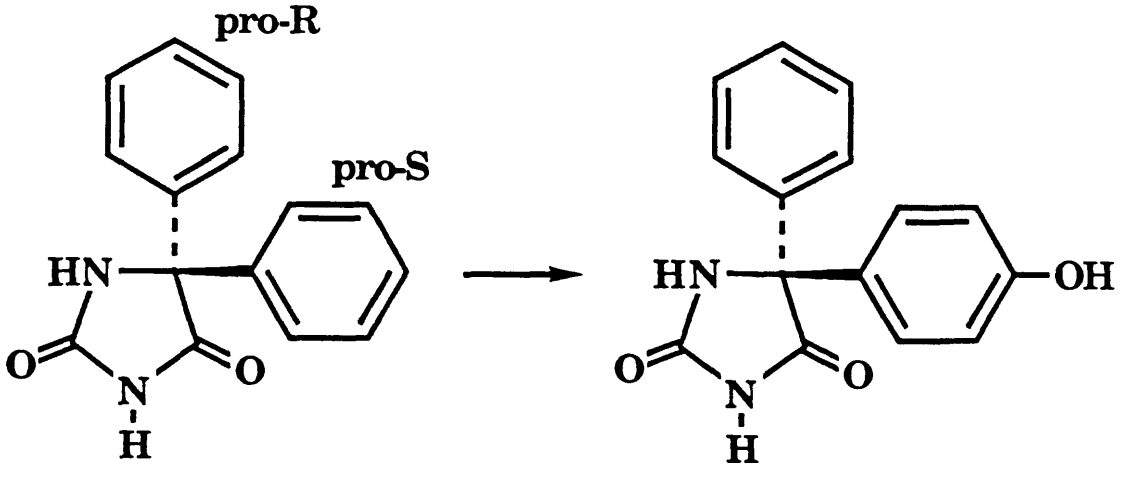
The product enantioselectivity displayed in the hydroxylation of diphenylhydantoin appears dependent on the relative affinities of the prochiral substrate to the two isozymes. It has been suggested (Testa 1988) that each isozyme involved only exhibits a single mode of binding, and that each binding mode brings one of the two enantiomeric phenyl rings into the proximity of the active site.

Figure 1.2

Substrate and product enantioselectivity in the para-hydroxylation of mephenytoin and diphenylhydantoin.



(+)-(S)-mephenytoin



diphenylhydantoin (-)-(S)

Mechanism of product enantioselectivity

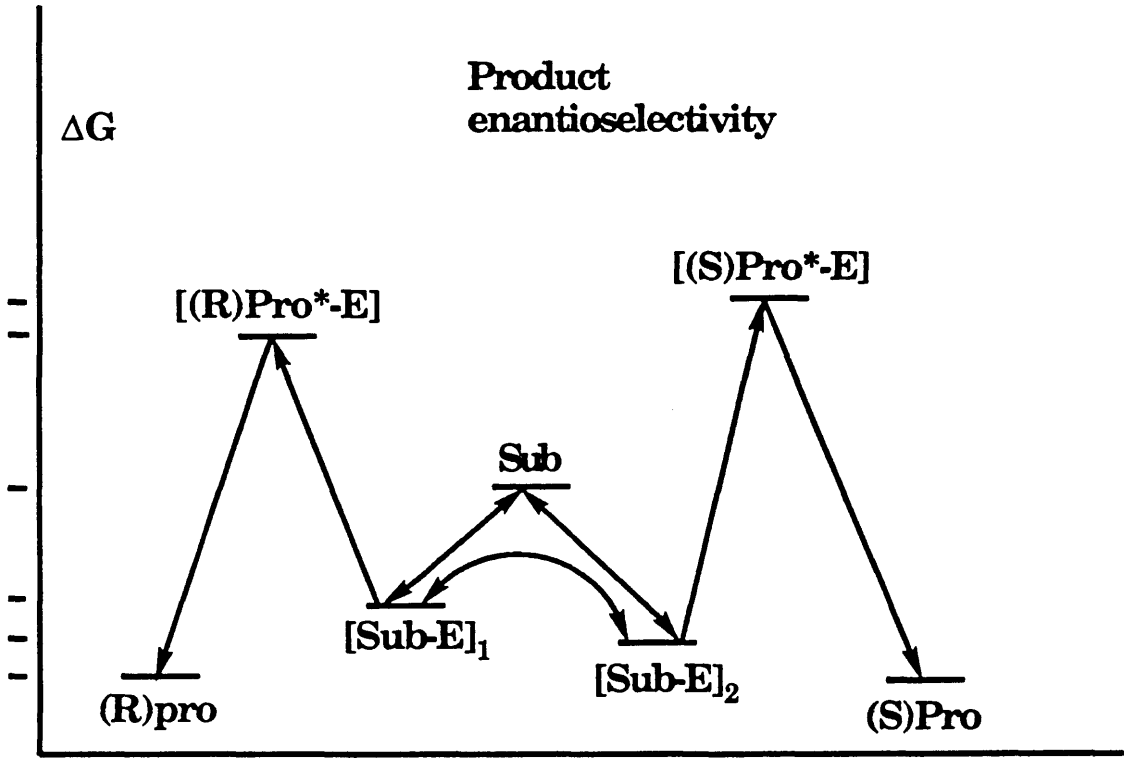
Product enantioselectivity arises from the metabolic discrimination of enantiotopic target groups in a prochiral molecule. This discrimination results from two different modes of binding of the prochiral substrate to the enzyme, which will in turn influence which of the two enantiotopic target groups enter the vicinity of the active site. Thus, the ratio of products may first depend on the relative probability of the two binding modes. In addition, binding of the prochiral substrate to the enzyme will produce two enzyme-substrate complexes which are diastereomeric and hence of different energies. Thus the ratio of products will also depend on relative energy differences between the two diastereomeric transition states, as summarised (Fig 1.3) proposed by Testa 1989. Finally when the enzyme itself exists and acts in multiple forms (isozymes), the isomeric composition of the enzyme mixture involved and the relative affinities of the prochiral substrate for each isozyme are factors determining the ratio of the products (Trager 1989).

Product regioselectivity

Very broadly, product regioselectivity may be defined as metabolism of a given substrate, at a particular site within the substrate molecule, when a number of similar sites are open to metabolism. Regioisomeric metabolites result from an enzymic ability to discriminate between two or more closely related (regiotopic) sites in the substrate molecule and may be produced by the same enzyme (or isozyme) or by two distinct enzymes catalysing identical reactions. Attack upon similar groups or atoms differently positioned in the substrate molecule, eg the ortho,

Figure 1.3

General thermodynamic profile of product enantioselective reactions in drug metabolism.



E : enzyme; Sub : prochiral substrate;
(R)pro and (S)pro : enantiomeric products;
* : transition state.

(Testa 1989)

meta and para positions in a phenyl substituent, will generate regioisomeric metabolites.

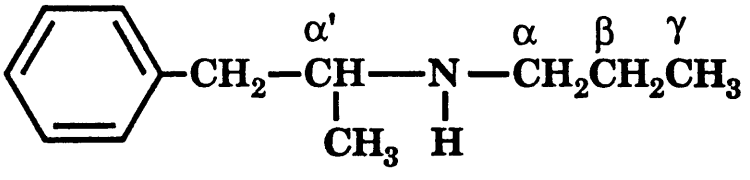
For example diphenylhydantoin is metabolised in man forming its S-para-hydroxyphenyl metabolite (see above), however in the dog the R-meta-hydroxyphenyl product is the major metabolite (Maguire *et al* 1978). This inter species variation in the metabolism of diphenylhydantoin not only shows reversed regioselectiviy (meta vs para) but also reversed stereoselectivity and indicates that in each species different metabolic constraints are involved.

Hydroxylation of molecules containing a hetero atom such as nitrogen and oxygen, exhibit marked regioselectivity towards the carbon atom in the α position, as compared to their regiotopic sites (Cβ ,Cγ..).

Consider the metabolism of N-n-propylamphetamine (Fig 1.4) by rat liver homogenates. 3-7% of all metabolites result from α'-hydroxylation followed by deamination, 20-40% result from N-oxidation and 35-40% and 3-6% of all metabolites result from Cα- and Cβ-hydroxylation respectively. For this reaction there is a 10 fold predominance of Cα-and-Cβ hydroxylation (Coutts *et al* 1976).

Figure 1.4

The structure of N-n-propylamphetamine.



Mechanism of product regioselectivity

Product regioselectivity arises from the enzymatic discrimination between regiotopic groups within a substrate molecule. If an enzyme exhibits a single mode of binding of the substrate to the active site, then the relative transition states of the regiotopic groups will determine the regioselectivity of the reaction, with the proviso that the enzyme is flexible enough to attack the more reactive or labilised sites. If an enzyme exhibits two or more distinct modes of binding, each orientating the substrate in such a manner as to bring one regiotopic group close to the active site, then the relative binding energies of the various modes will determine the regioselectivity of the reaction. If a substrate binds to two or more enzymes or isozymes, each of which catalyse the same reaction but differ in their selectivity for regiotopic sites, then the regioselectivity observed will be determined by the relative concentrations of the enzyme, by the relative enzyme-substrate affinity constants and by any differences in the transition state energies (Testa and Jenner 1980).

1.2 Cytochrome P450

Over the last 30 years there has been intense study of the microsomal multisubstrate mixed function monooxygenase system. The key components of this system are flavoprotein NADPH-cytochrome P450 oxidoreductase and cytochrome P450. A reduced pigment that had an absorption band with a λ_{max} at 450 nm, after binding to carbon monoxide, was first identified by Klingenberg (1958) and Garfinkel (1958). This pigment was further characterised as a haemoprotein in

1961 by Omura and Sato (1961). A key study was the demonstration of the role of this newly characterised cytochrome P450 (Omura and Sato 1964a,1964b) as the terminal oxidase in adrenal mixed function oxidation (Cooper *et al* 1965). Lu and Coon (1968) were able to solubilise liver microsomes with detergent, separate the fractions containing P450, NADPH-cytochrome P450 oxidoreductase and phospholipid and reconstitute catalytic activity by recombining the fractions. In the mid 1960's the idea that more than one form of liver microsomal P450 might exist began to find support and by the mid 1970's, several P450 forms had been isolated. The use of purified P450 and the reconstituted system revealed that individual forms of P450 can exhibit either highly specific or less specific overlapping substrate positional or stereospecificities.

P450's are found in prokaryotes and eukaryotes, in plants as well as animals. Most animal tissues contain some P450, with the exception of striated muscle and erythrocytes and the enzyme appears to be present in several subcellular organelles. Among the compounds metabolised by P450's are endogenous substances such as leukotrienes, prostaglandins, vitamins, fatty acids, steroids and xenobiotic substances that may be intentially or inadvertantly ingested such as drugs.

P450-mediated reactions include N-hydroxylation and N-oxidation, oxidative deamination, S-, N- and O-dealkylation and aliphatic and aromatic hydroxylation. The net result of the multiplicity of P450's and their diverse and overlapping substrate specificities is the ability to metabolise a plethora of chemicals. Since 1982, over 100 P450 amino acid primary sequences have been published, representing at least 53 unique P450 proteins from various species and many variants (Nebert *et al*

1987,1989), indicating the magnitude of the research undertaken in the last 30 years and the importance of this enzyme system.

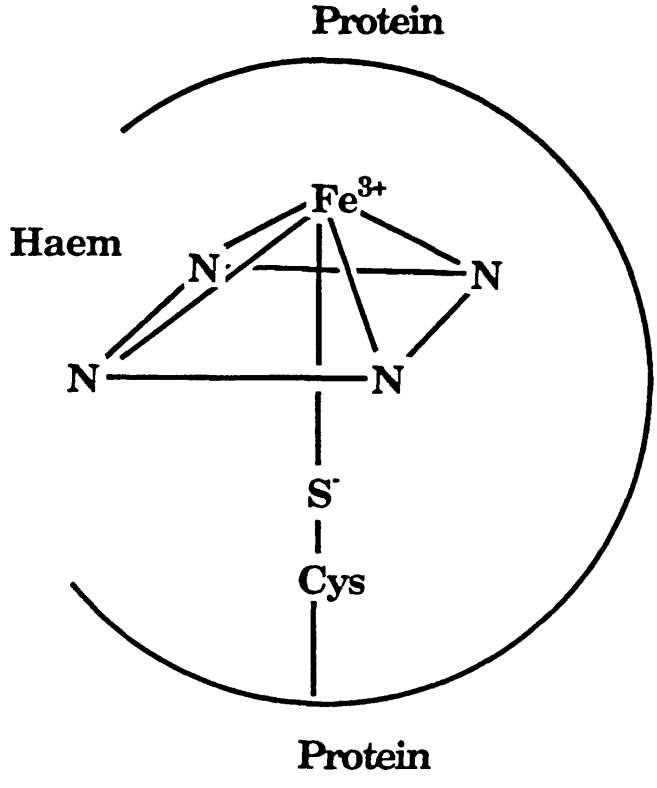
From the amino acid sequences of 12 cytochrome P450's, Gibson (1989) has shown there is a highly conserved cysteinyl-containing peptide towards the carboxyl terminus of the protein, around residues 450±15. This sequence similarity is centred around a cysteine residue which is totally conserved in all P450's (Nelson *et al* 1989) and suggests a common ancestral gene for all P450's.

The active site of all cytochromes P450 contains iron III protoporphyrin IX, in which the haem iron forms four bonds with the pyrrole nitrogen atoms of the protoporphyrin IX and anchors itself to the apoprotein via an axially iron-cysteine link (Fig 1.5). This fifth ligand has been deduced from several spectroscopic studies from various sources but was unequivocally established from X-ray analysis of bacterial cytochrome P450 ($P450_{cam}$) from *Pseudomonas putida* grown on camphor (Poulos *et al* 1985, 1987). X-ray analysis of this enzyme-substrate complex gives a clear picture of the active site, with the iron axially bound to the sulphur atom at cysteine 357. These studies are limited to $P450_{cam}$, as no mammalian enzymes have been crystallised for X-ray analysis. Thus although their tertiary and quaternary structures are unknown, from spectroscopic data it appears that the cysteine-iron porphyrin part of the active site is always present.

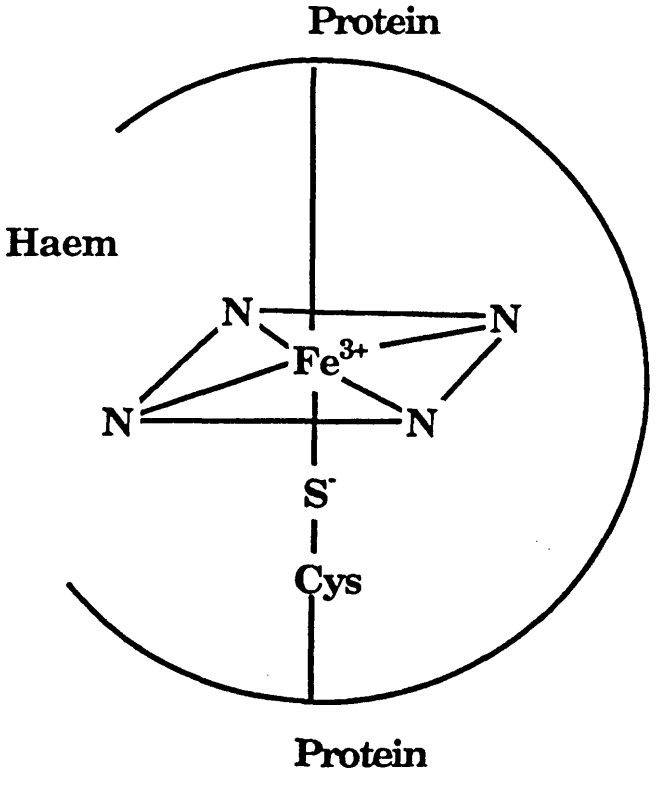
Cytochrome P450 exists in two forms, a hexacoordinated low spin iron III complex with two axial ligands, one being the cysteine residue and the other presumably an OH residue (Fig 1.5) and a penta-coordinated

Figure 1.5

Haem iron co-ordination in cytochrome P450.



Penta-coordinated, high-spin P450 with out-of -plane iron.



Hexa-coordinated, low-spin P450 with in-plane iron.

high spin iron III complex with cysteine as the only axial ligand. In its resting state an equilibrium is maintained between these two forms.

Cytochrome P450 is thought to function through the reaction cycle shown (Fig 1.6), in which six steps are apparent :-

(1) Upon binding of a substrate to a hydrophobic site close to the haem, the equilibrium shifts in favour of the penta-coordinated (high spin) state.

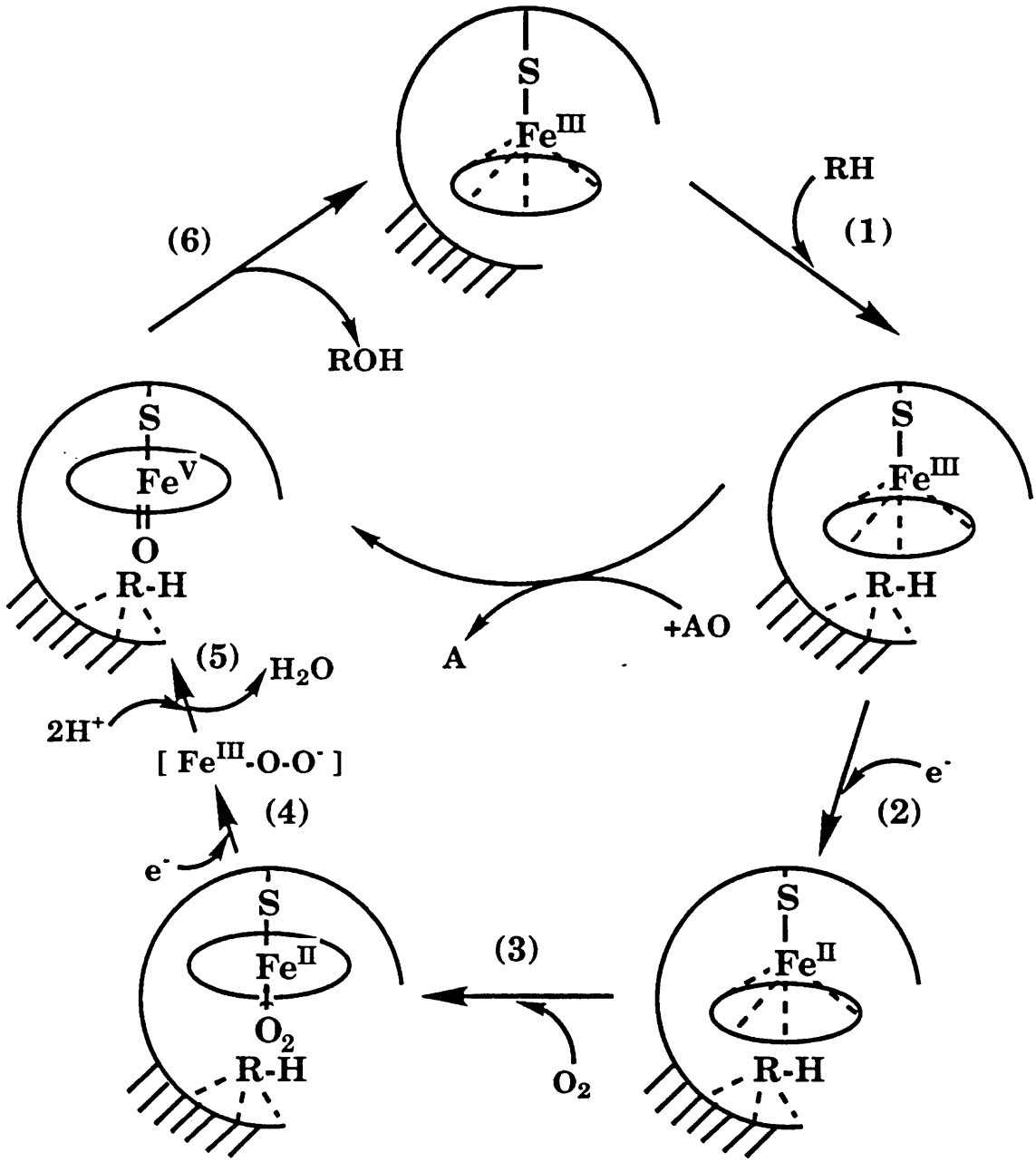
(2) The high spin-P450 substrate complex in the ferric (Fe^{3+}) oxide state is reduced by one electron to the ferrous (Fe^{2+}) state. This electron originates from NADPH, and transferred to the complex by NADPH-cytochrome P450 reductase.

(3) The high spin penta-coordinated ferrous complex reacts with molecular oxygen (dioxygen) to form a relatively stable hexa-co-ordinated low spin complex. Alternatively many other ligands such as isocyanides, nitrogenous bases, phosphine and CO can bind to the complex.

A detailed view of the variations of coordination and spin state of the iron during the catalytic cycle of the first three steps has been achieved using model iron-porphyrin complexes and X-ray analysis. However due to the short lifetime of the oxidising species involved, the final steps of the catalytic cycle are based on indirect evidence and on comparisons with better known active oxygen complexes.

Figure 1.6

Catalytic cycle of cytochrome P450.



(Mansuy *et al* 1989)

(4) One electron reduction of the Fe(II)-O_2 complex is thought to form a peroxoferric state (Fe(III)-O-O$^{\cdot}$). The source of the second electron can be NADPH-cytochrome P450 reductase or perhaps at least *in vivo*, NADH linked cytochrome b_5 (Hildebrandt and Estabrook 1971).

(5) Heterolytic cleavage of the O-O bond of a possible Fe(II)-O-OH intermediate to form a high-valent iron-complex; Fe(V)=O (oxenoid) derived formally from a two-electron oxidation of the ferric state, and the binding of an oxygen to the iron, with the release of H_2O.

(6) The oxygen of this iron-oxo complex is transferred to the substrate, with subsequent product release from the enzyme complex and the iron haemprotein to the ferric state. (Trager 1980, Mansuy *et al* 1989)

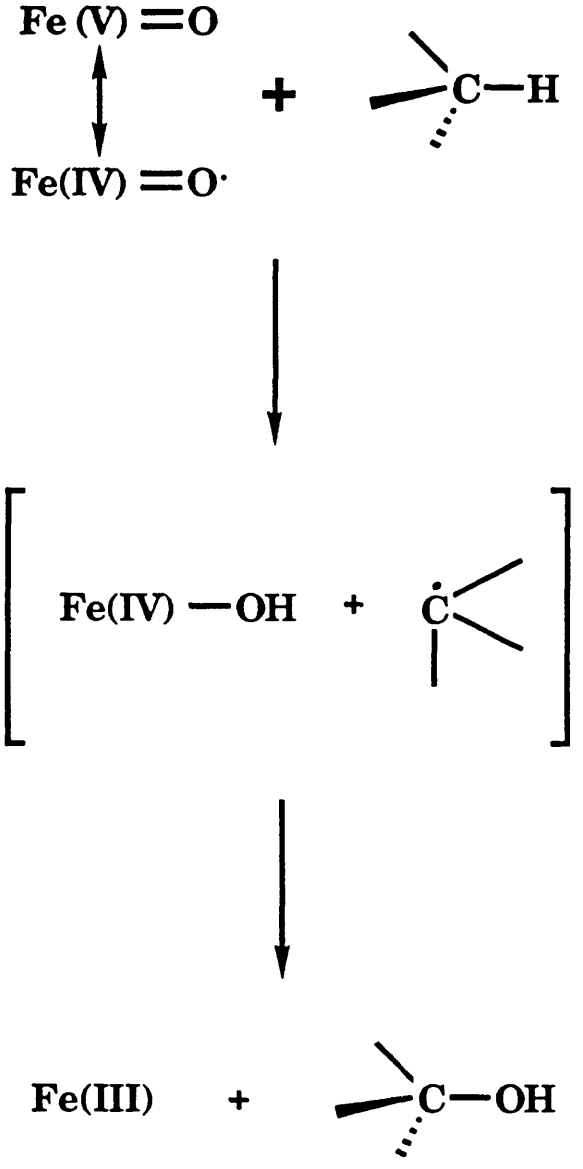
One of the main monooxygenase reactions performed by cytochrome P450 active-oxygen species is the hydroxylation of C-H bonds, undergoing the possible mechanism shown in Figure 1.7. Hydroxylation of the C-H bond is considered to involve two steps :-

(a) Hydrogen abstraction from the substrate C-H bond , by the high-valency iron-oxo intermediate having freeradical-like reactivity, would produce a substrate radical and an iron-hydroxo complex, followed by,

(b) oxidation of the intermediate free radical by the iron-hydroxo complex, resulting in C-O bond formation for the observed product alcohol. (White *et al* 1986, Groves *et al* 1988 and Mansuy *et al* 1989).

Figure 1.7

The possible mechanism of alkane bond hydroxylation catalysed by cytochrome P450.



The cytochrome P450 system is known to metabolise thousands of chemicals and thus shows a lack of substrate specificity. In contrast the individual isozymes often display significant regio-and stereo-selectivities. It is reasonable to assume that the basic hydroxylation chemistry will be the same for all P450's, given that all these enzymes have exactly the same haem group and conserved cysteine-haem ligand. Therefore their selectivities must be dependent on the nature of the amino acid residues from the protein which form the architecture of the active site and thus govern which part of a given substrate could gain the necessary access.

Detailed information is limited with regard to the exact nature of the amino acid residues of the polypeptide chain which are responsible for the binding of the substrate and for its positioning relative to the iron catalytic site. Working on the data obtained from the 2.6 Å crystal structure of $P450_{cam}$, Poulos *et al* (1985) showed that this enzyme's secondary structure consisted of 12 helical regions (A to L) and 7 β-pair regions, which was amended to 13 helical regions in 1987 (Poulos *et al* 1987). The earlier study had divided $P450_{cam}$ into a helix-rich domain, where most of the helices are located and a helix-poor domain containing a majority of antiparallel β-pairs. The majority of the protein-substrate contact points for the camphor molecule covers both domains, in particular the distal helix or helix I (residues 244-253) and the inner strand of the beta 3 segment (residues 295-299). Noticeable exceptions are those of Phen 87 and Tyr 96, the latter occupying the newly found B' helix close to the active site and, which is suggested in the case of camphor, to aid in controlling stereospecificity.

Nelson and Strobel (1989) predicted the secondary structures of 52 aligned cytochrome P450 sequences, all of which were membrane bound, and collectively compared with the crystal structure of the soluble $P450_{cam}$. This predicted that 10 of the 13 helical regions of $P450_{cam}$, 6 of the 7 β-pair region and beta-structure corresponding to the known β-bulge near the active site of $P450_{cam}$, all existed in the membrane P450's, and thus $P450_{cam}$ could be used as a model structure for membrane bound cytochrome P450. With particular reference to:-

(1) Helix I, containing 34 amino acid residues, which contains a distorted centre to allow a site for oxygen binding.

(2) A β structure analogous to the β bulge which contains the 100% conserved cysteine residue forming a thiolate pocket.

Poulos *et al* (1987) postulated a link between changes in the helix-poor domain and the control of the enzymic and substrate-binding properties, which in turn should lead to regio- and stereo-specificities of the substrate. Further substrate selectivities must be controlled by the enzyme in allowing entry of the substrate to the active site. In $P450_{cam}$ a loop centred at Thr-185 together with Phe-87 and Ileu-395 form a small opening above the camphor molecule, but the camphor molecule is too large to fit into the gap. It has been suggested (Poulos *et al* 1986) that this opening may be capable of undergoing dynamic fluctuation and thus provide an entrance channel to the active site.

Interestingly, a recent study by Lindberg and Negishi (1989) has shown that a single amino acid can profoundly effect the substrate specificity of mammalian P450. Cytochromes $P450_{coh}$ and $P450_{15\alpha}$ catalyse 7-

hydroxylation of coumarin and 15α-hydroxylation of testosterone respectively in the mouse, but whose sequences differ in only 11 amino acid residues (Lindberg *et al* 1989). Working with these isozymes they individually changed each of the amino acids in $P450_{coh}$ to the corresponding residues in $P450_{15\alpha}$ using site-directed mutagenesis. The most significant substitution was that of leucine 209 in $P450_{coh}$ for that of phenylalanine 209 in $P450_{15\alpha}$, which resulted in a variant $P450_{coh}$ having a greater affinity for testosterone than for coumarin, (in wild-type $P450_{coh}$, coumarin is 170 times better substrate than is testosterone). The reverse experiment, Phen 209 in $P450_{15\alpha}$ substituted for Leu 209 in $P450_{coh}$, also resulted in the switching of affinities, in that variant $P450_{15\alpha}$ is able to operate on coumarin (wild-type $P450_{15\alpha}$ does not hydroxylate coumarin) and showed approximately 125 times less activity towards testosterone. Using $P450_{cam}$'s X-ray structure as a guide, residue 209 should have originated from helix E, which is too far away from the haem-substrate pocket and the proposed access channel to have any direct effect. Thus it may be concluded that residue 209 either has a long range structural effect on the substrate binding or that the predictions of the mammalian P450 structure from comparisons of $P450_{cam}$ are not very close to the truth (Poulos 1989).

1.3 Product Regioselectivity in Aliphatic Hydroxylation

A well recognised phenomenon in drug metabolism is the preferential hydroxylation of carbon-hydrogen bonds at tertiary and secondary, rather than at primary, sites in substrates where multiple sites are available. Assuming the hydrogen abstraction mechanism of hydroxylation, involving radical-like reactive groups, these observations

would be expected from classical free radical mechanisms, in such that the ease of hydrogen abstraction follows thus:-

$$\text{Allylic} > 3^{\circ} > 2^{\circ} > 1^{\circ} > CH_4 \text{, vinylic.}$$

These preferential hydroxylation patterns have been well illustrated using the simple hydrocarbons n-pentane, n-heptane and 2-methylbutane as substrates in a rat liver microsome system (Ullrich *et al* 1972, Frommer *et al* 1972, and Ullrich 1969 respectively). Quantitive analysis of the products gives a clear picture of the regioselectivity involved (Table 1.1).

Table 1.1

Microsomal Oxidation of Aliphatic Hydrocarbon Substrates.

	Position of hydroxylation : alcohols (%)			
Substrates	ω	ω–1	ω–2	ω–3
2-Methylbutane:	6	20	74	--
n-Pentane:	0.5	84	16	--
n-Heptane:	9.5	73.8	11.1	5.6

(Data from Ullrich *et al* 1972, Frommer *et al* 1972, and Ullrich 1969)

As a general rule, for aliphatic substrates, hydroxylation would be expected to be regioselective for (ω-1)-hydroxylation, but this simplistic rule does not account for the undetermined number of isozymes, differing in their product selectivities, present in rat liver microsomes.

From inducer and inhibitor studies Frommer *et al* (1972) showed that the hydroxylation of n-heptane was mediated by at least 3 monooxygenases, each displaying its own regioselectivity. Thus, the regioselectivity observed in the hydroxylation of alkanes is determined primarily by the enzymes involved, whilst the properties of the substrate e.g. molecular size only has a secondary effect in "selecting" some enzymes.

These simple alkanes are relatively uncomplicated and as such provide only a limited model for aliphatic hydroxylation, as compared with alkyl side chains or more complex molecules which are of more practical significance. The hydroxylation of 5-dialkyl-substituted barbituric acids which contain side chains of four or more carbons display preferential (ω-1)-hydroxylation, whereas side chains containing three carbons are hydroxylated more extensively in the ω- than the (ω-1) positions, and those containing six carbons are hydroxylated at the (ω-2) position (Gilbert *et al* 1975), suggesting that steric factors, due either to the alkyl side chains or the barbituric acid moiety influence the enzymes involved.

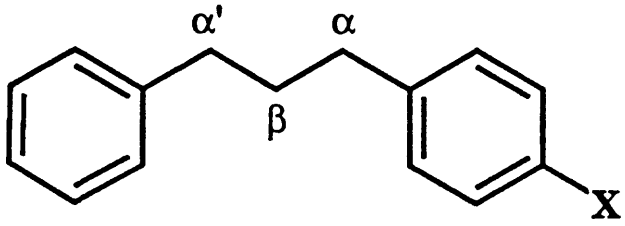
Further regioselective patterns are seen in the metabolism of molecules containing alkyl side chains adjacent to aromatic rings, and in particular the preferential hydroxylation of the benzylic carbon next to the ring of short chain alkyl substituted aromatics (Testa 1976). For example, McMahon and Sullivan (1966), investigating the metabolism of ethylbenzene, observed that hydroxylation occurs with high regioselectivity at the 1- (benzylic) position. Insight into the mechanism responsible for this benzylic hydroxylation has been obtained by Hjelmeland *et al* (1977), using mono-4-substituted 1,3-diphenylpropane derivatives (Fig 1.8). Three sites of aliphatic hydroxylation are available,

two of which are benzylic (α and α') and one methylene (β). As expected, hydroxylation at the β position was only slight, with the majority occurring at the α and α' positions. As the electron withdrawing capacity of the para-substituent increased, the hydroxylation of the α'-position, relative to the α–position, increased. The decrease in electron density at the α benzylic position renders it less reactive. Thus, the greater reactivity of benzylic carbon atoms relative to aliphatic carbon is believed to be due to resonance stabilisation of either radical or carbonium ion intermediates formed in the transition states of the reaction.

Although preferential benzylic hydroxylation is generally observed for short alkyl groups adjacent to the aromatic ring, this ceases to be the case when longer alkyl groups are involved, as seen for example with Δ^9-tetrahydrocannabinol (Δ^9 THC , Fig 1.9). Preferential hydroxylation at position 11 has consistently been found to be the major metabolic route, but it is the regioselectivity of hydroxylation along the n-pentyl moiety which is of interest. Hydroxylation at position 4' is only observed by dog (liver and perfused lung, Widman *et al* 1975), whilst the 3'-hydroxy derivative is formed by both dog (liver and lung, Widman *et al* 1975) and mouse (liver, Harvey *et al* 1977). The rabbit produces the 1'- and 2'-hydroxy derivatives of Δ^9 THC-11-oic acid (Burnstein *et al* 1972), whilst the mouse generates the 2'- and 3'-hydroxylated derivatives of the same acid (Harvey and Paton 1976), and the 2'- and 3'-hydroxylated derivatives of 11-hydroxy Δ^9 THC-6-one (Harvey *et al* 1977). It is likely that ω-hydroxylation also takes place as this reaction can partly account for the formation of several metabolites in which the pentyl side chain has been shortened, presumably by β-oxidation, e.g. 4',5'-bisnor 5-Δ^9 THC-3'-oic acid formed by the mouse *in vivo* (Martin *et al* 1976).

Figure 1.8

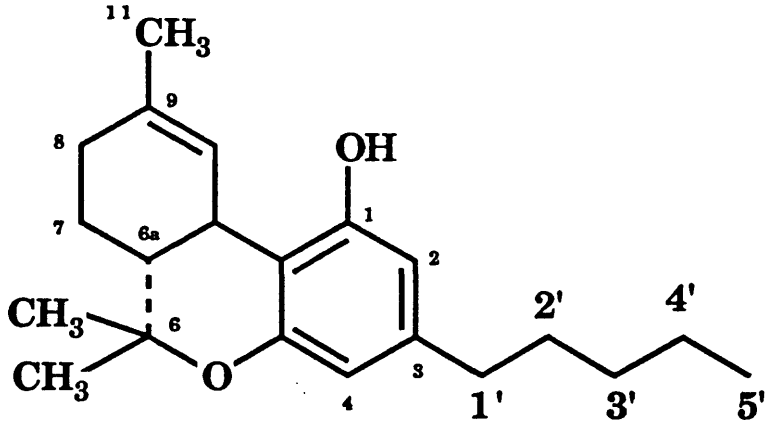
The structure of 4'-substituted 1,3-diphenylpropane derivatives.



X= H, F, CH_3 or CF_3

Figure 1.9

The structure of Δ^9 tetrahydrocannabinol.



Interestingly, dog (liver) generates the 1'- and 3'-hydroxy metabolites when the structural analogue, Δ^8-tetrahydrocannabinol is used as a substrate (Maynard *et al* 1971). These differences in regioselectivity observed between species indicate that enzymatic factors are predominant.

Lauric acid, like other fatty acids is hydroxylated at the ω and (ω-1) positions by microsomes from the liver, kidney, intestinal mucosa and lung. The prostaglandins, prostacyclins, thromboxanes and leukotrienes, complex and highly important derivatives of arachidonic acid, are also subject to ω- and (ω-1)-hydroxylation (Kupfer 1980). Parallel changes in renal ω- and (ω-1)-hydroxylation of lauric acid were observed in inducer and inhibitor studies, suggesting that a single cytochrome P450 enzyme mediates both reactions in the kidney (Ellin and Orrenius 1975, Moldeus *et al* 1974). In contrast, preferential elevation of hepatic (ω-1)-hydroxylation by phenobarbitone pretreatment (Bjorkhem and Danielsson 1970, Okita and Masters 1980) and preferential inhibition of this same activity by CO, SKF-525A, metyrapone, aminopyrine and α-naphthoflavone, suggests that different cytochrome P450 isozymes catalyse laurate ω- and (ω-1)-hydroxylation in the liver (Okita and Masters 1980). The preferential (ω-1)-hydroxylation of hydrocarbon chains is catalysed by several cytochrome P450 isozymes and is therefore considered nonspecific. Recent evidence has shown that a number of known isozymes of cytochrome P450 specifically ω-hydroxylate endogenous compounds. The number, specificity and physiological role of these ω-hydroxylases remains ambiguous, but it is clear from the available evidence that distinct isozymes catalyse the different ω-hydroxylations. For example, the rat liver P450IVA1 is active in the ω-hydroxylation of lauric acid but does not metabolise

prostaglandins, whereas the rabbit lung P450IVA4 catalyses the ω-hydroxylation of several prostaglandins but not lauric acid (Tamburini *et al* 1984, Matsubara *et al* 1987).

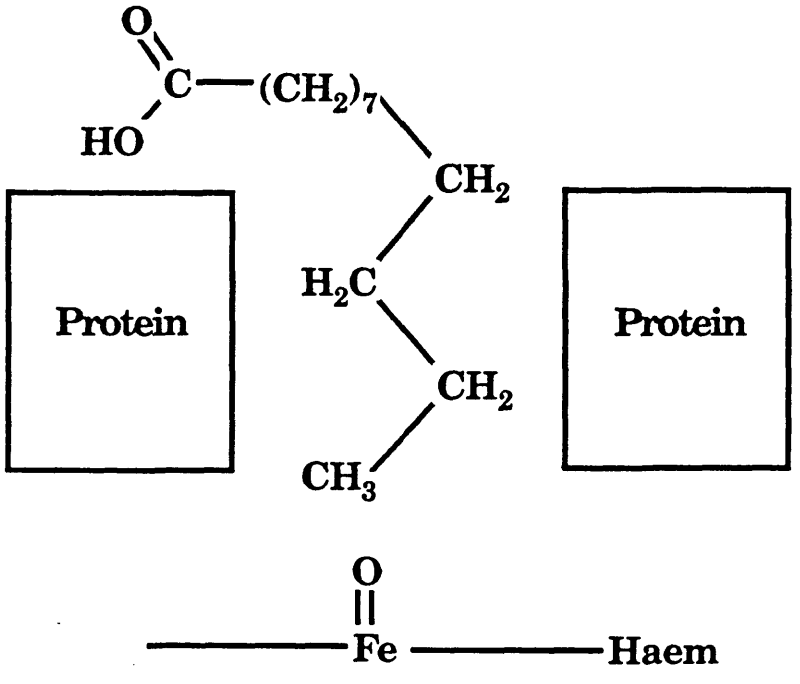
The regioselectivity of the fatty acid ω-hydroxylases are particularly interesting, because they oxidise the thermodynamically disfavoured terminal methyl group, and therefore must be capable of overriding the inherent preference of the catalytic activated oxygen species of P450 for the weaker C-H bonds situated along the remainder of the alkyl chain. CaJacob *et al* (1988), postulated that the lauric acid ω-hydroxylases must contain active sites that are highly structured, which only allow the terminal methyl group to reach the activated oxygen, thus suppressing (ω-1)-hydroxylation. The terminal methyl specificity is unlikely to be controlled by specific interactions of the protein with the carboxyl group, as the enzyme can accommodate fatty acids of different chain lengths. A schematic representation of the catalytic site has been put forward (CaJacob *et al* 1988) illustrating the steric constraints controlling access to the oxygen (Fig 1.10).

1.4 Product Stereoselectivity in Aliphatic Hydroxylation

Hydroxylation of substrates containing straight or branched alkyl or alicyclic groups is a commonly encountered event. However, when the substrate in question contains a prochiral centre, hydroxylation may generate two enantiomeric products. If these products are produced in unequal amounts the reaction is said to be stereoselective.

Figure 1.10

Schematic representation of the active site of lauric acid ω-hydroxylase.

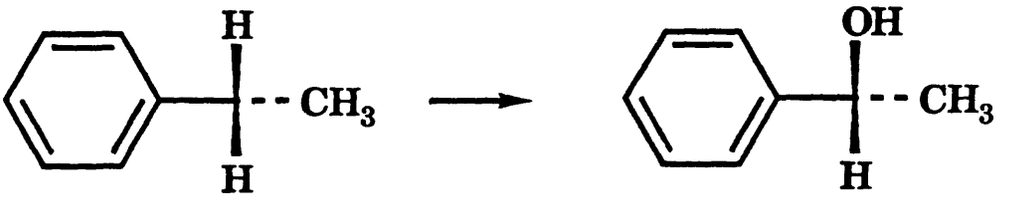


(CaJacob *et al* 1988)

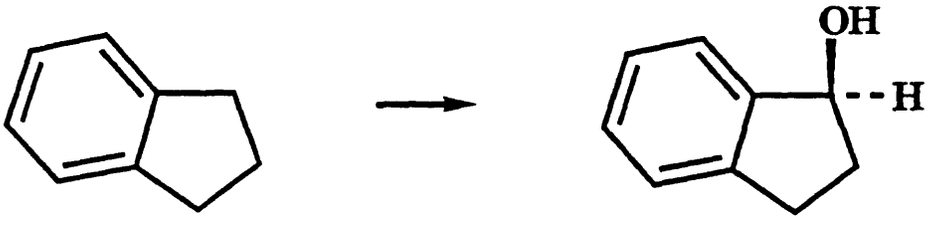
McMahon and Sullivan (1966, 1969) carried out extensive work on the metabolism of ethylbenzene, which is selectively hydroxylated at the prochiral benzylic position. Results show that the two enantiotopic hydrogen atoms are not equivalent with respect to monooxygenase activity, in that the (+)-(R)isomer is always produced preferentially (Fig 1.11). However, this stereoselectivity was partially obscured *in vivo*, as (+)-(R)-phenylethanol is excreted as its glucuronide, whilst its (-)-(S)-antipode is further oxidised to mandelic acid. Indane is hydroxylated by rat liver microsomes to form indanol (Fig 1.11), this process is product stereoselective in that the (+)-(S)-enantiomer is predominantly formed (Billings *et al* 1970). If this reaction is compared to that of ethylbenzene, it is evident that the metabolites generated have opposite absolute configurations, ie (+)-(S)-indanol and (+)-(R)-1-phenylethanol. However, the direction in which they rotate the plane of polarized light is the same. If the two substrates are compared in their preferred configurations (Fig 1.12), it is evident that in both cases the C-H bond involved in hydroxylation is located in the same position relative to the phenyl ring. Thus, when investigating the stereochemistry of a molecule, it must be remembered that the sequence rule nomenclature system follows a set of consistant but arbitrary rules which allows an hierarchical assignment of the substituents on the chiral centre. This in turn allows the researcher to discriminate between the two enantiomers (Appendix 1.1). However when comparing enantiomers of different chiral molecules, comparison of their 3-dimensional structures must be the prime consideration and not their assigned sequence rule nomenclatures, due to the inherent arbitrary nature of the rules.

Figure 1.11

The metabolism of ethylbenzene and indane.



ethylbenzene (+)-(R)-phenylethanol



indane (+)-(S)-indanol

Figure 1.12

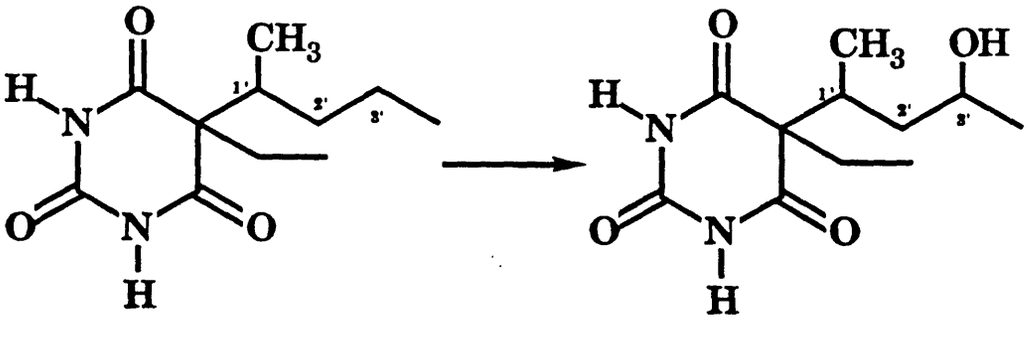
The preferred conformation of ethylbenzene and indane.

ethylbenzene indane

If the prochiral centre occurs in a compound already containing a chiral centre then hydroxylation may generate diastereomeric products. For example, pentobarbitone (Fig 1.13), which contains a chiral centre at position 1' and prochiral centres at positions 2' and 3', undergoes (ω–1)-hydroxylation in the butyl side chain yielding 5-ethyl-5-(3'-hydroxy-1'-methylbutyl)barbituric acid (Fig 1.13). This process creates a new centre of chirality, thus giving rise to four possible diastereoisomers. The stereochemical aspects of the reaction were studied in the dog upon administration of (+)-(R)-, (-)-(S)-, and (±)-pentobarbitone (Palmer *et al* 1969, 1970). Both enantiomers yielded comparable total amounts (35±3%) of 3'-hydroxypentobarbitone, indicating that substrate stereoselectivity for the overall hydroxylation is small or nonexistent. Product selectivity on the other hand varied with substrate, as the (R)-enantiomer yielded the (1'R;3'S)- and (1'R;3'R)-diastereomers in a 1:1 ratio and the (S)-enantiomer yielded the (1'S;3'R)- and (1'S;3'S)-diastereomers in a 1:5 ratio. Thus, product selectivity in this reaction is dependent upon the substrate configuration.

Figure 1.13

The metabolism of pentobarbitone.

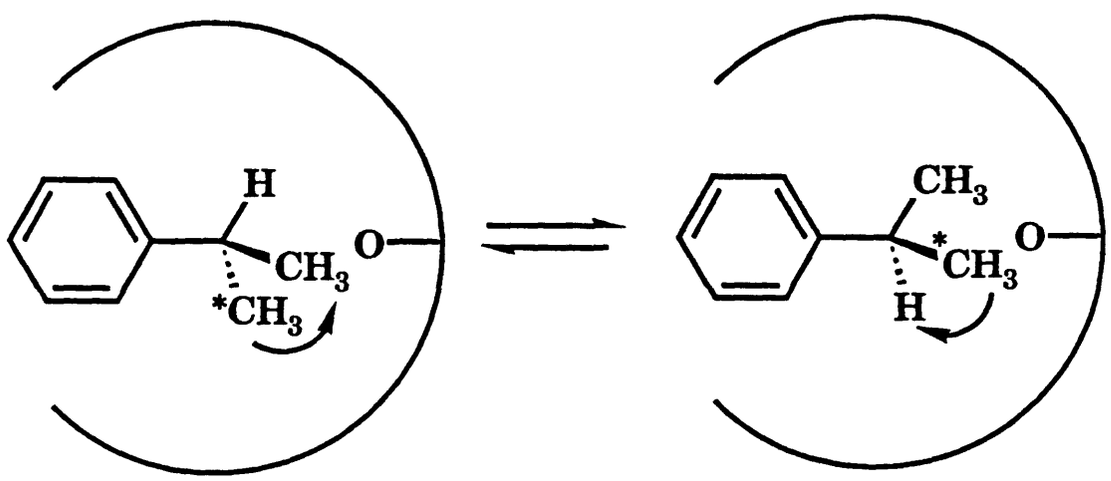


pentobarbitone **5-ethyl-5-(3'-hydroxy-1'-methylbutyl)barbituric acid**

More recently, the stereoselectivity associated with the hydroxylation of the pro-(R) versus the pro-(S) methyl groups of 2-phenylpropane (cumene) has been explored using liver microsomal preparations from untreated, phenobarbitone-treated and β-naphthoflavone-treated male rats (Sugiyama and Trager 1986). It was considered that the formation of two enantiomers from a prochiral substrate would directly reflect the topography of the active site of cytochrome P450, since the architecture of the active site is the only physical feature in the system capable of leading to differences in the rates of formation. Theoretically, the two methyl groups will experience different chemical environments when they are each aligned in a catalytically susceptible configuration (Fig 1.14). Thus the observed stereoselectivity depends primarily on the differences in the ground state energies between the substrate-enzyme complexes, and secondly on the differences in transition state energies leading to bond breaking between the two complexes. The values calculated for the R/S product stereoselectivities associated with the ω-hydroxylation of 2-phenylpropane by various rat liver microsome preparations were, uninduced, 2.23±0.27, phenobarbitone-pretreated, 1.65±0.21, and β-naphthoflavone-pretreated, 0.89±0.02. From these results it is clear that the hydroxylation reaction shows product stereoselectivity and that the composition of the products is highly dependent on the specific composition of isozymes present in each microsomal preparation. The deuterium isotope effect for the ω-hydroxylation was found to be approximately 8, irrespective of the microsomal preparation used. These results are entirely consistent with the radical abstraction mechanism for aliphatic hydroxylation (Groves *et al* 1978) and suggest that the apoprotein fraction of cytochrome P450 dictates stereoselectivity, whilst the porphyrin-Fe-O complex dictates the mechanism (Trager 1989).

Figure 1.14

Two distinct catalytically sensitive orientations of cumene at the active site of cytochrome P450 formed by a 120^0 rotation.



Protein Protein

The pro-R methyl group is identified by an asterisk

Sugiyama and Trager, (1986)

The cytochrome P450-catalysed aliphatic hydroxylations of steroids are generally highly stereoselective if not totally stereospecific (Waxman 1988). For example, Cheng and Schenkman (1983) studied the monohydroxylation of testosterone by purified cytochromes P450 IIC11 (RLM5) and RLM3 (strain-dependent). The former generated 16α–hydroxy as a major metabolite, 6β-hydroxy and both the 2α-and 2β-hydroxy metabolites in a 9:1 ratio. RLM3 converted the substrate to 6β-hydroxy metabolite to a major extent and to 7α- and 16β-hydroxy metabolites to a minor extent.

The phenobarbitone-inducible P450 forms IIB1 and IIB2 are identical in sequence except for 14 amino acids within the carboxy-terminal half of the molecule (Suwa *et al* 1985). Although their substrate specificities are virtually identical, IIB1 shows approximately 5-10 fold higher turnover for most monooxygenated substrates (Rynan *et al* 1982). Both oxygenate testosterone to yield 4 major metabolites; the 16α-hydroxy, 16β-hydroxy, 17-keto and 16β-hydroxy-7-keto. Recently Aoyama *et al* (1989) has isolated a variant IIB2 cDNA from untreated rat liver, which encoded a protein that produced the 16α-hydroxy and 17-keto metabolites of testosterone but no 16β-hydroxylated products. Sequence analysis of the variant cDNA revealed three amino acid substitutions, Leu58 > Phe, Ileu114 > Phe and Glu322 > Val, compared with the gene sequence derived by Mizukani *et al* (1983). Two of these substitutions occurred in the N-terminal half of the protein, in contrast to both IIB1 and IIB2 whose published sequences are identical in this region. When the changes Leu58 > Phe and Ileu114 > Phe were incorporated into IIB1, via construction of a chimeric cDNA, the expressed enzyme did not catalyse the 16β-hydroxylation of testosterone, but did generate the 16α-hydroxy and 17-keto metabolites, albeit at slightly reduced levels compared to the parent IIB1.

