MECHANISTIC STUDIES OF VALPROIC ACID HEPATOTOXICITY: IDENTIFICATION AND CHARACTERIZATION OF THIOL CONJUGATES

by

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ABSTRACT

The severe hepatotoxicity of the antiepileptic drug valproic acid (VPA) is believed to be mediated through chemically reactive metabolites. The monounsaturated metabolite 4-ene VPA is steatogenic in the rat, and in a similar fashion to the hepatotoxin 4-pentenoic acid, is thought to be oxidized by mitochondria to a highly reactive α,β-unsaturated ketone, 3-keto-4-ene VPA. The tripeptide thiol, glutathione (GSH), is known to react with a variety of electrophilic compounds that have the potential to interact with cellular macromolecules. The identification and structural characterization of GSH conjugates provides a means of identifying short-lived unstable electrophiles and thus an insight into the mechanisms of toxicity. This thesis describes the synthesis and characterization of thiol conjugates of reactive metabolites derived from the *in vivo* metabolism of VPA, 4-ene VPA, (E)-2,4-diene VPA, and 4-pentenoic acid.

A negative ion chemical ionization gas chromatographic/mass spectrometric (NICI/GC/MS) method for the determination of VPA and 14 of its metabolites in a single chromatographic run was developed. A combination of pentafluorobenzyl and trimethylsilyl derivatization resulted in the $[M-181]^-$ anion as the base peak for all the metabolites measured. When these ions were monitored sensitivities in the low picogram range were achieved. The VPA metabolite profile was determined in pediatric patients on VPA monotherapy and on combined therapy with either carbamazepine or clobazam. 4-Ene- and (E)-2,4-diene-VPA were found to be minor metabolites with serum levels below 1% that of VPA. In patients on combined therapy with carbamazepine, the ω and ω-1 pathways

of VPA metabolism were induced, while products of β-oxidation were significantly decreased. Polytherapy had no significant effect on the serum levels of 4-ene- or (E)-2,4-diene-VPA.

Rats were dosed intraperitoneally with 100 mg/kg of the sodium salts of VPA, 4-ene-, (E)-2,4-diene-VPA, 4-pentenoic or (E)-2,4-pentadienoic acids. Methylated bile extracts were analyzed by high pressure liquid chromatography and liquid chromatography/tandem mass spectrometry (LC/MS/MS) for GSH conjugates while urine samples were analyzed by GC/MS and LC/MS for N-acetylcysteine (NAC) conjugates and other metabolites. The GSH conjugate of (E)-2,4-diene VPA was detected in the bile of rats treated with 4-ene- and (E)-2,4-diene-VPA. The NAC conjugate was a major urinary metabolite of rats given (E)-2,4-diene VPA and was a prominent urinary metabolite of those animals given 4-ene VPA. The structures of these metabolites were confirmed by comparing GC/MS or LC/MS properties of the isolated metabolites to those of synthetic standards.

The GSH and NAC conjugates of (E)-2,4-diene VPA were chemically synthesized and their structures established to be (E)-5-(glutathion-S-yl)-3-ene VPA and (E)-5-(N-acetylcystein-S-yl)-3-ene VPA by nuclear magnetic resonance spectroscopy and mass spectrometry. In contrast to the very slow reaction of the free acid of (E)-2,4-diene VPA with GSH, the methyl ester reacted rapidly with GSH to yield the adduct. *In vivo* it appears the diene forms an intermediate with enhanced electrophilic reactivity to GSH as indicated by the facile reaction of the diene with GSH *in vivo* (about 40% of the (E)-2,4-diene VPA administered to rats was excreted as the NAC conjugate in 24 hr).

In rats treated with 4-pentenoic and/or (E)-2,4-pentadienoic acids the following conjugates were identified and characterized by synthesis: GSH and cysteine conjugates of 3-oxo-4-pentenoic acid, GSH and NAC conjugates of (E)-2,4-pentadienoic acid, and the NAC conjugate of acrylic acid. The results thus provided the first direct biochemical evidence for the *in vivo* formation of the metabolite of 4-pentenoic acid considered responsible for the irreversible inhibition of fatty acid metabolism. The results also revealed basic differences between the mitochondrial metabolism of 4-ene VPA and 4-pentenoic acid.

The 3-keto-4-ene VPA and its GSH and NAC conjugates were synthesized in order to facilitate the *in vivo* identification of these compounds following the administration of VPA, 4-ene-, or (E)-2,4-diene-VPA to rats. However, neither the 3-keto-4-ene VPA nor its thiol derivatives were evident in any of the treatments.

The NAC conjugate of (E)-2,4-diene VPA was also found to be a metabolite of VPA in patients. The level of the conjugate appeared to be higher in two patients who recovered from VPA-induced liver toxicity. The characterization of GSH and NAC (in humans and rats) conjugates of (E)-2,4-diene VPA suggests that VPA is metabolized to a chemically reactive intermediate that may contribute to the hepatotoxicity of the drug.

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LIST OF ABBREVIATIONS

API	atmospheric pressure ionization
ATP	adenosine triphosphate
bp	boiling point
bs	broad singlet
C-18	octadecylsilane
CAD	collision activated dissociation
CBZ	carbamazepine
CI	chemical ionization
CoA	coenzyme A thio ester
CoASH	coenzyme A
CLBZ	clobazam
D	deuterium
d	doublet (NMR), deuterium
DBU	1,8-diazabicyclo[5.4.0.]undec-7-ene
δ	chemical shift
dd	doublet of doublets
E	trans
EI	electron impact
FAB	fast atom bombardment
FAD	flavine adenine dinucleotide
GC/MS	gas chromatography/mass spectrometry
GSH	glutathione
GSSG	glutathione disulfide
hr	hour(s)
HMPA	hexamethylphosphoramide

HPLC	high performance liquid chromatography
Hz	Hertz
i.d.	internal diameter
i.p.	intraperitoneal
i.v.	intravenous
J	coupling constant
kg	kilogram
LC	liquid chromatography
LC/MS	liquid chromatography/mass spectrometry
LDA	lithium diisopropylamide
m	multiplet, meter
M^+	molecular ion
mg	milligram
2-MGA	2-methylglutaric acid
min	minutes
mL	milliliter
mmoles	millimoles
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
MW	molecular weight
m/z	mass to charge ratio
NAC	N-acetyl-L-cysteine
NAD	nicotinic acid adenine dinucleotide
NICI	negative ion chemical ionization
NMR	nuclear magnetic resonance spectroscopy
PCI	positive chemical ionization

PFBB	pentafluorobenzyl bromide
PFB	pentafluorobenzyl
ppm	parts per million
psi	pounds per square inch
q	quartet
s	singlet
SIM	selected ion monitoring
t	triplet
t-BDMS	tertiarybutyldimethylsilyl
THF	tetrahydrofuran
TIC	total ion current
TLC	thin layer chromatography
TMS	trimethylsilyl
VPA	valproic acid, valproate
$[^2H]_6$VPA	$[^2H]_6$valproic acid
2,3′-diene VPA	2-(1′-propenyl)-2-pentenoic acid
2,4-diene VPA	2-propyl-2,4-pentadienoic acid
2,4′-diene VPA	2-(2′-propenyl)-2-pentenoic acid
2-ene VPA	2-propyl-2-pentenoic acid
$[^2H]_3$2-ene VPA	$[^2H]_3$2-propyl-2-pentenoic acid
3-ene VPA	2-propyl-3-pentenoic acid
4-ene VPA	2-propyl-4-pentenoic acid
3-keto VPA	2-propyl-3-oxopentanoic acid
$[^2H]_3$3-keto VPA	$[^2H]_3$2-propyl-3-oxopentanoic acid
4-keto VPA	2-propyl-4-oxopentanoic acid
3-OH VPA	2-propyl-3-hydroxypentanoic acid
4-OH VPA	2-propyl-4-hydroxypentanoic acid

5-OH VPA	2-propyl-5-hydroxypentanoic acid
3-OH-4-ene VPA	2-propyl-3-hydroxy-4-pentenoic acid
3′-OH-4-ene VPA	2-(1′-hydroxypropyl)-4-pentenoic acid
3-keto-4-ene VPA	2-propyl-3-oxo-4-pentenoic acid
3′-keto-4-ene VPA	2-(1′-oxopropyl)-4-pentenoic acid
2-PGA	2-propylglutaric acid
2-PSA	2-propylsuccinic acid
Z	cis

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I. INTRODUCTION

The antiepileptic properties of valproic acid (VPA) were discovered serendipitously (Meunier *et al.*, 1963) nearly a century after the compound was first synthesized and used as an organic solvent. This branched-chain fatty acid is unique among antiepileptic drugs both in its structure and mechanism of action. VPA is very effective in a variety of seizure types and thus is regarded as an important new type of antiepileptic drug, and today enjoys widespread use throughout the world. VPA is a valuable drug for the treatment of primary generalized seizures (Gram and Bentsen, 1985; Wilder *et al.*, 1983) and is the drug of choice for children with absence seizures (Jeavons *et al.*, 1977). Emerging indications for VPA are psychiatric ailments such as bipolar disorders (Penry and Dean, 1989).

Initially VPA was considered a safe drug with only minor side effects, but with more widespread use it has been shown to cause fatal hepatotoxicity in man (Zimmerman and Ishak, 1982; Zafrani and Berthelot, 1982). The fatal hepatotoxicity is considered to be an idiosyncratic reaction primarily occurring in children under two years of age who were taking VPA along with other anticonvulsant drugs (Dreifuss *et al.*, 1987).

The mechanism of VPA hepatotoxicity is not understood, although the possible involvement of a reactive metabolite has been suggested (Zimmerman and Ishak, 1982). Because the monounsaturated metabolite 4-ene VPA is a structural analog of the fatty acid oxidation inhibitor 4-pentenoic acid, and is steatogenic in the rat, it has been proposed that 4-ene VPA contributes to the hepatotoxicity of VPA through metabolic activation to a reactive intermediate.

The biotransformation of xenobiotics can lead to reactive intermediates and metabolites that are capable of interactions with cellular components. The formation of chemically reactive metabolites from drugs is well documented, and it is generally accepted that reactive metabolites mediate the toxic reactions to many drugs and other xenobiotics (Nelson, 1982). The tripeptide thiol, glutathione (GSH), is known to react with a variety of electrophilic compounds thus preventing to a certain extent their reaction with essential components of the cell (Chasseaud, 1976).

The main objective of the present study was to determine whether chemically reactive metabolite(s) of VPA, which might contribute to the hepatotoxic response to the drug, are formed and can be trapped as their thiol conjugates *in vivo*.

As a background to the rationale and objectives of the thesis, the following topics will be briefly described. The metabolism of VPA and/or 4-ene VPA appears to play a crucial role in the hepatotoxicity of VPA, and because VPA shares common metabolic pathways with fatty acids, **fatty acid metabolism** will be briefly discussed. This will be followed by a discussion of the **metabolism of VPA and 4-ene VPA. Hypoglycin** and **4-pentenoic acid** are structurally related to 4-ene VPA and are known to be liver toxins, and hence the mechanism by which these analogs of 4-ene VPA cause hepatotoxicity will be presented. An outline of the **metabolic disturbances caused by VPA** and the **teratogenicity of VPA** will be presented because some of the consequences of the metabolic disturbances caused by VPA might be related to the hepatotoxicity of VPA. Then a literature survey of the **incidence and pathological manifestations of VPA hepatotoxicity** and a discussion of the proposed biochemical

mechanisms will be presented. Finally, there will be a short discussion of glutathione conjugation and its toxicological significance as this would be relevant to the reactive metabolite hypothesis of VPA hepatototoxicity.

1. Fatty acid metabolism

A. Mitochondrial β-oxidation

The sequence of reactions by which fatty acids are degraded is called β-oxidation. A single oxidation cycle results in the shortening of the chain by two carbons through the splitting off of an acetyl CoA unit (Devlin, 1986; Stryer, 1988). The initial step in the oxidation of fatty acids is their conversion to CoA thio ester derivatives. This reaction is catalyzed by acyl CoA synthetase and occurs in the endoplasmic reticulum or outer mitochondrial membrane. Fatty acids that are present in the mitochondria can also be activated; however, the physiological significance of this mitochondrial process is unknown.

Fatty acyl CoAs are formed outside of the mitochondria but are oxidized in the mitochondrial matrix. Since long-chain acyl CoAs do not readily cross the mitochondrial membrane, a transport mechanism is required. This is accomplished by forming acyl carnitine, between carnitine and the acyl CoA, which is shuttled across the inner mitochondrial membrane. Medium-chain (C_8-C_{10}) acyl CoAs can permeate into the mitochondrial matrix, and hence their oxidation is not dependent on carnitine. Also, short-chain fatty acids can cross the inner mitochondrial membrane directly i.e. without forming CoA thio esters.

In the first step of β-oxidation, a double bond of the trans-configuration is formed between carbons 2 and 3 of the acyl CoA, and in the process the oxidizing agent, FAD, is reduced to $FADH_2$. This reaction is catalyzed by several acyl dehydrogenases the specificity of which is dependent on chain length.

The second step is hydration of the double bond to a hydroxyacyl CoA by the enzyme enoyl CoA hydratase. The reaction is stereospecific resulting in only the L-isomer. This step precedes the second oxidation reaction, which converts the hydroxy group into a keto group. The reaction is catalyzed by L-3-hydroxyacyl CoA dehydrogenase and the cofactor is NAD^+.

The final step of β-oxidation is cleavage of the carbon-carbon bond of the acyl CoA by a molecule of CoASH to yield acetyl CoA and an acyl CoA shortened by two carbon atoms. This thiolytic cleavage is catalyzed by β-ketoacyl CoA thiolase, an enzyme with a broad specificity with regard to chain length.

Beta-oxidation of unsaturated fatty acids requires two additional enzymes; an isomerase which converts a cis- 3-ene double bond to a trans- 2-ene double bond, and an epimerase which catalyzes conversion of the D-3-hydroxyacyl CoA configuration to L-3-hydroxyacyl CoA. The enzyme, 2,4-dienoyl CoA reductase, which is present in the mitochondria as well as in peroxisomes, catalyzes the reduction of polyunsaturated fatty acids possessing a 2,4-dienoyl CoA structure (Osmundsen and Hovik, 1988). This results in the 3-ene analog which is β-oxidized following isomerization to 2-enoyl CoA. The level of 2,4-dienoyl CoA reductase is known to be induced by clofibrate treatment of rats (Borrebaek *et al.*, 1980).

B. Peroxisomal β-oxidation

It is now well known that in the cell the β-oxidation system is also present in peroxisomes (Lazarow, 1987). However, the physiological significance of the peroxisomal β-oxidation is considered to be relatively low with most of the oxidation of fatty acids occurring in the mitochondria (Devlin, 1986; Yamada *et al.*, 1987).

Peroxisomal β-oxidation is involved in the metabolism of dicarboxylic acids, bile acid, and very long-chain fatty acids which are poorly oxidized by mitochondria (Yamada *et al.*, 1987). Like the mitochondria, the peroxisomes also participate in the metabolism of xenobiotics (Yamada *et al.*, 1984; Yamada *et al.*, 1987; Suzuki *et al.*, 1990).

The mammalian peroxisomal fatty acid system differs from the mitochondrial β-oxidation in three respects. First, carnitine is not required for the entry of fatty acyl CoAs into peroxisomes. Second, the initial dehydrogenation step is accomplished by a cyanide-insensitive oxidase system and leads to the formation of H_2O_2, which is eventually eliminated by catalase. Third, the specificity of peroxisomal enzymes is for longer chain fatty acids. The observation that β-oxidation in peroxisomes does not proceed below C_8 (Devlin, 1986) has led to the hypothesis that the function of peroxisomes is to shorten long-chain fatty acids for eventual mitochondrial β-oxidation.

C. Omega and Omega-1 oxidations

Hydroxylation of the methyl carbon at the far end of the molecule from the carboxyl group (ω-oxidation) or on the carbon next to the methyl end (ω-1-oxidation) are minor pathways for fatty acid metabolism

(Devlin, 1986; van Hoof *et al.*, 1988). The reactions are catalyzed by the mixed-function oxidase system and require cytochrome P-450, molecular O_2, and NADPH. By this metabolic pathway, monocarboxylic acids are converted to hydroxyacids in the endoplasmic reticulum. These hydroxyacids are subsequently converted to dicarboxylic acids by the actions of cytosolic alcohol- and aldehyde-dehydrogenases. The dicarboxylic acids are then excreted, or are subject to β-oxidation from either end, depending on the tissue and its metabolic state.

Organic aciduria (excretion of medium-chain dicarboxylic acids) is increased in conditions of excessive fatty acid degradation (eg. ketosis and diabetes mellitus) or defective mitochondrial fatty acid metabolism (eg. inherited metabolic diseases, hypoglycin poisoning, riboflavin deficiency)(van Hoof *et al.*, 1988).

2. Metabolism of VPA and 4-ene VPA

A. VPA

Metabolism is the major route of elimination of VPA in both animals and humans with renal excretion of the unchanged drug accounting for less than 5% of the administered dose (Gugler *et al.*, 1977). The metabolic pathways of VPA are similar to those of endogeneous fatty acids. In spite of this fact and its simple structure, the metabolism of VPA has proved to be very complex, and complete elucidation of its metabolic pathways has proved elusive. The metabolism of VPA has been extensively studied in man and various animals and has been reviewed by Gugler and von Unruh (1980) and more recently by Eadie *et al.*(1988). These studies indicate four main metabolic pathways: glucuronidation, β-

oxidation, ω-oxidation and (ω-1)-oxidation. The human metabolic pathways of VPA are summarized in Figs. 1A and 1B.

In both man and the rat, β-oxidation and glucuronidation are the two primary pathways. Although VPA metabolism is qualitatively similar in humans and the rat, the level of β-oxidation metabolites in rats given high doses of VPA is lower than in humans (Granneman *et al.*, 1984a). Glucuronidation is quantitatively the most important route of VPA transformation, with 15-20% of the administered dose in man being excreted as VPA glucuronide (Bialer *et al.*, 1985). Metabolism of VPA is dose-dependent; with increasing doses of VPA, glucuronidation is increased at the expense of β-oxidation (Granneman *et al.*, 1984b). In the rhesus monkey the principal pathways of VPA metabolism in decreasing quantitative importance were glucuronide formation, ω-oxidation, β-oxidation and (ω-1)-oxidation (Rettenmeier *et al.*, 1986a).

Beta-oxidation of VPA produces trans- and cis-2-ene-, 3-OH- and 3-keto-VPA with 3-keto- and 2-ene-VPA being major metabolites in serum (Abbott *et al.*, 1986). 3-Keto VPA is also a major urinary metabolite and is considered to arise through mitochondrial β-oxidation. Thus, β-oxidation of VPA is incomplete, and this has been attributed to its branched-chain structure (Rettenmeier *et al.*, 1985). Although for many xenobiotics β-oxidation results in a chain shortened by two carbons (Suzuki *et al.*, 1990; Vyas *et al.*, 1990; Tsaconas and Padieu, 1989; Grindel *et al.*, 1979), for VPA such a chain-shortening has yet to be demonstrated. The involvement of 2-methyl-branched chain acyl CoA dehydrogenase, but not other acyl dehydrogenases, in the dehydrogenation of VPA to 2-ene VPA has recently been demonstrated (Ito *et al.*, 1990).

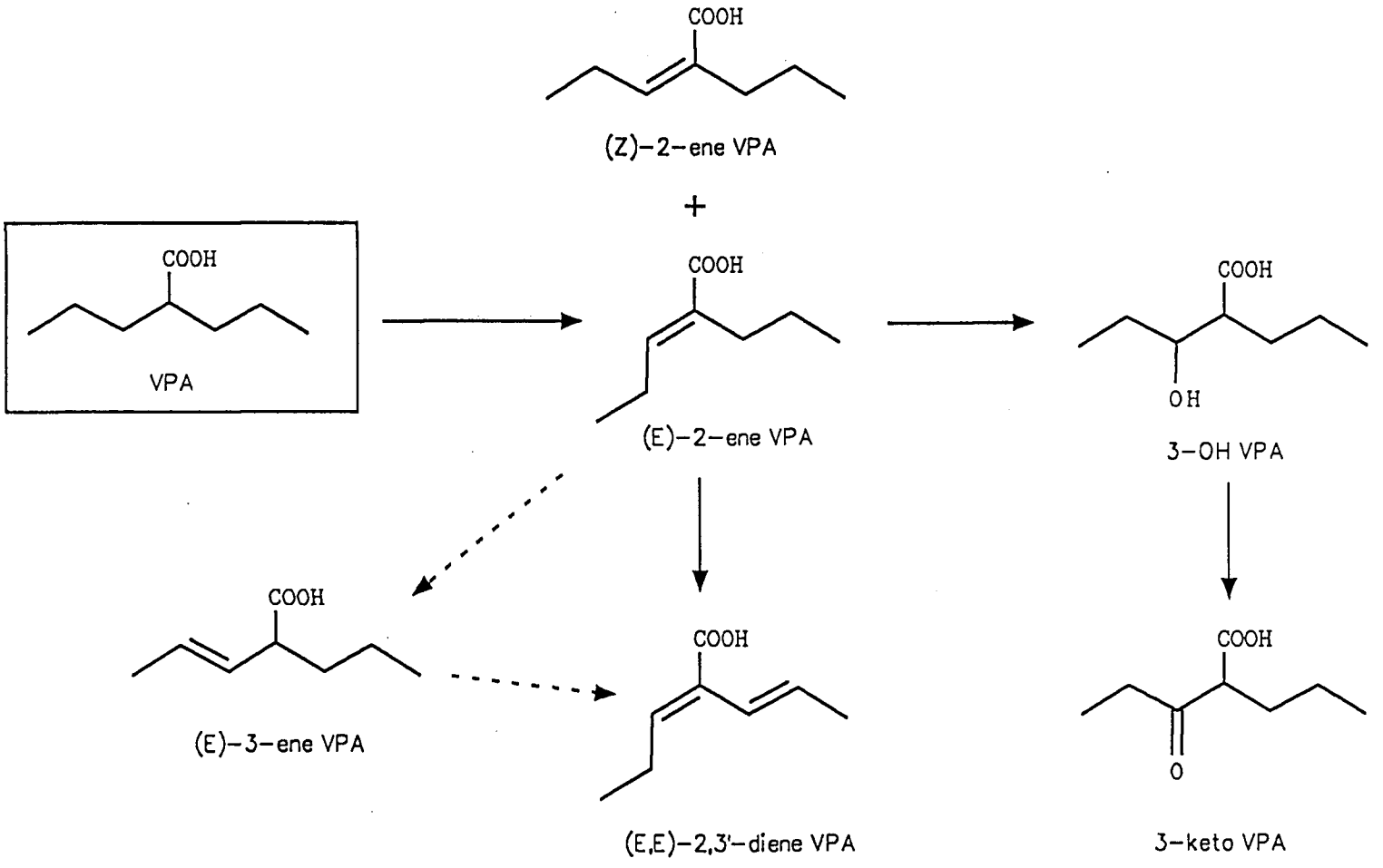


Figure 1A. The beta-oxidation pathway of VPA metabolism in humans. The broken lines indicate likely metabolic routes not yet confirmed.

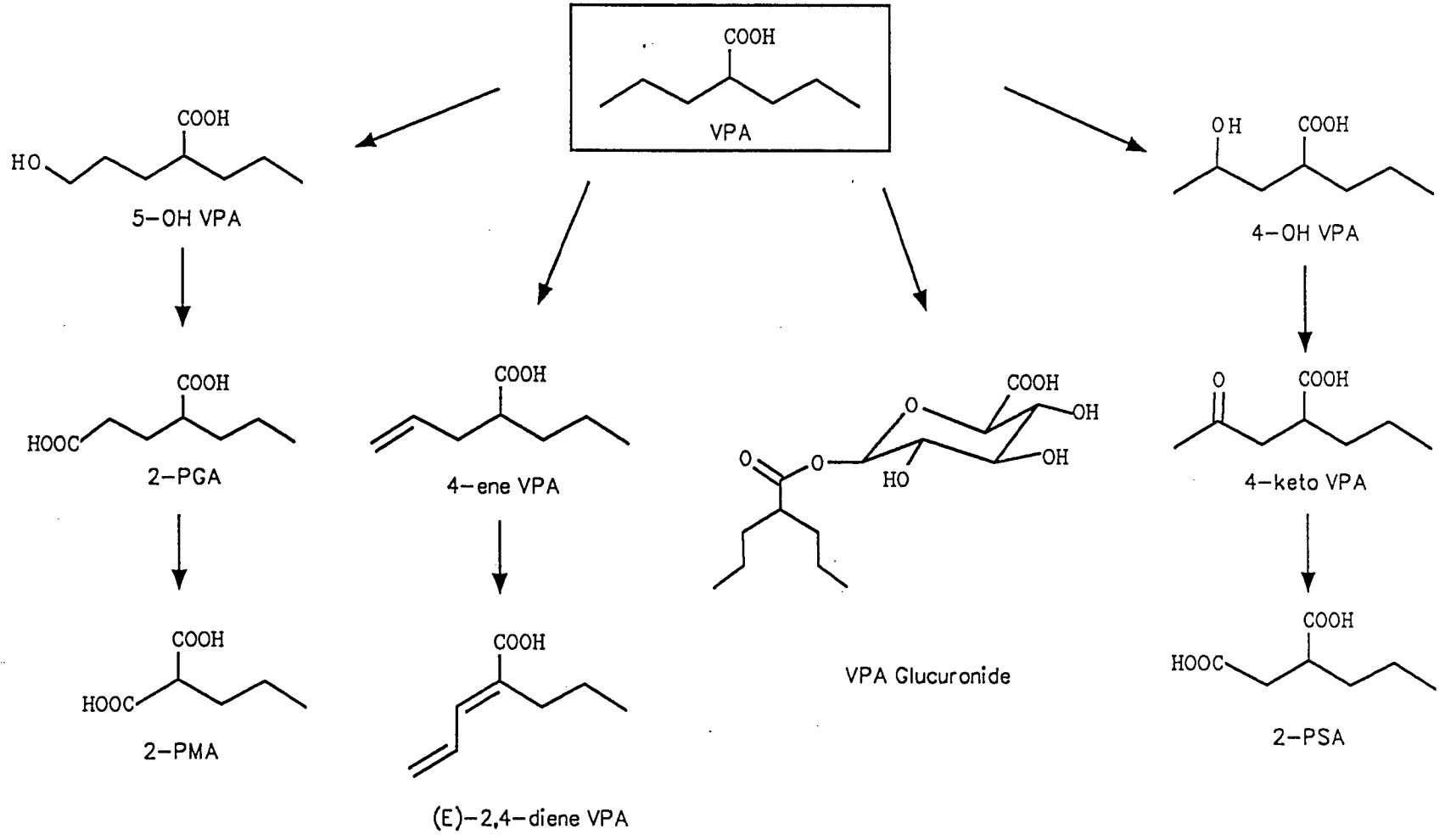


Figure 1B. Microsomal oxidation, desaturation and glucuronidation pathways of VPA metabolism in humans.

The ω-oxidation pathway leads to the formation of 5-OH VPA which is further oxidized in the cytosol to give 2-PGA. The (ω-1)-pathway results in the formation of 4-OH-, 4-keto-VPA and 2-PSA. Products of ω- and (ω-1)-oxidation of VPA are found in minor quantities in the serum of epileptic patients (Abbott *et al.*, 1986). The ω-and (ω-1)-pathways are cytochrome P-450 mediated; Prickett and Baillie (1984) showed that incubation of VPA with rat liver microsomes led to the formation of 3-, 4- and 5-OH-VPA. This indicated that the metabolite 3-OH VPA can be formed by both cytochrome P-450 dependent direct hydroxylation and β-oxidation, and thus may not be a good indicator of the extent of β-oxidation of VPA. Indeed, it has been shown, using stable isotope techniques, that 3-OH VPA has this dual origin (Rettenmeier *et al.*, 1987).

Gamma- and delta-dehydrogenation of VPA gives rise to 3-ene- and 4-ene-VPA, respectively. The cellular site of the formation of 3-ene VPA has not been established although there is evidence from studies using stable isotopes that 3-ene VPA is formed reversibly from 2-ene VPA (Rettenmeier *et al.*, 1987). It can also be speculated that 3-ene VPA and 2,4-diene VPA (see below) might have a product-precursor relationship since it is known that fatty acids having the 2,4-dienoyl structure are reduced by 2,4-dienoyl CoA reductase to the analog with the double bond at the 3-position (Osmundsen and Hovik, 1988).

Rettie *et al.*(1987) have demonstrated that cytochrome P-450 catalyzes the formation of 4-ene VPA from VPA. The proposed mechanism involves formation of a carbon centered free radical intermediate at C_4, via hydrogen abstraction, which partitions between recombination

(formation of 4-OH VPA) and elimination (formation of 4-ene VPA) pathways (Rettie *et al.*, 1988).

The monounsaturated metabolites, which also are excreted as glucuronide conjugates, are further metabolized to give dienes of VPA. The 2,4-diene VPA is formed from 4-ene VPA and is found as the minor diene metabolite in the serum of patients (Granneman *et al.*, 1984a; Rettenmeier *et al.*, 1985). The major diunsaturated metabolite in the serum of subjects treated with VPA has been characterized as (E,E)-2,3′-diene VPA by Acheampong and Abbott (1985) and probably arises from 3-ene VPA (Rettenmeier *et al.*, 1987). New VPA metabolites continue to be identified as exemplified by the recent identification of 4′-keto-2-ene VPA as a minor serum metabolite (Kassahun *et al.*, 1989).

Interest in VPA metabolism emanates from the possible contribution of metabolites to both the therapeutic and toxic effects of the drug. Thus, the unsaturated metabolites 2-ene VPA (Loscher, 1981a; Loscher and Nau, 1985) and 2,3′-diene VPA (Abbott and Acheampong, 1988) were found to have significant anticonvulsant activity in rodent models. The 4-ene VPA metabolite is thought to be involved in the hepatic damage of VPA (Zimmerman and Ishak, 1982; Rettenmeier *et al.*, 1985).

B. 4-ene VPA

The 4-ene VPA metabolite has been implicated in the hepatotoxicity of VPA, and consequently its metabolism has been extensively studied (Granneman *et al.*, 1984a; Rettenmeier *et al.*, 1985, 1986b). Eight metabolites were identified by GC/MS in rat bile and perfusate from isolated rat liver. The metabolites were identified as (E)-2,4-diene

VPA, 4,5-diOH VPA-γ-lactone, 2-propyl-2-enylglutaric acid, 2-PGA, 3′-keto-4-ene-, 5′-OH-4-ene-, 5-OH- and 3-OH-4-ene-VPA.

In the rhesus monkey, the fraction of the dose of 4-ene VPA recovered in urine in the form of metabolites and parent compound was much lower than that of VPA implying that 4-ene VPA or one or more of its metabolites tend to be incorporated into triglycerides (examples of xenobiotics that are incorporated into the adipose tissue are known (Fears, 1985)) or bind covalently to cellular components. In the rhesus monkey 19 metabolites were characterized indicating that the metabolism of 4-ene VPA is extremely complex and involves mitochondrial/peroxisomal, microsomal and cytosolic enzymes. The major metabolites identified were (E)-2,4-diene VPA, 4-ene VPA glucuronide and 3′-keto-4-ene VPA. Beta-oxidation on the propenyl side chain would lead to (E)-2,4-diene VPA and 3-OH-4-ene VPA. The latter metabolite could also arise from cytochrome P-450 dependent hydroxylation (Prickett and Baillie, 1986). Thus far no information is available on the identity of the enzyme(s) involved in the formation of (E)-2,4-diene VPA. Mitochondrial/peroxisomal β-oxidation has been implicated based on such factors as the stereospecificity of the product, i.e. the (E)-isomer.

By analogy to the metabolism of VPA, the end product of the β-oxidation of 4-ene VPA should be the electrophilic α,β-unsaturated ketone, 3-keto-4-ene VPA. However, this compound was not detected in any of the studies of 4-ene VPA metabolism (Granneman *et al.*, 1984a; Rettenmeier *et al.*, 1985, 1986b). On the other hand, β-oxidation on the saturated side chain of 4-ene VPA gave rise to the expected keto compound (3′-keto-4-ene VPA) and the hydroxy intermediate (3′-OH-4-ene

VPA) but not the diene intermediate (2,4′-diene VPA) (Rettenmeier *et al.*, 1986b).

Like VPA, conjugation with glucuronic acid was a major route of 4-ene VPA metabolism. The glycine conjugate of (E)-2,4-diene VPA was identified in significant amounts indicating that, unlike VPA, glycine conjugation plays a significant role in the metabolism of 4-ene VPA.

The minor pathways of 4-ene VPA metabolism in the rhesus monkey included hydroxylation at positions 3, 3′, 4′, and 5′ to give a series of regioisomeric hydroxy metabolites and their derivatives. There was also evidence for the formation of an epoxide intermediate and/or its precursor free radical species during the oxidation of the double bond of 4-ene VPA by cytochrome P-450 (Prickett and Baillie, 1986). This reaction was accompanied by alkyation of cytochrome P-450 and formation of 4,5-diOH VPA-γ-lactone, detected both in microsomal incubations and the urine of monkeys treated with 4-ene VPA.

From the available evidence both mitochondrial β-oxidation and cytochrome P-450 mediated oxidation appear to be involved in the generation of chemically reactive intermediates of 4-ene VPA.

3. Inhibition of fatty acid metabolism by analogs of 4-ene VPA

A. Hypoglycin

Hypoglycin (L-(methylenecyclopropyl)alanine) is the toxic hypoglycemic principle of the unripe fruit of the Jamaican ackee tree (Sherratt, 1986). Consumption of this fruit causes widespread disturbances in carbohydrate and lipid metabolism (Sherratt and Osmundsen, 1976), the clinical, biochemical, and histopathological

manifestations of which resemble those of Reye's syndrome and VPA hepatotoxicity.

The main symptoms of hypoglycin poisoning are severe hypoglycemia, vomiting, depletion of liver glycogen, fat deposits in the liver, increased plasma free fatty acids, and massive dicarboxylic aciduria (Sherratt, 1986). This is often followed by death.

Hypoglycin is metabolized to methylenecyclopropylpyruvic acid which is then oxidatively decarboxylated to methylenecyclopropylacetic acid (MCPA) in the mitochondria by the enzymes that catalyze metabolism of amino acids (Sherratt, 1986). The MCPA is activated to its CoA thio ester, part of which can be conjugated with glycine and eliminated from the body. The primary metabolic effect of hypoglycin, inhibition of fatty acid oxidation, is a consequence of the inactivation of several dehydrogenases by MCPA-CoA.

The MCPA-CoA is considered to be a suicide, mechanism-based inhibitor of dehydrogenases (Wenz *et al.*, 1981). The initial step of the dehydrogenation of acyl CoA esters by the general acyl CoA dehydrogenase enzyme is abstraction of an α-proton. This would give a transient α-carbanion which would be stabilized by the terminal double bond present in the molecule, but eventually lead to ring opening and covalent addition of the MCPA-CoA to the flavin electrophile of the enzyme.

Hypoglycin toxicity is attenuated by the administration of glycine since the latter promotes excretion of MCPA as the glycine conjugate (Al-Bassam and Sherratt, 1981). Clofibrate pretreatment of rats protects rats from the toxic effects of hypoglycin (van Hoof *et al.*, 1985) presumably because clofibrate induces mitochondrial β-oxidation.

B. 4-Pentenoic acid

The hypoglycin analog, 4-pentenoic acid, is a hypoglycemic agent and strong inhibitor of mitochondrial fatty acid oxidation (Billington *et al.*, 1978a; Billington *et al.*, 1978b). In rats, 4-pentenoic acid produces most of the features of Reye's syndrome, i.e. fatty liver, hypoglycemia, abnormal mitochondria, elevation of serum transaminase, hyperammonemia, etc.

In an effort to elucidate the mechanism by which 4-pentenoic acid inhibits fatty acid metabolism, the metabolism of 4-pentenoic acid has been studied by a number of investigators (Brendel *et al.*, 1969; Holland *et al.*, 1973; Hiltunen and Davis, 1981; Schulz, 1983). 4-Pentenoic acid is initially converted to its CoA thio ester and β-oxidized to 2,4-pentadienoyl CoA, which is then channeled into two pathways. In the major pathway a key step is the NADPH-dependent reduction of 2,4-pentadienoyl CoA to 3-pentenoyl CoA, which is subsequently β-oxidized to propionyl CoA and acetyl CoA. The minor pathway leads to the direct hydroxylation of 2,4-pentadienoyl CoA to give 3-hydroxy-4-pentenoyl CoA, which is then oxidized to 3-oxo-4-pentenoyl CoA. The formation of acryloyl CoA, which should be the end-product of this pathway, has been reported (Brendel *et al.*, 1969).

The inhibition of β-oxidation by 4-pentenoic acid appears to involve the irreversible inactivation of 3-ketoacyl CoA thiolase by 3-oxo-4-pentenoyl CoA (Schulz, 1983; Schulz, 1987). It has been proposed that 3-oxo-4-pentenoyl CoA acts as a suicide inhibitor of the thiolase enzyme while being cleaved to acryloyl CoA and acetyl CoA. The inactivation is accompanied by Michael addition of the highly

electrophilic α,β-unsaturated ketone to a nucleophilic center on the thiolase enzyme.

Studies of the inhibition of thiolase by 3-oxo-4-pentenoyl CoA were based on *in situ* formation of 3-oxo-4-pentenoyl CoA by incubating 2,4-pentadienoyl CoA with the necessary enzymes and co-factors. Thus, there is only indirect evidence for the formation of 3-oxo-4-pentenoic acid; an authentic standard had not been used in those studies.

Although the thiolase enzyme is inhibited much more strongly by 3-oxo-4-pentenoyl CoA, 2,4-pentadienoyl CoA (without the cofactors required to convert it to 3-oxo-4-pentenoic acid) does inhibit this enzyme to some extent (Holland *et al.*, 1973).

3-Oxo-4-pentenoic acid is also an inhibitor of carnitine acetyltransferase (Zhong *et al.*, 1985). There is also evidence that *in vivo* the site of inhibition of fatty acid β-oxidation by 4-pentenoic acid could be at the level of the acyl dehydrogenase (Thayer, 1984).

4. Metabolic disturbances caused by VPA

By virtue of its fatty acid structure, VPA interferes with several aspects of fatty acid metabolism. VPA inhibits β-oxidation of long- and medium-chain fatty acids (Turnbull *et al.*, 1983; Coude *et al.*, 1983; Thurston *et al.*, 1983a). Peroxisomal β-oxidation is increased in the liver of rats and mice treated with VPA (Horie and Suga, 1985; Draye and Vamecq, 1987); this appears to be a compensatory mechanism for the inhibition of mitochondrial function.

Administration of VPA results in an increased lipid content of the liver (Jezequel *et al.*, 1984; Olson *et al.*, 1986), inhibition of ketogenesis, and decreased plasma levels of β-hydroxybutyrate in young

rats (Kesterson *et al.*, 1984) and humans (Thurston *et al.*, 1983b). Increased excretion of C_6-C_{10} dicarboxylic acids is observed in the urine of patients given VPA (Mortensen *et al.*, 1980a). All the above biochemical effects of VPA are consistent with inhibition of fatty acid β-oxidation. VPA itself is thought to inhibit β-oxidation by sequestering CoA due to the accumulation of valproyl CoA (Kesterson *et al.*, 1984; Thurston *et al.*, 1985). The inhibition of fatty acid synthesis in isolated hepatocytes by VPA may also be related to the accumulation of valproyl CoA (Becker and Harris, 1983).

VPA therapy results in decreased liver and plasma carnitine levels (Bohles *et al.*, 1982; Ohtani *et al.*, 1982; Thurston *et al.*, 1983b; Murphy *et al.*, 1985). Valproylcarnitine has been identified as a minor metabolite in the urine of patients taking VPA (Millington *et al.*, 1985). Sequestration of hepatic CoA by VPA may result in fatty acids forming excess acyl carnitines, thus producing a deficiency of carnitine (Eadie *et al.*, 1988). Bohles *et al.*(1982) found reduced serum carnitine levels in a fatal hepatotoxic case, but another incident of VPA-induced hepatic failure (Laub *et al.*, 1986) was not associated with decreased carnitine levels, and carnitine administration did not reverse the toxicity.

The level of glucose in the blood of young rats, mice, and starved rats is decreased by administration of VPA (Turnbull *et al.*, 1983; Kesterson *et al.*, 1984; Graf *et al.*, 1985). Treating isolated rat hepatocytes with VPA and its metabolites (2-ene-, 4-ene-, 4-OH-, 5-OH-VPA and 2-PSA) causes concentration-dependent inhibition of gluconeogenesis (Turnbull *et al.*, 1983; Rogiers *et al.*, 1985). The mechanism by which VPA inhibits gluconeogenesis is not known, but it has

been suggested that gluconeogenesis might be inhibited at the pyruvate carboxylase level. Acetyl CoA which induces pyruvate carboxylase is decreased after VPA administration (Cotariu and Zaidman, 1988).

VPA also interferes with energy metabolism by inhibiting pyruvate oxidation and the activities of the enzymes pyruvate dehydrogenase and pyruvate decarboxylase (Turnbull *et al.*, 1983). In isolated liver mitochondria, oxidative phosphorylation is inhibited by VPA (Hayasaka *et al.*, 1986).

VPA therapy in humans is associated with hyperammonemia, which is mainly of renal origin, and is reversible in the absence of hepatic dysfunction (Coulter and Allen, 1981; Rawat *et al.*, 1981; Warter *et al.*, 1983; Zaccara *et al.*, 1987). A VPA-induced increase in plasma ammonia may be dose-related (Coulter and Allen, 1981) or non-dose-related (Zaret *et al.*, 1982). One cause of the hyperammonemia may be the inhibition of carbamoyl-phosphate synthetase by the high acyl CoA to free CoA ratio caused by VPA (Turnbull *et al.*, 1983); VPA is known to inhibit urea synthesis in healthy adults (Hjelm *et al.*, 1986, 1987) and in isolated hepatocytes (Turnbull *et al.*, 1983; Coude *et al.*, 1983).

VPA induces hyperglycinemia (Bartlett, 1977; Cherruau *et al.*, 1981), an effect attributed to the inhibition of the glycine cleavage system by both VPA and valproyl CoA (Kochi *et al.*, 1979; Mortensen *et al.*, 1980b; Martin-Gallardo *et al.*, 1983).

One possible mechanism of the antiepileptic activity of VPA is related to its ability to induce changes in the metabolism of γ-aminobutyric acid (GABA). The drug significantly raises GABA levels in the whole brain of rodents (Horton *et al.*, 1977) in a dose-dependent manner (Miyazaki *et al.*, 1988). GABA levels have been shown to be

increased in the plasma of patients on VPA therapy (Loscher and Schmidt, 1980). The effect of VPA on GABA levels is related to its inhibitory action on GABA-transaminase (Loscher *et al.*, 1981) and succinate semialdehyde dehydrogenase (Sawaya *et al.*, 1975), enzymes involved in the degradation of GABA.

Thus, it is clear from the preceding account that VPA interferes with several aspects of intermediary metabolism and energy production. However, the evidence available does not implicate the involvement of any single metabolic effect of the drug as the cause of the fatal hepatotoxicity of the drug.

5. Teratogenicity of VPA

Genetic factors related to epilepsy, seizures during pregnancy, and the teratogenic potential of antiepileptic drugs can all contribute to a high rate of teratogenicity in epileptic patients (Tsuru *et al.*, 1988). Next to hepatotoxicity, teratogenicity is the major toxic effect of VPA (Brown *et al.*, 1985). VPA is teratogenic in the mouse, rat, rabbit, and monkey (Hauck and Nau, 1989). VPA crosses the placenta and can affect the fetus (Dickinson *et al.*, 1979). Published case reports of fetal malformation in epileptic mothers on VPA include neural tube defects (spina bifida) and other defects collectively known as fetal valproate syndrome (Nau *et al.*, 1981a; Tein and MacGregor, 1985; Jager-Roman *et al.*, 1986).

In a prospective study of 172 deliveries, the incidence of malformations in infants exposed to VPA polytherapy was higher than that of VPA monotherapy or monotherapy with any of the antiepileptic drugs. The incidence was also higher than combinations that did not include VPA

(Kaneko *et al.*, 1988). In an animal study where six VPA metabolites were tested, only 4-ene VPA was clearly teratogenic (Nau and Loscher, 1986). Since both VPA and 4-ene VPA have teratogenic activity (Brown *et al.*, 1985; Hauck and Nau, 1989), it appears there are multiple biochemical mechanisms underlying the teratogenic activity of these compounds.

In a comparative study of the teratogenicity of VPA and the enantiomers of 4-ene VPA, it was found that the S-enantiomer was more potent than VPA while the R-enantiomer was less potent (Hauck and Nau, 1989). It is interesting to note at this point that the production of (E)-2,4-diene VPA was found to be higher in hepatocyte incubations containing (S)-4-ene VPA compared to that of its antipode (Porubek *et al.*, 1988). Whether the biochemical mechanism of the teratogenicity is similar to the hepatotoxicity is not clear at present. However, since electrophilic intermediates are known to cause the teratogenicity of other agents (e.g. the arene oxide metabolite of thalidomide (Nelson, 1982)), it would be of interest to determine whether reactive metabolites derived from VPA or 4-ene VPA are involved in the teratogenicity of these agents. In light of what is known about the metabolism of (S)-4-ene VPA, and the fact that it is more teratogenic than VPA, it could be speculated that (E)-2,4-diene VPA or its further metabolite might be involved in the teratogenicity of both 4-ene VPA and VPA.

6. The hepatotoxicity of VPA

A. Incidence and pathological manifestations

Many investigators classify VPA associated hepatotoxicity into two forms (Gram and Bentsen, 1983; Dreifuss and Langer, 1988; Cotariu and

Zaidman, 1988). The first form is a reversible, dose-related condition in which laboratory markers of hepatotoxicity disappear upon withdrawal of VPA therapy while the second form is a non-dose-related idiosyncratic liver failure that results in death. However, there are a number of problems associated with such a classification. Several cases of seemingly irreversible hepatotoxicity were reversed (Jeavons, 1984; Scheffner *et al.*, 1988), and thus it is not clear whether the two forms of hepatotoxicity are distinct diseases or merely manifestations of the degree of severity. In a recent case report (Kimura *et al.*, 1989), two patients who developed hepatotoxicity related to VPA therapy and who presented with Reye-like syndrome and coma, recovered after discontinuation of VPA. Necrosis, which is a frequent feature of VPA hepatotoxicity, was present in one of the patients. Microvesicular fat, a common histological finding of fatal cases, was the dominant histological feature in two subjects with non-fatal liver injury (Powell-Jackson *et al.*, 1984). In the non-fatal cases clinical and laboratory findings do not allow differentiation from those of fatal cases.

The moderately frequent incidence of minor hepatic toxicity, the reversibility of some of the severe cases, and the rarity of fatal cases, suggests that VPA hepatotoxicity in general is common. The hepatotoxicity appears to become fatal in those patients with a decreased capacity to detoxify the drug and its metabolites either because of illness or an inherited metabolic disease. Thus, by definition, the hepatotoxicity becomes idiosyncratic.

The fatal hepatotoxicity associated with VPA has been described in terms of a Reye-like syndrome and has similarities to Jamaican vomiting

sickness (hypoglycin poisoning)(Gerber *et al.*, 1979; Zimmerman and Ishak, 1982). Prior to 1987 more than 100 patients had died world-wide from VPA-induced liver failure (Scheffner *et al.*, 1988). A majority of the cases of hepatotoxicity occurred in children, most of them developing their first symptoms of toxicity within six months of initiation of VPA treatment. Many of the affected children, 34 out of 67 cases reviewed by Jeavons (1984), had other conditions such as mental retardation, metabolic disorders, neurological diseases and genetic abnormalities. In almost all cases the symptoms that patients presented with were vomiting, nausea, abdominal pain and apathy.

Of the 67 cases analyzed by Jeavons (1984) and for those for which data were available, biluribin levels were abnormal in 40 and normal in seven. The serum glutamic-oxaloacetic transaminase level was abnormal in 56 cases, normal in one and unknown in eight, while serum glutamic-pyruvic transaminase levels were abnormal in 40 and unknown in 23. There was also evidence of a blood coagulation disorder in almost all cases where there was information. For 20 patients having serum ammonia data, ammonia levels were high in 16 and normal in four.

In the 23 cases of VPA-associated fatal liver failure analyzed by Zimmerman and Ishak (1982), the most frequent histological finding was microvesicular steatosis, which was present in 17 of 21 cases for which data were available. The review by Jeavons (1984) identified 22 cases of steatosis accompanied by necrosis, 8 steatosis only, 20 necrosis only, and four steatosis with cirrhosis.

According to a review of United States cases (Dreifuss *et al.*, 1987), the overall rate of fatal hepatotoxicity related to VPA was 1 in 10000. Risk factors for fatal hepatotoxicity were identified as patient

age, co-medication with other antiepileptic drugs and the presence of other diseases. Patients at high risk (incidence of 1 in 500) were children under two years of age who were on polytherapy and who had other medical problems such as mental retardation, developmental delay, congenital abnormalities, and metabolic disorders. In a follow-up study (fatalities in 1985-1986) a four-fold decrease in hepatic fatalities associated with VPA therapy was reported (Dreifuss *et al.*, 1989; Dreifuss, 1989).

On the basis of a German study, the incidence of VPA-related fatal liver failure was placed at 1 in 5000 (Scheffner *et al.*, 1988). These authors point out that based on the criteria set out by Dreifuss *et al.*(1987), they were unable to single out any high risk group from a review of 16 fatal cases. Hence they argue that all patients taking VPA should be considered at risk for fatal hepatotoxicity.

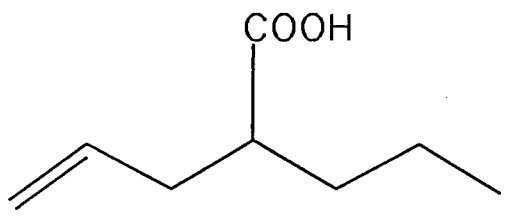
B. Biochemical mechanism

The mechanism by which VPA causes hepatotoxicity is unknown although one of its metabolites, 4-ene VPA, has been heavily implicated (Gerber *et al.*, 1979; Zimmerman and Ishak, 1982; Rettenmeier *et al.*, 1985, 1986b), and various biochemical mechanisms have been proposed (Eadie *et al.*, 1988). Because microvesicular steatosis, which is the most frequent lesion in VPA-related hepatotoxicity, is also the histological hallmark of Jamaican vomiting sickness caused by MCPA and of 4-pentenoic acid toxicity in the rat, it has been suggested that similar biochemical mechanisms underlie the liver toxicity caused by these three agents. Support for this comes from the close structural

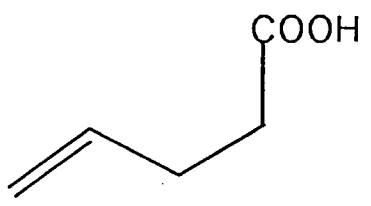
similarity between the VPA metabolite 4-ene VPA, 4-pentenoic acid, and MCPA (Fig. 2).

As mentioned earlier 4-pentenoic acid is a potent inhibitor of fatty acid metabolism and is apparently metabolized to the reactive electrophilic species, 3-oxo-4-pentenoic acid (Fig. 3), which alkylates 3-ketoacyl CoA thiolase (Schulz, 1983). Because 4-ene VPA is the 2-propyl analog of 4-pentenoic acid, it has been postulated by Rettenmeier *et al.*(1985) that 4-ene VPA may undergo a similar bioactivation (Fig. 4). This may explain the liver steatosis observed in patients with VPA-induced liver damage. It has indeed been shown that 4-ene VPA is a strong steatogen in rats (Kesterson *et al.*, 1984), is toxic to cultured hepatocytes in a dose-dependent fashion (Kingsley *et al.*, 1983) and inhibits β-oxidation of fatty acids *in vitro* (Bjorge and Baillie, 1985) and *in vivo* (Granneman *et al.*, 1984c). More recently, the covalent binding of radiolabelled 4-ene VPA to proteins of rat liver has been documented (Porubek *et al.*, 1989). The role of 4-ene VPA in VPA hepatotoxicity has recently been reviewed by Baillie (1988).

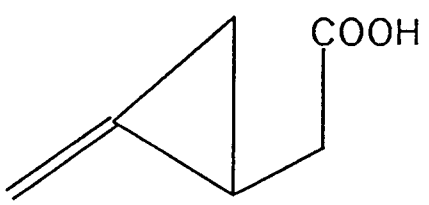
A major metabolic pathway of 4-ene VPA in the rat and monkey is β-oxidation leading to the formation of (E)-2,4-diene VPA (Rettenmeier *et al.*, 1985, 1986b). It was hypothesized that (E)-2,4-diene VPA undergoes further β-oxidation to give the ultimate toxic electrophilic species 3-keto-4-ene VPA (Baillie, 1988). However, in metabolic studies involving rats (Granneman *et al.*, 1984a; Rettenmeier *et al.*, 1985) and the rhesus monkey (Rettenmeier *et al.*, 1986b), there was no evidence for the formation of 3-keto-4-ene VPA. This lack of detection was presumed to result from the unstable and highly reactive nature of this compound.



4–ENE VALPROIC ACID



4–PENTENOIC ACID



METHYLENECYCLO–
PROPYLACETIC ACID

Figure 2. Structurally related liver toxin carboxylic acids.

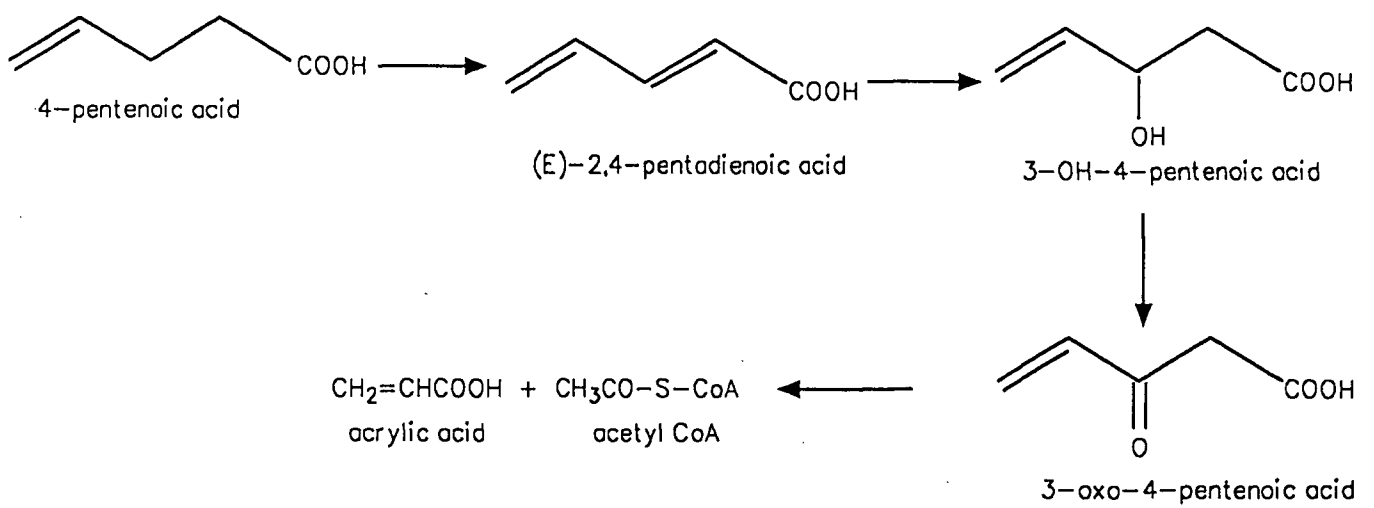


Figure 3. Mitochondrial metabolism of 4-pentenoic acid leading to the generation of chemically reactive intermediates.

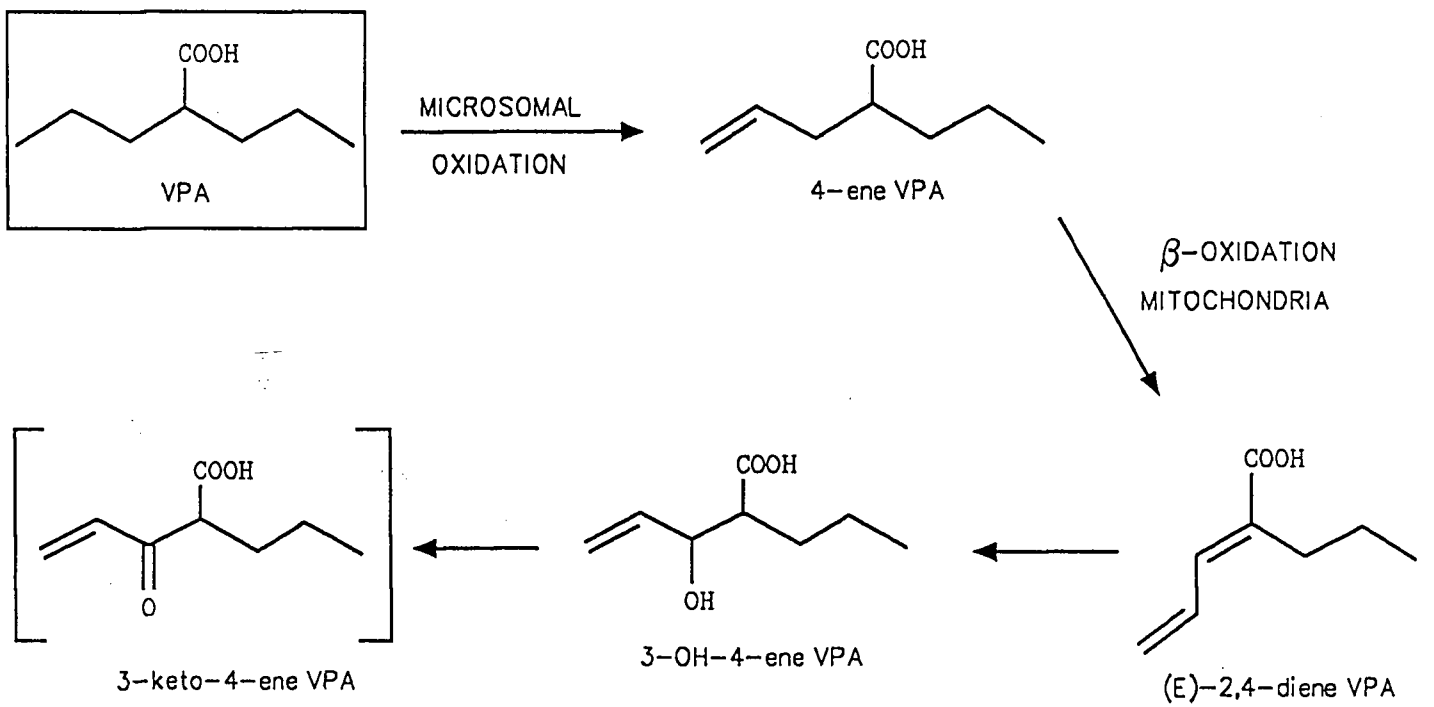


Figure 4. Metabolic pathway of VPA proposed to lead to the production of reactive and hepatotoxic metabolites.

The finding that VPA hepatotoxicity is more prevalent in polytherapy than monotherapy (Dreifuss *et al.*, 1987) is consistent with the view that a metabolite(s) of VPA produced in large quantity by the induced liver might be partly responsible for the hepatotoxicity. However, little has been published on VPA metabolite profiles in patients who had liver damage coincident with VPA therapy. In one report of a child who died of VPA hepatotoxicity, the plasma and urine levels of 4-ene- and 2,4-diene-VPA were highly elevated (Kochen *et al.*, 1983). In another case of fulminant hepatic failure (Kuhara *et al.*, 1990), 2-PGA was detected at abnormally high levels whereas VPA glucuronide and 3-keto VPA were undetectable indicating the impairment of VPA glucuronidation and β-oxidation. However, in the absence of additional information it is not clear whether the increased formation of certain metabolites is an effect or cause of the hepatotoxicity.

Since the centrizonal necrosis of the liver observed in VPA-induced liver toxicity is not seen in other lesions associated with microvesicular steatosis (Jamaican vomiting sickness, Reye's syndrome, 4-pentenoic acid toxicity), some investigators (Zimmerman and Ishak, 1982) have been prompted to conclude that the necrosis part of the VPA lesion may be caused by a reactive metabolite different from that which causes microvesicular steatosis.

Other mechanisms that do not involve formation of a chemically reactive metabolite, but that have been proposed to contribute to the toxicity are: carnitine deficiency (Coulter, 1984), interference of VPA with intermediary metabolism in a compromised liver, and depletion of CoA (Kesterson *et al.*, 1984).

7. Glutathione conjugation and its toxicological significance

The formation of chemically reactive metabolites from drugs and other xenobiotics is responsible for the initiation of many types of tissue lesions. Mitchell *et al.*(1982) classified chemically reactive metabolites into electrophiles, organic free radicals, and those that generate active O_2 species such as O_2^-. By binding covalently to proteins and nucleic acids, electrophilic intermediates can cause toxic effects, mutations, and cancer. There is a good correlation between covalent binding of electrophiles to tissue components and tissue necrosis (Gillette, 1974).

Many of the agents that are chemically reactive or are metabolically converted to chemically reactive intermediates affect cellular levels of GSH (Boobis *et al.*, 1989). This includes compounds that are substrates for GSH conjugation and those responsible for the oxidation of GSH to GSH disulfide (GSSG). GSH acts as a nucleophilic scavenger by converting electrophilic centers to S-linked conjugates and as a substrate in the GSH redox system that is involved in the destruction of hydroperoxides. GSH is an essential constituent of the cell, and failure to maintain adequate levels is detrimental to the survival of the cell. The protective role of GSH against chemically induced injury to tissues can be compromised by the depletion of GSH, which may lead to cell injury and death (Reed and Fariss, 1984).

A. Glutathione

GSH (L-γ-glutamyl-L-cysteinylglycine) constitutes 90% of the non-protein thiol in the cell (Reed, 1985). The cellular concentration of GSH varies between 0.5 to 10 mM depending on the cell type (Kosower and

Kosower, 1978). The GSH level in the liver is close to twice that of the kidneys and over threefold higher than in the lung. Most of the intracellular GSH is present in the reduced form with less than 5% present as GSSG.

Chemically GSH is characterized by the γ-glutamyl linkage which makes it resistant to degradation by typical proteases. The cysteinyl residue provides a nucleophilic thiol group that reacts with electrophiles and metabolically produced oxidizing species. The high number of hydrophilic groups of GSH ensures high water solubility for its conjugates. Its relatively high molecular weight provides for the preferential biliary excretion of GSH adducts. GSH and its thiolate anion are soft and strong nucleophiles that react well with soft electrophiles but poorly with both weak and hard electrophiles (Ketterer *et al.*, 1983).

B. Glutathione S-transferases

GSH S-transferases are a group of isoenzymes with overlapping substrate specificity and widespread distribution in nature. In mammals, GSH transferases are found in large quantities, representing 10% of the cytosolic protein of rat liver, which can reach greater than 20% following induction (Jakoby, 1985). Although the highest level of transferase is in the cytosol (0.2 to 0.5 mM), membrane-bound transferases have been detected in the microsomes and mitochondria (Ketterer *et al.*, 1983). In the rat liver there are at least seven soluble isoenzymes with similar dimeric structure and in which the monomers fall into three differing molecular weight categories. In addition to catalysis, these enzymes serve storage and transport

functions (Kaplowitz, 1980). The transferases have a low-affinity, broad-specificity site for lipophilic compounds and another site which is specific for GSH and its β-alanine analog (Jakoby, 1985).

Transferases catalyze the initial step of NAC adduct (mercapturic acid) synthesis in which a thioether bond is formed between GSH and the carbon atom of an electrophilic substrate (Habig *et al.*, 1974). The GSH conjugate is normally excreted into bile and is also metabolized by a series of reactions to the NAC conjugate which may be excreted in the bile or urine.

The broad specificity of the transferases has perhaps evolved to accommodate the wide spectrum of structural types of toxic substances to which the organism is exposed. The conjugation reactions catalyzed by transferases fall into two basic categories: nucleophilic substitution and nucleophilic addition to a polarized double bond. Transferases also catalyze the reaction of GSH with substrates having heteroatom electrophilic centers.

The basis of the catalytic mechanism of transferases appears to be to promote the formation of the thiolate anion (GS^-) from GSH (Jakoby and Habig, 1980). This seems reasonable since the rate of reaction of GS^- with electrophiles is three orders of magnitude greater than that of GSH. The mechanism proposed for the catalytic activity is related to the proximity effect (Jakoby and Habig, 1980). The active site of the enzyme consists of a specific binding area for GSH (the effect of binding to which is the reduction of the pKa of GSH) and another electrophilic binding site, which is close to the GSH binding site and is characterized by a lipophilic spacious surrounding. Thus, when a sufficiently reactive electrophilic substrate is in proximity to the

thiolate anion a reaction takes place. Formation of GSH conjugates can also occur nonenzymatically. However, *in vivo*, it is assumed that the contribution of the noncatalytic reaction is negligible owing to the slow formation of electrophiles by enzymes with slow rates of reaction such as cytochrome P-450 (Ketterer *et al.*, 1983).

