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# Synthesis of conformationally constrained glutamate analogues and their preliminary evaluation as glutamate transport inhibitors

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# SYNTHESIS OF CONFORMATIONALLY CONSTRAINED GLUTAMATE ANALOGUES AND THEIR PRELIMINARY EVALUATION AS GLUTAMATE TRANSPORT INHIBITORS

by

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Synthesis of conformationally constrained glutamate analogues and their preliminary evaluation as glutamate transport inhibitors

Director: Charles M. Thompson

The primary goals of the research effort were to assess the reactivity of dimethyl 4-oxoglutaconate in the normal electron demand Diels-Alder reaction. Once the Diels-Alder reaction was deemed a success, the adducts were transformed into conformationally constrained analogues of 2-oxoglutarate by saponification. The cyclic 2-oxoglutarate analogues were evaluated as substrates and inhibitors of a number of transaminases and dehydrogenases. One transaminase was found to convert the oxoglutarate analogues to glutamate analogues. The Diels-Alder adducts were also chemically transformed into conformationally constrained analogues of glutamate by preliminary formation of the corresponding N, N-dimethylhydrazones and subsequent reduction with sodium hydrosulfite and saponification. The glutamate analogues were tested at four glutamate transporters and two compounds, namely 6-(amino-carboxy-methyl)-cyclohex-3-enecarboxylic acid and 3-(amino-carboxy-methyl)-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid, 38 ± 2 and 35 ± 5 percent of control, respectively, were found to be potent and selective inhibitors of the excitatory amino acid transporter 2 (EAAT2).

# Dedication

It is with extreme delight that I dedicate this thesis to the loving memory of my recently departed Grandfather Clifford Denton, my grandmother Wilda Denton, my mother Karen Denton and my father James C. Denton. Without these people my life would not be complete. Thank you!!

# Acknowledgements

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And last but by no means least, I have to thank my wife Monica for all her love and support throughout my Ph. D. pursuit.

## Contents

**Chapter 5: Conclusions and Future Directions**

**List of Abbreviations**

| | |
|---|---|
| anal. calc | elemental composition; calculated value |
| ACPD | *trans*-1-aminocyclopentane-1,3-dicarboxylate |
| AMPA | (R,S)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)proprionic acid |
| anhyd | anhydrous |
| atm | atmosphere |
| ATP | adenosine 5'-triphosphate |
| br | broad |
| BSA | bovine serum albumin |
| calcd | calculated |
| CBZ | carbobenzyloxy |
| CBZCl | carbobenzyloxy chloride |
| concd | concentrated |
| cpm | counts per minute |
| $CH_2Cl_2$ | methylene chloride or dichloromethane |
| $\delta$ | chemical shift in parts per million |
| d | day(s); doublet (spectral) |
| dd | doublet of doublets |
| DME | dimethoxyethane |
| DMF | dimethylformamide |
| DMSO | dimethylsulfoxide |
| td | triplet of doublets |

| | |
|---|---|
| EAAT | excitatory amino acid transporter |
| ESI | electron spray ionization |
| Et | ethyl |
| EtOH | ethyl alcohol |
| $Et_2O$ | diethyl ether |
| EtOAc | ethyl acetate |
| FT | Fourier Transform |
| g | gram(s) |
| glu | glutamate |
| GVT | glutamate vesicular transporter |
| h | hour(s) |
| HCl | hydrogen chloride |
| HEPES | (N-[2-hydroxylethyl]piperazine-N'-[2-ethanesulfonic acid) |
| Hz | hertz |
| HRMS | high-resolution mass spectrum |
| IR | infrared |
| *J* | coupling constant (NMR) in hertz |
| KA | kainate |
| KCl | potassium chloride |
| μ | micro |
| m | multiplet (spectral), milli |
| M | moles per liter |
| m-CPBA | m-chloroperoxybenzoic acid |

| | |
|---|---|
| Me | methyl |
| MeOH | methyl alcohol |
| mg | milligram(s) |
| MHz | megahertz |
| min | minute(s) |
| mL | milliliter(s) |
| mol | mole(s) |
| mp | melting point |
| m/z | mass to charge ratio (mass spectrometry) |
| NaI | sodium iodide |
| NMDA | N-methyl-D-aspartate |
| NMR | nuclear magnetic resonance |
| ppm | parts per million |
| q | quartet |
| QDC | quinoline-2,4-dicarboxylate |
| Rf | retention factor (in chromatography) |
| rt | room temperature |
| s | singlet (NMR); second(s) |
| $S_N2$ | bimolecular nucleophilic substitution |
| t | triplet(spectra) |
| TEA | triethylamine |
| TFA | trifluoroacetic acid |
| THF | tetrahydrofuran |

| | |
|---|---|
| TLC | thin layer chromatography |
| TMSBr | trimethylsilylbromide |
| Torr | 1 mm Hg, 1/760 atm |
| Ts | tosyl, p-toluenesulfonyl |
| UV | ultraviolet |

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## Chapter 1:Overview of the Glutamate Neurosystem

### I. Glutamate as a Neurotransmitter

L-Glutamate (Figure 1.1) is an excitatory amino acid (EAA) that is responsible for neuronal signaling in the mammalian central nervous system (CNS). Immunostaining has revealed the involvement of L -glutamate utilizing proteins in optic nerve terminals and terminals of the cortico-fugal axons in the thalamus (Salt, 1996; Kechagias, 1995) as well as the hippocampus, medial striatum, and cortex regions of the brain (Cotman, 1995). Excitatory amino acids regulate the stimulation of the postsynaptic terminal of neurons by mediating changes in electric potential (caused by a flow of charged ions, i.e., $Na^{+}$, $Ca^{++}$ and $K^{+}$). The involvement of glutamate as an EAA ranges from participation in excitatory neurotransmission to memory to neuropathology. Neuronal signaling is highly dependent on the transport and metabolism of neurotransmitters. The most common signal termination pathways being either enzymatic conversion to a non-transmissible compound or transport of the neurotransmitters back out of the synaptic cleft by neurotransmitter transporters. The glutamate neurosystem follows the latter mechanism. Neurotransmitter signaling can also be directly affected by agonists and/or antagonists interacting at the receptors or transporters, which will affect the associated biological response.

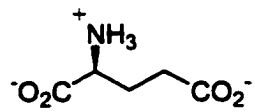


**Figure 1.1**. Structure of L-glutamate

The receptors, transporters, and enzymes that bind L-glutamate during the process of excitatory neurotransmission comprise one of the most diverse and broadly functional protein groups in the mammalian central nervous system (Cotman, 1995). L -Glutamate (Figure 1.1) is a conformationally flexible molecule containing three distinct functional groups. Owing to the three $sp^3$ hybridized carbons in its structure, L -glutamate can exist in nine low energy conformations. The pKa's of the acidic and basic functional groups of L-glutamate play an important role in the overall chemistry of the molecule and must be considered: pKa ($\alpha$-$CO_2H$) = 2.10; pKa ($\alpha$-$NH_3^+$) = 9.47; and pKa ($\delta$-$CO_2H$) = 4.07 (Carrigan, 1999). At physiological pH, L-glutamate is completely ionized and has a net charge of -1. These pKa characteristics play an important role upon binding to various receptors, transporters, and enzymes.

Within the glutamate pre-synaptic neuron, the glutamate molecules exist in the cell cytosol at a concentration of 1-3 mM (Arriza, 1994). From the cytosol, glutamate is transported and packaged into synaptic vesicles where it is stored until the signaling event arrives (Figure 1.2) (Naito, 1985). Upon nerve stimulation, the vesicles are transported via fusion proteins (Cousin, 1999; Betz, 1994) to the pre-synaptic nerve terminal where they dock and release glutamate in a calcium-dependent manner into the synaptic cleft. Once glutamate is released into the cleft, it freely binds to various post-synaptic receptors and therefore, enables signals to be potentiated. The post synaptic EAA receptors can be placed into two groups: (a) the metabotropic receptors (where activation leads to the generation of a cascade of second messengers) and (b) the ionotropic receptors (where activation leads to opening of ion channels which gate $Na^+$-,

$K^+$-, and $Ca^{++}$- currents) (Monaghan, 1986). It is hypothesized that glutamate may bind and trigger unique responses at the corresponding

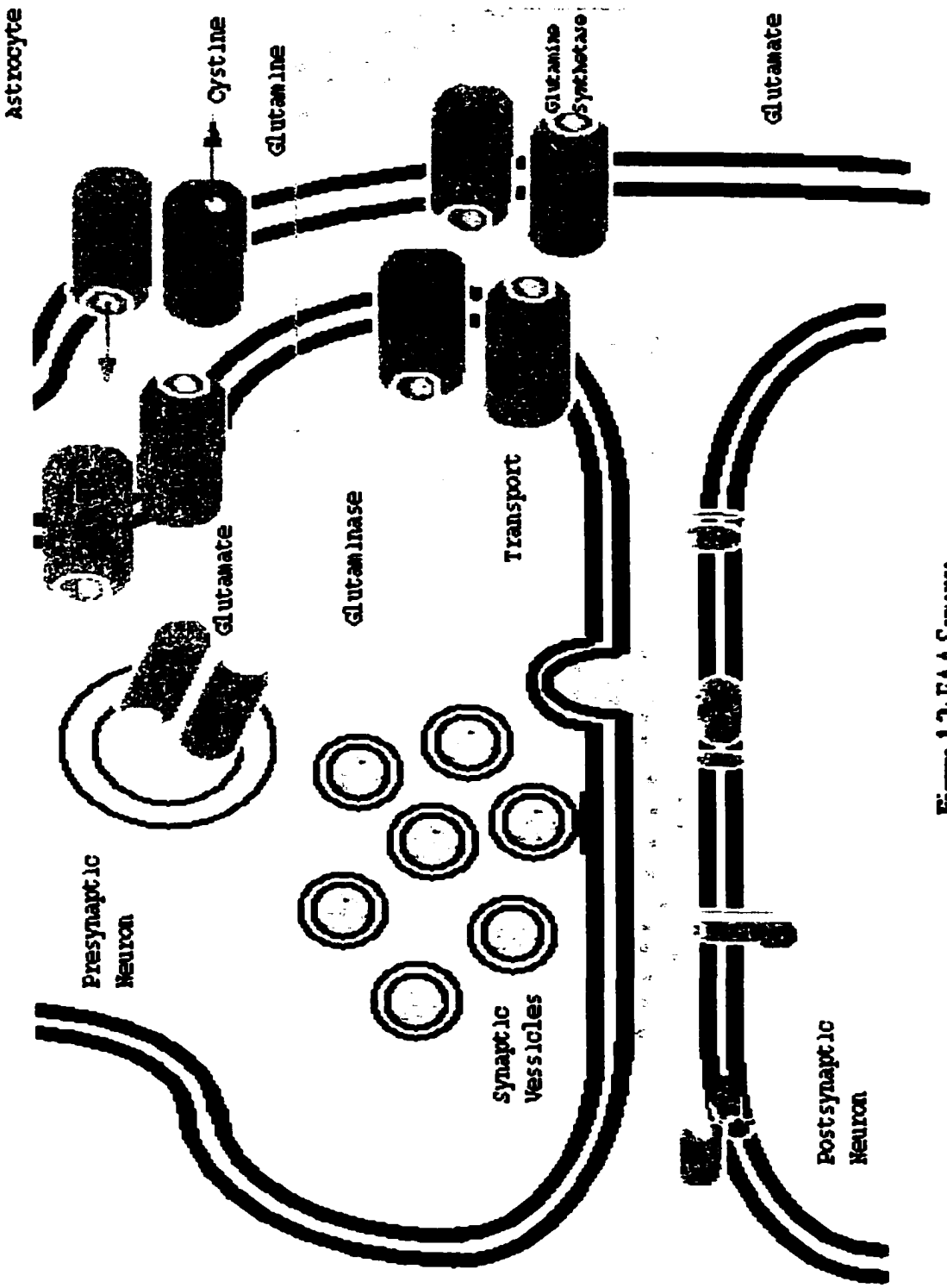



Figure 1.2: EAA Synapse

This figure was graciously donated by Richard J. Bridges of the University of Montana.

receptors through its distinct conformations (Chamberlin, 1993). Once L-glutamate binds to the individual receptors and induces a signal, the high concentrations (1-10 μM) of L-glutamate must be cleared from the synaptic cleft and returned to the pre-synaptic neuron (Bridges, 1994; Garlin, 1995). The clearance of glutamate from the synaptic cleft is accomplished by high-affinity glutamate transporters. There are two groups of "high affinity" transporters currently classified: (a) synaptosomal transporters (those which directly transport glutamate back to the presynaptic cytosol where it can be taken up by vesicles), or (b) astroglial transporters (those which relocate glutamate from the synapse into astrocytes). Once in astrocytes glutamate is concurrently transformed into glutamine via glutamine synthetase (Figure 1.2) (Cotman, 1995). Glutamine is not an excitatory amino acid and is thus rendered inactive to post synaptic receptors and, in turn, may be transported back to the presynaptic neuron via transporters specific for glutamine (Chaudhry, 1999). Once the glutamine is relocated to the presynaptic neuron it is enzymatically converted back into glutamate by glutaminase. The glutamate is subsequently transported back into synaptic vesicles to await the next excitatory impulse.

L-Glutamate neurotransmission has critical impact on numerous biochemical processes (Cotman, 1995). In addition to mediating fast excitatory synaptic signaling, glutamate receptor activation participates in higher-order processes, such as development, learning and memory. L-Glutamate is also involved in neuropathology, as excessive stimulation of the post-synaptic neuron by excitatory amino acids trigger events which can lead to cell death (Rothman, 1995; Choi, 1988). This process, referred to as excitotoxicity, is thought to play a role in a number of neurological insults and diseases

including ischemia, stroke, anoxia, hypoglycemia, epilepsy, Huntington's disease, and Alzheimer's disease (Rothman, 1995).

The focus of most of the L-glutamate EAA pharmacology over the last decade has resided in two areas: (1) differentiating the pharmacology of the ligand binding sites to the various EAA receptors and transporters, and (2) cDNA expression and structural studies of the receptors and transporters (Cotman, 1995). Major advances in the pharmacology of glutamate binding sites has been added by the design and synthesis of conformationally restricted and isosterically similar analogues of L-glutamate of which mimic low energy conformations exist (Figure 1.3) (Chamberlin, 1993). These analogues have provided key information about the receptor types and the three dimensional pharmacophore models of the receptor and transporter binding sites. These analogues have also played a pivotal role in differentiating the structure and function of the various receptors and transporters.

The study and design of conformationally restricted analogues of L-glutamate have led to selective and potent analogues which bind to and differentiate the receptor and transporter substrate binding sites. N-Methyl-D-aspartate (NMDA), (RS)-2-amino-(3-hydroxyl-5-methyl-4-isoxazol-4-yl) propionic acid (AMPA), and kainic acid (KA) are conformationally restricted and isosterically related analogues of L-glutamate which were used to identify and distinguish the ionotropic L-glutamate receptors. The glutamate analogues, 1-aminocyclopentane-trans-1,3-dicarboxylate (trans-ACPD) and L-2-amino-4-phosphonobutyrate (L-AP4) were the lead compounds (Salt, 1996; Cotman, 1995) in defining the pharmacology and function of the metabotropic L-glutamate receptors (mGluR's) (Conn, 1997). Several families of analogues have proven valuable in

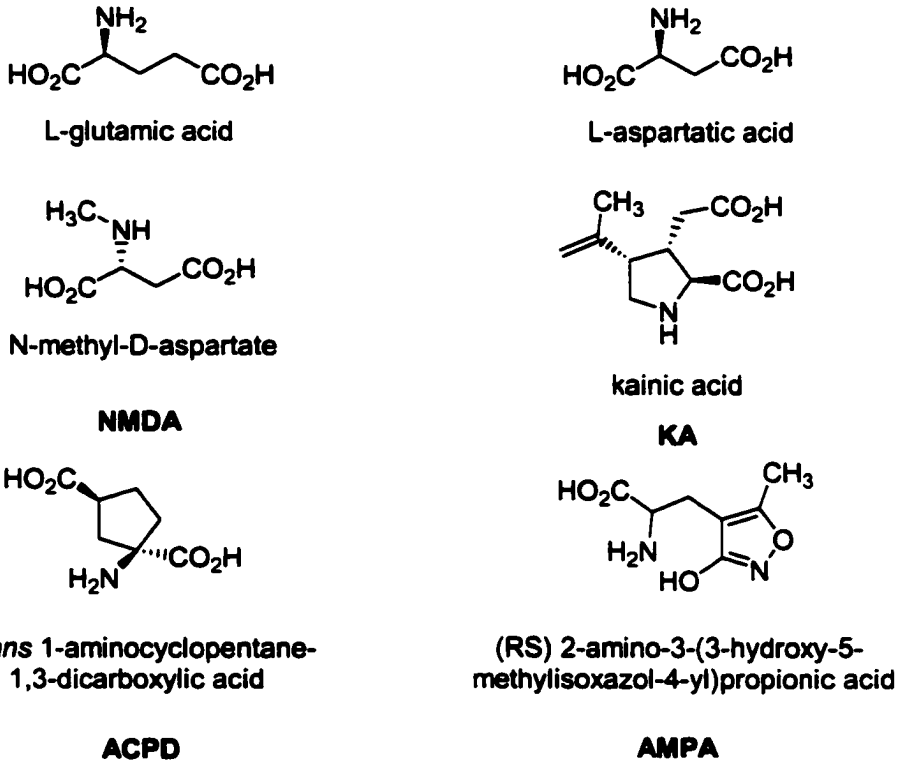
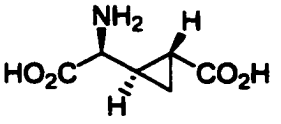


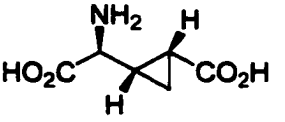

**Figure 1.3.** Examples of structurally constrained and unconstrained agents patterned after L-glutamate utilized in characterizing the glutamate receptors

characterizing the high-affinity, sodium dependent excitatory amino acid transporters (EAATs) that mediate the majority of glutamate uptake in the CNS, including: 2,4-pyrrolidinedicarboxylic acids (2,4-PDCs), 2-(carboxycyclopropyl)glycines (L-CCG -I-IV), β-hydroxyaspartate derivatives, methyl-substituted glutamate analogues, and oxazoline-based derivatives (Bridges, 1991; Chamberlin, 1998; Shimamoto, 1991). In particular, the conformationally restricted PDC- and CCG-based mimics are locked into specific configurations at the substituent sites C3 and/or C4 (Figure 1.4) (Bridges, 1991; Chamberlin, 1998; Shimamoto, 1991). Transport assays have demonstrated that L-*trans*-

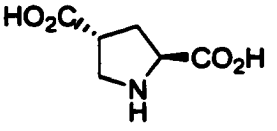
2,4-PDC acts as a substrate of the excitatory amino acid transporter subtypes EAAT1-4 and as a non-substrate inhibitor of the EAAT5 subtype (Arriza, 1997; 1994; Fairman,



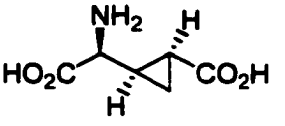
**L-CCG-II**
L-2-(carboxycyclopropyl)glycine II



**L-CCG-III**
L-2-(carboxycyclopropyl)glycine III



**2,4-PDC**
L-*trans* 2,4-pyrrolidine-dicarboxylic acid



**L-CCG-IV**
L-2-(carboxycyclopropyl)glycine IV

**Figure 1.4**. Structurally constrained analogues of L-glutamate utilized in characterizing the EAATs

1995; Koch, 1999). Similarly, the individual stereoisomers of the L-CCGs also exhibit interesting activities at these transporters. For example, L-CCG-II and L-CCG-III (Figure 1.4) have both been reported to block the uptake of [$^3$H]- L-glutamate into synaptosomal preparations (Robinson, 1993). More detailed studies using cellular expression systems have demonstrated that L-CCG-III potently inhibits EAAT1, EAAT2, and EAAT3, while L-CCG-IV preferentially inhibits EAAT2 (Shimamoto, 1998; Yamashita, 1995).

The analogues described either mimic L-glutamate with structural locking of the backbone into specific orientations or utilize a functional group isostere (a substituted group which exhibits similar but unique characteristics and/or properties to the original functional group which may affect binding affinities). The affinities of these analogues

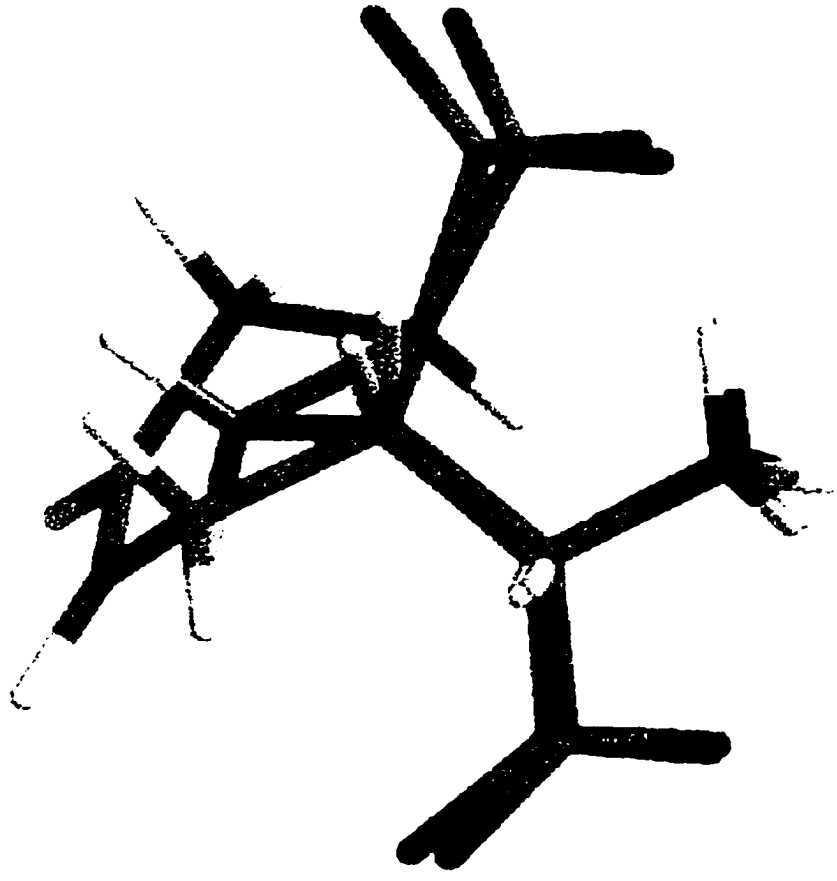


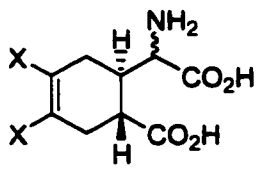
**Figure 1.5.** Overlay of L -CCG-II and R, R-3, 4-(4,5-cyclohexenyl)-L-glutamate rendered in PC Spartan Pro The numerical values from PC Spartan Pro for L -CCG-II was -40.88 and for R, R-3, 4-(4,5-cyclohexenyl)-L-glutamate was -6.26

to bind to various receptors and transporters are likely influenced by the relative positions of the charged functional groups and orientation of the hydrocarbon backbone as evidenced by the structurally diverse L-glutamate analogues (Figures 1.3 and 1.4) (Chamberlin, 1993).

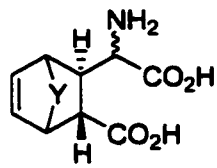
It is our goal to investigate cyclohexenyl locked analogues as glutamate mimics resembling 2, 4-PDC and the L-CCGs. A minimized molecular modeling rendition performed in PC Spartan Pro (Version 1.0.5, Irvine CA) is provided in Figure 1.5. It is

shown that the glutamate backbone in both L-CCG-II and the 3, 4-(4,5-cyclohexenyl)- L-glutamate (one of our theoretical targets, Figure 1.5) overlay very well.

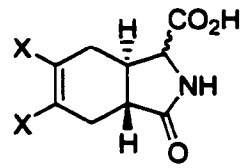
The fact that the molecules overlay well is key preliminary data promoting the synthesis of a new class of analogues for the study of the glutamate neurosystem. It can be predicted, by the modeling overlays, that at least one of the stereoisomers of 3,4-(4,5-cyclohexenyl)-D/ L -glutamates should show some activity at a transporter or receptor in the glutamate




3,4-cyclohexenyl-fused glutamate; X = H, alkyl, etc.




3,4-cyclohexenyl-fused glutamate; Y = $CH_2$, $CH_2CH_2$, etc.




3,4-cyclohexenyl-fused pyroglutamate; X = H, alkyl, etc.

**Figure 1.6**: Proposed target 3,4-(4,5-cyclohexenyl)-D/L- glutamate molecules

neurosystem. It has been shown by Robinson and co-workers (1993) that L-CCG-II inhibits glutamate uptake in crude synaptasomes (presynaptic neuronal transport, EAAT2) from rat cerebellum. With this data as groundwork, as well as the good overlay of the glutamate backbones of L -CCG-II and the 3,4-(4,5-cyclohexenyl)-L-glutamate shown in figure 1.6, it was decided to synthesize the 3,4-(4,5-cyclohexenyl)-D/L-glutamates which are good initial targets to explore as inhibitors at various transporters of the glutamate neurosystem, including EAAT2.

The various glutamate receptors and transporters tolerate a range of chemical variation, and perhaps these tolerances can be exploited in identifying a new class of

compounds whose structures can be attenuated and tailored for specific or multi-receptor / transporter binding.

Our approach to further elucidate the stereochemical binding requirements of the transporters of the L-glutamate neurosystem is to conformationally lock the structure of the L -glutamate molecule about the 3, 4 position utilizing a cyclohexenyl moiety. This hypothesis, to increase the size of the conformational locking group relative to the L-CCG compounds referred to above, may further help delineate the critical regions of space and the structural elements of ligands must occupy to interact with specific transporters of the glutamate neurosystem. Again, Figure 1.6 shows some representative examples of the types of molecules that are envisioned to further delineate the three-dimensional specificities of the transport binding sites. The design and chemical synthesis of this structural class of molecules, their intermediate structures and the biological implications of the products will be discussed in the following chapters.

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## Chapter 2: Organic Synthesis

### I. Introduction

Our development of the 3, 4-conformationally restricted analogues of L-glutamate is based on the retrosynthetic analysis depicted in Figure 2.1. The key of the design is the Diels-Alder reaction between dimethyl-4-oxoglutaconate. Once the adducts are accessed

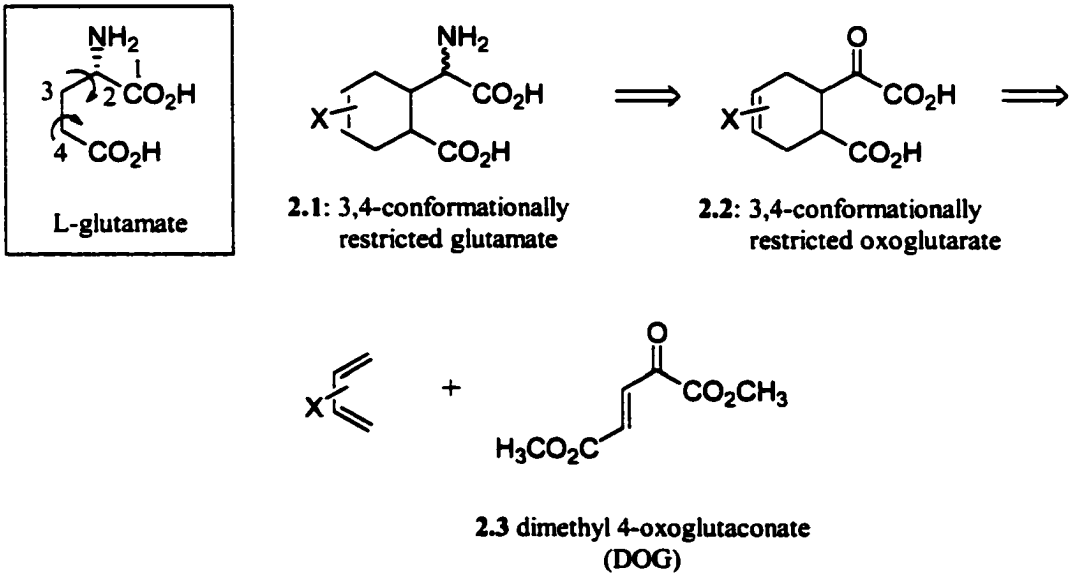



**Figure 2.1**: Retrosynthetic disconnection scheme towards 3,4-conformationally restricted glutamates

a chemical or enzymatic reductive amination was conceived to introduce the alpha-amino moiety.

One key feature of the synthesis is the need to develop of a multigram synthesis of the key intermediate, dimethyl 4-oxoglutaconate (DOG, **2.3**). The synthesis of the

ethyl analogue of DOG was first prepared by Cornforth et. al. by the oxidation of diethyl glutaconate with selenium dioxide (Cornforth, 1946). The percent yield was reported as 2% and the molecule was characterized by boiling point, elemental analysis and UV/visible spectroscopy. In 1981, an alternate route for the synthesis of DOG was disseminated by Corey and co workers (Corey, 1981). The synthesis described is shown in Figure 2.2, although only the general experimental procedure was reported.

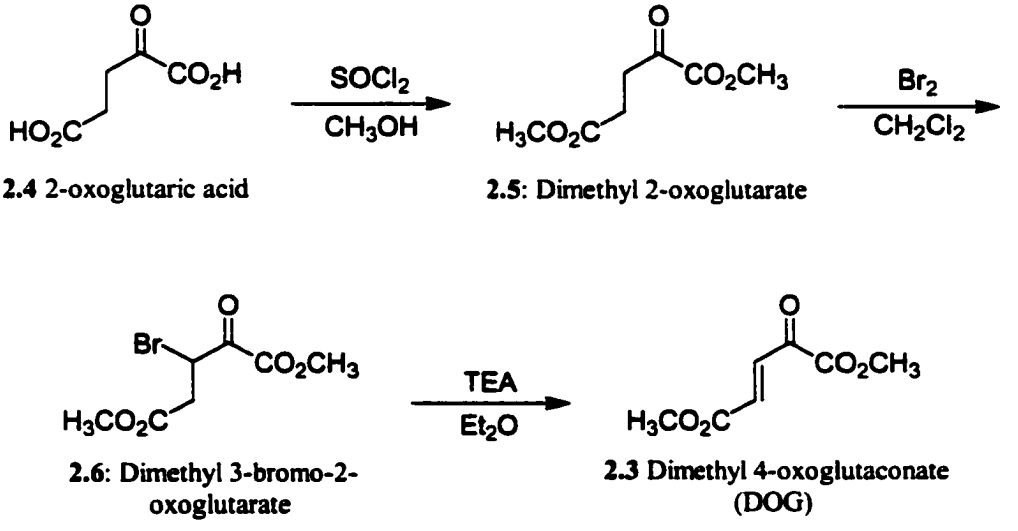



**Figure 2.2**: Synthesis of DOG (**2.3**)

In 1987, it was discovered by Ananda, et. al. that the oxidation of dimethyl glutaconate with activated carbon could generate DOG in modest yields (Ananda, 1987). In this report, DOG was characterized by $^{1}H$ NMR for the olefinic protons, melting point and UV/visible spectroscopy. In 1996, a detailed analysis of the $^{13}C$ NMR spectra of DOG in organic and aqueous media was performed by Kato and co workers (Kato, 1996).

The authors reported that the molecule could be purchased from Fluka Chemical Company (Buchs, Switzerland), but it is no longer available.

Taken together, DOG has been characterized by its spectroscopic and physical properties, but a reliable, concise chemical synthesis with detailed spectral data for the individual steps had yet to be performed. Carrigan (1999) reported a multigram synthesis of DOG from readily available starting materials in modest yield, according to the original sequence reported by Corey (1981) (Figure 2.2). In the following sections we will report our synthesis of DOG on a multigram scale and it's utilization in chemical reactions with detailed spectral interpretation of the products and key chemical intermediates.

The Diels-Alder reaction is a fundamental carbon-carbon bond forming reaction in synthetic organic chemistry (DeGraw, 1960; Tokoroyama, 1978; Danishefsky, 1979; Snowden, 1986; Grieco, 1990). It is synonymously termed a [4+2]-cycloaddition reaction in reference to the formation of a cyclic system upon reaction of a $4\pi$-electron system with a $2\pi$-electron system (Carey, 1984). The products formed are typically referred to as cycloadducts and the significance of this reaction in organic chemistry owes to the fact that two carbon-carbon bonds are formed and the reaction is very highly regio- and stereoselective. Experimental data supports the premise that the reaction occurs via a concerted mechanism. The stereoselective *syn* products resulting from this reaction strengthen this argument. If it were to occur in a stepwise mechanism, the 2nd addition step would have to occur at a rate faster than that of a rotation about a carbon-carbon bond, which is not likely.

The Diels-Alder reaction is also under stereo- and regioselective control in a number of ways. First, in the construction of bicyclic adducts, there are two possibilities; a favored endo adduct, shown for the maleic anhydride cyclopentadiene adduct (Fig 2.3, A.), as well as an exo product (Fig 2.3, B). This selectivity is governed by the Alder rule, or the rule of maximum accumulation of unsaturation. The comprehensive interpretation of this rule is such that when the maximum amount of unsaturated orbitals interact in the

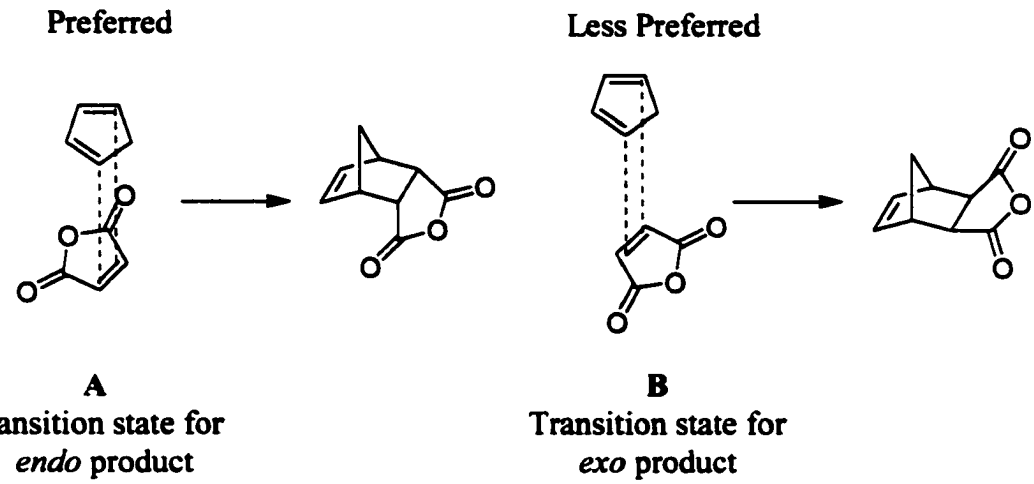



**Figure 2.3.** Transition states for endo vs. exo regioisomers

transition state, the stereospecific isomer corresponding to this transition state will predominate. To fully interpret the stereo- and regioselectivity of this reaction, a closer look into the reaction mechanism is required. The regioselectivity incorporated into the formation of the unsymmetrical adducts must also be acknowledged.

The Diels-Alder reaction is an example of a pericyclic reaction. The conventional definition of a pericyclic reaction envelops the idea that the mechanism involves the reorganization of molecular orbitals within two systems rather than the combination of ionic (i.e. carbanions, $S_N2$) or radical species. Pericyclic reactions occur via the interactions of the highest occupied molecular orbital (HOMO) of one species with the lowest unoccupied molecular orbital (LUMO) of another (Fig 2.4).