Replacement of just Leu114 > Phe in IIB1 resulted in a protein capable of producing all four testosterone metabolites with only slightly different product ratios compared with the parent enzyme. These results indicate that only small changes in the amino acid sequence can result in profound changes in the stereoselectivity of the enzyme. It has been postulated (Aoyama *et al* 1989) that P450 IIB1 displays multiple binding configurations for the steroid testosterone and that the bulky Phe residue prevents orientation of the molecule into a position that can be hydroxylated in the β plane.

1.5 Chromone-2-Carboxylic Acids

Several classes of natural compounds, including the flavones (2-phenylchromone) and isoflavones (3-phenylchromone), contain the chromone nucleus (4H-1-benzopyran-4-one, Fig 1.15). The pharmacological study of chromones began with Khellin (Fig 1.16), extracted from parts of *Amni visnaga*, a plant from the Middle East. This compound was used as a coronary vasodilator in cardiac diseases and as a bronchodilator for the treatment of asthmatic conditions. However, it produced unpleasant side effects, so that the chromone nucleus was used as the basis for extensive research, in an attempt to mimic the actions of Khellin. This work included chromone-2-carboxylic acid and its derivatives, certain of which possessed anticonvulsant, anti-allergy, anti-secretory, antispasmodic, anti-inflammatory, anticoagulant, analgesic and uricosuric properties.

Figure 1.15

The chromone nucleus.

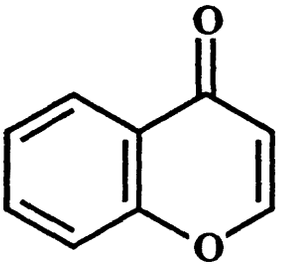
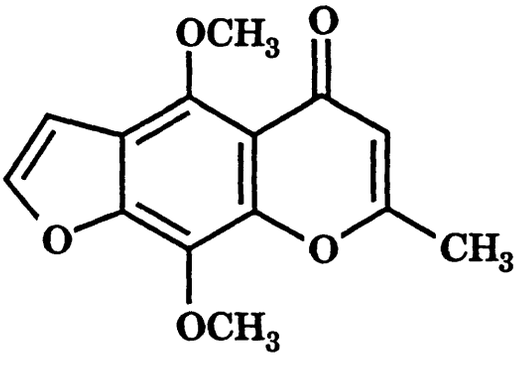


Figure 1.16

The structure of Khellin.



The nomenclature recommended by IUPAC for this structure is 4-oxo-4H-chromen-2-carboxylic acid but is referred to as 4-oxo-4H-1-benzopyran-2-carboxylic acid in "Chemical Abstracts". In this thesis for the acid (Fig 1.17) the trivial name chromone-2-carboxylic acid will be used.

The first synthetic chromone to be used clinically was 3-methylchromone (Crodimyl) which possessed both antispasmodic and coronary vasodilator actions, and for some time this and Khellin were the only two useful compounds containing the chromone ring system. The discovery that certain chromone-2-carboxylic acids possess antiallergic properties resulted in the synthesis and testing of a large number of compounds of this type and the eventual marketing of sodium cromoglycate (Intal, Cromolyn sodium, Fig 1.18), an important drug in the treatment of asthma which has also been shown to have activity against a number of allergic diseases including rhinitis, ulcerative colitis and psoriasis. Sodium cromoglycate is somewhat unusual in that no metabolism has been recorded in any animal species or man (Ashton *et al* 1973). The likely reasons for this biological inertness can be found in its physicochemical properties. Hydroxyl and carboxy groups are evenly distributed around the molecule resulting in a molecule that is very water soluble, with virtually no lipophilicity at physiological pH (log D of -4.8 at pH 7.4 in octanol-aqueous buffer system, Scherrer and Howard 1977). Consequently, absorption from the gastrointestinal tract is limited (1-5%) and migration into a membrane environment or active site of oxidative enzymes such as cytochrome P450 is extremely unlikely. Conjugation of this compound is a possibility but is not observed, most likely due to the very low pKa's of the acidic groups (less than 2). In the clinic, sodium cromoglycate is used by inhalation as a fine aerosol

Figure 1.17

The structure of chromone-2-carboxylic acid.

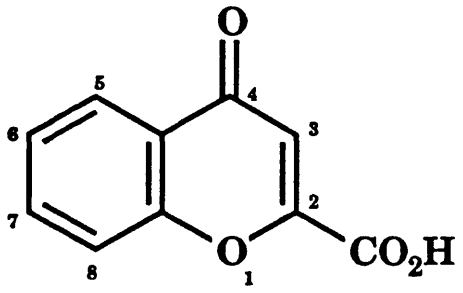
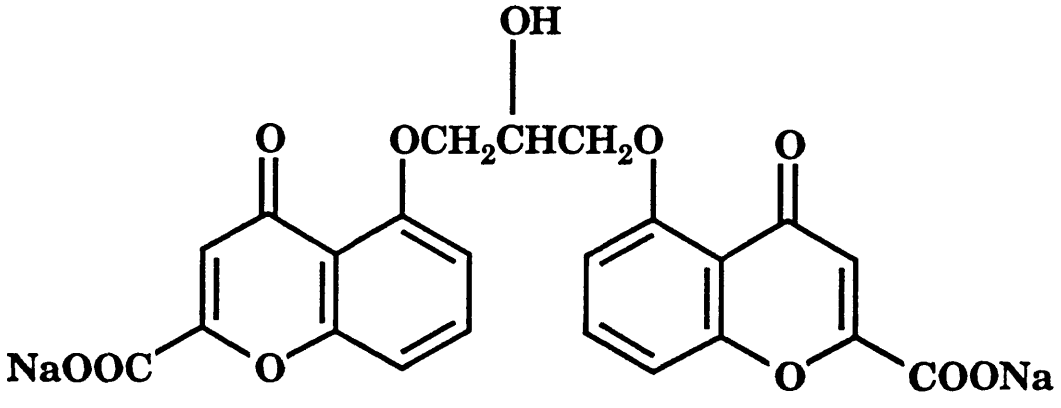


Figure 1.18

The structure of sodium cromoglycate.



powder or by topical administration. The success of this compound in the treatment of allergic diseases has prompted further study, using this structure as a basis for drug design, in particular the incorporation of alkyl and similar substituents on the chromone nucleus to increase lipophilicity (Augstein *et al* 1977).

The synthesis of chromone-2-carboxylic acids

Syntheses of chromone-2-carboxylic acids and their esters may be divided into two main types :-

(1) Direct syntheses , in which the C-2 substituent is present at the cyclisation stage.

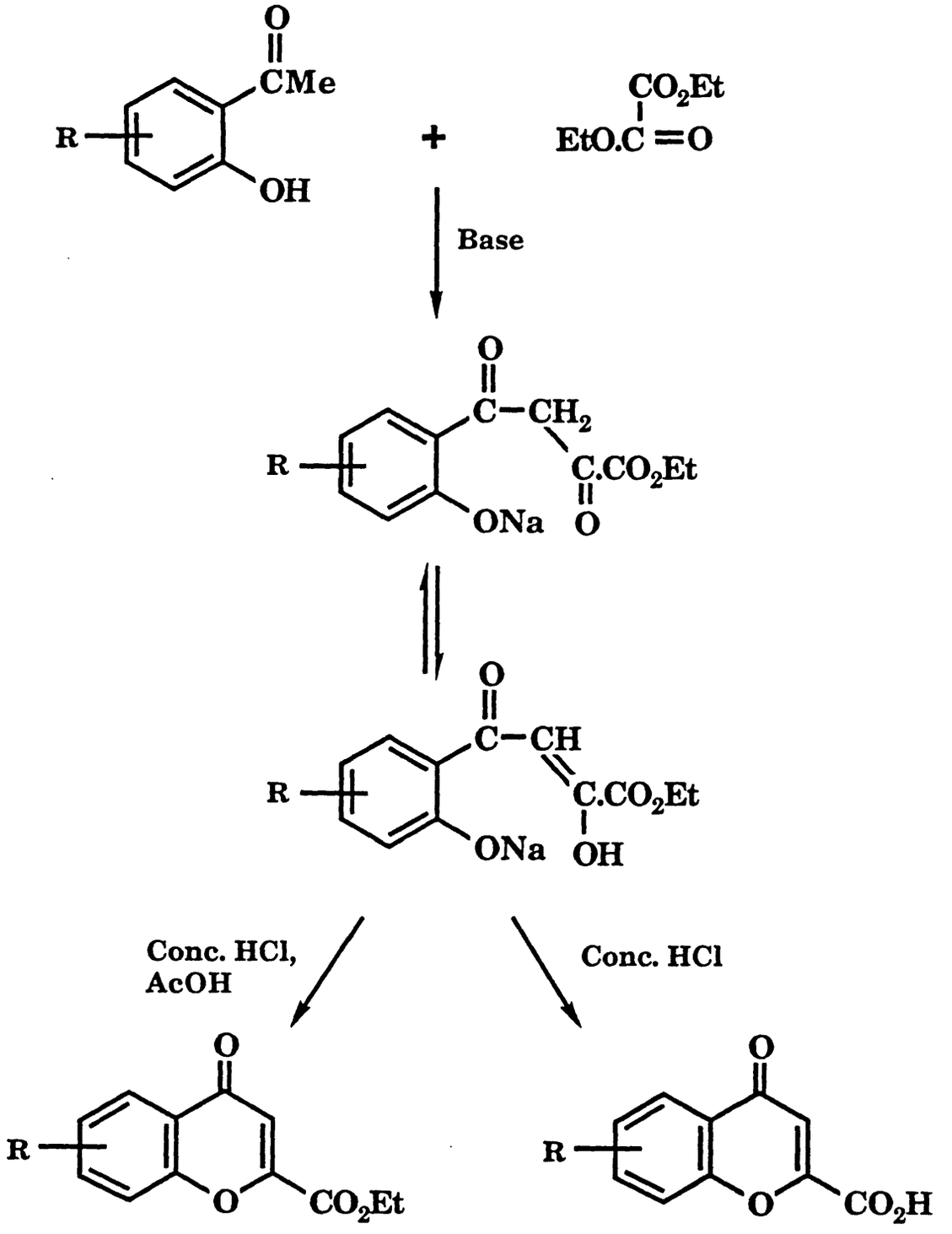
(2) Indirect syntheses , in which the C-2 substituent is formed from another group after formation of the pyrone ring.

The former is by far the most commonly used.

The Kostanecki method (Kostanecki *et al* 1901) for the synthesis of chromone-2-carboxylic acids from benzenoid precursors is probably the most important and frequently encountered method. 2-Hydroxyacetophenone undergoes Claisen condensation with ethyl oxalate in the presence of a strong base (in excess) such as sodium hydride,to give the highly coloured ethyl 3-(2-hydroxybenzoyl)-2-oxopropanoate (Fig 1.19). The oxopropanoate is cyclised either to chromone-2-carboxylic acid with concentrated hydrochloric acid or to ethyl chromone-2-carboxylate with a concentrated hydrochloric acid and acetic acid mixture. However, the product obtained depends on the quantity of mineral acid used and on the reaction time. The method

Figure 1.19

Kostanecki's synthesis of chromone-2-carboxylic acids.



enables 5-,6-,7-and 8-chromone-2-carboxylic acids to be substituted simply, providing they are unaffected by the reaction conditions. Substituents which may be incorporated include alkyl, alkoxyl, substituted alkoxy, halogen, ester, hydroxyl, acyl, nitro, aryl, cyano, amino, benzylic, heterocyclic and alicyclic.

When electron-withdrawing substituents such as nitro or halogen are present it is essential that the base be in excess. Hydroxyl substituents (other than that which reacts in the Kostanecki cyclisation) should be protected.

The Baker and Ollis method (Baker and Ollis 1952, 1953) is another direct synthesis of chromone-2-carboxylic acids, but has been used mainly in the synthesis of the 2-carboxylated derivatives, such as the naturally occurring 3-arylchromones (flavones). The reaction involves acylation of an aryl alkyl ketone with ethyl oxalyl chloride in pyridine under mild conditions and probably follows the mechanism as shown in Figure 1.20. When the aryl alkyl ketone contains more than one phenolic group, an additional molar equivalent of acyl chloride should be added and the ester so formed cleared at the end to regenerate the hydroxyl derivative.

The Ruhemann method for the synthesis of chromone-2-carboxylic acids dates from 1900 (Ruhemann and Stapleton, 1900). When allowed to stand with concentrated sulphuric acid, at room temperature for some hours, substituted aryloxyfumaric acids cyclise to chromone-2-carboxylic acids. These acids are obtained by heating a phenol with acetylene dicarboxylic acid or ester (Fig 1.21).

Figure 1.20

Baker and Ollis's synthesis of ethyl chromone-2-carboxylates.

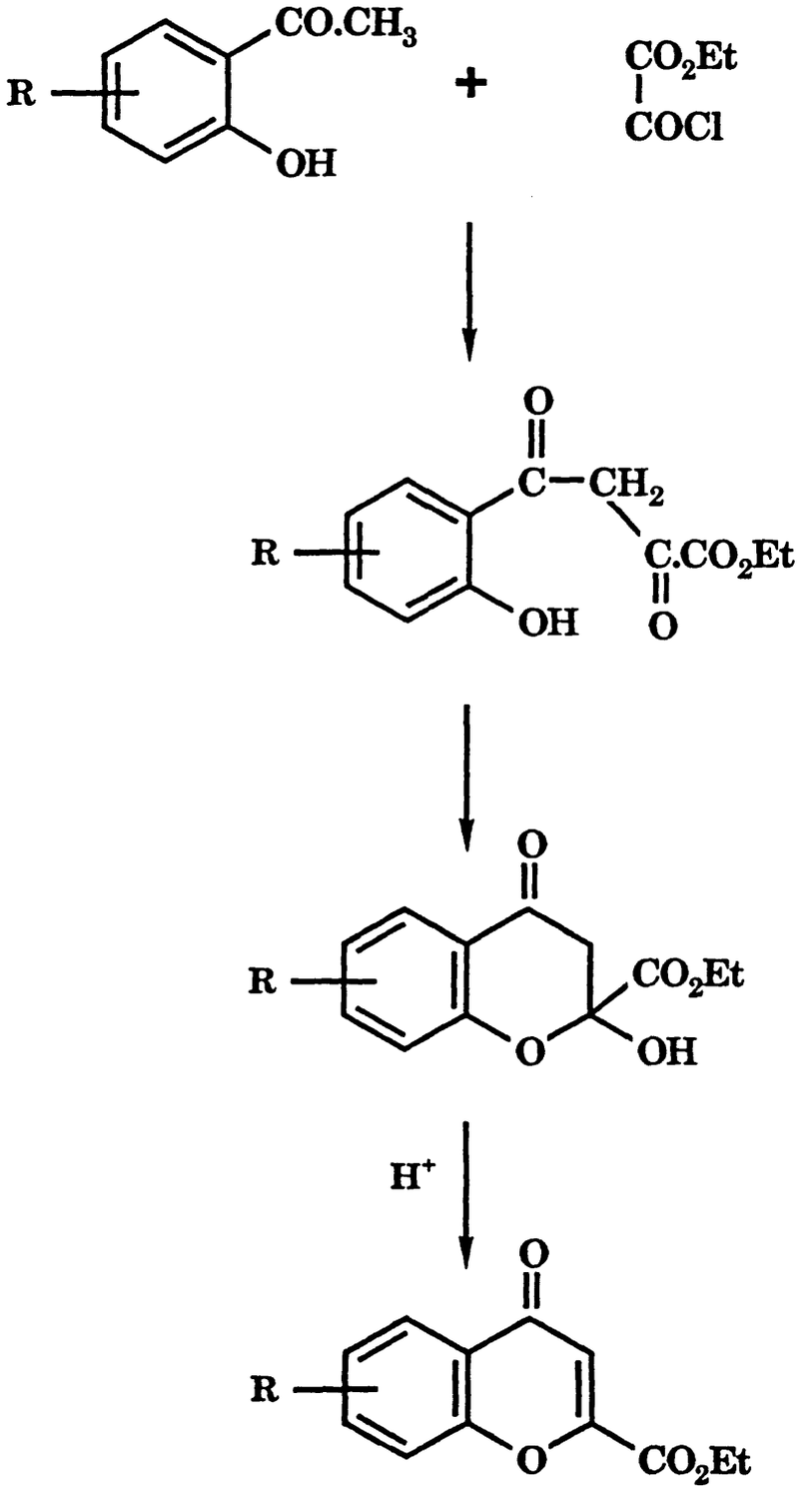
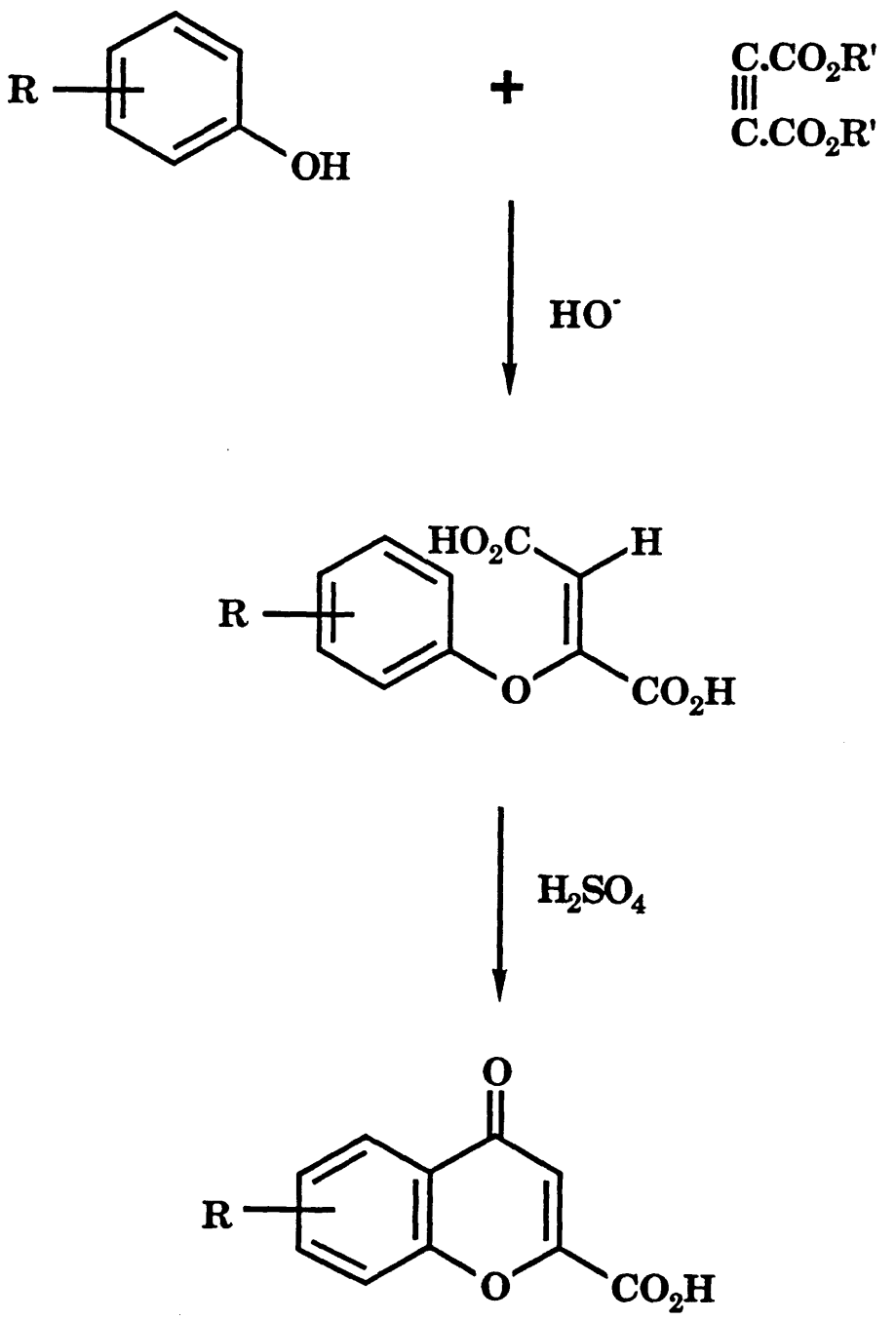


Figure 1.21

Ruhemann's synthesis of chromone-2-carboxylic acids.



R'= H or Et

Chromone-2-carboxylic acids carrying alkyl or halogen substituents on the benzene ring have been synthesised by this method.

The indirect syntheses have not been extensively used in the preparation of chromone-2-carboxylic acids, but chromones containing suitable substituents at C-2 (methyl, aldehyde, or cyano groups) have potential precursors of a carboxyl function. In practice, methods involving indirect synthesis generally result in poor yields due to insufficient control over the reaction and multistage reaction sequences. For example, oxidation of a methyl side chain is not an attractive route to chromone-2-carboxylic acids because of the ease with which the pyrone ring is attacked by oxidising agents.

Ellis and Baker (1972) have extensively reviewed the synthesis, physical and spectral characteristics, chemical reactions and biological proterties of both chromone-2- and -3-carboxylic acids and their derivatives.

Metabolism of chromone-2-carboxylic acids

Smith *et al* (1986) have reviewed extensive studies on the metabolism of a series of chromone-2-carboxylic acids, containing aliphatic or alicyclic substituents. These provide information concerning the influence of chemical and physicochemical properties upon biological fate, and has resulted in the following general conclusions :-

(1) The primary metabolism of substituted chromone-2-carboxylic acids occurs only by oxidative processes, limited to aliphatic or alicyclic

substituents, with no metabolism to the chromone-2-carboxylic acid nucleus occurring.

(2) The carboxylic acid function of the chromone-2-carboxylic acid is metabolically inert, undergoing no conjugation.

The mechanism of glucuronidation is a SN_2 reaction (Axelrod *et al* 1958), the acceptor group of the substrate attacking the C-1 of the pyranose ring to which UDP is attached, resulting in inversion of configuration. The attacking group should have sufficient nucleophilic character for a high rate of glucuronidation. In general, only weak carboxylic acids are good substrates for conjugation reactions (Williams 1959), whereas the strong acidity (pKa < 2 in all cases) of the chromone-2-carboxylic acid appears to prevent them acting as nucleophiles. Interestingly, Smith *et al* (1986) postulate a possible role for the pKa of the acidic group in determining a drug's susceptibility to conjugation, in that it is unusual for acids with a pKa lower than 3 to participate in glucuronic acid conjugation.

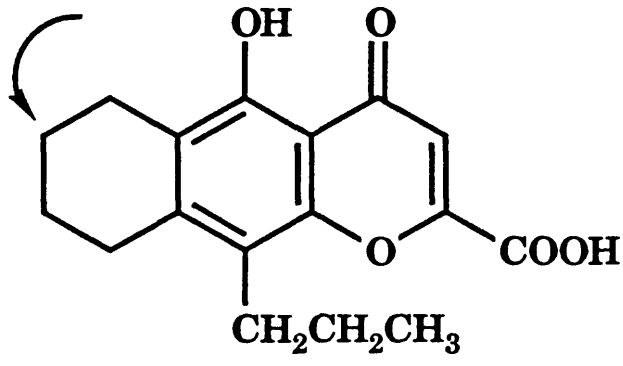
(3) Hydroxylation of aliphatic and alicyclic substituents is regioselective, attacking the group at the greatest distance from the carboxylic acid function.

This regioselectivity is associated with increasing lipophilicity when alkyl groups are attached to the 5-, 6-, and 7- positions. These compounds are "detergent-like", possessing a lipophilic region formed by the alkyl substituents and a strongly acidic function. Similar to detergents, these molecules will orientate themselves in a membrane environment, such that the lipophilic region will enter the membrane,

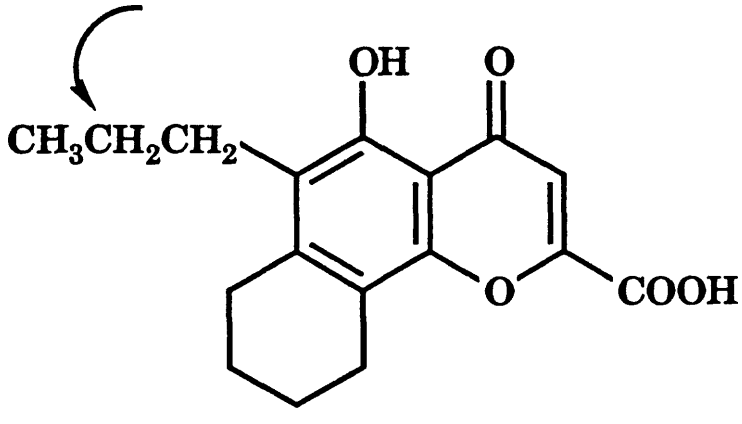
whilst the acidic function remains in the aqueous phase. If oxidative enzymes are present only in the lipid phase or have a lipophilic site, oxidative metabolism will be restricted to the end opposite the acidic function. However, the carboxyl group could play a more critical role, binding to a distinct part of the enzyme active site, orientating the alkyl moiety towards the activated oxygen in the active site. This regioselectivity has been observed in all chromone-2-carboxylic acids studied (Smith *et al* 1986) and appears to be a significant factor in their metabolism, to the extent that propyl substituents introduced into the 8-position of the chromone are resistant to oxidative metabolism. The metabolism of 6,7,8,9-tetrahydro-5-hydroxy-4-oxo-10-propyl-4H-naphtho[2,3-b]pyran-2-carboxylic acid (proxicromil) and its regio-isomer 7,8,9,10-tetrahydro-5-hydroxy-4-oxo-6-propyl-4H-naphtho[1,2-b]pyran-2-carboxylic acid provide a good illustration of this regioselective hydroxylation. Proxicromil (Fig 1.22) is metabolised only in the cyclohexenyl ring, at position 7-, the furthest from the carboxylic acid function, with no metabolism of the propyl group. This site of hydroxylation is unusual in itself, as aliphatic side chains attached to or alicyclic ring fused to, an aromatic ring are normally hydroxylated at the carbon attached to the aromatic ring. Hydroxylation of its isomer occurs mainly on the propyl chain forming the 2-hydroxypropyl derivative, with some hydroxylation of the cyclohexenyl ring. The strongly acidic carboxyl function therefore has profound effects on determining the regioselectivity of metabolism.

Figure 1.22

The major sites of hydroxylation of proxicromil and its positional isomer.



proxicromil (6,7,8,9-tetrahydro-5-hydroxy-4-oxo-10-propyl-4H naphtho[2,3-b]-pyran-2-carboxylic acid)



7,8,9,10-tetrahydro-5-hydroxy-4-oxo-6-propyl-4H naphtho[1,2-b]-pyran-2-carboxylic acid.

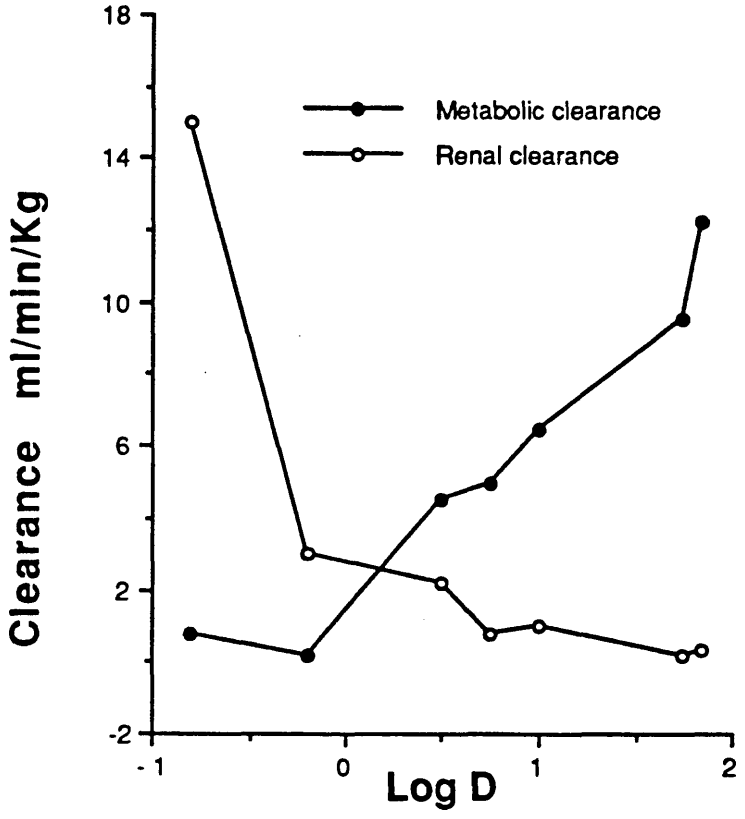
(4) The lipophilicity of chromone-2-carboxylic acids has a direct influence on their rates of metabolism and renal clearance, in the rat *in vivo*.

Smith *et al* (1986) showed that increase in lipophilicity increased metabolic clearance (blood clearance x fraction of dose excreted as metabolite) and decreased the renal clearance (blood clearance x fraction of dose excreted as parent compound in urine) in a parabolic relationship (Fig 1.23). The increase in metabolic clearance can be explained by hydrophobic effects, since all metabolism is by aliphatic or alicyclic carbon hydroxylation. The active site of cytochrome P450 has been shown to be hydrophobic in nature (Imai and Sato 1967, Cohen and Mannering 1973 and Al-Gailany *et al* 1974) and lipophilicity has been correlated with metabolism rate or binding *in vitro* (Jansson *et al* 1972 and Al-Gailany *et al* 1978). This decrease in renal clearance may reflect several processes. For instance, the degree of protein binding of the chromone-2-carboxylic acids is related to both the acidic function and lipophilicity. However, for compounds of moderate to high lipophilicity (log D > -1.0), protein binding is greater than 99% in the rat. It is therefore considered that this decrease in renal clearance reflects increase in tubular reabsorption, in that the compounds are excreted by the renal acidic transport system and reabsorbed due to their inherent lipophilicity. Weiner *et al* (1960) observed a similar inverse relationship between lipophilicity and renal clearance for homologues of probenicid.

(5) The chromone-2-carboxylic acids in general show strong similarities in their metabolism between species, with the rate of metabolism rather than route being the only major difference.

Figure 1.23

Relationship between lipophilicity (Log D) and renal (O) and metabolic (●) clearance for a series of chromone-2-carboxylic acids.



Smith et al (1986)

It is noteworthy that proxicromil not only undergoes regioselective hydroxylation, but that its metabolites were found to be optically active, indicating that the hydroxylation proceeded with some degree of stereoselectivity.

6-n-Alkylchromone-2-carboxylic acids

Winter (1987) studied the metabolic pathways of a homologous series of 6-n-alkylchromone-2-carboxylic acids (methyl to pentyl) *in vivo* in the rabbit. This work confirmed that 6-n-alkylchromone-2-carboxylic acids are only metabolised by aliphatic oxidation (Smith *et al* 1986). In addition it was observed that 6-n-propylchromone-2-carboxylic acid (6-n-PCCA) underwent (ω-1) hydroxylation exclusively, with 77% of the dose excreted in the urine, 53% as 6-n-PCCA and 47% as 6-(2'-hydroxypropyl) chromone-2-carboxylic acid (6-2'-HPCCA). This apparently simple oxidative prochiral-chiral transformation made 6-n-PCCA a suitable probe to investigate the enantioselectivity of alkyl hydroxylation. Administration of 6-n-PCCA to rabbits revealed that hydroxylation generated one of the enantiomers in excess of its antipode, in the ratio 76:24. Assignment of absolute configuration of the enantiomers showed that (S)-6-2'-HPCCA predominated. Administration of synthetic racemic 6-2'-HPCCA, resulted consistently in a 1:1 enantiomeric ratio in the urine, suggesting that any discrimination in the metabolism of 6-n-PCCA is indeed due to product stereoselective hydroxylation.

1.6 Aims of the Present Research

The metabolism of 6-n-PCCA in the rabbit involves hydroxylation at the 2- (ω-1) position on the propyl moiety exclusively. Like other alkyl

substituted chromone-2-carboxylic acids, oxidative metabolism of the aliphatic or alicyclic moiety occurs at positions furthest away from the acid function, which itself undergoes no conjugation. This hydroxylation of a prochiral methylene group also exhibits enantioselectivity. In the rabbit, both parent compound and metabolite are excreted in the urine, both being recovered to give high yields. These properties make 6-n-PCCA a suitable probe for the investigation of biological factors influencing the regioselectivity and enantioselectivity of aliphatic hydroxylation.

The present study sets out to use this compound with the following objectives :-

(1) To investigate the influence of animal species on the regio- and stereo-selective metabolism of 6-n-PCCA.

(2) To investigate the effect of variations in the hepatic complement of P450 isozymes upon the regio- and stereo-selective metabolism of 6-n-PCCA, by the pretreatment of known inducers giving different and characteristic patterns of P450 induction.

Chapter 2

Species Differences in the Regioselective Metabolism of 6-n-Propylchromone-2-Carboxylic Acid

2.1 Introduction

The metabolism of 6-n-propylchromone-2-carboxylic acid (6-n-PCCA) in the female rabbit was first described by Winter in 1987, as part of a systematic study on a homologous series of 6-n-alkylchromone-2-carboxylic acids , from methyl- to pentyl-. The single metabolite observed was isolated using a series of liquid-liquid extractions and TLC and was identified as 6-(2'-hydroxypropyl)chromone-2-carboxylic acid, (6-2'-HPCCA). The simple metabolic pattern and high urinary recovery of 6-n-PCCA thus suggested it as a suitable probe for the investigation of aliphatic hydroxylation mechanisms.

This chapter reports the results of further investigations into the metabolism of 6-n-PCCA in a number of different species of mammal (rabbit, rat, and guinea pig).

2.2 Materials and Methods

Compounds

General laboratory chemicals and solvents were purchased from usual U.K. commercial sources. All chemicals used in syntheses are listed with their sources in Appendix 2.1

6-(2'-hydroxypropyl)chromone-2-carboxylic acid was a sample synthesised by Winter (1987).

[2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid, specific activity 473 KBq mg^{-1}, and radiochemical purity > 99% by TLC in 7 systems, was synthesised by Fisons plc, Pharmaceutical Division, Research and Development Laboratories, Loughborough, UK.

6-n-propylchromone-2-carboxylic acid

6-n-propylchromone-2-carboxylic acid was prepared by Ruhemann's direct synthesis, involving the Michael addition of 4-propylphenol to dimethyl acetylenedicarboxylate and cyclisation using concentrated sulphuric acid.

4-propylphenol (10.0g) was dissolved in dioxan (88 ml), and to this solution dimethylacetylene dicarboxylate (11.0g) and N-benzyl trimethylammonium hydroxide (Triton B, 8 drops) were added. The mixture was refluxed on a steam bath for 30 min, after which 20% w/v sodium hydroxide solution (60ml) was added and the whole refluxed for

45 min. During this time the formation of a white solid was observed. After cooling, the reaction mixture was acidified to pH 1 with hydrochloric acid (4M) and extracted with chloroform (3 x 200ml). The combined chloroform extracts were washed with distilled water (3 x 300ml), dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo* yielding a yellow solid. This intermediate compound was then added to conc. sulphuric acid (60ml) and stirred until dissolved. The mixture was then carefully poured into ice-water (300ml) to precipitate the resulting solid, which was filtered, washed with boiling water and recrystallised three times from ethanol. This yielded 6-n-propylchromone-2-carboxylic acid (5.93g) as a white crystalline solid, mp 214°C (213-214°C Winter 1987).

^{1}H NMR analysis (360 MHz; $CDCl_3$/d_6-DMSO) of the compound is shown in Figure 2.1 which yielded the following results:

ppm (multiplicity, integration, assignment), 0.95 (t, 3H, $\underline{CH_3}CH_2CH_2$), 1.22 (t, EtOH), 1.67-1.73 (m, 2H, $CH_3\underline{CH_2}CH_2$), 2.61 (m, d_6-DMSO), 2.71 (t, 2H, $CH_3CH_2\underline{CH_2}$), 3.66 (q, EtOH), 7.01 (s, 1H, 3-H), 7.42 (s, $CDCl_3$), 7.53-7.59 (m, 2H, 7-H, 8-H), 7.96 (d, 1H, 5-H).

MS analysis (EI, Fig 2.2) revealed the following ions:
EI: m/z (% rel. abund.) 232 (32, molecular ion), 203 (100, base peak), 133 (19).

Both mass and NMR spectra were consistent with the structure proposed, and identical with those obtained with the authentic standards.

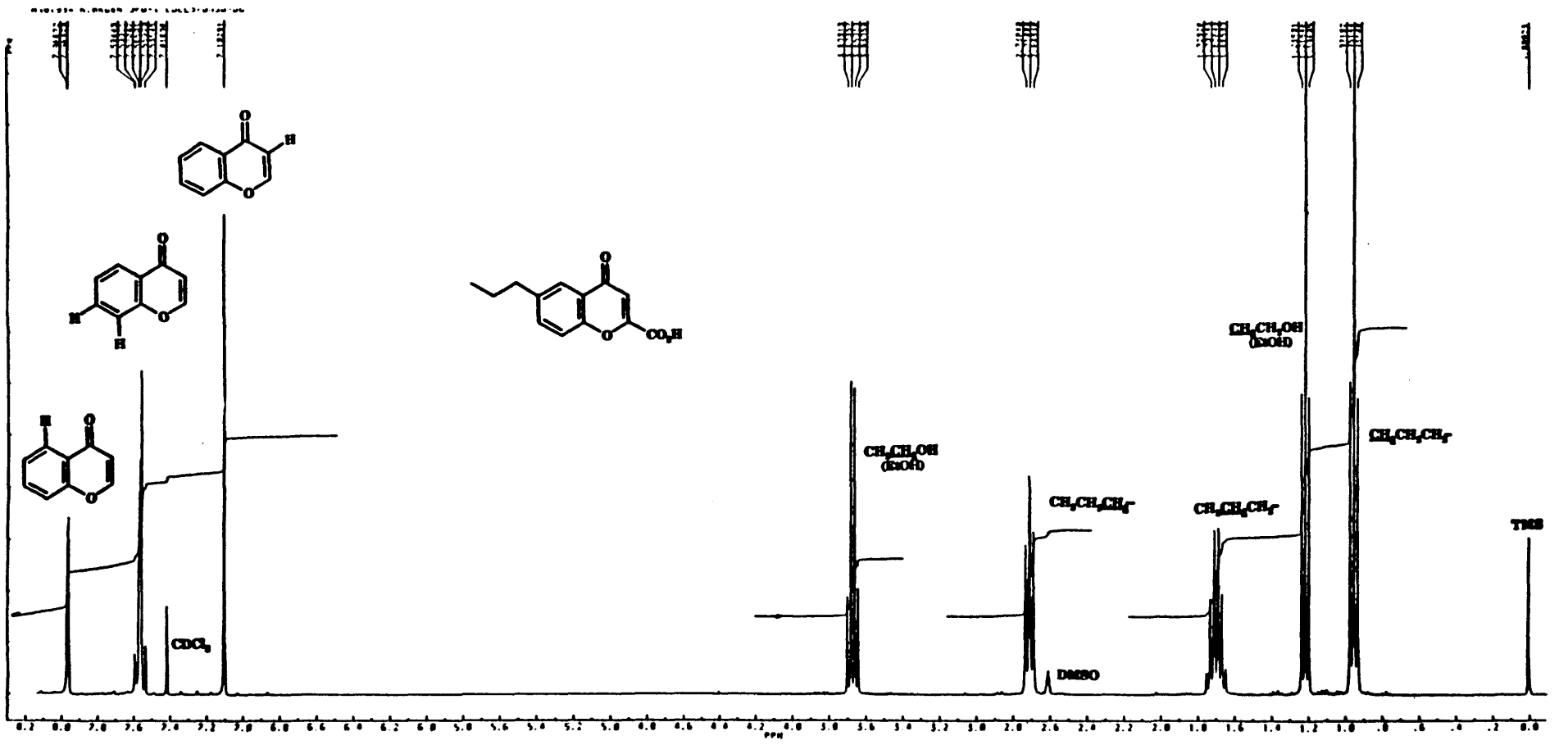


Figure 2.1 ^{1}H NMR spectrum (360MHz) of 6-n-propylchromone-2-carboxylic acid.

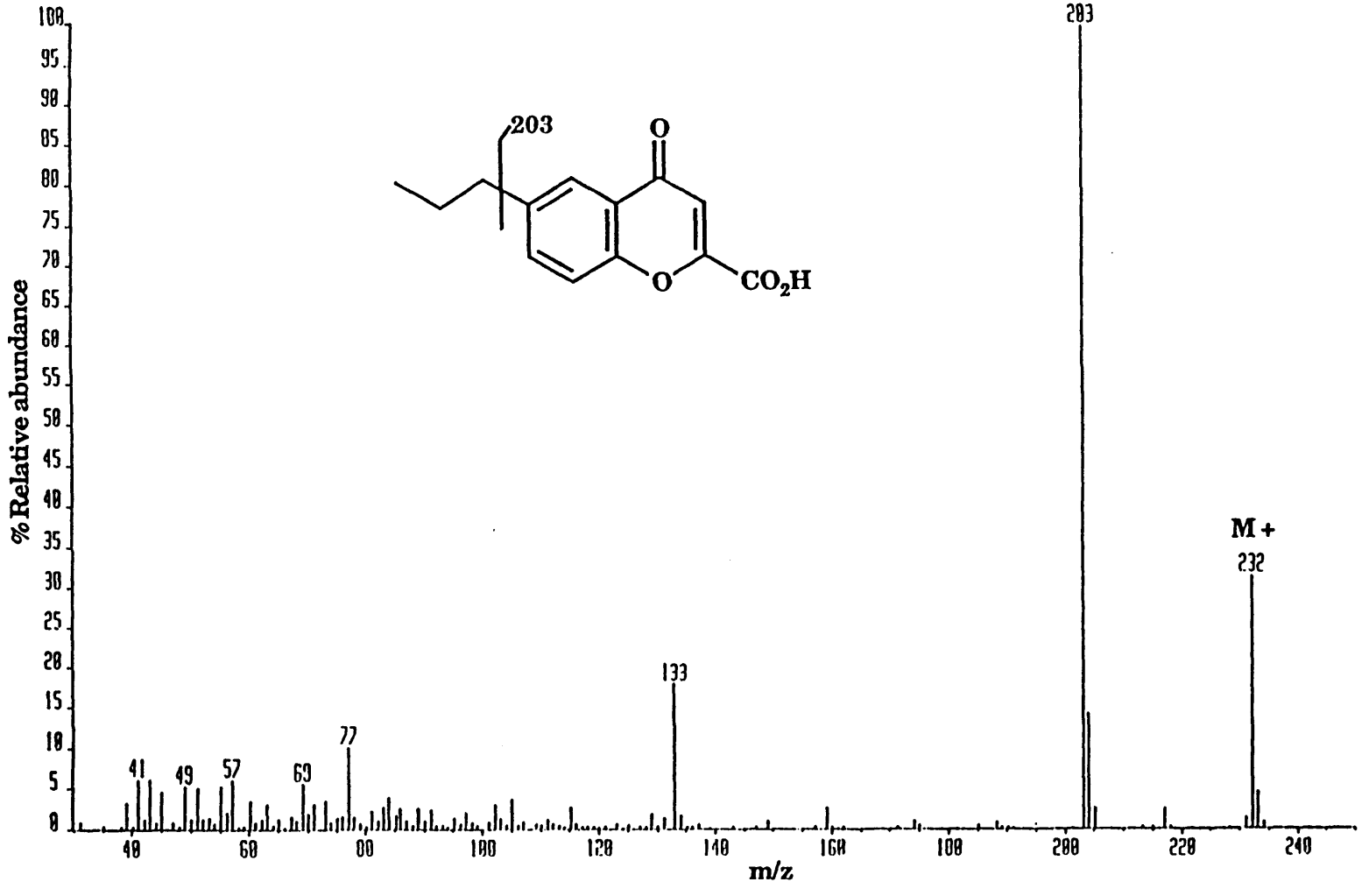


Figure 2.2 Mass spectrum of 6-n-propylchromone-2-carboxylic acid

Methyl 6-propylchromone-2-carboxylate.

6-propylchromone-2-carboxylic acid (45mg) was dissolved in BF_3 : methanol complex (14%, 6ml) and the solution refluxed for 1h at 90 °C. After cooling, the pH was adjusted to 7 with saturated sodium hydrogen carbonate solution and the resulting solution extracted into chloroform (2 x 1 vol). The combined extracts were dried over anhydrous sodium sulphate, reduced to dryness *in vacuo*, and the product recrystallised from water/methanol yielding methyl 6-propylchromone-2-carboxylate (34mg) as an off white crystalline solid. This synthesised compound gave an identical retention time (27.9 min, system RP 2) to that prepared and authenticated by Winter (1987).

Methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate.

The above methylation procedure was repeated using 6-(2'-hydroxypropyl)chromone-2-carboxylic acid (45mg), yielding methyl 6-propylchromone-2-carboxylate (36.1mg) as a white crystalline solid. This compound was authenticated by reference to a known standard synthesised by Winter (Rt 7.3 min, system RP 2).

Thin layer chromatography (TLC)

Thin layer chromatography was performed using System A, glass backed, pre-coated silica-gel F254 plates (20 x 20 x 0.2cm) (cat.no. 5717, Merck, FRG) developed with;

Chloroform : ethyl acetate (1 : 1 v/v), and/or

Systems B and C, aluminium backed, pre-coated silica-gel F254 plates (20 x 20cm x 0.25mm), (cat.no. 5554, Merck, FRG), developed with;

Ethyl acetate : hexane (1 : 1 v/v) and
Ethyl acetate : hexane (3 : 1 v/v) respectively.

Plates were developed by the ascending technique to 15cm from the origin, and compounds were located, as dark quenching spots under a U.V. lamp (254nm).

High performance liquid chromatography (HPLC)

HPLC analysis was performed using a Waters Associates pump (Model 6000A), a Rheodyne valve loop injector (No 7205), and a Waters Associates U.V. detector (model 441), operating at 254nm; the column (125mm x 4.0mm ID) was a HIBAR LiChrocart cartridge, prepacked with LiChrosorb RP-18 standard (Merck 15539). A Philips chart recorder (Model PM 2521) and a Shimadzu integrator (Model CR3A) was used for peak registration, calculation of retention times, and calculation of areas under peaks.

The following mobile phases (all compositions by volume) were used;

RP 1 30% aqueous methanol containing 0.1% trifluoroacetic acid (TFA);
After 18 mins, manually switched to
60% aqueous methanol containing 0.1% TFA

RP 2 45% aqueous methanol containing 0.1% TFA
After 18 mins, manually switched to
60% aqueous methanol containing 0.1% TFA

All flow rates were 1.0ml/min.

Mass spectrometry (MS) and gas chromatography-mass spectrometry (GC-MS).

All analyses were performed by D.J. Wilkinson of Fisons plc, Pharmaceutical Division, Loughborough.

Electron impact (E.I.,70eV) mass spectrometry was performed with a VG Model 70SEQ mass spectrometer, (VG Analytical Ltd, Withenshaw, Manchester, UK.), equipped with a Deak data system (Model PDP11-73), with a source temperature of 250°C using direct insertion into the ionisation chamber at 70eV.

Gas chromatographic separation was performed using a HP Model 5890 gas chromatograph. The 0.9m glass column (0.4cm I.D.) consisted of 3% (w/v) OV101 on a Chromosorb WHP (80-100 mesh) support. The temperature program for the column was 120°C for 20 secs, followed by a 8°C/min temperature rise to 300°C.

Preparation of trimethylsilyl (TMS) derivatives

Samples were treated with N,O-bis(trimethylsilyl)trifluoro-acetamide (BSTFA, 400µl) in tetrahydrofuran (50µl) prior to GC-MS analysis.

Nuclear magnetic resonance spectroscopy (NMR)

All samples were analysed by D.Hunter and D.J. Wilkinson of Fisons plc, Pharmaceutical Division, Loughborough.

^{1}H Nuclear magnetic resonance spectra were recorded at 360 MHz, using a Bruker AM 360 spectrometer (Bruker Spectrospin Ltd, Coventry, UK.). Samples were dissolved in $CDCl_3$, d_6-DMSO or $CDCl_3$/d_6-DMSO mixture, using tetramethylsilane as the internal standard.

Radiochemical techniques

Radioactivity in urine, cage washings, dose solutions, and chromatographic eluates were determined by liquid scintillation spectrometry, using a Tri-Carb Minaxi 4000 instrument (Canberra Packard, Pangbourne, Berks, UK). Samples (10-500µl) were counted in minivials using 3ml of Ecoscint scintillation cocktail, (National Diagnostics, Manville, New Jersey, USA). For samples < 500µl, (e.g. 0-24 h urine, and dose solutions) an appropriate volume of methanol was added in order to achieve a final total volume of 3.5ml.

Radioactivity in faeces was also determined by liquid scintillation counting but using a Tri-Carb 4640 instrument (Canberra Packard, Pangbourne, Berks, UK.). Samples (500µl) were also counted using 10ml Ecoscint.

Counting efficiency in both cases was assessed by reference to an external standard, and corrected using pre-determined quench correction curves stored in the data system of the spectrometers.

Radioactivity on TLC plates was located using a Series 900 mini monitor G.M. tube (Mini-Instruments Ltd, Burnham on Crouch, Essex, U.K.)

Radioactivity in HPLC eluent was determined by collecting fractions (0.5ml), using an LKB Redirac 2112 fraction collector, adding Ecosint (3ml) to each and counting using the Tri-carb Minaxi 4000.

Determination of radioactivity in faeces

This method was adapted from the procedure described by Caldwell (1972).

The entire faeces sample collected over a specific time period was placed into a plastic Stomacher bag, and a minimum amount of distilled water added in order to generate a manageable and consistent homogenate. The bag and contents were then placed into the Stomacher (Lab-Blender 80, model BA6020, Steward Medical, London, UK.) and homogenised for 1 min. Aliquots (2ml) in triplicate of homogenate were then placed into 10ml measuring cylinders, hydrogen peroxide (2ml) and sodium hydroxide (5M, 1ml) added to bleach the faeces, with iso-amyl alcohol (100 µl) to control foaming. The measuring cylinders were then capped with aluminium foil and left to stand at room temperature for 3 h. The samples were then neutralised with acetic acid (200µl) and ethanol added to give a final volume of 10.0ml. The measuring cylinders were than placed in a water bath for 30 min at 60 °C, and again capped to reduce evaporation. After cooling any reduction in total volume was corrected for by the further addition of ethanol. Aliquots (500µl) were then taken for liquid scintillation counting.

Animals and dosing

Six male Wistar rats, weighing approximately 200g (Oxford Laboratory Animals Co), were maintained on a commercially available pellet diet (Labsure CRM, Biosure Ltd, Manea, Cambridge).

Six male Duncan-Hartley guinea pigs, weighing approximately 200g (Porcellus Animal Breeding Ltd), were maintained on a pellet diet (Labsure RGP).

One female Dutch rabbit, weighing approximately 2.5kg (Ranch Rabbits, Sussex), was maintained on a pellet diet (Labsure R14B).

[2,2'-^{14}C]-6-n-Propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg) was administered to the rats and guinea pigs by intraperitoneal injection of a solution prepared by the addition of the appropriate quantity of [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid to its unlabelled equivalent which was then dissolved in a mixture of ethanol and isotonic sodium hydrogen carbonate (2:3 v/v, 1ml per dose).

