UNSATURATED ANALOGUES OF VALPROIC ACID: STRUCTURE ACTIVITY RELATIONSHIPS AND INTERACTION WITH GABA METABOLISM

by

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ABSTRACT

Valproic acid (VPA) is a versatile clinical antiepileptic drug which is also characterized by rare but potentially fatal side effects such as hepatotoxicity and teratogenicity. Its principal metabolite, the α,β-unsaturated acid 2-ene VPA, appears to share most of VPA's therapeutic properties while lacking its toxicity and is thus a useful lead compound for the development of safer antiepileptic drugs. The objectives of this thesis were to shed some light on the anticonvulsant properties of 2-ene VPA analogues by an investigation of their influences on GABA metabolism and membrane fluidity.

A group of α,β-unsaturated acids were synthesized by established methods or minor modifications thereof. The compounds were then evaluated for anticonvulsant activity in mice using the subcutaneous pentylenetetrazole test. Cyclooctylideneacetic acid (compound **17**) exhibited a potency markedly exceeding that of VPA itself with no more than modest levels of sedation. Potency, as log(ED50), was highly correlated with both volume and lipophilicity rather than with one of the shape parameters calculated by molecular modelling techniques, arguing against the existence of a specific receptor site. These relationships remained essentially intact when ED50 was replaced with the brain concentration of the drug 15 min following an ED50 dose.

Subsequent studies focused on the properties of nerve terminals from whole brain homogenates prepared from mice administered an ED50 dose of each drug. GABA levels were generally found to be elevated, supporting the central role of this neurotransmitter in the anticonvulsant properties of VPA and its analogues. Selectivity for regional or functional pools of GABA was suggested as a cause for the variability. The activity of GABA's synthesizing enzyme glutamate decarboxylase was mostly unchanged but some drugs, notably compound **17**, showed a significant decrease in activity compared to the control. These influences on GAD activity were unrelated to the extent of binding of the enzyme's co-factor, pyridoxal 5'-phosphate, following *in vivo* administration of VPA, 2-ene VPA and compound

17. Finally, the activity of the GAD was found to be inhibited in a non-competitive manner by compound **17** with $K_i = 9$ mM.

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LIST OF ABBREVIATIONS

A^b	where A = X, Y, Z and b = -, +: shape descriptors
Å	Ångstroms
Anal.	elemental analysis
apo-GAD	apoenzyme form of GAD
aq.	aqueous
ATP	adenosine triphosphate
bp	boiling point
Bu	butyl
°C	degrees Celsius
calc.	calculated
CBZ	carbamazepine
CLZ	clonazepam
cm^{-1}	wavenumber
CNS	central nervous system
CSF	cerebrospinal fluid
d	doublet
δ	chemical shift (NMR)
Δ	heat or change
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DPH	diphenylhexatriene
$[drug]_{brain}$	concentration of drug in brain at 15 min following an ED50 dose
ED50	dose producing given effect in 50% of animals
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
2-ene VPA	(*E*)-2-propyl-2-pentenoic acid
eq.	equivalents

Et	ethyl
et al.	*et alia*
ETH	ethosuximide
g	gram(s)
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamate decarboxylase
GC-MS	gas chromatography-mass spectrometry
GHB	γ-hydroxybutyrate
h	hour(s)
HMPA	hexamethylphosphoramide
^{1}H-NMR	proton nuclear magnetic resonance
holo-GAD	holoenzyme form of GAD
HPLC	high pressure liquid chromatography
i.c.v.	intracerebroventricular
Im/Ac	imidazole-acetate (buffer)
i.p.	intraperitoneal
i-Pr	isopropyl
IR	infrared
I_{vv}	intensity of fluorescence in plane of excitation
I_{vh}	intensity of fluorescence perpendicular to plane of excitation
J	coupling constant
°K	degrees Kelvin
k'	capacity factor (chromatography)
K_i	inhibition constant
kg	kilogram(s)
kJ	kilojoules
K_m	Michaelis constant

log P	logarithm of *n*-octanol/water partition coefficient
m	multiplet
M	molar
M^+	molecular ion
mA	milliamperes
Me	methyl
MES	maximal electroshock
mg	milligram
MHz	megahertz
min	minute(s)
mL	millilitre
mM	millimolar
mm Hg	millimetres mercury (pressure)
mmol	millimoles
MsCl	methanesulfonyl chloride
MSTFA	*N*-trimethylsilyl-*N*-methyltrifluoroacetamide
MTBSTFA	*N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide
mp	melting point
mV	millivolts
m/z	mass-to-charge ratio
n	number of replicates
nmol	nanomoles
PB	phenobarbital
PBS	phosphate-buffered saline
PHT	phenytoin
PLP	pyridoxal 5'-phosphate
pmol	picomoles
ppm	parts per million

PTZ	pentylenetetrazole
Pr	*n*-propyl
q	quartet
Q	brain-to-body partition coefficient
QSAR	quantitative structure-activity relationship
r	fluorescence anisotropy
r	correlation coefficient
R	universal gas constant
RPLC	reverse phase liquid chromatography
s	singlet
s	standard error of the estimate
scPTZ	subcutaneous PTZ
SD	standard deviation
SIM	selected ion monitoring
SUCA	spiro[4.6]undecane-2-carboxylic acid
t	triplet
T	temperature in Kelvin
tert-BDMS	*tert*-butyldimethylsilyl
THF	tetrahydrofuran
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3(*2H*)-one
TLC	thin layer chromatography
TMA-DPH	(trimethylamino)diphenylhexatriene
TMS	trimethylsilyl
t_r	retention time
ug	microgram(s)
ul	microlitre
uM	micromolar
v	solvent volume

V	molecular volume
VPA	valproic acid
vs.	*versus*
w	weight

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1. INTRODUCTION

1.1 EPILEPSY

1.1.1. CLINICAL AND THERAPEUTIC ASPECTS

The commonly used term "epilepsy" refers to "a group of CNS disorders having in common the repeated occurrence of sudden and transitory episodes (seizures) of abnormal phenomena of motor (convulsion), sensory, autonomic or psychic origin" (Rall and Schleifer, 1990). Epilepsy occurs in 0.5-1.0% of the population (Zielinsky, 1982), being particularly prominent in children. These seizures can be classified into partial or generalized, depending on whether or not the onset can be traced to a discrete region, as well as primary (or idiopathic, having no apparent cause) or secondary (symptomatic, apparently resulting from a specific insult). Further descriptions are listed in Table 1.

Table 1
Properties, occurrence and therapy of the most common seizure types

Type	Symptoms and ictal EEG activity[a]	Relative abundance[b] (%)	Drugs of choice[c]
Partial			
Simple	No loss of consciousness; symptoms reflect affected brain region; usually no change in EEG	9.2	CBZ, PHT, (VPA), (PB)
Complex	Consciousness lost or blunted; may become generalized; automatisms typical; variable EEG	27.9	CBZ, PHT, (VPA), (PB)
Generalized			
Primary tonic-clonic	Loss of consciousness; stiffening then jerking of limbs; EEG: burst of spikes followed by spike-wave activity	28.1	VPA, PB
Secondary tonic-clonic	As above, but originates in discrete region	30.8	CBZ, PHT, VPA, PB

Table 1 (continued)
Properties, occurrence and therapy of the most common seizure types

Type	Symptoms and ictal EEG activity[a]	Relative abundance[b] (%)	Drugs of choice[c]
Generalized			
Primary absence	Blank stare ± brief automatisms; characteristic generalized 3 Hz spike-wave discharges in EEG	1.7	ETH, VPA
Myoclonic	Rapid jerks of face or extremities; multiple spikes or spike-wave EEG patterns	1.3	VPA, CLZ

a) Fukuzako and Izumi, 1991
b) Keränen *et al.*, 1988
c) CBZ = carbamazepine; PHT = phenytoin; VPA = valproic acid; PB = phenobarbital; ETH = ethosuximide; CLZ = clonazepam. Secondary agents are listed in parentheses. Adapted from Fisher, 1991.

As the table indicates, most seizures are caused by unusually high levels of brain activity. For example, tonic-clonic seizures are controlled by drugs such as phenytoin and carbamazepine that serve to limit the high-frequency neuronal discharges through voltage-dependent inhibition of sodium channels. Absence seizures, on the other hand, are effectively controlled only by drugs such as ethosuximide, a blocker of the Ca^{2+} T current. It is particularly important to note that carbamazepine and phenytoin are as ineffective in dealing with absence seizures as ethosuximide is with convulsive seizures. Nevertheless, valproic acid (VPA) has proven to be effective in both types of disorders, despite this apparent paradox. The means by which VPA exerts its antiepileptic action will be the subject of this thesis.

1.1.2. ANIMAL MODELS

Before delving deeper into the subject of antiepileptic drugs, it would be useful to discuss some of the properties of actual human epilepsy and how these are modelled when

evaluating anticonvulsants. As mentioned, epilepsy refers to a tendency to suffer recurrent seizures, meaning that a seizure resulting from an acute situation such as an alcohol overdose obviously does not represent epilepsy. Yet, the most common tests for antiepileptic activity, the subcutaneous pentylenetetrazole injection (scPTZ) and the maximal electroshock seizure (MES)(Swinyard *et al.*, 1989), are both clearly acute seizure models. In the scPTZ test, a rodent is administered 80-85 mg/kg PTZ into the loose skin at the back of the neck and then observed for tonic-clonic seizure activity. The MES test is similar except that electrical current is administered *via* corneal or auricular electrodes. The scPTZ test acts primarily to screen for anti-absence drugs because carbamazepine and phenytoin are ineffective here. On the other hand, this test also detects barbiturates that are ineffective for treating absence seizures. The MES test, on the contrary, is quite specific for drugs targetted against tonic-clonic seizures.

Table 2
Potencies of clinical anticonvulsants in rats using common screening methods (mg/kg i.p.)

Drug	MES[a]	scPTZ[b]	GHB[c]	GEPR-9[d]	Kindling[e]
Phenytoin	29	inactive	-	6	>100
Carbamazepine	8	inactive		3	30
VPA	490	180	+	150	120
Ethosuximide	>1200	54	+	230	inactive[f]
Clonazepam	186	0.06			
	inactive[g]	0.082[h]			
Diazepam	15[g]	1.8[h]			

a) ED50 for abolition of tonic hindlimb extension upon administration of supramaximal electroshock via corneal electrodes (50 mA: Swinyard *et al.*, 1989)
b) ED50 for abolition of tonic hindlimb extension upon administration of subcutaneous pentylenetetrazole (125 mg/kg: Swinyard *et al.*, 1989)
c) Ability to antagonize γ-hydroxybutyrate-induced absence seizure (Snead and Bearden, 1980; Vayer *et al.*, 1987b)
d) Dose for prevention of stimulated seizures (generalized tonic-clonic) in 50% of highly seizure-prone rats (Dailey and Jobe, 1985)
e) ED50 for prevention of stage 5 seizure following stimulus in rats kindled with corneal electrodes (Edafiogho *et al.*, 1992)
f) Sato *et al.*, 1990
g) As in a), but 150 mA via auricular electrodes (Loscher and Nolting, 1991)
h) As in b), but 90 mg/kg scPTZ (Loscher and Nolting, 1991)

1.2. VALPROIC ACID

1.2.1. CLINICAL USE AND TOXICOLOGY

VPA, also known as 2-propylpentanoic acid, is a simple α-branched aliphatic carboxylic acid whose anticonvulsant properties were discovered fortuitously in 1962 by Meunier *et al.*. Further work established its effectiveness in a wide range of seizure disorders (Davis *et al.*, 1994) leading up to its approval for therapeutic use in the United States in 1978. Recently, the drug has also been used successfully for bipolar illnesses (Hopkins and Gellenberg, 1994). VPA is well tolerated, only infrequently giving rise to gastrointestinal complaints and minor CNS effects such as sedation, ataxia and tremor (Rall and Schleifer, 1990). However, the drug has been found to produce a potentially fatal hepatotoxicity, particularly in young children on polytherapy with other medical conditions (Kaneko *et al.*, 1988; Omzigt *et al.*, 1992). This toxicity has been linked to the metabolism of VPA to 4-ene VPA and 2,4-diene VPA (Figure 1) leading to hepatic steatosis presumably through covalent binding to proteins (Prickett and Baillie, 1986; Porubek *et al.*, 1989) and/or depletion of glutathione *via* a Michael-type addition (Kassahun *et al.*, 1991). Nevertheless, it should be emphasized that since the vulnerability of this group has been recognized, the occurrence of VPA-associated fatal hepatotoxicity has declined to 1 in 50,000 (Dreifuss, 1989). Acute pancreatitis has also occurred occasionally, but although the symptoms are severe they are also usually reversible upon withdrawal from VPA therapy. Finally, it has been shown that children of epileptic mothers receiving VPA have a tendency to exhibit neural tube defects such as spina bifida although the occurrence is quite rare (Dreifuss, 1989). It has been shown (Nau *et al.*, 1991; Collins *et al.*, 1992) that unlike anticonvulsant activity, teratogenicity is strongly dependent on the configuration of C(2) in asymmetric VPA analogues (Hauck *et al.*, 1991), indicating a possible stereospecific drug-protein interaction.

As a consequence of these potentially serious side effects of VPA, there has been some interest recently in the use of 2-ene VPA, a major metabolite of VPA, as a replacement for the parent drug (Loscher, 1992). This metabolite is equipotent with the parent drug but

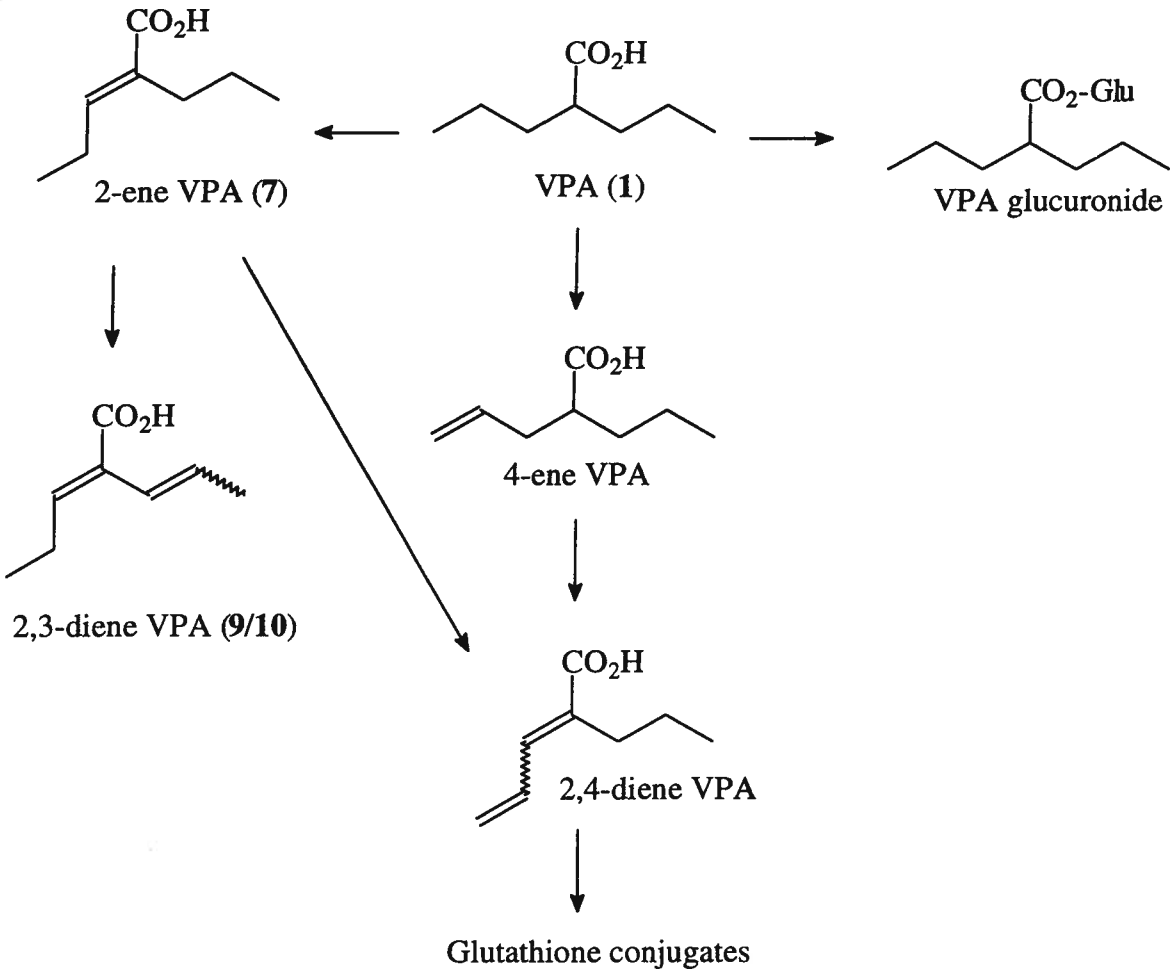


Figure 1. Some metabolic pathways of valproic acid (VPA).

does not exhibit apparent teratogenicity (Lewandowski *et al.*, 1986; Vorhees *et al.*, 1991) The hepatotoxicity of this compound also appears to be negligible (Kesterson *et al.*, 1984) despite its known conversion to the toxic 2,4-diene VPA (Kassahun and Baillie, 1993). This apparent contradiction is resolved by the fact that the formation of 2,4-diene VPA from 4-ene VPA occurs in the mitochondria while conversion from 2-ene VPA takes place in the cytosol. Consequently, 2-ene VPA shows promise as a less toxic successor to VPA.

Unlike other antiepileptic drugs, VPA has an intrinsically low potency. Initial daily doses are usually 15 mg/kg, increasing up to 60 mg/kg over several weeks (Rall and Schleifer, 1990). The human pharmacokinetics of VPA are characterized by high bioavailability and plasma protein binding (90%) at therapeutic concentrations of 0.21-0.69 umol/mL (Levy and Lai, 1982). Once inside the brain, the drug does not show any appreciable specificity as Goldberg and Todoroff (1980) have reported that VPA does not bind to rabbit brain homogenates or subcellular fractions of brain tissue. In patients, the brain levels were 6.8-27.8% of total plasma levels (Vajda *et al.*, 1981).

Following i.p. injection in mice, peak drug brain concentrations were reached within minutes (Hariton *et al.*, 1984), with CSF concentrations being generally equal to the plasma unbound fraction. The distribution of VPA and 2-ene VPA in the dog brain were studied in detail by Loscher and Nau (1983). They found 2-ene VPA to be the sole significant metabolite of the parent following either acute or prolonged VPA administration. Interestingly, it can be detected in the brain longer than VPA and while an MES anticonvulsant effect is still present. This has also been observed in patients and monkeys (Lockard and Levy, 1976). With regard to distribution, these workers noted that VPA failed to show any significant accumulation in any given brain region 7 h after a 40 mg/kg bolus. When the drug was administered at 225 mg/kg/day *via* osmotic minipumps and the brains analyzed at 1, 3 and 14 days, VPA and 2-ene VPA showed some accumulation in the substantia nigra but VPA was otherwise quite evenly distributed. In addition, 2-ene VPA showed elevated levels in the hippocampus, colliculi and medulla, which in some cases were up to one quarter of the VPA concentrations. These findings are supported by

autoradiography studies in the rat with [^{14}C]VPA that showed an even distribution throughout the brain, with some localization to the olfactory bulb (Schobben *et al.*, 1980).

The uptake of VPA into the brain has been assumed to occur *via* a saturable mechanism as the whole brain concentration was not found to increase proportionately with serum concentration 15 min postdose (Pollack and Shen, 1985). The "monocarboxylic acid" carrier is probably not involved here as Cornford (1983) failed to observe saturation of valproate uptake at 100 ug/mL. Likewise, Frey and Loscher (1978) demonstrated the existence of an active transport mechanism operating in the opposite direction across the blood-brain barrier because pretreatment with probenecid, which blocks carrier-mediated transport out of the brain, led to an increase in the CSF/serum concentration by 40% during i.v. infusion in dogs. Following efflux of the drug from the brain, elimination from the body is by hepatic processes that account for >96% of the administered dose (Levy and Shen, 1989).

This carrier-dependent mechanism of blood-brain barrier crossing has been questioned recently by Lucke *et al.* (1994) in light of data acquired using VPA-sensitive microelectrodes simultaneously sampling both blood and CSF *in vivo*. They found that the two VPA concentrations were in an equal equilibrium by 5 minutes, suggesting that the drug is able to rapidly and freely cross the barrier. It was argued that these results are in fact fully consistent with those described above, as the latter were based on measurements of VPA in whole brain rather than the extracellular compartment where the bulk of the drug resides (Lucke *et al.*, 1993). When this correction was made, a "good accord" was obtained with the microelectrode data.

An intriguing property of VPA that has defied explanation to date is why its potency increases with repeated dosing. For example, the protection of rats from amygdala-kindled seizures increased from 12% following a single dose at 200 mg/kg i.p. to 88% after seven such doses given three times per day (Loscher *et al.*, 1988). In humans, this phenomenon is manifested in the lack of a correlation between plasma VPA levels and seizure protection (Baruzzi *et al.*, 1977; Guelen and van der Kleijn, 1978; Dickinson *et al.*, 1979; Johannessen

and Henriksen, 1980; Nau *et al.*, 1981; Nau and Loscher, 1982; Snead and Miles, 1985). Steady state conditions are reached within 2 days but the onset of a useful anticonvulsant effect is several weeks. Similarly, there is a carryover effect lasting 2 weeks after cessation of therapy (Schobben *et al.*, 1980). The effect does not appear to be the result of an accumulation of more potent metabolites, Loscher (1981a) having shown that none are more potent than the parent, but bears an interesting resemblance to the drug's previously mentioned property of providing sustained seizure protection well after its plasma levels have fallen below the limit of detection and suggests an irreversible effect of some kind (Lockard and Levy, 1976).

The idea that the action of VPA is dependent on the accumulation of active metabolites was studied by Liu *et al.* (1990) using spiro[4.6]undecane-2-carboxylic acid (SUCA, Figure 2), a potent (scPTZ ED50 = 0.42 mmol/kg in mice) anticonvulsant chemically related to VPA but lacking significant Phase I metabolism. Thus, the anticonvulsant properties for this compound cannot be attributed to its metabolites. The study found that SUCA, like VPA, failed to show a correlation between serum levels and the time-course of the anti-PTZ anticonvulsant effect. Therefore, it was concluded that VPA's prolonged duration of action need not depend on the formation of active metabolites.

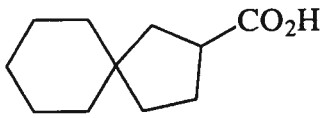


Figure 2. Spiro[4.6]undecane-2-carboxylic acid

A final note of caution needs to be made with regard to the markedly different pharmacokinetics of VPA in rodents compared to humans (reviewed by Loscher, 1985). This difference is especially prominent in mice, where it takes the form of a short half-life (0.8 *vs.* 9.3-18.4 h), reduced bioavailability (34-47 *vs.* 70-100%) and a lesser degree of protein binding (12 *vs.* 80-95%). Thus, there are limitations to using the mouse as an *in vivo* model for the behavior of antiepileptic drugs in humans.

1.2.2. PHARMACODYNAMICS

1.2.2.1. VPA AND GABA

1.2.2.1.1. THE GABA HYPOTHESIS

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the mammalian brain. It is a target for various anticonvulsants and sedatives-hypnotics, such as benzodiazepines and barbiturates, most of which influence the GABA receptor rather than GABA release. GABA's life cycle is depicted in Figure 3. It is synthesized from glutamate by glutamate decarboxylase (GAD) as part of the "GABA shunt" from the tricarboxylic acid (TCA) cycle prior to release and uptake by neurons and glia. In neurons it then undergoes transamination by GABA transaminase (GABA-T) to succinic semialdehyde which is then primarily oxidized to succinate and leads back into the TCA cycle. A small fraction of the semialdehyde is reduced to γ-hydroxybutyrate, a reaction that would be considered insignificant were it not for the fact that γ-hydroxybutyrate is a known convulsant (Marcus *et al.*, 1967) as well as being a neurotransmitter in its own right (Vayer *et al.*, 1987a). This compound also has strong links to absence seizures as illustrated by the markedly enhanced binding of [^{3}H]γ-hydroxybutyrate in lateral thalamic nuclei of the genetic Wistar rat model of absence epilepsy, in comparison with control animals (Snead *et al.*, 1990).

The above depiction of GABA is somewhat complicated by the existence of at least two distinct pools within the nerve terminal. This idea evolved from the experiments of Abe and Matsuda (1983) where [^{14}C]glutamate and [^{3}H]GABA were injected intracerebroventricularly in mice and the relative content of [^{14}C]GABA and [^{3}H]GABA then monitored in synaptosomes on the assumption that the former represented newly synthesized GABA and the latter newly taken up GABA, respectively. It was found that the GABA-T inhibitor aminooxyacetic acid caused a three-fold increase in the levels of [^{3}H]GABA with no change in the [^{14}C]GABA. Also, the release of [^{14}C]GABA from synaptosomes was 50% higher than that of [^{3}H]GABA. These results suggested that newly taken up GABA is simply degraded by GABA-T and is not recycled significantly back into the pool responsible for the

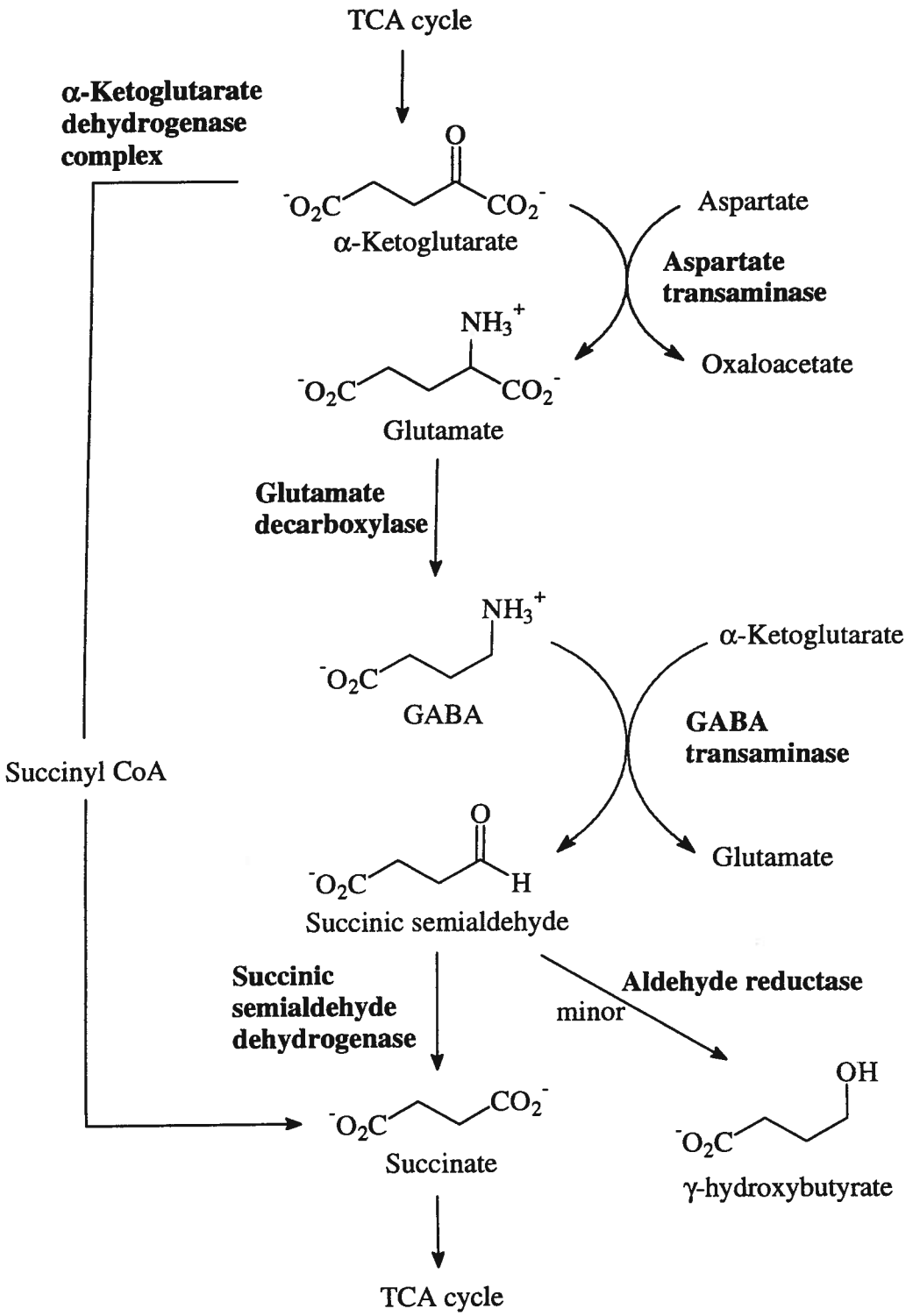


Figure 3. The metabolism of GABA (adapted from Meldrum, 1985).

GABAergic signal across the synapse. This scheme was further elaborated by Sihra and Nicholls (1987) and Wood *et al.* (1988), who proposed the existence of an uptake pool that equilibrates slowly with a neurotransmission pool of comparable size and, unlike the latter, releases GABA in a Ca^{2+}-independent manner upon depolarization.

Although the finding that VPA increases brain GABA levels has become generally accepted since it was first reported by Simler *et al.* (1968) and Godin *et al.* (1969), it is still unclear how this is accomplished and whether a simple increase in this inhibitory neurotransmitter can account for the drug's anticonvulsant properties. It has been found that ED50 doses of VPA and 2-ene VPA can significantly elevate GABA levels in whole brain synaptosomes (Loscher *et al.*, 1981), indicating an increase of this neurotransmitter in the nerve terminal compartment. Furthermore, VPA can also enhance the potassium-induced release of GABA from both cortical slices (Ekwuru and Cunningham, 1990) and cortical neurons in culture (Gram *et al.*, 1988). This was supported by the finding of Concas *et al.* (1991) that while the drug has no effect *in vitro* on the binding of [^{3}H]GABA, [^{3}H]flunitrazepam or [^{35}S]TBPS (a competitive antagonist at the GABA recognition site), when administered intraperitoneally prior to the assay the binding of [^{35}S]TBPS was markedly inhibited, an effect greatly potentiated by the co-administration of very low doses of diazepam, a facilitator of GABA binding. As there was no effect on the binding affinity, this result was interpreted as an increase in GABA release.

A temporal correlation between anticonvulsant effect and GABA levels was demonstrated in a more extensive study by Nau and Loscher (1982) who followed GABA, GAD and GABA-T in mouse brain homogenates, as well as electroconvulsive seizure threshold, up to 16 h following a 200 mg/kg (1.4 mmol/kg) i.p. dose of VPA. They observed parallel sharp rises in GABA, GAD and the seizure threshold that peaked at about 30 min before commencing first a rapid decay ending at 2 h and then a much slower one over the course of the remainder of the experiment. GABA and GAD were significantly elevated up to at least 8 h and returned to control levels by 16 h. No change in GABA-T was noted throughout the experiment. Similar results were obtained by Horton *et al.* (1977), Schechter

et al. (1978) and Chapman *et al.* (1984), also pointing to a distinct relationship between GAD, GABA and anticonvulsant activity.

Further evidence for the central role of GABA in the actions of VPA was provided by Kerwin *et al.* (1980) in a study that demonstrated altered GABAergic activity without measuring GABA itself. These workers showed that in rats the onset of full anticonvulsant efficacy (MES test) varied in a dose-dependent manner from 2 min (400 mg/kg i.p. VPA) to 10 min (100 mg/kg VPA) but did not appear to coincide with a reduction in firing of GABA sensitive neurons in the pars reticulata of the substantia nigra, that appeared at a fairly constant latency of 4-6 minutes. Unfortunately, the number of nigral cells sampled never exceeded five for any dose (100, 200 or 400 mg/kg) and none of these showed an unambiguous trend in activity. The situation for cortical cells was identical except for the 200 mg/kg dose where the decrease in firing rate was unequivocal (n = 16, of which only 3 showed no change) and coincided with the onset of seizure protection. Because these cells were inhibited by microiontophoretic application of GABA, this positive temporal correlation provides strong support for GABA as the prime effector of the anticonvulsant effect.

The uptake of GABA was unaffected in rat brain homogenate, cortical brain slices, or synaptosomes (Balcar and Mandel, 1976; Loscher, 1980; Ross and Craig, 1981; Slevin and Ferrara, 1985). While Hackman *et al.* (1981) observed a nearly complete blockade of GABA uptake in frog lumbar spinal cord slices at 10 μM VPA, a similar effect was also observed for glycine uptake, casting doubts on the specificity of this inhibition as well as the validity of this model system. Inhibition of GABA uptake was also inferred by Klee *et al.* (1985) on the basis that the chloride current elicited by microiontophoretically applied GABA in frog dorsal root ganglion neurons was enhanced by 1-10 mM VPA much more effectively in 90 mM external sodium than in a sodium-free solution. Like other neurotransmitters, the uptake of GABA is driven by the sodium potential gradient.

The above observations are not consistent with a post-synaptic site of action resulting in feedback inhibition and an accumulation of GABA in the presynaptic nerve ending, implying that the GABA increase could only be effected by either an inhibition of GABA

degradation or an increase in GABA synthesis (Loscher, 1993a). In contrast, both pre- and post-synaptic mechanisms are apparently viable for 2-ene VPA because this drug exhibited a 44 ± 6% displacement of [^{3}H]GABA from its $GABA_A$ receptor at 1 mM *in vitro*, a concentration at which VPA was ineffective (Nau and Loscher, 1984).

The VPA/GABA hypothesis has been elaborated by the suggestion of Iadarola and Gale (1981), based on the abilities of drugs such as aminooxyacetic acid (AOAA) to greatly increase GABA levels without possessing significant anticonvulsant activity, that GABA exists in both metabolic and neurotransmitter pools with VPA acting specifically on the latter. This was demonstrated in an experiment where the substantia nigra of rats was transected on one side of the brain in order to destroy the nerve terminals and hence the neurotransmitter pool of GABA. The substantia nigra on the transected side contained twice as much GABA following a dose of AOAA than an equipotent one of VPA, where the level was comparable to that of the control. Thus, VPA was ineffective in enhancing GABA outside the nerve terminal. On the other hand, when the transected GABA levels were subtracted from the total GABA levels of the intact side, the resultant (nerve terminal) concentration of GABA following VPA was much higher than for AOAA, directly demonstrating the specificity of VPA for the nerve terminal pool. This experimental model was further used to show a linear relationship between the GABA level in the nerve terminal compartment and the degree of protection against MES seizures, indicating that this GABA pool is in fact the one involved in seizure protection. Finally, AOAA, unlike VPA, showed essentially constant increases in GABA across the brain regions. Because the density of GABAergic terminals varies appreciably, this once again showed VPA's unique selectivity.

The mechanism by which VPA elevates GABA concentrations is unclear. A decrease in GABA catabolism could be achieved initially by inhibition of GABA-T, the enzyme that catalyzes a transamination between GABA and α-ketoglutarate. Because the bulk of GABA-T activity is contained in glia and neuronal cell bodies, rather than the nerve terminals, these represent the site of most of the degradation of GABA (Iadarola and Gale, 1981). However, VPA inhibits this enzyme significantly only at concentrations well above those normally

found in the brain (K_i = 9.5-40 mM, depending on source, for purified enzyme: Maitre *et al.*, 1978, Whittle and Turner, 1978), although it is interesting to note that it it is markedly more potent against neuronal GABA-T (IC_{50} = 0.63 mM for cultured cerebral neurons), than the predominant glial GABA-T (IC_{50} = 1.2 mM for cultured astrocytes)(Larsson *et al.*, 1986). This would explain why inhibition of GABA-T activity is observed in synaptosomes but not in whole brain homogenates (Loscher, 1993a). Interestingly, Nau and Loscher (1984) found that while 1 mM VPA had no effect on rat GABA-T activity *in vitro*, 1 mM 2-ene VPA caused a 27 ± 1 % inhibition. The inhibition of the human enzyme by 2-ene VPA is even more pronounced as indicated by K_i = 4.5 mM (corresponding K_i for VPA was 40 mM: Maitre *et al.*, 1978). Unfortunately, GABA-T activity has shown no temporal correlation with seizure protection in rats (Nau and Loscher, 1982).

VPA has also been shown to inhibit two other enzymes in the GABA degradation pathway, succinic semialdehyde dehydrogenase (SSADH) and non-specific succinic semialdehyde reductase (SSAR) with K_i values of 0.5-1.5 mM (Harvey *et al.*, 1975; van der Laan *et al.*, 1979) and 38-85 uM (Whittle and Turner, 1978), respectively. In principle, the resulting accumulation of succinic semialdehyde could either promote its conversion back to GABA by GABA-T or merely inhibit the forward reaction of this enzyme. However, the nearly complete inhibition of SSADH by *p*-hydroxybenzaldehyde was found to have no effect on GABA levels (Simler et al, 1981). The situation is similar for SSAR, where various flavonoids which are known potent inhibitors fail to show any anticonvulsant activity in the MES test (Whittle and Turner, 1981), except for the fact that the normal enzyme product, γ-hydroxybutyrate, produces absence-like epileptogenic effects (Snead *et al.*, 1980). This is particularly intriguing because such seizures are normally promoted in animal absence models by direct or indirect GABA agonists (Vergnes *et al.*, 1985 and references cited within). Consequently, this feature of VPA could explain how it could potentiate GABAergic neurotransmission while still being an effective drug for absence seizures. Unfortunately for this theory, it has been shown that VPA *in vivo* increases, rather than decreases brain levels of γ-hydroxybutyrate (Snead *et al.*, 1980).

To complete the list of VPA's inhibition of various enzymes involved in GABA metabolism, there is a report by Luder *et al.* (1990) showing that both VPA and 2-ene VPA inhibit the α-ketoglutarate dehydrogenase complex as their CoA esters. Thus, inhibition of this enzyme system would decrease flux through the TCA cycle and thus divert α-ketoglutarate into the GABA shunt. The potencies for both are very respectable but their mechanisms appear different as VPA acted solely in a competitive manner (K_i = 2.6 uM) while 2-ene VPA behaved as a mixed-type inhibitor (K_{is} = 6.1 uM and K_{ii} = 1.0 uM). This fundamental difference was further demonstrated by the irreversible inactivation of the enzyme when incubated with 2 mM 2-ene VPA. The authors suggested this as the explanation for the duration of the anticonvulsant effect after discontinuation of VPA therapy. No such effect was observed with up to 20 mM VPA.

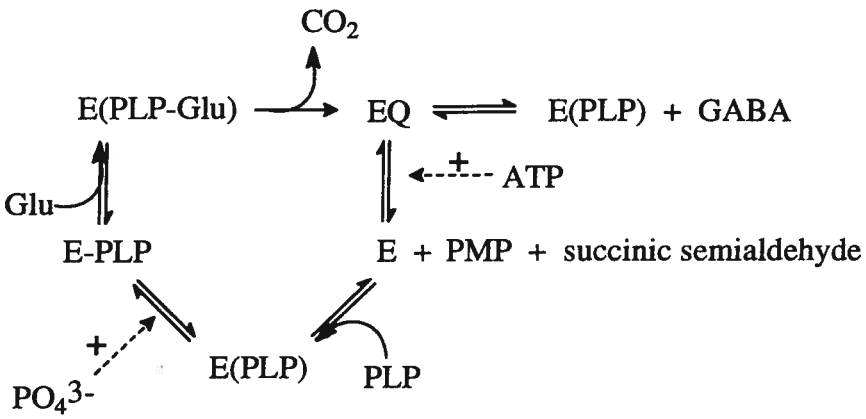
The evidence for a link betweem the anticonvulsant effects of VPA and enhancement of GABA synthesis is more encouraging. Injection of VPA (80 mg/kg i.p.) following administration of [^{14}C]-glucose was found to result in a significant increase in the production of labelled GABA (Taberner *et al.*, 1980). This line of work was recently extended by Bolanos and Medina (1993), who incubated neonatal brain slices with [2-^{14}C], [3,4-^{14}C] and [6-^{14}C]glucose, respectively, and measured the amount of $^{14}CO_2$ produced. Recall that glucose carbon atoms 3 and 4 are lost as CO_2 during oxidation of pyruvate to acetyl CoA immediately prior to entry into the TCA cycle, carbon atoms 1 and 6 are lost during the oxidation of the TCA intermediate isocitrate to α-ketoglutarate and carbon atoms 2 and 5 are lost during either the successive oxidation to succinate or the conversion of glutamate to GABA. They found the production of labelled CO_2 from [3,4-^{14}C]glucose and [[6-^{14}C]glucose to be unaffected by VPA and 2-ene VPA, indicating that these drugs do not influence glucose utilization and the TCA cycle, respectively. On the other hand, these drugs substantially enhanced the amount of $^{14}CO_2$ derived from [2-^{14}C]glucose. The authors estimated the activity of the GABA shunt pathway to be increased by 44 and 141%, respectively. In addition to supporting an enhancement of GABA synthesis as the cause of the elevated GABA levels, this result suggests that neither GABA-T nor α-ketoglutarate

dehydrogenase are likely targets of the two drugs as their inhibition would result in smaller amounts of $^{14}CO_2$ being detected.

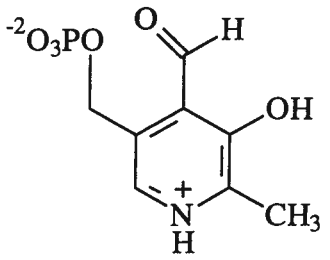
There is good evidence for this increased GABA shunt activity being caused by activation of glutamate decarboxylase, whose time course parallels those of the GABA increase and anticonvulsant effects (Nau and Loscher, 1982). Interestingly, this increase in activity is generally observed only in brain homogenates, slices and synaptosomes following *in vivo* VPA administration (Loscher, 1993a). Bolanos and Medina (1993) found no effect on enzyme activity when crude or "partially purified" enzyme was incubated with VPA, although Taylor *et al.* (1992) reported a marked enhancement of GAD activity using 0.25 mM VPA and >90% pure enzyme. Similarly, Nau and Loscher (1984) reported that 1 mM of either VPA or 2-ene VPA had no effect on GAD or GABA-T activity *in vitro*. The rapid onset of this activation could be explained by the conversion of the inactive apoenzyme to the active holoenzyme (see below), although this has yet to be proven. In fact, the minimal investigation into the mechanism of this activation by ED50 doses of VPA and 2-ene VPA (Loscher *et al.*, 1981) to date would make a summary of the properties of this enzyme appropriate at this point.

1.2.2.1.2. GLUTAMATE DECARBOXYLASE

GAD (reviewed by Martin and Rimvall, 1993) exists primarily as two isozymes of molecular weights 65.4 kDa (GAD_{65}) and 66.6 kDa (GAD_{67}) (Erlander *et al.*, 1991). GAD_{65} is localized primarily in neuronal terminals whereas the other isozyme is more evenly distributed throughout the cell. However, most differences between the two forms are generally minor. A significant feature of the enzyme, from a regulatory standpoint, is the fact that it is found primarily in the apoenzyme form, without bound pyridoxal 5'-phosphate (PLP) co-factor. Consequently, the cell has a large reserve capacity for synthesizing GABA when a sudden demand arises. In synaptosomes, the bulk of this apoenzyme can be attributed to the GAD_{65} species. It is this apo-GAD/holo-GAD equilibrium that is primarily responsible for



E = apo-GAD
E(PLP) = GAD with reversibly associated PLP
E-PLP = holo-GAD
E(PLP-Glu) = holo-GAD with bound glutamate
EQ = quinoid intermediate

PLP =  pyridoxal 5'-phosphate

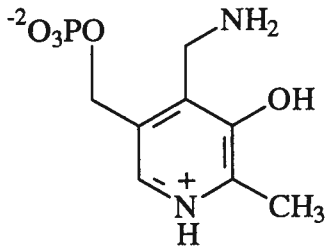
PMP =  pyridoxamine 5'-phosphate

Figure 4. The regulation of glutamate decarboxylase (GAD). Modified from Martin and Rimvall, 1993.

the regulation of the enzyme, as both substrate and co-factor are believed to be present at levels well above their effective concentrations. For example, in brain slices K^+-stimulated GABA release was unaffected by elevated concentrations of glutamate precursors glucose and glutamine, indicating an intracellular concentration of glutamate well above its K_m value (Szerb and O'Regan, 1984, 1986).

Normally the enzyme is present in the apoenzyme form and it combines tightly with PLP to form the holoenzyme that proceeds to form the quinoid intermediate EQ with glutamate as shown, a process promoted by phosphate through an allosteric mechanism (Figure 4). At this point, the species usually continues to form GABA and the regenerated holoenzyme, but it may also dissociate into apoenzyme, pyridoxamine 5'-phosphate and succinic semialdehyde. The apoenzyme may then be converted back to the holoenzyme except in the presence of ATP which binds to the enzyme in competition with PLP (Sze *et al.*, 1983; Porter and Martin, 1988) and stabilizes the protein against thermal decomposition, as the apoenzyme is normally unstable at physiological temperatures (Porter and Martin, 1988).

Evidence for the operation of this mechanism *in vivo* comes primarily from a comparison of how apo-/holo-GAD levels are influenced by the concentrations of glutamate, phosphate and ATP. For example, the normal concentration of phosphate found in cells is sufficient to promote the decarboxylation reaction while the concentration of free ATP (about 100 uM: Meeley and Martin, 1983) is well above the K_i value for inhibiting the binding of PLP to apo-GAD. It should be noted that this role of ATP can also be played by other anionic species (Tursky, 1970; Martin and Martin, 1982) as synaptosomes, which have low levels of ATP, have high levels of apo-GAD. Similarly, postmortem holo-GAD levels rise rapidly when ATP levels drop and the phosphate concentration rises (Miller *et al.*, 1977). In addition, the apo-/holo-GAD equilibria are influenced by factors such as depolarization, which converts apo-GAD to holo-GAD in synaptosomes (Miller and Walters, 1979). Finally, there has not been any evidence to date that PLP plays a role in this regulatory process, although it would seem to be a likely candidate. Unfortunately, this molecule is highly bound to protein

within the cell, making it difficult to measure changes in its free available concentration (Martin and Rimvall, 1993).

