THESIS

PREPARATION OF AZIDOBREVIANAMIDE A AND SYNTHETIC AND BIOLOGICAL STUDIES OF TETRAZOMINE

Submitted by
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In partial fulfillment of the requirements
for the Degree of Master of Science
Colorado State University
Fort Collins, Colorado
Summer 1995

COLORADO STATE UNIVERSITY

March 30, 1995

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY TRACY N. TIPPIE ENTITLED PREPARATION OF AZIDOBREVIANAMIDE A AND SYNTHETIC AND BIOLOGICAL STUDIES OF TETRAZOMINE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work

Advisor

Department Head

ABSTRACT OF THESIS

PREPARATION OF AZIDOBREVIANAMIDE A

AND

SYNTHETIC AND BIOLOGICAL STUDIES

OF TETRAZOMINE

The regioisomers 5-azidobrevianamide A and 7-azidobrevianamide A were synthesized from natural (+)-brevianamide A. The synthesis involved nitration, reduction to the amine, formation of the diazonium ion, and reaction with azide ion. These compounds might be of potential use as photo-affinity labeling agents for the isolation of a potential enzyme catalyzing a Diels-Alder reaction in the proposed biosynthesis of brevianamide A and B.

Synthetic studies were conducted towards the total synthesis of tetrazomine. A methodology was devised which allows regioselective introduction of nitrogen functionality to the aromatic ring of an isoquinolone system which is an intermediate in a synthetic pathway to the structurally related quinocarcin. The methodology involves the use of a chloride atom as a "blocking" group, followed by regioselective nitration, and simultaneous cleavage of the chloride and reduction of the nitro moiety furnishing the desired amine.

The synthesis of the β-hydroxypipecolinic acid (1 R, 2 R)-1-carboxy-2-hydroxypiperidine was achieved *via* a stereoselective aldol condensation with a chiral glycine template and 4-pentenal. Following reductive ozonolysis the amino acid was generated by a novel one pot transformation in which a protecting group was cleaved,

reductive amination realized and the chiral auxiliary of the template cleaved to furnish the substituted piperidine.

It was found that under anaerobic conditions tetrazomine undergoes a redox self-disproportionation of the oxazolidine moiety in an analogous manner to quinocarcin. The oxidized and reduced products were isolated by HPLC and characterized. It was proposed that under aerobic conditions tetrazomine generates superoxide *via* the proposed disproportionation which could ultimately generate hydroxyl radical by Haber-Weiss/Fenton chemistry. Elucidation of this mechanism provided the first evidence of the relative stereochemical configuration of tetrazomine. It was also shown using a ^{32}P-5'-end labeled synthetic oligionucleotide and high-resolution polyacrylamide gel electrophoresis that tetrazomine is capable of DNA nicking in a non-sequence-specific oxygen dependent fashion characteristic of Fenton-mediated DNA damage.

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Chapter 1

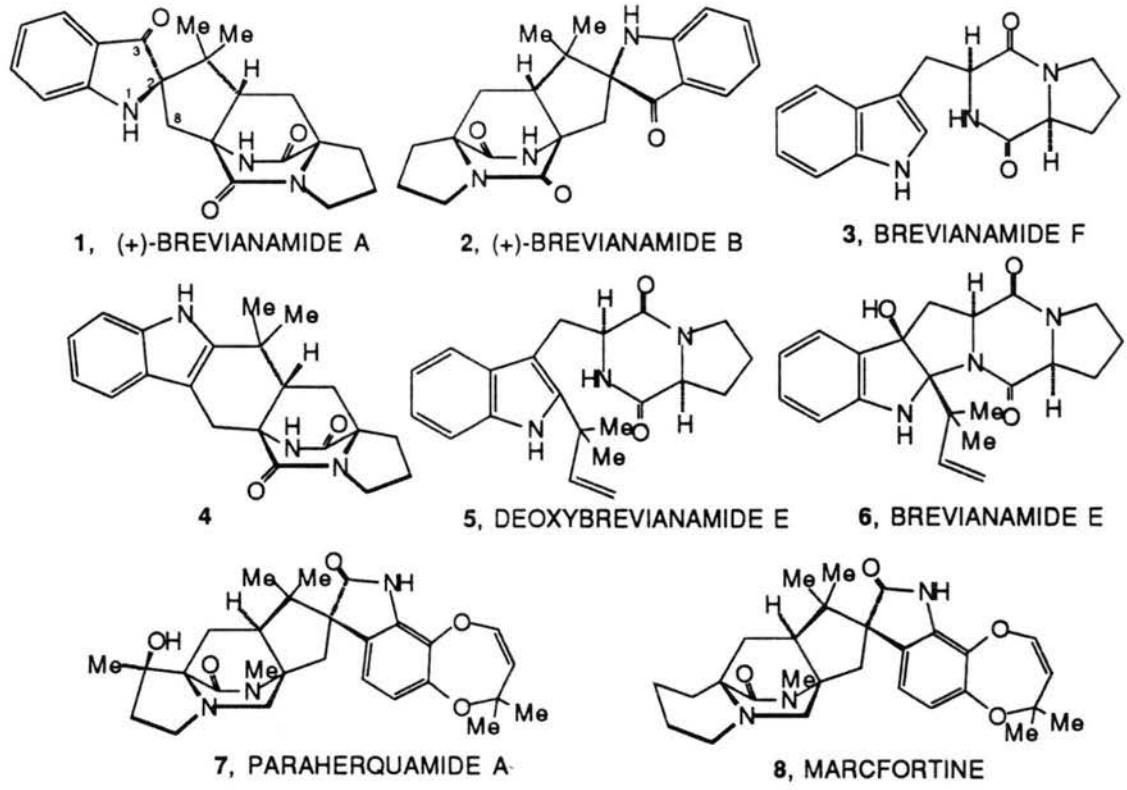
A. Introduction

In 1969 Birch isolated several neutral metabolites from a culture of the fungus *Pencillium brevicompactum* which were subsequently named brevianamides A-E[1]. An additional metabolite was later isolated from the same culture and designated brevianamide F[2]. Brevianamide A, the major metabolite isolated from *P. Brevicompactum*, has since been isolated from the cultures of *P. viridicatum*[3] and *P. ochraceum*[4] as well. The structure of Brevianamide A (**1**), as shown in Figure 1, was proposed by Birch based on spectroscopic analysis, chemical modification, and biogenetic considerations[5]. This structure was confirmed by single x-ray analysis of the 5-bromo derivative[6]. Furthermore, the relative and absolute stereochemistry was also established by the x-ray structure. Further isolation work resulted in the realization that brevianamides C and D were irradiation products of brevianamide A since neither compound was isolated from cultures of *P. brevicompactum* which were grown and isolated in the dark[2].

The brevianamides are members of a class of mycotoxins which include the paraherquamides[7] (**7**, paraherquamide A) and the marcfortines[8] (**8**, marcfortine A). Brevianamide A has shown potent insecticidal and antifeedant properties[9]. Many of the members of the paraherquamide family have displayed considerable anti-parasitic properties[10].

Beyond the biological activity of these compounds the distinctive feature of compounds **1**, **2**, **7**, and **8** is the existence of a bicyclo[2.2.2] ring system. It has been proposed that these structural systems may arise from a [4+2] cylcoaddition reaction[11]. Additionally, such a cycloaddition reaction may occur by enzyme catalysis[12].

Figure 1:



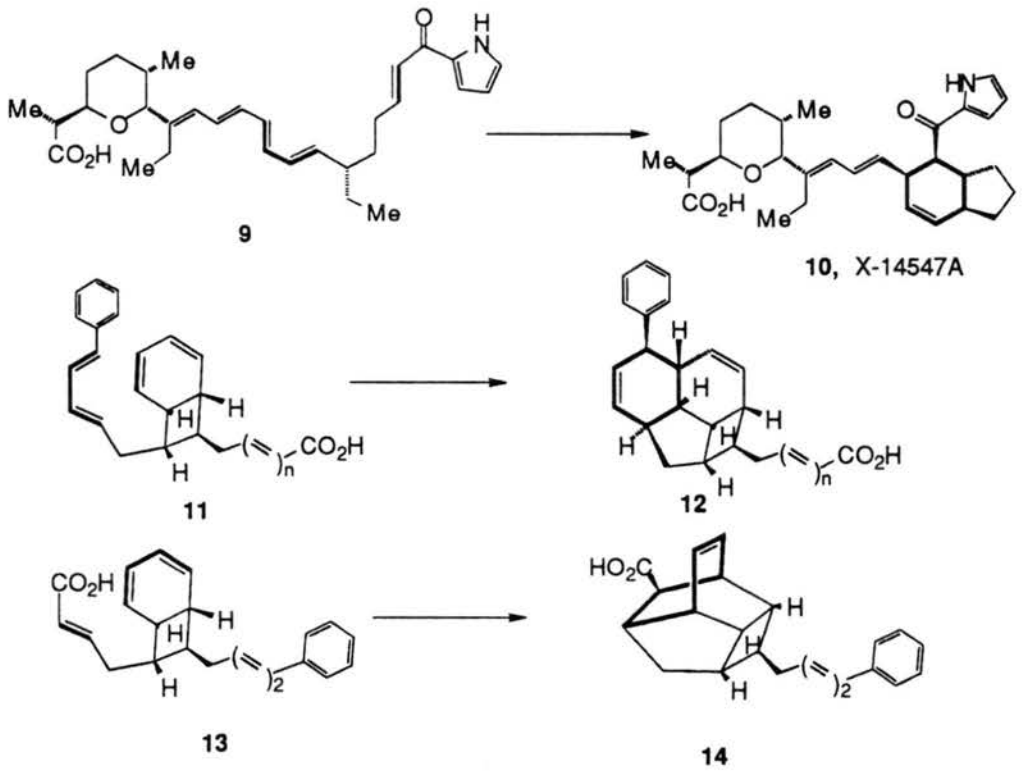
1. Biosynthetic Diels-Alder Reactions

Perhaps the most important ring-forming reaction in all of synthetic organic chemistry is the Diels-Alder reaction[13]. This transformation is a member of the pericyclic reaction class and involves a [4π + 2π] cylcoaddition reaction of a diene and dienophile. Numerous variations of this remarkably stereoselective reaction, including heteroatom variants, have been employed in the synthesis of a myriad of natural products.

However, only a limited number of examples appear in the literature documenting the proposed biosynthetic construction of substrates *via* a Diels-Alder reaction. A few examples appear whose construction is proposed to be a result of either an intramolecular [4+2] cycloaddition reaction or an intermolecular Diels-Alder reaction. Only a few cases have been rigorously proven to involve a Diels-Alder reaction.

Experimental evidence does exist for the construction of some systems by a [4+2] cycloaddition reaction. Rousch has shown that the antibiotic X-14547 A (**10**), was obtained by the spontaneous cyclization of polyene **9**[15]. Under ambient conditions the racemic substrates **11** and **13** cyclized to form the racemic endiadric acids **12** and **14** respectively[16]. It must be noted that in both cases evidence exists only for non-catalyzed [4+2] cycloaddition reactions.

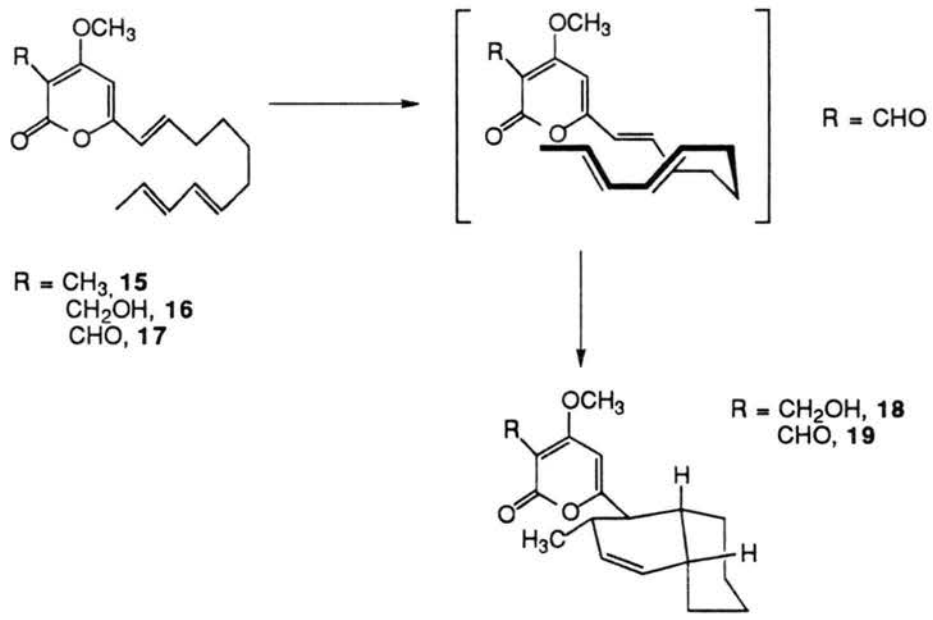
Scheme 1:



Oikawa, Ichihara, and coworkers have provided experimental evidence for an enzyme-catalyzed Diels-Alder reaction[17]. Feeding experiments conducted with *Alternaria solani* have shown that radio-labeled derivatives of the acyclic trienes **15**, **16** and **17** were incorporated into solanapyrone A (**18**) and B (**19**). Furthermore, the formation of the decalin ring system occurs in an enantioselective manner presumably *via* the *exo* transition state. The authors attribute this selectivity to an enzyme-catalyzed Diels-Alder reaction.

However, to date no report details the isolation of a natural enzyme catalyzing a Diels-Alder reaction.

Scheme 2:

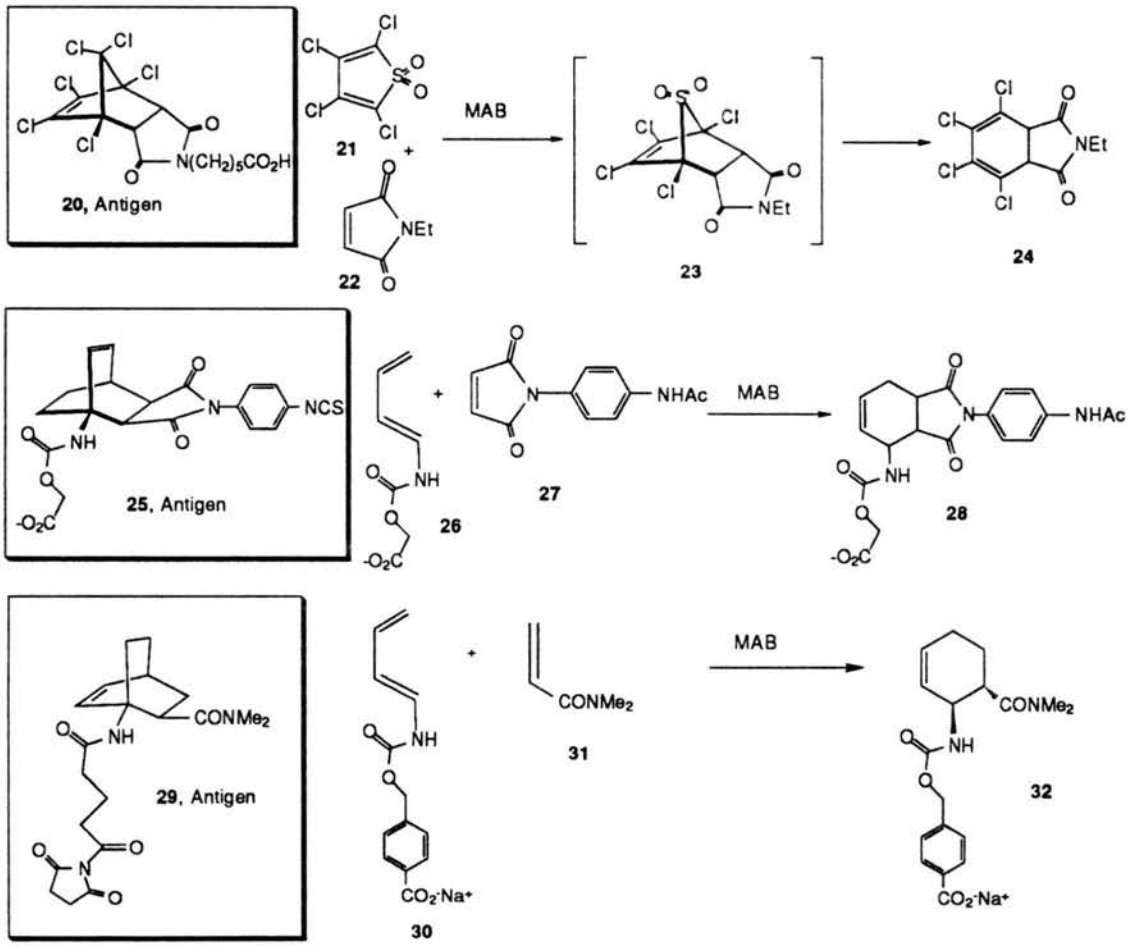


Enzymes normally catalyze transformations by lowering the transition state energy through stabilization of charge and structure[18]. In general, the fleeting transition state structure of an enzyme catalyzed transformation is often quite electronically and structurally dissimilar to either the starting material or product. Thus, catalysis is observed by the enzyme stabilizing the structure and charge of the transition state and the expeditious release of product from the active site of the enzyme. In the case of the Diels-Alder reaction the product, whose structure is quite similar to the highly ordered transition state, would likely bind to the active site inhibiting turnover.

2. Catalytic Antibody Catalyzed Diels-Alder Reactions

Although no enzyme-catalyzed examples are known, several recent examples of protein catalyzed Diels-Alder reactions do appear in the literature. Antibodies, which possess a specific chiral binding pocket have been used to catalyze Diels-Alder reactions. Although no natural proteins have yet been identified in the catalysis of a Diels-Alder reaction catalytic antibodies have been produced which are capable of such catalysis.

Scheme 3:



Employing a catalytic monoclonal antibody (MAB) raised using antigen **20** Hilver catalyzed the intermolecular [4+2] cycloaddition of **21** and **22**[19a]. Upon formation, the

strained [4+2] adduct **23** immediately extruded SO_2. Product **24**, no longer being structurally similar to the transition state, was released from the antibody-combining site.

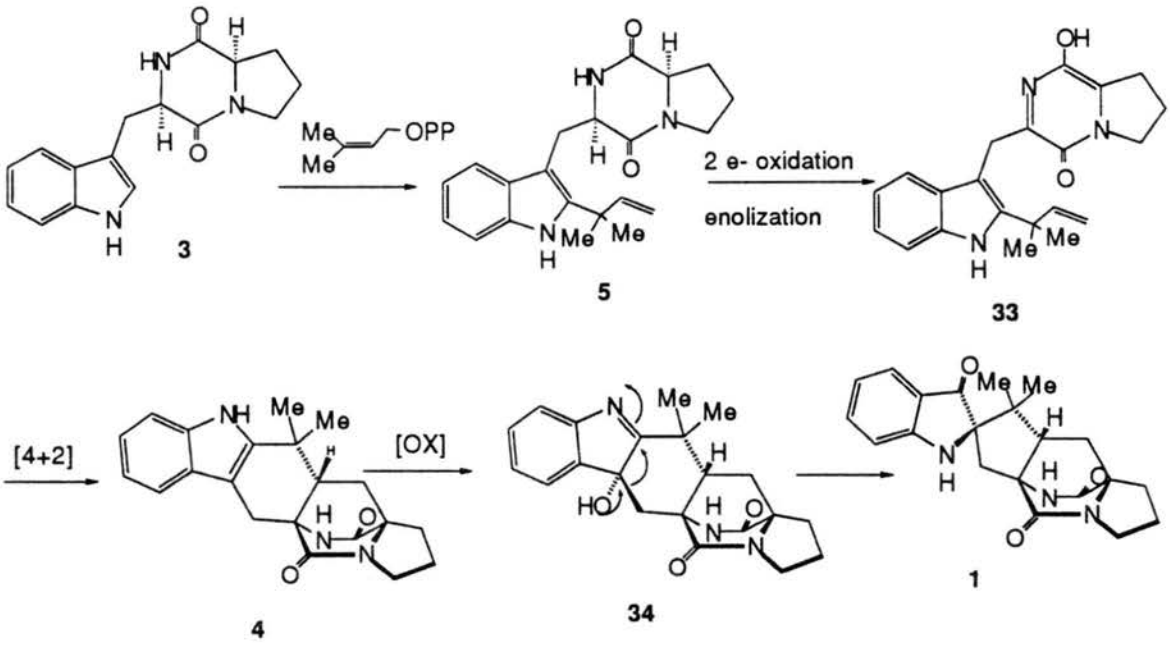
Shultz catalyzed a [4+2] cycloaddition reaction by raising monoclonal antibodies from antigen **25** which resembles the boat-like geometry of the transition state[19b]. However, once formed the [4+2] adduct **28** undergoes a significant change in geometry resulting in its release from the antibody-combining site.

Most recently a catalytic antibody was shown to catalyze an unfavorable Diels-Alder reaction[19c]. The cycloaddition of **30** with **31** under non-catalyzed reaction conditions gave an 85:15 mixture of *endo* (*cis*) and *exo* (*trans*) **32** products. However, when **29**, which mimics the transition state of the *exo* cylcoaddition, was employed to generate antibodies the catalyzed cycloaddition reaction yielded exclusively product **32.**

3. Biosynthesis of Brevianamide A and B

In 1970 Sammes initially proposed the possibility that the piperazine-dione moiety of Brevianamide A (**1**) might be formed by a [4+2] cycloaddition reaction between the isopentyl and pyranizone units[11]. Soon thereafter, Birch found that [^{3}H]tryptophan and [^{3}H]proline were biosynthetically incorporated into brevianamide A (**1**) and brevianamide F (**3**) and that [15-^{3}H,-^{14}C]brevianamide F also was incorporated into brevianamide A by *P. Brevicompactum*[20]. Based on this evidence Birch proposed a biosynthetic pathway for the synthesis of brevianamide A involving a unique intramolecular [4+2] cycloaddition reaction. Brevianamide F (**3**), obtained from the condensation of proline and tryptophan could be prenylated with dimethylallyl pyrophosphate to yield deoxybrevianamide E (**5**). Following two electron oxidation and enolization of the tryptophanyl moiety of **5** to yield achiral **33** the bicyclo [2.2.2] dioxopiperazine **4** could arise from an intramolecular [4+2] cycloaddition reaction. The hexacyclic indole **4** could then undergo oxidation to yield **34** followed by a pinacol-type rearrangement furnishing **1**.

Scheme 4:

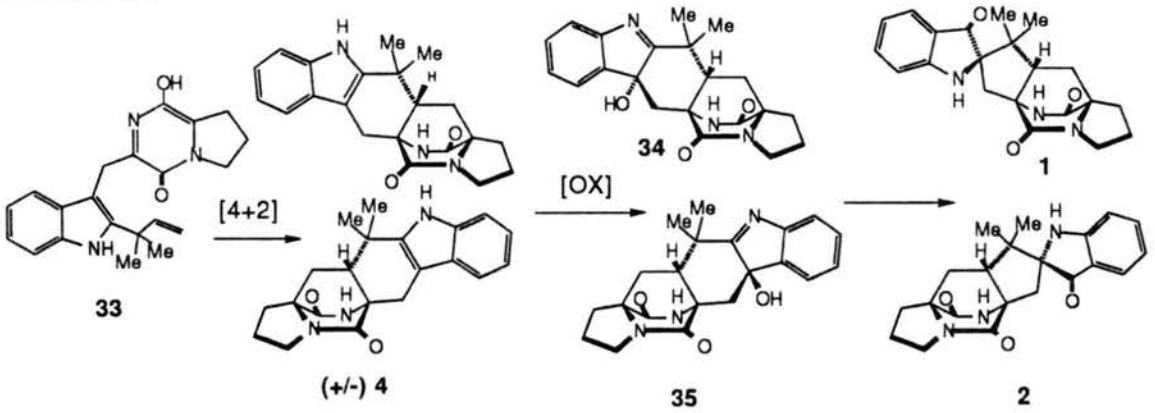


Several years later the William's group successfully synthesized (-)-brevianamide B[21]. Comparison of the synthetic material to the natural product revealed that they were enantiomers. Where Birch's incorrectly proposed structure for (+)-brevianamide B shared the same stereochemistry as that of (+)-brevianamide A with respect to the bicyclo [2.2.2] ring system it was elucidated that the ring system of natural (+)-brevianamide B (**2**) was actually enantiomorphic to the bicyclo [2.2.2] ring system of (+)-brevianamide A.

The William's group then modified the proposed biosynthesis to accommodate the synthesis of (+)-brevianamide B (**2**)[22]. From achiral intermediate **33** it is possible that the Diels-Alder cyclization could occur from either the top or bottom face yielding the two enantiomers of **4**. It was proposed that the optically pure indoxyl might be formed by an oxidative enzyme which only delivered oxygen from the pro-R face furnishing **34** and **35** which could then undergo spiro-rearrangement to afford **1** and **2**.

The William's group then prepared several radio-labeled intermediates in the proposed biosynthetic pathway to validate the proposed pathway[23]. A racemic mixture of **4**

Scheme 5:



incorporating a ^{13}C label was not incorporated into either **1** or **2**. Furthermore, **4** was not identified in the fermentation extracts of *P. brevicompactum*. Taken together these results suggested that **4** was not a precursor of brevianamide A or B.

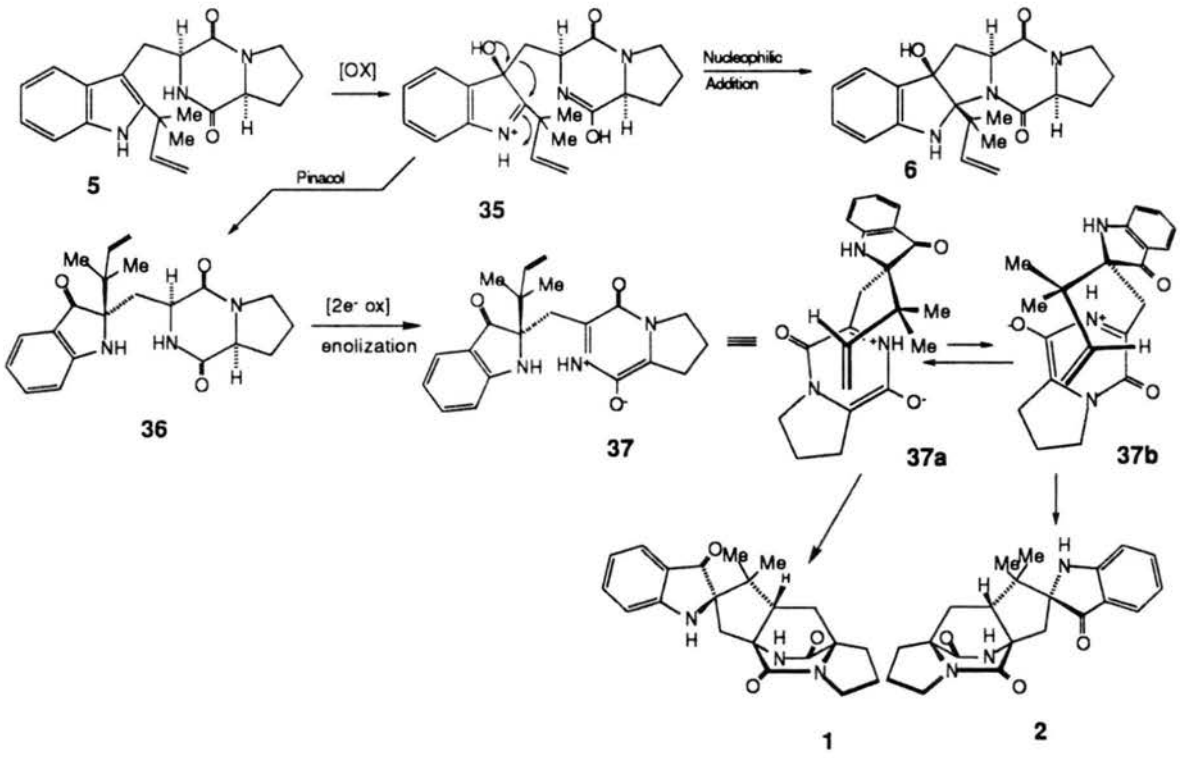
A ^{3}H-labeled deoxybrevianamide E (**5**) substrate when introduced into cultures of *P. Brevicompactum* showed incorporation of the radio-label into **1**, **2** and **6** suggesting it was an intermediate in the biosynthetic pathway of both brevianamide A and B. However, when a ^{3}H-labeled brevianamide E (**6**) was introduced into the cultures no incorporation was observed into either **1** or **2** suggesting that it was not a metabolite involved in the biosynthesis.

Although it had been suggested that brevianamide E (**6**) was merely an artifact of autoxidation of deoxybrevianamide E (**5**) the William's group proposed that it was more likely a dead end in the biosynthetic pathway. They found that deoxybrevianamide E was rather stable and was consistently recovered in both good yield and a constant ratio in comparison to the brevianamide A formed by *P. Brevicompactum.*

Based on these observations an alternative brevianamide biosynthetic pathway was proposed[23]. Unlike previous proposals it was hypothesized that oxidation of the indole nucleus to the indoxyl occurred early in the biosynthesis. Following an R selective oxidation of **5** to **35** it could undergo a pinacol-type rearrangement setting the

stereochemistry at the indoxyl quaternary center of **36** as R. Likewise, **35** could suffer nucleophilic addition *via* the dead end pathway furnishing brevianamide E (**6**). Following

Scheme 6:

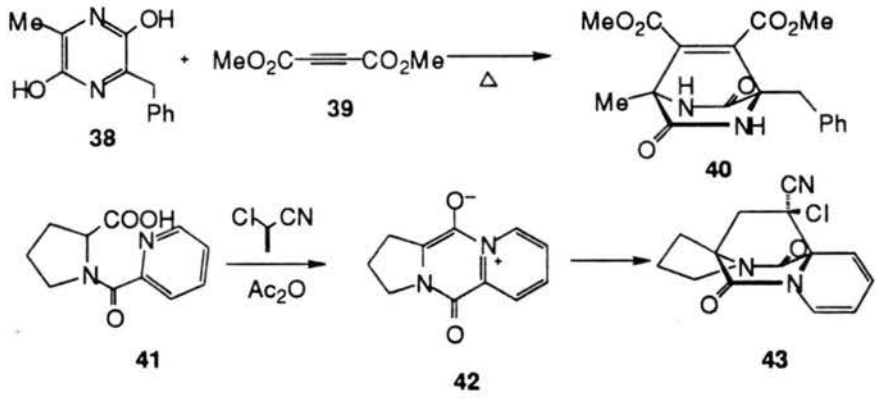


oxidation of the dioxopiperazine portion of **36** to the azadiene **37** it might undergo a intramolecular [4+2] cyclization. The major rotamer **37a** would furnish **1** and the minor rotamer **37b** would furnish **2**.