6-n-Propylchromone-2-carboxylic acid (500μmol/kg) was administered to the rabbit by intraperitoneal injection, dissolved in a mixture of ethanol and isotonic sodium hydrogen carbonate as above.

The animals were housed in individual metabolic cages at room temperature and were allowed food and water *ad libitum* throughout the experiments. Excreta were collected daily for three days after the administration of the dose. After each collection the cages were washed, and the washings retained for analysis.

Identification of urinary metabolites

Urines and cage washes were centrifuged (3000 x g, 10mins) and their ^{14}C content determined by liquid scintillation counting of aliquots of the supernatants in triplicate. Samples of neat 0-24 h urine from each animal were examined by reverse phase HPLC (system RP 1). Quantitation of unchanged drug and its metabolites was achieved by collection of discrete HPLC fractions and liquid scintillation counting, with a HPLC column efficiency of 90%. The pH of all samples was adjusted to 5 with 2M hydrochloric acid before storage at -20°C.

Isolation of urinary metabolites

The method for the isolation of the metabolites is illustrated in Scheme 2.1

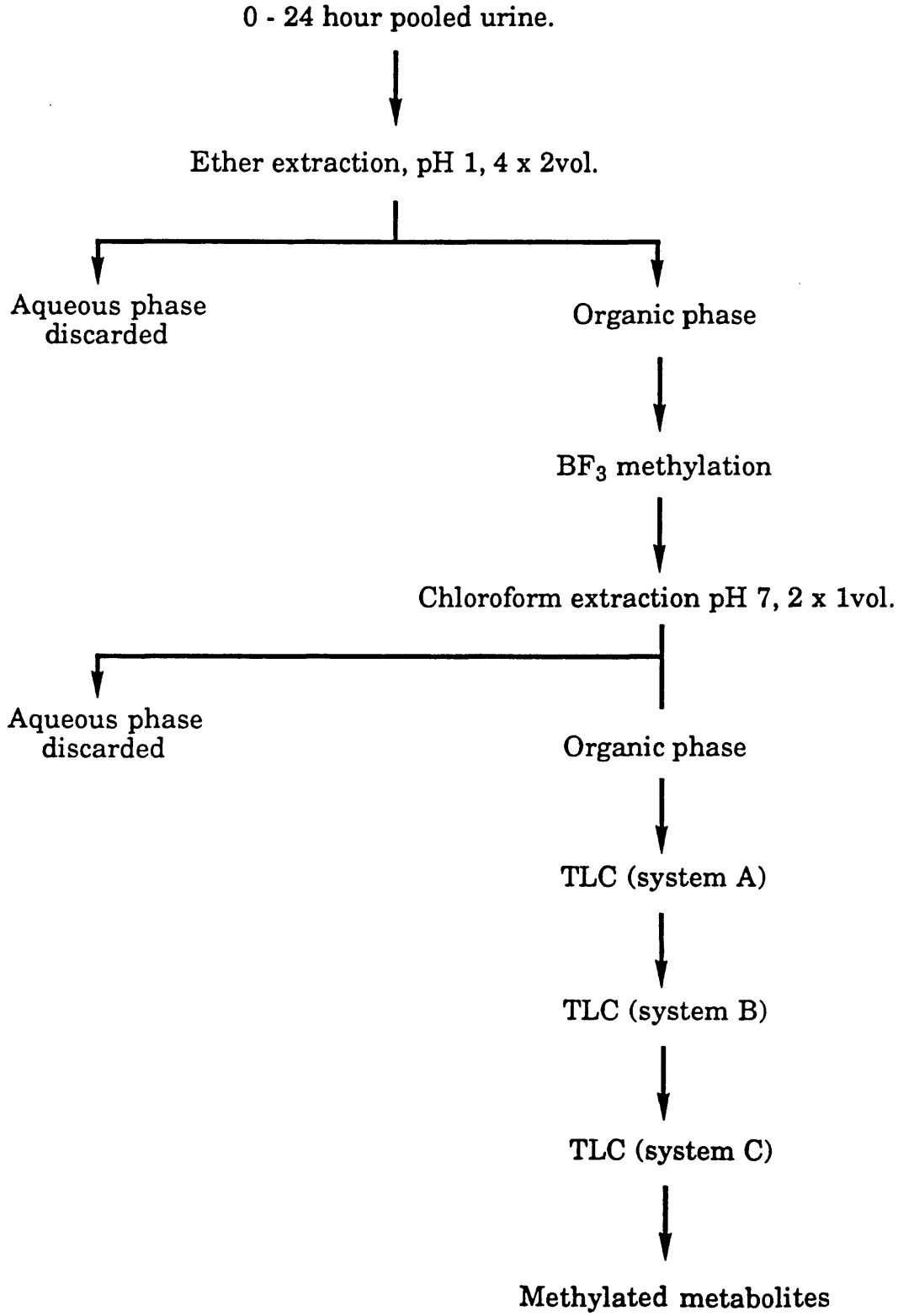
The 0-24 h urine from each animal was acidified to pH 1, by dropwise addition of conc. hydrochloric acid, and extracted with ether (4 x 2 vol). A few drops of propan-2-ol were added to prevent formation of emulsions. The combined extracts were dried over anhydrous sodium sulphate and the solvent removed *in vacuo*. The residue obtained was redissolved in methanol (4ml) and transferred to a thick-walled screw-top vial, BF_3 : methanol complex (20ml, 14%) added and the whole heated to 90 ^{0}C for 1 h. After cooling, the reaction mixture was neutralised with saturated sodium hydrogen carbonate solution and extracted with chloroform (2 x 1 vol). The combined extracts were dried over anhydrous sodium sulphate, reduced to dryness *in vacuo*, taken up in methanol (4ml), and an aliquot (100µl) removed for reverse phase HPLC analysis

(system RP 2). All subsequent procedures were thus carried out on the methyl esters and not the free acids.

The remaining methanol was again reduced to dryness, the residue taken up in chloroform (1ml) and applied to silica-gel TLC plates. The plates were developed in system A and the areas of silica corresponding to the position of the radioactive bands removed and eluted with ethyl acetate (40ml). The samples were centrifuged (3000 x g, 10min) to remove any suspended silica, and the supernatant concentrated as before and rechromatographed on TLC in system B. Again the areas of silica-gel containing the radioactive metabolites were removed, eluted with ethyl acetate (10ml), centrifuged and concentrated. The samples were then rechromatographed for the third and final time on TLC in system C, the appropriate areas of silica-gel removed and extracted with ethyl acetate (10ml). After centrifugation the supernatant was filtered through cotton wool to remove any traces of silica and concentrated under a stream of nitrogen.

Scheme 2.1

Isolation procedure for urinary metabolites of 6-n-propylchromone-2-carboxylic acid.



2.3 Results

NMR and MS of 6-propylchromone-2-carboxylic acid

Identification of any unknown metabolites isolated after the administration of the probe compound 6-n-PCCA will be made easier if both NMR (Fig 2.1) and MS (Fig 2.2) spectra of the compound are fully understood.

NMR of 6-propylchromone-2-carboxylic acid

The protons associated with the propyl side chain, attached to the chromone nucleus, give clear resonance signals as expected. As electron density around the individual protons decreases, the protons resonate at lower fields, thus the protons of the 3'-C CH_3 substituent, (resonating as a triplet), produced this signal at 0.95 ppm, whilst the protons of the 1'-C CH_2 substituent (resonating as a triplet), produced this signal at 2.71ppm. The protons of the 2'-C CH_2 group (resonating as a multiplet), produced this signal between the other two at approximately 1.70 ppm. The synthetic sample unfortunately contained traces of ethanol, as a consequence of recrystallisation, the protons of which produced signals at 1.21 and 1.67 ppm, $\underline{CH_3}CH_2OH$ and $CH_3\underline{CH_2}OH$, respectively. These signals were comparatively simple to assign, as their integrals were not consistent with those of the molecule under investigation.

The C-3 proton of the chromone moiety resonates as an uncoupled singlet at a relatively high field (7.10 ppm) compared to the three aromatic protons, due to the shielding by the anisotropic effect of the

pyrone carbonyl group and the reduced aromatic ring current. The three aromatic protons, C-5, C-7 and C-8, constitute an ABX coupling system, in which C-7 and C-8 can be regarded as the A and B protons and C-5 as X. The signal for the C-5 proton is split to a partially resolved doublet by meta coupling (2-3 Hz), with the C-7 proton, and resonates at a lower field (7.96 ppm) to that of C-7 and C-8 due to deshielding by the anisotropic effect of the pyrone carbonyl group.

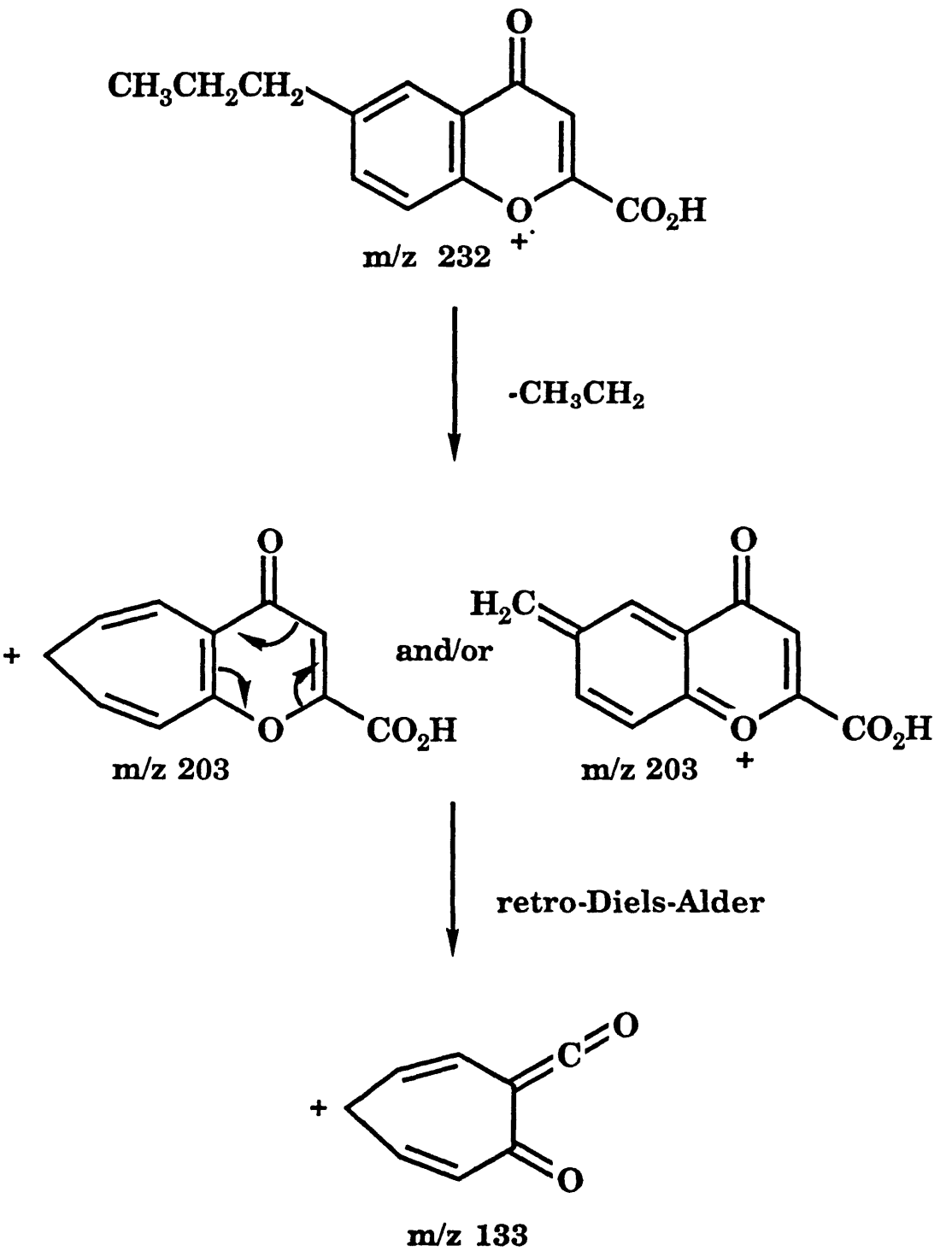
Ideally, the C-7 and C-8 protons should first generate two doublet AB system signals (ortho coupling). The doublet of proton C-7 should then be further split by meta coupling with C-5. Unfortunately the spectra obtained for 6-n-PCCA showed the signals for C-7 and C-8 as an unresolved multiplet, indicating little discrimination between the two protons.

Mass spectrum of 6-n-propylchromone-2-carboxylic acid

This compound was subjected to analysis by direct insertion (probe) mass spectrometry and is shown as Figure 2.2. The prominent ions occur at m/z 232 (molecular ion), 203 (base peak), and 133. The molecular ion (m/z 232) which fragments to give the base peak (m/z 203), results from cleavage of the 1'C-2'C bond of the 6-n-propyl substituent, yielding either the conjugated oxonium ion or the tropylium ion (Fig 2.3). Further fragmentation via the retro-Diels-Alder reaction, with the loss of an acetylene derivative, results in the ion fragment at m/z 133.

Figure 2.3

Proposed mass spectral fragmentation of 6-n-propylchromone-2-carboxylic acid.



Metabolism in the rabbit.

Although radiochemical detection was not employed, HPLC analysis (system RP 1) of neat urine (0-24 h), obtained from the treated rabbit, revealed the presence of a single metabolite, having a retention time of 8.97 min. On comparison with reference standards, this compound was identified as 6-(2'-hydroxypropyl)chromone-2-carboxylic acid (6-2'-HPCCA). Unchanged 6-n-propyl-chromone-2-carboxylic acid (6-n-PCCA) was also present in the neat urine (Rt 26.9 min) and the peak area ratio calculated to be 66.0 : 34.0, 6-2'-HPCCA/6-n-PCCA respectively (Fig 2.4).

Isolation of methyl 6-2'-HPCCA was achieved using solvent extraction and TLC as previously described, with R_F values of 0.40, 0.24, and 0.53 recorded for solvent systems A, B, and C respectively. The enantiomeric composition of this compound was investigated later (see Chapter 3).

Metabolism in the rat.

The excretion of radioactivity by male rats receiving 500μmol/kg, 20μCi/kg of 6-n-propylchromone-2-carboxylic acid is presented in Table 2.1.

74% of the administered dose was excreted in the urine over the 72 h period, identifying this as the major route of excretion. Elimination was rapid, with some 79% of the administered dose excreted during the first 24 h, with much smaller quantities being voided during the following two days. The total recovery of radioactivity after 72 h was 87%.

Figure 2.4

HPLC analysis (system RP 1) of 0-24 h urine from the female rabbit dosed with 6-n-propylchromone-2-carboxylic acid (500μmol/kg).

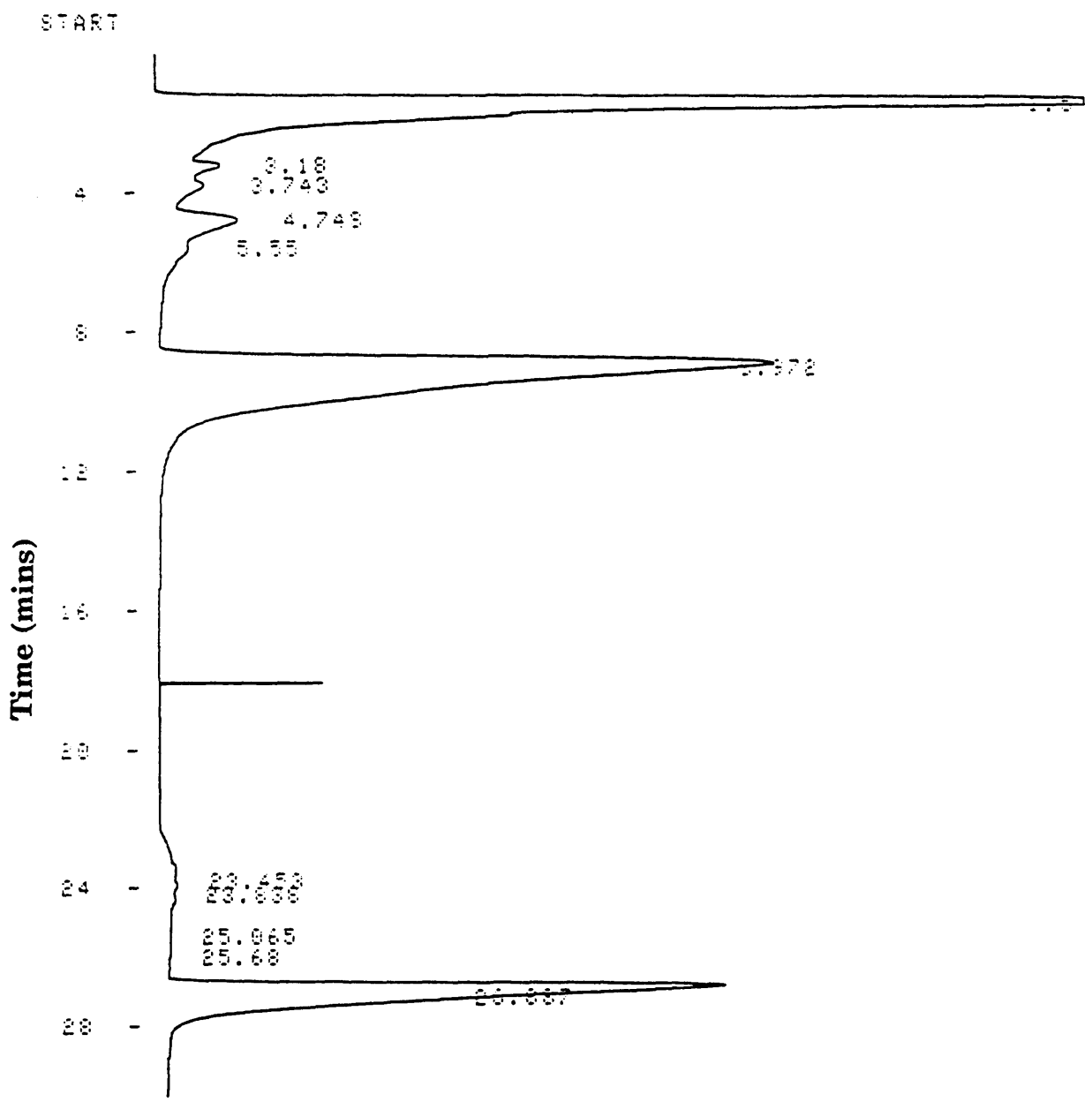


Table 2.1

Excretion of radioactivity after intraperitoneal administration of [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid to male rats and male guinea pigs, at a dose level of 500μmol/kg, 20μCi/kg.

	% Excretion of radioactivity			
Period after dosing (h)	rat		guinea pig	
	urine	faeces	urine	faeces
0-24	68.0 ±6.8	10.7 ±9.9	80.2 ±7.7	4.9 ±3.0
24-48	4.7 ±3.4	3.1 ±3.7	2.7 ±2.4	0.8 ±0.8
48-72	1.0 ±1.0	0.0 ±0.0	1.3 ±1.4	0.5 ±0.7
Total	73.7	13.8	84.2	6.2
Σ	87.5		90.4	

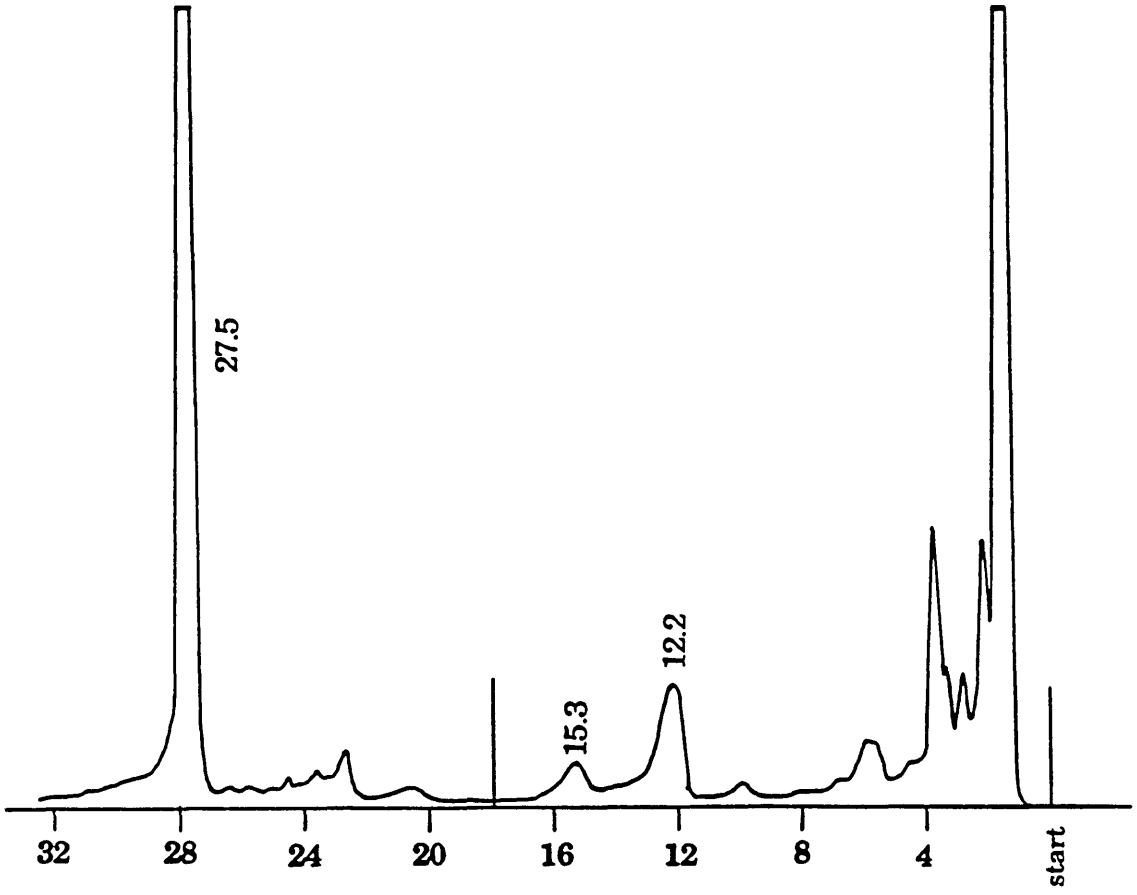
Mean ± S.D. of 5 animals for each species.

Radio HPLC analysis of the neat 0-24 h urine (system RP 1), showed the presence of three ^{14}C peaks (Fig 2.5). The peak at Rt 27.5 min, accounted for 80% ±1.3 (n=5) of the 0-24 h urinary radioactivity, and was identified by comparison with standards as unchanged 6-n-propylchromone-2-carboxylic acid (6-n-PCCA). The radioactive metabolite observed with Rt 12.2 min, was identified as 6-(2'-hydroxypropyl)chromone-2-carboxylic acid (6-2'-HPCCA) and accounted for 13% ±0.7 (n=5) of the 0-24 h urinary radioactivity, whilst a second metabolite whose retention time (15.3 min) did not correspond to an available reference compound, accounted for 7% of urinary radioactivity.

After extraction and methylation with BF_3 : methanol complex, the samples were analysed by radio HPLC (system RP 2), which resolved the radioactivity present into four peaks (Fig 2.6), two corresponding to 6-n-PCCA and 6-2'-HPCCA and two unknowns. Quantification of these peaks is presented in Table 2.2.

Figure 2.5

A typical U.V. absorbance trace and radiochromatogram from HPLC analysis (system RP 1) of 0-24 h urine from male rats dosed with [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg).



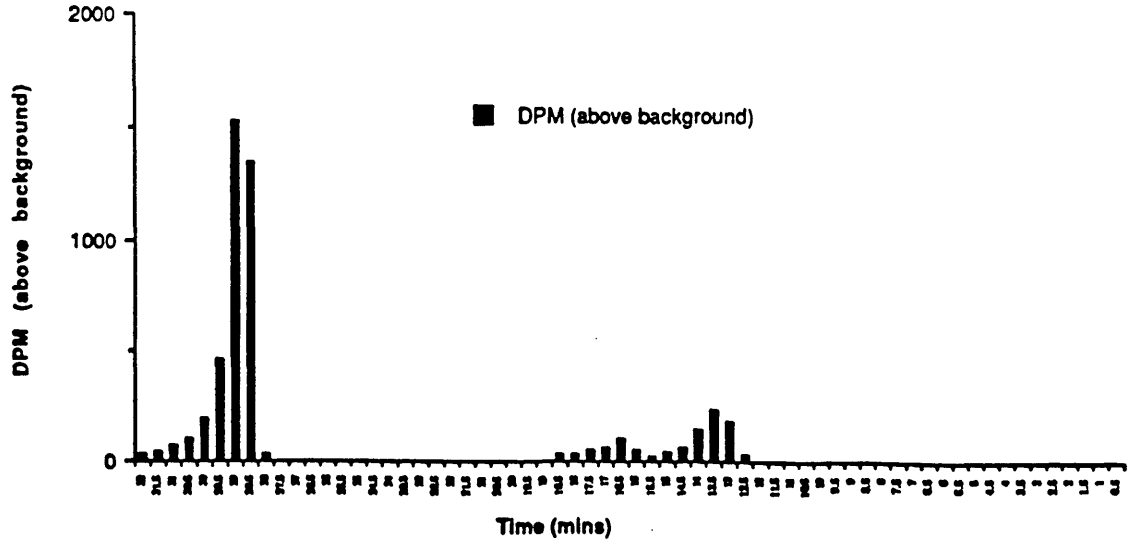
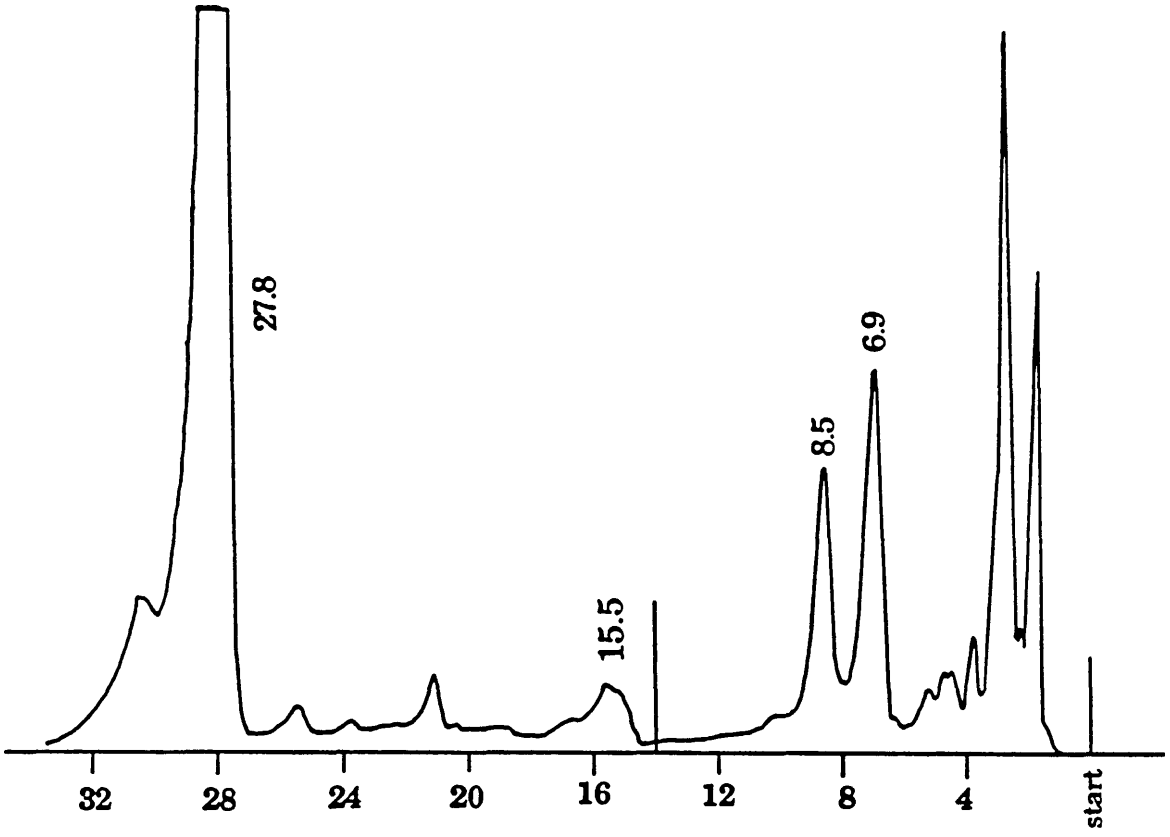


Figure 2.6

U.V. absorbance trace and radiochromatogram from HPLC analysis (system RP 2) of 0-24 h urine from male rats dosed with [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg) after methylation with boron trifluoride.



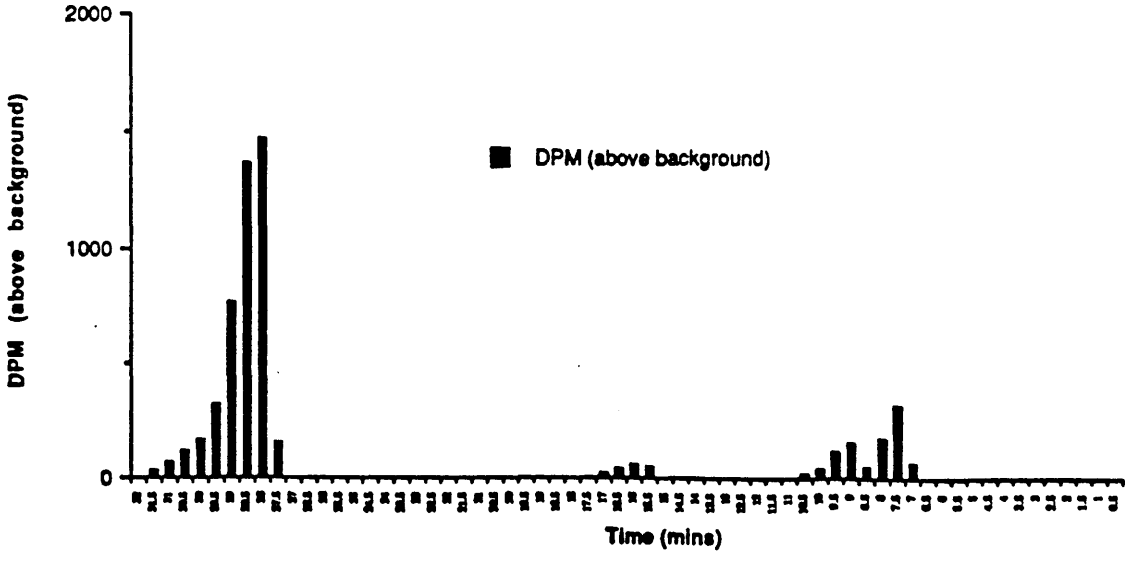


Table 2.2

Percentage radioactivity of methylated compounds present in the 0-24 h urine excreted by male rats after administration of [2,2'^{14}C] 6-n-propylchromone-2-carboxylic acid at dose level of 500μmol/kg, 20μCi/kg.

Peak Rt (mins)	Compound (as methyl ester)	% 0f 0-24 h urinary radioactivity
27.8	6-n-PCCA	79.3 ± 2.4
6.9	6-2'-HPCCA	11.3 ± 1.7
8.5	Unknown (X)	6.0 ±1.2
15.5	Unknown (Y)	3.0 ± 1.0

Mean ± S.D. of 5 animals

Isolation of the methylated metabolites was achieved using solvent extraction and TLC as previously described.

Unknown X

This compound had R_F values of 0.58, 0.45, and 0.75 in TLC solvent systems A, B, and C respectively. This compound was isolated as a white solid, in sufficient quantity for mass spectrometry and NMR analysis.

The ^1H NMR spectrum (360 MHz; $CDCl_3$) of the compound is shown in Fig 2.7 The signals were assigned as follows:

ppm (multiplicity, integration, assignment), 0.93 (t, 3H, $\underline{CH_3}CH_2CHOH$), 1.86 (m, 1H, $CH_3\underline{CH_2}CHOH$), 4.02 (s, 3H, $CO_2\underline{CH_3}$), 4.76 (t, 1H, $CH_3CH_2\underline{CH}OH$), 7.12 (s, 1H, 3-H), 7.27 (s, $CDCl_3$), 7.60-7.63 (d, 1H, 8-H), 7.79-7.82 (q, 1H, 7-H), 8.12-8.13 (d, 1H, 5-H).

MS analysis (EI, Fig 2.8) revealed the following:
EI: m/z (% rel.abund.) 264 (6), 233 (100), 231 (10), 149 (8).

NMR of unknown X

Figure 2.7 shows the NMR spectra obtained from the methylated and isolated unknown X. On comparison with the NMR from the synthetic 6-PCCA, it is clear that changes to the 6-propyl substituent have occurred.

The 3'C methyl three proton triplet at 0.93 ppm remains unchanged from that observed in the parent compound, indicating that the 2'C CH_2 group is also present in the unknown metabolite. This 2'C CH_2 group, resonating as a multiplet, centred at 1.86 ppm, is shifted downfield relative to the corresponding group in the parent compound, suggesting a new electron withdrawing group attached to the 1'-carbon, as well as the proton resonating as a triplet at 4.76 ppm. The methylated carboxyl function at the C-2 position on the chromone moiety, produces a new three proton singlet at 4.02 ppm but the C-3 proton singlet at 7.12 ppm remains virtually unaffected. All the signals from the aromatic ABX coupling system, are shifted downfield, due to the influence of the new group at the 1'C position.

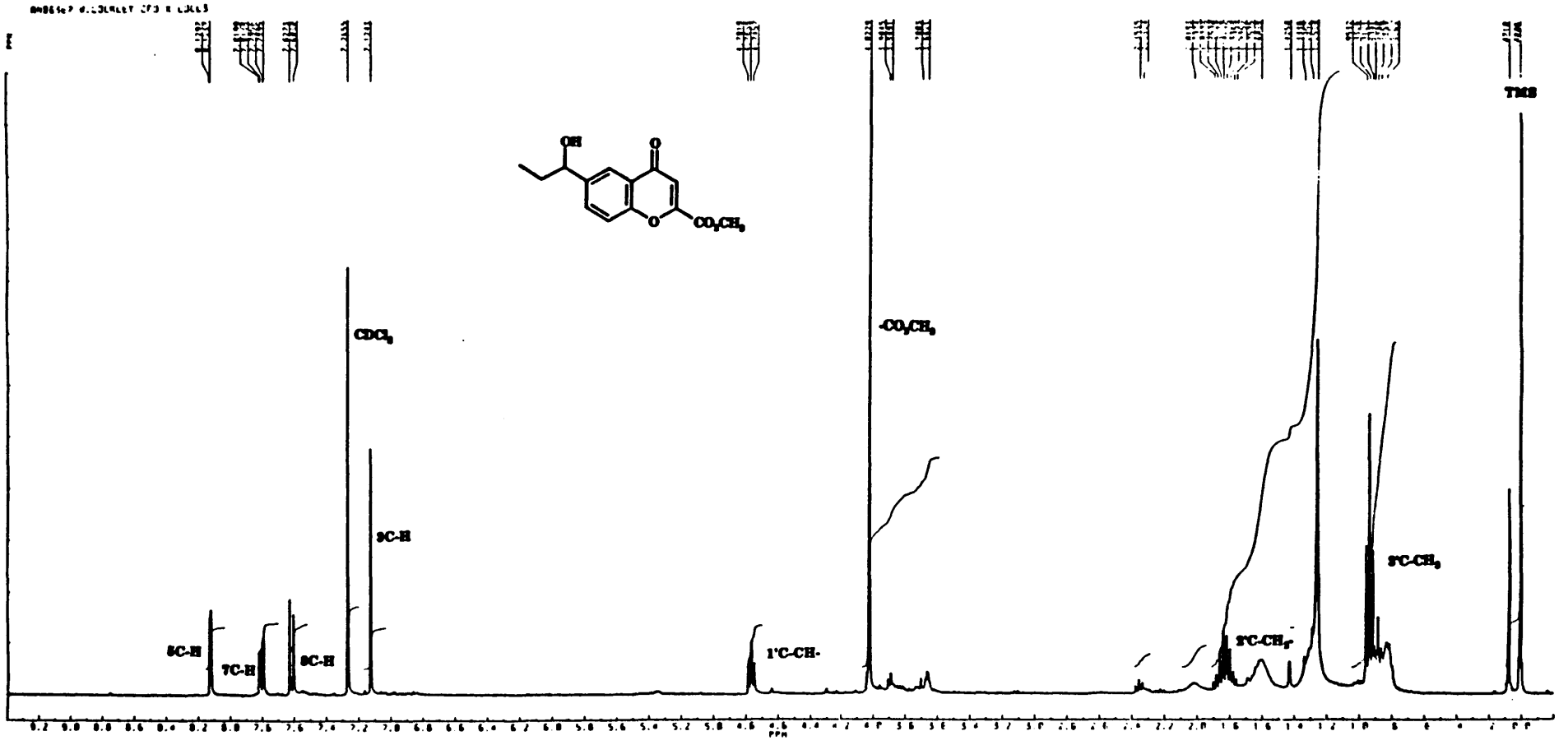


Figure 2.7 ^{1}H NMR spectrum (360MHz) of unknown X isolated from the 0-24 h urine of male rats dosed with 6-n-propylchromone-2-carboxylic acid (500μmol/kg).

This has resulted in the resolution of the C-7 and C-8 signals, forming a single proton quartet at 7.79-7.82 ppm and a single proton doublet at 7.60-7.62 ppm respectively. The spectrum also contains some impurity signals.

The NMR spectrum therefore suggests unknown X to be methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate.

Mass spectrum of unknown X.

The mass spectrum of this compound is shown in Figure 2.8. The prominant ions occur at m/z 262 (molecular ion), 233 base peak), 231, and 149. The methylated parent ion (methyl 6-propylchromone-2-carboxylate), would have a molecular ion of 246 amu, but the spectrum reveals the molecular ion of this compound to be 262 amu, an increase of 16 amu over the parent compound, suggesting the addition of one oxygen to the molecule. This, in conjunction with the loss of 29 amu (C_2H_5) by β cleavage of the 1'C-2'C bond to give the base peak, suggest a hydroxylated metabolite, with the oxygen inserted at the 1'C position of the 6-n-propyl substituent.

The compound was trimethylsilylated and the GC-mass spectrum of this derivative is shown in Figure 2.9. The ion at highest mass occurred at m/z 319, corresponding to the loss of 15 amu (CH_3) from a mono-TMS derivative. The prominent ion at m/z 305, corresponding to the loss of 29 amu (C_2H_5), again suggests a hydroxylated metabolite with oxygen inserted at the 1'C position of the parent compound.

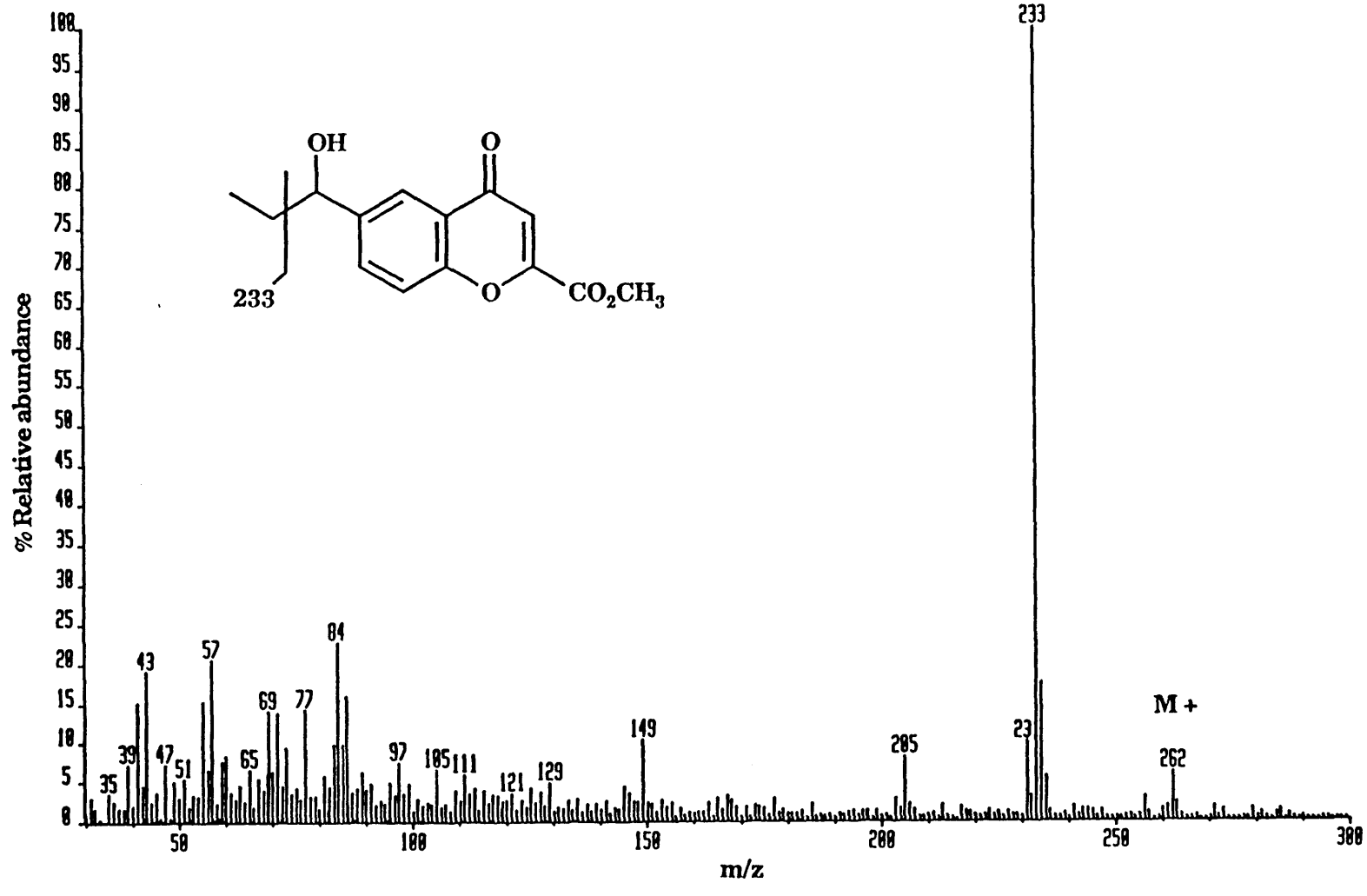


Figure 2.8 Mass spectrum of unknown X isolated from the 0-24 h urine of male rats dosed with 6-n-propylchromone-2-carboxylic acid (500μmol/kg).

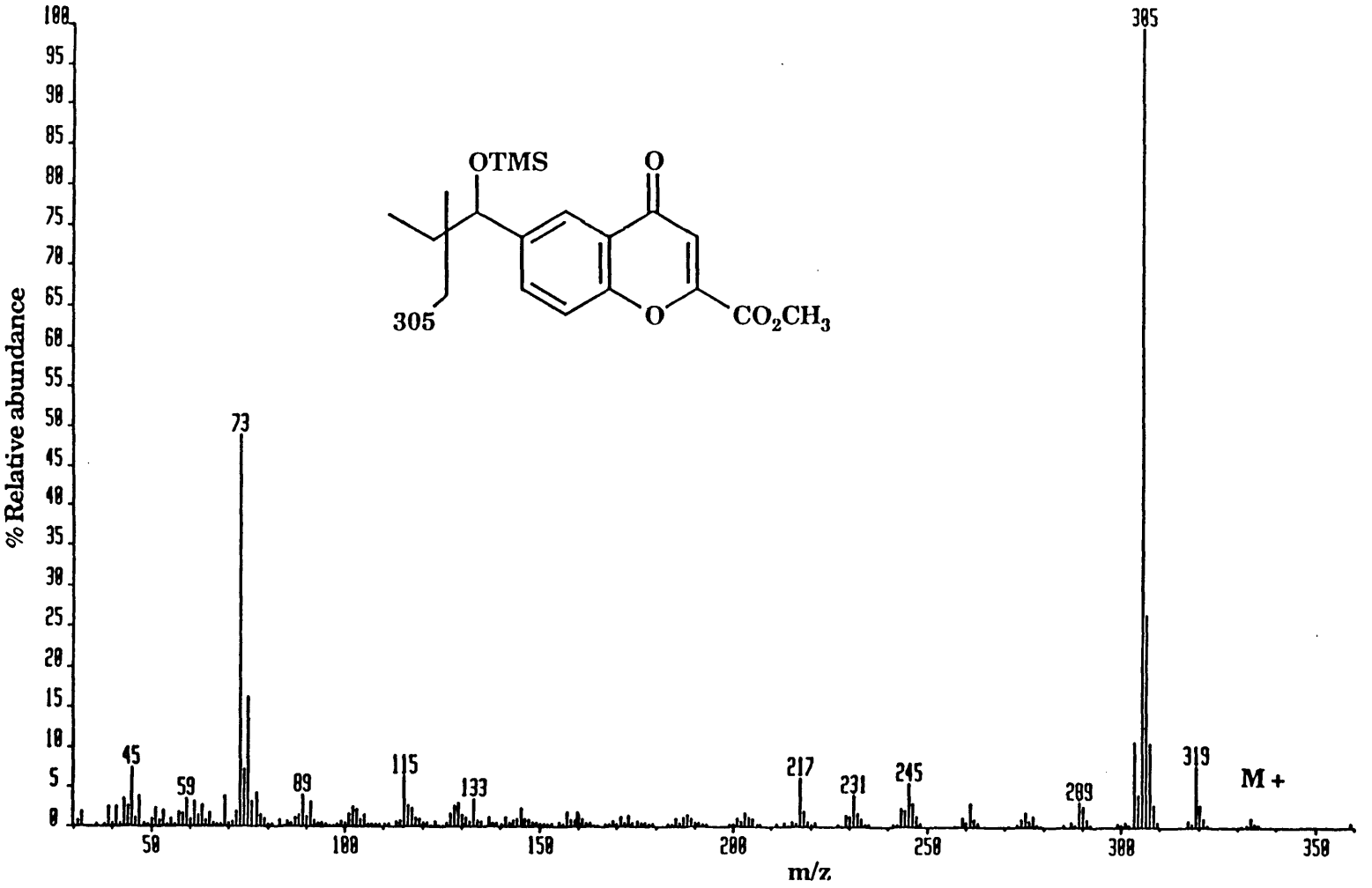
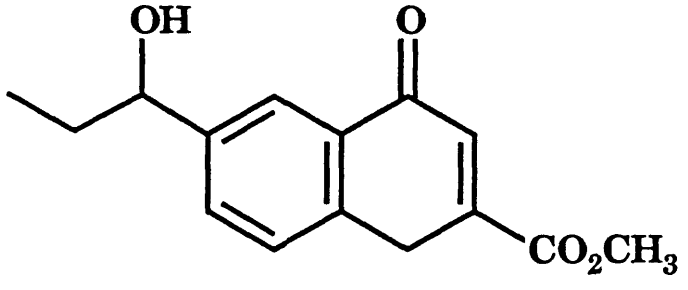


Figure 2.9 Mass spectrum of TMS derivative of unknown X.

The conclusions derived from the mass spectral data are consistent with those from the NMR, and together they indicate that unknown X, the methylated metabolite of 6-n-propylchromone-2-carboxylic acid to be methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate (Fig 2.10).

Figure 2.10

The structure of methyl 6-(1'-hydroxypropyl)-chromone-2-carboxylate.



Unknown Y

This compound had R_F values of 0.79, 0.64 and 0.83 in TLC solvent systems A, B and C respectively. This compound was isolated as a pale yellow solid, in sufficient quantity for mass spectrometry and NMR analysis.

The ^1H NMR spectrum (360 MHz; $CDCl_3$) of this compound is shown in Figure 2.11. The signals were assigned as follows:

ppm (multiplicity, integration, assignment), 2.70 (t, 2H, $CH_3O_2CH_2\underline{CH_2}$), 3.07, (t, 2H, $CH_3O_2\underline{CH_2}CH_2$), 3.68 (s, 3H, $\underline{CH_3}O_2CH_2CH_2$), 4.02 (s, 3H, $CO_2\underline{CH_3}$), 7.11 (s, 1H, 3-H), 7.27 (s, $CDCl_3$), 7.54-7.56 (d, 1H, 8-H), 7.60-7.63 (d, 1H, 7-H), 8.01-8.02 (d, 1H, 5-H).

MS analysis (EI, Fig 2.12) revealed the following:
EI: m/z (rel. abund.) 290 (31), 259 (8), 231 (100), 217 (25).

NMR of unknown Y

Figure 2.11 shows the NMR spectrum obtained from the methylated metabolite. On comparison with the NMR from the synthesised 6-PCCA it is clear that again changes to the 6-propyl substituent have occurred.

The 3'C methyl three proton triplet centered at 0.95 ppm of the parent compound is lost in the spectrum of the unknown Y, whilst a new three proton singlet at 3.67 ppm corresponding to a methylated carboxyl group at 3'C is seen. The two proton of the 1'C CH_2 group remains virtually uneffected at 2.70 ppm, whereas the 2'C signal is shifted downfield to 3.07 ppm and becomes a two proton triplet. This downfield shift and reduction in resonance is consistent with the proposed methylated carboxyl group at the 3'C position. The methylated carboxyl function at the 2-C position of the chromone nucleus produces a new three proton signal at 4.02 ppm. The characteristic 3-C single proton signal remains unaffected at 7.10 ppm, as does the 5-C single proton doublet at 8.01-8.02 ppm, whilst the C-7 and C-8 signals from the aromatic ABX coupling system are shifted downfield. This has resulted in the resolution of the two signals; a single proton quartet at 7.60-7.63 ppm and a single proton doublet at 7.54-7.56 ppm, C-7/C-8 respectively, due to the influence of the new methylated carboxyl group at the 3'C position.

The NMR therefore suggests the unknown Y to be methyl 6-(methyl-3'-carboxypropyl)chromone-2-carboxylate.

