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THE SYNTHESIS AND PHARMACOLOGY OF NOVEL EXCITATORY AMINO ACID MIMETICS

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The synthesis and pharmacology of novel excitatory amino acid mimetics

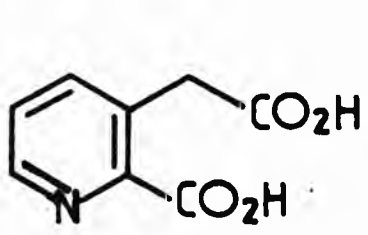
M. Joseph 1984

Glutamic and aspartic acids are considered to be potent excitatory amino acid neurotransmitters of vertebrates. Receptors for these amino acids may be sub-divided into three types: (i) Quisqualate/glutamate, (ii) N-methyl-D-aspartate (NMDA) and (iii) Kainate type. The aim of this project has been to develop specific mimetics which act only at one defined site. In particular, attention has been focused on the two former sub-groups.

It has been shown that 2-amino-5-phosphonopentanoic (APP) and 2-amino-7-phosphonoheptanoic (APH) acids are very potent NMDA antagonists and anticonvulsants and in order to further the pharmacological study of this class of compound, efficient synthetic routes have been developed which afford the compounds in high overall yields. APB(2-amino-4-phosphono-butanoic acid), APP and APH can now be obtained in 10-50 g quantities which has permitted the evaluation of these compounds in a variety of animal models of epilepsy closely related to those in man. A study focused on photically stimulated seizures in the baboon has shown APP and APH to be particularly effective. This model is thought to be one of the closest to human seizures.

Quinolinic acid (Quin), an endogenous excitant and neurotoxin, may be classified as an NMDA type compound. The aim of this project has been to develop analogues of Quin, some of which may well be specific antagonists of NMDA. The higher homologue (1) of Quin has been synthesised and has very recently been shown to be at least twelve times more potent than Quin as a neurotoxin. A series of homologues are to be synthesised, the synthesis of homologue (2) is now being completed. The peptide derivative (3) and its phosphonate analogue (4) have recently been synthesised and experiments are now in progress to determine their potency.

Tricholomic acid (TA)(5), an amino acid extracted from Tricholoma muscarium, has been suggested to be a very potent neuroexcitant of the quisqualate/glutamate sub-division by some Japanese workers. However, studies on this compound and the development of putative mimetics have been hindered by the lack of any reasonable synthetic route to TA. Such a route has now been devised and TA has been synthesised.



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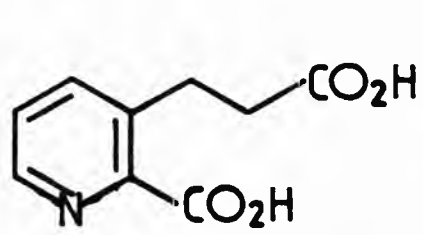

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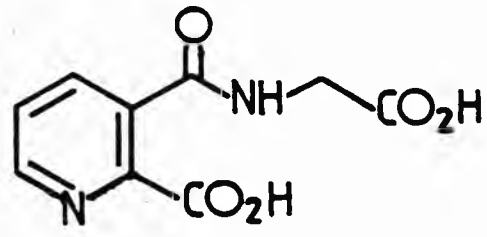

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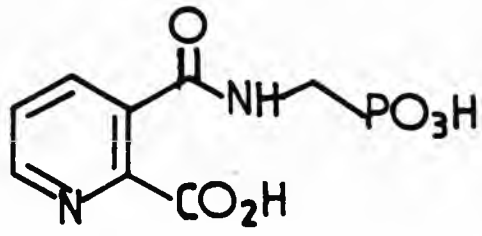

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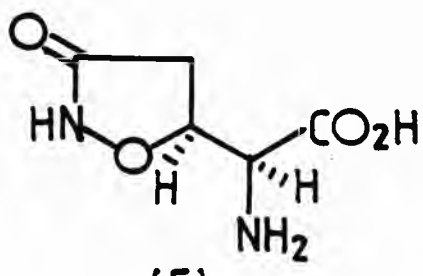

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CONTENTS

CHAPTER ONE

INTRODUCTION

1.1 Introduction

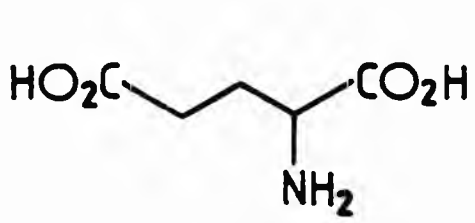
1.1.1 Historical

In the 1960s several criteria were put forward as necessary for the identification of a neurotransmitter[65,149]:

Firstly, the substance must be synthesised and/or stored pre-synaptically in the nerve endings from which it is released. The substance must be released upon presynaptic stimulation and appear in the extracellular fluid in the vicinity. Thirdly, postsynaptic applications of the substance must mimic the action seen when the presynaptic system is stimulated. A mechanism must exist for the removal of the transmitter from the postsynaptic region. Finally, specific antagonists should be able to block both natural transmission and the action of exogenously applied transmitter.

Neurochemical studies aimed at outlining a possible transmitter role for glutamate (1) have been fraught with difficulties because of the multitude of roles this amino acid must perform, e.g. it is incorporated into proteins and peptides; it contributes (along with glutamine (2) to the regulation of ammonia levels and the control of osmotic or anionic balance; it is involved in fatty acid synthesis; it serves as a precursor for γ-aminobutyric acid (GABA) (3) and for various Krebs cycle intermediates and it is a constituent of at least two important cofactors (glutathione and folic acid).

The excitatory effect of glutamate on the cerebral cortex was first reported by Hayashi in 1952[78]. Eight years later Curtis *et al*[43,44] demonstrated the excitation of single neurones in the Central Nervous System (CNS) by L-glutamate (1), L-aspartate (4) and structurally related acidic amino acids.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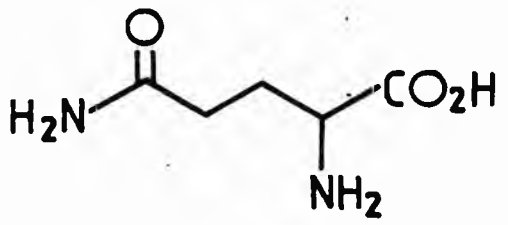
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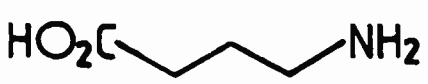
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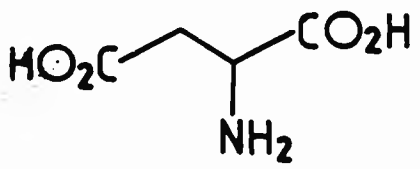
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For the next ten years or so much of the evidence to support a transmitter role for glutamate and aspartate came mainly from iontophoretic studies[90]. One of the main difficulties has been the lack of specific antagonists which could be used to compare the actions of excitatory amino acids with those of a natural transmitter released synaptically at discrete populations of neurones.

1.1.2 Blood-brain barrier and general entry of amino acids into the brain

The blood-brain barrier (BBB) is formed by the brain capillary endothelial cells being fused together by tight functions, which convert the brain capillary wall into an epithelial barrier. The BBB segregates the cerebral and systematic extracellular fluids and is effectively a plasma membrane for the entire brain; due to the presence of the BBB, circulating compounds entering the brain are either lipid soluble, such as drugs, or are water soluble, such as metabolic substrates[133]. Transport systems for neutral and basic amino acids have been known for some time[132], but only more recently for acidic amino acids[134]. Systemic administration of glutamate fails to raise brain levels of this amino acid[81]. Carrier systems for amino acids are bidirectional and the net influx for neutral and basic amino acids, is approximately zero[56], with the

exception of leucine and isoleucine, which have positive net uptakes of up to 40% of the rate of influx. The rate of glutamate influx into brain is 1.0 nmoles/min/g, the rate of net flux is 5.9 moles/min/g from brain to plasma[56]. Thus the rate of unidirectional efflux of glutamate is seven times greater than the rate of influx. If the glutamate carrier were symmetric, on both sides of the BBB, then the glutamate concentration in the brain interstitial space would have to be greater than 1 mM. Since this seems unlikely, it is probable that the carrier system is asymmetrical, and that the brain actively transports glutamate from the interstitium to the blood against a concentration gradient.

The rate of influx of a substrate into the brain is proportional to the rate of cerebral blood flow as long as the BBB permeability constant (PS in ml/min/g) is within an order of magnitude of the rate of flow (F in ml/min/g). The relationship between PS and F is given by the fractional extraction (E) of the unidirectional influx of the substrate into brain. When E is greater than about 15%, the influx changes with flow[18]; the E value for glutamate or aspartate is never generally higher than 2 or 3%[132], therefore cerebral blood flow does not normally influence the brain uptake of glutamate or aspartate. However, there are several circumventricular organs (CVOs) of the brain which lack a BBB, where the regional E value for glutamate may exceed 15%. Any condition, such as a seizure, which is induced by a high glutamate level, increasing F, may accelerate the uptake of glutamate into the CVOs.

The rate of brain uptake of amino acid is proportional to the plasma concentration as long as the Km (half-saturation constant) of BBB amino acid transport is greater than or equal to the plasma level. Postprandial plasma amino acid levels are a function of dietary amino acid composition and transport across splanchnic (gut and liver)

barriers. Physiological doses of glutamate and aspartate are metabolised to alanine via transamination by gut epithelial cells[151]. Larger, pharmacological doses exceed the capacity of the gut transamination sites and the acidic amino acids enter the portal circulation; from the portal system they must clear the hepatocyte bed before entering the systemic circulation. In the case of neutral amino acids a specific high capacity and low affinity uptake system operates[143]; thus the liver is rarely, if ever, saturated by neutral amino acids.

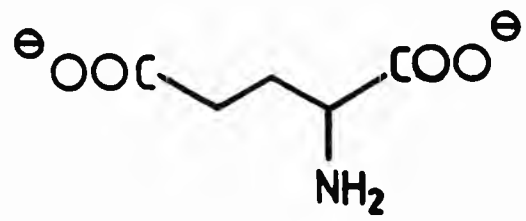
The Km of aspartate and glutamate transport is 1.9 mM and 2.7 mM, respectively. Normal portal plasma level of glutamate is 0.3 mM[151] and thus no saturation occurs; however, levels may reach 2.5 mM following oral doses of 2 g/kg of glutamate and the system may well become saturated. Since the capacity of the splanchnic barriers to clear high doses of glutamate is limited, the major factor protecting the brain from toxic levels of glutamate is the BBB.

1.1.3 Metabolism of Glutamate

Glutamate's intracellular concentration could be as high as 20 mM in view of its low concentration in blood and cerebral spinal fluid (CSF), and therefore presumably in the extracellular fluid[114,162]. It readily replaces glucose as an energy source, *in vitro*, and, though less effective, *in vivo*, due to limitations on entry (see 1.1.2), systemically-administered glutamate enhances recovery from insulin-induced coma. Endogenous glutamate may be rapidly and efficiently utilised for energy production when glucose supplies are limited[114,161].

Under normal conditions, *in vivo*, there is a rapid conversion of glucose to glutamate; within minutes of systemic administration of labelled glucose (intraperitoneally or subcutaneously), over 80% of the carbon is found in the non-essential amino acid fraction in which

glutamate predominates[156,126]. Comparison of the specific radioactivities of glutamate and of α-ketoglutarate (5) showed that the labelling of the glutamate had occurred via the normal reactions of transamination and dehydrogenation.



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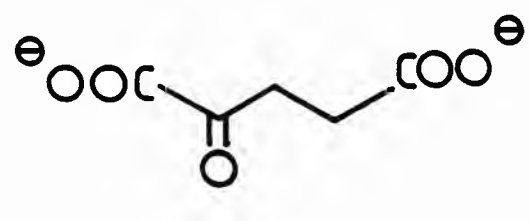

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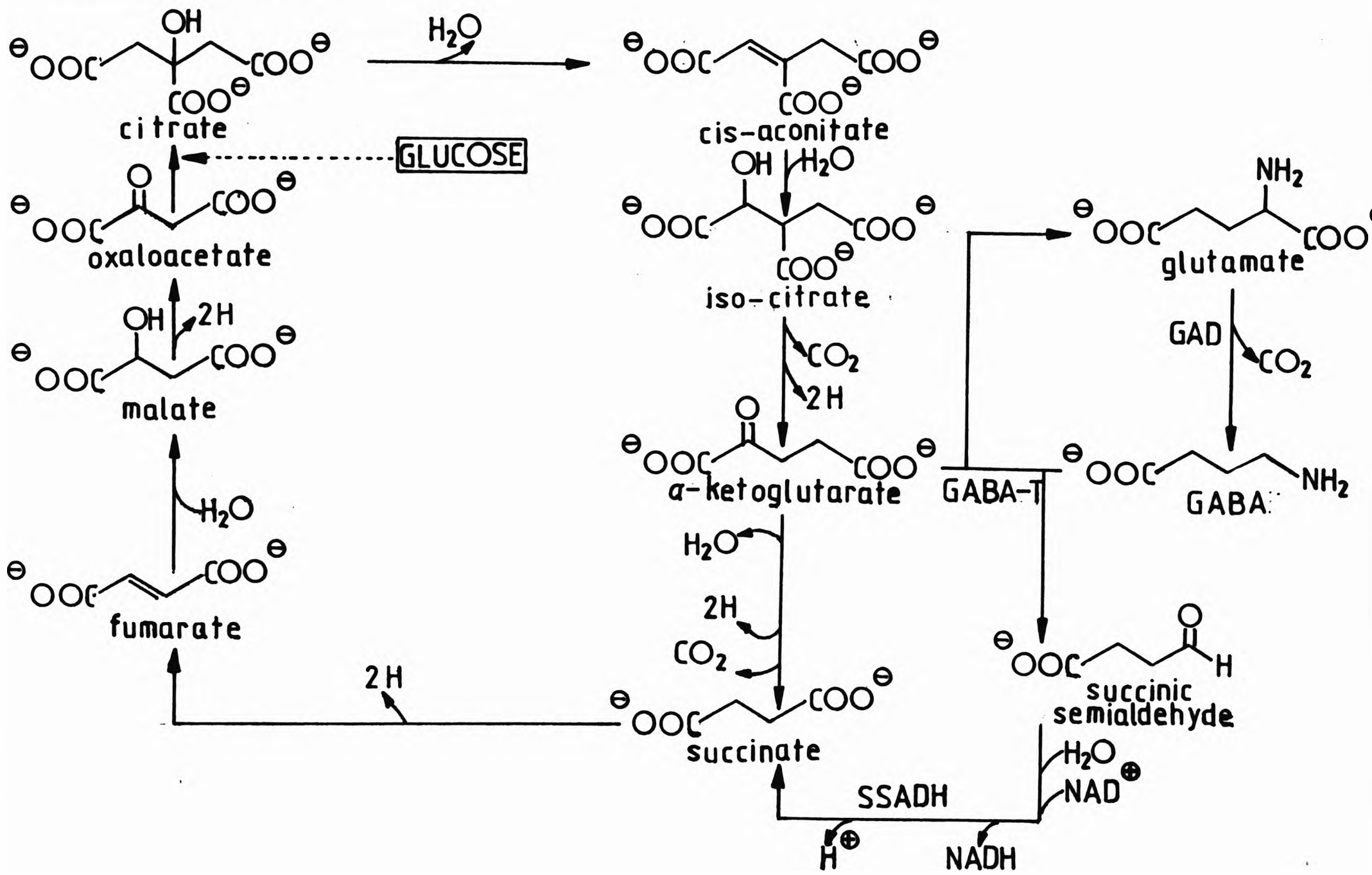
Glutamate also plays a key role in ammonia removal; in the absence of a fully functional urea cycle in the brain[114], major routes of ammonia detoxication involve glutamate formation, through amination of α-ketoglutarate via glutamate dehydrogenase[7], and glutamine formation via the glutamine synthetase reaction, both of which utilise the ammonium ion directly[19]. Glutamate shows both high and low affinity uptake to synaptosomes and glial cells (Km = 10 μM and 300-700 μM, respectively), whereas glutamine transport seems to be solely low affinity (Km = 250-600 μM). While no significant differences are observed for the transport of these two amino acids into nerve endings or glial cells, relevant differences in the activities of related enzymes have emerged. Cerebral synaptosomes and separated cerebellar Purkinje neurons, appear to be very enriched in glutaminase but low in glutamine synthetase whereas the reverse occurs in glial cells: these showed high synthesising ability but were almost devoid of glutaminase[71,72,73]. These observations fit in well with earlier studies on compartmentation of glutamate and glutamine metabolism[8,9,177].

Thus, two separate metabolic pools or compartments exist: one (presumably neuronal) where glutamate is preferentially fed into the Krebs cycle and the GABA shunt (Scheme 1), the other (presumably glial),

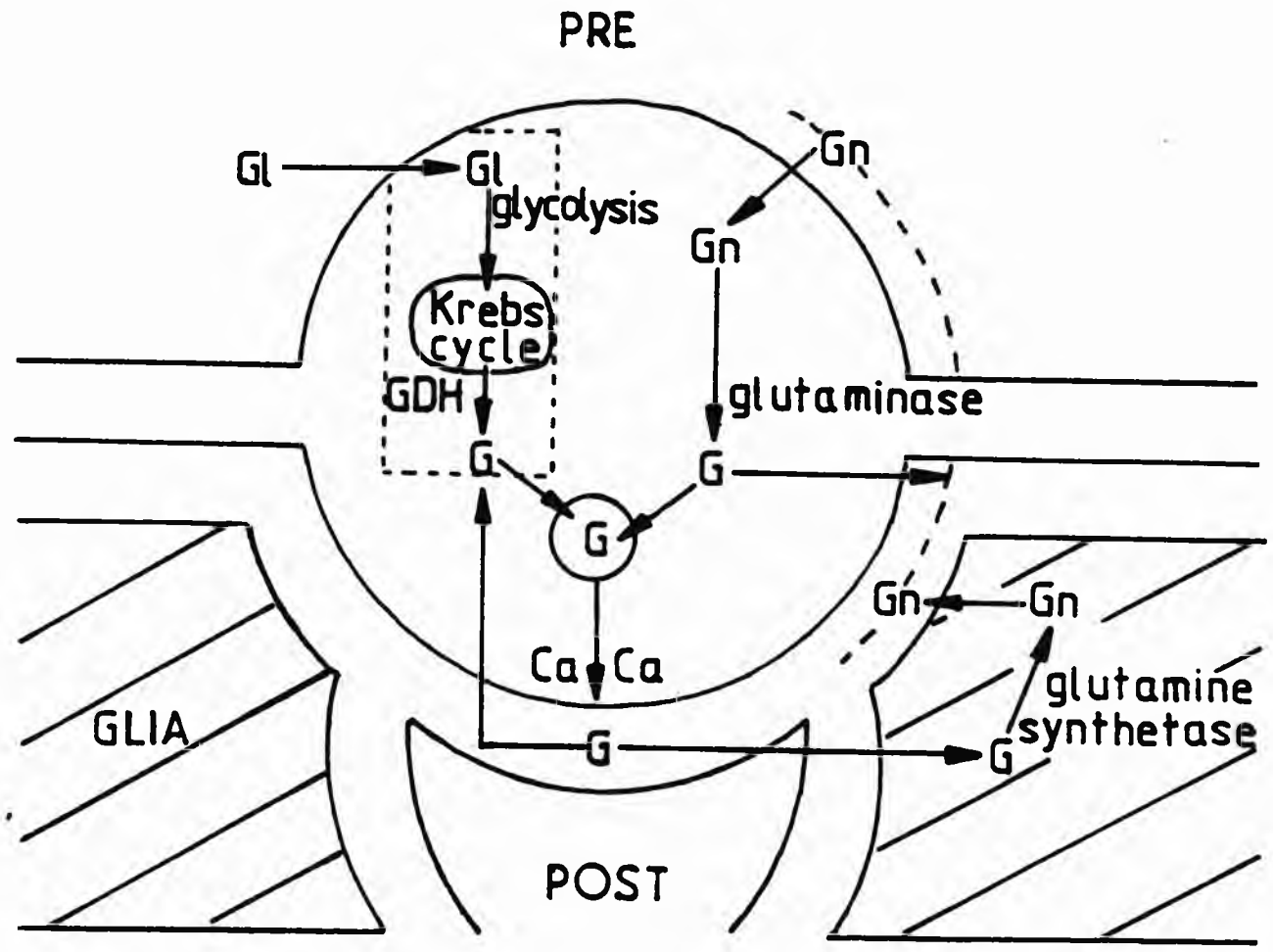
Scheme 1 Krebs cycle and GABA shunt



where glutamate is preferentially converted to glutamate (Fig. 1) 9,160,174.

The synthesis of glutamate derived from glucose or glutamine increases when the nerve terminals release glutamate in response to depolarisation and Ca influx. Glutamate release from this compartment is reduced by a unilateral entorhinal lesion so that it appears related to perforant path efferents to the dentate gyrus. Nearly all of the releasable glutamate is newly synthesised (70% is derived from glutamine and the rest from glucose)[31].

Fig. 1 Metabolic relationships at a glutamatergic bouton



Depolarisation and Ca influx triggers a substantial glutamate release which lowers the pool of glutamate. This relieves presynaptic glutaminase from end product inhibition and results in an activation of the enzyme. Ca dependent release of glutamate also stimulates glutamine influx from the extracellular space. The glutamate released is recaptured from the presynaptic ending via

high affinity transport systems and made available again for release. In addition glutamate probably enters glial cells where it is converted into glutamine which is released into the extracellular space and made available to the boutons to serve again as a glutamate precursor[73].

1.1.4 Action of L-Glutamate

Glutamatergic fibres may constitute the major central afferent and descending pathways in the mammalian CNS. Iontophoretic application of L-glutamate causes a fast onset excitation of most neurones tested in the CNS[39]. Dorsal root ganglion cell bodies, fibres and glia cells are insensitive to glutamate. However, afferent terminals in the spinal cord of cat seem to be depolarised by excitatory amino acids[42]. Very little data have been recorded where L-glutamate or agonists caused inhibitory actions. During the application the depolarisation reaches a plateau (dose-dependent) where the neuronal excitability is increased. Due to the decrease in the input resistance, associated with this depolarisation, excitatory and inhibitory postsynaptic transients are reduced in amplitude. Spike initiation which increases at lower levels of depolarisation decreases when higher concentrations of glutamate are applied which are sufficient to induce conductance changes detectable with electrodes lodged in the soma of the neuron[192]. This depolarisation block is presumably due to an inactivation of the voltage-dependent sodium channels. Small phoretic currents applied to dendritic regions of spinal neurons caused rapid onset depolarisations suggesting a dendritic localisation of glutamate receptors.

Recent studies suggest that the L-glutamate-operated ionophore in the mammalian CNS regulates the permeability of the postsynaptic membrane preferentially for Na^+ ions (and K^+ ions). Tetrodotoxin

does not block the depolarising response of L-glutamate, indicating that glutamate does not interfere with the voltage-sensitive Na^+ channel that is blocked by tetrodotoxin[40,191]. Additional Ca^{2+} influx triggered by this depolarisation might activate a K^+ conductance and could be involved in the hyperpolarisations observed after L-glutamate application. However, there are complicating factors to be considered, such as, ion movements, linked with amino acid uptake and alterations of intra- and extracellular ion concentrations induced by prolonged activation of receptors[79] and the possibility of an electrical component contained in some excitatory postsynaptic potentials (EPSPs)[185].

Most depolarising responses to L-glutamate in the vertebrate CNS do not desensitise. After termination of application there is a rapid repolarisation which usually exceeds the control values for some 5-10 mV and lasts for several minutes. The decay rate of the amino acid action is most likely not regulated by mere diffusion[167]. The other contributing factors may be low and high affinity, Na^+-dependent active uptake; activation of pump mechanisms; activation of K^+ conductance following Ca^{2+} influx; desensitisation; actions as neighbouring (inhibitory) neurones and peripheral receptors which mediate inhibitory responses.

L-glutamate is a flexible molecule existing in aqueous solution in a variety of low-energy conformations, ranging from partially folded to fully extended states[70]. There is evidence that different L-glutamate receptors as found in invertebrates, also exist in neurones of the mammalian CNS and that these receptors are not homogeneously distributed over the surface of the neurone.

Thus, the use of various analogues, especially conformationally restricted analogues e.g. kainic acid, can yield information as to the distribution of these receptor types, whereas the natural ligand

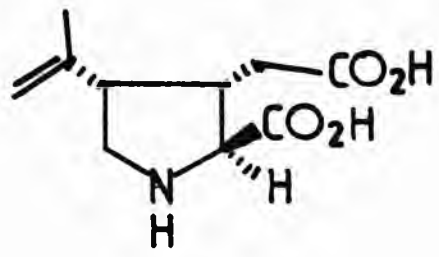
would be expected to interact equally well with the different sub-classes of receptors.

1.2 Elucidation of receptors for excitatory amino acids

To date the various approaches to the identification of the receptor or receptors involved in synaptic excitation involve four methods: electrophysiological studies, binding studies and the determination of neurotoxic and convulsant activity. (Convulsant action is discussed in 1.3 and 2.3.)

1.2.1 Electrophysiological studies

Recent electrophysiological studies[52,58,117,184] indicate the existence of three groups of receptors for excitatory amino acids in the vertebrate CNS based on the effects of a range of selective antagonists which block the actions of some excitants but not others[11,15,60,62,68,116]. The actions of these antagonists suggest that the excitants N-methyl-D-aspartic (NMDA) (6), kainic (7) and quisqualic (8) acids preferentially act as separate receptors.



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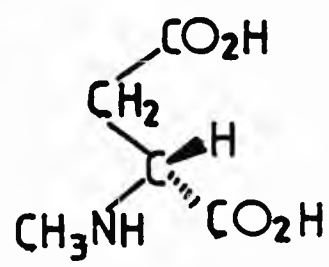

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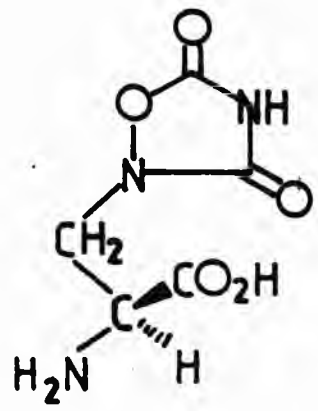

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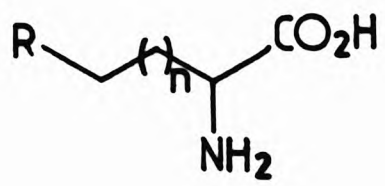
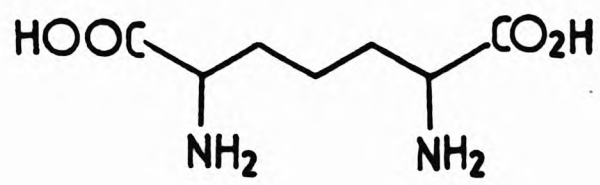
Several agents exist which are able to antagonise responses to NMDA in the absence of marked depressant effects on quisqualate or kainate-induced responses[183] (Table 1): 2-amino-5-phosphonopentanoic acid (APP) (11) has marked depressant action on NMDA-induced responses in low micromolar concentration while 0.5 - 1 mM has little or no effect on quisqualate or kainate-induced responses. APP has no action on non-amino acid receptors[50].

A group of compounds, D-α-aminoadipic (D-αAA) (14), D-α-amino-pimelic (D-αAP) (15), D-α-aminosuberic (D-αAS) (16) and D,D-α-ε-dia-

minopimelic (D,D-α,εDAP) (17) acids[52,117], are slightly less potent and less specific than APP, in that depressant actions on kainate and quisqualate-induced responses are sometimes observed at higher concentrations[104]. However, they are highly selective for amino acid as against non-amino acid receptors[52,58].



R = COOH n = 2 (14)
n = 3 (15)
n = 4 (16)
R = PO_3H n = 2 (11)



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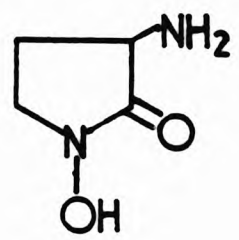
3-Amino-1-hydroxypyrrolid-2-one (HA-966) (18) is reported to be an antagonist of NMDA[182].



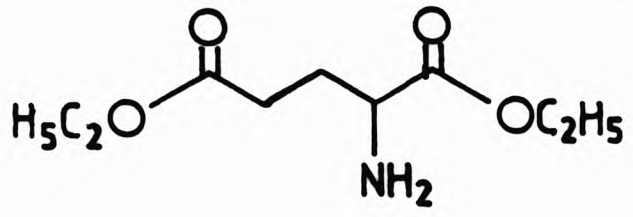
(18)

HA-966 has no action on muscarinic cholinoceptors or on noradrenalin or substance P receptors[62] but it does depress acetylcholine-induced excitation of cat Renshaw cells[10].

Divalent cations (Mg^{2+} [33], Co^{2+}, Mn^{2+} and Ni^{2+}) produce a similar pattern of amino acid antagonism but the mechanism of this action is thought to involve different sites in the NMDA receptor-ionophore complex[61].

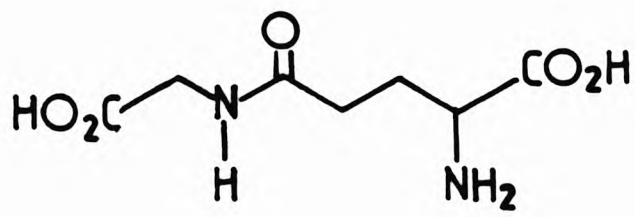
The ability of L-glutamic acid diethyl ester (GDEE) (19) to depress quisqualate - but not kainate-induced responses in the cat CNS, <u>in vivo</u>[52,117], suggests that kainate and quisqualate act

predominantly on separate receptors from each other. However, in vitro,[49] GDEE does not have this action. Also, GDEE is not specific for excitatory amino acid receptors, blocking cholinoceptors on Renshaw cells[52,117].



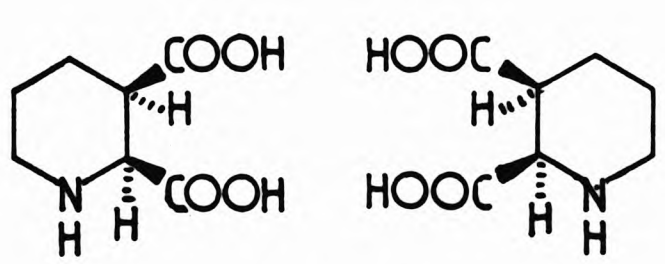
(19)

The dipeptide, γ-D-glutamyl-glycine (γ-DGG) (20), antagonises excitation due to kainic acid or to NMDA but not that due to quisqualic acid[41].



(20)

A relatively pure population of 'kainate' receptors exists in fibres within the dorsal root of the neonatal rat[49]; they are depolarising, non-synaptic and insensitive to NMDA while quisqualate is a relatively weak agonist compared to kainate. The most effective antagonist at these fibre receptors is cis-2,3-piperidine dicarboxylic acid (cis-2,3-PDA) (21)[183].



(21)

cis forms

The existence of discrete quisqualate receptors has been inferred from the actions of GDEE (19) and γ-DGG (20)[52,117,49]. The

most potent known antagonist for quisqualate-induced responses is cis-2,3-PDA, but since this compound also blocks NMDA and kainate receptors, and also has partial NMDA agonist activity[53,183], cis-2,3-PDA should be used in conjunction with NMDA and kainate antagonists in order to draw reliable conclusions from its actions.

TABLE 1 Apparent dissociation constants for NMDA receptor-antagonist complex [183]

Antagonist	App. K_D μM
(±)-2-Amino-5-phosphonopentanate	2.4
D-α-Aminosuberate (DαAS)	15.9
γ-D-Glutamylglycine (γDGG)	21.4
β-D-Aspartyl-β-alanine	21.7
D-α-Aminoadipate (DαAA)	42.4
(±)-cis-2,3-Piperidine dicarboxylate (PDA)	53.8
(±)-cis-2,3-Piperazine dicarboxylate	104
(±)-α,ε-Diaminopimelate (α,ε-DAP)	120
(±)-Mono-N-acetyl-α,ε-DAP	120
γ-L-Glutamylglycine	147
(±)-2-amino-5-phosphonobutyrate (2APB)	>200
L-Glutamic acid diethyl ester (GDEE)	>1000

1.2.2 Binding studies

Binding studies are now accepted as a standard procedure for the, in vitro, study of the interaction of neurotransmitters, drugs or hormones with their receptors. However, a number of criteria need to be satisfied before any observed binding can be ascribed a physiological significance: binding must be specific, of high affinity and exhibit saturability, consistent with a finite number of receptor

sites; the binding should be readily reversible with a time course consistent with the rate of termination of the physiological action, and the subcellular distribution of binding sites should be compatible with that for neurotransmitter receptors, i.e. a preferential localisation in the synaptic membrane fraction.

The most important criterion for receptor identification is pharmacological specificity. Thus, drugs which from 'indirect' studies have been found to mimic or to antagonise the effects of the natural ligands, should also be effective displacers of the binding of the ligand.

The early proposals of Johnson[91] stated that at least four populations of excitant amino acid agonist binding sites are associated with rat brain membrane.

L-glutamic acid preferring (extended) binding sites[33] which show a preference for kainic acid over L-glutamic acid and interact poorly with L-aspartic acid, D-aspartic acid and NMDA; these sites are studied using kainic acid as the binding ligand. L-glutamic acid preferring (partially folded) binding sites which prefer L-glutamic acid to either kainic acid or L-aspartic acid; these are the bulk of the sites studied using L-glutamic acid as the binding ligand.

L-aspartic acid preferring binding sites[33] show a strong preference for L-aspartic acid over L-glutamic acid and show little interaction with kainic acid or D-aspartic acid; these sites are studied using L-aspartic acid as the binding ligand.

NMDA interacts very strongly and kainic acid interacts only poorly with the fourth class of binding site, L-glutamic acid and L-aspartic acid binding sites; these sites show little selectivity between L-glutamic, or D- or L-aspartic acids and are studied using D-aspartic acid as the binding ligand.

Many <u>in vitro</u> studies have been directed towards the physiological receptor of L-glutamic acid. One approach has been to study

the binding of labelled kainic acid to whole membrane or crude synaptic membrane preparations, where saturable, high affinity sites have been detected.

Simon et al[164] were the first to describe the specific, reversible, high affinity binding of [^{3}H]-kainic acid to rat brain membranes. They defined a binding site with an apparent K_D of 50 nM in the rat forebrain, using a relatively low specific activity ligand (0.4 Ci/mmol). The use of a more highly radioactive [^{3}H]-kainic acid, although the specific activity is still low (2.1 - 5.7 Ci/mmol), has revealed the presence of at least two sites: one with a K_D of approximately 50 nM, similar to that reported by Simon et al[164], and a second higher affinity site with a K_D of approximately 3-5 nM[105]. Other investigators have described either the higher or lower affinity binding site[5,175].

The binding sites for kainic acid appear to have a predominantly neuronal localisation since specific binding could not be demonstrated in non-neuronal tissues such as liver and muscle. The higher and lower affinity sites had an uneven and independent distribution in rat brain with high affinity sites contributing up to 30% of total binding in the frontal cortex and striatum but representing 10% or less of total binding in the cerebellum, brain-stem regions and the retina[105]. At a subcellular level, synaptic functions contribute the majority of the 'synaptic population' of kainic acid binding sites and in the hippocampus these sites are present in high density in close opposition to the mossy fibres - CA3 pyramidal cell synapse. Neither glutamate nor aspartate seem to be transmitters at this synapse[66].

Kainic acid lesion to the striatum, which caused a virtually complete degeneration of striatal intrinsic neurones, resulted in a 50% reduction in both the higher and lower affinity binding sites within the lesioned striatum. In contrast, decortication, which

ablated a major excitatory, presumably glutamatergic, input from the overlying cerebral cortex, did not significantly reduce the specific binding of kainic acid in the underlying striatum[14].

Extensive structural activity studies of the neurophysiological effects of kainic acid, kainic acid derivatives and other conformationally restricted analogues of glutamate have been carried out in the vertebrate CNS[12,158]. The results of these studies can be used to identify critical features of the kainate molecule responsible for binding to and activation of its recognition site; the results of competition studies can be used in the same way (Table 2).

From Table 2, kainic acid (7) itself, was seen to be the most effective inhibitor of specific binding. Dihydrokainic acid (23), which has the double bond in the isopropylene side-chain reduced, and α-allo kainic acid (24), which has the isopropylene side chain in the opposite plane had at least a 400-fold lower affinity for the binding site.

If the conformational rigidity of the kainic acid molecule, in particular the two carboxyl groups, confer its potent action at glutamate receptors, it is surprising that alteration of the isopropylene side chain, which has no homology in the glutamate molecule, so dramatically affects its affinity for the receptor sites.

Both agents are virtually inactive as neuroexcitants and neurotoxins[12,92].

α-Keto-kainic acid (25), which has a keto group in the place of the methylene group, exhibited an affinity approximating that of quisqualic acid.

TABLE 2 Inhibition of the specific binding of [^{3}H] kainic acid in cerebellum

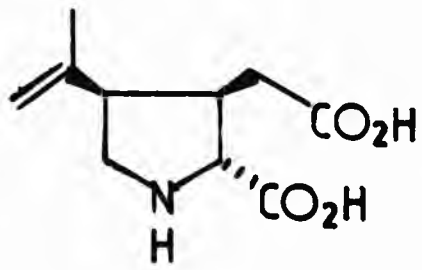
	K_I (μm)	Hill coefficient	Excitation
Kainic acid	0.025	0.98	4+
Alpha-allo kainic acid	>>10.0	-	0
Dihydrokainic acid	59	0.38	±
Alpha-keto-kainic acid	0.60	0.91	?
Quisqualic acid	0.98	0.90	4+
Ibotenic acid	>>10.0	-	3+
Cis-1-amino-1,3-dicarboxy-cyclopentane (ADCP)	>>10.0	-	2+
L-glutamic acid	0.44	0.61	1+
D-glutamic acid	49.0	0.63	1+
D,L-homocysteic acid	>>10.0	-	2+
N-methyl-D,L-aspartic acid	>>10.0	-	3+
2-amino-4-phosphono-butyric acid (APB)	>>10.0	-	I
D-alpha-amino-adipic acid (DαAA)	>>10.0	-	I
l-glutamate diethylester (GDEE)	>>10.0	-	I

Receptor binding values are taken from London and Coyle[105], K_I

K_I indicated as >>10 μM, no inhibition of the specific binding of 50 nM [^{3}H] kainic acid was observed at 10 μm of the drug.

I = reputed inhibitor of glutamate-induced excitation

+ = excitatory action; - = depressant action; 0 = no effect.



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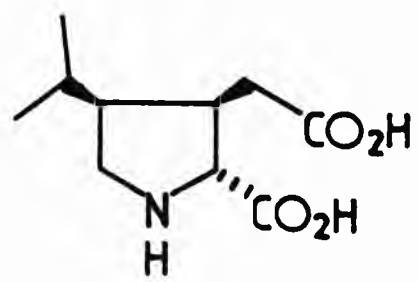

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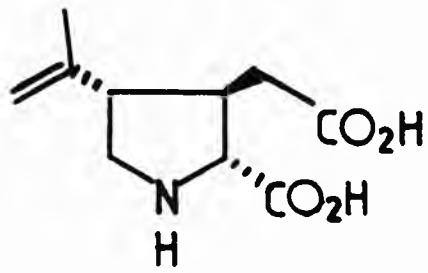

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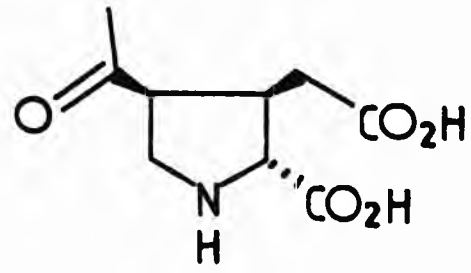


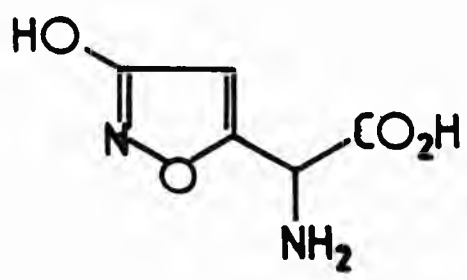
(25)

L-glutamic acid showed a 20-fold lower affinity for the site than kainic acid. D-glutamic acid was 100-fold less effective than L-glutamic acid.

The distance between the carboxyl groups on the compounds also played a major role since the dicarboxylic acids, D- and L-aspartic acids[105] and NMDLA were virtually inactive at the receptor site.

The importance of the dicarboxylic nature of the molecule was further emphasised by the rather poor results of substitution with the electrophilic sulphur or phosphate moiety for the γ-carboxyl group i.e. D,L-homocysteic acid (22) and APB (10), respectively,

Ibotenic acid (26) and cis-1-amino-1,3-dicarboxycyclopentane (ACDP) (27) a conformationally restricted analogue of glutamic acid had very poor affinity for the site.



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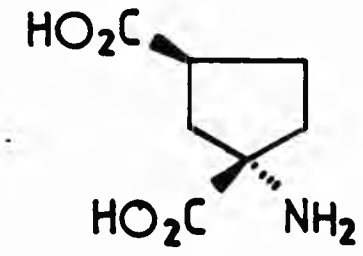

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Reputed glutamate antagonists GDEE (19) and DαAA (14)[52,117] did not exert any inhibition of specific binding at concentrations 200-fold greater than that of the radioligand.

The Hill coefficients of approximately 1.0 for kainic acid, quisqualic acid and α-keto-kainic acid and of approximately 0.5 for dihydrokainic acid, L- and D-glutamic acid, suggested that the double bond in the isopropylene side-chain or a keto group as occurs in α-keto-kainic acid and quisqualic acid served as essential moieties in the binding to the recognition site for [^{3}H]-kainic acid. Reduction or orientation in the opposite plane of the isopropylene side-chain or the absence of a pi-electron group at the appropriate site resulted in compounds which lacked an essential component for interaction with the recognition site for [^{3}H]-kainic acid.

Thus, the recognition site mediating kainate's action on neurones appears to be highly selective and is also unlikely to be a recognition site for the excitatory effects of glutamic acid. A view which is reinforced by the marked stereoselectivity for the L-form of glutamic acid in contrast to the nearly equipotent excitatory efficacy of the D- and L-isomers in the mammalian CNS[12]. Also, although quisqualic acid (8), a potent neuroexcitant[12], exhibits a relatively high affinity for the kainate receptor binding sites, ibotenic acid is nearly inactive as a competitor.

An alternative approach to the elucidation of the L-glutamic acid receptor has been to use labelled glutamic acid itself as the receptor probe.

The binding of [^{3}H]-L-glutamic acid to brain synaptic membranes was first described by Roberts[152]. Further studies indicated a family of sodium independent binding sites for L-glutamic acid with multiple K_Ds of approximately 10,000 and 70 nM[67,15]. (The existence of a 2-amino-4-phosphonobutyric (APB)-sensitive L-glutamic acid binding site has recently been reported[153].)

A neuronal localisation is supported by the observations that peripheral tissues exhibit negligible specific binding of [^{3}H]-L-glutamate and that prior kainate lesion of the striatal intrinsic reduced specific binding in this region by 45%[15]. Cortical regions, including frontal and parietal cortex, and hippocampus have high levels of specific binding, whereas, hypothalamus, medulla-pons and cerebellum have considerably lower levels.

Competitive inhibition studies have provided correlations which suggest that the binding site is a recognition site mediating the excitatory effects of glutamate. There is a significant stereo-selectivity for the L-form of glutamate. L-aspartate exhibits activity at the binding site, whilst, NMDLA, an agonist at aspartate preferring receptors, has a negligible affinity for the site[67].

From Table 3 it is observed that quisqualic acid exhibits a two-fold greater affinity, and cis-1,3-ADCP an eight-fold lower affinity, than L-glutamate. Ibotenic acid, with a Hill coefficient of nearly 1.0, is surprisingly weak (Foster[67] found quisqualic acid and ibotenic acid to be equipotent). DαAA and DαAP exhibited significant activity for the binding site. Neither kainic acid nor dihydrokainic acid inhibited the specific binding of [^{3}H]-L-glutamic acid at concentrations of 0.1 mmol. However, α-keto-kainic acid did show some activity which

indicates that the isopropylene side-chain, with or without a double bond, offers severe steric hindrance that interferes with the binding at the recognition site for [^{3}H]-glutamic acid. However, the isopropylene side-chain is a major determinant for binding to the kainic acid receptor, as was seen above. The keto group of quisqualic acid does not hinder binding to the recognition site for L-glutamic acid and it also partially satisfied the pi-electron requirement of the kainate site. Ibotenic acid and cis-1,3-ADCP were both relatively effective competitors for the [^{3}H]-L-glutamic acid recognition site with neither showing any effective affinity for the [^{3}H] kainic acid site.

Studies of [^{3}H]-L-glutamic acid and [^{3}H]-kainic acid binding have revealed two distinct receptor sites. NMDA has virtually no affinity for either of these sites[15,67,105], but it is a potent neuroexcitant[12] with neurotoxic effects[158], which suggests the presence of at least a third receptor.

The study of L-aspartate binding is not very advanced and so far none of the binding sites identified satisfy the requirements of an aspartate receptor. Using [^{14}C]-aspartate three distinct sites (K_D = 0.2, 10 and 50 μm) have been detected[64]. Recently, Sharif and Roberts[153,163], investigating the binding of [^{3}H]-L-aspartate to cerebellar synaptic membranes, have identified a single binding site with a K_D of 874 nM. Quisqualic acid, a good inhibitor of glutamate binding, was only weakly active on the aspartate system. Kainic acid was devoid of any inhibitory activity, showing the same minimal affinity for the L-aspartate site as it did for the L-glutamate site. Binding was inhibited by DαAA (14), DαAS (16) and HA-996 (18) but not by NMDA. The surprising lack of inhibitory effects of NMDA, a proposed aspartate-preferring agonist[111] and of the D- and L-isomers of homosysteic acid (22), means that many more results will have to be

TABLE 3 Inhibition of the specific binding of [^{3}H]-L-glutamic acid in cerebellum

	K_I (μM)	Hill-efficient	Excitation
Glutamic acid	1.43	0.93	1+
D-glutamic acid	88.2	0.75	1+
Kainic acid	>>100	-	4+
Alpha-keto-kainic acid	74.0	0.60	?
Alpha-allo-kainic acid	>>100	-	0
Dihydrokainic acid	>>100	-	±
Quisqualic acid	0.60	0.74	4+
Ibotenic acid	37.0	0.98	3+
Cis-1-amino-1,3-dicarboxy-cyclopentane	11.6	0.52	2+
D-alpha-amino Adipic acid	2.45	0.58	I
D-alpha-amino pimelic acid	6.60	0.53	I

Cerebellar membranes were incubated with 700 nM [^{3}H]-L-glutamic acid in the presence of various concentrations of the compounds listed above.

K_I indicated as >> 100 μM, no inhibition of the specific binding was observed at this concentration of the drug.

I = reputed inhibitor of glutamate-induced excitation.

presented before one can accept that the binding of [^{3}H]-L-aspartate, observed, <u>in vitro</u>, is in fact to a synaptic receptor.

NMDA exhibits high activity when applied iontophoretically to

spinal neurones[111]. Unfortunately the binding of [^{3}H]-NMDA to cerebellar membranes has not yet been shown.

1.2.3 Neurotoxicity of amino acids

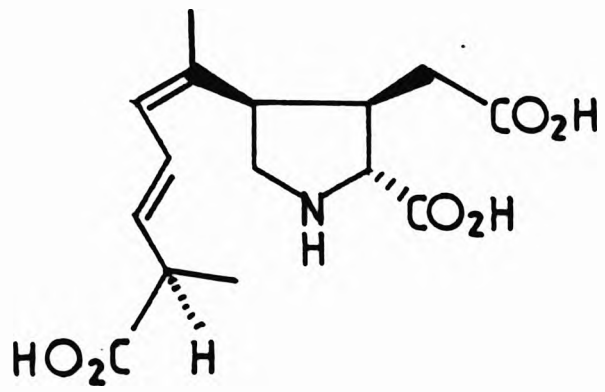
Glutamate has excitatory[41,43,44] and neurotoxic[135,139,141] properties. Circumventricular organs (CVOs) are selectively damaged by systemic administration of glutamate[136,141]. Glutamate lesions cause rapid swelling of neuronal dendrites and cell bodies followed by acute degenerative changes in intracellular organelles and coarse clumping of nuclear chromatin.

Olney[138,137] used the term 'excitotoxic' amino acids to refer to those structural analogues of glutamate which exhibit both neuro-excitatory and neurotoxic activity and have the same order of potency for their excitatory and toxic activities. An excitotoxic hypothesis of neuronal death was proposed[137,139,142] which stated that glutamate, when present in unphysiological concentrations in the vicinity of dendritic or somal surfaces of the neuron, effects a state of continuous depolarisation and sustained increased in plasma membrane permeability, possibly resulting in cell death.

It was established [91,137,138] that when systemically administered to immature rats, excitatory amino acids act as convulsants that vary in potency in direct proportion to their known excitatory and toxic activities. However, more recent studies[6,190] show a poor correlation between excitatory potency and neurotoxic action as well as little correspondence between neurotoxic effects and epileptogenic action in the hippocampal formation.

Compounds interacting with the kainate receptor determined by the specific binding of [^{3}H]-kainic acid[105], exhibited neurotoxic effects in the striatum which correlated reasonably well with their affinities for the receptor site.

Domoic acid (28), reputed to be 2 to 3-fold more potent than kainic acid (7)[12], has a 3-fold higher affinity for the kainate receptor binding site. In the striatum, domoate has a neurotoxic action comparable to that of kainate, making the two compounds the most potent excitotoxins studied.



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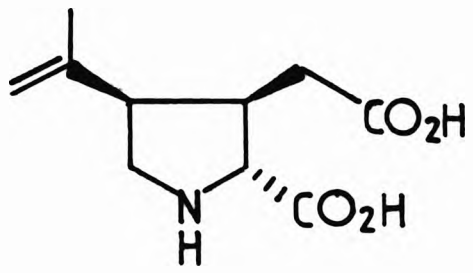


(7)

The neurotoxic activity of α-keto-kainate (25) was in good agreement with its excitatory activity.[36] Schwarcz[158] has shown that dihydrokainic acid (23) which has weak excitatory effects and a 300-fold lower affinity for the kainate receptor, is devoid of neurotoxic activity at doses fifty times greater than the threshold dose of kainate. Unlike dihydrokainic acid, α-allo-kainic acid (24) was neurotoxic although with only 2% of the potency of kainate. The excitatory amino acids thought to act at receptors different from that of kainate (for example, ibotenic acid, cis-1,3-ADCP, quisqualic acid and NMDA) were substantially less potent as neurotoxins.

A more compelling pattern of results emerged when rather than using striatal neuronal markers, intrahippocampal injection was used[190], to study neurotoxic and convulsant properties.

Kainate, domoate and α-allo-kainate caused intermittent episodes of seizures, predominantly of Stage I, totalling 30-40% of the recording

period. The duration of seizure activity for α-keto-kainate was nearly twice that occurring with kainate. The pattern of seizure activity with quisqualate resembled that observed with kainate-like substances; quisqualate has shown some affinity for the kainate receptor[105], NMDA was the most effective convulsant, causing EEG seizures for 95% of the recording period. The NMDA-treated animals exhibited the greatest percentage of time in Stage I and Stage II seizures and experienced the greatest number of generalised tonic-clonic convulsions, which occurred only rarely with kainate. Ibotenic acid and cis-1,3-ADCP were virtually devoid of epileptogenic effects.

