QUANTITATIVE STRUCTURE-ANTICONVULSANT ACTIVITY STUDIES OF VALPROIC ACID ANALOGUES

By

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We accept this thesis as conforming

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ABSTRACT

Valproic acid (2-propylpentanoic acid) is an antiepileptic drug widely used for treatment of absence seizures. Valproic acid has a unique chemical structure which does not contain the imide structure found in most conventional antiepileptic drugs. An in vivo study of the antagonism of pentylenetetrazol-induced clonic seizures by alkyl-substituted carboxylic acids and tetrazoles was of interest owing to the known bioisosterism between the carboxylic and the tetrazolyl moiety. The main objective of this study was to investigate the role played by the lipophilicity, the electronic properties and the steric influence of compounds on their anticonvulsant potency.

Quantitative structure-activity relationships of the aliphatic and alicyclic substituted carboxylic acids and tetrazoles have been performed using the Hansch linear free-energy relationships model. The study proceeded by synthesis of compounds using known procedures. The diunsaturated derivatives of valproic acid, 2-[(E)-1'-propenyl]-(E)-2-pentenoic acid and 2-[(Z)-1'-propenyl]-(E)-2-pentenoic acid were prepared via a stereoselective synthetic route. The synthesized diunsaturated acids were used in identification of the major diunsaturated metabolite of valproic acid as 2-[(E)-1'-propenyl]-(E)-2-pentenoic acid.

The anticonvulsant potency of test compounds was determined in mice (CD1 strain, 20-32g) by the standard subcutaneous pentylenetetrazole seizure threshold test. The pentylenetetrazole clonic seizure test was found to be more sensitive to structural effects than the pentylenetetrazole mortality assay. The lipophilicity (octanol-water partition coefficient) of compounds was determined indirectly by reversed phase liquid chromatography employing an octadecylsilane column (Hypersil ODS)

and mobile phase as 70% methanol : 30% phosphate buffer (pH 3.5). The electronic character of the compounds was monitored by the apparent acid ionization constant obtained from potentiometric titration in 10% methanol-water system.

The ED_{50} of 0.70 mmol/kg found for valproic acid was similar to literature values. 5-Heptyltetrazole was found to be the most potent compound in the series of analogues studied. The carboxylic plus tetrazole group gave a low correlation ($r = 0.63$) between the anticonvulsant potency and a linear combination of lipophilicity and apparent ionization constant. However, in the series of active carboxylic acids, the anticonvulsant activity was noted to be significantly correlated with lipophilicity and apparent ionization constant ($r = 0.91$).

The usefulness of the electronic parameters, acid ionization constant and dipole moment, were explored in an extensive set of alkyl-substituted anticonvulsant compounds with different polar moieties. Addition of the dipole moment term to the lipophilicity term led to significantly better correlations ($r = 0.81$) as compared to that with an added pKa term. The negative dependency of anticonvulsant activity on dipole moment supported previous findings in studies of 1,4-benzodiazepines and phenyl-substituted anticonvulsant compounds.

There were some exceptions to the dependence of anticonvulsant activity on lipophilicity and dipole moment or pKa. N,N-dibutylsuccinamic acid showed convulsant properties at sublethal doses. The lack of activity of cyclohexylacetic acid and 5-cyclohexylmethyltetrazole, in comparison to the active 1-methylcyclohexanecarboxylic acid, has some pharmacological significance. It shows a certain degree of molecular specificity in the anticonvulsant action of valproic acid analogues. The cyclohexylmethyl conformation was suggested, from a

proposed model, to be less effective in hydrophobic binding due to a steric effect at a stereoselective position on the hydrophobic site of the GABA receptor complex. Thus it can be concluded that while lipophilicity governed access to sites of action, the dependence of activity on the polar character may explain the diverse structures of anticonvulsants provided that the steric requirements of the hydrophobic binding site are met. Steric effects may lead to inactivity or even convulsant properties of alkyl-substituted compounds.

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SYMBOLS AND ABBREVIATIONS

P	octanol-water partition coefficient
pKå	negative logarithm of the apparent acid ionization constant
ED_{50}	effective dose in 50% of mice
TD_{50}	toxic dose in 50% of mice
k'	capacity factor
tR	retention time of retained solute
t_o	elution time of unretained solute
i.p.	intraperitoneal
s.c.	subcutaneous
r	correlation coefficient
S	standard error of estimate
μ	dipole moment
E_S	Taft steric factor
σ*	polar substituent constant
E	trans
Z	cis
m/z	mass to charge ratio
f	fragmental constant
s	singlet
t	triplet
d	doublet
dd	doublet of doublets
q	quadruplet
m	multiplet
δ	chemical shift
J	coupling constant

o/w	octanol-water
i.d.	internal diameter
ACN	acetonitrile
BDZ	benzodiazepine
C-18	octadecylsilane
CNS	central nervous system
DHP	dihydropicrotoxinin
diene	diunsaturated
GABA	gamma-aminobutyric acid
GABA-T	Gamma-aminobutyrate transaminase
GAD	glutamic acid decarboxylase
GCMS	gas chromatography mass spectrometry
HMPA	hexamethylphosphoramide
HPLC	high-performance liquid chromatography
IR	infra red
LDA	lithium diisopropylamide
lit.	literature
MES	maximal electroshock seizure test
NMR	nuclear magnetic resonance
ODS	octadecylsilane
PTZ	pentylenetetrazole
QSAR	quantitative structure-activity relationships
RP	reversed phase
SAR	structure-activity relationships
s.c. PTZ	subcutaneous pentylenetetrazole seizure threshold test
SSA	Succinic semialdehyde

t-BDMS	tertiarybutyldimethylsilyl
TBPS	t-butylbicyclophosphothionate
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
2,3'-diene VPA	2-(1'-propenyl)-2-pentanoic acid
2,4-diene VPA	2-propyl-2,4-pentadienoic acid
2E-3'E Diene	2-[(E)-1'-propenyl]-(E)-2-pentenoate
2E-3'Z Diene	2-[(Z)-1'-propenyl]-(E)-2-pentenoate
2Z-3'E Diene	2-[(E)-1'propenyl]-(Z)-2-pentenoate
2Z-3'Z Diene	2-[(Z)-1'-propenyl]-(Z)-2-pentenoate
3Z-3'Z Diene	2-[(Z)-1'-propenyl]-(Z)-3-pentenoate
3Z-3'E Diene	2-[(E)-1'-propenyl]-(Z)-3-pentenoate
3E-3'E Diene	2-[(E)-1'-propenyl]-(E)-3-pentenoate
3,3'-Diene	2-(1'-propenyl)-3-pentenoate
VPA	valproic acid
2-ene VPA	2-propyl-2-pentenoic acid
4-ene VPA	2-propyl-4-pentenoic acid
3-ene VPA	2-propyl-3-pentenoic acid
4-OH VPA	2-propyl-4-hydroxypentanoic acid
3-OH VPA	2-propyl-3-hydroxypentanoic acid
4-Keto VPA	2-propyl-4-oxopentanoic acid

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INTRODUCTION

The medium branched-chain fatty acid, valproic acid, I,(2-propyl-pentanoic acid) is a relatively new antiepileptic drug, introduced in North America in 1978. It is used in treatment of absence seizures and in combination therapy for generalized tonic-clonic seizures. The discovery of the anticonvulsant properties of valproic acid by Meunier et al. (1) has directed some attention toward similar potential anticonvulsant compounds without the imide structure as found in most conventional antiepileptic drugs.

Valproic acid presents a good lead compound for structure-activity studies because it appears to have a novel mechanism of action involving augmentation of GABAergic activity. It has been reported to increase brain GABA levels in vivo and inhibit enzymes involved in GABA degradation pathways (2). Elucidation of the molecular actions of valproic acid that are directly related to its anticonvulsant effect would be facilitated by the development of structure-activity relationships (SAR) within a series of closely-related analogues of valproic acid. Investigation of the molecular specificity of the anticonvulsant action of valproic acid analogues is also of interest in determining structural requirements at the site of action.

Earlier studies on the relationships between structure and activity explored the possibility of modifying the carboxylic functional group to enhance anticonvulsant activity. Most of the compounds tested for anticonvulsant activity were dipropylacetic acid derivatives such as amides (3-5), ureides (6), esters (3,5) including an oxazepam derivative (7) and a hydantoin derivative of valproic acid (6). Ureas (8), alco-

hols (5,9), carbamates (9) and ketones (9) with the 1-propylbutyl chain, as in valproic acid, were also examined for anticonvulsant activity.

A number of aliphatic and alicyclic-substituted carboxylic acids have been evaluated for anticonvulsant activity but only at a single dose (5,10,11) or biological response was determined using a less specific test, i.e. protection against pentylenetetrazole (PTZ) -induced mortality (10). However, there have been recent studies which reported the dose-dependency of the anti-PTZ clonic seizure activity of homologous straight-chain and alpha-branched fatty acids (12,13,14).

Anticonvulsant drugs have diverse chemical structures which suggest they may have different mechanisms of action or that they may interact at a similar site of action by virtue of having some similar pharmacophoric groups. There have been several attempts to uncover the pharmacophoric structural features of the conventional antiepileptic drugs. Andrews (15) reported that there was no relationship between the anticonvulsant activity and the effective atomic charges at the quaternary carbon common to the anticonvulsant drugs with the imide or ureide structure. Some investigators have looked at the variety of functional groups that will confer anticonvulsant activity on compounds with alkyl or phenyl substituents (16). Patrick and Bresee (17) reported that hydrogen-bonding strengths of the major antiepileptic drugs, measured by the hydrogen-bonding enthalpies with phenol, were the same for the compounds studied and thus unrelated to activity. Camerman and Camerman (18) examined the x-ray structures of some conventional antiepileptic drugs including phenytoin, diazepam and phenylacylurea and proposed that the spatial configurations of these compounds allow superposition of the phenyl groups and also the carbonyl groups or an equivalent electron-donor group.

Valproic acid has the basic structural features, a polar moiety with an electron-donor group or hydrogen-bonding group and hydrophobic substituents, in common with conventional anticonvulsant drugs. It also possesses a carboxylic group and alkyl chain as in the structure of gamma-aminobutyric acid (GABA). However, valproic acid does not have a nitrogen function as found in the structure of GABA. Different aliphatic and alicyclic groups may enhance or have an adverse effect on the interaction of the carboxylic acids at the site of action. This has been shown in some barbiturates where the presence of an isoalkyl, isoalkenyl and β-cyclohexylidene-ethyl chain at the quaternary carbon results in convulsant activity (19).

From studies on a homologous alpha-branched aliphatic carboxylic acid series, Keane et al. (13) and Meldrum et al. (12) reported that there was a significant correlation between the anticonvulsant potency and the length of side-chain. They also found good correlations between the increase in GABA brain levels and anticonvulsant potency. In a different study, Perlman and Goldstein (14) used a fluorescent probe to show that the ability of these homologous carboxylic acids to disorder synaptosomal plasma membranes correlated well with their anticonvulsant potency. From the fluorescent polarization studies, they suggested that the anticonvulsant effect of valproic acid is mediated by nonspecific mechanisms similar to those of general anesthetics. It seems that a wide variety of structures are required to investigate the structural specificity of valproic acid analogues.

The present investigation is concerned with the effect of diverse substituents on anticonvulsant activity of valproic acid analogues. The physicochemical properties, namely lipophilicity, electronic properties

and steric factors have been determined to find whether activity in vivo is determined by a nonspecific property such as lipophilicity or whether there is a stereoelectronic factor determining anticonvulsant activity. Multiparametric relationships increase the likelihood of structural specificity in a class of structurally-related compounds. This approach has been used by various investigators to reconcile the high structural specificity of drug-receptor interactions and the common physicochemical properties of disparate structures with a common biological activity (20).

Different tests have been used to evaluate the anticonvulsant potency of compounds for development of SAR. Currently two in vivo experimental models of epilepsy have been widely employed for such purposes. These are the maximal electroshock seizure test (MES) and the subcutaneous pentylenetetrazole seizure threshold test. In this study, the anticonvulsant potency of alkyl-substituted carboxylic acids and tetrazoles have been determined by the subcutaneous pentylenetetrazole seizure threshold test. Several investigators have pointed out the bioisosterism between the carboxylic group and the tetrazole nucleus (21-23). Comparative studies on substituted carboxylic acid and tetrazoles have revealed similar, greater or inferior biological activity of the tetrazole analogues (23). Kraus (24) found both valproic acid and its corresponding tetrazole, 4-tetrazolylheptane, inhibited succinic-semialdehyde dehydrogenase in the GABA metabolic shunt with inhibitory constants (K_i) of 0.7 mM and 0.75 mM respectively. Both compounds have a polar acidic group and alkyl substituents. However, pentylenetetrazole is a convulsant and has no acidic properties. The effect of acidic properties of compounds on anticonvulsant potency has made it necessary that the electronic effect of the polar moiety, which

is influenced by the alkyl substituents, be quantified by physical methods.

By expressing physicochemical properties of structural features and the anticonvulsant activity in quantitative terms, multiple regression analysis is used to obtain quantitative structure-activity relationships (QSAR). This linear free-energy relationships model was pioneered by Hansch and co-workers (29). The use of statistical analysis in the regression equation appears to allow much more objective establishment of SAR. Several researchers have used the Hansch approach to identify molecular properties that account for the anticonvulsant activity of the structurally diverse antiepileptic drugs (15,19,26,27). Lien and co-workers (26,28) found that 1,4-benzodiazepines, which are known to have specific binding sites, could not be included with other antiepileptic drugs to develop a significant QSAR. There have been suggestions from QSAR studies that CNS-acting drugs have differing lipophilicity requirements which determine their distributional localization and hydrophobic binding at the active site (20). Hansch and co-workers (29) developed quantitative structure-activity relationships for a series of hypnotic barbiturates and suggested an optimal octanol-water partition coefficient of about 100 (log P = 2.0).

This QSAR approach has not been applied to either alkyl substituted carboxylic acids or tetrazoles which may specifically antagonize PTZ-induced clonic seizures because of the insimilarity in structure and the known bioisosterism between carboxylic and tetrazole groups. It appears that the semi-rigid cycloalkyl-substituted compounds are potential structures to investigate the structural requirements at the site of action for valproic acid analogues. Apparently 5-alkyl-

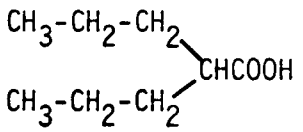
tetrazoles (21,23) and diunsaturated derivatives of valproic acid (2) have not been evaluated for their anticonvulsant activity.

Unidentified diunsaturated metabolites of valproic acid have been reported to be present in the serum and urine of patients on valproic acid therapy (2). The availability of synthetic reference material will help characterize the stereochemical configuration of these diunsaturated metabolites. Their synthesis requires stereoselective methods owing to the number of positional isomers and multiplicity of the stereoisomers. Compounds with polar groups in the alkyl chain, in addition to the terminal carboxylic function, have also been included in the study.

Specific Objectives

1. Valproic acid has been reported to exhibit selective actions that may mediate its anticonvulsant effects. A wide variety of structural analogues of valproic acid were to be used to investigate the degree of structural specificity of the anticonvulsant actions. The series of valproic acid analogues (Figure 1) include compounds with γ-alkyl substituents; α,α-dialkyl substituents; β-alkyl substituents; α-alkyl substituent, alicyclic substituents; polar groups in alkyl chain; unsaturated alkyl groups. The first part of the study was to apply synthetic methods to prepare the substituted carboxylic acids and tetrazoles.

2. In the course of the study, it was of interest to employ a stereoselective synthetic method to prepare the diunsaturated analogues of valproic acid, 2-[(E)-1'-propenyl]-(E)-2-pentenoic acid and 2-[(Z)-1'-propenyl]-(E)-2-pentenoic acid, for evaluation of the anticonvulsant activity and identification of the major di-



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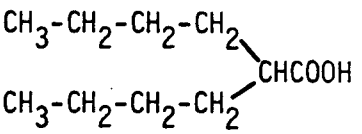

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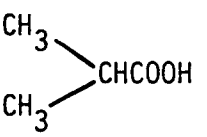

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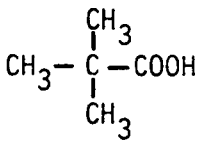

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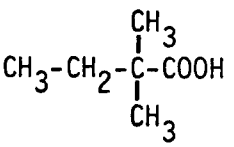

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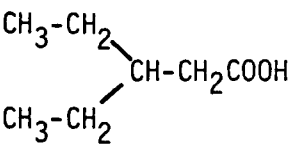

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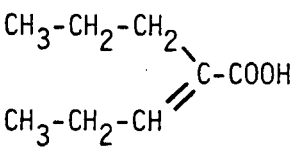

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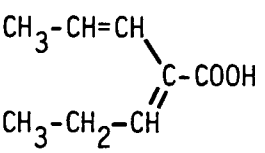

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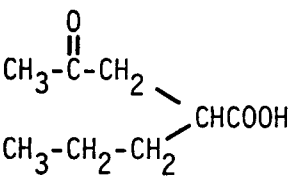

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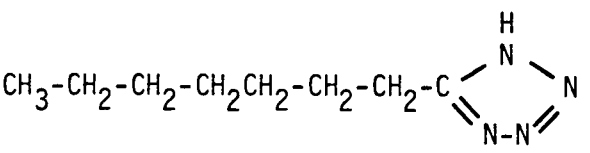

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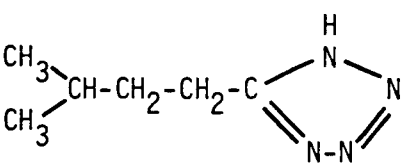

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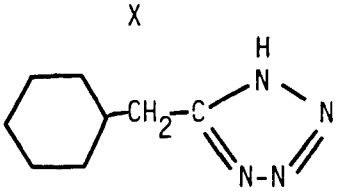

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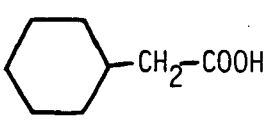

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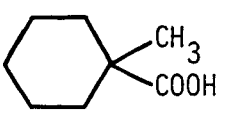

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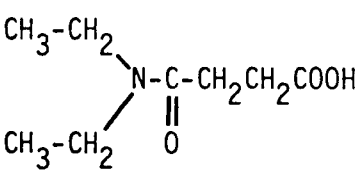

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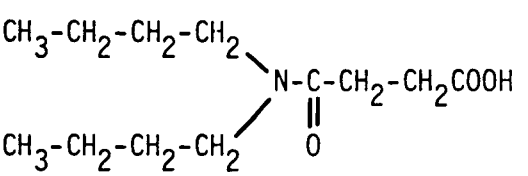


XVI

Figure 1. Chemical Structures of Valproic Acid and analogues

unsaturated metabolites of valproic acid.

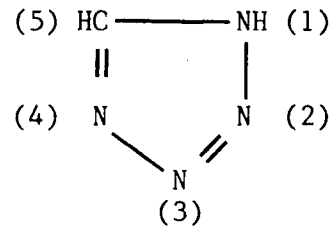
3. Aside from application of reverse-phase HPLC as an analytical tool, one can relate the observed chromatographic retention parameters of compounds to physicochemical properties such as lipophilicity. Octanol-water partition coefficients indirectly determined by reverse phase (RP) - HPLC have been shown to be in close agreement with values obtained by the traditional shake-flask procedure. Since chromatographic methods are generally more rapid than the static method owing to the higher rate of equilibration of solute between the phases, this study utilizes a RP-HPLC procedure to determine the octanol-water partition coefficients of valproic acid analogues. The effects of different mobile phases and compounds of diverse chemical structures on the accuracy of the RP-HPLC method were examined.

4. Variation of the substitution pattern in the carboxylic acids and tetrazoles is manifested by the resultant electronic effects at the acidic groups of the polar moiety. While the apparent ionization constants (pKa) of the compounds may have been variously reported in the literature, the conditions used for measurement of pKa values usually differ from one laboratory to the other. It was in the interest of accuracy and consistency to employ an appropriate method to determine the pKa of valproic acid and analogues.

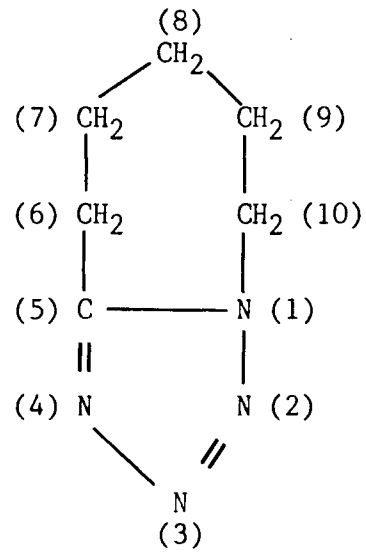
5. Valproic acid and alkyl-substituted compounds have been reported to be more effective against PTZ-induced clonic seizures compared to other chemical-induced seizures or electrically induced seizures. Since there have been relatively few studies on the dose-dependent anti-PTZ activity of valproic acid analogues, the aim of the study

was to determine the anti-PTZ potency of not only the homologous α-branched acids but also compounds with other substitutional characteristics.

6. Since the QSAR approach has not been applied to alkyl-substituted carboxylic acids and tetrazoles, it was of interest to evaluate the role played by the electronic properties of the polar group and the hydrophobicity of the alkyl group on the anticonvulsant potencies of test compounds. Additional structural properties considered in the present study were the conformational or steric effects and the molecular dipole moments.



Tetrazole



Pentylenetetrazole (PTZ)

Although this study pertains specifically to alkyl substituted carboxylic acids and tetrazoles, comparative studies with other alkyl-substituted heterocyclic compounds were made possible by the available literature data on their anti-PTZ potencies and physicochemical properties. The basis of such comparison is the possibility of a common molecular action of the alkyl substituted compounds as suggested from biochemical, pharmacological and neurophysiological studies by several researchers (30-34).

LITERATURE SURVEY

1. Clinical Use and Anticonvulsant Properties of Valproic Acid (VPA)

The therapeutic efficacy of VPA (Depakene®) has been demonstrated in several clinical studies (35,36). VPA is now widely used in primary generalized epilepsies, particularly those of the absence seizure type. Valproic acid is considered to be at least as effective as ethosuximide in the treatment of absence seizure (37). Its broad spectrum of anti-epileptic effects has, however, proven valuable in combination therapy for myoclonic epilepsy and generalized tonic-clonic seizures (35,36).

VPA shows selective activity against several types of chemically or electrically-induced seizures in a variety of species. VPA has a weak activity against maximal electroshock seizures in mice compared to the activity of phenobarbital and phenytoin (38). VPA is more effective in prevention of clonic or tonic seizures induced by PTZ and picrotoxin (38-40). High doses are reported to block tonic-clonic, bicuculline and strychnine-induced seizures (38-40).

2. Pharmacological Testing

a. Experimental Models of Epilepsy

Quantitative effects of structural variants on the pharmacological activity of the antiepileptic drugs have been obtained by the use of numerous tests in experimental animals. The common experimental techniques for inducing seizure in rodents include electroshock and systemic administration of convulsants such as PTZ. The maximal electroshock seizure test (MES) and the subcutaneous pentylenetetrazole seizure threshold test (s.c. PTZ) are

widely recognized models for determining the anticonvulsant activity of compounds. Activity in the MES test has been correlated with a compound's ability to modify maximal seizures or inhibit the seizure spread through the brain. In contrast the s.c. PTZ test measures the ability of a compound to elevate the degree of seizure threshold (41). The MES test is thus a model of generalized tonic-clonic seizure while the s.c. PTZ test is a model of absence seizures. Other models have been developed to simulate partial seizures.

Extensive investigations have been carried out to standardize the MES test (41-43) and the s.c. PTZ test (41,44,45). These two methods have been preferred over other tests since they are reported to be rapid, simple, easily controlled and non-erratic in producing the clonic or tonic seizure component (38). The value of these tests has been shown by good correlation between test results and efficacy in clinical epilepsy (38,46). In the MES test, maximal seizures are induced by passing high current (five to seven times threshold value, i.e. 50 mA, 60 Hz) through corneal electrodes for 0.2 sec in mice (46). In the MES test, active compounds protect against the tonic extension of hind limbs. PTZ is administered in mice or rats at doses ranging from frank convulsant to nearly lethal doses. In the s.c. PTZ test, threshold clonic seizures are produced when PTZ is administered subcutaneously in a dose of 85 mg/kg in mice. This is the reported CD_{97} dose in mice (46). Protection in the s.c. PTZ test is defined as absence of clonic spasms of duration greater than 5 sec. Another PTZ seizure threshold test, the timed i.v. infusion method,

has also been used to determine anticonvulsant potency in rats (45).

Whereas there is a need for the use of in vitro models of neuronal discharge, especially in identifying the selective molecular actions of antiepileptic drugs (31), the empirical in vivo models have also been discriminative in showing differential actions of antiepileptic drugs. Ethosuximide and trimethadione, both alkyl-substituted compounds, are effective only for absence seizures and in the s.c. PTZ test (44,47). Phenytoin is ineffective for absence seizures and in the s.c. PTZ test, but it prevents generalized tonic-clonic seizures, partial seizures and maximal electroshock seizures (42-44). Phenytoin is thought to suppress the spread of seizures. 1,4-Benzodiazepines, which block the tonic seizure component in MES test and the clonic spasms in s.c. PTZ test at approximately the same dose, may act by elevation of seizure threshold (48). Barbiturates are effective in the MES test and s.c. PTZ test at separated doses suggesting that the mechanism of action involves both elevation of seizure threshold and inhibition of seizure spread (41,48). The effect of valproic acid is similar to barbiturates, except that it is more effective in elevation of seizure threshold (39,40).

b. Mechanism of Action of PTZ

PTZ is known to act on the whole CNS but there are conflicting reports as to whether the PTZ-induced convulsions originate in specific areas such as the cerebral cortex (49). The mechanism of PTZ convulsant actions is still not defined. However, the direct effect on cell membrane excitability, together with its ability to

selectively block the effect of GABA on chloride conductance may account for the convulsant properties of PTZ (49,50).

A number of workers have reported that PTZ enhances the excitatory system through direct effect on membrane properties to increase spontaneous discharge by altering ionic conductance (51,52). Using cultured mouse spinal cord neurons, Macdonald and Barker (53) noted that PTZ reversibly antagonizes in a dose-dependent manner the conductance produced by iontophoresed GABA, without an effect on the resting membrane properties. The response was selective for GABA since PTZ did not affect the response due to glycine, β-alanine and glutamic acid. The synaptic action of PTZ is reported to block the increase in chloride conductance induced by iontophoresed GABA in neurons of Aplysia Californicus (54). In the same study (54), PTZ was observed to show minimal effect on excitatory response of inward sodium current and inhibitory effect of outward potassium current. Unlike valproic acid, PTZ is reported to have no significant effect on brain GABA levels (55). PTZ inhibited the activity of glutamic acid decarboxylase in vitro at concentrations related to physiological levels (55). In vivo, PTZ inhibited the activity of glutamic acid decarboxylase at twice the CD_{97} dose in mice (55).

It has been observed that PTZ seizures are similar to those induced by picrotoxin but unlike those of strychnine (50). Ticku and Olsen (33) reported that PTZ shows a significant affinity for the picrotoxin binding site (IC_{50} = 30 μM) hence it is likely to act at the benzodiazepine-GABA-chloride ionophore receptor complex. Recently Squires et al. (56) examined a series of convulsant tetra-

zoles for their potencies in displacing ^{35}S-t-butylbicyclo phosphonothionate (TBPS) from the picrotoxin site on the BDZ-GABA-Cl^- ionophore receptor complex. A good correlation between relative affinities for ^{35}S-TBPS binding site and convulsant potencies (IC_{50} of PTZ = 0.54 mM) was found.

Tetrazole derivatives have been investigated for their convulsant activity. Potent convulsant effects were obtained when 1,5-positions are bridged by a penta to heptamethylene chain (57). Tetrazole derivatives possessing a methyl or ethyl group at position-5 and a substituent at position 1(N) with four to six carbon atoms (aliphatic or alicyclic) are active as convulsants (58). Depressant effects of some 1,5-disubstituted tetrazoles have been obtained with substitution of aminophenyl groups at position 1 or 5 and aliphatic groups at the unsubstituted position (59). Ahmad and Shunkla (60) investigated 5-acetamido-substituted tetrazoles and reported anticonvulsant activity of 5-(N-phenylacetamido)-tetrazole with an i.p. dose of 100 mg/kg.

c. Pharmacokinetics of PTZ

The pharmacokinetic properties of PTZ are attributed to its ready solubility in both lipids and water. PTZ is rapidly absorbed from all sites of administration in mice and rats and is rapidly distributed in total body water (61,62). Brain ^{14}C-PTZ uptake is rapid within 10 min after s.c. administration in rats and the half-life of PTZ in brain ranged from 16-21 min (191). The serum half-life of radiolabelled PTZ in rats was found to be 2-4 hr (61,62). Metabolism is the major route of PTZ elimination. A low percentage of unchanged drug is excreted in urine (61,64). The major metab-

olites in rats are 6-hydroxy PTZ and 8-hydroxy PTZ (23,61). Binding to tissue constituents is almost negligible and approximately 9% of the drug is bound to plasma proteins (65).

3. Chemistry and Physicochemical Properties of Valproic Acid

Valproic acid (2-propylpentanoic acid) was first synthesized by Burton (66) in 1882 by alkaline decomposition of 2,2-dipropylacetoacetate. The physical and chemical properties of valproic acid have been reviewed by Chang (67). VPA has a solubility of 1.27 mg/ml in water. The critical micellar concentration of its straight chain isomer, octanoic acid is reported to be 8.0 mmol/L (68). The partition coefficient of VPA between various organic solvents and phosphate buffer (pH 7.4) has been reported to be 0.013 for heptane, 0.064 for benzene and 0.21 for chloroform (69). Valproic acid showed a pKa value of 4.56 when determined by potentiometric titration of the acid in aqueous medium (70).

4. Mechanism of Action of Valproic Acid

The basic mechanism by which valproic acid exerts it anticonvulsant effect is not yet clear. Several biochemical and neurophysiological studies have shed some light on its mode of action (2,71,72). The biological specificity of valproic acid, like that of other anticonvulsants, is broad. At high doses, valproic acid shows nonspecific actions such as inhibition of mitochondrial oxidative phosphorylation (73), actions shared by non-anticonvulsants (73). Some investigators have used the selective actions of anticonvulsants, such as valproic acid, at lower drug concentrations to pinpoint their

mechanism of action (31,74). The possibility of a nonspecific anticonvulsant action of valproic acid has received some attention (14) although more direct effects have been observed for valproic acid.

Four possible modes of action have been postulated from direct effects observed for valproic acid. These are elevation of brain GABA levels, enhancement of GABAergic inhibition at the post-synaptic GABA receptor complex, reduction of excitatory action of aspartate and increase in membrane conductance to potassium ions.

Valproic acid has been repeatedly shown to raise levels of GABA in the brain (75,76). The increase in GABA levels induced by valproic acid appears to take place in nerve terminals (77). In vitro studies showed that valproic acid levels up to 1 mM had no effect on the release of preloaded radiolabelled GABA from rat brain synaptosomal preparations (78). GABA is synthesized by GAD-catalyzed decarboxylation of glutamate and is metabolized to succinic semialdehyde by the reversible GABA-T catalyzed reaction. Succinic semialdehyde (SSA) is metabolized either to succinic acid by SSA-dehydrogenase catalyzed oxidation or metabolized by aldehyde reductase-catalyzed reduction to 4-hydroxybutyric acid. The increase in GABA brain levels produced by valproic acid was attributed from early studies to inhibition of GABA-T (75). It appears that VPA more potently inhibits SSA-dehydrogenase (K_i, 0.5 mM) compared to inhibition of GABA-T, K_i >20 mM (79,80). Thus an increase in succinic semialdehyde levels might elevate GABA levels by indirectly inhibiting GABA-T. Valproic acid has also been shown to increase the activity of the GABA biosynthetic enzyme, glutamic acid decarboxylase, in whole brain and in brain synaptosomes in mice (77).

Recent studies suggest that VPA may rather act at the post-synaptic

membrane of the GABAergic synapse. The administration of valproic acid by iontophoresis potentiates the inhibition effect of GABA on neurons in the brain (81,82), in mouse spinal cord neuronal culture (74) and in cuneate fibre preparations (83). Iontophoresed valproic acid had no significant effect on responses from either iontophoresed glycine or glutamate (74). The concentration of valproic acid acting on the neuronal membrane during iontophoretic application of valproate was not ascertained in some of the studies. Harrison and Simmonds (83) reported that valproic acid enhances GABA effects at 3.0 mM in vitro.

Benzodiazepines and barbiturates are also reported to potentiate the postsynaptic inhibitory effects of GABA (31). This action of anticonvulsant barbiturates and valproic acid is suggested to occur at the picrotoxin binding site, i.e. the chloride ionophore (30,33,34). The GABA receptor appears to be part of a protein complex containing receptor sites for GABA, BDZ and picrotoxin as well as the chloride ionophore (30). Loscher (84) has reported that valproic acid does not inhibit nor enhance the binding of ^{3}H-GABA to rat brain membranes at concentrations up to 1 mM. Valproic acid also does not bind to benzodiazepine receptors at concentrations up to 1 mM (34). The component of the GABA-receptor-chloride ionophore, the picrotoxin "receptor" is a site at which several convulsants (including pentylenetetrazole) and anticonvulsants appear to act (30,33,34). Ticku and Davis (34) found that valproic acid inhibits the binding of ^{3}H-dihydropicrotoxinin to rat brain membrane (IC_{50} = 0.5 mM). Valproic acid is reported to have no affinity for the binding site of ^{3}H-phenytoin to rat brain membranes (85).

In an intracellular study of the effects of valproatec on non-

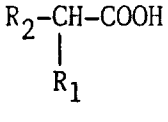
mammalian species, Johnson (71) found that concentrations of VPA (5-30 mM), 15-50 times the serum level of patients on VPA therapy, caused an increase in potassium membrane conductance to produce hyperpolarization of the resting membrane.

Valproic acid is reported by several researchers to reduce brain aspartate in vivo (12,75,86). The time course for the decrease in aspartate concentrations correlated with the period of valproic acid-induced protection against audiogenic seizure in mice and with the increase in brain GABA levels (86).

So far it is not certain whether valproic acid exerts its anticonvulsant effects exclusively through one of the four possible modes of action. Potentiation of GABAergic post-synaptic inhibition may act together with elevated GABA levels resulting from inhibition of SSA-dehydrogenase. Moreover there appear to be inconsistencies as to whether the suggested molecular actions of valproate occur at therapeutically relevant valproic acid brain and serum levels in mammalian species. In patients receiving therapeutic doses of valproic acid, serum levels of 0.3-0.8 mM have been reported to be effective (2). The present study, by investigating the SAR of closely-related analogues of valproic acid, may be useful in determining which of the molecular actions of valproic acid can be correlated with anticonvulsant activity.

5. Studies on Anticonvulsant Activity of Valproic Acid Analogues

Valproic acid is not the only active aliphatic carboxylic acid among its congeners. Carraz (10) reported protection of PTZ-induced mortality by some alpha-branched fatty acids of the general form, XVII. These studies were conducted at a single dose, 200 mg/kg i.p. in mice.

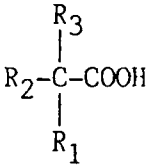


XVII

$R_1 = R_2 = CH_3$
$R_1 = R_2 = C_2H_5$
$R_1 = R_2 = C_3H_7$
$R_1 = R_2 = C_4H_9$

Activity rose with increases in chain length up to valproic acid. Dibutylacetic acid was reported to be inactive. Recent studies by Chapman et al. (12) and Keane et al. (13) showed that the activity of alpha-branched fatty acids continued to increase with elongation of the side-chain. However, they found straight-chain fatty acids (C_4-C_6) to be inactive up to 4.0 mmol/kg doses.

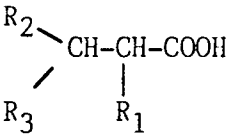
Anticonvulsant activities were also noted with trisubstituted fatty acids (10,87) of the structural form, XVIII. Taillandier et al. (5) investigated the anti-PTZ activity of a series of β-substituted and



XVIII

$R_1 = R_2 = R_3 = CH_3$
$R_1 = R_3 = CH_3$, $R_2 = C_2H_5$
$R_1 = R_3 = CH_3$, $R_2 = C_3H_7$

α,β-disubstituted acids of the type XIX.

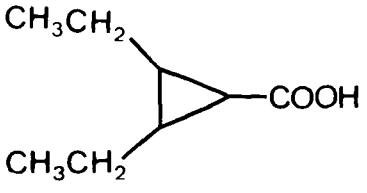


XIX

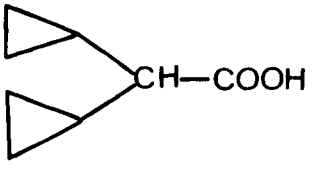
$R_2 = R_3 = CH_3$, $R_1 = H$
$R_2 = R_3 = C_2H_5$, $R_1 = H$
$R_2 = R_3 = C_3H_7$, $R_1 = H$
$R_2 = R_3 = C_3H_7$, $R_1 = C_2H_5$
$R_2 = R_3 = C_3H_7$, $R_1 = C_4H_9$
$R_2 = R_3 = R_1 = C_3H_7$

As in most of these structure-activity studies, the PTZ test was performed at a single dose in mice, 0.9 mmol/kg in this instance. According to the investigators (5), isopentanoic acid, 3-ethylpentanoic acid, 2-ethyl-3-propylhexanoic acid and 2,3-dipropylhexanoic acid were inactive while 3-propylhexanoic and 2-butyl-3-propylhexanoic acid were as active as valproic acid.

Cyclic analogues of valproic acid have also been studied. Cycloheptane carboxylic acid is inactive compared to valproic acid when administered at a dose of 200 mg/kg i.p. in mice (11). Both α-cyclopentylpentanoic acid and α-cyclohexylpentanoic acid are weaker anticonvulsants than valproic acid (5). Two recent studies explored the possibility of rigid alicyclic carboxylic compounds revealing the nature of the active configuration of the alkyl groups that interact at the site of action of valproic acid. Brana and co-researchers (5) found compounds, XX and XXI, to be as active as valproic acid in the s.c. PTZ

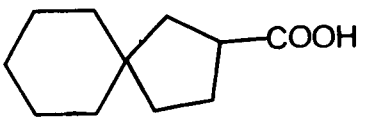


XX

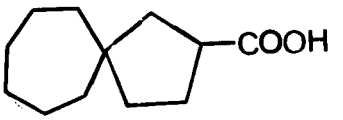


XXI

test when compounds were administered i.p. at a dose of 200 mg/kg in mice. Scott et al. (88) looked at the anti-PTZ activity of supposedly metabolically stable spirocarboxylic acids, including XXII and XXIII. Compound XXIII, Spiro[4,6]undecane-2-carboxylic acid, the most active



XXII



XXIII

compound in the series, was slightly more potent than valproic acid (ED_{50} of 0.42 mmol/kg compared to 0.9 mmol/kg for sodium valproate).

Early published studies on anticonvulsant activity of valproic acid analogues focused on various derivatives of the carboxylic acid such as esters, amides and ureas (3-9). Most of the compounds tested were barely active except the primary amide of valproic acid which was equally potent. However, dialkylureides have been previously reported to be active compounds (89). The secondary and tertiary amides of valproic acid were found to be mostly convulsants (3,4). Conversion of the carboxylic acids to the primary alcohols in a dialkylalkanoic acid series did not reduce drastically their anticonvulsant activities while esterification of the resulting alcohol destroyed their activity (5). The anticonvulsant properties of the primary amide and the primary alcohols were partly attributed to the active carboxylic acid metabolites (5).

Some workers have evaluated analogues with bifunctional groups. 1,3-Propanediols and dicarbamates such as 2,2-dipropyl-1,3-propanediol and meprobamate are known anticonvulsants (38,90). The malonic acid derivative of the spirocarboxylic compound, XXIII, was observed to be slightly less active than valproic acid (88). Schwartz and coworkers (91) reported that 2-ethyl-2-propylcyanoacetamide and 2-ethyl-2-

workers (91) reported that 2-ethyl-2-propylcyanoacetamide and 2-ethyl-2-butylmalondiamide possessed anticonvulsant activity with minimal sedative properties at a dose of 400 mg/kg in the s.c. PTZ test.

A number of valproic acid metabolites with greater polarity possessed anticonvulsant activity weaker than that of valproic acid (92). Among the known metabolites of valproic acid, the unsaturated metabolites, namely 4-ene VPA, 3-ene VPA and 2-ene VPA were the most potent. However, these metabolites occur in serum and urine of human and rodents in relatively small quantities (<10%) when compared to levels of valproic acid (92). 3-Keto VPA, the hydroxy metabolites and 2-propylglutaric acid had slight anticonvulsant properties. There are no data on the anticonvulsant properties of the recently described metabolites of valproic acid, 4-Keto VPA and the diunsaturated compounds (93).

While no rational basis for the prediction of anticonvulsant activity of alkyl substituted carboxylic acid derivatives has been established, the anticonvulsant data suggest that the nature of the alkyl group is of basic importance to pharmacological action. The significance of determining the QSAR of valproic acid analogues is highlighted by the experimental observations that valproic acid, valproamide, dipropylureide and 5,5-dipropylbarbituric acid are potent anticonvulsants (4,6) despite differences in lipid solubility.

6. General SAR of Antiepileptic Drugs

Although valproic acid differs from the traditional antiepileptics (Figure 2) which contain a nitrogen functional group, there are similarities in structure. Common structural features of the antiepileptic

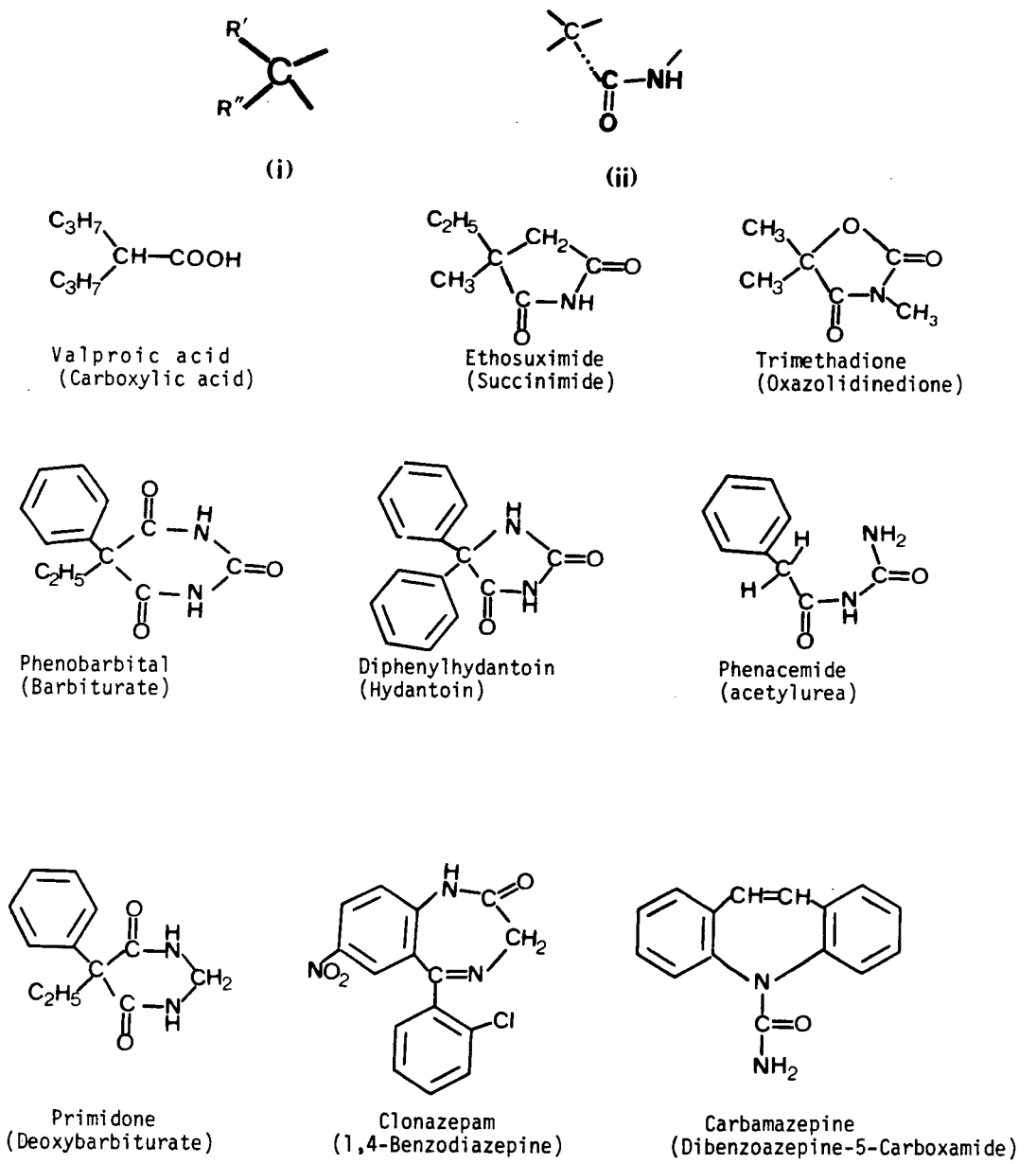


Figure 2. Chemical structures of traditional antiepiletic agents.

drugs are embodied in the polar carboxyl or imide functional group and the disubstituted quaternary or tertiary carbon, i and ii in Figure 2. Anticonvulsant activities of classes of compounds have been compiled from the literature and summarized to determine SAR among the anticonvulsant drugs (94). Similar structural requirements appear to exist in the conventional anticonvulsant drug groups such as the barbiturates, hydantoins, oxazolidinediones, succinimides and acyclic ureides.

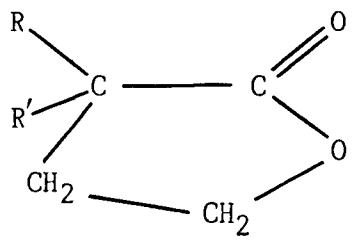
Certain distinguishing features in the SAR of these antiepileptic drugs have been recognized. The introduction of at least one phenyl or similar aromatic group at R_1 or R_2 (Figure 2) is required for optimal activity against MES-induced seizures. In contrast, alkyl substituents at R_1 or R_2 confer high activity against s.c. PTZ-induced seizures. Increasing the length of the alkyl chain usually maximizes the hypnotic activity. Most of the classical antiepileptic drugs produce maximal activity in the s.c. PTZ test when the sum of carbon atoms in the substituents is between C_6 and C_{10}. Diphenylacetic acid is active in the MES test (16). 5,5-Diphenyloxazolidinedione, 5,5-diphenylhydantoin, 2,2-diphenylsuccinimide and 5,5-diphenylbarbiturate have minimal activity (or even inactivity) in the s.c. PTZ test (94). 2-Methyl-2-butylsuccinimide is inactive in the MES test (94).

The nitrogen atom may be substituted preferably by a methyl group, lower alkyl groups and alkoxyalkyl groups without reducing significantly the anticonvulsant activity, e.g. metharbital, N-methyldipropylacetamide (5,8), mephenytoin, methsuximide and trimethadione (5,8).

Rigid analogues of alkyl substituents have been tested for anticonvulsant activity. These compounds can alter the steric properties and metabolism of the flexible alkyl groups. Thus cyclo C_5-C_{12} spiro com-

pounds of hydantoin (95), oxazolidinediones (95), succinimides (95), carboxylic acids (88) and barbiturates (94) have been studied. There have been no generalizations as to whether certain spirocycloalkyl groups confer desirable pharmacological properties.

The 1,4-benzodiazepines and dibenzazepines have more rigid structures with different structure-activity requirements. 5-Phenyl substitution is considered to be necessary for all active 1,4-benzodiazepines (96). Diazepam and carbamazepine (Figure 2) both have lipophilic groups and the typical amido structure containing the electron-donor group or hydrogen-bonding group as in other antiepileptic drugs. According to Camerman and Camerman (18), the phenyl groups in phenytoin overlap with those of diazepam when crystal structures are examined by x-ray crystallography. Other classes of compounds with anticonvulsant activity are the γ-butyrolactones (XXIV) and glutarimides. Klunk



XXIV

et al. (47) recently found α,α-disubstituted γ-butyrolactones to be potent anticonvulsants when examined in the s.c. PTZ test. Somers (98) has described the anticonvulsant properties of β-methyl-β-butyl glutarimide and convulsant properties of β-methyl-β-ethylglutarimide (Bemegride®). Klunk and co-workers (97) have found β,β-dialkyl γ-butyrolactones such as β-ethyl-β-methyl-γ-butyrolactone to be con-

vulsants.