1.2.2.2. VPA AND POST-SYNAPTIC EFFECTS

A post-synaptic site of action for VPA, whereby it would potentiate the action of GABA, has also been investigated. Macdonald and Bergey (1979) reported that VPA applied microiontophoretically to cultured mouse spinal cord neurons voltage clamped at -90 mV augmented the depolarizing response to GABA but not glycine or glutamate. Similarly, Hackman *et al.* (1981) observed an increase in the GABA-induced response of frog spinal cord in the presence of 10 uM VPA. There have been numerous reports since of a similar effect of VPA on the GABA response using microiontophoretic application to cortical (Schmutz *et al.*, 1979; Kerwin *et al.*, 1980; Baldino and Geller, 1981) and nigral neurons (Kerwin *et al.*, 1980), potentiation of the depressant effect of muscimol in spontaneously firing locus coeruleus neurons (Olpe *et al.*, 1988) and potentiation of the responses of both GABA and muscimol in medullary reticular formation neurons (Gent and Phillips, 1980).

However, there have also been a similar number of studies where no effect of VPA on the GABA response was found. For example, McLean and Macdonald (1986) were unable to detect a significant enhancement of the response of cultured mouse spinal cord neurons to GABA when VPA was applied by microiontophoresis. Buchhalter and Dichter (1986) found that up to 3 mM VPA had no effect on the increased chloride conductance of dissociated cortical neurons in response to GABA. Similarly, no potentiation of the GABA response was obtained by Preisendorfer *et al.* (1987) with 5 mM VPA using hippocampal neurons, and Gallagher *et al.* (1981), using 0.1-1 mM VPA applied to cat dorsal root ganglion cells. This latter result is significant because the preparation is inherently free of synapses. Thus, any potentiation would have been the result of VPA acting at the level of the GABA receptor complex. It has even been reported that VPA promotes spontaneous firing of neurons in the rat cortex and dorsal hippocampus (Blume *et al.*, 1979), an effect that was found to be

antagonized by GABA. However, these results should be treated with caution as the animals were anesthetized with pentobarbital, an indirectly acting GABA agonist. Finally, Harrison and Simmonds (1982) found that while VPA did indeed enhance the muscimol-induced depolarization of rat cuneate afferent fibres, as determined by extracellular recording, this effect was only apparent in the 3-10 mM range, well above the normal concentration of unbound drug in the brain.

A different approach to demonstrate the lack of an exclusive interaction between VPA and GABAergic synaptic transmission was provided by Agopyan *et al.* (1985), who studied rhythmic synchronous bursts in hippocampal slices in a low-Ca^{2+} and high-Mg^{2+} medium where synaptic transmission is virtually eliminated. VPA (0.5 mM) markedly decreased these bursts even in the additional presence of the GABA site antagonist bicuculline. It should be noted that this experiment merely showed that the effects of VPA are not exerted exclusively on the GABA receptor itself.

1.2.2.3. INFLUENCE OF VPA ON MEMBRANE ION CHANNELS

Like other antiepileptic drugs, VPA also influences the permeability of the cell membrane to ions. Most studies to date have shown that sodium conductance is reduced and the gating of its channel is slowed down (Zona and Avoli, 1990 and references cited below). For example, van Dongen *et al.* (1986) found that 0.5 mM VPA produced a 5-40% reduction in the peak sodium current obtained in the sciatic nerve of *Xenopus laevis* under voltage clamp conditions. The decreased excitability of the nerve was further demonstrated by the decreased action potential amplitude and maximal rate of rise, increased threshold potential and diminished ability to transmit high frequency signals. The latter finding is consistent with the work of McLean and Macdonald (1986), where even 30 uM VPA, along with phenytoin and carbamazepine, inhibited both spontaneous and stimulated sustained repetitive firing of dissociated mouse spinal cord neurons, and is clearly of particular relevance to the ability of a drug to prevent the spread of seizure activity. However, a common site of action is probably

not involved as VPA, unlike phenytoin and carbamazepine, is unable to reduce the batrachotoxin-induced influx of $^{22}Na^+$ into neuroblastoma cells and rat brain synaptosomes (Willow *et al.*, 1984) and has a much longer latency to inhibition of repetitive firing in area CA1 of the rat hippocampal slice (Franceschetti *et al.*, 1986). Van den Berg *et al.* (1993), in their detailed investigation of the sodium channel gating effects of 1 mM VPA in cultured rat hippocampal neurons, explained this property as the result of a 200% increase in the recovery time constant for the sodium current. In addition, they found a marked reduction in the peak conductance and the current decay time constant and showed that VPA's inhibition of Na^+ influx was both use- and voltage-dependent, being more pronounced at more positive potentials. Again, this is consistent with the actions of a drug that acts to inhibit elevated levels of neuronal activity. Furthermore, the voltage-dependence indicates that VPA's mechanism did not involve simple channel blockade, as seen with the local anesthetics (Fohlmeister *et al.*, 1986).

This promotion of sodium channel inactivation and delay of recovery has also been reported for *Myxicola* giant axons (Schauf, 1987) and squid giant axons (Fohlmeister *et al.*, 1986). However, in both studies the drugs were applied internally and in the latter report no effect was observed when up to 20 mM VPA was added to the perfusion medium.

There is little agreement between studies on the effect of VPA on potassium channels. Some have seen little or no effect on potassium conductance or channel gating (Fohlmeister *et al.*, 1986; Franceschetti *et al.*, 1986 (indirectly, as these workers monitored extracellular potassium levels); Schauf, 1987; Zona and Avoli, 1990). One early study (Slater and Johnston, 1978) even claimed to observe an increase in potassium conductance in *Aplysia* neurons. However, the concentrations of VPA employed were very high (5-30 mM) and the relevance of the model system to human epilepsy is questionable. On the other hand, it has also been shown (van Dongen et al, 1986; van Erp *et al.*, 1990) that in the sciatic nerve of *X. laevis* 2.4 mM VPA had a biphasic, voltage-dependent effect on potassium conductance, increasing it at potentials more negative than -50 mV and reducing it at more positive potentials. Although a reduced potassium conductance would hardly seem compatible with

the mode of action of an antiepileptic drug, the authors suggested that this may serve to limit the concentration of extracellular potassium whose accumulation may promote sustained repetitive firing (Dichter and Ayala, 1987). This idea, however, is not supported by the work of Franceschetti *et al.* (1986), who found only a very minor reduction by 1 mM VPA of the extracellular potassium accumulation during spontaneous epileptic activity in rat hippocampal slices.

There is good evidence that VPA reduces the low threshold (T) Ca^{2+} current that is believed to play an important role in absence seizures. It has been suggested that it is the de-inactivation of these channels by $GABA_B$ receptor-mediated hyperpolarization that is the cause of their aggravation of absence seizures (Hosford *et al.*, 1992). Kelly *et al.* (1990) found that VPA reduced the total Ca^{2+} (T) current in dissociated rat nodose ganglion neurons at approximately therapeutic concentrations (0.1-0.5 mM) but the reductions (10-27%) were less than the values observed with the exclusively antiabsence drug ethosuximide in the thalamic relay neurons that are believed to play a critical role in generalized absence seizures (Coulter *et al.*, 1989). Because ethosuximide was not tested by Kelly *et al.* (1990), one cannot say whether or not their results can explain VPA's antiabsence properties. By contrast, Coulter *et al.* (1989) failed to observe an effect of even 1 mM VPA on the low threshold Ca^{2+} current in isolated ventrobasal complex (thalamic) neurons from rats, but found ethosuximide to be a most effective blocker.

1.2.2.4. VPA AND MEMBRANE FLUIDITY

Even if the GABA hypothesis of VPA's mode of action is true, there still remains the question of the actual effector mechanism in the absence of any known binding sites. An appealing candidate for this role is the plasma membrane itself, whose perturbation by the incorporation of VPA could lead to altered ionic conductances and thus altered synthesis and release of GABA.

Seeing that VPA has no known binding sites and distributes throughout the brain in an essentially homogeneous manner (Schobben *et al.*, 1980), it is certainly relevant to ask how it exerts its effects on ion channels and GABA-metabolizing enzymes. One possibility is that the drug acts *via* the plasma membrane to increase its fluidity and hence the properties of the proteins embedded in it. In other words, the greater the degree to which the drug can disturb the membrane, the greater its potency.

The best illustration of such a mechanism is provided by the volatile anesthetics that possess no activity-shape dependence, exhibit a very high correlation between potency and lipophilicity and require quite high concentrations to be effective. For example, the minimum alveolar concentrations of most clinical anesthetics are in the 0.01-0.1 atm range which corresponds to 0.39-3.9 mM in the lung. At equilibrium, this will be the brain concentration as well, owing to the anesthetics' high lipophilicity that allows them to readily cross the blood-brain barrier. Such properties, in fact, compare favourably with those observed following therapeutic doses of VPA. The mechanism by which the anesthetic effect is exerted remains unclear, however. One explanation offered is based on the anesthetics' promotion of membrane disordering leading to a decrease in the gel-liquid crystalline transition temperature. Because this transition is accompanied by a change in volume, the effect of the anesthetics would be to decrease the magnitude of the resultant changes in volume. Because integral membrane proteins such as ion channels would be expected to be sensitive to such changes in their environment, the incorporation of a membrane disordering agent could thus markedly influence their activity, though the exact effect would be open to speculation (Kennedy and Longnecker, 1990).

There has been remarkably little interest in exploring this particular facet of VPA's action, even though Perlman and Goldstein (1984) have shown that when corrected for brain concentrations, ED50 values of a wide range of linear and branched fatty acids show a very high degree of correlation with their ability to increase the fluidity of synaptosomal membranes measured using fluorescence polarization spectroscopy. A bothersome aspect of this study, however, was the very high concentration (>10 mM for VPA) of drugs needed to

detect a significant change in membrane fluidity. This may have been due to the fact that the probe used in this study was embedded in the middle of the membrane bilayer and might thus not be able to detect changes in membrane structure near the surface where such anionic drugs might be expected to act. Alternately, it is possible that changes in fluidity that would exert a profound effect on an integral membrane protein might simply not be detected with this assay, necessitating the use of higher drug concentrations. Nevertheless, there is solid support for a role of increased membrane fluidity in the anticonvulsant action of VPA. Lucke *et al.* (1993) used the diffusion properties of VPA and 2-ene VPA to conclude that the drugs are unlikely to significantly penetrate the plasma membrane and thus reside primarily in it or in the extracellular space. This was demonstrated by measuring the drugs' diffusion coefficients in agar (D, representing a strictly aqueous medium) and cortical tissue (D^*). The resultant ratio $\lambda = D/D^*$, where λ is known as the tortuosity factor, then represents the relative impedance of the biological medium to the diffusion of the analyte. A substance that freely crosses biological membranes will have λ approaching unity, whereas the value of a strictly extracellular species such as the tetramethylammonium ion will be much larger. Lucke *et al.* found that the tortuosity values for VPA (1.90) and 2-ene (1.70) were similar to those reported for the tetramethylammonium ion, indicating a predominantly extracellular distribution of these species. This finding also implies that determinations of brain concentrations of VPA- and 2-ene VPA-related drugs using whole brain homogenate (such as the one described in this thesis) will greatly underestimate the true effective concentrations, as the extracellular space represents only about 20% of the total brain volume.

Indirect evidence for such a mechanism has also been provided by Hauck *et al.* (1991) in their study of the effect of configuration on two VPA analogues (2-(2'-propenyl)pentanoic and 2-(2'-propynyl)pentanoic acids). The anticonvulsant potencies of each compound's enantiomers were evaluated separately and found to be independent of configuration, arguing against the existence of an enantioselective receptor site. Instead, this role could be served by the asymmetric medium of the interior of the plasma bilayer that would be sensitive to the drug's basic structure but not to its chirality.

A more clinically relevant example was offered by Tangorra *et al.* (1991), who found that the fluidity of erythrocyte membranes from epileptic patients was significantly affected by therapeutic doses of various antiepileptic drugs including VPA. However, these workers employed the cationic probe TMA-DPH which is tethered to the membrane surface and whose fluorescence is thus a function of the fluidity of the environment near the surface of the membrane. They found that patients receiving antiepileptic drugs showed decreased membrane fluidity which appeared to correlate with the lipoperoxide content of the membrane. However, it is difficult to draw conclusions from the data as the authors presumably used non-epileptic patients not receiving drug therapy as a control. Thus, one cannot distinguish the effects of epilepsy itself from those of the medication.

1.2.3. STRUCTURE-ACTIVITY STUDIES

Early structure-activity studies (Carraz, 1967; Taillandier *et al.*, 1975) demonstrated that while many carboxylic acids and their derivatives possessed significant anticonvulsant effects, VPA represented the optimum balance between seizure protection and sedation. Specifically, increasing size by the use of longer alkyl chains increased anticonvulsant potency but at the cost of enhanced sedation.

There have been few recent structure-activity studies of VPA and its analogues, which is hardly surprising in view of the proven efficiency of the prototype drug. Loscher and Nau (1985) examined a fairly broad range of VPA analogues, focusing primarily on their anticonvulsant potency and toxicity. They found that an alkyl substituent at the α carbon atom was virtually essential for activity, which also increased with the size of the substituent. Interestingly, there was no further significant increase in potency for α-monosubstituted aliphatic acids larger than VPA, although α-disubstituted acids were shown to be even more potent than VPA, further emphasizing the requirement for a branched structure. Unfortunately, this latter group frequently caused delayed deaths after several days, demonstrating that VPA represented the optimum structure for this group of compounds in

terms of both anticonvulsant efficacy and toxicity. Qualitatively similar results were obtained by Keane and Morre (1985).

Table 3
Anticonvulsant properties of VPA and its analogues

Compound	i.p. ED50 (mmol/kg)	i.c.v. ED50 (umol)
Loscher and Nau (1985)[a]		
VPA	1.7	
(*E*)-2-ene VPA	1.6	
2-Propylhexanoic acid	1.6	
2-Butylhexanoic acid	1.5	
1-Methyl-1-cyclohexanoic acid	1.9	
2-Ethyl-2-methylpentanoic acid	1.3	
2,2-Dimethylpentanoic acid	1.5	
Keane *et al.* (1983)[a]		
VPA	0.81	
Butyric acid	inactive	
2-Ethylbutyric acid	2.23	
2-Ethylpentanoic acid	1.21	
2-Ethyl-hexanoic acid	1.14	
Pentanoic acid	inactive	
2-Methylpentanoic acid	3.64	
2-Butylpentanoic acid	0.75	
2-Pentylpentanoic acid	0.6	
Hexanoic acid	inactive	
2-Butylhexanoic acid	0.77	
Chapman *et al.* (1984)[b]		
VPA	1.25	6.0
2-Ethylhexanoic acid	0.66	10.2
2-Propylhexanoic acid	0.68	5.0
Pentanoic acid	5.80	> 15

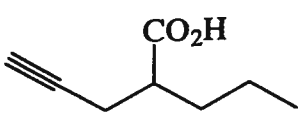
a) Endpoint: prevention of scPTZ-induced seizures in mice
b) Endpoint: prevention of audiogenic seizures in DBA/2 mice

Chapman *et al.* (1984) studied the potencies of VPA analogues using both i.p. and intracerebroventricular (i.c.v.) routes of injection. They found that the enhanced potencies of the larger acids were due to pharmacokinetic rather than pharmacodynamic effect because the most potent compound by the i.p. route was amongst the least effective when intrinsic potency was evaluated by i.c.v. administration. This suggests that pharmacokinetic factors

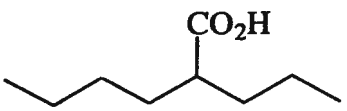
make a significant contribution to the observed anticonvulsant effect. Similar results have been obtained by Perlman and Goldstein (1984), who found that on the basis of brain concentrations, both heptanoic and octanoic acids were up to seven times more potent than VPA and thus emphasized the need for normalization of anticonvulsant data.

More recently, Elmazar *et al.* (1993) looked at 2-substituted pentanoic acids as VPA analogues. As shown, substitution with various alkyl and alkenyl groups did not significantly affect potency. However, the ED50 dropped dramatically when a triple bond was introduced into one of the branches. In addition, the protective index (TD50/ED50, using rotorod and scPTZ tests, respectively) was greatly improved. It is interesting to note that the calculated lipophilicity of this compound was quite low, in contrast to the usual trend where potency is a linear function of log P.

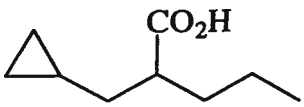
There have been even fewer quantitative structure-activity studies of this family of compounds. Abbott and Achaempong (1988) examined a broad array of carboxylic acids and tetrazoles for anti-scPTZ activity in mice, using Taft's steric parameter, log P, $(\log P)^2$ and pK_a as physicochemical descriptors in the Hansch linear free energy model. The Taft parameter was found to correlate with log P and was not used. The best correlation was found for log P and pK_a, which indicated optimal activity with increasing lipophilicity and decreasing acidity. Because the brain concentrations of these drugs were not measured, it is not known if log P and pK_a influenced primarily the interaction of drug with the effector or simply the brain's drug uptake ability.



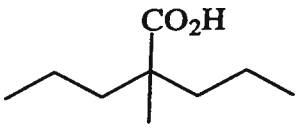
2-(2'-Propynyl)-pentanoic acid



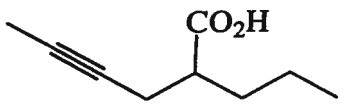
2-Propylhexanoic acid



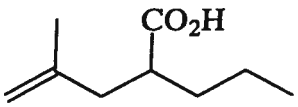
2-(Methylcyclopropyl)-pentanoic acid



2-Methyl-2-propyl-pentanoic acid



2-(2'-Butynyl)-pentanoic acid



2-(2'-Methyl-2'-propenylpentanoic acid

Figure 5. Anticonvulsant 2-substituted pentanoic acids (Elmazar et al., 1993).

Table 4
Anticonvulsant and lipophilicity properties of some 2-substituted pentanoic acids (Elmazar *et al.*, 1993)

Compound	log P	ED50	Neurotoxicity[a]
VPA	2.72	0.71	33
2-(2'-Propynyl)pentanoic acid	1.31	1.78*	20
4-Ene VPA	2.18	1.29*	29
2-Methyl-2-propylpentanoic acid	3.12	0.40	80
2-Propylhexanoic acid	3.25	0.78	80
2-Ene VPA	2.59	0.68	60
2-(Methylcyclopropyl)pentanoic acid	2.63	0.96	20
2-(2'-Butynyl)pentanoic acid	1.84	0.28*	0
2-(2'-Methyl-2'-propenyl)pentanoic acid	2.57	0.84	0

*) Significantly different ($p < 0.05$) from VPA.
a) Percentage of mice ($n = 7$) showing minimal neurological deficits on a rotating rod 15 min following i.p. administration of 1.5 mmol/kg drug.

1.3. OBJECTIVES

At this point, we can summarize VPA as a clinically useful drug with two significant drawbacks. The first is practical, for although VPA's side effects are generally few, those which may occur, such as its hepatotoxicity and teratogenicity, can be fatal. The incidence of such events can be minimized by not prescribing the drug to children on polytherapy or to pregnant women, but it clearly indicates the potential for improving the therapeutic utility of VPA. The second problem with VPA is primarily academic in that its mechanism of action still contains unanswered questions. For example, no study has sought a correlation for a series of VPA analogues between anticonvulsant activity and synaptosomal GABA levels or the activities of enzymes in the GABA pathway. Working on the assumption of an identical mechanism of action, such a correlation could not only prove that elevated GABA was the key to the anticonvulsant properties, but would also indicate VPA's biochemical target.

Both problems could theoretically be addressed by studying the properties of 2-ene VPA and its analogues. This compound, formed by the ß-oxidation of VPA, exhibits no teratogenicity and minimal hepatoxicity and it may be that its analogues exhibit similar properties. Furthermore, its centrally located double bond makes it ideal for QSAR work as it imparts a degree of rigidity lacking in VPA itself. Combined with the fact that it has many of the same biological properties as its parent, including equivalent anticonvulsant potency, 2-ene VPA would thus appear to be an ideal starting point to probe the mechanisms of VPA and perhaps develop more efficient anticonvulsant analogues.

The objectives of this project were the following:

1) prepare at least 10-15 analogues of 2-ene VPA: specifically, aliphatic α,β-unsaturated acids with alkyl or alkenyl substituents
2) for the above compounds, determine:
    a) anticonvulsant and sedative properties
    b) lipophilicity and solution conformation

c) membrane-disordering potency

d) effect of ED50 dose on nerve terminal GABA levels and GAD activities

3) develop quantitative relationships between these physicochemical and biological parameters

4) investigate the mechanism of GAD interaction by the effect of drugs on

a) saturation of GAD with its cofactor PLP

b) GAD activity *in vitro*

It should be stressed that the primary objective of this study was to shed light on the mechanism of action of 2-ene VPA, and thus presumably VPA itself, rather than to search for a more potent anticonvulsant drug, which could be done more efficiently by a more broadly based screening protocol.

2. EXPERIMENTAL

2.1. MATERIALS

Flash chromatography was performed with silica gel 60 (Merck 9285, 230-400 mesh). Melting points were determined with a Thomas-Hoover melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on Bruker 200 MHz or 300 MHz instruments at the Chemistry Department, UBC. IR spectra were recorded on a Bomem MB-100 instrument.

Qualitative GC-MS analyses were performed on a Hewlett-Packard HP5700A gas chromatograph (packing: 3% Dexsil 300; oven: 50°C initial to 260°C @ 16°C/min or 32°C/min) interfaced to a Finnegan MAT-111 mass spectrometer. Where noted, alcohols and acids were derivatized with MSTFA or MTBSTFA in ethyl acetate prior to analysis. Quantitative GC-MS analyses were performed using a Hewlett-Packard HP 5890 gas chromatograph interfaced to a HP 5989A mass spectrometer. GC: 34.5 kPa helium head pressure, Hewlett-Packard HP-1 capillary column (12 m x 0.2 mm ID x 0.33 um film). Injection: 1 ul. MS: electron impact ionization potential 70 eV, single ion monitoring, dwell time 75 msec, source 275°C, quad 100°C.

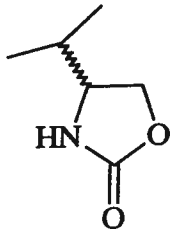
Elemental analyses were performed by Mr. Peter Borda at the Department of Chemistry, UBC.

Male CD-1 mice were obtained from University of British Columbia Animal Services or Charles River, PQ: in the latter case, the animals were allowed one day of recovery upon arrival. GABA-d_6 was purchased from MSD Isotopes (Montreal, PQ). TMA-DPH was purchased from Molecular Probes (Eugene, OR). Linear regression calculations were performed using QSAR-PC (BIOSOFT, Cambridge, MA).

2.2. SYNTHESIS

Acids **3**, **9** and **10** were prepared as described by Lee (1991) and Lee *et al.* (1989). Acids **2** and **4** were obtained commercially (Aldrich).

2.2.1. (±)-4-(1-Methylethyl)-2-oxazolidinone (**1a**)



DL-Valinol (25.06 g, 243 mmol) was dissolved in diethyl carbonate (170 mL). Potassium *tert*-butoxide (6.95 g, 62 mmol) was added and the mixture was brought to reflux over 15 min. Distillate was collected until the stillhead temperature rose to 126°C (total distillation time: 30 min). The solution was allowed to cool and then diluted with diethyl ether (150 mL) and washed with saturated ammonium chloride (3 x 30 mL) and brine (30 mL). The organic extract was dried over magnesium sulfate, filtered and evaporated *in vacuo*. The solid residue was decanted with petroleum spirit (2 x 30 mL) and 1:9 and 1:4 diethyl ether/petroleum spirit solutions (30 mL each). The resultant suspension was refluxed briefly with a 2:3 solution (30 mL), cooled at -10°C, filtered and washed with 1:4 diethyl ether/petroleum spirit (2 x 15 mL) to afford the oxazolidinone (21 g, 68%) as a white powder.

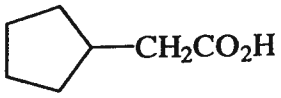
mp 69-72°C

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.90 (6H, m, -CH_3), 1.68 (1H, m, -C<u>H</u>$(CH_3)_2$), 3.62 (1H, m, -C<u>H</u>NH), 4.08 (1H, dd, J = 9, 8 Hz, -CH_2O-), 4.40 (1H, dd, J = 9, 9 Hz, -CH_2O-), 6.90 (1H, br s, NH).

IR ($CHCl_3$, cm^{-1}): 3244, 2950, 1749, 1474, 1405.

GC-MS (*m/z*): 129 (M^+), 86, 42.

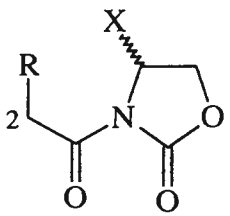
2.2.2. Cyclopentylacetic acid



Cyclopentylacetic acid was prepared from diethyl malonate and cyclopentyl bromide by the general procedure of Furniss *et al.* (1987a). Fractional distillation afforded 2-cyclopentylacetic acid (9.26 g, 39%, bp 106-112°C / 6 mm Hg: lit. bp 226-230°C / 760 mm Hg, bp 139-140°C / 26 mm Hg (Buckingham, 1982)) as a clear oil.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.05-1.26 (2H, m, cyclopentyl), 1.42-1.70 (4H, m, cyclopentyl), 1.70-1.91 (2H, m, cyclopentyl), 2.04 (1H, m, H(3)), 2.09-2.31 (2H, m, H(2)).
IR (neat, cm^{-1}): 3140-3000, 2950, 1709, 1427, 1292, 1217, 1136.
GC-MS (*m/z*, as TMS derivative): 200 (M^+), 185, 132, 117, 73, 75.

2.2.3. (±)-3-(1-Oxoalkyl)-4-(1-methylethyl)-2-oxazolidinones (**2a/a-f**) and (±)-3-(1-oxoalkyl)-2-oxazolidinones (**2b/e-f**)



2aa: X = i-Pr; R = Me
2ab: X = i-Pr; R = Et
2ac: X = i-Pr; R = Pr
2ad: X = i-Pr; R = Bu
2ae: X = i-Pr; R = *i*-Pr
2af: X = i-Pr; R = cyclopentyl

2be: X = H; R = i-Pr
2bf: X = H; R = cyclopentyl

Cyclopentylacetyl chloride (bp 57-58°C / 6 mm Hg: lit. bp 55°C / 10 mm Hg (Seubold, 1954)) and pentanoyl chloride (bp 127-132°C: lit. 124-127°C (Furniss *et al.*, 1987b)) were prepared from their respective acids by the procedure described by Furniss *et al.* (1987b). The remaining acid chlorides were obtained commercially (Aldrich).

The oxazolidinone (**1a/b**: 46.5 mmol) was dissolved in dry THF (180 mL) under a nitrogen atmosphere and the solution cooled to -78°C. Butyllithium (1.6 M in hexanes, 32.0 mL, 51.2 mmol, 1.1 eq.) was added and the solution stirred for 5 min. A solution of the acid

chloride (51.2 mmol, 1.1 eq.) was then added as a solution in THF (10 mL) and stirring continued for 10 min. The reaction was quenched with saturated aqueous ammonium chloride (100 mL) and the mixture warmed to room temperature. The layers were separated and the aqueous phase extracted with diethyl ether (180 mL). The combined organic extracts were washed with saturated aqueous sodium bicarbonate (40 mL) and brine (40 mL) before being dried over magnesium sulfate, filtered and evaporated *in vacuo*. The crude product was then purified by distillation using a 10 cm Vigreaux column.

(±)-3-(1-Oxopropyl)-4-(1-methylethyl)-2-oxazolidinone (**2aa**)

7.35 g (85%, bp 82-87°C / 0.1 mm Hg).

^{1}H-NMR (200 MHz, acetone d_6) δ: 0.85 (3H, d, J = 7.0 Hz, -CH($C\underline{H}_3$)$_2$), 0.91 (3H, d, J = 7 Hz, -CH($C\underline{H}_3$)$_2$), 1.08 (3H, t, J = 7.5 Hz, H(3)), 2.20-2.43 (1H, m, -C$\underline{H}$(CH$_3$)$_2$), 2.70-3.05 (2H, m, H(2)), 4.20-4.50 (3H, m, -CHCH_2O-).

IR (neat, cm^{-1}): 2956, 1778, 1704, 1487, 1463, 1382, 1224, 1072.

GC-MS (*m/z*): 185 (M$^+$), 142, 130, 100, 85, 68, 57.

(±)-3-(1-Oxobutyl)-4-(1-methylethyl)-2-oxazolidinone (**2ab**)

7.62 g (82%, bp 83°C / 0.05 mm Hg).

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.80-1.00 (9H, m, 3 x -CH_3), 1.65 (2H, m, H(3)), 2.31 (1H, septet of d, J = 5, 4 Hz, -C$\underline{H}$(CH$_3$)$_2$), 2.67-3.02 (2H, m, H(2)), 4.26-4.52 (3H, m, -CHCH_2O-).

IR (neat, cm^{-1}): 2953, 1777, 1701, 1486, 1463, 1382, 1216.

GC-MS (*m/z*): 199 (M$^+$), 184, 171, 156, 130, 86, 71.

(±)-3-(1-Oxopentyl)-4-(1-methylethyl)-2-oxazolidinone (**2ac**)

8.77 g (89%, 97-105°C / 0.05 mm Hg).

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.80-1.00 (9H, m, -CH(C<u>H</u>$_3$)$_2$, H(5)), 1.28-1.48 (2H, m, H(4)), 1.48-1.70 (2H, m, H(3)), 2.20-2.44 (1H, m, -C<u>H</u>(CH$_3$)$_2$), 2.70-3.09 (2H, m, H(2)), 4.28-4.56 (3H, m, -CHCH$_2$O-).

IR (neat, cm^{-1}): 2948, 1779, 1708, 1463, 1384, 1205.

GC-MS (*m/z*): 213 (M^+), 198, 184, 171, 130, 85.

(±)-3-(1-Oxohexyl)-4-(1-methylethyl)-2-oxazolidinone (**2ad**)

7.49 g (64%, bp 104-6°C / 0.05 mm Hg).

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.68-0.80 (9H, m, -CH(C<u>H</u>$_3$)$_2$, H(6)), 1.09-1.23 (4H, m, H(5), H(4)), 1.38-1.60 (2H, m, H(3)), 2.02-2.29 (1H, m, -C<u>H</u>(CH$_3$)$_2$), 2.51-2.88 (2H, m, H(2)), 4.03-4.39 (3H, m, -CHCH$_2$O-).

IR (neat, cm^{-1}): 2944, 1772, 1702, 1486, 1464, 1383, 1215.

GC-MS (*m/z*): 228 (M^+), 198, 184, 171, 130, 99.

(±)-3-(1-Oxo-3-methylbutyl)-4-(1-methylethyl)-2-oxazolidinone (**2ae**)

6.78 g (91%, bp 94-98°C / 0.05 mm Hg) at 3/4 scale of above.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.80-1.00 (12H, m, -CH(C<u>H</u>$_3$)$_2$), 2.00-2.12, 2.12-2.41 (1H, 1H, m, m, -C<u>H</u>(CH$_3$)$_2$, H(3)), 2.60 (2H, dd, J = 7.0, 15.0 Hz, H(2)), 4.22-4.55 (3H, m, -CHCH$_2$O-).

IR (neat, cm^{-1}): 2954, 1778, 1700, 1466, 1388, 1305, 1210.

GC-MS (*m/z*): 213 (M^+), 198, 171, 130, 85, 69.

(±)-3-(1-Oxo-2-cyclopentylethyl)-4-(1-methylethyl)-2-oxazolidinone (**2af**)

9.13 g (82%, bp 127-130°C / 0.2 mm Hg).

mp 33-37°C

^{1}H-NMR (300 MHz, acetone-d_6) δ: 0.88, 0.95 (3H, 3H, d, d, J = 7 Hz each, -CH(C<u>H</u>$_3$)$_2$), 1.10-1.32 (2H, m, cyclopentyl), 1.46-1.73 (4H, m, cyclopentyl), 1.73-1.92 (3H, m,

cyclopentyl, H(3)), 2.21-2.40 (1H, m, -C<u>H</u>$(CH_3)_2$), 2.77 (1H, dd, J = 7, 16 Hz, H(2)), 3.04 (1H, dd, J = 7, 16 Hz, H(2)), 4.28-4.52 (3H, m, -$CHCH_2O$-).

IR ($CHCl_3$, cm^{-1}): 3030, 2957, 1777, 1699, 1486, 1383.

GC-MS (*m/z*): 239 (M^+), 196, 171, 130, 111.

(±)-3-(1-Oxo-3-methylbutyl)-2-oxazolidinone (**2be**)

10.44 g (66%, bp 89-92°C / 0.05 mm Hg)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.95 (6H, d, J = 7 Hz, (C<u>H</u>$_3)_2$CH-), 2.0-2.2 (2H, m, $(CH_3)_2$C<u>H</u>-, H(3)), 2.75 (2H, d, J = 7 Hz, H(2)), 4.00 (2H, t, J = 8 Hz, -NCH_2CH_2O-), 4.44 (2H, d of t, J = 1, 8 Hz, -NCH_2CH_2O-)

IR ($CHCl_3$, cm^{-1}): 2964, 1781, 1699, 1482.

GC-MS (*m/z*): 156 (M^+-15), 129, 114, 101.

(±)-3-(1-Oxo-2-cyclopentylethyl)-2-oxazolidinone (**2bf**)

mp 44-46°C

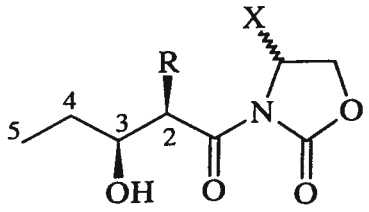
1.91 g (58%, bp 118°C / 0.05 mm Hg)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.05-1.29 (2H, m, cyclopentyl), 1.44-1.72 (4H, m, cyclopentyl), 1.72-1.94 (3H, m, cyclopentyl, H(3)), 2.27 (1H, br septet, J = 7 Hz, C<u>H</u>$(CH_2)_2$), 2.88 (2H, d, J = 7 Hz, H(2)), 4.00 (2H, br t, J = 8 Hz, -NCH_2CH_2O-), 4.45 (2H, br t, J = 8 Hz, -NCH_2CH_2O-).

IR ($CHCl_3$, cm^{-1}): 2960, 1781, 1702.

GC-MS (*m/z*): 197 (M^+), 177, 168, 154, 129, 111, 101, 88, 83.

2.2.4. Aldol addition: (±)-(*erythro*)-3-[3-hydroxy-2-alkyl-1-oxopentyl]-4-(1-methylethyl)-2-oxazolidinone (**3a/a-f**) and (±)-(*erythro*)-3-[3-hydroxy-2-alkyl-1-oxopentyl]-2-oxazolidinone (**3b/e-f**)



3aa: X = *i*-Pr; R = Me
3ab: X = *i*-Pr; R = Et
3ac: X = *i*-Pr; R = Pr
3ad: X = *i*-Pr; R = Bu
3ae: X = *i*-Pr; R = *i*-Pr
3af: X = *i*-Pr; R = cyclopentyl
3be: X = H; R = *i*-Pr
3bf: X = H; R = cyclopentyl

To a solution of the carboximide **2a/b** in dry dichloromethane (0.2 M, 30-40 mmol) at -78°C was added dibutylboron triflate (1.0 M solution in dichloromethane, 1.1 eq.) followed by triethylamine (1.4 eq.) in dichloromethane (5 mL). The pale yellow solution was stirred at -78°C for 1 h and then at 0°C for 15 min. The solution was re-cooled to -78°C and freshly-distilled propionaldehyde (1.5 eq.) in dichloromethane (5 mL) was added: the solution was then stirred at -78°C for 1 h and 0°C for 1 h before being partitioned between aqueous sodium bisulfate (1 M, 150 mL) and ethyl acetate/petroleum spirit (300 mL, 1:1 v/v). The organic layer was washed with brine (45 mL), evaporated in vacuo and combined with diethyl ether (225 mL), saturated aqueous ammonium chloride (75 mL) and hydrogen peroxide (30%, 7.5 eq.) at 0°C. The mixture was stirred at 0°C for 1 h and then diluted with diethyl ether (150 mL). The phases were separated and the organic extract was washed with saturated aqueous sodium bicarbonate (35 mL) and brine (2 x 35 mL), dried over magnesium sulfate and evaporated *in vacuo*. Samples of the adducts were further purified by flash chromatography.

(±)-(*Erythro*)-3-[3-hydroxy-2-methyl-1-oxopentyl]-4-(1-methylethyl)-2-oxazolidinone (**3aa**)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.80-0.95 (9H, m, 3 x -CH_3), 1.20 (3H, d, J = 7 Hz, H(3')), 1.31-1.50 (2H, m, H(4)), 2.30 (1H, septet of d, J = 4, 7 Hz, -$CH(CH_3)_2$), 3.61 (1H, d, J = 5 Hz, -OH), 3.62-3.85 (2H, m, H(2), H(3)), 4.25-4.55 (3H, m, -$CHCH_2O$-).

IR ($CHCl_3$, cm^{-1}): 3538, 3033, 2964, 1779, 1684, 1382.

GC-MS (*m/z*, TMS ether): 300 (M^+-15), 230, 170, 158, 143, 130.

(±)-(*Erythro*)-3-[3-hydroxy-2-ethyl-1-oxopentyl]-4-(1-methylethyl)-2-oxazolidinone (**3ab**)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.79-1.05 (12H, m, 4 x -CH_3), 1.35-1.90 (4H, m, H(3'), H(4)), 2.20-2.43 (1H, m, -C<u>H</u>$(CH_3)_2$), 3.67 (1H, s, -OH), 3.78-3.88 (1H, m, H(3)), 3.90-4.05 (1H, m, H(2)), 4.30-4.59 (3H, m, -$CHCH_2O$-).

IR (neat, cm^{-1}): 3436, 2933, 1764, 1693, 1462, 1384, 1211.

GC-MS (*m/z*, TMS ether): 314 (M^+-15), 270, 228, 204, 184, 143, 130.

(±)-(*Erythro*)-3-[3-hydroxy-2-propyl-1-oxopentyl]-4-(1-methylethyl)-2-oxazolidinone (**3ac**)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.85-0.98 (12H, m, 4 x -CH_3), 1.22-1.55 (4H, m, H(4), H(4')), 1.63-1.89 (2H, m, H(3')), 2.31 (1H, septet of d, J = 7, 4 Hz, -C<u>H</u>$(CH_3)_2$), 3.56-3.68 (1H, m, H(3)), 3.72 (1H, d, J = 6 Hz, -OH), 4.00-4.12 (1H, m, H(2)), 4.30-4.60 (3H, -$CHCH_2O$-).

IR (neat, cm^{-1}): 3445, 2923, 1771, 1690, 1462, 1382, 1302, 1220, 1098.

GC-MS (*m/z*, TMS ether): 328 (M^+-15), 284, 258, 198, 186, 158, 143, 125.

(±)-(*Erythro*)-3-[3-hydroxy-2-butyl-1-oxopentyl]-4-(1-methylethyl)-2-oxazolidinone (**3ad**)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.83-0.98 (12H, m, 4 x -CH_3), 1.21-1.54 (6H, m, H(3'), H(4'), H(5')), 1.72-1.86 (2H, m, H(4)), 2.34 (1H, septet of d, J = 7, 4 Hz, -$CH(CH_3)_2$), 3.55-3.70 (1H, m, H(3)), 3.73 (1H, d, J = 6 Hz, -OH), 3.99-4.10 (1H, m, H(2)), 4.28-4.60 (3H, m, -$CHCH_2O$-).

IR (neat, cm^{-1}): 3474, 2921, 1770, 1690, 1457, 1381, 1205.

GC-MS (*m/z*, TMS ether): 342 (M^+-15), 298, 272, 212, 200, 158, 143.

(±)-(*Erythro*)-3-[3-hydroxy-2-(1-methylethyl)-1-oxopentyl]-4-(1-methylethyl)-2-oxazolidinone (**3ae**)

mp 88-93°C

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.85-1.04 (15H, m, 5 x -CH_3), 1.20-1.60 (2H, m, H(4)), 2.14-2.43 (2H, m, 2 x -C<u>H</u>$(CH_3)_2$), 3.71-3.87 (2H, m, H(3), -OH), 4.12 (1H, dd, J = 6, 8 Hz, H(2)), 4.28-4.58 (3H, m, -$CHCH_2O$-).

IR ($CHCl_3$, cm^{-1}): 3414, 2967, 1768, 1678.

GC-MS (*m/z*): 213, 198, 171, 130, 111, 85. Extremely broad peak indicative of decomposition in instrument.

GC-MS (*m/z*, *tert*-BDMS ether): 328 (M^+-57), 284, 242, 198, 125.

(±)-(*Erythro*)-3-[3-hydroxy-2-cyclopentyl-1-oxopentyl]-4-(1-methylethyl)-2-oxazolidinone (**3af**)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.88-1.05 (9H, m, 3 x -CH_3), 1.1-2.4 (12H, m, cyclopentyl, -C<u>H</u>$(CH_3)_2$, H(4)), 3.69-3.85 (2H, m, -OH, H(2)), 4.19 (1H, dd, J = 6, 10 Hz, -$CHCH_2O$-), 4.29-4.57 (3H, m, H(3), -$CHCH_2O$-).

IR ($CHCl_3$, cm^{-1}): 3511, 2961, 1766, 1692.

GC-MS (*m/z*, TMS ether): 354 (M^+-15), 340, 311, 211.

(±)-(*Erythro*)-3-[3-hydroxy-2-(1-methylethyl)-1-oxopentyl]-2-oxazolidinone (**3be**)

^{1}H-NMR (200 MHz, acetone-d_6): δ: 0.84-1.00 (9H, m, CH_3), 1.2-1.6 (2H, m, H(4)), 2.23 (1H, septet of d, J = 7 Hz, H(3')), 3.69 (1H, d, J = 6 Hz, -OH, disappears with D_2O), 3.73-3.88 (1H, m, H(3)), 4.00-4.15 (3H, m, -$CHCH_2O$-, H(2)), 4.39-4.50 (2H, dd, J = 9, 9 Hz, -$CHCH_2O$-)

IR ($CHCl_3$, cm^{-1}): 3525, 2968, 1776, 1684.

GC-MS (*m/z*, TMS ether): 286 (M^+-15), 258, 242, 162, 144, 125.

(±)-(*Erythro*)-3-[3-hydroxy-2-cyclopentyl-1-oxopentyl]-2-oxazolidinone (**3bf**)

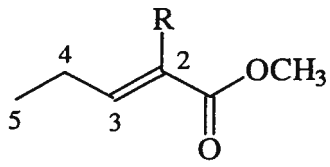
mp 91-94°C

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.92 (3H, t, J = 8 Hz, H(5)), 1.2-2.0 (10H, m, cyclopentyl, H(4)), 2.13-2.38 (1H, m, H(3')), 3.50-3.85 (2H, m, -OH, H(2)), 4.00-4.20 (3H, m, H(3), $-CH_2CH_2O-$), 4.38-4.52 (2H, m, $-CH_2CH_2O-$).

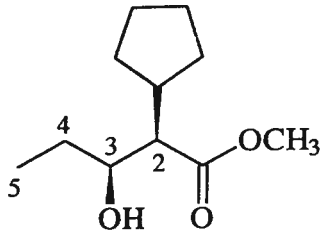
IR ($CHCl_3$, cm^{-1}): 3535, 2954, 1772, 1694, 1383.

GC-MS (*m/z*, TMS ether): 312 (M^+-15), 284, 268, 162, 151, 123.

2.2.5. Methyl (±)-(*E*)-2-alkylpent-2-enoate (**5a-e**) and (±)-(*erythro*) methyl 2-cyclopentyl-3-hydroxypentanoate (**4f**)



5a: R = Me
5b: R = Et
5c: R = Pr
5d: R = Bu
5e: R = *i*-Pr



4f

The crude adduct **3a/b** was dissolved in THF/water (400 mL, 3:1 v/v) and cooled to 0°C. 3,5-Dibutyl-4-hydroxytoluene (80 mg, 0.4 mmol), hydrogen peroxide (30%, 16.4 mL, 145 mmol) and lithium hydroxide monohydrate (3.06 g (73 mmol) in 5 mL water) were then added and the solution stirred for 3 h at 0°C. Aqueous sodium sulfite (1.5 M, 106 mL, 160 mmol) and saturated aqueous sodium bicarbonate (50 mL) were then added and the THF evaporated *in vacuo*. The aqueous residue was extracted with chloroform (200 mL), acidified with hydrochloric acid and extracted with ethyl acetate (2 x 200 mL). The ethyl acetate extract was washed with brine (40 mL), dried over magnesium sulfate, filtered and evaporated *in vacuo* to afford the 3-hydroxyacid. The hydroxyacid was then dissolved in anhydrous diethyl ether and treated with diazomethane using the standard procedure (Furniss *et al.*, 1987c). The ethereal solution was evaporated and the ester redissolved in dry dichloromethane (0.14 M) at 0°C. Triethylamine (2 eq.) and methanesulfonyl chloride (2 eq.) were added and the solution was stirred for 1 h and then evaporated *in vacuo* and filtered with THF in a volume equal to that of the initial dichloromethane solution. DBU (2 eq.) was then

added and the solution refluxed for 1 h. The mixture was diluted with an equal volume of petroleum spirit and washed with 1 M hydrochloric acid, saturated aqueous sodium bicarbonate and brine. The extract was dried over magnesium sulfate, filtered and evaporated *in vacuo* to afford a clear oil which was then purified by fractional distillation.

Methyl (*E*)-2-methyl-2-pentenoate (**5a**)

2.18 g (55% from carboximide **2aa**, bp 133-136°C, lit. 51°C / 11 mm Hg (Lide, 1994))

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.90 (3H, t, J = 8 Hz, H(5)), 1.70 (3H, m, H(3')), 2.08 (2H, m, H(4)), 3.56 (3H, s, -OCH_3), 6.60 (1H, t, J = 8 Hz, H(3)).

IR (neat, cm^{-1}): 2950, 1720, 1450, 1345.

GC-MS (*m/z*): 128 (M^+), 113, 97.

Methyl (*E*)-2-ethyl-2-pentenoate (**5b**)

1.21 g (31% from carboximide **2ab**, bp 112-117°C / 20 mm Hg)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.90-1.10 (6H, m, H(4'), H(5)), 2.10-2.30 (4H, m, H(3'), H(4)), 3.68 (3H, s, -OCH_3), 6.68 (1H, t, J = 15 Hz, H(3)).

IR (neat, cm^{-1}): 2961, 2876, 1714, 1647, 1440, 1297, 1239, 1149.

GC-MS (*m/z*): 142 (M^+), 127, 113, 111, 95, 83, 67.

