The Design and Synthesis of Antiepileptic Agents Based on Neurotransmitter and Neural Metal Mediated Inhibition

by

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ABSTRACT

Conventional anticonvulsant drugs suffer from serious drawbacks. They are effective in only 60-65% of patients, their use is associated with significant side-effects in 40-45% of patients, and they treat the symptoms of epilepsy (*ie.* seizures, ictogenesis) but not the underlying disease process (*ie.* epileptogenesis). The need for a useful antiepileptic drug, defined as efficacious, non-toxic, anti-ictogenic and antiepileptogenic, is thus a neuropharmacologic priority. To meet that need, an unprecedented rational drug design strategy, based solidly on a mechanistic understanding of neurotransmitter and neural metal mediated inhibition, has been used to design antiepileptic agents with hybrid anti-ictogenic/antiepileptogenic activity.

A survey of the pathogenesis of epilepsy has revealed that: i) the neurochemical aetiology of epileptogenesis (epilepsy focus generation) arises from an altered ratio of excitatory/inhibitory neurotransmitter stimulation, and ii) ictogenesis (seizure generation) and epileptogenesis are distinct phenomena which act as reciprocal determinants. Thus, the discovery of an antiepileptic drug was postulated to involve the design and synthesis of molecules with combined anticonvulsant and antiepileptogenic properties that exploit agonists and antagonists of inhibitory and excitatory neurotransmitters, respectively.

After extensive literature evaluation, β-alanine was identified as a prototype anti-ictogenic/antiepileptogenic neurotransmitter analogue which derives its biological activity from simultaneously (i) inhibiting excitatory processes via antagonism of the NMDA receptor glycine co-agonist site and (ii) stimulating inhibitory processes via blockade of glial GABA uptake. This presumably accounts for β-alanine's anti-

ictogenic/antiepileptogenic activity, confirmed using the Spontaneous Recurrent Seizure (SRS) model of epileptogenesis. Consequently, a versatile, novel synthesis was developed to manufacture β-alanine analogues: alkyl β-aminothiophenecarboxylates were N-acylated, reductively desulfurized and doubly deprotected to yield α- or β-substituted β-amino acids in reasonable yield. Four of eight β-amino acids demonstrated anti-ictogenic activity in rat seizure models. Furthermore, α-(4-phenylcyclohexyl)-β-alanine • HCl, anti-ictogenic at ip doses of 50 mg/kg, demonstrated significant antiepileptogenic activity in the SRS model, reducing seizure frequency by 80%. The design of congeners with optimal antiepileptic activity was also proposed.

The second approach to the design and synthesis of antiepileptic drugs was based on neural metal mediated inhibition. Zn(II) metalloenzymes mediate neurotransmitter synthesis and degradation. Abstraction of brain Zn(II) initiates a cascade of biochemical events resulting in a decrease of L-glutamate levels and an increase in GABA levels. This may have an antiepileptogenic effect. On the other hand, anti-ictogenic activity is effected by Cu(II) supplementation. Since combined anti-ictogenic/antiepileptogenic activity is required in a useful antiepileptic drug, Cu(II) complexes of cyclic dipeptides were designed and synthesized to translocate Cu(II) across the blood-brain-barrier, release it, then chelate and remove Zn(II). Two compounds, chloro(*cyclo*-L-methionyl-L-phenyl-alaninato)copper(II) hydrate and chlorobis(*cyclo*-L-leucyl-L-methioninato)copper(II) dihydrate, demonstrated *in vitro* ability to translocate Cu(II) and Zn(II) across a membrane. Both compounds displayed anticonvulsant activity in the MES seizure model at ip doses of 100 mg/kg. A determination of antiepileptogenic activity is pending.

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Fruitful scientific endeavour is never a solitary affair. Without the assistance and support of my colleagues, friends and family, this thesis would never have been completed. For all the help I have received, I am truly grateful.

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Finally, I thank Jesus Christ, my Lord, who has provided wisdom, revelation, blessing and ultimate meaning for my research.

STATEMENT OF ORIGINAL CONTRIBUTION

This document contains a number of original contributions to the field of medicinal chemistry and antiepileptic drug design.

Chapter 2 reports:

- a presentation of the neurochemical basis of ictogenesis and epileptogenesis.
- the first proposal of β-amino acids as combined anti-ictogenic/antiepileptogenic agents which antagonize GABA uptake and NMDA glycine site mediated excitation *simultaneously*.
- a tabular comparison of the 26 most useful β-amino acid syntheses in terms of efficiency and reaction scope (i.e. number of synthetic steps, total yield, substrates, backbone substitution modes, advantages, disadvantages and chiral selectivity).
- the design of a novel four step synthesis of β-amino acids involving the Raney nickel desulfurization of alkyl acetamidothiophenecarboxylates. The new synthesis is unparalleled in reaction scope and utility as an analogue synthesis of β-amino acids.
- the synthesis of 17 diprotected β-amino acids and 8 unprotected β-amino acids (6 novel) using the new protocol; 50% of the unprotected β-amino acids displayed anticonvulsant activity in animal models.

- the discovery of α-(4-phenylcyclohexyl)-β-alanine hydrochloride salt as a β-amino acid with significant anticonvulsant activity (active in >2/4 rats at a dose of 50 mg/kg).
- the first determination of hybrid anti-ictogenic/antiepileptogenic activity using the Spontaneous Recurrent Seizure model of epileptogenesis: β-alanine and α-(4-phenyl-cyclohexyl)-β-alanine hydrochloride salt demonstrated significant hybrid bioactivity.
- the proposal of a β-amino acid analogue series, α-[1-arylmethyl-ω-(diaryl-methoxy)alkyl]-β-alanines, where affinity to the GABA uptake receptor and the NMDA glycine co-agonist site is optimized or maximized. As a consequence, anti-ictogenic/antiepileptogenic potency should be enhanced.
- the first proposal of β-amino acids as putative therapeutic agents for stroke, AIDS dementia, psychosis, Huntington's chorea, Alzheimer's disease, head trauma, Parkinson's disease, anxiety, pain, schizophrenia, wound healing and some forms of cancer.

Chapter 3 reports:

- the synthesis and anticonvulsant activity of 9 Zn(II) complexes and 9 Cu(II) complexes to assist the design of a Cu(II)/Zn(II) chelator.
- the design and synthesis of two methionine containing cyclic dipeptides as Cu(II) releasing agents in the CNS; chloro(*cyclo*-L-methionyl-L-phenylalaninato)Cu(II)

hydrate and chlorobis(*cyclo*-L-leucyl-L-methioninato)Cu(II) dihydrate were synthesized.

- the synthesis and X-ray crystal structure determination of bis(5,5-diphenyl-hydantoinato)diamminezinc(II) hemihydrate; this has been published in *Acta Cryst.* C**51**, 1297-1300 (1995).
- the first semi-empirical AM1 molecular orbital study of Zn(II) - phenytoin complexation.

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The following abbreviations are used throughout this document.

ACDs:	Anticonvulsant drugs
AEDs:	Antiepileptic drugs
ATPase:	Adenosine triphosphatase
BBB:	Blood-brain barrier
BOC:	*tert*-Butoxycarbonyl
BOC-ON:	2-(*tert*-Butoxycarbonyl-oxyimino)-2-phenylacetonitrile
$CDCl_3$:	Chloroform-d
cMMCu:	dichloro(*cyclo*-L-methionyl-L-methionato)copper(II)
CNS:	Central nervous system
DCHA:	Dicyclohexylamine
DMF:	Dimethylformamide
DMSO:	Dimethyl sulfoxide
ED_{50}:	Effective therapeutic dose for 50% of test subjects
EDTA:	Ethylenediaminetetraacetic acid
EEDQ:	2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
GABA:	γ-Aminobutyric acid
GAD:	Glutamate decarboxylase
HSAB:	Hard and soft acid-base
^{1}H nmr:	Proton nuclear magnetic resonance
IC_{50}:	Inhibitory concentration for 50% of test subjects
IR:	Infrared
K:	Stability constant
Leu:	Leucine
LFSE:	Ligand field stabilization energy
log P:	Logarithm of the octanol-water partition coefficient
LD_{50}:	Lethal dose at which 50% of test subjects die
MEK:	Methyl ethyl ketone
MES:	Maximal electroshock assay
Met:	Methionine
MS:	Mass spectrometry
NIH:	National Institutes of Health
NMDA:	N-Methyl-D-aspartate
PHT:	Phenytoin
PIS:	Pilocarpine induced seizures
PLP:	Pyridoxal-5'-phosphate
ps:	Picoseconds
PTZ:	Pentylenetetrazole
QSAR:	Quantitative structure-activity relationship
TEA:	Triethylamine
TLC:	Thin layer chromatography
TMS:	Tetramethylsilane

CHAPTER 1

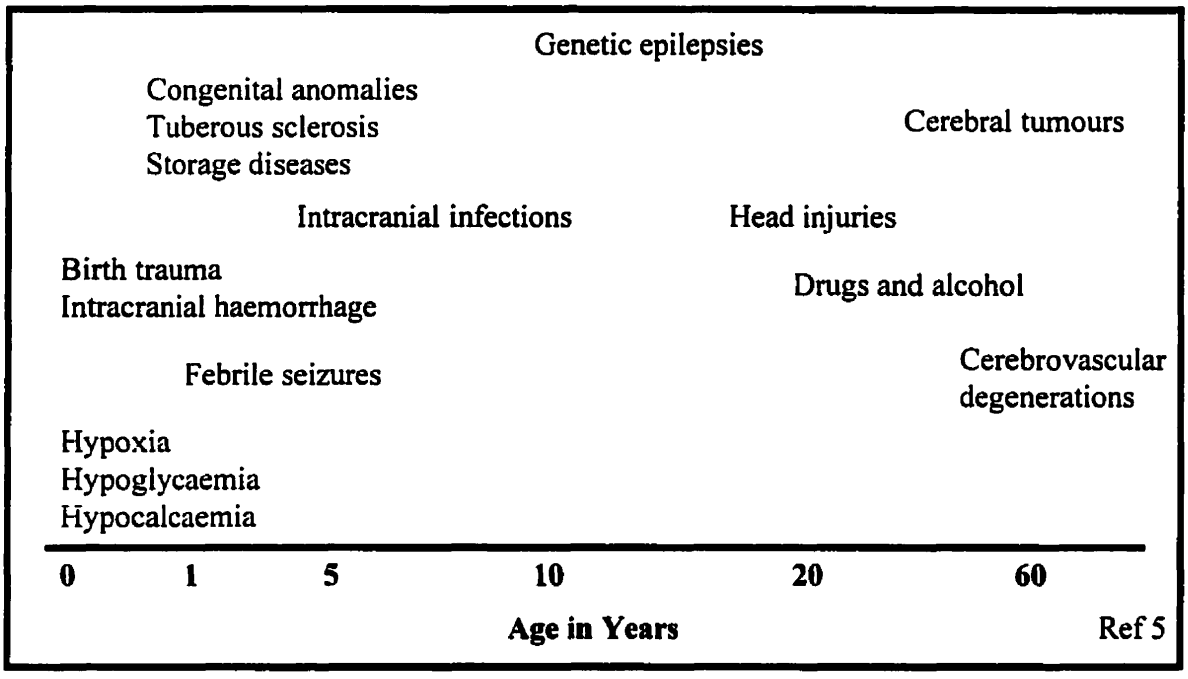
INTRODUCTION

1.1 EPILEPSY DEFINED

Epilepsy is a progressive, chronic brain disorder characterized symptomatically by recurrent seizures which arise from excessive electrical discharge in the epileptic brain [1]. The disorder has numerous causes and often strikes early in life (Figure 1.1) thereby impairing the epileptic's ability to become an integrated, productive member of society. The global rate of incidence is 1.3% [2] and increasing. Sadly, the epileptic condition has no medical "cure" and conventional treatments are less than satisfactory. Current anticonvulsant drugs effectively suppress seizures in only 65% of patients [3] and 40-45% of these patients experience significant toxic side-effects [4]. Thus, the design and synthesis of novel, efficacious antiepileptic drugs emerges as a neuropharmacological priority. In particular, there is a need to rationally develop new drugs which are not only seizure-suppressant but are antiepileptogenic — drugs that will actually treat the underlying epilepsy.

The primary symptom of epilepsy is seizures. Thus, any drug rationally designed to suppress seizures necessarily requires an understanding of seizure pathogenesis. Seizures originate when two or more neurons forming an epileptic focus hyperexcite and randomly fire. As adjacent neurons are affected, aberrent electrical activity spreads into the surrounding cortex. The initiation and propagation of the seizure (Latin=*ictus*) in time and space is known as ictogenesis; it is a rapid, electrical, chemical event which occurs over seconds or minutes. Ictogenesis involves an excitatory upregulation of ordinary neuronal communication.

Figure 1.1: Causes of seizures and epilepsy by age



In normal neurons, communication proceeds via electrical transmission known as the action potential (Figure 1.2). This is an electrical/chemical process dependent on voltage- and receptor-regulated macromolecular ion channel proteins. A neuron at rest possesses a transmembrane charge asymmetry of 65 millivolts with the intracellular compartment being negative with respect to the extracellular compartment. Electrical stimulation opens voltage-regulated ion channels and permits Na^+ ions to enter the neuron. This produces a local depolarization in the transmembrane potential and induces conformational change in the adjacent Na^+ channels. Consequently a wave of depolarization travels along the neuron until it reaches the terminal bouton where the voltage-gated Ca^{2+} channels open; this mediates the release of neurotransmitters which diffuse across the synapse and dock with receptors on the adjacent neuron. Excitatory

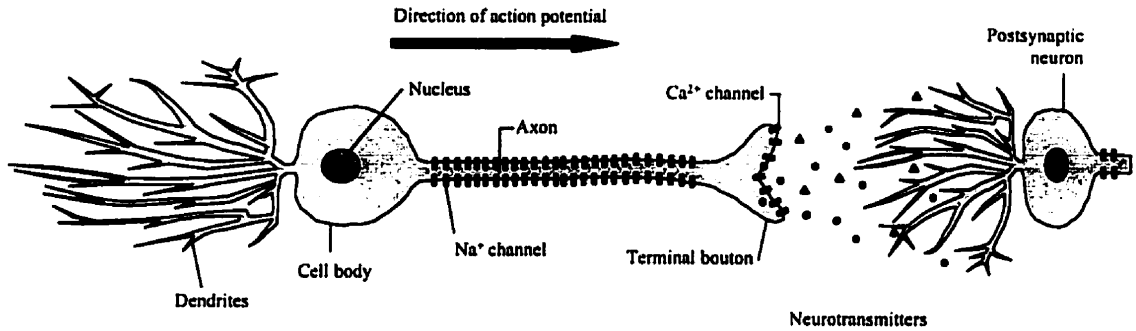


Figure 1.2: Representation of synapsis

neurotransmitters, such as glutamate or aspartate, dock with and open postsynaptic receptor-regulated ion channels. This permits cations to enter the postsynaptic neuron and reestablishes the process of electrical excitation. In epileptogenic neurons, this mechanism is recruited into producing the paroxysmal electrical activity resulting in seizures.

Seizures promulgate from epileptogenic foci — collections of neurons predisposed to hyperexcitability. Epileptogenic foci develop via a gradual process (months to years) whereby normal brain is transformed into a state susceptible to spontaneous, episodic, time-limited recurrent seizures. A causal factor (*ie.* brain insult) initiates focal, ictal predisposition by lowering the normal seizure threshold. Evidence suggests that an imbalance in the concentration of inhibitory (γ-aminobutyric acid) and excitatory (glutamate) neurotransmitters is responsible [6,7]. Eventually a seizure occurs resulting from a matured epileptogenic focus. Often the seizures become more frequent and more severe over time. The locale of focal origin from which abnormal electrical

activity spreads produces behavioural manifestations at the clinical level dependent on the function of the source neuroanatomical region. Seizures may be classified accordingly.

When ictogenesis is restricted, partial seizures ensue and may be subdivided into three subgroups: simple, complex and secondarily generalized. Simple partial seizures may emerge from any part of the cortex, however, the frontal lobe (motor cortex) is a common point of origin. These seizures are associated with jerking movements and normal consciousness is maintained. Complex partial seizures, usually originating in the temporal lobe, are associated with an altered state of consciousness complete with various automatisms. Finally, either a simple or complex partial seizure may progress secondarily to a generalized tonic-clonic seizure [8].

When ictogenesis spreads simultaneously and equally throughout both hemispheres of the brain, primary generalized seizures occur. Classically termed *grand mal*, primary generalized tonic-clonic seizures occur with a sudden vocalization (the epileptic cry), loss of consciousness, tonic extensor rigidity of the trunk and extremeties, clonic movements and heavy stertorous breathing. Absence seizures, formerly *petit mal*, are characterized by brief, vacant staring with minimal motor accompaniment; they probably emanate from the diencephalon region of the brain. Myoclonic seizures are associated with sudden clonic jerking. The incidence of particular seizure types is shown in Table 1.1.

Knowledge of a particular seizure type is useful in selecting appropriate drug therapy since current drugs are seizure specific. Still, therapeutic success varies

Table 1.1: Incidence of seizure types

	Incidence (%)[a]
Partial Seizures	67.9
Simple partial	9.2
Complex partial	27.9
Secondarily generalized	30.8
Generalized Seizures	32.1
Generalized tonic-clonic	28.2
Absence	1.2
Myoclonic	1.3
Other	1.4

a. Distribution from a sample of 1005 patients with epilepsy [10].

depending on the seizure type. For example, complex partial seizures remain the most refractory class of seizures [9]. The need for novel drugs to treat ictogenesis (using anticonvulsant drugs) and the underlying epilepsy (using antiepileptogenic drugs) persists. This drug design process is consequent to a historical and mechanistic evaluation of existing anticonvulsants.

1.2 Discovery of Existing Anticonvulsants

Useful epileptic therapy is a relatively recent phenomenon. Prior to the 19th century, the treatment of epilepsy was predicated on a combination of ignorance, superstition, charlatanism and "magical prescriptions". All manner of bizarre "remedies" were employed including the use of enemas, purgatives, herbal extracts and animal

excrement. Epileptics suffered and occasionally died [11] in the face of such futile methods. In 1857, Sir Charles Locock pulled therapeutic epileptology out of the Dark Ages with the discovery of the first genuine anticonvulsant drug — potassium bromide. Antiquated remedies were widely replaced as bromide therapy became the method of choice. In spite of toxic side-effects such as dermatitis and psychosis, inorganic bromides became the mainstay of antiepileptic therapy for the next 55 years.

In 1912, the German physician Hauptmann prescribed the cyclic ureide, phenobarbital, to sedate an unusually active ward of epileptic patients. To his amazement, their seizure frequency decreased dramatically. Through serendipity and keen clinical observation, Hauptmann had discovered the first major organic anticonvulsant molecule (5-ethyl-5-phenylbarbituric acid). Quite rapidly, phenobarbital replaced bromide as the premier anticonvulsant drug due to its greater efficacy and reduced toxicity [12]. Nevertheless, phenobarbital's hypnotic properties prevented clinicians from using sufficient doses to ensure full seizure protection in all patients.

Twenty-five years passed until the next major discovery — phenytoin, another cyclic ureide. During the mid 1930s, Tracy Putnam subjected hundreds of heterocyclic phenyl compounds (phenobarbital analogues) to a sceening program using protection against electro-shock induced convulsions as the selection criterion. Only phenytoin combined high activity with low toxicity. In 1938, Putnam's colleague, Houston Merritt, conducted a successful clinical evaluation of phenytoin, also known as diphenylhydantoin, which led to its market introduction later that same year. The discovery of phenytoin by Merritt and Putnam was important for several reasons: it

established the fact that an anticonvulsant drug need not be a sedative (like KBr and phenobarbital); it demonstrated the value of systematic preclinical testing of putative anticonvulsants; it encouraged the search for drugs with selective anticonvulsant action; it opened a new era for the study of structure-activity relations; and it provided some basic knowledge about the mechanism of drug action [13].

The success of phenytoin initiated a period of intense activity in the search for new anticonvulsant compounds. Between 1946 and 1960, 13 new antiepileptic drugs were marketed in the United States. Most were succinimide, benzodiazepine and hydantoin-like drugs; the most effective were ethosuximide and primidone. The large number of new discoveries was accompanied by research that revealed that anticonvulsant effects were caused by a variety of mechanisms [14,15,16]. Compounds able to raise the seizure threshold in animals were effective against absence seizures in humans; drugs effective against partial and generalized tonic-clonic seizures acted to prevent seizure spread. Knowledge about the epileptic condition was increasing.

Unfortunately, the next fifteen years (1961-1975) saw no new drugs introduced specifically for the treatment of epilepsy. Two factors account for this silence: an absence of lead compounds and regulatory changes. In the United States, the 1962 Drug Amendments Act required not only accurate labelling and proof of safety but also proof of efficacy before a new pharmaceutical could be marketed. Consequently, this substantially delayed the introduction of new antiepileptic drugs since extended, costly

Table 1.2: History of anticonvulsant drug discovery in North America

Year	Anticonvulsant	Trade Name	Company
1857	Potassium bromide		
1912	Phenobarbital	Luminal	Winthrop
1935	Mephobarbital	Mebaral	Winthrop
1938	Phenytoin	Dilantin	Parke-Davis
1946	Trimethadione	Tridione	Abbott
1947	Mephenytoin	Mesantoin	Sandoz
1949	Paramethadione	Paradione	Abbott
1950	Phethenylate*	Thiantoin	Lilly
1951	Phenacemide	Phenurone	Abbott
1952	Metharbital	Gemonil	Abbott
1952	Benzchlorpropamide†	Hibicon	Lederle
1953	Phensuximide	Milontin	Parke-Davis
1954	Primidone	Mysoline	Ayerst
1957	Methsuximide	Celontin	Parke-Davis
1957	Ethotoin	Peganone	Abbott
1960	Aminoglutethimide‡	Elipten	Ciba
1960	Ethosuximide	Zarontin	Parke-Davis
1968	Diazepam§	Valium	Roche
1974	Carbamazepine	Tegretol	Geigy
1975	Clonazepam	Clonopin	Roche
1978	Valproic acid	Depakene	Abbott
1981	Clorazepate dipotassium§	Tranxene	Abbott
1992	Clobazam	Frisium	Hoescht
1993	Vigabatrin	Sabril	Merril Dow
1994	Gabapentin	Neurontin	Parke-Davis
1995	Lamotrigine	Lamictal	Glaxo-Wellcome

Withdrawn in * 1952, † 1955, ‡ 1966; § Approved by the FDA as an adjunct.

clinical trials became a legal necessity. Nevertheless, during this period, the anticonvulsant properties of carbamazepine and valproic acid were realized. Carbamazepine was synthesized in 1954 as part of a tricyclic iminodibenzyl analogue series in the search for compounds with anesthetic and antihistaminic properties. Its anticonvulsant properties were accidently discovered 9 years later after a series of routine neuro-screening tests. Valproic acid, on the other hand, was synthesized in 1882; however, its anticonvulsant properties were not noted until 1963 when it was used as a solvent in the anticonvulsant testing of an inactive compound [17]. Due to regulatory constraints, the North American market introduction of both compounds was delayed until after 1975.

Another anticonvulsant, clobazam, was discovered during research into 1,4-benzodiazepine analogues. Clobazam, a 1,5-congener, recently emerged from clinical development and was marketed in Canada in 1992 but was not evaluated in the U.S. In addition to sedative side-effects, tolerance develops to clobazam's anticonvulsant action [18].

To date, less than 30 drugs with significant anticonvulsant properties (Table 1.2) have enjoyed widespread human use; most have a cyclic ureide or similar heterocyclic structure (Figure 1.3). Currently, only six compounds find extensive clinical use (Table 1.3, Figure 1.4). Carbamazepine tends to be the drug of first choice for partial seizures while valproic acid is most efficacious for generalized seizures. Evidence suggests that most current anticonvulsants exert their effects at the level of neuronal ion channels or

through the modulation of γ-aminobutyric acid (GABA), an inhibitory neurotransmitter (Table 1.4).

X	Drug Class
-NH-	Hydantoins
-NHCO-	Barbiturates
-O-	Oxazolidinediones
$-CH_2-$	Succinimides

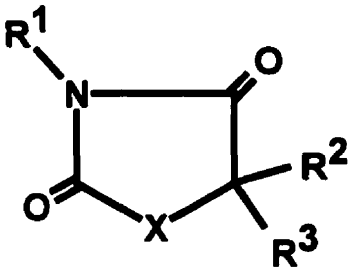
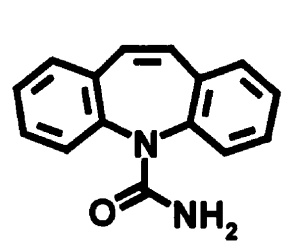


Figure 1.3: Heterocyclic anticonvulsants

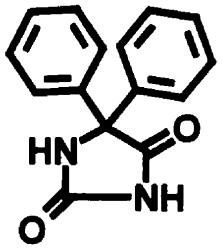
Table 1.3: Recommended traditional anticonvulsants for seizure types (Canada)

Decreasing likelihood of efficacy	Partial Seizures			Primary Generalized Seizures		
	Simple partial	Complex partial	Secondarily generalized	Generalized tonic-clonic	Absence	Myoclonic
1	CBZ	CBZ	CBZ	VPA	VPA	VPA
2	PHT	PHT	PHT	CBZ	ETX	VPA/CLB
3	VPA	VPA	VPA	PHT	VPA/CLB	
4	VPA/CBZ	VPA/CBZ	VPA/CBZ	VPA/PHT	VPA/ETX	
5	CBZ/CLB	CBZ/CLB	PRIM	VPA/CLB		
6	PRIM	PRIM				

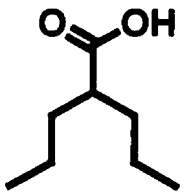
CBZ: carbamazepine PHT: phenytoin VPA: valproic acid
CLB: clobazam PRIM: primidone ETX: ethosuximide



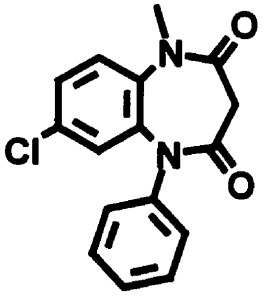
Carbamazepine



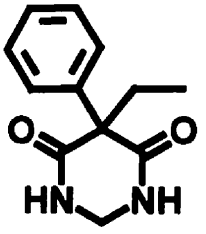
Phenytoin



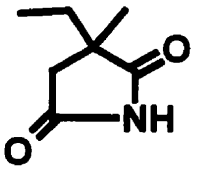
Valproic acid



Clobazam



Primidone



Ethosuximide

Figure 1.4: Structures of commonly used, current anticonvulsants

Sodium channel modulators, which include carbamazepine, phenytoin, valproic acid and lamotrigine, bind to the inactive state of the sodium channel and stabilize it thus preventing depolarization and the propagation of seizure activity, especially activity which leads to generalized tonic-clonic seizures. Sodium channel blockers are the most common anticonvulsants and more than 50% of the experimental drugs in clinical trials have been shown to prevent seizures via this mechanism (Table 1.5).

Calcium channel blockers such as ethosuximide, the principal anti-absence drug, operate analogously to Na^+ channel blockers. Possibly, other calcium channel blockers

Table 1.4: Current anticonvulsant therapies: indication and site of action

Anticonvulsant	Indication	Site of Action
Carbamazepine	gen. t/c, c.p.	Sodium channel
Valproic Acid	gen. t/c, c.p. absence	Sodium channel, GABA mediated inhibition, T-type calcium channel
Phenytoin	gen. t/c, c.p.	Sodium channel
Clobazam	all indications	GABA-benzodiazepine receptor (chloride channel)
Primidone	gen. t/c, c.p.	GABA-benzodiazepine receptor (chloride channel)
Clonazepam	absence, myoclonic	GABA-benzodiazepine receptor (chloride channel)
Phenobarbital	gen. t/c, c.p.	GABA-benzodiazepine receptor (chloride channel), sodium channel
Ethosuximide	absence	T-type calcium channel
Lamotrigine	gen. t/c, c.p., absence	Sodium channel
Vigabatrin	gen. t/c, c.p.	GABA transaminase inhibitor
Gabapentin	gen. t/c, c.p.	Large neutral amino acid transporter

Epilepsy types: gen.t/c: generalized tonic/clonic; and c.p.: complex partial seizures.

may be instrumental in controlling absence seizures [19,20].

Clobazam, primidone, clonazepam and phenobarbital act to enhance post-synaptic binding of GABA at the receptor-gated chloride channel. GABA is an inhibitory neurotransmitter which acts to suppress hyperexcitable neuronal activity [21].

Table 1.5: Anticonvulsants in clinical trial: indication and site of action

Anticonvulsant	Indication	Site of Action
Zonisamide	gen. t/c, c.p.	Sodium channel
Topiramate	gen. t/c, c.p.	Sodium channel, GABA receptor, carbonic anhydrase enzyme
Milacemide	gen. t/c, c.p.	Sodium channel
Oxcarbazepine	gen. t/c, c.p.	Sodium channel
Tiagabine	gen. t/c, c.p.	GABA uptake inhibitor
Remacemide	gen. t/c, c.p.	Sodium channel, NMDA antagonist

Epilepsy types: gen.t/c: generalized tonic/clonic; and c.p.: complex partial seizures.

The discovery of anticonvulsant mechanisms has provided drug targets in the search for new anticonvulsants. The 1980s has witnessed a new mechanistic approach to antiepileptic drug discovery based on the pharmacological enhancement of GABA-mediated inhibition and excitatory amino acid neurotransmission. Vigabatrin has been shown to bind irreversibly to GABA transaminase, an enzyme which catabolizes GABA. Tiagabine, currently in clinical trials, has been designed to prevent the uptake of GABA into glial cells. In both cases, synaptic GABA concentrations are elevated to produce anticonvulsant effects. Another promising drug candidate, remacemide, has been shown to be an NMDA excitatory amino acid antagonist leading to diminished electrical activity and restricted seizures.

The history of anticonvulsant drug discovery has proceeded through 3 phases (ignorance, serendipity, animal screens) and is currently in transition to a 4th

(mechanistic design). Despite advances, epilepsy researchers are faced with six major problems as they contend to find truly therapeutic agents:

1. Few drugs

Sadly, after more than a century, only a limited number of drugs have been discovered either by serendipity and/or mass animal screening [17]. Only six drugs comprise the bulk of useful epileptic therapeutics (Figure 1.4).

2. Lack of efficacy

Of all patients taking currently available anticonvulsant drugs, approximately 50% are well controlled, 30% may suffer 1-2 seizures a year and the remaining 20% are refractory [22]. The effectiveness of specific drugs varies.

3. Toxicity

None of the drugs currently available are free from untoward adverse effects (Table 1.6) and there is a gross lack of information correlating chemical structure to toxic effects. Toxicity tends to be the hardest aspect of drug development to predict.

Table 1.6: Toxic effects of commonly used anticonvulsant drugs

Anticonvulsant	Toxic Effects[1]
Carbamazepine	ataxia, diplopia, bone marrow suppression, hyponatremia, dermatitis
Valproic Acid	tremor, nausea, vomiting, anorexia, hepatitis, pancreatitis, weight gain, hair loss
Phenytoin	ataxia, diplopia, vertigo, aplastic anaemia, hirsutism, gingival hyperplasia, osteoporosis
Primidone	drowsiness, ataxia, diplopia, emotional lability, personality change, vertigo, nausea, vomiting, anorexia
Clobazam	sedation, depression, nausea, ataxia, diplopia, confusion, personality changes, generalized fatigue, weight gain
Ethosuximide	nausea, abdominal pain, vomiting, hiccups, headache, drowsiness, skin rash
Phenobarbital	drowsiness, ataxia, altered mood, generalized fatigue, hyperactivity, chemical dependence, hepatitis

1. References 23,24,25,26,27,28.

4. Lack of knowledge of biochemical mechanisms

Despite developments leading to the current mechanistic understanding of anticonvulsant drug action, much more work needs to be done. Anticonvulsant drugs may operate via alternative, as yet undiscovered, mechanisms. Or they may operate through complementary mechanisms. More exhaustive research is required.

Even so, a key problem in the search for accurate mechanistic descriptions is the fact that the body is under multifactorial control. Amplification systems, modulating systems, feedback inhibitory mechanisms, ion fluxes, multiple messenger systems, etc.

provide natural checks and balances for any given physiological function. If one pathway is blocked by drug action, another pathway is likely to take over. Consequently, one can never be sure from the outset that designing a drug to act on a particular receptor or enzyme will provide treatment for a particular condition. This mitigates, in part, against the rational approach to drug design.

5. High Cost

The present cost of development represents a significant hurdle in bringing a drug from discovery to market. In 1938, phenytoin was discovered and brought to market at minimal expense. In 1976, a minimum of 8 years and $54 million were required to bring a drug to market [29]. By 1992, costs had soared to $231 million and 10-12 years were required for design, development and testing [30].

6. Symptomatic cures

At the present time, there are no clinically proven antiepileptogenic drugs. The currently available so-called "antiepileptic drugs" [AEDs] are simply anti-ictogenic agents with no clinically demonstrated antiepileptogenic efficacy. These presently marketed AEDs suppress the seizure symptoms of epilepsy but do not affect the natural course of the epileptogenic process.

A concerted attack on each of the six problems is the goal of rational antiepileptic drug design.

1.3 Approaches to the Rational Design of Future Antiepileptic Drugs

Modern day medicinal chemistry utilizes interdisciplinary, complementary techniques (Table 1.7) to effect rational antiepileptic drug design. A fundamental understanding of the disease process permits the selection and synthesis of promising drug candidates which interrupt the disease mechanism and exert a therapeutic effect at suitable sub-cellular drug targets. Numerous design criteria must be satisfied for the compound to be active *in vivo*.

Table 1.7: Techniques of rational drug design

1. **Mechanistic understanding of medical condition**
2. **Rational hypothesis of drug action at drug target**
3. **Identification of lead compounds**
4. **Synthesis of analogues**
5. **Bioassay to measure drug activity**
6. **Computational studies**
   a) **Molecular modelling**
      i) **Quantum mechanics**
      ii) **Molecular mechanics**
      iii) **Molecular dynamics**
   b) **Quantitative structure activity relationship studies**

Ref 31

1.3.1 Design Criteria

Rational molecular design must satisfy biological design criteria (Table 1.8) to ensure that a drug survives its journey to the target receptor where it produces a therapeutic effect. During the pharmacokinetic phase, an orally active drug (by far the most patient compliant) must be resistant to gastro-intestinal assault and must be

Table 1.8: Biological criteria for rational drug design

Phase	Molecular Requirements
Pharmacokinetic	Stability in the gastrointestinal tract[1] • Resistance to acid in the stomach (pH = 1.8 - 2.2) • Resistance to base in the duodenum (pH = 7 - 9) • Resistance to proteolytic enzymes in saliva and gastric juices Ability to access intestinal uptake systems[1] • Solubility • Passive diffusion Active transportMetabolic stability during first pass through the liver • Resistance to catabolic enzymes Ability to traverse the blood-brain-barrier • Passive diffusion Molecular weight ≤ 800 g•mol^{-1} 1.3 ≤ LogP ≤ 3.0 • Active transport Mimic of molecules known to be shuttled by active transport
Pharmacodynamic	Receptor affinity • Correct conformation • Correct configuration Enantiomers preferred

1. Assumes oral administration

Table 1.9: Criteria for commercial attractiveness of rational drug design

Therapeutic compound	Synthetic process
• High therapeutic ratio • Multiple indications • No or few side-effects • No or few alternatives • Proprietary	• Inexpensive • Efficient (few synthetic steps, high yield) • Rapid • Scalable for mass production • Proprietary
Disorder or disease state	**Patients**
• Chronic • Recurrent	• Large population • Economic strength • High compliance

systemically absorbed. As the drug travels from the point of entry to the receptor microenvironment, the compound must be robust enough to survive metabolism and elimination. Furthermore, the compound must have properties suitable for diffusion or active transport through the blood-brain barrier (BBB), a tight epithelial cell layer which excludes most drugs from the brain [32]. Finally, the putative therapeutic agent must be designed to maximize the effects of drug interaction at the receptor (the pharmacodynamic phase). This may involve the manipulation of chemical structure to increase receptor affinity.

In addition to biological criteria, numerous commercial criteria (Table 1.9) must be met to realistically bring a drug to market. A therapeutic agent is only useful if it is efficacious, non-toxic and *used by a patient*. This implies that the drug has been marketed and is available by prescription or over-the-counter. This means that a company was willing to develop and commercialize the product contingent upon meeting a minimum number of financially favourable commercial criteria. Thus, medicinal chemists who are

serious about designing and discovering truly useful therapeutic agents should consider these criteria (as many as possible, as early as possible) as an additional guide for the rational drug design phase of a medicinal chemistry research program.

1.3.2 Drug Targets

Within the CNS, the physiological mechanisms of ictogenesis and epileptogenesis become the primary targets of antiepileptic drug design. Ictogenesis or seizure propagation involves excessive, aberrant electrical communication between neurons while epileptogenesis is a gradual process involving the initiation and maturation of an epileptogenic focus. Thus any neuronal component which participates in the propagation of the action potential or the growth of a seizure focus becomes a legitimate drug target in the attack on epilepsy. Neurotransmitters, receptors, enzymes and ion channels qualify as targets as well as any cellular entities which influence these targets. Various brain metals have been shown to influence each of the targets mentioned. Thus, a modulation of brain metals represents one drug target. Since the manipulation of neurotransmitter function holds promise in the quest for a true antiepileptogenic drug, neurotransmitter mediated inhibition constitutes another viable drug target.

1.3.2.1 Neurotransmitter Mediated Inhibition

Current strategies aimed at preventing epilepsy seek to enhance inhibition or inhibit excitation through an influence of neurotransmitter function. GABA (the major inhibitory neurotransmitter) and glutamate (the major excitatory neurotransmitter) play key roles in influencing synaptic transmission, normal or epileptiform.

1.3.2.1.1 Enhanced GABA Inhibition

GABA is the major inhibitory neurotransmitter in the CNS where it activates postsynaptic $GABA_A$ receptors causing chloride flux, membrane hyperpolarization and the inhibition of burst firing. GABA-mediated inhibition is crucial for proper neuronal communication and its deficiency has been linked to epileptic seizures [33]. Compounds which impair GABA synthesis (*ie.* allylglycine) or block its action postsynaptically (*ie.* penicillin) are pro-convulsant. Thus, it would appear beneficial that levels of GABA be increased in the brain of epileptic patients.

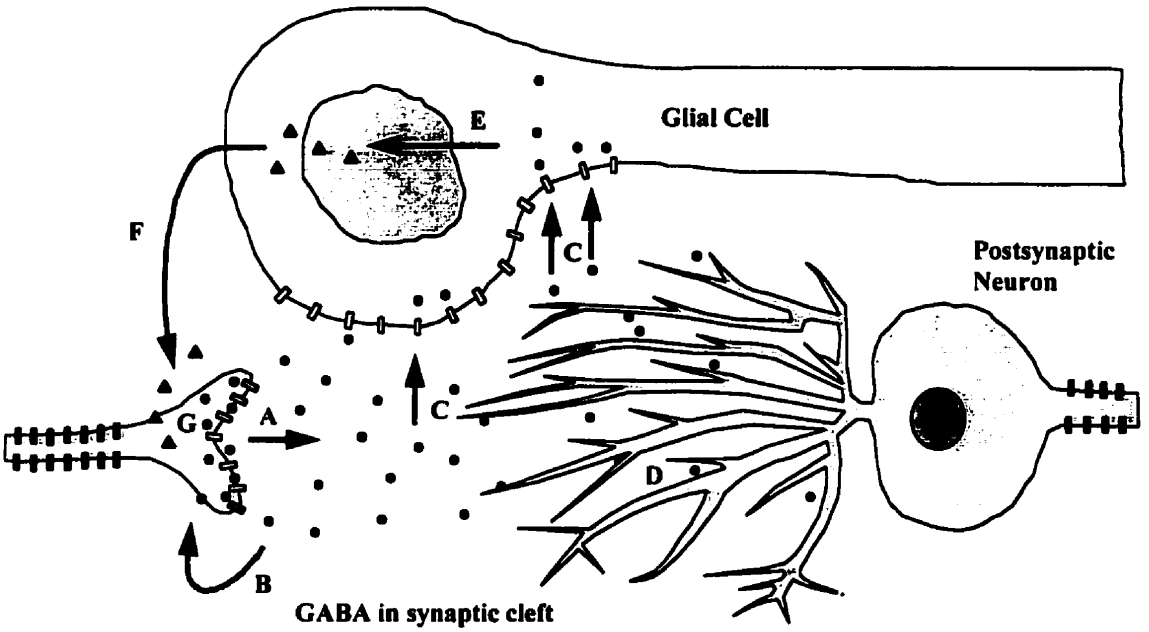
Since the discovery that benzodiazepines and barbiturates potentiate GABAergic inhibition at the chloride ionophore [34,35], other inhibition strategies have been developed (Table 1.10), some with limited success. Muscimol (5-aminomethyl-3-hydroxyisoxazole) and THIP (4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]-pyridin-3-ol) have been synthesized as GABA agonists but unacceptable side-effects in animal models prohibit

Table 1.10: Pharmacological enhancement of GABA-mediated inhibition

Inhibition Strategy	Examples
Direct action on chloride ionophore	Benzodiazepines, barbiturates
True GABA agonist	Muscimol, THIP
GABA prodrug	Progabide
Facilitation of GABA release	Vitamin B_6
Enhancement of GABA efficacy	Benzodiazepines
Inhibition of GABA-transaminase	Vigabatrin
Inhibition of GABA reuptake into neurons or glia	Tiagabine

clinical use. Progabide has been synthesized as a GABA prodrug since GABA does not penetrate the BBB [36]. Vitamin B_6 is thought to mediate inhibition by stimulating GABA synthesis and has been used sparingly to treat infantile seizures. Vigabatrin, an inhibitor of GABA-transaminase, has shown the most promise [37] and has recently been marketed (1993) as a potent anticonvulsant.

One of the more attractive strategies for GABA-mediated inhibition is the blockade of GABA uptake into neurons or glia. Ordinarily, the action of GABA in the synapse is terminated by temperature- and ion-dependent reuptake by a high affinity, bidirectional, 70- to 80-kDa glycoprotein located in the terminal bouton and surrounding glial cells (Figure 1.5). GABA taken up into nerve terminals is available for reutilization whereas GABA in glia is converted to glutamine. The glutamine is taken up by the neuron, enters the GABA shunt and is transformed into GABA. Under normal physiological conditions, the ratio of internal to external GABA is about 200:1; uptake



A. Presynaptic GABA release
B. Presynaptic GABA reuptake
C. Glial GABA uptake
D. Postsynaptic binding at GABA receptors
E. GABA metabolism to glutamine
F. Glutamine transfer to neuron
G. Multistep transformation of glutamine to GABA

Figure 1.5: Glial GABA uptake system

inhibitors decrease this ratio. Thus uptake inhibition increases synaptic GABA concentration and enhances its inhibitory effects. Therefore the GABA transporter represents an important target for therapeutic intervention.

Research in this area is promising. Nipecotic acid and guvacine, conventional GABA uptake blockers, demonstrate anticonvulsant activity when injected intracerebroventricularly, however these drugs do not cross the BBB [38]. Recently, several lipophilic nipecotic acid derivatives have been found active when administered to

animals systemically: tiagabine, SKF 89976A and CI-966 show potent inhibition of pentylenetetrazole (PTZ) clonic seizures, less effective prevention of maximal electroshock (MES) seizures, and very effective, potent anticonvulsant action in kindled rat models of partial seizures [39,40,41,42]. Notably, these compounds cause sedation, ataxia and myoclonus in animals at doses that are five to ten times higher than those producing anticonvulsant effects [43]. Tiagabine recently passed human clinical trials and demonstrates excellent anticonvulsant activity [44].

Relative to the other GABA-mediated inhibition strategies, GABA uptake inhibitors appear to be far more attractive therapeutically. A receptor agonist, for example, may produce a continuous, non-physiological pattern of receptor stimulation. An uptake inhibitor, on the other hand, involves the enhancement of an endogenous transmitter which ensures that the physiological specificity and selectivity of drug action will be maintained. Reduced side-effects are the probable result.

1.3.2.1.2 Excitatory Neurotransmission Blockade

Glutamate and aspartate are excitatory neurotransmitters which depolarize neurons and play a role in the initiation and spread of seizure activity. Thus, a rational approach to reduce and possibly prevent seizures would involve the inhibition of these acidic, excitatory amino acids. Presently, three drug strategies have been advanced (Table 1.11) to reduce the concentration of glutamate and aspartate at the synapse, however the pharmacological manipulation of the synthesis, release and uptake of excitatory amino

Table 1.11: Pharmacological diminution of excitatory amino acid neurotransmission

Reduce concentration of excitatory amino acid at synapse
• **Decrease maximal rate of synthesis of glutamate and aspartate**
• **Decrease synaptic release of glutamate and aspartate**
• **Enhance uptake of glutamate**
Antagonize receptors
• **Decrease post-synaptic action of glutamate at NMDA and other receptors**

acids has often led to severe toxic side-effects. Consequently, post-synaptic glutamate receptors have emerged as the most promising drug targets in the quest to decrease the excitatory action of glutamate.

Three post-synaptic glutamate receptor subtypes have been identified according to the preferential binding of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate and NMDA (N-methyl-D-aspartate). Due to its widespread distribution in the CNS and its unusual properties, the NMDA receptor has garnered the most interest.

The NMDA receptor is a ligand-controlled ion channel, likely pentameric, composed of high molecular weight, protein subunits (NR, NR1, NR2). The channel mediates transmembrane passage of sodium, calcium and potassium ions (Figure 1.6) and has distinct domains which bind glutamic acid, phencyclidine (PCP), glycine, Zn(II), polyamines and various spider and wasp toxins [45,46,47].

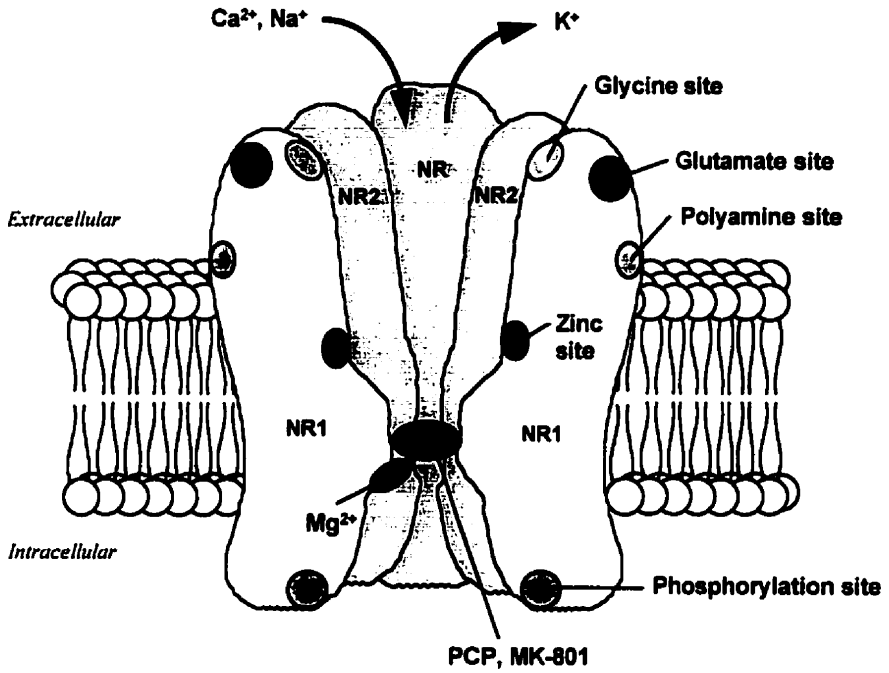


Figure 1.6: Model of the NMDA receptor [48]

Epilepsy research is particularly focussed on the NMDA receptor for four reasons. First, *in vitro* physiologic studies indicate that NMDA receptors are critical to epileptiform activity [49,50] and epileptogenesis [51]. Second, NMDA antagonists are potent anticonvulsants in a broad spectrum of animal seizure models and are particularly effective against maximal electroshock seizures. Unfortunately, poor CNS penetration and neurotoxicity have limited their effectiveness. Third, the NMDA receptor is unique among the family of ligand-gated excitatory amino acid receptors in that it is voltage dependent, requiring some membrane depolarization to relieve a Mg^{2+} block within the ion channel. Finally, the NMDA receptor possesses numerous binding domains that have been well documented [52]. One such domain is the glycine "co-agonist" binding site. A

unique requirement among neurotransmitter receptors, glycine acts as an obligatory co-agonist in concert with the neurotransmitter glutamic acid to effect receptor activation and channel opening [53].