Figure 2.4 shows generic representations of the molecular orbitals in a Diels-

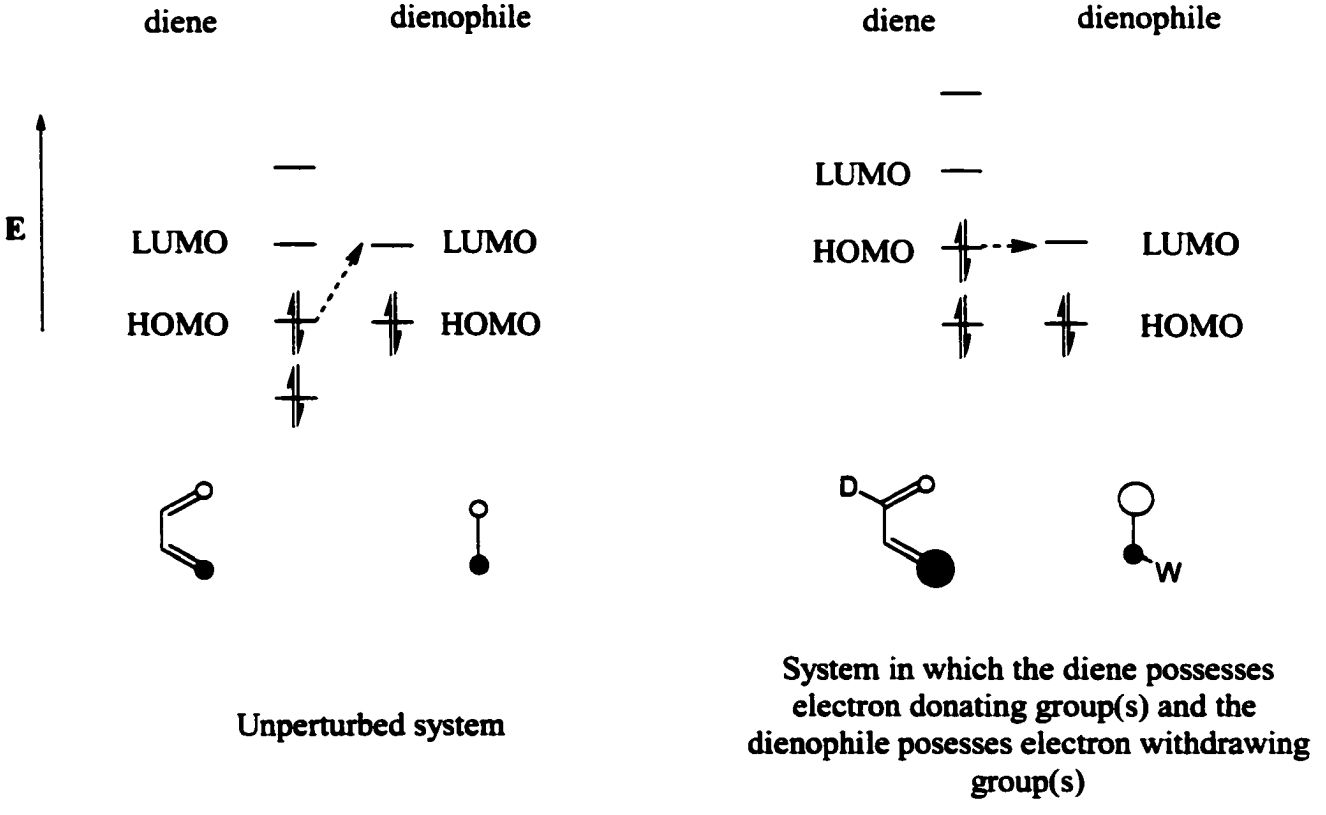



**Figure 2.4.** Frontier orbital interactions in [4+2] cycloaddition reactions

Alder cycloaddition reaction. In the case of a cycloaddition reaction, orbitals of proper symmetry and energy are uniquely postured for interaction. In the case of Diels-Alder cycloaddition reactions, it is the interaction of the HOMO of the 4π system (the diene) and the LUMO of the 2π system (the dienophile). As shown in Figure 2.5, the symmetry of the 4π systems HOMO correlates to the symmetry of the LUMO 2π system. The second factor, the similar energy element, is also uniquely set up for a cycloaddition reaction. The most similar molecular orbitals, in relation to energy, are the HOMO of the diene and the LUMO of the dienophile. These two factors together make for a favorable orbital interaction and subsequent reaction.

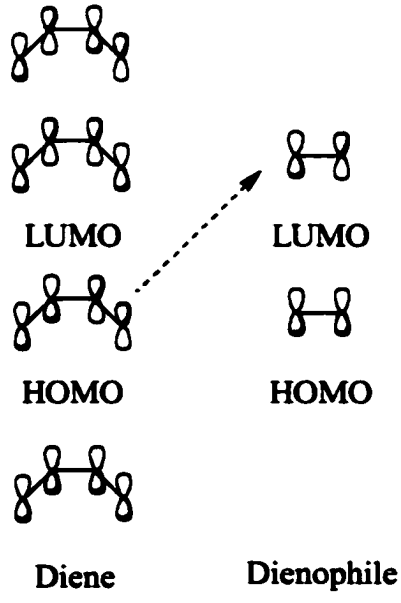



**Figure 2.5.** Orbital symmetry of 2 and 4 π systems

It has been shown that in the presence of electron withdrawing substituents on the $2\pi$ system as well as electron donating substituents on the $4\pi$ system, the relative rates of reactivity increase as shown in Table 2.1 (Carey, 1984). The overall reactivity as well as the regioselectivity of the Diels-Alder reaction can be delineated using Figures 2.4, 2.5 and 2.6. Figure 2.6 shows the predicted outcome when dienes bear an electron donating group on either the 1 or 2 position and a single electron withdrawing group is present on the dienophile. The increased electron density on the $\alpha$-carbon relative to the donating group at the 1 position increases the overall energy of the diene's HOMO as well as

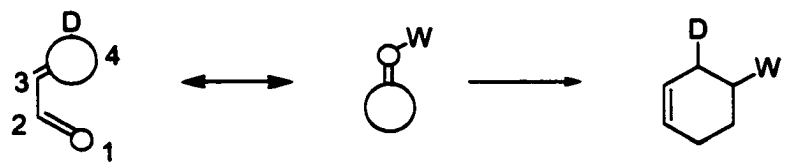
**Table 2.1**: Relative Reactivity Toward Cyclopentadiene in the Diels Alder Reaction[a]

| Dienophile | Relative Rate |
|---|---|
| Tetracyanoethylene | 4.3 X 107 |
| 1,1-Dicyanoethylene | $4.5 \times 10^5$ |
| p-Benzoquinone | $5.6 \times 10^4$ |
| Maleonitrile | 91 |
| Fumaronitrile | 81 |
| Dimethyl Fumarate | 74 |
| Dimethyl Maleate | 0.6 |
| Methyl Acrylate | 1.2 |
| Acrylonitrile | 1.0 |

[a] From second order rate constants in dioxane at 20 °C (Wuest, 1964).

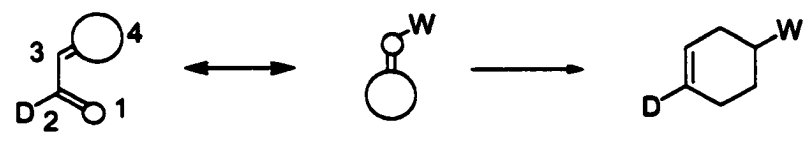
directing the major amount of electron density on the α-carbon, thus regioselectively governing the outcome of the reaction. The increased overall energy of the HOMO in the 2- substituted case remains the same as the 1- substituted case although the regioselectivity has been reversed. The increased electron density is now on the β-carbon relative to the electron donating group thus giving rise to the 1, 4 cyclohexenyl adduct.

The aim of this chapter is to assess the reactivity of DOG as a dienophile in the Diels-Alder reaction. The combination of some well known dienes and DOG will be examined. Specifically, the reactivity and regiochemical results obtained from the reaction of DOG with symmetrical and unsymmetrical dienes will be examined. The




3,4-disubstituted cyclohexene

Favorable orbital overlap




1,4-disubstituted cyclohexene

**Figure 2.6.** Representative regioselective outcomes due to substituent effects

structure of DOG (**2.6** on page 14) contains all the essential elements to be a productive dienophile in the Diels-Alder reaction. The olefinic portion of the compound is flanked

by two electron withdrawing groups, which should decrease the energy of its LUMO (relative to an unsubstituted olefin) and activate it as a dienophile. Although the two groups adjacent to the olefin are electron withdrawing, they are not identical and, therefore, the molecule is not symmetrical and a regioselective reaction is expected. The keto-ester moiety is more electron withdrawing than is the ester group and thus should partially govern the regioselectivity of the reaction with unsymmetrical dienes. Namely, with 2-methyl-1,3-butadiene, the major regioisomer will should have the methyl group on the 4 position relative to the ketoester moiety etc.

Solvents are of particular interest in the Diels-Alder reaction. The Diels-Alder reaction is theorized to progress through an aromatic transition state (Carey, 1984). In the case of a pericyclic reaction, the new carbon-carbon bonds are formed by the combination of $\pi$ molecular orbitals of the diene and dienophile. In general, this may be compared to that of electron delocalization in an aromatic system, therefore, an aromatic solvent allows the transition state to be more easily reached than a non-aromatic solvent. Although this rational tends to hold true, it has also been shown that various polar solvents can be just as effective in the Diels-Alder reaction, although the proposed interactions differ from that of an aromatic solvent Carey, 1984).

## II. Results and Discussion

*1. Multigram synthesis of the key intermediate dimethyl 2-oxoglutaconate (DOG) (2.3) (for a detailed analysis and spectral data see: Carrigan, 1999.*

2-Oxoglutarate (89.4 g) was reacted with thionyl chloride in MeOH to afford dimethyl 2-oxoglutarate (**2.5**) in quantitative yield. The resulting diester was treated with

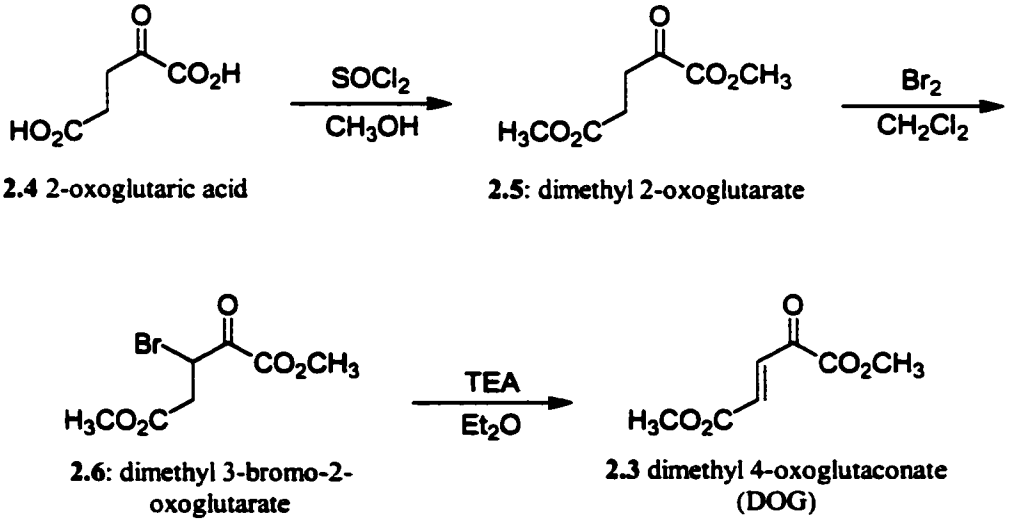


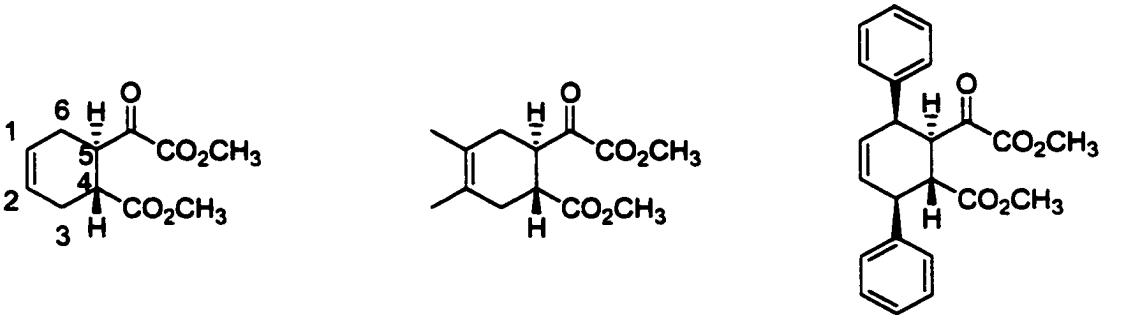

**Figure 2.7**: Synthesis of DOG as described by Corey and Co-Workers

bromine in $CH_2Cl_2$ and stirred at reflux to afford the crude dimethyl 3-bromo-2-oxoglutarate (**2.4**) in quantitative yield. In the final step in the preparation of DOG, dimethyl 3-bromo-2-oxoglutarate was dehydrohalogenated with triethylamine in diethyl ether to afford DOG (**2.3**, 102.5 g, 97.3% overall) after filtration through a plug of silica

gel and subsequent recrystallization with ethyl acetate and hexanes. The product DOG showed physical and spectral details identical to that reported (Carrigan, 1999).

*2. Survey of DOG as a dienophile in the Diels-Alder cycloaddition reaction*

Figures 2.8 - 2.11 show the products of the reactions that have been attempted in the laboratory. The reaction conditions and percent yields (Table 2.2) reveal the high



**2.7** (58 - >95%) **2.8** (71 - >95%) **2.9** (>95%)

Figure **2.8.** Monocyclic Diels-Alder adducts from symmetrical dienes

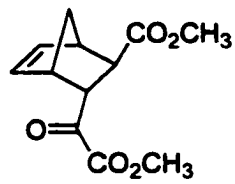
reactivity and success of DOG as a dienophile in the Diels-Alder reaction. As is evident in the table, high temperatures and pressures are not needed for this reaction to proceed. In the cases where a non-aromatic solvent was used, either the diene was lower boiling than benzene, or was so reactive that an aromatic solvent was not required. In the case of the reaction utilizing furan as the diene, increased temperatures were required to overcome, or break, the aromaticity of the diene. In utilizing anthracene as the diene, high temperature was required to solubilize the anthracene.

The *trans* orientation of DOG presents additional interesting features in this reaction. With symmetrical open chain dienes, the adducts obtained will have two newly

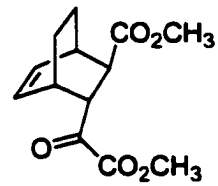
**Table 2.2.** Conditions and yields for Diels-Alder reactions between DOG and select dienes

| Adduct number | Diene | Solvent | Conditions | Percent Yield |
|---|---|---|---|---|
| **2.7** | Sulfolene | m-xylene | reflux | 58 |
| **2.7** | 1,3-butadiene | $CH_2Cl_2$ | rt | ≥ 95 |
| **2.8** | 2,3-dimethyl-1,3-butadiene | hexanes | 0 °C → reflux | 71 |
| **2.8** | 2,3-dimethyl-1,3-butadiene | benzene | sealed tube 80 °C | 88 |
| **2.8** | 2,3-dimethyl-1,3-butadiene | $CH_2Cl_2$ | reflux | ≥ 95 |
| **2.9** | 1,4-diphenyl-1,3-butadiene | $CH_2Cl_2$ | rt | ≥ 95 |
| **2.10** | Cyclopentadiene | $CH_2Cl_2$ | rt | 90 |
| **2.10** | Cyclopentadiene | benzene | 0 °C | 86 |
| **2.11** | 1,3-cyclohexadiene | benzene | sealed tube 150 °C | 83 |
| **2.11** | 1,3-cyclohexadiene | benzene | reflux | 58 |
| **2.12** | Furan | furan | sealed tube 150 °C | 28 |
| **2.12** | Furan | $CH_2Cl_2$ | reflux | 8 |
| **2.12** | Furan | $CH_2Cl_2$ | rt $AlCl_3$ | 67 |
| **2.13** | 2-methylfuran | $CH_2Cl_2$ | reflux | ≥ 95 |
| **2.14** | 2,5-dimethylfuran | benzene | sealed tube 110 °C | N/R[a] |
| **2.14** | 2,5-dimethylfuran | $CH_2Cl_2$ | reflux | N/R[a] |
| **2.14** | 2,5-dimethylfuran | $CH_2Cl_2$ | rt $AlCl_3$ | N/R[a] |
| **2.15** | 2,3,4,5-tetraphenyl-cyclopentadienone | benzene | sealed tube 150 °C | N/R[a] |
| **2.16** | Anthracene | benzene | sealed tube 150 °C | 67 |
| **2.17** | Acridine | benzene | sealed tube 150 °C | N/R[a] |
| **2.18** | Phenazine | $CH_2Cl_2$ | sealed tube 150 °C | N/R[a] |
| **2.19** | Isoprene | $CH_2Cl_2$ | reflux | ≥ 95 |
| **2.20** | furfural amine | $CH_2Cl_2$ | rt | 84 |

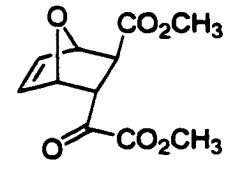
[a]N/R = no reaction



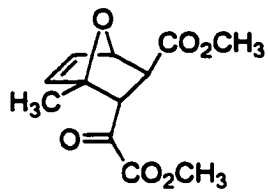
**2.10** (86 - 90%)



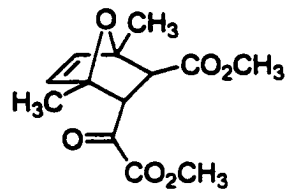
**2.11** (58 - 83%)



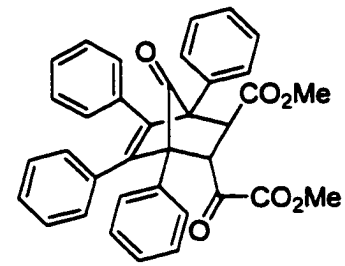
**2.12** (8 -67%)



**2.13** (>95%)

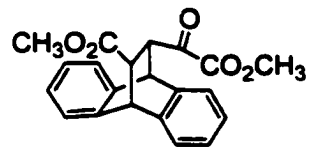


**2.14** (N/R)

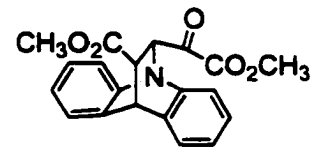


**2.15** (N/R)

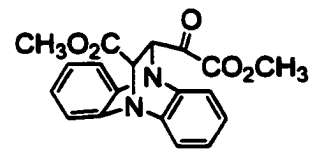
**Figure 2.9.** Bicyclic Diels-Alder adducts



**2.16** (67%)



**2.17** (N/R)



**2.18** (N/R)

**Figure 2.10.** Diels-Alder adducts from extended aromatic dienes

formed chiral centers. The interesting feature of this system is that only two stereoisomeric products are obtained. Since the dienophile is the *trans* isomer, the

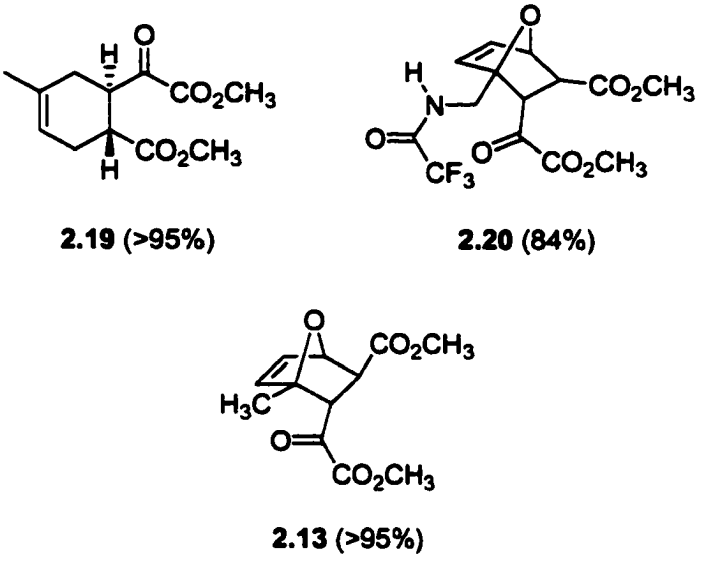



**Figure 2.11**. Diels-Alder adducts derived from unsymmetrical dienes

cycloaddition adducts will be trans across the 4-5 bond of the cyclohexenyl products, therefore, producing a mixture of only two enantiomers. For the adducts **2.7** and **2.8** the $^1$H NMR spectra indicate the *trans* ring junction by affording a doublet of triplets at 2.81 and 2.95 ppm, respectively with coupling constant values of 11.1 and 14.6 Hz, respectfully. In the case of the diphenyl substituted cyclohexenyl adduct (**2.9**), there will be four total chiral centers, but the product mixture will contain only four stereoisomers. In the formation of the adduct, the phenyl rings at the 3 and 6 position of the cyclohexene will be *cis* relative to one another, in turn, limiting the number of stereoisomers obtained. With this limitation, as well as the *trans* orientation about the 4-5 bond, indicated by the

coupling constant of the triplet corresponding to the proton alpha to the ester of 11.4 Hz, the number of isomers is well short of that which would be obtained if a non-stereospecific reaction sequence was used (i.e. $2^n = 2^4 = 16$).

Table 2.2 also reveals that the relative rates of reaction for furan, 2-methylfuran and 2, 5-dimethylfuran differ considerably. The interaction of the dienes with DOG in the transition state is highly dependent on the energy of the HOMO of the dienes. The HOMO energies (calculated using PC Spartan Pro 1.0.5, Irvine CA) for the dienes are given in Table 2.6. The consistent feature of the calculations is that the respective HOMO energies of furan, 2-methylfuran and 2, 5-dimethylfuran increase with the addition of electron donating substituents and this, in turn, should increase the reactivity with DOG, although the reactions utilizing furan and 2, 5-dimethylfuran as the diene are extremely more sluggish than that with 2-methylfuran. This is most likely due to the fact that the HOMO of furan is too low in energy to interact constructively with the LUMO of DOG and that the HOMO of 2, 5-dimethylfuran may have been increased so much that it has surpassed the level of energy that is required to interact favorably with DOG in the Diels-Alder reaction. Another piece of evidence that adds confidence to this hypothesis is that when a catalytic amount of $AlCl_3$ was added to the reaction mixture of furan and DOG, the reaction was complete in less than five minutes and the yield was increased to 68% (not optimized). This supports the theory that the LUMO of DOG is too high to interact with furan under non-catalytic conditions but once the catalyst is added, the coordination of the ketone of DOG with the aluminum atom leads to a decrease in DOG's LUMO enough to interact considerably with the HOMO of furan. Also, when a catalytic amount of $AlCl_3$ was added to the reaction mixture of 2, 5-dimethylfuran and DOG, no

increase in percent yield was achieved and more by-products were detected by thin layer chromatographic (TLC) analysis. This adds further evidence in support of the

**Table 2.3**. Energies and yield of substituted furan Diels-Alder adducts

| Diene | HOMO Energy (eV)[a] | Percent Yield | Percent Yield with $AlCl_3$ |
|---|---|---|---|
| 2, 5-dimethylfuran | -8.755 | N/R[b] | N/R[b] |
| 2-methylfuran | -9.015 | ≥ 95 | N/A[c] |
| Furan | -9.312 | 8 | 68 |

[a] as calculated with PC Spartan Pro, [b] N/R = no reaction,
[c] N/A = not assessed due to the high reactivity in the absence of catalyst

interpretation that the HOMO of 2, 5-dimethylfuran has risen above that of DOG and a productive HOMO / LUMO interaction can not be achieved.

The low reactivity of 2, 3, 4, 5-tetraphenylcyclopentadienone may be due to a collection of factors including the crowded nature about the diene due to the four aromatic groups, the slight electron withdrawing nature of the phenyl groups as well as the electron withdrawing nature of the ketone moiety (Figure 2.12).

The aromatic molecules anthracene, phenazine and acridine also show much different reactivities in the Diels-Alder reaction with DOG. As is shown in Table 2.5, anthracene was able to undergo the Diels-Alder reaction with DOG in modest yield but the reaction with phenazine and acridine resulted in no reaction. The incorporation of the nitrogen atoms in the ring may slow the reaction by having the aromatic nature removed

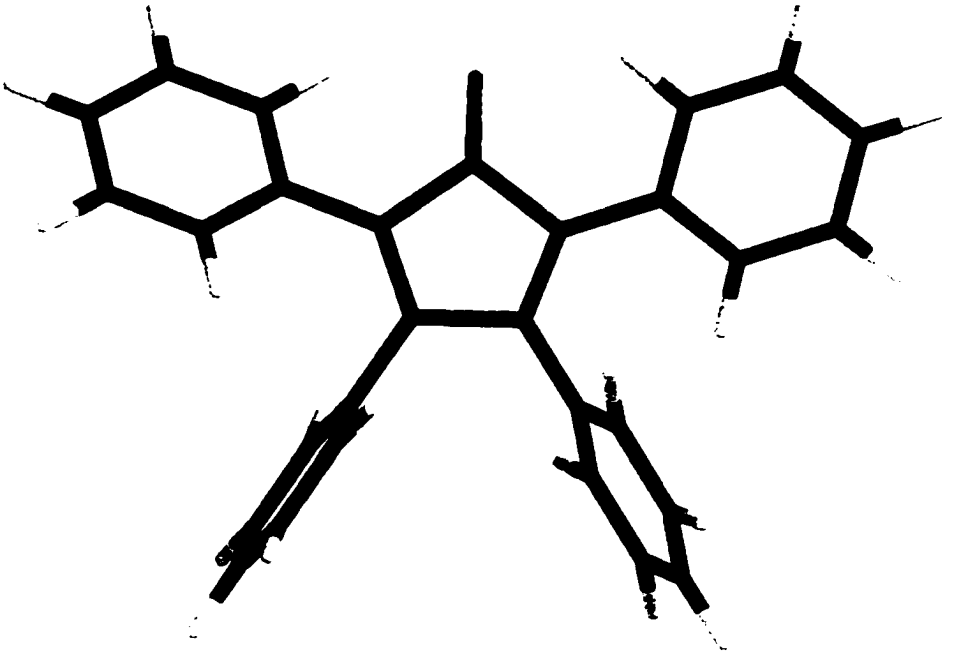


**Figure 2.12.** Structure of 2, 3, 4, 5-tetraphenylcyclopentadienone

at the end of the reaction. Although the two rings next to the nitrogens are aromatic, the activation energy needed to achieve the transition state may be too high with phenazine and acridine.

Since the overall goal of the project was the synthesis of structurally constrained amino acid analogues, the survey of the regioselective nature of DOG in the Diels-Alder reaction with DOG was examined only briefly. Three dienes were used: isoprene, 2-methylfuran and N-trifluoroacetyl furfuralamine (Figure 2.11). With isoprene, the reaction mixture produced two regioisomers collectively with excellent yield and a regioisomer ratio of 66:34 as determined by integration of the ester α-proton doublet of triplets in the $^1$H NMR (Figure 2.13).

The ketone group appears to control the regioselectivity of the reaction as evidenced by the $^1$H NMR chemical shifts and coupling patterns of the DOG adducts with furan, 2-methylfuran and N-trifluoroacetylfurfural amine. Figure 2.14 shows a stack of the three $^1$H NMR spectra of the resulting adducts. As is seen in the spectra of the furan-DOG adduct there is a broad doublet of doublets at 7.34 ppm assigned to the bridgehead proton ($H_a$) adjacent to the proton alpha to the ketone functionality. The spectra of the 2-methylfuran-DOG adduct contains no resonance between 7 and 8 ppm but there is an additional singlet appearing at 2.18 ppm corresponding to the newly introduced bridgehead methyl group. In the spectra for the N-trifluoroacetylfurfuralamine-DOG adduct, there is a broad triplet at 7.48 ppm corresponding to the amide hydrogen and a new resonance at 4.36 ppm corresponding to the methylene carbon alpha to the amide nitrogen. These data suggest that the regiochemistry of the reaction is consistent and controlled by the carbonyl groups such

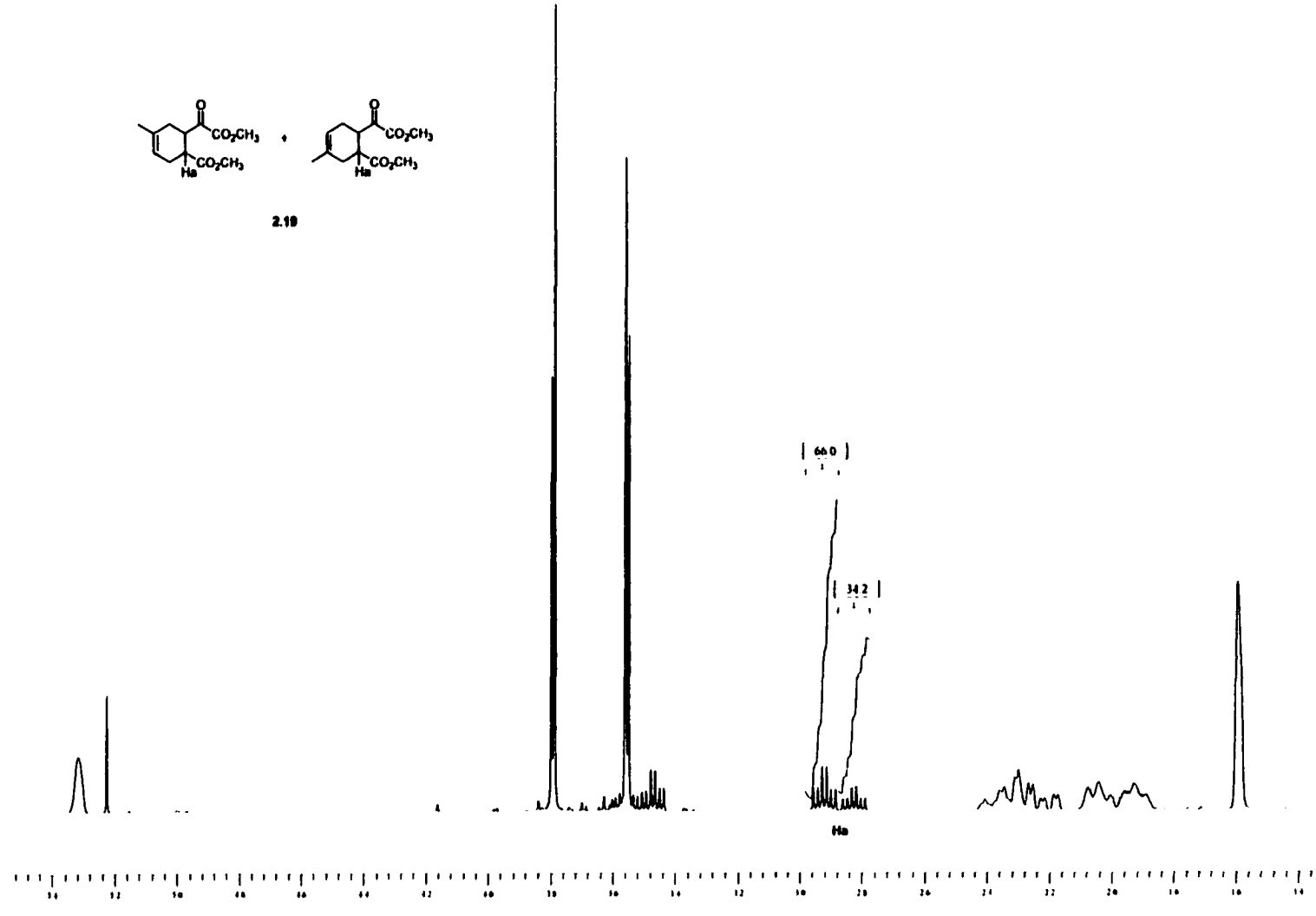



**Figure 2.13.** $^{1}$H NMR of the regioisomer mixture obtained from the Diels-Alder reaction between DOG and isoprene

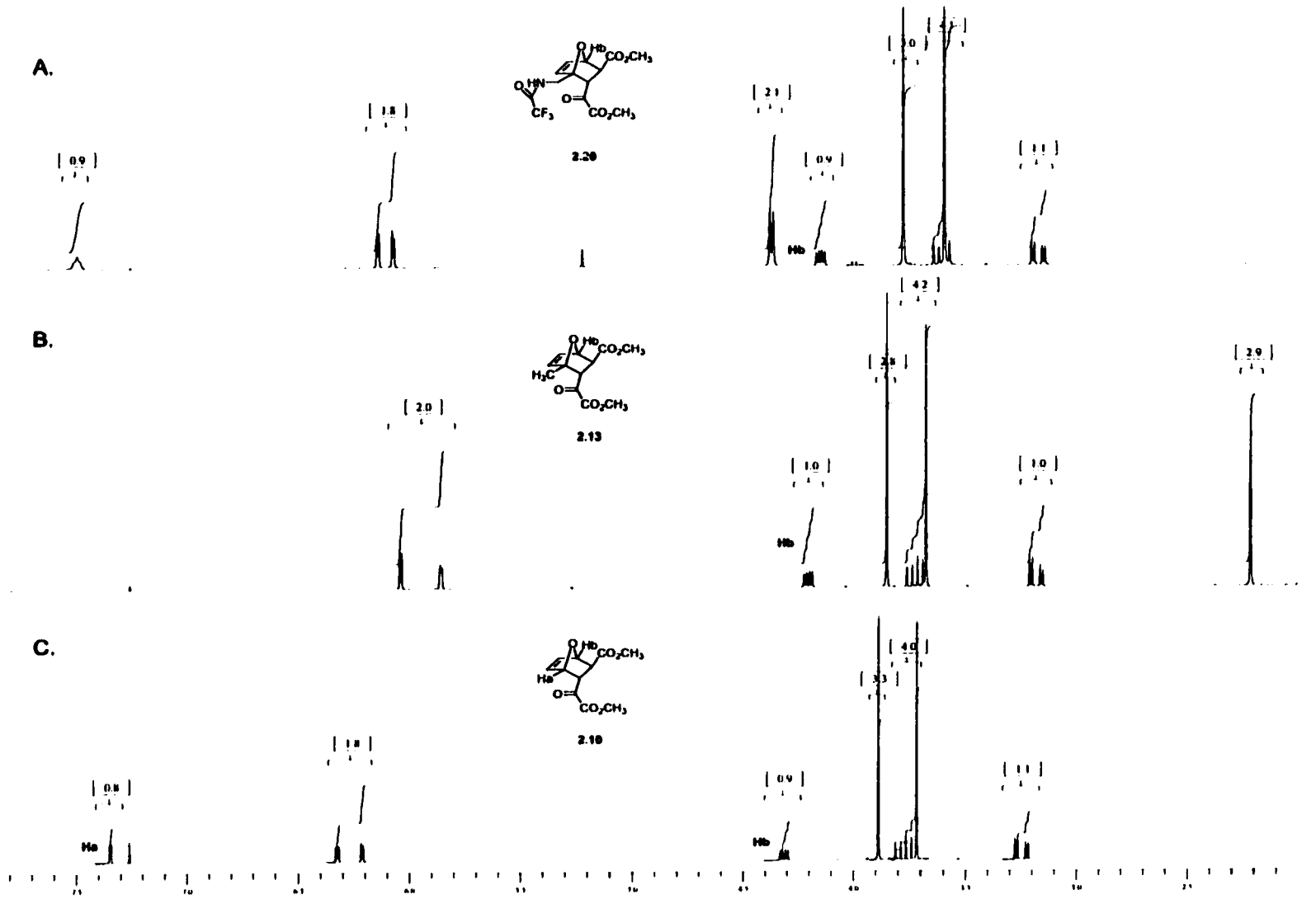



**Figure 2.14.** $^{1}$H NMR comparison of N-trifluoroacetylfurfuralamine (**2.20**), 2-methylfuran (**2.13**) and furan (**2.10**)-DOG adducts.

that the donor group in the diene aligns with the ketoester moiety (Figure 2.14). Although the regiochemistry of this reaction was only slightly researched, the potential to synthesize regiochemically specific keto-esters in route to the corresponding glutamate analogues may be of tremendous benefit in the future.

It was shown that DOG is a highly reactive dienophile in the Diels-Alder reaction. The reaction was successful with the dienes sulfolene (1, 3-butadiene), 2, 3-dimethyl-1, 3-butadiene, 1, 4-diphenyl-1, 3-butadiene, cyclohexadiene, cyclopentadiene, furan, 2-methylfuran, 2, 3, 4, 5-tetraphenylcyclopentadienone, anthracene, isoprene and N-trifluoroacetyl furfuralamine. It was also shown that the dienes acridine, phenazine and 2, 5-dimethylfuran were not of sufficient reactivity to partner with DOG in the Diels-Alder reaction.