Thus, substantial differences in the neurotoxic and epileptogenic effects of the potent excitatory amino acid analogues exist. Kainic acid and its structurally related analogues possessing a double bond in the isopropylene side chain appear to be disproportionately more potent as neurotoxins than is suggested by their neurophysiologically-defined excitatory effects[12]. One notable exception is α-allo-kainic acid which has a low affinity for the kainic receptor site but substantial neurotoxic properties (Table 4).

The receptor that mediates the effects of NMDA has been well characterised by neurophysiological and neuropharmacological techniques[49]. The convulsant and neurotoxic effects of NMDA diverge considerably from those observed with the other agents. NMDA appears to be disproportionately more potent as a convulsant at doses that produce limited lesions. Quisqualic acid, which does not interact at the NMDA site, also possesses weak neurotoxic effects in comparison to its neurophysiologically defined excitatory actions[12] and to its epileptogenic effects in the hippocampal formation. Ibotenate and cis-1,3-ADCP exhibited negligible convulsant effects at doses that produced substantial and relatively uniform lesions. Neurophysiological experiments indicate that ibotenic acid activates an excitatory

TABLE 4 Comparison of the neuroexcitatory, neurotoxic and epileptogenic properties of excitatory amino acid analogues

	Neuro-excitatory [12]	[^{3}H] kainate receptor [36]	Neuro-toxic	Convulsant/ neurotoxic
Domoic acid	2 - 3	3	1	1+
Kainic acid	1	1	1	1+
α-allo-kainic acid	0.01 - 0.1	<<0.005	0.02	1+
α-keto-kainic acid	ND	0.05	0.03	2+
Dihydrokainic acid	0.01	0.001	<<0.02	-
Quisqualic acid	1 - 2	0.03	0.01	3+
Ibotenic acid	0.01 - 0.3	0.01	0.02	±
cis-1,3-ADCP	ND	<<0.001	0.01	±
NMDA	0.03 - 1	<<0.001	0.01	4+

Neurotoxic potencies calculated relative to kainic acid represent the approximate molar ratio producing equivalent lesions in the striatum. Convulsant/neurotoxic ratio represents the severity of cortical seizures produced by intrahippocampal injection of doses that cause roughly comparable lesions in the striatum[190].

receptor as well as an additional receptor that results in hyperpolarisation[131]. The combination of excitatory and inhititory effects of ibotenic acid and cis-1,3-ADCP may account for the negligible epileptogenic effects of these two compounds.

Zaczek's study of the effect of these excitatory amino acids in both the corpus striatum and hippocampal formation indicate that the neurotoxic effects of acidic excitatory amino acids correlate poorly with their neurotoxic effects. So, neuronal excitation or Olney's 'excitotoxic' hypothesis, appear to be insufficient explanation for neuronal degeneration.

Decortication, which causes degeneration of the reputed cortico-striate excitatory glutamate pathway[55], prevents the neurotoxic effects of kainic acid in the striatum[14,113] without altering the excitatory effects[115]. The disparities between the neurotoxic and convulsant properties of these agents would therefore be more likely to be present at the level of specific receptors, which differentially alter neuronal membrane conductance.

1.2.4 Three-point attachment site?

The actions of many of the compounds discussed in this chapter suggest that receptors are not completely rigid templates to which agonist molecules must conform but rather that some degree of flexibility in the chemical structure of the receptors themselves is possible.

Curtis and Watkins[44] put forward a fundamental requirement for the various receptor types: a three-point attachment site involving both of the acidic and the single cationic groups which all of the agonists possess.

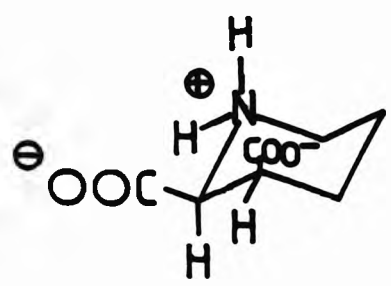
Investigations have been made of the structural features of agonists acting on NMDA receptors in attempts to elucidate the nature of the transmitter postulated to activate synaptic receptors that are sensitive to blockade by NMDA antagonists.

α-Aminomalonate* (30) is an NMDA agonist; the fact that a substance with as short a molecule as this compound is such an agonist (susceptible to blockade by D-α-amino-adipic acid (D-αAA) and Mg^{2+})[182] suggests that if the postulated transmitter is L-glutamate or L-aspartate it probably acts in a 'folded' conformation.

Some idea of this active conformation has been gained by the study of the cyclic aspartate and glutamate analogues, 2,3- and 2,4-piperidine dicarboxylates (PDA), respectively. The trans forms of these

* $H_3\overset{+}{N}CH(COO^-)_2$

substances are both NMDA agonists of quite high potency. Cis-2,3-PDA (21) is an NMDA antagonist with partial agonist activity, while cis-2,4-PDA (32) appears to be relatively inactive either as an agonist or antagonist[48]. Since cis-2,4-PDA is an 'extended' glutamate analogue (with reference to the relative disposition of the two carboxylate groups) and trans-2,4-PDA (33) is a 'folded' glutamate analogue, it seems that glutamate and glutamate analogues might need to possess or be able to assume a 'folded' conformation (carboxyl groups less than maximally separated) in order to interact effectively with NMDA receptors.



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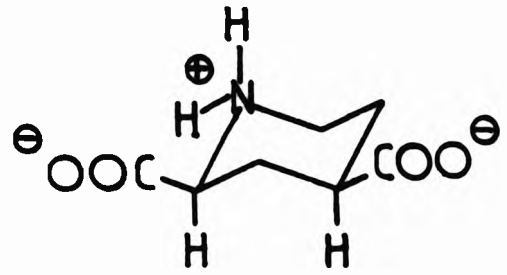

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Trans-2,3-PDA (31) and trans-2,4-PDA (33) could both act on the NMDA receptor by way of a 3-point ionic binding[44], if the distal carboxylate groups in the two compounds could each provide an anionic oxygen atom in a similar position in space to allow binding to the same membrane cationic group (Fig. 3)[182].

Fig. 3

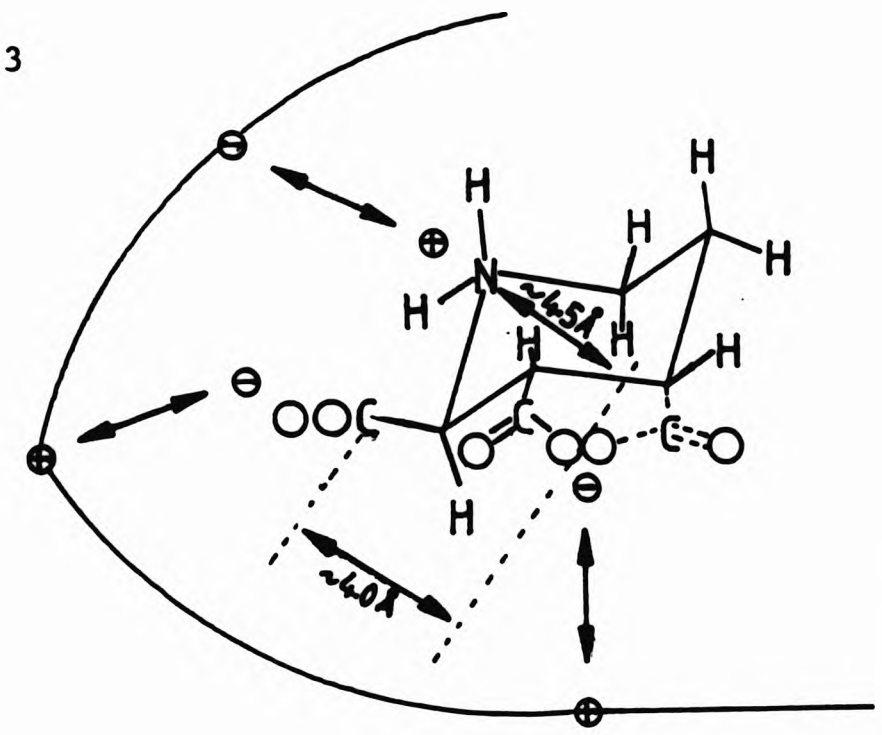
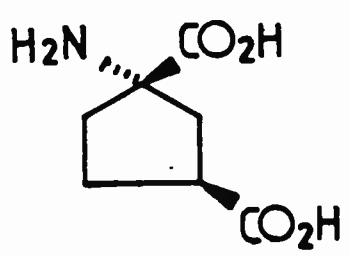


Fig. 3 shows that an oxygen atom from either an equatorial C-3 or an axial C-4 carboxylate group could occupy a similar position in space and thus could interact equally with an appropriately positioned membrane cationic group. An equatorial C-4 carboxylate group, as in the inactive isomer cis-2,4-PDA could not interact effectively with this membrane group.

It has been suggested[182] that such an anionic oxygen would be separated by approximately 3.8 - 4.2 Å from the centre of the α-carboxylate carbon atom and approximately 4.2 - 4.8 Å from the centre of the N atom, and this may well be a feature conducive to NMDA agonist activity.

Cis-1-amino-1,3-dicarboxyclopentane (cis-1,3-ADCP) (27) has a similar arrangement of amino and carboxyl groups to trans-2,4-PDA (33) and can be regarded in the same light. Cis-1,3-ADCP is a DαAA[69] and Mg^{2+}-susceptible[182] agonist and interestingly trans-1,3-ADCP (29) (with intercharge relationships more similar to cis-2,4PDA) is considerably less potent[69].



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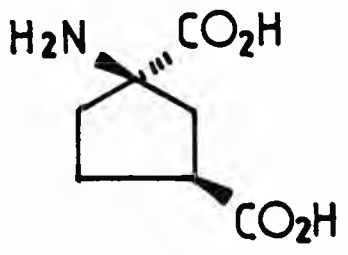

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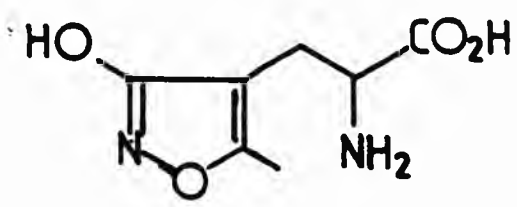
McLennan using observed differences between glutamic acid diethyl ester (GDEE) and D-α-aminoadipate (DαAA)-sensitive receptors, attempted to define the likely conriguration of the three-point site. Glutamate, quisqualate and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (34) [100] are examples of molecules which are most sensitive to GDEE blockade but are little affected by DαAA, they possess one common feature, a considerable flexibility in the permissible distance between the



(34)

acid groups as well as between the distal acid function and the amino nitrogen.

Ibotenic and 1,3-ADCP are examples of molecules with inflexible structures, the three active substitutes form part of or are closely attached to planar rings such that interatomic mobilities are restricted, they are DαAA-sensitive but are little affected by GDEE[117]. In these molecules the spacing between the carbon atoms of the acidic groups is relatively fixed at ca. 0.4 nm, while in molecules such as glutamate the analogous distance can vary from ca. 0.2 nm to 0.4 nm or more. Thus, it appears that the GDEE-sensitive receptor accepts its ligands in a folded configuration in which the inter-carboxylate spacing optimally is ca. 0.2 nm. In DαAA-antagonised receptors that distance is greater, approximately 0.4 nm.

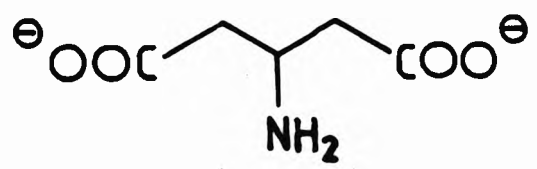
D- and L-glutamate can exist in both folded and extended configurations. D-glutamate has an excitatory potency about one half that of the L-isomer[69] and unlike L-glutamate its effects are preferentially blocked by DαAA. It is likely therefore that the excitatory effect of D-glutamate is due to reaction with the extended receptor, while L-glutamate reacts preferentially with the folded form.

Consideration of the carbon-nitrogen distance which is very similar to the carbon-carbon bond length suggests that the carbon-nitrogen spacing may in fact be the distinguishing factor for GDEE-sensitive from DαAA-sensitive receptors (Table 5).

TABLE 5 Maximal and minimal distances (nm) between carboxyl carbon atoms (C_α and C_ω) and the amino nitrogen atom in three amino acids.

		Glutamate	3-aminoglutarate	Aspartate
$C_\alpha - C_\omega$	Max	0.46	0.46	0.38
	Min	0.18	0.18	0.25
$C_\alpha - N$	Max	0.25	0.38	0.25
	Min	0.25	0.25	0.25
$C_\omega - N$	Max	0.46	0.38	0.38
	Min	0.18	0.25	0.25

3-aminoglutarate (35) is a symmetrical molecule and a neuronal excitant[44]. From the table it is evident that if the carbon-carbon distances are critical 3-aminoglutarate should resemble glutamate pharmacologically. The carbon-nitrogen distances are identical to those for the distal-carbon-nitrogen spacing in aspartate.



(35)

Experimental testing has indicated that 3-aminoglutarate is indistinguishable from aspartate but differs from glutamate in terms of its pattern of blockade by GDEE and DαAA and therefore as stated above the carbon-nitrogen spacing appears to be the one distinguishing GDEE-sensitive from DαAA-sensitive receptors.

1.3 Clinical Considerations

Epilepsy

In reflex epilepsy, in focal epilepsy and in primary generalised epilepsy with clonic or tonic motor signs, the development of clinically evident convulsive activity is associated with the excessively synchronous or sustained discharge of a group of neurones. Since this process depends on excitatory neurotransmission it can be prevented by antagonists of excitatory neurotransmitters. If applied iontophoretically glutamic and aspartic acids are each excitatory on most central neurones[41] but precise evidence of physiological role is still lacking. Similarly, an exact role for these acids is epileptogenesis, or anti-convulsant drug action has yet to be defined. In an effort to define this role, Croucher and Meldrum have since carried out extensive studies examining the effects of the phosphono derivatives of aliphatic amino acids on seizures in baboons and mice.

In an early study Croucher[37] investigated the role of excitatory amino acids in the development of seizure responses by administering selective antagonists of these excitants to DBA/2 mice, an inbred strain in which, within a critical age range, a fixed sequence of seizure responses can be induced by a loud sound[28]. It was found that the amino phosphono acids that antagonise NMDA-induced excitation blocked all stages of the audiogenic seizure response. APH was the most potent of the series. The relative anti-convulsant potencies of these compounds matched their relative potencies as antagonists of NMDA to the rat cortex[148,169]. The greater activity of the D-(-)-isomer of APH compared with the (+)-isomer also corresponded to the relative activities of the two isomers after iontophoretic application. This correlation suggests that excitatory neurotransmission mediated by the NMDA receptor plays an important role in the spread of epileptic neuronal hyperactivity.

The recent findings of Meldrum and Croucher[121,121a] have indicated that selective antagonists of amino acid-induced excitation provide an anticonvulsant action comparable both in terms of efficacy and acute toxicity to that of some drugs in clinical use. The testing of such antagonists in man for their efficacy against reflex epilepsy and focal or generalised seizures must await further study of their selectivity of action and short- and long-term toxicity.

Huntington's Chorea

Huntington's Chorea[35] is a heriditary condition, confined to humans, characterised by severe mental deterioration and bizarre motor abnormalities. Although nothing is known about what induces neuronal changes in the disease it is thought that neuronal cell loss may occur through a mechanism analogous to the mechanism of neurotoxicity[34,137] involving kainate and glutamate systems of brain (interaction between kainate- and glutamate-induced depolarisation has been well documented[14,15,62a]).

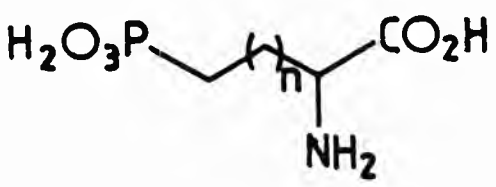
Although Huntington's Chorea is a rare disease (occurring in 5 in 100,000 in the United States and United Kingdom[35]), an elucidation of its pathophysiology could lead to fundamental advances in our understanding of brain mechanisms underlying other behavioural and motor disorders.

1.4 Aims of Project

The concept of at least three types of excitatory amino acid receptor has been developed throughout this chapter; the receptors being NMDA (antagonised by DαAA, DαAS and Mg^{2+}); kainate (antagonised by γDGG and cis-2,3-PDA) and quisqualate (antagonised by GDEE). It still remains uncertain whether any or all of these receptors are involved in the actions of endogenous synaptic transmitters, e.g. glutamic acid and it has therefore become increasingly important to try to define the precise molecular requirements necessary for activating the different receptors and to develop specific mimetics which act only at one defined site (focusing attention on NMDA and quisqualate type receptors).

To date only a few compounds, antagonists or agonists, have been found which exhibit a marked degree of selectivity. One approach to this problem is to examine structure-activity relationships e.g. the limits of chain length of the straight chain amino acids; the effects of altering groups within a compound or of conformationally restricted analogues.

A series of long chain alkyl α-amino acids with substitution which terminates in a phosphonic acid residue have been synthesised[38].

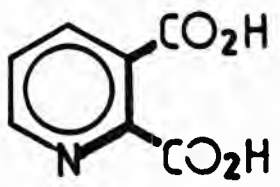


Subsequent pharmacological testing showed that 2-amino-5-phosphonopentane acid (APP) (11), n = 2, and 2-amino-7-phosphonoheptanoic acid (APH) (13), n = 4, were highly potent and selective antagonists of NMDA. Thus, replacement of the distal carboxylic acid groups by a phosphonic acid group had an obviously significant effect[38,140,148,169].

It was decided, therefore, to employ the amino phosphonic acids in further biological tests. Many of the experiments previously carried out would be repeated to examine the reproducibility of their results. Also, studies would be carried out on different systems e.g. seizure in baboons.

APP (11) and APH (13) would be synthesised based on the tried and tested method outlined by Curry[38]. The lower homologue, 2-amino-4-phosphonobutyric acid (APB) (10), n = 1, which exhibited weak and unspecific antagonism[182], would also be synthesised, in order that its action on different regions of the CNS could be studied.

Quinolinic acid (44), a rigid aspartic acid (4) analogue and an endogenous metabolite[107,130], is a potent excitant at amino acid receptors[80,168].



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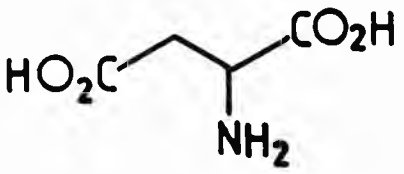
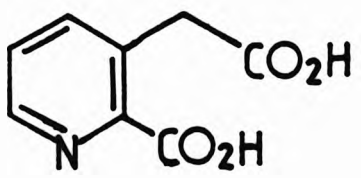


(4)

However, except for the recent cursory work of Perkins and Stone[146,147], the role of quinolinic acid in the study of excitatory amino acids has remained virtually unexplained. It was decided that the action of quinolinic acid would be further investigated utilising various quinolinic acid derivatives.

The higher homologues, (45) and (46), of quinolinic acid (44)



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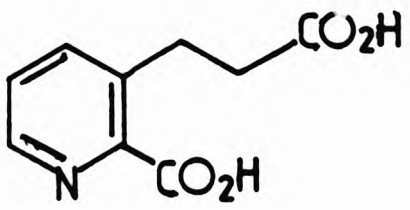

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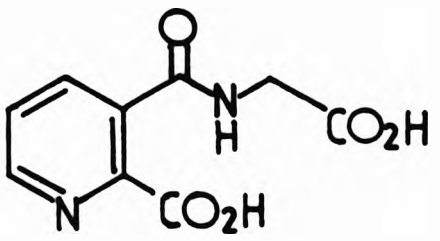
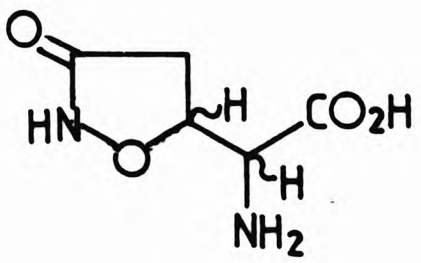


(47)

and the peptide (47) were to be synthesised.

The inherent scope of this area of synthesis is extensive e.g. the side chain at position 3 of the ring can be lengthened; various degrees of unsaturation can be introduced and peptides of the various homologues can be prepared.

Tricholomic acid (50), is a conformationally restricted analogue of glutamic acid (1) and a saturated analogue of the widely-studied ibotenic acid (26)[92,155,181].



(5)

However, with respect to the study of receptors for the excitatory amino acids, very little work has been done employing tricholomic acid, due, presumably, to its scarcity.

Thus, it was decided to synthesise tricholomic acid to afford larger quantities of the acid for biological testing.

CHAPTER TWO

AMINO PHOSPHONO ACIDS

2.1 Introduction

2.1.1 Straight chain acidic amino acids

A range of straight chain acidic amino acids, and derivatives, have been tested iontophoretically to assess their excitatory and inhibitory tendencies[38,45] (Table 1).

For the carboxylic, acidic amino acids, L-glutamic and L-aspartic acids were the most potent, followed by their respective D-isomers, showing about half the potency. D-α-aminoadipate had little or no activity as an excitant. This pattern was followed by cysteic acid (48), in which the difference between L and D activity was slightly more pronounced. However, for the sulphono containing glutamic acid analogue, homocysteic acid (22), the L-form was a good agonist whilst the D isomer was the most potent straight chain analogue to be tested.

Generally, N-alkylation produced an increase in activity for aspartic acid and a decrease for glutamic acid and the sulphur-containing amino acids. In the case of aspartic acid, N-methylation, increased the activity of the D-isomer, whilst decreasing it for the L-isomer[180].

The longer chain analogues, far from being excitants, seemed to antagonise the excitatory effects of other acidic amino acids, particularly N-methyl-D-aspartic acid (NMDA) and homocysteic acid[58]. The L-isomers of these analogues retained a modicum of excitatory potency whilst the D-forms of α-aminoadipic, pimelic and suberic acids were all relatively powerful antagonists of a specific nature, in that they diminished responses due to NMDA and homocysteic acid, whilst having no effect on excitations produced by glutamic and aspartic acids (Table 1).

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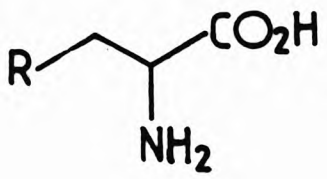
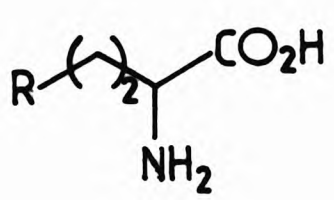
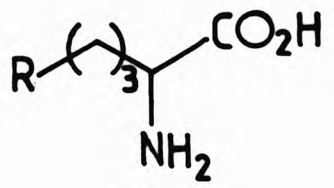
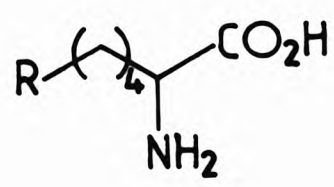
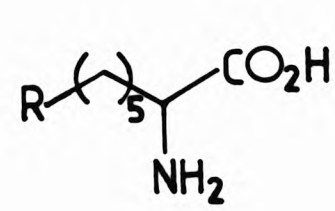
TABLE 1 Table of inhibitory and excitatory tendencies for straight chain acidic amino acids[38]

General formula	Compound	R	Excitatory potency (1 - 10)	Inhibitory potency (1 - 10 L-Glu	L-Asp	NMDA	Homocys.
	L-asp	CO_2H	5	0	0	0	0
	D-asp	CO_2H	3	0	0	0	0
	L-cys	SO_3H	7	0	0	0	0
	D-cys	SO_3H	2	0	0	0	0
	L-glu	CO_2H	5	0	0	0	0
	D-glu	CO_2H	3	0	0	0	0
	L-homocys	SO_3H	6	0	0	0	0
	D-homocys	SO_3H	10	0	0	0	0
	L-αAA	CO_2H	slight	0	0	0	0
	D-αAA	CO_2H	0	1	1	4	2
	L-αAP	CO_2H	0	0	0	0	0
	D-αAP	CO_2H	0	1	1	4	2
	L-αAS	CO_2H	0	0	0	0	0
	D-αAS	CO_2H	0	0	slight	2	1

cys = cysteic acid homocys = homocysteic acid

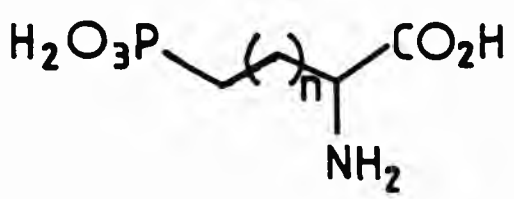
asp = aspartic acid glu = glutamic acid

AA = amino adipic; AP = aminopimelic; AS = aminosuberic acids.

Thus, it appears that excitatory potency falls as chain length of the D-isomers is increased. The relationship between chain length and antagonist activity was not so well defined; adipic and suberic acids were both antagonists of similar potency, whilst pimelic acid was considerably less potent.

2.1.2 Structure of amino phosphono acids

Replacement of the terminal γ-carboxyl group of straight chain carboxylic amino acids, with an isoteric phosphonic acid group, α-amino-ω-phosphono acid (36).



(36)

n = 1, 2-amino-4-phosphonobutyric acid (APB) (10)
n = 2, 2-amino-5-phosphonopentanoic acid (APP) (11)
n = 3, 2-amino-6-phosphonohexanoic acid (APHex) (12)
n = 4, 2-amino-7-phosphonoheptanoic acid (APH) (13)

2.1.3 Previous pharmacological evaluation

Stone[169] and Perkins[148] tested the effects of the microiontophoretic application of several amino phosphono acids on rat cerebral cortex. The results of their experiments showed that APH and APP were more potent and selective antagonists of NMDA than compounds tested previously[50,53,183] and also suggested that a distinct population of receptors exists in the vertebrate CNS for NMDA on which glutamate, kainate and quisqualate have little activity; (±)APH (13) was found to be the most potent member of the phosphonic acid series, requiring currents of only 10 nA or less for 30-60 seconds to greatly reduce NMDA responses. The lower homologue (±)APP (11) needed ejection currents of 30-50 nA to reduce NMDA responses. (±)APHex (12) had poor antagonistic effect (Table 2).

TABLE 2 Depression of excitatory effects of amino acids and analogues by phosphonate derivatives

Agonists	Antagonists (±)-APP	(±)-APHex	(±)-APH
NMDA	78.2 ± 4.0 (18)	52.5 ± 6.8 (10)	88.1 ± 5.4 (14)
DLH	73.5 ± 6.2 (10)	39.5 ± 9.4 (8)	79.6 ± 4.0 (35)
Aspartate	60.8 ± 5.3 (11)	46.9 ± 9.4 (8)	36.2 ± 6.7 (32)
Glutamate	22.7 ± 3.9 (8)	41.3 ± 8.1 (9)	24.2 ± 6.7 (23)
Kainate	31.6 ± 6.4 (8)	16.4 ± 7.3 (5)	6.6 ± 2.9 (9)
Quisqualate	2.4 ± 3.8 (3)	not tested	7.0 ± 5.1 (3)

± indicates mean error

Figures in parentheses are number of cells tested.

(±)-APH was able to distinguish between NMDA and aspartate, glutamate and kainate responses better than (±)-APP, whilst (±)-APHex showed little or no ability to distinguish between the various agonists[148].

The (-)-isomers of APP[148] and APH[38] and not the (+)-isomers exhibit antagonistic activity (Tables 3 and 4).

TABLE 3 Antagonistic efficacy of isomers of APP against excitatory responses to NMDA, kainate and glutamate[148]

	AGONIST NMDA	Kainate	Glutamate
APP isomer			
(-) 2 - 10 nA	92.5 ± 3.6 (26)	33.9 ± 8.4 (8)	13.4 ± 8.6 (14)
(±) 30 - 60 nA	78.2 ± 4.0 (18)	31.6 ± 6.4 (8)	22.7 ± 3.9 (8)
(+) < 20 nA	9.5 ± 12.3 (10)	3.4 ± 7.2 (5)	4.6 ± 6.2 (8)
(+) > 20 nA	31.3 ± 11.0 (9)	16.2 ± 5.4 (4)	18.8 ± 9.7 (6)

TABLE 4 Mean ratios of agonist response[38]

	% reduction ratio (+)-APH/(-)-APH		Cells tested
	Mean	SEM	
NMDA	0.66	±0.12	6
cis-cyclopentylglutamate	0.75	±0.07	6
4-fluoroglutamate	0.49	±0.07	11
D-homocysteate	0.63	±0.13	7

Also, it has been reported, that in cortex, (-)-APP is much more powerful than D-αAA in antagonising either excitatory or neurotoxic activities of NMDA. (-)-APP (7.5 mg/Kg) afforded protection (98%) against the neurone-necrotising activity of NMDA (Table 5)[140].

TABLE 5 Relative antagonist potencies of D-αAA and (-)-APP

n	Agents	Dose (mg/Kg)	Lesion severity
24	NMDA	50	23.8 ± 1.2
10	NMDA + D-αAA	50 + 250	13.3 ± 2.1
5	NMDA + D-αAA	50 + 500	1.5 ± 0.7
3	NMDA + D-αAA	50 + 700	0.7 ± 0.6
6	NMDA + (-)-APP	50 + 2.5	17.0 ± 3.0
6	NMDA + (-)-APP	50 + 5.0	5.5 ± 2.1
6	NMDA + (-)-APP	50 + 7.5	0.5 ± 0.2
9	NMDA + (-)-APP	50 +>7.5	0.0 ± 0.0

Interestingly, Davies and co-workers[50], using spinal cord preparations, also found APP to be considerably more potent than DαAA.

However, unlike the results of Stone[169], Davies found APP and not APH to be the most potent substance of the amino phosphono acid series[59], in isolated spinal cord preparations of immature rat or frog. This anomaly can probably be explained in terms of methodological differences combined with regional CNS differences or species differences[184].

Measurement of the stimulation of cyclic GMP (guanosine monophosphate) formation in cerebellar slices by excitatory amino acids has shown that APP is more potent than APH against NMDA, whilst APH is more potent against aspartate[154].

Generally, the long chain amino phosphono acids showed the predicted trend of antagonism to straight chain amino acid excitants. The antagonist activity was directed toward NMDA type agonists and resided in the D-isomers.

APB (10), a glutamic acid analogue, exhibited little excitatory action and weak and unspecific antagonism. APP (11) and APH (13) showed much more powerful and specific antagonist action, with the anomalous, APHex being rather weaker and less specific in its action.

As these preliminary studies[148,169] had shown that it was possible to improve the potency and selectivity of amino acid antagonists by using phosphonate analogues it was decided that APB, APP and APH would be synthesised and used in more rigorous pharmacological tests.

2.2 Synthesis of Amino Phosphono Acids

2.1.1 Introduction

In the 1940s, Kosolopoff[98,99] published several syntheses of alkyl phosphonates from phosphite esters.

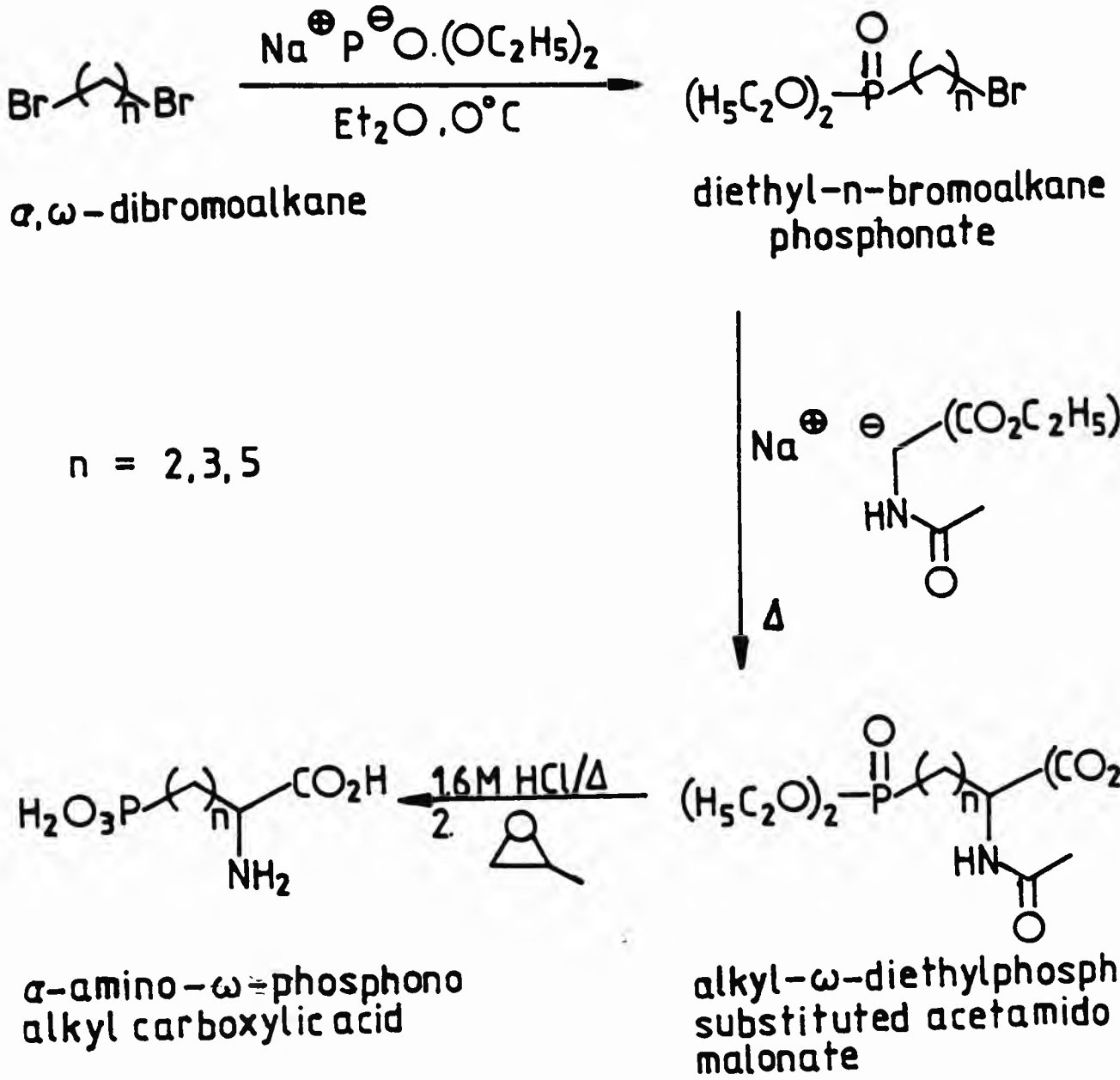
Alkyl phosphonates, prepared from diethyl and triethyl phosphites, have been used in syntheses of amino phosphono acids[25,38].

Although a synthetic route could be designed based on earlier

reports[25,39], several refinements were necessary if more efficient yields were to be obtained. Reactions were carried out on larger scales and under milder conditions.

2.2.2 General synthesis

Scheme 1



2.2.3 Preparation of alkyl phosphonates

Diethyl-3-bromopropane phosphonate (38), n = 3, and diethyl-5-bromopentane phosphonate (39), n = 5, were prepared by the reaction of 1,3-dibromopropane and 1,5-dibromopentane, respectively, with sodium diethyl phosphite, in dry diethyl ether at 0°C (Michaelis Becker reaction) (Scheme 1).

Theprogress of the reaction was followed by thin-layer chroma-

tography (TLC). Sodium bromide formed was filtered and the pure phosphonates were isolated after column chromatography. There was no need for the additional distillation step as described by Curry[38].

The ^{13}C spectra were complex due to the coupling of phosphorus to carbon. ^{31}P resonances for phosphonates (38) and (39) were at δ 29.888 and δ 30.698 respectively.

(Diethyl-2-bromoethane phosphonate (37), n = 2, was supplied by Aldrich.)

2.2.4 Preparation of alkyl-ω-diethylphosphono substituted acetamido malonate

The acetamido malonate adducts (40), n = 2; (41), n = 3 and (42), n = 5, were synthesised by straightforward malonic ester syntheses. The reaction of sodium acetamidomalonate and a bromophosphonate (Scheme 1) was followed by TLC. The adducts (40), (41), (42) were obtained, after column chromatography, in 51, 50 and 52% yields, respectively. (Unreacted starting materials were recovered.)

2.2.5 Synthesis of (±)-α-amino-ω-phosphono acids

The acetamido malonate adducts (40), (41) and (42) were hydrolysed in 6M HCl within 12 hours. The reactions were clean and the amino acids isolated as the chlorides were dissolved in methanol. The slow addition of propylene oxide afforded the precipitation of the free amino acids.

The racemic amino acids were recrystallised from water-ethanol and were obtained in high yield: (±)APB (10) 68%; (±)APP (11) 90% and (±)APH (13) 70%. The purity of these acids was determined by ^{13}C and ^{31}P NMR; analysis; melting-point and optical rotation (Table 6).

Generally, purer and more easily purified products were isolated

than in previous reports by Curry[38] and Chambers et al[24] who treated products of the acid hydrolyses with decolourising charcoal. Also, the amino acids had to be treated with ion-exchange resin before recrystallisation.

2.2.6 Resolution of (±)-α-amino-ω-phosphono acids (10),(11),(13)

The racemic amino phosphono acids, (±)APB, (±)APP and (±)APH were resolved using a modification of the method described by Evans et al[58].

The L-lysine salts of the acids were easily formed in aqueous solution. However, subsequent precipitation of these salts required much care and methanol and diethyl ether were cautiously added. Increasing the scale of the resolution did not pose any problems.

Degradation of these salts was achieved on ion-exchange resin (Dowex50-pyridinium form), which tightly bound lysine ($H_2N(CH_2)_4CH(NH_2)CO_2H$) (49) and allowed the elution of the acidic acids with aqueous pyridine. (TLC of the eluant showed that lysine was not eluted.)

The optical rotations of the isomers (+) and (-) were measured on an automatic polarimeter at the mercury line. The rotation values obtained for (+) and (-) amino phosphono acids showed that the resolutions had been successful (Table 6). (Slightly higher values have been reported by Evans et al[59], where rotations were determined in 6M HCl rather than in water.)

Circular dichroism (CD) spectra (supplied by Dr. P.M. Scopes of Westfield College) of the (+) and (-) amino phosphono acids showed that the (-) isomer and (+) isomer have D- and L-configurations, respectively.

Table 6

Amino phosphono acid	mpt °C (with	Analysis found/required decomposition)	α^{25}_{546}	^{31}P (δ)
APB (10)	225	C, 26.08; H, 5.48; N, 7.37; P, 16.87	(D)-14.8°	26.65
		C, 26.23; H, 5.50; N, 7.65; P, 16.92	(L)+11.3°	
APP (11)	230	C, 30.09; H, 6.14; N, 6.81; P, 15.62	(D)-10°	29.95
		C, 30.46; H, 6.14; N, 7.10; P, 15.71	(L)+8°	
APH (13)	213	C, 37.13; H, 7.29; N, 6.11; P, 13.76	(D)-7°	31.77
		C, 37.33; H, 7.16; N, 6.22; P, 13.76	(L)+7.5°	

^{13}C See experimental

2.3 Methods and Results of Pharmacological Tests

2.3.1 Introduction

The antagonist actions of APP (11) and APH (13) were compared on the excitatory activity of NMDA or aspartate on rat cortical neurones and also against seizures induced by sound in DBA/2 mice[37,121a] or seizure induced by NMDA or by 3-mercaptopropionic acid (3MPA) (43) in Swiss S mice[121a].

The effects of APP and APH (and other excitatory amino acid antagonists) on photically-induced epileptic responses in baboons, Papio papio, were also examined[121].

The above-mentioned tests were carried out by B.S. Meldrum of the Institute of Psychiatry, London.

APB (10), APP (11) and APH (13) were also tested on retina of albino rabbits[129], by M. Neal of the School of Pharmacy, University of London. Experiments were designed to investigate the relative importance of ON- and OFF-channels as inputs to the cholinergic amacrine cells.

2.3.2 Microiontophoretic studies in rat cortex

Male Wistar rats were anaesthetised and secured in a stereotaxic frame. The cerebral cortex was exposed and the dura nater removed; the exposed surface was covered with warm saline throughout the experiment.

All compounds (NMDA, glutamate, aspartate, APP, APH) were applied by microiontophoresis[85] from seven-barrelled micropipettes, the tips of which were broken back to 4-8 μm under microscopic observation. A separate single glass microelectrode was glued alongside the multi-barrel for recording unit activity.

The ratios of the potency of APH to that of APP were determined against NMDA, L-glutamate and L-aspartate (Table 6). Whereas APH and APP were essentially equipotent against L-glutamate, APH showed greater activity than APP against NMDA and aspartate.

TABLE 6 Ratios of the antagonist potency of AP:APP against amino acid excitants on cortical neurones

Method*	Potency ratios APH:APP		
	NMDA	Glutamate	Aspartate
IC_{50}	1.52 ± 0.35 (11)	1.14 ± 0.40 (6)	1.80 ± 0.38 (10)
Plateau	0.94 ± 0.14 (7)	0.90 ± 0.21 (8)	1.31 ± 0.29 (6)
$t\frac{1}{2}$	1.39 ± 0.18 (22)	not tested	1.21 ± 0.32 (8)

* For explanation of method see Meldrum et al[121a]

2.3.3 Protection against sound-induced seizures

The investigation of the protection afforded by APP and APH against sound-induced seizures used 21-28 days old DBA/2 mice. DBA/2 mice are an inbred strain in which, within a critical age range, a fixed sequence of seizure responses can be induced by a loud sound[28].

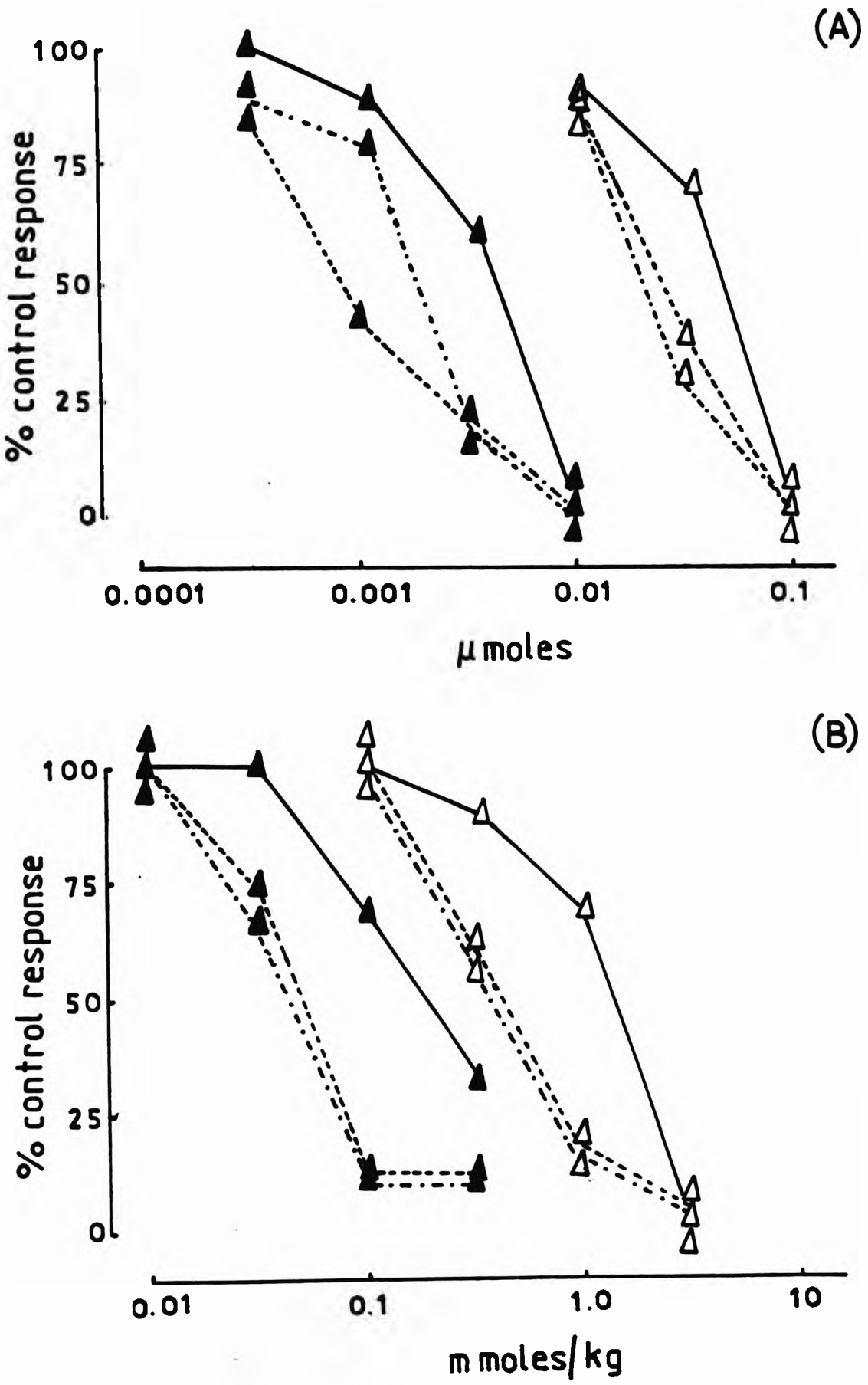
Animals recived drugs either intraperitoneally (ip) or intracerebroventricularly (icv) and were placed individually under a hemispheric perspex dome for behavioural and postural assessment. Auditory stimulation, an electric bell generating 109 dB at mouse level, was subsequently applied for one minute or until tonic extension occurred. Responses were graded according to the severity of the seizure. Control seizure responses consisted of an initial wild running phase (WR; score 1) followed by fore- and hind-limb myoclonus (score 2), tonic flexion and extension (score 3) and occasionally respiratory arrest (RA; score 4). Absence of any abnormal motor response was scored as zero and only the maximal score for each animal was recorded.

The relative anticonvulsant activities of APP and APH against the principal phases of the audiogenic seizure response are shown in Table 7. Figure 1, shows the log dose response curves for APH are to the left of those for APP, suggesting that APH is approximately one order of magnitude more active than APP against all phases by the response, following either ip or icv administration. The (-)-isomer of each antagonist showed greater anticonvulsant activity than the (+)-isomer.

TABLE 7 Relative anticonvulsant potencies of APP and APH in DBA/2 mice following central or systemic administration

Antagonist	Minimum dose to suppress WR($p < 0.01$)	ED_{50} versus		
		WR	Clonus	Tonus
ICV ADMIN (μmoles/Kg)				
(±)-APP	0.10	0.046	0.022	0.025
(±)-APH	0.01	0.004	0.002	0.001
Potency ratio APH:APP	10	11.5	11	25
IP ADMIN (nmoles/Kg)				
(±)-APP	3.3	1.40	0.43	0.43
(±)-APH	0.33	0.18	0.05	0.05
Potency ratio APH:APP	10	7.8	8.6	8.6
APP isomers				
(+) isomer	0.33	0.10	0.021	0.022
(-) isomer	0.10	0.033	0.018	0.018
Potency ratio (-):(+)	3.3	3.0	1.2	1.2
APH isomers				
(+) isomer	0.033	0.014	0.002	0.002
(-) isomer	0.003	0.002	0.001	0.001
Potency ratio (-):(+)	11	7	2	2

Fig. 1 Log dose-response curves for (±)APP(Δ) and (±)APH(▲) against the individual phases of the audiogenic seizure response in DBA/2 mice



A. Intracerebroventricular injection. B. Intraperitoneal injection. Siezure responses were assessed following administration of progressive doses of antagonist and ED_{50} values for each phase of the seizure response were estimated.
(—— wild running; —●— clonus; ----- tonus).

2.3.4 Protection against chemically-induced seizures

Experiments on protection against chemically-induced seizures were carried out on Swiss S mice; mice which are not genetically seizure prone[121a]. The albino Swiss male mice were housed in colony cages and were randomly assigned to groups (8-10 animals).

APP and APH were administered (ip) 45 minutes before NMDA or 3MPA ($HSCH_2CH_2CO_2H$) (43). Animals injected with convulsant compounds were put separately into plastic cages and observed for 30 minutes. The occurrence and timing of either clonic or tonic seizures were noted.

The effect of pretreatment with APP or APH on the ED_{50} for clonic or tonic seizures due to NMDA or 3MPA is shown in Tables 8, 9 and 10.

APH was more than twice as potent than APP with respect to the suppression of the clonic seizure phases. The potency ratio for suppression of tonic seizures after 3MPA was even greater.

TABLE 8 Calculated doses (mmol/Kg) of (±)APP and (±)APH necessary to double the ED_{50} values of NMDA or 3MPA

Compound	NMDA	3MPA	
	Clonic seizures	Clonic seizures	Tonic seizures
APP	1.73	1.76	1.40
APH	0.64	0.73	<0.026

TABLE 9 Effect of (±)-APP and (±)-APH on NMDA-induced convulsions in mice

Treatment	Clonic seizures	% of Control
NMDA	1.41 (1.28 - 1.55)	-
NMDA +(±)-APH (0.165 mmol/Kg)	1.62 (1.46 - 1.80)	115
NMDA	1.40 (1.26 - 1.55)	-
NMDA +(±)-APH (0.33 mmol/Kg)	2.12** (1.78 - 2.52)	151
NMDA	1.52 (1.37 - 1.69)	-
NMDA +(±)-APP (0.33 mmol/Kg)	1.80* (1.67 - 1.94)	118
NMDA	1.41 (1.28 - 1.55)	-
NMDA +(±)-APP (0.66 mmol/Kg)	1.75* (1.55 - 1.98)	124

* $p < 0.05$;

** $p < 0.01$ vs NMDA treated groups

TABLE 10 Effect of (±)APP and (±)APH on 3MPA-induced induced convulsions in mice

	Clonic seizures	% of control	Tonic seizures	% of control
3-MPA	0.32 (0.29 - 0.35)	-	0.34 (0.31 - 0.37)	-
3MPA +(±)APH (0.165 mmol/Kg)	0.43* (0.37 - 0.49)	134	1.89*** (1.15 - 2.65)	529
3MPA	0.33 (0.37 - 0.38)		0.39 (0.39 - 0.44)	
3MPA +(±)APH (0.33 mmol/Kg)	0.48* (0.43 - 0.54)	145	>5.0***	>1282
3MPA	0.32 (0.29 - 0.35)	-	0.34 (0.31 - 0.37)	-
3MPA +(±)APP (0.33 mol/Kg)	0.38* (0.35 - 0.42)	119	0.42* (0.38 - 0.47)	124
3MPA	0.32 (0.29 - 0.35)	-	0.34 (0.38 - 0.47)	-
3MPA +(±)APP (0.66 mmol/Kg)	0.41* (0.34 - 0.50	128	0.64** (0.47 - 0.88)	188

* $p < 0.05$; :: $p < 0.01$; ::: $p < 0.001$ vs 3MPA treated groups.

All table values are ED_{50} with 95% confidence limits in parentheses, calculated according to Litchfield[103].

TABLE 10 Effect of (±)APP and (±)APH on 3MPA-induced induced convulsions in mice

	Clonic seizures	% of control	Tonic seizures	% of control
3-MPA	0.32 (0.29 - 0.35)	-	0.34 (0.31 - 0.37)	-
3MPA +(±)APH (0.165 mmol/Kg)	0.43* (0.37 - 0.49)	134	1.89*** (1.15 - 2.65)	529
3MPA	0.33 (0.37 - 0.38)		0.39 (0.39 - 0.44)	
3MPA +(±)APH (0.33 mmol/Kg)	0.48* (0.43 - 0.54)	145	>5.0***	>1282
3MPA	0.32 (0.29 - 0.35)	-	0.34 (0.31 - 0.37)	-
3MPA +(±)APP (0.33 mol/Kg)	0.38* (0.35 - 0.42)	119	0.42* (0.38 - 0.47)	124
3MPA	0.32 (0.29 - 0.35)	-	0.34 (0.38 - 0.47)	-
3MPA +(±)APP (0.66 mmol/Kg)	0.41* (0.34 - 0.50	128	0.64** (0.47 - 0.88)	188

* $p < 0.05$; :: $p < 0.01$; ::: $p < 0.001$ vs 3MPA treated groups.