The glycine co-agonist NMDA binding site represents an especially attractive drug target [54]. Antagonists operating at the NMDA glycine site have advantages over the channel-blocking NMDA antagonists in that behavioural side-effects such as impaired learning or muscle relaxation [55,56,57,58,59,60] are absent at systemic anticonvulsant doses. In fact, NMDA glycine site antagonists have been found to be antipsychotic [61], neuroprotective [62] and more importantly anticonvulsant [63]. As an example of the latter, felbamate was recently marketed as an extremely effective anticonvulsant. It was found to antagonize NMDA receptor function by interacting with the NMDA strychnine-insensitive glycine binding site [64,65]. Unfortunately, felbamate caused the deaths of 24 patients due to its chemical similarity to the toxic compound, chloramphenicol. Felbamate's NMDA glycine site antagonism was not responsible for the toxicity and therefore remains an extremely attractive target in the continued development of novel antiepileptic agents.

1.3.2.2 Neural Metal Mediated Inhibition

1.3.2.2.1 Metals of the Transition Series

Metals of the transition series are present in human brain in trace quantities: 27 ppm wet mass for Fe^{2+}, 16 ppm for Zn^{2+}, 2.4 ppm for Cu^{2+}, 0.3 ppm for Mn^{2+} and 0.05 ppm for Co^{2+} [66]. Although their role in brain function is not fully understood, the transition metals do participate in several aspects of synaptic transmission [66]. Hence, their presence or absence may affect the propagation of electrical information between two neurons.

Transition metals are often bound in metalloenzymes where they may exert specific catalytic properties operating through several possible mechanisms. Metal ions can accept or donate electrons from ligands via π or σ bonds, thereby promoting acid-base or covalent catalysis. Alternatively, they can serve as a bridge through common coordination to bring an enzyme and substrate into proximity. Unfortunately, a detailed knowledge of the chemistry of transition metals does not permit the determination of their roles in the CNS. However, the well-described capacity of metal ions to induce epilepsy [66] demonstrates the need to identify possible sites at which metal ions may regulate neuronal function thereby interfering with normal synaptic transmission.

Excess concentration of divalent metal ions, such as Cu^{2+} and Zn^{2+}, can inhibit enzymes which catalyze the synthesis and degradation of neurotransmitters, thus

disturbing the bioavailable pools of various chemical messengers. Moreover, after intense neuronal activity, terminal bouton extrude Zn^{2+} into the extracellular space [67,68,69] where it prolongs the action of excitatory neurotransmitters [70] and renders the action of inhibitory GABA less effective [71]. Clearly, divalent transition metals influence neurotransmitter function which in turn affects synaptic transmission. Thus, these metals constitute plausible targets in the search for antiepileptic drugs.

1.3.3 Techniques in Anticonvulsant Drug Discovery

1.3.3.1 Computer Modelling

Due to recent advances in computer technology, computer modelling has emerged as a powerful tool in the drug design process. Theories of particle motion, classical and quantum mechanical, have been married to the high speed performance of super-computers to effect the efficient and precise mathematical modelling of molecules and their interactions. Information derived from these molecular physics methodologies holds great promise in the rational design of antiepileptic agents.

Typically, a molecular modelling system consists of three key components: an initial geometry generator, a graphical interface and an energy function with an associated minimization procedure. The initial molecular geometry is specified in terms of atomic or Cartesian coordinates and graphics permits visualization of the molecule. An appropriate mathematical technique — quantum mechanics or molecular mechanics — is used to

optimize the molecule's geometry by energy minimization to obtain pertinent geometric and electronic information. Simulations may be extended to analyze intermolecular interactions, molecular flexibility at physiological temperatures, or conformational changes due to solvation in aqueous or lipid environments. Structure-activity relationship studies may also be performed for any series of molecules. The desired molecular information depends on the technique used.

Quantum mechanics is a non-empirical calculational method used to define the energy and properties of a molecule by solving the Schrödinger partial differential equation: $H\Psi = E\Psi$, where H is the Hamiltonian differential operator and Ψ is the wave function. Molecular quantum mechanics may be *ab initio* (non-empirical) or semi-empirical. *Ab initio* calculations consider all electrons in the molecule and attempt a rigorous solution to the Hamiltonian equation while semi-empirical calculations consider only valence electrons and incorporate parameters adjusted to fit experimental data.

Quantum mechanics derives its strength from the high quality geometric and electronic information that may be obtained, however, the method is restricted to small drug molecules due to the technique's mathematical complexity [72]. Severe computational demands (and expensive computing time) restrict semi-empirical and *ab initio* methods to molecules of not more than 200 atoms and 75 atoms, respectively. Calculations on larger molecules (*ie.* proteins and nucleic acids) must be performed using molecular mechanics, an empirical calculational technique based on classical Newtonian equations of motion.

Calculational methods use energy minimization schemes to identify a molecule's global energy minimum. The optimized geometry at the global energy minimum is assumed to model the molecule's native conformation. Importantly, the properties of known or suspected bioactive and toxic conformers may be studied. Alternatively, molecular modelling may be used to engineer molecules for desired receptor site geometries. New antiepileptic drugs may be designed using these techniques. Clearly, computer assisted rational drug design (CARDD), especially when used in conjunction with other rational drug design techniques, has great potential to significantly advance the antiepileptic drug design effort.

1.3.3.2 Animal Testing

A putative anticonvulsant compound may be rationally designed, possibly with the aid of a computer, to operate at a specific drug target. However, the compound's activity and toxic effects remain uncertain unless tested in a biological system. Appropriate animal screens are necessary before the drug may be promoted to human drug trials and used clinically.

Presently, the clinical usefulness of available antiepileptic drugs is indicated by their ability to prevent the spread of seizures and/or increase the minimum seizure threshold in humans and animals. Animal screens used over the last 60 years have led to the development of animal models which are fair predictors of human seizure drug efficacy. In rats, drugs effective against the pilocarpine induced seizure model (PIS) or

against the maximal electroshock model (MES) are often useful against secondary generalized tonic-clonic and complex partial seizures in humans. Drugs effective against subcutaneous pentylenetetrazole (PTZ) are effective against absence seizures [20]. These models provide excellent screens for novel anticonvulsant drugs effective against specific seizure types.

More recently, research has focussed on the development of antiepileptogenic drugs since no drug currently on the market prevents the progression of epilepsy. A new model, the Spontaneous Recurrent Seizure (SRS) model, has been used to produce epileptogenesis in rats. Pilocarpine (380 mg/kg ip) induces *status epilepticus* which is followed by spontananeous recovery after 10-12 hours. Over the next 2 weeks, progressive behavioural responses culminate in generalized tonic-clonic seizures. Spontaneous seizures recur over the months that follow. Administration of a putative therapeutic agent immediately after recovery from *status epilepticus* permits a compound to be evaluated as an antiepileptogenic and reflects ability to prevent the initiation of spontaneous seizures; administration after 1-2 months permits a compound to be evaluated as an anti-ictogenic and reflects ability to suppress an established seizure disorder.

In this Thesis, three anticonvulsant models (MES, PIS, PTZ) and one antiepileptogenic (SRS) model are used in rodents to test putative antiepileptic agents; details are provided in Appendix B.

1.4 AIMS OF THIS THESIS

To date, the fruits of antiepileptic research have been less than satisfactory. Pharmacotherapy is limited to a few agents which possess toxicities ranging from cosmetic to life-threatening. Drug efficacy remains moderate to poor because antiepileptic drug therapies targets symptoms, not root causes. An ignorance of biochemical mechanisms contributes to high research costs. These problems persist despite valiant efforts using traditional and contemporary research methods. In response, this Thesis aims to address each of these "design challenges" through the design and synthesis of peptides and amino acids as novel antiepileptic agents based on neurotransmitter (Chapter 2) and transition metal (Chapter 3) mediated inhibition.

Chapter 2 reports the design, synthesis and biological testing of β-amino acids as neurotransmitter analogues with hybrid anti-ictogenic/antiepileptogenic properties. The design of congeners with optimal antiepileptic activity is also proposed.

Chapter 3 evaluates Zn(II) and Cu(II) complexes leading to the design and synthesis of Cu(II)/Zn(II) chelators. Cyclic dipeptides are designed to translocate Cu(II) across the blood-brain-barrier, release it, then chelate and remove Zn(II) to produce an antiepileptic effect.

Ultimately, the goal of this Thesis is the discovery and development of a truly useful antiepileptic agent which not only prevent seizures but also arrests the development of the underlying epilepsy. Such a drug is not only efficacious and safe, but also can be patented, mass produced and has high patient compliance. Hopefully, by

using novel drug design methodologies, a truly curative antiepileptic agent will be found – an agent which will revolutionize the therapeutic expectations of a large and significant patient population.

CHAPTER 2

RATIONAL DESIGN AND SYNTHESIS OF ANTIEPILEPTIC DRUGS BASED ON NEUROTRANSMITTER MEDIATED INHIBITION

2.1 BACKGROUND

Incremental advances in epilepsy research since the serendipitous discoveries of phenobarbital and phenytoin have done little to improve the plight of the epileptic patient; epilepsy remains a condition without a cure, a disorder resistant to control. At present, antiepileptic chemotherapy is plagued by poor efficacy (65%) and unacceptable levels of toxicity (45%). Furthermore, the drugs currently available for treating epilepsy are little more than "symptomatic" agents, suppressing the symptoms of epilepsy (*ie.* seizures) while failing to contend meaningfully with the fundamental pathological process that initially caused (and continues to cause) the underlying susceptibility to seizures. No current anticonvulsant drug has demonstrated ability to affect the natural course of the epileptogenic process. For example, Temkin *et al.* have shown that conventional anticonvulsant drugs do not prevent seizure development in patients who have experienced neurologic haemorrhage or trauma [73]; this huge patient population remains unserviced by conventional drugs. Accordingly, future rationally designed drugs for epilepsy must extend beyond the scope of simple ictogenesis and should encompass the greater mandate of epileptogenesis. The development of novel antiepileptic drugs (safe, efficacious and antiepileptogenic) is a medical imperative.

Traditionally, ureide and heterocyclic anti-seizure drugs operating at neuronal cation channels have been discovered by serendipity and animal screens. These drug discovery "techniques" must be superseded by drug design based on mechanistic understanding if truly useful antiepileptic molecules (anti-seizure and antiepileptogenic

combined) are to be discovered in the near future. Accordingly, the evolution and understanding of concepts concerning the aetiology and pathogenesis of epilepsy is requisite for the rational design of a novel antiepileptic drug, one which has the capacity to positively influence the natural history of epilepsy curatively, not merely symptomatically.

2.2 ICTOGENESIS VS. EPILEPTOGENESIS: IMPLICATIONS FOR DRUG DESIGN

An important first conceptual step in target selection for future curative drug design is the differentiation between the notions of "ictogenesis" and "epileptogenesis". Ictogenesis is the initiation and propagation of a seizure in time and space; it is a rapid, electrical/chemical event which occurs over seconds or minutes. Epileptogenesis, on the other hand, is the gradual process whereby normal brain is transformed into a state susceptible to spontaneous, episodic, time-limited recurrent seizures through the initiation and maturation of an "epileptogenic focus"; this is a slow biochemical/histological process which occurs insidiously over months to years. From an electrophysiological perspective, epileptogenesis is a process in which a normal neural network is transformed into a hyperexcitable network with an abnormal seizure threshold; ictogenesis is simply an event associated with discrete electrical discharges within this altered network. If the ictogenic event remains restricted to an anatomically limited hyperexcitable neuronal network, the clinical correlate is a partial seizure; if the ictogenic event overtakes the

entire forebrain, a generalized seizure occurs. Obviously, the chemistries of ictogenesis and epileptogenesis have unique differences which may be reflected in the therapeutics targeting these two processes. Rational drug design strategies require an explicit understanding of both processes at the molecular level of refinement.

Ictogenesis is a fast, short-term event heuristically divided into the sequential phases of initiation and elaboration. Elaboration arises from the extension of the seizure in time and space while initiation involves excessive brain electrical discharges propagated by the sequential opening of neuronal Na^+ channels [74,75,76,77]. The electrical activity passes from neuron to neuron by the Ca^{2+} channel mediated release of neurotransmitters (glutamate and GABA) which diffuse across synapses and dock with receptors; glutamate interacts with a variety of receptors including the NMDA (N-methyl-D-aspartate) receptor, γ-aminobutyric acid interacts with $GABA_{A,B}$ receptors.

Unlike ictogenesis, epileptogenesis is a gradual two phase process showing dynamic changes over the course of time: Phase 1 – the initiation of the epileptogenic focus/predisposition, and Phase 2 – the maturation of the epileptogenic focus or predisposition. Phase 1 epileptogenesis refers to the events which take place prior to the occurrence of the first seizure. There may be a considerable delay of months to years between the occurrence of the brain injury (*ie.* stroke, trauma) and the onset of spontaneous, recurrent seizures; during this latent period, epileptogenesis is evolving, culminating in active epilepsy in which recurrent seizures occur. Phase 2 epileptogenesis refers to the events which take place after the first seizure has occurred. This also is a long, protracted process in which seizures may become more frequent, more severe, more

refractory to treatment, or phenomenologically different in their clinical manifestations. At the clinical level, chronic epileptogenesis presents itself as a significant patient population which shows chronic progressive epilepsy uninfluenced by the administration of conventional anticonvulsant drugs. Since the design of antiepileptogenics is central to the discovery of truly curative agents, understanding the pathogenesis of epileptogenesis is crucial.

The cascade of histological/biochemical events which characterize epileptogenesis differ from those of ictogenesis. At the histological level, epileptogenesis involves cellular alterations which, in the drug-resistant partial epilepsies, are usually at the level of the limbic system. Chronic epileptogenesis manifests as neuronal loss in a variety of limbic structures, including the dentate gyrus, CA1, CA3, entorhinal cortex and endopyriform nucleus, together with sprouting and reorganization of the synaptic connections of the mossy fibre pathway in the dentate gyrus [78]. At the biochemical level, studies of partial seizures originating in limbic structures indicate that epileptogenesis involves abnormalities of synaptic chemistry: glutamate toxicity mediated via NMDA or AMPA receptors, and/or γ-aminobutyric acid insufficiency mediated via $GABA_{A,B}$ receptors [79a-dd]. From these studies, two theories of epileptogenesis have emerged: the mossy fibre sprouting hypothesis and the dormant basket cell hypothesis.

The mossy fibre sprouting hypothesis, which has emerged from work by Sutula *et al.*, postulates that an upregulation of excitatory coupling between neurons leads to epileptogenesis [6]. This excitatory upregulation is mediated by N-methyl-D-aspartate

glutamatergic receptors which are activated in chronic epileptic brain under circumstances that would not lead to activation in normal brain. Five studies support the mossy fibre sprouting hypothesis: Urban *et al.* showed that the excitatory synaptic input to dentate granule cells in hippocampi from epileptic temporal lobe contains an increased NMDA receptor mediated component [80]; Mody *et al.* found the same increase in granule cells from kindled rats, but no NMDA component in granule cells from non-kindled control rats [81]; Geddes *et al.* showed an increased number of NMDA receptors in the dentate gyrus in hippocampi taken from patients with temporal lobe epilepsy [82]; Dragunow *et al.* demonstrated that seizures activate immediate early genes (IEGs) and that this activation can be attenuated by NMDA receptor antagonism [83]; and, McNamara and co-workers showed that the NMDA antagonist MK-801 prevents kindling and the neuronal degeneration normally caused by recurrent seizures [84].

In contrast to the mossy fibre sprouting hypothesis, the dormant basket cell hypothesis suggests a downregulation of inhibitory coupling between neurons. Studies by Sloviter and Lothman *et al.* indicate that the connections which normally drive GABA releasing inhibitory interneurons are disturbed thereby rendering them functionally disconnected or dormant [7,85,86,87]; the resultant loss of GABAergic inhibition induces the epileptogenic state.

Thus the mossy fibre sprouting hypothesis invokes augmentation of glutamatergic excitation while the dormant basket cell hypothesis invokes reduction of GABAergic inhibition. Each theory, mutually exclusive in mechanism, proposes an abnormality (excessive glutamatergic excitation or diminished GABAergic inhibition) which could in

isolation predispose to epileptiform paroxysms however the fundamental disturbance that yields epileptogenesis seems to involve NMDA mediated enhanced function of excitatory synapses augmented by a superimposed, concomitant enduring loss of GABAergic inhibition. This suggests that a novel antiepileptogenic drug may be achieved through the design and synthesis of a single molecule capable of blocking NMDA function while augmenting GABA inhibition.

Still another theory of epileptogenesis implicates ictogenesis itself as a causal factor. Gowers observed in the 1880's that "seizures beget seizures" [88] and "epilepsy is a progressive disorder". These observations and recent evidence from the epilepsy kindling model support the idea that the recurrence of seizures can be responsible at least in part for the progression of epilepsy. Thus, the events of ictogenesis may constitute a sub-set of additive factors contributing to the process of epileptogenesis. Clearly, this potential "cause-and-effect" interdependency implies that both ictogenesis and epileptogenesis must be *simultaneously* targetted for effective antiepileptic drug therapy. Therefore an antiepileptogenic drug must also restrict ictogenesis or it will be unsatisfactory.

A comparison of general strategies for the rational design of anti-ictogenic and antiepileptogenic agents (Table 2.1) reveals that the mediation of neurotransmitters constitutes a common avenue to attack both problems. Hence, an agent capable of simultaneously inhibiting excitatory processes and stimulating inhibitory processes would not be purely antiepileptogenic, but would be capable of hybrid antiepileptogenic/anti-ictogenic activity. Such a hybrid would represent a significant advance in the field of

Table 2.1: General strategies for the rational design of anti-ictogenic and antiepileptogenic agents

Anti-ictogenic[a]	Antiepileptogenic
• Antagonize excitatory neurotransmitters • Mimic inhibitory neurotransmitters • Influence postsynaptic receptors • Block voltage gated ion channels	• Antagonize excitatory neurotransmitters • Mimic inhibitory neurotransmitters • Influence postsynaptic receptors

a. Reference 89.

antiepileptic drug design since the drug would be the first with antiepileptogenic properties and would have clinical utility beyond that of either an anticonvulsant or an antiepileptogenic in isolation; the hybrid would block seizure expression while simultaneously inhibiting the progression of epileptogenesis.

2.3 Design of Anti-ictogenic/Antiepileptogenic Agents

2.3.1 Design Goal and Criteria

The survey of the pathogenesis of epilepsy has revealed that: (i) the neurochemical aetiology of epileptogenesis (epilepsy focus generation) arises from an altered ratio of excitatory/inhibitory neurotransmitter stimulation, and (ii) ictogenesis (seizure generation) and epileptogenesis are distinct phenomena which act as reciprocal determinants. Therefore, the successful discovery of an antiepileptic drug must involve the design and synthesis of molecules with combined anticonvulsant (*i.e.* anti-ictogenic)

and antiepileptogenic properties that exploit agonists and antagonists of inhibitory and excitatory neurotransmitters, respectively. Accordingly, the goal of this research is the design and synthesis of anticonvulsant/antiepileptogenic hybrid drugs capable of simultaneously (i) inhibiting excitatory processes via antagonism of the NMDA receptor and (ii) stimulating inhibitory processes via blockade of glial GABA uptake. Additionally, to be truly useful pharmaceutical agents, these hybrid drugs must also satisfy the criteria for biological activity (Table 1.8) and commercial viability (Table 1.9).

2.3.2 Design Methodology

Molecules which satisfy the design criteria may be designed through the iterative use of the techniques of rational drug design as outlined in Table 1.7. One approach starts with detailed knowledge of the appropriate target receptor. A lead molecule may be designed to interact with the receptor to produce the desired antiepileptic effect and to fulfil other design criteria. Molecular refinement using structure activity relationships follows.

An alternate approach begins with a lead molecule or compound class which is known to interact with the target receptor and which already fulfils a majority of the design criteria. Molecular modification is used to increase receptor affinity and to meet unsatisfied design criteria.

The latter approach has been used effectively to identify and design a class of molecules with putative anti-ictogenic/antiepileptogenic properties.

2.3.3 Lead Molecule: β-Alanine

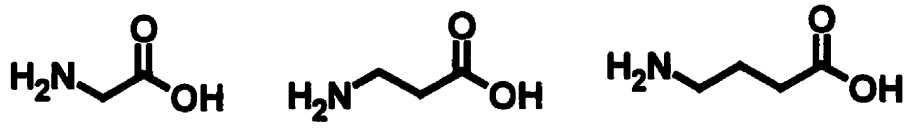
2.3.3.1 Rationale

After extensive literature evaluation and experimentation, β-amino acids have been selected as excellent candidates to meet the design goal. β-Alanine, the simplest β-amino acid, has been selected as a prototype lead compound for the following nine reasons:

1. Neuro-inhibitory properties

β-Alanine is intermediate to glycine and GABA (γ-aminobutyric acid) in the homologous amino acid series (Figure 2.1). Since both glycine and GABA are inhibitory neurotransmitters, β-alanine likely possesses similar properties.

A MEDLINE search discovered numerous studies which suggest that β-alanine might be an inhibitory neurotransmitter in the CNS: β-alanine occurs naturally in the



Glycine **β-Alanine** **γ-Aminobutyric Acid**

Figure 2.1: α, β, γ - Homologous amino acid series

CNS, is released by electrical or high potassium stimulation, has uptake or binding sites, and inhibits neuronal excitability [90a-l]. Of anatomical significance, β-alanine binding sites have been identified throughout the hippocampus and other limbic structures central to the epileptogenesis of complex-partial seizures [91] however it is uncertain whether these sites are specific to β-alanine [92].

2. Simultaneous action at multiple target receptors

Numerous studies have verified the action of β-alanine on both glycine receptors and GABAergic processes [93,94,95]. β-Alanine increases GABAergic inhibition by the preferential blockade of glial GABA uptake [96,97,98,99,100,101] resulting in an increase in interstitial GABA concentrations capable of depolarizing adjacent neurons [99]. β-Alanine also produces inhibition by binding to the glycine co-agonist site on the excitatory amino acid NMDA cation channel complex [102,103].

3. Anticonvulsant activity

Eight studies, listed chronologically in Table 2.2 have demonstrated the efficacy of β-alanine as an anticonvulsant. Remarkably, β-alanine suppresses seizures against a variety of convulsion models (ouabain, penicillin, MES, 3-mercaptopropionic acid and strychnine) in a variety of species (mouse, rat and cat) when administered centrally or parenterally.

Table 2.2: Literature precedence for β-alanine anticonvulsant activity

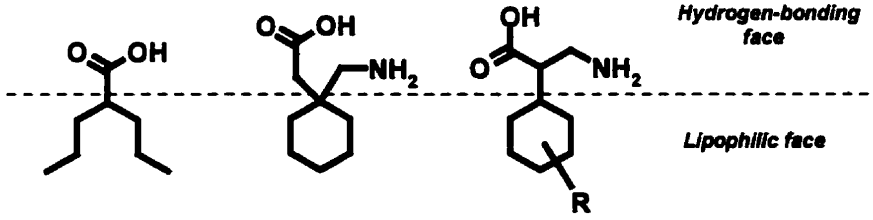
Year	Reported β-alanine anticonvulsant activity	Ref.
1974	50 μL of 0.1M β-alanine given intracerebroventricularly totally blocked ouabain induced seizures in rats	104
1977	Intrathecal injection of β-alanine inhibited strychnine induced seizures in cats whereas intracerebroventricular injection of β-alanine depressed rhinencephalic excitability and produced sleep	105
1979	Intravenous injection of β-alanine retarded penicillin induced focal limbic seizures in cats	106
1984	β-alanine enhanced GABA-T anticonvulsant effects against myoclonic seizures induced by 3-mercaptopropionic acid in rodents	107
1985	Intraperitoneal administration of β-alanine increased the strychnine convulsive threshold by 26% in mice	108
1986	Intracortical injection of β-alanine decreased penicillin induced seizures in rat	109
1988	β-alanine significantly decreased the incidence and duration of strychnine induced convulsions in rats	110
1995	Z-β-alanine (synthesized as a lipophilic inhibitory glycine analogue) protected against MES seizures in animals	111

The eight articles which attribute anticonvulsant effects to β-alanine were published between 1974 and 1995. Notably, no author has published twice and only one article has been published in the last eight years. Obviously, the relationship between β-alanine and anticonvulsants has been overlooked (or neglected). Moreover, no comment has been made concerning the putative antiepileptogenic effects of β-alanine specifically or β-amino acids in general.

4. Structural similarity to known anticonvulsants

β-Alanine and its congeners bear structural correspondence with known anticonvulsants and may exert their anticonvulsant effects by similar mechanisms. Taurine, the sulphonic acid analogue of β-alanine, is anticonvulsant [112,113,114] and antagonizes glial GABA uptake in the mammalian nervous system [98,115]. Conceivably, β-alanine's anticonvulsant properties arise from similar central GABAergic action.

Lipophilic β-alanine derivatives are structurally similar to the clinical anticonvulsants, valproic acid and gabapentin (Figure 2.2). In addition to blocking Na^+ channels, valproic acid increases the GABA concentration in the brain by increased synthesis and reduced degradation of GABA [116]. Valproic acid also decreases brain aspartate levels in adult mice and decreases aspartate and glutamate levels in developing mice [117,118]. Gabapentin may also influence these excitatory amino acids [119] but at present its mechanism of action is undefined. Perhaps β-alanine analogues also exert anti-seizure activity through similar mechanisms: pro-GABAergic and anti-glutamatergic.



Valproic acid **Gabapentin** **Lipophilic β-amino acid**

Figure 2.2: Lipophilic β-amino acids as analogues of clinical anticonvulsants

5. Antiepileptogenic activity

Preliminary studies in this laboratory (Section B.1.1.2.2) demonstrated that β-alanine is anticonvulsant against pilocarpine induced seizures at intraperitoneal (ip) doses of less than 20 mg/kg; more importantly however, at the same ip doses, β-alanine also shows antiepileptogenic activity blocking onset of kindling by greater than 50% [120]. These results clearly indicate that β-alanine is a superb starting point for the design of an anti-ictogenic/antiepileptogenic agent.

6. Metabolic stability

The limited metabolism of known β-amino acids *in vivo* [126] suggests that β-amino acid analogues with lipophilic side chains will also be metabolically stable. Furthermore, the incorporation of β-amino acids into peptide factors have resulted in increased resistance to enzymatic degradation [121,122].

7. Ability to cross the blood-brain-barrier

The anticonvulsant activity of parenterally administered β-alanine (Table 2.2) affirms its ability to cross the BBB, a property not shared with the other inhibitory neurotransmitters (glycine and GABA). This suggests that uptake into the brain occurs by active transport rather than by passive diffusion. Indeed, studies have identified a β–amino acid active transport shuttle capable of transporting β-alanine and related

analogues across the BBB [123,124,125]. Importantly, the active uptake mechanism permits greater flexibility in molecular design — analogues need not be designed within strict logP limits.

8. Low toxicity

Molecular structure and concentration are the key determinants of drug toxicity. β-Alanine, the simplest β-amino acid, is an endogenous neurotransmitter which is non-toxic under normal physiological conditions. Three other β-amino acids are endogenous to mammals and non-toxic: (*R* and *S*)-β-aminoisobutyrate and β-leucine [126]. This implies that the β-amino acid functionality is not a toxicophore (a molecular structure which gives rise to toxic effects).

Nonetheless, antiepileptic drug design using β-alanine analogues should proceed with discretion. Disorders such as neurolathyrism and amyotrophic lateral sclerosis (ALS) have been linked to the dietary consumption of N-oxalyl-β-alanine and N-methyl-β-alanine, respectively. Apparently, N-substitution forms a toxicophore which overactivates AMPA and NMDA receptors [127]. This suggests that a non-toxic anti-ictogenic/antiepileptogenic hybrid drug must remain free of an amino substituent.

9. Neglect

A survey of MEDLINE has identified a remarkable lack of interest in β-amino acids and β-alanine. In the last 24 years, only 342 articles in the medical and biochemical

Table 2.3: MEDLINE Hits for α- and β- amino acids (1972-1996)

Years	α-Amino Acids	β-Amino Acids
1992-96	62,101	121
1987-91	42,312	104
1982-86	22,279	64
1977-81	12,477	38
1972-76	5,300	15
1972-96	144,469	342

Search criteria: key word is found in article abstract.

Table 2.4: MEDLINE Hits for glycine, β-alanine and GABA (1972-1996)

Years	Glycine	β-Alanine	GABA
1992-96	6,117	284	5,120
1987-91	4,409	290	4,605
1982-86	2,838	259	3,331
1977-81	2,048	172	1,475
1972-76	864	56	258
1972-96	16,276	1,061	14,789

Search criteria: key word is found in article abstract.

literature have mentioned β-amino acids in the abstract (Table 2.3). By contrast, 144,469 articles referencing amino acids in the abstract were published in the same period. Similarly, the interest in β-alanine has been far exceeded by the interest in glycine and GABA (Table 2.4).

In this decade, considerable effort in anticonvulsant drug design has been expended in producing glycine analogues (as glutamate NMDA antagonists) and γ-amino acids (as GABA mimetics) yet β-alanine analogues have been completely neglected. No β-amino acid series has been prepared or evaluated in models of seizures/epilepsy.

Obviously, this lack of interest favours new entrants into the field and favourably supports the commercial design criteria.

In summary, the focus of this research is the design and synthesis of anticonvulsant/antiepileptogenic hybrid drugs capable of simultaneous antagonism of the NMDA glycine co-agonist site and blockade of glial GABA uptake. β-amino acids have been selected as excellent candidates to meet these design goals. Nine strong reasons support β-alanines as the molecular candidates with the best known potential to meet the challenges of antiepileptic drug design.

2.3.3.2 Current Problems with β-Amino Acids

Despite many excellent reasons to pursue β-amino acids as antiepileptic agents, two major problems seriously hamper research efforts: the lack of availability of β-amino acids and their high cost to buy or to synthesize. Both problems have severely restricted the widespread biological evaluation of β-amino acids as neuroactive drugs. Previously, only β-alanine and a few racemic β-aminocarboxylic acids were commercially available; however, more recently, limited arrays of expensive, enantiomeric β-amino acids have become commercially accessible. These problems of unavailability and high price find their source in the synthetic process. Balenovic in 1958 [128] and Drey in 1986 [129] have reviewed the syntheses of β-amino acids and have concluded that most synthetic routes have only a limited capacity to generate a diversity of β-amino acid structures. A

recent, more extensive review (Table 2.6) based on a literature search of CHEMICAL ABSTRACTS (1950-96) concurs.

Table 2.6 compares the top 26 β-amino acid syntheses based on the commercial and practical attractiveness of the synthetic process. Specifically, the number of synthetic steps, typical yield and scope of reaction (substrates, substitution, advantages, disadvantages and enantioselectivity) are evaluated. The syntheses are grouped according to the key synthetic reaction required to produce the β-amino acid structure (Table 2.5). To standardize the comparison, syntheses are traced from readily available substrates (if any) through to the free β-amino acid. Necessarily, some literature protocols have been extended to fit this criterion.

Table 2.5: List of key reactions which produce β-amino acids

A) Michael addition of nitrogen nucleophiles to α,β-unsaturated acids, esters or nitriles
B) Alkylation of activated β-homoserine equivalents via organolithiums and organocuprates
C) Addition of phosphorus ylids to ethyl N-thioacylurethanes
D) Condensations involving derivatives of malonic acid or cyanoacetic acid
E) Hydrolysis of uracils, dihydroxyuracils, pyrimidines or pyrimidinones
F) Reformatsky reaction of α-bromoesters
G) Curtius rearrangement of α-substituted succinates
H) Cycloaddition of alkenes with chlorosulphonyl isocyanate or nitrones
I) Addition of activated imines to ketene silyl acetals or silyl enolates
J) Arndt-Eistert homologation of N-protected α-amino acids
K) Palladium-catalyzed enamide carboacylation
L) Reduction of oximes of β-keto esters

Table 2.6: Comparison of β-amino acid syntheses

Protocol[a]	No. of Synthetic Steps[b]	Typical Yield (%)	Scope				Ref
			Substrates	Substitution	Advantages (+) / Disadvantages (-)	Selectivity	
1[A]	2 or 3	25-60	α- or β-substituted acrylates[c] or acrylonitriles, (chiral) benzylamines or hydroxylamine HCl	α- or β-substituted	+ General utility + Few steps, rapid + Trivial synthetic methodology - Strong acidic conditions - Few α-substituted acrylates and acrylonitriles - Limited structural diversity	<38% ee	130, 131, 132
2[A]	3	15	α-substituted acrylonitriles, (chiral) benzylamines	α-substituted	+ Few steps, rapid + Trivial synthetic methodology - Few substrates - Strong acidic conditions - Low yield - Limited structural diversity	racemic	130

Table 2.6 continued

Protocol[a]	No. of Synthetic Steps[b]	Typical Yield (%)	Scope				Ref
			Substrates	Substitution	Advantages (+) / Disadvantages (-)	Selectivity	
3[B]	7	25-50	Aspartic acid, R_2CuLi	β-alkyl or β-arylalkyl	+ R added in step 5 + High enantioselectivity - Long and tedious - Limited structural diversity	99% ee	133
4[B]	5	40-60	Z-Aspartic acid *t*-butyl ester, R_2CuLi	β-alkyl or β-arylalkyl	+ R added in step 3 + High enantioselectivity - Long and tedious - Limited structural diversity	>98% ee	134
5[B]	9	<10	α-Amino acid, RLi	α,β-di(alkyl or arylalkyl)	+ High diastereoselectivity - Steric sensitivity in key hydroboration step - Long and tedious - Low yield	diastereomers	135
6[B]	8	20-35	Asparagine, R_2CuLi	β-alkyl or β-arylalkyl	+ High enantioselectivity + R added in step 6 - Long and tedious - Limited structural diversity	>99% ee	136

Table 2.6 continued

Protocol[a]	No. of Synthetic Steps[b]	Typical Yield (%)	Scope: Substrates	Scope: Substitution	Scope: Advantages (+) / Disadvantages (-)	Selectivity	Ref
7[C]	4	40-50	Ethoxycarbonyliso-thiocyanate, Grignard reagent, (carbethoxy-methylene)triphenyl-phosphorane	β-alkyl	+ Few steps - Strong acidic conditions - Limited structural diversity	racemic	137, 138
8[D]	1*	6-69	Arylaldehyde, malonic acid, ammonium acetate	β-aryl	+ Simple and rapid + Readily available, inexpensive substrates and reagents - Strong *para* electron withdrawing groups may hinder reaction - workup varies according to substrate - Cinnamic acid by-products - Limited structural diversity	racemic	139, 140

Table 2.6 continued

Protocol[a]	No. of Synthetic Steps[b]	Typical Yield (%)	Scope				Ref.
			Substrates	Substitution	Advantages (+) / Disadvantages (-)	Selectivity	
9[D]	1*	15-75	Arylaldehyde, alkylmalonic acid, ammonia	α-alkyl, β-aryl	+ Simple and rapid - Strong *para* electron withdrawing groups may hinder reaction - Malonamide by-products - workup varies according to substrate - No diastereoselectivity	diastereomers	141
10[D]	3	NA	α-cyanoester or α-haloester, RBr or NaCN	α-alkyl or α-arylalkyl	+ Few steps + Trivial synthetic methodology - Limited structural diversity	racemic	126
11[D]	2	60-70	N-Chloromethyl-phthalimide, $RCH(CO_2Et)_2$[d]	α-substituted	+ Simple and rapid + General utility + High yield - Limited structural diversity	racemic	142
12[E]	3*	30-85	5-Bromouracil, N-alkylbenzylamine or dialkylamine	α-(alkyl or dialkyl)amino	+ Few steps - Strong acidic conditions - Limited structural diversity	racemic	143, 144

Table 2.6 continued

Protocol[a]	No. of Synthetic Steps[b]	Typical Yield (%)	Scope			Selectivity	Ref
			Substrates	Substitution	Advantages (+) / Disadvantages (-)		
13[E]	1*	90	5- or 6-Substituted dihydrouracil[c]	α- or β-substituted	+ High yield and efficiency - Strong acidic conditions - Limited dihydrouracil substrate - Limited structural diversity	racemic	145
14[E]	6	20	β-Alanine, RX	α-alkyl or α-arylalkyl	+ R introduced in step 5 + High enantioselectivity - Long and tedious - Limited structural diversity	>95% ee	146
15[E]	4	45	Asparagine, aryliodide	β-aryl	+ Aryl added in step 3 + High enantioselectivity - Strong acid conditions - Limited structural diversity	>95% ee	147
16[F]	5	25-35	Phenylglycinol, alkylaldehyde, $BrZnCH_2CO_2Et$	β-alkyl or β-arylalkyl	+ Good enantioselectivity + Mild conditions - Limited structural diversity	60-90% ee	148

Table 2.6 continued

Protocol[a]	No. of Synthetic Steps[b]	Typical Yield (%)	Scope: Substrates	Scope: Substitution	Scope: Advantages (+) / Disadvantages (-)	Selectivity	Ref
17[F]	3	35-65	α-Halocarboxylic acid[f], allylzinc bromide, N-benzylarylimines	β-aryl, α-(alkyl or aryl)	+ Few steps - No diastereoselectivity - HgI_2 used as a catalyst - Strong acidic conditions - Limited number of acid substrates	diastereomers	149
18[F]	3	10-40	Benzylamine derivatives, aldehyde, $BrZnCH_2CO_2R$	β-substituted	+ General utility + Few steps, rapid + Trivial synthetic methodology - Limited structural diversity	<56% ee	150
19[G]	5	40	Alkyl aldehyde, dialkyl succinates	α-alkyl	+ High enantioselectivity - Limited structural diversity	95 % ee	151
20[G]	7	30-40	Itaconic anhydride, arylhalide	α-arylmethyl	+ High enantioselectivity - Long and tedious - Limited structural diversity	95% ee	151

Table 2.6 continued

Protocol[a]	No. of Synthetic Steps[b]	Typical Yield (%)	Scope				Ref
			Substrates	Substitution	Advantages (+) / Disadvantages (-)	Selectivity	
21[H]	4[*]	20	Benzylhydroxylamine oxalate[g], aldehyde, vinylacetate or α-chloroacrylonitrile[h]	β-aryl or β-alkyl	+ Few steps - Significant side reactions reduce yield during the oxidation step - Limited structural diversity	25-80% ee	152
22[H]	3	40-50	Chlorosulfonyl isocyanate, 1-alkene	β-alkyl or β-arylalkyl	+ Few steps - Strong acidic conditions - Up to 50% olefin by-product may be produced - Hazardous substrate - Limited structural diversity	racemic	153, 154
23[I]	3 or 4	50	Aldehyde, benzylamine, silyl enolates[i]	β-, α,β-, or α,α,β-substitution	+ Few steps + Structural diversity + High diastereoselectivity - Limited disubstituted silyl enolates	diastereomers or racemic	155

Table 2.6 continued

Protocol[a]	No. of Synthetic Steps[b]	Typical Yield (%)	Scope: Substrates	Scope: Substitution	Scope: Advantages (+) / Disadvantages (-)	Selectivity	Ref
24[J]	4	35-85	BOC-α-amino acid	β-substituted	+ High enantioselectivity + Few steps + Readily available, inexpensive substrate - Limited structural diversity	high % ee	121, 122, 154
25[K]	5	30-40	Acryloyl chloride, nucleophile, carbon monoxide	β-substituted	+ Numerous nucleophile substrates + General utility - Hazardous substrate - Limited structural diversity	racemic	156
26[L]	3	10-30	β-Ketoesters, benzylamines	β-alkyl or β-aryl	- Limited substrate - Limited structural diversity	<50% ee	157

a. Superscripts on scheme numbers denote the key synthetic reaction which produces the β-amino acid structure, listed in Table 2.5; b. All protocols have been extended to the free β-amino acid; syntheses where protected β-amino acids are not relays are denoted by *; c. The reaction of aldehydes with (carbethoxymethyl)triphenylphosphonium bromide gives β-substituted unsaturated esters [158]; d. Substituted malonate may be derived from the enolate and alkylhalide in one step at high yield; e. Some dihydrouracils are commercially available and may be prepared by condensation of 2-alkenoic acids (or unsaturated esters; see c) with ureá [159]; f. See ref. 160 for preparation; g. See ref. 161 for preparation; h. Use of ketene acetals eliminates the problematic oxidation step; i. Made in one step from the corresponding ester.

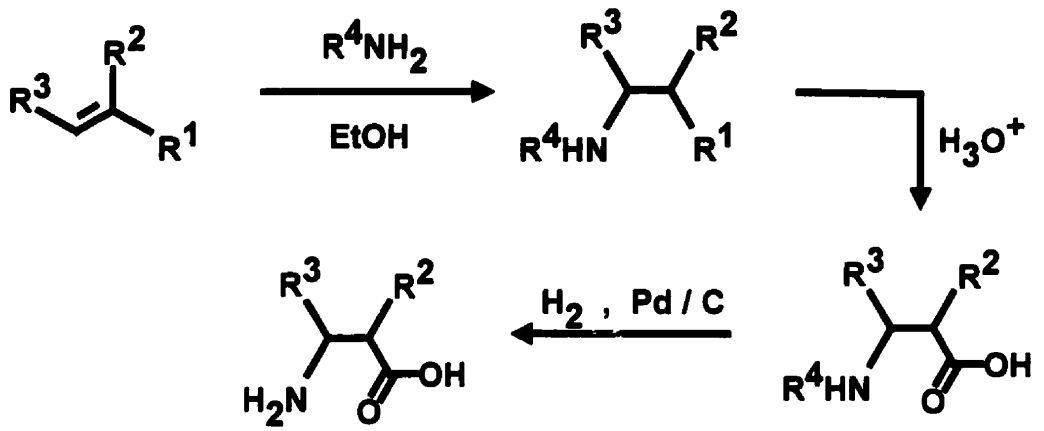
Table 2.7: Major problems of literature syntheses of β-amino acids

Primary Synthetic Problems	Consequence
• Lack of available substrates • Synthetic procedure limited to one substitution mode: α or β	• Limited diversity in the β-amino acid structure
• Numerous synthetic steps (> 5 steps) • Non-trivial procedures • Synthetic limitations (*ie.* stereoelectronic restrictions, by-products) • Methodological problems (*ie.* hazards, special requirements) • Expensive substrates and reagents	• High expense to synthesize product • Protracted, tedious procedures • Low overall yield (less than 20%)

Table 2.6 demonstrates that even the best β-amino acid literature protocols are deficient due to their inability to produce structural diversity or due to their inherent high cost. One or more primary problems (Table 2.7) contribute to these deficiencies. The reactions which demonstrate the greatest general utility, given their limitations, are protocols 1,11,18 and 25 (depicted in Scheme 2.1). α-Substituted β-alanines in which R is alkyl or aryl are obtained in good yields via protocols 1 and 11; β-substituted β–alanines in which R is alkyl or aryl are obtained in reasonable yields via protocols 1, 18 and 25. These protocols are generally useful and acceptable for selected, small scale syntheses, however, a wide range of the required bromoesters, substituted malonates and α-substituted acrylates are not readily available and may require multiple steps to produce. These disadvantages make these protocols impractical for the synthesis of an extensive, diverse analogue series.

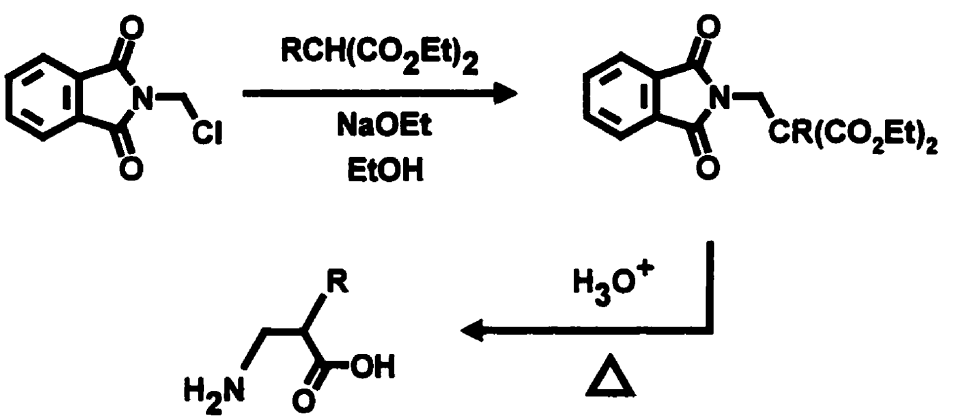
Scheme 2.1: General utility literature syntheses for β-amino acids

Protocol 1 from Table 2.6



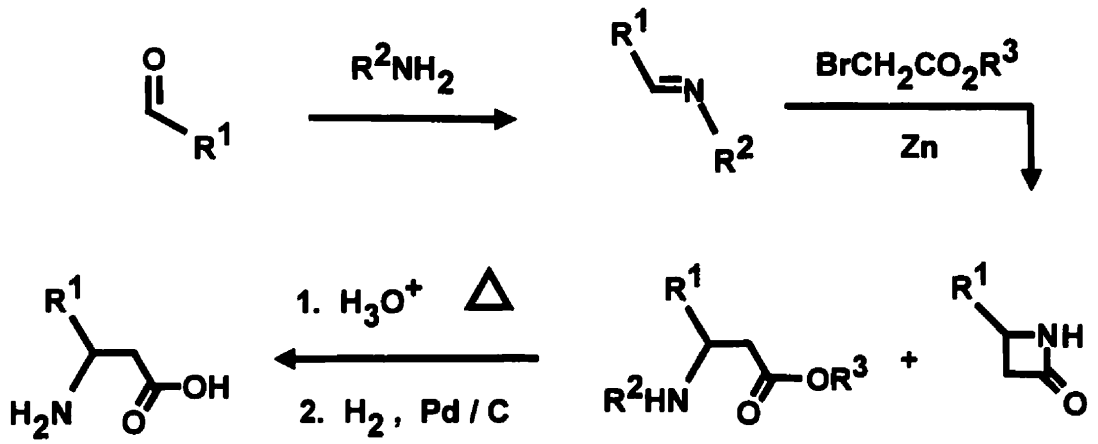
R^1= CN, $COOR^5$; R^2, R^3= H, aryl, alkyl; R^4= benzyl derivative

Protocol 11 from Table 2.6



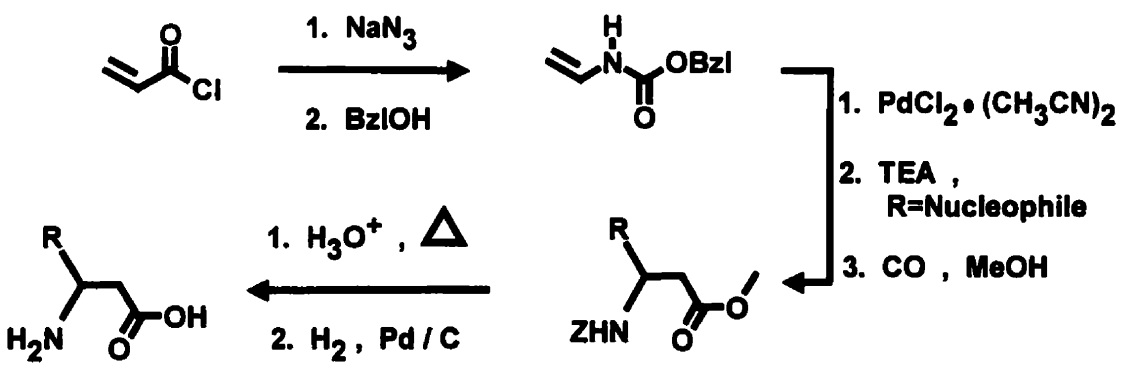
R= aryl, alkyl

Protocol 18 from Table 2.6



R^1= aryl, alkyl; R^2= benzyl derivative; R^3= Et, *l*-menthyl

Protocol 25 from Table 2.6



R= aryl, alkyl, heteroaryl, heteroalkyl

2.4 Design of a Novel Synthesis of β-Amino Acids

The 12 major reactions (A-L, Table 2.5) which form the basis for the top 26 β-amino acid syntheses are plagued with unacceptable difficulties and limitations which frustrate attempts to design and synthesize hybrid anticonvulsant/antiepileptogenic drugs. In response to these problems, an efficient, low cost synthesis, capable of generating a wide variety of substituted β-amino acids, has been developed: alkyl β-amino-thiophenecarboxylates are N-acylated, reductively desulfurized and doubly deprotected to yield α- or β-substituted β-amino acids in reasonable yield.