*3. Ketone to amine transformation: Formal reductive amination*

The justification for the use of DOG as a dienophile in the Diels-Alder reaction was predicted on the fact that the keto-ester cyclic products are potential starting materials for the synthesis of 3, 4-conformationally-restricted L-glutamate analogues. The key chemical transformation to access the conformationally constrained glutamate analogues is the conversion of the α-keto ester moiety into an α-amino ester. The reductive amination of ketones is well documented (for a review see, Hutchins, 1979). One common route used to convert a ketone into an amine is reduction of an imine formed by the condensation of a ketone with a $1^{\circ}$ amine (Figure 2.15) including: a) hydrogenation over a nickel (Norton, 1954), b) platinum (Freifelder , 1966) or c) palladium catalyst (Eleveld, 1986) , d) $NaBH_4$ (Hutchins, 1983, and references therein) and one of the most mild and selective, to date, e) $NaCNBH_3$ (for a good review see, Lane, 1975). The reductive amination success relies on high conversion of the carbonyl and the amine to the imine and many different procedures have been adapted to increase the formation of the imine including the use of: a) dehydrating agents such as molecular sieves (Bonnett, 1965), b) removal of water under azeotropic conditions using benzene or toluene (Wrobel, 1981) c) acid catalysis utilizing acetic acid (Hutchins, 1979) and d) $TiCl_4$ is used as a Lewis acid catalyst as well as the dehydrating agent (Weingarten, 1967).

A vast number of different conditions were employed in the laboratory to effect the reductive amination of the structurally constrained keto esters via an imine

intermediate, but, the structures do not appear to be good substrates for this reaction. There are a number of reasons that the reaction may fail. The steric constraints

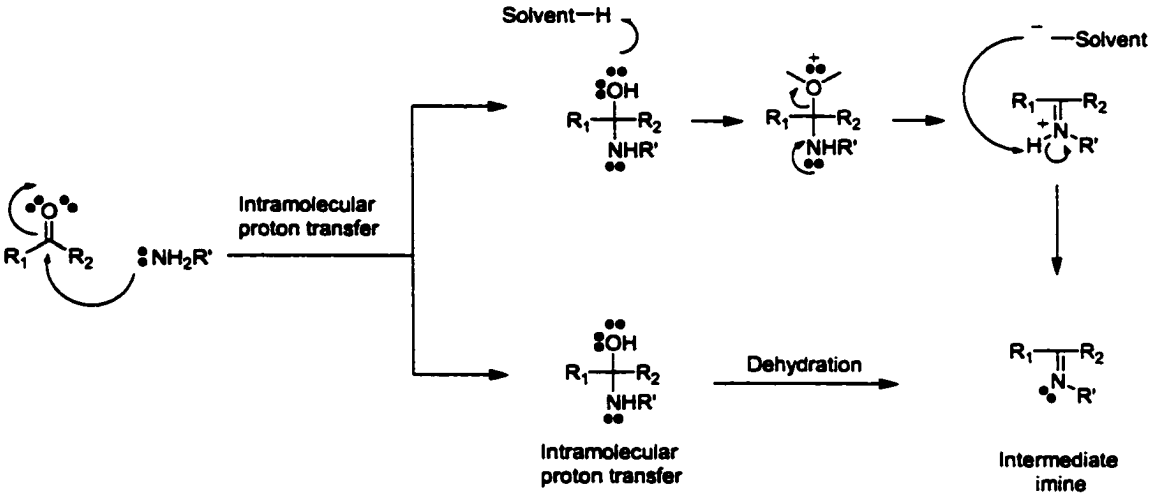



**Figure 2.15.** Formation of the imine intermediate in route to reductive amination

imposed about the ketone moiety make the region too crowded for an effective imine formation. This seems to be highly unlikely due to the fact that the ketone can be converted to the corresponding oxime and dimethylhydrazone, which proceed by a similar mechanism (discussed later). Another factor that may effect the imine formation is that the ketone may readily enolize in the presence of certain acid catalysts that prevent the nucleophilic addition of the amine to the ketone functionality but, once again, this argument may be contradicted, in part, by the relative ease of formation of the oxime and dimethylhydrazone products. In the case where ammonia and primary amines were used as the initial nucleophile, there is a large opportunity for amide formation with the ester functionalities of the starting materials. Although this is a distinct possibility, it was not researched exhaustively due to the fact that, although the problem is an interesting one in

mechanistic organic chemistry, the product amino acids were highly desired and the search for a procedure to produce them in good yields predominated over a mechanistic investigation. Table 2.4 summarizes some of the conditions we explored unsuccessfully in route to the imine.

**Table 2.4.** Conditions employed for chemical reductive amination

| Starting Material | Amine Eq. | Reducing agent | Solvent | Conditions |
|---|---|---|---|---|
| dimethyl 2-oxoglutarate | $NH_4Br$ | $NaBH_3CN$ | $CH_3OH$ | HCl (cat.) rt |
| dimethyl 2-oxoglutarate | benzylamine | $NaBH_3CN$ | $CH_3OH$ | HOAc (cat.) rt |
| dimethyl 2-oxoglutarate | benzylamine | $NaBH_3CN$ | $CH_3OH$ | HOAc (cat.) rt 4A sieves |
| dimethyl 2-oxoglutarate | benzylamine | $NaBH_3CN$ | $CH_3OH$ | HOAc (cat.) reflux |
| 2-oxoglutarate | benzylamine | $NaBH_3CN$ | $CH_3OH$ | HOAc (cat.) reflux |

The ketoxime of compound **2.8** (Figure 2.16) was synthesized by the addition of ammonium acetate trihydrate to a solution of **2.8** and hydroxylamine hydrochloride in ethanol. The resultant ketoxime was isolated as a white solid in 42 % yield after

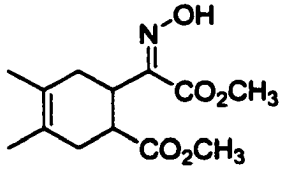



**Figure 2.16.** Ketoxime (**2.21**) of compound **2.8**

recrystallization from ethanol As mentioned above, it has been shown that ketoximes can be reduced to their corresponding amines by hydrogen and Raney nickel (Norton, 1954). This reaction was attempted in the lab, but was initially unsuccessful and not highly researched according to the arguments stated above as well as the fact that there is a high potential for the reduction of the olefin moiety in the molecule, which is a necessary component of our final amino acid molecules to impose the conformational restriction about the 1, 2 bond. In addition, the reduction of the olefin double bond would introduce two new centers of chirality which, even though the reduction is stereoselective, will double the number of product stereoisomers and render product isolation more difficult, therefore, a more selective reagent was sought.

Serckx-Poncin and co-workers (1982) have shown that the nitrogen-nitrogen bond of a hydrazine can be broken using zinc in acetic acid. With this in mind, it was postulated that the double bond of a hydrazone could also be reduced under these conditions, therefore, introducing a new hydrazine bond which would be subsequently reductively cleaved under the reaction conditions to afford the desired amino diesters. With this in mind, we set out to synthesize the N, N-dimethylhydrazone derivatives of the keto diester adducts previously obtained in the Diels-Alder reaction and survey their potential as substrates for the reductive cleavage reaction in the preparation of the corresponding conformationally restricted analogues of glutamate (figure 2.17). The

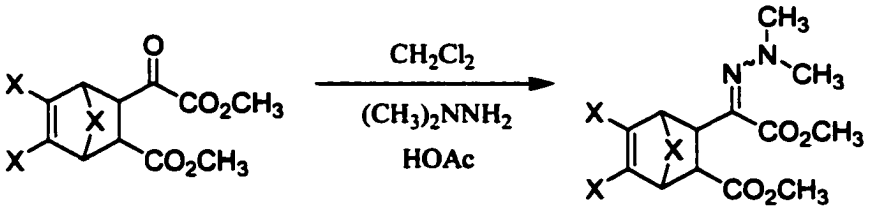
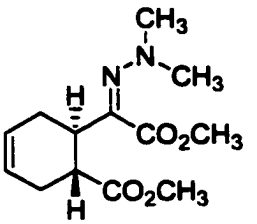
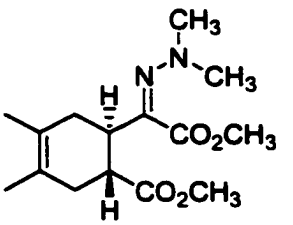



**Figure 2.17**. Synthesis of N, N-dimethylhydrazones of the Diels-Alder adducts. The identity of X is described in table 2.2

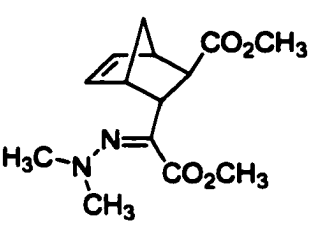
hydrazones were accessed by the condensation of N, N-dimethylhydrazine with the keto diester adducts, catalyzed by acetic acid in dichloromethane, ranging from 79-91 percent yield. One very important factor in this reaction is its convenience and ease of execution. The dichloromethane solvent and the hydrazine reagent are both very low boiling and thus readily removed *in vacuo*. The acetic acid catalyst is readily removed by an aqueous workup and the hydrazone products are readily obtained via flash chromatography (see the Experimental section). Four hydrazones were successfully synthesized from the ketone precursors (Figure 2.18).



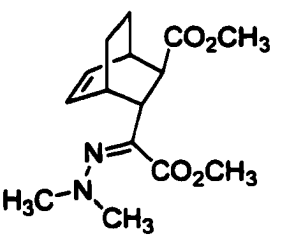
**2.22**



**2.23**



**2.24**



**2.25**

**Figure 2.18.** Hydrazone products from select Diels-Alder adducts

Once the hydrazones were synthesized, their ability as substrates was assessed in a tandem reduction, reductive cleavage reaction. It was brought to our attention that the tandem reduction, reductive cleavage reaction has been accomplished with the use of sodium hydrosulfate under aqueous conditions (DeGraw, personal communication). With this information, as well as the evidence that under the conditions using zinc and acetic acid, double bonds are readily reduced (Serckx-Poncin, 1983), conditions to perform the sequence with sodium hydrosulfate were first considered. The reader is advised that sodium hydrosulfate may also be called sodium thiosulfite.

It is essential that a solvent capable of dissolving the sodium hydrosulfate to a substantial amount that it can interact with the hydrazone is used. It was determined that a mixture of water and methanol (1 : 1) under reflux was a favorable set of conditions.

Figure 2.19 shows the reagents and products of the reactions of compounds **2.22 – 2.24** with sodium hydrosulfate in water-methanol (1 :1).

The products arising from the reduction of the monocyclic cases are lactam esters and lactam acids **2.26 - 2.29,** resulting from initial formation of the desired amine followed by cyclization and hydrolysis. In the bicyclic cases, the products **2.30 - 2.31** are obtained as amino diesters. It is assumed that the lactams are not formed in the bicyclic cases due to the extra rigidity of the molecules exerted by the bridging methylene groups.

Due to the nature of these compounds to form lactams, the number of glutamate analogues has increased due to an intermediate structure showing activity in biological preparations.

For compounds **2.24** and **2.25,** the intermediate amino esters (**2.30** and **2.31**) were isolated by a simple aqueous extraction, identified by electrospray mass spectrometry

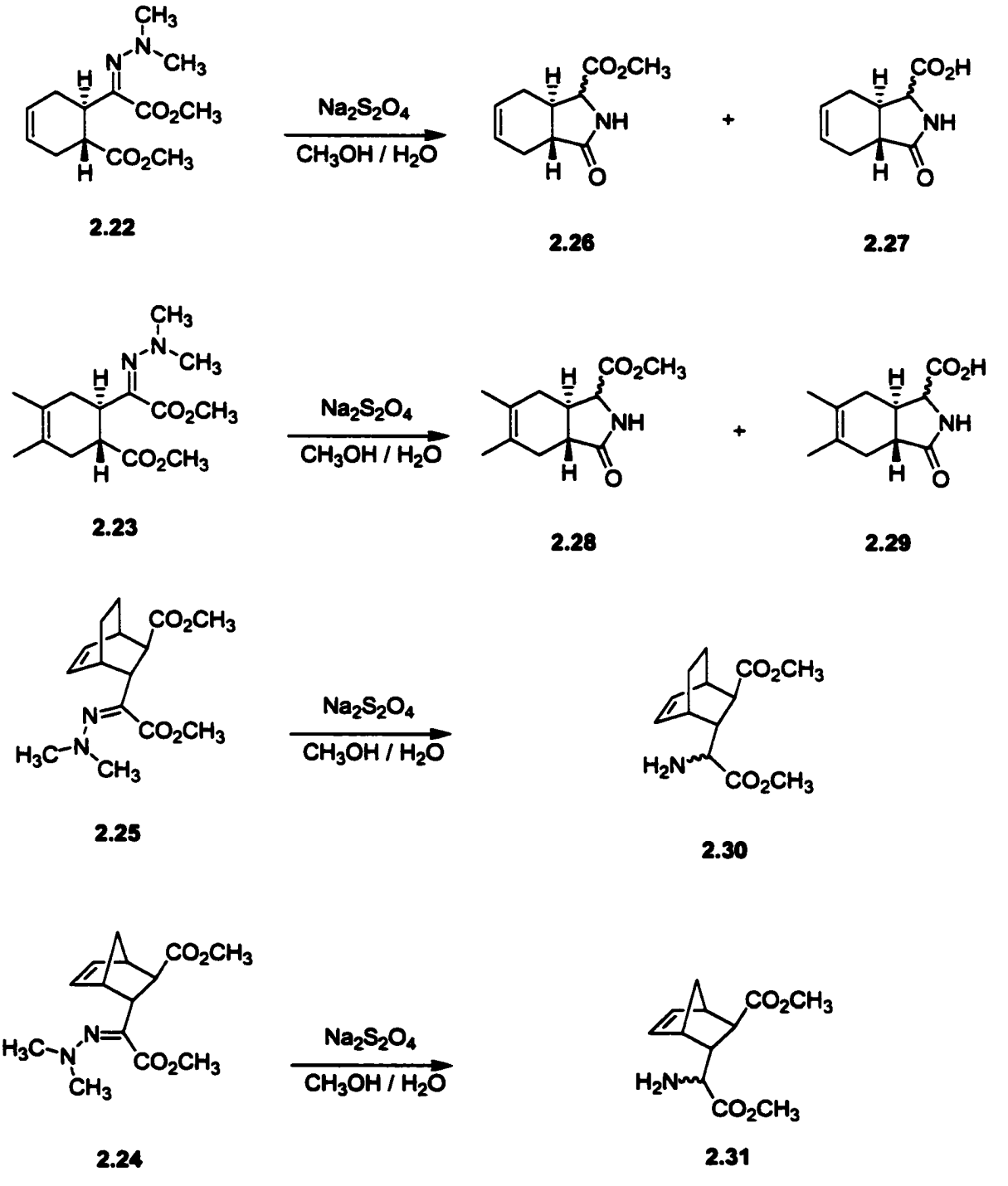



**Figure 2.19. Sodium hydrosulfate reduction of hydrazones**

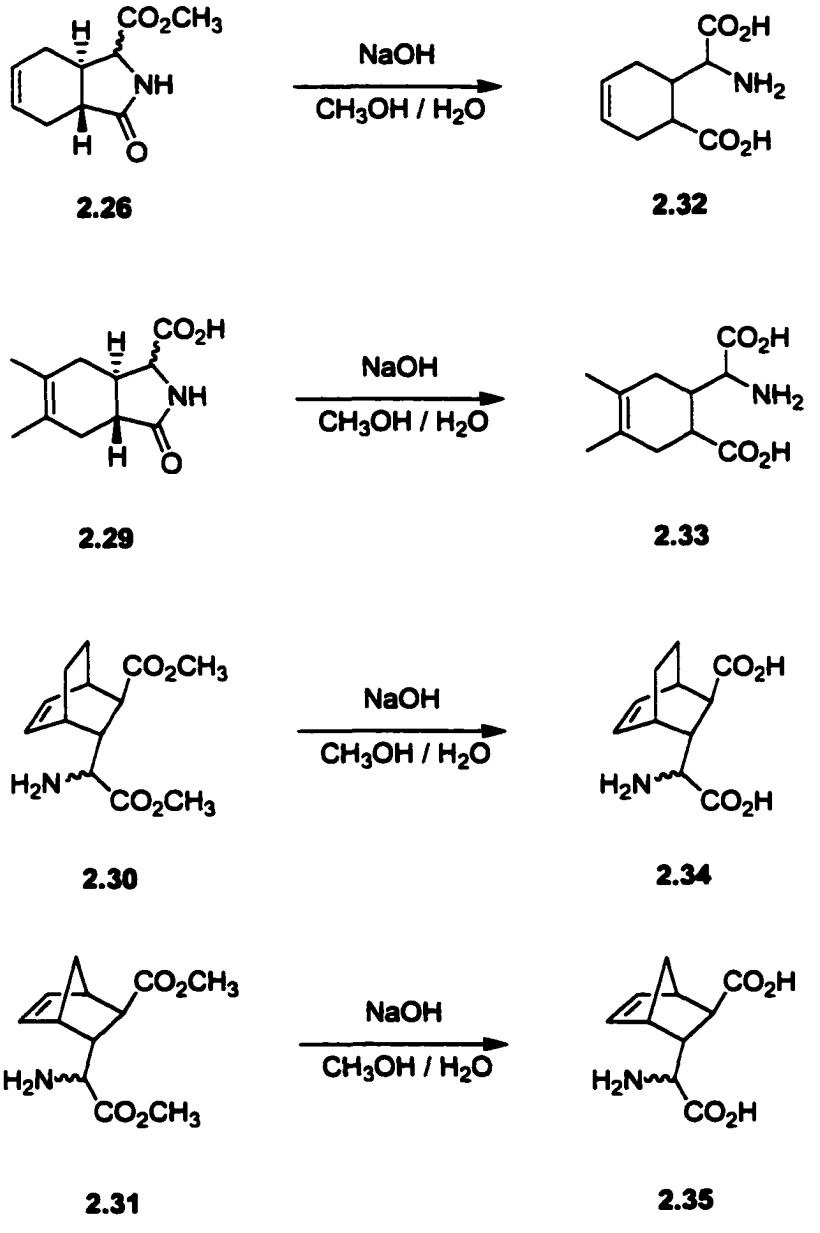



**Figure 2.20.** Saponification to afford the target amino acids

(ESI-MS) and used directly in the next reaction without further purification or characterization.

To access the amino acid analogues, the lactam acids, lactam esters or amino esters were subsequently saponified with sodium hydroxide (Figure 2.20) and purified by

ion exchange chromatography to afford the amino acid hydrochorides **2.32**, **2.33**, **2.34** and **2.35** in 58, 85, ≥95 and 76 % yield, respectively. These newly formed, conformationally restricted glutamate analogues were tested as inhibitors of the sodium dependent excitatory amino acid transporters EAAT2 and EAAT3, the chloride dependent glial cystine/glutamate exchanger system $x_c^-$, and the glutamate vesicular transport system (VGLUT) (for details, see Chapter 4).

*4. Synthesis of 1-dimethylamino-3-methoxyoxalyl-1,2,3,4-tetrahydro-pyridine-2-carboxylic acid methyl esters. Reaction of DOG with azadienes (α, β-unsaturated N, N-dimethylhydrazones).*

Once it was determined that DOG was a reactive dienophile in the Diels-Alder reaction and the subsequent adducts were starting materials for the synthesis of conformationally restricted glutamates with biological activity, it was in our interest to synthesize a number of amino acid derivatives that will mimic glutamate. It has been shown that α, β-unsaturated hydrazones derived from α, β-unsaturated aldehydes readily undergo the Diels-Alder reaction with electrophilic dienophiles. Data has supported the theory that the mechanism of the reaction is not truly a 4 + 2 cycloaddition reaction, but an initial 1, 4 addition of the hydrazone with the double bond of the dienophile, followed by an intramolecular nucleophilic ring closure of the intermediate enolate onto the newly formed iminium ion (Figure 2.21).

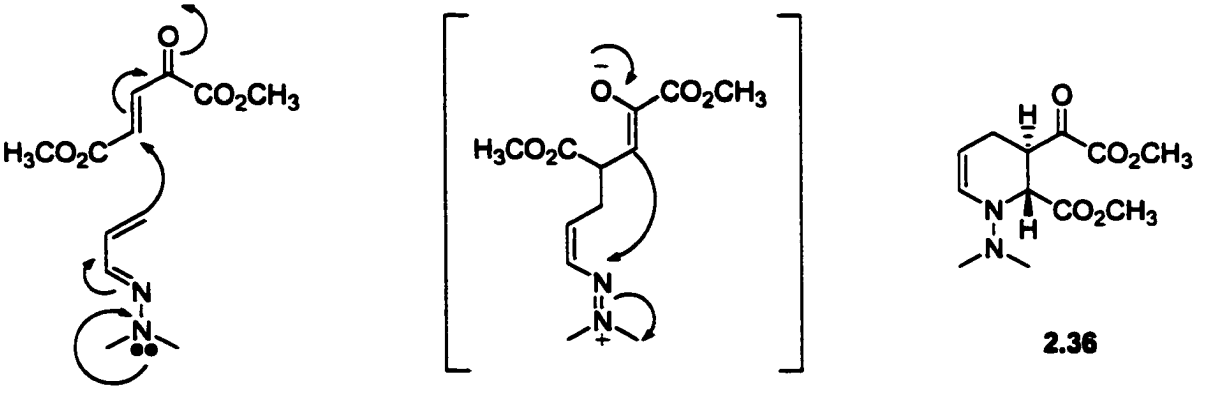



**Figure 2.21. Proposed mechanism and reaction outcome between DOG and α, β-unsaturated N, N-dimethylhydrazones**

The mechanism of this reaction has been substantiated by a number of methods. Serckx-Poncin and co workers (1983) have shown when the N, N-dimethylhydrazone derived from methacrolein (Compound 2.36) undergoes reaction with electrophilic dienophiles, specifically, acrylonitrile and methyl vinylketone, the regiochemistry of the reaction is inverse to that which is expected in a typical, molecular orbital driven, Diels-Alder cycloaddition reaction (Figure 2.22). Further evidence that the mechanism is following the path described in Figure 2.21 is that when the cycloaddition is performed between the N, N-dimethylhydrazone of acrolein with dimethyl fumarate and dimethyl maleate, the resulting cycloadducts have the same *trans* configuration about the 5-6 bond

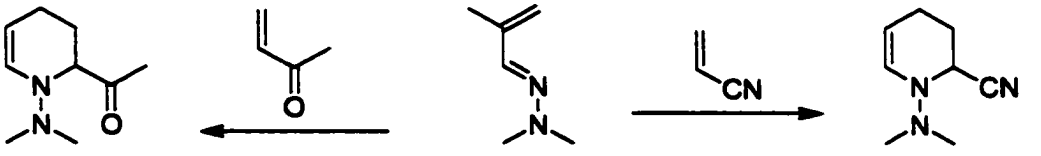
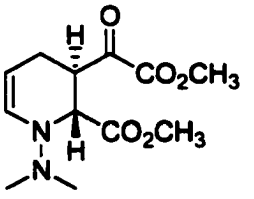



**Figure 2.22.** Regiochemical outcomes of the reaction of methacrolein N, N-dimethylhydrazone with acrylonitrile and methylvinylketone (Serckx-Poncin, 1983)

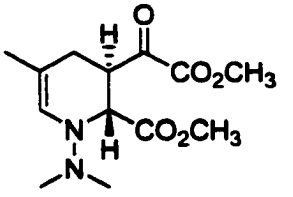
as determined by the coupling constants of the vicinal protons on carbons $C_5$ and $C_6$ ($J$ = 8 Hz). The authors further confirmed the *trans* stereochemistry by the synthesis of the *cis* isomer and subsequent analysis of the coupling constants of the vicinal vicinal protons on carbons $C_5$ and $C_6$ ($J$ = 4 Hz) (Serckx-Poncin, 1983). The *trans* orientation could only have resulted in a stepwise mechanism with dimethyl fumarate as an electrophile.

With this data in hand, we set out to perform the cycloaddition reaction of the hydrazones of methacrolein and acrolein as an exploratory but direct route to a new class

of structurally constrained glutamate analogues, namely, 1-dimethylamino-3-methoxyoxalyl-1,2,3,4-tetrahydro-pyridine-2-carboxylic acid methyl esters. Figure 2.23 shows the two model adducts (**2.36** and **2.37**) formed by the reaction between the N, N-dimethylhydrazones of acrolein and methacrolein and DOG. The regiochemistry was determined to be consistent with the structure depicted in Figure 2.23 by utilizing the knowledge of the reaction mechanism (figure 2.21) for the 1,4 addition-cyclization as well as interpretation of the $^1$H NMR of the adducts. Adducts **2.36** and **2.37** both have a




**2.36**




**2.37**

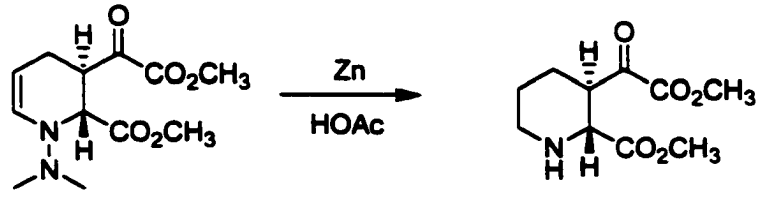
**Figure 2.23**. Adducts of the reaction between the N, N-dimethylhydrazones of acrolein and methacrolein and DOG

triplet of doublets appearing at 3.41 and 3.21 ppm, respectively. This indicates that the proton giving rise to this resonance is alpha to the ketone, for example, in compound **2.7** the chemical shift for the proton alpha to the ketone is 3.45 ppm while the chemical shift for the proton alpha to the ester resonates at 2.81 ppm and in the adduct **2.8** the chemical shift of the proton alpha to the ketone is 3.56 ppm where the proton alpha to the ester resonates at 2.95 ppm.

The adducts formed in this sequence provide the intermediate materials for the synthesis of a new class of structurally constrained glutamates which incorporate the nitrogen of the amino acid within the ring. Besides having the conformation of glutamate

locked about the 2-3 bond of the glutamate backbone, there is also a new feature introduced into the chemical structure, a ketone at the 4 position. This additional feature in the glutamate backbone may serve to further elucidate the structural requirements for analogues to bind constructively at glutamate transporters. The introduction of the ketone group in the intermediates also adds a functional group that can be further manipulated numerous ways to incorporate many new functional groups into the amino acid structure (see Chapter 5).

To access the final amino acid analogue structure requires the reductive cleavage of the hydrazine bond. This reaction has been accomplished with zinc in acetic acid, although the olefin moiety was also reduced. Although this potentially detracts from the structural rigidity of




**2.36** **2.38**

**Figure 2.24.** Reduction of hydrazine and olefin with zinc in acetic acid

the analogues, there is one positive note. In the case of the acrolein adduct, if the tandem reduction-reductive cleavage were to occur without reduction of the olefin, the new product will be an enamine, which has a high potential of ring opening to form the aldehyde containing molecule 2-amino-4-oxo-3-(2-oxo-ethyl)-pentanedioic acid dimethyl ester. This molecule itself is a new analogue of glutamate with potential for further

**manipulation, but it's synthesis, characterization and manipulation is only theoretical to date.**

*5. Investigations into the synthesis of 4-oxoglutamate*

Once it was determined that the Diels-Alder reaction with α, β-unsaturated hydrazones was a success and had potential for accessing 4-oxoglutamate analogues, the synthesis of the most simple 4-oxoglutamate (Compound **2.40**, Figure 2.25) was undertaken. Although the synthesis as shown in figure 2.25 is logical, the actual

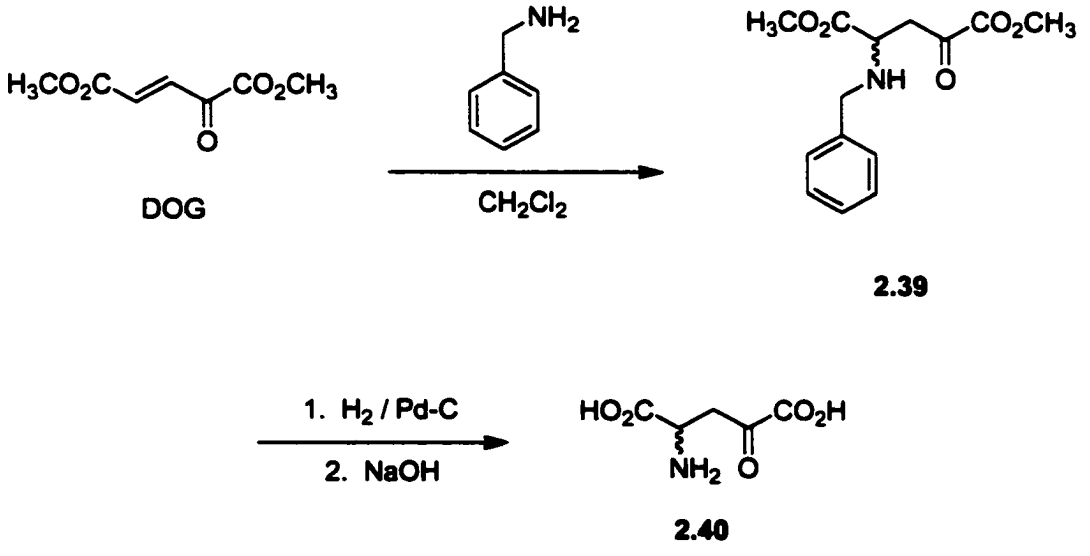



**Figure 2.25.** Proposed synthesis of 4-oxoglutamate

sequence of events which take place are outlined in Figure 2.26. Upon initial 1, 4 addition of benzylamine to DOG, the subsequent amino compound readily cyclizes to form the substituted pyrrolidine (**2.41**) which subsequently reacts with a second equivalent of amine to form the pyrrolidinoenamine (**2.42**). This molecule is easily hydrolyzed back to the pyrrolidine under aqueous conditions. When only 1 equivalent of amine was used, the yield of product, 1-benzyl-4-benzylamino-5-oxo-2,5-dihydro-1H-

pyrrole-2-carboxylic acid methyl ester (**2.41**), was isolated in less than 50 % yield. If two equivalents of amine were used, the yield was increased substantially. This indicates that the formation of the enamine is equally or more rapid as the initial 1, 4 addition,

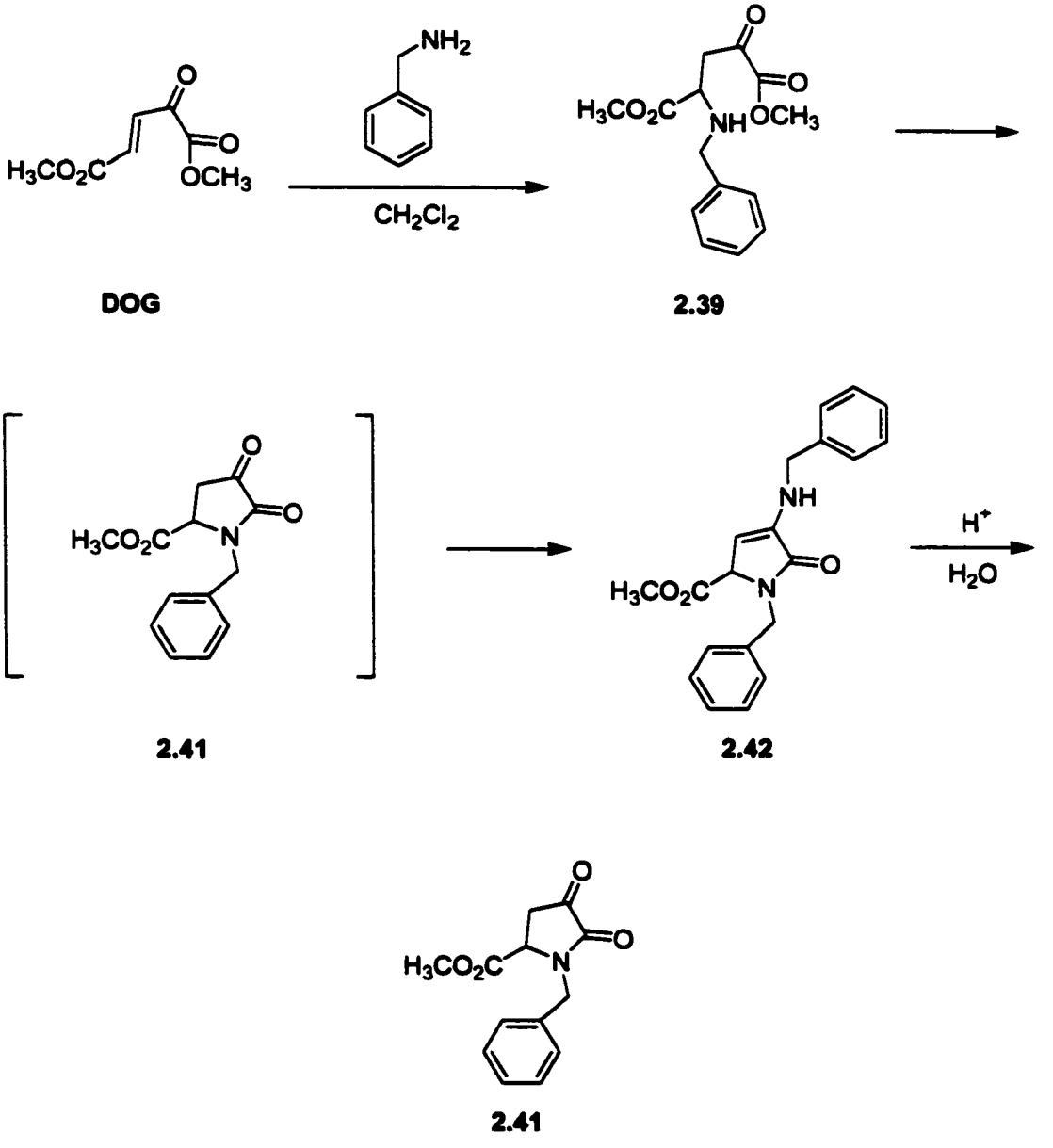



**Figure 2.26.** 1,4 Addition-cyclization-enamine formation of benzylamine with DOG

therefore, to access the pyrrolidine **2.41**, it is necessary to use two equivalents of amine and subsequently hydrolize the enamine product (**2.42**) back to the intermediate target molecule, 1-benzyl-4,5-dioxo-pyrrolidine-2-carboxylic acid methyl ester (**2.41**).

Preliminary investigations into the hydrogenolysis of compound (**2.41**) have been attempted, and reduction of the ketone is evident in the $^{1}H$ NMR and ESI-MS of the crude reaction mixture. Future manipulations of compound (**2.41**) will be discussed in Chapter 5.

*6. Synthesis of structurally constrained 2-oxoglutarate analogues: potential substrates of dehydrogenases and aminotransferases*

One of the initial goals of the project was to synthesize the conformationally constrained glutamate analogues in a stereoselective manner utilizing an enzymatic transformation to convert the keto ester to an amino acid. For this route to be researched, it was necessary to synthesize the substrate 2-oxoglutarates. A brief panel of substrates (**2.43**, **2.44**, **2.45**, **2.46** and **2.47**) was synthesized by the saponification of the Diels-Alder adducts **2.7**, **2.8**, **2.10** and **2.11** (Figure 2.25). A solution of the keto ester in MeOH was saponified with an excess of aqueous sodium hydroxide (NaOH). The resultant ketocarboxylate salts were protonated with hydrochloric acid, extracted with diethyl ether, dried over anhydrous magnesium sulfate and the crude oils were obtained after evaporation of the solvent. Further purification of the keto diacids by ion exchange or traditional chromatography (below) was very difficult; therefore, the crude keto diacids were used as substrates in the transamination reaction (see Chapter 3).

Purification of the ketoacids was attempted by a number of methods. First, the acids were subjected to anion exchange chromatography. The aqueous solutions of the ketoacid sodium salts were prepared by the addition of NaOH to a vigorously stirred slurry/solution of ketoacid in water until all of the oil was dissolved (ca. pH 8). The solution was added to a cation exchange column (BioRad, AG 1-X2, , 20 g resin / gram ketoacid), and washed with water to remove any inorganic ions. It was attempted to elute the keto acids with an increasing gradient of aqueous acetic acid, but the acids were only sparingly soluble in aqueous solution and became virtually inseparable from the resin.