All table values are ED_{50} with 95% confidence limits in parentheses, calculated according to Litchfield[103].

2.3.5 Suppression of photically-induced epilepsy

Baboons, Papio papio, from the Casamance region of Senegal show a genetically-determined syndrome of photosensitive epilepsy that is manifest as myoclonic responses; and sometimes tonic-clonic seizures, during stroboscopic stimulation[126].

Adolescent baboons were tested whilst seated in a primate chair by exposure to stroboscopic stimulation for up to five minutes, in a standardised fashion. Compounds APP(11), APH (13), GDEE (19) and cis-2,3-PDA (21) were administered intravenously and the animals were observed for the following five hours with testing of myoclonus responses[120] to stroboscopic stimulation at hourly intervals.

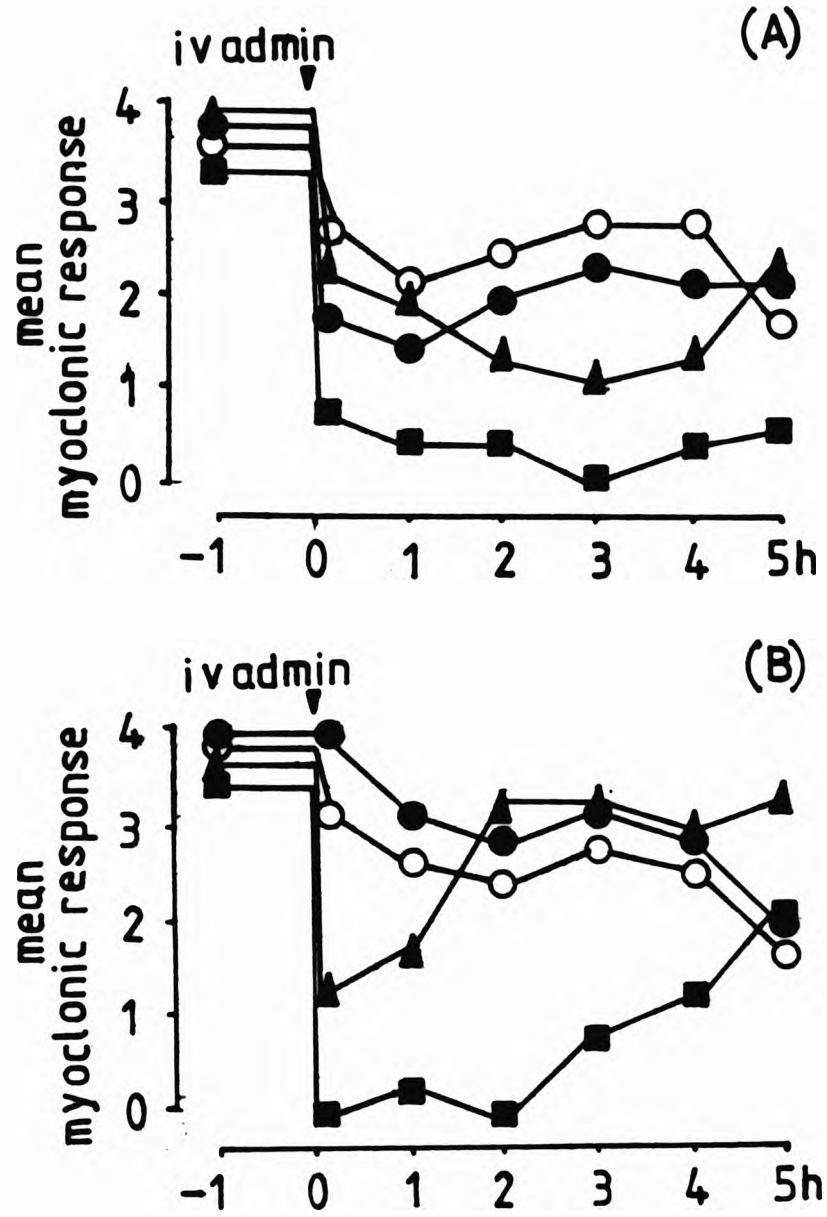
Administration of APH (0.1 - 1.0 mmol/Kg) resulted in a dose dependent suppression of photically-induced myoclonus. (Fig. 2). Control animals showed diffuse myoclonus of the limbs and trunk in response to intermittent light stimulation (ILS). Ten minutes after APH, 1.0 mmol/Kg, stimulation-induced only intermittent eyelid flickering in all animals. The myoclonic response was maintained at this level, or was totally abolished, for the following five hours.

APH action produced no marked neurological impairment. At the highest dose, one baboon showed some retching and excess salivation, but all animals appeared alert during photic stimulation.

APP showed a shorter time-course of action than APH; myoclonic responses were suppressed within ten minutes of the administration of APP, 1.0 mmol/Kg, but returned to control levels after 1-2 hours. Following APP (3.3 mmol/Kg) responses to ILS were markedly reduced or absent for 2-4 hours, but diffuse myoclonic responses returned on subsequent stimulation.

At the lower dose levels no behavioural or postural impairment was evident. At 3.3 mmol/Kg, however, signs of sedation appeared immediately after injection with drooping eyelids, a loss of muscle

FIG. 2 The suppression of photically-induced myoclonic responses in the baboon, Papio papio, by APP(A) and APH(B)



The mean myoclonic response to photic stimulation is plotted aginst time (hours) relative to drug administration at time zero.

Groups of n animals were tested at each dose level (n = 3 at all doses except APP control group where n = 5)

The following dose levels (mmol/Kg) are shown:

A: ⊙ saline; ● 0.1; ▲ 0.33; ■ 1.0
B: ○ saline; ● 0.1; ▲ 1.0; ■ 3.3

tone in all four limbs and a lack of responsiveness to aggressive stimuli. Excessive salivation was noted in two out of three drug-treated animals. Normal responsiveness returned within one hour although mild sedation persisted for the duration of the experiments.

Cis-2,3-PDA (21) resulted in a similar but weaker protection against photically-induced myoclonus as APH. Few signs of toxicity were noted, with only three of nine drug administrations inducing periods of yawning and transient drowsiness.

GDEE (19) administration caused very little suppression of photosensitivity. A transient reduction in the severity of the myoclonic response was observed after GDEE, 1.0 - 3.3 mmol/Kg, which was preceded by a period of respiratory distress and cardiac arrhythmias.

2.3.6 APB-effect on acetylcholine release from rabbit retina

Adult albino rabbits were anaesthetised and a stainless steel ring was sutured to the episclera to prevent collapse of the eye-cup. The cornea, iris and lens were removed, followed by vitrectomy. The resulting eye-cup was filled with Krebs carbonate ringer solution.

A solution of ringer containing [^{3}H]-choline was placed in the eye-cup for 30 minutes and then the retina was continuously irrigated for one hour with medium containing eserine sulphate. The medium in the cup was replaced every five minutes and the total radioactivity in the removed medium was measured. During this period, the retina was exposed to light flashes (3 Hz, 25% duty cycle, 800 lux). The electroretinogram (ERG) was recorded[129].

The compounds under test, a series of amino phosphono acids (36), (n = 0 to n = 4), were dissolved in the medium and their effects were plotted to obtain the concentration of compound needed to produce a reduction in the release of ACh or the amplitude of the b-wave by 50% (EC_{50}). (Table 11).

TABLE 11 The effect of ω-phosphono-2-aminocarboxylic acids on the light-evoked release of ACh and on the ERG b-wave

Compound	EC_{50} (μm)	
	ACh release	ERG b-wave
(±)2-amino-3-phosphonopropionic acid	NE	NE
L-(+)-2-amino-4-phosphonobutyric acid	7.5	5.8
D-(-)-2-amino-4-phosphonobutyric acid	115	140
DL-(±)-2-amino-4-phosphonobutyric acid	16	11
L-(+)-2-amino-5-phosphonopentanoic acid	230	155
D-(-)-2-amino-5-phosphonopentanoic acid	660	680
(±)-2-amino-6-phosphonohexanoic acid	NE	NE
(±)-2-amino-7-phosphonoheptanoic acid	NE	NE

Each result was obtained from graphs of log concentration against effect using at least 3 drug concentrations. Each drug concentration was the mean of at least 4 separate experiments.

NE = no effect up to 1 mM.

(±)APB (200 μm) reversibly blocked the ON-response of the ganglion cells, whilst the OFF-response was increased in size. The b-wave of the ERG was abolished. At a lower concentration (100 μm) the ERG was reduced by 80% and the ON-response by 75%. (+)-L-APB was far more potent than (-)-D-APB in blocking ON-responses. Both isomers reduced the light-evoked release of ACh and had no effect on the spontaneous resting release. The (+)-isomer was much more potent, the EC_{50}s for the (+) and (-) isomers being 7.5 μm and 115 μm respectively. For the racemate the EC_{50} = 16 μm.

APP also reduced the light evoked release of ACh and the b-wave of the ERG but it was less potent than APB. The (+)-isomer was most

effective, the EC_{50}s for the (+) and (-)-isomers being 230 μm and 660 μm respectively.

(±)2-amino-3-phosphonopropionic acid (9) (n = 0), APHex and APH, at concentrations up to 1 mM, had no obvious effect on either the light evoked release of ACh or the ERG.

2.4 Discussion

2.4.1 Microiontophoretic studies and suppression of seizures

The results of the microiontophoretic experiments on rat cerebral cortex showed that APH and APP inhibited the excitatory action of NMDA and aspartate (Table 6) (IC_{50} method was probably the most accurate, being least affected by reuptake and other processes.)

APH was approximately 1.5 times as potent as APP against excitation due to NMDA and perhaps rather more potent when assessed against aspartate[59,121a,154].

Experiments testing the anticonvulsant action of the amino phosphono acids indicated that APH was approximately 10 times more potent than APP by both intracerebroventricular and intraperitoneal routes. This constancy of relative effect suggested that the two compounds are closely similar in their pattern of absorption and their capacity to enter the brain from the circulation.

The (-)-isomers of APP and APH were more potent than the (+)-isomers at suppressing sound-induced seizures. The proconvulsant (myoclonus-inducing) properties of (+)-APH suggested a partial agonist action.

Evaluation of chemically-induced seizures in Swiss S mice showed an increase in the potency ratio, APH:APP, for suppression of tonic seizures after the administration of 3MPA. Biochemical studies have indicated that 3MPA owes its convulsant effect to a reduction in

GABAergic transmission subsequent to inhibition of the enzyme synthesising GABA (3) glutamic acid decarboxylase (GAD). It has recently been shown in preloaded rat brain slices that the release of [^{3}H]-D-aspartate is enhanced by 3MPA[165]. A special role for excitatory amino acid transmission in 3MPA seizures must be considered[46].

The development of the successive stages of a seizure involves the progressive recruitment of different neural populations into a synchronous firing pattern. Any factor which can reduce excitatory transmission will hamper this process of recruitment. The element of positive feedback in the recruitment process will enhance even a small difference in potency between two antagonists; which explains why the late tonic seizures after 3MPA, or the complex segmented elements of the sound-induced seizure response are more potently suppressed by APH (relative to APP) than would have been predicted solely on the basis of single unit responses.

Having shown that APP and APH potently block excitation due to NMDA and suppress epileptic responses in a genetically determined syndrome of reflexepilepsy (sound-induced seizures in DBA/2 mice) it was decided to investigate the effect of these compounds on the baboon, Papio papio, which exhibits a genetically determined syndrome of photosensitive epilepsy.

APH abolished myoclonic responses for 5 hours and APP whilst giving less prolonged protection had toxic side effects. Thus, the greater anticonvulsant potency of APH against APP was consistent with earlier observations[121a].

The epileptic responses manifested by Papio papio are blocked by anticonvulsant drugs, such as barbiturates, benzodiazepines and sodium valproate, which are effective against primary generalised seizures in man[119]. So the importance of certain amino phosphono acids which have such a marked effect on this photosensitive syndrome,

a valuable model for testing novel anticonvulsant agents, cannot be stressed enough.

Convulsions induced by NMDA may be due to a direct action of NMDA on a subgroup of excitatory receptors. Receptors of this type are prominent in the cerebral cortex but it cannot be assumed that this is the site of epileptogenesis.

The effectiveness of specific NMDA receptor antagonists, in each of the models studied, support a major role for NMDA receptor-mediated excitation in the generation or propagation of epileptic neuronal hyperactivity within the CNS. The endogenous agonist or agonists acting at the NMDA receptor have not been identified but it is possible that both glutamate and aspartate are involved.

The regional site of anticonvulsant action of APP and APH is unknown. Although it is likely that reduction in excitatory transmission in the cortex modifies seizure responses, anatomical and electrophysiological evidence indicates that brain stem and mid-brain systems play a major role in the development of audiogenic seizure and in tonic seizures[121a].

Testing of the possible clinical usefulness of this new class of anticonvulsant agent must await further studies of the anticonvulsant profile of these agents and of their acute and chronic toxicity in both rodents and primates.

2.4.2 ON- and OFF-channels in amacrine cells

APB has a relatively weak and non-selective antagonist action on NMDA; quisqualate- and kainate-induced depolarisations, compared to APP and APH[182] and therefore the later compounds are used preferentially in studies on brain. However, it has been found that APB is very useful, pharmacologically, in retina research.

The retina is an integral part of the CNS, having an overall metabolic organisation resembling that in the brain[173]. Retina can be isolated without much injury and kept for hours, in a viable condition, responding to light[1]. Responses are monitored by recording the ERG or by intracellular recording. The retina is a very thin, layed structure, having very short diffusion pathways and the extracellular fluid, therefore, can equilibrate rapidly with the bathing fluid, an obvious advantage in release studies. The absence of any barrier in diffusion pathways ensures that compounds are applied to cellular elements in known concentrations. It is reasonable to assume that retina utilises transmitter substances which occur in the rest of the CNS and therefore may provide clues to an understanding of central synaptic mechanism in general.

In the outer retina, photoreceptors appear to communciate with a dark released agent which provides excitatory input at both the onset and termination of a light stimulus[47]. Two post-synaptic elements, horizontal cells (HCs) and OFF-bipolars, are depolarised in the dark. A third cell type, ON-bipolar, is hyperpolarised in the dark. A light stimulation which hyperpolarises the photoreceptors causes a reduction in the rate of transmitter release resulting in a depolarisation of ON-bipolars and a hyperpolarisation of HCs and OFF-bipolars.

Exogenously applied glutamate and aspartate on second order neurons mimic the photoreceptor transmitter[127,128,144]. In mudpuppy, Necturus maculosus, retina, Slaughter and Miller[166] attempting to characterise the synaptic receptors of second order neurons found APB to be a highly selective agonist which appeared to interact with the synaptic receptors of ON-bipolars. In low concentrations APB action in the outer retina was restricted to ON-bipolars. In the inner retina ON responses or response components were eliminated but OFF responses persisted. The loss of ON-activity in the inner retina

probably reflects the action of APB at the bipolar cell level. In fairly high concentrations (10-20 nM) APB acted as an antagonist to horizontal cells.

The results of these experiments investigating the role of ON- and OFF-channels in amacrine cells indicated that the release of ACh from amacrine cells is mediated mainly via the ON-channels. It has been shown that exposure of the retina to chloride-free medium also selectively abolished responses mediated via the ON-channels[122]. Chloride-free medium also abolished the light-evoked release of ACh and produced charges in the ERG similar to those produced by high concentrations of APB. However, unlike APB chloride-free medium increased the spontaneous resting release of ACh approximately two-fold.

Thus, APB selectively abolishes ON-channels in the rabbit retina and there is a strong possiblity that the activity of the cholinergic amacrine cells is determined mainly by the depolarising bipolar cells. The ON-channels appear to require the presence of chloride ions[122].

The mechanism by which APB blocks transmission of ON-channels in the rabbit retina is unknown although in the mudpuppy it appears to mimic the endogenous photoceptor transmitter[166], which may be glutamate and/or asparte[127]. Results of work carried out with APB and other amino phosphono acids are consistent with this suggestion.

An alternative possibility that APP and APH are acting as antagonists of aspartate or glutamate is very unlikely because antagonistic activity is greater in the (-)-isomers, rather than in the (+)-isomers which were most potent in the retina experiments. Furthermore, the order of potency in blocking NMDA receptors in the brain is APH > APP > APB[148] again the reverse of these results.

The binding of [^{3}H]-APB has recently been characterised[22]. The L-(+)-isomer is shown to be fifteen times more active than the D-(-)-form in inhibiting the binding of DL-[^{3}H]-APB, which is in close

agreement with the ability of these compounds to produce depression of synaptic transmission. The most potent inhibitor of binding was quisqualic acid. It has been suggested[22] that APB may interact with a quisqualate-'preferring' class of excitatory amino acid receptors, possibly localised predominantly on presynaptic terminals.

2.5 Summary

Efficient synthetic routes to the amino phosphono acids, APB, APP and APH, have been developed and all of these compounds can now be obtained in high overall yields.

Evaluation of these compounds in a variety of animal models of epilepsy has shown that administration, both icv and ip, of APP and APH suppresses photically-stimulated seizures in the baboon. Sound- and chemically-induced seizures in mice can also be suppressed. APB has proved useful in the elucidation of the mechanism of transmission of ON-channels in the rabbit retina.

CHAPTER THREE

SYNTHESIS OF SOME QUINOLINIC ACID ANALOGUES

3.1 Introduction

Quinolinic acid (2,3-pyridine dicarboxylic acid) (44) is an endogenous metabolite of L-tryptophan (51)[107,130].

The demonstration of a cerebral pathway for the synthesis of quinolinic acid is incomplete, but the biosynthetic mechanisms for 3-hydroxyanthranilic acid (57), its immediate metabolic precursor, in the brain have been established. Quinolinic acid has been unequivocally identified in both rat and human brain tissue[186a].

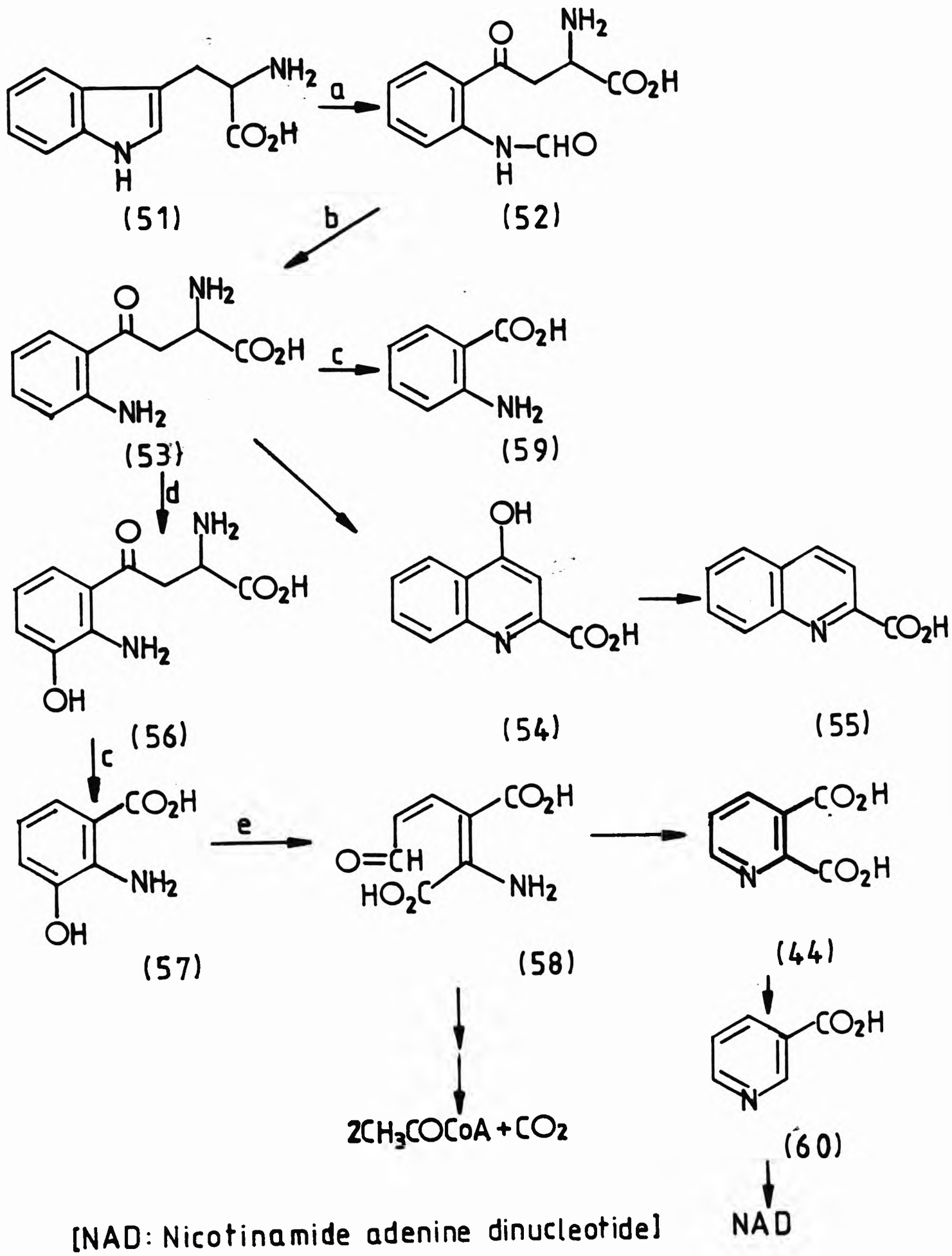
3.1.1 Biosynthesis

The biosynthesis of 3-hydroxyanthranilic acid (57) in brain, as in other organs, occurs via the kynurenine pathway (Scheme 1)[186], which constitutes a minor route of tryptophan catabolism in cerebral tissue. In peripheral organs, 3-hydroxyanthranilic acid is rapidly converted to quinolinic acid and further to nicotinic acid (60).

Tryptophan is oxidised to L-N-formylkynurenine (52) which is converted to kynurenine (53) by a liver enzyme, kynurenine formylase. Kynurenine is hydroxylated at position 3 to form 3-hydroxykynurenine (56).

A pyridoxal phosphate requiring enzyme, kynureninase, catalyses cleavage of 3-hydroxykynurenine and kynurenine to 3-hydroxyanthranilic (57) and anthranilic (59) acids, respectively. Alanine is the other reaction product in both instances. 3-hydroxykynurenine is split approximately twice as rapidly as kynurenine. The latter also transaminates; the corresponding keto acid undergoes ring closure to yield kynurenic acid (54) which is dehydroxylated to quinaldic acid (55).

Scheme 1 Metabolic relationships among tryptophan and its metabolites



(a) tryptophan pyrrolase; (b) kynurenine formylase;
(c) kynureninase; (d) kynurenine 3-hydroxylase;
(e) 3-hydroxyanthranilic acid oxidase.

α-Amino-β-carboxymuconic-Δ-semialdehyde (58) is the oxidation product of 3-hydroxyanthranilic acid (57). The semi-aldehyde (58) is a branch point in tryptophan degradation; it may be converted to quinolinic acid (44) or follow a pathway to glutaric acid and thus to acetyl CoA.

3.1.2 Quinolinic acid; a potent endogenous excitant

Quinolinic acid is a potent excitant of neurones in rat brain[168] and in cat caudate nucleus[80].

Stone and Perkins [168] applied quinolinic acid (44), quisqualic acid (8), N-methyl-D-aspartic acid (NMDA) (6), 2-amino-5-phosphono-pentanoic acid (APP) (11) and glutamic acid diethyl ester (GDEE) (19), iontophoretically to rat brain. A comparison of ejecting currents indicated that quinolinate was about as potent as glutamate and aspartate, about one quarter as potent as NMDA and one tenth as potent as quisqualate. Both antagonists, APP and GDEE, blocked quinolinate responses which suggests that quinolinate like L-glutamate, may activate both the NMDA and quisqualate type of receptor. However, APP exhibited a far greater ease of antagonism than GDEE, so it is possible that quinolinate may act preferentially on the NMDA receptor.

Surprisingly, it has taken more than twenty years for the importance of quinolinic acid, a rigid aromatic analogue of NMDA, to be realised. In 1978, Watkins[181] stated (in a footnote) that quinolinic acid showed little or no activity on the frog spinal cord. Unfortunately, it has since taken five years for the proposal that quinolinic acid should merit special attention as a potential neurotransmitter to appear to be valid.

A study of neuronal sensitivity of quinolinic acid in different regions of the CNS has shown that this acid is unique among amino acid-related excitants described to date, being excitatory in some

areas of the CNS such as cortex and hippocampus and almost completely inactive in others such as spinal cord (Table 1) [147].

TABLE 1 Relative sensitivity to cells in CNS regions to glutamate and quinolinate

Area	Cells tested	Excited by glutamate	Excited by quinolinate	Glu:Quin current ratio for comparable responses
Neocortex	25	25	25	0.71 ± 0.05 (25)
Spinal cord	17	17	4	0.14 ± 0.014 (4)
Neocortex	29	29	29	0.75 ± 0.07 (29)
Hippocampus	23	23	23	0.47 ± 0.06 (23)
Neocortex	21	21	20	0.86 ± 0.08 (21)
Cerebellum	36	36	12	0.15 ± 0.03 (12)
Ant striatum	31	31	31	0.75 ± 0.06 (31)
Post striatum	29	29	29	0.33 ± 0.04 (29)

± indicates mean error

Figures in parentheses are number of cells tested.

Kynurenic acid (54), a metabolite of tryptophan[186], is a relatively powerful antagonist of quinolinic acid[145]. However, kynurenic acid also antagonises NMDA and quisqualic acid; this lack of preferential antagonism suggests that the effect is not a specific antagonism at one of the proposed populations of excitatory amino acid receptors[182].

The presence of an endogenous antagonist could have many implications for the role of quinolinic acid in the control of neuronal excitability[168].

3.1.3 In vitro release of [^{3}H]-purines by quinolinic acid

L-glutamate and L-aspartate can evoke [^{3}H]-adenosine release from the rat cortical surface in vivo[89]. Recently, it has been shown that quinolinate also produces a large increase in basal release of [^{3}H]-purines. See Table 2[146].

N-methyl-DL-aspartate (NMDLA) and potassium chloride produced large increases, while glutamate, quisqualate and kainate had lesser effects.

TABLE 2 Effect of amino acids and KCl on [^{3}H]-purine release

	Concentrations (mM)	Percentage increase in release above baseline
Potassium chloride	56	245 ± 48 (21)
L-glutamate	5.0	89 ± 23 (8)
Kainate	1.0	56 ± 7 (5)
	0.5	40 ± 5 (5)
	0.1	87 ± 9 (5)
	0.05	40 ± 12 (5)
Quisqualate	0.5	51 ± 10 (5)
NMDLA	5.0	277 ± 70 (5)
	1.0	152 ± 19 (8)
Quinolinate	5.0	291 ± 39 (11)
	1.0	257 ± 39 (11)
	0.5	143 ± 18 (4)
	0.3	64 11 (5)

Figures in parentheses are the number of observations.

2-amino-7-phosphonoheptanoic acid (APH)(13), the NMDA-preferring receptor antagonist, significantly reduced the release evoked by quinolinate and NMDLA but not that produced by the other agonists (glutamate, quisqualate and kainate), this gives further support to the postulate that quinolinate acts on NMDLA-preferring receptors[168].

3.2 Structure-activity Relationships

3.2.1 Effects of cyclic dicarboxylic acids on the CNS

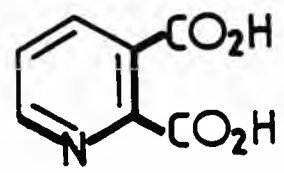
Davies et al[48] reported that (±)cis-2,3-piperidine dicarboxylic acid (cis-2,3-PDA) (21) was an antagonist at excitatory amino acid receptors in the spinal cord. A study by Birley et al[13] examined the actions of a series of cyclic dicarboxylic acids, including cis-2,3-PDA and quinolinic acid (44), in the cerebral cortex (Table 3).

Cis-2,3-PDA exhibited antagonistic properties towards excitatory amino acids which were not shared by the cis-2,4-, 2,5- or 2,6-dicarboxylic analogues; this antagonism appeared to be relatively selective for NMDA. Davies et al[48] found cis-2,3-PDA to be most effective in antagonising responses to quisqualate. (The disparity is possibly due to some subtle differences between the spinal cord and cerebral cortex receptors i.e. in cortex either quisqualate excitations are more resistant or NMDA is more susceptible to blockade by cis-2,3-PDA.) Such selectivity was only observed at lower currents, at higher currents non-selective antagonism was reported.

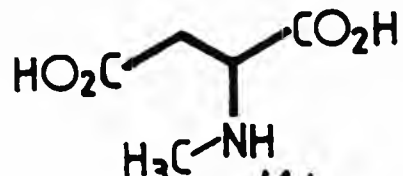
(±)Cis-2,3-PDA showed some selectivity against responses to NMDA, presumably, as it is a highly structured, semi-rigid analogue of NMDA. However, the failure of 2,3-piperazine dicarboxylic acid (63) to show any activity does imply that the electronic distribution in the ring is of importance for activity, and the three-dimensional structure is not the only criterion.

Cis-2,3-PDA is also a partial agonist[48] and caused a weak increase of firing of a small proportion of neurones.

Quinolinic acid (40), a fully rigid aromatic analogue of NMDA (6), is an effective excitant of cortical neurones.



(40)



(6)

TABLE 3

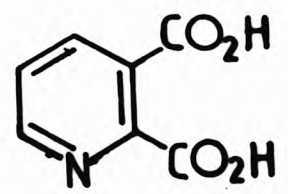
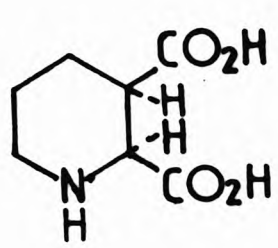
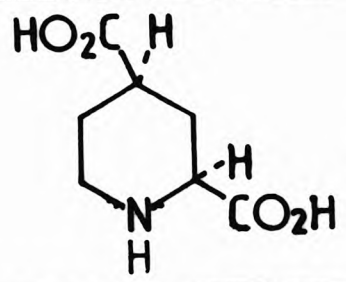
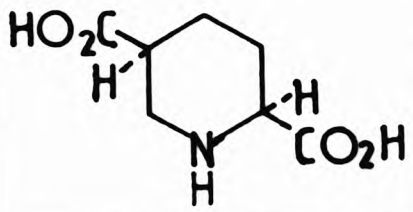
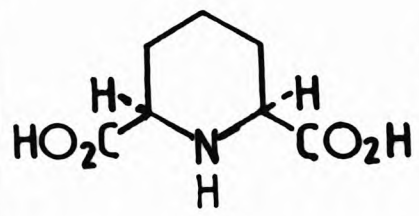
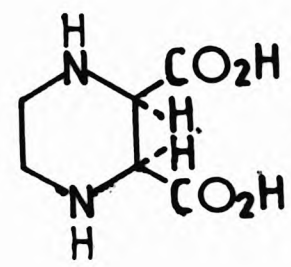
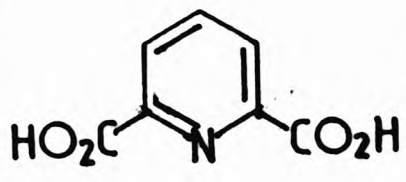
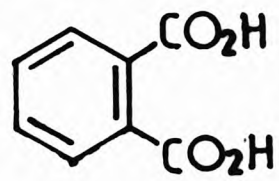
Compound (±)	Agonistic Effect	Antagonistic Effect
 (44)	Excitation of all of 55 neurones tested (20 - 65 nA, 5 - 15 seconds). Response abolished by (-)APP.	Not tested because of own excitant activity.
 (21)	Not detectable (< 25 nA). Very slow and weak increase of firing of 4 of 14 cells (> 60 nA).	Complete antagonisation of responses to NMDA with no change of responses to kainate or quisqualate on 16 of 18 neurons (10 - 25 nA). Nonselective reduction in size of responses to NMDA, glutamate, kainate and quisqualate on all 12 cells tested 60 - 100 nA).
 (34)	Weak depression of 1 and excitation of 2 of 12 cells.	None.
 (61)	No effect on 14 cells	None.

TABLE 3 continued

Compound (±)	Agonistic Effect	Antagonistic Effect
 (62)	Weak excitation of 12 of 32 cells (50 nA and > 50 nA)	Partial reduction of excitation evoked by glutamate or quisqualate on 5 of 25 cells. No change of response to NMDA (40 - 60 nA) Greater reduction in size of response and reduction of response to NMDA (80 or 100 nA)
 (63)	Excitation of 9 of 10 cells	Partial, nonselective reduction in size of responses to NMDA and quisqualate on 1 of 10 cells. (Effectively antagonised NMDA-induced excitation of frog spinal neurones[48,182].)
 (64)	Weak excitation of 4 of 12 cells tested (> 60 nA)	Reduction to 50% in responses to NMDA, but not in responses to glutamate or to quisqualate, on 5 cells. Conversely, antagonism of responses to glutamate but not to NMDA on 3 cells and approximately equal antagonism of responses to NMDA, quisqualate, and kainate on 8 cells (> 60 nA).
 (65)	No effect (100 nA for 6 min) (Stone[169b] has recently reported excitation of 14 of 16 units.)	

3.2.2 Convulsant action of quinolinic acid

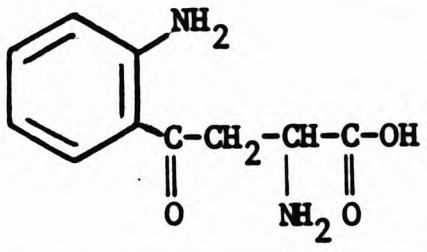
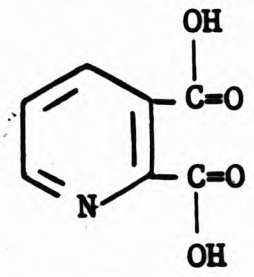
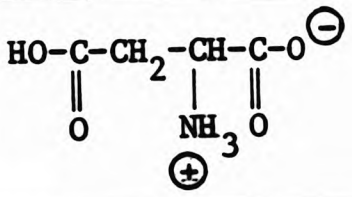
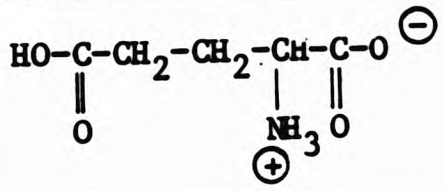
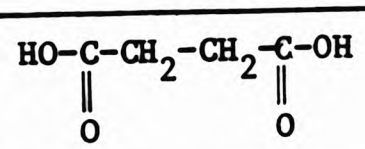
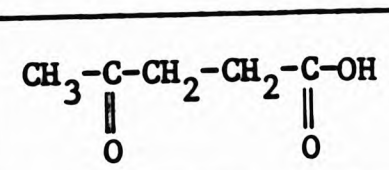
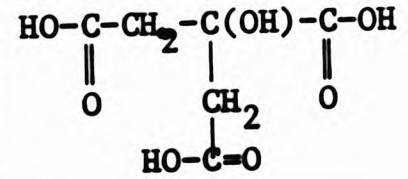
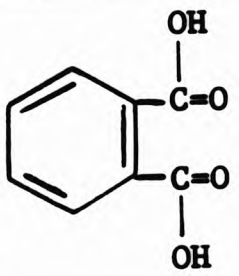
Quinolinic acid and L-kynurenine sulphate (69) induced seizures after intracerebroventricular (icv) injection in mice[102]. Kynurenine (53) like quinolinic acid is an endogenous tryptophan metabolite (Scheme 1). It is possible that a relationship exists between the chemical structures of these metabolites and their strong convulsant action.

Lapin[102] administered quinolinic acid (44), L-kynurenine sulphate (69), and a series of other dicarboxylic acids (Table 4) intracerebroventricularly in mice, inducing clonic seizures. When administered intraperitoneally (ip) or orally they were reported to antagonise strychnine-induced seizures. The convulsant effect of L-kynurenine sulphate was observed only when the pH of the solution was 4.0 or lower, while the actions of the other drugs were not dependent on pH. Control injections of sulphuric acid induced seizures in 25% of the mice.

As all the compounds administered (icv) induced seizures, the O=C-C-C-C=O moiety, presumably was responsible for the convulsant actions of quinolinic acid and L-kynurenine sulphate. The greater convulsant action of quinolinic acid could be related to the stability of its stereoconfiguration which provides a constant distance between the C=O group (6.81 Å between two oxygen atoms), whereas the side-chain of L-kynurenine is flexible and, thefore, this distance may not always be optimal.

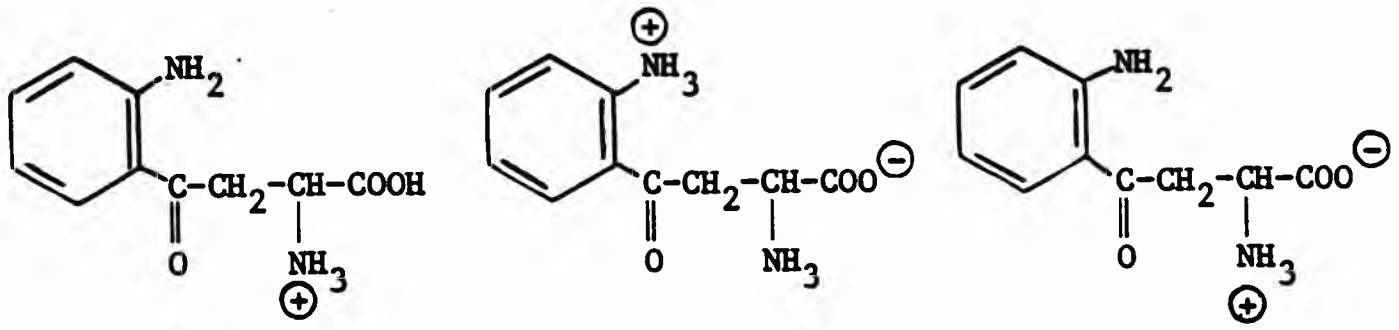
Although the role of pH was not clear, it was reported that the action of L-kynurenine sulphate consisted of effects of the cation of kynurenine (75-80%) and of the anion (20-25%). The action required a low pH in the medium therefore, the charges on L-kynurenine sulphate salt must affect its activity. At pH 2.0 -2.5, L-kynurenine, which exists mainly in two betaine forms, I and II, had maximum pharmacological activity. At pH 5.0 and above, L-kynurenine exists mainly in

TABLE 4 Convulsant and anticonvulsant actions[102]

Compound	Structural formula	Threshold doses	
		Convulsant (icv)(µg)	Anti-convulsant (ip)(mg Kg)
L-kynurenine sulphate* (69)		20	100
Quinolinic acid (44)		1	50
L-Aspartic acid (4)		20	200
L-Glutamic acid (1)		5	200
Succinic acid (66)		50	100
Laevulinic acid (67)		50	400
Citric acid (68)		20	400
Phthalic acid (65)		2	600

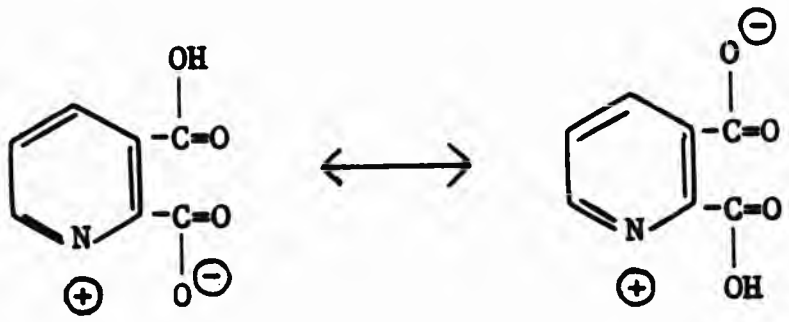
* Presented in neutral form

zwitter-ionic form III and its activity disappears. Consequently, if the charge on the molecule is functionally important, the greater the positive charge, the stronger the action.



I II III

Quinolinic acid, at pH 2.5, exists mainly as two canonical zwitter-ions, IV and V,



IV V

the proportions of which, together with the neutral form of the molecule, change when the pH is increased.

Phthalic acid (65) is devoid of these charges and was much less active than quinolinic acid, which suggested that the charges on quinolinic acid are important for convulsant activity. The independence of the activity of quinolinic acid from pH may be due to the presence of adequate charges in spite of charges in zwitter-ions. Succinic acid (without charges) was also less active than aspartic acid (with charges).

The effect of L-kynurenine sulphate could be reduced by taurine, inhibitory amino acids such as glycine and γ-aminobutyric acid (GABA), and interestingly, the side chain of the kynurenine molecule includes the structure of glycine ($-CH(NH_2)COOH$). However, these amino acids could not counteract the effects of quinolinic acid. Thus, inspite of

the presence of the O=C-C-C-C=O moiety in the structure of quinolinic acid its mechanism of action appears to be dissimilar to that of L-kynurenine.

Stone[145] has proposed that the convulsant activity of quinolinic acid may be a reflection of the direct excitatory action of this compound on central neurones. A view which Stone points out is apparently contradicted by Lapin's report that the icv injection of kynurenine induced seizures with a shorter latency than did quinolinic acid. However, Lapin has also stated that quinolinic acid is 25 times more potent than kynurenine at inducing seizures in mice and conceivably the relatively large dose of kynurenine needed could be disrupting other pathways.

3.3 Quinolinic Acid: a Neurotoxic Amino Acid

3.3.1 Toxic effects of excitatory amino acids

Injection of an exogenous 'excitotoxin'[137] into the brains of experimental animals produces effects that have been considered as models of human degenerative disorders. Thus, striatal lesions caused by these agents are thought to closely resemble the neurochemical and neuropathological changes observed in Huntington's disease[112]. The intraventricular, intrahippocampal or systemic administration of kainate[6,125,190] results in seizures and nerve cell changes characteristic of temporal lobe epilepsy in man.

The structural resemblance of exogenous amino acids such as kainic and ibotenic acids, to endogenous excitatory amino acids, e.g. glutamic and aspartic acids, has led to the hypothesis that hyperfunction of the body's own 'excitotoxins' may be related to neuronal damage in certain neuropsychiatric diseases[35]. Endogenous excitatory amino acids have been shown to exhibit only marginal neurotoxic potency in the mature nervous system when compared to the experimental neurotoxins, as they would have to be transported across the blood brain barrier.

3.3.2 Axon-sparing lesions produced by quinolinic acid

It has been reported[159] that quinolinic acid can produce axon-

sparing neuronal lesions after intracerebral injection in rats. Neurochemical, neuropathological and gross behavioural analyses show that quinolinic acid mimics some of the effects of kainic acid and ibotenic acid in both character and intensity.

The unilateral intrastriatal application of quinolinic acid (> 150 nmol) resulted in tonic-clonic movements of the contralateral forelimb which lasted approximately five hours. This behaviour was dose dependent and was usually accompanied by episodic barrel-like rotations, which have also been observed with kainate and ibotenate treatments[112]. No abnormal behaviour was reported to be displayed after the operation.

The general appearance of the striatum, with the exception of neuronal degeneration, was unchanged. The number of glial cells were not reduced. 'Distant' nerve cell loss, as observed with striatal kainate lesions[91], were not seen, and with the administration of nicotinic acid (60) (800 nmol) the decarboxylated product from quinolinic acid, healthy neurones were plentiful close to the injection site.

Ultrastructural analysis of the striatum four days after the injection of quinolinic acid (60 nmol) revealed disturbance of neuropil and nerve cells. The number of synaptic complexes was decreased and dendritic swelling was evident. Axons were well preserved.

Neurochemical measurements[159] confirmed the axon-sparing qualities of striatal quinolinic acid lesions. Ascompared to the contralateral side, the activities of striatal glutamic acid decarboxylase (GAD) and choline acetyltransferase, marker enzymes for intrinsic GABAergic and cholinergic neurones, decreased in dose-dependent fashion with increasing amounts of quinolinic acid. Tyrosine hydroxylase activity, an indicator of the presence of the dopaminergic terminal network originating from cell bodies in the substantia nigra[157], remained unchanged (Table 5).

Intrahippocampal injections of quinolinic acid (> 500 nmol) results in generalised convulsions, characterised by intermittent jumping, running fits, ipsilateral turning and pronounced exophthalmos, lasting

TABLE 5 Effects of intrastriatal quinolinic and nicotinic acid on local neurotransmitter related enzymes[159]

Injected amino acid (nmol)	Glutamic acid decarboxylase		Choline acetyltransferase		Tyrosine hydroxylase	
	Activity	Percent of contra-lateral striatum	Activity	Percent of contra-lateral striatum	Activity	Percent of contra-lateral striatum
			QUINOLINIC ACID			
60	307 ± 15	96	797 ± 40	96	2.5 ± 0.1	102
150	158 ± 13	55	557 ± 62	78	2.6 ± 0.3	92
300	85 ± 17	26	424 ± 56	50	3.0 ± 0.1	112
600	44 ± 14	14	116 ± 31	14	2.9 ± 0.1	86
			NICOTINIC ACID			
800	298 ± 4	96	790 ± 45	95	2.5 ± 0.1	94

for two to four hours[159]. Histological examination showed lesions of the dorsal hippocampus and underlying thalamic nuclei; pyramidal cells were preferentially damaged over granule cells. (Nicotinic acid did not damage the hippocampal formation.)

The absence of distant lesions, the production of circumscribed lesions and the lack of pronounced convulsant properties indicate an ibotenate-like activity[112]; whilst the selective vulnerability of pyramidal cells and the absence of neurotoxic effects in seven-day old rat striata or hippocampi indicate a kainate-like neurotoxicity[125,190].

Since kainic and ibotenic acids provide an animal model for Huntington's disease, it is possible that quinolinic acid has a role in the etiology of this disorder. A metabolic defect in the brain or increased transport from the periphery could provide an excess of quinolinic acid sufficient to induce neuronal degradation of the type seen experimentally[159].

3.3.3 Summary of pharmacology

Evidence to support the suggestion that quinolinic acid may act preferentially at NMDA receptors has been provided by the results of iontophoretic studies on rat brain[168] (APP blocked quinolinate's action more easily than GDEE) and also by the assessment of [^{3}H]-purine release evoked by quinolinic acid[146] (APH reduced the release evoked by quinolinate and NMDLA but not that due to the other agonists, e.g. glutamate and quisqualate).

Quinolinic acid exhibits neuronal sensitivity in different regions of the CNS i.e. it is excitatory in the cortex and hippocampus but it is inactive in the spinal cord[147].

Intracerebral injection of quinolinic acid produced axon-sparing neuronal lesions[159]. Neurochemical, neuropathological and gross behavioural analyses showed quinolinic acid to be both kainate-like and ibotenate-like in its actions.

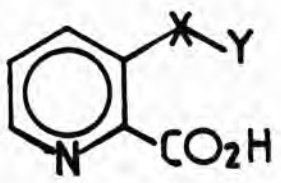
Quinolinic acid induced convulsant action after intracerebroventricular injection in mice[102]; structurally-related compounds were also examined and the results suggested that the presence of the O=C-C-C=O moiety may be necessary for the induction of this action. Stone[145] suggested that quinolinic acid's convulsant action may be a result of its direct excitatory action on central neurones.

As a result of the various reports of the tremendous activity of quinolinic acid it was decided to develop analogues of quinolinic acid (some of which, it was hoped, would be specific antagonists of NMDA) which could be submitted for pharmacological testing.

3.4 Synthesis of Some Quinolinic Acid Analogues

3.4.1 Introduction

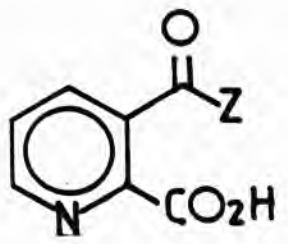
From the foregoing introduction, quinolonic acid is seen to be pharmacologically interesting, for example, it causes neuronal excitation[80,168]; induces seizures in mice[102] and produces axon-sparing lesions[159]. However, if the full significance of quinolinic acid's activity is to be realised, especially with respect to the identification of an excitatory amino acid receptor, extensive studies (e.g. structure activity relationships) need to be carried out. It was thought that examination of the biological effects of analogues having the general structures shown below would enhance the quinolinic acid 'field';



(75 a) $X = (CH_2)_n$; $Y = CO_2H$

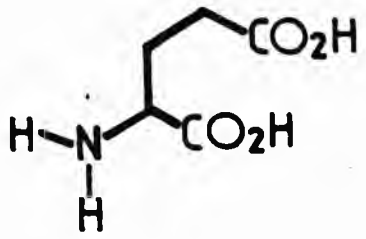
(75 b) $X = (CH_2)_n$; $Y = PO_3H$

(75 c) $X = (CH_2)_n$; $Y = SO_3H$

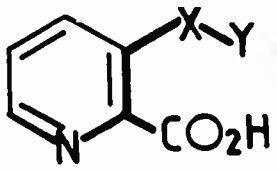


(108) $Z = -NH(CH_2)_n-Y$

As with the straight chain acidic amino acids (see 2.1.1) the length of the alkyl chain could be increased and the acid group (Y) could be either a carboxylic, phosphonic or sulphonic acid group. The acid (75a) would afford a relatively rigid glutamic acid (1) analogue.



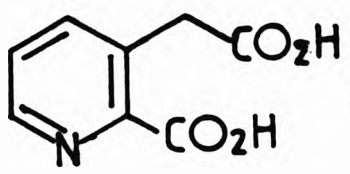
(1)



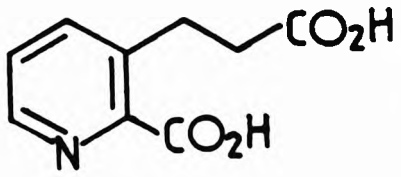
X = CH_2
Y = CO_2H

(75a)≡(45)

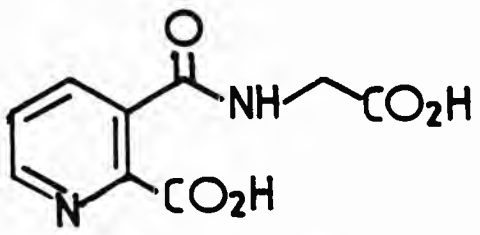
Peptide analogues could also be synthesised (e.g. (108)). Hence, a diverse choice of synthetic possibilities exists and therefore it was decided to undertake syntheses of the following compounds: the acids (45) and (46) and the peptide (47).



(45)



(46)



(47)

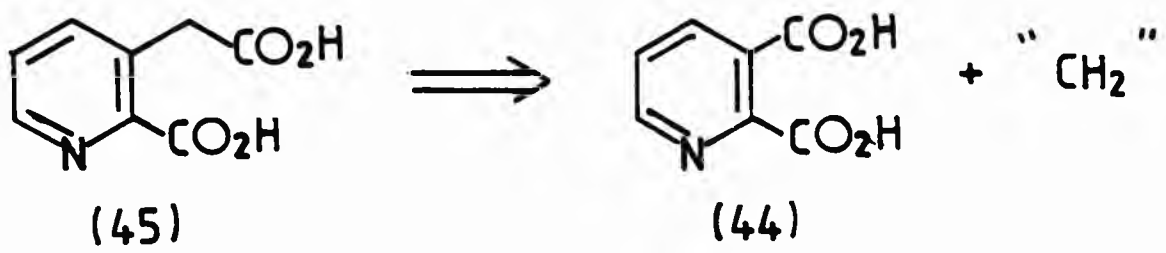
The acid (45) is a known compound[75] and its preparation would allow us to explore the chemistry of difunctional pyridine compounds and would provide an analogue for pharmacological testing. The diacid (46) is not a known compound but our knowledge gained from synthesising acid (45) would benefit its preparation.