Anticonvulsant or convulsant properties may be obtained by minor structural changes in the side chains. There are structural features that may confer convulsant properties in the SAR of anticonvulsant drugs (94). Certain benzylethylbarbiturates (94), dibenzylbarbiturates (94), branched chain alkylethylbarbiturates (17,99), branched chain alkenylalkylbarbiturates (17,99), N-alkylated and di-N-alkylated compounds, e.g. secondary and tertiary amides of valproic acid (3,4), 1,3,5,5-tetraalkylbarbiturates (94). 2,2,3,3-tetraalkyl succinimides (97) and 5-methyl-1,4-benzodiazepine (30) are known convulsants. According to studies by Andrews et al. (19) and Downes et al. (100), the terminal isopropyl and isopropenyl groups in 5,5-dialkylbarbiturates (Figure 3) appear to be necessary for convulsant action.

7. Structural Specificity of Anticonvulsants

Antiepileptic drugs appear to show low structural and biological specificity. This may be due to the few pharmacophoric groups in these compounds and their ability to modulate GABA effects. Although few drugs show absolute biological specificity, most drugs act by interaction with specific bioreceptors. Specificity of biological action is determined by the combination and stereochemical arrangement of chemical groups and their physical interaction with specific binding sites or receptors.

By virtue of their nonspecific perturbation of cell membranes and lack of structural specificity, general anesthetics have been classified as structurally nonspecific drugs (101). General anesthetics have been regarded as non-polar, inert and nonionizable compounds which do not

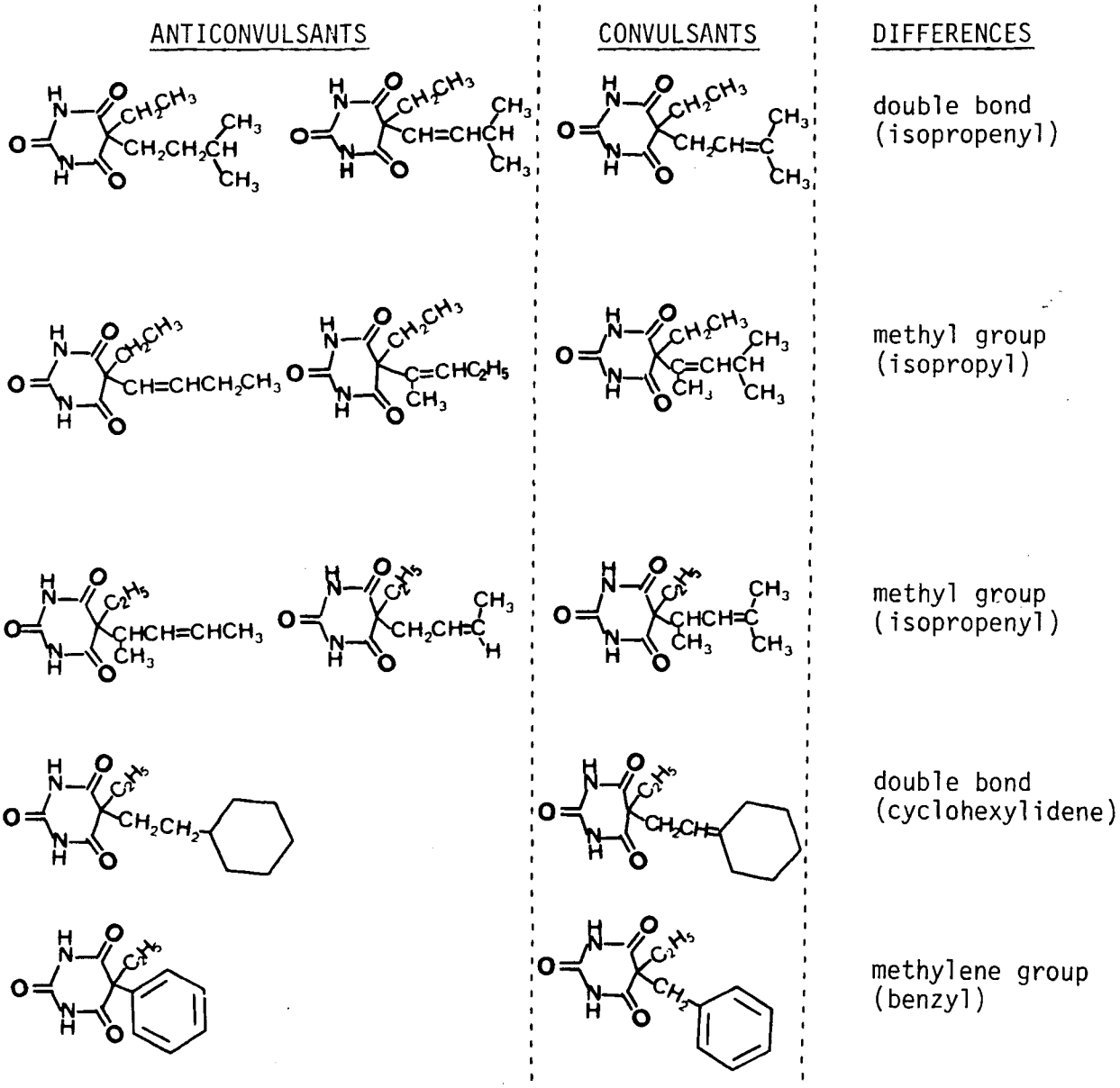


Figure 3. Structurally-related convulsant and anticonvulsant barbiturates.

have highly reactive polar groups to interact with specific receptors but partition into cell membranes to secondarily disrupt ionic channels in the membrane structure (101).

Most antiepileptic drugs, however, show sedative effects. It is generally assumed that sedative, hypnotic and anesthetic actions of anticonvulsants occur with increasing the dose of the compound. In some compounds, sedative and anticonvulsant effects overlap although phenytoin is reported to exert its anticonvulsant effect at non-sedative doses (42). It is known that anticonvulsants are not the only class of compounds with sedative actions. Most CNS-active agents show sedative properties probably because they have sufficient lipophilicity to penetrate the blood brain barrier to interact nonspecifically with brain membranes. Even certain convulsants have been reported to show sedative properties (59). It may be premature to conclude that valproic acid and analogues act by nonspecific mechanisms as suggested by Perlman and Goldstein (14). These workers indicated that the ability of ethanol, valproic acid and homologous congeners to disorder cell membranes correlated well with their sedative and anticonvulsant potencies. Some investigators studied other direct physiological and biochemical effects of valproic acid analogues and also reported good correlation with anticonvulsant potencies (12,13,20,81,82).

Antiepileptic drugs can be described as more structurally specific than anesthetics. Barbiturates are known to show marked specificity. Small changes in the alkyl side chain of certain anticonvulsant barbiturates can produce convulsant properties (Figure 3). The selective nature of antiepileptic drug therapy with anticonvulsants such as phenytoin, phenobarbital, valproic acid and ethosuximide attest to some degree of structural specificity. These findings suggest that anticon-

vulsants may show discrete or selective effects on neuronal cell structures.

Recent advances in neurophysiological and biochemical techniques have allowed extensive investigations of the selective synaptic or cellular actions of some of the antiepileptic drugs (74,102). Furthermore, radioligand binding studies (30) have added support to the suggestion that different subgroups of anticonvulsants exist, each with a different binding site but in close association and probably each with its own structure-activity relationships. The observation of high affinity, saturable and in some cases stereospecific binding of 1,4-benzodiazepines (103), GABA and analogues (30), dihydropicrotoxinin (33) and phenytoin (85) have added weight to proposals of specific molecular mechanisms of antiepileptic and convulsant actions.

1,4-Benzodiazepines have been documented to enhance GABA binding and also vice-versa (30). Barbiturates are known to enhance the binding of benzodiazepines (EC_{50} of pentobarbital ~100 μM) and GABA(30). Picrotoxin at concentrations of 1 μM is reported to inhibit the enhancement of benzodiazepine binding by barbiturates (30). Barbiturates and valproic acid are suggested to act on the same sites as convulsants such as picrotoxin, 1,5-alkyl-substituted tetrazoles and tert-butylbicyclophosphonothionate (30,33,34,56). The convulsant picrotoxin has been suggested to exert its effects at the site controlling the flux of chloride ions, the chloride ionophore (30). ^{3}H-Dihydropicrotoxinin (DHP) and ^{35}S-tert-butylbicyclophosphothionate (TBPS) have been used to assay picrotoxin binding sites (30,33,56,104). Barbiturates competed with ^{3}H-DHP binding with relative potencies that correlated with their ability to enhance ^{3}H-GABA binding (33,104). Pentobarbital, pheno-

barbital and valproic acid are reported to inhibit the binding of ^{3}H-DHP with IC_{50} of 50 μM, 400 μM and 500 μM respectively (33,34). Valproic acid, in comparison to barbiturates, did not inhibit the binding of ^{3}H-GABA (87) or ^{3}H-diazepam (34) at concentrations up to 1 mM. Studies on other anticonvulsants have shown that ethosuximide inhibited ^{35}S-TBPS binding by 30% at 1 mM while α-ethyl-α-methylbutylrolactone inhibited ^{35}S-TBPS binding by 23% at 0.5 mM (32).

Phenytoin inhibited ^{3}H-DHP binding with an IC_{50} of 100 μM (30). The binding of phenytoin to its recognition site is reported to interact with the GABA-BDZ-receptor Cl^{-} ionophore complex (85). Using spinal cord neuronal culture, Macdonald and McLean (102) indicated that phenytoin augmented postsynaptic GABA response at higher concentrations (>8μM) than inhibition of post-tetanic potentiation (high frequency repetitive firing of neurons) which occurred at therapeutic free serum concentrations (4-8 μM). Phenytoin and carbamazepine, which show significant effect on post-tetanic potentiation compared to other anti-epileptics, are reported to decrease calcium influx across synaptosomes and affect significantly calcium-calmodulin dependent protein phosphorylation (105).

Figure 4 depicts a model of the GABA receptor-benzodiazepine-chloride ionophore complex developed by various investigators for sites of action of some anticonvulsants and convulsants. The functional coupling between the GABA receptor and the 1,4-benzodiazepine receptor and picrotoxinin sites have given suggestive evidence of a common mechanism of action involving modulatory effects on the inhibitory neurotransmitter, GABA. The model also shows the wide variety of structures which interact on each recognition site. Detailed studies have indicated multiple recognition sites for the GABA and benzodiazepine

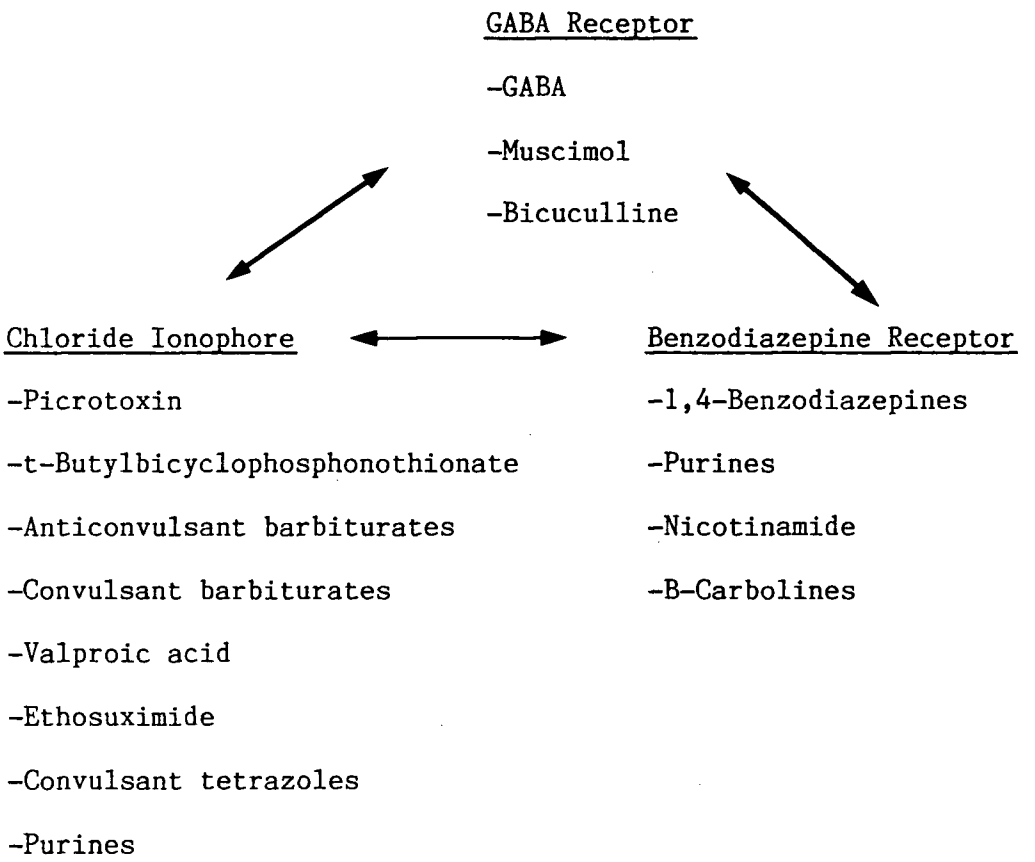


Figure 4. Model of GABA-benzodiazepine-receptor chloride ionophore complex

receptor complex (30,103). A recent study (107) investigated the kinetics of inhibition of ^{35}S-TBPS by barbiturates for evidence that there is one class of binding sites for picrotoxin and barbiturates. It was suggested that picrotoxin/TBPS binding sites are allosterically linked to barbiturate sites. There are also reports of differences between aliphatic-substituted barbiturates and phenyl-substituted barbiturates in their action to augment GABA responses (31,108).

8. Pharmacokinetics of Valproic Acid

a. Human

Valproic acid is rapidly and nearly completely absorbed following oral clinical doses of 15-60 mg/kg with peak blood levels occurring within 1-4 hr (109). The elimination half-life is in the range of 6-18 hr, with shorter half-lives obtained in the presence of antiepileptic drugs which induce the metabolism of valproic acid (109). Total plasma clearance is 0.07-0.14 ml/min/kg with an apparent volume of distribution of 0.1-0.4L/kg (109-111). Plasma protein binding of the drug at therapeutic plasma concentrations (50-100 mg/L) ranges from 80-95% (111). Protein binding sites become saturated at concentrations greater than 80 mg/L (111,112). Plasma clearance of VPA is dependent on free fraction and intrinsic clearance.

These pharmacokinetic parameters indicate that VPA distribution is limited mostly to extracellular fluids with only minor tissue uptake. Such distributional characteristics are attributed to the low pKa of VPA and the high plasma protein bind-

ing. Studies on single dose and multiple dose kinetics of VPA by Acheampong et al. (113) and Bowdle et al. (114) indicate increased plasma clearance at higher doses due to an increase in free fraction. There is a tendency for a decrease in intrinsic clearance at higher doses probably as a result of saturation of metabolism or autoinhibition of metabolism (113,114).

b. Rodents

After oral administration of 200 mg/kg in mice, valproate is reported to be rapidly absorbed with maximum serum concentrations reached within 5-15 min (70). There are quantitative differences in pharmacokinetic properties of the drug in rodents compared to human, apparently due to differences in protein binding. The half-life of valproic acid is 0.8 hr in mouse and 1-4 hr in rats (70,115). Volume of distribution is about 0.66 L/kg in rat and 0.33 L/kg in mice with total body clearance of 4.17 ml/min/kg in rats and 4.33 ml/min/kg in mice (110). At plasma concentrations of 50-80 μg/mL, the plasma protein-binding of the drug is about 90% in human, 63% in rat and 12% in mice (110).

c. Kinetics of Valproic Acid in the CNS

Valproic acid has been shown to enter the CNS rapidly with maximal brain concentrations reached at 5-10 min in mice (70). Several studies have demonstrated that regional distribution of the drug in the brain of mice and rats is relatively homogenous (116,117). Analysis of discrete brain areas, however, showed gradual accumulation of the drug in olfactory bulbs (116,117). Valproic acid does not bind to brain

tissues (118). Studies on the subcellular distribution of the drug by Aly and Abdel-Latif (119) indicated that it is mainly associated with the soluble and mitochondrial fractions. It has also been documented that the drug is rapidly cleared from the brain (120). The rapid brain uptake of valproic acid is suggested to be due to an active-transport mechanism (120) or rapid dissociation of protein-bound drug within the brain capillaries (121). Human brain valproic levels were found to be 7-28% of levels in plasma (122). In mice, brain drug concentrations (5-60 mg/L) were found to be about 15-20% of those in serum (20-250 mg/L) after oral administration of 200 mg/kg of sodium valproate (70,117).

9. Metabolism of Valproic Acid

Metabolism is the major route of valproic acid elimination in human and rodents (123). Several metabolites have been identified in a number of metabolism studies [reviewed in (123)]. These studies indicate four main metabolic pathways, namely glucuronidation, β-oxidation, w-oxidation and (w-1)-oxidation. The metabolic pathways are summarized in Figure 5. Glucuronidation and β-oxidation are the major pathways. The major metabolites in human are the glucuronide and 3-keto VPA (123). 3-Keto VPA and 2-ene VPA are major plasma metabolites in human, rats and mice (115). Other metabolites that occur in significant quantities in serum of patients are 2,3'-diene VPA, 4-OH VPA and 5-OH VPA. Acheampong et al. (93) have identified a new metabolite, 4-keto VPA in human serum and urine. The urinary compounds, 2-propylsuccinic acid and 2-propylmalonic acids were confirmed as valproic acid metabolites by stable-isotope and GCMS techniques (93).

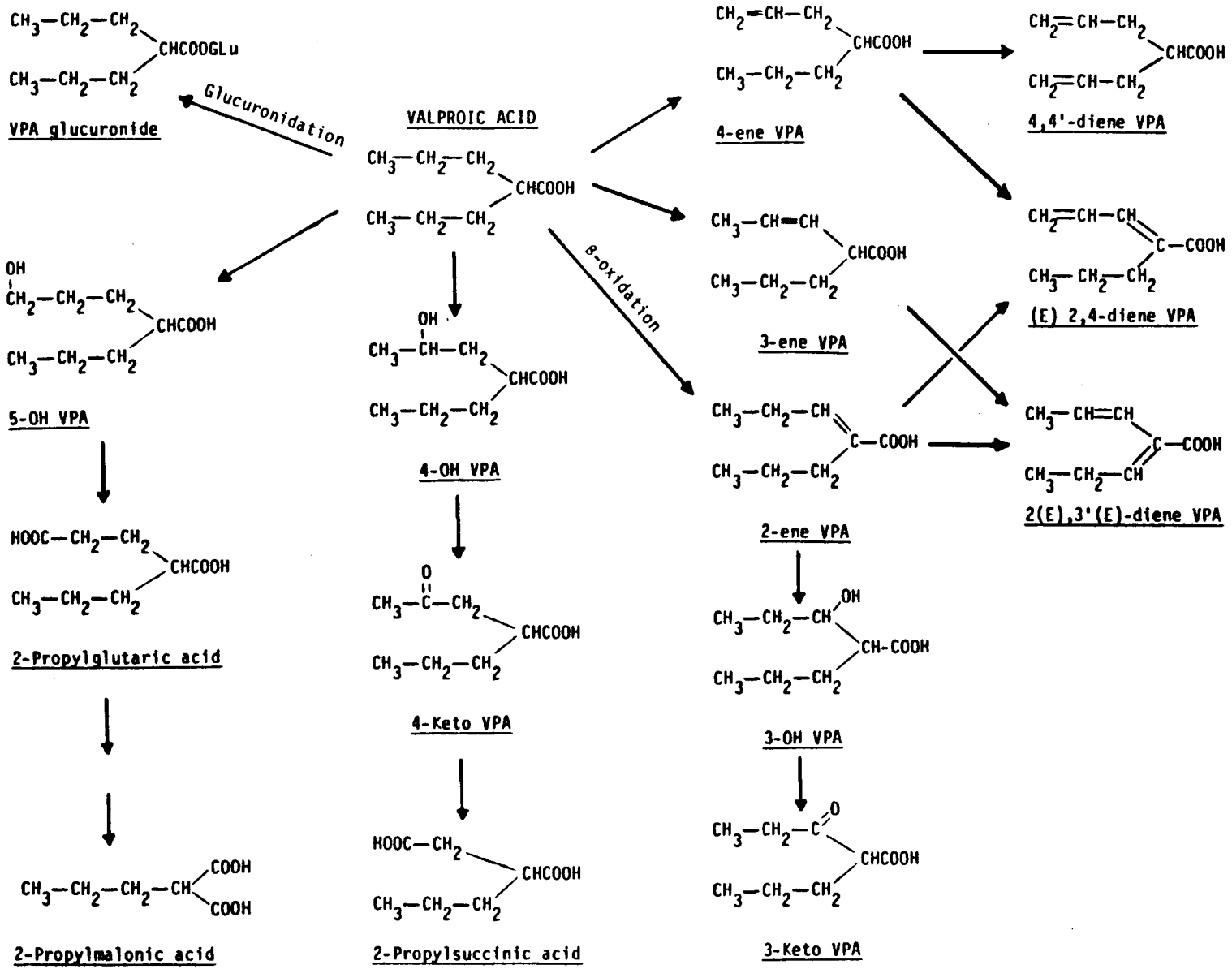


Figure 5. Metabolic pathways of valproic acid (VPA).

There have been more recent studies which sought to further delineate the metabolic pathways of the drug, with emphasis on the identity of diunsaturated metabolites detected in human serum and urine (124,125). Only two diene metabolites, 2-[2'-propenyl]-4-pentenoic acid and 2-propyl-(E)-2,4-pentadenoic acid have been identified (128,129). Identification of the major diene metabolite has been elusive, primarily due to multiplicity of the possible diunsaturated acid isomers and also the unavailability of synthetic reference material.

10. Toxicity of Valproic Acid

Nausea, vomiting, abdominal cramps and diarrhea are the most commonly reported side effects (35). Drowsiness and sedation occur, especially at high doses and with combination therapy (35). Hyperammonemia and hyperglycinemia have been observed to occur during valproic acid therapy (127). Side effects associated with the drug have generally been mild (35). However, in more recent years more serious side effects have been observed. These include thrombocytopenia (128), pancreatitis (126) and hepatic damage (128-131). The potential hepatic toxicity is of major concern. In most serious cases, lethal effects of valproic acid due to hepatic failure have been variously reported (128-130). The proposed mechanism for the hepatic toxicity and/or the Reye-like syndrome (131) induced by valproic acid include direct toxic effect of a metabolite, possibly 4-ene VPA which is a structural analogue of the hepatotoxin, 4-pentenoic acid (130,132).

11. A Quantitative Structure-Activity Model

One appropriate method of examining the relationship between structures of closely-related congeners and anticonvulsant activity was to

determine the quantitative relationship between structural properties or substructural descriptors and pharmacological activity. Hansch and collaborators (25,29) have applied the linear free energy relationship approach to in vitro and in vivo conditions where physicochemical interactions between drugs and biomacromolecular sites of action can be quantitatively expressed in terms of regression equations involving hydrophobic (π), electronic (σ), steric (E_S) and other structural parameters.

The Hansch method models the dynamic situation in a biological system where a drug is applied at a dose C and proceeds through several biophases to reach the target site with probability of access, A and effects response reaction with a rate or equilibrium constant, K_x. Thus the rate of biological response at the reaction site can be expressed mathematically as

$$d(\text{response})/dt = ACK_x \qquad .....(i)$$

For most drugs the transport process or probability of reaching the site of action is determined largely by lipid solubility or partition coefficient (π or log P), i.e.

$$A = f(\pi) \qquad .....(ii)$$

On the basis of an empirical parabolic relationship between a drug's biological activity and partition coefficient, Hansch (25) expresses the function in the form of a normal Gaussian distribution.

$$f = s\sqrt{2\pi}\,\frac{1}{}\,e^{-(\pi-\pi_o)^2/2s^2} \qquad \text{....(iii)}$$

From equations (i), (ii), (iii)

$$d(\text{response})/_{dt} = ae^{-(\pi-\pi_o)^2/b.C.K_x} \qquad \text{.....(iv)}$$

where $a = s\sqrt{2\pi}\,\frac{1}{}$, $b = 2s^2$, s = standard deviation parameter

At a fixed time interval after administration of equieffective doses C (e.g. ED_{50}), a constant biological response is determined under steady state conditions, i.e.

$$\frac{d\ (\text{response})}{dt} = \text{constant} = ae^{-(\pi-\pi_o)^2/b.K_xC}$$

Taking logarithms,

$$\log\ (\text{constant}) = \log\ [ae^{-(\pi-\pi_o)^2/b.C.K_x}]$$

and

$$\log\ 1/C = -n_1\pi^2 + n_2\pi.\pi_o - n_3\pi_o^2 + \log\ K_x + n_4 \qquad \text{.....(v)}$$

The Hansch method relies on the physicochemical interaction phenomenon at the critical site of action which is determined by the hydrophobic (π), electronic (s) and steric (E_S) factors.

$$\log\ K_x = k\pi + k_3\sigma + k_4E_s \qquad \text{.....(vi)}$$

Thus

$$\log 1/C = k_1\pi + k_2\pi^2 + k_3\sigma + k_4E_s + k_5 \quad \text{.....(vii)}$$

Equations (vi) and (vii) are linear free energy relationships showing the additive combination of the free-energy related parameters. The first two terms in π describe the transport process. The π^2 variable in equation (vii) is generally agreed to be essential in complex biological systems, for instance in whole animal studies. In simpler biological systems π^2 may not be required and π describes hydrophobic effects (25). On the basis of the additive-constitutive properties of partition coefficient (P) revealed by Hansch (133), equation (vii) can also be expressed as

$$\log 1/C = k_1 \log P + k_2 (\log P)^2 + k_3\sigma + k_4E_s + k_5 \quad \text{.....(viii)}$$

where

$$\log P = \Sigma\pi \text{ or } \log P\ (R\text{-}X) = \pi(R) + \pi(X)$$

Lipophilicity can be considered as the relative affinity of a drug for the lipid biophase and is quantitatively defined in terms of partition coefficient (P) which characterizes the equilibration between the aqueous and lipid phases.

12. Physicochemical Parameters Used in QSAR

Steric and electronic effects in drug structures are not as well defined as lipophilicity. Examination of the structures of valproic acid analogues in Figure 2 shows that the lipophilic parameter describes the hydrophobic character of the alkyl groups, the electronic parameter evaluates the electronic properties of the polar moiety and the sub-

stitution pattern or steric effects of the alkyl chain can be characterized by the steric parameter. Since the regression equation (viii) is a linear free energy relationship, other free-energy related parameters can be used. The electronic parameters used frequently in QSAR are the Hammett's substitution constant (σ^*), pKa, ΔpKa and dipole moment (μ). Ionization constant (pKa) is generally considered as a function of polar effects describing the electron-withdrawing or electron-releasing effects (134).

There are various methods for the determination of pKa values of acidic compounds. These include potentiometric titration methods, spectrophotometric methods, and conductivity methods. For many drugs such as valproate analogues, solubility and UV absorption characteristics appear to determine the methods of choice. In one study (69), the pKa of valproic acid was determined by potentiometric titration using acetone-water media. Dipole moments of a number of anticonvulsant drugs have also been calculated by measurement of the dielectric constant and molar refraction of the drug solutions and solvents (135). Lipophilicity of compounds is either determined experimentally or calculated using substituent constants.

13. Hydrophobic Parameters

a. Determination of lipophilicity by the shake-flask procedure

Determination of the lipophilicity of a drug usually requires measurement of the equilibrium constant or partition coefficient between the non-polar and aqueous phases using shake-flask procedures. The choice of a model for the lipid phase in biomembranes has been arbitrary, ranging from highly nonpolar solvents to mod-

erately nonpolar solvents (133). Octan-1-ol is the most frequently used nonpolar solvent. Apart from preferred advantages of octanol in terms of availability, purity and bifunctional properties (133), octanol is probably as appropriate as other nonpolar solvents for modelling biomembranes. Collander (136) has showed that partition values can be converted between organic solvent-water systems. It has been found, however, that for a successful conversion, consideration ought to be given to differences in hydrogen-bonding properties between octanol and other organic solvents as well as hydrogen-donor or acceptor properties of the solutes (133,136).

b. HPLC determination of lipophilicity

The traditional shake-flask procedure of determining octanol-water partition coefficient (log P) has been observed to have many analytical problems (137-141). It is tedious and time-consuming. Solubility difficulties in aqueous media, instability of compounds in liquid phases and loss of material by adsorption or other mechanisms have been a major problem. Alternative methods, especially chromatography methods (TLC, HPLC) that provide a rapid and accurate estimate of lipophilicity have been sought. In recent years, reverse-phase (RP)-HPLC, with a hydrophobic column consisting of silica particles coated with covalent-bonded C-18 and C-8 alkyl chain, has been used to study hydrophobic effects (137-143) and structure-activity relationships (144).

The reverse-phase HPLC method is based on the determination of retention parameters which are then correlated with log P values. It is also required that the retention parameter be reproducible

and accounted for by a mechanism to reflect only partitioning behaviour of solute in liquid phases. The linear relationships reported between log P and capacity factor of compounds found by numerous investigators (137-150) appear to be a close parallel to the linear regression equation found by Collander (136) to exist between partition coefficients determined in two different nonpolar solvent-aqueous partitioning systems.

The unique features of the monomeric bonded-phase chromatographic column is the presence of a nonpolar C-18 or C-8 hydrocarbon chain chemically bonded to the silica support. The various methods for attaching a covalently-bonded phase to the porous or pellicular silica support rely on the conversion of surface silanol groups to silicate esters (-Si-OR), aminosilanes (-Si-N-) and siloxanes (-Si-O-Si-R_3). The most widely used chemically bonded stationary phases are those based on siloxanes [(-O-)$_3$-Si-O-Si(OH)$_2$-$C_{18}H_{37}$]. These phases are known to be relatively stable against hydrolysis within the pH range of 2-8 (151). Siloxane bonded phase packings are commercially available with microparticular supports and a monomeric C-18 organic layer such as Hypersil ODS used in this study.

Different reverse phase HPLC procedures of determining log $P_{o/w}$ have emerged over the years of its application. Both untreated (138,140,141) and silylated (140) octadecylsilane reverse phase columns have been used as the stationary phase. In a different procedure, Mirrlees et al. (139) coated silylated silica with a layer of n-octanol and used n-octanol-saturated water as the mobile phase. This was suggested to be closely analogous to the octanol-water partitioning system. Unger and co-workers (137) used

octanol-coated C-18 bonded silica as the stationary phase and octanol-saturated phosphate buffer as the eluent.

HPLC methods have been used to demonstrate good correlations between capacity factors and log $P_{o/w}$ values of straight-chain alkylcarboxylic acids (145,146). In a study of long chain n-aliphatic acids (C_7-C_{20}), d'Amboise and Hanai (145) reported excellent correlations with unacidified 50% acetonitrile-water as the mobile phase among different compositions of methanol, acetonitrile and tetrahydrofuran. Tanaka and Thorton (153) studied the hydrophobic effects of various carboxylic acids using a μ-Bondapak C_{18}-column and methanol-0.01M sodium phosphate buffer (pH 3.0-3.5) of different compositions. To suppress ionization (low pH) and minimize adsorption (NH_4+) of a series of aromatic acids in one study (147), 50% methanol-ammonium phosphate (pH 2.15) and Hypersil ODS was used to determine the capacity factors. In this study the HPLC method is used to determine the capacity factors of the compounds as well as the log $P_{o/w}$ values. This requires use of reference compounds and optimization of the analytical procedure to be sensitive to structural hydrophobic effects of varied carboxylic acids and tetrazoles. The log P values obtained in the HPLC method are evaluated in terms of accuracy by comparison to results from the shake-flask procedure.

Although the nature of the stationary phase during the chromatographic separations and the mechanism of retention in chemically-bonded phases is still not completely understood, a partition-adsorption mechanism has been postulated by various investigators (148-150) for the retention of solutes. According to

the proposed mechanism, the stationary phase is a combination of C-18 hydrocarbon layer, residual silanol present on the silica surface and associated solvent molecules from the mobile phase. The solute molecules are suggested to adsorb on a modified silica surface with an adsorbed layer of mobile phase components or partition into a liquid phase formed by the silica bonded-C_{18} layer with associated solvent molecules from the bulk eluent.

The application of liquid chromatography to measurement of physicochemical properties such as log P has been based on chromatographic theory. A theoretical treatment of a pure partition process in relation to the retention parameter by Snyder and Kirkland (151) showed, in the equilibrium situation, the relationship between the observed chromatographic retention parameter and the distribution coefficient

$$k' = \frac{K\ V_s}{V_m}$$

where k' is the capacity factor, V_m is the total volume of solvent within the column, V_s is the total volume of the stationary phase and K is the distribution coefficient which defines the equilibrium distribution of solute between the stationary phase and the mobile phase.

c. Determination of lipophilicity using substituent constants

Other methods of estimating log P values are the Hansch π method (133,152) and Rekker fragmental method (153). Both methods are based on the additive-constitutive properties of partition coefficient of compounds, but the approaches are different. Hansch π

values are calculated from the relation,

$$\pi_x = \log P\ (R\text{-}X) - \log P\ (R\text{-}H).$$

The Hansch method usually requires the octanol-water partition coefficient of the parent compound (R-H). Rekker adopted an empirical approach based on the relation

$$\log P\ (R\text{-}X) = \Sigma\ a_n f_n \text{ or } \log P\ (R\text{-}X) = f(R) + f(X)$$

Thus f_n is the fragment constant for the molecular fragment, n and a_n is the numerical factor indicating the number of times a given fragment n appears in the structure. The fragment constants are obtained from statistical data reduction procedures (153) in which contributions from the molecular fragment, n, in various molecular structures are averaged. Thus, the linear regression method has a_n as the independent parameter, log P as the dependent variable and the f_n values are determined as the regression coefficient. One result of the Rekker estimation method is the prediction that the hydrogen atom in a molecular structure contributes significantly to the partition coefficient. Thus compared to Hansch π-values, f-values differ for C, CH, CH_2 and CH_3.

EXPERIMENTAL

A. Chemicals and Materials

1. Synthesis

Chemicals were reagent grade and procured from the following sources.

a. Aldrich Chemical Co. (Milwaukee, Wisconsin)

Aluminium chloride (anhydrous), n-Butyl bromide, n-Butyl-lithium (1.6M in hexane), Calcium hydride, Cyclohexylmethylbromide, Deuterochloroform (gold label), Diisopropylamine, 2-Ethylbutyric acid, 2-Ethylhexanoic acid, 2-Ethoxyethanol, n-Heptyl bromide, Hexamethylphosphoramide, Isobutyric acid, Lithium aluminium hydride, Magnesium sulfate (anhydrous), N-Methyl-N-nitroso-p-toluenesulfonamide (Diazald®), 1-Methylcyclohexanecarboxylic acid, Pentan-2-one, (E)-2-Pentenoic acid, Potassium hydride (35% oil dispersion), n-Propyl bromide, n-Propyl iodide, Sodium hydride (50% oil dispersion), Tetrahydrofuran, Triethylamine.

b. British Drug House (Poole, U.K.)

Diethyl malonate, Diethylamine, Pyridine, Sodium cyanide.

c. Eastman Kodak Co. (Rochester, New York)

Sec-Butyl alcohol, 1-Bromo-3-methylbutane, Di-n-Butylamine, Ethyl acetoacetate, Methanesulfonyl chloride, Propionaldehyde, Sodium azide, Succinic anhydride.

d. Fisher Scientific Co. (Fairlawn, New Jersey)

Bromine, t-Butanol, Hydrogen bromide (48%), Potassium cyanide, Quinoline.

e. Mallinkrodt Chemicals (St. Louis, Missouri)

Ethyl bromide, Ethyl iodide, Potassium carbonate (anhydrous), Sodium bicarbonate, p-Toluenesulfonyl chloride.

f. Matheson Coleman and Bell Co. (Norward, Ohio)

Dimethylsulfoxide (anhydrous), Phosphorus pentoxide, Phosphorus tribromide.

2. Thin-layer Chromatography

Glass plates (20 x 20 cm) - CAMAG, Berlin, Germany.

Ether solvent - USP grade

Petroleum ether, 30^{o}-60^{o}C - USP grade

Benzene - USP grade

Silica gel G - E. Merck, Darmstadt, FR Germany

Sulfuric acid (96%) - British Drug House

Silver nitrate - Nichols Chemical Co., Vancouver, B.C.

TLC Streaking Apparatus - Applied Science Lab Ltd., State College, Pennsylvania

Spreader - Desaga, Heidelberg, FR Germany

3. High-Performance Liquid Chromatography

Norganic cartridges, membrane filters (for preparation of HPLC

grade water from deionized distilled water) - Millipore Corporation, Bedford, Massachusetts.

Sodium dihydrogen orthophosphate, monohydrate - British Drug House (Canada) Ltd., Toronto, Ontario.

2-Ethylbutyric acid, 2-Ethylhexanoic acid, Trimethylacetic acid, n-Valeric acid - Aldrich Chemical Co.

n-Heptanoic acid, n-Hexanoic acid - Eastman Kodak Co.

n-Butyric acid, Octanoic acid - Nutritional Biochemicals Corporation, Cleveland, Ohio.

4. Potentiometric Titrimetry

Potassium hydrogen phthalate - British Drug House.

Potassium hydroxide - American Scientific and Chemical Co., Seattle, Washington.

Phosphate pH 7.0 buffer - VWR Scientific Inc., San Francisco, California.

Potassium dihydrogen orthophosphate - Mallinkrodt Chemicals.

5. Gas Chromatography - Mass Spectrometry

3% Dexsil 300 on 100/120 Supelcoport - Supelco Inc., Bellefonte, Pennsylvania.

Fused silica capillary column (25 m x 0.3 mm i.d.) coated with SE-54 - Hewlett Packard.

Fused silica capillary column (25 m x 0.3 mm i.d.) with bonded OV-1701 - Quadrex Co.

t-Butyldimethylsilyl chloride - Applied Science Lab., State College, Pennsylvania.

N-Methyl-N-trimethylsilyltrifluoroacetamide, trimethylanilinium hydroxide (0.2M in methanol) - Pierce Chemical Co., Rockford, Illinois.

2-Propyl-(E)-2,4-pentadienoic acid was a gift from Dr.T.A. Baillie, School of Pharmacy, University of Washington, Seattle, Washington.

6. Pharmacological Testing

Pentylenetetrazole - Aldrich Chemical Co.

Sodium chloride - British Drug House.

Hydrochloric acid - American Scientific and Chemical Co.

B. Instrumentation

1. Nuclear Magnetic Resonance Spectrometry

Proton NMR spectra were recorded on a Bruker WP-80, Varian XL-100, Nicolet-Oxford-270 or Bruker WH-400 spectrometer at the NMR facility in the Department of Chemistry, U.B.C. Spectra were taken with $CDCl_3$ as solvent and tetramethylsilane as an internal standard.

2. Infra Red Spectrometry

IR spectra were obtained with sodium chloride disks either as liquid films or nujol mulls on a Unicam SP1000 spectrometer.

3. Ultraviolet Spectrometry

UV spectra were recorded in 50% methanol on a Beckman Model 24 UV-visible spectrophotometer.

4. Gas Chromatography Mass Spectrometry (GCMS)

a. Packed Column

GCMS analysis was performed on a Hewlett Packard 5700A gas chromatograph interfaced to a Varian MAT-111 mass spectrometer via a variable slit separator. Electron impact mass spectra were recorded at 70eV, ion source pressure of 5.0×10^{-6} Torr and emission current of 300μA. Computerized background subtractions were made to plot mass spectra. The scan range was 5 to 500 daltons with one scan taken every 5 sec. Total ion chromatographic plots were based on m/z 50 to 500. Mass chromatograms were plotted in scan mode. The data were processed by an on-line Varian 620L computer system.

GCMS analysis was carried out under the following conditions: 3% Dexsil 300 column (1.8 m x 2 mm i.d.); Oven temperature, 50°C to 280°C at 8°C/min; Helium (carrier gas) flow rate, 25 mL; Injection port temperature, 250°C; Separator temperature, 250°C; Inlet line temperature, 250°C.

b. Fused-silica Capillary Columns

Capillary GCMS analysis was done on a Hewlett Packard 5987A instrument equipped with an HP gas chromatograph, mass spectrometer and on-line data system. Electron impact mass spectra were recorded at 70eV, ion source pressure of 2.0×10^{-6} Torr and emission

current of 300 μA. The gas chromatograph was interfaced to the mass spectrometer via an open-split interface.

GCMS analysis of t-BDMS derivatives was performed under the following conditions: Dimethylsilicone column (12.5 m x 0.2 mm i.d.), OV-1701 column (25 m x 0.3 mm i.d.); Oven temperature, 50°C to 100°C at 30°C/min, 100°C to 260°C at 8°C/min; Helium flow rate, 1 mL/min; Splitless mode of injection; Source temperature, 200°C; transfer line temperature, 240°C; injection port temperature, 240°C.

C. Synthesis of Alkyl Carboxylic Acids, Tetrazoles and Succinamic Acids

1. Synthesis of Alpha-Substituted Aliphatic Acids

a. Synthesis of 2-Butylhexanoic Acid (II)

A flame-dried 500 mL three-necked flask, equipped with a graduated separatory funnel with septum inlet and reflux condenser connected to a mercury bubbler, was immersed in an ice-water bath. The reaction vessel was flushed with nitrogen and maintained under a nitrogen atmosphere throughout the reaction. Diisopropylamine (0.13 mol) in 100 mL anhydrous THF was placed in the flask. After cooling the mixture to 0°C, n-butyllithium in hexane (81 mL of 1.6 M, 0.13 mol) was added dropwise and with stirring. The mixture was stirred for 20 min and then n-hexanoic acid (0.055 mol) added at a slow rate to maintain the reaction temperature below 0°C. A milky white precipitate formed and then HMPA (0.13 mol) in THF was added. The resulting homogenous mixture was stirred at 0°C for an additional 20 min. n-Butylbromide (0.055 mol) was added rapidly to

the dianion of hexanoic acid, during which time the reaction temperature approached room temperature. The reaction was allowed to proceed for a further 90 min before being quenched with 25% HCl (80 mL). The organic layer was separated and the aqueous layer extracted twice with petroleum ether, 30°-60°C (100 mL portions). The combined organic layers were washed in turn with 10% HCl, saturated $NaHCO_3$ and saturated NaCl solution. The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvents removed with a rotary evaporator. The residue was then subjected to fractional distillation under reduced pressure to obtain 2-butylhexanoic acid (70% yield), bp 96°-98°C/0.35 mm [Lit (154), 89°C/0.1 mm].

NMR Spectrum: δ 0.7-1.0 (t, 6H, $2CH_3$), 1.1-1.8 (m, 12H, $6CH_2$), 2.1-2.5 (m, 1H, CH), 8.3-10 (broad s, 1H, COOH).

b. Synthesis of Valproic Acid (I)

Valproic acid was prepared, in a similar fashion to that described above for 2-butylhexanoic acid, by reaction of n-valeric acid (0.084 mol) with n-propylbromide. The final product, valproic acid, was separated from unreacted valeric acid by fractional distillation [bp of valproic acid 110°-112°C/1.4 mm; literature (69) bp 221°C at 760 mm].

NMR Spectrum: δ0.8-1.1 (t, 6H, $2CH_3$), 1.1-1.8 (m, 8H, $4CH_2$), 2.2-2.5 (m, 1H, CH), 8.0-10 (broad s, 1H, COOH).

c. Synthesis of 2-Propyl-(E)-2-Pentenoic Acid (VII)

Valproic acid (0.15 mol), synthesized as described above, was placed in a 250 mL flask equipped with a reflux condenser the top

of which was connected to a gas absorption device. Bromine (0.16 mol) was added into the flask followed by 0.7 mL of phosphorus tribromide. The mixture was stirred and heated with an oil bath at 70°C for 30 min and then at 100°C for 4 hours until all the bromine had reacted. The reaction mixture was then distilled under reduced pressure using a water pump to remove residual hydrogen bromide. The distillation assembly was then connected to an oil pump and the fraction containing 2-bromovalproic acid and 2-propyl-2-pentenoic acid collected at 70°-80°C/0.01 mm.

The acids were converted to the ethyl ester derivatives by refluxing a mixture of the acids for 12 hours in an excess of ethyl alcohol in benzene and catalyzed by a small amount of concentrated sulfuric acid. A Dean-Stark apparatus was used to separate out the water. The isolated ethyl ester derivatives and quinoline were placed in a flask connected to a Vigreux column set for downward distillation. The reaction mixture was heated to 160°C with stirring during a 15 min period, after which heating was increased rapidly to distill the resulting 2-propyl-2-pentenoate. The fraction with bp 188°-196°C was collected and purified by washing with dilute sulfuric acid. The unsaturated ester was hydrolyzed and the unsaturated acid obtained after acidification. The unsaturated acid was extracted with diethyl ether. The ether extract was washed with water, dried with anhydrous Na_2SO_4, filtered and solvent removed with a rotary evaporator. The product was recrystallized from a chloroform solution by keeping the solution at -20°C for a prolonged period. After two successive recrystallization steps, pure 2-propyl-(E)-2-pentenoic acid was

obtained, mp 32°C [lit. (155) mp 35°C]. These purification steps were necessary to remove small amounts of the low-melting Z-isomer.

NMR Spectrum: δ0.8-1.1 (m, 6H, $2CH_3$), 1.2-1.7 (m, 2H, CH_3-$\underline{CH_2}$-CH_2), 2.1-2.5 (m, 4H, $\underline{CH_2}$-CH = and $\underline{CH_2}$-C=), 6.8-7.1 (t, 1H, - CH = C, trans).

d. Synthesis of 2-Propyl-4-oxopentanoic acid (IX)

2-Bromopentanoic acid was synthesized, in a similar fashion to that described above for synthesis of 2-bromovalproic acid, by reaction of pentanoic acid with bromine in the presence of a catalytic amount of phosphorus tribromide. The crude product was distilled to give 2-bromopentanoic acid, bp 102°-105°C/2.5 mm. Mass spectrum: m/z 55 (100%), 138 (30%), 140 (28%), 27 (20%), 41 (18%), 29 (15%), 94 (12%), 43 (10%).

The ethyl ester of 2-bromopentanoic acid was prepared by refluxing a mixture of 2-bromopentanoic acid (0.43 mol), ethanol (1.0 mol), benzene (100 mL) and concentrated sulfuric acid (1.5 mL) for 12 hour using a Dean-Stark water separation unit. Pure ethyl 2-bromopentanoate was obtained by distillation after the usual work-up. Bp 60°-62°C/3.0 mm. Mass spectrum: m/z 29 (100%), 166 (26%), 168 (24%), 101 (22%), 140 (12%), 138 (10%).

For synthesis of 2-propyl-4-oxopentanoic acid, anhydrous THF (100 mL) was placed in a 250 mL flask and sodium hydride (0.084 mol) added. Ethyl acetoacetate (0.08 mol) was added dropwise over 30 min and the mixture stirred for an additional 10 min. Ethyl 2-bromopentanoate (0.08 mol) was added drop by drop and the solution refluxed for 5 hours. Distilled water (30 mL) was added and the resulting mixture filtered under suction. The organic

layer was separated and the aqueous phase extracted twice with ether. The combined organic layer was dried over Na_2SO_4, filtered and ether removed by a rotary evaporator. The residue was distilled to give ethyl 2-propyl-3-acetylsuccinate (75% yield) bp 115°C/0.2 mm.

A mixture of the acylsuccinate (0.018 mol) and concentrated HCl (0.18 mol) was heated under reflux for 10 hours to effect hydrolysis and decarboxylation. The product was extracted three times with ether and the ether extract dried over Na_2SO_4. The residue left after evaporation of the solvent was distilled to give pure 2-propyl-4-oxopentanoic acid, bp 135°-140°C/9 mm [lit. (156) bp 165°C/20 mm].