8. Rationale and objectives

While there may be multiple mechanisms by which VPA causes hepatotoxicity, there is a strong biochemical basis to implicate the 4-ene VPA pathway as discussed in preceding sections. The putative reactive metabolite, 3-keto-4-ene VPA, that would arise from this pathway has not been identified in serum or urine, presumably because of its chemical instability. The 3-keto-4-ene VPA, akin to other α,β-unsaturated carbonyl compounds, is highly reactive and can theoretically undergo Michael additions to give covalent adducts of nucleophiles. As alluded to in an earlier section, reactive intermediates produced in the body are usually detoxified by the formation of GSH conjugates. In view of the nature of the putative reactive metabolite in the case of VPA, it is possible that a GSH conjugate may be formed *in vivo*. Hence the logical derivative of 3-keto-4-ene VPA in urine will be the NAC conjugate, while it may be possible to detect the GSH conjugate in bile.

The (E)-2,4-diene VPA metabolite is presumably a β-oxidation product of 4-ene VPA and is a key intermediate in the metabolism of 4-ene VPA. A detailed study of the metabolism of this diene will not only enable the possible detection of the putative α,β-unsaturated ketone but also the detection of other metabolites that point to additional sources of toxicity.

The metabolism of 4-pentenoic acid will be studied in a parallel fashion to that of 4-ene VPA with the aim of characterizing thiol conjugates of reactive metabolites of 4-pentenoic acid. The information that will be obtained from this study has a twofold significance. Firstly, it may provide the answer to the question of whether the parallel drawn between the metabolism of 4-ene VPA and 4-pentenoic acid and hence the proposed mechanism of hepatotoxicity is realistic. Secondly, the detection of the thiol conjugates of the α,β-unsaturated keto metabolite of 4-pentenoic acid will provide the first direct biochemical evidence for the formation of the metabolite of 4-pentenoic acid responsible for the irreversible inhibition of β-ketoacyl CoA thiolase, one of the enzymes in the fatty acid β-oxidation pathway.

Hence the present study will focus on the metabolism of 4-ene VPA, (E)-2,4-diene VPA, and 4-pentenoic acid and/or (E)-2,4-pentadienoic acid with particular emphasis on proving the formation of reactive metabolites of VPA and their thiol conjugates and devising methods for their detection in humans when applicable.

Specific objectives

1. To study the metabolism of 4-ene- and (E)-2,4-diene-VPA in rats with particular emphasis on pathways generating reactive metabolites and to characterize any novel metabolites spectroscopically and by synthesis.

2. To chemically synthesize 3-keto-4-ene VPA and its NAC adduct and to investigate the formation of these metabolites in rats following the administration of VPA, 4-ene- and (E)-2,4-diene-VPA.

3. To synthesize the GSH conjugate of 3-keto-4-ene VPA both chemically and from *in situ* metabolism of (E)-2,4-diene- and 4-ene-VPA by rat liver mitochondrial fraction.

4. To study the metabolism of 4-pentenoic acid and/or (E)-2,4-pentadienoic acid with the objective of trapping the electrophilic 3-keto-4-pentenoic acid *in vivo* in the form of its thiol conjugates.

5. To develop a highly sensitive NICI assay for the quantitation of the known VPA metabolites, including 4-ene- and (E)-2,4-diene-VPA, in pediatric patients and to use the method for the detection of new metabolites.

6. To investigate the formation of NAC conjugates of reactive metabolites in patients on VPA therapy and to develop a method to quantitate these conjugates as a measure of exposure of patients to toxic metabolites of VPA.

II. EXPERIMENTAL

1. Chemicals and materials

Chemicals were reagent grade and obtained from the following sources.

A. Aldrich Chemical Co. (Milwaukee, Wisconsin)

Acrolein, allyl bromide, Amberlite XAD-2 resin, p-benzoquinone, n-butyllithium (1.6 M in hexane), calcium hydride, chloroform-d, deuterium oxide, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), Diazald^{R}, diisopropylamine, diisopropylethylamine, hexamethylphosphoramide (HMPA), lithium aluminium hydride, methanesulfonyl chloride, methanol-d_4, 2-methylglutaric acid (2-MGA), palladium (II) chloride, pentanoic acid, (E)-2-pentenoic acid, 4-pentenoic acid, potassium hydride (35% dispersion in mineral oil), propionaldehyde

B. Alfa Products (Danvers, Massachusetts)

Pentafluorobenzyl bromide (PFBB)

C. J. T. Baker Inc. (Phillipsburg, New Jersey)

Silica gel for flash chromatography, solid phase extraction cartridges (6 mL size)

D. BDH Chemicals (Toronto, Ontario)

Acetone, benzene, ether (anhydrous), hydrochloric acid, sodium dihydrogen orthophosphate, sodium hydroxide, sodium sulfate (anhydrous), petroleum ether, pyridine, tetrahydrofuran (THF)

E. Boehringer Mannheim (Montreal, Quebec)

Adenosine triphosphate (ATP), bovine serum albumin

F. Caledon Laboratories Ltd. (Georgetown, Ontario)

All solvents from this supplier were of distilled-in-glass grade-acetonitrile, dichloromethane, ethyl acetate, methanol

G. ICN Biochemicals Inc. (Plainview, New York)

Di-n-propylacetic acid (VPA)

H. Mallinckrodt Chemicals (St. Louis, Missouri)

Acetic acid (glacial), sodium bicarbonate

I. Matheson Ltd. (Edmonton, Alberta)

Hydrogen chloride gas

J. Pierce Chemical Co. (Rockford, Illinois)

N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), triethylamine

K. Sigma Chemical Co. (St. Louis, Missouri)

Coenzyme A (CoA), glutathione (GSH), N-acetyl-L-cysteine (NAC), urethane

L. Supelco (Oakville, Ontario)

3% Dexsil 300 on 100/120 mesh Supelcoport

M. Whatman Ltd. (Maidstone, Kent, England)

Silica gel plates

N. Compounds synthesized in our laboratory

2-Propyl-2-pentenoic acid (E- and Z-isomers, 2-ene VPA), 2-propyl-3-pentenoic acid (3-ene VPA, stereochemistry not determined), 2-(1'-propenyl)-2-pentenoic acid (E,E-and E,Z-isomers, 2,3'-diene VPA), 2-propyl-3-oxopentanoic acid (3-keto VPA), 2-propyl-4-oxopentanoic acid (4-keto VPA), 2-propyl-3-hydroxypentanoic acid (3-OH VPA), 2-propyl-4-hydroxypentanoic acid as the γ-lactone (4-OH VPA), 2-propylglutaric acid (2-PGA), 2-propylsuccinic acid (2-PSA), di-([3,3,3-^{2}H]propyl)acetic acid ([$^{2}H_6$]VPA), [3,5,5-^{2}H]3-heptene-4-carboxylic acid ([$^{2}H_3$]2-ene VPA), [3',3',3'-^{2}H]2-propyl-3-oxopentanoic acid ([$^{2}H_3$]3-keto VPA) (Acheampong *et al.*, 1983; Acheampong *et al.*, 1984; Abbott *et al.*, 1986; Lee *et al.*, 1989).

2. Animals and surgical equipment

Animals and surgical equipment were purchased from the following suppliers.

Animal Care Facility (University of British Columbia, Vancouver, B.C)

Sprague Dawley male rats (250-350 g)

Becton Dickinson (Rutherford, New Jersey)

Yale needle 23G, 1", 19G 1.5"; tuberculin syringe 1 cc

Clay Adams (Parsippany, New Jersey)

Polyethylene tubing PE-10

Ethicon Sutures Ltd. (Peterborough, Ontario)

4-0 Silk

3. Instrumentation

A. Capillary column GC/MS

A Hewlett-Packard 5987A quadrupole GC/MS was used in electron impact (EI), negative ion chemical ionization (NICI), and positive chemical ionization (PCI) modes with the following conditions and settings.

EI analysis: column, DB-1701 (J and W Scientific, Folsom, California), 30 m x 0.32 mm x 0.25 μm film thickness; oven, 50-140°C at 30°/min, 140-260°C at 5°/min, hold 10 min; source, 240°C; injection port, 240°C; open split interface, 250°C. Electron energy was 80 eV and source pressure 10^{-6} torr. The carrier gas was helium at a flow rate of 1 mL/min with a back pressure of 10 psi.

NICI analysis: column, DB-1, 30 m x 0.32 mm x 0.25 μm. Oven temperature was programmed from 200-250°C at 30°/min, hold 20 min, 250-260°C at 2°/min, hold 12 min, for conjugated metabolites and 110-140°C at 30°/min, 140-260°C at 5°/min for non-conjugated metabolites. Other temperatures were the same as for EI except for the source temperature which was set at 200°C. NICI spectra were recorded at an ionization energy of 120 eV and source pressure of ca. 1 torr using methane as the moderating gas.

B. Packed column GC/MS

Intermediates and end products of synthetic reactions were characterized using a Hewlett-Packard 5700A gas chromatograph interfaced to a Varian MAT-111 mass spectrometer via a variable slit separator. Data were processed using a Packard Bell computer (IBM AT clone) and a program written in our laboratory. Mass spectra were obtained from m/z

14 to 750 at 12 scans per minute. Total ion current (TIC) chromatograms were plotted from m/z 50 to 500. Ionization energy was 70 eV and source pressure 5 x 10^{-6} torr. Other operating conditions: column, 1.8 m x 2 mm i.d. packed with 3% Dexsil 300 on 100/200 mesh Supelcoport; oven temperature, initial 50°C, rate 8°/min to 270°C, hold 10 min at 270°C. The injection port, transfer line and separator temperatures were held at 250°C.

C. HPLC

HPLC analysis was carried out on an HP-1050 series LC with a quaternary pump and variable wavelength UV detector fitted with either a Hypersil ODS analytical column (4.6 x 200 mm) or a Whatman Partisil 10 ODS-2 preparative column (9 x 250 mm). The wavelength of detection was 210-220 nm. The mobile phase consisted of various proportions of 0.01 or 0.02 M NaH_2PO_4 buffer (adjusted to pH 3.5 with H_3PO_4) and methanol. All analyses were performed isocratically at a flow rate of 1 mL/min (analytical column) or 3 mL/min (preparative column).

D. LC/MS and LC/MS/MS

LC/MS and LC/MS/MS were performed at Cantest Ltd. of Vancouver on a Sciex atmospheric pressure ionization (API) tandem mass spectrometer coupled to a heated nebulizer LC/MS interface. The LC system consisted of an Applied Bio System 140A pump and a 4.6 X 200 mm ODS column connected to a Valco VE106C injector. The mobile phase consisted of acetonitrile and 10 mM ammonium acetate buffer, programmed 20 to 100% acetonitrile over a 10 min period. The nebulizing gas was N_2 and vapor temperature 150°C. Collision activated dissociation (CAD) daughter ion

spectra were obtained using argon as the collision gas (collision energy 20-60 eV) in the second quadrupole. Detection was both in the positive and negative ion modes.

E. FAB/MS

FAB spectra were obtained in the Department of Chemistry and were recorded on an AEI MS9 mass spectrometer with an in-house built saddle field gun and ion source. Ionization was effected using xenon gas at 1-1.5 mA current and 8 kV accelerating potential. Spectra were obtained at a scan rate of 10 sec/decade. Samples in methanolic solutions were applied to the 3-nitrobenzyl alcohol matrix.

F. NMR spectroscopy

All NMR spectra were performed in the NMR facility of the Chemistry Department. High field ^{1}H-NMR spectra were recorded on a Bruker WH-400 spectrometer using tetramethylsilane as the reference. ^{13}C-NMR were obtained on either a Varian XL-300 or Bruker AC-200 spectrometer operating at 75 MHz, by broad-band decoupling on the proton frequency and by an attached proton test. Samples were dissolved in methanol-d_4 and D_2O in the case of conjugated metabolites and $CDCl_3$ for all other compounds.

4. Chemical syntheses

A. General

Purification of synthetic products was carried out either by fractional distillation or medium pressure (flash) chromatography according to the method of Still *et al.* (1978). Up to 2 g of material

were purified using a 2.5 X 20 cm silica gel column. The eluent composition was selected on the basis of an rf value of about 0.3 on TLC. The sample was dissolved in the less polar component of the mobile phase and eluted at 2 mL/min with approximately 5 mL fractions being collected.

The syntheses described in this thesis that involve alkylation reactions are based on the general procedure of the alkylation of ester enolates described by R.J. Cregge *et al.* (1973).

B. Synthesis of 3-keto-4-ene VPA

This compound was synthesized as the methyl ester by oxidizing methyl 3-OH-4-ene VPA with tert-butyl hydroperoxide in the presence of chromium trioxide. The 3-OH-4-ene VPA was prepared using the following procedure. n-Butyllithium (69 mL, 0.11 mol) was added dropwise to diisopropylamine (15.5 mL, 0.11 mol) in THF (100 mL) at 0°C. The mixture was stirred for 20 min and cooled in a dry ice acetone bath to -78°C, and methyl pentanoate (11.6 g, 0.1 mol) in THF (10 mL) was added dropwise over a period of 30 min. After stirring the reaction mixture for another 30 min, acrolein (6.7 mL, 0.1 mol) in 10 mL THF was added dropwise and the reaction allowed to continue for 60 min before being quenched with 15% HCl solution. The mixture was extracted with ether, the etheral extract washed with $NaHCO_3$ and water, and dried over anhydrous Na_2SO_4. The product (3-OH-4-ene VPA methyl ester) was purified by fractional distillation (bp 62°C/0.1 mm, yield 72%).

GC/MS: (MW=172) m/z 87(100%), 55(82%), 116(32%), 130(3%), 141(2%), 155(2%).

^{1}H-NMR: δ 0.92(t,3H,CH_3-), 1.28-1.73(m,4H,-CH_2-CH_2-), 2.47-2.57(m,1H,-CH-), 2.60-2.73(bs,1H,-OH), 3.70(s,3H,-OCH_3), 4.31(t,1H,-CH-O), 5.15-5.35(m,2H,CH_2=), 5.80-5.92(m,1H,=CH-).

tert-Butyl hydroperoxide (9.6 mL, 70% solution, 0.07 mol) and methyl 3-OH-4-ene VPA (1.72 g, 0.01 mol) were successively added to a stirred solution of chromium trioxide (100 mg, 0.001 mol) in 20 mL dichloromethane (Muzart, 1987a, 1987b). After stirring for 6 hr at room temperature the mixture was treated with saturated $NaHCO_3$ solution and the organic layer dried (Na_2SO_4). The product (3-keto-4-ene VPA methyl ester) was purified by flash chromatography using 10% ethyl acetate in pet. ether (yield 75%).

GC/MS: (MW=170) m/z 55(100%), 128(9%), 84(7%), 96(5%), 139(3%), 170(1%).

^{1}H-NMR: δ 0.94(t,3H,CH_3-), 1.30-1.37(m,2H,CH_3-CH_2-), 1.82-1.95(m,2H,-CH_2-), 3.71(t,1H,-CH-), 3.73(s,3H,-OCH_3), 5.86-6.51(m,3H,CH_2=CH-).

C. Synthesis of the GSH conjugate of 3-keto-4-ene VPA methyl ester

To a solution of GSH (921 mg, 3 mmol) in 5 mL of water was added a solution of 3-keto-4-ene VPA methyl ester (510 mg, 3 mmol) in 10 mL of methanol. The pH of the mixture was adjusted to 7.8 with 10 M NaOH solution and the mixture stirred at room temperature for 30 min at which time all substrate was converted to product as determined by HPLC. The product was purified by preparative HPLC. Repeated injections (200 μL) of a methanolic solution of the crude product (70 mg/mL) were made on the preparative column using a mobile phase of 0.02 M NaH_2PO_4-methanol (60:40). The conjugate eluted at 4.02 min.

FAB/MS diagnostic ions: m/z 171$(M-GS)^+$, 478$(M+H)^+$, 500$(M+Na)^+$.

^{1}H-NMR: δ 0.94(t,3H,CH_3-), 1.27-1.36(m,2H,CH_3-CH_2-), 1.81(t,2H,-CH_2-CH_2-), 2.15-2.23(m,2H,Glu$\beta\beta'$), 2.55-2.62(m,2H,Glu$\gamma\gamma'$), 2.79(t,2H,-S-CH_2-CH_2-CO-), 2.83-2.90(m,1H,Cysβ), 2.94(t,2H,-S-CH_2-CH_2-CO-), 3.05-3.12(m,1H, Cysβ'), 3.71(t,1H,Gluα), 3.75(s,3H,-OCH_3), 3.80(s,2H,Gly$\alpha\alpha'$), 4.57(1H,dd ,Cysα).

^{13}C-NMR: δ 14.2(CH_3-), 21.5(CH_3-CH_2-), 26.3(-CH_2-CH_2-), 27.7(-S-CH_2-CH_2-CO-), 31.1(-S-CH_2-CH_2-CO-), 32.9(Gluβ), 34.6(Cysβ), 44.2(Gluγ), 48.2(Glyα), 53.2(-OCH_3), 54.3(Cysα), 55.3(Gluα), 59.8(-CH-$COOCH_3$, weak signal), 172.2(Cys-CO-), 172.5(Glu-CO-), 174.3(Gly-COOH), 175.3(Glu-COOH), 176(-C$OOCH_3$), 206.9(-CH_2-CO-CH-).

D. Synthesis of the methyl NAC conjugate of 3-keto-4-ene VPA methyl ester

This adduct was synthesized by refluxing the substrate with the methyl ester of NAC according to the method of Delbressine *et al.* (1981) for the synthesis of the methyl ester of the NAC conjugate of methyl crotonate. The methyl ester of NAC was synthesized by stirring NAC in excess methanol in the presence of thionyl chloride (van Bladeren *et al.*, 1980). To a solution of methyl 3-keto-4-ene VPA (1.72 g, 10 mmol) in 50 mL p-dioxane, methyl NAC (1.77 g, 10 mmol) and triethylamine (2.8 mL, 20 mmol) were added and the mixture refluxed for 1 hr. The solvent was removed by flash evaporation and the residue purified by HPLC using 0.01 M NaH_2PO_4-methanol (40:60) as the mobile phase with which the conjugate eluted at 6.7 min.

GC/MS: PCI: m/z 348(100%)$(M+H)^+$, 376(40%)$(M+C_2H_5)^+$, 388(18%)$(M+C_3H_5)^+$.

^{1}H-NMR: δ 0.94(t,3H,CH_3-), 1.27-1.36(m,2H,CH_3-CH_2-), 1.80(t,2H,-CH_2-CH_2), 2.01(s,3H,-$NHCOCH_3$), 2.78(t,2H,-S-CH_2-CH_2-CO-), 2.82-2.86(m,1H, Cysβ), 2.89(t,2H,-S-CH_2-CH_2-CO-), 2.98-3.04(1H,m,Cysβ'), 3.73(s,3H,-OCH_3), 3.75(s,3H,-S-CH_2-CH-$COOCH_3$), 4.63(1H,dd,Cysα).

^{13}C-NMR: δ 14.2(CH_3-), 21.6(CH_3-CH_2-), 22.4(-$NHCOCH_3$),26.7(-CH_2-CH_2-), 31.2(-S-CH_2-CH_2-CO-), 34.6(-S-CH_2-CH_2-CO-), 43.0(Cysβ), 52.8(Cysα), 52.9(-OCH_3), 53.7(-S-CH_2-CH-$COOCH_3$), 59.5(-CO-CH-$COOCH_3$, weak signal), 171.9(-$NHCOCH_3$), 172.5(-$COOCH_3$), 173.2(-S-CH_2-CH-$COOCH_3$), 205.5(-CH_2-CH_2-CO-CH-).

E. Synthesis of (E)-2,4-diene VPA

Methyl 3-OH-4-ene VPA synthesized as described in section B above was converted to 2,4-diene VPA employing two synthetic approaches. The hydroxy compound (8.6 g, 0.05 mol), triethylamine(7.7 mL, 0.055 mol) and dichloromethane (100 mL) were cooled to 0°C and methanesulfonyl chloride (4.3 mL, 0.055 mol) was added dropwise and the mixture stirred for 45 min at room temperature (Lee *et al.*, 1989). The precipitate formed was filtered off and following the removal of the solvent the residue was reconstituted with THF (80 mL), cooled to 0°C and potassium hydride (11.4 g, 0.1 mol) added. The reaction mixture was stirred at room temperature for 7 hr before being quenched cautiously at -78°C with water. The product was isolated by extraction with ether and purified by fractional distillation (bp 63°C/1 mm) to give 3.3 g (yield 42%) of a 3:1 mixture of the E and Z isomers of 2,4-diene VPA methyl ester. The product was further purified by flash chromatography (5% ethyl acetate in pet. ether) and afforded 94% of the E isomer as determined by NMR. To obtain the free acid, a portion of the ester was refluxed in two

equivalents of aqueous NaOH at 60°C for 24 hr. The mixture was then treated with dilute HCl solution, extracted with ether and, following the removal of ether, was purified by column chromatography (5% methanol in chloroform). The purity of the diene, as judged by HPLC relative peak area, was 98%.

In the second synthetic route the mesylate synthesized as described above was subjected to elimination using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). To a solution of the mesylate in THF (5.0 g, 0.02 mol), DBU (3.8 mL, 0.025 mol) was added and the mixture refluxed for 2 hr (Rettenmeier *et al.*, 1989). The solution was then allowed to cool to room temperature, quenched with water and extracted with ether. The etheral layer was washed successively with dilute HCl and NaOH and dried (Na_2SO_4). Following column purification as described above, the methyl ester of (E)-2,4-diene VPA was obtained in 76% yield.

GC/MS: (MW=154)(methyl ester): m/z 95(100%), 154(83%), 93(63%), 79(59%), 59(54%), 123(49%), 67(45%), 139(25%), 111(22%).

GC/MS: (MW=140)(free acid): m/z 140(100%), 95(97%), 67(82%), 41(75%), 111(50%), 125(34%).

^{1}H-NMR: (methyl ester): δ 0.92(t,3H,CH_3-), 1.41-1.52(m,2H,CH_3-C$\underline{H}_2$-), 2.40(t,2H,-CH_2-C$\underline{H}_2$-), 3.76(s,3H,-OCH_3), 5.44(dd,1H,CH_2=), 5.56(dd, 1H,CH_2=), 6.61-6.72(m,1H,=CH-), 7.17(d,1H,-CH=).

^{1}H-NMR: (free acid) δ 0.93(t,3H,CH_3-), 1.45-1.57(m,2H,CH_3-C$\underline{H}_2$-), 2.41(t,2H,-CH_2-C$\underline{H}_2$-), 5.50(dd,1H,CH_2=), 5.62(dd,1H,CH_2=), 6.62-6.74(m, 1H,=CH-), 7.30(d,1H,-CH=).

F. Synthesis of the GSH conjugate of (E)-2,4-diene VPA

To a solution of (E)-2,4-diene VPA (140 mg, 1 mmol) in methanol (5 mL), GSH (614 mg, 2 mmol) in 0.1 M NaH_2PO_4 (pH 7.4, 20 mL) was added and the mixture stirred at room temperature. The progress of the reaction was monitored by TLC using the solvent system of chloroform/methanol/acetic acid (7:3:1) for development and spraying with Young's reagent (Knight and Young, 1958). One hr after the commencement of the reaction a product could be detected on TLC and the stirring was stopped after another 7 hr. The reaction mixture was extracted with ether to remove unreacted starting material and the aqueous portion was transferred onto Sep-pak C-18 cartridges, washed with water and eluted with methanol/H_2O (1:2). Following lyophilization of the eluate, the residue was analyzed by FAB/MS.

FAB/MS diagnostic ions: m/z 448$(M+H)^+$, 470$(M+Na)^+$.

G. Synthesis of the GSH conjugate of the methyl ester of (E)-2,4-diene VPA

This conjugate was synthesized essentially following the procedure described in section C, the main difference being that stirring was continued for 5 hr instead of 1. At that time 50% of the substrate was converted to product as determined by HPLC. The crude product was purified by preparative HPLC using the solvent system 0.02 M NaH_2PO_4-methanol (50:50) with which the conjugate eluted at 5.28 min.

FAB/MS diagnostic ions: m/z 155$(M-GS)^+$, 462$(M+H)^+$, 484$(M+Na)^+$.

^{1}H-NMR: δ 0.92(t,3H,CH_3-), 1.24-1.36(m,2H,CH_3-CH_2-), 1.48-1.59(m,1H,-CH_2-CH_2-), 1.66-1.77(m,1H,-CH_2-CH_2-), 2.15-2.23(m,2H,Glu$\beta\beta'$), 2.51-2.64 (m,2H,Glu$\gamma\gamma'$), 2.72-2.81(m,1H,Cysβ), 2.96-3.03(m,1H,Cysβ'), 3.10(q,1H,-

CH=CH-C<u>H</u>-), 3.19(d,2H,-S-C<u>H</u>$_2$-CH=CH-), 3.70(s,3H,-OCH$_3$), 3.73(t,1H, Gluα), 3.79(s,2H,Glyα), 4.55(dd,1H,Cysα), 5.51-5.67(m,2H,-CH=CH-).

^{13}<u>C-NMR</u>: δ 14.1(CH$_3$-), 21.1(CH$_3$-<u>C</u>H$_2$-), 27.7(-CH$_2$-<u>C</u>H$_2$-), 32.9(Gluβ), 34.1(Cysβ), 35.4(Gluγ), 44.0(Glyα), 49.8((-CH=CH-<u>C</u>H-), 52.7(Cysα), 54.0(Gluα), 55.3(-OCH$_3$), 129.7(-<u>C</u>H=CH-), 132.0(-CH=<u>C</u>H-), 172.4(Cys-CO-), 174.2(Glu-CO-), 175.3(Gly-COOH), 175.7(Glu-COOH), 176.9(-<u>C</u>OOCH$_3$).

H. <u>Synthesis of the dimethyl ester of the NAC conjugate of (E)-2,4-diene VPA</u>

To a solution of methyl (E)-2,4-diene VPA (1.54 g, 10 mmol) in 50 mL p-dioxane, methyl NAC (1.77 g, 10 mmol) and triethylamine (5.6 mL, 40 mmol) were added and the mixture refluxed for 3 hr. Following the removal of the solvent the crude product was purified by preparative HPLC. With the mobile phase of 0.01 M NaH$_2$PO$_4$-methanol (40:60) the conjugate eluted at 10.09 min.

<u>GC/MS</u>: PCI: m/z 332(100%)(M+H)$^+$, 360(24%)(M+C$_2$H$_5$)$^+$, 372(12%)(M+C$_3$H$_5$)$^+$.

^{1}<u>H-NMR</u>: δ 0.92(t,3H,CH$_3$-), 1.27-1.38(m,2H,CH$_3$-C<u>H</u>$_2$-), 1.49-1.58(m,1H,-CH$_2$-C<u>H</u>$_2$-), 1.68-1.78(m,1H,-CH$_2$-C<u>H</u>$_2$-), 2.02(s,3H,-NHCOCH$_3$), 2.70-2.79 (m,1H,Cysβ), 2.91-2.99(m,1H,Cysβ′), 3.10(q,1H,-CH=CH-C<u>H</u>-), 3.17 (d,2H,-S-C<u>H</u>$_2$-CH=CH-), 3.68(s,3H,-OCH$_3$), 3.75(s,3H,-S-CH$_2$-CH-COOC<u>H</u>$_3$), 4.58(dd, 1H,Cysα), 5.49-5.64(m,2H,-CH=CH-).

^{13}<u>C-NMR</u>: δ 14.1(CH$_3$-), 21.3(CH$_3$-<u>C</u>H$_2$-), 22.4(-NHCO<u>C</u>H$_3$), 32.5(-CH$_2$-<u>C</u>H$_2$-), 34.1(Cysβ), 35.6(-S-<u>C</u>H$_2$-CH=CH-), 49.8(Cysα), 52.4(-OCH$_3$), 53.0(-S-CH$_2$-CH-COO<u>C</u>H$_3$), 53.2(-CH=CH-<u>C</u>H-), 129.7(-<u>C</u>H=CH-), 132.5(-CH=<u>C</u>H-), 172.7(-NH<u>C</u>OCH$_3$), 173.3(-<u>C</u>OOCH$_3$), 176.3(-S-CH$_2$-CH-<u>C</u>OOCH$_3$).

I. Synthesis of methyl 3-keto-4-pentenoate

A solution of diisopropylamine (12.5 mL, 0.09 mol) in THF was cooled to 0°C and n-butyllithium (56 mL, 0.09 mol) added in a dropwise fashion. The mixture was stirred for 20 min, cooled to -78°C and methyl acetate (7.1 mL, 0.08 mol) in THF was added dropwise. After stirring for 10 min acrolein (5.2 mL, 0.08 mol) was added and the mixture stirred for a further 60 min before being quenched with 15% HCl solution. Following extraction with ether, the extract was washed with $NaHCO_3$ and water. The ether was removed and the product purified by fractional distillation (bp 75°C/8 mm) to yield 5.2 g of ester (yield 50%).

GC/MS: (MW=130) m/z 43(100%), 57(83%), 74(51%), 98(32%), 81(4%), 87(3%), 113(2%), 130(2%).

^{1}H-NMR: δ 2.50-2.65(m,2H,-CH_2-), 2.85-3.05(bs, 1H,-OH), 3.73(s,3H,-OCH_3), 4.50-4.60(m,1H,-CH(OH)-), 5.15(dd,1H,CH_2=), 5.35(dd,1H,CH_2=), 5.85-5.95(m,1H,=CH).

A 4.5 g portion (0.035 mol) was oxidized to the final product in a fashion similar to that described for the synthesis of methyl 3-keto-4-ene VPA in section B above. Pure methyl 3-keto-4-pentenoate was obtained in 41% yield by distillation under reduced pressure (70°C/23 mm).

GC/MS: (MW=128) m/z 55(100%), 69(18%), 96(16%), 97(8%), 128(7%), 127(3%).

^{1}H-NMR: (keto and enol forms with a ratio of 2:1) (1). keto form; δ 3.63(s,2H,-C$\underline{H}_2$-$COOCH_3$), 3.75(s,3H,-OCH_3), 5.85-6.50(m,3H,CH_2=CH-); enol form; δ 5.10(s,1H,=C$\underline{H}COOCH_3$).

J. Synthesis of the thiol conjugates of methyl 3-keto-4-pentenoate

Both GSH and NAC conjugates were synthesized in a manner similar to that described for the GSH conjugate of methyl 3-keto-4-ene VPA by mixing a methanolic solution of the substrate and aqueous solutions of the conjugating agents (pH of mixture=7.8) and stirring for 30 min. The NAC conjugate was also synthesized as the dimethyl ester by refluxing methyl NAC with methyl 3-keto-4-pentenoate in dioxane.

For the GSH conjugate the reaction mixture was lyophilized and characterized without further purification since it was deemed to be pure by HPLC.

LC/MS: m/z 436(100%)$(M+H)^+$, 309(51%)$(GS+3H)^+$, 308(49%)$(GS+2H)^+$, 404(12%)$(M-OCH_3)^+$.

^{1}H-NMR: δ 2.07-2.20(m,2H,Glu$\beta\beta'$), 2.45-2.57(m,2H,Glu$\gamma\gamma'$), 2.75(d,2H,-S-CH_2-C$\underline{H}_2$-CO-), 2.80-2.89(m,1H,Cysβ), 2.90-2.98(m,2H,-S-C$\underline{H}_2$-CH_2-CO-), 3.07-3.13(m,1H,Cysβ'), 3.68-3.83(m,6H,Gluα,-OCH_3,Gly$\alpha\alpha'$), 4.56(dd,1H, Cysα).

^{13}C-NMR: δ 25.1(-S-CH_2-$\underline{C}H_2$-CO-), 26.5(Gluβ), 31.7(-S-$\underline{C}H_2$-CH_2-CO-), 33.4(Cysβ), 43.6(Glyα), 53.1(-OCH_3), 53.3(Cysα), 54.4(Gluα), 170.5(Cys-CO-), 172.2(Glu-CO-), 174.6(Gly-COOH), 175.2(Glu-COOH), 177.1(-$\underline{C}OOCH_3$), 206.5(-CH_2-$\underline{C}O$-CH_2-).

The NAC conjugate of methyl 3-keto-4-pentenoate was purified by HPLC using 0.01 M NaH_2PO_4-methanol (50:50) (retention time 3.72 min).

GC/MS: (MW=305) (dimethyl ester) PCI: m/z 178(100%)(N-acetylcysteinyl moiety+2H)$^+$, 248(48%)$(M-NHCOCH_3+H)^+$, 276(17%)$(M-NHCOCH_3+C_2H_5)^+$, 288(5%) $(M-NHCOCH_3+C_3H_5)^+$.

^{1}H-NMR: (monomethyl ester) δ 2.00(s,3H,-NHCOCH$_3$), 2.75(t,2H,-S-CH$_2$-CH$_2$-CO-), 2.78-2.87(m,1H,Cysβ), 2.90(t,2H,-S-CH$_2$-CH$_2$-), 3.03-3.09(m,1H, Cysβ'), 3.73(s,3H,-OCH$_3$), 4.40(dd,1H,Cysα).

^{13}C-NMR: δ 22.8(-NHCOCH$_3$), 26.5(-S-CH$_2$-CH$_2$-CO-), 35.8(-S-CH$_2$-CH$_2$-CO-), 42.9(Cysβ), 52.8(Cysα), 55.6(-OCH$_3$), 170.2(-NHCOCH$_3$), 172.9(-COOCH$_3$), 177.2(-COOH), 204.1(-S-CH$_2$-CH$_2$-CO-).

K. Synthesis of 2,4-pentadienoic acid, its methyl ester and the thiol conjugates of the ester

Acrolein (14.7 mL, 0.22 mol) was added to a solution of malonic acid (21.8 g, 0.2 mol) in pyridine (30 mL) containing 100 mg hydroquinone. The temperature was increased to 80°C and the mixture stirred for 3 hr. The reaction was quenched by pouring the mixture into ice cold water followed by dilute acid (Worley and Young, 1972). The product was isolated by ether extraction and purified by recrystallization from pet. ether (yield 57%, 100% E-isomer).

GC/MS: (MW=98) m/z 98(100%), 97(72%), 53(71%), 42(47%), 70(35%), 81(21%).

^{1}H-NMR: δ 5.55(dd, 1H,CH$_2$=), 5.65(dd,1H,CH$_2$=), 5.95(d,1H,J=17Hz,trans,-CH=CH-), 6.45-6.55(m,1H,CH$_2$=CH-), 7.35-7.45(m,1H,-CH=CH-), 11.6-12.0 (bs,1H,-OH).

The acid was converted to its methyl ester by refluxing a mixture of acid (10 g, 0.1 mol), methanol (30 mL) and H_2SO_4 (0.3 mL) for 2 hr. The mixture was treated with water and the product extracted with ether and dried (Na_2SO_4). Pure ester was obtained by distillation at reduced pressure (37°C/10 mm, yield 42%).

GC/MS: (MW=112) m/z 53(100%), 81(92%), 112(43%), 97(32%).

^{1}H-NMR: δ 3.05(s,3H,-OCH_3), 4.85(dd,1H,CH_2=), 4.95(dd,1H,CH_2=), 5.25(d,1H,J=17Hz,trans,-CH=CH-), 5.70-5.85(m,1H,CH_2=CH-), 6.55-6.65 (m,1H,-CH=CH-).

To synthesize the GSH conjugate of the above ester, a methanolic solution of the ester (0.67 g, 6 mmol) and an aqueous solution of GSH (1.84 g, 6 mmol) (pH of mixture = 7.8) were stirred for 4 hr at which time 70% of the substrate was converted to product. Pure GSH conjugate was obtained by preparative HPLC (solvent system, 40% methanol, 60% 0.02 M NaH_2PO_4; retention time 4.49 min).

LC/MS: m/z 420(100%)$(M+H)^+$, 147(25%)(ammoniated glutamyl residue), 129(20%)(glutamyl moiety).

^{1}H-NMR: δ 2.10-2.25(m,2H,Glu$\beta\beta'$), 2.50-2.65(m,2H,Glu$\gamma\gamma'$), 2.70-2.80(m,1H,Cysβ), 2.95-3.05(m,1H,Cysβ'), 3.13(d,2H,-CH_2-$COOCH_3$), 3.17 (d,2H,-S-CH_2-CH=CH-), 3.70(s,3H,-OCH_3), 3.73(t,1H,Gluα), 3.78 (s,2H, Glyα), 4.55(dd,1H,Cysα), 5.52-5.62(m,1H,J=16Hz,-CH=CH-), 5.65-5.75 (m,1H,J=16Hz,-CH=CH-).

^{13}C-NMR: δ 27.7(Gluβ), 32.8(Cysβ), 34.1(Gluγ), 38.0(-CH_2-$COOCH_3$), 44.2 (Glyα), 52.9(Cysα), 54.0(-OCH_3), 55.3(Gluα), 126.3(-CH=CH-), 131.1(-CH=CH-), 172.6(Cys-CO-), 174.4(Glu-CO-), 175.1(Gly-COOH), 175.4(Glu-COOH), 176.1(-$COOCH_3$).

The dimethyl ester of the NAC conjugate of (E)-2,4-pentadienoic acid was prepared by refluxing methyl NAC with the substrate in a manner similar to that of the formation of the NAC conjugate of (E)-2,4-diene VPA. Pure conjugate was obtained by preparative HPLC using the solvent system of 0.01 M NaH_2PO_4 and methanol (50:50) (retention time 10.55 min).

GC/MS: (MW=289) PCI: m/z 290(100%)$(M+H)^+$, 318(24%)$(M+C_2H_5)^+$, 330(8%)$(M+C_3H_5)^+$.

^{1}H-NMR: δ 2.0(s,3H,-NHCOCH$_3$), 2.68-2.78(m,1H,Cysβ), 2.90-3.00(m,1H,Cysβ'), 3.12(d,2H,-CH$_2$-COOCH$_3$), 3.17(d,2H,-S-CH$_2$-CH=CH-), 3.68(s,3H,-OCH$_3$), 3.75(s,3H,-CH-COOCH$_3$), 4.61(dd,1H,Cysα), 5.50-5.60 (m,1H,J=15.9 Hz,-CH=CH-), 5.65-5.75(m,1H,J=15.90Hz,-CH=CH-).

^{13}C-NMR: δ 22.3(-NHCOCH$_3$), 32.6(Cysβ), 34.3(-CH$_2$-COOCH$_3$), 52.5(-CH-COOCH$_3$), 52.9(Cysα), 53.3(-CH$_2$-COOCH$_3$), 126.6(-CH=CH-), 130.9(-CH=CH-), 172.7(-NHCOCH$_3$), 173.2(-CH-COOCH$_3$), 173.7(-CH$_2$-COOCH$_3$).

L. Attempted synthesis of the CoA ester of (E)-2,4-diene VPA

The anhydride of (E)-2,4-diene VPA was prepared by adding triethylamine (14 μL, 100 μmol) to a solution of (E)-2,4-diene VPA (14 mg, 100 μmol) in THF (Goldman and Vagelos, 1961). After stirring the mixture for 15 min ethyl chloroformate (10 μL, 100 μmol) in THF was added and the reaction allowed to proceed for 30 min at 0°C. The precipitate formed was filtered off and the product analyzed by GC/MS.

GC/MS: (MW=212) m/z 123(100%), 95(45%), 67(41%), 167(2%), 139(1%), 140(1%).

CoASH (70 mg, 90 μmol) was dissolved in 2 mL of THF-H_2O mixture (7:3) and the pH adjusted to 8 with 1 M NaOH solution. The anhydride was dissolved in 2 mL THF and added dropwise to the CoASH solution under N_2, while maintaining the pH of the reaction mixture at 8 by the addition of NaOH solution. After 30 min the pH of the mixture was adjusted to 5 with 1% $HClO_4$. Following lyophilization of the mixture, the product was analyzed by HPLC and LC/MS.

M. Synthesis of 4′-keto-2-ene VPA

This new VPA metabolite was prepared as the ethyl ester by oxidizing the terminal double bond of synthesized ethyl 2-(2′-propenyl)-2-pentenoate to the corresponding keto function. n-Butyllithium (28 mL, 0.045 mol) was added dropwise to diisopropylamine (6.2 mL, 0.045 mol) in THF (80 mL) at 0°C. The mixture was stirred for 15 min and cooled to -78°C, and ethyl 4-pentenoate (5.12 g, 0.04 mol) in THF was added dropwise over a period of 20 min. After stirring the reaction mixture for another 20 min, propionaldehyde (2.9 mL, 0.04 mol) was added and the reaction allowed to continue for 45 min before being quenched with 15% HCl solution. The product, ethyl 2-(1′-hydroxypropyl)-4-pentenoate, was isolated by extraction with ether and purified by fractional distillation (bp 85°C/1.5 mm).

The dehydration of the hydroxy ester was carried out using methanesulfonyl chloride and potassium hydride. Hydroxy ester (4.10 g, 0.022 mol), triethylamine (3.3 mL, 0.024 mol) and dichloromethane (60 mL) were cooled to 0°C and methanesulfonyl chloride (1.9 mL, 0.024 mol) was added dropwise and the mixture stirred for 60 min. The precipitate formed was filtered off and the solvent removed. The mesylate was then taken up in THF and potassium hydride (5.03 g, 0.044 mol) was added at 0°C and the reaction mixture stirred for 4 hr at room temperature. Following neutralization of the excess potassium hydride, the mixture was extracted with ether and pure ethyl 2-(2′-propenyl)-2-pentenoate was obtained by distillation (bp 44°C/0.5 mm, yield 52%).

The final product was obtained by palladium-catalyzed oxidation of the terminal double bond of ethyl 2-(2′-propenyl)-2-pentenoate (Rettenmeier *et al.*, 1986a; Tsuji, 1984). Palladium(II) chloride (21 mg,

0.12 mmol) and p-benzoquinone (0.71 g, 6.6 mmol) were added to a 7:1 mixture of dimethylformamide/water (16 mL) and then the diene (1.0 g, 6 mmol) was added over a period of 5 min. The reaction was allowed to proceed for 24 hr, at which time the reaction mixture was poured into cold 10% HCl (15 mL) and extracted with ether. The ether extract was washed with 10% NaOH and dried over Na_2SO_4. Following the removal of the ether, the residue was distilled (bp 94°C/1 mm) to yield a mixture of E- and Z-isomers of ethyl 4'-keto-2-ene VPA (the E:Z ratio as determined by GC/MS was 95:5; yield 41%). The major isomer was assigned the E-configuration on the basis of stereoselectivity of the synthetic procedure (Lee *et al.*, 1989).

GC/MS: (MW=184) m/z 43(100%), 138(61%), 67(59%), 69(53%), 113(42%), 95(33%), 143(32%), 139(24%), 169(2%), 184(2%).

^{1}H-NMR: δ 1.07(t,3H,CH_3-CH_2-CH=), 1.29(t,3H,CH_3-CH_2O-), 2.11-2.25(m,2H, -CH_2-CH=), 2.22(s,3H,CH_3-CO-), 3.44(s,2H,-CO-CH_2-), 4.29(q,2H,-OCH_2-), 6.99(t,1H,J=6Hz,-CH=).

N. Synthesis of 4'-keto-3-ene VPA

This compound was synthesized in a manner similar to that described for 4'-keto-2-ene VPA by oxidizing the corresponding diene. To a solution of diisopropylamine (8.0 mL, 0.055 mol) at 0°C, n-butyllithium (34.0 mL, 0.055 mol) and HMPA (9.5 mL, 0.055 mol) were added successively and the mixture stirred for 20 min. The mixture was then cooled to -78°C and (E)-ethyl 2-pentenoate (6.4 g, 0.05 mol) added dropwise over a period of 20 min and stirring continued for 30 min. This was followed by the addition of allyl bromide (4.5 mL, 0.05 mol) and further stirring for 30 min. Finally the reaction mixture was quenched

with 15% HCl solution and extracted with ether. Pure ethyl 2(2'-propenyl-3-pentenoate was obtained by distillation at reduced pressure (bp 60°C/4 mm; yield 56%). The ratio of the Z:E isomers was determined by GC/MS to be 97:3, the major isomer assigned the Z-configuration on the basis of the known stereoselectivity of the synthetic route (Kende and Toder, 1982). A portion of the diene was oxidized to the keto function in the same way as that described for 4'-keto-2-ene VPA, to give 4'-keto-3-ene VPA (bp 98°C/2 mm; yield 38%).

GC/MS: (MW=184) m/z 43(100%), 138(48%), 67(46%), 95(22%), 139(18%), 155(1%), 169(1%), 184(1%).

^{1}H-NMR: δ 1.23(d,3H,CH_3-CH=), 1.26-1.28(t,3H,CH_3-CH_2O-), 1.72(d,2H,-CO-CH_2-), 2.17(s,3H,CH_3-CO-), 3.00-3.07(m,1H,-CH=CH-CH-CH_2-), 4.15(q,2H,-OCH_2-), 5.25-5.33(t,1H,$J_{3,4}$=10Hz,CH_3-CH=CH-,cis), 5.60-5.80(m,1H,CH_3-CH=).

O. Synthesis of 4-ene VPA

n-Butyllithium (94 mL, 0.15 mol), was added to a THF solution of diisopropylamine (21 mL, 0.15 mol) at 0°C and the mixture stirred for 20 min. The mixture was cooled to -78°C and HMPA (26 mL, 0.15 mol) and ethyl pentanoate (18.2 g, 0.14 mol) were added successively and stirring continued for 45 min. Allyl bromide (12 mL, 0.14 mol) was added and the reaction mixture stirred for 1.5 hr. Following the usual work-up and fractional distillation (bp 55°C/3 mm) 4-ene VPA ethyl ester was obtained in 61% yield. A portion of the ester was converted to the free acid by refluxing the ester in two equivalents of aqueous NaOH. Distillation afforded pure 4-ene VPA (107°C/4.5 mm).

GC/MS: (MW=142) m/z 57(100%), 56(83%), 85(64%), 41(47%), 100(7%), 142(6%), 113(3%).

^{1}H-NMR: δ 0.92(t,3H,CH_3-), 1.28-1.70(m,4H,-CH_2-CH_2-), 2.21-2.51(m,3H,-CH-CH_2-CH=), 5.01-5.13(m,2H,CH_2=), 5.72-5.85(m,1H,-CH=).

P. Synthesis of 5-OH VPA

This metabolite was synthesized by hydroboration of 4-ene VPA (Rettenmeier *et al.*, 1985). To a stirred solution of ethyl 4-ene VPA (2.5 g, 0.015 mol)in THF (20 mL) at 0°C, borane (1 mL) was added dropwise and the mixture stirred for 30 min at room temperature. The mixture was cooled and excess hydride neutralized with water. Then 3 M NaOH (7 mL, 0.02 mol) and 30% H_2O_2 (2.4 mL) were added dropwise at a rate that allowed for the temperature of the reaction mixture to remain below 35°C. The mixture was then refluxed for 60 min. The product was isolated by ether extraction and purified by distillation (bp 88°C/0.1 mm; yield 32%).

GC/MS: (MW=188) m/z 101(100%), 55(90%), 143(43%), 128(41%), 113(40%), 159(8%), 188(2%).

Q. Synthesis of the dimethyl ester of the NAC conjugate of acrylic acid

This adduct of acrylate was synthesized for use as an internal standard, following the same procedure for the synthesis of other NAC adducts described in preceding sections.

GC/MS: (MW=263) m/z 43(100%), 172(51%), 204(47%), 55(46%), 88(43%), 190(12%).

5. Animal experiments

A. *In vivo* metabolism studies

A group of 3 rats was used for each treatment. For urine collections the animals were housed in stainless steel metabolic cages and were allowed free access to food and water. For the bile duct cannulation, each rat was anesthesized with urethane (1.1 g/kg) which was administered i.p. as an aqueous solution (0.3 g/mL). A midline incision was made and the portion of the bile duct at the junction with the duodenum located. A puncture was made into the duct and a cannula of PE-10 tubing inserted through the opening directed toward the liver. The cannula was ligated at the duodenal junction with silk thread and exteriorized through the lower abdominal wall with the help of a bore of an externally inserted 19G1 needle. After the surgical procedure, the incision was sutured and the animal placed in a metabolism cage. Control bile and urine were collected for 1 hr and following the administration of test compounds, bile and urine were collected for 8 and 24 hr, respectively. Samples were kept frozen at -20°C until analyzed.

Each group of animals was given a 100 mg/kg i.p. dose of the sodium salts of the following compounds: VPA, 4-ene VPA, (E)-2,4-diene VPA (isomeric purity 94%), 4-pentenoic acid and (E)-2,4-pentadienoic acid (isomeric purity 100%). To prepare solutions of these compounds for administration to rats, 200 mg of each compound was weighed out and an equivalent amount of NaOH (3 M solution) added. When necessary, the pH of the solution was adjusted to 7.4 using 3 M HCl solution and the volume made up to 2 mL with water.

To study the metabolic disposition of the GSH conjugates of (E)-2,4-diene- and 3-keto-4-ene-VPA, the conjugates (methyl ester form) were

dissolved in normal saline and a dose of 40 mg/kg injected into a tail vein of each rat.

B. Preparation of bile and urine for mass spectral analysis

To identify GSH conjugates in bile, 40 mL of pooled bile (from each treatment group and control animals) was diluted with 40 mL of distilled water and passed through a 2.5 x 20 cm Amberlite XAD-2 column. The bile was allowed to percolate slowly through the column packing. The column was then washed with 100 mL distilled water and eluted with 150 mL methanol. The eluate was evaporated to dryness and a portion of the residue dissolved in methanol. The solution was treated with methanolic hydrogen chloride gas, dried under the flow of N_2, dissolved in methanol and treated with diazomethane generated from Diazald^R. The sample was then analyzed by HPLC, LC/MS and/or LC/MS/MS. An authentic sample of each conjugate (2-4 mg) was also methylated in the same way as the bile sample.

For EI/GC/MS analysis, a 1 mL urine sample was taken from treated or control animals and acidified to pH 1.5-2.0 with 3 M HCl solution. The sample was saturated with 1 g NH_4Cl and extracted with ethyl acetate (3 mL) by gentle rotation of the phases for 30 min. The layers were separated by centrifugation, the organic portion dried over anhydrous Na_2SO_4 and the volume reduced to 100 μL under N_2. The concentrated extract was methylated with diazomethane.

C. *In vitro* metabolism studies

Animals were sacrificed by decapitation and the livers immediately removed. The liver was sheared with scissors and rinsed in 0.9% NaCl

solution and homogenized in Tris-HCl (0.05 M, pH 7.5 containing 1.15% KCl) buffer with a Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 600 g for 20 min and the pellet discarded. The supernatant was centrifuged at 7,500 g for 10 min, the pellet recovered and recentrifuged at 7,500 g for 10 min and the pellet (mitochondrial fraction) suspended in sucrose, homogenized and stored at -80°C until used. The supernatant from the 7,500 g spin was centifuged at 17,000 g for 10 min and the pellet discarded. The supernatant was further centrifuged at 100,000 g for 65 min and the resulting supernatant was saved as a source of cytosolic enzymes. The pellet was suspended in EDTA/KCl and centrifuged at 100,000 g for 70 min. The resulting pellet was resuspended in sucrose, homogenized, and saved as the microsomal fraction. Protein concentrations in all cellular fractions were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Biological synthesis of the CoA thio ester of (E)-2,4-diene VPA: Microsomal incubations of the substrate were carried out at 37°C for 0, 30, and 60 min in a total volume of 2 mL (Yamaoka *et al.*, 1988). (E)-2,4-diene VPA (100 μM) was incubated with the microsomal fraction (3 mg protein) suspended in 2.0 mL of 60 mM potassium phosphate buffer (pH=7.4) containing 1 mM CoASH, 5.0 mM ATP, and 5.0 mM $MgCl_2$. After the indicated incubation times the reaction was stopped by adding 25 μL acetic acid and 6 mL methanol. The mixture was centrifuged at 1000 g, the supernatant evaporated to dryness and the residue analyzed by LC/MS.

6. Human study

Patients studied were regular visitors to the Seizure Clinic of British Columbia's Children's Hospital and included 26 receiving VPA only and 33 receiving VPA in combination with carbamazepine (CBZ) or clobazam (CLBZ). The ages of the patients ranged from 1.0 to 19.8 years. The mean VPA daily doses were 18.6 mg/kg for the monotherapy group and 32.9 mg/kg for the polytherapy group. VPA metabolite concentrations were determined in serum (trough) and overnight urine samples. One healthy male volunteer weighing 70 kg participated in the study in order to facilitate metabolite identification by the twin ion technique. The volunteer was given a 50:50 mixture of VPA and [$^2H_6$]VPA until steady state was reached i.e. 350 mg doses of each drug given twice daily for three days.

7. Quantitation of metabolites

A. Quantitation of non-thiol metabolites

Serum and urine standards: A reference standard was prepared by spiking VPA and the 14 metabolites mentioned below in either control serum or urine. Six standards including a blank sample were prepared for each run by taking aliquots of standard serum or urine and making up to a final volume of 250 μL with control serum or urine. The concentrations for the serum standards making up the calibration curves were as follows: 24.8, 49.6, 74.4, 99.2 and 124 μg/mL for VPA; 0.030, 0.300, 0.600, 0.900 and 1.20 μg/mL for 4-ene VPA; 0.394, 0.788, 1.18, 1.58 and 1.97 μg/mL for 3-ene VPA; 0.016, 0.162, 0.324, 0.486 and 0.648 μg/mL for (Z)-2-ene VPA; 3.07, 6.16, 9.23, 12.3 and 15.4 μg/mL for (E)-2-ene VPA; 0.016, 0.160, 0.320, 0.480 and 0.640 μg/mL for (E,Z)-2,3′-diene VPA;

3.10, 6.20, 9.30, 12.4 and 15.5 μg/mL for (E,E)-2,3′-diene VPA; 0.204, 0.408, 0.612, 0.816 and 1.02 μg/mL for (E)-2,4-diene VPA; 0.380, 0.760, 1.14, 1.52 and 1.90 μg/mL for 3-OH VPA; 0.320, 0.640, 0.960, 1.28 and 1.60 μg/mL for 5-OH VPA; 0.208, 0.416, 0.624, 0.832 and 1.04 μg/mL for 4-keto VPA; 2.0, 4.0, 6.0, 8.0 and 10.0 μg/mL for 3-keto VPA; 0.200, 0.400, 0.600, 0.800 and 1.0 μg/mL for 2-PGA; and 0.020, 0.198, 0.396, 0.594 and 0.792 μg/mL for 2-PSA.

Stock solutions of the internal standards were prepared in distilled water at a concentration of 50 μg/mL for each of [$^2H_3$]2-ene VPA, [$^2H_3$]3-keto VPA and [$^2H_6$]VPA and 10 μg/mL for 2-MGA. Equal volumes of each internal standard from the above solutions were mixed and a 100 μL aliquot was added to each sample.

The calibration curves were obtained by plotting the peak area ratio of VPA or metabolite to that of the internal standard versus the concentration of the compound by a computer program written in our laboratory and which also provided a reading of sample concentration. [$^2H_6$]VPA and [$^2H_3$]2-ene VPA served as the internal standards for VPA and the unsaturated metabolites, respectively. [$^2H_3$]3-keto VPA was used to quantitate 3-keto VPA while 2-MGA served as the internal standard for the dicarboxylic acid metabolites. [$^2H_3$]2-ene VPA also served as the internal standard for the hydroxy metabolites and 4-keto VPA.

Sample preparation: Depending on the amount of available serum, 50-250 μL of serum sample were taken, internal standards added and the pH was adjusted to 2 with 3 M HCl solution. The sample was allowed to sit at room temperature for 10 min and extracted with 500 μL of ethyl acetate by mechanical rotation of the phases for 20 min. To increase the recovery of the analytes the extraction step was repeated with another

500 μL of ethyl acetate. After each extraction step the sample was centrifuged at 450 g for 10 min in order to break the emulsion formed. The combined organic layer was then transferred to a 3.5 mL screw cap vial containing anhydrous Na_2SO_4, vortex mixed, and centrifuged at 450 g for 20 min. The supernatant was then transferred to another vial and the volume reduced to approximately 200 μL under N_2.

To determine total urinary levels of VPA and metabolites, urine samples (50-250 μL) were treated with 3 M NaOH (pH 12-13) and incubated at 60°C for 60 min before their pH was adjusted to 2 with 3 M HCl solution and extracted with ethyl acetate. For free urine concentration measurements, urine samples were treated as for serum except for a single extraction step. The concentration of metabolites as conjugates was detemined from the difference between total and free urine concentrations.

To form the pentafluorobenzyl (PFB) derivative of the carboxyl groups the extract was transferred to a 1 mL conical reaction vial and 10 μL of diisopropylethylamine and 10 μL of 30% PFBB solution in ethyl acetate were added and the sample heated in a heating block at 50°C for 60 min. The PFB esters were then treated with 50 μL of MSTFA and heated at 60°C for 30 min to silylate metabolites containing the hydroxyl and 3-oxo moiety. A 1 μL volume of the derivatized sample was injected into the GCMS system in the splitless mode using an automatic sample injector (Hewlett-Packard Model 7673A) under NICI conditions.

B. Quantitation of the NAC conjugate of (E)-2,4-diene VPA

Calibration curve: The dimethyl ester of the NAC conjugate of (E)-2,4-diene VPA was hydrolyzed by stirring the conjugate in two equivalents of NaOH at room temperature for 10 hr. An aliquot of the de-esterified conjugate was taken and made up to volume with water to obtain a stock solution of 5 mg/mL. 50 and 200 and μL samples of the stock solution were then taken and the volume adjusted to 100 mL with control urine. An appropriate volume was taken from either of these standard solutions to yield standard concentrations of 0.05, 0.10, 0.50, 2.0, 4.0, and 6.0 μg/mL in a final volume of 1 mL.

An internal standard, the NAC conjugate of acrylic acid, was prepared by hydrolyzing the methyl NAC derivative of methyl acrylate in aqueous NaOH in the same way as that of the dimethyl ester of (E)-2,4-diene VPA. An aliquot of the hydrolyzed sample was utilized to prepare a stock solution of 2.5 mg/mL, from which an amount was taken to give an internal standard concentration of 5 μg/mL in water.

Sample preparation: A 1 mL urine sample was taken from each of 28 pediatric patients on VPA therapy and 200 μL of the internal standard solution added. The samples were acidified, saturated with NH_4Cl and extracted with ethyl acetate in a similar way as that described in an earlier section. To determine the amount of (E)-2,4-diene VPA dose recovered as the NAC conjugate, urine was obtained from three rats for 24 hr, the volume recorded, and a 200 μL sample assayed for the NAC conjugate. To determine the extent of the reversal of the conjugation of (E)-2,4-diene VPA under alkali, samples were treated with 3 M NaOH to pH=12 and heated at 60°C for 60 min before the usual sample work-up.

For derivative formation the concentrated extract was treated with 20 μL of 20% PFBB in ethyl acetate and 10 μL of diisopropylethylamine and heated at 50°C for 60 min. A 1 μL volume of the derivatized sample was injected into the GC/MS in NICI mode and selected ion monitoring (SIM) of the ions m/z 414 and 482.

III. RESULTS

1. Chemical syntheses

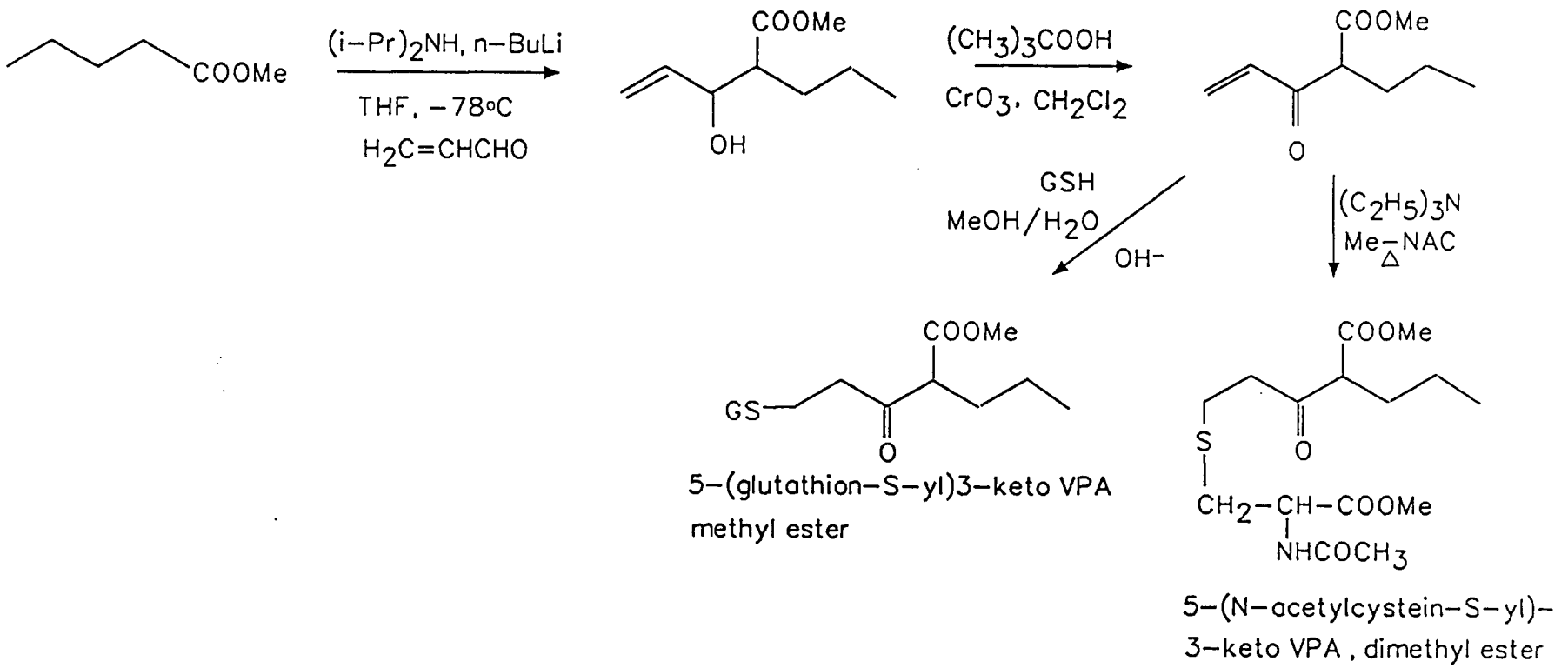
A. 3-Keto-4-ene VPA

This putative metabolite of VPA was synthesized as the methyl ester by oxidizing methyl 3-OH-4-ene VPA according to Scheme 1. The product gave satisfactory GC/MS (Fig. 5) and ^{1}H-NMR (App. 1) data. Methyl 3-OH-4-ene VPA was first synthesized by alkylating the α-carbanion of methyl pentanoate, generated with lithium diisopropylamide (LDA) by acrolein, according to the general procedure for the alkylation of ester enolates (Cregge *et al.*, 1973).

An attempt to synthesize the title compound by alkylating methyl pentanoate with acryloyl chloride in the presence of LDA resulted in a product showing addition of $(i\text{-}Pr)_2NH$ across the double bond of 3-keto-4-ene VPA. Similarly, an effort to synthesize the free acid form of 3-keto-4-ene VPA by oxidizing the free acid of 3-OH-4-ene VPA was not successful; the product obtained apparently being the decarboxylated 3-keto-4-ene VPA. A method previously reported for the synthesis of the ethyl ester of 3-keto-4-ene VPA (Rettenmeier *et al.*, 1985) did not prove useful for preparing the free acid form of this β-keto acid.

B. GSH and NAC conjugates of methyl 3-keto-4-ene VPA

The GSH adduct was synthesized by mixing a methanolic solution of the substrate with an aqueous solution of GSH and adjusting the pH of the mixture to 7.8 (Scheme 1). The reaction was instantaneous and the conversion quantitative; no substrate was detected 30 min after commencement of the reaction. Following purification by preparative



Scheme 1. Synthetic route for 3-keto-4-ene VPA methyl ester and its GSH and NAC conjugates.