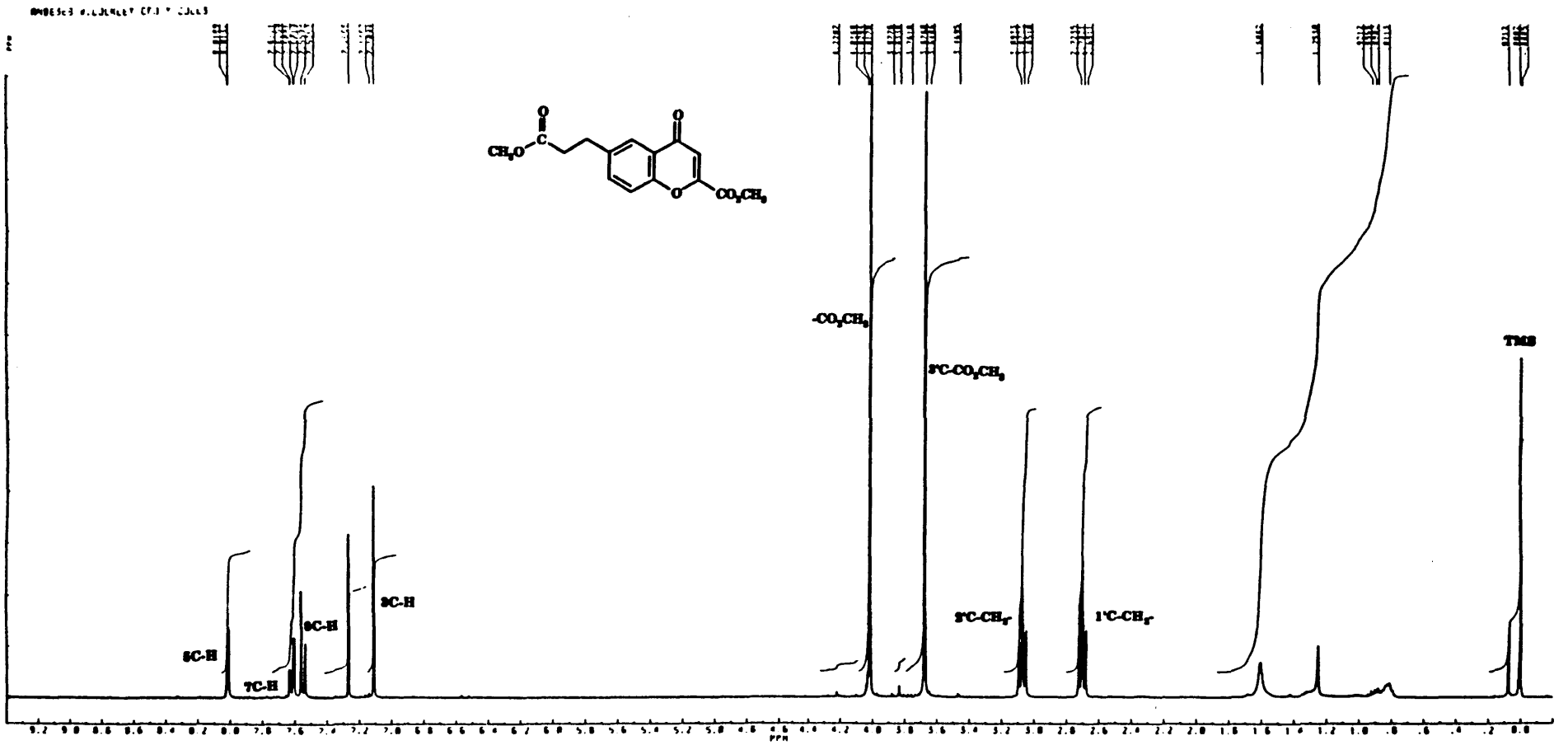


Figure 2.11 ^{1}H NMR spectrum (360MHz) of unknown Y isolated from the 0-24 h urine of male rats dosed with 6-n-propylchromone-2-carboxylic acid (500μmol/kg).

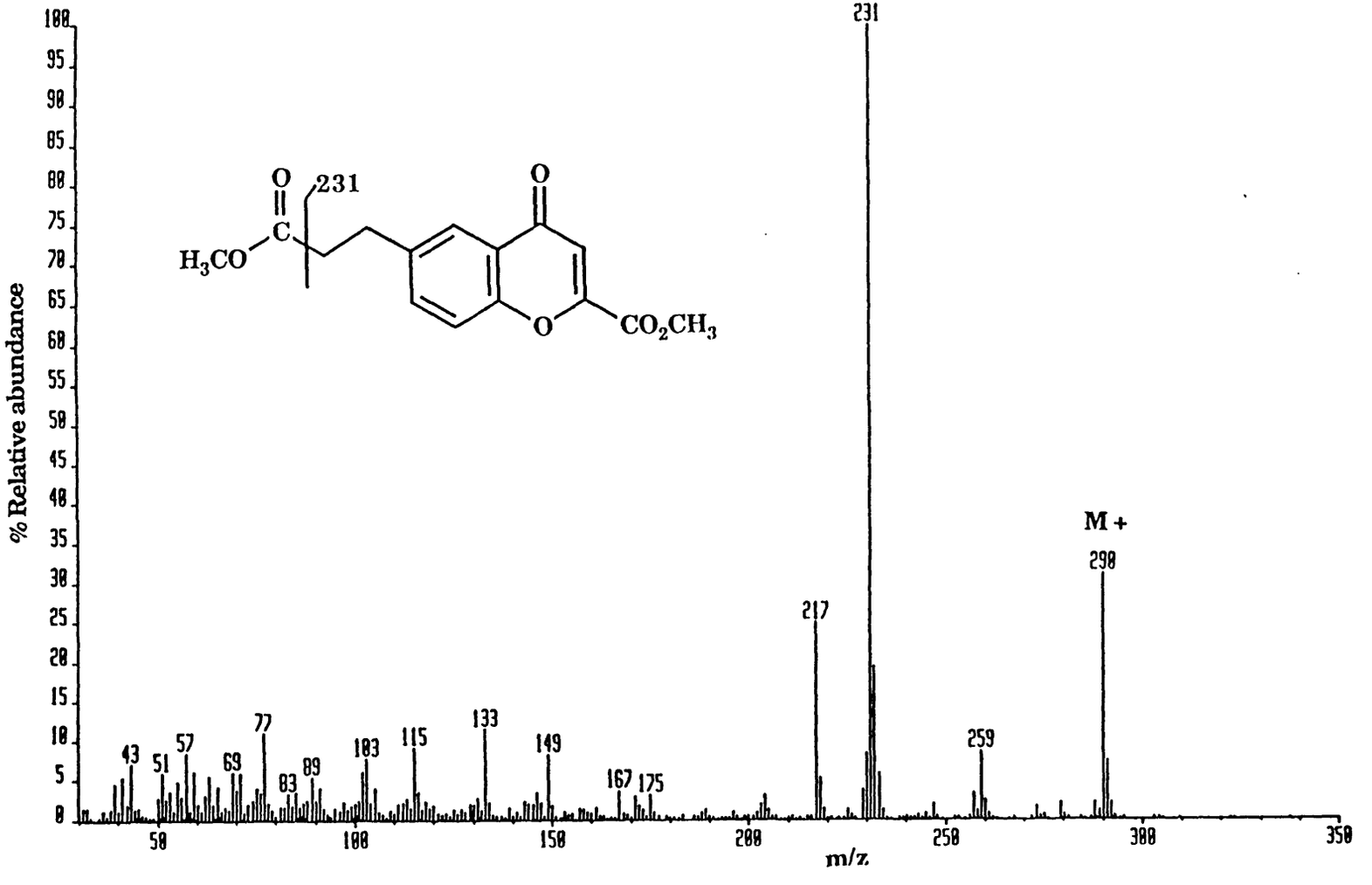


Figure 2.12 Mass spectrum of unknown Y isolated from the 0-24 h urine of male rats dosed with 6-n-propylchromone-2-carboxylic acid (500μmol/kg).

Mass spectrum of unknown Y

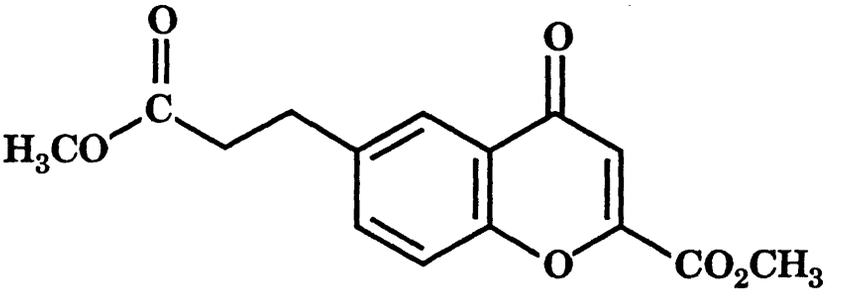
The mass spectrum of the compound is shown as Figure 2.12 The prominent ions occur at m/z 290 (molecular ion), 259, 231 (base peak), and 217. The methylated parent ion (methyl 6-propylchromone-2-carboxylate) would have a molecular ion of 246 amu, but the spectrum reveals the molecular ion of this compound to be 290 amu, an increase of 44 amu over the parent compound. The loss of 59 amu ($C_2H_3O_2$) by β cleavage of the 2'C-3'C bond to give a base peak at m/z 231, in conjunction with the initial fragmentation with the loss of 31 amu (CH_3O), suggests the presence of a methylated carboxyl group at the terminal 3'C position of the 6-n-propyl substituent.

The compound was trimethylsilylated and the GC- mass spectrum of the derivative obtained. This spectrum was identical to that of the underivatised compound, confirming the conclusion, that there were no alcohol groups in the molecule.

The conclusions derived from the mass spectral data are consistent with those of the NMR data, and together they indicate that unknown Y, the methylated metabolite of 6-n-propylchromone-2-carboxylic acid to be methyl 6-(methyl-3'-carboxyethyl)chromone-2-carboxylate (Fig 2.13).

Figure 2.13

The structure of methyl 6-(methyl-3'-carboxyethyl)-chromone-2-carboxylate.



Metabolism and excretion in the guinea pig.

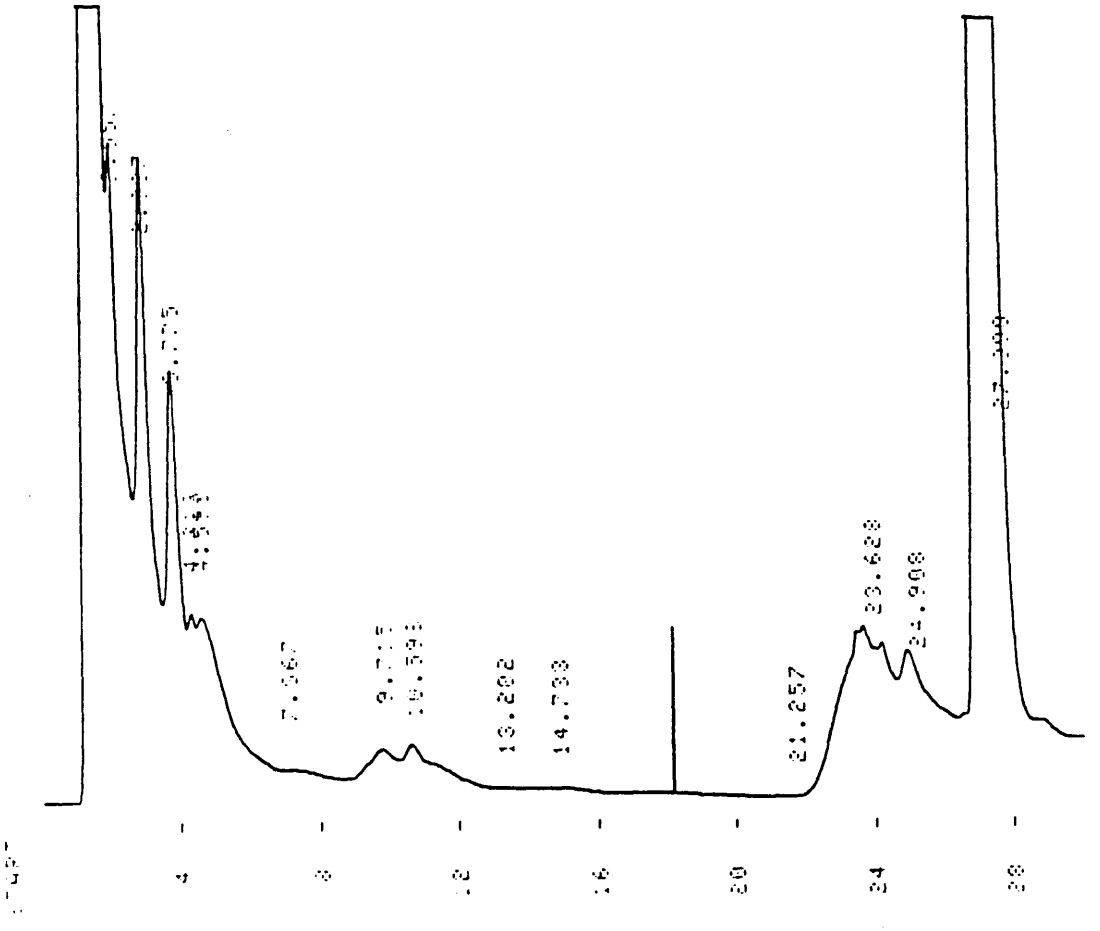
The excretion of radioactivity by guinea pigs receiving 500μmol/kg, 20μCi/kg of 6-n-propylchromone-2-carboxylic acid is presented in Table 2.1

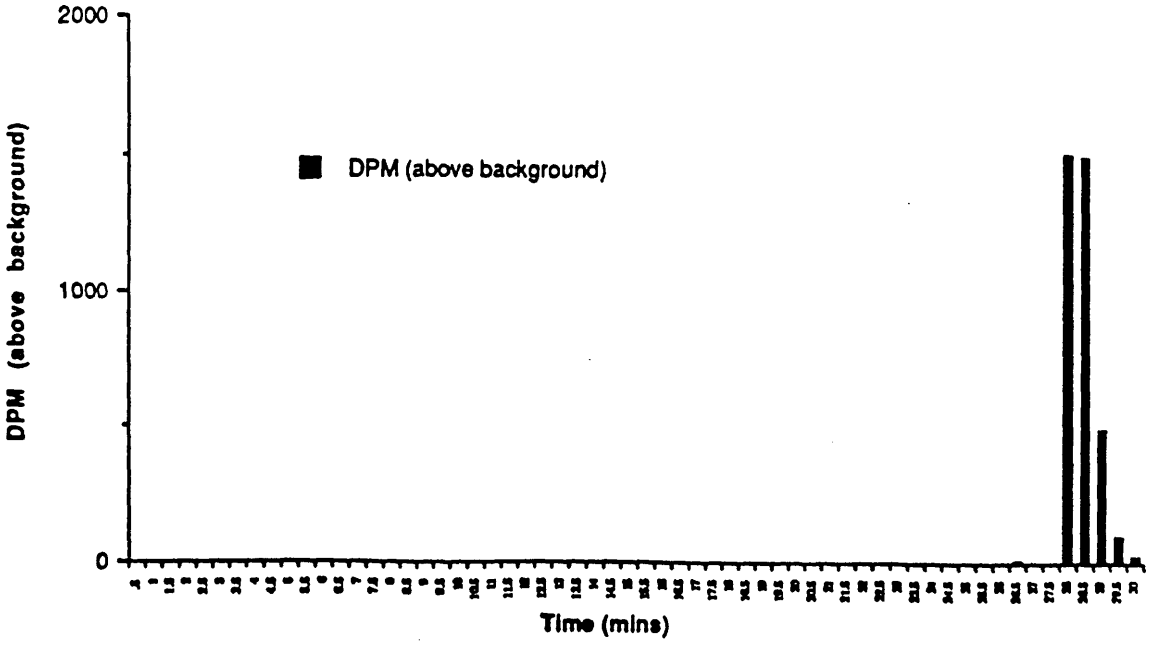
84% of the administered radioactive dose was excreted in the urine over the 72 h period, showing this to be the major route of excretion. Elimination was rapid with some 85% of the administered dose excreted during the first 24 h with much smaller quantities being voided during the following two days. The total recovery of radioactivity after 72 h was 90%.

Radio HPLC analysis (system RP 1) of neat 0-24 h urine showed only one radioactive peak to be present (Fig 2.14). The peak at Rt 27.1 min accounted for 100% of the 0-24 h radioactivity and was identified as unchanged 6-n-propylchromone-2-carboxylic acid.

Figure 2.14

U.V. absorbance trace and radiochromatogram from HPLC analysis (system RP 1) of 0-24 h urine from male guinea pigs dosed with [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg).





2.4 Discussion

The metabolism of 6-n-PCCA in the female rabbit gave rise to a single metabolite identified on HPLC as 6-2'-HPCCA, arising from (ω-1)-hydroxylation of the n-propyl substituent.

This result and that obtained by Winter (1987) is somewhat surprising, in that no (ω) or (ω-2) oxidation was observed in such a multicomponent and complex system. Straight chain alkanes generally show a predominance of (ω-1)-hydroxylation (Introduction 1.3), but usually show some (ω), and somewhat more (ω-2)-hydroxylation. In the case of short alkyl side chains adjacent to an aromatic ring (as with 6-n-PCCA), preferential hydroxylation of the benzylic carbon is usually observed (e.g ethylbenzene).

The absence of (ω-2)-hydroxylation may be in part due to; (a) the inability of the aromatic ring contained in the chromone nucleus to resonance stabilise either radical or carbonium ion intermediates formed in the transition state of the hydroxylation, (b) the constraints of the active site of the enzyme(s).

The metabolism of proxicromil also exhibits this unusual site of hydroxylation (Smith and Neale 1983, Introduction 1.5), in that alicyclic ring systems fused to an aromatic ring are normally hydroxylated in the carbon attached to the aromatic ring, but the major metabolite observed in man, rabbit, and rat was the product of hydroxylation in the cyclohexenyl ring, at position 7-.

Oxidation of proxicromil with potassium dichromate yielded the expected 6-carbon hydroxylated product, suggesting that the more reactive site had not been accessable for biological oxidation. Unlike proxicromil, 6-n-PCCA does not contain a electron withdrawing hydroxyl substituent attached to the C-5 position of the chromone nucleus. This relative increase in the electron density associated with the aromatic ring would resonance stabilise either radical or carbonium ion intermediates formed at the benzylic position, making it a strong candidate for the site of metabolism.

This would suggest that the limited regioselective pattern of hydroxylation observed after the administration of 6-n-PCCA to the female rabbit is due to biological constraints such as the topology of the active site of the enzymes involved. For example, if 6-n-PCCA binds to a single cytochrome P450 isozyme, enzymatic discrimination between regiotopic groups within the molecule can only occur if the isozyme is flexible enough to attack the more reactive or labilised sites. As no (ω-2) metabolite was observed, a single isozyme system would need to possess a rigid active site, in order to restrict hydroxylation of this regiotopic group.

In contrast, Clark *et al* (1982) reported the metabolism of 6,8-diethyl-5-hydroxychromone-2-carboxylic acid in various species. The principal site of hydroxylation was the benzylic (ω-1) position of the 6-ethyl substituent, with only limited hydroxylation occurring at the ω site.

After the administration of 6-n-propylchromone-2-carboxylic acid to male guinea pigs, no metabolites were observed, only unchanged dose compound. Smith *et al* (1986) observed strong similarities in the

metabolism of the chromone-2-carboxylic acids between various species (not including the guinea pig), with the rate of metabolism rather than the route being the major difference. The dog does not metabolise 6,8,-diethyl-5-hydroxychromone-2-carboxylic acid, (Clark *et al* 1982) or proxicromil (Smith and Neale, 1983). This may suggest that the guinea pig and the dog do not possess the specific cytochrome P450's required for the metabolism of these compounds.

A relevant anomaly exhibited by the guinea pig exists for the metabolism of amphetamine. Amphetamine can undergo either aromatic hydroxylation giving 4-hydroxy amphetamine, or side chain degradation to benzoic acid (Caldwell 1980) In the guinea pig side chain breakdown is the only route of metabolism due to a deficiency in aromatic hydroxylation, whereas in other species both routes are significant, particularly in the rat where ring hydroxylation predominates.

The metabolism of 6-n-propylchromone-2-carboxylic acid in the male rat gave rise to three metabolites; 6-2'-HPCCA, 6-1'-HPCCA and 6-3'-CPCCA accounting for 7.7 %, 4.1 %, and 2.0 % of the dose respectively.

This pattern of regioselectivity is more consistent with those reported in the literature for short alkyl side chains adjacent to an aromatic ring system. For example. El Masry *et al* (1956), studied the fate of propylbenzene in the rabbit and showed the 3-carbon side chain to undergo (ω-2)- and (ω-1)-hydroxylation, as well as side chain cleavage to benzoic acid. Sangster *et al* (1983) observed that p-propylanisole was metabolised in rat and mouse, by (ω-2)-and (ω-1)-hydroxylation, forming the 1'- and 2'-hydroxy-p-propylanisole, as well as p-methoxyhippuric acid. It was proposed that although p-methoxyhippuric acid could be

formed via a 1'.2' diol intermediate, it more likely arises via 3'-hydroxy-p-propylanisole, which was presumed to be rapidly oxidised to p-methoxyphenylpropionic acid and converted to p-methoxybenzoic acid by β-oxidation. This proposed route of metabolism may indicate the possible origin of the 3'-carboxylic acid metabolite, in that initial hydroxylation may generate a 3'-hydroxy intermediate (not detected), which is subsequently further oxidised forming the dicarboxylic acid. The absence of β-oxidation would account for its excretion and detection in the urine. The apparent absence of β-oxidation is perhaps surprising but a similar metabolic profile was reported for the metabolism of 6-n-pentylchromone-2-carboxylic acid in the female rabbit by Winter (1987). In contrast after the administration of 6-butylchromone-2-carboxylic acid, 6-(2'-carboxymethyl)chromone-2-carboxylic acid was isolated, suggesting degradation by β-oxidation had occurred.

Although the major site of hydroxylation was again the (ω-1) position, other regiotopic groups on the 6-n-substituent were susceptible to hydroxylation, namely the ω, and (ω-2) positions. These different sites of hydroxylation might indicate that the metabolism of 6-n-PCCA in the male rat involves a range of cytochrome P450 isozymes, and/or a single enzyme with a very flexible active site and/or allowing multiple modes of binding. Although the cytochrome P450 monooxygenases collectively display a broad range of substrate specificities, the individual isozymes are characterised by a high degree of substrate and product selectivity. Waxman (1988) established that several of the testosterone metabolites corresponded to P450 enzyme-characteristic metabolites, in that some P450 isozymes exhibit pronounced regioselectivity. For example P45011C11, P45011A1, and P45011B1, hydroxylate testosterone exclusively at the 2α-, 7α-, and 16β- positions respectively. Additionally,

Frommer *et al* (1972), from inducer and inhibitor studies, showed that n-heptane was hydroxylated by at least 3 monooxygenases, each displaying its own regioselectivity (Introduction 1.3). The regioselective pattern observed for the hydroxylation of 6-n-PCCA may therefore depend on the relative concentrations of the enzymes involved, the relative enzyme-substrate affinity constants and any differences in the transition state energies.

However, a single enzyme with a flexible active site cannot be ruled out. White *et al* (1984) showed that the substrates, D-camphor, adamantanone and adamantane were hydroxylated by $P450_{cam}$ to give the 5-exo-, 5- and 1-hydroxylated derivatives respectively, indicating there to be a tight enzyme-substrate complex. In contrast, the same substrates yielded two or more isomeric products when hydroxylated by cytochrome P450 IIB4. This suggests that within this enzyme-substrate complex there is considerable movement of the substrate.

Chapter 3

Species Differences in the Stereoselective Metabolism of 6-n-Propylchromone-2-Carboxylic Acid

3.1 Introduction

The single metabolite, 6-2'-HPCCA excreted in the 0-24 h urine after administration of 6-n-PCCA to the female rabbit was purified as its methyl ester (Winter 1987) and subsequent polarimetric analysis showed that it possessed a slight dextroratory activity, suggesting that one enantiomer was present in excess of its antipode. Further investigation revealed the two enantiomers to be present in the ratio 24:76, and the assignment of absolute configuration, using the method described by Dale and Mosher (1973) showed that the (S)-enantiomer predominated. This observed preferential metabolism was attributed to product stereoselective hydroxylation, rather than stereoselective distribution and excretion, as the enantiomeric composition of the methylated 6-2'-HPCCA isolated after administration of synthetic racemic 6-2'-HPCCA was consistently found to be 1:1.

This chapter reports results of further investigations into the enantiomeric composition of the 1'- and 2'-hydroxy chiral metabolites excreted in the urine after the administration of 6-n-PCCA to the female rabbit and the male rat.

3.2 Materials and Methods

Materials and methods as preceding chapter unless otherwise stated.

Compounds

All chemicals used in the syntheses are listed with their sources in Appendix 3.1

(+)-S-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) was purchased from JPS Chimie, Bevaix, Switzerland.

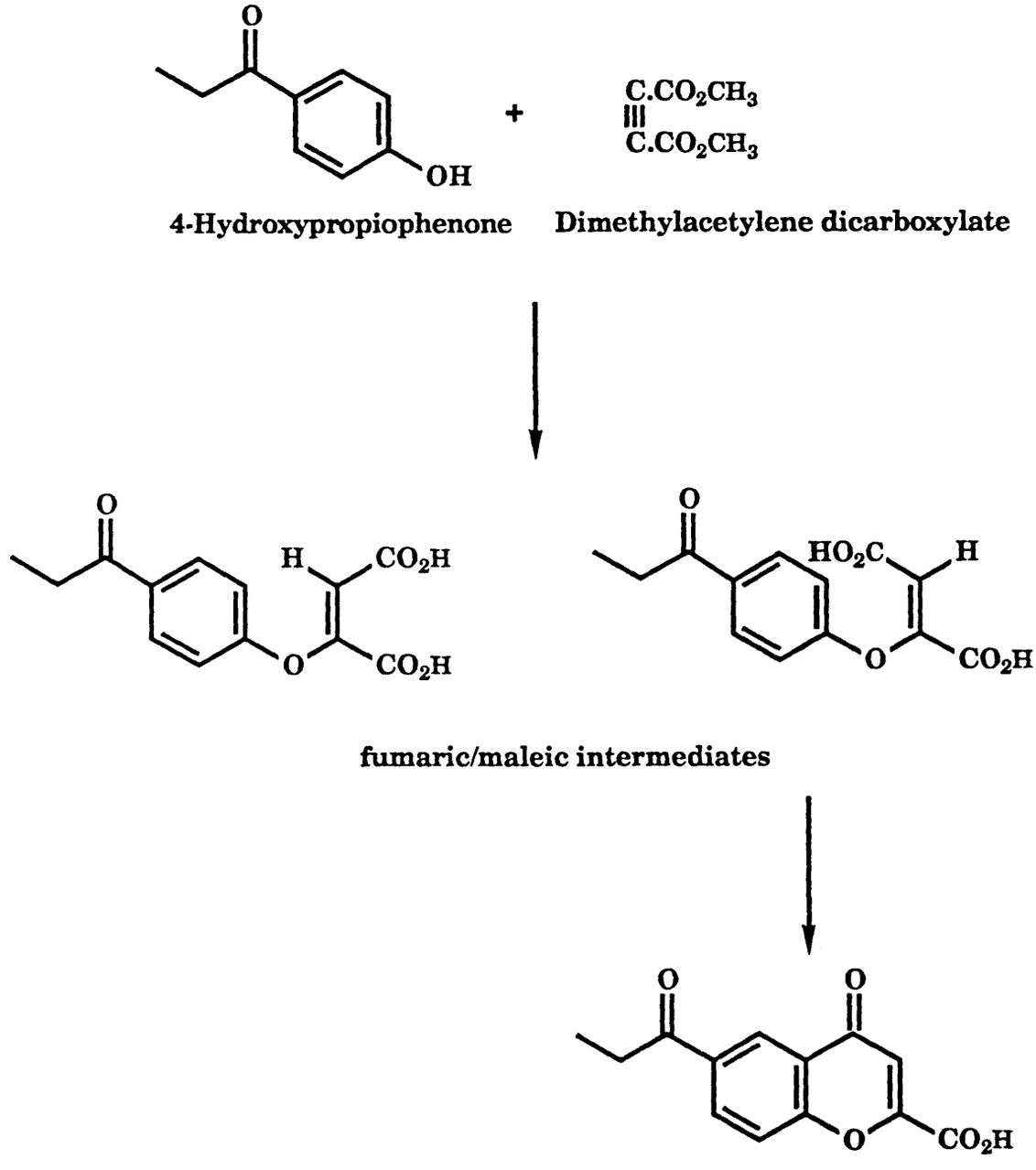
Methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate was isolated from the urine of a female Dutch rabbit after the administration of 500μmol/kg 6-n-propylchromone-2-carboxylic acid (Chapter 2).

6-(1'-Oxopropyl)chromone-2-carboxylic acid

6-(1'-Oxopropyl)chromone-2-carboxylic acid was prepared by a Ruhemann synthesis, involving the Michael addition of 4-hydroxy-propiophenone to dimethyl acetylenedicarboxylate to yield a fumaric/maleic intermediate. Cyclisation of the trans isomer with chlorosulphonic acid completes the reaction sequence yielding 6-(1'-oxopropyl)chromone-2-carboxylic acid (Fig 3.1).

Figure 3.1

The Ruhemann synthesis of 6-(1'-oxopropyl)chromone-2-carboxylic acid.



4-Hydroxypropiophenone (10.0g), dimethyl acetylenedicarboxylate (9.4g) and N-benzyl trimethylammonium hydroxide (Triton B, 25 drops) were dissolved in dioxan (80ml), and the reaction mixture heated over a steam bath for 1 h. Sodium hydrogen carbonate (13.3g), dissolved in methanol/water (330ml, 1 : 1 v/v), was added and the entire mixture refluxed on an electric mantle for 24 h. Upon cooling, distilled water (300ml) was added and the whole then washed with ether (1 vol), acidified to pH 1 with hydrochloric acid (2M) and washed again with chloroform (1 vol). The resulting mixture was then extracted into ether (3 x 1 vol) and the combined extracts washed with distilled water (1 vol). The ether was then dried over anhydrous sodium sulphate and the mixture reduced to dryness *in vacuo*, to yield the fumaric/maleic intermediate (1.1372g) as a pale yellow/orange solid. The mass and NMR spectra of the product were consistant with the proposed structure.

Cold chlorosulphonic acid (10ml) was added drop-wise to the fumaric/maleic intermediate (1.0g), in an ice bath and stirred for 1 h. The reaction mixture was then poured slowly and cautiously on to crushed ice (approximately 300ml) and stirred for a further 1 h. The resulting precipitate was filtered, washed with cold distilled water and cold chloroform and recrystallised twice from ethanol. This yielded 6-(1'-oxopropyl)chromone-2-carboxylic acid (0.2131g) as a white crystalline solid. The mass and NMR spectra of the product were consistant with the proposed structure and shown in Appendices 3.2 and 3.3 respectively.

6-(1'-Hydroxypropyl)chromone-2-carboxylic acid

6-(1'-Oxopropyl)chromone-2-carboxylic acid (200mg) and sodiun hydrogen carbonate (34mg) were dissolved in 12ml of a carbonate buffer pH 10 (1.32g sodium carbonate and 0.95g sodium hydrogen carbonate in 100ml water). Sodium borohydride (32mg) was then added and the reaction mixture stirred for 2 hs at room temperature. The pH was then adjusted to 1 with conc. hydrochloric acid and the whole extracted with ether (3 x 2 vol). The combined extracts were dried over anhydrous sodium sulphate, reduced to dryness *in vacuo*, yielding a light-brown solid material (0.1624g). This material, containing the required product 6-(1'-hydroxypropyl)chromone-2-carboxylic acid was not purified further, to restrict losses to a minimum. The sructure of this compound was authenticated by mass spectrometry and NMR, which are shown in Appendices 3.4 and 3.5 respectively.

Methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate

The crude 6-(1'-hydroxypropyl)chromone-2-carboxylic acid (0.1624g), was methylated using BF_3 : methanol complex (14%), as described in Chapter 2, and the products extracted into chloroform (2 x 1 vol). After drying (anhydrous Na_2SO_4) and reducing to dryness *in vacuo*, the residue obtained was redissolved in a mixture of methanol/water (1:1 ratio). This solution was then applied to a C18 endcapped Clean-up Cartridge, (100mg/1ml, Technicol, No CEC18111), and eluted with a mixture of methanol/water (1ml, 1.5:1 v/v). Any impurities present in the solution were retained on the column whilst the required compound was eluted directly. The initial "loading solution" and the eluent were pooled and reduced to dryness under a stream of nitrogen, to yield

methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate (0.1039g) as a white solid.

Methylation using diazomethane

N-Nitrosomethyl urea (300mg) was placed in a smooth-walled test tube and dissolved in cold ether (4ml). Cold aqueous potassium hydroxide (2ml, 50% w/v) was carefully added and the reaction mixture allowed to stand for 1 h in ice. The diazomethane produced from this reaction dissolves directly into the ether layer, which consequently turns a straw-yellow colour. On completion of the reaction the ether layer was then removed cautiously and stored briefly on ice prior to use.

Samples for methylation were placed in a smooth-walled test tube, reduced to dryness under nitrogen and redissolved in methanol (100µl). Ethereal diazomethane (300µl) was then added and the reaction mixture allowed to stand for 30 min on ice. If no colour was observed in the ether layer, an additional 300µl of diazomethane solution was added. Methylation was considered complete when the ether remained coloured, indicating the presence of excess diazomethane. The samples were then reduced to dryness under nitrogen.

Derivatisation using (+)-S-methyl-α-(trifluoromethyl) phenylacetyl chloride (MTPA-Cl).

Methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate or methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate was dissolved in pyridine (350µl). (+)-(S)-MTPA-Cl (35µl) was then added and the reaction mixture shaken for 24 h at room temperature. On completion, the reaction

mixtures were solvated with chloroform (10ml) and washed successively with;

(a) 5ml saturated sodium hydrogen carbonate solution
(b) 5ml distilled water
(c) 5ml hydrochloric acid (0.5M)
(d) 2x5ml distilled water

The chloroform extract was then dried over anhydrous sodium sulphate and reduced to dryness with a stream of nitrogen

Preparation of methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate-α-methoxy-α-trifluoromethyl-phenylacetates (MTPA derivatives).

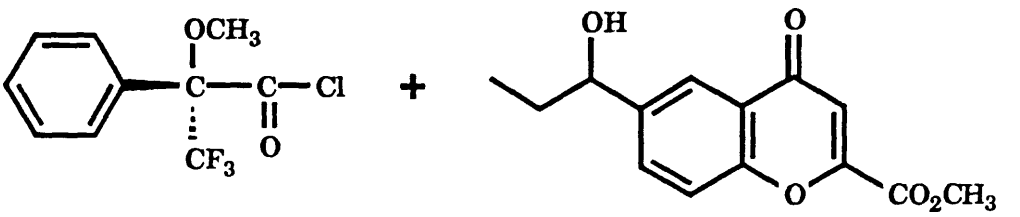
Racemic methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate (24mg) was esterified with (+)-(S)-MTPA-Cl (35µl) as described above. This yielded the two diastereomeric esters (22.7mg combined) as a pale yellow solid, which was then analysed using normal phase HPLC (system NP 1, see later)

Preparation of methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate-α-methoxy-α-trifluoromethyl-phenylacetates (MTPA-derivative) standards.

Racemic methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate (40mg) was esterified with (+)-S-MTPA-Cl (60µl) as described above for methyl-6-(2'-hydroxypropyl)chromone-2-carboxylate, (proportionally increasing all compounds) and illustrated in Figure 3.2.

Figure 3.2

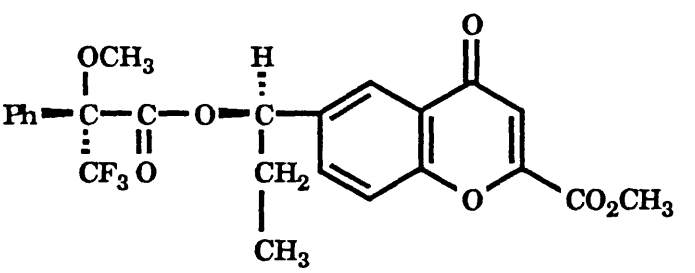
Derivatisation of racemic methyl 6-(1'-hydroxypropyl)-chromone-2-carboxylate (R,S-I) with (+)-(S)-MTPA-Cl, (Mosher's reagent) to yield the diastereomeric esters S-I-R-MTPA and R-I-R-MTPA.



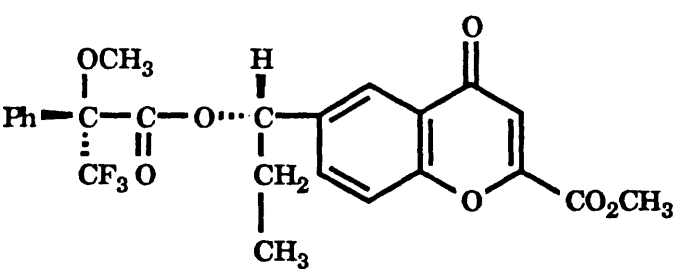
(+)-S-MTPA-Cl

methyl 6-(1'-hydroxypropyl)-chromone-2-carboxylate (R,S-I)

pyridine @ room temperature for 24 hours



R-I-R-MTPA ester



S-I-R-MTPA ester

The resulting chloroform solution was reduced to dryness under a stream of nitrogen, yielding a straw-yellow liquid containing the two diastereomeric esters, which was than analysed using normal phase HPLC (system NP 2), giving the chromatogram as shown in Figure 3.3. The individual diasteromeric esters were then isolated by normal phase semi-preparative HPLC (system NP 3), using the peak shaving technique, followed by recrystallisation from methanol.

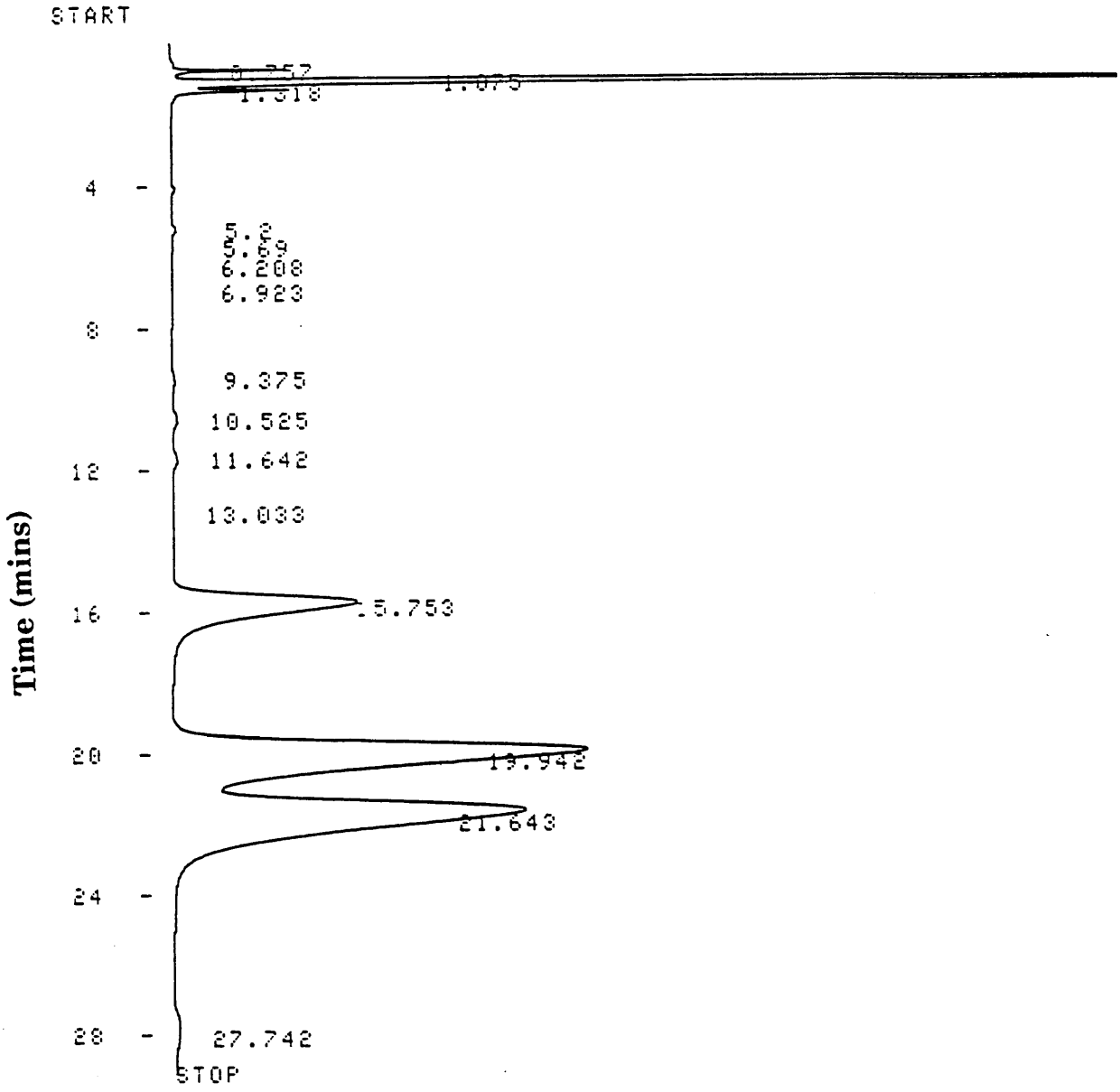
^{1}H NMR analysis (360 MHz,$CDCl_3$) of the individual diastereomers are shown in Figure 3.4 (the MPTA ester having the shorter retention time on HPLC (system NP 2, peak 2), and Figure 3.5 (the MTPA ester having the longer retention time on HPLC (system NP 2, peak 3), which yielded the following results :

ppm (multiplicity, integration, assignment), 0.84 (t, 3H, $\underline{CH_3}CH_2CHO$), 1.84-2.03 (m, 2H, $CH_3\underline{CH_2}CHO$), 3.45 (s, 3H, $O\underline{CH_3}$), 4.02 (s, 3H, $CO_2\underline{CH_3}$), 5.96 (t, 1H, $CH_3CH_2\underline{CH}O$), 7.13 (s, 1H, 3-H), 7.26 (s, $CDCl_3$), 7.33-7.45 (m, 5H, phenyl), 7.59-7.62 (d, 1H, 8-H), 7.62-7.72 (q, 1H, 7-H), 8.17-8.18 (d, 1H, 5-H), for the R-MTPA ester having the shorter retention time on HPLC (peak 2).

ppm (multiplicity, integration, assignment), 0.94 (t, 3H, $\underline{CH_3}CH_2CHO$), 1.88-2.07 (m, 3H, $CH_3\underline{CH_2}CHO$), 3.56 (s, 3H,$O\underline{CH_3}$), 4.02 (s, 3H, $CO_2\underline{CH_3}$), 5.90 (t, 1H, $CH_3CH_2\underline{CH}O$), 7.12 (s, 1H, 3-H), 7.26 (s, $CDCl_3$), 7.30-7.39 (m, 5H, phenyl), 7.54 (s, 2H, 7-H, 8-H), 8.07 (s, 1H, 5-H), for the R-MTPA ester having the longer retention time on HPLC (peak 3).

Figure 3.3

The U.V. absorbance trace from HPLC analysis (system NP 2) of the diastereomeric MTPA esters of methyl 6-(1'-hydroxypropyl)-chromone-2-carboxylate synthesised from racemic 6-(1'-hydroxy-propyl)chromone-2-carboxylic acid.



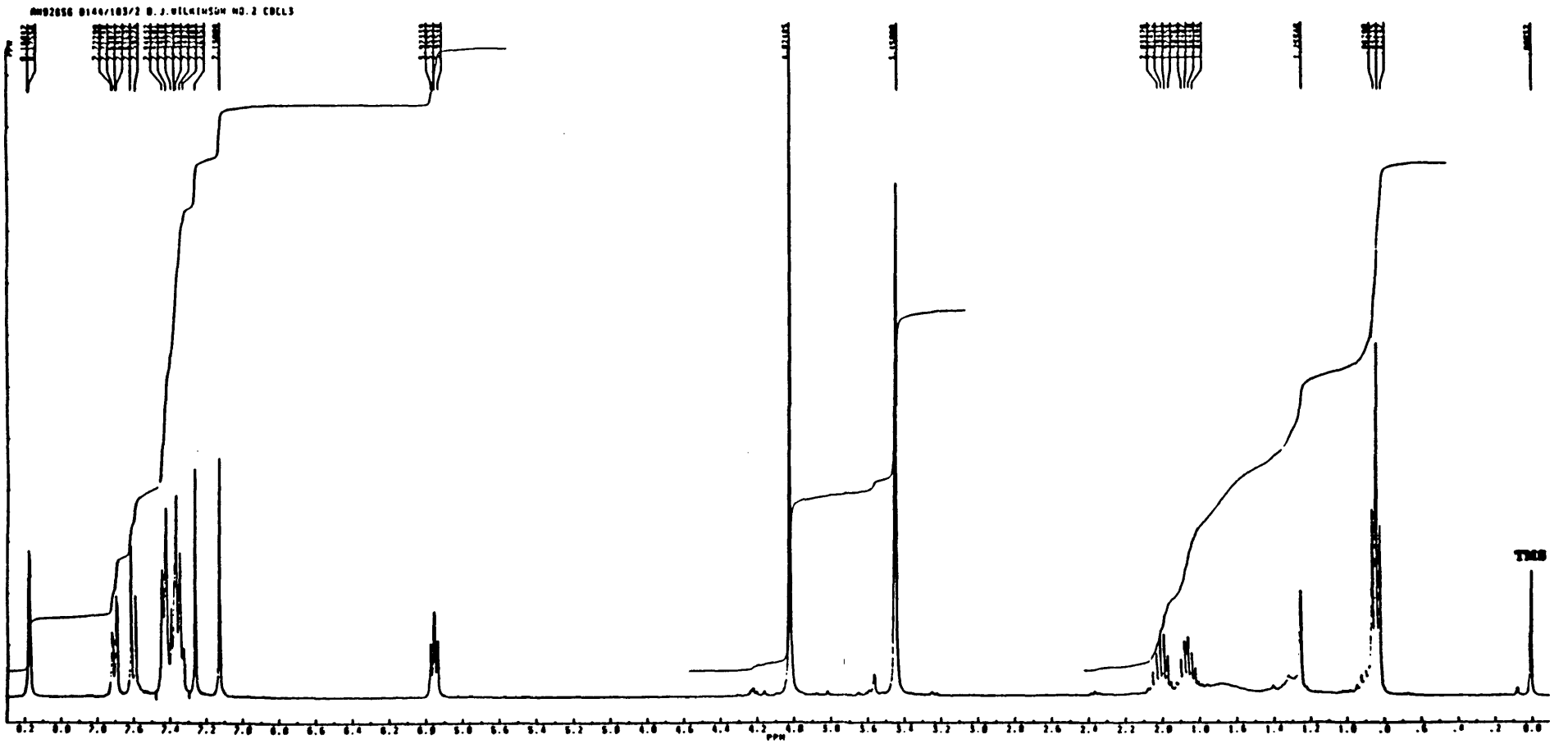


Figure 3.4 ^{1}H NMR spectrum (360MHz) of the MTPA ester of methyl 6-(1'-hydroxypropyl)-chromone-2-carboxylate having the shorter retention time (19.9 min) on HPLC (system NP 2, peak 2).

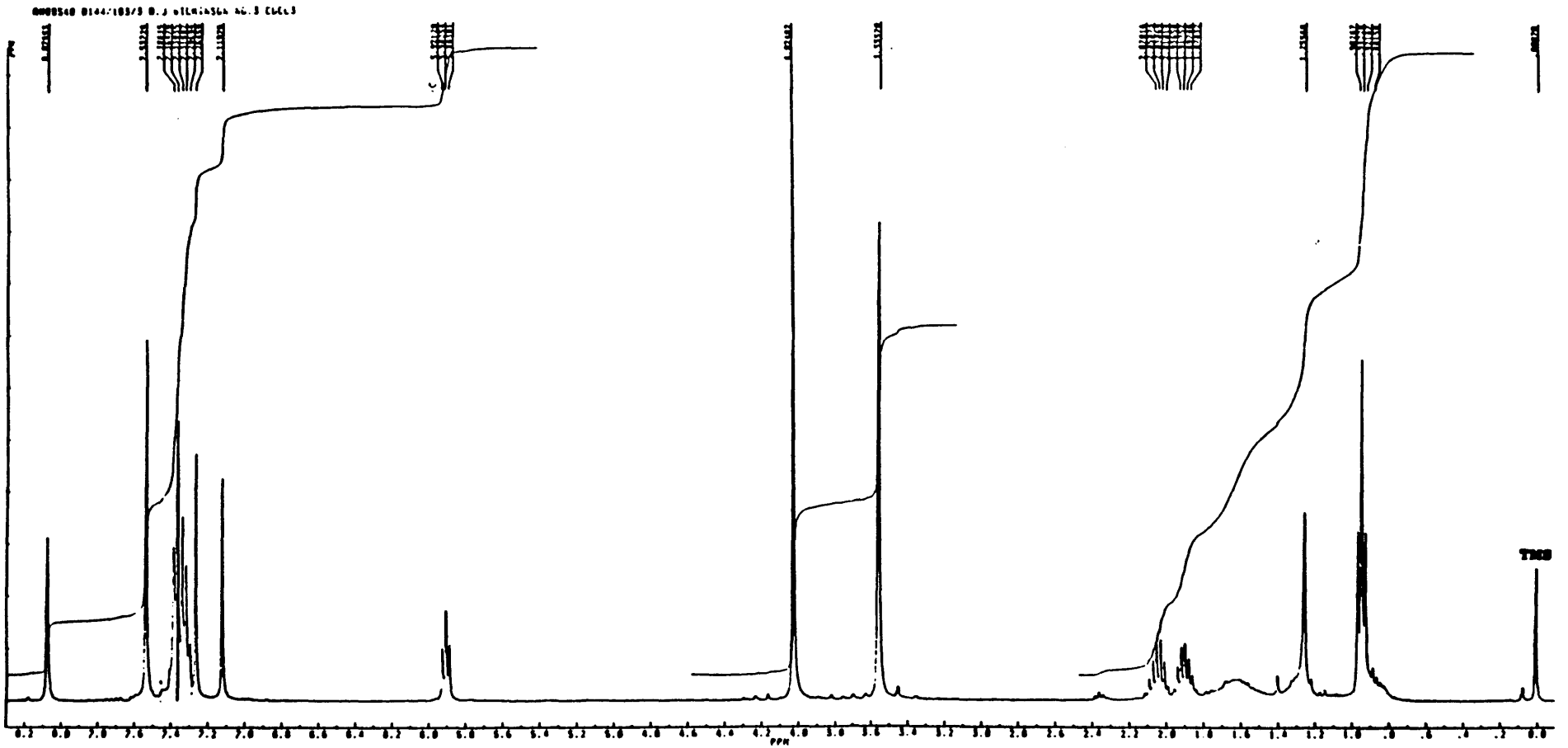


Figure 3.5 ^{1}H NMR spectrum (360MHz) of the MTPA ester of methyl 6-(1'-hydroxypropyl)-chromone-2-carboxylate having the longer retention time (21.6 mins) on HPLC (system NP 2, peak 3).

^{19} F NMR analysis (131.7 MHz, $CDCl_3$) of the individual diastereomers are shown in Figure 3.6 (the MTPA ester peak 2, HPLC system NP 2), and Figure 3.7 (the MPTA ester peak 3 HPLC system NP 2), which yielded the following results ;

ppm (multiplicity, integration, assignment), -71.65 (s, 3F, $\underline{CF_3}$), and -71.94 (s, 3F, $\underline{CF_3}$), for peaks 2 and 3 respectively.

High performance liquid chromatography (HPLC)

Normal phase HPLC analysis was performed using a Waters Associates pump (model 6000A), a Rheodyne valve loop injector (No 7205), and a Waters Associates U.V. detector (model 441), opperating at 254nm; the column (125mm x 4.0mm I.D.) was a HIBAR LiChrocart cartridge, prepacked with LiChrospher Si-60 standard (Merck 50820). A Shimadzu integrator (model CR3A) was used for peak registration and the calculation of retention times and area under the peaks.