NMR Spectrum: δ0.8-1.1 (t, 3H, CH_3), 1.2-1.7 (m, 4H, $2CH_2$), 2.15 (s, 3H, CH_3-C=O), 2.5-3.1 (complex m, 3H, CH_2 - C = O, CH - C = O).

2. Synthesis of Alpha, Alpha-Disubstituted Aliphatic Acids

a. Synthesis of 2,2-Dimethylbutyric Acid (V)

Anhydrous THF (80 mL) and diisopropylamine (0.1 mol) were added to a dry, nitrogen-flushed flask immersed in an ice-water bath. n-Butyllithium in hexane (62 mL of 1.6 M, 0.1 mol) was added drop by drop to the well-stirred solution. Isobutyric acid (0.045 mol) was then added to the lithium diisopropylamide solution. The resulting milky white solution was turned into a clear homogenous solution by addition of tetrahydrofuran. Stirring was continued for 60 minutes at room temperature. Ethyl iodide (0.048 mol) was then added at once into the reaction flask. After

2 hours of additional stirring at room temperature, the reaction mixture was quenched by addition of 15% HCl (65 mL) at 0°C. The organic layer was separated and the aqueous layer extracted twice with diethyl ether. The combined organic layers were washed with 10% HCl, and saturated NaCl solution. The organic layer was then dried with anhydrous Na_2SO_4, filtered and solvent removed using an oil bath. The residue was distilled to give 2,2-dimethylbutyric acid (50% yield), bp 183°-185°C/760 mm [lit (157) bp 79°-81°C/11 mm].

NMR Spectrum: δ0.8-1.0 (t, 3H, CH_3), 1.2 (s, 6H, $2CH_3$), 1.4-1.8 (m, 2H, CH_2), 10.5-11.0 (broad s, 1H, COOH).

b. Synthesis of 2,2-Dimethylvaleric Acid

This compound was prepared using a procedure similar to that described above for 2,2-dimethylbutyric acid, by reaction of isobutyric acid (0.05 mol) and n-Propyl iodide (0.055 mol). The 2,2-dimethylvaleric acid thus obtained was purified by distillation, bp 203°-204°C/760 mm. [Lit. (158) bp 110°C/20 mm].

NMR Spectrum: δ0.8-1.0 (m, 3H, CH_3), 1.2 (s, 6H, $2CH_3$), 1.3-1.7 (m, 4H, $2CH_2$), 10.8 (broad s, 1H, COOH).

3. Synthesis of Beta-Substituted Aliphatic Acids

a. Synthesis of 3-Ethylpentanoic Acid (VI)

Lithium aluminium hydride (0.48 mol) and sodium-dried ether (700 mL) were introduced into a 2 litre flask equipped with mechanical stirrer, dropping and reflux condenser. After stirring the mixture for 15 min, ethyl 2-ethylbutyrate (0.70 mol) in anhydrous

ether was added such that the ether refluxed gently. On addition of the ester, the mixture was stirred for 20 min to complete the reaction. Excess hydride was destroyed by addition of water. Following filtration and separation of the phases, the ethereal solution was dried over $MgSO_4$, filtered and the ether removed by flash evaporation. Distillation of the residue afforded 2-ethylbutan-1-ol (80% yield), bp 146°-149°C. Mass spectrum (eight intense ions): m/z 43, 70, 71 (M-31), 55, 41, 56, 29, 84 [M-18].

To cold 2-ethylbutan-1-ol (0.54 mol), phosphorus tribromide (0.30 mol) was added dropwise and with stirring over 2 hours at -10°C. The solution was then stirred at ambient temperature for 2 hours before being heated for 20 min with a steam bath. Water was added to the cooled reaction mixture and the product extracted with hexane. The hexane extract was dried over P_2O_5 and the solvent removed. Distillation of the residue gave 1-bromo-2-ethylbutane (50% yield), bp 145°-148°C/760 mm. Mass spectrum (eight most intense ion, relative intensity): m/z 43 (100%), 85 (51%), 55 (48%), 71 (27%), 29 (23%), 116 (15%), 135 (12%), 137 (10%).

1-Bromo-2-ethylbutane (0.18 mol) in methanol was added to an aqueous-methanol solution of KCN (0.26 mol). The reaction mixture was refluxed for 24 hours. After distilling of the alcohol, the product was extracted three times with hexane and the combined organic extracts washed successively with 30% HCl, saturated $NaHCO_3$, water and dried over anhydrous $MgSO_4$. Distillation afforded 2-ethylpentanenitrile (50% yield), bp 100-102°C/ca 20 mm. Mass spectrum: m/z 43 (100%), 28 (40%), 71 (35%), 41 (30%), 55 (28%), 29 (20%), 54 (16%), 82 (8%).

2-Ethylpentanenitrile (0.05 mol) was hydrolyzed to the corresponding acid by refluxing with 50% sulfuric acid (25 mL) for 9 hours. On extraction of the acid with ether, the ether extract was dried with anhydrous Na_2SO_4 and the ether distilled off. Distillation of the residue produced 3-ethylpentanoic acid (80% yield), bp 80°-82°C/4 mm [lit. (5) bp 87°C/1 mm].

Mass Spectrum: m/z 60 (100%), 43 (64%), 70 (61%), 55 (58%), 61 (23%), 71 (18%), 83 (12%), 101 (10%).

NMR Spectrum: δ0.8-1.0 (m, 6H, $2CH_3$), 1.2-1.5 (m, 4H, $2CH_2$), 1.6-1.9 (m, 1H, CH), 2.2-2.4 (d, 2H, CH_2-COOH), 9.5-10.5 (broad s, 1H, COOH).

b. Synthesis of Cyclohexylacetic Acid (XIII)

Cyclohexylmethylcyanide (bp 76°-80°C/7 mm) was prepared from cyclohexylmethyl bromide and hydrolyzed to cyclohexylacetic acid in a similar procedure to that described above for synthesis of 3-ethylpentanoic acid (a). Distillation of crude product under reduced pressure gave cyclohexylacetic acid, bp 136°C/0.15 mm [lit.(159) bp 137°C/0.2 mm] which solidified under ambient temperature.

Mass Spectrum: m/z 60 (100%), 82 (75%), 55 (65%), 83 (52%), 61 (51%), 67 (43%), 41 (39%), 27 (17%).

NMR Spectrum: δ0.8-1.4 (m; 5H; C_2, C_6 protons on ring and CH), 1.5-2.0 (m, 6H, C_3-C_5 protons on alicyclic ring), 2.2 (d, 2H, CH_2-COOH), 10.2 (broad s, 1H, COOH).

c. Synthesis of 3-Methylvaleric Acid

Diethyl 1-methylpropylmalonate was synthesized from diethyl

malonate and sec-butyl bromide. Ethanol and sodium ethoxide (prepared in situ) were used as the solvent and base, respectively. Saponification of diethyl 1-methylpropylmalonate, followed by acidification gave upon decarboxylation 3-methylvaleric acid, bp 190°-196°C/760 mm [lit. (160) bp 187°-200°C/760mm].

NMR Spectrum: δ0.8-1.1 (m, 6H, $2CH_3$), 1.2-1.5 (m, 2H, CH_2), 1.6-2.0 (m, 1H, CH), 2.1-2.5 (m, 2H, CH_2COOH), 10.2 (broad s, 1H, COOH).

d. Synthesis of 3-Methylhexanoic Acid

Diethyl 1-methylbutylmalonate was synthesized from diethyl malonate and 2-bromopentane (from pentan-2-ol). Ethanol and sodium ethoxide were used as the solvent and base respectively. Saponification of the monoalkylmalonate, followed by acidification gave upon decarboxylation 3-methylhexanoic acid bp 91°-94°C/5 mm [lit. (5) bp 60°-70°C/0.5 mm].

NMR Spectrum: δ0.8-1.1 (m, 6H, $2CH_3$), 1.2-1.5 (m, 4H, $2CH_2$), 1.7-2.0 (m, 1H, CH), 2.1-2.5 (m, 2H, CH_2COOH), 10.1 (broad s, 1H, COOH).

e. Synthesis of 2-propyl-4-pentenoic acid (4-ene VPA) and 2-propyl-3-hydroxypentanoic acid (3-OH VPA) was accomplished as described in a previous work (93).

4. Synthesis of 5-Alkyltetrazoles

a. Synthesis of 5-Isoamyltetrazole (XI)

5-Methylpentanenitrile was prepared in a similar manner to that described for synthesis of 3-ethylpentanoic acid, from 1-bromo-3-methylbutane and an aqueous-methanol solution of potassium cyanide. Fractional distillation gave 5-methylpentanenitrile, bp 150°-155°C/760 mm.

Mass Spectrum: m/z 55 (100%), 41 (51%), 43 (47%), 27 (35%), 29 (30%), 54 (21%), 39 (15%), 57 (13%), 82 ($M-CH_3$, 11%).

5-Methylpentanenitrile (0.06 mol) and sodium azide (0.18 mol) in anhydrous THF (60 mL) were treated at room temperature with anhydrous aluminium chloride (0.063 mol) dissolved in cold anhydrous THF. The mixture was refluxed for 24 hours and with stirring. The solvent was then distilled off under reduced pressure using a steam bath.

Water (80 mL) was added to the residue and the solution acidified to pH 2 with concentrated HCl. Evaporation was carried out again under reduced pressure to remove residual hydrazoic acid. The solution was further diluted with water to dissolve the aluminium chloride and sodium chloride formed. The oily organic layer was separated and the aqueous phase extracted with ethyl acetate. The organic layers were left in the refrigerator until the 5-alkyltetrazole crystallized out. The crystalline solid was filtered and recrystallized from petroleum ether-diethylether solvent mixture to give 5-isoamyltetrazole, mp 95°-96°C [lit. (161) mp 95°-96°C].

NMR Spectrum: δ0.95 (d, 6H, $2CH_3$), 1.5-2.0 (m, 3H, $CH-CH_2$), 3.0-3.3 (t, 2H, $CH_2-C=$), 11.4 (s, 1H, NH).

b. Synthesis of 5-Cyclohexylmethyltetrazole (XII)

Cyclohexylethanenitrile was prepared, as described in the synthesis of cyclohexylacetic acid, from cyclohexylmethylbromide. The nitrile was converted into 5-cyclohexylmethyltetrazole, in a similar fashion to that described for synthesis of 5-isoamyltetrazole. This required use of cyclohexylethanenitrile and aluminium azide (prepared in situ from sodium azide and anhydrous aluminium chloride). The isolated crude product was recrystallized from ethyl acetate to give 5-cyclohexylmethyltetrazole, mp 109-110°C [lit. (161) mp 109°C].

NMR Spectrum: δ0.9-1.4 (m; 5H; C_2, C_6 protons on alicyclic ring and CH), 1.5-2.0 (m, 6H, C_3-C_5 protons on ring), 2.9-3.2 (d, 2H, CH_2C=), 10.5 (broad s, 1H, NH).

c. Synthesis of 5-Heptyltetrazole (X)

1-Bromoheptane (0.32 mol) was added slowly to a warm, well-stirred solution of sodium cyanide (0.41 mol) in dimethylsulfoxide (130 mL). Stirring was continued for 1 hour until the solution temperature cooled from 130°C to ambient temperature. The reaction mixture was poured into water and the product extracted three times with ether. The combined ether extract was washed with saturated NaCl and dried over anhydrous $MgSO_4$. The liquid left upon evaporation of ether was distilled under reduced pressure to give octanenitrile, bp 85°-88°C/10 mm.

Octanenitrile was reacted with aluminium azide in refluxing THF, employing the procedure for synthesis of 5-isoamyltetrazole.

The crude solid product isolated was recrystallized from acetonitrile to give 5-heptyltetrazole, mp 41°-42°C [lit. (161) mp 41.5°C].

NMR Spectrum: δ0.7-1.0 (t, 3H, CH_3), 1.1-1.5 (m, 8H, $4CH_2$), 1.6-2.0 (m, 2H, $\underline{CH_2}$ CH_2-C=), 2.9-3.3 (t, 2H, CH_2-C=), 10-11 (broad s, 1H, NH).

5. Synthesis of Succinamic Acids

a. Synthesis of N,N-Diethylsuccinamic Acid (XV)

Succinic anhydride (0.1 mol) was added with stirring to diethylamine (0.1 mol) in cold absolute ethanol (25 mL). Stirring was continued until the reaction mixture attained room temperature. N,N-Diethylsuccinamic acid crystallized out of the homogenous solution on cooling and was filtered under suction before recrystallization from benzene, mp 83°-85°C [lit. (162) mp 82-84°C].

IR Spectrum (mull): 1690-1720 cm^{-1} (C = O in COOH), broad), 1630 (C = O in amide).

NMR Spectrum: δ1.1-1.5 (distorted t, 6H $2CH_3$), 2.6 (s, O=C-CH_2CH_2-C=O), 2.8-3.3 (q, 4H, CH_2-N-CH_2), 5.0-6.0 (broad s, 2H, COOH).

b. Synthesis of N,N-Dibutylsuccinamic Acid (XVI)

Succinic anhydride (0.15 mol) was added to anhydrous benzene (120 mL) and the solution warmed to partially dissolve the anhydride. Dibutylamine (0.15 mol) in benzene (30 mL) was rapidly added to the mixture at a controlled rate to contain the heat generated. The reaction mixture was then refluxed until the

reaction mixture turned homogenous. The resulting reaction mixture was allowed to cool to ambient temperature and left in the refrigerator until the solid product crystallized out. N,N-Dibutylsuccinamic acid was filtered under suction and recrystallized with benzene, mp 78°-80°C.

IR Spectrum (mull): 1720 cm^{-1} (C = O in COOH), 1615 (C = O in amide).

NMR Spectrum: δ0.8-1.1 (t, 6H, $2CH_3$), 1.2-1.9 (m, 8H, $2CH_2$-CH_2), 2.6 (s, 4H, O = C-CH_2 CH_2 C = O), 2.8-3.1 (t, 4H, CH_2-N-CH_2), 8.2-8.9 (broad s, 2H, COOH).

6. Synthesis of Diunsaturated Analogues of Valproic Acid

The diunsaturated derivatives of valproic acid were prepared by dehydration of the β-hydroxy unsaturated esters followed by hydrolysis of the diunsaturated esters. The β-hydroxy unsaturated esters were prepared by deconjugative aldol condensation of the lithium dienolates of ethyl 2-pentenoate and propionaldehyde. The dienolate of ethyl (E)-2-pentenoate was expected to give mainly the β-hydroxy-(Z)-3-pentenoate whereas β-hydroxy-(E)-3-pentenoate was expected from reaction with ethyl (Z)-2-pentenoate.

Ia. Synthesis of Ethyl 2-(1'-hydroxypropyl)-3-Pentenoate from Ethyl (E)-2-Pentenoate

Esterification of (E)-2-pentenoic acid (EtI, K_2CO_3, THF, reflux, 6 hr) gave ethyl-(E)-2-pentenoate, bp 36-38°C/0.07 mm.

NMR Spectrum: δ1.0 (t, 3H, CH_3), 1.3 (t, 3H, CH_3), 2.2 (q, 2H, CH_2-C=), 4.2 (q, 2H, OCH_2), 5.6-6.0 (d, 1H, = CH, J = 16Hz), 6.8-7.3 (dt, 1H, HC ==, J = 16 Hz).

n-Butyllithium in hexane (69 mL of 1.6M, 0.11 mol) was added dropwise with stirring to a solution of diisopropylamine (0.11 mol) in anhydrous THF (120 mL) at 0°C. The solution was stirred for 20 min at 0°C and then cooled to -78°C. Hexamethylphosphoramide (0.11 mol) was added to the solution followed by dropwise addition of ethyl-(E)-2-pentenoate (0.10 mol). After 30 min, propionaldehyde (0.10 mol) was added. The reaction mixture was stirred for a further 30 min at -78°C before the reaction was quenched with 15% HCl. The product was extracted with ether and the ether extract washed successively with 10% HCl, saturated $NaHCO_3$ and saturated NaCl. The organic layer was dried over Na_2SO_4, and solvents removed with a rotary evaporator. The residue after fractional distillation under reduced pressure gave the major product, ethyl 2-(1'-hydroxypropyl)-3-pentenoate, bp 85-90°C/0.25 mm (48% yield).

IR Spectrum: 3400 cm^{-1} (O-H), 1735 cm^{-1} (C=O), 1635 cm^{-1} (C=C), 1175 cm^{-1} (C-O), 985 cm^{-1} (medium intensity, =CH, trans), 745 cm^{-1}.

NMR Spectrum: δ0.95 (t, 3H, CH_3), 1.25 (t, 3H, $OCH_2\underline{CH_3}$), 1.55 (m, 2H, CH_2), 1.75 (d, 3H, $\underline{CH_3}$-CH=), 2.55 (broad s, 1H, OH), 3.40 (m, 1H, CH-C=O), 3.8 (m, 1H, CH-O), 4.2 (q, 2H, $O\underline{CH_2}CH_3$), 5.3-5.9 (m, 2H, CH = CH).

Ib. Synthesis of Ethyl 2-(1'-hyrodxypropyl)-3-pentenoate from Ethyl-(Z)-2-Pentenoate

Ethyl (Z)-2-Pentenoate was prepared in three stages. (i) A mixture of 2-pentanone (0.5 mol) and precooled 48% HBr (50 mL) was chilled to 0°C and bromine (1.0 mol) added dropwise. After addition of bromine, water (200 mL) was added and the heavier organic

layer distilled to give a fraction with bp 84-89°C/10 mm. GCMS analysis showed 1,3-dibromo-2-pentanone to be the main product. (ii) 1,3-dibromo-2-pentanone (0.3 mol) was added to a solution of $KHCO_3$ (2.1 mol) in 1200 mL water. The reaction mixture was stirred for 24 hours. Nonacidic by-products in basic solution were discarded after extraction with ether. The basic solution was acidified to pH 2 with dilute HCl and the product extracted with ether. The ether phase was in turn dried over $MgSO_4$, filtered and concentrated with a rotary evaporator. Distillation of the residue gave (Z)-2-pentenoic acid, bp 39°-41°C/0.4 mm. (iii) Esterification of the acid (EtI, K_2CO_3, THF, reflux, 6 hr) gave ethyl (Z)-2-pentenoate, bp 50°-52°C/12 mm.

NMR Spectrum: δ1.0 (t, 3H, CH_3), 1.3 (t, 3H, $OCH_2\underline{CH_3}$), 2.65 (q, 2H, CH_2-C=), 4.2 (q, 2H, OCH_2-), 5.6-5.9 (d, 1H, CH=, J = 10 Hz), 6.0-6.4 (dt, 1H, CH=, J = 10 Hz).

The lithium dienolate of ethyl (Z)-2-pentenoate (0.05 mol) was prepared at -78°C using lithium diisopropylamide (0.055 mol) in 60 mL THF and hexamethylphosphoramide (0.055 mol). Proprionaldehyde (0.05 mol) was added dropwise to the solution and the reaction stirred for 30 min and finally quenched with 10% HCl. The major product, ethyl 2-(1'-hydroxypropyl)-3-pentenoate, was isolated, bp 95-100°C/1 mm (50% yield).

IR Spectrum: 3400 cm^{-1} (O-H), 1735 cm^{-1} (C=O), 1640 cm^{-1} (C=C), 985 cm^{-1} (strong intensity, = CH, trans), 745 cm^{-1}.

II. Dehydration of ethyl 2-(1'-hydroxypropyl)-3-pentenoate

a. Phosphorus Pentoxide

Phosphorus pentoxide (36 mmol) was added to the β-hydroxy unsaturated ester (39 mmol, from ethyl (E)-2-pentenoate) in 60 mL of anhydrous benzene and the mixture refluxed for 4 hours. The diunsaturated esters isolated were saponified with dilute NaOH and subsequently acidified with dilute H_2SO_4 to give a mixture of seven isomeric dienoic acids, bp 105°-110°C/2.5 mm.

b. Toluenesulfonyl chloride-pyridine

Dehydration of the β-hydroxyunsaturated ester (33 mmol, from ethyl (E)-2-pentenoate) in 20 mL of pyridine with p-toluenesulfonylchloride (45 mmol) was carried out by first stirring the reaction mixture at room temperature for two days, diluting with ice-water and extracting the p-toluenesulfonate derivative with $CHCl_3$. The isolated p-toluenesulfonate was refluxed with dilute NaOH and acidified to give a mixture of four isomeric dienoic acids, bp 120°-128°C/6 mm.

NMR Spectrum (270 MHz) of mixture: δ0.95-1.1 (t, CH_3), 1.55 (dd, CH_3-C=, 2E-3'Z), 1.64 (dd, CH_3-C=, 3Z-3'Z), 1.70 (dd, CH_3-C=, 2Z-3'E), 1.85 (d, CH_3-C=, 2E-3'E), 2.16 (m, CH_2-C=, 2E-3'Z), 2.35 (m, CH_2-C=, 2E-3'E), 2.55 (m, CH_2-C=, 2Z-3'E), 4.05 (t, CH, 3Z-3'Z), 5.65 (m, CH=CH, 3Z-3'Z), 5.73 (m, CH=CH, 2E-3'Z), 5.85 (m, CH=CH, 2Z-3'E), 5.95 (t, CH=, 2Z-3'E), 6.03 (m, CH=, 2E-3'E), 6.14 (d, CH=, 2E-3'E), 6.83 (t, CH=, 2E-3'E), 7.00 (t, CH=, 2E-3'Z).

IR Spectrum of mixture: 1690 cm^{-1} (C=O), 1633 cm^{-1} (C=C), 965 cm^{-1} (=CH, trans), 935 cm^{-1}, 735 cm^{-1}.

NMR assay of the four dienoic acid isomeric mixture, using appropriate integrated areas, gave the ratio of 2-(1'-propenyl)-2-pentenoic acid and 2-(1'-propenyl)-3-pentenoic acid isomers 3Z-3'Z, 2Z-3'E, 2E-3'Z, 2E-3'E as approximately 12 : 14 : 56 : 18.

c. Methanesulfonyl chloride - Potassium hydride

(i) Dehydration of the β-hydroxy unsaturated ester (0.05 mol), derived from ethyl (E)-2-pentenoate, was carried out by forming the sulfonate with methanesulfonyl chloride (0.06 mol) in the presence of triethylamine (0.08 mol) and methylene chloride (40 mL) at 0°C. KH (0.10 mol) was added at 0°C to the isolated sulfonate and reaction mixture stirred for 12 hours at room temperature. Excess hydride was decomposed by addition of t-butanol and water. Following the usual work-up, the isolated product mixture, bp 75-80°C/2 mm, contained three isomeric dienoates.

IR Spectrum of mixture: 1716 cm^{-1} (C=O), 1636 cm^{-1} (C=C), 968 cm^{-1} (=CH, trans), 756 cm^{-1}, 690 cm^{-1} (=CH, cis more intense than =CH, trans).

NMR assay of the ethyl esters of the three dienoic acid mixture, using appropriate integrated areas, gave the ratio of isomers 2Z-3'E: 2E-3'Z: 2E-3'E as 7:71:22.

NMR Spectrum of isomeric mixture (270 MHz): δ1.0-1.1 (m, CH_3), 1.3 (m, CH_3), 1.55 (dd, CH_3-C=, 2E-3'Z), 1.7 (dd, CH_3-C=, 2Z-3'E), 1.83 (d, CH_3-C=, 2E-3'E), 2.12 (m, CH_2-C=, 2E-3'Z), 2.3 (m, CH_2-C=, 2E-3'E), 2.52 (m, CH_2-C=, 2Z-3'E), 4.2 (q, CH_2O), 5.75 (m, CH =, 2E-3'Z), 5.98 (d, CH=, 2E-3'Z), 6.04 (m, CH=, 2E-3'E), 6.13 (d, CH=, 2E-3'E), 6.55 (t, CH=, 2E-3'E), 6.8 (t, CH=, 2E-3'Z).

The isomeric dienoates mixture was saponified with dilute NaOH and subsequently acidified with dilute H_2SO_4 to give the isolated product mixture of the corresponding acids of two major isomers 2E-3'Z, 2E-3'E and trace amounts of the isomer 2Z-3'E.

NMR Spectrum (400 MHz) of the mixture: δ1.0-1.1 (m, CH_3), 1.56 (dd, CH_3-C=, 2E-3'Z), 1.73 (dd, CH_3-C=, 2Z-3'E), 1.84 (d, CH_3-C=, 2E-3'E), 2.15 (m, CH_2-C=, 2E-3'Z), 2.23 (m, CH_2-C=, 2E-3'E), 2.50 (m, CH_2-C=, trace, 2Z-3'E), 5.8 (m, CH=, 2E-3'Z), 5.96 (d, CH=, 2E-3'Z), 6.07 (m, CH=, 2E-3'E), 6.13 (d, CH=, 2E-3'E), 6.78 (t, CH=, 2E-3'E), 6.97 (t, CH=, 2E-3'Z).

(ii) Dehydration of the β-hydroxyunsaturated ethyl ester (0.02 mol), derived from ethyl (Z)-2-pentenoate, was carried out by treatment of the mesylate derivative with KH (0.04 mol). The isolated product mixture, bp 65-70°/0.1 mm contained three isomeric dienoates, 3Z-3'Z, 2E-3'Z, 2E-3'E in the ratio of approximately 44:8:48 (determined by NMR analysis).

IR Spectrum: 1733 cm^{-1} (C=O), 1716 cm^{-1} (C=O), 1655-1640 cm^{-1} (C=C), 985-965 cm^{-1} (CH=, trans), 745 cm^{-1}.

NMR Spectrum of the isomeric mixture (270 MHZ): δ1.0-1.1 (m, CH_3), 1.2-1.25 (m, CH_3), 1.53 (d, CH_3-C=, 2E-3'Z), 1.64 (dd, CH_3-C=, 3Z-3'Z), 1.68 (dd, CH_3-C=, 3Z-3'Z), 1.82 (d, CH_3-C=, 2E-3'E), 2.1 (m, CH_2-C=, 2E-3'Z), 2.35 (m, CH_2-C=, 2E-3'E), 3.5 (m, CH, 3Z-3'Z), 5.5-5.7 (m, CH=CH, 3Z-3'Z), 6.04 (m, CH=, 2E-3'E), 6.13 (d, CH=, 2E-3E), 6.57 (t, CH=, 2E-3'E), 6.77 (t, CH=, 2E-3'Z).

III. Semi-Preparative Argentation TLC

Silica gel G was impregnated with $AgNO_3$ (20% w/w) and TLC

plates prepared with 0.5 mm thickness. A mixture of isomeric dienoates (20-40 mg) in $CHCl_3$ was applied across the plates (20 x 20 cm) in a narrow band. Benzene: petroleum ether, 30°-60°: diethyl ether (80 : 20 : 5) was selected as the mobile phase after experimenting with different solvent systems. The plates were developed in a rectangular glass tank. The solvent front was allowed to run to 15 cm on the plates, from the origin. The plates were then sprayed with 50% H_2SO_4 and heated at 200°C for 10 min. R_f values of three charred bands were determined. Another series of plates was developed under the same conditions. With pre-knowledge of band positions, strips of support material corresponding to the three bands were scraped off the unheated plates into centrifuge tubes. The ethyl esters were extracted with methanol. Following centrifugation and filtration of the methanolic solutions, the three fractions were concentrated and reconstituted in $CDCl_3$ for GCMS and NMR analysis.

IV. In vivo metabolism - isolation procedure

a. Serum. A serum sample (2.0 mL) from a patient on VPA, was acidified to pH 2.0 with 4N HCl. Extraction was carried out twice with 2.0 mL of ethyl acetate, each time centrifuging at 2000 rpm for 20 min to separate the phases. The ethyl acetate layers were combined, dried over anhydrous Na_2SO_4 and then concentrated at room temperature using a gentle stream of dry nitrogen. The concentrated extracts were derivatized and aliquots injected into the gas chromatograph mass spectrometer.

b. Urine. To 5 mL of a urine sample was added 3N NaOH to bring

the pH to 13.0 and hydrolysis of conjugates was effected by heating the solution at 60°C for 1 hr. After cooling, the solution was acidified to pH 2.0 with 4N HCl. The acidified urine was extracted twice with ethyl acetate. The combined ethyl acetate layers were dried, concentrated and derivatization carried out.

V. Derivatization of Acids

t-BDMS derivatives were prepared by adding 30-50 µL of t-BDMS reagent to synthesized sample (3-5mg) or urine extraction samples (see above) and warming at 60°C for 5 min. The t-BDMS derivatives were extracted with 100-150 µl of solvent (ethyl acetate-hexane) and aliquots injected into the GCMS.

TMS-derivatives were formed by treating the concentrated urine extraction samples or synthesized samples with 30-50 µl of N-methyl-N-trimethylsilyl-trifluoroacetamide at 50°C for 5 min.

Ethyl esters of acids were formed using appropriate volumes of trimethylanilinium hydroxide solution and ethyl iodide. The mixture was warmed at 50°C for 5 min.

Methyl esters of acids were prepared by reaction of the acid sample with diazomethane generated from Diazald® (dissolved in ether/2-ethoxyethanol) and 60% KOH.

VI. Photochemical Isomerization

A mixture of seven isomeric dienoic acids (1g), derived from dehydration of ethyl 2-(1'-hydroxypropyl)-3-pentenoate with P_2O_5, was dissolved in 150 mL of hexane and added to a pyrex well. 2-Propyl-(E)-2-pentenoic acid (2-ene VPA) was added as an internal

standard. A quartz immersion well with a 450W unfiltered Hanovia lamp was set into place and irradiation carried out for 6 hours. Aliquots of the reaction mixture were removed periodically and the t-BDMS esters of photolyzed acids analyzed by capillary GCMS.

VII. Capillary GCMS resolution of 2,3'-diene VPA and 2,4-diene VPA

Samples of synthetic diene acids, 2-propyl-(E)-2,4-pentadienoic acid and 2-[(Z)-1'-propenyl]-(E)-2-pentenoic acid, or the urine extract from one patient on VPA therapy, were made with ethyl acetate and derivatives formed. Urine extracts were also spiked with synthetic diene acids prior to TMS or t-BDMS derivatization. Capillary GCMS analysis of the TMS derivatives was performed using a 25 m x 0.3 mm i.d. SE 54 column at oven temperatures from 50°C to 90°C at 30°/min and then held at 90°C for 10 min. Capillary GCMS analysis of the t-BDMS derivatives was performed using either the SE-54 column or OV-1701 column. Oven temperatures were programmed from 50°C to 100°C at 30°/min and then 100°C to 200°C at 8°/min.

D. Subcutaneous Pentylenetetrazole (PTZ) Seizure Threshold Test

1. Animals

Adult male Swiss mice (CD1 strain, 20-32g, Charles River, Quebec, Canada) were used as experimental animals. All animals were allowed free access to food and water except during the time of test. Mice were housed after arrival for at least 24 hours before use.

2. Drugs

Isobutyric acid, trimethylacetic acids, 1-Methylcyclohexane-1-carboxylic acid and pentylenetetrazole (PTZ) were obtained from Aldrich Chemical Company, Milwaukee, USA. Valproic acid was purchased from Abbott Laboratories, Illinois, USA. 2-Butylhexanoic acid, 2,2-Dimethylbutyric acid, 3-Ethylpentanoic acid, Cyclohexylacetic acid, 2-Propyl-4-oxopentanoic acid (4-keto VPA), 2-Propyl-(E)-2-pentenoic acid (2-ene VPA), 2-(1'-Propenyl)-2-pentenoic acid (2,3'-diene VPA), as a mixture of 2E-3'Z and 2E-3'E in a ratio of 7 : 3, N,N-Diethylsuccinamic acid, N,N-Dibutylsuccinamic acid, 5-Cyclohexylmethyltetrazole, 5-Isoamyltetrazole and 5-Heptyltetrazole were synthesized as described in the experimental section. The purity and identity of these compounds were verified by IR, NMR spectroscopy and GCMS analysis.

3. Drug Solutions

Pentylenetetrazole was dissolved in 0.9% sodium chloride to make a concentration of 1.7%. The succinamic acids, which are soluble in water, were dissolved in 0.9% sodium chloride. The solutions of the fatty acids and tetrazoles were prepared by neutralizing a known amount of the drug with 1N NaOH and the pH of the resulting solution adjusted to 7.4 with 1.0N HCl. All drugs were administered to mice in concentrations which permitted optimal accuracy in dosage. Drugs were administered i.p. in a volume not more than 7 mL/kg body weight in mice and PTZ injected s.c. in a volume of 5 mL/kg.

4. Experimental Procedure

a. Characterization of PTZ Seizures (85 mg/kg dose)

Mice received a subcutaneous injection of PTZ, 85 mg/kg, in a loose fold of skin, on the back of the neck. This is the CD_{97} dose of PTZ in mice (46). Control mice, in a group of 12, were injected s.c. with PTZ, 85 mg/kg. After PTZ administration, animals were placed in individual cages for observation. Observation time was 30 min following the s.c. PTZ injection.

The reaction of each mouse to PTZ (85 mg/kg) followed a definite pattern, characterized by a brief episode of body twitches which passed into a series of clonic jerkings usually accompanied by audible squeaks and Straub tail phenomenon. The clonic spasms occurred between resting phases. The convulsive syndrome usually ended with the swimming phase, characterized by coordinated flexor-extensor movement of the legs. The post seizure phase usually ended fatally with tonic extension of hind limbs or was characterized by a stupor phase after which mice recovered.

During the 30 minute observation period, sustained synchronous clonic jerkings occurred in 100% of control mice. The onset to clonic episodes (episodes of 5 second duration or longer) was between 3-9 minutes. Seventy-five percent of control mice died within the 30 min observation period.

b. Antagonism of PTZ clonic seizures

Mice in groups of eight per dose of drug, received i.p. injections of test drug in a maximum of four doses between 0.2 mmol/kg and 2.0 mmol/kg. For each drug, a fixed time interval of 15 min

after i.p. administration was chosen before mice received a s.c. 85 mg/kg PTZ injection.

Each mouse was individually caged for observation after the s.c. PTZ injection. Each mouse was observed for a maximum time of 30 min after PTZ administration but clonic seizures rarely occurred after times longer than 20 min. During the period of observation, the incidence and timing of clonic convulsions was recorded with absence of sustained clonic spasms (5 sec duration or longer) being defined as protection.

Eight mice were used for each point on the dose-effect curves. At least three points were established between the limits of complete protection and no protection. The probit of the percentage protected was plotted against the log dose. The data were analyzed by the statistical method of Litchfield and Wilcoxon (192). The ED_{50} values, slope of the curves, and their 95% confidence limits were then recorded for each anticonvulsant drug.

c. Mortality Test

The percentage of mice which survived within 30 minutes after the s.c. 85 mg/kg PTZ injection was determined at the 1.0 mmol/kg i.p. dose level of test compound. Test drugs were administered i.p. 15 minutes before PTZ administration.

d. Toxic effects of drugs

Specific toxicity tests were not conducted. However, observations of toxic signs were made during the 15 minute time interval before administration of PTZ. Neurotoxic effects were indicated by

sedation, abnormal gait or ataxia, hyperactivity, rapid-circling activity, abnormal body posture, abnormal spread of the limbs, prostration (immobility or resting on belly), fasciculation (movement of muscles on the back), tremors, and convulsions.

e. Convulsant Activity Test

Compounds which induced tremors, hyperexcitability and convulsions in mice were tested for convulsant activity by i.p. administration of sublethal doses and the mice observed for 30 minutes without administration of PTZ.

E. HPLC Method for Determination of Octanol-Water---Partition Coefficients

1. Instrumentation

Analyses were performed with a Hewlett-Packard Model 1082B HPLC equipped with an HP 79850B LC terminal. Detection of the acidic compounds was at 210 nm with a Hitachi Model 155-10 variable wave length spectrophotometer. The Absorbance Units Full Scale (AUFS) was set at 0.05 AU.

2. Column

The analyses were carried out using a 20 cm x 4.6 mm i.d. column packed with 5 μm, Hypersil ODS (Hewlett-Packard).

3. Eluents

HPLC-grade acetonitrile and methanol were used in the preparation of the mobile phases. Distilled and deionized water (Milli-Q system, Millipore Corp., Bedford, MA) was used to prepare

0.01M NaH_2PO_4 buffer, pH 3.5. The eluents were 0.01M NaH_2PO_4 buffer containing different percentages of methanol or acetonitrile. The solvents were mixed and degassed with stirring for 5 min under a water-aspirator vacuum. Eluent flow rates were between 1.0-1.5 mL/min requiring a pressure of 900-1500 psi.

4. Compounds

Seven aliphatic acids whose octanol-water partition coefficients had been determined in the same laboratory served as reference compounds. The log $P_{o/w}$ of these acids ranged from 0.98 to 3.20. Straight-chain fatty acids obtained commercially, provided a homologous series for estimation of void time. Isobutyric acid, trimethylacetic acid and 1-methylcyclohexane-1-carboxylic acid were purchased from Aldrich Chemical Company, Milwaukee, USA. The other acidic compounds used were synthesized as described in the experimental section.

5. Sample Preparation

Standards of acidic compounds were prepared in either methanol or acetonitrile to give concentrations of saturated fatty acids of 0.2-0.6 µg/µL, unsaturated fatty acids of 0.01-0.05 µg/µL, N,N-dibutylsuccinamic acid of 0.1-0.3 µg/µL, N,N-diethylsuccinamic acid of 0.8-1.2 µg/µL and tetrazoles of 0.02-0.07 µg/µL.

6. Retention Time Measurements

The acidic compounds were studied individually. Sample injection volumes were 20 µL. The retention times were expressed in

terms of log (capacity factors) or log k'. Log k' = log $\left(\frac{t_R - t_o}{t_o}\right)$ where t_R represents the retention time of the compound and t_o, the elution time of unretained peak generated by methanol or acetonitrile. The recorder chart speed was 1.0 cm/min and the flow rate of the eluent was between 1.0 mL/min and 1.5 mL/min. Retention times were measured by the integrator and the average of two replicate injections was used.

7. Column Void Time

While the dead volume (v_o) is independent of flow rate, the column void (or dead) time is dependent on the eluent composition and flow rate. It was therefore determined by injection of a substance that is expected to be unretained, i.e. methanol. The void time was compared to the void time determined by dead time iteration of the retention times of homologous n-alkylcarboxylic acids (181). The equation is expressed by $t_{R,N+1} = \alpha t_{R,N} - t_o(\alpha-1)$. By plotting $t_{R,N+1}$ (retention time of the N+1 = carbon homologue) versus $t_{R,N}$ (retention time of the N-carbon homologue), t_o can be calculated from the slope of the regression line, α (relative retention) and the intercept. Values of t_o and t_R were not corrected for the small time lag between column and detector.

F. Determination of Octanol-Water Partition Coefficients by the Shake-Flask Procedure

The partition coefficient of four compounds, trimethylacetic acid, 5-isoamyltetrazole, 5-cyclohexylmethyltetrazole and N,N-dibutylsuccinamic acid, were determined in 1-octanol-0.1N HCl. For the partitioning studies, 1-octanol saturated with 0.1N HCl and

0.1N HCl saturated with 1-octanol were used.

The amount of compound and volume of the aqueous phase were chosen such that the initial concentrations of compounds in 0.1N HCl saturated with 1-octanol were in the range, 1.0×10^{-3}M to 5.0×10^{-3}M. Trimethylacetic acid and N,N-dibutylsuccinamic were soluble in the volume of aqueous phase used. The weighed amount of the tetrazoles were rendered soluble in the aqueous phase by first dissolving in a small volume of MeOH (2%, v/v) and the calculated volume of 0.1N HCl added. The ratios of the volumes of the aqueous and octanol phase were 2:1 for trimethylacetic acid and 10:1 for the other compounds. The ratios were chosen so that analytical errors can be decreased.

The separatory funnel (50-120 mL capacity) was shaken gently and inverted several times for 15 min. The aqueous phase was collected into centrifuge tubes and centrifuged at 2000 rpm for 20 min. The concentration of the compounds in the aqueous phase after partitioning was determined by HPLC analysis. Calibration curves for concentrations in the range (0.01 mg/mL to 0.6 mg/mL) were obtained for each compound studied. Duplicate injections were made for standard and sample solutions. The concentration of compounds in the aqueous phase was deduced from the calibration curves.

HPLC analyses were conducted with a Hewlett Packard Model 1082B, HPLC. Detection of the acidic compounds was accomplished at 210 nm with a Hitachi Model variable wave length spectrophotometer. The spectrophotometer was set at 0.05 absorbance units full scale. A 20 cm x 4.6 mm i.d. column packed with 5 µm Hypersil ODS (Hewlett-Packard) was used in the analysis. Mobile phase of 70% MeOH : 30% 0.01M NaH_2PO_4 was used for trimethylacetic acid, 5-

isoamyltetrazole and N,N-dibutylsuccinamic acid analyses. Mobile phase of 50% CH_3CN : 50% 0.01M NaH_2PO_4 was used for 5-cyclohexylmethyltetrazole analysis. Eluent flow rate was 1 mL/min and volumes of 20 µL were injected. Quantitation was by peak height or peak area measurements. HPLC analyses were conducted at room temperature.

The partition experiment for each compound was done in quadruplicate. The partition coefficient (p) of a compound was calculated from the relationship.

$$P = \frac{V_{aq}}{V_o}\left(\frac{X^i_{aq}}{X_{aq}} - 1\right)$$

where X^i_{aq} and X_{aq} are the respective amount of compound in the aqueous phase before and after partitioning and V_{aq} and V_o are the volumes of the aqueous and organic phases respectively.

G. Determination of apparent ionization constants (pKå) by potentiometric titration

The pKå values of the acidic compounds were determined by the method of A. Albert and E. Serjeant (185). Potentiometric titration was carried out with an automatic potentiometric titration instrument containing a motor-driven microburet and a recorder (Beckman). The electrode system was a glass electrode in combination with a saturated calomel electrode. Before each titration the instrument was calibrated against potassium hydrogen phthalate (0.05M, pH 4.00) and phosphate buffer (pH 7.00). Solvents were deionized water and HPLC-grade methanol.

The apparent ionization constants were determined in aqueous methanol (10% and 50% MeOH, v/v) by potentiometric titration with standard KOH (standardized with potassium hydrogen phthalate), delivered from a microburet. Samples of compounds were weighed out into beakers and dissolved in the solvent mixture (47.5 mL) such that the mean concentration of acids during the titration (or concentration at half-neutralization) was approximately 0.002M for low molecular weight acids and 0.001M for high molecular weight (>130 Daltons) acids. Values of pKa were calculated at several points in each titration by applying the Henderson-Hasselbalch equation and a mean pKå determined. An average of 6 pKå values was used.

RESULTS AND DISCUSSION

A. Chemistry

1. Alpha-alkylsubstituted aliphatic acids

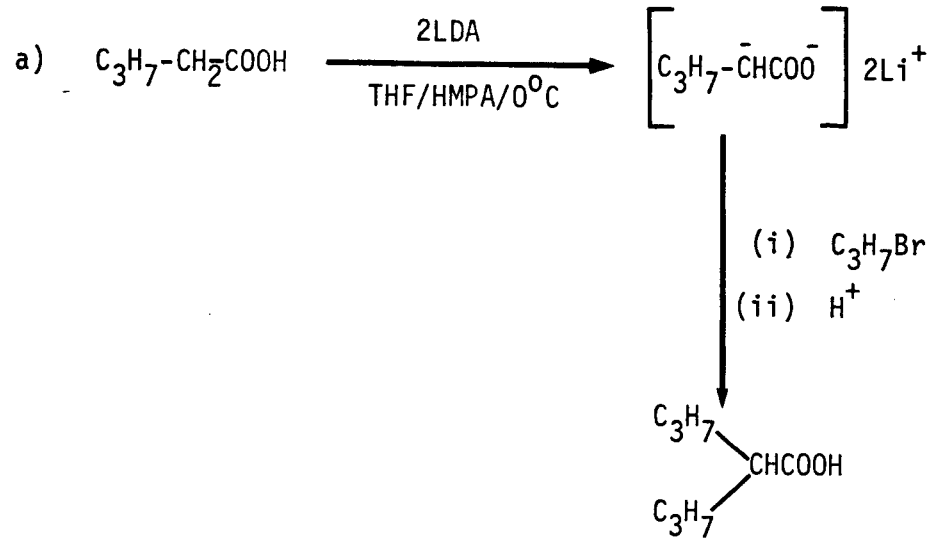
Valproic acid, I, and 2-butylhexanoic acid, II, were prepared according to the method of Pfeffer and co-workers (154). The synthetic sequence is shown in Scheme 1. It is a one-pot synthetic method involving treatment of alkylcarboxylic acids with the strong base, lithium diisopropylamide (LDA) to generate the dianions. Alkylation of the dianions with the appropriate alkyl bromide occurred regiospecifically at the α-anionic site to produce the α-substituted aliphatic acids in good yields.

2. α,α-Dialkylsubstituted aliphatic acids

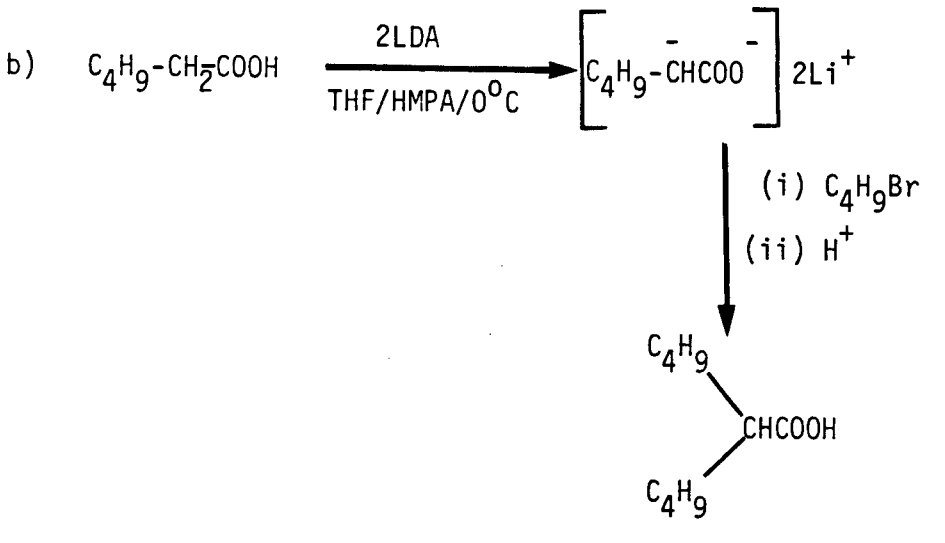
The synthetic procedure of Creger (163) was used to make the α,α-disubstituted acids, 2,2-dimethylvaleric acid (XXV) and 2,2-dimethylbutyric acid (V) (Scheme 2). The reactive centres are usually less accessible with other bases (e.g. NaH) or require multiple reaction steps in other synthetic methods such as the classical malonic ester synthesis.