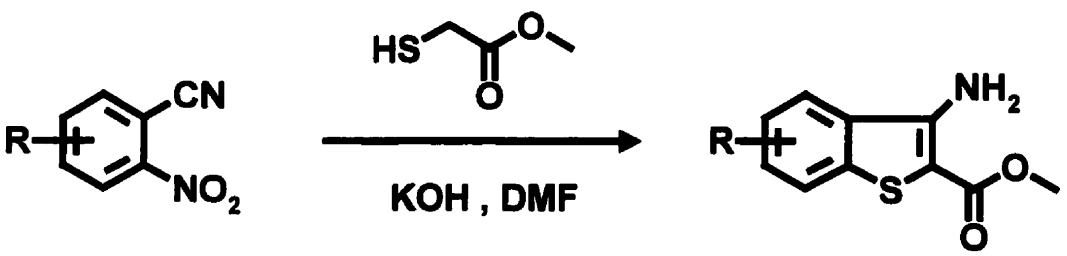
Synthesis of Alkyl β-Aminothiophenecarboxylates

The initial alkyl β-aminothiophenecarboxylates are synthesized using procedures by Beck, Gewald and Fiesselmann (Scheme 2.2 to Scheme 2.5). See Appendix A for detailed procedures.

Beck Method

Following the method of Beck [162], methyl 3-aminobenzo[b]thiophene-2-carboxylates are synthesized from *o*-nitrobenzonitriles in one step (Scheme 2.2) via the nucleophilic, thiolate displacement of an activated nitro function followed by a base-

catalyzed ring closure. Advantage is taken of the displacement lability of an arene nitro group ortho to a cyano function.

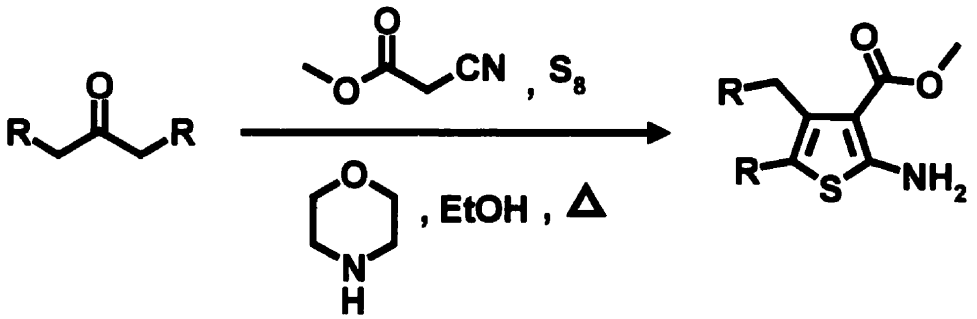


Scheme 2.2: 3-Aminobenzo[b]thiophene-2-carboxylates via nitro displacement

Substituted *o*-nitrobenzonitriles are available commercially or may be easily prepared from 1-chloro-2-nitrobenzenes by reaction with cuprous cyanide.

Gewald Method

In one step, the Gewald reaction [163,164] produces alkyl 2-aminothiophene-3-carboxylates, substituted in position 4 and/or 5, from α-methylenecarbonyl compounds which undergo base-catalyzed Knoevanegal condensation with activated nitriles (*ie.* methyl cyanoacetate) followed by cyclization with sulfur (Scheme 2.3). Morpholine is used as base; ethanol is used as solvent.



R= alkyl, cyclic alkyl or aryl

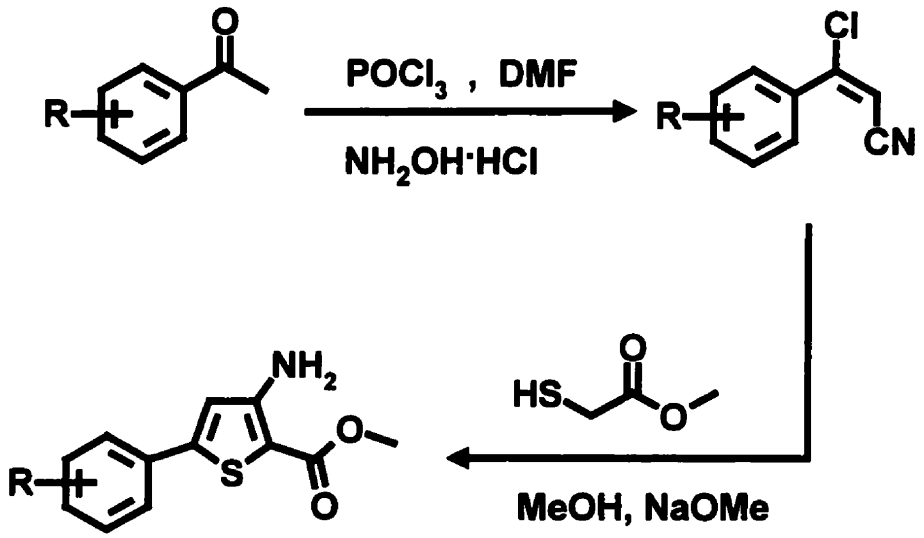
Scheme 2.3: 2-Aminothiophene-3-carboxylates from alkyl or arylalkyl ketones.

Fiesselmann Method

Through the use of the Fiesselmann reaction, alkyl 3-amino-5-arylthiophene-2-carboxylates are synthesized in one step by the base-catalyzed nucleophilic addition of α-mercaptoacetic esters to arylhaloacrylonitriles followed by a Dieckmann-Thorpe cyclization of the resulting 3-aryl-3-alkoxycarbonylmethylmercaptoacrylonitriles. The arylhaloacrylonitriles are synthesized from acetophenones or arylaldehydes.

Fiesselmann Method using Acetophenones

Methyl 3-amino-5-arylthiophene-2-carboxylates are synthesized from acetophenones by a 2-step method [165,166] (Scheme 2.4). Dimethylformamide, phosphoryl chloride, acetophenone and hydroxylamine hydrochloride react stepwise to

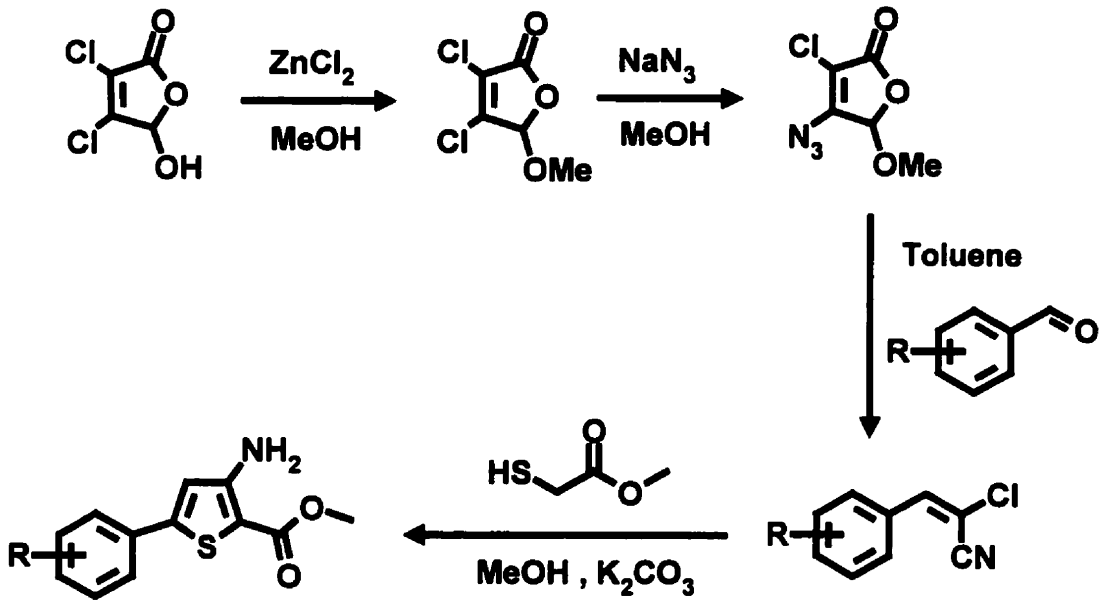


Scheme 2.4: 3-Aminothiophene-2-carboxylates from β-chlorocinnamonitriles

produce β–chlorocinnamonitrile which, in a second step, reacts with methyl thioglycolate in the presence of base to yield the Fiesselmann product.

Fiesselmann Method using Arylaldehydes

Methyl 3-amino-5-arylthiophene-2-carboxylates are synthesized by way of arylaldehydes in a four step process (Scheme 2.5). Mucochloric acid (a vinyl chloride) is converted to its pseudomethyl ester [167] which, upon treatment with sodium azide in methanol, gives the azidofuranone [168]. Thermolysis of 4-azido-3-chloro-5-methoxy-2(5H)-furanone produces a highly reactive chlorocyanoketene *in situ* which reacts with aromatic aldehyde to give α-halo-β-arylacrylonitrile [169], a Fiesselmann precursor. This

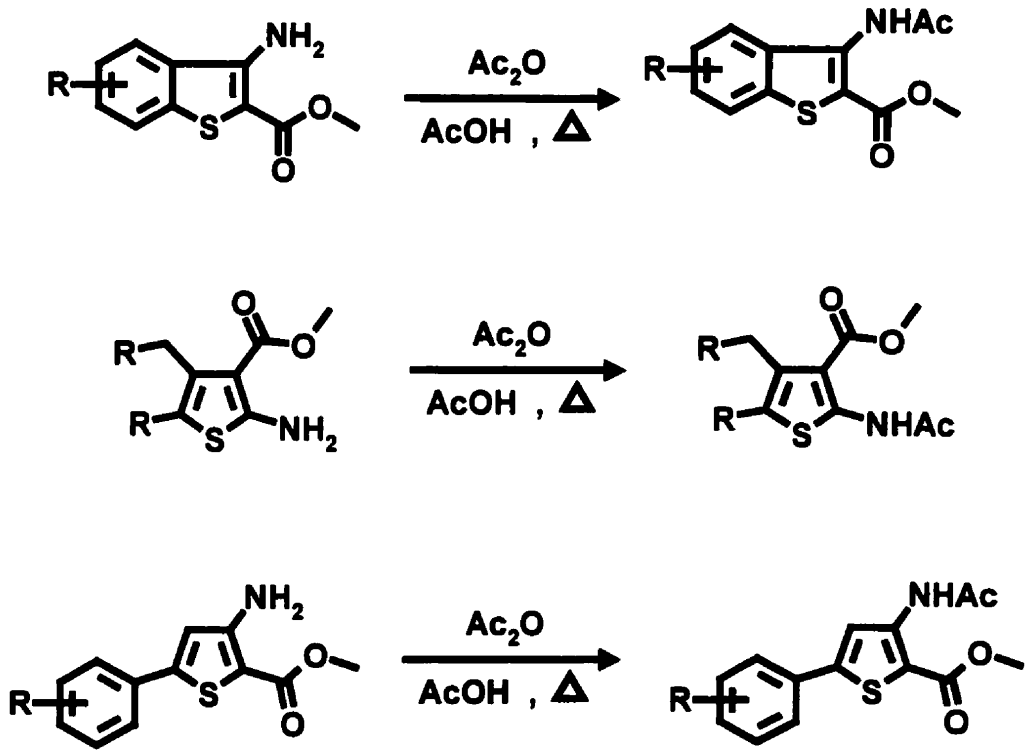


Scheme 2.5: 3-Aminothiophene-2-carboxylates from α-halo-β-arylacrylonitriles

reacts with methyl thioglycolate in the presence of base to yield the Fiesselmann product [170].

N-Acetyl Protection of Alkyl β-Aminothiophenecarboxylates

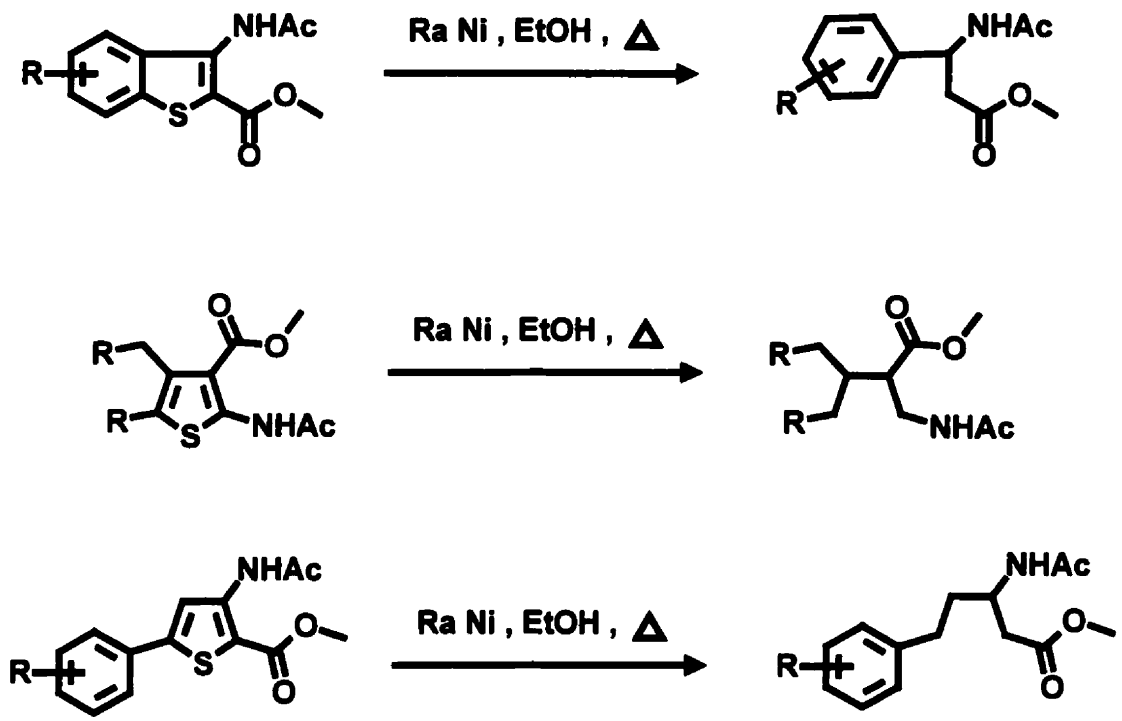
Alkyl β-acetamidothiophenecarboxylates are prepared in one step by reacting alkyl β-aminothiophenecarboxylates with acetic anhydride according to standard procedure [171,172] (Scheme 2.6). N-Acyl protection is effected for three reasons: Raney nickel reduction in the presence of EtOH has reportedly ethylated amino groups [173,174], N-acylation facilitates the workup of the desulfurized product and N-acylation significantly increases the yield of Raney nickel reduction [175].



Scheme 2.6: Acetylation of methyl β-aminothiophenecarboxylates

Raney Nickel Reductive Desulfurization of Alkyl β-Acetamidothiophene-carboxylates

The critical chemical transformation in this novel synthesis of β-alanines is the reductive desulfurization of alkyl β-acetamidothiophenecarboxylates by Raney nickel in EtOH to yield N-acetyl-β-alanine alkyl esters. Alkyl 2-acetamidothiophene-3-carboxylates are reduced to racemic α-substituted-N-acetyl-β-alanine alkyl esters; alkyl 3-acetamidothiophene-2-carboxylates are reduced to racemic β-substituted-N-acetyl-β-alanine alkyl esters (Scheme 2.7). Raney nickel catalyst (W-5), prepared by the method of Billica and Adkins [176], adsorbs sulfur compounds via sulfur's unshared electrons,



Scheme 2.7: Raney nickel reductive desulfurization of methyl β-acetamidothiophenecarboxylates to yield racemic α- and β-substituted N-acetyl-β-alanine methyl esters

weakens C-S bonds which break to form free radicals, and saturates unfilled valences and double bonds with H from the catalyst surface [177,178]. Sulfur is bound as nickel sulfide; ester and acetamido functions remain intact.

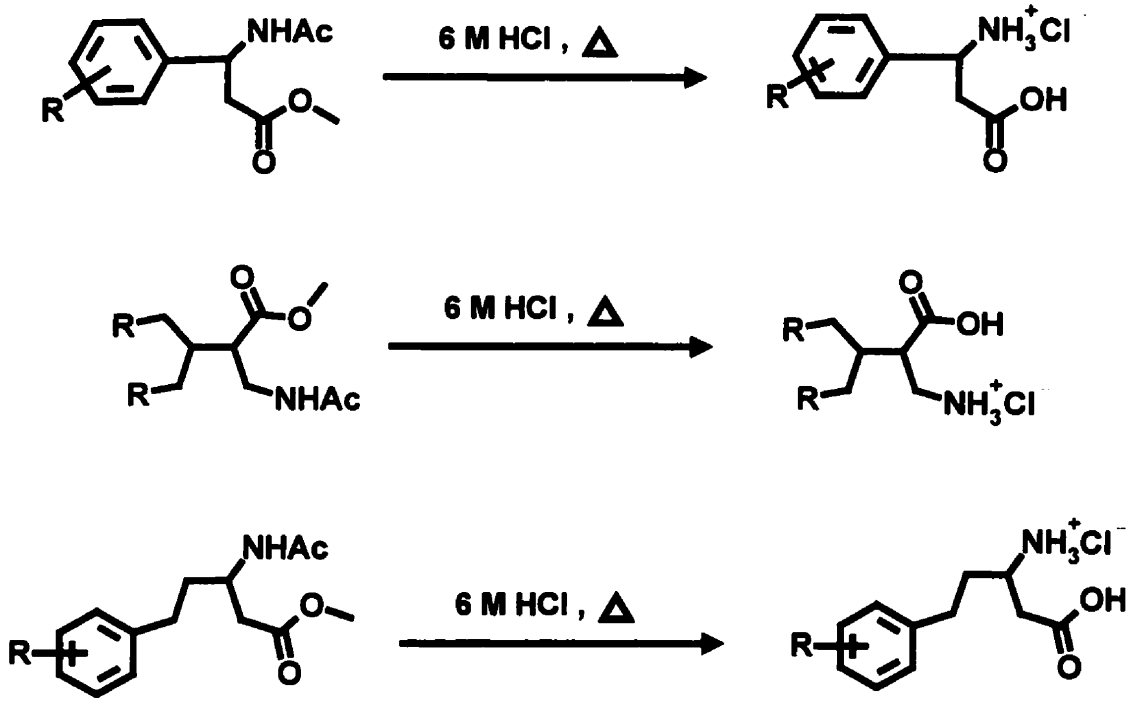
To date, Raney nickel (Ra Ni) reduction has not been used to construct a conformationally unrestricted β-amino acid backbone from functionalized thiophene; nevertheless, the proposed synthesis of N-acetyl-β-alanine alkyl esters is theoretically sound based on previous reports of amino acids synthesized from thiophene precursors.

Between 1956 and 1962, the Russian group Gol'dfarb, Fabrichnyi and Shalavina published 3 syntheses of substituted ω-amino acids derived from the Ra Ni reductive desulfurization of substituted thiophenes: 5-alkyl-4-nitro-2-thiophenecarboxylic acids were reduced to γ-alkyl-γ-amino acids in 46-84% yield [179]; ω-[5-(1-oximinoalkanoic acid)-2-thienyl]-alkanoic acids were desulfurized to produce α-amino-α,ω-dicarboxylic acids in 24-64% yield [180,181]; and ω-[5-(1-oximinoalkyl)-2-thienyl]-alkanoic acids were reduced to ω-alkyl-ω-aminoalkanoic acids in 24-50% yield [182]. In the only previous report of a β-amino acid produced by the reduction of a substituted thiophene, the 1 step malonic acid synthesis of β-amino acids (refer to protocol 8, Table 2.6) was used by Gol'dfarb *et al.* to produce β-thienyl-β-amino acids which were subsequently desulfurized by Ra Ni to yield β-alkyl-β-amino acids [175].

The Gol'dfarb reactions demonstrate that the presence of thiophene, amino and carboxylate should pose no problem to the synthesis of β-amino acids from alkyl β-acetamidothiophenecarboxylates.

Acid Deprotection of Substituted N-Acetyl-β-Alanine Alkyl Esters

De-acylation and de-esterification of substituted N-acetyl-β-alanine alkyl esters are simultaneously accomplished by acidolysis (Scheme 2.8). Substituted β-amino acid racemates are isolated free or as the hydrochloride salt.



Scheme 2.8: Acid deprotection to yield α- and β-substituted β-alanine hydrochloride salts

In this novel, patented synthesis [183], β-amino acids are prepared from β-aminothiophenecarboxylate precursors in 3 steps with the precursors being prepared in 1-4 steps. Due to the abundance of inexpensive β-aminothiophenecarboxylate derivatives that may be readily synthesized, a wide variety of β-amino acids may be produced. This is ideal for the synthesis of an analogue series. Accordingly, the novel synthesis has been applied to the design of two β-amino acid analogue series to (i) rigorously evaluate the chemistry and antiepileptic activity of the compounds produced by this novel protocol, and to (ii) refine the molecular structure of analogues to increase compound affinity to

target receptors with the ultimate goal of maximizing anti-ictogenic/antiepileptogenic activity.

2.5 Design and Synthesis of a β-Amino Acid Series to Evaluate the Novel Protocol

A simple series of β-amino acid analogues has been selected to explore the scope of the novel synthesis and to systematically explore the influence of varying steric and electronic substituent properties upon the anticonvulsant bioactivities of β-amino acid analogues. The α- and β-substituted β-amino acid analogues were selected to meet the following criteria:

1) β-Amino acid analogues must be synthesized from inexpensive, commercially available precursors.

2) To ease work-up and analysis, β-amino acid precursors must be selected to minimize the number of chiral centres in the product.

As a consequence of the Raney nickel reductive desulfurization, thiophene C_3 is transformed into an asymmetric carbon on the β-amino acid backbone. Precursors must be selected with care to ensure that reduction does not produce a second asymmetric carbon (originally thiophene C_4). Therefore,

- only symmetric ketones were selected as Gewald precursors.
- aminothiophenecarboxylates were limited to the 2,3 or 3,2 amino acid substitution pattern (as opposed to the 3,4 substitution pattern).

- Gewald precursors which are diastereoselective were deemed acceptable.

For practical reasons, racemic products need not be resolved until antiepileptic activity can be verified. During the early phases of drug design and synthesis, enantiomeric separations would be counterproductive since an efficient, large scale analogue synthesis requires rapidity, low cost and high throughput.

3) A sufficient subset of analogues must be selected and synthesized to evaluate the chemical and antiepileptic influence of alkyl, aromatic, hetero-aromatic, electron-donating and electron-withdrawing substituents in key positions.

Given these criteria, a search of the Aldrich chemical catalog (a library of over 66,000 chemicals) was performed and all starting materials for the syntheses of β-aminothiophenecarboxylates were identified. Without synthesizing special starting materials, 261 β-amino acid analogues may be readily synthesized using commercially available materials: 6 by the Beck method, 28 by the Gewald method, 21 by the Fiesselmann method using acetophenones, and 206 by the Fiesselmann method using arylaldehydes. From this basis set, a subset of 40 precursors was selected (see Figure 2.3 to Figure 2.6 for precursor structures) to afford a systematic spectrum of substituent patterns on the β-amino acid end products.

Figure 2.3: *o*-Nitrobenzonitriles as Beck precursors for the synthesis of β-aryl-β-alanines

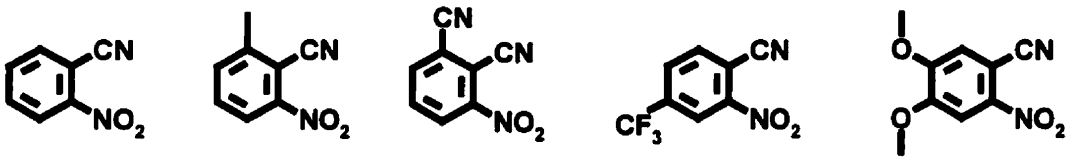


Figure 2.4: Symmetric ketones as Gewald precursors for the synthesis of α-substituted β-alanines

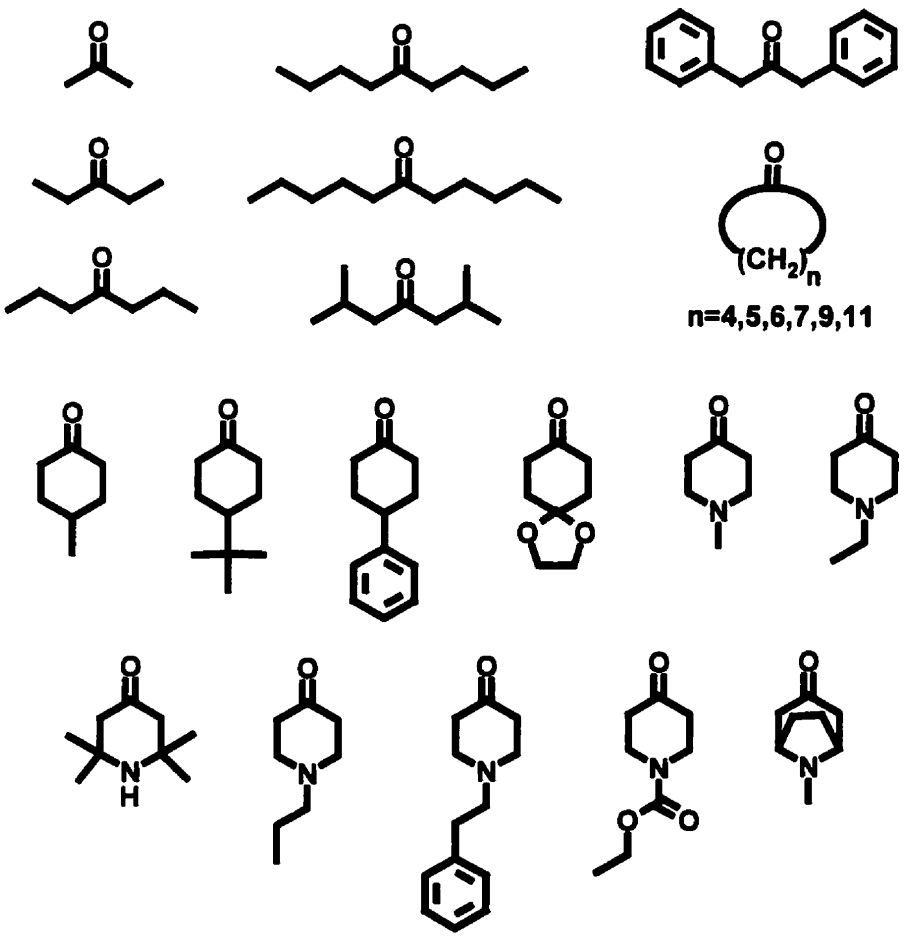


Figure 2.5: Aromatic aldehydes as Fiesselmann precursors for the synthesis of β-substituted β-alanines

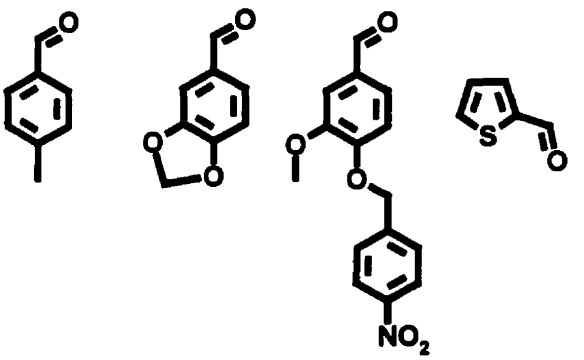
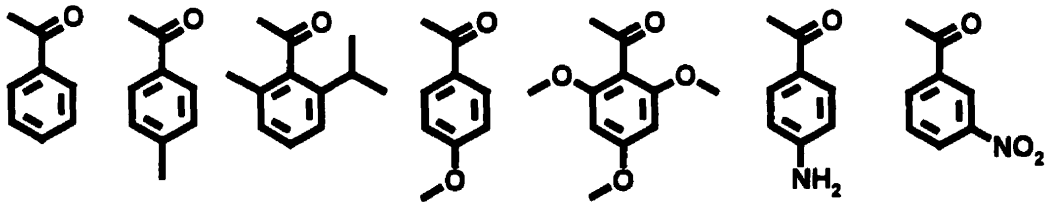


Figure 2.6: Acetophenones as Fiesselmann precursors for the synthesis of β-substituted β-alanines



All β-amino acids and intermediates were subject to ^{1}H NMR, IR, TLC and melting point analyses to assess product identity and product purity. Elemental analysis were performed on β-amino acids.

Furthermore, all β-amino acids were biologically evaluated to determine anti-ictogenic activity. Test compounds were subjected to a series of complementary seizure models [184]: (1) maximal electroshock seizures (MES), (2) pentylenetetrazol induced seizures (PTZ), and (3) pilocarpine induced seizures (PIS). Tests for the MES and PTZ seizure models were performed by the Anticonvulsant Drug Development Program at the

National Institute of Health in Bethesda, MD. PIS seizure tests were performed in-house at Queen's University. Refer to Appendix B for details of the experimental procedures. Compound efficacy was determined by seizure protection in more than 2/4 rats at doses of 300, 100, 50 and 10 mg/kg ip.

β-Amino acids with strong anti-ictogenic activity were tested for antiepileptogenic activity in the Spontaneous Recurrent Seizure (SRS) model of chronic epileptogenesis (See Appendix B).

2.5.1 Experimental Results and Discussion

2.5.1.1 Novel Chemistry

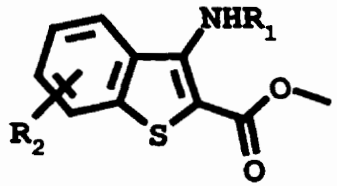
Using the newly developed, 4 step protocol, the analogue synthesis of β-amino acids commenced with 40 precursors and resulted in the synthesis of 17 doubly protected β-alanines of which a subset of 8 were fully deprotected to yield free α- and β-substituted β-amino acids. Seven different reaction types were employed of which six demonstrated a high level of synthetic success (Table 2.8) in moderate to high yield. All intermediates and end products were characterized by NMR, IR, TLC, and melting point analyses (Table 2.9 to Table 2.18). β-Amino acid purity was confirmed primarily by NMR and by elemental analysis (Table 2.19). Deliberate discontinuations (Table 2.20) or reaction failures (Table 2.21) abbreviated the 4 step reaction sequence in some cases; the

occasional reaction failure was to be expected and contributed positively to an understanding of the scope of the novel synthesis.

Table 2.8: Synthetic productivity of the novel protocol

Step	Reaction Product	Associated Chemistry	Number of successful reactions	Range of Yields (%)
1	β-Aminothiophene-carboxylate alkyl esters	Beck reaction Gewald reaction Fiesselmann using $ArCOCH_3$ Fiesselmann using ArCHO	4/5 11/24 4/7 4/4 23/40	40-77 34-87 33-51 12-50 12-87
2	Acetamidothiophene-carboxylate esters	Acetylation	19/20	24-95
3	N-Acetyl-β-alanine alkyl esters	Ra Ni reductive desulfurization	17/18	60-100
4	β-Alanines	Acid deprotection	8/8	34-98

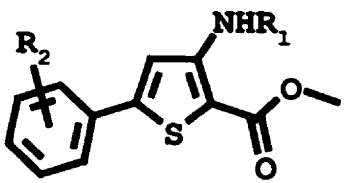
Table 2.9: Analytical data for substituted methyl 3-amino or 3-acetamidobenzo[b]thiophene-2-carboxylate



Compound	R_1	R_2	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν (NHR_1)	IR (cm^{-1}) ν (C=O)	^1H nmr ($CDCl_3$) δ
B3P107	H	H	49.8[c]	104[d]	0.70 (I)	3431, 3337	1663	7.75-7.36 (m, 4H), 5.92 (br s, 2H), 3.89 (3, 3H)
B3P109	H	4-Me	40.4	91	0.92 (K)	3488, 3338	1671	7.55 (d, 1H, J=11Hz), 7.30 (t, 1H, J=11Hz), 7.02 (d, 1H, J=11Hz), 6.30 (br s, 2H), 3.88 (s, 3H), 2.82 (s, 3H)
B3P141	H	6-CF_3	59.0[e]	120[f]	0.67 (I)	3469, 3343	1671	8.02 (s, 1H), 7.74 (d, 1H, J=8.6Hz), 7.59 (d, 1H, J=8.6Hz), 5.94 (br s, 2H), 3.92 (s, 3H)
B5P9	H	4-CN	77.2	171-172	0.70 (I)	3445, 3339	1684	7.94 (dd, 1H, J=8.2Hz,1.1Hz), 7.69 (dd, 1H, J=7.4Hz,0.9Hz), 7.49 (t, 1H, J=7.9Hz), 6.62 (br s, 2H), 3.91 (s,3H)
B6P43	Ac	H	65.9[g]	178-180	0.63 (I)	3271	1716, 1670	9.46 (br s, 1H), 8.08 (dd, 1H, J=7.0, 2.2 Hz), 7.76 (dd, 1H, J=7.5, 1.0Hz), 7.48 (d of t, 1H, J=6.9, 1.4Hz), 7.39 (d of t, 1H, J=7.0, 1.0Hz), 3.94 (s, 3H), 2.33 (s, 3H)
B6P49	Ac	6-CF_3	88.6[g]	204-205	0.72 (I)	3274	1720, 1676	9.81 (br s, 1H), 8.06 (s, 1H), 7.94 (d, 1H, J=8.7Hz), 7.51 (dd, 1H, J=8.7, 1.4Hz), 3.85 (s, 3H), 2.20 (d, 3H, J=4.2 Hz)

a. Products recrystallized from EtOH, EtOH/H_2O, or petroleum ether/EtOH/H_2O; b. Solvent systems used: I: EtOAc:MeOH 9:1; K: MeOH:AcOH 5:1; c. 72 % [162]; d. 110-111°C [162]; e. 69 % using methylcyclohexane as recrystallization solvent [162]; f. 126-127°C [162]; g. Yield quoted for acetylation of free amino compound.

Table 2.10: Analytical data for substituted methyl 3-amino- or 3-acetamido-5-phenylthiophene-2-carboxylate



Compound	R_1	R_2	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν (NHR_1)	IR (cm^{-1}) ν (C=O)	^{1}H nmr ($CDCl_3$)[c] δ
B3P171[d]	H	3'-NO_2	50.5	216	0.52 (Q)	3483, 3350	1648	8.32 (m, 1H), 8.20 (dd, 1H, J=2.0, 8.0Hz), 8.04 (dd, 1H, J=2.0, 8.0Hz), 7.71 (t, 1H, J=8.0Hz), 7.15 (s, 1H), 3.73 (s, 3H)
B3P177[d]	H	H	50.2[e]	146[f]	0.76 (Q)	3482, 3371	1666	7.58 (m, 2H), 7.36 (m, 3H), 6.77 (s, 1H), 5.18 (br s, 1H), 3.85 (s, 3H)
B4P51[d]	H	4'-OMe	34.5[g]	179[h]	0.19 (Q)	3490, 3377	1665	7.52 (m, 2H), 6.90 (m, 2H), 6.67 (s, 1H), 5.49 (br s, 2H), 3.84 (s, 3H), 1.61 (s, 1H)
B4P55[d]	H	4'-Me	32.6[i]	135[j]	0.54 (Q)	3487, 3369	1664	7.48 (dd, 2H, J=6.5Hz,1.7Hz), 7.19 (d, 2H, J=8.0Hz), 6.73 (s, 1H), 5.42 (br s, 2H), 3.84 (s, 3H), 2.37 (s, 3H)
B4P91	H	4'-Me	31.7[i]	134[j]	0.59 (Q)	3487, 3369	1664	7.47 (d, 2H, J=8.2Hz), 7.19 (d, 2H, J=8.0Hz), 6.73 (s, 1H), 5.42 (br s, 1H), 3.84 (s, 3H), 2.37 (s, 3H)
B4P95	H	H**	15.3	99	0.23 (Q)	3464, 3353	1663	7.30 (m, 2H), 7.05 (t, 1H, J=3.0Hz), 6.63 (s, 1H), 5.48 (br s, 2H), 3.83 (s, 3H)

Table 2.10 continued

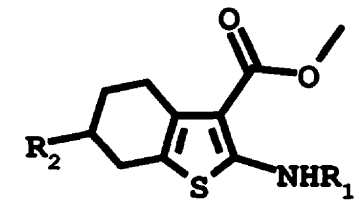
Compound	R_1	R_2	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν (NHR$_1$)	IR (cm^{-1}) ν (C=O)	^{1}H nmr ($CDCl_3$)[c] δ
B4P103	H	3',4'-OCH_2O-	*12.1*	184	0.23 (Q)	3464, 3361	1661	7.12 (m, 2H), 6.91 (m, 1H), 6.83 (s, 1H), 6.50 (br s, 2H), 6.05 (s, 2H), 3.71 (s, 3H)
B4P107	H	3'-OMe, 4'-(4''-NO_2 $PhCH_2O$)	50.4	179	0.31 (Q)	3464, 3351	1668	8.26 (d, 2H, J=8.7Hz), 7.70 (d, 2H, J=8.6Hz), 7.18 (s, 1H), 7.14 (d, 1H, J=9.5Hz), 7.05 (d, 1H, J=8.8Hz), 6.91 (s, 1H), 6.54 (br s, 2H), 5.30 (s, 2H), 3.86 (s, 3H), 3.71 (s, 3H)
B6P41	Ac	4'-OMe	93.7[m]	148-149	0.68 (I)	3303	1705, 1663	10.19 (br s, 1H), 8.27 (s, 1H), 7.60 (d of m, 2H, J=8.9Hz), 6.93 (d of m, 2H, J=8.8Hz), 3.89 (s, 3H), 3.84 (s, 3H), 2.24 (s, 3H)
B6P45	Ac	H	93.8[m]	115[n]	0.70 (I)	3319	1715, 1680	10.18 (br s, 1H), 8.38 (s, 1H), 7.66 (m, 2H), 7.41 (m, 3H), 3.90 (s, 3H), 2.25 (s, 3H)
B6P53	Ac	H**	83.6[m]	102	0.78 (I)	3314	1697, 1679	10.16 (br s, 1H), 8.22 (s, 1H), 7.32 (m, 2H), 7.04 (dd, 1H, J=4.9, 3.5Hz), 3.89 (s, 3H), 2.23 (s, 3H)
B6P55	Ac	3',4'-OCH_2O-	85.6[m]	185	0.81 (J)	3284	1707, 1682	10.16 (br s, 1H), 8.22 (d, 1H, J=2.7 Hz), 7.17 (dd, 1H, J=8.2, 1.8Hz), 7.12 (d, 1H, J=1.7Hz), 6.82 (d, 1H, J=8.1Hz), 6.00 (s, 2H), 3.88 (s, 3H), 2.23 (s, 3H)

Table 2.10 continued

Compound	R_1	R_2	Yield[a] (%)	m.p. (^{0}C)	TLC[b] (R_f)	IR (cm^{-1}) ν (NHR_1)	IR (cm^{-1}) ν (C=O)	^{1}H nmr ($CDCl_3$)[c] δ
B6P69	Ac	3'-NO_2	89.5[m]	165-168	0.72 (I)	3324	1702, 1676	10.15 (br s, 1H), 8.48 (s, 1H), 8.46 (s, 1H), 8.20 (dd, 1H, J=8.3, 2.0Hz), 7.97 (dd, 1H, J=7.8, 1.0Hz), 7.60 (t, 1H, J=8.0Hz), 3.92 (s, 3H), 2.26 (s, 3H)
B6P71	Ac	4'-Me	94.6[m]	127-129	0.70 (I)	3316	1710, 1675	10.18 (br s, 1H), 8.33 (s, 1H), 7.56 (d, 2H, J=8.2Hz), 7.21 (d, 2H, J=8.0Hz), 3.89 (s, 3H), 2.38 (s, 3H), 2.24 (s, 3H)
B6P73	Ac	3'-OMe, 4'-(4''-NO_2 $PhCH_2O$)	92.6[m]	193-194	0.68 (I)	3326	1705, 1671	10.19 (br s, 1H), 8.28 (d, 2H, J=2 Hz), 8.23 (s, 1H), 7.62 (d, 2H, J=8.7Hz), 7.19 (d, 2H, J=5.6Hz), 6.85 (d, 1H, J=8.9Hz), 5.27 (s, 2H), 3.97 (s, 3H), 3.90 (s, 3H), 2.24 (s, 3H)

** Substituent at position 5 on the thiophene ring is 2'-thienyl, not phenyl; a. Products recrystallized from EtOH, EtOH/H_2O, or petroleum ether/EtOH/H_2O; b. Solvent systems used: I: EtOAc:MeOH 9:1; J: $CHCl_3$:acetone:H_2O 88:12:15; Q: $CHCl_3$; c. DMSO-*d*6 used for B3P171 and B4P107; d. Compounds were synthesized using substituted $ArCOCH_3$, other 3-amino compounds were synthesized from ArCHO intermediates; f. 94.1 % [170]; g. 150-151^{0}C [185]; h. 55 % [166]; i. 181-182^{0}C [166], 185^{0}C [186]; j. 93.7 % [170]; k. 124^{0}C [186]; l. 76 % [166]; m. 139-41^{0}C [166]; n. Yield quoted for acetylation of free amino compound; p. 124-125^{0}C [185].

Table 2.11: Analytical data for substituted methyl 2-amino- or 2-acetamido-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate



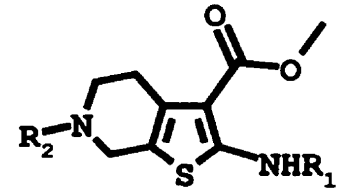
Compound	R_1	R_2	Yield[a] (%)	m.p. (^{0}C)	TLC[b] (R_f)	IR (cm^{-1}) ν (NHR_1)	IR (cm^{-1}) ν (C=O)	^{1}H nmr ($CDCl_3$) δ,
B3P99	H	H	87.4	96	0.68 (I)	3414, 3305	1650	5.92 (br s, 2H), 3.78 (s, 3H), 2.69 (m, 2H), 2.49 (m, 2H), 1.77 (m, 4H)
B3P113	H	$C(CH_3)_3$	60.2	110	0.78 (Q)	3473, 3338	1664	5.92 (br s, 2H), 3.78 (s, 3H), 2.94 (d of m, 2H, J=16Hz), 2.56 (dd, 2H, J=15Hz, 4Hz), 1.95 (d of m, 2H, J=12Hz), 1.34 (m,1H), 0.91 (s, 9H)
B3P135	H	CH_3	78.8	79	0.64 (I)	3420, 3309	1652	5.91 (br s, 2H), 4.25 (q, J=7.2Hz)**, 3.78 (s, 3H), 2.86 (d of m, 1H), 2.55 (d of m, 2H), 2.15 (t of m, 1H), 1.84 (m, 2H), 1.33 (t, J=7.2Hz)**, 1.03 (d, 3H, J=6.5Hz)
B3P147	H	Ph	58.6	114	0.74 (Q)	3447, 3323	1668	7.29 (m, 5H), 5.94 (br s, 2H), 4.28 (q, J=7.2Hz)**, 3.81 (s, 3H), 2.93 (m, 2H), 2.74 (m, 2H), 2.67 (m, 1H), 2.06 (m, 1H), 1.90 (m, 1H), 1.35 (t, J=7.2Hz)**
B6P37	Ac	H	94.0[c]	103-106	0.68 (I)	3248	1698, 1668	11.22 (br s, 1H), 3.86 (s, 3H), 2.74 (m, 2H), 2.63 (m, 2H), 2.25 (s, 3H), 1.79 (m, 2H), 1.76 (m, 2H)

Table 2.11 continued

Compound	R_1	R_2	Yield[a] (%)	m.p. (^{0}C)	TLC[b] (R_f)	IR (cm^{-1}) ν (NHR$_1$)	IR (cm^{-1}) ν (C=O)	^{1}H nmr ($CDCl_3$) δ
B6P47	Ac	Ph	82.8[c]	116-117	0.79 (I)	3255	1686, 1668	11.25 (br s, 1H), 7.28 (m, 5H), 3.88 (s, 3H), 3.00 (m, 2H), 2.89 (m,2H), 2.78 (m, 1H), 2.27 (s, 3H), 2.08 (m, 1H), 1.94 (m, 1H)
B6P63	Ac	$C(CH_3)_3$	58.8[c]	117-118	0.74 (I)	3271	1674	11.20 (br s, 1H), 3.85 (s, 3H), 3.00 (d of m, 1H, J=17.1Hz), 2.68 (d of m, 1H, J=15.7Hz), 2.50 (d of m, 1H, J=17.3Hz), 2.34 (d of m, 1H, J=14.2Hz), 2.25 (s, 3H), 2.00 (d of m, 1H, J=10.8Hz), 1.49 (dd, 1H, J=12.0, 5.0Hz), 1.27 (dd, 1H, 12.1, 5.1Hz), 0.93 (s, 9H)

** Resonances attributed to ethyl ester; partial Et-Me ester exchange occurred due to solvolysis; consider yield to be crude.
a. Amino products recrystallized from EtOH or EtOH/H_2O, acetamido products recrystallized from EtOH; b. Solvent systems used: I: EtOAc:MeOH 9:1; Q: $CHCl_3$; c. Yield quoted for acetylation of free amino compound.

Table 2.12: Analytical data for 6-substituted methyl 2-amino- or 2-acetamido-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate



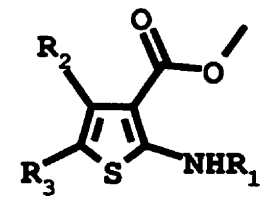
Compound	R_1	R_2	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν (NHR_1)	IR (cm^{-1}) ν (C=O)	^1H nmr ($CDCl_3$) δ
B3P145	H	CO_2Et	79.3	138[c]	0.26 (Q)	3415, 3310	1677	6.03 (br s, 2H), 4.39 (s, 2H), 4.16 (q, 2H, J=7.0Hz), 3.79 (s, 3H), 3.66 (t, 2H, J=5.6Hz), 2.80 (m, 2H), 1.27 (t, 3H, J=7.0Hz)
B3P165	H	Me	58.4	108	0.20 (I)	3490, 3380	1664	5.96 (br s, 2H), 4.27 (q, 2H)**, 3.79 (s, 3H), 3.37 (s, 3H), 2.80 (m, 2H), 2.65 (m, 2H), 2.44 (s, 3H), 1.33 (t, 3H)**
B4P177	H	Et	53.7	111-112	0.27 (I)	3499, 3389	1657	5.97 (br s, 2H), 3.77 (s, 3H), 3.40 (s, 2H), 2.81 (m, 2H), 2.70 (t, 2H, J=5.3Hz), 2.56 (q, 2H, J=7.1Hz), 1.14 (t, 3H, J=7.1Hz)
B6P39	Ac	Me	24.2[d]	108-109	0.33 (I)	3282	1728, 1655	11.30 (d, 1H, J=9.8Hz), 4.42 (q, 2H)**, 3.95 (s, 3H), 3.45 (m, 2H), 3.07 (m, 2H), 2.27 (d, 3H, J=3.1Hz), 2.07 (s, 3H), 2.02 (s, 2H), 1.42 (t, 3H)**
B6P67	Ac	CO_2Et	88.8[d]	139-141	0.72 (I)	3254	1682	11.19 (br s, 1H), 4.53 (s, 2H), 4.16 (q, 2H, J=7.0Hz), 3.86 (s, 3H), 3.68 (t, 2H, J=5.7Hz), 2.85 (t, 2H, J=5.7Hz), 2.26 (s, 3H), 1.27 (t, 3H, J=7.0Hz)

Table 2.12 **continued**

Compound	R_1	R_2	Yield[a] (%)	m.p. (^{0}C)	TLC[b] (R_f)	IR (cm^{-1}) ν (NHR_1)	ν (C=O)	^{1}H nmr ($CDCl_3$) δ
B6P75	Ac	Et	39.4[d]	oil	0.35, 0.94 (J)	3280	1714, 1676	11.19 (br s, 1H), 4.33 (q, 2H)**, 3.87 (s, 3H), 3.80 (br s, 2H), 3.40 (m, 1H), 3.25 (m, 1H), 2.99 (m, 2H), 2.82 (q, 2H, J=7.2Hz), 2.27 (s, 3H), 1.38 (t, 3H)**, 1.24 (t, 3H, J=7.3Hz)

** Resonances attributed to ethyl ester; partial Et-Me ester exchange occurred due to solvolysis; consider yield to be crude.
a. EtOH, H_2O or a mix used for recrystallization; b. Solvent systems used: I: EtOAc:MeOH 9:1; J: $CHCl_3$:Acetone:H_2O 88:12:15: K: MeOH:AcOH 5:1; Q: $CHCl_3$; c. 147-150^{0}C [187]; d. Yield quoted for acetylation of free amino compound.