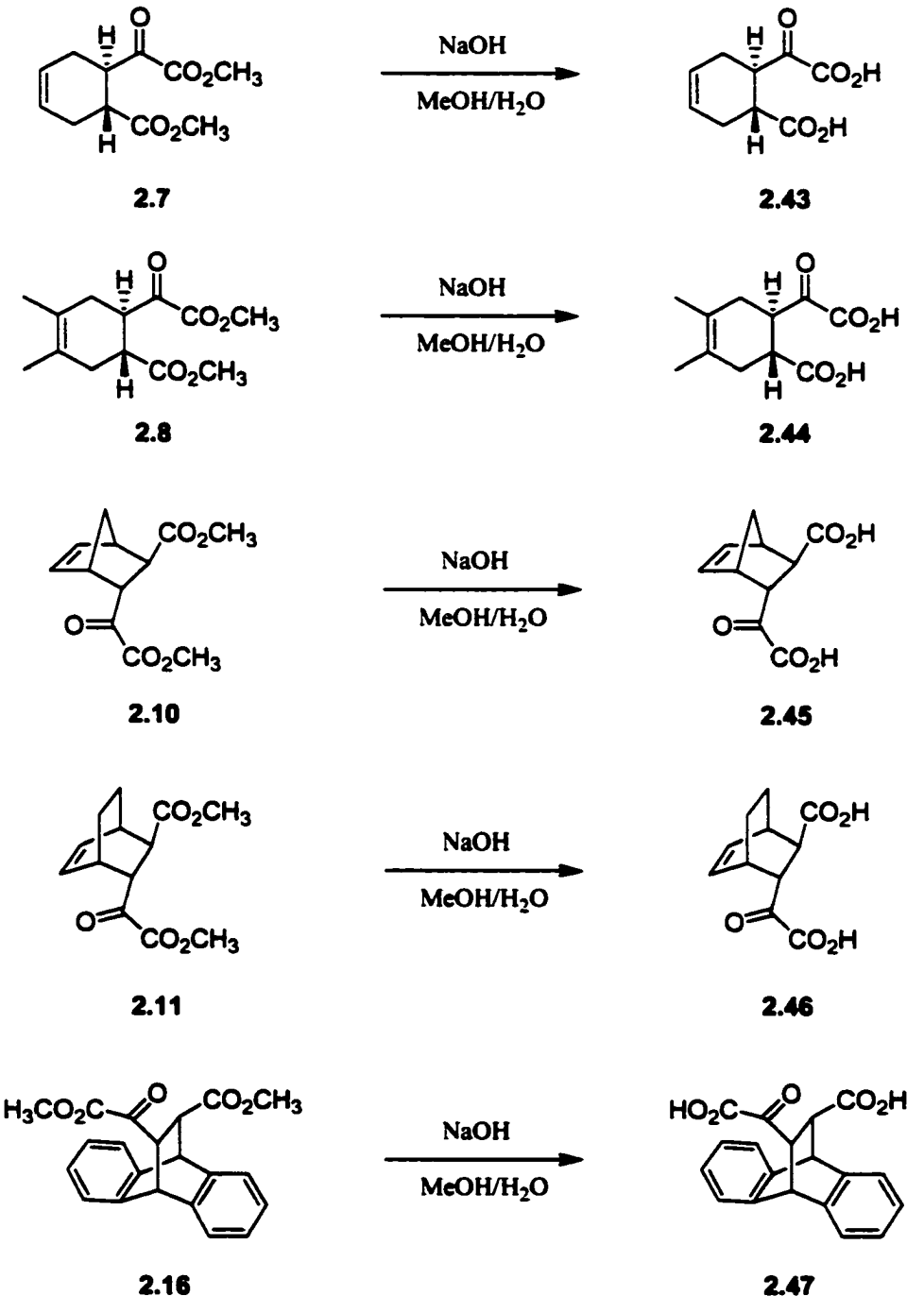



**Figure 2.27.** Saponification of keto ester Diels-Alder adducts

Attempts to extract the keto acids from the resin with hydrochloric acid and methanol or as their pyridinium salts with aqueous pyridine also failed.

It was also attempted to form a salt that may be isolated by crystallization. In an attempt to form an insoluble barium salt a solution/slurry of keto acid in water was treated with saturated $Ba(OH)_2$ until pH > 10. The resultant solution was let stand at rt for up to thirty days with no appreciable precipitation.

In an attempt to form the salt with the organic base N, N-dicyclohexylamine, a solution of keto diacid in diethyl ether was treated with the amine and the resultant solution was allowed to stand at rt °C in excess of 60 days with no appreciable precipitation of the salt.

It was then attempted to purify the keto diacids by high performance liquid chromatography (HPLC). Because the keto diacids have a poor chromophore, it was very difficult to detect them with a UV/visible detector coupled to the HPLC, although it was attempted. The keto diacids were injected into the HPLC eluting with various ratios of MeOH in water (0-50 %) containing 0.08 % trifluoroacetic acid (TFA). Conditions for the preparative purification of the keto diacids were not optimized due to the fact that the use of TFA in the mobile phase spawned a new idea for the isolation of the keto diacids, the use of TFA as the proton source in the isolation of the keto diacids after saponification.

It was attempted to precipitate the previously synthesized keto diacids by the neutralization of their sodium salts with TFA in water. This method was unsuccessful, however, it lead to the discovery that the keto diacids could be obtained as brown solids

after saponification of the keto diesters with 2.1 equivalents of NaOH, evaporation of the solvent, reconstitution in a minimum amount of water and subsequent protonation with dilute TFA.

Once the initial crude keto diacids were obtained, it was necessary to choose an enzyme capable of converting the ketone moiety of our keto diacids into an amine group. Upon discussion with Dr. Richard Bridges about which enzyme would be the most logical starting point, we contacted Dr. Arthur Cooper of the Weill Medical College of Cornell University (an expert in the field of aminotransferases and dehydrogenases). A collaboration was established with Dr. Cooper in which he would preliminarily survey our keto diacids as substrates and/or inhibitors of a panel of aminotransferases, dehydrogenases and dehydrogenase complexes (for experimental details, see Chapter 3).

*7. Synthesis of phosphono analogues of 2-oxoglutarate, 2-oxoisocaproate, 2-oxoisovalerate and 2-oxo-3-methylvalerate*

The collaboration with Dr. Cooper (section 2.6)initiated a research plan to investigate the synthesis of a number of phosphonate analogues of 2-oxoglutarate in the search for a potent and selective inhibitor of the 2-oxoglutarate dehydrogenase complex as a model for neurodegenerative diseases (for an overview of this subject, see: Brown, 2000; Cooper, 1996, 1998). Compounds **2.48** – **2.50** (Figure 2.28) and **2.51** and **2.52** (Figure 2.29) were identified as targets and were synthesized using a modification of previously

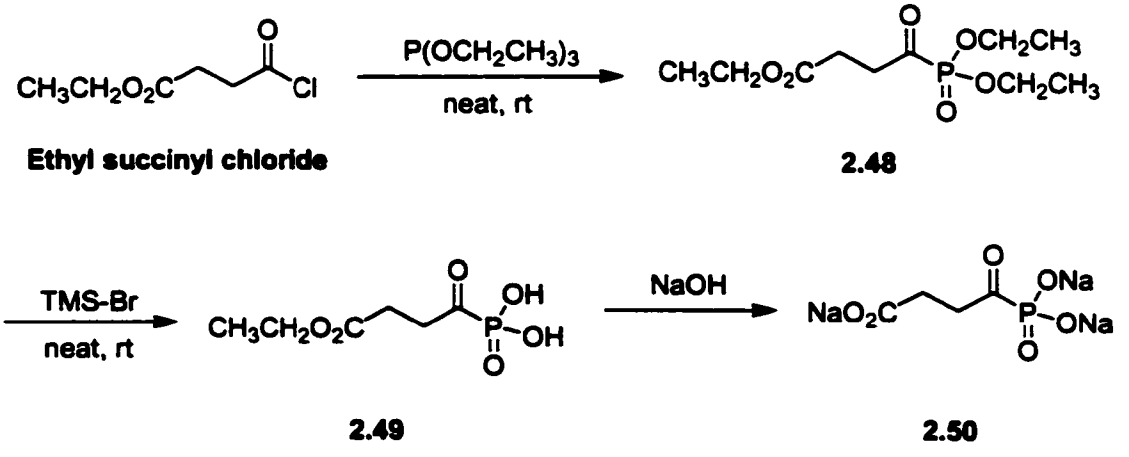
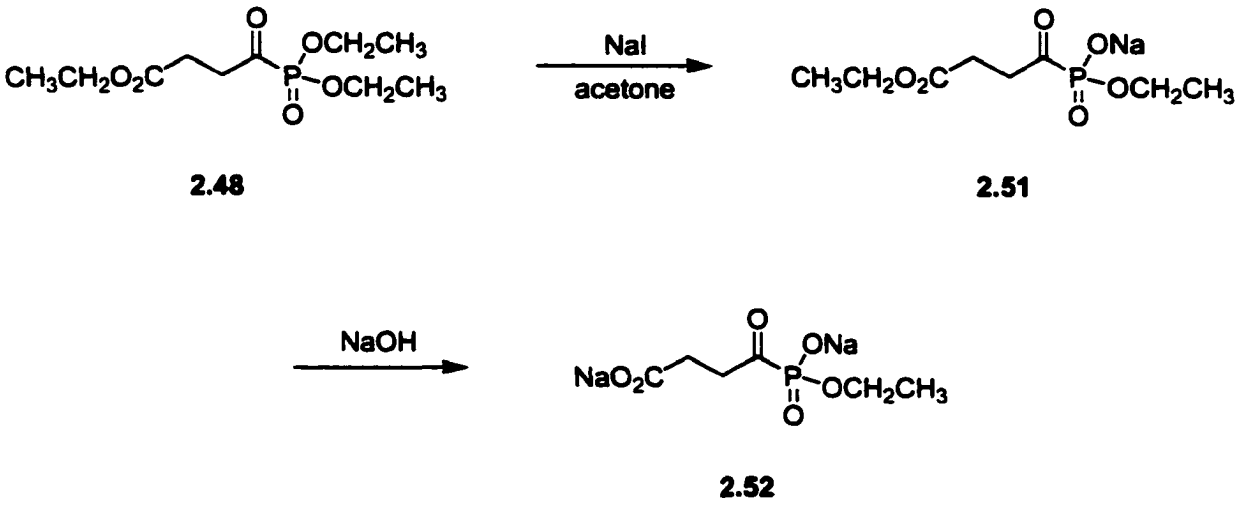



**Figure 2.28**: Synthesis of phosphonate analogues of 2-oxoglutarate

published work (Khomutov, 1978). Compound **2.48** is obtained via a modified Arbuzov reaction between ethyl succinylchloride and triethyl phosphite. Compound **2.49** is obtained via the dealkylation of the phosphonate esters with bromotrimethylsilane followed by treatment of **2.49** with 3 equivalents of NaOH in water to afford the trisodio

salt **2.50**. Compound **2.51** was synthesized from triester **2.48** by monodealkylation with sodium iodide (NaI) in refluxing acetone. The disodium salt **2.52** was synthesized by the




**2.48** **2.51** **2.52**

**Figure 2.29**: Synthesis of phosphono analogues of 2-oxoglutarate

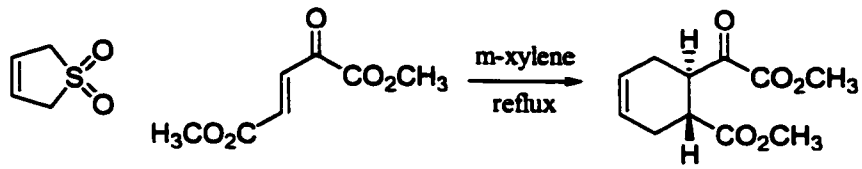
saponification of the carboxylate ester of compound **2.51** with NaOH in water. All of the products gave satisfactory $^{1}H$ NMR, $^{13}C$ NMR and ESI-MS properties (see Experimental Section).

The molecules described above are phosphono analogues of 2-oxoglutarate, which is a substrate for the enzyme complex of 2-oxoglutarate dehydrogenase. The reduced capacity of this enzyme complex is implicated in neurodegenerative diseases. The selective inhibition of this complex with a synthetic molecule will decrease its activity and mimic the disease state which can then be studied. The biological evaluation of these compounds to inhibit 2-oxoglutarate dehydrogenase *in vitro* and *in vivo* is being done by collaborators Dr. Arthur J. L. Cooper and Dr. Gary E. Gibson at the Weill

Medical College of Cornell University. The results are pending at the time of this thesis preparation.

## III. Experimental

Unless otherwise noted, reactions were run in flame-dried apparatus under argon atmosphere. Anhydrous reagent grade solvents (methylene chloride, $CH_2Cl_2$: dimethoxyethane, DME: dimethyl formamide (DMF): tetrahydrofuran (THF)), toluene, methanol (MeOH), triethylamine (TEA), were purchased from Aldrich Chemical Co. Melting points were determined on a Mel-Temp II metal block apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was performed on silica gel (Whatman) aluminum-backed plates. Flash chromatography was performed with silica gel. Compounds were detected using UV absorption at 254 nm and/or stained with ninhydrin or $I_2$ (iodine). $^1H$ NMR (400 MHz), $^{13}C$ NMR (100 MHz) and $^{31}P$ (161 MHz) spectra were recorded with a Varian 400 MHz Unity Plus spectrometer. Chemical shifts are reported in parts per million (ppm, δ) using residual solvent signals as internal standards. High resolution mass spectra (HRMS) were obtained from the University of Montana Mass Spectrometry facility with a Micromass LCT HRMS. Elemental analyses for C, H, and N were performed by Midwest Microlab, Indianapolis, IN. For most compounds described in this section, analytical (HRMS or elemental analysis) data are presented. For those compounds devoid of this data it is because at the time of synthesis either the spectrometer was dysfunctional or the amount of material obtained was insignificant for an elemental analysis.




**2.3** **2.7**

**Methyl 6-methoxyoxalyl-cyclohex-3-enecarboxylate (2.7)**

A solution of DOG (1.19 g, 6.89 mmol) and butadiene sulfone, (8.41 g, 68.90 mmol) in m-xylene (20 mL) was heated to reflux and stirred for 6 h. The solution was allowed to cool to rt and the solvent was removed *in vacuo*. The resultant brown oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.7** (1.17 g, 58.8%) as a gold oil:

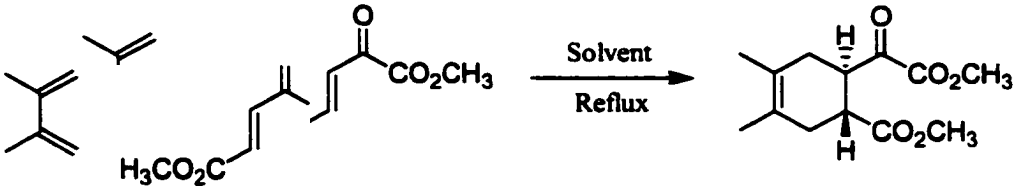
$^1$H NMR ($CDCl_3$, 400 MHz): δ 5.55 (t, $J$ = 1.9 Hz, 2 H), 3.72 (s, 3 H), 3.48 (s, 3 H), 3.45 (td, $J$ = 5.7, 11.1 Hz, 1 H), 2.81 (td, $J$ = 5.7 Hz, 11.1 Hz, 1 H), 2.40-2.25 (m, 2H), 2.06-1.85 (m, 2 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 196.8, 175.3, 161.5, 125.3, 125.0, 52.8, 52.0, 43.0, 41.4, 27.5 (2 C);

IR (liquid cell, $CDCl_3$): 3156, 1727, 1438, 1302, 1267, 1232, 1200, 1179, 1081 $cm^{-1}$;

Anal. Calcd for $C_{11}H_{14}O_5$: C, 58.40; H, 6.24. Found: C, 58.44; H, 6.25;

HRMS Calcd for $C_{11}H_{15}O_5$ 227.0919. Found: 227.0925 $(M+H)^+$.




**2.3** **2.8**

**Methyl 6-methoxyoxalyl-3,4-dimethyl-cyclohex-3-enecarboxyate (2.8)**

**A.** To a solution of DOG (9.02 g, 51.85 mmol) in hexanes (30 mL) was added 2,3-dimethyl-1,3-butadiene (11.73 mL, 103.7 mmol) and the solution was heated slowly to reflux until TLC indicated consumption of starting material. The solvent was removed *in vacuo* and the resultant brown oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.8** (9.39 g, 70.7%) as a gold oil:

**B.** To a solution of DOG (1.14 g, 6.6 mmol) in $CH_2Cl_2$ (30 mL) was added 2,3-dimethyl-1,3-butadiene (3.97 mL, 33.1 mmol) and the solution was heated to reflux and stirred for 5 min. The solvent was removed *in vacuo* and the resultant brown oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.8** (1.66 g, 98.1%) as a gold oil:

**C.** To a sealable pressure tube was added DOG (1.14 g, 6.61 mmol), 2,3-dimethyl-1,3-butadiene (1.49 mL, 13.21 mmol) and benzene (5 mL). The solution was heated to 80 ° C and stirred for 2 h. The solvent was removed *in vacuo* and the resultant brown oil was

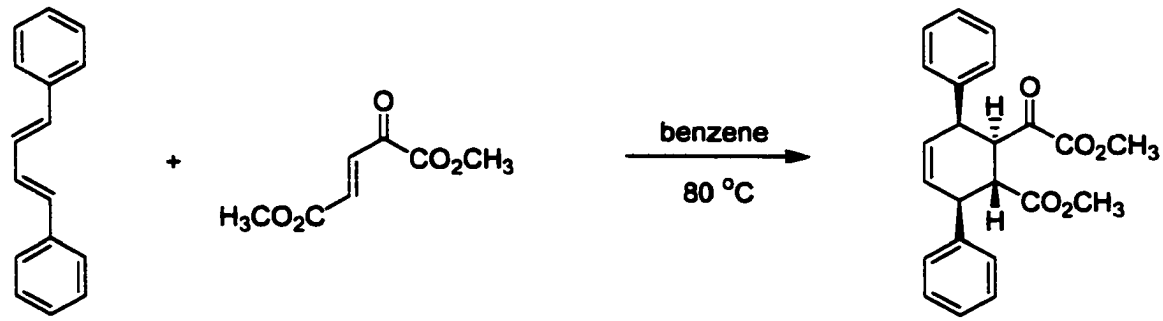
chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.8** (1.49 g, 88.3%) as a gold oil:

$^1$H NMR ($CDCl_3$, 400 MHz): δ 3.87 (s, 3 H), 3.63-3.56 (m, 4 H), 2.95 (td, $J$ = 5.7, 14.6 Hz, 1 H); 2.37-2.24 (m, 2 H); 2.17-1.98 (m, 2 H); 1.53 (s, broad, 6 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 197.5, 175.9, 162.0, 124.8, 124.5, 53.3, 52.4, 44.4, 42.6, 34.1 (2 C), 18.7 (2 C);

IR (liquid cell, $CDCl_3$) 3155, 2998, 2954, 2917, 2844, 1722, 1438 $cm^{-1}$;

Anal. Calcd for $C_{13}H_{18}O_5$: C, 61.41; H, 7.13. Found: C, 61.29; H, 7.13.




**2.3** **2.9**

**Methyl 6-methoxyoxalyl-2,5-diphenyl-cyclohex-3-enecarboxylate (2.9)**

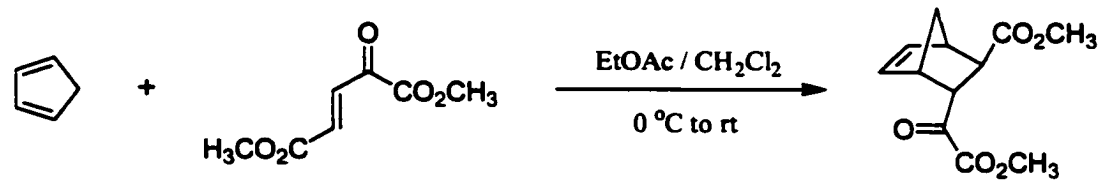
To a solution of DOG (0.708 g, 4.07 mmol) in benzene (10 mL) was added 1,4-diphenyl-1,3-butadiene (1.68 g, 8.14 mmol) and the resulting solution was heated to reflux and stirred for 12 h. The solvent was removed *in vacuo* and the residue was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.9** (1.20 g, 77.4%) as an off white solid:

mp 109-113 °C

$^1H$ NMR ($CDCl_3$, 400 MHz): δ 7.39-7.13 (m, 10 H), 5.98-5.88 (m, 2 H), 4.49-4.46 (br m, 1 H), 4.30 (dd, $J$ = 11.4, 6.2 Hz, 1 H), .3.87 (s, 6 H), 3.64-3.61 (m, 1 H), 3.33 (s, 6 H), 3.02 (t, $J$ = 11.4 Hz, 1 H);

$^{13}C$ NMR ($CDCl_3$, 100 MHz): δ 193.2, 175.1, 160.4, 141.9, 138.6, 131.9, 131.0, 128.8, 128.8, 128.6, 128.0, 127.8, 127.6, 127.3, 53.0, (51.3, 51.1), 46.5, 44.7, 42.3;

Anal. Calcd for $C_{23}H_{22}O_5$: C, 73.00; H, 5.86. Found: C, 72.83; H, 6.08.




**cyclopentadiene** **2.3** **2.10**

**3-Methoxyoxalyl-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid methyl ester (2.10)**

Two methods were used to prepare compound **2.10**, which is isolated as a mixture of diastereomers:

**A.** To a solution of DOG (3.00 g, 14.5 mmol) in benzene (25 mL) at 0 °C was added freshly cracked cyclopentadiene dimer (> 1.75 mL, 20.94 mmol) and the reaction was complete upon addition as determined by TLC analysis. The solvent was removed *in vacuo* and the resultant clear/yellow oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.10** (3.57 g , 85.8 %) as a clear/yellow oil:

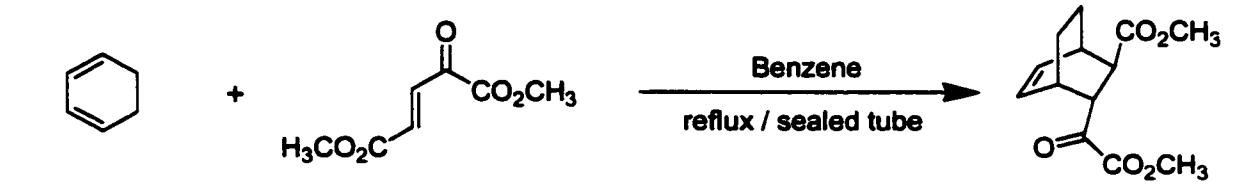
**B.** To a solution of DOG (0.44 g, 2.5 mmol) in $CH_2Cl_2$ (10 mL) at rt was added freshly cracked cyclopentadiene dimer (> 0.425 mL, 5.1 mmol) and the solution was stirred for 10 min. The solvent was removed *in vacuo* and the resultant clear/yellow oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford (**2.7.a**) (0.05 g , 90.2%) as a yellow oil:

$^1$H NMR ($CDCl_3$, 400 MHz): δ 6.31 (dd, $J$ = 3.1, 5.7 Hz, 0.5 H), 6.23 (dd, $J$ = 3.1, 5.7 Hz, 0.5 H), 6.10 (dd, $J$ = 2.5, 5.7 Hz, 0.5 H), 5.91 (dd, $J$ = 2.5, 5.7 Hz, 0.5 H), 3.96, (dd, $J$ = 3.5, 4.5 Hz, 1 H), 3.86 (s, 1.5 H), 3.83 (s, 1.5 H), 3.67 (s, 1.5 H), 3.60 (s, 1.5 H),

3.44-3.39 (m, 1 H), 3,26 (s, 0.2 H) 3.14 (s, 0.8 H), 2.72 (dd, $J$ = 1.6, 4.8 Hz, 1 H); 1.71 (d, $J$ = 8.8 Hz, 0.5 H); 1.48 (dd, $J$ = 1.6, 8.8 Hz, 0.5 H), 1.38 (s, 1 H);

$^{13}C$ NMR ($CDCl_3$, 100 MHz): δ (194.3, 193.4), (175.0, 173.8), (162.1, 162.0) (138.3, 137.5), (136.2, 134.4), (53.1, 52.8), 52.0, 51.7, (47.7, 47.6), (46.2, 46.1), (45.9, 45.6), 45.1

IR (liquid cell, $CDCl_3$) 3155, 2986, 2956, 1727, 1438, 1381, 1253, 1091 $cm^{-1}$;




**1, 3-cyclohexa-diene** **2.3** **2.11**

**Methyl 3-methoxyoxalyl-bicyclo[2.2.2]oct-5-ene-2-carboxylate (2.11)**

Two methods were used to prepare compound **2.11**, which was isolated as a diastereomer mixture:

**A.** To a solution of DOG (0.742 g, 4.27 mmol) in benzene (6 mL) was added 1,3-cyclohexadiene (0.577 mL, 8.52 mmol) and the resulting solution was heated to reflux and stirred for 24 h. The solvent was removed *in vacuo* and the resultant brown oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the compound **2.11** (0.63 g, 58.1%) as mixture of endo/exo isomers as a gold oil:

**B.** To a sealable pressure tube was added DOG (1.51 g, 8.78 mmol), 1,3-cyclohexadiene (1.25 mL, 13.17 mmol) and benzene (10 mL). The solution was heated to 150 ° C and stirred for 72 h. The solvent was removed *in vacuo* and the resultant black tar was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford compound **2.11** (1.84 g, 82.5%) as a gold oil:

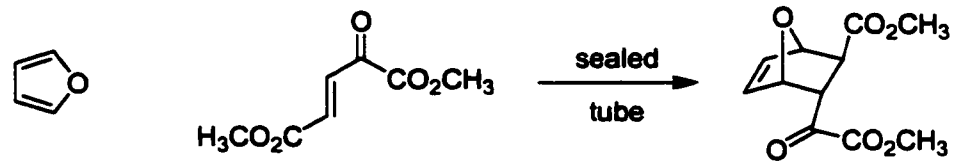
$^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 6.33 (t, $J$ = 8.3 Hz, 1 H), 6.22 (t, $J$ = 7.6, 1 H), 3.85 (s, 3 H), 3.83 (s, 3 H), 3.78 (dd, $J$ = 1.9, 5.7 Hz, 1 H), 3.17 (dd, $J$ = 1.9, 5.4 Hz, 1 H),

3.00-2.89 (m, 2 H), 1.78-1.70 (m, 1 H), 1.58-1.50 (m, 1 H), 1.36-1.27 (m, 1 H), 1.17-1.09 (m, 1 H);

$^{13}C$ NMR ($CDCl_3$, 100 MHz): δ (195.2, 194.4), (175.2, 174.9), 162.8, (136.2, 135.1), (134.2, 132.3), (54.1, 54.0), (53.2, 53.1), (51.5, 50.7), (44.6, 44.3), (33.4, 33.2), (32.8, 32.6), (25.9, 25.1), (21.5, 21.0);

IR (liquid cell, $CDCl_3$) 3156, 2955, 1728, 1466, 1437, 1382, 1273, 1225, 1094 $cm^{-1}$;

Anal. Calcd for $C_{13}H_{16}O_5$: C, 61.90; H, 6.39. Found: C, 61.99; H, 6.41.




**furan** **2.3** **2.12**

**Methyl 3-methoxyoxalyl-7-oxa-bicyclo[2.2.1]hept-5-ene-2-carboxylate (2.12)**

Three methods were used to prepare compound **2.12:**

**A.** To a sealable pressure tube was added DOG (0.415 g, 2.39 mmol) and furan (5 mL) as the solvent. The solution was heated to 120 °C and stirred for 72 h. The solvent was removed *in vacuo* and the resultant black tar was chromatographed ($SiO_2$, 33% EtOAc : hexanes) to afford the product **2.12** (0.14 g, 24.8%) as a yellow oil.

**B.** To a solution of DOG (0.50 g, 2.88 mmol) in $CH_2Cl_2$ (10 mL) was added furan (0.69 mL, 14.40 mmol) and the resulting solution was heated to reflux and stirred for 14 h. The solvent was removed *in vacuo* and the resultant yellow oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.12** (0.06 g, 8.0%) as a gold oil.

**C.** To a solution of DOG (0.504 g, 2.93 mmol) and furan (0.703 mL, 14.67 mmol) in $CH_2Cl_2$ (25 mL) was added $AlCl_3$ (ca. 0.1 g) and the resulting solution turned black in 5 min. The reaction mixture was filtered through a pad of Celite, the solvent was removed

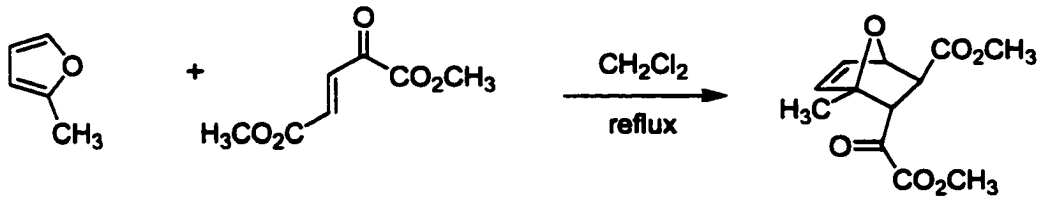
*in vacuo* and the brown oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.12** (0.47 g, 66.7%) as a gold oil:

$^1$H NMR ($CDCl_3$, 400 MHz): δ 7.34 (dd, $J$ = 0.6, 1.9 Hz, 1 H), 6.32 (dd, $J$ = 1.9, 3.5 Hz, 1 H), 6.21 (d, $J$ = 3.5 Hz, 1 H), 4.29 (dd, $J$ = 4.6, 9.7 Hz, 1 H), 3.88 (s, 3H), 3.77 (dd, $J$ = 9.8, 19.0 Hz, 1 H), 3.72 (s, 3 H), 3.24 (dd, $J$ = 4.8, 19.0 Hz, 1 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz) δ 192.1, 171.9, 161.5, 150.8, 143.2, 111.2, 108.0, 53.5, 53.1, 40.4, 40.0;

IR (liquid cell, $CDCl_3$) 3154, 1793, 1735, 1472, 1382, 1095 $cm^{-1}$;

Anal. Calcd for $C_{11}H_{12}O_6$: C, 55.00; H, 5.04. Found: C, 55.05; H, 5.09



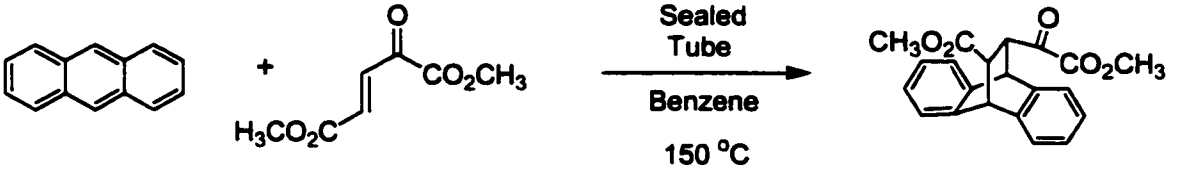

**2-methylfuran** **2.3** **2.13**

**Methyl 3-methoxyoxalyl-4-methyl-7-oxa-bicyclo[2.2.1]hept-5-ene-2-carboxylate (2.13)**

To a solution of DOG (1.05 g, 6.11 mmol) in $CH_2Cl_2$ (25 mL) was added 2-methylfuran (2.76 mL, 30.55 mmol) and the resulting solution was heated to reflux and stirred for 14 h. The solvent was removed *in vacuo* and the resultant gold oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.13** (1.54 g, 99.0%) as a gold oil:

$^1$H NMR ($CDCl_3$, 400 MHz): δ 6.05 (d, br, $J$ = 3.2 Hz, 1 H), 6.04 (dd, br, $J$ = 3.2, 1.0 Hz, 1 H), 4.21 (dd, J = 4.5, 10.0 Hz, 1 H), 3.85 (s, 3 H), 3.73 (dd, J = 10.0, 19.1 Hz, 1 H), 3.68 (s, 3 H), 3.18 (dd, J = 4.5, 19.1 Hz, 1 H), 2.23 (s, 3 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 191.1, 171.0, 160.5, 152.0, 147.9, 107.9, 106.3, 53.0, 52.6, 40.2, 39.7, 13.4




**Anthracene** **2.3** **2.16**

**Dimethyl 9,10-dihydroanthraceno-3,10-[3,4-(2-oxo)]-glutarate (2.16)**

To a sealable pressure tube was added DOG (1.55 g, 9.02 mmol), anthracene (1.78 g , 10.0 mmol) and benzene (30 mL). The solution was heated to 150 °C and stirred for 48 h. The heat was removed, the tube was opened and the unreacted anthracene was removed by filtration. The mixture was recrystallized with benzene to remove more anthracene and the mother liquor was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the compound **2.16** (2.22 g, 67.4 %) as a yellow solid:

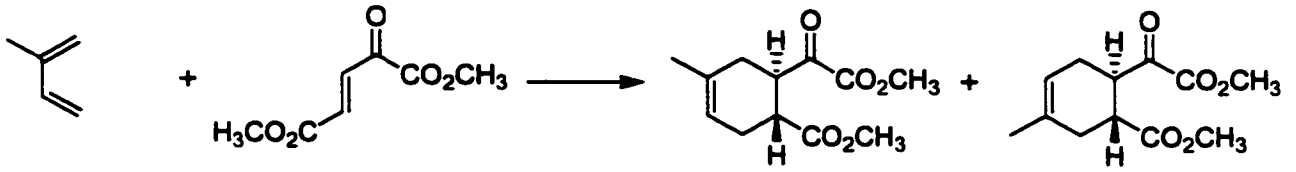
m.p. 131-133 °C;

$^1$H NMR ($CDCl_3$, 400 MHz): δ 7.25 (m, 8 H), 4.84 (d, $J$ = 2.5 Hz, 1 H), 4.79 (d, $J$ = 2.5 Hz, 1 H), 4.08 (dd, $J$ = 1.9, Hz, 1 H), 3.93 (s, 3 H), 3.63 (s, 3 H), 3.48 (dd, $J$ = 3.2, 5.1 Hz, 1 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 192.8, 173.3, 162.4, 142.7, 142.4, 141.2, 139.7, 127.5, 127.2, 127.2, 125.5, 125.3, 124.5, 124.4, 53.4, 52.5, 46.7, 46.3, 46.2;

IR (liquid cell, $CDCl_3$) 1730.1, 1260.5, 1221.9, 1087.7 cm$^{-1}$;

Anal. Calcd for $C_{21}H_{18}O_5$: C, 71.99; H, 5.18. Found: C, 71.17; H, 5.14.




**isoprene** **2.3** **2.19.a** **2.19.b**

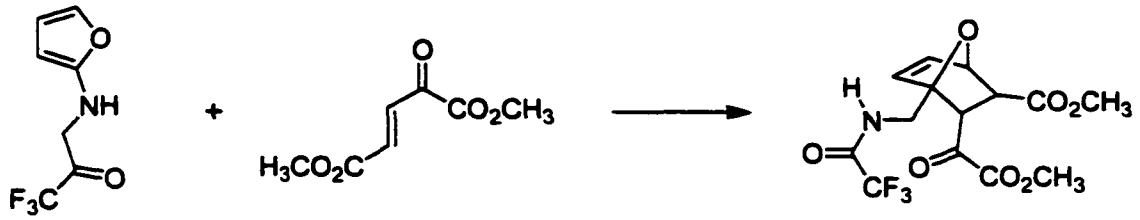
**Methyl 6-methoxyoxalyl-4-methyl-cyclohex-3-enecarboxylate (2.19.a) and Methyl 6-methoxyoxalyl-3-methyl-cyclohex-3-enecarboxylate (2.19.b)**

To a sealable pressure tube was added DOG (0.53 g, 3.09 mmol), isoprene (1.55 mL, 15.45 mmol) and $CH_2Cl_2$ (30 mL). The solution was stirred at rt for 1 h and subsequently heated to 75 °C and stirred for 0.5 h. The solvent was removed *in vacuo* and the resultant oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the products (**2.19.a** and **2.19.b**) as a mixture of regioisomers (0.71 g, 95.4 % combined). A 64 : 36 regioisomer ratio was determined for the crude isolate by integration of the ester α-protons doublets of triplets arising at 2.92 and 2.83 ppm in the $^1$H NMR, respectively.