The glycyl peptide analogue (47) is the first member of the series (and may be biologically active). Once the methodology had been devised, the phosphonic and sulphonic compounds could be derived by the same route.

3.4.2 Preparation of β-homoquinolinic acid (45)

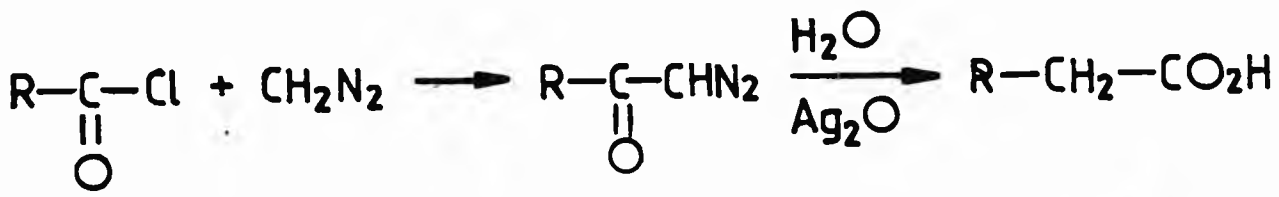
Quinolinic acid (44) was an obvious starting material; homologation, in principle, would only require the insertion of a methylene group (Scheme 2).

Scheme 2



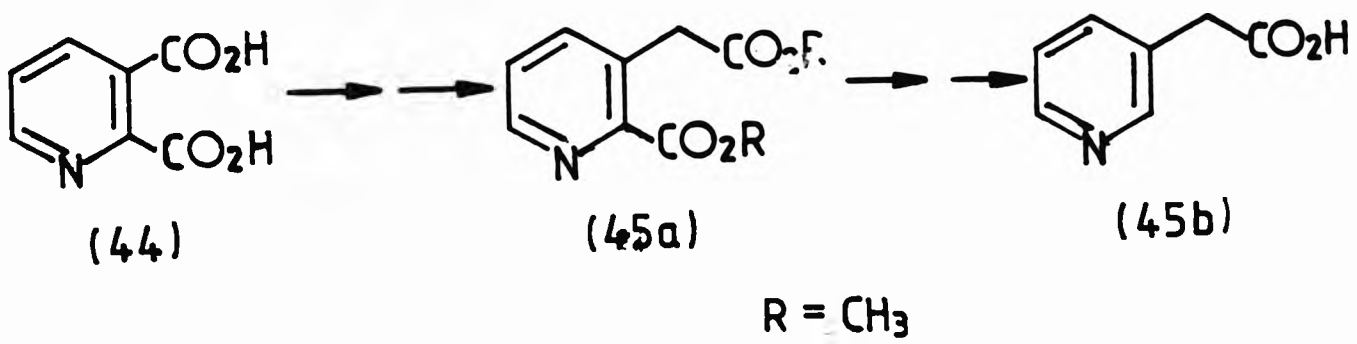
Several methods exist for increasing the length of a carbon chain by one carbon[108]. The best general method is the Arndt-Eistert synthesis[16] which converts an acyl halide to homologous carboxylic acid. In the first step the acyl halide is converted to a diazoketone in the presence of excess diazomethane. The actual rearrangement (Wolff rearrangement[84,118]) occurs in the second step (Scheme 3). If an alcohol, R'OH, is used instead of water, the ester, RCH_2COOR' is isolated.

Scheme 3



A literature search revealed that a patent issued in 1946[75] reported the preparation of a decarboxylated derivative of β-homoquinolinic acid, pyridine-3-acetic acid (45b), which employed the Arndt-Eistert homologation of quinolinic acid (Scheme 4).

Scheme 4



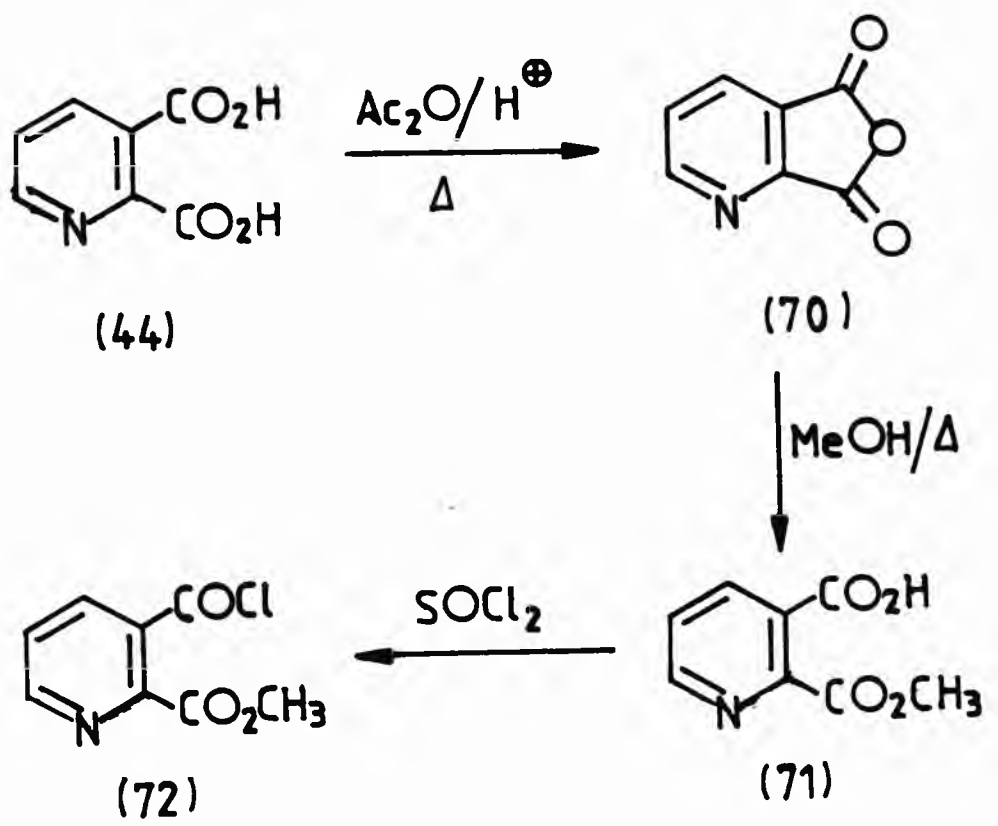
3.4.2.1 Synthesis of the diazoketone (73)

The anhydride (70) was synthesised in good yield, 67% after recrystallisation, by stirring a solution of quinolinic acid (44) in acetic anhydride with the slow addition of hydrochloric acid. The anhydride's infrared spectrum had absorptions at 1630 and 1690 cm^{-1} indicative of an anhydride. (Typically at 1710 cm^{-1}.)

Selective esterification of the anhydride in methanol afforded the 2-carbomethoxy acid (71) in 60% yield (melting point 122-125°C, literature melting point 125-126°C[17]). The methoxy protons appeared as a singlet (δ 4.48) in the ^1H NMR spectrum of the monoester (71). Infrared absorptions at 3540 (broad stretch) and at 1620 and 1730 cm^{-1} indicated the presence of carboxylic acid and ester groups.

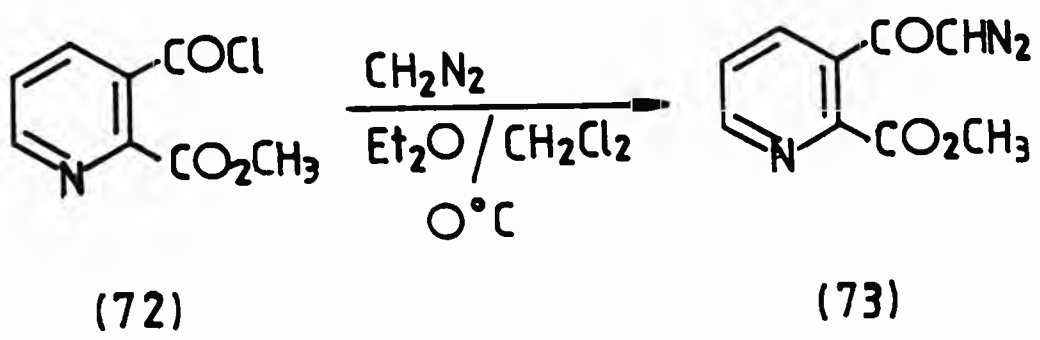
The monoester (71) was then heated under reflux with thionyl chloride to prepare the acyl chloride (72); the progress of the reaction being followed by TLC and infrared. The acid chloride has a characteristic absorption at 1785 cm^{-1}. The crude acid chloride (72) was isolated in 98% yield on evaporation of solvent (Scheme 5).

Scheme 5



The acyl chloride (72) in dichloromethane was carefully added to a solution of diazomethane[2,63] in diethyl ether, at 0°C (Scheme 6). The progress of the reaction was again followed by TLC and infrared spectroscopy (absorptions at 2100 cm^{-1} (CHN_2) and 1620 cm^{-1} ($COCHN_2$)). Excess diazomethane was destroyed with acetic acid and after the evaporation of solvent the diazoketone (73) was isolated. It was found to be stable at 0°C; there being no evidence (TLC) of decomposition within 12 hours at 0°C or 60 hours at -10°C.

Scheme 6



(72) (73)

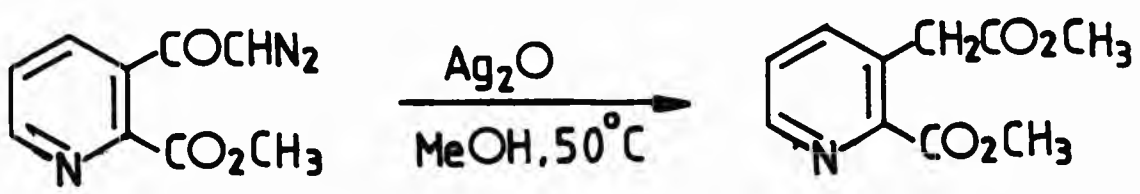
2.4.2.2 Conversion of diazoketone (73) to diester (74)

The diazoketone (73), stirred as a solution in methanol at 50°C, was treated with two equivalents of silver (1) oxide over a period of 2 hours. When the evolution of nitrogen ceased the reaction mixture was carefully filtered, several times, to remove all traces of silver (1) oxide. The residue had to be immediately purified by chromatography on silica as the crude residue readily decomposed at room temperature and 0°C.

Despite the difficulty in handling the reaction mixture, (with care), the diester (74) could be isolated as a pale yellow oil in good yield (72%). An accurate mass spectrum of the diester (74) established the molecular formula as $C_{10}H_{11}NO_4$ (molecular ion at m/e 209.071). Confirmation of its structure came from the NMR spectra: methylene proton resonances at δ 4.61 and methoxy protons at δ 3.71 ($CH_2CO_2\underline{CH}_3$)

and δ 3.96 ($CO_2C\underline{H}_3$); ^{13}C absorptions were at δ 38.73 ($\underline{C}H_2CO_2CH_3$), δ 52.06 ($CH_2CO_2\underline{C}H_3$), δ 52.22 ($CO_2\underline{C}H_3$).

Scheme 7



(73) (74)

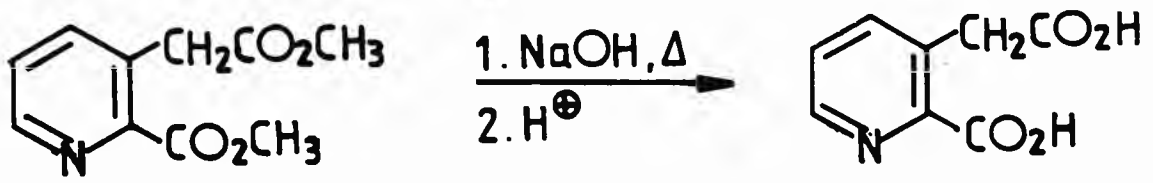
As silver (1) oxide is a relatively expensive material, it was decided to examine the use of a cheaper alternative: copper(I) iodide is a less expensive catalyst and has been reported to catalyse the Wolff rearrangement[187] successfully.

Copper(I) iodide in acetonitrile/methanol, at 65°C, successfully catalysed the conversion of diazoketone (73) to diester (74) (TLC evidence). However, the work-up of the reaction mixture was much more difficult and required more filtrations than with silver oxide. The diester was isolated in low yield (15%). Silver (1) oxide, therefore, was a more suitable catalyst than copper iodide.

3.4.3.3 Hydrolysis of diester (74)

The diester (74) was smoothly hydrolysed by treatment with two equivalents of sodium hydroxide for 4 hours. The reaction mixture was acidified and β-homoquinolinic acid (45) isolated after ion exchange (Dowex 50, H^+) column chromatography and recrystallisation, in good yield (78%) (Scheme 8). On larger scales the reaction mixture had to be purified by silica column chromatography due to the presence of impurities (a possible result of decarboxylation) before recrystallisation of the acid (45).

Scheme 8



(74) (45)

Elemental analysis of β-homoquinolinic acid confirmed the molecular formula as $C_8H_7NO_4$. Its ^{13}C NMR spectrum showed singlets due to carboxy groups at δ 164.09 (CO_2H) and δ 174.71 (CH_2CO_2H) and its mass spectrum showed a molecular ion at m/e 181.

Quinolinic acid, therefore, could be homologated using the Arndt-Eistert methodology. Silver oxide was found to be essential for the rearrangement of the intermediate diazoketone (73) and although on larger scales longer purification procedures were required it was still possible to prepare relatively large amounts of β-homoquinolinic acid.

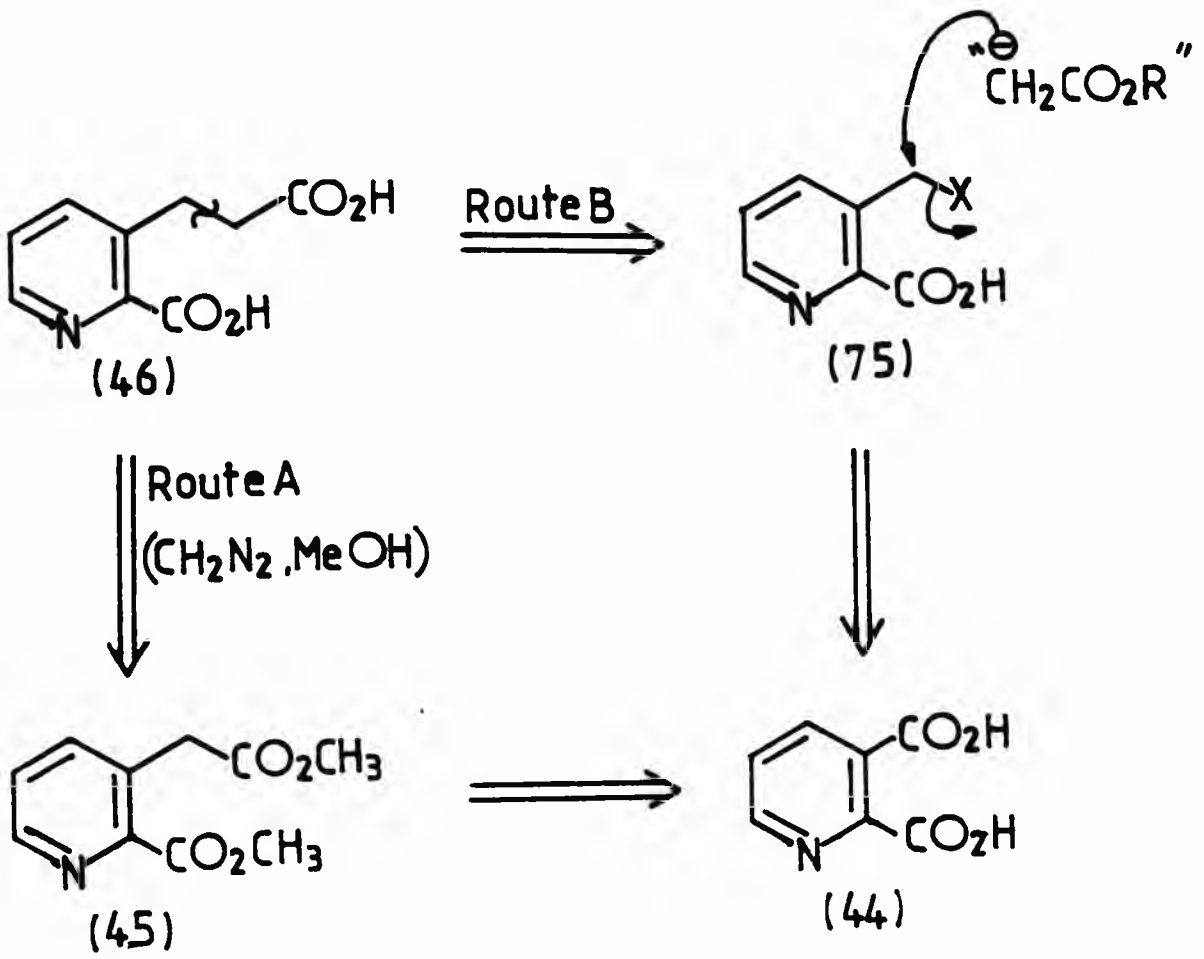
3.4.3 Approaches to the synthesis of 2-carboxy-3-(-2-carboxyethyl) pyridine (46)

3.4.3.1 Introduction: synthetic considerations

β-Homoquinolinic acid (45) could in theory be converted to the next higher homologue (46) using, once again, the Arndt-Eistert method (Route A). However, this would result in a lengthy route. Also, although the conversion of individual steps was efficient, the overall yield from quinolinic acid (twelve steps) would be very low. Therefore, since we could always revert to this route, alternative approaches were examined to achieve better conversion of quinolinic acid.

One possible avenue (Route B) required that the carboxylic acid group (3-position) be "converted" to a halomethyl moiety (CH_2-X, where X is a leaving group (e.g. halogen or Otosyl) which could be derived from an alcohol) which would then be reacted with a two carbon homologating nucleophile (e.g. diethyl potassio malonate or ethyl lithio acetate). (Scheme 9).

Scheme 9



Initially, we attempted to synthesise, selectively, the alcohol (76), hoping to convert the hydroxyl to either X or Otosyl.

3.4.3.2 Synthesis of 2-carbomethoxy-3-hydroxymethyl pyridine (76)

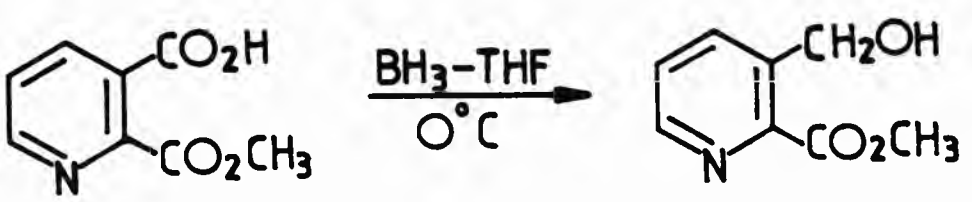
The diester (74) could not be used as it lacked selectivity, therefore, 3-carboxy-2-carbomethoxy pyridine (71), easily available, had to be used.

The selective reduction of a carboxylic acid to the corresponding primary alcohol, in the presence of an ester or other substituents, by

borane in tetrahydrofuran (THF) has been well documented[20,21,188,189]. Lithium aluminium hydride or sodium borohydride are non-selective, and would give 2,3-dihydroxymethyl pyridine.

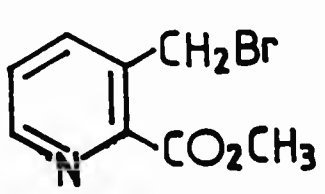
Using borane in dry THF (Scheme 10) the acid (71) smoothly afforded the alcohol (76) in good yield, 65% after ion exchange column chromatography and recrystallisation. The alcohol's (76) ^{1}H NMR spectrum showed a singlet at δ 7.92 - 8.83 due to pyridyl protons. Its mass spectrum exhibited molecular ions at m/e 167 (m) and 149 (M-H_2O) which confirmed its structure.

Scheme 10

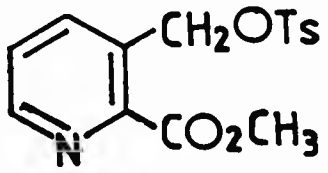


(71) (76)

All attempts to synthesise the bromo (77) or tosyl (78) derivatives of the alcohol (76) were unsuccessful. A variety of conditions and reagents were used (e.g. PBr_3 or CBr_4/PPh_3 or TsCl/py), but to no avail. The reactions were difficult to follow by TLC and there was a possibility of deleterious reaction of the products due to acidic by-products.



(77)



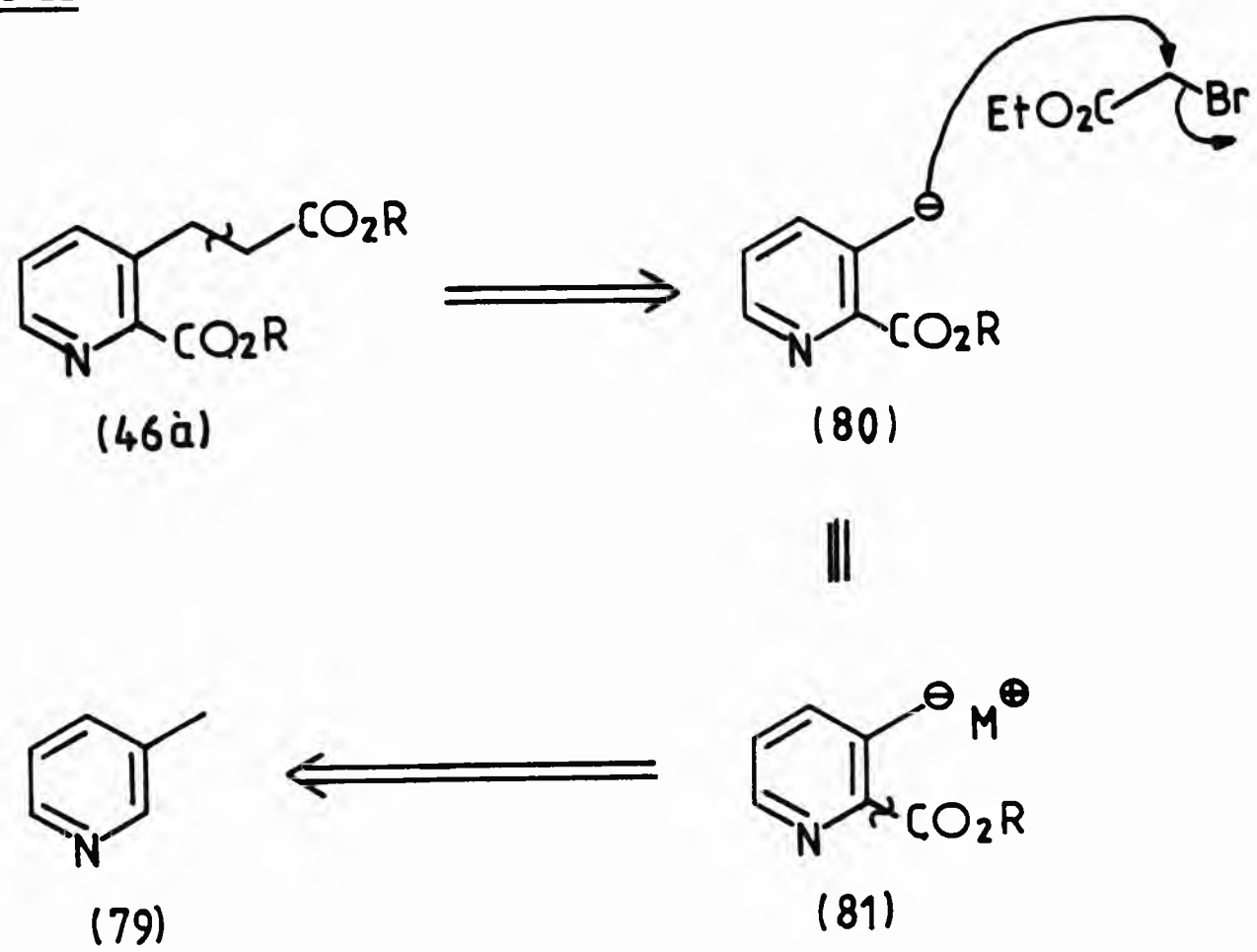
(78)

As the alcohol (76) could not, under the conditions used, be converted to the bromide or tosylate an alternative approach to the synthesis of 2-carboxy-3-(2-carboxyethyl) pyridine (46) had to be sought.

3.4.3.3 Attempted C-3 metallation of 2,3-lutidene

Since we could not obtain a methylene group activated toward nucleophilic displacement (i.e. pyridine (78)) it was decided to explore the possibility of achieving the desired homologation via the opposite strategy. If we could obtain a synthon for pyridine (80), then the reaction between it and, for example, ethyl bromoacetate ought to furnish the desired product. None of the intermediates prepared thus far could serve this purpose. A new starting material had to be sought. (Scheme 11).

Scheme 11

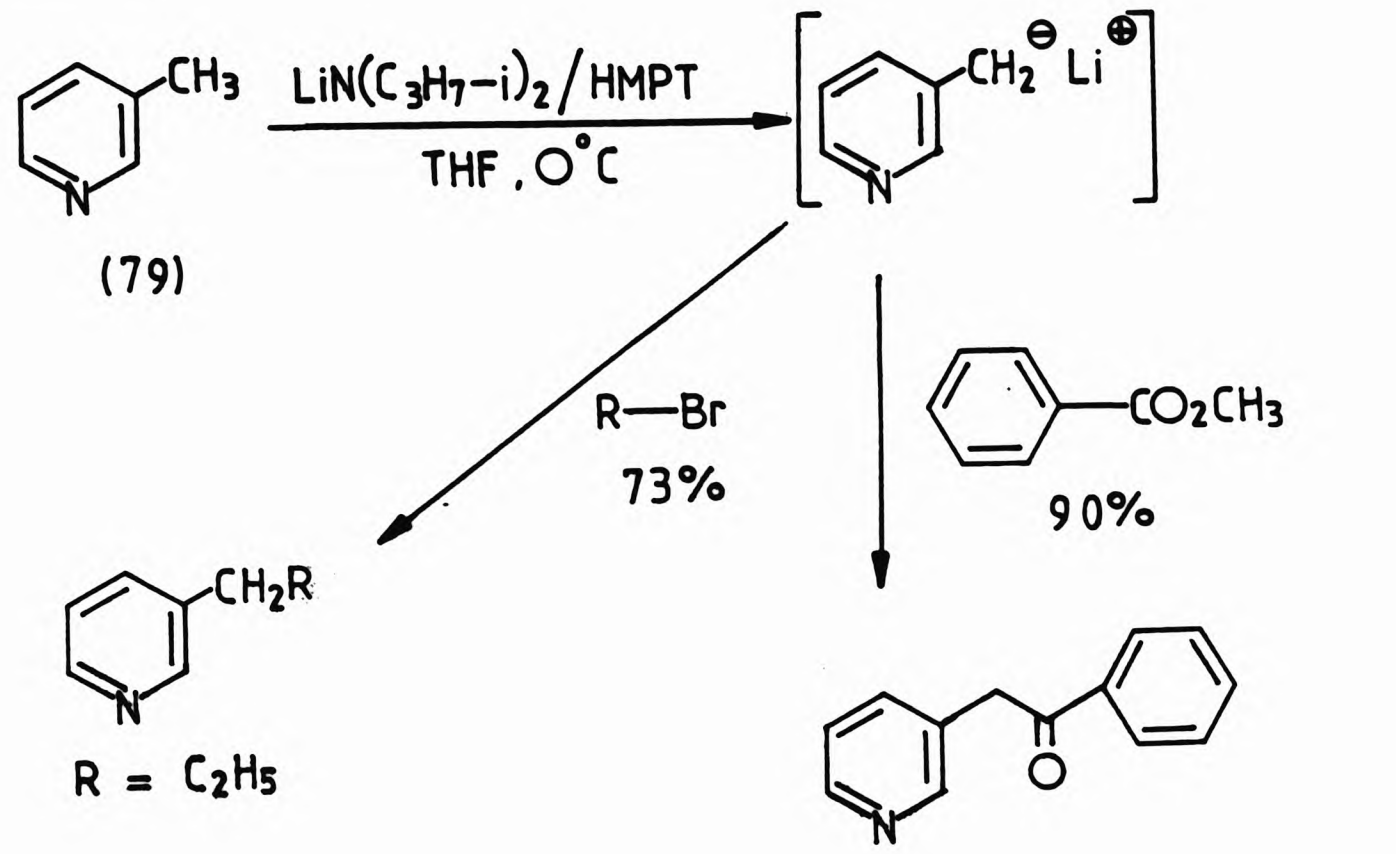


Since radical ethoxycarboxylation of 3-picoline (79)[123], in particular (and of pyridines in general) is possible in very low yield, we turned our attention to achieving the preparation of 'anion' (81) (and hence (80)), which could then be alkylated to afford the desired product (46a).

Although 3-picoline had been metalated and subsequent products obtained in poor yield, Kaiser[93] has shown that it can be metalated

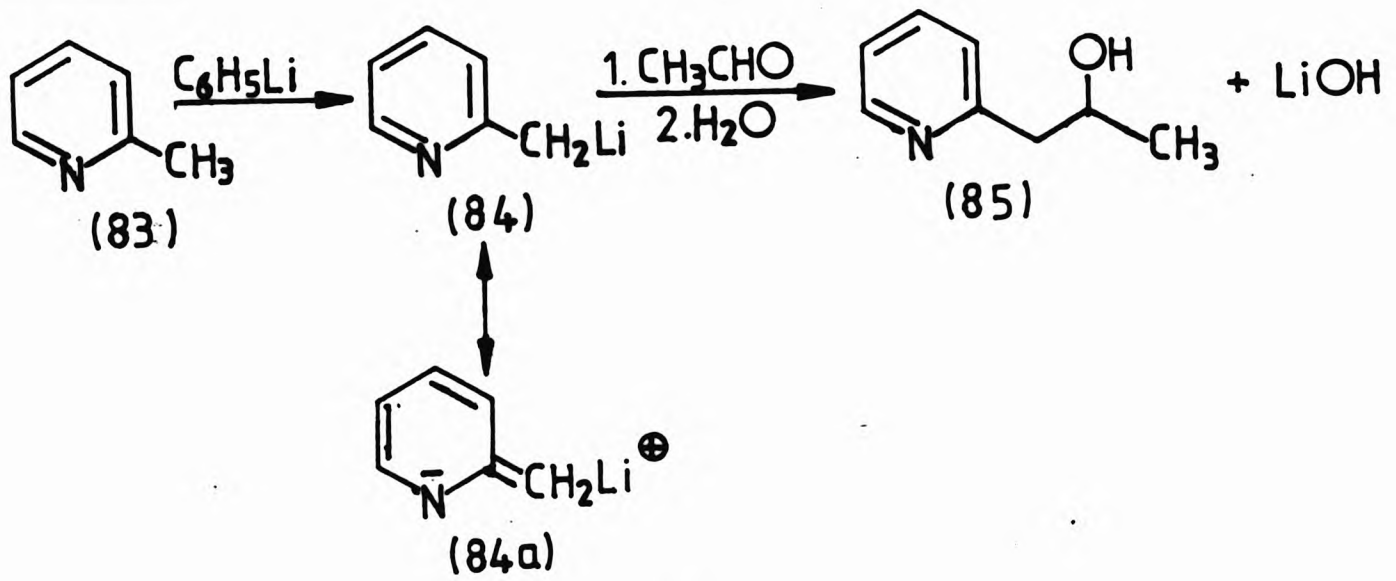
with a complex of lithium diisopropylamide (LDA): hexamethylphosphoric triamide (HMPT) in tetrahydrofuran at 0°C. Subsequent condensation with a variety of electrophiles afforded 3-substituted pyridines in good yields (Scheme 12).

Scheme 12



It is well known[179] that 2-picoline (83) for example can be converted to 2-(β-hydroxy-propyl)-pyridine (85) via the reaction of its lithium salt with acetaldehyde (Scheme 13).

Scheme 13

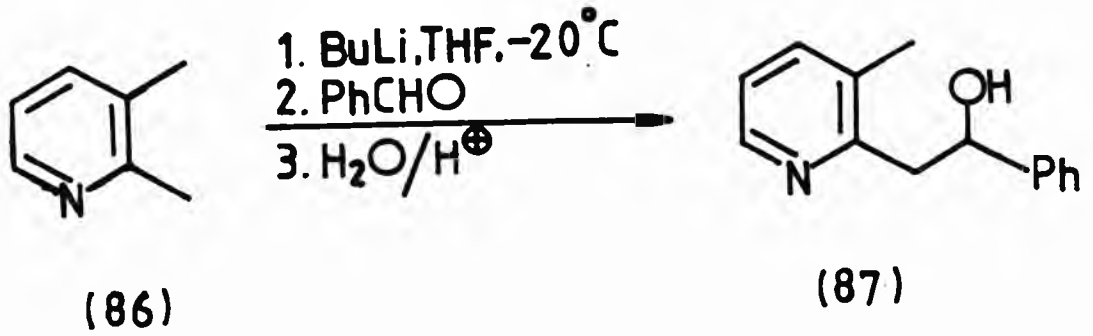


Similar work is well documented[76,93,123,179]. As a result of these facts the readily available 2,3-lutidine (86)[32] was used as starting material for the proposed synthesis of the acid (46). If the 2-methyl group, due to its greater acidity, could be selectively converted to a carboxy function, then subsequent selective metallation would achieve preparation of synthon (80).

3.4.3.3.1 Preparation of alcohol (87)

2,3-Lutidine (86) was treated with one equivalent of butyl lithium at -20°C; the resulting anion smoothly underwent condensation with benzaldehyde at 0°C (Scheme 14). The alcohol (87) was isolated in very good yield, 85% after column chromatography. The NMR spectrum of the alcohol (87) with a methylene singlet (3 protons) at δ 2.20 showed that only one methyl 'group' was still present; resonances due to the methylene protons appeared as a multiplet (δ 3.05); absorptions of the side chain methine proton at δ 5.28 and benzyl (and pyridyl) protons in the region, δ 7.10 - 8.38 indicated that benzaldehyde had reacted with only one methyl group. The presence of a strong hydroxyl group absorption at 3400 cm^{-1}, in the infrared spectrum of the alcohol (87) and its mass spectrum with a molecular ion at m/e 214 (m^+) confirmed the structure of the alcohol; analysis confirmed its molecular formula as $C_{14}H_{15}NO$.

Scheme 14



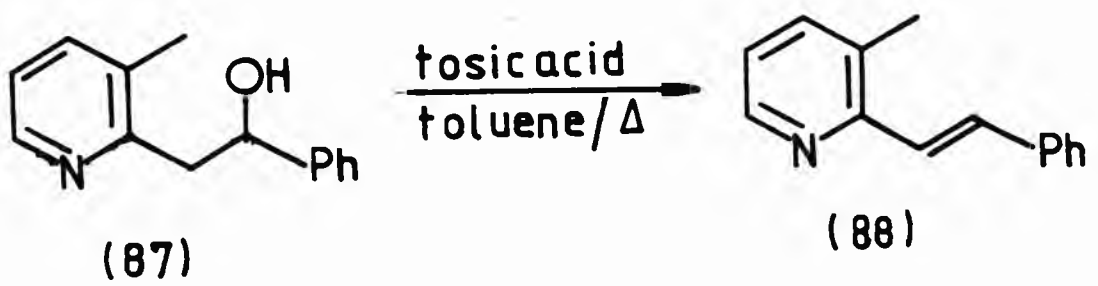
(86) (87)

3.4.3.3.2 Dehydration of alcohol (87)

Alcohol (87) was heated under reflux with toluene and one equivalent of para-toluenesulphonic acid over night (Scheme 15). The dehydration product, the alkene (88), a yellow oil, was afforded in good yield, 98% after column chromatography.

Analysis and an accurate mass spectrum of the alkene (88) established the molecular formula as $C_{14}H_{13}N$ (molecular ion at m/e 195.104). Absence of any absorption assignable to a hydroxyl group in the infrared spectrum of the alkene and the absence of the methine proton resonance (at δ 5.28), together with the presence of olefinic protons at δ 6.9 and the benzyl and pyridyl resonances (δ 7.05 - 8.46) confirmed its structure.

Scheme 15



(87) (88)

3.4.3.3.3 Synthesis of the carboxylic acid (89)

Pyridyl compounds containing a vinyl substituent have been converted to the carboxylic acid and/or aldehyde, by ozonolysis[3,108]. The carboxylic acid (89) was, thus, synthesised by passing ozone through a methanol solution of the alkene (88) at room temperature (Scheme 16). It was isolated in good yield (65% after recrystallisation); melting point 110°C (literature melting point 110°C[21a]). The NMR spectrum of the acid (89), which did not show absorptions due to the benzylidine moiety (Ph) i.e. no olefinic or phenyl protons, did have a methylene singlet at δ 2.71 and pyridyl resonances (three protons) in the δ 7.95 - 8.52.

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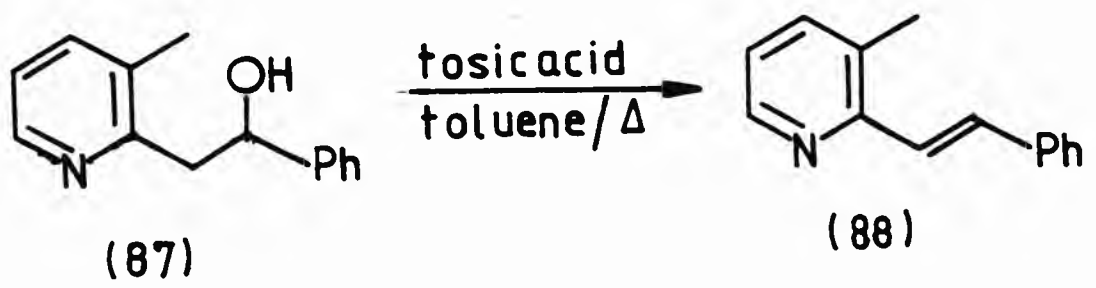
Scheme 15



(87) (88)

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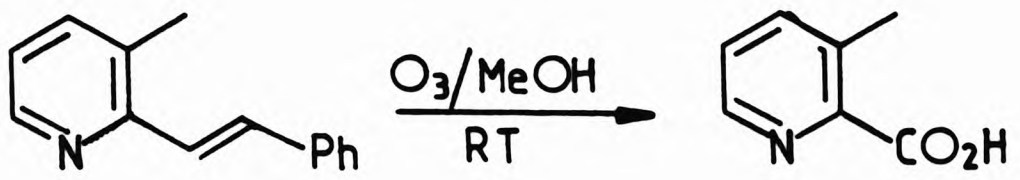
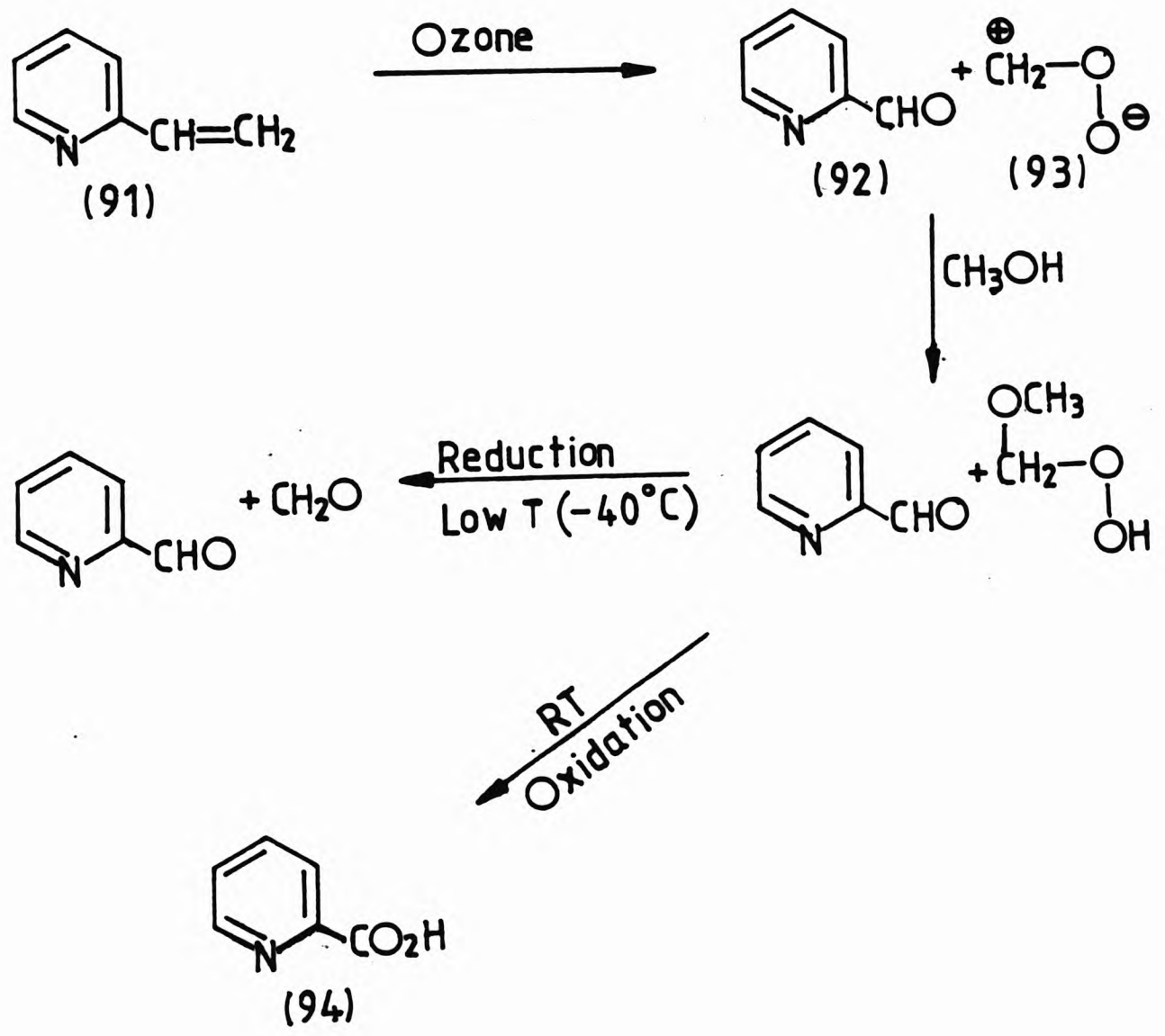
Scheme 16



(88) (89)

Analogous to the above reaction, the ozonolysis of vinylpyridine (91) in methanol at room temperature, was reported by Callighan[23]. At room temperature, the available oxygen converts the aldehyde (92) to pyridine carboxylic acid (94). Methanol stabilised the peroxidic zwitterion intermediate (93) by reacting with it (Scheme 17). Pyridine is thought to act as an internal base which increases the rate of oxidation.

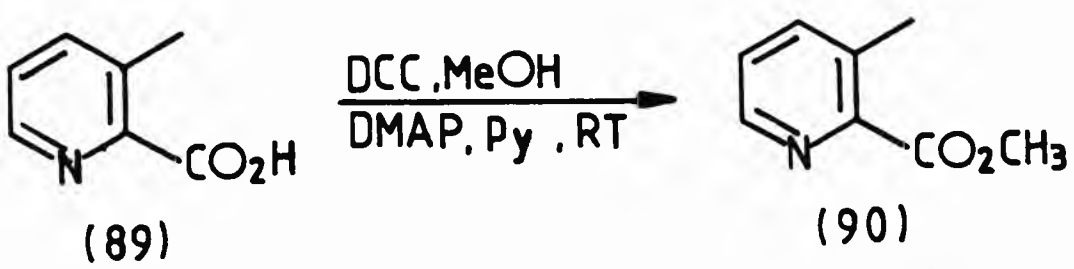
Scheme 17



3.4.3.3.4 Esterification of acid (89)

The acid (89) was not soluble in protic solvent e.g. THF or hexamethylphosphoramide (HMPA), which would be used in the subsequent metallation reactions, and was, therefore, converted to the methyl ester (90) using dicyclohexylcarbodiimide (DCC), methanol and pyridine (Scheme 18). Other methods, for example treatment with methanolic hydrochloric acid gave markedly lower yields (15 - 25%).

Scheme 18



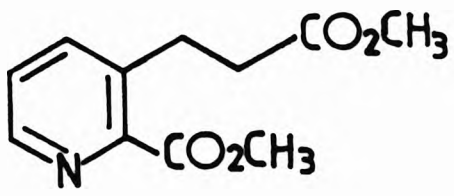
(89) (90)

The ester (90) was isolated in 80% yield after column chromatography. Proton resonances in its NMR spectrum were due to the C-3 methyl, a singlet at δ 2.60; the ester methyl, a singlet at δ 3.98 and pyridyl protons in the region δ 7.35 - 8.56.

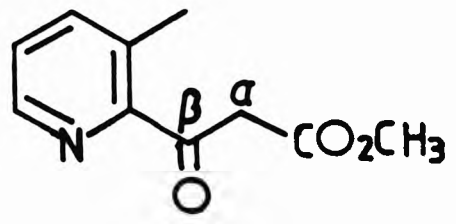
3.4.3.3.5 Attempted metallations of ester (90)

Ester (90) was added to a solution of freshly prepared lithium diisopropylamide:hexamethylphosphoramide (1:1) complex in tetrahydrofuran at 0°C (Kaiser's conditions); methylbromoacetate (the electrophile) was added and after work-up a product was isolated in 10% yield after column chromatography. Its ^1H NMR spectrum indicated that the expected product (96) had not been isolated i.e. the C-3 methyl singlet (at δ 2.57) was still present. An extra absorption due to methylene protons appeared at δ 3.8 (2H, multiplet) but as there were still three ring (pyridyl) protons the methylene group had to be attached to the C-2 functionality. An absorption at 1730 cm^{-1} indicated an ester was still

present and an accurate mass spectrum established the molecular formula as $C_{10}H_{11}NO_3$ (molecular ion at m/e 193.603) and a peak at m/e 92 (M-$COCH_2CO_2Me$) confirmed the structure of the product as β-keto-pyridyl ester (98).

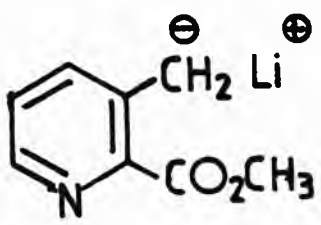


(96)

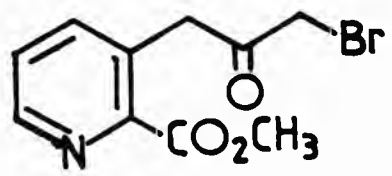


(98)

Alkylation at the 3-methyl group presumably had not occurred as neither of the two possible products; the alkylated pyridine (96) nor the acylated pyridine (97) had been in evidence. Pyridine (97) would arise from attack by anion (95) at the bromoacetate carbonyl.



(95)



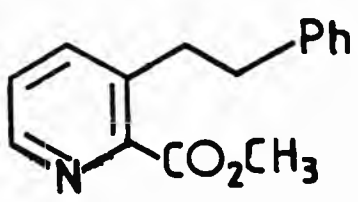
(97)

All attempts to carry out the reaction using modified reaction conditions e.g. solvent, time of reaction, temperature, more equivalents of base (for generating "anion") were equally unsuccessful.

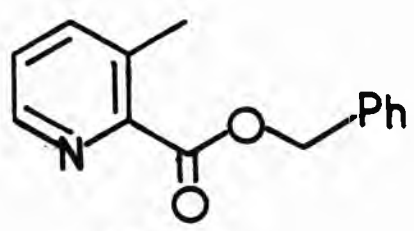
The initial reaction was repeated using the same conditions but a 'simpler model', mono- rather than difunctional alkylating agent, in order to determine whether failure to obtain the desired product was due to failure to metallate or due to some untoward reaction of the difunctional electrophile. Benzyl bromide was used instead of methyl bromoacetate.

Column chromatography afforded a product in 50% yield. However,

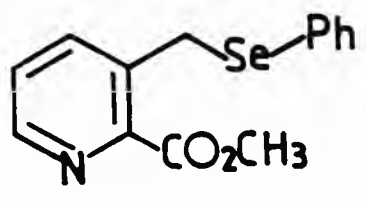
as with methyl bromoacetate its NMR spectrum showed C-3 methyl resonances (δ 2.55). Benzyl protons appeared as δ 5.44 and phenyl protons (and pyridyl) at δ 7.3 - 8.54 (8H). Its infrared spectrum showed ester absorption at 1730 cm^{-1} and mass spectrum, with molecular ions at m/e 228 (M^{+}) and 121 ($M^{+}-OCH_2Ph$), were consistent with the product having the structure of benzyl ester (100) and not the expected alkylated pyridine structure (99).



(99)



(100)



(101)

It was now clear that reaction was preferentially occurring at the ester carbonyl. To determine whether any "anion" (95) was being generated, phenylselenyl chloride, a very reactive carbanion-trapping agent[26a], was used as the electrophilic species (under conditions employed above). The carbanion derived product (101) was not obtained (starting material ∿ 60% was recovered).

Therefore, under conditions required to metallate the methyl group the ester group reacted preferentially. Work is in progress to determine under what conditions, if any, the C-3 methyl of 2,3-lutidine can be selectively metallated.

3.4.3.4 Attempted metallation of alkene (88)

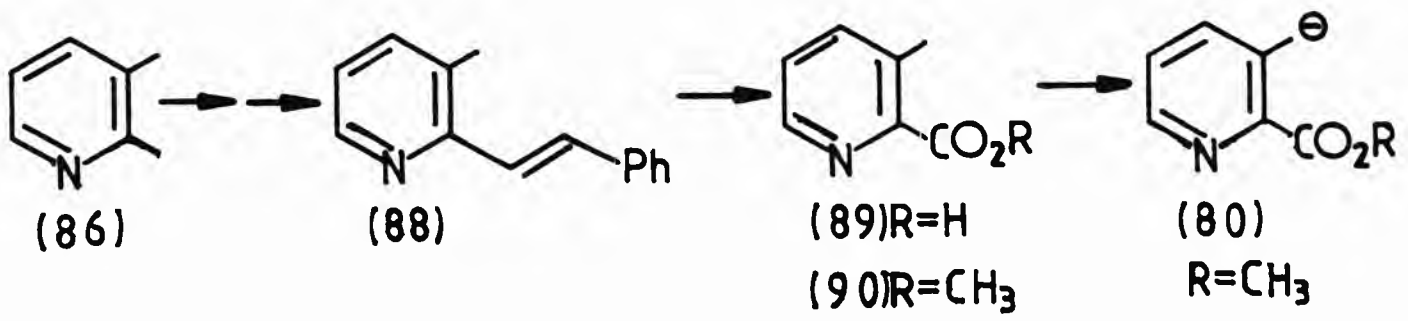
Using the same conditions as above, metallation of the alkene (88) was attempted, but no reaction occurred at -78°C, 0°C or higher temperatures.