Table 2.13: Analytical data for 4,5-substituted methyl 2-amino- or 2-acetamido-thiophene-3-carboxylate



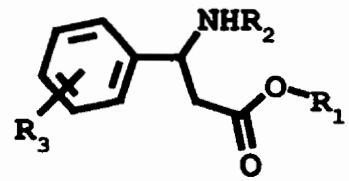
Compound	R_1	R_2	R_3	Yield[a] (%)	m.p. (^{0}C)	TLC[b] (R_f)	IR (cm^{-1}) ν (NHR_1)	IR (cm^{-1}) ν (C=O)	^{1}H nmr ($CDCl_3$) δ
B3P91	H	Et	Me	37.8[c]	70	0.69 (I)	3410, 3302	1652	5.86 (br s, 2H), 3.81 (s, 3H), 2.65 (q, 2H, J=7.4Hz), 2.16 (s, 3H), 1.05 (t, 3H, J=7.4Hz)
B3P101	H	$-CH_2CH_2CH_2-$		67.9	49-63	0.76 (J)	3412, 3297	1657	[d]7.18 (m, 2H), 4.11 (q, J=7.0Hz)**, 3.65 (s, 3H), 2.62 (m, 4H), 2.22 (quintet, 2H, J=6.9Hz), 1.22 (t, J=7.0Hz)**
B3P137[e]	H	$-CH_2(CH_2)_3CH_2-$		33.7	73	0.78 (Q)	3398, 3296	1652	5.76 (br s, 2H), 4.28 (q, 2H, J=7.3Hz), 2.97 (t, 2H, J=5.5 Hz), 2.57 (t, 2H, J= 5.3Hz), 1.81 (m, 2H), 1.63 (m, 4H), 1.35 (t, 3H, J=7.2Hz)
B3P157[e]	H	$-CH_2(CH_2)_8CH_2-$		62.5	45-46	0.46 (Q)	3444, 3331	1702	5.96 (br s, 2H), 4.27 (q, 2H, J=7.1Hz), 2.67 (t, 2H, J=6.9Hz), 2.59 (t, 2H, J=7.3Hz), 2.47 (m, 2H), 2.44 (m, 2H), 1.71 (m, 4H), 1.61 (m, 4H), 1.34 (t, 3H, J=7.0Hz), 1.30 (m, 4H)
B6P59	Ac	Et	Me	88.5[f]	58	0.73 (J)	3246	1669	11.25 (br s, 1H), 3.88 (s, 3H), 2.72 (q, 2H, J=7.4Hz), 2.26 (s, 3H), 2.25 (s, 3H), 1.06 (t, 3H, J=7.4Hz)

Table 2.13 continued

Compound	R_1	R_2 R_3	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν (NHR$_1$)	ν (C=O)	^{1}H nmr ($CDCl_3$) δ
B6P61	Ac	$-CH_2CH_2CH_2-$	32.9[f]	118-121	0.79 (I)	3294	1700, 1659	10.94 (br s, 1H), 3.85 (s, 3H), 2.85 (d of q, 4H, J=1.6, 6.4Hz), 2.38 (q, 2H, J=6.7Hz), 2.26 (s, 3H)
B6P65[c]	Ac	$-CH_2(CH_2)_3CH_2-$	90.8[f]	87-89	0.79 (I)	3264	1682, 1653	11.15 (br s, 1H), 4.34 (q, 2H, J=7.1Hz), 3.02 (t, 2H, J=5.4Hz), 2.71 (t, 2H, J=5.4Hz), 2.24 (s, 3H), 1.84 (m, 2H), 1.67 (m, 2H), 1.63 (m, 2H), 1.39 (t, 3H, J=7.2Hz)
B6P123[c]	Ac	$-CH_2(CH_2)_8CH_2-$	82.3[f]	54-74	0.73 (I)	3358	1710, 1678	11.35 (br s, 1H), 4.33 (q, 2H, J=7.3Hz), 2.75 (t, 2H, J=6.9Hz), 2.69 (t, 2H, J=7.6Hz), 2.47 (m, 2H), 2.44 (m, 2H), 2.24 (s, 3H), 1.74 (m, 4H), 1.62 (m, 4H), 1.38 (t, 3H, J=7.2Hz), 1.30 (m, 4H)

** Resonances attributed to ethyl ester; partial Et-Me ester exchange occurred due to solvolysis; consider yield to be crude.
a. Amino products recrystallized from EtOH or EtOH/H_2O, acetamido products recrystallized from EtOH; b. Solvent systems: I: EtOAc:MeOH 9:1; J: $CHCl_3$:Acetone:H_2O 88:12:15; Q: $CHCl_3$; c. 50% [188]; d. ^{1}H nmr solvent: DMSO-*d6*; e. Ethyl ester analogue synthesized (ester exchange was total); f. Yield quoted for acetylation of free amino compound.

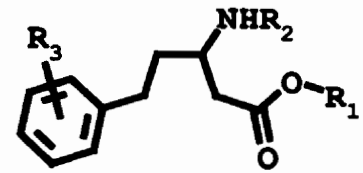
Table 2.14: Analytical data for β-aryl-β-alanines and precursors



Compound	R_1	R_2	R_3	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν	^1H nmr[c] δ
B5P65	CH_3	Ac	H	97.4	58-61	0.42 (I)	3322 (NH), 1741 (C=O), 1649 (C=O)	[d]7.30 (m, 5H), 6.62 (br d, 1H, J=6.0Hz), 5.43 (q, 1H, J=6.0Hz), 3.62 (s, 3H), 2.89 (dd, 2H, J=5.9, 8.5Hz), 2.02 (s, 3H)
B6P140	CH_3	Ac	ρ-F_3C	87.1	oil	0.52 (I)	3340 (NH), 1736 (C=O), 1654 (C=O)	[e]8.45 (d, 1H, J=8.0Hz), 7.59 (d, 2H, J=8.3Hz), 7.49 (d, 2H, J=8.1Hz), 5.25 (q, 1H, J=7.6, 15Hz), 3.55 (s, 3H), 2.75 (m, 2H), 1.82 (s, 3H)
B5P91	H	H•HCl	H	61.1[f]	220-221	0.75 (I)	3305 (OH), 1627 (C=O)	[g]7.32 (s, 5H), 4.49 (t, 1H, J=7.9Hz), 2.71 (d of t, 2H, J=6.5, 1.3Hz)

a. EtOH, H_2O or a mix was used for recrystallization; b. Solvent systems: I: EtOAc:MeOH 9:1; H: MeOH:AcOH 5:1; c. ^1H nmr solvents: d: $CDCl_3$, e: DMSO-*d6*, g: D_2O; f. 48% [139].

Table 2.15: Analytical data for aryl substituted β-phenethyl-β-alanine and precursors



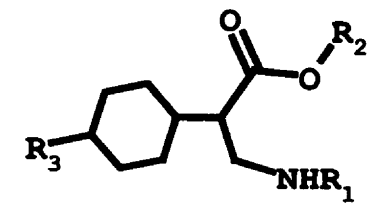
Compound	R_1	R_2	R_3	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν	^1H nmr[c] δ
B5P69	CH_3	Ac	ρ-CH_3O	93.8	oil	0.54 (I)	3285 (NH), 1735 (C=O), 1651 (C=O)	[d]7.08 (d, 2H, J=8.5Hz), 6.81 (d, 2H, J=8.7Hz), 6.03 (br d, 1H, J=8.7Hz), 4.27 (m, 1H), 3.77 (s, 3H), 3.67 (s, 3H), 2.59 (t, 2H, J=8.2Hz), 2.55 (d, 2H, J=8.4Hz), 1.96 (s, 3H), 1.84 (q, 2H, J=8.2Hz)
B5P73	CH_3	Ac	H	98.6	gum	0.68 (I)	1735 (C=O), 1654 (C=O)	[d]7.23 (m, 5H), 6.10 (br d, 1H, J=8.8Hz), 4.30 (t of d, 1H, J=8.9, 5.4Hz), 3.68 (s, 3H), 2.66 (t, 2H, J=8.2Hz), 2.57 (dd, 2H, J=4.9, 3.0Hz), 1.96 (s, 3H), 1.87 (m, 2H)
B6P89	CH_3	Ac	ρ-CH_3	99.1	50-51	0.63 (I)	3288 (NH), 1731 (C=O), 1639 (C=O)	[d]7.07 (s, 4H), 6.08 (br d, 1H, J=8.8Hz), 4.28 (sextet, 1H, J=5.3Hz), 3.67 (s, 3H), 2.63 (d, 2H, J= 8.2Hz), 2.55 (m, 2H), 2.30 (s, 3H), 1.96 (s, 3H), 1.84 (quintet, 2H, J=7.9Hz)
B6P101	CH_3	Ac	*m*-NHEt	100	oil	0.62 (I)	1731 (C=O), 1653 (C=O)	[d]7.11 (t, 1H, J=7.5Hz), 6.48 (br t, 3H), 6.05 (br d, 1H, J=8.4Hz), 4.31 (m, 1H), 3.67 (s, 3H), 3.33 (q, 2H, J=7.0Hz), 2.59 (t, 2H, J=8.4Hz), 2.56 (d, 2H, J=4.4Hz), 2.39 (br s, 1H), 1.94 (s, 3H), 1.87 (m, 2H), 1.14 (t, 3H, J=7.0Hz)

Table 2.15 continued

Compound	R_1	R_2	R_3	Yield[a] (%)	m.p. (^{0}C)	TLC[b] (R_f)	IR (cm^{-1}) ν	^{1}H nmr[c] δ
B6P113	CH_3	Ac	*m*,ρ-OCH_2O-	97.5	oil	0.53 (I)	1729 (C=O), 1654 (C=O)	[d]7.01(d, 1H, J=8.4Hz), 6.75 (d, 1H, J=8.4Hz), 6.65 (m, 1H), 6.16 (m, 1H), 5.90 (s, 0.5H), 4.25 (m,1H), 3.68 (s, 3H), 2.57 (m, 2H), 2.53 (m, 2H), 1.97 (s, 3H), 1.77 (m, 2H), 1.51 (impurity), 1.24 (impurity)
B6P119	CH_3	Ac	ρ-OH *m*-CH_3O	60.0	oil	0.80 (L)	3498 (OH), 1743 (C=O), 1663 (C=O)	[d]6.82 (d, 1H, J=7.9Hz), 6.67 (m, 2H), 6.10 (br d, 1H, J=8.6Hz), 5.56 (br s, 1H), 4.28 (m, 1H), 3.88 (s, 3H), 3.68 (s, 3H), 2.60 (d, 2H, J=8.4Hz), 2.55 (t, 2H, J=2.2Hz), 1.97 (s, 3H), 1.85 (m, 2H)
B5P95	H	H	H	39.6[e,f]	211-214[g]	0.37 (I)	3310 (OH), 1663 (C=O)	[h]8.36 (d, 5H, J=15.6Hz), 4.92 (br s, 1H), 4.14 (br s, 2H), 3.95 (br d, 2H, J=8.0Hz), 3.32 (br s, 2H)[j]
B5P111	H	H	ρ-CH_3	66.9	206-207	0.89 (K)	3280 (OH), 1706 (C=O)	[h]8.20 (m, 4H), 4.89 (m, 1H), 4.10 (m, 2H), 3.87 (m, 2H), 3.38 (s, 3H), 3.28 (quintet, 2H, J=6.3Hz)
B6P145	H	H•HCl	ρ-OH *m*-CH_3O	98.4	oil	0.32 (I)	3447 (OH), 1718 (C=O)	[m]7.79 (br d, 1H, J=8.3Hz), 6.68 (s, 1H), 6.65 (d, 1H, J=9.5Hz), 6.49 (d, 1H, J=8.0Hz), 4.00 (m, 1H), 3.69 (s, 3H), 2.43 (m, 2H), 2.30 (d, 2H, J=6.6Hz), 1.76 (s, 3H, solvent), 1.63 (m, 2H)

a. EtOH, H_2O or a mix used for recrystallization, where possible; b. Solvent systems: I: EtOAc:MeOH 9:1; L: EtOH:AcOH 50:1; K: MeOH:AcOH 5:1; c. ^{1}H nmr solvents: d: $CDCl_3$, h: TFA-*d*, m: DMSO-*d6*; e. B5P95 was synthesized in the R configuration from R-aspartic acid in 52% yield (7 steps) by Jefford *et al.* [133]; f. B5P95 was synthesized in 3 steps in 65% yield using the malonic acid synthesis by Ashton *et al.* [189]; g. 226-228^{0}C (dec.) [189]; j. ^{1}H nmr in D_2O provided by Jefford *et al.* [133].

Table 2.16: Analytical data for 4'-substituted α-cyclohexyl-β-alanine and precursors



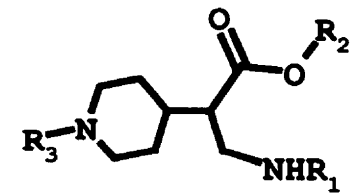
Compound	R_1	R_2	R_3	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν	^1H nmr[c] δ
B6P77	Ac	CH_3	H	93.5	oil	0.80 (I)	1738 (C=O), 1674 (C=O)	[d]5.91 (br s, 1H), 4.14 (q, J=7.1Hz)**, 3.69 (s, 3H), 3.53 (m, 1H), 3.32 (m, 1H), 2.46 (m, 1H), 1.94 (s, 3H), 1.69 (m, 5H), 1.26 (t, J=7.2Hz)**, 1.14 (m, 6H)
B6P81	Ac	CH_3	Ph	95.8	75-80	0.79 (L)	3259 (NH), 1730 (C=O), 1647 (C=O)	[d]7.29 (m, 3H), 7.19 (m, 2H), 5.94 (br s, 1H), 3.73 (s, 3H), 3.58 (m, 1H), 3.44 (m, 1H), 2.53 (m, 1H), 2.46 (m, 1H), 1.97 (s, 3H), 1.91 (m, 2H), 1.75 (m, 3H), 1.50 (m, 2H), 1.26 (m, 2H)
B6P109	Ac	CH_3	$C(CH_3)_3$	98.3	73-75	0.70 (I)	3261 (NH), 1735 (C=O), 1648 (C=O)	[d]5.88 (br s, 1H), 3.69 (s, 3H), 3.53 (m, 1H), 3.37 (m, 1H), 2.44 (m, 1H), 1.94 (s, 3H), 1.77 (m, 2H), 1.63 (m, 1H), 1.50 (m, 2H), 1.27 (t, 1H, J=7.1Hz), 1.00 (m, 4H), 0.82 (s, 9H)
B5P107	H•HCl	H	Ph	33.5	268-270	0.74 (I)	3300-2500 (OH) 1701 (C=O)	[c]8.09 (br s, 0.5H), 7.18 (m, 5H), 3.01 (m, 1H), 2.87 (dd, 1H, J=4.0, 12.8Hz), 2.57 (t, 1H, J=4.5Hz), 2.46 (m, 2H), 1.75 (m,5H), 1.29 (m, 3H)

Table 2.16 continued

Compound	R_1	R_2	R_3	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν	^1H nmr[c] δ
B5P119	H	H	H	51.9	238-240	0.75 (I)	3300-2700 (OH) 1635 (C=O)	[f]4.58 (quintet, 2H), 4.01 (m, 1H), 3.11 (m, 1H), 2.83 (m, 5H), 2.32 (m, 5H)
B5P127	H•HCl	H	$C(CH_3)_3$	62.7	230 (dec)	0.91 (K)	3400-2700 (OH) 1732 (C=O)	[e]8.02 (br s, 3H), 2.97 (m, 1H), 2.84 (m, 1H), 2.51 (m, 1H), 1.71 (m, 3H), 1.63 (m, 3H), 0.95 (m, 4H), 0.79 (s, 9H)

** Resonances attributed to ethyl ester; partial Et-Me ester exchange occurred due to solvolysis; consider yield to be crude.
a. EtOH, H_2O or a mix was used for recrystallizations; b. Solvent systems: I: EtOAc:MeOH 9:1; L: EtOH:AcOH 50:1; K: MeOH:AcOH 5:1; c. ^1H nmr solvents: d: $CDCl_3$, e: DMSO-*d*6, f: TFA-*d*.

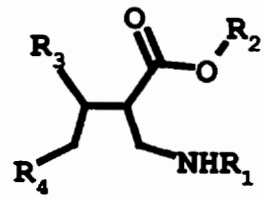
Table 2.17: Analytical data for 4'-substituted N-acetyl-α-piperidinyl-β-alanine methyl ester



Compound	R_1	R_2	R_3	Yield (%)	m.p. (°C)	TLC[a] (R_f)	IR (cm^{-1}) ν	^1H nmr ($CDCl_3$) δ
B6P105	Ac	CH_3	CO_2Et	96.8	gum	0.65 (I)	1743 (C=O), 1708 (C=O), 1673 (C=O)	5.92 (br s, 1H), 4.16 (q, J=6.6Hz)**, 4.10 (q, 2H, J=7.1Hz), 3.70 (s, 3H), 3.52 (m, 1H), 3.41 (m, 1H), 2.69 (m, 2H), 2.51 (m, 1H), 2.01 (m, 2H), 1.95 (s, 3H), 1.79 (m, 1H), 1.71 (d of m, 2H), 1.55 (d of m, 2H), 1.30 (t, J=6.6Hz)**, 1.23 (t, 3H, J=7.0Hz)

** Resonances attributed to ethyl ester; partial Et-Me ester exchange occurred due to solvolysis.
a. Solvent system: I: EtOAc:MeOH 9:1.

Table 2.18: Analytical data for α-substituted N-acetyl-β-alanine alkyl ester and α-substituted β-alanine



Compound	R_1	R_2	R_3	R_4	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν	^1H nmr (DMSO-*d6*) δ
B6P85	Ac	CH_3	$-CH_2CH_2$	CH_2-	94.1	oil	0.54 (I)	1720 (C=O), 1660 (C=O)	7.78 (br s, 1H), 4.03 (q, J=7.0Hz)**, 3.57 (s, 3H), 3.30 (m, 1H), 3.09 (m, 2H), 2.35 (m, 2H), 1.87 (m, 2H), 1.76 (s, 3H), 1.49 (m, 5H), 1.17 (t, J=7.0Hz)**
B6P93	Ac	CH_3	Et	CH_3	83.4	oil	0.75 (I)	3189 (NH), 1723 (C=O), 1665 (C=O)	7.80 (br m, 1H), 3.58 (s, 3H), 3.26 (m, 1H), 3.04 (m, 1H), 2.59 (m, 1H), 1.76 (s, 3H), 1.5-1.1 (m, 5H), 0.9-0.7 (m, 6H)
B6P97	Ac	CH_3	H	Bu	99.6	gum	0.53 (I)	1739 (C=O), 1658 (C=O)	7.54 (br d, 1H, J=8.1Hz), 4.17 (quintet, 1H, J=6.5Hz), 3.70 (s, 3H), 2.51 (br d, 2H, J=6.3Hz), 1.94 (s, 3H), 1.51 (br m, 2H), 1.33 (br m, 8H), 0.94 (m, 3H)

Table 2.18 continued

Compound	R_1	R_2	R_3 R_4	Yield[a] (%)	m.p. (°C)	TLC[b] (R_f)	IR (cm^{-1}) ν	^1H nmr (DMSO-*d6*) δ
B6P117	Ac	Et	$-CH_2(CH_2)_3CH_2-$	79.7	oil	0.77 (I)	3216 (NH), 1727 (C=O), 1666 (C=O)	[c]5.89 (br s, 1H), 4.16 (d of q, 2H, J=7.0, 4.0Hz), 3.62 (d of q, 1H, J=3.7, 13.5Hz), 3.25 (d of q, 1H, J=5.2, 13.5Hz), 2.52 (d of q, 1H, J=3.7, 9.5Hz), 1.94 (s, 3H), 1.7-1.3 (br m, 11H), 1.27 (t, 3H, J=7.0Hz)
B6P133	Ac	Et	$-CH_2(CH_2)_8CH_2-$	98.5	oil	0.75 (I)	3316 (NH), 1725 (C=O), 1661 (C=O)	7.88 (br s, 1H), 4.05 (q, 2H, J=8.1Hz), 3.59 (m, 2H), 2.45 (m, 1H), 1.74 (s, 3H), 1.50 (m, 1H), 1.28 (m, 22H), 1.15 (t, 3H, J=8.1Hz)
B5P131	H•HCl	H	$-CH_2(CH_2)_8CH_2-$	36.7	201-204	0.79 (I)	3400-2700 (OH) 1722 (C=O)	12.72 (br s, 1H), 7.99 (br s, 3H), 2.98 (m, 1H), 2.82 (m, 1H), 2.68 (m, 1H), 1.91 (m, 1H), 1.28 (m, 24H)

** Resonances attributed to ethyl ester; partial Et-Me ester exchange occurred due to solvolysis; consider yield to be crude.
a. Yield of last synthetic step; b. Solvent system I: EtOAc:MeOH 9:1; c. ^1H nmr solvent: $CDCl_3$.

Table 2.19: Elemental analysis of α- and β-substituted β-alanines

Compound Code	Compound Name	Formula		% C	% H	% N
B5P119	α-Cyclohexyl-β-alanine	$C_9H_{17}NO_2$	Found	63.11, 63.22	10.23, 10.42	8.19, 8.22
			Calculated	63.13	10.01	8.18
B5P131	α-Cyclododecyl-β-alanine Hydrochloride Salt	$C_{15}H_{29}NO_2$•HCl	Found	62.18, 62.00	10.79, 10.64	4.53, 4.50
			Calculated	61.73	10.36	4.80
B5P127	α-(4-*tert*-Butylcyclohexyl)-β-alanine Hydrochloride Salt	$C_{13}H_{25}NO_2$•HCl	Found	59.50, 59.79	10.38, 10.47	5.26, 5.31
			Calculated	59.19	9.93	5.31
B5P107	α-(4-Phenylcyclohexyl)-β-alanine Hydrochloride Salt	$C_{15}H_{21}NO_2$•HCl	Found	63.86, 63.65	7.81, 7.94	4.81, 4.80
			Calculated	63.48	7.81	4.94
B5P91	β-Phenyl-β-alanine Hydrochloride Salt	$C_9H_{11}NO_2$•HCl	Found	54.14, 53.96	6.00, 6.15	7.34, 7.29
			Calculated	53.61	6.00	6.95
B5P95	β-Phenethyl-β-alanine	$C_{11}H_{15}NO_2$	Found	67.97, 67.62	7.87, 7.87	7.14, 7.16
			Calculated	68.37	7.82	7.25
B5P111	β-(*p*-Methylphenethyl)-β-alanine	$C_{12}H_{17}NO_2$•0.33EtOH	Found	68.19, 68.19	8.21, 8.30	6.40, 6.53
			Calculated	68.31	8.59	6.29
B6P145	β-[2-(4-Hydroxy-3-methoxyphenyl)ethyl]-β-alanine Hydrochloride Salt	$C_{12}H_{17}NO_4$•HCl•MeOH	Found	51.03, 50.88	6.88, 7.02	4.22, 4.29
			Calculated	50.72	7.20	4.55

Table 2.20: Rationale for the non-completion of a reaction sequence

Last Successful Chemical Reaction	Substrate	Reason for Discontinuation
Gewald reaction	Methyl 2-amino-4,5,6,7-tetrahydro-6-methylbenzo[b]thiophene-3-carboxylate (B3P135)	Major ethyl ester impurity
Beck reaction	Methyl 3-amino-4-methylbenzo[b]thiophene-2-carboxylate (B3P109)	Incomplete reaction due to low reaction temperature
Fiesselmann reaction	Methyl 3-amino-5-(4-methylphenyl) thiophene-2-carboxylate (B4P91)	B4P91 is identical to another Fiesselmann product, B4P55.
Acetylation	Methyl 2-acetamido-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (B6P75) Methyl 2-acetamido-4,5,6,7-tetrahydro-6-methylthieno[2,3-c]pyridine-3-carboxylate (B6P39)	Major ethyl ester impurity
Ra Ni reductive desulfurization	N-Acetyl-α-(1-ethylpropyl)-β-alanine methyl ester (B6P93) N-Acetyl-α-cyclopentyl-β-alanine ethyl and methyl esters (B6P85) N-Acetyl-α-cycloheptyl-β-alanine ethyl ester (B6P117) N-Acetyl-α-[4-(N-carbethoxypiperidinyl)]-β-alanine methyl ester (B6P105) N-Acetyl-β-[*m*-(ethylamino)phenethyl]-β-alanine methyl ester (B6P101) N-Acetyl-β-hexyl-β-alanine methyl ester (B6P97)	Work is still in progress

Table 2.21: Rationale for reaction failures

Chemical Reaction Attempted	Substrate	Reason for Failure
Beck reaction	4,5-Dimethoxy-2-nitrobenzonitrile	Methoxy groups are deactivating[a]
Gewald reaction	Acetone 4-Heptanone 5-Nonanone 6-Undecanone 2,6-Dimethyl-4-heptanone 1,3-Diphenylacetone Cyclooctanone Cyclodecanone 1,4-Cyclohexanedione mono-ethylene ketal 1-Propyl-4-piperidone 1-Phenethyl-4-piperidone Tropinone 2,2,6,6,-Tetramethyl-4-piperidone	Precursors failed to react under the *prescribed reaction conditions* due to one of the following reasons: • low reactivity • steric hindrance • presence of a protic functionality • aldol side reactions • low reaction temperature • inappropriate reaction solvent
Fiesselmann reaction	2-Isopropyl-4,6-dimethylacetophenone 2,4,6-Trimethoxyacetophenone	Steric hindrance
Fiesselmann reaction	4-Aminoacetophenone	Protic functionality
Acetylation	Methyl 3-amino-4-cyanobenzo[b]thiophene-2-carboxylate (B5P9)	Electron withdrawal by 4-cyano substituent reduced reactivity
Ra Ni reductive desulfurization	Methyl 3-acetamido-5-(3,4-methylenedioxy phenyl)thiophene-2-carboxylate (B6P55)	Partial cleavage of the 3,4-methylenedioxy ring leading to impurity[b]

a. Beck performed the analogous synthesis of methyl 5,6-dimethoxybenzo[b]thiophene-2-carboxylate (16% yield) under much more vigorous conditions (18 hr/100^{0}C vs 0.5 hr/0^{0}C) [162]; b. The thiophene moiety was reduced as expected.

Synthesis of Alkyl β-Aminothiophenecarboxylates

Using established chemistry, the Beck, Gewald and Fiesselmann reactions were used to construct a wide variety of "thiophene-constrained" β-amino acids in a rapid and efficient manner. Chemical analyses and yields were in accordance with literature results. The Beck and Fiesselmann reactions presented no synthetic difficulties however the Gewald reaction experienced two problems.

First, the Gewald reaction worked in only half of the attempts. Under the standard reaction conditions, some α-methylenecarbonyl compounds were not reactive enough. Procedural modification [163] may be used to enhance precursor reactivity by 1) using a different solvent such as DMF or dioxane, 2) raising the reaction temperature to 70-90^{0}C, or 3) completing the reaction in two steps: first, the α-methylenecarbonyl compound undergoes base-catalyzed Knoevanegal condensation with an activated nitrile; second, the resulting alkylidene derivative reacts with sulfur in a separate step. Despite the number of Gewald precursors that failed to react, the Gewald reaction supplied nearly half of the alkyl β-aminothiophenecarboxylate intermediates.

A second problem involved the solvolysis of methyl β-aminothiophene-carboxylates in refluxing EtOH which resulted in partial to total ester exchange (methyl for ethyl). In some cases, syntheses were discontinued due to ethyl ester impurity; in other cases, the impurity was tolerated since the final step in the reaction sequence involves ester deprotection. The use of ethyl cyanoacetate as a Gewald precursor would eliminate the solvolysis problem.

Synthesis of Alkyl β-Acetamidothiophenecarboxylates

β-Aminothiophenecarboxylates readily acetylated, often in high yield, when subjected to acetic anhydride reflux. Electron withdrawing groups (CF_3, CN) on β-aminobenzo[b]thiophenecarboxylates demonstrated the ability to retard or prevent acetylation.

N-Acetyl protection was easily confirmed by standard analytical methods: acetyl hydrogens resonated as a singlet at δ2.20-2.33 for 3-acetamido compounds and at δ 1.76-2.26 for 2-acetamido compounds; IR was characterized by a single amide NH stretch and by two strong carbonyl absorptions; TLC was ninhydrin-insensitive; and in most cases melting points were constant or elevated (by as much as 75^0C) due to an increase in compound lipophilicity promoting charge transfer interactions.

Synthesis of Substituted N-Acetyl-β-alanine Alkyl Esters

In all 17 attempts, thiophene or benzo[b]thiophene moieties in the precursors were reduced in high yield to produce substituted β-alanines with acetamido and ester groups intact. H_α and H_β were identified by ^{1}H NMR (Table 2.22) and IR demonstrated that, with the loss of thiophene aromaticity, conjugation and constraint, ester carbonyl absorbed infrared radiation at a frequency up to 50 cm^{-1} higher while amide carbonyl absorbed at a slightly lower frequency. Reduction of the thiophene ring resulted in a loss of UV absorption.

Table 2.22: Range of chemical shifts for backbone protons on N-acetyl-β-alanine alkyl esters

Substituents	^{1}H NMR δ		Solvent
	H_α	H_β	
α-*sec*-alkyl, α-cycloalkyl[a]	2.44-2.59	3.04-3.62	$CDCl_3$
α-cyclopentyl	3.30	3.09	DMSO-*d6*
β-(arylethyl)	2.53-2.63	4.25-4.31	$CDCl_3$
β-aryl	2.75-2.89	5.25-5.43	$CDCl_3$

a. cyclohexyl or higher

Despite its tedious preparation and pyrophoric nature, Raney nickel demonstrated exceptional utility as a versatile reducing agent. It was inexpensive, not poisoned by sulfur, and allowed the retention or modification of many of the functional groups listed in Table 2.23. Notably, the reduction of the nitro substituent in B6P69 to amino followed by ethylation (see B6P101) confirmed that the N-acylation protection of alkyl β-aminothiophenecarboxylates was justified.

Table 2.23: Summary of the effects of Raney Nickel reduction on functional groups

Functional Groups Transformed[a]	Functional Groups Unaffected
• Carbon halides except fluorides are hydrogenated • Olefins are saturated • Ketones are converted to alcohols • Benzyl is cleaved from heteroatoms • All sulfur functionalities are reduced • Strained heterocycles are broken • Aryl nitrogen groups are reduced to primary amines which may be monoalkylated in alcohol	• Amides • Esters • Carboxylic acids • Aliphatic and aromatic ethers • Hydroxyl • Ketones[b] • Fluorides • Arenes and heteroarenes without S

a. Refer to references 190 and 191; b. No reaction occurs in the presence of lower aliphatic ketones [192].

In all 8 attempts, the simultaneous deprotection of acetyl and ester afforded free β-amino acids in moderate to high yield. Work is in progress to complete the deprotection of the remaining protected β-alanines. Physical analyses confirmed the synthesis of novel β-amino acids: broad OH absorptions appeared in the IR spectra, compounds were ninhydrin-active, and melting points were consistently 60-80^{0}C lower than melting points for α-amino acid analogues.

In general, isolation of β-amino acids as the hydrochloride salt is superior to isolation of the free amino acid because work-up proceeded in higher yield, less physical manipulation was required, the product was often soluble in H_2O or DMSO (the free product usually was not), and the product simply had a greater mass.

Resolution of racemates was not performed.

2.5.1.2 Scope of Reaction

Number of Synthetic Steps and Typical Total Yield

β-Alanines were synthesized in 4 steps with an overall synthetic yield of 16-40% for β-alanines with α-substituents, 9-42% for β-alanines with β-substituents, and 20-42% for β-alanines with β-aryl substituents. While not yet optimized, these yields are comparable to the yields of most other β-alanine protocols, even the 1 step synthesis of β-aryl-β-amino acids using malonic acid.

The protection/deprotection steps decreased the overall yield but were necessary since Ra Ni ethylates amino groups in the presence of EtOH. Conceivably, these protection and deprotection steps could be eliminated if a solvent, perhaps aqueous ammonia, was found compatible with Ra Ni reduction and the dissolution of aminothiophenecarboxylic acids.

Substrates and Substitution Patterns

Since the β-amino acid backbone was constructed from substituted thiophene, the substrates and substitution patterns of β-alanines are dependent on the chemistry of alkyl β-aminothiophenecarboxylates (Table 2.24) which has been extensively reviewed in the 5 volume series "*Thiophene and Its Derivatives*" (John Wiley & Sons, New York, 1985), edited by Salo Gronowitz.

Note that β-alanines derived from Beck, Gewald and Fiesselmann precursors are excluded from α-aryl substitution or any form of disubstitution at C_α or C_β.

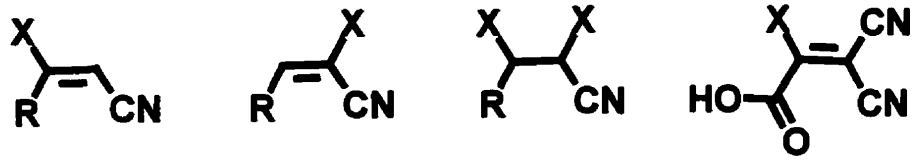
Derivations of the 3 β-aminothiophenecarboxylate syntheses may be used to expand the scope of the β-alanine synthesis. Cyano may replace alkyl ester in the Gewald synthesis since both functional groups are converted to carboxylic acid during acidolysis. Derivatized and/or functionalized thiophenecarboxaldehyde may be used as a Fiesselmann precursor to ultimately produce a β-alkyl-β-amino acid. Other Fiesselmann precursors (Figure 2.7) may be used to expand the spectrum of substituents. Furthermore,

Table 2.24: Substrate parameters and β-alanine substitution effects based on the synthesis of alkyl β-aminothiophenecarboxylates

Name Reaction	Substrate	Restrictions	β-Alanine Substitution Pattern
Beck	*o*-nitrobenzonitriles	• No strong or multiple electron donating groups • No protic functional groups	β-aryl
Gewald	α-methylenecarbonyl compounds	• No substituents β to carbonyl (due to steric hindrance) • No protic functional groups	α-alkyl
Fiesselmann	acetophenones	• No substituents ortho to the acetyl group (due to steric hindrance) • No strong or multiple electron withdrawing groups[a] • No protic functional groups	β-arylethyl
Fiesselmann	arylaldehydes	• No strong or multiple electron withdrawing groups[a] • No protic functional groups	β-arylethyl

a. Reaction may be enhanced by the presence of electron donating groups

α,β-disubstituted β-alanines may be synthesized by reducing alkyl 2,5-substituted-3-acetamidothiophene-4-carboxylate.



X= chloro, bromo; R= alkyl, aryl, NO_2, halo, cyano, H

Figure 2.7: Fiesselmann precursors

Ultimately, the suitability of a substrate is limited by the chemistry of the particular alkyl β-aminothiophencarboxylate synthesis. A second limitation is imposed by the use of Raney nickel: some functional groups may be reduced or cleaved (Table 2.23). Finally, substituents must be stable to acidolysis.

Advantages and Disadvantages

The total synthesis of β-amino acids has the following advantages:

- Readily available, inexpensive substrates and reagents
- High derivatizability; rich in synthetic potential
- Rapid synthesis
- Few steps (4-5); procedurally simple
- Trivial synthetic methodology; syntheses of thiophenes are well documented
- Acceptable yields up to 42% (Note: yield optimization not performed yet)
- Structural diversity unmatched by any other β-amino acid synthesis
- Scalable
- S_8, thiophene and NiS_2 do not produce a stench. This is of particular interest to the synthetic practitioner and co-workers!
- Ability to incorporate deuterium into the β-amino acid backbone

Disadvantages of the synthesis include:

- Strong acidic conditions required for deprotection
- No enantioselectivity
- Thioglycolate produces a stench

Overall, the numerous advantages and few disadvantages of the novel synthesis position it as the premier protocol for the synthesis of β-amino acids. Remarkably, the synthesis is free from nearly *all* of the problems inherent in the literature protocols. More importantly, it has the ability to meet the challenges of antiepileptic drug design. Specifically, the novel synthesis of β-amino acids has been used to synthesize anti-ictogenic/anti-epileptogenic agents.

2.5.1.3 Antiepileptic Activity

Anti-ictogenic Activity

Eight β-alanine analogues, isolated free or as the hydrochloride salt, were subjected to biological tests in 3 seizure models (Table 2.25). No compounds were active in the PTZ model which tests for anti-absence seizure activity. On the other hand, 4 compounds, depicted in Figure 2.8, demonstrated anti-ictogenic activity in either the PIS or MES models which suggest that these compounds may be active against secondary

generalized tonic-clonic and complex partial seizures in humans. One compound, α-(4-phenylcyclohexyl)-β-alanine hydrochloride salt (B5P107), showed strong activity, preventing seizures at an ip dose of 50 mg/kg in rats.

Table 2.25: Anti-ictogenic activity of β-alanine analogues in various seizure models

β-Alanine Analogue	Seizure Model[a]		
	PTZ	PIS	MES
α-Cyclohexyl-β-alanine (B5P119)	0	0	1+
α-Cyclododecyl-β-alanine hydrochloride salt (B5P131)	0	0	0
α-(4-*tert*-Butylcyclohexyl)-β-alanine hydrochloride salt (B5P127)	0	0	0
α-(4-Phenylcyclohexyl)-β-alanine hydrochloride salt (B5P107)	0	0	3+
β-Phenyl-β-alanine hydrochloride salt (B5P91)	0	0	0
β-Phenethyl-β-alanine (B5P95)	0	2+	1+
β-(*p*-Methylphenethyl)-β-alanine (B5P111)	0	0	0
β-[2-(4-Hydroxy-3-methoxyphenyl)ethyl]-β-alanine hydrochloride salt (B6P145)	0	2+	1+

a. test compound is active in > 2/4 rats at 10 mg/kg, 4+; 50 mg/kg, 3+; 100 mg/kg, 2+; 300 mg/kg, 1+; or inactive, 0.

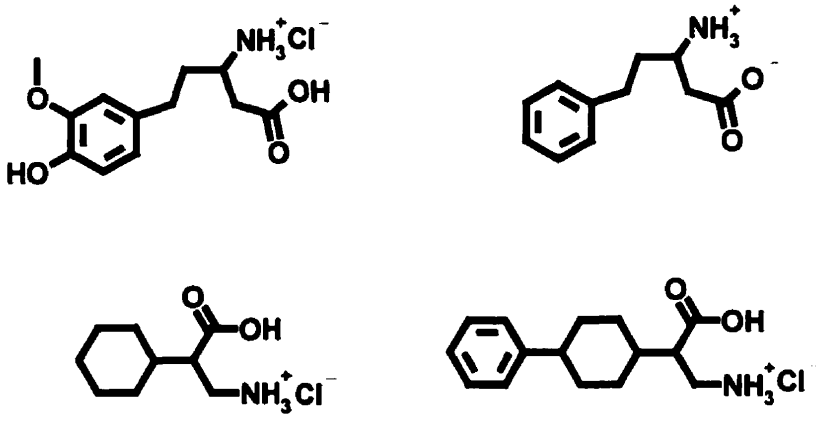
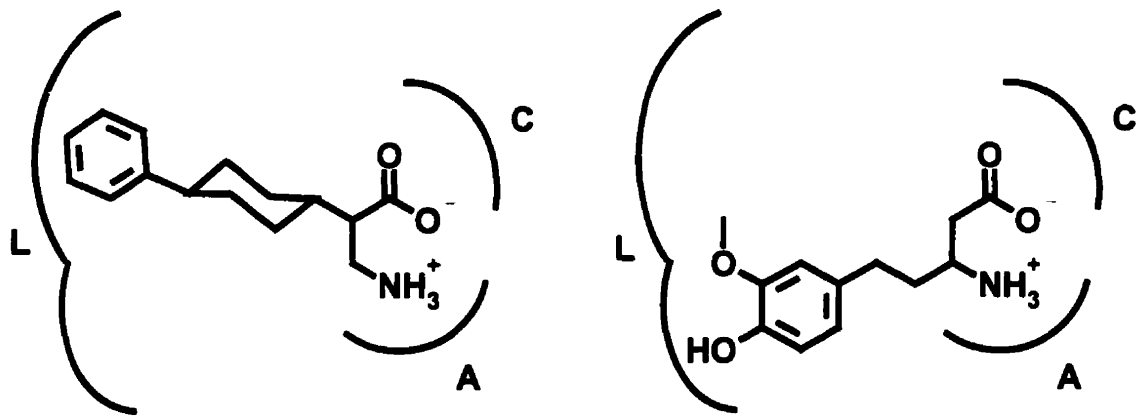


Figure 2.8: Anti-ictogenic β-amino acids

Active β-amino acids are drawn as they would be found in the physiological milieu.

Structure analysis suggests that β-substitution is important in the control of PIS whereas the type of side chain, not the locus of substitution, is important in the control of MES seizures. Size-limited lipophilic side chains appear to be the important structures: 1) cyclohexyl but not cyclododecyl, 2) 4-phenylcyclohexyl but not 4-*t*-butylcyclohexyl (therefore, activity likely comes from π interaction), and 3) phenethyl with a para substituent OH or smaller. A putative receptor site is shown in Figure 2.9. B5P107 is assumed to exist in its low energy conformation with both cyclohexyl substituents equatorial.



A=Amine Acceptor, C=Carboxylate Acceptor, L=Large Hydrophobic Region

Figure 2.9: Putative receptor site for anti-ictogenic β-amino acids

Numerous reasons may account for the inactivity of 4 of the β-alanine analogues: poor receptor fit, short duration of action, poor solubility leading to low plasma levels, poor transport to receptor within the brain, metabolic inactivation, or inability to cross the BBB. Further experimentation is necessary to determine the precise cause of inactivity.

Combined Anti-ictogenic/Antiepileptogenic Activity

β-Alanine (the anti-ictogenic lead compound) and α-(4-phenylcyclohexyl)-β-alanine • HCl (B5P107; the β-amino acid with the greatest anti-ictogenic activity) were tested for hybrid anti-ictogenic/antiepileptogenic activity in a variant of the Spontaneous Recurrent Seizure (SRS) model of epilepsy (See Appendix B). Pilocarpine chemoconvulsant produced spontaneous recurrent seizures in 94% of control rats, in 37% of rats on β-alanine and in only 20% of rats on B5P107. Both β-amino acids demonstrated significant antiepileptogenic activity. Given the anti-ictogenic properties of the two β-amino acids, these findings represent the inaugural discovery of hybrid anti-ictogenic/antiepileptogenic compounds. The goal of antiepileptic drug design based on neurotransmitter mediation has been achieved.

Anti-ictogenic/antiepileptogenic testing of other β-alanine analogues is still in progress.

Selected Issues

Optical Resolution

The US Food and Drug Administration allows drug companies to choose whether to develop chiral drugs as racemates or as single enantiomers; however, the use of asymmetric syntheses is encouraged because one racemate may be inactive, marginally active or even toxic [193,194]. Consequently, the percentage of all therapeutic agents

marketed and administered as isomeric mixtures in the U.S. has fallen to 25% [195]. Since the Ra Ni synthesis of β-amino acids produced racemates, the question of whether enantiomeric resolution is necessary needs to be addressed.

To test individual enantiomers of β-amino acids for biological activity, racemic β-amino acids must be resolved in a fifth step. However, in the early stages of a research project where high throughput is required, any additional synthetic or procedural manipulation is inefficient and costly in terms of time and resources. Therefore, rapid, high efficiency, cost-effective racemic syntheses are performed first then, depending on compound activity, optical resolutions may be performed. Table 2.26 presents the spectrum of antiepileptic activity and toxicity that may be found in a racemic mixture of β-amino acids. Resolution of racemates is warranted only if both antiepileptic activity and toxicity is present. The toxic enantiomer must be identified. If the biologically active enantiomer is non-toxic, it remains a candidate for therapeutic use.

Table 2.26: Resolution of racemates based on permutations of antiepileptic activity and toxicity

#	R Enantiomer		S Enantiomer		Resolve?	#	R Enantiomer		S Enantiomer		Resolve?
1	I	T	I	T	No	9	I	T	A	T	Yes
2	I	N	I	T	No	10	I	N	A	T	Yes
3	I	T	I	N	No	11	I	T	A	N	Yes
4	I	N	I	N	No	12	I	N	A	N	No
5	A	T	I	T	Yes	13	A	T	A	T	Yes
6	A	N	I	T	Yes	14	A	N	A	T	Yes
7	A	T	I	N	Yes	15	A	T	A	N	Yes
8	A	N	I	N	No	16	A	N	A	N	No

A: Antiepileptic activity; I: Antiepileptic inactivity; T: Toxic activity; N: Non-toxic

Current strategies for optical resolution [196] utilize 2 methods: 1) the conversion of racemates into diastereomeric salts with subsequent separation and liberation of the separated enantiomers, and 2) chromatographic separation using a chiral stationary phase. Both methods have been used to resolve DL-β-amino acids. For example, the racemates (±)-β-(2-furyl)-β-alanine and (±)-β-(2-thienyl)-β-alanine were successfully resolved through selective crystallization by treating the N-benzyloxycarbonyl derivatives with quinine in ethyl acetate or ethanol [197]. The enantiomers of both α- and β-substituted β-alanines, derivatized as alkyl esters, were chromatographically separated using silica-bonded chiral stationary phases [198]. Davankov *et al.* reported a method for the preparative enantiomeric separation of β-amino acids [199] and Yamazaki *et al.* reported the direct enantiomeric separation of β-amino acids (including β-phenyl-β-alanine) by ligand-exchange chromatography [200]; octadecylsilanized silica coated with N-*n*-dodecyl-L-hydroxyproline served as the stationary phase, acetate buffer containing Cu(II) served as the mobile phase.

One of the two methods will be applied for the chiral separation of β-alanine analogues where hybrid anti-ictogenic/antiepileptogenic activity and toxicity are simultaneously indicated.

Ligand Binding Studies

In theory, β-alanine analogues possess hybrid anti-ictogenic/antiepileptogenic activity due to the blockade of GABA uptake glycoproteins and the antagonism of

NMDA glycine co-agonist receptor sites. To confirm to a high degree of probability the role of these receptors in the mechanism of β-amino acid antiepileptogenics (and β-alanine's capacity to bind), competitive receptor binding studies using radiolabelled glycine and radiolabelled GABA will be performed.

Ligand binding studies may determine whether β-alanine has its own unique receptor site or if it binds only to GABA and glycine receptors.

2.5.1.4 Ancillary Applications

β-Amino acids show potential to be therapeutic agents for neurological conditions which are caused, in part, by abnormal synaptic concentrations of inhibitory and excitatory neurotransmitters. Neurodegeneration associated with epilepsy, stroke [201], AIDS dementia [202], psychosis [203,204], Huntington's chorea [205,206], Alzheimer's disease [205,206,207] and head trauma [208,209,210] has been linked to excitotoxicity arising from excessive stimulation of NMDA receptors. β-Alanine, acting at the glycine co-agonist site, antagonizes the NMDA receptor and may prevent or restrict neurodegeneration. Dysfunctional GABAergic processes have been associated with Parkinson's disease [211,212], Huntington's chorea [213], anxiety [214], pain [214] and some forms of schizophrenia [215]. β-Alanine and its analogues may mitigate these conditions by elevating synaptic GABA concentration thereby promoting inhibition. β-Alanine's combined anti-glutamatergic/pro-GABAergic action in concert with its

ability to cross the BBB uniquely positions β-amino acids as putative therapeutic agents for numerous neurological conditions.

The therapeutic potential of β-amino acids may be extended further. β-Alanine promotes nucleic acid and collagen synthesis in wound healing [216] and, when used as an adjuvant treatment, improved physical comfort, improved immunocompetency and increased the survival rate of patients with squamous cell carcinoma [217]. β-Alanine analogues also find utility in the preparation of therapeutically enhanced peptidomimetics [218,219] and as precursors of β-lactams [220a-i] which may be used in the preparation of antibiotics.

Finally, other applications include the use of β-amino acids in peptide combinatorial libraries and the use of β-alanine analogues to perform mammalian β-amino acid metabolism studies.