Methyl (*E*)-2-propyl-2-pentenoate (**5c**)

4.02 g (54% from carboximide **2ac**, purified by flash chromagraphy using 1:19 diethyl ether/petroleum spirit, v/v)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.88 (3H, t, J = 8 Hz, H(5)), 1.04 (3H, t, J = 8 Hz, H(5')), 1.25-1.51 (2H, m, H(4')), 1.75-1.84 (2H, m, H(3')), 2.17-2.34 (2H, m, H(4)), 3.69 (3H, s, -OCH_3), 6.71 (1H, t, J = 8 Hz, H(3)).

IR (neat, cm^{-1}): 2951, 2874, 1715, 1646, 1451, 1354, 1279, 1223, 1148.

GC-MS (*m/z*): 156 (M^+), 127, 113, 95, 67, 55.

Methyl (*E*)-2-butyl-2-pentenoate (**5d**)

4.12 g (64% from carboximide **2ad**: bp 64-90°C / 8 mm Hg).

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.80-1.00 (3H, m, H(6')), 1.04 (3H, t, J = 8 Hz, H(5)), 1.20-1.40 (4H, m, H(4'), H(5')), 2.15-2.40 (4H, m, H(4), H(3')), 3.68 (3H, s, -OCH_3), 6.72 (1H, t, J = 15 Hz, H(3)).

IR (neat, cm^{-1}): 2946, 2869, 1716, 1646, 1458, 1439, 1261, 1205, 1148, 1112.

GC-MS (*m/z*): 170 (M^+), 155, 141, 127, 109, 95, 81, 69.

Methyl (*E*) 2-(1-methylethyl)-2-pentenoate (**5e**)

Using 1.6 g of the hydroxyester **4e** (obtained in 4.47 g (70%) yield from carboximide **2be**) gave only 100 mg of the methyl ester **5e** using the above procedure.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.02 (3H, t, J = 8 Hz, H(5)), 1.2-1.4 (6H, m, H(4'), H(4")), 2.20 (2H, q, J = 8 Hz, H(4)), 2.2-2.3 (1H, m, H(3')), 3.65 (3H, s, -OCH_3), 6.68 (1H, t, J = 8 Hz, H(3)).

IR ($CHCl_3$, cm^{-1}): 2960, 1708, 1449.

GC-MS (*m/z*): 156 (M^+), 141, 127, 109, 95, 81.

(±)-(*Erythro*) methyl 2-cyclopentyl-3-hydroxypentanoate (**4f**)

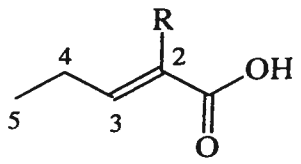
0.94 g (62% from carboximide **2bf**)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.0-1.9 (10H, m, cyclopentyl, H(4)), 2.12-2.28 (1H, m, -CHC<u>H</u>$(CH_2)_2$), 3.50-3.64 (5H, m, -OCH_3, H(2), -OH), 3.78-3.82 (1H, m, H(3)).

IR ($CHCl_3$, cm^{-1}): 3450, 2954, 1725, 1443.

GC-MS (*m/z*): 142 (M^+-58), 111, 99, 83, 74.

2.2.6. (*E*)-2-Alkyl-2-pentenoic acid (**5-8**)



5: R = Me
6: R = Et
7: R = Pr
8: R = Bu

The ester **5a-d** was dissolved in methanol (8.2 mL/mmol). Aqueous potassium hydroxide (2 M, 8.2 mL/mmol) was added and the solution refluxed for 2 h before the methanol was removed *in vacuo*. The solution was acidified with 6 M hydrochloric acid and extracted with diethyl ether. The organic extract was washed with brine, dried over magnesium sulfate and evaporated *in vacuo*. The residue was purified by distillation or flash chromatography on silica using diethyl ether/petroleum spirit as an eluent to afford the pure acid.

(*E*)-2-Methyl-2-pentenoic acid (**5**)

1.17 g crystalline solid (60%; bp 79-88°C / 0.7 mm Hg; lit. 106.5°C (Lucas and Prater, 1937)); melted upon warming to room temperature.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.03 (3H, t, J = 8 Hz, H(5,*E*)), 1.17 (3H, t, J = 7 Hz, H(5,*Z*)), 1.64 (3H, d, J = 5 Hz, H(3',*Z*)), 1.78 (3H, d, J = 1 Hz, H(3')), 2.20 (2H, q of d, J = 8, 8 Hz, H(4,*E*)), 2.46 (2H, m, H(4,*Z*)), 5.55 (1H, m, H(3,*Z*)), 6.75 (1H, t of d, J = 1, 8 Hz, H(3,*E*)). *E*:*Z* = 85:15.

IR (neat, cm^{-1}): 3200-3040, 2849, 1693, 1645, 1421, 1282, 1165.

GC-MS (*m/z*, TMS ester): 186 (M^+), 171, 157, 127, 97, 73, 75.

Anal. calc. for $C_6H_{10}O_2$: C, 63.14; H, 8.83, found: C, 63.15; H, 8.75.

(*E*)-2-Ethyl-2-pentenoic acid (**6**)

1.01 g clear oil (62%: bp 88°C / 0.5 mm Hg)

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.89-1.08 (6H, m, H(4'), H(5)), 2.10-2.38 (4H, m, H(3'), H(4)), 6.70 (1H, t, J = 9 Hz, H(3)).

IR (neat, cm^{-1}): 3200-3040, 2964, 1688, 1642, 1457, 1418, 1288, 1255, 1166.

GC-MS (*m/z*, *tert*-BDMS ester): 185 (M^+-57), 141, 111.

Anal. calc. for $C_7H_{12}O_2$: C, 65.60; H, 9.44, found: C, 65.55; H, 9.38.

(*E*)-2-Propyl-2-pentenoic acid (**7**)

1.78 g crystalline solid (49%: purified by flash chromatography)

mp 33-4°C

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.90 (3H, t, J = 8 Hz, H(5')), 1.04 (3H, t, J = 8 Hz, H(5)), 1.3-1.4 (2H, m, H(4')), 2.10-2.26 (4H, m, H(3'), H(4)), 6.75 (1H, t, J = 9 Hz, H(3)).

IR (neat, cm^{-1}): 3200-3040, 2932, 1674, 1632, 1458, 1418, 1274, 1166.

GC-MS (*m/z*, TMS ester): 214 (M^+), 199, 185, 169, 124.

Anal. calc. for $C_8H_{14}O_2$: C, 67.57; H, 9.92, found: C, 67.74; H, 10.08.

(*E*)-2-Butyl-2-pentenoic acid (**8**)

2.74 g crystalline solid (76%: bp 135-139°C / 4 mm Hg).

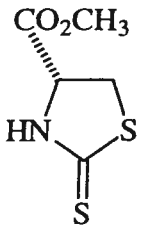
^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.90 (3H, t, J = 7 Hz, H(6')), 1.05 (3H, t, J = 8 Hz, H(5)), 1.20-1.47 (4H, m, H(4'), H(5')), 2.14-2.36 (4H, m, H(3'), H(4)), 6.75 (1H, t, J = 7.6 Hz, H(3)).

IR (neat, cm^{-1}): 3200-3040, 2898, 1692, 1639, 1459, 1418, 1279, 1214, 1163.

GC-MS (*m/z*, *tert*-BDMS ester): 213 (M^+-57), 179, 139, 105.

Anal. calc. for $C_9H_{16}O_2$: C, 69.19; H, 10.32, found: C, 68.87; H, 10.33.

2.2.7. 4(*R*)-(Methoxycarbonyl)-1,3-thiazolidine-2-thione (**9x**)



The following esterification procedure was adapted from Brenner and Huber (1953) and the subsequent cyclization step from Hsiao *et al.* (1987).

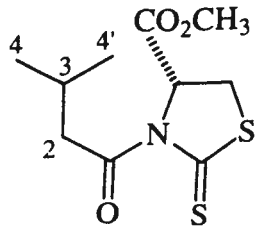
Thionyl chloride (5.0 g, 42 mmol) in chloroform (50 mL) was added slowly to dry methanol (80 mL) at room temperature. L-Cysteine (1.82 g, 15 mmol) was added gradually and the solution then stirred for 20 min before being evaporated *in vacuo*. The residue was dissolved in methanol (15 mL) and evaporated again to afford crude methyl L-cysteine hydrochloride (2.79 g) as a sticky white foam. The ester was suspended in dry dichloromethane (25 mL): triethylamine (1.87 g, 18.5 mmol) and carbon disulfide (1.20 g, 15.8 mmol) were added, each as a solution in dichloromethane (1 mL), and the bright yellow suspension stirred for 1 d at room temperature. The solvent was evaporated *in vacuo* and the residue suspended in ethyl acetate (30 mL) and filtered through a 10 cm column of silica gel (60-120 mesh). The product was eluted with further portions of ethyl acetate (2 x 50 mL) and the filtrate evaporated to afford a foul-smelling yellow oil (1.05 g) which was subjected to flash chromatography (1:1 ethyl acetate/petroleum spirit) to give pure 4(*R*)-methoxycarbonyl)-1,3-thiazolidine-2-thione (0.28 g, 11% overall) as a translucent oil.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 3.77 (1H, dd, J = 5, 11 Hz, CH_2S), 3.80 (3H, s, -OCH_3), 4.01 (1H, dd, J = 10, 11 Hz, CH_2S), 5.04 (1H, dd, J = 5, 10 Hz, NCH), 9.25 (1H, br s, NH).

IR (neat, cm^{-1}): 3350-3114, 2954, 1710, 1453, 1264, 1200, 1025.

GC-MS (*m/z*): 124, 118, 78 (unidentified fragments). Distinct peak in total ion current scan.

2.2.8. *N*-(3-Methylbutanoyl)-4(*R*)-(methoxycarbonyl)-1,3-thiazolidine-2-thione (**10x**)



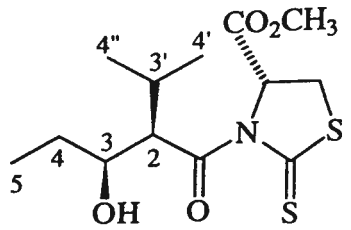
To a solution of the thiazolidinethione **9x** (1.00 g, 5.65 mmol) in dry dichloromethane (60 mL) at -78°C was added pyridine (0.50 g, 6.5 mmol), the cloudy suspension stirred 5 min and then isovaleryl chloride (0.83 g, 6.8 mmol) added. The yellow suspension was stirred at -78°C for 0.75 h, following which the cold bath was removed and the suspension warmed to room temperature over 2 h. The clear yellow solution was then diluted with dichloromethane (40 mL) and washed with water (10 mL), 5% aqueous oxalic acid (10 mL) and water (10 mL) before being dried over magnesium sulfate, filtered, evaporated *in vacuo* and purified by flash chromatography using diethyl ether/petroleum spirit (2:3, v/v) gave *N*-(3-methylbutanoyl)-4(*R*)-(methoxycarbonyl)-1,3-thiazolidine-2-thione (1.13 g, 77%) as oily crystals.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.96 (6H, d, J = 7 Hz, H(4), H(4')), 2.10-2.30 (1H, m, H(3)), 3.08 (1H, dd, J = 7, 16 Hz, H(2)), 3.25 (1H, dd, J = 7, 16 Hz, H(2)), 3.47 (1H, dd, J = 2, 12 Hz, -NCHCH_2S-), 3.80 (3H, s, -OCH_3), 3.93 (1H, dd, J = 9, 12 Hz, -NCHCH_2S-), 5.75 (1H, dd, J = 2, 9 Hz, -NCHCH_2S-).

IR ($CHCl_3$, cm^{-1}): 2962, 1750, 1705, 1318, 1266, 1174.

GC-MS: broad peak indicative of decomposition

2.2.9. (*Erythro*)-*N*-[3-hydroxy-2-(1-methylethyl)-1-oxopentyl]-4(*R*)-(methoxycarbonyl)-1,3-thiazolidine-2-thione (**11x**)



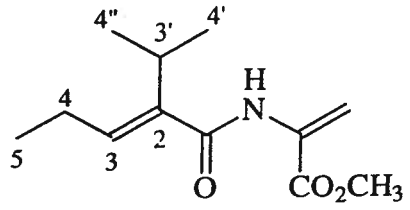
To a solution of the imide **10x** (1.00 g, 3.83 mmol) in dichloromethane (60 mL) at 0°C was added dibutylboron triflate (1.0 M in) dichloromethane, 4.45 mL, 4.45 mmol). After stirring for 5 min, triethylamine (0.49 g, 4.8 mmol) was added as a solution in dichloromethane (1 mL) and stirring was continued for 30 min at 0°C. The lime-green solution was cooled to -78°C, propionaldehyde (0.31 g, 5.3 mmol) in dichloromethane (1 mL) was added and stirring continued for 30 min at -78°C before the solution was allowed to warm to 0°C over 20 min. Phosphate buffer (pH 7, 60 mL) was added and the mixture stirred vigorously for 3 min prior to separation of the layers. The organic phase was separated, dried over magnesium sulfate, filtered and evaporated *in vacuo*. Flash chromatography (1:2-1:1 diethyl ether/petroleum spirit) afforded starting material (0.35 g, 35%) and the adduct (0.65 g, 53%) as a bright yellow oil.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.96 (3H, t, J = 8 Hz, H(5)), 1.04, 1.05 (3H, 3H, d, d, J = 1 Hz, H(4'), H(4")), 1.10-1.60 (2H, m, H(4)), 2.21 (1H, septet of d, J = 7 Hz, H(3')), 3.36-3.39 (1H, m, -NCHCH_2S-), 3.40 (1H, d, J = 2 Hz, -OH), 3.50-3.65 (1H, m, H(3)), 3.80 (3H, s, -OCH_3), 3.82-3.90 (1H, m, H(2)), 5.05 (1H, dd, J = 8, 8 Hz, -NCHCH_2S-), 5.73 (1H, dd, J = 2, 8 Hz, -NCHCH_2S-).

IR ($CHCl_3$, cm^{-1}): 3508, 2976, 1760, 1696, 1264, 1160.

GC-MS: broad peak indicative of decomposition.

2.2.10. *N*-(2-(Methoxypropenoyl))-2-(1-methylethyl)-2-pentenamide (**12x**)



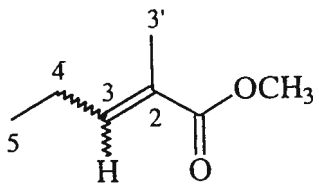
A solution of the adduct **11x** (480 mg, 1.5 mmol), triethylamine (0.17 g, 1.7 mmol) and methanesulfonyl chloride (0.19 g, 1.7 mmol) in dry dichloromethane (12 mL) was stirred 1.5 h at 0°C before being evaporated, filtered with THF (12 mL) and stirred for 3 h at room temperature in the presence of DBU (0.91 g, 6.0 mmol). Petroleum spirit (12 mL) was added and the mixture washed with 1 M hydrochloric acid (2 x 3 mL) and brine (3 mL). The solution was dried over magnesium sulfate, filtered and evaporated *in vacuo* prior to purification by flash chromatography using 1:1 diethyl ether/petroleum spirit (v/v) afforded 240 mg (71%) of the amide **12x** as a clear oil.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.95-1.04 (9H, m, -CH_3), 1.63-1.92 (2H, m, H(4)), 2.05-2.11 (1H, m, H(3')), 3.88 (3H, s, -OCH_3), 4.90-5.02 (1H, m, H(3)), 5.84, 6.50, (1H, 1H, s, s, C=CH_2), 8.52 (1H, br s, NH).

IR ($CHCl_3$, cm^{-1}): 3395, 2965, 1723, 1687, 1516, 1442.

GC-MS (*m/z*): 225 (M^+), 210, 182, 154, 141, 125, 109.

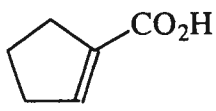
2.2.11. Attempted preparation of methyl (Z)-2-methyl-2-pentenoate (**15x**)



To a solution of carboximide **2aa** (0.60 g, 3.2 mmol) in dry dichloromethane (16 mL) at -78°C was added dibutylboron triflate (3.6 mL of 1 M solution in dichloromethane, 3.6 mmol) and triethylamine (0.46 g, 4.5 mmol) and the resultant yellow solution stirred for 1 h at -78°C and 15 min at 0°C before being re-cooled to -78°C and added through a double-tipped needle to a solution of diethylaluminum chloride (9.7 mL of a 1 M solution in dichloromethane, 9.7 mmol) and freshly-distilled propionaldehyde (0.28 g, 4.9 mmol) in dichloromethane (12 mL) at -78°C. The resultant solution was stirred for 2 h at -78°C before the reaction was quenched by the addition of water (5 mL) and the solution allowed to warm to room temperature. The layers were separated and the aqueous phase re-extracted with diethyl ether (40 mL). The combined organic extracts were washed with saturated sodium carbonate (8 mL) and brine (8 mL) prior to being dried over magnesium sulphate, filtered and evaporated *in vacuo* to afford the crude adduct (1.35 g). The hydrolysis and dehydration steps were carried out as described above to afford, following flash chromatography using diethyl ether/petroleum spirit (1:40, v/v), the unsaturated ester **15x** (200 mg, 49% from carboximide) as a clear oil.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 0.92 (3H, t, J = 7 Hz, H(5)), 1.70 (3H, s, H(3',*E*)), 1.75 (3H, s, H(3',*Z*)), 2.09 (2H, m, H(4,*E*)), 2.30 (2H, m, H(4,*Z*)), 3.56 (3H, s, -OCH_3), 5.84 (1H, t, J = 7 Hz, H(3,*Z*)), 6.60 (1H, t, J = 7 Hz, H(3,*E*)). The ratio of *E*/*Z* signals was 86:14.

2.2.12. 1-Cyclopentenyl-1-carboxylic acid (**12**)



To a solution of ethyl (2-oxocyclopentyl)carboxylate (6.00 g, 38.4 mmol) in methanol (100 mL) at 0°C was added sodium borohydride (1.60 g, 42.3 mmol). The solution was stirred for 1 h at 0°C, then water (30 mL) was added, followed by acidification with 6 M hydrochloric acid. The solution was condensed *in vacuo*, extracted with diethyl ether (2 x

100 mL) and the extract washed with brine (20 mL), dried over magnesium sulfate, filtered and evaporated *in vacuo* to afford an orange oil, which was diluted with diethyl ether (80 mL) and washed with saturated aqueous sodium bicarbonate (2 x 10 mL) and water (10 mL) before being dried over magnesium sulfate, filtered and evaporated to afford a yellow oil (4.94 g). Owing to apparent decomposition during distillation in a previous experiment, the crude material was used directly in the next (dehydration) step. Dry dichloromethane (100 mL) was added, followed by triethylamine (3.48 g, 34.3 mmol) and methanesulfonyl chloride (3.94 g, 34.3 mmol), each as dichloromethane solutions (5 mL). The solution was stirred 1 h at room temperature, then evaporated in vacuo and filtered with THF (50 mL). DBU (5.22 g, 34.3 mmol) in THF (10 mL) was added and the mixture stirred 1 d at room temperature before being diluted with diethyl ether (100 mL), washed with 1 M hydrochloric acid (2 x 15 mL) and brine (15 mL), dried over magnesium sulfate, filtered and evaporated *in vacuo*. Distillation afforded ethyl cyclopent-1-enyl-1-carboxylate (bp 82-105°C / 10 mm Hg; 3.02 g, 56% overall) as a clear liquid.

IR ($CHCl_3$, cm^{-1}): 2941, 1716, 1448.
GC-MS (*m/z*): 140 (M^+), 112, 95, 67.

Ethyl cyclopent-1-enyl-1-carboxylate (1.04 g, 7.43 mmol) was dissolved in 95% ethanol (30 mL). Aqueous potassium hydroxide (2M, 30 mL, 60 mmol) was added and the solution refluxed 2 h before being cooled, acidified with hydrochloric acid and extracted with diethyl ether (100 mL). The organic extract was washed with brine (2 x 15 mL), dried over magnesium sulfate, filtered and evaporated to afford cyclopentenylcarboxylic acid as a crystalline white solid (0.80 g, 96%).

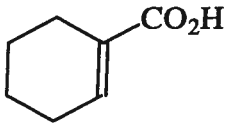
mp 114-117°C (lit. 123-124°C (Philp and Robertson, 1978))
^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.82-2.00 (2H, m, $CH_2C\underline{H}_2CH_2$), 2.40-2.56 (4H, m, $C\underline{H}_2CH_2C\underline{H}_2$), 6.69-6.77 (1H, m, C=CH).

IR ($CHCl_3$, cm^{-1}): 3040, 2950, 1691, 1621, 1428, 1288.

GC-MS (*m/z*, *tert*-BDMS ester): 169 (M^+-57), 125, 95.

Anal. calc. for $C_6H_8O_2$: C, 64.27; H, 7.19, found: C, 64.26; H, 7.25.

2.2.13. 1-Cyclohexenyl-1-carboxylic acid (**13**)



1-Cyclohexenyl-1-carboxylic acid was prepared by hydrolysis of methyl 1-cyclohexenyl-1-carboxylate: the crude product was purified by flash chromatography using diethyl ether/petroleum spirit (1:2, v/v) to afford the acid 1.98 g (87%) as a white crystalline solid.

mp 36-38°C (lit. 38°C (Boorman and Linstead, 1935))

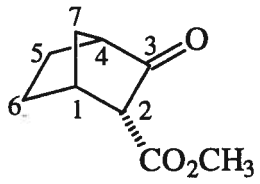
^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.5-1.7 (4H, m, $-CH_2CH_2-$), 2.1-2.3 (4H, m, $-CH_2-C=$), 6.9-7.0 (1H, m, -CH=), 10.1 (1H, br s, $-CO_2H$).

IR ($CHCl_3$, cm^{-1}): 3200-3000, 2936, 1686, 1644, 1425.

GC-MS (*m/z*, *tert*-BDMS ester): 183 (M^+-57), 139, 109.

Anal. calc. for $C_7H_{10}O_2$: C, 66.65; H, 7.99, found: C, 66.50; H, 8.10.

2.2.14. (±)-Methyl bicyclo[2.2.1]hept-3-oxo-2-carboxylate (**17x**)



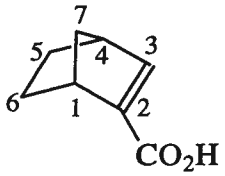
(±)-Methyl bicyclo[2.2.1]hept-3-oxo-2-carboxylate was prepared as described by Mander and Sethi (1983) to afford the product **17x** (5.39 g, 89%) as a yellowish oil.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.4-1.9 (6H, m, H(5), H(6), H(7)), 2.1-2.3, 2.4-2.6 (1H, 1H, m, m, H(1), H(4)), 2.9 (1H, m, H(2)), 3.75 (3H, s, -OCH_3).

IR ($CHCl_3$, cm^{-1}): 3035, 2961, 1764, 1731, 1442, 1272.

GC-MS: broad peak indicative of decomposition.

2.2.15. (±)-Bicyclo[2.2.1]hept-2-ene-2-carboxylic acid (**11**)



The methyl ester **18x** was prepared from **17x** as described for ethyl cyclopentenylcarboxylate. Distillation afforded a crude product (1.14 g, 36%, bp 93-123°C/10 mm Hg) which was hydrolyzed in refluxing potassium hydroxide as described above to the acid (0.40 g, 14% from **17x**, bp 132-135°C / 0.4 mm Hg) as a clear oil which crystallized upon standing.

mp 30-32°C (lit. 21.5-22.5°C (Finnegan and McNees, 1964))

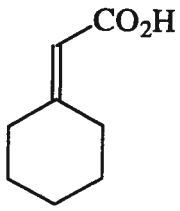
^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.0-1.9 (6H, m, H(5), H(6), H(7)), 3.00 (1H, m, H(4)), 3.20 (1H, m, H(1)), 6.92 (d, 1H, J = 3 Hz, H(3)).

IR ($CHCl_3$, cm^{-1}): 3038, 2948, 1691, 1597, 1411, 1283.

GC-MS (*m/z*, *tert*-BDMS ester): 237 (M^+-15), 195, 167, 123.

Anal. calc. for $C_8H_{12}O_2$: C, 69.55; H, 7.29, found: C, 69.74; H, 7.33.

2.2.16. Cyclohexylidineacetic acid (**15**)



The ester **20b** was prepared according to Wadsworth and Emmons (1973) and the crude product then hydrolyzed in refluxing potassium hydroxide as above to afford the crystalline white acid (1.28 g, 46%) after decanting with petroleum spirit.

mp 86-88°C (lit. 89°C (Wolinsky and Erickson, 1965))

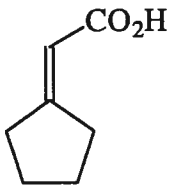
^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.52-1.74 (6H, m, -$CH_2CH_2CH_2$-), 2.2-2.3 (2H, m, -CH_2C=), 2.8-2.9 (2H, m, -CH_2C=), 5.63 (1H, t, J = 1 Hz, -C=CH), 10.3 (1H, br s, -CO_2H).

IR ($CHCl_3$, cm^{-1}): 3200-3000, 2935, 2859, 1688, 1643.

GC-MS (*m/z*, *tert*-BDMS ester): 197 (M^+-57), 153, 123.

Anal. calc. for $C_8H_{12}O_2$: C, 68.55; H, 8.63, found: C, 68.45; H, 8.64.

2.2.17. Cyclopentylideneacetic acid (**14**)



Cyclopentylideneacetic acid was prepared from cyclopentanone using the method described for cyclohexylideneacetic acid. The final product was purified by flash chromatography using 1:2 diethyl ether/petroleum spirit (v/v) to afford the acid (1.60 g, 84%) as a clear oil which crystallized upon standing.

mp 58-60°C (lit. 60-61°C (Weiland and Arens, 1956))

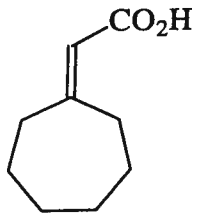
^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.5-1.7 (4H, m, -CH_2CH_2-), 2.12-2.30 (4H, m, -CH_2C=), 6.95-7.05 (1H, m, -C=CH), 10.4 (1H, br s, -CO_2H).

IR ($CHCl_3$, cm^{-1}): 3200-3000, 2911, 1688, 1644, 1424, 1283.

GC-MS (*m/z*, *tert*-BDMS ester): 183 (M^+-57), 139, 109.

Anal. calc. for $C_7H_{10}O_2$: C, 66.65; H, 7.99, found: C, 66.74; H, 7.94.

2.2.18. Cycloheptylideneacetic acid (**16**)



Cycloheptylideneacetic acid was prepared from cycloheptanone using the method described for cyclohexylideneacetic acid. The final acid product was purified by flash chromatography using 1:2 diethyl ether/petroleum spirit (v/v) to afford the acid as a clear oil which crystallized upon standing.

mp 50-2°C (lit. 54-55°C (Wolinsky and Erickson, 1965))

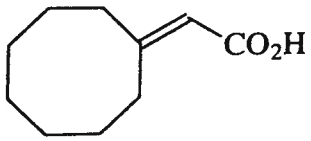
^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.5-1.8 (8H, m, -$(CH_2)_4$-), 2.40 (2H, br t, J = 7 Hz, -CH_2C=), 2.85 (2H, br t, J = 7 Hz, -CH_2C=), 5.66 (1H, t, J = 1 Hz, C=CH-).

IR ($CHCl_3$, cm^{-1}): 3200-3000, 2930, 1685, 1628, 1420.

GC-MS (*m/z*, *tert*-BDMS ester) : 211 (M^+-57), 167, 137.

Anal. calc. for $C_9H_{14}O_2$: C, 70.10; H, 9.15, found: C, 70.24; H, 9.18.

2.2.19. Cyclooctylideneacetic acid (**17**)



Cyclooctylideneacetic acid was prepared from cyclooctanone using the method described for cyclohexylideneacetic acid. The final product was purified by flash chromatography using 1:2 diethyl ether/petroleum spirit (v/v) to afford the acid as a clear oil which crystallized upon standing.

mp 81-3°C (lit. 89-89.5°C (Wolinsky and Erickson, 1965))

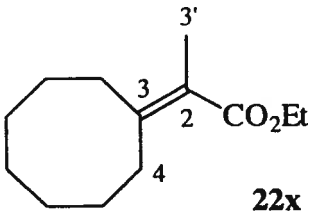
^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.4-1.9 (10H, m, -$(CH_2)_5$-), 2.38 (2H, t, J = 7 Hz, -CH_2C=), 2.78 (2H, t, J = 7 Hz, -CH_2C=), 5.78 (1H, s, C=CH-).

IR ($CHCl_3$, cm^{-1}): 3200-3000, 2913, 1687, 1626, 1450.

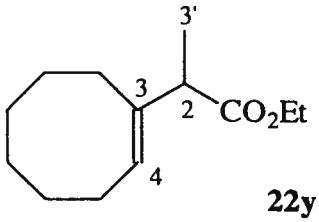
GC-MS (*m/z*, *tert*-BDMS ester): 225 (M^+-57), 181, 151.

Anal. calc. for $C_{10}H_{16}O_2$: C, 71.39; H, 9.59, found: C, 71.33; H, 9.63.

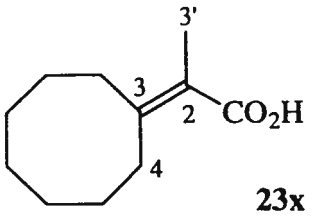
2.2.20. Attempted preparation of cyclooctylidenepropanoic acid (**23x**)



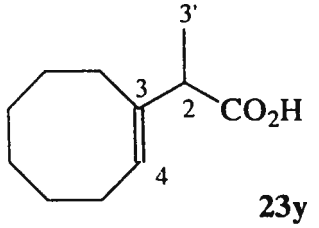
22x



22y



23x



23y

Cyclooctylidenepropanoic acid was prepared as above except that triethyl 2-phosphonopropionate was used instead of the acetate. A sample of the intermediate ester **22** was purified by flash chromatography using 1:24 diethyl ether/petroleum spirit (v/v).

^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.0-1.2 (m, -CH_2-, -$OCH_2C\underline{H}_3$), 1.3, 1.5 (m, -CH_2-, -$CHC\underline{H}_3$), 1.74 (s, CH_3C=), 2.2, 2.3 (m, CH_2C=), 2.96 (q, J = 8 Hz, -C<u>H</u>-CH_3), 3.8-4.2 (m, -$OC\underline{H}_2CH_3$), 5.40 (t, J = 8 Hz, C=CH). The $\Delta^{2,3}$ and $\Delta^{3,4}$ isomers were present in a 3:1 ratio.
GC-MS (*m/z*): two peaks, t_r 5.25 min (210 (M^+), 195, 182, 167, 153, 137, 109, 102, 95) and t_r 5.67 min (210 (M^+), 195, 182, 165, 149, 136, 109) in approximately 1:3 ratio.

The mixture of esters was hydrolyzed with refluxing potassium hydroxide as described previously. The crude product was purified by flash chromatography using 1:2 diethyl ether/petroleum spirit (v/v) to afford a mixture of the acids **23x** and **23y** as a clear oil.

^{1}H-NMR (200 MHz, acetone-d_6) δ: 1.19 (3H, d, J = 7 Hz, -CH_3, **23y**), 1.4-2.2 (m, -CH_2-), 2.32, 2.60 (2H, 2H, m, m, CH_2C=, **23x**), 3.12 (1H, q, J = 8 Hz, H(2), **23y**), 5.52 (1H, t, J = 7 Hz, H(4), **23y**).
IR (neat, cm^{-1}): 3050, 2920, 1691, 1605, 1453, 1409, 1280.
GC-MS (*m/z*, *tert*-BDMS esters) : two peaks, t_r 6.08 min (239 (M^+-57), 195, 167, 153) and t_r 6.33 min (239 (M^+-57), 165, 143, 95) in approximately 1:3 ratio.

2.3. PHYSICOCHEMICAL PROPERTIES

Molecular modelling was performed using MacroModel v. 3.5 on a Silicon Graphics IRIS workstation. The input structure was minimized using the MM2* force field in an aqueous medium and the van der Waals volume calculated for this structure. A Monte Carlo conformational search was then conducted for 500 conformations using a 10 kJ/mol energy cutoff. The atomic Cartesian coordinate for each conformer were then transformed with a custom PASCAL program (written with the assistance of Mr. Roland Burton) so as to place the carbonyl atom at the origin, C(2) on the *x*-axis and the C(2) substituent in the *xy* plane (Figure 34). The population-weighted mean internuclear dimensions, expressed as the maximal *x*, *y* or *z* values, were calculated based on a Boltzmann distribution at 37°C.

The lipophilicity of most compounds were initially obtained by an HPLC method (Achaempong, 1985). Methanolic solutions of a series of compounds with previously determined log P values (butyric, valeric, hexanoic, 2-ethylbutyric, 2-ethylhexanoic, VPA) were injected into an HP 1090 liquid chromatograph equipped with an Altex Ultrasphere-ODS column and a 50 mM pH 3.5 phosphate/acetonitrile (54:46, v/v) mobile phase. The capacity factor for each was calculated and used to derive a calibration curve for the log P values of **2-15**.

Lipophilicity was also calculated for the undissociated species using the CLOGP program (Daylight Chemical Information Systems, Irvine, CA, v. 3.54) by Dr. Eric Bigham at Burroughs Wellcome Co. (Research Triangle Park, NC). The program was not able to distinguish *cis* and *trans* isomers.

2.4. ANTICONVULSANT POTENCY

Anticonvulsant potency (Swinyard *et al.*, 1989) was determined for each drug using five intraperitoneal doses (4 mL/kg) of its sodium salt and eight 6 week old mice per dose. Pentylenetetrazole (85 mg/kg, 10 mL/kg) was injected subcutaneously at 10 min and the

animals observed for a further 30 min. An animal was considered to be unprotected if it showed a 5 s clonus with loss of balance. ED50 was determined from a graph of percentage protection *vs* log(dose) following the method of Litchfield and Wilcoxon (1949), where percentage protection refers to the percent of animals in each dose group which were protected against seizures.

2.5. MOUSE BRAIN DRUG DETERMINATION

The frozen brain homogenate samples (500 ul: from homogenates used for GAD assay) were thawed, mixed with sodium octanoate solution (500 ul, 22.4 nmol) and 1.5 M hydrochloric acid (1000 ul) and 380 ul aliquots combined with ethyl acetate (2000 ul). The mixtures were vortexed thoroughly and then subjected to gentle rotation for 30 min. The vials were centrifuged (2060*g*, 10 min) and a 1000 ul portion of the organic layer removed and dried over sodium sulfate for 10 min. A fraction (800 ul) of the supernatant was removed, evaporated to approximately 100 ul under a flow of dry nitrogen in a 40°C water bath and finally derivatized with 500 ul of an ethyl acetate solution containing 7% MTBSTFA, 0.07% *tert*-BDMSCl (v/v) and 2.38 nmol/mL cyclohexylcarboxylic acid for 1 h at 65°C. Upon cooling, the solutions were analyzed by GC-MS as described below.

The corresponding calibration curve was prepared by combining 47.5 ul aliquots of stock aqueous solutions of the sodium salts of each drug (0, 2.5, 12.5, 25, 50, 100, 200 and 400 nmol/mL) and octanoic acid (100 nmol/mL) with control brain homogenate (95 ul) and 1.2 M hydrochloric acid (238 ul). Ethyl acetate (2000 ul) was added and the samples processed as described above.

Recovery values were evaluated by running a second set of calibration standards to determine the actual amount of drug in each of the above calibration samples. In this case, 100 ul aliquots of solutions of each drug in ethyl acetate (0, 1.875, 3.75, 7.500, 15.00, 25.00 and 50.00 nmol/mL) containing octanoic acid (17.02 nmol/mL) were combined with the

MTBSTFA solution described above (500 ul) and heated for 1 h at 65°C prior to analysis by GC-MS.

The acids in the above assays were analyzed by GC-MS as noted in Section 2.1 with the following additional specifications. Oven: 50°C initial, increasing to 260°C @ 10°C/min with final hold time 5 min. Equilibration time: 0.5 min. MS: single ion monitoring at *m/z* (M^+ - 57) for *tert*-butyl(dimethylsilyl) esters of drug and the two internal standards. A standard auto-tuning sequence was used.

2.6. MEMBRANE FLUIDITY

Membrane fluidity studies were initially performed using erythrocyte ghosts prepared by the general method of Dodge *et al.* (1963). Freshly collected human whole blood was centrifuged (913*g*, 10 min), decanted, the cells washed three times with phosphate-buffered saline (PBS: 10 mM sodium phosphate, 0.9% NaCl (w/v), pH 7.4), lysed with hypotonic buffer (10 mM sodium phosphate, pH 7.4), centrifuged (2060*g*, 20 min), decanted, washed with PBS and resuspended to afford a 10% ghost suspension. For the fluidity assay, the ghost suspension was combined with PBS, membrane probe (DPH or TMA-DPH as THF-PBS or ethanol-PBS solutions, respectively) and drug solution in PBS and incubated in the dark at room temperature for 2 h. Final concentrations: ghosts 1.3%; probe 2 uM; drug 20 mM. Fluorescence was recorded at 20°C using a Shimadzu RF-540 polarizer-equipped spectrofluorophotometer. Excitation 355 nm (DPH) or 363 nm (TMA-DPH); emission 428 nm; slit widths 10 nm. The parallel (I_{vv}) and perpendicular (I_{vh}) fluorescence components for each sample (five per drug) were measured three times without stirring and the fluorescence anisotropy r calculated using corrections for scatter (4%) and grating polarization G (0.9757). Fluorescence anisotropy showed the expected temperature dependence in all preparations.

Synaptic membranes were prepared as described by Jones and Matus (1974).

2.7. GABA AND GAD ASSAYS

Synaptosomes from mouse whole brain were prepared by a modification of the method of Dodd *et al.* (1981). All steps were carried out at 0-4°C. For the assay of GABA, all sucrose solutions were 1 mM in sodium 3-mercaptopropionate (3-MP) to inhibit the large post-mortem GABA increase (Loscher *et al.*, 1981). Ten mice (five per assay) were injected with 4 mL/kg of either 0.9% saline or VPA as an aqueous solution of its sodium salt (0.83 mmol/kg), decapitated at 15 min postdose into 0.9% saline, the brains were quickly removed, homogenized in 0.32 M sucrose (4 mL, pH 7.0), diluted with sucrose (2 mL) and centrifuged at 1,000*g* for 10 min. A 500 ul sample was taken prior to centrifugation from each 3-MP-free homogenate for subsequent drug analysis (Section 2.5). The supernatant was layered onto 1.2 M sucrose (9.5 mL, pH 7.0) and then centrifuged (220,000*g*, $\omega^2 t = 1.6 \times 10^{10}$ rad^2/s): the interface was collected by pipet, made up to 10 mL, layered onto 0.8 M sucrose (9.5 mL, pH 7.0) and centrifuged as before. The supernatant was removed and the pellet resuspended in either water (GAD assay, 5 mL) or buffer (GABA assay, 5 mL: 0.5 M KCl, 0.4 M sodium phosphate, 10 mM EDTA, 0.5% Triton X-100, 47.4 nmol GABA-d_6, pH 6.4, 5 mL) (Holdiness *et al.*, 1981). The protein content of the synaptosomes was 19.6 ± 2.1 mg/g tissue for the GABA assay (n = 10) and 16.3 ± 2.0 mg/g tissue for the GAD assay (n = 10), using a modified Lowry assay (Markwell *et al.*, 1978). The GABA and protein assays were conducted promptly, whereas the GAD assays were performed on samples stored at -78°C.

In the GAD assay (Loscher, 1981), each synaptosome sample (50 ul) was incubated in triplicate for 1 h at 37°C with 50 ul of a solution containing glutamate (1 umol) and PLP (50 nmol) in 0.1 M pH 6.4 sodium phosphate buffer. Final concentrations were 5 mM and 0.25 mM for glutamate and PLP, respectively. For the blank (run in duplicate), the glutamate/PLP mixture was replaced with 50 ul of buffer. The reaction was quenched by the addition of 1.7% trichloroacetic acid (53 ul) containing GABA-d_6 (660 pmol), the precipitated material separated by centrifugation (13,600*g*, 10 min), the supernatant lyophilized at ambient temperature and derivatized with an acetonitrile solution (300 ul, containing 30 ul MTBSTFA

and 0.3 ul *tert*-BDMSCl (Mawhinney *et al.*, 1986)) at 65°C for 1 h prior to GC-MS analysis. GAD activity was defined as the amount of GABA (per mg protein) in the sample incubated with glutamate and PLP less the amount obtained in the blank. In separate control experiments, the glutamate and/or PLP were omitted from the phosphate buffer solution. A blank experiment (n = 4) was performed that retained the Glu/PLP mixture but replaced the synaptosomes with an equivalent volume of water.

Experiments designed to test the conditions of the above assays were also conducted using the procedure described above except for the changes noted below. The same synaptosome preparation was used throughout. The activity for each set of conditions was measured in triplicate and then subtracted from an appropriate blank measured in duplicate. In the first set, the final concentration of PLP was fixed at 0.25 mM and final glutamate concentrations varied as 10, 7.5, 5, 2.5, 1.25 and 0.5 mM. In the second, glutamate was fixed at 5 mM and PLP varied as 0.5, 0.375 and 0.125 mM. Finally, the assay was conducted using the standard glutamate and PLP concentrations for 5, 10, 20, 30, 45, 60, 90 and 120 min timepoints.

The dependence of activity on protein concentration was determined from a synaptosome pellet which that been resuspended in 2.5 mL, rather than the usual 5 mL, of water. In addition to an undiluted sample, the GAD assay was then run with samples diluted 1:0.5, 1:1, 1:2, 1:3, 1:4 and 1:5 (v/v) with water.

In the brain GABA assay, run in triplicate for each synaptosome preparation, resuspended synaptosome samples (50 ul) were acidified with 1.7% trichloroacetic acid (60 ul) and centrifuged (13,600g, 10 min). An aliquot (80 ul) of the supernatant was then removed, lyophilized and derivatized as described above. A blank experiment (n = 5) was conducted where the synaptosomes were replaced with the resuspension buffer.

The GABA contents of the above assays were analyzed by capillary GC-MS as noted in Section 2.1 with the following additional specifications. Oven: 50°C initial, increasing to 130°C @ 30°C/min then to 200°C @ 10°C/min and finally to 280°C @ 30°C/min, with a final hold time of 2 min. Equilibration time: 1 min. MS: single ion monitoring at m/z 274

(GABA) and 280 (GABA-d_6). The tune was optimized for *m/z* 264 with the high-energy dynode set at 10 kV.

Individual standard curves were prepared for the GABA and GAD assays using aqueous solutions of GABA and GABA-d_6 containing the appropriate amounts of buffer and trichloroacetic acid. These solutions were evaporated to dryness and derivatized as above. For the GAD blank and the GABA assay, a range of 400-1900 pmol GABA was used, while a range of 3-20 nmol was employed for the GAD assay.

2.8. GAD INHIBITION ASSAY

The inhibition of GAD activity by **17** *in vitro* was studied by incubation of frozen synaptosomes from saline-treated mice with varying concentrations of the drug. An aliquot of synaptosomes (50 ul) was mixed with a substrate solution containing **17** (final concentrations: 0, 1, 5 mM), glutamate (final concentrations: 1, 3, 5, 10 mM) and PLP (final concentration: 0.25 mM) and incubated at 37°C for 10 min prior to being quenched and processed as described in Section 2.7. For each set of conditions (n = 3), a blank (n = 2) containing synaptosomes and buffer only was also run and subtracted from the former to afford the enzyme activity. A double-reciprocal plot of activity *vs.* glutamate concentration was then constructed to obtain the various kinetic parameters.

2.9. GAD SATURATION ASSAY

The GAD saturation assay was based on procedures described by Martin (1986) with the modifications of Nathan *et al.* (1994). All operations were carried out at 0-4°C. Mature male CD-1 mice were given saline or an ED50 dose of VPA, **7** or **17** and decapitated at 15 min postdose into ice-cold 0.9% saline. The cranium was quickly exposed underwater and the head cooled briefly. The brain was removed, homogenized and processed as described for synaptosome preparation except that all sucrose solutions contained 5 mM ATP rather than 3-

MP. The final pellet was resuspended in a mixture of 5 mM ATP (1 mL, pH 6.5) and 80 mM Im/Ac buffer (1 mL, 80 mM 1:1 imidazole/acetic acid, 4 mM 2-aminoethylisothiouronium bromide, 2 mM EDTA·Na_4, 1% Triton X-100, pH 6.5 with concentrated NaOH). A 500 ul aliquot was applied to a column of Sephadex G-25 (medium, 4.0 g packing, 20 mL total volume) and eluted at 0.2 mL/min with 40 mM Im/Ac buffer (a 1:1 (v/v) dilution of the 80 mM buffer with water). The protein fractions were combined and 150 ul aliquots mixed with either pyridoxal phosphate (25 ul, 0.8 mM, $n = 4$) or buffer ($n = 6$). Following pre-incubation for 5 min at 37°C, glutamate (25 ul, 8 mM) was added to all except two control tubes, where buffer (25 ul) was added. The samples were incubated for a further 5 min at 37°C and then quenched (50 ul, 6% trichloroacetic acid, 8.78 uM GABA-d_6) and centrifuged (13,600*g*, 10 min). The supernatant (230 ul) was removed, lyophilized, treated with MTBSTFA solution (400 ul, 10% MTBSTFA and 0.1% *tert*-BDMSCl, v/v, in acetonitrile) at 65°C for 1 h and analyzed by GC-MS as described before. Controls were also run using the pyridoxal phosphate and glutamate solutions individually and in combination ($n = 3$ each). Calibration standards were made up with the appropriate amounts of buffers, trichloroacetic acid and GABA-d_6 and treated identically as the samples.