Their is little precedent for Diels-Alder reactions occurring in dioxopiperazines except when the dienophile is highly electron-deficient. The viability of such a cycloaddition reaction was confirmed by the formation of bicyclic adduct **40** upon heating **38** and the electron deficient dimethyl acetylene-dicarboxylate (**39**) in DMF[11], and the synthesis of **43** by condensation of 1-cyano-1-chloroethylene with **42**[24]. FMO theory predicts that an electron-rich diene, such as a dioxopiperazine, could only effectively interact with a dienophile possessing electron-withdrawing groups; unlike the isolated

electron-neutral vinyl group in the proposed cycloaddition reaction of **37**[23]. Thus, if indeed the brevianamide biosynthetic pathway is that shown it is unlikely that the Diels-Alder reaction would occur spontaneously.

Scheme 7:



Williams has speculated that the preponderance of **1** over **2** might be the result of the affinity of a single enzyme for each conformer or due to the relative activities of two unique enzymes[23]. Molecular mechanics calculations have shown that rotamer **37a** is 1 kcal lower in energy than **37b**[25]. This difference in energy is accounted for by a hydrogen bonding interaction between the indoxyl carbonyl and the amide proton. It is unlikely that if the cycloaddition is catalyzed by a single enzyme that rotation could occur.

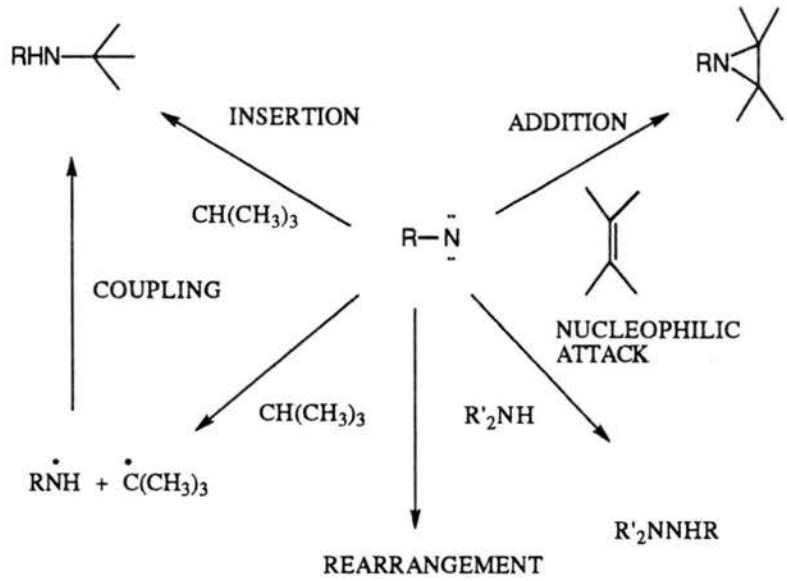
Williams has also speculated that the proposed Diels-Alder reaction may be catalyzed by the oxidase that converts **36** to **37**. If an iron-containing oxidase catalyzed oxidation to **37** it is reasonable that the metal may then coordinate to the amide carbonyl. Formation of **1** and **2** may actually occur as a result of a transition-metal catalyzed Diels-Alder reaction[25].

4. Photo-Affinity Labels

A vast array of important biological processes involve the interaction of a small organic molecule with a biological macromolecule. Hormones interact with their receptors; antibodies interact with their haptens; and enzymes catalyze chemical reactions of specific substrates.

It is often of interest as to how a ligand and receptor interact at the molecular level. Affinity labels have been developed to provide insight into this interaction[26]. Affinity labels achieve this by presenting a receptor with a chemically reactive analog of the natural ligand by which the receptor binding site may be tagged by formation of a covalent bond. To be effective, the chemically modified analog must mimic the natural ligand so as to maintain the specificity of the interaction. Furthermore, the affinity label often incorporates a radioactive label to aid in isolation of the label-receptor complex.

Figure 2:



Nitrenes are commonly used as photo-activated affinity labels[26] and can be generated by the photolysis of aryl and alkyl azides as well as isocyanates. Nitrenes can

couple to macromolecules *via* several mechanisms. Nitrenes are capable of abstraction (normally of a hydrogen from a carbon), cycloaddition, direct insertion (often into a carbon-hydrogen bonds), attack by nucleophiles, and rearrangement. Abstraction reactions followed by radical coupling also yield the insertion product. Direct intermolecular insertion products are rare, but are common in the intramolecular case. In the instance when a nitrene is generated *in situ* at the enzyme active site the intramolecular model is most likely more appropriate. Addition reactions, abstraction-coupling, or direct insertion all may result in a covalent attachment to the label site.

Aryl azides are the most commonly employed sources of nitrenes for photo-affinity labeling for several reasons. Under photolytic conditions acyl azides can smoothly undergo Curtius or Shmidt rearrangements to isocyanates decreasing their labeling efficiency. Aryl nitrenes are much less likely to undergo rearrangement. The nitrene must also be easily generated and be relatively stable to be a useful photo-affinity label. Alkyl azides have absorption maximum at approximately 290 nm, and in some systems it is not possible to effect photolysis without subsequent damage to the receptor. Aryl azides are commonly chemically stable at 37°C and can often be generated by photolysis at wavelengths above 350 nm. Thus, given their stability, ability to generate nitrenes at low energy, and their inertness towards rearrangement aryl azides have been commonly used as photo-affinity agents.

B. Results and Discussion

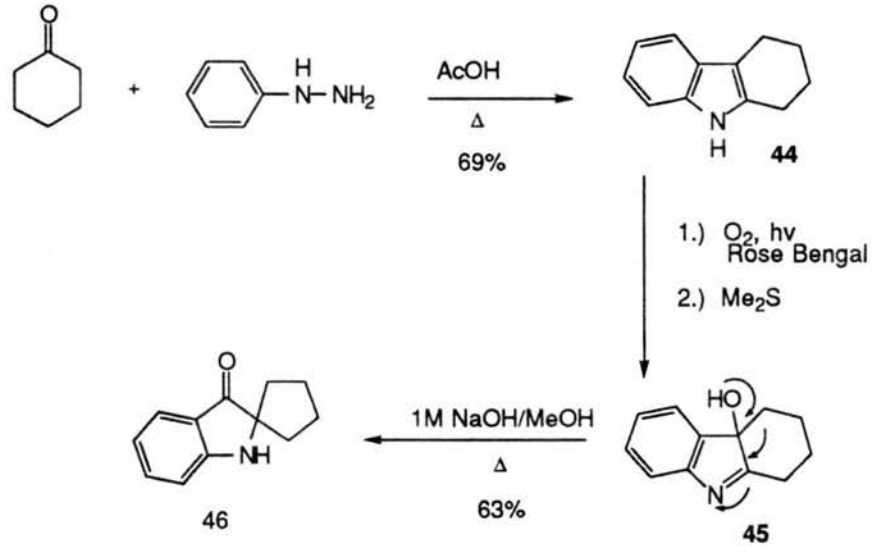
It was thought that the synthesis of an azidobrevianamide A derivative might serve as a photo-affinity label allowing the identification of the proposed enzyme catalyzing the biosynthetic Diels-Alder reaction. Based on the principle of microscopic reversibility it is possible that the proposed Diels-Alder product, brevianamide A, might reversibly bind to the proposed Diels-Alderase. If the enzyme serves solely to catalyze the cycloaddition, the

likelihood of such binding is greater given the probable similarity between the proposed transition state and product. If an azidobrevianamide A derivative could be prepared from brevianamide A, photolysis of this derivative in the presence of a cell-free preparation of *P. Brevicompactum* might covalently label the enzyme. Thus, it was of interest to develop a methodology to synthesize an aryl azide derivative of brevianamide A.

1. Model Studies for the Synthesis of Azidobrevianamide A

Since cultures of *P. Brevicompactum* yield only minute amounts of the brevianamide metabolites it was desirable to optimize reaction conditions using a model system. The spiro-indoxyl compound[27] **46,** which is structurally similar to the aromatic

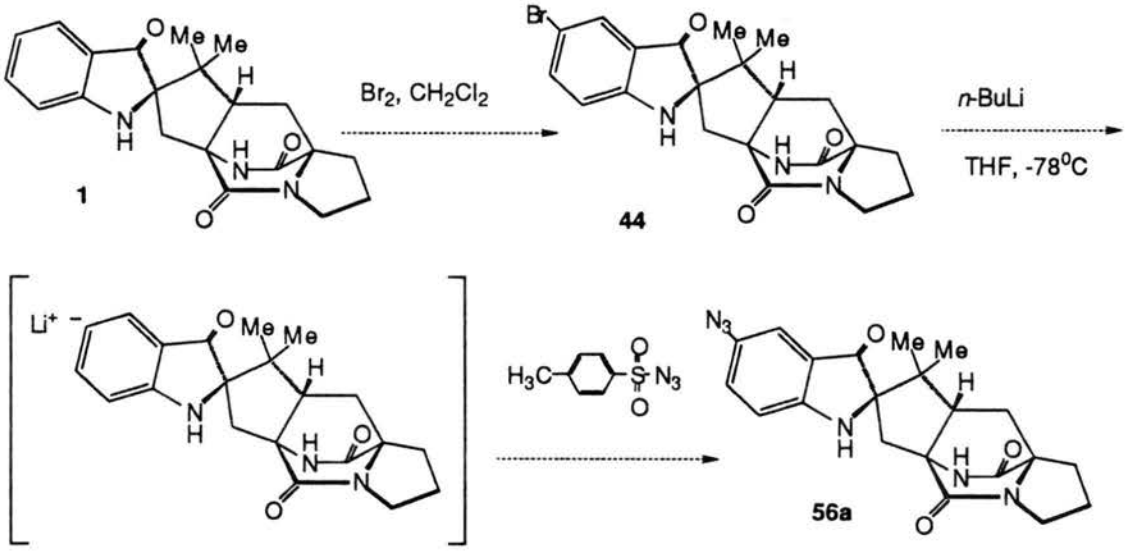
Scheme 8:



portion of brevianamide A, was efficiently prepared in 3 steps and utilized in the model studies. Reaction of cyclohexanone and phenylhydrazine under Fischer indole reaction conditions, as described in the literature[28], yielded 1,2,3,4-tetrahydrocarbazole **44** in good yield. Photolytic oxidation of the carbazole followed by gentle reduction of the peroxide yielded 11-hydroxytetrahydrocarbazolenine **45**. It was crucial that the temperature of the

photolytic oxidation be kept as close to 0°C as possible to maximize the yield of **45**. Substrate **45** was carried on in crude form and following treatment with base underwent a pinacol-type rearrangement yielding the indoxyl **46**.

Scheme 9:

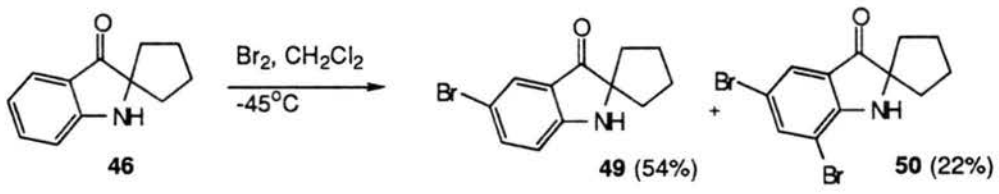


Initially introduction of the azide moiety was examined by reaction of an aromatic anion with trisyl azide. The aryl anion could in theory be obtained by halogen-metal exchange or formation of a Grignard reagent from an aryl halide. In his pioneering studies Birch had brominated the aromatic ring of **1** obtaining a dibrominated product[5]. If reaction conditions were judiciously controlled it might be possible to obtain a monobrominated product such as **44**. When treated with a strong alkyl lithium base **44** might undergo halogen-metal exchange to furnish the aromatic anion which upon addition of tosyl or trisyl azide might furnish **56a**[29]. Furthermore, William's total synthesis of (-)-brevianamide B illustrated the stability of brevianamide analogs to *t*-BuLi[21].

Attention was then turned to the obtainment of a monobrominated indoxyl substrate. The brominated indoxyl system was synthesized by treatment of indoxyl **46** with bromine and the 5-bromo indoxyl product **49** was obtained as well as the

dibrominated product **50**. When the reaction was not monitored by TLC for the disappearance of starting material **50** was obtained almost exclusively.

Scheme 10:

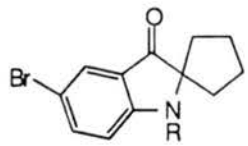


All attempts at formation of the azide product from **49** were unsuccessful whether by formation of the Grignard reagent or halogen-metal exchange. Furthermore, all attempts at inducing halogen-metal exchange were unsuccessful. All reactions of **49** with an alkyl lithium base followed by quenching resulted in exclusive recovery of **49** and no **46** which would be expected if lithium-halogen exchange were occurring. The difficulty encountered might be a result of the fact that halogen-metal exchange would result in the formation of a dianion since the secondary amine likely suffers deprotonation. Once the amine is deprotonated, the aromatic system of the indoxyl might be so electron rich that formation of the aryl anion is disfavored.

The secondary amine of **49** was then protected as the *t*-BOC derivative to circumvent the potential problem of dianion formation. However, no product was observed from initial reactions employing the N-protected indoxyl **45.** This approach was then abandoned for an alternative approach.

Simultaneously with the methodology described above a synthetic strategy involving nitration, reduction to the amine, and conversion to the azide *via* the diazonium salt was investigated. The classic method for the synthesis of azides by this methodology

Table 1:



1.) Reagent 1
2.) Reagent 2

R = H, **49**
R = *t*-BOC, **45**

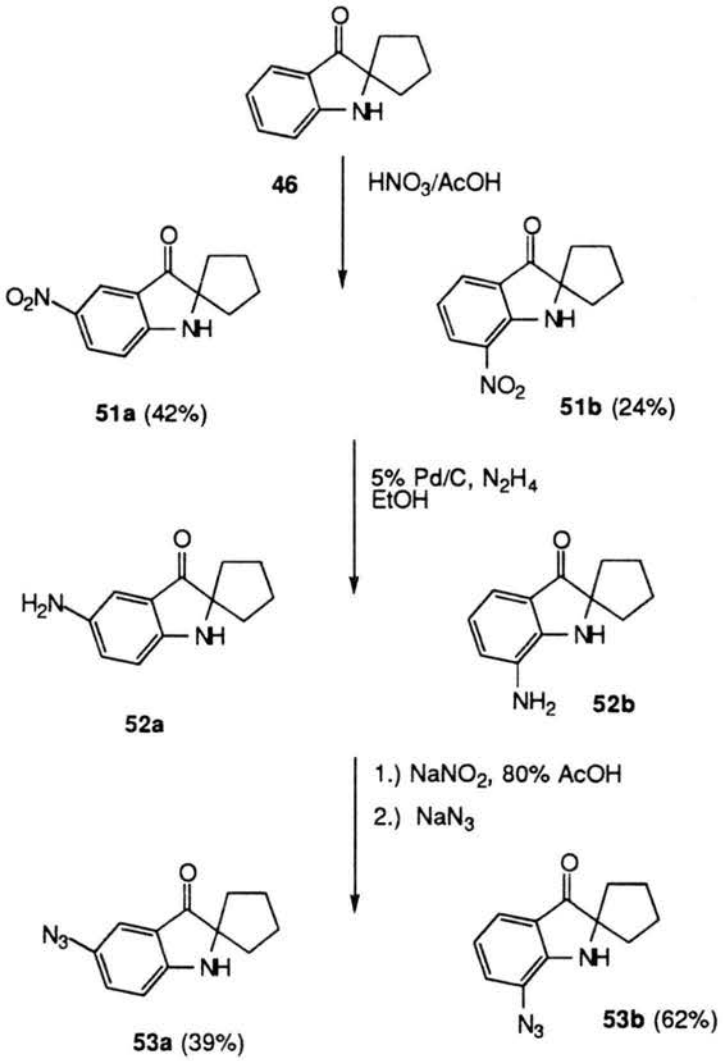
Starting Material	Reagent 1	Reagent 2	Product
1.) **49**	n-BuLi (3eq.) THF, -78°C	Trisyl Azide	Starting Material , **49**
2.) **49**	n-BuLi (3eq.) DMF, KBr, -78°C	Trisyl Azide	Starting Material , **49**
3.) **49**	n-BuLi (4eq.) THF, -78°C	Trisyl Azide	Starting Material , **49**
4.) **49**	n-BuLi (4eq.) THF, -78°C	AcOH	Starting Material , **49**
5.) **45**	Mg/I_2 THF, -78°C	Trisyl Azide	Starting Material , **45**
6.) **45**	sec-BuLi (4 eq.) THF, -78°C	AcOH	Starting Material , **45**
7.) **45**	sec-BuLi (6 eq) THF,TMEDA, -78°C	AcOH	Starting Material , **45**
8.) **45**	sec-BuLi (4 eq.) THF, -100°C	AcOH	Starting Material , **45**

involved harsh acidic conditions such as concentrated sulfuric acid. Previous work had shown that some brevianamide derivatives were unstable to strong acid. However, several alternative methods for such conversions have been published which involve much milder reaction conditions.

Procedures in the literature describe the use of organic acids to generate NO_2^+, the active nitrating agent[30], by protonation and dissociation of nitric acid. Optimal nitration conditions for **46** using nitric acid and the organic solvent acetic acid[31] yielded a mixture

of regioisomers with the 5-nitro indoxyl **51a** predominating over the 7-nitro regioisomer **51b**. This lack of regioselectivity was not considered a problem since the position of the azide on the aromatic ring would not likely affect the labeling ability of the nitrene once generated.

Scheme 11:



Reduction of the nitro group to the amine was then investigated. The facile reduction of the nitro moiety of both **51a** and **51b** to amines **52a** and **52b** was achieved using hydrazine and 5% Pd/C[32]. However, isolation of the amine products by silica gel column chromatography proved to be problematic. Both regioisomers were prone to what

was believed to be oxidation and ultimately were carried on crude to the next step. Although not isolated, both reductions appeared to furnish high yields of both **52a** and **52b**.

To form the azide conversion to the diazonium salt and addition by an azide ion would be necessary. A report in the literature described such conversions with a large variety of indoles using sodium nitrite in 80% AcOH as the diazotizing agent followed by treatment with sodium azide in the same flask to yield the azide[33]. Both amine **52a** and **52b** were smoothly converted to their corresponding diazonium salt and after stirring for 1.5 hours at 0°C were treated with sodium azide to furnish the corresponding azides **53a** and **53b**. Both regioisomers were purified by chromatography, but care was taken to perform the work-up and isolation with limited exposure to light and heat given the inherent photolytic and thermal instability of azides.

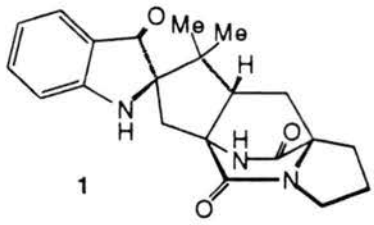
2. Synthesis of 5-Azidobrevianamide A and 7-Azidobrevianamide A

Given the applicability of the synthetic route for introduction of the azide moiety into the model system it was then applied towards the analogous transformation with brevianamide A. Brevianamide A was previously obtained from the cultures of *P. Brevicompactum* which were grown and isolated by extraction and silica gel column chromatography as previously described[21].

Nitration of brevianamide A (**1**) furnished a mixture of regioisomers **54a** and **54b** whose isolated ratio corresponded to that observed in the model study. The nitro regioisomers were purified by silica gel preparative thin layer chromatography. Care was taken to minimize the exposure of the nitro compounds to light during isolation since brevianamide A itself is photolabile.

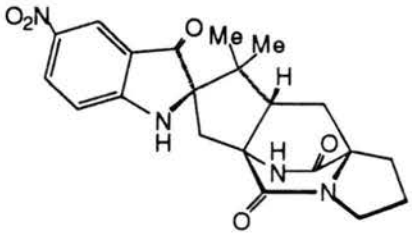
Both **54a** and **54b** were individually reduced to their corresponding amines **55a** and **55b** in an analogous fashion as with the model system. Purification was not attempted

Scheme 12:

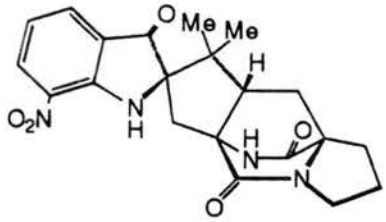


1

HNO_3, AcOH

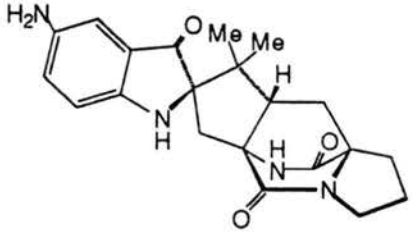


54a (53%)

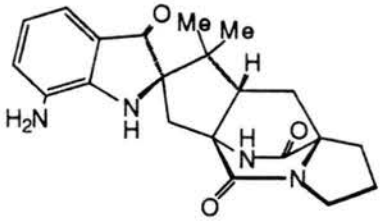


54b (23%)

N_2H_4, 5% Pd/C

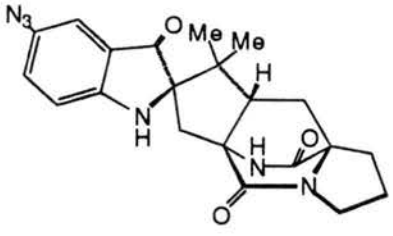


55a

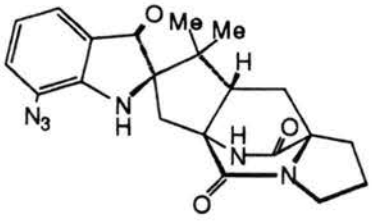


55b

1.) $NaNO_2$, 80% AcOH
2.) NaN_3



56a (60%)



56b (80%)

with either **55a** or **55b** due to a fear of oxidation. Both amino regioisomers were carried on in their crude form to the final step of the transformation.

The azide regioisomers **56a** and **57a** were obtained using the same reaction conditions as for the model system. Azide **56a** was obtained in a 32% yield for the 3 step transformation from brevianamide A, and **56b** was obtained in an 18% overall yield. Both compounds exhibited an IR stretch in the 2100 cm^{-1} region typical of an azide.

Extreme care was taken to minimize the exposure of the photo-affinity labels to light during work-up and purification. Rigorous purification by preparative thin layer chromatography proved problematic due to the instability of the compounds. For the purpose of biological studies HPLC purification will most likely be a necessity.

3. Conclusion

A strategy has been developed which allows for the introduction of the azide moiety onto the aromatic ring of (+)-brevianamide A. The synthesis of **56a** and **56b** is achieved in 3 short steps, involves limited purification, and is applicable for small-scale transformations.

In theory, the photo-reactivity of aryl azides should allow for the employment of **56a** and **56b** as photo-affinity labels. When photolyzed the aryl azides should form a nitrene which can then form a covalent linkage with another substrate. If in fact an enzyme does catalyze the Diels-Alder reaction as proposed in Scheme 6, and (+)-brevianamide A has an affinity for the active site, then the nitrene might show selectivity in labeling the proposed catalyst.

Ultimately it will be necessary to introduce a radio-label into (+)-brevianamide A to provide a mechanism for isolation of the labeled enzyme. One possible means to achieve this would be feeding of ^{14}C-labeled tryptophan to a culture of *P. Brevicompactum* as described by Birch[20]. Following isolation of the radio-labeled brevianamide A it could

then be converted to the aryl azide following the sequence developed for non-labeled brevianamide A. Introduction of the radio-labeled photo-affinity label into a cell-free extract of *P. Brevicompactum* under conditions of photolysis, separation of the proteins by molecular weight using gel electrophoresis, and analysis of the bands by autoradiography or scintillation counting might provide the enzyme-labeled complex. Thus, application of the synthetic strategy outlined towards the synthesis of a radio-labeled azidobrevianamide A derivative might afford the isolation of the proposed Diels-Alderase.

C. Experimental

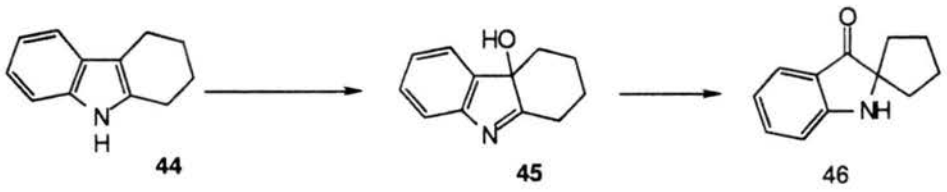
General Methods

^1H NMR spectra were recorded on a Bruker AC 300 MHz NMR and chemical shifts are reported in parts per million relative to TMS. Infared spectra were collected on a Perkin-Elmer 1600 FT IR as a thin film from methylene chloride and are reported in cm^{-1}.

Analytical thin-layer chromatography (TLC) was performed using Merck Kieselgel 60 F 254 alumina plates. Flash chromatography was carried out using Grade 60 230-400 mesh silica gel.

All reagents were commercial grade and were used as supplied with the following exception. Dry methylene chloride was obtained by distillation over CaH_2 and tetrahdyrofuran was freshly distilled from sodium benzophenone ketyl. DMF and HMPA were dried over 4Å molecular sieves.

All reactions were stirred with teflon coated stir bars. The following low temperature baths were used: 0°C (ice/water), -45°C (CCl_4, dry ice), -78°C (acetone, dry ice), -100°C (liquid N_2, dry ice). All glassware was oven-dried. All air-sensitive reactions were performed under an atmosphere of N_2 or Ar.



44 45 46

2,2-tetramethylene-Ψ-indoxyl (46)

Tetrahydrocarbazole **44** (1.4 g, 0.008 mol) was dissolved in MeOH (225 mL) with stirring. The solution was cooled to 0°C, rose bengal (0.002 g) was added, and O_2 was bubbled through the solution. The reaction was photolyzed with a 250 W lamp for 7.5 hours. The reaction was concentrated to approximately 100 mL after which Me_2S (0.66 mL, 0.009 mol) was added and the reaction stirred for 14 hours at 4°C. The reaction was then concentrated and **45** was dissolved in crude form in 0.5 M NaOH (20 mL) and MeOH (5 mL). The reaction was heated for 1.25 hours at reflux, the reaction was allowed to cool, and then quenched with 1 M HCl (10 mL) . The aqueous layers was extracted 4 times with CH_2Cl_2, the organic layers combined, and dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by silica gel column chromatography (100:1 CH_2Cl_2:MeOH) yielding 0.022 g of a yellow crystalline material in 63.2% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.67-1.74 (8 H, m), 4.80 (1 H, bs), 6.78 (2 H, m), 7.40 (1 H, m), 7.59 (1 H, dd, J = 8.42 Hz, J = 1.21 Hz).

IR (NaCl, CH_2Cl_2) 3331, 2915, 1687, 1488, 1284, 1165 cm^{-1}.

46 49

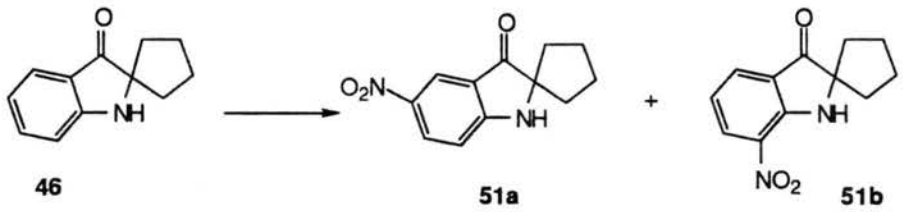
5-bromo-2,2-tetramethylene-Ψ-indoxyl (49)

Indoxyl **46** (0.22 g, 0.001 mol) was dissolved in CH_2Cl_2 and cooled to 0°C. The reaction was cooled to -45°C and Br_2 (2 mL, 0.6 M solution of Br_2 in CH_2Cl_2) was added dropwise by syringe. The reaction was stirred for 20 minutes and then quenched with 1 M sodium thiosulfate. The reaction was extracted 3 times with CH_2Cl_2, the organic

layers combined, washed once with brine, dried over $MgSO_4$, filtered, and concentrated. The crude reaction mixture was purified by silica gel column chromatography (4:1 hexanes:EtOAc) to yield 0.17 g of product in 54.5% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.67-1.74 (8 H, m), 4.78 (1 H, bs), 6.71 (1 H, d, J = 8.60 Hz), 7.47 (1 H, dd, J = 8.61 Hz, J = 2.12 Hz), 7.71 (1 H, d, J = 2.14 Hz).

IR (NaCl, CH_2Cl_2) 3401, 2922, 1704, 1607, 1456, 1172 cm^{-1}.



46 51a 51b

5-nitro-2,2-tetramethylene-Ψ-indoxyl (51a) and 7-nitro-2,2-tetramethylene-Ψ-indoxyl (51b)

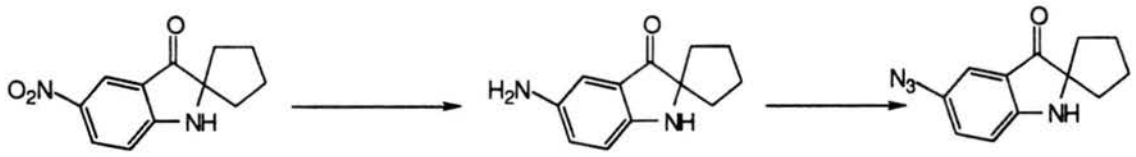
Indoxyl **46** (0.10g, 0.54 mmol) was dissolved in a solution of HNO_3 (3 mL) and AcOH (7 mL) at room temperature. The reaction was stirred for 2 hours after which it was quenched with saturated K_2CO_3. The reaction mixture was extracted 5 times with CH_2Cl_2, the organic layers combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction mixture was purified by preparative thin layer chromatography (100:1 CH_2Cl_2:MeOH) to yield 0.031 g of the 7-nitro indoxyl (**51b**) in 24.3% yield and 0.053 g of the 5-nitro indoxyl (**51a**) in 41.7% yield.