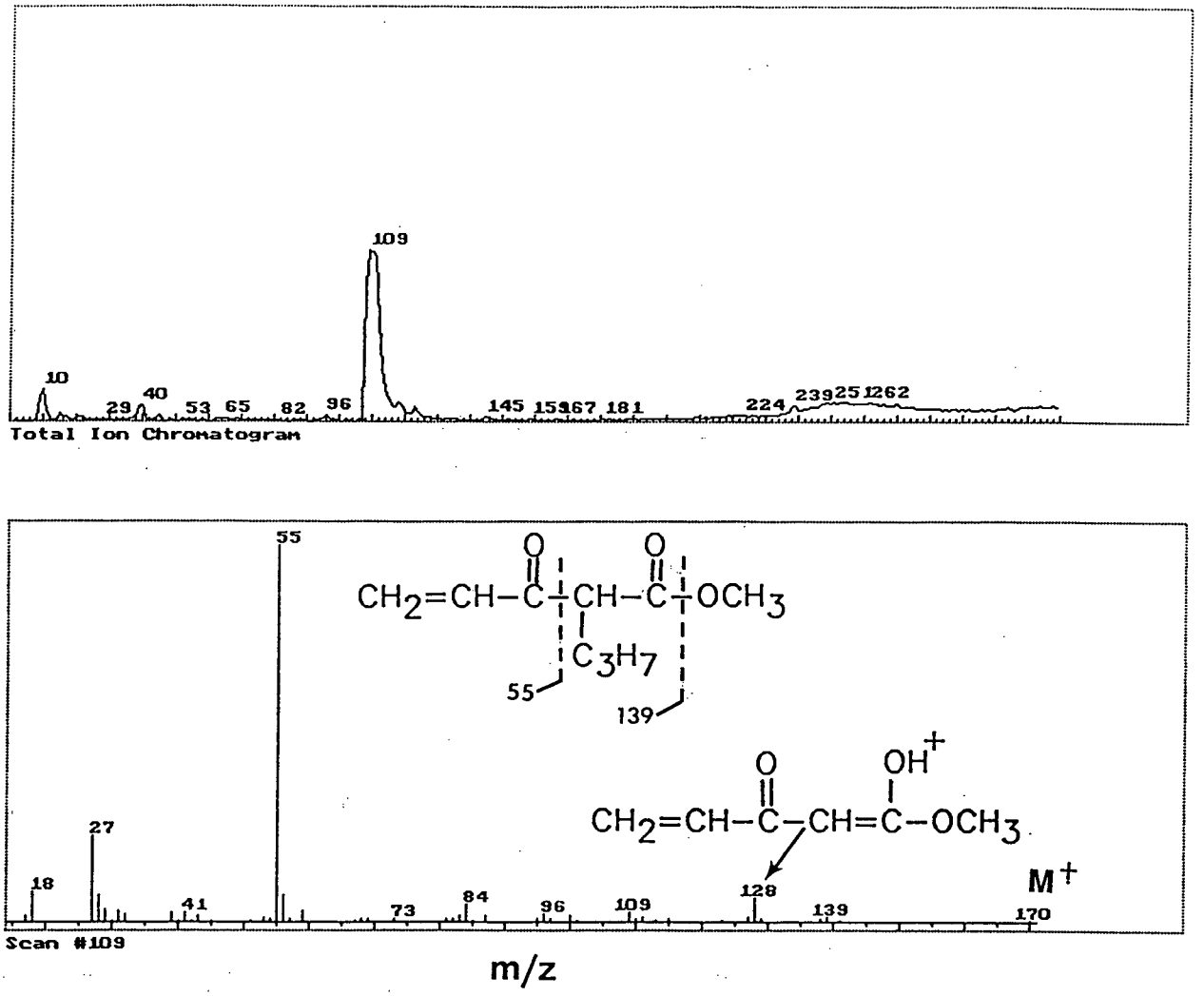


Figure 5. EI/GC/MS TIC plot and spectrum of synthesized methyl 3-keto-4-ene VPA.

HPLC, the structure of the adduct was determined to be 5-(glutathion-S-yl)-3-keto VPA by NMR and FAB/MS. The ^{1}H-, ^{13}C-NMR and FAB/MS spectra are shown in Figs. 6, 7 and 8, respectively. The signals for the CH_2=CH- protons in the ^{1}H-NMR spectrum of the substrate (App. 1) were replaced by signals for CH_2-CH_2- in the spectrum of the GSH conjugate (Fig. 6). Because the methine proton (-COC<u>H</u>($COOCH_3$)C_3H_7) exchanged with deuterium of the NMR solvent (methanol-d_4 and D_2O), there was no signal for this proton in the ^{1}H-NMR spectrum and there was only a weak signal for the -CH-(or -CD-) carbon in the ^{13}C-NMR spectrum (Fig. 7).

Refluxing the methyl ester of NAC with the methyl ester of 3-keto-4-ene VPA in the presence of two equivalents of $(C_2H_5)_3N$ afforded the NAC conjugate in a good yield. The NMR spectra (App. 2 and 3) were consistent with the structure 5-(N-acetylcystein-S-yl)-3-keto VPA. The NAC conjugate decomposed thermally in the GC/MS injector as determined by GC/MS and LC analysis of the same sample (Fig. 9). The decomposition of the conjugate was a reversal of the Michael addition (retro-Michael reaction) resulting in the substrate and methyl NAC. A similar decomposition has been reported for other mercapturates upon GC analysis (Onkenhout *et al.*, 1982). As the EI mass spectrum (Fig. 10A) did not furnish molecular weight information, for purposes of positive MS identification the conjugate was analyzed by PCI (Fig. 10B).

C. <u>GSH and NAC conjugates of (E)-2,4-diene VPA</u>

The synthetic pathway to these compounds is depicted in Scheme 2. The (E)-2,4-diene VPA was synthesized in a manner similar to a published procedure (Lee *et al.*, 1989). However, another reagent (DBU) was also tried for the elimination of the mesylate. DBU elimination of the

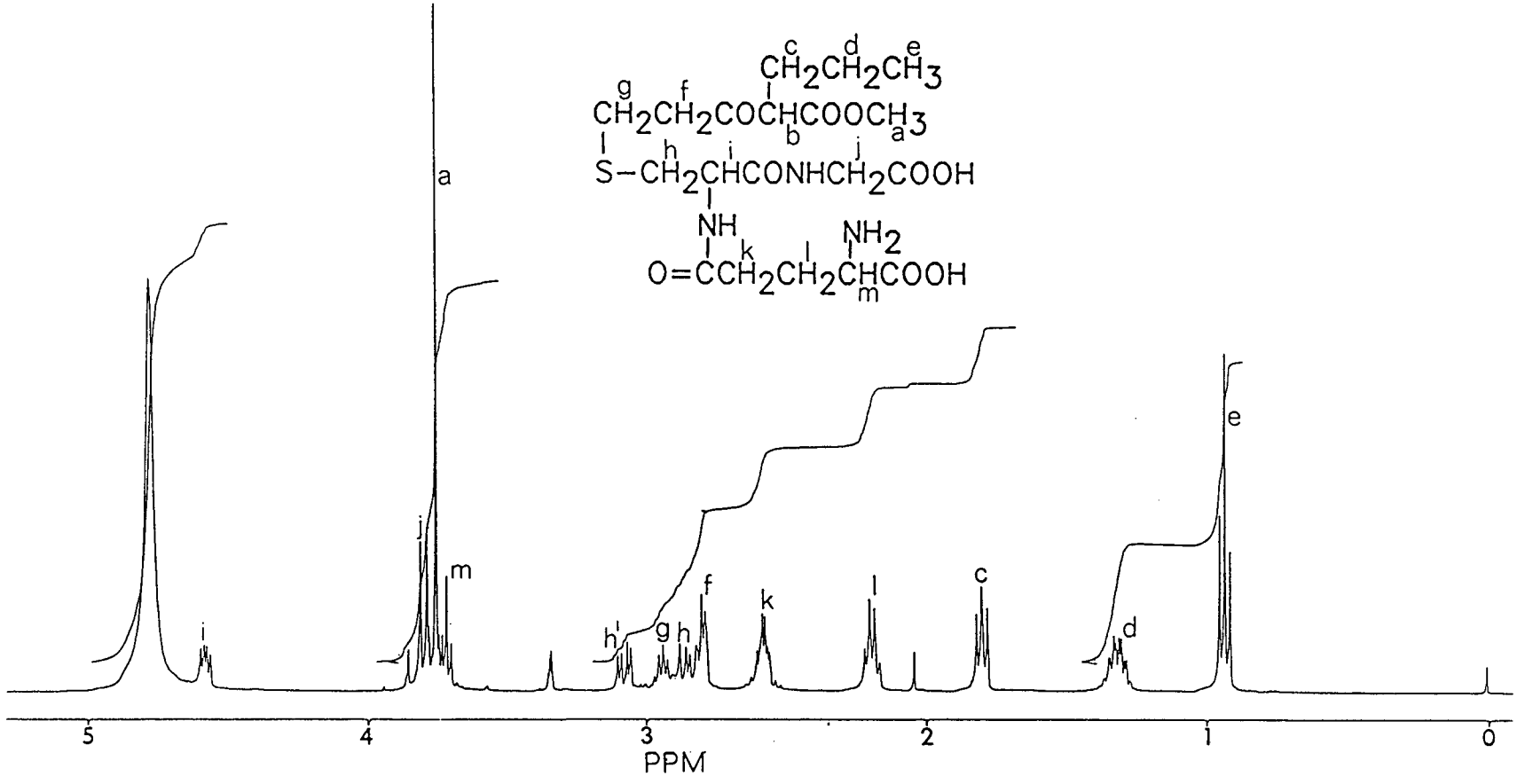


Figure 6. ^{1}H-NMR spectrum of synthetic GSH conjugate of methyl 3-keto-4-ene VPA.

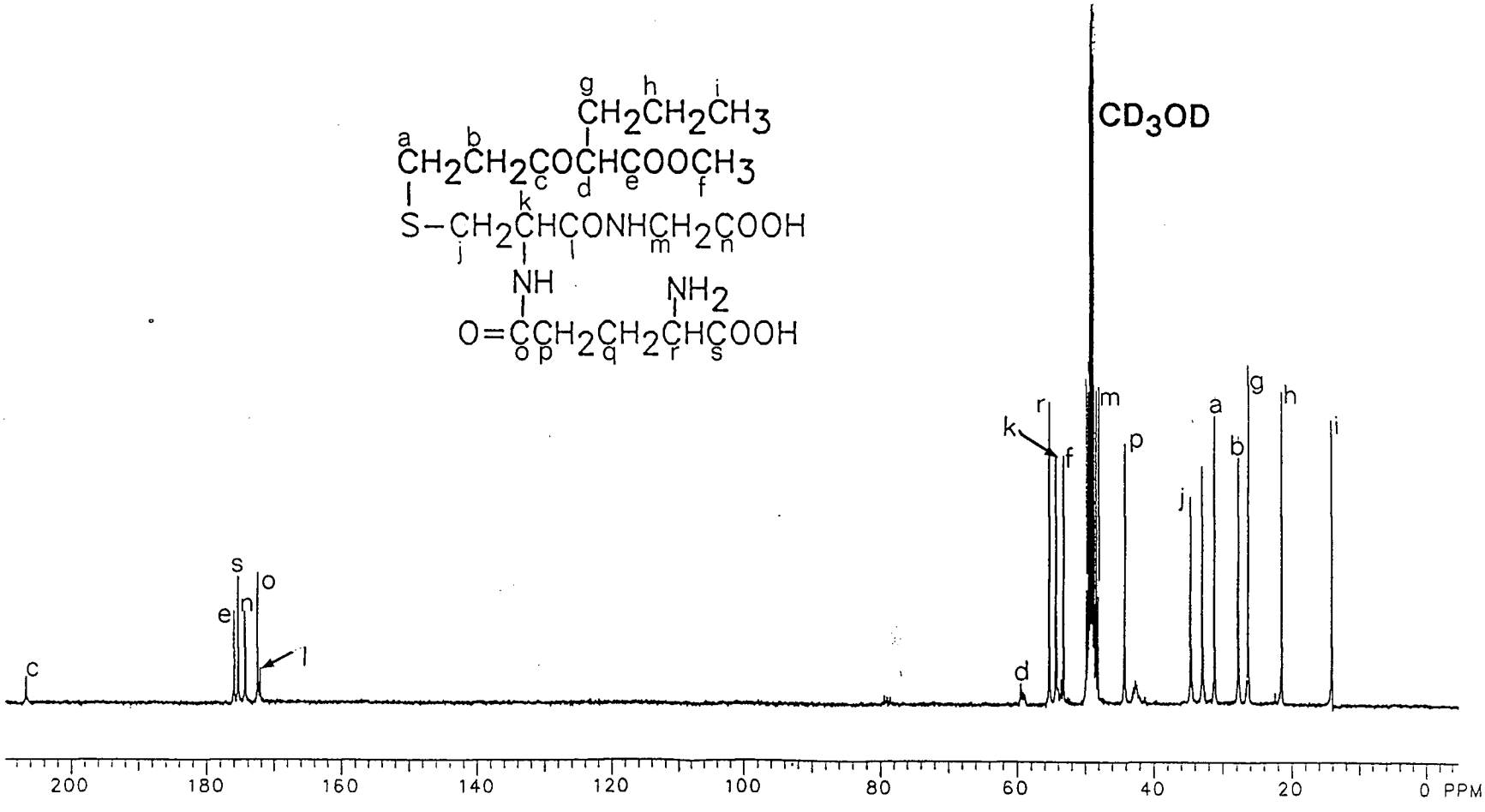


Figure 7. ^{13}C-NMR spectrum of synthetic GSH conjugate of methyl 3-keto-4-ene VPA.

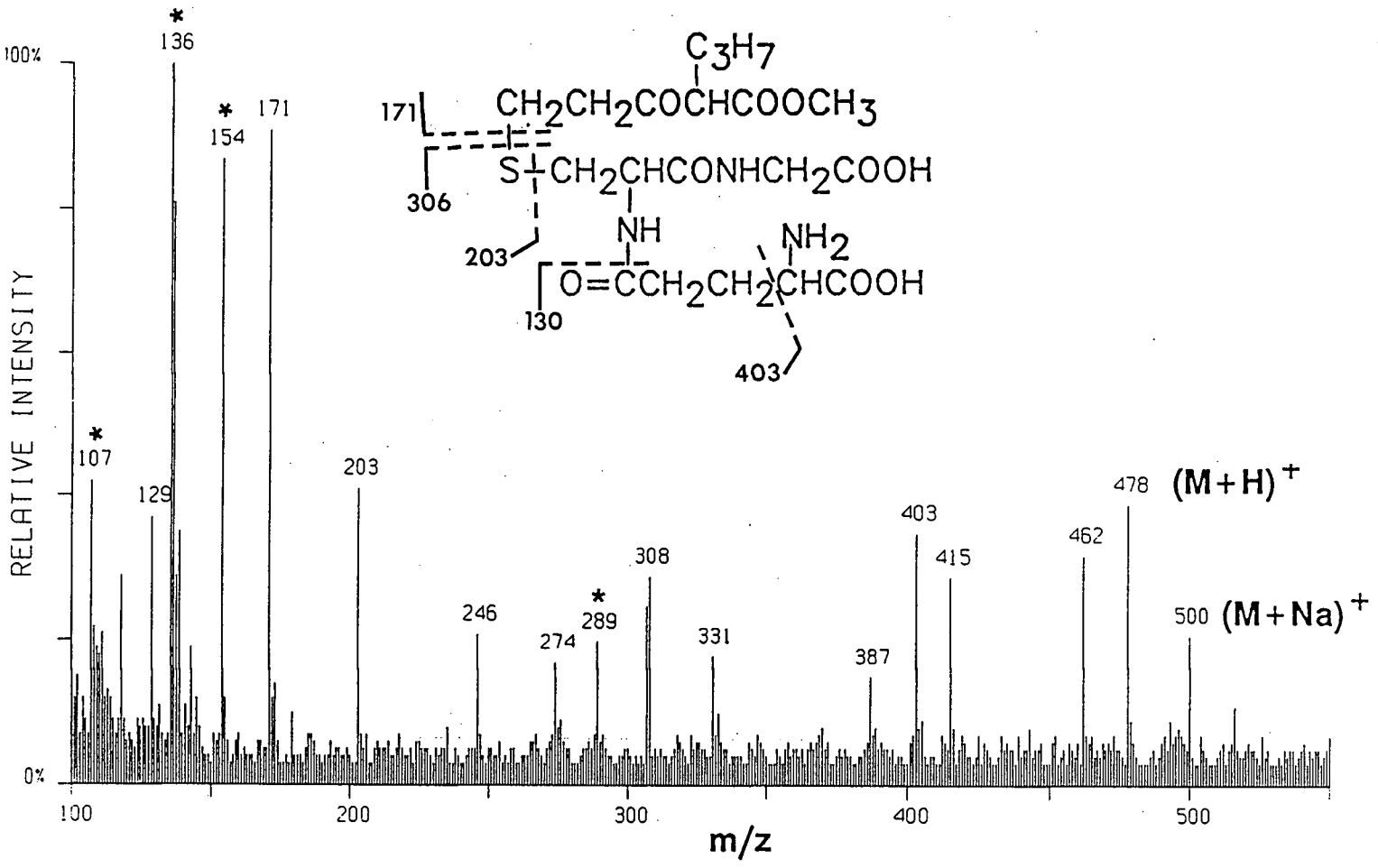


Figure 8. Reconstructed FAB mass spectrum of the GSH conjugate of methyl 3-keto-4-ene VPA in 3-nitrobenzyl alcohol matrix.

* matrix ions

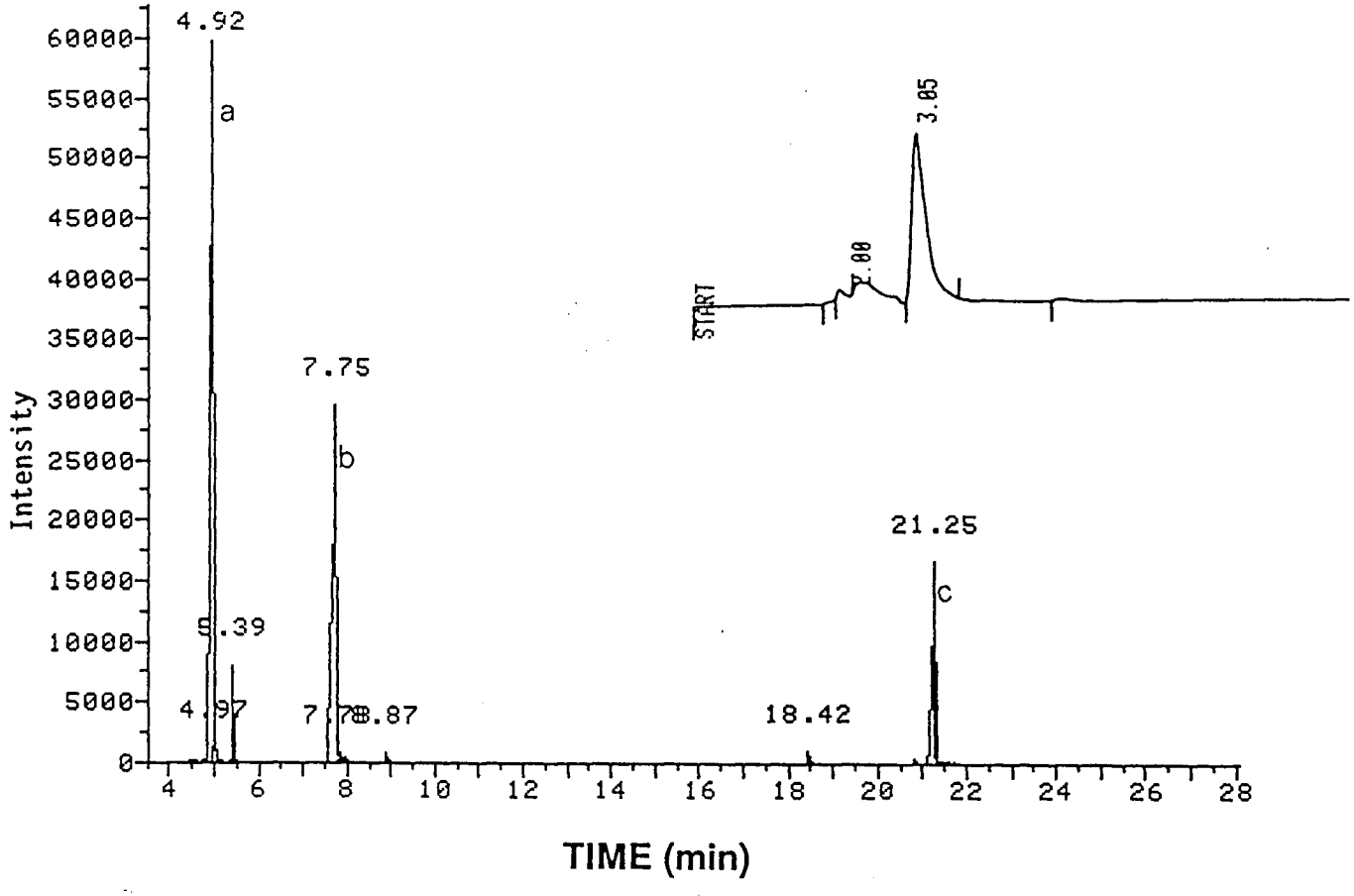


Figure 9. EI/TIC chromatogram of an HPLC purified synthetic sample of the dimethyl ester of the NAC conjugate of 3-keto-4-ene VPA showing the decomposition of the compound upon GC/MS analysis. Peaks: a = methyl 3-keto-4-ene VPA, b = methyl NAC and c = methyl NAC conjugate of methyl 3-keto-4-ene VPA. The inset is the HPLC chromatogram obtained from the same sample.

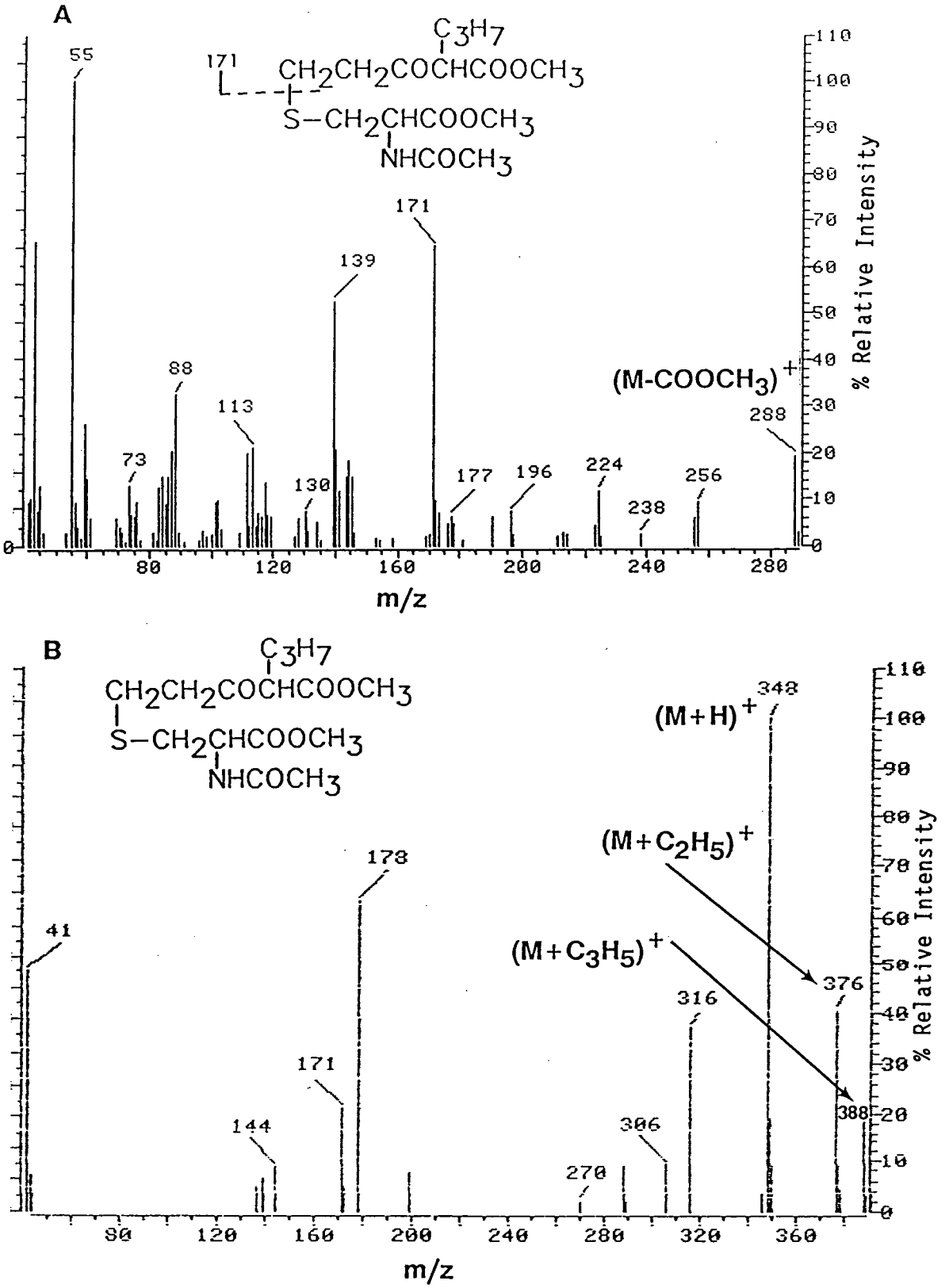
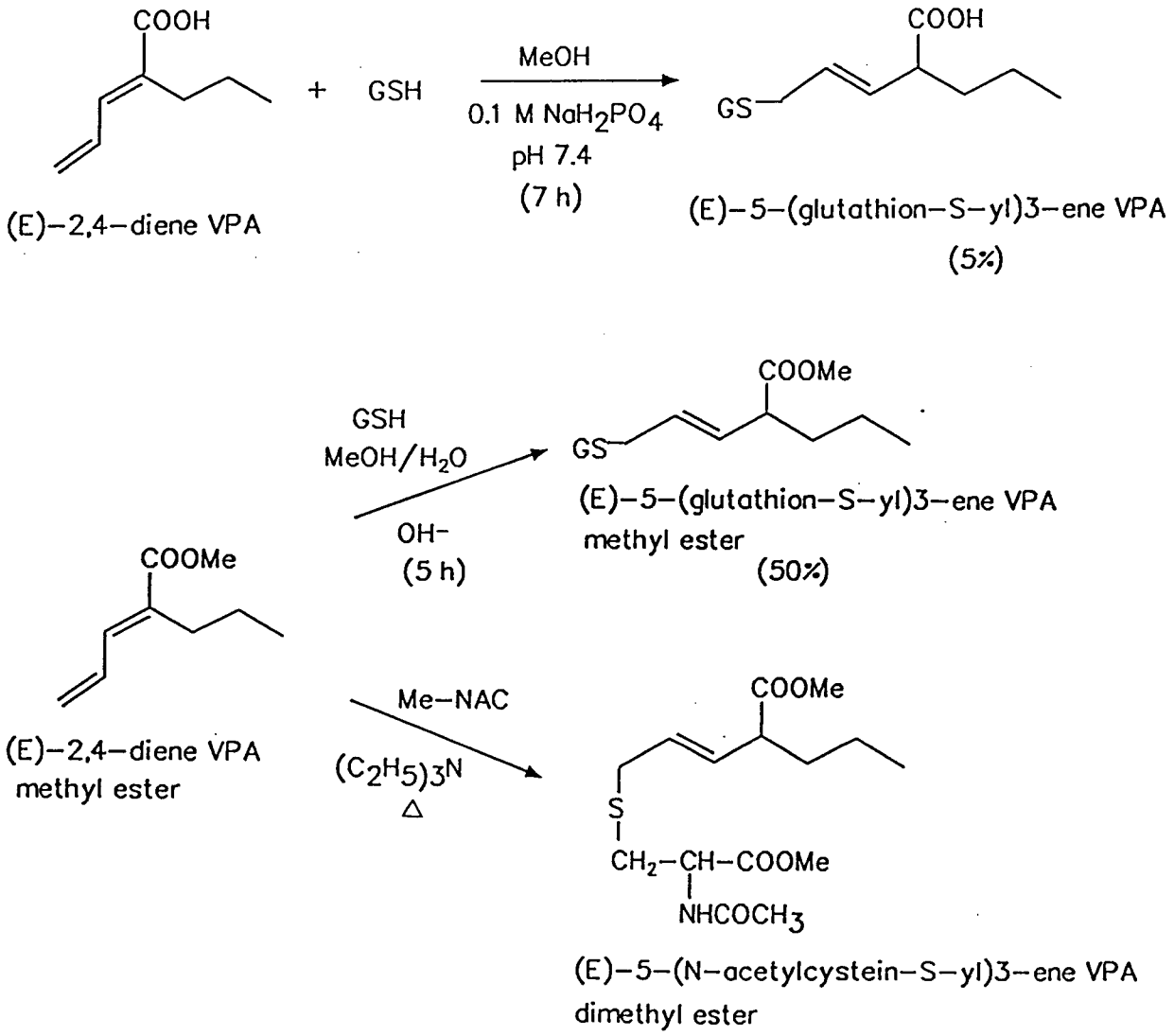


Figure 10. A) mass spectrum of peak c, Fig. 9, corresponding to the dimethyl ester of the NAC conjugate of 3-keto-4-ene VPA. B) PCI mass spectrum of the dimethyl ester of the NAC conjugate of 3-keto-4-ene VPA.



Scheme 2. Outline of the synthetic route of the thiol conjugates of (E)-2,4-diene VPA and its methyl ester.

mesylate resulted in a higher overall yield than did KH but gave a similar isomeric composition of the product. Column chromatography aided in the enrichment of the isomeric content of 2,4-diene VPA to 94% of the E isomer.

Efforts to synthesize the GSH conjugate of the free acid of (E)-2,4-diene VPA in alcoholic aqueous solution (pH=7.8) were not successful. The use of a phosphate buffer (pH=7.4), however, resulted in the production of the desired product albeit in very low yields; 5% in 7 hr. On the other hand, the methyl ester of (E)-2,4-diene VPA readily reacted with GSH in methanolic aqueous solution (pH=7.8) with 50% of the substrate being converted to product within 5 hr. The product was purified by preparative HPLC and characterized by FAB/MS, ^{1}H- and ^{13}C-NMR. The FAB/MS spectrum showed structurally informative ions at m/z 484$(M+Na)^+$, 462$(M+H)^+$ and 155$(M-GS)^+$ (Fig. 11). The most important structural evidence came from the NMR spectrum. The proton chemical shift data are presented in Table 1 and Fig. 12. Signals at 5.44, 5.56, 6.66 and 7.17 δ which were present for the substrate were absent in the GSH adduct while new signals at 3.10, and 5.51-5.67 δ appeared for the substrate portion of the adduct. The methylene protons (=C-$C\underline{H}_2CH_2CH_3(COOCH_3)$) which have a single resonance in the spectrum of 2,4-diene VPA (App. 4) are non-equivalent in the GSH adduct (Fig. 12) indicating that the methylene carbon might be adjacent to a chiral center in the latter. The ^{1}H- and ^{13}C-NMR (Fig. 13) signals of the glutathionyl moiety of the conjugate were similar to literature values of GSH conjugates (Nocerini *et al.*, 1985; te Koppele *et al.*, 1986; Ishida *et al.*, 1989; Nakagawa and Tayama, 1989). From these data plus the data of decoupling experiments the structure of the GSH conjugate of

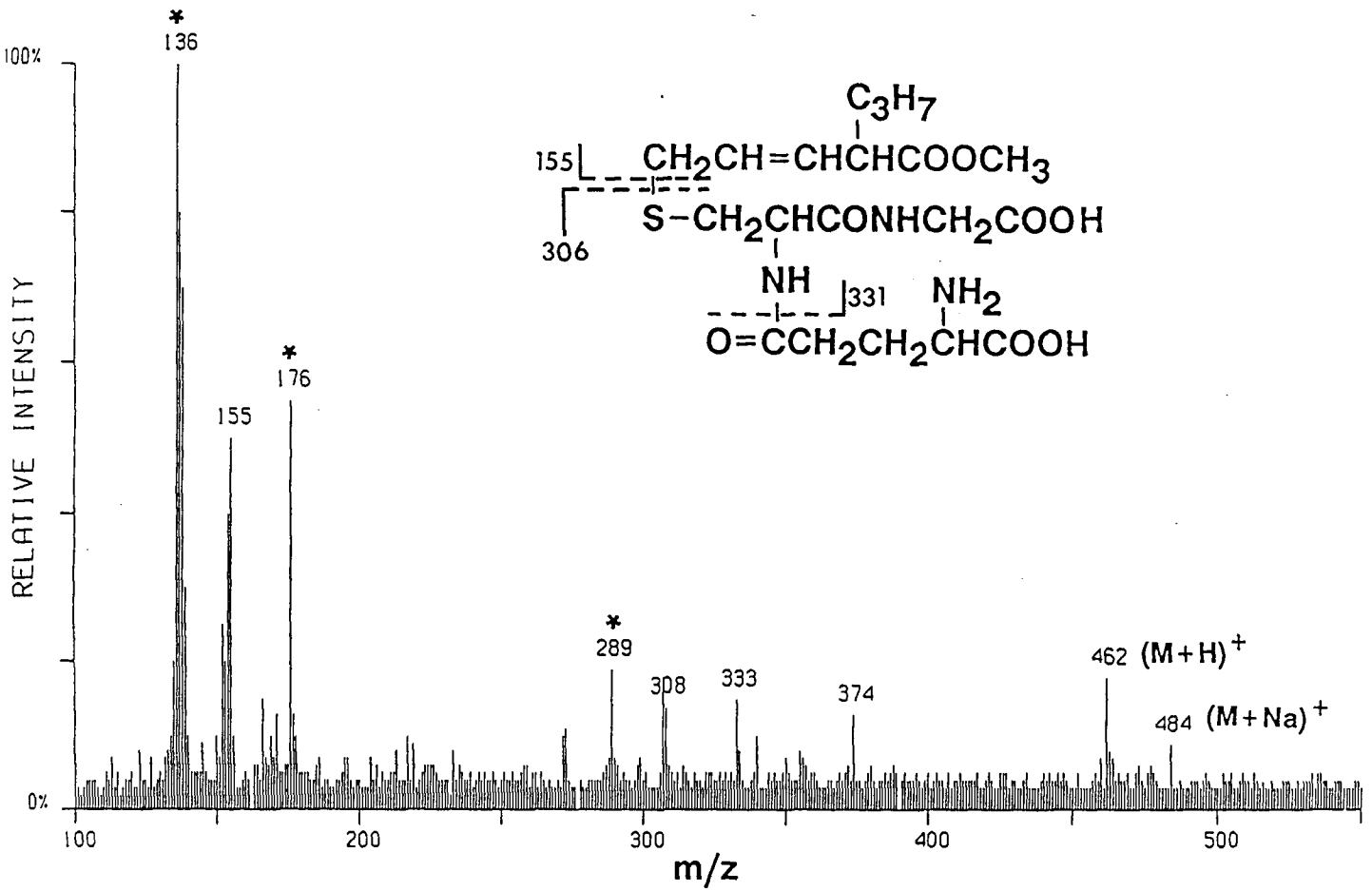
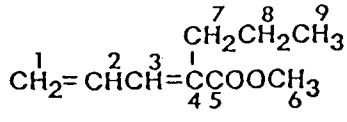
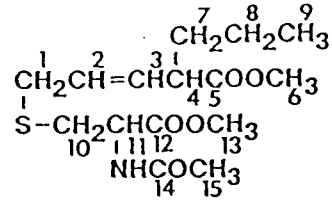


Figure 11. Reconstructed FAB mass spectrum of the GSH conjugate of methyl (E)-2,4-diene VPA in 3-nitrobenzyl alcohol matrix.
Some fragment ions have m/z values of 2 amu higher than shown on the structure as a result of double proton transfer.

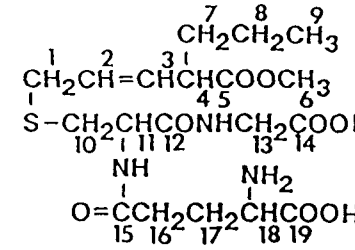
* matrix peaks



I



II



III

TABLE 1

^{1}H-NMR data of (E)-2,4-diene VPA methyl ester and its NAC (methyl ester) and GSH conjugates

I		II		III	
0.92[a](3H,t[b])	9[c]	0.92 (3H,t)	9	0.92 (3H,t)	9
1.46 (2H,m)	8	1.34 (2H,m)	8	1.31 (2H,m)	8
2.40 (2H,t)	7[d]	1.54 (1H,m)	7	1.54 (1H,m)	7
3.76 (3H,s)	6	1.73 (1H,m)	7	1.71 (1H,m)	7
5.44 (1H,dd)	1[e]	2.02 (3H,s)	15	2.17 (2H,m)	17
5.56 (1H,dd)	1[e]	2.75 (1H,m)	10	2.56 (2H,m)	16
6.66 (1H,m)	2	2.94 (1H,m)	10	2.76 (1H,m)	10
7.17 (1H,d)	3[f]	3.10 (1H,q)	4	3.01 (1H,m)	10
		3.17 (2H,d)	1	3.10 (1H,q)	4
		3.68 (3H,s)	6	3.19 (2H,d)	1
		3.75 (3H,s)	13	3.70 (3H,s)	6
		4.58 (1H,dd[g])	11	3.73 (1H,t)	18
		5.49-5.64 (2H,m)	3,2	3.79 (2H,s)	13
				4.55 (1H,dd[h])	11
				5.51-5.67 (2H,m)	3,2

[a] Chemical shift (ppm)

[b] Multiplicity

[c] Assignment

[d] Coupling constant, J=7 Hz

[e] J_{BX}=10 Hz, J_{AB}=2 Hz
J_{AX}=17 Hz, J_{AB}=2 Hz

[f] J=12 Hz

[g] J=4 and 8.0 Hz

[h] J=4.2 and 8.4 Hz

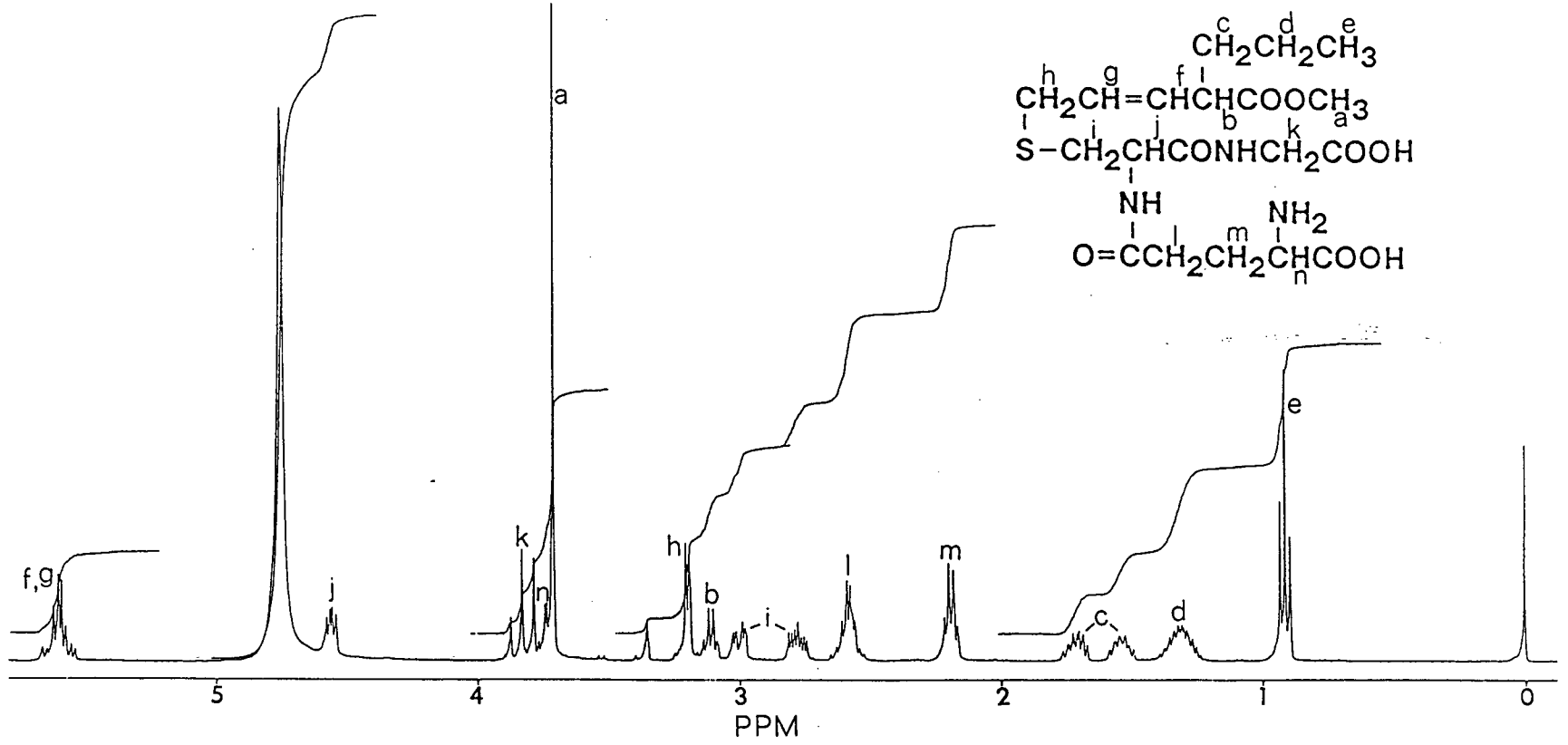


Figure 12. ^{1}H-NMR spectrum of the GSH conjugate of methyl (E)-2,4-diene VPA.

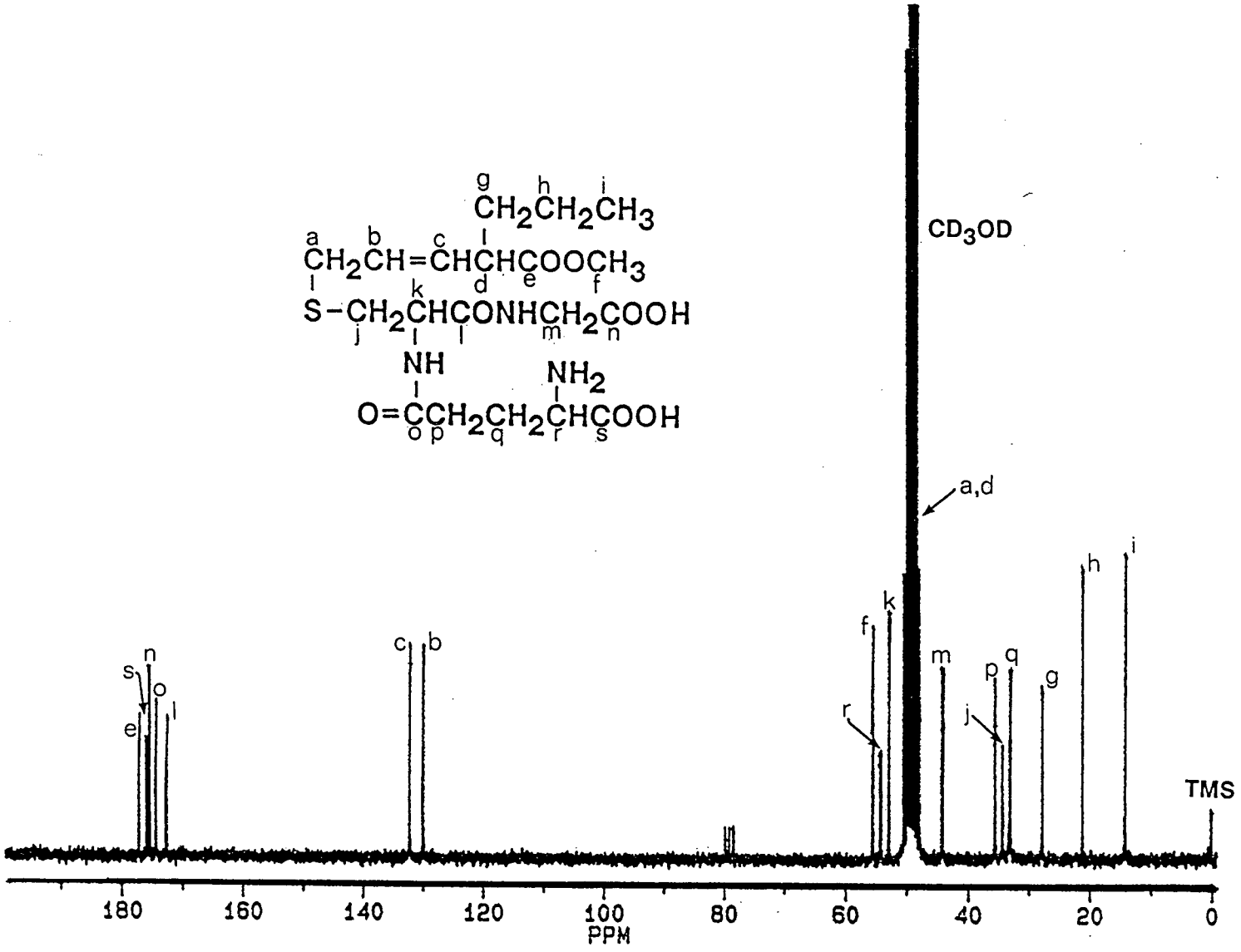


Figure 13. ^{13}C-NMR spectrum of the GSH conjugate of methyl (E)-2,4-diene VPA.

(E)-2,4-diene VPA was concluded to be 5-(glutathion-S-yl)-3-ene VPA. The ^{13}C-NMR, shown in Fig. 13 also supported this structural assignment for the conjugate. The signals for the -CH=CH- carbons are present at 132.1 and 129.8 δ. The chemical shift for -$\underline{C}$H-($COOCH_3$)$CH_2CH_2CH_3$ occurs at 49.0 δ as verified by the attached proton test.

The methyl ester of (E)-2,4-diene VPA reacted rapidly with methyl NAC and yielded a single product when analyzed by LC and EI/GC/MS. The GC/MS spectrum of the conjugate is given in Fig. 14. Structurally informative ions are present at m/z 331(M^+), 300($M-OCH_3$)$^+$, 272($M-COOCH_3$)$^+$, 176($SCH_2CH(NHCOCH_3)COOCH_3$)$^+$, 155($M-(SCH_2CH(NHCOCH_3)COOCH_3)$)$^+$ and 144($CH_2CH(NHCOCH_3)COOCH_3$)$^+$. The ^1H-NMR and ^{13}C-NMR (App. 5 and 6) data confirmed the position of the double bond to be at the same position as in the GSH conjugate i.e. at position 3. From these spectra and a series of homonuclear decoupling experiments the structure of the adduct was concluded to be 5-(N-acetylcystein-S-yl)-3-ene VPA. The quartet for -C$\underline{H}$-($COOCH_3$)$CH_2CH_2CH_3$ at 3.10 (Table 1) collapsed into a doublet and triplet respectively, upon decoupling of the signals at 1.73 and 5.55 δ. Irradiation of the signals at 3.10 and 3.17 δ resulted in the appearance of doublets on the downfield (3.10) and upfield (3.17) side of the 5.49-5.64 δ signal assigned to the -CH=CH- protons. The coupling constant of the vinylic protons was 16 Hz indicating a trans configuration for the double bond (Silverstein *et al.*, 1981). A similar coupling constant value was obtained for the vinylic protons in the GSH conjugate.

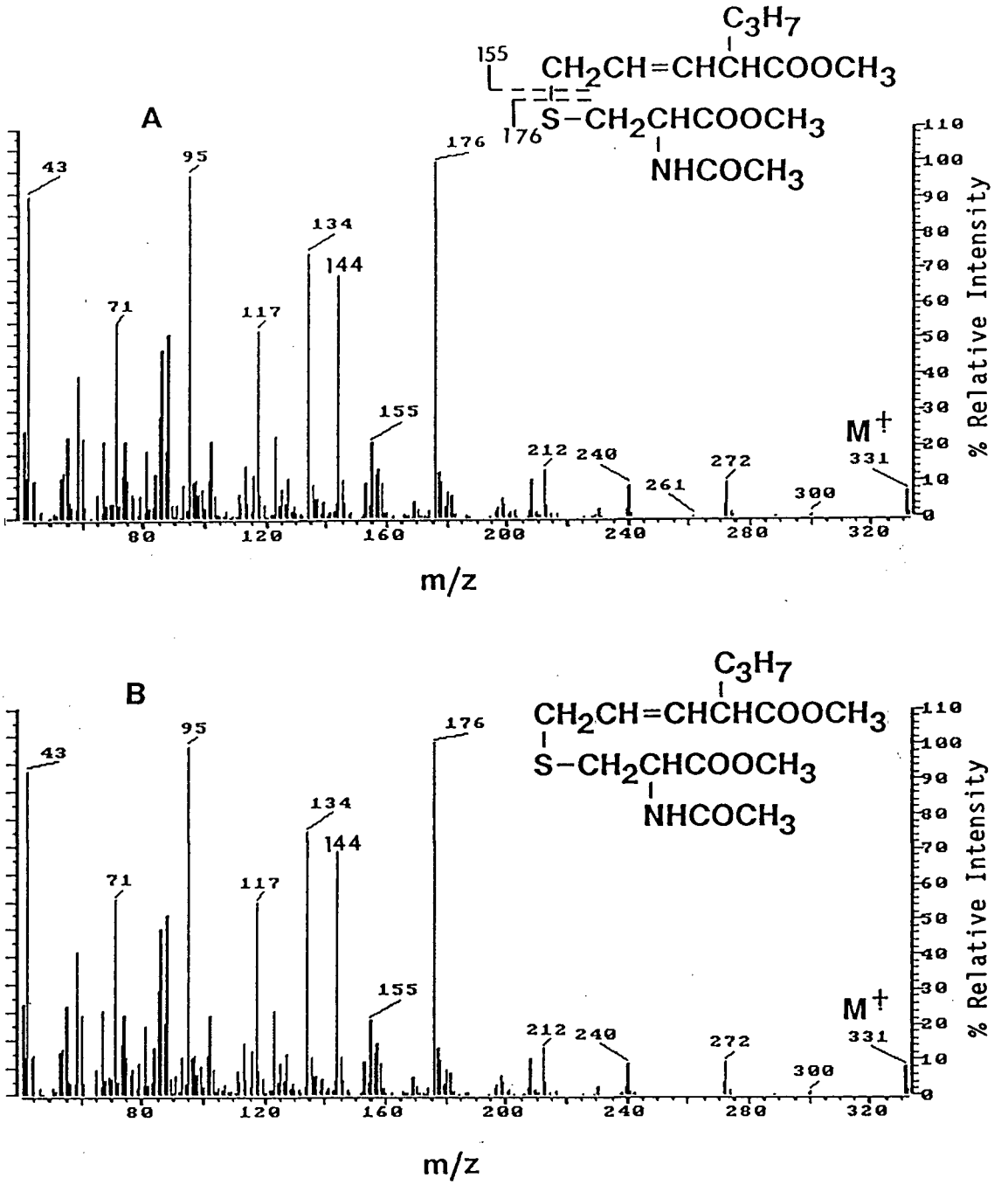


Figure 14. EI mass spectra of A) authentic dimethyl ester of the NAC conjugate of (E)-2,4-diene VPA and B) peak marked with an asterisk, Fig. 29).

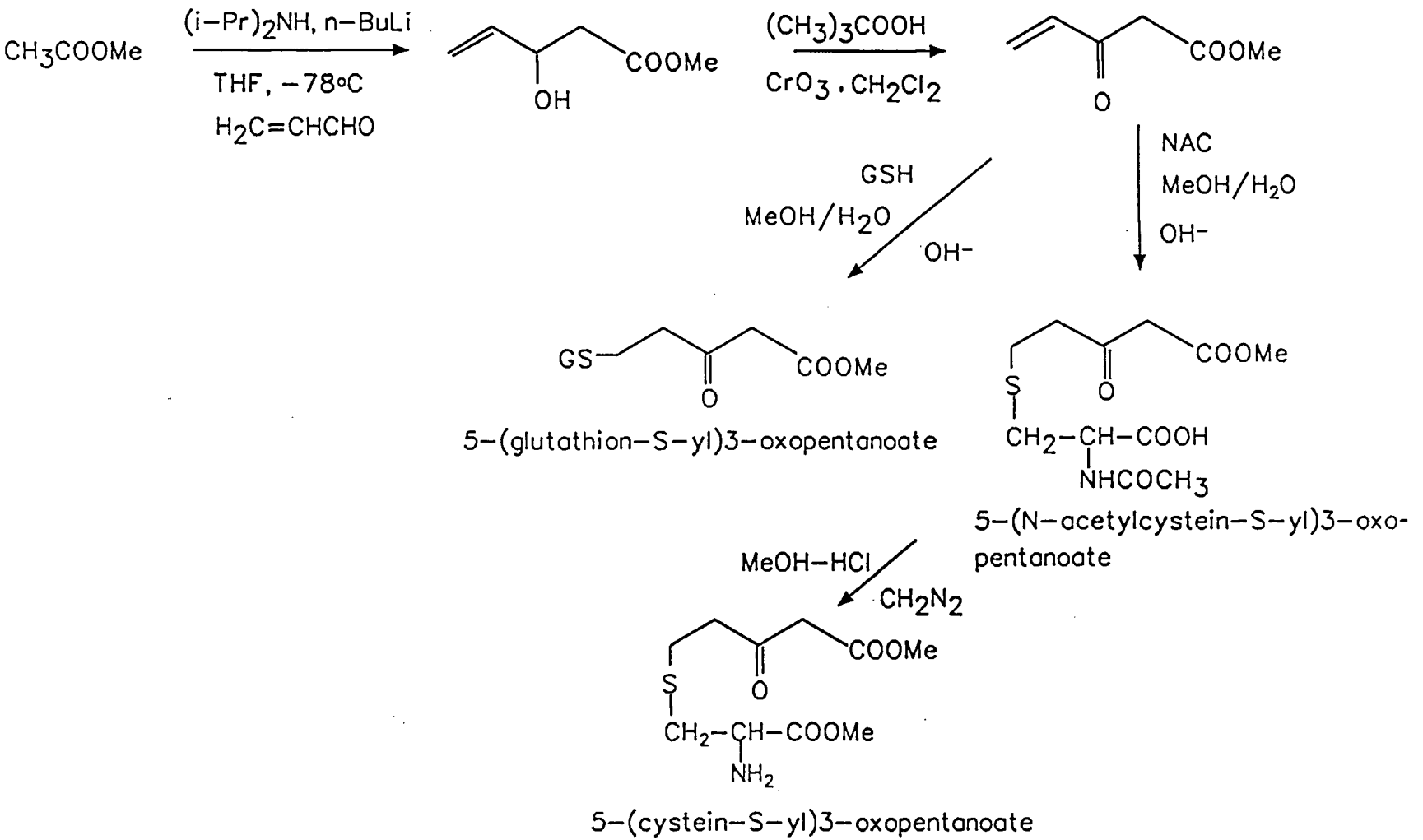
D. Methyl 3-oxo-4-pentenoate

This α,β-unsaturated keto ester is a known compound and has previously been synthesized as the ethyl ester by a synthetic route that involved several steps which were tedious and inefficient (Collins and Tomkins, 1977). In the present study the methyl ester was synthesized by an efficient, two-step synthetic route as shown in Scheme 3. The spectroscopic data of the compound was in agreement with that in the literature (Collins and Tomkins, 1977) with the ^{1}H-NMR showing the keto and enol forms of the compound in a ratio of 2:1 (App. 12). The GC/MS spectrum of the compound is shown in Fig. 15 and is consistent with the structure of the desired product.

E. GSH and NAC conjugates of 3-oxo-4-pentenoate

The 3-oxo-4-pentenoate reacted readily with GSH in alkaline aqueous/alcohol solution (Scheme 3). The structure of the product was determined to be 5-(glutathion-S-yl)-3-oxopentanoate using LC/MS (Fig. 16) and ^{1}H- and ^{13}C-NMR (Figs. 17 and 18) spectroscopy. As was the case with the GSH conjugate of 3-keto-4-ene VPA, there was no signal for the methylene protons adjacent to the two carbonyl groups in the GSH conjugate of 3-oxo-4-pentenoate.

The NAC conjugate was also synthesized in a manner similar to that of the GSH conjugate by stirring NAC and the substrate in aqueous methanolic solution. The NMR spectra of the purified product are shown in App. 13 and 14. For GC/MS analysis an attempt was made to methylate the free carboxyl group; however, upon treatment with methanolic HCl gas followed by CH_2N_2 the NAC conjugate was deacetylated to give the cysteine conjugate. The NAC conjugate was also synthesized as the



Scheme 3. Synthetic pathway of methyl 3-oxo-4-pentenoate and its thiol conjugates.

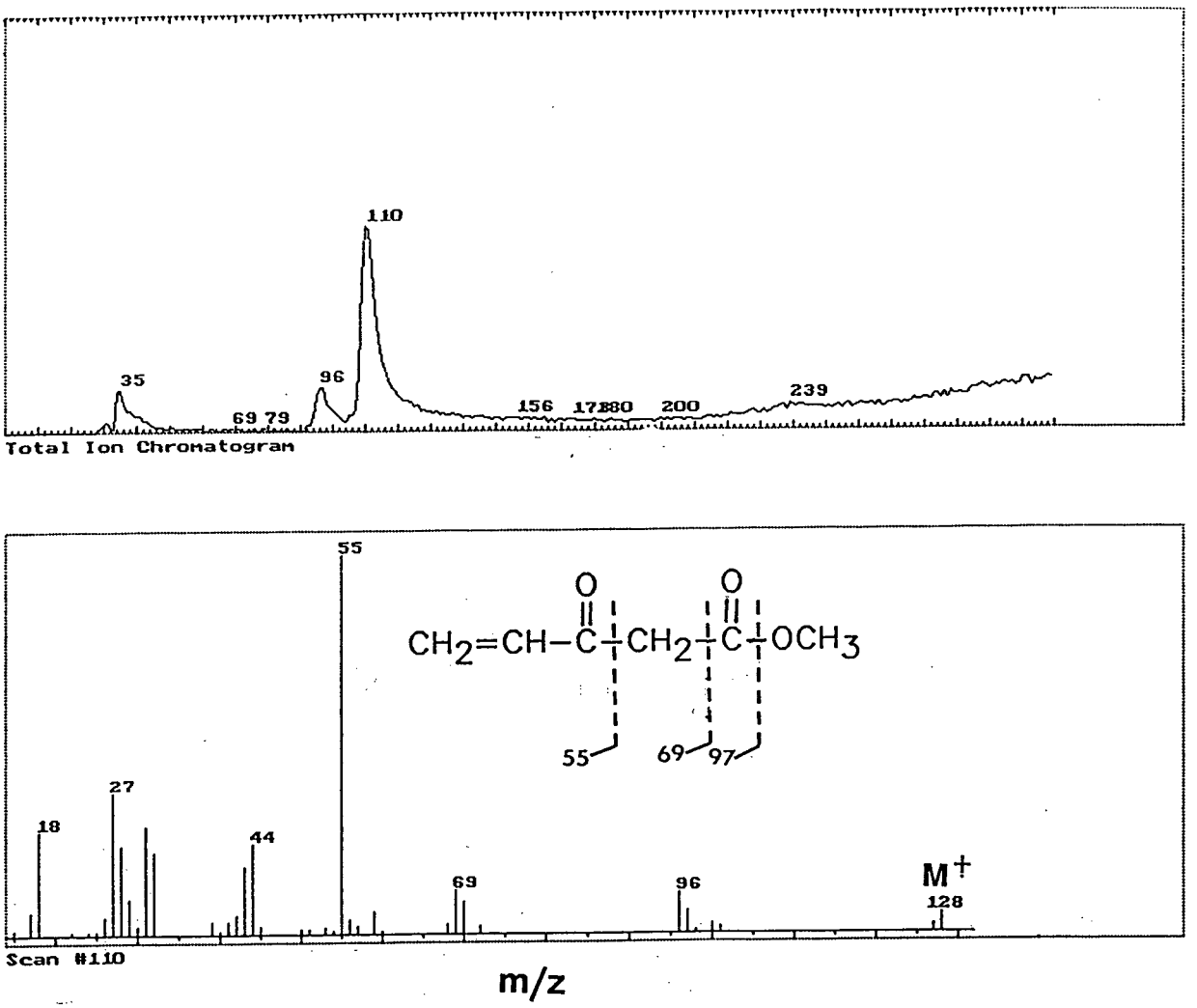


Figure 15. EI/GS/MS TIC chromatogram and spectrum of a synthetic sample of methyl 3-oxo-4-pentenoate.

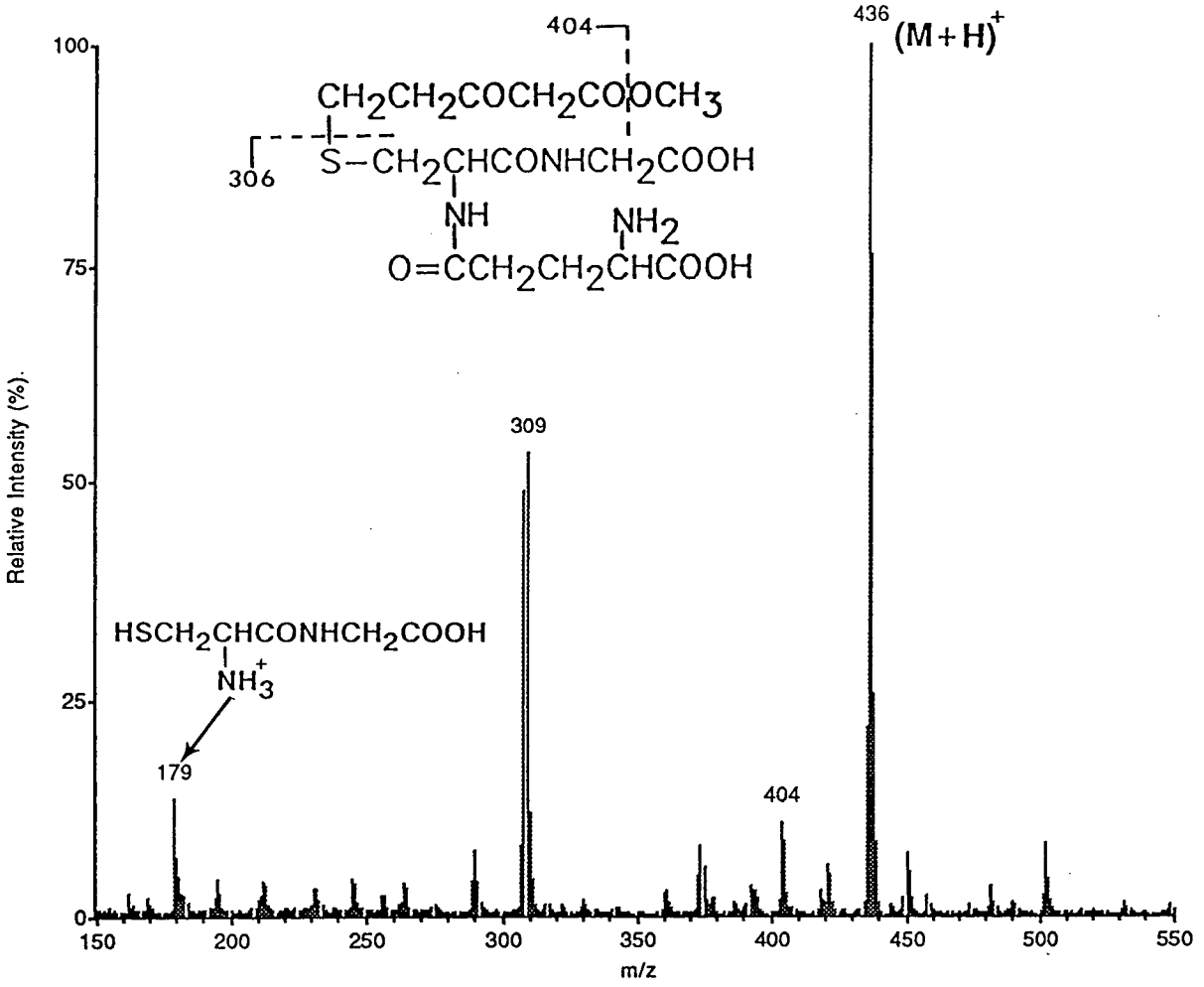


Figure 16. LC/MS spectrum, in the positive ion mode, of synthesized GSH conjugate of methyl 3-oxo-4-pentenoate.