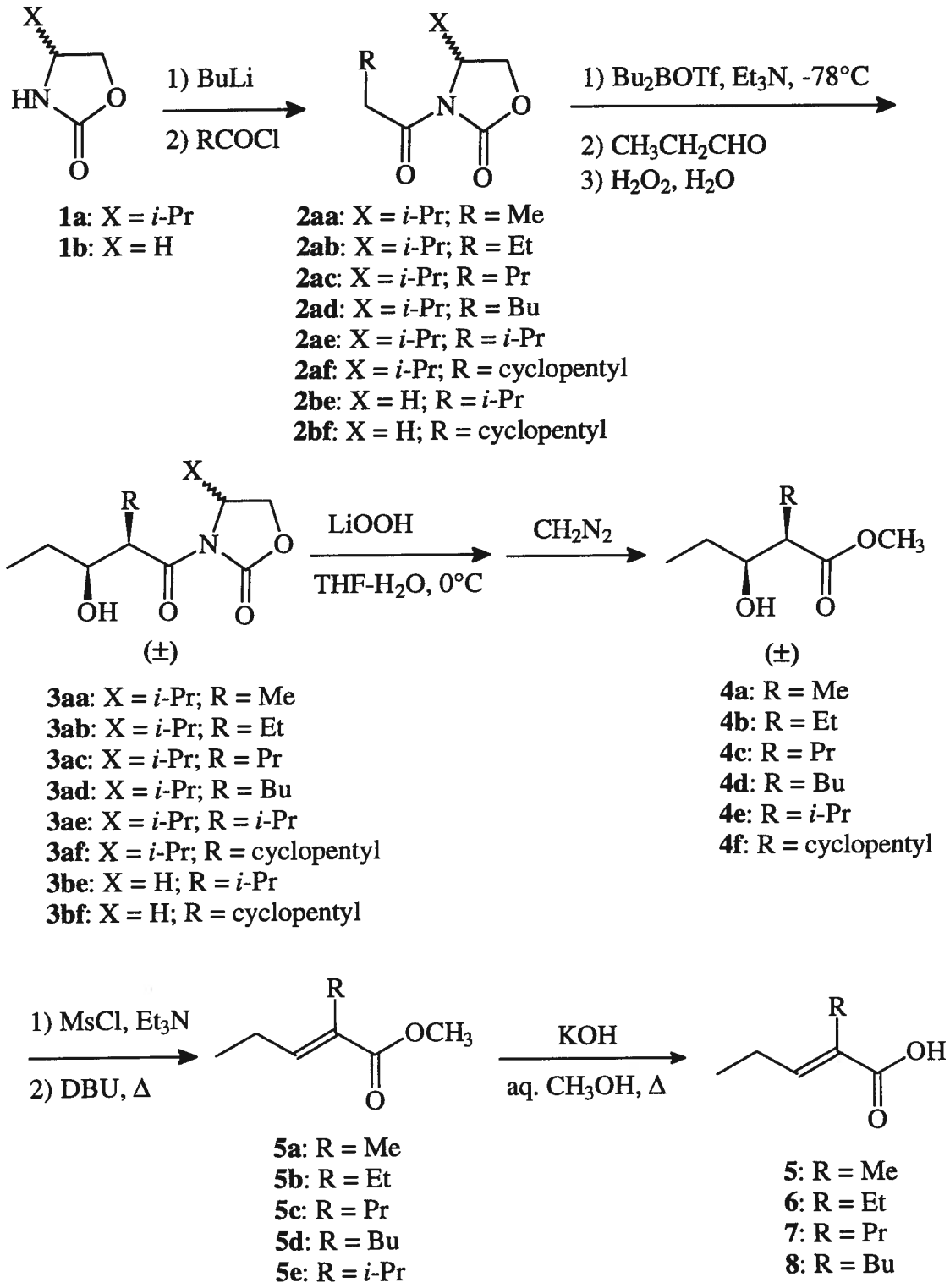
Synaptosome GAD activity was determined using samples frozen for several days at -78°C, with the assay being performed as described in Section 2.7 except that the 0.1 M phosphate buffer was replaced by 40 mM Im/Ac buffer containing 5 mM ATP. All protein concentrations were measured using the method of Markwell *et al.* (1978).

3. RESULTS

3.1. SYNTHESIS

The initial choice of 2-ene VPA analogues for study was a series of (*E*)-2-alkyl-2-pentenoic acids (**5-8**). Their preparation was based on the highly stereoselective addition of propionaldehyde to dibutylboryl enolates derived from racemic *N*-acylisopropyloxazolidinones (Figure 6) using the procedure described by Evans *et al.* (1986). The carboximides **2a/a-f** were first prepared according to Evans *et al.* (1981) from the oxazolidinone **1a** synthesized by a modification of the method of Newman and Kutner (1951). The adducts **3a** were then isolated and characterized by ^{1}H-NMR and by GC-MS of their silyl ethers, the spectra being consistent with the presence of a pair of presumably *erythro* enantiomers. Note that all structures in Figure 6 are racemic. This finding was supported by the fact that all of the subsequent unsaturated acids **5-8**, where the double bond had been formed by a stereospecific E2 elimination mechanism, were found to exist predominantly as their *E* isomers, based on the chemical shift δ of their H(3) ^{1}H-NMR signals. Specifically, δ(H(3)) for our preparation of acid **7** agreed well with not only the published value but also with those for the other acids **5, 6** and **8** (see Figure 14 for a full list of final structures).

Hydrolysis of the adducts **3a** with lithium hydroperoxide (Evans *et al.*, 1987) followed by treatment with diazomethane gave high yields of the methyl hydroxyesters **4a-f** . However, the branched side-chain adducts **3ae** and **3af** hydrolyzed very slowly (isopropyl: 29%; cyclopentyl: 15%) even when the reaction was conducted at room temperature. The probable explanation is the steric crowding in the region of the "amide" carbonyl by these groups. Assuming that the dibutylboryl enolate *Z* stereochemistry alone would be sufficient to direct the reaction to the *erythro* adduct (Evans *et al.*, 1982), we synthesized the isopropyl-substituted adduct **3be** derived from 2-oxazolidone **1b** itself. The stereoselectivity of the process had not been compromised, as indicated by ^{1}H-NMR spectroscopy (Figure 7), but the overall conversion of carboximide **2be** to hydroxyester **4e** proceeded in 70% yield. A similar yield was obtained with the cyclopentyl analogue **2bf**. This indicates that the stereochemical



1a: X = *i*-Pr
1b: X = H

2aa: X = *i*-Pr; R = Me
2ab: X = *i*-Pr; R = Et
2ac: X = *i*-Pr; R = Pr
2ad: X = *i*-Pr; R = Bu
2ae: X = *i*-Pr; R = *i*-Pr
2af: X = *i*-Pr; R = cyclopentyl
2be: X = H; R = *i*-Pr
2bf: X = H; R = cyclopentyl

(±)

3aa: X = *i*-Pr; R = Me
3ab: X = *i*-Pr; R = Et
3ac: X = *i*-Pr; R = Pr
3ad: X = *i*-Pr; R = Bu
3ae: X = *i*-Pr; R = *i*-Pr
3af: X = *i*-Pr; R = cyclopentyl
3be: X = H; R = *i*-Pr
3bf: X = H; R = cyclopentyl

(±)

4a: R = Me
4b: R = Et
4c: R = Pr
4d: R = Bu
4e: R = *i*-Pr
4f: R = cyclopentyl

5a: R = Me
5b: R = Et
5c: R = Pr
5d: R = Bu
5e: R = *i*-Pr

5: R = Me
6: R = Et
7: R = Pr
8: R = Bu

Figure 6. Synthesis of 2-substituted 2-pentenoic acids.

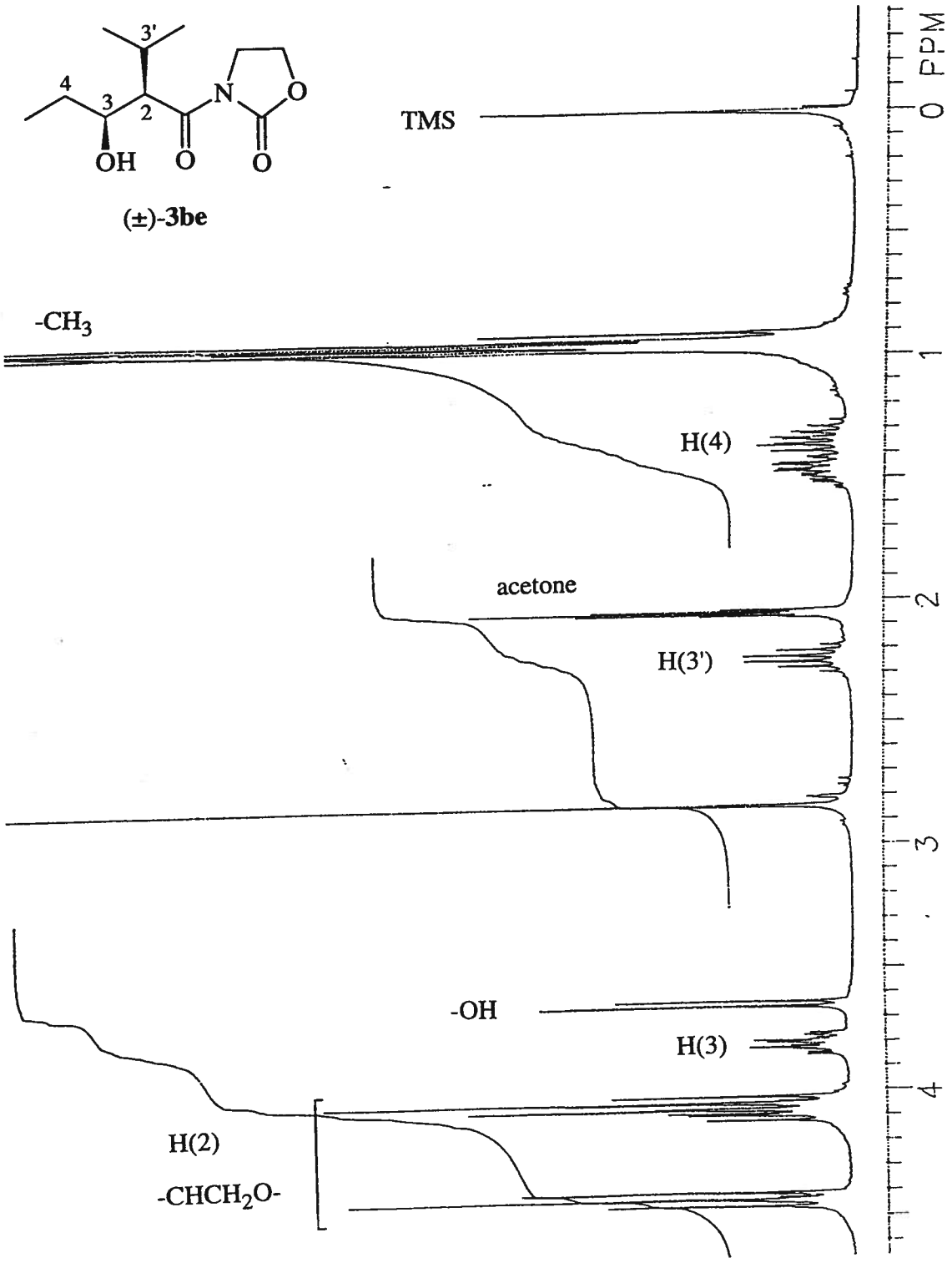


Figure 7. 300 MHz ^{1}H-NMR spectrum of adduct **3be**.

control of the reaction is determined by the dibutylboryl enolate geometry rather than the structure of the carboximide, allowing for the use of inexpensive 2-oxazolidinone rather than costly 4-isopropyl-2-oxazolidinone.

The subsequent "dehydration" step proved to be surprisingly difficult with the saturated analogues although many earlier reactions with the aldol adducts themselves as well as with precursors of the bis-unsaturated acids **9** and **10** invariably proceeded in high yields using 1.1 equivalents of all reagents (triethylamine, methanesulfonyl chloride and DBU) and stirring overnight at room temperature (Lee, 1991). The hydroxyester **4c**, however, did not show more than approximately 50% conversion (as judged by GC-MS) by this protocol, even when excess DBU was added and the solution refluxed for several hours. It was found that using 4 equivalents of all reagents and conducting the elimination step under reflux for 1 h afforded reasonable yields of esters **5a-d**, although only trace amounts were obtained for **5e**. This is consistent with the conditions reported elsewhere for the elimination of mesylates leading to isolated double bonds (Williams and Maruyama, 1987). All products were isolated as single isomers, indicating that the integrity of the E2 elimination was maintained at the elevated temperature. The final acid products were then obtained by basic hydrolysis as single isomers with the exception of **5**, whose ^{1}H-NMR spectrum indicated the presence of two isomers in an 85:15 (*E:Z*) ratio.

Unfortunately, TLC and GC-MS analysis of the dehydration step for the cyclopentyl ester **4f** indicated no conversion, probably due to excessive steric congestion. In view of this result and the poor yield obtained for **5e**, an alternative approach seemed desirable.

The dehydration step could be performed on the adduct itself, which had previously been shown to be a very facile process, and then hydrolyzing this species to our final product, the 2,3-unsaturated acid (Figure 8). However, such a hydrolysis reaction would involve an even less electrophilic "amide" carbon atom, owing to its conjugation with the double bond, thus making the desired hydrolysis of the branched side-chain adducts even less favourable. One solution to this problem was the use of an oxazolidinone-related auxiliary group that would likewise allow formation of the dibutylboryl enolate and the aldol addition to

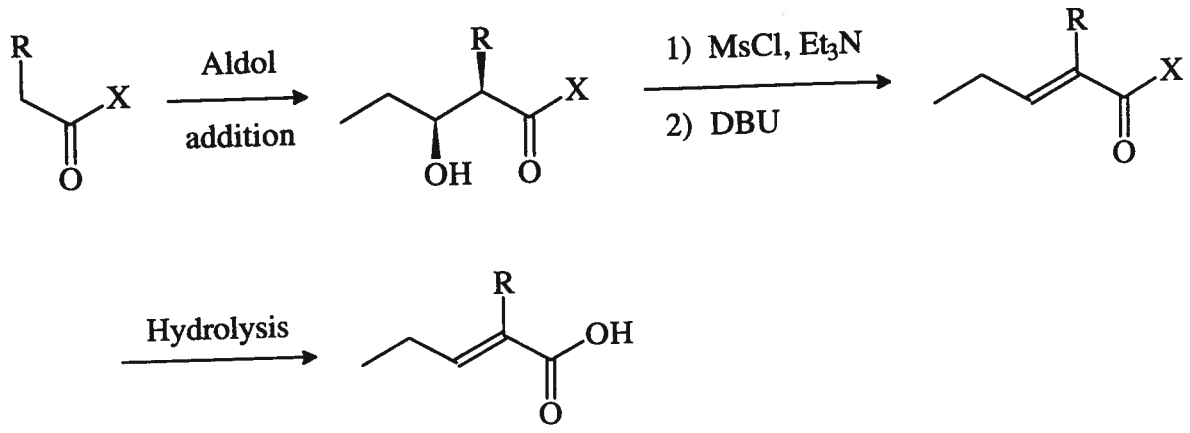


Figure 8. Envisaged alternative pathway to final acid product.

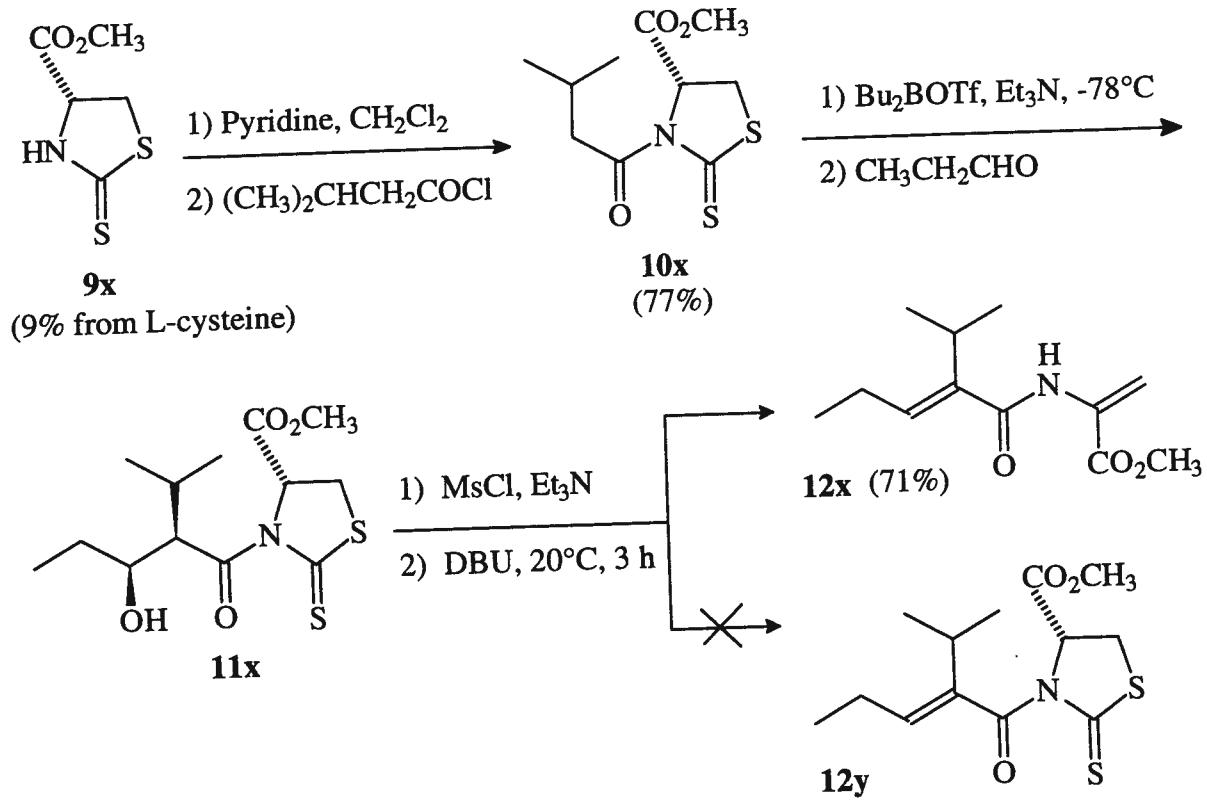


Figure 9. Thiazolidinethione-based approach to 2-substituted-2-pentenoic acids.

proceed with high selectivity and facilitate the formation of the 2,3-unsaturated adduct, but would simultaneously be much more susceptible to removal by basic hydrolysis. The thiazolidinethiones reported by Hsiao *et al.* (1987) appeared to meet these requirements, leading to the sequence of reactions described in Figure 9. Although the dehydration reaction proceeded quantitatively at room temperature, the major product was apparently the di-unsaturated species shown, indicative of a facile elimination of CS_2. Further studies to determine if the mesylate alone could be selectively eliminated were not performed.

We briefly investigated the feasibility of the *threo*-selective aldol addition, described by Walker and Heathcock (1991), using the 2-methyl carboximide **2aa**. The aldol addition of the carboximide to diethylaluminum chloride-complexed propionaldehyde, along with the subsequent steps (Figure 10), proceeded in reasonable yields but the ^{1}H-NMR spectrum of the final unsaturated ester **15x** revealed an 86:14 mixture of isomers. Because the dehydration was stereospecific, as shown previously by the presence of only one set of signals in the ^{1}H-NMR spectrum of the ester **4a** prepared by the route described in Figure 6, this ratio corresponded to the degree of stereoselectivity of the aldol addition itself.

To further study the effects of chemical structure on anticonvulsant activity, we also synthesized the acids **11-13** shown in Figure 11. The norcamphor derivative was prepared by the methoxycarbonylation of norcamphor (Mander and Sethi, 1983) followed by reduction with sodium borohydride, dehydration and hydrolysis. A similar approach was used for **12** and **13** except that the starting materials were the commercially available ethyl cyclopentanonecarboxylate and ethyl cyclohexenylcarboxylate, respectively.

Compounds **14-17**, where the double bond is exocyclic to the ring, were prepared by reaction of the corresponding ketone with the sodium salt of triethyl phosphonoacetate (Wadsworth and Emmons, 1973) followed by hydrolysis of the resultant ester (Figure 12). We also attempted to prepare the acid **23x** using an identical approach with triethyl phosphonopropionate. Unfortunately, the ^{1}H-NMR spectra of both the ester and the final acid, even after purifications by flash chromatography, indicated inseparable 3:1 mixtures of regioisomers **22x/22y** and **23x/23y**, respectively (Figure 13).

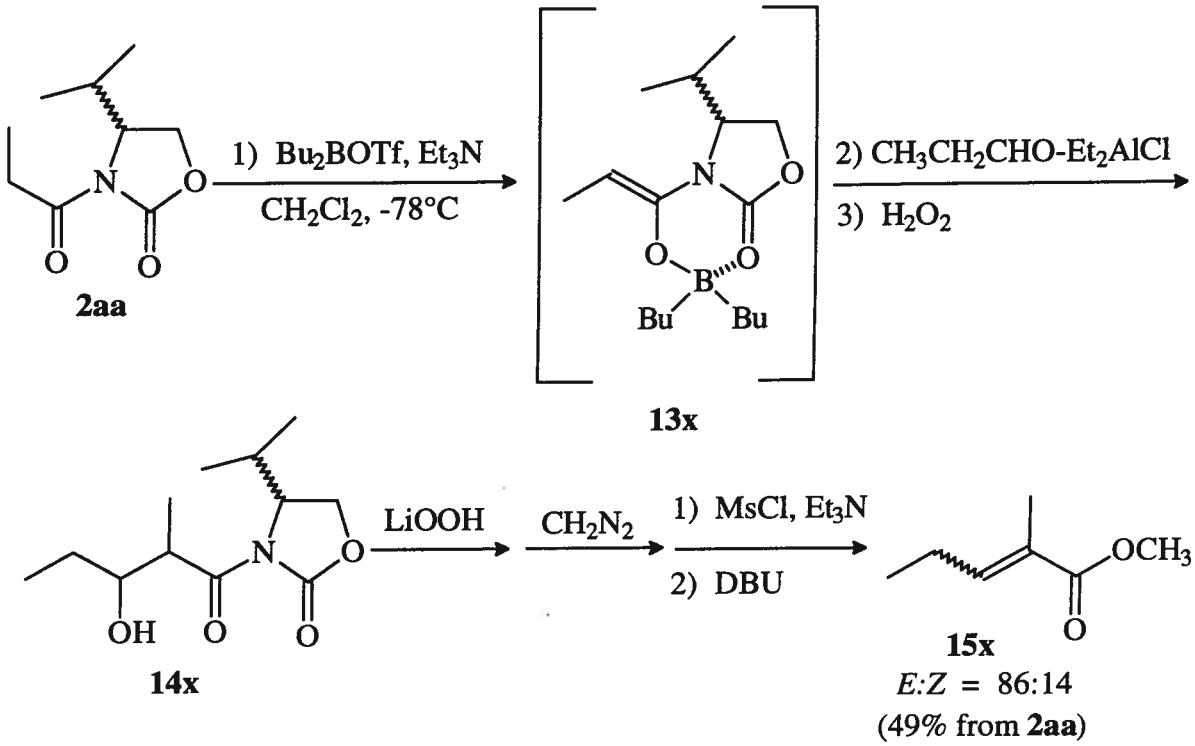


Figure 10. Attempted preparation of methyl(*Z*)-2-methyl-2-pentenoate.

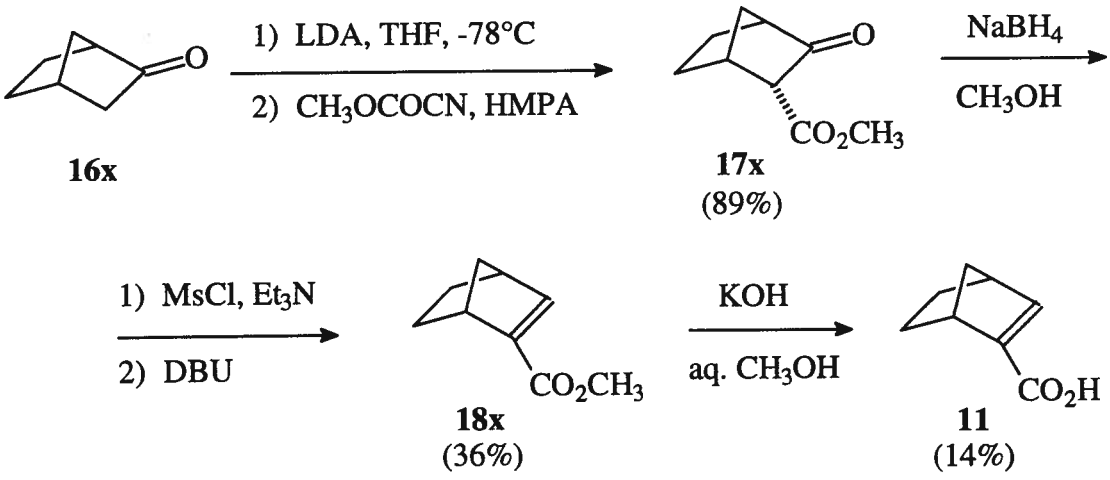
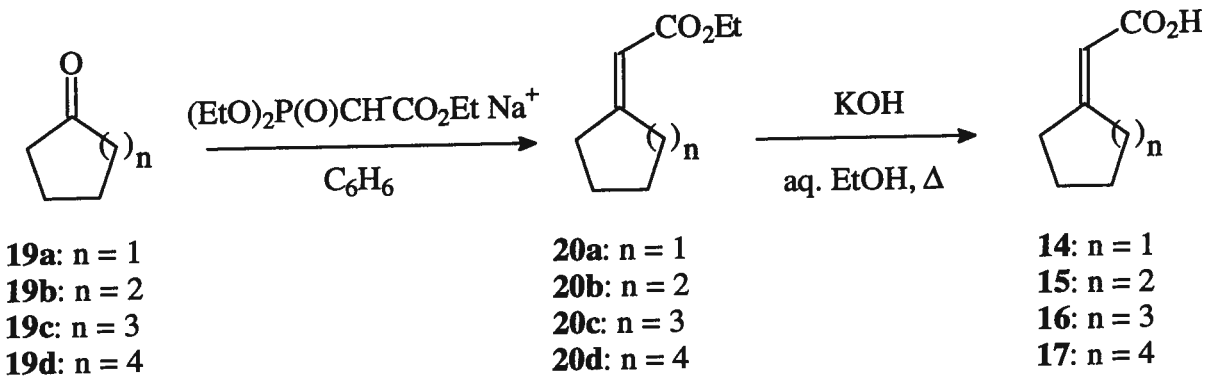


Figure 11. Preparation of acid **11**.

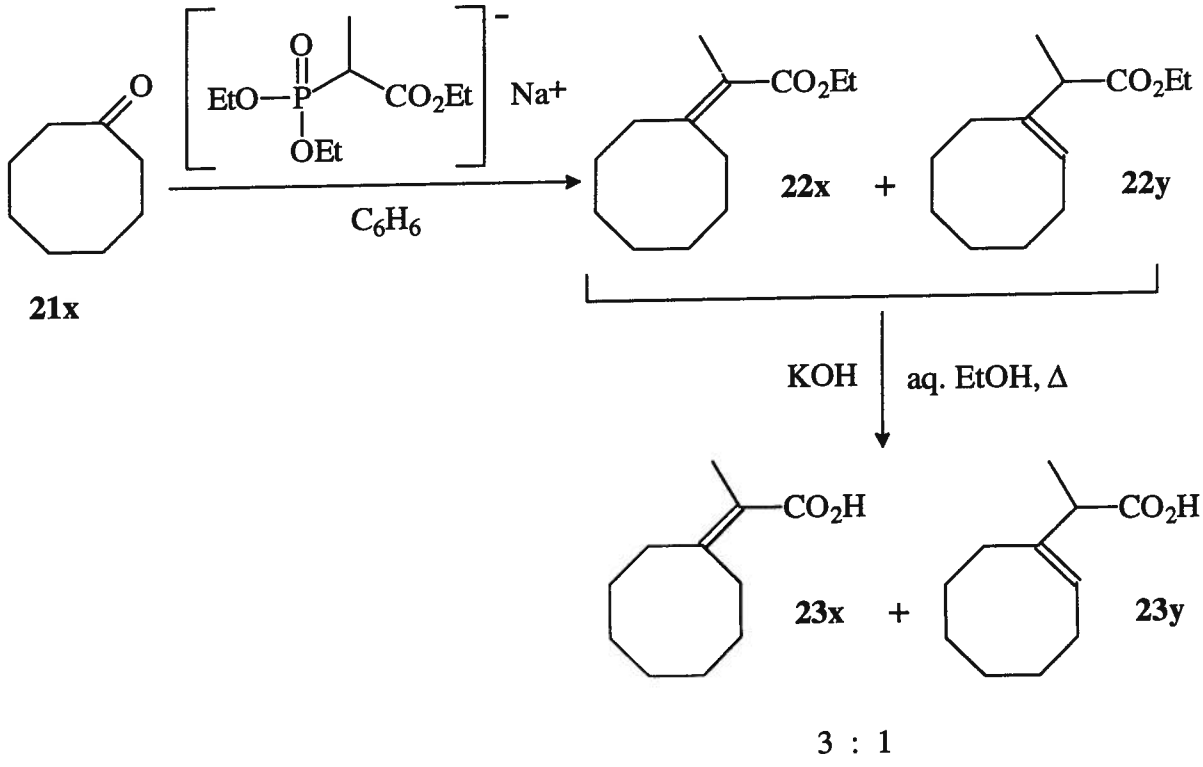


19a: n = 1
19b: n = 2
19c: n = 3
19d: n = 4

20a: n = 1
20b: n = 2
20c: n = 3
20d: n = 4

14: n = 1
15: n = 2
16: n = 3
17: n = 4

Figure 12. Preparation of cycloalkylideneacetic acids **14-17**.



21x

3 : 1

Figure 13. Attempted preparation of 2-cyclooctylidenepropanoic acid **23x**.

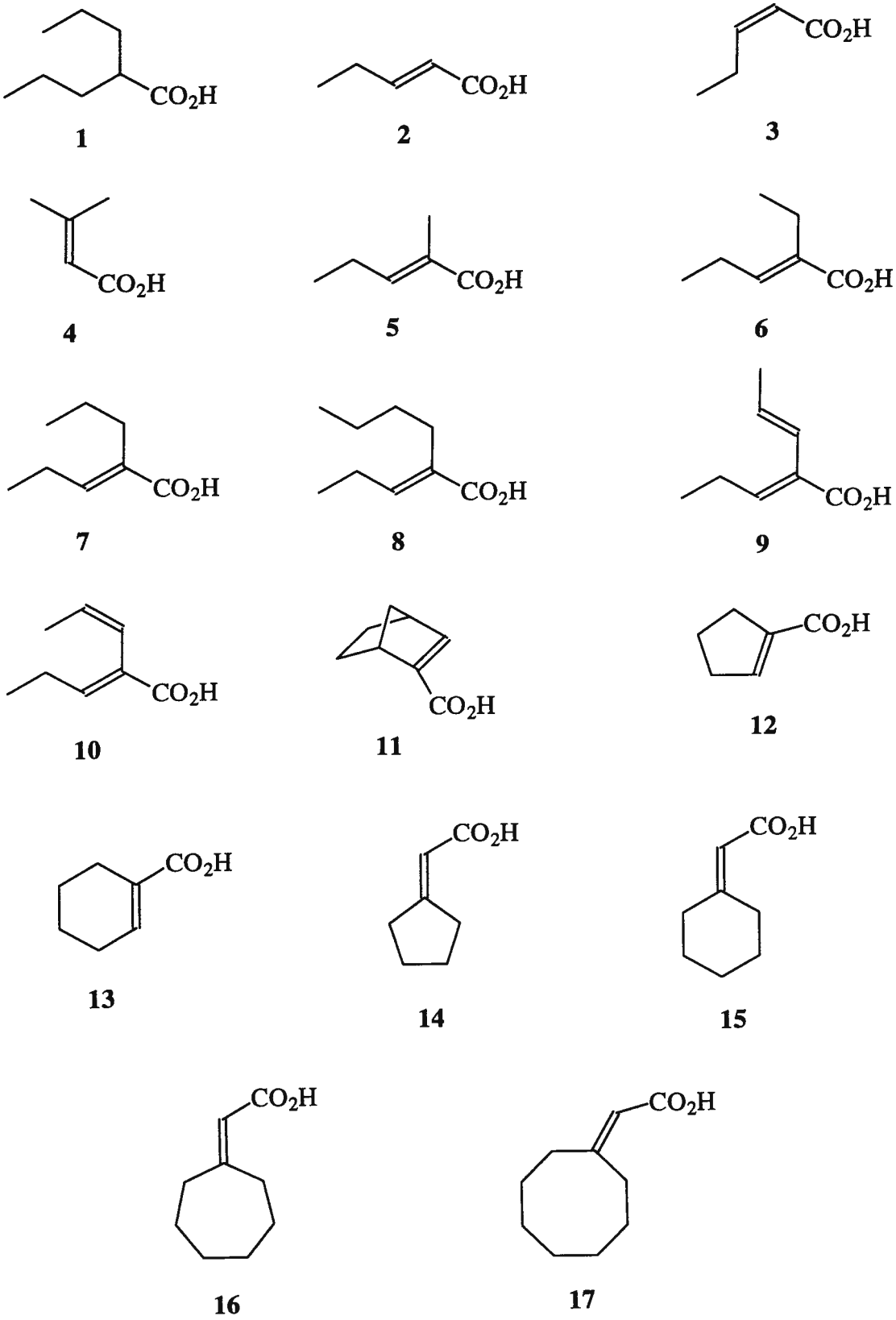


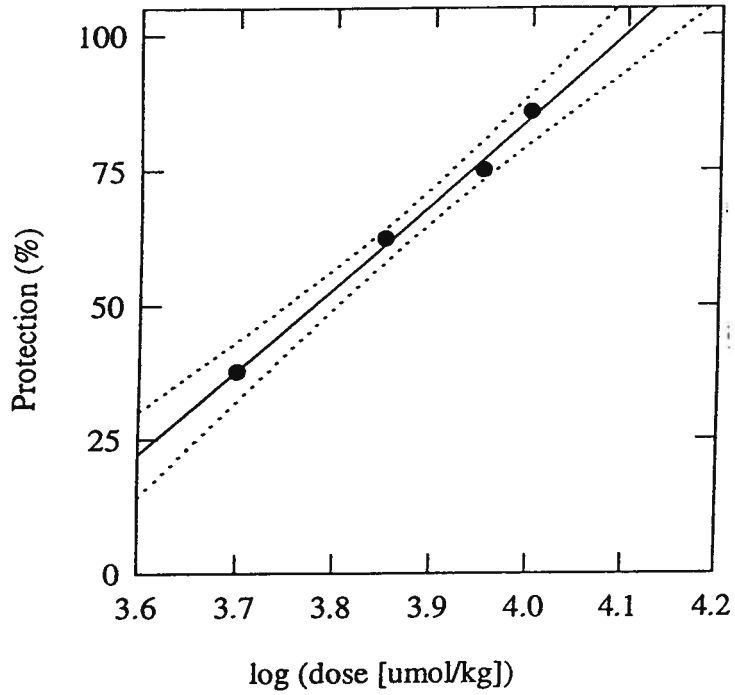
Figure 14. Compounds selected for evaluation.

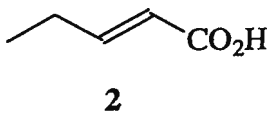
3.2. ANTICONVULSANT ACTIVITY EVALUATION

Compounds **2-17** were evaluated for anticonvulsant activity by subcutaneous injection of 85 mg/kg PTZ 10 min following i.p. administration of each drug as its sodium salt in aqueous solution. The criterion for a convulsion was a tonic-clonic seizure lasting several seconds accompanied by a loss of balance. Administration of PTZ alone produced seizures in 87% of a group of 48 mice, with a mean latency period of 7.2 ± 4.3 min and a median period of 6 min. Using the method of Litchfield and Wilcoxon (1949), the percentage protection was plotted against the log dose to obtain the dose corresponding to 50% protection (ED50), as shown in Figures 15-30 and summarized in Table 5. These graphs show all data collected, with outlying points not used in the construction of the regression line depicted as empty circles. Error was determined from the 95% confidence interval of each plot. Sedation was evaluated qualitatively by the degree of activity and muscle tone present during handling when the mice were administered PTZ. "No sedation" (-) implies normal activity and uncooperative behavior whereas moderate (+) to high (++) sedation describes states where the animal lies increasingly limp and motionless (Swinyard *et al.*, 1989). This parameter is obviously subjective and should be considered only in a most qualitative way. More reliable tests, such as the Rotorod, were not available.

Examination of Table 5 reveals that potency increased with molecular weight. Although this trend was less evident with the most potent drugs, the highest molecular weight drug (**17**) was also the most potent. The sedative potencies of the drugs also followed size but the effect was much more noticeable for the acyclic drugs because, with the exception of compound **17**, all cyclic compounds showed no sedative effects at the ED50 dose. This property was exploited in compounds **16** and **17**, which preserved or even exceeded the potency of 2-ene VPA while remaining free of substantial sedation.

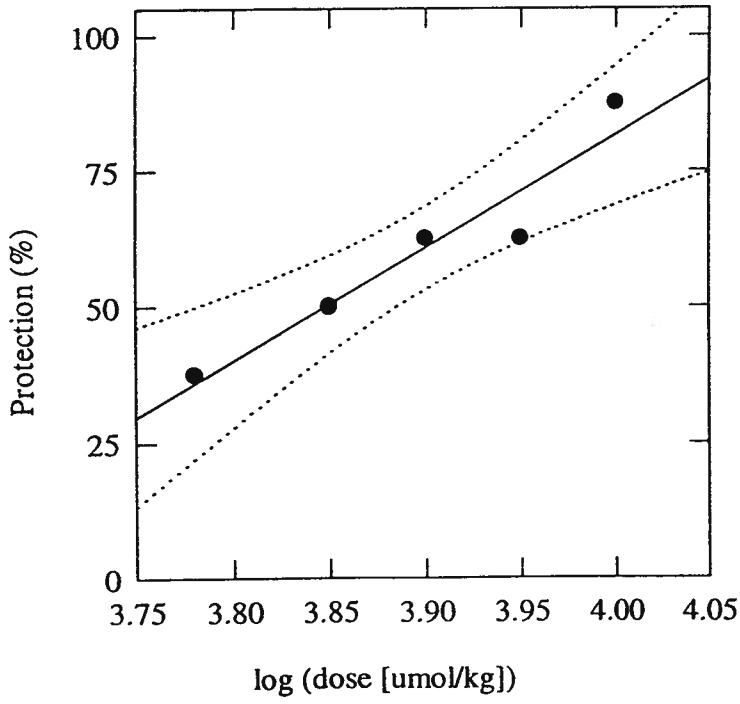
As has been noted earlier by Lee (1991), compound **9** ((*2E,3'E*)-diene VPA) induced a profound neurotoxicity characterized primarily by rigidity. This was in marked contrast to the ataxia and decreased muscle tone observed as the sedative effects of the other drugs.

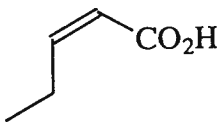




2

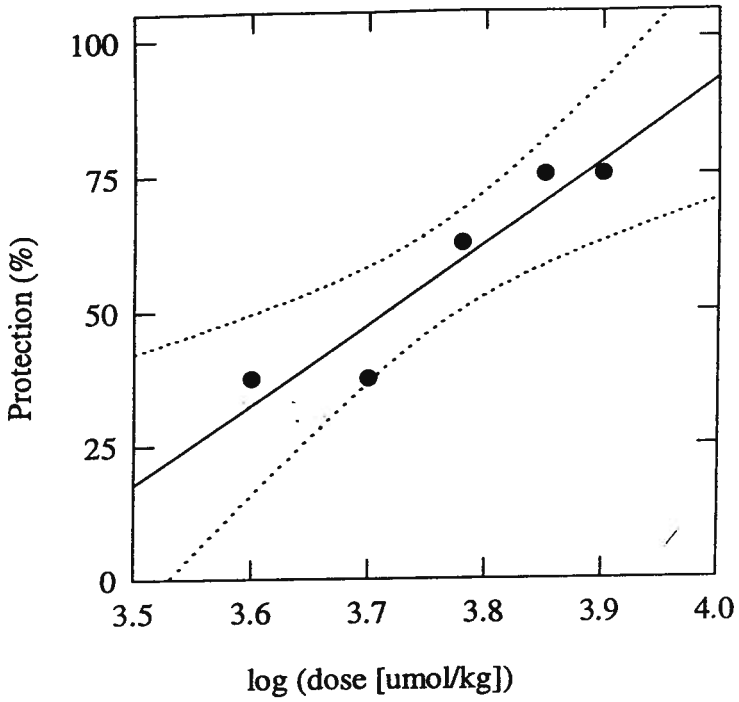
Figure 15. Determination of ED50 dose for acid **2**.

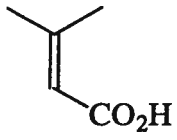




3

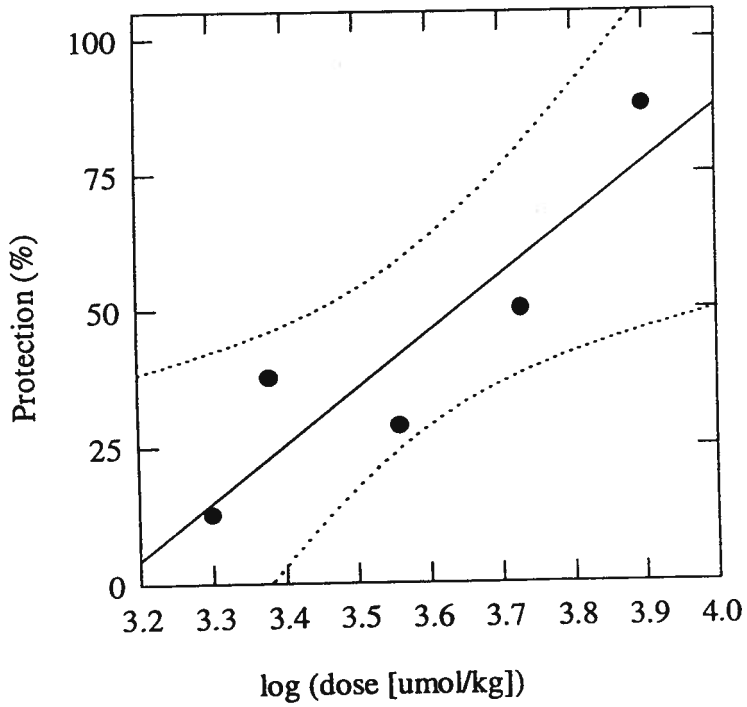
Figure 16. Determination of ED50 dose for acid **3**.

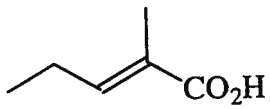




4

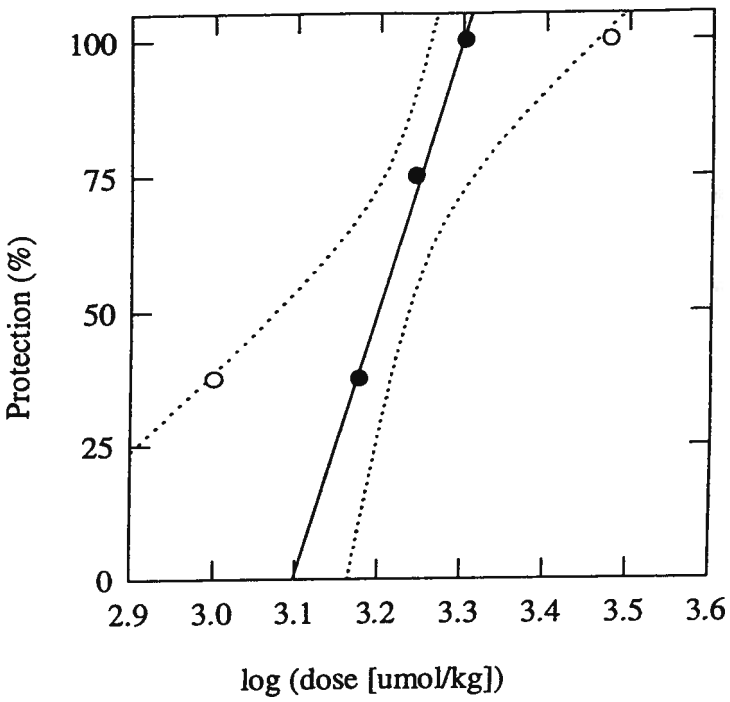
Figure 17. Determination of ED50 dose for acid **4**.

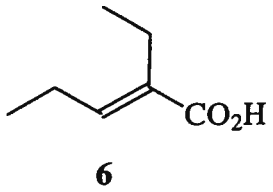




5

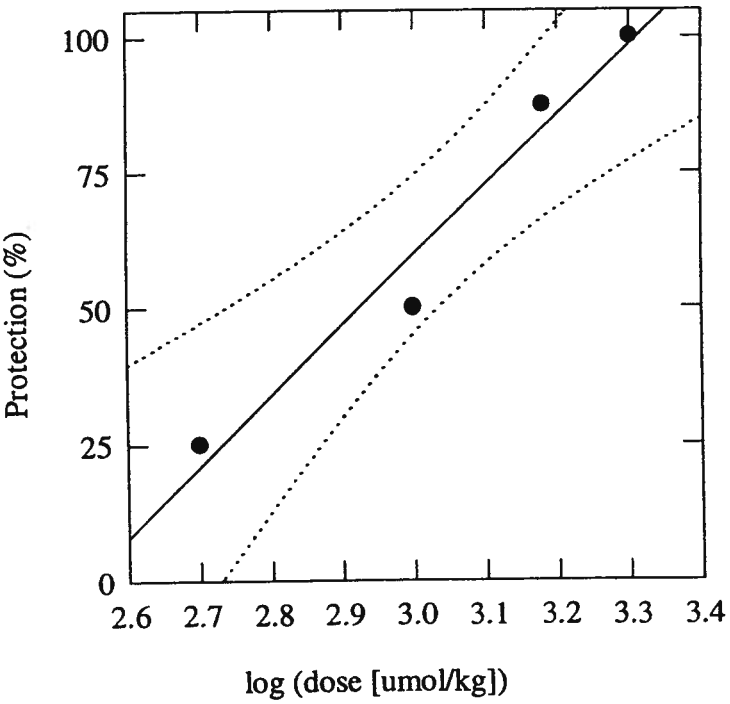
Figure 18. Determination of ED50 dose for acid **5**.