3. α-Alkylsubstituted aliphatic acids with functionality in the carbon chain

2-Propyl-(E)-2-pentenoic acid was prepared employing standard procedures (Scheme 3). The first step involves bromination of valproic acid, I, in the α-position. The bromo-acid was converted to the ester and dehydrobromination carried out successfully with

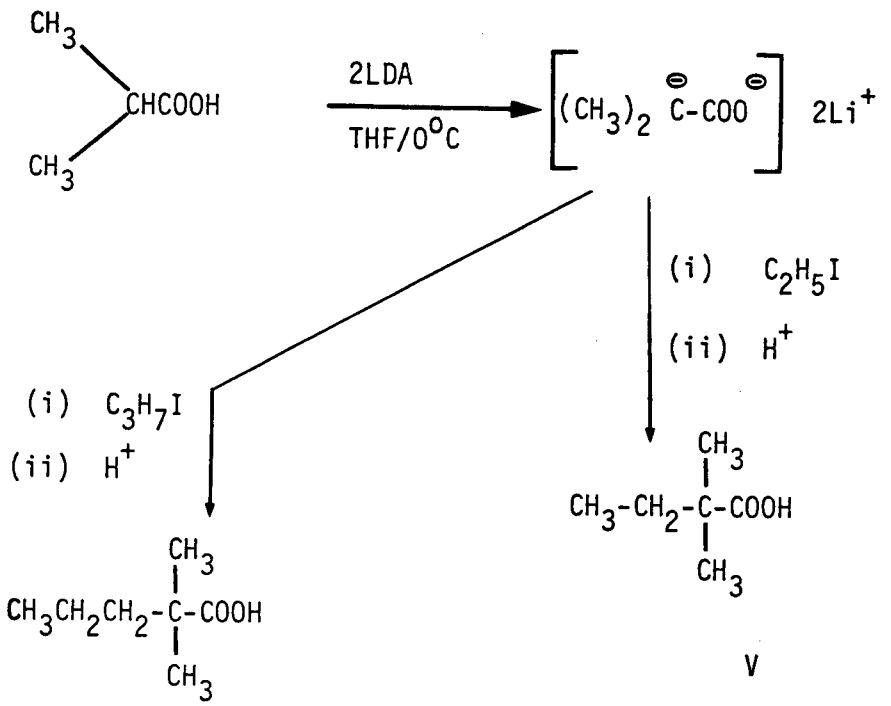


I



II

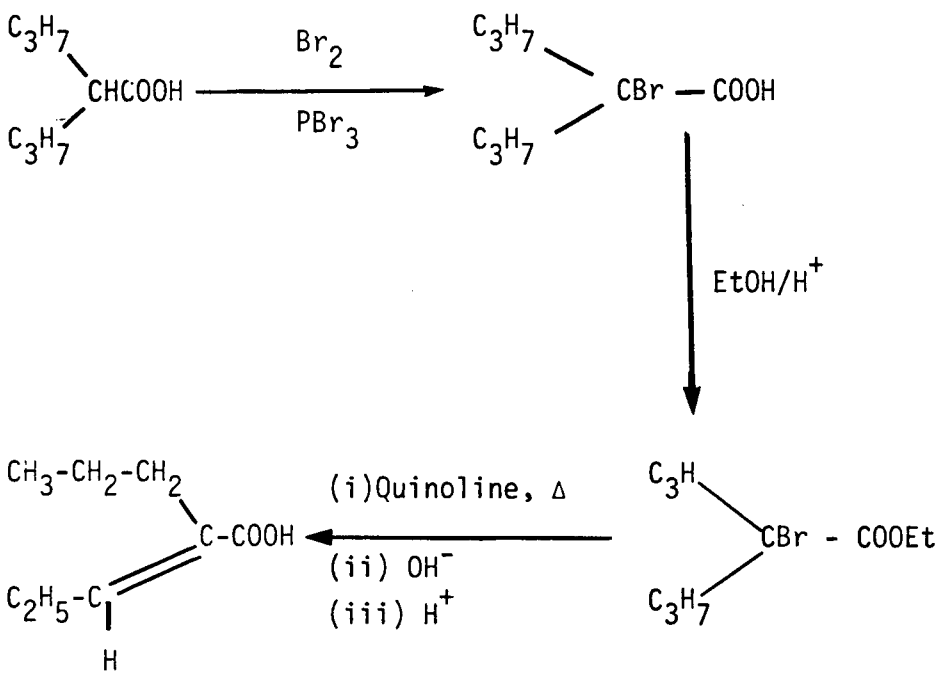
Scheme 1. Synthetic pathway for alpha-substituted aliphatic acids.



XXV

V

Scheme 2. Synthetic route for alpha,alpha-disubstituted aliphatic acids



VII

Scheme 3. Outline for synthesis of 2-propyl-(E)-2-pentenoic acid (VII)

quinoline. The synthetic product which occurs as a mixture of E:Z geometric isomers (~5:1) was purified by fractional crystallization to give the thermodynamically stable E isomer.

The synthesis of 4-keto VPA (Scheme 4), IX, as reported elsewhere (93) involved application of the classical acetoacetic acid synthesis.

4. β-substituted carboxylic acids

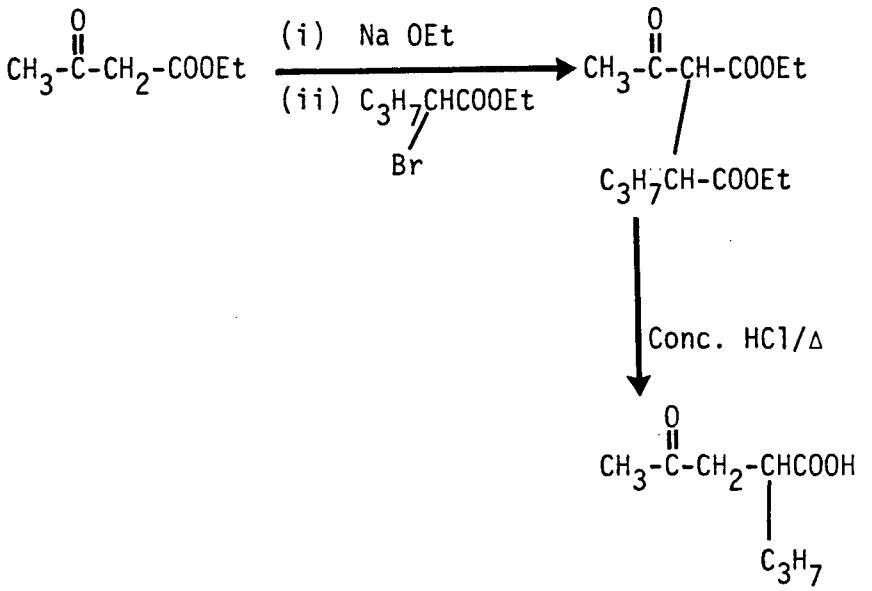
The malonic ester synthesis was employed to synthesize 3-methylvaleric acid and 3-methylhexanoic acid. The familiar pathways of the synthesis involved alkylation of malonate with secondary halides, followed by base and acid hydrolysis to convert the diester to a monoacid through deesterification and decarboxylation.

3-Ethylpentanoic acid VI, and cyclohexylacetic acid XIII, were synthesized using a one-carbon homologation method as outlined in Scheme 5, starting from available chemical reagents.

5. N,N-Dialkylsuccinamic acids

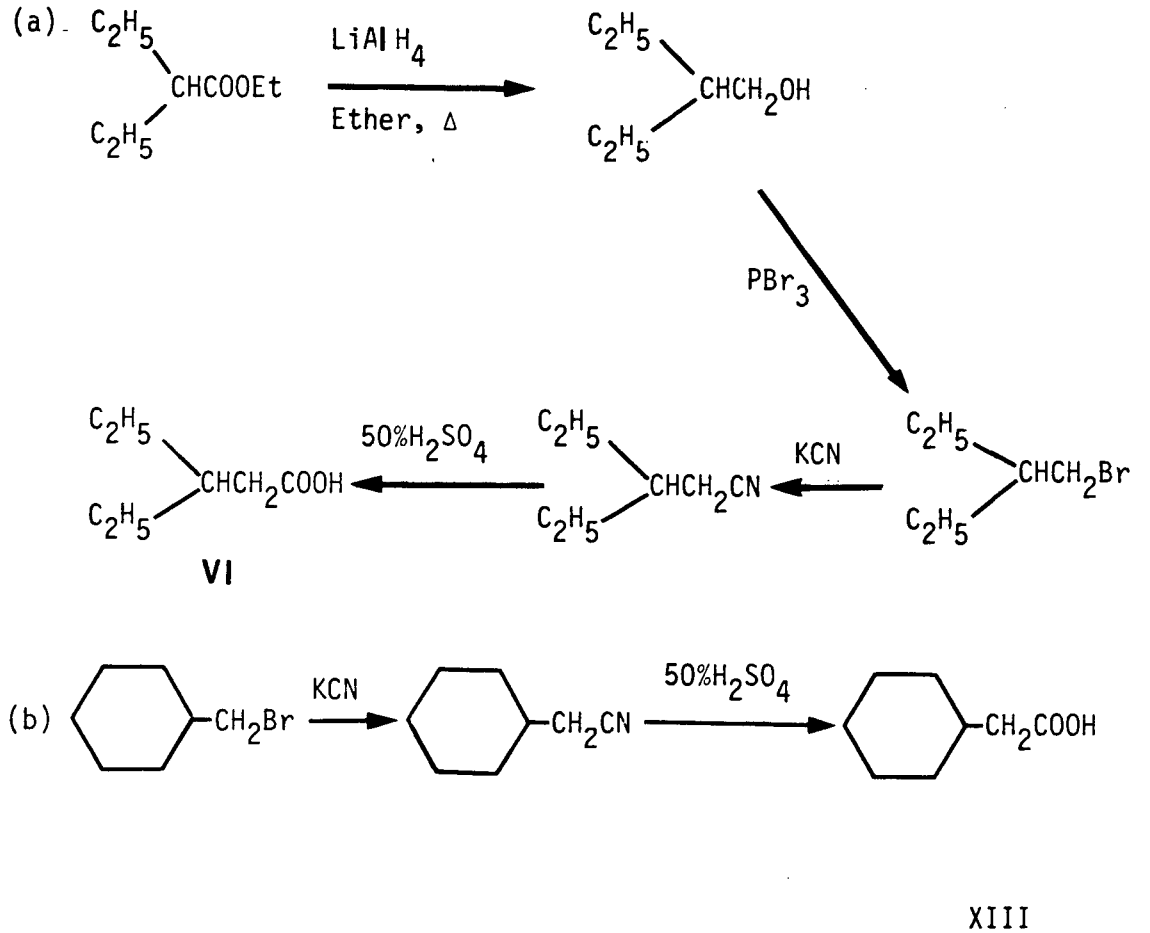
Formation of N,N-diethylsuccinamic acid XV, and N,N-dibutylsuccinamic acid XVI, proceeded readily by reaction of succinic anhydride and the secondary bases (Scheme 6). Various solvents, ether (162), ethanol (164) and benzene have been used for these reactions. In this study, dissolution of succinic anhydride and isolation of crystalline product proceeded more readily with ethanol than benzene.

The IR spectroscopic data of the tertiary succinamic acids are

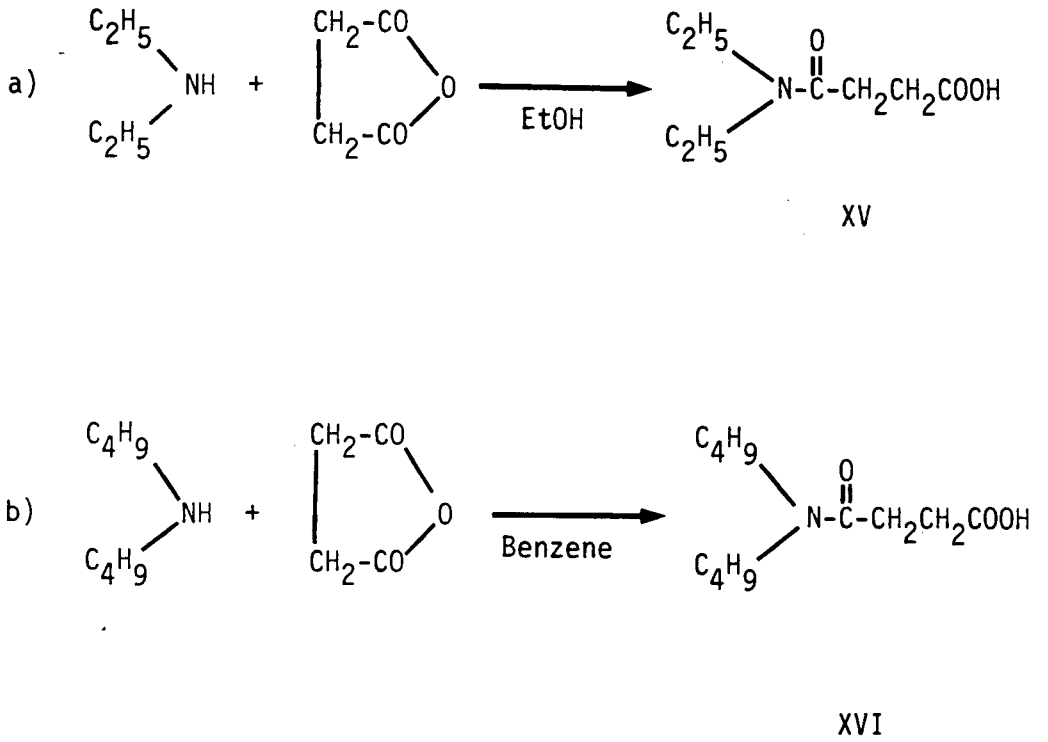


IX

Scheme 4. Synthetic sequence for preparation of 2-propyl-4-oxopentanoic acid (IX)



Scheme 5. Pathways for Synthesis of the beta-substituted Carboxylic acids



Scheme 6. Synthetic route for preparation of the succinamic acids

in agreement with literature data (165). The IR spectra of the succinamic acids show two characteristic bands at 1690-1725 cm^{-1} and 1610-1640 cm^{-1} corresponding to the carboxylic and tertiary amide carbonyl respectively. The NMR spectra (Figure 6) of the succinamic acids show differences between N,N-diethylsuccinamic acid (XV) and N,N-dibutylsuccinamic acid (XVI). Both compounds have two CH_2 units between the amide and carboxylic functional groups. While N,N-dibutylsuccinamic acid gives a singlet at 2.6δ corresponding to four equivalent hydrogens of the two CH_2 units (Figure 6b), N,N-diethylsuccinamic acid spectrum shows a singlet at 2.6δ equivalent to less than 4 protons. The ^{1}H-NMR spectrum of N,N-dibutylsuccinamic acid shows a broad peak at 8.5δ, for the hydrogen-bonded carboxylic proton, equivalent to two protons. In contrast, the ^{1}H-NMR of N,N-diethylsuccinamic acid shows a broad peak at 5.5δ for the hydrogen-bonded carboxylic proton, equivalent to two protons. It appears likely that the amide and carboxylic group interact intramolecularly, especially in N,N-diethylsuccinamic acid. In a study of molecular interactions of N,N-diethylsuccinamic acid and N,N-diisobutylsuccinamic acid by IR spectroscopy, Antonenko (165) reported that, in dilute solutions, the amides probably exist in a cyclic form through interaction of the acidic function with the amide group. Other workers (162) have also suggested the existence of a cyclic structure in N-alkylsuccinamic acid and N,N-dialkylsuccinamic acids.

6. 5-Alkyltetrazoles

The synthesis of 5-isoamyltetrazole (XI) 5-heptyltetrazole (X) and 5-cyclohexylmethyltetrazole (XII) has been reported in the

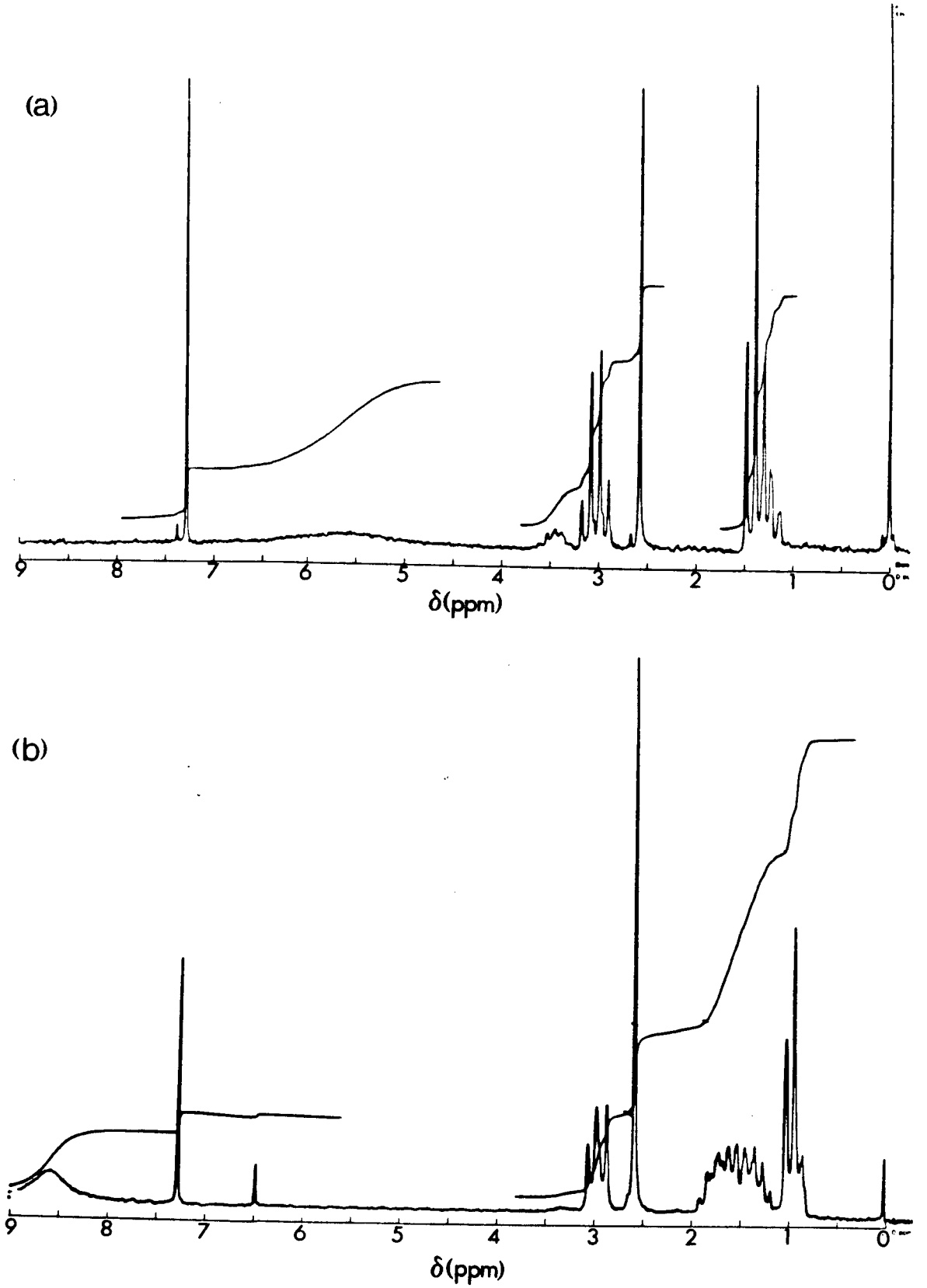


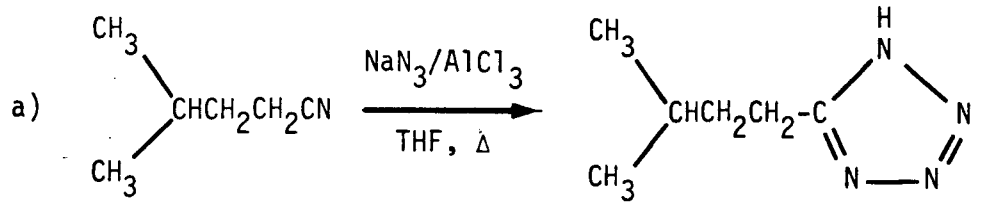
Figure 6. NMR spectra of (a) N,N-diethylsuccinamic acid and (b) N,N-dibutylsuccinamic acid.

literature (161) and involved reaction of alkylcyanide with hydrazoic acid (from NaN_3 and acetic acid) in benzene at 150°C for 96-120 hrs in a sealed tube. The most frequently employed open-reaction method requires reaction of nitriles with ammonium azides in dimethylformamide (166). Attempts to apply this method for the synthesis of the 5-alkyltetrazoles proved unsuccessful. The reaction products were apparently not acidic and possessed different melting points from expected values (161). In addition, ^{1}H-NMR data did not conform to expectations. It is known that electron-releasing alkyl groups attached to the cyano functionality decreases the reactivity of the alkyl nitrile towards hydrazoic acid.

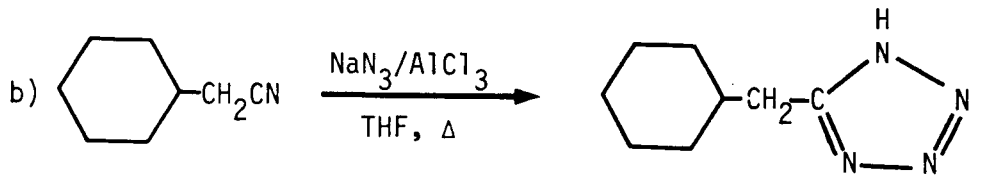
An alternative procedure (167), requiring reaction of alkylnitrile with aluminium azide (prepared in situ from anhydrous aluminium chloride and sodium azide) in tetrahydrofuran (Scheme 7) was successful in producing desired products. The disadvantages associated with this method include the excess of sodium azide used and the presence of inorganic aluminium salt in the crude product. As a result of the known difficulties (161,166) encountered in isolation and purification of the 5-alkyltetrazole, the yields of products were not determined.

7. Diunsaturated derivatives of valproic acid-synthesis and metabolism study

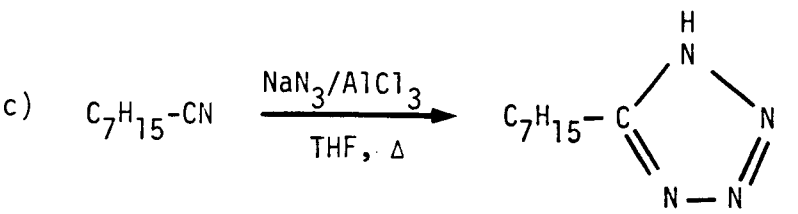
The synthesis of selected analogues of valproic acid have been reported in the literature except for the synthesis of 2-(1'-propenyl)-2-pentenoic acid (VI) and 2-(1'-propenyl)-3-pentenoic acid (XXVI). These two dienoic acids have been previously proposed as possible structures for the major diunsaturated metabolite of



XI

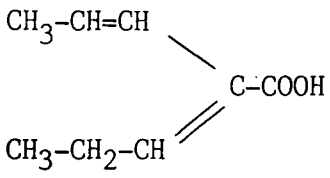


XII

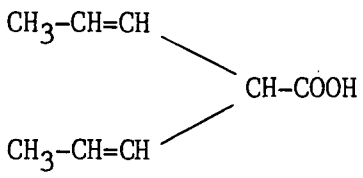


X

Scheme 7. Synthetic pathway for 5-alkyltetrazoles



VI



XXVI

valproic acid (93). There are four geometric isomers of VI and three stereoisomers of XXVI. Due to the multiplicity of the stereoisomers, stereoselective synthetic methods are necessary to characterize these dienoic acid isomers.

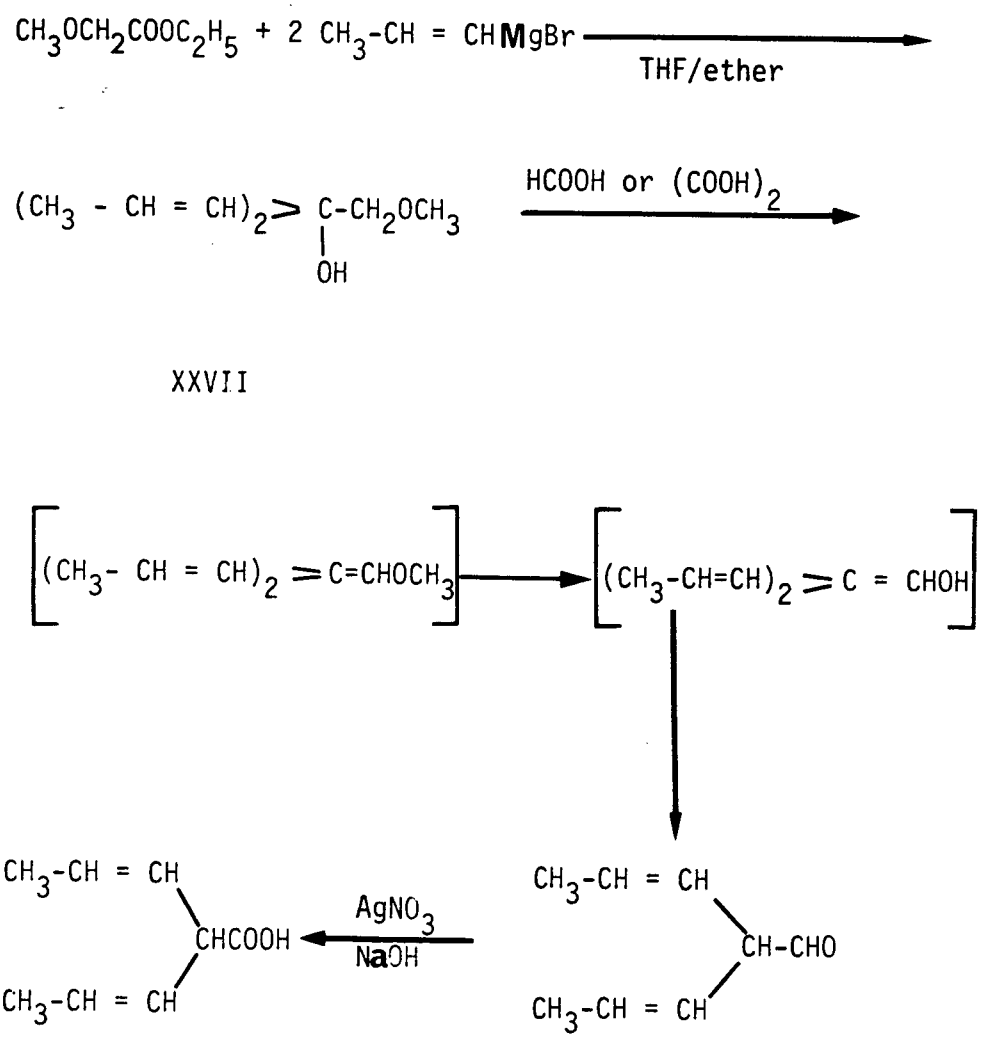
a. Attempted synthesis of 2-(1'-propenyl)-3-pentenoic acid (XXVI)

In attempts to synthesize 2-(1'-propenyl)-3-pentenoic acid, the procedure of Normant (168) was followed. Ethyl α-methoxy-acetate was treated with two equivalents of the Grignard reagent, propenylmagnesium bromide to afford the di-olefin tertiary alcohol, XXVII (Scheme 8) whose structure was established by MS, ^{1}H-NMR and IR spectroscopic data.

Mass spectrum of dienol ether, XXVII: m/z 111 ($M-CH_2OCH_3$, 100%), 43 (35%), 55 (33%), 41 (27%), 77 (22%), 91 (20%), 45 (17%), 123 (14%), 138 (M-HOH, 9%).

NMR spectrum of dienol ether, XXVII: δ1.8 (m, 6H, $2CH_3$-C=), 2.45 (broad peak, 1H, OH), 3.43 (m, 5H, CH_2OCH_3), 5.4-5.8 (m, 4H, 2CH=CH).

However, dehydration of the dienol ether followed by



XXVII

XXVI

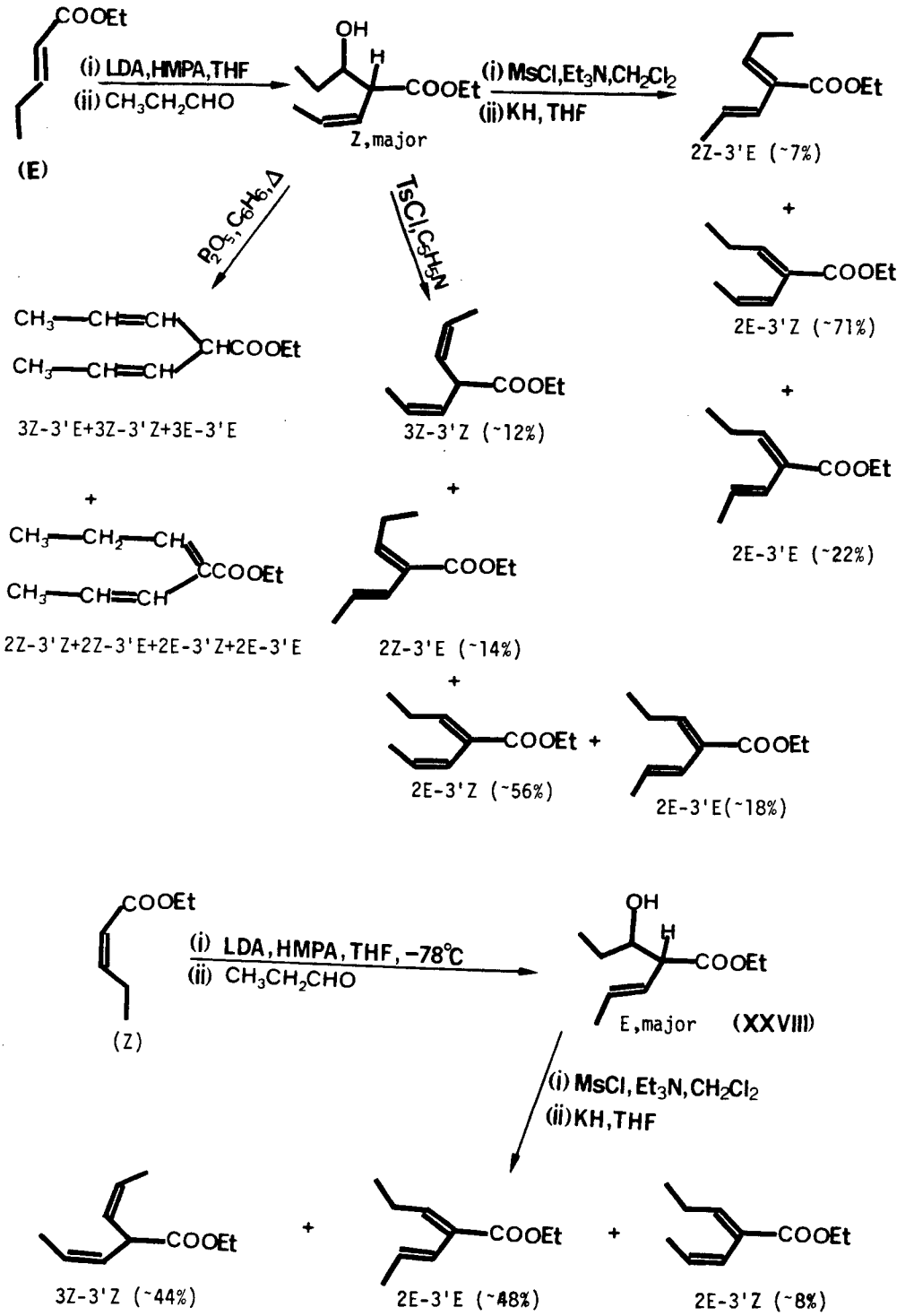
Scheme 8. Outline for synthesis of dienol ether in an attempt to prepare 2-(1'-propenyl)-3-pentenoic acid.

hydrolysis with either anhydrous oxalic acid or formic acid (168) yielded a dark viscous polymeric product. The polymolecular product was presumed to result from polymerization of the diene aldehydes, although this problem was not reported in similar conditions for synthesis of monounsaturated aldehydes (168).

b. Aldol Condensation reactions towards synthesis of 2-(1'-propenyl)-2-pentenoic acid, VI

A recent study by Kochen et al. (124) attempted synthesis of 2-(1'-propenyl)-2-pentenoic acid via nucleophilic acylation of 1-bromopropane with "umpolung" or carbonyl carbanion equivalent of crotonaldehyde, followed by saponification. They reported, however, that the synthetic products consisted of a mixture of diunsaturated acid isomers of unknown stereochemistry.

The synthetic strategic sequence used for the synthesis of 2-(1'-propenyl)-2-pentenoic acid proceeded via an aldol condensation of ethyl 2-pentenoate (E or Z isomer) with propionaldehyde, followed by dehydration of the β-hydroxy-β',γ'-unsaturated ester with dehydration agents of varied stereoselectivity (Scheme 9). NMR and IR spectral analysis demonstrated that under the kinetically-controlled reaction procedure, propionaldehyde adds regioselectively at the α-position of the α,β-unsaturated ester enolates to afford ethyl 2-(1'-hydroxypropyl)-3-pentenoate (XXVIII) as the major product. Addition of aldehydes to enolates of α,β-unsaturated esters is reported to occur at either the γ- or α-carbon of the esters (169-173). The geometric aspects of the aldol condensation reaction have been subjected to several investigations (170-173).



Scheme 9. Stereoselective synthetic routes for preparation of 2-(1'-propenyl)-2-pentenoic acid.

$CH_3 - \overline{C}H - CH = CH - COOEt$ $CH_3 - CH = CH - \overline{C}H - COOEt$

γ-anion α-anion

In the present study, NMR and IR analysis of the aldol condensation products indicated that ethyl 2-(1'-hydroxypropyl)-(Z)-3-pentenoate predominated over the corresponding (E)-isomer when ethyl (E)-2-pentenoate was the starting material (Scheme 9). On the other hand, ethyl 2-(1'-hydroxypropyl)-(E)-3-pentenoate predominated over the (Z)-isomer when ethyl (Z)-2-pentenoate was the starting reagent. In particular, the (Z)-isomer showed a medium intensity IR absorption band at 985 cm^{-1} and a strong band at 690 cm^{-1} corresponding to the Z-configuration at the β,γ-ethylene group in XXVIII. The E-isomer showed a strong IR absorption band at 985 cm^{-1} characteristic of an E-configuration at the β,γ-ethylenic group (see Experimental 5-Ib). These results are in accord with similar findings reported for aldol condensation and alkylation reactions (170,172,173).

The mechanism of the inversion of the geometrical (olefinic) configuration from (Z)-precursor to (E)-product is probably analogous to that proposed by Kende and Toder (170) as shown in Figure 6. The stereochemical course of the reaction has been based on product stereoselectively and to factors stabilizing the incipient intermediates. Kende and Toder (170) have asserted that the base-induced deprotonation of the 2Z-precursor occurs preferably from the conformation XXIXa to afford the more stable carbanion, XXXIa and subsequently the 3E-product. The carbanion

intermediate XXXIIa was considered to be less stable due to 1,3-allylic steric repulsion between R and COOEt (Figure 7). The geometry of the product from the 2E-precursor was considered to arise from the greater stability of the carbanion XXXIb although 1,3-allylic non-bonded interactions are not apparent in XXXIIb. While it seems less ob vious that the carbanion XXXIb is more stable than the carbanion XXXIIb as put forward by the authors (170), the possibility of slight differences in stability between XXXIb and XXXIIb could explain reports (172,173) that the reaction with 2Z-precursor is more stereoselective than that with 2E-precursor. Moreover, this study showed the presence of 3E-product together with the major 3Z-product using ethyl (E)-2-pentenoate as starting material (see IR spectral data in Experimental 6-Ia).

c. Dehydration of β-hydroxyunsaturated esters (XXVIII)

Various dehydration agents were used for dehydration of the β-hydroxyunsaturated ester formed from the aldol condensation reaction. Phosphorus pentoxide dehydration of ethyl 2-(1'-hydroxy-propyl)-3-pentenoate, derived from ethyl (E)-2-pentenoate, gave the seven possible isomeric acids of 2,3'-diene VPA (VI) and 3,3'-diene VPA, XXVI (Scheme 9). Figure 8a shows the total ion chromatogram of the t-BDMS esters of the dienoic acid isomeric mixture after conversion of the ethyl esters to the dienoic acids.

Dehydration of ethyl 2-(1'-hydroxypropyl)-3-pentenoate, derived from ethyl (E)-2-pentenoate, with p-toluenesulfonyl chloride afforded four diunsaturated isomeric esters (Scheme 9). Figure 9a shows the mass chromatogram of the $[M-57]^+$ ion of the t-BDMS esters of the four diene VPA isomers in the synthetic product mixture.

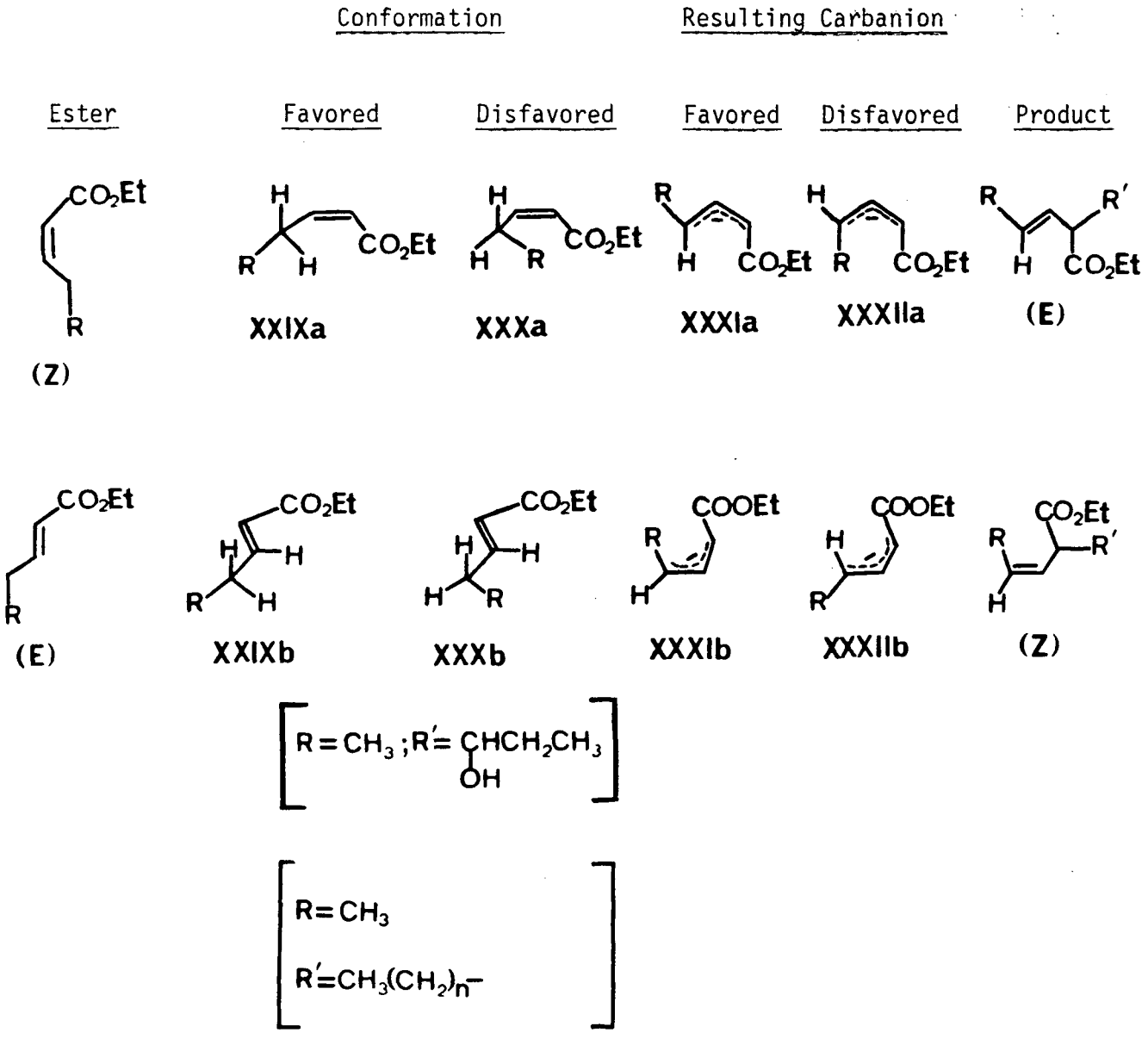


Figure 7. Stereoisomers in alkylation and aldol reactions of ester enolates.

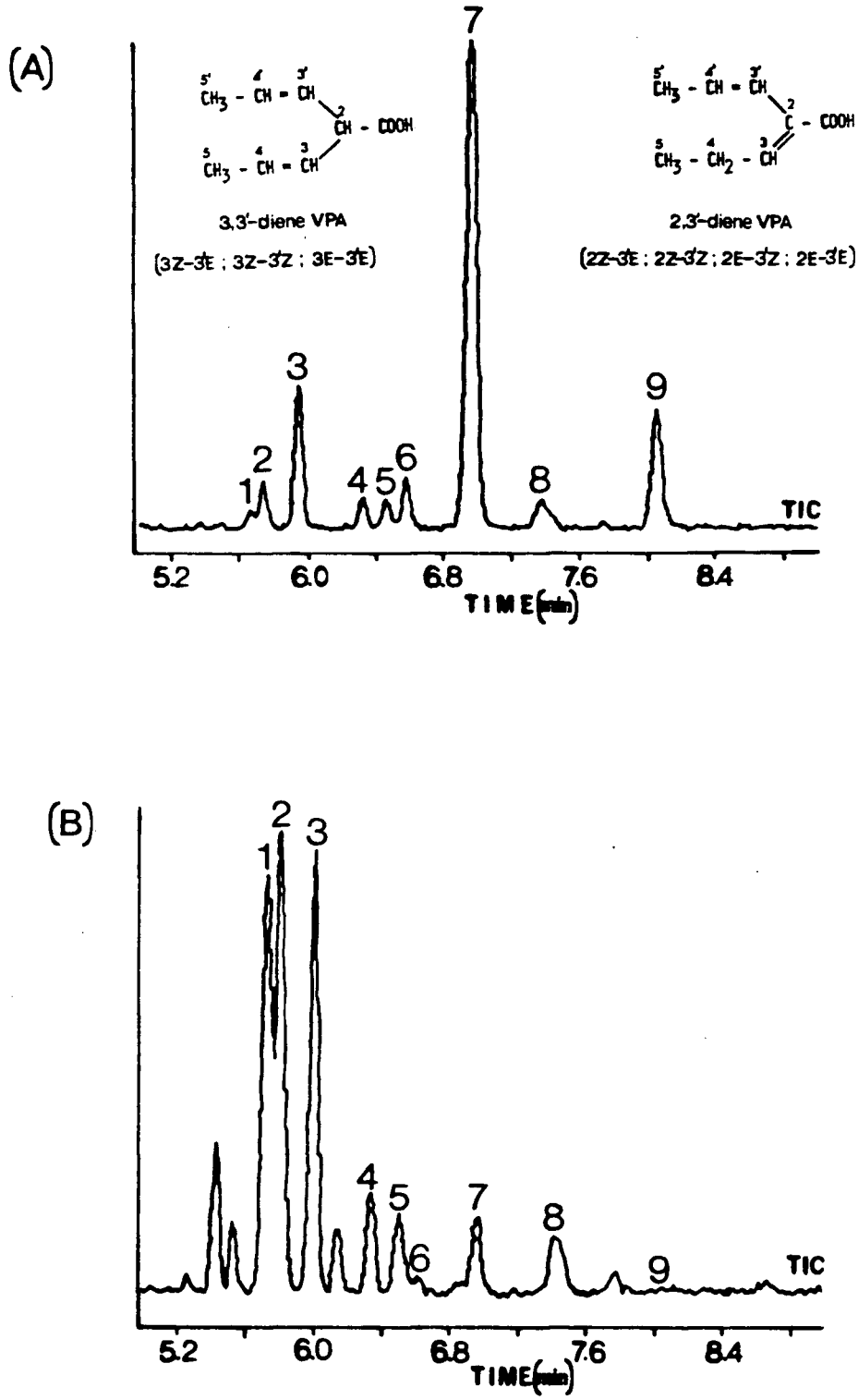


Figure 8. Capillary GCMS separation of t-BDMS esters of isomeric diene-VPA. a) before UV irradiation, b) after 6 hr UV irradiation. Peak numbers correspond to 1: (Z,E)-3,3'-diene; 2: (Z,E)-3,3'-diene; 3: (Z,Z)-3,3'-diene; 4: (E,E)-3,3'-diene; 5: (Z,E)-2,3'-diene; 6: (E)-2-ene VPA; 7: (E,Z)-2,3'-diene; 8: (Z,Z)-2,3'-diene; 9: (E,E)-2,3'-diene.

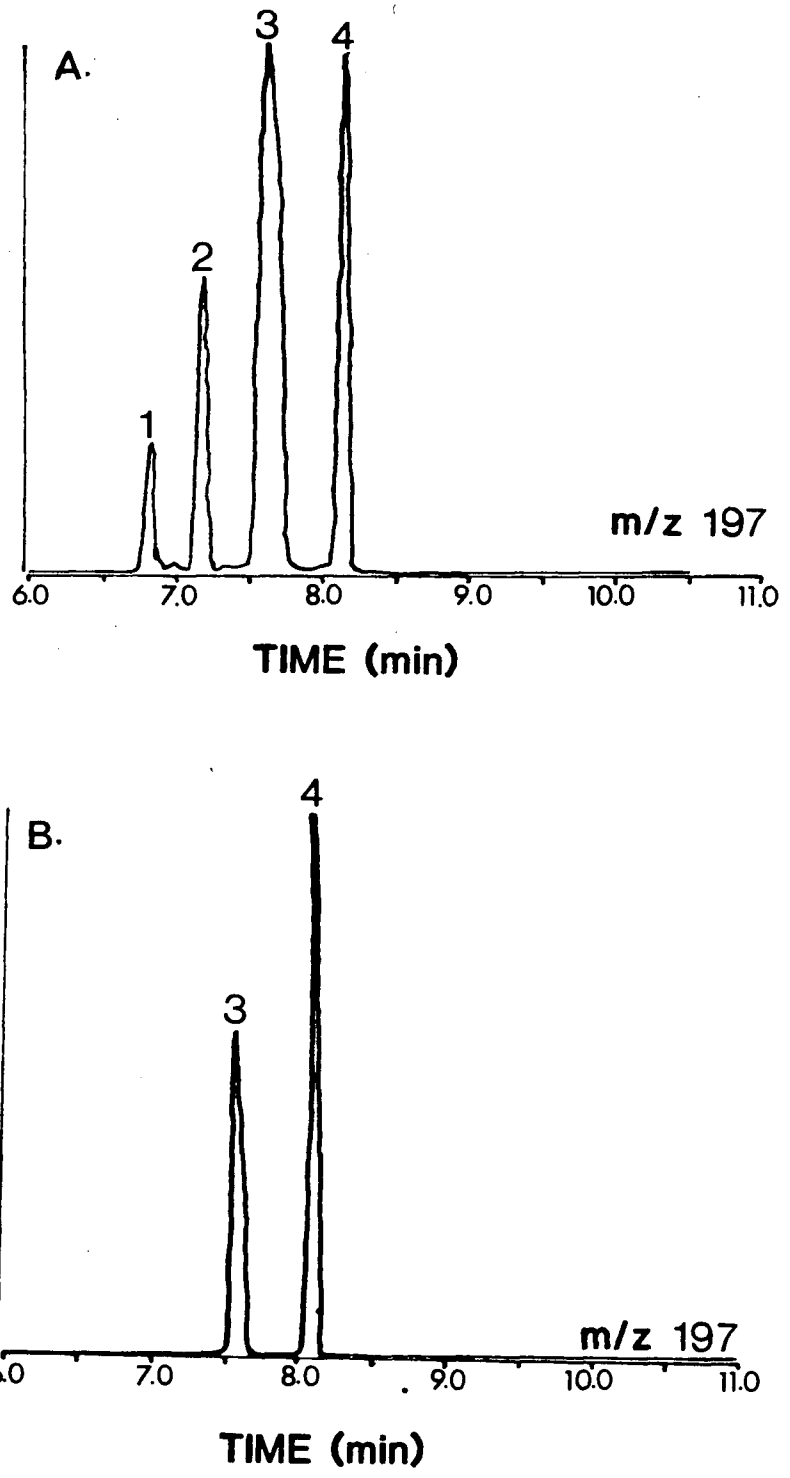


Figure 9. Mass chromatograms of t-BDMS esters of a) four diene-VPA isomeric mixture prepared using p-TsCl, b) diene-VPA metabolites in urine extract. Peak 1: (Z,Z)-3,3'-diene; 2: (Z,E)-2,3'-diene; 3: (E,Z)-2,3'-diene; 4: (E,E)-2,3'-diene.