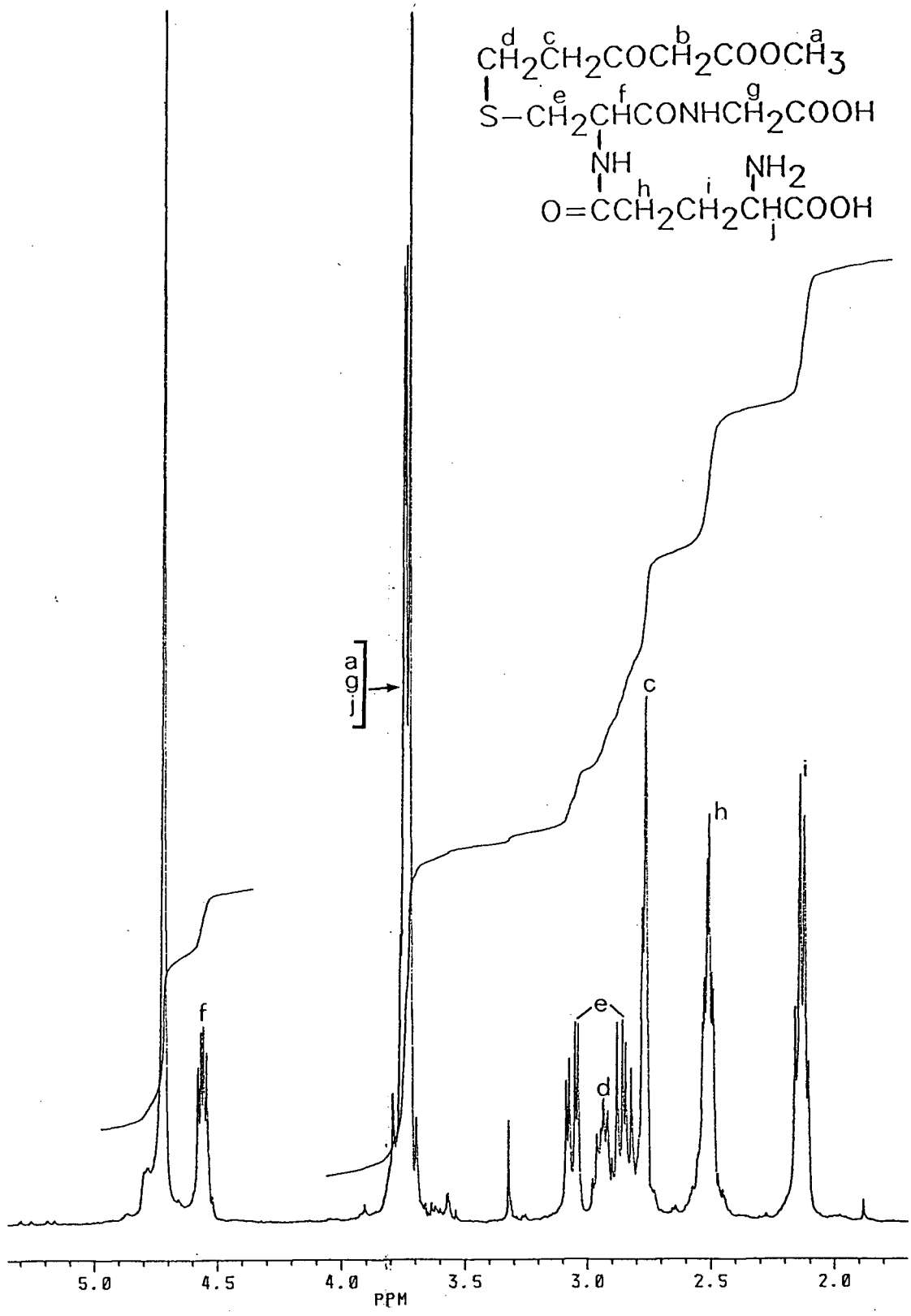


Figure 17. ^{1}H-NMR spectrum of the GSH conjugate of methyl 3-oxo-4-pentenoate.

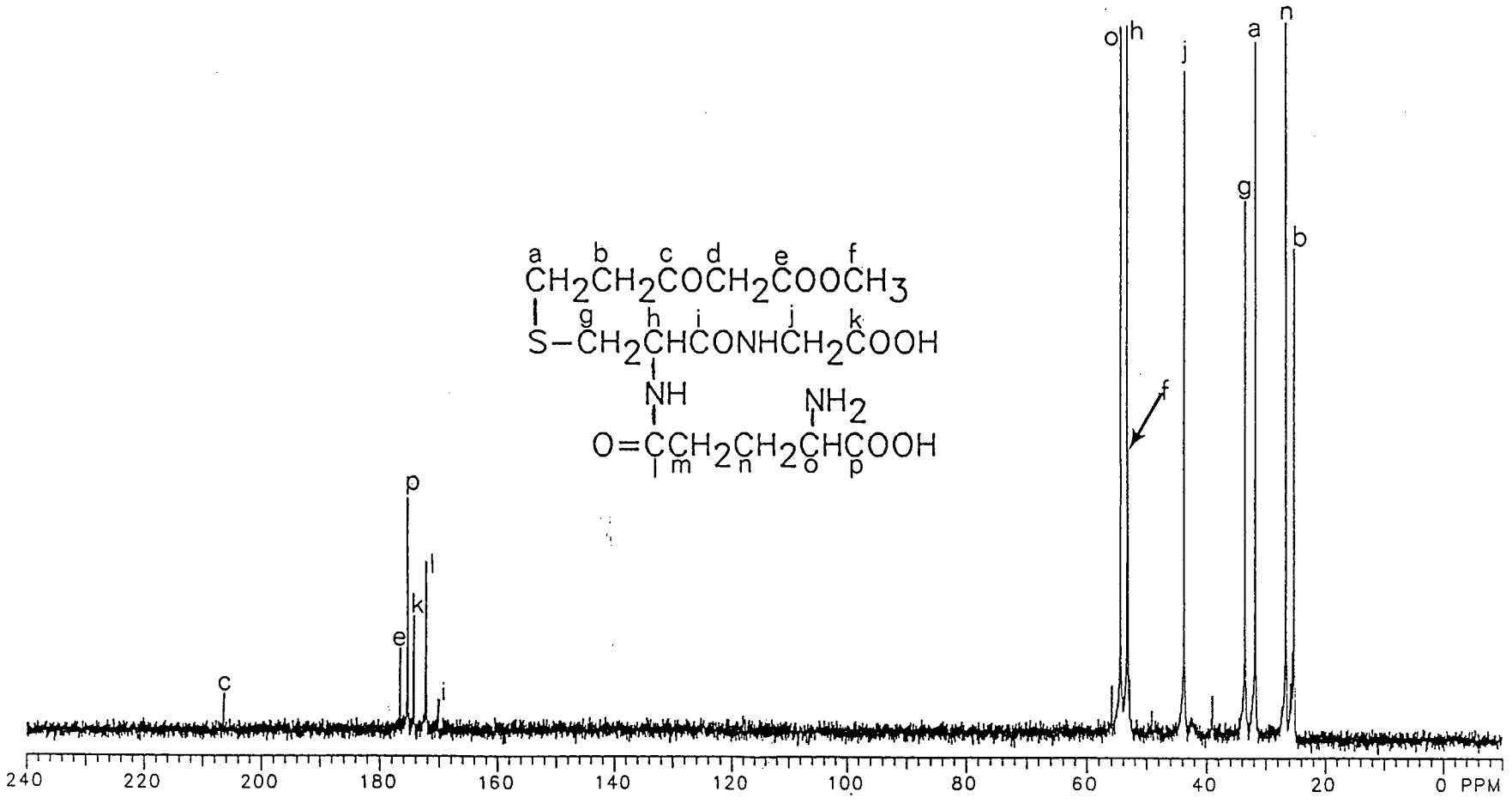


Figure 18. ^{13}C-NMR spectrum of the GSH conjugate of methyl 3-oxo-4-pentenoate.

dimethyl ester starting with methyl NAC. Compared to other conjugates studied, the dimethyl ester of the NAC conjugate of 3-oxo-4-pentenoate underwent extensive MS fragmenation upon GC/MS analysis (Fig.19A). Even the PCI spectrum did not furnish molecular weight information (Fig. 19B). Satisfactory mass spectral identification was obtained only using LC/MS/MS (Fig. 20).

F. GSH and NAC conjugates of (E)-2,4-pentadienoate

Methyl 2,4-pentadienoate was synthesized stereospecifically (100% E isomer) following a literature method (Worley and Young, 1972). The ester reacted readily with GSH according to Scheme 4 with 70% of the substrate converted to product in 4 hr. The LC/MS, ^{1}H- and ^{13}C-NMR data of the HPLC purified product are illustrated in Figs. 21, 22 and 23, respectively. From these spectroscopic data and a series of homonuclear decoupling experiments, the structure of the conjugate was established to be (E)-5-(glutathion-S-yl)-3-pentenoate. Thus, this analog of (E)-2,4-diene VPA reacted with GSH by the same mechanism as that of (E)-2,4-diene VPA. Unlike that of (E)-2,4-diene VPA, however, the reaction of (E)-2,4-pentadienoate produced two isomers detectable by LC/MS (Fig. 24) and having the same MS/MS mass spectra (Fig. 25).

The dimethyl ester of the NAC conjugate of (E)-2,4-pentadienoate was prepared in a manner similar to that described for the NAC conjugate of (E)-2,4-diene VPA methyl ester. The mass chromatogram (m/z 176) obtained from a crude sample is illustrated in Fig. 26A. There are four isomeric peaks with the same mass spectra. Figure 26B shows the mass spectrum of the major peak and contains several ions which are informative of the structure- m/z 289(M^+), 258($M-OCH_3$)$^+$, 230($M-COOCH_3$)$^+$,

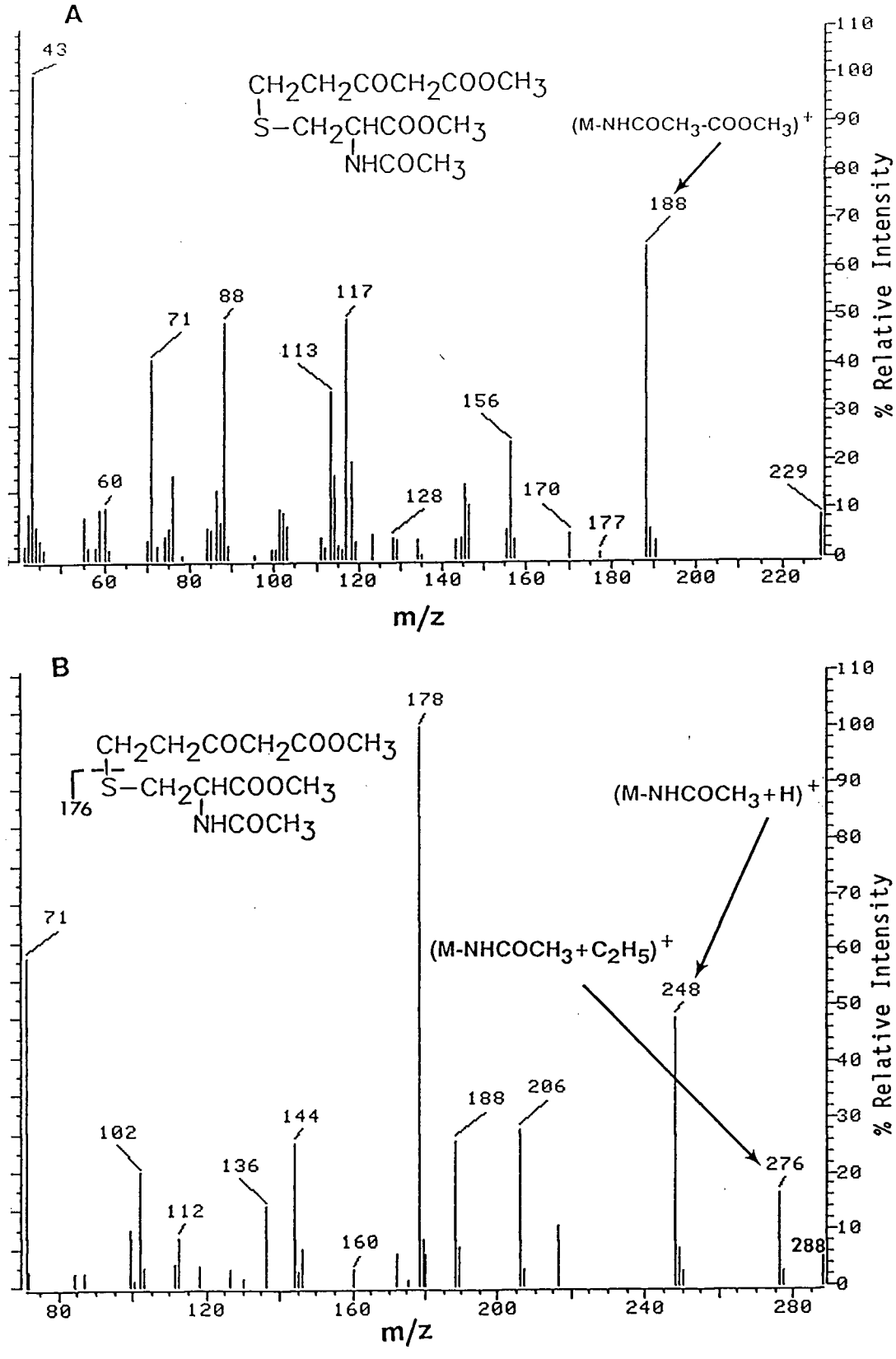


Figure 19. EI (A) and PCI (B) mass spectra of the NAC conjugate of 3-oxo-4-pentenoate, dimethyl ester.

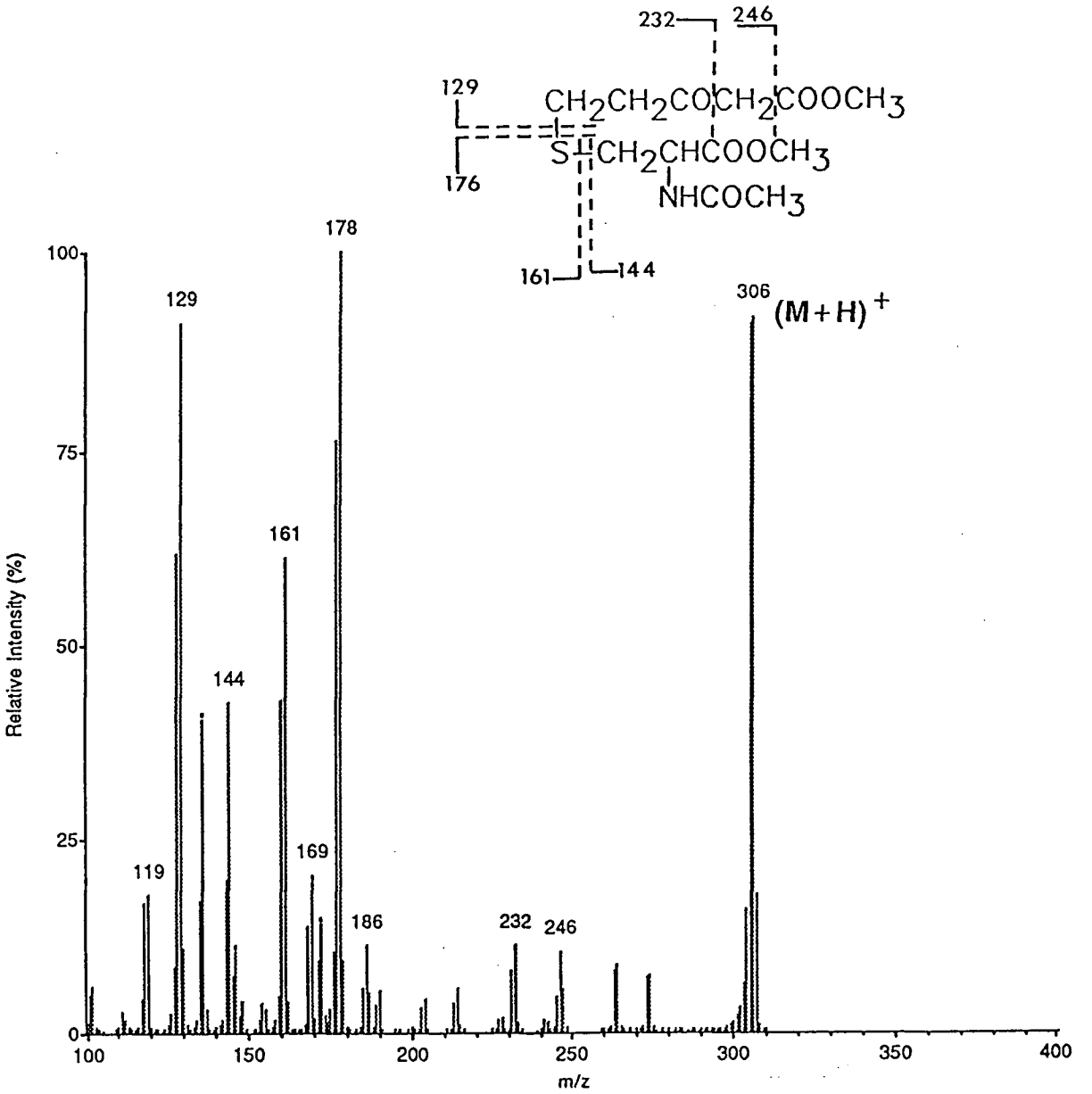
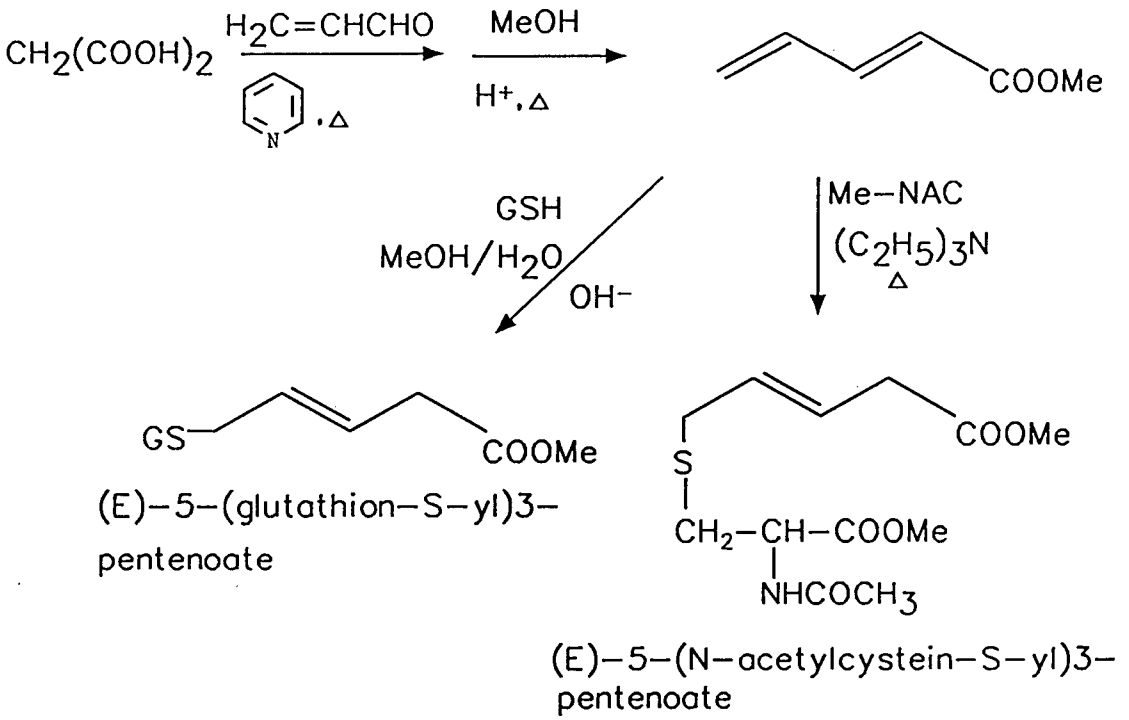


Figure 20. LC/MS/MS of the $(M+H)^+$ of the dimethyl ester of the NAC conjugate of 3-oxo-4-pentenoate.



Scheme 4. Synthesis of methyl (E)-2,4-pentadienoate and its GSH and NAC adducts.

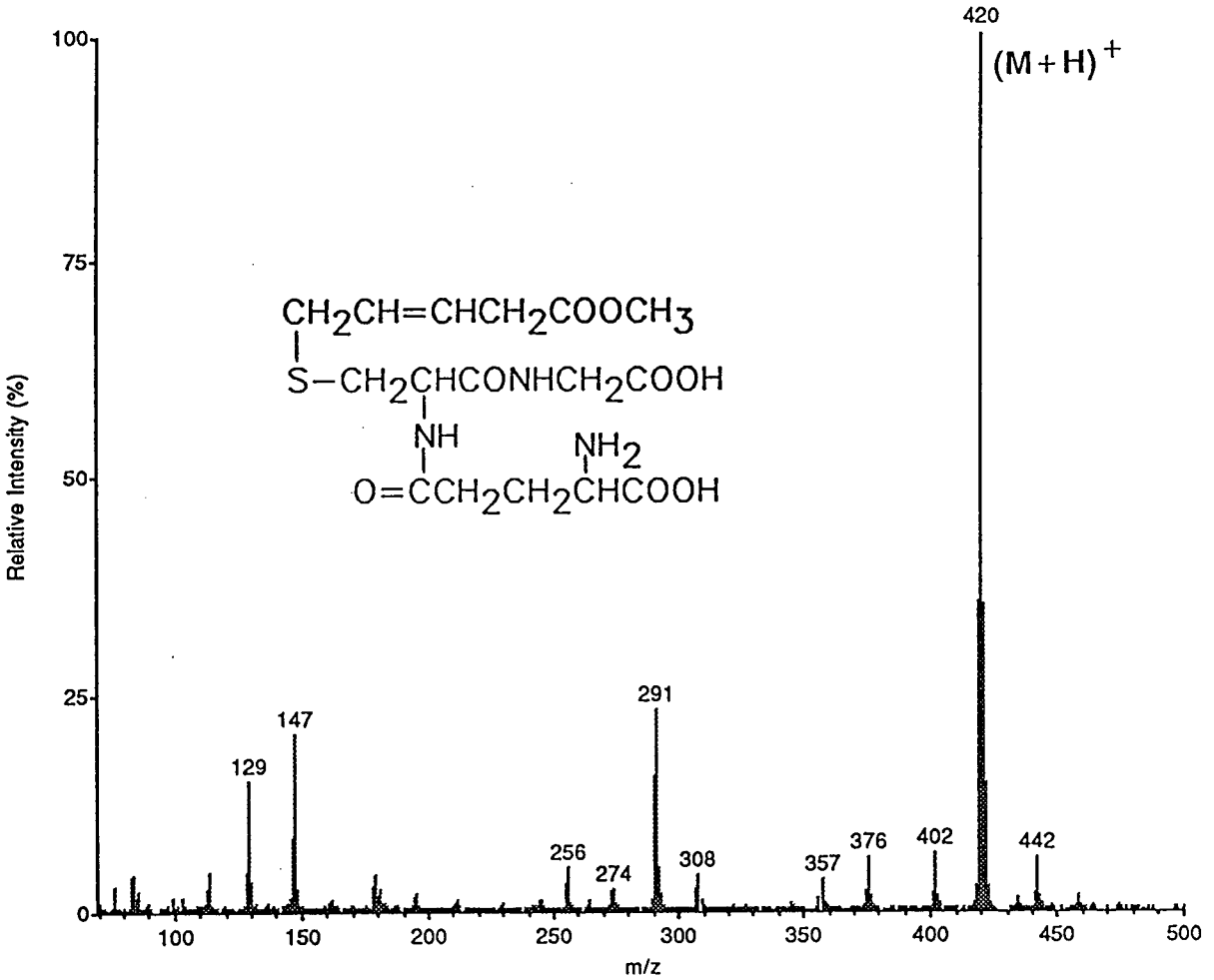


Figure 21. LC/MS mass spectrum of the GSH conjugate of methyl (E)-2,4-pentadienoate.

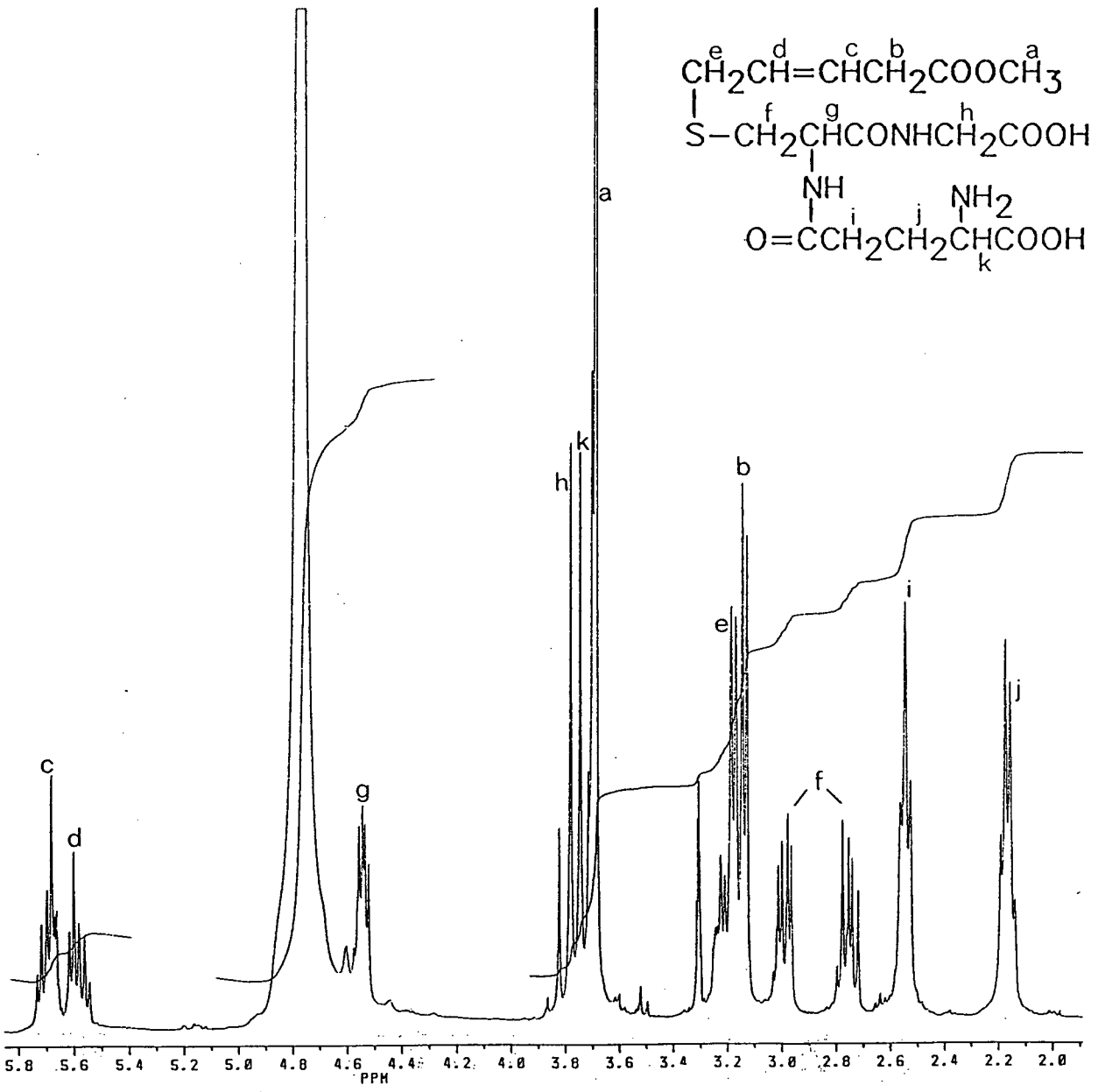


Figure 22. ^{1}H-NMR spectrum of the GSH conjugate of methyl (E)-2,4-pentadienoate.

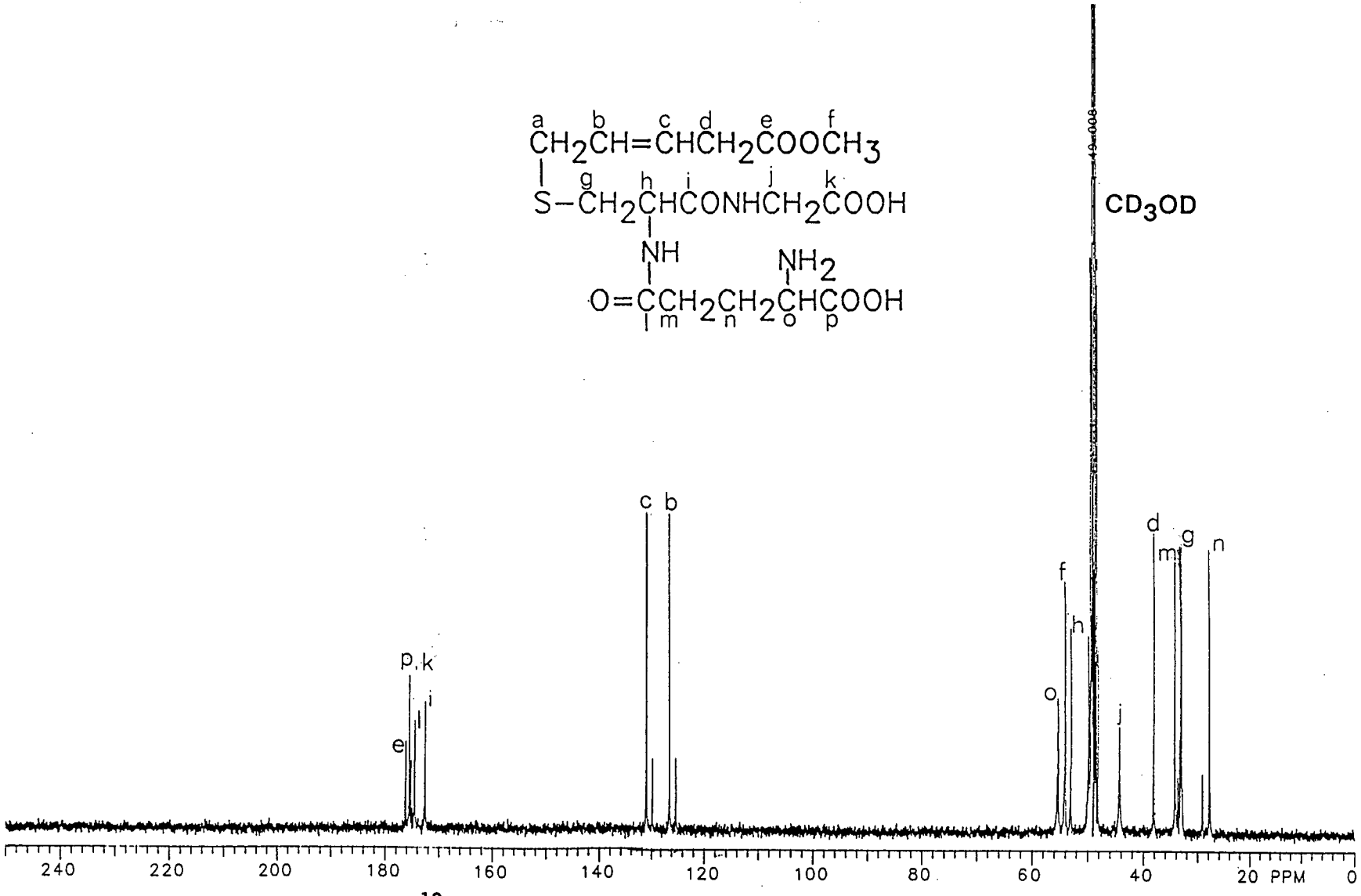


Figure 23. ^{13}C-NMR spectrum of the GSH conjugate of methyl (E)-2,4-pentadienoate.

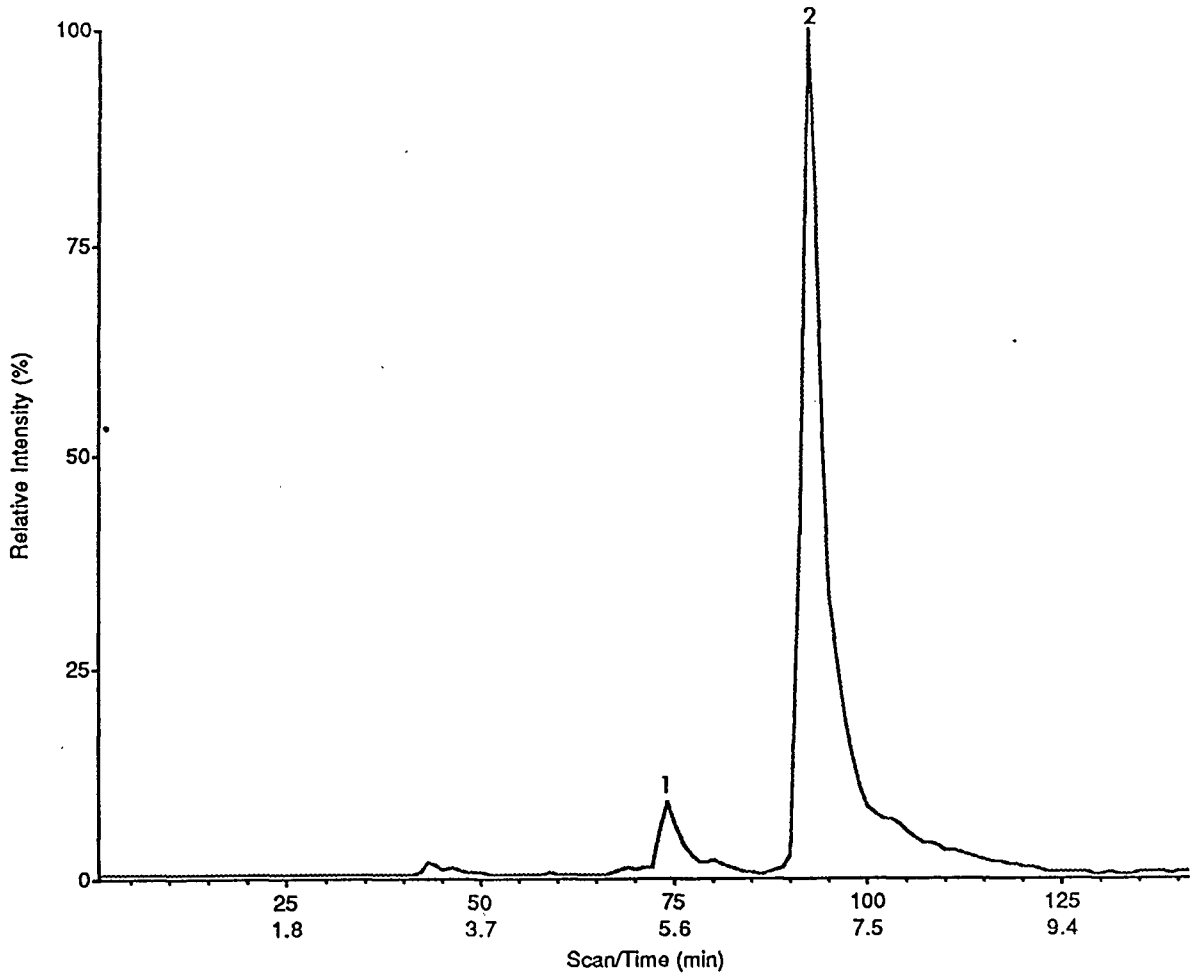


Figure 24. LC/MS profile of a fully methylated sample of the GSH conjugate of (E)-2,4-pentadienoate. Peaks 1 and 2 were identified as isomeric GSH conjugates of (E)-2,4-pentadienoate.

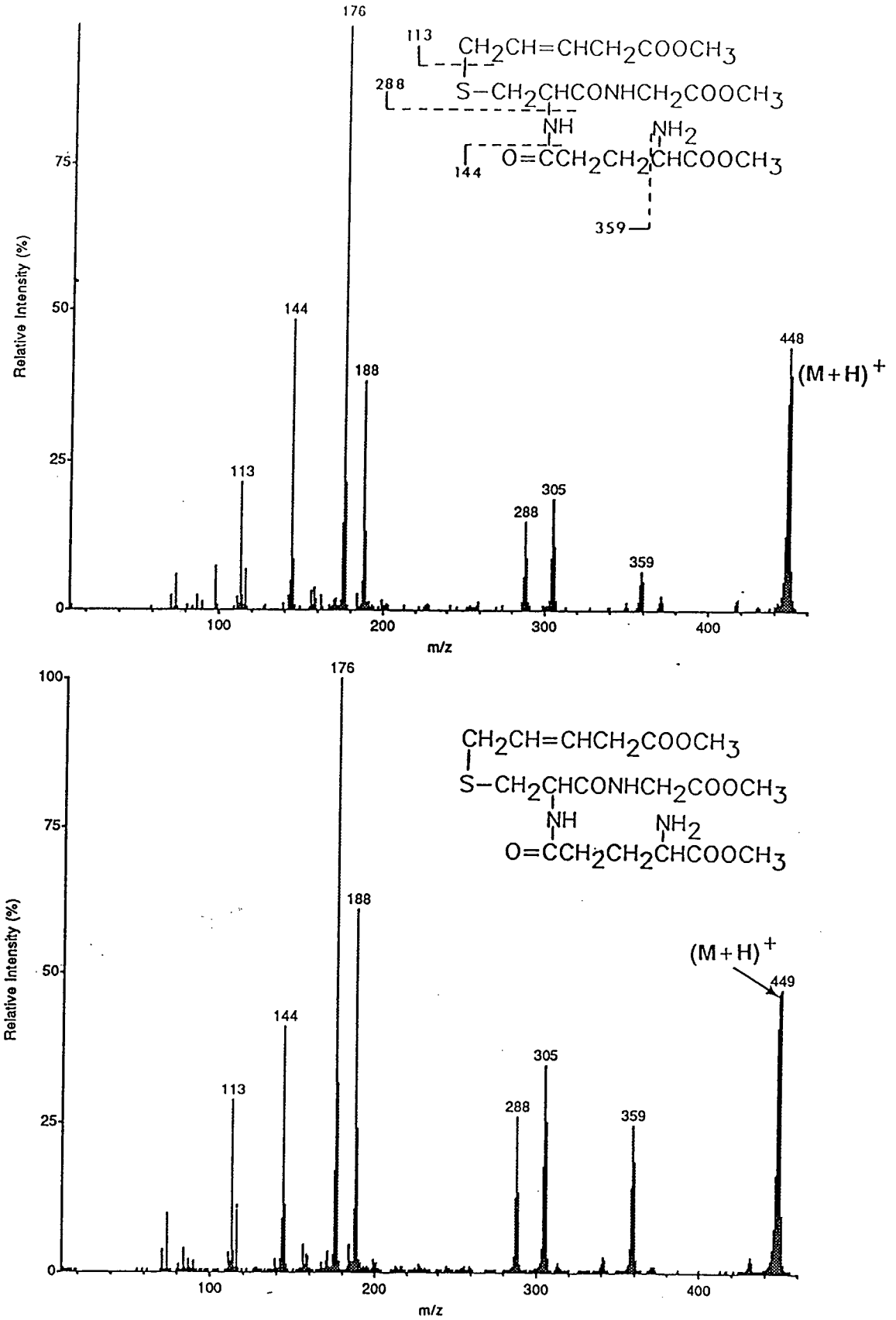


Figure 25. Daughter ion (parent, m/z 448) MS/MS spectra of peaks 1 (top) and 2 (bottom) in Fig. 24 corresponding to isomeric GSH conjugates of (E)-2,4-pentadienoate.

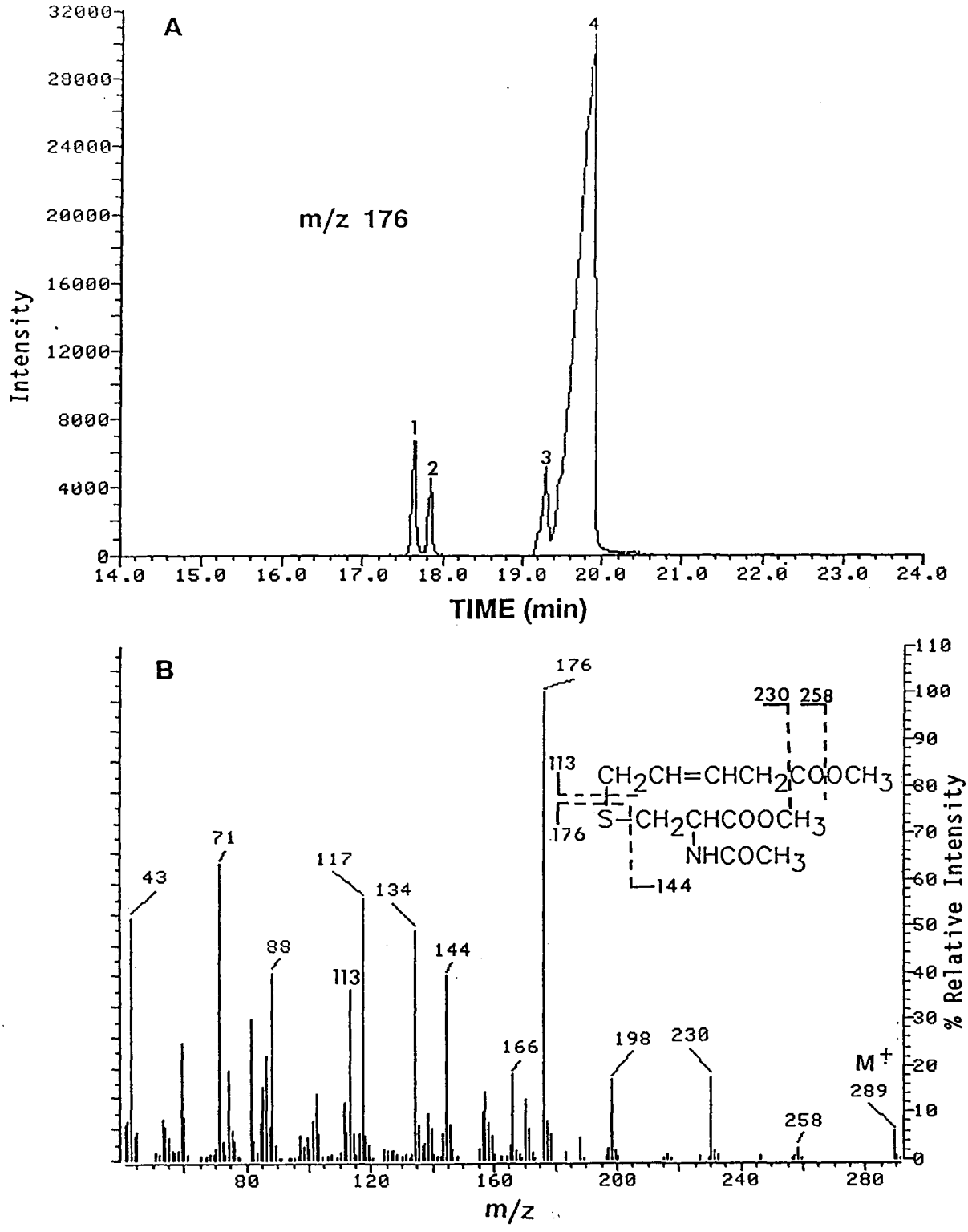


Figure 26. A) Mass chromatogram of m/z 176 obtained from a crude synthetic sample of the NAC conjugate of (E)-2,4-pentadienoate (dimethyl ester) and B) EI/GC/MS spectrum of the peak labelled 4.

176($SCH_2CH(NHCOCH_3)COOCH_3)^+$ and 113($M-(SCH_2CH(NHCOCH_3)COOCH_3))^+$. From this spectrum and ^1H-NMR (App. 9, obtained from an HPLC purified sample) and ^{13}C-NMR (App. 10) plus decoupling experiments the structure of the conjugate was determined to be 5-(N-acetylcystein-S-yl)-3-pentenoate. The coupling constant between the vinylic protons was 15.90 Hz indicating a trans configuration for the double bond.

2. Development of an analytical method for profiling VPA metabolism in patients

A sensitive and selective GC/MS assay was required for the simultaneous quantitation of VPA and 14 of its metabolites in order to assess the 4-ene VPA pathway relative to other VPA metabolic pathways in pediatric patients. In a previous communication (Kassahun *et al.*, 1989), the use of NICI/GC/MS for the identification of VPA metabolites as their pentafluorobenzyl (PFB) derivatives was described. In the present study the NICI methodology was used to determine the levels of the putative hepatotoxic metabolites of VPA along with the other known metabolites.

A. Negative ion GC/MS properties of VPA metabolites

The PFB derivatives of the metabolites and the parent drug have excellent negative ion response as reported earlier (Kassahun *et al.*, 1989). With the DB-1 column, however, the PFB derivatives of the hydroxy metabolites chromatographed poorly and sensitivity was compromised. Trimethylsilylation of the hydroxyl groups after PFB derivatization of the carboxyl moiety greatly improved both peak shape and sensitivity of detection. The PFB derivative of 3-keto VPA, unlike the other metabolites, fragmented to yield the $[M-181-CO_2]^-$ (M-181 corresponds to

the loss of the pentafluorobenzyl moiety) ion as the base peak (Kassahun *et al.*, 1989). This fragmentation was blocked with TMS derivatization of the 3-oxo moiety, resulting in the $[M-181]^-$ ion as the major ion in the mass spectrum. Hence, SIM chromatograms were obtained by monitoring the $[M-181]^-$ anions which were the base peaks of all the metabolites studied i.e. both PFB esters and O-TMS-PFB derivatives. The metabolites assayed and the negative ions monitored are given in Table 2. The negative ion spectra obtained for VPA and its metabolites are typified by the example in Fig. 27, with the $[M-181]^-$ as almost the only ion in the mass spectrum. Thus, the combination of PFB and TMS derivatization produced suitable negative ions and chromatographic characteristics which allowed the development of a simultaneous assay of VPA and 14 metabolites. Typical SIM chromatograms representing all the metabolites assayed are shown in Figs. 28A and 28B.

The use of the DB-1 column and PFB derivatization enabled the complete separation of 4-ene VPA from VPA as well as the separation of the monounsaturated metabolites as seen in Fig. 28A, m/z 141. PFB derivatization also resulted in baseline separation of the geometric and/or positional isomers of the diunsaturated metabolites (Fig. 28A, m/z 139). The putative toxic metabolite, (E)-2,4-diene VPA is clearly separated from (E,Z)-2,3'-diene VPA. When the t-BDMS and TMS derivatives were analyzed under similar conditions, these two metabolites overlapped and being isobaric were counted as one.

In the case of 4-OH VPA only the open chain form was monitored since the γ-lactone would be transparent to NICI. Because the base diisopropylethylamine was used for the derivatization reaction, it was assumed that most of the 4-OH VPA would be in the open chain form.

Table 2. Ions monitored (m/z) in the NICI mode for VPA and metabolites derivatized with PFB and TMS

COMPOUND	$[M-181]^-$
(E,Z)-2,3′-Diene VPA	139
(E)-2,4-Diene VPA	139
(E,E)-2,3′-Diene VPA	139
4-Ene VPA	141
3-Ene VPA	141
(Z)-2-Ene VPA	141
(E)-2-Ene VPA	141
VPA	143
4-Keto VPA	157
3-Keto VPA[a]	229
4-OH VPA[a,b]	231
3-OH VPA[a]	231
5-OH VPA[a]	231
2-PSA[c]	339
2-PGA[c]	353

[a] O-TMS-PFB derivatives

[b] two isomers

[c] di-PFB derivatives

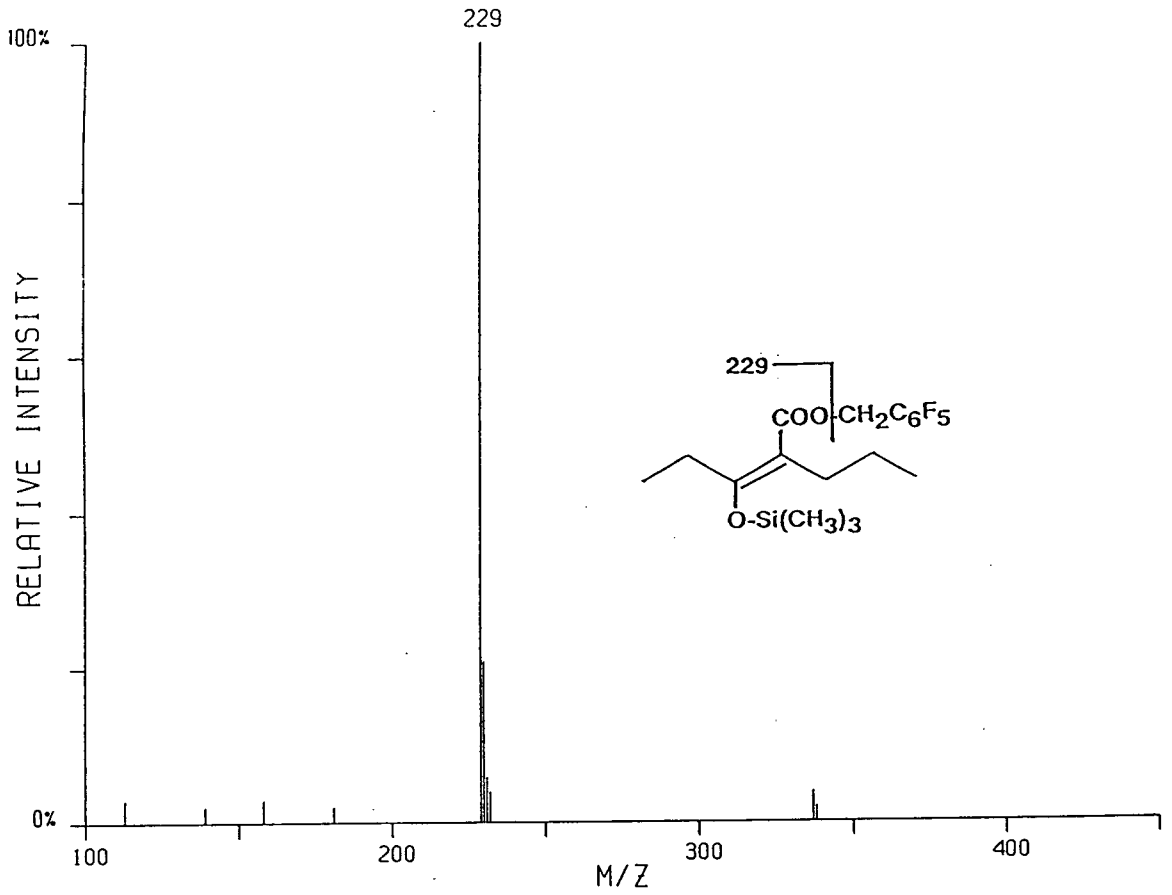


Figure 27. NICI/GC/MS spectrum of the O-TMS-PFB derivative of 3-keto VPA.

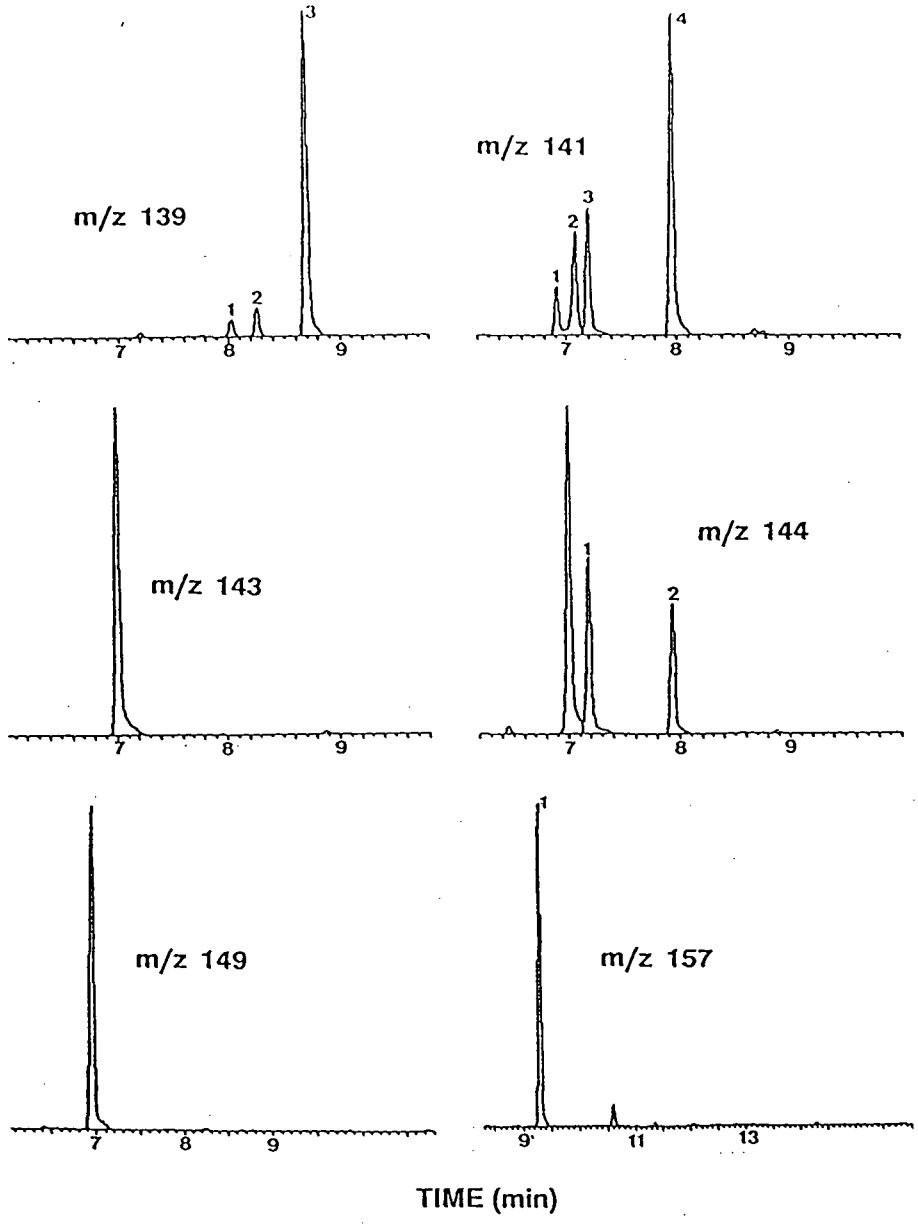


Figure 28A. Typical SIM chromatograms obtained from the serum extract of a patient on VPA to which internal standards have been added. Peaks: m/z 139, 1 = (E,Z)-2,3′-diene VPA, 2 = (E)-2,4-diene VPA, 3 = (E,E)-2,3′-diene VPA; m/z 141, 1 = 4-ene VPA, 2 = 3-ene VPA, 3 = (Z)-2-ene VPA, 4 = (E)-2-ene VPA; m/z 143, VPA; m/z 144, 1 = (Z)-[$^2H_3$]2-ene VPA, 2 = (E)-[$^2H_3$]2-ene VPA, m/z 149, [$^2H_6$]VPA; m/z 157, 1 = 4-keto VPA.

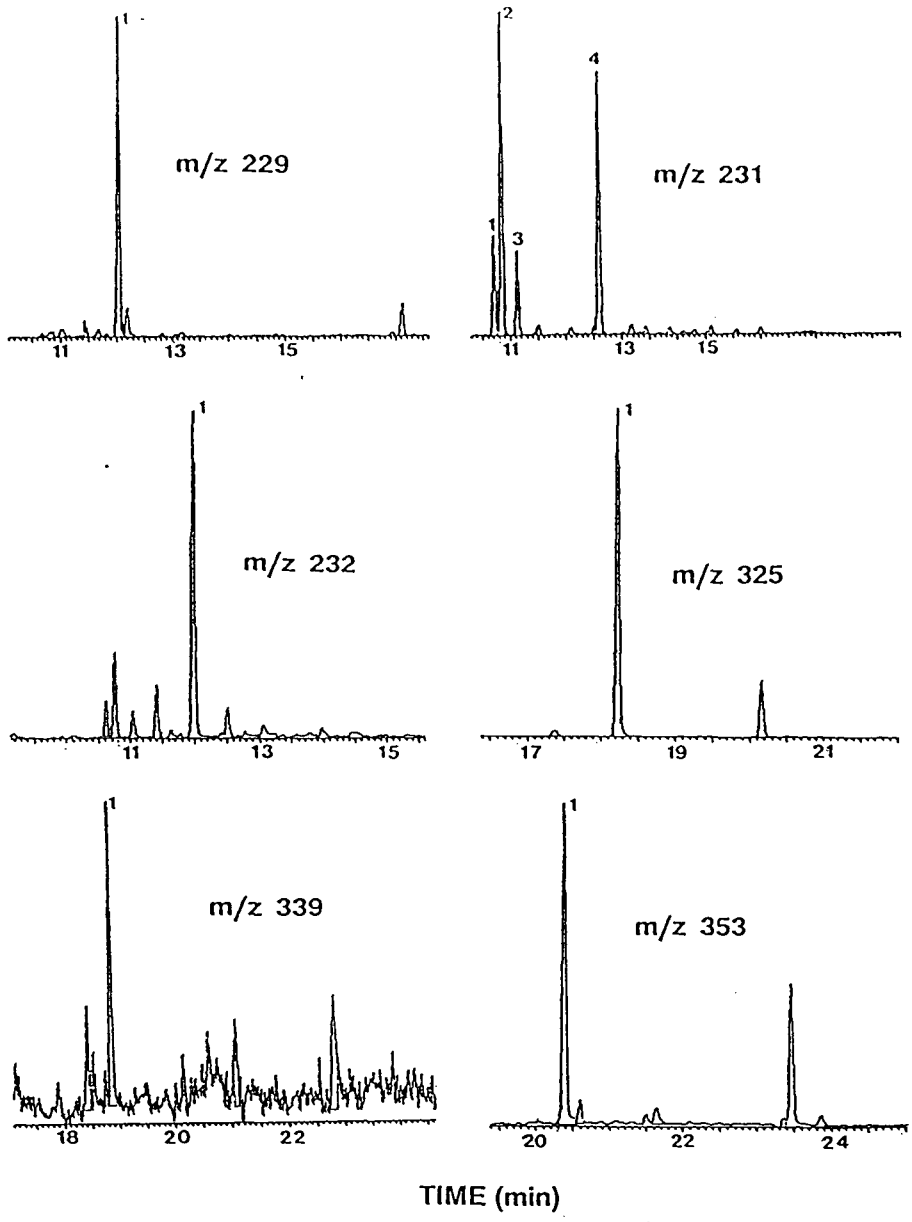


Figure 28B. Typical SIM chromatograms obtained from the serum extract of a patient on VPA to which internal standards have been added (same run as in Fig. 28A). Peaks: m/z 229, 1 = 3-keto VPA; m/z 331, 1 & 3 = 4-OH VPA, 2 = 3-OH VPA, 4 = 5-OH VPA; m/z 232, 1 = [$^{2}H_{3}$]3-keto VPA; m/z 325, 1 = 2-MGA; m/z 339, 1 = 2-PSA; m/z 353, 1 = 2-PGA.

Greater than 90% of the 3-keto VPA formed a diderivative i.e. O-TMS-PFB. Formation of a small amount of 3-keto VPA monoderivative did not present a problem because a deuterated analog was used as the internal standard. Even though the two geometric isomers of the O-TMS-PFB derivative of 3-keto VPA were formed, only one isomer, which accounted for 93% of the ion current, was monitored.

B. Analytical parameters

Calibration curve linearity was observed over the concentration ranges tested. Non-linear response was evident in the case of VPA when serum volumes of greater than 250 μL were used. Correlation coefficients were typically greater than 0.99 for all standard curves. The method showed good precision with a day to day variation of less than 15% for all 15 analytes. The day to day precision and accuracy of the assay for individual metabolites is shown in Table 3. The precision of the assay for analytes having deuterated analogs as internal standards (VPA, 2-ene-, 3-keto-VPA) was especially high, although this could be attributable to the higher levels measured for these metabolites. For 4-OH VPA it was necessary to maintain the extraction pH fairly constant in order to obtain reproducible results. A deuterated internal standard for 4-OH VPA would eliminate this problem.

Trace metabolites such as 4-ene- and (E,Z)-2,3'-diene-VPA were readily quantitated from 50 μL of serum. The lower limit of detection of the metabolites (signal to noise ratio $\geq$ 3) varied between 1 to 8 ng/mL based on a 250 μL sample of serum.

Table 3. Day to day precision and accuracy data for VPA and metabolites in serum (n = 5)

Compound	Spiked (μg/mL)	Found (μg/mL)	C.V. (%)
4-Ene VPA	0.30	0.31	6.2
	1.20	1.24	1.4
3-Ene VPA	0.39	0.38	10.2
	1.97	2.04	2.2
(Z)-2-Ene VPA	0.16	0.15	6.0
	0.65	0.67	1.1
(E)-2-Ene VPA	3.07	3.06	2.7
	15.39	15.52	0.8
(E,E)-2,3′-Diene VPA	3.10	3.20	6.2
	15.50	16.26	1.8
(E,Z)-2,3′-Diene VPA	0.18	0.21	11.0
	0.64	0.62	2.0
(E)-2,4-Diene VPA	0.20	0.19	12.3
	1.02	1.08	3.7
VPA	24.88	27.70	2.0
	124.40	125.50	1.5
4-OH VPA	0.38	0.37	11.8
	1.90	2.01	1.3
3-OH VPA	0.19	0.22	6.1
	0.98	0.98	3.6

Table 3. (continued). Day to day precision and accuracy data for VPA and metabolites in serum

Compound	Spiked (μg/mL)	Found (μg/mL)	C.V (%)
5-OH VPA	0.80	0.95	13.8
	1.60	1.50	6.1
4-Keto VPA	0.21	0.23	14.6
	1.04	1.04	3.9
3-Keto VPA	2.00	2.5	2.8
	10.00	9.72	1.3
2-PSA	0.19	0.17	15.6
	0.60	0.58	8.5
2-PGA	0.20	0.20	15.3
	1.00	1.05	2.4

C. VPA metabolite profiles in pediatric patients

The assay was employed to determine the levels of VPA and 14 of its metabolites in the serum and urine of 59 pediatric patients. The serum concentrations for 26 patients on VPA monotherapy are shown in Table 4. The major metabolites in serum were (E)-2-ene-, (E,E)-2,3'-diene- and 3-keto-VPA. The putative toxic metabolite, 4-ene VPA, was present at low levels (mean = 0.20 μg/mL). The mean concentration of the diene metabolite ((E)-2,4-diene VPA) suspected to be a hepatotoxin was 0.65 μg/mL.

The concentrations of metabolites (normalized to dose) in the patient groups on combined therapy with other antiepileptic drugs is shown in Table 5. The serum levels of 4-keto- and 5-OH-VPA were significantly ($P<0.05$) higher in the VPA + CBZ group compared to the monotherapy group. Also, the serum concentrations of VPA and its β-oxidation products were lower in the VPA + CBZ group. CLBZ had no significant effect on the VPA metabolite profile.

In urine the major metabolites were 3-keto-, 4-OH-VPA and 2-PGA (Table 6). The unsaturated metabolites were excreted mainly as conjugates, while the keto metabolites were predominantly excreted in the free form. The hydroxy metabolites and 2-PGA were found in urine in both the conjugated and free forms.

The levels of 4-ene- and (E)-2,4-diene-VPA were determined in the urine of two patients who had developed VPA-induced hepatotoxicity and were found not to be different from the levels in other patients with comparable VPA urinary concentrations.