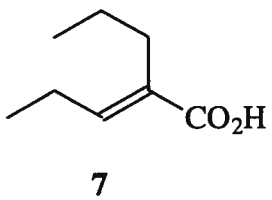




6

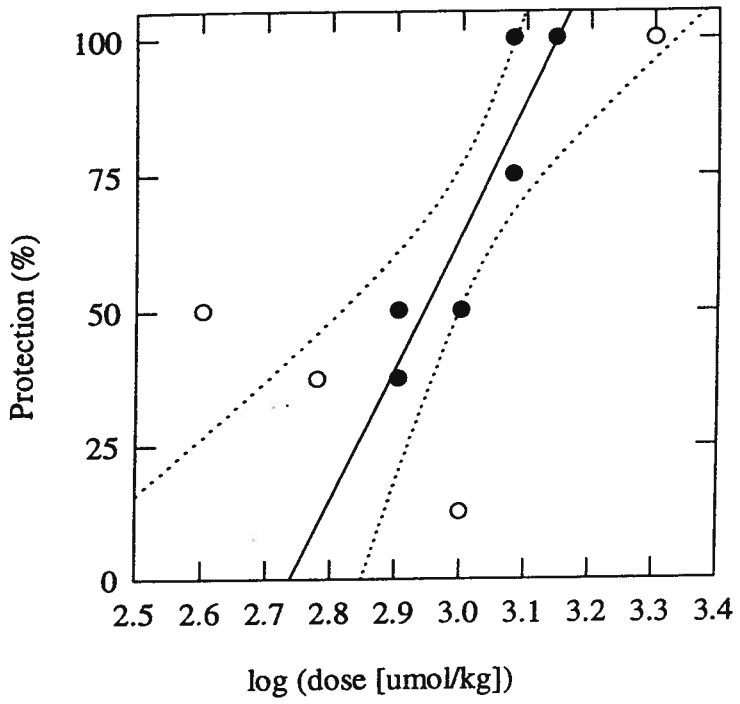
Figure 19. Determination of ED50 dose for acid **6**.

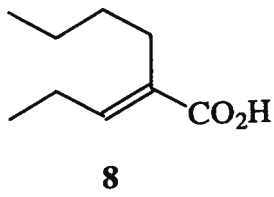




7

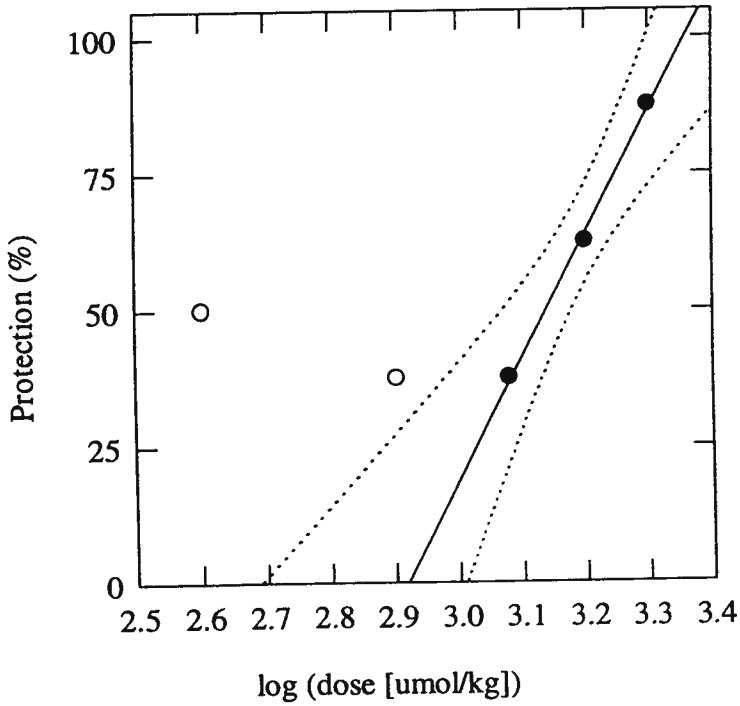
Figure 20. Determination of ED50 dose for acid **7**.

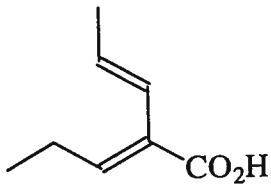




8

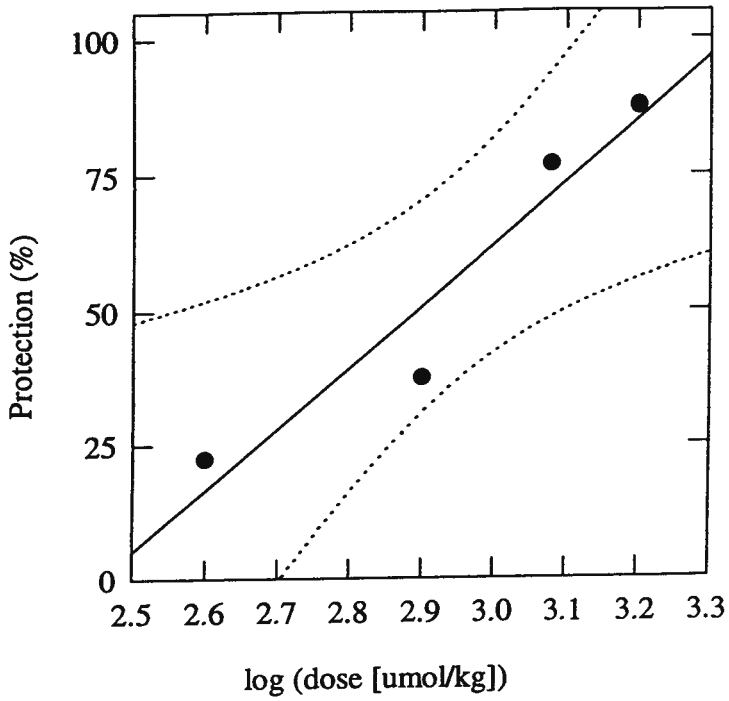
Figure 21. Determination of ED50 dose for acid **8**.

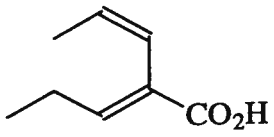




9

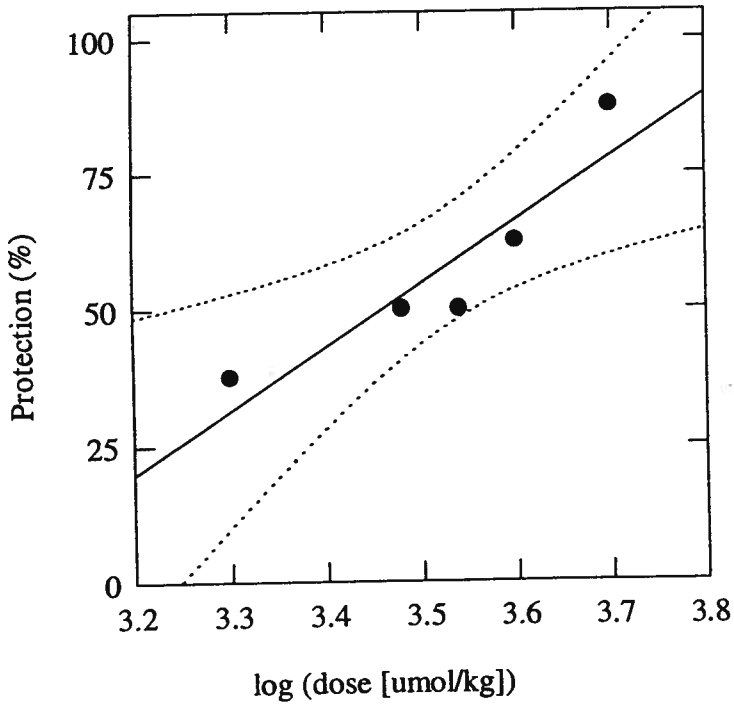
Figure 22. Determination of ED50 dose for acid **9**.

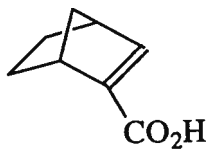




10

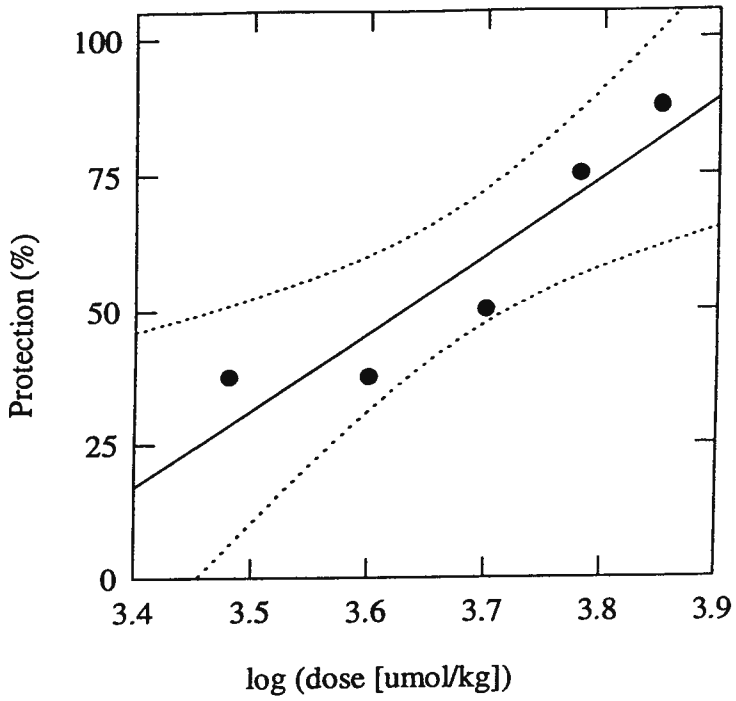
Figure 23. Determination of ED50 dose for acid **10**.

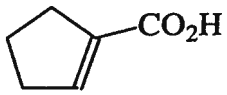




11

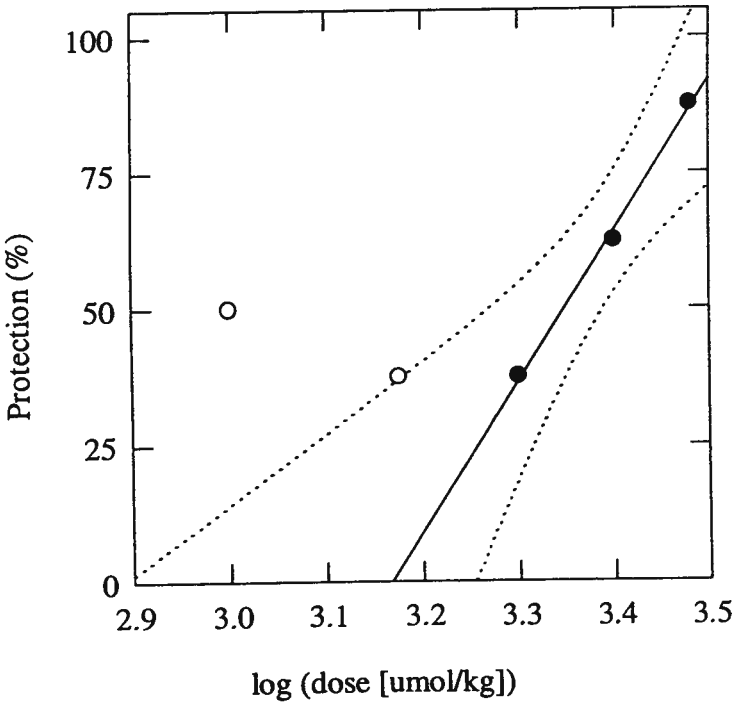
Figure 24. Determination of ED50 dose for acid **11**.

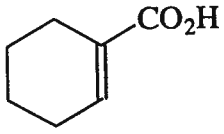




12

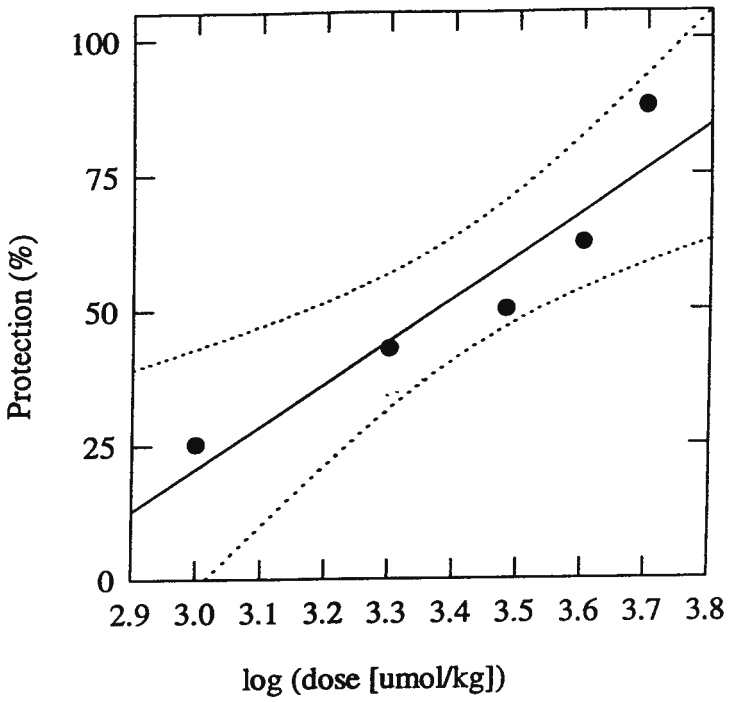
Figure 25. Determination of ED50 dose for acid **12**.





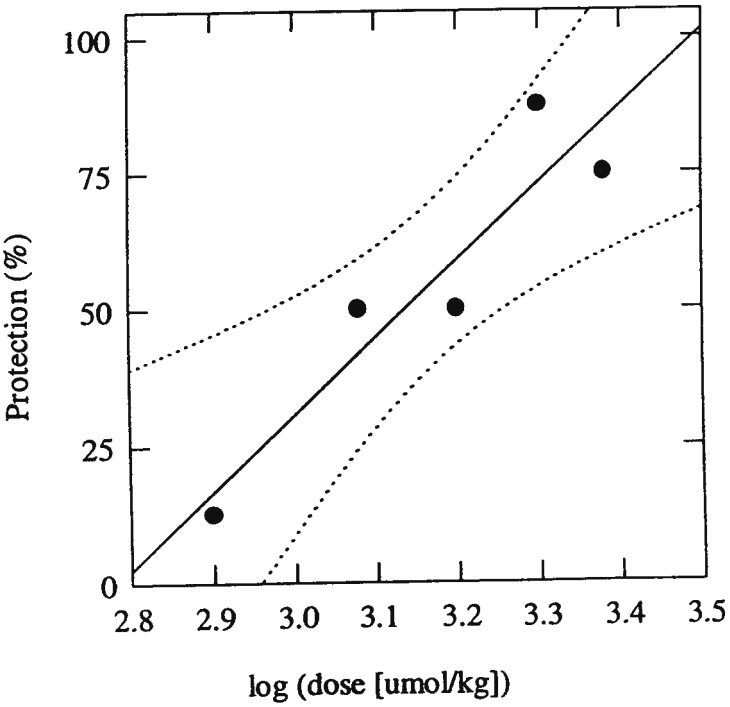
13

Figure 26. Determination of ED50 dose for acid **13**.



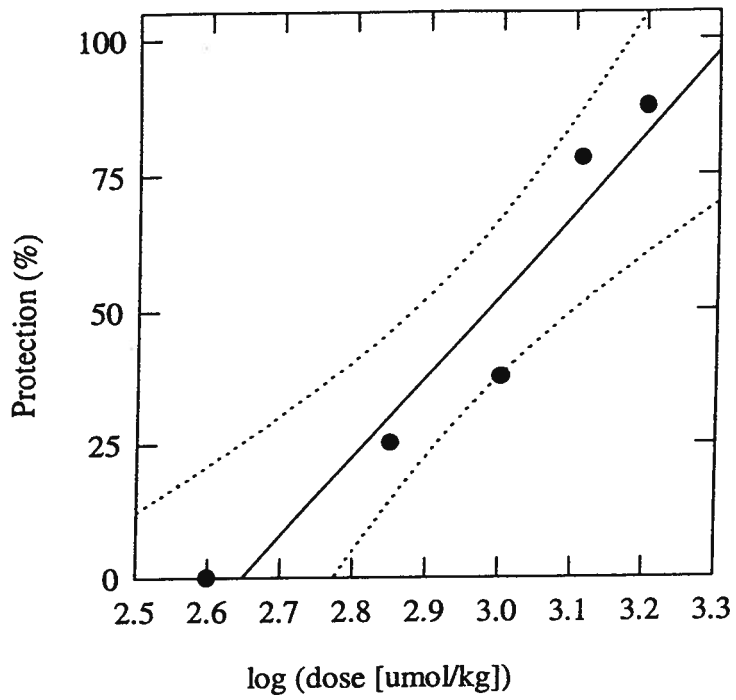
14

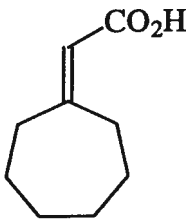
Figure 27. Determination of ED50 dose for acid **14**.



15

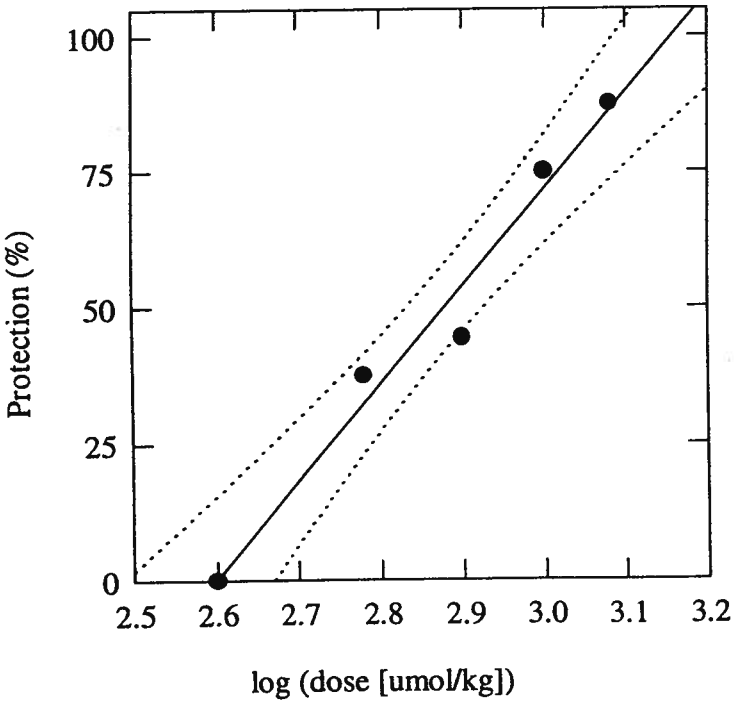
Figure 28. Determination of ED50 dose for acid **15**.

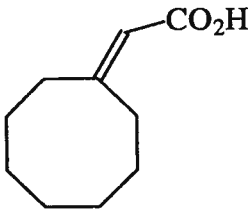




16

Figure 29. Determination of ED50 dose for acid **16**.





17

Figure 30. Determination of ED50 dose for acid **17**.

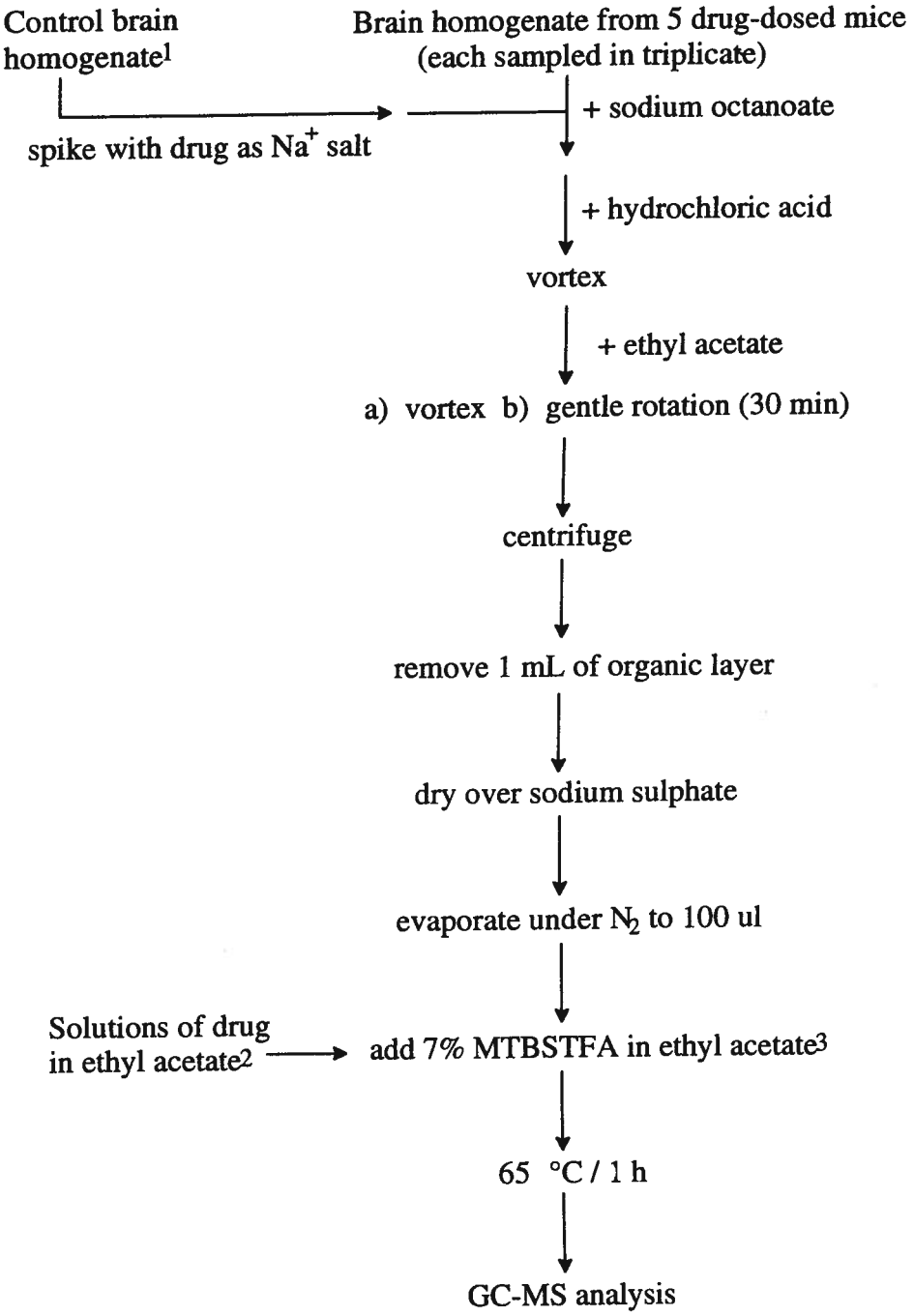
Table 5
Anticonvulsant and sedative properties and brain concentrations of VPA and its analogues

Compound	ED50 (mmol / kg)	95% confidence interval (mmol / kg)	Brain concentration (nmol / g tissue)[a]	Sedation[b]
1	0.83[c]	0.48 - 1.14	617 ± 239	+
2	6.0	5.2 - 6.5	2222 ± 943	-
3	7.0	5.6 - 9.8	4460 ± 842	-
4	5.2	3.5 - 6.1	2710 ± 594[d]	-
5	4.4	2.4 - 17	1520 ± 468	-
6	1.6	0.68 - 1.75	664 ± 41	-
7	0.84	0.44 - 1.15	261 ± 71	+
8	0.87	0.59 - 1.00	144 ± 54	+/++
9	1.4	0.46 - 1.65	684 ± 95	[e]
10	0.78	0.26 - 1.66	348 ± 44[d]	-
11	2.9	1.2 - 3.8	2150 ± 326[d]	-
12	4.7	2.3 - 5.5	496 ± 80[d]	-
13	2.2	1.6 - 2.5	1210 ± 243	-
14	2.3	1.3 - 3.7	455 ± 61[d]	-
15	1.4	0.77 - 1.9	320 ± 105	-
16	0.96	0.73 - 1.3	159 ± 31[d]	-
17	0.73	0.65 - 0.80	94 ± 37	+

a) $[Drug]_{brain}$: mean ± SD drug levels at 15 min postdose in whole brain homogenates from five mice given an ED50 dose and sampled in triplicate (Section 3.3)
b) -: no sedation; +: some loss of muscle tone; ++: significant loss of muscle tone;
c) W. Tang, personal communication
d) As in a), but four brain samples only: a single deviant value was omitted if the resultant SD was decreased by more than 50%
e) Animals exhibited severe ataxia and rigidity rather than sedation

3.3. BRAIN CONCENTRATIONS AT ED50 DOSE

The concentration of each drug at 15 min following an ED50 dose was determined from samples of frozen whole brain homogenates (obtained during the assay of synaptosomal GAD activity: Section 3.9.5) as summarized in Figure 31. The final results are included in Table 5 in the previous section for ease of comparison with the ED50 data. Octanoic acid was used as an internal quantitation standard at a concentration comparable to that of VPA itself. The final extracts were treated with MTBSTFA to produce the acids as *tert*-butyl(dimethylsilyl) esters (Figure 32: Abbott *et al.*, 1986) characterized by strong $M^+ - 57$



1) Standards for quantitation calibration curve
2) Standards for recovery calibration curve
3) Containing cyclohexylcarboxylic acid

Figure 31. Quantitation and recovery determination of drugs in brain homogenate.

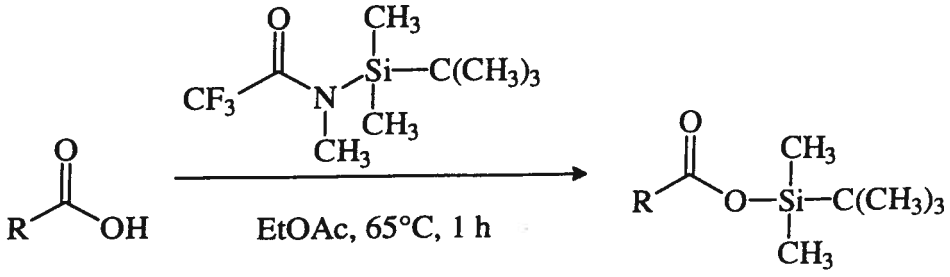


Figure 32. Derivatization of carboxylic acids with MTBSTFA.

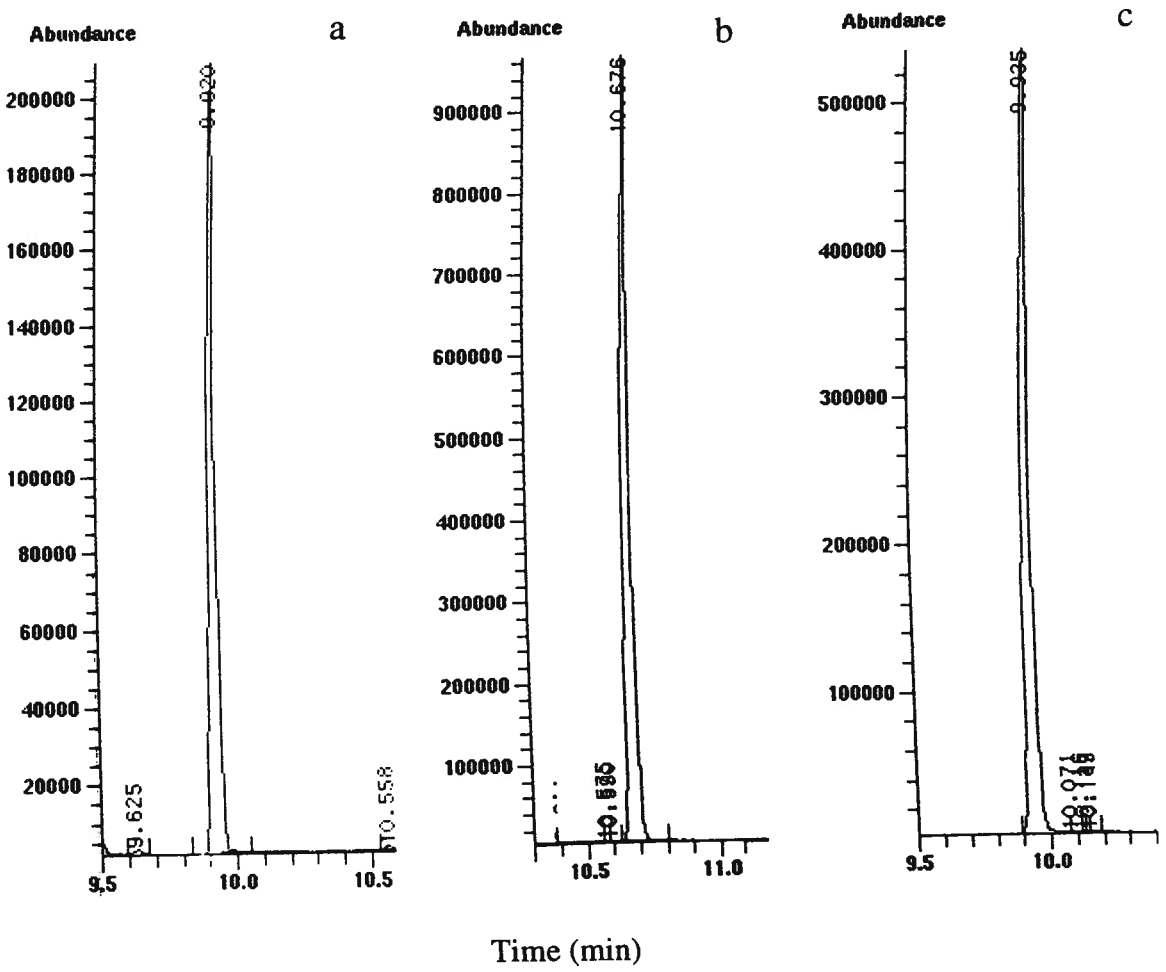


Figure 33. SIM chromatograms of MTBSTFA-treated 2-ene VPA (a) and internal standards octanoic acid (b) and cyclohexylcarboxylic acid (c).

peaks, where the molecular ion had lost the labile tert-*butyl* substituent. Despite the similar or even identical masses of the internal standards (octanoic acid and cyclohexylcarboxylic acid) to some drugs, there were no problems with interference or inadequate sensitivity. Typical SIM chromatograms are shown in Figure 33.

The brain concentrations of all drugs, with the exception of compounds **2** and **4**, fell within the boundaries of the calibration curves. The brain concentrations of these drugs were estimated by extrapolation from the standard calibration curve. However, the overall error is likely minimal in view of both the small extrapolations from highly linear standard plots (Table 6) as well as the fact that the primary source of variation for these or any other drugs was between the individual brain samples. The latter was the reason that the full set of standards for some drugs was not always used (Table 5). Values from a single sample were rejected whenever their omission produced a greater than 50% reduction in the overall standard deviation.

The number of data points used in the calibration curves is variable in order to minimize skewing. For example, calibration curves for compounds present in the brain at high concentrations omit the lowest concentration standards and *vice versa*.

Table 6
Brain concentration assay parameters

Compound	Calibration curve[a]	*n*	*r*
1	$y = -0.01827 + 0.6528x$	7	0.99961
2	$y = 0.01213 + 0.1094x$	4	0.99898
3	$y = 0.01703 + 0.1055x$	4	0.99942
4	$y = 0.007725 + 0.1321x$	4	0.99999
5	$y = 0.0006771 + 0.1842x$	5	0.99973
6	$y = -0.013828 + 0.3913x$	6	0.99984
7	$y = 0.001472 + 0.2369x$	6	0.99954
8	$y = 0.03999 + 0.2007x$	6	0.99979
9	$y = -0.01652 + 0.1356x$	6	0.99996
10	$y = -0.001103 + 0.1457x$	5	0.99999
11	$y = 0.006322 + 0.07153x$	4	0.99963
12	$y = -0.006050 + 0.1467x$	5	0.99983
13	$y = 0.006177 + 0.2134x$	5	0.99954
14	$y = 0.01829 + 0.1918x$	4	0.99983
15	$y = 0.008353 + 0.2028x$	4	0.99872
16	$y = -0.01481 + 0.2441x$	5	0.99961

Table 6 (continued)
Brain concentration assay parameters

Compound	Calibration curve[a]	*n*	*r*
17	$y = -0.009561 + 0.3071x$	6	0.99892

a) Where: y = area ratio [(drug M^+ - 57) / (octanoic acid M^+ - 57)] and x = nmol drug

Although the quantitation protocol clearly compensated for losses, it was still important to estimate recovery in order to demonstrate the validity of the method. Thus, the actual amount of drug present in the final MTBSTFA solutions of the quantitation calibration curve standards was determined using a second set of drug standards directly dissolved in ethyl acetate and treated with MTBSTFA. For each quantitation calibration curve standard, the drug's SIM chromatogram peak area, expressed relative to the area of the common internal standard cyclohexylcarboxylic acid, was divided by the corresponding ratio expected based on the recovery calibration curve. This quotient represents the recovery. The individual recovery values are presented in Table 7.

Table 7
Drug recovery as a function of amount per vial[a]

Drug (pmol)	Recovery (%)			
	1	**2**	**3**	**4**
19,000	68.1	93.1	81.6	98.3
9500	72.9	89.0	94.6	93.0
4750	74.7	97.3	95.1	95.4
2375	66.5	94.1	96.3	94.8
1188	37.2	83.4	101.4	93.9
594	60.9		113.9	89.0
297	94.7		133.0	83.5
	5	**6**	**7**	**8**
38,000		90.4	81.7	
19,000	98.2	87.1	81.3	93.9
9,500	103.2	95.2	82.0	105.7
4,750	105.7	99.8	95.9	88.7
2,375	113.8	89.1	82.9	91.6
1,188	107.9	93.8	81.7	102.3
594	113.7	87.9	78.3	114.3
297	118.6			141.3

Table 7 (continued)
Drug recovery as a function of amount per vial[a]

Drug (pmol)	Recovery (%) 9	10	11	12
19,000	111.5	87.9	90.2	82.6
9,500	112.7	73.9	89.1	81.7
4,750	107.9	84.9	99.1	85.3
2,375	109.2	86.3	98.6	78.4
1,188	112.6	84.0	88.7	93.3
594	130.3	101.7	107.9	78.0
297	156.3	137.5	134.1	111.0
	13	**14**	**15**	**16**
19,000	100.4	107.6	101.5	99.7
9,500	106.9	113.2	114.1	104.4
4,750	100.9	97.9	(240.3)	108.7
2,375	93.4	99.9	109.3	110.4
1,188	94.5	104.4	117.6	94.2
594	115.8	116.5	170.4	104.7
297	38.5	146.7	163.8	126.9
	17			
19,000	122.9			
9,500	115.4			
4,750	132.9			
2,375	117.7			
1,188	119.4			
594	136.4			
297	159.9			

a) Approximately 8.7 ug of brain tissue (as homogenate in 0.32 M sucrose) was added to each vial.

As shown, the recovery values were predominantly 80% or better, but there are a few cases where the values were consistently above 100%. Considering that the signal from the aqueous blank standard was invariably non-zero, this phenonomenon was likely the result of interference from endogenous compounds.

Having both the ED50 values as well as the resultant brain concentrations allows for an estimate of the relative ability of the drugs to penetrate into the brain at their therapeutic dose (Table 8). This parameter Q can be considered as a distribution ratio between the brain and overall tissue compartments under the non-steady-state conditions of the experiment.

Q = Brain concentration (umol / g wet brain tissue) / ED50 (umol / g body weight) (1)

Table 8
Brain/body distribution ratio Q

Compound	Q
1	0.74
2	0.37
3	0.64
4	0.52
5	0.35
6	0.41
7	0.31
8	0.17
9	0.49
10	0.45
11	0.75
12	0.11
13	0.55
14	0.20
15	0.23
16	0.17
17	0.13

3.4. MOLECULAR MODELLING

The objective of molecular modelling using the MacroModel program was to determine the structures in an aqueous solution of not only the most stable conformation of each molecule but all stable conformations whose energies were within a certain range of the global minimum. Using the initial coordinates provided by the user, the relative energy for the input structure was calculated using the MM2* force field (Mohamadi *et al.*, 1990), which is simply an equation expressing the potential energy of the molecule as a function of the positions of its constituent atoms. It contains terms describing the vibrational, torsional, bending and van der Waals and Coulombic interaction potential energy contributions. The solvation energy of the molecule was determined by treating the surrounding medium as a continuum, or shell, beginning near the van der Waals surface of the molecule. The solvation energy was then calculated as the sum of the van der Waals solute-solvent interaction energy and the electrostatic polarization.

The computer program then rotated the specified bonds until it found the nearest potential energy well, or the "local minimum". This was done by calculating the energy of the current conformation, shifting the atomic coordinates in a systematic way and then recalculating the energy. By taking the first derivative of the potential energy with respect to the coordinates, the program determined how to move the individual atoms so as to minimize the energy. When this derivative equalled zero, a local minimum had been found.

However, a typical molecule will have many such minima across its conformational space and there is no guarantee that this particular one is the "global minimum". A more exhaustive search of the entire conformational space is thus necessary in order to find the lowest possible energy. Furthermore, we still required knowledge of all other conformations within a reasonable separation from the global minimum because the free energy of a drug's binding to a receptor is greater than the free energy difference between similar conformers (Crippen, 1979). In other words, the actual pharmacophore's energy can be well above that of the global minimum, while still retaining a favourable overall free energy change upon receptor binding.

If the inter-conformational energy and structural differences are relatively small, a molecule will possess many conformations within a reasonable energy limit of the global minimum. Consequently, we chose to estimate effective solution structure dimensions by taking a weighted mean of all conformers within 10 kJ/mol of the global minimum. This energy range was chosen in order to retain at least 95% of the conformations of a given molecule while keeping their total number within a reasonable limit.

The actual conformational search was conducted using the Monte Carlo routine (see Chang *et al.,* 1989), which generates conformations in a fairly random manner and then minimizes the energies of these structures as described above to evaluate the entire potential energy hypersurface. The Monte Carlo routine began with the structure corresponding to the initial minimum. Several bonds capable of exhibiting independent torsions were then selected. Because this number usually exceeded the limits of the computer, the program randomly chose a set from this group for each iteration. Each of these chosen bonds was then

varied by some random amount between 0 and 180 degrees. The program checked that the resultant structure was plausible, in terms of non-bonded interactions and ring closures, and then proceeded with the energy minimization routine to find the local minimum. Once the minimum had been reached, the new structure was checked to ensure that its energy, interatomic distances and torsional angles were within the specified limits, compared with stored structures from previous iterations and used for the next cycle. The number of these cycles required will clearly vary with the size of the molecule and the number of possible minima. As the process is a random one, a more extensive search will not only improve the chances of sampling the entire hypersurface but will also increase the number of times a given conformation is found, thus ensuring that the conformation is in fact valid. In our searches, a 500-iteration paradigm was used, with a structure being considered valid only if found at least five times. Each search typically produced at least one conformation that was found fewer than five times.

However, the Cartesian coordinates for these final structures described molecules oriented randomly in space, making it necessary for them to be converted to a common orientation in order for their dimensions to be easily calculated. We chose an orientation with the carbon atom at the origin, C(2) on the *x*-axis and the C(2) substituent in the *xy* plane, as shown in Figure 34. The transformation calculations were performed by a custom PASCAL program. The various parameters for the individual conformations *i* were then simply read off from this list of final coordinates as the maximum *x* value for $X^{+}(i)$, minimum *x* value for $X^{-}(i)$ and so on. These parameters correspond to the STERIMOL scheme proposed by Verloop *et al.* (1976) as a means of quantifying shape as well as size in a Hansch-type linear free energy relationship.

To arrive at an average molecular size, it was assumed that the populations p of conformations *i* would follow a Boltzmann distribution:

$$[p_i] / [p_{i+1}] = \exp(E_{i+1} - E_i) / RT) \qquad (2)$$

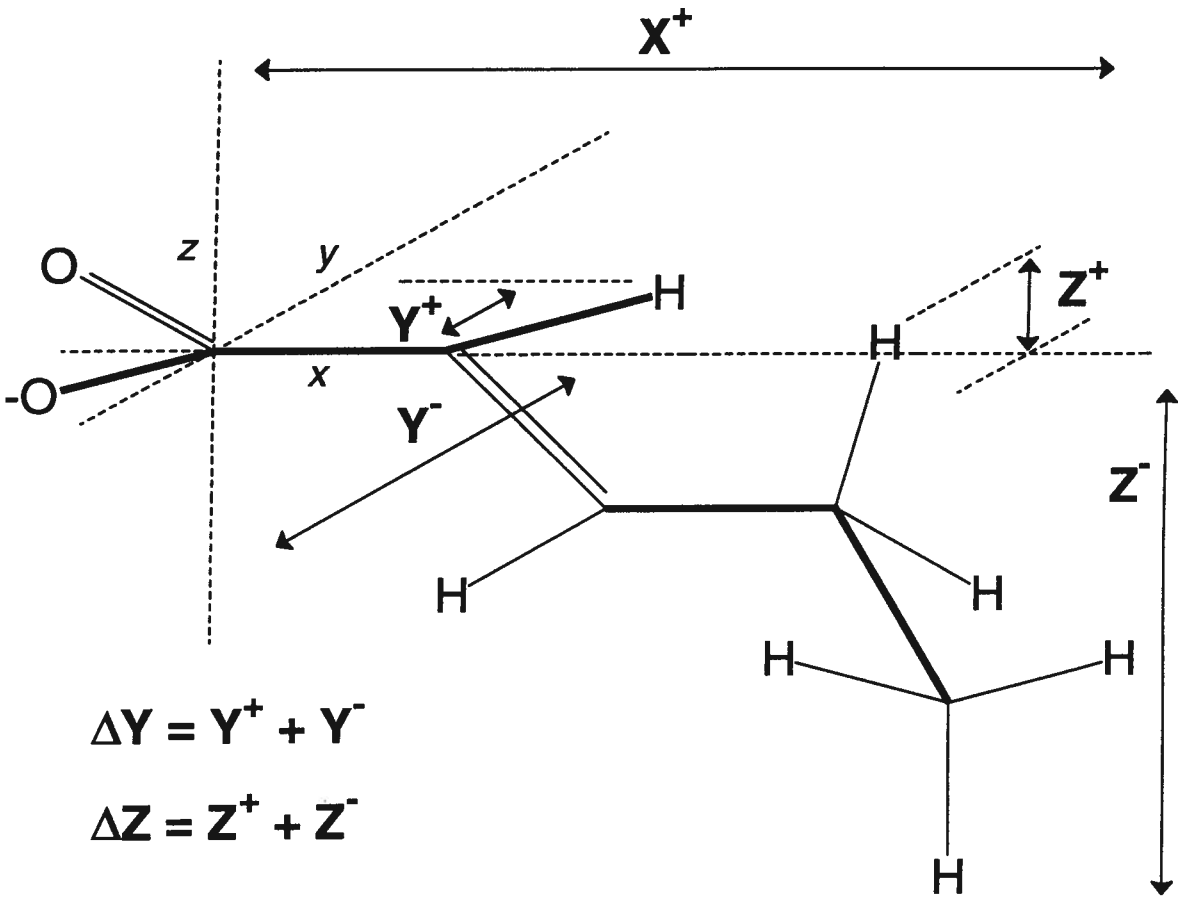


Figure 34. STERIMOL scheme illustrated for *(E)*-2-pentenoate.

where R is the universal gas constant, T is temperature (37°C) and E is potential energy. From these ratios, the fraction P of the overall population corresponding to each conformation was then calculated and used to determine the final parameters listed in Table 9 below. For example,

$$X^+ = \Sigma(P_i \cdot X^+_i) \qquad (3)$$

Table 9
Molecular modelling of VPA analogues as carboxylates in an aqueous medium: population-weighted mean dimensions and number of conformers within 10 kJ of global minimum

Compound	Number of conformers	X^+ (Å)	Y^+ (Å)	Y^- (Å)	Z^+ (Å)	Z^- (Å)	Volume (Å^3)
2	4	5.42	0.99	2.66	1.36	1.36	94.0
3	5	3.73	0.99	4.45	1.25	1.37	93.9
4	2	4.27	1.01	3.02	0.90	0.90	94.2
5	3	5.18	1.97	3.19	1.40	1.17	110.0
6	2	5.18	2.86	2.90	1.33	2.18	127.7
7	6	5.19	3.46	2.85	2.46	1.99	144.7
8	26	5.21	4.22	2.89	3.31	2.32	161.9
9	10	5.29	3.76	2.69	1.93	1.93	138.0
10	9	5.31	2.77	3.16	2.27	2.91	139.1
11	2	4.99	2.20	2.14	1.86	2.31	123.1
12	2	4.58	1.99	2.14	1.29	1.30	99.8
13	2	5.60	2.11	2.08	1.56	1.33	117.1
14	2	5.22	0.98	4.35	1.59	1.27	117.4
15	2	5.56	1.01	4.31	1.56	1.55	133.3
16	13	5.41	0.99	4.31	1.56	1.55	150.5
17	14	5.56	1.01	4.27	2.45	2.50	166.6

Calculations on the protonated forms of some acids were also performed for the cases where protonation might be expected to influence structure. As shown in Table 10, the main effect was to increase the conformational lability, as indicated by the number of conformers below the energy cut-off, with little effect on the actual molecular shape as measured by Y^+. As most remaining structures are either cyclic or possess very limited conformational mobility regardless of the conditions, it is thus probably safe to assume that any drug's shape is relatively independent of its degree of dissociation.

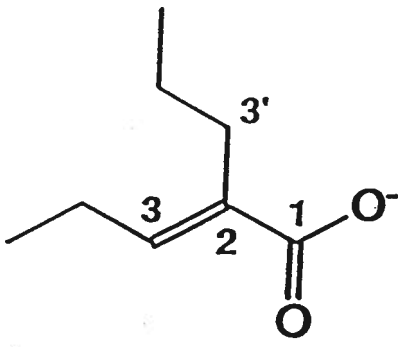
Table 10
Molecular modelling of VPA analogues as protonated acids in an aqueous medium: number of conformers within 10 kJ of global minimum and population-weighted mean Y^+

Compound	Number of conformers	Y^+ (Å)
2	4	0.97
3	4	0.99
5	5	1.96
6	4	2.86
7	12	3.64
8	24	4.15
9	12	4.07
10	7	2.81

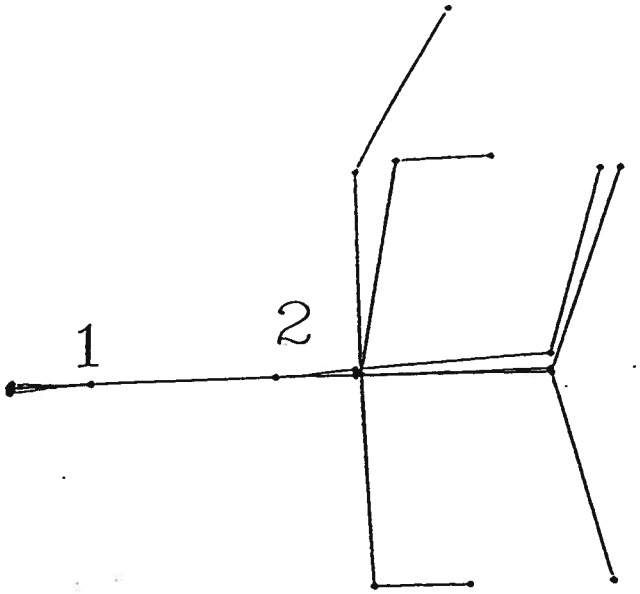
Finally, similar calculations were performed on selected *Z* isomers from the 2-substituted 2-pentenoate group (Table 11). Again, the compounds are as their carboxylates in an aqueous medium and the number of conformers refers to the number of stable conformations found within 10 kJ of the global minimum. As the data suggests, the *Z* isomers are inherently more conformationally labile than the *E* series. This is further illustrated in Figure 35.