51a: ^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.91-2.16 (8 H, m), 5.73(1 H, bs), 6.77 (1 H, d, J = 8.81 Hz), 8.27(1 H, dd, J = 8.92 Hz, J = 2.32 Hz), 7.28 (1 H, d, J = 2.18 Hz).

IR (NaCl, CH_2Cl_2) 3381, 2957, 1728, 1608, 1532, 1443, 1343, 1267, 1216, 1129, 757 cm^{-1}.

51b: ^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.77-2.07 (8 H, m), 5.28(1 H, bs), 6.77 (1 H, t, J = 8.84 Hz), 7.82 (1 H, dd, J = 8.82 Hz, J = 2.02 Hz), 8.22 (1 H, dd, J = 8.14 Hz, J = 2.18 Hz).

IR (NaCl, CH_2Cl_2) 3381, 2943 1667, 1608, 1343, 1267, 1129 cm^{-1}.



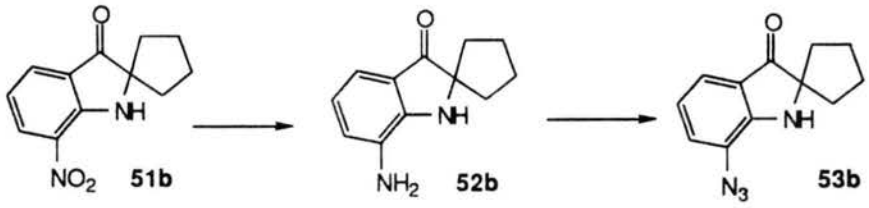
51a **52a** **53a**

5-azido-2,2-tetramethylene-Ψ-indoxyl (53a)

To a solution of **51a** (0.053 g, 0.0002 mol) in EtOH (12 mL) was added 5% Pd/C (0.105 g) with stirring. An excess of hydrazine (0.6 mL) was added to the reaction *via* syringe and the reaction was stirred for 14 hours under N_2. The reaction was filtered through a plug of celite and concentrated. Product **52a** was dissolved in crude form in 80% AcOH (8 mL) and cooled to 0°C under N_2. A solution of $NaNO_2$ (0.179 g, 0.003 mol, 10 eq.) in H_2O (0.75 mL) was added to the reaction vessel in a dropwise fashion *via* syringe. The reaction stirred for 1 hour at 0°C after which NaN_3 (0.169 g, 0.003 mol, 10 eq.) dissolved in H_2O (0.75mL) was added to the reaction vessel in a dropwise fashion *via* syringe and stirred for an additional 1.5 hours. The reaction was diluted with H_2O, extracted 4 times with CH_2Cl_2, the organic layers combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by silica gel preparative thin layer chromatography (100:1 CH_2Cl_2:MeOH) to yield 0.049 g of product in 39% yield for the 2 steps.

^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.88-2.16 (8 H, m), 6.79 (1 H, d, J = 8.96 Hz), 7.06 (1 H, dd, J = 8.69 Hz, J = 2.40 Hz), 7.28 (1 H, d, J = 2.38 Hz).

IR (NaCl, CH_2Cl_2) 3332, 2957, 2113, 1681, 1489 cm^{-1}.

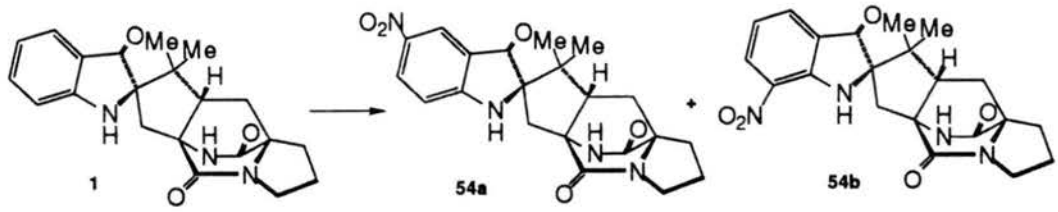


7-azido-2,2-tetramethylene-Ψ-indoxyl (53b)

To a solution of **51b** (0.024 g, 0.0001 mol) in EtOH (10 mL) was added 5% Pd/C (0.025 g) with stirring. An excess of hydrazine (0.2 mL) was added to the reaction *via* syringe and the reaction was stirred for 10 hours under an atmosphere of N_2. The reaction was filtered through a plug of celite and concentrated. Product **52b** was dissolved in crude form in 80% AcOH (3 mL) and cooled to 0°C under N_2. A solution of $NaNO_2$ (0.034 g, 0.0005 mol, 10 eq.) in H_2O (0.5 mL) was added to the reaction vessel in a dropwise fashion *via* syringe. The reaction stirred for 1 hour at 0°C after which NaN_3 (0.032 g, 0.0005 mol, 10 eq.) dissolved in H_2O (0.5 mL) was added to the reaction vessel in a dropwise fashion *via* syringe and stirred for an additional 1.5 hours. The reaction was diluted with H_2O, extracted 4 times with CH_2Cl_2, the organic layers combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by silica gel preparative thin layer chromatography (100:1 CH_2Cl_2:MeOH) to yield 0.008 g of product in 62% yield for the 2 steps.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.85-2.03 (8 H, m), 5.27(1 H, bs), 6.83 (1 H, t, J = 7.67 Hz), 7.18 (1 H, dd, J = 7.61 Hz, J = 1.09 Hz), 7.37 (1 H, dd, J = 7.90 Hz, J = 0.78 Hz).

IR (NaCl, CH_2Cl_2) 3302, 2910, 2115, 1692, 1490, 1283, 1256, 1174 cm^{-1}.



5-nitrobrevianamide A (54a) and 7-nitrobrevianamide A (54b)

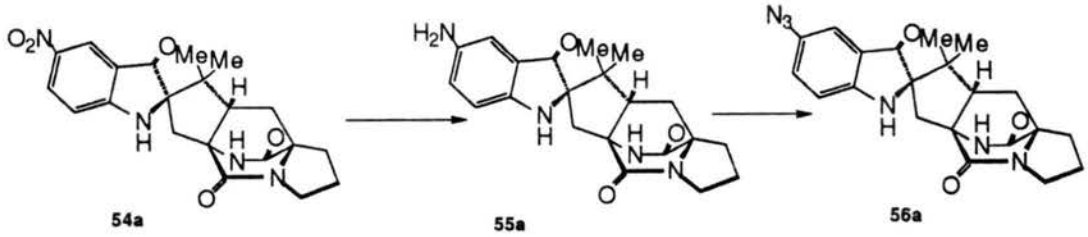
Brevianamide A (**1**) (0.019 g, 0.052 mmol) was dissolved in a solution of HNO_3 (1 mL) and AcOH (4 mL) at room temperature. The reaction was stirred for 4 hours in the dark after which it was quenched with saturated K_2CO_3. The reaction mixture was extracted 4 times with CH_2Cl_2, the organic layers combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction mixture was purified by preparative thin layer chromatography (15:1 CH_2Cl_2:MeOH) to yield 0.005 g of **54b** in 23.0% yield and 0.011 g of **54a** in 52.7% yield.

54a: ^{1}H NMR (300 MHz, d_6-Acetone vs TMS) δ 0.92 (3 H, s), 1.13 (3 H, s), 1.82-2.02 (4 H, m), 2.50 (1 H, 1/2 AB q, J = 15.62 Hz), 2.59-2.66 (3 H, m), 2.70 (2 H, m), 2.79 (1 H, 1/2 AB q, J = 15.61 Hz), 3.33 (3 H, m), 7.00 (1 H, d, J = 8.79 Hz), 7.81 (1 H, bs), 8.27 (2 H, m), 8.45 (1 H, bs).

IR (NaCl, CH_2Cl_2) 3269, 2926, 1682, 1664, 1493, 1399, 1286, 1177 cm^{-1}.

54b: ^{1}H NMR (300 MHz, d_6-Acetone vs TMS) δ 0.88 (3 H, s), 1.12 (3 H, s), 1.80-1.93 (4 H, m), 2.45 (1 H, 1/2 AB q, J = 15.62 Hz), 2.58 (2 H, m), 2.85 (1 H, 1/2 AB q, J = 16.2 Hz), 3.35-3.41 (3 H, m), 6.92 (1 H, t, J = 8.30 Hz), 7.88 (1 H, dd, J = 8.28, J = 1.14 Hz), 8.33 (1 H, dd, J = 8.26 Hz, J = 1.16 Hz).

IR (NaCl, CH_2Cl_2) 3258, 2925, 1682, 1676, 1619, 1505, 1447, 1283, 1101, 805 cm^{-1}.

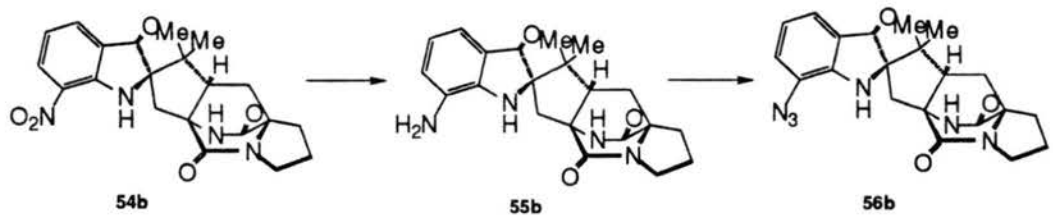


5-azidobrevianamide A (56a)

To a solution of **54a** (0.018 g, 0.043 mmol) in EtOH (5 mL) was added 5% Pd/C (0.050 g) with stirring. An excess of hydrazine (0.2 mL) was added to the reaction *via* syringe and the reaction was stirred for 22 hours under N_2. The reaction was filtered through a plug of celite and concentrated. Product **55a** was dissolved in crude form in 80% AcOH (4 mL) and cooled to 0°C under an atmosphere of N_2. A solution of $NaNO_2$ (0.029 g, 0.0004 mol, 10 eq.) in H_2O (0.5 mL) was added to the reaction vessel in a dropwise fashion *via* syringe. The reaction stirred for 2 hour at 0°C after which NaN_3 (0.028 g, 0.0004 mol, 10 eq.) dissolved in H_2O (0.5 mL) was added to the reaction vessel in a dropwise fashion *via* syringe and stirred for an additional 2.5 hours. The reaction was diluted with H_2O, extracted 4 times with CH_2Cl_2, the organic layers combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by silica gel preparative thin layer chromatography (15:1 CH_2Cl_2:MeOH) to yield 0.011 g of product in 60% yield for the 2 steps.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 0.90 (3 H, s), 1.10 (3 H, s), 1.86 (3 H, m), 2.37 (1 H, 1/2 AB q, J = 15.77 Hz), 2.40 (1 H, m), 2.70 (2 H, m), 2.78 (1 H, 1/2 AB q, J = 15.45 Hz), 3.46 (3 H, m), 6.81 (1 H, d, J = 8.65 Hz), 7.09-7.16 (2 H, m).

IR (NaCl, CH_2Cl_2) 3274, 2921, 2115, 1692, 1676, 1492, 1399, 1284 cm^{-1}.



7-azidobrevianamide A (56b)

To a solution of **54b** (0.009 g, 0.022 mmol) in EtOH (3 mL) was added 5% Pd/C (0.025 g) with stirring. An excess of hydrazine (0.2 mL) was added to the reaction *via* syringe and the reaction was stirred for 24 hours under N_2. The reaction was filtered through a plug of celite and concentrated. Product **55b** was dissolved in crude form in 80% AcOH (3 mL) and cooled to 0°C under N_2. A solution of $NaNO_2$ (0.015 g, 0.22 mmol, 10 eq.) in H_2O (0.5 mL) was added to the reaction vessel in a dropwise fashion *via* syringe. The reaction stirred for 1.5 hour at 0°C after which NaN_3 (0.015 g, 0.22 mmol, 10 eq.) dissolved in H_2O (0.5 mL) was added to the reaction vessel in a dropwise fashion *via* syringe and stirred for an additional 3 hours. The reaction was diluted with H_2O, extracted 4 times with CH_2Cl_2, the organic layers combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by silica gel preparative thin layer chromatography (15:1 CH_2Cl_2:MeOH) to yield 0.016 g of product in 80.2% yield for the 2 steps.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 0.88 (3 H, s), 1.02 (3 H, s), 1.78-1.191 (3 H, m), 2.28 (1 H, 1/2 AB q, J = 16.84 Hz), 2.28 (1 H, m), 2.72 (2 H, m), 2.78 (1 H, 1/2 AB q, J = 15.91 Hz), 3.43-3.62 (3 H, m), 6.79 (1 H, t, J = 7.75 Hz), 7.18 (1 H, dd, J = 7.80 Hz, J = 1.5 Hz), 7.33 (1 H, dd, J = 7.88 Hz, J = 0.9 Hz).

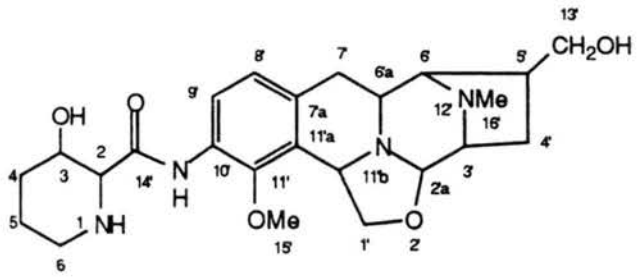
IR (NaCl, CH_2Cl_2) 3258, 2925, 2114, 1682, 1676, 1619, 1504, 1447, 1398, 1301, 1101 cm^{-1}.

Chapter 2

A. Introduction

Tetrazomine, a new alkaloid anti-tumor antibiotic, is a natural secondary metabolite that was recently isolated by the Yamanouchi Pharmaceutical Co. from the culture broth of *Saccharothrix mutabilis* subsp. *chichijiimaensis* .[34] Tetrazomine has shown broad activity against Gram-positive and Gram-negative bacteria *in vitro*. It has shown *in vitro* anti-tumor activity against P388 leukemia and lymphoid leukemia L1210 at 0.014 and 0.0427 ug/mL, respectively. Tetrazomine has also shown activity against P388 leukemia *in vivo*.

Figure 3:



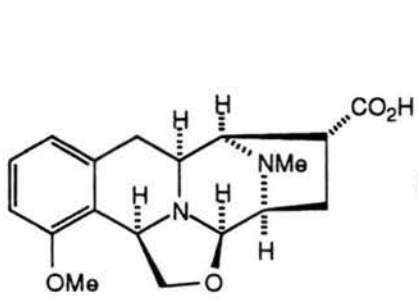
57, Tetrazomine

The structure of tetrazomine was determined as the hydrochloride salt using mass spectroscopy and a variety of NMR techniques[34]. Tetrazomine has a unique structure consisting of six ring systems including piperidine, piperazine, oxazole, and pyrrolidine ring systems. To date the stereochemistry of the 8 stereocenters of tetrazomine have not been spectroscopically elucidated.

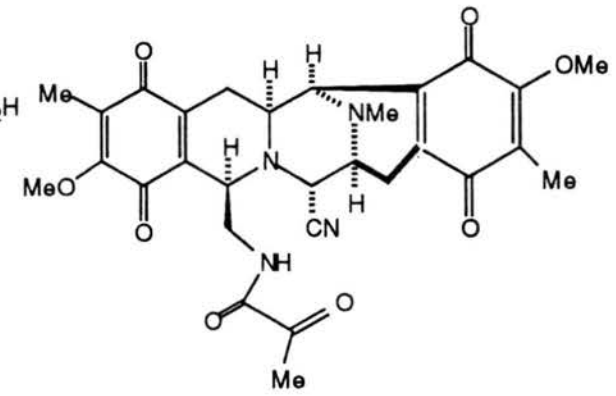
Tetrazomine shares structural similarities with the members of the quinocarcin[35a], napthyridinomycin[35b], saframycin[35c], and bioxalomycin[35d] family of antibiotics. All of these compounds have been isolated from actinomycete microorganisms. However, none of these compounds were produced by the tetrazomine producing bacteria strain. Each

compound shares an oxazolidine moiety, or its oxidative equivalent, at C-11'b and C-2'a based on tetrazomine's numbering system. With the exception of **59**, the isoquinolone alkaloids **57-63** share a common 3,7,8-diazabicyclo[3.2.1] octane substructure. Without regard to stereochemistry tetrazomine is structurally most similar to quinocarcin. Notable exceptions in structure are the oxidation state of C-13', and the linkage of a β-hydroxypipecolinic amino acid *via* a peptide bond at C-10'.

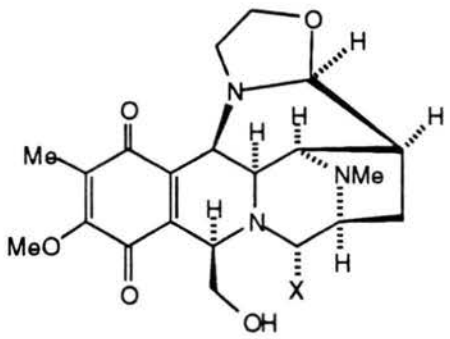
Figure 4:



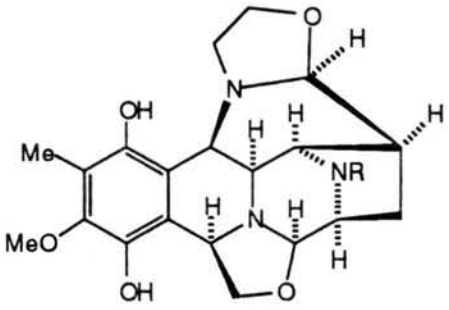
58, Quinocarcin



59, Saframycin A



60, Napthyridinomycin, X = OH
61, Cyanocycline, X = CN



62, Bioxalomycin α-1, R = H
63, Bioxalomycin α-2, R = Me

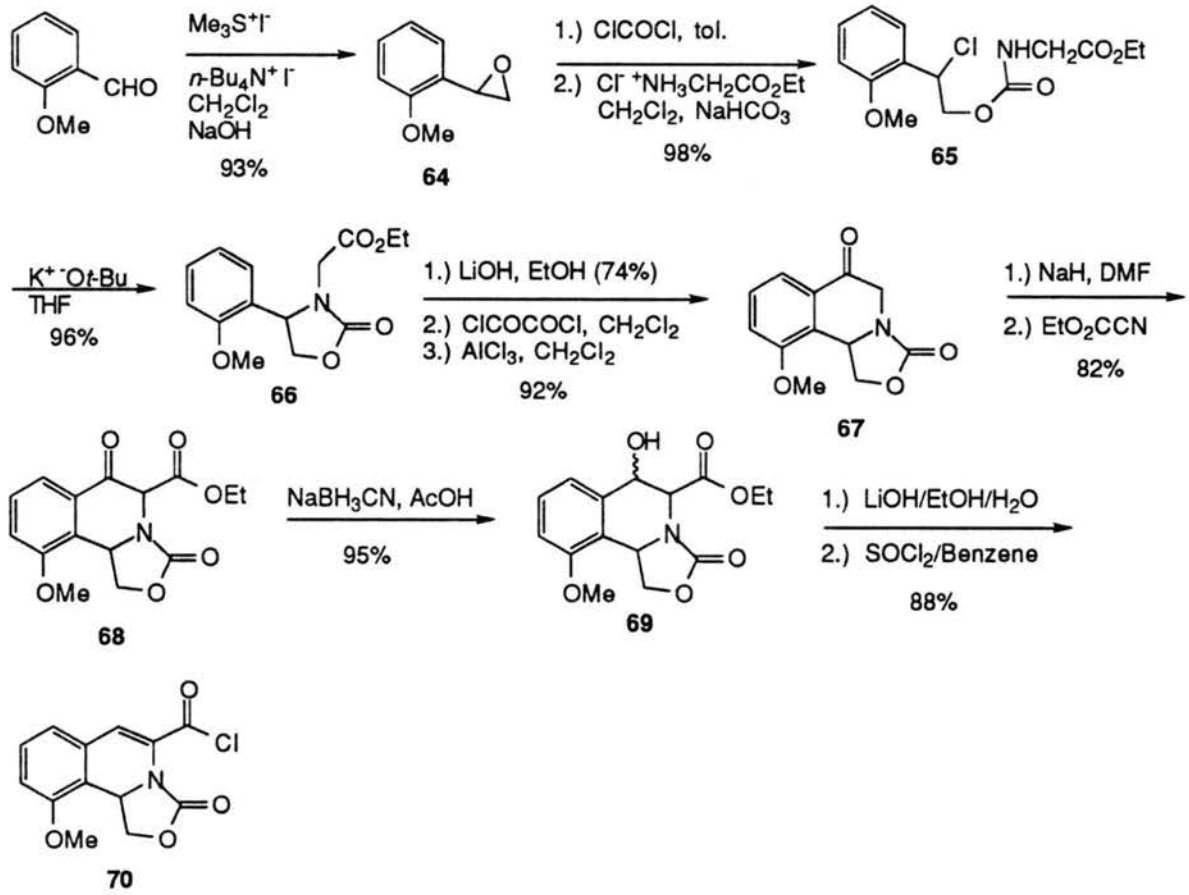
1. William's Synthesis of Quinocarcin and Analogs

To date no reports appear in the literature detailing synthetic studies in regard to tetrazomine. However, synthesis of the structurally related quinocarcin has been of

considerable interest and several reports now appear in the literature detailing both the racemic and asymmetric total synthesis of (-)-quinocarcin[36].

The total synthesis of quinocarcin, and mechanistic analogs, has been a focus of interest in the William's research group. The key intermediate in the preparation of quinocarcin derivatives is the isoquinolone intermediate **67** [37, 38]. The isoquinolone has been prepared on a multi-gram scale and has an infinite shelf-life. Treatment of *o*-anisaldehyde with trimethylsulfonium iodide under phase-transfer conditions yielded epoxide **64**. When treated with phosgene and a catalytic amount of H_2O epoxide **64**

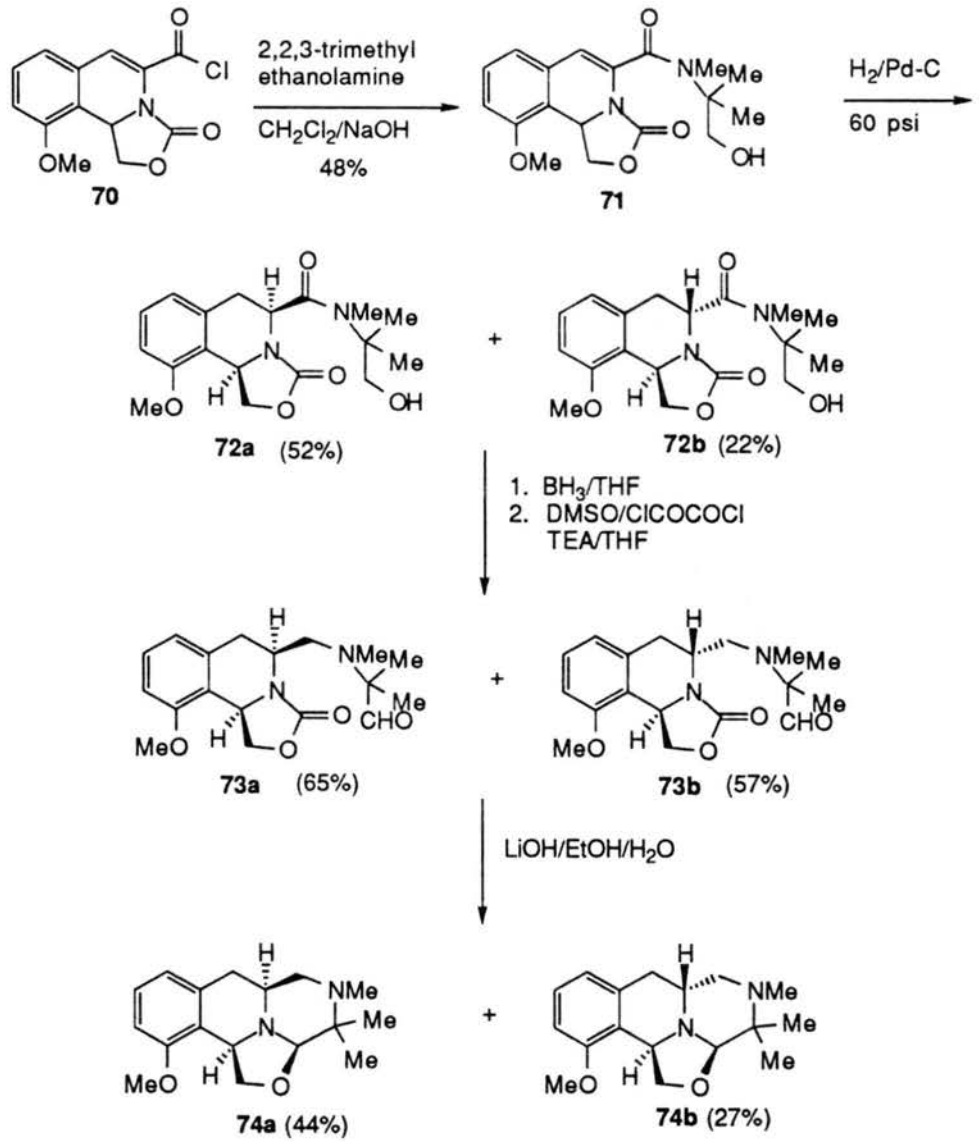
Scheme 13:



opened regioselectivity affording the chloroformate which was then acylated with glycine ethyl ester hydrochloride under Schotten-Baumann conditions providing **65**. Treatment of

65 with base furnished urethane **66** which was then saponified to the acid. Formation of the acid chloride followed by intramolecular Friedel-Crafts acylation afforded the key isoquinolone **67**.

Scheme 14:



Functionalization of **67** was achieved by formation of the sodium enolate and C-homologation with ethyl cyanoformate to yield the β-ketoester **68**. Adduct **68** was then reduced to the β-hydroxyester **69** with sodium cyanoborohydride. The β-hydroxyester was saponified to the acid and treatment of this substrate with thionyl chloride in refluxing

benzene converted the carboxylic acid to the α,β-unsaturated acid chloride **70** resulting in both elimination and acid chloride formation. Acid chloride **70** has proven to be an intermediate in both William's synthesis of tetracyclic quinocarcin analogs[37] as well as the formal synthesis of quinocarcin[38].

To investigate the role that the conformation of the oxazolidine moiety plays in quinocarcin's ability to oxidatively damage DNA the simpler oxazolidine containing tetracyclic analogs **74a** and **74b** were synthesized by Williams *et. al*[37]. Each analog mirrors one of the 2 possible conformations of the natural product.

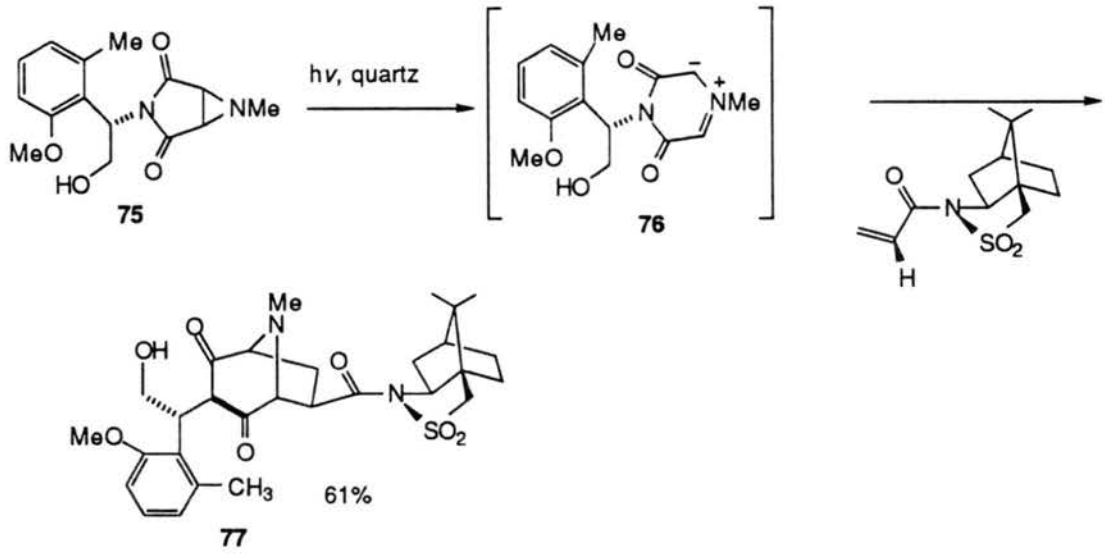
To obtain the tetracyclic analogs acid chloride **70** was treated with 2,2,3-trimethyl ethanolamine under Schotten-Baumann acylation conditions furnishing the unsaturated amide **71**. When **71** was subjected to conditions of catalytic hydrogenation a mixture of *syn* **72a** and *anti* **72b** isomers were obtained in approximately a 2:1 ratio. Following separation each stereoisomer was reduced to the corresponding tertiary amine utilizing borane and subsequently converted to the corresponding aldehydes **73a** and **73b** by Swern oxidation. Refluxing **73a** and **73b** in lithium hydroxide furnished the oxazolidine possessing tetracyclic analogs **74a** and **74b.** The pharmacophoric properties of these compounds will be discussed in Chapter 3.

Furthermore, a formal synthesis of racemic quinocarcin has been developed in the William's group from intermediate **70** employing a [3+2] cycloaddition strategy to furnish the pyrrolidine ring of quinocarcin[38]. The attractive feature of the [3+2] cycloaddition reaction is that the reaction allows the formation of 2 C-C bonds often in a stereoselective manner.

Such a methodology was first employed by Garner in his asymmetric synthesis of (-)-quinocarcin[36a]. Garner found that photolysis of **75** resulted in electrophilic ring opening yielding azomethine ylide **76** to which was added Oppolzer's chiral acryloyl sultam furnishing the single stereoisomer **77** from the cycloaddition reaction.