3.4.4 Alternative approach to preparation of acid (46)

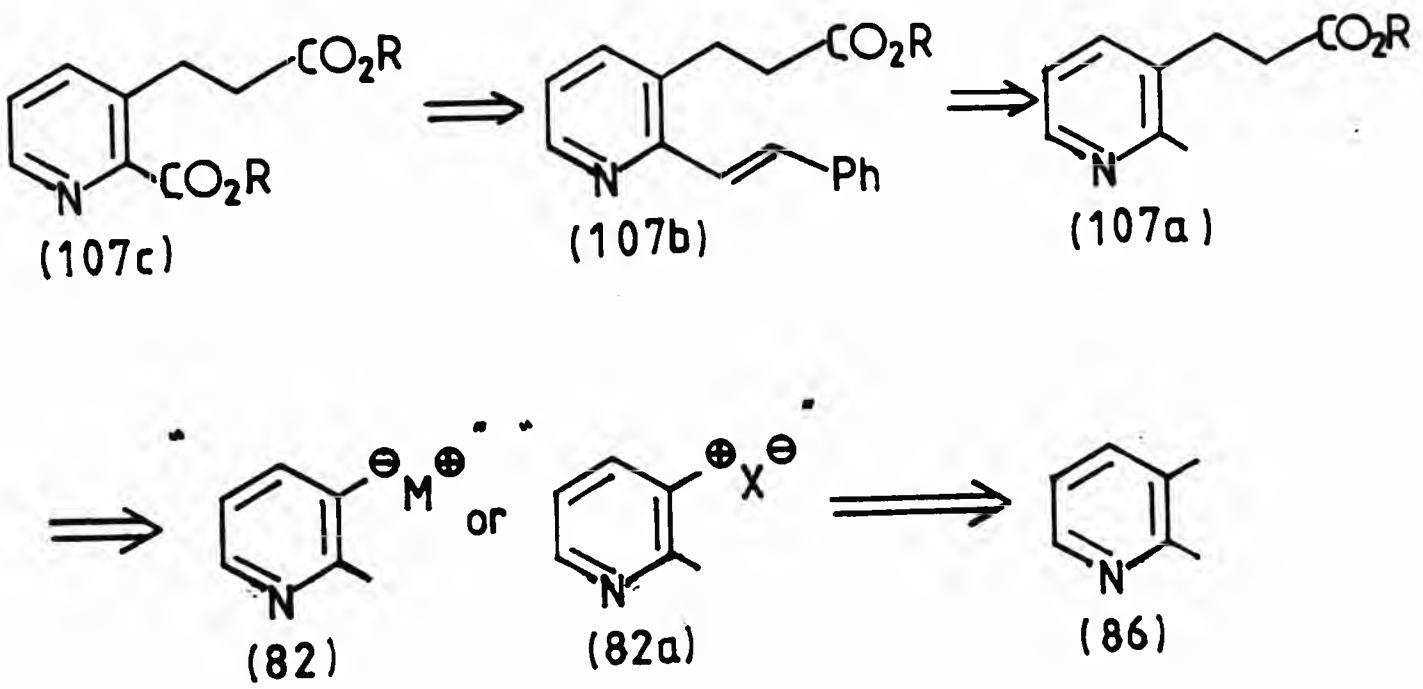
3.4.4.1 Selective functionalisation of the 3-methyl group in 2,3-lutidine (86)

Attempts to functionalise the ester (90) at the 3-methyl group had not succeeded due to preferential reaction at the ester carbonyl, under the reaction conditions employed. Therefore, a strategy was sought, whereby the 3-methyl moiety could be homologated initially and the 2-methyl group oxidised to a carboxy function (Scheme 20) (using the methodology developed previously (Scheme 19) thereafter:

Scheme 19



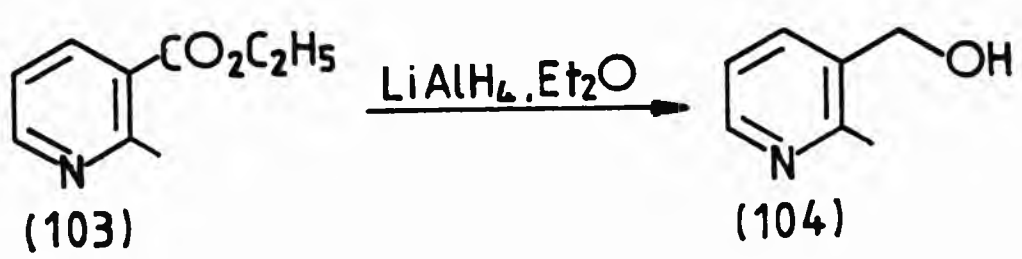
Scheme 20



A method had to be found, therefore, to convert the less reactive 3-position in 2,3-lutidine (86) into an electrophilic centre (i.e., (82a)) (since the converse approach had failed). A literature search revealed that 3-bromomethyl-2-methyl pyridine (105) had been prepared[67a] by two methods, though it had not been rigorously characterised. It was decided to prepare this intermediate:

Reduction of the readily available ethyl ester (103) with lithium aluminium hydride (three equivalents) furnished the alcohol (104) in 92% yield, after chromatography (Scheme 21).

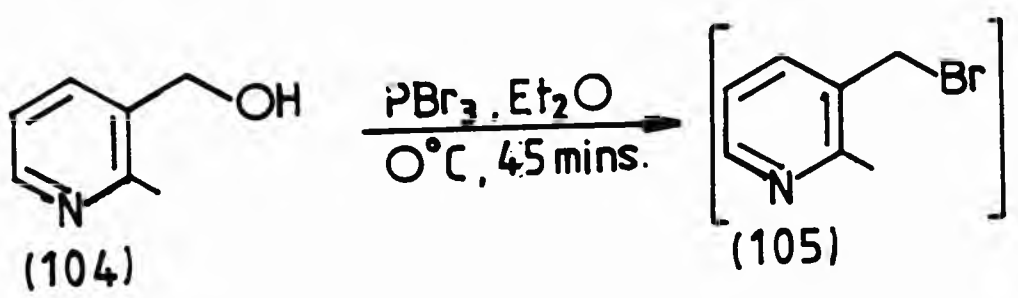
Scheme 21



(103) (104)

Treatment of an ethereal solution of the alcohol (104) with one equivalent of phosphorus tribromide, led to the total disappearance of starting material (TLC) and the appearance of a single, less polar, product. After standard extractive work-up this single spot was still apparent. However, all attempts to isolate the product were unsuccessful (Scheme 22). Evaporation of the solvent (diethyl ether), even at

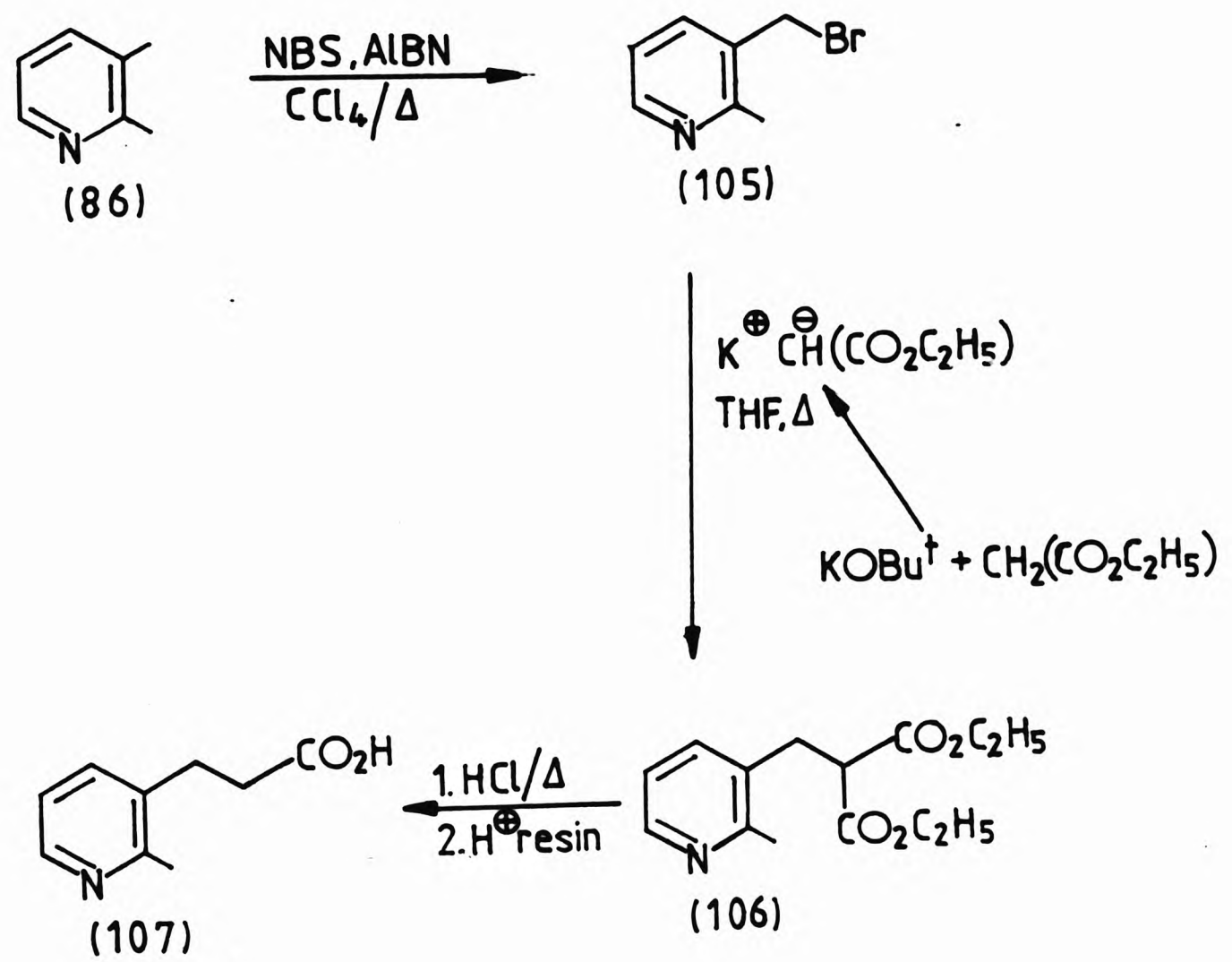
Scheme 22

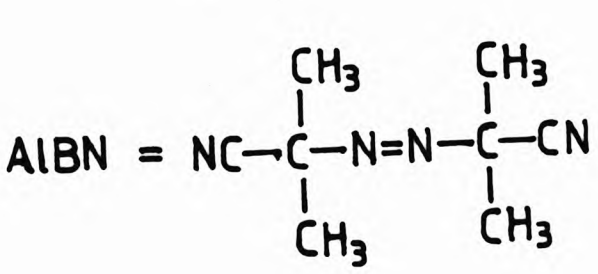


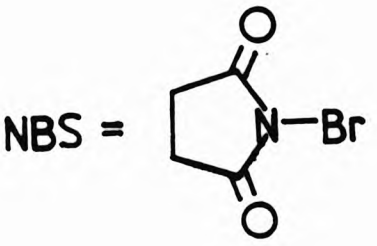
(104) (105)

0°C, only yielded a red solid, soluble now only in hydroxylic solvents (e.g. ethanol). It was apparent that the bromide was only stable in dilute solutions. Even on standing, these solutions were seen to slowly deposit a red solid over a period of 1 - 2 hours. Since it was impracticable to react the bromide <u>in situ</u>, due to the presence of acidic by-products (and because it was difficult to estimate the extent of decomposition and, hence, purity of the bromide), it was decided to use the bromide (105) via the other available method (Scheme 23).

Scheme 23





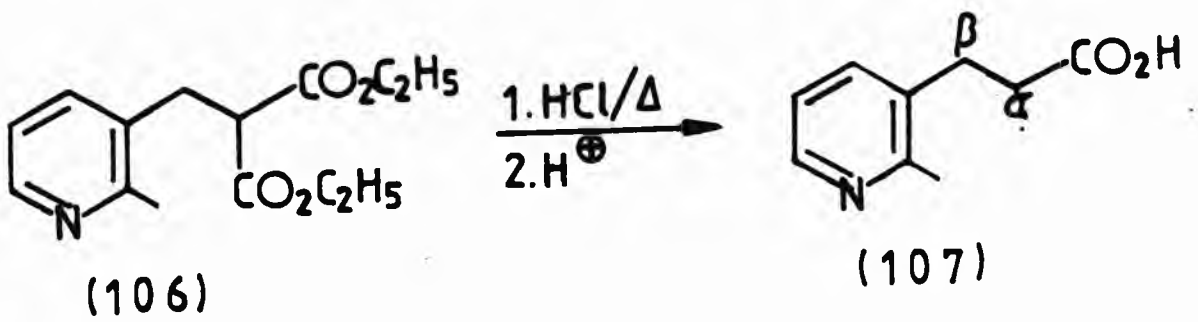


Reaction of 2,3-lutidine (86) with N-bromosuccinimide (NBS) in the presence of azobisisobutyronitrile (AIBN), with lamp irradiation, for 90 minutes, resulted in the disappearance (TLC) of starting material. A single, less polar reaction product, identical to that observed from the bromination of alcohol (104) appeared as the amount of starting material decreased. After concentration of the reaction mixture, the solution was added dropwise to a refluxing solution of potassiodiethyl malonate in dry THF. After 2½ hours no bromide was apparent (TLC). Standard extractive work up gave a 56% yield of the malonate adduct (106).

The selective C-3 bromination of 2,3-lutidine is extremely surprising in view of the fact that 2-picoline was found to be much more reactive than 3-picoline toward radical bromination[100a]. No explanation has been put forward to account for this anomalous reaction.

A ^{1}H NMR spectrum of the malonate adduct (106) exhibited peaks due to the 2-methyl group (δ 2.58) and the three pyridyl protons (C5 proton δ 7.05), C4 proton δ 7.5 and C6 proton δ 8.4). In addition to these absorptions and those of the ethyl groups of the ester (δ 1.2 and δ 4.2), the methine proton shift was observed at δ 3.5 and that of the "malonate" methylene group at δ 3.24. The adduct was further characterised by elemental (C,H,N) analysis.

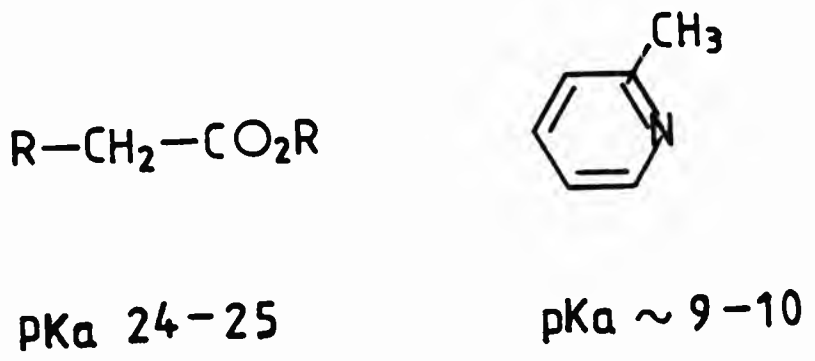
Scheme 24



(106) (107)

The adduct (106) was then successfully hydrolysed and decarboxylated to the acid (107), obtained in 88% yield after ion exchange chromatography and recrystallisation. The β methylene protons exhibited absorptions at δ 2.55, in the NMR spectrum of acid (107), and the α methylene protons at δ 3.05. A methyl singlet (δ 2.74) and three pyridyl proton multiplets (C5H: δ 7.7; C4H and C6H: δ 8.3) confirmed the presence of the 2-methyl pyridyl moiety. Once again, the acid was further characterised by C,H,N analysis.

Having successfully functionalised and homologated 2,3-lutidine at the C-3 position it should now be possible to oxidise the 2-methyl group of the acid (107) (presumably via an ester derivative) to a carboxyl function using the approach employed previously. The pKa of model systems[129a] would tend to lend support to this expectation:



Work is now in progress to achieve this end.

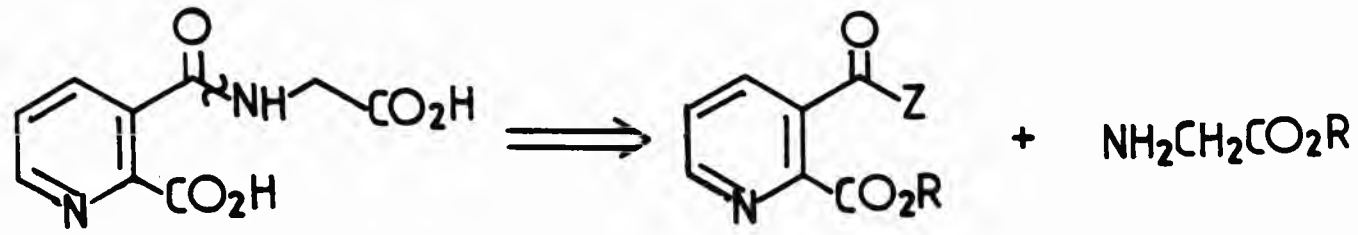
3.4.5 Synthesis of some peptide analogues of quinolinic acid

3.4.5.1 Synthesis of "glycine" analogue (47)

3.4.5.1.1 Introduction

From Scheme 25 an obvious disconnection indicates that the obvious starting material is quinolinic acid (44). But C-2 needs protection and therefore the C-2 ester of quinolinic acid, (71), prepared before, ought to be used. An activated acid is required and glycine (109) needs to be protected and therefore the ethyl ester was used.

Scheme 25



(47)

R=CH3

Z= activated carbonyl

(108)

R= C2H5

(109)

3.4.5.1.2 Preparation of peptide acid (47)

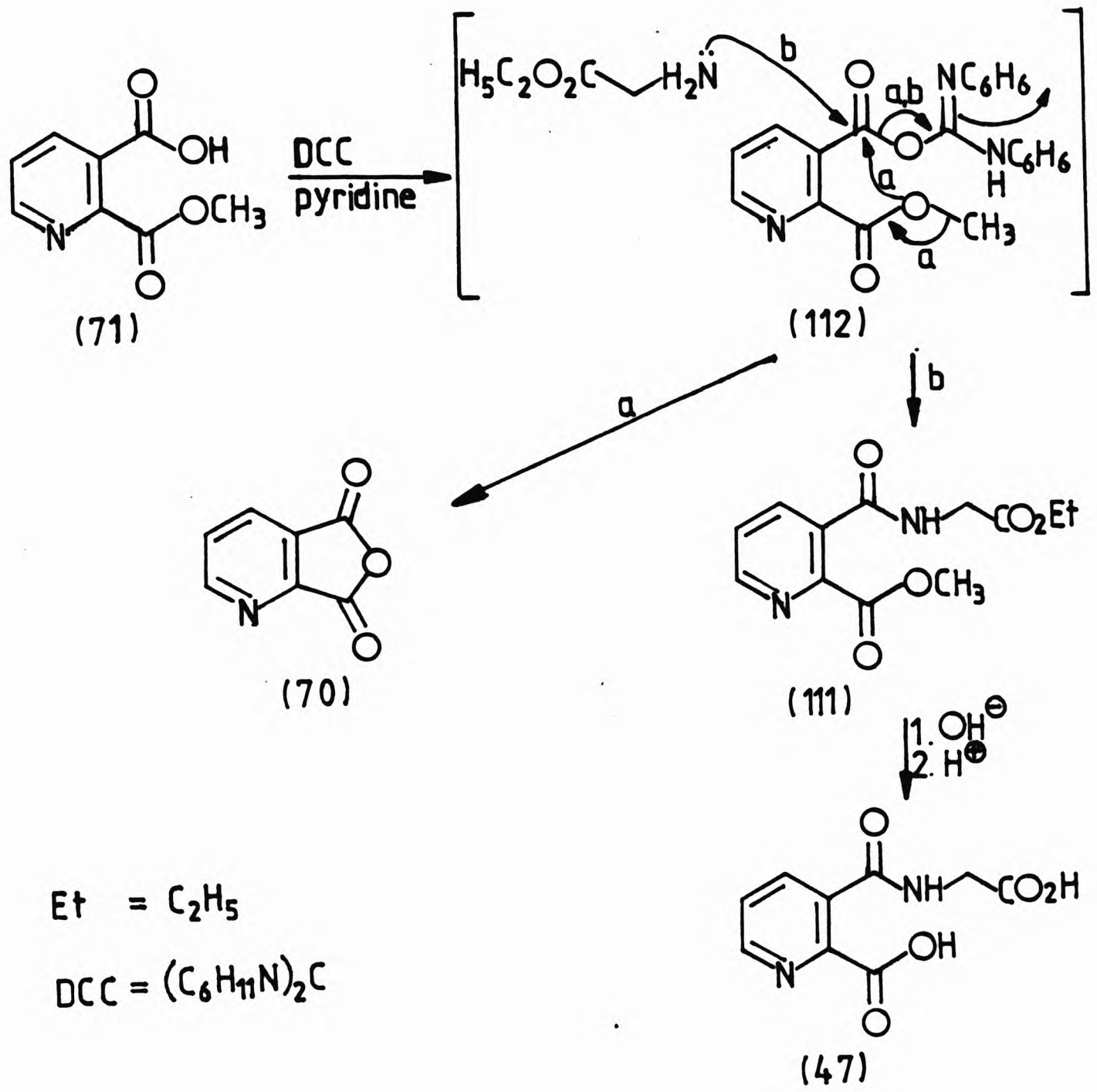
Initially, the ester (71) and glycine ethyl ester hydrochloride (110) were stirred at room temperature in dichloromethane, in the presence of one equivalent of dicyclohexylcarbodiimide (DCC) and three equivalents of pyridine. After work up and column chromatography, two products were isolated. The less polar product (67%) was identified as the anhydride (70) on the basis of its mp (136°C) and by TLC comparison of an authentic sample. The other product was characterised as the diester-amide (111) (24%) (Scheme 26).

An NMR spectrum of the amide (111) exhibited characteristic peaks due to the ethyl (three proton triplet at δ 1.32 and a methylene quartet at δ 4.20), and methyl esters (singlet at δ 4.0). In addition, a two proton singlet at δ 4.27 confirmed the presence of the methylene of the glycine residue. The structural assignment was further confirmed by mass spectroscopy (molecular ion at m/e 251).

Formation of the anhydride (70), no doubt, arises from intramolecular nucleophilic attack by the ester oxygen on the intermediate, activated ester (112). The fact that this is an intramolecular process accounts for the relatively high yield.

It was found that when the reaction was carried out at lower temperature (0°C) the yield of peptide (111) increased to (44%) but the

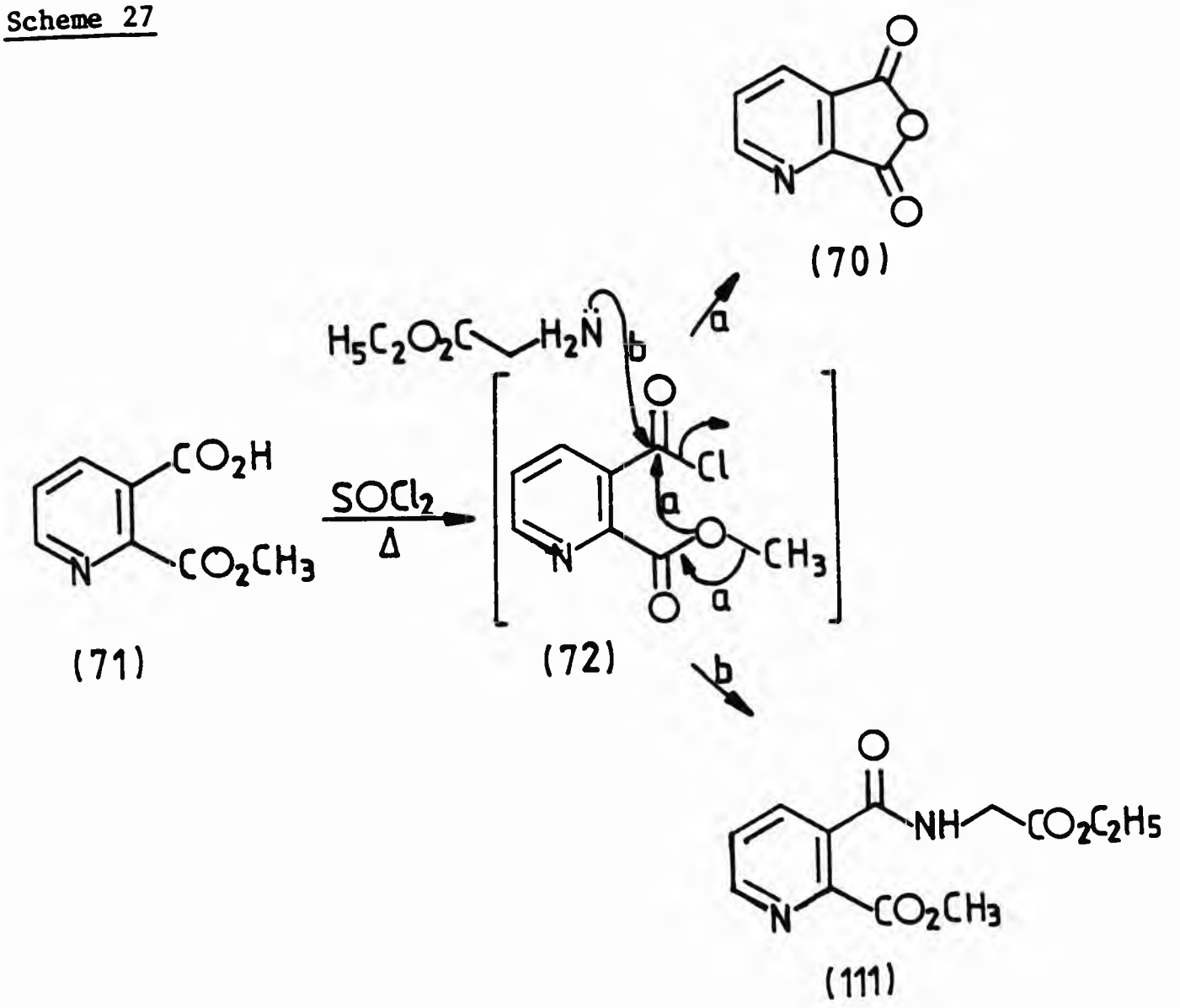
Scheme 26



anhydride (70) was still isolated in 28% yield.

Treatment of the acid chloride (72) with glycine ethyl ester hydrochloride, however, at 0°C, resulted in a 62% yield of peptide (111). The yield of anhydride had decreased to 12% (Scheme 27). Formation of anhydride (70) seems unavoidable when the methyl ester (71) is used. Perhaps the use of a more hindered ester (e.g. t-butyl) might be more efficacious.

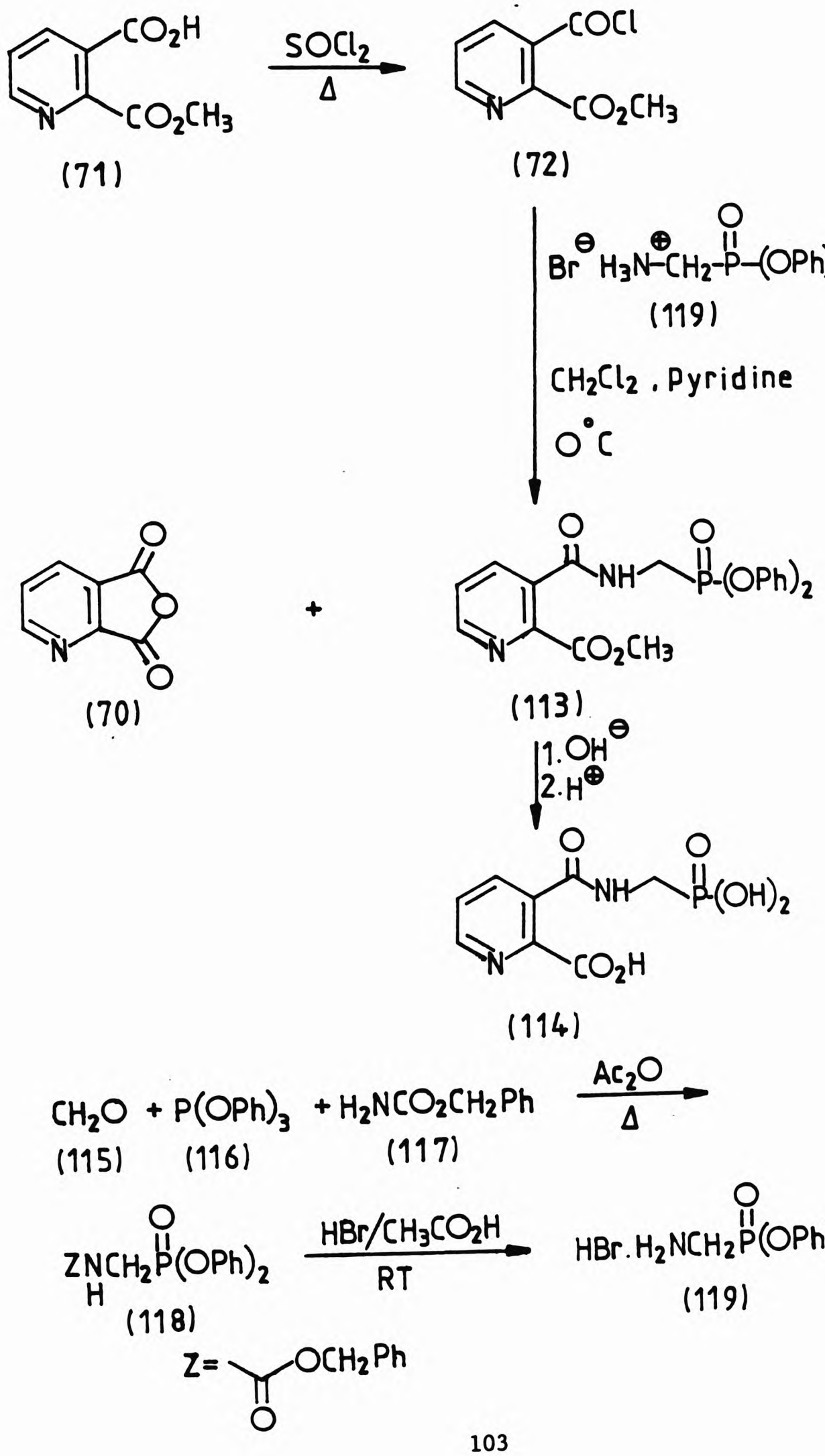
Scheme 27



Base hydrolysis of peptide (111) followed by ion exchange column chromatography afforded the acid (47) in good yield (86%). Elemental analysis of the acid (47) established the molecular formula as $C_9H_8NO_5$ and its mass spectrum exhibited a molecular ion at m/e 224. Absence of any absorptions, in the ^1H NMR spectrum of the acid (47) due to methyl or ethyl esters together with the presence of a methylene singlet at δ 4.24 and pyridyl resonances at δ 7.78 - 8.99 confirmed its structure.

3.4.5.1.2 Preparation of peptide acid (114)

Scheme 28



The diphenylphosphono-peptide (113) was prepared by the method developed previously for the carbon analogue (111). Reaction of the acid chloride (72), at 0°C, with the diphenylaminomethylphosphonate hydrobromide (119) afforded a 56% yield of the diester-amide (113), after column chromatography. Some anhydride (70) (20%) was again isolated. Base hydrolysis of the peptide (113), followed by ion exchange chromatography, gave the phosphonic acid (114) in 78% yield. The presence of phosphorus was confirmed by ^{31}P spectroscopy (singlet at δ 10.8 relative to phosphoric acid (H_3PO_4)).

3.4.6 Conclusion

β-Homoquinolinic acid (45) was successfully synthesised and although the higher homologue, the acid (46) was difficult to make due to the problem of dicarbonyl selectivity, this has been largely overcome. The way is now open for the preparation of more carboxylic analogues.

The peptide derivatives (47) and (114) were easier to make and therefore this series of analogues can be easily extended.

Preliminary studies by T. Stone[169a] of St. George's Hospital, London, have found β-homoquinolinic acid (45) to be at least twelve times more potent than quinolinic acid as a neurotoxin. Further pharmacological testing of acid (45) is in progress. Also, the effect of the amido (NH-C(=O)) moiety on the potency of the quinolinic derivatives (47) and (114) is currently being examined.

CHAPTER FOUR

APPROACHES TO THE SYNTHESIS OF TRICHOLOMIC ACID

4.1 Introduction

4.1.1 Isolation and purification

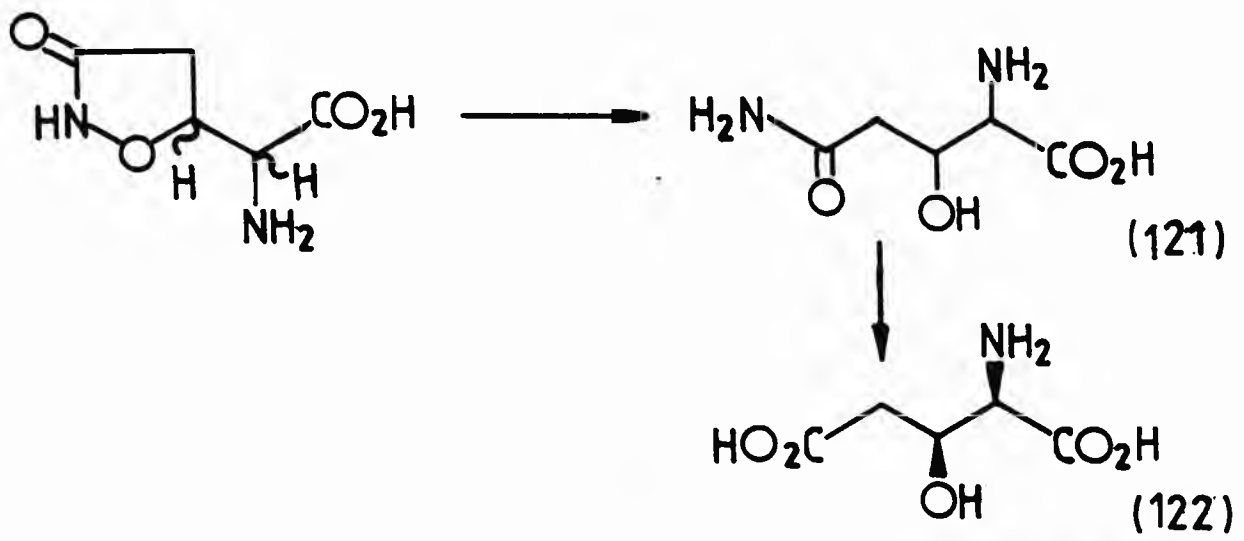
Takemoto[170,171], in the 1960s, investigated the fly-killing component of Tricholoma muscarium, a tasty mushroom which is widely distributed in Japan. The ready passage of the fly-killing component through cellophane membranes and its solubility properties suggested that it had a relatively low molecular weight, while its behaviour toward cation exchange resins, on paper electrophoresis and its reaction with ninhydrin suggested it was an acidic amino acid.

Chromatography of an aqueous extract of the mushroom (80 g of pulverised mycelium) on ion-exchange resin columns yielded a fraction (10 mg) which gave a positive ninhydrin test and had fly-killing activity. Recrystallisation of the fraction gave colourless prisms melting at 207°C (with decomposition) and an optical rotation of $[\alpha]_D$ +80.0° (c, 0.2, H_2O). The composition corresponded to a formula of $C_5H_8O_4N_2$. The new substance was named tricholomic acid after the mushroom from which it was obtained.

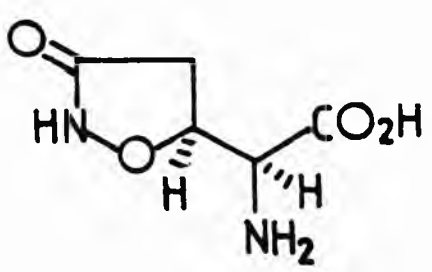
4.1.2 Structure elucidation

The structure of tricholomic acid was proposed as α-amino-3-oxo-5-isoxazolidine acetic acid. It rapidly decolourised aqueous potassium permanganate or bromine, indicating some unsaturation in the molecule. On hydrogenation over PtO_2, amide (121) was isolated which when hydrolysed gave the α-amino acid (122) (Scheme 1). The degradation product (122) was identified as erythro-3-hydroxy-L-glutamic acid[170,172].

Scheme 1

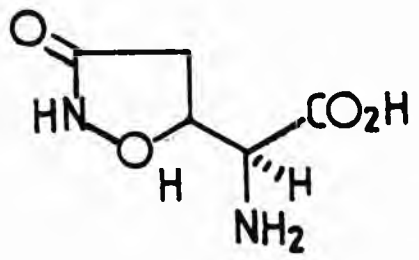


NMR spectroscopy had indicated the presence of a CH_2CHCH moiety, therefore, the only possible structures to fit the evidence were the erythro- and threo-isoxalidines, (50) and (120) respectively.



ERYTHRO

(50)



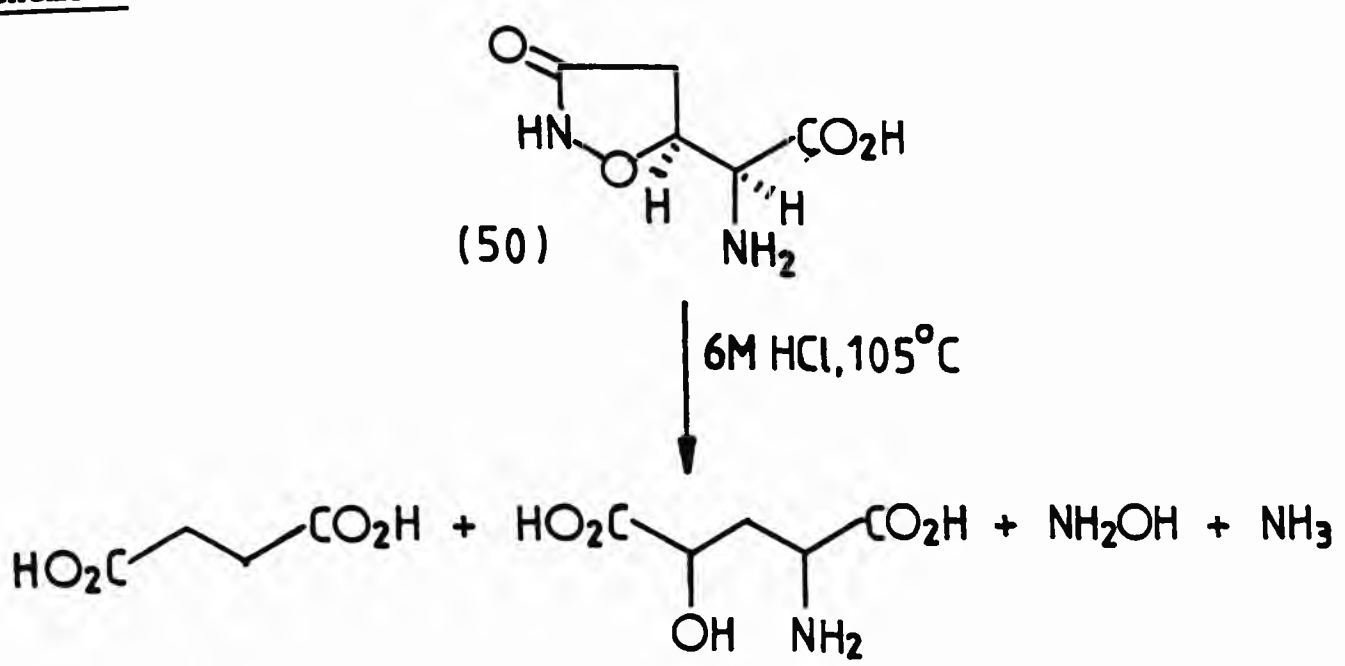
THREO

(120)

The postulated structure was confirmed by ultraviolet spectra and by the treatment of tricholomic acid with 6M HCl. The products of the hydrolysis were γ-hydroxyglutamic acid (123), succinic acid (66), hydroxylamine and ammonia (Scheme 2).

The D-erythro- and D- and L-threo-isomers of tricholomic acid, unlike the L-erythro-isomer are not biologically active[87] as excitants/ taste enhancers.

Scheme 2



(50) (66) (123)

4.1.3 Biosynthesis

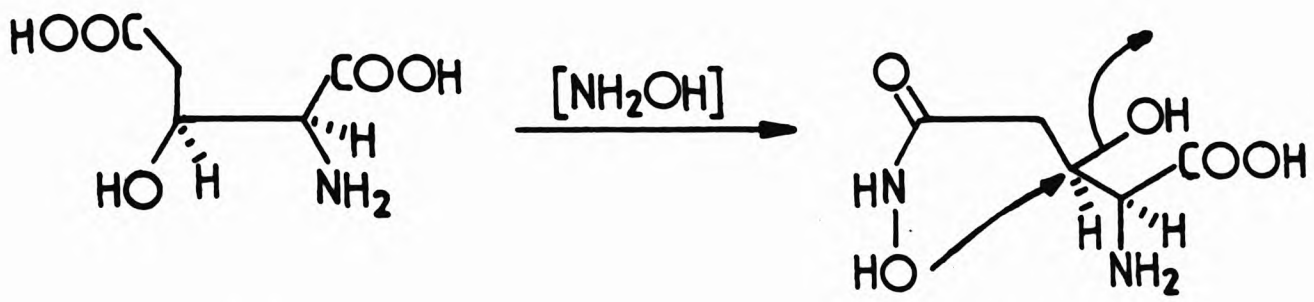
Apart from tricholomic acid (50) only a few other compounds containing modified isoxazole rings have been isolated. Ibotenic acid (26), muscimol (125) and muscazone (126) have been isolated from the mushroom, Amanita. The structural similarity between tricholomic acid and ibotenic acid (except for the oxidation state) prompted the proposal of a common mode of biogenesis[57a] (Scheme 3).

β-Hydroxyglutamic acid (122) is thought to be converted to the hydroxylamine (124) which is then cylised to tricholomic acid. Subsequent loss of a proton and a hydride ion gives ibotenic acid which can be decarboxylated to muscimol or be transformed in a number of steps to muscazone.

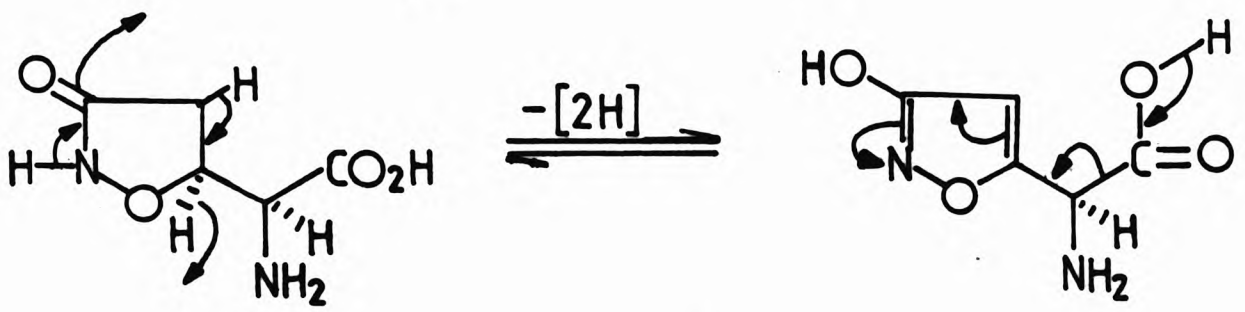
4.1.4 Pharmacological aspects of tricholomic acid related compounds

Recent Japanese studies[170a] suggest that tricholomic acid is a very potent neuroexcitant of the quisqualate/ glutamate receptor. Tricholomic acid (50) can be thought of as a conformationally restricted analogue of glutamic acid (1).

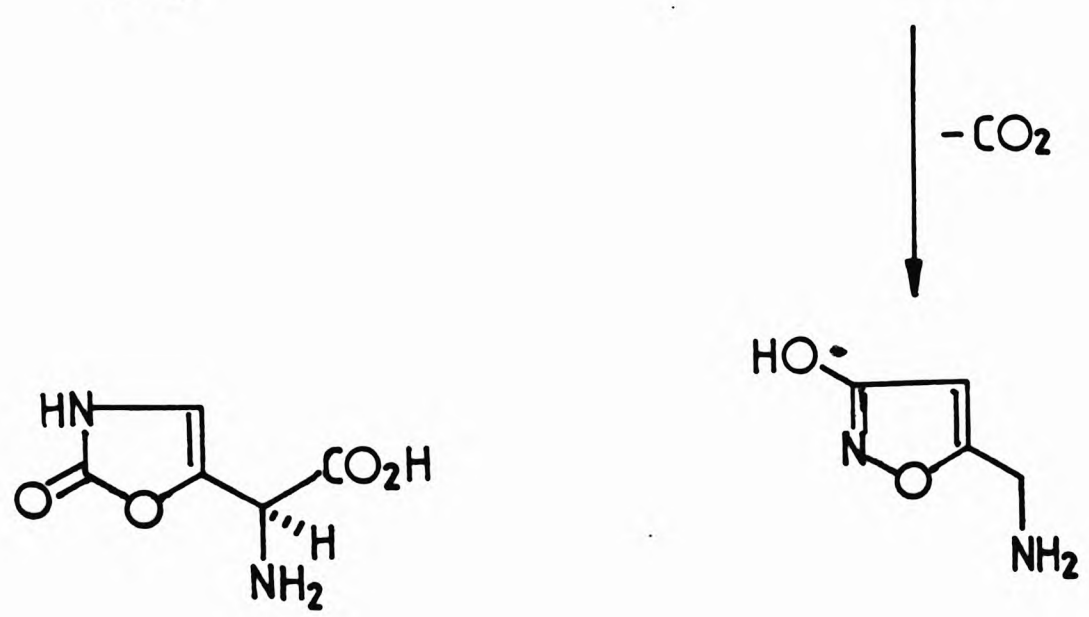
Scheme 3



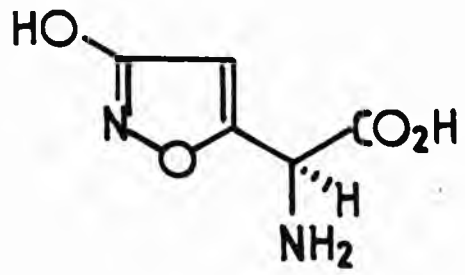
(122) (124)



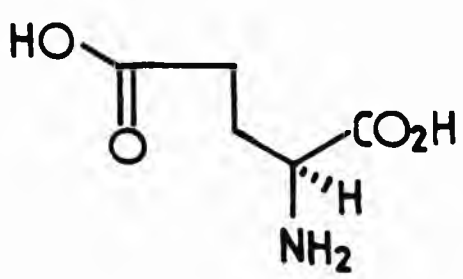
(50) (26)



(126) (125)



(26)

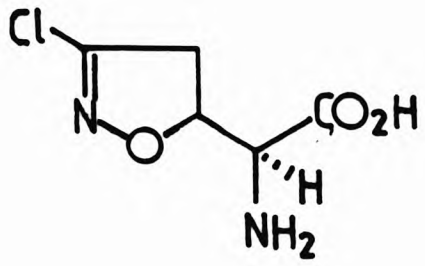


(1)

Ibotenic acid (26), an unsaturated analogue of tricholomic acid, is also a glutamic acid analogue, which has a strong neuroexcitant and neurotoxicant action[92,112,181]. Although ibotenate has been discussed theoretically as a selective agonist for receptors showing a preference for glutamate, (in an 'extended' conformation)[92], it is in fact a preferential NMDA agonist[52,117,182]. Ibotenic acid's effective interaction with a NMDA-receptor is thought to be a function of its carboxyl groups being less than maximally separated.

Iontophoretic application of ibotenic acid to cat spinal neurons in vivo produces a biphasic action on neuronal excitability, i.e. an initial excitation followed by a long lasting inhibition[106]. This inhibition is antagonised by bicuculline (a known antagonist of muscimol (125) and γ-aminobutyric acid GABA (3)[57]), which suggests that this unusual effect, not seen with glutamic acid or other related analogues is the result of ibotenic acid forming its decarboxylated derivative, muscimol. This decarboxylation easily occurs in vitro.

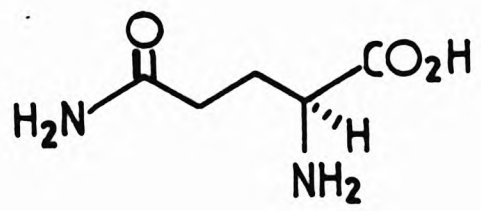
(αS, 5S)-α-Amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid (127) sometimes referred to as AT-125, is another structural analogue of tricholomic acid, though again in a different oxidation state.



(127)

AT-125, isolated from the actinomycete, Streptomyces sviceus[110], is an antimetabolite and an exceptional antitumor agent. It significantly increased the life span of tumor (L-120 or P388) bearing mice[74] and more interestingly, it significantly increased the life span of immune deficient mice implanted with a solid human mammary tumor. The toxicity of AT-125, as measured by weight change, was virtually nil at dose levels which significantly prolonged the life span of leukemic mice[74].

AT-125 has been shown to act as a glutamine antagonist in mammalian cells and as an inhibitor of various bacterial and mammalian enzymes which catalyse the transfer of the amide group of L-glutamine (2)[88].



(2)

L-Glutamine, a biological source of nitrogen, is utilised in the biosynthesis of a large number of essential cellular constituents. Thus, AT-125, as a glutamine antagonist, inhibits DNA (Deoxyribose nucleic acid) biosynthesis in tumor cells, presumably by impairing purine and pyrimidine biosynthesis.

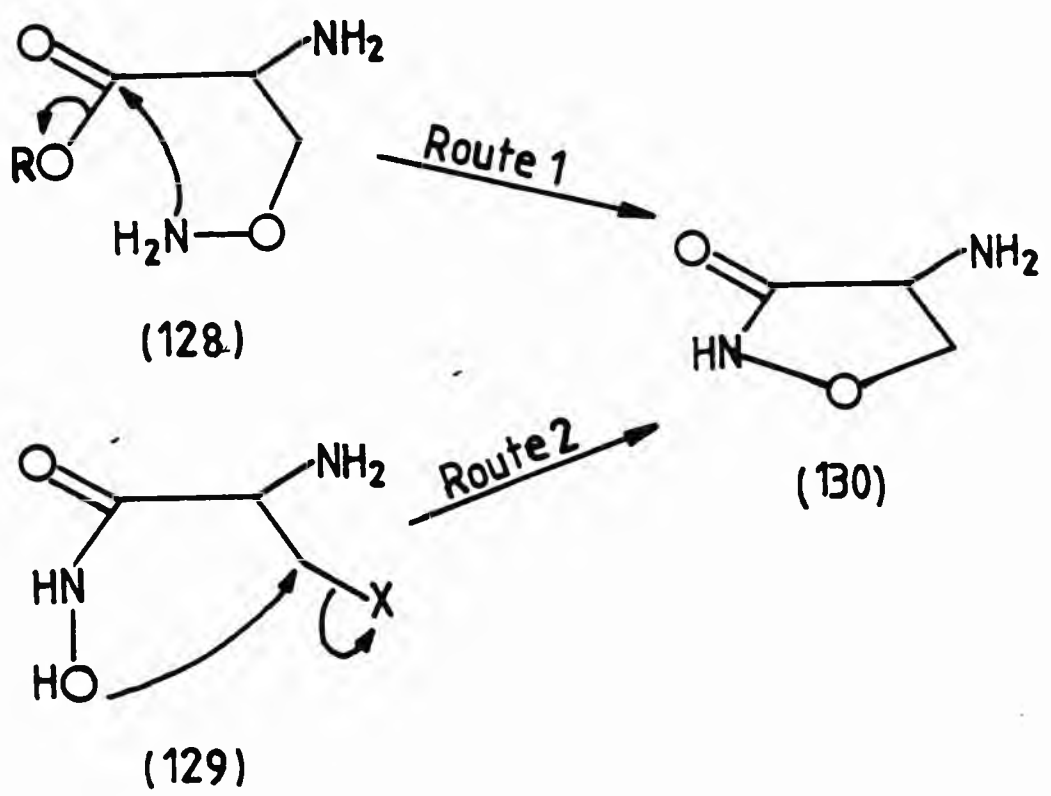
As the related compounds, ibotenic acid and AT-125 exhibit such marked biological activity it seems highly probable that tricholomic acid too is an 'active' compound. Thus, it was decided to subject tricholomic acid to pharmacological testing. However, a total lack of availability of the acid prompted us to undertake a literature search to determine whether tricholomic acid could be obtained by synthetic methods.