2.5.2 Conclusions

With intent to break away from traditional anticonvulsant research and to focus on the important target of epileptogenesis, the goal of this research program was the inaugural design and synthesis of a small molecule with combined anticonvulsant/anti-epileptogenic properties. This has been accomplished. β-Alanine was identified as a prototype compound with the ability to simultaneously (i) inhibit excitatory processes via antagonism of the NMDA receptor at the glycine site and (ii) stimulate inhibitory processes via blockade of glial GABA uptake. Consequently, a novel and efficient

synthesis of β-amino acids was devised, patented and used to produce the first β-amino acids evaluated in a model of anti-ictogenic/antiepileptogenic activity. Notably, 4 of 8 β-amino acids possessed anti-ictogenic activity in the MES/PIS models of epilepsy and 2 of 2 compounds (β-alanine and α-(4-phenylcyclohexyl)-β-alanine hydrochloride salt (B5P107)) possessed antiepileptogenic activity in the SRS model of epileptogenesis. B5P107 was found to be the most efficacious anti-ictogenic β-amino acid and it also demonstrated significant antiepileptogenic activity. It constitutes the most promising lead compound for further antiepileptic research.

These results are very promising. By applying the techniques of rational drug design (subject to the constraints imposed by numerous biological and commercial criteria), novel neurotransmitter analogues were synthesized using a novel synthetic protocol and were shown to possess novel biological activity in a novel animal epilepsy model. For the first time, molecules with hybrid anti-ictogenic/antiepileptogenic activity have been identified. In concert with the novel synthesis, these discoveries represent a significant advance in antiepileptic drug design. With development, a β-amino acid antiepileptic molecule has the potential to clinically surpass conventional "antiepileptic" agents — drugs which are anticonvulsant but not antiepileptogenic, drugs which treat symptoms but do not deter the development of epileptogenic lesions. The discovery of truly curative antiepileptic agents has become a legitimate possibility; further research and development in this area is imperative.

Development

Further research and development of β-amino acids will concentrate on the following areas:

1) The novel synthesis of β-amino acids using Ra Ni will be optimized. Efforts will be made to eliminate the protection/deprotection steps and to explore the use of Ni-Al alloy in aqueous alkali solution.
2) More β-alanines (α-, β- and α,β- substituted) will be synthesized and biologically tested.
3) Deuteriated β-amino acids will be synthesized and used to acquire receptor binding data.
4) Selected β-amino acids, inactive after ip administration, will be tested icv to ascertain the influence of the BBB upon bioactivity.
5) Substituted N-acetyl-β-alanine alkyl esters will be tested for biological activity to identify whether uptake occurs by shuttle or diffusion.
6) β-Amino acids will be evaluated in a bovine model of the β-shuttle.
7) The structural requirements of the β-shuttle will be elucidated.
8) A toxicity profile for β-alanine analogues will be compiled.

Another key area in this research program is the design of β-amino acid analogues to optimize anti-ictogenic/antiepileptogenic activity. Preliminary research in this area is the focus of the next section.

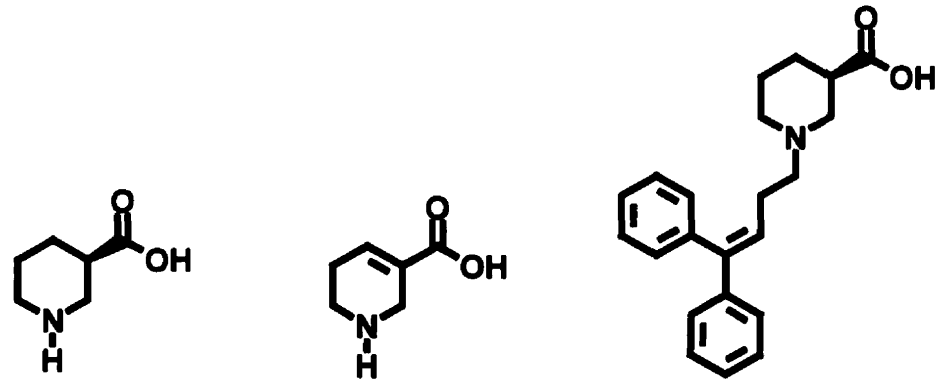
2.6 Design of a β-Amino Acid Series to Optimize Anti-ictogenic / Antiepileptogenic Activity

The previous section reported the antiepileptic activity of β-amino acids. This section reports the use of molecular design to increase the affinity of β-amino acids to target receptors with the goal of maximizing anti-ictogenic/antiepileptogenic activity. The structures of the GABA uptake receptor and the NMDA glycine co-agonist site will be used to design β-alanine analogues with optimum NMDA antagonist and GABA agonist properties.

In vivo and *in vitro* structure-activity data of GABA uptake inhibitors (Figure 2.10) has been used by Murali Dhar *et al.* [221] and N'Goka *et al.* [222] (with molecular modelling) to construct general pharmacophore models of GABA uptake inhibition. Their results distill into a pharmacophore model (Figure 2.11A) with the following parameters:

- An amine is 4.1-4.3 Å from the centre of a carboxylic acid.
- A lipophilic, aromatic region is located 9.5-10.5 Å from the carboxylate centre.

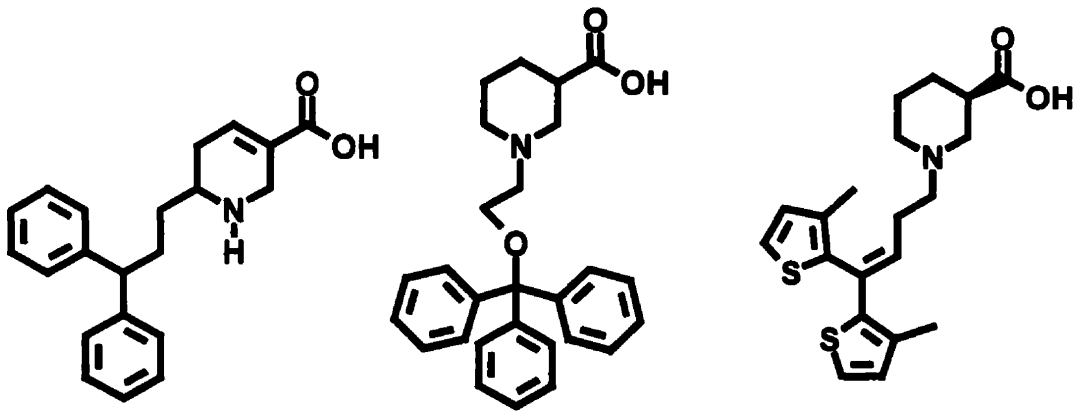
- A functionality bearing 2 p doublets (double bond or an oxygen atom) is located 3/4 of the distance between the centre of the amine to carboxylate link and the lipophilic region.
- Secondary amino substitution negates *in vivo* activity.



a. (R)-Nipecotic acid IC_{50} = 1.7 μM

b. Guvacine IC_{50} = 4.9 μM

c. SKF-89976A IC_{50} = 0.11 μM

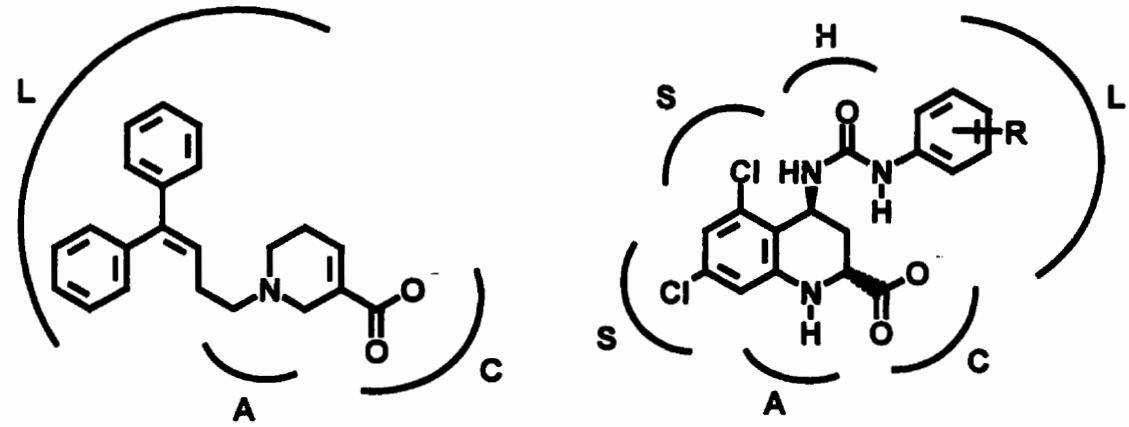


d. 6-(3,3-Diphenyl-propyl)guvacine IC_{50} = 0.1 μM

e. (±)-1-[2-(Triphenylmethoxy) ethyl]-3-piperidine-carboxylic acid IC_{50} = 1.4 μM

f. Tiagabine IC_{50} = 0.07 μM

Figure 2.10: GABA uptake inhibitors



A. GABA-Uptake Inhibitor Receptor **B. NMDA Glycine Co-agonist Site**

A=Amine Acceptor; C=Carboxylate Acceptor; L=Large Hydrophobic Region; S=Small Hydrophobic Region; H=Hydrogen Bonding

Figure 2.11: Models of general receptor sites

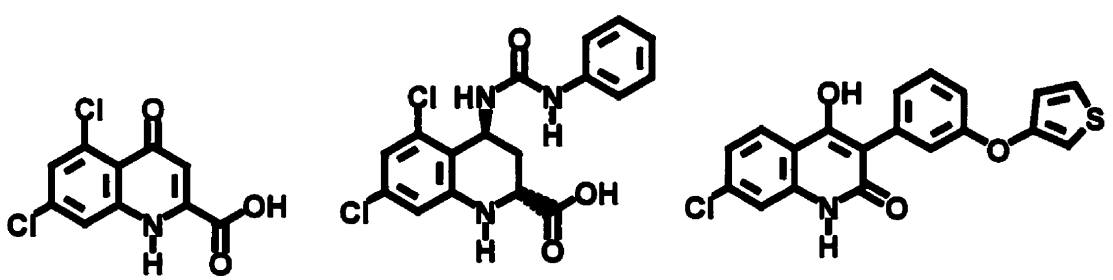
Having established parameters to aid the design of β-alanine analogues with optimum GABA uptake inhibition, the next step is the optimization of β-alanine NMDA antagonism. β-Alanine binds to both the strychnine sensitive glycine receptor and the strychnine insensitive NMDA glycine co-agonist site. Binding to the NMDA site will be optimized since this is the receptor central to ictogenesis and epileptogenesis. Leeson [54] studied structure-activity data of kynurenic acid analogues (Figure 2.12) with NMDA glycine site antagonism activity and formulated a model receptor site. The general NMDA glycine site pharmacophore (Figure 2.11B) had the following features:

- An amine is 3.4 Å from the centre of a carboxylate group.
- Two small size limited hydrophobic regions are located (i) 4.4 Å from the amine and 7.5 Å from the acid group and (ii) 4.4 Å from the amine and 6.8 Å

from the acid group; potency increases as substituents range through H, CH_3 and Cl.

- A hydrogen bond acceptor is located on the periphery of one of the small hydrophobic regions, 5.8 Å from the amine and 7.3 Å from the carboxylate.
- The centre of a large hydrophobic region is located 4.8 Å from the hydrogen bond aceptor and and 7.2 Å from the carboxylate, opposite the small size limited hydrophobic regions; considerable bulk is tolerated without loss of affinity.
- Secondary amino substitution negates *in vivo* activity unless the α-amino acid function is replaced with a δ-lactam function.

Using parameters and features from both putative pharmacophores, β-alanine analogues capable of interacting with both of these receptor sites (Figure 2.13) have been

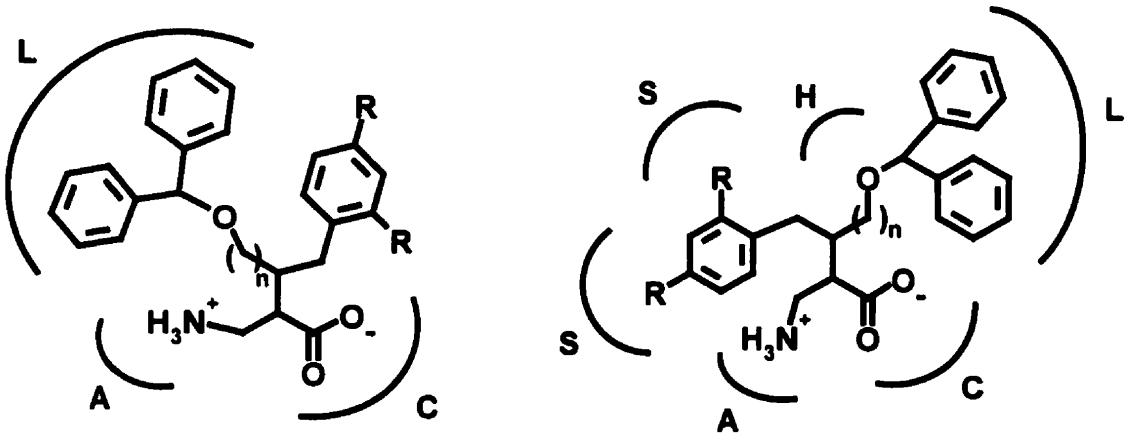


a. 5,7-Dichloro-kynurenic acid
$IC_{50} = 0.20\ \mu M$

b. 5,7-Dichloro-4-(phenylureido)-2-carboxytetrahydroquinoline
$IC_{50} = 0.0078\ \mu M$

c. 7-Chloro-4-hydroxy-3-[3'-(3-thienyloxy)phenyl]-2-quinolone
$IC_{50} = 0.0014\ \mu M$

Figure 2.12: NMDA co-agonist glycine site antagonists



A. GABA-Uptake Inhibitor Receptor **B. NMDA Glycine Co-agonist Site**

A=Amine Acceptor; C=Carboxylate Acceptor; L=Large Hydrophobic Region; S=Small Hydrophobic Region; H=Hydrogen Bonding; R=H, F, CH_3; n=2-4

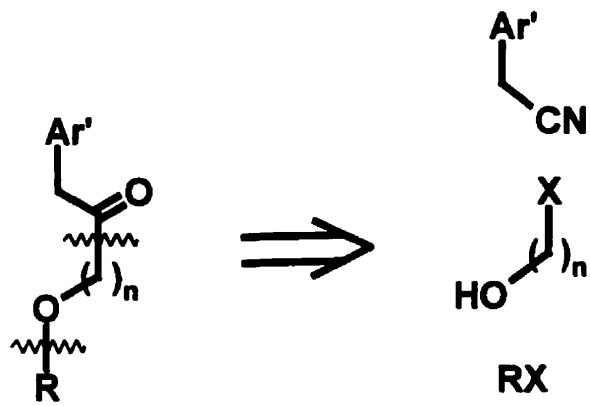
Figure 2.13: Optimized β-amino acid derivatives with putative anti-glutamatergic and pro-GABAergic activity

designed. A seven step synthesis for these analogues is shown in Scheme 2.9.

The acid catalyzed condensation of ω-bromoalcohol and benzyl alcohol using a Dean Stark trap yields benzyl ω-bromoalkyl ether (an alkyl halide). The alkyl halide is converted to a Grignard reagent in anhydrous diethyl ether. Arylacetonitrile is slowly added to the Grignard solution. Addition of 1 M HCl precipitates arylmethyl ω-benzyloxyalkyl ketone, a Gewald synthon. Reaction of the synthon with ethyl cyanoacetate and elemental sulfur in the presence of base produces ethyl 2-amino-4-arylmethyl-5-(ω-benzyloxyalkyl)thiophene-3-carboxylate. The substituted thiophene is protected with BOC-ON then subjected to Raney nickel reductive desulfurization to produce N-butoxycarbonyl-α-(1-arylmethyl-ω-hydroxyalkyl)-β-alanine ethyl ester. Saponification followed by the addition of 2 equivalents of the highly reactive

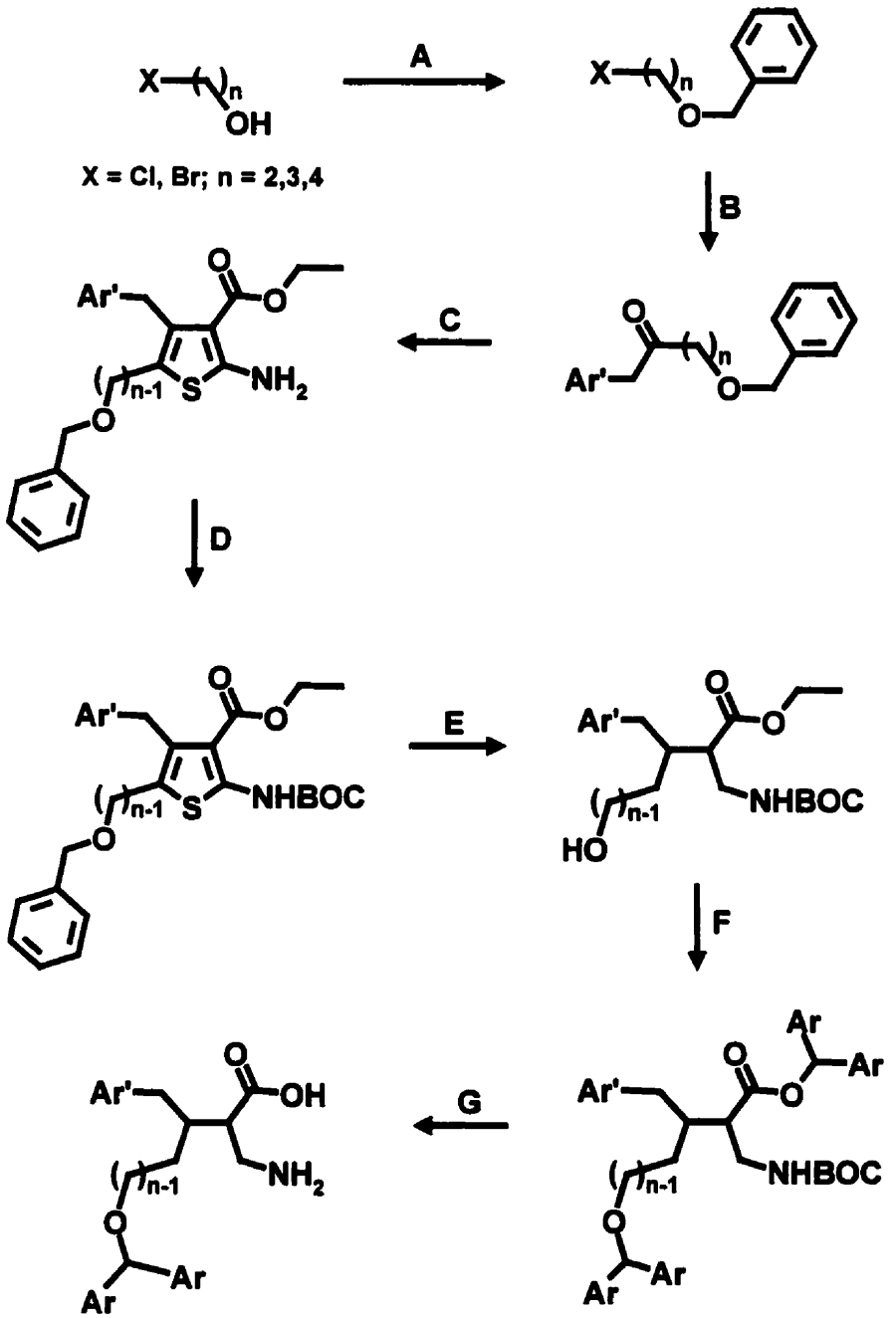
diarylmethylbromide leads to simultaneous esterification and etheration. Selective deprotection using formic acid [223,224,225] yields α-[1-arylmethyl-ω-(diaryl-methoxy)alkyl]-β-alanine.

This synthesis was designed using a SYNTHON retrosynthetic strategy (Figure 2.14). A three segment disconnection of the ketone synthon leads to variability in the diaryl, arylmethyl and alkyl linkages enabling numerous analogues to be readily prepared. These compounds will be evaluated orally, iv and icv for anti-ictogenic/antiepileptogenic activity in animal models.



X = Cl, Br; R = Bzl-, Ar_2CH-; n = 2-4

Figure 2.14: Synthons for the synthesis of α-substituted β-amino acid derivatives with anti-glutamatergic and pro-GABAergic activity



A) BzlOH, $PhCH_3$, p-TsOH, Δ; B) 1. Mg, Et_2O, 2. Ar'CH_2CN, 3. H_3O^+; C) EtO_2CCH_2CN, Et_3N, S_8, EtOH, Δ; D) BOC-ON, Et_3N, dioxane/H_2O; E) Raney Nickel, EtOH, Δ; F) 1. NaOH, 2. Ar_2CHBr (2 equiv.); G) Formic acid.

Scheme 2.9: Synthesis of α-[1-arylmethyl-ω-(diarylmethoxy)alkyl]-β-alanine

CHAPTER 3

RATIONAL DESIGN AND SYNTHESIS OF ANTIEPILEPTIC DRUGS BASED ON NEURAL METAL MEDIATED INHIBITION

3.1 INTRODUCTION

Combined anti-ictogenic/antiepileptogenic activity has surpassed mere anti-seizure activity as the design goal for the next generation of efficacious antiepileptic agents. At least two mechanism-based strategies may afford antiepileptogenic protection. As reported in Chapter 2, β-amino acids were used to simultaneously antagonize glycine-assisted NMDA excitation and glial GABA uptake. In contrast to this "receptor antagonism" approach, the neuronal concentrations of excitatory and inhibitory neurotransmitters may be manipulated in favour of inhibition by targetting the zinc metalloenzymes which mediate neurotransmitter synthesis and degradation. Thus the zinc cation represents an alternative, important target for antiepileptogenic drug design. Additionally, the well known anticonvulsant activity of Cu(II) complexes positions the copper cation as an important target for anti-ictogenic drug design. Since combined anti-ictogenic/antiepileptogenic activity is required in a useful antiepileptic drug, the goal of the research presented in this chapter is the design and synthesis of an antiepileptic agent which simultaneously manipulates neural Cu(II) and Zn(II) concentrations as a mechanism of therapeutic action. Since rational drug design is preceded by an understanding of the relevant biochemical mechanisms, the first step in this research program is to examine the relationship between epilepsy and the transition metals, Zn(II) and Cu(II).

3.2 Role of Zn(II) and Cu(II) in Epilepsy

Zn(II)

As a catalytic, structural or regulatory co-factor in more than 200 metalloenzymes, zinc cation is an essential requirement for the normal development, operation and regulation of the human brain. When Zn(II) concentrations in the CNS are disturbed, Zn(II)-dependent brain processes are compromised and neurodegenerative disorders may occur. In particular, altered Zn(II) levels may contribute to epileptogenesis and convulsions.

Zinc's role in the pathogenesis of epilepsy is supported by at least six neurophysiological studies: 1) Zn(II) is concentrated in the nerve terminal boutons of mossy-fibre axons in the hippocampus [226], a seizure-prone area of the brain; 2) electrical stimulation of the mossy-fibre axons selectively facilitates Zn(II) uptake [69] and release [67,69]; 3) dense-core zinc-rich vesicles undergo exocytosis during epileptic seizures [227,228,229]; 4) Zn(II) affects the metabolism of neuro-active amino acids [66]; 5) Zn(II) modulates inhibitory and excitatory amino acid receptor ion channels [230]; and 6) brain zinc concentration is elevated in audiogenic mice [231]. Zn(II) appears to be a vital component of the cellular machinery appropriated by the epileptic process.

Pharmacological evidence further establishes the link between Zn(II) and epilepsy. Mice maintained on zinc deficient diets displayed protection against electrically

induced seizures [232] whereas direct administration of Zn(II) salts to the brain has induced convulsions in a variety of animal models [233,234,235]. Systemic zinc loading facilitated [232] or inhibited [236] seizure activity although inhibition has been disputed by some researchers [237,238]. Zinc loading iv or ip at 100 mg/kg has also had no effect on seizure activity [239] although this may be due to case specific instances in which Zn(II) was unable to cross the BBB.

Numerous zinc chelators display anticonvulsant properties: D-penicillamine, administered orally, protects seizure-prone baboons against photic-induced convulsions [240]; diphenylthiocarbazone decreases mossy fibre excitatory transmission [241]; and cycloserine's anticonvulsant activity is due, in part, to its ability to chelate Zn(II) [242].

Taken together, the neurophysiological and pharmacological evidence suggests that, while Zn(II) is necessary for brain function, an excess has a predominantly proconvulsant action on the CNS. Furthermore, epilepsy causes and is caused by altered Zn(II) levels in the brain. This implies that Zn(II) is a source of ictogenesis and perhaps epileptogenesis.

The biochemical basis for Zn(II)'s proconvulsant/epileptogenic activity have emerged from morphological and biochemical investigations which illustrate the neurological effects of altered zinc metabolism [243,244].

a) Effect on Neurotransmitter Metabolism

Zn(II) influences the aetiology and pathogenesis of epilepsy through the modulation of glutamine synthetase, glutamate dehydrogenase, glutamate decarboxylase (GAD) and GABA-glutamate transaminase — four essential enzymes involved in the metabolism of inhibitory and excitatory amino acids (Figure 3.1).

Zn(II) significantly inhibits the action of glutamine synthetase [66], the enzyme which catalytically converts L-glutamate to L-glutamine. Zn(II) also regulates the conversion of α-ketogluturate to L-glutamate by glutamate dehydrogenase [245]. In both

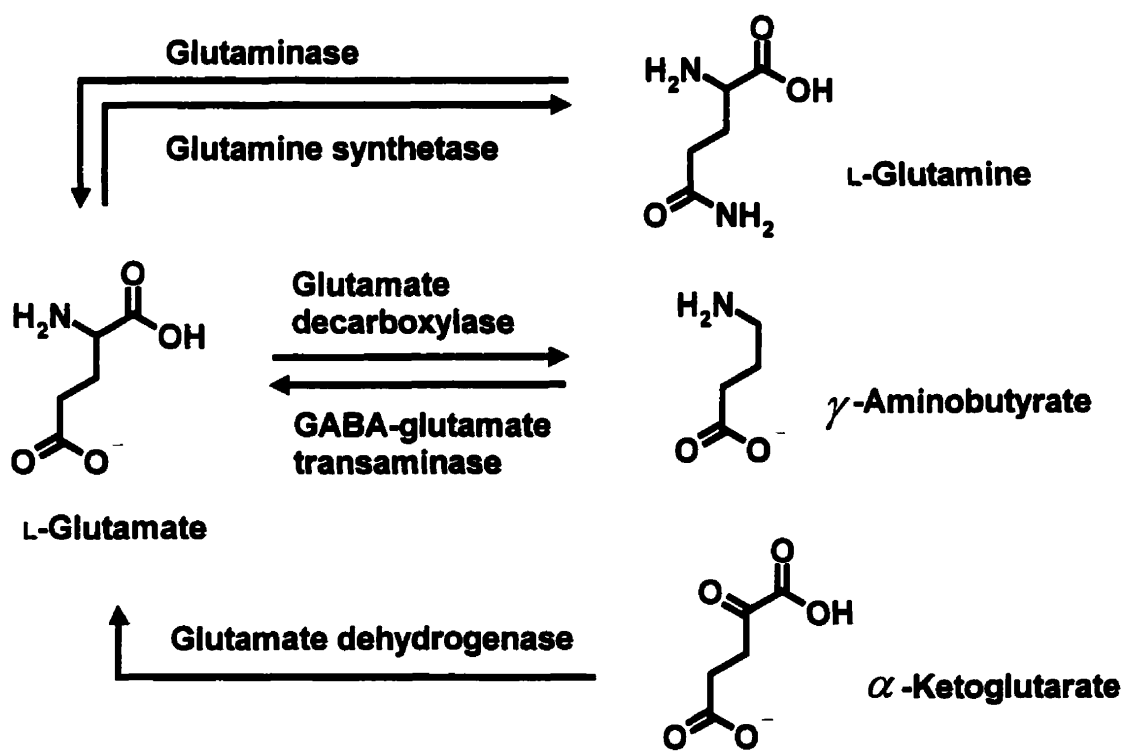


Figure 3.1: Biosynthetic pathways of L-glutamate and GABA in the CNS

cases, high Zn(II) concentrations lead to elevated levels of L-glutamate, an excitatory neurotransmitter which in high concentrations is convulsant.

GAD catalyzes the conversion of L-glutamate to GABA. Studies have shown that GAD's activity is inversely dependent on the concentration of Zn(II) [246,247]; large concentrations of Zn(II) inhibit the production of GABA and result in an accumulation of L-glutamate [248]. Conversely, the activity of GABA-glutamate transaminase, the enzyme which catalyzes the reverse reaction, is enhanced by excessive levels of Zn(II) [249]. Again, the net result is that high Zn(II) levels give rise to high L-glutamate concentrations.

Zn(II) affects L-glutamate levels in another way. Zn(II) activates pyridoxal phosphokinase [250], the enzyme which converts pyridoxal to pyridoxal-5'-phosphate (PLP). In turn, PLP functions as an essential coenzyme which critically regulates the activity of GABA-glutamate transaminase [247] and, by extension, the synthesis of L-glutamate from GABA. Zn(II)'s role in this 3 step biosynthetic pathway is that the activity of pyridoxal phosphokinase is enhanced by Zn(II) by as much as 50% [247]. This shifts reaction equilibria in the pathway and ultimately results in an accumulation of L-glutamate and a depletion of GABA [251,252]. Consequently, the brain is rendered more susceptible to epileptogenic activity.

In summary, the action of high Zn(II) levels on the essential enzymes involved in the metabolism of L-glutamate and GABA produces high L-glutamate levels and low GABA levels. As a result, the inhibitory potential of synaptic transmission is impaired and the excitatory potential is enhanced. Epileptogenesis is more likely to occur.

b) Effect in Extracellular Space

Zn(II) is released into the extracellular space when hippocampal mossy fibres undergo electrical stimulation [67] and when dense-core zinc-rich vesicles undergo exocytosis during epileptic seizures [229]. Elevated extracellular Zn(II) levels retard the binding of GABA [253] and neuroactive amino acids [66] on the postsynaptic membrane and significantly enhance the release of neuroexcitatory amino acids from the presynaptic terminals. This may account for the prolongation of the excitatory postsynaptic potential [70] and the increase in the spontaneous firing rate [254] in brain tissue placed in a Zn(II) bath. The effect of elevated extracellular Zn(II) concentration is a reduction in GABA function and an increase in L-glutamate concentration in the synapse; epileptogenesis may be the result.

c) Effect on Sodium-Potassium Pump

Energy dependent ion pumps, Na^+-K^+-adenosine triphosphatase (Na^+-K^+-ATPase), function to maintain large ionic gradients across the surface of neuronal membranes such that high concentrations of sodium ions and low concentrations of potassium ions exist extracellularly relative to the intracellular fluid. These gradients render nerve cells electrically excitable. High Zn(II) concentration interferes with these gradients by significantly decreasing regional Na^+-K^+-ATPase [233], especially in the hypothalamus and hippocampus (a seizure prone area of the brain) [234]. This is highly

relevant to the seizure-prone state since the disruption of mechanisms which maintain correct intraneuronal Na^+ and K^+ ratios is known to be associated with neuronal depolarization and paroxysmal discharges [255]. A complementary study by Zuckerman and Glaser demonstrated that administration of single shocks to the hippocampus during a period of high potassium application causes convulsions [256]. Thus, the evidence suggests that high Zn(II) levels contribute to ictogenesis by way of action on the sodium-potassium pump.

In summary, the effect of altered Zn(II) metabolism — on neurotransmitter metabolism, in the extracellular space and on the sodium-potassium pump — clearly demonstrates that zinc, in excess, interferes with normal synaptic transmission and possibly facilitates the development of epileptogenic activity. Accordingly, the control of Zn(II) concentration is a rational approach to the control of ictogenic/epileptogenic activity.

Cu(II)

Copper(II), a metal cation essential for neurogenesis and proper brain function, has been implicated in the aetiology and neuropathology of seizures. For example, seizures occur in Cu(II) deficient animals: quaking mice and mottled mice exhibit tremors and neural degeneration due to genetic Cu(II) deficiency [257]; deliberate dietary Cu(II) deficiency produces convulsions and neural degeneration in rats [257]. Following

treatment with seizure-inducing drugs, the copper level decreases concomitantly with seizures [258,259]; complexing agents which produce tremors reduce brain copper levels [260]. Low Cu(II) levels appear to be convulsant.

This Cu(II) hypothesis is supported by additional human evidence. *Post-mortem* samples of the brain tissue from epileptic patients have markedly depressed levels of copper [261]. Children with severe copper deficiency due to inadequate intake or Menkes' Syndrome (characterized by markedly decreased brain Cu(II) levels) suffer convulsions. These observations suggest that "impaired copper metabolism in the CNS may lead to the seizure state" [262] and prolonged reduction of some copper-dependent process may be relevant to the chronic epileptic state.

The influence of Cu(II) concentration on seizure activity may be tied to Cu(II)'s effect on the activity of dopamine-β-hydroxylase, an enzyme which catalyzes the hydroxylation of dopamine to produce norepinephrine — a neural hormone central to synaptic transmission. Studies show that low Cu(II) levels yield low norepinephrine levels [263] and seizures occur with the depletion of norepinephrine synthesis in the brain [264,265,266,267,268,269,270]. Thus, an increase in brain Cu(II) concentration would elevate norepinephrine synthesis which would subsequently act to impede ictogenesis.

The neural concentration of Cu(II) may be raised by the systemic administration of Cu(II). It crosses the BBB. For example, Chutkow demonstrated that inorganic copper injected into the carotid artery crosses the BBB within 15 seconds; coadministration (and complexation) with amino acids accelerates Cu(II) uptake [271]. This shows that Cu(II)

complexes may be used to supplement Cu(II) to Cu(II)-deficient test subjects to alleviate seizures.

This has precedent. Copper complexes of salicylates, acylsalicylates and amino acids demonstrate anticonvulsant activity [257]. Remarkably, the free ligands possess no activity and, in the cases of salicylate and acetylsalicylate (aspirin), actually cause convulsions at high doses. Clearly, Cu(II) has anticonvulsant properties.

Moreover, Cu(II) enhances the activity of known anticonvulsants. For example, the Cu(II) complex of phenytoin has a time of peak effect of 4 hours, 3 hours longer than the time of peak effect for phenytoin alone. Cu(II) may also moderate toxic effects. The copper complex of amobarbital is free of the hypnotic side-effects associated with the free ligand [257].

In conclusion, Cu(II) is anticonvulsant and crosses the BBB. Thus, the manipulation of Cu(II) concentration in the brain emerges as a potential rational approach to anti-ictogenic drug design.

3.3 Design and Synthesis of Zn(II)/Cu(II) Chelating Agents as Antiepileptic Drugs

3.3.1 Introduction

The goal of neural metal mediated antiepileptic drug research is the design and synthesis of an efficacious, non-toxic, anti-ictogenic/antiepileptogenic drug which

influences Zn(II) and Cu(II) concentration and function in the brain to affect epilepsy therapeutically. The role of Zn(II) and Cu(II) in the pathogenesis of epilepsy (presented in the previous section) supports the hypothesis that high Zn(II) concentrations are both proconvulsant and epileptogenic whereas low Cu(II) levels are ictogenic. Thus, it is reasonable to assume that to suppress seizures and the development of epilepsy, excess Zn(II) must be removed from the brain concomitant with Cu(II) supplementation. This has prompted research into the design and synthesis of a class of Cu(II) coordination compounds capable of translocating Cu(II) across the BBB, releasing it, then chelating Zn(II) for its removal from the CNS.

3.3.2 Design Strategy

A six step strategy was used to rationally design and synthesize antiepileptic drugs based on neural metal mediated inhibition.

Step 1: The relationship between Cu(II), Zn(II) and the widely prescribed, traditional anticonvulsant phenytoin was examined to assess whether it is reasonable to postulate that phenytoin's mechanism of therapeutic action involves neural metal mediated inhibition.

Step 2: A preliminary study on the biological effects of miscellaneous ligands and their transition metal complexes was performed: i) to verify hypotheses and literature data concerning the seizure-related effects of systemically administered ligands and

complexes, and ii) to obtain insight to direct the design process in terms of molecule class.

Step 3: An antiepileptic class of molecules (i.e. acyclic versus cyclic, peptide versus carbohydrate) was selected subject to the constraints of biological and commercial criteria (Table 1.8, Table 1.9).

Step 4: Structure refinement was performed to enhance and perhaps optimize desired antiepileptic properties in a molecule class.

Step 5: A facile and economically efficient synthesis of putative antiepileptic compounds was devised.

Step 6: All compounds were evaluated using *in vitro* and *in vivo* biological tests.

3.3.3 Evaluation of Cu(II) and Zn(II) Complexes of Phenytoin

3.3.3.1 Introduction

In theory, molecules which introduce Cu(II) into the brain are anti-ictogenic and molecules which remove Zn(II) are anti-ictogenic and possibly antiepileptogenic. Given these presuppositions, the aim of antiepileptic research based on neural metal mediated inhibition is the discovery of molecules which alter Zn(II) and Cu(II) levels in the brain as a mechanism of anti-ictogenic and/or antiepileptogenic action. As a first step to this end, a molecule with known anticonvulsant activity — phenytoin — was selected and evaluated to determine whether coordination occurs with Cu(II) and Zn(II) and, if so,

whether the interaction supports the presuppositions. Phenytoin (PHT) was selected for study because 1) it is a widely prescribed, traditional anticonvulsant, 2) its structural and biological properties have been extensively studied, and 3) PHT's "hydantoin" [imidazoline-2,4-dione] ring contains two amide functionalities which provide two chelatable electronegative atoms, N and O, in close proximity on three faces of the heterocycle.

3.3.3.2 Evaluation of the Phenytoin-Cu(II) Chelate

A literature search revealed that the Cu(II) complex of phenytoin, $Cu[(NH_3)_2PHT_2]$, has been synthesized and characterized by X-ray crystallography [272]. Dipositive copper resides in a distorted tetrahedral 4-N coordination environment and coordinates to PHT through deprotonated N3. Biological testing of the Cu(II) complex has shown prolonged anticonvulsant activity [257]. Phenytoin has a time of peak effect for 1 hour whereas the Cu(II) complex has a time of peak effect of 4 hours. This is consistent with the hypothesis that molecules which introduce Cu(II) into the brain are anti-ictogenic.

3.3.3.3 Evaluation of the Phenytoin-Zn(II) Chelate

PHT-Zn(II) complexation has been reported [273,274] however characterization has not been performed. Characterization is necessary to prove complexation and to determine the nature of the complexation. This is the focus of this section.

Zn(II) may complex two forms of the PHT ligand: the basic, deprotonated ligand or the neutral ligand. Both forms of the coordination complex may exist *in vivo*, however, at plasma pH 7.38, the complex containing neutral PHT likely predominates. Thus, the more probable *in vivo* complex, $Zn(PHT)_2A_2 \cdot xH_2O$ (where A is a counter anion), was examined by semi-empirical molecular orbital calculations.

3.3.3.3.1 Theoretical Calculations

3.3.3.3.1.1 Introduction

PHT-Zn(II) chelation may be a causal factor in PHT's anticonvulsant activity; therefore, the interaction must be rigorously examined. Accordingly, the purpose of this study is to apply the first semi-empirical AM1 molecular orbital calculations to the structural optimization of the Zn(II)-PHT interaction.

3.3.3.3.1.2 Method

All PHT-Zn(II) chelates (including hydrated forms) were geometrically optimized using the AM1 Hamiltonian, as implemented in the MOPAC 5.0 molecular orbital quantum mechanics package [275]. AM1 calculations [276], parameterized for Zn(II) [277], were performed on an IBM RS/6000 RISC 550 computer operating under AIX. The convergence criterion PRECISE was used with the MMOK keyword option enabling energy optimization accurate to 10^{-6} kcal/mol.

Initial calculations indicated that a 1:1 PHT-Zn(II) chelate could not form a stable complex. Crystal structure data of other transition metal - phenytoin complexes supports the presence of two PHT in the chelates [272,278]. Therefore, 2:1 PHT-Zn(II) chelates were examined. Zn(II) was systematically aligned along all "amide face" chelation sites of PHT — from the right (**r**), left (**l**), and bottom (**b**) faces of the hydantoin ring (see Figure 3.2). Bonding schemes are given in Table 3.1. Figure 3.3 displays the six possible configurations (**bb**,**br**,**bl**,**ll**,**rl**,**rr**) studied. To simulate *in vivo* solvation about the Zn(II) binding site, the three most stable configurations were further optimized in hydrated forms (**bbw**, **rlw**, and **brw**).

The starting geometry of the PHT molecules was taken from X-ray crystallographic data [288]. Zn(II) was placed 2.0 - 2.6 Å from the coordinating atoms in the starting geometries, in a tetrahedral environment between the two PHT molecules. The hydantoin rings and the Zn(II) binding environment were optimized. Counterions were not explicitly considered.

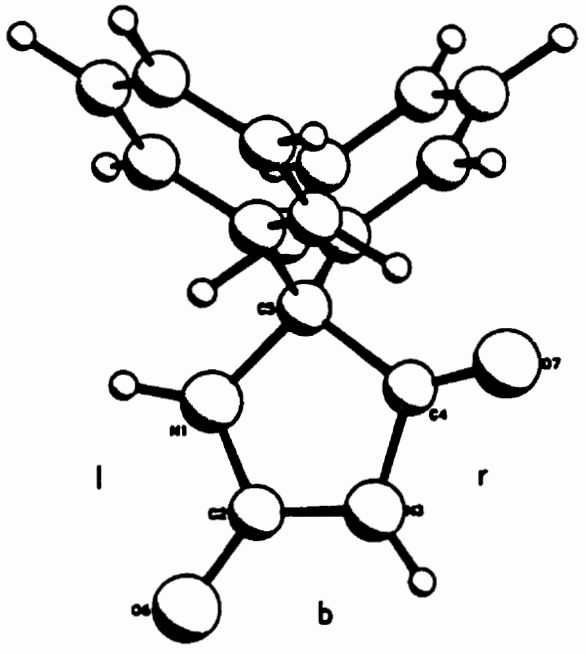


Figure 3.2: Phenytoin showing left (l), right (r) and bottom (b) amide faces

Table 3.1: Coordination in starting configurations

Starting Configuration	Coordinating Atoms in Zn(II) Binding Environment
rr	N3 / C4(O7) / N3' / C4'(O7')
rl	N3 / C4(O7) / N1' / C2'(O6')
ll	N1 / C2(O6) / N1' / C2'(O6')
bb	N3 / C2(O6) / N3' / C2'(O6')
bl	N3 / C2(O6) / N1' / C2'(O6')
br	N3 / C2(O6) / N3' / C4'(O7')

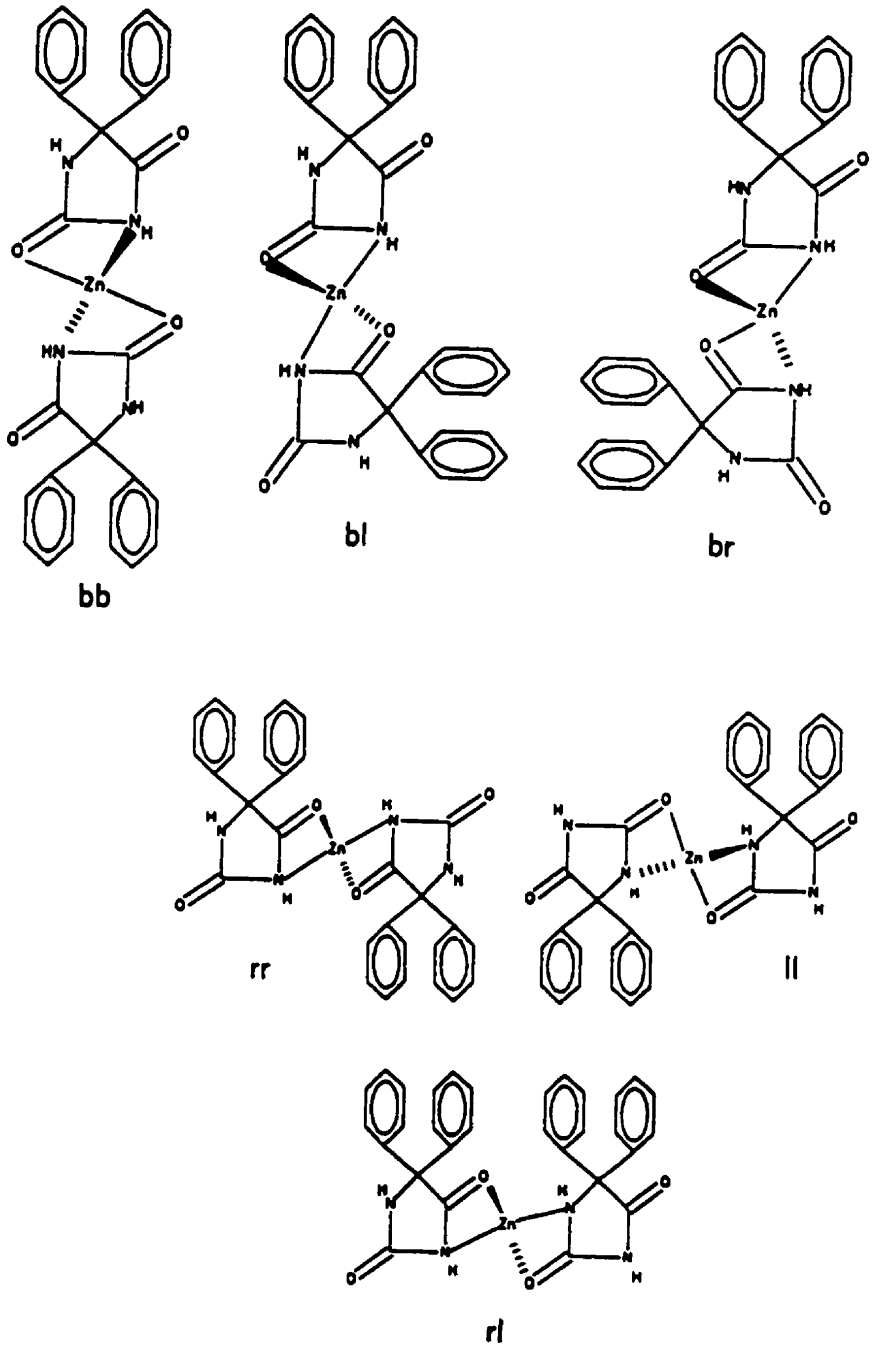


Figure 3.3: Starting conformations of phenytoin zinc complexes

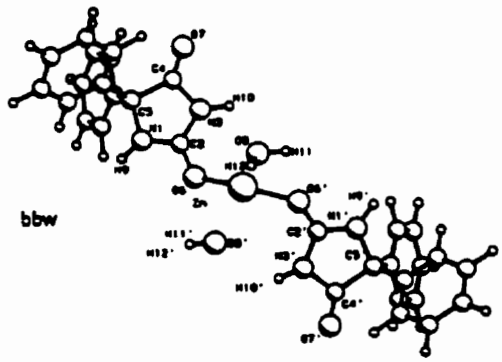
3.3.3.3.1.3 Results

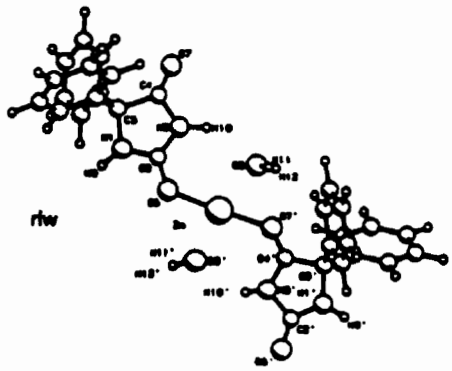
Using AM1, the six non-hydrated starting configurations (Figure 3.3) were fully optimized to yield five 2-coordinate complexes and one 4-coordinate complex. The three most stable complexes (**bb**, **rl**, and **br**) were then hydrated and re-optimized to mimic the first solvation sphere solvent effect in the vicinity of Zn(II). The chelating atoms in the Zn(II) binding site for the nine optimized complexes are listed in Table 3.2. All optimized configurations are shown in Figure 3.4. Pertinent bond lengths and bond angles of all optimized configurations are given in Table 3.3 and Table 3.4, respectively.