William's formal synthesis of quinocarcin intersects Garner's synthesis. From α,β-unsaturated acid chloride **70** treatment with $NaBH_4$ furnished the allylic alcohol **78** in

Scheme 15:

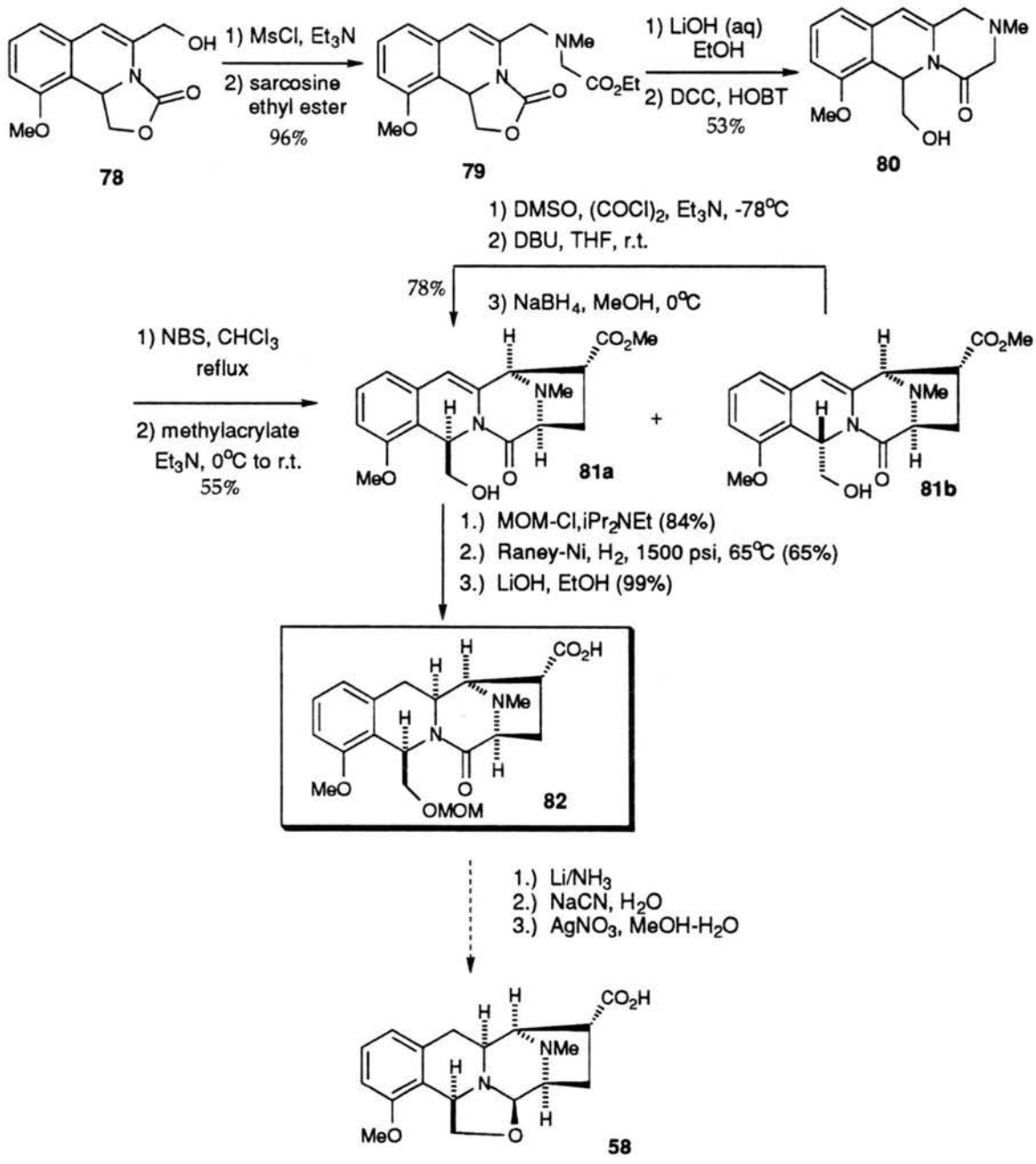


46% yield. Conversion of the alcohol to the mesyl group and condensation with sarcosine ethyl ester provided **79**. Saponification of the ethyl ester to the acid followed by deprotection of the oxazolidine furnished the alcohol which was then cyclized to **80.**

At this point the stage was set for the [3 +2] cycloaddition reaction. Firstly, the iminium ion was generated by oxidation with N-bromosuccinamide followed by formation of the azomethine ylide by treatment with TEA. To the ylide was added methylacrylate furnishing a 5:1 ratio of diastereomers **81b** and **81a**. The stereoselectivity of the cylcoaddition was rationalized by approach of the dipolarophile from the less sterically hindered face of the rigid ylide. The major diastereomer **81b** was converted to the desired diastereomer **81a** by Swern oxidation, and reaction with DBU resulting in conversion to the epimeric aldehyde. The alcohol moiety of **81** was then protected with the MOM group and treatment with Raney-Ni resulted in reduction of the olefin. Saponification of the

methyl ester to the carboxylic acid furnished **82** which intersected Garner's total synthesis of (-)-quinocarcin[36a]. From **82** dissolving lithium metal reduction results in partial

Scheme 16:

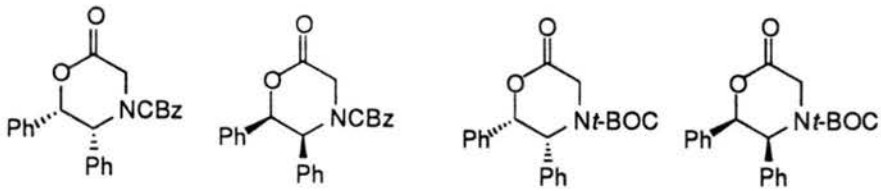


reduction of the amide which can in turn be trapped with cyanide ion. The oxazolidine moiety of quinocarcin can then be generated by either acidic or Lewis-acidic reaction conditions.

2. William's Lactone for Asymmetric Synthesis of Amino Acids

The synthesis of α-amino acids has been the focus of considerable interest in the William's research group. Numerous natural and unnatural amino acids have been synthesized employing the chiral glycine templates **83** and **84** which allow the synthesis of both **D**- and **L**-configured amino acids[39].

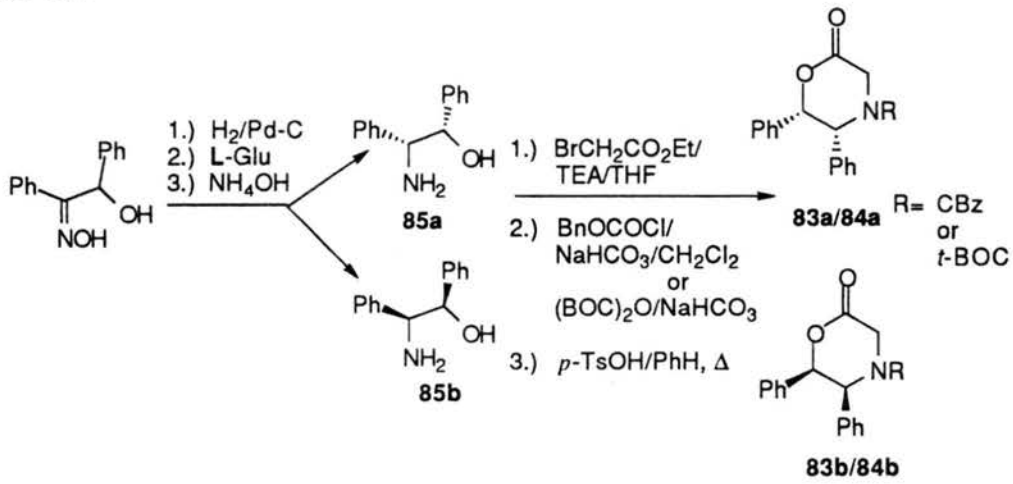
Figure 5:



83a **83b** **84a** **84b**

The templates, which have an indefinite shelf-life, can be prepared in six steps requiring no chromatography[39]. The templates are also commercially available.

Scheme 17:



Stereospecific hydrogenation of α-benzoin oxide yields a racemic mixture of *erthryo*-amino alcohols **85** which are resolved as the **L**-glutamate salts in greater than 98% ee providing **85a** and **85b**. Each optical isomer is individually alkylated with ethyl bromoacetate followed by acylation with either benzyl chloroformate or di-*t*-butyl carbonate. Finally, lactonization is induced employing catalytic *p*-toluenesulfonic acid in refluxing benzene or toluene to yield the optically active templates **83** and **84** in good yield.

The versatility of these oxazinones arises from the ability of the α-carbon, which is adjacent to a carbonyl and a nitrogen atom, to stabilize a variety of reactive intermediates through resonance and dipolar interactions. The formation of C-C bonds has relied on the ability of the α-carbon to form stable anionic, cationic, and radical reactive intermediates[39].

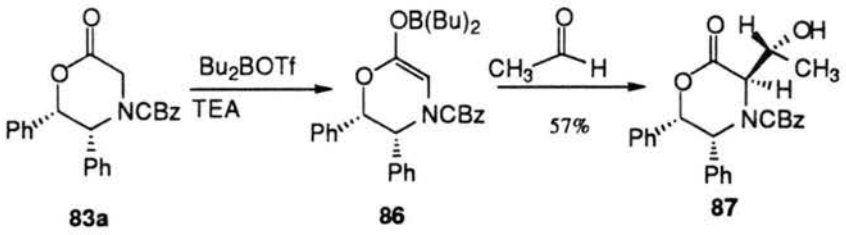
In addition to the ability of the lactones to stabilize a variety of reactive intermediates the physical properties of the templates make them quite useful[39]. The rigid geometry of the lactone templates allows C-C bonds to be formed in an extremely diastereocontrolled manner. Another advantage of the templates is the ease of unmasking the chiral auxiliary from the alkylated lactone to furnish the amino acid. Typical reaction conditions involve catalytic hydrogenation or dissolving metal reduction.

3. Aldol Condensations with William's Lactone

The formation of *B*-hydroxyl carbonyl compounds in theory can be achieved *via* aldol condensation reactions. However, under classic reaction conditions mixed aldols often form a mixture of condensation products which may undergo further transformation to form dehydration products. An important modification of the aldol condensation reaction is the use of boron enolates in directed aldol condensations[40]. Typically aldols generated in this manner do not suffer from further transformation to the α,β-unsaturated product. The stereoselectivity of these reactions is also quite high and a six-membered cyclic transition state has been proposed to account for this selectivity. In general Z

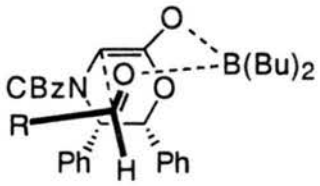
enolates furnish the *syn* stereoisomer while the E enolate furnishes the *anti* stereoisomer.

Scheme 18:



A report by Miller detailed the first aldol condensation reaction performed employing the William's glycine template **83a**[41]. The template was treated with di-(*n*-butyl)borontriflate and triethylamine in CH_2Cl_2 at 0°C to furnish the boron enolate. Treatment of the enolate with various aldehydes followed by work-up with pH 7 phosphate buffer stereoselectively furnished aldols. *Anti*-β-hydroxy adducts were predominately obtained and reduction of **87** afforded exclusively **L**-*allo*-threonine as determined by enzymatic assay. Interestingly, this was the first example of a chiral glycine template enolate exhibiting *anti* stereoselectivity.

Figure 6:

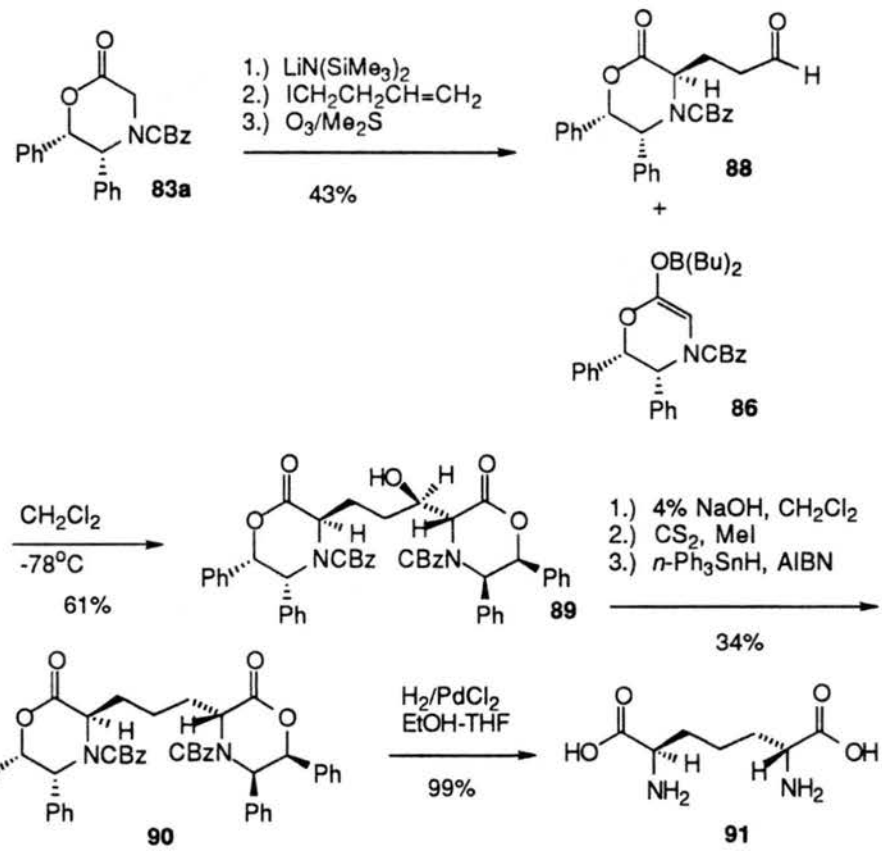


Miller proposed the *anti* selectivity was the result of the reaction proceeding through a Zimmerman-Traxler chair type transition state where the aldehyde approaches the fixed E-enolate from the less sterically encumbered face as shown in Figure 6[41]. The two *cis* phenyl substituents of the oxazinone serve to produce facial selectivity. Transition state selectivity was supported by variable temperature reactions which revealed that the stereoisomeric composition of the mixture did not change with a change in temperature

once the reaction was complete, but a change in product composition was observed when the reactions were conducted at different temperatures.

Additionally, the Williams group has employed this aldol condensation methodology to synthesize various 2,6-diaminopimelic acids (DAP) stereoisomers[42]. The synthesis of (R,R)-2,6-diaminopimelic acid, as shown in Scheme 19, first necessitated generation of the lithium enolate followed by stereoselective alkylation with homoallyl iodide from the less sterically hindered face of lactone **83a**. The terminal alkene was then

Scheme 19:



reductively ozonolyzed to furnish the aldehyde which was condensed with the boron enolate **86** to furnish aldol **89**. Although unimportant for the described synthesis the diastereoselectivity of the aldol condensation was excellent. Treatment of **89** with dilute aqueous base, carbon disulfide, and methyl iodide provided the xanthate ester which was

radically reduced to furnish **90**. Catalytic hydrogenation of **90** furnished the amino acid **91**.

B. RESULTS AND DISCUSSION

The development of a synthetic methodology for the synthesis of tetrazomine and analogs would be of interest for several reasons. The total synthesis of tetrazomine would provide a means to elucidate the stereochemistry of the natural product. The biological activity of synthetic analogs of tetrazomine could be analyzed to provide insight into the mechanism of tetrazomine's biological interactions. Of particular interest is the role that the β-hydroxypipecolinic acid plays in the biological activity of tetrazomine since it is the prominent structural difference between tetrazomine and quinocarcin.

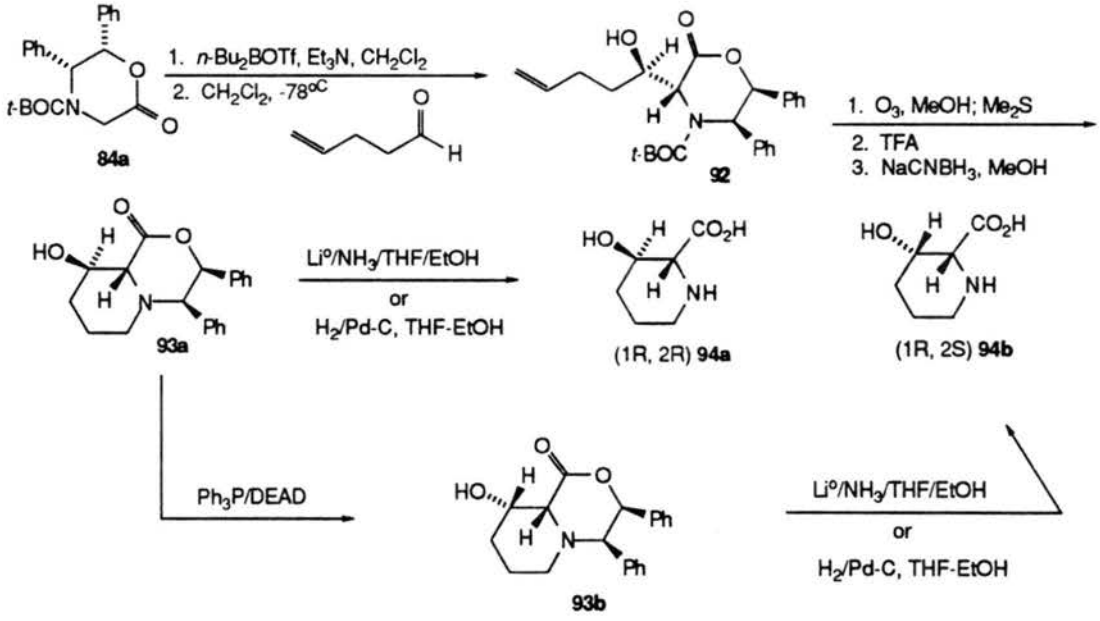
When one looks at tetrazomine form a synthetic view point it can be divided into 2 hemispheres. The left-hand side being the β-hydroxypipecolinic acid and the right-hand side being the portion of tetrazomine which is structurally similar to quinocarcin. The development of a methodology which would allow regioselective introduction of nitrogen functionality in the amine oxidation state *ortho* to the methoxy group at C-10' might intersect one of the intermediates of William's quinocarcin synthesis. The β-hydroxypipecolinic acid might be stereoselectively synthesized using William's glycine template and the aldol condensation methodology discussed.

1. Synthetic Studies of the Left Hemisphere of Tetrazomine

Since the stereochemistry of the β-hydroxypipecolinic acid moiety of tetrazomine is unknown it was necessary to devise a strategy which would allow access to all 4 of the possible stereoisomers. Given the stereoselectivity of the aldol condensation methodology

employing boron enolates and William's lactone it seemed a wise strategy to obtain the amino acids. It was envisioned that condensation of 4-pentenal with the boron enolate of the *t*-butyl carbamate protected lactone **84a** would stereoselectively furnish adduct **92.**

Scheme 20:

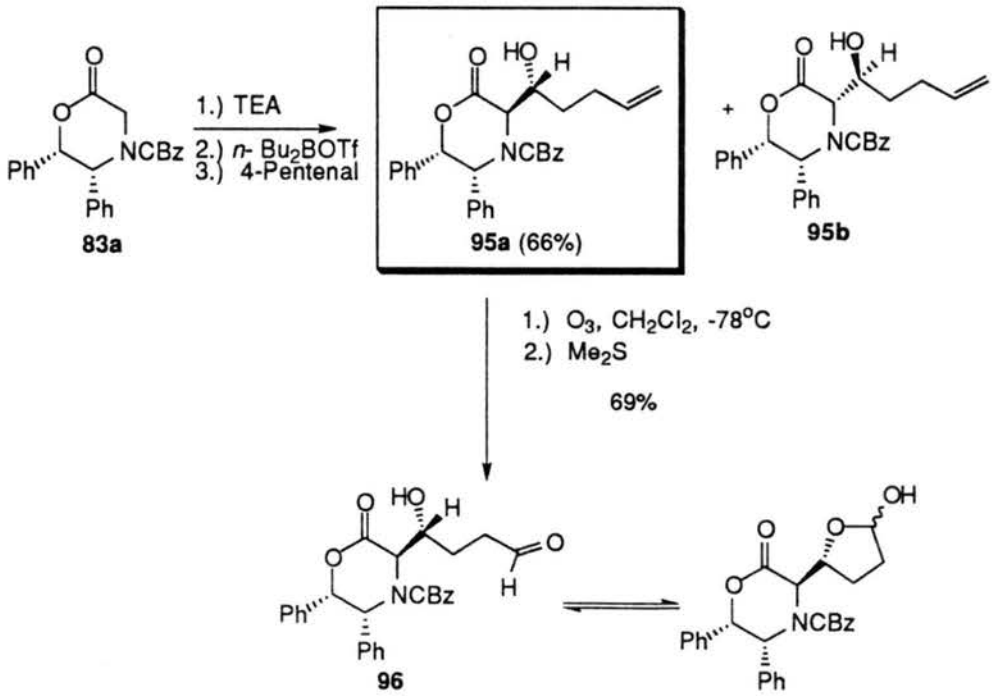


Ozonolysis of the terminal olefin of **92** followed by a reductive work-up would likely furnish the aldehyde which when treated with trifluoroacetic acid, the classic method for cleavage of *t*-BOC protected amines, would provide the secondary amine which could then undergo intramolecular reductive amination accessing compound **93a.** The epimeric hydroxy-carbon species **93b** might then be obtained by Mitsunobu inversion of **93a.** Lastly, it seemed reasonable that the β-hydroxypipecolinic acid would be obtained by cleavage of the chiral auxiliary using either dissolving metal or catalytic hydrogenation conditions to furnish either amino acid **94a** or **94b.** If successful, the (1S,2S) and (1S, 2R) stereoisomers of the amino acid should be accessible from lactone **84b** utilizing the same protocol.

To investigate the applicability of this synthetic approach it was first necessary to secure 4-pentenal. Optimal conditions for the synthesis of 4-pentenal were determined to be oxidation of commercially available 4-pentenol with freshly prepared pyridinium chlorochromate[43]. The low boiling point aldehyde could readily be purified by distillation when prepared in this manner. Rigorous purification of the product by distillation proved to be much more problematic when the aldehyde was alternatively prepared by Swern oxidation.

With 4-pentenal in hand, the aldol condensation was attempted using the *t*-BOC protected lactone **84a**. However, the *t*-BOC protected aldol **92** was not obtained when the condensation was attempted using the conditions described in the literature for the CBz protected lactone systems. A complex mixture of decomposition products was obtained; presumably the *t*-BOC moiety was not stable to the strong Lewis acid *n*-Bu2BOTf. Previous attempts at generating the boron enolate from **84a** and condensation with different aldehydes in the William's laboratory have proved unsuccessful in the past[44].

Scheme 21:



Given this initial failure at employing the *t*-BOC system, the aldol condensation of 4-pentenal was then attempted with the CBz protected lactone **83a**. Product **95a** was obtained in good yield by generation of the boron enolate according to Miller followed by condensation with 4-pentenal at low temperature in methylene chloride[41]. The reaction proved to be quite stereoselective providing the *B*-hydroxy lactone diastereomer **95a** in 66% yield following purification. Analysis of the crude reaction mixture by crude ^{1}H NMR revealed approximately an 11:1 ratio of **95a** to **95b** as shown in Figure 7. The minor diastereomer was not isolated.

The assignment of stereochemistry at the α carbon is based on the chemical shifts (Δδ) observed for the methine protons at the benzylic positions of the lactone as shown in Figure 7. Sinclair made the empirical observation that *anti*-isomers of the alkylated lactone from template have displayed larger Δδ's than the Δδ's observed for the *syn*-isomers[45]. The Δδ of the methine protons of diastereomer **95a** was 1.26 ppm (5.24 ppm and 6.53 ppm) which is indicative of *anti* alkylation in relation to the phenyl rings. The Δδ of the *syn* addition product was 0.64 ppm (5.88 ppm and 6.52 ppm). These differences in chemical shift are in agreement with values which have been previously observed[41, 42]. Furthermore, the small vicinal coupling constants observed for the C-2 and C-3 methines, based on tetrazomine numbering (2 Hz for **95a** and 3 Hz for **95b**), are indicative of *anti* aldol addition. This is in accord with previous observations by both Miller and Williams[41, 42].

Scheme 22:

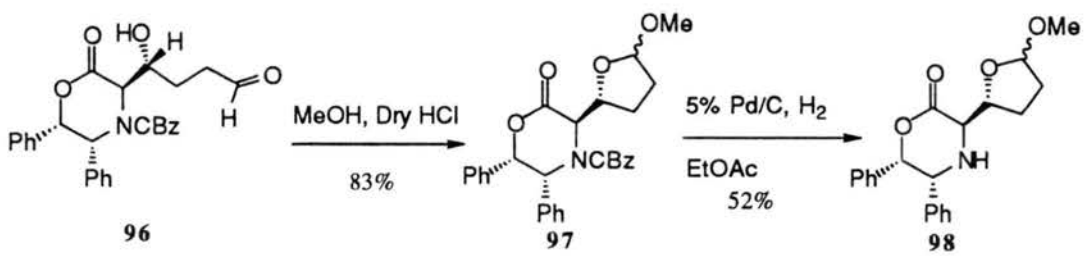
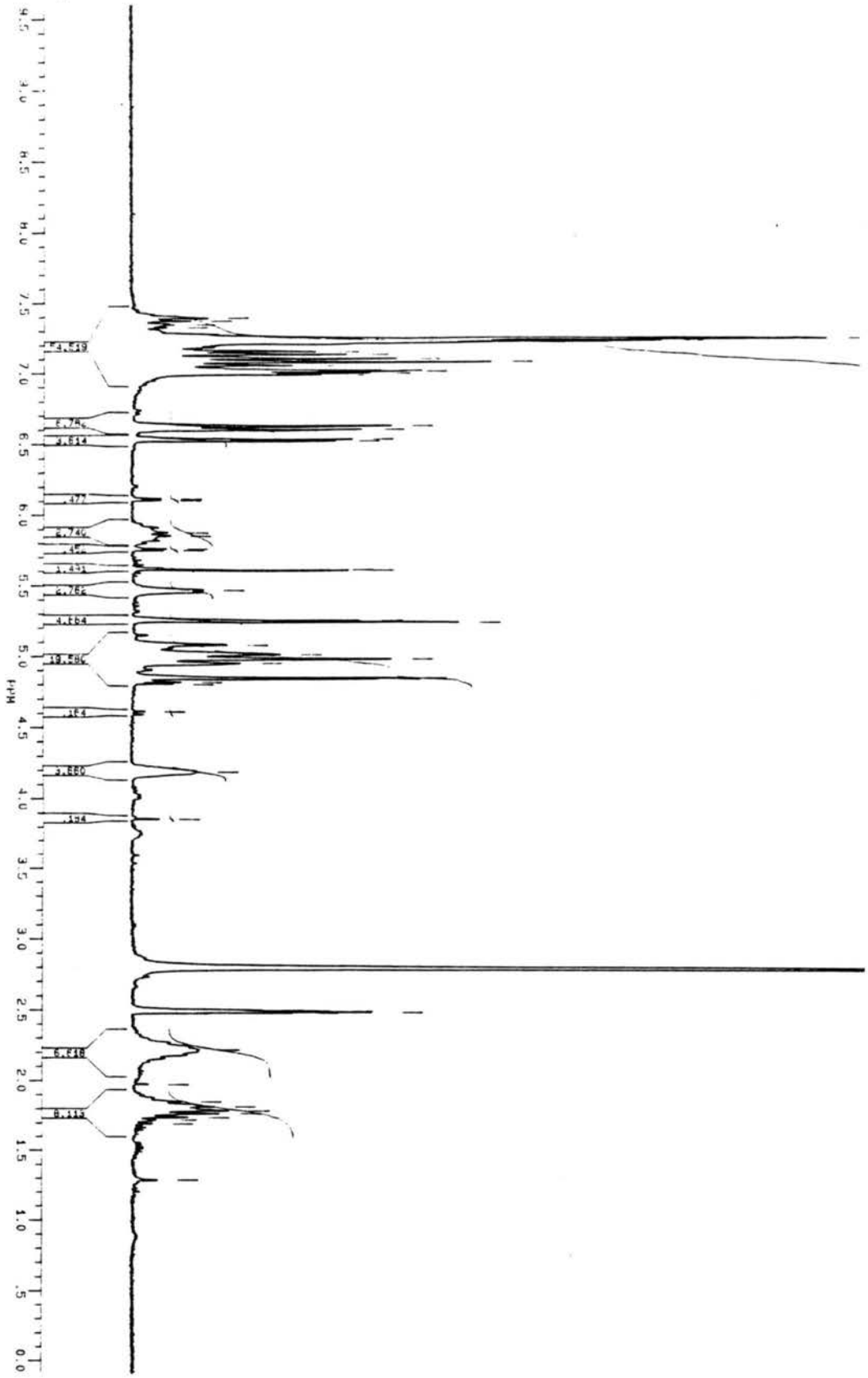


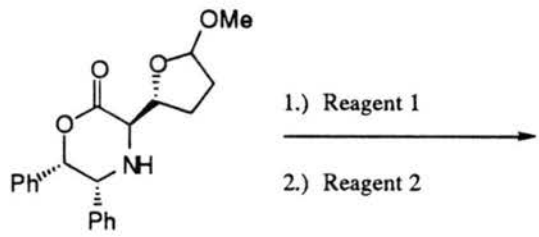
Figure 7: ^{1}H NMR Spectrum (300 MHz, d_6-DMSO, 393°K)



It was next necessary to unmask the aldehyde so that ring closure might be induced. Masking of the aldehyde as the olefin proved to be an effective means to avoid undesired transformations during the condensation reaction. The aldol product **95a** was uneventfully converted to the aldehyde **96** in good yield by low temperature ozonolysis in methylene chloride followed by a reductive work-up with dimethyl sulfide. The aldehyde product **96** existed in equilibrium with the hemi-acetal.

At this point methods for the cleavage of the CBz group were considered. Fearing that catalytic hydrogenation conditions, the most widely used method for cleavage of a CBz group, might reduce the aldehyde moiety to the alcohol prior to cleavage of the CBz group, conversion to the mixed acetal was investigated. It was thought that the acetal would prove inert towards the reductive conditions necessary to cleave the CBz group. The methyl mixed acetal **97** was obtained by treatment of a solution of **96** in MeOH with dry HCl gas.