4.2 Review of Previous Approaches to the Syntheses of Tricholomic Acid

4.2.1 General approaches to isoxazole ring formation

Generally the methodology employed for the construction of the isoxazole ring system falls into one of two categories. The first involves ring closure of a (β-aminoxy) amino acid derivative (Route 1) and the second, the cyclisation of an appropriate β-hydroxamic acid (Route 2) (Scheme 4).

Scheme 4

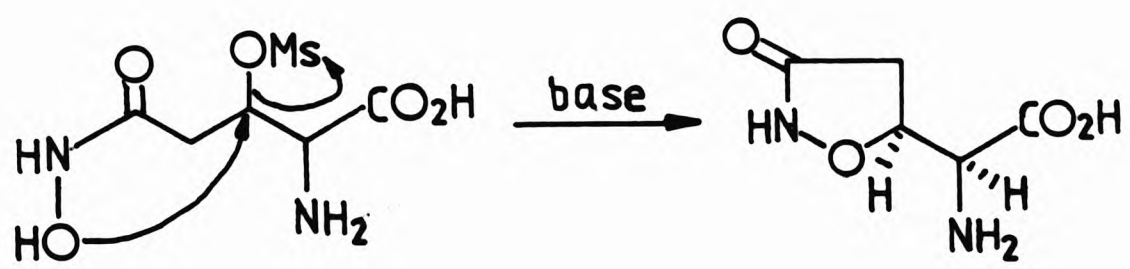


Brettschneider et al[24] prepared (β-aminooxy) amino ester (R=CH_3)(128) and subsequently racemic isoxazolidine (130) employing the synthetic strategy embodied in Route 1, while Plattner employed the alternative synthetic strategy (Route 2) in the cyclisation of β-halohydroxamic acid (X=Cl) (129)[24].

4.2.2 Previous syntheses of tricholomic acid

Kamiya[94] has reported several protracted synthetic routes to tricholomic acid (50) (Scheme 5), the key step being the cyclisation of an activated β-hydroxy-ω-hydroxamic acid (131). (Scheme 6)

Scheme 6

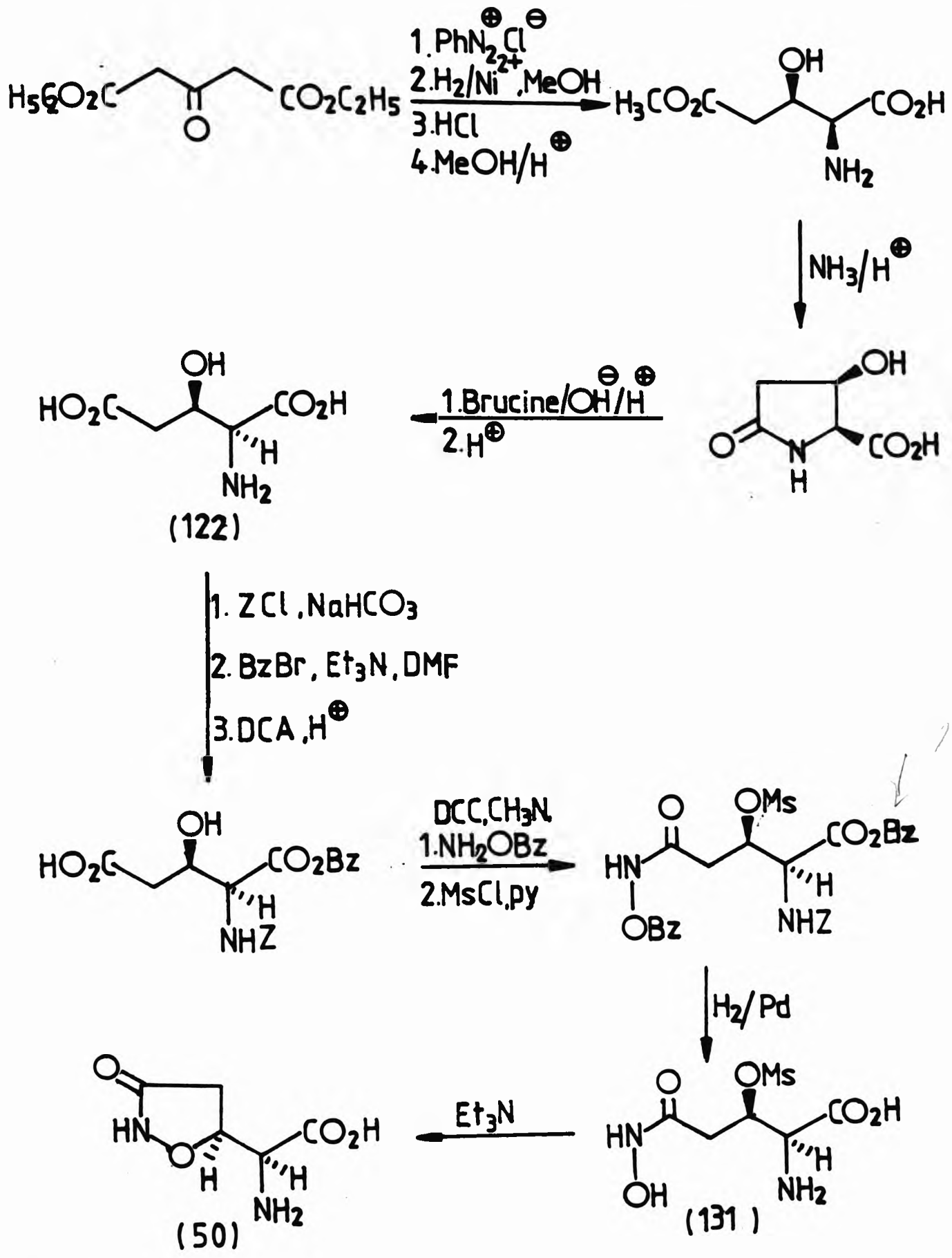


(130) (50)

It is very interesting to note that Kamiya[94] pointed out that neither threo- or erythro-tricholomic acid was actually isolated but that the diastereoisomers were merely "observed" by paper electrophoresis.

However, he stated that threo-tricholomic acid had been obtained from erythro-mesylates and erythro-tricholomic acid was obtained from threo-mesylates. It has been proposed that the formation of tricholomic acid from the cyclisation of the mesylate was due to an intramolecular rearrangement (Scheme 7).

Scheme 5



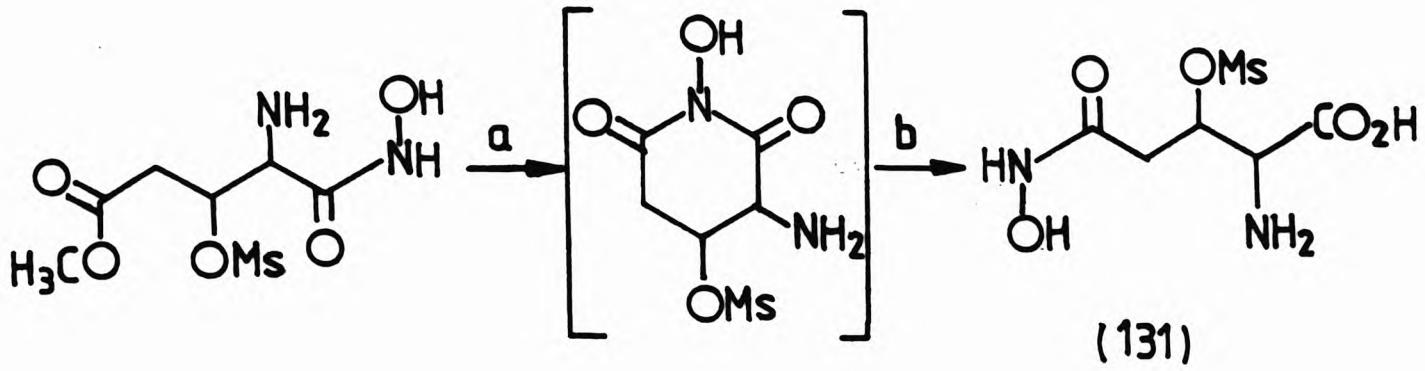
Bz = $-CH_2Ph$

Z = $-C(=O)-OCH_2Ph$

Ms = $-CH_3SO_2$

DCC = $(C_6H_{11}N)_2C$

Scheme 7



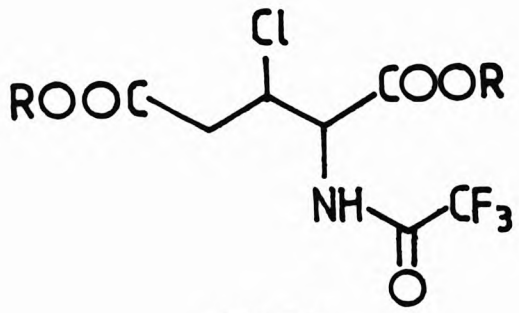
(131)

(a)-MeOH ; (b) H_2O

The final cyclisation was reported to occur with almost complete inversion of configuration at the β-asymmetric carbon.

In an alternative route using the N-trifluoroacetylated DL-dimethyl or diethyl 3-chloroglutamate (132), an isolated yield of only 1.7%, of racemic <u>erythro</u>-tricholomic acid (40), was reported.

R = CH_3
C_2H_5



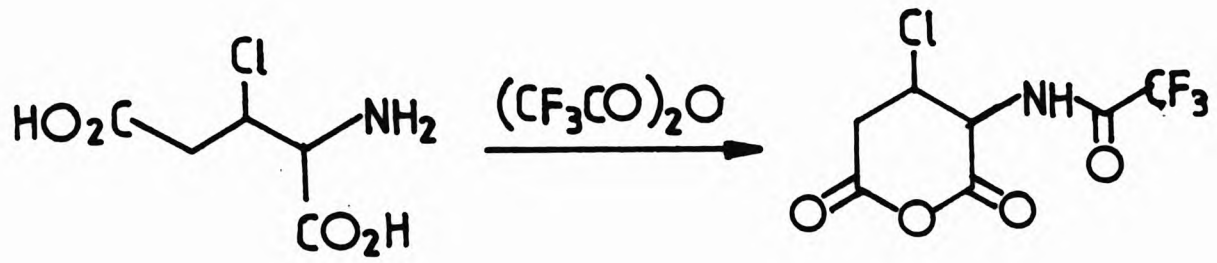
(132)

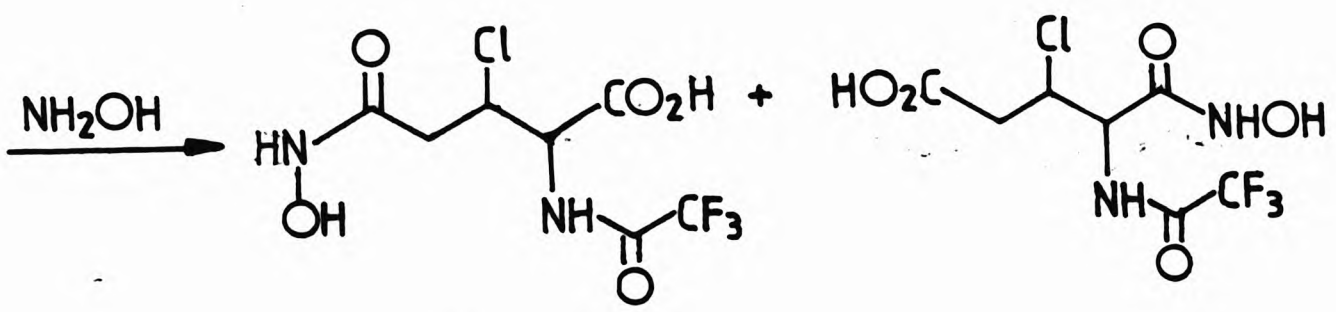
Another communication[86], which again does not disclose actual yields, gives the ratio of DL-<u>erythro</u> to DL-<u>threo</u>-tricholomic acid as 20:80. The melting points of the <u>erythro</u>- and <u>threo</u>-isomers are 195-198°C and 213-214°C (with decomposition), respectively. The melting point of natural tricholomic acid is 207°C[170]. Consequently, considerable doubt has been cast on the structure of the compounds Kamiya has prepared.

A similar synthesis involving β-chloro-glutamic hydroxamic acid

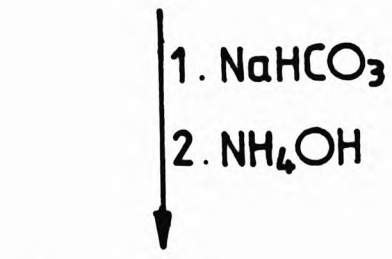
(133) as a key intermediate has been published, but without details[96]. (Scheme 8).

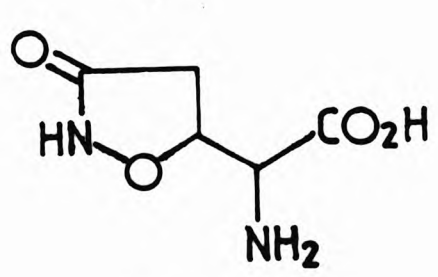
Scheme 8





(133)





(134)

The melting point of the final product (134) is given as 166-167°C, this is a huge discrepancy with the melting point, 207°C, of natural tricholomic acid, therefore tricholomic acid was obviously not prepared by this method.

The syntheses of racemic tricholomic acid described by Kamiya and Iwasaki were lengthy and low yielding and so too was the resolution procedure[87]. The separation of the D- and L-isomers utilised ethyl thiofluoroacetate, ammonia and quinine; total recovery of both isomers was only 13%.

In the fifteen years that have elapsed since these publications no reports of syntheses of tricholomic acid, using either Kamiya's unverified methods or similar routes have appeared. For this reason and the others mentioned above, it was decided that a synthetic route to tricholomic acid would have to be designed, which overcame these problems.

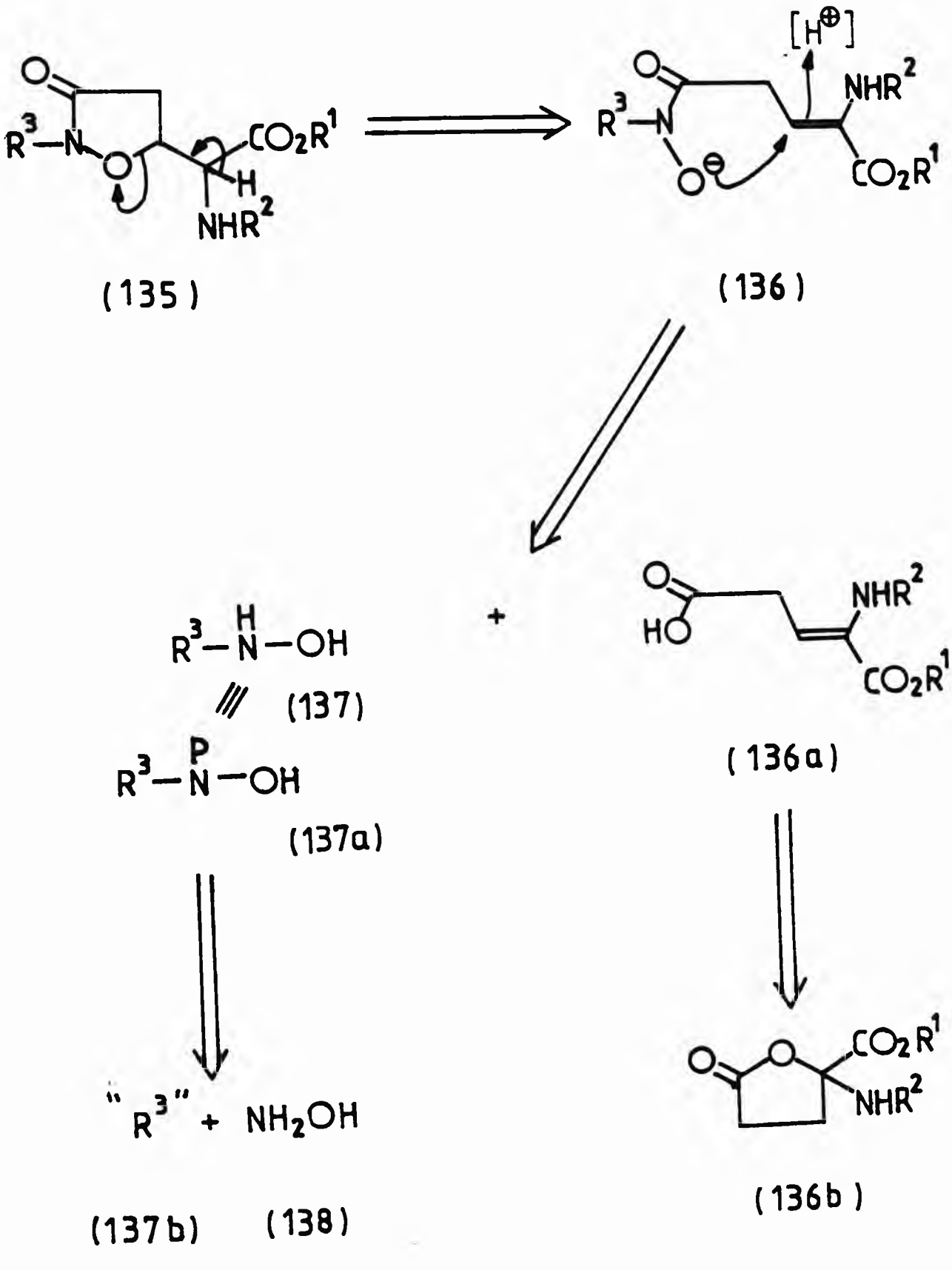
4.3 Synthesis of Tricholomic Acid

4.3.1 Retrosynthetic analysis

The simplest way to obtain tricholomic acid would be by the attempted hydrogenation of its unsaturated analogue, ibotenic acid (26). However, Takemoto has reported that hydrogenation of ibotenic acid gave an unstable product, due to ring opening, which could not be satisfactorily isolated[170]. Thus, it was decided to design a short, simple and flexible route to tricholomic acid which would require readily available starting materials and reagents (Scheme 9).

Coupling of the N-protected hydroxylamine (137a) and the di-protected glutamate (136a) would give the hydroxamic acid (136). Cyclisation of the hydroxamic acid to the triprotected isoxazolidine (135) (via an intramolecular Michael addition) is then followed by deprotections to give tricholomic acid. Such a triprotected tricholomic acid derivative had been prepared by Baldwin *et al*[4] in their elegant synthesis of AT-125.

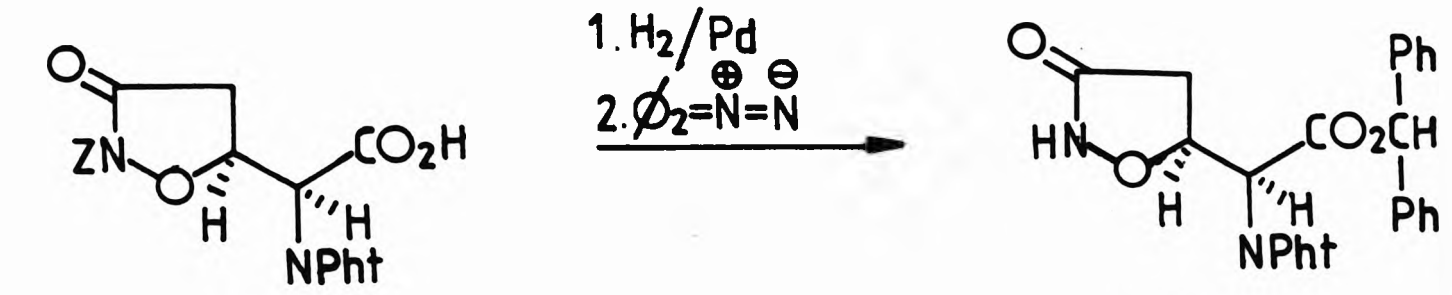
Scheme 9



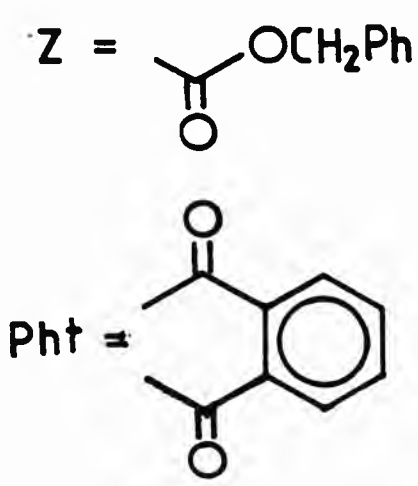
Apart from the use of simple starting materials, another attractive quality of this route is its flexibility with respect to the protecting groups, R^1, R^2 and R^3.

Kelly[95] has shown the nitrogen of the isoxazolidine ring is selectivity deprotected, without any cleavage of the sensitive N–O bond of the ring (Scheme 10).

Scheme 10



(139) (140)



Baldwin et al also showed that several different groups could be used to protect the isoxazolidine structure[4].

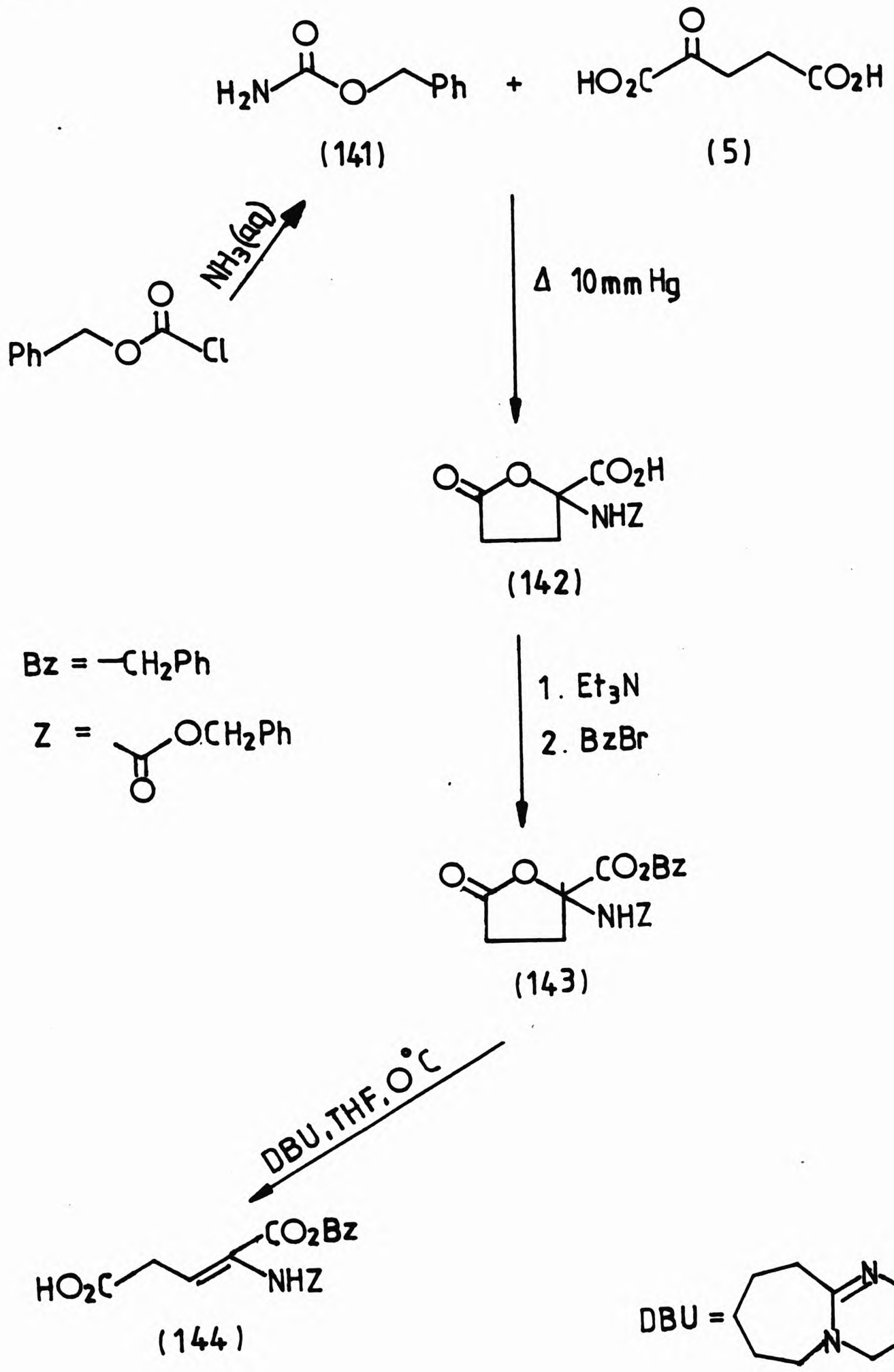
Thus, it was decided that the carboxylic acid would be protected as a benzyl ester (R^1) which could be easily removed with aluminium amalgam[24]. The two nitrogens would be protected with benzyloxy-carbonyl groups, deprotection would be simultaneously affected by dehydrogenation.

From the retrosynthetic scheme, the starting materials required are hydroxylamine (137) and a glutamic acid derivative (136a); the derivative can be easily prepared from the lactone acid (136b)[109].

4.3.2 Preparation of acid (144)

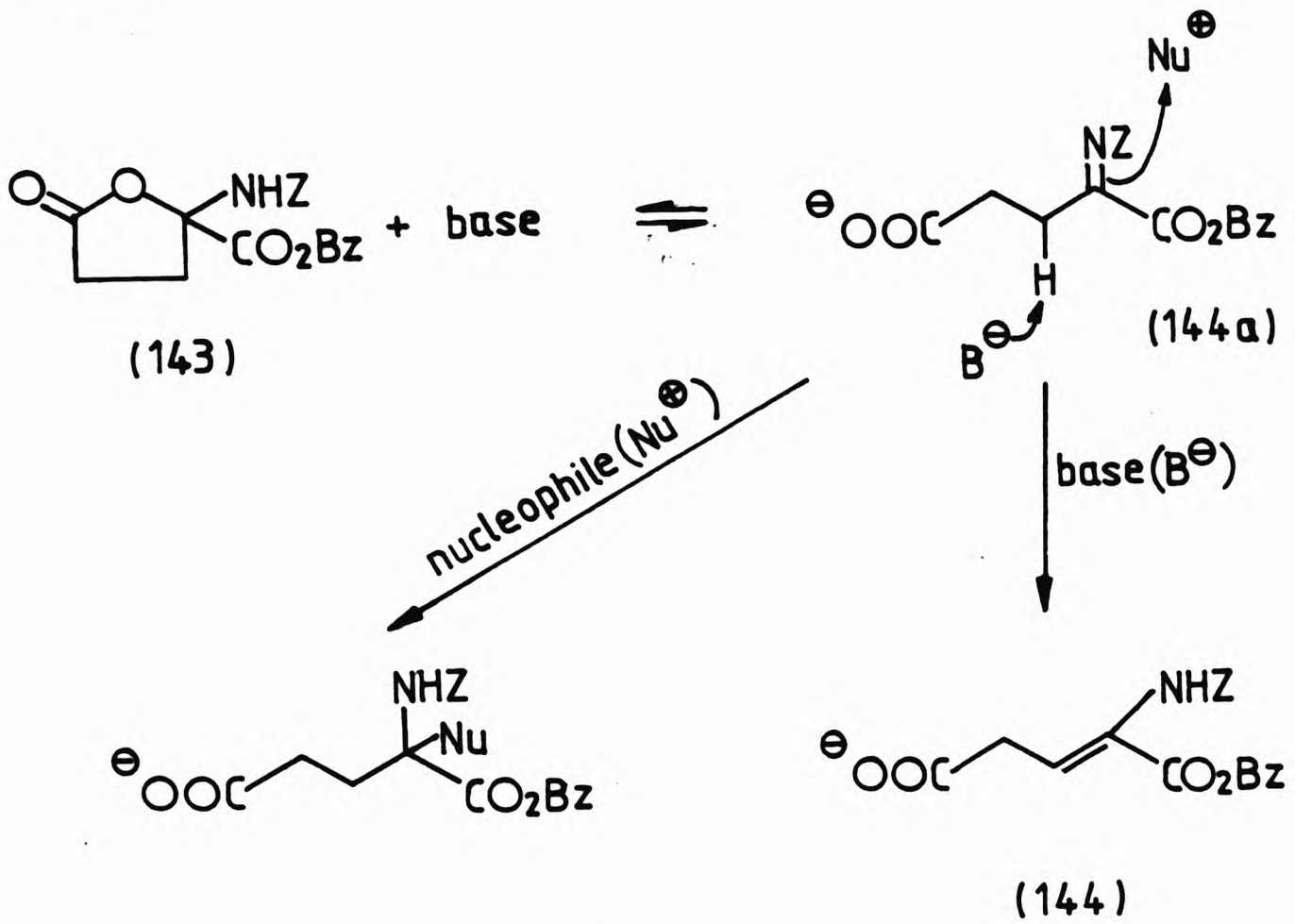
α-Ketoglutarate (5) and benzyl carbamate (141), prepared in high yield (85%) from ammonia and benzyl chloroformate, were heated under vacuum to give the lactone acid (142) (70%)[109], which was converted to the ester (143) with benzyl bromide and triethylamine (75%) (Scheme 11).

Scheme 11

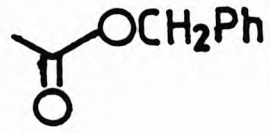

H2N
Ph
(141)
+
HO2C
CO2H
(5)
NH3(aq)
Ph
O
Cl
Δ 10mm Hg
CO2H
NHZ
(142)
Bz = CH2Ph
Z = OCH2Ph
1. Et3N
2. BzBr
CO2Bz
NHZ
(143)
DBU, THF, 0°C
CO2Bz
HO2C
NHZ
(144)
DBU =
N
N

The rearrangement of the lactone ester (143) to dehydroglutamic acid (144) was smoothly achieved by treatment with DBU. The reaction proceeded in high yield (71%) and the identity of the acid (144) was confirmed by the proton NMR spectrum: the methylene protons had a chemical shift of δ 3.3, the benzylic protons of δ 5.11 and 5.17 and the olefinic proton of δ 6.78. The mechanism of this rearrangement (Scheme 12) is thought to involve the initial formation of an acylamino ester (144a) as an unstable primary product which isomerises, in the absence of external nucleophiles or protic solvents, to the acylenamino acid ester (144).

Scheme 12



Bz = $-CH_2Ph$

Z = 

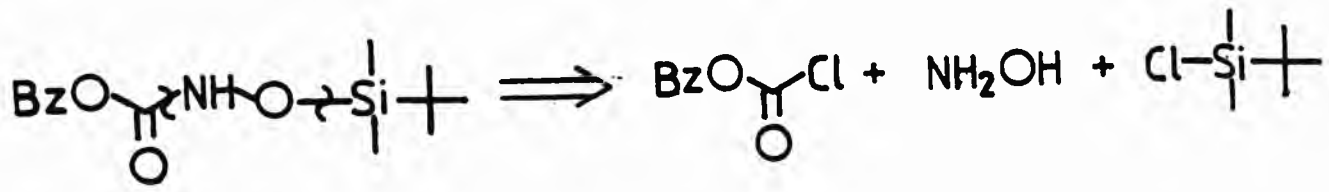
4.3.3 Preparation of hydroxylamine (146)

It has been shown that the presence of a substituent on nitrogen facilitates the cyclisation of the hydroxamic acid[24]. The difference between N-protected and unprotected hydroxamic acids has been attributed to the unsubstituted hydroxamic acid possessing several nucleophilic sites, whereas there is only one nucleophilic site in N-substituted hydroxamic acid.

Hydroxylamines are kinetically acylated on oxygen[4] and in order to avoid O-acylation the hydroxylamine would have to be O-protected.

See Scheme 13.

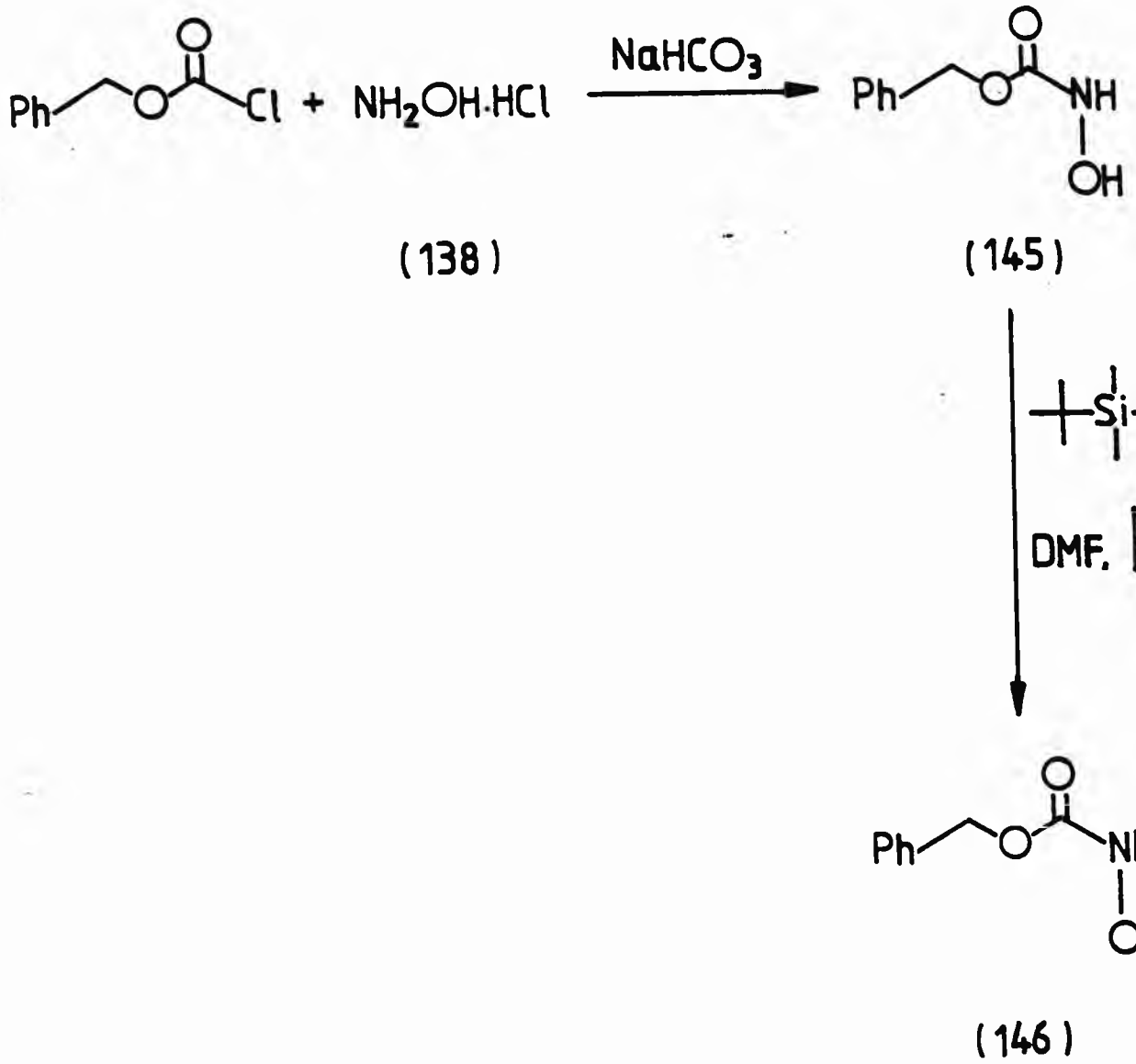
Scheme 13



Bz = $-CH_2Ph$

It was necessary, therefore, to N-protect hydroxylamine, with a benzyloxycarbonyl group, as the subsequent deprotection could be achieved by mild, facile hydrogenation over palladium[95]. The acylate (146) was easily prepared, in overall 68% yield, by initial reaction with benzyl chloroformate and hydroxylamine (138) and then with tert-butyldimethylsilyl chloride (TBDMS-Cl). (Scheme 14).

Scheme 14



(138)

(145)

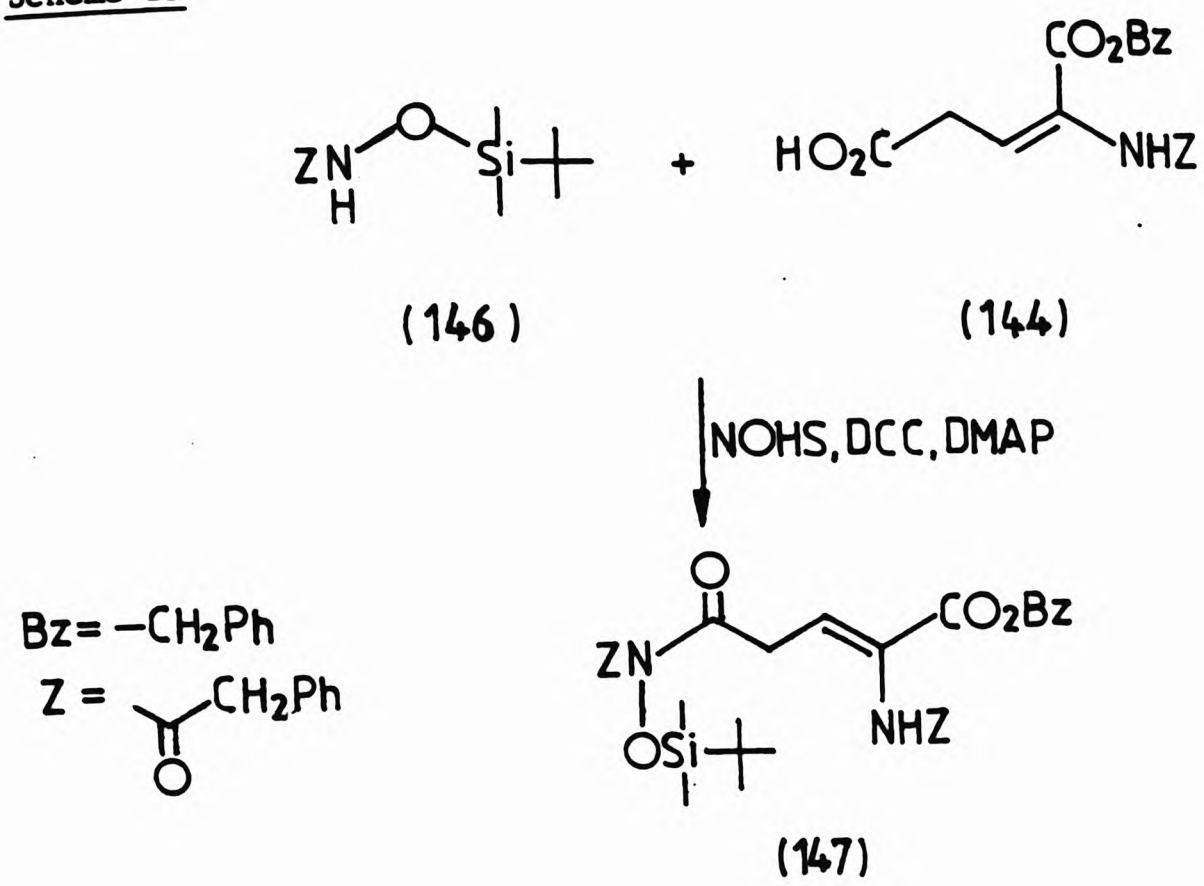
(146)

4.3.4 Attempted coupling of hydroxylamine (146) with acid (144)

Coupling of acylated hydroxylamine (146) with the acid (144) was attempted using N-hydroxysuccinimide (NOHS), DCC and dimethylamino pyridine (DMAP). DMAP was used to facilitate acylation[26,82,83] (Scheme 15).

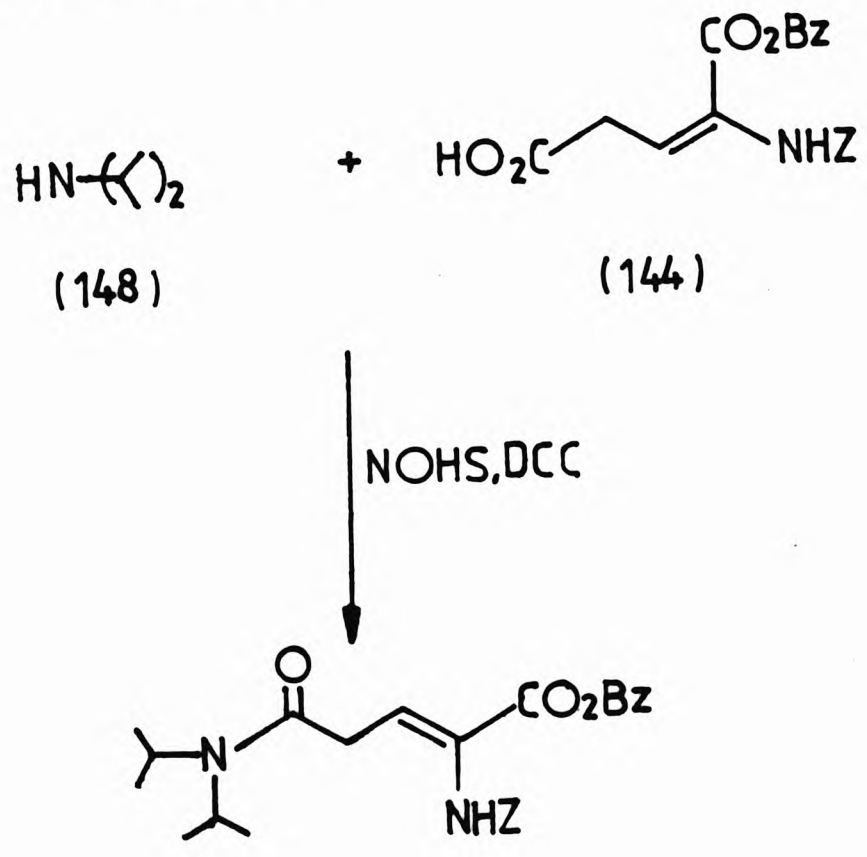
The "hydroxylamide" could not be coupled with the acid; increasing reaction time and the addition of pyridine (to ensure deprotonation of the acid) also had no effect.

Scheme 15



Using a model system in order to try to verify our methods it was decided to replace the nucleophile (146) with a hindered amine, diisopropylamine (146) (Scheme 16)

Scheme 16



The amide (149) was isolated (55% yield, after column chromatography) and its ^1H NMR spectrum showed the presence of both the hydroxamic acid moiety (i.e., olefinic proton at δ 6.83 - 6.9 and benzylic protons at δ 5.13 - 5.19) and significantly the characteristic doublet of the methyl protons (δ 1.22) of the isopropylamine moiety.

The fact that the hindered amine could be coupled with the acid suggested that the methodology i.e. technique and reagents, was not at fault. Presumably, the very weak nucleophilicity of amidic nitrogen, as exists in the acylated hydroxylamine was important. It was thought, therefore, that the coupling of the acylate (146) with a more active ester might be more successful.

Thus, it was decided to convert the acid (144) into an acyl chloride (150), where the chloride, a good leaving group, should facilitate the coupling reaction.

4.3.5 Acyl chloride (150)

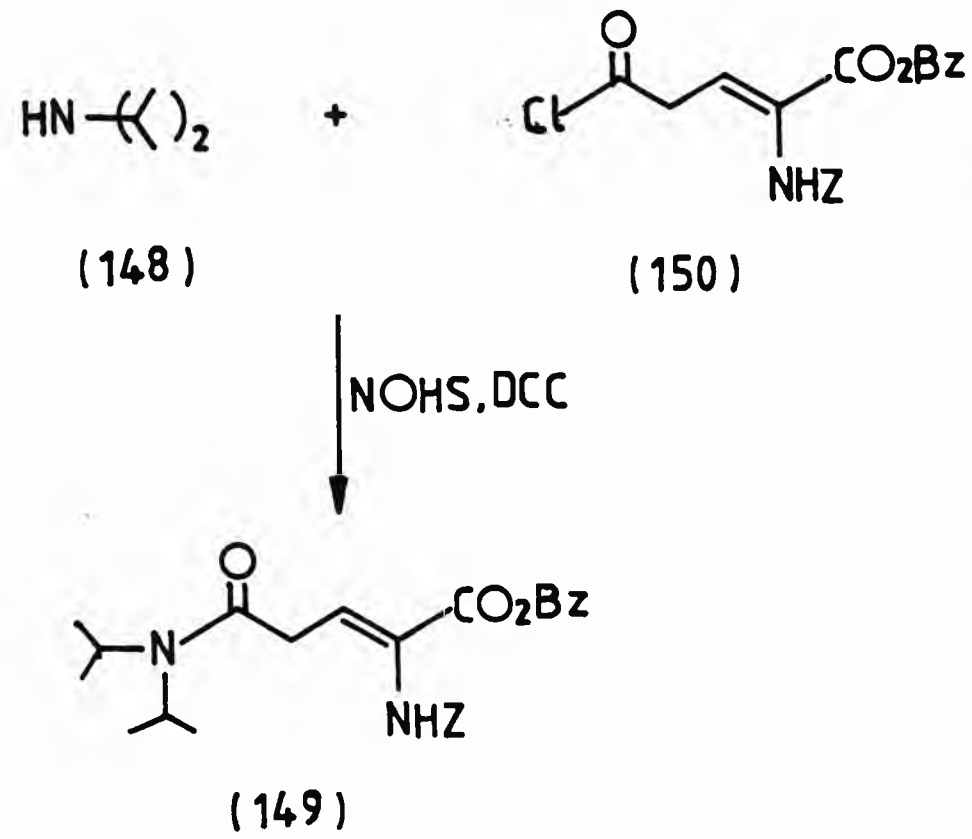
Initially, even though the acid (144) was reacted with thionyl chloride in dichloromethane at RT or heated under reflux at 60-80°C, the acyl chloride (150), could not be isolated. The reaction conditions employed were possibly too harsh. The mild reagent, 1-chloro-N,N-2-trimethylpropenylamine[54,77] was also unable to chlorinate the acid. However, it was found that the unstable acyl chloride (150) could be prepared by stirring a solution of the acid in toluene and DMF (a catalytic amount) with thionyl chloride at 0°C. TLC showed that no starting material remained after one hour.

4.3.6 Attempted coupling of hydroxylamine (146) with acyl chloride (150)

Using the same conditions (as previously described for coupling reactions), the amide (146) still could not be coupled with the acyl chloride (150).

Diisopropylamine (148) was successfully coupled with the acyl chloride; the product (149) was isolated in a yield of 25% (Scheme 17). It was the same as that isolated with the acid (144) (Scheme 16).

Scheme 17



(148) (150) (149)

The reduction in yield may have been due to competition between decomposition of the acyl chloride (150) and reaction of the amine (148).

It is probable that the amide (146) being much less nucleophilic than the amine (148) and, therefore, reacting much more slowly, was unable to couple with the acyl chloride (150) before the latter had fully decomposed.

4.3.7 N-Allyl-protecting group

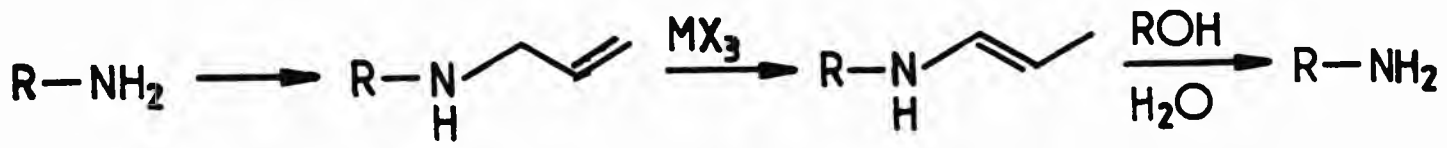
As increasing the reactivity of the acid (144), by conversion to the acyl chloride (150), did not facilitate the coupling of the acylate (146) it was decided that the hydroxylamine moiety rather than the acid should be modified.

The nucleophilic character of nitrogen is important in the acylation and so the benzyloxycarbonyl N-protecting group would have to be replaced

by a group which would have little effect on the nitrogen's basicity. Such a group, was the acid- and base-stable, allyl group[30,101].

Generally, the protection method involves reaction of an amine (151) with an allylic compound to give an N-allyl derivative (152). Deallylation, under neutral aprotic conditions, is catalysed by various transition metal complexes e.g. tris(triphenyl phosphine)rhodium chloride ($RhCl(PPh_3)_3$)[29]. The resulting enamine (152a) then undergoes facile hydrolysis to give the product (152b)(Scheme 18).

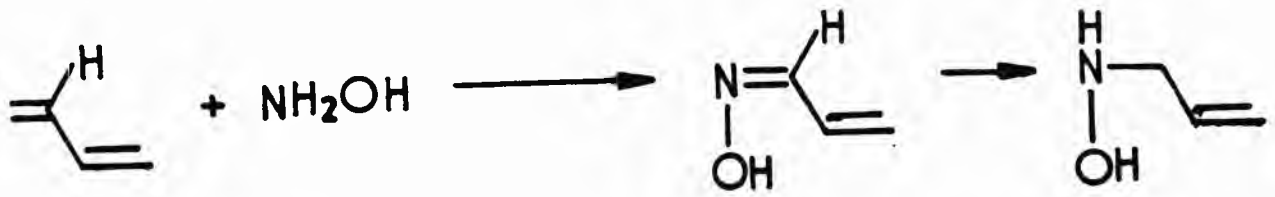
Scheme 18



(151) (152) (152a) (152b)

The N-allyl hydroxylamine (154) was synthesised[27]. However, it and its precursor, oxime (153), although stable in dilute solution, polymerised on attempted isolation, making them very difficult to purify (Scheme 19).

Scheme 19



(153) (154)

As the alkyl oxime, in our hands, was extremely difficult to handle an alternative N-protecting group was sought.