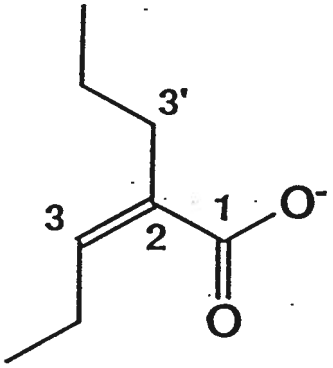
Table 11
Molecular modelling of selected VPA analogues as *Z* isomers

Compound	Number of conformers
(Z)-2-Pentenoate	5
(Z)-2-Methyl-2-pentenoate	4
(Z)-2-Ethyl-2-pentenoate	8
(Z)-2-Propyl-2-pentenoate ((Z)-2-ene VPA)	17



(*E*)-2-ene VPA





(*Z*)-2-ene VPA

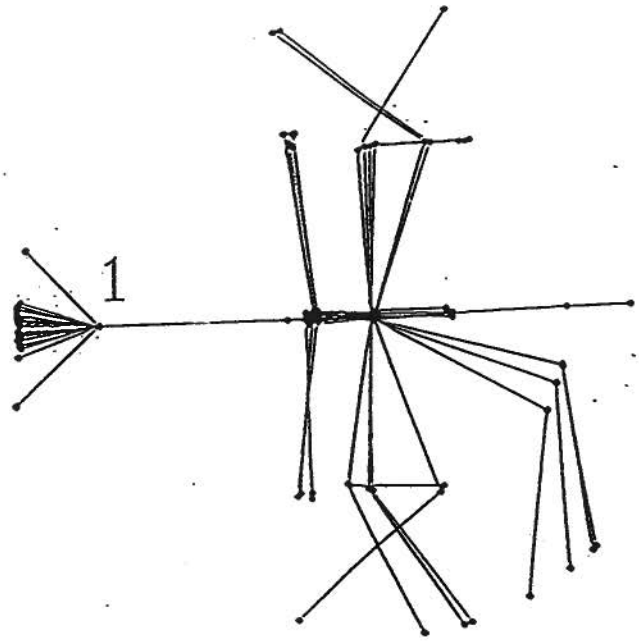


Figure 35. Conformations of *(E)*- and *(Z)*-2-ene VPA carboxylates in an aqueous medium. The structures at right are as seen looking along the *y* axis on to the *xz* plane.

3.5 LIPOPHILICITY

The lipophilicity of a molecule is usually described in terms of its partition coefficient (log P), which in turn generally refers to the logarithm of the ratio of the neutral unionized species in each phase of an *n*-octanol/water mixture (reviewed by Taylor, 1990; Kaliszan, 1986). The standard approach to measuring log P is by the "shake flask" method, where the compound is added to a mixture of *n*-octanol and an aqueous buffer solution of the appropriate pH, allowed to reach equilibrium and then assayed in both phases. The disadvantages of this method include the length of time required, the formation of emulsions and the need for appreciable amounts of a potentially scarce compound, but also a rapidly increasing error as log P rises above zero. The latter is the result of the parameter being a ratio, so that as log P increases, detection of the diminishing quantities of analyte in the aqueous phase will become increasingly uncertain. Although it may seem obvious to simply increase the overall amount of analyte in the mixture in these situations, the assay is also under the further constraint of having to keep concentrations in the organic (C_o) and aqueous phases (C_w) less than 1 M and 10 mM respectively in order for the activity coefficient to remain at unity and the fundamental equation $P = C_o / C_w$ to remain valid.

Consequently, much effort has been invested in the search for more efficient ways of determining log P. Reverse-phase liquid chromatography (RPLC) is one such example, due to the relationship between capacity factor k' and log P:

$$\log P = a \log k' + b \qquad (4)$$

$$k' = \log [\text{adjusted retention time} / \text{void time}] \qquad (5)$$

A further attraction of RPLC is the intuitive similarity of the analyte-stationary phase interactions with those that might be expected between the compound and a membrane bilayer (Baker *et al.*, 1977). In practice, this translates to using a series of standards of known

log P values to develop a calibration curve for the analytes of interest. The method is rapid and gives highly linear plots provided that the compounds share similar chemical structures. However, because the use of water as the mobile phase does not afford a log P / log k' correlation, a co-solvent (usually methanol) must be added, which clearly casts doubts on whether or not the method genuinely reflects log P for a strictly aqueous medium. This obstacle is often avoided by measuring k' as a function of the fraction of the organic solvent (f_m) in the mobile phase in order to obtain the "true" value (k_w):

$$\log k' = \log k_w - B \cdot f_m \qquad (6)$$

A more empirical approach to log P was pioneered most notably by Nys and Rekker (1973) and since refined by the Pomona group (Hansch and Leo, 1979) and incorporated into their ongoing CLOGP program. In this method, a molecule ABC is divided into fragments A, B and C such that:

$$\log P_{ABC} = f_A + f_B + f_C + \Sigma F_i \qquad (7)$$

where the sum of the lipophilicity values of the fragments *f*, plus factors F to compensate for non-additivity, equals log P for the molecule as a whole. The exact method whereby the CLOGP algorithm calculates these values has been described in detail recently by Leo (1990).

An important aspect of any of the log P methods discussed above that is particularly relevant to VPA analogues is their measurement of log P for the unionized species even though the VPA analogues are 99.9% ionized at the pH of the biological medium. This is clearly not a problem if the resultant QSAR equation is used simply to predict the effective *in vivo* activity (e.g. ED50), but raises serious questions if used to probe actual mechanisms, such as an interaction with an enzyme. However, while such an equation would assume the neutral species to be the active form, which is possible but unlikely, the relative lipophilicities of the molecules should be constant regardless of their state of dissociation.

Finally, in the debate over the validity of various log P assays one should not lose sight of the reason for using it in the first place. Namely, the need for an index of lipophilicity that can be correlated with biological results. If this need is fulfilled by log k', for example, then it is irrelevant to ask whether or not log k' is an accurate representation of log P.

In this study, lipophilicity was determined initially by the standard HPLC method as described by Acheampong (1985) where, using a set of compounds of known log P values as standards, lipophilicity was calculated from the capacity factor k'. A plot of this calibration curve is shown in Figure 36. The log P values of compounds **2-15** were then calculated from this plot. Because the principal source of error was in the calibration curve itself, the uncertainty associated with log P was calculated from the corresponding 95% confidence interval.

However, this approach suffered from the drawback of having to deal with a wide range of lipophilicity values such that HPLC conditions appropriate to the small C_5 acids produced extremely long retention times for large analogues such as **8** (**16** and **17** were not measured in this assay). Consequently, the CLOGP program was used instead to measure the log P values for all compounds, which are used in all subsequent calculations. As shown in Figure 37 and Table 12, there was good agreement between the values obtained by the two methods as the differences were generally similar to the 95% confidence interval for the values obtained by the HPLC method. Note that CLOGP does not distinguish *cis* and *trans* double bonds.

Table 12
Determination of lipophilicity (log P) by HPLC and CLOGP

Acid	mean t_r' (min)	log k'	log P Shake flask/HPLC[a]	CLOGP
VPA	14.0	0.483	2.75[b]	2.72
Butyric	1.65	-0.403	0.98[b]	
Valeric	3.22	-0.151	1.51[b]	
Hexanoic	5.30	0.104	1.93[b]	
2-Ethylbutyric	5.59	0.0460	1.68[b]	
2-Ethyl-Hexanoic	12.7	0.484	2.64[b]	
2	2.24	-0.240	1.27 ± 0.15	1.22
3	3.53	-0.183	1.39 ± 0.14	1.22
4	3.18	-0.213	1.32 ± 0.13	1.09
5	4.19	0.0328	1.79 ± 0.11	1.53
6	6.82	0.244	2.19 ± 0.11	2.06
7	10.8	0.446	2.59 ± 0.14	2.59
8	18.8	0.675	3.04 ± 0.24	3.11
9	10.5	0.329	2.37 ± 0.13	2.22
10	9.30	0.267	2.26 ± 0.12	2.22
11	5.52	0.0499	1.84 ± 0.10	1.42
12	3.43	-0.147	1.45 ± 0.13	1.10
13	5.89	0.0390	1.82 ± 0.11	1.66
14	5.75	0.0775	1.88 ± 0.11	1.72
15	7.68	0.483	2.68 ± 0.16	2.28
16				2.84
17				3.40

a) Expressed as 95% confidence interval determined from calibration curve
b) Values from Keane *et al.* (1985)

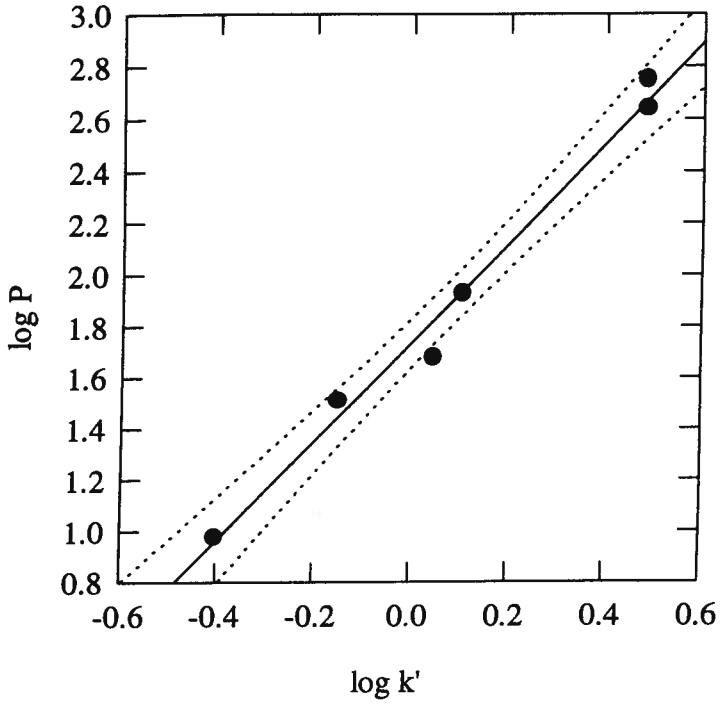


Figure 36. Log P *vs.* log (capacity factor) for some saturated carboxylic acids.

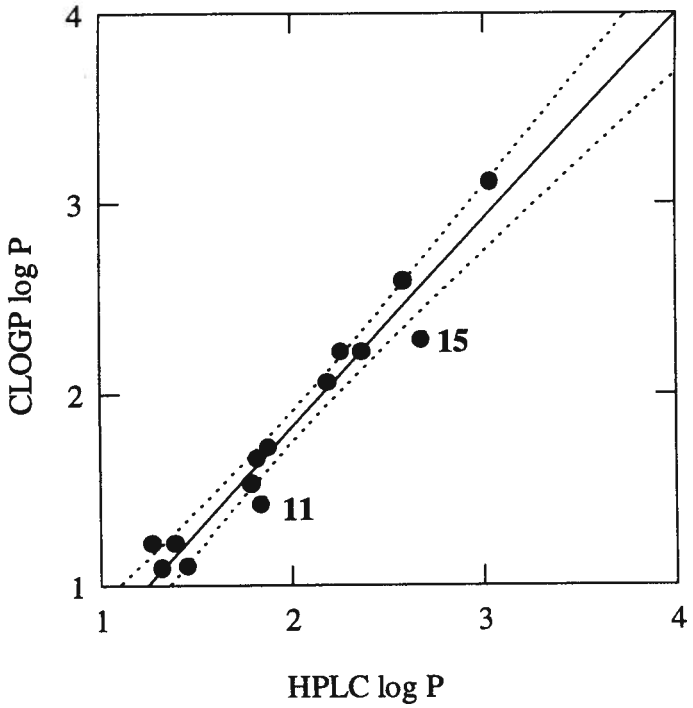


Figure 37. Log P comparison: CLOGP *vs.* HPLC.

3.6 QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS

Modern QSAR theory has largely resulted from the seminal papers of Hansch and co-workers in the early 1960's that showed how biological activity could be expressed as a sum of one or more physicochemical parameters related to free energy. The equations are usually linear as a function of at least one variable, and are thus collectively termed "linear free energy relationships" (LFERs). In view of its importance to our work, the derivation of the fundamental equation as described by Hansch and Fujita (1964) is shown below. Consider the rate-controlling step for the expression of a drug's biological activity as:

$$\text{Rate} = A \cdot C \cdot k_x \tag{8}$$

where k_x is a rate constant and A·C, the product of the probability of a drug molecule reaching the target site and the extracellular concentration, is the effective concentration at the site. Previous work (Collander, 1954, Hansch *et al.,* 1963) having shown that the migration of a drug with substituent X through a biological medium was dependent on lipophilicity (π) in a linear or, if log P was given a sufficiently broad range, second order manner with optimal activity at π_o, the probability A can be taken as a function of a drug's lipophilicity. Assuming this distribution to be normal,

$$A = a \cdot \exp(-(\pi - \pi_o)^2 / b) \tag{9}$$

$$\pi = \log (P_x / P_H) \tag{10}$$

where lowercase letters refer to constants and P_x and P_H to the *n*-octanol/water partition coefficients of the derivative and parent drugs, respectively. Substituting equation (9) for A affords:

$$\text{Rate} = a \cdot \exp(-(\pi - \pi_o)^2 / b) \cdot C \cdot k_x \quad (11)$$

Now, if the rate is taken as a constant biological endpoint, such as seizure protection, it will thus be equivalent to the concentration of drug required to reach that endpoint (e.g. ED50).

$$1 / C = d \cdot \exp(-(\pi - \pi_o)^2 / b) \cdot k_x \quad (12)$$

Taking the logarithm then gives:

$$\log (1 / C) = -d \cdot \pi^2 + 2d \cdot \pi \cdot \pi_o - d \cdot (\pi_o)^2 + \log k_x + e \quad (13)$$

The k_x term can also be expanded because of its relationship with the Hammett equation

$$\log (k_x / k_H) = \rho \cdot \sigma \propto \Delta G \quad (14)$$

which states that the ratio of the rate constant k_x for a molecule with substituent X to that of the unsubstituted parent (e.g. X = H) is equal to the product of the two parameters ρ and σ, related to the electronic properties of X and the type of reaction involved, respectively. Because the molecules under scrutiny are all derived from the same parent, k_H and σ will be constant:

$$\log (1 / C) = -d \cdot \pi^2 + 2d \cdot \pi \cdot \pi_o - d \cdot (\pi_o)^2 + \rho \cdot \sigma + f \quad (15)$$

At this point, the equation expresses biological potency of a series of related drugs in terms of the lipophilicity π and electronic inductive effect ρ. However, there are still two limitations. First, π refers to the lipophilicity of substituent X, which cannot be measured directly, rather than the derivative drug itself. By recognizing $\log P_H$ to be a constant, one obtains:

$$\log (1 / C) = -g \cdot (\log P)^2 + h \cdot \log P + \rho \cdot \sigma + i \quad (16)$$

The second problem arises from the lack of a stereochemical term describing shape. Based on the Hammett equation, one can derive a similar constant E_s such that:

$$E_s = \log (k_x / k_H) \quad (17)$$

Because the original k_x (equation (8)) is actually the product of all parameter-dependent rate constants involved, such that

$$k_x = k_x(\text{electronic}) \cdot k_x(\text{steric}) \quad (18)$$

the lipophilicity term already having been incorporated into the equation elsewhere as a free energy term in its own right, the final version can be written as:

$$\log (1 / C) = -g \cdot (\log P)^2 + h \cdot \log P + \rho \cdot \sigma + j \cdot E_s + i \quad (19)$$

Because E_s simply represents size, it can be replaced with molecular volume or a so-called STERIMOL parameter, as described in Section 3.4. In this study, there was no role for electronic parameters and examination of the data failed to show any sign of a second-order dependence of anticonvulsant activity on lipophilicity. Thus, the first and third terms of equation (19) were omitted to produce the following final version:

$$\log (1 / C) = h \cdot \log P + j \cdot (\text{steric descriptor}) + i \quad (20)$$

Links between biological and physicochemical parameters were then investigated by analyzing for all possible correlations as shown in Table 13. Not surprisingly, lipophilicity was found to be significantly correlated with molecular volume V and most width parameters:

consequently, it is not possible to distinguish the roles of shape and lipophilicity in drug potency which does not permit the use of both parameters simultaneously in equation (20).

Table 13
Correlation matrix of biological and physicochemical properties of VPA analogues[a]

	Dose[b]	C[c]	logP[d]	V	X^+	ΔY	Y^-	Y^+	ΔZ	Z^-
C	0.871	1.000								
logP	-0.920	-0.888	1.000							
V	-0.955	-0.874	0.973	1.000						
X^+	-0.636	-0.584	0.569	0.598	1.000					
ΔY	-0.683	-0.548	0.688	0.692	0.155	1.000				
Y^-	-0.186	-0.289	0.352	0.223	-0.055	0.252	1.000			
Y^+	-0.453	-0.258	0.331	0.433	0.178	0.676	-0.543	1.000		
ΔZ	-0.845	-0.703	0.816	0.882	0.442	0.680	0.014	0.581	1.000	
Z^-	-0.731	-0.512	0.627	0.721	0.358	0.556	-0.066	0.533	0.908	1.000
Z^+	-0.750	-0.674	0.772	0.816	0.406	0.704	-0.037	0.786	0.918	0.736

a) Correlation coefficient *r*
b) Log(ED50)
c) $Log([drug]_{brain})$

The best correlations of log(ED50) with these physicochemical properties for analogues **2-17** are shown below, along with the relevant statistical parameters. Specifically, *s* is the standard error of estimate (square root of residual mean square) and *r* is the correlation coefficient. F_{calc} is used to test the null hypothesis that slope = 0 (e.g. no correlation) by comparison with the corresponding table value of the F distribution for a 1-tailed test at a 1% significance level.

$$\log(ED50) = (-0.0139 \pm 0.0012)\cdot V + (5.0520 \pm 0.149) \quad (21)$$

($n = 16$; $s = 0.1069$; $r = 0.954$; $F_{calc} = 142.29$; $F_{table} = 8.86$)

$$\log(ED50) = (-0.4331 \pm 0.049)\cdot \log P + (4.1641 \pm 0.104) \quad (22)$$

($n = 16$; $s = 0.1403$; $r = 0.920$; $F_{calc} = 76.63$; $F_{table} = 8.86$)

Multiparameter equations are also quite possible. For the second parameter in the following equations, Y^+ and Y^- were chosen as they show the lowest correlation with volume/log P.

$$\log(ED50) = (-0.0136 \pm 0.0013)\cdot V + (-0.0153 \pm 0.028)\cdot Y^+ + (5.0437 \pm 0.153) \quad (23)$$

($n = 16$; $s = 0.1097$; $r = 0.955$; $F_{calc} = 67.70$; $F_{table} = 6.7$)

$$\log(ED50) = (-0.0140 \pm 0.0012)\cdot V + (0.0121 \pm 0.034)\cdot Y^- + (5.0254 \pm 0.171) \quad (24)$$

($n = 16$; $s = 0.1104$; $r = 0.955$; $F_{calc} = 66.75$; $F_{table} = 6.7$)

$$\log(ED50) = (-0.4076 \pm 0.050)\cdot \log P + (-0.0514 \pm 0.033)\cdot Y^+ + (4.2162 \pm 0.105) \quad (25)$$

($n = 16$; $s = 0.1336$; $r = 0.933$; $F_{calc} = 43.47$; $F_{table} = 6.7$)

$$\log(ED50) = (-0.4592 \pm 0.0051)\cdot \log P + (0.0638 \pm 0.044)\cdot Y^- + (4.0108 \pm 0.145) \quad (26)$$

($n = 16$; $s = 0.1350$; $r = 0.931$; $F_{calc} = 42.47$; $F_{table} = 6.7$)

Because the ED50 values in Table 5 are a measure of all the rate constants involved between the injection of drug and its interaction at the final site, they are a poor measure of the actual pharmacodynamic properties of the drug. Hence, the initial absorption steps were eliminated by replacing ED50 in the previous equations with the brain concentrations of the drug following an ED50 dose.

$$\log([drug]_{brain}) = (-0.0184 \pm 0.0027)\cdot V + (5.101 \pm 0.348) \quad (27)$$

($n = 16$; $s = 0.2496$; $r = 0.875$; $F_{calc} = 45.56$; $F_{table} = 8.86$)

$$\log([drug]_{brain}) = (-0.0197 \pm 0.0030)\cdot V + (0.0670 \pm 0.064)\cdot Y^+ + (5.1462 \pm 0.348) \quad (28)$$

($n = 16$; $s = 0.2488$; $r = 0.885$; $F_{calc} = 23.48$; $F_{table} = 6.7$)

$$\log([\text{drug}]_{\text{brain}}) = (-0.0179 \pm 0.0028)\cdot V + (-0.0577 \pm 0.079)\cdot Y^{-} + (5.2367 \pm 0.394) \quad (29)$$

($n = 16$; $s = 0.2539$; $r = 0.880$; $F_{calc} = 22.29$; $F_{table} = 6.7$)

$$\log([\text{drug}]_{\text{brain}}) = (-0.6029 \pm 0.0084)\cdot \log P + (3.997 \pm 0.176) \quad (30)$$

($n = 16$; $s = 0.2370$; $r = 0.888$; $F_{calc} = 52.09$; $F_{table} = 8.86$)

However, these equations could be misleading because the molecular weight of the drugs themselves is also highly variable. The question to ask is whether or not the differences in potency could have been accounted for by different masses of drug being injected. Should this have been the case, we would conclude that the anticonvulsant effect was the result of a completely non-specific interaction. As ED50 is a fixed endpoint this would require potency to increase in parallel with molecular weight in order to keep the mass of the injected drug constant. However, this possibility of a completely non-specific mechanism can be dismissed by the fact that ED50 values (0.73-7.0 mmol/kg) vary much more than molecular weights (100 g/mol (**2**-**4**) to 168 g/mol (**17**)), indicating that protection against seizures was a function of the drug itself rather than simply the weight of material administered. Further justification for the molar rather than mass basis was provided by the inferior correlations shown below.

$$\text{Log(ED50) \{mg/kg\}} = -(0.0109 \pm 0.0012)\cdot V + (3.793 \pm 0.148) \quad (31)$$

($n = 16$; $s = 0.1066$; $r = 0.929$; $F_{calc} = 88.8$; $F_{table} = 8.9$)

$$\text{Log}([\text{drug}]_{\text{brain}})\ \{\text{ug/g}\} = -(0.0154 \pm 0.0027)\cdot V + (3.853 \pm 0.347) \quad (32)$$

($n = 16$; $s = 0.2494$; $r = 0.835$; $F_{calc} = 32.3$; $F_{table} = 8.9$)

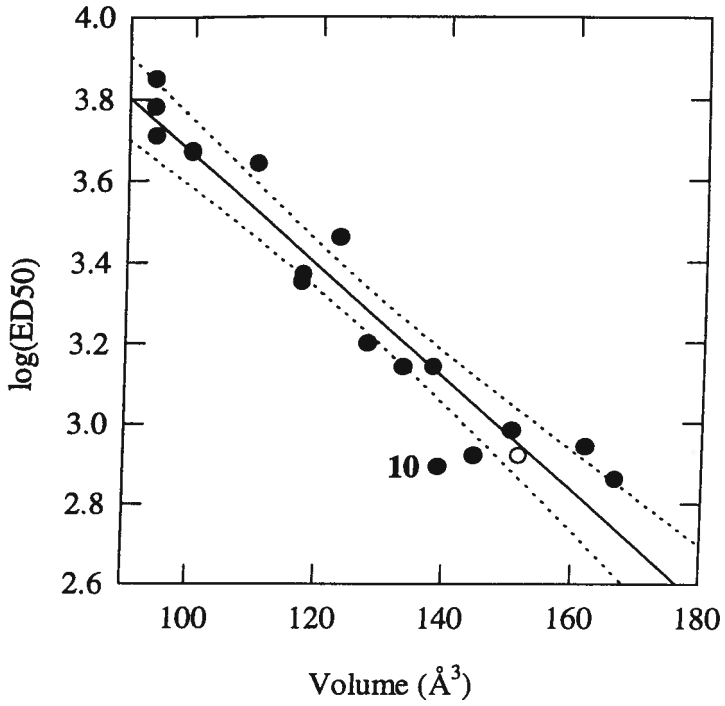


Figure 38. Log(ED50) *vs.* volume for acids **2-17**.
VPA (not used in equation) is shown as an empty circle.

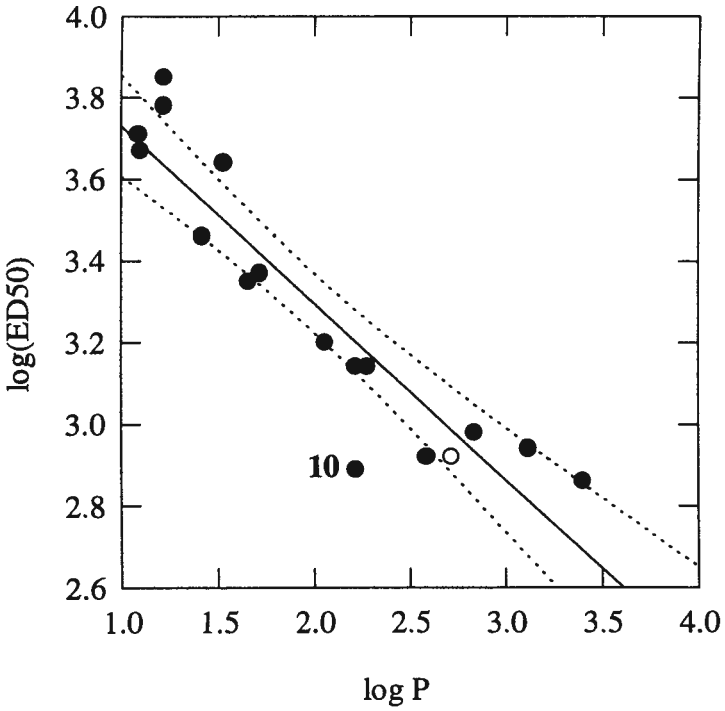


Figure 39. Log(ED50) *vs.* lipophilicity for acids **2-17**.
VPA (not used in equation) is shown as an empty circle.

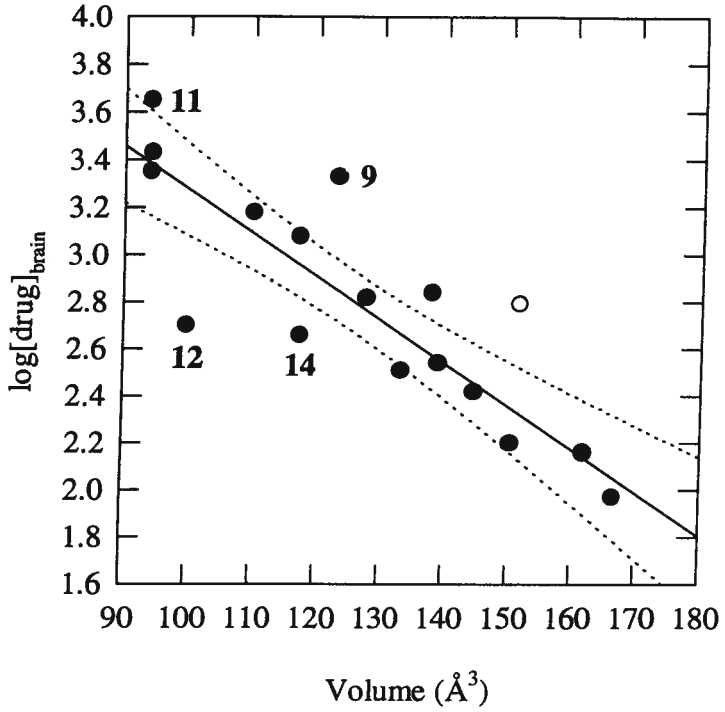


Figure 40. Log[drug]$_{brain}$ *vs.* volume for acids **2-17**. VPA (not used in equation) is shown as an empty circle.

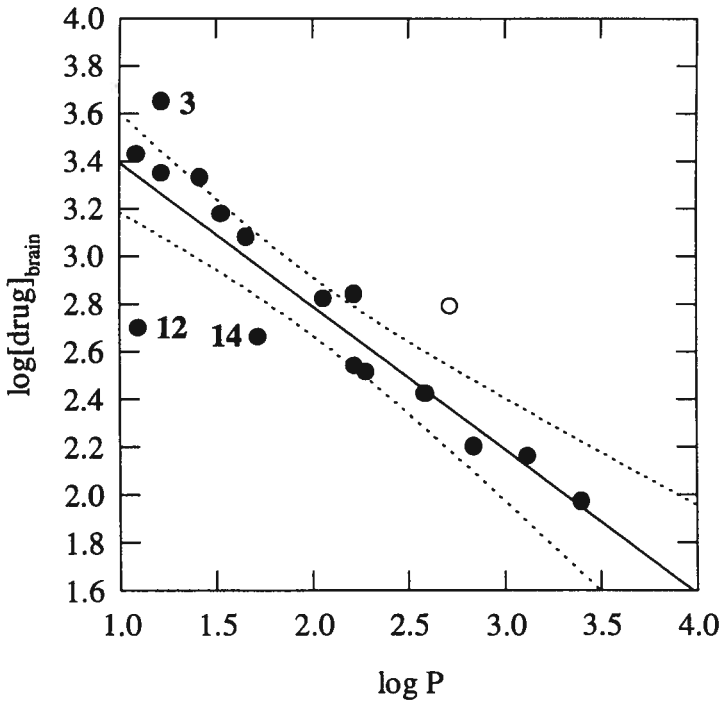


Figure 41. Log[drug]$_{brain}$ *vs.* lipophilicity for acids **2-17**. VPA (not used in equation) is shown as an empty circle.

3.7 MEMBRANE FLUIDITY

The fluidity assay employed in this project (reviewed by Shinitzky and Barenholz, 1978) is based on the degree of fluorescence depolarization when a hydrophobic fluorophore embedded in a membrane is excited with polarized light. A more fluid membrane environment will allow the probe to rotate more easily during its excited state and diminish the intensity of the emitted light polarized in the plane of the exciting beam (I_{vv}) by re-emitting the energy in other planes, such as the one perpendicular to the exciting beam (I_{vh}). This can be expressed as the fluorescence anisotropy r:

$$r = (I_{vv} - G \cdot I_{vh}) / (I_{vv} + 2G \cdot I_{vh}) \quad (33)$$

where G is an instrumental factor to correct for instrumental polarization of the emitted beam, calculated by:

$$G = I_{hh} / I_{hv} \quad (34)$$

when the excitation polarizer is set in the horizontal position. The parameter r is related to the actual membrane fluidity by the Perrin equation:

$$r_o / r = 1 + [C(r) \cdot T \cdot \tau / \eta] \quad (35)$$

r_o = limiting anisotropy, or that value of r which would be observed in a rigid matrix

C(r) = parameter relating to fluorophore shape and the location of its transition dipoles

τ = lifetime of excited state

η = membrane viscosity

T = temperature

Equation (35) suffers from several drawbacks. First, the C parameter will vary with r for a non-spherical fluorophore. Second, we cannot readily determine the lifetime of the excited state. Finally, as the viscosity will vary with the particular axis of symmetry because the membrane is an anisotropic medium, it needs to be replaced with the term "microviscosity" (ϕ), which is defined as the geometrical mean of the viscosities along the symmetry axes, but which may thought of as the "effective viscosity". If the Perrin equation is rewritten as

$$([r_o / r] - 1)^{-1} = \phi / [C(r) \cdot T \cdot \tau] \qquad (36)$$

we now effectively obtain

$$([r_o / r] - 1)^{-1} = \phi / \text{constant} \qquad (37)$$

because the variations of C(r) and τ with temperature or between individual preparations labelled with the same probe are considerably smaller than η. Because C(r) and τ decrease with increasing temperature, the result is that the denominator of equation (36) is essentially constant that allows for the relation of anisotropy to membrane fluidity.

The most commonly used membrane probe is diphenylhexatriene (DPH), a highly lipophilic compound with a simple spectrum that localizes in the centre of the membrane bilayer (Figure 42). Furthermore, it exhibits fluorescence only when in a non-polar medium, which minimizes background interference from molecules not incorporated into a membrane.

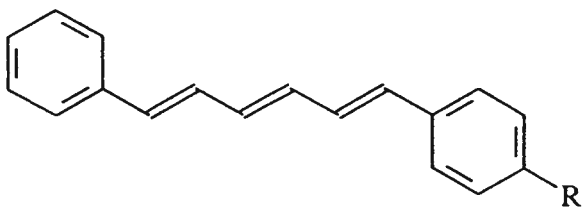


Figure 42. Structures of membrane probes DPH (R = -H) and TMA-DPH (R = $-N(CH_3)_3^+$)

The properties of the analogous compound trimethylammonium-diphenylhexatriene (TMA-DPH) are very similar to those of DPH with the important exception that the molecule is anchored in the region immediately below the membrane surface by its cationic substituent (Prendergast *et al.,* 1981).

Initial experiments using DPH with various membrane preparations (e.g. crude erythrocyte ghosts, synaptosomal membranes and intact synaptosomes) indicated a barely significant difference between controls and samples incubated with 10 mM VPA, a result in general agreement with the work of Perlman and Goldstein (1984). It thus appeared that the problem of low sensitivity was the result of the membrane-disordering potency of VPA being either very low or exerted in a region not sampled by the highly lipophilic probe DPH. Because VPA in its dominant dissociated state might be expected to interact primarily with the extracellular surface rather than the interior of the plasma bilayer, we thus investigated the use of the polar probe TMA-DPH which should be more responsive to changes in this region.

The parameters for this fluidity assay were established as follows. The erythrocyte ghosts were diluted to afford a OD_{366} value of about 0.1, which gave a maximum of 4% scattering at the emission wavelength of 428 nm (Figures 43 and 44). The integrity of the preparation was verified by Arrhenius plots of anisotropy for control and 20 mM VPA preparations (Figures 45 and 46). The negative slope indicates that anisotropy was decreasing with increasing temperature, as expected. Thus, the degree of membrane fluidity increased as the probe was more readily able to rotate during the period between absorption and emission.

The results of this TMA-DPH assay showed little or no effects of 20 mM concentrations of representative drugs on membrane fluidity, although a few exhibited anisotropy shifts that reached statistical significance (Table 14). In contrast, all drugs showed a significant decrease in anisotropy when the assay was repeated with DPH, although there were no apparent trends that could be correlated with any physicochemical properties of the compounds. For comparison, Perlman and Goldstein obtained $\Delta r = -0.0010$ for DPH in a 11 mM VPA/synaptic membrane preparation at 25°C. Seeing that they found r to be a linear function of concentration, this value agrees quite well with our own for 20 mM VPA.

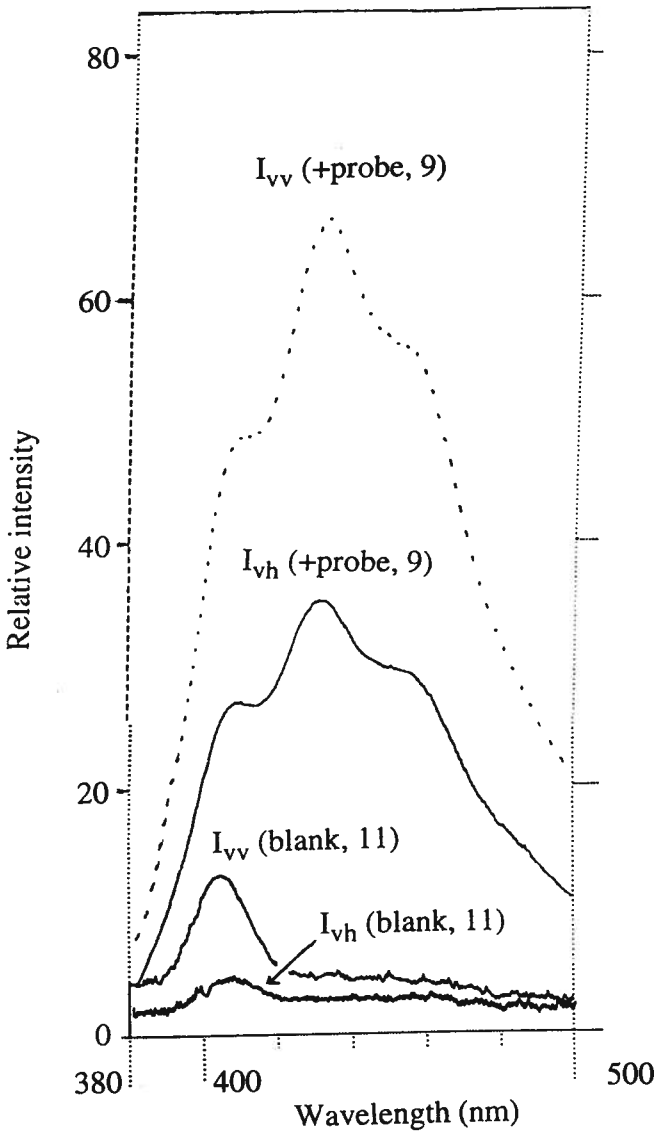


Figure 43. Emission spectra of erythrocyte ghosts with and without incorporated fluorescent probe TMA-DPH. Numbers in parentheses refer to recorder potentiation.

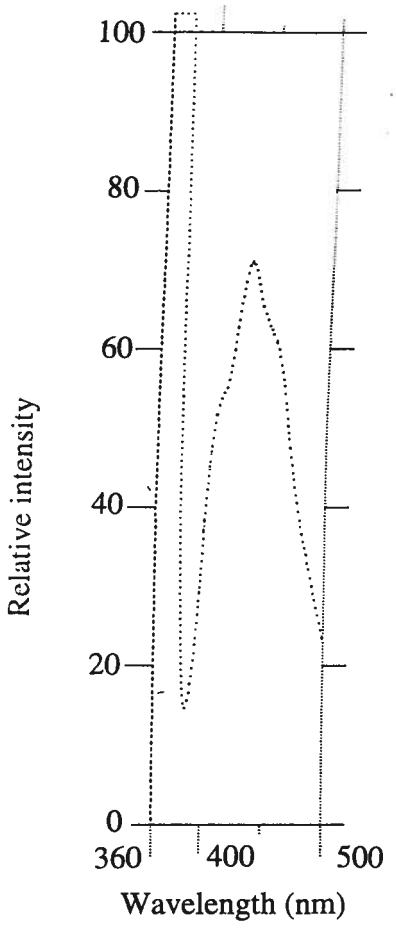


Figure 44. Emission spectrum of erythrocyte ghosts containing fluorescent probe DPH.

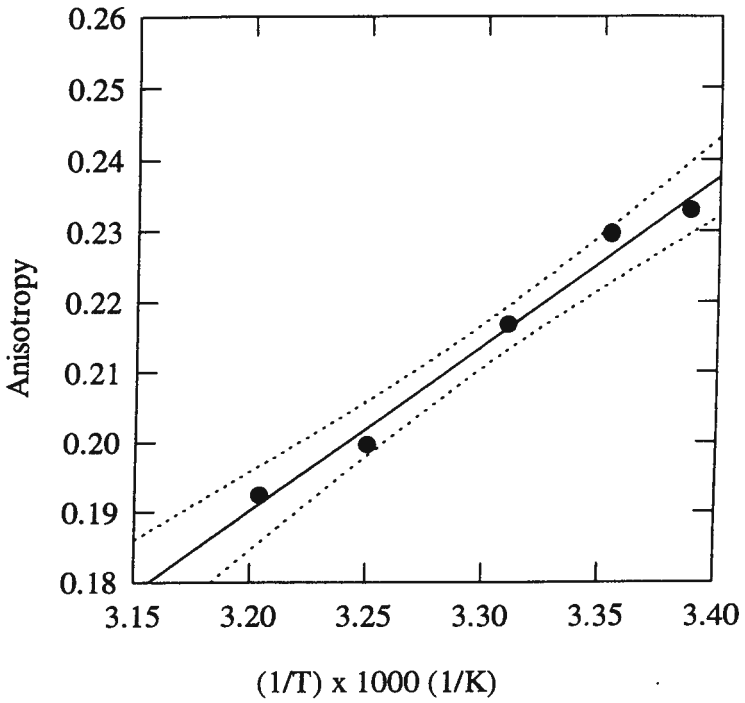


Figure 45. Fluorescence polarization of DPH in erythrocyte ghosts.

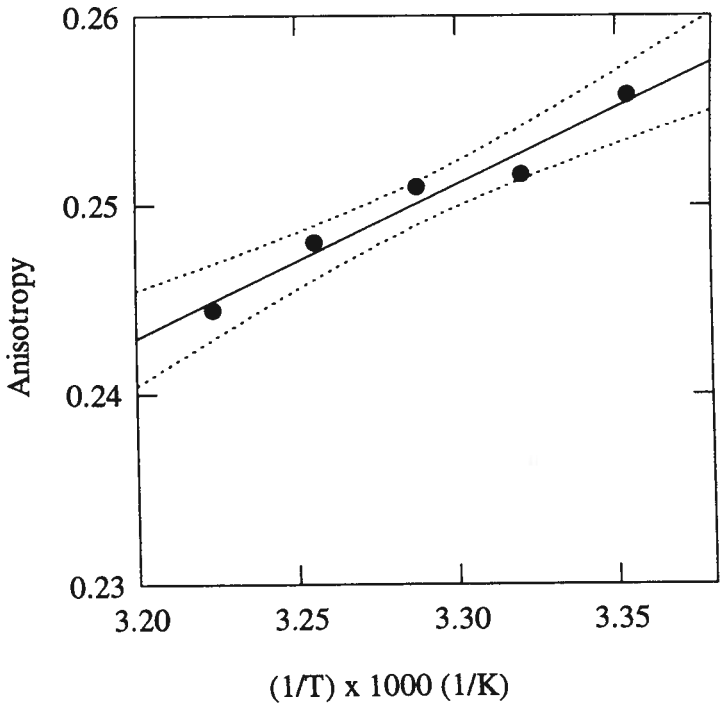


Figure 46. Fluorescence polarization of TMA-DPH in erythrocyte ghosts.

Statistical significance was determined by an independent two-tailed Student's t-test using the pooled method for calculating the standard error of the difference and using a significance level of 0.05.

Table 14
The effect of VPA analogues (20 mM) on membrane fluidity of human erythrocyte ghosts as measured by fluorescence anisotropy (r, x 10^3)

	TMA-DPH		DPH	
Compound	r	Δr	r	Δr
Control	279.2 ± 4.4	-	243.6 ± 5.9	-
1	281.8 ± 6.7	+2.6	226.9 ± 8.6*	-16.7*
7	278.0 ± 4.6	-1.2	229.6 ± 4.1*	-14.0*
8	277.9 ± 8.3	-1.3	237.5 ± 7.0*	-6.1*
9	274.9 ± 3.5*	-4.3*	208.3 ± 5.6*	-35.3*
10	281.3 ± 4.3	+2.1	208.4 ± 9.7*	-35.2*
14	275.9 ± 4.1*	-3.3*	224.9 ± 8.6*	-18.7*
15	278.5 ± 4.8	-0.7	163.2 ± 4.2*	-80.4*
16	281.4 ± 7.3	+2.2	237.3 ± 6.9*	-6.3*
17	271.0 ± 6.1*	-8.2*		

*) Significantly different ($p < 0.05$) from control.

Unfortunately, we were subsequently unable to reproduce the results obtained with DPH using several different, but identically prepared, ghost membrane suspensions. Similarly, there was no difference when both the incubation and the measurements were conducted at 37°C, or when the highly lipophilic drug tamoxifen (100 uM), a potent membrane-ordering agent (Wiseman *et al.*, 1993), replaced VPA. Thus, the membrane fluidity studies were considered inconclusive.

3.8 SYNAPTOSOME PREPARATION

The synaptosomes from the brains of mice administered ED50 doses of each drug (as described in Section 3.2) were prepared by an adaptation of the Loscher *et al.* (1981) version of the method of Dodd *et al.* (1981), as summarized in Figure 47. Although there was no clear evidence, such as that provided by electron microscopy, that the preparation did in fact

consist of resuspended synaptosomes, the GABA content is quite similar to that obtained by Loscher *et al.*. Furthermore, because GAD activity is found predominantly in nerve terminals (Martin, 1986), it can double as a marker enzyme for this region. Again, the activities calculated are not significantly different from those of Loscher *et al.*. In both cases, enzyme activity was determined by measuring the amount of GABA formed following incubation of the synaptosome suspension with glutamate and pyridoxal 5'-phosphate. Some differences are likely accounted for by the fact that these workers used a radioreceptor-type displacement assay for GABA and GAD. However, the synaptosomal protein content in the GAD assay was determined by the same literature method (Markwell *et al.*, 1978) and yet shows a large discrepancy.