Table 2:



98

Trial	Reagent 1	Reagent 2	Product
1.)	SiO_2, H_2O Oxalic Acid	----------	Starting Material
2.)	20% AcOH THF	----------	Starting Material
3.)	TFA, H_2O	$NaBH_3CN$ MeOH	Decomposition

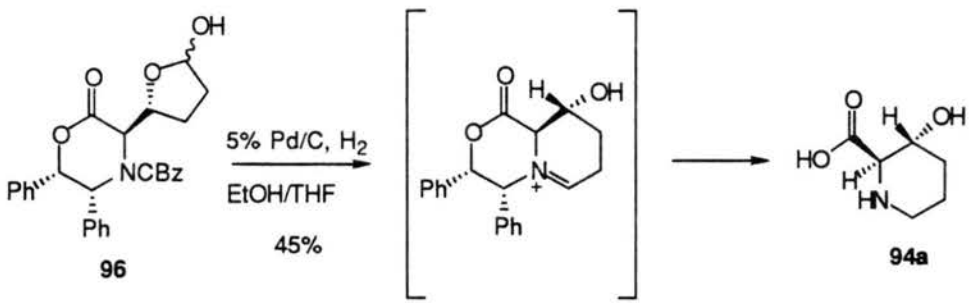
At this point cleavage of the CBz group was necessary. Detailed studies conducted in the William's laboratory had shown that cleavage of the CBz group preceded cleavage of the chiral auxiliary under reductive conditions[46]. The CBz group was cleaved from **97** by

catalytic hydrogenation at 1 atm to furnish **98** in moderate yield. The reaction was closely monitored by TLC and stopped once all of **97** had been consumed.

From **98** the hydrolysis of the mixed methyl acetal was desired. However, all attempts at hydrolysis of **98** followed by treatment with mild reducing agent to yield the reductive amination product **93a** were unsuccessful. Various conditions resulted in either recovery of starting material or decomposition products as compiled in Table 2.

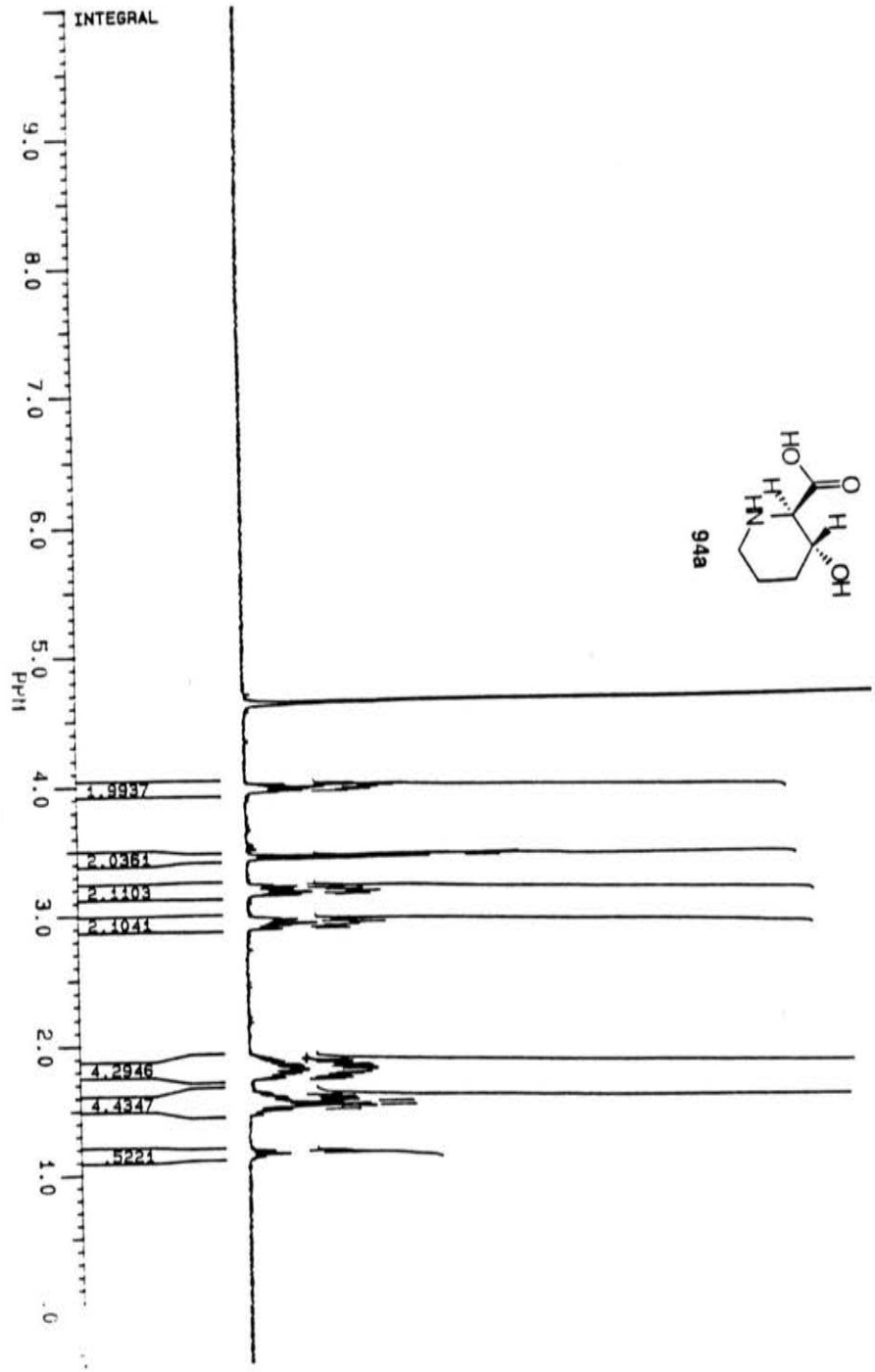
Returning to adduct **96** it was considered that if cleavage of the CBz group by catalytic hydrogenation preceded reduction of the aldehyde then reductive amination might occur. Cleavage of the CBz moiety by mild reduction would likely precede reduction of the aldehyde; especially since the aldehyde was in equilibrium with the hemi-acetal. Once deprotected, the secondary amine would then condense with the aldehyde and undergo dehydration to furnish the bicyclic iminium ion as shown in Scheme 23. Finally, under the reductive reaction conditions, the iminium ion might reduce to furnish the reductive amination product and chiral auxiliary cleavage realized to furnish the β-hydroxypipecolinic acid stereoisomer **94a**.

Scheme 23:



When the reaction was performed using 5% Pd/C and hydrogen gas at 1 atm the only organic soluble product isolated after 5 hours was the bibenzyl product indicating cleavage of the chiral auxiliary. Additional UV active products were observed when the reaction was monitored by TLC, but isolation was not attempted. Lyophilization of the

Figure 8: ^{1}H NMR Spectrum (300 MHz, D_2O)



aqueous layer of the reaction yielded a product whose molecular weight corresponded to that of the β–hydroxypipecolinic acid. As shown in Figure 8, the ^{1}H NMR spectrum of the product showed a single doublet at 3.42 ppm for the C-2 methine (based on tetrazomine numbering) with a vicinal coupling constant of 6.96 Hz. Taken together with the stereoselectivity observed in the formation of **95a** these results are indicative of the synthesis of a single stereoisomer whose configuration is most likely that of **94a**. However, rigorous purification of the amino acid was not realized allowing more detailed characterization. It is noteworthy that the cleavage of the CBz protecting group, reductive amination, and cleavage of the chiral auxiliary were realized in a single pot to furnish the substituted piperidine amino acid.

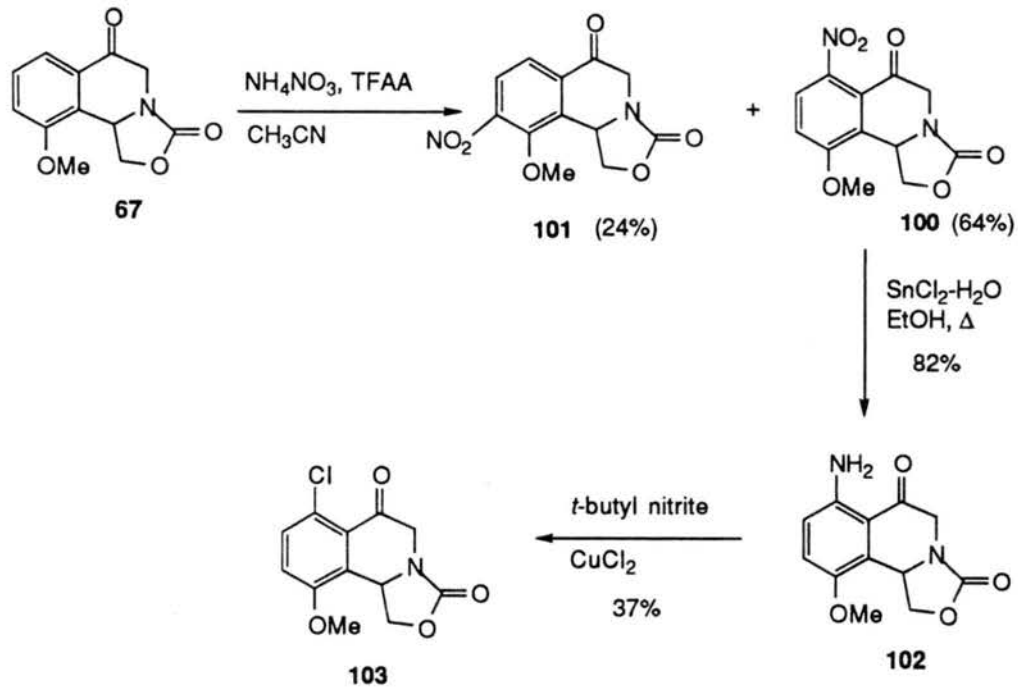
2. Synthetic Studies of the Right-Hemisphere of Tetrazomine

The most logical means to introduce nitrogen functionality at C-10' for the synthesis of tetrazomine and analogs is electrophilic aromatic nitration. Such reactions have been thoroughly studied and many experimental procedures appear in the literature which are suitable for large scale reactions[30]. Subsequent reduction of the nitro moiety to the amine results in the correct nitrogen oxidation state for tetrazomine. In theory the nitrogen of the relatively inert nitro moiety might be left in that oxidation state throughout the synthesis or reduced to the amine and protected. Nitration would be most logical following Friedel-Crafts acylation of intermediate **67** since the electron-withdrawing nitro moiety would deactivate the aromatic ring towards acylation if it were introduced earlier[47]. Friedel-Crafts reactions require electron rich aromatic rings and the nitro group would likely prohibit ring closure from occurring.

Isoquinolone **67** was prepared as described earlier in comparable yields. It was expected that the electron-donating methoxy moiety of **67** would act as an *ortho/para* director furnishing a mixture of regioisomers upon nitration. Initial attempts at nitrating **67**

employing nitric acid and acetic acid or sulfuric acid as a solvent were unsuccessful. The use of ammonium nitrate and TFAA, which is reported to give high *ortho:para* ratios with some systems, proved successful in nitrating the isoquinolone system[48]. However, the undesired regioisomer **100** was obtained in approximately a 3:1 ratio to the desired regioisomer **101**. This was not surprising since nitration *ortho* to the methoxy moiety would experience greater steric hindrance than the *para* position. Such a low yield was not practical at this juncture in the synthesis and other approaches were investigated.

Scheme 24:



The possibility of converting the undesired regioisomer to a substrate that would furnish the desired regioisomer was investigated since the overall nitration yield was excellent. Reduction of the nitro moiety of **100** to the amine, followed by conversion to the diazonium salt, and substitution with a chloride atom *via* Sandmeyer reaction might provide chloroisoquinolone **103**. The halogen could then in theory serve as a "blocking"

group and allow regioselective nitration *ortho* to the methoxy moiety (this approach will be discussed more thoroughly later in this chapter).

The first potential problem was reduction of the nitro moiety without concomitant reduction of the benzylic carbonyl. Use of the reagent stannous chloride[49] was reported to selectively reduce aromatic nitro groups in substrates bearing benzylic carbonyl groups. Reduction of **100** with stannous chloride selectively furnished the amine product **102** in good yield.

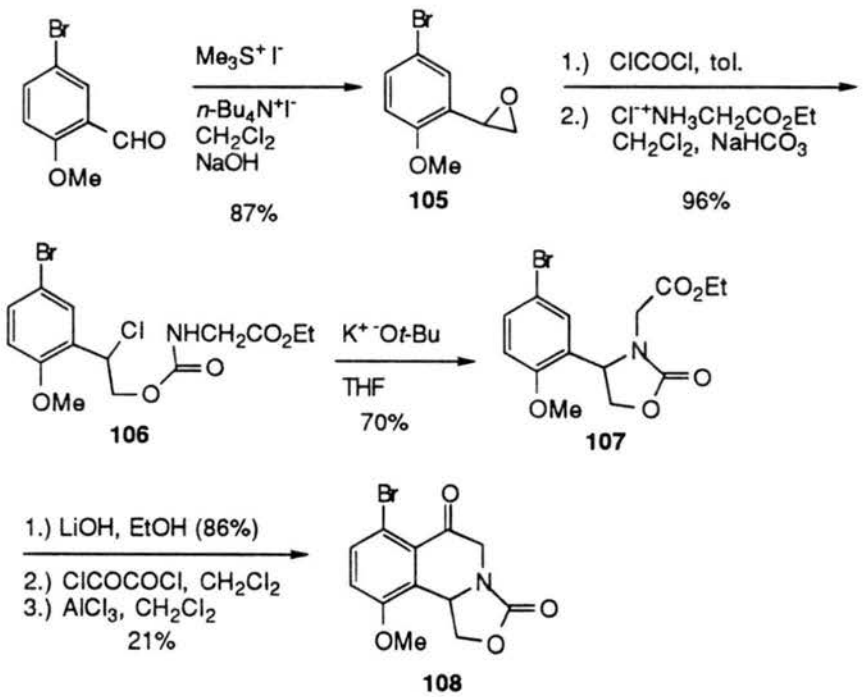
However, numerous attempts at generation of the chloride from the amine employing a variety of reaction conditions yielded product in only low yield as tabulated in Table 3. In most cases the major product obtained was reduction of **102** to the isoquinolone **67**. The chloroisoquinolone **103** was obtained in 37% yield with the reagents *t*-butyl nitrite and $CuCl_2$[50]. It was noted that many electron rich substrates employed in the original study conducted with the reagents *t*-butyl nitrite and $CuCl_2$ furnished a high percentage of arene product as well. Thus, given the low yields of **103** obtained from the Sandmeyer transformation this approach was abandoned.

Attention was then turned to developing a method that would allow regioselective nitration of any of the precursors of **70**. In principle, placement of a substituent *para* to the methoxy group should allow regioselective nitration at C-10' by "blocking" C-8'. The use of a halogen would be practical for two reasons. Firstly, a halogen is a weak *ortho/para* director so the methoxy moiety would be the primary directing functionality during electrophilic aromatic substitution and would activate C-10'. Secondly, halogens are often readily cleaved from aromatic rings making their cleavage following nitration feasible.

Many of the halogenated *o*-anisaldehyde derivatives are commercially available. It was thought that the presence of the deactivating halogen substituent on the aromatic ring might not inhibit the Friedel-Crafts acylation since the methoxy moiety is so electron rich. Following acylation the halogenated isoquinolone could in theory be regioselectively nitrated and the halogen cleaved.

A bromide atom at C-8' was initially investigated as a potential blocking group. Aromatic bromides are readily cleaved by treatment with the radical reducing agent triphenyltin hydride providing a facile means for the cleavage of the "blocking" group[50]. The commercially available 5-bromo-*o*-anisaldehyde was treated with trimethylsulfonium iodide under phase transfer conditions to yield the epoxide **105**. When **105** was treated

Scheme 25:



with a solution of phosgene in toluene and a catalytic amount of water the epoxide regioselectively opened yielding the chloroformate which was then reacted with glycine ethyl ester hydrochloride under Schotten-Bauman conditions yielding urethane **106**. Treatment of the urethane with base resulted in cyclization to **107** which was then saponified with lithium hydroxide furnishing the acid.

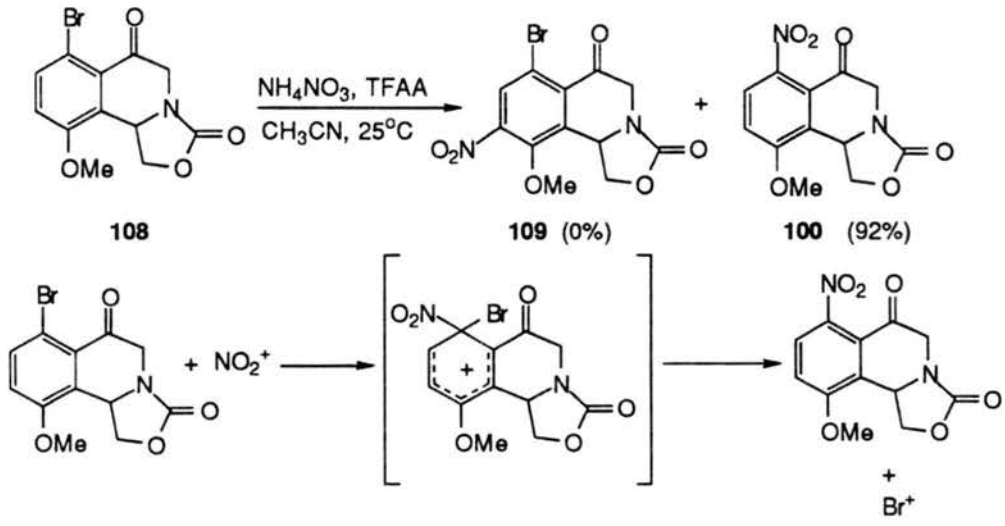
The acid was converted to the acid chloride with oxalyl chloride and an intramolecular Friedel-Crafts acylation was performed using the Lewis acid AlCl3 as a catalyst. The acylation reaction provided a low yield of cyclized product **108**. The low

yield is most likely a result of the deactivating nature of the bromide atom. Even though the bromide atom is not a highly deactivating moiety, the fact that the methoxy moiety does not directly activate the *meta* position towards acylation amplifies the electronic effect of the bromide. Efforts were not taken to maximize the yield of this reaction since the absolute inadequacy of this approach was realized when nitration was attempted.

Nitration of bromoisoquinolone yielded none of the desired nitration product **109**, but exclusively nitration product **100**. Nitroisoquinolone **100** was formed as a result of an *ipso* substitution reaction where the nitronium ion displaced the bromide atom as shown in Scheme 26.

Upon review of the literature it was found that such transformations are quite common in halogens substituted *para* to highly electron-donating substituents[52]. For example, nitration of *p*-bromoanisole furnishes the *ipso* substitution product *p*-nitroanisole in 30-40% yield. However, positively charged chloride atoms are much poorer leaving groups and are less likely to undergo *ipso* elimination. Indeed, no *ipso* substitution product is observed in the nitration of *p*-chloroanisole.

Scheme 26:



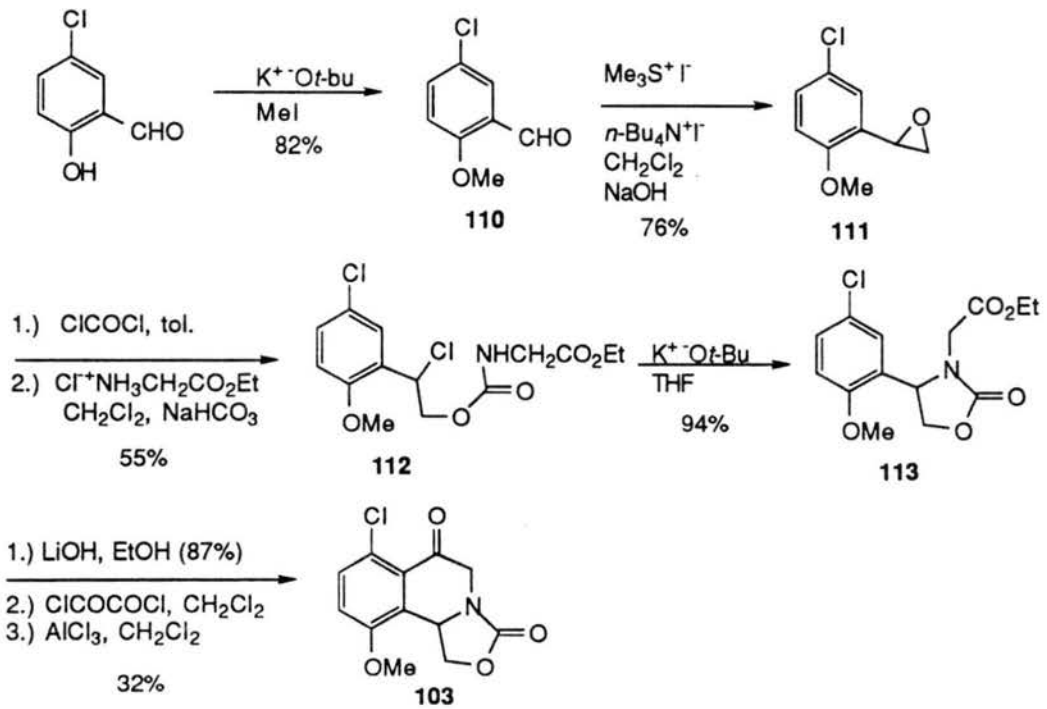
108 **109** (0%) **100** (92%)

Given the decreased likelihood of a chloride atom to suffer *ipso* displacement the use of a chloride atom as a blocking group was investigated. Starting with commercially

available 5-chlorosalicylaldehyde the methyl ester **110** was formed by treatment with methyl iodide and base. Urethane **113** was synthesized in an analogous manner to that described for the brominated derivative. The urethane could be converted to the acid and ultimately to isoquinolone **103** following the previously described protocol. Once again, the Friedel-Craft reaction provided a poor yield of the halogenated isoquinolone **103**. Thus, the sensitivity of this reaction to electron-withdrawing substituents was realized.

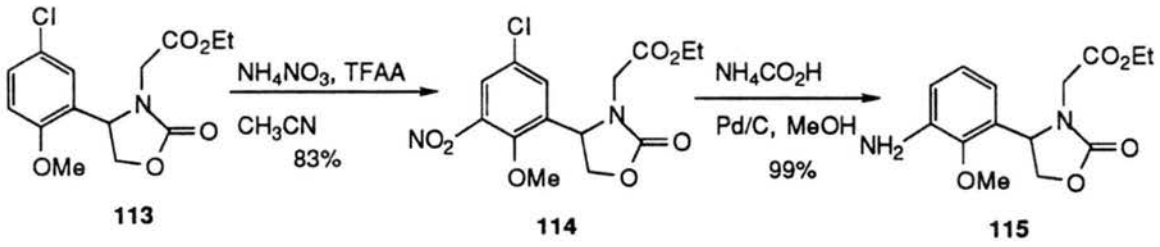
It was concluded that introduction of the nitro moiety and cleavage of the halogen "blocking group" must be realized prior to Friedel-Crafts acylation. Although the nitro moiety would inhibit Friedel-Crafts acylation it could be reduced to the electron-donating amine. The amine would require protection since the nitrogen of free amines tend to coordinate to aluminum ultimately deactivating the aromatic ring towards Friedel-Crafts reaction[47]. However, once protected the amine should activate the aromatic ring towards intramolecular Friedel-Crafts acylation since it would directly activate the *para* position.

Scheme 27:



Urethane **113** was regioselectively nitrated in excellent yield with ammonium nitrate and TFAA furnishing **114** without any evidence of *ipso* substitution. Given this desirable result attention was turned to finding a means by which to reduce the nitro moiety and to dechlorinate **114**. Since nitro groups are so labile towards reduction it was thought that the use of a hydrogen transfer agent and catalytic Pd/C should reduce the chloride and convert the nitro group to the amine in a single pot. In fact, treatment of **114** with ammonium formate and 5% Pd/C[53] yielded the dechlorinated amine product **115** in quantitative yield.

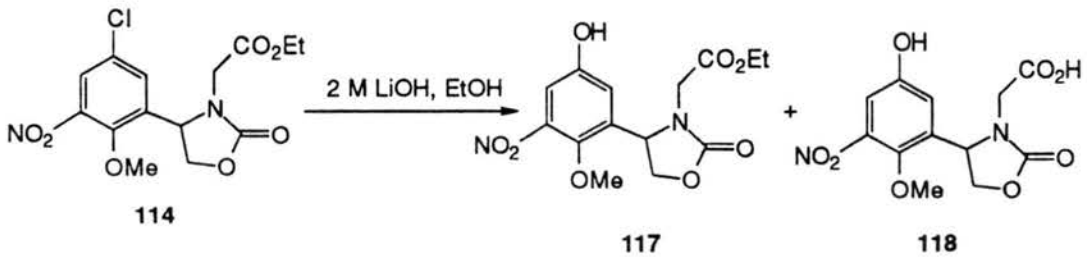
Scheme 28:



113 **114** **115**

Although unrelated to the synthetic sequences described in this thesis an unexpected transformation was realized when saponification of **114** was attempted which warrants identification. Saponification did not yield any of the expected nitro/chloro acid, but primarily a mixture of phenols **117** and **118**. This result was rationalized by the generation of a benzyne[54]. Deprotonation *ortho* to the chloride and elimination of the

Scheme 29:

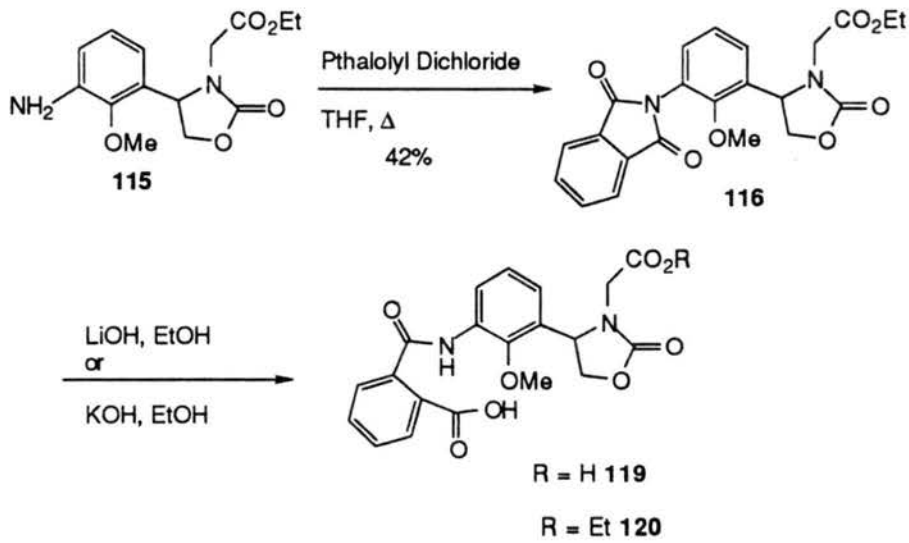


114 **117** **118**

chloride anion to form the "triple bond" intermediate followed by addition of hydroxide ion and protonation upon work-up would furnish the phenol.

To induce Friedel-Crafts acylation it was then necessary to protect the amine. Many of the common amine protecting groups such as the *t*-BOC or CBz group are unstable to the strong Lewis acid $AlCl_3$[55]. The pthalimide group as a protecting group for aliphatic amines is quite stable to most reaction conditions, including the Friedel-Crafts acylation, and can be selectively cleaved with hydrazine[55]. This protecting group was attractive for a total synthesis of tetrazomine because it was envisioned that it might be suitable for protection of the amine through the entire synthetic sequence. All attempts at protecting the aromatic amine with base and pthalic anhydride or carboethoxy-pthalimide[55], the classical methods for protecting aliphatic amines, proved fruitless. The amine was successfully protected as the pthalimide derivative **116** using pthaloyl dichloride. However, the protecting group proved to be unstable to saponification conditions needed to convert the ethyl ester to the acid and thus presumably furnished **119** and **120**. Given the numerous saponification transformations in the synthesis of quinocarcin further studies were not conducted with this protecting group.

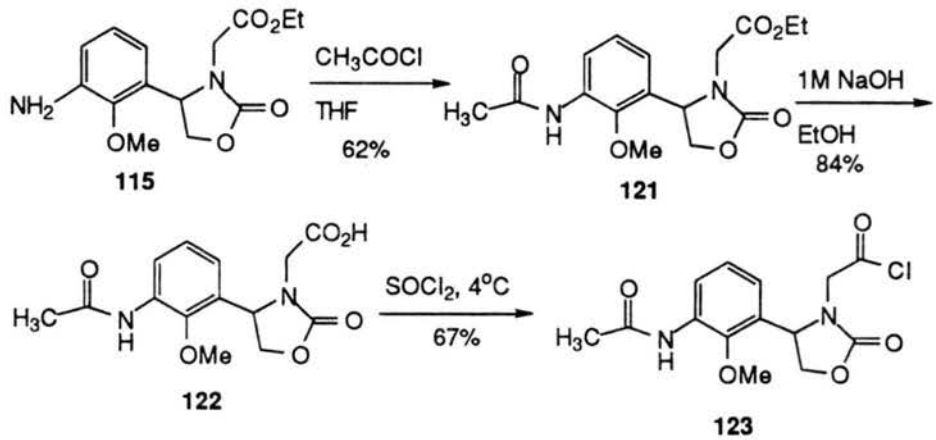
Scheme 30:



The feasibility of protecting the aromatic amine as the acetylated derivative was then investigated. The acetyl group has classically been employed for the protection of aromatic amines during Friedel-Crafts reactions[47]. The amine was acetylated using acetyl chloride furnishing **121**. The product proved to be stable to saponification conditions yielding acid **122**. Surprisingly **122** was quite water soluble and insoluble in most non-protic organic solvents. Isolation of **122** from the reaction mixture was not possible by extraction with organic solvent and could only be obtained by neutralization and lyophilization of the aqueous reaction mixture.

The physical properties of acid **122** proved to be problematic for formation of the acid chloride. Acid chloride formation was only realized with neat thionyl chloride at low temperature. The formation of the acid chloride **123** was confirmed by generation of the methyl ester when **123** was treated with MeOH.