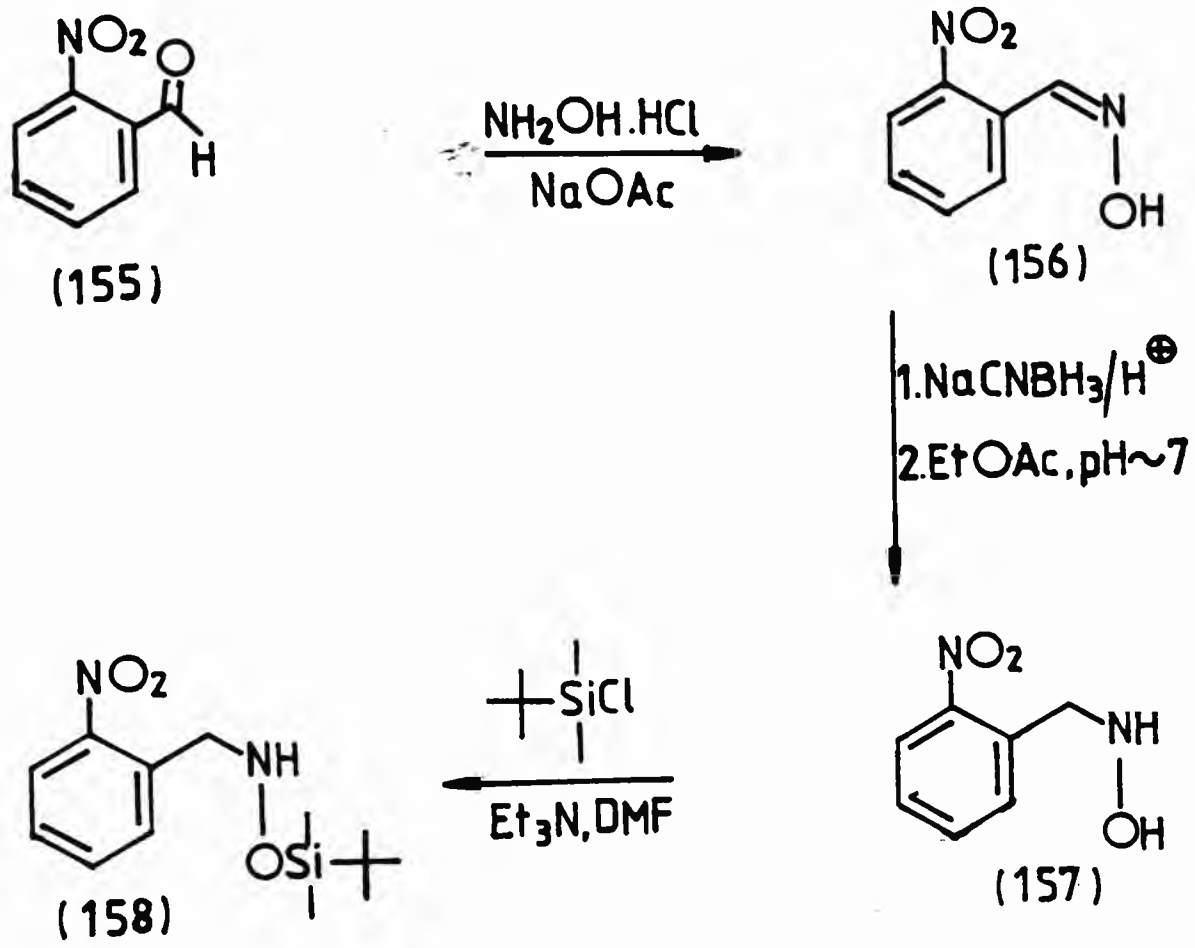
4.3.8 Alternative approaches towards N-protection of hydroxylamine

4.3.8.1 The ortho-nitrobenzyl protecting group

Ortho-Nitrobenzyl groups have been widely and successfully used as N-protecting groups[24]. A facile deprotection of this group can be achieved using aluminium amalgam or ultra violet radiation.

The silylated N-(ortho-nitrobenzyl)-hydroxylamine (158) was readily synthesised from ortho-nitrobenzaldehyde (155) in 79% overall yield (Scheme 20).

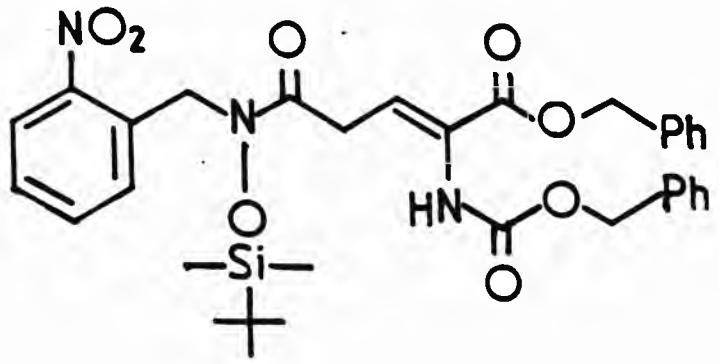
Scheme 20



The oxime (156), derived from the available aldehyde, was reduced with sodium cyanoborohydride. The work-up of the reaction mixture had to be carried out at approximately pH 7; pH greater than 7 lowered the yield of the hydroxylamine (157). Silylation was effected by tert-butyldimethylsilyl chloride under basic conditions.

The silylated hydroxylamine (158) was smoothly coupled to the acid (144) using DCC and NOHS.

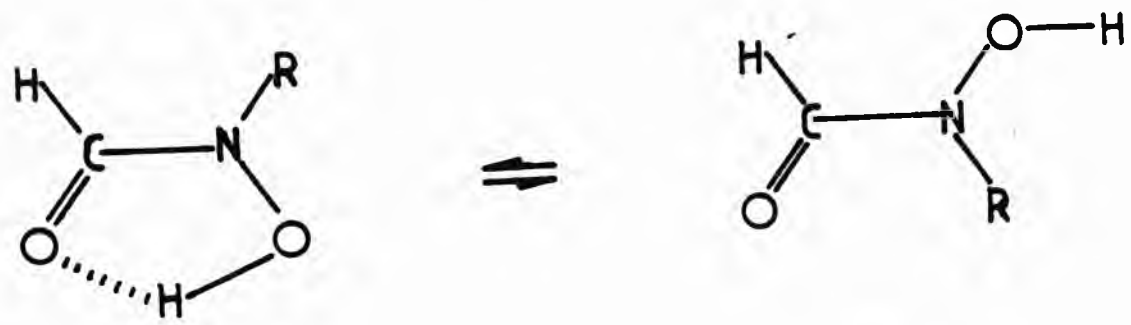
4.3.8.1.1 Structure and deprotection of coupled product (159)



(159)

Examination of the NMR spectrum of the coupled product (159) confirmed the presence of the O-tert-butyl-dimethylsilyl group (singlet absorptions at δ 0.038 and 0.87) and the αβ unsaturated glutamyl moiety (olefinic proton multiplet at δ 6.3 - 6.9, and a methylene multiplet at δ 3.6). The methylene protons of the unsubstituted acid (144) resonate at δ 3.3. In the benzylic methylene region (5.0 - 5.30), however, the NMR spectrum indicated the presence of three, not two, benzyl groups. This was confirmed by reference to the aromatic region (δ 7.25 - 7.35) which again indicated the presence of four extra protons (due to the ortho-nitrobenzyl aromatic protons).

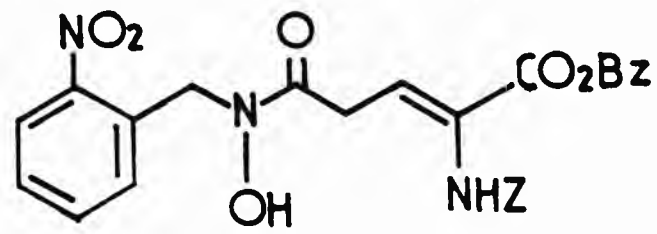
The widespread nature of the olefinic multiplet (δ 6.3 - 6.9) perhaps deserves some comment. Some hydroxamic acids have been shown[97] to exist as an Z/E equilibrium mixture of rotamers, the Z (cis) rotamer being capable of participating in intramolecular H-bonding. Bulky nitrogen substituents enhance the tendency for such



cis (Z) ⇌ trans (E)

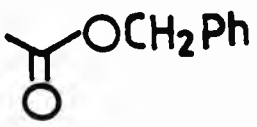
acids to exist as two different rotamers. In our case, presumably, the olefinic proton experiences two very differing electronic environments in each rotamer. Hence, the widespread nature of its chemical shift.

The silylated hydroxamic acid (159) was treated with p-toluenesulphonic acid in methanol at room temperature and hydroxamic acid (160) was afforded in good yield, 74% after column chromatography. ^1H NMR assignments for the hydroxamic acid (160) showed that the tert-butyldimethylsilyl group had been removed (i.e. gross spectrum same as above but no absorptions at 0 - 0.1 region due to protecting group).



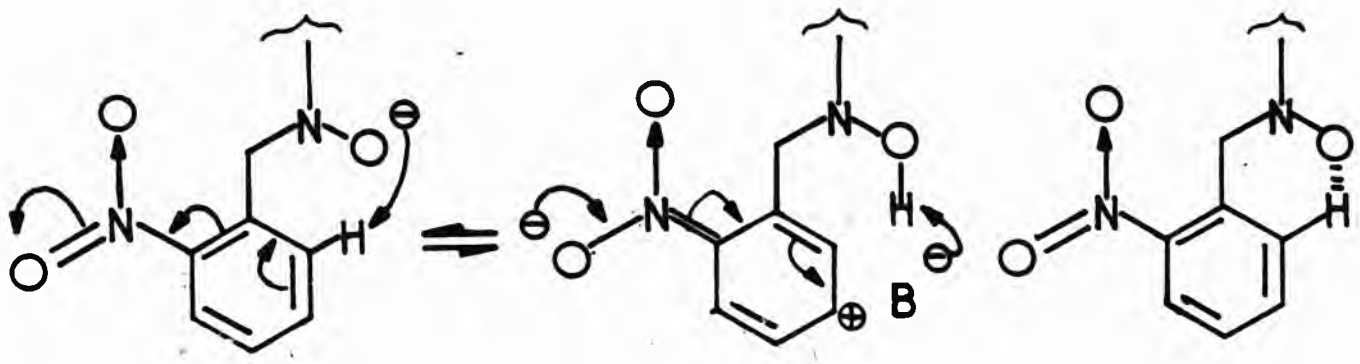
(160)

Bz = $-CH_2Ph$

Z = 

4.3.8.1.2 Attempted cyclisation of hydroxamic acid (160)

Hydroxamic acid (160) could not be cyclised in sodium bicarbonate or sodium hydroxide. Other reaction conditions were used; e.g. sodium bicarbonate and tetrahydrofuran (THF); triethylamine in a solution of THF:water (10:1); excess triethylamine or potassium hydroxide in methanol; longer reaction times; without success. In all cases the starting material was recovered in greater than 90% yield. This failure to cyclise the acid (160) under any of the conditions employed may be caused by the electron-withdrawing effect of the nitro group in increasing the acidity of the aromatic protons of the σ-nitrobenzyl protecting group.



(163) (164)

In neither case would the acid exist as its anion; hence the hydroxamic oxygen would not be an available nucleophile. The fact that starting material was recovered in high yield would tend to support this postulate, only internal salt formation, no other reactions had occurred. Neither of the two possible intermediates (163) or (164) would undergo internal Michael reactions as formation of the six-membered conformer (in basic media) would be easier and therefore more favourable than cylisation to the hydroxamic acid.

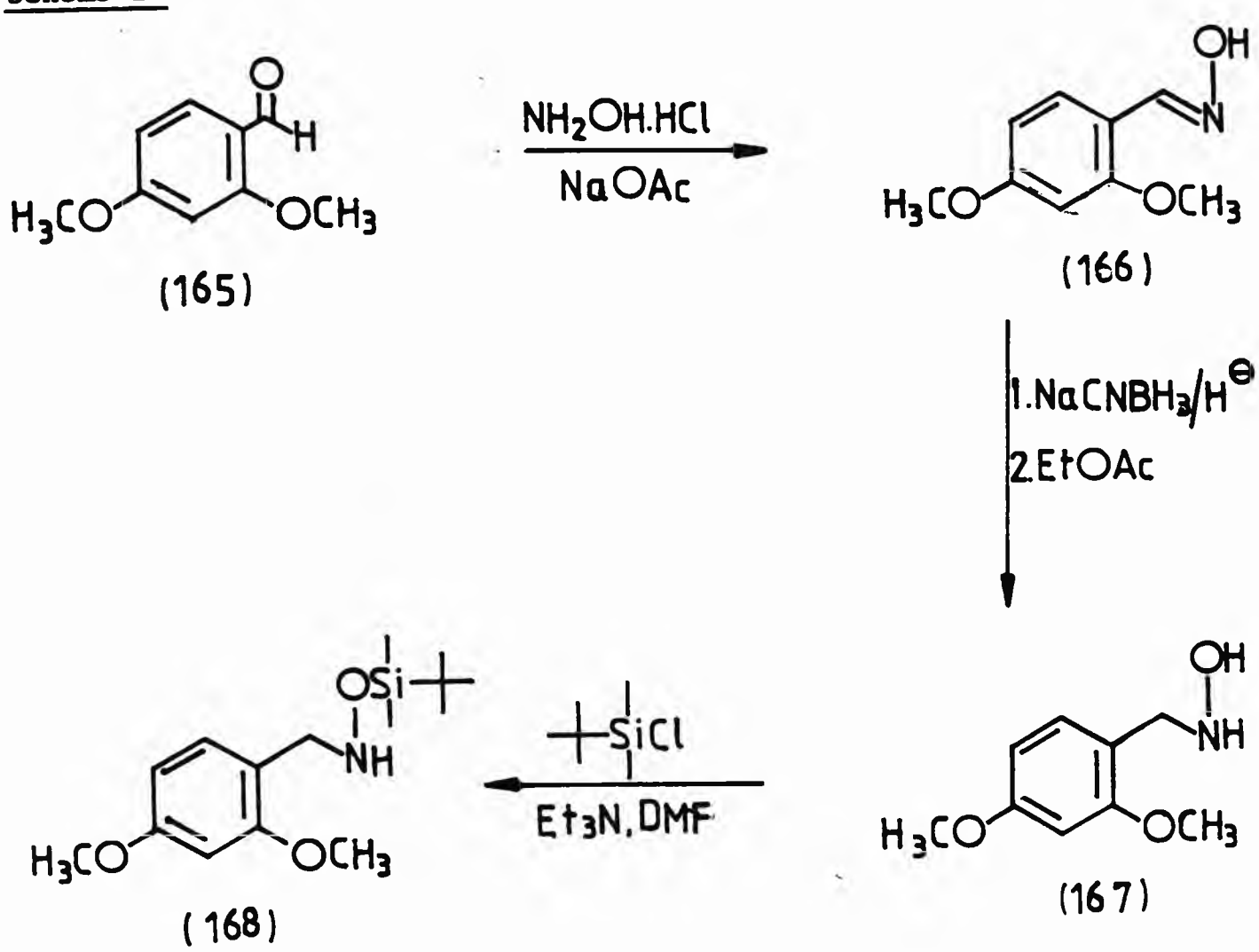
4.3.8.2 The 2,4-dimethoxybenzyl group

N-protection of an ortho-nitrobenzyl group had thus proved unsuitable and an alternative protecting group had to be employed. A 2,4-dimethoxybenzyl group would be used to N-protect hydroxylamine (138). Methoxybenzyl groups can be readily and efficiently removed with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ)[131a]; other methods e.g. treatment with trifluoroacetic acid, H_2/Pt and sodium in liquid ammonia are much more harsh, yet still effective.

Hydroxylamine (167) was isolated, after colomn chromatography, in 72% yield, The pH of the 'work-up' conditions was not critical.

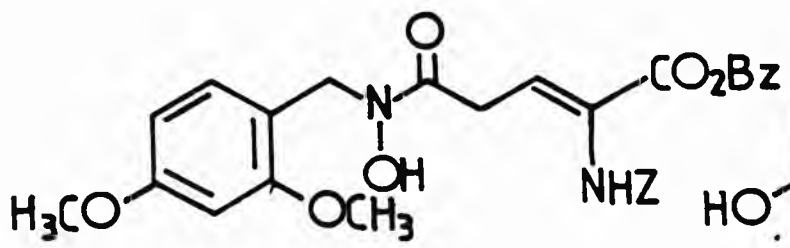
Singlets in the ^1H NMR spectrum of hydroxylamine (167) for the methoxy protons were at δ 3.78. The silylated hydroxylamine's (168) NMR spectrum also exhibited similar resonances for methoxy protons at δ 3.79 and the tert-butyldimethylsilyl group's methyl singlets at δ 0.09 and 0.92 (Scheme 22).

Scheme 22

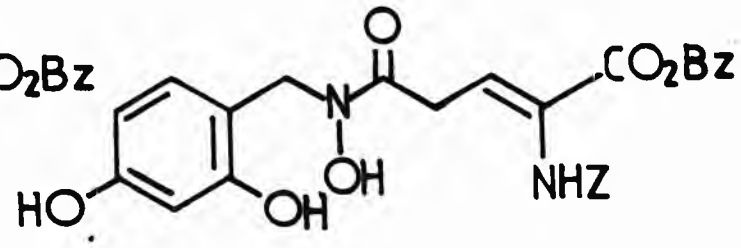


The silylated hydroxylamine (168) was successfully coupled (as before) with the acid (144) and the tert-butyldimethylsilyl group removed with tosic acid/methanol at room temperature. Column chromatography afforded a mixture of products, however, whose ^1H NMR spectrum indicated that a number of hydroxamic acids had been isolated. Presumably, the N-protecting group of the hydroxylamine moiety had either two methoxy groups (169), two hydroxy groups (161) or one methoxy and one hydroxy group (162). Separation of each of these compounds from the mixture, would be tedious and very difficult since all products were separated by an Rf of about 0.1, likewise, the alternative step of

treating the mixture with base would have yielded a mixture of all six possible diastereoisomers of cyclised products which would again require separation.



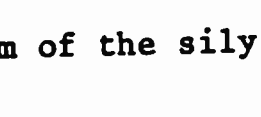
(169)



(161)

(162)

Bz = $-CH_2Ph$

Z = 

4.3.8.3 The para-methoxybenzyl protecting group

It was thought that a para-methoxybenzyl group would be more easily handled. Para-methoxybenzyl groups, as with the dimethoxybenzyl groups, can be easily removed with DDQ under mild conditions[131a]. A para-methoxybenzyl group had been used for N-protection in Baldwin's synthesis of AT-125 (127)[4] (although, there, it had served to activate the triprotected tricholomic acid toward imino chloride formation).

Hydroxylamine (172), prepared from para-methoxybenzaldehyde (170), in good yield (82%, after column chromatography) was smoothly silylated (Scheme 24).

The protons of the tert-butyldimethylsilyl group in the NMR spectrum of the silylated hydroxylamine (173), exhibited absorptions at

δ 0.05 and 0.92 and the methoxy protons at δ 3.78. The aromatic protons appeared as a multiplet at δ 6.79 - 7.29.

Coupling of the silylated hydroxylamine (173) and the acid (144) afforded a mixture of products, in low yield (24%). ^1H NMR resonances of this mixture (methylene protons at δ 3.55 - 3.66; benzylic protons at δ 5.03 - 5.19; olefinic protons at δ 7.32; methoxy group singlet at δ 3.78 was not equivalent to three protons) indicated that the coupling reaction had produced two hydroxamic acids: one with the para-methoxy-benzyl group intact and the other with a N-benzyl group, (174) and (175), respectively (in a 1:3 ratio) (Scheme 24).

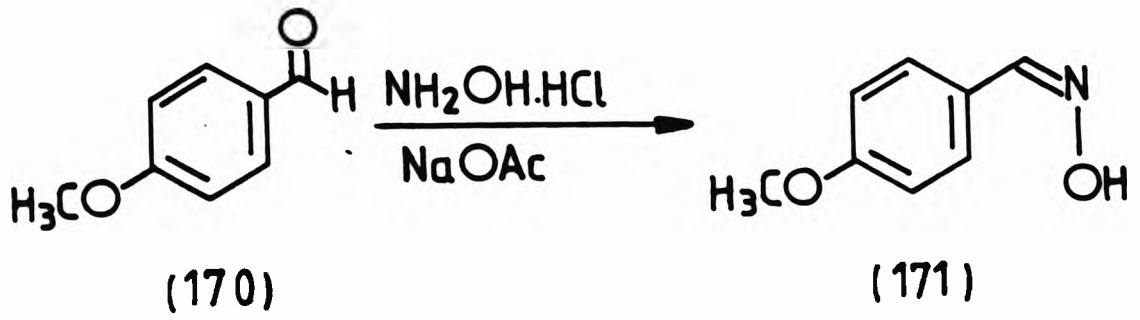
As an efficient separation of this mixture was very difficult it was decided to revert to a simpler system i.e. an N-benzyl protecting group. Use of a benzyl group for N-protection had previously been avoided as it was thought to be more difficult to deprotect the end product. However, at this stage it was most important to show that a route based on an intramolecular Michael reaction was a viable approach and therefore a hydroxamic acid had to be cyclised (subsequent deprotections being of lesser importance). Also, once a cyclised product had been isolated it had to be shown that the diastereoisomers could be separated.

4.3.8.4 The benzyl protecting group

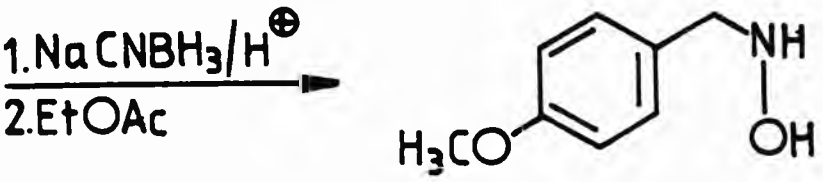
N-Benzylhydroxamic acid (175) was prepared from the coupling of acid (144) and a silylated hydroxylamine (179) using methods employed for the other substituted benzyl protected hydroxamic acids.

The ^1H NMR spectrum of the hydroxamic acid (175) had absorptions for the methylene protons at δ 3.26 - 3.35 and benzylic protons at δ 5.09 - 5.2; resonances for the olefinic proton occurred as a multiplet in the region δ 6.75 - 6.9 and the aromatic protons at δ 7.35.

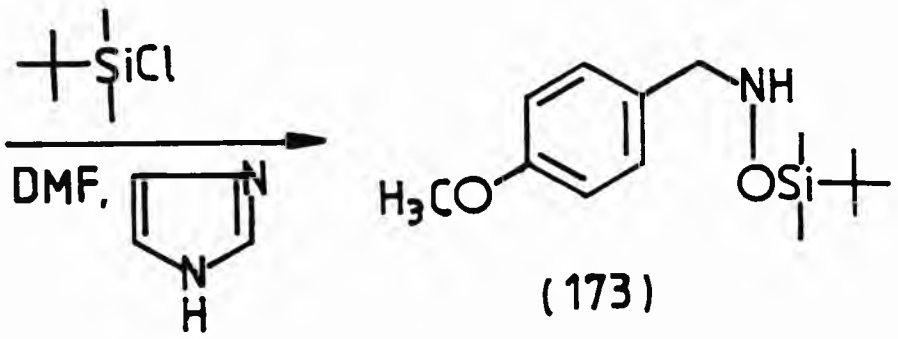
Scheme 24



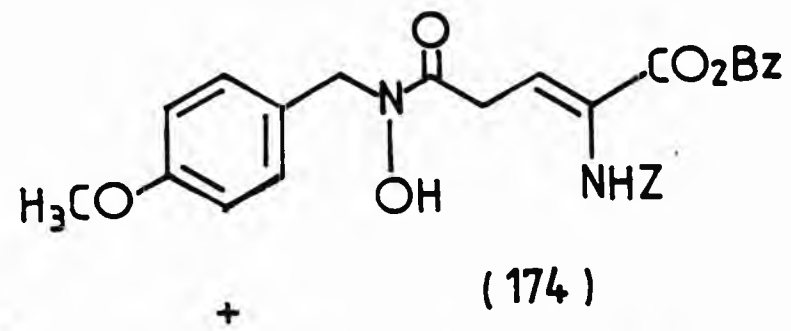
(170) (171)



(172)

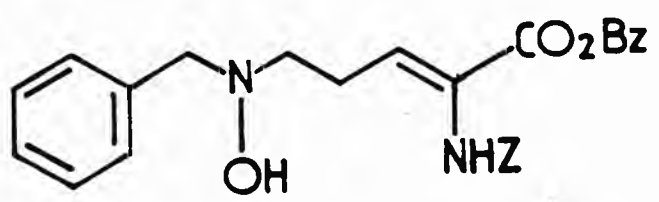


(173)



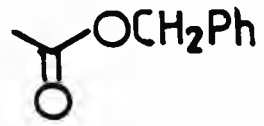
(174)

1. (144) NOHS.DCC
2. tosic acid/ MeOH



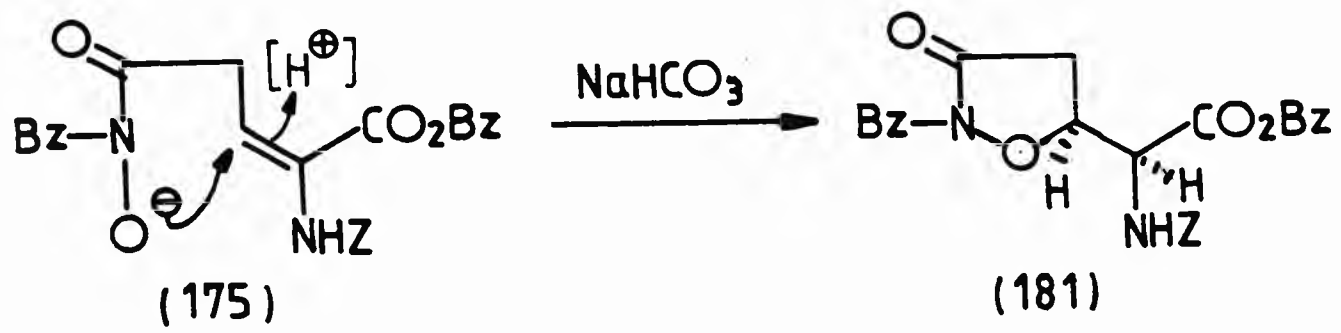
(175)

Bz = $-CH_2Ph$

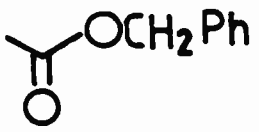
Z = 

The hydroxamic acid (175) was smoothly cyclised to the isoxazolidine (181) in sodium bicarbonate, which was isolated in 84% yield (Scheme 25).

Scheme 25

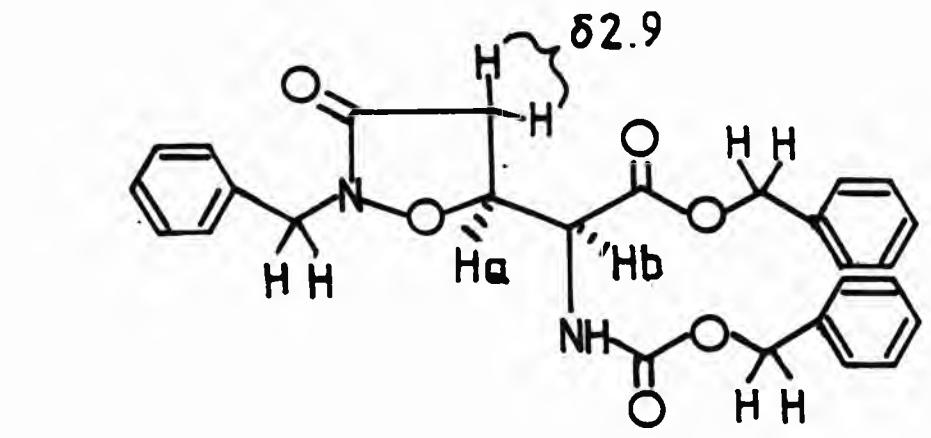


(175) (181)

Bz = $-CH_2Ph$; Z = 

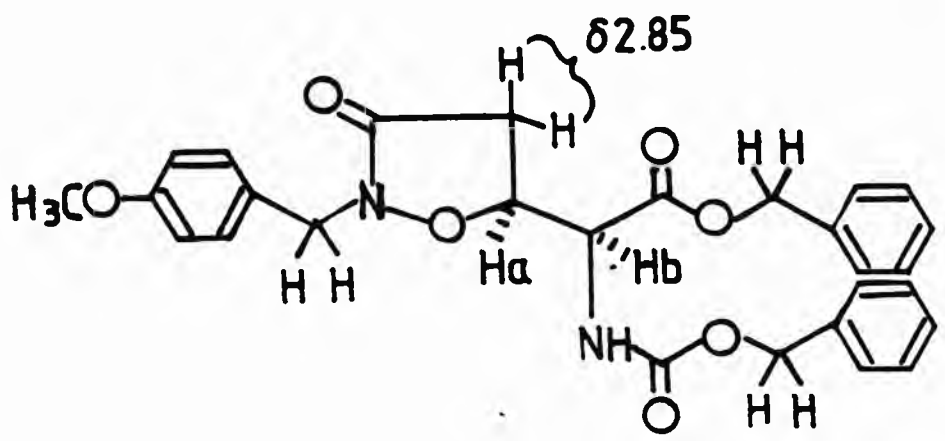
The NMR spectrum of the product (and TLC) indicated the disappearance of starting hydroxamic acid. No olefinic resonances were apparent and the methylene multiplet (δ 3.26 - 3.35) had disappeared, to be replaced by a similar 2-proton multiplet at δ 2.9. This was assigned to the isoxazolidine ring methylenes. The three benzyl groups (three methylenes at δ 5.0 - 5.38 and fifteen aromatic protons at δ 7.25 - 7.3) were still present. However, the presence of a two-proton multiplet in the region δ 4.40 - 4.88 was the most significant feature of the spectrum. The starting acid (175) exhibits no absorptions in this region. On the other hand, the diastereomeric methines (H_a and H_b) would, by analogy with previously known compounds[4, 24] (e.g. isoxazolidine (182)), have their chemical shifts in this region. TLC analysis confirmed the presence of two very closely differentiated spots (R_f 0.56 and 0.59 in ether/petrol 7:5).

The isoxazolidine (182) synthesised by Cha[24] was not deprotected or purified (an analytical sample was prepared for its characterisation).



Ha } δ4.40–4.88
Hb }

(181)



Ha } δ4.41–4.88
Hb }

(182)

So far, attempts to separate the <u>erythro</u>- and <u>threo</u>-diastereoisomers have proved difficult using conventional column chromatography. The triprotected tricholomic acid (181) decomposes on silica (10% recovery) and alumina (no recovery). Pre-elution or washing and drying of the silica with triethylamine or methylamine or precoating with silver nitrate has had no beneficial effect. Neither has the use of alumina in its basic, neutral or acidic forms. However, work is in progress to surmount this problem: high performance liquid chromatography studies are currently being undertaken to attempt the separation of the <u>erythro</u>-and <u>threo</u>-tricholomic acids.

CHAPTER FIVE

EXPERIMENTAL

Melting points were determined on an Electrochemical melting point apparatus. ^{1}H, ^{13}C and ^{31}P NMR spectra were measured with a Jeol FX 90 Q Fourier Transform spectrometer; the reported chemical shifts are relative to TMS (^{1}H and ^{13}C) and H_3PO_4 (^{31}P) as external standards.* IR spectra were recorded on a Perkin-Elmer 298 spectrometer and mass spectra on a JMS DX 300 spectrometer . Optical rotations were obtained on a NPL Automatic Polarimeter 143D.

Column chromatography with Kieselgel 60 (70 - 230 mesh) silica gel or Dowex 50 (H^+) ion-exchange resin (BDH) was used for the purification.

For reactions, diethyl ether, dichloromethane, chloroform, toluene and benzene were dried and distilled from phosphorus pentoxide; methanol and ethanol from magnesium. Tetrahydrofuran (THF) was dried and distilled from sodium benzophenone ketyl and N,N-dimethylformamide (DMF) was stirred over calcium hydride before being decanted and distilled under reduced pressure.

Lithium diisopropylamine (LDA) was prepared, in 1M concentration, according to the method of Reich et al[150].

(DCC = N,N'-Dicyclohexylcarbodiimide)

* δ-values are expressed in parts per million (ppm).

5.1 Amino Phosphono acids

Diethyl-2-acetamido-2-[diethyl-2-phosphonoethyl]-malonate (40)

To a solution of sodium (0.9 g, 0.94 mol) in ethanol (20 mls) was added diethyl acetamido malonate (8.5 g, 0.04 mol). Solvent was evaporated, in vacuo, and the residue washed with toluene (3 x 15 ml), to give diethyl sodiomalonate (9.6 g, 0.04 mol) which was suspended in toluene (40 ml) and diethyl carbonate (40 ml). Diethyl-2-bromoethyl phosphonate (37) (10.8 g, 0.04 mol) was added, the mixture heated under reflux overnight, filtered hot, and the solvent evaporated, in vacuo, to give a dark yellow oil (15.8 g). Chromatography on silica gel (500 g) using diethyl ether/chloroform 1:1 as eluant afforded acetamido adduct (40) (7.72 g, 51%) as a viscous yellow oil.

(±)-2-Amino-4-phosphonobutanoic acid (APB) (10)

A solution of the acetamido adduct (40) (7.72 g, 0.02 mol) in 6M HCl (100 ml) was heated under reflux for 12 hours. The solution was evaporated, in vacuo, and the residue dissolved in methanol. Propylene oxide was added until no further precipitate formation was apparent. Filtration and recrystallisation from water-ethanol gave (±)-APB (10) (2.5 g, 68%) as white crystals: mp 225°C (with decomposition) (lit. mp 225°C[25]); ^{13}C NMR (H_2O) δ 20.04 and 26.17 (d, J = 138 Hz, P$\underline{C}H_2$), 24.38 (d, J = 3.1 Hz, PCH$_2\underline{C}H_2$), 53.31 and 54.12 (d, J = 18.3 Hz, $\underline{C}HNH_2$), 172.11 (s, $\underline{C}O_2H$); ^{31}P NMR (H_2O) δ 26.65 (s).

Anal. calcd for $C_4H_{10}NO_5P$: C, 26.23; H, 5.50; N, 7.65; P, 16.92.

Found: C, 26.08; H, 5.48; N, 7.37; P, 16.87.

Resolution of (±)-2-Amino-4-phosphonobutanoic acid (APB) (10)

A solution of L-lysine (1.46 g, 0.01 mol) and (±)-APB (10) (183 g, 0.01 mol) in water (10 ml) was heated gently (60°C) for 15 minutes; methanol was added until the initial appearance of a flocculent precipitate. The mixture was cooled, diethyl ether added until the onset of a slight cloudiness and left at -10°C overnight. The precipitate was filtered to give the (-)-APB-lysine salt (1.48 g, 45%) which was dissolved in 2M pyridine and poured onto an ion-exchange resin column (Dowex 50, pyridinium form). The column was eluted with 2M pyridine, all ninhydrin positive fractions were combined, evaporated, in vacuo, the residue washed with methanol and solvent evaporated, in vacuo, to give (-)-APB (7.70 mg): $[\alpha^{25}_{546}] = -14.8°$ (c = 2.7, H_2O).

The mother-liquor from the above filtration was poured onto an ion-exchange resin column (Dowex 50, pyridinium form) and (+)-APB (620 mg) was isolated as above: $[\alpha^{25}_{546}] = +11.3°$ (c = 6.2, H_2O).

Diethyl-3-bromopropyl phosphonate (38)

Sodium (4.14 g, 0.18 mol) was added to a solution of diethyl phosphite (22.84 g, 0.18 mol) and diethyl ether (20 ml). The resulting solution was added dropwise to a stirred solution of 1,3-dibromopropane (55 g, 0.27 mol) in diethyl ether (25 ml) at 0°C. The mixture was allowed to warm to room temperature, left overnight, heated under reflux for three hours, cooled and then filtered. The solvent was evaporated, in vacuo, and the residue chromatographed on silica gel (500 g) using ethyl acetate eluant to give bromopropyl phosphonate (38) (17.82 g, 38%) as a colourless oil: ^{13}C NMR (neat) δ 16.79 and 17.06 (d, J = 6 Hz, $OCH_2\underline{C}H_3$), 21.51 and 27.74 (d, J = 140 Hz, $P\underline{C}H_2$), 26.92 and 27.09 (d, J = 3.7 Hz, $\underline{C}H_2Br$), 34.45 and 35.27 (d, J = 18.3 Hz, $PCH_2\underline{C}H_2$), 61.6 and 61.9 (d, J = 6 Hz, $O\underline{C}H_2$); ^{31}P NMR (neat) δ 29.88 (s).

Diethyl-2-acetamido-2-[diethyl-3-phosphonopropyl]-malonate (41)

To a solution of sodium (1.6 g, 0.07 mol) in ethanol (35 ml) was added diethyl acetamido malonate (14.9 g, 0.07 mol). Solvent was evaporated, in vacuo, and the residue washed with toluene (3 x 20 ml), to give diethyl sodiomalonate (16.9 g, 0.07 mol) which was suspended in toluene (60 ml) and diethyl carbonate (60 ml). Bromopropyl phosphonate (38) (17.8 g, 0.07 mol) was added, the mixture heated under reflux overnight, filtered hot, and solvent evaporated, in vacuo, to give a dark yellow oil (29 g). The acetamido adduct (41) was isolated as a viscous yellow oil (13.9 g, 50%) after column chromatography on silica gel (599 g) using diethyl ether/ethyl acetate 1:1 as eluant.

(±)-2-Amino-5-phosphonopentanoic acid (APP) (11)

A solution of the acetamido adduct (41) (13.9 g, 0.035 mol) in 6M HCl (100 ml) was heated under reflux for 12 hours. Solvent was evaporated, in vacuo, and the residue taken up in methanol; propylene oxide was added until no further precipitate formation was apparent. Filtration and recrystallisation from water-ethanol afforded (±)-APP (11) (6.24 g, 90%) as white crystals: mp 230°C (with decomposition) (lit. mp 250°C[38]); ^{13}C NMR (H_2O) δ 18.55 and 18.74 (d, J = 4.3 Hz, $\underline{C}H_2CHNH_2$), 23.16 and 29.20 (d, J = 136 Hz, $P\underline{C}H_2$), 30.66 and 31.39 (d, J = 16.5 Hz, $PCH_2\underline{C}H_2$), 53.17 (s, $\underline{C}HNH_2$), 172.30 (s, $\underline{C}O_2H$); ^{31}P NMR (H_2O) δ 29.95 (s). Anal. calcd for $C_5H_{12}NO_5P$: C, 30.46; H, 6.14; N, 7.10; P, 15.71. Found: C, 30.09; H, 6.14; N, 6.81; P, 15.62.

Resolution of (±)-2-amino-5-phosphonopentanoic acid (11)

A solution of L-lysine (2.92 g, 0.02 mol) and (±)-APP (11) (3.94 g, 0.02 mol) in water (20 ml) was heated gently (60°C) for 15 minutes and methanol was added until the initial appearance of a flocculent precip-

itate. The mixture was cooled, diethyl ether added until the onset of a slight turbidity and left at -10°C overnight. The precipitate was filtered to give the (-)-APP-lysine salt (3.3 g, 48%) which was then taken up in 2M pyridine and poured onto an ion-exchange resin column (Dowex 50, pyridinium form). The column was eluted with 2M pyridine, all ninhydrin positive fractions were combined, evaporated, <u>in vacuo</u>, the residue washed with methanol and the solvent evaporated, <u>in vacuo</u>, to afford (-)-APP (1.71 g): $[\alpha^{25}_{546}] = -10°$ (c = 10, H_2O).

The mother-liquor from the above filtration was poured onto an ion-exchange resin column (Dowex 50, pyridinium form) and (+)-APP (1.65 g) was isolated as above: $[\alpha^{25}_{546}] = +8°$ (c = 9.7, H_2O).

<u>Diethyl-5-bromopentyl phosphonate (39)</u>

To a solution of diethyl phosphite (20.64 g, 0.16 mol) in diethyl ether (20 ml) was added sodium (3.68 g, 0.16 mol). The resulting solution was added dropwise to a stirred solution of 1,5-dibromopentane (76.5 g, 0.33 mol) in diethyl ether (35 ml) at 0°C. The mixture was allowed to warm to room temperature, left overnight, heated under reflux for three hours, cooled and then filtered. The solvent was evaporated, <u>in vacuo</u>, and chromatography of the residue on silica gel (400 g) using petroleum ether (30-40°C)/diethyl ether 9:1 eluant yielded bromopentyl phosphonate (39) (15.59 g, 34%) as a colourless oil: ^{13}C NMR (neat) δ 16.96 and 17.23 (d, J = 6 Hz, $OCH_2\underline{C}H_3$), 22.32 and 22.54 (d, J = 4.9 Hz, $PCH_2CH_2\underline{C}H_2$), 22.81 and 29.04 (d, J = 140 Hz, $P\underline{C}H_2$), 29.15 and 29.85 (d, J = 16 Hz, $PCH_2\underline{C}H_2$), 32.99 (s, $\underline{C}H_2CH_2Br$), 34.56 (s, $\underline{C}H_2Br$), 61.33 and 61.60 (d, J = 6 Hz, $O\underline{C}H_2$); ^{31}P NMR (neat) δ 30.69 (s).

Diethyl-2-acetamido-2-[diethyl-5-phosphonopentyl]-malonate (42)

Diethyl acetamido malonate (10.7 g, 0.05 mol) was added to a solution of sodium (1.2 g, 0.05 mol) in ethanol (20 ml). The solvent was evaporated, in vacuo, and the residue washed with toluene (3 x 20 ml) to give diethyl sodiomalonate (13 g, 0.05 mol) which was suspended in toluene (40 ml) and diethyl carbonate (40 ml). Bromopentyl phosphonate (39) (15.59 g, 0.05 mol) was added, the mixture heated under reflux overnight, filtered hot, and solvent evaporated, in vacuo, to give a dark yellow oil (25 g). Chromatography on silica gel (400 g) using ethyl acetate/diethyl ether 4:10 eluant afforded the acetamido adduct (42) (12 g, 52%) as a viscous yellow oil.

(±)-2-Amino-7-phosphonoheptanoic acid (APH) (13)

A solution of the acetamido adduct (42) (12 g, 0.03 mol) in 6M HCl (100 ml) was heated under reflux for 12 hours. The solvent was evaporated, in vacuo, and the residue dissolved in methanol. Propylene oxide was added until no further precipitate formation was apparent. Filtration and recrystallisation from water-ethanol yielded (±)-APH (13) (4.5 g, 70%) as white crystals: mp 213°C (with decomposition(lit. mp 228°C[28]); ^{13}C NMR (H_2O) δ 22.05 and 22.27 (d, J = 4.9 Hz, $PCH_2\underline{C}H_2\underline{C}H_2$), 23.46 (s, $P(CH_2)_3\underline{C}H_2$), 24.16 and 30.12 (d, J = 134 Hz, $P\underline{C}H_2$), 29.20 and 30.12 (d, J = 20.8 Hz, $PCH_2\underline{C}H_2$), 30.12 (s, $\underline{C}H_2CHNH_2$), 53.63 (s, $\underline{C}HNH_2$), 172.87 (s, $\underline{C}O_2H$); $^{31}P(H_2O)$ δ 31.77 (s). Anal. calcd. for $C_7H_{16}NO_5P$: C, 37.33; H, 7.16; N, 6.22; P, 13.76. Found: C, 37.13; H, 7.29; N, 6.11; P, 13.76.

Resolution of (±)-2-amino-7-phosphonoheptanoic acid (13)

A solution of L-lysine (3.21 g, 0.022 mol) and (±)-APH (13) (5 g, 0.022 mol) in water (22 mls) was heated gently (60°C) for 15 minutes and methanol was added until the initial appearance of a flocculent precipitate. The mixture was cooled, diethyl ether added until the onset of a slight cloudiness and left at -10°C overnight. The precipitate was filtered to give the (-)-APH-lysine salt (3.80 g, 46%) which was taken up in 2M pyridine and poured onto an ion-exchange resin column (Dowex 50, pyridinium form). The column was eluted with 2M pyridine, all ninhydrin positive fractions were combined, evaporated, in vacuo, the residue washed with methanol and the solvent evaporated, in vacuo, to afford (-)-APH (2.1 g): $[\alpha^{25}_{546}] = -7°$ (c = 10, H_2O).

The mother liquor from the above filtration was poured onto an ion-exchange resin column (Dowex 50, pyridinium form) and (+)-APH (1.88 g) was isolated as above: $[\alpha^{25}_{546}] = +7.5°$ (c = 5, H_2O).

5.2 Quinolinic Acid Analogues

2,3-Pyridinecarboxylic anhydride (70)[17]

Concentrated HCl (8 ml) was added to a stirred solution of quinolinic acid (44) (50 g, 0.3 mol) in acetic anhydride (110 ml); over two hours at 65°C. The mixture was then poured into carbon tetrachloride (1 l) and the precipitated solid was crystallised from ethylacetate-hexane to give the anhydride (70) (29.7 g, 67%): mp 136°C (lit. mp 136-138°C[17]); IR (nujol) 3400, 3150, 1690, 1630, 1580, 760 cm^{-1}.

2-Carbomethoxy-3-carboxypyridine (71)[17]

A solution of anhydride (70) (21.74 g, 0.15 mol) in methanol (115 ml) was heated under reflux overnight, solvent was evaporated, *in vacuo*, and the oily residue dissolved in hot water. On cooling to 0°C, ester (71) precipitated and recrystallisation from water and then ethyl acetate gave ester (71) (15.84 g, 60%): mp 122-125°C (lit. mp 125-126°C[17]); IR (nujol) 3540, 1730, 1620, 1580, 1300, 1210, 1160, 1090, 900, 760 cm^{-1}; ^{1}H NMR (D_2O) δ 4.48 (s, 3H, $CO_2\underline{C}H_3$), 8.25 (dd, J = 5.13 Hz and 8 Hz, 1H, C5H), 8.81 (dd, J = 1.6 Hz and 8 Hz, 1H, C4H), 9.19 (dd, J = 1.6 Hz and 5.13 Hz, C6H); ^{13}C NMR (H_2O) δ 154.34 ($CO_2\underline{C}H_3$), 127.47 (C5), 129.86 (C3), 139.77 (C4), 147.25 (C2), 150.66 (C6), 167.72 ($\underline{C}O_2CH_3$), 169.99 ($\underline{C}O_2H$).

2-Carbomethoxy-3-[1'-chloro-1'-oxo-methyl]-pyridine (72)

A solution of ester (71) (6 g, 0.033 mol) in redistilled thionyl chloride (70 ml) was heated under reflux for five hours. The solvent was then evaporated, *in vacuo*, to give the acyl chloride (72) (6.5 g, 98%): IR (liq. film) 2950, 1785, 1750, 1560, 1300, 1200, 1140, 1100, 880, 710 cm^{-1}.

2-Carbomethoxy-3-[2'-diazo-1'-oxo-ethyl]-pyridine (73)[75]

A solution of the acyl chloride (72) (6.5 g, 0.033 mol) in dichloromethane (20 ml) was added drop-wise to a solution of diazomethane[2] (1.5 g, 0.036 mol) in ether (180 ml), with stirring at 0°C. Solvent was evaporated, in vacuo, to give the diazoketone (73) (6.7 g, 100%) as a dark brown oil: IR (liq. film) 3600, 3400, 3100, 2950, 2100, 1740, 1620, 1360, 1210, 1140, 880, 710 cm^{-1}.

2-Carbomethoxy-3-[2'-carbomethoxy-ethyl]-pyridine (74)

A paste of silver (1) oxide (15 g, 0.065 mol) in methanol was added, portion-wise, to a solution of diazoketone (73) (6.7 g, 0.033 mol) in methanol (120 ml) with stirring at 50°C. When evolution of nitrogen ceased, the mixture was filtered with Celite (3 times) and solvent evaporated, in vacuo. The residue was chromatographed on silica gel (100 g) using ethyl acetate as eluant to afford the diester (74) (4.99 g, 72%) as a pale yellow oil: IR (liq. film) 2940, 1730, 1560, 1430, 1300, 1200, 1170, 1090, 1000, 720 cm^{-1}; ^{1}H NMR (CCl_4) δ 3.71 (s, 3H, $CH_2CO_2\underline{CH}_3$), 3.96 (s, 3H, $CO_2\underline{CH}_3$), 4.4 (s, 2H, $\underline{CH}_2CO_2CH_3$), 7.38 (m, 1H, C5 H), 7.58 (m, 1H, C6H), 7.62 (m, 1H, C4 H); ^{13}C NMR (CCl_4) δ 38.73 ($\underline{CH}_2CO_2CH_3$), 52.06 ($CH_2CO_2\underline{CH}_3$), 52.22 ($CO_2\underline{CH}_3$), 125.79 (C5), 131.64 (C3), 139.77 (C4), 148.06 (C6), 148.44 (C2), 166.31 ($\underline{CO}_2CH_3$), 170.17 ($CH_2\underline{CO}_2CH_3$); mass spectrum, m/e 209 (M^+), 150 ($M - CO_2CH_3$), 92 ($M^+-2(CO_2CH_3)$), calcd. for $C_{10}H_{11}NO_4$ 209.206, found 209.0712. Anal. calcd. for $C_{10}H_{11}NO_4$: C, 57.41; H, 5.3; N, 6.69. Found: C, 57.21; H, 5.28; N, 6.44.

Preparation of diester (74) using copper (I) iodide

Copper (I) iodide (3.43 g, 18 mmol) was added to a solution of the diazoketone (73) (1.87 g, 9 mmol) in dry acetonitrile/methanol 5:1 (100 ml) heated at 65 - 70°C for four hours. The mixture was

cooled, filtered and the solvent evaporated, in vacuo, to give a dark brown residue. Chromatography using ethyl acetate eluant on silica gel (30 g) afforded the diester (74) (260 mg, 15%) as a yellow oil, which was characterised as above.

2-Carboxy-3-[2'-carboxy-ethyl]-pyridine (45) [Homoquinolinic acid]

A solution of the diester (74) (300 mg, 1.4 mmol) in sodium hydroxide (0.1M, 14 ml) was heated under reflux for four hours. The mixture was allowed to cool to room temperature, hydrochloric acid (0.1M, 15 ml) added and the solution poured onto an ion-exchange resin column (Dowex 50 (H^+)). The resin was eluted with water, the solvent evaporated, in vacuo, and the residue recrystallised from water-ethanol to yield the acid (45) (190 mg, 73%) as tan crystals: mp 186-187°C (lit. mp 187°C[75]); IR (nujol) 3450, 1710, 1640, 1600, 1335, 1240, 1210, 1180, 1160, 1100, 935, 800, 710 cm^{-1}; ^1H NMR (D_2O) δ 4.26 (s, 2H, $C\underline{H}_2CO_2H$), 8.04 (dd, J = 5.6 Hz and 8 Hz, 1H, C5H), 8.51 (dd, J = 1.5 Hz and 8 Hz, 1H, C4H), 8.67 (dd, J = 1.6 Hz and 5.6 Hz, C6H); ^{13}C (H_2O) δ 38.68 ($\underline{C}H_2CO_2H$), 128.01 (C5), 134.68 (C3), 139.99 (C4), 145.62 (C2), 150.39 (C6), 164.09 ($\underline{C}O_2H$), 174.71 ($CH_2\underline{C}O_2H$); mass spectrum, m/e 181 (M), 163 (M-H_2O), 137 (M-CO_2) 91 (M-CH_2CO_2H and CO_2H). Anal. calcd. for $C_8H_7NO_4.H_2O$: C, 48.24; H, 4.55; N, 7.11. Found: C, 48.44; H, 4.25; N, 7.11.

2-Carbomethoxy-3-hydroxymethyl-pyridine (76)

To a stirred solution of the ester (71) (1.6 g, 9 mmol) in THF (40 ml), borane-tetrahydrofuran complex (1M, 12 ml) was added, under nitrogen at 0°C. After 30 minutes the solution was allowed to warm to room temperature and left overnight. The mixture was diluted with water (40 ml), basified with sodium bicarbonate and evaporated, in vacuo. The residue was taken up in water (40 ml) and ethyl acetate

(50 ml) and the ethylacetate layer discarded. The aqueous layer was acidified with concentrated HCl, poured onto an ion-exchange resin column (Dowex 50 (H^+)), eluted with water and the eluate evaporated, in vacuo. Recrystallisation from water-ethanol gave the alcohol (76) (0.97 g, 65%) as white crystals: ^1H NMR (D_2O) δ 3.96 (s, 3H, $CO_2C\underline{H}_3$), 7.92 (dd, J = 5.13 Hz and 7.8 Hz, 1H, C5H), 8.47 (dd, J = 1.5 Hz and 7.8 Hz, 1H, C4H), 8.83 (dd, J = 1.5 Hz and 5.13 Hz, 1H, C6H); mass spectrum, m/e 167 (M), 149 ($M-H_2O$), 123 ($M-CO_2$), 105 ($M-CO_2CH_3$ and H_2O), 77 ($M-CH_2OH$ and CO_2CH_3).