$^1$H NMR ($CDCl_3$, 400 MHz): δ 5.31 (s, 1 H), 3.80 (d, 3 H), 3.61-3.44 (m, 4 H), 2.92 (td, $J$ = 5.8, 11.6 Hz, 0.64 H), 2.83 (td, $J$ = 5.8, 11.6 Hz, 0.36 H), 2.42-1.88 (m, 4 H), 1.59 (s, 3 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 198.5, 198.3, 176.9, 176.8, 163.0, 163.0, 134.1, 133.9, 120.8, 120.4, 54.2, 53.4, 45.0, 45.6, 43.4, 42.9, 33.5, 33.4, 24.1

HRMS Calcd for $C_{12}H_{17}O_5$: 241.1076. Found: Pending.

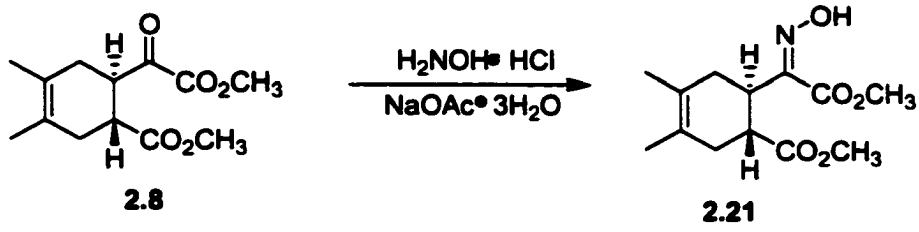


**2.50** **2.3** **2.20**

**Methyl 3-methoxyoxalyl-4-[(2,2,2-trifluoro-acetylamino)-methyl]-7-oxa-bicyclo[2.2.1]hept-5-ene-2-carboxylate (2.20)**

To a solution of DOG (0.46 g, 3.0 mmol) in $CH_2Cl_2$ (15mL) was added compound 2.50 N-trifluoroacetylfurfuralamine (0.57 g, 3.0 mmol) and the resulting solution was stirred at rt for10 min. The solvent was removed *in vacuo* and the resultant gold oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.20** (0.83 g, 84.0 %) as a gold oil:

$^1$H NMR ($CDCl_3$, 400 MHz): δ 7.49 (br, t, 1 H), 6.13 (d, $J$ = 3.2 Hz, 1 H), 6.06 (d, $J$ = 3.2 Hz, 1 H), 4.36 (d, $J$ = 5.8 Hz, 2 H), 4.14 (dd, $J$ = 9.1, 5.2 Hz, 1 H), 3.77 (s, 3 $^{13}$C NMR ($CDCl_3$, 100 MHz) δ 191.9, 170.7, 160.2, 156.9 (q, $J_{C-F}$ = 36.6 Hz), 149.8, 148.6, 109.6, 108.2, 53.0, 52.5, 39.9, 39.5, 36.4 (2 C)



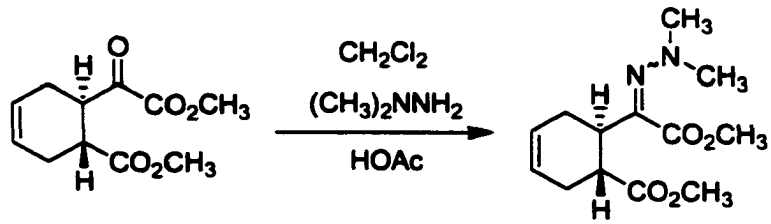

**2.8** **2.21**

**Methyl 6-(Hydroxyimino-methoxycarbonyl-methyl)-3,4-dimethyl-cyclohex-3-enecarboxylate (2.21)**

To a solution of ketoester **2.8** (0.33 g, 1.30 mmol) in absolute ethanol (6 mL) was added hydroxylamine hydrochloride (0.09 g, 1.20 mmol) and sodium acetate trihydrate (0.18 g, 1.30 mmol) and the resultant solution was stirred at rt for 21 h, heated to reflux and stirred an additional 3.5 h. The EtOH was removed *in vacuo* and the residue was partitioned between $CH_2Cl_2$ (10 mL) and brine (10 mL). The organic layer was collected and the aqueous was subsequently extracted with $CH_2Cl_2$ (3 x 10 mL), dried ($MgSO_4$), filtered and the solvent was removed *in vacuo* to afford the crude product (0.32 g) as a white solid. Recrystallization from EtOH afforded the product **2.21** (0.15 g, 41.5 % as a white solid.

$^1$H NMR ($CDCl_3$, 400 MHz): δ 3.78 (s, 3 H), 3.58-3.50 (m, 4 H), 3.39 (td, $J$ = 11.7 Hz, $J$ = 5.8 Hz, 1 H), 2.45-2.38 (br t, 1 H), 2.24-2.09 (br m, 2H), 2.01-1.95 (br m, 1 H), 1.60-1.58 (br d, 6 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 176.2, 164.6, 154.0, 125.1, 124.6, 52.8, 52.1, 41.7, 35,2, 35.1, 33.!, 18.8, 18.6;




**2.7** **2.22**

**Methyl 6-[(dimethyl-hydrazono)-methoxycarbonyl-methyl]-cyclohex-3-enecarboxylate (2.22)**

To a solution of ketoester **2.7** (4.42 g, 19.57 mmol) in $CH_2Cl_2$ (25 mL) was added HOAc (3.26 mL, 58.70 mmol) and the solution was stirred at rt for 5 min. To the resultant solution was added N,N-dimethylhydrazine (7.42 mL, 97.83 mmol) and the solution was heated to reflux and stirred for 24 h. The $CH_2Cl_2$ and HOAc were removed *in vacuo* and the resultant slurry was triturated with $CH_2Cl_2$ and filtered through a pad (40 g) of silica gel ($CH_2Cl_2$ ; 50 mL) then 50% EtOAc : hexanes (200 mL). The solvent was removed *in vacuo* and the resultant yellow oil was chromatographed ($SiO_2$, 50% EtOAc : hexanes) to afford the compound **2.21** (4.48 g, 77.4%) as a yellow oil:

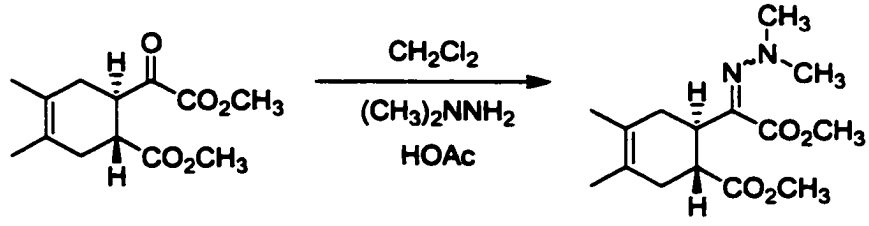
$^1$H NMR ($CDCl_3$, 400 MHz): δ 5.53 (d, $J$ = 3.2 Hz, 2 H), 3.65 (s, 3 H), 3.51 (s, 3 H), 2.81 (td, $J$ = 5.2, 11.0 Hz, 1 H), 2.68 (td, $J$ = 5.2, 11.0 Hz, 1 H); 2.46 (s, 6 H), 2.29–1.87 (m, 4 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz) δ 176.1, 165.9, 151.8, 125.7, 125.1, 51.6, 51.3, 47.3, 47.2, 39.9, 39.8, 29.6, 28.8;

IR neat 3025, 2951-2850, 1736, 1293-1126 cm;

Anal. Calcd for $C_{13}H_{20}N_2O_4$: C, 58.19; H, 7.51; N, 10.44. Found: C, 58.16; H, 7.59; N, 10.25;

HRMS Calcd for $C_{13}H_{20}N_2O_4$ 269.1501. Found: 269.1493 $(M+H)^+$.




**2.8** **2.23**

**Methyl 6-[(dimethyl-hydrazono)-methoxycarbonyl-methyl]-3,4-dimethyl-cyclohex-3-enecarboxylate (2.23)**

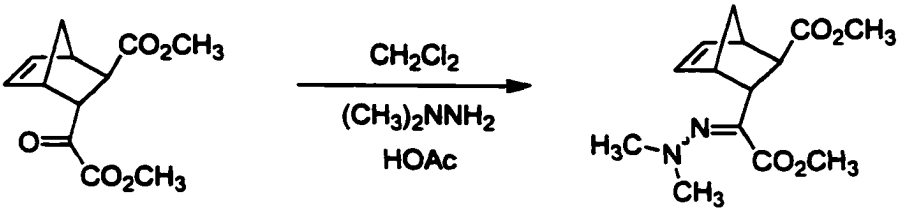
To a solution of keto diester **2.8** (5.67 g, 22.34 mmol) in $CH_2Cl_2$ (25 mL) was added HOAc (3.72 mL, 67.01 mmol) and the solution was stirred at rt for 5 min. To the resultant solution was added unsymmetrical N,N-dimethylhydrazine (8.47 mL, 111.68 mmol) and the solution was heated to reflux and stirred for 24 h. The $CH_2Cl_2$ and HOAc were removed *in vacuo* and the resultant slurry was triturated with $CH_2Cl_2$ and filtered through a pad (80 g) of silica gel with $CH_2Cl_2$ (100 mL) then 50% EtOAc : Hexanes (600 mL). The solvent was removed *in vacuo* and the resultant yellow oil was chromatographed ($SiO_2$, 50% EtOAc : hexanes) to afford the product **2.23** (6.02 g, 91.1%) as a yellow oil:

$^1$H NMR ($CDCl_3$, 400 MHz): δ 3.70 (s, 3 H), 3.56 (s, 3 H), 2.84 (td, $J$ = 5.5, 11.0, 1 H), 2.65 (td, $J$ = 6.5, 11.00, 1 H), 2.49 (s, 6 H), 2.16–1.91 (m, 4 H), 1.51 (s, 6 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 177.6, 167.4, 154.7, 125.9, 125.4, 53.0, 52.6, 48.7, 48.6, 44.5, 42.0, 37.1, 36.4, 19.7, 19.6;

Anal. Calcd for $C_{15}H_{24}N_2O_4$: C, 60.79; H, 8.16; N, 9.45. Found: C, 60.62; H, 8.04; N, 9.50.

HRMS Calcd for $C_{15}H_{25}N_2O_4$ 297.1814. Found: 297.1810 $(M+H)^+$.




**2.10** **2.24**

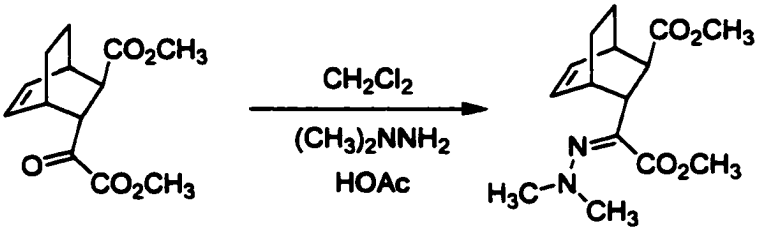
**Methyl 3-[(Dimethyl-hydrazono)-methoxycarbonyl-methyl]-bicyclo[2.2.1]hept-5-ene-2-carboxylate (2.24)**

To a solution of ketoester **2.10** (2.03 g, 8.52 mmol) in $CH_2Cl_2$ (20 mL) was added HOAc (1.42 mL, 25.57 mmol) and the solution was stirred at rt for 5 minutes. To the resultant solution was added N,N-dimethylhydrazine (3.23 mL, 42.61 mmol) and the solution was stirred at rt for 5 h when TLC indicated consumption of starting material. The solvent, HOAc and hydrazine were removed *in vacuo* and the resultant yellow oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford **2.24** (2.14 g, 89.5 %) as a yellow oil, as a $^1$H NMR discriminable mixture of E / Z isomers:

$^1$H NMR ($CDCl_3$, 400 MHz): δ6.13 (dd, $J$ = 5.8, 3.2 Hz, 0.3 H), 6.07 (dd, $J$ = 5.8, 3.2 Hz, 0.7 H), 5.93 (q, $J$ = 5.8, 3.2 Hz, 0.3 H), 5.90 (q, $J$ = 5.8, 3.2 Hz, 0.7 H) 3.67 (s, 0.9 H), 3.66 (s, 2.1 H), 3.57 (s, 2.1 H), 3.50 (s, 0.9 H) 3.42 (t, $J$ = 3.9 Hz, 0.3 H), 3.29 (t, $J$ = 3.2 Hz, 0.7 H), 3.08 (br s, 0.3 H), 2.98 (br s, 0.7 H), 2.93 (br s, 0.7H), 2.90 (br s, 0.3 H), 2.70 (dd, $J$ = 4.5, 1.9 Hz, 0.7 H), 2.63 (dd, $J$ = 4.5, 1.9 Hz, 0.3 H) 2.44 (s, 1.8 H), 2.38 (s, 4.2 H), 1.52 (bd t, $J$ = 9.1 Hz, 1H), 1.32 (br t, $J$ = 9.1, 3.9, 1.9 Hz, 1 H));

$^{13}C$ NMR ($CDCl_3$, 100 MHz) δ 176.0, 167.0, 154.9, 136.8, 135.5, 51.9, 51.6, 47.4, 47.0, 46.9, 46.5, 46.3, 45.7, 45.4;

HRMS Calcd for $C_{14}H_{21}N_2O_4$ 281.1501. Found: 281.1492 $(M+H)^+$.




**2.11** **2.25**

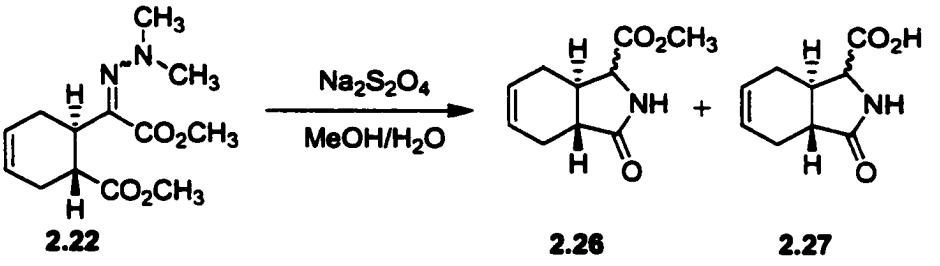
**Methyl 3-[(Dimethyl-hydrazono)-methoxycarbonyl-methyl]-bicyclo[2.2.2]oct-5-ene-2-carboxylate (2.25)**

To a solution of keto diester (**2.11**) (3.61 g, 14.20 mmol) in $CH_2Cl_2$ (30 mL) was added HOAc (2.37 mL, 42.60 mmol) and the solution was stirred at rt for 5 min. To the resultant solution was added N,N-dimethylhydrazine (5.39 mL, 71.00 mmol) and the solution was heated to reflux and stirred for 2 h. The $CH_2Cl_2$ and HOAc were removed *in vacuo* and the resultant slurry was triturated with $CH_2Cl_2$ and passed through a pad of silica gel (1% MeOH : $CH_2Cl_2$). The solvent was removed *in vacuo* and the resultant yellow oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford **2.25** (3.61 g, 86.4%) as a yellow oil:

$^1$H NMR ($CDCl_3$, 400 MHz): δ 6.25 (br t, $J$ = 7.1 Hz, 1 H), 6.06 (br t, $J$ = 7.1, 1 H), 3.71 (s, 3 H), 3.64 (s, 3 H), 3.18 (dd, $J$ = 6.5, 2.6 Hz, 1 H), 2.95 (m, 1 H), 2.84 (m, 1 H), 2.72 (m, 1 H), 2.42 (s, 6 H), 1.58 (m, 1 H), 1.47 (m, 1 H), 1.24 (m, 1 H), 1.07 (m, 1 H);

$^{13}C$ NMR ($CDCl_3$, 100 MHz): δ 175.8, 167.5, 158,6, 134.6, 133.4, 52.9, 52.7, 48.6, 48.5, 46.2, 45.1, 33.9, 26.1, 25.8, 21.7

HRMS Calcd for $C_{15}H_{23}N_2O_4$ 295.1658. Found: 295.1666 $(M+H)^+$.




2.22 2.26 2.27

**Methyl 3-oxo-2,3,3a,4,7,7a-hexahydro-1H-isoindole-1-carboxylate (2.25)**

**3-Oxo-2,3,3a,4,7,7a-hexahydro-1H-isoindole-1-carboxylic acid (2.26)**

To a solution of hydrazonoester (**2.22**) (1.24 g, 5.06 mmol) in MeOH : $H_2O$ (1 : 1, 20 mL) was added sodium hydrosulfite (1.85 g, 11.88 mmol) and the solution was stirred at reflux for 6.5 h. The MeOH was removed *in vacuo* and the remaining aqueous solution was brought to pH 9 with solid potassium carbonate, extracted with $Et_2O$ (3 x 50 mL), dried ($Na_2SO_4$) and filtered. The solvent was removed *in vacuo* and the resultant brown oil was chromatographed ($SiO_2$, 5% MeOH : $CH_2Cl_2$) to afford **2.26** (0.26 g, 25.8%) as a tan oil:

$^1$H NMR ($CDCl_3$, 400 MHz): δ 5.70-5.62 (m, 2 H), 4.07 (m, 1 H), 4.31-3.83 (m, 3 H), 2.43-1.75 (m, 6 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 179.5, 173.1, 128.3, 128.0, 61.4, 53.8, 45.0, 44.9, 30.4, 26.8;

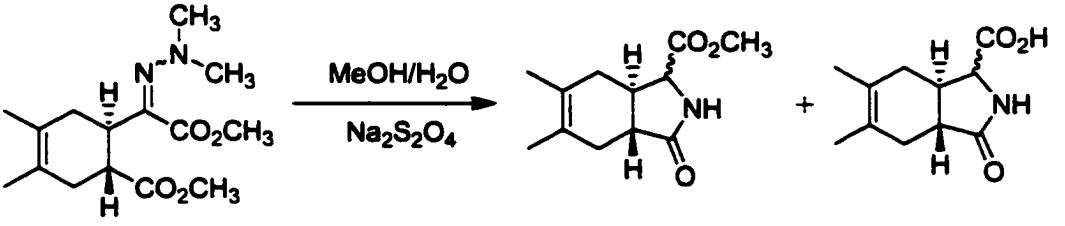
HRMS Calcd for $C_{10}H_{14}NO_3$ 196.0974. Found: 196.0973 $(M+H)^+$.

The remaining aqueous layer was brought to pH 3 with 6 N HCl, extracted with $Et_2O$ (3 x 50 mL), dried over $MgSO_4$ and filtered. The solvent was removed *in vacuo* and the resultant yellow/white solid was triturated with $Et_2O$ and the solid was collected by filtration to afford **2.27** (234 mg, 25.5%) as a white solid:

$^1H$ NMR ($CDCl_3$, 400 MHz): δ 7.88 (s, 1 H), 5.72-5.66 (m, 2 H), 4.04-3.87 (m, 1 H), 2.31-2.08 (m, 4 H), 1.95-1.77 (m, 2 H);

$^{13}C$ NMR ($CDCl_3$, 100 MHz): δ 178.6, 175.1, 129.0, 128.8, 61.2, 45.0, 44.5, 30.3, 27.3;

HRMS Calcd for $C_9H_{12}NO_3$ 182.0817. Found: 182.0819 $(M+H)^+$.




**2.23** **2.28** **2.29**

**Methyl 5,6-Dimethyl-3-oxo-2,3,3a,4,7,7a-hexahydro-1H-isoindole-1-carboxylate (2.27)**
**5,6-Dimethyl-3-oxo-2,3,3a,4,7,7a-hexahydro-1H-isoindole-1-carboxylic acid (2.28)**

To a solution of hydrazonoester (**2.23**) (1.40 g, 4.71 mmol) in MeOH : $H_2O$ (1 : 1, 20 mL) was added sodium hydrosulfite (2.09 g, 13.42 mmol) and the solution was stirred at reflux for 12.5 h. The MeOH was removed *in vacuo* and the remaining aqueous solution was brought to pH 9 with solid potassium carbonate, extracted with $Et_2O$ (3 x 50 mL), dried ($Na_2SO_4$) and filtered. The solvent was removed *in vacuo* and the resultant brown oil was chromatographed ($SiO_2$, 5% MeOH : $CH_2Cl_2$) to afford **2.28** (0.23 g, 21.6%) as a tan oil.

$^1$H NMR ($CDCl_3$, 400 MHz): δ 6.86 (s, 1 H), 3.95 (d, J = 9.7, 1 H), 3.74 (s, 3 H), 2.30-1.96 (m, 6 H), 1.62 (s, 6 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz); δ 178.4, 172.1, 126.19, 125.9, 60.2, 52.7, 44.5, 35.4, 31.5, 19.3;

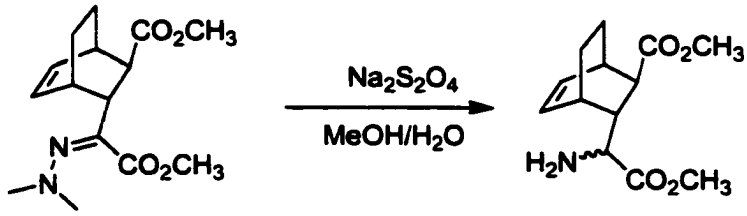
HRMS Calcd for $C_{12}H_{18}NO_3$ 224.1287. Found: 224.1281 $(M+H)^+$.

The remaining aqueous layer was titrated to pH 3 with 6 N HCl, extracted with $Et_2O$ (3 x 50 mL), dried ($MgSO_4$) and filtered. The solvent was removed *in vacuo* and the resultant yellow/white solid was triturated with $Et_2O$ and the solid was collected by filtration to afford **2.28** (0.30 g, 29.7%) as a white solid:

$^1H$ NMR ($CDCl_3$, 400 MHz): δ 7.83 (s, 1 H), 4.01-3.85 (m, 2 H), 4.04-3.87 (m, 1 H), 2.25-1.99 (m, 4 H), 1.60 (s, 6 H);

$^{13}C$ NMR ($CDCl_3$, 100 MHz): δ 178.6, 175.2, 127.5, 127.3, (61.1, 60.9), 45.7, 45.3, 36.5, 33.2, 20.8;

HRMS Calcd for $C_{11}H_{16}NO_3$ 210.1130. Found: 210.1139 $(M+H)^+$.

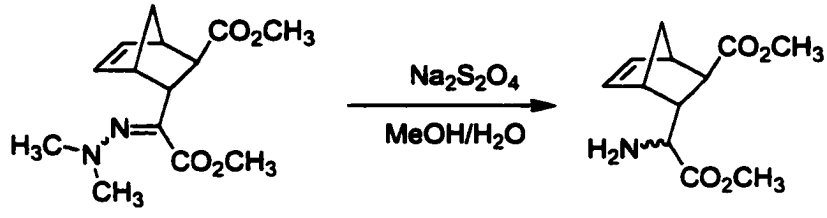



**2.25** **2.30**

**Methyl 3-(amino-methoxycarbonyl-methyl)-bicyclo[2.2.2]oct-5-ene-2-carboxylate (2.30)**

To a solution of hydrazono ester (**2.25**) (1.23 g, 4.19 mmol) in MeOH (10 mL) was added water (10 mL) and the resultant suspension was heated to reflux and $Na_2S_2O_4$ was added in one portion and the resultant solution was stirred for 4 h. The MeOH was removed *in vacuo* and the resultant slurry was partitioned between $Et_2O$ (20 mL) and water (20 mL) and the pH was adjusted to ca. 9 with $K_2CO_3$ (10%, aqueous) and extracted with $Et_2O$ (4 x 20 mL), dried ($MgSO_4$) and concentrated *in vacuo* to afford the crude amino diester (**2.29**) (0.90 g, 85.1 %) as a yellow semisolid. This material is used directly in the saponification reaction without further purification.

ESI-MS indicates 1 major product with an m/z of 254 which corresponds to the product amino diester.

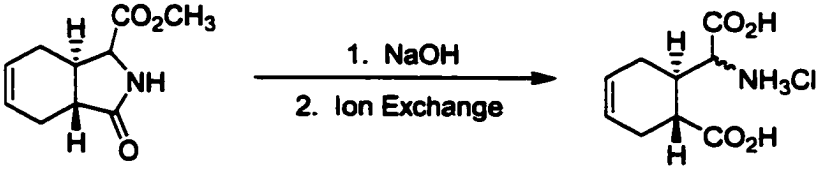



**2.24** **2.31**

**Methyl 3-(amino-methoxycarbonyl-methyl)-bicyclo[2.2.1]hept-5-ene-2-carboxylate (2.30)**

To a solution of hydrazono ester (**2.24**) (0.95 g, 3.39 mmol) in MeOH (10 mL) was added water (10 mL) and the resultant suspension was heated to reflux when $Na_2S_2O_4$ (1.43 g, 9.14 mmol) was added in one portion and the resultant solution was stirred for 4 h. The MeOH was removed *in vacuo* and the resultant slurry was partitioned between $Et_2O$ (20 mL) and water (20 mL) and the pH was adjusted to ca. 9 with $K_2CO_3$ (10%, aqueous) and extracted with $Et_2O$ (3 x 20 mL), dried ($MgSO_4$) and concentrated *in vacuo* to afford the crude amino diester (**2.31**) (0.76 g, 93.7 %) as a yellow semisolid. This material is used directly in the saponification reaction without further purification.

ESI-MS indicates 1 major product with an m/z of 240 which corresponds to the product amino diester.




**2.26** **2.32**

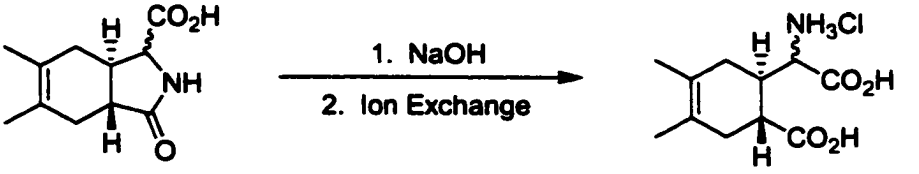
**6-(Amino-carboxy-methyl)-cyclohex-3-enecarboxylic acid (2.31)**

To a solution of lactam ester (**2.26**) (0.22 g, 1.13 mmol) in MeOH (10 mL) and water (4 mL) was added NaOH (2 N, 6.21 mL) and the resultant solution was stirred at rt for 10 min. The solution was heated to reflux and stirred for 5 h, cooled to rt, stirred an additional 12 h, re-heated to reflux and stirred for 3 h (HPLC/MS indicated consumption of starting materials). The solution was cooled to rt and the MeOH was removed *in vacuo*. The aqueous was titrated to pH 3 with 6 N HCl and washed with $Et_2O$ (20 mL) and loaded onto an ion exchange (Bio-Rad, AG-1X2, acetate form)column. The column was eluted with 1 N HCl to afford **2.32** (0.15 g, 58.0 %) as a white solid.

$^1$H NMR ($D_2O$, 400 MHz): δ 5.74-5.65 (br m, 2 H), 4.24 (d, $J$ = 3.2 Hz, 0.5 H), 4.07 (d, $J$ = 3.2 Hz, 0.5 H), 2.90-2.05 (br m, 2 H), 2.57-2.35 (br m, 3 H), 2.29-2.16 (br m, 2 H), 2.07 (br s, 1H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 179.9, 179.7, (126.5, 126.3), (126.1, 125.6), (56.6, 55.8), (43.1, 42.2), (37.0, 36.8), (30.3, 29.3), (26.1, 25.6)

HRMS Calcd for $C_9H_{14}NO_4$ 200.0923. Found: 200.0918 $(M+H)^+$.




**2.29** **2.33**

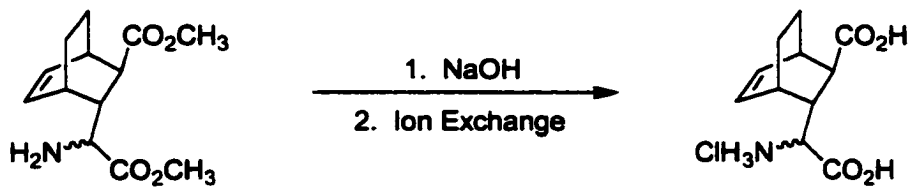
**6-(Amino-carboxy-methyl)-3,4-dimethyl-cyclohex-3-enecarboxylic acid (2.33)**

To a solution of lactam acid **2.29** (0.10 g, 0.47 mmol) in THF/water (1 : 1, 10 mL) was added NaOH (2 N, 2.34 mL, 4.67 mmol) and the reaction was heated to 50 °C and stirred for 7 h followed by stirring at rt for 10 h. The THF was removed *in vacuo* and the remaining aqueous was titrated to pH 3 with 6 N HCl and stirred for 15 min. The solution was washed with $Et_2O$ (3 x 20 mL) and the remaining aqueous layer was added to an ion exchange (Bio-Rad, AG-1X2, acetate form) column. Elution with 1 N HCl afforded **2.33** (0.09, 85.2 %) as an off white solid:

$^1$H NMR ($D_2O$, 400 MHz): δ 3.73-3.67 (br m, 1 H), 2.17-1.61 (br m, 6 H), 1.56-1.37 (br m, 6 H)

$^{13}$C NMR ($D_2O$, 100 MHz): δ 182.14, 179.35, 127.58, 126.52, 71.45, 64.08, (45.93, 45.26), 35.40, 31.91, 19.44:

HRMS Calcd for $C_{11}H_{18}NO_4$ 228.1236. Found: 228.1236 $(M+H)^+$.



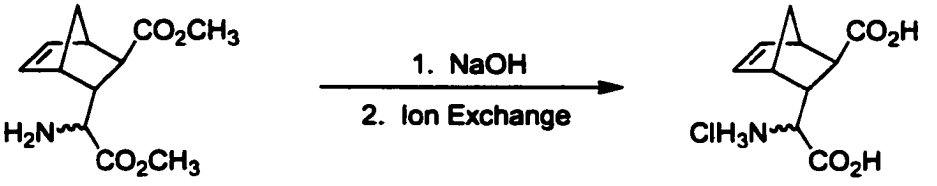

**2.30** **2.34**

**3-(Amino-carboxy-methyl)-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (2.34)**

To a solution of amino diester (**2.30**) (0.180 g, 0.712 mol) in MeOH (2.5 mL) was added NaOH (6 N, 475 μL, 2.847 mmol) and the resultant solution was stirred at rt for 30 min and subsequently heated to reflux and stirred for 5 min. The solution was cooled to rt and the MeOH was removed *in vacuo*. The remaining aqueous solution was brought to ca. pH 6 with 1 N HCl and loaded to an ion exchange (Bio-Rad, AG-1X2, acetate form) column and the amino acid eluted with 0.25 – 0.5 N HCl, the solvent was removed *in vacuo* to afford **2.34** (185.3 mg, 99.5%) as a white solid.

$^{1}$H NMR ($CDCl_3$, 400 MHz): δ 6.68-6.57 (m, 1 H), 6.41-6.34 (m, 1 H), 4.12 (d, $J$ = 4.8 Hz, 0.6 H), 3.78 (d, $J$ = 9.7 Hz, 0.4 H), 3.10-3.09 (m, 1 H), 2.89-2.66 (m, 3 H), 1.73-1.24 (m, 4 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ (179.7, 179.4), (173.8, 173.2), (138.0, 137.7), (133.6, 132.9), (58.2, 57.9), (49.0, 48.6), 47.2, (42.8, 42.6), (32.3, 31.8), (26.8, 26.3), 20.1;




**2.31** **2.35**

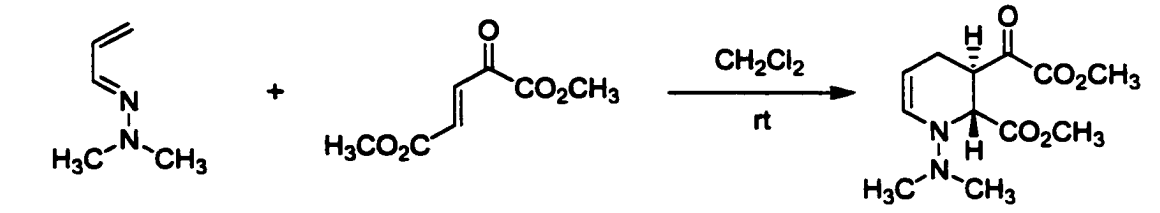
**3-(Amino-carboxy-methyl)-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid (2.34)**

To a solution of amino diester (**2.31**) (0.40 g, 0.15 mol) in MeOH (2.5 mL) was added NaOH (6N, 101 μL, 0.61 mmol) and the resultant solution was stirred at rt overnight. The MeOH was removed *in vacuo* and the remaining aqueous solution was titrated to ca. pH 6 with 6 N HCl. The crude amino acid was loaded onto an ion exchange (Bio-Rad, AG-1X2, acetate form) column and the amino acid eluted with 0.25 N HCl. The solvent was removed *in vacuo* to afford **2.35** (0.03 g, 75.7%) as a white solid.

$^1$H NMR ($CDCl_3$, 400 MHz): δ 6.35-5.98 (m, 2 H), 3.41 (br d, $J$ = 10.4 Hz, 0.5 H), 3.28 (br d, $J$ = 11.6 Hz, 0.5 H), 3.02 (br s, 1 H), 2.94 (br s, 1 H), 2.69-2.59 (br m, 1 H), 2.27 (br s, 0.5 H), 2.14 (br s, 0.5 H), 1.43-1.35 (br m, 2 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ (180.0, 180.1), (173.8, 173.2), (141.6, 139.7), (136.5, 134.5), (58.4, 57.3), (49.4, 48.9), (48.6, 48.5), (47.7, 47.5), (46.9, 46.2), (45.7, 45.1);

HRMS Calcd for $C_{10}H_{14}NO_4$ 212.0923. Found: 212.0914 $(M+H)^+$.




**Acrolein dimethylhydrazone** **2.3** **2.36**

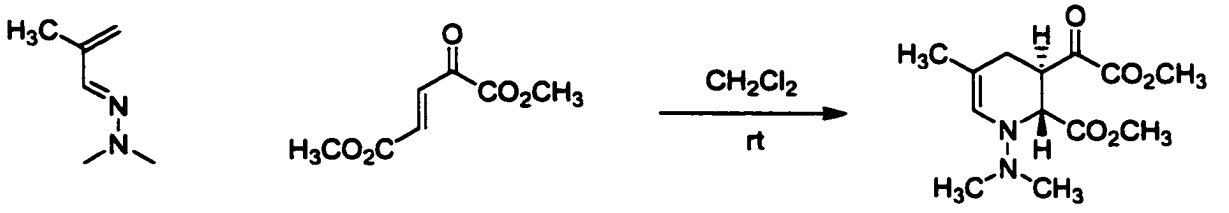
**Methyl 1-dimethylamino-3-methoxyoxalyl-1,2,3,4-tetrahydro-pyridine-2-carboxylate (2.36)**

To a solution of DOG **2.3** (0.32 g, 1.88 mmol) in $CH_2Cl_2$ (2.5 mL) was added a solution of acrolein dimethylhydrazone (0.28 g, 2.81 mmol) in $CH_2Cl_2$ (2.5 mL) and the resultant solution was stirred at rt for 3 h. The solvent was removed *in vacuo* and the crude red oil was chromatographed ($SiO_2$, 50% EtOAc : hexanes) to afford **2.36** (0.21 g, 41.4 %) as a red oil:

$^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 6.48 (d, $J$ = 5.8 Hz, 1 H), 6.11-6.10 (br m, 1 H), 4.51 (ddd, $J$ = 12.9, 5.2, 1.9 Hz, 1 H), 3.74 (s, 3 H), 3.68 (s, 3 H), 3.43-3.38 (m, 1 H), 2.78 (s, 6 H),2.31-2.24 (m, 1 H), 2.06-1.97 (m, 1 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ 173.6, 164.1, 145.5, 132.0, 109.2, 53.4 (2 C), 44.0, 43.5, 39.2, 34.8, 29.5;

HRMS Calcd for $C_{12}H_{19}N_2O_5$ 271.1294. Found: 271.1298 $(M+H)^+$.