The following mobile phases (all compositions by volume) were used;

NP 1	1 : 7	ethyl acetate : hexane at a flow rate of 5.0 ml/min
NP 2	1 : 8	ethyl acetate : hexane at a flow rate of 3.0 ml/min

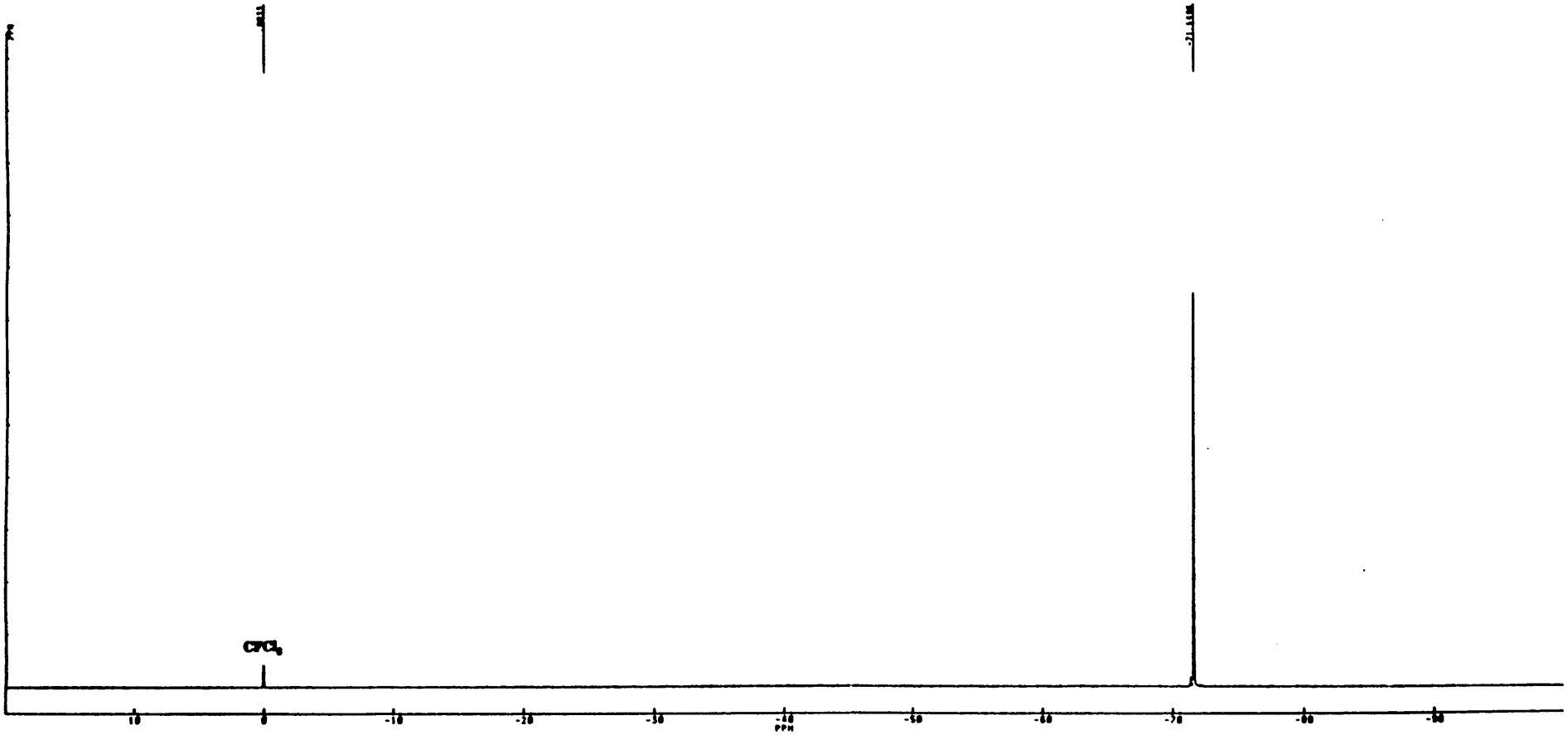


Figure 3.6 **^{19}F NMR spectrum (131.7MHz) of the MTPA ester of methyl 6-(1'-hydroxypropyl)-chromone-2-carboxylate having the shorter retention time (19.9 min) on HPLC (system NP 2, peak 2).**

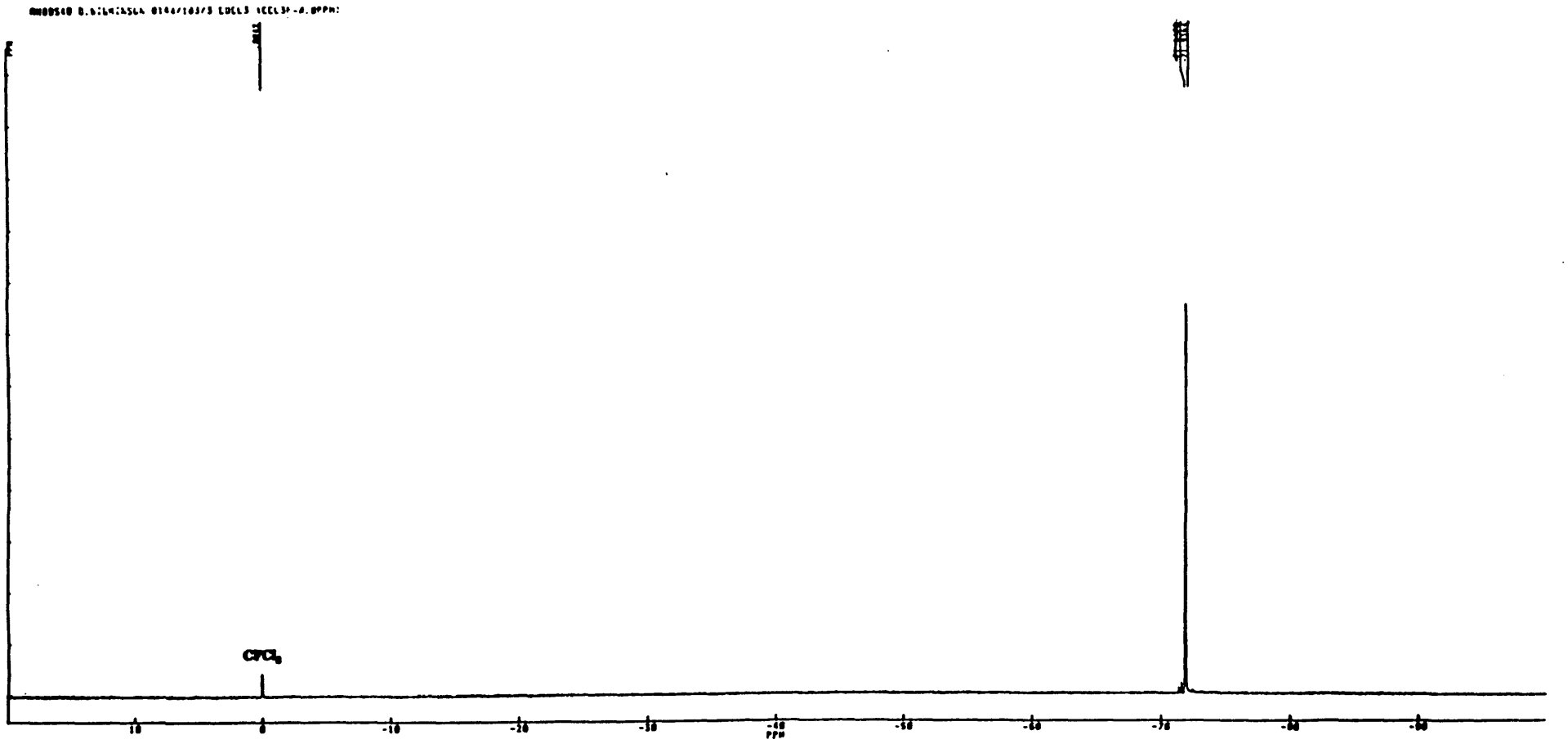


Figure 3.7 **^{19}F NMR spectrum (131.7MHz) of the MTPA ester of methyl 6-(1'-hydroxypropyl)-chromone-2-carboxylate having the longer retention time (21.6 min) on HPLC (system NP 2, peak 3).**

Normal phase semi-preparative HPLC was performed using a Waters Associates pump (Model 6000A), a Rheodyne valve loop injector (No 7205), a Waters Associates UV detector (Model 441), operating at 254nm; the column (25cm x 10mm ID) was prepacked with Spherisorb 5 Si (Technicol, No S5W-SP10). A Shimadzu integrator (model CR3A) was used for peak registration.

The mobile phase (all compositions by volume) used was;

System NP 3	1 : 5	ethyl acetate : hexane
		at a flow rate of 4.0 ml/min

Mass spectrometry and NMR spectroscopy

Mass spectrometry and ^{1}H NMR spectroscopy were performed as previously described.

^{19}F NMR spectra were recorded at 131.7 MHz using a Bruker AM 360 spectrometer (Bruker Spectrospin Ltd). The samples were dissolved in $CDCl_3$, using $CFCl_3$ as the internal standard.

Radiochemical techniques

Radioactivity in urine, faeces, cage washings, dose solution and chromatographic eluants were determined as described in Chapter 2.

Animals and dosing

Six male Wistar rats, weighing approximately 200g (Oxford Laboratory Animal Co), were maintained on a commercially available pellet diet (Labsure CRM, Biosure Ltd, Manea, Cambridge).

[2,2'-^{14}C] 6-n-Propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg) was administered by intraperitoneal injection of a solution prepared by the addition of the appropriate quantity of [2,2'-^{14}C] 6-n-propylchromone-2-carboxylic acid to its unlablled equivalent which was then dissolved in a mixture of ethanol and isotonic sodium hydrogen carbonate (2:3 v/v).

The animals were housed in individual metabolic cages and were allowed food and water *ad libitum*. Excreta were collected daily for three days as previously described in Chapter 2.

Identification of urinary metabolites

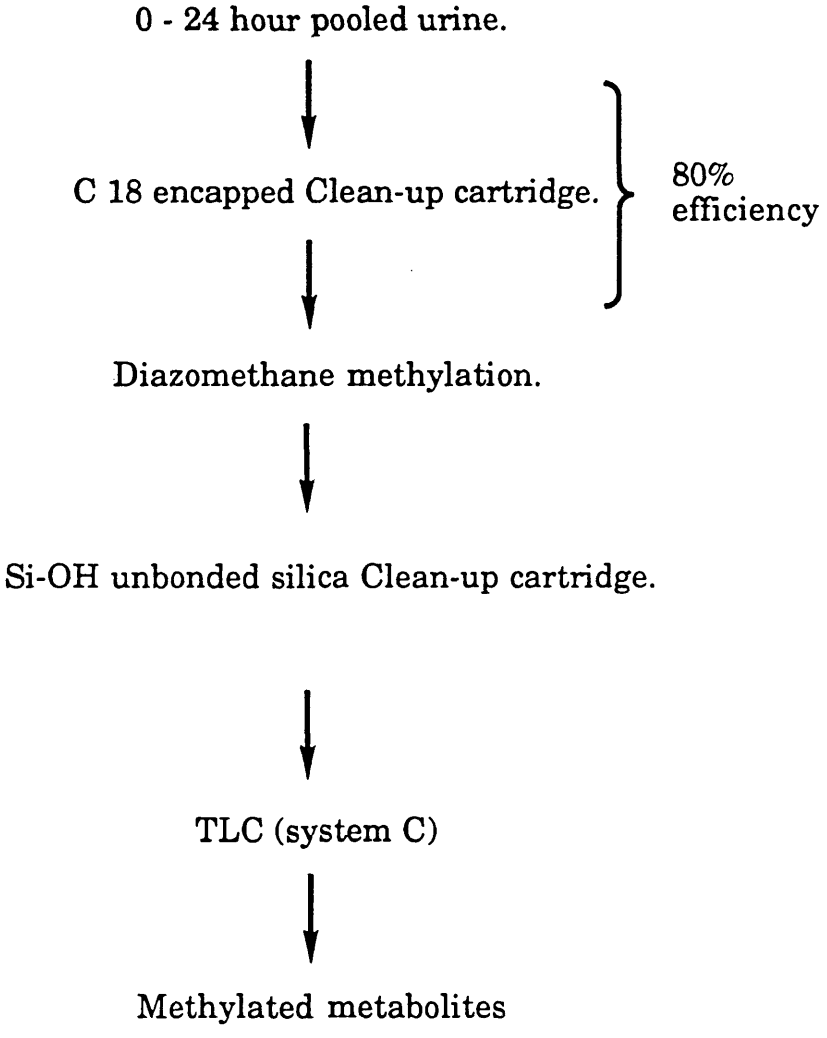
Urines and cage washes were centrifuged (3000xg, 10min) and their ^{14}C content determined as in Chapter 2. Samples of neat 0-24 h urine were filtered (0.45μm, Acrodisc LC13 PVDF, Gelman Sciences) and examined by reverse phase radio HPLC (system RP 1). The pH of all samples was adjusted from approximately pH 6.5 to 5 with 2M hydrochloric acid before storage at -20°C.

Isolation of urinary metabolites

The procedure for the isolation of metabolites from rat urine is illustrated in Scheme 3.1.

Scheme 3.1

New methodology for the isolation of the urinary metabolites of 6-n-propylchromone-2-carboxylic acid.



The urine (0-24 h) from each rat was acidified to pH 1 by drop-wise addition of conc. hydrochloric acid, and centrifuged (3000 x g, 10min). If the volume of supernatant obtained was > 20ml, an appropriate amount of water was added, in order to achieve a final total volume of 20ml or more in all the samples. 3/4 of the sample (approximately 15ml) was then removed, and applied to C 18 endcapped Clean-up cartridges, (3 x 1000mg/6ml, Technicol, No CEC181M6), in aliquots (3 x 5ml). The cartridges then were placed in a Vac-Elut SPS 24 collector (Model AT6500, Analytichem International, Harbor City, CA. 90710, USA.) and sequentially eluted as follows:

Applications

5ml methanol	---- to prime the cartridge
5ml water	

	Eluted fractions
5ml urine	1
5ml H_2O	2
5ml 10% methanol : 90% H_2O	3
5ml 20% methanol : 80% H_2O	4
5ml 30% methanol : 70% H_2O	5

Eluted fractions 4 and 5 were pooled, reduced to dryness *in vacuo* and redissolved in methanol (4ml). The samples were then methylated using diazomethane and the methyl esters analysed using reverse phase HPLC (system RP 2). The mother liquors were again reduced to dryness,

redissolved in chloroform (11.2ml), and applied to Si-OH unbonded Silica Clean-up Cartridges (4 x 500mg/3ml, Technicol, No CUSIL143), in aliquots (4 x 2.8ml). The cartridges underwent the following elutions using a Vac-Elut SPS 24 collector (as above).

Applications

2.8ml chloroform --- to prime cartridge

	Eluted fractions
2.8ml sample	1
2.8ml hexane	2
2.8ml 10% chloroform : 90% hexane	3
2.8ml 20% chloroform : 80% hexane	4
2.8ml 30% cholroform : 70% hexane	5
2.8ml 40% chloroform : 60% hexane	6
2.8ml 50% chloroform : 50% hexane	7
2.8ml 60% chloroform : 40% hexane	8
2.8ml 70% chloroform : 30% hexane	9
2.8ml 80% chloroform : 20% hexane	10
2.8ml 90% chloroform : 10% hexane	11
2.8ml chloroform	12

Eluted fractions 8 to 12 were pooled together and reduced to dryness *in vacuo*. Further purification was achieved using TLC; each sample was redissolved in chloroform (750µl), applied to silica-gel TLC plates, and developed in system C. The areas of silica-gel corresponding to the position of each radioactive metabolite band were removed and eluted

with ethyl acetate (10ml). After centrifugation (3000 x g, 10mins) and filtration through cotton wool, to remove any silica, the samples were concentrated under a stream of nitrogen and subjected to further analysis.

3.3 Results

Metabolism and excretion of [2-2'-^{14}C]-6-n-propylchromone-2-carboxylic acid in the male rat

Excretion

The excretion of radioactivity by male rats receiving 500μmol/kg, 20μCi/kg of 6-n-propylchromone-2-carboxylic acid is presented in Table 3.1.

Table 3.1

Excretion of radioactivity after intraperitoneal administration of [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid to male rats, at a dose level of 500μmol/kg, 20μCi/kg.

	% Excretion of radioactivity	
Time after dosing (h)	urine	faeces
0-24	66.4 ±2.8	4.2 ±2.5
24-48	8.0 ±4.4	2.1 ±2.2
48-72	4.2 ±3.5	0.6 ±0.9
Total	78.6	6.9
Σ	85.5	

Mean ± S.D. of 5 animals.

79% of the administered dose was excreted in the urine over the 72 h period, indicating this to be the major route of excretion. Elimination was rapid with some 71% of the administered dose excreted during the first 24 h, with much smaller quantities being voided during the following days. The total recovery of radioactivity after 72 h was 85%.

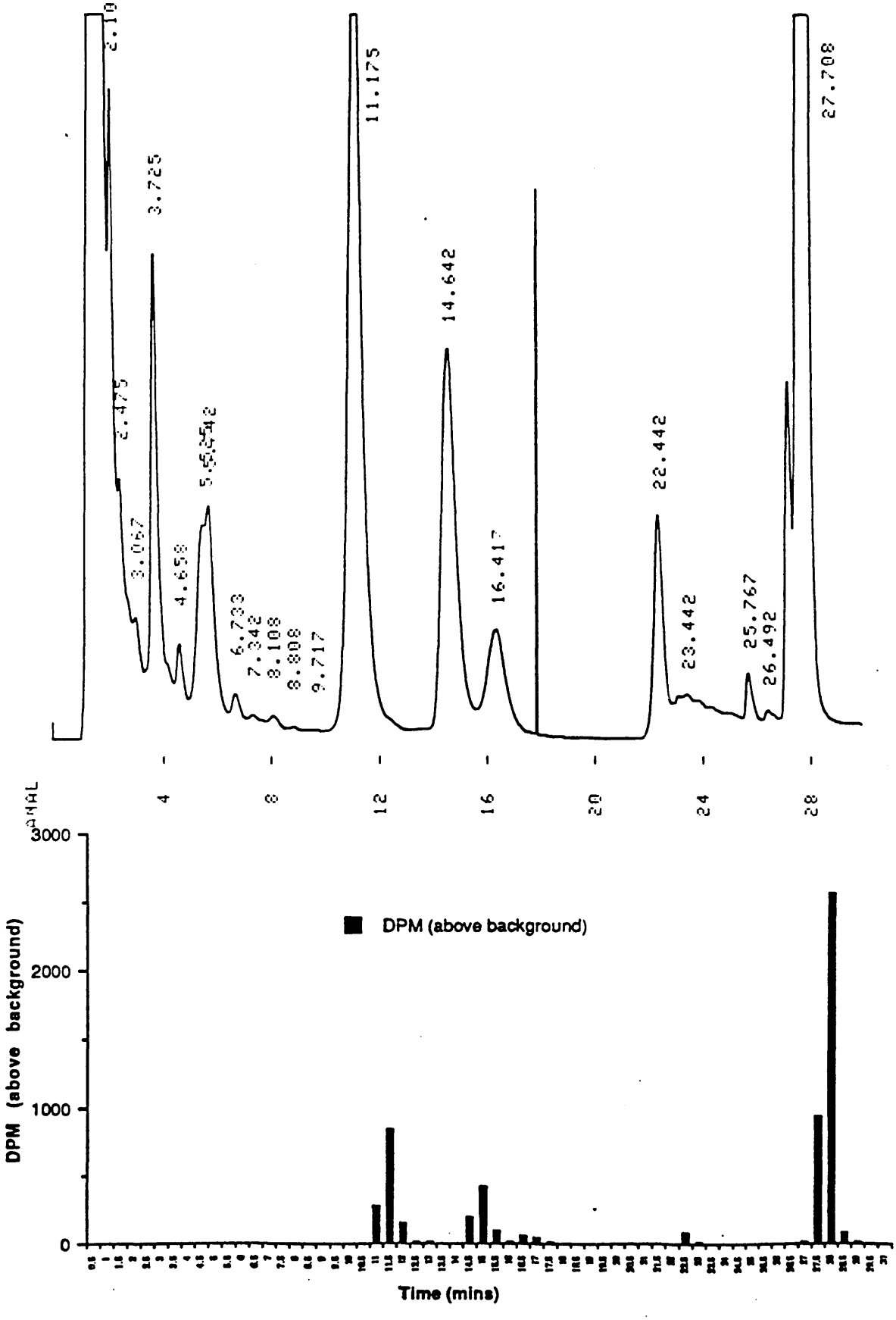
Urinary metabolites of 6-n-propylchromone-2-carboxylic acid in the male rat.

Metabolite identification and quantitation

Radio HPLC analysis of neat 0-24 h rat urine (system RP 1), enabled the radioactivity present to be resolved into five peaks (Fig 3.8). The peak at Rt 27.7 min accounted for 55.5 ± 4.3% (n=5) of the 0-24 h urinary radioactivity and was identified as unchanged 6-n-propylchromone-2-carboxylic acid (6-n-PCCA). The metabolites 6-(2'-hydroxypropyl)-chromone-2-carboxylic acid (6-2'-HPCCA) and 6-(3'-carboxypropyl)-chromone-2-carboxylic acid (6-3'-CPCCA), co-eluting at Rt 11.2 min accounted for 26.2 ± 2.9%, and 6-(1'-hydroxypropyl)chromone-2-carboxylic acid (6-1'-HPCCA), Rt 14.6 min, accounted for 14.9±3.0% of the 0-24 h urinary radioactivity. Two minor metabolites with unknown structures, assigned the letters A and Z, Rt 16.4 min and 22.4 min, accounted for 1.5±0.2% and 1.9 ±0.1% of the 0-24 h urinary radioactivity respectively.

Figure 3.8

U.V. absorbance trace and radiochromatogram form HPLC analysis (system RP 1) of 0-24 h urine from male rats dosed with [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg).



After C18 solid phase extraction, to remove unchanged 6-n-PCCA, the samples were again analysed using HPLC (system RP 1), combined with fraction collecting and liquid scintillation counting. The radioactivity present was resolved into three peaks (Fig 3.9). The peaks at Rt 13.4 min and 14.7 min representing metabolites 6-1'-HPCCA and A respectively, were not generally resolved sufficiently, and were therefore quantitated together.

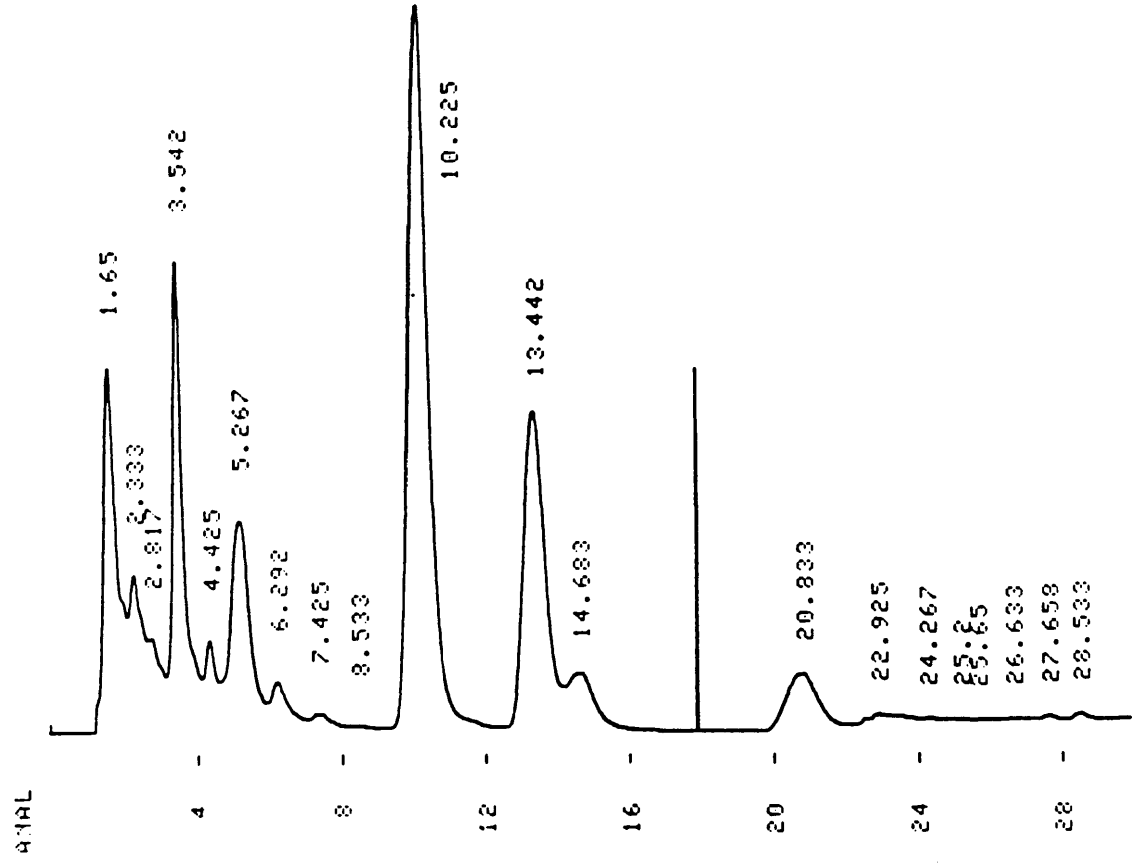
After methylation with diazomethane, the samples were again analysed by HPLC (system RP 2), combined with fraction collection and liquid scintillation counting. This enabled the radioactivity present to be resolved into three peaks (Fig 3.10). The metabolites, 6-1'-HPCCA and A remained quantitated together (Rt 9.1 min), as were the metabolites 6-3'-CPCCA and Z (Rt 14.6 and 15.7 min respectively), but 6-2'-HPCCA for the first time was eluted unhindered giving a retention time of 7.0 min.

It is noteworthy that the percentage of radioactivity associated with 6-1'-HPCCA and A remains unaltered after methylation, indicating that 6-3'-CPCCA co-eluted precisely with 6-2'-HPCCA.

Subtracting the percentage for Z, obtained after C18 solid phase extraction, from the combined percentage for 6-3'-CPCCA and Z, obtained after methylation, will give the percentage of 6-3'-CPCCA in the methylated sample. Knowing this and the percentage of 6-2'-HPCCA in the methylated sample, the ratio of the two metabolites can be calculated, and thus the true percentage of each in the neat urine. The metabolites and their percentages in the neat 0-24 h urine are presented in Table 3.2

Figure 3.9

HPLC analysis (system RP 1) of 0-24 h urine from male rats dosed with [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg) after C18 solid phase extraction.



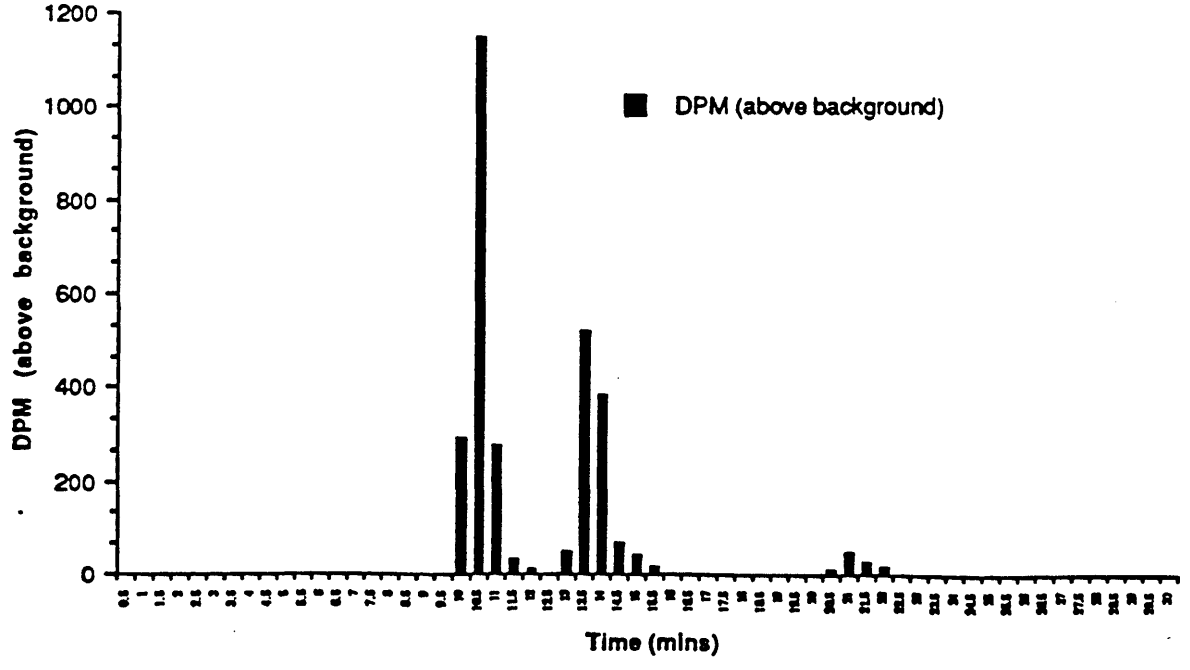
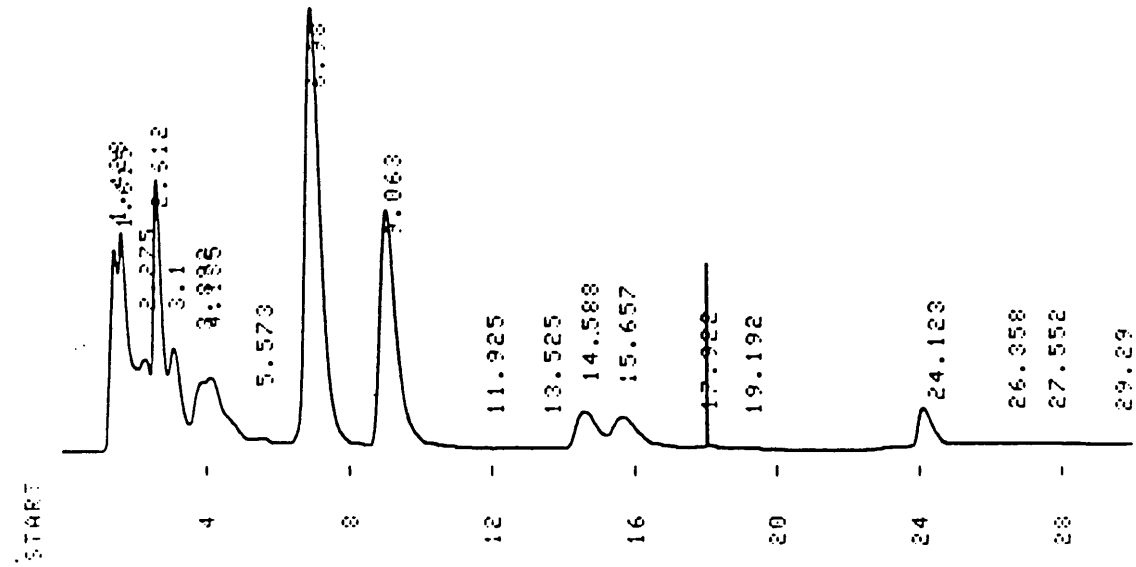


Figure 3.10

HPLC analysis (system RP 2) of 0-24 h urine from male rats dosed with [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg), after C18 solid phase extraction and methylation with diazomethane.



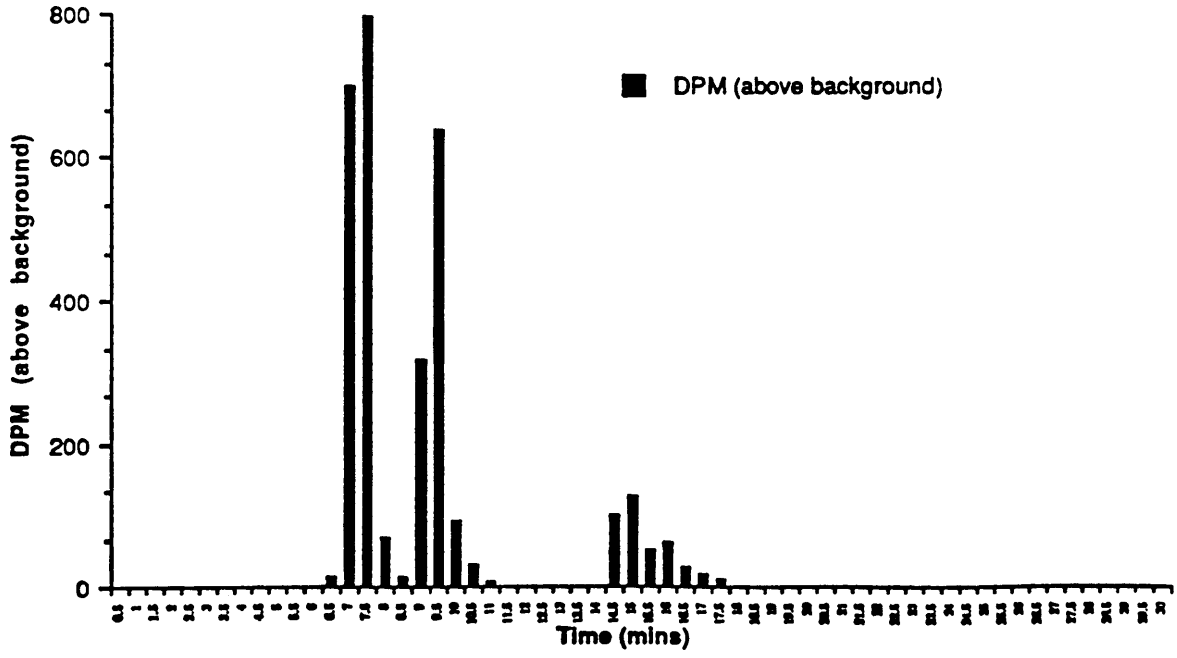


Table 3.2

Metabolites and their percentages in the 0-24 h urine of male rats after the administration of [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid, at a dose level of 500μmol/kg, 20μCi/kg.

Metabolite	Rt (min)	% of urinary radioactivity
6-2'-HPCCA	11.2	22.3 ± 2.3
6-1'-HPCCA	14.6	15.0 ± 3.0
6-3'-CPCCA	11.2	4.0 ± 0.9
A	16.4	1.5 ± 0.2
Z	22.4	2.0 ± 0.1
6-n-PCCA	27.7	55.5 ± 4.3

Isolation of 6-2'-HPCCA and 6-1'-HPCCA was achieved using solid phase extraction and TLC as previously described. R_F values of 0.53 and 0.75 respectively were recorded in TLC solvent system C.

Methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate-MTPA esters

Derivatisation of synthetic methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate with (+)-S-MTPA-Cl permitted the determination of the enantiomeric composition by HPLC separation of the diastereomeric MTPA esters. Determination of this enantiomeric composition using analytical HPLC (system NP 2) and peak area calculations, consistently gave a ratio of 1:1 (Fig 3.3).

Subsequent semi-preparative HPLC (system NP 3) purification yielded sufficient quantities of each of the individual diastereomeric MTPA esters for ^{1}H NMR and ^{19}F NMR, from which assignment of configuration was achieved.

Semi-preparative HPLC (system NP 3) purification also yielded a sufficient quantity of peak 1, having the retention time of 15.7 min on HPLC (system NP 2, Fig 3.3), for ^{1}H NMR spectroscopy to be undertaken (Appendix 3.6). This indicates that 6-(1'-hydroxypropyl)chromone-2-carboxylic acid, when reacted with boron trifluoride : methanol complex, cannot only form the required methyl ester but also small quantities of methyl 6-(1'-methoxypropyl)chromone-2-carboxylate.

Assignment of configuration from ^{1}H NMR data

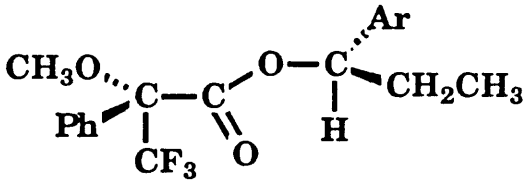
The assignment of absolute configuration to the MTPA esters was achieved by constructing the NMR configuration correlation model, described by Dale and Mosher (1973) for the MTPA esters of chiral alcohols.

In this model (Fig 3.11), the highly electronegative α-trifluoromethyl (CF_3) substituent more or less eclipses the carbonyl group, which maintains the chiral centre in a relatively fixed configuration based on strong dipole-dipole interactions between the two groups. The model positions the hydrogen attached to the carbinyl carbon in a region nonpreferentially shielded (Dale and Mosher 1973), and thus places different groups attached to the carbinyl carbon in the vicinity of the anisotropic shielding region (π cloud) of the α-phenyl substituent, in the two esters.

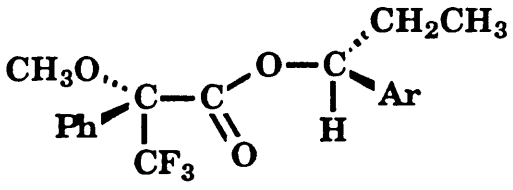
The major differences in the signals of functionalities attached to the carbinyl carbon, for the two esters are the 3'C (ω) carbon methyl triplet at 0.84 ppm (peak 2) and 0.94 ppm (peak 3) (Fig 3.4 and Fig 3.5 respectively). From the configuration correlation model, it is evident that in the
R-alcohol, the position of the 3'C methyl group is such that "shielding " by the aromatic ring of the α–phenyl substituent can occur, which should result in a relative upfield shift of the triplet signal (compared to that of the S-alcohol). In the S-alcohol, the shielded position is occupied by the chromone moiety, and the methyl group is uneffected by the aromatic ring of the α-phenyl group, which should result in a relative downfield shift of the triplet signal (compared to that of the R-alcohol). Therefore, in the ester having the shorter retention time on HPLC (peak 2), the methyl group (resonating as a triplet) produced this signal at 0.84 ppm, indicating it to be the R-alcohol, and in the ester having the longer retention time on HPLC (peak 3), the methyl group (resonating as a triplet) produced this signal at 0.94 ppm, indicating it to be the S-alcohol.

Figure 3.11

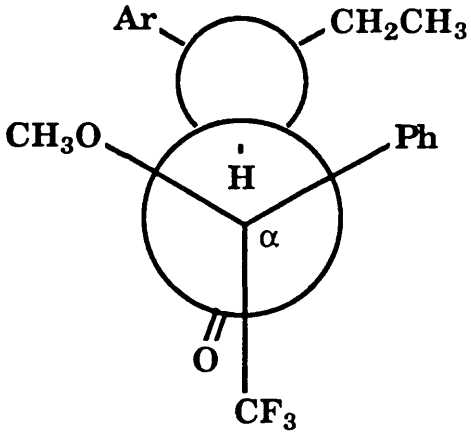
^{1}H NMR configuration model for the diastereomeric (R)-MTPA esters of methyl 6-(1'-hydroxypropyl)chromone-2-carboxylates.

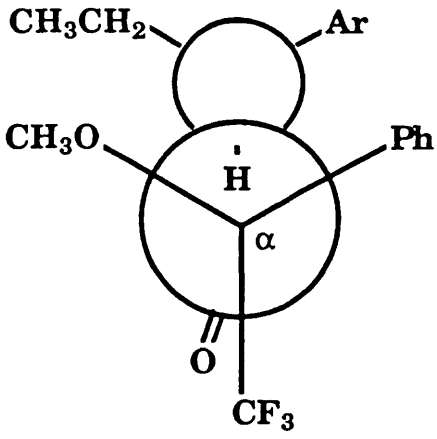


R-Alcohol-R-MTPA ester



S-Alcohol-R-MTPA ester





Ar = chromone-2-carboxylate

The α-OCH_3 substituent also showed significant chemical shift differences, in that, shielded by the aromatic region of the chromone nucleus (R-alcohol), the group (resonating as a singlet) produced a signal (3.45 ppm) which is upfield relative to the resonance of the corresponding group in the alternate diastereomer (S-alcohol, 3.56 ppm). The α-OCH_3 signal in MTPA derivatives is an unresolved quartet due to long-range coupling with the α-CF_3 group, and generally small diastereomeric chemical shift differences are observed (Dale and Mosher 1973).

Assignment of configuration from ^{19}F NMR data

The assignment of absolute configuration to the MTPA esters, was achieved by constructing the NMR correlation model described by Sullivan *et al* (1973), for MTPA esters of chiral alcohols. These models are constructed initially using the parameters described by Dale and Mosher (1973), but also incorporate steric and ecectronic interactions between some key functionalities within the molecule.

Figure 3.12 illustrates the NMR configuration correlation model for the diastereomeric R-MTPA esters of methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate, obtained using these total parameters.

In the R-alcohol, these is little or no interaction between the substituents attached to the carbinyl carbon and the α-methoxy and α-phenyl groups. This should result in the α-CF_3 substituent remaining coplanar with the ester carbonyl, and as a consequence the α-CF_3 group remains in the deshielded environment of the carbonyl group. Thus its resonance should be downfield relative to that of the S-alcohol. In the S-alcohol both

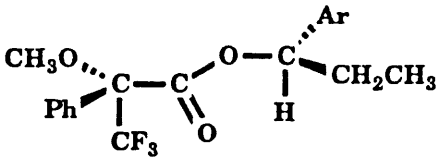
large groups, the chromone nucleus and the α-phenyl, are on the same side of the molecule. The overall result should be rotation of the α-CF_3 group out of coplanarity with the carbonyl group, placing the α-CF_3 substituent in a more shielded environment. Thus its resonance should be upfield relative to that of the R-alcohol.

Therefore, in the ester having the shorter retention time on HPLC (peak 2), the α-CF_3 group (resonating as a singlet) produced this signal at -71.65 ppm (Fig 3.6), indicating it to be the R-alcohol, and the ester having the longer retention time on HPLC (peak 3), the α-CF_3 group (resonating as a singlet), produced this signal at -71.94 ppm (Fig 3.7), indicating it to be the S-alcohol.

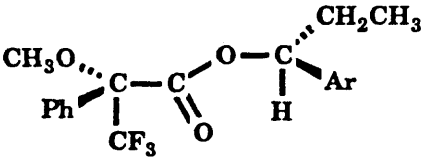
This more sophisticated model is in agreement with the conclusions results drawn from the ^1H NMR data, as, for effective shielding of the substituents attached to the carbinyl carbon by the α-phenyl group, the α-CF_3-carbonyl configuration need not be perfectly eclised but can be substantially skewed from coplanarity (Dale and Mosher, 1973).

Figure 3.12

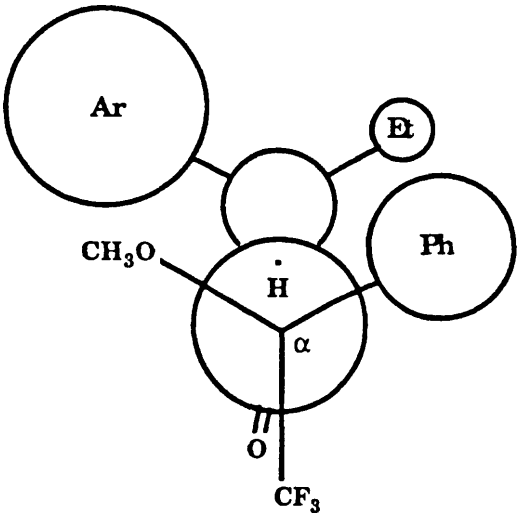
^{19}F NMR configuration correlation model for the diastereomeric (R)-MTPA esters of methyl 6-(1'-hydroxypropyl)chromone-2-carboxylates.

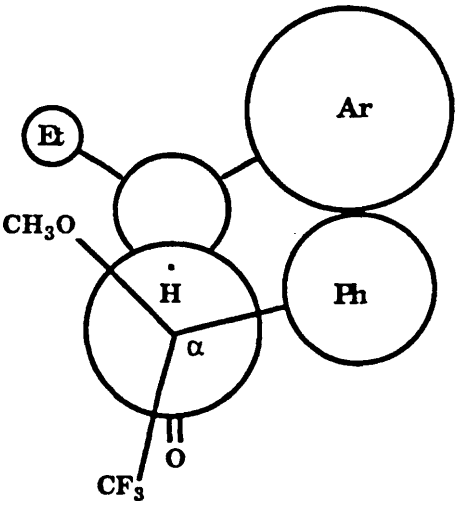


R-alcohol-R-MTPA ester



S-alcohol-R-MTPA ester





Ar = chromone-2-carboxylate
Et = CH_2CH_3

Enantiomeric composition of the chiral metabolites

(1) Female rabbit

Methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate-MPTA esters

Methylation of the 0-24 h urine extract, with purification by further solvent extraction and TLC as previously described in Chapter 2, resulted in the isolation of methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate. This was subsequently converted to the MTPA esters, using Mosher's reagent, and the enantiomeric composition of the diastereomers formed analysed by HPLC (system NP 1). From the chromatogram, a peak area ratio of R/S 20 : 80 (Fig 3.13) was calculated.

(2) Male rat

Methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate-MTPA esters

After isolation, methyl 6-(2'-hydroxyproply)chromone-2-carboxylate was converted to its MTPA esters using Mosher's reagent, and the enantiomeric composition of the diastereomers formed analysed by HPLC (system NP 1). The peak area ratio was 90.4 : 9.6 ± 0.6% (R/S, n=5) from the U.V. chromatogram and 89.6 : 10.4 ± 0.6 (R/S) from the radiochromatogram (Fig 3.14).

Figure 3.13

HPLC analysis (system NP 1) of the diastereomeric MTPA esters of methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate isolated from a female rabbit dosed with 6-n-propylchromone-2-carboxylic acid (500μmol/kg).

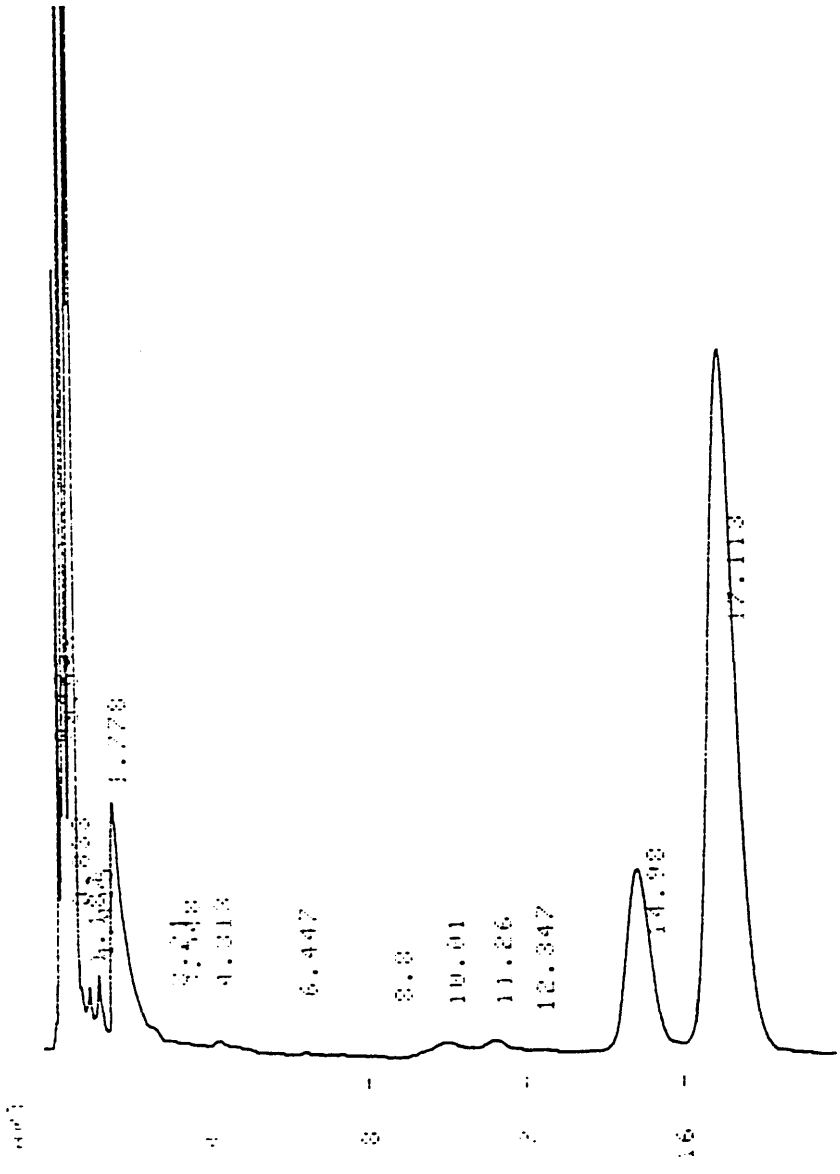
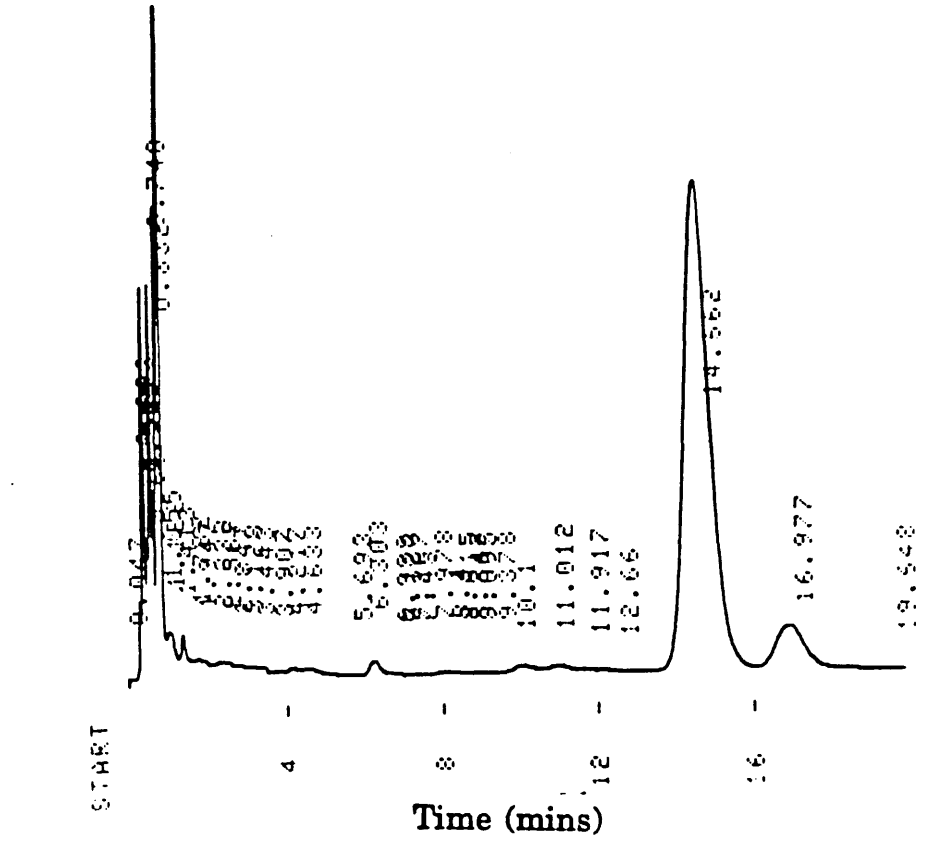
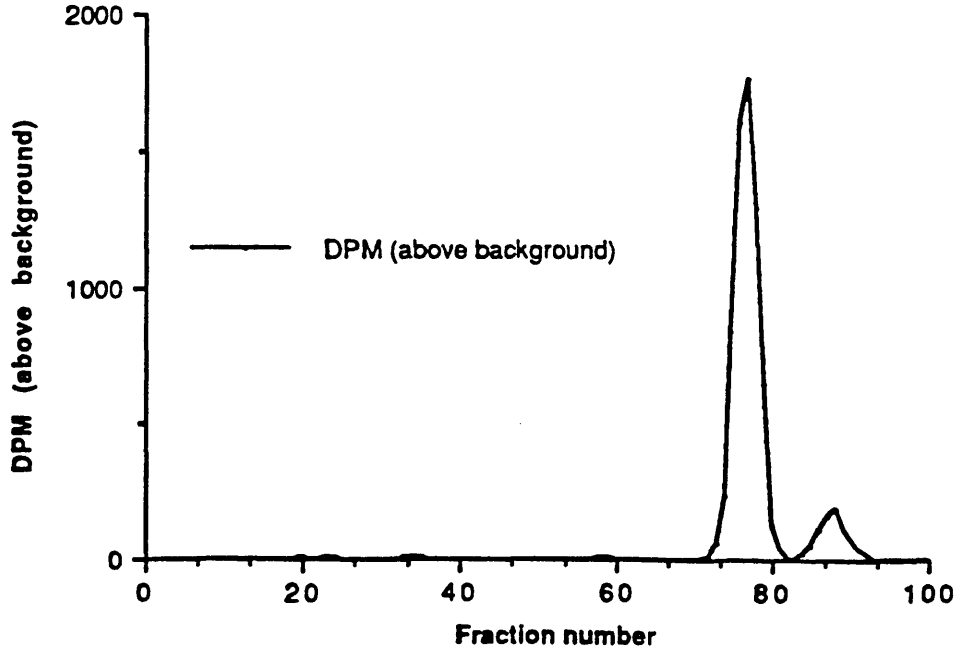


Figure 3.14

HPLC analysis (system NP 1) of the diastereomeric MTPA esters of methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate isolated from male rats dosed with [2,2'-^{14}C]-6-n-propyl-chromone-2-carboxylic acid (500μmol/kg, 20μCi/kg).