2-[2'-hydroxy-2'-phenyl-ethyl]-3-methyl-pyridine (87)

n-Butyllithium (55 ml, 93 mmol) was slowly added to a stirred solution of 2,3-lutidine (10 g, 93 mmol) in THF (50 ml) under nitrogen at -20°C. The mixture was allowed to warm to room temperature, after 30 minutes, benzaldehyde (15 ml, 93 mmol) was added dropwise at 0°C. The mixture was again allowed to warm to room temperature. The colourless mixture was poured into ethyl acetate (60 ml) and water (45 ml) and 1M HCl (45 ml) added. The aqueous layer was basified with .880 ammonium hydroxide and the organic layer was separated. The aqueous phase was extracted with more ethyl acetate (3 x 100 ml). The combined extracts were dried (Na_2SO_4), filtered and solvent evaporated, in vacuo. to afford a yellow oil. Chromatography on silica gel (260 g) using diethyl ether as eluant gave the pure alcohol (87) (17 g, 85%) as white crystals: mp 63°C; IR (CH_2Cl_2) 3400, 3070, 3040, 2930, 2870, 1725, 1690, 1630, 1590, 1565, 1455, 1275, 1210, 1110, 1080, 1045, 1030, 740, 700 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.20 (s, 3H, $C\underline{H}_3$), 3.05 (m, 2H, $C\underline{H}_2CHPh$), 5.28 (dd, J = 4.64 Hz and 7.56 Hz, 1H, $C\underline{H}Ph$), 7.10 (dd, J = 4.64 Hz and 7.57 Hz, 1H, C5H), 7.36 (m, 6H, $CH\underline{Ph}$ and C4H), 8.38 (dd, J = 1.2 Hz and 4.64 Hz, 1H, C6H); mass spectrum, m/e 214 (M^+), 196 (M^+-H_2O), 170 (M-CHOHPh), 92 (M^+-CH_2CHOPh). Anal. calcd. for $C_{14}H_{15}NO$:

C, 78,84; H, 7.09; N, 6.57. Found: C, 78.81; H, 7.2; N, 6.42.

3-Methyl-2[-2'-phenyl-ethenyl]-pyridine (88)

A 100 ml round bottomed flask fitted with a condenser and a Dean and Stark trap, was charged with the alcohol (87) (300 mg, 1.41 mmol), tosic acid (600 mg, 3.15 mmol) and toluene (15 ml). The mixture was heated under reflux overnight. On cooling, the mixture was poured into ethyl acetate (15 ml) and water (15 ml); the aqueous layer was basified with .880 ammonium hydroxide and the organic layer separated. The aqueous phase was extracted with more ethyl acetate (3 x 20 ml). The combined ethyl acetate extracts were dried (Na_2SO_4), filtered and solvent evaporated, in vacuo. Chromatography on silica gel (8 g) using hexane/diethyl ether 5:1 eluant afforded the alkene (88) (270 mg, 98%) as a yellow oil; IR (liq. film) 3060, 3030, 2980, 2930, 2860, 1690, 1635, 1590, 1580, 1560, 1490, 1450, 1420, 1380, 1330, 1275, 1220, 1100, 1075, 1030, 970, 770, 740, 690 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.43 (s, 3H, CH_3),* 7.05 (dd, J = 4.76 Hz and 8 Hz, 1H, C5H), 7.40 (m, 6H, CHPh and C4H), 8.46 (dd, J = 1.5 Hz and 4.64 Hz, 1H, C6H); mass spectrum, m/e 195 (M), 117 (M^+-Ph), 105 (M^+-CHPh), 91 (M-CHCHPh), calcd. for $C_{14}H_{13}N$ 195.265, found 195.1041. Anal. calcd. for $C_{14}H_{13}N$: C, 86.12; H, 6.71; N, 7.17. Found: C, 86.03; H, 6.68; N, 6.89.

* 6.9 (m, 2H, HC=CH)

2-Carboxy-3-methylpyridine (89)

Ozone was passed through a stirred solution of the alkene (88) (9.33 g, 0.05 mol) in methanol at room temperature, until the solution became colourless. The solvent was evaporated, in vacuo, and recrystallisation from dichloromethane-hexane yielded the acid (89) (4.3 g, 65%) as white crystals; mp 110°C (lit. mp 111°C [21a]); ^1H NMR ($CDCl_3$) δ 2.71 (s, 3H, CH_3), 7.95 (dd, J = 5.66 Hz and 8.3 Hz, 1H, C5H), 8.52 (m, 2H, C4H and C6H).

C, 78,84; H, 7.09; N, 6.57. Found: C, 78.81; H, 7.2; N, 6.42.

3-Methyl-2[-2'-phenyl-ethenyl]-pyridine (88)

A 100 ml round bottomed flask fitted with a condenser and a Dean and Stark trap, was charged with the alcohol (87) (300 mg, 1.41 mmol), tosic acid (600 mg, 3.15 mmol) and toluene (15 ml). The mixture was heated under reflux overnight. On cooling, the mixture was poured into ethyl acetate (15 ml) and water (15 ml); the aqueous layer was basified with .880 ammonium hydroxide and the organic layer separated. The aqueous phase was extracted with more ethyl acetate (3 x 20 ml). The combined ethyl acetate extracts were dried (Na_2SO_4), filtered and solvent evaporated, in vacuo. Chromatography on silica gel (8 g) using hexane/diethyl ether 5:1 eluant afforded the alkene (88) (270 mg, 98%) as a yellow oil; IR (liq. film) 3060, 3030, 2980, 2930, 2860, 1690, 1635, 1590, 1580, 1560, 1490, 1450, 1420, 1380, 1330, 1275, 1220, 1100, 1075, 1030, 970, 770, 740, 690 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.43 (s, 3H, CH_3),* 7.05 (dd, J = 4.76 Hz and 8 Hz, 1H, C5H), 7.40 (m, 6H, CHPh and C4H), 8.46 (dd, J = 1.5 Hz and 4.64 Hz, 1H, C6H); mass spectrum, m/e 195 (M), 117 (M^+-Ph), 105 (M^+-CHPh), 91 (M-CHCHPh), calcd. for $C_{14}H_{13}N$ 195.265, found 195.1041. Anal. calcd. for $C_{14}H_{13}N$: C, 86.12; H, 6.71; N, 7.17. Found: C, 86.03; H, 6.68; N, 6.89.

* 6.9 (m, 2H, HC=CH)

2-Carboxy-3-methylpyridine (89)

Ozone was passed through a stirred solution of the alkene (88) (9.33 g, 0.05 mol) in methanol at room temperature, until the solution became colourless. The solvent was evaporated, in vacuo, and recrystallisation from dichloromethane-hexane yielded the acid (89) (4.3 g, 65%) as white crystals; mp 110°C (lit. mp 111°C [21a]); ^1H NMR ($CDCl_3$) δ 2.71 (s, 3H, CH_3), 7.95 (dd, J = 5.66 Hz and 8.3 Hz, 1H, C5H), 8.52 (m, 2H, C4H and C6H).

2-Carbomethoxy-3-methylpyridine (90)

Dicyclohexylcarbodiimide (721 mg, 3.5 mmol), dimethylaminopyridine (3.8 mg, 0.004 mmol) and a solution of methanol/dichloromethane (3ml) were added to a suspension of the acid (89) (480 mg, 3.5 mmol) in pyridine (7 ml) with stirring for 12 hours at room temperature. The mixture was then filtered to remove dicyclohexyl urea. After the solvent had been evaporated, in vacuo, the residue was taken up in a minimum volume of dichloromethane, the solution cooled at -10°C for a few hours to precipitate any residual urea and filtered. The solvent was evaporated, in vacuo, and the residue chromatographed on silica gel (10 g) using hexane/ethyl acetate 1:1 eluant to give the ester (90) (424 mg, 80%) as a colourless oil: bp 105°C (lit. bp 102-103°C 15 mmHg[21a]); IR (liq. film) 2960-2860, 1730, 1675, 1570, 1440, 1385, 1300, 1230, 1200, 1140, 1100, 790, 710 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.60 (s, 3H, $C\underline{H}_3$), 3.98 (s, 3H, $CO_2C\underline{H}_3$), 7.35 (dd, J = 4 Hz and 7.8 Hz, 1H, C5H), 7.63 (dd, J = 1.5 Hz and 7.8 Hz, 1H, C4H), 8.56 (dd, J = 1.5 Hz and 4.4 Hz, 1H, C6H).

3-Methyl-2-[3'-carbomethoxy-1'-oxo-propyl]-pyridine (98)

Hexamethylphosphoramide (HMPA) (0.085 ml, 0.44 mmol) was added to a stirred solution of LDA (0.44 ml, 0.44 mmol) in THF (2 ml) under nitrogen at -10°C. A solution of the ester (90) (60 mg, 0.4 mmol) in THF (1 ml) was added dropwise to the mixture. After 30 minutes the mixture was allowed to warm to 0°C, methylbromoacetate (0.05 ml, 0.6 mmol) was added, the mixture allowed to warm to room temperature and left overnight. The solvent was evaporated, in vacuo, to give a dark brown oil (480 mg). Chromatography on silica gel (8 g) using diethyl ether/hexane 2:1 eluant afforded the ester (98) (10 mg, 12%) as a brown oil: ^1H NMR ($CDCl_3$) δ 1.01 (s, 3H, $OC\underline{H}_3$), 2.57 (s, 3H, $C\underline{H}_3$), 3.8 (m, 2H, $C\underline{H}_2$), 7.31 (dd, J = 4.6 Hz and 7.8 Hz, 1H, C5H), 7.60 (dd, J = 1.7 Hz and 7.8 Hz, 1H, C4H), 8.55 (dd, J = 1.7 Hz and 4.6 Hz, 1H, C6H);

mass spectrum, m/e 193 (M), 92 (M-$COCH_2CO_2CH_3$), calcd. for $C_{10}H_{11}NO_3$ 193.205, found 193.603.

3-Methylpyridine-2-carboxylic acid, benzyl ester (100)

To a stirred solution of LDA (0.36 ml of 1M solution) in THF (3 ml), HMPA (0.07 ml, 0.36 mmol) was added under nitrogen at -10°C. A solution of the ester (90) (50 mg, 0.33 mmol) in THF (2 ml) was added and after 50 minutes the solution was allowed to warm to 0°C. The mixture was recooled, benzyl bromide (0.1 ml, 0.72 mmol) added and after 2 hours allowed to warm to room temperature and stirred overnight. The solvent was evaporated, in vacuo, and the residue chromatographed on silica gel (5 g) using hexane/diethyl ether 2:1 eluant to give the benzyl ester (100) (40 mg, 50%): IR (liq. film) 3075, 2980-2860, 1730, 1540, 1500, 1450, 1375, 1300, 1230, 1200, 1140, 1100, 790, 740, 700 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.55 (s, 3H, CH_3), 5.44 (s, 2H, CH_2Ph), 7.3 (m, 7H, CH_2Ph, C4H and C5H), 8.54 (dd, J = 1.7 Hz and 4.6 Hz, 1H, C6H); mass spectrum, m/e 228 (M^+), 121 (M^+-OCH_2Ph), 93 ($M^+-CO_2CH_2Ph$).

3-Hydroxymethyl-2-methyl pyridine (104)

To a stirred suspension of lithium aluminium hydride (1.38 g, 38 mmol) in dry diethyl ether (200 ml) was added dropwise, at 0°C, a solution of the ester (103) (8 g, 48 mmol) in ether (100 ml). After the addition, the mixture was allowed to warm to room temperature over a period of 45 minutes, before being heated under reflux for 30 minutes. Water (5 ml) was cautiously added to the cooled solution and the resulting precipitate filtered and washed with more diethyl ether (2 x 100 ml). Drying (Na_2SO_4), filtration and evaporation, in vacuo, of the filtrate afforded a pungent oil (8.3 g). Chromatography on silica gel (80 g) using diethyl ether as eluant gave the alcohol (104) (4.9 g,

92%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 2.50 (s, 3H, $C\underline{H}_3$), 3.56 (broad s, 1H, O<u>H</u>), 4.7. (s, 2H, $C\underline{H}_2OH$), 7.13 (dd, J = 4.9 Hz and 7.8 Hz, 1H, C5 H), 7.74 (dd, J = 1.7 Hz and 7.8 Hz, 1H, C4 H), 8.32 (dd, J = 1.7 Hz and 4.9 Hz, 1H, C6 H).

<u>Reaction of alcohol (104) with phosphorus tribromide</u>

To a stirred solution of the alcohol (104) (369 mg, 3 mmol) in dry diethyl ether (12 ml) was added dropwise phosphorus tribromide (810 mg, 0.28 ml, 3 mmol), under nitrogen, at 0°C. The mixture was stirred for 45 minutes when no more starting material was apparent (TLC). The mixture was poured onto ice (10 g) and the whole extracted with diethyl ether (5 x 30 ml). The extracts were washed with aqueous sodium bicarbonate (2 x 20 ml), water (2 x 25 ml), dried (Na_2SO_4) and filtered. Evaporation of the solvent, <u>in vacuo</u>, led to a red solid. TLC analysis of the reaction mixture, before and after work-up, had shown a spot (R_f 0.8 in diethyl ether; starting material R_f 0.35 in diethyl ether) which was not in evidence on evaporation of solvent.

<u>3-[2',2'-Dicarbethoxyethyl]-2-methyl pyridine (106)</u>

A mixture of 2,3-lutidine (86) (2.14 g, 20 mmol) and N-bromo-succinimide (4.27 g, 22 mmol) in carbon tetrachloride (80 ml) containing a trace of azobisisobutyronitrile (660 mg, 4 mmol) was heated under reflux, with lamp irradiation for 90 minutes. The mixture was then concentrated to about 2/3 volume and added, dropwise, to a refluxing solution of diethyl potassiomalonate (prepared from potassium t-butoxide (6.72 g, 60 mmol) and diethyl malonate (9.6 g, 60 mmol)) in THF (150 ml), under nitrogen. The resulting mixture was stirred and heated under reflux for another 2½ hours. The solvent was evaporated, <u>in vacuo</u>, to

give the malonate adduct (106) (2.98 g, 56%) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.2 (t, J = 7 Hz, 6H, 2 x $OCH_2\underline{CH}_3$), 2.58 (s, 3H, $C\underline{H}_3$), 3.24 (d, J = 8 Hz, 2H, $C\underline{H}_2CH(CO_2Et)_2$), 3.58 (m, 1H, $CH_2\underline{CH}(CO_2Et)_2$), 4.20 (q, J = 7 Hz, 4H, 2 x $OC\underline{H}_2CH_3$), 7.05 (m, 1H, C5 H), 7.50 (m, 1H, C4 H), 8.4 (m, 1H, C6 H). Anal. calcd. for $C_{14}H_{19}NO_4$: C, 59.72; H, 7.94; N, 5.80. Found: C, 59.68; H, 8.21; N, 5.92.

A sample (∿ 250 mg) was chromatographed on silica gel (5 g) using diethyl ether/hexane (1:3) eluant, but the bulk of the adduct (106) was used in the next reaction without further purification.

3-[2'-Carboxyethyl]-2-methyl pyridine (107)

The malonate adduct (106) (2.6 g, 9.8 mmol) and concentrated hydrochloric acid (50 ml) were heated under reflux for 18 hours. The mixture was evaporated to dryness, the residue dissolved in water (25 ml) and chromatographed on ion exchange resin (Dowex 50 [H^+] using water as eluant. All UV (254 nm) active fractions were combined and evaporated, in vacuo, to afford a solid. Recrystallisation from water/ethanol gave the pure acid (107) (1.42 g, 88%): ^{1}H NMR (D_2O) δ 2.55 (t, J = 7.5 Hz, 2H, $C\underline{H}_2CH_2CO_2H$), 2.74 (s, 3H, $C\underline{H}_3$), 3.05 (t, J = 7.5 Hz, 3H, $CH_2C\underline{H}_2CO_2H$), 7.7 (m, 1H, C5 H), 8.3 (m, 2H, C4 H and C6 H). Anal. calcd. for $C_9H_{11}NO_2$: C, 65.43; H, 6.71; N, 8.48. Found: C, 65.29; H, 6.77; N, 8.61.

2-Carbomethoxy-3-[(N-2'-carbethoxy-ethyl)amido] pyridine (111)

Glycine ethyl ester hydrochloride (2.09 g, 15 mmol), dicyclohexyl-carbodiimide (4.12 g, 20 mmol) and dimethylaminopyridine (0.18 g, 1.5 mmol) were added to a stirred solution of the ester (22) (2.86 g, 15 mmol) in dichloromethane (60 ml) and pyridine (5 ml). The mixture was stirred overnight at room temperature. The solvent was evaporated,

in vacuo, the residue taken up in a minimum volume of dichloromethane and the solution cooled at -10°C for a few hours to precipitate dicyclohexyl urea and filtered. The solvent was evaporated, in vacuo, and the residue chromatographed on silica gel (20 g). Initially using diethyl ether/ethyl acetate 2:1 eluant afforded the anhydride (70) (1.26 g): mp 136°C. Diethyl ether/ethyl acetate 1:2 as eluant gave the ester (111) (900 mg, 24%): IR (liq. film) 3300, 3040, 2960, 2925, 1740, 1650, 1300, 1200, 1170, 1130, 1020, 990, 710 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.32 (t, 3H, $CH_2\underline{CH}_3$), 3.99 (s, 3H, $CO_2\underline{CH}_3$), 4.27 (m, 4H, $\underline{CH}_2CH_3$ and $\underline{CH}_2CO_2Et$), 6.69 (broad s, 1H, $\underline{NH}$), 7.52 (dd, J = 4.6 Hz and 7.8 Hz, 1H, C5 H), 7.97 (dd, J = 1.7 Hz and 7.8 Hz, 1H, C4 H), 8.77 (dd, J = 1.7 Hz and 4.6 Hz, 1H, C6 H); mass spectrum, m/e 251 (M), 233 ($M-H_2O$), 161 ($M-CO_2C_2H_5$), 106 ($M-CONHCH_2CO_2C_2H_5$).

2-Carboxy-3-[(N-2'-carboxyethyl)amido]pyridine (47)

A solution of the ester (111) (800 g, 3.6 mmol) in 4M sodium hydroxide (3 ml) was stirred overnight at 60°C. The mixture was allowed to cool to room temperature, poured onto an ion exchange resin column (Dowex 50 [H^+]) which was then eluted with water. Acidic fractions were combined, evaporated, in vacuo, and recrystallised from water-ethanol to give the acid (47) (690 mg, 86%) as white crystals: mp 203°C; IR (nujol) 3600, 3400, 1940, 1720, 1640-1560, 1230, 1160, 950, 825, 720 cm^{-1}; ^{1}H NMR (D_2O) δ 4.24 (s, 2H, $\underline{CH}_2CO_2H$), 7.78 - 8.99 (m, 3H, C4 H, C5 H and C6 H); ^{13}C NMR (H_2O) δ 42.0 ($\underline{CH}_2$), 128.3 (C5), 133.3 (C1), 145.2 (C4), 145.6 (C2), 147.6 (C6), 169.8 ($\underline{C}ONH$), 172.0 ($\underline{C}O_2H$); mass spectrum, m/e 224 (M), 207 ($M-H_2O+1$), 161 ($m-CO_2H$ and $-H_2O$), 105 ($M-CO_2H$ and $-NHCH_2CO_2H$), 78 ($M-CO_2H$ and $-CONHCH_2CO_2H$). Anal. calcd. for $C_9H_8NO_5$: C, 41.52; H, 4.65; N, 10.77. Found: C, 41.37; H, 4.57; N, 10.6

5.3 Tricholomic acid

Benzyl carbamate (141)[109]

Benzyl chlorofomate (15 ml, 0.11 mol) was added slowly to 0.880 ammonium hydroxide (60 ml) at 0°C, with vigorous stirring. The mixture was allowed to warm to room temperature, left for 30 minutes, filtered, the solid washed with water and dried, *in vacuo*, to give benzyl carbamate (141) (14.13 g, 85%) as white crystals: mp 86° (lit. mp 86°C)[109]; IR (nujol) 3400, 3320, 3260, 1690, 1610, 1400, 1070, 730 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.08 (s, 4H, $NH_2CO_2CH_2Ph$), 7.34 (s, 5H, CH_2Ph).

N-Benzyloxycarbonyl-2-aminotetrahydro-5-oxo-2-furan carboxylic acid (142)

A 250 ml, two-necked flask equipped with a thermometer and a condenser was charged with finely powdered benzyl carbamate (141) (16 g, 0.11 mol) and 2-keto-glutaric acid (22 g, 0.15 mol). The mixture was vigorously stirred and heated, at 85-90°C, under reduced pressure (10-15 mmHg) for two hours. The solid residue was recrystallised from ethyl acetate to give the lactone acid (142) (20.7 g, 70%) as white crystals: mp 175°C (lit. mp 176°C[109]).

Benzyl-N-benzyloxycarbonyl-2-aminotetrahydro-5-oxo-2-furan carboxylate (143)

To a stirred solution of lactone acid (142) (8.37 g, 30 mmol) in ethyl acetate (50 ml), triethylamine (6.9 ml, 50 mmol) was added dropwise at 60°C. Benzyl bromide (3.9 ml, 33 mmol) was added after 10 minutes. The mixture was heated under reflux for 3 hours, filtered and the solvent evaporated, *in vacuo*. The resulting oil was triturated with hexane and then recrystallised from dichloromethane-hexane to give the lactone ester (143) (8.3 g, 75%) as white crystals: mp 117-118°C; IR (nujol) 3260, 1745, 1710, 1500, 1130, 715 cm^{-1}; ^1H NMR ($CDCl_3$)

δ 2.73 (m, 4H, $C\underline{H}_2C\underline{H}_2$), 5.11 (s, 2H, $CCO_2C\underline{H}_2Ph$), 5.24 (d, J = 3 Hz, 2H, $CNHCO_2C\underline{H}_2Ph$), 6.51 (broad s, 1H, $CN\underline{H}CO_2CH_2Ph$), 7.35 (s, 10H, $CCO_2CH_2\underline{Ph}$ and $CNHCO_2CH_2\underline{Ph}$)

N-Benzyloxycarbonyl-1-benzyl-(Z)-2,3-dehydroglutamate (144)

To a solution of the lactone ester (143) (4.74 g, 13 mmol) in dry THF (20 ml) cooled to 0°C and under nitrogen, 1,5- diazabicyclo [5:4.0] undec-5-ene (DBU) (4 ml, 26 mmol) was added dropwise and the resulting solution stirred for 30 minutes. The mixture was allowed to warm to room temperature, diluted with water/ethyl acetate 1:1 (300 ml), the aqueous layer was acidified with 1M HCl and the ethyl acetate layer separated. The aqueous phase was extracted with more ethyl acetate (3 x 30 ml). The combined extracts were dried ($NaSO_4$), filtered and solvent evaporated, in vacuo, to give a pale yellow oil. Crystallisation from dichloromethane-hexane yielded the dehydroglutamate (144) (3.3 g, 71%) as slightly-yellow crystals: mp 115-116°C (lit. mp 115-117°C[24]); IR (nujol) 3400, 3260, 1720, 1680, 1060, 730 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.3 (d, J = 7 Hz, 2H, $C\underline{H}_2CO_2H$), 5.11 (s, 2H, $CO_2C\underline{H}_2Ph$), 5.17 (s, 2H, $NHCO_2C\underline{H}_2Ph$), 6.78 (t, 1H, $CH_2C\underline{H}$), 6.86 (broad s, 1H, $N\underline{H}$), 7.32 (s, 10H, 2x$\underline{Ph}$) 10.05 (s, 1H, $CO_2\underline{H}$). Anal. calcd. for $C_{20}H_{19}NO_6$: C, 65.03; H, 5.18; N, 3.79. Found: C, 64.92; H, 5.16; N, 3.79.

N-Benzyloxycarbonyl hydroxylamine (145)

To a vigorously stirred suspension of benzyl chloroformate (17.05 g, 0.1 mol) in ethyl acetate (80 ml) and water (80 ml) was added hydroxylamine hydrochloride (8.34 g, 0.12 mol), at 0°C. Sodium bicarbonate (25.44 g, 0.24 mol was slowly added, the mixture allowed to warm to room temperature and was left overnight. The aqueous layer was acidified with concentrated HCl and the ethyl acetate layer separated.

The aqueous phase was extracted with more ethyl acetate (3 x 100 ml). The combined extracts were washed with brine (60 ml), dried (Na_2SO_4), filtered and the solvent evaporated, in vacuo, to yield a colourless oil which solidified on standing. Recrystallisation from hexane-diethyl ether afforded the hydroxylamine (145) (10 g, 60%).

N-Benzyloxycarbonyl-O-t-butyldimethylsilyl hydroxylamine (146)

t-Butyldimethylsilyl chloride (2.89 g, 19.2 mmol) and imidazole (3.92 g, 57.6 mmol) were added to a stirred solution of the hydroxylamine (145) (3.2 g, 19.2 mmol) in dimethylformamide (50 mls), at room temperature, under nitrogen. The mixture was left for 24 hours, diluted with ethyl acetate (30 ml) and water (15 ml) and the ethyl acetate layer was separated. The aqueous phase was extracted with more ethyl acetate (3 x 30 ml). The combined ethyl acetate extracts were dried (Na_2SO_4), filtered, and the solvent evaporated, in vacuo. Chromatography on silica gel (80 g) using hexane/diethyl ether 3:1 eluant gave the silylated hydroxylamine (146) (3.54 g, 68%) as a yellow oil.

N-Benzyloxycarbonyl-1-benzyl-(Z)-2,3-dehydro-5-N,N-diisopropylamido glutamate (149)

A solution of dehydroglutamate (144) (55 mg, 0.15 mmol), N-hydroxysuccinimide (17 mg, 0.15 mmol) and DCC (31 mg, 0.15 mmol) in dichloromethane (3 ml) was stirred for 30 minutes under nitrogen at room temperature. Diisopropylamine (148) (0.033 ml, 0.24 mmol) was then added, the mixture left overnight, filtered and solvent evaporated, in vacuo. The residue was taken up in a minimum volume of dichloromethane, the solution cooled at -10°C for a few hours, filtered and the solvent evaporated, in vacuo. The residue was chromatographed on silica gel (5 g) using hexane/diethyl ether 2:1 eluant to give the amide (149)

The aqueous phase was extracted with more ethyl acetate (3 x 100 ml). The combined extracts were washed with brine (60 ml), dried (Na_2SO_4), filtered and the solvent evaporated, in vacuo, to yield a colourless oil which solidified on standing. Recrystallisation from hexane-diethyl ether afforded the hydroxylamine (145) (10 g, 60%).

N-Benzyloxycarbonyl-O-t-butyldimethylsilyl hydroxylamine (146)

t-Butyldimethylsilyl chloride (2.89 g, 19.2 mmol) and imidazole (3.92 g, 57.6 mmol) were added to a stirred solution of the hydroxylamine (145) (3.2 g, 19.2 mmol) in dimethylformamide (50 mls), at room temperature, under nitrogen. The mixture was left for 24 hours, diluted with ethyl acetate (30 ml) and water (15 ml) and the ethyl acetate layer was separated. The aqueous phase was extracted with more ethyl acetate (3 x 30 ml). The combined ethyl acetate extracts were dried (Na_2SO_4), filtered, and the solvent evaporated, in vacuo. Chromatography on silica gel (80 g) using hexane/diethyl ether 3:1 eluant gave the silylated hydroxylamine (146) (3.54 g, 68%) as a yellow oil.

N-Benzyloxycarbonyl-1-benzyl-(Z)-2,3-dehydro-5-N,N-diisopropylamido glutamate (149)

A solution of dehydroglutamate (144) (55 mg, 0.15 mmol), N-hydroxysuccinimide (17 mg, 0.15 mmol) and DCC (31 mg, 0.15 mmol) in dichloromethane (3 ml) was stirred for 30 minutes under nitrogen at room temperature. Diisopropylamine (148) (0.033 ml, 0.24 mmol) was then added, the mixture left overnight, filtered and solvent evaporated, in vacuo. The residue was taken up in a minimum volume of dichloromethane, the solution cooled at -10°C for a few hours, filtered and the solvent evaporated, in vacuo. The residue was chromatographed on silica gel (5 g) using hexane/diethyl ether 2:1 eluant to give the amide (149)

(35 mg, 54%): ^{1}H NMR ($CDCl_3$) δ 1.22 (dd, J = 4.88 Hz and 9.52 Hz, 6H, $N(C\underline{H}_3)_2$), 3.22 (d, J = 4.84 Hz, 2H, $COC\underline{H}_2$), 3.2 - 3.9 (m, 2H, NC<u>H</u>), 5.13 - 5.19 (m, 4H, $CO_2C\underline{H}_2Ph$ and $NHCO_2C\underline{H}_2Ph$), 6.83 - 6.9 (m, 2H, $CH_2C\underline{H}$ and N<u>H</u>), 7.34 (s, 10H, 2x<u>Ph</u>).

<u>Isolation of amide (149) via acyl chloride (150)</u>

To a stirred solution of dehydroglutamate (144) (40 mg, 0.11 mmol) in toluene (3 ml) and DMF (0.2 ml), a solution of thionyl chloride (10 µl, 0.11 mmol) in toluene (0.1 ml) was added at 0°C, under nitrogen. After 1 hour the mixture was allowed to warm to room temperature, diisopropylamine (148) (18 µl, 0.13 mmol) added and left overnight. The resulting solution was diluted with water (5 ml) and ethyl acetate (10 ml), the aqueous layer was acidified with concentrated HCl and the ethyl acetate layer separated. The aqueous phase was extracted with more ethyl acetate (4 x 20 ml). Ethyl acetate extracts were combined, dried (Na_2SO_4), filtered, the solvent evaporated, <u>in vacuo</u>, to give an oily residue. Chromatography on silica gel (5 g) using hexane/diethyl ether 1:1 eluant gave the amide (149) (13 mg, 25%).

<u>2-Nitrobenzaldoxime</u> (156)

To a solution of 2-nitrobenzaldehyde (155) (9.02 g, 60 mmol) in ethanol (95 mls) was added hydroxylamine hydrochloride (6 g, 90 mmol) and sodium acetate (6.56 g, 80 mmol). The mixture was heated under reflux for 2 hours and the solvent evaporated, <u>in vacuo</u>, to give the oxime (156) (9 g, 90%) as white crystals; ^{1}H NMR ($CDCl_3$) δ 4.29 (s, 1H, C<u>H</u>Ph), 7.52 - 8.05 (m, 4H, CH<u>Ph</u>).

N-(2-Nitrobenzyl)hydroxylamine (157)

To a stirred solution of the oxime (156) (10 g, 60 mmol) and sodium cyanoborohydride (3.8 g, 60 mmol) in methanol (90 ml), a trace of methyl orange was added. A solution of 2M methanolic-HCl was added to maintain the red colour. The mixture was left overnight, the solvent evaporated, in vacuo, the residue taken up in water (40 ml) and ethyl acetate (40 ml) and the organic layer separated. The aqueous layer was raised to pH 7.5 - 8 with sodium hydroxide pellets, extracted with ethyl acetate (4 x 35 ml), the extracts combined, dried (Na_2SO_4), filtered and solvent evaporated, in vacuo. Chromatography on silica gel (50 g) using diethyl ether/hexane 2:1 eluant gave the hydroxylamine (157) (5.7 g, 57%) as white crystals: ^1H NMR ($CDCl_3$) δ 4.33 (s, 2H, CH_2), 5 (broad s, 1H, NH), 7.41 - 8.04 (m, 4H, Ph).

t-Butyldimethylsilyl-N-(2-nitrobenzyl)-hydroxylamine (158)

t-Butyldimethylsilyl chloride (0.97 g, 6.4 mmol) was added to a stirred solution of the hydroxylamine (157) (1.08 g, 6.4 mmol) and triethylamine (1.2 ml, 8.5 mmol) in DMF (6 mls) under nitrogen at room temperature and left overnight. The mixture was diluted with water (10 ml) and ethyl acetate (20 ml). The aqueous layer was acidified with concentrated HCl and the ethyl acetate layer was separated. The aqueous phase was extracted with more ethyl acetate (3 x 40 ml). The extracts were combined, dried (Na_2SO_4), filtered and the solvent evaporated, in vacuo. The residue was chromatographed, using hexane/diethyl ether 1:1 eluant, on silica gel (20 g) to afford the silylated hydroxylamine (158) (1.63 g, 90%) as a yellow oil: ^1H NMR ($CDCl_3$) δ 0.03 (s, 6H, $OSi(CH_3)_2$), 0.87 (s, 9H, $OSi(CH_3)_2(CH_3)_3$, 4.26 (broad s, 1H, NH), 5.16 (m, 2H, CH_2), 7.51 (m, 4H, Ph).

N-Benzyloxycarbonyl-1-benzyl-(Z)-2,3-dehydroglutamic -5-N-(2-nitrobenzyl) hydroxamic acid (160)

A stirred solution of the dehydroglutamate (144) (590 mg, 1.6 mmol), N-hydroxysuccinimide (310 mg, 2.77 mmol) and dimethylaminopyridine (20 ml, 0.16 mmol) in dichloromethane (3ml) under nitrogen at room temperature was left for 15 minutes and DCC (550 mg, 2.7 mmol) was added. After a further 15 minutes a solution of the silylated hydroxylamine (158) (756 mg, 2.7 mmol) in dichloromethane (3 ml) was added. The mixture left for two days. The mixture was filtered, the solvent evaporated, in vacuo, the residue taken up in a minimum volume of dichloromethane and the solution cooled at -10°C for a few hours to precipitate residual dicylohexyl urea and filtered. The solvent was evaporated, in vacuo, and the residue diluted with water (15 ml) and ethyl acetate (20 ml): the aqueous layer was acidified with 1M HCl and the organic layer was separated. The aqeuous phase was extracted with more ethyl acetate (3 x 20 ml), the extracts were combined, dried (Na_2SO_4), filtered and solvent evaporated, in vacuo, to afford the silylated hydroxamic acid (159) (820 mg, 82%): ^1H NMR ($CDCl_3$) δ 0.032 (s, 6H, $OSi(C\underline{H}_3)_2$), 0.87 (s, 9H, $OSi(CH_3)_2(C\underline{H}_3)_3$); 3.56 - 3.66 (m, 2H, $COC\underline{H}_2$), 5.04 - 5.30 (m, 7H, 3x$C\underline{H}_2$Ph and N$\underline{H}$), 6.29 - 6.87 (m, 1H, $CH_2C\underline{H}$), 7.26 - 7.32 (m, 14H, 3$\underline{Ph}$).

To a stirred solution of the silylated hydroxylamine (159) (820 mg, 1.3 mmol) in ethyl acetate-methanol(5 ml) was added tosic acid (250 mg, 1.3 mmol) at room temperature. After 30 minutes, solvent was evaporated, in vacuo, and the residue chromatographed on silica gel (5g) using diethyl ether eluant to give the hydroxamic acid (160) (497 mg, 74%), as a tan foam; mp 95 - 100°C; IR (nujol) 1785, 1730-1690, 1280, 715 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.56 - 3.66 (m, 2H, $COC\underline{H}_2$), 5.04 - 5.30 (m, 7H, 3x$C\underline{H}_2$Ph and N$\underline{H}$), 6.29 - 6.87 (m, 1H, $CH_2C\underline{H}$), 7.26 - 7.35 (m, 14H, 3x$\underline{Ph}$).

N-Benzyloxycarbonyl-1-benzyl-(Z)-2,3-dehydroglutamic -5-N-(2-nitrobenzyl) hydroxamic acid (160)

A stirred solution of the dehydroglutamate (144) (590 mg, 1.6 mmol), N-hydroxysuccinimide (310 mg, 2.77 mmol) and dimethylaminopyridine (20 ml, 0.16 mmol) in dichloromethane (3ml) under nitrogen at room temperature was left for 15 minutes and DCC (550 mg, 2.7 mmol) was added. After a further 15 minutes a solution of the silylated hydroxylamine (158) (756 mg, 2.7 mmol) in dichloromethane (3 ml) was added. The mixture left for two days. The mixture was filtered, the solvent evaporated, in vacuo, the residue taken up in a minimum volume of dichloromethane and the solution cooled at -10°C for a few hours to precipitate residual dicylohexyl urea and filtered. The solvent was evaporated, in vacuo, and the residue diluted with water (15 ml) and ethyl acetate (20 ml): the aqueous layer was acidified with 1M HCl and the organic layer was separated. The aqeuous phase was extracted with more ethyl acetate (3 x 20 ml), the extracts were combined, dried (Na_2SO_4), filtered and solvent evaporated, in vacuo, to afford the silylated hydroxamic acid (159) (820 mg, 82%): ^1H NMR ($CDCl_3$) δ 0.032 (s, 6H, $OSi(C\underline{H}_3)_2$), 0.87 (s, 9H, $OSi(CH_3)_2(C\underline{H}_3)_3$); 3.56 - 3.66 (m, 2H, $COC\underline{H}_2$), 5.04 - 5.30 (m, 7H, 3x$C\underline{H}_2$Ph and N$\underline{H}$), 6.29 - 6.87 (m, 1H, $CH_2C\underline{H}$), 7.26 - 7.32 (m, 14H, 3$\underline{Ph}$).

To a stirred solution of the silylated hydroxylamine (159) (820 mg, 1.3 mmol) in ethyl acetate-methanol(5 ml) was added tosic acid (250 mg, 1.3 mmol) at room temperature. After 30 minutes, solvent was evaporated, in vacuo, and the residue chromatographed on silica gel (5g) using diethyl ether eluant to give the hydroxamic acid (160) (497 mg, 74%), as a tan foam; mp 95 - 100°C; IR (nujol) 1785, 1730-1690, 1280, 715 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.56 - 3.66 (m, 2H, $COC\underline{H}_2$), 5.04 - 5.30 (m, 7H, 3x$C\underline{H}_2$Ph and N$\underline{H}$), 6.29 - 6.87 (m, 1H, $CH_2C\underline{H}$), 7.26 - 7.35 (m, 14H, 3x$\underline{Ph}$).

N-(2,4-dimethoxy)benzaldoxime (166)

To a solution of 2,4-dimethoxybenzaldehyde (165) (10g, 60 mmol), in ethanol (100 ml) was added hydroxylamine hydrochloride (6.26 g, 90 mmol) and sodium acetate (7.38 g, 90 mmol). The mixture was heated under reflux for 2 hours and the solvent evaporated, in vacuo, to give the oxime (166) (9.3 g, 85%) as white crystals: mp 106 - 7°C.

N-2,4-Dimethoxybenzylhydroxylamine (167)

To a stirred solution of the oxime (166) (9 g, 50 mmol) and sodium cyanoborohydride (3.14 g, 50 mmol) in methanol (90 ml), a trace of methyl orange was added. 2M methanolic HCl was added to maintain the red colour. The mixture was stirred overnight, solvent evaporated, in vacuo, the residue diluted with water (40 ml) and ethyl acetate (40 ml) and the ethyl acetate layer separated. The aqueous layer was then basified to pH > 9 with sodium hydroxide pellets and extracted with ethyl acetate (4 x 35 ml). The ethyl acetate extracts were combined, dried (Na_2SO_4), filtered and the solvent evaporated, in vacuo. Chromatography on silica gel (50 g) using diethyl ether/ hexane 2:1 eluant yielded the hydroxylamine (167) (6.5 g, 72%) as white crystals: ^1H NMR ($CDCl_3$) δ 3.78 (s, 6H, 2xOC$\underline{H}_3$), 3.96 (s, 2H, C$\underline{H}_2$Ph), 6.00 (broad s, 2H, N$\underline{H}$O$\underline{H}$), 6.35 - 7.26 (m, 3H, $\underline{Ph}$).

N-t-Butyldimethylsilyl-N(-2,4-dimethoxybenzyl)hydroxylamine (168)

To a stirred solution of the hydroxylamine (167) (1.3 g, 7 mmol) in DMF (7 ml), t-butyldimethylsilylchloride (1.05 g, 7 mmol) and imidazole (0.48 g, 7 mmol) were added, at room temperature, under nitrogen. The mixture was stirred overnight, taken up in water (12 ml) and ethyl acetate (25 ml), the ethyl acetate layer was separated. The aqueous phase was extracted with more ethyl acetate (3 x 50 ml). The extracts

were combined, dried (Na_2SO_4), filtered and the solvent evaporated, in vacuo. The residue was chromatographed on silica gel (50 g) using hexane/diethyl ether 5:2 eluant to afford the silylated hydroxylamine (168) (1.9 g, 90%) as a yellow oil: ^1H NMR ($CDCl_3$) δ 0.09 (s, 6H, $OSi(C\underline{H}_3)_2$), 0.92 (s, 9H, $OSi(CH_3)_2(C\underline{H}_3)_3$), 3.79 (s, 6H, $2xOC\underline{H}_3$), 3.96 (s, 2H, $C\underline{H}_2Ph$), 5.34 (broad s, 1H, N<u>H</u>), 6.36 - 7.26 (m, 3H, <u>Ph</u>).

<u>Attempted synthesis of N-benzyloxycarbonyl-1-benzyl-(Z)-2,3-dehydroglutamic-5-N-(2,4-dimethoxybenzyl)hydroxamic acid (169)</u>

A stirred solution of the dehydroglutamate (144) (600 mg, 1.63 mmol), N-hydroxysuccinimide (187 mg, o.63 mmol) and dimethylaminopyridine (20 mg, 0.16 mmol) in dichloromethane (3 ml) under nitrogen, at room temperature, was left for 15 minutes and DCC (336 mg, 1.63 mmol) was added. After 15 minutes a solution of the silylated hydroxylamine (168) (484 mg, 1.63 mmol) in dichloromethane (3ml) was added; the resulting solution was stirred for 2 days, filtered to remove dicyclohexyl urea and the solvent evaproated, <u>in vacuo</u>. The residue was taken up in a minimum volume of dichloromethane, left at -10°C for a few hours to precipitate any residual urea and the solvent evaporated, <u>in vacuo</u>. The residue was diluted with water (20 ml) and ethyl acetate (25 ml), the aqueous layer was acidified with 1M HCl and the ethyl acetate layer was separated. The aqueous phase was extracted with more ethyl acetate (3 x 25 ml), the extracts were combined, dried (Na_2SO_4), filtered and the solvent evaporated, <u>in vacuo</u>, the residue was chromatographed on silica gel (10 g) using hexane/diethyl ether 2:1 as eluant to afford the silylated hydroxamic acid (169') (370 mg, 35%) as an oil: ^1H NMR ($CDCl_3$) δ 0.035 (s, $OSi(C\underline{H}_3)_2$), 0.87 (s, $OSi(CH_3)_2(C\underline{H}_3)_3$, 3.44 - 3.66 (m, $COC\underline{H}_2$), 3.75 - 3.82 (m, $OC\underline{H}_3$), 5.03 - 5.24 (m, $C\underline{H}_2Ph$), 6.27 - 6.86 (m, $CH_2C\underline{H}$ and N<u>H</u>), 7.30 - 7.32 (m, <u>Ph</u>).

4-Methoxybenzaldoxime (171)

Hydroxylamine hydrochloride (7 g, 100 mmol) was added to a stirred solution of 4-methoxybenzaldehyde (170) (9 ml, 74 mmol) in ethanol (20 ml) at room temperature. After 10 minutes the solution was cooled to 0°C, left for 2 hours and filtered. The white crystals were suspended in saturated aqueous sodium bicarbonate and extracted with ethyl acetate (3 x 20 ml). The ethyl acetate extracts were combined, dried (Na_2SO_4), filtered and the solvent evaporated, *in vacuo*. Recrystallisation from benzene-hexane gave the oxime (171) (8 g, 72%) as white crystals; mp 64°C (lit mp 63°C[24]).

N-(4-Methoxybenzyl)hydroxylamine (172)

To a stirred solution of the oxime (171) (8 g, 53 mmol) and sodium cyanoborohydride (3.33 g, 53 mmol) in methanol (55 ml), a trace of methyl orange was added. 2M methanolic HCl was added to maintain the red colour, the mixture stirred overnight and solvent evaporated, *in vacuo*. The residue was washed with diethyl ether, diluted with water (50 ml), raised to pH 8 with sodium hydroxide pellets and extracted with ethyl acetate (4 x 60 ml). The extracts were combined, dried (Na_2SO_4), filtered and solvent evaporated, *in vacuo*. Recrystallisation from ethyl acetate-hexane gave the hydroxylamine (172) (6.62 g, 82%) as white crystals; mp 76°C (lit mp 75.7°C[24]).

t-Butyldimethylsilyl-N-(4-methoxybenzyl)hydroxylamine (173)

t-Butyldimethylsilyl chloride (1.17 g, 7.78 mmol) and imidazole (2.12 g, 31 mmol) were added to a stirred solution of hydroxylamine (172) (1.19 g, 7.78 mmol) in dimethylformamide (9 mls) at room temperature, under nitrogen and the solution was left overnight. The solvent was evaporated, *in vacuo*, to give a yellow oil which was

distilled, in vacuo, to afford the silylated hydroxylamine (173) (1.18 g, 54%) as a colourless oil: bp 104°C/15 mmHg; ^{1}H NMR ($CDCl_3$) δ 0.05 (s, 6H, $OSi(C\underline{H}_3)_2$, 0.92 (s, 9H, $OSi(CH_3)_2(C\underline{H}_3)_3$), 3.78 (s, 3H, $OC\underline{H}_3$), 3.93 (s, 2H, $C\underline{H}_2Ph$), 4.75 (broad s, 1H, $N\underline{H}CH_2Ph$), 6.79 - 7.29 (m, 4H, $CH_2\underline{Ph}$).

Attempted synthesis of N-benzyloxycarbonyl-l-benzyl-(Z)-2,3-dehydroglutamic-5-N-(4-methoxybenzyl)hydroxamic acid (174)

A stirred solution of the dehydroglutamate (144) (427 mg, 1.16 mmol), N-hydroxysuccinimide (133 mg, 1.16 mmol) and dimethylamino-pyridine (15 mg, 0.012 mmol) in dichloromethane (3 ml) under nitrogen, at room temperature, was left for 15 minutes and DCC (240 mg, 1.16 mmol) was added. After 15 minutes a solution of the silylated hydroxylamine (173) (310 mg, 1.16 mmol) in dichloromethane (3 ml) was added and the mixture was left stirring for 2 days. The resulting mixture was filtered, the solvent evaporated, in vacuo, the residue taken up in a minimum volume of dichloromethane, left at -10°C for a few hours to precipitate any residual dicyclohexyl urea, filtered and the solvent evaporated, in vacuo. The residue was diluted with water (15 ml) and ethyl acetate (20 ml), the aqueous layer was acidified with 1M HCl and the ethyl acetate layer was separated. The aqueous phase was extracted with more ethyl acetate (3 x 25 ml). The combined extracts were dried (Na_2SO_4), filtered and solvent evaporated, in vacuo, to yield the silylated hydroxamic acid (180) (500 mg, 70%) as an oil: ^{1}H NMR ($CDCl_3$) δ 0.035 (s, 6H, $OSi(C\underline{H}_3)_2$), 0.86 (s, 9H, $OSi(CH_3)_2(C\underline{H}_3)_3$), 3.42 (d, J = 6.84 Hz, 2H, $COC\underline{H}_2$), 3.76 (s, 3H, $OC\underline{H}_3$), 4.7 (broad s, 1H, $N\underline{H}$), 5.08 - 5.29 (m, 6H, $3C\underline{H}_2Ph$), 6.68 - 6.84 (m, 1H, $CH_2C\underline{H}$), 7.34 (s, 14H, $3\underline{Ph}$).

To a stirred solution of the silylated hydroxylamine (180) (500 mg, 0.97 mmol) in ethylacetate/methanol 1:1 (8 ml), tosic acid (184 mg,

0.97 mmol) was added at room temperature and left for 30 minutes. The solvent was evaporated, in vacuo, and the residue chromatographed on silica gel (8 g), using ethyl acetate eluant, afforded N-(4-methoxybenzyl)hydroxamic acid (174) and N-(benzyl)hydroxamic acid (175) (90 mg): ^{1}H NMR ($CDCl_3$) δ 3.55 - 3.66 (m, $COC\underline{H}_2$), 3.76 (s, $OC\underline{H}_3$), 5.03 - 5.19 (m, $C\underline{H}_2Ph$), 5.50 (broad s, C-O<u>H</u>) 6.28 - 6.85 (m, $CH_2C\underline{H}$), 7.32 - 8.04 (m, <u>Ph</u>).

N-Benzyloxycarbonyl-2-benzyl-(Z)-2,3-dehydroglutamic -4-N-(benzyl)hydroxamic acid (175)

To a stirred solution, under nitrogen, of the dehydroglutamate (144) (5.54 g, 15 mmol) and N-hydroxysuccinimide (1.73 g, 15 mmol) in dry dichloromethane (60 ml) was added DCC (3.09 g, 15 mmol) and the mixture stirred at room temperature for 25 minutes. The silylated hydroxylamine (179)[27] (3.91 g, 16.5 mmol) in dichloromethane (5 ml) was then added and the resulting mixture stirred for a further 48 hours at room temperature. After cooling to 0°C, ethyl acetate (75 ml) was added, the mixture filtered and the filtrate evaporated, in vacuo, to give a red oil which was dissolved in a minimum of dichloromethane and kept at -10°C overnight. Filtration of the precipitated dicyclohexyl urea, evaporation of the solvent followed by chromatography of the residual oil, on silica gel (60 g) using ethyl acetate/hexane 3:2 eluant afforded the hydroxamic acid (175) (3.6 g, 50%): R_f 0.52 (ethyl acetate/hexane 3:2); ^{1}H NMR ($CDCl_3$) δ 3.26 - 3.35 (d, J = 7.3 Hz, 2H, $COC\underline{H}_2$), 5.09 - 5.2 (m, 6H, $NC\underline{H}_2Ph$, $CO_2C\underline{H}_2Ph$, $NHCO_2C\underline{H}_2Ph$), 6.75 - 6.9 (m, 1H, $COCH_2C\underline{H}$), 7.35 (m, 15H, $NCH_2\underline{Ph}$, $CO_2CH_2\underline{Ph}$, $NHCO_2CH_2\underline{Ph}$).

Erythro,threo-DL-N-benzyloxycarbonyl-2-amino-2-benzyl-3-oxo-5-isoxazolidine acetic acid benzyl ester (181)

To a solution of the hydroxamic acid (175) (237 mg, 0.5 mmol) in THF/water (10:1) (5 ml) was added saturated aqueous sodium bicarbonate

(3 ml). The resulting suspension was stirred and heated under reflux for 75 minutes. On cooling, ethyl acetate (25 ml) was added and the mixture washed with 1M hydrochloric acid (5 ml) and water (2 x 10 ml). Drying (Na_2SO_4), filtration and evaporation, <u>in vacuo</u>, of the solvent gave the isoxazolidine (181), a yellow oil, (200 mg, 84%), as a mixture of diastereoisomers: R_f 0.56 and 0.59 (ethyl acetate/ hexane 7:5); ^1H NMR ($CDCl_3$) δ 2.9 (m, 2H, $COCH_2$), 4.40 - 4.88 (m, 2H, CH_2CH and $CH(NHCO_2CH_2Ph)$), 5.0 - 5.38 (m, 6H, NCH_2Ph, CO_2CH_2Ph and $NHCO_2CH_2Ph$).

CHAPTER SIX

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Statement of Advanced Studies Undertaken

Attendance of meetings of the Amino Acid Discussion Group and lectures at the Royal Society of Chemistry and colleges of the University of London. Also, participation in research seminars at the City of London Polytechnic.

Publications

The Effect of 2-amino-4-phosphonobutyrate (APB) on acetylcholine release from the rabbit retina: evidence for ON-channel input to cholinergic amacrine cells.

M.J. Neal, J.R. Cunningham, T.A. James, M. Joseph and J.F. Collins.

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