**methacrolein dimethylhydrazone** **2.3** **2.37**

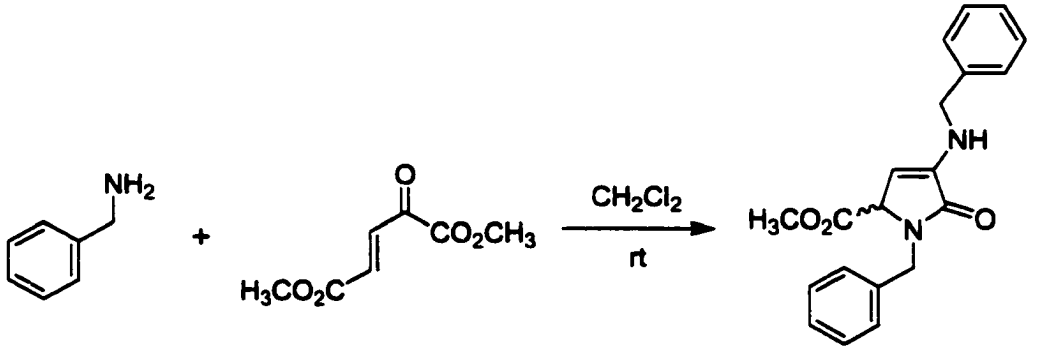
**1-Dimethylamino-3-methoxyoxalyl-5-methyl-1,2,3,4-tetrahydro-pyridine-2-carboxylic acid methyl ester (2.36)**

To a solution of DOG **2.3** (0.72 g, 4.21 mmol) in $CH_2Cl_2$ (10 mL) was added a solution of methacrolein dimethylhydrazone (0.28 g, 2.81 mmol) in $CH_2Cl_2$ (2 mL) and the resultant solution was stirred at rt for 4 h. The solvent was removed i*n vacuo* and the crude red oil was chromatographed ($SiO_2$, 25% EtOAc : hexanes) to afford the product **2.35** (0.88 g, 73.3 %) as a red oil:

$^1$H NMR ($CDCl_3$, 400 MHz): δ 6.44 (s, 1 H), 6.10 (d, $J$ = 3.2 Hz, 1 H), 3.71 (s, 3 H), 3.64 (s, 3 H), 3.21 (td, $J$ = 8.4, 3.2 Hz, 1 H), 2.67 (s, 6 H), 2.22 (q, $J$ = 14.2, 8.4 Hz, 1 H), 1.99 (q, $J$ = 14.2, 7.1 Hz, 1 H), 1.34 (s, 3 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 172.4, 163.2, 142.9, 108.2, 107.1, 52.1, 52.0, 42.5, 42.4, 35.8, 32.3, 27.2, 23.0

HRMS Calcd for $C_{13}H_{21}N_2O_5$ 285.1450. Found: 285.1458 $(M+H)^+$.




**benzylamine** **2.3** **2.42**

**Methyl 1-benzyl-5-oxo-4-phenylamino-2,5-dihydro-1H-pyrrole-2-carboxylate (2.42)**

To a solution of benzyl amine (6.92 mL, 63.41 mmol) in $CH_2Cl_2$ (20 mL) was added DOG (**2.3**) (5.19 g, 30.19 mmol) in $CH_2Cl_2$ (30 mL) dropwise over 20 min. The resultant solution was stirred at rt for 10 min., the solvent was removed *in vacuo* and the resultant crude oil was chromatographed (SiO2, 25 % EtOAc : hexanes) to afford the **2.42** (6.73 g, 90.2 %) as a yellow oil:

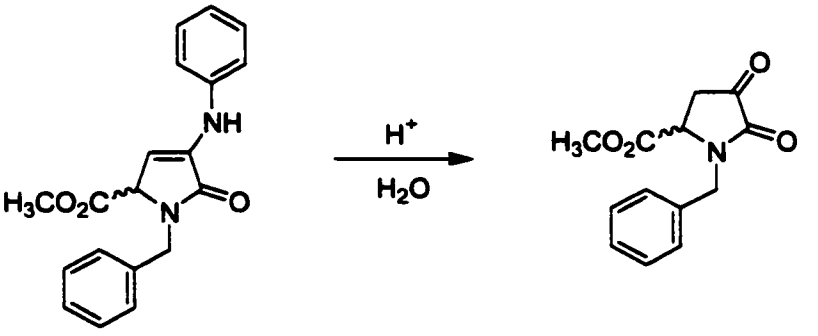
$^1$H NMR ($CDCl_3$, 400 MHz): δ 7.38-7.19 (m, 10 H), 5.19 (d, $J$ = 14.9, 1 H), 5.06 (d, $J$ = 2.6 Hz, 1 H), 4.36 (d, $J$ = 2.6 Hz, 1 H), 4.26-4.14 (m, 3H), 3.68 (s, 3 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 171.8, 169.8, 142.3, 139.6, 130.6, 130.4, 130.2, 129.5, 129.3, 97.5, 61.6, 53.9, 46.8;

HRMS Calcd for $C_{20}H_{21}N_2O_3$: 337.1552. Found: 337.1542 $(M+H)^+$.

HRMS Calcd for $C_{20}H_{20}N_2O_3Na$: 359.1372. Found: 359.1388 $(M+Na)^+$.

HRMS Calcd for $C_{22}H_{23}N_3O_3$: 400.1637. Found: 400.1636 $(M+CH_3CN+Na)^+$.




**2.42** **2.41**

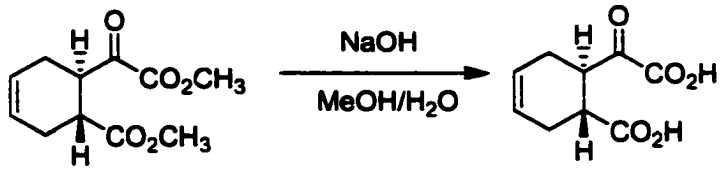
**Methyl 1-benzyl-4,5-dioxo-pyrrolidine-2-carboxylate (2.41)**

To a solution of enamine **2.41** (0.16 g, 0.48 mmol) in wet MeOH (3 mL) was added HCl (1 N, 10 drops) and the solution was stirred at rt for 5 min. The MeOH was removed i*in vacuo* and the resultant slurry was partitioned between $Et_2O$ (10 mL) and HCl (0,25 N, 5 mL) and the organic layer was collected and the aqueous layer was extracted with $Et_2O$ (10 mL), the organics were combined, dried over $MgSO_4$ and filtered to afford **2.41** (0.11 g, 93.4 %) as a brown oil:

$^1$H NMR ($CDCl_3$, 400 MHz): δ 7.36-7.24 (m, 5 H), 5.16 (d, $J$ = 14.2 Hz, 1 H), 4.33-4.27 (m, 2 H), 3.68 (s, 3 H), 2.89-2.82 (m, 1 H), 2.72-2.67 (m, 1 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 196.4, 170.9, 160.0, 134.5, 129.8, 129.6, 129.4, 53.2, 47.9, 35.2, 29.8;

HRMS Calcd for $C_{13}H_{14}NO_4$: 248.0923. Found: 248.0917 $(M+H)^+$.




**2.7** **2.43**

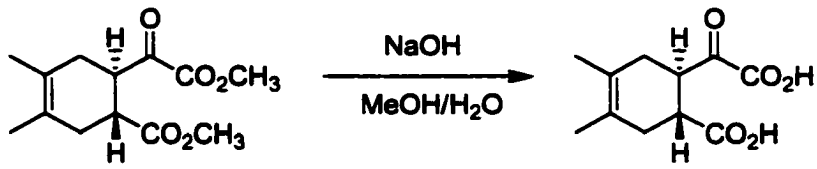
**6-Oxalyl-cyclohex-3-enecarboxylic acid (2.43)**

To a solution of **2.7** (0.32 g, 1.44 mmol) in MeOH (2.5 mL) was added NaOH (2 N, 2.0 mL, excess) and the solution was stirred at rt overnight. The MeOH was removed *in vacuo* and the aqueous solution was extracted with $Et_2O$ (3 x 7 mL) then titrated to pH 2 with 6 N HCl and extracted with $Et_2O$ (3 x 20 mL), dried ($MgSO_4$) and the solvent was removed *in vacuo* to afford the diacid **2.43** (0.26 g, 90.4 %) as a yellow oil.

$^1$H NMR ($D_2O$, 400 MHz); δ 7.64 (br s, 2 H), 5.25 (br s, 1 H), 4.77-4.70 (m, 1 H), 4.36-4.30 (m, 2 H), 4.12-4.04 (m, 1 H), 3.95-3.88 (m, 1 H);

$^{13}$C NMR ($D_2O$, 100 MHz); δ 180.7, 170.9 (2 C), 126.2, 126.0, 45.3, 41.5, 28.1, 27.0

HRMS Calcd for $C_9H_{11}O_5$ 199.0606. Found: 199.0602 $(M+H)^+$.




**2.8** **2.44**

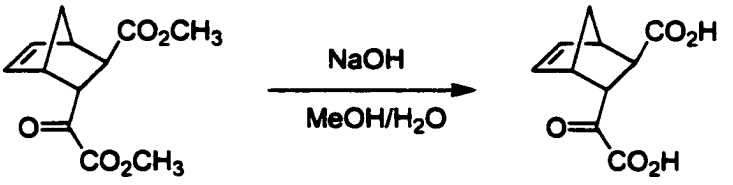
**3,4-Dimethyl-6-oxalyl-cyclohex-3-enecarboxylic acid (2.44)**

To a solution of **2.8** (0.45 g, 1.76 mmol) in MeOH (3.6 mL) was added NaOH (1N, 3.62 mL, 3.62 mmol) dropwise over 5 min and the resultant solution was heated to 50 °C and stirred for 48 h. The solvent was removed with a stream of air and the resultant solid was dissolved in a minimum amount of water and brought to pH 3 by the addition of 25% aqueous TFA. The precipitate was isolated by centrifugation to afford **2.44** (0.40 g, 67.0%) as an off white solid.

$^{1}$H NMR ($D_2O$, 400 MHz): δ 3.25 (br s, 1 H), 2.70 (td, $J$ = 10.4, 5.8 Hz, 1 H), 2.21-1.84 (m, 4 H), 1.51 (s, 6 H);

$^{13}$C NMR ($D_2O$, 100 MHz): δ 180.6, 170.9 (2 C), 125.5, 125.2, 46.0, 43.3, 42.0, 33.9, 32.7, 18.9, 18.8;

HRMS Calcd for $C_{11}H_{15}O_5$ 227.0919. Found: 227.0920 $(M+H)^+$.




**2.10** **2.45**

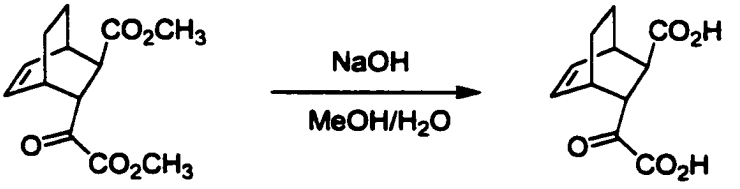
**3-Oxalyl-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid (2.45)**

To a solution of **2.10** (0.52 g, 2.20 mmol) in MeOH (4.5 mL) was added NaOH (1 N, 4.50 mL, 4.50 mmol) and the resultant solution was stirred at rt overnight. The solvent was removed *in vacuo* and the resultant solid was dissolved in a minimum amount of water and brought to pH 3 by the addition of 25% aqueous TFA. The precipitate was isolated by centrifugation to afford **2.45** (0.40 g, 67.0%) as a brown solid. 1H NMR indicates an 80 : 20 mixture of stereoisomers by integration of the resolved alkene protons:

$^1$H NMR ($CDCl_3$, 400 MHz) δ 6.26 (q, $J$ = 5.8 Hz, 0.2 H), 6.18 (q, $J$ = 5.8 Hz, 0.8 H), 6.06 (q, $J$ = 5.8 Hz, 0.2 H), 5.92 (q, $J$ = 5.8 Hz, 0.8 H) 3.71 (t, $J$ = 5.2 Hz, 1 H), 3.25 (br s, 1 H), 3.06 (br s, 1 H), 2.54 (d, $J$ = 5.2 Hz, 1 H), 1.53 (br d, $J$ = 9.1 Hz, 1 H), 1.39 (br d, $J$ = 9.1 Hz, 1 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz) δ 205.7, 179.6, 171.6, 138.8, 135.4, 54.1, (48.3, 48.2), 47.7, 47.1, 46.8, (46.4, 46.3);

HRMS Calcd for $C_{10}H_{11}O_5$ 211.0606. Found: 211.0597 $(M+H)^+$.




**2.11** **2.46**

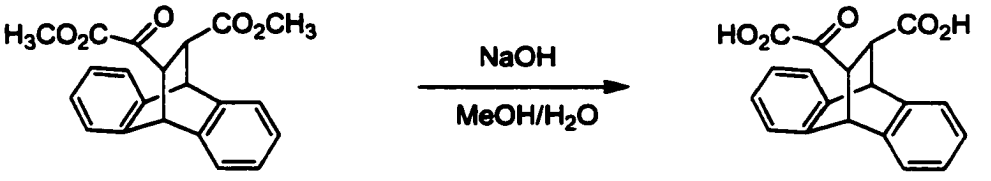
**3-Oxalyl-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (2.46)**

To a solution of **2.11** (0.45 g, 1.79 mmol) in MeOH (2.5 mL) was added NaOH (1 N, 2 mL) and the resultant solution was stirred at rt overnight. The MeOH was removed *in vacuo* and the remaining aqueous was washed with $Et_2O$ (3 x 7 mL), adjusted to pH 2 with HCl (6 N, aqueous) and extracted with $Et_2O$ (5 x 20 mL), dried ($MgSO_4$) and concentrated *in vacuo* to afford the diacid **2.44** (0.38 g, 96.6%) as a tan oil:

$^1$H NMR ($D_2O$, 400 MHz): δ 6.53-6.14 (m, 2 H), 3.90-2.90 (m, 4 H), 1.85-1.17 (m, 4 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ (197.5, 196.8), (177.5, 176.1), (163.5, 163.3), 136.9, 136.2), (134.7, 133.5), (50.2, 50.1), (44.9, 44.5), (33.7, 33.6), (33.0, 32.7), (25.8, 25.2), (21.3, 20.8);

HRMS Calcd for $C_{11}H_{13}O_5$ 225.0763. Found: 225.0763 $(M+H)^+$.




**2.16** **2.47**

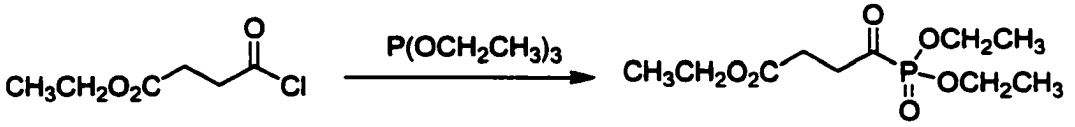
**9,10-Dihydroanthraceno-3,10-[3,4-(2-oxo)]-glutaric acid (2.47)**

To a solution of **2.16** (0.18 g, 0.51 mmol) in MeOH (5 mL) was added NaOH (2 N, 2.0 mL) and the solution was stirred at rt overnight. The MeOH was removed *in vacuo* and the aqueous was washed with $Et_2O$ (3 x 7 mL) then titrated to pH 2 with 6 N HCl and extracted with $Et_2O$ (5 x 20 mL), dried ($MgSO_4$) and the solvent was removed *in vacuo* to afford **2.47** (0.13 g, 76.8 %) as a white solid.

$^1$H NMR ($CD_3CN$, 400 MHz): δ 7.41-7.07 (m, 8 H), 4.85 (d, $J$ = 2.6 Hz, 1 H), 4.78 (d, $J$ = 2.6 Hz, 1 H), 4.00 (dd, $J$ = 5.2, 1.9 Hz, 1 H), 3.32 (dd, $J$ = 5.2, 2.6 Hz, 1 H);

$^{13}$C NMR ($CD_3CN$, 100 MHz): δ 195.2, 174.6, 162.7, 144.3, 143.9, 142.5, 141.4, 128.2, 126.5, 125.4, 119.0, 52.2, 47.6, 47.0, 46.5

HRMS Calcd for C19H15O5 323.0919. Found: 323.0926 $(M+H)^+$.




**Ethyl Succinyl Chloride**

**2.48**

**4-(Diethoxy-phosphoryl)-4-oxo-butyric acid ethyl ester (2.48)**

To triethylphosphite (3.97 mL, 23.16 mmol) under argon was added ethyl succinyl chloride (3.00 mL, 21.05 mmol) dropwise. The resultant solution was stirred at rt for 8 h. The by-products were removed by distillation (below 85 °C, 0.050 mm Hg) and **2.48** was collected by distillation (98-100 °C, 0.050 mm Hg, 5.37 g, 95.6%) as a colorless oil:

$^{1}$H NMR ($CDCl_3$, 400 MHz): δ 4.12 (m, $J$ = 7.1 Hz, 4 H), 4.03 (q, $J$ = 7.1 Hz, 2 H), 3.04 (td, $J_{P\text{-}H}$ = 2.6, $J$ = 6.5 Hz, 2 H), 2.51 (t, $J$ = 6.5 Hz, 2 H), 1.26 (t, $J$ = 7.1 Hz, 6 H), 1.13 (t, $J$ = 7.1 Hz, 3 H);

$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 210.0, 208.3, 63.6 (d, $J_{C\text{-}P}$ = 6.0 Hz), 60.5, 37.9 (d, $J_{C\text{-}P}$ = 57.7 Hz), 26.6 (d, $J_{C\text{-}P}$ = 6.1 Hz); 16.1 (d, $J_{C\text{-}P}$ = 6.1 Hz), 13.8;

$^{31}$P NMR ($CDCl_3$, 161 MHz) δ -2.7;

HRMS Calcd for $C_{10}H_{20}O_6P$ 267.0998. Found: 267.0993 $(M+H)^+$.


**2.48** **2.49**

**4-Oxo-4-phosphono-butyric acid ethyl ester (2.49)**

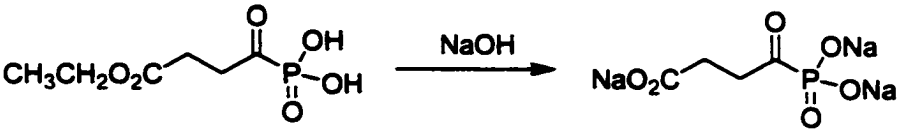
To **2.46** (1.27 g, 4.78 mmol) under argon was added bromotrimethylsilane (5.68 mL, 43.04 mmol) dropwise over 5 min. The resultant solution was stirred at rt overnight. The excess bromotrimethylsilane and ethyl bromide were removed *in vacuo* and the remaining residue was partitioned between water (50 mL) and EtOAc (50 mL). The aqueous layer was collected and subsequently washed with EtOAc (2 x 50 mL) and the combined organic fractions were back extracted with water (2 x 20 mL). The combined aqueous portions were concentrated *in vacuo* to afford **2.49** as a clear semisolid in about 80% purity as determined by $^{1}$H NMR analysis.

$^{1}$H NMR ($D_2O$, 400 MHz): δ 3.41 (q, $J$ = 7.1, 5.2 Hz, 2 H), 2.46 (br t, 2 H), 1.93 (br t, 2 H), 0.50 (br t, 3 H);

$^{13}$C NMR ($D_2O$, 100 MHz): δ 218.1 (d, $J_{C\text{-}P}$ = 167.1 Hz); 175.6, 62.7, 38.3 (d, $J_{C\text{-}P}$ = 51.1 Hz), 27.8, 14.0;

$^{31}$P NMR ($D_2O$, 161 MHz): δ -3.97;

HRMS Calcd for $C_6H_{12}O_6P$ 211.0372. Found: 211.0364 $(M+H)^+$.




**2.49** **2.50**

**trisodium 4-oxo-4-phosphonobutyrate (2.50)**

To a solution of **2.49** (0.28 g, 1.38 mmol) in deionized water (10 mL) was added NaOH (1 N, 4.40 mL, 3.20 mmol) and the resultant solution was heated to 50 °C and stirred overnight. The solvent was removed *in vacuo* to afford trisodium 4-oxo-4-phosphono-butyrate, as a clear semisolid, >95% purity as determined by $^{1}$H NMR analysis.

$^{1}$H NMR ($D_2O$, 400 MHz): δ 2.94 (t, $J$ = 7.1, 6.5 Hz, 2 H), 2.22 (t, $J$ = 7.1, 6.5 Hz, 2 H);

$^{13}$C NMR ($D_2O$, 100 MHz) δ 228.6 (d, $J_{C\text{-}P}$ = 156.6 Hz); 183.4, 40.4 (d, $J_{C\text{-}P}$ = 42.8 Hz); 31.5 (d, $J_{C\text{-}P}$ = 3.6 Hz);

$^{31}$P NMR ($D_2O$, 161 MHz) δ 0.5;

HRMS Calcd for $C_4H_6O_6P$ 180.9902. Found: 180.9904 $(M\text{-}H)^-$.

HRMS Calcd for $C_4H_5O_6NaP$ 202.9721. Found: 202.9713 $(M+Na\text{-}H)^-$.


**2.48** **2.51**

**Ethyl 4-(ethoxy-sodium-hydroxy-phosphoryl)-4-oxo-butyrate (2.51)**

To a solution of **2.48** (0.72 g, 2.71 mmol) in acetone (15 mL) under argon was added a solution of NaI (0.43 g, 2.84 mmol) in acetone (2 mL) over 10 min. The resultant solution was heated to reflux and stirred 14 h. Solid NaI (0.43g, 2.84 mmol) was added in one portion and the resultant solution was heated to reflux and stirred for 6 h when all starting material was consumed as indicated by TLC. The solvent was removed *in vacuo* to afford **2.51** as a pale yellow solid with > 99 % purity as determined by $^{1}$H NMR analysis. This material was used in biological assays and additional reactions without further purification:

$^{1}$H NMR ($d_6$-acetone, 400 MHz): δ 4.01 (q, $J$ = 7.1 Hz, 2 H), 3.86 (t, $J$ = 7.1 Hz, 2 H), 3.10 (br s, 2 H), 2.54 (b t, $J$ = 5.8, 3.9 Hz, 2 H), 1.14-1.10 (m, 6 H):

$^{13}$C NMR ($d_6$-acetone, 100 MHz): δ 208.6, 174.2, 62.6, 61.7, 38.2 (d, $J_{C\text{-}P}$ = 45.6 Hz), 28.1, 17.3 (d, $J_{C\text{-}P}$ = 6.1 Hz), 14.9;

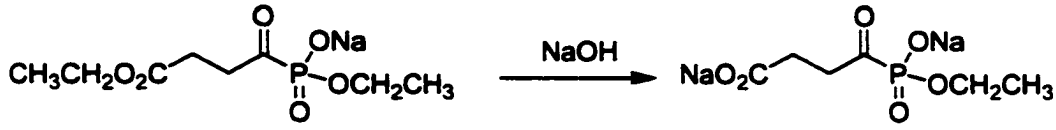
$^{31}$P NMR ($d_6$-acetone, 161 MHz): δ 4.7:

$^{1}$H NMR ($D_2O$, 400 MHz): δ 4.05 (q, $J$ = 7.1 Hz, 2 H), 3.85 (p, $J$ = 7.1 Hz, 2 H), 3.07 (td, $J$ = 6.5, 5.8, 1.9 Hz, 2 H), 2.54 (t, $J$ = 6.5, 5.8 Hz, 2 H), 1.15 (qd, $J$ = 7.1, 1.9 Hz, 6 H):

$^{13}$C NMR ($D_2O$, 100 MHz): δ 220.5 (d, $J_{C\text{-}P}$ = 165.1 Hz), 176.2, 63.7 (d, $J_{C\text{-}P}$ = 6.1 Hz), 63.0, 39.3, 38.8, 28.2 (d, $J_{C\text{-}P}$ = 4.1 Hz), 17.0 (d, $J_{C\text{-}P}$ = 5.1 Hz), 14.4;

$^{31}$P NMR ($D_2O$, 161 MHz): δ 1.35:

HRMS Calcd for $C_8H_{16}O_6P$ 239.0685. Found: 239.0684 $(M+H)^+$.




**2.51** **2.52**

**Sodium 4-oxo-4-(ethyl, sodium phosphono)-butanoate (2.52)**

To a solution of **2.49** (1.35 mmol, used directly from the previous reaction) in deionized water (3 mL) was added NaOH (1 N, 1.35 mL, 1.35 mmol) and the resultant solution was stirred at rt for 4 h. The solvent was removed *in vacuo* to afford **2.52** as a yellow oil with > 95 % purity as determined by $^{1}$H NMR analysis.

$^{1}$H NMR ($D_2O$, 400 MHz): δ 3.95 (m, $J$ = 7.1 Hz, 2 H), 3.07 (t, $J$ = 7.1 Hz, 2 H), 2.41 (m, 2 H), 1.25 (t, $J$ = 7.1, 6.5 Hz, 3 H);

$^{13}$C NMR ($D_2O$, 100 MHz): δ 221.8, 183.2, 63.9 (d, $J_{C\text{-}P}$ = 6.4 Hz), 41.0 (d, $J_{C\text{-}P}$ = 46.7 Hz), 31.4 (d, $J_{C\text{-}P}$ = 4.2 Hz), 17.5 (d, $J_{C\text{-}P}$ = 6.4 Hz);

$^{31}$P NMR ($D_2O$, 161 MHz): δ -0.6.

HRMS Calcd for $C_6H_{10}O_6P$ 209.0215. Found: 209.0203 $(M\text{-}H)^-$.

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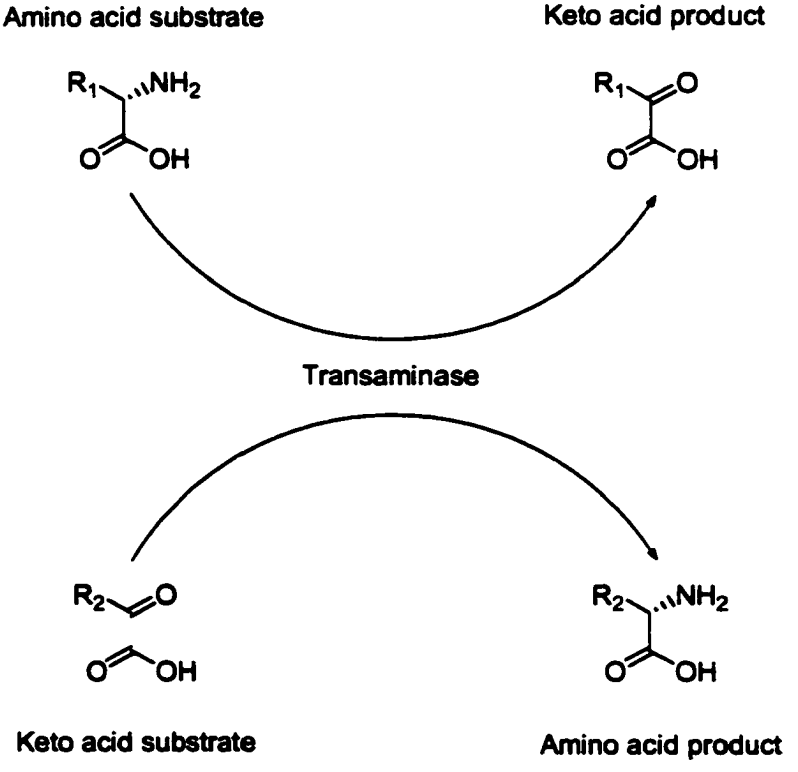
## Chapter 3: Enzymatic transformation of keto diacids to glutamic acid analogues

### I. Introduction

Over the past few decades, explosive growth has occurred in the use of enzymes in organic synthesis (Davies, 1992). The chemoselectivity, enantioselectivity and, in general, benign reaction conditions utilized in enzyme-mediated transformations remain unrivaled by chemical reagents. Some of the most difficult transformations and assemblies have been accomplished using enzymes at both analytical and preparative levels. Although enzyme-mediated transformations in organic synthesis hold great promise, the application of a given biocatalyst is often limited to a narrow range of substrate possibilities. Specifically, only modest structural variation to the natural substrate is tolerated owing to restrictions in the active site topology. A more thorough understanding of the structural limits for potential substrates is often helpful in determining the synthetic diversity of an enzyme or enzyme class, and its catalytic mechanism. The evaluation of substrate tolerance is especially useful when crystal structures are lacking or several engineered mutants have been derived that show subtle variances in activity. Based on the possible favorable outcomes for biocatalysts, it is important to prepare and screen potential enzyme substrates that have been custom-tailored with incremental changes in structure such that the rate and extent of product formation may be used to derive salient differences in the topographies of the active sites among different enzymes. Likewise, the relative rates of conversion to products and the

$K_m$ values derived from a structurally similar panel of substrates can reveal important elements of the catalytic mechanism.

One of the most strategically important enzyme-mediated organic reactions is the conversion of an α-keto acid to an α-amino acid (Equation **3.1**). The substrate α-keto acids may be obtained by a variety of chemical and biochemical procedures (Cooper, 1983), but importantly, the product α-amino acid contains a new stereogenic center.




**Equation 3.1**: Reaction cycle catalyzed by a transaminase

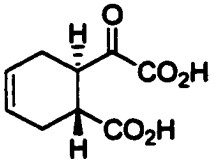
Because microbiological production of natural α-amino acids is now commonplace, an interesting challenge at this point is to prepare innovative unnatural α-amino acids. An important example of a class of unnatural α-amino acids resides in the glutamate

neurotransmitter system in which the conformationally-restricted analogues of glutamic acid have been shown to selectively regulate various receptors and transporters (Chamberlin, 1993). Surprisingly, very few analogues of glutamic acid have been prepared via enzyme-mediated syntheses despite the fact that a number of enzyme systems are capable of producing or using glutamic acid. Importantly, in an enzyme-based strategy, a single stereoisomer can be produced, a likely structural requirement for selectivity in the glutamate neurotransmitter system.

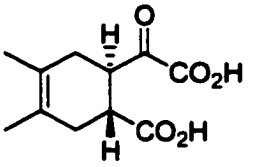
The goal of this section is two fold: (1) to identify an enzyme capable of transaminating the 3, 4-conformationally restricted analogues of α-ketoglutarate compounds **2.43 - 2.47**, Figure 3.1)] to produce structurally constrained analogues of L-glutamate with the S configuration at the new stereogenic center, and (2) to test the ability of the constrained ketoglutarate analogues to inhibit the action of certain enzymes. Due to the principle of microscopic reversibility, if the structurally constrained α-keto acid is an inhibitor of an enzyme, it is thought that the product α-amino acid will, in turn, inhibit the enzymatic transformation. If the α-keto acids are found not to inhibit these glutamate-utilizing enzymes it is a good indication that these 3,4-conformationally restricted analogues of L-glutamate will not be metabolized by this system *in vivo*.

In this section, five 3,4-conformationally restricted analogues of α-ketoglutarate (Figure 3.1) were surveyed as substrates of cytosolic aspartate aminotransferase (AspAT), alanine aminotransferase (AlaAT), glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH), α-ketoglutarate dehydrogenase complex (KGDHC), L-leucine dehydrogenase (LeuDH), glutamine transaminase K (GTK), L-phenylalanine

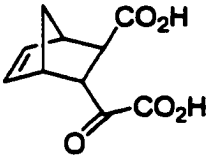
dehydrogenase (PDH) and five thermostable aminotransferases obtained as a kit (CloneZyme™) from Sigma Chemical company designated as AT-1 through AT-5.



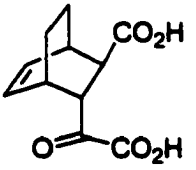

2.43



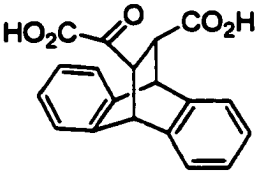

2.44




2.45




2.46




2.47

**Figure 3.1**: 3,4-Conformationally restricted analogues of α-ketoglutarate

The 3,4-conformationally restricted analogues of α-ketoglutarate were also tested as inhibitors of AlaAT, AspAT, GDH and GTK.

## II. Results and Discussion

### *1. Analysis of Compounds* **2.43 – 2.47** *as Substrates of L-Amino Acid Dehydrogenases*

Because compounds **2.43 – 2.47** (Figure 3.1) are rigid analogues of αKG, it seemed possible that they would be substrates of GDH. Moreover, GDH converts hydrophobic α-keto acids, such as α-keto-γ-methiolbutyrate (αKMB) and α-ketobutyrate to their corresponding amino acids (Cooper, 1981 and refs. therein). However, compounds **2.43 – 2.47** were not converted to the amino acids even in the presence of 50 μg of GDH (see Experimental Section). Presumably, compounds **2.43 – 2.47** are too large to be productively accommodated at the active site of GDH. Although the idea that compounds **2.43 – 2.47** might be substrates of GDH proved to be untenable, the result was useful nevertheless. Since compounds **2.43 – 2.47** did not interact with GDH, the findings show that GDH could be used as an indicator enzyme to quantitate transamination between GLU and the 3,4-conformationally restricted analogues of α-ketoglutarate.

Two of the α-keto acid substrates of LeuDH, namely α-ketoisovalerate and α-keto-β-methylvalerate, contain a hydrophobic branch point immediately adjacent to the keto group and, therefore, bear some resemblance to compounds **2.43 – 2.47**. However, no activity could be detected when the natural substrates (i.e. α-ketoisocaproate) were replaced by compounds **2.43 – 2.47** even when 50 μg of enzyme was present in the reaction mixture (see Experimental Section). Either the compounds are too large to fit

into the active site or the additional carboxylic acid group is involved in a charge repulsion at the active site.

In addition to phenylpyruvate, PDH exhibits some activity toward *p*-hydroxyphenylpyruvate, indolyl 3-pyruvate, αKMB and certain branched chain α-keto acids (Asano, 1987). Therefore, it was reasoned that PDH has a somewhat large and flexible active site and might show activity toward compounds **2.43 – 2.47**. However, no activity of PDH toward compounds **2.43 – 2.47** could be detected in the presence of 50 μg of enzyme within the assay mixture (see Experimental Section). The result was useful because it showed that, again, since the compounds did not interact with the protein, PDH could be used as an indicator enzyme to verify the formation of phenylpyruvate in transamination reactions between L-phenylalanine (PHE) and compounds **2.43 – 2.47**.

*2. Analysis of Compounds* **2.43 – 2.47** *as Substrates of LDH, GTK, AspAT, AlaAT and KGDHC*

As first shown by Meister fifty two years ago, LDH catalyzes the reduction of many α-keto acids with NADH to the corresponding α-hydroxy acid (Meister, 1950). However, no reduction of compounds **2.43 – 2.47** could be detected with LDH at enzyme concentrations as high as 150 μg per assay (see Experimental Section). The results show that LDH could be used as an indicator enzyme in an assay to determine whether the αKG analogues could participate in transamination reactions using L-alanine (ALA) as the amine donor.

Compared to many other aminotransferases, GTK has a relatively broad specificity. For example, it may be regarded as a fully reversible glutamine (methionine) aromatic aminotransferase (Cooper, 1982). In addition, GTK catalyzes transamination reactions with phenylglyoxylate (Cooper, 1982) and cyclohexylpyruvate (Cooper, 1982). Moreover, the enzyme utilizes cystine, cystathionine and lanthionine as amino donor substrates (Cooper, 1989 and ref. therein). These amino acids contain charged groups distal to the binding site for the α-amino carboxyl grouping. Inasmuch as GTK can accommodate large hydrophobic groups and distally charged groups at the active site it was reasoned that compounds **2.43 – 2.47** might be substrates for GTK. As was found with the dehydrogenases, however, no conversion to products was evidenced with GTK (PHE as amino donor) for compounds **2.43 – 2.47** - even at an enzyme concentration of 25 μg per 50-μl assay mixture (see Experimental Section).

AspAT exhibits some reactivity toward aromatic α-keto acids and L-α-amino acids (Shrawder, 1972). Therefore, it was considered that the active site of this enzyme might be large enough to accommodate compounds **2.43 – 2.47**. However, the ability of these compounds to act as amino acceptors in a reaction catalyzed AspAT (or AlaAT) was not observed - even at a concentration of enzyme as high as 50 μg per 200-μl assay (see Experimental Section). In a separate experiment, it was found that the 3,4-conformationally restricted analogues of α-ketoglutarate had no effect on malate dehydrogenase (MDH). Therefore, the inability to detect AspAT-catalyzed transamination between ASP and αKG analogues was not due to inhibition of the coupling enzyme MDH. During the transamination reaction catalyzed by AspAT, the active site becomes closed over (Saier, 1967). Presumably, the 3,4-conformationally restricted analogues of α-ketoglutarate are too large to bind productively at the active site of AspAT or the active site cannot attain a closed conformation.