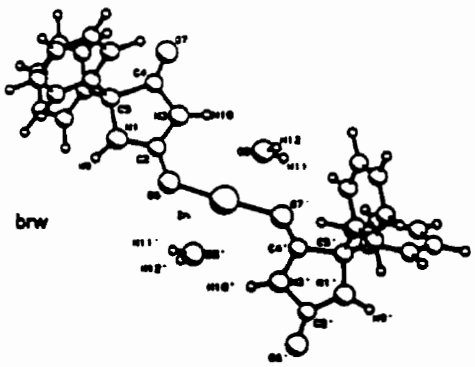
Table 3.2: Stabilities and coordination in optimized configurations

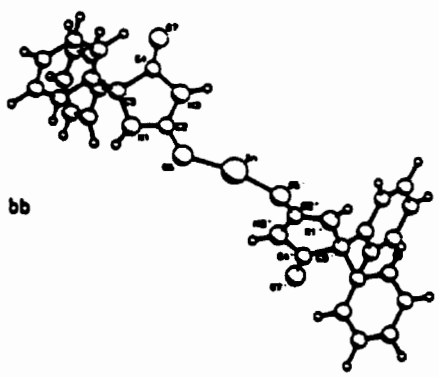
Optimized Configuration	Coordinating Atoms in Zn(II) Binding Environment	Heat of Formation (kcal/mol)
bbw	C2(O6) / C2'(O6') / O8 / O8'	325.58
rlw	C2(O6) / C4'(O7') / O8 / O8'	334.11
brw	C2(O6) / C4'(O7') / O8 / O8'	339.66
bb	C2(O6) / C2'(O6')	486.27
rl	C2(O6) / C4'(O7')	499.93
br	C2(O6) / C4'(O7')	502.69
ll	C2(O6) / N1'	508.02
bl	C2(O6) / N1'	508.70
rr	N3 / C4(O7) / N3' / C4'(O7')	632.89

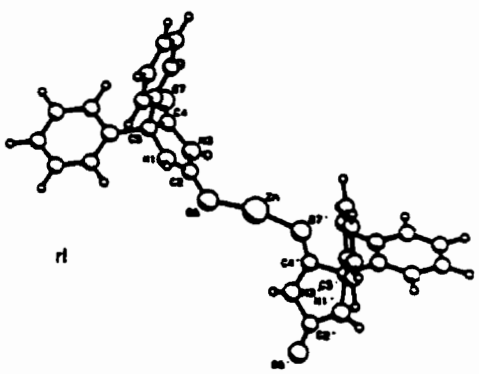
Figure 3.4: Optimized configurations of phenytoin zinc complexes











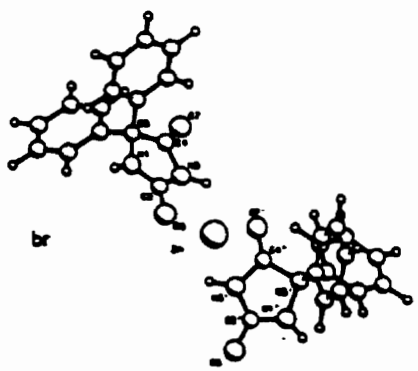
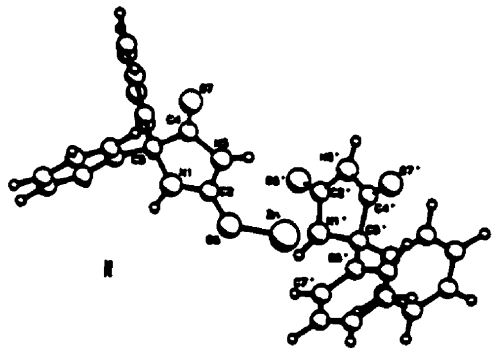
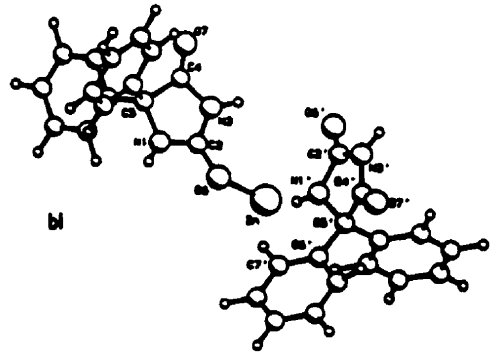


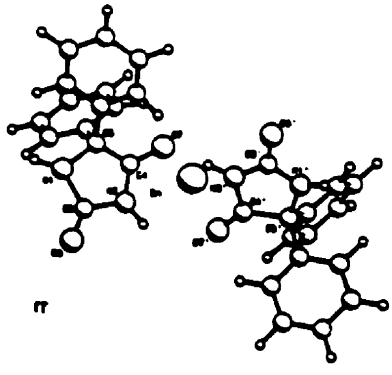
Figure 3.4 continued ...



II



III



IV

Table 3.3: Bond lengths (Å) in optimized configurations

Hydantoin Rings

	bbw	rlw	brw	bb	rl	br	ll	bl	rr	PHT[288]
N1-C2	1.36	1.37	1.37	1.36	1.37	1.36	1.37	1.37	1.57	1.33
N1-C5	1.49	1.50	1.51	1.50	1.50	1.51	1.50	1.50	1.50	1.46
C2-N3	1.38	1.39	1.39	1.38	1.38	1.38	1.39	1.39	1.46	1.39
N3-C4	1.45	1.45	1.44	1.45	1.46	1.45	1.45	1.45	1.38	1.34
C4-C5	1.57	1.57	1.56	1.57	1.57	1.56	1.57	1.57	1.36	1.55
C4-O7	1.22	1.22	1.22	1.22	1.22	1.22	1.22	1.22	1.33	1.22
C2-O6	1.33	1.31	1.32	1.33	1.33	1.33	1.32	1.32	1.22	1.21
N1'-C2'	1.36	1.39	1.39	1.35	1.38	1.39	1.47	1.47	1.36	1.33
N1'-C5'	1.50	1.48	1.48	1.51	1.48	1.48	1.51	1.51	1.50	1.46
C2'-N3'	1.38	1.47	1.47	1.39	1.48	1.49	1.41	1.41	1.38	1.39
N3'-C4'	1.46	1.36	1.36	1.45	1.35	1.35	1.42	1.42	1.45	1.34
C4'-C5'	1.57	1.59	1.59	1.57	1.60	1.61	1.58	1.58	1.57	1.55
C4'-O7'	1.22	1.29	1.29	1.22	1.30	1.30	1.22	1.23	1.22	1.22
C2'-O6'	1.33	1.23	1.23	1.33	1.23	1.23	1.23	1.23	1.33	1.21

Zn(II) Binding Environment

O6-Zn	2.04	2.03	2.04	1.98	1.96	1.97	2.00	2.00		
O7-Zn									2.26	
O6'-Zn	2.03			1.98						
O7'-Zn		2.09	2.08		2.00	2.00			2.11	
N1'-Zn							2.27	2.27		
N3-Zn									2.54	
N3'-Zn									2.56	
O8-Zn	2.16	2.18	2.18							
O8'-Zn	2.19	2.18	2.18							
Zn-C6'							2.59	2.58		
Zn-C7'							2.28	2.28		

Hydrogen Bonds

O6-H11'	2.35		2.62
O6-H12'	2.66	2.87	2.81
O8'-H10'	2.53	2.28	2.36
O6'-H11	2.30		
O8-H10		2.31	2.37
O7'-H11		2.34	2.46
O7'-H12		2.43	2.33

Table 3.3 continued

Other atomic distances

	bbw	rlw	brw	bb	rl	br	ll	bl	rr
O8-H10	3.91								
O6'-H12	3.05								
O6'-Zn							3.95	3.94	
N3-Zn	3.56	3.46	3.44	3.60	3.90	3.61	3.73	3.61	
N3'-Zn	3.54	3.53	3.55	3.47	3.49	3.51			
C5-C5'	10.21	9.74	9.84	10.42	9.64	8.45	7.83	8.04	7.51

Table 3.4: Bond angles (^{0}) in optimized configurations

Hydantoin Rings

	bbw	rlw	brw	bb	rl	br	ll	bl	rr	PHT[288]
C2-N1-C5	110.9	111.0	111.0	110.9	111.0	110.8	110.8	110.8	101.1	114.2
N1-C5-C4	101.5	101.4	101.1	101.3	101.2	101.2	101.4	101.4	111.1	99.1
C5-C4-N3	106.4	106.5	107.1	106.4	106.3	106.8	106.5	106.4	112.2	107.3
C5-C4-O7	132.0	131.6	131.2	132.0	131.9	131.8	131.7	131.8	119.8	125.6
N3-C4-O7	121.6	121.8	121.7	121.5	121.7	121.4	121.7	121.7	128.0	127.0
C4-N3-C2	108.9	109.3	109.3	109.0	109.1	109.0	109.1	109.1	108.9	112.6
N3-C2-N1	112.1	111.6	111.5	112.2	112.2	112.0	111.9	111.9	106.4	106.6
N3-C2-O6	127.6	127.3	126.9	128.9	123.8	127.6	126.3	128.1	121.6	125.0
N1-C2-O6	120.2	121.1	121.7	118.8	123.8	120.3	121.6	120.0	131.9	128.4
C2'-N1'-C5'	111.2	113.1	112.8	111.2	114.0	113.3	107.9	107.7	110.8	114.2
N1'-C5'-C4'	101.2	100.4	100.6	100.9	99.6	100.1	97.8	97.9	101.2	99.1
C5'-C4'-N3'	106.4	108.7	108.6	106.6	108.9	108.8	106.8	106.8	106.4	107.3
C5'-C4'-O7'	131.9	125.7	125.3	131.6	123.3	123.1	128.0	128.0	132.0	125.6
N3'-C4'-O7'	121.6	125.7	126.1	121.8	127.8	128.1	125.1	125.0	121.6	127.0
C4'-N3'-C2'	108.9	110.6	110.6	108.7	110.8	110.5	111.1	111.0	109.0	112.6
N3'-C2'-N1'	112.2	107.2	107.3	112.4	106.7	107.1	105.7	105.9	112.1	106.6
N3'-C2'-O6'	128.0	122.0	121.9	127.3	121.2	120.7	129.5	129.5	127.7	125.0
N1'-C2'-O6'	119.8	130.8	130.8	120.3	132.1	132.1	124.6	124.3	120.2	128.4

Table 3.4 continued

Zn(II) Binding Environment

	bbw	rlw	brw	bb	rl	br	ll	bl	rr
C2-O6-Zn	128.9	128.7	127.8	134.1	132.5	131.6	132.0	133.1	
C2'-O6'-Zn	126.9			127.4					
C4-O7-Zn									84.7
C4'-O7'-Zn		133.0	133.6		130.9	132.6			92.5
N3-Zn-O6									
O6-Zn-O8	123.3	106.4	108.6						
O6-Zn-O6'	154.5			166.8					
O6-Zn-N1'							141.3	151.4	
O6-Zn-N3'									
O6-Zn-O8'	73.3	90.0	79.8						
O6-Zn-O7'		162.8	178.0		166.0	171.0			
O7-Zn-O6'									
O7-Zn-O7'									149.1
O6'-Zn-O8	77.3								
O6'-Zn-O8'	102.4								
O6'-Zn-N3'									
O7'-Zn-O8		69.3	69.9						
O7'-Zn-O8'		98.8	101.7						
N3-Zn-O7'									
N3-Zn-N3'									
Zn-N1'-C2'							113.3	112.7	
Zn-N1'-C5'							92.2	92.0	

Description of Non-Hydrated Optimized Configurations

Four distinct configurational types emerged from the optimization of the non-hydrated structures: (1) a structure with tetra-coordination, (2) structures involving "phenyl trapping", (3) structures involving coordination through C4'(O7'), and (4) a structure involving coordination through C2'(O6').

Structure with tetra-coordination

The **rr** optimized configuration (632.89 kcal/mol) is the only structure with tetra-coordination. It is also the least stable energetically. The starting and final optimized geometries of **rr**'s Zn(II) binding environment are similar, although changes in hydantoin ring geometries occurred with optimization.

Due to Zn(II) coordination through the N3-C4-O7 and N3'-C4'-O7' amides, various bond length and bond angle changes occurred in the hydantoin rings, relative to PHT's X-ray geometry. Electron donation through C4(O7) to Zn(II) caused an elongation of C4-O7 from 1.22 Å to 1.33 Å. This produced a compression within the hydantoin ring; the C2-N3 face was pulled toward C5 as C2-N3, C2-N1, and N1-C5 lengthened and the C5-C4 bond decreased by 0.19 Å to 1.36 Å.

Structures involving "phenyl trapping"

The **ll** and **bl** orientations optimized into nearly identical structures with heats of formation of 508.02 and 508.70 kcal/mol, respectively. Both structures involve phenyl trapping. In the Zn(II) binding environment, Zn(II) bonds to C2(O6) and N1' but it is also attracted to the electron-rich phenyl ring; the Zn(II) atom optimized to a position 2.28 Å from C7' on the phenyl ring.

Zn(II) coordination to C2(O6) lengthened C2-O6 by 0.10 Å to 1.32 Å. This also produced an increase in the C-N bonds of both amide groups; C4-N3 lengthened to 1.45

Å from 1.34 Å, N1-C2 lengthened to 1.37 Å from 1.33 Å. Bond angles about each carbonyl carbon were also altered.

Zn(II) coordination through N1' produced fewer geometric effects. Carbonyl bond lengths remained constant at 1.23 Å while the bridge between the two amide groups, N1'-C2', elongated from 1.33 Å to 1.47 Å. Also, angle C2'-N1'-C5' decreased 6.5^0 to 107.7^0 as electron donation through N1' produced ring compression.

In the **bl** complex, angle O6-Zn-N1' is 151^0 and the hydantoin ring planes are nearly perpendicular. In the **ll** orientation, which is a slightly more stable configuration, the ring planes intersect at 60^0 and the "bite angle", O6-Zn-N1', is lower at 141.3^0.

Structures involving coordination through C4'(O7')

Phenytoin, in the near linear complexes **rl** and **br**, coordinates with Zn(II) through C2(O6) and C4'(O7'). This increases the stability of the optimized structures compared to the aforementioned configurations, **rr**, **ll**, and **bl**. The O6-Zn bond length is 1.97 Å in **br**, 1.96 Å in **rl**. In both structures, O7'-Zn is 2.00 Å.

The O7'-Zn bond is marginally weaker than the O6-Zn bond; consequently, there is less electron donation from the carbonyl oxygen. C4'-O7' (1.30 Å) retains a larger bond order.

Zn(II) coordination to C2(O6) produces geometric changes in the hydantoin ring similar to those mentioned in the previous section: C-N bonds are lengthened and bond

angles about carbonyl carbons are affected. Once again, the C2-O6 bond length is 1.33 Å (for both **rl** and **br**).

In the **br** orientation, angle O6-Zn-O7' is 171.0^0 and the hydantoin ring planes are nearly planar making an angle of 176^0. In the **rl** complex, which is a slightly more stable configuration, the ring planes intersect at 90^0, and angle O6-Zn-O7' is smaller at 166.0^0.

Structure involving coordination through C2'(O6')

The **bb** optimized configuration (486.27 kcal/mol) is the only structure that involved Zn(II) coordination through the C2(O6) carbonyl of both hydantoin rings. Angle O6-Zn-O6' is nearly linear at 166.8^0 and both Zn(II) - oxygen bonds are 1.98 Å. The ring planes are skewed about 120^0. The stereochemistry of complexation allows for the greatest separation of the aromatic moities and consequently produces the lowest energy non-hydrated configuration.

Coordination of Zn(II) through C2(O6) and C2'(O6') elongates these bonds to 1.33 Å from 1.21 Å in native PHT. This serves to lengthen N3-C4 and N3'-C4' by 0.11 Å to 1.45 Å. Again, due to carbonyl complexation, bond angles about all four carbonyl carbons are significantly altered (Table 3.4).

Description of Hydrated Optimized Configurations

Two water molecules were added to the Zn(II) binding environment of the three most stable 2-coordinate complexes: **br**, **rl**, and **bb**. Further optimization was performed to allow for maximum hydrogen-bonding.

brw *configuration*

In the **brw** complex, Zn(II) is bound by four oxygens in a distorted square planar arrangement. The hydantoin rings bind Zn(II) linearly (179^0) through C2(O6) and C4'(O7'); N atoms do not act as chelators. The two water molecules produce six hydrogen bond interactions.

Although **brw**'s hydantoin ring geometries are identical to those in **br**, the presence of two water molecules produces geometric differences in the Zn(II) binding environment. Relative to **br**, **brw**'s O6-Zn bond lengthened from 1.97 to 2.04 Å. Similarly, O7'-Zn lengthened from 2.00 to 2.08 Å. Some bond angles changed appreciably: C2-O6-Zn decreased from 131.6^0 in **br** to 127.8^0 in **brw**, C4'-O7'-Zn changed from 132.6^0 to 133.6^0, and O6-Zn-O7' changed from 171.0^0 to 178.0^0. Several bond angles in the hydantoin rings were also slightly altered ($\leq 2.2^0$).

rlw *configuration*

As in **brw**, **rlw** possesses a distorted square planar Zn(II) binding site. The co-planar hydantoin rings chelate Zn(II) through C2(O6) and C4'(O7') forming an angle of 162.8^0. Two water molecules complete the binding environment and allow for five hydrogen bond interactions.

The **rlw** complex has nearly identical geometries to **brw**: for example, hydantoin ring bond angles differ by 0.6^0 or less, and bond lengths differ by not more than 0.01 Å.

bbw *configuration*

Among the optimized non-hydrated configurations, **bb** is most stable (486.27 kcal/mol). Correspondingly, **bbw** is the most stable hydrated complex (325.58 kcal/mol).

In the **bbw** configuration, Zn(II) is bound by four oxygens in a distorted square planar complexation environment. The hydantoin rings, skewed 160^0 to each other, bind Zn(II) through the C2(O6) and C2'(O6') forming an angle of 154.5^0. Zn(II) does not bind to the ring nitrogens. The two water molecules provide two chelating oxygen atoms which arrange to maximize stabilizing hydrogen bond interactions. Four hydrogen bonds are formed.

The bond lengths and bond angles of PHT in **bbw** are nearly identical to the X-ray crystal data for PHT itself. The major exception concerns the geometry of the N1-C2-O6 amide. In PHT, the N1-C2 bond is 1.33 Å whereas in the complex it is 1.36 Å. A much

greater elongation occurs in C2-O6: for PHT, 1.21 Å; for **bbw**, 1.33 Å. Also, the N1-C2-O6 angle decreases from 128^0 in PHT to 120^0 in the **bbw** hydantoin rings.

3.3.3.3.1.4 Discussion

Computational Analysis

Semi-empirical calculations show that Zn(II) demonstrates affinity for each heteroatom in PHT. In the non-hydrated bidentate complexes, Zn(II) is coordinated to one PHT molecule through C2(O6) and to the second PHT through N1', C4'(O7'), or C2'(O6'). When the non-hydrated complexes are ranked in order of stability (Table 3.2), the following is evident: i) Zn(II) prefers to complex to amide oxygen instead of amide nitrogen, ii) complexation to the C2'(O6') carbonyl is preferred to C4'(O7'), and iii) an N1' chelate is preferred to the N3' adduct. The zinc cation electrophile coordinates to oxygen in preference to nitrogen because O has two sterically accessible electron pairs whereas the protonated amide nitrogen resides in a relatively crowded environment. In support of the theoretical results, experimental studies have shown that metal cation chelates neutral amide through carbonyl oxygen [279]. For example, Zn(II) is complexed by biuret via two carbonyl oxygen atoms [280].

Table 3.2 shows that Zn(II) prefers to coordinate through C2'(O6') instead of C4'(O7'). Steric hindrance accounts for this observation. The bulky phenyl moieties on the hydantoin rings are farthest apart and do not repel one another when Zn(II) chelates to

the C2(O6) carbonyl (compare C5-C5' atomic distances in **bb** and **rl**). Steric hindrance also accounts for the high energy of the 4-coordinate rr complex. The two hydantoin rings (and phenyl groups) in **rr** are much closer to each other compared to the bidentate complexes.

Zn(II) coordination to PHT causes geometric changes in hydantoin due to electron donation from both the chelating atoms and the ring. Regardless of the mode of complexation, hydantoin ring bond lengths increased and bond orders decreased (Table 3.3). Literature support for these results remains ambiguous. An earlier theoretical study found that metalation at a neutral amide nitrogen weakens and lengthens the C-N bond [281]. Experimental [282] and theoretical results [281,283,284] found that metal ion complexation at amide oxygen shortens amide C-N while lengthening the carbonyl. Nevertheless, all Zn-O and Zn-N bond lengths are within acceptable limits when compared to similar bond lengths taken from a survey of crystal data of Zn(II) complexes [285].

Hydration of the three most stable bidentate complexes resulted in minor geometric changes in the Zn(II) binding environment. Coordination and electron donation by the two water molecules loosened the Zn-O coordinate bonds by 0.06 - 0.09 Å and left Zn(II) in a distorted square planar environment. Changes in the hydantoin rings were negligible. Hydrogen bond interactions stabilized the complexes though not enough to offset instability due to steric hindrance. The **bbw** complex, with four hydrogen bonds, was more stable than **rlw** with its five H-bonds. Similarly, **rlw** was more stable than **brw**

which possesses six H-bonds. All hydrogen bond lengths [286] and angles [287] were in acceptable agreement with experimental values.

In vivo, the native conformation of a Zn(II)-PHT chelate is one in which the energy of the complex is a minimum. Assuming that hydration occurs, this study demonstrates that such a chelate exists in the **bbw** configuration where Zn(II) bridges two PHT molecules by coordinating through C2(O6).

Implications for Antiepileptic Drug Design

PHT-Zn(II) coordination associates anticonvulsant activity with Zn(II) chelation. The theoretical results of this study have identified PHT's C2(O6) carbonyl as the Zn(II) binding site. Zn(II) chelation has been demonstrated theoretically but not empirically. This is the focus of the next section.

3.3.3.3.2 X-ray Crystal Structure

3.3.3.3.2.1 Introduction

The PHT-Zn(II) complex has been synthesized previously [273,274] and characterized computationally but not empirically. Thus, as part of the ongoing investigation of the Zn(II)-PHT interaction, diamminebis(5,5-diphenylhydantoinato)

zinc(II) hemihydrate (Figure 3.5 $\bullet^{1}/_{2}\ H_2O$) was synthesized and structurally examined to establish its coordination geometry and mode of bonding.

Notably, the PHT-Zn(II) complex, diamminebis(5,5-diphenylhydantoinato) zinc(II) hemihydrate, differs from the PHT-Zn(II) complexes that were studied by molecular orbital calculations. Solvation difficulties precluded the synthesis of a PHT-Zn(II) complex at neutral pH.

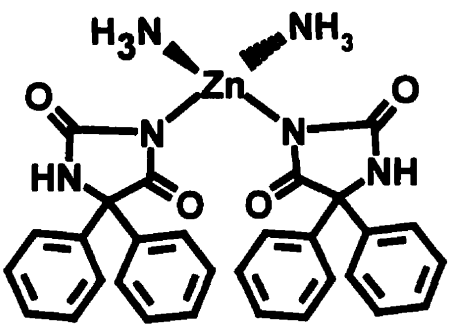


Figure 3.5: Diamminebis(5,5-diphenylhydantoinato)zinc(II)

3.3.3.3.2.2 Method

Single crystals of the PHT-Zn(II) complex were obtained by slow evaporation, at constant 50^0C, of aqueous ammonia containing phenytoin and $[Zn(NO_3)_2]\bullet 6H_2O$. Synthetic details are provided in Appendix A. Elemental analysis revealed the presence of a half molecule of water per molecule of complex in the crystal structure.

X-ray analysis was performed by Dr. Aleksander Roszak using an Enraf-Nonius CAD-4 diffractometer. Specific details of X-ray experimental may be found in *Acta Cryst.* **C51**, 1297-1300 (1995).

3.3.3.3.2.3 Results

The complex (Figure 3.6) has tetrahedral 4-N Zn coordination with the Zn ion and ammonia N7 and N8 atoms sitting on a mirror plane perpendicular to the crystallographic *y* axis. The interaction of phenytoin with Zn(II) parallels Cu(II) and Ni(II) complexation [272,278]. All of these complexes have a deprotonated N3 atom in the coordination sphere of the transition metal. The Zn-N distances are equal but the tetrahedral geometry is distorted due to the large size of the phenytoin ligand, the N3-Zn-$N3^i$ angle [$N3^i$ is the mirror image of N3; symmetry code: (i) x, $^3/_2 - y$, z] being about 10° greater than the ideal value. The hydantoin ring is not symmetrically oriented with respect to the Zn-N3 bond and the two ammonia ligands have O2···$O2^i$ and O4···$O4^i$ distances of 5.177(4) and 3.155(4) Å, respectively. The N7···O4 and N8···O2 distances are similar [3.273(3) and 3.218(3) Å, respectively], but due to the symmetrically constrained positions of the ammonia H atoms, only the second contact can be considered a weak intramolecular hydrogen bond (Table 3.6). The dihedral angle between the hydantoin ring and the mirror plane of the complex is 57.2(1)°, and the angle between the two hydantoin rings is 114.4(1)°.

The geometry of the hydantoin ring is similar, within experimental error, to that found in the analogous Cu and Ni complexes [272,278], but shows some differences in bond angles when compared to the free phenytoin of the first determination by Camerman & Camerman [288]. When compared to free phenytoin of the latest more accurate determination by Chattopadhyay *et al.* [289], significant differences can be observed, namely, an increase in the N1-C2 and C4-C5 bond lengths and concerted changes in all endocyclic bond angles which can be rationalized by the effect of coordination to the Zn atom. The N3 atom is being pulled out of the hydantoin ring so that the C2-N3-C4 angle decreases from 112.2(2)° in free phenytoin to 109.0(2)° in the Zn complex; the adjacent angles at atoms C2 and C4 increase by about 2.6° and the angles at N1 and C5 decrease slightly. The same effect can be seen by comparing the endocyclic distances N3···N1 and N3···C5 in the two compounds; they are 2.194 and 2.326 Å, respectively, in the free phenytoin and 2.248(3) and 2.374(3) Å in the Zn complex, an increase of about 0.050 Å. Consistent with these changes, the bonds N1-C2 and C4-C5 increase by about 0.02 and 0.01 Å, respectively.

Another significant difference occurs in the arrangement of the phenyl rings with respect to the hydantoin ring. In free phenytoin, one of the phenyl rings almost eclipses the N1-C5 bond and the second eclipses the C4-C5 bond (torsion angles N1-C5-C_{Ph1}-C_{Ph1} and C4-C5-C_{Ph2}-C_{Ph2} are 2.3° and -1.9°, respectively, according to Camerman & Camerman [288] and 1.5° and -3.6° in the paper by Chattopadhyay *et al.* [289]). Such arrangements result in a dihedral angle of about 90° between the phenyl rings. A similar situation is observed in the Cu complex with corresponding torsion angles of 7.9° and

-4.2°, and in the Ni complex is somewhat distorted with angles of -21.4° and 16.9°; the phenyl-phenyl angle is still close to 90° in both these complexes. In the Zn complex, the phenyl-ring bond C61-C66 is relatively close to the eclipsing position with a torsion angle N1-C5-C61-C66 of 17.3(4)°, but the other phenyl-ring bond, C51-C56, forms a torsion angle of -68.9(3)° [C4-C5-C51-C56] with the C4-C5 bond resulting in a dihedral angle of 63.4(2)° between the two phenyl rings. This different arrangement of phenyl rings in the Zn complex is probably due to the tetrahedral coordination and reflection symmetry of the molecule which place the C51-C56 phenyl ring in close proximity to its mirror counterpart; the shortest C···C distance observed between these rings (C55···C55^{i}) is 3.589(9) Å. It should be noted that this arrangement of phenyl rings does not fall into one of the potential energy minima allowed for free phenytoin calculated by Chattopadhyay *et al.* [289].

The packing of molecules in the crystal is determined by hydrogen bonding between the N1 amino function and the carbonyl O2 atom, and by several other hydrogen bonds between the ammonia ligands and the carbonyl O2 and O4 functions (Table 3.6 provides details of the hydrogen bonding). Formally, a half molecule of the solvent water sits on the mirror plane of the complex and is disordered over two positions, OW1 and OW2, 0.77(5) Å apart and each with 0.25 occupancy. OW1 and OW2 form hydrogen bonds to the hydantoin O atoms O2 and O2^{i} on both sides of the mirror plane.

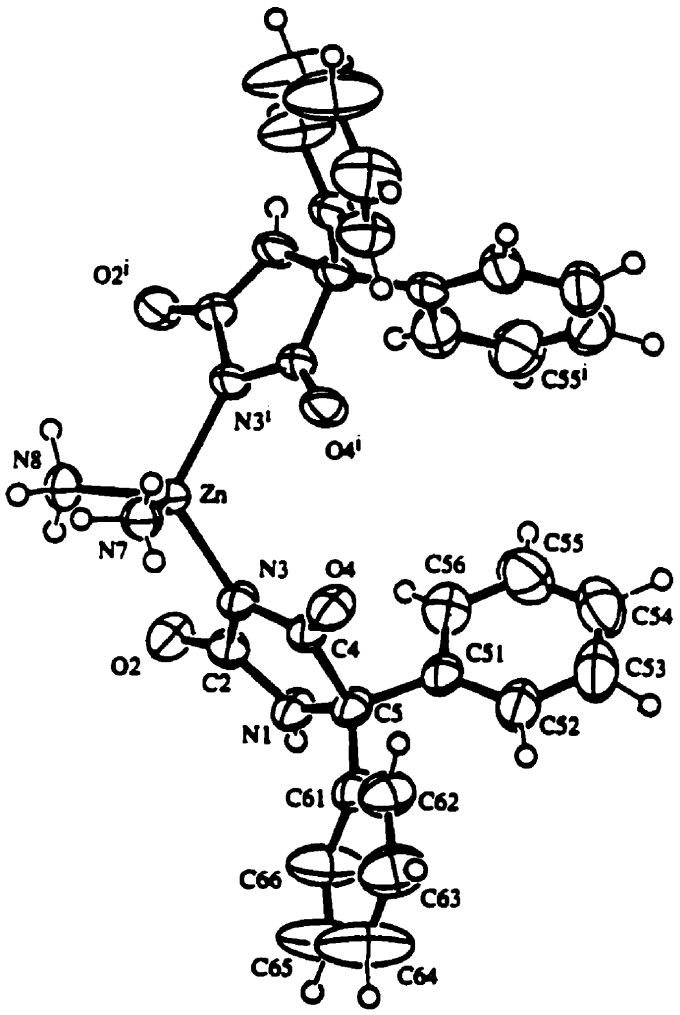


Figure 3.6: Molecular structure of $[Zn(NH_3)_2 (C_{15}H_{11}N_2O_2)_2]\cdot\frac{1}{2}H_2O$ showing 50% probability displacement ellipsoids

Table 3.5: Selected geometric parameters of $[Zn(NH_3)_2 (C_{15}H_{11}N_2O_2)_2]\cdot\frac{1}{2}H_2O$

Bond Lengths (Å)

Zn-N3	2.004(2)	C2-N3	1.390(3)
Zn-N7	2.010(3)	N3-C4	1.348(3)
Zn-N8	2.006(3)	C4-O4	1.220(3)
N1-C2	1.358(3)	C4-C5	1.554(3)
N1-C5	1.445(3)	C5-C51	1.550(4)
C2-O2	1.233(3)	C5-C61	1.523(4)

Bond Angles (^{0})

N3-Zn-N3^i	118.99(12)	O4-C4-C5	124.1(2)
N7-Zn-N3	106.31(7)	N3-C4-C5	109.6(2)
N3-Zn-N8	106.94(7)	N1-C5-C4	99.4(2)
N7-Zn-N8	111.38(12)	N1-C5-C51	111.1(2)
C2-N1-C5	112.2(2)	N1-C5-C61	113.2(2)
O2-C2-N1	125.3(2)	C4-C5-C51	106.8(2)
O2-C2-N3	125.0(2)	C4-C5-C61	111.9(2)
N1-C2-N3	109.7(2)	C51-C5-C61	113.5(2)
C4-N3-C2	109.0(2)	C5-C51-C52	123.9(3)
C4-N3-Zn	120.89(15)	C5-C51-C56	118.4(2)
C2-N3-Zn	130.1(2)	C5-C61-C62	121.7(2)
O4-C4-N3	126.4(2)	C5-C61-C62	119.8(3)

Torsion Angles (^{0})

N1 - C2 - N3 - Zn	175.2(2)	N1 - C5 - C51 - C52	-143.3(3)
O2 - C2 - N3 - Zn	-4.3(4)	N1 - C5 - C51 - C56	38.5(3)
N3^i - Zn - N3 - C2	-106.6(2)	C4 - C5 - C51 - C52	109.3(3)
N3^i - Zn - N3 - C4	70.7(2)	C4 - C5 - C51 - C56	-68.9(3)
N7 - Zn - N3 - C2	133.7(2)	C61 - C5 - C51 - C52	-14.5(3)
N7 - Zn - N3 - C4	-49.1(2)	C61 - C5 - C51 - C56	167.3(3)
N8 - Zn - N3 - C2	14.6(3)	O4 - C4 - C5 - C61	57.6(3)
N8 - Zn - N3 - C4	-168.2(2)	N3 - C4 - C5 - C61	-123.3(2)
Zn - N3 - C4 - O4	5.0(4)	N1 - C5 - C61 - C62	-162.7(3)
Zn - N3 - C4 - C5	-174.1(2)	N1 - C5 - C61 - C66	17.3(4)
C2 - N1 - C5 - C51	-110.1(2)	C4 - C5 - C61 - C62	-51.5(3)
C2 - N1 - C5 - C61	120.9(2)	C4 - C5 - C61 - C66	128.6(3)
O4 - C4 - C5 - C51	-67.1(3)	C51 - C5 - C61 - C62	69.5(3)
N3 - C4 - C5 - C51	112.0(2)	C51 - C5 - C61 - C66	-110.4(3)

Table 3.6: Hydrogen-bonding geometry (Å, °) in $[Zn(NH_3)_2(C_{15}H_{11}N_2O_2)_2]\cdot\frac{1}{2}H_2O$

D-H···A	D-H	H···A	D···A	D-H···A
N1 - H1···O2^{i}	0.86	2.20	3.037(3)	164
N7 - H7A···O4^{ii}	0.89	2.38	2.980(3)	125
N8 - H8B···O2	0.89	2.50	3.218(3)	138
N8 - H8A···O4^{ii}	0.89	2.34	2.938(3)	125
OW1···O2^{iii}			3.074(16)	
OW2···O2^{iii}			3.029(16)	

H atoms of the ammonia atoms N7 and N8 are denoted with A for atoms in the mirror plane and B for out-of-mirror-plane atoms.

3.3.3.3.2.4 Discussion

The crystal structure of $[Zn(NH_3)_2(C_{15}H_{11}N_2O_2)_2]\cdot\frac{1}{2}H_2O$ has been rigorously defined. Zn(II) coordinates PHT through deprotonated N3. The empirical confirmation of PHT-Zn(II) chelation would be expected if Zn(II) chelation is a mechanism of anticonvulsant action.

3.3.3.4 Conclusions

Semi-empirical molecular orbital methods and X-ray crystallography have shown that PHT chelates Zn(II) through the C2 carbonyl oxygen at neutral pH or via deprotonated N3 at high pH. The former coordination compound is the more probable form in the physiological milieu. PHT-Zn(II) coordination, affirmed by these studies, associates anticonvulsant activity with Zn(II) chelation; however, this does not prove

causation. A review by Woodbury [290] points to numerous mechanisms of action including sodium channel blockade and the inhibition of calcium influx but Zn(II) chelation was not implicated. This remains a possibility — a potential contributory factor — but further studies are necessary to firmly establish a link. Many more ligands need to be examined. This is the focus of the next section.

3.3.4 Evaluation of Miscellaneous Zn(II) and Cu(II) Complexes as Antiepileptic Agents

3.3.4.1 Introduction

To supplement anticonvulsant activity data of Cu(II) and Zn(II) complexes available from the National Institutes of Health (NIH), Cu(II) or Zn(II) complexes of miscellaneous organic ligands were synthesized and evaluated for anticonvulsant activity. Qualitative structure-activity relationships afforded clues for the design of Cu(II) releasing agents capable of Cu(II)/Zn(II) transposition.

3.3.4.2 Method

Organic ligand (2 equiv.) and either metal chloride (1 equiv.) or metal nitrate (1 equiv.), where the metal cation is Cu(II) or Zn(II), were dissolved in EtOH, MeOH or H_2O and refluxed for 4 to 24 hours. Crystals of Cu(II) or Zn(II) complex were isolated

either by filtering crystals from the cooled solution or by evaporating the solution to dryness. The product was washed with EtOH and Et_2O, dried *in vacuo*, then analyzed by melting point, elemental analysis and/or atomic absorption spectroscopy.

All products were biologically evaluated using MES, PTZ or PIS seizure models to determine anti-ictogenic activity. Refer to Appendix B for details of the experimental procedures. Compound efficacy was determined by seizure protection in more than 2/4 rats at doses of 300, 100, 50 and 10 mg/kg ip.

3.3.4.3 Results

Using commercially available organic ligands, 9 Cu(II) complexes and 9 Zn(II) complexes were synthesized and physically characterized (Table 3.7 and Table 3.8). All compounds including precursors were biologically evaluated for anticonvulsant activity.

NIH data concerning the anticonvulsant activity for an additional 20 Cu(II) complexes, 10 Zn(II) complexes, and associated ligands was also compiled (Table 3.9).

Table 3.7: Physical data and anticonvulsant activity of Cu(II) complexes and ligands

Code	Cu(II) Complex	Form[a]	Yield (%)	m.p. (°C)	Formula	Elemental Analysis				Biological Activity[b,c]	
							%C	%H	%N	Free ligand	Metal Complex
B2P132	Dichlorobis(triphenyl-phosphine oxide)copper(II)	bt ye cr	99.0	173	$C_{36}H_{30}O_2P_2Cl_2Cu$ MW = 691.03	Found: Calc:	62.21 62.57	4.21 4.38	0 0	0	0
B2P144	Dichloro(N,N,N',N'-tetramethylethylenediamine) copper(II)	dk bl cr	100	120-124	$C_6H_{16}N_2Cl_2Cu$ MW = 250.66	Found: Calc:	28.58 28.75	7.00 6.43	11.18 11.18	0	0
B2P160	Bis(benzimidazole)dinitrato copper(II) hemihydrate	tur cr	100	221	$C_{14}H_{12}N_6O_6Cu \cdot 0.5H_2O$ MW = 432.84	Found: Calc:	38.82 38.85	2.74 3.03	19.17 19.42	0[d]	1+[e]
B2P164	Bis(N-benzoyl-N-phenyl-hydroxylamino)copper(II)	lt gr cr	48.5	208	$C_{26}H_{20}N_2O_4Cu$ MW = 485.99	Found: Calc:	64.18 64.26	3.88 3.73	5.71 5.76	0	1+
B2P178	(Diphenylthiocarbazonato) copper(II)	br pw	97.1	203	$C_{13}H_{10}N_4SCu$ MW = 317.85	Found: Calc:	49.49 49.12	3.43 3.17	17.66 17.63	2+	1+
B2P182	Bis[tris(carboxymethyl)-amino]copper(II) dihydrate	bl cr	70.3	250	$C_{12}H_{16}N_2O_{12}Cu \cdot 2H_2O$ MW = 477.83	Found: Calc:	30.08 30.16	4.21 3.80	5.85 5.86	NA	0
B1P140	Glycylglycinatocopper(II) hydrate[f]	bl cr	83.0	216 (dec)	$C_4H_6N_2O_3Cu \cdot H_2O$ MW = 211.66	Found: Calc:	22.47 22.70	3.62 3.81	13.20 13.23	1+	3+

Table 3.7 continued

Code	Cu(II) Complex	Form[a]	Yield (%)	m.p. (°C)	Formula	Elemental Analysis				Biological Activity[b,c]	
							%C	%H	%N	Free ligand	Metal Complex
B1P148	(L-Phenylalanyl-L-tryptophanato)copper(II) hemihydrate[g]	bl cr	65.2	261 (dec)	$C_{20}H_{19}N_3O_3Cu \cdot 0.5H_2O$ MW = 420.93	Found: Calc:	56.97 57.07	4.46 4.55	9.88 9.98	0	2+
B1P151	(L-Leucyl-L-tryptophanato)copper(II) sesquihydrate	bl cr	67.4	437 (dec)	$C_{17}H_{21}N_3O_3Cu \cdot 1.5H_2O$ MW = 405.94	Found: Calc:	50.07 50.30	5.77 5.96	10.14 10.35	0	2+

a. bl: blue; br: brown; bt: bright; cr: crystal; dk: dark; gr: green; lt: light; pw: powder; tur: turquoise; ye: yellow.
b. Using maximal electroshock, compound is active in > 2/4 rats at 10 mg/kg, 4+; 50 mg/kg, 3+; 100 mg/kg, 2+; 300 mg/kg, 1+; or inactive, 0.
c. No compounds were active in the pentylenetetrazole animal model.
d. Inactive in pilocarpine model.
e. Active in pilocarpine model.
f. TLC: R_f=0.58 (EtOH/AcOH 20:1), 0.44 (CH_2Cl_2/EtOAc 9:1).
g. TLC: R_f=0.52 (EtOH/AcOH 20:1), 0.79 (CH_2Cl_2/EtOAc 9:1).

Table 3.8: Physical data and anticonvulsant activity of Zn(II) complexes and ligands

Code	Zn(II) Complex	Form[a]	Yield (%)	m.p. (°C)	Formula	Elemental Analysis				Biological Activity[b,c]	
							%C	%H	%N	Free ligand	Metal Complex
B2P32	Dichlorobis(triphenyl-phosphine oxide)zinc(II)	wh so	74.4	232	$C_{36}H_{30}Cl_2O_2P_2Zn$ MW = 692.87	Found: Calc:	62.40 62.41	4.46 4.36	0 0	0	0
B2P34	Sodium adenosine triphosphatozinc(II) dihydrate	wh pw	75.2	200 (dec)	$C_{10}H_{16}N_5O_{13}P_3$ $Na_2Zn{\bullet}3H_2O$ MW = 713.72	Found: Calc:	16.57 16.83	2.75 2.82	9.46 9.81	0	0
B2P52	Dichloro(N,N,N',N'-tetramethylethylenediamine) zinc(II)	col tr cr	39.2	174-175	$C_6H_{16}N_2Cl_2Zn$ MW = 252.49	Found: Calc:	28.54 28.55	6.39 6.31	11.09 10.88	0	0
B2P56	Chloro(diethanolamino) zinc(II)	wh fl	78.1	180	$C_4H_{10}NO_2ClZn$ MW = 204.96	Found: Calc:	23.61 23.44	4.77 4.92	6.83 6.83	NA	0
B2P70	Bis(3,3'-dimethoxy-benzidine)dinitratozinc(II)	gy pw	72.6	235 (dec)	$C_{28}H_{32}N_6O_{10}Zn$ MW = 677.98	Found: Calc:	49.21 49.60	4.48 4.76	12.25 12.40	0	0
B2P72	Bis(benzimidazole)zinc(II) hemihydrate	wh cr	28.2	>400 (dec)	$C_{14}H_{10}N_4Zn$ $\bullet 0.5H_2O$ MW = 306.63	Found: Calc:	54.47 54.84	3.43 2.96	18.55 18.27	0	0
B2P76	Bis(N-benzoyl-N-phenyl-hydroxylamine)dinitrato zinc(II)	wh so	11.1	259 (dec)	$C_{26}H_{20}N_4O_{10}Zn$ MW = 487.82	Found: Calc:	63.62 64.02	4.14 3.72	5.71 5.74	0	0

Table 3.8 continued

Code	Zn(II) Complex	Form[a]	Yield (%)	m.p. (°C)	Formula	Elemental Analysis				Biological Activity[b,c]	
							%C	%H	%N	Free ligand	Metal Complex
B2P80	Bis(2-aminobenzothiazole) dinitratozinc(II)	lt gr cr	100	196 (dec)	$C_{14}H_{12}N_6O_6S_2Zn$ MW = 489.79	Found: Calc:	34.28 34.33	2.39 2.47	16.80 17.16	1+	0
B2P122	(12-Crown-4)dinitrato zinc(II) trihydrate	wh fl	86.4	207	$C_8H_{16}N_2O_{10}Zn \cdot 3H_2O$ MW = 419.65	Found: Calc:	22.58 22.90	4.83 5.28	6.53 6.68	1+	0

a. col: colourless; cr: crystal; fl: flakes; gr: green; gy: grey; lt: light; pw: powder; so: solid; tr: transparent; wh: white.
b. Using maximal electroshock, compound is active in > 2/4 rats at 10 mg/kg, 4+; 50 mg/kg, 3+; 100 mg/kg, 2+; 300 mg/kg, 1+; or inactive, 0.
c. No compounds were active in the pentylenetetrazole animal model.

Table 3.9: Anticonvulsant activity of ligands and their Cu(II) or Zn(II) complexes from the NIH database

Ligand	Biological Activity[a,b] Free ligand	Cu(II) Complex	Ligand	Biological Activity[a,b] Free ligand	Cu(II) Complex
biuret	2+	3+[d]	isatin	0	0
12-crown-4	1+[d]	3+[c]	2,4-pentanedione	0	0
diazald	1+	1+	3,3'-dimethoxybenzidine	0	0
2-mercaptobenzimidazole	1+	0			
furildioxime	1+	0	**Ligand**	**Free ligand**	**Zn(II) Complex**
dibenzoylmethane	1+	0	ethyl acetamidocyanoacetate	1+	0
ethyl acetamidocyanoacetate	1+	0	2-mercaptobenzimidazole	1+	0
thioacetamide	0[c]	2+[d]	diazald	1+	0
D-penicillamine	0[c]	2+[c]	12-crown-4	1+	0
guanidineacetic acid	0	1+	N-methyl-N-nitroso-ρ-toluenesulfonamide	1+	0
thiosemicarbazide	0	1+			
N,N'-dimethylaniline	0	1+	thioacetamide	0	0
sulfosalicylic acid	0[c]	1+[d]	3,3'-dimethoxybenzidine	0	0
2-mercaptobenzothiazole	0	1+	sulfosalicylic acid	0	0
benzotriazole	0	1+	nitrilotriacetic acid	0	0
3,3'-dithiodipropionic acid	0	1+	thiosemicarbazide	0	0
5-nitrobenzotriazole	0	0	tris(hydroxymethyl)amino-methane	0	0

a. Using maximal electroshock, compound is active in > 2/4 rats at 10 mg/kg, 4+; 50 mg/kg, 3+; 100 mg/kg, 2+; 300 mg/kg, 1+; or inactive, 0; b. No compounds were active in the pentylenetetrazole animal model; c. Inactive in pilocarpine model; d. Active in pilocarpine model.

3.3.4.4 Discussion

Zn(II) and Cu(II) complexes possess vastly different anticonvulsant activity profiles (Table 3.7 to Table 3.9). No Zn(II) complex in the study sample was anticonvulsant and, for more than a third (7/20) of the ligands examined, activity was suppressed upon complexation with Zn(II). In contrast, Cu(II) complexation enhanced anticonvulsant activity for more than half (16/30) of the ligands. Furthermore, 68% (13/19) of the active complexes were derived from inactive ligands. These results support the claim that systemically administered Cu(II) is often anticonvulsant while Zn(II) is not. Incidentally, Zn(II)'s lack of anticonvulsant activity does not prove that Zn(II) is proconvulsant, however, it is congruent with that notion.

With respect to Cu(II) complexes, the nature of the organic ligand strongly influences the level of anticonvulsant activity. Maximum activity (i.e. active in >2/4 rats at 50mg/kg) was found in the Cu(II) complexes of biuret, 12-crown-4 and glycylglycine. The greatest increase in activity for a Cu(II) complex relative to its organic ligand was found in the Cu(II) complexes D-penicillamine, thioacetamide, 12-crown-4, glycylglycine, L-phenylalanine-L-tryptophan and L-leucyl-L-tryptophan. Certain structural features in this subset of organic ligands appear to be responsible for enhanced anticonvulsant activity: cyclic ligands with chelatable heteroatoms (stability is enhanced by the chelate effect [291]), heteroatoms on amino acid and peptide backbones, and chelatable sulfur atoms. Accordingly, these features may be used to guide the anticonvulsant drug design process.

In particular, peptides merit further consideration because they satisfy two important design criteria: 1) Cu(II) transforms inactive or poorly active peptide ligands into complexes with strong anticonvulsant activity, and 2) both Cu(II) and Zn(II) chelate peptides — Cu(II) chelation was demonstrated; Zn(II) chelation has been reported [292,293]. Therefore, based on the evaluation of miscellaneous Cu(II) and Zn(II) complexes, peptides when complexed with Cu(II) are recommended as the molecule class with the greatest promise of anticonvulsant activity.