Table 4. Serum concentrations (μg/mL) of VPA metabolites in 26 pediatric patients on VPA monotherapy

Compound	Mean	Range	% of VPA
4-Ene VPA	0.20	0.04-0.40	0.2
3-Ene VPA	1.80	0.72-3.30	2.0
(Z)-2-Ene VPA	0.11	0.05-0.18	0.1
(E)-2-Ene VPA	6.44	2.51-10.30	7.2
(E,E)-2,3′-Diene VPA	6.31	2.47-13.50	7.1
(E,Z)-2,3′-Diene VPA	0.28	0.06-0.38	0.3
(E)-2,4-Diene VPA	0.65	0.15-1.16	0.7
VPA	89.2	39.8-134.0	100
4-OH VPA	0.71	0.14-1.64	0.8
3-OH VPA	0.97	0.10-2.27	1.1
5-OH VPA	0.45	0.13-0.96	0.5
4-Keto VPA	0.60	0.16-1.27	0.7
3-Keto VPA	3.63	0.78-8.24	4.1
2-PSA	0.02	Tr.-0.15	0.02
2-PGA	0.16	0.02-0.30	0.2

Table 5. Mean concentrations normalized to dose (μg/mL/mg/kg) of VPA and metabolites in the serum of patients

Compound	VPA only n = 26	VPA + CBZ n = 21	VPA + CLBZ n = 12
4-Ene VPA	0.009	0.007	0.008
3-Ene VPA	0.109*	0.042	0.086
(Z)-2-Ene VPA	0.005*	0.002	0.005
(E)-2-Ene VPA	0.311*	0.136	0.274
(E,E)-2,3′-Diene VPA	0.304*	0.129	0.201
(E,Z)-2,3′-Diene VPA	0.012*	0.004	0.007
(E)-2,4-Diene VPA	0.035	0.029	0.029
VPA	4.88*	2.31	3.82
4-OH VPA	0.053	0.051	0.046
3-OH VPA	0.064	0.049	0.038
5-OH VPA	0.022	0.031*	0.028
4-Keto VPA	0.030	0.049*	0.026
3-Keto VPA	0.311*	0.126	0.102
2-PSA	0.005	0.006	0.004
2-PGA	0.010	0.007	0.006

* Significantly different at $p<0.05$ (VPA only vs. VPA + CBZ)

Table 6. Urinary concentrations (μg/mg creatinine) of the conjugated and unconjugated fractions of VPA metabolites in 9 pediatric patients on VPA monotherapy

Compound	Mean (Range)[a]	Mean (Range)[b]
4-Ene VPA	0.25 (0.02-1.53)	0.03 (Tr-0.17)
3-Ene VPA	0.21 (0.03-0.93)	0.05 (Tr-0.28)
(Z)-2-Ene VPA	0.94 (0.10-2.77)	0.03 (Tr-0.22)
(E)-2-Ene VPA	21.5 (1.0-54.3)	0.92 (Tr-5.73)
(E,E)-2,3′-Diene VPA	17.2 (3.85-56.9)	1.12 (Tr-6.12)
(E,Z)-2,3′-Diene VPA	1.23 (0.24-3.56)	0.16 (Tr-0.26)
(E)-2,4-Diene VPA	3.68 (0.18-11.5)	0.52 (Tr-3.17)
VPA	262 (15.6-1191)	15.3 (0.43-96)
4-OH VPA	17.2 (0.45-79.4)	29.7 (3.16-94.9)
3-OH VPA	10.6 (0.83-26.7)	10.1 (5.01-18.4)
5-OH VPA	5.21 (Tr-16.30)	12.6 (2.56-34.8)
4-Keto VPA	0.51 (Tr-3.60)	9.64 (1.09-28.1)
3-Keto VPA	7.93 (Tr-28.0)	160 (33.9-362)
2-PSA	0.23 (Tr-1.48)	1.57 (0.23-5.66)
2-PGA	6.45 (1.49-25.1)	32.0 (4.26-66.2)

[a] Conjugated fraction
[b] Unconjugated fraction

3. Metabolism studies involving VPA, 4-ene- and (E)-2,4-diene-VPA

A. Animal studies

Identification of the NAC conjugate of (E)-2,4-diene VPA in urine: When the urine extract from rats treated with (E)-2,4-diene VPA was methylated and analyzed by capillary GC/MS, a substantial part of the ion current was carried by the peak at retention time of 21.95 min. A representative chromatogram is shown in Fig. 29. The mass spectrum of this compound was identical to that of the synthetic NAC adduct of (E)-2,4-diene VPA (Fig. 14) and the compound had identical retention times with the synthetic standard when analyzed on two columns of different polarity. Using the same techniques the conjugate was also positively identified to be a prominent urinary metabolite of rats treated with 4-ene VPA (Fig. 30A). The conjugate could be detected in the urine extract of rats given a single dose of VPA but required the use of SIM because of the low levels of the conjugate.

Of the (E)-2,4-diene VPA dose administered to rats, 40.2% $\pm$ 8.7 (n=3) was excreted in urine as the NAC conjugate in 24 hr. The reversibility of the conjugation of (E)-2,4-diene VPA with NAC was investigated by heating the conjugate at 60°C in alkaline aqueous solution (pH=12). Under these conditions there was approximately 50% decomposition of the conjugate back to (E)-2,4-diene VPA and NAC.

The GSH conjugate of (E)-2,4-diene VPA in rat bile: The bile extract was methylated with diazomethane before analysis by HPLC and the GSH conjugate of (E)-2,4-diene VPA was identified as the methyl ester. Methylation was necessary as efforts to synthesize a relatively pure conjugate of the free acid of (E)-2,4-diene VPA were not successful. The reaction of the free acid with GSH was very slow and purification of the

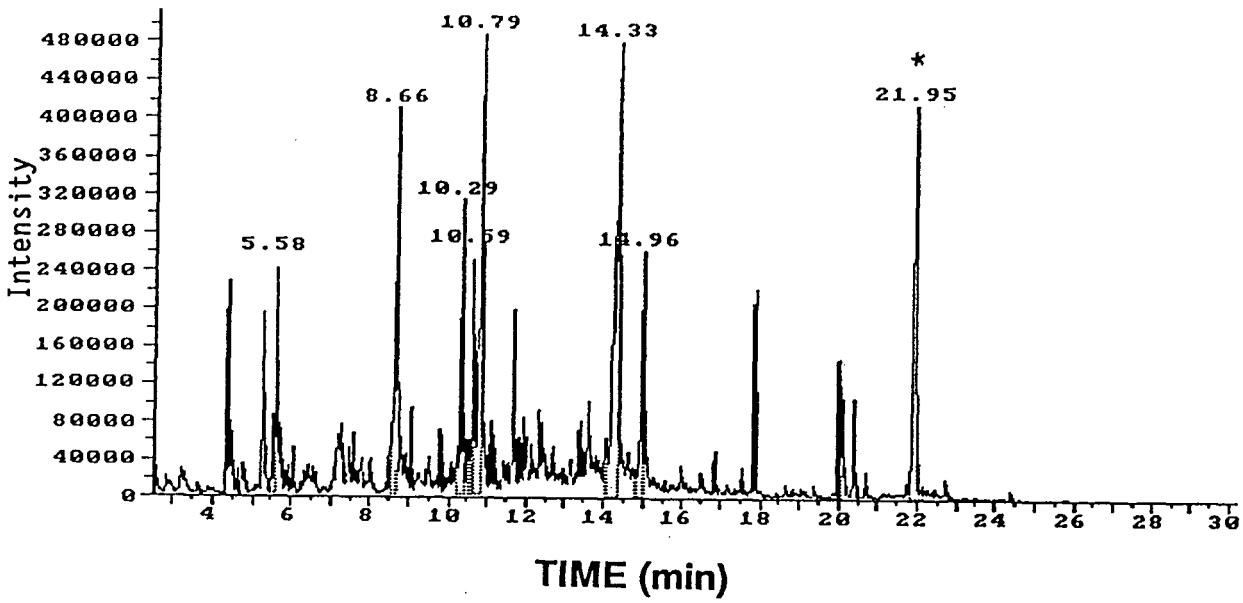


Figure 29. A representative TIC chromatogram (EI mode) from the methylated urine extract of a rat dosed with (E)-2,4-diene VPA. The peak marked with an asterisk was identified as the dimethyl ester of the NAC conjugate of (E)-2,4-diene VPA.

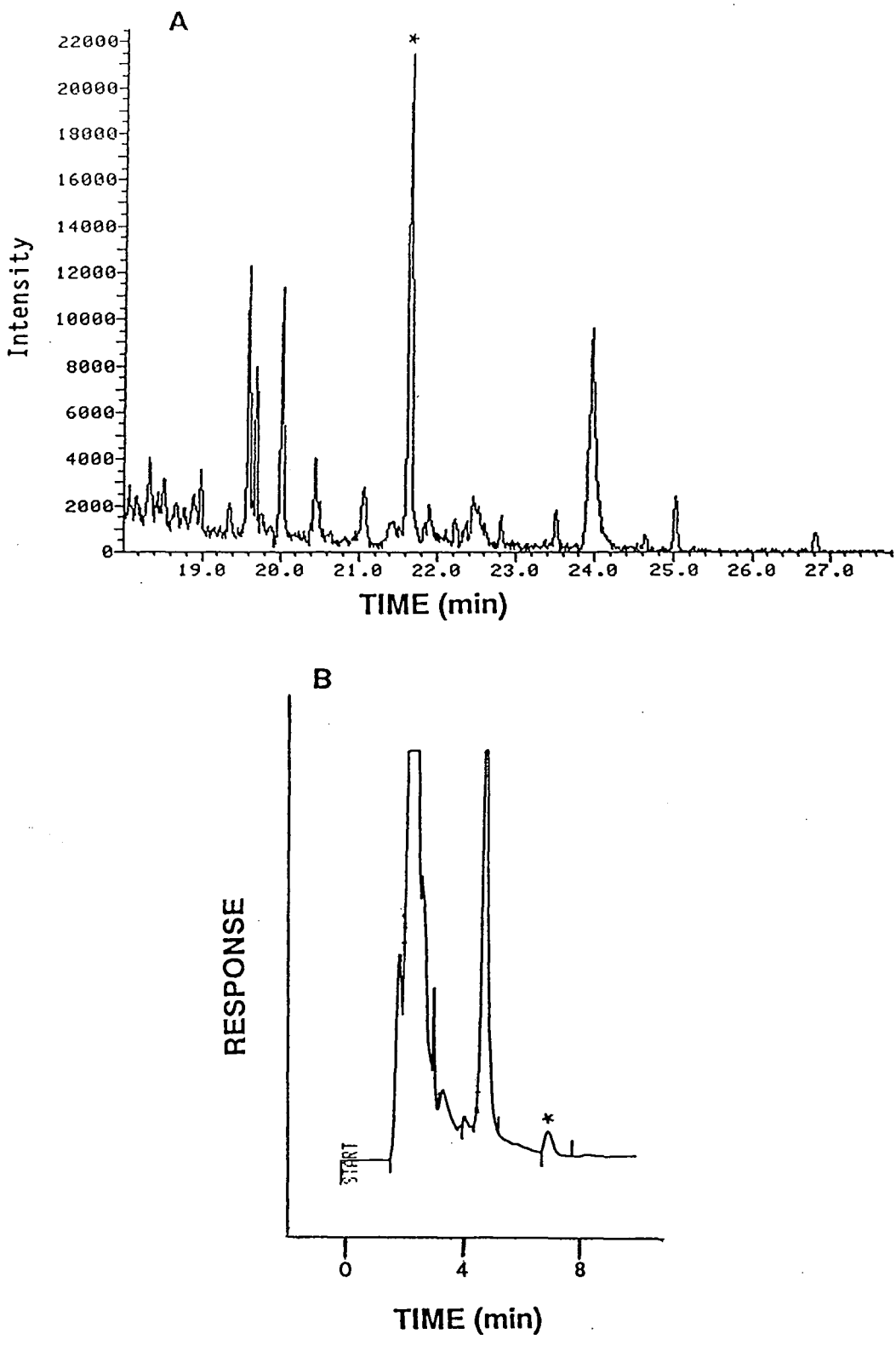


Figure 30. A) EI/TIC chromatogram from the methylated urine extract of a rat given 4-ene VPA. The peak marked with an asterisk was characterized as the methylated NAC conjugate of (E)-2,4-diene VPA. B) HPLC profile of the methylated bile extract of rats dosed with 4-ene VPA. The peak marked with an asterisk was identified as the methylated GSH conjugate of (E)-2,4-diene VPA.

conjugate by HPLC proved difficult because of co-eluting polar contaminants. The HPLC profile of the methylated bile extract from rats treated with (E)-2,4-diene VPA is shown in Fig. 31 together with a tri-methylated sample of synthetic GSH conjugate of (E)-2,4-diene VPA. The peak marked with an asterisk had the same retention time as the authentic standard. The peak from the bile extract co-chromatographed with the synthetic standard but was absent in control bile. An identical peak was present in the methylated bile extract of animals administered 4-ene VPA (Fig. 30B).

For spectral characterization these samples were analyzed by LC/MS/MS. Figure 32 is the LC/MS trace of the methylated bile extract from rats dosed with (E)-2,4-diene VPA. The mass spectra of the conjugate from synthetic and biological samples matched with each other and contained intense acetate attachment ions (m/z 548) of the methylated GSH conjugate of (E)-2,4-diene VPA (App. 15 and 16). The daughter ion (parent, m/z 548) spectra from the two sources were nearly identical as shown in Fig. 33. The spectra contained several ions which were structurally informative. Of particular significance was the fragment m/z 127 which was indicative of the position of the double bond.

Non-thiol metabolites of (E)-2,4-diene VPA: The following monounsaturated acids were identified and characterized as metabolites of (E)-2,4-diene VPA in rat urine: 3-ene- (stereochemistry not determined), 4-ene- and (E)-2-ene-VPA (Fig. 34). On the basis of peak height the level of 3-ene VPA was higher than that of (E)-2-ene VPA and that of 4-ene VPA was intermediate between the two.

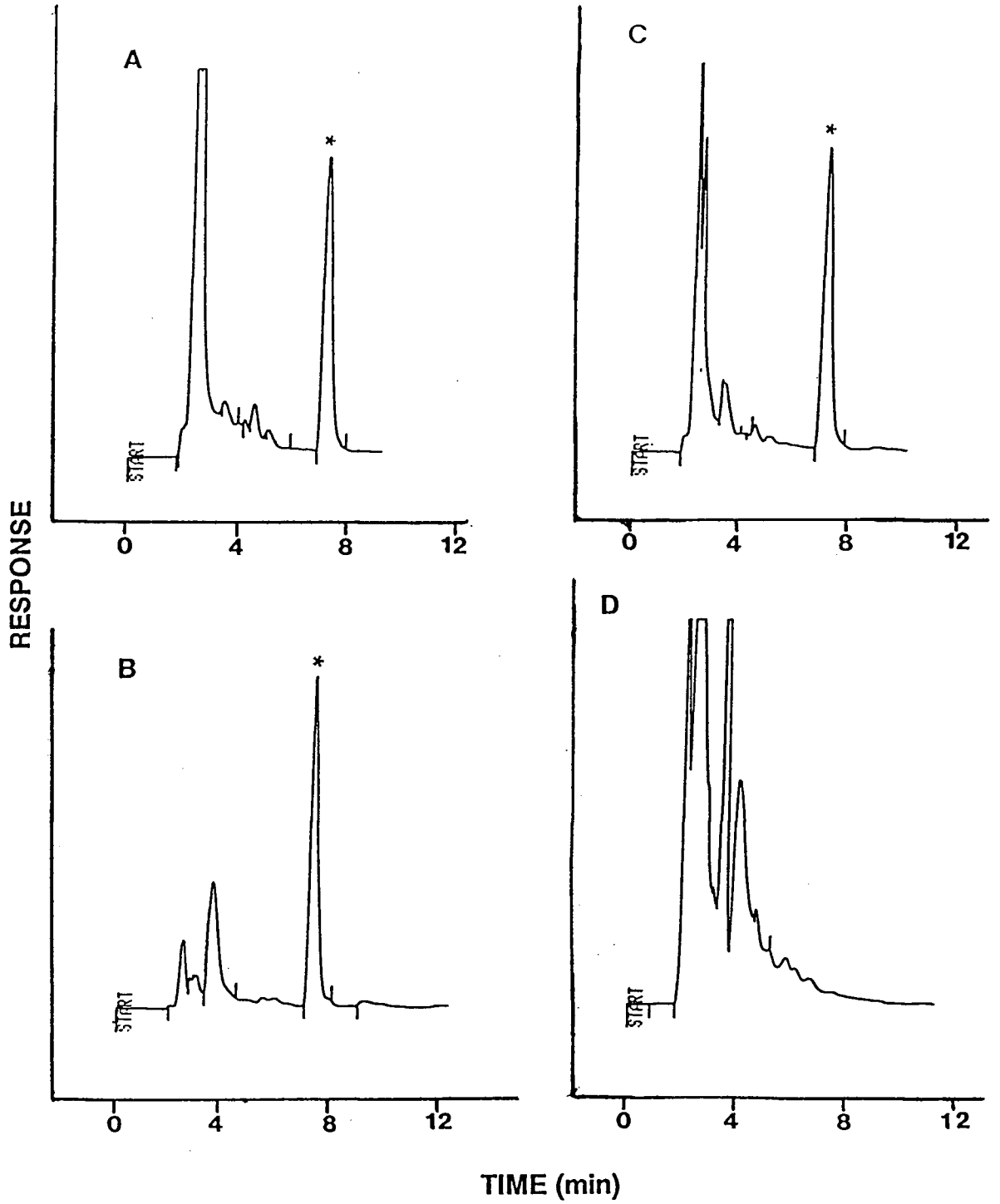


Figure 31. HPLC profile of the methylated bile extract of rats treated with (E)-2,4-diene VPA (A). The peak marked with an asterisk had the same retention time as the methylated synthetic sample of the GSH conjugate of (E)-2,4-diene VPA shown in (B) but was absent in the methylated control bile extract (D). Chromatogram C was obtained from a mixture of equivalent amounts of the conjugate in synthetic and bile samples.

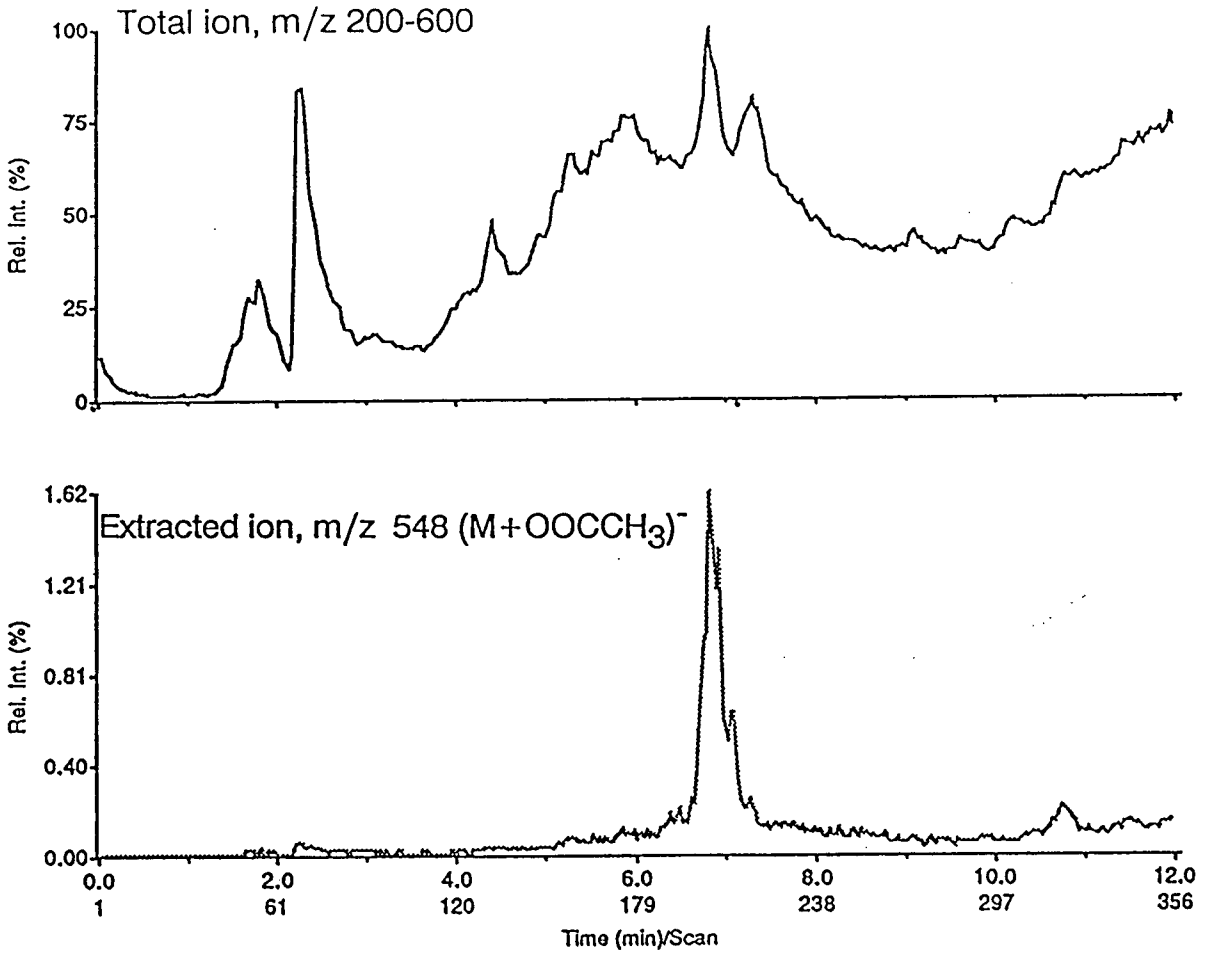


Figure 32. LC/MS chromatogram (negative ion mode) obtained from the methylated bile extract of rats dosed with (E)-2,4-diene VPA.

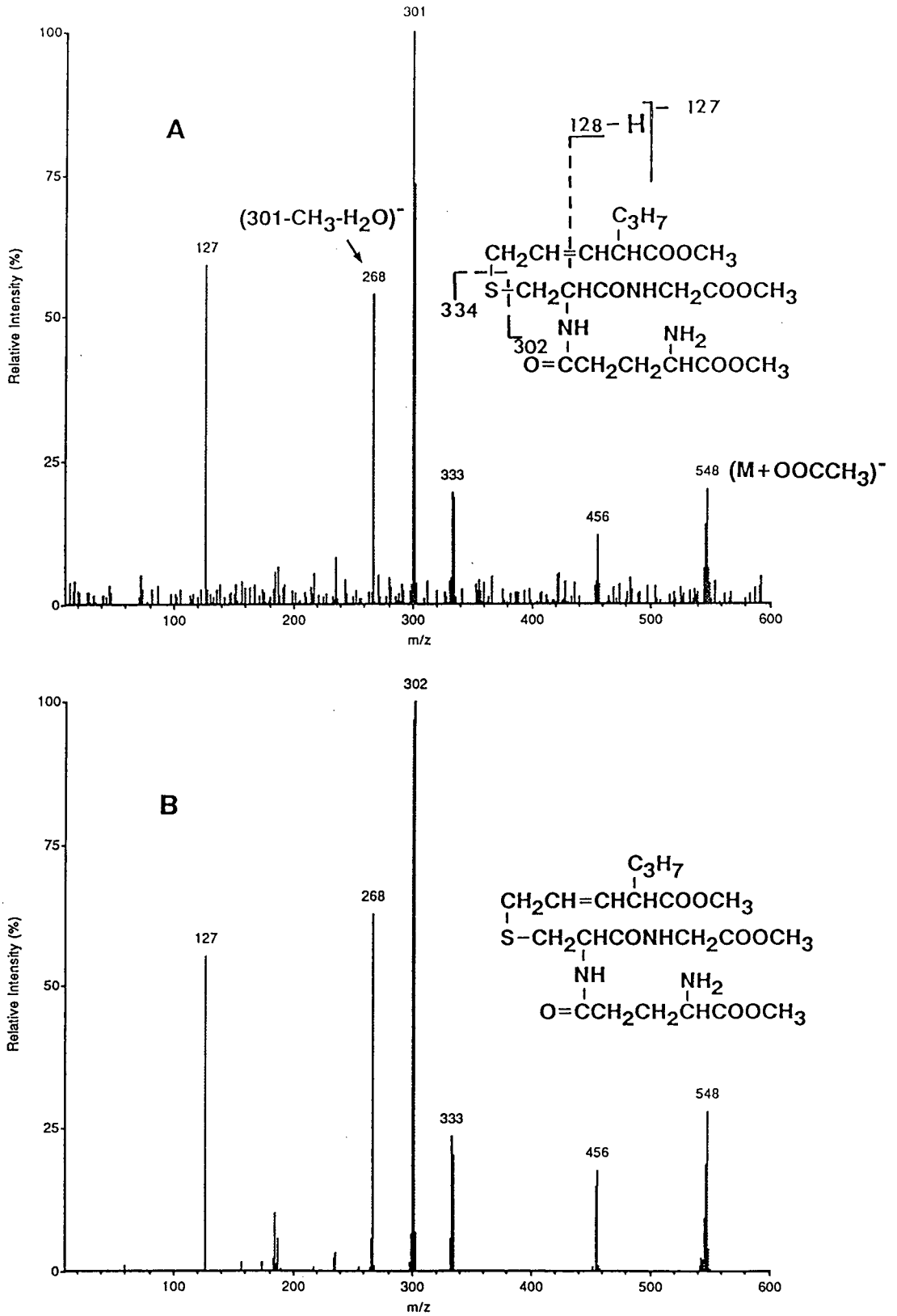


Figure 33. Daughter ion spectra obtained by argon CAD of the $(M+OOCCH_3)^-$ anion of the negative ion LC/MS spectrum of A) methylated bile extract from rats dosed with (E)-2,4-diene VPA and B) authentic methylated GSH conjugate of (E)-2,4-diene VPA.

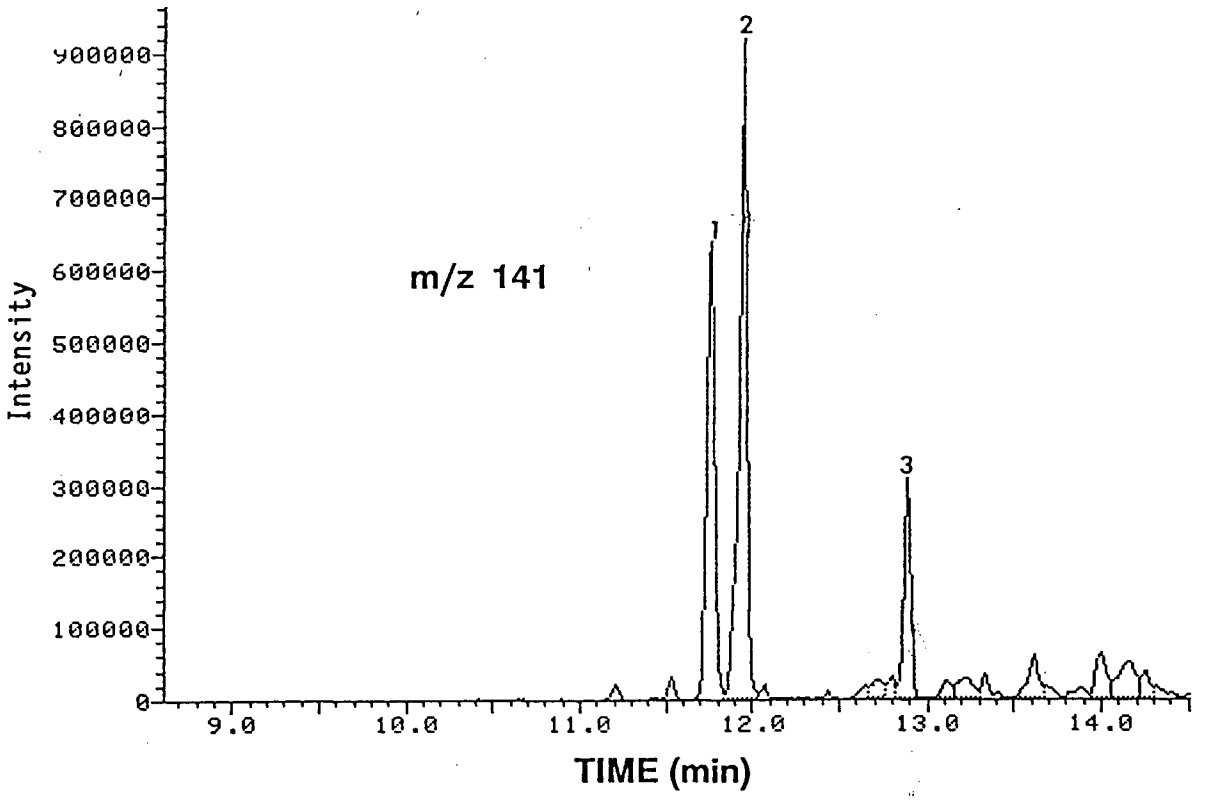


Figure 34. Mass chromatogram of m/z 141 obtained from the PFB derivatized urine extract of rats treated with (E)-2,4-diene VPA. Peaks 1, 2 and 3 were identified as 4-ene-, 3-ene- and (E)-2-ene-VPA, respectively.

The 3-OH-4-ene VPA was identified as a urinary metabolite of (E)-2,4-diene VPA (Fig. 35) with the mass chromatogram of m/z 229 ($[M-181]^-$) showing a single major peak. A similarly derivatized synthetic sample of 3-OH-4-ene VPA had the same GC/MS properties. A synthetic sample of another regioisomer, 3-OH'-4-ene VPA, was available and gave two diastereomeric peaks upon GC/MS analysis. This isomer could not be detected in urine samples.

Mass spectral evidence was obtained for the presence of the glycine amide conjugate of (E)-2,4-diene VPA in rat urine. The NICI mass spectrum of the PFB-derivatized urine extract (Fig. 36) contained the expected $[M-181]^-$ ion and another ion at $[M-181-CO_2]^-$ which appeared to be diagnostic for the glycine amide metabolite. The presence of a heteroatom adjacent to the α-carbon seems to result in the decarboxylation of the carboxylate anion fragment as was also demonstrated for 3-keto VPA (Kassahun *et al.*, 1989).

3-Keto-4-ene VPA and its thiol conjugates: The 3-keto-4-ene VPA and its thiol conjugates were sought in the biological fluids of rats treated with VPA, 4-ene-, and (E)-2,4-diene-VPA. The search for these compounds was made easier by the availability of synthetic standards. However, neither the 3-keto-4-ene VPA itself nor its thiol conjugates were evident in any of the treatment groups.

Disposition of the GSH conjugates of 3-keto-4-ene VPA and (E)-2,4-diene VPA in rats: The GSH conjugates of 3-keto-4-ene VPA and (E)-2,4-diene VPA (methyl esters of the carboxyl groups of the substrates) were administered i.v. to rats in order to determine the type of S-linked urinary metabolites of these conjugates. For both GSH conjugates the only detectable conjugated metabolites were the respective NAC

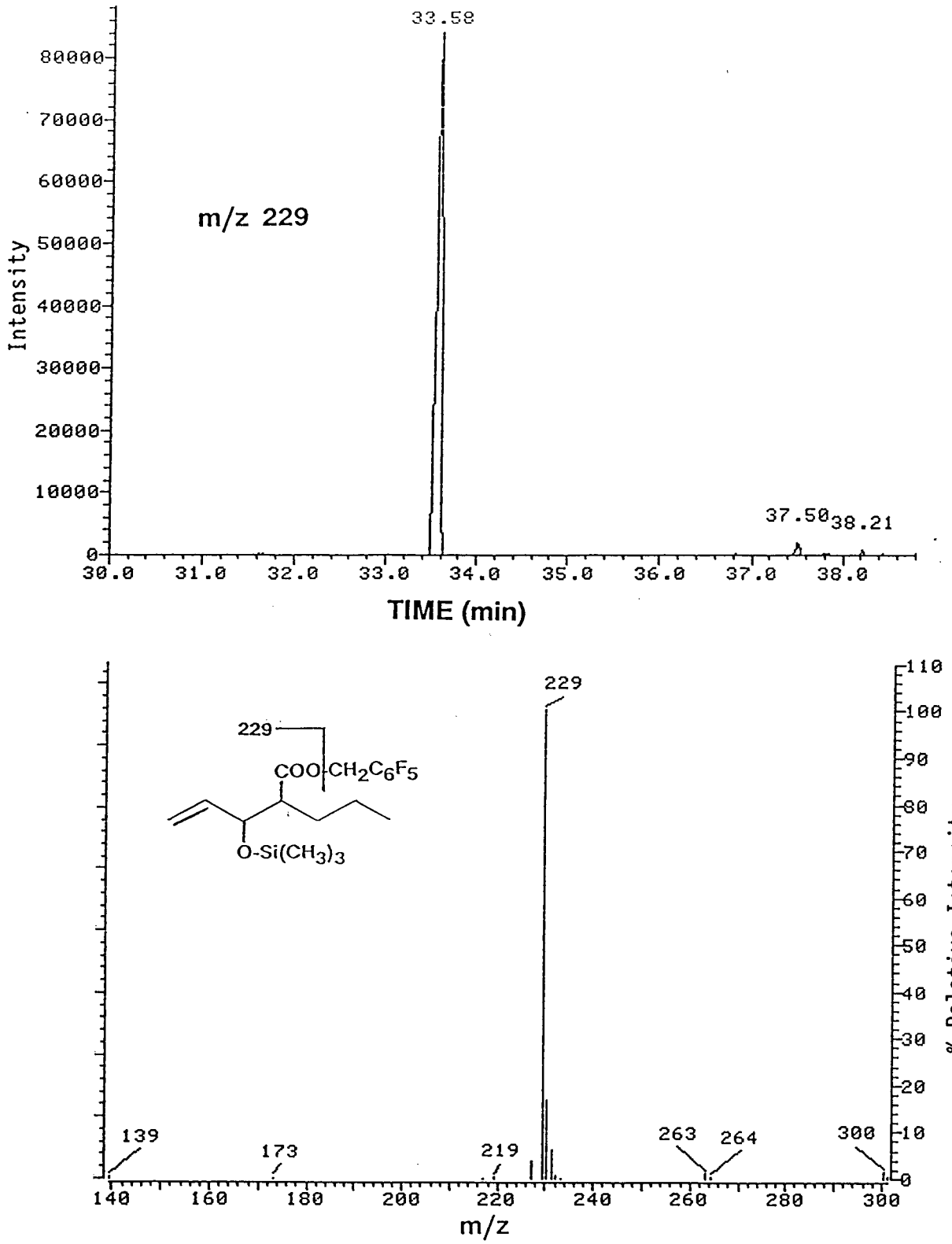


Figure 35. Extracted ion (m/z 229) from the PFB and TMS derivatized urine extract of rats given (E)-2,4-diene VPA (above) and the NICI mass spectrum of the peak with a retention time of 33.58 min (below).

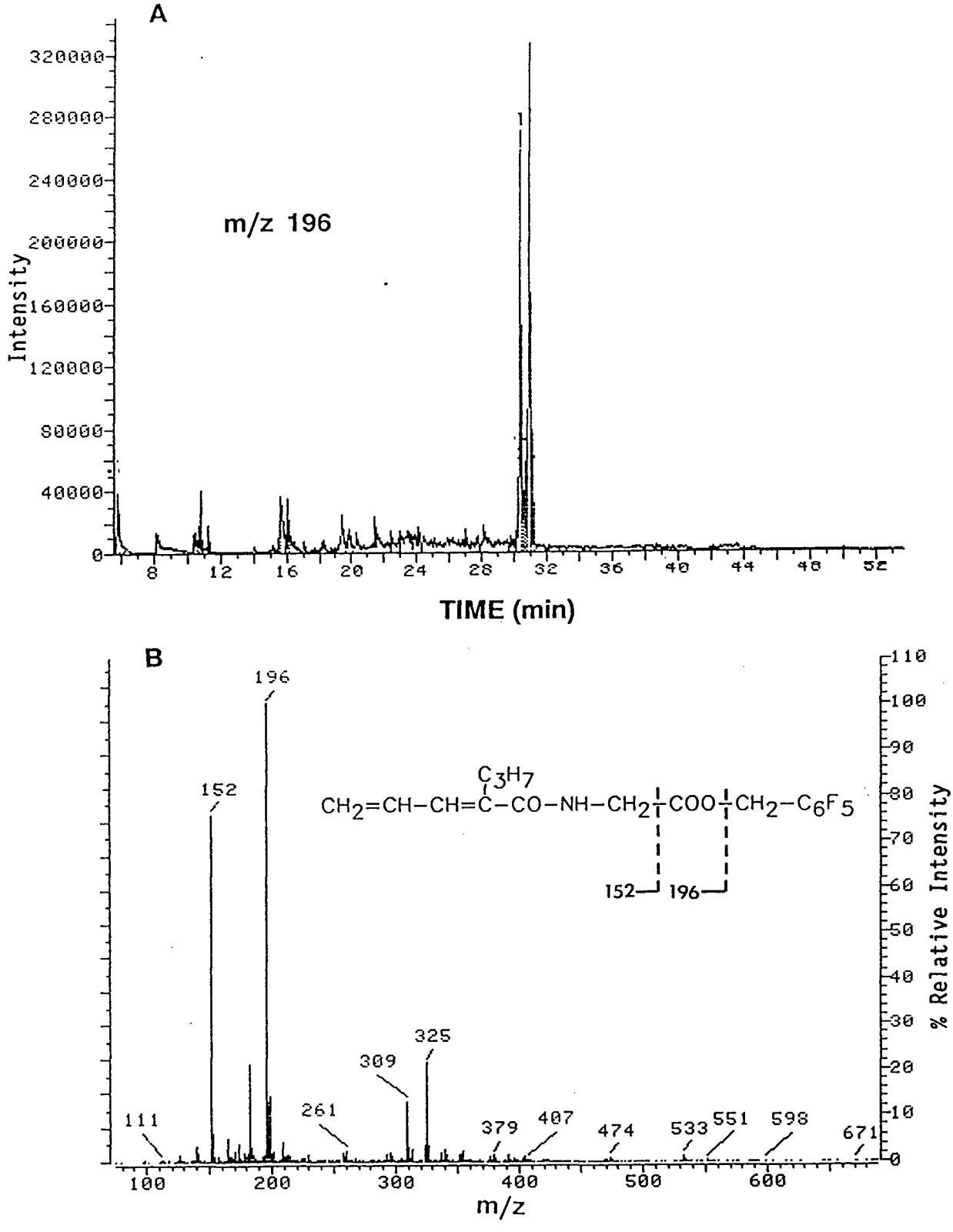


Figure 36. A) mass chromatogram (m/z 196, ($[M-181]^-$)) from the PFB derivatized urine extract of rats treated with (E)-2,4-diene VPA, B) NICI mass spectrum of peak 1 which on a mass spectral basis appears to be the glycine amide conjugate of (E)-2,4-diene VPA.

conjugates (Figs. 37 and 38). There was no evidence for the cysteine conjugates of these compounds. For the NAC conjugate of 3-keto-4-ene VPA GC/MS was not an appropriate method of analysis since the conjugate decomposed into the substrate and methyl NAC upon GC/MS analysis with no intact conjugate being observed. A satisfactory result was obtained only by LC/MS as shown in Fig. 38. The two peaks in Fig. 38 had the same MS/MS spectra and are assumed to be diastereomeric NAC conjugates of 3-keto-4-ene VPA. Although the monomethyl esters of the GSH conjugates were administered to rats, the respective NAC conjugates were recovered in the urine only as free acids.

B. Human study

Identification of the NAC conjugate of (E)-2,4-diene VPA in patients on VPA therapy: The NAC conjugate of (E)-2,4-diene VPA was found to be a urinary metabolite of VPA in patients. Figure 39, m/z 482, is a typical selected ion current chromatogram in the NICI mode obtained from the PFB derivatized urine extract of a patient. The ion monitored was the M-pentafluorobenzyl fragment anion of the di-PFB derivative of the conjugate. A full scan mass spectrum of the conjugate could also be obtained, in the NICI mode, from the derivatized urine extract of some of the patients.

Further evidence of the formation of the conjugate in humans was obtained from the urine extract of a volunteer who was given a 50:50 mixture of VPA and [$^2H_6$]VPA to steady state. By monitoring m/z 482, a mass chromatogram similar to that seen for patient samples (Fig. 39 top) was obtained. When the ion at m/z 487 [M-181+$^2H_5$]$^-$ was monitored (Fig. 39 bottom) a peak appeared at the expected retention time (25.82 min)

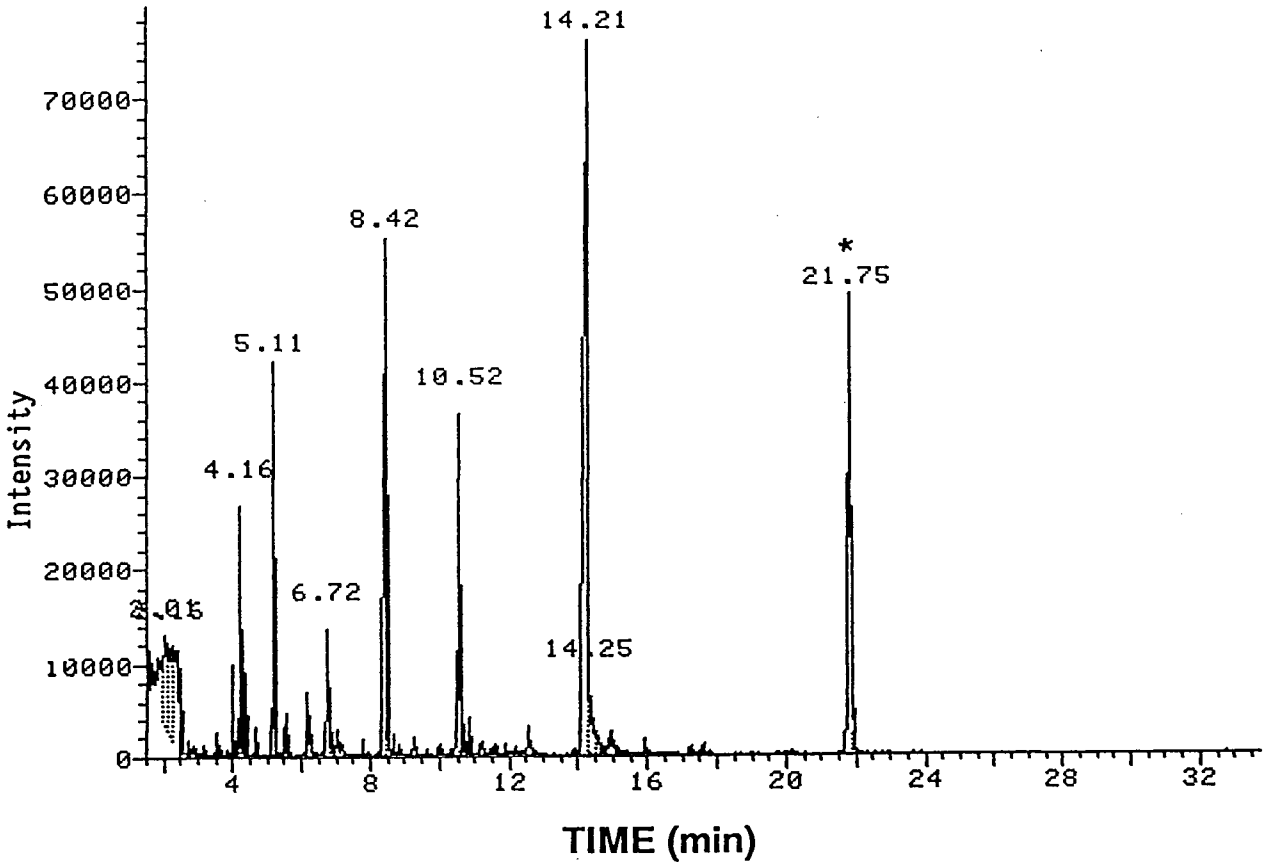


Figure 37. EI/TIC chromatogram of the methylated urine extract of rats dosed with the GSH conjugate of methyl (E)-2,4-diene VPA. The peak marked with an asterisk was identified to be the dimethyl ester of the NAC conjugate of (E)-2,4-diene VPA.

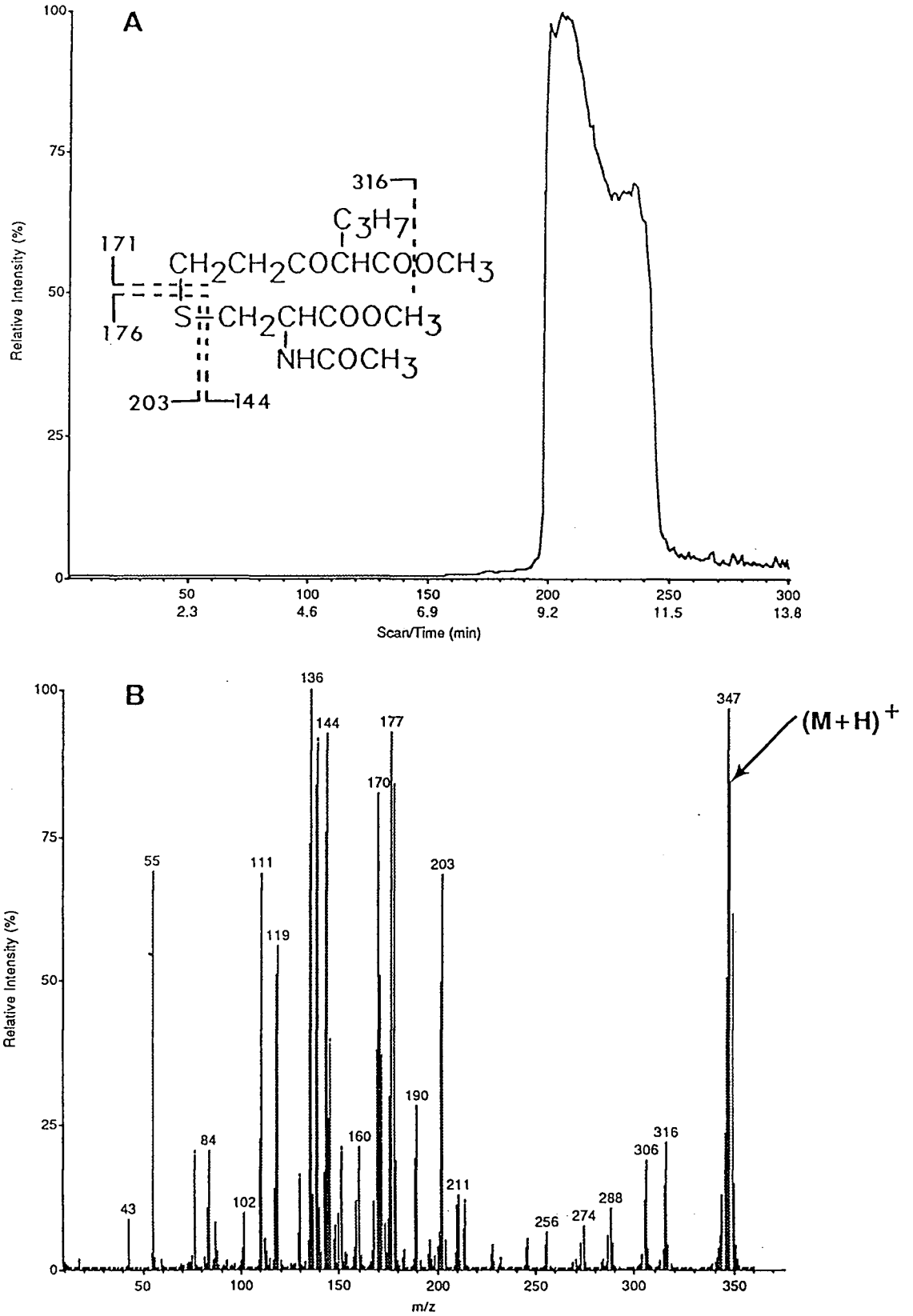


Figure 38. A) LC/MS extracted ion (m/z 348) obtained from the methylated urine extract of rats given the GSH conjugate of methyl 3-keto-4-ene VPA, B) MS/MS of scans 198-218 which were characterized to represent the dimethyl ester of the NAC conjugate of 3-keto-4-ene VPA.

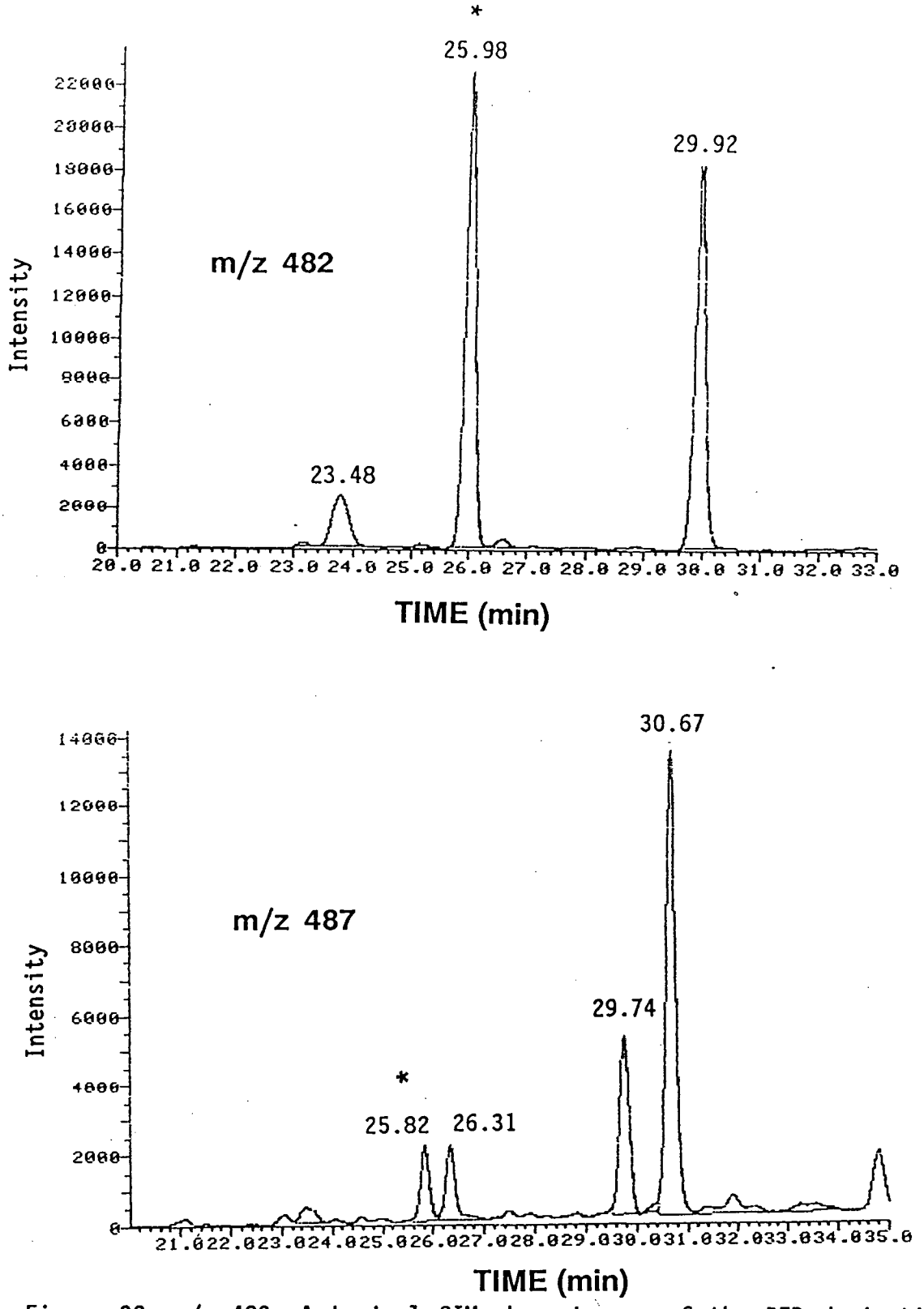


Figure 39. m/z 482: A typical SIM chromatogram of the PFB derivatized urine extract of an epileptic patient on VPA therapy. The peak marked with an asterisk corresponds to the di-PFB derivative of the NAC conjugate of (E)-2,4-diene VPA. m/z 487: Peak marked with an asterisk is the pentadeuterated analog of the di-PFB derivative of the NAC conjugate of (E)-2,4-diene VPA obtained from the urine extract of a volunteer administered six doses of a 50:50 mixture of VPA and [$^2H_6$]VPA.

i.e. slightly before the unlabelled metabolite (25.98 min). It is to be noted that the deuterated analogs of VPA metabolites elute slightly ahead of their unlabelled counterparts on capillary columns used for their analysis (Kassahun *et al.*, 1989). In the bottom chromatogram of Fig. 39 the peaks with retention times of 26.31 and 30.67 min are background peaks also present in control urine. The peak with a retention time of 29.74 min appears to be an isomer of the conjugate (see below).

From the ion chromatogram obtained from the urine extract of the volunteer the m/z 482 to 487 ratio was not unity even though a 50:50 mixture of VPA and [$^2H_6$]VPA was administered. Instead the level of the deuterated conjugate was 65% of the undeuterated analog. This small isotope effect was also observed in the metabolism of [$^2H_6$]VPA to (E)-[$^2H_5$]2,4-diene VPA.

As seen in Fig. 39 there was a second peak at m/z 482 (retention time 29.92 min), about equal in intensity to the peak identified as the NAC conjugate of (E)-2,4-diene VPA (retention time 25.98 min). This peak was absent in control urine and is thought to be an isomer of the conjugate. The synthetic sample of the NAC conjugate of (E)-2,4-diene VPA also contained this peak but at less than 10% of the intensity of the peak for the major isomer (App. 22). A corresponding isomer was also found in the urine extract of the subject administered labelled VPA (Fig. 39, m/z 487, 29.74 min).

Quantitation of the NAC conjugate of (E)-2,4-diene VPA in human urine: The urinary concentration of the NAC conjugate of (E)-2,4-diene VPA was determined in 28 pediatric patients on VPA therapy. Quantitation of the conjugate was carried out using an NICI/GC/MS method developed

for this purpose. Figure 40 shows the SIM chromatograms of the $[M-181]^-$ anions obtained from the internal standard and synthetic sample extracted from urine. A typical calibration curve is shown in Fig. 41. The assay had good precision with intra- and inter-day variation of less than 5 and 10%, respectively. The lower limit of detection (signal-to-noise ratio=3) was 10 pg injected on column. The mean concentration of the conjugate in the urine of these 28 patients was 1.38 μg/ml with a range of trace to 5.74 μg/ml. The level of the conjugate when normalized to creatinine was 3-4 times higher in two patients who had developed VPA induced liver failure compared to those without this side effect. There was no difference in the urinary level of the (E)-2,4-diene VPA itself among the patients studied.

4. <u>Identification and characterization of the thiol conjugates of reactive metabolites of 4-pentenoic acid</u>

Urinary metabolites: Figure 42 illustrates a partial EI/TIC chromatogram obtained from the methylated urine extract of a rat dosed with 100 mg/kg of (E)-2,4-pentadienoic acid. The peak labelled 1 was identified as the cysteine conjugate of 3-oxo-4-pentenoic acid using various mass spectral techniques and by comparison with a synthetic standard. The retention times (GC/MS, LC/MS (Fig. 43)) as well as mass spectra (LC/MS (Fig. 44), PCI/GC/MS, EI/GC/MS (Fig. 45)) of the isolated metabolite were the same as those of the synthetic standard. The synthesized monomethyl NAC conjugate of 3-keto-4-pentenoate was converted to the dimethyl ester by treating the conjugate with methanolic HCl gas followed by CH_2N_2. This, however, resulted not only in the formation of the dimethyl ester but also caused deacetylation of

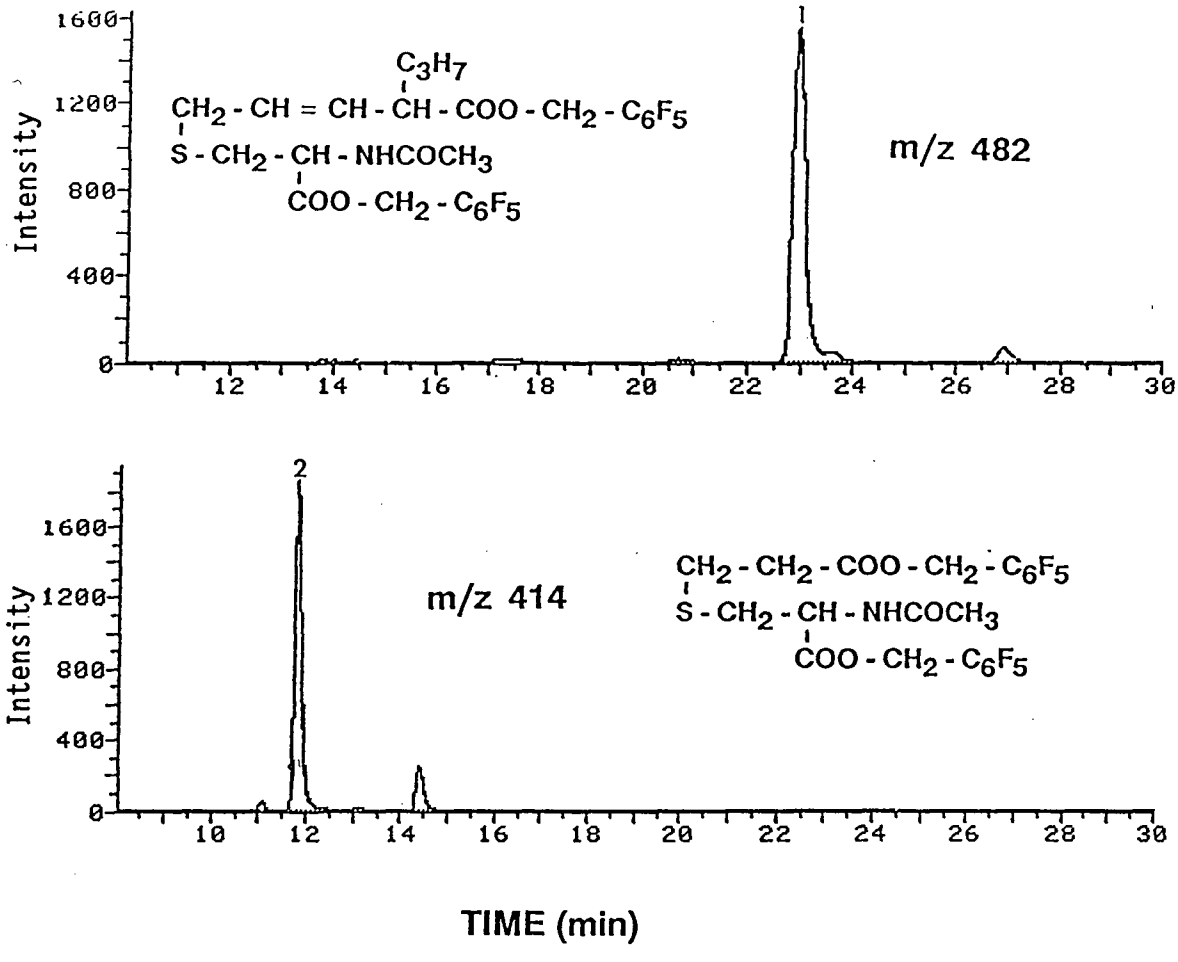


Figure 40. SIM chromatograms ($[M-181]^-$) of the PFB derivatives of the analyte (m/z 482, peak 1) and internal standard (m/z 414, peak 2) extracted from control urine.

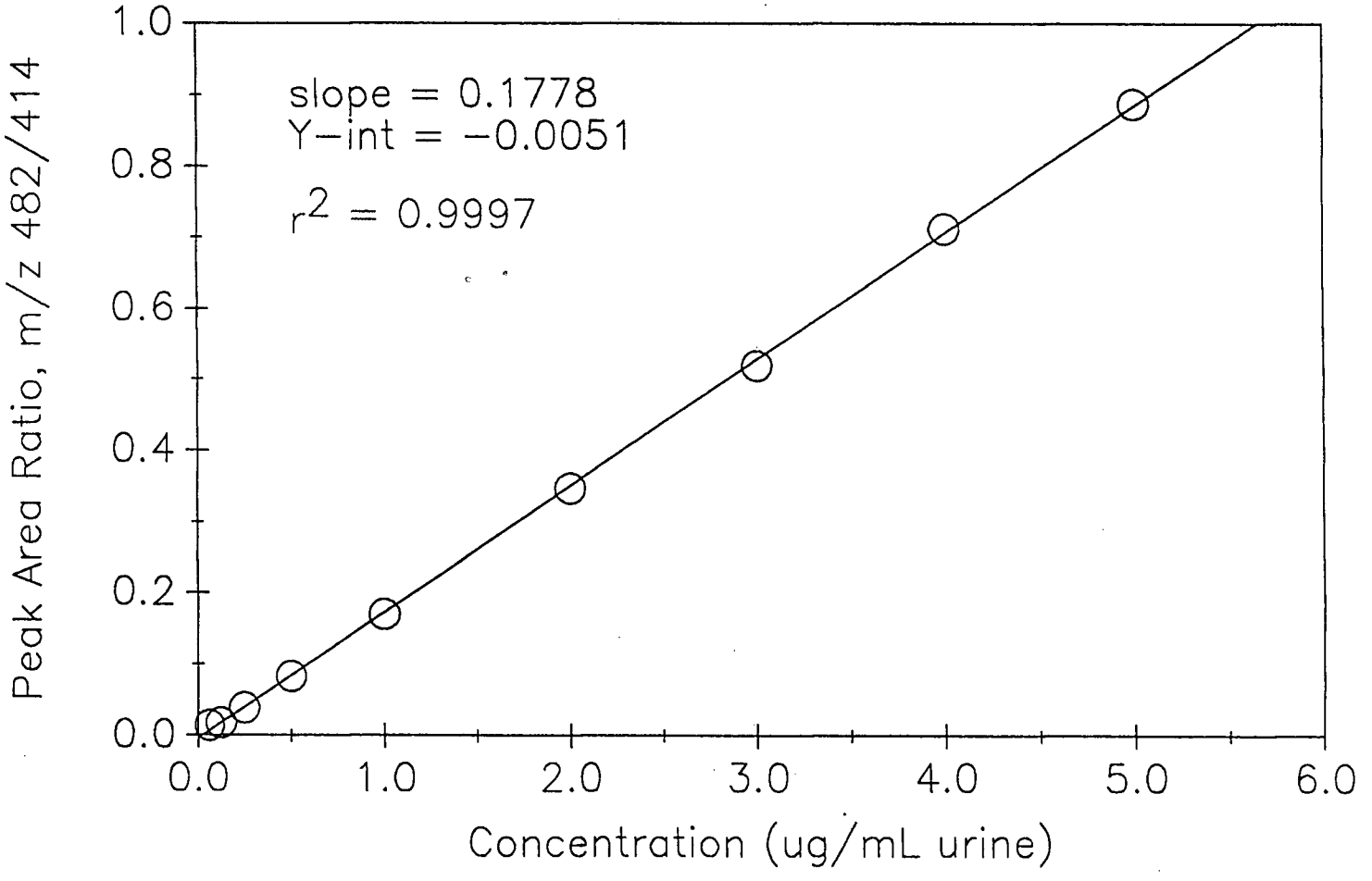


Figure 41. A typical calibration curve for the quantitation of the NAC conjugate of (E)-2,4-diene VPA obtained from the PFB derivative of the conjugate using the NAC conjugate of acrylic acid as the internal standard.

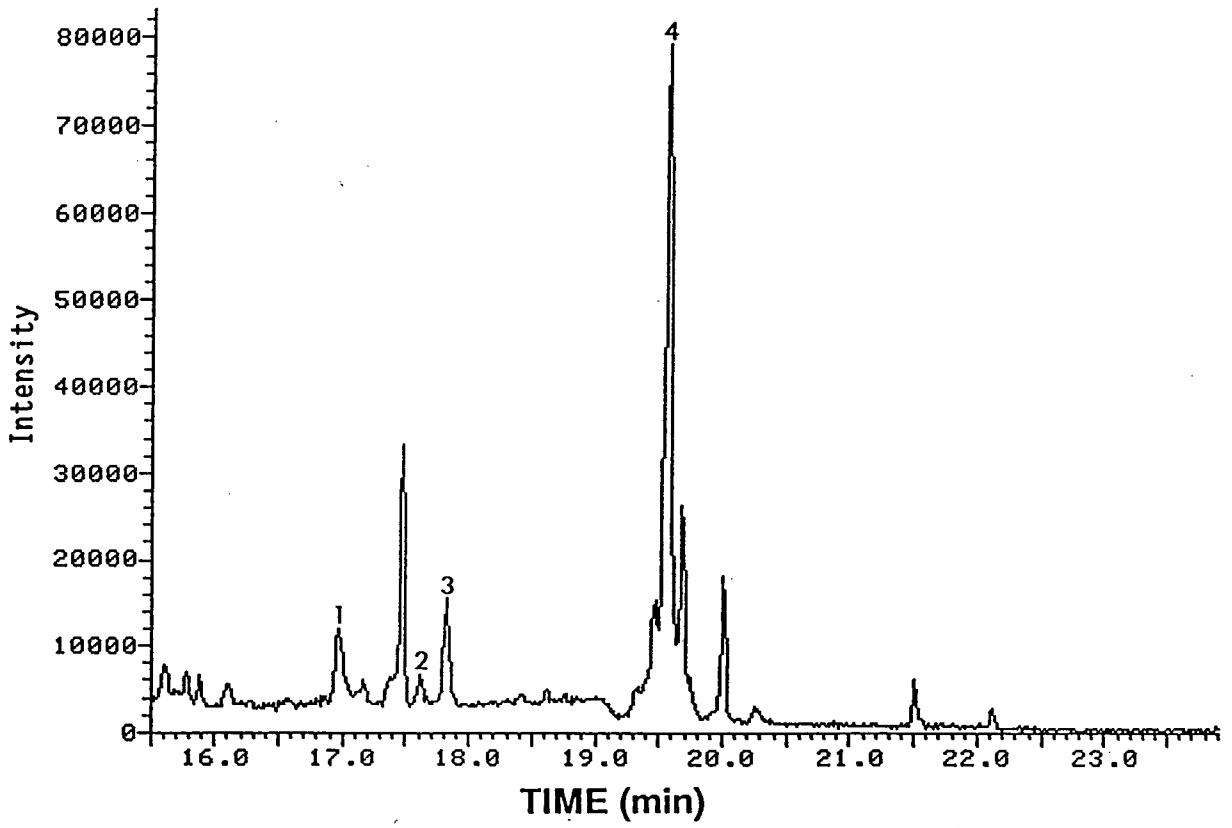


Figure 42. EI/TIC chromatogram of the methylated urine extract of rats dosed with (E)-2,4-pentadienoic acid. Peaks labelled were identified to be the cysteine* conjugate of 3-oxo-4-pentenoate (peak 1) and isomeric NAC conjugates of (E)-2,4-pentadienoate (peaks 2, 3 and 4).