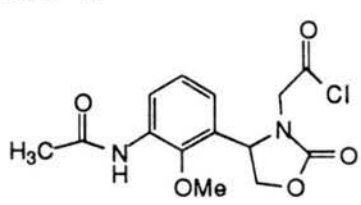
Scheme 31:



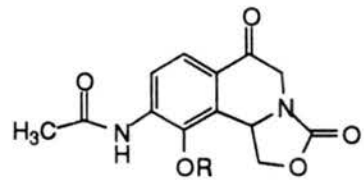
Numerous attempts at intramolecular Friedel-Crafts acylation proved surprisingly unsuccessful with substrate **123**. It was expected that this reaction would be facile due to the activated aromatic ring. A variety of different Lewis acids and solvents commonly employed for Friedel-Craft reactions were used as compiled in Table 4. In most cases the use of $AlCl_3$ resulted in decomposition. In one instance (entry 6) a trace product was

isolated which appeared to be the demethylated isoquinolone product **152**. However, this result proved to be irreproducible.

Table 4:



123

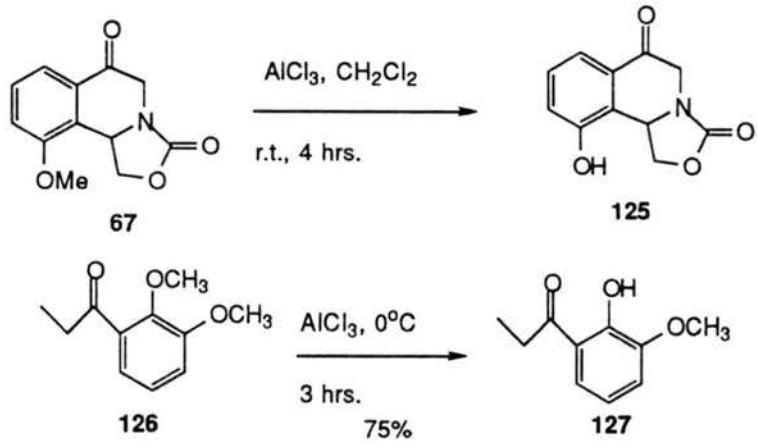


R = Me, 151
R = H, 152

Trial	Conditions	Product
1.)	$AlCl_3$ (3 eq.), CH_2Cl_2 1/2 hr. 0°C, 3.5 hrs. r.t.	Decomposition
2.)	$AlCl_3$ (4 eq.), CH_2Cl_2 1/2 hr. 0°C, 3.5 hrs. r.t.	Decomposition
3.)	$AlCl_3$ (4 eq.), $C_2H_4Cl_2$ 1/2 hr. 0°C, 3.5 hrs. r.t.	Decomposition
4.)	$AlCl_3$ (4 eq.), CH_2Cl_2 6 hrs. Δ	Decomposition
5.)	$AlCl_3$ (2eq.), $C_2H_4Cl_2$ 2.5 hrs. Δ	Decomposition
6.)	$AlCl_3$ (4eq.), $C_2H_4Cl_2$ 2.5 hrs. Δ	**152** < 10%
7.)	$AlCl_3$ (1.2 eq.), $C_2H_4Cl_2$ 1/2 hr. 0°C, 3.5 hrs. r.t.	Decomposition
8.)	$TiCl_4$ (6 eq.), $C_2H_4Cl_2$ 36 hrs.	Acid, **122**
9.)	$SnCl_4$ (4 eq.), CH_2Cl_2 1/2 hr. 0°C, 3.5 hrs. r.t.	Acid, **122**
10.)	$AlCl_3$ (2 eq.), 1,1,2,2-$C_2H_2Cl_4$ 8 hrs., r.t.	Decomposition
11.)	$AgBF_4$ (4 eq.) $C_2H_4Cl_2$ 4 hrs., r.t.	Acid, **122**

Analysis of the aqueous layer of the reactions following work-up by crude ^1H NMR suggests that the methyl ether might become demethylated during the reaction. Such a possibility is not without experimental precedent. Conversion of **67** to the phenol **125** was observed when reaction times exceed 4 hours[38]. Furthermore, $AlCl_3$ is commonly used to demethylate aromatic methyl esters adjacent to carbonyl moieties such as the transformation of **126** to **127**[56]; presumably by coordination of the $AlCl_3$ with the ether and carbonyl oxygens. A similar mechanism could be proposed for substrate **123** where the methyl ester is demethylated by coordination with the nitrogen of the acetyl group. If the Al-O complex forms prior to Friedel-Crafts acylation the aromatic ring would likely become highly electron deficient excluding the possibility of Friedel-Crafts acylation. In the case of entry 6 in Table 4 some of **123** may have underwent acylation prior to demethylation.

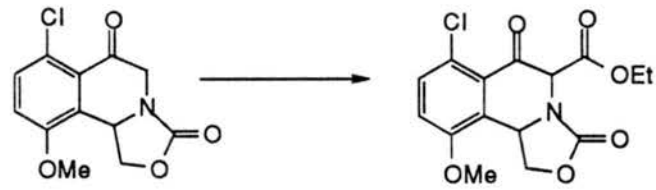
Scheme 32:



Simultaneous with the last approach, methods were investigated to regioselectively nitrate **67** since the sensitivity of the Friedel-Crafts acylation reaction was becoming apparent. Initially, halogenation by electrophilic substitution of the isoquinolone was not considered since it was thought that α-halogenation of the carbonyl would be a competitive reaction. However, the chlorinated isoquinolone **103** was regioselectively obtained in 66% yield when chlorination was attempted using $Cl_2/FeCl_3$[57].

Attempts at nitrating **103** with the same reaction conditions used to nitrate **113** were unsuccessful. This was somewhat surprising and could only be rationalized by deactivation of the aromatic ring by the benzylic carbonyl since that is the main structural difference between **113** and **103**. However, if the chloroisoquinolone was carried on to β-hydroxy ester **129,** the alcohol might prove to be more amenable towards nitration.

Table 5:



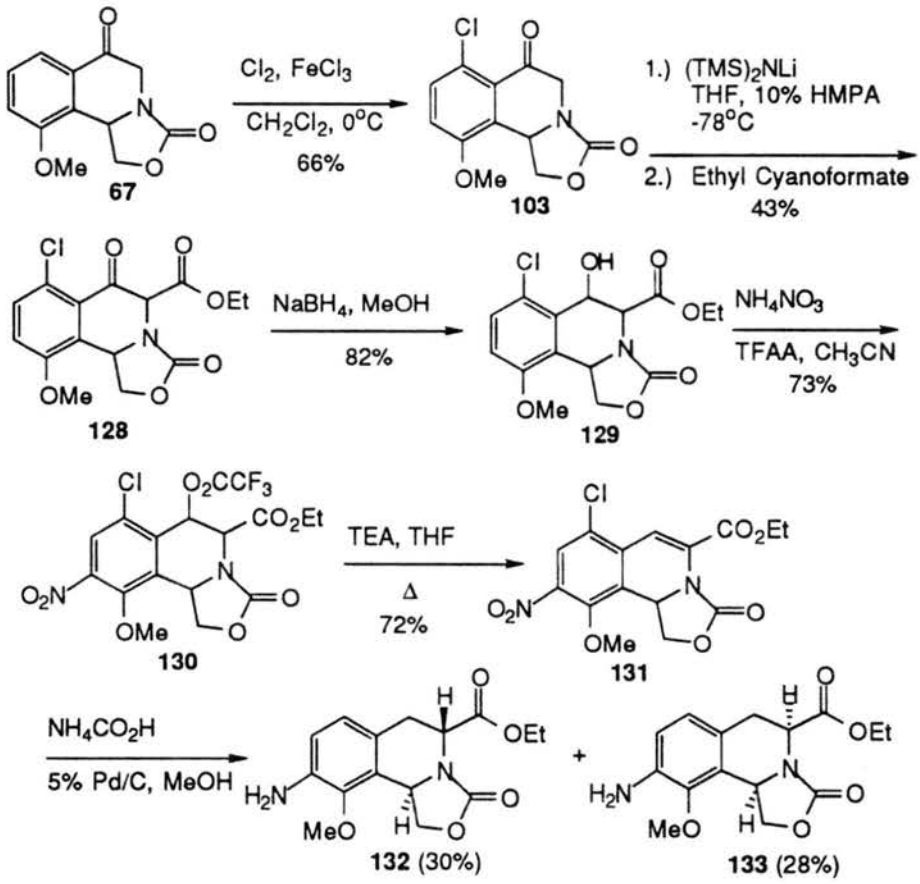
103 **128**

Trial	Reagents	Yield
1.)	1.)$(Si(CH_3)_3)_2NLi$ (1.5 eq.), THF -78°C 2.) Ethyl Cyanoformate (1.5 eq.)	17%
2.)	1.)$(Si(CH_3)_3)_2NLi$ (1.3 eq.), THF/10% HMPA -78°C 2.) Ethyl Cyanoformate (1.5 eq.)	20%
3.)	1.)$(Si(CH_3)_3)_2NLi$ (1.3 eq.), THF/10% HMPA -78°C 2.) Ethyl Cyanoformate (1.2 eq.)	43%
4.)	1.)$(Si(CH_3)_3)_2NLi$ (1.05eq.), THF/10% HMPA -78°C 2.) Ethyl Cyanoformate (1.05 eq.)	39%
5.)	1.)$(Si(CH_3)_3)_2NLi$ (1.2 eq.), THF/10% HMPA -78°C 2.) Ethyl Cyanoformate (1.1 eq.)	36%
6.)	1.) NaH (1.5eq), DMF 0°C-r.t. 2.) Ethyl Cyanoformate (1.2 eq.)	Decomp.
7.)	1.) NaH (1.5eq), DMF -40°C 2.) Ethyl Cyanoformate (1.2 eq.)	Decomp.
8.)	1.) K^+ ^-O*t*-Bu (1.2 eq.) 2.) Ethyl Cyanoformate (1.1 eq.)	Starting Material

Formation of the β-keto ester proved to be problematic. Substrate **128** was often obtained in very low yield when alkylation was attempted using Mander's reagent[58]. The

reaction proved to be quite temperature and solvent dependent as shown in Table 5. The optimal reaction conditions involved lithium enolate generation in THF/10% HMPA at -78°C. One possible explanation for the observed results is that the α-proton of the

Scheme 33:



product is more acidic than the α-protons of **103** so in the presence of excess base and ethyl cyanformate **128** might undergo a second alkylation. The chloride atom may further increase the acidity of **128** due to its inductive nature. The use of the sterically cumbersome base lithium bis(trimethylsilyl)amide might limit the amount of dialkylation due to increased steric hindrance in effecting deprotonation of **128** in comparison to the smaller NaH. The β-hydroxy ester **129** was then formed by reduction of **128** with $NaBH_4$.

Figure 9: ^{1}H NMR Spectrum (300 MHz, $CDCl_3$)

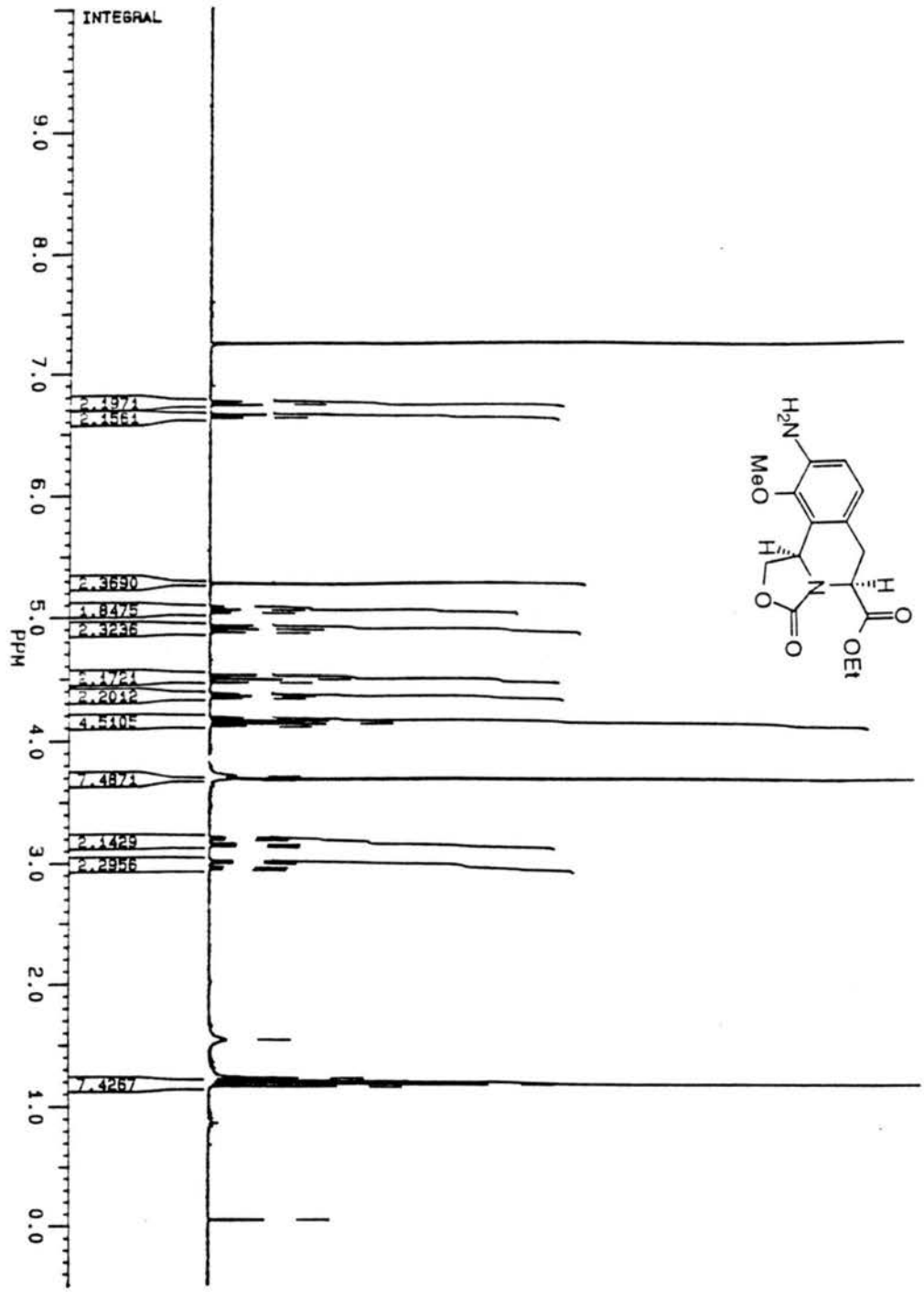
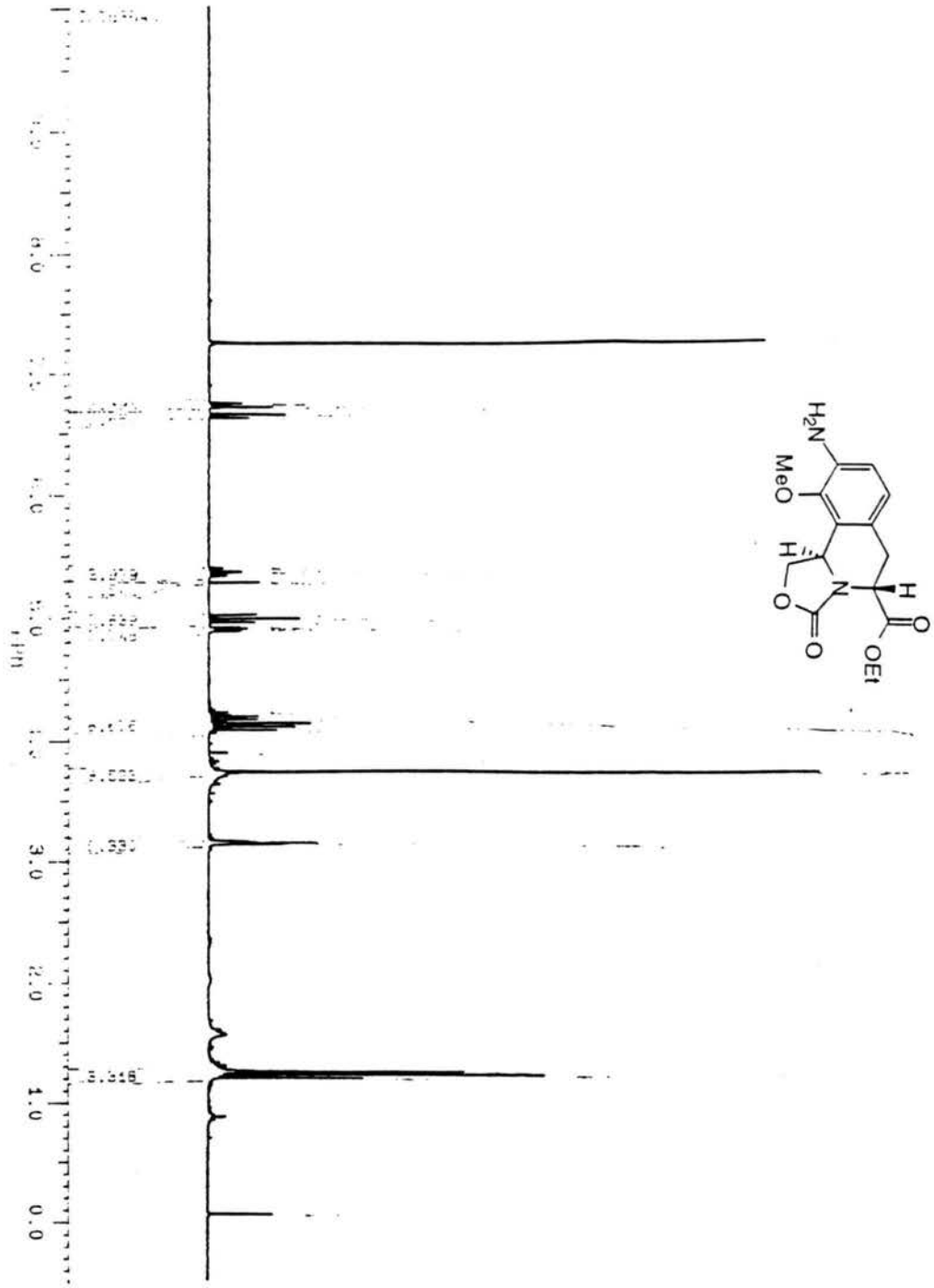


Figure 10: ^{1}H NMR (300 MHz,$CDCl_3$)



Nitration of the more electron-rich **129** was successful, but the free hydroxyl compound was not obtained. Under the nitration reaction conditions the hydroxyl group was converted to the trifluoroacetate, though **130** was obtained in 70% yield. Attempts at nitration of **129** using nitric acid and acetic acid were once again unsuccessful. Compound **130** proved to be quite unstable and eliminated to form **131** upon exposure to silica gel. As such, it was carried on in crude form.

At this point it was necessary to investigate methods for removal of the chloride and conversion of the nitro group to the amine. Given the instability of **130** it was thought that it would be best to convert it to **131**. When treated with TEA **130** eliminated to form the α,β-unsaturated ester **131**.

Finally, it was predicted that the reductive conditions necessary for the chloro/nitro reduction would likely reduce the olefin as well; this was indeed the case. Treatment **131** of with ammonium formate and 5% Pd/C yielded a mixture of *cis* **133** and *trans* **132** diastereomers readily separated by preparative thin layer chromatography. The stereochemistry of these compounds was assigned by ^{1}H NMR as shown in Figure 9 and 10. Stereochemical assignments for **133** and **132** were based on comparison to previously reported spectral data for the structurally analogous compounds lacking the aromatic amine[59]. It must be noted that the ratio of diastereomers might be somewhat misleading since NH_3 is generated in the reaction mixture and might epimerize the newly formed acidic stereocenter. Thus, in a single pot three reductive transformations were realized in good yield to furnish **132** and **133** which possess the nitrogen functionality at C-10' in the same oxidation state as that of tetrazomine.

C. Conclusion

A single stereoisomer of β-hydroxypipecolinic acid has been synthesized employing a stereoselective aldol condensation reaction and William's chiral lactone to establish the stereochemistry of the 2 asymmetric centers of the amino acid. Furthermore, a novel one pot procedure that results in the cleavage of an amine protecting group, intramolecular reductive amination, and finally cleavage of the chiral auxiliary has been developed furnishing a substituted piperidine ring. The enantiomer of **94a** should be accessible by the same reaction sequence by employing the alternative enantiomer of the chiral glycine template. The other two stereoisomers in theory should be obtainable by performing Mitsunobu inversion of the hydroxyl moiety of substrate **95a** and its enantiomer. Thus, a methodology has been developed which might allow access to all of the possible stereoisomers of the β-hydroxypipecolinic acid portion of tetrazomine.

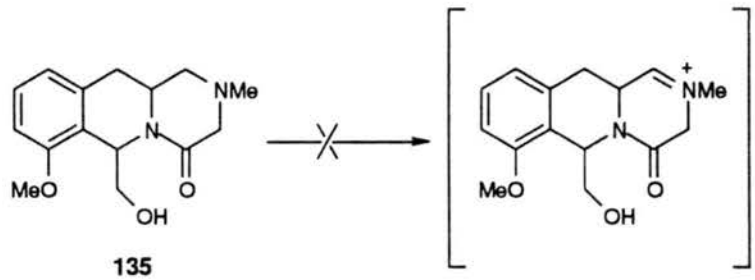
A synthetic route has been developed which allows regioselective introduction of the nitrogen functionality at C-10' necessary for the synthesis of tetrazomine and tetrazomine analogs. The use of a chloride has proven to be an effective "blocking" group, and introduction of this moiety is best achieved following intramolecular Friedel-Crafts acylation to yield isoquinolone **67**. Nitration of β-hydroxy ester **129** furnishes the chloro/nitro product and the use of transfer hydrogenation conditions can, in one pot, dechlorinate the substrate and reduce the nitro to the desired amine oxidation state.

However, at the conclusion of these studies it became evident from studies conducted on the formal synthesis of quinocarcin in our group that the presence of the olefin at C-6'a was crucial for the synthetic methodology described for the total synthesis of quinocarcin. Attempts at generation of the iminium ion **151** from **135**, the saturated analog of **80**, were unsuccessful implicating the necessity of *sp2* hybridization at C-6'a.

Therefore, in the total synthesis of tetrazomine reduction of the olefin moiety of

131 simultaneously with reduction of the nitro and chloride will not be acceptable. Furthermore, based on the behavior observed with similar substrates lacking the amine

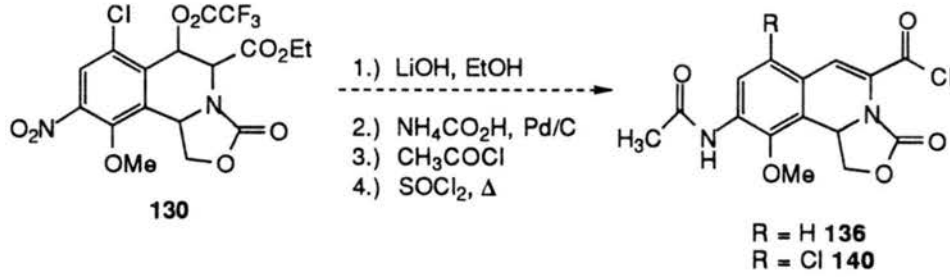
Scheme 34:



135 **151**

moiety, it is likely that saponification of **133** will epimerize the α-hydrogen to the more thermodynamically favorable *trans* configuration.

Scheme 35:



130

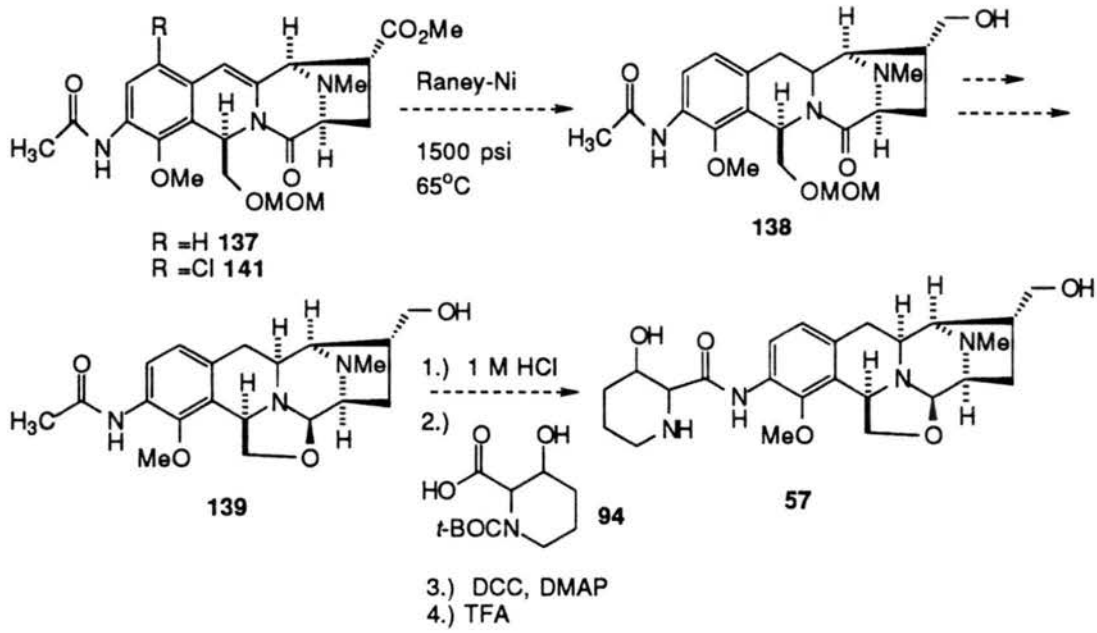
R = H **136**
R = Cl **140**

Two alternatives might be proposed to circumvent this problem. If the trifluoroacetate ester can be saponified to the hydroxyl group, reduction of the nitro and chloro groups could be initiated prior to generation of the olefin. Thus, reduction of the aromatic functionality would occur prior to generation of the requisite alkene. If benzyne formation is not a problem during saponification, then **136** should be accessible as shown in Scheme 35. Adduct **136** is analogous to **70** and could be carried on in a similar manner to **137** as in the quinocarcin synthesis described in Scheme 16.

Garner and associates reported in their synthesis of quinocarcin[36a] that during Raney-Ni olefin reduction of an intermediate similar to the MOM-protected **81a,** a

byproduct was the reduction of C-13' to the alcohol oxidation state; the correct oxidation state for tetrazomine. If **137** could be converted to **138** by extended reduction then generation of the oxazolidine of **139** might be achieved following the protocol outlined in Scheme 16. To obtain tetrazomine treatment of **139** with 1 M HCl would likely hydrolyze the acetylated amine. It then might be condensed with a *t*-BOC protected β-hydroxypipecolinic acid using standard peptide coupling conditions. Removal of the *t*-

Scheme 36:



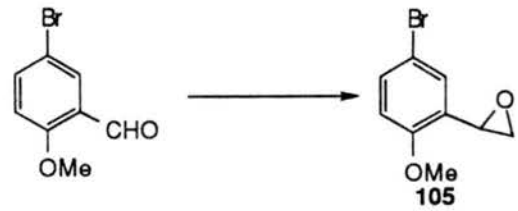
BOC could be effected by treatment with TFA thus furnishing tetrazomine with the same stereochemistry as that of quinocarcin. If tetrazomine and quinocarcin do share the same stereochemistry at their related stereocenters, each of the stereoisomers of the amino acid might be coupled to determine the stereochemistry of the natural product.

Another alternative may be selective reduction of the nitro group of **130** with stannous chloride followed by acetylation of the amine. The ester could then be saponified to the acid and the α,β-unsaturated acid chloride **140** formed as previously described. It

has also been reported that Raney-Ni dechlorinates aromatic chlorides[60] so it is plausible that **141** might be directly converted to **139.**

Given the synthetic transformations realized in this study and those in the synthesis of quinocarcin the total synthesis of tetrazomine might be achieved.

D. Experimentals



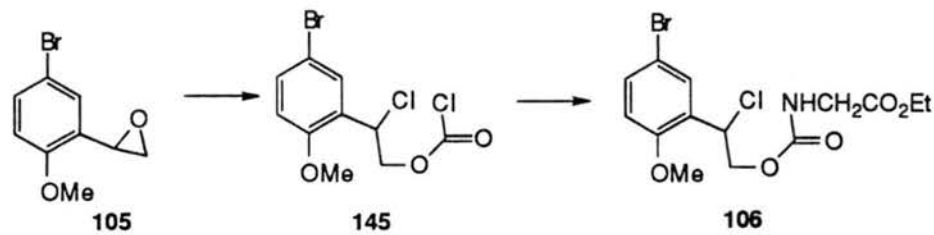
105

(5-bromo-2-methoxyphenyl)oxirane (105)

A nonhomogenous mixture of 5-bromo-*o*-anisaldehyde (10.0 g, 0.0465 mol), trimethylsulfonium iodide (11.39 g, 0.0558 mol), tetrabutyl ammonium iodide (0.21 g, 0.0006 mol), methylene chloride (160 mL), and 50% NaOH (105 mL) was stirred vigorously at room temperature for 3.5 days. The reaction mixture was transferred to a separatory funnel and the aqueous NaOH was allowed to settle (bottom layer) and drained. The organic layer was then carefully washed with water, dried over $MgSO_4$, filtered, concentrated, and purified by high vacuum short-path distillation to yield 9.24 g of a clear oil in 86.7% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 2.63 (1 H, q, J = 2.50 Hz), 3.10 (1 H, q, J = 4.11 Hz), 3.83 (3 H, s), 4.11 (1 H, t, J = 2.56 Hz), 6.71 (1 H, d, J = 8.68 Hz), 7.23 (1 H, d, J = 2.51 Hz), 7.31 (1 H, dd, J = 8.70 Hz, J = 2.54 Hz).

IR (NaCl, CH_2Cl_2): 3053, 2991, 2939, 2839, 1596, 1491, 1256, 1137, 1029, 808 cm^{-1}.