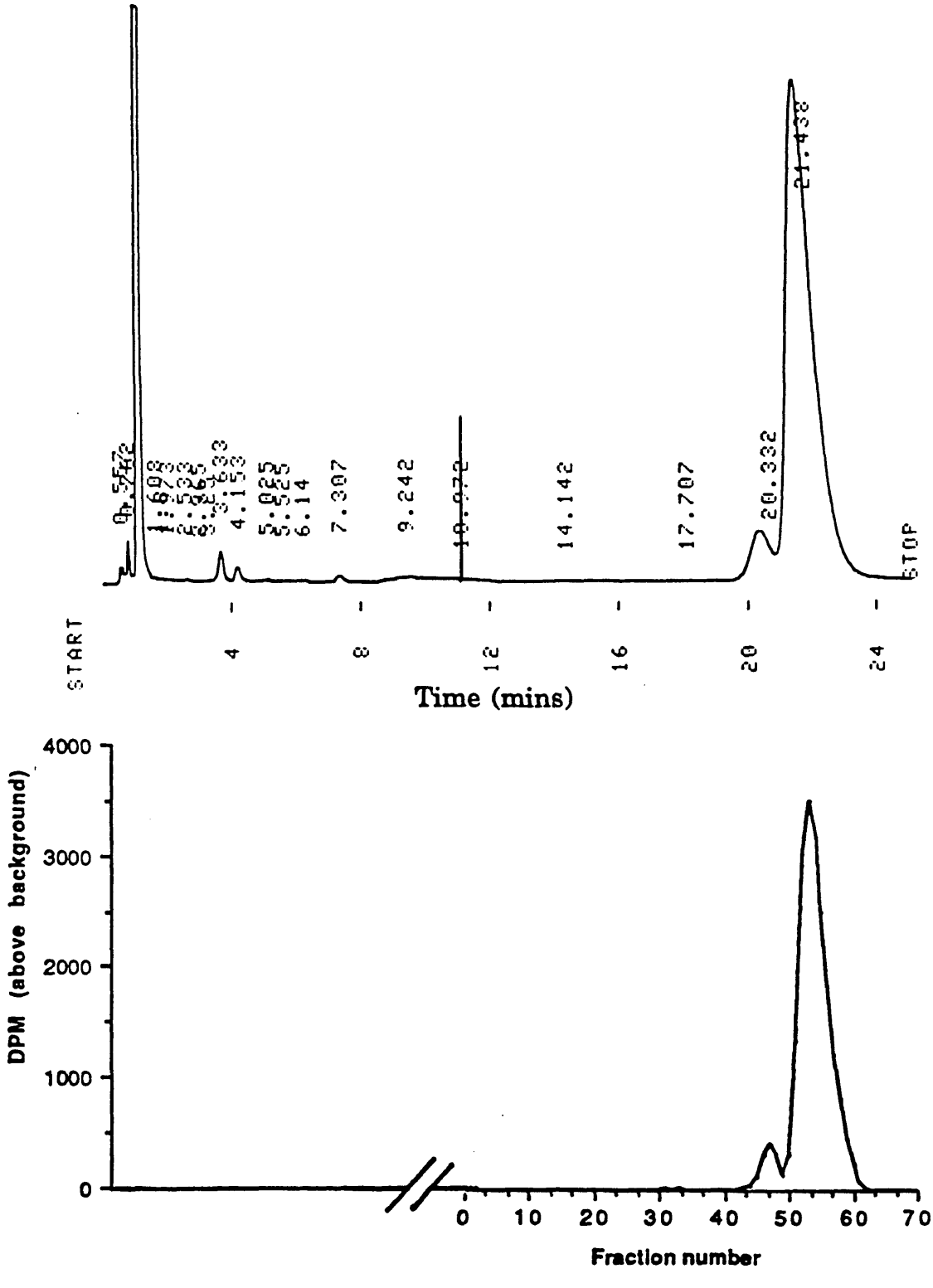


Methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate-MTPA esters

After isolation, methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate was converted to its MTPA esters using Mosher's reagent, and the enantiomeric composition of the diastereomers formed analysed by HPLC (system NP 2). The peak area ratio was calculated at 7.1 : 92.9 ± 0.5 (R/S, n=5) from the U.V chromatogram and 7.9 : 92.1 ± 0.4 (R/S) from the radiochromatogram (Fig 3.15)

Figure 3.15

HPLC analysis (system NP 2) of the diastereomeric MTPA esters of methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate isolated from male rats dosed with [2,2'-^{14}C]-6-n-propyl-chromone-2-carboxylic acid (500μmol/kg, 20μCi/kg).



3.4 Discussion

Chromatographic resolution of the chiral metabolite, isolated from the 0-24 h urine, revealed that the female rabbit metabolised 6-n-PCCA to give an enantiomeric ratio 20:80, with preponderance of the (S)-6-2'-HPCCA metabolite. This result is in accord with the findings of Winter (1987, enantiomeric ratio 24:76, R/S) allowing for experimental error and the absence of the more accurate radiochromatogram.

This preferential formation of the (S)-enantiomer can be attributed to product stereoselective hydroxylation (Introduction 3.1), and implies enzymatic discrimination between enantiotopic proton at the (ω-1) prochiral centre on the 6-propyl substituent. This discrimination may result from the binding of 6-n-PCCA to a single enzyme in two distinct modes, each mode influencing the specific enantiotopic hydrogen allowed to enter the vicinity of the active site, to facilitate oxidative attack. Thus the enantiomeric ratio of the product will depend on the relative probabilities of the two binding modes. The simplest enzymatic model for this situation would require a flexible binding site, to accomodate the steric and electrostatic properties of 6-n-PCCA, in two binding modes.

Alternatively, 6-n-PCCA could exhibit a single mode of binding to an enzyme, then any discrimination between the enantiomeric hydrogens will depend on the relative energy differences between the two diastereomeric transition states of the two enzyme-substrate complexes. This situation would require an enzyme with a rigid binding site. If 6-PCCA binds to two or more enzymes (or isozymes), in one or more

modes, then the specific concentration of each enzyme (isozyme) will have a direct effect on the product enantiomeric ratio.

Chromatographic resolution of the enantiomeric MTPA esters of 6-2'-HPCCA isolated from the 0-24 h urine revealed that the male rat metabolised 6-n-PCCA to give an enantiomeric ratio R/S 90:10. The mechanism for the discrimination between the enantiomeric hydrogens of 6-n-PCCA in the male rat remains unresolved, as it does for the female rabbit, but clearly the rat shows a marked difference in the preferentially formed enantiomer.

The assignment of absolute configuration to the synthetic racemic MTPA esters of methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate was achieved by assigning NMR signals to key functionalities, based on the NMR configuration correlation models for MTPA esters of chiral secondary alcohols described by Dale and Mosher (1973) and Sullivan *et al* (1973). This together with chromatographic resolution revealed that the 6-1'-HPCCA metabolite of 6-n-PCCA in the male rat was excreted in the urine predominantly as the (S)-enantiomer (R/S ratio 8:92).

This result is in direct contrast to that of the 6-2'-HPCCA metabolite of 6-n-PCCA which is excreted in male rat urine predominantly as the (R)-enantiomer (R/S ratio 90:10).

White *et al* (1986) studied the stereochemistry of the 1'-hydroxylation of (R)- and (S)-1'-deuterated phenylethane by a single isolated rabbit enzyme P450IIB4 (LM2). This work suggested that product-stereoselectivity relies on the constraints of the substrate binding site enforced by the surrounding protein tertiary structure rather than on

the intrinsic hydroxylation reaction mechanism of cytochrome P450. Therefore, the extreme differences between the enantiomeric ratio of the excreted hydroxylated metabolite of 6-n-PCCA in the rabbit and rat may be due to apoprotein of the cytochrome P450(s) involved. Differences in the apoproteins would result in contrasting binding sites, allowing either the pro-(R) or pro-(S) hydrogens to predominantly enter the vicinity of the active site.

Sugiyama and Trager (1986) studied the metabolism of 2-phenylpropane using rat liver microsomes isolated from control and induced animals and showed that the enantiomeric composition of the products, was highly dependent on the specific composition of the isozymes present (Introduction 1.4). If the metabolism of 6-n-PCCA in both rabbit and rat is carried out by two isozymes, each specifically producing one enantiomer, then the concentration of the isozymes in each species must be unique to that species and in direct contrast to each other.

This would be a remarkable coincidence and highly unlikely. Later in Chapter 5 the enantiomeric composition of the chiral metabolites of 6-n-PCCA are investigated in the male rat after pretreatment with various inducers. These studies may indicate the possible number of cytochrome P450 isozymes involved in the stereoselective hydroxylation.

Closer inspection of the 3-dimensional structure of these metabolites reveals that their newly acquired hydroxy groups, in (R)-6-2'-HPCCA and (S)-6-1'-HPCCA are attached to the same side of the molecule.

As 6-n-PCCA is almost entirely planar, apart from a slight deviation situated in the 6-n-propyl substituent (established with molecular

mechanics using the MM2 force field), it would be convenient to label the different faces of the molecule. Therefore we can assign; A, as the side or face directly accessible in Figure 3.16 (ie, coming out of the paper), and B, as the side of face directly accessible in Figure 3.17 The predominantly excreted enantiomeric metabolites: (R)-6-2-HPCCA and (S)-6-1-HPCCA, can therefore be viewed as both arising from attack from the B side or face.

Some cytochromes P450 participating in steroid metabolism selectively attack either the α or β face of their substrates. For example, cytochrome $P450_{11\beta}$, isolated from bovine adrenal cortex mitochondria, catalyses the hydroxylation of deoxycorticosterone forming the 11β- and 18- hydroxy metabolites, as well as 4-androstene-3,17-dione, forming the 11β–and 19-hydroxy metabolites (Sato *et al* 1978). In contrast cytochrome $P450_{17\alpha\text{-}lyase}$ purified from guinea pig adrenal microsomes, catalysed the hydroxylation of progesterone yielding 17α-hydroxyprogesterone (Kominami *et al* 1982).

Cytochrome $P450_{14DM}$ (lanosterol 14α-demethylase) of *Saccharomyces cerevisiae*, catalyses the oxidative removal of the 14α–methyl group (C-32) of lanosterol. In addition to face-specific contact, between the β-surface and the substrate-site apoprotein Aoyama *et al* (1989a, 1989b) concluded that the 3-hydroxy group of lanosterol forms hydrogen bond interactions with a specific amino acid residue, contained within the substrate binding site. This hydrogen bond formation may play a critical role in orientating the substrate within the substrate site, such that the 14α-methyl group is located near the haem iron.

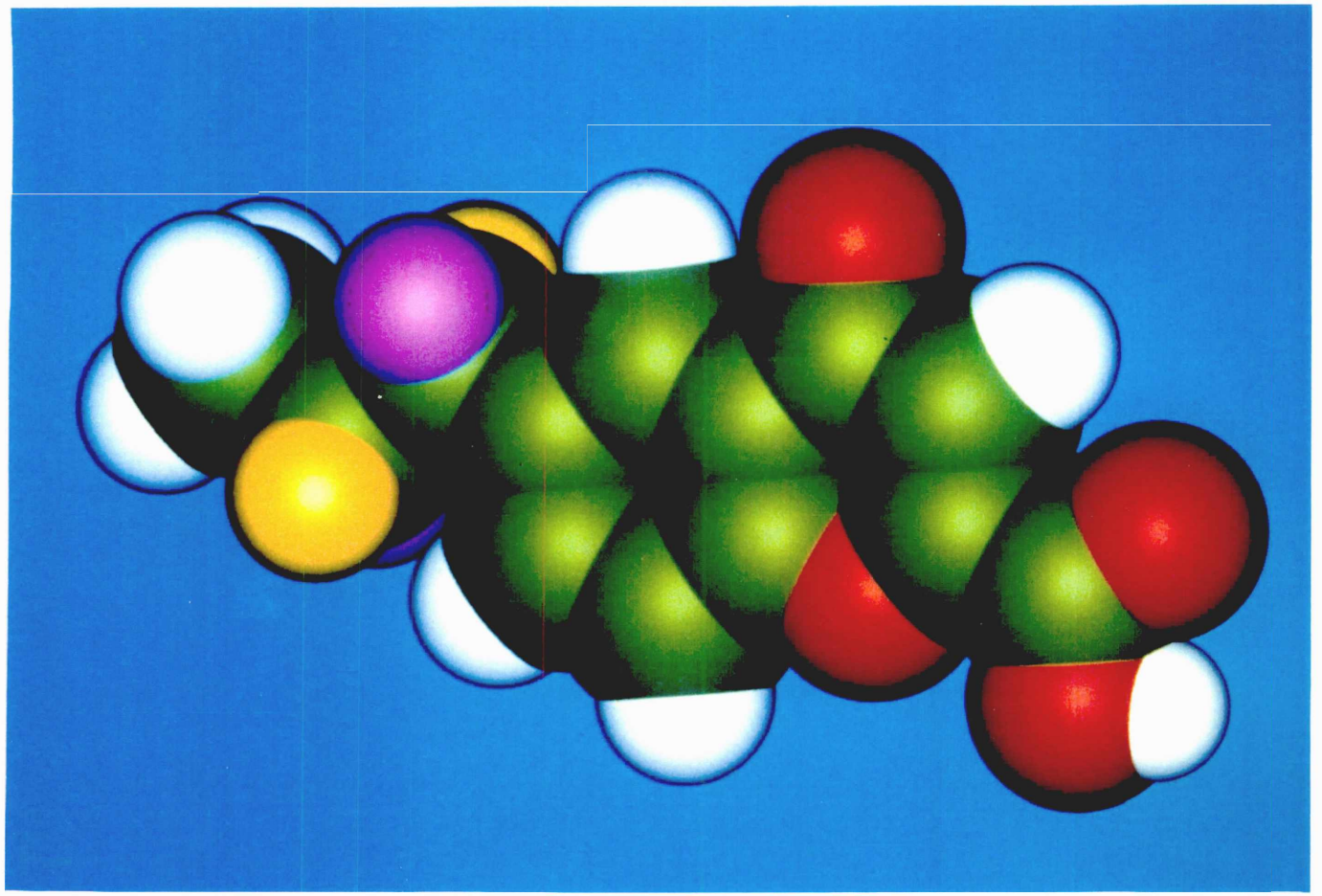


Figure 3.16 Space filling model of 6-n-propylchromone-2-carboxylic acid showing the A side or face, with the pro-R and pro-S hydrogens coloured purple and yellow respectively.

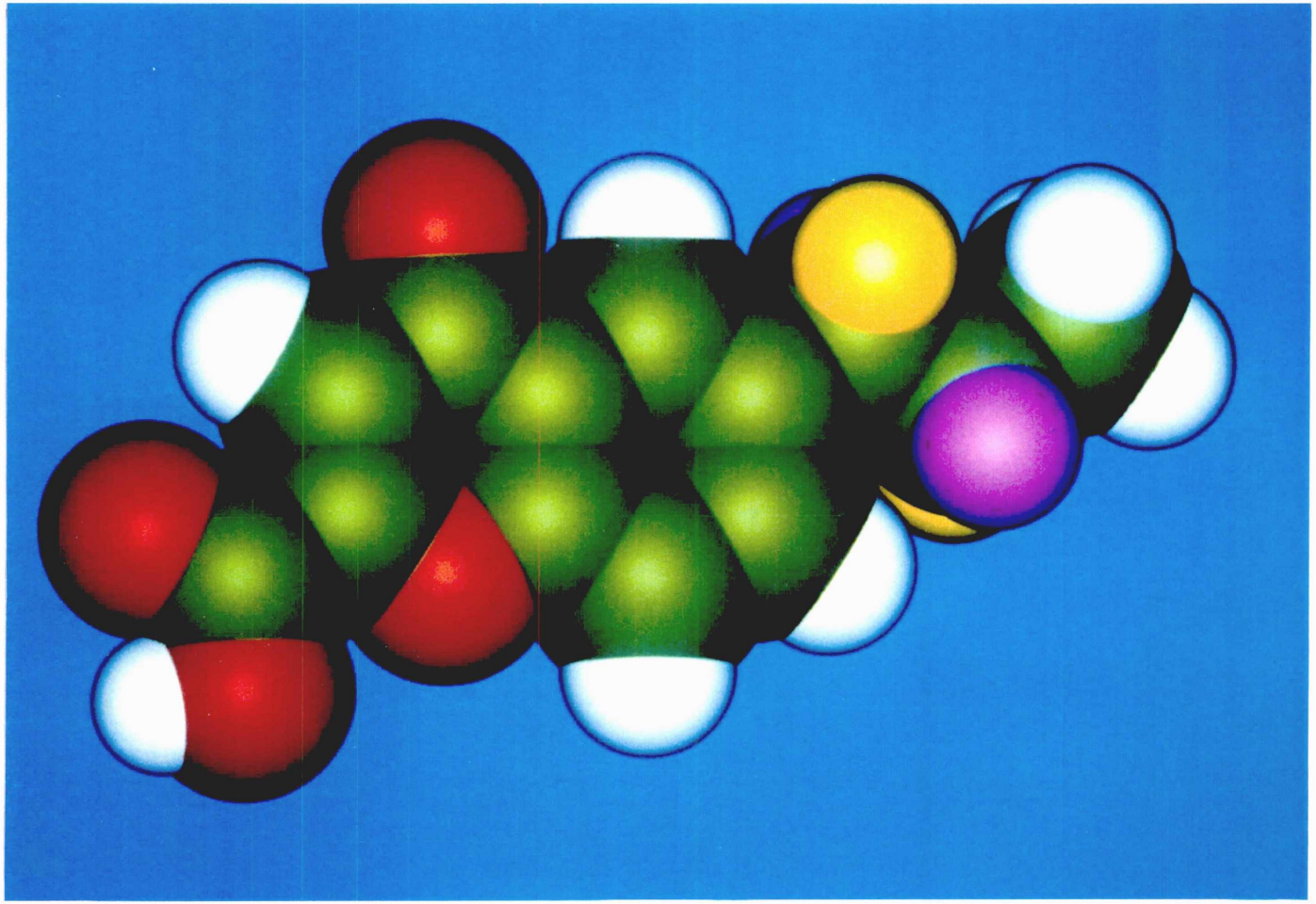


Figure 3.17 Space filling model of 6-n-propylchromone-2-carboxylic acid showing the B side or face, with the pro-R and pro-S hydrogens coloured purple and yellow respectively.

Cytochrome $P450_{cam}$ from *pseudomonas putida* catalyses the regio- and stereo- specific hydroxylation of camphor to yield the 5-exohydroxy-camphor as the sole product, (Gelb *et al* 1982). The X-ray crystal structure for camphor-bound cytochrome $P450_{cam}$ (Poulos *et al* 1985, 1987), revealed an unique hydrogen bond between the carbonyl moiety of camphor and tyrosine 96, as well as hydrophobic contacts between several neighbouring residues, including phenylalanine 87, leucine 244, valine 247 and valine 295. Working with the substrate analogues thiocamphor and camphane, Atkins and Sligar (1988) suggested that the carbonyl moiety of camphor plays a critical steric role in addition to its interaction as a hydrogen bond acceptor.

Any oxygen functional groups of 6-n-PCCA may therefore contribute to any regio- or stereo-selectivity, due to hydrogen bonding or steric interactions, in particular the pyran oxygen at position 1 of the chromone nucleus and the carbonyl moiety attached to the C-4 carbon. The "detergent like" lipophilicity of the alkyl substituted chromone-2-carboxylic acids, suggests that they will orientate themselves in the membrane environment, such that the lipophobic regions will enter the membrane, whilst the acidic function will remain in the aqueous phase (Smith *et al* 1986).

White *et al* (1980) suggested that substrate binding to cytochrome P450 LM was stabilized by hydrophobic interactions of the substrate with the protein in the vicinity of the haem.

Therefore if cytochrome P450 does contains a lipophilic binding site, it is unlikely that the carboxylic acid moiety will enter.

The tolerance of the cytochrome P450s for fatty acids of somewhat different chain lengths, decanoic (C10), lauric (C12), palmatic (C16) and stearic (C18), (Preiss and Bloch 1964, Bjorkhem and Danielssan 1970, Hamberg and Bjorkhem 1971) suggests that hydroxylation specificity is not governed by specific interactions of the protein with the carboxyl group. Thus we may tentatively assume that the carboxyl substituent of 6-n-PCCA does not directly influence any stereoselective metabolism, it does however have a profound effect on determining the regioselectivity of metabolism due to the steering effect it imposes on the molecule (Introduction 1.5).

From the electron density map (Fig 3.18) it is clear that the carbonyl moiety of the chromone nucleus is likely to participate in strong hydrogen bond interaction. Therefore, upon binding of the substrate to a cytochrome P450, hydrogen-bond formation between a specific amino acid residue and the carbonyl moiety (as well as Van der Waal's contact with an unknown number of residues), may be such that the (B) face of the 6-n-propyl substituent is orientated into the vicinity of the active site. This binding position may be further strengthened by weak interactions between the pyran oxygen and other amino acid residues. This binding may allow limited (A) face attack or be totally exclusive to the (B) face. If the latter were the case rotation of the substrate through 180^0 , ie a second mode of binding would be required to place the (A) face into the vicinity of the active site. From the results this second mode of binding is not as favoured, perhaps due to steric hindrance by the carbonyl moiety.

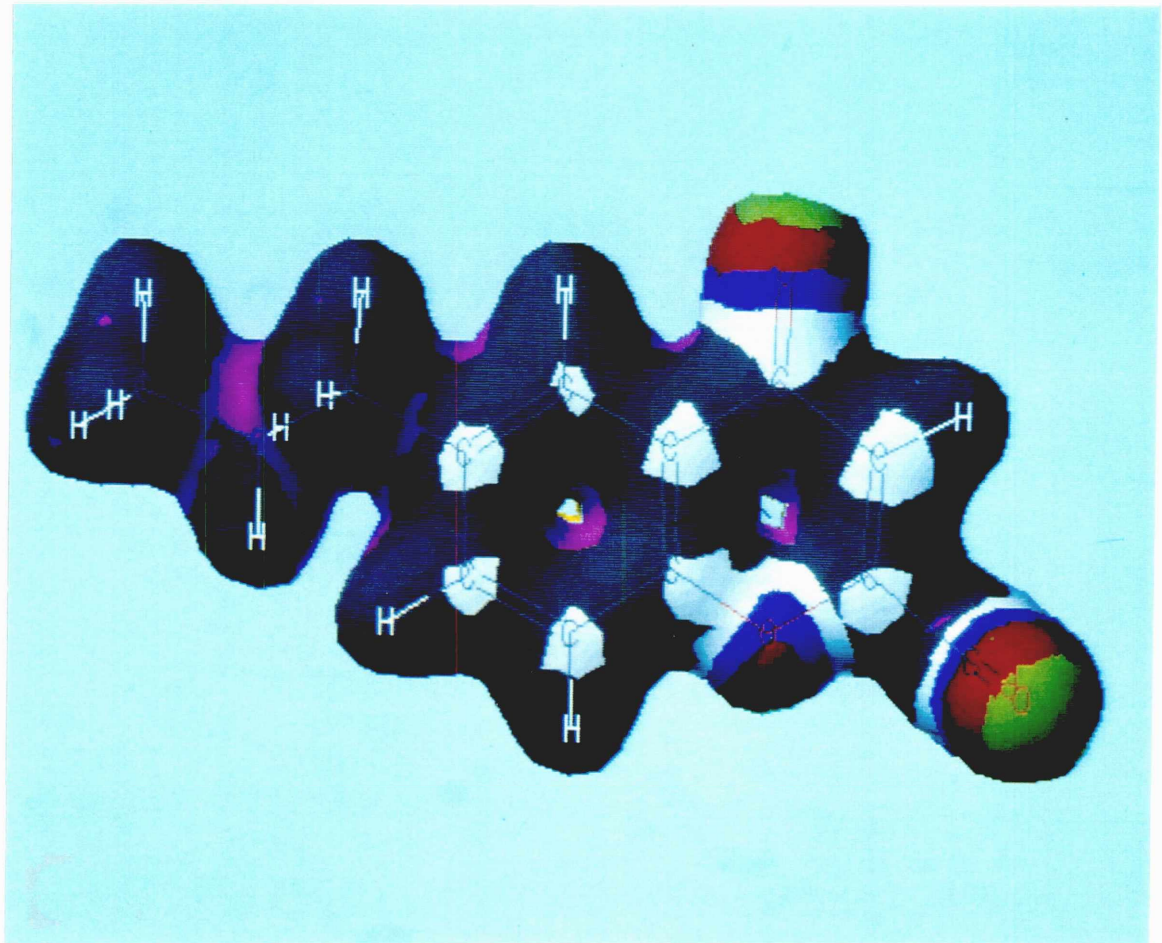


Figure 3.18 Electron density map of 6-n-propylchromone-2-carboxylic acid.

Alternatively, 6-n-PCCA may bind to two cytochrome P450 isozymes, each isozyme being responsible for either (A) or (B) face attack, then the specific concentrations of each isozyme would effect the product enantiomeric ratio. The results suggest that if two isozymes were involved their specific concentrations are somewhat different. However, any variation in this specific cytochrome P450 concentration would theoretically result in a change in enantiomeric composition of the observed metabolites. This is further investigated in detail in Chapter 5.

Chapter 4

Metabolism of Racemic 6-(1'-Hydroxypropyl)- and 6-(2'-Hydroxypropyl)chromone-2-Carboxylic Acid in the Male Rat.

4.1 Introduction

Drug absorption, distribution, metabolism and excretion are all processes which may involve interaction between the drug molecule and chiral biological macromolecules. Enantiomers of chiral drugs which will ultimately form diastereomeric complexes with these macromolecules may exhibit differing affinities for these molecules, resulting in a certain degree of enantioselective drug disposition.

Drug absorption may occur via a number of different mechanisms, but the majority of drugs undergo passive diffusion. This process depends upon the physicochemical properties of the drug notably its lipophilicity and pKa, and in such cases no differences in the rate and extent of absorption between enantiomers of chiral drugs would be expected. However enantioselective absorption has been demonstrated for methotrexate and dopa (Hendel and Brodthagen 1984, Wade *et al* 1973). The L-forms of these two compounds undergo active absorption, whilst the D-enantiomers only undergo passive absorption resulting in preferential uptake of the L-enantiomer.

Many drugs are reversibly bound to plasma proteins in appreciable proportions, but it is generally assumed that the drug in the plasma not bound to proteins is responsible for the pharmacological effects. As plasma proteins are composed of L-amino acids, enantiomers of chiral molecules will interact differently with these proteins, forming pairs of diastereomeric drug enantiomer-protein complexes. Thus any differences in the affinity of plasma proteins for these enantiomers will cause in the free or active concentration of the enantiomers at the site of action to differ.

The renal clearance of a drug is affected by its plasma protein binding, as renal glomerular filtration is a product of glomerular filtration rate and its free fraction in plasma. Thus any enantioselectivity in plasma protein binding can result in differences in total renal clearance of the enantiomers.

If the renal clearance of a drug is substantially facilitated by active renal secretion and reabsorption, or if substantial quantities of the dose is excreted as parent drug, enantioselective renal secretion may be observed, as has been demonstrated for the enantiomers of pindolol and metoprolol (Hsyu and Giacomini 1985, Lennard *et al* 1983).

It has been established that the male rat, when administered 6-n-PCCA, excretes two major metabolites in the urine, and that the enantiomeric composition of these chiral urinary metabolites are unequal. This may reflect product stereoselective hydroxylation. Alternatively enantioselective plasma protein binding and/or renal clearance may be responsible for, or contribute to, this observed phenomenon.

Winter (1987) considered the preferential excretion of the S-6-2'-HPCCA metabolite in the 0-24 h urine, after the administration of 6-n-PCCA to the female rabbit reflected product selectivity, as the enantiomers of the racemic metabolite standard were excreted in the urine in equal proportions when administered to the rabbit.

This chapter reports on the enantiomeric composition of the chiral compounds excreted in the urine of the male rat following the administration of racemic 6-2'-HPCCA and 6-1'-HPCCA and attempts to identify any metabolites formed. It further investigates any ketone

reductase activity on the possible intermediate metabolites 6-(2'-oxopropyl)chromone-2-carboxylic acid, (6-2'-OPCCA) and 6-(1'-oxopropyl)chromone-2-carboxylic acid (6-1'-OPCCA).

4.2 Materials and Methods

Materials and methods were as described in the preceding chapters unless otherwise stated.

Compounds

6-(2'-hydroxypropyl)chromone-2-carboxylic acid and 6-(2'-oxopropyl)-chromone-2-carboxylic acid were samples synthesised by Winter (1987) and further purified by recrystallisation (aqueous ethanol), Their mass spectra and NMR's are shown in Appendices 4.1 to 4.4.

6-(1'-Hydroxypropyl)chromone-2-carboxylic acid

Preparation of this compound, was achieved by following the same sequence of reactions described in Chapter 3, and illustrated in Figure 3.1.

Fumaric/maleic intermediate

Formation of the fumaric/maleic intermediate was undertaken using identical compounds and quantities stated in Chapter 3. The experimental procedure was carried out in an identical manner, using the same equipment as before. As a result the fumaric/maleic intermediate (3.4701g) was obtained as a pale orange solid.

6-(1'-Oxopropyl)chromone-2-carboxylic acid

In order to maximise yields, all this fumaric/maleic intermediate (3.4701g) was used in the formation of the 6-(1'-oxopropyl)chromone-2-carboxylic acid. Again the experimental procedure and compounds used were identical to those stated in Chapter 3, but the quantities involved were increased appropriately. The final product was recrystallised four times from ethanol, to yield 6-(1'-oxopropyl)chromone-2-carboxylic acid (0.8945g) as a white solid.

6-(1'-Hydroxypropyl)chromone-2-carboxylic acid

6-(1'-Oxopropyl)chromone-2-carboxylic acid (0.8g) and sodium hydrogen carbonate (136mg) were dissolved in a carbonate buffer pH 10 (48ml). Sodium borohydride (136mg) was then added and the reaction mixture stirred for 2 h at room temperature. The solution was then acidified (pH 1) with hydrochloric acid (12M) and extracted into ether (3 x 2vol). The combined extracts were dried over anhydrous sodium sulphate, reduced to dryness *in vacuo*, and the product recrystallised three times in ethanol, yielding 6-(1'-hydroxypropyl)chromone-2-carboxylic acid (0.1563g) as a white solid. However, due to the difficulties in obtaining 6-(1'-hydroxypropyl)chromone-2-carboxylic acid in sufficient quantity and purity for dosing, the final recrystallised product contained approximately 5% 6-(1'-oxopropyl)chromone-2-carboxylic acid.

Animals and dosing

24 male Wistar rats, weighing approximately 200g (Oxford Laboratory Animals Co) were maintained on a commercially available diet (Labsure CRM, Biosure Ltd, Cambridge).

These animals were divided into four groups of six, each group receiving one of the following :-

(1) 6-(2'-hydroxypropyl)chromone-2-carboxylic acid

(2) 6-(2'-oxopropyl)chromone-2-carboxylic acid

(3) 6-(1'-hydroxypropyl)chromone-2-carboxylic acid

(4) 6-(1'-oxopropyl)chromone-2-carboxylic acid

The animals received the appropriate acid (500μmol/kg) dissolved in a mixture of ethanol/isotonic sodium hydrogen carbonate (2 : 3 v/v), by intraperitoneal injection. A control animal in each group received the vehicle only.

The animals were housed in individual metabolism cages and allowed food and water *ad libitum* throughout the experiment. All excreta were collected for three days after the administration of the dose.

Identification of metabolites

Urine (0-24 h), from each animal was centrifuged (3000xg, 10mins), and an aliquot (100µl) of supernatant removed. This aliquot was filtered (0.45µm, Acrodisc LC13 PVDF, Gelman Sciences) and examined by reverse phase HPLC (system RP 1). The remaining supernatant was decanted, acidified (pH 5) with hydrochloric acid (2M) and stored at -20°C, until further analysis.

Isolation of metabolites

The procedure for the isolation of metabolites from rat urine was as described in Chapter 3 and illustrated in Scheme 3.1.

4.3 Results

Metabolism of 6-(2'-hydroxypropyl)chromone-2-carboxylic acid

HPLC analysis (system RP 1) of the neat 0-24 h rat urine revealed the administered compound; 6-(2'-hydroxypropyl)chromone-2-carboxylic acid was the only xenobiotic component of the urine. 6-n-Propylchromone-2-carboxylic acid and 6-(2'-oxopropyl)chromone-2-carboxylic acid were not observed, indicating that no Phase 1 metabolism of this compound had occurred (Fig 4.1).

Methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate-MTPA esters

Enantiomeric composition

6-(2'-Hydroxypropyl)chromone-2-carboxylic acid was isolated as its methyl ester from the 0-24h urine of each treated rat. This was subsequently converted to the MTPA esters (Chapter 3) and the enantiomeric composition of the diastereomers analysed by HPLC (system NP 1). The resulting chromatograms (Fig 4.2), revealed a peak ratio of R/S, 48.0 : 52.0 ± 1.7 (n=5).

Metabolism of 6-(2'-oxopropyl)chromone-2-carboxylic acid

HPLC analysis (system NP 1) of the neat 0-24 h urine obtained from the treated rats showed that unchanged 6-(2'-oxopropyl)chromone-2-carboxylic acid was by far the major compound excreted.

Figure 4.1

A typical U.V. absorbance trace from HPLC analysis (system RP 1) of 0-24 h urine from male rats dosed with racemic 6-(2'-hydroxypropyl)chromone-2-carboxylic acid (500μmol/kg).

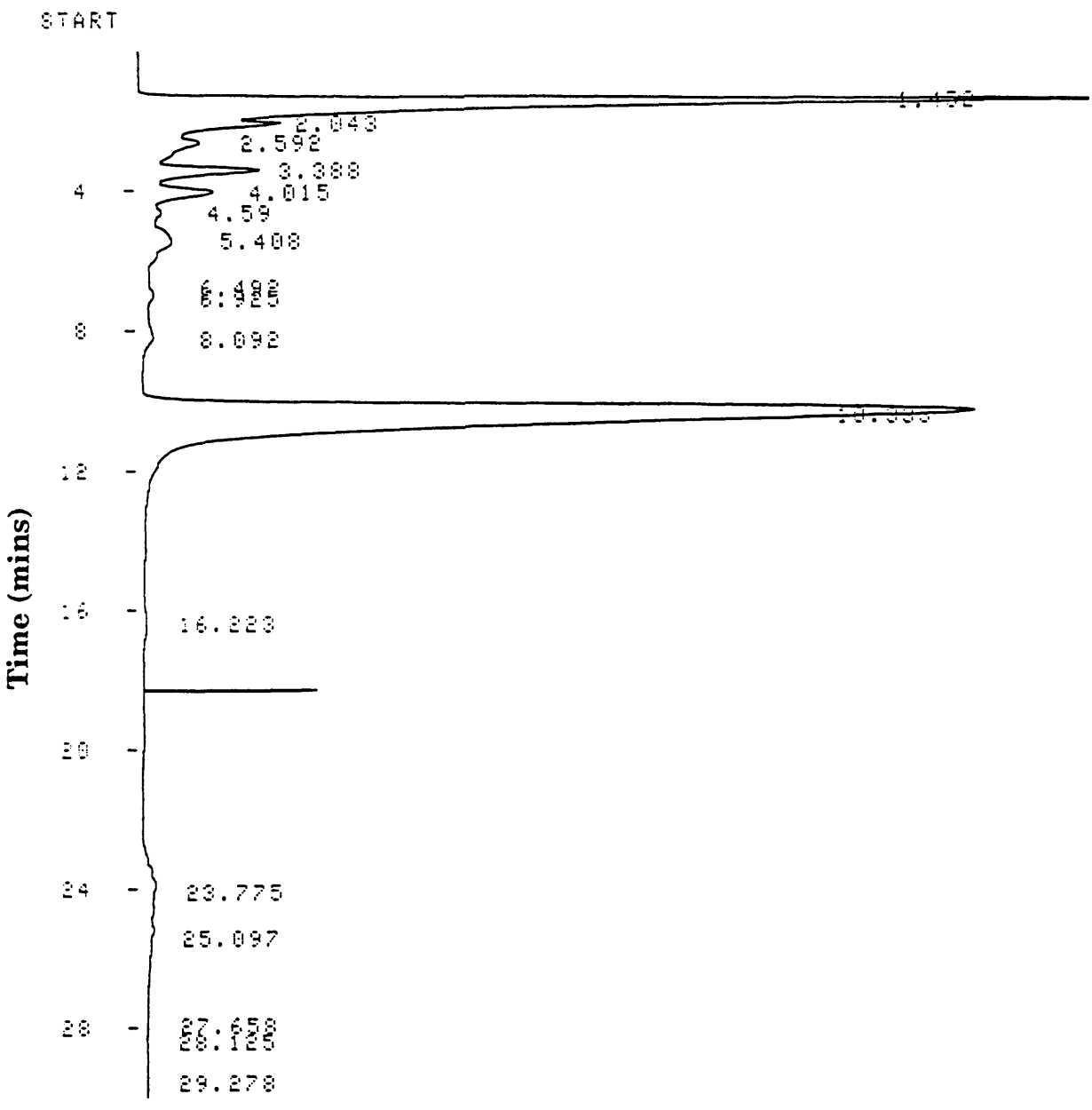
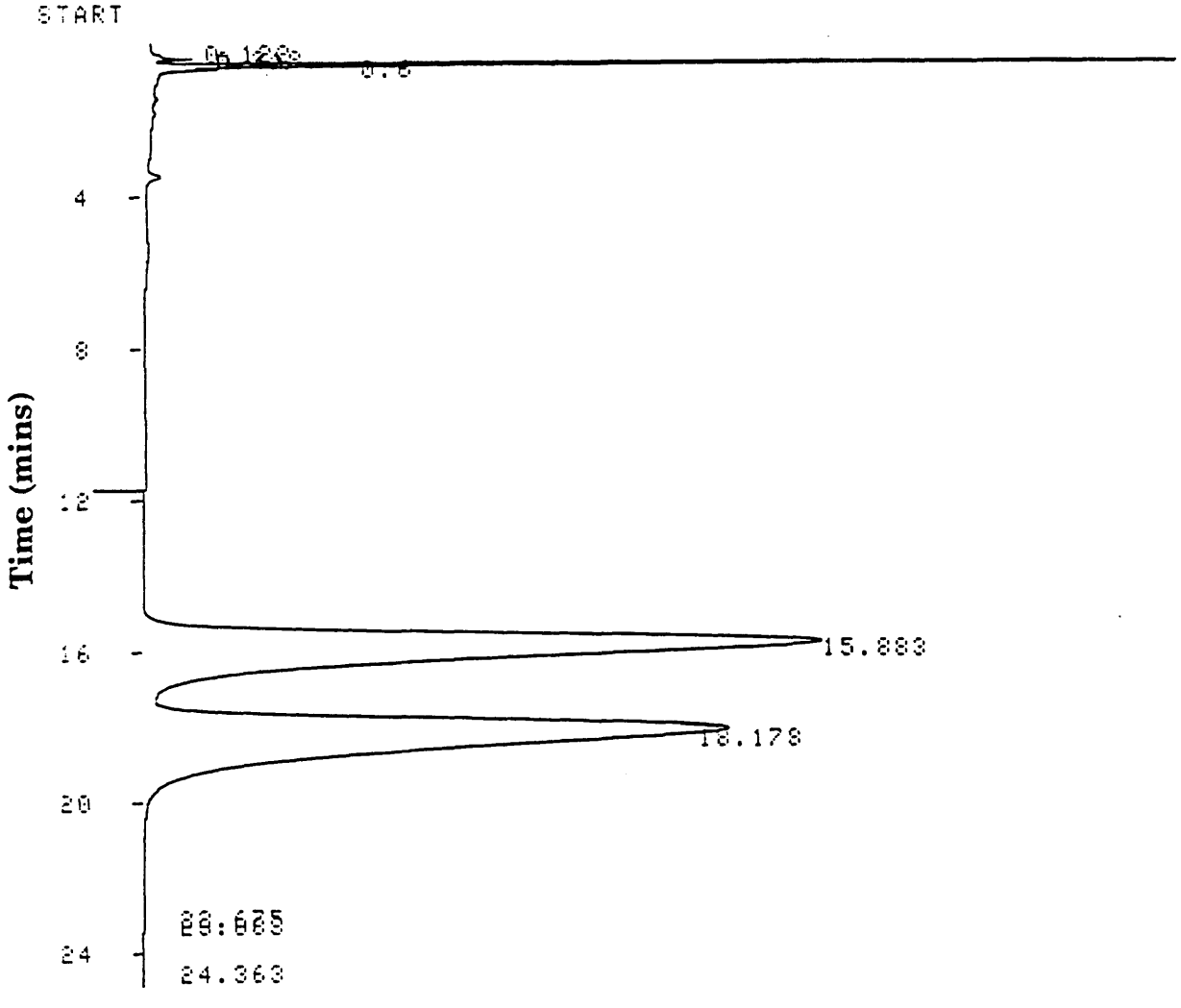


Figure 4.2

U.V. absorbance trace from HPLC analysis (system NP 1) of the diastereomeric MTPA esters of methyl 6-(2'-hydroxypropyl)-chromone-2-carboxylate isolated from the urine of male rats dosed with racemic 6-(2'-hydroxypropyl)chromone-2-carboxylic acid (500μmol/kg).



A minor metabolite with a retention time identical to that of 6-(2'-hydroxypropyl)chromone-2-carboxylic acid was also observed (Fig 4.3). The peak area ratio of these two compounds was calculated to be 94.0 : 6.0 ±1.2 (n=4) respectively.

Metabolism of 6-(1'-hydroxypropyl)chromone-2-carboxylic acid

HPLC analysis (system RP 1) of neat 0-24 h urine, from the animals dosed, showed that 6-(1'-hydroxypropyl)chromone-2-carboxylic acid was excreted to a large extent unchanged (Fig 4.4). Evidence of a minor metabolite was observed, having an identical retention time to that of 6-(1'-oxopropyl)chromone-2-carboxylic acid. The peak area ratio of these two compounds was calculated to be 94.0 : 6.0 ±0.9 (n=5) respectively.

Methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate-MTPA esters

Enantiomeric composition

6-(1'-Hydroxypropyl)chromone-2-carboxylic acid was isolated from the 0-24 h urine of rats, in the form of its methylated derivative, and converted to its MTPA esters, using Mosher's reagent (Chapter 3). The enantiomeric composition of the diastereomers generated was determined by HPLC (system NP 2), as shown in Figure 4.5 and revealed a peak area ratio of R/S, 51.0 : 49.0 ±0.4 (n=5).

Figure 4.3

U.V. absorbance trace from HPLC analysis (system RP 1) of 0-24 h urine from male rats dosed with 6-(2'-oxopropyl)-chromone-2-carboxylic acid (500μmol/kg).

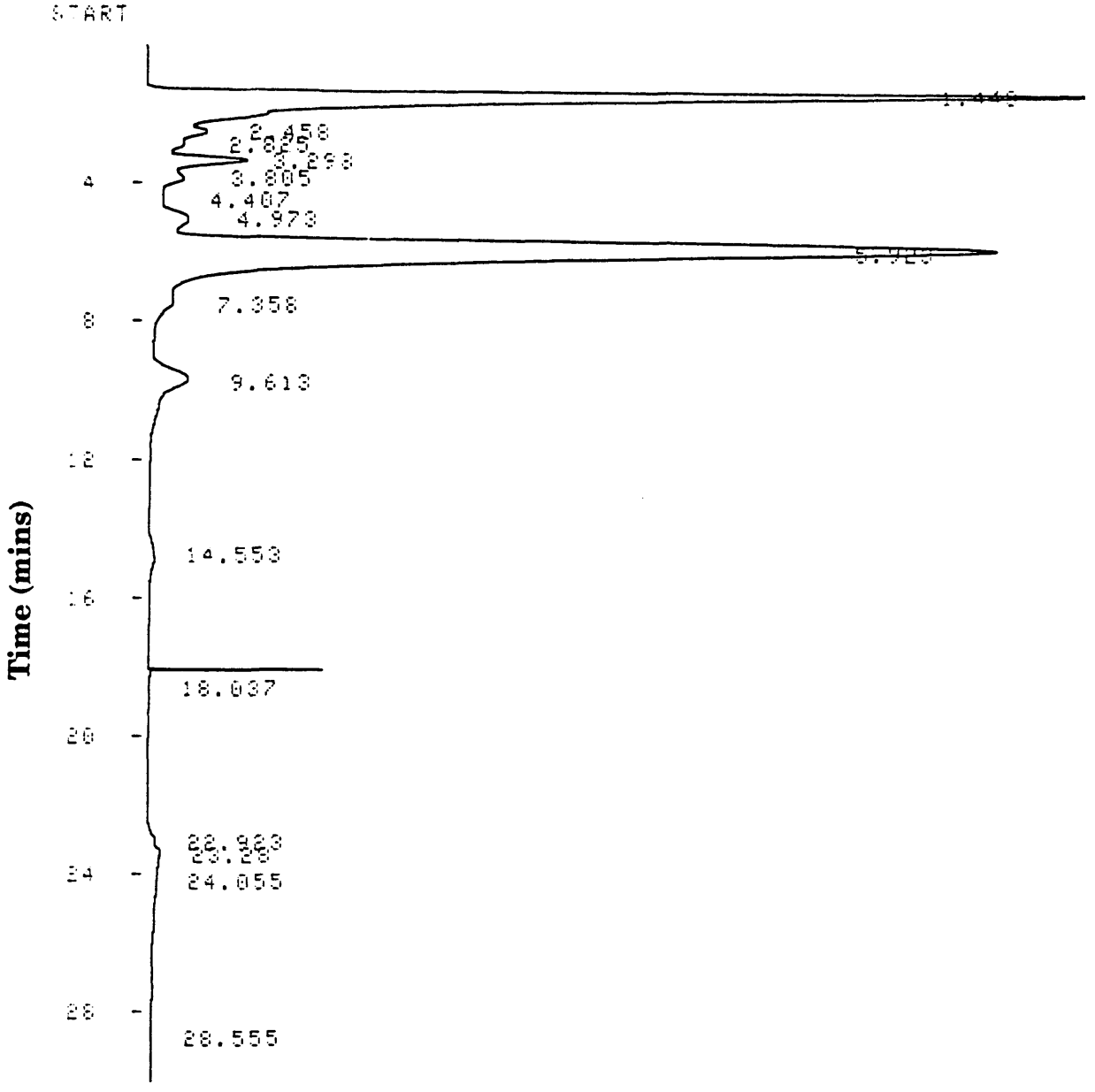


Figure 4.4

U.V. absorbance trace from HPLC analysis (system RP 1) of 0-24 h urine from male rats dosed with racemic 6-(1'-hydroxypropyl)chromone-2-carboxylic acid (500μmol/kg).