* the NAC conjugate was shown to be deacetylated upon treatment with methanolic HCl gas followed by CH_2N_2.

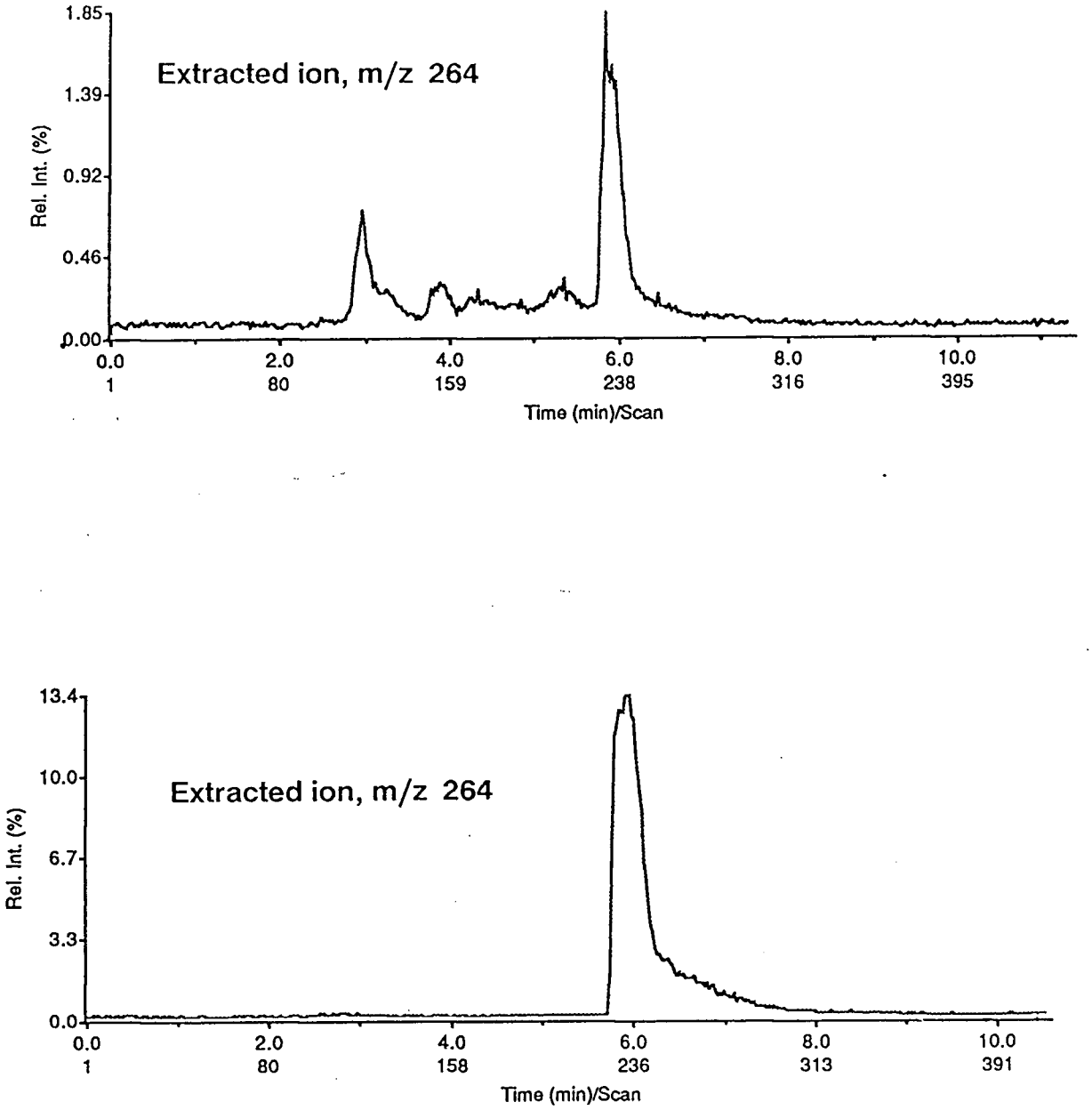


Figure 43. LC/MS trace (extracted ion): from the methylated urine extract of rats given (E)-2,4-pentadienoic acid (top) and synthetic cysteine conjugate of methyl 3-oxo-4-pentenoate (bottom).

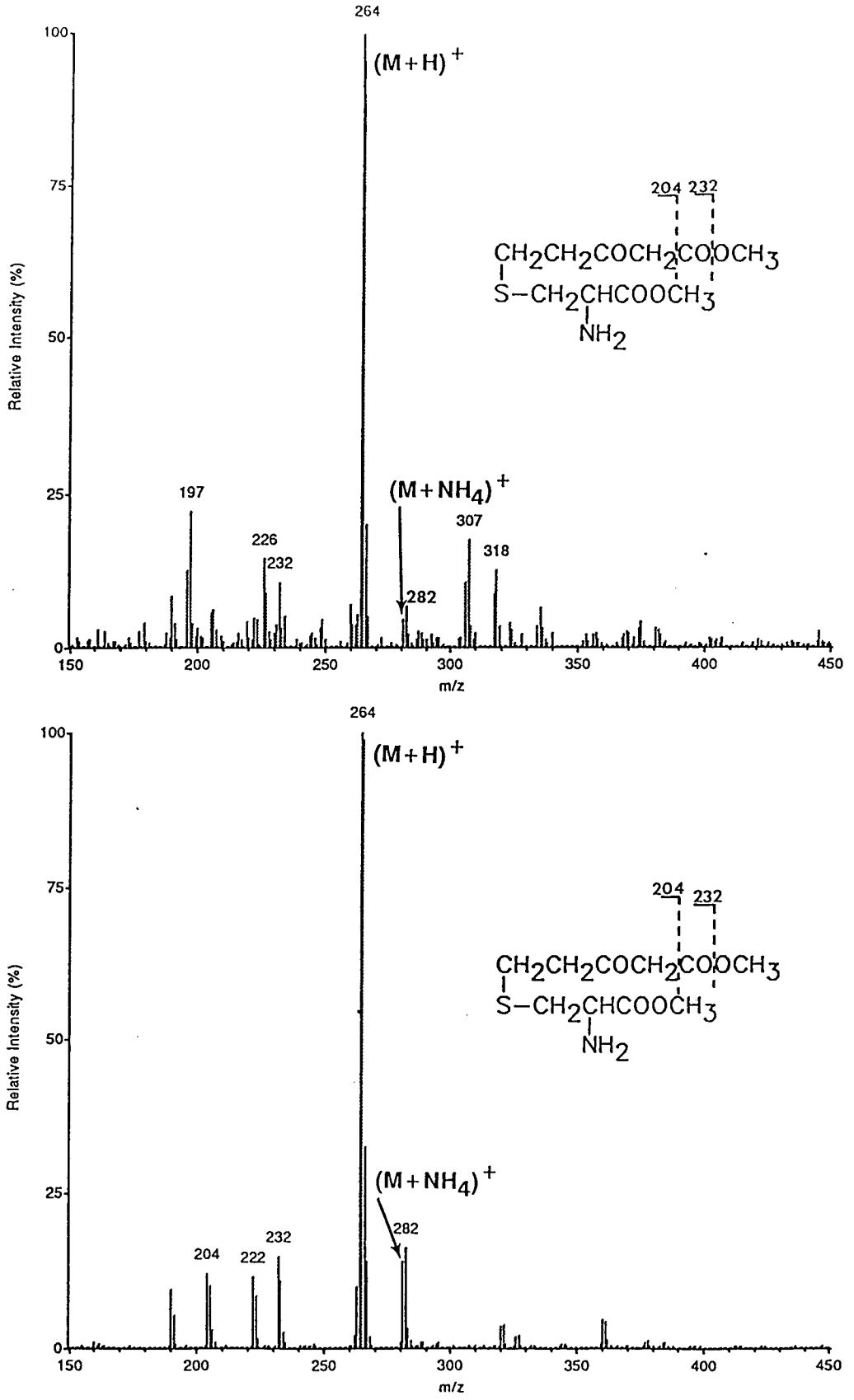


Figure 44. LC/MS spectra: scans 231-237 (Fig. 43, top) and scans 228-239 (Fig. 43, bottom).

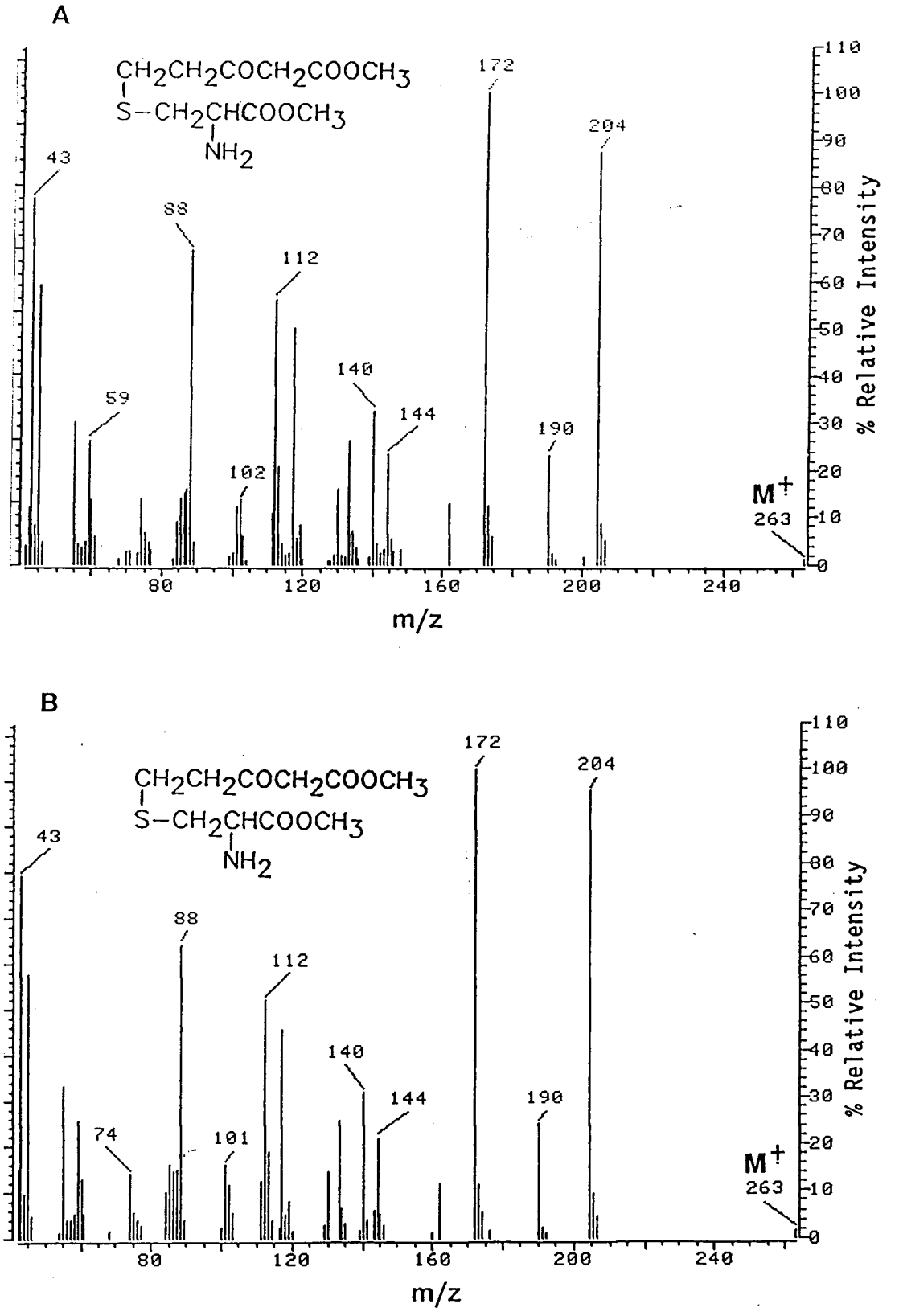


Figure 45. EI/GC/MS spectra of A) synthetic cysteine conjugate of methyl 3-oxo-4-pentenoate and B) peak 1, Fig. 42.

the NAC conjugate to yield the cysteine conjugate. The identity of this S-linked compound was established using EI, PCI and LC/MS mass spectral techniques. Because the urine extract was also treated the same way (i.e. methanolic HCl gas followed by CH_2N_2) it appears that the cysteine conjugate was formed artificially from the NAC conjugate *in vitro*.

In Fig. 42 the peaks marked 2, 3 and 4 were identified as isomeric NAC conjugates of (E)-2,4-pentadienoic acid by comparing retention times and mass spectral data (Fig. 46) with an authentic standard. The major peak was assigned the structure (E)-5-(N-acetylcystein-S-yl)3-pentenoate on the basis of the spectroscopic data of the synthetic compound. Thus the main NAC conjugate of (E)-2,4-pentadienoic acid in the rat is (E)-5-(N-acetylcystein-S-yl)3-pentenoic acid. The same conjugate was identified as a urinary metabolite of rats treated with 4-pentenoic acid. The amount of the NAC conjugate of (E)-2,4-pentadienoic acid recovered in urine of rats treated with either (E)-2,4-pentadienoic acid or 4-pentenoic acid was much less than that of the NAC conjugate of (E)-2,4-diene VPA recovered after comparable doses of (E)-2,4-diene VPA or 4-ene VPA.

The NAC conjugate of a third chemically reactive metabolite of 4-pentenoic acid, namely acrylic acid, was also identified and characterized in the urine of rats dosed with (E)-2,4-pentadienoic acid. The level of this conjugate, as judged by its peak height, was much smaller than that of the NAC conjugate of (E)-2,4-pentadienoic acid.

Biliary metabolites: Bile from (E)-2,4-pentadienoic acid treated rats was purified by solid phase extraction, methylated and analyzed by LC and LC/MS/MS. Figure 47A is the LC/MS trace (extracted ion, m/z 478) of the methylated bile extract obtained in the positive ion mode using a

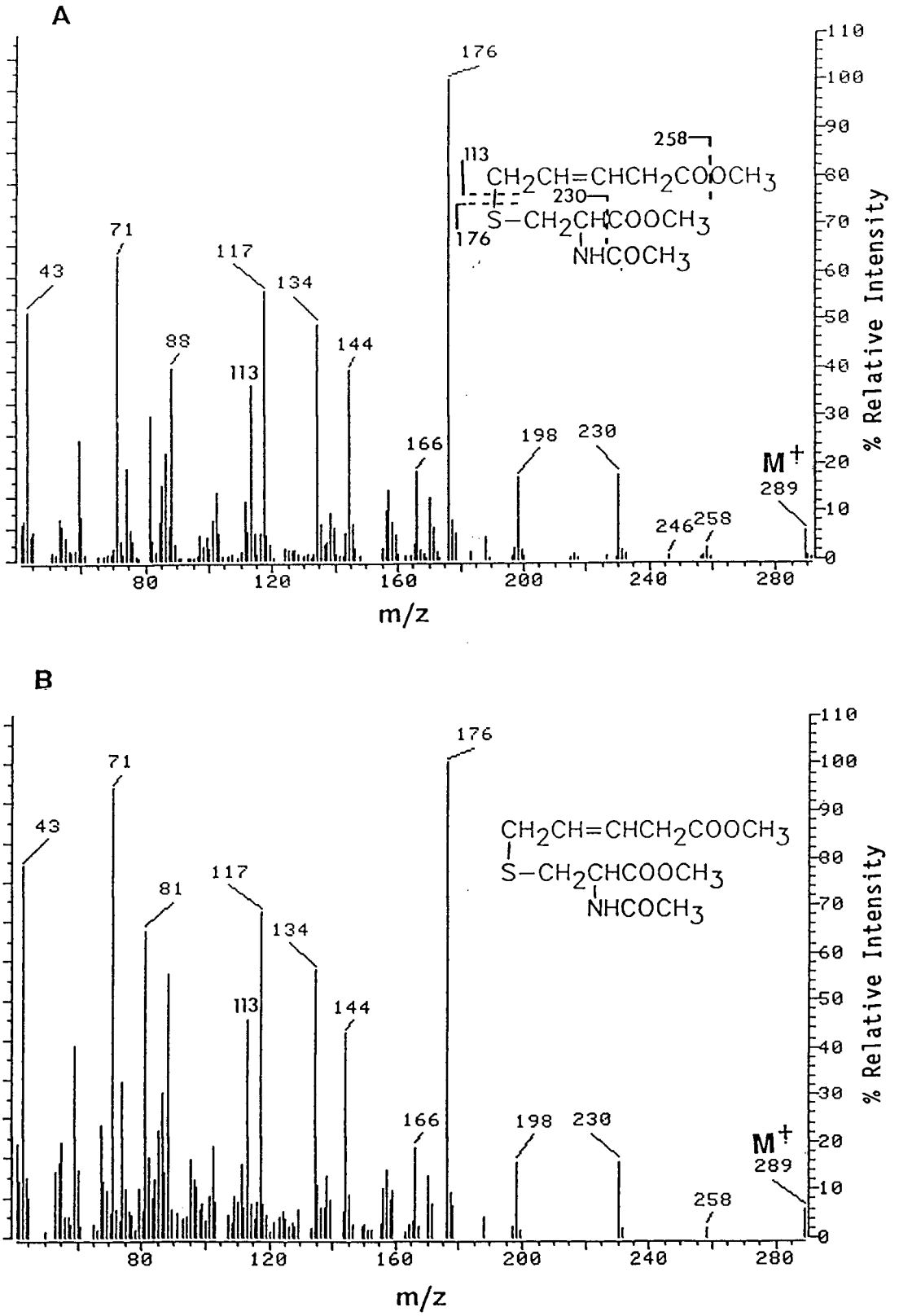


Figure 46. EI/GC/MS spectra of A) authentic NAC conjugate of (E)-2,4-pentadienoate (dimethyl ester) and B) peak 4, Fig. 42.

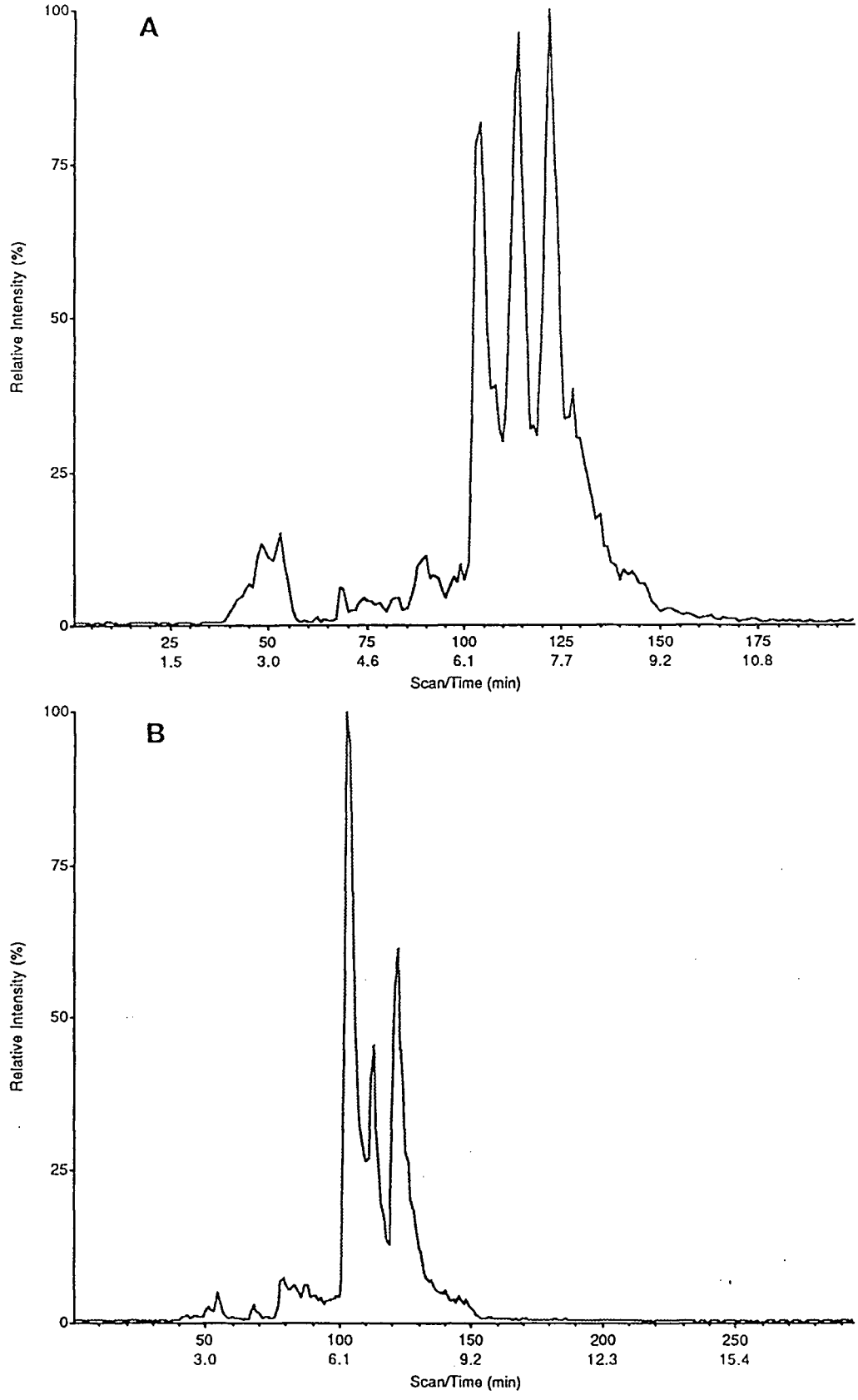


Figure 47. Positive ion LC/MS chromatogram (extracted ion, m/z 478), A) from the methylated bile extract of rats dosed with (E)-2,4-pentadienoic acid, B) methylated authentic GSH conjugate of 3-oxo-4-pentenoate.

mixture of CH_3CN and NH_4COOCH_3 buffer as the mobile phase. The corresponding extracted ion chromatogram from the methylated synthetic standard is shown in Fig. 47B. The ion at m/z 487 represents $(M+H)^+$ of the tetra-methylated GSH conjugate of 3-oxo-4-pentenoate. There were three major peaks at m/z 478 for both the synthetic and biological samples. The daughter ion spectra of the peaks from the bile extract were identical to those from the synthetic sample as shown in Fig. 48 for one pair of these peaks. The three peaks had the same MS/MS fragmentation patterns except for minor differences in the intensity of some of the fragment ions. Thus isomeric GSH conjugates of 3-oxo-4-pentenoate were present in the methylated bile extract of rats dosed with (E)-2,4-pentadienoic acid.

In addition to the two free carboxyl groups, a third carbon of the GSH adduct of methyl 3-oxo-4-pentenoate was methylated upon treatment with diazomethane to give $(M+H)^+$ ion of m/z 478. The fragment ion, m/z 143 (Fig. 48), indicated that the additional methyl group was on the substrate portion of the conjugate. Because the methylene protons of the carbon between the two carbonyl groups are acidic, it appears that this carbon was the site of the additional methylation. This would give a pair of diastereomers and would account for two of the three isomers observed. The keto function of the substrate moiety of the molecule may be enolized and consequently the hydroxyl group methylated. This enolized isomer may account for the third isomer observed.

Figure 49A represents an LC/MS extracted ion chromatogram (m/z 448) from the methylated bile extract of rats given (E)-2,4-pentadienoic acid. In Fig. 49B the LC/MS trace (extracted ion, m/z 448) representing the $(M+H)^+$ of the the methylated synthetic GSH conjugate of (E)-2,4-

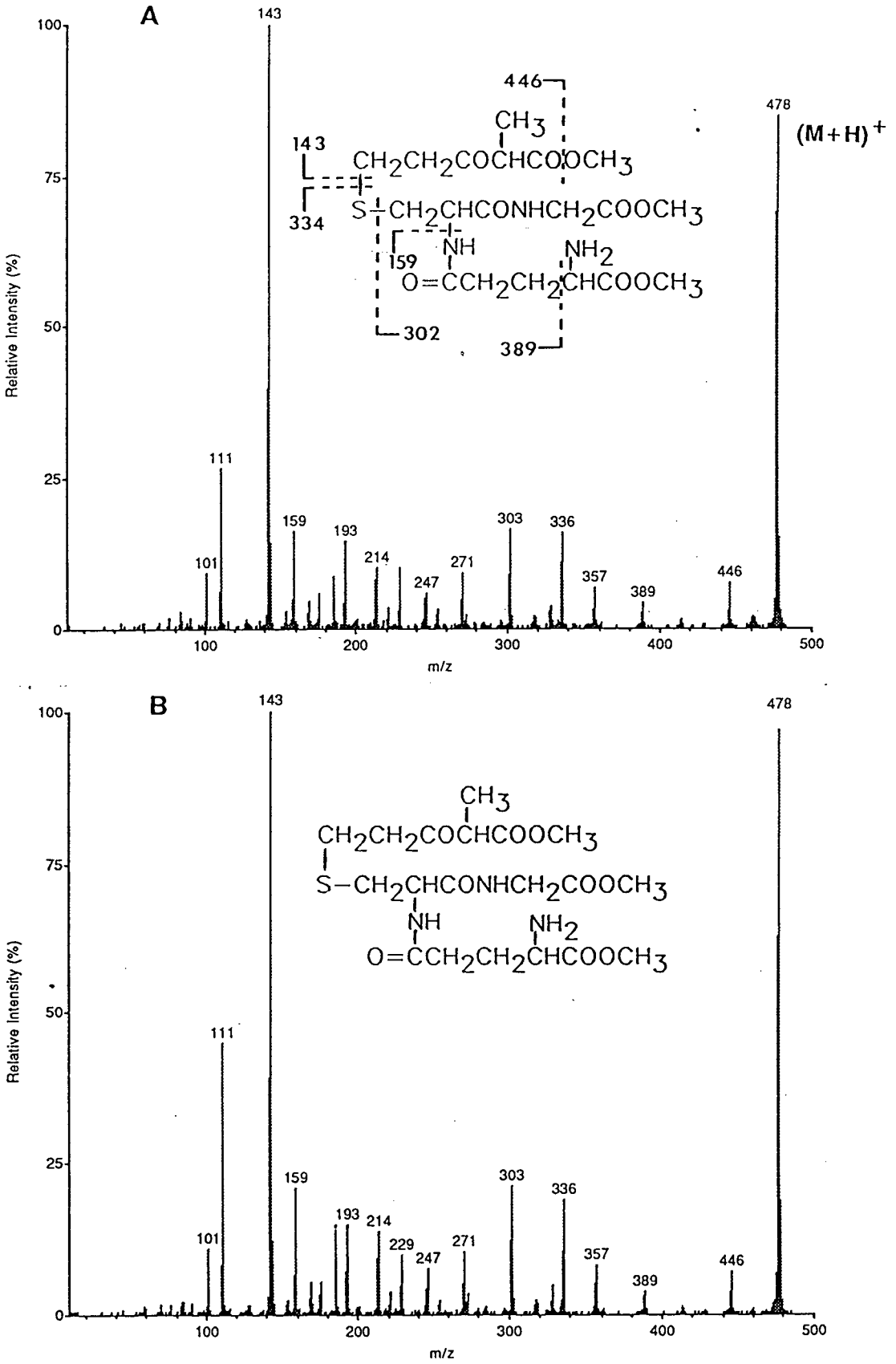


Figure 48. Daughter ion spectra (parent, m/z 478) of A) Scans 102-106 (Fig. 47A) and B) Scans 102-106 (Fig. 47B) representing tetra-methylated GSH conjugate of 3-oxo-4-pentenoate.

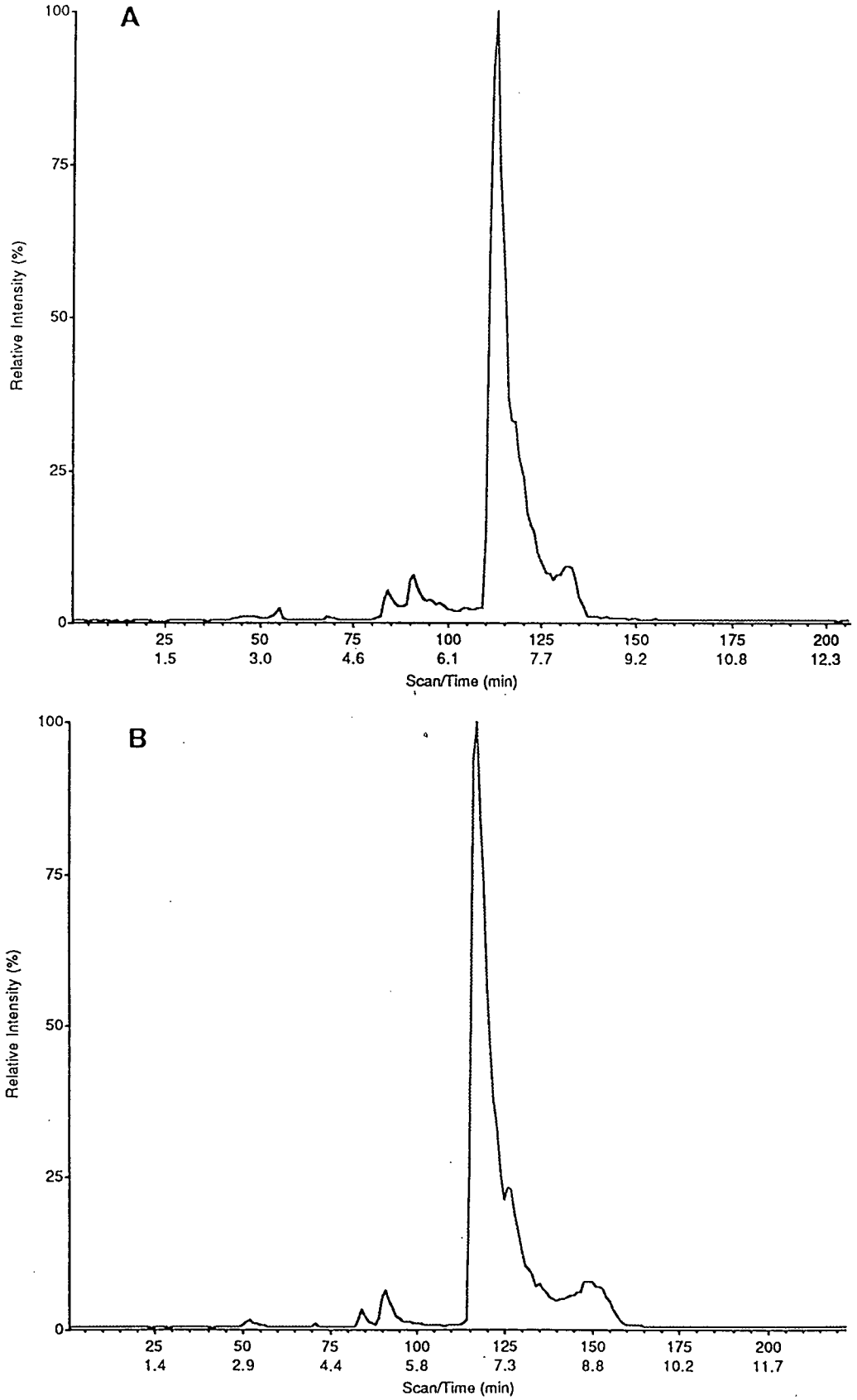


Figure 49. LC/MS extracted ion chromatogram (m/z 448): A) methylated bile extract of rats treated with (E)-2,4-pentadienoic acid, B) methylated synthetic GSH conjugate of (E)-2,4-pentadienoate.

pentadienoate is shown. The retention times of the major peaks from the two sources were the same with nearly identical MS/MS fragmentation patterns as shown in Fig. 50. Hence (E)-5-(glutathion-S-yl)3-pentenoic acid was the major GSH conjugate of (E)-2,4-pentadienoic acid in the rat.

5. Attempted synthesis of the CoA thio ester of (E)-2,4-diene VPA

The chemical synthesis of the CoA ester of (E)-2,4-diene VPA was attempted by reacting the anhydride of the diene with CoASH under slightly alkaline conditions. The LC/MS spectrum of the synthetic sample did not show the pseudomolecular ion of the expected molecule. A possible explanation for the lack of mass spectral identification of the product is that the diene may have formed a di-CoA derivative (MW=1657) which was probably susceptible to thermal degradation when the heated nebulizer LC/MS interface was used. In addition to the formation of the more conventional CoA ester derivative the diene can act as a Michael acceptor to form a conjugate of CoA where CoA is added across the double bond of the terminal carbon of (E)-2,4-diene VPA.

The microsomal fraction of rat liver was used as a source of synthetase for the biological synthesis of the CoA ester of (E)-2,4-diene VPA. The LC/MS spectrum obtained from the incubation mixture contained a number of ions that could arise from the thio ester of (E)-2,4-diene VPA. However, confirmation of the structure was not possible due to the unavailability of an authentic standard.

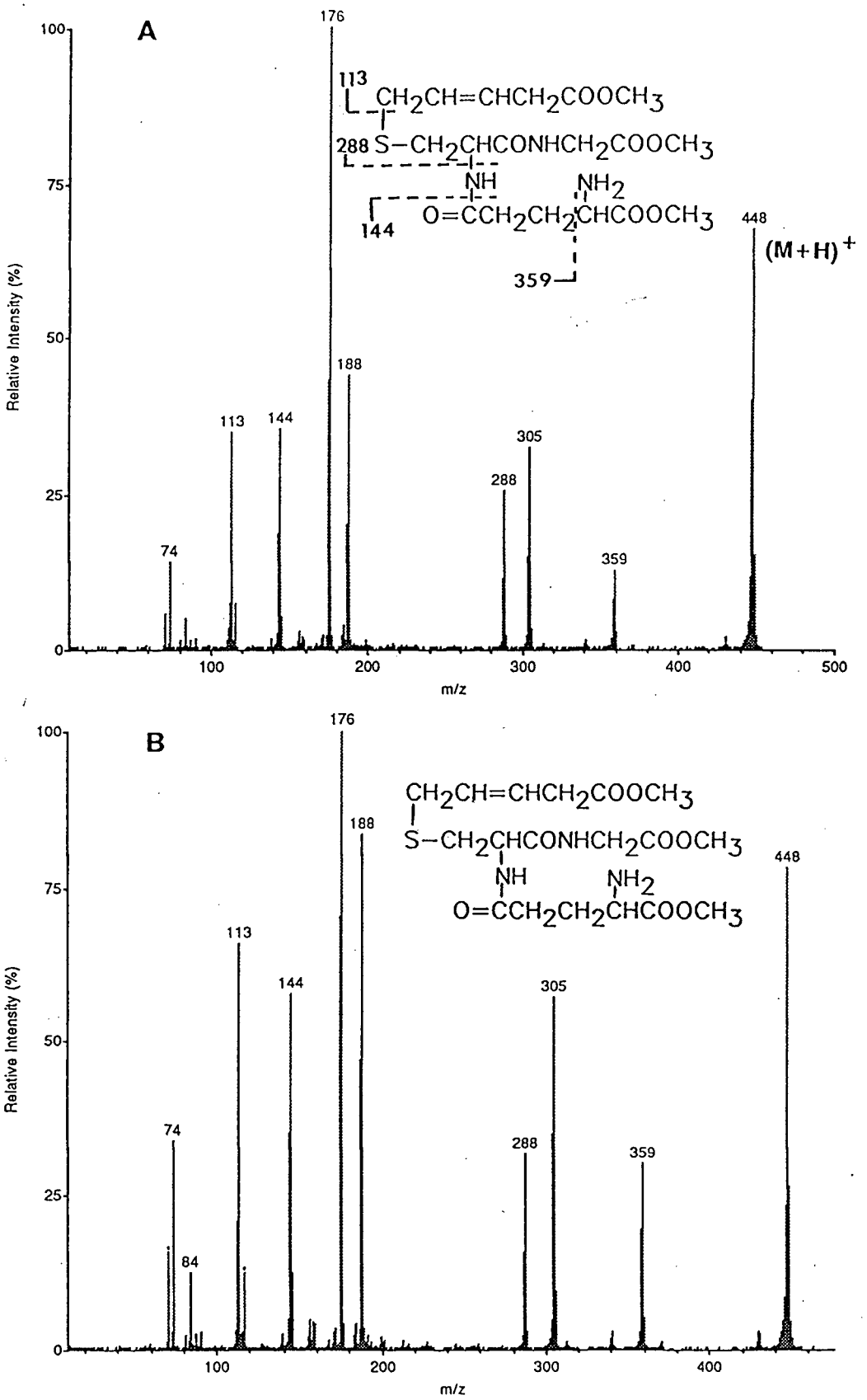


Figure 50. Daughter ion spectra (parent, m/z 448): A) Scans 111-115 (Fig. 49A), B) Scans 115-121 (Fig. 49B).

IV. DISCUSSION

1. Chemistry

Several compounds were synthesized in anticipation of proposed metabolic schemes while others were prepared as the need arose to characterize substances identified as potential metabolites by mass spectrometry.

A. α,β-Unsaturated keto metabolites and their thiol conjugates

The putative metabolites of 4-ene VPA and 4-pentenoic acid, 3-keto-4-ene VPA and 3-oxo-4-pentenoic acid respectively, are characterized by two salient chemical properties: they are α,β-unsaturated carbonyl compounds and are also β-keto acids. These chemical features largely determined the synthetic routes that could be successfully used for their synthesis, and gave chemical instability to the compounds under conditions normally used for the analysis of VPA metabolites.

Considerable difficulty was encountered in the synthesis of 3-keto-4-ene VPA. Several synthetic approaches were tried to obtain the free acid form of the compound in an amount sufficient for the desired experiments. However, partly because of the β-keto acid nature of 3-keto-4-ene VPA the product obtained was mostly the decarboxylated analog (dienol). For this reason the methyl ester of 3-keto-4-ene VPA was synthesized since, for purposes of comparison of biological samples with that of a synthetic standard, the biological sample could easily be methylated using diazomethane. The ester was synthesized by alkylating the enolate of methyl pentanoate with acrolein and oxidizing the resulting hydroxy compound using tert-butyl hydroperoxide in the

presence of a catalytic amount of chromium trioxide (Scheme 1). The use of tert-butyl hydroperoxide for the oxidation of alcohols to carbonyl compounds, as reported by Muzart (1987a, 1987b), obviates the need for large quantities of chromium (VI) reagents. This has advantages in that residual chromium would not interfere with final sample work-up and also that the oxidation would take place under milder conditions.

An attempt to de-esterify a sample of 3-keto-4-ene VPA methyl ester under alkaline conditions was not successful, the ester being converted to an undetermined product(s). Because the strong UV absorbance observed for methyl 3-keto-4-ene VPA disappeared after the above sample manipulation, the product may be a result of addition of water across the double bond of 3-keto-4-ene VPA to give 5-hydroxy-3-keto VPA. Unfortunately GC/MS analysis of the product(s) did not furnish adequate information on their identity.

The 3-oxo-4-pentenoate was also synthesized as the methyl ester using a procedure similar to that followed for 3-keto-4-ene VPA. Reported syntheses of 3-oxo-4-pentenoate have employed rather long and tedious synthetic routes (eg. Collins and Tomkins, 1977; Stork and Guthikonda, 1972; Pelletier *et al.*, 1968). In the present study methyl 3-oxo-4-pentenoate was synthesized by an efficient two-step method (Scheme 3). Alkylation of the α-carbanion of methyl acetate with acrolein and oxidation of the resulting hydroxy compound afforded the 3-oxo-4-pentenoate in 41% yield.

Synthesis of the GSH conjugate of methyl 3-keto-4-ene VPA was first attempted using triethylamine as a catalyst. NMR analysis of the resulting product showed that the conjugate had formed a triethylamine salt. On the other hand triethylamine was not a problem in the synthesis

of the NAC conjugate since methyl NAC was used as one of the starting materials. The GSH conjugates of methyl 3-keto-4-ene VPA and 3-oxo-4-pentenoate were prepared by mixing a methanolic solution of the substrate with an aqueous solution of GSH and adjusting the pH of the mixture to 7.8. Under these conditions the reaction was instantaneous and almost quantitative. A facile reaction of these compounds with GSH and NAC is to be expected as the reaction of α,β-unsaturated carbonyl compounds with Michael donors such as thiols is a well known fact (Friedman *et al.*, 1965).

The GSH and NAC conjugates of methyl 3-keto-4-ene VPA and 3-oxo-4-pentenoate were fully characterized using various spectral techniques (Figs. 6-8, 10, 16-20). In each case the adduct was the result of conjugate addition where the thiolate ion of GSH and/or NAC attacked the terminal carbon of the chain bearing the keto function.

The methine and methylene protons which are present between the two carbonyl groups of the substrate moieties of the thiol conjugates of methyl 3-keto-4-ene VPA and 3-oxo-4-pentenoate exchanged with deuterium quite readily and thus gave no ^{1}H-NMR signals when D_2O was used as the NMR solvent (Figs. 6 and 17). These protons were so acidic that the methylene carbon of methyl 3-oxo-4-pentenoate was methylated upon treatment with diazomethane (Fig. 48). For 3-keto-4-ene VPA such a propensity for methylation was not observed probably because of the steric hindrance of the propyl substituent at position 2.

A. 2,4-Diunsaturated carboxylic acid metabolites and their thiol conjugates

(E)-2,4-diene VPA and (E)-2,4-pentadienoic acid were synthesized using modifications of the methods of Lee *et al.* (1989) and Worley and Young (1972), respectively. When these acids were reacted with either GSH or NAC under conditions similar to that described in the preceding section for the α,β-unsaturated keto metabolites, the rate of reaction was very slow and consequently little product was obtained. The poor reactivity of the free acids appears to be due to the fact that at the pH (7.4) used for the synthesis the acids would be largely in the ionized form. Charge repulsion would preclude activation of the terminal double bond to Michael addition by GSH. On the contrary, the methyl ester derivatives of these diene metabolites reacted with GSH and NAC fairly rapidly resulting in good yields of the respective adducts. Compared to the rate of reaction of the α,β-unsaturated keto metabolites however, the reaction of the diene esters with GSH was slower as indicated by a 50% (in 5 hr) and 90% (in 30 min) yield for the GSH conjugates of methyl (E)-2,4-diene- and methyl 3-keto-4-ene-VPA, respectively.

Methyl esters of (E)-2,4-diene VPA and (E)-2,4-pentadienoate belong to the category of compounds with a general structure of C=C-C=C-Z where Z is COOR, CHO, COR, CN, NO_2, etc. Such systems can undergo Michael-type of reactions by a mechanism that involves either a 1,2-, 1,4-, or 1,6-addition (March, 1985). In the synthesis of the GSH conjugate of methyl (E)-2,4-diene VPA only one product was observed by LC/MS and NMR (Figs. 12 and 13) and was established to be the result of the addition of the thiolate ion at the terminal carbon with the

remaining double bond appearing at position 3. In a 1,4-addition the thiolate ion would be attached to carbon 3 and the terminal double bond (position 4) would remain intact (Fig. 51). Methyl (E)-2,4-diene VPA or (E)-2,4-pentadienoate were not expected to undergo additions of the 1,2-type because a 1,2-addition is generally speaking not likely for compounds with structures similar to these diene esters (Oare and Heathcock, 1989).

The major GSH conjugate of (E)-2,4-pentadienoate methyl ester had a similar structure with the thiolate attached at the terminal carbon and the double bond appearing at position 3. Thus, both diene analogs underwent nucleophilic addition reactions mainly via the 1,6-mechanism.

The configuration of the double bonds of the major GSH conjugates of methyl (E)-2,4-diene VPA and (E)-2,4-pentadienoate was determined by NMR to be trans, i.e. the structures of the conjugates were (E)-5-(glutathion-S-yl)-3-ene VPA and (E)-5-(glutathion-S-yl)-3-pentenoate. This was established from the coupling constant of the vinylic protons which was 16 Hz. To the best of our knowledge there is very little information in the literature regarding the stereochemistry of products arising from similar reactions. In the mammalian liver the enzyme 2,4-dienoyl CoA reductase catalyzes the NADPH-dependent addition of two hydrogen atoms to the double bonds of fatty acyl CoAs having a 2,4-dienoyl CoA structure (Osmundsen and Hovik, 1988). The double bond of the product appears at position 3 and in some cases was shown to have the trans configuration (Dommes and Kunau, 1984). Although the mechanism of the reaction of a diene ester with GSH i.e. nucleophilic addition is likely to be different from that of the reduction of a 2,4-dienoyl CoA fatty acid, the two reactions are similar in as much as that in the

products of both reactions the remaining double bond appears at position 3.

In view of the mechanism of the reaction of methyl (E)-2,4-diene VPA or methyl (E)-2,4-pentadienoate with GSH it is not clear why the reaction should be stereoselective for the trans isomer. A plausible explanation is that such an orientation would result in the more thermodynamically stable product, with the two bulky groups on either side of the double bond farther apart than they would be in the cis isomer.

Methyl (E)-2,4-diene VPA is prochiral and forms a chiral center upon reaction with GSH. Hence the reaction of (E)-2,4-diene VPA with GSH can result in either diastereomers, geometric or regio-isomers. The structures of the regioisomers and the chiral centers in the conjugates are shown in Fig. 51. Similar isomers for the GSH conjugate of methyl (E)-2,4-pentadienoate will be observed if both 1,4- and 1,6-addition mechanisms are followed.

For the synthesized GSH conjugate of (E)-2,4-diene VPA only one isomer was detected by LC/MS whereas NICI/GC/MS of the NAC conjugate revealed two isomers with the minor isomer having a longer retention time (App. 22). The major isomer was characterized to be (E)-5-(N-acetylcystein-S-yl)-3-ene VPA. The minor isomer is not likely to be the (Z) or cis isomer of the above compound because cis isomers normally elute before their trans counterparts on the semipolar capillary columns used for the analysis of VPA metabolites. Therefore the minor isomer might be a diastereomer or regioisomer. The pattern of isomers arising from the metabolism studies will be discussed in the metabolism section.

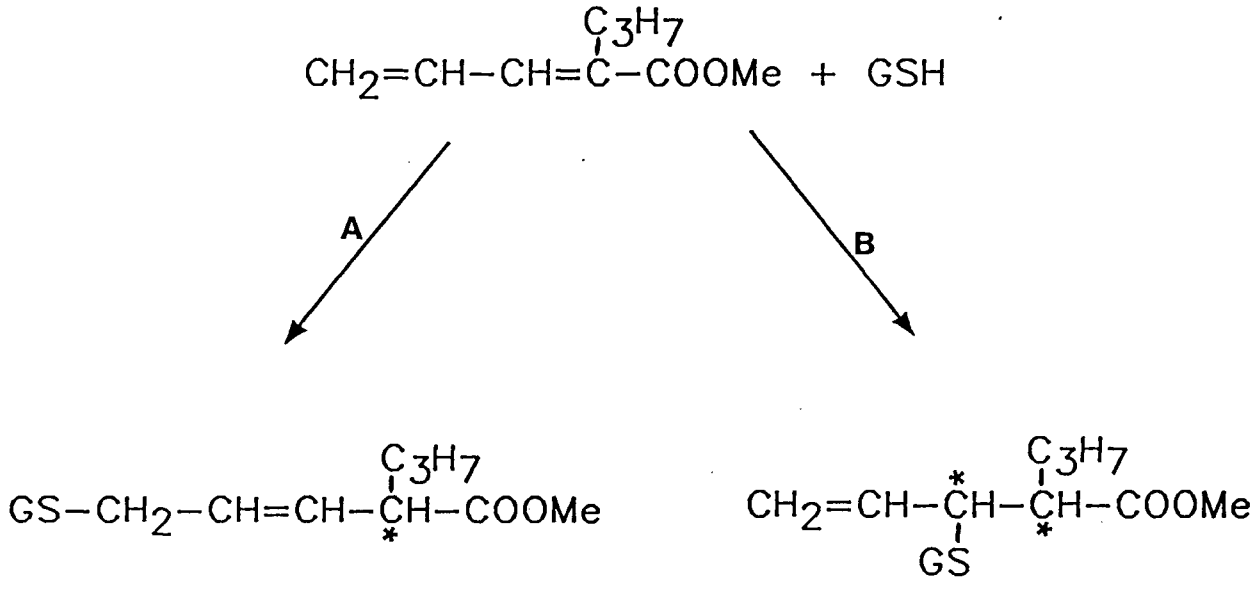
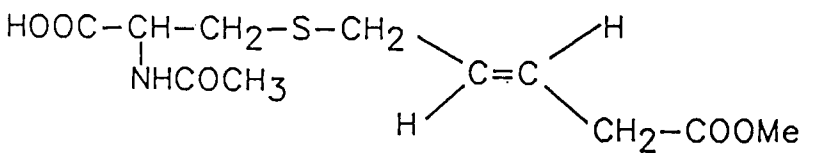


Figure 51. Scheme showing the possible regioisomeric GSH conjugates of methyl (E)-2,4-diene VPA as a result of 1,6- (A) and 1,4- (B) additions. The chiral centers that can be formed are marked with asterisks.

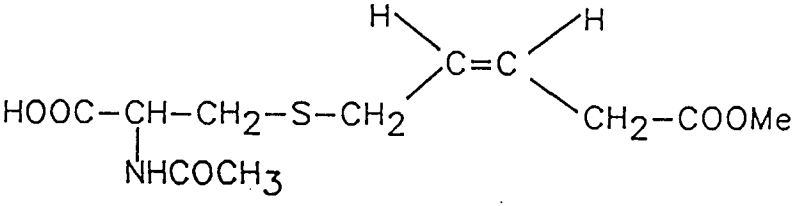
The reaction of methyl (E)-2,4-pentadienoate with GSH produced two isomers as detected by LC/MS (Fig. 24) while the reaction with NAC resulted in four isomers which were detected by GC/MS (Fig. 26A). With a 1,6-addition mechanism the reaction of methyl (E)-2,4-pentadienoate with GSH is expected to produce at most two geometric isomers. The ^{13}C-NMR spectrum (Fig. 23) of the GSH conjugate of methyl (E)-2,4-pentadienoate appears to indicate two such isomers with a pair of signals for the -CH=CH- carbons. When the nucleophlic addition follows a 1,4-mechanism (for structures see Fig. 52) a chiral center would be created at position 3 of the substrate moiety of the GSH or NAC adduct again resulting in a maximum of two isomers (Fig. 52). The four isomers of the NAC conjugate of methyl (E)-2,4-pentadienoate observed by GC/MS therefore may be the two geometric isomers of 5-(N-acetylcystein-S-yl)-3-pentenoate (peaks 3 and 4, Fig. 26A) and the two diastereomers of the conjugate with the structure of 3-(N-acetylcystein-S-yl)-4-pentenoate (peaks 1 and 2, Fig. 26A). Unfortunately the EI spectra of these compounds were the same and did not enable the discrimination between regioisomers. Definitive structural assignment of these compounds will have to await the preparative isolation of the compounds in sufficient quantities for NMR analysis.

The apparent lack of formation of the analogous conjugate of (E)-2,4-diene VPA i.e. 3-(N-acetylcystein-S-yl)-4-ene VPA might be attributable to the steric interference of the propyl substituent at position 2, making 1,4-addition unlikely.

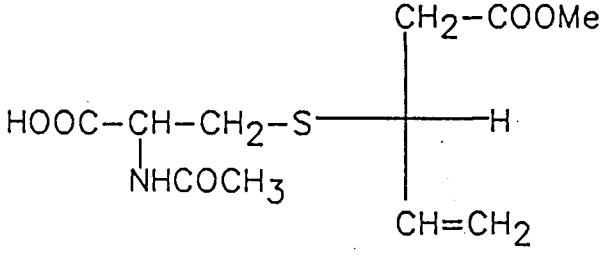
Reversibility of the conjugation reaction with thiols and the *in vitro* stability of GSH and NAC conjugates is an important consideration when dealing with such conjugates. Toxicity of some GSH conjugates can



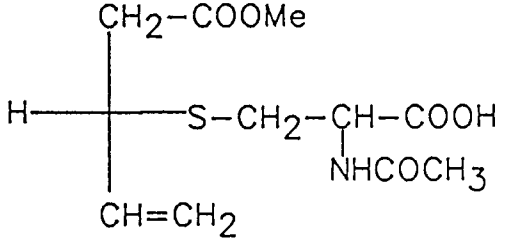
(E)–5–(N–acetylcystein–S–yl)3–pentenoate



(Z)–5–(N–acetylcystein–S–yl)3–pentenoate



(3R,2'R)–3–(N–acetylcystein–S–yl)4–pentenoate



(3S,2'R)–3–(N–acetylcystein–S–yl)4–pentenoate

Figure 52. Structures of the possible isomeric NAC conjugates of methyl (E)-2,4-pentadienoate. Four isomers were detected by GC/MS for the synthetic sample while three isomers were identified in the urine extract of rats treated with (E)-2,4-pentadienoic acid.

result from the reversal of the conjugation reaction (van Bladeren, 1988). An example of a reversible conjugation is that of the conjugates of N-(1-methyl-3,3-diphenylpropyl)isocyanate which were found to release the isocyanate under mild alkaline conditions (Mutlib *et al.*, 1990). Knowledge of the factors influencing the stability of GSH and NAC conjugates is also important from the analytical point of view. Heating the NAC conjugate of (E)-2,4-diene VPA with alkali resulted in some reversibility of the conjugation reaction as evidenced by the amount of conjugate before and after alkaline treatment. The NAC conjugate of (E)-2,4-diene VPA was found to be quite stable under the GC conditions used for its analysis, with no trace of decomposition products of the NAC conjugate being detected. This is in contrast to the NAC conjugate of methyl 3-keto-4-ene VPA which readily underwent decomposition to NAC and methyl 3-keto-4-ene VPA in the GC inlet (Fig. 9).

2. Development of analytical methods

A. Assay development for non-thiol metabolites and profiles in patients

Despite the importance given to the metabolic profile of VPA due to the potential anticonvulsant activity and toxicity of some VPA metabolites, very little has been published on VPA metabolite profiles in epileptic patients. In this study it was sought to obtain an assessment of the 4-ene VPA pathway relative to other metabolic pathways in epileptic patients on monotherapy and combined therapy with CBZ and CLBZ.

Partly because of their large number, the wide concentration range at which they are found in biological fluids, and similarities to endogeneous compounds, the simultaneous quantitation of VPA metabolites

is of considerable difficulty. Published GC/MS methods for the analysis of VPA metabolites are based on either t-BDMS (Abbott *et al.*, 1986) or TMS (Nau *et al.*, 1981b; Tatsuhara *et al.*, 1987; Rettenmeier *et al.* 1989) derivatives. Two methods which employed TMS derivatives (Nau *et al.*, 1981b; Tatsuhara *et al.*, 1987) did not include all the important metabolites, while in a third method (Rettenmeier *et al.*, 1989) separation was achieved by using a much longer column and GC run time compared to the others. Although the previously reported assay from our laboratory (Abbott *et al.*, 1986) was satisfactory for most metabolites, there were chromatographic problems with some of the hydroxy metabolites.

In the present study a GC/MS method for the simultaneous quantitation of VPA and 14 of its metabolites was developed using PFB derivatives and negative ion detection. The assay made use of the inherent sensitivity and soft ionization nature of electron-capture NICI for a successful quantitation of this large array of metabolites. By a combination of PFB (carboxyl group) and TMS (hydroxyl and 3-oxo moiety) derivatization and the use of a non-polar capillary column, adequate chromatographic separation and sensitivity were achieved.

The NICI assay developed was superior to published methods in many respects. Separation of 14 metabolites and the parent compound was achieved in a reasonable GC run time of 25 min with all compounds giving sharp peaks as shown in Fig. 28. VPA was completely resolved from 4-ene VPA as were the monounsaturated metabolites. PFB derivatization resulted in baseline separation of the geometric and regioisomeric diunsaturated metabolites. This is significant because with t-BDMS and TMS derivatives of the toxic metabolite (E)-2,4-diene- and (E,Z)-2,3'-diene-VPA overlap

and having the same $[M-57]^+$ or $[M-15]^+$ fragment ions are counted as one. Other metabolites, such as 3-OH VPA, which chromatograph poorly as t-BDMS derivatives were analyzed quite readily by using TMS and PFB derivatives. The NICI method was highly sensitive for VPA being 30-50 times more sensitive than the t-BDMS derivative by EI. The lower limit of detection for metabolites varied between 1-8 ng/mL based on a signal to noise ratio of ≥ 3.

The assay was successfully employed for the metabolic profiling of VPA in pediatric patients and for tissue distribution studies of VPA metabolites in animals.

The major serum metabolites in pediatric patients on VPA monotherapy were (E)-2-ene-, (E,E)-2,3′-diene-, and 3-keto-VPA as shown in Table 4. (E)-2-Ene- and 3-keto-VPA are β-oxidation products (Loscher, 1981b), whereas the origin of (E,E)-2,3′-diene VPA is unknown although its concentration pattern suggests it might be a derivative of (E)-2-ene VPA probably via 3-ene VPA (Rettenmeier *et al.*, 1987). The 4-ene VPA metabolite was found at low levels (mean = 0.20 μg/mL) while (E)-2,4-diene VPA had a mean serum concentration of 0.65 μg/mL. The serum concentration values obtained for most of the metabolites in this study compare favorably to those previously reported by Abbott *et al.* (1986) and Tatsuhara *et al.* (1987). Notable exceptions were the levels of 4-ene- and (E,E)-2,3′-diene-VPA which were found to be lower and higher, respectively, than in the previous study (Abbott *et al.*, 1986). The reasons for these discrepancies appear to be the better resolution of 4-ene VPA from VPA and the availability of a synthetic standard for preparing the calibration curve of (E,E)-2,3′-diene VPA in the present assay.

The serum levels of 4-keto- and 5-OH-VPA were significantly higher in the VPA + CBZ group compared to the monotherapy group (Table 5), suggesting the induction of ω (5-OH VPA) and ω-1 (4-keto VPA) pathways, consistent with the fact that CBZ induces VPA metabolism (Panesar *et al.*, 1989). The serum concentrations of VPA and its β-oxidation products were lower in the VPA + CBZ group. The decrease in the serum concentration of 2-ene VPA, when VPA was coadministered with CBZ, was also documented in a study in adult volunteers (Panesar, 1987).

Polytherapy had no significant effect on the serum levels of 4-ene- and (E)-2,4-diene-VPA. Similar results were obtained in a recent report of VPA urine metabolite profiles (Levy *et al.*, 1990) where CBZ had no significant effect on the urinary levels of 4-ene VPA.

B. Assay of the NAC conjugate of (E)-2,4-diene VPA

Once the presence of the NAC conjugate of (E)-2,4-diene VPA in the urine of patients taking VPA was established, it was necessary to develop a method for the quantitative determination of the conjugate in those patients.

The measurement of NAC conjugates in urine allows for non-invasive monitoring of the exposure of subjects to toxic agents. Determination of NAC conjugates in a readily available biological fluid such as urine, also enables the estimation of the extent of GSH conjugation and the characterization of the chemistry of GSH conjugation without the need for surgical procedures in animals.

The NAC conjugate of (E)-2,4-diene VPA was isolated from urine by acidifying urine to pH 1-2, adding NH_4Cl, and extracting with ethyl acetate. It was not possible to determine recovery because the authentic

NAC conjugate was available as the dimethyl ester and had to be hydrolyzed and then extracted from aqueous solution. Using a similar extraction procedure, the recoveries (concentration range low μg/mL) for benzyl and phenyl mercapturic acids were 94 and 70%, respectively (Laham and Potvin, 1987; Jongeneelen *et al.*, 1987). For future quantitative work it will be important to preparatively isolate the free acid form of the NAC conjugate of (E)-2,4-diene VPA to use as the standard.

The quantitation of NAC conjugates has been carried out using various analytical instruments such as UV spectrophotometry, HPLC, and GC (Vermeulen, 1989). The most selective and sensitive assay for the NAC conjugates of (Z-) and (E)-1,3-dichloropropene was GC/MS by SIM (Onkenhout *et al.*, 1986).

The NAC conjugate of (E)-2,4-diene VPA was found to be sufficiently thermostable for GC analysis. The assay was based on PFB derivatization and negative ion detection. The conjugate underwent efficient derivatization with PFBB to yield a di-PFB derivative with suitable GC/MS properties as shown in Fig. 40. The di-PFB derivative of the conjugate gave a sharp peak and its NICI mass spectrum (App. 17) was characterized by an intense ion at m/z 482 which corresponded to the loss of one PFB moiety with the remaining PFB retained on the carboxylate anion fragment.

The dimethyl ester of the NAC conjugate of acrylic acid was synthesized and, after hydrolysis with alkali, was used as the internal standard. Being an NAC conjugate as well as a dicarboxylic acid, the internal standard had similar chromatographic and mass spectral properties to that of the analyte and was expected to behave in a similar way in solution. This enabled the development of an assay for

the NAC conjugate of (E)-2,4-diene VPA in patient urine using 1 mL samples. The sensitivity of the assay was 2 ng/mL with a signal to noise ratio of >3.

To our knowledge this is the first report of the use of PFB derivatization and negative ion detection for the analysis of thiol conjugates. The concentration of the conjugate in some of the patients studied was in the low ng/mL level. However, the sensitivity of the assay, which was mainly due to the type of derivative and use of NICI, was adequate for the analysis.

The mean concentration of the conjugate in patients on VPA was found to be 1.38 μg/mL (n=28) with a range of trace to 5.74 μg/mL. Using the NICI methodology, a comparison of the levels of the conjugate in VPA monotherapy and combined therapy with other antiepileptic drugs is currently underway in our laboratory.

3. Metabolism

A. 4-Ene- and (E)-2,4-diene-VPA

The serum levels of the toxic metabolites of VPA namely, 4-ene- and (E)-2,4-diene-VPA were found to be respectively, 0.2 and 0.7% that of VPA (Table 4) indicating that they are only minor metabolites in the serum of epileptic children. Although polytherapy is known to be a predisposing factor to VPA hepatotoxicity, the level of either 4-ene- or (E)-2,4-diene-VPA was not increased in combination therapy as shown in Table 5. In rats too, phenobarbital treatment did not greatly enhance the formation of 4-ene VPA from VPA (Granneman *et al.*, 1984c). This does not necessarily mean that polytherapy with microsomal enzyme inducers fails to lead to an increased formation of 4-ene VPA. An alternate

reasoning is that the increased formation of 4-ene VPA is not necessarily reflected by its serum levels; an increased formation clearance of 4-ene VPA was demonstrated after CBZ administration in a drug interaction study of CBZ and VPA (Panesar, 1987) and also in patients given VPA in combination with either CBZ or phenytoin (Levy *et al.*, 1990). In a previous study (Tennison *et al.*, 1988), the monitoring of 4-ene VPA serum levels was not found to predict risk of fatal hepatotoxicity.

Despite the lack of a sufficient number of studies of metabolite levels from patients who died or recovered from VPA-related hepatotoxicity, the available evidence suggests that 4-ene VPA concentration in serum is not predictive of potential VPA hepatotoxicity. This raises the possibility that 4-ene VPA may be metabolized to secondary metabolites that are covalently bound to tissue macromolecules. The metabolism studies of 4-ene- and (E)-2,4-diene-VPA in the rat were therefore undertaken in part to address this apparent dissociation between the 4-ene VPA metabolite level in patients and VPA hepatotoxicity.

As discussed in the introduction to 4-ene VPA and its hepatotoxic analogs, 4-pentenoic acid and hypoglycin are substrates for enzymes of the fatty acid β-oxidation system. It is well known that the endoplasmic reticulum is the site for several Phase I and some Phase II routes of drug metabolism. However, for drugs with acyl and alkyl side chains β-oxidation is also an important metabolic pathway (Yamada *et al.*, 1987). For example, various hypolipidemic drugs are activated to their acyl CoA derivatives and are subsequently β-oxidized to various metabolites (Aarsland *et al.*, 1990). Xenobiotic CoA thio esters play a critical role

in the metabolism, activity, and toxicity of a wide variety of xenobiotic carboxylic acids (Caldwell, 1984). Thus, it is not surprising that 4-ene VPA is capable of activation to its CoA ester and subsequent β-oxidation.

Detection of thiol conjugates and other metabolites of (E)-2,4-diene VPA: In the present study it was found that 4-ene VPA was bioactivated to a metabolite that avidly reacted with GSH. The product of this interaction of reactive metabolite with GSH was determined to be (E)-5-(glutathion-S-yl)-3-ene VPA i.e. the GSH conjugate of (E)-2,4-diene VPA. The NAC conjugate was subsequently confirmed as a metabolite in the urine of patients administered therapeutic doses of VPA.

The presence of these thiol conjugates in the respective species and biological fluids was established using various spectral techniques as presented in the results section. The interaction of (E)-2,4-diene VPA with GSH and NAC *in vitro* produced the respective adducts, the structures of which were determined using NMR and mass spectrometry. The site of attachment of GSH or NAC with the substrate molecule was unequivocally demonstrated to be at the terminal carbon resulting in a shift of the double bond to the 3 position. Several isomers, including geometric, diastereomeric, and regio-isomers could theoretically be formed from the interaction of GSH with (E)-2,4-diene VPA. Only one isomer was observed by HPLC for both the bile extract (Fig. 32) and synthetic standard. On the other hand, GC/MS of the NAC conjugate isolated from the urine of patients on VPA therapy appears to indicate a second isomer of the conjugate (Fig., 39 top). Whether this isomer is geometric, diastereomeric or positional to the identified NAC conjugate remains to be established.