3.3.5 Lead Compounds: Peptides

As lead compounds in the search for antiepileptic drugs, peptides are a good starting point; their advantages are multifold. First, peptide synthesis, characterization and conformation are well described in the chemical, biochemical and pharmacological literature. Second, toxicity is less likely to be a problem: peptides are easily metabolized after performing their desired biological function, thereby avoiding buildup of toxic metabolites. Furthermore, the chance of designing a peptide drug which produces an unacceptably toxic metabolite is lower since peptide metabolism and pharmacokinetics are predictable [294,295]. Third, peptides mediate a vast array of biochemical processes [296]. This provides a rational physiological basis for the design of neuroactive antiepileptic drugs. Indeed, peptides are a reasonable target for antiepileptic drug design since endogenous neuropeptides are postulated to modulate epileptic phenomena [297]. Finally (and most importantly), peptides are able to complex transition metals via main

chain and side chain heteroatoms. Chelating ability is a prerequisite for a drug which functions to inhibit ictogenesis and epileptogenesis through neural metal mediation.

Thus, peptides are promising lead compounds; however, they also suffer serious deficiencies in the pharmaceutical, pharmacokinetic and pharmacodynamic phases of drug action which limit their application as therapeutic agents. Linear peptides are rapidly degraded (proteolyzed) by peptidases in the gastrointestinal tract [298], in the blood and in various tissues. They are also rapidly excreted by the kidneys [299]. Peptide half-lives can be on the order of seconds [300], but are typically minutes. Linear peptides also suffer solubility problems which manifest as poor transport from the gastrointestinal tract to the blood and from the blood to the brain. With the exception of N-tyrosine peptides, the penetration of peptides across the BBB is, in general, poor [301]; polar zwitterionic termini hinder passage through lipid membranes. Potency is lost. Even if access to the brain is achieved, linear peptides are conformationally flexible and thus lack the precise geometric integrity crucial for drug receptor interactions. Again potency is lost. Finally, the stability of peptide coordination compounds follows the Irving-William series [302]. As indicated by stability or formation constants, nearly all types of donor ligands chelate Cu(II) in preference to Zn(II). This seemingly presents a major problem for a drug design strategy where ligands must transpose Cu(II) for Zn(II). Thus, given these numerous and substantial problems, linear peptides complexed with Cu(II) appear to be disqualified as lead antiepileptic compounds; clearly, they fail to meet biological design criteria. However, none of the problems are intractable.

A simple and potentially elegant solution is to use cyclic dipeptides (diketopiperazines, 2,5-piperazinediones [303]) as ligands for Cu(II) complexation (Figure 3.7) because they precisely address each deficiency inherent in the drug action of linear peptides. Upon cyclization, ionizable C- and N-termini on the peptide are eliminated. This greatly enhances enzymic stability, peptide solubility, lipophilicity, conformational rigidity, receptor specificity and potency. Enzyme stability [304] and conformational rigidity are also enhanced by the constraints of Cu(II) complexation. The net result is that transport to the brain, diffusion or uptake across the BBB [294, 305], and pharmacodynamic drug action is facilitated. Thus, cyclic dipeptides satisfy important biological design criteria whereas linear peptides do not.

Moreover, cyclic dipeptide construction may allow manipulation of the metal coordination environment to favour Zn(II) coordination and disfavour Cu(II) coordination. This requires astute control of coordination geometry and the appropriate

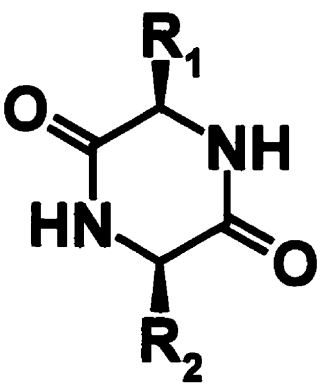


Figure 3.7: Structure of a cyclic dipeptide (in the *cis* conformation)

selection of donor atoms.

First, an understanding of coordination geometry is required. In the dipositive state, copper and zinc reside in a d^9 and d^{10} electronic configuration, respectively. This has a profound effect on the coordination stereochemistries of the two cations. Due to the d^9 configuration, Cu(II) is subject to Jahn-Teller distortion [306] if the cation is placed within an environment of cubic symmetry (regular octahedral or tetrahedral). In the six-coordinate case, distortion manifests as an elongation (or contraction) of two trans Cu-L bonds, which in the limit becomes indistinguishable from square planar coordination. Thus, Cu(II) coordinates in square planar and tetragonally distorted octahedral geometries but almost never in regular octahedral or tetrahedral geometries. In contrast, Zn(II) prefers a four-coordinate tetrahedral bonding environment. Stability is the primary reason. When metals with unfilled d orbitals are coordinated by ligands, the ligands create an electrostatic field around the metal ion which produces additional stability known as crystal field stabilization energy. Hence, d^9 Cu(II) is stabilized, d^{10} Zn(II) is not. Because Zn(II)'s d^{10} configuration affords no crystal field stabilization, coordination stereochemistry is determined solely on the bases of size, electrostatic forces and covalent bonding forces. For Zn(II), tetrahedral coordination is often most stable. Accordingly, this must be the goal in the design of a coordination environment where Zn(II) is to replace Cu(II).

Second, the appropriate selection of donor atoms can facilitate the Cu(II)/Zn(II) replacement process. Here, hard and soft acid-base (HSAB) theory may be helpful. According to the theory [307,308], hard metal ions have low polarizability, small radii

and high positive charge; they form the most stable complexes with donor atoms which have high electronegativity, low polarizability and small radii (i.e. N and O). Soft metal ions have a low positive charge, are readily polarized and form stable complexes with readily oxidized, polarizable ligands with S or P donor atoms. According to the HSAB classification, Cu(II) is a borderline hard acid and Zn(II) is a soft acid. Advantage may be gained by this small difference. "Soft" thioethers coordinate with "borderline hard" Cu(II) only weakly in dipolar solvents [309]; this should facilitate decomplexation [310]. On the other hand, "soft" Zn(II) would display significant affinity for a "soft" thioether ligand. Thus, a cyclic dipeptide with a thioether side chain (i.e. methionine side chain) could enhance the possibility that the Cu(II) cation is sufficiently labile to allow Cu(II)/Zn(II) translocation. Moreover, with a decrease in the difference between the stability constants (K) for Cu(II) and Zn(II) complexation, the rate of Cu(II)/Zn(II) transposition would increase, even though $K_{Cu} > K_{Zn}$.

3.3.6 Design Criteria for a Peptide Antiepileptic Drug

The design criteria for a peptide antiepileptic drug, summarized from the preceding sections, are as follows:

1. Cu(II) must transform the inactive or poorly active peptide ligand into a complex with strong anticonvulsant activity.
2. Both Cu(II) and Zn(II) must chelate the peptide.

3. The peptide must be cyclized (to satisfy the standard biological criteria for neuroactive drugs).
4. The peptide must be sufficiently lipophilic to traverse the BBB.
5. The Cu(II) cation should reside in an environment of cubic symmetry in order that it may be displaced more easily by Zn(II).
6. The environment of cubic symmetry should be tetrahedral as opposed to octahedral because of Zn(II)'s preference for coordination four and Cu(II)'s preference for a distorted octahedral environment.
7. The donor ligand should have a thioether functionality to ensure that the Cu(II) cation is sufficiently labile to allow Cu(II)/Zn(II) translocation.

These seven design criteria must be used to guide the rational design of a novel antiepileptic drug that uses neural metal mediated inhibition as its therapeutic mechanism of action.

3.3.7 Synthesis of Cyclic Dipeptide Copper (II) Complexes as Antiepileptic Drugs

3.3.7.1 Introduction

Using the principles of rational drug design, Cu(II) complexes of cyclic dipeptides possessing one or more thioether side chains were identified as a class of putative

antiepileptic drugs. Thus, the next logical step in this research program was to examine the structure and activity of the Cu(II) complex of *cyclo*-L-methionyl-L-methionine.

Here, previous studies proved useful. Both Ettorre *et al.* [311] and Khalil [312] synthesized dichloro(*cyclo*-L-methionyl-L-methionato)copper(II) and used ^1H nmr spectroscopy to confirm that the copper cation was coordinated to the cyclic dipeptide through one thioether sulfur atom. Furthermore, Khalil used molecular mechanics (parameterized specially for peptides and metal-ligand interactions) to theoretically determine the coordination geometry. He found that the diketopiperazine ring resides in a flat conformation (as opposed to a chair or boat conformation), the *cis* side chains are antiperiplanar with respect to the C_{sp3}-C_{sp2} bond of the ring, and diketopiperazine ligand coordinates Cu(II) and Zn(II) in a tetrahedral environment. Finally, Khalil found that dichloro(*cyclo*-L-methionyl-L-methionato)copper(II), hereafter symbolized as cMMCu, possessed anticonvulsant activity against the MES seizure model.

Remarkably, cMMCu meets all of the design criteria for a putative antiepileptic drug. Not surprisingly, it possessed significant anticonvulsant activity, however, cMMCu displayed activity only up to the sixth phase of NIH's eight phase Anticonvulsant Screening Project. Therefore, the task of this research program was to apply structure refinement to improve and possibly maximize antiepileptic activity. Through the design and evaluation of an analogue series, pharmacologic potency may be enhanced.

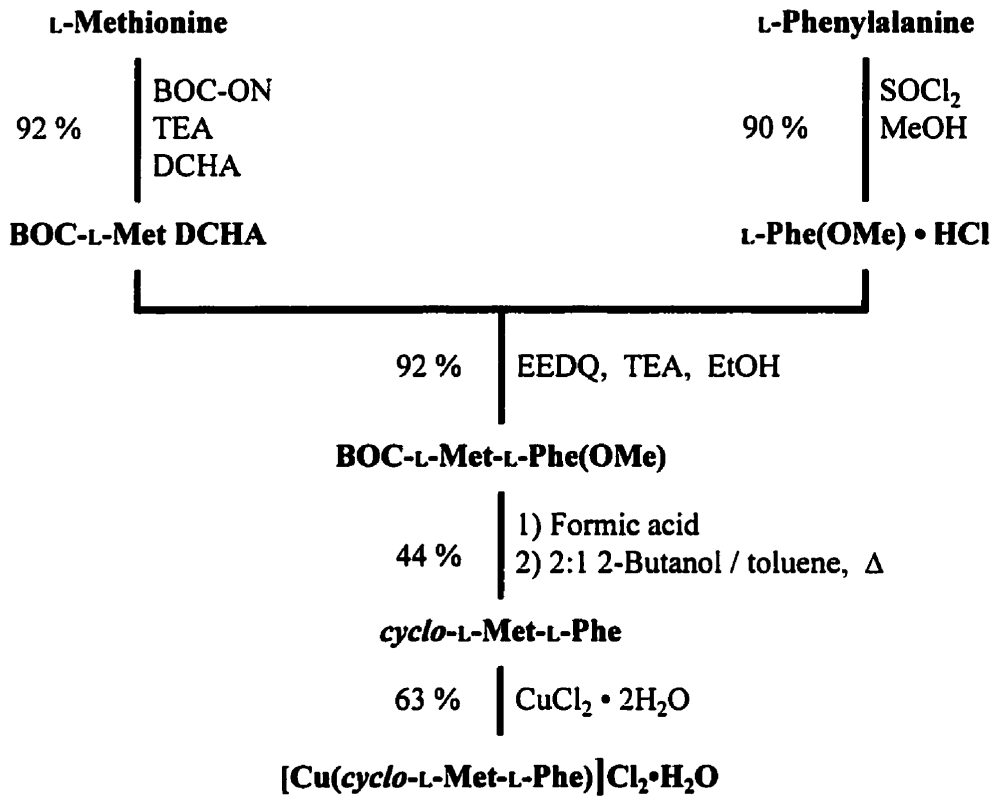
Since cMMCu functions, in theory, by depositing Cu(II) inside the brain and by removing Zn(II) from the brain, its potency is likely related to its ability to cross the BBB. Possibly, the polar nature of the two methionyl side chains hinder the passage of

cMMCu through the BBB resulting in a loss of efficacy. Since only one methionyl side chain is necessary for Cu(II) and Zn(II) coordination, the other side chain may be varied to influence compound lipophilicity in a bid to increase anticonvulsant activity. Therefore, copper(II) complexes of *cyclo*-L-methionyl-L-X (where X is an amino acid with a lipophilic side chain) are proposed as improved antiepileptic agents. Accordingly, Cu(II) complexes of *cyclo*-L-methionyl-L-phenylalanine and *cyclo*-L-leucyl-L-methionine were synthesized and evaluated for anticonvulsant activity. The ability of these Cu(II) complexes to traverse the BBB was measured *in vitro* and by an increase in *in vivo* anticonvulsant activity.

3.3.7.2 Method

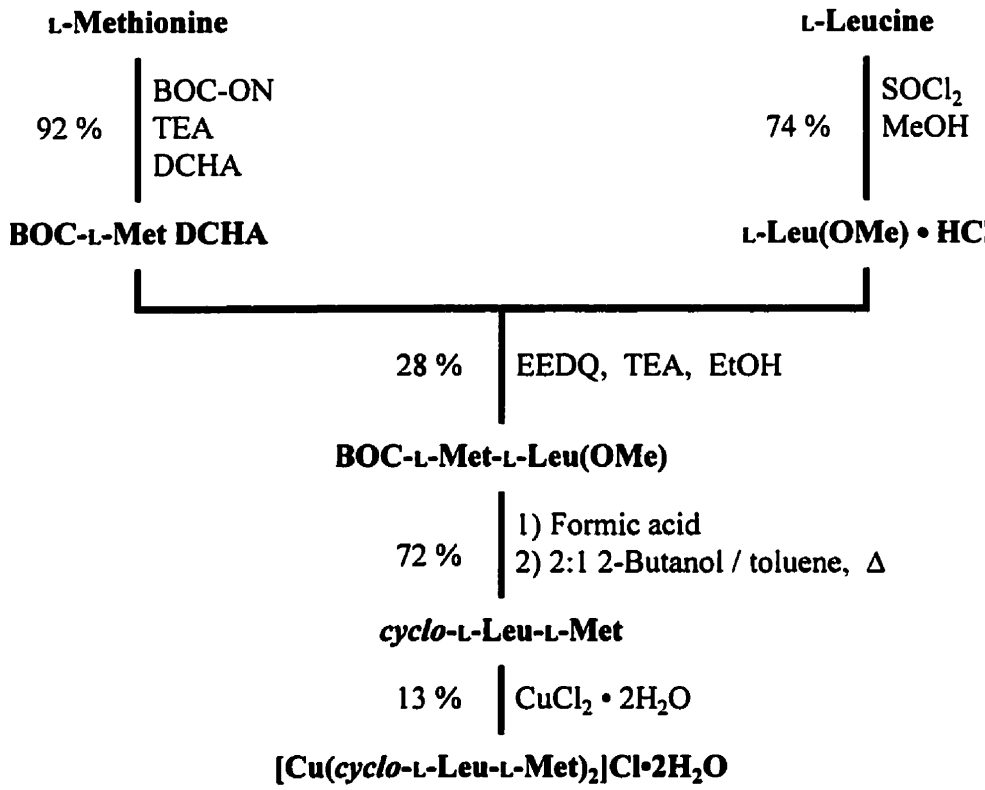
A five step, convergent synthesis was used to produce the copper complexes of *cyclo*-L-methionyl-L-phenylalanine (Scheme 3.1) and *cyclo*-L-leucyl-L-methionine (Scheme 3.2).

The synthesis of *cyclo*-L-methionyl-L-phenylalanine commenced with the N- and C-terminal protection of L-methionine and L-phenylalanine, respectively. Using the protocol developed by Itoh *et al.* [313], N-BOC-L-methionine was synthesized by treating L-methionine with 2-(*tert*-butoxycarbonylimino)-2-phenylacetonitrile (BOC-ON) in 50% aqueous dioxane. The N-protected amino acid was then isolated as the dicyclohexylamine (DCHA) salt. Following the procedure by Boissonnas *et al.* [314], L-phenylalanine



Scheme 3.1: Synthesis of [Cu(*cyclo*-L-methionyl-L-phenylalaninato)]Cl_2•H_2O

methyl ester hydrochloride salt was synthesized by treating L-phenylalanine with thionyl chloride in dry methanol. The N- and C-protected amino acids were then coupled using N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) to yield the fully protected dipeptide [315]. Anhydrous formic acid removed the N-protecting group [316], then the dipeptide methyl ester was simultaneously C-deprotected and cyclized in refluxing toluene/*sec*-butanol as described by Nitecki *et al.* [317]. The cyclic dipeptide and $CuCl_2$•$2H_2O$ were dissolved in an acetone/ethanol solution and heated to reflux for 24



Scheme 3.2: Synthesis of [Cu(*cyclo*-L-leucyl-L-methioninato)$_2$]Cl•2H$_2$O

hours. Upon cooling, the copper complex of *cyclo*-L-methionyl-L-phenylalanine precipitated; it was isolated and analyzed by mp, TLC, IR, ^{1}H nmr and elemental analysis.

The synthesis and analysis of the Cu(II) complex of *cyclo*-L-leucyl-L-methionine was analogous to the synthesis described above.

The synthetic strategy was devised to maximize efficiency (i.e. high yield, few steps) and maintain chiral integrity. The BOC group was used because N-protection proceeds in high yield, is free from racemization [318], and the *tert*-butyl ester group is

easily removed by anhydrous acid under mild conditions. Methyl ester was used for C-protection because the methoxy terminus is an excellent leaving group that facilitates concomitant C-deprotection and cyclization without racemization [317]. EEDQ was used because it allows the coupling of protected amino acids in high yield, in a single step, and without racemization [315].

The copper complexes were also tested for Zn(II)/Cu(II) transportability. In an *in vitro* experiment to mimic cation transport across the BBB, a Divided Beaker Cell Transport System was used to test the ability of a cyclic dipeptide to transport Cu(II) from one aqueous phase to another aqueous phase across an octanol "membrane phase" and subsequently to translocate Zn(II) in the opposite direction. Section B.2.1 in Appendix B provides details. Copper complexes were also tested for *in vivo* anticonvulsant activity against MES and PTZ seizure models. Cu(II) complexes which demonstrate significant anticonvulsant activity will be tested further for antiepileptogenic activity.

3.3.7.3 Results

Syntheses

The 5 step protocol was used to successfully synthesize chloro(*cyclo*-L-methionyl-L-phenylalaninato)Cu(II) hydrate and chlorobis(*cyclo*-L-leucyl-L-methioninato)copper(II) dihydrate as pure green crystals in overall yields of 23% and 2%, respectively. The Cu(II)

complexes (and their cyclic dipeptide precursors) were characterized (Table 3.10) to confirm structure and purity. Experimental details and analysis of other intermediates and substrates are provided in Appendix A.

The syntheses were facile and rapid. Product yields in the reaction sequences were high (>65%, near 90% in most cases) with the exception of three reactions: BOC-L-Met-L-Phe(OMe) was cyclized in 44% yield; BOC-L-Met DCHA and L-Leu(OMe)•HCl were coupled by EEDQ in 28% yield, and *cyclo*-L-leucyl-L-methione was complexed to Cu(II) in 13% yield. In part, these yields were low due to product retention in recrystallization solvent.

^{1}H nmr, TLC and mp analyses affirmed product purity. Fully assigned ^{1}H nmr was free from extraneous peaks caused by impurity. TLC gave homogeneous single spots and was ninhydrin negative for BOC-methionine and cyclic dipeptides, as expected. Melting points were sharp or within a few degrees C.

A comparison of the ^{1}H nmr spectra of the cyclic dipeptides and their Cu(II) complexes (Figure 3.8, Figure 3.9) reveals that the presence of paramagnetic copper broadens and shifts resonances and occasionally obscurs peak multiplicity. Cu(II) produced an upfield shift up to 0.04 ppm in the resonances of *cyclo*-L-methionyl-L-phenylalanine and *cyclo*-L-leucyl-L-methionine although, in the latter case, some differences in shift may be due to different ^{1}H nmr solvents.

Finally, the infrared spectra of the cyclic dipeptides and the copper(II) complexes were identical indicating that Cu(II) does not affect bond vibrations in the cyclic dipeptide.

Biological Activity

Cu(II)/Zn(II) transposition across a membrane phase was accomplished through the use of cyclic dipeptides. Using the Divided Beaker Cell Transport System, chloro(*cyclo*-L-methionyl-L-phenylalaninato)Cu(II) hydrate deposited Cu(II) in the "intra-CNS" aqueous phase then transported Zn(II) across the membrane phase to the "extra-CNS" aqueous phase at a rate of 0.71 mmol•day^{-1}. The Zn(II) transport rate using chlorobis(*cyclo*-L-leucyl-L-methioninato)copper(II) dihydrate was 0.61 mmol•day^{-1}. Thus, Cu(II)/Zn(II) exchange by cyclic dipeptides is theoretically possible *in vivo*. This may explain the anticonvulsant activity of the Cu(II) cyclic dipeptides in the MES seizure model — both Cu(II) complexes were active when administered ip at doses of 100 mg/kg. Neither Cu(II) cyclic dipeptide complex was active against PTZ-induced seizures. Furthermore, neither *cyclo*-L-methionyl-L-phenylalane or *cyclo*-L-leucyl-L-methionine was anticonvulsant; this shows that copper cation is a necessary component of Cu(II) cyclic dipeptide anticonvulsant drugs.

Table 3.10: Physical and biological data of *cyclo*-L-methionyl-L-phenylalanine, *cyclo*-L-leucyl-L-methionine, and their Cu(II) complexes

Code	Compound	Yield (%)	m.p. (°C)	TLC[a] (R_f)	IR (cm^{-1}) ν (NH)	IR (cm^{-1}) ν (C=O)	^{1}H nmr (DMSO-*d6*) δ
B4P20	*cyclo*-L-Methionyl-L-phenylalanine	43.9	263-264	0.39 (B) 0.52 (E)	3300	1650	8.24 (s, 1H), 8.10 (s, 1H), 7.25 (m, 3H), 7.18 (m, 2H), 4.22 (m, 1H), 3.72 (t, 1H), 3.18 (dd, 1H), 2.83 (dd, 1H), 1.88 (s, 3H+2H), 1.29 (m, 1H), 0.98 (m, 1H).
B4P28	*cyclo*-L-Leucyl-L-methionine	72.0	242	0.31 (B) 0.70 (C)	3420, 3300	1650	[b]7.50 (br s, 1H), 7.20 (br s, 1H), 4.17 (m, 1H), 3.98 (br d, 1H), 2.69 (m, 2H), 2.28 (m, 1H), 2.10 (s, 3H), 2.04 (m, 1H), 1.81 (m, 2H), 1.61 (m, 1H), 0.95(t, 6H).
B4P29	Chloro(*cyclo*-L-methionyl-L-phenylalaninato) copper(II) hydrate	62.7	238	0.46 (B) 0.72 (C)	3300	1650	8.21 (br s, 1H, NH), 8.08 (br s, 1H, NH), 7.22 (m, 3H, ArH), 7.15 (m, 2H, ArH), 4.20 (br s, 1H, αCH), 3.70 (br s, 1H, αCH), 3.14 (br d, 1H, CH_aHCH_2S), 2.81 (br d, 1H, CHH_bCH_2S), 1.88 (s, 3H+2H, SCH_3+$ArCH_2$), 1.25 (br s, 1H, CH_2CH_aHS), 0.98 (br s, 1H, CH_2CHH_bS).
B4P30	Chlorobis(*cyclo*-L-leucyl-L-methioninato)copper(II) dihydrate	12.7	199-201	0.37 (B) 0.79 (F)	3420, 3300	1650	8.19 (br s, 2H, 2NH), 3.89 (br s, 1H, αCH), 3.74 (br s, 1H, αCH), 2.52 (br s, 2H, CH_2S), 2.03 (s, 3H, SCH_3), 1.85 (m, 2H, CH_2CH_2S), 1.57 (m, 2H, $CH_2CH(CH_3)_2$), 1.45 (m, 1H, $CH_2CH(CH_3)_2$), 0.85 (t, 6H, $2CH_3$).

Table 3.10 continued

Code	Compound	Form	Formula		Elemental Analysis %C	%H	%N	Biological Activity[c,d]
B4P20	*cyclo*-L-Methionyl-L-phenylalanine	white crystals	-	-	-	-	-	0
B4P28	*cyclo*-L-Leucyl-L-methionine	white crystals	-	-	-	-	-	0
B4P29	Chloro(*cyclo*-L-methionyl-L-phenylalaninato)copper(II) hydrate	green crystals	$C_{14}H_{17}N_2O_2S$ $ClCu•H_2O$ MW = 394.37	Found: Calc:	42.54 42.64	4.52 4.86	7.00 7.10	2+
B4P30	Chlorobis(*cyclo*-L-leucyl-L-methioninato)copper(II) dihydrate	green crystals	$C_{22}H_{39}N_4O_4S_2$ $ClCu•2H_2O$ MW = 621.72	Found: Calc:	42.45 42.50	7.14 6.81	9.50 9.01	2+

a. Solvent systems used: B: CH_2Cl_2 / Acetone / AcOH 100:100:0.5; C: EtOH / AcOH 20:1; E: MEK / AcOH / H_2O 4:1:1; F: CH_2Cl_2 / MeOH 5:2.

b. $CDCl_3$

c. Using maximal electroshock, compound is active in > 2/4 rats at 10 mg/kg, 4+; 50 mg/kg, 3+; 100 mg/kg, 2+; 300 mg/kg, 1+; or inactive, 0.

d. No compounds were active in the pentylenetetrazole animal model.

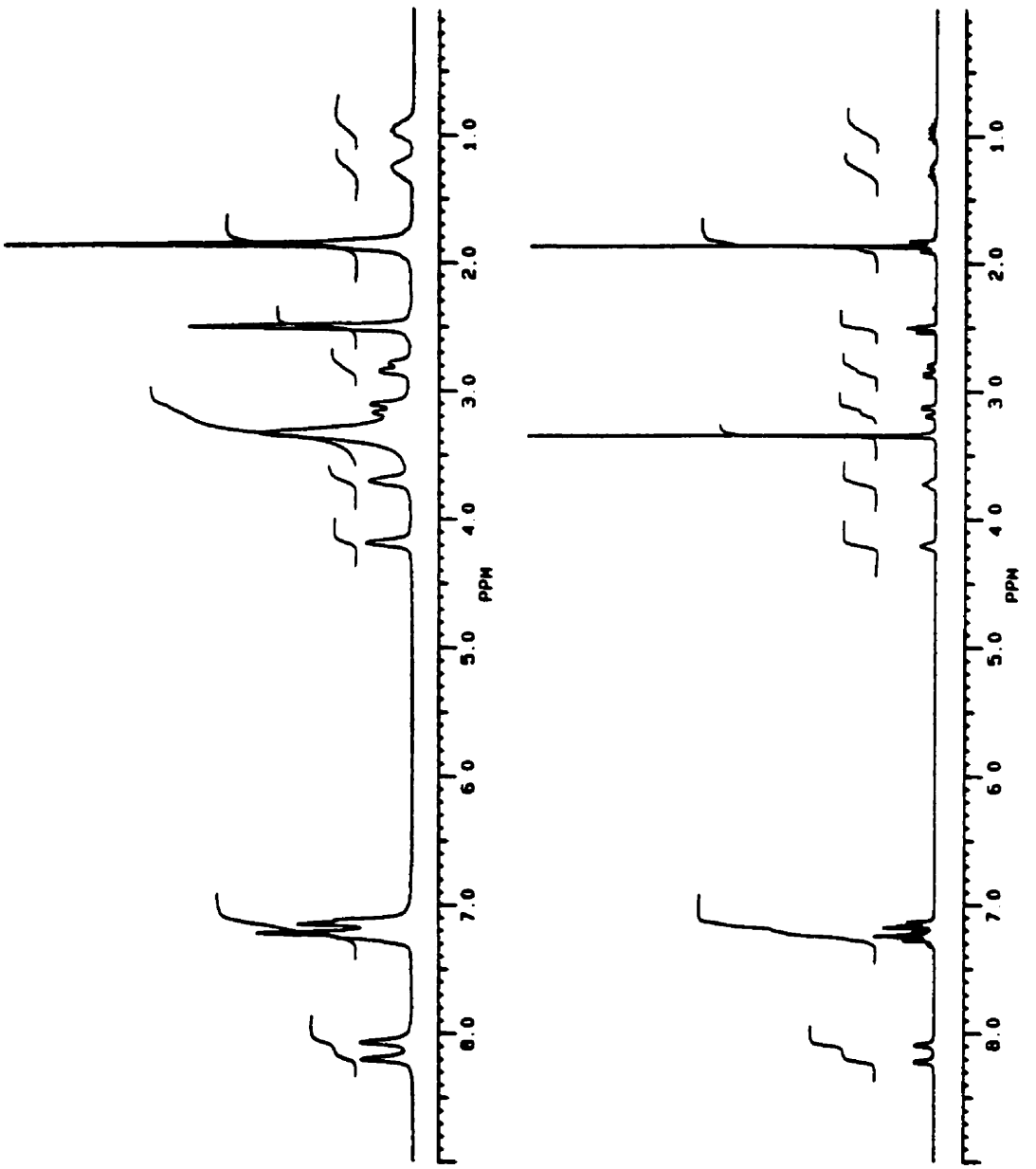


Figure 3.8: ^{1}H nmr spectra of *cyclo*-L-methionyl-L-phenylalanine and its Cu(II) complex (left), both in DMSO-*d*6

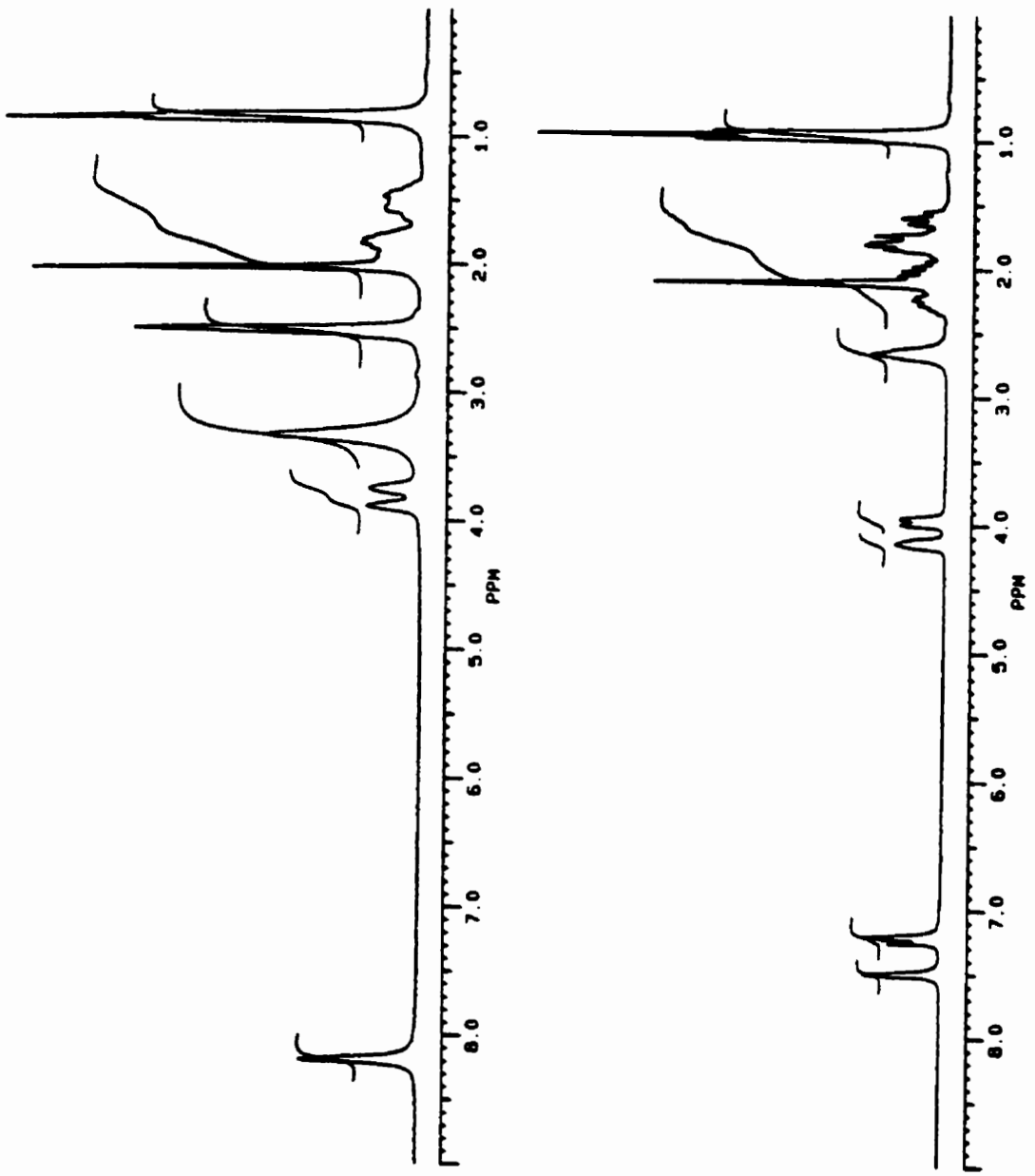


Figure 3.9: ^{1}H nmr spectra of *cyclo*-L-leucyl-L-methionine in $CDCl_3$ and its Cu(II) complex (left) in DMSO-*d*6

3.3.7.4 Discussion

Chloro(*cyclo*-L-methionyl-L-phenylalaninato)copper(II) hydrate (B4P29) and chlorobis(*cyclo*-L-leucyl-L-methioninato)copper(II) dihydrate (B4P30) demonstrate anticonvulsant activity at ip doses of 100 mg/kg. At this level of potency, these compounds are more active than Cu(II) complexes of non-peptides (in general), equally active with Cu(II) linear dipeptides, and less active than cMMCu. Although they were designed to be more resistant to degradation than Cu(II) linear dipeptides and more lipophilic and more active than cMMCu, anticonvulsant potency has not been enhanced. At least four reasons may account for this: 1) B4P29 and B4P30 require modification to enhance lipophilicity (and the ability to cross the BBB) further, 2) the difference in stability constants for the Cu(II) and Zn(II) complexes is too large; perhaps Cu(II) is not labile enough to allow replacement by Zn(II), 3) the Zn(II) transport rate is too slow; thus, the effect on brain Zn(II) concentrations is negligible, or 4) B4P29 and B4P30 may not have attained peak effect during the time scale of the MES seizure model. Reasons 3 and 4 may be a consequence of reasons 1 and 2; nevertheless, since the anticonvulsant activity of B4P29 and B4P30 is marginally less, and not greater, than the activity of cMMCu, an improvement in *in vivo* activity must be made.

To increase anticonvulsant activity, further structure refinement is required. Assuming that a cyclic dipeptide remains the base structure, structure refinement can be applied to the metal ion, the donor atom, or the ligand's side chains. First, Cu(II) should not be replaced or removed; Cu(II) affords anticonvulsant protection. Without Cu(II), the

cyclic dipeptide is inactive. Second, the donor atom, thioether sulfur, should not be changed (at least not initially) since the results of the Divided Beaker Transport System clearly demonstrate that the cyclic dipeptides B4P29 and B4P30 possess the ability to transpose Cu(II) and Zn(II) through a membrane phase. Third, replacement of one of the side chains with a more lipophilic side chain is the best next step in the drug design process. Analogues should be synthesized — the general classs dichloro(*cyclo*-L-methionyl-L-X)Cu(II), where X = tyrosine, isoleucine, valine should display considerable anticonvulsant activity. Syntheses of these analogues followed by complete physical and biological characterization should result in the analogue of optimal activity and should ascertain the nature of future modifications.

3.4 CONCLUSIONS

The primary goal of the research presented in this chapter was the rational design and synthesis of antiepileptic drugs based on neural metal mediated inhibition. Specifically, cyclic dipeptides complexed to Cu(II) were designed and synthesized to deposit Cu(II) in the brain, chelate Zn(II) then remove it as a mechanism of therapeutic antiepileptic action. A 6 step rational drug design strategy was used to synthesize chloro(*cyclo*-L-methionyl-L-phenylalaninato)copper(II) hydrate and chlorobis(*cyclo*-L-leucyl-L-methioninato)copper(II) dihydrate. Both compounds were anticonvulsant at ip doses of 100mg/kg. The synthesis of analogues with greater anticonvulsant activity was proposed.

A critical evaluation of the antiepileptic research presented in this chapter reveals both strengths and weaknesses. The weaknesses give rise to recommendations for further work.

Strengths

1. This research departs from traditional anticonvulsant (anti-ictogenic) research to focus, more importantly, on molecules and mechanisms of action to treat epileptogenesis.
2. A rational drug design process was used to synthesize compounds with moderate anticonvulsant activity.
3. Cyclic dipeptides were identified as attractive lead compounds. Their synthesis and characterization is simple and allows for the production of numerous analogues.

Weaknesses and recommendations for future work

1. Although the stoichiometry is known, the structure of the Cu(II) cyclic dipeptides has not been determined. Also, the geometry of the metal coordination environment remains unclear. X-ray crystallography would be quite useful for structure determination.

2. The stabilities of the metal complexes remain unknown. Stability constants should be measured and cyclic dipeptide analogues should be selected so as to minimize the difference in the stabilities of their Cu(II) and Zn(II) complexes.
3. The role of Cu(II) as an anticonvulsant is clear, however, the role of Zn(II) — the manipulation of its concentration in the brain — is not clear. Brain concentrations of Cu(II), Zn(II), L-glutamate and GABA need to be measured before and after rats ingest putative Cu(II) cyclic dipeptide antiepileptic drugs.

CHAPTER 4

CONCLUSIONS

From the outset of this research program, the goal was to use the techniques of rational drug design to devise and synthesize antiepileptic agents which would address the deficiencies of currently marketed epilepsy medications. While acknowledging that the accomplishment of that goal is really the domain of a multinational pharmaceutical company, nevertheless, the research presented in this thesis represents a significant and substantial step in that direction.

First, epileptogenesis was identified as the point of attack for an antiepileptic research program intent on actually treating epilepsy curatively and not merely symptomatically. Consequently, the mechanistic basis of epileptogenesis was delineated and used as the foundational assumption for rational drug design. The simultaneous agonism and antagonism of inhibitory and excitatory neurotransmitters, respectively, was identified as the most promising way to produce both an anti-ictogenic and antiepileptogenic effect. This required the design of a bifunctional molecule capable of unprecedented hybrid biological activity. To meet this challenge, two rational drug design strategies were conceived — inhibition mediated by neurotransmitters (a direct approach) and inhibition mediated by neural metals (an indirect approach).

The indirect approach attempted to simultaneously increase brain Cu(II) levels (potentially anticonvulsant) and decrease brain Zn(II) levels (potentially anticonvulsant and antiepileptogenic). In theory, removal of Zn(II) from the CNS would promote neural inhibition by decreasing L-glutamate concentration and by increasing GABA concentration. Cyclic dipeptide Cu(II)/Zn(II) chelators, capable of translocating Cu(II) and Zn(II) across a membrane phase, were designed, synthesized and biologically

evaluated; however, the complexes demonstrated only modest anti-ictogenic activity and therefore were not immediately evaluated in the time- and labour-intensive SRS model of epileptogenesis.

In contrast to the indirect approach, the direct inhibitory mediation of neurotransmitters by neurotransmitter analogues (β-amino acids) was exceptionally successful. In numerous ways, antiepileptic drug research and medicinal chemistry have been advanced significantly.

First, rational drug design, often maligned as more of a dream than reality, was used to pioneer the discovery of anti-ictogenic/antiepileptogenic molecules capable of simultaneously (i) inhibiting excitatory processes via antagonism of the NMDA receptor glycine co-agonist site and (ii) stimulating inhibitory processes via blockade of glial GABA uptake. For the first time, molecules with a reasonable potential to cure epilepsy have been identified.

Second, a simple but elegant synthesis of β-amino acids, unequalled in scope, was developed and used to synthesize numerous structurally diverse β-amino acids. This was necessary to define the parameters of the novel protocol and to make numerous analogues for biological evaluation.

Third, the novel SRS model of epileptogenesis was used to confirm the antiepileptic bimodal bioactivity of two β-amino acids, β-alanine and α-(4-phenylcyclo-hexyl)-β-alanine • HCl. Only two β-amino acids were evaluated due to the time- and labour-intensive nature of the SRS model; yet, both compounds were active. This clearly demonstrates the enormous potential for further research, discovery and development.

Accordingly, future efforts within this emerging antiepileptic research paradigm (Table 4.1) should focus on molecular design to increase the affinity of β-amino acids to the GABA uptake receptor and to the NMDA glycine co-agonist site with the goal of maximizing anti-ictogenic/antiepileptogenic activity. The β-amino acid analogue series, α-[1-arylmethyl-ω-(diarylmethoxy)alkyl]-β-alanines, has been proposed as a point of departure.

The development of this research culminating in the market introduction of a truly useful, curative antiepileptic drug is imperative. Enormous benefits would accrue. Health care systems would experience a substantial reduction in costs and society would experience significant gains in productivity. The total lifetime cost of all persons with epilepsy onset in the US in 1990 was estimated at $3.0 billion [319]; 38% of the cost was attributed to direct medical care, 62% was attributed to lost productivity due to inactivity, unemployment or excess mortality. With the introduction of curative antiepileptic agents, these costs would be reduced significantly.

Finally, the most important benefactors of antiepileptic drugs would be the people afflicted with epilepsy. They would benefit from drugs that attack root causes, not mere symptoms. They would experience a significant reduction in seizures, drug costs, side-effects and discrimination. Furthermore, as benefactors of a curative treatment, these people would experience a significant increase in societal re-integration, economic productivity, self esteem and general happiness. In short, people formerly afflicted with epilepsy would be granted a vastly improved quality of life. Lest any forget, this is the

end state of successful antiepileptic R&D; in the final analysis, this will always remain the true goal of antiepileptic drug design.

Table 4.1: Evolution of dominant antiepileptic research paradigms

	Traditional (1912 to 1970s)	Transitional (1970s to 1990s)	Emergent (21st Century)
Drug discovery techniques	Ignorance Serendipity Animal screens	Mechanistic understanding Computer assisted design	Mechanistic understanding Computer assisted design‡ Combinatorial libraries‡ Novel syntheses
Molecular structures	Ureides Heterocycles	Ureides Heterocycles Neurotransmitter analogues	Metal chelates Neurotransmitter analogues β-amino acids
Therapy	Anti-ictogenic (symptomatic)	Anti-ictogenic (symptomatic)	Anti-ictogenic (symptomatic) Antiepileptogenic (curative)
Major drug targets	Neuronal ion channels	Neuronal ion channels Neurotransmitter receptors	Neurotransmitter receptors Neurotransmitter pools
Therapeutic mechanisms	Ion channel blockade	Ion channel blockade GABAergic inhibition NMDA antagonism	Combined GABAergic and anti-glutamatergic inhibition Neural metal mediated inhibition
Drug efficacy	Moderate to low	Moderate	High
Drug toxicity	Moderate to high	Moderate	Low
Animal models	Chemoconvulsant Electroconvulsant	Chemoconvulsant Electroconvulsant	Chemoconvulsant Electroconvulsant Kindling‡ Spontaneous recurrent seizures

‡ Not described in this thesis.

APPENDIX A

CHEMICAL SYNTHESIS

A.1 General Experimental

NMR spectra were recorded on either a Bruker AM400 400 MHz or Bruker AM200 200 MHz FT-NMR spectrometer. Samples were dissolved in one of $CDCl_3$, DMSO-*d*6 or D_2O. Chemical shifts are reported as δ parts per million downfield of TMS. Chemical shifts were calibrated based on solvent peaks. Infrared spectra were recorded with either a Perkin-Elmer 598 spectrometer or a BOMEM Michelson series IR spectrometer using a KBr disk for solid samples or a Nujol mull for liquids. Melting points were determined using a Thomas Hoover Capillary Melting Point Apparatus and are uncorrected. Atomic absorption spectroscopy was performed using a Perkin-Elmer 1100B Atomic Absorption Spectrophotometer. All reagents were obtained from commercial sources (Aldrich, Fluka, or BDH) and were used as supplied. Super dry methanol was prepared by the method of Lund and Bjerrum [320]. Thin-layer chromatography (TLC) was performed using pre-coated Brinkmann silica gel 60 F_{254}-plates with aluminum backing. Solvent systems used for TLC are shown in Table A.1. TLCs were visualized using ultraviolet light, ninhydrin or iodine vapour. Elemental analyses were performed by Guelph Chemical Laboratories Ltd. of Guelph, Ontario, Canada.

Table A.1: List of solvent systems for TLC

Solvent	System	Ratio
A	CH_2Cl_2 / MeOH	9:2
B	CH_2Cl_2 / Acetone / AcOH	100:100:0.5
C	EtOH / AcOH	20:1
D	CH_2Cl_2 / EtOAc	9:1
E	MEK / AcOH / H_2O	4:1:1
F	CH_2Cl_2 / MeOH	5:2
G	CH_2Cl_2 / MeOH	5:1
H	MeOH / AcOH	5:1
I	EtOAc / MeOH	9:1
J	$CHCl_3$ / Acetone / H_2O	88:12:15
K	MeOH / AcOH	5:1
L	EtOH / AcOH	50:1
M	$CHCl_3$ / MeOH / NH_4OH	70:25:5
N	2-Butanol / AcOH / H_2O	4:1:1
O	Cyclohexane / EtOAc	1:1
P	MeCN / H_2O / AcOH	8:1:1
Q	$CHCl_3$	

A.2 Synthesis of β-Aminothiophenecarboxylic Acid Alkyl Esters

A.2.1 Beck Method

A.2.1.1 General Procedure for the Synthesis of Methyl 3-Aminobenzo[b]thiophene-2-carboxylates

Following the procedure by Beck [162], a solution of KOH (3 g) in water (15 ml) was added dropwise to a cold solution of DMF (60 ml) containing substituted *o*-nitrobenzonitrile (30 mmol) and methyl thioglycolate (2.68 ml, 30 mmol). The mixture was stirred in the cold for 1 hour then poured into ice water. The solid crude product was collected and recrystallized from EtOH or EtOH/H_2O.

Procedural recommendation: To eliminate stench, use Javex© as a source of sodium hypochlorite to oxidize sulfur contained in unreacted methyl thioglycolate in the aqueous filtrate.

A.2.1.2 List of 4 Compounds Synthesized

Using the appropriate *o*-nitrobenzonitrile precursors, the following methyl 3-aminobenzo[b]thiophene-2-carboxylates were synthesized via the Beck method:

1. Methyl 3-aminobenzo[b]thiophene-2-carboxylate (B3P107)
2. Methyl 3-amino-4-methylbenzo[b]thiophene-2-carboxylate (B3P109)
3. Methyl 3-amino-6-(trifluoromethyl)benzo[b]thiophene-2-carboxylate (B3P141)
4. Methyl 3-amino-4-cyanobenzo[b]thiophene-2-carboxylate (B5P9)

Analytical and biological activity data is presented in Table 2.9 on page 80.

A.2.2 Gewald Method

A.2.2.1 General Procedure for the Synthesis of Methyl 2-Aminothiophene-3-carboxylates

Following Gewald's method [163,188], a mixture of alkyl ketone (30 mmol), methyl cyanoacetate (2.65 ml, 30 mmol), sulfur (1.0 g, 31 mmol) and morpholine (25 ml) in EtOH (200 ml) was stirred at 60^0C until the sulfur was consumed. The solution was reduced by half then poured into ice water (200 ml). The resulting precipitate was filtered, washed with water and dried *in vacuo*, then recrystallized from EtOH, EtOH/H_2O or EtOAc. If not all of the sulfur was consumed, the isolated precipitate (2-aminothiophene-3-carboxylate methyl ester plus sulfur impurity) was dissolved in $CHCl_3$ and the insoluble S_8 impurity was filtered off . Sulfur has only a 1.5 % solubility in $CHCl_3$ at 18^0C.

Note that in some cases, solvolysis resulted in the exchange of the methyl ester for ethyl ester.