Table 15
Comparison of synaptosome parameters with results of Loscher *et al.* (1981)

	Experimental	Loscher *et al.*
Protein (GABA assay) (mg / g wet tissue)	19.6 ± 2.1[a]	-
Protein (GAD assay) (mg / g wet tissue)	16.3 ± 2.0[a]	5.29 ± 0.65[d]
GABA concentration (nmol / mg protein)	20.0 ± 1.4[b]	16.6 ± 4.2[d]
GAD activity (nmol / mg protein / h)	325 ± 23[c]	442 ± 134[d]

a) Mean ± SD of two separate experiments using 5 mice: each individual synaptosome suspension was sampled in duplicate
b) As in a), but one experiment only and sampling in triplicate
c) As in a), but sampling in triplicate
d) $n = 48$

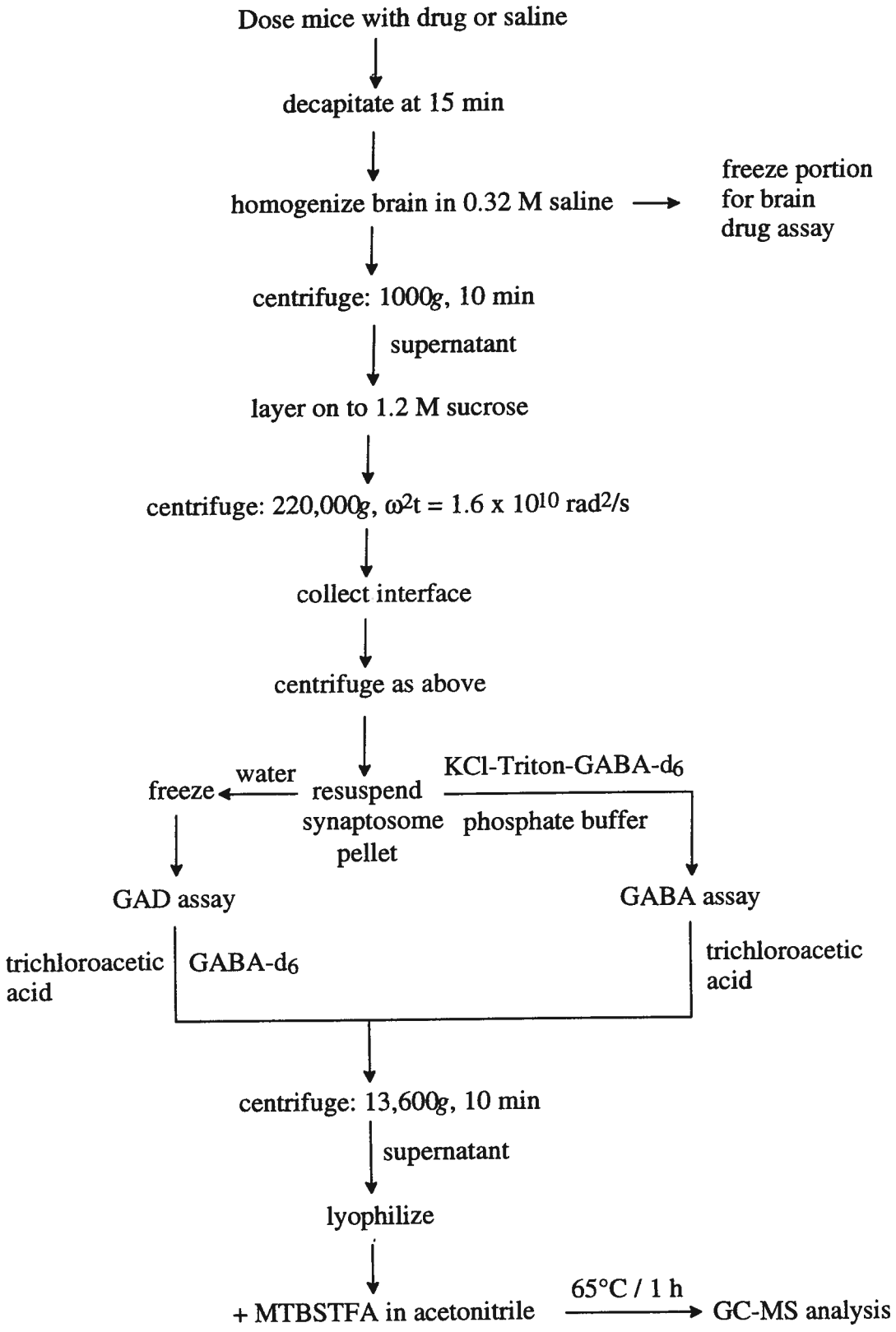


Figure 47. Preparation of synaptosomes for GABA and GAD assays..

3.9 GABA AND GAD ASSAYS

3.9.1 GENERAL

We initially employed the method of Bertilsson and Costa (1976) for the derivatization of GABA with pentafluoropropionic anhydride and hexafluoroisopropanol followed by GC-MS analysis. However, the peak shapes were poor and it was felt that the length of the derivatization procedure and its acidic column-degrading by-products were not fully compatible with our requirements. Consequently, other methods were investigated. Our initial treatment of GABA with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) afforded the *N,O*-bis(trimethylsilyl) GABA derivative. The compound showed sharp peaks and good sensitivity with the *m/z* 232 ion, corresponding to a species having lost one methyl group presumably from the amino substituent. When MSTFA was replaced with its *tert*-butyl analogue, MTBSTFA (containing 1% *tert*-butyldimethylsilyl chloride (*tert*-BDMSCl)), GABA was derivatized to the *N,O*-bis((*tert*-butyl)dimethylsilyl) compound. Sensitivity improved approximately ten-fold using the analogous ion *m/z* 274 (molecular ion less a *tert*-butyl substituent) that appeared as the base peak (Figures 48 and 49).

3.9.2 ASSAY CHARACTERISTICS

Owing to differences in the buffer medium and concentrations of GABA, separate calibration curves were required for the synaptosomal GABA and GAD assays. Both curves showed good linearity with $r^2 > 0.99$. Coefficients of inter-day variation for the GC-MS portion of the method were 7.7% and 7.9% for the GABA and GAD assays ($n = 7$), respectively, calculated from their respective calibration curves. The coefficients of intra-day variation were 0.95% (five replicates of five samples) and 1.9% (three replicates of ten samples) for the GABA and GAD assays, respectively. The response was essentially linear from 0.1 to at least 20 nmol GABA.

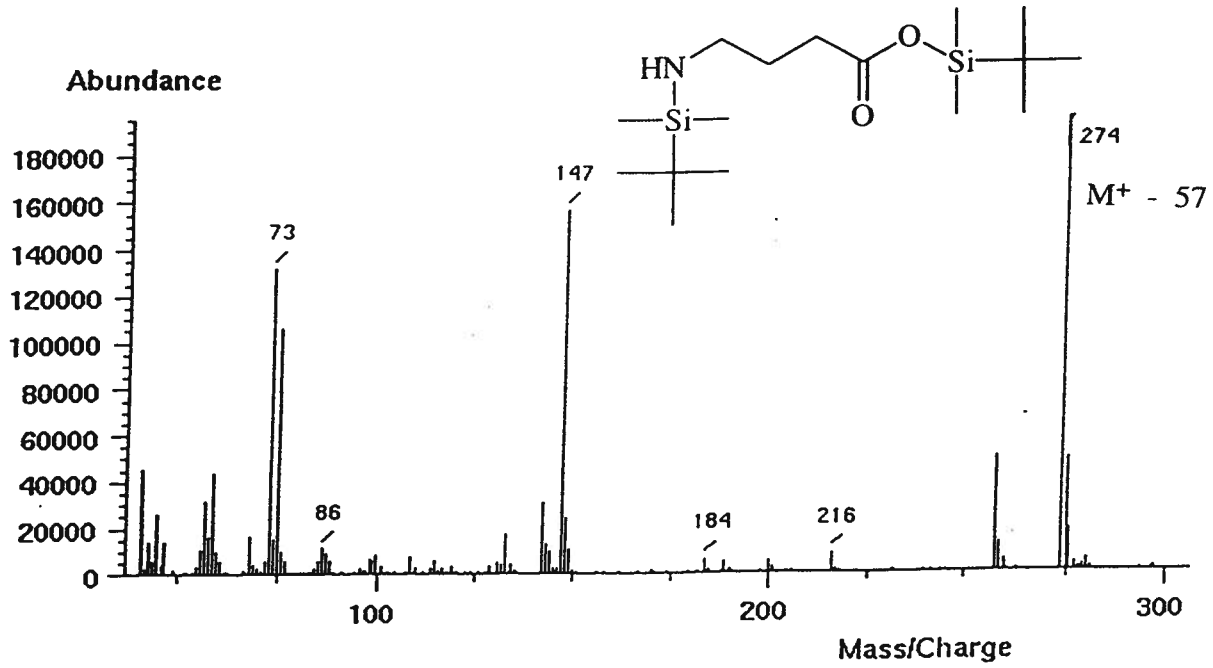


Figure 48. Electron impact mass spectrum of GABA disilylated with MTBSTFA.

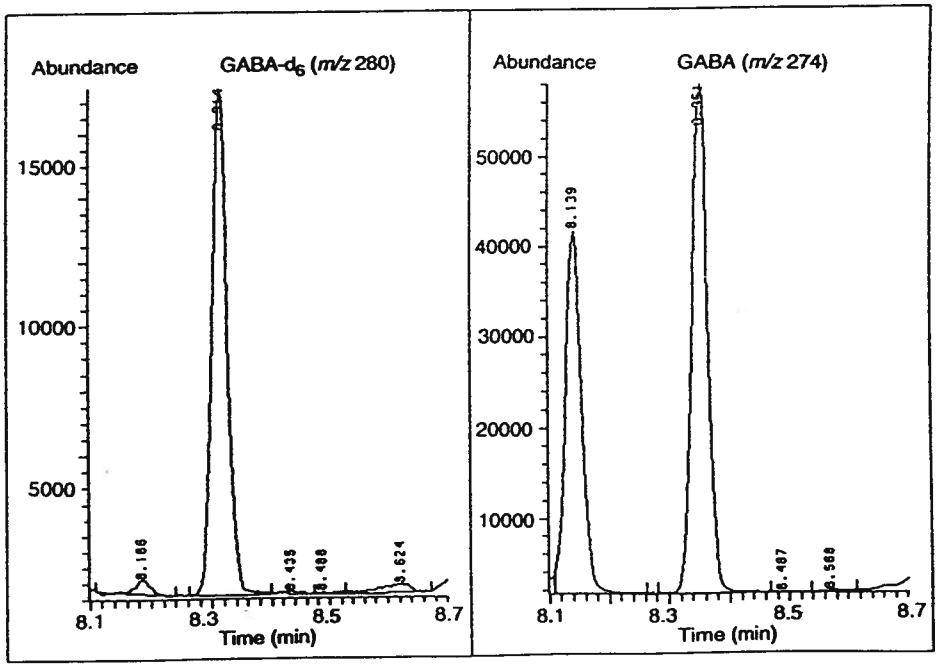


Figure 49. SIM chromatograms for GABA-d_6 and GABA from synaptosome GABA assay.

For a 3:1 signal/noise ratio, the detection limit was about 10 fmol of injected GABA derivative, corresponding to about 6 pmol GABA per 50 ul synaptosome aliquot. The calibration curve ($y = 0.00424 + 0.8182x$) for this range (6.7-107 fmol GABA per injection) was linear with $r^2 = 0.9998$. This sensitivity was more than adequate for the GABA and GAD assays because the average synaptosome sample in either assay contained at least 1000 pmol GABA.

The derivatized samples were stable for at least several weeks at room temperature.

3.9.3 APPLICATION TO SYNAPTOSOMES

GABA levels and GAD activity for both fresh and frozen synaptosomes are shown in Table 16. As might be expected, GABA concentrations were relatively similar for both fresh and frozen samples. Encouragingly, this was also the case for GAD activity. The noticeably higher GABA levels recorded in our assay, in comparison with Loscher *et al.* (1981), may be the result of the detergent in the resuspension buffer, which may have permitted a more efficient extraction of GABA from vesicle sites although this factor was not investigated further. Note the similarity of the values for GABA and the GAD blank, in terms of nmol/mg protein, indicating that the latter (e.g. synaptosomes incubated with buffer only) does not contain appreciable amounts of glutamate and PLP and thus represents primarily endogenous GABA.

The question of GAD assay reproducibility was further examined by separate preparations of synaptosomes. The first trial afforded a (fresh) activity of 335 ± 29 nmol/mg/h, while the second gave 315 ± 9.0 nmol/mg/h, representing a difference of about 6%. These values also agree well with the control activity determined in the GAD saturation assay (Section 3.10).

There were no significant interferences for either the GABA or GABA-d_6 peaks. This was demonstrated by the blanks run using only the resuspension buffer (GABA assay) or the Glu/PLP/phosphate solution (GAD assay). For the former, the GABA/GABA-d_6 ratio was

0.68 ± 0.38 % of the mean value for the intact synaptosome assay. For the GAD assay, the blank gave a ratio that was about 0.081 ± 0.023 % of the value obtained when the synaptosomes were incubated with the Glu/PLP solution.

The recovery of GABA-d_6 from the resuspended synaptosomal pellet in the GABA assay was 78 ± 3 % (n = 3), based on the ratio of the GABA-d_6 peak area obtained in the assay to the peak area recorded when this internal standard was incorporated into the MTBSTFA derivatization mixture rather than into the resuspension buffer. Although the recovery value is modest, the associated error is apt to be minimal owing to the chemical similarity of GABA-d_6 to GABA. It is unlikely that protein binding was the cause of these losses as the recovery was still only 86.9 ± 4.3 % (n = 6) when GABA-d_6 was added immediately prior to lyophilization rather than at the initial pellet resuspension. The determination of the recovery of GABA itself using a spiking method might not be reliable because the endogenous compound is in vesicles rather than simply in solution.

Table 16
Analysis of GABA and GAD in mouse whole brain synaptosomes[a]

Conditions	GABA (nmol/mg protein)	GAD activity[b] (nmol GABA/mg protein/h)
Fresh synaptosomes + Glu + PLP	20.0 ± 1.4	314.9 ± 9.0
Frozen synaptosomes + Glu + PLP	18.9 ± 1.5	329 ± 18
Frozen synaptosomes + PLP		7.9 ± 3.5
Frozen synaptosomes + Glu		32.0 ± 4.4

a) $n = 5$
b) Corrected for a blank value of 25.9 nmol GABA/mg protein/h.

3.9.4. DEMONSTRATION OF LINEARITY AND SATURATION CONDITIONS

Although the GAD assay conditions used in the project were those of Loscher (1981b), evaluations of the dependence of GAD activity on time and concentrations of substrate, co-factor and protein were also conducted, albeit subsequently to the results reported in Section 3.9.5.. The results are shown graphically in Figures 50-53: circled points correspond to the standard assay conditions used subsequently.

It is clear that while the samples contained saturating levels of PLP, the same could not be said for glutamate. This might be accounted for by the higher protein content of our synaptosomes compared to those of Loscher et al.. Figure 52 indicates a gradual decrease in rate with time over the standard 60 min incubation period, suggesting that a shorter duration, on the order of 10-20 min, would have been more appropriate. Finally, Figure 53 demonstrates that activity was in fact linear with respect to protein concentration.

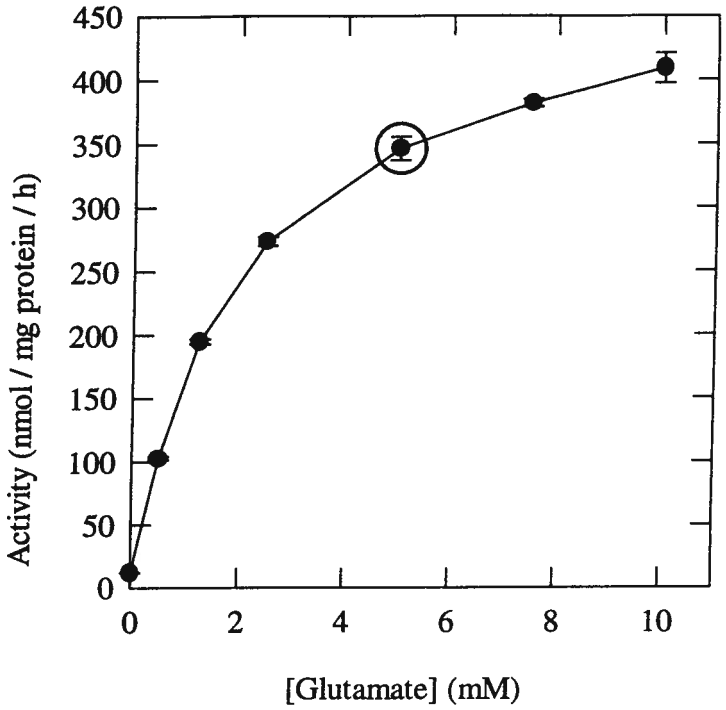


Figure 50. Effect of glutamate concentration on GAD activity.

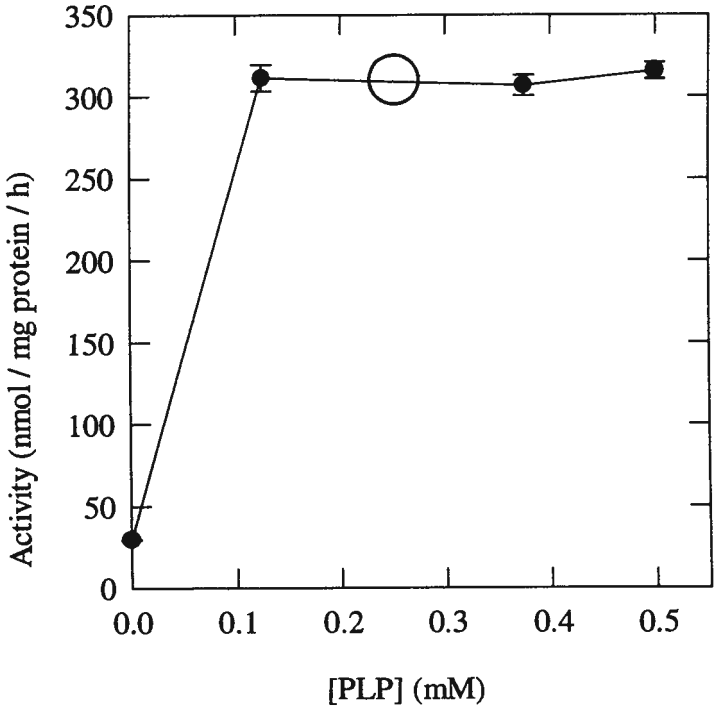


Figure 51. Effect of PLP concentration on GAD activity.

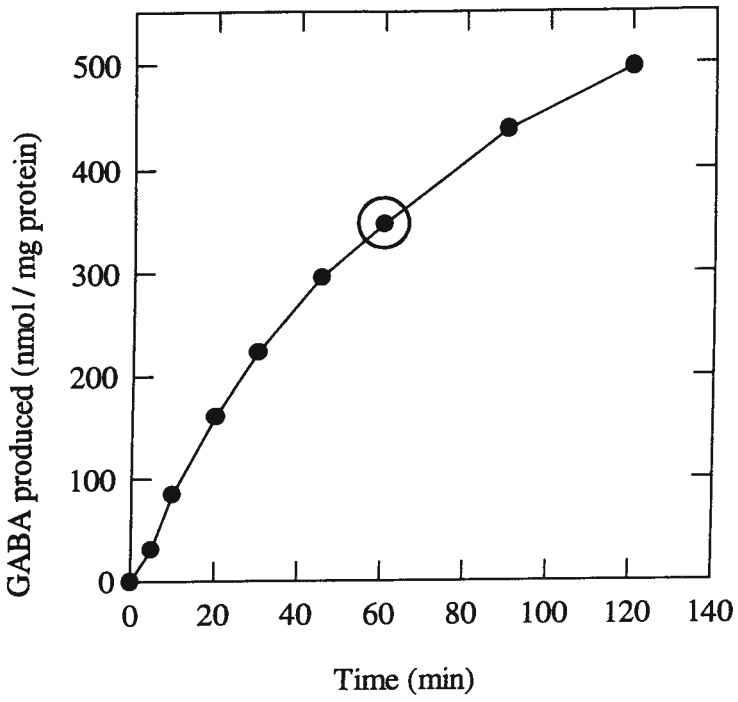


Figure 52. Time-dependence of GAD activity.

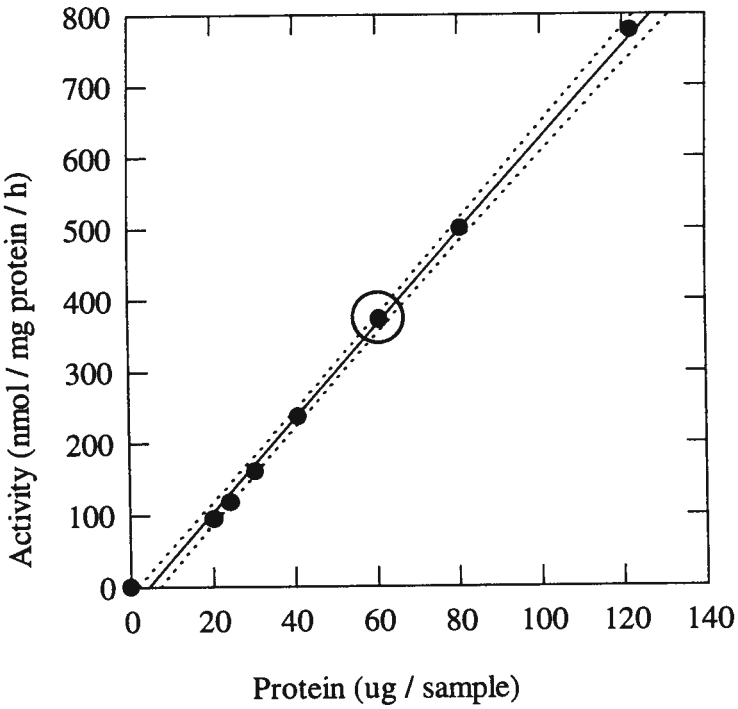


Figure 53. Effect of protein concentration on GAD activity.

3.9.5 RESULTS FROM DRUG-TREATED MICE

Using an ED50 dose of each drug, the levels of GABA and activities of GAD were determined in mouse brain synaptosomes as indicated. GABA was measured using freshly prepared synaptosomes while GAD activity was determined from samples stored at -78°C.

Table 17
Interaction with synaptosomal GABA metabolism after an ED50 dose

Compound	GABA[a] (nmol / mg protein)	% change	GAD activity[a] (nmol / mg protein / h)	% change
15 min				
Control	20.0 ± 1.4	-	329 ± 18	-
1	22.2 ± 1.6*	+11*	351 ± 21	+6.7
2	22.4 ± 3.7	+12	318 ± 44	-3.3
3	22.2 ± 3.4	+11	310 ± 12	-5.5
4	20.7 ± 1.9	+3.4	370 ± 22*	+12*
5	17.7 ± 1.1*	-11*	269 ± 38*	-18*
6	18.8 ± 1.8*	-6.2*	293 ± 45	-11
7	22.1 ± 1.8*	+10*	308 ± 44	-6.4
8	20.6 ± 1.9	+2.9	297 ± 27	-9.7
9	22.2 ± 1.1*	+11*	328 ± 16	-0.3
10	21.3 ± 2.8	+6.7	278 ± 10*	-15*
11	22.3 ± 3.0	+11	299 ± 23*	-9.1*
12	19.6 ± 1.1	-2.1	276 ± 64	-16
13	20.3 ± 1.4	+1.6	337 ± 7	+2.4
14	23.4 ± 2.8*	+17*	355 ± 31	+7.9
15	21.4 ± 2.6	+7.0	294 ± 18*	-11*
16	19.1 ± 1.8	-4.5	338 ± 27	+2.7
17	16.4 ± 2.2*	-18*	273 ± 15*	-17*
30 min				
1	23.7 ± 2.1*	+18*		
7	22.7 ± 3.6*	+13*		
8	19.8 ± 2.6	-1.0		
9	21.85 ± 1.1*	+9.2*		
10	20.8 ± 2.1	+4.2		
120 min				
Control	19.35 ± 1.35	-		

*) Significantly different from control ($p < 0.05$) by one-tailed Student's t-test.
a) Mean ± SD

Synaptosomal GABA levels were also assayed in mice administered higher doses of VPA in order to demonstrate dose-dependence.

Table 18
Effect of high VPA doses on synaptosomal GABA

Time (min)	*n*	Dose (mmol/kg)	GABA[a] (nmol/mg protein)	% change
15	3	2.08	24.2 ± 2.4*	+21*
30	3	2.08	25.4 ± 3.2*	+27*
15	1	4.17	26.72 ± 0.27*	+34*

a) Mean ± SD

A correlation matrix analogous to the one shown in Table 13 was constructed with the additional entries for changes in GABA levels and GAD activities (Table 19).

Clearly, there were no significant correlations for GABA with physicochemical parameters or GAD activity, although the relationship of GAD as a function of GABA and Z^- (mutually unrelated parameters as indicated in the Table 19) deserves mention. It is noteworthy that where the changes in GABA and GAD were both significant, their directions were usually identical, as indicated by the positive value of *r*. Some of the more interesting equations are shown below.

$$\text{GAD activity (nmol/mg/h)} = (6.54 \pm 3.58)\cdot\text{GABA} + (172 \pm 75) \quad (38)$$

($n = 16$; $s = 28.1$; $r = 0.438$; $F_{calc} = 3.33$; $F_{table} = 8.9$)

$$\text{GAD activity (nmol/mg/h)} = -(26.4 \pm 12.3)\cdot Z^- + (354 \pm 23) \quad (39)$$

($n = 16$; $s = 27.1$; $r = 0.498$; $F_{calc} = 4.6$; $F_{table} = 8.9$)

$$\text{GAD activity (nmol/mg/h)} = (5.60 \pm 3.28)\cdot\text{GABA} - (23.6 \pm 11.7)\cdot Z^- + (233 \pm 74) \quad (40)$$

($n = 16$; $s = 25.5$; $r = 0.621$; $F_{calc} = 4.1$; $F_{table} = 6.7$)

Table 18
Full correlation matrix of biological and physicochemical properties of VPA analogues[a]

	Dose[b]	C[c]	logP	V	X^+	ΔY	Y^-	Y^+	ΔZ	Z^-	Z^+	GAD[d]	GABA[e]
Dose	1.00												
C	0.87	1.00											
logP	-0.92	-0.90	1.00										
V	-0.95	-0.87	0.97	1.00									
X^+	-0.64	-0.58	0.57	0.60	1.00								
ΔY	-0.68	-0.55	0.69	0.69	0.15	1.00							
Y^-	-0.19	-0.29	0.35	0.22	-0.05	0.25	1.00						
Y^+	-0.45	-0.26	0.33	0.43	0.18	0.68	-0.54	1.00					
ΔZ	-0.84	-0.70	0.82	0.88	0.44	0.68	0.01	0.58	1.00				
Z^-	-0.73	-0.51	0.63	0.72	0.36	0.56	-0.07	0.53	0.91	1.00			
Z^+	-0.75	-0.67	0.77	0.82	0.41	0.70	-0.04	0.79	0.92	0.74	1.00		
GAD	0.16	0.22	-0.21	-0.23	-0.07	-0.23	0.04	-0.22	-0.38	-0.50	-0.32	1.00	
GABA	0.27	0.40	-0.40	-0.36	-0.17	-0.06	-0.11	0.04	-0.15	-0.14	-0.07	0.44	1.00

a) Correlation coefficient *r*
b) Log(ED50)
c) $Log([drug]_{brain})$
d) GAD activity (nmol/mg/h)
e) Nmol/mg

3.9.6. INHIBITION OF GAD BY ACID 17

The simultaneous decrease in GABA levels and GAD activity for acid **17** prompted us to examine whether or not this compound could also inhibit the enzyme *in vitro*.

Control synaptosomes were incubated with a saturating concentration of PLP and variable amounts of glutamate and compound **17** (as its sodium salt) for 10 min, a length of time previously shown to afford a good estimate of the initial rate (Figure 50). The concentrations of glutamate (1-10 mM) spanned the range from saturating to those affording GABA levels only about half those present in the blank. The results are expressed as a double-reciprocal plot in Figure 54, which clearly shows the enzyme being inhibited at both 1 and 5 mM concentrations of compound **17**. Based on the apparently common *x*-intercept but different *y*-intercept values for the three lines, we conclude the mode of inhibition to be non-competitive. The following equations were then used in conjunction with Figure 54 to obtain the parameters listed in Table 20.

$$\text{x-intercept} = -1 / K_m \tag{41}$$

$$V_{max} = K_m / \text{slope} \tag{42}$$

$$V_{max}^I = V_{max} / (1 + [I]/K_i) \tag{43}$$

$$V_{max}^I = V_{max} \text{ in presence of inhibitor I at concentration [I]} \tag{44}$$

Table 20
Effect of acid **17** on activity of GAD *in vitro*

[**17**] (mM)	K_m (glutamate) (mM)	V_{max}^I (nmol / mg protein)	K_i (mM)
0	2.1	98	-
1	2.0	83	5.5
5	2.4	70	12 (mean: 9 mM)

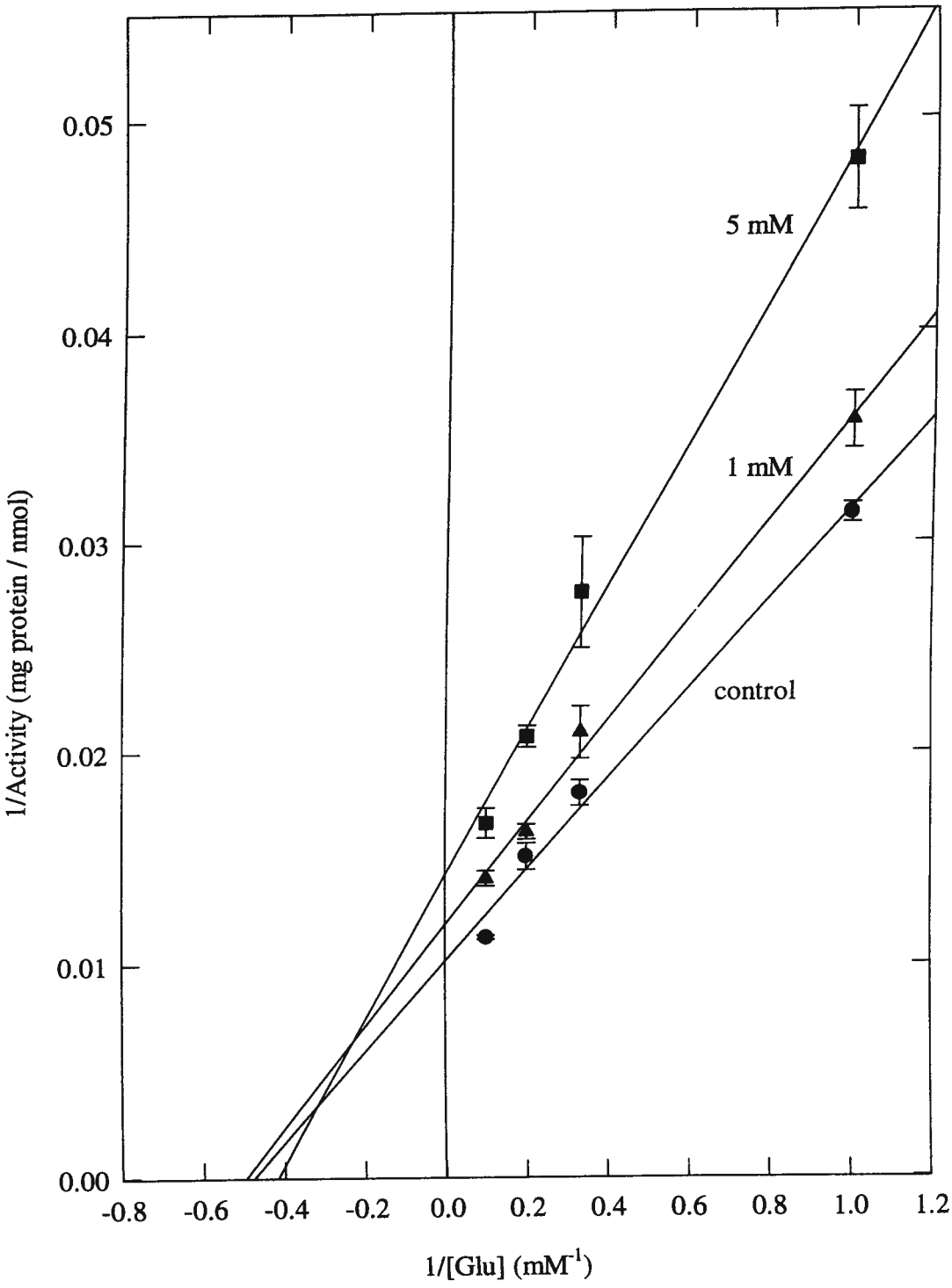


Figure 54. *In vitro* inhibition of synaptosomal GAD by **17**.

Error bars indicate SD for one sample evaluated in triplicate.

3.10. GAD SATURATION

Finally, it was of interest to determine the mechanism by which these drugs might lead to GAD activation or inhibition. Because GAD is only partially saturated with PLP cofactor under normal physiological conditions, an intuitively obvious way to increase its activity is to increase the extent of cofactor binding. This would be manifested as enhanced enzyme activity when the preparation is assayed following removal of all unbound cofactor, in this case by gel filtration. Thus, our approach was to isolate synaptosomes by the standard method from mice given ED50 doses of VPA, 2-ene VPA and compound **17**, remove unbound low-molecular weight species by passage through a Sephadex G-25 size exclusion column and conduct a modified enzyme activity assay. This enzyme assay differed from the standard version used above in its short reaction time (5 min *vs.* 60 min), low concentration of glutamate (1 mM *vs.* 5 mM: K_m = 1 mM (Martin and Rimvall, 1993)) and avoidance of a phosphate buffer in an attempt to minimize apo-GAD/holo-GAD interconversions. GABA production was then analyzed by GC-MS as before. However, it was found that the recommended concentration of imidazole-acetate buffer (100 mM: Martin, 1986) did not afford satisfactory or reproducible derivatization of GABA to its disilylated form, presumably because both imidazole and acetic acid, unlike phosphate, can serve as substrates for MTBSTFA. Increasing the amount of MTBSTFA was unsuccessful, but reducing the buffer concentration gave a concentration-dependent improvement in the response.

An initial trial run was conducted in order to test the effectiveness of the gel filtration column. Individual column fractions were assayed for protein concentration and approximate relative GAD activity (expressed only as the GABA/GABA-d_6 area ratio) as shown in Figure 55. Protein recovery was approximately 87% (fractions 7-11) and there was good agreement between the protein concentration and GAD activity curves. The level of GABA in the peak fraction was minimal (Table 21: compare with Table 16), demonstrating the integrity of the gel filtration protocol. Finally, the enzymatic origin of the produced GABA was proven by comparing solutions containg only Glu and/or PLP as well as blanks where both were omitted

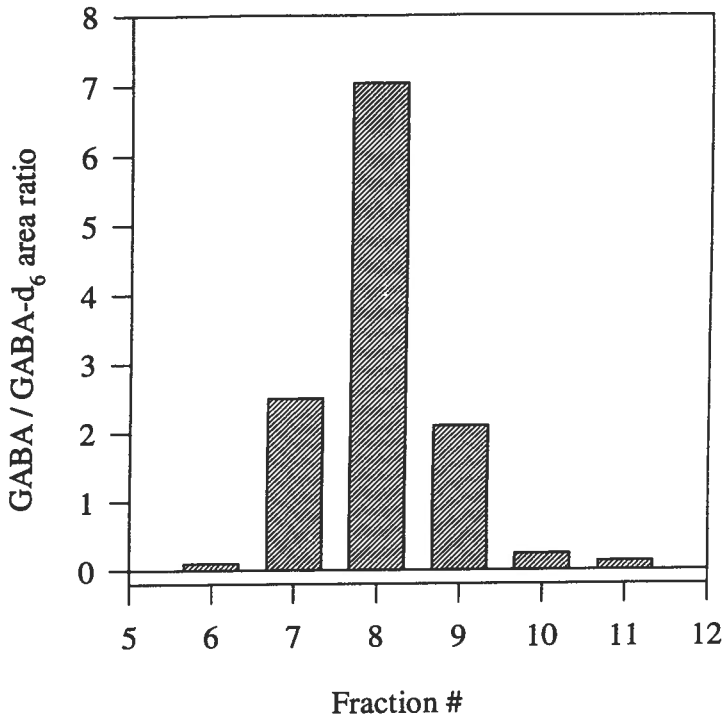


Figure 55a. GAD saturation assay: approximate relative GAD activity *vs.* column fraction #.

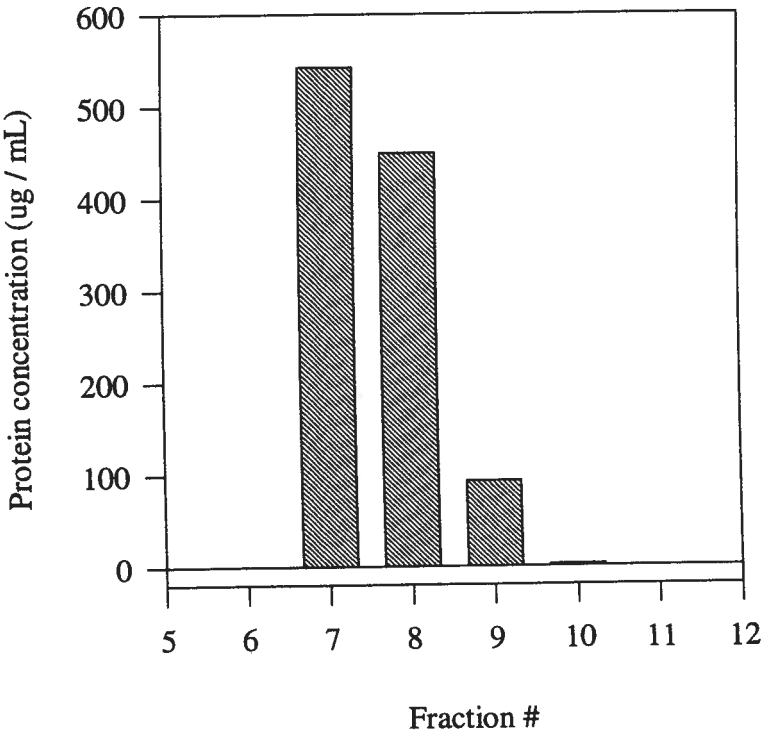


Figure 55b. GAD saturation assay: protein concentration *vs.* column fraction #.

(Table 21) with the complete mixture. These results show that the maximal interference with the GAD activity was only about 3% and was apparently derived mainly from the buffer itself.

Table 21
Interference with holoenzyme activity in post-gel filtration GAD assay[a]

Conditions	GABA/GABA-d_6 area ratio	Interference[b] (%)
Eluate + Glu + PLP	1.20 ± 0.81	-
PLP	0.075 ± 0.044	6.2 ± 4.4
Glu	0.039 ± 0.010	3.2 ± 1.2
PLP + Glu	0.032 ± 0.0027	2.7 ± 0.4
Eluate only	0.0174 ± 0.0099	1.5 ± 0.9

a) $n = 3$
b) (GABA/GABA-d_6 area ratio in "Conditions") / (GABA/GABA-d_6 area ratio (eluate + Glu + PLP))

The full procedure was carried out using 3 mice per drug. In the initial brain isolation step, decapitation into ice-cold saline was promptly followed by cutting of the scalp in order to cool the brain as rapidly as possible prior to its equally rapid removal from the cranium. It has been shown that prolonged exposure to room temperature at this point will markedly affect the saturation of the enzyme (Miller et al., 1977). Synaptosomes were then prepared as described and an aliquot from each was purified by the size exclusion column. The combined protein fractions were assayed for GABA production in the presence and absence of added PLP. The degree of saturation of the enzyme with cofactor was then calculated as the ratio of these two values following correction for endogenous GABA (Table 22).

The large uncertainty associated with the control value for synaptosome activity was the result of one apparently anomalously high result. All three synaptosomal (un-filtered) activity measurements from this sample were in agreement, indicating that the preparation itself was suspect. When this value was omitted, the activity for synaptosomes from VPA-treated mice reached statistical significance.

Table 22
Effect of ED50 doses of VPA, 2-ene VPA and compound **17** on saturation of synaptosomal GAD 15 min postdose[a]

Treatment	Synaptosomal GAD activity (nmol GABA/mg/h)	Gel-filtered GAD activity (nmol GABA/mg/5 min) With PLP	Without PLP	Saturation (%)
Individual results				
Saline	309	14.18 ± 0.37	6.06 ± 0.13	42.7
	327	12.17 ± 0.15	5.34 ± 0.10	43.9
	383	14.45 ± 0.31	6.11 ± 0.14	42.3
VPA	373	14.39 ± 0.16	6.189 ± 0.059	43.0
	365	13.48 ± 0.21	5.68 ± 0.15	42.1
	347	11.36 ± 0.39	4.77 ± 0.17	42.0
2-Ene VPA	309	11.96 ± 0.41	4.53 ± 0.10	37.9
	357	9.692 ± 0.095	3.710 ± 0.082	38.3
	336	11.94 ± 0.18	5.057 ± 0.064	42.3
17	343	11.87 ± 0.85	5.10 ± 0.33	43.0
	366	9.96 ± 0.46	3.64 ± 0.11	36.6
	396	10.00 ± 0.41	3.51 ± 0.13	35.1
Mean results				
Saline	339 ± 38	13.60 ± 1.25	5.84 ± 0.43	42.97 ± 0.82
VPA	361 ± 14	13.08 ± 1.55	5.55 ± 0.71	42.38 ± 0.55
2-Ene VPA	334 ± 24	11.20 ± 1.30	4.10 ± 0.41	39.50 ± 2.47
17	369 ± 27	10.61 ± 1.09	4.08 ± 0.88	38.21 ± 4.19

a) Mean ± SD for mice sampled in triplicate.

4. DISCUSSION

4.1. SYNTHESIS

Because the initial objective of this study was to focus on *E* and *Z* isomers of 2-substituted 2-pentenoic acids, most of the described synthetic efforts are aimed in this direction. First, in any study examining the effect of chemical substitution on biological activity it is clearly essential that the compounds be available as single isomers. Furthermore, in our case, it was desirable that the method give consistent results with all types of substrates with only minimal modification. For these reasons, we chose as a general approach the dehydration of 3-hydroxy carbonyl compounds, which can be prepared with high degrees of diastereoselectivity through the highly stereoselective aldol addition established by Evans *et al.* (1981) involving the addition of dibutylboryl enolates of *N*-acyloxazolidinones to aldehydes. This method affords the *erythro* adducts with consistently high yields and selectivity for a wide variety of substrates. When dehydrated by an E2 mechanism-based process, these adducts should then lead to the *E*-unsaturated derivatives (Figure 6). Furthermore, it has recently been reported by Walker and Heathcock (1991) that the *threo* isomers are also available *via* minor modifications of this approach, thus allowing access to the *Z* isomers as well.

The hydrolysis of the aldol adducts to the free acid was reported to be highly efficient using lithium hydroperoxide. However, it was doubtful that the same would apply to the corresponding α,β-unsaturated adduct. Thus, it seemed advisable to perform the dehydration step following adduct hydrolysis. In order to prevent side-reactions such as ketene formation in the dehydration step, the carboxylic acid would then be protected as the methyl ester with diazomethane. Subsequent dehydration *via* elimination of the corresponding mesylate and saponification should then afford the free acid. Indeed, the results for compounds bearing unbranched substituents at C(2) demonstrate that this overall approach was successful for the *E* isomers. The only apparent failings of this method were in the case of compound **5**, which was found to undergo *E/Z* isomerization upon basic hydrolysis, and the branched side-chain

compounds that could not be induced to undergo methanesulfonyl chloride-mediated dehydration in any but the lowest yields.

The previously unreported syntheses of the other analogues are unremarkable, being based on either a similar approach starting from a β-keto ester or a routine Wadsworth-Emmons reaction. As mentioned, acids **3**, **9** and **10** were prepared as described in the literature.

4.2. ANTICONVULSANT EVALUATION AND CORRELATION WITH PHYSICOCHEMICAL PROPERTIES

The next step was to evaluate the anticonvulsant potencies of compounds **2-17** as their sodium salts and compare these values with that of VPA. The test used was the standard subcutaneous pentylenetetrazole injection administered 10 min following the drug (Swinyard *et al.*, 1989). We found that the convulsant alone produced seizures in 87% of all animals, which was in agreement with the test's objective of evaluating how the drugs influence seizure threshold rather than seizure spread, as would be the case with the MES test (Piredda et al, 1985). The evaluation of VPA and its analogues *in vivo* supported earlier findings (Tables 3-4) that increasing the size of the aliphatic group attached to a carboxylic acid fragment will also increase anticonvulsant potency. This was shown especially clearly in the cases of the 2-substituted 2-pentenoic acids (compounds **2** and **5-8**) and the cycloalkylideneacetic acids (compounds **14-17**). Quantitative support for this phenonomenon is provided by equation (21), depicted graphically in Figure 38, which shows a high degree of correlation between log(ED50) and volume. This relationship largely persists when log(ED50) is replaced by the logarithm of the brain concentration (Figure 40) although the correlation parameters are clearly inferior. The situation could not be improved significantly by the further introduction of shape parameters Y^-, Y^+ and ΔY describing the C(2) substituent, chosen on the basis of their independence of volume/log P. This suggests that the pharmacodynamics of the drugs are governed to a lesser degree by shape-independent

properties such as lipophilicity than in the overall situation where the pharmacokinetics of drug access into the brain must be considered. Although this evidence is less than robust, it supports the involvement of a receptor site with some minor shape requirements that mediates the anticonvulsant effect.

These results showing good correlations of anticonvulsant potency with volume and lipophilicity are in agreement with the previously mentioned studies of Abbott and Acheampong (1988) which examined a series of VPA analogues. Interestingly, Lien *et al.* (1979) reported similar correlations (r = 0.72-0.76) with lipophilicity and molecular weight for a group of 12 hydantoins and barbiturates. The equations were unsuccessful in approximating the potencies of diazepam and clonazepam, however, and the use of ED50 rather than actual brain concentration indicates that the equation may represent nothing more than the relative abilities of the drugs in reaching the brain, rather than their interaction with their effector sites.

What was the influence of a ring system on anticonvulsant potency? Comparing cyclic and acyclic compounds with equal numbers of carbon atoms indicates that the introduction of a ring closure caused a reduction in activity most readily explained by parallel decreases in both volume and lipophilicity as revealed by Tables 9 and 12. Thus, structure is apparently less important than the simple bulk properties of volume and lipophilicity. This relationship is demonstrated particularly clearly by the C_8 acids 2-ene VPA (**7**), **15** and **11** with their varying number of ring systems.

The finding that the volume and log P dependence of potency persists largely undiminished when actual ED50 brain concentrations are evaluated is quite significant. Much of the work to date (Section 1.2.3) has focused strictly on ED50 itself as the biological indicator of potency that does not allow for reliable conclusions about mechanisms of action as it incorporates pharmacokinetic as well as pharmacodynamic terms. Thus, this study clearly shows that a drug's lipophilicity is important in its interaction with the still unknown receptor site as well as in its passage from the periphery into the brain.