The combination of methanesulfonyl chloride and potassium hydride in the dehydration of the β-hydroxyunsaturated esters gave the minimum number of isomeric dienoic acids (Scheme 9). These results, along with the studies of Kende and Toder (170), demonstrate the highly stereoselective nature of CH_3SO_2Cl-KH in the dehydration reactions.

d. Photochemical Isomerization

Photochemical isomerization of the unsaturated acids provided an indirect method of determining the positional isomers and their capillary GC retention times. Cis and trans α,β-unsaturated acids have been reported to isomerize photochemically to cis and/or trans β,γ-unsaturated acids (174,175). α,β-Unsaturated acid esters usually possess a strong absorption band in the 220-250 nm region compared to less strong absorption of their isomeric β,γ-unsaturated compounds. UV irradiation of the seven isomeric acid mixture (from dehydration of β-hydroxyunsaturated esters with P_2O_5) resulted in a striking build-up of four peaks (peaks 1, 2, 3, 4 in Fig. 7) which were assigned to β,γ-β',γ'-diunsaturated acids (VII). Peaks 1 and 2 can be described as diastereoisomers of 3Z-3'E-diene VPA or one of the peaks may be due to a diene VPA with a terminal double bond. The peaks whose height decreased with UV irradiation (peaks 7, 9 in Fig. 8) were described as trans α,β-diunsaturated acids. The peaks whose height did not show any significant change (peaks 5, 8) were described as cis α,β-diunsaturated esters since under the conditions of photoisomerization they can be formed from the corresponding trans α,β-isomers and in turn isomerize to the β,γ-isomers (174,175).

e. GC Elution Order in GCMS Analysis

Additional support for a tentative assignment of stereochemical configuration of the dienoic acid peaks in Figure 8 was obtained from the GC retention data. Shorter retention times for the cis-isomer of a given position compared to the trans-isomer on non-polar columns have been frequently observed (176,177). The two peaks before peak 1 in Figure 8b, which were confirmed using authentic samples to be 3-ene VPA and cis 2-ene VPA, were produced by photochemical isomerization of trans 2-ene VPA (peak 6) added as an internal standard.

With a tentative description of the GC elution order (12.5m dimethylsilicone column) of the seven isomeric acids, peaks 3 and 4 in Figure 9a were due to trans α,β-β,γ'-diunsaturated acids. This was supported by NMR spectra of the four diene VPA isomeric mixture obtained from dehydration of the β-hydroxyunsaturated ester with p-toluenesulfonyl chloride. The intensity of the ^{1}H-NMR triplets at δ6.83 and δ7.0 (see Experimental IIb) due to the trans β-vinyl proton indicated the high proportion of 2E-3'Z and 2E-3'E isomers in the mixture (Scheme 9).

f. Argentation TLC

The synthesized isomeric mixtures of dienoates obtained from dehydration of the major products, ethyl 2-(1'-hydroxypropyl)-(Z)-3-pentenoate and ethyl 2-(1'-hydroxypropyl)-(E)-3-pentenoate with CH_3SO_2Cl-KH were subjected to purification by preparative argentation thin layer chromatography. The objective was to

isolate the isomers from the bands on the TLC plates, characterize the NMR spectral data for each isomer and confirm stereochemical designation of the isomeric dienoates and dienoic acids.

Table 1 shows the identity, proportion and R_f values of the isomers in the three bands, following argentation TLC. The proportion of isomers in the mixture of dienoates before and after argentation TLC was determined by NMR analysis. Figure 10 shows the profile of components in the three bands following argentation TLC and GCMS analysis of the ethyl esters on a Dexsil 300 packed column. The order of elution of isomeric dienoates on the Dexsil 300 column and the mobility of the isomers on the TLC plate (Table 1) agree with the chromatographic properties of the stereoisomers. Trans-trans diunsaturated esters are reported to be less polar (high R_f) than their corresponding cis-cis, trans-cis or cis-trans isomers (177-178). Moreover conjugated dienoates have been reported to have higher R_f values than their non-conjugated congeners using similar eluents (181,182).

Mass spectra of the four dienoic acid ethyl esters show the intense M^+, m/z 168 of the 2,3'-dienes compared to the 3,3'-dienes (Table 1) indicating the effect of conjugation in 2,3'-dienes. Diene ethyl esters eluted from the TLC bands could be isolated either as a single isomer or a mixture of isomers (Figure 10). Purified synthesized products were characterized by NMR spectroscopy and Table 2 summarizes the NMR data for the dienoate isomers. The chemical shifts assigned the olefinic protons, β-vinyl proton, methylene and methyl protons adjacent to the double bands are characteristic of the stereochemical features of the dienoate

Table 1

Composition of chromatographic data of a mixture of synthesized isomeric dienoates

Diene VPA Ethyl Ester	Product-ratio with reactant, ethyl 2-pentenoate[a] E	Z	TLC[b] R_f	t_R[c] (min)	Mass spectrum (70ev) m/z (rel. intensity)
3Z-3'Z	-	0.44	0.42-0.49 (Band Ia)	9.37	95 (100%), 168 (7%), 139 (2%), 122(1%)
2Z-3'E	0.07	-	0.53-0.62 (Band IIIb)	10.0	95 (100%), 168 (50%), 140 (41%), 122 (24%)
2E-3'Z	0.71	0.08	0.45-0.53 (Band IIb)	10.5	95 (100%), 168 (57%), 140 (41%), 122 (28%), 153 (4%)
2E-3'E	0.22	0.48	0.53-0.62 (Band IIIa, b)	11.1	95 (100%), 168 (54%), 140 (35%), 122 (32%), 153 (4%)

a ratio of isomers in synthetic product mixture before TLC using either ethyl (Z) or (E)-2-pentenoate as reactant.

b band in which isomer is concentrated.

c GCMS retention time of isomeric peaks analyzed with 3% Dexsil 300 column (1.8m x 2 mm i.d.) Helium, 25 ml/min. Column temp of 50°C to 280°C at rate of 8°C/min.

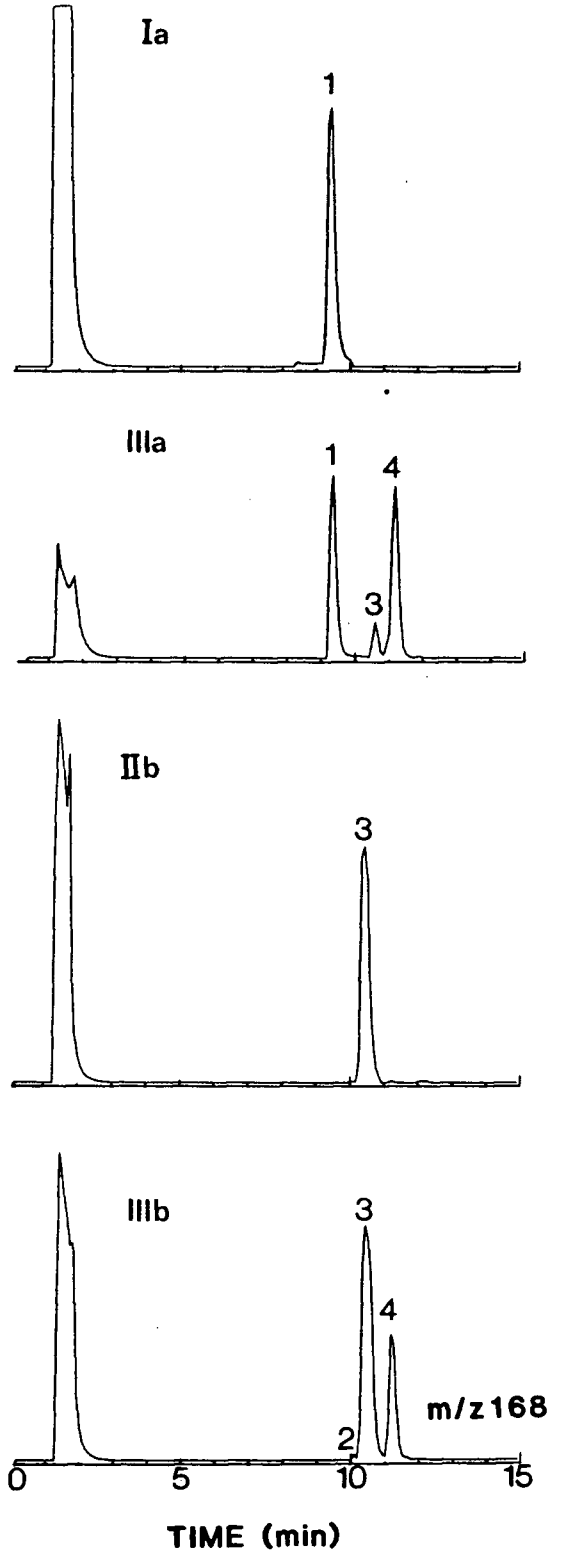


Figure 10. GCMS analysis of dienoates eluted from TLC plates. Total ion chromatograms I, II, III correspond to ethyl esters in bands I, II, III respectively. a and b refer to esters synthesized from (Z) and (E)-2-pentenoate respectively. Peak 1: (Z,Z)-3,3'-diene; 2: (Z,E)-2,3'-diene; 3: (E,Z)-2,3'-diene; 4: (E,E)-2,3'-diene.

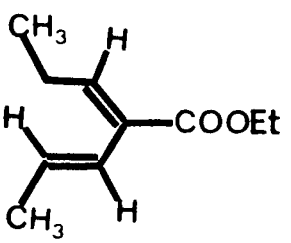
Table 2

NMR (400 MHz) data for diene VPA ethyl esters

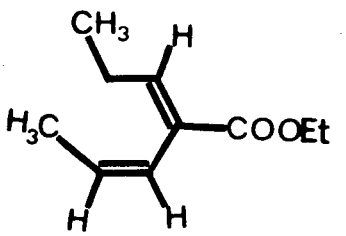
Dienote	CH_3	CH_3-C=	CH_2	CH	H(3')[a]	H(4')[a]	H(3)[a]
2E-3'E	1.04(t)	1.84(d)	2.31(m)		6.17(d) J=16Hz	6.08(dq) J=16Hz	6.59(t)
2E-3'Z	1.04(t)	1.54(dd)	2.11(m)		6.01(d) J=11.4Hz	5.79(dq) J=11.4Hz	6.79(t)
2Z-3'E	1.04(t)	1.70(d)	2.44(m)		b	b	5.92(t)
3Z-3'Z		1.66(dd) 1.69(d)		3.5(t)	5.5-5.6 (m)	5.6-5.7 (dq)	

[a]Position of hydrogen in the branched-carboxylic acid ester (Scheme 9).

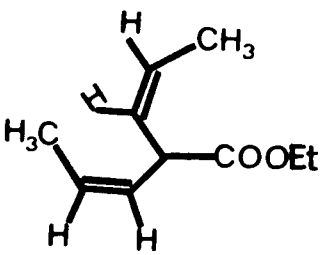
[b]Resonance peaks were very weak due to small amounts of isomer obtained.



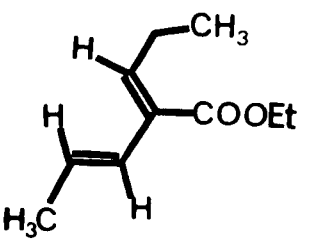
2E - 3'E



2E - 3'Z



3Z - 3'Z



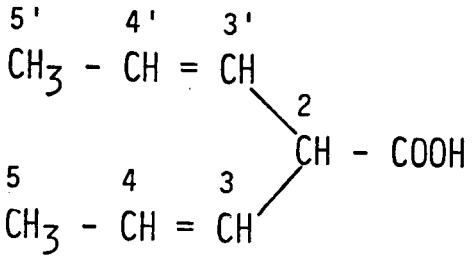
2Z - 3'E

isomers and agree with literature precedence (170,179,180). The structures of the 2,3'-dienes and 3,3'-dienes are shown in Scheme 9. The stereochemistry of the isomers is clarified by the observation that the 2E-3'Z and the 3Z-3'Z isomers have the less deshielded methyl (CH_3-C=) doublets while the 2E-3'E and 2Z-3'E isomers have the more deshielded methyl (CH_3-C=) doublets. Similarly the 2E-3'Z has the least deshielded methylene (CH_2-C=) multiplets in comparison with those of 2E-3'E and 2Z-3'E. The 2E-3'E and 2E-3'Z have the more deshielded β-vinyl proton occurring as a triplet at δ6.59 and δ6.79 respectively. 2E-3'E and 2E-3'Z can also be differentiated by the coupling constants obtained for the coupling of the olefinic protons, H(3') and H(4') in Table 2.

g. Identification of the Major and Minor Diene VPA Metabolites

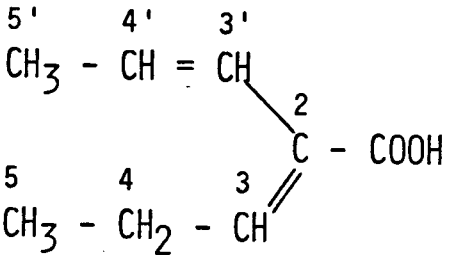
The identification of one of the minor diene VPA metabolites has been reported to be 2-propyl-(E)-2,4-pentadienoic acid (124,125). In this study, the t-BDMS derivatives of 2E-3'Z-diene VPA and 2E-3'E diene VPA were found to have identical retention times with the minor and major diene VPA metabolites respectively in human urine (Figures 8 and 9). With the acquisition of a synthetic sample of 2-propyl-(E)-2,4-pentadienoic acid, it was included in the GCMS analysis of synthetic dienoic acid derivatives and urine metabolites.

Separation of the t-BDMS and TMS derivatives was accomplished using a 25m long SE-54 and OV-1701 columns. Figure 11 shows the structures of the diunsaturated derivatives of valproic acid which were considered in the identification of the diunsaturated metabolites. The retention times of the t-BDMS derivatives of 2-propyl-



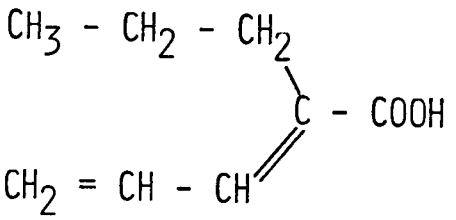
3,3'-DIENE VPA

(3Z-3'E ; 3Z-3'Z ; 3E-3'E)



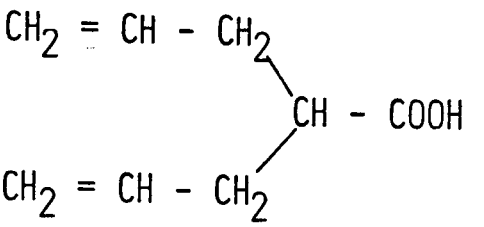
2,3'-DIENE VPA

(2Z-3'E ; 2Z-3'Z ; 2E-3'Z ; 2E-3'E)



2,4-DIENE VPA

(2E ; 2Z)



4,4'-DIENE VPA

Figure 11. Chemical structures of diunsaturated derivatives of valproic acid investigated as the potential metabolites of valproic acid.

(E)-2,4-pentadienoic acid and 2-[(Z)-1'-propenyl]-(E)-2-pentenoic acid as well as the minor diene VPA metabolite were all identical. To resolve the problem of which of the two dienoic acids can be ascribed to the minor metabolite, TMS derivatives were analyzed by capillary GCMS. Separation of the TMS derivatives of the dienoic acids was accomplished (Figure 12) and indicated that one of the two minor diene VPA metabolites and 2-propyl-(E)-2,4-pentadienoic acid had identical retention times which was different from that of 2-[(Z)-1'-propenyl]-(E)-2-pentenoic acid (peak 3 in Figure 12a). The major diene VPA metabolite (peak 5 in Figure 12b) had the same retention time as 2-[(E)-1'-propenyl]-(E)-2-pentenoic acid. This confirmed earlier results obtained with the t-BDMS derivatives of dienoic acids in a synthesized product sample and in a urine extract (Figures 8, 9). The small peak in Figure 12b had a relative retention time similar to that of 2-[(E)-1-propenyl]-(Z)-2-pentenoic acid (peak 4 in Figure 12a), a by-product in the synthesis of 2-[(Z)-1'-propenyl]-(E)-2-pentenoic acid. This diene has been tentatively suggested to be another diene VPA metabolite.

On examination of the stereochemical outcome of the aldol condensation and subsequent dehydration reactions, it is clear that there is an inversion of the geometric configuration in transforming the αβ-unsaturated ester to the β'γ'-unsaturated β-hydroxy ester while the 2E-isomer is favoured over the 2Z-isomer in the dehydration reactions with the more selective dehydration agents. However, the presence of significant amounts of the 3Z-3'Z-diene VPA in the dehydration of 2-[-1'-hydroxypropyl]-(E)-3-pentenoate (XXVIII) is in contrast to the established direction of the

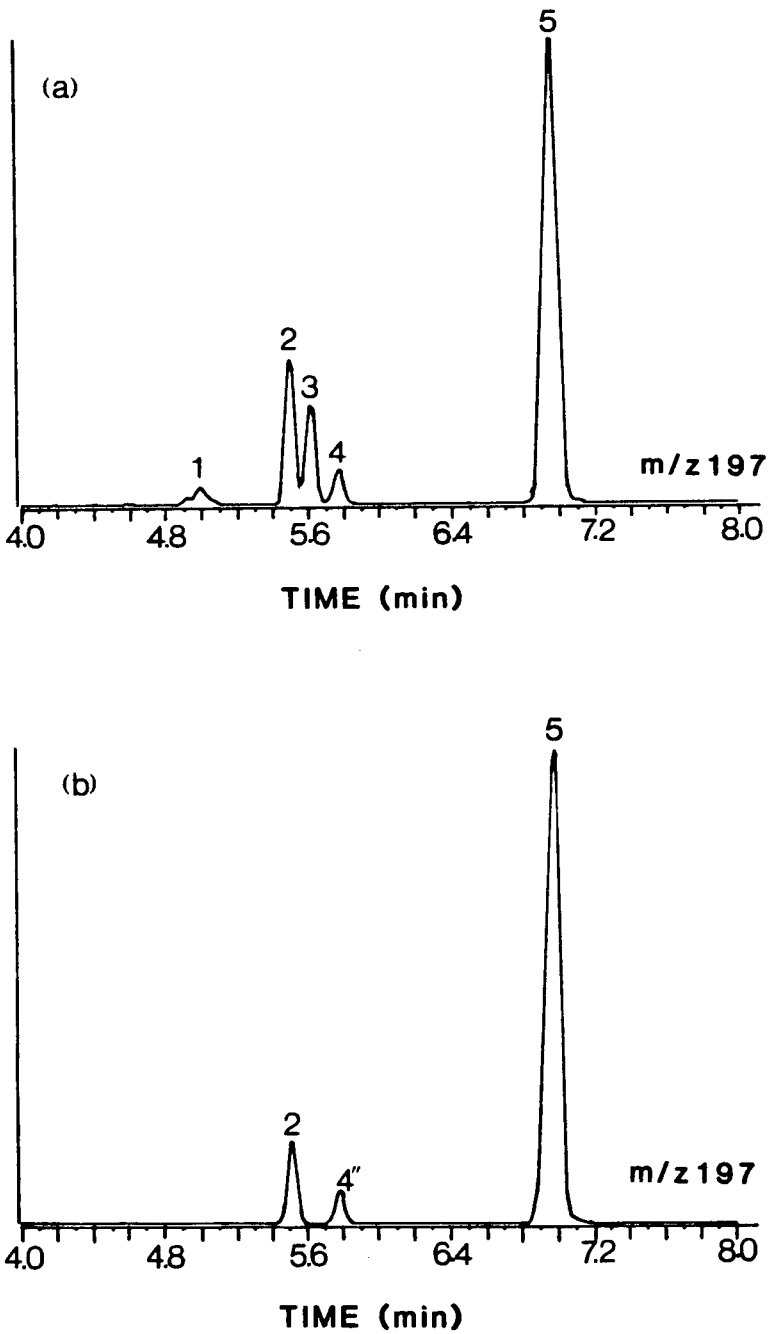


Figure 12. Capillary GCMS separation of TMS derivatives of (a) synthesized 2,3'-diene VPA and 2,4-diene VPA; (b) diene VPA metabolites in urine extract. Peak 1: (Z)-2,4-diene VPA; 2: (E)-2,4-diene VPA; 3: (E,Z)-2,3'-diene VPA; 4: (Z,E)-2,3'-diene VPA; 5: (E,E)-2,3'-diene VPA.

dehydration reaction of 2-[-1'-hydroxypropyl]-(Z)-3-pentenoate. This unexpected by-product could arise from deconjugation and inversion of geometric configuration during elimination of mesylate with KH.

It is evident from the ^{1}H-NMR spectra of synthesized dienoates that the chemical shifts are consistent with the stereochemical assignment of the 2,3'-diene VPA isomers. This was not the case with the 3,3'-diene VPA isomers. The geometry about the double bond could not be determined from the NMR pattern alone except when combined with expected GC elution order (Figures 8 and 9) and photoisomerization results (Figure 8).

B. HPLC determination of lipophilicity

1. Assay method

The RP-HPLC procedure was developed systematically by isocratic chromatographic analysis of reference compounds under different conditions. Detection of the compounds was accomplished by UV absorption. The selection of the wave length of 210 nm was based on the absorption spectra of those compounds which show maximum absorption in the range of 205-215 nm (Figure 13). Methanol and acetonitrile were chosen as the organic co-solvents, in anticipation of selectivity differences between these two solvents and their individual effects on the octanol-water partition coefficient values as determined by RP-HPLC.

RP-HPLC is generally considered to be a combination of partition (dynamic equilibrium conditions) and adsorptive (non-equilibrium conditions) processes. Minimization of the adsorption

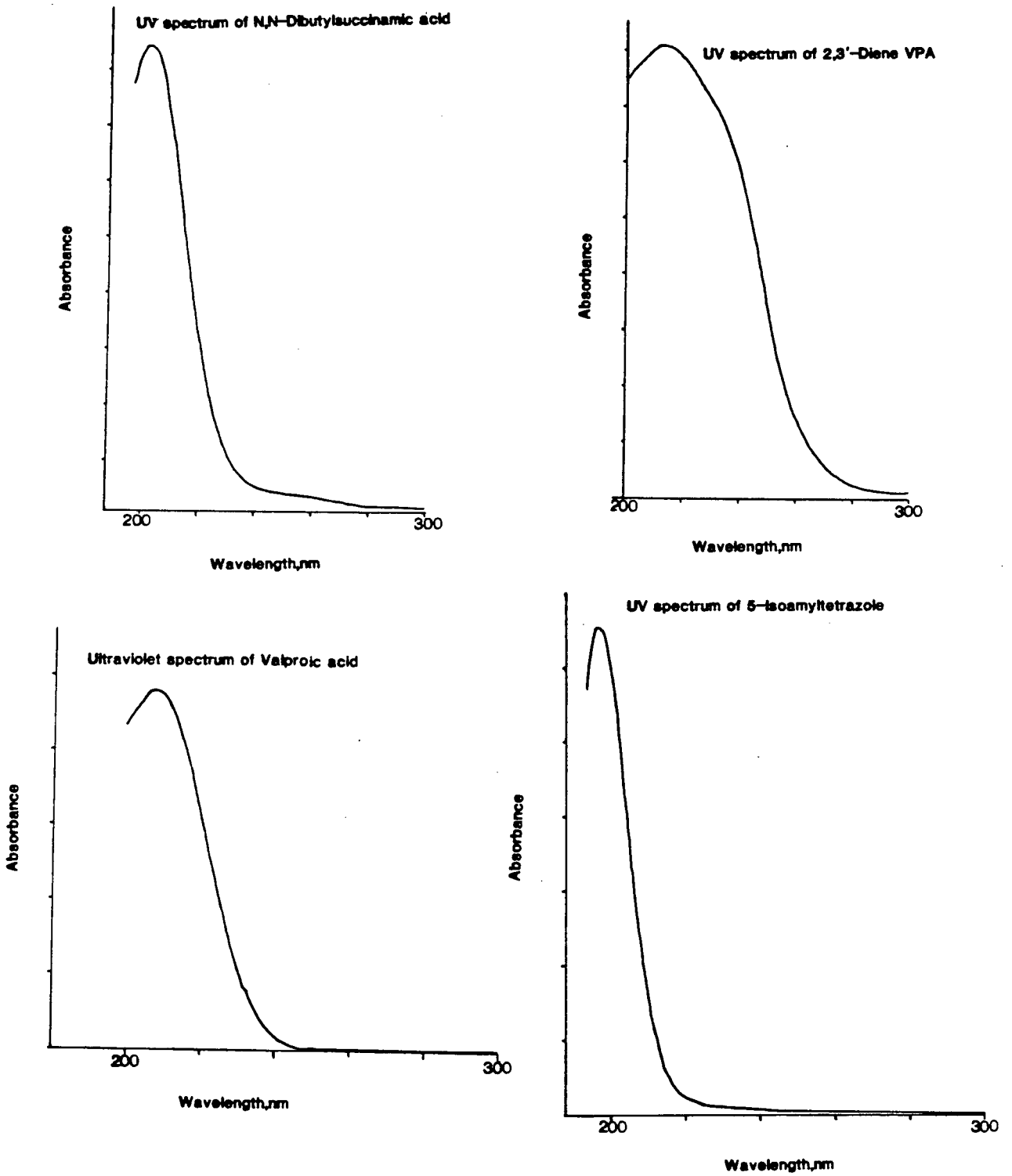


Figure 13. UV absorption spectra of four acidic compounds.

of compounds on residual silanol sites ensures hydrophobic interactions as the sole process in retention of compounds evidenced by the correlation between retention factors and log P. The seven reference compounds selected for the correlation of retention factors and log P are valproic acid and several analogues. The log P values for these compounds have been determined from the shake-flask procedure by Keane et al. (13).

d'Amboise and Hanai (145) used unbuffered mobile phases to study hydrophobic effects of medium chain and long chain normal aliphatic acids in RP-HPLC. In this study, the retention times (t_R) of the polar and ionizable valproic acid analogues were very close using a low percentage of organic modifier in unbuffered mobile phase (Table 3). Figure 14 shows asymmetric peak shapes or even double peaks for the ionizable compounds using the Hypersil ODS column and the unbuffered acetonitrile-water mobile phase. Reducation of solute adsorption with a higher percentage of organic co-solvent in unbuffered water (higher eluting strength) caused them to elute closely to each other and to the unretained solvent (Table 4).

Sodium phosphate buffer (pH 3.5) was used to suppress formation of ionized forms which interacted strongly with the ODS stationary phase. By selecting the appropriate flow rates and varying the composition of the methanol-buffer and acetonitrile-buffer mobile phases, the retention times of the reference compounds were determined (Tables 5-7). Longer retention times and broad, tailing peaks were observed for the highly lipophilic compound 2-butylhexanoic acid in methanol-buffer compositions below

Table 3

Retention times of reference compounds using unbuffered mobile phase (CH_3CN/H_2O)

Compounds	Retention Time (min) % CH_3CN (v/v)		
	20%	22%	25%
MeOH	1.68	-	2.23
1. Butyric acid	1.66	-	2.03
2. Valeric acid	1.76	1.72	2.09
3. 2-Ethylbutyric acid	1.87	1.79	2.16
4. Hexanoic acid	1.99*	1.94	2.38
5. Valproic acid	2.87*	2.35	-
6. 2-Ethylhexanoic acid	-	2.42	-
7. 2-Butylhexanoic acid	3.55*	-	-

*Broad peak

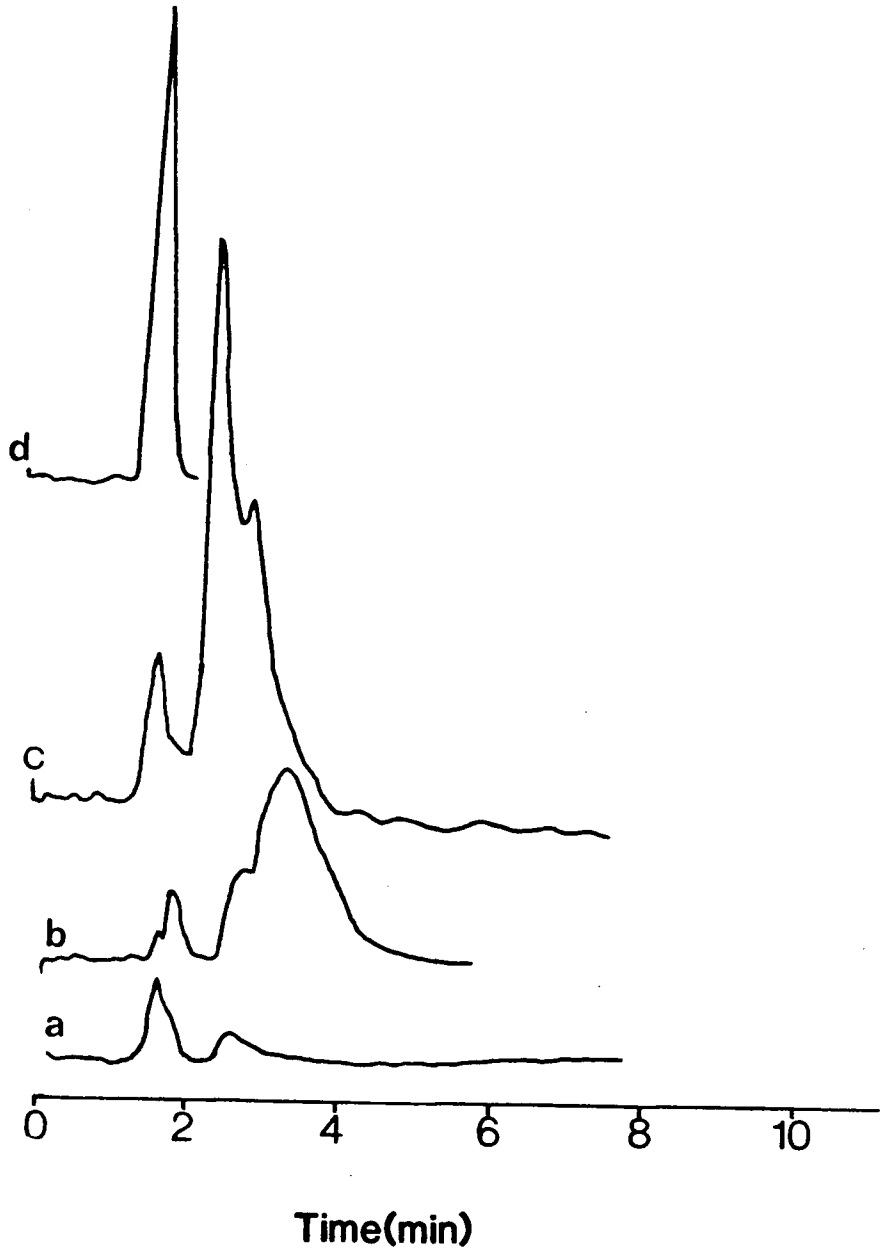


Figure 14. Superimposed HPLC chromatograms of acidic compounds using unbuffered mobile phase. Column; Hypersil ODS reverse phase column (20cm x 4.6mm). Mobile phase; 20% acetonitrile in water (v/v). Flow rate, 1.0 mL/min. a) solvent, b) valproic acid in solvent, c) heptanoic acid in solvent, d) valeric acid in solvent.

Table 4

Effect of addition of phosphate buffer (pH 3.5), in mobile phase ($MeOH/H_2O$), on the retention times of reference compounds. Flow rate is 1.0 mL/min

Compounds	Retention Time (min)			
	Presence of Buffer		Absence of Buffer	
	60% MeOH	70% MeOH	60% MeOH	70% MeOH
Methanol	2.40	2.39	2.28	2.39
1. Butyric acid	3.23	2.94	1.92	2.09
2. Valeric acid	4.36	3.37	2.19	2.27
3. 2-Ethylbutyric acid	5.50	3.91	2.29	2.34
4. Hexanoic acid	6.28	4.18	2.30	2.39
5. Valproic acid	13.85	6.53	3.24*	2.65*
6. 2-Ethylhexanoic acid	13.96	6.58	-	2.95*
7. 2-Butylhexanoic acid	>30.0	13.70	-	2.89*

*Broad and tailing peaks.

Table 5

Retention times of seven reference compounds at different percentages of MeOH in the mobile phase (MeOH/0.01M NaH_2PO_4). Flow rate of mobile phase is 1.0 mL/min.

Compound	Retention Time (min) % MeOH (v/v)		
	50%	60%	70%
Methanol	1.54	2.40	2.39
1. Butyric acid	2.49	3.23	2.94
2. Valeric acid	3.80	4.36	3.37
3. 2-Ethylbutyric acid	5.54	5.50	3.91
4. Hexanoic acid	6.85	6.28	4.18
5. Valproic acid	20.45	13.85	6.53
6. 2-Ethylhexanoic acid	19.45	13.96	6.58
7. 2-Butylhexanoic acid	>50.0	>30.0	13.70

Table 6

Retention times of seven reference substances at different flow rates

(a) In 70% MeOH: 30% 0.01M NaH_2PO_4 mobile phase

Compound	Retention Time (min) Flow Rates		
	1.0mL/min	1.2mL/min	1.5mL/min
Methanol	2.39	2.03	1.54
1. Butyric acid	2.94	2.45	1.90
2. Valeric acid	3.37	2.83	2.21
3. 2-Ethylbutyric acid	3.91	3.27	2.54
4. Hexanoic acid	4.18	3.50	2.73
5. Valproic acid	6.53	5.49	4.37
6. 2-Ethylhexanoic acid	6.58	5.49	4.40
7. 2-Butylhexanoic acid	13.70	11.46	9.50

(b) In 60% MeOH: 40% 0.01M NaH_2PO_4 mobile phase

Compound	Retention Time (min) Flow Rates	
	1.0mL/min	1.5mL/min
Methanol	2.40	1.63
1. Butyric acid	3.23	2.19
2. Valeric acid	4.36	2.94
3. 2-Ethylbutyric acid	5.50	3.71
4. Hexanoic acid	6.28	4.25
5. Valproic acid	13.85	9.26
6. 2-Ethylhexanoic acid	13.96	9.32
7. 2-Butylhexanoic acid	>30.0	>23.0

Table 7

Retention times of seven reference compounds at different percentages of acetonitrile in the mobile phase

Compounds	Retention Time (min)					
	(Flow Rate=1.0mL/min) % CH_3CN (v/v)			(Flow Rate=1.5mL/min) % CH_3CN (v/v)		
	50%	55%	60%	40%	45%	50%
Methanol	2.00	2.00	2.00	1.47	1.42	1.37
1. Butyric acid	2.72	2.78	2.70	2.06	1.93	1.85
2. Valeric acid	3.17	3.16	3.00	2.67	2.33	2.18
3. 2-Ethylbutyric acid	3.65	3.52	3.25	3.24	2.72	2.47
4. Hexanoic acid	3.96	3.73	3.40	3.73	3.02	2.69
5. Valproic acid	6.32	5.51	4.63	7.71	5.31	4.30
6. 2-Ethylhexanoic acid	6.34	5.50	4.63	7.77	5.35	4.33
7. 2-Butylhexanoic acid	13.51	10.25	7.88	23.23	13.39	9.40

60% and acetonitrile-buffer compositions below 40%.

2. Void time and retention mechanism in RP-HPLC

The unretained elution time or void time (t_o) in the RP-HPLC method was determined by the elution time of the mobile phase component, methanol or acetonitrile. Table 8 shows the values of t_o obtained by organic co-solvent and linear regression of homologous straight-chain carboxylic acids (C_3H_7COOH-$C_7H_{15}COOH$) according to the method of Berendsen et al. (181). For the same flow rate, the void times in the two methods appear to be similar and stay reasonably constant with increases in the volume percentage of acetonitrile in the buffered mobile phase. For the same flow rate, anomalous values are obtained for the methanol-buffer mobile phase. The void times determined by either of the two methods do not appear to decrease or stay constant with increases in the proportion of methanol in the mobile phase. Similar findings of void times in methanol-water mixtures, especially in the region of 60-70% methanol, have been reported by several researchers (149,150,181). However, for the same mobile phase, the void time decreases with an increase in the flow rate.

Berendsen et al. (181), in a study of retention mechanisms in RP-HPLC, have proposed that the normal effect of decrease in void time with increase in volume percentage of methanol or acetonitrile could be explained by solvophobic effects. Thus, with a decrease in volume percentage of organic modifier, the solvation of the C-18 monomeric layer by the organic modifier decreases. Solvation was suggested to be minimal for pure water, hence interaction between

the hydrocarbon chain and water eluent is minimal. According to the authors, minimal solvation implied a maximal internal porosity of the microparticulate supports and hence a maximal hold-up or void time.

In comparison of methanol-water and acetonitrile-water effects on void time, Yonker et al. (149,150) explained the anomalous values of void time in methanol-water mixtures as due to a stationary phase phenomenon. They also proposed a model of the stationary phase composed of the C-18 monomeric layer, silica support and a solvation layer from mobile phase components. With increases in the volume of percentage of methanol up to 70%, they postulated that the mobile phase components, methanol or water, hydrogen-bond to the exposed silica support between the C-18 chains and this phenomenon predominates over that of solvation. They explained the plateau with approximately 70% methanol-water (instead of a decrease in void time) to be due to the dominating effect of solvation layer on C-18 hydrocarbon chain. Void times of the mobile phase component, methanol is then held up by interaction with the solvation layer on the C-18 chain. On the other hand, the void time determined in acetonitrile-water mixtures decreases slightly or stays nearly constant with an increase in volume percentage of acetonitrile (Table 8). The effects of acetonitrile-water mobile phase in this study could be explained accordingly by the lower hydrogen-bonding properties of acetonitrile with the effects of the solvation layer in opposition to the higher eluting strength of acetonitrile.

In view of the differing stationary phase conditions, void times were determined experimentally for each mobile phase compos-

Table 8

Comparison of void times (t_o) determined from
(a) injection of methanol, and
(b) dead time iteration of the retention times of the homologous series from C_3H_7COOH to $C_7H_{15}COOH$

Mobile Phase using 0.01M NaH_2PO_4 as co-solvent (v/v)	Flow Rate (mL/min)	t_o(MeOH) (min)	t_o(iteration)α (min)
50% MeOH	1.5	1.54	1.38
60% MeOH	1.0	2.40	2.13
70% MeOH	1.0	2.39	2.33
70% MeOH	1.2	2.03	1.86
70% MeOH	1.5	1.54	1.51
60% MeOH	1.5	1.63	1.33
50% CH_3CN	1.0	2.00	2.06
50% CH_3CN	1.5	1.37	1.43
55% CH_3CN	1.0	2.00	2.02
60% CH_3CN	1.0	2.00	1.80
40% CH_3CN	1.5	1.47	1.41
45% CH_3CN	1.5	1.42	1.38

[a]determined using equation $t_{R,N+1} = \alpha t_{R,N} - t_o(\alpha-1)$

ition. Capacity factor (k') for each reference compound in a different mobile phase was then calculated.

3. Eluent effects on capacity factor

Mobile phases containing various percentages of organic co-solvent were used to determine which mobile phase provides a significant high correlation for the linear regression equation log P = a + b log k'. Tables 9-11 show the log k' values and the correlation parameters obtained for the various percentages of organic modifier in buffered mobile phases.

Several interesting points emerge from the regression data. For the same organic co-solvent and flow rate, the slope of the plot of log P versus log k' decreased with a decrease in the percentage of organic co-solvent in the mobile phase. Thus sensitivity to changes in the lipophilic character increased with the more aqueous mobile phase, suggesting that the hydrophobic effect is a function of the polarity difference between the mobile and stationary phases. On the other hand, there was virtually no change in the slope with the same mobile phase but differing flow rates between 1.0 ml/min and 1.5 ml/min.

The regression equations with methanol-buffer mixture between 50-70% methanol and 40-60% acetonitrile-buffer mixture as mobile phases gave significantly high correlation coefficients. The linear model explained greater than 97% of the variations observed. The correlation coefficient increased at lower organic modifier volume percentage due to the greater sensitivity to changes in lipophilic character. However, lower percentages could not elute

Table 9

Correlation of log k' and log $P_{o/w}$ for seven reference compounds at various compositions of the mobile phase (MeOH/0.01M NaH_2PO_4).
log $P_{o/w}$ = a + b log k'

Compound		50% MeOH (FR*=1.0) log k'	60% MeOH (FR=1.0) log k'	70% MeOH (FR=1.0) log k'	70% MeOH (FR=1.2) log k'	70% MeOH (FR=1.5) log k'	log $P_{o/w}$ **
1. Butyric acid		-0.2098	-0.4611	-0.6380	-0.6842	-0.6312	0.98
2. Valeric acid		0.1666	-0.0879	-0.3872	-0.4044	-0.3614	1.51
3. 2-Ethylbutyric acid		0.4145	0.1111	-0.1965	-0.2141	-0.1875	1.68
4. Hexanoic acid		0.5376	0.2086	-0.1255	-0.1402	-0.1120	1.93
5. Valproic acid		1.089	0.6786	0.2386	0.2316	0.2643	2.75
6. 2-Ethylhexanoic acid		1.065	0.6827	0.2438	0.2316	0.2688	2.64
7. 2-Butylhexanoic acid		>1.498	>1.061	0.6751	0.6670	0.7134	3.20
	a	1.2336	1.6279	2.1955	2.2223	2.1576	
n = 6	b	1.3348	1.5218	1.9461	1.8822	1.9179	
(excluding compound #7)	r	0.9957	0.9944	0.9930	0.9928	0.9944	
	s	0.070	0.080	0.090	0.091	0.080	
	F	467	355	281	273	357	
	a			2.1463	2.1755	2.1098	
	b			1.7618	1.7222	1.726	
n = 7	r			0.990	0.991	0.990	
	s			0.122	0.117	0.120	
	F			246	270	255	

* FR is Flow Rate (mL/min) of eluent, s is standard error of estimate, $F_{0.01}$ = 16.0, r is correlation coefficient, F statistic from analysis of variance

** Values obtained from P.E. Keane et al. (13).

Table 10

Correlation of log k' and log $P_{o/w}$ for seven reference compounds at various compositions of the mobile phase (CH_3CN/0.01M NaH_2PO_4).

$$\log P_{o/w} = a + b \log k'$$

Compound		50% (FR*=1.0) log k'	55% (FR=1.0) log k'	60% (FR=1.0) log k'	40% (FR=1.5) log k'	45% (FR=1.5) log k'	50% (FR=1.5) log k'	** log $P_{o/w}$
1. Butyric acid		-0.4437	-0.4089	-0.4559	-0.3965	-0.4447	-0.4555	0.98
2. Valeric acid		-0.2328	-0.2366	-0.3010	-0.0881	-0.1932	-0.2282	1.51
3. 2-Ethylbutyric acid		-0.0835	-0.2744	-0.2041	0.0806	0.03834	-0.09533	1.68
4. Hexanoic acid		-0.00877	-0.063	-0.1549	0.1870	0.05183	-0.01615	1.93
5. Valproic acid		0.3345	0.2443	0.1189	0.6279	0.4377	0.3301	2.75
6. 2-Ethylhexanoic acid		0.3365	0.2430	0.1189	0.6320	0.4421	0.3346	2.64
7. 2-Butylhexanoic acid		0.7600	0.6154	0.4683	1.1703	0.9258	0.7680	3.20
	a	1.9178	2.0623	2.2436	1.6309	1.7941	1.9266	
	b	1.9104	2.1190	2.4780	1.4793	1.6945	1.8879	
n=7	r	0.9880	0.9798	0.9834	0.9895	0.9836	0.9877	
	s	0.133	0.173	0.157	0.125	0.156	0.135	
	F	205	120	146	235	148	200	
	a	1.9506	2.1153	2.3472	1.6236	1.8003	1.9623	
	b	2.1840	2.4244	2.9534	1.6765	1.9276	2.1740	
n=6	r	0.9958	0.9822	0.9968	0.9967	0.9875	0.9968	
(excluding compound #7)	s	0.069	0.143	0.061	0.062	0.120	0.061	
	F	478	109	616	607	157	613	

* FR is Flow Rate (mL/min) of eluent, s is standard error of estimate, $F_{0.01} = 16.0$, r is correlation coefficient, F statistic from analysis of variance

** Values obtained from P.E. Keane et al. (13).

Table 11

Summary of linear regression parameters for log P versus log k'
Log P = a + b log k'

n* = 6

Mobile Phase (Aqueous buffer-organic solvent)	Flow Rate (mL/min)	a	b	r	s
50% MeOH	1.0	1.2336	1.3348	0.9957	0.070
60% MeOH	1.0	1.6279	1.5218	0.9944	0.080
70% MeOH	1.0	2.1955	1.9461	0.9930	0.090
70% MeOH	1.2	2.2223	1.8822	0.9928	0.091
70% MeOH	1.5	2.2389	2.0530	0.9944	0.080
50% AcN	1.0	1.9506	2.1840	0.9958	0.069
55% AcN	1.0	2.1153	2.4244	0.9822	0.143
60% AcN	1.0	2.3472	2.9534	0.9968	0.961
40% AcN	1.5	1.6236	1.6765	0.9967	0.062
45% AcN	1.5	1.8327	1.9334	0.9965	0.120
50% AcN	1.5	1.9623	2.1740	0.9968	0.961

n* = 7

70% MeOH	1.0	2.1463	1.7618	0.990	0.122
70% MeOH	1.2	2.1755	1.7222	0.991	0.117
70% MeOH	1.5	2.1561	1.7278	0.990	0.120
50% AcN	1.0	1.9178	1.9104	0.9880	0.133
55% AcN	1.0	2.0623	2.1190	0.9798	0.173
60% AcN	1.0	2.2436	2.4780	0.9834	0.157
40% AcN	1.5	1.6309	1.4793	0.9895	0.125
45% AcN	1.5	1.8144	1.6842	0.9879	0.156
50% AcN	1.5	1.9266	1.8879	0.9877	0.135

*number of reference compounds (solutes) used;
r is correlation coefficient, s is standard error of estimate.

the highly lipophilic 2-butylhexanoic acid without peak broadening. Consequently when this compound was included, the correlation coefficient decreased.

Thus reduction of the extent of adsorption in the partition process using optimum flow rate and eluting strength produced high correlation coefficients for the set of compounds. A decrease in the adsorption phenomenon then ensures optimum rate of exchange and mass transfer of solute between the liquid phases.

4. HPLC log P values of valproic acid and analogues

Mobile phases selected for the determination of log P values were 70% methanol-buffer and 50% acetonitrile-buffer. The two mobile phases gave high correlation coefficients in the regression equation and were able to elute the highly lipophilic 2-butylhexanoic acid without excessive peak broadening. Having selected the mobile phases and experimental conditions the retention times of reference compounds and remaining compounds were determined (Table 12, 13). Figure 15 shows the HPLC chromatograms of the compounds, superimposed for comparison, using 70% methanol-buffer mobile phase. Twenty-three compounds were analyzed including the reference compounds. All the compounds showed symmetrical and sharp peaks. Values of log P were calculated from the respective regression equation below and are shown in Tables 12, 13.

$\log P = 2.1463 + 1.7618 \log k'$

$n = 7$, $r = 0.990$, $s = 0.122$, $F = 246$

(Mobile phase: 70% MeOH-30% NaH_2PO_4, 0.01M)

$\log P = 1.9506 + 2.1840 \log k'$

$n = 7$, $r = 0.9958$, $s = 0.069$, $F = 478$

(Mobile phase: 50% CH_3CN - 50% NaH_2PO_4, 0.01M)

Table 12

HPLC method for determining the lipophilicities of the acidic compounds using 70% MeOH: 30% 0.01M NaH_2PO_4 as mobile phase[a]

Compounds	t_R (min)	log k'	log P_{HPLC}	log $P^b_{o/w}$
1. Isobutyric acid	3.00	-0.5931	1.10	
2. Butyric acid	2.94	-0.6380	1.02	0.98
3. Trimethylacetic acid	3.47	-0.3450	1.54	
4. Valeric acid	3.37	-0.3872	1.46	1.51
5. 2-Ethylbutyric acid	3.91	-0.1965	1.80	1.68
6. 2,2-Dimethylbutyric acid	4.12	-0.1403	1.90	
7. Hexanoic acid	4.18	-0.1255	1.93	1.93
8. Heptanoic acid	5.49	0.1130	2.35	
9. 3-Ethylpentanoic acid	4.93	0.02644	2.19	
10. Cyclohexylacetic acid	5.35	0.09289	2.31	
11. 1-Methylcyclohexane-1-carboxylic acid	5.65	0.1348	2.38	
12. Valproic acid (VPA)	6.53	0.2386	2.57	2.75
13. 4-Keto VPA	3.19	-0.4753	1.31	
14. 2-Ene VPA	5.89	0.1658	2.44	
15. 2,3'-Diene VPA	5.21	0.07185	2.27	
16. 2-Ethylhexanoic acid	6.58	0.2438	2.58	2.64
17. Octanoic acid	7.66	0.3434	2.75	
18. 2-Butylhexanoic acid	13.70	0.6751	3.34	3.20
19. 5-Isoamyltetrazole	3.17	-0.4863	1.29	
20. 5-Heptyltetrazole	4.39	-0.07737	2.01	
21. 5-Cyclohexylmethyl-tetrazole	3.54	-0.3177	1.59	
22. N,N-Diethylsuccinamic acid	5.20	0.07031	2.27	
23. N,N-Dibutylsuccinamic acid	5.31	0.08699	2.30	

[a]Flow rate is 1.0 mL/min, t_o(MeOH) is 2.39 min.