105 145 106

O-2-chloro-2-(5'-bromo-2'-methoxyphenyl)-N-ethoxyacetylcarbamate (106)

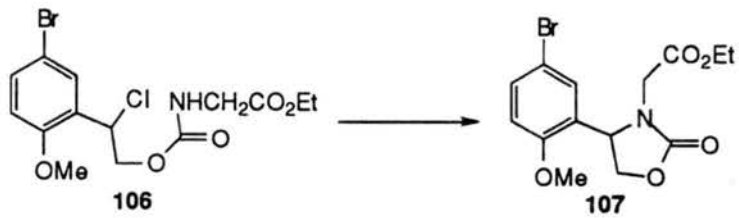
A solution of **105** (9.08 g, 0.0396 mol) was prepared by dissolving in toluene (100 mL) in a sealed flask fitted with a phosgene inlet, a pressure balloon, and an aqueous alkali scrubber. Phosgene was bubbled through the solution for 25 minutes and the reaction was

stirred for an additional 40 hours. The reaction flask was purged of phosgene by bubbling nitrogen through the system until the reaction mixture was concentrated to half of its original volume. The product **145** was then concentrated and dissolved in methylene chloride (125 mL). To this solution was added a saturated solution of $NaHCO_3$ (125 mL), followed by glycine ethyl ester hydrochloride (5.43 g, 0.0389 mol) dissolved in a minimum volume of water. The nonhomogenous mixture was vigorously stirred at room temperature for 20 minutes. The organic layer was separated from the aqueous layer and washed twice with water, dried over $MgSO_4$, filtered, and concentrated in vacuum to yield 14.77 g of a crystalline product in 96.2% yield for the reaction.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.26 (3 H, t, J = 7.22 Hz), 3.81 (3 H, s), 3.92 (2 H, m), 4.18 (2 H, q, J = 7.11 Hz), 4.36 (2H, m), 5.26 (2 H, bs), 5.48 (1 H, t, J = 5.73 Hz), 6.72 (1 H, d, J = 8.79 Hz), 7.36 (1H, dd, J = 8.79 Hz, J = 2.50 Hz), 7.57 (1H, d, J = 2.45 Hz).

IR (NaCl, CH_2Cl_2): 3354, 2982, 2942, 1732, 1538, 1488, 1255, 1200, 1053, 1026, 811 cm^{-1}.

^{13}C NMR ($CDCl_3$) δ 14.11, 42.79, 53.62, 55.87, 61.50, 67.56, 112.52, 113.04, 127.90, 131.49, 132.59, 155.33, 169.36, 169.72.



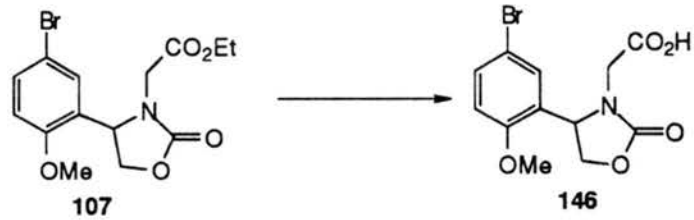
1-(carboethoxy)methyl-5-(5'bromo-2'-methoxy)phenyloxazolidin-2-one (107)

A solution of **106** (14.69 g, 0.0372 mol) in THF (225 mL) was cooled to 0°C and stirred for 30 minutes. A solution of potassium *t*-butoxide (5.72 g, 0.0400 mol) in THF (100 mL) was added to the reaction flask and stirred for 40 minutes at 0°C. The reaction was quenched with 0.5 M HCl (85 mL). The product was extracted 3 times with CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated. The product was purified by silica gel flash

column chromatography (100:1 CH_2Cl_2:MeOH) to yield 10.3 g of a crystalline product in 70.0% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.28 (3H, t, J = 8.01 Hz), 3.40 (1H, d, J = 17.96 Hz), 3.80 (3H, s), 4.15 (2H, m), 4.40 (1H, d, J = 18.01 Hz), 4.70 (1H, t, J = 7.96 Hz), 5.30 (1H, q), 6.80 (1H, d, J = 8.23 Hz), 7.35 (1H, d, J=1), 7.45 (1H, dd, J = 8.64 Hz, J = 2.15 Hz).

IR (NaCl, CH_2Cl_2) 2978, 2942, 1748, 1489, 1252, 1207, 1027 cm^{-1}.



107 146

1-(carboxy)methyl-5-(2'-methoxy-5'-bromo)phenyloxazolidin-2-one (146)

A solution of **107** (3.92 g, 0.011 mol) in 95% ethanol (100 mL) was cooled to 0°C with stirring. A solution of 2 M LiOH (15 mL) was added to the reaction flask and stirred for 60 minutes at 0°C. The reaction was concentrated and redissolved in 20 mL of 1 M NaOH. The organic layer was washed 2 times by extraction with EtOAc. The aqueous layer was adjusted to pH 3 by the addition of 1 M HCl. The product was extracted 3 times with CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated to yield 3.13 g of a white crystalline product in 86.2% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 3.45 (1 H, d, J = 18.35 Hz), 3.79 (3 H, s), 4.10 (1 H, dd, J = 8.59 Hz, J = 6.54 Hz), 4.36 (1 H, d, J = 18.34 Hz), 4.67 (1 H, t, J = 8.75 Hz), 5.26 (1 H, dd, J = 9.32 Hz, J = 6.44 Hz), 6.78 (1 H, d, J = 8.79 Hz), 7.28 (1 H, d, J = 2.45 Hz), 7.40 (1 H, dd, J = 8.70 Hz, J = 2.54Hz).

IR (NaCl, CH_2Cl_2) 3063, 2940, 2942, 1755, 1489, 1442, 1253, 1200, 1025, 813 cm^{-1}.

^{13}C NMR ($CDCl_3$) δ 43.69, 54.79, 56.14, 69.32, 113.14, 113.70, 127.33, 130.38, 133.25, 156.88, 159.35, 173.48.

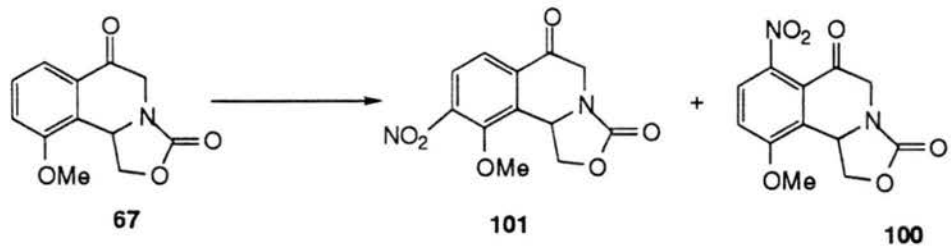
146 108

1-hydroxymethyl(2,2'carbonyl)-4-keto-5-bromo-8-methoxytetrahydroisoquinolone (108)

To a solution of **146** (0.23 g, 0.00068 mol) in CH_2Cl_2 (15 mL) was added DMF (0.20 mL) by syringe. Oxalyl chloride (0.07 mL, 0.00081 mol) was diluted with CH_2Cl_2 (2 mL) and added dropwise by syringe to the reaction flask at room temperature. The reaction was stirred for 2 hours, concentrated, and redissolved in CH_2Cl_2 (30 mL), after which alkaline activated carbon was added and vigorously stirred for 1 minute. The solution was filtered through a celite plug supported on a fritted glass filter funnel, and washed with additional CH_2Cl_2 (30 mL). The filtrate was cooled to 0°C after which $AlCl_3$ (0.54 g, 0.0041 mol) was added to the reaction flask and stirred for 30 minutes at 0°C, followed by an additional 44 hours at room temperature. The reaction mixture was then poured over ice, transferred to a separatory funnel, and the reaction mixture extracted 4 times with CH_2Cl_2. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by silica gel column chromatography (3:1 EtOAc:hexanes) to yield 0.044 g of product in 20.6% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 3.78 (1 H, d, J = 17.95 Hz), 3.89(3 H, s), 4.18 (1 H, t, J = 7.98 Hz), 4.62 (1 H, d, J = 18.05 Hz), 5.21 (1 H, t, J = 7.15 Hz), 6.96 (1 H, d, J = 8.42 Hz), 7.71 (1 H, d, J = 8.49 Hz).

IR (NaCl, CH_2Cl_2) 2919, 2848, 1756, 1706, 1574, 1458, 1437, 1271, 1241, 1034 cm^{-1}.

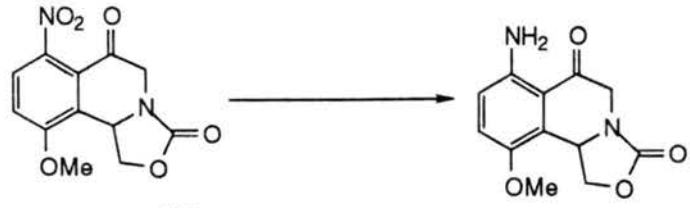


1-hydroxymethyl(2,2'carbonyl)-4-keto-7-nitro-8-methoxytetrahydroisoquinolone (101) and 1-hydroxymethyl(2,2'carbonyl)-4-keto-5-nitro-8-methoxytetrahydroisoquinolone (100)

Isoquinolone **67** (0.032 g, 0.00014 mol) and NH_4NO_3 (0.011 g, 0.00014 mol) was dissolved in CH_3CN (1 mL) with stirring. TFAA (0.5 mL) was added dropwise by syringe to the reaction vessel and stirred for 5 hours at room temperature. The reaction was quenched with H_2O (5 mL) and the aqueous layer was extracted 3 times with CH_2Cl_2. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by silica gel preparative thin layer chromatography (3:1 EtOAc:hexanes) to yield of 0.009 g of the 7-nitro product **101** in 24% yield and 0.023 g of the 5-nitro product **100** in 62% yield.

100: ^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 3.86 (1 H, d, J = 19.99 Hz), 3.97 (3 H, s), 4.24 (1 H, dd, J = 8.17 Hz, 1.00 Hz), 4.66 (1 H, d, J = 18.54 Hz), 4.99 (1 H, t, J = 9.10 Hz), 5.18 (1 H, t, J = 8.46), 7.12 (1 H, d, J = 8.79 Hz), 7.55 (1 H, d, J = 8.72 Hz). IR (NaCl, CH_2Cl_2) 3095, 2946, 2852, 1756, 1711, 1580, 1535, 1419, 1277, 1035 cm^{-1}.

101: ^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 3.87 (1 H, d, J = 19.93 Hz), 3.94 (3 H, s), 4.29 (1 H, dd, J = 8.34 Hz, 0.89 Hz), 4.72 (1 H, d, J = 18.42 Hz), 5.06 (1 H, t, J = 8.60), 5.27 (1 H, t, J = 8.72 Hz), 7.86 (1 H, d, J = 8.60 Hz), 7.96 (1 H, d, J = 8.57 Hz). IR (NaCl, CH_2Cl_2) 2925, 2852, 1760, 1712, 1592, 1532, 1423, 1351, 1294, 1271, 1236, 1113, 1021 cm^{-1}.



100 102

1-hydroxymethyl(2,2'carbonyl)-4-keto-5-amino-8-methoxytetrahydroisoquinolone (102)

To a solution of nitroisoquinolone **100** (0.065 g, 0.00023 mol) dissolved in EtOH (10 mL) was added stannous chloride (0.264 g, 0.0012 mol, 5 eq.). The reaction was heated to reflux under an atmosphere of N_2 with stirring for 1.5 hours. The reaction was allowed to cool, quenched with H_2O, and the pH of the reaction adjusted to 8 with 1 M NaOH. The reaction was extracted 5 times with CH_2Cl_2, the organic layers were combined, dried over $MgSO_4$, filtered, and concentrated to yield 0.053 g of crystalline product in 91.2 % yield. The product was carried on in crude form.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 3.79 (3 H, s), 3.82 (1 H, d, J = 17.80 Hz), 4.20 (1 H, t, J = 8.49 Hz), 4.54 (1 H, t, J = 17.88 Hz), 4.99 (1 H, t, J = 8.80 Hz), 5.13 (1 H, t, J = 8.61 Hz), 6.20 (2 H, bs), 6.63 (1 H, d, J = 9.13 Hz), 7.05 (1 H, d, J = 9.09 Hz).

IR (NaCl, CH_2Cl_2) 3449, 3339, 2936, 2838, 1748, 1656, 1557, 1442, 1260, 1188, 1033 cm^{-1}.

110

5-chloro-*ortho*-anisaldehyde (110)

Commercially available 5-chloro-salicylaldehyde (17.7 g, 0.11 mol) was dissolved in THF (400 mL) and cooled to 0°C. Potassium *t*-butoxide (23.9 g, 0.21 mol) was added to the reaction vessel and after stirring for 10 minutes MeI (13.3 mL, 0.213 mol) was added. The

reaction vessel was fitted with a reflux condenser and the reaction heated to reflux for 8 hours with stirring. The reaction was allowed to cool to room temperature and quenched with H_2O (400 mL). Saturated NaCl (100 mL) was added to the aqueous layer and was extracted 3 times with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. Following recrystallization (9:1 hexanes:EtOAc) 15.9 g of a white crystalline product was obtained in 82.2% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 3.86 (3 H, s), 6.73 (1 H, d, J = 8.76 Hz), 7.16 (1 H, dd, J = 8.73 Hz, J = 2.64 Hz), 7.70 (1 H, d, J = 2.76 Hz), 10.33 (1 H, s).

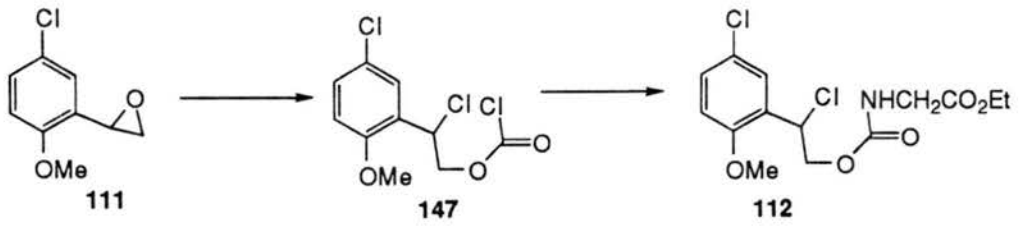
IR (NaCl, CH_2Cl_2) 3098, 2942, 2905, 2839, 1682, 1595, 1481, 1389, 1245, 1179, 1022, 963, 824 cm^{-1}.

111

(5-chloro-2-methoxyphenyl)oxirane (111)

A nonhomogenous mixture of 5-chloro-*o*-anisaldehyde (15.9 g, 0.093 mol), trimethylsulfonium iodide (22.8 g, 0.112 mol), tetrabutylammonium iodide (0.34 g, 0.0009 mol), methylene chloride (160 mL), and 50% NaOH (105 mL) was stirred vigorously at room temperature for 4 days. The reaction mixture was transferred to a separatory funnel and the aqueous NaOH was allowed to settle (bottom layer) and drained. The organic layer was then carefully washed with water, dried over $MgSO_4$, filtered, concentrated, and purified by high vacuum short-path distillation to yield 13.1 g of a clear oil in 76.0 % yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 2.64 (1 H, q, J = 2.61 Hz), 3.12 (1 H, q, J = 4.17 Hz), 3.85 (3 H, s), 4.11 (1 H, q, J = 2.56 Hz), 6.78 (1 H, d, J = 8.72 Hz), 7.11 (1 H, d, J = 2.67 Hz), 7.18 (1 H, dd, J = 8.73, J = 2.73 Hz).

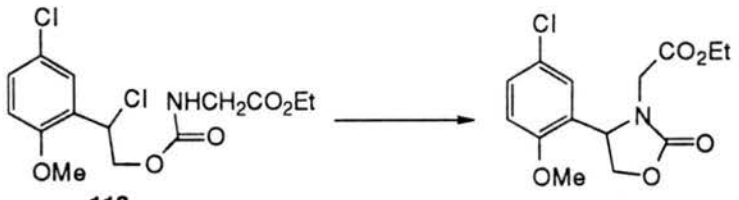


O-2-chloro-2-(5'-chloro-2'-methoxyphenyl)-N-ethoxyacetylcarbamate (112)

To a solution of **111** (19.4 g, 0.105 mol) in toluene (100 mL) was added 150 mL of a 20% phosgene solution. The sealed reaction was stirred for 42 hours at room temperature. The reaction flask was purged of phosgene by bubbling nitrogen through the system until the reaction mixture was concentrated to half of its original volume. Product **147** was then concentrated in vacuum to yield 27.2 g of an oil which was dissolved in CH_2Cl_2 (450 mL) and added to a saturated solution of $NaHCO_3$ (450 mL). Glycine ethyl ester hydrochloride (17.42 g, 0.13 mol) dissolved in a minimum volume of water was then added and the nonhomogenous mixture was vigorously stirred at room temperature for 20 minutes. The organic layer was separated from the aqueous layer and washed twice with water, dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by silica gel column chromatography (1:1 EtOAc:hexanes) to yield 20.4 g of a white crystalline product in 60.7 % yield.

^1H NMR (300 MHz,$CDCl_3$ vs TMS) δ 1.23 (3 H, t, J = 8.63 Hz), 3.84 (3 H, s), 3.92 (2 H, m), 4.18 (2H, q, J = 1.31 Hz), 4.38 (2 H, m), 5.29 (1 H, bs), 5.46 (1 H, t, J = 5.90 Hz), 6.77 (1 H, d, J = 8.79 Hz), 7.21 (1 H, dd, J = 8.87 Hz, J = 2.47 Hz), 7.43 (1 H, d, J = 2.57 Hz).

IR (NaCl, CH_2Cl_2) 3356, 2981, 2942, 1731, 1573, 1490, 1278, 1253, 1203, 1027 cm^{-1}.

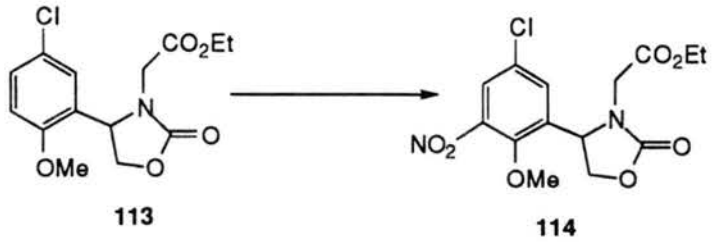


1-(carboethoxy)methyl-5-(5'chloro-2'-methoxy)phenyloxazolidin-2-one (113)

To a solution of **112** (20.3 g, 0.058 mol) in THF (175 mL) cooled to 0°C was added potassium *t*-butoxide (7.16 g, 0.064 mol) in THF (100 mL) and stirred for 45 minutes at 0°C. The reaction was quenched with 5% HCl (200 mL). The product was extracted 3 times with CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated. The product was purified by silica gel column chromatography (3:2 hexanes:EtOAc) to yield 14.8 g of a crystalline product in 81.3% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.20 (3 H, t, J = 7.10 Hz), 3.37 (1 H, d, J = 17.96 Hz), 4.13 (3 H, s), 4.03-4.20 (3 H, m), 4.30 (1 H, d, J = 17.70 Hz), 4.65 (1 H, t, J = 8.62 Hz), 5.26 (1 H, q, J = 6.36 Hz), 6.81 (1 H, d, J = 8.77 Hz), 7.12 (1H, d, J = 2.56 Hz), 7.24 (1 H, dd, J = 8.71 Hz, J = 2.56 Hz).

IR (NaCl, CH_2Cl_2) 2978, 2941, 1748, 1486, 1428, 1252, 1208, 1026 cm^{-1}.



1-(carboethoxy)methyl-5-(5'chloro-3'-nitro-2'-methoxy)phenyloxazolidin-2-one (114)

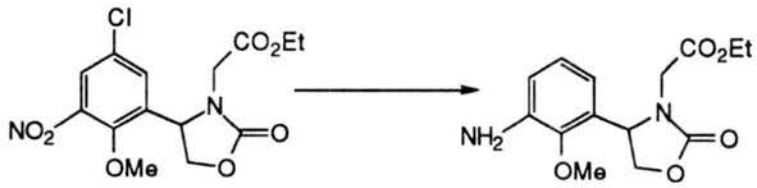
To a stirred solution of **113** (5.14 g, 0.018 mol) in CH_3CN (28 mL) cooled to 0°C was added NH_4NO_3 (2.17 g, 0.027 mol) followed by excess TFAA (7.5 mL) in a dropwise manner by syringe. After 15 minutes the ice bath was removed and the reaction was allowed to warm to room temperature. After 10 hours the reaction was poured over an ice/water mixture and the aqueous layer was extracted 3 times with CH_2Cl_2. The organic

layers were combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction was then redissolved in CH_2Cl_2 and passed through a plug of silica gel to yield 5.36 g of a yellow crystalline product in 83% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.22 (3 H, t, J = 4.2 Hz), 3.39 (1 H, d, J = 17.98 Hz), 3.84 (3 H, s), 4.08-4.27 (3 H, m), 4.34 (1 H, d, J = 17.97 Hz), 4.75 (1 H, t, J = 8.97 Hz), 5.41 (1 H, q, J = 6.48 Hz), 7.49 (1 H, d, J = 2.70 Hz), 7.82 (1 H, d, J = 2.62 Hz).

^{13}C NMR ($CDCl_3$) δ 14.04, 43.71, 53.75, 61.81, 63.34, 68.94, 126.03, 130.15, 131.62, 135.60, 143.84, 150.56, 158.23, 168.00.

IR (NaCl, CH_2Cl_2) 3082, 2984, 1765, 1746, 1537, 1476, 1431, 1351, 1211, 1094, 1025, 984 cm^{-1}.



114 **115**

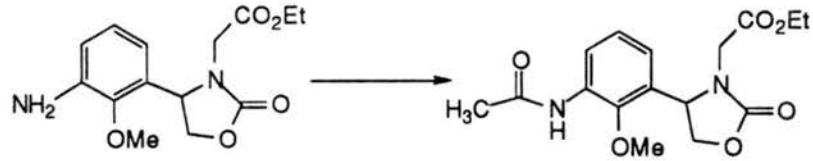
1-(carboethoxy)methyl-5-(3'-amino-2'-methoxy)phenyloxazolidin-2-one (115)

To a stirred solution of **114** (0.045 g, 0.15 mmol) in MeOH (7 mL) with stirring was added ammonium formate (0.048 g, 0.77 mmol) and 5% Pd/C (0.035 g). The reaction was stirred for 24 hours under an atmosphere of nitrogen after which it was passed through a plug of celite which was rinsed with MeOH. The product was concentrated to yield 0.035 g of a brownish crystalline product in 99% yield which did not require further purification.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.19 (3 H, t, J = 7.12 Hz), 3.36 (1 H, d, J = 17.91 Hz), 3.63 (3 H, s), 3.89 (2 H, bs), 4.11 (3 H, m), 4.22 (1 H, d, J = 17.98 Hz), 4.65 (1 H, t, J = 8.60 Hz), 5.35 (1 H, t, J = 7.48 Hz), 6.54 (1 H, dd, J = 7.80 Hz, J = 1.49 Hz), 6.68 (1 H, dd, J = 7.70 Hz, J = 1.51 Hz), 6.92 (1 H, t, J = 7.8 Hz).

^{13}C NMR ($CDCl_3$) δ 13.92, 43.18, 53.79, 59.98, 61.22, 69.55, 115.73, 116.41, 125.53, 129.76, 140.47, 145.56, 158.50, 168.34.

IR(NaCl, CH_2Cl_2) 3468, 3362, 2983, 2939, 1745, 1621, 1488, 1424, 1316, 1212, 1098, 1026, 998 cm^{-1}.



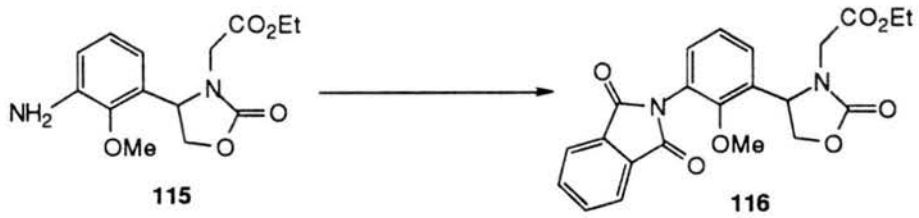
1-(carboethoxy)methyl-5-(2'-methoxy-3'-N-acetyl)phenyloxazolidin-2-one

To a stirred solution of **115** (0.10 g, 0.00038 mol) in THF (5 ml) at 0°C under an N_2 atmosphere was added acetyl chloride (0.11 mL, 0.0015 mol) by syringe. The reaction was allowed to warm to room temperature and stirred for 14 hours. The reaction was quenched with H_2O and the aqueous layer was extracted 3 times with CH_2Cl_2, the organic layers combined, and dried over $MgSO_4$. The crude reaction was filtered, concentrated, and purified by silica gel preparative thin layer chromatography (3:1 EtOAc:hexanes) to yield 0.31 g of a white foamy product in 62% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.25 (3 H, t, J = 7.19 Hz), 2.24 (3 H, s), 3.35 (1 H, d, J = 17.98 Hz), 3.72 (3 H, s), 4.17-4.22 (3 H, m), 4.30 (1 H, d, J = 18.03 Hz), 4.74 (1 H, t, J = 8.69 Hz), 5.45 (1 H, t, J = 8.90 Hz), 7.01 (1 H, d, J = 7.69 Hz), 7.22 (1 H, t , J = 8.01 Hz), 7.54 (1 H, bs), 8.28 (1H, d, J = 7.80 Hz).

^{13}C NMR ($CDCl_3$) δ 13.86, 24.43, 43.06, 53.69, 61.28, 61.75, 69.35, 121.81, 122.09, 125.64, 129.42, 131.87, 147.86, 158.26, 168.18, 168.30.

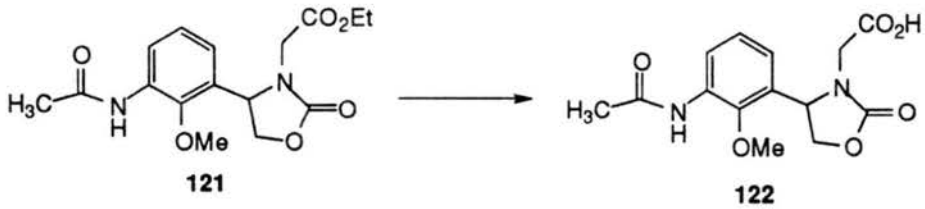
IR (NaCl, CH_2Cl_2) 3313, 2983, 2936, 1760, 1744, 1605, 1527, 1477, 1427, 1346, 1373, 1210, 1096, 1025, 995, 763 cm^{-1}.



1-(carboethoxy)methyl-5-(2'-methoxy-3'-N-pthalimido)phenyloxazolidin-2-one (116)

To a stirred solution of **115** (0.07 g, 0.30 mmol) partially dissolved in THF (7 mL) under an N_2 atmosphere was added pthaloyl dichloride (0.06 mL, 0.375 mmol) by syringe. The reaction vessel was fitted with a condenser and heated to reflux for 2 hours. The reaction was allowed to cool and quenched with H_2O. The aqueous layer was extracted 3 times with CH_2Cl_2, the organic layers combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction mixture was purified by silica gel preparative thin layer chromatography (3:1 EtOAc:hexanes) to yield 0.05 g of product in 43% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.26 (3 H, t, J = 7.17 Hz), 3.47 (1 H, d, J = 18.03 Hz), 3.60 (3 H, s), 4.14-4.23 (3 H, m), 4.35 (1 H, d, J = 18.02 Hz), 4.78 (1H, t, J = 8.78 Hz), 5.45 (1 H, dd, J = 6.93 Hz, J = 2.11 Hz), 7.28-7.37 (2 H, m), 7.41(1 H, dd, J = 5.42 Hz, J = 2.08 Hz), 7.84 (2 H, m), 7.98 (2 H, m).



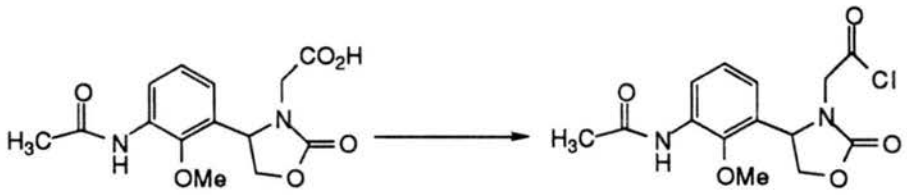
1-(carboxy)methyl-5-(2'-methoxy-3'-N-acetyl)phenyloxazolidin-2-one (122)

Ester **121** (0.44g, 0.0013 mol) was dissolved in EtOH (30 mL) and cooled to 0°C with and ice/H_20 bath with stirring. A 1M NaOH solution (3.0 mL, 0.003 mol, 2.3 eq.) was added and the reaction stirred for 45 minutes. The reaction was concentrated, redissolved in H_2O (10 mL), and then washed 2 times with CH_2Cl_2. The pH of the aqueous layer

was then adjusted to 7 with 1M HCl and the aqueous layer lyophilized. The solid product was triturated with MeOH and filtered to yield 0.40 g of product in 84.4% yield.

^{1}H NMR (300 MHz, MeOH-d_4 vs TMS) δ 2.18 (3 H, s), 3.39 (1 H, d, J = 18 Hz), 3.72 (3 H, s), 4.14 (2 H, m), 4.75 (1 H, t), 5.38 (1H, t), 7.13 (2H, m), 7.82 (1H, d, J = 6.3 Hz).

^{1}H NMR (300 MHz, DMSO-d_6 vs TMS) δ 2.09 (3 H, s), 2.75 (1 H, d, J = 13.86 Hz), 3.60 (3 H, s), 3.86 (1 H, t, J = 7.72 Hz), 4.60 (1 H, t, J = 8.46), (1 H, d, J = 18 Hz), 5.48 (1 H, t, J = 8.75 Hz), 6.96 (1 H, d, J = 7.49 Hz), 7.12 (1 H, t, J = 7.90 Hz), 7.84 (1H, d, J = 7.66 Hz), 9.43 (1 H, s).



1-(carbochloro)methyl-5-(3'-N-acetyl-2'-methoxy)phenyloxazolidin-2-one (123)

A solution of **122** (0.13 g, 0.00042 mol) was dissolved in excess $SOCl_2$ (5 mL) and cooled to 4°C. The reaction was stirred for 18 hours, concentrated, dissolved in CH_2Cl_2, and filtered through a plug of celite to yield 0.09 g of product in 67% yield.

^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 2.22 (3 H, s), 3.71 (3.5 H, s), 3.60 (3 H, s), 3.79 (0.5 H, s), 4.20 (1 H, t, J = 8.34 Hz), 4.73 (2 H, m), 5.31 (1 H, t, J = 7.84 Hz), 6.96 (1 H, d, J = 8.83 Hz), 7.54 (1 H, bs), 8.32 (1H, d, J = 8.92 Hz).