Considering the chemical similarity of the compounds tested, it is relevant to ask whether or not a QSAR study is justifiable in this case. The only physicochemical terms involved here are highly correlated parameters describing volume and lipophilicity because all compounds are identical in the electrostatic or inductive properties of their substituents on the basic α,β-unsaturated carboxylic acid unit, with the minor exception of compounds **9** and **10**. Thus, is there enough diversity in the set to obtain a balanced picture of the cause of the anticonvulsant effect? We believe the answer to be in the affirmative if the ranges of lipophilicities (Table 12) and potencies (Table 5) are considered. For example, the compounds vary from those that are nearly water-miscible in the undissociated form (compounds **2** and **3**) to one that is barely water-soluble even as its sodium salt (compound **17**). Similarly, ED50 potencies vary from 0.7 to 7.0 mmol/kg, demonstrating that acids **2-17** represent a diverse group of physical and biological, if not chemical, properties.

More importantly, our primary objective here was to study the interaction of the drugs with the effector leading to seizure protection. For this assumption of a common mechanism to hold, it is evident that the tested analogues must not stray far from the fundamental structure of 2-ene VPA. The simplicity of this molecule clearly restricted allowed substituents on the α,β-unsaturated carboxylic acid moiety to those of an aliphatic non-polar nature, although some liberties were taken by the use of cyclic, branched or unsaturated substituents. Introduction of electrostatic charges or strongly electron-withdrawing species, for example, would profoundly alter the properties of the molecule that could lead to altered pharmacokinetics (notably transfer across the blood-brain barrier) and quite possibly different pharmacodynamics as well. When combined with the fact that the scPTZ anticonvulsant test is fairly non-specific (recall that the mechanistically unrelated drugs ethosuximide and diazepam are both effective here: Table 2), this could clearly lead to considerable ambiguity about how a drug is exerting its anticonvulsant effect. Consequently, a correlation equation with ED50 or $[drug]_{brain}$ for a group of pharmacologically diverse compounds would no longer carry implications about the mechanism through which seizure protection was being achieved.

It should be noted that all tested compounds were achiral with the exception of acid **11** which had been prepared from (±)-norcamphor and was thus a racemate. This clearly raises the question of the relative pharmacokinetics and pharmacodynamics of the two enantiomers, but is likely largely academic in view of the findings of Hauck *et al.* (1991) that show a lack of enantioselectivity in the anticonvulsant properties of various asymmetric VPA analogues.

A note of caution about the data presented in Table 5 concerns the reliability of the ED50 values. It is important to realize that the experiment is subject to a high degree of variability, as is clearly demonstrated by the large ED50 confidence intervals. In some cases, in fact, the data collected from the group of 40 mice for a given drug gave a plot with an infinite confidence interval, requiring that the entire experiment be repeated. This variability was also the reason for routinely omitting data points, despite the already low initial number, in an effort to obtain meaningful results from an experiment. This editing was unavoidable in some experiments because the percent protection is obviously a linear function of log(dose) across only a limited range of doses. That is, there will be two continuums of dose ranges affording zero and full protection, respectively. Elimination of data points will influence the reliability of the result, making it vital to consider the respective confidence intervals whenever comparing potencies. Finally, this variability would be expected to affect subsequent experiments involving an initial ED50 dose. For example, one would not expect to observe a small coefficient of variation for a synaptosomal GABA increase following an ED50 dose with a large confidence interval, especially considering that ED50 by definition is an inherently uncertain quantity (e.g. only half of the animals receiving this dose will be protected against seizures) made even more so when small values of n are involved. Were it not for the comparable coefficients of variation for ED50 and brain levels, the fact that such small variations were routinely found in the GABA and GAD assays would suggest that the principal cause of ED50 variation lies downstream from GABA production, perhaps in the form of individual differences in GABA receptor densities or neural architecture.

Whereas VPA remained as one of the most effective overall anticonvulsant drugs as measured by its ED50 value, its intrinsic potency, that is inversely related to its brain

concentration at 15 min following an ED50 dose (Table 5), was remarkably low in comparison with a large number of other analogues, including 2-ene VPA. This was shown by the compact group of analogues with ED50 values less than 1 mmol/kg, whose intrinsic potencies must be very good indeed if they could afford anticonvulsant protection equal to that of VPA at essentially the same dose but at substantially lower effective brain concentrations. The therapeutic advantage of VPA must therefore be derived mainly from its ability to be efficiently transported into the brain rather than from an exceptionally effective interaction with its final neurochemical target. A truly significant reduction in ED50 can thus be achieved only by dealing with the issue of blood-brain barrier transport as well as intrinsic potency. Specifically, a rational step to further improve the effectiveness of the most potent compound, acid **17**, would not be to increase volume or log P, for example by increasing the size of the ring or adding further substituents, but rather to incorporate the molecule into a prodrug such as a diacylglyceryl ester (Mergen *et al.,* 1991), which has been shown to be an efficient carrier of VPA into the brain. The need to consider primarily pharmacokinetics rather than intrinsic potency is perhaps best illustrated by the considerable difficulties encountered in preparing an aqueous solution of compound **17** as its sodium salt for an ED50 dose, which demonstrated that lipophilicity cannot be increased significantly beyond this point if aqueous solubility is to be retained.

Dividing the ED50 brain concentration by the ED50 dose was taken as an index of the drug's ability to penetrate into the brain from the initial site of injection at its therapeutic dose (Q: Table 8). This value represents a drug's instantaneous distribution ratio between the two compartments because the system is not at equilibrium. Although the results appear randomly distributed at first, some distinct trends do emerge. First, VPA clearly is very efficient in reaching the brain, as noted above. This is likely due to the lack of a double bond conjugated with the carboxyl moiety, although it is less clear how this feature is expressed biochemically. It may represent a lower degree of plasma protein binding due to altered charge densities on the carboxyl group or reduced dissociation leading to a greater free concentration and thus a potentially larger gradient with the CSF compartment. This notion is

undermined by the fact that the dienes **9** and **10** have above-average values of Q despite being the most acidic compounds in the table (pK_a VPA = 4.95; pK_a **7** = 4.36; pK_a **9/10** = 4.02: Abbott and Acheampong, 1988). Also, the unsaturated analogues **3** and **11** have Q values comparable to that of VPA itself. Alternative explanations for the facile transport of VPA across the blood-brain barrier are hard to conceive as these compounds have very similar potencies and physicochemical properties (such as shape and lipophilicity).

The remaining compounds appear to have similar Q values within their own sub-groups with an inverse relationship with molecular weight that suggests increased difficulties in penetrating the blood-brain barrier for the larger molecules. There is no apparent difference between the 2-pentenoic acids (compounds **5-8**) and the cyclic analogues (compounds **14-17**) when one compares compounds with identical numbers of carbon atoms. Compounds **11-13** are unusual in exhibiting a wide range of Q values although they possess consistently compact and rigid structures. For example, it is difficult to see how **12** and **13** could behave so differently despite their structural similarity.

A comparison of Tables 5 and 9 reveals an appreciable correlation between a drug's sedative properties and the number of conformers occurring within 10 kJ of its global minimum. Thus, the greater a molecule's floppiness, the greater its accompanying sedation. This is shown clearly when one compares the 2-substituted-2-pentenoic acids, which show rapidly increasing sedation with size, with the cyclic compounds, which show noticeable sedation only at the level of the flexible cyclooctylidene ring. The literature generally supports this trend but not without some exceptions. While both Loscher and Nau (1985) and Liu and Pollack (1994) reported that the neurotoxicity of 1-methyl-1-cyclohexylcarboxylic acid was somewhat less than that of VPA, the latter workers noted that the closely-related 1-cyclohexylcarboxylic acid was only about one half as toxic as VPA. Furthermore, Scott *et al.* (1985) found that while the large rigid molecule spiro[4.6]undecane-2-carboxylic acid (Figure 2) gave an scPTZ ED50 value of 0.42 mmol/kg, its neurotoxicity was about twice that of VPA. This suggests that sedation may simply be a function of volume/lipophilicity that increases in parallel with conformational lability. This idea would account for the seemingly

contradictory results presented in Table 9 that while **16** and **17** have an equally high number of conformations, only the latter shows sedative effects. However, the acid **16** has a greater volume/lipophilicity than 2-ene VPA and yet only the latter is noticeably sedating. One possible explanation is that it is the spatial range of the conformational interconversions that must be considered, rather than simply the volume for a given conformation. Specifically, the coordinates of given carbon atoms in compound **16**, unlike those in 2-ene VPA or compound **17**, do not show appreciable variation between conformations, resulting in a relatively small time-averaged volume. Therefore, the decreased toxicity of compound **16** may in fact stem from the corresponding reduction in volume, as noted previously.

Nevertheless, it is clear that although both anticonvulsant potency and sedation increase with volume, regardless of the structure, the presence of a ring system in the molecule can selectively diminish the dependence of sedation. Thus, a C_{10} homologue of acid **8** would be profoundly sedating unless it was a cyclic molecule such as compound **17**. As shown in Figures 38-41, the absence of outliers representing different structural classes indicates that there is no such relationship with anticonvulsant potency. This partial dissociation of sedation from molecular size was demonstrated above by 1-cyclohexylcarboxylic acid and VPA, the former showing half the neurotoxicity of the latter despite their nearly identical molecular formulas.

The molecular modelling calculations for compounds **5-8** also revealed that the *Z* isomers show much more conformational lability than the *E* isomers. Considering the strong dependence of the *in vivo* effects of these drugs on similar bulk properties such as lipophilicity, this may explain why the *Z* isomer of compound **7** has been reported to be less potent than the *E* form (Loscher, 1992). However, the potency difference might also be simply due to the shape resulting from the orientation of the double bond itself.

Having shown that both anticonvulsant activity and sedation increase with size/lipophilicity, it is pertinent to now ask what the actual effector site might be. The previously mentioned studies of Perlman and Goldstein (1984) as well as the repeatedly demonstrated effects of VPA on ion channel kinetics (Section 1.2.2.4) justify proposing a role

for membrane disordering in the pharmacodynamics of VPA, analogous to the one for volatile anesthetics. Such a target site is supported by the work of Lucke *et al.* (1993) who found that neither VPA nor 2-ene VPA readily cross the plasma membrane. Furthermore, it has been reported that epileptic patients on VPA therapy exhibit markedly higher fluidity of their erythrocyte membranes, showing the existence of an *in vivo* effect following normal doses (Tangorra *et al.*, 1991).

The specific advantage of the fluorescence polarization assay is that while it evaluates the lipophilicity of a drug, like the log P determinations, it does so in the anisotropic environment of a cell membrane. This is in marked contrast to the isotropic medium in a shake-flask or HPLC log P experiment. Thus, the fluorescence polarization method examines the actual biological target site rather than a synthetic model.

In this project, erythrocyte ghosts rather than the synaptosomal membranes of Perlman and Goldstein were employed for several reasons. One such issue was membrane purity: whereas the ghosts represent a single pure plasma membrane, synaptosomal membranes probably contain contributions from the plasma membrane, mitochondria and endoplasmic reticulum. From a more practical viewpoint, erythrocyte ghosts are also preferable because their preparation is more rapid and easily yields the amounts of membrane required for the assay. In contrast, our attempts to produce synaptic membranes by the laborious method of Jones and Matus (1974) consistently gave negligible amounts of the final product even with large amounts of starting material (16 g of brain from 8 rats).

Unfortunately, the results of the membrane fluidity assays can only be described as inconclusive on account of the repeated failures to reproduce the initial encouraging data (Table 14) using the same conditions. We had hoped to be able to show a correlation between anticonvulsant potency and/or sedation but, even if one accepts the data in Table 14 as valid, it is evident that no such relationship exists.

Results notwithstanding, there remain serious questions about the suitability of the approach used. For example, there is the issue of the very high concentrations of drugs used in the experiment due to the inherently low potency of the analogues in this assay. Second,

there is the issue of the similarity of erythrocyte membranes to those of a neuron. Human erythrocyte membranes have a cholesterol/phospholipid molar ratio of about 0.92 (Cunnane *et al.,* 1989; Baldini *et al.,* 1989; Sumikawa *et al.,* 1993; Tangorra *et al.,* 1991), compared with about 0.54 for murine synaptic plasma membranes (Hitzemann and Johnson, 1983; North and Fleischer, 1983; Koblin *et al.,* 1980). Thus, it is possible that the erythrocytes' membrane fluidity was too high for a weakly membrane-disordering agent such as VPA to exert an appreciable effect, whereas this influence might have been readily apparent in a more structured system such as a synaptic plasma membrane. While it might therefore be inappropriate to consider erythrocyte ghosts as behaving identically to synaptic plasma membranes, this difference in membrane composition would not be expected to significantly influence the relative membrane disordering potencies of the drugs. Finally, one might ask whether a membrane protein could be sufficiently affected by fluidity changes far too subtle to be detected by the present method, especially in light of its high degree of associated error. For example, the standard error of the anisotropy alone in Table 14 is about ± 0.002. This result contrasts sharply with those of Perlman and Goldstein, who reported standard errors for anisotropy *differences* on the order of ± 0.0005 or less using a similar number of samples.

4.3. EFFECT OF 2-ENE VPA ANALOGUES ON GABA LEVELS

The best-proven mechanism for VPA's anticonvulsant effects to date remains its enhancement of brain GABA. Prior to studying the effects of our drugs on GABA metabolism, however, the development of a simple but sensitive GC-MS-based method was required to evaluate how therapeutic doses of these drugs would affect the neurotransmitter pool of GABA located in the nerve terminals. Monitoring drug-induced changes in GABA levels in nerve terminals, rather than whole tissue, has the advantage of reducing interference from metabolic pools of GABA that do not participate in seizure protection and thus provides a better reflection of the pharmacodynamics of the drug (Gale, 1992).

There are numerous reports in the literature describing the analysis of GABA by GC-MS (for example, see Schaaf *et al.*, 1985 and references cited within), the most common of which is based on the derivatization of GABA with pentafluoropropionic anhydride and hexafluoroisopropanol developed by Bertilsson and Costa (1976) to assay for GABA and glutamate in rat cerebellum. Although its sensitivity is good (detection limit about 24 fmol injected GABA derivative: Schaaf et al.), this method employs fairly low molecular mass (*m/z* 204) ions for selective ion monitoring in electron ionization mode that increases the risk of interference from other species. Furthermore, the length of the derivatization procedure, the low stability of the product (Singh and Ashraf, 1988) and the formation of acidic by-products, that may degrade the column's stationary phase, make this approach less than ideal for large numbers of samples.

An alternative derivatization procedure is silylation of GABA to *N,N,O*-tris(trimethylsilyl)-GABA (Cattabeni *et al.*, 1976). This derivatization and associated GC-MS assay overcomes the drawbacks of the Bertilsson and Costa method although the detection limit (60 fmol) is inferior. The silylation protocol has subsequently been modified by the use of the versatile reagent MTBSTFA to afford *N,O*-bis((*tert*-butyl)dimethylsilyl)-GABA (Mawhinney *et al.*, 1986; Kapetanovic *et al.*, 1990, 1993). However, these procedures used either aqueous standards or tissue homogenates and did not fully evaluate the recovery, variability and detection limit.

The reliability of the GC-MS portion of the method was demonstrated by the favourable coefficients of variation both for intra-day and inter-day experiments. Similarly, there was good agreement for the GAD activities between completely separate assays. This was accompanied by a reasonable recovery of internal standard (78%) for the GABA assay. The value for the GAD assay, where the GABA-d_6 is added at the quenching step rather than during resuspension of the synaptosomal pellet, would likely be higher still. Finally, it is worth re-emphasizing the value of this method in a project where many hundreds of samples need to be analyzed in a time-efficient manner.

Using this method, the GABA concentration in a control synaptosomal preparation was found to be 20.0 nmol/mg protein, which compares favourably with published values (Table 15) and, in conjunction with the 19.4 nmol/mg obtained in a separate experiment for 2 h postdose, demonstrates the validity and reliability of the synaptosome preparation protocol and subsequent analysis. An ED50 dose of VPA elicited a significant increase in synaptosomal GABA 15 min postdose, demonstrating that the drug affects GABAergic neurotransmission at a therapeutic dose. Our values for VPA and 2-ene VPA compare favourably with the respective data of 22.1 ± 2.3 nmol/mg and 21.6 ± 2.2 nmol/mg for 30 min postdose reported by Loscher *et al.* (1981) for VPA (1.18 mmol/kg) and 2-ene VPA (1.41 mmol/kg), although the magnitude of our observed increase is significantly smaller (VPA: 11% vs. 33%; 2-ene VPA: 10% vs 30%). The increase in GABA levels was temporally consistent with the onset of seizure protection. As the median time to the first seizure in the PTZ test was also about 15 min, this finding implies that the drug had reached therapeutically effective levels in the brain by this time and had begun to exert its anticonvulsant effect. While this does not prove a causal relationship between the two, it does at least provide better proof than the 30 min postdose data of Loscher *et al.*

In order to investigate the time course of this GABA increase for a few drugs of particular interest, as well as to provide a further check on the reliability of the data, the synaptosomal GABA levels for VPA and compounds **7**-**10** at 30 min following an ED50 dose were also examined (Table 17). The results indicate a sustained elevation of GABA levels consistent with the demonstrated continuing seizure protection at this time point. As a further test of reliability it was also shown that the GABA increase is dose-dependent (Table 18).

On the assumption that drugs **1**-**17** all exert their anticonvulsant effect by an identical GABAergic mechanism, we expected to find that equipotent (i.e. ED50) doses of these compounds produced identical increases in GABA. Although at 15 min postdose both VPA and 2-ene VPA showed significantly elevated GABA concentrations, the overall values were highly variable (20.7 ± 1.9 nmol/mg protein), with many compounds showing no significant change and several others showing a marked decrease. This apparent lack of consistency in

the GABA data could also be explained more simply by the low sample number and the fact that the individual experiments were conducted separately without a simultaneous control. Furthermore, because subtle changes in GABA concentrations were being sought following administration of a drug dose (e.g. ED50) incorporating a very large uncertainty in its biological endpoint, there was no guarantee that a drug would actually convey seizure protection to a given mouse. This situation could have been improved by using higher doses (up to 4.2 mmol/kg for mice are routinely employed in the literature) but at the cost of physiological relevance. Despite the variability, though, the data appears to be sound as demonstrated, albeit indirectly, by the 30 min post-dose GABA assays that are all fully consistent with their 15 min counterparts.

The resultant question of how anticonvulsants expected to exert their effect through promotion of GABAergic transmission can do so while decreasing levels of this neurotransmitter presents a strong, though not definitive, argument against our central assumption. Furthermore, it is valid to ask if GABA is responsible for the anticonvulsant properties of VPA itself, given the diversity of effects on the GABA system produced by such closely-related compounds.

The hypothesis of GABA's dominant role in the anticonvulsant effect of VPA has been questioned occassionally. Perry and Hansen (1978) found no effect on GABA concentrations when VPA was administered orally to rats for 7 d at 315-365 mg/kg/d, although this could be due to their analysis of whole brain homogenate rather than more specific preparations. However, this is not in agreement with other studies using rodent brain homogenate (Simler *et al.,* 1968; Iadarola *et al.,* 1979). In fact, some workers have even found increases in GABA in the CSF (Zimmer *et al.,* 1980; Loscher and Siemes, 1984, 1985) and plasma (Loscher and Schmidt, 1980, 1981) of patients on VPA therapy. Second, it has been reported that VPA exerts clear anticonvulsant effects at i.p. doses that fail to influence GABA levels (Anlezark *et al.,* 1976). Most recently, Wolf and Tscherne (1994) found that 0.56 mM VPA applied *via* a push-pull cannula both outside and inside the pars reticulata of the substantia nigra in rats significantly decreased GABA release into the perfusate.

However, this decrease was judged to be unaccompanied by modified GABAergic transmission as the animals failed to show the circling behavior characteristic of unilateral GABA transmission in the extrapyramidal motor system. The authors thus concluded that the action of VPA was to simultaneously suppress presynaptic nigral GABA release and to synergistically enhance the post-synaptic response to released GABA.

Hackman *et al.* (1981) also failed to observe any enhancement of GABA released from frog lumbar spinal cord slices, as did Farrant and Webster (1989) using a push-pull cannula embedded in the substantia nigra of VPA-dosed rats. A similar conclusion regarding GABA release was also reached by de Boer *et al.* (1982) using rat cortical slices, as well as by Abdul-Ghani *et al.* (1980) who examined superfused sensorimotor cerebral cortex. Unfortunately, the latter study was marred by these workers' inability to detect GABA in the control superfusion fluid and their very low readings even for rats pretreated with the GABA-T inhibitors γ-vinyl GABA and γ-acetylenic GABA that increase GABA levels four- to five-fold at the concentrations used (Schechter *et al.,* 1977). Wolf *et al.* (1988), on the other hand, reported that perfusion of the preoptic area of rats with 0.3-1.4 mM VPA produced a highly significant decrease in GABA release. The magnitude of this effect decreased with increasing VPA concentration until, at 11 mM, enhancement of GABA release was observed. A potentiation of the post-synaptic GABA response leading to decrease of GABA release by negative feedback was suggested.

To further cloud the issue, Biggs *et al.* (1992) recently reported that VPA has a biphasic dose-dependent effect on extracellular GABA levels measured by *in vivo* microdialysis. They found that while 400 mg/kg i.p. VPA doubled basal GABA levels, 200 mg/kg had no effect and 100 mg/kg actually cut the level in half.

Finally, no discussion on VPA and GABA would be complete without examining the role of VPA in treating absence seizures. While VPA is an effective drug for absence seizures both in humans and genetic rodent models, there is good evidence that seizures in the latter are in fact promoted by GABAergic agonists. Vergnes *et al.* (1985) reported that VPA potentiated seizure enhancement by the $GABA_A$ agonist THIP in the genetic Wistar rat model

despite VPA being an effective anticonvulsant alone. Similar observations were made by Smith and Bierkamper (1990) using a different rat model wherein the animals were chronically treated with the cholesterol biosynthesis inhibitor AY-9944. They found that GABA agonists induced seizures, whereas GABA antagonists such as bicuculine and picrotoxin, ethosuximide and the clinical anti-absence benzodiazepines diazepam and clonazepam had protective effects. VPA was an anticonvulsant at 30 mg/kg i.p. but enhanced seizure occurence at 60 and 120 mg/kg. In an attempt to explain this biphasic effect of VPA as well as the behavior of the benzodiazepines, whose fundamental mechanism of action is well-established, the authors proposed the existence of two distinct pools of GABA with different sensitivity to such anticonvulsants: a synaptosomal one concerned with anticonvulsant effects, and a larger pool that promotes seizures. Nevertheless, they did not rule out the possibility that the anticonvulsant mechanism of VPA in absence seizures is distinct from its GABAergic properties.

Concluding that the acids **1-17** could not be considered as a single homogeneous group of GABA-elevating agents, we sought to at least define some distinct trends in the data of Table 17. First, the data as a whole was examined using a correlation matrix (Table 19), but no links to any of the previous physicochemical parameters were revealed. Next, we looked at the individual sub-groups. The 2-pentenoic acids (compounds **2, 5-10**) show a fairly consistent GABA increase although there are some irregularities with compounds **5** and **6**. Interestingly, the highly neurotoxic acid **9** elevated GABA to a greater extent than its benign isomer **10** at both 15 and 30 minutes, but it is doubtful that this difference was truly significant if one compares the absolute GABA concentrations. Thus, an unusually high level of this neurotransmitter is unlikely to be the cause of the effects seen with compound **9** although one cannot dismiss the possibility of the drug's selective action on GABAergic transmission a particular brain region. In fact, Lee (1991) has shown that compound **9** is found in particularly high concentrations in the substantia nigra, which is a major source of GABAergic fibers. Thus, if the observed GABA increase with this drug treatment is confined to this region, the local augmentation might be sufficiently large to produce the noted

neurotoxicity. However, this does not agree with the previously discussed finding of Wolf and Tscherne (1994) that VPA applied directly into the substantia nigra had no apparent effect on behavior.

The other principal sub-group, the cyclic acids **14-17**, shows a noticeable, though possibly coincidental, trend in GABA levels. Specifically, GABA levels dropped evenly from an initial marked increase (compound **14**) to a profound decrease (compound **17**), suggesting that while the drugs may be interacting with the effector site in a structure-dependent manner, the effect could simply be the result of their declining brain concentration. This may also represent influences on a non-neurotransmitter pool of GABA that is not directly involved in seizure protection (Gale, 1992). In fact, such a result is not inconsistent with the literature as Smith and Bierkamper (1990) have shown that VPA itself, which normally targets the neurotransmitter pool, exerts a biphasic effect in a rat absence epilepsy suggestive of both a GABA agonist and antagonist properties. Similarly, one can consider the case of the benzodiazepines diazepam and clonazepam. Although both would presumably act exclusively at the GABA receptor, the former is used solely for status epilepticus and the latter only for absence seizures (Rall and Schleifer, 1990), illustrating the diverse physiological roles of GABA. The difference between these two drug types is further demonstrated in Table 2.

This subdivision of GABA may be regional as well as functional. Although neither Lee (1991) nor Loscher and Nau (1983) found significant localization of VPA or 2-ene VPA in rat brain, Loscher and Vetter (1985) observed widely varying degrees of GABA elevation in a study examining this property in the synaptosomal fraction of 11 different brain regions in rats administered 200 mg/kg i.p. VPA. Even at 5 min postdose, they noted increases from 60-80% in the hippocampus, hypothalamus, and tectum down to essentially none for the striatum, medulla and, oddly enough, substantia nigra. This variation did not appear to be derived from different control levels as these were all quite similar, indicating the existence of functionally distinct (e.g. VPA-sensitive *vs.* -insensitive) pools of GABA.

The influence of 2-ene VPA on whole-tissue GABA levels in various brain regions was markedly different in the study of Weissman *et al.* (1978). Specifically, these workers found a broad range of increases from 10% in the hippocampus to 80% in the substantia nigra. In view of the even distribution of this compound, it can be concluded that the magnitudes of the GABA increases appear to be the result of specificity for certain GABA pools rather than the drug concentration in a particular region.

This heterogeneity of drug-GABA interactions can be used to explain the apparent decrease of the levels of this neurotransmitter obtained in the experiments with acids **5**, **6**, **16** and **17**. First, a change in nerve terminal GABA does not carry any specific implications for the traffic across the synapse (e.g. GABA release), which would be the ideal measure of the influence of VPA and its analogues. Instead, this experiment considered the sum of newly-synthesized GABA, GABA taken up from the synapse and GABA present in non-transmitter pools (Iadarola and Gale, 1981; Wood *et al.,* 1988), in addition to GABA from contaminating glial and neuronal cell body fragments. Drug inhibition of GABA re-uptake, for example, would result in an enhanced trans-synaptic signal that would be recorded as a GABA decrease in our procedure. Alternately, the overall reduction in GABA release might be very real but might be accompanied by increases in specific brain regions that could still discourage seizure activity if appropriately localized, as suggested by the results of Weissman *et al.* (1978). It could even be argued that seizure protection was the result of a suitably located decrease in GABAergic output. Such mechanisms for the action of the aforementioned drugs are of course hypothetical but at least provide an explanation of how a decrease in GABA need not be incompatible with an anticonvulsant effect.

While our GABA measurements alone cannot be used to infer a specific anticonvulsant mechanism for drugs **1-17**, it is at least clear that they do not all function in the same manner. They may all still act *via* GABA but the variable change in the levels of this neurotransmitter implies there is no single unique mode of interaction.

The central assumption of GABA playing the critical role in the expression of an anticonvulsant effect is worth examining: could not other neurotransmitters also be involved?

The scattered studies performed to date on the possible role of such alternate neurotransmitters have been generally negative. Zeise *et al.* (1991) reported that neuronal responses to glutamate were unaffected by VPA *in vitro* but transient depolarizations evoked by *N*-methyl-D-aspartate were suppressed by 0.1-1 mM VPA. Acute VPA also promotes a decrease in glutamate levels in the hippocampus and striatum but an increase in the neocortex and cerebellum (Chapman *et al.,* 1982). Likewise, it is known that VPA decreases the levels of the excitatory amino acid aspartate in rats and mice (Chapman *et al.,* 1982; Sarhan and Seiler, 1979), albeit at high (200-400 mg/kg) doses, but the fact that this change is confined to the non-synaptosomal compartment implies interaction with a metabolic rather than neurotransmitter pool of aspartate. Chronic administration of VPA (100 mg/kg i.p. for 3 weeks) failed to influence the uptake or binding of aspartate or glutamate (Slevin and Ferrara, 1985).

There is evidence for an increase in the inhibitory neurotransmitter glycine (Martin-Gallard *et al.,* 1985; Similae *et al.,* 1979). There have also been clear demonstrations of the enhancing effects of the drug on serotonin and dopamine levels by Horton *et al.* (1977) and others (Hwang and van Woert, 1979; Whitton and Fowler, 1991; Biggs *et al.,* 1991; Zimmer *et al.,* 1980; Nagao *et al.,* 1979). However, this study showed the anticonvulsant effect to be not only temporally uncorrelated with the concentrations of these neurotransmitters but also retained following administration of their respective biosynthesis inhibitors.

However, these studies invariably sought to find a connection between neurotransmitter levels and anticonvulsant rather than sedative activity. While evidence for the influence of these neurotransmitters on the former is weak, there is good support for their contribution to the behavioral effects. For example, Horton *et al.* (1977) found a vague temporal correlation between behavior (as judged by degree of sedation) and brain levels of the serotonin metabolite 5-hydroxyindoleacetic acid. This is particularly interesting in view of the propensity of modest doses of VPA (200 mg/kg), but not 2-ene VPA, to induce wet-dog shakes, that have been repeatedly linked to elevated serotonin concentrations (Pagliusi and Loscher, 1985). The relevance of these results to our data lies in both the clear dissociation of

sedative properties with GABA levels and the exceptional neurotoxicity of the diene **9**, leading to the question of whether these discrepancies might be explained by the drugs' influence on serotonin levels.

Alternately, seizure protection might not be the result of synaptic mechanisms at all, but rather an influence on the kinetics of membrane ion channels. This explanation is appealing on the grounds that such effects have been shown to occur at very low concentrations of VPA and that this is also the mechanism of several other anticonvulsants, namely phenytoin, carbamazepine and ethosuximide. Nevertheless, this idea does not explain the proven increase in GABA (although this might be a purely secondary effect) and encounters some difficulties in explaining how one drug combines the channel-blocking properties of both phenytoin (Na^+) and ethosuximide (Ca^{2+}).

4.4. INTERACTION OF 2-ENE VPA ANALOGUES WITH GLUTAMATE DECARBOXYLASE

Thus, GABA remains the most likely source of the anticonvulsant effect of VPA and 2-ene VPA, although the situation is clearly more ambiguous for some other analogues. The most obvious means of controlling synaptosomal GABA levels is through the activity of its synthesizing enzyme GAD. Unfortunately, the regulation of this enzyme, beyond the apo-/holo-GAD equilibria discussed earlier, is poorly characterized to say the least. For example, there is no evidence for direct and rapid regulation by membrane depolarization or any second messenger systems and no group has yet addressed the question of how VPA would interact with GAD seeing that the V_{max} and K_m values of GAD activity are unaffected *in vitro*. There is an elevation of holoenzyme activity in synaptosomes upon incubation in a high-K^+ medium, but the effect takes at least 20 min to become apparent (Miller and Walters, 1979). Similarly, a Ca^{2+}-dependent enhancement of GAD activity by K^+-induced depolarization of striatal slices was observed by Gold and Roth, 1979) but only after a pre-incubation of at least 10 min in the depolarizing medium.

A solution to this question of control may be the long-known property of GAD to bind irreversibly to membranes in the presence of calcium, albeit at the unphysiological concentration of 1 mM. Nathan *et al.* (1994) have recently demonstrated that GAD occurs in both soluble (SGAD) and membrane (MGAD-I, MGAD-II, MGAD-III) forms in the porcine brain. MGAD contributed 53% of the total brain homogenate GAD activity, with each form representing about one third of this value. Of particular interest was their finding that MGAD-III binds to membranes in a reversible calcium-dependent manner, rising to a plateau at 10 uM calcium, indicating that MGAD-III, unlike MGAD-I and MGAD-II, is a peripheral rather than an integral membrane protein. Nathan *et al.* thus proposed that because GAD is apparently activated by dephosphorylation by a Ca^{2+}-dependent phosphatase (Bao *et al.*, 1993) and a subpopulation of GAD is associated with synaptic vesicles (McLaughlin *et al.*, 1975), the Ca^{2+} influx accompanying the arrival of the action potential at the nerve terminal may simultaneously activate the enzyme and favor its association with the delivery site of its product. All this is, of course, highly speculative because the MGAD proteins have not been sequenced and it is uncertain how, or even if, they correspond to the soluble GAD_{65} and GAD_{67} isoforms mentioned above. Nevertheless, a GAD enzyme sensitive to the calcium levels expected to follow depolarization of the nerve terminal membrane clearly offers several potential points of enzyme regulation.

To retain the validity of our initial assumption of a common mechanism, such as the elevation of GABA as a result of GAD activity enhancement being the key to anticonvulsant activity, we would have expected to observe a fixed constant increase for both parameters because the ultimate endpoint (ED50) was also being kept constant. As Tables 17 and 19 show, however, the changes in GAD activity were neither constant nor did they correlate with the changes in GABA levels (r = 0.44), although the two generally coincided in direction if not magnitude. Still, it is worrisome that while most of the significant GABA decreases were accompanied by a decrease in GAD activity, there is no case showing a significant increase in both indices. This indicates that while GABA elevation might be the principal mechanism of the anticonvulsant effect, although this is by no means proven by our data, it is certainly not

achieved exclusively through the enhancement of GAD activity. Rather, it may be the result of the drugs' interacting with one or more additional enzymes or sites as well. One likely target site would be the GABA-degrading enzyme GABA transaminase (Figure 3), which has been shown by Loscher (1993b) to be inhibited by VPA (200 mg/kg i.p.) in synaptosomal preparations from numerous brain regions by up to 25% (substantia nigra). This was in marked contrast to whole tissue samples, that were unchanged compared to the controls, and was taken to prove that VPA was specific for the neuronal, rather than the extraneuronal, form of GABA-T. Thus, it may be that the resultant GABA level is the product of the interactions of the drugs in Table 17 with multiple enzymes, such as GAD and GABA-T. The possibility that the drugs may be selectively acting on particular subspecies of GAD, such as those distinguished by sequence or location, should also be considered. It is conceivable that the measured production of GABA involved in seizure protection is the domain of only a small GAD sub-group whose change in activity cannot be detected when the entire population is assayed. The possibility of selective localization of the drugs themselves is unlikely in the light of the finding of Lee (1991), who found that at the time of peak brain concentration (30 min), a 150 mg/kg i.p. dose of either VPA or 2-ene VPA to rats gave essentially constant (± 10%) levels of the drug in the ten brain regions studied. However, this does not rule out some kind of subcellular selectivity that might target the drugs to a specific pool of GAD in the cell. The potential for such selectivity is shown by the fact that GAD_{65} binds PLP more weakly than GAD_{67}. Evidence favouring such distinct forms of GAD was provided by Phillips and Fowler (1982) who found that homogenates of five different regions of rat brain had very similar GAD activities but varied greatly in their response to 400 mg/kg i.p. VPA. The medulla/pons, cerebellum and midbrain showed an approximately 22% increase in GAD activity but the striatum and cerebral cortex showed no significant change.

How might the drugs be interacting with the enzyme? Do the most active compounds share any common structural features that might point to a specific binding site on the enzyme? Or could it be that they do not directly interact with the enzyme at all, but rather exert their influence at some point further upstream in the pathway that translates the initial

action potential signal into an increased synthesis of GABA? These questions are difficult to address for several reasons. First, only a handful of the enzyme activities reach statistical significance presumably because of the modest, but therapeutically relevant, doses used in the experiment. Second, a trustworthy structure-activity relationship can be devised only if either GAD activity or drug concentration is kept fixed, which is clearly not the case in this experiment where the constant parameter was chosen to be anticonvulsant activity. Consequently, the individual changes in GAD activity might be as much the result of variable drug concentrations as due to a particular physicochemical parameter.

Having verified that therapeutic doses of some drugs influenced the activity of GAD, albeit in a variable manner, the project was concluded with investigations of this mechanism. In synaptosomes (Miller and Walters, 1979), depolarization with a high potassium medium required at least 20 min to influence the apo-/holo-GAD ratio, suggesting that a direct membrane effect is probably not responsible. This is unfortunate, as such a mechanism would have the advantage of not requiring the drug to enter the intracellular compartment which, as Lucke *et al.* (1993) have shown, is not a favourable process. A more promising approach would be alteration of PLP binding. Because about half of GAD exists in the apoenzyme form, this would represent an easy means of modifying the activity in either direction.

This idea of VPA analogues interacting directly with GAD receives only mixed support from the literature. While Bolanos and Medina (1993) reported that 1 mM VPA does not affect the kinetics (V_{max} and K_m) of crude or "partially purified" enzyme, Taylor *et al.* (1992) found that even 0.25 mM VPA enhanced the activity of a >90% pure enzyme preparation by 19% through an allosteric site. In either case, this still leaves the possibility of an allosteric mechanism operating *in vivo* whose effects might be partially retained *in vitro*. For example, the drug could inhibit ATP from promoting the conversion of the quinoid intermediate (Figure 4) to apo-GAD, leading to an effective increase in the overall saturation of the enzyme population. Arguing in favor of such a possibility are the fact that this role can also be performed by nucleotides other than ATP, demonstrating a lack of absolute

specificity, and that both VPA and ATP are anionic. Such an interaction would alter the degree of saturation of the enzyme in the pre-assay environment that could persist into the assay. It is important to note that even though the standard assay used in Table 17 allegedly measures the total GAD activity, where the initial degree of saturation should be irrelevant, the combination of enzyme with substrate is by no means instantaneous. For example, Miller *et al.* (1977) showed that the total activity decreased upon modification of the initial brain homogenate preparation conditions to those inhibiting conversion of apo-GAD to holo-GAD, thus producing a lower degree of PLP saturation in the final enzyme assay. Conversely, this suggests that a greater initial degree of saturation would ultimately be manifested as a greater enzyme activity.

The results of the saturation assay suggested that the observed changes in GAD activity elicited by VPA, 2-ene VPA and compound **17** cannot be explained by alterations in the binding of endogenous PLP. First, we found that the GAD activities in the control and VPA- and 2-ene VPA-treated frozen synaptosomes were in good agreement with our previously obtained values (Tables 16-17), although the same could not be said for those obtained from mice dosed with compound **17**. This raises the question of whether or not the drug-enzyme-PLP interactions in this study were truly comparable to those observed in the standard GAD assay (Table 17).

The values for the saturation of GAD with PLP for the drug-treated samples were not significantly different from the control, possibly on account of the low value of n (Table 22). The obvious solution to this problem would have been to use more samples but this would have resulted in highly variable processing times for individual samples. Furthermore, the experiment is very laborious so repetition did not seem worthwhile in light of actual magnitude of the change in saturation, if any.

The numbers calculated for the ratio of holoenzyme to total GAD compare well with those obtained by Miller *et al.* (1977), who found 43 ± 3% saturation (total GAD: 7.3 ± 0.5 nmol/mg/5 min; holo-GAD: 3.2 ± 0.4 nmol/mg/5 min) for whole brain GAD from rats decapitated into liquid nitrogen. As our numbers indicate, there was no apparent effect on

PLP binding *in vivo* by VPA, but there was a suggestion of decreased PLP binding by 2-ene VPA and compound **17**. Interestingly, both total and holoenzyme activities were also decreased relative to the control for the latter two compounds, which is intriguing in view of their previously observed inhibition of intact synaptosomal GAD activity and would appear to support our initial hypothesis of a mechanism involving altered PLP binding.

At this point it was evident that our initial assumption of a common mechanism of action of drugs **1-17** was likely false. Consequently, even though VPA had been shown to have no effect on GAD activity *in vitro*, this no longer implied that other drugs would act identically and thus rationalized a study of the effects of compound **17** on the GAD activity *in vitro*. This compound was chosen on account of its high anticonvulsant activity despite profound depression of GABA levels and GAD activity. Such behavior was not only paradoxical for an antiepileptic drug expected to promote GABAergic activity, but was suggestive of a direct inhibition of the enzyme. Thus, we sought to ascertain whether or not there was an effect on V_{max} or K_m. Accordingly, a double-reciprocal plot (Figure 54) was constructed using the standard saturating level of co-factor, variable glutamate concentrations, a short reaction time (in order to estimate the initial rate) and two concentrations of compound **17** (1 and 5 mM). The slopes yield a mean K_m for glutamate of about 2.1 mM, that is not inconsistent with the approximately 1 mM cited by Martin and Rimvall (1993) and demonstrates the GAD enzyme in this assay to be showing expected behavior. The graph indicates a concentration-dependent inhibition of GABA production by compound **17** that was noticeable even at 1 mM. This inhibition was likely non-competitive based on the convergence of the three lines at the *x*-axis rather than the *y*-axis. The two K_i values obtained (Table 20) are in as good agreement with each other as might be expected with the minimal data obtained. Seeing that the mean value of about 9 mM is well above the effective brain concentration found following an ED50 dose for this compound (94 nmol/g wet tissue, e.g. about 0.094 mM), one might question the relevance of this finding to the *ex vivo* results. These numbers could be reconciled by a selectivity of compound **17** for certain brain regions or specific sub-populations of GAD. The former idea does not find much support in

previously mentioned studies showing an essentially homogeneous distribution of VPA and 2-ene VPA throughout the brain. However, while the 94 nmol/g refers to the concentration of the drug across all compartments (e.g. intracellular, extracellular and membrane), Lucke *et al.* (1993) have shown that VPA and 2-ene VPA do not readily cross membranes into the 80% of brain volume contained in intracellular spaces, a property that may be shared by compound **17**. Consequently, localized extracellular concentrations may be much higher. Furthermore, because the therapeutic effects of the drug likely involve only minor inhibition of this important enzyme, rather than the 50% produced by the K_i concentration, it is evident that the concentrations required for effective inhibition and those obtained following a normal dose may in fact be quite similar.

The alternative explanation invoking enzyme specificity is compatible with reports that VPA does not affect GABA levels (Loscher and Vetter, 1985) or GAD activities (Phillips and Fowler, 1982) equally in all brain regions. Furthermore, it is now clear that GAD is not a universally cytosolic enzyme with little functional diversity between the individual isoforms, as the reports of Nathan *et al.* (1994) and Christgau *et al.* (1992) have emphasized. Thus, it might be that compound **17** selectively inhibits a GAD subspecies involved with a convulsant, rather than anticonvulsant, GABA pool as has been postulated for the seizure-promoting properties of GABA agonists in rat models of absence epilepsy (Vergnes *et al.,* 1985).

A most interesting feature of Figure 54 is its implication of a non-competitive mode of GAD inhibition. Combined with the apparent, though not statistically significant, decrease in the enzyme's saturation with PLP, this provides indirect evidence for a mechanism by which binding of co-factor to enzyme is inhibited. This might take the form of competition for the PLP site or compound **17** acting as an inverse agonist at the ATP site. The finding is noteworthy in light of the reported interaction of VPA and various (*R,S*)-3-alkylglutamic acids at an allosteric site of the enzyme leading to increased V_{max} without affecting K_m of glutamate (Taylor *et al.,* 1992).

5. CONCLUSIONS

The structure-activity results are consistent with previous reports of increasing anticonvulsant potency with increasing molecular size/lipophilicity for saturated aliphatic carboxylic acids. High degrees of correlation were obtained between activity (ED50) and either volume or log P, with the introduction of further parameters failing to significantly improve the correlation parameters. As it was not possible to find shape parameters independent of volume/log P that also showed a linear relationship with activity, we were unable to determine whether or not a distinct receptor site is involved. However, the fact that the correlation with volume/log P was largely undiminished when ED50 was replaced with the actual brain concentration following an ED50 dose strongly argues against this receptor site possessing distinct stereochemical requirements. If the drugs therefore interact with this site in a shape-independent manner, we might tentatively identify the site as the plasma membrane and the mode of action as one analogous to that of the volatile anesthetics. We thus sought to find a correlation between a drug's potency and its ability to alter plasma membrane fluidity as measured by fluorescence polarization spectroscopy, but were unable to obtain reproducible results.

One analogue, cyclooctylideneacetic acid (compound **17**), was significantly more potent than the lead compound VPA while exhibiting only moderate sedative effects. This potency difference was even more pronounced when actual brain levels were compared, where compound **17** was found to be more than six times as potent as VPA. However, VPA retained a high affinity for brain tissue as indicated by the brain/body distribution ratio Q, suggesting that the key to further increase anticonvulsant potency is enhancement of drug delivery into the brain as well as increased intrinsic potency. This was emphasized by the difficulty in preparing aqueous solutions of compound **17**, even as its Na^+ salt, indicating that a further increase in drug lipophilicity would likely come at the expense of sufficient aqueous solubility.

With regard to the means by which the anticonvulsant effect is exerted, there was only mixed support for a link to increased levels of GABA following therapeutic doses. As expected, VPA and 2-ene VPA showed significant increases in synaptosomal GABA but the most potent analogue (compound **17**), amongst others, showed a prominent decrease. This suggests either participation by other neurotransmitters or the drugs' selectivity for synaptosomal GABA in specific pools dedicated to various types of neurotransmitter actions. Such pools might be distinguished by their regional distribution or functions. GABA levels failed to show a correlation with sedative effects, indicating that their cause must be sought elsewhere. Attempts to explain sedation as the outcome of altered cell membrane fluidity were unsuccessful for reasons mentioned earlier.

There were similar inconsistencies in the effects of the drugs on synaptosomal GAD activity, ranging from inhibition to activation and with many showing no significant effect at all. Enzyme activity failed to correlate significantly with either GABA level or any physicochemical parameter, although a positive correlation coefficient was at least obtained in the former case. Again, these discrepancies might be accounted for by regional and/or functional pools of GAD, a subgroup of which might promote seizure protection but whose change in activity might be masked by the overall value from the nerve terminals as a whole.

There was weak evidence that the drugs interacted with GAD by influencing pyridoxal 5'-phosphate binding. Administration of ED50 doses of VPA, 2-ene VPA and compound **17** failed to give significant changes in the extent of PLP binding, although there were slight decreases for 2-ene VPA and compound **17**.

In vitro studies showed a clear inhibition of GAD with 1 mM of compound **17** and a K_i value of about 9 mM. The non-competitive nature of the kinetics suggests the PLP or ATP binding sites as possible targets of the drug. Further studies would be useful in order to determine the exact region as well as to compare compounds such as acid **17** with the known GAD-activating 3-alkyl-4-aminobutanoic and 3-alkyl-4-glutamic acids (Taylor *et al.,* 1992).

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