A.2.2.2 List of 11 Compounds Synthesized

Using the appropriate alkyl ketone precursors, the following methyl or ethyl 2-aminothiophene-3-carboxylates were synthesized via the Gewald method:

1. Methyl 2-amino-4-ethyl-5-methylthiophene-3-carboxylate (B3P91)
2. Methyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (B3P99)
3. Methyl 2-aminocyclopenta[b]thiophene-3-carboxylate (B3P101)

4. Methyl 2-amino-6-*tert*-butyl-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (B3P113)
5. Methyl 2-amino-4,5,6,7-tetrahydro-6-methylbenzo[b]thiophene-3-carboxylate (B3P135)
6. Ethyl 2-aminocyclohepta[b]thiophene-3-carboxylate (B3P137)
7. Methyl 2-amino-6-carbethoxy-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (B3P145)
8. Methyl 2-amino-4,5,6,7-tetrahydro-6-phenylbenzo[b]thiophene-3-carboxylate (B3P147)
9. Ethyl 2-aminocyclododeca[b]thiophene-3-carboxylate (B3P157)
10. Methyl 2-amino-4,5,6,7-tetrahydro-6-methylthieno[2,3-c]pyridine-3-carboxylate (B3P165)
11. Methyl 2-amino-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (B4P177)

Analytical and biological activity data is presented in Table 2.11 to Table 2.13 starting on page 84.

A.2.3 Fiesselmann Method using Acetophenones

A.2.3.1 General Procedure for the Synthesis of Methyl 3-Aminothiophene-2-carboxylates

Following an adaptation [165,166] of Fiesselmann's method, phosphoryl chloride (9.3 ml, 100 mmol) was added dropwise from a dropping funnel to cold, dry DMF (50 ml) in a three-neck flask, while maintaining solution temperature below 25^0C. To this formamide chloride solution, acetyl arene (50 mmol) was added such that the solution temperature did not exceed 60^0C. After 0.5 hours of stirring at room temperature, the mixture was warmed to 50^0C then treated with NH_2OH•HCl (13.9 g, 200 mmol) in small portions (temperature rose to 100-150^0C). After 30 minutes, the mixture was added to water (150 ml) and extracted with ether (3 x 50 ml). The combined extracts were dried ($MgSO_4$), filtered and reduced *in vacuo* to yield a β-chlorocinnamonitrile product.

A basic, methanolic solution was prepared by stirring sodium methoxide (50 mmol) into dry MeOH (50 ml). To this basic solution, methyl thioglycolate (50 mmol) was added dropwise then the β-chlorocinnamonitrile, dissolved in MeOH or DMF, was added and the mixture was heated to reflux for 10-60 minutes. The solution was cooled, diluted with ice water (200 ml) and filtered. After drying, the methyl 3-aminothiophene-2-carboxylate product was recrystallized from EtOH or EtOH/H_2O.

A.2.3.2 List of 4 Compounds Synthesized

Using the appropriate acetophenone precursors, the following methyl 3-aminothiophene-2-carboxylates were synthesized via the Fiesselmann method:

1. Methyl 3-amino-5-(3-nitrophenyl)thiophene-2-carboxylate (B3P171)
2. Methyl 3-amino-5-phenylthiophene-2-carboxylate (B3P177)
3. Methyl 3-amino-5-(4-methoxyphenyl)thiophene-2-carboxylate (B4P51)
4. Methyl 3-amino-5-(4-methylphenyl)thiophene-2-carboxylate (B4P55)

Analytical and biological activity data is presented in Table 2.10 on page 81.

A.2.4 Fiesselmann Method using Arylaldehydes

A.2.4.1 General Procedure for the Synthesis of Methyl 3-Aminothiophene-2-carboxylates

A.2.4.1.1 Synthesis of 2,3-Dichloro-5-methoxy-2(5H)-furanone (B3P103)

Following an adaptation of Mowry's procedure [321], mucochloric acid (527.1 g, 3.12 mol), dry MeOH (1.30 L) and anhydrous $ZnCl_2$ (212.7 g, 1.56 mol) were refluxed for 7 hours then added to water (1.50 L) and extracted with ether (3 x 600 ml). The combined ether extracts were reduced *in vacuo* and the residue was fractionally distilled (bp: 126.5-128.5^{0}C at 9 mm Hg) to yield pure product as a clear yellow liquid (465.3 g,

2.54 mol, 81.5 %); lit. yields=85 % [167,322], 77 % [321]; IR (cm^{-1}): 2926 (CH aliphatic), 1806 (lactone C=O); ^1H nmr ($CDCl_3$): δ 5.76 (s, 1H), 3.58 (s, 3H).

Caution: Distillation products are extremely potent lachrymators.

A.2.4.1.2 Synthesis of 4-Azido-3-chloro-5-methoxy-2(5H)-furanone (B3P123)

Following the procedure by Moore *et al.* [168,323], sodium azide (136.1 g, 2.09 mol) was added to a cold stirred solution of 2,3-dichloro-5-methoxy-2(5H)-furanone (383.0 g, 2.09 mol) in dry MeOH (600 ml). After 1 hour, the mixture was diluted with water (1.00 L) and filtered. The off-white crystalline product was washed with H_2O, air dried and dried *in vacuo* to yield pure product as white crystals (372.0 g, 1.96 mol, 93.9 %); lit. yield=91 % [168]; mp: 51-55^{0}C; TLC: R_f=0.70 (I), 0.58 (J); IR (cm^{-1}): 2150 (N_3), 1777 (lactone C=O); ^1H nmr ($CDCl_3$): δ 5.76 (s, 1H), 3.63 (s, 3H).

A.2.4.1.3 Synthesis of Methyl 3-Aminothiophene-2-carboxylates

4-Azido-3-chloro-5-methoxy-2(5H)-furanone (5.69 g, 30 mmol) and arylaldehyde (30 mmol) were refluxed in toluene (60 ml) for 5 hours then reduced to yield β-aryl-α-chloroacrylonitrile, in accordance with the procedure by Moore *et al.* [324].

Then, following the procedure by Ishizaki *et al.* [170], methyl thioglycolate (2.68 ml, 30 mmol) was added to a stirred solution of K_2CO_3 (8.3 g, 60 mmol) in MeOH (170 ml) maintained at 0-10^{0}C. β-Aryl-α-chloroacrylonitrile (30 mmol) in MeOH (30 ml) was added dropwise from a dropping funnel over 1 hour. The solution was stirred at ambient

temperature for 5 hours, was diluted with ice water (200 ml) and was filtered. After drying, the product was recrystallized from EtOH or EtOH/EtOAc.

A.2.4.2 List of 4 Compounds Synthesized

Using the appropriate arylaldehyde precursors, the following methyl 3-aminothiophene-2-carboxylates were synthesized via the Fiesselmann method:

1. Methyl 3-amino-5-(4-methylphenyl)thiophene-2-carboxylate (B4P91)
2. Methyl 3-amino-5-(2-thienyl)thiophene-2-carboxylate (B4P95)
3. Methyl 3-amino-5-(3,4-methylenedioxyphenyl)thiophene-2-carboxylate (B4P103)
4. Methyl 3-amino-5-[3-methoxy-4-(4-nitrobenzyloxy)phenyl]thiophene-2-carboxylate (B4P107)

   Note: due to poor solubility in MeOH, the β-aryl-α-chloroacrylonitrile was dissolved in DMF and the solution was refluxed for 4 hours.

Analytical and biological activity data is presented in Table 2.10 on page 81.

A.3 Synthesis of Alkyl Acetamidothiophenecarboxylates

A.3.1 General Procedure for N-Acetyl Protection via Acetic Anhydride

Using a standard procedure [171,172], acetamidothiophenecarboxylic acid alkyl esters were prepared by refluxing the corresponding amino compound with excess Ac_2O (4 equiv.) in anhydrous AcOH for 1 hour. The mixture was poured in cold water and the product was isolated by filtration, washed with water and recrystallized from EtOH.

A.3.2 List of 18 Compounds Synthesized

N-Acetylation of the appropriate alkyl β-aminothiophenecarboxylates produced the following compounds:

1. Methyl 3-acetamidobenzo[b]thiophene-2-carboxylate (B6P43)
2. Methyl 3-acetamido-6-(trifluoromethyl)benzo[b]thiophene-2-carboxylate (B6P49)
   Note: B3P141 required reflux in Ac_2O for 2.5 hours to produce B6P49.
3. Methyl 2-acetamido-4-ethyl-5-methylthiophene-3-carboxylate (B6P59)
4. Methyl 2-acetamido-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (B6P37)
5. Methyl 2-acetamidocyclopenta[b]thiophene-3-carboxylate (B6P61)
6. Methyl 2-acetamido-6-*tert*-butyl-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (B6P63) [*Cautionary note: Compound is a mild sternutator.*]
7. Ethyl 2-acetamidocyclohepta[b]thiophene-3-carboxylate (B6P65)
8. Ethyl 2-acetamidocyclododeca[b]thiophene-3-carboxylate (B6P123)

9. Methyl 2-acetamido-6-carbethoxy-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (B6P67)
10. Methyl 2-acetamido-4,5,6,7-tetrahydro-6-phenylbenzo[b]thiophene-3-carboxylate (B6P47)
11. Methyl 2-acetamido-4,5,6,7-tetrahydro-6-methylthieno[2,3-c]pyridine-3-carboxylate (B6P39)
12. Methyl 3-acetamido-5-(3-nitrophenyl)thiophene-2-carboxylate (B6P69)
13. Methyl 3-acetamido-5-phenylthiophene-2-carboxylate (B6P45)
14. Methyl 3-acetamido-5-(4-methoxyphenyl)thiophene-2-carboxylate (B6P41)
15. Methyl 3-acetamido-5-(4-methylphenyl)thiophene-2-carboxylate (B6P71)
16. Methyl 3-acetamido-5-(2-thienyl)thiophene-2-carboxylate (B6P53)
17. Methyl 3-acetamido-5-(3,4-methylenedioxyphenyl)thiophene-2-carboxylate (B6P55)
18. Methyl 3-acetamido-5-[3-methoxy-4-(4-nitrobenzyloxy)phenyl] thiophene-2-carboxylate (B6P73)

Analytical and biological activity data is presented in Table 2.9 to Table 2.13 beginning on page 80.

A.4 Synthesis of Substituted N-Acetyl-β-alanine Ethyl and Methyl Esters

A.4.1 Synthesis of Raney Nickel Catalyst

A solution of NaOH (320.0 g, 8 mol) in water (1.2 L) was mechanically stirred in a 2.0 L flask. After cooling to 10^0C in an ice-bath, nickel aluminum alloy (250 g) was added in small portions over 90 minutes. The resulting suspension was stirred at room temperature for 1 hour and at 50^0C for an additional 8 hours. The suspension was transferred to a graduated cylinder and the aqueous supernatant was decanted. The resulting slurry was shaken with 2.5 M aqueous NaOH solution (200 ml) then decanted. The nickel catalyst was washed 30 times by suspension in water (150 ml) followed by decanting. The washing was repeated 3 times with absolute EtOH (100 ml) and the resulting Raney nickel (W-5 [176]) was stored under absolute EtOH.

A.4.2 General Procedure for Raney Nickel Reductive Desulfurization

Alkyl acetamidothiophenecarboxylate (20 mmol) and freshly prepared W-5 Raney nickel (8 equiv.) were refluxed in EtOH (75 ml) with vigorous stirring for 16 hours. The hot mixture was filtered through diatomaceous earth (Celite) and the nickel residue was washed with hot EtOH (50 ml). The filtrate was concentrated to yield pure N-acetyl-β-alanine alkyl ester as a clear oil, a gum or white crystals.

A.4.3 List of 17 Compounds Synthesized

Raney nickel reductive desulfurization was used to synthesize the following compounds:

1. N-Acetyl-α-(1-ethylpropyl)-β-alanine methyl ester (B6P93)
2. N-Acetyl-α-cyclohexyl-β-alanine ethyl and methyl esters (B6P77)
3. N-Acetyl-α-cyclopentyl-β-alanine ethyl and methyl esters (B6P85)
4. N-Acetyl-α-cyclododecyl-β-alanine ethyl ester (B6P133)
5. N-Acetyl-α-(4-*tert*-butylcyclohexyl)-β-alanine methyl ester (B6P109)
6. N-Acetyl-α-cycloheptyl-β-alanine ethyl ester (B6P117)
7. N-Acetyl-α-[4-(N-carbethoxypiperidinyl)]-β-alanine methyl ester (B6P105)
8. N-Acetyl-α-(4-phenylcyclohexyl)-β-alanine methyl ester (B6P81)
9. N-Acetyl-β-phenyl-β-alanine methyl ester (B5P65)
10. N-Acetyl-β-(4-trifluoromethylphenyl)-β-alanine methyl ester (B6P140)
11. N-Acetyl-β-[*m*-(ethylamino)phenethyl]-β-alanine methyl ester (B6P101)
12. N-Acetyl-β-phenethyl-β-alanine methyl ester (B5P73)
13. N-Acetyl-β-(*p*-methoxyphenethyl)-β-alanine methyl ester (B5P69)
14. N-Acetyl-β-[2-(4-methylphenyl)ethyl]-β-alanine methyl ester (B6P89)
15. N-Acetyl-β-hexyl-β-alanine methyl ester (B6P97)
16. N-Acetyl-β-[2-(3,4-methylenedioxyphenyl)ethyl]-β-alanine methyl ester (B6P113)
17. N-Acetyl-β-[2-(3-methoxy-4-hydroxyphenyl)ethyl]-β-alanine methyl ester (B6P119)

Addendum to Procedure: After B6P119 was reductively desulfurized using W-5 Raney nickel, the filtered solution was dissolved in hot EtOAc then washed with 0.5 N HCl (2 x 30 mL) and H_2O. The organic layer was dried ($MgSO_4$), filtered and concentrated to yield the title compound as a yellow oil.

Analytical and biological activity data is presented in Table 2.14 to Table 2.18 beginning on page 90.

A.5 Synthesis of α- and β-Substituted β-alanines

A.5.1 General Procedure for N-Acetyl and Ester Deprotection via Acidolysis

The doubly protected α- or β-substituted β-alanine was refluxed in 6 M HCl for 5 hours. The solution was evaporated (to remove H_2O, HCl, MeOH and AcOH) and the residue was twice dissolved in distilled H_2O and concentrated (to remove residual HCl). The product was recrystallized from EtOH to yield the hydrochloride salt as white crystals. Alternatively, the crude product was dissolved in a minimum volume of hot H_2O and titrated with NH_4OH until the free β-amino acid precipitated. Two volumes of EtOH or MeOH were added to aid the separation of the product and prevent clumping. The mixture was cooled (4^0C) for 24 hours to encourage further precipitation then was filtered. The product was washed with ice cold H_2O and EtOH then was recrystallized from MeOH to yield pure substituted β-alanine as white crystals.

A.5.2 List of 8 Compounds Synthesized

Acidolysis was used to synthesize the following β-alanines:

1. α-Cyclohexyl-β-alanine (B5P119)
2. α-Cyclododecyl-β-alanine hydrochloride salt (B5P131)
3. α-(4-*tert*-Butylcyclohexyl)-β-alanine hydrochloride salt (B5P127)
4. α-(4-Phenylcyclohexyl)-β-alanine hydrochloride salt (B5P107)
5. β-Phenyl-β-alanine hydrochloride salt (B5P91)
6. β-Phenethyl-β-alanine (B5P95)
7. β-(*p*-Methylphenethyl)-β-alanine (B5P111)
8. β-[2-(4-Hydroxy-3-methoxyphenyl)ethyl]-β-alanine hydrochloride salt (B6P145)

Analytical and biological activity data is presented in Table 2.14 to Table 2.18 beginning on page 90.

A.6 Synthesis of Zn(II) Complexes

A.6.1 Organic Ligand Zn(II) Complexes

A.6.1.1 General Procedure

Organic ligand (2 equiv.) and either $ZnCl_2$ (1 equiv.) or $Zn(NO_3)_2 \cdot 6H_2O$ (1 equiv.) were dissolved in EtOH, MeOH or H_2O and refluxed for 4 - 24 hours. Crystals of Zn(II) complex were isolated either by filtering crystals from the cooled solution or by evaporating the solution to dryness. Product was washed with EtOH and Et_2O to remove unreacted starting material. The white crystals were dried *in vacuo* then were analyzed by melting point and elemental analysis.

A.6.1.2 List of 9 Compounds Synthesized

The chelation of organic ligand by Zn(II) produced the following compounds:

1. Dichlorobis(triphenylphosphine oxide)zinc(II) (B2P32)
2. Sodium adenosine triphosphatozinc(II) dihydrate (B2P34)
3. Dichloro(N,N,N',N'-tetramethylethylenediamine) zinc(II) (B2P52)
4. Chloro(diethanolamino)zinc(II) (B2P56)
5. Bis(3,3'-dimethoxybenzidine)dinitratozinc(II) (B2P70)
6. Bis(benzimidazole)zinc(II) hemihydrate (B2P72)
7. Bis(N-benzoyl-N-phenylhydroxylamine)dinitratozinc(II) (B2P76)
8. Bis(2-aminobenzothiazole)dinitratozinc(II) (B2P80)

9. (12-Crown-4)dinitratozinc(II) trihydrate (B2P122)

Analytical and biological activity data is presented in Table 3.8 on page 167.

A.6.2 Bis(5,5-diphenylhydantoinato)diamminezinc(II) Hemihydrate (B1P134)

Long, clear, needle-shaped crystals of title compound (0.2405 g, 0.39 mmol, 38.6%) were obtained by slow evaporation (at a constant temperature of 50°C) of aqueous ammonia containing phenytoin (0.5135 g, 2.03 mmol) and $Zn(NO_3)_2 \cdot 6H_2O$ (0.3024 g, 1.02 mmol). Elemental analysis revealed the presence of a half molecule of water per one molecule of the complex. Calculated: %C 59.0; %H 4.8; %N 13.8; %O 11.8; Found: %C 59.0; %H 4.8; %N 13.7; %O 10.7. mp: 230^0C (dec); TLC: R_f=0.77 (L).

A.7 Synthesis of Cu(II) Complexes

A.7.1 Organic Ligand Cu(II) Complexes

A.7.1.1 General Procedure

Organic ligand (2 equiv.) and either $CuCl_2 \cdot 2H_2O$ (1 equiv.) or $Cu(NO_3)_2 \cdot 3H_2O$ (1 equiv.) were dissolved in EtOH, MeOH or H_2O and refluxed for 4 to 24 hours. Crystals of Cu(II) complex were isolated either by filtering crystals from the cooled solution or by evaporating the solution to dryness. Product was washed with EtOH and Et_2O to remove unreacted starting material. The blue or green crystals were dried *in vacuo* then were analyzed by melting point and elemental analysis.

A.7.1.2 List of 6 Compounds Synthesized

The chelation of organic ligand by Cu(II) produced the following compounds:

1. Dichlorobis(triphenylphosphine oxide)copper(II) (B2P132)
2. Dichloro(N,N,N',N'-tetramethylethylenediamine)copper(II) (B2P144)
3. Bis(benzimidazole)dinitrato copper(II) hemihydrate (B2P160)
4. Bis(N-benzoyl-N-phenylhydroxylamino)copper(II) nitrate (B2P164)
5. Bis(diphenylthiocarbazonato)copper(II) (B2P178)
6. Bis[tris(carboxymethyl)amino]copper(II) dihydrate (B2P182)

Analytical and biological activity data is presented in Table 3.7 on page 165.

A.7.2 Linear Dipeptide Cu(II) Complexes

A.7.2.1 General Procedure

According to the method of Manyak *et al.* [325], $Cu(NO_3)_2 \cdot 3H_2O$ (1 equiv.) was dissolved in H_2O and converted to the hydroxide by treatment with an excess of 10 N $NaOH_{(aq)}$. The blue precipitated cupric hydroxide was washed with H_2O by decantation until free of inorganic salts then was treated in aqueous suspension with dipeptide (1 equiv.). The excess cupric hydroxide was filtered off and the resulting deep blue solution was reduced to 50 ml. Addition of EtOH:EtOAc (1:1) or acetone precipitated the linear dipeptide Cu(II) complex as blue crystals which were subsequently dried *in vacuo* and analyzed by melting point and elemental analysis.

A.7.2.2 List of 3 Compounds Synthesized

Using the method of Manyak *et al.* [325], Cu(II) was complexed to linear dipeptide to produce the following compounds:

1. Glycylglycinatocopper(II) hydrate (B1P140)
2. (L-Phenylalanyl-L-tryptophanato)copper(II) hemihydrate (B1P148)
3. (L-Leucyl-L-tryptophanato)copper(II) sesquihydrate (B1P151)

Analytical and biological activity data is presented in Table 3.7 on page 165.

A.7.3 Cyclic Dipeptide Cu(II) Complexes

A.7.3.1 Chloro(*cyclo*-L-methionyl-L-phenylalaninato)copper(II) hydrate (B4P29)

cyclo-L-Methionyl-L-phenylalanine (0.5617 g, 2.02 mmol) was added to a solution of $CuCl_2 \cdot 2H_2O$ (0.3456 g, 2.03 mmol) in acetone / EtOH (1:1, 18 ml) and the solution was refluxed for 24 hrs. Green crystals were obtained by vacuum filtration. The crystals were crushed to a fine powder, then added to a solution of $CuCl_2 \cdot 2H_2O$ (0.3394 g, 1.99 mmol) in acetone / EtOH (1:1, 30 ml). The solution was again refluxed for 24 hrs, then filtered to yield green crystals. The crystals were dried *in vacuo* yielding chloro(*cyclo*-L-methionyl-L-phenylalaninato)copper(II) hydrate (0.4996 g, 1.27 mmol, 62.7%); mp: 238^{0}C; TLC: R_f=0.46 (B), 0.72 (C); IR (cm^{-1}): 3300 (N-H), 1650 (C=O amide), 1445 (C=C), 1335 (C=C), 1095 (C-N), 700 (C=CH); ^{1}H nmr (DMSO-*d6*): δ 8.21 (br s, 1H), 8.08 (br s, 1H), 7.22 (m, 3H), 7.15 (m, 2H), 4.20 (br s, 1H), 3.70 (br s, 1H), 3.14 (br d, 1H), 2.81 (br d, 1H), 1.88 (s, 3H+2H), 1.25 (br s, 1H), 0.98 (br s, 1H); Calculated: %C 42.64; %H 4.86; %N 7.10; Found: %C 42.54; %H 4.52; %N 7.00.

A.7.3.2 Chlorobis(*cyclo*-L-leucyl-L-methioninato)copper(II) dihydrate (B4P30)

cyclo-L-Leucyl-L-methionine (0.7447 g, 3.05 mmol) was added to a solution of $CuCl_2 \cdot 2H_2O$ (0.5202 g, 3.05 mmol) in acetone / EtOH (1:1, 18 ml) and the solution was refluxed for 24 hrs. Green crystals were isolated by vacuum filtration. The crystals were crushed to a fine powder, then added to a solution of $CuCl_2 \cdot 2H_2O$ (0.5328 g, 3.12 mmol) in acetone / EtOH (1:1, 35 ml) The solution was again refluxed for 24 hrs, then filtered to yield green crystals. The crystals were dried *in vacuo* yielding chlorobis(*cyclo*-L-leucyl-L-methioninato)copper(II) dihydrate (0.1204 g, 0.19 mmol, 12.7 %); mp: 199-201^{0}C; TLC: R_f=0.37 (B), 0.79 (F); IR (cm^{-1}): 3420 (NH), 3300 (NH), 1650 (amide C=O), 1090 (CN); ^{1}H nmr (DMSO-*d6*): δ 8.19 (br s, 2H), 3.89 (br s, 1H), 3.74 (br s, 1H), 2.52 (br s, 2H), 2.03 (s, 3H), 1.85 (m, 2H), 1.57 (m, 2H), 1.45 (m, 1H), 0.85 (t, 6H).

A.8 Synthesis of Cyclic Dipeptides

A.8.1 *cyclo*-L-Methionyl-L-phenylalanine

A.8.1.1 BOC-L-Methionine Dicyclohexylammonium Salt

L-Methionine (3.1238 g, 20.94 mmol), BOC-ON (5.3047 g, 21.54 mmol), triethylamine (4.3 ml, 31 mmol) and dioxane / H_2O (1:1, 50 ml) were stirred for 24 hrs then concentrated. After the addition of water (40 ml), the solution was washed with Et_2O (3 x 20 ml) and the pH of the aqueous layer was adjusted to 3.2 by the dropwise addition of 10 % $KHSO_{4\ (aq)}$. The solution was then extracted with Et_2O (3 x 20 ml) and the

combined organic extracts were dried ($MgSO_4$), filtered and concentrated to yield crude BOC-amino acid as a brown oil. The oil was dissolved in a minimum of CH_2Cl_2 and an equimolar amount of dicyclohexylamine was added. The mixture was stirred 24 hrs then filtered and the product dried *in vacuo* to yield BOC-L-methionine dicyclohexylammonium salt as light brown crystals (8.2700 g, 19.26 mmol, 91.7 %); mp: 134-136^{0}C; TLC: R_f=0.83 (A), 0.54 (B); IR (cm^{-1}): 3420 (NH), 1700 (ester C=O), 1615 (amide C=O), 1160 (C-O); ^{1}H nmr (DMSO-*d*6): δ 6.17 (d, 1H), 3.67 (m, 1H), 2.92 (br s, 2H), 2.50 (s, 1H), 2.38 (m, 2H), 2.00 (s, 3H), 1.92 (br m, 5H), 1.69 (br m, 5H), 1.58 (d, 2H), 1.36 (s, 9H), 1.22 (m, 9H), 1.08 (t, 2H).

A.8.1.2 L-Phenylalanine Methyl Ester Hydrochloride Salt

Thionyl chloride (1.15 ml, 15.83 mmol, 1.1 equiv.) was slowly added to a vigorously stirred mixture of L-phenylalanine (2.5718 g, 15.56 mmol, 1 equiv.) and cold super dry methanol (20 ml). The solution was refluxed for 2 hrs then concentrated *in vacuo* to yield a light yellow solid. The solid was dissolved in the minimum amount of methanol (at 20^{0}C) and precipitated by the dropwise addition of ether. The product was washed with ether and dried *in vacuo* to yield L-phenylalanine methyl ester hydrochloride salt as fluffy white crystals (3.0189 g, 14.00 mmol, 89.9 %); mp: 159-161^{0}C; TLC: R_f=0.72 (A), 0.60 (C); IR (cm^{-1}): 3470 (NH), 1730 (ester C=O), 1575 (C=C), 1485 (C=C), 1235 (C-O), 740 (=CH), 705 (=CH); ^{1}H nmr (DMSO-*d*6): δ 8.47 (br s, 2H), 7.27 (m, 5H), 4.20 (t, 1H), 3.63 (s, 3H), 3.10 (m, 2H).

A.8.1.3 BOC-L-Methionyl-L-phenylalanine Methyl Ester

BOC-L-methionine DCHA (3.6135 g, 8.39 mmol) was partitioned between EtOAc (40 ml) and 10 % $KHSO_{4\,(aq)}$ (30 ml). To the organic layer was added 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 2.4441 g, 9.88 mmol), L-phenylalanine methyl ester HCl (1.8061 g, 8.37 mmol), TEA (1.17 ml, 8.39 mmol) and EtOH (30 ml). The solution was stirred at room temperature for 24 hrs then it was concentrated, dissolved in EtOAc (50 ml) and washed with 10% $KHSO_{4\,(aq)}$ (3 x 30 ml), brine (25 ml), saturated $NaHCO_{3\,(aq)}$ (3 x 30 ml) and again with brine (25 ml). The organic layer was dried ($MgSO_4$) and concentrated to yield crude product. This was recrystallized twice from EtOAc, washed with cold diethyl ether and dried *in vacuo* to yield BOC-L-methionyl-L-phenylalanine methyl ester as off-white crystals (3.1710 g, 7.72 mmol, 92.3 %); mp: 77-79^{0}C; TLC: R_f=0.76 (B), 0.37 (D); IR (cm^{-1}): 3330 (NH), 1730 (ester C=O), 1660 (amide C=O), 1515 (C=C), 1170 (C-O), 700 (C=C), 625 (C=C); ^{1}H nmr (DMSO-*d*6): δ 8.30 (m,1H), 7.30 (m, 5H), 7.00 (d, 1H), 4.55 (m, 1H), 4.06 (m, 1H), 3.64 (s, 3H), 3.05 (m, 2H), 2.43 (t, 2H), 2.08 (s, 3H), 1.79 (m, 2H), 1.43 (s, 9H).

A.8.1.4 *cyclo*-L-Methionyl-L-phenylalanine

BOC-L-methionyl-L-phenylalanine methyl ester (2.0005 g, 4.87 mmol) was stirred in formic acid (35 ml) for 1.5 hrs. The formic acid was removed under high vacuum, then 2:1 2-butanol / toluene (60 ml) was added and the mixture was refluxed 24 hrs. During

reflux, crystals formed which increased as the solution was cooled. The isolated white crystals (1.1173 g, 82.4%, impure) were dissolved in DMSO then precipitated with addition of absolute ethanol. The crystals were dried *in vacuo* to yield *cyclo*-L-methionyl-L-phenylalanine as white crystals (0.5938 g, 2.14 mmol, 43.9 %); mp: 263-264^{0}C; TLC: R_f=0.39 (B), 0.52 (E); IR (cm^{-1}): 3300 (NH), 1650 (amide C=O), 1445 (C=C), 1335 (C=C), 1095 (C-N), 700 (=CH); ^{1}H nmr (DMSO-*d6*): δ 8.24 (s, 1H), 8.10 (s, 1H), 7.25 (m, 3H), 7.18 (m, 2H), 4.22 (m, 1H), 3.72 (t, 1H), 3.18 (dd, 1H), 2.83 (dd, 1H), 1.88 (s, 3H+2H), 1.29 (m, 1H), 0.98 (m, 1H).

A.8.2 *cyclo*-L-Leucyl-L-methionine

A.8.2.1 L-Leucine Methyl Ester Hydrochloride Salt

Thionyl chloride (2.00 ml, 27.53 mmol, 1.1 equiv.) was slowly added to a vigorously stirred mixture of L-leucine (3.1663 g, 24.13 mmol, 1 equiv.) and cold super dry methanol (30 ml). The solution was refluxed for 2 hrs then concentrated *in vacuo* to yield a light yellow solid. The solid was dissolved in the minimum amount of methanol (at 20^{0}C) and precipitated by the dropwise addition of ether. The product was washed with ether and dried *in vacuo* to yield L-leucine methyl ester hydrochloride salt as a fluffy white precipitate (3.2488 g, 17.88 mmol, 74.1 %); mp: 147^{0}C; TLC: R_f=0.75 (A), 0.68 (E); IR (cm^{-1}): 3480 (NH), 1725 (ester C=O), 1250 (C-O), 1225 (C-O); ^{1}H nmr ($CDCl_3$): δ 4.10 (t, 1H), 3.84 (s, 3H), 1.97 (m, 2H), 1.86 (m, 1H), 1.01 (s, 3H), 0.99 (s, 3H).

A.8.2.2 BOC-L-Methionyl-L-leucine Methyl Ester

BOC-L-methionine DCHA (4.8264 g, 11.21 mmol) was partitioned between EtOAc (40 ml) and 10 % $KHSO_{4\ (aq)}$ (30 ml). L-Leucine methyl ester HCl (2.0365 g, 11.21 mmol), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 3.1858 g, 12.88 mmol), triethylamine (1.57 ml, 11.26 mmol) and EtOH (30 ml) were added to the organic layer and the solution was stirred at ambient temperature for 24 hrs. The solution was concentrated, dissolved in EtOAc (50 ml) and washed with 10% $KHSO_{4\ (aq)}$ (3 x 30 ml), brine (25 ml), saturated $NaHCO_{3\ (aq)}$ (3 x 30 ml) and again with brine (25 ml). The organic layer was dried ($MgSO_4$) and concentrated to yield crude product. This was recrystallized twice from EtOAc, washed with cold Et_2O and dried *in vacuo* to yield the terminally protected peptide, BOC-L-methionyl-L-leucine methyl ester, as pure white crystals (1.7823 g, 4.73 mmol, 42.2 %); mp: 108^0C; TLC: R_f=0.45 (B), 0.76 (F); IR (cm^{-1}): 3320 (NH), 3260 (NH), 1740 (ester C=O), 1640 (amide C=O), 1360 (C-N), 1150 (C-O); ^{1}H nmr ($CDCl_3$): δ 6.59 (m,1H), 5.21 (m, 1H), 4.64 (m, 1H), 4.32 (m, 1H), 3.75 (s, 3H), 2.63 (t, 2H), 2.15 (s, 3H), 2.03 (d of m, 2H), 1.68 (m, 2H), 1.59 (m, 1H), 1.46 (s, 9H), 0.95 (s, 3H), 0.93 (s, 3H).

A.8.2.3 *cyclo*-L-Leucyl-L-methionine

A solution of BOC-L-methionyl-L-leucine methyl ester (1.6914 g, 4.49 mmol) in formic acid (35 ml) was stirred for 1.5 hrs. The formic acid was removed under high vacuum, then 2-butanol / toluene (2:1, 60 ml) was added. This mixture was refluxed 24 hrs, forming crystals. Purification by recrystallization with 2-butanol produced white crystals which were dried *in vacuo* to yield pure *cyclo*-L-leucyl-L-methionine (0.7902 g, 3.23 mmol, 72.0 %); mp: 242^{0}C; TLC: R_f=0.31 (B), 0.70 (C); IR (cm^{-1}): 3420 (NH), 3300 (NH); 1650 (amide C=O); 1090 (C-N); ^{1}H nmr ($CDCl_3$): δ 7.50 (br s, 1H), 7.20 (br s, 1H), 4.17 (m, 1H), 3.98 (br d, 1H), 2.69 (m, 2H), 2.28 (m, 1H), 2.10 (s, 3H), 2.04 (m, 1H), 1.81 (m, 2H), 1.61 (m, 1H), 0.95(t, 6H).

APPENDIX B

BIOLOGICAL TESTING

B.1 In Vivo

B.1.1 Antiepileptic animal screens

B.1.1.1 National Institutes of Health

The anticonvulsant activity of synthesized compounds was evaluated by the Epilepsy Branch of the National Institutes of Health in Bethesda, MD, according to the established protocol of the Antiepileptic Drug Development Program [326].

All compounds tested were either dissolved in 0.9% sodium chloride or suspended in a mixture of 30% polyethylene glycol 400 and 70% water. Although an increased activity of certain test compounds results when they are suspended in polyethylene glycol in the Metrazol test, no significant bias is introduced by the solvents. The compounds were administered intraperitoneally (i.p.) or orally (p.o.) to Carsworth Farms #1 mice (in a volume of 0.01 ml/g of body weight) or Spraque-Dawley rats (in a volume of 0.004 ml/g of body weight). Times on peak effect and peak neurologic deficit were determined before the anticonvulsant tests were administered.

In the *maximal electroshock seizure test (MES)*, corneal electrodes primed with a drop of electrolyte solution (0.9% sodium chloride) were applied to the eyes and an electrical stimulus (50 mA in mice, 150 mA in rats; 60 Hz) was delivered for 0.2 seconds at the time of peak effect of the test compound. The animals were restrained by hand and released at the moment of stimulation in order to permit observation of the entire seizure. Abolition of the hind-leg tonic-extensor component (hind-leg tonic extension does not

exceed a 90° angle to the plane of the body) indicated that the compound prevented MES-induced seizure spread.

In the *subcutaneous pentylenetetrazol seizure threshold test* (PTZ), the convulsant dose (CD_{97}) of pentylenetetrazol (85 mg/kg in mice and 70 mg/kg in rats) was injected at the time of peak effect of the test compound. The animals were isolated and observed for 30 minutes to see whether seizures occurred. Absence of clonic spasms persisting for at least five seconds indicated that the compound could elevate the pentylenetetrazol-induced seizure threshold.

Drug-induced toxicity is usually characterized by some type of neurologic abnormality. In mice, these abnormalities are easily detected by the *rotorod ataxia test.* The neurologic deficit is indicated by inability to maintain equilibrium for one minute in each of three trials on a knurled rod rotating at 6 rpm. Rats were examined by the *positional sense test* and *gait and stance test.* In the *positional sense test*, one hind leg is gently lowered over the edge of a table, whereupon the animal will quickly lift it back to a normal position. Inability to do so rapidly indicates a neurologic deficit. In the *gait and stance test*, a neurologic deficit is indicated by a circular or zigzag gait, ataxia, abnormal spread of the legs, abnormal body posture, tremor, hyperactivity, lack of exploratory behavior, somnolence, stupor, or catalepsy.

Testing was carried out in 12 mice at doses of 30, 100, 300 mg/kg (4 mice apiece) 30 minutes and four hours after administering the compound. Based on the results of this test, compounds were divided into four groups: (a) those with no anticonvulsant activity at doses up to 300 mg/kg, which were not tested further; (b) those showing activity at 100

mg/kg, which were tested further; (c) those showing activity at 300 mg/kg, which may or may not be tested further depending on the novelty of the structure; and (d) those demonstrating activity and/or toxicity at 30 mg/ kg, which were usually retested and may or may not be evaluated further.

B.1.1.2 Queen's University

Compounds were evaluated for anticonvulsant activity at Queen's University by Sandra Marone under the supervision of Dr. Donald F. Weaver.

B.1.1.2.1 Protocol for Systemic Convulsants

All test compounds were dissolved in standard saline (0.9% NaCl (w/v)) or dimethyl sulfoxide or a mixture of both. The compounds were administered intraperitoneally (ip) to Spraque-Dawley rats at a dose of 100 mg/kg. Twenty to thirty minutes later – the time of peak effect of the test compound – the convulsant dose (CD_{97}) of pentylenetetrazol (70 mg/kg) or pilocarpine (350 mg/kg) was injected. The animals were isolated and observed for 30 minutes to see whether seizures occurred. In the case of PTZ injection, the absence of clonic spasms persisting for at least five seconds indicated that the test compound could elevate the pentylenetetrazol-induced seizure threshold. In the case of pilocarpine injection, the absence of tonic clonic seizures indicated that the test compound could prevent pilocarpine-induced spread of seizures.

If the rat entered a sustained generalized tonic clonic seizure due to inefficacy of the test compound, the rat was quickly euthanized with an overdose of sodium pentobarbital (100-120 mg/kg) injected ip.

B.1.1.2.2 Spontaneous Recurrent Seizure Model: β-Amino Acids vs Phenytoin

To evaluate agents with hybrid anti-ictogenic/antiepileptogenic activity distinct from conventional anticonvulsants, a novel *in vivo* biological evaluation paradigm was devised[‡]. This is a variation of the emerging SRS [Spontaneous Recurrent Seizure] model of epilepsy [327,328]. A 260 g Sprague Dawley rat is given pilocarpine (380 mg/kg ip). Within 15 minutes, the animal enters *status epilepticus* lasting 10-12 hrs. The rat is allowed to recover spontaneously and then remains in a 16 hr/8 hr light/dusk cycle receiving food and water *ad libitum*. Commencing on day 13-15, the rat develops spontaneous recurrent seizures, subsequently experiencing 4-7 seizures per week. Rats are videotaped 16 hr/day and behavioural seizures (head nodding, forelimb clonus, rearing) are counted by a blinded observer. Test rats are monitored for 3 months, permitting an average of 65 seizures to be counted per rat. In addition 1 of 4 rats has electroencephalography recordings 3 hrs per day. Experimental compounds for evaluation may be administered commencing at one of two time points during this protocol: Time 1 is during day 1, immediately after the *status epilepticus* (at time = 10 hrs), but 12-14 days prior to the onset of SRS's; Time 2 is at Day 60 when the rats have been having SRSs for

[‡] This research has been submitted to *Science* (D. F. Weaver, S. Marone, P. Milne. "Pharmacological Differentiation between Ictogenesis and Epileptogenesis in a Spontaneous Recurrent Seizure Model of Epilepsy).

approximately 6 weeks. Administration at Time 1 permits a compound to be evaluated as an antiepileptogenic and reflects ability to prevent the initiation of spontaneous seizures; administration at Time 2 permits a compound to be evaluated as an anti-ictogenic and reflects ability to suppress an established seizure disorder. β-Alanine was selected for evaluation as a prototype agent with hybrid anti-ictogenic/anti-epileptogenic activity.

Four groups (A,B,C,D) of rats were studied. Groups A,B, and C all received pilocarpine as described above. The 16 rats of Group A received only pilocarpine but no phenytoin or β-alanine. The 8 rats of Group B received phenytoin 20 mg/kg/day intraperitoneally [ip] starting at Time 1 and on days 2-7; the 8 rats of Group C received β-alanine 20 mg/kg/day ip starting at Time 1 and on days 2-7; the 4 rats of Group D were an untreated control group. In Group A, 15/16 rats developed SRSs with an average of 6.4 observed seizures per week; in group B, 8/8 rats developed SRSs with an average of 5.8 seizures per week; in Group C, 3/8 rats developed SRSs with an average of 2.7 seizures per week. No seizures occurred in Group D. At Time 2 (time = day 60), the 15 rats with SRSs in Group A were divided into three groups of 5 rats. Group A1 received phenytoin 20 mg/kg/d ip for 10 days; Group A2 received β-alanine 20 mg/kg/d ip for 10 days; Group A3 was untreated. Seizure counts were performed in all 3 groups for the time period days 63-69. Group A1 rats demonstrated an average of 1.8 seizures/rat/week; Group A2 demonstrated an average of 3.3 seizures/rat/week; Group A3 demonstrated 5.9 seizures/rat/week.

The pilocarpine protocol produced SRSs in 94% of rats. Treatment at Time 1 with phenytoin had no influence on the development of SRSs; however, in the rats treated with

β-alanine at Time 1, only 37% of rats developed SRSs. This is a significant reduction in the development of epilepsy. At Time 2, phenytoin produced a 69% reduction in seizures, while β-alanine produced a 44% reduction in seizures. Both phenytoin and β-alanine showed anti-ictogenic activity.

Using the same protocol, α-(4-phenylcyclohexyl)-β-alanine hydrochloride salt (B5P107), anti-ictogenic in rats at ip doses of 50 mg/kg, was tested for antiepileptogenic activity. Only 20% of rats treated with B5P107 at time 1 developed SRSs.

These results demonstrate that β-alanine and B5P107 had significant anti-epileptogenic activity in the SRS model of epilepsy; phenytoin had none. Since both β-amino acids are anti-ictogenic, β-alanine and B5P107 have been shown to possess hybrid anti-ictogenic/anti-epileptogenic activity (an unprecedented bimodal bioactivity). It is notable that while anti-ictogenic activity for β-alanine has been previously documented (Table 2.2), antiepileptogenic activity for β-alanine or B5P107 has not been previously reported.

The implications of these findings are multifold. First, they emphasize that the future development of drugs for epilepsy must recognize the uniqueness yet potential interdependency of ictogenesis and epileptogenesis. True antiepileptic drugs should have combined activity against both of these processes. Second, the results identify the utility of the SRS assay as a model of Phase 1 epileptogenesis which has a role in future drug development in addition to the standard pentylenetetrazole and maximal electroshock models. Finally, the findings recognize the importance of β-amino acids as future starting points in the development of drugs for epilepsy.

B.2 In Vitro

B.2.1 Cation Transfer Using the "Divided Beaker Cell" Transport System

The transport of copper (II) from an extracellular phase to an intracellular phase and the subsequent translocation of zinc (II) across a membrane phase in the opposite direction were determined in the Divided Beaker Cell Transport System (Figure B.1) [329]. In the left side of the cell (extracellular phase), 0.008 grams of the Cu(II) cyclic dipeptide was placed in 60.0 ml of water. In the right side of the cell (intracellular phase), 20 mmole of $Zn(SCN)_2$ was dissolved in 60.0 ml of water. *n*-Octanol (membrane phase) was gently layered on top of both sides of the cell until the level of this reached the top of the partition; 35.0 ml of *n*-octanol was then added.

The cell was incubated in a warm water bath maintained at 37^0C. All phases were stirred simultaneously at a constant rate. Samples of the extracellular and intracellular phases were taken at intervals of 24 hours for 10 days. Concentrations of copper (II) and zinc (II) in the two phases were determined by atomic absorption spectroscopy: copper (II) absorbs light at 324.8 nm, zinc (II) absorbs at 213.9 nm. Calibration standards were prepared by diluting 1000 μg/ml copper and zinc standard solutions (Aldrich). Copper (II) stock solutions were made to 5.0, 4.0, 2.0, 1.0 and 0.5 ppm. Zinc (II) stock solutions were made to 1.0, 0.8, 0.5, 0.2 and 0.1 ppm.

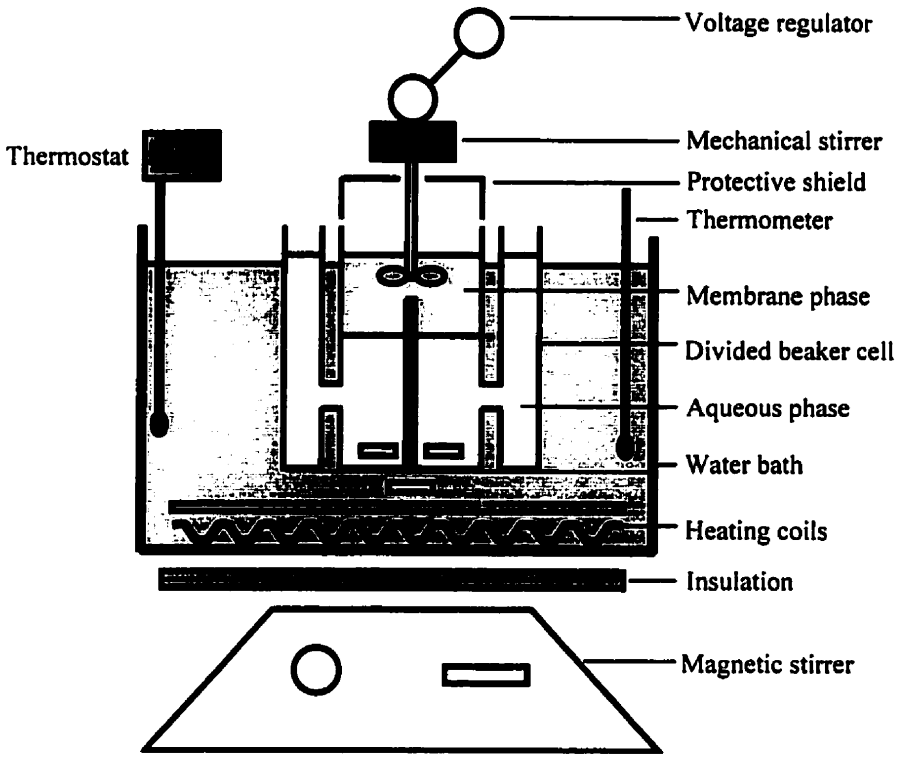


Figure B.1: The "Divided Beaker Cell" Transport System

A number of control studies were performed by D. F. Weaver. First, the cell was evaluated for evidence of outward Zn(II) leakage. The extracellular phase contained no $CuCl_2$ and the intracellular phase contained 20 mmol of $Zn(SCN)_2$. After 10 days, no Zn(II) had leaked into the extracellular phase. Second, the cell was evaluated for evidence of inward Cu(II) leakage. The extracellular phase contained 0.02 mmol $CuCl_2$ and the intracellular phase contained no $Zn(SCN)_2$. After 10 days, no Cu(II) had leaked into the intracellular phase. Third, the divided beaker cell was evaluated for concomitant Zn(II) to Cu(II) transmembrane exchange in the absence of a cyclic dipeptide. The extracellular phase contained 0.02 mmol $CuCl_2$ and the intracellular phase contained 20 mmol

$Zn(SCN)_2$. After 10 days, there was no evidence of Cu(II) leakage inward or of Zn(II) leakage outward.

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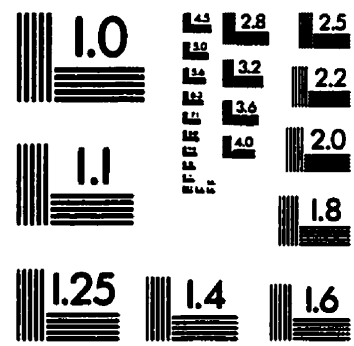

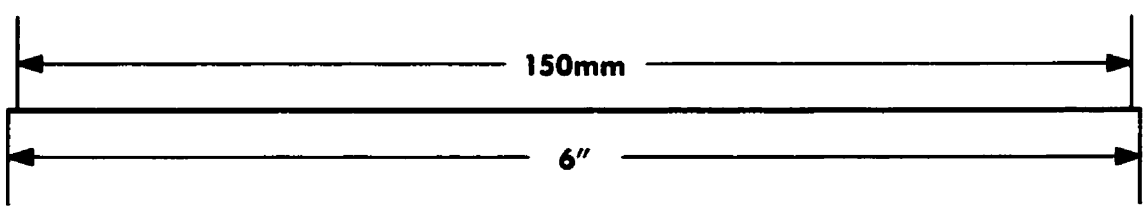

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