The detection of these thiol metabolites provided evidence that VPA is metabolized *in vivo* to a chemically reactive intermediate capable of reaction with cellular nucleophiles most probably in the liver. The capacity of (E)-2,4-diene VPA to form tissue bound residues is supported by a recent tissue distribution study in rats wherein (E)-2,4-diene VPA could not be detected in extracts of liver tissue homogenates even though it was present in serum (Lee *et al.*, 1991). In contrast, VPA and other metabolites were detectable in both tissues.

The NAC conjugate of (E)-2,4-diene VPA was a major urinary metabolite of (E)-2,4-diene VPA as indicated by the nearly 40% of the dose of the diene recovered as the NAC conjugate. Other metabolites of (E)-2,4-diene VPA that were identified in urine were 3-OH-4-ene-, 3-ene- (stereochemistry not determined), 4-ene-, and (E)-2-ene-VPA. The metabolic origin of 3-ene VPA from (E)-2,4-diene VPA could be rationalized by invoking the action of the 2,4-dienoyl CoA reductase enzyme on (E)-2,4-diene VPA. The subsequent action of isomerase, the enzyme responsible for converting a 3-ene double bond to a trans 2-ene double bond, would then give (E)-2-ene VPA. (E)-2-ene VPA is expected to undergo further β-oxidation to yield other VPA metabolites such as 3-OH- and 3-keto-VPA, but no effort was made to detect these metabolites. 3-Ene- and (E)-2-ene-VPA were also detected in urine following the administration of 4-ene VPA to monkeys (Rettenmeier *et al.*, 1986b).

The detection of 4-ene VPA as a metabolite of (E)-2,4-diene VPA can be rationalized by invoking the 1,4-addition of hydrogens to (E)-2,4-diene VPA catalyzed by 2,4-dienoyl CoA reductase. The formation of a 1,4-addition product (4-ene VPA) in addition to the 1,6-addition product (3-ene VPA) may be due to the branched nature of the diene as opposed to

endogeneous substrates of the enzyme. Branching may cause the enzyme to lose some of its regio-selectivity. Alternatively, 4-ene VPA may be formed by the action of 2-enoyl CoA reductase on (E)-2,4-diene VPA. This enzyme is known to act on fatty acids with a 2-enoyl CoA structure (Kunau and Dommes, 1978).

The amount of 3-ene VPA recovered from urine after (E)-2,4-diene VPA administration to rats was not quantitated. However, the reduction of (E)-2,4-diene VPA to 3-ene VPA is not likely to be the major pathway of (E)-2,4-diene VPA metabolism as the diene was found to be largely excreted as the NAC conjugate. In contrast, the major metabolic pathway of 4-pentenoic acid was the reduction of its diene metabolite (E)-2,4-pentadienoic acid to 3-pentenoic acid and oxidation of the latter to acetyl CoA and propionyl CoA (Schulz, 1983). Thus, (E)-2,4-diene VPA tended to bind with GSH or accumulate to a greater extent than (E)-2,4-pentadienoic acid. The relatively inefficient reduction of (E)-2,4-diene VPA to 3-ene VPA might be related to the hepatotoxic properties of (E)-2,4-diene VPA. This is supported by the observation that inhibition of fatty acid oxidation by 4-pentenoic acid and hence the resulting toxicity is abolished by treating rats with clofibrate (Borrebaek *et al.*, 1980; van Hoof *et al.*, 1985). By its strong inducing effect on 2,4-dienoyl CoA reductase, clofibrate treatment channels the metabolism of (E)-2,4-pentadienoyl CoA toward the 3-pentenoyl CoA pathway. In this connection it would be interesting to determine if treatment of rats with clofibrate or glycine would decrease or abolish the toxicity of (E)-2,4-diene VPA. Hypoglycin toxicity is attenuated by the administration of glycine (Al-Bassam and Sherratt, 1981). Similarly, administration of glycine might stimulate the excretion of (E)-2,4-diene

VPA as its glycine amide conjugate. Of course administration of NAC would be expected to decrease the toxicity of (E)-2,4-diene VPA by increasing GSH in the liver. GSH precursors such as NAC are effective against acetaminophen toxicity largely through provision of GSH for conjugation with the reactive metabolite of acetaminophen (Boobis *et al.*, 1989).

3-Keto-4-ene VPA and its thiol conjugates: The metabolism studies in rats involving 4-ene- and (E)-2,4-diene-VPA were conducted primarily to try and isolate the thiol derivatives of 3-keto-4-ene VPA. The experimental findings were somewhat surprising in that a major metabolite of (E)-2,4-diene- and 4-ene-VPA was in fact the GSH conjugate of (E)-2,4-diene VPA. The finding that (E)-2,4-diene VPA can be activated to an electrophilic intermediate capable of rapid reaction with GSH was not anticipated.

Because intact 3-keto-4-ene VPA may not be sufficiently stable to survive in urine or under sample work-up conditions, it was hoped that detection in the form of its thiol conjugates would be feasible. *In vitro*, 3-keto-4-ene VPA reacted readily with GSH and NAC and consequently synthetic samples were available to facilitate the search for these metabolites. However, neither the 3-keto-4-ene VPA nor its thiol conjugates were evident in any of the treatment groups. In patient urine the 3-keto-4-ene VPA was not detected even though the more sensitive NICI technique was used and the ions expected to arise from the PFB derivative of 3-keto-4-ene VPA were monitored. Other forms of 3-keto-4-ene VPA that were searched for by GC/MS but not detected included the decarboxylated derivative of the acid.

It is generally accepted that NAC conjugates that appear in the urine are the major end products of the GSH pathway although cysteine conjugates, S-methyl derivatives, and sulphoxides can also be formed (Onkenhout, 1983). Metabolites derived from the GSH conjugate of methyl 3-keto-4-ene VPA were determined by administering the GSH conjugate to rats. The NAC conjugate was the only GSH-derived metabolite that could be identified in urine (Fig. 38). Hence if the GSH conjugate of 3-keto-4-ene VPA was formed *in vivo*, the NAC conjugate should have been detected in urine.

The initial proposal to synthesize the GSH conjugate of 3-keto-4-ene VPA from *in situ* metabolism of 4-ene VPA in mitochondrial fractions of rat liver was not attempted as this would offer no mechanistic information in the absence of *in vivo* formation of 3-keto-4-ene VPA. One technique that might be useful to establish the *in vivo* formation of 3-keto-4-ene VPA would be to isolate and characterize the protein (enzyme) adduct of the compound after administration of a labelled analog of 4-ene- or (E)-2,4-diene-VPA to rats.

There are a number of possible reasons why 3-keto-4-ene VPA or its thiol addition metabolites were not detected in the present study. The 3-keto-4-ene VPA and its thiol conjugates could have been formed, but may have been at levels below the detection limit of our analytical method which was based on EI/GC/MS of the dimethyl ester in the case of the NAC conjugate. It was not possible to develop an NICI method based on the PFB esters for a more sensitive detection of the NAC conjugate of 3-keto-4-ene VPA. Attempts to prepare the free acid form of the conjugate were unsuccessful.

The detection of 3-OH-4-ene VPA, the immediate precursor to 3-keto-4-ene VPA, after (E)-2,4-diene VPA administration to rats is the best evidence for the *in vivo* formation of 3-keto-4-ene VPA. The 3-OH-4-ene VPA was also detected in previous metabolism studies of 4-ene VPA (Rettenmeier *et al.*, 1985, 1986b). It should nevertheless be noted that 3-OH-4-ene VPA could also be formed by a direct microsomal hydroxylation of 4-ene VPA. The latter was identified as a urinary metabolite of (E)-2,4-diene VPA as discussed below. As well, 3-OH-4-ene VPA having a substituent at position 2 and a terminal double bond may not be a good substrate for the β-oxidation enzyme responsible for oxidation of hydroxy acyl CoAs to keto acyl CoAs.

Another reason for the lack of detection of 3-keto-4-ene VPA or its derivatives in the metabolism studies that were undertaken is that 4-ene-/(E)-2,4-diene-VPA may not be metabolized at all to 3-keto-4-ene VPA. (E)-2,4-diene- and 3′-keto-4-ene-VPA, which is a product of β-oxidation on the saturated side chain of 4-ene VPA (for metabolic scheme see Fig. 53), are major metabolites of 4-ene VPA. (E)-2,4′-diene VPA, an intermediate in the metabolism of 4-ene VPA to 3′-keto-4-ene VPA, was not detected as a metabolite of 4-ene VPA (Fig. 53) (Rettenmeier *et al.*, 1985). In the present study a major urinary metabolite of (E)-2,4-diene VPA in the rat was the NAC conjugate of (E)-2,4-diene VPA with apparently little β-oxidation of the diene taking place. Taken together, these observations suggest that while 4-ene VPA undergoes β-oxidation on the saturated side chain readily to give 3′-keto-4-ene VPA, the diene structure of (E)-2,4-diene VPA is somehow an impediment to β-oxidation and hence there is less likelihood of the formation of 3-keto-4-ene VPA from (E)-2,4-diene VPA. This conclusion is supported by the relative

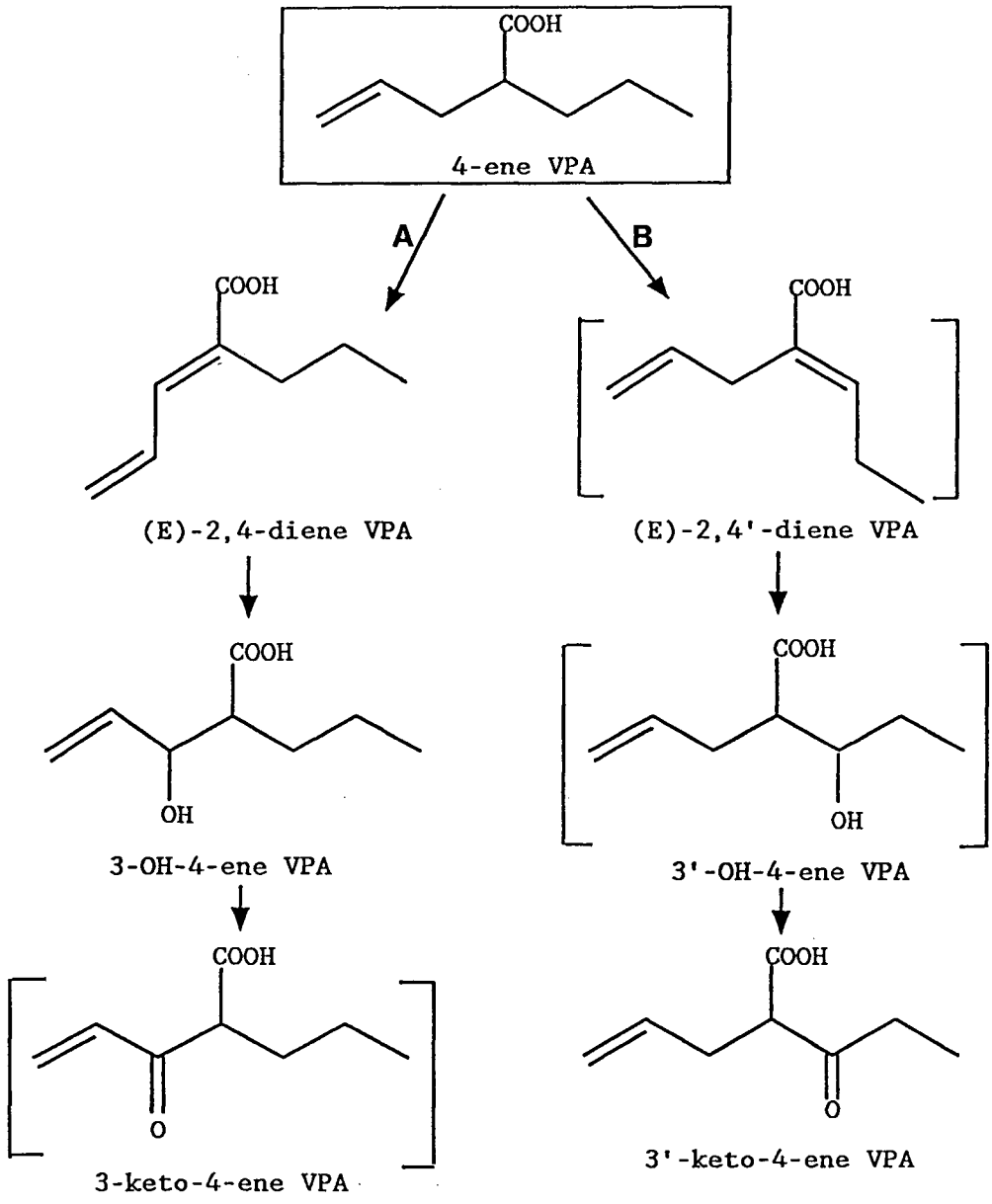


Figure 53. Proposed mitochondrial beta-oxidation of 4-ene VPA on the propenyl (A) and propyl (B) chains (Rettenmeier *et al.*, 1985). The compounds in brackets could theoretically be formed but were not detected in the study.

amounts of the NAC conjugates of (E)-2,4-diene VPA and (E)-2,4-pentadienoic acid recovered after equivalent doses of the acids. The amount of the NAC conjugate of (E)-2,4-diene VPA recovered in urine was much higher than that of the NAC conjugate of (E)-2,4-pentadienoic acid based on a comparison of peak height and area. (E)-2,4-Pentadienoic acid is known to be efficiently β-oxidized to give acetyl CoA and propionyl CoA (Schulz, 1983) and was metabolized to 3-oxo-4-pentenoic acid as found in the present study. These factors explain why a relatively small amount of the NAC conjugate of (E)-2,4-pentadienoic acid was recovered in urine.

B. 4-Pentenoic and (E)-2,4-pentadienoic acids

The metabolism studies involving 4-pentenoic and (E)-2,4-pentadienoic acids were carried out for two reasons. The first was to determine if parallels can realistically be drawn between the metabolism of 4-ene VPA and 4-pentenoic acid and hence a similar proposed mechanism of hepatotoxicity. The second reason was to isolate and characterize spectroscopically the thiol conjugates of 3-oxo-4-pentenoic acid, the putative metabolite of 4-pentenoic acid considered to be responsible for the irreversible inhibition of the β-ketoacyl CoA thiolase enzyme.

The NAC adduct of (E)-2,4-pentadienoic acid was found to be a metabolite of both 4-pentenoic and (E)-2,4-pentadienoic acids (Figs. 42 and 46), demonstrating a similarity between 4-pentenoic acid and 4-ene VPA with respect to the metabolic fate of their diene derivatives.

The putative α,β-unsaturated keto metabolite of 4-pentenoic acid, 3-oxo-4-pentenoic acid, was isolated and detected in the form of its

thiol conjugates following the administration of (E)-2,4-pentadienoic acid to rats (Figs. 42-45, 47, 48).

The thiol derivative of 3-oxo-4-pentenoic acid that was characterized in urine extracts was the cysteine conjugate (Fig. 42). Treatment of the urine extract with HCl gas and CH_2N_2 prior to GC/MS analysis not only resulted in the esterification of the NAC conjugate but also caused deacetylation to give the cysteine conjugate of 3-oxo-4-pentenoate. Similar treatment of an authentic sample of the NAC conjugate of 3-oxo-4-pentenoate also yielded the cysteine conjugate, thus verifying that the deacetylation of the NAC conjugate had occurred *in vitro*.

The GSH conjugate of 3-oxo-4-pentenoate was detected in the bile of rats treated with (E)-2,4-pentadienoic acid. Three isomeric conjugates (Fig. 47) with similar MS/MS fragmentation patterns were identified. The bile extract was methylated with diazomethane prior to analysis and as a result the spectra indicated that in addition to the methylation of three carboxyl groups there was one additional methyl group in the molecule. The daughter ion spectra of the compounds indicated that the methyl group was attached to a carbon of the substrate moiety of the conjugate (Fig. 48). The site of this methylation is likely to be the methylene carbon between the two carbonyl groups. The protons of this methylene carbon were found to be highly acidic and readily exchanged with deuterium of D_2O.

Introduction of a methyl group on this α-carbon will render that carbon chiral and together with the chiral carbons of L-cysteine and L-glutamic acid will result in the formation of two diastereomers. These diastereomers probably account for two of the three isomers observed for

the tetra-methylated GSH conjugate of 3-oxo-4-pentenoate. The third isomer detected can be accounted for by assuming enolization of the conjugate and methylation of the resulting hydroxyl group. The ^{1}H-NMR spectra of methyl 3-oxo-4-pentenoate did show both enol and keto forms of the compound as indicated in the experimental section and shown in App. 12. Enolization of the molecule can theoretically give rise to a pair of geometric isomers. But in this case only one isomer was clearly seen. This is not unusual since in the case of the O-TMS ether of 3-keto VPA it was found that one of the geometric isomers accounted for 93% of the GC/MS ion current observed as mentioned in the results section.

The unsaturated keto metabolite, 3-oxo-4-pentenoic acid, is generally believed to be the cause of the hepatotoxicity of 4-pentenoic acid (Schulz, 1983), although it has never been characterized in the studies conducted thus far. The results of the present study thus provide the first direct biochemical evidence for the formation of the metabolite of 4-pentenoic acid considered responsible for the irreversible inhibition of fatty acid metabolism.

In comparing the metabolism of 4-ene VPA and 4-pentenoic acid or metabolites derived therefrom in rats the following differences can be noted:

1) Following equivalent doses of the two diene metabolites, the amount of NAC conjugate of (E)-2,4-pentadienoic acid recovered in urine was much less than that of the NAC conjugate of (E)-2,4-diene VPA.

2) Beta-oxidation of (E)-2,4-pentadienoic acid resulted in the formation of 3-oxo-4-pentenoic acid as detected in the form of its thiol derivatives whereas the corresponding metabolite from 4-ene VPA could not be detected.

3) In the case of 4-pentenoic and (E)-2,4-pentadienoic acids it was difficult to detect the parent compound in urine collections taken over 24 hr. In the urine of rats treated with 4-ene- or (E)-2,4-diene-VPA, the administered compounds were readily detectable.

Thus the results suggest that there is a fundamental difference between the mitochondrial metabolism of 4-ene VPA and that of 4-pentenoic acid.

4. Significance of the findings of the metabolism studies to the mechanism of the hepatotoxicity of VPA

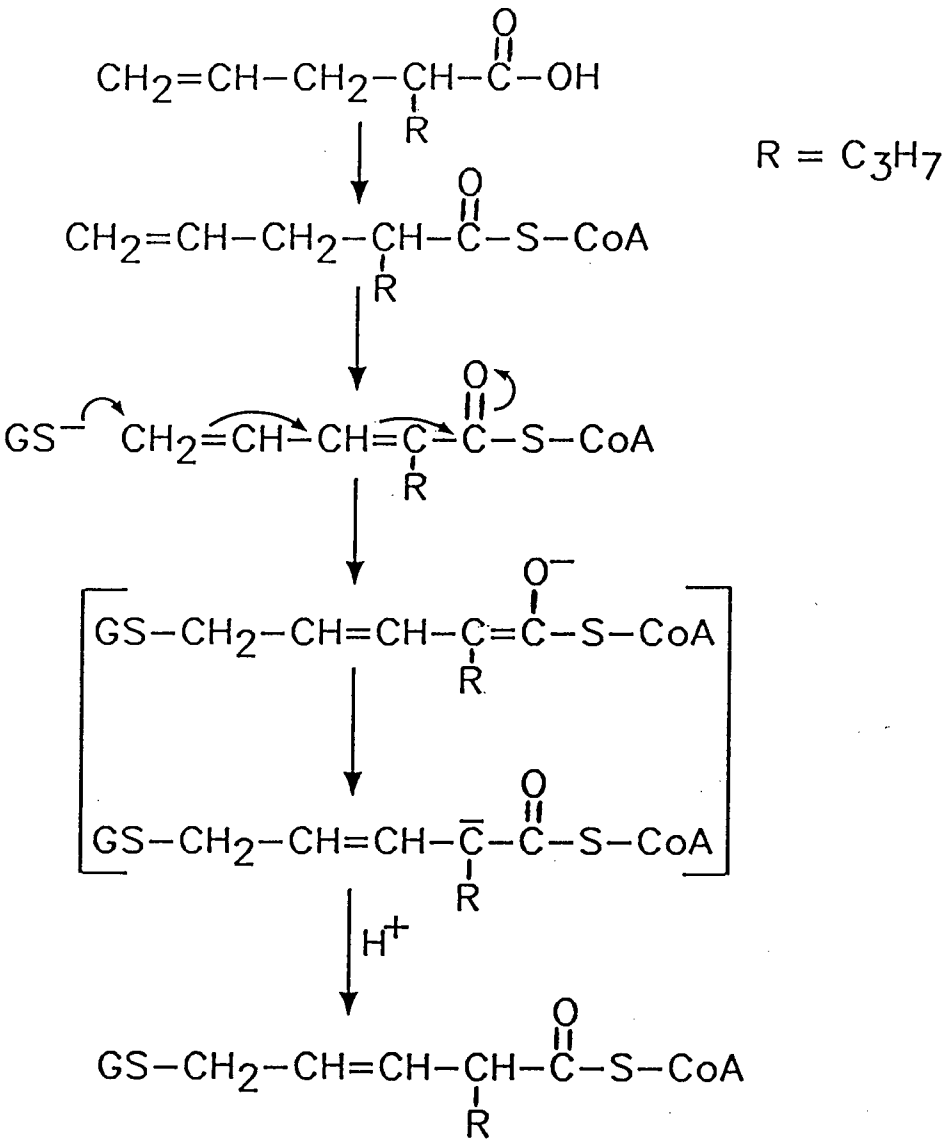
A novel metabolite of VPA, 5-(N-acetylcystein-S-yl)-3-ene VPA, has been identified in rat and human urine. 5-(Glutathion-S-yl)-3-ene VPA was detected in rat bile following the administration of either (E)-2,4-diene- or 4-ene-VPA. The identification and characterization of these thiol adducts marks the first real evidence for the formation of chemically reactive intermediates from VPA metabolism *in vivo*.

Among the well established class of compounds that are substrates for GSH conjugation are the α,β-unsaturated carbonyl compounds (Chasseaud, 1979); although not entirely unique the formation of the GSH conjugate of (E)-2,4-diene VPA is unusual since it is a 1,6-addition rather than the 1,4-Michael addition commonly observed with α,β-unsaturated carbonyl compounds.

A high percentage of the (E)-2,4-diene VPA related material recovered from the urine of rats dosed with the diene was in the form of the NAC conjugate. Compared to the methyl ester of the diene which reacted readily with GSH to form the adduct, the *in vitro* reaction of the free acid of the diene with GSH was very slow. This result is

similar to the finding that acrylic acid was less reactive than its esters when reacted with GSH *in vitro* (Sanders *et al.*, 1988). The Michael reaction of GSH with α,β-unsaturated acids is bound to be slow because the ionization of acids at physiological pH prevents activation of the double bond for nucleophilic attack. Because the *in vitro* reaction of the free acid of (E)-2,4-diene VPA with GSH was slow and yet urinary recovery of the NAC conjugate of the diene was high it is most likely that GSH reacted *in vivo* with an activated form of the diene, namely with the CoA ester form as shown in Scheme 5. This is a reasonable assumption based on the fact that (E)-2,4-diene VPA formed a glycine amide conjugate previously characterized by Rettenmeier *et al.*(1986b) after 4-ene VPA administration to monkeys and also detected in the present study. Formation of a CoA thio ester intermediate is an obligatory step for glycine conjugation (Fears, 1985).

The apparent facile reaction *in vivo* of (E)-2,4-diene VPA with GSH raises the question of the possible contribution of this diene to the hepatotoxicity of VPA. (E)-2,4-diene VPA is a potent hepatotoxin in the rat with one of its toxic manifestations being the induction of mitochondrial lesions (Kesterson *et al.*, 1984). Since this diene is considered to be a mitochondrial oxidation product of 4-ene VPA CoA (Rettenmeier *et al.*, 1985, 1986b) it appears that mitochondria are both the site of formation and the target for toxicity of (E)-2,4-diene VPA and/or (E)-2,4-diene VPA CoA. It is conceivable then that, by virtue of its electrophilic nature, (E)-2,4-diene VPA CoA may covalently bind to a nucleophilic site of a mitochondrial enzyme which would account for the potent inhibition of mitochondrial β-oxidation of fatty acids observed for (E)-2,4-diene VPA in rats (Kesterson *et al.*, 1984).



Scheme 5. Proposed mechanism for the biosynthesis of the GSH conjugate of (E)-2,4-diene VPA from 4-ene VPA.

Before considering an alternate mechanism for the hepatotoxicity of (E)-2,4-diene VPA CoA, a brief description of the cellular compartmentalization of GSH seems relevant. In the liver GSH is compartmentalized to the cytoplasm (85-90%) and the mitochondrial matrix (10-15%) (Jocelyn, 1975; Reed, 1985). The presence of a discrete mitochondrial pool of GSH was first proposed by Vignais and Vignais (1973) and confirmed by other investigators (Jocelyn and Kamminga, 1974; Jocelyn, 1975; Meredith and Reed, 1982, 1983). Liver mitochondria contain a complete GSH redox system consisting of GSH, GSH reductase, GSH peroxidase, and an NADPH generating system (Reed, 1990).

There is considerable experimental evidence to support the concept that mitochondrial GSH is metabolically distinct from that of the cytoplasm. The low mitochondrial rate of GSH turnover ($t_{1/2}$ = 30 hr) compared to that of the cytoplasm ($t_{1/2}$ = 2 hr) indicates the two pools are not free to equilibrate (Meredith and Reed, 1982). Moreover, the rate of incorporation of [^{35}S]methionine or cysteine into the mitochondrial pool was found to be 15 times less than that of the cytoplasmic pool. In incubations containing the GSH depleting agent, diethylmaleate (185 μM), mitochondrial levels were not changed after 60 min whereas cytopasmic levels of GSH were decreased to 40% of control values.

Mitochondria depend largely on GSH peroxidase to detoxify peroxides because they do not contain catalase. Consequently GSH plays a protective role against mitochondrial cytotoxicity by providing reducing equivalents to GSH peroxidase (Meredith and Reed, 1982). Several reports indicated that cell injury occurred when cellular GSH levels fell below 10-15% of control values which is the amount of GSH associated with the

mitochondria (Anundi *et al.*, 1979; Younes and Siegers, 1981; Mitchell *et al.*, 1985; Casini *et al.*, 1985). It was shown that, although diethylmaleate depleted cytoplasmic GSH, the mitochondrial GSH level was unaltered and there was no resulting cellular injury. On the other hand, ethacrynic acid, a compound that can penetrate the mitochondrial membrane, produced marked cytotoxicity coincident with mitochondrial GSH depletion (Meredith and Reed, 1982, 1983). These findings indicated that not only is the GSH pool in the mitochondria metabolically distinct but also is the critical GSH pool influencing cytotoxicity.

An alternate mechanism for the hepatotoxicity of the reactive (E)-2,4-diene VPA CoA ester could then be the localized depletion of GSH in mitochondria. Such a condition would lead to oxidative stress within the cell and result in subsequent cellular injury. The cellular pool of GSH in mitochondria is relatively small and when depleted can lead to significant cytotoxicity as discussed above. Thus a situation whereby (E)-2,4-diene VPA is formed in unusually high levels by susceptible individuals might explain the idiosyncratic nature of VPA-induced hepatotoxicity. Evidence in support of this theory was obtained from four patients described as having VPA-associated hepatotoxicity (Farrell and Abbott, 1991). All patients survived with three successfully treated with NAC. The lymphocytes from these patients demonstrated a significantly higher cell kill when incubated with VPA plus microsomes than did lymphocytes from controls. The addition of GSH to the incubation medium fully protected the cells. The overall evidence suggests that abnormalities in the GSH cell defence mechanisms may have played a role in the predisposition of these patients to VPA hepatotoxicity.

The potential role of GSH in VPA-associated hepatotoxicity is supported by recent studies in the rat where single toxic doses of VPA were observed to significantly depress the GSH peroxidase and reductase enzymes responsible for GSH homeostasis (Cotariu *et al.*, 1990). Thus a combination of enhanced production of one or more reactive electrophilic metabolites, and a deficiency in the ability to maintain a normal GSH level resulting either from genetic factors or a drug induced effect could well form the basis for the idiosyncratic nature of VPA hepatotoxicity.

The observation that phenobarbital pretreatment of animals induces the formation of 4-ene VPA from VPA (Rettie *et al.*, 1987) also sugggests an increased formation of the (E)-2,4-diene VPA in the induced liver. This of course would be true in the presence of an intact β-oxidation system since (E)-2,4-diene VPA is considered to be a mitochondrial oxidation product of 4-ene VPA (Rettenmeier *et al.*, 1985, 1986b). However, the expected increased formation of these metabolites is not always reflected by their serum or urine levels as discussed elsewhere in the thesis. This may be rationalized for example by further metabolism of (E)-2,4-diene VPA to its thiol conjugates. The data from the two patients who recovered from VPA-related hepatotoxicity tends to support this view. Our finding that (E)-2,4-diene VPA is activated *in vivo* to an electrophilic species that readily reacts with GSH and that the GSH conjugate of (E)-2,4-diene VPA is a metabolite of 4-ene VPA is consistent with the report of Porubek *et al.* (1989) of the covalent binding of 4-ene VPA to proteins of the liver. What needs to be established is whether polytherapy leads to increased urinary levels of the NAC conjugate of (E)-2,4-diene VPA in patients. The measurement of

this metabolite may prove to be more indicative of exposure to a toxic species than that of a readily metabolized precursor such as 4-ene- or even (E)-2,4-diene-VPA itself.

Like 4-ene VPA, the (E)-2,4-diene VPA is hepatotoxic in the rat (Kesterson *et al.*, 1984; Granneman *et al.*, 1984c) and was highly elevated in one reported hepatic failure case (Kochen *et al.*, 1983). In the present study, the level of the NAC conjugate of (E)-2,4-diene VPA was found to be 3-4 times higher in two patients with hepatic failure compared to those without this side effect. Taken together, these observations point to the potential role of (E)-2,4-diene VPA in the toxicity of VPA and suggest that this diene should exhibit greater hepatotoxicity than 4-ene VPA. In a previous study (Kesterson *et al.*, 1984) that compared the hepatotoxic properties of 4-ene- and (E)-2,4-diene-VPA in the rat, 4-ene VPA was in fact found to be the more steatogenic. This *in vivo* result might be explained by reasoning that (E)-2,4-diene VPA produced *in situ* from 4-ene VPA CoA would be present at the site of toxicity (mitochondria) in the activated form. Administered (E)-2,4-diene VPA on the other hand would be conjugated with GSH in the cytosol and made less available for intramitochondrial enzymes. Such a hypothesis needs further clarification.

It has been postulated that β-oxidation of (E)-2,4-diene VPA leads to formation of the ultimate hepatotoxin, the highly electrophilic 3-keto-4-ene VPA (Rettenmeier *et al.*, 1985). This metabolite of VPA has yet to be detected (Rettenmeier *et al.*, 1985, 1986b; Granneman *et al.*, 1984a). In the present metabolic study of (E)-2,4-diene VPA neither the 3-keto-4-ene VPA itself nor its NAC conjugate could be demonstrated under our experimental conditions even though the thiol conjugates of 3-

oxo-4-pentenoate were identified and characterized in rat urine and bile. In light of this finding, the contribution of (E)-2,4-diene VPA to the hepatotoxicity of VPA may be more significant than thought previously. However, the formation of 3-keto-4-ene VPA *in vivo* can not be completely ruled out since it may bind avidly to tissue macromolecules with thiol conjugates being below the level of detection.

Future studies with (E)-2,4-diene VPA should include experiments on GSH depletion and resulting toxicity, the testing of the toxicity of (E)-2,4-diene VPA CoA using a suitable *in vitro* model, and the monitoring of the NAC conjugate in patients on VPA mono- and poly-therapy. It would also be important and mechanistically informative to determine if any of the enzymes in the fatty acid oxidation pathway are inactivated (covalently modified) by the (E)-2,4-diene VPA CoA.

V. SUMMARY AND CONCLUSIONS

A possible mechanism for the hepatotoxicity of valproic acid was investigated based on the metabolism of this drug to reactive electrophilic intermediates. The formation of the GSH and NAC conjugates of reactive metabolites derived from VPA, 4-ene-, or (E)-2,4-diene-VPA was studied in the rat. Metabolism of the hepatotoxin 4-pentenoic acid which is an analog of 4-ene VPA was also studied.

Because of the hepatotoxic potential of 4-ene- and (E)-2,4-diene-VPA in humans the levels of these metabolites were determined in the serum of epileptic patients. For this purpose a highly sensitive and selective NICI/GC/MS assay, which enabled the simultaneous quantitation of VPA and 14 of its metabolites, was developed. 4-Ene- and (E)-2,4-diene VPA were minor serum metabolites with average concentrations below 1 μg/mL. Polytherapy with CBZ and CBLZ had no significant effect on the levels of either 4-ene- or (E)-2,4-diene VPA. In the patient groups taking VPA in combination with CBZ there was an induction of ω and ω-1 pathways of VPA metabolism while the levels of the β-oxidation products were significantly decreased.

Several compounds were synthesized as metabolite standards. The putative metabolite of 4-ene VPA, 3-keto-4-ene VPA and its analog 3-oxo-4-pentenoic acid were synthesized as their methyl esters by an efficient two-step method. The GSH conjugates of these two α,β-unsaturated keto compounds were prepared in almost quantitative yields by adding methanolic solutions of the substrates to an alkaline aqueous solution of GSH. The GSH and NAC conjugates of methyl 3-keto-4-ene VPA and 3-oxo-4-pentenoate were fully characterized using NMR and mass spectrometry.

In each case the adduct formed was the result of conjugate addition across the activated double bond.

When the free acids of (E)-2,4-diene VPA and (E)-2,4-pentadienoic acid were reacted with either GSH or NAC under conditions similar to that described for the α,β-unsaturated keto metabolites, the rate of reaction was very slow and consequently little product was obtained. The poor reactivity of the free acids appears to be due to the fact that at the pH (7.4) used for the synthesis the acids would be largely in the ionized form. This would prevent activation of the terminal double bond to Michael addition by GSH. In contrast, the methyl ester derivatives of these diene metabolites reacted relatively rapidly with GSH and NAC to give good yields of the respective adducts. Compared to the rate of reaction of the α,β-unsaturated keto metabolite standards, however, the reaction of the diene esters with GSH was slower as indicated by a 50% (in 5 hr) and 90% (in 30 min) yield for the GSH conjugates of methyl (E)-2,4-diene- and methyl 3-keto-4-ene-VPA, respectively.

The structure of the GSH conjugate of methyl (E)-2,4-diene VPA was established to be (E)-5-(glutathion-S-yl)-3-ene VPA by NMR and mass spectrometry. The major GSH conjugate of (E)-2,4-pentadienoate had a similar structure with the thiolate attached to the terminal carbon and the double bond appearing at position 3. Thus, both diene analogs underwent nucleophilic addition reactions mainly via the 1,6-mechanism.

The GSH conjugate of (E)-2,4-diene VPA was found as a biliary metabolite of rats dosed with 4-ene- and (E)-2,4-diene VPA. The NAC conjugate of (E)-2,4-diene VPA was a major metabolite in the urine of rats treated with these compounds. The NAC conjugate was subsequently confirmed as a metabolite of VPA in the urine of patients. The detection

of these thiol metabolites provided evidence that VPA is metabolized *in vivo* to a chemically reactive intermediate capable of reaction with cellular nucleophiles most probably in the liver.

(E)-2,4-diene VPA is prochiral and consequently the interaction of the diene with GSH can produce diastereomers, geometric- or regio-isomers. Two isomers of the NAC conjugate of (E)-2,4-diene VPA were detected by NICI/GC/MS both for the synthetic standard and as metabolites in human urine. The major synthetic isomer and metabolite was spectroscopically characterized to be (E)-5-(N-acetylcystein-S-yl)-3-ene VPA. Based on retention time, the second isomer appears to be a diastereomer of the conjugate resulting from a 1,6-addition of GSH to the diene.

The urinary concentration of (E)-5-(N-acetylcystein-S-yl)-3-ene VPA was determined in 28 patients on VPA therapy. Quantitation of the conjugate was carried out using an NICI/GC/MS method developed for this purpose. The mean concentration of the conjugate in the urine of these patients was 1.38 μg/mL with a range from trace to 5.74 μg/mL. The level of the conjugate when normalized to creatinine was found to be 3-4 times higher in two patients who had developed VPA-induced liver failure compare to those without this side effect. There was no difference in the urinary levels of the (E)-2,4-diene VPA itself among the patients studied.

The metabolism studies in rats involving 4-ene- and (E)-2,4-diene-VPA were conducted primarily to isolate and characterize the thiol derivatives of 3-keto-4-ene VPA. This was based on the analogy of 4-ene VPA to 4-pentenoic acid which is metabolized to the ultimate hepatotoxin 3-oxo-4-pentenoic acid. The 3-keto-4-ene VPA or its thiol conjugates

could not be demonstrated as metabolites even though synthetic standards were available to facilitate their detection. Thus 3-keto-4-ene VPA either does not arise as a metabolite of 4-ene VPA, or reacts rapidly with cellular macromolecules so that thiol conjugates, if formed, were below our limit of detection.

The metabolism of 4-pentenoic acid was studied in order to examine the parallel drawn between the metabolism of 4-ene VPA and 4-pentenoic acid and to determine if the proposed mechanism of hepatotoxicity was realistic. In rats dosed with 4-pentenoic and (E)-2,4-pentadienoic acids, the thiol conjugates of (E)-2,4-pentadienoic acid were detected, demonstrating a similarity between the metabolism of 4-pentenoic acid and 4-ene VPA. The putative α,β-unsaturated keto metabolite of 4-pentenoic acid, 3-oxo-4-pentenoic acid, was isolated and detected in the form of its thiol conjugates following the administration of (E)-2,4-pentadienoic acid to rats. Three isomers of the GSH conjugate were detected by LC/MS/MS in methylated bile extract. The isomers appeared to arise through the enolization of the molecule and subsequent methylation of the hydroxyl group of the enol, and the introduction of a chiral center at the α-carbon of the substrate portion of the conjugate. These experiments provided the first direct biochemical evidence for the formation of the reactive metabolite of 4-pentenoic acid thought responsible for the irreversible inhibition of fatty acid metabolism. The results also indicated that there was a fundamental difference between the mitochondrial metabolism of 4-ene VPA and that of 4-pentenoic acid.

In summary, a novel metabolite of VPA, the NAC conjugate of (E)-2,4-diene VPA has been identified in rat and human urine. The

identification and characterization of this thiol adduct marks the first real evidence for the formation of chemically reactive intermediates from VPA metabolism *in vivo*. Although not entirely unique, the formation of the GSH conjugate of (E)-2,4-diene VPA is unusual since it is a 1,6-addition rather than the 1,4-Michael addition commonly observed with α,β-unsaturated carbonyl compounds. Because the *in vitro* reaction of the free acid of (E)-2,4-diene VPA with GSH was slow and yet urinary recovery of the NAC conjugate of the diene in rats was high it was hypothesized that (E)-2,4-diene VPA was activated to nucleophilic addition by the formation of the CoA thio ester.

The apparent facile reaction *in vivo* of (E)-2,4-diene VPA with GSH raises the question of the possible contribution of this diene to the hepatotoxicity of VPA. (E)-2,4-diene VPA is a potent hepatotoxin in the rat, with one of its toxic manifestations being the induction of mitochondrial lesions. Thus, it appears that mitochondria are both the site of formation and the target for toxicity of (E)-2,4-diene VPA/(E)-2,4-diene VPA CoA. It is conceivable then that, by virtue of its electrophilic nature, (E)-2,4-diene VPA CoA may covalently bind to a nucleophilic site of a mitochondrial enzyme which would account for the reported potent inhibition of mitochondrial β-oxidation of fatty acids by (E)-2,4-diene VPA in rats.

An alternate mechanism for the hepatotoxicity of the reactive (E)-2,4-diene VPA CoA ester could be the localized depletion of GSH in mitochondria. Such a condition would lead to oxidative stress within the cell and result in subsequent cellular injury. The cellular pool of GSH in mitochondria is relatively small and when depleted can lead to significant cytotoxicity. Thus a situation whereby (E)-2,4-diene VPA is

formed in unusually high levels by susceptible individuals, and a deficiency in the ability to maintain a normal GSH level, resulting either from genetic factors or a competition for detoxification when VPA is given in combination with other drugs, might explain the idiosyncratic nature of VPA-induced hepatotoxicity.

VI. REFERENCES

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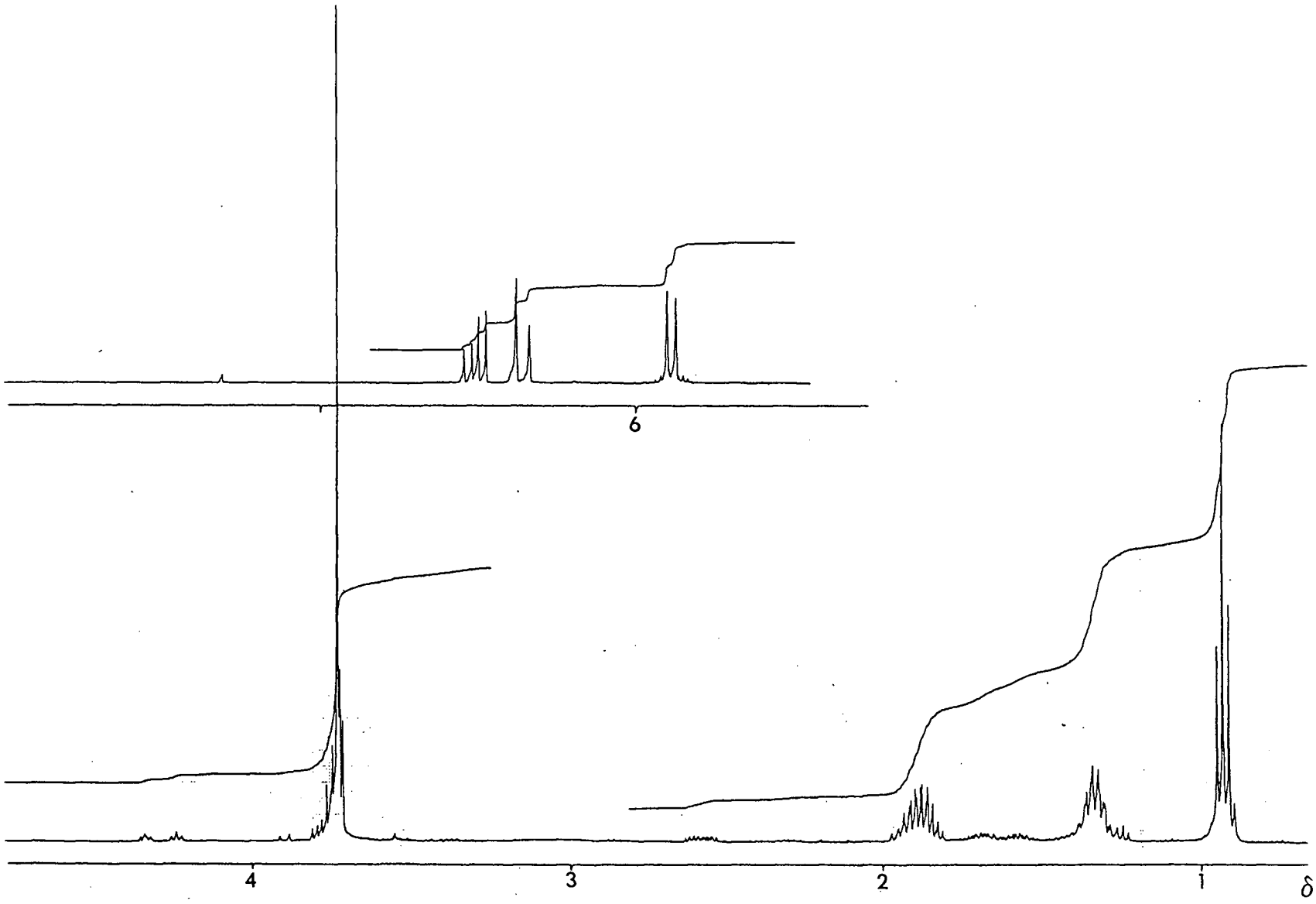
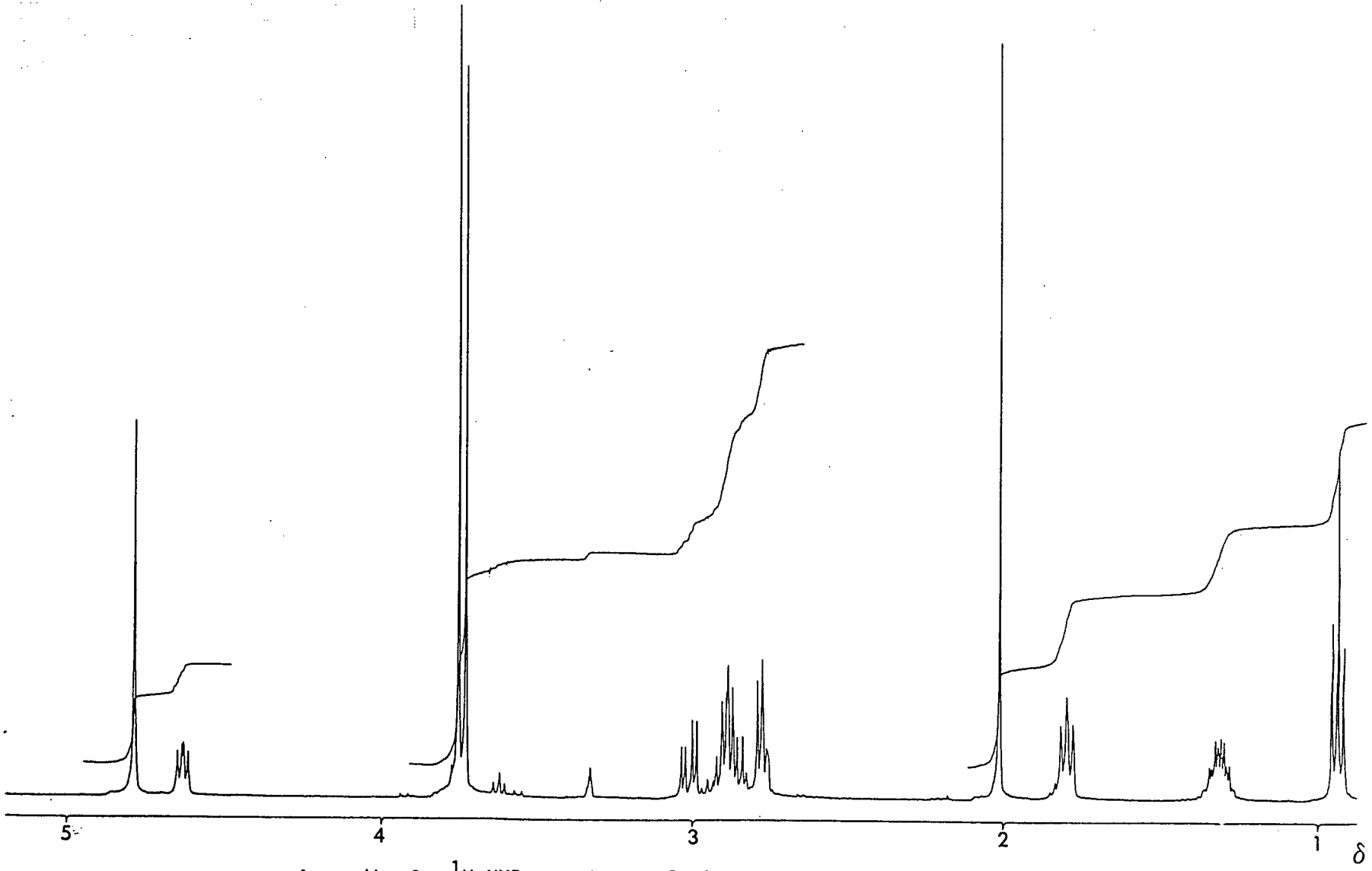
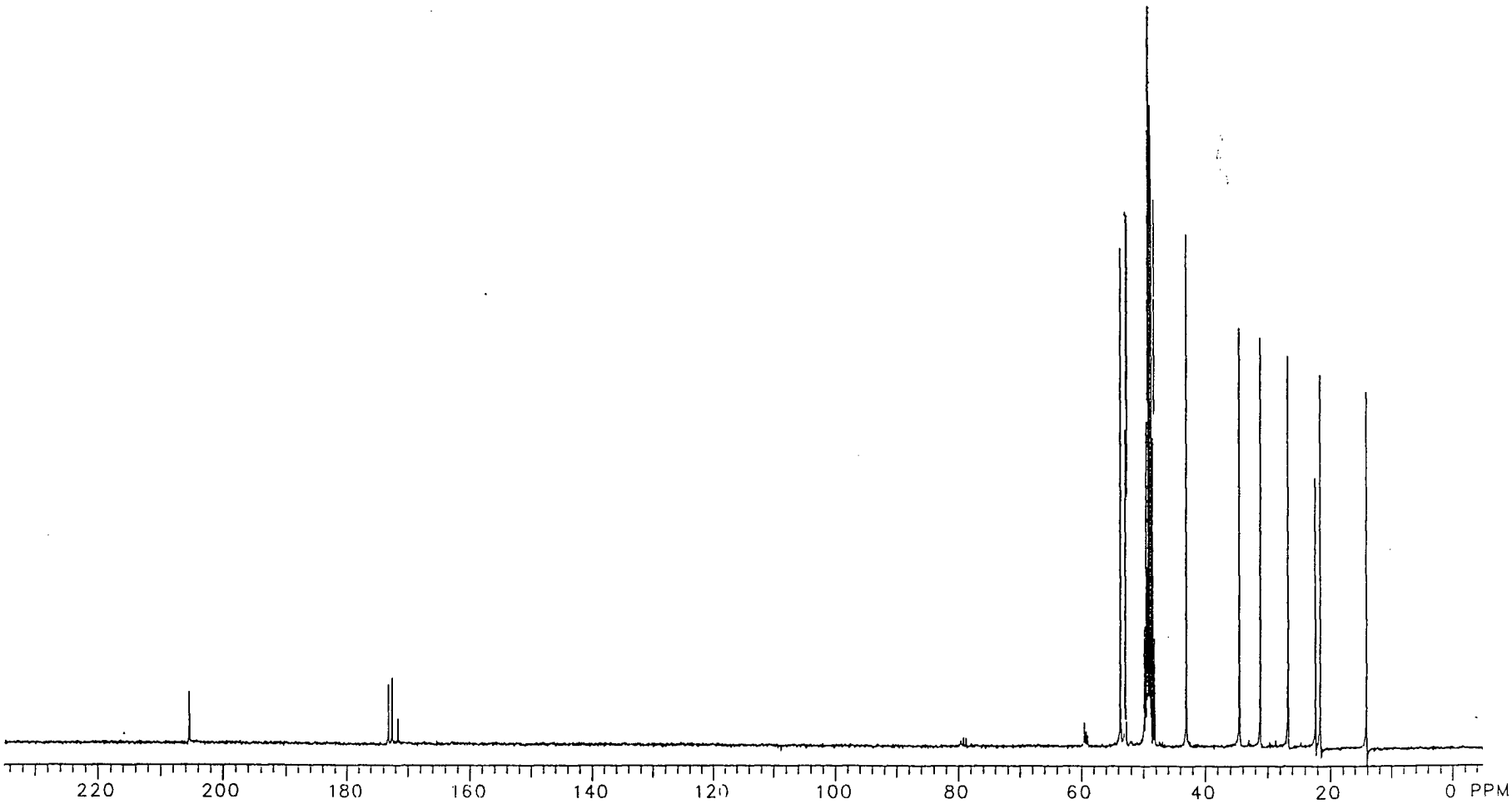
VII. APPENDICES



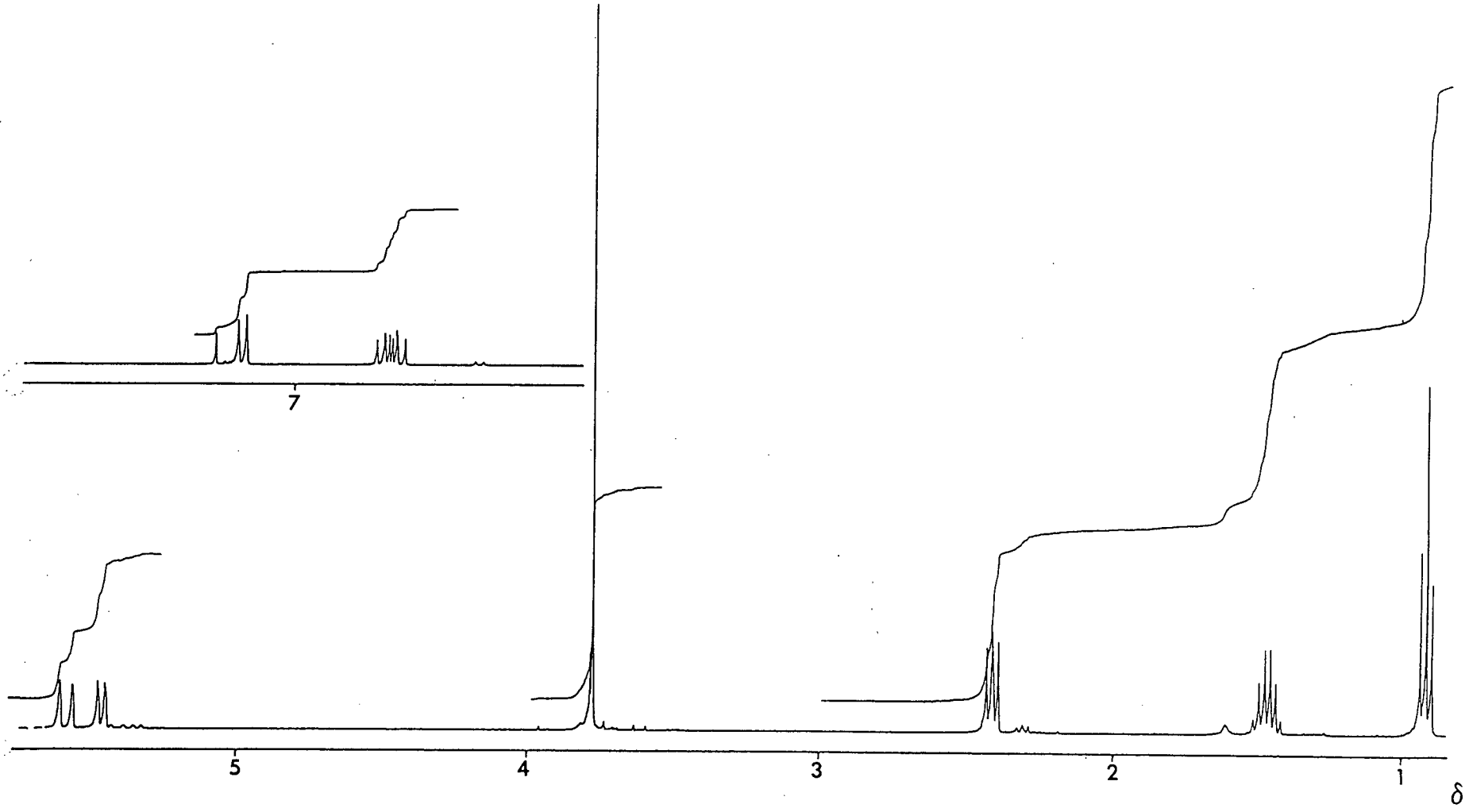
Appendix 1. ^{1}H-NMR spectrum of methyl 3-keto-4-ene VPA.



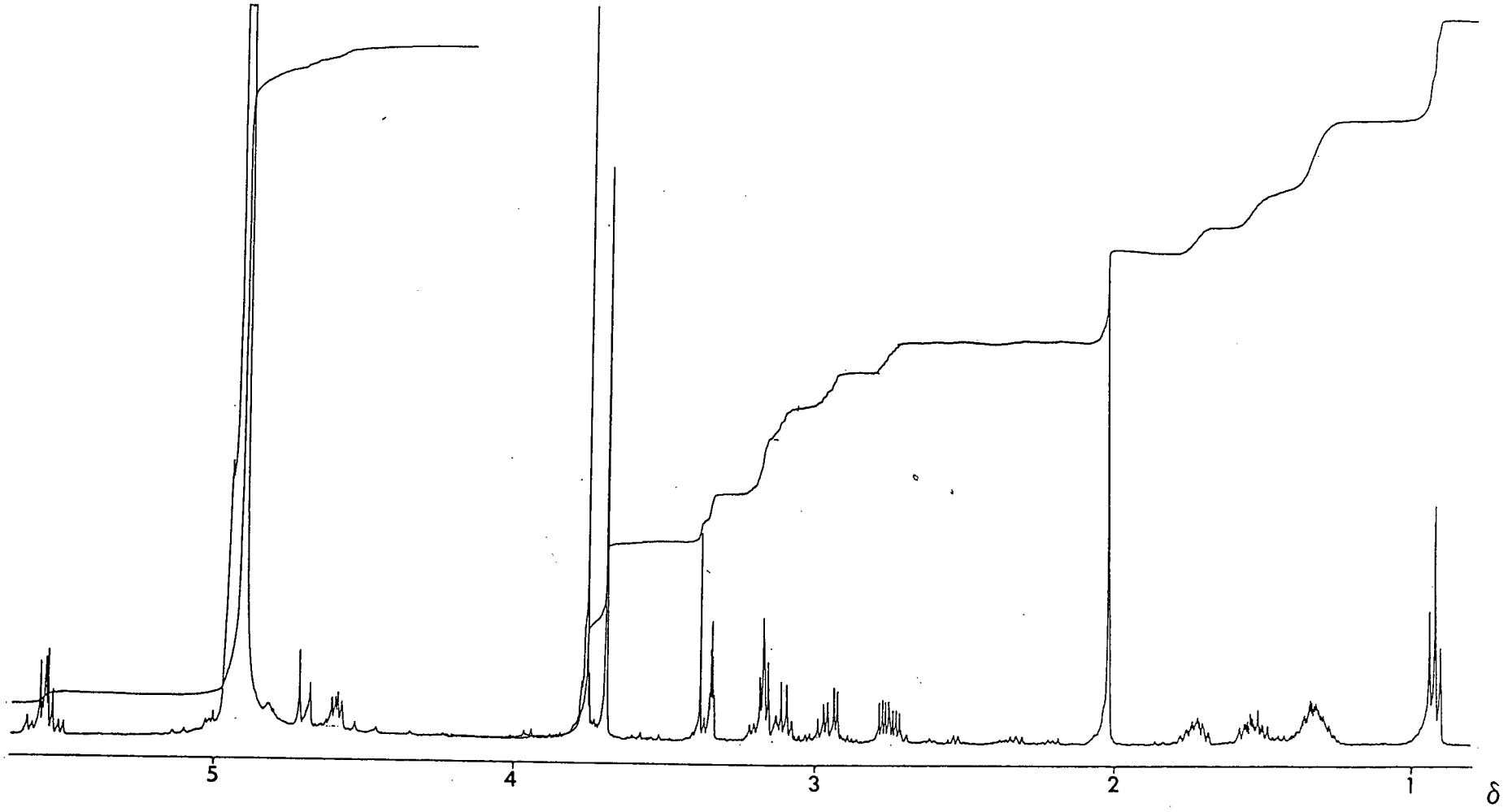
Appendix 2. ^{1}H-NMR spectrum of the NAC conjugate of 3-keto-4-ene-VPA, dimethyl ester.



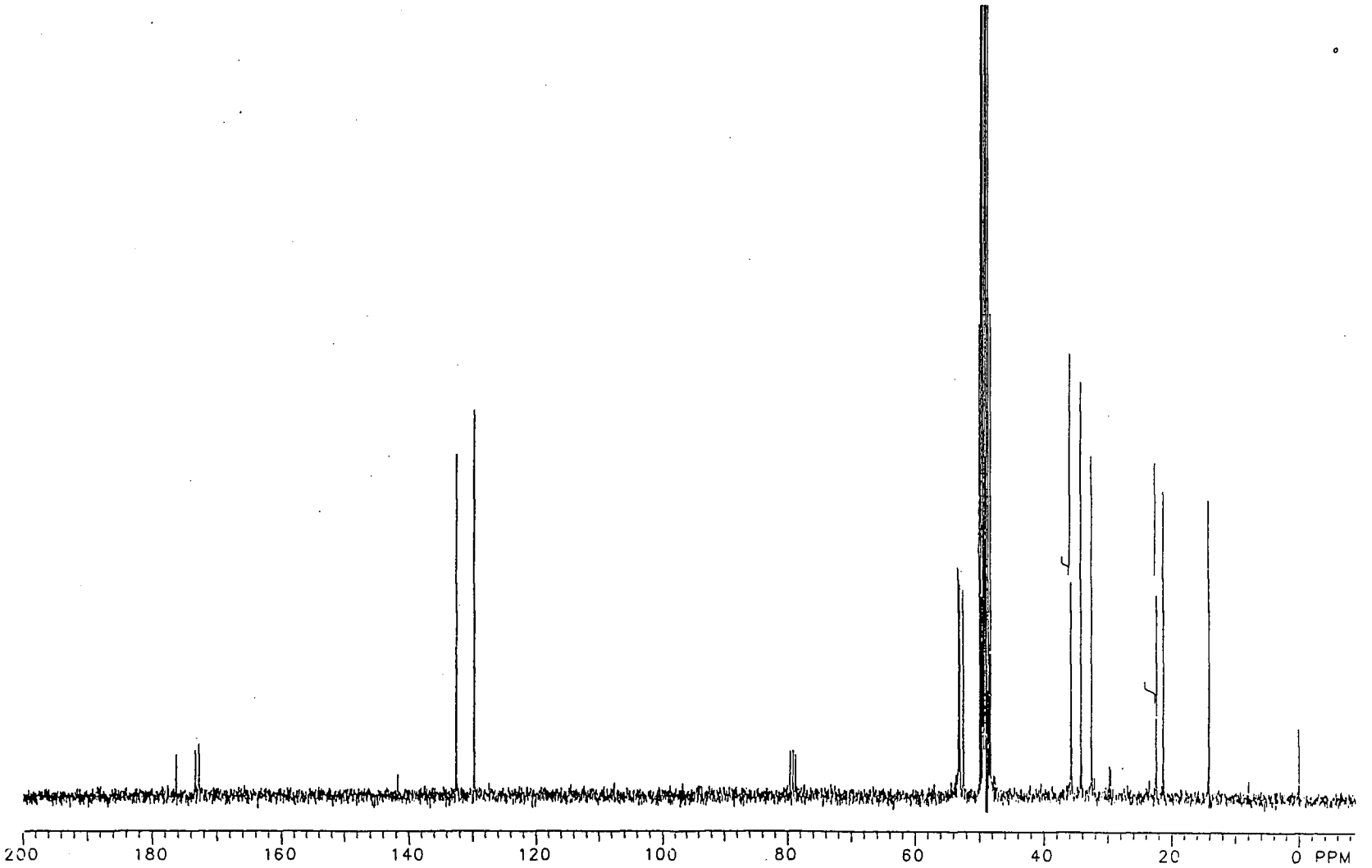
Appendix 3. ^{13}C-NMR spectrum of the dimethyl ester of the NAC conjugate of 3-keto-4-ene VPA.