[b]Used in linear correlation of log $P_{o/w}$ versus log k'. Values taken from P.E. Keane et al. (13).

Table 13

HPLC method for determining the lipophilicities of the acidic compounds using 50% CH_3CN: 50% 0.01M NaH_2PO_4 as mobile phase[a]

Compounds	t_R (min)	log k'	log P_{HPLC}	log $P^b_{o/w}$
1. Isobutyric acid	2.72	-0.4437	1.07	
2. Butyric acid	2.72	-0.4437	1.07	0.98
3. Trimethylacetic acid	3.24	-0.2076	1.52	
4. Valeric acid	3.17	-0.2328	1.47	1.51
5. 2-Ethylbutyric acid	3.65	-0.0835	1.76	1.68
6. 2,2-Dimethylbutyric acid	3.82	-0.04096	1.84	
7. Hexanoic acid	3.96	-0.00877	1.90	1.93
8. Heptanoic acid	5.17	0.2000	2.30	
9. 3-Ethylpentanoic acid	4.61	0.1156	2.14	
10. Cyclohexylacetic acid	4.97	0.1717	2.25	
11. 1-Methylcyclohexane-1-carboxylic acid	5.24	0.2095	2.32	
12. Valproic acid (VPA)	6.32	0.3345	2.56	2.75
13. 4-Keto VPA	3.03	-0.2882	1.37	
14. 2-Ene VPA	6.08	0.3096	2.51	
15. 2,3'-Diene VPA	4.99	0.1746	2.25	
16. 2-Ethylhexanoic acid	6.34	0.3365	2.56	2.64
17. Octanoic acid	7.31	0.4241	2.72	
18. 2-Butylhexanoic acid	13.51	0.7600	3.37	3.20
19. 5-Isoamyltetrazole	3.00	-0.2967	1.35	
20. 5-Heptyltetrazole	4.09	0.01912	1.95	
21. 5-Cyclohexylmethyl-tetrazole	3.52	-0.1192	1.69	
22. N,N-Diethylsuccinamic acid	4.92	0.1651	2.23	
23. N,N-Dibutylsuccinamic acid	5.35	0.2240	2.35	

[a]Flow rate is 1.0 mL/min, t_o(MeOH) is 2.0 min.

[b]Taken from P.E. Keane et al. (13). Values used in linear correlation of log $P_{o/w}$ versus log k'.

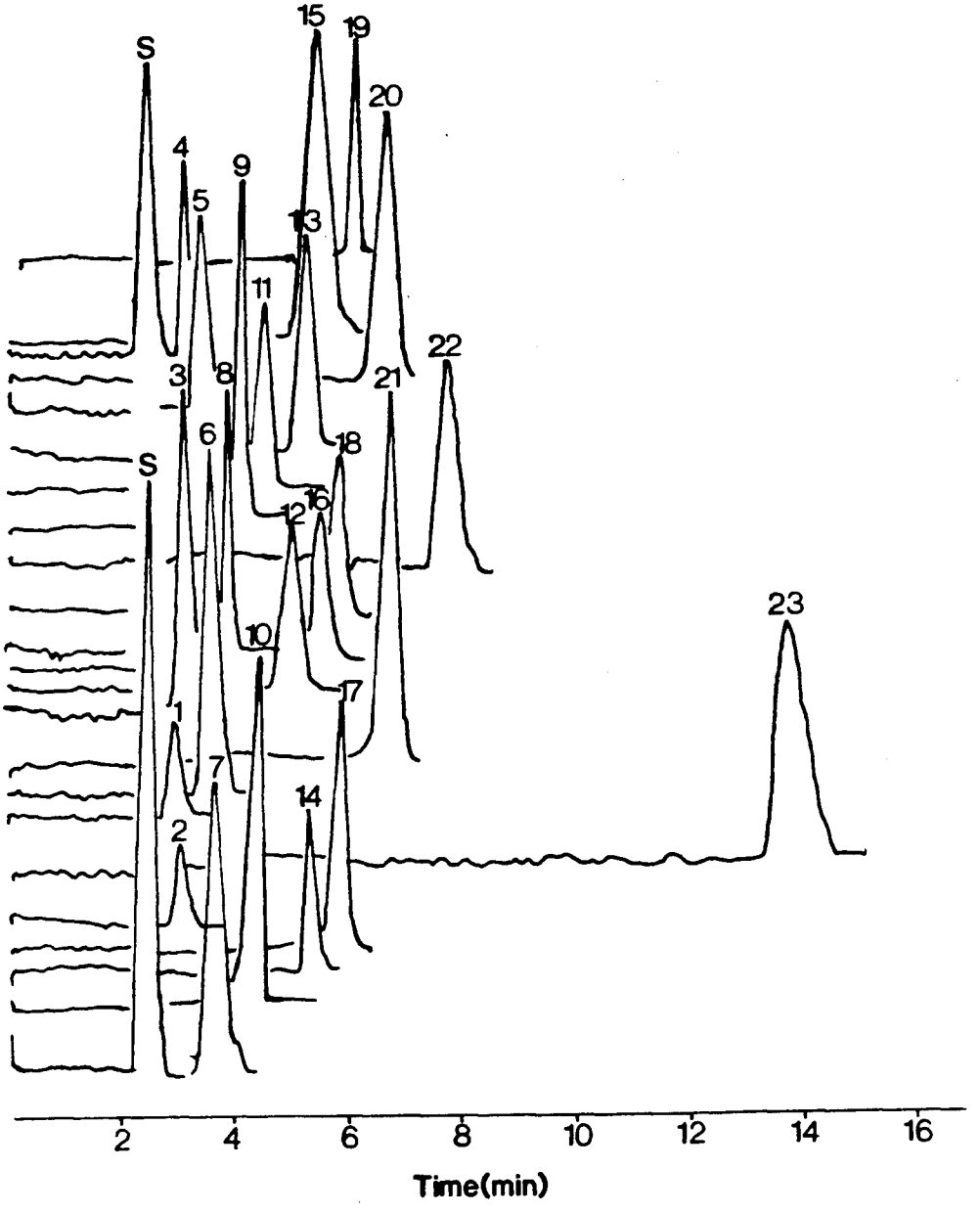


Figure 15. Superposed HPLC chromatograms of acidic compounds. Column; hypersil ODS reverse phase column (20cm x 4.6mm). Mobile phase; 70% Methanol:30% 0.01M NaH_2PO_4 (pH3.5). Flow rate, 1.0ml/min. S,solvent;

1. Butyric acid 2. Isobutyric acid 3. 5-Isoamyltetrazole
4. 4-Keto VPA 5. Valeric acid 6. Trimethylacetic acid
7. 5-Cyclohexylmethyltetrazole 8. 2-Ethylbutyric acid
9. 2,2-Dimethylbutyric acid 10. Hexanoic acid 11. 5-Heptyltetrazole
12. 3-Ethylpentanoic acid 13. N,N-Diethylsuccinamic acid
14. 2,3-Diene VPA 15. N,N-Dibutylsuccinamic acid 16. Cyclohexylacetic acid
17. Heptanoic acid 18. 1-Methylcyclohexane carboxylic acid
19. 2-Ene VPA 20. Valproic acid (VPA) 21. 2-Ethylhexanoic acid
22. Octanoic acid 23. 2-Butylhexanoic acid

5. Comparison of lipophilicity from RP-HPLC and other methods

Retention of the test compounds on the RP-HPLC column appeared to be based on the hydrocarbon structure, the presence of polar functional groups, the type of alkyl substitution, the number of double bonds, the presence of a ring structure and molecular size (Tables 12, 13). As shown in Tables 12 and 13, the HPLC log P values determined from both mobile phases are in good agreement with an average difference of $\pm$ 0.06 log units.

The shake-flask method was used to determine the octanol-water partition coefficient of four compounds of diverse structures in order to verify the accuracy of the HPLC log P values. Tables 14-17 show the calibration data for the four compounds determined using HPLC analysis. High correlation coefficients were obtained for the calibration curves. The log P values of the four compounds determined by the shake-flask method are shown in Table 18. The log P values are for the unionized form of the acidic compounds since 0.01N HCl was used as the aqueous phase. The precision of the log P values was high with average standard deviations less than 0.05 log units, except for N,N-dibutylsuccinamic acid with a deviation of 0.08 log units. Probable sources of error include low sensitivity for detection of these compounds by HPLC and propensity of N,N-dibutylsuccinamic acid to be unstable in an acidic aqueous phase. In addition, dissolution of tetrazoles in the 0.01N HCl phase was difficult and had to be effected using small volumes of methanol.

Table 14

Calibration curve data of trimethylacetic acid in 0.1N HCl

Concentration mg/mL	Mean Peak Height	Linear Regression Parameters[a]
0.05	193	
0.1	392	$a^o = 22.81$
0.2	767	$a^1 = 3639$
0.4	1465	$r = 0.9999$
0.6	2210	$r^2 = 0.9998$

a r^2 is the coefficient of determination, a^1 is the slope and a^o is the intercept. Equation for the line is $y = a^1x + a^o$ where y is the peak height and x is concentration of compound.

Table 15

Calibration curve data of N,N-Dibutylsuccinamic acid in 0.1N HCl

Concentration mg/mL	Mean Peak Height	Linear Regression Parameters[a]
0.1	128	$a^o = 3.14$
0.2	260	$a^1 = 1321$
0.4	530	$r = 0.99994$
0.6	787	$r^2 = 0.99988$

a r^2 is the coefficient of determination, a^1 is the slope and a^o is the intercept. Equation for the line is $y = a^1x + a^o$ where y is the peak height and x is concentration of compound.

Table 16

Calibration curve data of 5-Isoamyltetrazole in 0.1N HCl

Concentration mg/mL	Mean Peak Height	Linear Regression Parameters[a]
0.01	1099	a^o = 235.9
0.02	2153	a^1 = 92742
0.05	4889	r = 0.9999
0.1	9496	r^2 = 0.9998

a r^2 is the coefficient of determination, a^1 is the slope and a^o is the intercept. Equation for the line is $y = a^1x + a^o$ where y is the peak height and x is concentration of compound.

Table 17

Calibration curve data of 5-cyclohexylmethyltetrazole in 0.1N HCl

Concentration mg/mL	Mean Peak Height	Linear Regression Parameters[a]
0.01	455	a^o = -72.67
0.02	974	a^1 = 52782
0.05	2581	r = 0.99999
0.1	5200	r^2 = 0.99998

a r^2 is the coefficient of determination, a^1 is the slope and a^o is the intercept. Equation for the line is $y = a^1x + a^o$ where y is the peak height and x is concentration of compound.

Table 18

Octanol-water partition coefficients of selected acidic compounds determined by the shake-flask procedure

Compound	log $P^{a}_{o/w}$	($\pm$SD[b])
1. Trimethylacetic acid	1.54	$\pm$ 0.01
2. N,N-Dibutylsuccinamic acid	2.27	$\pm$ 0.08
3. 5-Isoamyltetrazole	1.38	$\pm$ 0.03
4. 5-Cyclohexylmethyltetrazole	1.61	$\pm$ 0.04

a Shake-flask method used with 1-octanol as organic phase and 0.01N HCl as aqueous phase.

b Standard deviation in log units, from four separate experimental measurements.

Table 19
Hansch-π - values[a] used in calculating log $P_{o/w}$

Aliphatic Group	π
1. CH_3	0.5
2. CH_2	0.5
3. -OH	-1.16
4. -O-	-0.98
5. COOH	-0.65
6. C=O	-1.21
7. $CONH_2$	-1.71
8. NH_2	-1.19
9. -CON-	-2.27
10. N	-1.32
11. Tetrazole	-1.04[b]
12. NMe_2	-0.32
13. Double bond	-0.30 ($\Delta\pi$)
14. Chain branch (single)	-0.20 ($\Delta\pi$)
15. Branching in ring closure	-0.09 ($\Delta\pi$)
16. Ring closure (per bond)	-0.09 ($\Delta\pi$)
17. Intramolecular H-bonding	-0.65 ($\Delta\pi$)

[a]Taken from A. Leo et al. (133).

[b]π(tetrazole) obtained from C. Hansch and A. Leo (152).

Table 20

Rekker's fragmental values (f)[a] used in calculating log $P_{o/w}$

Fragment	f(aliphatic)
1. CH_3	0.702
2. CH_2	0.527
3. CH	0.236
4. C	0.14
5. H	0.175
6. CH_2 = CH	0.93
7. COOH	-1.003
8. C = O	-1.69
9. NH_2	-1.38
10. NH	-1.864
11. -N-	-2.133
12. CON	-2.894
13. $CONH_2$	-1.99
14. Tetrazole	-2.93
15. NH (heterocyclic)	-0.70
16. N (heterocyclic)	-1.06
17. C (heterocyclic)	0.157
18. H (heterocyclic)	0.199
19. CH (heterocyclic)	0.35
20. Single conjugated pattern	0.314 (Δf)
21. Proximity effect for 1 C separation	0.80
22. Proximity effect of electronegative group for 2C separation	0.46
23. -N = C-NH	-0.79
24. -N = N	-2.14

[a]Taken from R. F. Rekker (153).

Table 21
Lipophilicities (log $P_{o/w}$) of the acidic compounds obtained by different methods

Compounds	log $P_{o/w}$				
	HPLC[a] (MeOH)	HPLC[b] (CH_3CN)	Hansch[c]	Rekker[d]	Shake-Flask
1. Isobutyric acid	1.10	1.07	0.65	0.64	
2. Butyric acid	1.02	1.07	0.85	0.76	0.98[e]
3. Trimethylacetic acid	1.54	1.52	0.95	1.24	1.54[f]
4. Valeric acid	1.46	1.47	1.35	1.28	1.51[e]
5. 2-Ethylbutyric acid	1.80	1.76	1.65	1.69	1.68[e]
6. 2,2-Dimethylbutyric acid	1.90	1.84	1.45	1.45	
7. Hexanoic acid	1.93	1.90	1.85	1.81	1.93[e]
8. Heptanoic acid	2.35	2.30	2.35	2.33	
9. 3-Ethylpentanoic acid	2.19	2.14	2.15	2.22	
10. Cyclohexylacetic acid	2.31	2.25	2.22	2.39	
11. 1-Methylcyclohexane-1-carboxylic acid	2.38	2.32	2.13	2.47	
12. Valproic acid (VPA)	2.57	2.56	2.65	2.75	2.75[e]
13. 4-keto VPA	1.31	1.37	1.14	0.99	
14. 2-Ene VPA	2.44	2.51	2.35	2.56	
15. 2,3-Diene VPA	2.27	2.25	2.05	2.26	
16. 2-Ethylhexanoic acid	2.58	2.56	2.65	2.75	2.64[e]
17. Octanoic acid	2.75	2.73	2.85	2.86	
18. 2-Butylhexanoic acid	3.34	3.37	3.65	3.80	3.20[e]
19. 5-Isoamyltetrazole	1.29	1.35	1.26	-1.03	1.38[f]
20. 5-Heptyltetrazole	2.01	1.95	2.46	0.83	
21. 5-Cyclohexylmethyl-tetrazole	1.56	1.69	1.85	0.50	1.61[f]
22. N,N-Diethylsuccinamic acid	2.27	2.23	0.08	0.08	
23. N,N-Dibutylsuccinamic acid	2.30	2.35	2.08	2.18	2.27[f]

[a] determined using 70% MeOH: 30% 0.01M NaH_2PO_4
[b] determined using 50%CH_3CH: 50% 0.01M NaH_2PO_4
[c] calculated using Hansch π-approach
[d] calculated using Rekker's fragment constant
[e] experimentally determined by P. E. Keane et al. (13)
[f] octanol-water partition coefficient determined in this study

Hansch-π and Rekker-f values (Tables 19, 20) have been used to predict log P values of the compounds studied (Table 21). Comparison of the HPLC log P values with Hansch and Rekker values in Table 21 shows that there is good agreement for the homologous straight-chain and alpha-branched aliphatic acids, C_4H_9COOH-$C_7H_{15}COOH$. The discrepancies in log P values for the HPLC and calculated methods are, however, for highly substituted compounds (e.g. trimethylacetic acid), substituted alicyclic compounds (e.g. 1-methylcyclohexanecarboxylic acid), tetrazoles, intramolecular bonded compounds e.g. N,N-diethylsuccinamic acid (165) and 4-keto VPA (162).

The shake-flask log P values of the highly-substituted trimethylacetic acid, highly lipophilic 2-butylhexanoic acid, tetrazoles and less lipophilic butyric acid agree better with HPLC log P values than the calculated log P values (Table 21). This indicates that the Hansch and Rekker log P values may lead to less accurate values for compounds showing constitutive effects on hydrophobicity. Obviously, additivity of group contribution does not hold for the tetrazoles and the log P value of the unsubstituted tetrazole would have to be used, as noted in Table 19. There is close agreement among the various methods with the shake-flask value for N,N-dibutylsuccinamic acid (XVI). Lower values from additivity methods were obtained for N,N-diethylsuccinamic acid (XV) which may show significant intramolecular effects to increase the log P value.

6. Intramolecular bonding effects of amic acids

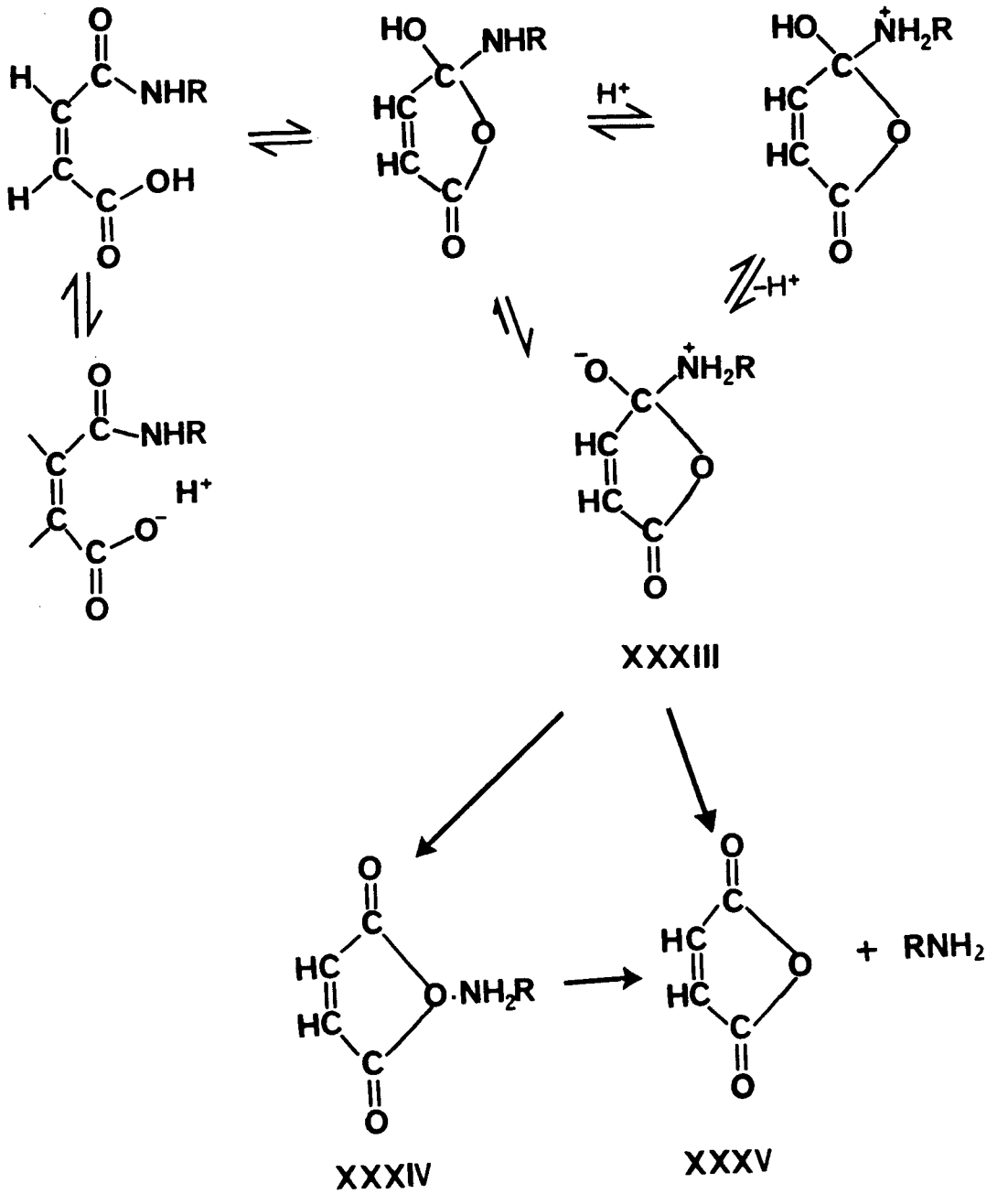
The HPLC log P value for N,N-diethylsuccinamic acid (XV) was considerably higher than predicted (Table 21). This could be explained by a greater intramolecular bonding for N,N-diethylsuccinamic acid than for N,N-dibutylsuccinamic acid. In addition, the NMR spectra of N,N-diethylsuccinamic appear to indicate an interaction between the carboxylic group and the amido group compared to that of N,N-dibutylsuccinamic acid (Figure 6). A study (165) of NMR spectra of N,N-dialkylsuccinamic acids also showed evidence of an interaction between the amido and carboxyl functional groups in N,N-diethylsuccinamic acid through hydrogen bonding in dilute solutions.

Another aspect of amic acids involves their stability in acidic media. Both N,N-dibutylsuccinamic and N,N-diethylsuccinamic are soluble in water. N,N-dibutylsuccinamic acid was relatively stable in 0.1N HCl and 0.01M NaH_2PO_4 as shown by the high correlation coefficient of its linear calibration curves used to determine the concentration in the aqueous phase of the octanol-0.1N HCl partition system. The long-term stability of N,N-dibutylsuccinamic acid was not investigated. On the other hand, N,N-diethylsuccinamic was not stable in 0.1N HCl and its partition coefficient in the octanol/0.1N HCl system could not be determined. In order to observe chromatographic peaks, amounts of 20μg or greater of N,N-diethylsuccinamic acid in methanol or acetonitrile had to be injected.

There is literature precedence (183,184) for the instability of some amic acids in acidic media. It has been documented that

the presence of a carboxylic group adjacent to an amide within the molecular structure usually leads to hydrolysis of the amic acid. The degree of hydrolysis appears to depend on the type of amic acid and N-alkyl substituents (183,184). Thus the hydrolysis is reported to be even greater in N-alkylmaleamic acids (182,183) where the cis-configuration favours intramolecular interactions. In a kinetic study of N-alkylmaleamic acid hydrolysis, Kluger and Chin (184) proposed a mechanism where for compounds with more basic leaving groups, the amine group elimination XXXIV-XXXV is the rate-determining step in formation of the internal anhydride, XXXV (Scheme 10). Aldersley et al. (183) proposed a similar mechanism to account for the rapid hydrolysis of the N-alkylmaleamic acids. However, the rate-determining step was suggested to be cleavage of the C-N bond, XXXIII-XXXV. The HPLC chromatograms, following injection of N,N-diethylsuccinamic acid, indicated a bigger than expected solvent peak. This was probably due to co-elution of the degraded products, succinic acid, diethylamine and methanol. The amide group has also been reported to give underestimated log P values, calculated by the Hansch method, in compounds with multiple functional groups (182).

In comparative studies, both HPLC and shake-flask methods have advantages and limitations. The shake-flask method is laborious, time-consuming, subject to errors from insolubility and instability of compounds in the octanol-0.1N HCl partition system and requires sensitive analytical techniques to determine the concentration of solute in the aqueous phase. Advantages associated with the shake-flask procedure include high sensitivity to hydrophobic effects



Scheme 10. Kinetic model proposed by some investigators (182, 183) for the hydrolysis of maleamic acids.

since aqueous solutions were used instead of mixed solvents. The shake-flask method can also be used for compounds of widely different chemical structures. The HPLC method, on the other hand, is a rapid technique. It offers high reproducibility of log P values since the only parameter, retention time, can be determined accurately. It does not require quantitative analysis of compounds. It may be appropriate for compounds prone to degradation in octanol-water systems or for compounds in less pure samples.

The RP-HPLC method, however, requires reference compounds with log P values determined by the standard shake-flask method. The range of lipophilicity is such that different chromatographic conditions have to be employed for compounds of high or low lipophilicity.

In this study the HPLC method under isocratic conditions was limited to compounds with log P of about 0.8-3.2. One notable shortcoming of the HPLC method observed in this study is that the order of retention time between structural isomers, for example butyric acid and isobutyric acid or valproic acid and 2-ethyhexanoic acid, can change depending on the composition and type of mobile phase. Some workers (141,147) have tackled this problem by extrapolation of the plot of log k' versus mobile phase composition to obtain log k' for water. Apparently this was not possible for the compounds studied since the mobile phase composition needed to elute all the test compounds while maintaining dynamic equilibrium conditions was limited to a range of 40-60% acetonitrile-buffer and 60-70% methanol-buffer. Nevertheless, the accuracy of HPLC log P values was high, and values were comparable to shake-flask log P values. The HPLC method could also be applied to a variety of

chemical structures unlike the additivity methods of Hansch and Rekker.

C. Electronic Structural Effects - Determination of Apparent Ionization Constants

1. Analytical method

The apparent ionization constants (pKå) of test compounds were determined by potentiometric titration in aqueous-methanol media due to the limited solubility of most of the compounds in water. Potentiometric and UV spectrophotometric methods have been frequently used to determine the ionization constants of acidic compounds (185,187). The conductivity method has been used to determine the ionization constants of aliphatic acids (188). Spectrophotometric methods were not appropriate for the compounds studied since there is no change in the UV spectra upon ionization.

In the potentiometric procedure, solutions of the compounds were progressively neutralized with quantities of standard KOH and the pH recorded. Tables 22-28 show typical results obtained in the potentiometric titration procedure. The correction factor in the Henderson-Hasselbalch equation, which is reported (185) to be more significant at pH values lower than 5 and higher than 9, was uniformly applied at all pH values. As shown in Tables 23, 26, 28, values of pKå at the beginning of the titration occasionally deviated from expected values. Table 29 shows the pKa values of 23 compounds determined in either 10% methanol-water or 50% methanol-water. The ionization constants were determined at $23^{o}C \pm 1.0^{o}C$. Values of pKå were determined to within a precision of $\pm$ 0.05 pKa

Table 22

Determination of the ionization constant of a monobasic acid, valproic acid in 10% MeOH
Temperature - 24°C

Titrant 0.0105M KOH (mL)	pH	$[HA] \times 10^3$ (mol. L^{-1})	$[A^-] \times 10^3$ (mol. L^{-1})	$\frac{[HA] - \{H^+\}}{[A^-] + \{H^+\}}$	pKa
0.5	4.24	0.9218	0.105	5.325	4.97
1.0	4.47	0.8178	0.210	2.930	4.94
1.5	4.64	0.7128	0.315	2.042	4.95
2.0	4.82	0.6078	0.420	1.362	4.95
2.5	4.99	0.5028	0.525	0.9204	4.95
3.0	5.18	0.3978	0.630	0.6147	4.97

Table 23

Determination of the ionization constant of 5-Isoamyltetrazole
in 10% MeOH
Temperature - 23°C

Titrant 0.0105M KOH (mL)	pH	$[HA] \times 10^3$ (mol. L^{-1})	$[A^-] \times 10^3$ (mol. L^{-1})	$\frac{[HA] - \{H^+\}}{[A^-] + \{H^+\}}$	pKa
0.5	4.36	0.888	0.105	5.7043	5.12
1.0	4.69	0.783	0.210	3.3099	5.21
1.5	4.91	0.678	0.315	2.0339	5.22
2.0	5.12	0.543	0.420	1.3223	5.24
2.5	5.28	0.468	0.525	0.8729	5.22
3.0	5.46	0.363	0.630	0.5672	5.21
3.5	5.65	0.258	0.735	0.3470	5.19

Table 24

Determination of the ionization constant of trimethylacetic acid in 10% MeOH
Temperature - 23°C

Titrant 0.01997M KOH (mL)	pH	$[HA] \times 10^3$ (mol. L^{-1})	$[A^-] \times 10^3$ (mol. L^{-1})	$\frac{[HA] - \{H^+\}}{[A^-] + \{H^+\}}$	pKa
0.5	4.27	1.8983	0.1997	7.2794	5.10
1.0	4.52	1.6986	0.3994	3.8836	5.10
1.5	4.77	1.4989	0.5991	2.4053	5.15
2.0	4.91	1.2992	0.7988	1.5866	5.11
2.5	5.08	1.0995	0.9985	1.0838	5.11
3.0	5.22	0.8998	1.1982	0.7422	5.09
3.5	5.38	0.7001	1.3979	0.4963	5.08

Table 25

Determination of the ionization constant of dibutylacetic acid
in 50% MeOH
Temperature - 23°C

Titrant 0.0105M KOH (mL)	pH	$[HA] \times 10^3$ (mol. L^{-1})	$[A^-] \times 10^3$ (mol. L^{-1})	$\frac{[HA] - \{H^+\}}{[A^-] + \{H^+\}}$	pKa
0.5	5.11	0.8776	0.105	7.7110	6.00
1.0	5.38	0.7726	0.210	3.5873	5.93
1.5	5.58	0.6676	0.315	2.0938	5.90
2.0	5.74	0.5626	0.420	1.3295	5.86
2.5	5.98	0.4576	0.525	0.8681	5.92
3.0	6.29	0.3526	0.630	0.5584	6.04
3.5	6.53	0.2476	0.735	0.3363	6.06

Table 26

Determination of the ionization constant of N,N-diethylsuccinamic acid in 50% MeOH
Temperature - 24°C

Titrant 0.0098M KOH (mL)	pH	$[HA] \times 10^3$ (mol. L^{-1})	$[A^-] \times 10^3$ (mol. L^{-1})	$\frac{[HA] - \{H^+\}}{[A^-] + \{H^+\}}$	pKa
0.5	6.29	0.896	0.098	9.0304	7.24
1.0	6.39	0.792	0.196	4.0305	7.00
1.5	6.50	0.694	0.294	2.3571	6.87
2.0	6.61	0.596	0.392	1.5191	6.79
2.5	6.76	0.498	0.490	0.0155	6.77
3.0	6.91	0.400	0.588	0.6800	6.74
3.5	7.12	0.302	0.686	0.4404*	6.76
4.0	7.43	0.204	0.784	0.2607*	6.85

*calculated using the correction factor $\frac{[HA] + \{OH^-\}}{[A^-] - \{OH^-\}}$

Table 27

Determination of the ionization constant of 5-cyclohexylmethyltetrazole in 50% MeOH
Temperature - 23°C

Titrant 0.0105M KOH (mL)	pH	$[HA] \times 10^3$ (mol. L^{-1})	$[A^-] \times 10^3$ (mol. L^{-1})	$\frac{[HA] - \{H^+\}}{[A^-] + \{H^+\}}$	pKa
0.5	4.75	0.9552	0.105	7.658	5.63
1.0	5.05	0.8502	0.210	3.843	5.63
1.5	5.27	0.7452	0.315	2.309	5.63
2.0	5.45	0.6402	0.420	1.503	5.62
2.5	5.62	0.5352	0.525	1.010	5.62
3.0	5.79	0.4302	0.630	0.6786	5.61
3.5	5.97	0.3252	0.735	0.4403	5.57

Table 28

Determination of the ionization constant of N,N-dibutylsuccinamic acid in 50% MeOH
Temperature - 24°C

Titrant 0.0098M KOH (mL)	pH	$[HA] \times 10^3$ (mol. L^{-1})	$[A^-] \times 10^3$ (mol. L^{-1})	$\frac{[HA] - \{H^+\}}{[A^-] + \{H^+\}}$	pKa
0.5	6.21	0.9060	0.0980	9.1825	7.17
1.0	6.33	0.8080	0.1960	4.1094	6.94
1.5	6.42	0.7100	0.2940	2.4103	6.80
2.0	6.56	0.6120	0.3920	1.5593	6.75
2.5	6.69	0.5140	0.4900	1.0481	6.71
3.0	6.82	0.4160	0.5880	0.7069	6.67
3.5	7.01	0.3180	0.6860	0.4635	6.68
4.0	7.23	0.2200	0.7840	0.2809*	6.68
4.5	7.58	0.1220	0.8820	0.1388*	6.72
5.0	8.29	0.0240	0.9800	0.0265*	6.71

*calculated using the correction factor $\frac{[HA] + \{OH^-\}}{[A^-] - \{OH^-\}}$

Table 29
pKå Values of valproic aid and analogues

Compound	pKå ± s.d. (n=6)	
	(in 10% MeOH)	(in 50% MeOH)
1. Isobutyric acid	4.96 ± 0.02	5.85 ± 0.06
2. Trimethylacetic acid	5.11 ± 0.02	5.94 ± 0.06
3. 3-Methylpentanoic acid	4.85 ± 0.03	5.76 ± 0.05
4. Diethylacetic acid	4.83 ± 0.03	6.02 ± 0.02
5. 3-Methylhexanoic acid	5.02 ± 0.01	5.72 ± 0.05
6. 2,2-Dimethylbutyric acid	5.26 ± 0.03	6.52 ± 0.02
7. 2,2-Dimethylpentanoic acid	5.26 ± 0.05	6.36 ± 0.01
8. Valproic acid (VPA)	4.95 ± 0.01	6.03 ± 0.01
9. 3-OH VPA	4.39 ± 0.02	5.56 ± 0.04
10. 4-keto VPA	4.58 ± 0.01	5.28 ± 0.06
11. 4-Ene VPA	4.63 ± 0.01	5.51 ± 0.06
12. 2-Ethylhexanoic acid	5.05 ± 0.03	6.14 ± 0.13
13. Dibutylacetic acid	5.05 ± 0.06	5.95 ± 0.08
14. 1-Methylcyclohexane-1-carboxylic acid	5.17 ± 0.05	6.58 ± 0.01
15. 3-Ethylpentanoic acid	4.78 ± 0.08	5.99 ± 0.02
16. Cyclohexylacetic acid	4.78 ± 0.02	5.77 ± 0.04
17. N,N-Diethylsuccinamic acid	5.58 ± 0.05	6.80 ± 0.05
18. N,N-Dibutylsuccinamic acid	5.58 ± 0.05	6.72 ± 0.03
19. 5-Isoamyltetrazole	5.22 ± 0.02	5.60 ± 0.01
20. 5-Cyclohexylmethyltetrazole	5.37 ± 0.03	5.62 ± 0.01
21. 5-Heptyltetrazole	5.31 ± 0.05	5.60 ± 0.01
22. 2,3'-Diene VPA	4.02 ± 0.02	5.47 ± 0.06
23. 2-Ene VPA	4.36 ± 0.05	5.51 ± 0.06

units. Standard deviations of the pKa values were in a few cases higher than $\pm$ 0.06 pKa.

The pKa of valproic acid, 4.95, determined in 10%-methanol-water is higher than the reported values of 4.82 in 5% methanol-water (189) and 4.6 as the aqueous value from extrapolated acetone-water mixtures (67). The pKa values in Table 29 are higher in 50% methanol-water than in 10% methanol-water due to the lower dielectric constant of 50% methanol-water. However, the order of ionization strength in 50% methanol-water did not reflect the same order in 10% methanol-water. Similar findings on the non-linearity between pKå and the composition of organic solvent have been reported for some compounds (185-187). There have also been reports that the conventional approach of extrapolating values of pKå to aqueous values may be useful when lower solvent compositions are used (185,186). The pKå values in 10% methanol-water were considered to be more reliable than values in 50% methanol-water. Compared to pKa values of aliphatic acids reported by Dippy (188) in conductivity methods (pKa of trimethylacetic acid = 5.05, pKa of diethylacetic acid = 4.75, pKa of isobutyric acid = 4.86), the pKa values in 10% methanol-water are 0.1-0.2 pKa units above the aqueous values.

2. Effects of structural constitution on ionization constants

To the extent that the pKå values in 10% methanol are most likely to be different from the aqueous values by a constant amount, the electronic effects can be determined by the pKå values. This is supported by the observation that the pKå values in Table 29 are consistent with the inductive effects of substituent groups

expressed in Table 30 by the polar substituent constants. Electron-withdrawing substituents have a positive polar substituent constant while electron-repelling groups have negative values.

The ionization constants of the tetrazoles (Table 29) conform to the generalization (21) that they are about a tenth to one-half as large as their corresponding carboxylic acid values. The pKa values of the tetrazoles are similar to results obtained by Mihina and Herbst (161) in 25% by weight methanol-water.

The influence of substitution pattern, polar groups, alicyclic groups, unsaturation and intramolecular bonding are reflected in the pKa values. Beta-substitution in the alkyl chain, exemplified by 3-ethylpentanoic acid and cyclohexylacetic acid, leads to relatively low pKa values compared to alpha-substituted compounds. Diethylacetic and valproic acid, both alpha-branched acids, have comparable or slightly higher ionization strength than isobutyric acid, in agreement with results of Dippy (188). Compounds such as α,α-dimethylvaleric acid, trimethylacetic acid and 1-methylcyclohexane carboxylic acid have relatively high pKa values due to combined inductive effects of the methyl groups. The decrease in pKa of the unsaturated compounds compared to their saturated analogues is known to be due to polarization of the double bond. Values of the polar substituent constants indicate that cyclohexylmethyl and cyclohexyl substitution diminishes the ionization strength of the unsubstituted carboxylic acids and the tetrazoles (Table 30).

The presence of the keto group or hydroxy group in the alkyl chain increases the ionization strength in accordance with the

Table 30

Polar effect of substitution in an aliphatic series, R-Y where Y is the functional group.

R	σ^* (Polar Substituent Constant)[a]
Cl_3C	2.65
CH_3CO	1.65
CH_3COCH_2	0.60
$HOCH_2$	0.55
H	0.49
CH_3-CH=CH	0.36
CH_3-CH=CH-CH_2	0.13
CH_3	0
C_2H_5	-0.1
i - C_4H_9	-0.125
n - C_4H_9	-0.13
n - C_3H_7	-0.115
i - C_3H_7	-0.19
Cyclo - $C_6H_{11}CH_2$	-0.06
Cyclo - C_6H_{11}	-0.15
s - C_4H_9	-0.21
$(C_2H_5)_2$ CH	-0.225
t - C_4H_9	-0.30

[a]taken from R. W. Taft (134).

polar substituent constants. The presence of an amido group close to a carboxyl group usually leads to a decrease in pKa relative to an alkyl substituent because of the inductive effect. The opposite results are obtained for the N,N-dialkylsuccinamic acids. The relatively high pKa values could be explained by hydrogen-bonding between the carboxylic proton and the amido group. Intramolecular bonding is even stronger in N-propylmaleamide, pKa of 10.53 (184) where the cis configuration favours such an interaction.

The pKa values for most of the compounds studied are available in the literature but the values are usually determined under different conditions and by different workers. This consideration prompted the determination of the pKa values of test compounds by the potentiometric titration method using the same solvent-mixture for all the compounds.

D. Pharmacological Studies

a. Evaluation of anticonvulsant activity

The anticonvulsant activity of each test compound was determined using the in vivo s.c. PTZ seizure threshold test. The test appears to be suitable for valproic acid and analogues. Valproic acid and alkyl-substituted antiepileptic drugs are known to be more effective in the s.c. PTZ threshold test than in maximal electroshock seizure tests. In addition, valproic acid and PTZ appear to exert opposing actions at the GABA post-synaptic site (53,54,81). Recent studies (30,31,33,34,57) have suggested that valproic acid and PTZ may act at the picrotoxin site of the

GABA-Benzodiazepine-receptor-chloride ionophore complex. The specifity of the s.c. PTZ test has been recently re-investigated and suggested to be related to the dosage level of PTZ used (190). Clonic threshold seizures were found to be induced reproducibly with 85 mg/kg s.c. PTZ (CD_{97}) in mice (190).

The control test in this study showed that 100% of the mice responded to the s.c. injection of 85 mg/kg PTZ with well-defined clonic spasms. The time of onset of clonic seizures was between 3-9 minutes which is consistent with previous studies on the onset of clonus at different PTZ doses (191). PTZ was injected s.c. 15 minutes after i.p. injection of each test compound. The selected time interval was based on literature reports where 2 min (14), 15 min (5,12,13) and 30 min (10,11,88) have been chosen as suitable elapsed times before s.c. injection of PTZ. The i.p. dose of test compounds ranged from 0.2 mmol/kg to 2.0 mmol/kg. The observation time of 30 min after s.c. PTZ injection was chosen since clonic seizures rarely occurred after times longer than 20 min with i.p. doses of compounds that prevent the initial series of clonic activity.

The dose-response data for test compounds are presented in Table 31. Nine compounds prevented the clonic seizures induced by the threshold dose of PTZ in a dose-dependent fashion. Isobutyric acid, 4-keto VPA, cyclohexylacetic acid, 5-cyclohexylmethyltetrazole and 5-isoamyltetrazole were inactive in the dose range studied. N,N-Diethylsuccinamic acid was inactive at doses between 0.2-2.0 mmol/kg but induced hyperactivity (rapid-circling activity) when injected alone in mice at doses of 3.0 mmol/kg. On the other hand, N,N-dibutylsuccinamic acid exhibited convulsant activity at

Table 31

Protection against PTZ-induced seizures[a] in mice by valproic acid and its analogues

Compounds	% Protected against clonic seizures Dose, mmol/kg					
	0.2	0.3	0.5	1.0	1.5	2.0
1. Valproic acid (VPA)	-	-	25	87.5	87.5	100
2. 2-Butylhexanoic acid	-	12.5	37.5	87.5	100	-
3. 1-Methylcyclohexane-1-carboxylic acid	-	-	12.5	37.5	75	87.5
4. 2,2-Dimethylbutyric acid	-	-	0	50	37.5	62.5
5. 3-Ethylpentanoic acid	-	-	-	12.5	25	62.5
6. Trimethylacetic acid	-	-	0	25	50	37.5
7. 5-Cyclohexylmethyltetrazole	-	-	-	12.5	12.5	0
8. Cyclohexylacetic acid	-	-	-	12.5	12.5	0
9. Isobutyric acid	-	-	-	0	-	12.5
10. 5-Isoamyltetrazole	-	-	-	12.5	-	0
11. 2,3'-Diene VPA	-	-	-	50	37.5	62.5
12. 2-Ene VPA	-	-	-	37.5	50	62.5
13. 4-keto VPA	-	-	-	12.5	-	0
14. 5-Heptyltetrazole	25	37.5	87.5	100	-	-
15. N,N-Diethylsuccinamic acid	-	-	-	12.5	-	0
16. N,N-Dibutylsuccinamic acid	-	-	Convulsant		-	-

[a] Anticonvulsant activity of compounds was evaluated 15 min after i.p. administration. Mice were observed for clonic spasms (>5 sec duration) within 30 min after 85 mg/kg s.c. PTZ administration. Adult male Swiss mice (CD1 strain, 20-32g) were used. Eight mice were used per dose of test compounds.

sub-lethal doses of 1.0 mmol/kg or less. The dose-response curves of active compounds are depicted in Figure 16. The dose-response curves of the most potent drugs, valproic acid, 2-butylhexanoic acid, 5-heptyltetrazole and 1-methylcyclohexane-1-carboxylic acid are relatively steep compared with the moderately active drugs.

Calculation of ED_{50} values was based on the method of Litchfield and Wilcoxon (192). Goodness-of-fit of the estimated curve to the observed dose-response data was tested using the Chi-Square test. Tables 32 and 33 show the ED_{50} values, slopes of dose-response curves and chemical structures of active compounds. The slope values in Tables 32 and 33 were determined by the method of Litchfield and Wilcoxon (192) and are the antilogarithm of the inverse of the slope constant in the dose-response curves. The dose-response curves did not deviate significantly from parallelism. The estimated ED_{50} of valproic acid, 0.70 mmol/kg is in the range of values of 0.57 mmol/kg (14) and 0.90 mmol/kg (40) reported in previous studies using the s.c. PTZ test. The anticonvulsant activity of five aliphatic acids studied have previously been examined in a single-dose study (10) where the endpoint used was the protection of mortality. The results of the mortality study are summarized in Table 34 along with the results of this study at 1 mmol/kg i.p. dose. As shown in Table 34, the mortality endpoint does not reveal the quantitative differences in activity of the five aliphatic acids compared to the discriminative clonic seizure test. Moreover, dibutylacetic acid was reported to be inactive at 1.39 mmol/kg, which is contrary to the results of the present

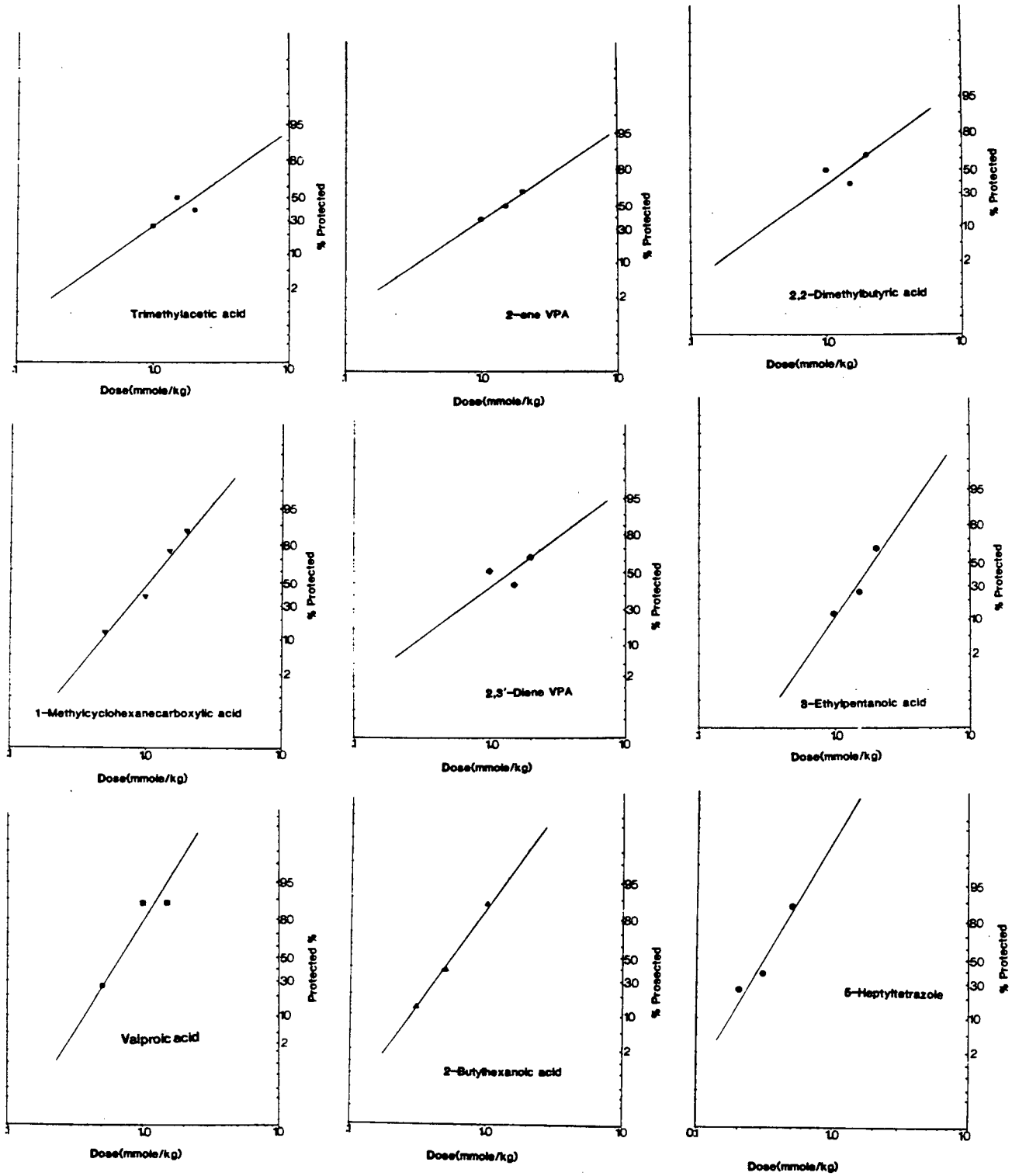


Figure 16. Dose-response curves of valproic acid and analogues using the subcutaneous pentylenetetrazole seizure threshold test in mice.