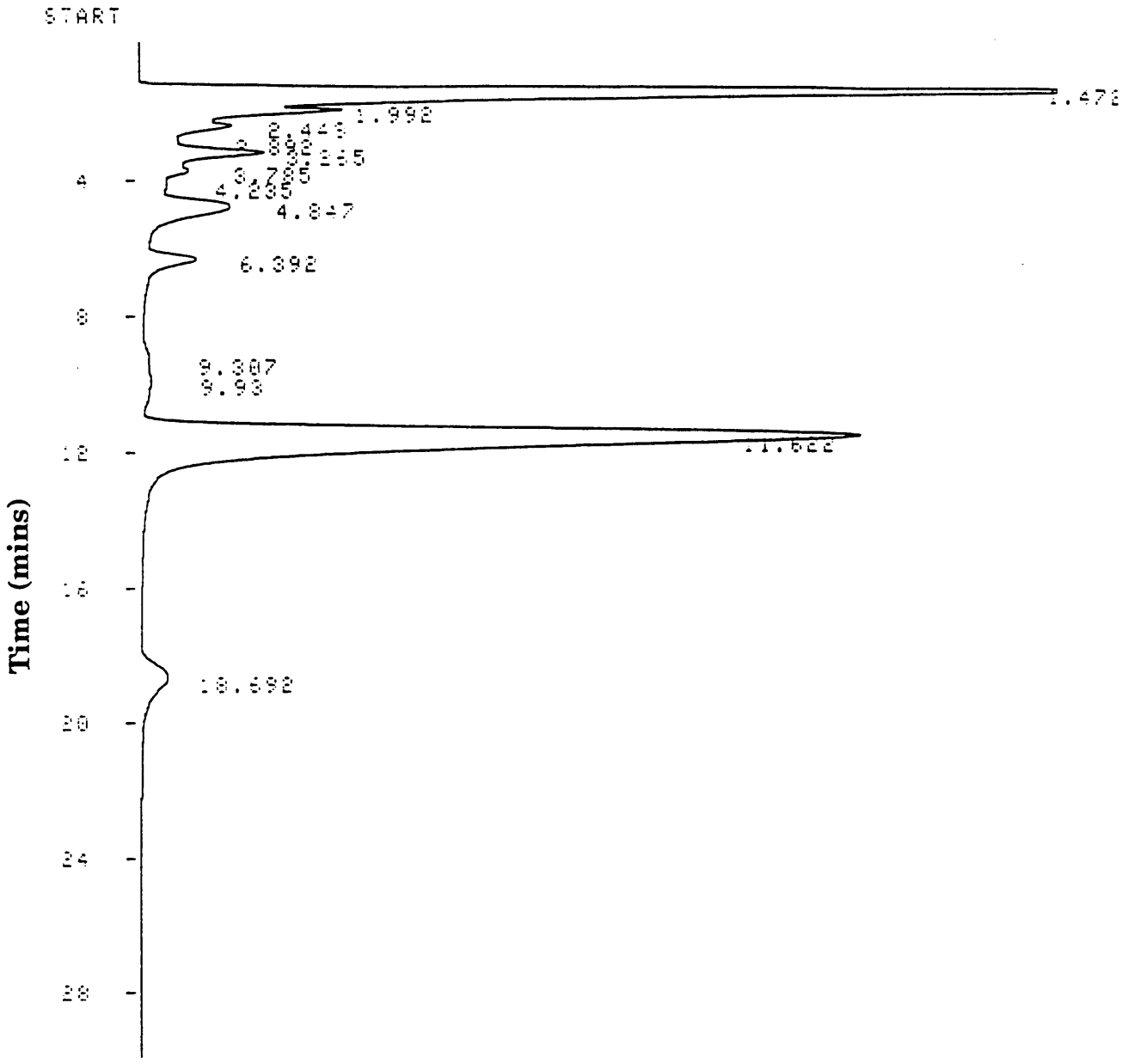
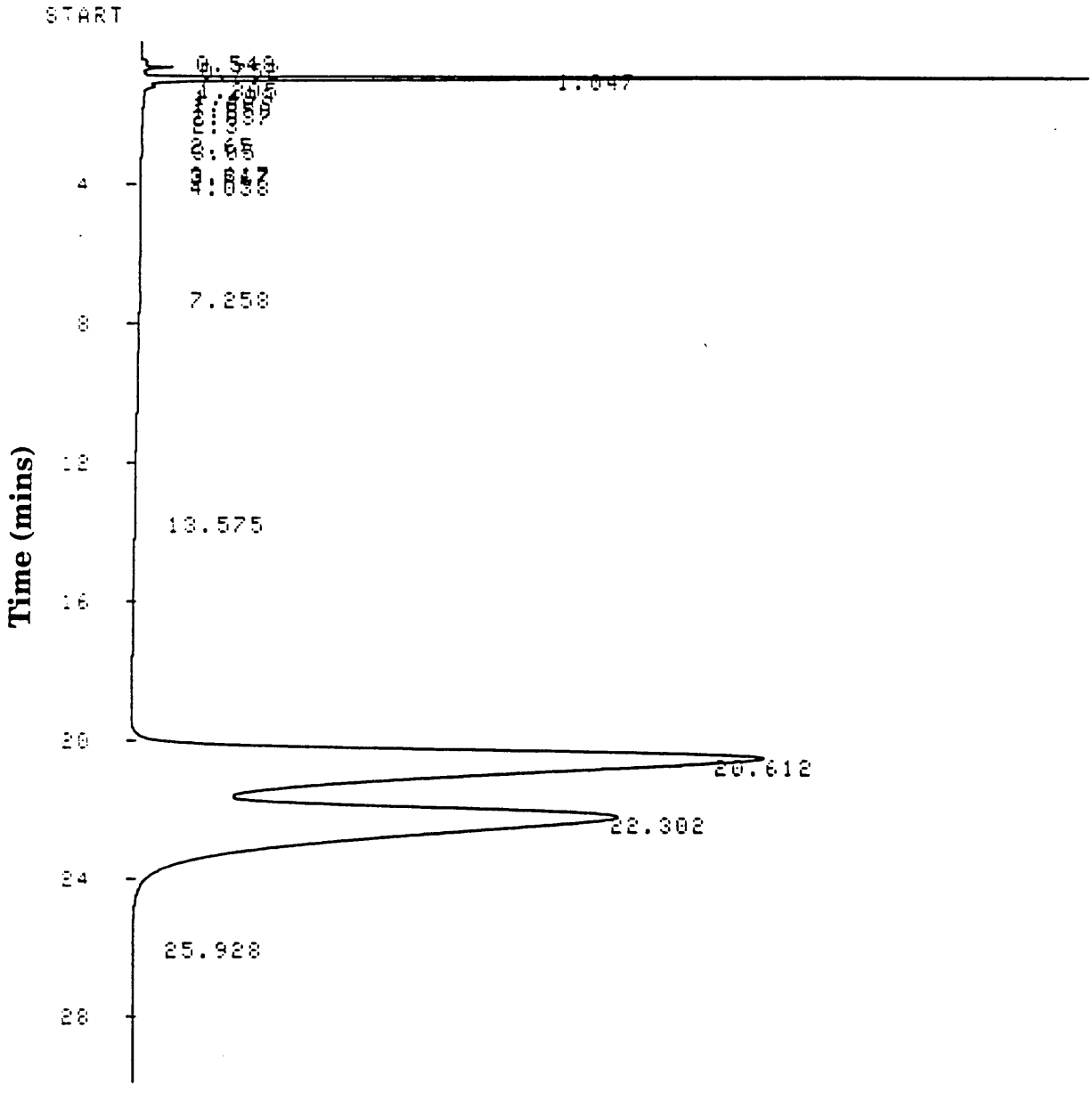


Figure 4.5

U.V. absorbance trace from HPLC analysis (system NP 2) of the diastereomeric MTPA esters of methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate isolated from male rats dosed with racemic 6-(1'-hydroxypropyl)chromone-2-carboxylic acid (500μmol/kg).

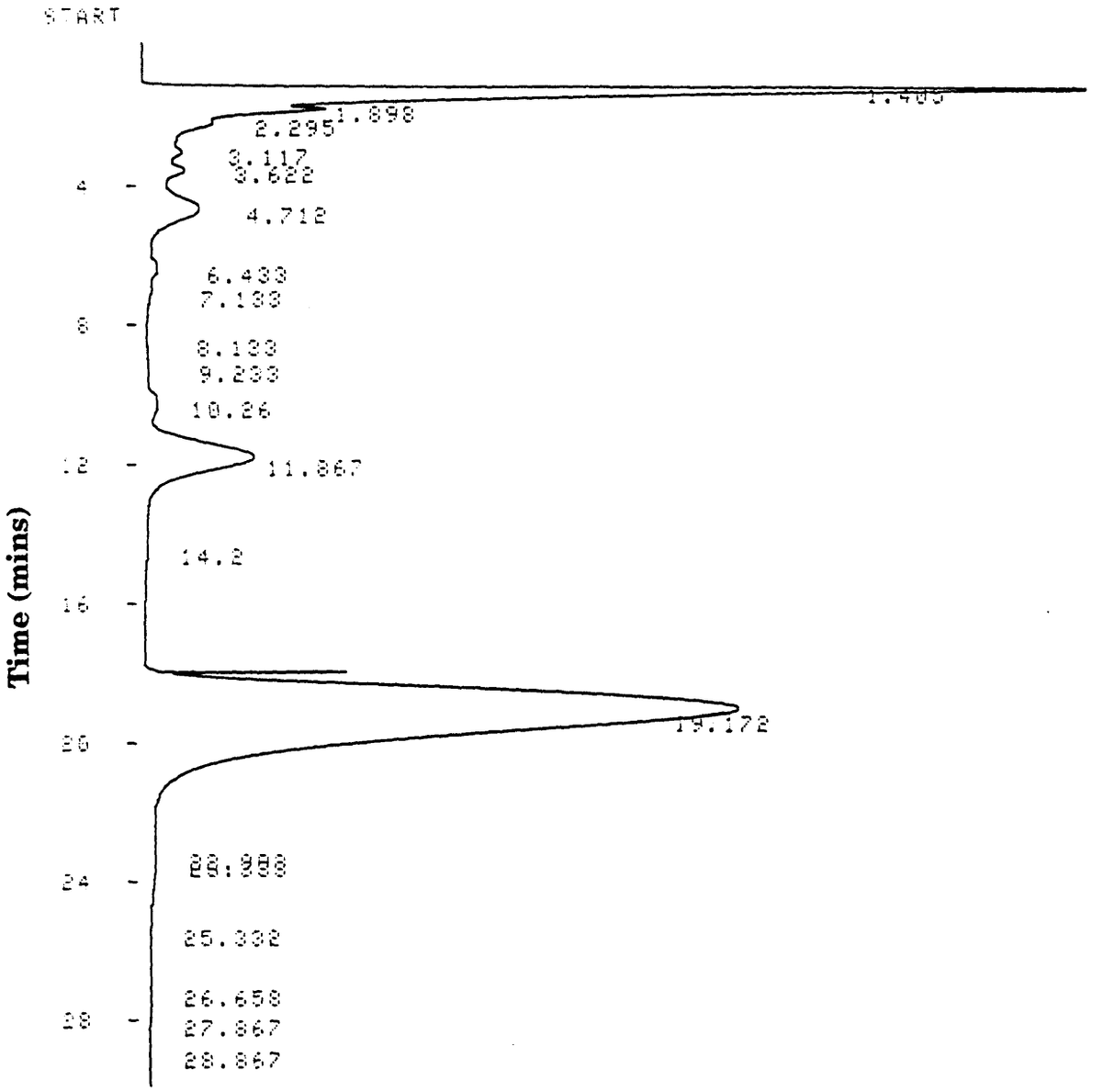


Metabolism of 6-(1'-oxopropyl)chromone-2-carboxylic acid

Neat 0-24 h urine from the treated rats was analysed using HPLC (system RP 1). The chromatograms obtained clearly indicated that some metabolism of the dose compound had occurred (Fig 4.6), with a peak having an identical retention time to that of 6-1'-HPCCA being present in all cases. However, the major compound detected in the urine was the dose compound 6-(1'-oxopropyl)chromone-2-carboxylic acid (6-1'-OPCCA). The peak area ratio of the two compounds was calculated to be 11.9 : 88.0 ± 1.7 (n=5) 6-1'-HPCCA/6-1'-OPCCA respectively.

Figure 4.6

U.V. absorbance trace from HPLC analysis (system RP 1) of 0-24 h urine from male rats dosed with 6-(1'-oxopropyl)-chromone-2-carboxylic acid (500μmol/kg).



4.4 Discussion

The administration of synthetic racemic 6-2'-HPCCA and 6-1'-HPCCA to male rats revealed that the enantiomeric composition of the 6-2'-HPCCA and 6-1'-HPCCA recovered in the urine and determined as their methyl MTPA esters, was essentially racemic for both standards and that there was little or no metabolism of the dose compound. These results indicate that absorption, distribution, and renal clearance of the two racemic standards were not enantioselective.

Tsai *et al* (1985) documented a process termed "non-chiral differentiation", which refers to non-racemic mixtures of enantiomers being progressively altered in favour of the more abundant enantiomer, by typical work-up procedures, which do not involve chiral-chiral interactions, eg solvent extractions and achiral chromatography. Therefore these results also indicate that "non-chiral enantiodifferentiation" does not occur either in the biological system or during the isolation procedure.

However, product selective hydroxylation (i.e. hydroxylation via a single enzyme step) may not be the only possible route of metabolism. A multi-component route, involving two or more metabolising enzymes is also conceivable. This may involve oxidation of the alcohol to yield the corresponding ketones in which the chirality of the molecule is lost, and possible reduction of the prochiral ketone back to the alcohol. Thus substrate and product enantioselectivity may contribute to the observed unequal excretion of the enantiomeric metabolites.

For example, it is known that the *in vivo* conversion of ethylbenzene to mandelic acid is stereoselective, with only the R-enantiomer being excreted in the urine (Sullivan *et al* 1976, Drummond *et al* 1989). Further studies by Drummond *et al* (1990) to establish the mechanism of the conversion of the reaction suggested that the preferential excretion of R-mandelic acid can be attributed to a number of reactions, including racemisation, chiral inversion and enantioselective dehydrogenation of S-mandelic acid forming phenylgloxylic acid, which is excreted in the urine.

The reduction of a considerable number of ketones has been examined and the formation of the secondary alcohol with a new chiral centre has been shown to be highly stereoselective, forming the S-alcohol in 80% or greater excess (Culp and McMahon 1968, Gal *et al* 1981).

After the administration of the racemic metabolic standard, 6-2'-HPCCA, no further metabolism of this compound was observed and the enantiomeric composition of the 6-2'-HPCCA excreted was essentially racemic. This indicates that the formation of 6-2'-HPCCA, in the male rat, from the parent compound 6-n-PCCA is most likely to occur by a single stereoselective hydroxylation, with the ketone apparently playing no role in the excretion of the compound.

Although a small quantity of 6-1'-OPCCA was detected in the urine after the administration of 6-1'-HPCCA to the male rat, it was not considered to be a metabolite. Due to difficulties in obtaining a sufficient quantity of the compound, the final purity of 6-1'-HPCCA was considered to be approximately 95%, with the other 5% being 6-1'-OPCCA. We can therefore conclude that the formation of 6-1'-HPCCA from the parent

compound 6-n-PCCA is likely to occur by a single stereoselective hydroxylation process.

After the administration of 6-2'-OPCCA and 6-1'-OPCCA to the male rat, the metabolites 6-2'-HPCCA (6.0 %) and 6-1'-HPCCA (12.0 %) were detected respectively. Reduction of aldehydes or ketones to their corresponding alcohols plays a role in the metabolism of endogenous as well as exogenous compounds, eliminating reactive carbonyl groups by forming alcohols (Bachur 1976). The enzymes attributed for this type of reaction are found in microsomal and cytosolic fractions of various tissues, but mainly in the liver, with the data available suggesting that the reductases in the two fractions are different enzyme systems (Hermans and Thijssen 1989).

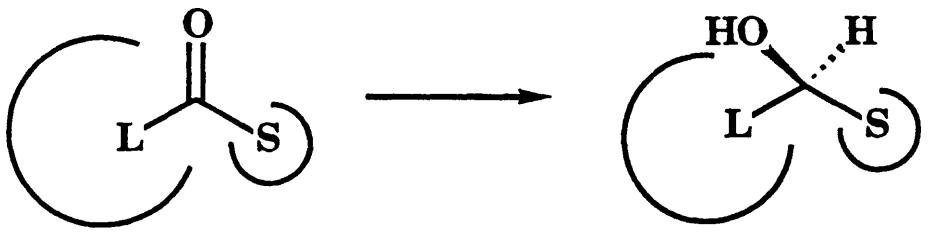
Product stereoselectivity in enzymic ketone reduction is a well known phenomenon (Kabuto *et al* 1978, Prelusky *et al* 1982). For example the reductive metabolism of ketanserin, a $5HT_2$-receptor antagonist, at the prochiral carbonyl group is mainly (>95%) reduced to the corresponding R-(+) carbinol (Meese and Eichelbaum 1989). It would therefore be highly probable that any ketone reduction of either 6-2'-OPCCA or 6-1'-OPCCA, forming 6-2'-HPCCA and 6-1'-HPCCA respectively, would exhibit product selectivity. Further isolation and enantiomeric analysis of these metabolites was not undertaken due to the small quantities excreted in the urine.

Prelog (1964), studying the bacterial reduction of ketones and formulated a rule to predict the configuration of the resulting alcohol, which was affirmed by Prelusky (1982). In the simple scheme (Fig 4.7), the ketone is projected in the plane with the bulky group (L) adjacent to the carbonyl

placed on the left and the small group (S) placed on the right. The resulting alcohol predominantly will have the configuration with the hydroxyl group above the plane.

Figure 4.7

Steric course of the microbial reductions of carbonyl compounds.



The reduction of 6-2'-OPCCA and 6-1'-OPCCA, according to this rule, will yield predominantly the (R) and (S) hydroxy metabolites respectively. However, as little or no ketone intermediates were detected after the administration of the synthetic racemic 2'- and 1'-alcohols, this stereoselective ketone reduction is not considered to be a significant factor in the metabolism of 6-n-PCCA in the rat.

Hermans and Thijssen (1989) studied the *in vivo* metabolic ketone reduction of warfarin and its analogues 4'-nitrowarfarin and 4'-chlorowarfarin using microsomal and cytosolic fractions of several species. Prelog's rule inferred the formation of the R,S and S,S alcohols from the R and S enantiomers respectively, which was generally found to be the case for the R but not the S-enantiomer in the cytosolic system and for the S but less the R in the microsomal system. From these results Hermans and Thijssen (1989) have questioned the validity of

Prelog's rule for predicting product stereoselectivity of mammalian ketone reductases.

Chapter 5

The Effect of Variations in the Hepatic Complement of Cytochrome P450 Isozymes upon the Regio- and Stereo-selective Metabolism of 6-n-Propylchromone-2-Carboxylic Acid in the Male Rat.

5.1 Introduction

After the administration of 6-n-propylchromone-2-carboxylic acid (500μmol/kg) to male rats, three major urinary metabolites were detected and identified. These metabolites, which were excreted in various amounts in the urine had all resulted from oxidations along the 6-n-propyl moiety, indicating that the metabolism of 6-n-PCCA exhibited some degree of regioselectivity (Chapter 2).

The formation of new chiral centres within two of the metabolites prompted investigations into their urinary enantiomeric composition (Chapter 3). This revealed that preferential excretion of one enantiomer had occurred for both metabolites and suggested that the metabolism of 6-n-PCCA exhibits some degree of enantioselective hydroxylation. Other possible enantioselective mechanisms, both biological and non-biological, were later eliminated as possible explanations of this preferential enantiomeric excretion (Chapter 4), confirming initial speculations. However, speculation concerning the possible number of cytochrome P450 isozyme involved in both the regio- and stereo-selective metabolism has already been aired in Chapters 2 and 3.

This chapter now reports on further investigations of the regio- and stereo-selective metabolism of 6-n-propylchromone-2-carboxylic acid in the male rat after pretreatment of the animals with known inducers of cytochrome P450 isozymes. It is hoped that this work will give an insight into the possible number(s) of cytochrome P450 isozymes involved in the metabolism of 6-n-PCCA, as previously speculated in Chapters 2 and 3.

5.2 Materials and Methods

Materials and methods were as described in preceding chapters unless otherwise stated.

Compounds

All chemicals used in the pre-treatment of animals are listed with their sources in Appendix 5.1.

Animals and dosing

Metabolism of 6-n-propylchromone-2-carboxylic acid

24 Male Wistar rats, weighing approximately 200g (Oxford Laboratory Animal Co), were maintained on a commercially available diet (Labsure CRM, Biosure Ltd, Manea, Cambridge.).

These animals were divided into 4 groups of 6, each group receiving one of the following pre-dose treatments :-

Phenobarbitone (0.1% w/v) for 8 days in the drinking water.
Clofibrate (400mg/kg daily), intraperitoneal injection for 3 days.
Isosafrole (150mg/kg daily), intraperitoneal injection for 3 days.
β-Naphthoflavone (80mg/kg daily), intraperitoneal injection for 3 days.
(taken from Waxman and Walsh (1982), Tamburini et al (1984), Fisher et al (1981) and Lau and Strobel (1982) respectively). Data from this laboratory confirms the specific patterns of cytochrome P450 induction using the above dose regimes (T.Reed personal communication).

The vehicle for all intraperitoneal injections was trioctanoin (C 8:0).

The animals received [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg) by intraperitoneal injection 24 h after the last administration of the pretreatment. The dose solution was prepared as in Chapter 2.

The animals were housed in individual metabolism cages and allowed food and water *ad libtum* throughout the experiment. All excreta were collected daily for three days after the administration of the dose.

Identification of urinary metabolites

Urines and cage washes were centrifuged (3000 x g, 10min) and their ^{14}C content determined as in Chapter 2. Samples of neat 0-24 h urine were filtered (0.45μm, Acrodisc LC13 PVDF, Gelman Sciences) and examined by reverse phase radio HPLC (system RP 1). The pH of all samples was adjusted to 5 with 2M hydrochloric acid before storage at -20°C.

Isolation of urinary metabolites

The isolation of urinary metabolites was carried out as described in Chapter 3.

Derivitisation using (+)-(S)-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl)

Both chiral metabolites from each group of animals (isolated as their methyl esters), were derivatised using Mosher's reagent (MTPA-Cl) as described in Chapter 3.

5.3 Results

Metabolism and excretion of [2-2'.-^{14}C]-6-n-propylchromone-2-carboxylic acid in the male rat after pretreatment with the various inducers.

Excretion

The excretion of radioactivity by pretreated male rats 500μmol/kg (20μCi/kg) of 6-n-propylchromone-2-carboxylic acid i.p. is presented in Table 5.1.

Typically, > 72% of the administered doses were excreted in the urine over the 72 h period, again identifying this as the major route of metabolism. Elimination was rapid, with > 71% of the administered doses excreted during the first 24 h, with much smaller quantities being voided during the following two days. The total recovery of radioactivity after 72 h was always > 80%.

Urinary metabolites of 6-n-propylchromone-2-carboxylic acid in the male rat.

Metabolite identification and quantitation was achieved using the procedures described in Chapter 3. The metabolites and their percentages in the neat 0-24 h urine are presented in Table 5.2.

The pretreatment of male rats with phenobarbitone prior to the administration of 6-n-PCCA resulted in increased levels of the (ω-2)-hydroxylated metabolite in the 0-24 h urine and a substantial decrease in the excretion of parent compound. Pretreatment with β–naphthoflavone

also caused an increase in the observed excretion of the (ω-2)-hydroxylated metabolite, but reduced the excretion of 6-2'-HPCCA. However, pretreatment with isosafrole did not alter the observed urinary excretion of the (ω-2)-hydroxylated metabolite but did reduce the excretion of 6-2'-HPCCA and increase the amount of unchanged parent compound detected in the urine. Pretreatment with clofibrate reduced the excretion of all metabolites with the exception of the ω–oxidised product, which was substantially increased.

Enantiomeric composition of the chiral metabolites

Methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate-MTPA esters

After isolation, methyl 6-(1'-hydroxypropyl)chromone-2-carboxylate from each treated group of animals was converted to its MTPA esters using Mosher's reagent and the enantiomeric composition of the diastereomers formed analysed by HPLC (system NP 2). The peak area ratios (R/S) were calculated from the U.V. and radio-chromatograms and are presented in Table 5.3.

Methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate-MPTA esters

After isolation, methyl 6-(2'-hydroxypropyl)chromone-2-carboxylate from each group of treated animals was converted to its MTPA esters using Mosher's reagent and the enantiomeric composition of the dastereomers formed analysed by HPLC (system NP 1). The peak areas ratio (R/S) were calculated from the U.V. and radio-chromatograms and are presented in Table 5.3

Table 5.1 **Excretion of radioactivity after intraperitoneal administration of [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg) to male rats pretreated with various inducers.**

Time (h) after dosing	% excretion of radioactivity							
	Phenobarbitone		Clofibrate		Isosafrole		β–Naphthoflavone	
	urine	faeces	urine	faeces	urine	faeces	urine	faeces
0-24	67.0 ±11.3	10.0 ±13.4	71.3 ±6.2	6.3 ±7.2	67.1 ±4.2	8.1 ±4.6	61.9 ±5.0	9.9 ±4.1
24-48	3.5 ±1.5	1.6 ±1.5	5.7 ±3.9	4.9 ±2.4	3.5 ±2.2	3.2 ±2.6	2.8 ±0.4	3.6 ±4.1
48-72	1.7 ±1.2	1.7 ±2.5	0.9 ±0.6	2.1 ±2.6	1.9 ±3.0	1.1 ±1.8	1.2 ±0.8	0.3 ±0.2

Mean ± S.D. of 5 animals

Table 5.2

Metabolites and their percentages in the 0-24 h urine of male rats, after pretreatment with various inducers and intraperitoneal administration of [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg).

	% of 0-24 h urinary radioactivity					
Pretreatment	1HPCCA	2HPCCA	3CPCCA	A	Z	PCCA
Control	15.0±3.0	22.3±2.3	4.0±0.9	1.5±0.2	2.0±0.1	55.5±4.3
Phenobarbitone	28.2±4.1	27.2±4.2	2.8±0.7	2.3±0.7	2.7±0.5	36.9±8.3
β-Naphthoflavone	27.3±6.8	10.9±2.7	2.0±0.6	0.0	1.5±0.5	58.1±9.2
Isosafrole	14.0±2.0	8.4±1.6	4.6±0.8	0.0	1.3±0.3	72.4±3.1
Clofibrate	9.8±2.3	11.0±3.0	11.1±5.4	0.0	0.8±0.2	67.3±7.1

1HPCCA= 6-1'-HPCCA, 2HPCCA= 6-2'-HPCCA, 3CPCCA= 6-3'-CPCCA, A= unknown, Z= unknown, PCCA= 6-n-PCCA

Mean ± S.D. of 5 animals.

Table 5.3 **The effect of pretreatment with enzyme inducers on the enantiomeric composition of 6-(1'-hydroxypropyl)- and 6-(2'-hydroxypropyl)chromone-2-carboxylic acids excreted in the 0-24 h urine of male rats dosed with [2,2'-^{14}C]-6-n-propylchromone-2-carboxylic acid (500μmol/kg, 20μCi/kg).**

Pretreatment	6-2'-HPCCA Enantiomeric Ratio (R/S)		6-1'-HPCCA Enantiomeric Ratio (R/S)	
	U.V.	^{14}C	U.V.	^{14}C
Control	90.4 / 9.6 ±0.58	89.6 / 10.4 ±0.58	7.1 / 92.9 ±0.49	7.9 / 92.1 ±0.39
Phenobarbitone	89.1 / 10.9 ±0.55	89.4 / 10.6 ±1.33	5.9 / 94.1 ±0.67	5.6 / 94.4 ±0.54
β–Naphthoflavone	90.3 / 9.7 ±0.65	90.4 / 9.6 ±2.0	2.0 / 98.0 ±0.10	1.9 / 98.1 ±1.1
Clofibrate	89.4 / 10.6 ±1.2	87.9 / 12.1 ±0.51	7.0 / 93.0 ±0.86	9.0 / 91.0 ±0.40
Isosafrole	88.7 / 11.2 ± 2.0	88.0 / 12.0 ±1.13	10.4 / 89.6* ±0.96	11.1 / 88.9* ±0.87

Mean ± S.D. of 5 animals unless otherwise stated, * (n=4)

5.4 Discussion

Induction of cytochrome P450 by drugs and other xenobiotics was first reported from observations on the pharmacological responses (Conney 1967). For example, the sedative effect of phenobarbitone was gradually reduced during chronic administration to rats, due to increased barbiturate metabolism and clearance (Remmer 1962, Remmer and Merker 1963). Since these early observations many more drugs and xenobiotics have been shown to cause induction of cytochrome P450, and depending upon the specific isozymes induced, they can generally be assigned to one of five major inducer categories (Table 5.4).

Table 5.4 Major inducible forms of P450 in the rat.

Inducer category	P450 forms induced
Polycyclic aromatic hydrocarbons	P450IA1 P450IA2
Phenobarbitone	P450IIB1 P450IIB2
Ethanol.	P450IIE1
Pregnenolone-16α carbonitrile and glucocorticoids.	P450IIIA1
Peroxisome proliferators.	P450IVA1

(adapted from Okey 1990)

However not all known P450 inducers necessarily fit into any of these categories.

Although a compound may induce two or more isozymes, the extent of induction is not uniform. The compounds used as inducers in this chapter are no exceptions and Table 5.5 identifies the major forms of P450 known to be induced by them.

Table 5.5

Summary of the major forms of cytochrome P450 isozymes induced by the pretreatment regimes used in the present chapter.

	Cytochrome P450 isozymes				
Pretreatments	IIB1	IIB2	IA1	IA2	IVA1
Phenobarbitone	[*]	*			
β–Naphthoflavone			[*]	*	
Isosafrole	*	*	*	[*]	
clofibrate					[*]

* induced isozymes, [ ] major form induced

Data from Guengerich (1987)

It is noteworthy that isosafrole does not fit neatly into any single category, in that it induces P450IIB1 and P450IIB2 (Yeowell *et al* 1985, Thomas *et al* 1987) as well as P450IA1 and P450IA2.

The oxidative metabolism of 6-n-PCCA in the male rat after pretreatment with various inducers shows quite distinctive regioselective patterns. This suggests that more than one cytochrome P450 is catalysing the production of these metabolites. Frommer *et al* (1972) concluded that at least three monooxygenases were active in the hydroxylation of n-heptane, forming the ω, (ω-1), (ω-2) and (ω-3) metabolites. Due to the complexities of an *in vivo* system it is almost impossible to identify an individual isozyme responsible for the formation of a particular metabolite, if indeed that were the case, as cytochrome P450's in general have broad and overlapping substrate specificities. However some interesting observations can be made (Table 5.6).

The administration of clofibrate to rats has been shown to induce P450IVA1 (Tamburini *et al* 1984), which is known to catalyse lauric acid ω-hydroxylation (Orton and Parker 1982), but has also been reported to decrease the levels of cytochromes P450IIB1 and P450IA1 (Sharma *et al* 1988). After administration of 6-n-PCCA, the metabolite composition of the urine showed a decrease in the (ω-1) and (ω-2) hydroxylated metabolites but a marked increase in the ω oxidised product. This may suggest that 6-n-PCCA undergoes ω-hydroxylation catalysed by P450IVA1 and further cytosolic metabolism resulting in the formation of the 6-3'-CPCCA (Robbins 1968).

Table 5.6

Summary of regioselective metabolism relative to control.

	6-1'-HPCCA	6-2'-HPCCA	6-3'-CPCCA
Control	0	0	0
Phenobarbitone	▲	0	0
β-Naphtho-flavone	▲	▼	0
Isosafrole	0	▼	0
Clofibrate	▼	▼	▲▲

▼-indicates reduced formation, ▲-indicates increased formation of metabolite, 0- indicates relative to control.

The repression of both cytochromes P450IIB1 and P450IA1 and the decrease in both (ω-1) and (ω–2) hydroxylated metabolites suggests that an interelationship between these isozymes and the metabolites may exist.

Administration of isosafrole (a "mixed-inducer") to rats has been shown to predominantly induce cytochrome P450IA2 (Ryan *et al* 1980). This induction has little or no effect on the relative abundance of either ω or (ω−2) metabolites, but has a profound effect on reducing (ω-1)-hydroxylation, which is paralleled with an increase in the excretion of

unchanged parent compound. This result suggests that although isosafrole induces a number of different isozymes, to some extent, it has " switched off " the formation of 6-2'-HPCCA, indicating that as well as inducer properties, it also exhibits the ability to repress levels of some specific cytochrome P450 isozymes. This would not be unprecedented, as 3-methylcholanthrene is known to reduce the levels of P450IIB1 (Baron *et al* 1984). Isosafrole is known to induce cytochromes P450IIB1 and P450IIB2 (Yeowell *et al* 1985, Thomas *et al* 1987) which would suggest that neither of these isozymes are responsible for the formation of the (ω-1) metabolite, as its formation was clearly reduced after pretreatment with isosafrole.

Chronic administration of phenobarbitone has been shown to predominantly induce P450IIB1 as well as P450IIB2. After the pretreatment of phenobarbitone, a significant increase in the hydroxylation activity was observed for the metabolism of 6-n-PCCA, with a marked increase in the (ω-2) metabolites.

However, the regioselective pattern of metabolism of 6-n-PCCA after pretreatment with phenobarbitone does not show any substantial increase in the formation of the (ω-1) metabolite, suggesting that the isozymes P450IIB1 and P450IIB2 are not responsible for its formation. This confirms the conclusions arising from the results after pretreatment with isosafrole.

Morohashi *et al* (1983) studied the hydroxylation of n-hexane by rat liver microsomes, and observed the formation of 1-, 2- and 3-hexanols in untreated microsomes. Microsomes prepared from phenobarbitone-

treated rats resulted in an increase in formation of all three metabolites, but particularly the formation of the (ω-1) and (ω-2) metabolites.

Bjorkhem and Danielsson (1970) reported that phenobarbitone treatment induced the rat liver microsomal (ω-1) hydroxylase activities for laurate, palmitate and stearate, but not the liver microsomal ω-hydroxylase activity.

From these and other reports (Frommer *et al* 1972) its seems unusual that the formation of the (ω−1) metabolite was not substantially increased. However short chain alkyl groups adjacent to aromatic rings are known to favour benzylic hydroxylation. Thus any preferential benzylic hydroxylation would result in an increased formation of the (ω-2) metabolite to the detriment of the (ω-1) product. This may account for the marked increase in the (ω-2) metabolite and suggests that P450IIB1 and/or P450IIB2 are responsible for its formation.

Administration of β-naphthoflavone (a methylcholanthrene type inducer) predominantly induces P450IA1 and P450IA2. This induction resulted in a marked increase in the (ω-2) metabolite, but a decrease in the (ω-1) metabolite.

Morohashi *et al* (1983) observed the microsomes from rats treated with 3-methylcholanthrene stimulated the formation of 3-hexanol from n-hexane with the formation of the other two isomeric alcohols being reduced. This result closely parallels those for 6-n-PCCA and suggest that P450IA1 and/or P450IA2 are responsible for the formation of the (ω-2)-hydroxy metabolite in the treated rat.

Although the treatment of rats with inducers increases a specific range of cytochrome P450's, there are a number of isozymes which are not susceptible to induction and which contribute to the total P450 content in the untreated rat. These include P450IIC7 (Ryan *et al* 1984) and P450IID1 (Larrey *et al* 1984) as well as sex-specific forms. In the untreated rat many of the isozymes susceptible to induction only occur at very low levels (e.g. P450IIB1 and P450IA1 account for < 1% of total P450). Thus the metabolism seen in the untreated rats (Chapter 2) is likely due to P450 isozymes which are not susceptible to induction, in particular the formation of the (ω-1) metabolite, as no substantial increase in levels of excretion was observed after any of the pretreatments.

If only one cytochrome P450 isozyme were involved in the oxidative metabolism of 6-n-PCCA, the same pattern of metabolites quantities would be expected, irrespective of the pretreatment of the animals. The results indicate that this is not the case and that a number of isozymes are involved.

In summary, these results suggest that the major cytochrome P450 isozymes inducible by phenobarbitone and β-naphthoflavone may be responsible for the formation of the (ω-2) metabolite and that the ω-metabolite is formed by P450IVA1. This overlapping substrate specificity indicates that the active site within the two P450 families P450IIB and P450IA are similar enough to be able to accomodate the 6-n-PCCA molecule. This would not be unprecedented, as Lu and West (1980) also reported that structurally distinct cytochrome P450's have overlapping reactivities.

It has been established that 6-n-PCCA is metabolised in the male rat by more than one P450 isozyme and that the regioselectivity observed depends on the relative concentrations of these isozymes. However, this regioselectivity will also depend on the relative enzyme-substrate affinity constants and any differences in the transition state energies. An indication of the latter has been proposed, in that the (ω-2) hydroxylated product is preferentially formed even after pretreatment with phenobarbitone. This is probable due to the relative lowering of the transition energy at the benzylic position by resonance stabilisation from the aromatic ring.

The oxidative metabolism of 6-n-PCCA in the male rat yields two metabolites (6-2'-HPCCA and 6-1'-HPCCA), which contain chiral centres and as such may be expressed in terms of their enantiomeric composition (Chapter 3). After pretreatment with various inducers the enantiomeric compositions of the individual metabolites were generally comparable, with the exception of the 6-1'-HPCCA metabolite formed after pretreatment with β–naphthoflavone (Fig 5.3). This indicates that, irrespective of the relative concentration of specific cytochrome P450 isozymes present, the parameters determining enantiomeric hydroxylation are constant.

In contrast, Sugiyama and Trager (1986) calculated the R/S product stereoselectivities associated with the ω-hydroxylation of 2-phenyl-propanol by various liver preparations to be; uninduced 2.23± 0.27, phenobarbitone treated 1.65± 0.21 and β-naphthoflavone induced 0.89± 0.2. These results clearly indicate that the specific cytochrome P450 isozyme content of each microsomal preparation has a profound effect on the stereoselectivity observed in the metabolism of this substrate.

White *et al* (1984) studied the hydroxylation of three alicyclic compounds, D-camphor, adamantanone, adamantane by the cytochrome P450 isozymes $P450_{cam}$ and P450IIB4. $P450_{cam}$ catalysed only one product formation from each of the substrates, namely 5-exo hydroxycamphor, 5-hydroxyadamantanone and 1-adamantanol respectively. These three positions are topographically congruent and indicate a single and tight enzyme-substrate complex. P450IIB4 catalysed the formation of two or more isomeric products from each substrate. In particular the metabolism of D-camphor resulted in the formation of 3-endo-, 5-exo-, and 5-endo-hydroxycamphors, indicating that the substrate-enzyme complex allows considerable movement of the substrate and less limited access to the enzymic hydrogen abstractor, resulting in the loss of rigid regio- and stereo-selective product formation.

Sugyama and Trager (1986) and White *et al* (1986) working with deuterated 2-phenylpropanone and deuterated 1-phenylethanol respectively both suggested that the product stereoselectivity is primarily dependent upon the cytochrome P450 apoprotein structure and the chirality of the catalytic site and not the intrinsic characteristic of the P450 hydroxylation mechanism. This would be consistent with White's earlier report (see above).

If hydroxylation stereoselectivity is enforced by the surrounding protein tertiary structure of the cytochrome P450 isozyme involved, then varying the levels of specific isozymes using inducers should result in variation in enantiomeric composition as it is known that most isozymes have distinct structures, reflected in their substrate and product specificities. However, the enantiomeric metabolism of 6-n-PCCA in the male rat

after pretreatment with various inducers does not show any significant variation in enantiomeric composition of either chiral metabolites.

This suggests that the structure of the substrate may also play a critical role in influencing the extent of stereoselective metabolism.

It has been shown that liver microsomes from 3-methylcholanthrene treated rats catalyse the epoxidation of benzo[a]pyrene with high stereoselectivity. The 4,5-oxide formed consists of > 97% of the (4S,5R)-enantiomer (Armstrong *et al* 1981), whilst the 7,8-oxide formed consists of at least 95% of the (7R,8S)-enantiomer (Levin *et al* 1980, Thakker *et al* 1977). From these results Jerina *et al* (1982) proposed a model for the catalytic site of cytochrome P450IA1 in which only one face of the benzo[a]pyrene can point towards the activated oxygen of the haem complex, but allows multiple binding orientations for the substrate necessary for the observed positional isomeric metabolites.

Due to the "steering effect" of the carboxylic acid function at the 2-position on the chromone nucleus of 6-n-PCCA (Chapter 3), we postulate that the molecule may only gain access to the enzyme by insertion of the 6-n-propyl substituent. This single binding orientation prevents metabolism occurring around the chromone nucleus but determines regioselective metabolism along the 6-n-propyl substituent. However, we also postulate that the steric constraints imposed by the carbonyl substituent at the 4- position of the chromone nucleus inhibit horizontal (x-axis) rotation of the molecule and therefore restrict the enantiotopic hydrogens gaining access to the activated oxygen of the haem complex.

The lack of any significant (A) face attack (Chapter 3) even after treatment with known inducers indicates that in all cases the enzyme-substrate complex formed upon binding of 6-n-PCCA to the enzyme results in a rigid, non-flexible structure restricting or prohibiting (A) face attack. This is probably primarily enforced by hydrogen bonding between unknown amino acid residues within the enzyme and the carbonyl moiety of the chromone nucleus.

If 6-n-PCCA exhibits a single mode of binding to the enzyme, limited movement of the substrate within the active site must occur in order to achieve (A) face attack. Thus the relative energy differences between the two diastereomeric transition states will determine the ratio of the products. However if 6-n-PCCA exhibits two modes of binding, in which one rotates the molecule 180° relative to the other, each dedicated to either (A) or (B) face attack then the relative probabilities of the two binding modes will determine the ratio of the products.

Chapter 6

General Discussion

Although cytochrome P450's are found in prokaryotes and eukaryotes, in plants as well as animals, to date the available literature generally concentrates on mammalian derived P450's. The isolation and sequencing of many of these mammalian cytochrome P450 proteins, c DNA's and genes has resulted in the development of a classification system for cytochrome P450's based on their primary amino acid sequence alignment data (Nebert et al 1987).

In this system, the P450's are comprise of a gene superfamily which is subdivided into gene families and further subdivided into subfamilies. The amino acid sequence of a protein in one gene family is $\leq$ 36% similar to that from another gene family, and the amino acid sequence of a protein in a given subfamily is about 40 to 60% similar to a protein in another subfamily. Those P450 genes considered to be in the same subfamily have $\geq$ 68% similarity to other genes in the same subfamily. However this system of nomenclature, based solely on sequence similarities, does not in many cases determine whether a specific form of P450 in one species has a functional counterpart in another species.

The interspecies variation in particular cytochrome P450 isozymes is highlighted from the results obtained after the administration of 6-n-PCCA to the rabbit, rat, and guinea pig. (Chapter 2).

The rabbit exhibits rigid regioslective hydroxylation metabolism, allowing the formation of the 6-2'-HPCCA metabolite only, whereas the rat generates metabolites by oxidative metabolism along the whole lenght of the 6-n-propyl substituent.

This would suggest that either the cytochrome P450 isozyme responsible for the metabolism of 6-n-PCCA in the rabbit forms a rigid enzyme-substrate complex only steering the regiotopic hydrogens attached at the (ω-1) position into the vicinity of the activated oxygen of the iron haem complex or that the rabbit does not pocssess the compliment of P450 isozymes that are present in the rat.

The induction of specific cytochrome P450 isozymes in the rat by pretreatment with known inducers and the subsequent administration of 6-n-PCCA, resulted in marked changes in the regioselectivity of metabolism (Chapter 5), strongly suggesting that more than one isozyme was responsible for the metabolism of 6-n-PCCA in the rat. Therefore the limited metabolism observed in the rabbit would suggest that it does not possess homologues of these specific isozymes.

The primary amino acid sequence alignment data show that this is not the case. For example the gene family P450I contains two subfamilies A1 and A2, both of which are specific P450 isozymes that have structural counterparts in species including rat and rabbit. Pretreatment of rats with β-naphthoflavone caused a significant increase in the (ω-2) metabolite (Chapter 5) which suggested the formation of this metabolite was probably catalysed by either P450IA1 or IA2. If this is so and these specific isozymes are also present in the rabbit, we must question the absence of the (ω-2) metabolite in the rabbit urine. The most likely explanation for its conspicuous absence is that although the specific P450's from each animal are very structrally similar they are generally functionally distinct and any changes in the amino acid sequence, however small have considerable functional consequences (Lindberg and Negishi (1989), Introduction 1.2).

The gene family P450IV contains the isozyme IVA1 responsible for lauric acid ω-hydroxylation in the rat and IVA5, IVA6 and IVA7, responsible for lauric acid ω-hydroxylation in the rabbit. Pretreatment with clofibrate caused a significant increase in the ω–oxidised metabolite excreted by the rat, suggesting that formation of the ω–hydroxylated metabolite intermediate was probably catalysed by P450IVA1. However no ω-oxidised metabolite was isolated from the rabbit urine, indicating that although these isozymes share a common substrate in lauric acid, 6-n-PCCA is not a substrate for these rabbit-specific P450 isozymes.

This variation in species-specific cytochrome P450 isozymes is again evident when considering the enantiomeric composition of the metabolites (Chapter 3). The isozyme(s) responsible for the stereoselective metabolism in the rabbit have been shown to favour (A) face attack on the 6-n-PCCA molecule, forming the S-6-2'-HPCCA metabolite predominantly, in comparison to the (B) face attack which gives rise to both R-6-2'-HPCCA and S-6-1'-HPCCA in the rat.

This differing stereoselective metabolism may be due to differing concentrations of the specific isozymes responsible for each enantiomeric product or to the P450 isozymes in each species having significantly different binding sites such that the chirality of the products is reversed.

Induction of specific cytochrome P450 isozymes did not significantly alter the enantiomeric composition of either chiral metabolite excreted in rat urine (Chapter 5), suggesting that the product stereoselectivity is not highly dependent on the concentrations of specific P450 isozymes.

These results are in direct contrast to those of Sugiyama and Trager (1986) with the simple hydrocarbon 2-phenylpropane. From this we suggest that structural feactures of the 6-n-PCCA molecule has a profound effect on determining product stereoselectivity (see later).

However this also indicates that, although specific P450 isozymes in the rabbit and rat are capable of accomodating 6-n-PCCA within their binding sites, the tertiary protein structures of theses isozymes must be substantially different resulting in either (A) or (B) face attack. This discrimination may be due to differing amino acid residues in an enzyme exhibiting a single binding site or to the enzyme exhibiting two binding modes. Thus the diastereomeric enzyme substrate-complexes generated upon binding of the substrate in both rabbit and rat must favour opposite enantiotropic hydrogens.

The absence of any further metabolism of the secondary alcohol metabolites, either by enantioselective oxidation forming achiral ketones or any subsequent further possible product stereoselective reduction of the prochiral ketones back to the respective alcohols, clearly indicates that the preferential enantiomeric excretion of both metabolites formed after the administration of 6-n-PCCA to the rat was governed by the stereoselectivity of the hydroxylation process (Chapter 3). This, combined with the adoption of a more manageable and routine methodology, which was shown not to exhibit any "non-chiral enantiodifferentation", made 6-n-PCCA a suitable probe to investigate in greater detail regio- and stereo-selective metabolism in the rat.

The induction of specific P450 isozymes in the rat using known inducers, and the subsequent administration of 6-n-PCCA resulted in

marked and characteristic variation in the regioselective metabolite pattern. This indicated that more that one P450 isozyme was responsible for the formation of the metabolites observed. In particular pretreatment with clofibrate resulted in a substantial increase in 6-3'-CPCCA and indicated that P450IVA1 may catalyse the formation of the intermediate 6-3'-HPCCA which is further oxidised by cytosolic metabolism forming the observed 6-3'-CPCCA. If this proves to be the case, it will be the first reported exogenous substrate found that is oxidised by P450IVA1. However due to the complex nature of the *in vivo* system it is impossible to pinpoint exactly which cytochrome P450 isozymes are responsible for each regioselective metabolite.

For future studies it would be desirable to incubate purified P450 isozymes with 6-n-PCCA in a reconstituted *in vitro* system. Analysis of the metabolites generated would establish exactly which cytochrome P450 isozymes could accommodate the 6-n-PCCA molecule and thus increase our understanding of the topology of the active site of these specific P450 isozymes.

Analysis of the enantiomeric composition of both chiral metabolites excreted in the urine of rats after pretreatment with various known P450 inducers revealed little or no change in their stereochemistry. This result is somewhat surprising as specific changes in the proportions of cytochrome P450 have been shown to change the product selectivities of other prochiral substrates. However in the case of 6-n-PCCA these results suggest that the structure of the substrate molecule plays an important role in the determination of product stereoselectivity. In the case of 6-n-PCCA the caboxylic acid substituent positioned at carbon 2 on the chromone nucleus "steers" the 6-n-propyl substituent into the active

site of cytochrome P450, restricting regioselective metabolism to this substituent. The carbonyl group is then presumed to be hydrogen bonded with specific residues within the binding site, holding the molecule in a rigid substrate-enzyme complex. The predominant (B) face attack suggests that either the enzyme has a single binding mode of limited flexibility allows the (B) face enantiotropic hydrogens substantially more occupation of the vicinity of the activated oxygen, or that the enzyme has two binding modes which only allow very restricted access of the molecule in such an orientation as to accomodate (A) face attack, presumably due to the steric effects imposed by the carbonyl group.

For further studies it would be desirable to investigate the product stereoselective metabolism associated with compounds structurally related to 6-n-PCCA, such as 6-propylnaphthalene-2-sulphonic acid (Fig 6.1) and 6-propylchromene-2-carboxylic acid (Fig 6.2). Both these compounds are similar to 6-n-PCCA in that they contain a planar fused ring nucleus and a propyl substituent attached to this nucleus, at the furthest possible distance from the strongly acidic function. However, these do not possess a carbonyl group so that they could not hydrogen bond with residues in the active site of the P450. The absence of what seems to be the major determinant of enantioselective hydroxylation should result in markedly less product stereoselectivity in thier aliphatic hydroxylation.

Figure 6.1

The structure of 6-propylnaphthalene-2-sulphonic acid.

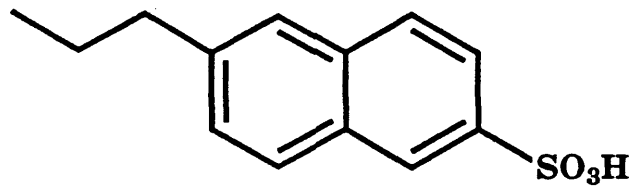
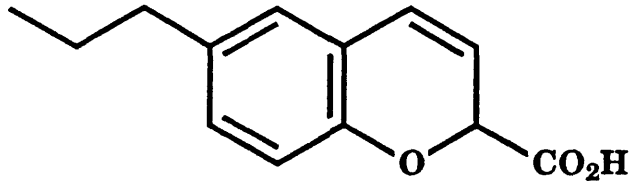


Figure 6.2

The structure of 6-propylchromene-2-carboxylic acid.



Appendix

Appendix 1.1

The R and S nomenclature.

The R and S nomenclature for chiral centres was first proposed by Cahn and Ingold (1951) and the procedure has been consolidated and extended subsequently (Cahn, Ingold and Prelog 1956, 1966).

The essential part of the R and S nomenclature is known as the Sequence Rule, which is in fact a set of arbitrary but consistant rules which allow a hierarchical assignment of the substituents on the chiral centre. The sequence rule contains five sub-rules which are applied, in succession, as long as necessary to reach a decision on the hierarchical assignment.

To assign an R or S configuration to a chiral atom;

(1) rank the four groups (or atoms) attached to the chiral atom in order of priority by the sequence rules (contained in above references),

(2) project the molecule with the group of *lowest priority* to the rear,

(3) follow the path from the group of *highest priority* to the group of next highest priority,

(4) if this gives a clockwise course the configuration is R (latin rectis, right), if this gives a anticlockwise course the configuration is S (latin sinister,left).

Further nomenclature and a list of 76 groupings arranged according to the sequence rule have been published in the IUPAC Recommendations for Fundanental Stereochemistry (1976).

Appendix 2.1

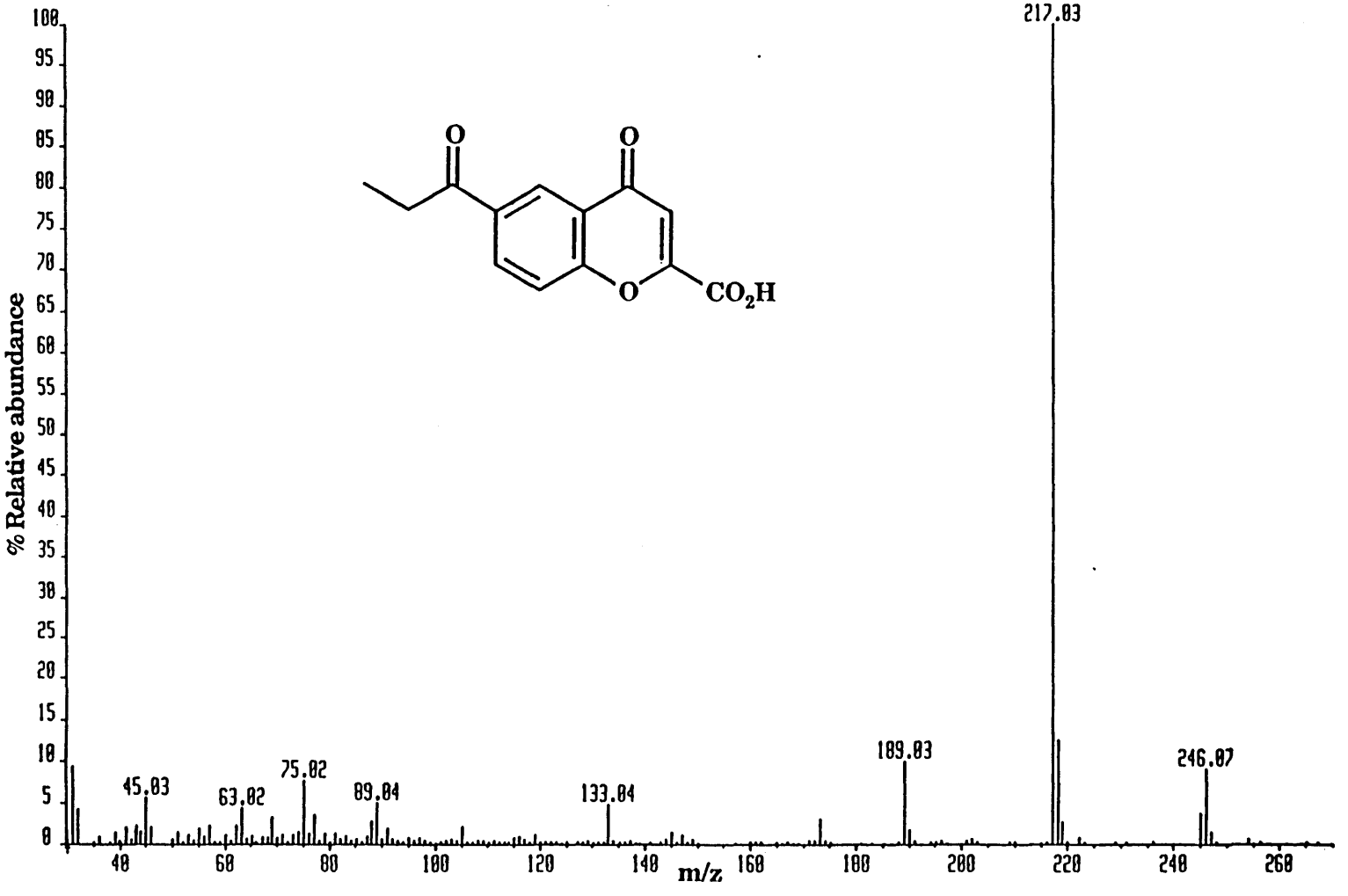
Chemicals used in Chapter 2 and their sources :

4-Propylphenol.	Aldwich Chemical Company Ltd, Gillingham, Dorset, U.K.
Dimethylacetylene dicarboxylate.	
N-Benzyltrimethylamonium hydroxide.	
Boron trifluoride-methanol complex (14%).	