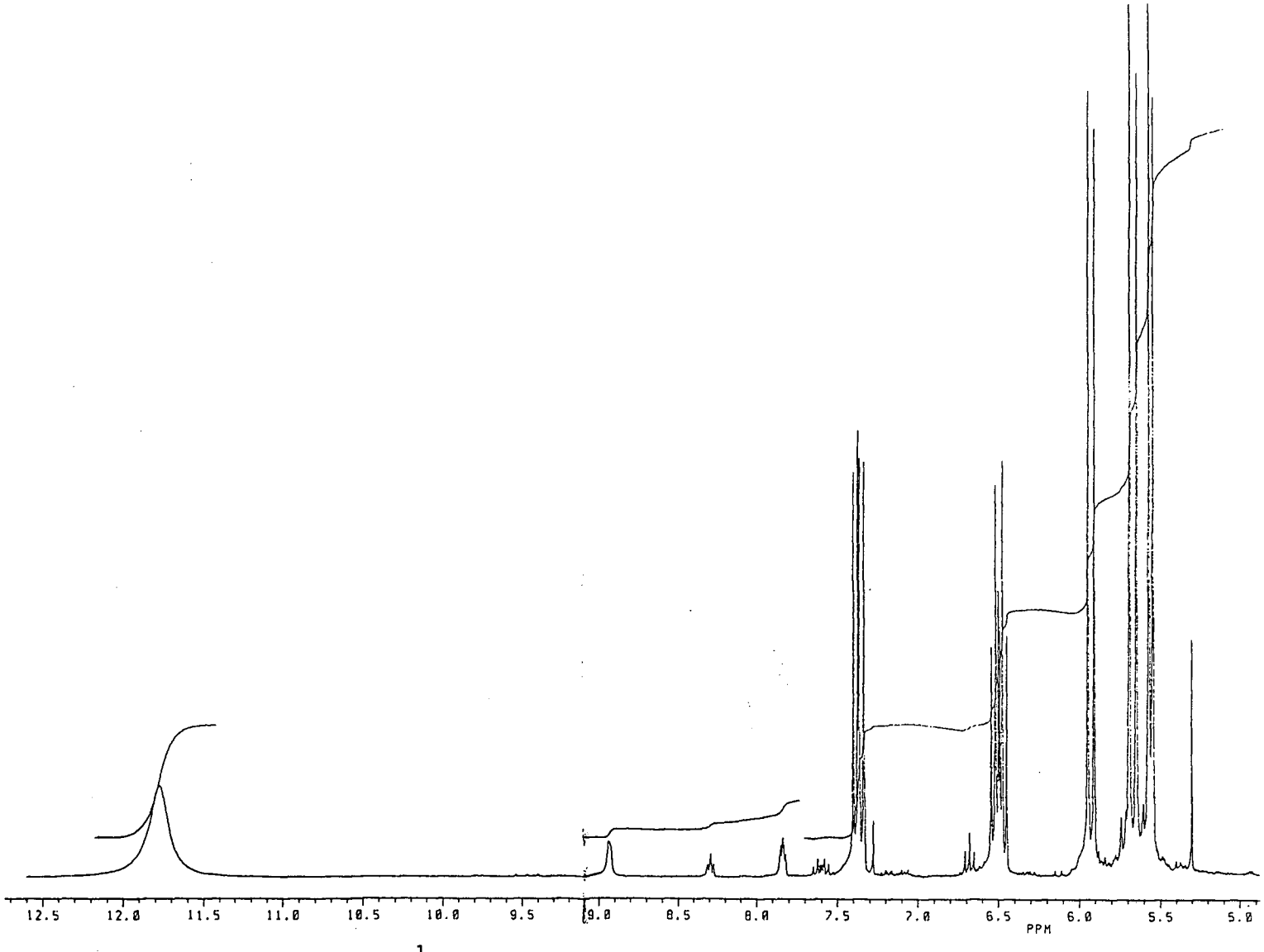
Appendix 4. ^{1}H-NMR spectrum of the methyl ester of (E)-2,4-diene VPA.



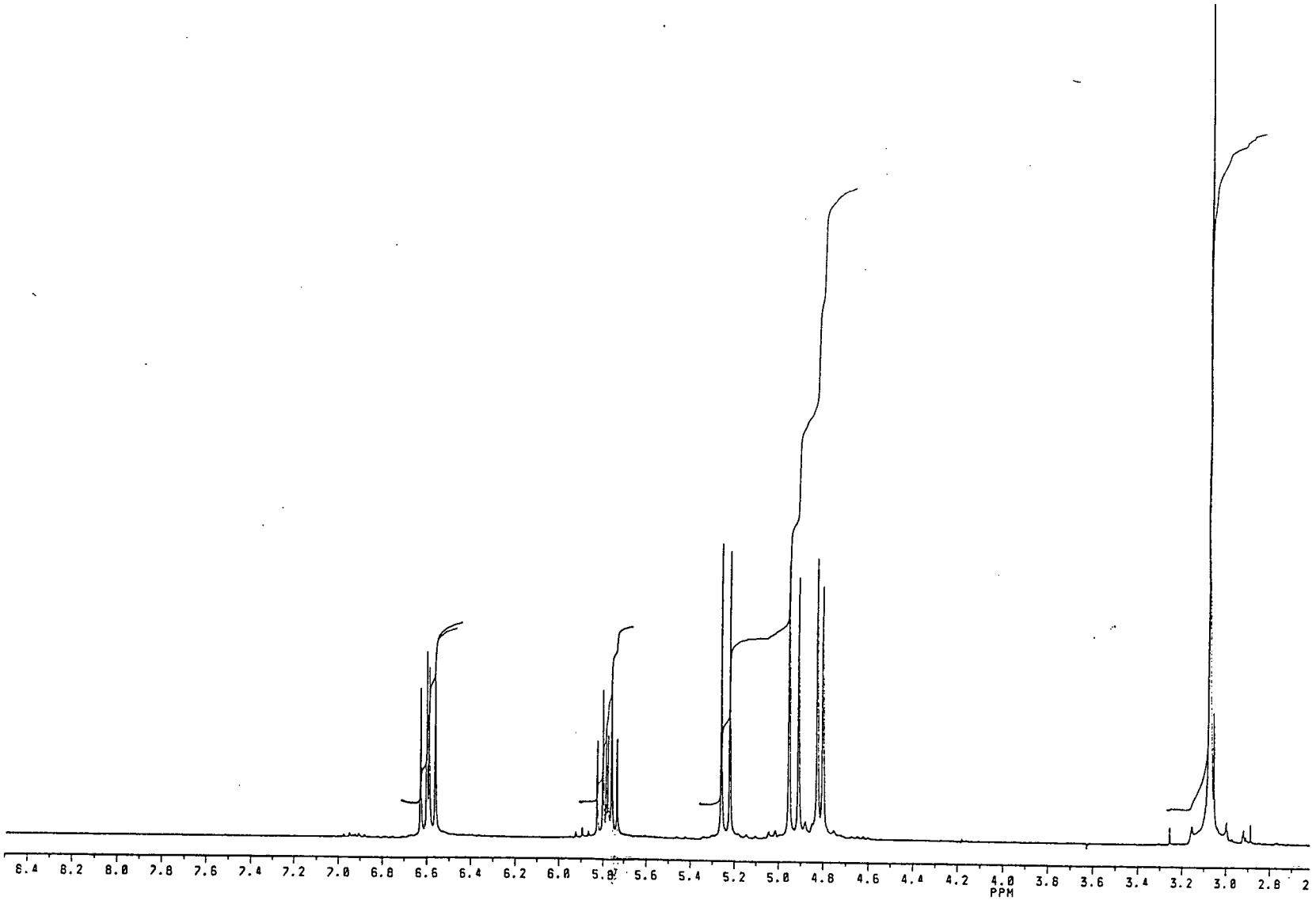
Appendix 5. ^{1}H-NMR spectrum of the methyl NAC conjugate of methyl (E)-2,4-diene VPA.



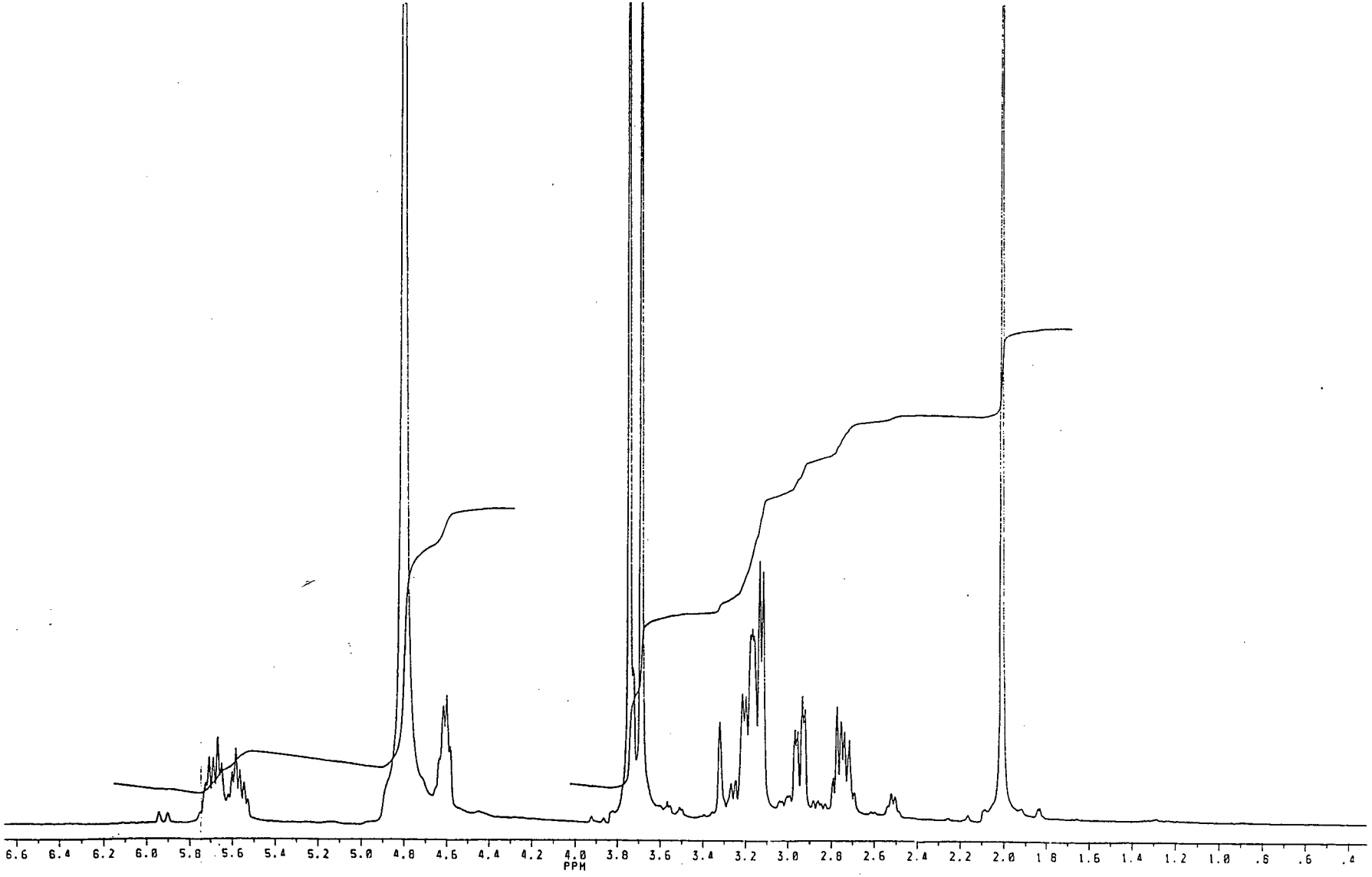
Appendix 6. ^{13}C-NMR spectrum of the NAC conjugate of (E)-2,4-diene VPA, dimethyl ester.



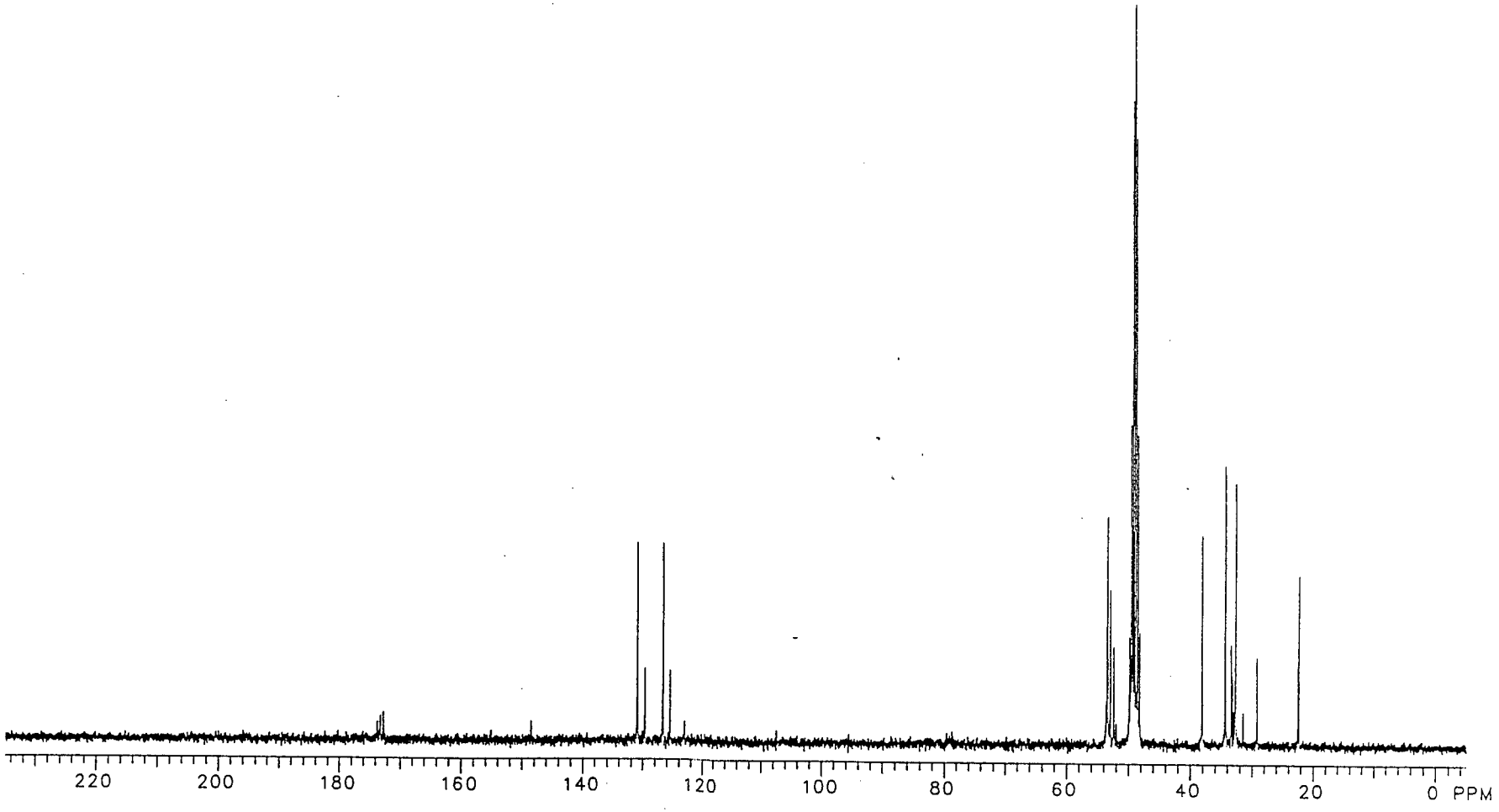
Appendix 7. ^{1}H-NMR spectrum of (E)-2,4-pentadienoic acid.



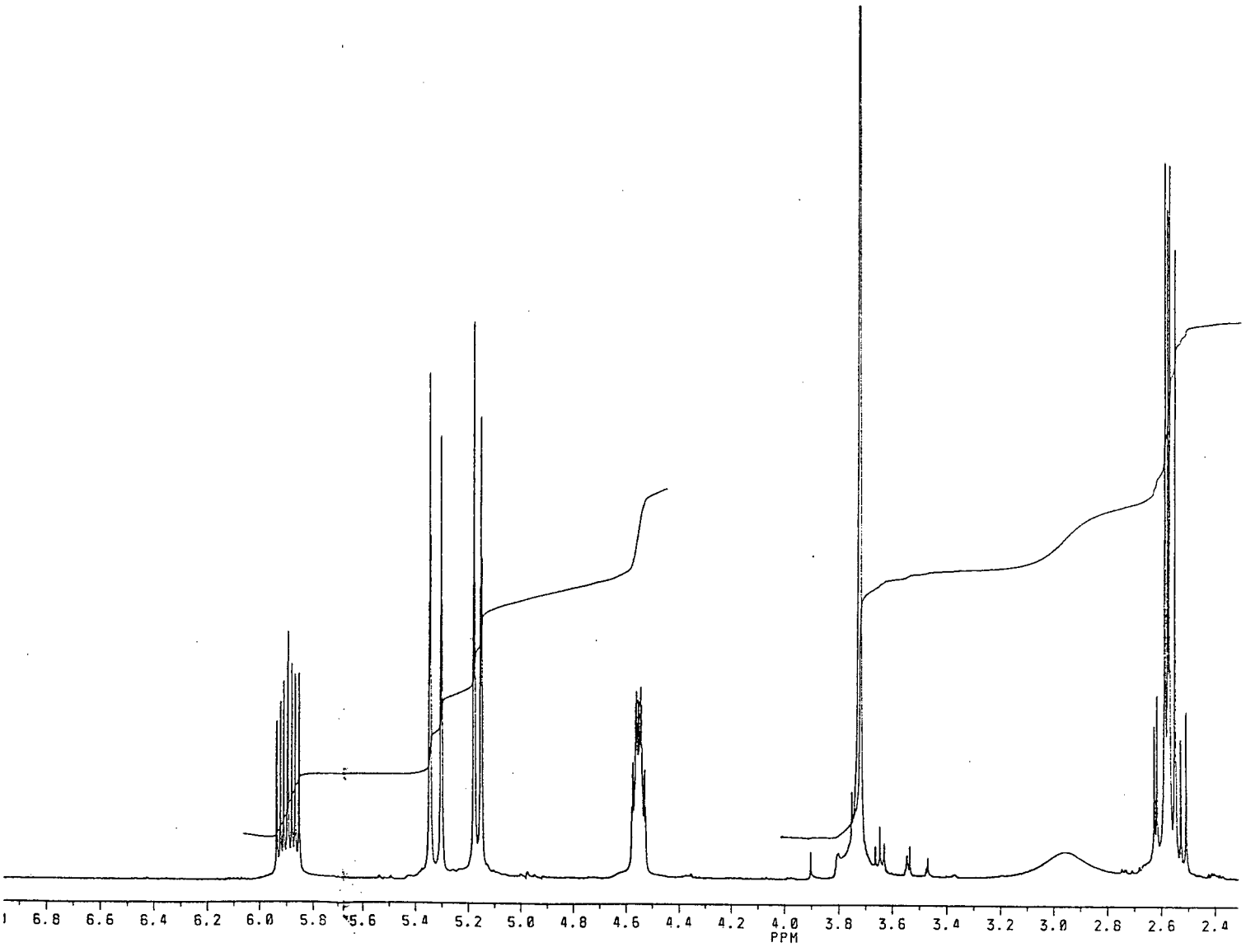
Appendix 8. ^{1}H-NMR spectrum of methyl (E)-2,4-pentadienoate.



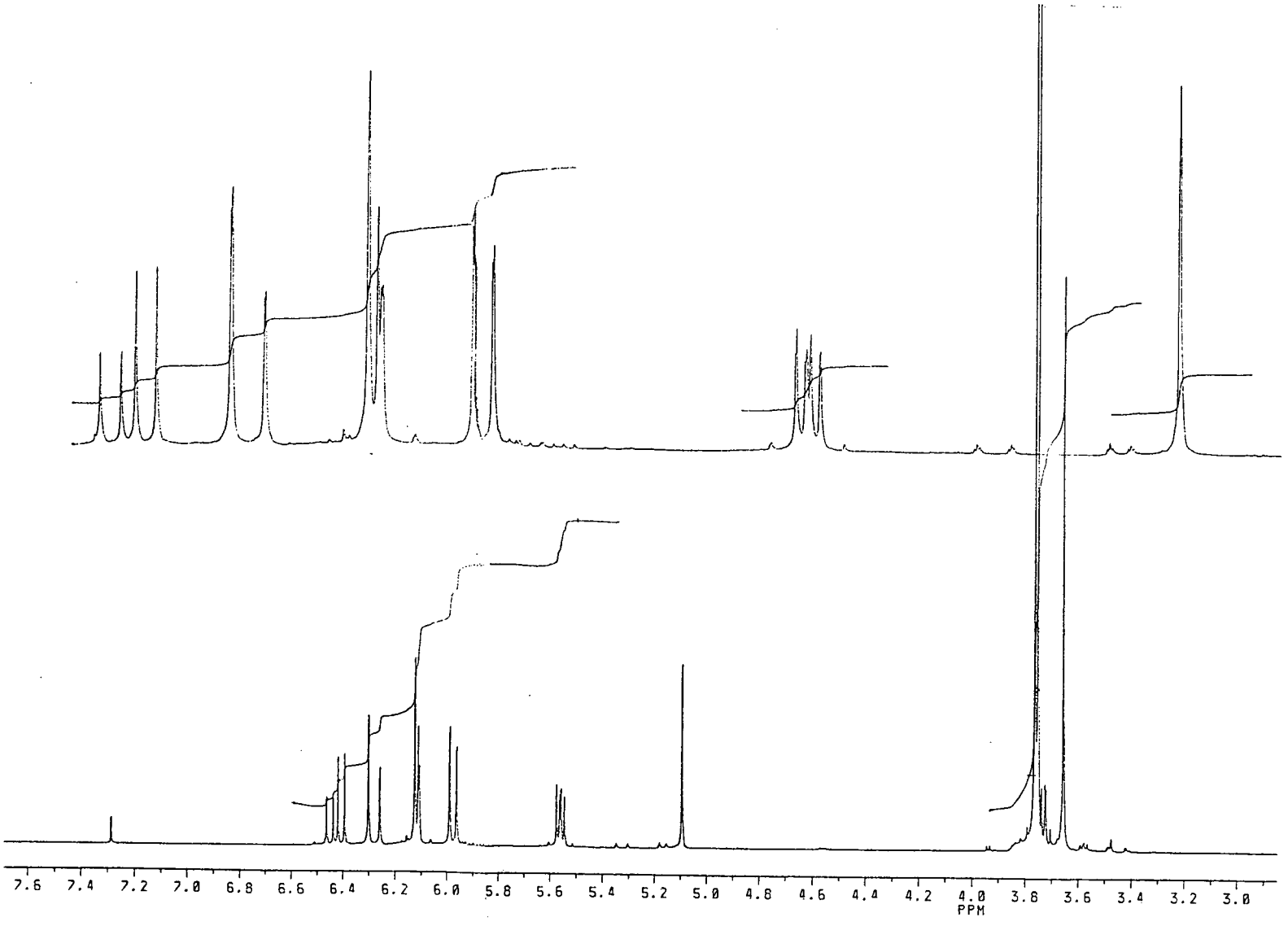
Appendix 9. ^{1}H-NMR spectrum of (E)-5-(N-acetylcystein-S-yl)3-pentenoate, dimethyl ester.



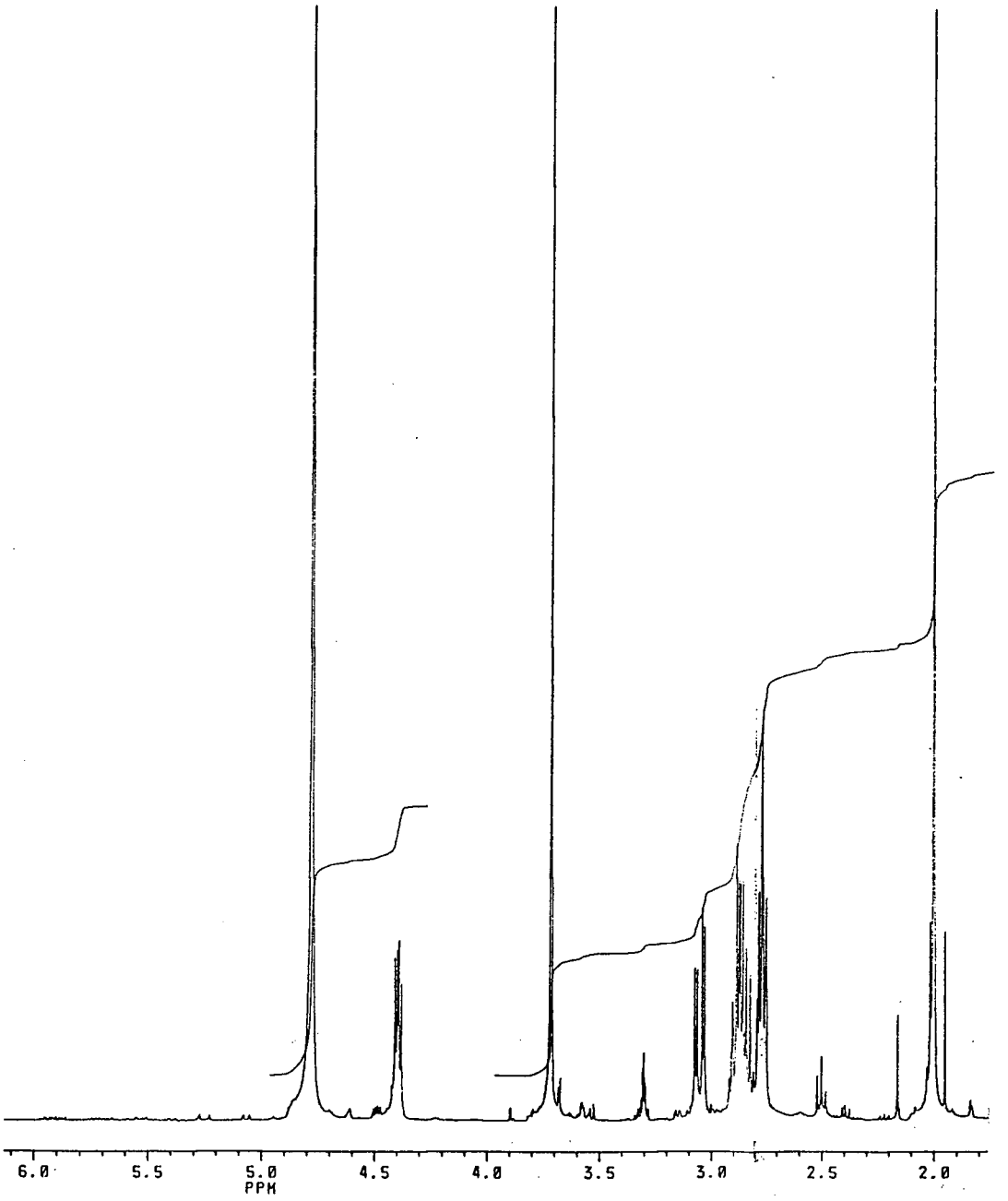
Appendix 10. ^{13}C-NMR spectrum of (E)-5-(N-acetylcystein-S-yl)3-pentenoate, dimethyl ester.



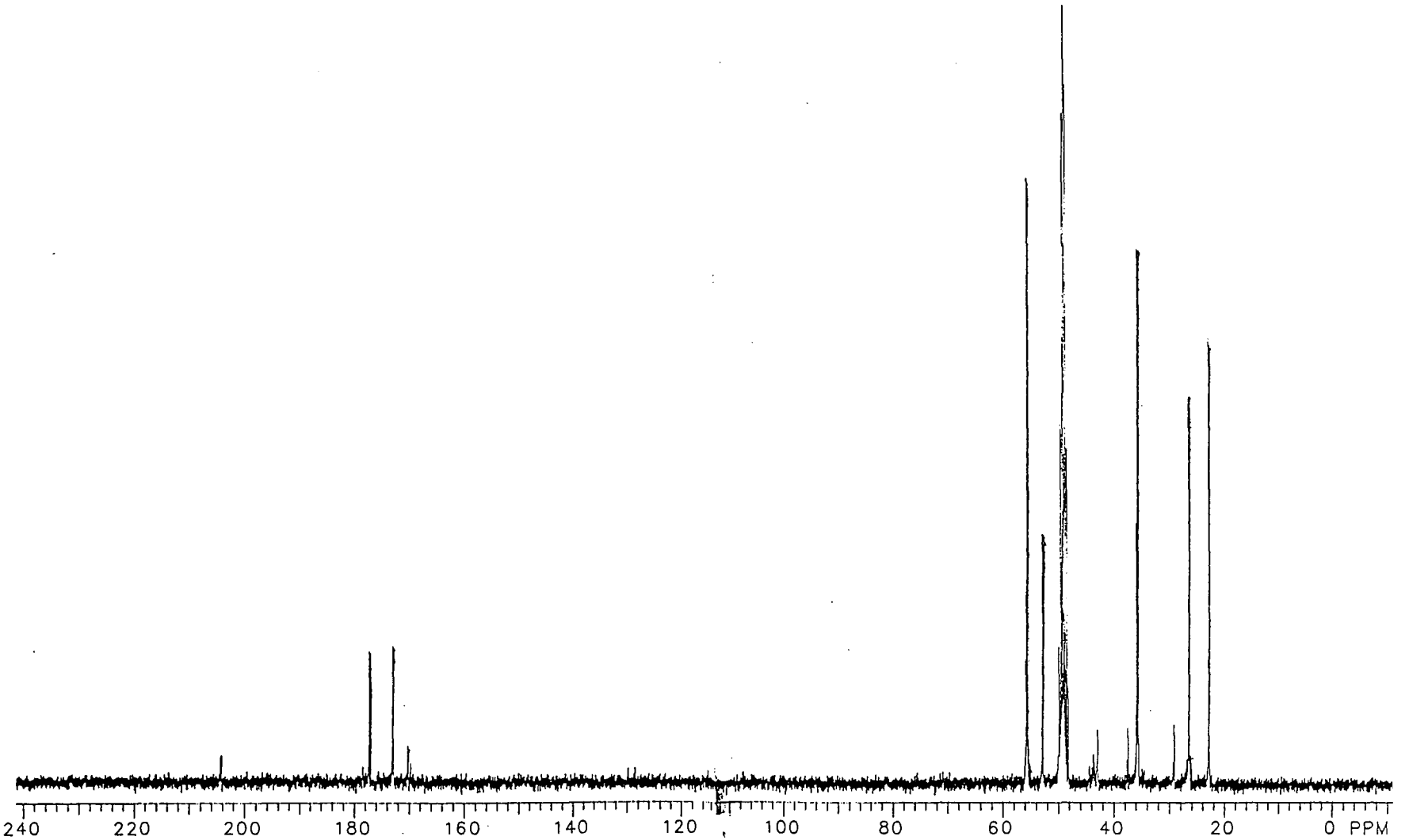
Appendix 11. ^{1}H-NMR spectrum of methyl 3-hydroxy-4-pentenoate.



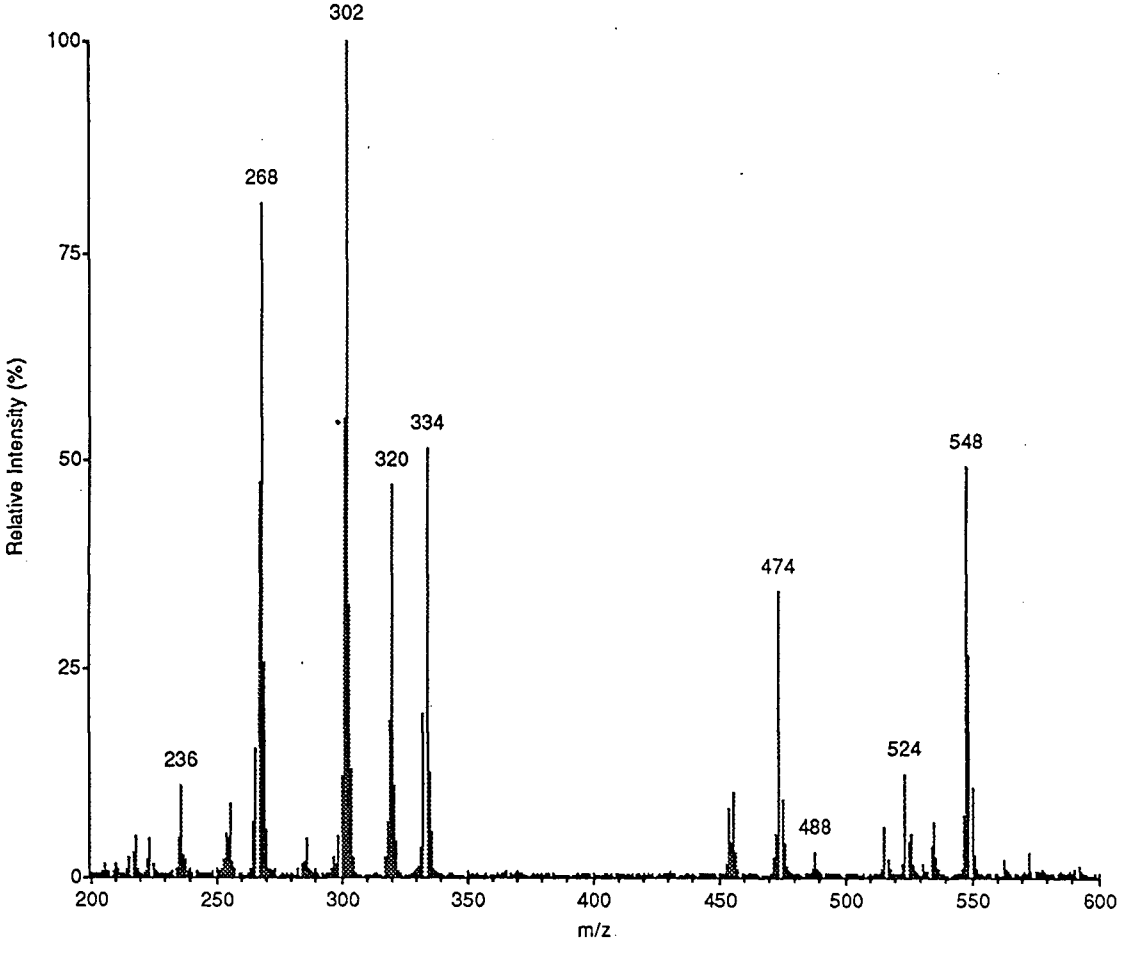
Appendix 12. ^{1}H-NMR spectrum of methyl 3-oxo-4-pentenoate.



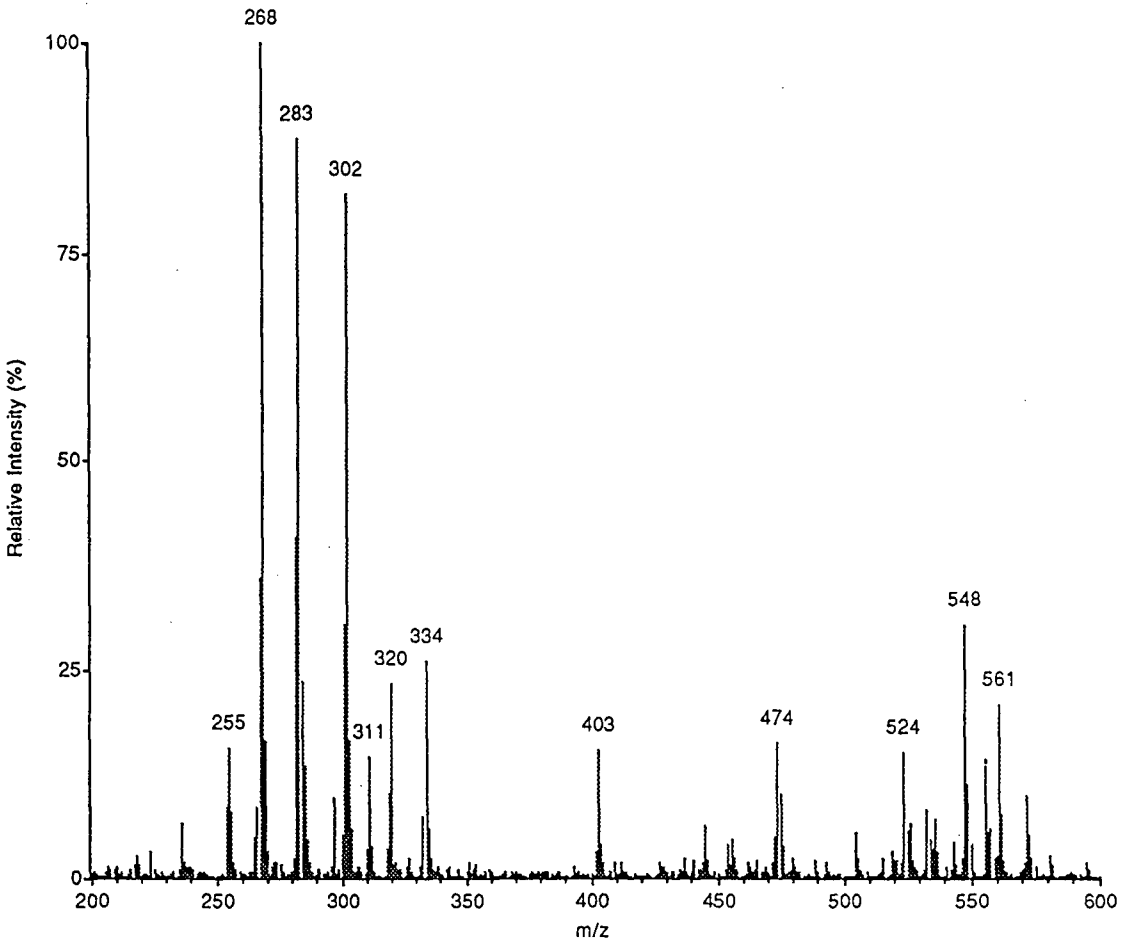
Appendix 13. ^{1}H-NMR spectrum of 5-(N-acetylcystein-S-yl)3-oxopentanoate, mono-methyl ester.



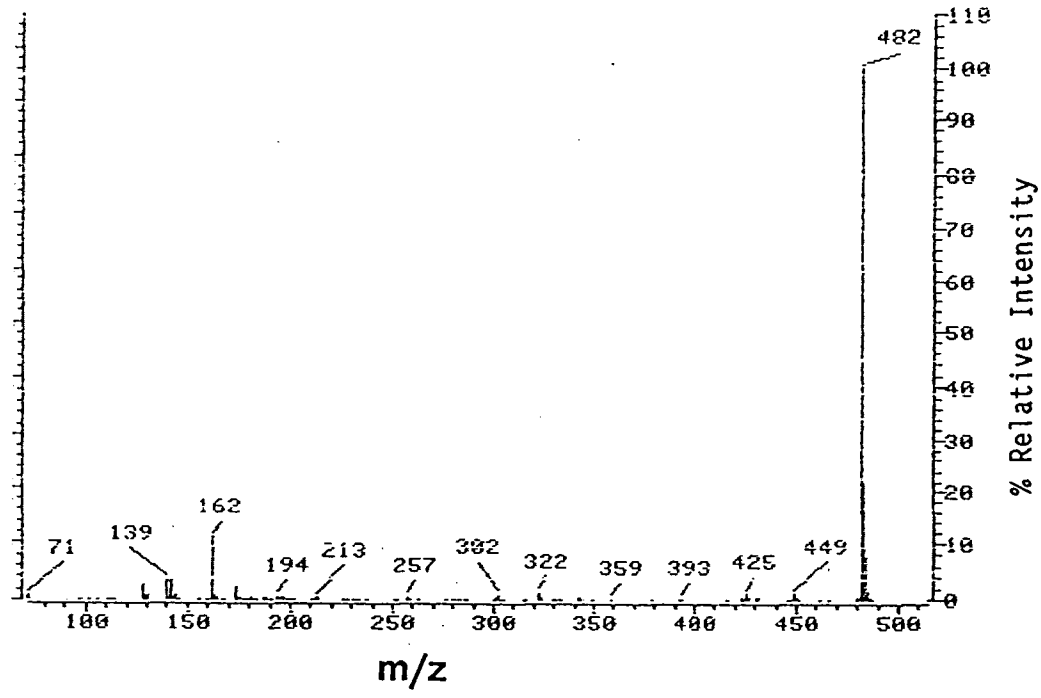
Appendix 14. ^{13}C-NMR spectrum of 5-(N-acetylcystein-S-yl)3-oxopentanoate, mono-methyl ester.



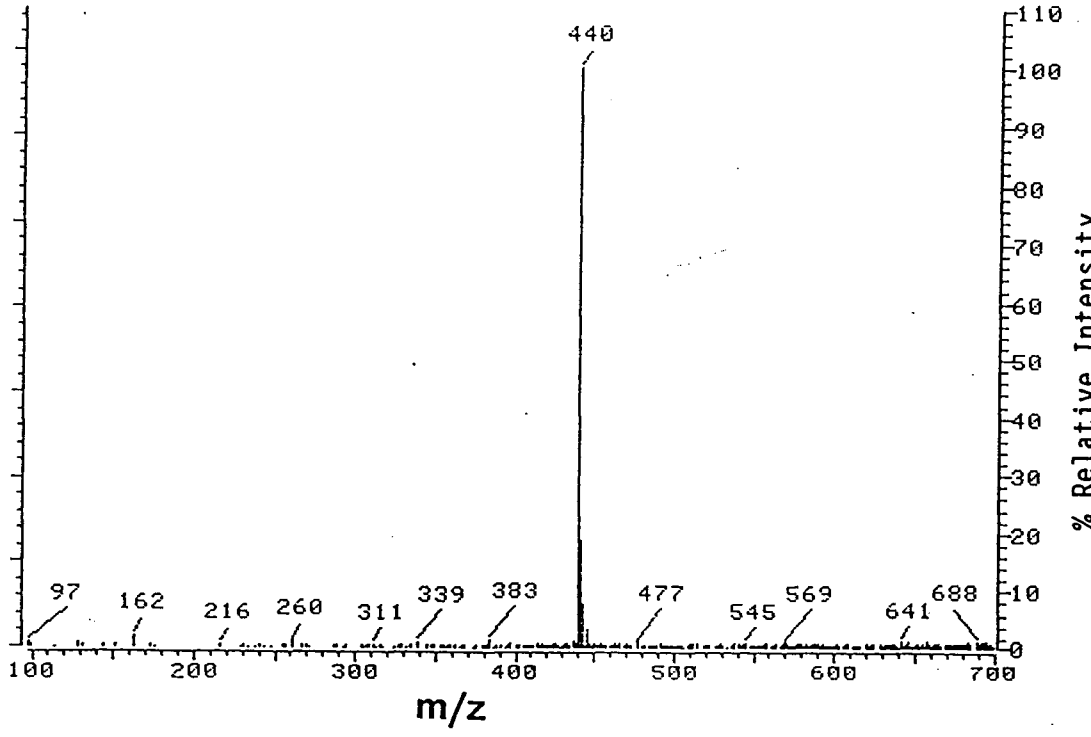
Appendix 15. LC/MS mass spectrum (negative ion mode) of the trimethylated GSH conjugate of (E)-2,4-diene VPA. The ion at m/z 548 is the $(M+OOCCH_3)^-$ anion of the conjugate.



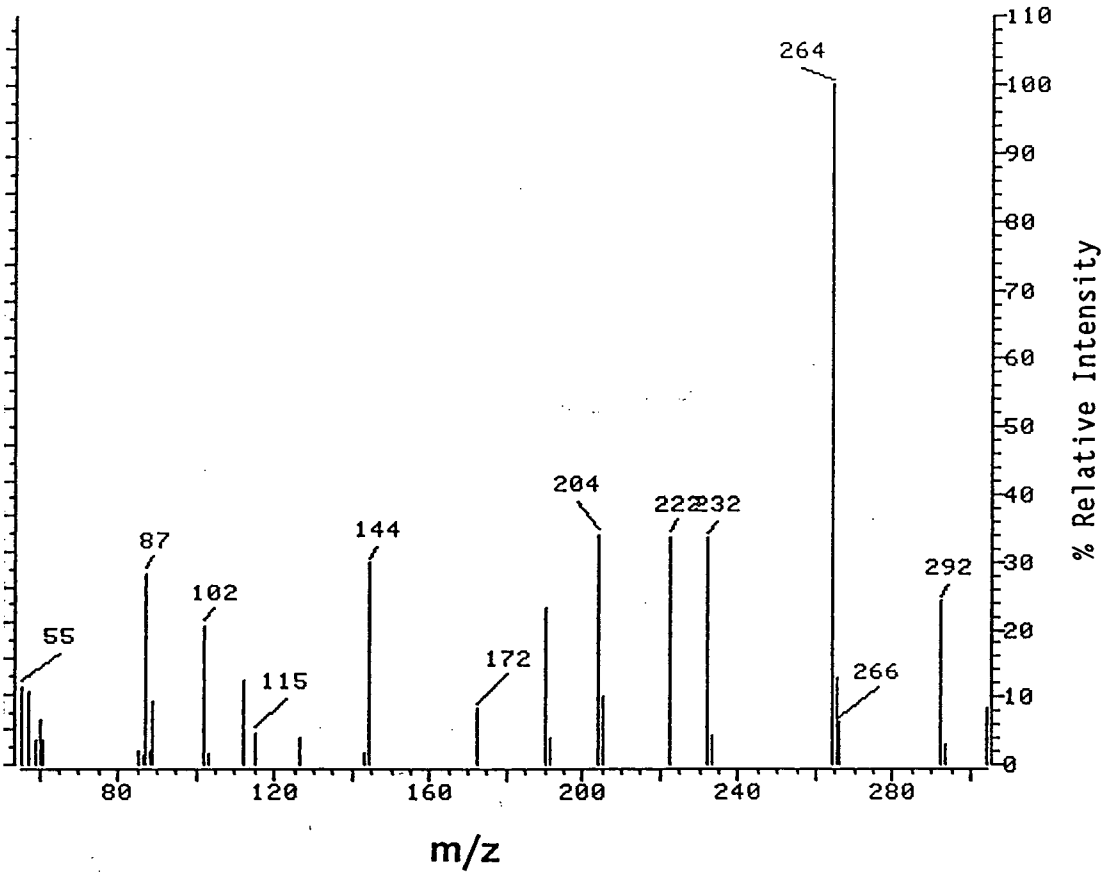
Appendix 16. LC/MS mass spectrum (negative ion mode) of a bile component (methylated) from rats dosed with (E)-2,4-diene VPA that was identified as the GSH conjugate of (E)-2,4-diene VPA. The ion at m/z 548 is the $(M+OOCCH_3)^-$ anion of the fully methylated conjugate.



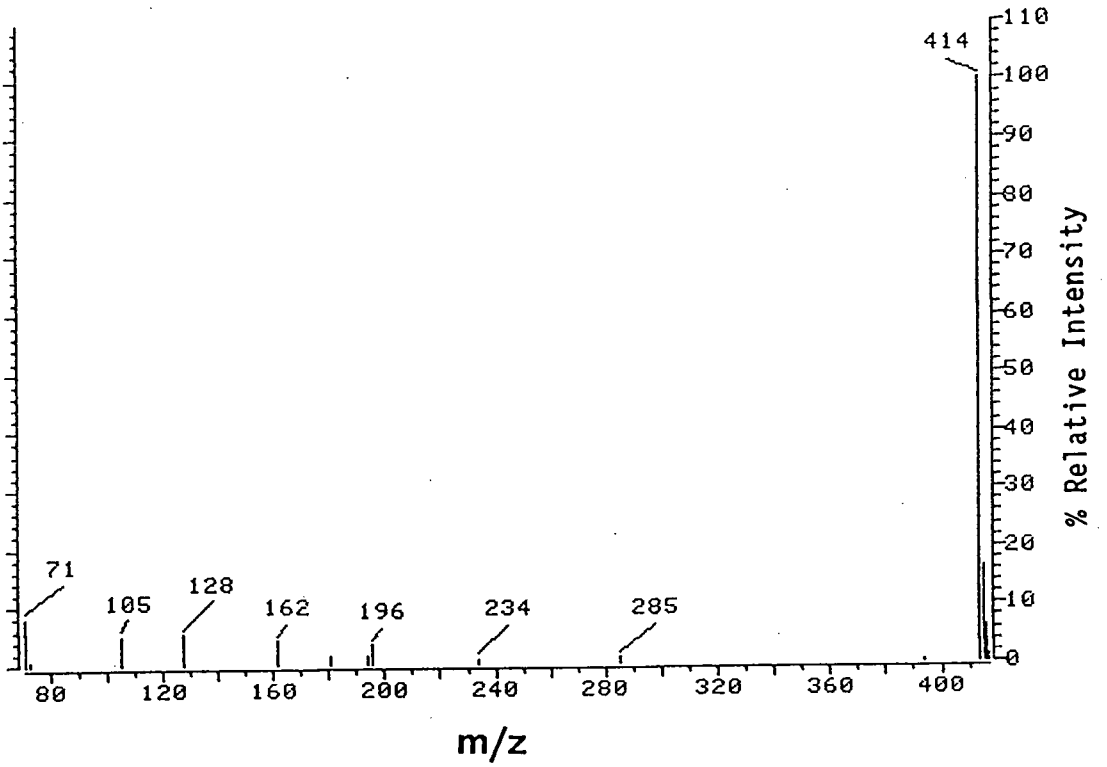
Appendix 17. NICI/GC/MS mass spectrum of the PFB derivative of NAC conjugate of (E)-2,4-diene VPA. The ion m/z 482 is the $[M-181]^-$ anion of the di-PFB derivative of the conjugate.



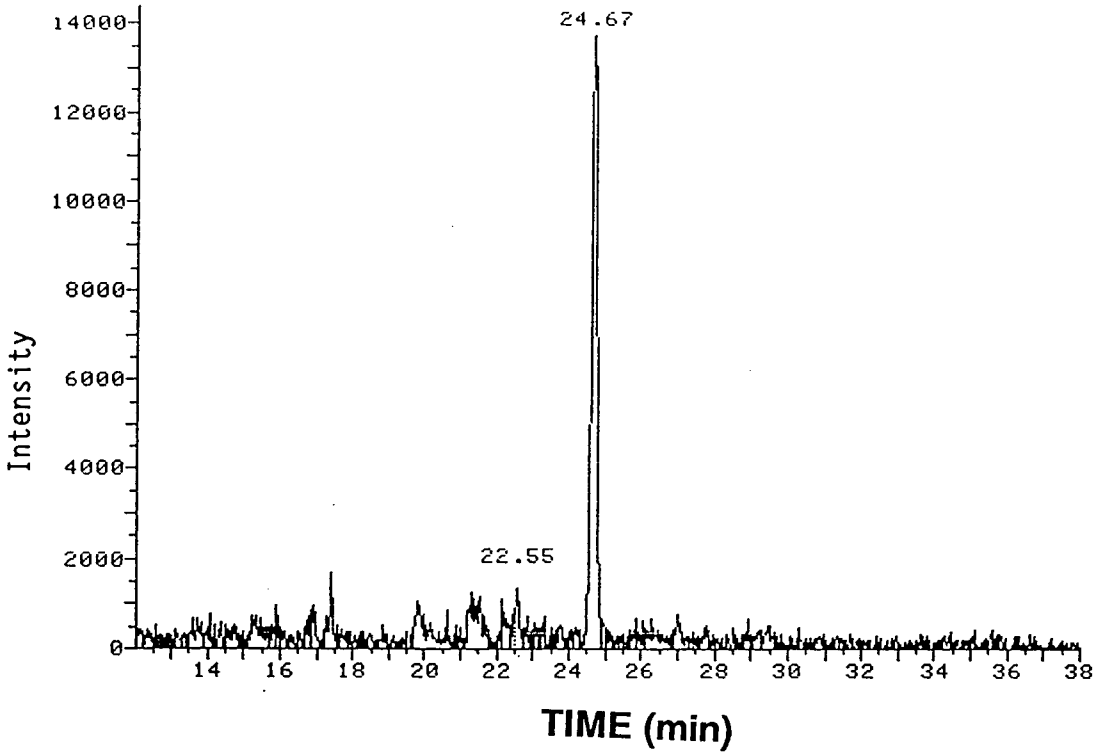
Appendix 18. NICI/GC/MS mass spectrum of the PFB derivatized NAC conjugate of (E)-2,4-pentadienoic acid. The icn at m/z 440 is the $[M-181]^-$ anion of the di-PFB derivative of the conjugate.

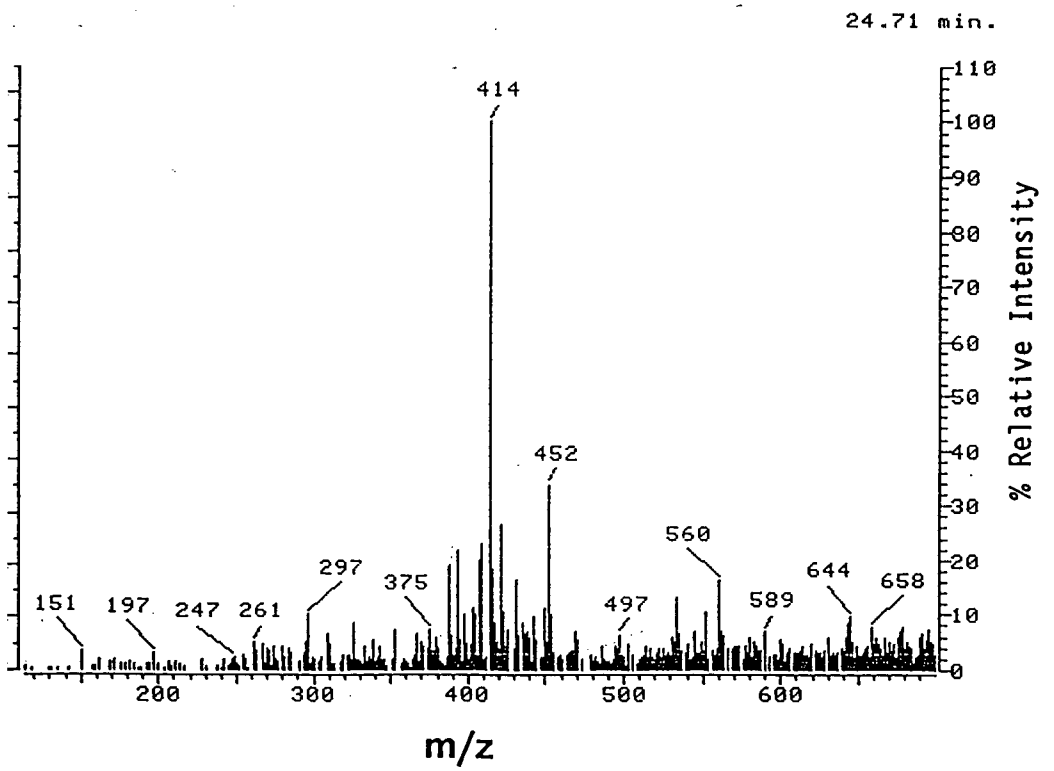


Appendix 19. PCI/GC/MS mass spectrum of the methylated cysteine conjugate of 3-oxo-4-pentenoate. The ion at m/z 264 is the $[M+H]^+$ ion of the conjugate.

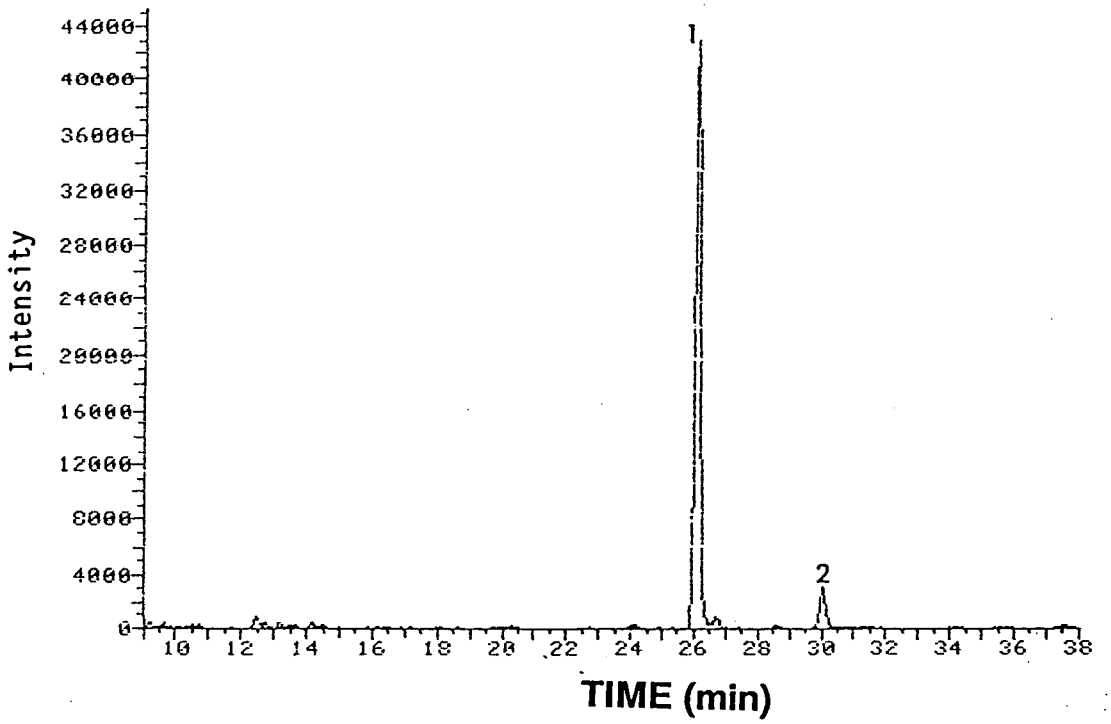


Appendix 20. NICI/GC/MS mass spectrum of the PFB derivatized NAC conjugate of acrylic acid. The peak at m/z 414 is the $[M-181]^-$ of the di-PFB derivative of the conjugate.

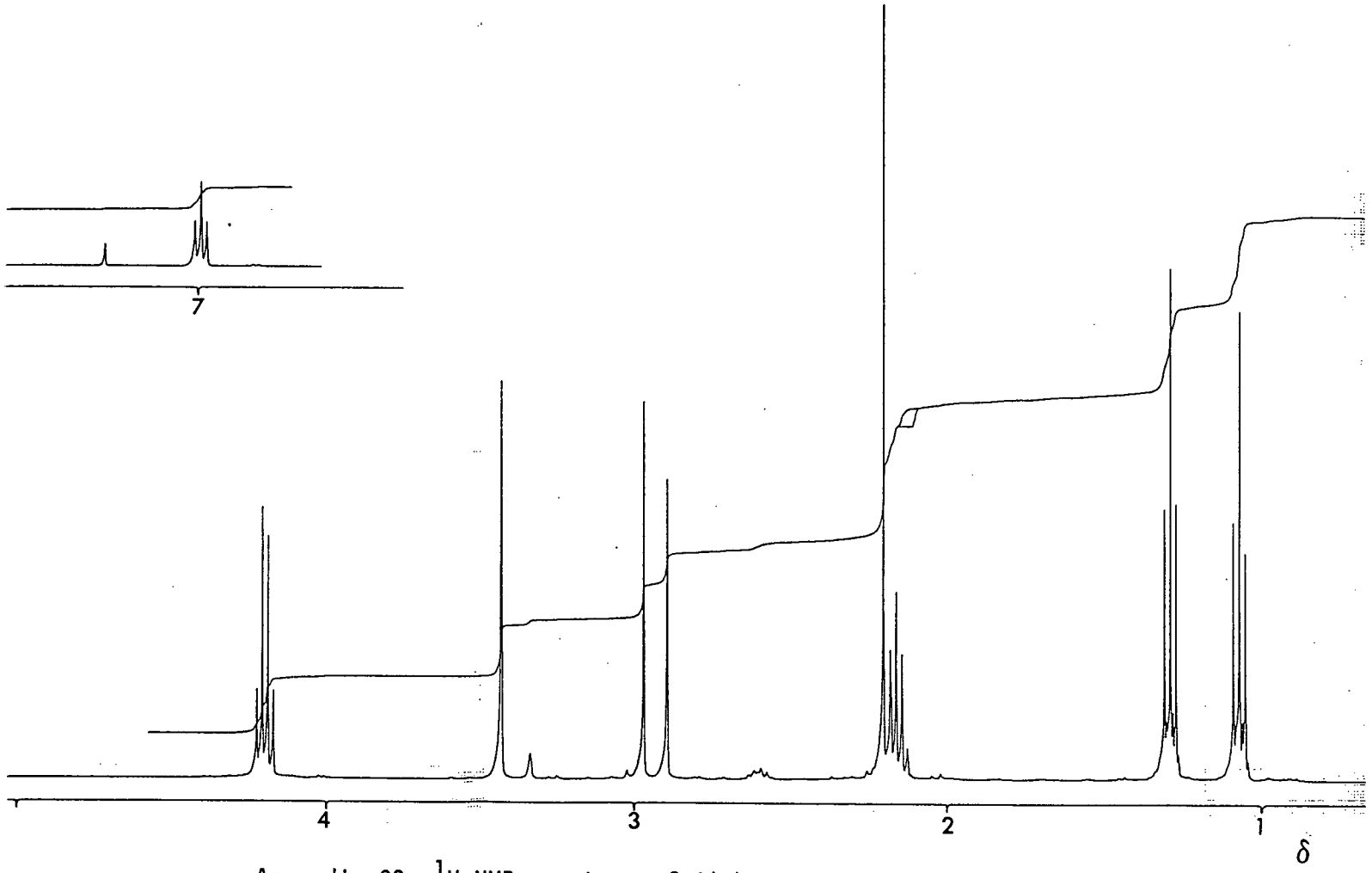




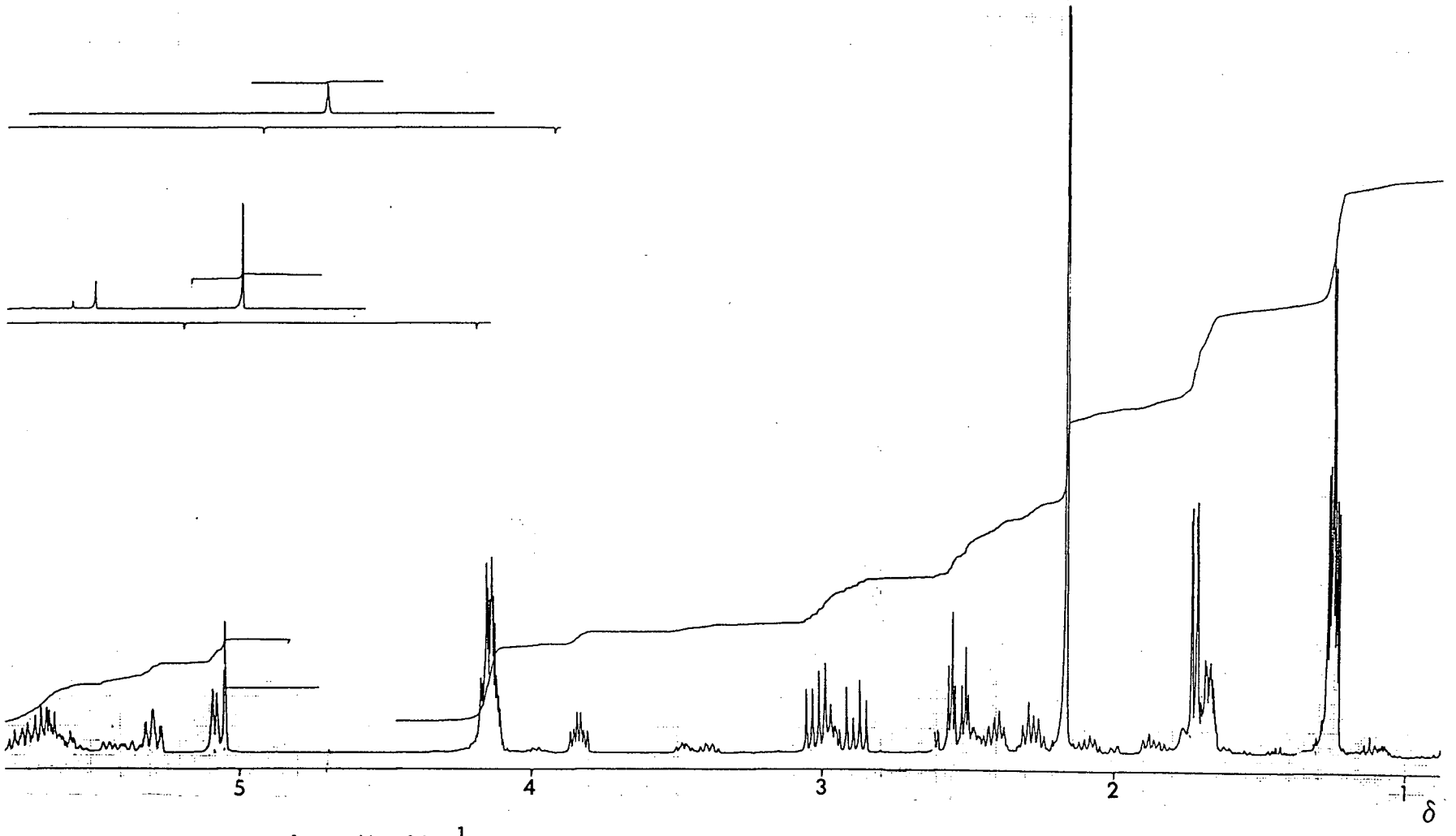
Appendix 21. NICI/GC/MS extracted ion (m/z 414) chromatogram and spectrum from rat urine treated with (E)-2,4-pentadienoic acid. The peak at 24.67 min was identified as the NAC conjugate of acrylic acid.



Appendix 22. TIC chromatogram, in the NICI mode, of a PFB derivatized synthetic sample of (E)-2,4-diene VPA. Peaks 1 and 2 are isomeric NAC conjugates of (E)-2,4-diene VPA.



Appendix 23. ^{1}H-NMR spectrum of 4'-keto-2-ene VPA ethyl ester.



Appendix 24. ^{1}H-NMR spectrum of the ethyl ester of 4'-keto-3-ene VPA.