Table 32

Anticonvulsant potency of valproic acid and its analogues against the clonic phase of PTZ-induced seizures in mice

Compounds[a]	ED_{50}, mmol/kg, i.p. (95% confidence limits)[b]	Slope (95% confidence limits)[b]
1. 5-Heptyltetrazole	0.31 (0.23-0.42)	1.56 (1.22-2.00)
2. 2-Butylhexanoic acid	0.57 (0.33-0.97)	1.72 (1.02-2.91)
3. Valproic acid (VPA)	0.70 (0.50-0.98)	1.63 (1.14-2.33)
4. 1-Methylcyclohexane-1-carboxylic acid	1.08 (0.71-1.64)	1.83 (1.06-3.16)
5. 2,2-Dimethylbutyric acid	1.43 (0.56-2.58)	2.84 (0.72-11.2)
6. 2,3'-Diene VPA	1.45 (0.82-2.57)	2.76 (0.29-25.9)
7. 2-Ene VPA	1.46 (0.78-2.74)	3.06 (0.24-38.8)
8. 3-Ethylpentanoic acid	1.91 (1.43-2.56)	1.69 (0.81-3.52)
9. Trimethylacetic acid	2.02 (1.11-3.68)	2.88 (0.77-10.8)
10. Isobutyric acid	inactive	-
11. Cyclohexylacetic acid	inactive	-
12.5-Cyclohexylmethyltetrazole	inactive	-
13. 5-Isoamyltetrazole	inactive	-
14. 4-keto VPA	inactive	-
15. N,N-Diethylsuccinamic acid	inactive[c]	-
16. N,N-Dibutylsuccinamic acid	convulsant	-

[a]Drugs administered 15 min before s.c. 85 mg/kg PTZ injection. Dose range of 0.2-2.0 mmol/kg.

[b]Data analyzed by the method of Litchfield and Wilcoxon (192).

[c]Rapid-circling activity of test drug at 3.0 mmol/kg.

Table 33

Anticonvulsant potency of valproic acid and its analogues against the clonic phase of PTZ-induced seizures in mice. Dose range of acids, 0.2-2.0 mmol/kg

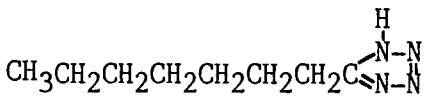
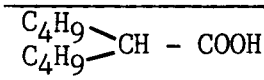
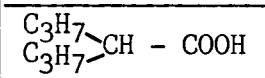
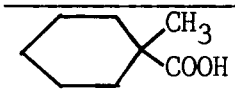
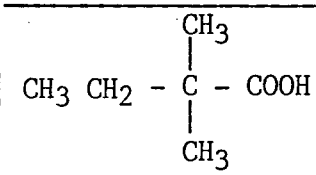
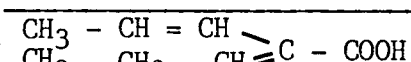
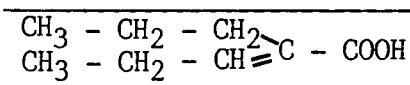
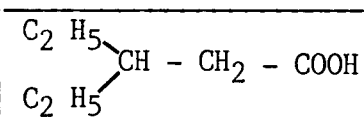
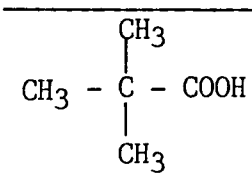
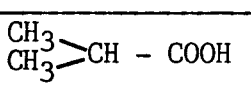
Compounds	ED_{50}, mmol/kg, i.p. (95% confidence limits)	Slope (95% confidence limits)
	0.31 (0.23-0.42)	1.56 (1.22-2.00)
	0.57 (0.33-0.97)	1.72 (1.02-2.91)
	0.70 (0.50-0.98)	1.63 (1.14-2.33)
	1.08 (0.71-1.64)	1.83 (1.06-3.16)
	1.43 (0.56-2.58)	2.84 (0.72-11.2)
	1.45 (0.82-2.57)	2.76 (0.29-25.9)
	1.46 (0.78-2.74)	3.06 (0.24-38.8)
	1.91 (1.43-2.56)	1.69 (0.81-3.52)
	2.02 (1.11-3.68)	2.88 (0.77-10.8)
	inactive	-

Table 33 (Cont'd)

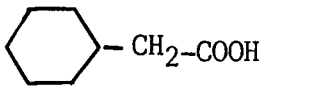
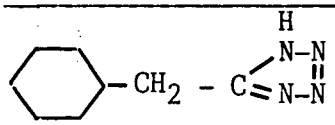
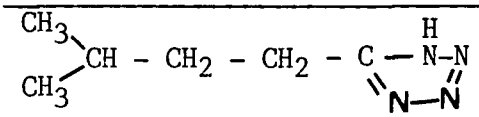
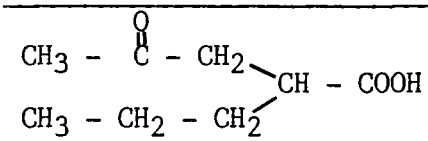
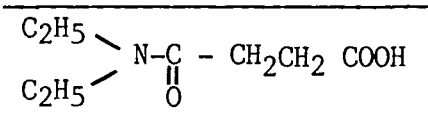
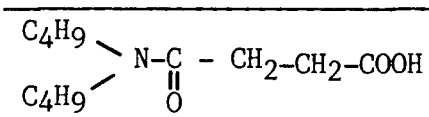
Compounds	ED_{50}, mmol/kg, i.p. (95% confidence limits)	Slope (95% confidence limits)
	inactive	-
	inactive	-
	inactive	-
	inactive	-
	inactive	-
	convulsant	-

Table 34

Anticonvulsant activity of VPA and its analogues on the threshold for PTZ-induced seizures determined by protection against clonic seizures and by percent mortality in mice

Compounds	% Protected Against (n = 8/dose)		
	Clonic Seizures[a]	Mortality[b]	Mortality[c]
1. Valproic acid (VPA)	87.5	100	100
2. 2-Butylhexanoic acid	87.5	100	0
3. 2,2-Dimethylbutyric acid	50	100	80
4. Trimethylacetic acid	25	100	80
5. Isobutyric acid	0	75	20
6. 3-Ethylpentanoic acid	12.5	100	
7. 1-Methylcyclohexane-1-carboxylic acid	37.5	100	
8. Cyclohexylacetic acid	12.5	100	
9. 2-Ene VPA	37.5	62.5	
10. 2,3'-Diene VPA	50	100	
11. 4-keto VPA	12.5	25	
12. 5-Isoamyltetrazole	12.5	62.5	
13. 5-Cyclohexylmethyltetrazole	12.5	75	
14. 5-Heptyltetrazole	100	100	
15. N,N-Diethylsuccinamic acid	12.5	62.5	
16. N,N-Dibutylsuccinamic acid	0	0	

[a]Activity at 1.0 mmol/g i.p. of test compounds administered 15 min before 85 mg/kg s.c. PTZ injection.

[b]Conditions same as in (a) above with mortality determined within 30 min after PTZ administration.

[c]Taken from G. Carraz (4) where 1.39 mmol/kg i.p. administered 30 min before 80 mg/kg s.c. PTZ administration.

study. Table 34 also shows that compounds inactive against clonic seizures would have been described as active by the mortality test.

2. Toxicity of compounds

The acute toxic effects of the test compounds were noted during the 15 min interval before injection of PTZ (Table 35). Sedation was a common side effect of the anticonvulsant compounds. Valproic acid showed remarkable sedation at 2 mmol/kg doses. The TD_{50} of valproic acid is reported to be 2.56 mmol/kg in mice (40). The unsaturated analogues of valproic acid and dibutylacetic acid exhibited marked sedation even at 1 mmol/kg doses while the most potent anticonvulsant 5-heptyltetrazole showed sedation at much lower doses. Dibutylacetic acid appeared to have the lowest protective index with its lethal effects occurring at 2.0 mmol/kg. Compared to the carboxylic acids, the tetrazoles possessed greater toxic properties with ataxia occurring at low doses. N,N-Dibutylsuccinamic acid had convulsant properties at sublethal doses of 0.5-1.0 mmol/kg.

Among the compounds studied, valproic acid appeared to have the most desirable pharmacological properties since it possessed high anticonvulsant activity with marked sedation appearing at doses greater than 2.0 mmol/kg. It is apparently not yet clear whether anticonvulsant and sedative effects occur at the same site in the CNS (14,30). It has been suggested that the distributional localization of anticonvulsant drugs may be the predominating factor to determine the sedative side effects (20,29). The unsaturated analogues of valproic acid showed more sedative effects and less anticonvulsant activity than valproic acid (Table 35).

Table 35
Observed toxic effects of test compounds in mice

Compounds	Observed Toxic Effects
A. Isobutyric acid 4-keto VPA Trimethylacetic acid 2,2-Dimethylbutyric acid 3-Ethylpentanoic acid Cyclohexylacetic acid	Minimal sedative effects at doses not greater than 2.0 mmol/kg.
B. Valproic acid (VPA) 1-Methylcyclohexane-1-carboxylic acid	Sedative effects at 2.0 mmol/kg dose.
C. 2,3'-Diene VPA 2-Ene VPA	Sedative effects at doses greater than 1.0 mmol/kg.
D. 2-Butylhexanoic acid	Sedation and ataxia at doses greater than 1.0 mmol/kg. Lethal dose at 2.0 mmol/kg.
E. 5-Cyclohexylmethyltetrazole 5-Isoamyltetrazole	Ataxia at doses greater than 1.0 mmol/kg. Prostration and abnormal spread of hind limbs at 2.0 mmol/kg dose.
F. 5-Heptyltetrazole	Sedative effects and ataxia at doses greater than 0.2 mmol/kg. Abnormal body posture. Occasional fasciculation at 1.0 mmol/kg dose.
G. N,N-Diethylsuccinamic acid	Abnormal spread of hind limbs at doses greater than 0.5 mmol/kg. Hyperactivity (rapid-circling activity) at doses of 3.0 mmol/kg
H. N,N-Dibutylsuccinamic acid	Tremors, jumping or hopping movements and convulsions at doses tested (0.5-1.0 mmol/kg). Tonic-clonic spasms. Lethal dose at 2.0 mmol/kg.

E. Structure-Activity Relationships

1. Quantitative Structure-Activity Relationships

The investigation of the molecular specifity of the anticonvulsant action of valproic acid analogues was one of the major objectives of this study. Towards this end, the Hansch linear free-energy model was applied to study the SAR among the structurally-diverse valproate analogues. Since the classical work of Meyer and Overton, it has become evident that lipophilicity plays a predominant role in determining the in vivo activity of many CNS-active drugs. According to the Hansch model, the probability that CNS-active drugs reach their site of action is determined by lipophilicity (log P). In addition, pharmacokinetic factors such as protein-binding, distribution, metabolism and excretion which play a major role in determining the efficacy of these drugs are at least governed by lipophilicity (193,194). On the other hand, steric and electronic factors appear to be critical for the pharmacodynamic action of drugs that arise by interaction with specific receptors.

In this study, the lipophilicity of compounds was described by the octanol-water partition coefficient (log P) values, obtained from HPLC studies using 70% methanol-phosphate buffer as the mobile phase. The electronic effects were represented by the pKa values of the compounds as determined in 10% methanol-water. The pKa values express the relative ionization strength of the compounds. The biological activity evaluated was the antagonism of PTZ-induced clonic seizures in mice. Table 36 shows the structure, anti-

Table 36

Biological data and physicochemical properties of compounds tested

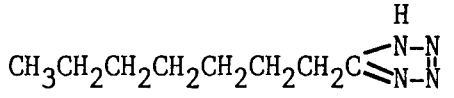
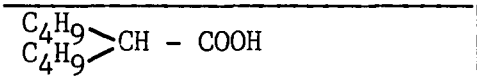
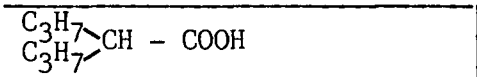
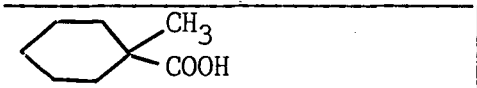
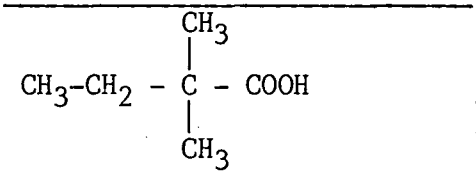
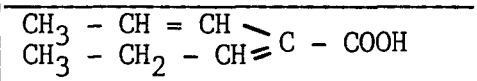
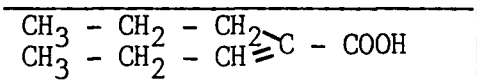
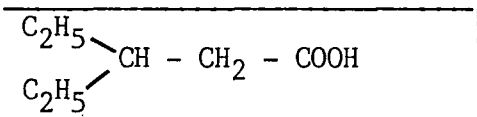
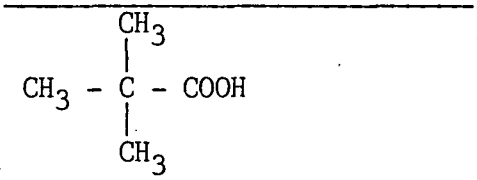
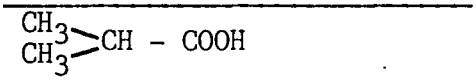
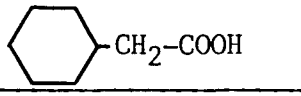
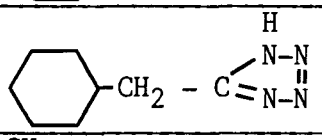
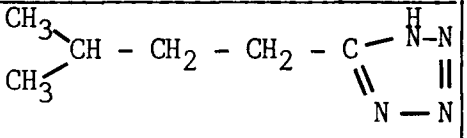
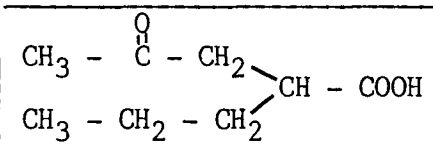
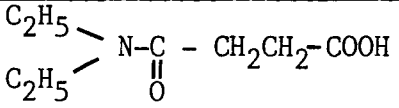
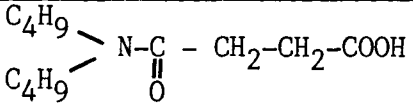
Compounds	ED_{50} mmol/kg i.p.	$E_s(R)$ *	log $\frac{1}{ED_{50}}$	log P (HPLC)	pKa (10% MeOH)	$E_s(R)$ **
	0.31	-0.35	0.509	2.01	5.31	-1.54
	0.57	-2.23	0.243	3.34	5.05	-3.47
	0.70	-2.11	0.155	2.57	4.95	-3.35
	1.08	-2.03	-0.0334	2.38	5.17	-3.27
	1.43	-1.60	-0.156	1.90	5.26	-3.41
	1.45		-0.161	2.27	4.02	-
	1.46		-0.164	2.44	4.36	-
	1.91	-2.00	-0.281	2.19	4.78	-2.17
	2.02	-1.54	-0.305	1.54	5.11	-2.78
	inactive	-0.47		1.10	4.96	-1.71

Table 36 (Cont'd)

Compounds	ED_{50} mmol/kg i.p.	$E_s(R)$ *	log $\frac{1}{ED_{50}}$	log P (HPLC)	pKa (10% MeOH)	$E_s(R)$ **
	inactive	-0.98		2.31	4.78	-3.22
	inactive	-0.98		1.59	5.37	-3.22
	inactive	-0.35		1.29	5.22	-1.59
	inactive			1.31	4.58	
	inactive			2.27	5.58	
	convul-sant			2.30	5.58	

* Taken from R. W. Taft (134).

** Taken from C. Hansch and A. Leo (152).

convulsant potency and physicochemical properties of valproic acid and the other analogues tested. The log P values range from 1.0 to 3.4 and pKa values from 4.0 to 5.6. The steric bulk of the alkyl substituents was described by the Taft steric constant, E_s* or Hansch steric constant, E_s**.

In accordance with the linear free-energy relationship model, log $1/ED_{50}$ was correlated with log P, pKa and E_s using multiple regression analysis (Michigan Interactive Data Analysis System, MIDAS). In Table 37 are listed the regression equations along with the multiple correlation coefficient, r (from residual and predicted values), the standard error of the regression, (S) and the attained significance level, p (from analysis of variance for the regression). From the correlation matrix in Table 35, it is observed that all interdependence among the variables of the nine active compounds are insignificant except between log P and E_s (r = -0.54). This is expected from the increase in log P with molecular size which in turn correlates with E_s (195).

For the nine active compounds, it can be seen from Table 37 that the regression equations 1-4 are not significant. The anti-PTZ potency could not be described by either log P (equation 1) or pKa (equation 2) alone. The correlation improved in equation 3 where both log P and pKa are included. However, equation 3 accounts for only 40% (r^2) of the variations in anticonvulsant potency.

Examination of the graphic plot of log $1/ED_{50}$ versus log P or pKa revealed that 5-heptyltetrazole was actually more active than anticipated. When 5-heptyltetrazole was deleted from the QSAR, the potency was better correlated with log P (Table 38, equation 5).

Table 37

Equations obtained correlating the anti-PTZ effect of valproic acid and analogues with their physicochemical parameters

Equation	n[a]	r[b]	S[c]	F	p[d]
1. $-\log ED_{50} = 0.223 \log P - 0.534$ ($\pm$0.186)[e] ($\pm$0.435)	9	0.413	0.264	1.442	0.269
2. $-\log ED_{50} = 0.254$ pKa $- 1.263$ ($\pm$0.215) ($\pm$1.05)	9	0.408	0.265	1.398	0.276
3. $-\log ED_{50} = 0.263 \log P +$ ($\pm$0.173) 0.300 pKa $- 2.09$ ($\pm$0.199) ($\pm$1.11)	9	0.631	0.243	1.986	0.218
4. $-\log ED_{50} = -0.265\ E_s - 0.517$	7	-0.291			

[a]number of compounds studied, [b]correlation coefficient,
[c]standard error of estimate, [d]level of significance of F-value,
[e]number in parentheses gives standard error of coefficient.

Correlation matrix for the physicochemical parameters with nine observations

	log P	pKa	E_s
log P	1		
pKa	-0.153	1	
E_s	-0.540	0.298	1

Table 38

Equations obtained correlating the anti-PTZ effects of VPA and analogues (excluding 5-heptyltetrazole) with their physicochemical parameters

Equation	n	r	S	F	p
5. $-\log ED_{50} = 0.322 \log P - 0.837$ $(\pm 0.079) \quad (\pm 0.188)$	8	0.856	0.110	16.48	0.0067
6. $-\log ED_{50} = 0.102\ pKa - 0.582$ $(\pm 0.180) \quad (\pm 0.876)$	8	0.225	0.207	0.321	0.59
7. $-\log ED_{50} = 0.331 \log P + 0.136\ pKa$ $(\pm 0.171) \quad (\pm 0.086)$ -1.516 (± 0.461)	8	0.907	0.098	11.56	0.013
8. $-\log ED_{50} = 0.065 \log P$ (± 0.622) $+0.052 (\log P)^2 - 0.536$ $(\pm 0.126) \quad (\pm 0.751)$	8	0.861	0.118	7.190	0.034
9. $-\log ED_{50} = 0.478 \log P$ (± 0.647) $-0.0297 (\log P)^2 + 0.147\ pKa$ $(\pm 0.130) \quad (\pm 0.107)$ -1.743 (± 1.12)	8	0.908	0.109	6.263	0.054

For meaning of n, r, s, F, P: refer to Table 35.

Correlation Matrix

	log P	$(\log P)^2$	pKa
log P	1		
$(\log P)^2$	0.991	1	
pKa	-0.0841	-0.0206	1

The addition of a pKa term to equation 5, gave a significant and better correlation (equation 7). The positive coefficient for log P and pKa in equation 7 indicated that an increase in log P and pKa enhanced the anticonvulsant activity. The same conclusion was apparent in equation 3 for Table 36. The relationship expressed by equation 7 agrees with earlier studies (196) which showed that lipid solubility and ionization constants of acidic and basic drugs are of greater importance in drug entry into the CNS. Since anticonvulsant activity of the carboxylic acids increases with high log P values, realization of an optimum log P value could be obtained from the parabolic relationship between in vivo biological activity and log P suggested by Hansch and co-workers (19,199). However, as shown in equation 9 (Table 38), the parabolic relationship in log P was not significant, probably because the log P values of the active compounds are less than the optimum log P value but fall into the linear portion of the curve.

The failure of log P and pKa to explain the variation in antagonism of PTZ by the tested compounds including 5-heptyltetrazole could be due to the absence of a physicochemical parameter to characterize the intrinsic activity of compounds arising from interaction with the recognition site. The Hansch model has been extended to replace the Hammet constant, σ, with other electronic parameters such as pKa, dipole moment and net atomic charges (205). However, the electronic effects expressed by the pKa values have been noted to have a dual function (197). The pKa affects the proportion of unionized drug which penetrates the blood brain barrier. It also describes the electronic effects of the alkyl

substituent on the charge density of the carboxylic group or the other polar moiety. Dipole moments have been shown to be useful in characterizing the polar moiety which may interact electrostatically at the recognition site (26-28,182).

Due to the possibility that alkyl-substituted anticonvulsant compounds act via interaction at the picrotoxin site of the GABA receptor complex, the physicochemical properties that determine anti-PTZ activity were investigated. The anti-PTZ potency following standard procedures and physicochemical properties of the other alkyl-substituted compounds, including the molecular dipole moments, were obtained from literature sources and are summarized in Table 39. The results of the QSAR are presented in Table 40.

It can be seen that the regression equations 1-7 are insignificant. Equations 1-3 show that log P, pKa or dipole moment (M) alone, do not account for the variations in anticonvulsant potency. Addition of a term in $(\log P)^2$ to equation 1 gives a slight improvement in the correlation (equation 4). The combination of log P and pKa (equation 5) or log P and μ (equation 6) also slightly improves the correlation in equation 1. Equation 8 gave the best correlation. Comparison of equations 7 and 8, demonstrates that equation 8 is superior in terms of statistical significance ($p = 0.01$) and reduction of the standard error of the estimate. Thus the regression equation with the added dipole moment term accounts better for the variation in anticonvulsant activity than that with the added pKa term. The optimum log P value in equation 8 below was 1.45. The negative coefficient of

Table 39

Anticonvulsant activity of various drugs against clonic seizures induced by PTZ (s.c. 85 mg/kg) in mice and their physicochemical constants

Compound	ED_{50} mmol/kg	$\log \frac{1}{ED_{50}}$	$\log P_{o/w}$	pKa	μ,[a,b] (debyes)
1. Valproic acid	0.70[c]	0.155	2.57[c]	4.95[c]	1.15
2. 2-Butylhexanoic acid	0.57[c]	0.243	3.34[c]	5.05[c]	1.15
3. Ethosuximide	0.922[d]	0.035	0.016[b]	9.1[e]	1.47
4. α,α-Dimethyl-succinimide	4.2[e]	-0.623	-0.49[f]	9.1	1.47
5. Pentobarbital	0.057[d]	1.244	2.19[g]	7.93[g]	1.13
6. Barbital	0.293[h]	0.533	0.68[g]	7.75[g]	1.13
7. Metharbital	0.051[h]	2.973	1.21[b]	8.45[i]	1.13
8. Butobarbital	0.085[h]	1.070	1.70[g]	7.81[g]	1.13
9. 5-Heptyltetrazole	0.31[c]	0.509	2.01[c]	5.31[c]	2.65
10. Dimethadione	5.75[b]	-0.760	-0.93[b]	6.13[i]	1.74
11. Trimethadione	2.083[d]	-0.319	-0.37[b]	-	1.74
12. Paramethadione	0.399[d]	0.399	0.13[b]	-	1.69
13. α,α-Dimethyl-γ-butyrolactone	3.51[e]	-0.545	1.01[f]	-	4.13
14. α,α-Ethylmethyl-γ-butyrolactone	1.17[e]	-0.157	1.51[f]	-	4.13

[a]Taken from A. L. McClellan (198).
[b]Taken from E. J. Lien et al. (28).
[c]Taken from this study.
[d]Taken from R. L. Krall et al. (38).
[e]Taken from W. E. Klunk et al. (47).
[f]Calculated using Hansch π-parameters, or fragmental constants (152).
[g]Taken from S. Toon and M. Rowland (194).
[h]Taken from A. Raines et al. (200).
[i]Taken from D. M. Woodbury et al. (46).

Table 40
Equations correlating anti-PTZ activity and physicochemical properties of anticonvulsants in Table 39

Equations	n[a]	s[b]	r[c]	F	p[d]
1. $-\log ED_{50} = 0.2961 \log P + 0.0327$	14	0.925	0.387	2.12	0.17
2. $-\log ED_{50} = 0.150\ pKa - 0.537$	10	1.10	0.232	0.454	0.52
3. $-\log ED_{50} = -0.37\mu + 1.03$	14	0.917	-0.406	2.37	0.15
4. $-\log ED_{50} = -0.873 \log P - 0.262 (\log P)^2 + 0.103$	14	0.859	0.573	2.70	0.11
5. $-\log ED_{50} = 0.586 \log P + 0.431\ pKa - 3.263$	10	0.900	0.668	2.81	0.13
6. $-\log ED_{50} = 0.286 \log P - 0.360\mu + 0.710$	14	0.874	0.552	2.41	0.14
7. $-\log ED_{50} = 1.11 \log P - 0.302 (\log P)^2 + 0.205\ pKa - 1.31$	10	0.830	0.772	2.95	0.12
8. $-\log ED_{50} = 1.14 \log P - 0.392 (\log P)^2 - 0.559\mu + 1.19$	14	0.643	0.811	6.40	0.01

[a]number of compounds used, [b]standard error of estimate,
[c]correlation coefficient, [d]level of significant of F-value and degrees of freedom.

Correlation matrix

	log P	$(\log P)^2$	pKa	μ
log P	1	0.8723	-0.5691	-0.0336
$(\log P)^2$		1	-0.7342	-0.1971
pKa			1	-0.3076
μ				1

$$\log 1/ED_{50} = 1.14 \log P - 0.392 (\log P)^2 - 0.559 \mu + 1.19$$
$$(n = 14, r = 0.811, s = 0.643, \log P_o = 1.45)$$

the dipole moment variable in equation 8 implied that an increase in dipole moment without the necessary log P value would decrease the anticonvulsant potency. Similar findings, indicating the role of dipole moment in anticonvulsant activity, have been reported in the literature. Lien et al. (26) reported that the best equation for phenyl- and alkyl-substituted heterocyclic antiepileptic drugs was

$$\log 1/ED_{50} = 0.852 \log P - 0.301 (\log P)^2 - 0.629\mu + 4.139$$
$$(n = 12, r = 0.915, s = 0.227, \log P_o = 1.42)$$

As in this study, highly active compounds in different classes of antiepileptic drugs were used in the regression analysis. Blair and Webb (27) investigated the relationship between anti-PTZ activity and physicochemical properties in a set of 1,4-benzodiazepines. The best equation was indicated to be

$$\log 1/ED_{50} = -0.50\mu + 3.26$$
$$(n = 52, r = 0.626, s = 0.866)$$

The equation showed that in a series of 1,4-benzodiazepines with similar log P values or with almost equal access to the CNS sites of action, dipole moment played the major role in determining the anticonvulsant activity.

The dependence of activity on dipole moment may explain the low structural specificity of anticonvulsant compounds in which steric effects are not apparent. Thus there is a continuous spectrum of structurally diverse compounds with varying molecular dipole moments. The physical meaning of the negative dependence of anticonvulsant activity on dipole moment is not clear. Those compounds with a low molecular dipole moment are not necessarily the most potent anticonvulsant compounds. The 1,4-benzodiazepines are reported to have higher dipole moments and log P values than other traditional antiepileptic drugs (26). The dipole moment of diazepam was found to be 2.65 debyes (26). However, the benzodiazepines show very high activity against PTZ-induced clonic seizures. The effectiveness of these compounds may be due to favourable conformations for hydrophobic binding and electrostatic interaction at target sites. Since the benzodiazepines could not be included in a series of anticonvulsants to develop QSAR (26), it appears that different classes of anticonvulsant drugs have characteristic molecular dipole moments that determine their electrostatic interaction at specific binding sites. It has been suggested that in whole animal studies, the significance of dipole moment in QSAR of anticonvulsant drugs may be indicative of dipole-dipole or charge-dipole interaction not only at sites of action but also at sites of nonspecific binding (20).

Pentylenetetrazole and 5-heptyltetrazole show opposing pharmacological effects, and have different physicochemical properties. 5-Alkyltetrazoles have an average dipole moment of 2.65 whereas 1,5-dialkyltetrazoles have an average value of 5.30 (198). Both

compounds are lipid-soluble while PTZ alone is readily soluble in water. PTZ, however, lacks acidic properties. It is obvious that in such comparisons, differences exist in hydrophilicity, dipole moment values and acidic properties (pKa). Also steric differences cannot be ruled out.

The dominating influence of dipole moment in the preceding regression equation does not rule out the role of pKa in determining anticonvulsant activity. It would be expected that among structural congeners such as the alkylcarboxylic acids that there will be high collinearity between pKa and μ as reported for homologous alkyl-substituted compounds (134). However, as shown in Table 40, for compounds with diverse polar groups the correlation between pKa and μ is low ($r = 0.3076$).

In this study, approximately 66% (r^2) of the variance in anticonvulsant activity of the structurally diverse alkyl-substituted anticonvulsants could be accounted for by lipophilicity and dipole moment (equation 8, Table 38). It appears unlikely that a near-perfect correlation could be obtained since the anticonvulsant activity and physicochemical properties were determined from different sources. In addition, the correlation in the QSAR study is limited by the complex nature of the biological test system, whole animal. The variability in the anticonvulsant potency could be influenced by structural effects on absorption, metabolism, distribution and excretion. These processes have also been variously reported to be governed by physicochemical parameters such as lipophilicity and pKa (193,194). Furthermore, steric factors could explain the residual variance unaccounted for by regression equations.

2. Structural features that enhance or diminish anticonvulsant activity

a. Aliphatic substituents

The lack of activity of isobutyric acid and reported inactivity of non-branched C_3H_7COOH to $C_5H_{11}COOH$ (12,13,20) may be due to their low lipophilicity. It appears that α or β-branching may be as effective as straight-chain alkyl substitution in conferring anticonvulsant activity. 5-Heptyltetrazole, with a straight-chain alkyl substituent possessed potent anticonvulsant activity. From QSAR studies it is known that aliphatic substituents increase the lipid solubility of compounds for entry into the CNS. The anticonvulsant activity of trimethylacetic acid and 2,2-dimethylbutyric acid probably reflect the stabilizing effect of multiple α-branching on oxidative metabolism or increased hydrophobic groups for binding to a hydrophobic portion of the recognition site. Increased hydrophobic binding by the C_7-chain may also account for the high activity of 5-heptyltetrazole.

Valproic acid and its analogues probably bind to a protein complex in the GABA-metabolizing enzyme system or the reported (30) protein complex of the GABA-benzodiazepine picrotoxin receptor complex. In vitro studies have shown that valproic acid (112) and other medium-chain fatty acids (199) bind with high affinity to bovine or human serum albumin. Bovine or human serum albumin has been used as a convenient model to study the molecular basis of specific-ligand protein interactions (112,199), since many drug receptors have recognition sites on protein subunits. Valproic acid and other medium chain fatty acids have been suggested, from in vitro protein-binding studies, to bind with high affinity to a

site on human albumin identical or close to the indole/benzodiazepine site (112,199). In a recent study, Brown et al. (199) investigated the albumin binding of normal aliphatic acids (pentanoic acid up to nonanoic acid) using ultrafiltration techniques. A single high affinity site was observed for each fatty acid with an increase in number of secondary sites with chain elongation. From the binding affinities and competitive binding data, the authors suggested that there are distinct albumin binding sites for the short-chain ($<C_7$) and the medium chain (C_8-C_9) fatty acids. On the other hand, the high affinity sites of long chain fatty acids are reported to be different from that of medium-chain fatty acids (112,199).

5-Isoamyltetrazole was inactive. The carboxylic bioisosteric analogue, isohexanoic acid has also been reported to be inactive (5). This could be due to the low lipophilicity of these compounds. Thus the effect of the isopropyl terminus could not be investigated in this series. The convulsant properties of barbiturates with the isopropyl and isopropenyl terminus (Figure 3) have been attributed to either a steric influence from conformation of these groups at the recognition site or to steric influence on oxidative metabolism at the (ω-1) position (19,99,100).

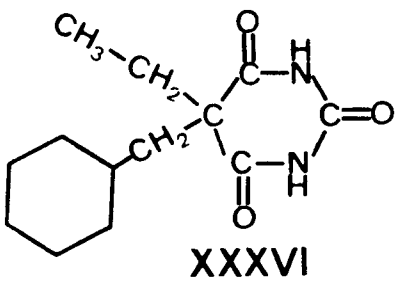
b. Alicyclic-substituents

Cyclohexylacetic acid and its tetrazole analogue, 5-cyclohexylmethyltetrazole were found to be inactive in the dose range studied (Table 29). However, 1-methylcyclohexanecarboxylic acid was relatively active even though the combined effect of log P and

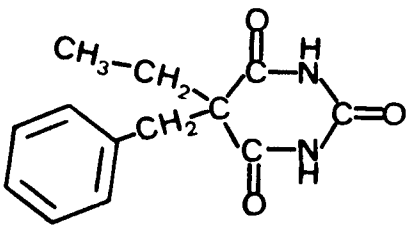
pKa values is similar to that of the cyclohexylmethyl-substituted compounds. Owing to the enormous difference in activity between the cyclohexyl and cyclohexylmethyl-substituted compounds, it is apparent that subtle steric effects were responsible for the difference in activity. The Taft steric parameter, E_s, has been frequently found to be inadequate in describing steric properties that influence biological activity (195). Literature E_s values showed covariance with log P values (Table 37).

The uniqueness of the cyclohexylmethyl group in reducing anticonvulsant activity was evident in other classes of anticonvulsants although it appears to have been overlooked. 5-Cyclohexylmethyl barbiturate, XXXVI, has been reported to have limited biological activity compared to barbital (201), while 5-ethyl-5-benzylbarbiturate, XXXVII, has convulsant properties (201). The urea derivative, XXXVIII (8), acylurea derivative, XL (89), and spirocarboxylic acid derivative, XLI (98), have been reported to have limited activity against PTZ-induced seizures. On the other hand, 1-methylcyclohexylurea, XXXIX (205), and the spiro carboxylic derivative, XXIII (88), have been found to be active against PTZ-induced seizures at comparative doses.

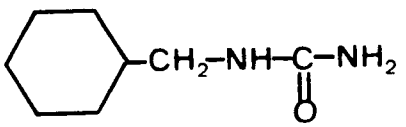
The lack of activity of cyclohexylmethyl-substituted compounds may be due to their inability to assume a required conformation to interact with binding sites. It seems possible that this may be due to in vivo metabolic effects. However, it is difficult to see how metabolic effects can uniformly apply to all the different classes and also not to those with cyclohexyl groups. The effects of structural features such as the cyclohexylmethyl group, benzyl group, isopropyl or isopropenyl group may due to steric



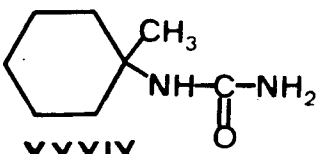
XXXVI



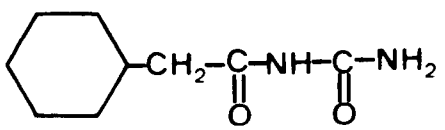
XXXVII



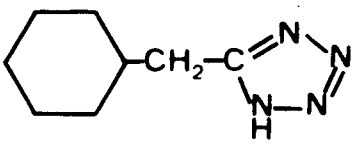
XXXVIII



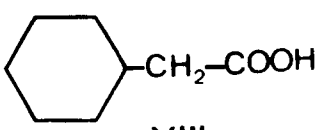
XXXIX



XL



XII



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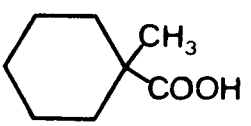
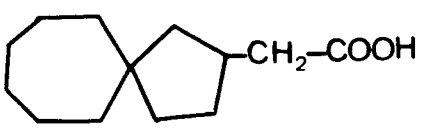
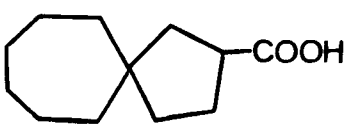


XIV



XLI



XXIII

Figure 17. Active and inactive alicyclic and alicyclicalkyl-substituted compounds.

interactions at the site of action. The molecular conformation of the convulsant barbiturates with an isopropyl or isopropenyl terminus in the butyl side chain (Figure 3) has been investigated using NMR spectroscopy (100) and molecular orbital calculations (99). It was suggested from these studies that the convulsant properties may be due to the steric influence of the isopropyl or isopropenyl terminus. Confirmation of the steric influence of these structural features will have to await results of further in vitro and in vivo structure-activity studies.

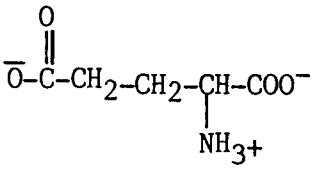
Recent studies on spiro analogues of valproic acid (88) and rigid cycloalkyl compounds (11) have been aimed at revealing configurations which would confer high activity of valproic acid analogues. In analogy to structures of the GABA agonists, isoguvacine and THIP (4,5,6,7-tetrahydro-isoxazolopyridin-3-ol), 1-methylcyclohexanecarboxylic acid derivatives will be of interest for future studies.

c. Effects of a polar functionality in the alkyl chain

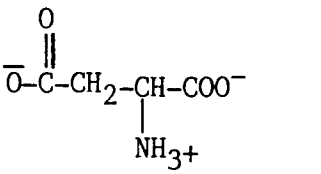
N,N-dibutylsuccinamic acid was found to possess convulsant properties while N,N,-diethylsuccinamic acid appeared to induce hyperactivity at the dose range tested. Both compounds have relatively high pKa and log P values when compared to the other compounds studied. Secondary and tertiary amides of aliphatic acid amides (3,4,50), succinic acid-bis-dialkylamides, tetra alkylureas (49), and polymethylene lactams (26) have been reported to have stimulant and/or convulsant properties. The N,N-dialkylsuccinamic acids and these compounds share a common functional

group, the substituted amido group. According to Lien et al. (26) the convulsant activity of some alkyl-substituted ureas and lactams increased with higher molecular dipole moments.

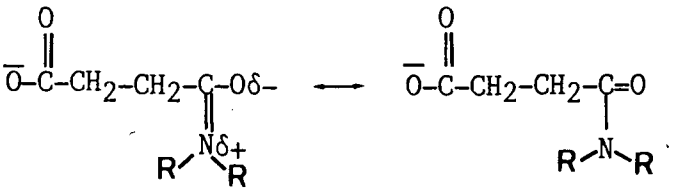
The two succinamic acids studied appear to show some structural similarities to the putative excitatory transmitters, aspartic and glutamic acids. It has been proposed that glutamic-like activity required a cationic and two anionic centres in the molecular structure (204). Due to amide resonance in the succinamic acids there could be two centres of negative charge and a positive centre but with charge separation different from that of glutamic acid or aspartic acid.



glutamic acid



aspartic acid



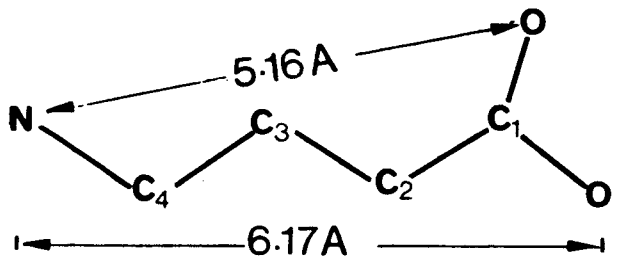
N,N-dialkylsuccinamic acid

Like PTZ, the two succinamic acids are water-soluble and lipid-soluble. It has been pointed out that an optimal balance of water and lipid solubility may also be important in the development

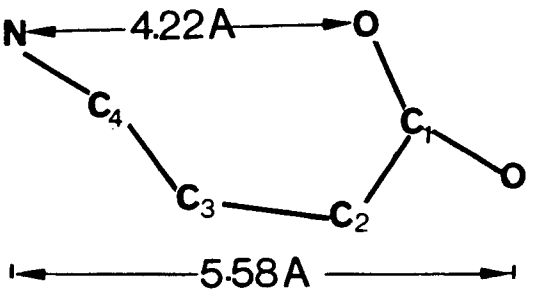
of convulsant activity due to distributional localization of compounds in the CNS (49). Succinamic acids and glutaramic acid are the open-chain forms of the anticonvulsant succinimides and glutarimides, respectively. They are reported to be formed in minor amounts from in vivo transformation of the succinimides and glutarimides (49,98). Published studies have indicated the lack of anticonvulsant activity of a series of N-alkyl-2-phenylsuccinamic acids (203) and slight convulsant activity of 3,3-ethylmethylglutaramic acid, the open-chain form of the convulsant bemegride^{R} (49).

d. Model showing selective effects of aliphatic and alicyclic substituents at the hydrophobic binding site

The results of x-ray or theoretical analysis on GABA structure have indicated the presence of two preferential conformers, one planar extended conformation, the other a non-planar folded conformation (Figure 18a). Ferrandes et al. (205) also determined the conformation of crystalline amides of valproic acid and pointed out that the molecular conformation of valproic acid may overlap with the GABA conformers. The dipropyl branched-chain was shown to adopt a planar extended conformation which is symmetrical with respect to the amido group (Figure 18b). Cohen-Addad (206) determined the molecular conformation of crystalline amides of 3-ethylpentanoic acid and indicated that the β,β-diethyl branched chain was not oriented symmetrically with respect to the amido group as in valproic acid (Figure 18c). However, the conformation of a segment of 3-ethylpentanoic acid could be compared to that observed for valproic acid. As shown in Figure 18, the geometry of the

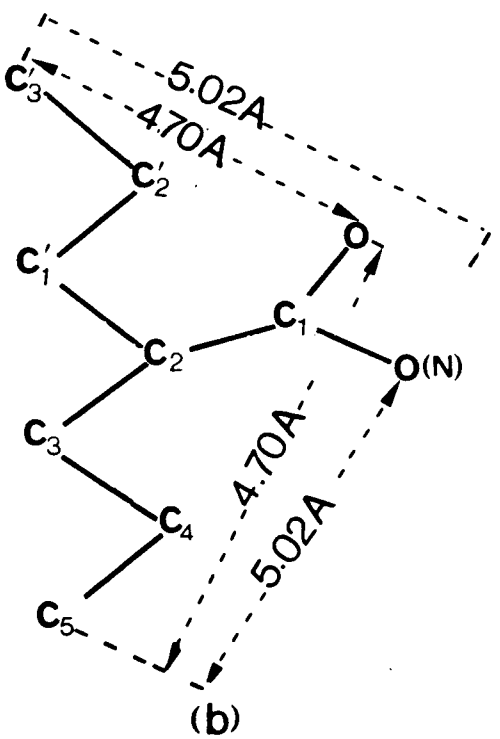


GABA extended planar conformation



GABA folded conformation

(a)



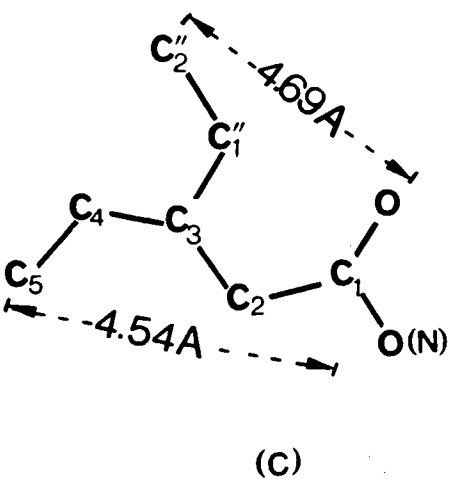


Figure 18. Conformation of (a) GABA, (b) Valproic acid amide, and (c) 3-ethylpentanoic amide.

atoms [C_1,C_2,C_3,C''_1,C''_2] of 3-ethylpentoic acid was shown to be equivalent to that of [C_1,C_2,C'_1,C'_2,C'_3] from valproic acid, and also the folded conformation of GABA. However, the ethyl groups in 3-ethylpentanoic are not stereochemically identical. In a previous single dose study (5), 3-ethylpentanoic acid was found to be inactive. This result is contrary to the present study which has shown its effectiveness in antagonizing PTZ-induced seizures (Table 31).

From the conformations shown in Figure 18, it is likely that the more stable conformations of the anticonvulsant compounds in this study will have segments of the alkyl chain almost indistinguishable from the folded or extended conformations of GABA. The flexible normal aliphatic side chain in 5-heptyltetrazole would likely adopt an equilibrium conformation that superimposes with the extended GABA conformation. Convulsant tetrazoles have been reported to interact at the picrotoxin site of the GABA receptor complex like valproic acid (7). Kraus (24) has also reported that valproic acid and its tetrazole analogue, 4-tetrazolylheptane inhibited succinic semi-aldehyde dehydrogenase, an enzyme in the GABA metabolism shunt, with inhibitory constants of 0.7 mM and 0.75 mM respectively. The conformation of the amide of the highly active dibutylacetic acid was also reported to be similar to that of valproic acid with the dibutyl alkyl chain oriented symmetrically to the amide group (206). Similarly the conformations of the alkyl chain in the unsaturated active compounds may overlap with either the extended or folded GABA conformation. The alkyl configurations in 2,2-dimethylbutyric acid and trimethylacetic acid would likely overlap with either the

extended or the folded GABA conformation.

In the active alicyclic-substituted compound, 1-methylcyclohexanecarboxylic acid, the semi-rigid cyclohexyl chain conformation (Figure 19) would likely conform to the GABA extended or folded conformation with overlap of the atoms [C_2,C_3,C_4] in Figure 18a. The cyclohexylmethyl group in the inactive compounds, 5-cyclohexylmethyltetrazole and cyclohexylacetic acid, appears to have segments which overlap with atoms [C_2, C_3, C_4] of the GABA extended or folded conformation (Figure 18a). However, in comparison with 1-methylcyclohexanecarboxylic acid, the presence of the methylene group between the polar functional group and the alicyclic ring may cause the terminal portion of the cyclohexyl ring to appear in the position occupied by the nitrogen group of GABA. Thus, unlike the β,β-diethyl chain in 3-ethylpentanoic acid, the presence of two methylene groups connecting C_2" and C_4 (Figure 18c) may explain the unexpected inactivity of cyclohexylacetic acid and 5-cyclohexylmethyltetrazole.

The structural model (Figure 19) proposed for the pharmacophoric features in valproic analogues is based on the inactivity and anticonvulsant activity of the carboxylic acids and tetrazoles studied. Both alkylsubstituted carboxylic acids and tetrazoles were included in the pharmacophoric model because of reports of possible similar binding sites of valproic acid and tetrazoles (33,34,55,57,104).