Finally, the ability of compounds **2.43 – 2.47** to act as substrates of KGDHC was determined. No reduction of $NAD^+$ was noted under conditions in which KG was very rapidly oxidatively decarboxylated, which indicates the inability of compounds **2.43 – 2.47** to act as substrates of this enzyme complex.

3. *Preliminary Screening of the CloneZyme™ Library*

The recommended amino donor for screening of potential α-keto acid substrates for the CloneZyme™ library is L-glutamate (GLU). However, according to the suppliers information, each of the enzymes in the CloneZyme™ library (i.e., AT-1 through AT-5) is active with phenylalanine (PHE) as amino donor and L-methionine (MET) serves as a good substrate of all the enzymes in the library except AT-3. Therefore, by invoking the principle of microscopic reversibility, it was assumed that the enzymes AT-1, AT-2, AT-4, and AT-5 would catalyze transamination between PHE and αKMB (the α-keto acid analogue of MET). The transamination product of PHE (i.e., phenylpyruvate) is easier to quantitate than is the transamination product of GLU (i.e., αKG). The PHE – αKMB transamination procedure measures appearance of product (phenylpyruvate) rather than disappearance of substrate and is 2.0 to 2.5 times more sensitive than the procedure in which loss of NADH absorbance is measured. In typical reactions, the product of the transamination with αKG is coupled to an indicator enzyme which reduces the newly generated α-keto acid with NADH. In preliminary experiments with PHE as amino donor, αKMB as amino acceptor and AT-1 (or AT-5) as catalyst, it was established that pyridoxal 5'-phosphate (PLP) is essential for maximal activity. Moreover, activity in the presence of 132 mM potassium borate buffer (pH 8.0) is considerably greater than the activity in 100 mM potassium phosphate buffer (pH 7.4) or ammediol buffer (pH 9.0) (data not shown). Based on the above considerations, procedures were devised as convenient standard assays to screen the usefulness of the CloneZyme™ library for the transamination of compounds **2.43 – 2.47**. The enzyme (0.02 to 5 μg) is incubated at

45°C in a 50-µl reaction mixture containing 10 mM PHE, 5 mM αKMB, 20 µM PLP and 132 mM potassium borate buffer (pH 8.0). After 2 to 10 min, 150 µl of 1 N NaOH is added and the absorbance due to phenylpyruvate-*enolate* is immediately determined at 320 nm ($\varepsilon \approx 16{,}000\ cm^{-1}$). By using this procedure, high PHE - αKMB transaminase activity was found with AT-1 and AT-5. Surprisingly, no activity was detected with AT-2 and AT-4, but the reasons were not investigated. When αKMB was replaced by αKG, transamination was somewhat slower. The results obtained with AT-1 and AT-5 as catalysts for PHE – αKMB/αKG transamination are shown in Table 3.1. [Note that in order to prevent problems with evaporation, the well plate should be covered with a tight fitting lid. Wells surrounding the "test" wells should be filled with water.]

**Table 3.1**. Transamination Between PHE and αKMB (or αKG) Catalyzed by AT-1 and AT-5[a]

| | µmol/min/mg of enzyme | |
|---|---|---|
| α-Keto acid substrate | AT-1 (n=3) | AT-5 (n=3) |
| αKMB (5 mM) | 0.51 ± 0.01 | 2.20 ± 0.02 |
| α-KG (20 mM) | 0.29 ± 0.01 | 0.76 ± 0.01 |
| α-KG (40 mM) | 0.60 ± 0.04 | 1.31 ± 0.05 |

[a] The 50-µl reaction mixture containing α-keto acid at the concentration shown, 10 mM PHE, 20 µM PLP, 132 mM potassium borate buffer (pH 8.0) and 2.5 µg of either AT-1or AT-5 (batch 1) was incubated at 45°C. After 2 min (αKMB as substrate) or 5 min (α-ketoglutarate as substrate), phenylpyruvate-*enolate* formation was measured as indicated in the experimental section.

In preliminary experiments, it was found that AT-5 is several times more effective than is AT-1 in catalyzing transamination reactions involving glutamate, phenylalanine,

αKMB and the various αKG analogues (data not shown). A comparison of GLU versus PHE as an amino donor in the AT-5-catalyzed transamination of αKMB is shown in Table 3.2. The activity exhibited by AT-5 toward PHE in Table 3.2 is somewhat greater than that shown in Table 3.1. This may be due to the fact that a different batch of AT-5 was used in the experiment described in Table 3.1 (batch 1) from that used in the experiment described in Table 3.2 (batch 2). Clearly, although the $V_{max}$ values with PHE and GLU are comparable, AT-5 exhibits a much higher affinity toward PHE than toward GLU. Based on the above findings, it was decided to use AT-1 and AT-5 (batch 2) and 10 mM PHE (or in some cases 320 mM GLU) as amino donor for screening 3,4-conformationally restricted analogues of α-ketoglutarate as α-keto acid substrates.

4. *Ability of AT-5 to Catalyze Amine Transfer from PHE to Compounds 2.43 – 2.47*

In preliminary experiments, compound **2.44** was chosen as the prototype to establish a general procedure for measuring transamination of compounds **2.43 – 2.47**. Both AT-1 and AT-5 were found to catalyze transamination of compound **2.44** (Figure 3.1). When 4 mM compound **2.44** was incubated in a 50-μl reaction mixture containing 10 mM PHE, 132 mM potassium borate buffer (pH 8.0), 20 μM PLP and 20 μg of enzyme at 45 °C for 1h, the amount of phenylpyruvate formed (as measured as the *enolate* after addition of base) was 7.2 ± 0.2 and 24 ± 1.6 nmol (n = 3) for AT-1 and AT-5 (batch 1), respectively. An additional experiment was also conducted in which phenylpyruvate formed after the

**Table 3.2.** Comparison of PHE and GLU in the AT-5-Catalyzed Transamination of α-KMB[a]

| L-Amino acid | μmol/min/mg of enzyme |
|---|---|
| PHE (10 mM) | 4.61 ± 0.07 |
| PHE (50 mM) | 5.60 ± 0.06 |
| GLU (10 mM) | 1.61 ± 0.05 |
| GLU (100 mM) | 4.35 ± 0.15 |
| GLU (320 mM) | 5.60 ± 0.21 |
| GLU (560 mM) | 5.83 ± 0.31 |

[a] The 50-μl reaction mixture containing L-amino acid at the indicated concentration, 5 mM αKMB, 20 μM PLP, 132 mM potassium borate buffer (pH 8.0) and 0.2 μg of AT-5 (batch 2) was incubated for 10 min at 45°C. Phenylpyruvate formed from PHE was measured as its *enolate* after addition of 150 μl of 1 M NaOH. αKG formed from GLU was measured by addition of a 150-μl reaction mixture containing GDH, ammonia and NADH. See the Experimental Section. αKMB exhibits slight activity with GDH and therefore a correction was made for the drift in the blank lacking AT-5.

1 h incubation was quantitated using PDH (see Experimental section). The amount of phenylpyruvate found using this procedure was 10.1 ± 0.3 and 22 ± 1.2 nmol (n = 3), respectively. The two methods are in reasonably good agreement. Finally, it was shown that the transamination reaction between 10 mM PHE and 4 mM compound **2.44** was linear for 4 h at 45 °C when carried out in the presence of either 20 μg of AT-1 or 20 μg of AT-5. These findings, together with those summarized in Tables 3.4 – 3.6, show that at a concentration of 4 mM, compound 3-1 is about 1 % as effective as 5 mM αKMB in transamination reactions with PHE catalyzed by both AT-1 and AT-5. However, because of the generally greater activity exhibited by AT-5 than AT-1 toward α-keto acids (αKMB, αKG, compound **2.44**), AT-5 was chosen for most studies involved in the screening of the ability of compounds **2.43, 2.45, 2.46** and **2.47** to participate in transamination reactions. In addition to compound **2.44**, compounds **2.43, 2.45, 2.46** and **2.47** were found to be amine acceptors in the presence of 10 mM PHE or 320 mM GLU and 10 μg of AT-5 (batch 2) in the 50-μl reaction mixture (Table 3.4). In general, the effectiveness of compounds **2.43 – 2.47** as substrates was similar whether PHE or GLU was used as the amine donor.

In a separate set of experiments, conditions were developed to characterize by HPLC/MS the conformationally-restricted analogues of L-glutamate generated by transamination of the 3, 4-conformationally restricted analogues of α-ketoglutarate with PHE. Borate buffer is not recommended in these experiments because nonvolatile buffer salts interfere with the electrospray ionization process and can cause contamination. Ammonium acetate buffer (pH 7.5) was used in its place but this buffer is not optimal for AT-5 activity. Therefore, to increase product formation, the temperature of the assay

mixture was increased from 45 °C to the optimal temperature of 80 °C. Because the heating chamber of the well plate analyzer cannot be set to 80 °C, the reaction at this temperature was performed at the bench. The 250-µl reaction mixture contained 10 mM PHE, 20 µM PLP, 100 mM ammonium acetate buffer (pH 7.5), 10 µg of AT-5 and compounds **2.43 – 2.47**. After a 1h incubation at 80 °C in a screw-capped vial in a heating block, 50 µl was transferred to a 96-well plate containing 150 µl 1 N NaOH and the phenylpyruvate formed from transamination was measured as its *enolate* at 340 nm (see the Experimental section). Table 3.3 shows the amount of phenylpyruvate formed per h/mg of AT-5 at 80 °C for compounds **2.43 – 2.47**. The relative transamination rates between PHE and the 3,4-conformationally restricted analogues of α-ketoglutarate (as measured by phenylpyruvate formation/h/mg of enzyme) are slightly different between those shown in Table 3.3 (borate buffer, pH 8.0; 45 °C) and Table 3.4 (ammonium acetate

**Table 3.3**. Effectiveness of Compounds **2.41 – 2.45** as Substrates of AT-5 in the Presence of 10 mM PHE

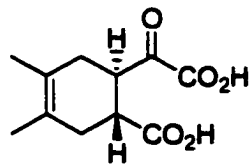
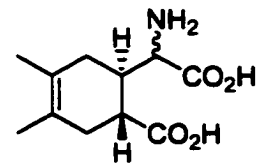
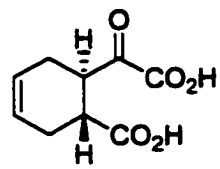
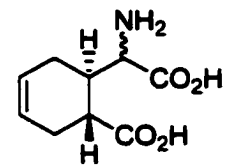
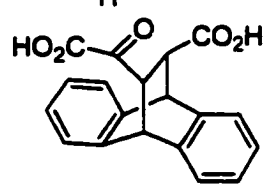
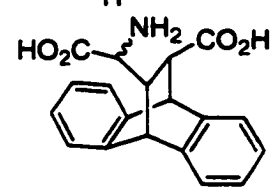
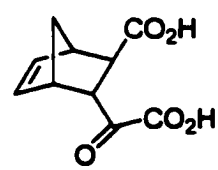
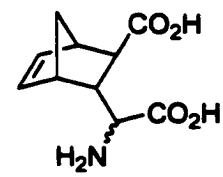
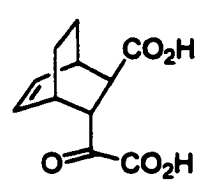
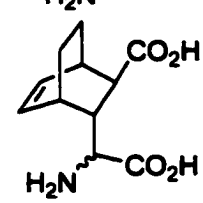
| Compound | Conc. (mM) | µmol/h/mg of enzyme (n=3) |
|---|---|---|
| **2.43** | 4 | 0.84 ± 0.08 |
| **2.44** | 4 | 1.23 ± 0.08 |
| **2.45** | 25 | 0.26 ± 0.01 |
| **2.46** | 25 | 0.60 ± 0.02 |
| **2.47** | 1 | 0.42 ± 0.04 |

[a] The 250-µl reaction mixture contained 10 mM PHE, 20 µM PLP, 100 mM ammonium acetate buffer (pH 7.5), 10 µg of AT-5 and compounds **2.43 – 2.47** (concentrations indicated in Table). After a 1-h incubation at 80°C in a screw capped vial in a heating block, 50 µL was transferred to a 96 well plate containing 150 µl 1 N NaOH and phenylpyruvate formed from PHE transamination was measured as its *enolate*. See the Experimental section.

buffer, pH 7.5; 80 °C). This slight discrepancy may be due in part to different activation energies for transamination of the various α-keto acids and different buffer conditions. Nevertheless, for three 3,4-conformationally restricted analogues of α-ketoglutarate, the yield of transamination product was increased significantly at 80 °C relative to 45 °C despite the fact that the buffer used at the higher temperature and for mass spectral determinations was not optimal.

The remainder of the reaction mixture (i.e., 200 μl) was quenched by submersion in a −10 °C salt bath. Aliquots (5 μl) of the solution were injected into the HPLC/MS eluting at 1 ml/min. Two peaks were detected corresponding to PHE (*m/z* = 166) and newly formed amino acid (m/z values indicated in Table 3.4). The 3,4-conformationally restricted analogues of α-ketoglutarate substrates and phenylpyruvate product were not observed owing to poor ionization under the experimental conditions. In accordance with the above observation, the mass spectrometer was set to the ion selective mode. The *m/z* values monitored correspond to the amine donor (PHE, *m/z* 166) and the newly formed amino acid (Figure 3.2). Representative examples of the HPLC/MS (compound **2.44**) chromatogram and spectra are shown in Figures 3.2 and 3.3, respectively.

**Table 3.4.** Structures of Compounds **2.43 – 2.47** and Their Corresponding Amino Acids

| Compound | Keto Acid Structure | Amino Acid Structure | m/z (of the Amino Acid) |
|---|---|---|---|
| **2.42** |  |  | 228 |
| **2.41** |  |  | 200 |
| **2.45** |  |  | 324 |
| **2.43** |  |  | 212 |
| **2.44** |  |  | 226 |

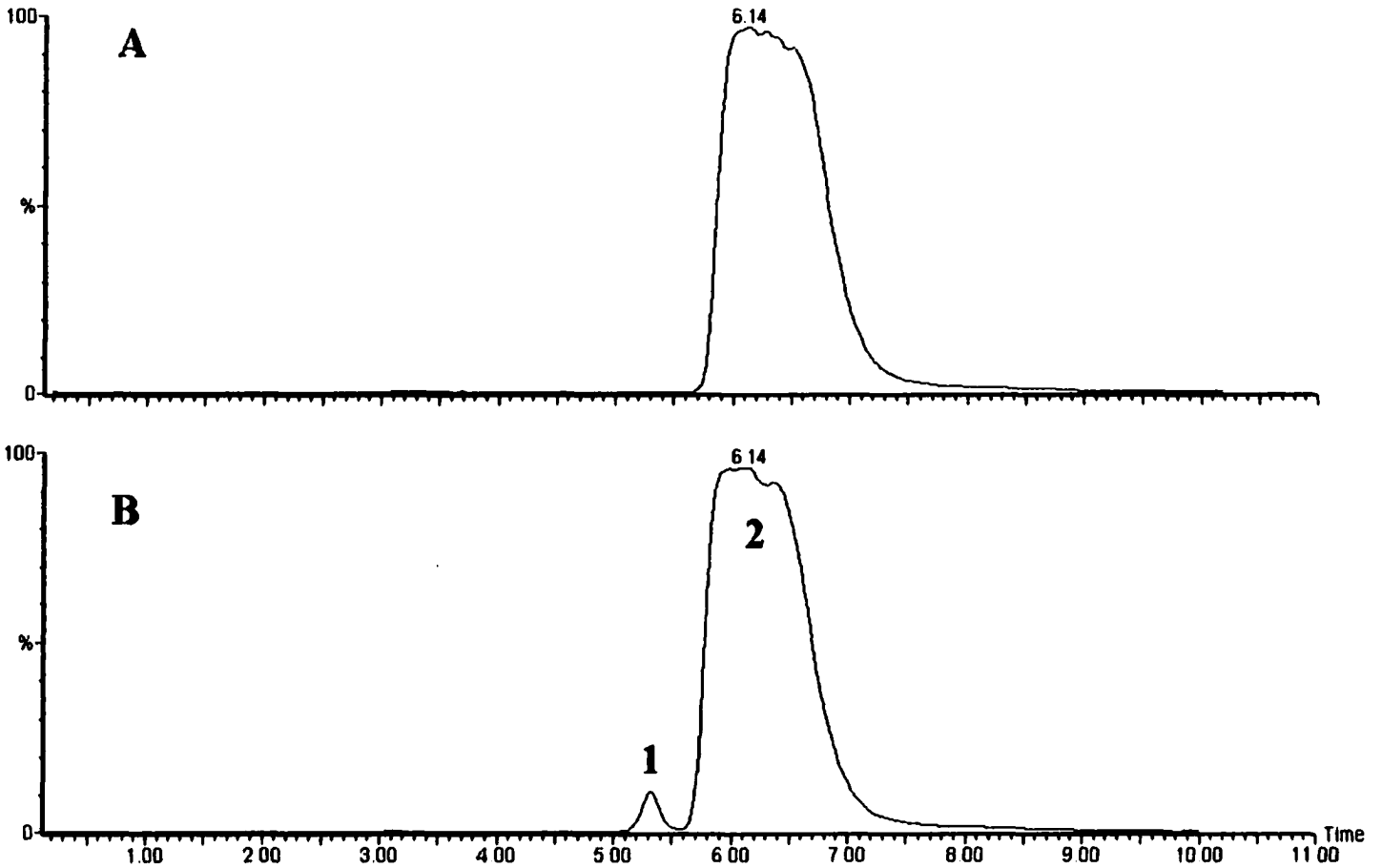



**Figure 3.2**: Selected ion recording of the amino acids in a reaction mixture in which PHE was transaminated with compound **2.41.** The reaction mixture (same as described in the legend to Table 3.5) was incubated for 1h (A) without of (B) in the presence of AT-5. After removal of 50 μL for the determination of phenylpyruvate, the mixture was cooled in a brine/ice bath and subjected to HPLC/MS analysis with selected ion recording of phenylalanine (m/z 166) and conformationally restricted GLU (m/z 228).

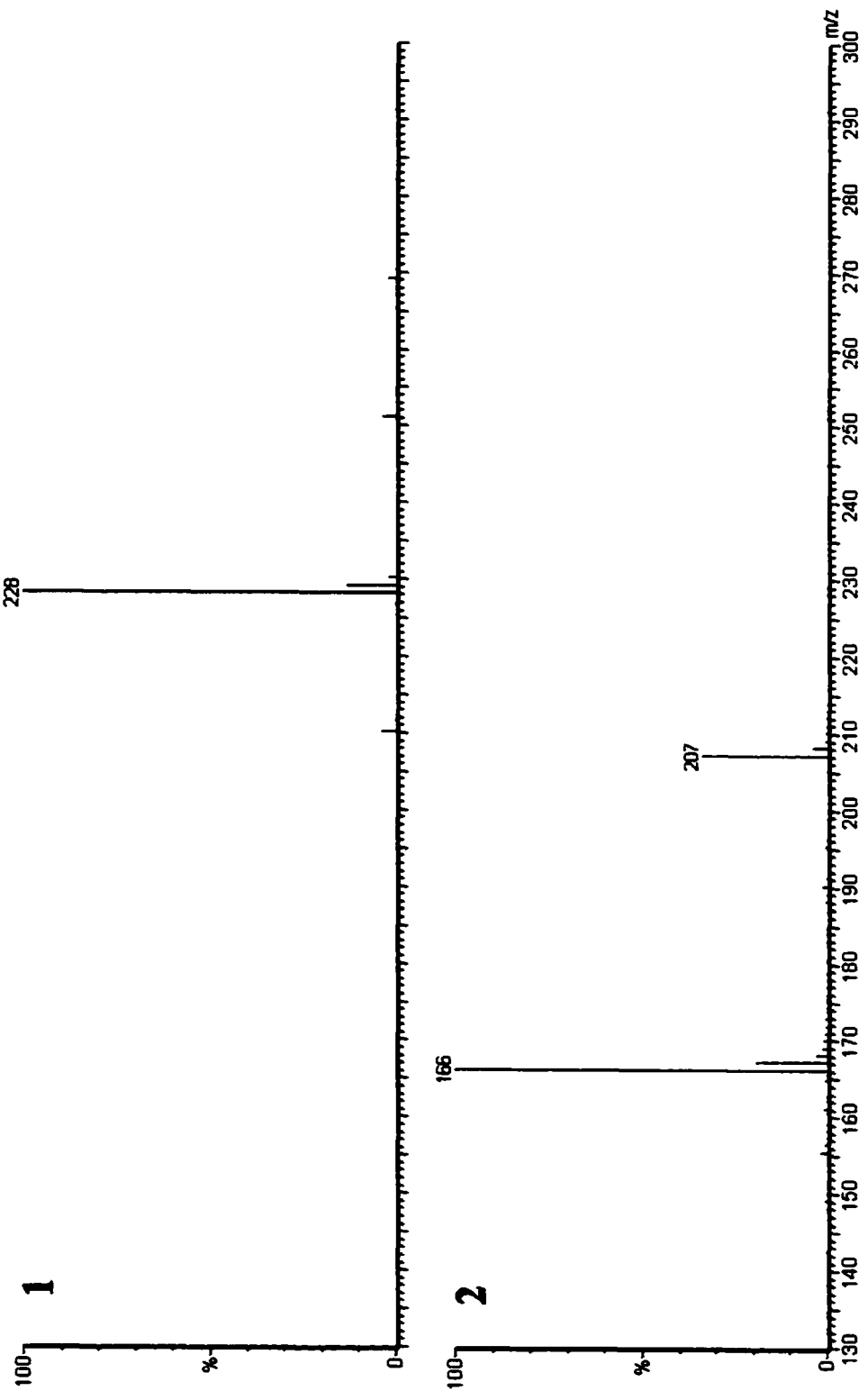



**Figure 3.3**: Mass spectra corresponding to peaks 1 and 2 from Figure 3.2 (207 *m/z corresponds to the* (PHE - H+)(CH3CN) adduct).

*5. Survey of α-Ketoglutarate Analogues as Inhibitors of Dehydrogenases and Aminotransferases*

Some or all of compounds 3-1 - 3-5 were found to be inhibitors of GTK, AspAT, AlaAT or GDH (Table 3.5). Presumably, the compounds can bind non-productively through their carboxyl groups. However, inhibition of GTK, AspAT, AlaAT and GDH by compounds **2.43 – 2.47** is at best moderate. In this regard, it is interesting to note that simple monocarboxylic acids are weak inhibitors of AlaAT (Saier, 1967), and dicarboxylate analogues of αKG are weak inhibitors of AspAT (Velick, 1962).

**Table 3.5**: Inhibition of Various Enzymes by Compounds **2.43 – 2.47**

| Cmpd | Conc. (mM) | GTK | AspAT | AlaAT | GDH |
|---|---|---|---|---|---|
| None | | [100 ± 2] | [100 ± 3] | [100 ± 3] | [100 ± 3] |
| **2.44** | 4 | 96 ± 2 | 98 ± 4 | 75 ± 4[b] | 111 ± 13 |
| **2.43** | 8 | 92 ± 2[b] | 102 ± 3 | 83 ± 4[b] | 104 ± 4 |
| **2.47** | 1 | 93 ± 2[b] | 97 ± 1 | 62 ± 5[b] | 99 ± 6 |
| **2.45** | 20 | 73 ± 1[b] | 77 ± 4[b] | 47 ± 5[b] | 91 ± 2[b] |
| **2.46** | 20 | 64 ± 1[b] | 42 ± 3[b] | 48 ± 2[b] | 40 ± 1[b] |

[a] The assay mixtures contained 10 mM PHE/5 mM αKMB (GTK), 10 mM ASP/10 mM αKG (AspAT), 20 mM ALA/10 mM αKG (AlaAT), and 10 mM αKG (GDH). Details provided in the Experimental Section.
[b] Different from control (no addition) with $p \leq 0.05$; n = 3 or 4.

In addition, the effectiveness of compounds **2.43 – 2.47** to act as inhibitors of KGDHC was tested. None of the αKG analogues were found to inhibit KGDHC when added to the standard assay mixture. Moreover, prior incubation of KGDHC with 2-10 mM αKG analogue for 30 min at 30 °C had no effect on enzyme activity.

## III. Summary

In summary, an exhaustive survey of L-amino acid dehydrogenases and aminotransferases as potential catalysts for the conversion of rigid αKG analogues to rigid GLU analogues was carried out. This search led to the discovery that two enzymes in the Sigma CloneZyme™ kit (AT-1 and AT-5) are capable of catalyzing the conversion of compound 1 to its cognate L-amino acid in the presence of PHE. AT-5 was the more effective of the two and was shown to be capable of catalyzing transamination of all five rigid αKG analogues with PHE or GLU as amino donor.

## IV. Conclusion

The fact that compounds **2.43 – 2.47** are not substrates of major mammalian enzymes that can utilize αKG/GLU turns out to be an important finding in regard to our long-range goals. Because the 3,4-conformationally restricted analogues of α-ketoglutarate (**2.43 – 2.47**) are not substrates of GDH, AspAT or AlaAT, it is unlikely that the corresponding amino acids will be substrates. The compounds will at best be moderate inhibitors of these enzymes. Thus, experiments designed to use the corresponding amino acids as probes for GLU receptor/uptake systems will be unlikely to suffer from complications due to interference with major αKG/GLU-metabolizing enzymes.

The fact that PHE is a good amine donor for AT-5 should prove useful in the isolation of pure rigid GLU analogues. Because GLU and the rigid GLU analogues each have one amino group and two carboxyl groups, separation by ion-exchange chromatography may be difficult. On the other hand, separation of PHE (one carboxyl) from the rigid GLU analogues (two carboxyls) by ion-exchange chromatography should be relatively easy. Moreover, the co-product of the transamination reaction (i.e., phenylpyruvate) is strongly absorbed by charcoal whereas the rigid GLU analogues are not expected to bind strongly.

The present results show that it is possible to use thermostable aminotransferases to bring about transamination of some very bulky α-keto acids. As noted above, GTK is able to catalyze transamination of some remarkably elongated amino acids containing charged groups distal to the amino acid portion that binds to the active site. This finding

suggests that the active site is groove-like and relatively open-ended. However, the present findings suggest that there is a limit to the width (i.e., bulkiness) of the α-keto acid or α-amino acid that can bind within the active site "groove".

To our knowledge, the aminotransferase that catalyzes transamination reactions with the largest substrates is 7, 8-diaminopelargonic acid aminotransferase (Equation 3.2) (Eisenberg, 1971; Stoner, 1975). This aminotransferase, however, is unusual on two counts. First, the "oxo" substrate (i.e. 7-oxo-8-aminopelargonic acid) is not an α-keto acid. Secondly, the amine donor is *S*-adenosyl-L-methionine. This amino acid is obviously much larger than the more common amino donors in transamination reactions (e.g. GLU, ALA, ASP). Moreover, in solution, there is no restriction to rotation at the 3 and 4 positions in *S*-adenosyl-L-methionine. In the present work, we showed that AT-5

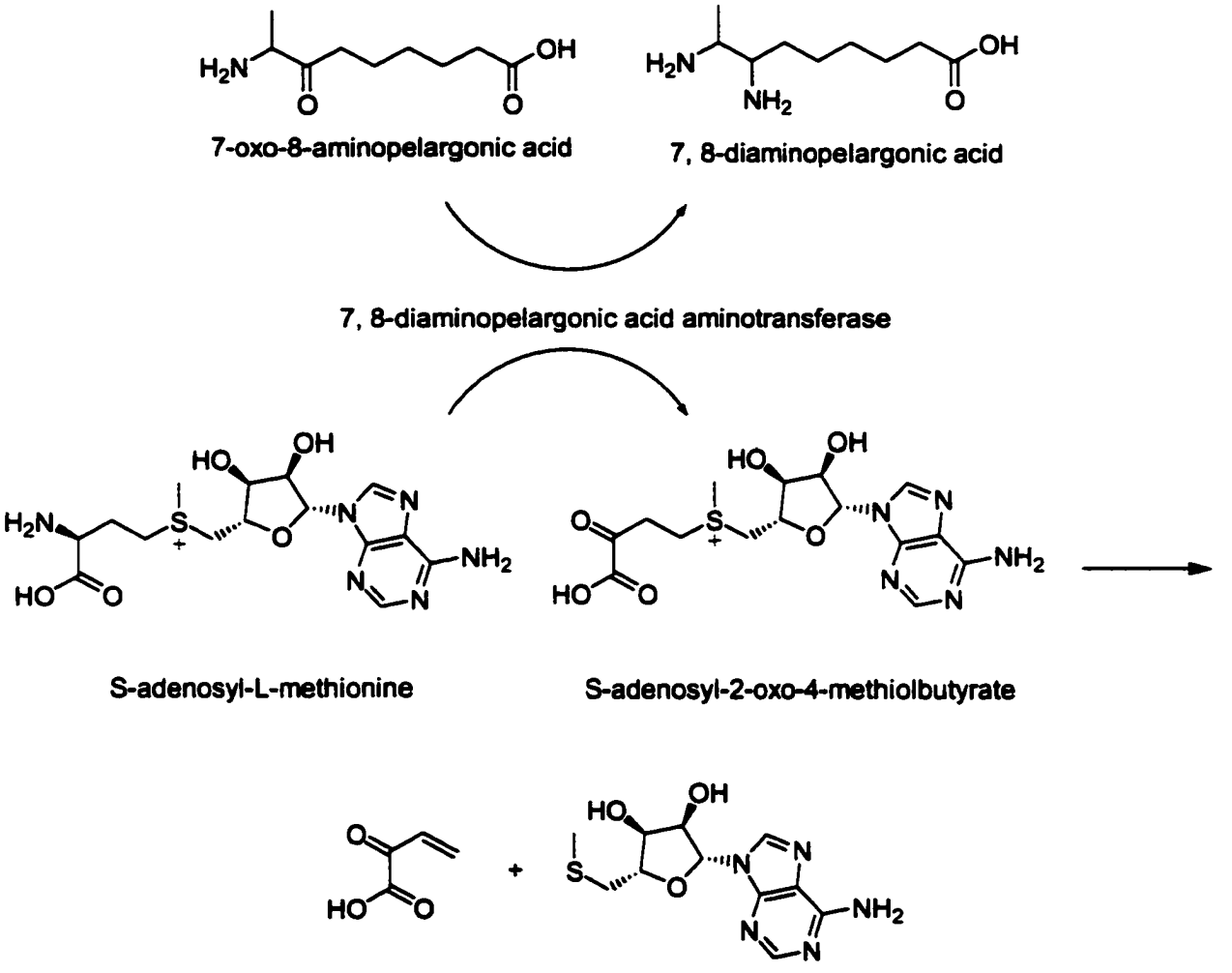



**Figure 3.4.** Transamination of very bulky substrates

is able to catalyze some remarkable transamination reactions with conformationally restricted αKG analogues that contain large, bulky groups at the 3– and 4 carbons. To our knowledge, compound 3.3 is by far the largest α-keto acid with bulky substitutions at the 3- and 4 carbons that has been demonstrated to undergo an enzyme-catalyzed transamination. Our findings indicate that AT-5 must have a large and flexible active site. Thus, the enzyme may be of general utility in the synthesis of bulky amino acids from their corresponding α-keto acids.

AT-5 may also prove useful in other regards. Each of the compounds shown in Table 3.5 is composed of a mixture of stereoisomers. Because of the flexibility of the active site, AT-5 may be able to accommodate all of the stereoisomers at its active site. Thus, the synthesis of a number of diastereoisomers of conformationally restricted glutamates may be possible using AT-5.

## V. Experimental

### *1. Enzymes and Reagents*

Crystalline pig heart cytosolic aspartate aminotransferase (AspAT; 270 U/mg of protein at 37°C; suspension in 2 M ammonium sulfate), crystalline pig heart alanine aminotransferase (AlaAT; 80 U/mg of protein at 37°C; suspension in 2 M ammonium sulfate), beef liver glutamate dehydrogenase (GDH; 50 U/mg of protein at 37°C in 50% glycerol), rabbit muscle lactate dehydrogenase (LDH; type XXXIX; 720/U mg of protein at 37°C in 50% glycerol), mitochondrial pig heart malate dehydrogenase (MDH; 910 U/mg of protein at 37°C in 50% glycerol), pig heart α-ketoglutarate dehydrogenase complex (KGDHC; 0.8 U/mg of protein at 30°C in 50% glycerol), pyridoxal 5'-phosphate (PLP), NADH, ammonium acetate ($NH_4OAc$), ADP, L-aspartate (ASP), L-alanine (ALA), L-phenylalanine (PHE), monosodium L-glutamate (GLU), phenylpyruvate and the sodium salts of α-ketoisocaproate, α-ketoglutarate (αKG) and α-keto-γ-methiolbutyrate (αKMB) were obtained from Sigma Chemical Co. (St. Louis, MO). An aminotransferase library kit (CloneZyme™) was also obtained from Sigma (Warren 1998; 1999; 2000). This kit consists of five unique thermostable aminotransferases (60 to >95% purity) supplied as lyophilized powders together with a solution of 660 mM potassium borate buffer (pH 8.0). Each enzyme (1 mg of protein per vial) was dissolved in 1 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v). For simplicity, the aminotransferases designated as AMN-001-01 through AMN-001-05 by the manufacturer

are referred to as AT-1 through AT-5. AT-5 was also purchased in bulk (500 mg) directly from the Diversa Corporation (San Diego, CA). In preliminary experiments with AT-1 and AT-5, the enzymes were found to be stable in solution for one week at 4°C. However, the two enzymes lost all activity by 2 weeks. Storage at room temperature in the glycerol/phosphate buffer did not improve the stability. L-Leucine dehydrogenase (LeuDH; lyophilized powder with a specific activity of 38 U/mg of protein at 37°C) was obtained from Toyobo Co., LTD., Osaka, Japan. The concentrations of solutions of AT-1 through AT-5 and specific activities of the commercial enzymes were verified by means of standard assay procedures (data not shown). Cytosolic glutamine transaminase K (GTK) of rat kidney was purified essentially as described by Cooper (Cooper, 1978). The preparation has a specific activity at 37 °C of 5 U/mg of protein (PHE – αKMB transaminase assay) (Cooper, 1978). L-Phenylalanine dehydrogenase from *Sporosarcina ureae* (PDH; crystalline suspension in 3 M ammonium sulfate with a specific activity in the direction of PHE oxidation of ~100 U/mg of protein at 25°C (Asano, 1987) was a generous gift from Professor Yasuhisa Asano (Toyama Prefectural University, Japan). A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of one μmol of product per minute at the specified temperature.

*2. Enzyme assays*

To test the αKG analogues as substrates of various L-amino acid dehydrogenases, the α-keto acid was dissolved in a 200-μl reaction mixture containing 100 mM Tris-HCl buffer (pH 8.4), 0.1 mM NADH, 40 mM ammonium sulfate, 0.1 mM ADP and dehydrogenase (1 – 50 μg). The rate of decrease in absorbance at 340 nm due to loss of NADH (ε = 6,230) and concomitant with the reductive amination of α-keto acid was continuously recorded at 37°C. The rates of reductive amination were compared to those obtained with 5 mM standard α-keto acid substrates, namely αKG (for GDH), α-ketoisocaproate (for LeuDH) or phenylpyruvate (for PDH) in place of the αKG analogues. To test the αKG analogues as substrates of LDH, the α-keto acid was dissolved in a 200-μl reaction mixture containing 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM NADH, and LDH (1 – 150 μg). The rate of disappearance of NADH at 21°C was compared to that obtained with a standard assay mixture containing 5 mM pyruvate in place of the αKG analogues. To test the αKG analogues as substrates of GTK the α-keto acid was dissolved in a 50-μl reaction mixture containing 10 mM PHE, 100 mM ammediol (2-amino-2-methyl-1,3-propan-diol) buffer (pH 9.0) and 25 μg of enzyme. After incubation at 37 °C for 1 h, 150 μl of 1 N NaOH was added and the absorbance at 320 nm was determined immediately in a 96-well plate analyzer and compared to that obtained with 5 mM αKMB in place of the αKG analogues. The extinction coefficient of phenylpyruvate-*enolate* under these conditions is 16,000 $cm^{-1}$. In some experiments, the absorbance at 340 nm was determined (extinction coefficient of

phenylpyruvate-*enolate* at this wavelength = 10,400 $cm^{-1}$). To test the αKG analogues as substrates of AspAT, the α-keto acid was dissolved in a 200-µl reaction mixture containing 20 mM ASP, 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM NADH, 1 µg of MDH and 1-50 µg of AspAT. The rate of disappearance of NADH at 21°C was compared to that of a standard assay mixture containing 10 mM αKG in place of the αKG analogues. To test the αKG analogues as substrates of AlaAT, the α-keto acid was dissolved in a 200-µl reaction mixture containing 20 mM ALA, 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM NADH, 1 µg of LDH and 1-50 µg of AlaAT. The rate of disappearance of NADH at 21°C was compared to that of a standard assay mixture containing 10 mM αKG in place of the αKG analogues. To test the αKG analogues as substrates of KGDHC, the standard 200-µl assay mixture (Park, 1999) was used except that αKG was replaced by the α-keto acid analogue. The rate of increase of absorbance at 340 nm due to reduction of $NAD^+$ to NADH at 30°C was compared to that obtained with αKG. For all enzyme assay determinations, blanks lacked enzyme.