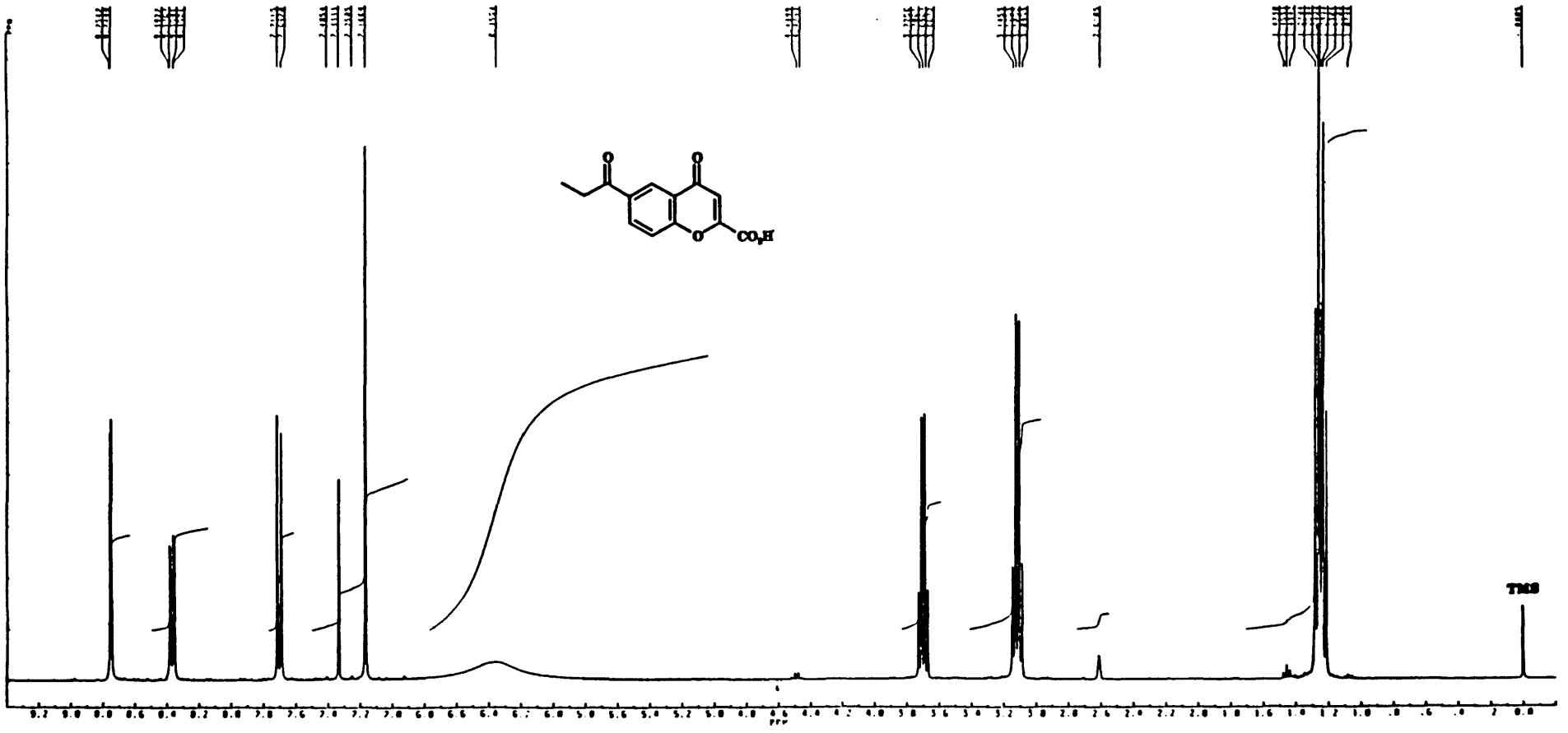
Appendix 3.1

Chemicals used in Chapter 3 and their sources :

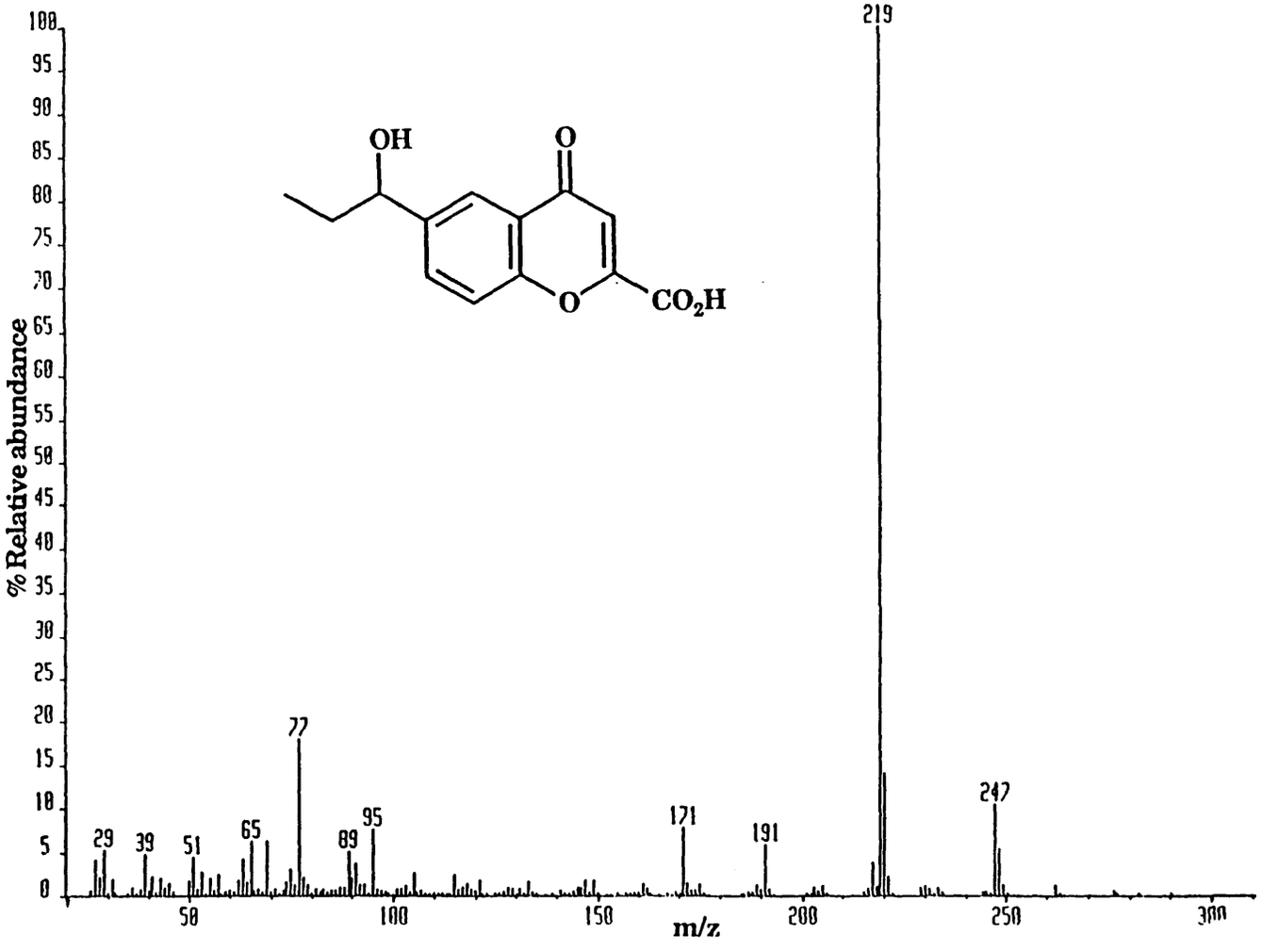
4-Hydroxypropiophenone	Aldwich Chemical Company, Ltd, Gillingham, Dorset, U.K.
Sodium borohydride	
N-Nitrosomethyl urea	Sigma Chemical Company Ltd, Poole, Dorset, U.K.
(+)-(S)-Methoxy-α-(trifluoromethyl)-phenylacetyl chloride.	J.P.S. Chemie, Bevaix, Switzerland.



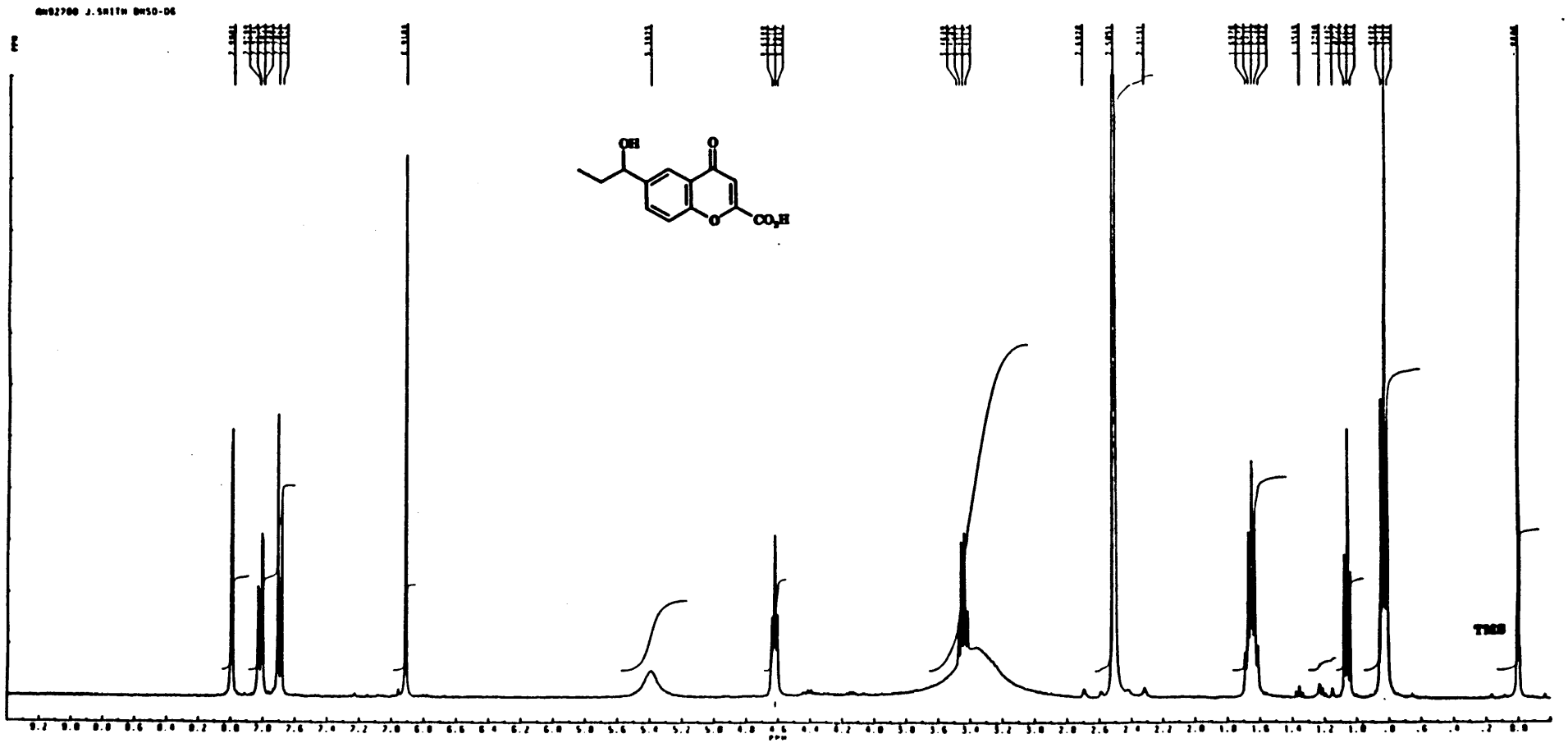
Appendix 3.2 Mass spectrum of 6-(1'-oxopropyl)chromone-2-carboxylic acid.



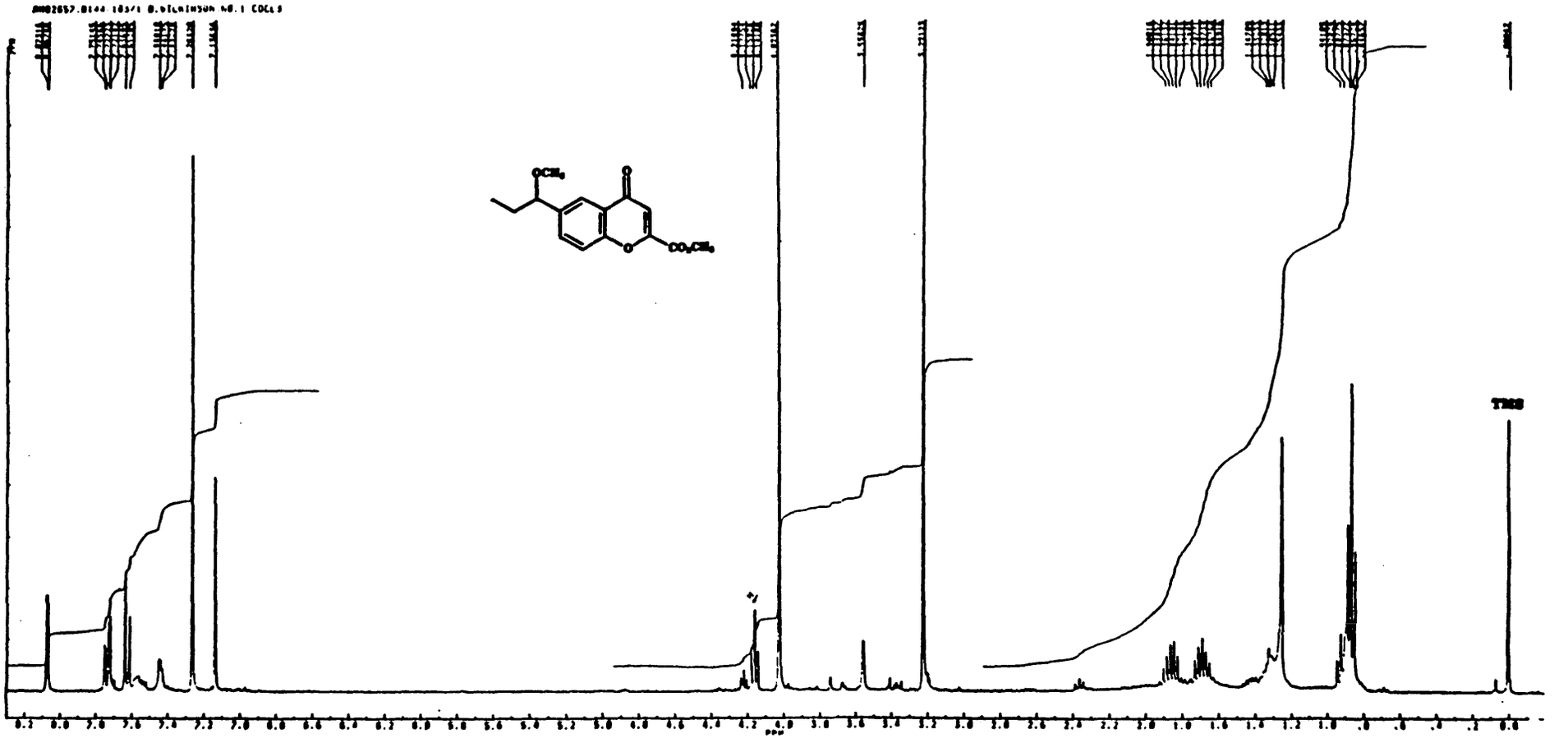
Appendix 3.3 ^{1}H NMR spectrum (360MHz) of 6-(1'-oxopropyl)chromone-2-carboxylic acid.



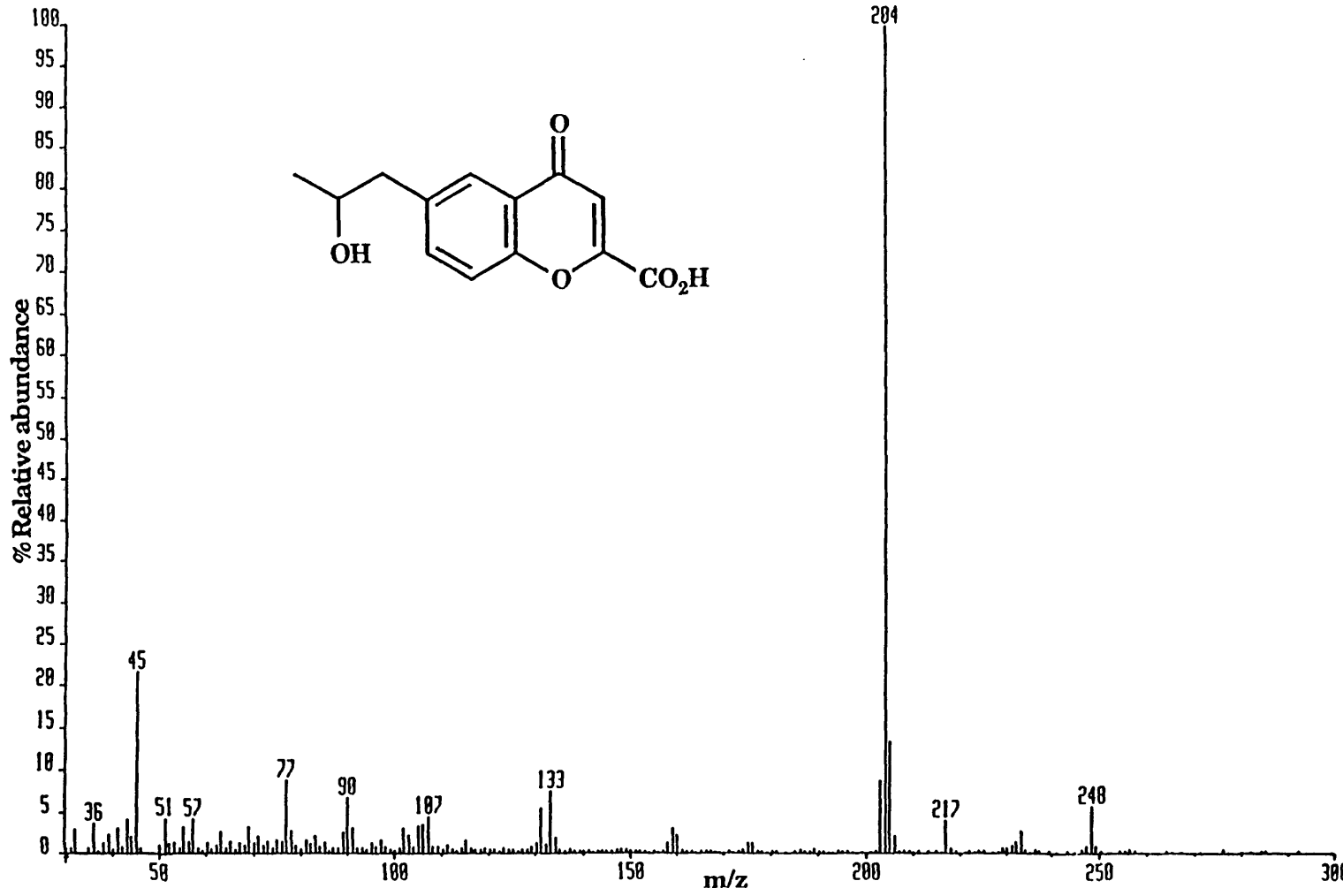
Appendix 3.4 **Mass spectrum of 6-(1'-hydroxypropyl)chromone-2-carboxylic acid.**



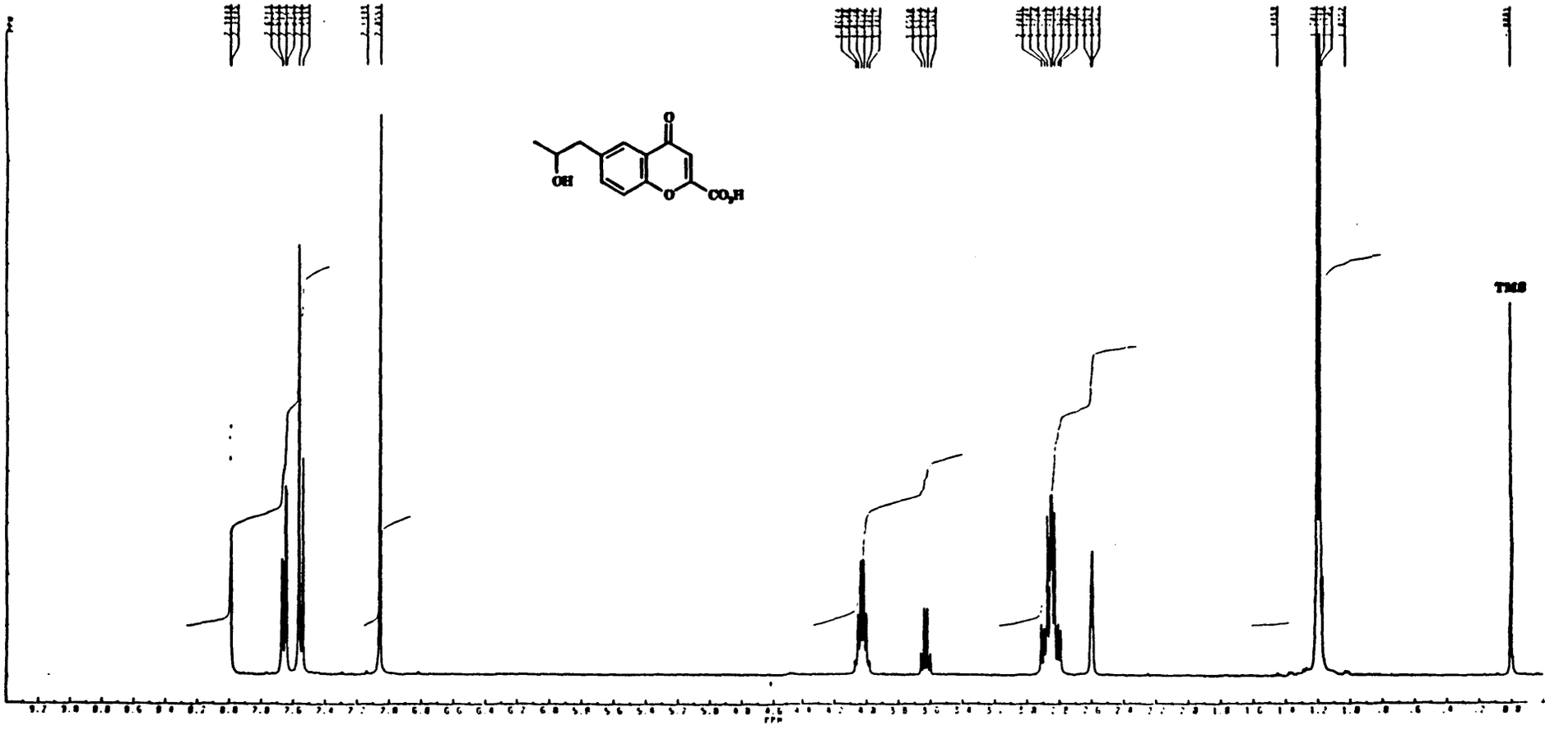
Appendix 3.5 **^{1}H NMR spectrum (360MHz) of 6-(1'-hydroxypropyl)chromone-2-carboxylic acid.**



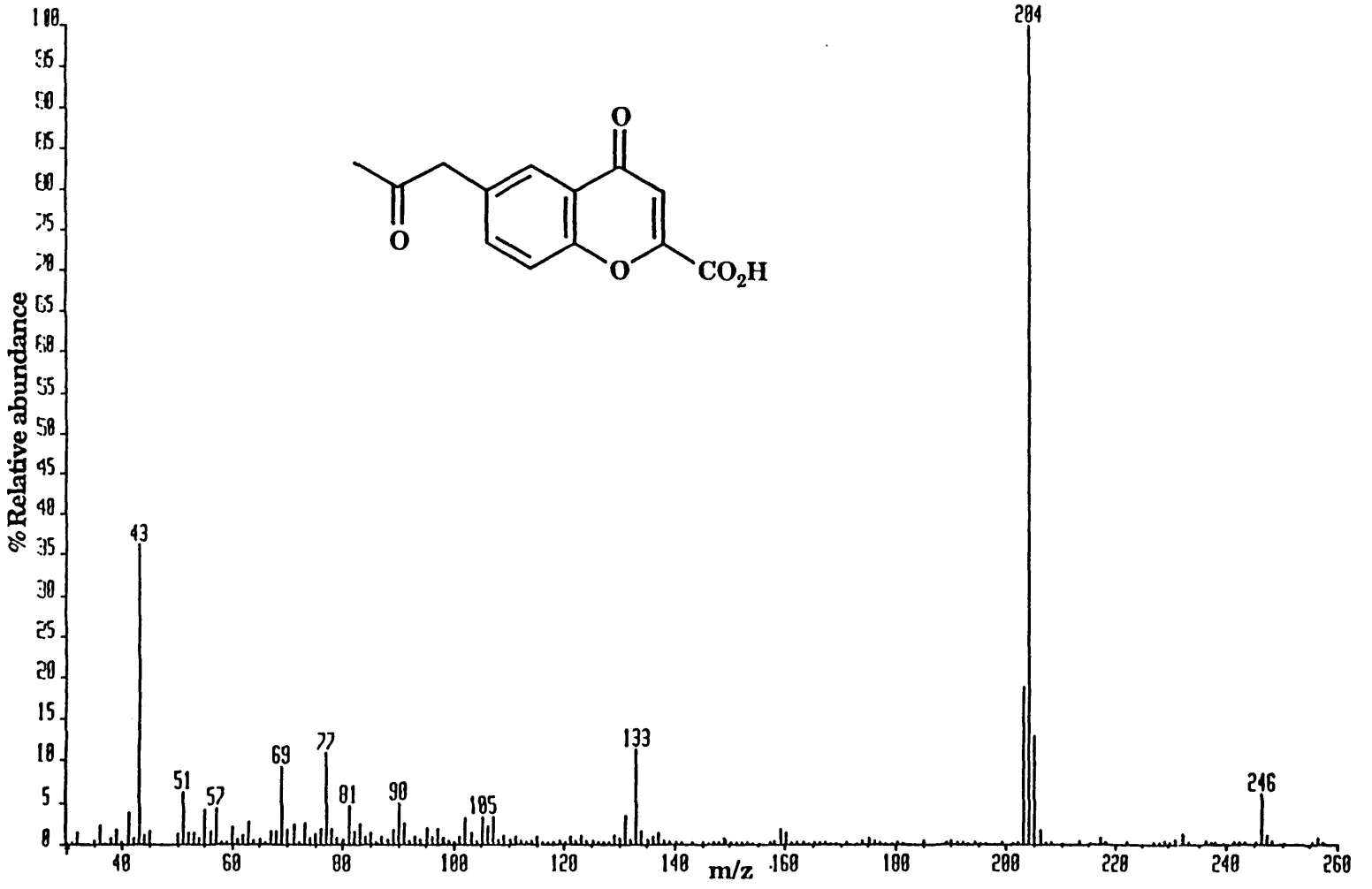
Appendix 3.6 **^{1}H NMR spectrum (360MHz) of methyl 6-(1'-methoxypropyl)chromone- 2-carboxylic acid, having a retention time of 15.7 min on HPLC (system NP 2, peak 1).**



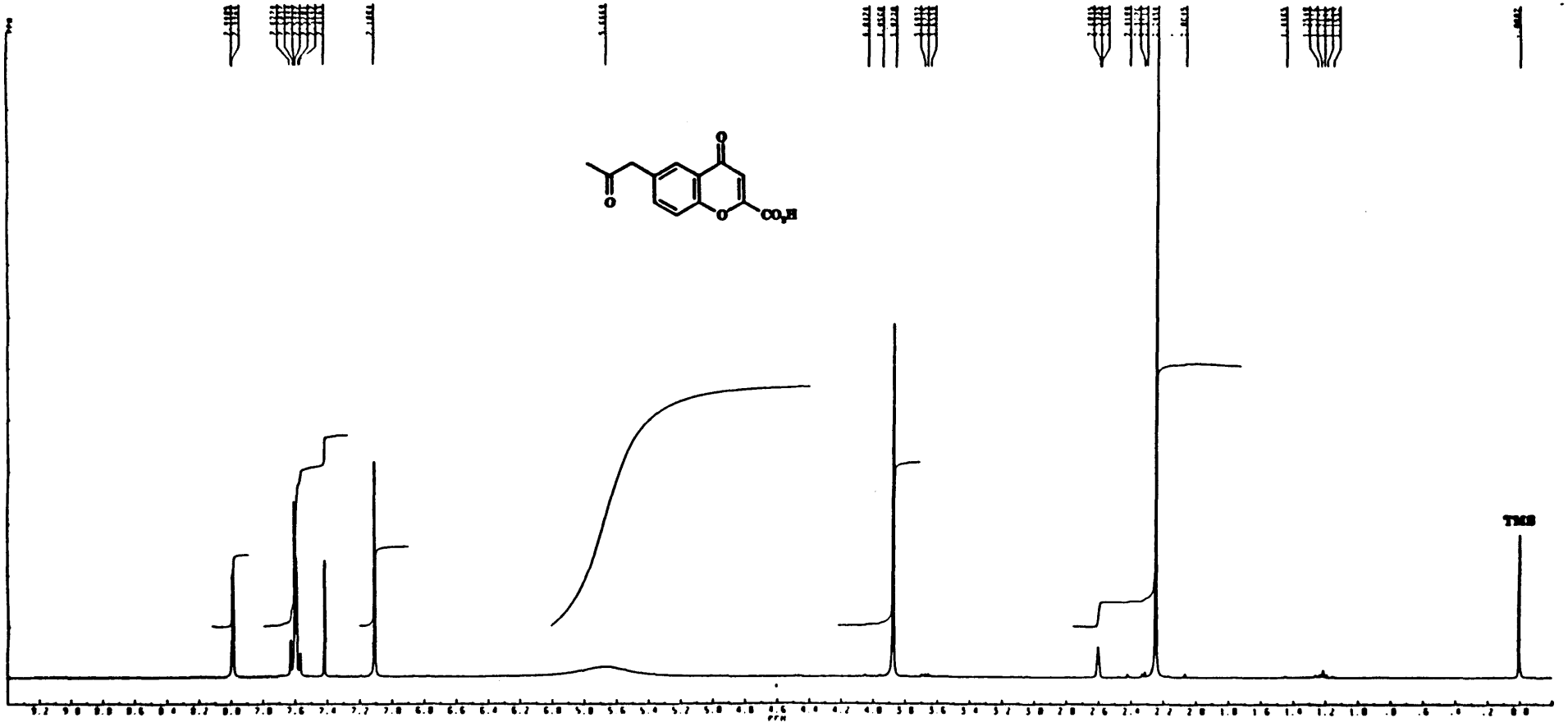
Appendix 4.1 **Mass spectrum of 6-(2'-hydroxypropyl)chromone-2-carboxylic acid.**



Appendix 4.2 **^{1}H NMR spectrum (360MHz) of 6-(2'-hydroxypropyl)chromone-2-carboxylic acid.**



Appendix 4.3 Mass spectrum of 6-(2'-oxopropyl)chromone-2-carboxylic acid.



Appendix 4.4 ^{1}H NMR spectrum (360MHz) of 6-(2'-oxopropyl)chromone-2-carboxylic acid.

Appendix 5.1

Chemicals used in Chapter 5 and their sources :

β–Naphthoflavone.	Aldwich Chemical Company Ltd, Gillingham, Dorset, U.K.
Clofibrate.	
Isosafrole.	
Phenobarbitone (sodium salt)	BDH Limited, Poole, Dorset, U.K.
Trioctanoin (C 8:0)	Sigma Chemical Company Ltd, Poole, Dorset, U.K.

References

Al-Gailany K.A.S., Wilson A.G.E., and Bridges J.W., (1974),
Biochem. Soc. Trans., 2: 113.

Al-Gailany K.A.S., Houston J.B., and Bridges J.W., (1978),
Biochem. Pharmacol., 27: 783.

Aoyama T., Korzerkwa K., Nagata K., Adesnik M., Reiss A., Lapenson D.P., Gillette J., Gelboin H.V., Waxman D.J., and Gonzalez K.J., (1989),
J. Biol. Chem., 264: 21327.

Aoyama Y., Yoshida Y., and Sonoda Y., (1989a),
Biochim. Biophys. Acta., 1001: 196.

Aoyama Y., Yoshida Y., and Sonda Y., (1989b),
Biochim. Biophys. Acta., 1006: 206.

Armstrong R.N., Levin W., Ryan D.E., Thomas P.E., Mah H.D., and Jerina D.M., (1981),
Biochem. Biophys. Res. Commun., 100: 1077.

Ashton M.J., Clark B., Jones K.M., Moss G.F., Neale M.G., and Ritchie J.T., (1973),
Toxicol. Appl. Pharmacol., 26: 319.

Atkins W.M. and Sligar S.G., (1988),
J. Biol. Chem., 263: 18842.

Augstein J., Cairns H., Hunter D., Lee T.B., Suschitzky J., Altounyan R.E.C., Jackson D.M., Mann J., Orr T.S.C., and Sheard P., (1977),
Agents and Action, 7: 443.

Axelorod J., Inscoe J.K., and Tomkins G.M., (1958),
J. Biol. Chem., 232: 835.

Bachur N.R., (1976),
Science N.Y., 193: 595.

Baker W., and Ollis W.D., (1952),
Nature, 169: 706.

Baker W., Chadderton J., Harborne J.B., and Ollis W.D., (1953),
J. Chen. Soc., 1852.

Baron J., Kawabata T.T., Knapp S.A., and Voigt J.M., Redick J.A., Jakoby W.B., and Guengerich F.P., (1984),
In Foreign Compound Metabolism, (Caldwell J., and Paulson G.D., eds), Taylor & Francis, p 17.

Billings R.E., Sullivan H.R., and McMahon R.E., (1970),
Biochemistry, 9: 1256.

Bjorkhem I., and Danielsson H., (1970),
Eur. J. Biochem., 14: 473.

Bray H.G., Franklin T.J., and James S.P., (1959),
Biochem. J., 73: 465.

Burstein S., Rosenfeld J., and Wittstruck T., (1972),
Science N.Y., 176: 422.

Cahn R.S., and Ingold C.K., (1951),
J. Chem. Soc., 612.

Cahn R.S., Ingold C.K., and Prelog V., (1956),
Experientia, 12: 81.

Cahn R.S., Ingold C.K., and Prelog V., (1966),
Angew. Chem. Intern. Ed., 5: 385.

CaJacob C.A., Chan W.K., Shepard E., and Ortiz de Montellano P.R., (1988),
J. Biol. Chem., 263: 18640.

Caldwell J., (1972),
Ph.D. Thesis, University of London.

Caldwell J., (1980),
In Enzymatic Basis of Detoxication, Vol 1, (Jakoby W.B., ed), Academic Press, New York, p 85.

Caldwell J., Winter S.M., and Hutt A.J., (1988),
Xenobiotica, 18: 59.

Chang K., and Schenkman J., (1983),
J. Bol. Chem., 258: 11738.

Clark N., Smith D.A., Eason C.T., and Parke D.V., (1982),
Xenobiotica, 12: 147.

Cohen G.M., and Mannering G.T., (1973),
Mol. Pharmac., 9: 383.

Cooney A.H., (1967),
Pharmacol. Rev., 19: 317.

Cooper D.Y., Levin S.S., Narasimeulu S., Rosenthal O., and Estabrook R.W., (1965),
Science N.Y., 147: 400.

Corner E.D.S., and Young L., (1954),
Biochem. J., 58: 647.

Coutts R.T., Dawson G.W., and Beckett A.H., (1976),
J. Pharm. Pharmacol., 28: 815.

Culp H.W., and McMahon R.E., (1968),
J. Biol. Chem., 243: 848.

Dale J.A., and Mosher H.S., (1973),
J. Am. Chem. Soc., 95: 512.

Drummond L., Caldwell J., and Wilson H.K., (1989),
Xenobiotica, 19: 199.

Drummond L., Caldwell J., and Wilson H.K., (1990),
Xenobiotica, 20: 159.

Ellin A., and Orrenius S., (1975),
Mol. Cell. Biochem., 8: 69.

Ellis G.P., and Baker G., (1972),
In Progress in Medicinal Chemistry, Vol 9, Butterworth, London, p 65.

El Masry A.M., Smith J.N., and Williams R.T., (1956),
Biochem. J., 64: 50.

Fisher G.J., Fukushima H., and Gaylor J.L., (1981),
J. Biol. Chem., 256:4388.

Fritz S., Lindner W., Roots I., Frey B.M., and Kupfer A., (1987),
J. Pharmacol. Exp. Ther., 241: 615.

Frommer U., Ullrich V., Staudinger H.J., and Orrenius S., (1972),
Biochim. Biophys. Acta., 208: 487.

Gal J., De Vito D., and Harper T.W., (1981),
Drug Metab. Dispos., 9: 557.

Garfinkle D., (1958),
Arch. Biochem. Biophys., 77: 493.

Gelb M.H., Heimbrook D.C., Malkonen P., and Silgar S.G., (1982),
Biochemistry, 21: 370.

Gibson G.G., (1989),
Xenobiotica, 19: 1123.

Gilbert J.N.T., Powell J.W., and Templton J., (1975),
J. Pharm. Pharmacol., 27: 923.

Groves J.T., McClusky G.A., White R.E., and Coon M.J., (1978),
Biochem. Biophys. Res. Commun., 81: 154.

Groves J.T., and Watanabe Y., (1988),
J. Am. Chem. Soc., 110: 8443.

Guengerich F.P., (1987),
In Prgress in Drug Metabolism Vol 10, (Bridges J.W., Chasseaud L.F., and Gibson G.G., eds), Taylor & Francis, p 1.

Hamberg M., and Bjorkhem I., (1971),
J. Biol. Chem., 246: 7411.

Harvey D.J., and Patson W.D.M., (1976),
Res. Commun. Chem. Path. Pharmac., 13: 585.

Harvey D.J., Martin B.R., and Patson W.D.M., (1977),
J.Pharm. Pharmac., 29: 482.

Hendel J., and Brodthagen H., (1984),
Eur. J. Clin. Pharmacol., 26: 103.

Hermans J.J.R., and Thijssen H.H., (1989),
Biochem. Pharm., 38: 3365.

Hildebrandt A., and Estabrook R.W., (1971),
Arch. Biochem. Biophys. 143: 66.

Hjelmeland L.M., Aronow L., and Trundell J.R., (1977),
Mol. Pharmacol., 13: 634.

Hsyu P., and Giacomini K.M., (1985),
J. Clin. Invet., 76: 1720.

Imai Y., and Sato R., (1967),
J. Biochem., 62: 239.

IUPAC Commision on Nomenclature of Organic Chemistry, 1974 Recomendation for Section E, Fundamental Stereochemistry, (1976),
Pure Appl. Chem., 45: 11.

Jansson I., Orrenius S., Ernster L., and Schenkman J.B., (1972),
Arch. Biochem. Biophys., 151: 391.

Jerina D.M., Michaud R.J., Feldmann R.N., Armstrong R.N., Vyas K.P., Thakker D.R., Yagi H., Thomas P.E., Ryan D.E., and Levin W., (1982), In Microsomes, Drug Oxidation and Drug Toxicity, (Sato R., and Kato R., eds), p 195.

Kabouto K., Imuta M., Kempner E.S., and Ziffer H., (1978),
J. Org. Chem., 43: 2357.

Kalow W., (1986),
Xenobiotica, 16: 379.

Klingenberg, M., (1958),
Arch. Biochem. Biophys., 75: 376.

Kominami S., Shinzawa K., and Takemori S., (1982),
Biochem. Biophys., 109: 916.

Kostanecki S.V., Paul L., and Tambor J., (1901),
Ber., 34: 2475.

Kupfer D., (1980),
Pharmacol. Ther., 11: 469.

Kupfer A., Desmond P.V., Schenker S., and Branch R.A., (1982),
J. Pharmacol. Exp. Ther., 221: 590.

Larrey D., Distlerath L.M., Dannan G.A., Wilkinson G.R., and Guengerich F.P., (1984),
Biochemistry, 23: 2787.

Lau P.P., and Strobel H.W., (1982),
J. Biol. Chem., 257: 5257.

Lennard M.S., Tucker G.T., Silas J.H., Freestone S., Ramsay L.E., and Woods H.F., (1983),
Clin. Pharmacol. Ther., 34: 732.

Lindberg R.L.P., and Negishi M., (1989),
Nature, 339: 632.

Levin W., Buening M.K., Wood A.W., Chang R.L., Kedzierski B., Thakker D.R., Boyd D.R., Gadaginamath G.S., Armstrong R.M., Yagi H., Karle J.M., Slaga T.J., Jerina D.M., and Conney A.H., (1980),
J. Biol. Chem., 255: 9067.

Lindberg R., Burkhert B., Ichikawa T., and Negishi M., (1989),
J. Biol. Chem., 264: 6465.

Lu A.Y.H., and Coon M.J., (1968),
J. Biol. Chem., 243: 1321.

Lu A.Y.H., and West S.B., (1980),
Pharmacol. Rev., 31: 277.

Maguire J.A., Butler T.C., and Dudly K.K., (1978),
J. Med. Chem., 21: 1294.

Mansuy D., Battioni P., and Battioni J.P., (1989),
Eur. J. Biochem., 184: 267.

Martin B.R., Harvey D.J., and Patson W.D.M., (1976),
J. Pharm. Pharmac., 28: 773.

Matsubara S., Yamamoto S., Sogawa K., Yokotani N., Fuji-Kuriyama Y., Hanui M., Shively J.E., Gotoh O., Kusuhose E., and Kusunose M., (1987),
J. Biol. Chem., 262: 13366.

Maynard D.E., Gurny O., Pitcher R.G., and Kierstead R.W., (1971),
Expreientia, 27: 1154.

McMahon, R.E., and Sullivan H.R., (1966),
Life Sci., 5: 921.

McMahon R.E., Sullivan H.R., Craig J.C., and Pereira W.E., (1969),
Arch. Biochem. Biophys., 132: 575.

McMahon R.E., (1982),
In Metabolic Basis of Drug Detoxication, (Jakoby W.B., Bend J.R., and Caldwell J., eds), Academic Press, New York, p 91.

Meese C.O., and Eichelbaum M., (1989),
In Xenobiotic Metabolism and Disposition, (Kato R., Estabrook R.W., and Caeyen M.N., eds), Taylor & Francis, London, p 179.

Mizukami Y., Sogawa K., Suwa Y., Muramatsu M., Fujii-Kuriyama Y., (1983),
Proc. Nattl. Acad.Sci., 80: 3958.

Moldeus P., Ellin A., Thor H., and Orrenius S., (1974),
In Regulation Of Hepatic Metabolism, (Lundquist F., and Tygstrup N., eds), Munksgaard, Copenhagen, P 659.

Morohashi K., Sadano H., Okada Y., and Omura T., (1983),
J. Biochem., 93: 413.

Nebert D.W., Adesnik M., Coon M.J., Estabrook R.W., Gonzalaz F.T., Guengerich F.P., Gunsalus I.C., Johnson E.F., Kemper B., Levin W., Phillips I.R., Sato R., and Waterman M.R., (1987),
DNA, 6: 1.

Nebert D.W., Nelson D.R, Adesnik M., Coon M.J., Estabrook R.W., Gonzalaz F.T., Guengerich F.P., Gunsalus I.C., Johnson E.F., Kemper B., Levin W., Phillips I.R., Sato R., and Waterman M.R., (1989), *DNA*, 8: 1.

Nelson D.R., and Strobel H.W., (1989), *Biochemistry*, 28: 656.

Okey A.B., (1990), *Pharmac. Ther.*, 45: 241.

Okita R.T., and Masters B.S.S., (1980), *Drug Metab. Dispos.*, 8: 147.

Omura T., and Sato R., (1961), *J. Biol. Chem.*, 237: 1375.

Omura T., and Sato R., (1964a), *J. Biol. Chem.*, 239: 2370.

Omura T., and Sato R., (1964b), *J. Biol. Chem.*, 239: 2379.

Orton T.C., and Parker G.L., (1982), *Drug Metab. Dispos.*, 10:110.

Palmer K.H., Fowler M., Wall M., Rhodes L., Waddell W., and Buggett B., (1969), *J. Pharmacol. Exp. Ther.*, 170: 355.

Palmer K.H., Fowler M., and Wall M., (1970), *J. Pharmacol. Exp. Ther.*, 175: 38.

Poulos T.L., Finzel B.C.,Gunsalus I.C., Wagner G.C., and Kraut J., (1985), *J. Biol. Chem.*, 260: 16122.

Poulos T.L., Finzel B.C., and Howard A.J., (1986),
Biochemistry, 25: 5314.

Poulos T.L., Finzel B.C., and Howard A.J., (1987),
J. Mol. Biol., 195: 687.

Poulos T.L., (1989),
Nature, 339: 580.

Poupaert J.H., Cavalier R., Claessen M.H., and Dumont P.A., (1975),
J. Med. Chem., 18: 1268.

Preiss B., and Bloch K ., (1964),
J. Biol. Chem., 239: 85.

Prelusky D.B., Coutts R.T., and Pasutto T.M., (1982),
J. Pharm. Sci., 71: 1390.

Prelog V., (1964),
Pure Appl. Chem., 9: 119.

Remmer H., (1962),
In Ciba Foundations Symposium of Enzymes and Drug Action, (Monger J.L., and De Reuck A.V.S., eds), Little Brown, Boston, P 276.

Remmer H., and Merker H.J., (1963),
Science N.Y., 142: 1657.

Robbins K.C., (1968),
Arch. Biochem. Biophys., 123: 531.

Ruhemann S., and Stapleton H.E., (1900),
J. Chem. Soc., 77: 1179.

Ryan D.E., Thomas P.E., and Levin W., (1980),
J. Biol. Chem., 255: 7941.

Ryan D.E., Thomas P.E., and Levin W., (1982),
Arch. Biochem. Biophys., 216: 272.

Ryan D.E., Iidia S., Wood A.W., Thomas P.E., Lieder C.S., and Levin W., (1984),
J. Biol. Chem., 259: 1239.

Sato H., Ashida N., Suhara K., Itagaki E., Takemori S., and Katagiri M., (1978),
Arch. Biochem. Biophys., 190: 307.

Sangster S.A., Caldwell J., Hutt A.J., and Smith R.L., (1983),
Fed. Chem. Toxic., 21: 263.

Scherrer R.A., and Howard S.M., (1977),
J. Med. Chem., 20: 53.

Sharma R., Lake B.G., Foster J., and Gibson G.G., (1988),
Biochem. Pharmacol., 37: 1193.

Smith D.A., and Neale M.G., (1983),
Eur. J. Drug Met. Pharmacokin., 8: 225.

Smith D.A., Brown K., and Neale M.G., (1986),
Drug Metab. Rev., 16: 365.

Sugiyama K., and Trager W.F., (1986),
Biochemistry, 25: 7336.

Sullivan H.R., Miller W.M., and McMahon R.E., (1976),
Xenobiotica, 6: 49.

Sullivan G.R., Dale J.A., and Mosher H.S., (1973),
J. Org. Chem. 38: 2143.

Suwa Y., Mizukami Y., Sogawa K., and Fuji-Kuriyama Y., (1985),
J. Biol. Chem., 260: 7980.

Tamburini P.P., Masson H.A., Bains S.K., Makowski R.T., Morris B., and Gibson G.G., (1984),
Eur. J. Biochem., 139: 235.

Thomas P.E., Bandiera S., Reik L.M., Maines S.L., Ryan D.E., and Levin W., (1987),
Federation Proc., 46: 2563.

Testa B., and Jenner P., (1976),
In Drug Metabolism: Chemical and Biological Aspects, Dekker, New York.

Testa B., (1980),
In Concepts in Drug Metabolism, Part A, Marcel Dekker, New York, p 53.

Testa B., and Trager W.F., (1983),
In Topics in Pharmaceutical Sciences, (Breimer D.D. and Speiser P., eds.), Elsevier Science Publishers, B.V., p 99.

Testa B., (1988),
Biochem. Pharmacol., 37: 85.

Testa B., (1989),
In Xenobiotic Metabolism and Disposition, (Kato R., Estabrook R.W., and Cayen M.N., eds), Taylor & Francis, London, P 153.

Testa B., (1990),
In Chirality and Biological Activity, (Holmsted B., Testa B., and Frank H., eds), Alan R. Liss inc, New York.

Trager W.F., (1980),
In Concepts in Drug Metabolism, Part A, Marcel Dekker, New York, p 177.

Trager W.F., (1989),
In Xenobiotic Metabolism and Disposition, (Kato R, Estabrook R.W., and Cayen M.N., eds.), Taylor & Francis, London, p 171.

Tsai W.L. Hermann K., Hug E., Rhode B., and Dreiding A.S., (1985),
Helv. Chim. Acta., 68: 2238.

Ullrich V., (1969),
Z. Naturforsch, 246: 699.

Ullrich V., Raf H.H., and Mimoum H., (1972),
In Biological Hydroxylation Mechanisms, (Boyd G.S., and Smellie R.M.J., eds), Academic Press, New York, p 11.

Wade D.N., Mearrick P.T., and Morris J.L., (1973),
Nature, 242: 463.

Waxman D.J., and Walsh C., (1982),
J. Biol. Chem., 257: 10446.

Waxman D.J., (1988),
Biochem. Pharmacol., 37: 71.

Weil A., Caldwell J., Strolin Benedetti M., and Dostert P., (1986),
10 th European Drug Metabolism Workshop, Guilford, U.K., Abst. no 1/5.

Weiner I.M., Waharington J.A., and Mudge G.H., (1960),
Bull. John Hopkins Hosp., 106: 337.

White R.E., Oprian D.D., and Coon M.J., (1980),
In Microsomes, Drug oxidation and Chemical carcinogensis, (Coon M.J., Conney J.R., Estabrook R.W., Gelboin H.V., Gillette J.R., and O'Brian P.J., eds), Academic Press, New York, p 243.

White R.E., McCarthy M.B., Egeberg K.D., and Silgar S.G., (1984),
Arch. Biochem. Biophys., 228: 493.

White R.E., Miller J.P., Farreau L.V., and Bhattacharyya A., (1986),
J. Am. Chem. Soc., 108: 6024.

Widman M., Nordquist M., Dollery C., and Briant R.H., (1975)
J. Pharm. Pharmac., 27: 842.

Williams R.T., (1959),
In Detoxication Mechanisms, Chapman & Hall, London, p 357.

Winter S.M., (1987),
Ph.D. Thesis, University of London.

Yeowell H.N., Linko P., Hodgson E., and Goldstein J.A.,
(1985),
Arch. Biochem. Biophys., 243: 408.