Although the evidence that valproic acid and convulsant tetrazoles enhance or diminish GABA-mediated postsynaptic inhib-

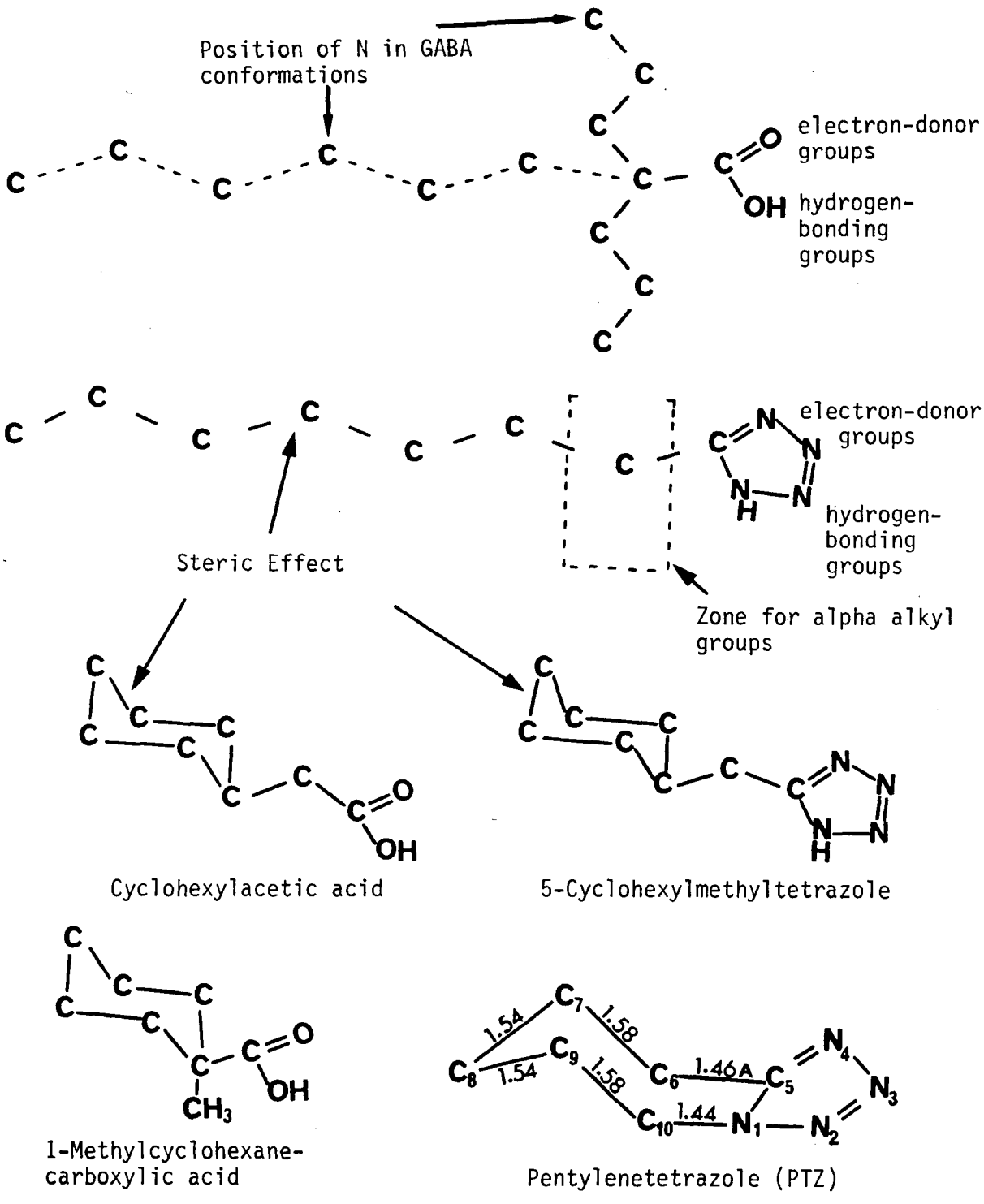


Figure 19. Model of pharmacophoric structural features in carboxylic acids and tetrazoles.

ition may not be strong or complete (compared to 1,4-benzodiazepines and barbiturates), there has been a growing body of evidence implying inhibition of GABA metabolism by valproic acid at synaptic sites (2). Valproic acid may be a potential substrate of GABA-transaminase or succinic semialdehyde dehydrogenase. Valproic acid and its tetrazole analogue, 4-tetrazolylheptane were shown to inhibit succinic semialdehyde dehydrogenase (24).

In the structural model (Figure 19), the alkyl chain probably binds to a hydrophobic site on the GABA recognition site. This hydrophobic site is probably between binding sites for the nitrogen and carboxylic groups in GABA. The possibility that GABA exists in either the extended or folded conformation has been considered in the proposed structural model. The lack of anticonvulsant activity of cyclohexylacetic acid and 5-cyclohexylmethyltetrazole is suggested to be due to steric effects at the hydrophobic site, close to the GABA nitrogen binding site on the GABA-receptor complex. The zone of alkyl groups at the α-position influences anticonvulsant activity as shown by the activity of α-cyclohexylpentanoic acid which was, however, reported to be less than that of VPA (5). Thus if metabolism effects on the cyclohexylmethyl-substituted and cyclohexylsubstituted compounds are similar, then the difference in activity is suggested to be due to conformational effects at the site of action.

The polar moiety plays a significant role in determining the anticonvulsant activity of compounds as shown by the presence of the dipole moment term in the QSAR. It is considered that favourable orientation of the electron-donor or hydrogen-bonding groups

in the carboxylic and tetrazole moieties, in addition to hydrophobic binding of alkyl chain, influence anticonvulsant activity. Valproic acid is strongly bound to the serum protein, albumin (112). A theoretical evaluation of the interaction between valproic acid and human serum albumin has been made by Andrews et al. (207). Valproic acid was indicated to meet the geometric requirements of the specific binding sites and the carboxylic group was implicated in the high strength of the non-covalent bonding.

The presence of polar functions in the alkyl chain of the succinamic acids may imply different dipole-dipole or dipole-charge interactions compared with that of valproic acid. It is likely that the convulsant or stimulatory succinamic acids interact at a different site from that of alkylsubstituted carboxylic acids and tetrazoles. The in vivo antagonism between valproic acid and N,N-dibutylsuccinamic acid was, however, not investigated.

The structure-activity correlations in this study implicate both the carboxylic and tetrazole analogues of valproic acid in the structural pharmacophore model shown in Figure 19. X-ray analysis of crystals of pentylenetetrazole-iodine monochloride complex by Baenziger et al. (208) indicated that the tetrazole ring is planar with the C_6-C_7-C_8-C_9-C_{10} alicyclic ring in the chair conformation (Figure 19). The electron-donor group, bound to the iodine-monochloride was found to be the nitrogen atom at position-4 of the tetrazole ring.

It has been reported that substitution of a methyl or isopropyl group at C_8 of pentylenetetrazole increased the stimulatory activity while substitution of similar alkyl groups at C_9 and C_{10} led to a decrease in stimulatory activity (58,59). It is conceiv-

able that substitution of alkyl groups in the pentylene chain of PTZ exerts stereochemical effects similar to that proposed for the carboxylic and tetrazole analogues of valproic acid (Figure 19). However, these possibilities can only be evaluated adequately when the x-ray structures of these compounds have been determined. It is also worth investigating if the preferential conformations in solution phase correspond to those in the solid state.

It appears that further structure-activity correlations of analogues of GABA, valproic acid,picrotoxin and pentylenetetrazole may provide supporting evidence for the active conformations and structural basis for interactions at the GABA-receptor complex. This could lead to compounds with greater specificity in their anticonvulsant actions.

SUMMARY AND CONCLUSIONS

A wide range of structurally-related analogues of valproic acid have been synthesized and examined for anticonvulsant activity. The series of analogues included compounds with α-alkyl substituents, β,β-dialkyl substituents, β-alkyl substituents, γ-alkyl substituents, alicyclic and alicyclicalkyl groups, an unsaturated alkyl chain and the tetrazole functional group as a bioisosteric function of the carboxylic group. The compounds were synthesized using known procedures.

A stereoselective method was used to prepare the diunsaturated analogues of valproic acid, 2-[(E)-1'-propenyl]-(E)-2-pentenoic acid and 2-[(Z)-1'-propenyl]-(E)-2-pentenoic acid. The stereochemical course of the deconjugative aldol-type reaction of ethyl 2-pentenoate with propionaldehyde was investigated using the (E) or (Z)-isomer of ethyl 2-pentenoate. The addition reaction was shown to occur with high regioselectivity and deconjugation occurred with high stereoselectivity. The β'-hydroxy-β',γ'-unsaturated products were dehydrated with various dehydration agents. The combination of methanesulfonyl chloride and KH resulted in the minimum number of diunsaturated VPA ethyl ester isomers. NMR spectroscopic data for each dienoate isomer was characterized following purification of the product mixture by argentation TLC. Using synthesized reference diunsaturated acids, the unidentified major diunsaturated VPA metabolite has been assigned the chemical structure, 2-[(E)-1'-propenyl]-(E)-2-pentenoic acid.

Lipophilic and electronic properties of test compounds have been determined experimentally to identify the molecular properties relevant to their anticonvulsant activity. Lipophilicity was described by the

octanol-water partition coefficient. The possibility of estimating octanol-water partition coefficients (P) of compounds of diverse structures by RP-HPLC was explored. A 5μm Hypersil ODS column was used. Different compositions of methanol-phosphate buffer (pH 3.5) or acetonitrile-phosphate buffer were investigated for their suitability as mobile phase to determine hydrophobic effects.

Analysis of the linear regressions of known log P values of reference substances and their log [capacity factors] for the different mobile phases culminated in the selection of 70% methanol-phosphate buffer and 50% acetonitrile-phosphate buffer as the mobile phases to be used in determining the HPLC log P values. The two mobile phases showed high correlation coefficients, good sensitivity to hydrophobic effects and eluted highly lipophilic compounds with minimization of non-equilibrium adsorption phenomena. HPLC log P values, determined from the regression equations, were found to be better than Hansch or Rekker predicted log P values in determining accurate log P values for highly substituted aliphatic and alicyclic carboxylic acids, heterocyclic tetrazoles and intramolecularly-bonded compounds. The accuracy of the HPLC log P values was verified by determining the shake-flask log P values of four compounds of diverse structure. An HPLC method was described for measuring compounds in aqueous solutions. Owing to its speed, high reproducibility, and ability to determine the log P values of unstable compounds, the RP-HPLC method complements and provides an alternative to the conventional shake-flask procedure.

The electronic properties of the test compounds were described by the apparent ionization constants. The ionization strength was determined with good precision by a potentiometric titration method. Mixed aqueous-methanol solvents were used because of the limited aqueous

solubility of the compounds. pKa Values in 10% methanol-water were found to be more meaningful than values in 50% methanol-water. The isosterism between carboxylic acid and tetrazole was indicated by the pKa values of tetrazoles which were just slightly higher than the corresponding carboxylic acids.

The standardized s.c. PTZ seizure threshold was used to establish the anticonvulsant potency of test compounds in mice. Anti-PTZ activity of each compound was determined at i.p. doses between 0.2 and 2.0 mmol/kg. The reliability of the pharmacological testing was shown by the close agreement of the measured ED_{50} of valproic acid to literature values. The s.c. PTZ seizure test was noted to be more selective in discrimination of the anticonvulsant potency than the PTZ mortality test used in previous studies of some aliphatic acids. 5-Heptyltetrazole was the most potent compound tested. However, valproic acid had the most desirable pharmacological properties in terms of high anticonvulsant activity and minimum qualitative toxic effects.

QSAR studies showed that the dependency of anticonvulsant potency on a combination of log P and pKa was statistically significant when 5-heptyltetrazole was deleted from the series of active compounds. The stated relationship suggested that the in vivo activity was governed by the proportion and lipophilicity of the unionized form for entry into the CNS. The failure of log P and pKa to account for the variation in potency of the nine active compounds including 5-heptyltetrazole was attributed to lack of an effective parameter to measure the electronic properties of the polar functional group which electrostatically interacts with the recognition site.

A comparison between pKa and dipole moment in describing the elec-

tron properties was undertaken using highly active alkyl-substituted compounds from different classes of anticonvulsant drugs. These alkyl substituted compounds have been suggested by various workers to have a common mechanism of action. The physicochemical properties and anti-PTZ ED_{50} values were obtained from literature sources. Regression analysis of the QSAR showed that for the different classes of alkyl-substituted compounds, the dependence of activity on log P and μ terms was statistically significant compared to dependence of activity on log P and pKa. Dipole moment values showed low covariance with pKa values. Similar findings of negative dependence of anticonvulsant activity on dipole moment have been reported for 1,4-benzodiazepines and other groups of anticonvulsants. Further studies may be required for elucidation of the negative dependence of activity on dipole moment.

A major finding of the structure-activity studies was the unique structural property of the cyclohexylmethyl configuration in greatly reducing the anticonvulsant activity of cyclohexylacetic acid and 5-cyclohexylmethyltetrazole. A model of the structural requirement at the site of action has been proposed to account for the activity and inactivity of valproic acid analogues. This model is based on the reported X-ray structure of GABA, crystalline amides of valproic acid, 3-ethylpentanoic acid, dibutylacetic acid, pentylenetetrazole and the hypothesized mechanism of action of valproic acid and pentylenetetrazole. The modulatory influence of the different aliphatic and alicyclic substituents probably results from binding to a hydrophobic site on the GABA-receptor complex or the GABA metabolizing enzyme system. The cyclohexylmethyl conformation was suggested to be less effective in hydrophobic bonding due to its steric effect at a stereoselective position on the hydrophobic recognition site.

The significance of this research lies in the delineation of the dependence of anticonvulsant activity on the physicochemical properties: lipophilicity, apparent acid ionization constant, dipole moment and steric factors. While the proportion and lipophilicity of unionized species governed their access to CNS sites of action, the dependence of activity on dipole moment may explain the diverse structures of anticonvulsants. There appears to be a provision that steric requirements of the hydrophobic binding site are accommodated in active compounds. Steric effects, as suggested to be present in cyclohexylmethyl-substituted compounds, may lead to inactivity or probably convulsant properties as in barbiturates with an isopropyl terminus or benzyl substituents.

A survey of the available literature data indicated that there has been no report of the high potency of a 5-alkyltetrazole (e.g. 5-heptyltetrazole) in antagonizing the convulsant effects of a closely-related analogue, pentylenetetrazole. These findings have pharmacological significance in elucidating the nature of the active site of valproic acid and its analogues. It can be postulated from the results of this structure-activity study that the activity of valproic acid, 5-heptyltetrazole, cyclohexylacetic acid, 5-cyclohexylmethyltetrazole, pentylenetetrazole and the other tetrazole and carboxylic acids may be governed by common structural requirements at a similar site of action.

This study also reports the stereochemical course of a stereoselective synthesis of 2-[(Z)-1'-propenyl]-(E)-2-pentenoic acid and 2-[(E)-1'-propenyl]-(E)-2-pentenoic acid. The major diunsaturated metabolite of valproic aid was identified. Studies of the toxic properties of the diunsaturated analogues of valproic acid could be

undertaken with due regard to their importance as metabolites of valproic acid.

The in vivo structure-activity studies are useful, despite the limitations of whole animal studies, for establishing correlations between in vivo and in vitro actions of valproic acid analogues.

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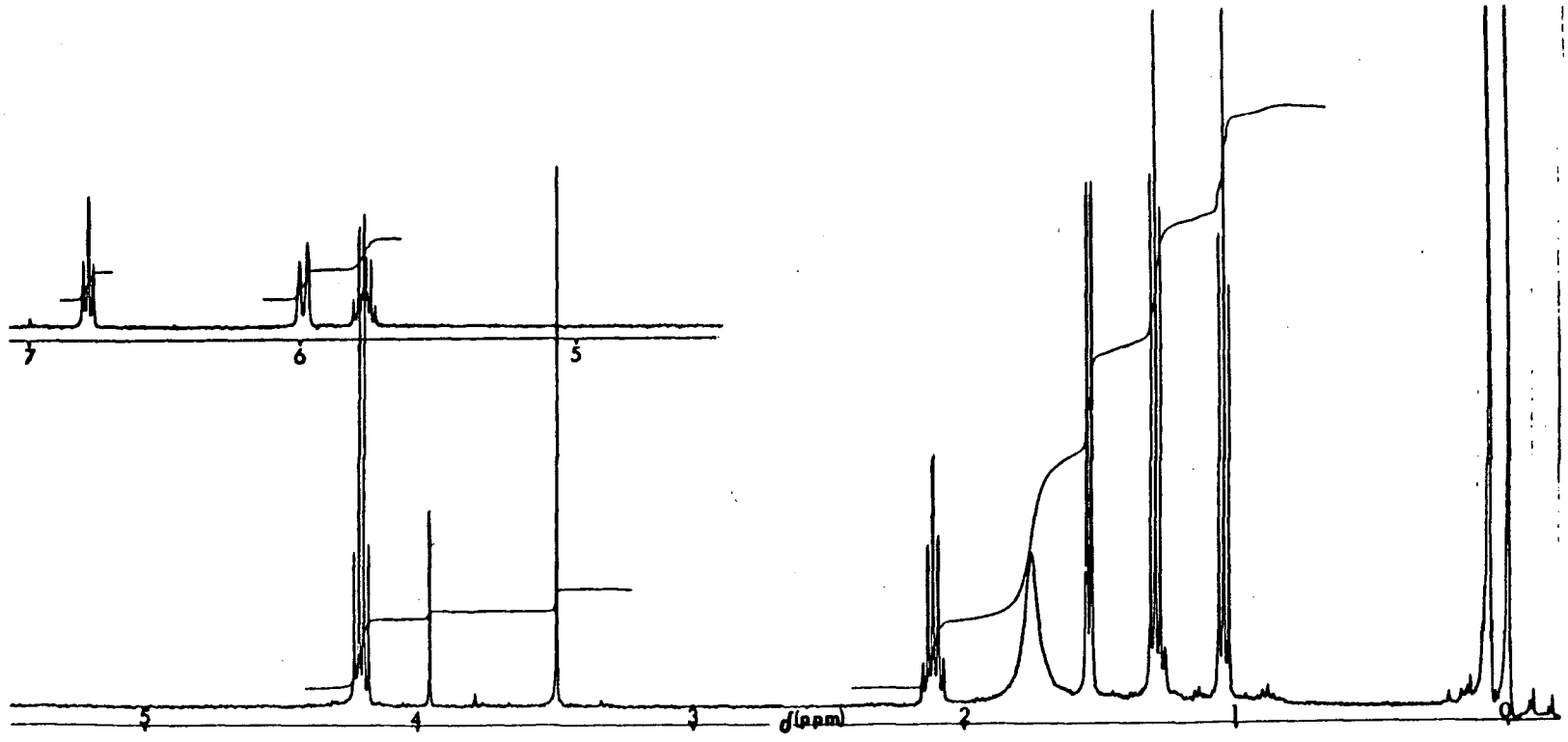
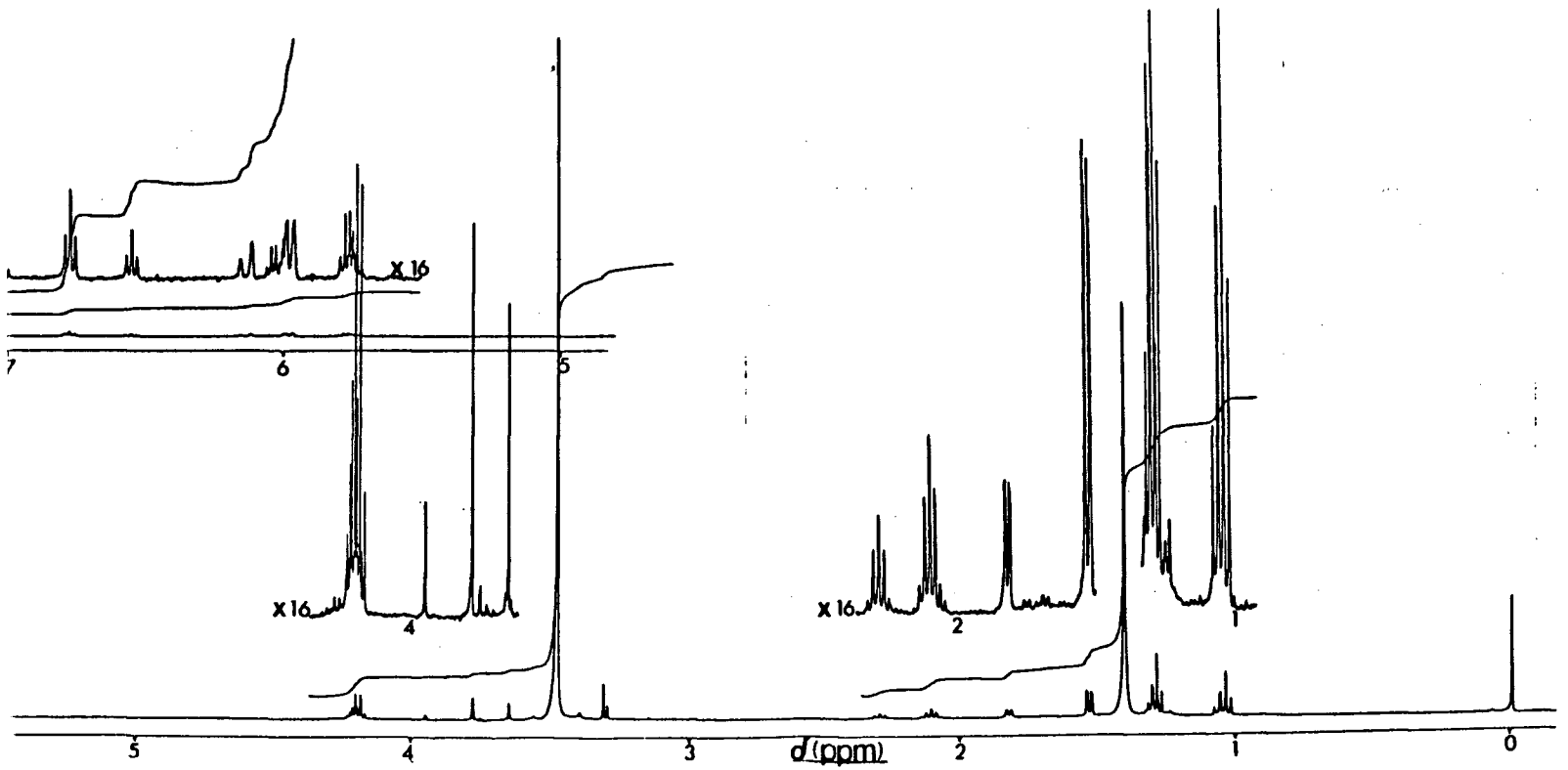
APPENDIX

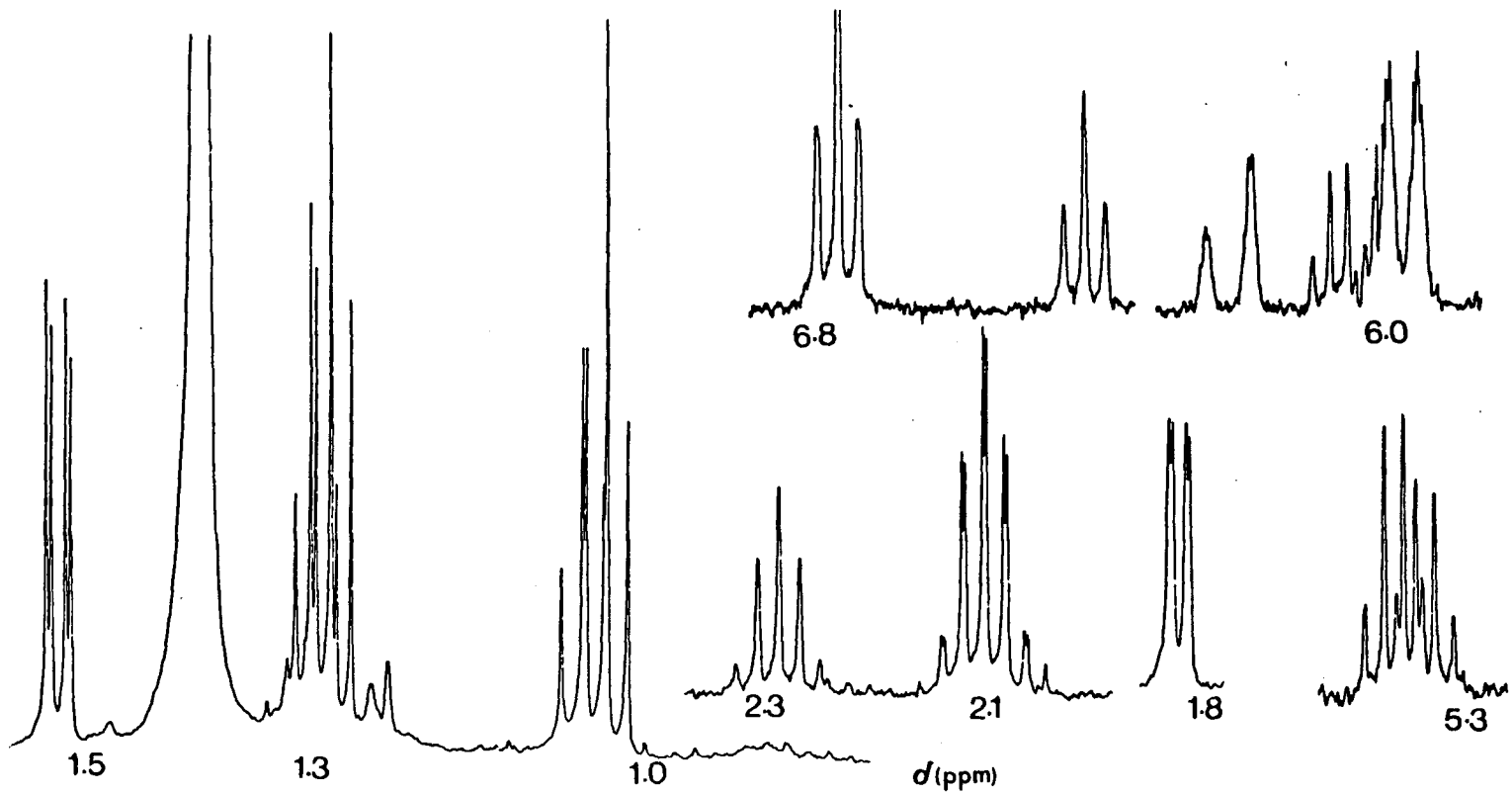
NMR spectra of some of the synthesized compounds.



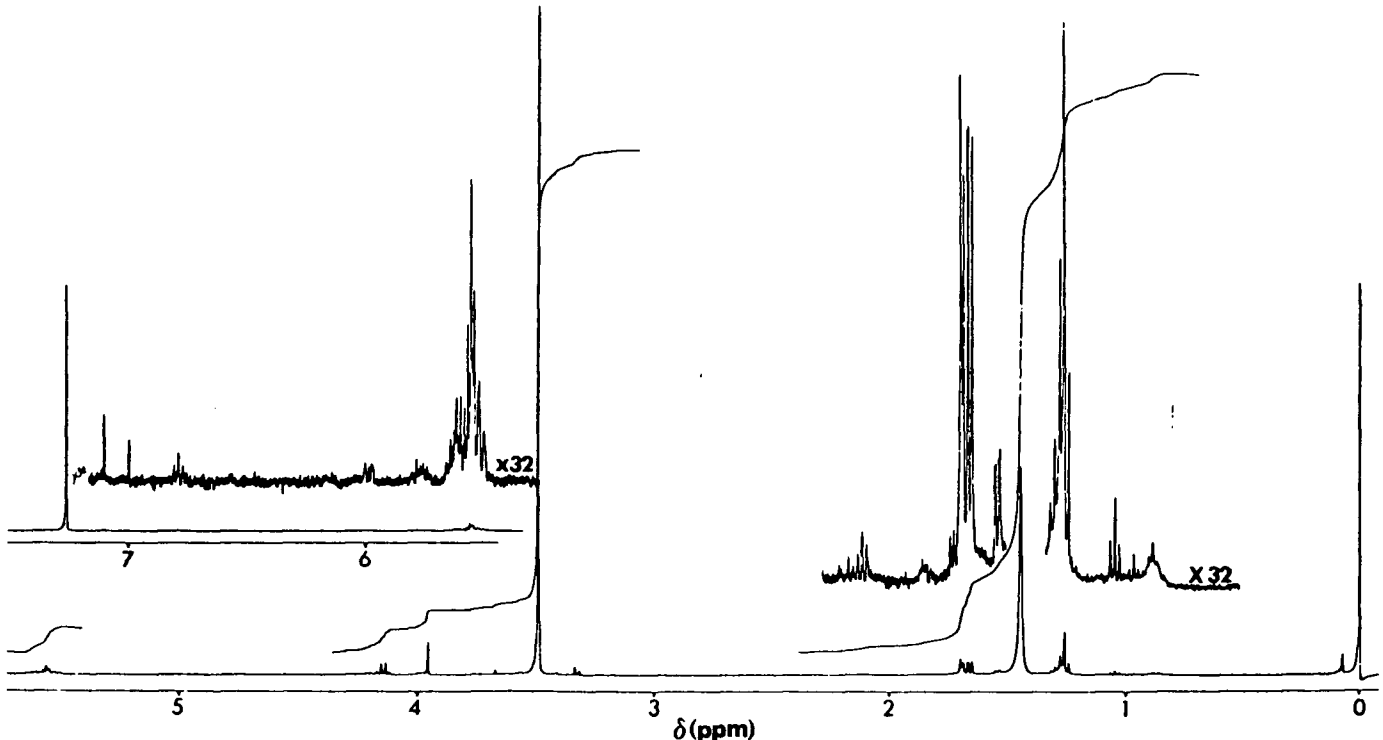
^{1}H-NMR (400 MHz) spectrum of ethyl 2-(Z-1'-propenyl) -E-2-pentenoate.



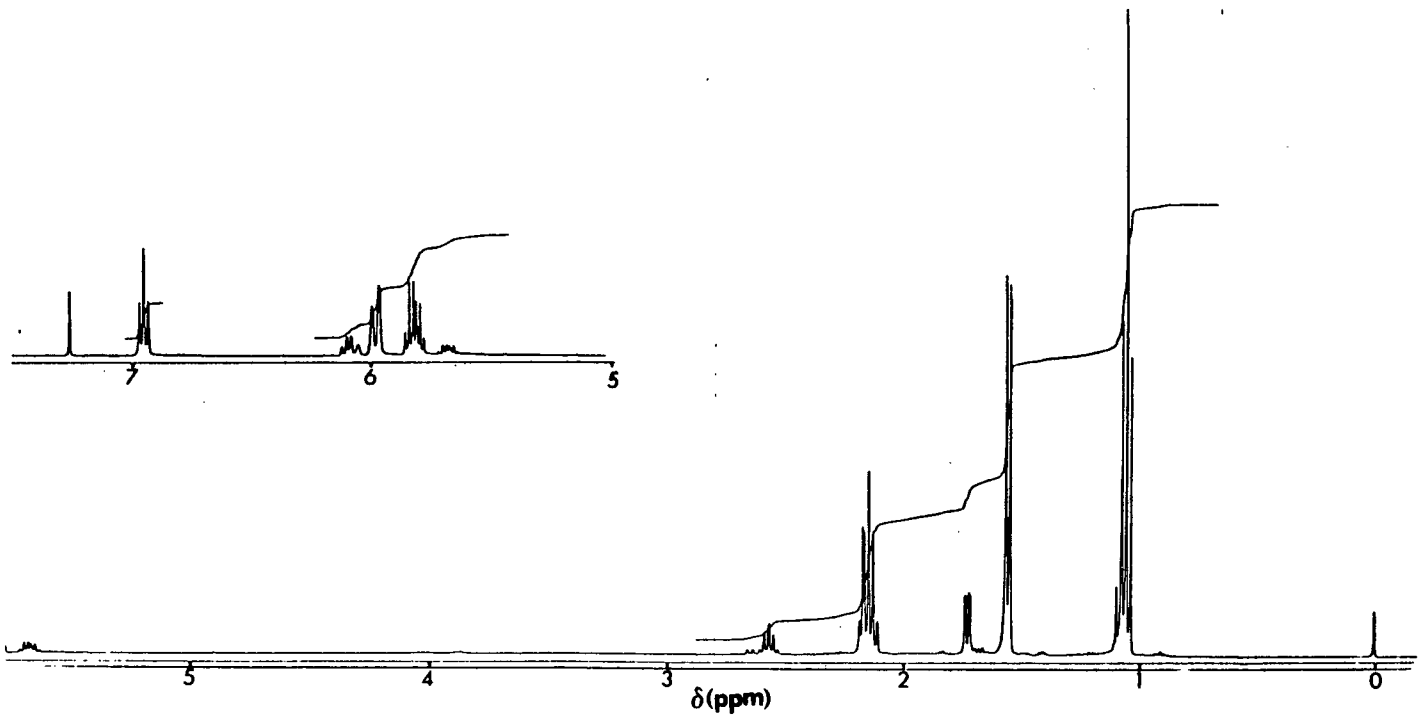
^{1}H-NMR (400 MHz) spectrum of isomeric mixture of ethyl 2-(Z-1'-propenyl)-E-2-pentenoate and ethyl 2-(E-1'-propenyl)-E-2-pentenoate.



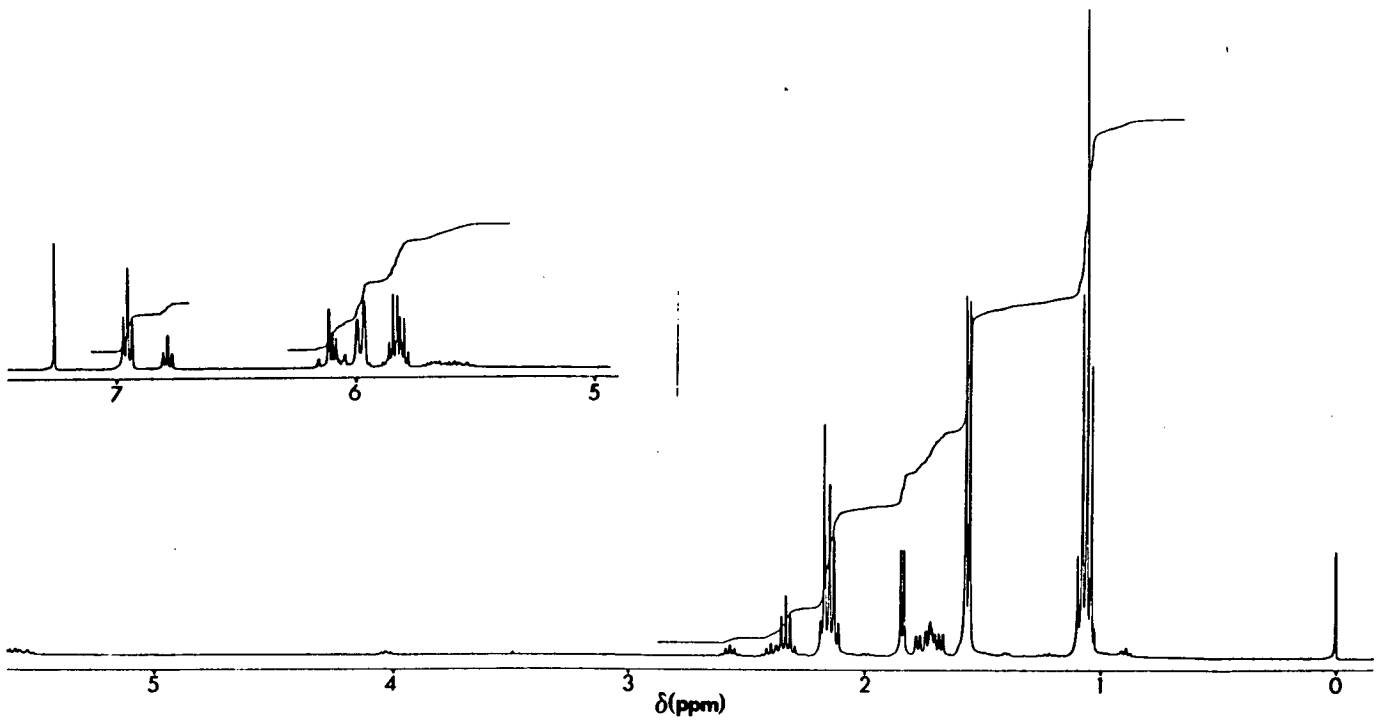
^{1}H-NMR (400 MHz) magnified spectrum of isomeric mixture of ethyl 2-(Z-1'-propenyl)-E-2-pentenoate and ethyl 2-(E-1'-propenyl)-E-2-pentenoate.



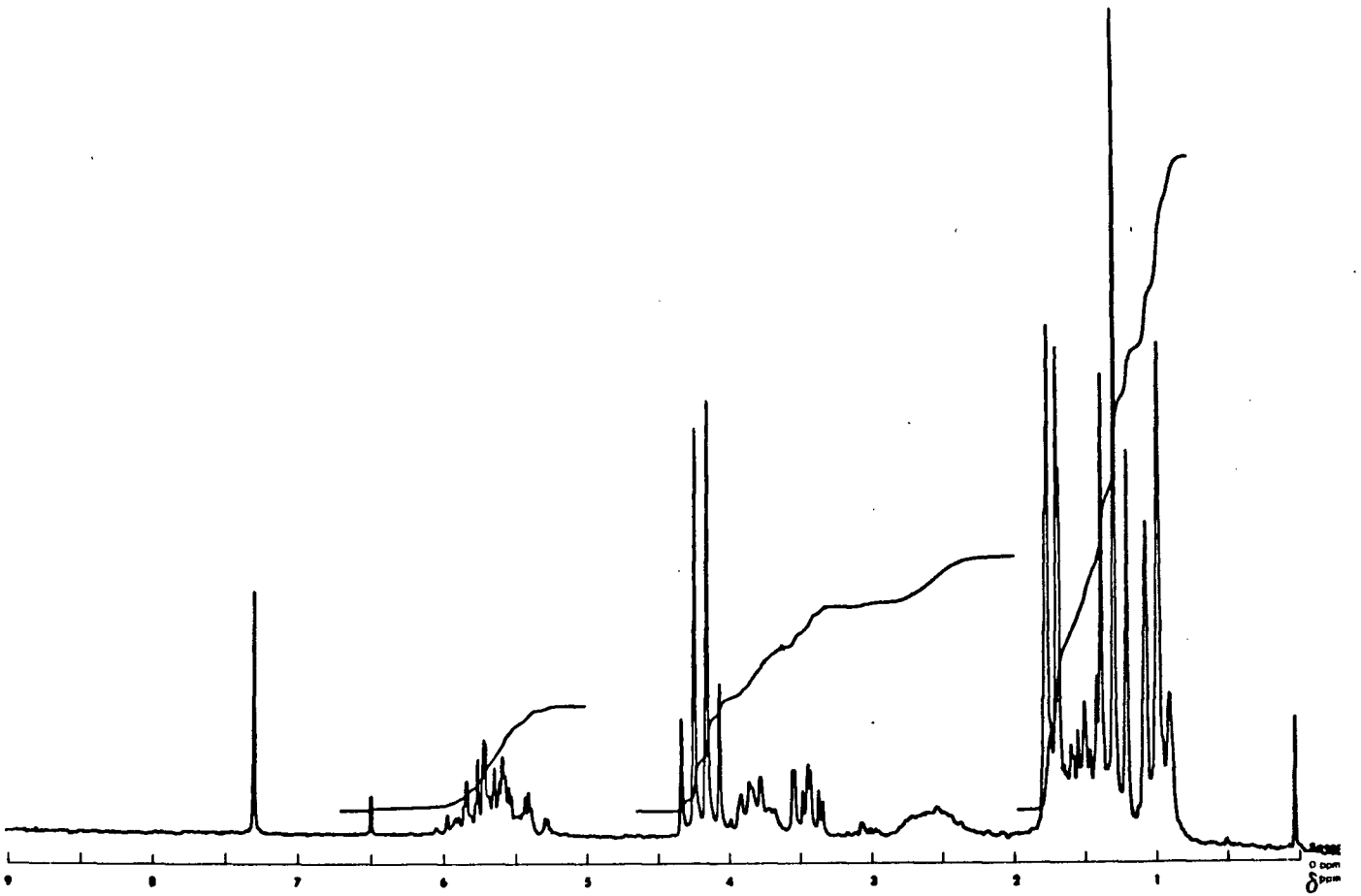
^{1}H-NMR (400 MHz) spectrum of ethyl 2-(Z-1'-propenyl)-Z-3-pentenoate with trace amount of ethyl 2-(Z-1'-propenyl)-E-2-pentenoate.



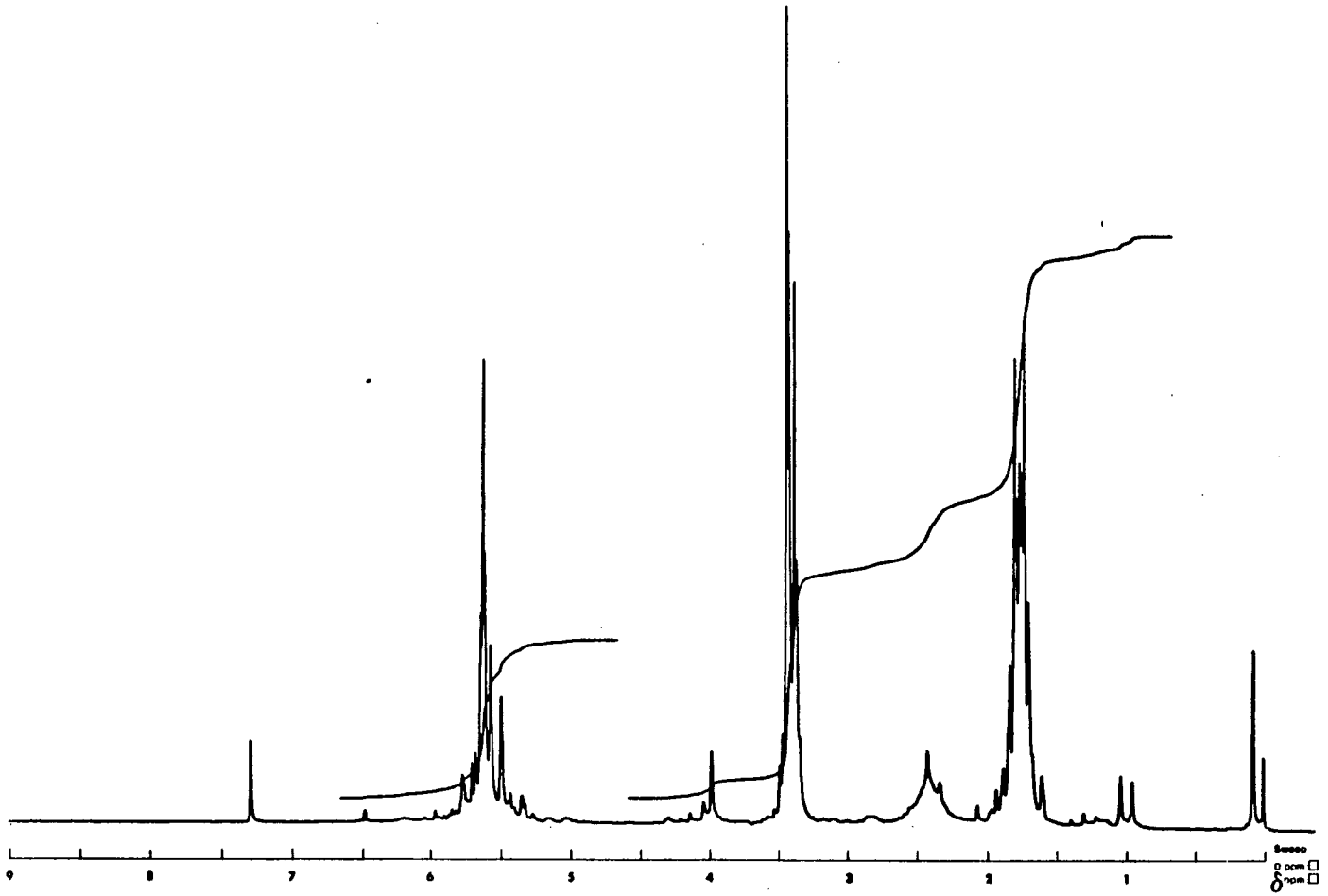
^{1}H-NMR (400 MHz) spectrum of 2-(Z-1'-propenyl)-E-2-pentenoic acid with trace amount of 2-(E-1'-propenyl)-Z-2-pentenoic acid.



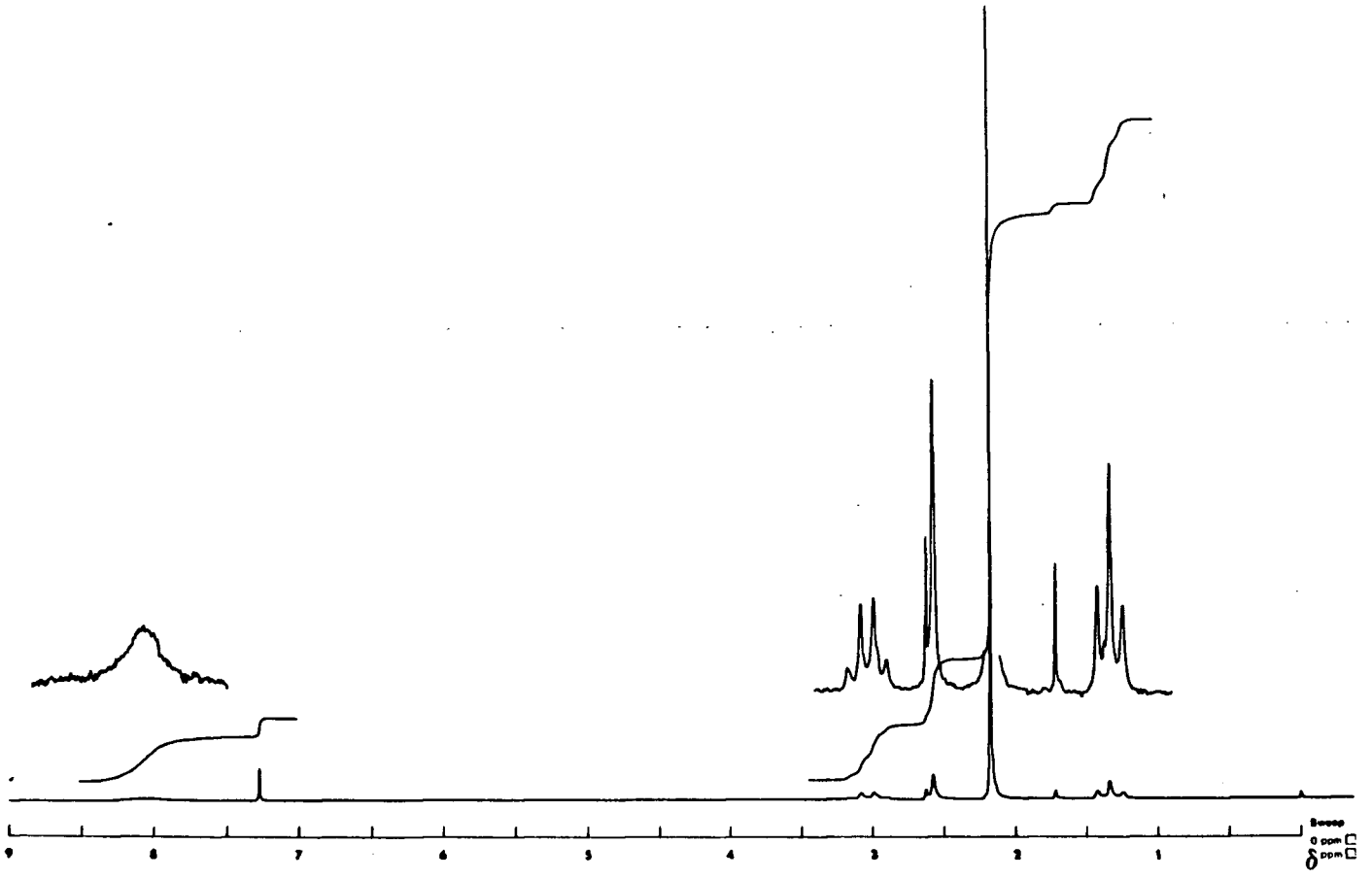
^{1}H-NMR (400 MHz) spectrum of isomeric mixture of 2-(Z-1'-propenyl)-E-2-pentenoic acid, 2-(E-1'-propenyl)-E-2-pentenoic acid, 2-(E-1'-propenyl)-Z-2-pentenoic acid, and 2-(Z-1'-propenyl)-Z-3-pentenoic acid.



^{1}H-NMR (80 MHz) spectrum of ethyl 2-(1'-propenyl)-3-hydroxypentanoate.



^{1}H-NMR (80 MHz) spectrum of 4-hydroxy-4-methoxymethylhepta-2,5-diene.



^{1}H-NMR (80 MHz) spectrum of N,N-diethylsuccinamic acid from the same synthesized product shown in Figure 6a.