In a separate series of experiments, assays were developed for the aminotransferases in the Sigma CloneZyme™ kit as a prerequisite for the search for enzymes within the kit capable of catalyzing transamination of compounds 3-1 - 3-5 (Figures 1 and 2). Although the enzymes in the Sigma kit have high temperature optima (80 °C for AT-1, 2, 3, and 5; 50 °C for AT-4) preliminary experiments were conducted at 45 °C [maximum temperature setting in the well plate analyzer]. The development of the assays (final volume = 50 µl) with either PHE or GLU as amino donor is described in the Results and Discussion section. The product of the transamination of GLU (i. e., αKG)

was quantitated with GDH. To the 50-µl reaction mixture was added 150 µl of a solution containing 0.05 mM NADH, 0.1 mM ADP, 40 mM ammonium sulfate and 10 µg of GDH. The disappearance of NADH due to reductive amination of αKG was monitored at 340 nm at room temperature. The amount of αKG formed in the initial 50-µl reaction mixture was calculated from the Δ absorbance relative to a control reaction mixture lacking thermostable aminotransferase. Reductive amination of αKG was complete in about one minute. The product of the transamination of PHE (i.e., phenylpyruvate) was routinely determined as its *enolate* as described above. However, in some cases, phenylpyruvate was also determined with PDH. In this case, the 50-µl reaction mixture was treated with a 150-µl solution containing 0.05 mM NADH, 40 mM ammonium sulfate and 10 µg of PDH. The disappearance of NADH due to reductive amination of phenylpyruvate was monitored at 340 nm at room temperature. Reductive amination of phenylpyruvate was complete in about one minute. The amount of phenylpyruvate formed in the initial 50-µl reaction mixture was calculated from the Δ absorbance relative to a control reaction mixture lacking thermostable aminotransferase.

Enzyme activities are reported as the mean ± S. E. M.

*3. Apparatus*

Spectrophotometric assays were carried out in either a SpectraMax or VersaMax 96-well plate analyzer (Molecular Devises). High performance liquid chromatography/mass spectroscopy (HPLC/MS) was carried out using a MicroMass LCT mass spectrometer running in the positive ion mode. The HPLC was performed with a Waters 2790 pump on an (R, R) WHELK-0 1 column (5μM, 25 cm X 4.6 mm i.d.) (REGIS Technologies, Inc. Morton Grove, IL, USA), 5 μl injection with a flow rate of 1ml/min (50/50 acetonitrile:water v/v containing 1% v/v formic acid) with post column splitting to allow 180 μl/min of eluent into the MS source. Analytes of interest were observed as the protonated species yielding mass spectral peaks corresponding to $M^{+}+1$.

In many cases, analytes were also observed as the protonated species clustered with acetonitrile yielding mass spectral peaks corresponding to $M^{+} + 42$.

The mass spectrometer settings were: capillary voltage = 2500 V; sample cone = 4 V; extraction cone = 4 V; $R_f$ lens = 150 V; desolvation temperature = 400 °C; source temperature = 150 °C; gas flow (cone) ~ 2 – 10 l/hr; desolvation ~ 650 l/hr.

*4. α-Ketoglutaric Acid Analogues*

The analogues tested were: 6-oxalyl-cyclohex-3-enecarboxylic acid (**2.43**); 3,4-dimethyl-6-oxalyl-cyclohex-3-enecarboxylic acid (**2.44**), 3-oxalyl-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid; and (**2.45**), 3-oxalyl-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid (**2.46**) and 9,10-dihydroanthraceno-3,10-[3,4-(2-oxo)]-glutaric acid (**2.47**). Compounds **2.43 – 2.47** were prepared as described in Chapter 2. Stock solutions of the α-keto acids at 40 mM (compound **2.43**), 20 mM (compound **2.44**), 100 mM (compounds **2.45** and **2.46**) and 5 mM (compound **2.45**) were made up in distilled water and stored frozen at – 20 °C.

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# Chapter 4: Evaluation of *trans*-3, 4-Conformationally Constrained Glutamate Analogues (2.31, 2.32, 2.33, 2.34) as Excitatory Amino Acid Transport Inhibitors

## I. Introduction

The conformationally constrained analogues of glutamate synthesized in Chapter 2 have many useful features in the field of glutamate pharmacology. These compounds are very similar to that of known glutamate transport inhibitors such as 2, 4-PDCs and L-CCGs (Chapter 1). The constrained analogues may prove beneficial in locking the glutamate backbone into a conformation that may interact specifically with one transporter. For example, if a compound is found that is specific for EAAT2, it is possible preferentially block the transport of glutamate through that protein and study the biological implications of this state. A potent and selective inhibitor may give valuable insight into the mechanisms and actions of specific that transporter.

Specifically, the conformationally constrained analogues of glutamate **2.27**, **2.29**, **2.32 - 2.35** (Figure 4.1) were screened as potential inhibitors of the sodium dependent excitatory amino acid transporters 2 (EAAT2) and 3 (EAAT3), the chloride dependent glial cystine/glutamate exchanger system $x_c^-$, and the glutamate vesicular transport system (VGLUT). These transporters are responsible for the regulating of synaptic glutamate concentrations and if a transporter is found that the analogues are specific for, there is high potential for scientific and therapeutic use.

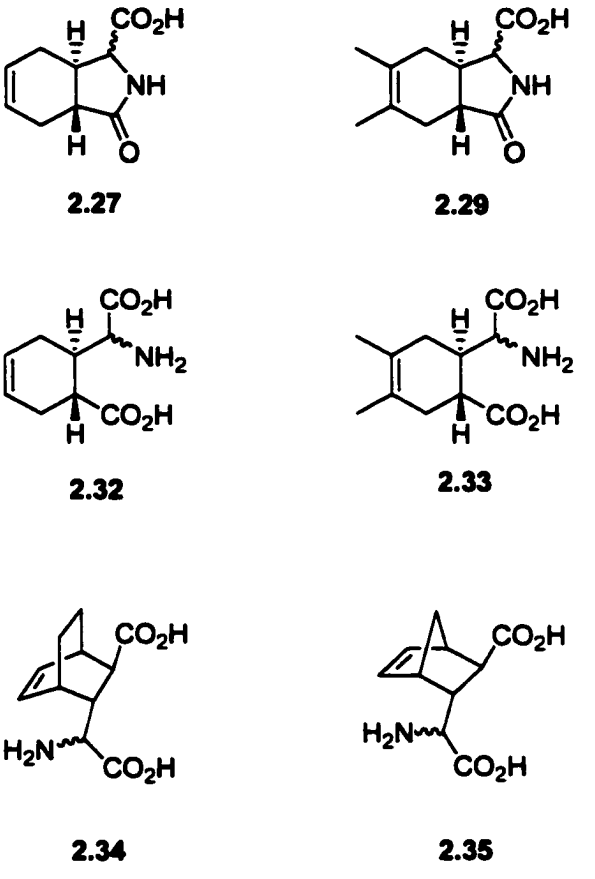


**Figure 4.1**. Glutamate analogues tested as potential transport inhibitors.

In reference to Chapter 1, transport assays have demonstrated that L-*trans*-2,4-PDC acts as a substrate of the excitatory amino acid transporter subtypes EAAT1-4 and as a non-substrate inhibitor of the EAAT5 subtype (Arriza, 1997; 1994; Fairman, 1995; Koch, 1999). Similarly, the individual stereoisomers of the L-CCGs also exhibit interesting activities at these transporters. For example, L-CCG-II and L-CCG-III (Figure 1.4) have both been reported to block the uptake of [$^3$H]-L-glutamate into synaptosomal preparations (Robinson, 1993). More detailed studies using cellular expression systems

have demonstrated that L-CCG-III potently inhibits EAAT1, EAAT2, and EAAT3, while L-CCG-IV preferentially inhibits EAAT2 (Shimamoto, 1998; Yamashita, 1995).

## II. Biological Activity

The well characterized transport inhibitor of EAAT2, dihydrokainate (Figure 4.2; Bridges, 1991) is used as a standard for comparison of inhibition of glutamate transport. The natural substrate for the transporter, L-glutamate is also included to as a standard reference for the inhibition of EAAT3.

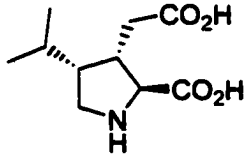


**Figure 4.2**. Dihydrokainate

Testing of the potential inhibitors of EAAT2 was conducted by quantifying the ability of the analogues to attenuate the uptake of [$^3$H]-D-aspartate into synaptosomes prepared from rat forebrain. Under the assay conditions employed, the $K_m$ value for the uptake of [$^3$H]- D -aspartate into the synaptosomes was 4.9 ± 1 μM (Koch, 1999). While EAAT2 is generally considered a glial transporter, previous studies have demonstrated that synaptosomal preparations clearly exhibit a pharmacological profile consistent with EAAT2 (Koch, 1999). As reported in Table 4.1, compounds **2.27**, **2.32**, and **2.34** markedly reduced uptake below Control values. In particular, **2.32** and **2.34** proved to be comparable in activity (i.e., 62% and 65% inhibition, respectively) to dihydrokainate (DHK), a well-characterized selective EAAT2 inhibitor. Further, as these analogues were tested as a stereoisomeric mixture, it is quite possible that the observed activity may reside in a single species that would, consequently, possess considerably greater activity. The

**Table 4.1.** Activity of conformationally constrained glutamate analogs (**2.27, 2.29, 2.32-2.35**) as transport inhibitors

| Compound (conc.) | | Synaptosomal (EAAT2). Uptake of $^3$H-D-Asp (5 μM) | C6 Cellular (EAAT3). Uptake of $^3$H-D-Asp (25 μM) | SNB-19 Cellular ($x_C^-$).Uptake of $^3$H-L-Glu (100 μM) | Synaptic Vesicular (VGLUT). Uptake of $^3$H-D-Glu (250 μM) |
|---|---|---|---|---|---|
| **2.27** | (250 μM) | 53 ± 6 (n =3) | | | 103 ± 2 (n =3) |
| | (500 μM) | | 93 ± 2 (n =3) | 89 ± 7 (n =3) | |
| **2.29** | (250 μM) | 77 ± 7 (n =3) | | | 96 ± 3 (n =3) |
| | (500 μM) | | 97 ± 3 (n =3) | 103 ± 9 (n =3) | |
| **2.32** | (250 μM) | 38 ± 2 (n =3) | | | 85 ± 5 (n =3) |
| | (500μM) | | 95 ± 3 (n =3) | 101 ± 7 (n =3) | |
| **2.33** | (250 μM) | 71 ± 14 (n =3) | | | 99 ± 5 (n =3) |
| | (500 μM) | | 100 ± 1 (n =3) | 104 ± 8 (n =3) | |
| **2.34** | (250 μM) | 70 ± 4 (n =3) | | | 104 ± 4 (n =3) |
| | (500 μM) | | 97 ± 3 (n =3) | 88 ± 5 (n =3) | |
| **2.35** | (250 μM) | 35 ± 5 (n =3) | | | 93 ± 5 (n =3) |
| | (500 μM) | | 87 ± 9 (n =3) | 88 ± 5 (n =3) | |
| **DHK** | (250 μM) | 22 ± 3 (n =3) | - | - | - |
| **L-Glu** | (25 uM) | 28 ± 5 (n ≥ 3) | | | |

Compounds were evaluated at the concentrations indicated as inhibitors of several different glutamate transport systems. Na-dependent uptake of [$^3$H]-D-aspartate into synaptosomes (Koch, 1999) and C6 glioma cells (Palos, 1996) was used to evaluate activity at EAAT2 and EAAT3, respectively. Cl-dependent uptake of [$^3$H]-L-glutamate into SNB19 (Ye, 1999; Martin, 1979) was used to determine activity at system $x_C^-$, while the ATP-dependent uptake of [$^3$H]-L-glutamate by isolated synaptic vesicles (Maito, 1985) was used to quantify activity at the vesicular glutamate transporter. Values shown are reported as mean % of Control ± SEM (n ≥ 3). Dihydrokainate (**DHK**) and L-glutamate (L -Glu) are included as a reference for inhibition of EAAT2 and EAAT3. Control rates of uptake (pmol/min/mg protein) were as follows: synaptosomes, 1827 ± 184 (n = 8); C6 cells, 26 ±1 (n = 8); SNB19 cells, 629 ± 23 (n= 7); synaptic vesicles, 3328 ± 373 (n = 5). All values have been corrected for non-specific uptake.

potential cross reactivity of the analogues was also assessed at: a) EAAT3, by quantifying the uptake of [$^3$H]-D-aspartate into rat C6 glioma cells (Palos, 1996); b) the system $x_c^-$ glutamate/cystine exchanger, by quantifying the uptake of [$^3$H]-L-glutamate into SNB-19 human astrocytoma cells (Ye, 1999; Martin, 1979) and c) the vesicular glutamate transporter, by quantifying the uptake of [$^3$H]-L-glutamate into synaptic vesicles prepared from rat forebrain (Carrigan, 1999; Naito, 1985) To provide an indication of activity at these other systems, the Km values for the respective substrates have been determined to be: 9 ± 2 μM (n = 9) for D-aspartate at EAAT3 in C6 cells, 111 ± 10 μM (n = 4) for L - glutamate at system $x_c^-$ in SNB-19 cell, and 2.8 ± 0.2 mM for L-glutamate at the vesicular transporter in isolated synaptic vesicles. In each instance (Table 4.1), the compounds exhibited little or no activity at these alternative transporters. The activity and selectivity shown by inhibitors **2.32** and **2.35** at EAAT2 suggests a well-defined steric relationship. Analogs **2.32** and **2.35** differ from analogs **2.33** and **2.34** in the incorporation of two methyl groups and insertion of a ring methylene yet **2.33** and **2.34** show significantly lower activity than analogs **2.32** and **2.35**. Molecular modeling studies and the separation of the stereoisomers and testing each one individually will even further refine the pharmacophore model of the EAAT2 transport binding site. For the most selective and potent analogues, the assay mixture contained four stereoisomers. If these were to be tested individually, their potency could be as much as four times what we have seen in the assays. Molecular modeling of the inhibitors against the most potent and selective EAAT2 inhibitor, **DHK**, would also provide good insight into the elements of the molecules required of inhibition at EAAT2. Taken together, these results identify the conformationally restricted glutamate analogs as a new structural class of compounds

that should prove useful in developing novel probes of EAAT2 and help to further delineate a pharmacophore model for the substrate-binding site on the transporter protein.

## III. Experimental

1. *EAAT2 uptake (Koch, 1999)*

$Na^+$-dependent uptake of [$^3$H]-D-aspartate (Dupont NEN, Boston, MA) into synaptosomes was quantified as follows. Synaptosomes were prepared from forebrains of male Sprague-Dawley rats (200-300 g) using a discontinuous Ficoll gradient. The synaptosomal pellet was suspended in assay buffer (see below) at a final concentration of approximately 0.2 mg protein/mL. Uptake of [$^3$H]-D-aspartate through the high-affinity, sodium-dependent EAAT2 was quantified in assay buffer containing 128 mM NaCl, 10 mM glucose, 5 mM KCl, 1.5 mM $NaH_2PO_4$, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 10 mM Tris (pH 7.4). Following a preincubation (5 min, 25 °C), uptake was initiated by the simultaneous addition of [$^3$H]-D-aspartate (final concentration 5 μM) and the conformationally constrained glutamate analogues. Uptake was allowed to proceed for 2 min, after which time the reaction was stopped by the addition of ice-cold assay buffer (6 mL) and rapidly filtered on Whatman GF/F glass fiber filters. Following a rinse with ice-cold buffer (6 mL), the radioactivity retained on the filters was quantified by liquid scintillation counting. All values were corrected for background by subtracting [$^3$H]-D-aspartate accumulated at 4 °C and are reported as mean ± SEM. Previous experiments have demonstrated that under these conditions, uptake is sodium-dependent and linear with respect to both protein content and time. Protein concentrations were determined by the Pierce BCA (bicinchoninic acid) assay.

*2. EAAT3 (Palos, 1996) and system $x_c^-$ uptake (Ye, 1999; Martin, 1979)*

The uptake of (a) [$^3$H]-L-glutamate or (b) [$^3$H]-D-aspartate into cultured cells was quantified as follows. Individual wells, after removal of culture media, were rinsed three times and pre-incubated in 1 mL HEPES buffered (pH 7.4) Hank's balanced salt solution (HBHS) at 30 °C for 5 min. The HBHS was altered by ionic substitution to isolate the $Na^+$ and $Cl^-$ -dependent transport systems. The buffers contained: (a) *$Cl^-$-dependent (EAAT3):* 137.5 mM Choline Cl, 5.36 mM KCl, 0.77 mM $KH_2PO_4$, 0.71 mM $MgSO_4.7H_2O$, 1.1 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES or (b) *total $Na^+$ and $Cl^-$ -dependent (system $x_c^-$):* 137 mM NaCl, 5.1 mM KCl, 0.77 mM $KH_2PO_4$, 0.71 mM $MgSO_4.7H_2O$, 1.1 mM $CaCl_2$, 10 mM D -glucose, 10 mM HEPES and uptake was initiated by aspiration of the preincubation buffer and the addition of a 500 µl aliquot of the appropriate transport buffer containing (a) [$^3$H]-L-glutamate (100 µM) or (b) [$^3$H]-D-aspartate (25 µM). A 500 µl aliquot of transport buffer contained both the (a) [$^3$H]-L-glutamate, (b) [$^3$H]-D-aspartate and conformationally constrained glutamate analogues (50, 500 µM, final concentration) to ensure simultaneous addition. Following a 5 minute incubation at 30 °C, the assays were terminated by three sequential 1 mL washes with ice cold buffer and then the cells were dissolved in 1 mL of 0.4 M NaOH for 24 hours. An aliquot (200 µl) was then transferred into a 5 mL glass scintillation vial and neutralized with the addition of 5 µl glacial acetic acid followed by 3.5 mL scintillation fluid to each sample. Incorporation of radioactivity was quantified by liquid scintillation counting. Values reported are corrected for non-specific uptake by subtracting the amount of radioactive substrate accumulation at 4 °C. Previous experiments have demonstrated that

under these conditions, uptake is sodium-dependent and linear with respect to both protein content and time. Protein concentrations were determined by the Pierce BCA (bicinchoninic acid) assay.

*3. VGLUT uptake (Carrigan, 1999; Naito, 1985)*

Vesicular glutamate transport (VGLUT) was quantified in a pH 7.4 solution containing 5 mM HEPES, 2 mM ATP, 4 mM KCl, 4 mM $MgCl_2$ and [$^3$H]-L-glutamate (0.25-8 mM). The assays were initiated by the addition of [$^3$H]- L -glutamate ± inhibitors (0.01-5 mM) to the synaptic vesicles (total vol. 100 µl, 0.1 mg protein). Uptake was allowed to proceed at 30 °C for 1.5 min, after which the vesicles were collected by vacuum filtration through Millipore HAWP filters. The filters were rinsed twice, after which the retained radioactivity was quantified by liquid scintillation counting. Non-specific uptake was determined in the absence of ATP.

## IV. Collaborators

All of the pharmacological data was collected in collaboration with Todd Seib (EAAT2), Fred Rhoderick (EAAT3), Brady Warren (system $x_c^-$) and Kimberly Cybulski (VGLUT) under the advisement of Dr. Richard J. Bridges, Department of Pharmaceutical Sciences, COBRE Center for Structural and Functional Neuroscience, The University of Montana, Missoula, MT 59812, USA.

## V. Rererences


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## Chapter 5: Conclusions and Future Directions

### I. Conclusions

The goal of this project was to develop a novel synthetic route to conformationally constrained glutamate analogues using a novel dienophile to explore its reactivity in a normal electron demand Diels-Alder sequence. Exploration of the use of a chemical and/or enzyme mediated reductive amination. The goals that were achieved are:

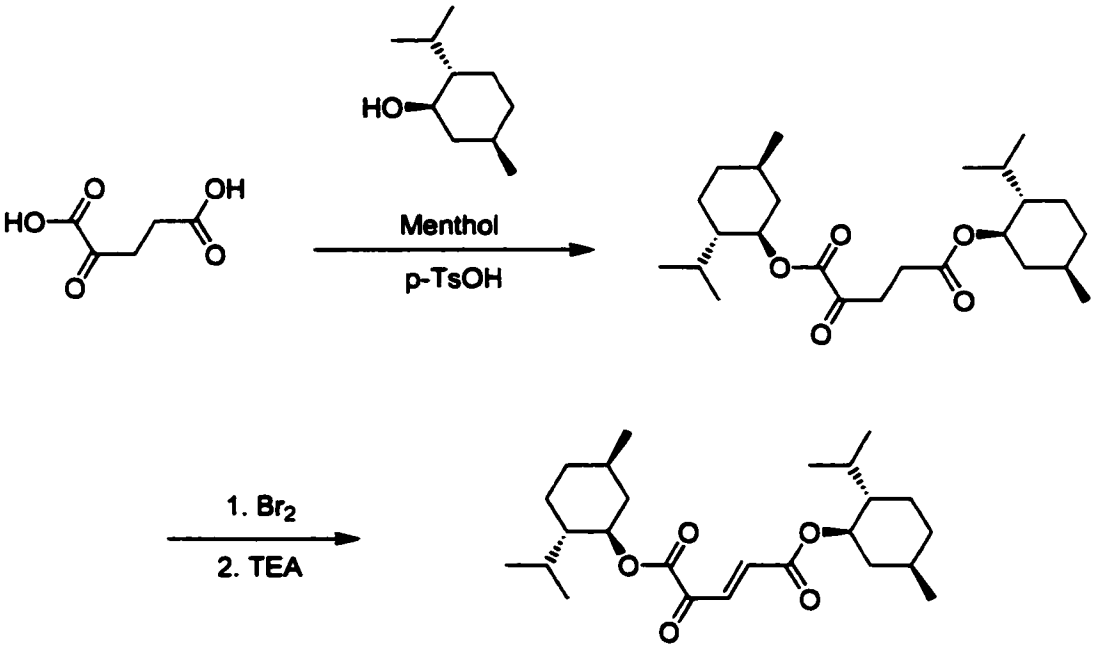
- It was shown that dimethyl 4-oxoglutaconate is a highly reactive dienophile in the normal electron demand Diels-Alder reaction;
- dimethyl 4-oxoglutaconate underwent formal Diels-Alder reactions with azadienes to form heterocyclic glutamate analogues;
- several methods were found that chemically transform the ketone of the Diels-Alder adducts into an amino group;
- the conformationally-constrained analogues of glutamate were synthesized by the reaction of the Diels-Alder adducts with N, N-dimethylhydrazine to form the hydrazones which were subsequently reduced using sodium hydrosulfite
- conformationally-constrained analogues of 2-oxoglutarate were synthesized and a procedure for their purification was developed
- a broad survey of the 2-oxoglutarate analogues as substrates of aminotransferases and dehydrogenases was performed

- an aminotransferase was found that converts the conformationally constrained analogues of 2-oxoglutarate to glutamate analogues
- two conformationally constrained analogues of glutamate were found to be potent and selective inhibitors of EAAT2 and inactive at EAAT3, system $x_c^-$ and VGLUT

## II. Future directions

It is important to expand the versatility of the Diels-Alder reaction with DOG as a dienophile. The resultant Diels-Alder adducts are excellent precursors for the synthesis of conformationally constrained glutamate analogues that are not normally accessible. A broadened panel of Diels-Alder adducts should include more regiodirecting dienes and the interpretation of their reactivity with DOG in both percent yield and regioselectivity.

With the utilization of DOG as the dienophile, the product mixture of the reaction has at least one pair of enantiomers which, in turn, are inseparable by traditional chromatographic techniques. If the synthesis was modified to bear the chiral alcohol menthol in the initial ester formation followed by bromination and dehydrohalogenation, a chiral dienophile will be generated (Figure 5.1). This new dienophile can then be assessed for reactivity and stereoselectivity that goes above and beyond the retention of regiochemistry about the olefin bond. This dienophile has the potential for controlling the enantioselectivity of the Diels-Alder reaction as well as producing a product pool that will contain chromatographically resolvable mixture of stereoisomers. Once resolved, each stereoisomer may be used in the hydrazone formation and reductive amination producing a new resolvable set of amino esters or lactam esters. Further, each resolved amino ester or lactam ester can be saponified and tested as an individual stereoisomer of a conformationally constrained glutamate.




**dimenthol 4-oxoglutaconate**

**DMOG**

**Figure 5.1**: Synthesis of dimenthol 4-oxoglutaconate

With the testing of individual stereoisomers being a very worthwhile cause, there a number of ways in which the stereoisomers can be resolved to obtain optically pure constrained glutamate analogues. For example, there are two stereoisomeric products of the reaction between 2, 3-dimethyl-1, 3-butadiene and DOG (Figure 5.2). Once the hydrazone is formed and reduced to produce the lactams, there are four stereoisomeric products. In either circumstance, it may be possible to transesterify with a chiral alcohol (S-2-butanol, for example) to form chromatographically separable stereoisomers (Figures 5.2 and 5.3).

As is shown in Table 4.1, two stereoisomer mixtures of conformationally constrained glutamate analogues (**2.31** and **2.33**) were shown to be selective and modestly potent inhibitors of EAAT2. Therefore, if the individual stereoisomers can be

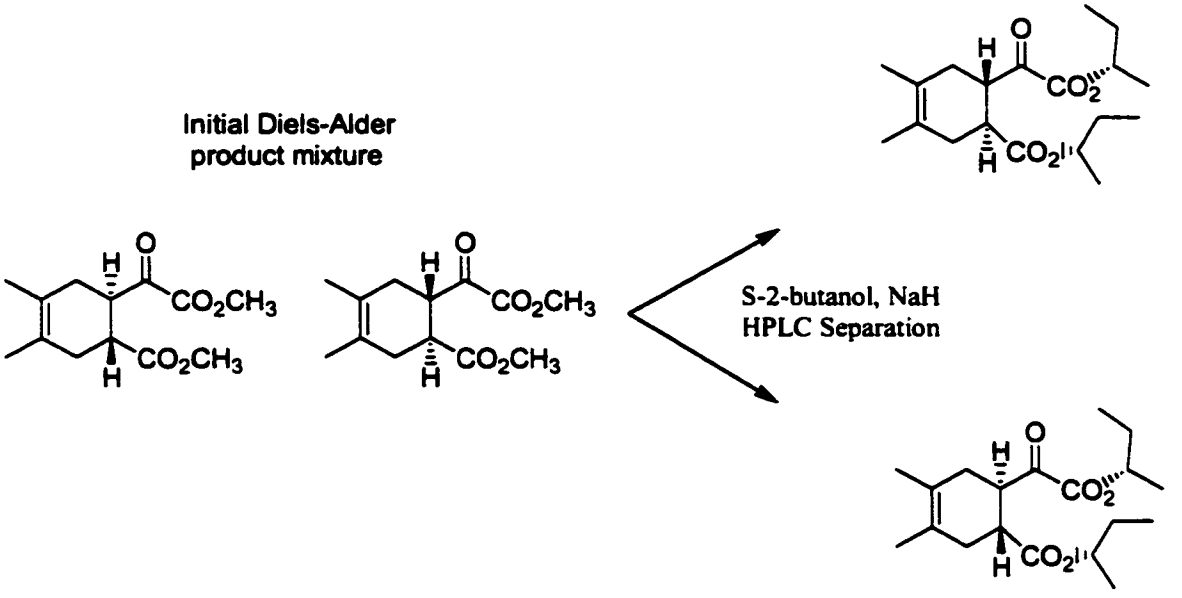


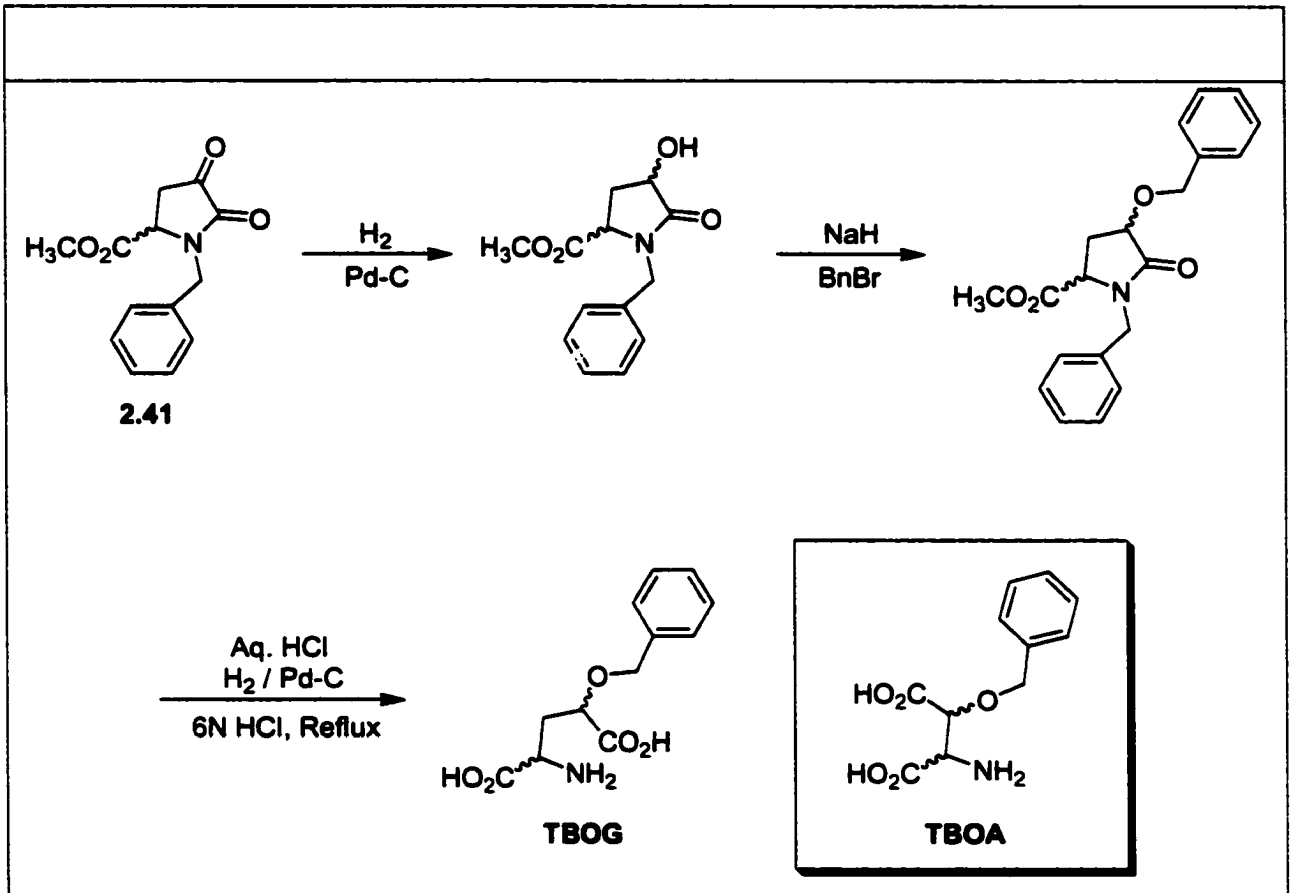

**Figure 5.2**: Functionalization and separation of keto diesters

obtained and tested, there is a distinct possibility that a single stereoisomer will possess considerably greater activity.

The reactivity of the α, β-unsaturated N, N-dimethylhydrazones with DOG to form a new class of conformationally constrained glutamate analogues will also be of great interest. With the distinct regiochemistry of the reaction, the products that can be accessed by the reductive cleavage of the hydrazine bond followed by saponification of the esters are much fewer than that of a reaction under no regiocontrol. This is a very valuable method for the synthesis of a vast number of conformationally constrained

glutamate analogues with minimal synthetic variation, i.e. a large number of analogues can be accessed by simply varying the α, β-unsaturated aldehydes purchased for the synthesis of the α, β-unsaturated N, N-dimethylhydrazones to be used as azadienes.

The initial studies into the synthesis of 4-oxoglutamate (Chapter 2) produced mixed results. The desired target molecule has yet to be synthesized, however, the intermediate structures may be of potential benefit in the study of conformationally constrained glutamate analogues. Figures 5.4 and 5.5 illustrate some potential glutamate analogues that can be accessed from pyrrolidone (**2.41**) formed by the 1, 4-addition of benzylamine to DOG. As depicted in Figure 5.4, the proposed synthesis of the glutamate analogue DL-*threo*-β-benzyloxyglutamate (**TBOG**), which incorporates similar features to the well known competitive, non-transportable inhibitor of glutamate transporters, DL-*threo*-β-benzyloxyaspartate (**TBOA**), is outlined. Another possible addition to the chemistry proposed in figures 5.4 and 5.5 is that the chiral amine R-(+)-α-methylbenzylamine can be used as the nucleophilic addition reagent and the products obtained will possess the potential for chromatographic, stereoisomer resolution. The utility of the synthetic strategy of this thesis has much potential for further exploration in the field of medicinal chemistry. The synthesis of novel analogues of glutamate is of great potential for experimental and therapeutic use in the glutamate neurosystem.




**Figures 5.3**: Proposed synthesis of DL-*threo*-β-benzyloxyglutamate

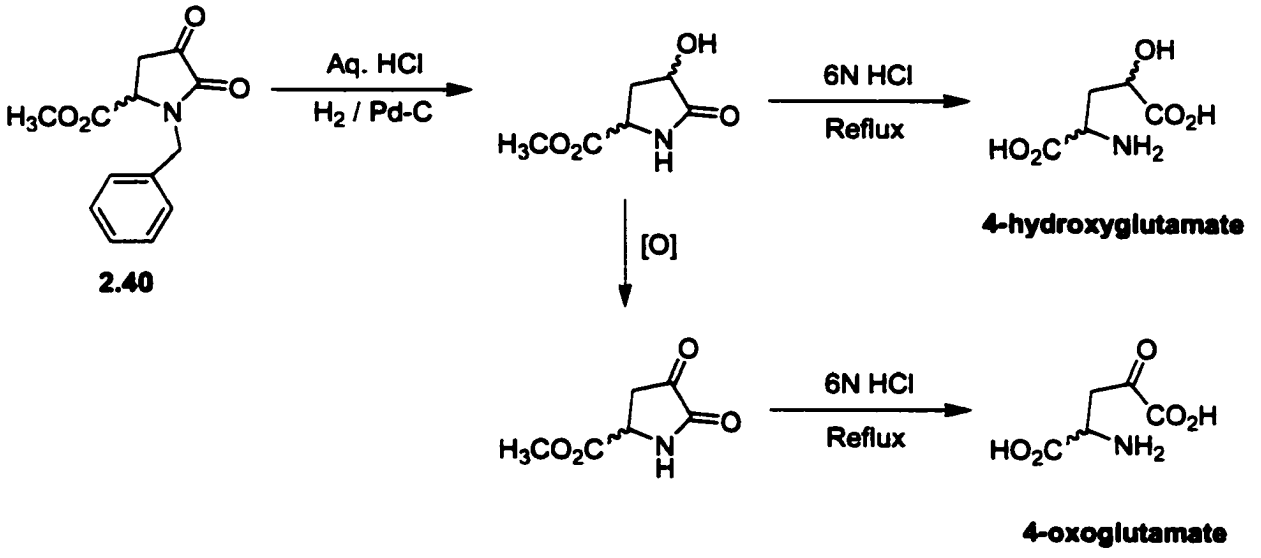



**Figure 5.4**: Proposed synthesis of 4-hydroxyglutamate and 4-oxoglutamate