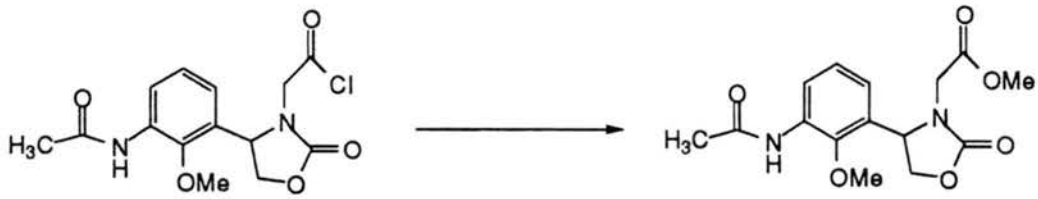
113 148

1-(carboxy)methyl-5-(5'-chloro-2'-methoxy)phenyloxazolidin-2-one (148)

A solution of **113** (0.2 g, 0.63 mmol) in 95% EtOH (10 mL) was cooled to 0°C with stirring. A solution of 2 M LiOH (0.7 mL) was added to the reaction flask and stirred for 1 hour at 0°C. The reaction was concentrated and redissolved in 1 M NaOH (4 mL). The aqueous layer was washed 2 times with CH_2Cl_2 and acidified to approximately pH 3 with 1 M HCl. The product was extracted 3 times with EtOAc, dried over $MgSO_4$, filtered, and concentrated to yield 0.16 g of a white crystalline product in 86.7% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 3.44 (1 H, d, J = 18.34 Hz), 3.80 (3 H, s), 4.11 (1 H, t, J = 8.41 Hz), 4.36 (1 H, d, J = 18.32 Hz), 4.70 (1 H, t, J = 8.81 Hz), 5.29 (1H, q), 6.82 (1 H, d, J = 8.78 Hz), 7.14 (1 H, d, J = 2.56 Hz), 7.26 (1 H, dd, J = 8.74 Hz, J = 2.57 Hz).

IR (NaCl, CH_2Cl_2) 3585, 2932, 2847, 1732, 1441, 1251, 1200, 1025 cm^{-1}.

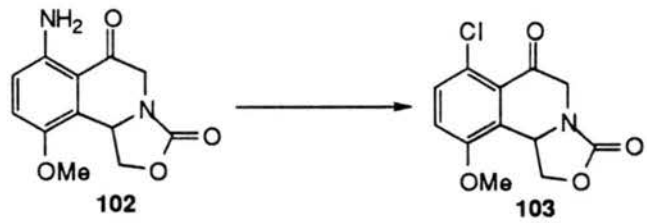


123 149

1-(carbomethoxy)methyl-5-(3'-N-acetyl-2'-methoxy)phenyloxazolidin-2one (102)

To **123** (0.01 g, 0.031 mmol) was added MeOH (5 mL) and stirred for 4 hours. The reaction was quenched with H_2O, concentrated, and extracted 3 times with CH_2Cl_2. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated to yield 0.008 g of product in 83% yield requiring no further purification.

^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 2.22 (3 H, s), 3.37 (1 H, d, J = 17.80 Hz), 3.69 (6 H, s), 4.09 (1 H, t, J = 8.62 Hz), 4.73 (1 H, t, J = 8.85 Hz), 4.73 (1 H, t, J = 8.85 Hz), 5.41 (1 H, t, J = 8.21 Hz), 7.00 (1 H, d, J = 7.65 Hz), 7.17 (1 H, t , J = 7.99 Hz), 7.58 (1 H, bs), 8.26 (1H, d, J = 8.01 Hz).



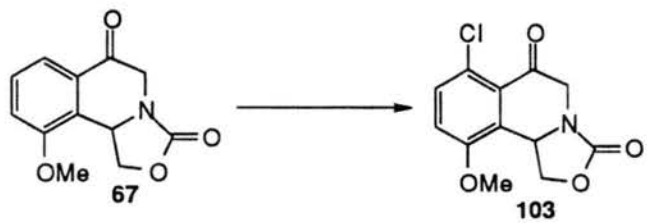
1-hydroxymethyl(2,2'carbonyl)-4-keto-5-chloro-8-methoxytetrahydroisoquinolone (103)

Copper (II) chloride (0.016 g, 0.12 mmol) and *t*-butyl nitrite (0.018 mL, 0.15 mmol) were dissolved in CH_3CN (2 mL) under N_2 and cooled to 0^oC with an ice/H_2O bath. Amine **102** dissolved in CH_3CN (2 mL) was added dropwise by syringe to the reaction vessel over a 5 minute period. The reaction was stirred for 2 hours at 0^oC, and an additional 8 hours at room temperature. The reaction was quenched with 20% HCl, the aqueous layer extracted 4 times with CH_2Cl_2, combined, dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by preparative thin layer chromatography (3:2 EtOAc:hexanes) to yield 0.01 g of product in 37% yield.

148 103

1-hydroxymethyl(2,2'carbonyl)-4-keto-5-chloro-8-methoxytetrahydroisoquinolone (103)

To a solution of **148** (0.058g, 0.0002 mol) in CH_2Cl_2 (10 mL) was added DMF (0.20 mL) by syringe. Oxalyl chloride (0.04 mL, 0.0004 mol) was diluted with CH_2Cl_2 (2 mL) and added dropwise by syringe to the reaction flask at room temperature. The reaction was stirred for 2 hours, concentrated, and redissolved in CH_2Cl_2 (10 mL), after which alkaline activated carbon was added and vigorously stirred for 1 minute. The solution was filtered through a celite plug supported on a fritted glass filter funnel, and washed with additional CH_2Cl_2 (10 mL). The filtrate was cooled to 0°C after which $AlCl_3$ (0.11 g, 0.0008 mol) was added to the reaction flask and stirred for 30 minutes at 0°C, followed by an additional 24 hours at room temperature. The reaction mixture was then poured over ice and the reaction mixture extracted 3 times with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by silica gel column chromatography (3:2 EtOAc:hexanes) to yield 0.017 g of product in 32.0% yield.



67 103

1-hydroxymethyl(2,2'carbonyl)-4-keto-5-chloro-8-methoxytetrahydroisoquinolone (103)

Isoquinolone **67** (1.1g, 0.005 mol) and $FeCl_3$ (0.3g, 0.002 mol) was dissolved CH_2Cl_2 (250 mL) and cooled to 0°C. Chlorine gas was bubbled through the reaction solution for 5

minutes and the reaction was stirred at 0°C for 45 minutes and 30 minutes at room temperature. The reaction volume was then reduced to approximately 200 mL and saturated $NaHCO_3$ (20 mL) was added. The reaction mixture was diluted with water (200 mL) and extracted four times with CH_2Cl_2. The organic layers were combined, dried over $MgSO_4$, filtered and concentrated. The product was isolated by flash silica gel column chromatography (7:3 EtOAc:hexanes). Following purification, 0.83g of product was obtained as a white crystalline product in 66% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 3.81 (1 H, d, J = 17.90 Hz), 3.87 (3 H, s), 4.13 (1 H, t, J = 8.10 Hz), 4.56 (1 H, d, J = 18.46 Hz), 4.94 (1 H, t, J = 8.95 Hz), 5.15 (1 H, d, J = 6.89 Hz), 7.03 (1 H, d, J = 8.87 Hz), 7.41 (1 H, d, J = 8.94 Hz).

^{13}C NMR ($CDCl_3$) δ 49.33, 51.76, 56.18, 69.37, 115.90, 126.21, 127.83, 131.99,132.56, 153.53, 156.41, 188.06.

IR (NaCl, CH_2Cl_2) 3083, 2943, 2843, 1761, 1705, 1577, 1462, 1440, 1274, 1240, 1034, 823 cm^{-1}.

HRMS (FAB) calc. for $C_{12}H_{10}NO_4Cl$ (MH^+): 267.0298. found 267.0283.

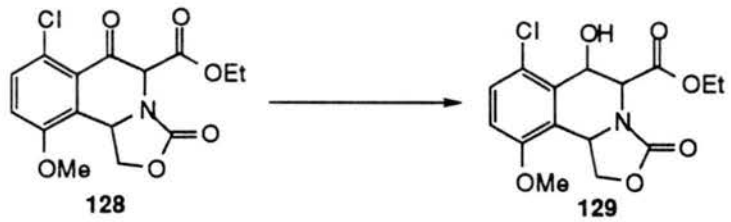
1-hydroxymethyl(2,2'-carbonyl)-3-carboethoxy-4-keto-5-chloro-8-methoxytetrhydroisoquinolone (128)

Chloroisoquinolone **103** (0.052 g, 0.0002 mol) was dissolved in THF (2mL) and HMPA (0.2 mL) and cooled to -78°C under N_2. To the reaction was added a 1M solution of $(Si(CH_3)_3)_2NLi$ (0.22 mL, 0.22 mmol) in THF *via* syringe and the reaction was stirred for 45 minutes after which ethyl cyanoformate (0.03 mL, 0.0003 mol) was added and the reaction was stirred for an additional 2 hours. The reaction was quenched with saturated NH_4Cl (2 mL) and allowed to warm to room temperature. The reaction was diluted with

H_2O (5 mL) and the reaction mixture was extracted 3 times with CH_2Cl_2. The organic layers were combined and washed one time with H_2O and with brine. The organic layer was dried over $MgSO_4$, filtered, concentrated and the product purified by silica gel preparative thin layer chromatography (1:1hexanes:EtOAc). Following purification 28 mg of product was obtained in a 43% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.24 (3 H, t, J = 7.07 Hz), 3.88 (3 H, s), 4.10-4.22 (3 H, m), 5.01 (1 H, t, J = 8.97 Hz), 5.28 (1 H, s), 5.54 (1 H, t, J = 8.87), 7.04 (1 H, d, J = 8.87 Hz), 7.43 (1 H, d, J = 8.82 Hz).

IR (NaCl, CH_2Cl_2) 3084, 2979, 2942, 1766, 1711, 1578, 1463, 1438, 1275, 1219, 1205, 1084, 1024, 823 cm^{-1}.



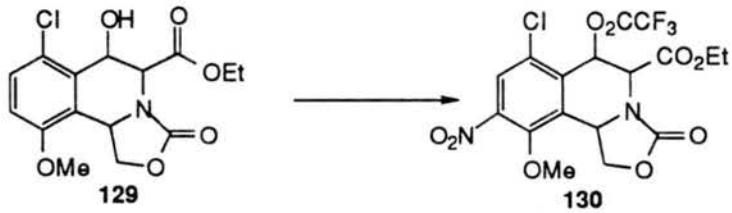
1-hydroxymethyl(2,2'-carbonyl)-3-carboethoxy-4-hydroxy-5-chloro-8-methoxytetrahydroisoquinolone (129)

β-keto ester **128** (0.037g, 0.00011 mol) was dissolved in MeOH (5 mL) and cooled to 0°C under an atmosphere of N_2. To the reaction vessel was added $NaBH_4$ (0.008g, 0.00022 mol) after which the reaction was allowed to warm to room temperature and stirred for 11 hours. To the reaction was added 1M HCl (2 mL) and the reaction was concentrated. To the reaction mixture was added H_2O (10 mL) and the aqueous layer was extracted 3 times with CH_2Cl_2. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. Following purification by silica gel preparative thin layer chromatography (1:1 hexanes:EtOAc) 0.031 g of a white crystalline product was obtained in 82% yield.

^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.27 (3 H, t, J = 7.11 Hz), 3.05 (1 H, d, D_2O exchange, J = 4.52 Hz), 3.81 (3 H, s), 4.15-4.29 (3 H, m), 5.34 (1 H, t, J = 8.82 Hz), 5.52 (1 H, q, J = 4.50 Hz), 6.79 (1 H, d, J = 8.88 Hz), 7.30 (1 H, d, J = 8.85 Hz).

^{13}C NMR ($CDCl_3$) δ 14.10, 50.39, 55.21, 55.82, 61.71, 63.50, 69.63, 111.66, 125.20, 126.44, 130.03, 133.56, 154.11, 156.53, 168.42.

IR (NaCl, CH_2Cl_2) 3398, 2937, 2843, 1738, 1584, 1475, 1440, 1262, 1198, 1093, 1060, 1027 cm^{-1}.

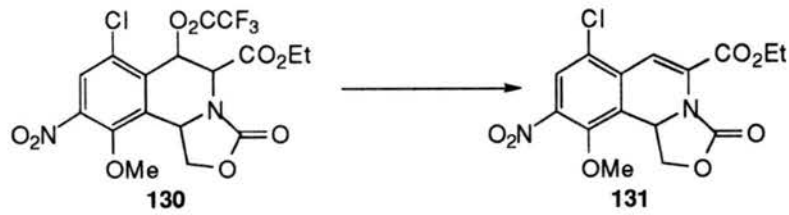


1-hydroxymethyl(2,2'-carbonyl)-3-carboethoxy-4-triflouroacetate-5-chloro-7-nitro-8-methoxytetrahydroisoquinolone (130)

β-hydroxy ester **129** (0.12, 0.0004 mol, 1 eq.) was dissolved in CH_3CN (2 mL) to which was added NH_4NO_3 (0.04g, 0.0005 mol, 1.5 eq.) and cooled to 0°C. To the reaction vessel was added TFAA (1 mL) *via* syringe and the reaction was slowly allowed to warm to room temperature. The reaction was stirred for 18 hours after which it was quenched with a 1M HCl/ice slurry. The aqueous layer was extracted 3 times with CH_2Cl_2 and the organic layers were combined. The organic layer was dried over $MgSO_4$, filtered, and concentrated to yield 0.12g of product in 70% yield which was carried on in crude form to the following reaction.

^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.26 (3 H, t, J = 7.19 Hz), 4.02 (3 H, s), 4.17 (3 H , m), 5.02 (1 H, t, J = 9.07 Hz), 5.36 (1 H, t, J = 8.96 Hz), 5.74 (1 H, t, J = 8.76 Hz), 6.62 (1 H, d, J = 8.97)), 7.93 (1 H, s).

IR (NaCl, CH_2Cl_2) 3090, 2986, 2944, 1766, 1748, 1661, 1537, 1466, 1440, 1414, 1349, 1276, 1202, 1171, 1107, 1021, 949, 848, 825 cm^{-1}.



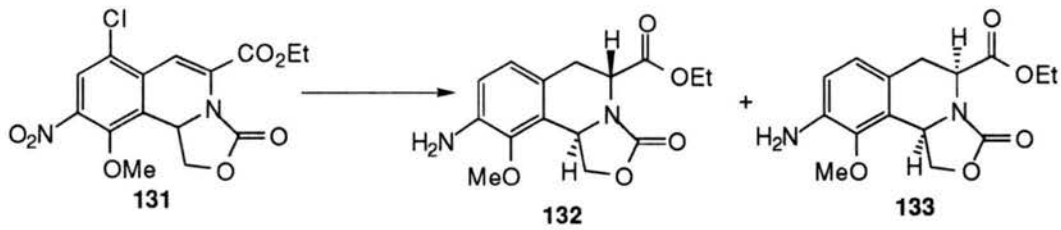
1-hydroxymethyl(2,2'-carbonyl)-3-carboethoxy-4-triflouroacetate-5-chloro-7-nitro-8-methoxy-1,2-dihydroisoquinolone (131)

To a 0°C solution of **130** (6 mg, 0.012 mmol) dissolved in dry THF (3 mL) was added TEA (0.006 mL, 0.05 mmol, 4 eq.). The reaction was stirred for 3 hours after which the reaction was quenched with H_2O. The aqueous layer was extracted 3 times with CH_2Cl_2 and the organic layers were combined. The organic layer was dried over $MgSO_4$, filtered, and concentrated. The crude reaction was purified by silica gel preparative thin layer chromatography (3:2 hexanes:EtOAc) to yield 3.4 mg of product in 74% yield.

^1H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.36 (3 H, t, J = 7.19 Hz), 3.85 (3 H, s), 4.34 (2 H , q), 4.65 (1 H, t, J = 9.45 Hz), 5.13 (1 H, t, J = 9.45 Hz), 5.45 (1 H, t, J = 8.91 Hz), 7.15 (1 H, s), 7.91 (1 H, s).

^{13}C ($CDCl_3$) δ 14.39, 53.82, 62.40, 62.90, 70.56, 115.89, 127.03, 127.86, 131.20, 132.01, 133.87, 142.50, 148.52, 154.29, 161.85.

IR (NaCl, CH_2Cl_2) 3083, 2978, 2924, 1777, 1731, 1465, 1300, 1243, 1092, 1039, 958, 752, 735 cm^{-1}.



***trans*-1-hydroxymethyl(2,2'-carbonyl)-3-carboethoxy--7-amino-8-methoxytetrahydroisoquinolone (132) and *cis*-1-hydroxymethyl(2,2'-carbonyl)-3-carboethoxy--7-amino-8-methoxytetrahydroisoquinolone (133)**

To a stirred solution of **131** in MeOH (5 mL) was added NH_4CO_2H (22.0 mg, 0.035 mmol, 10 eq.) and 5% Pd/C (100 mg). The solution was stirred under N_2 for 22 hours

after which the crude reaction was filtered through celite and rinsed with MeOH. The crude reaction was then concentrated and purified by silica gel preparative thin layer chromatography (3:1 EtOAc:hexanes) to yield 3.2 mg of **132** in 29.9% yield and 3.0 mg of **133** in 28.0% yield.

133: ^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.18 (3 H, J = 7.11 Hz), 2.94 (1 H, dd, J = 15.63 Hz, J = 5.37 Hz), 3.68 (3 H, s), 4.11 (2 H, q), 4.34 (1 H, t, J = 6.03 Hz), 4.87 (1 H, t, J = 8.69 Hz), 4.93 (1 H, t, J = 8.15 Hz), 6.62 (1 H, d, J = 7.93 Hz), 6.73 (1 H, d, J = 8.03 Hz).

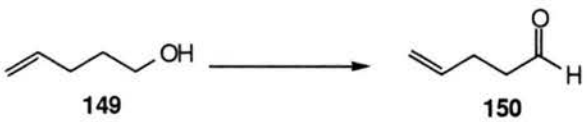
IR (NaCl, CH_2Cl_2) 3450, 3359, 2924, 2852, 1750, 1622, 1498, 1283, 1236, 1201, 1083, 1036 cm^{-1}.

HRMS (FAB) calc. for $C_{15}H_{19}N_2O_5$ (MH^+): 307.1294. found 307.1296.

132: ^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.20 (3 H, J = 7.24 Hz), 3.11 (1 H, m), 3.70 (3 H, s), 4.06 (3 H, m), 4.89 (1 H, t, J = 3.40 Hz), 4.97 (1 H, t, J = 8.72 Hz), 5.36 (1 H, t, J = 8.21 Hz), 6.63 (1 H, d, J = 8.31 Hz), 6.72 (1 H, d, J = 8.22 Hz).

IR (NaCl, CH_2Cl_2) 3447, 3359, 2923, 2851, 1738, 1625, 1501, 1415, 1240, 1204, 1074, 1024 cm^{-1}.

HRMS (FAB) calc. for $C_{15}H_{19}N_2O_5$ (MH^+): 307.1294. found 307.1295.



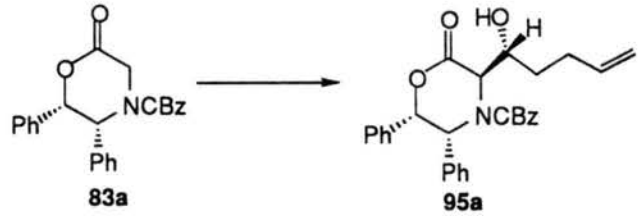
149 **150**

4-pentenal

A solution of PCC (7.76 g, 0.36 mol) and CH_2Cl_2 (75 mL) was vigorously stirred at room temperature. After stirring for 5 minutes **149** was added to the reaction vessel *via* syringe and stirred for 2 hours. The reaction mixture was filtered through a plug of celite, concentrated to 25% of its original volume, and filtered through a plug of silica gel. The

reaction mixture was concentrated to a volume of 40 mL and then purified by fractional distillation to yield 1.44 g of a clear pungent oil in 63.7% yield.

^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 2.44 (2 H, m), 2.52 (2 H, m), 5.06 (2 H, m), 5.82 (1 H, m), 9.78 (1 H, t).



83a **95a**

(3S, 5R, 6S)-4-(Benzyloxycarbonyl)-5,6-diphenyl-3-(1'R-4'-pentenyl)-2,3,5,6-tetrahydro-4*H*-1,4-oxazin-2-one (95a)

To a stirred solution of lactone **83a** dissolved in CH_2Cl_2 (18 mL) under a N_2 atmosphere at 0°C was added dibutyl boron triflate (3.5 mL, 3.5 mmol) followed by TEA (0.51 mL, 3.63 mmol). After 15 minutes the reaction mixture was cooled to -78°C. A solution of CH_2Cl_2 (2 mL) and 4-pentenal (**150**) (0.15 g, 1.81 mmol) was added by syringe and the reaction mixture was stirred for 1.25 hours at -78°C. The reaction mixture was quenched with a phosphate buffer solution (0.025M, pH=7) and poured over H_2O. The aqueous layer was extracted 3 times with CH_2Cl_2 and the organic layers combined and dried over $MgSO_4$, filtered, and concentrated. The crude reaction mixture was purified by flash silica gel column chromatography using an initial eluent system of 1:1 CH_2Cl_2:hexanes followed by 5:4:1 hexanes:CH_2Cl_2:EtOAc to yield 0.38 g of a white solid product in 66% yield.

^{1}H NMR (300 MHz) (d_6-DMSO, 393°K) TMS: 1.69-1.85 (2 H, m), 2.14-2.28 (2 H, m), 4.17 (2 H, m), 4.84 (1 H, d, J = 2.25 Hz), 4.95-5.18 (4 H, m), 5.25 (1 H, d, J = 3.18), 5.47 (1 H, d, J = 5.34 Hz), 5.82 -5.88 (1 H, m), 6.53 (1 H, d, J = 3.16 Hz), 6.60 (2 H, d, J = 7.24 Hz), 6.99-7.39 (15 H, m).

IR (NaCl, CH_2Cl_2) 3526, 3064, 3031, 2947, 1744, 1704, 1681, 1455, 1411, 1305, 1215, 1120, 1082, 1057 cm^{-1}.

Anal calc. for C, 63.87; N, 2.97; H, 6.23. found C, 64.04; N, 2.85; H, 6.14.

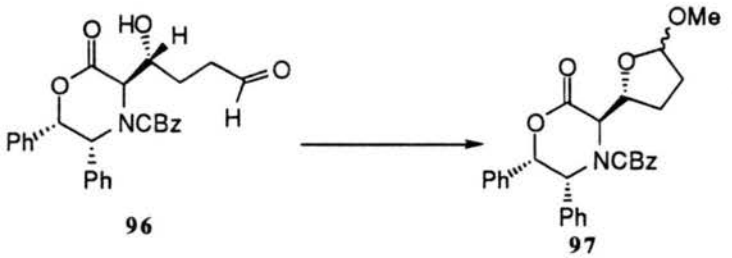
(3S, 5R, 6S)-4-(Benzyloxycarbonyl)-5,6-diphenyl-3-(1'R-4'-butanal)-2,3,5,6-tetrahydro-4*H*-1,4-oxazin-2-one (96)

Ozone was bubbled through a solution of **95a** (0.14 g, 0.30 mmol) and CH_2Cl_2 (5 mL) at -78°C for 10 minutes. The reaction mixture was then purged with N_2 until the solution was clear and allowed to warm to room temperature. The reaction mixture was quenched with excess Me_2S and after stirring for 12 hours was concentrated. The crude reaction was purified by column chromatography on silica gel (1:1 hexanes:EtOAc) and yielded 0.1 g of a white solid product in 69% overall yield.

^1H NMR (300 MHz) (d_6-DMSO vs TMS, 393°K) δ 2.04 (2 H, m), 2.29 (2 H, m), 4.53 (1 H, m), 4.99 (1 H , d, J = 2.52 Hz), 5.027 (4 H, m), 5.24 (2 H, m), 6.71 (1 H, d, J = 3.24 Hz), 7.02 (15 H, m), 9.70 (1 H, s).

IR (NaCl, CH_2Cl_2) 3467, 3063, 3033, 2953, 1751, 1670, 1455, 1404, 1269, 1241, 1173, 1062, 973, 736 cm^{-1}.

HRMS (FAB) calc. for $C_{28}H_{28}NO_6$ (MH^+): 474.1917. found: 474.1914.



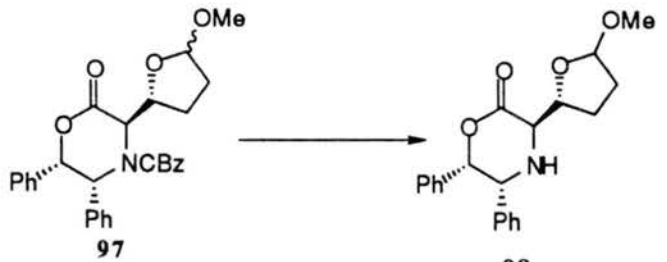
(3S, 5R, 6S)-4-(Benzyloxycarbonyl)-5,6-diphenyl-3-(1'R-4'-methoxy-tetrahydrofuran)-2,3,5,6-tetrahydro-4*H*-1,4-oxazin-2-one (97)

Dry HCl gas generated from H_2SO_4 and NaCl was bubbled through a stirred solution of **96** (0.062 g, 0.13 mmol) in MeOH (5 mL) at room temperature for 5 minutes. The reaction was allowed to stir for an additional hour and a white precipitate came out of

solution. The MeOH was decanted off, the precipitate rinsed with MeOH, and the white precipitate dried *in vacou* to yield 0.053 g in 83% yield of product which was carried on crude to the following reaction.

^{1}H NMR (300 MHz) (d_6-DMSO vs TMS, 393°K) δ 1.78-2.22 (4 H, m), 3.29 (3 H, s) 4.61 (1 H, m), 5.00-5.04 (5 H, m), 5.32 (1 H, d, J = 2.99 Hz), 6.36 (1 H, d, J = 2.99 Hz), 6.60 (2 H, d, J = 16.10 Hz), 7.06-7.35 (15 H, m).

IR (NaCl, CH_2Cl_2): 2958, 2888, 1723, 1652, 1488, 1463, 1406, 1364, 1300, 1255, 1114, 1024, 961 cm^{-1}.



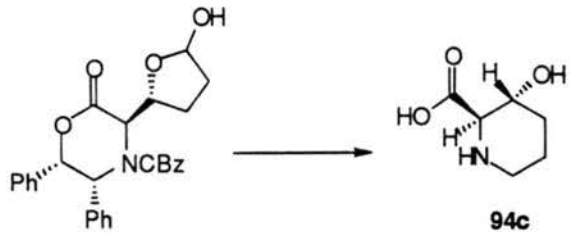
97 **98**

(3S, 5R, 6S)-4H-5,6-diphenyl-3-(1'R-4'-methoxy-tetrahydrofuran)-2,3,5,6-tetrahydro-4*H*-1,4-oxazin-2-one (98)

To a stirred solution of **97** (0.032 g, 065 mmol) in EtOAc (6 mL) was added 5% Pd/C (0.05 g). The reaction was then purged with H_2 gas and stirred for 1.5 hours under a H_2 atmosphere at 1 atmospheric pressure. The reaction vessel was purged with N_2, the reaction filtered through a plug of celite, and concentrated. The product was purified by silica gel preparative thin layer chromatography (5:4:1 hexanes:CH_2Cl_2:EtOAc) to yield 0.012 g of a white crystalline product in 52% yield.

^{1}H NMR (300 MHz, $CDCl_3$ vs TMS) δ 1.88-2.26(4 H, m), 2.65 (1 H, m) 3.32 (3 H, s), 4.18 (1 H, d, J = 2.93 Hz), 4.62 (1 H, m), 4.82 (1H, d, J = 3.00 Hz), 5.08 (1H, d, J = 3.18 Hz), 5.88 (1H, d, J = 3.01 Hz), 6.98-7.24 (10 H, m).

IR (NaCl, CH_2Cl_2): 3325, 3031, 2917, 2830, 1738, 1496, 1454, 1363, 1206, 1101, 1039, 950, 700 cm^{-1}.



96 94c

(1R, 2R)-1carboxy-2-hydroxypiperidine

To a solution of **96** (0.032 g, 0.068 mol) in EtOH (3 mL) and THF (2 mL) was added 5% Pd/C (0.05 g) with stirring. The solution was saturated with H_2 and stirred under an atmosphere of H_2 for 5 hours. The reaction mixture was purged with N_2, and filtered through celite. The reaction was concentrated and dissolved in H_2O (10 mL). The aqueous layer was washed 2 times with CH_2Cl_2 and then lyophilized to yield 0.0055 g of a white fluffy solid in 56.1% crude yield.

^1H NMR (300 MHz, D_2O vs TMS) δ 1.16-1.73 (2 H, m), 1.75-1.86 (2 H, m), 2.87-2.96 (1 H, m), 3.14-3.21 (1 H, m), 3.44 (1 H, d, J = 6.96 Hz), 3.94-4.00 (1 H, m).

HRMS (FAB) calc. for $C_6H_{12}NO_3$ (MH^+): 146.0817. found: 146.0822.

Chapter 3

A. Introduction

It is widely recognized that a variety of clinically significant antitumor antibiotics can mediate oxygen-dependent cleavage of the ribose phosphate backbone of both cellular DNA and RNA[61]. This oxidative strand scission is effected by a enormous array of structural motifs. However, the ability of many drugs to exercise oxidative damage to tumor DNA is often inseparable from the damage that these antitumor drugs effect on healthy DNA. Therefore, the recognition of new mechanisms for the production of oxygen-dependent cleavage, and methods to control this reactivity in harmony with other mechanisms of DNA toxicity is crucial for the development of more specific antitumor agents.

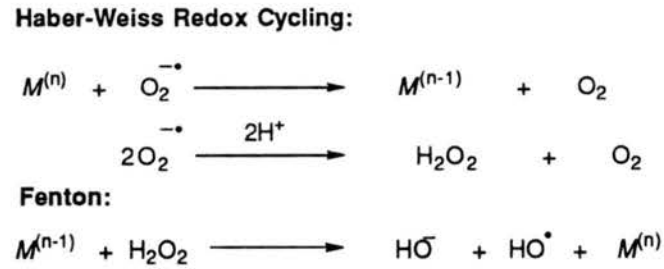
Oxidative strand scission occurs through three main families of reactions. Firstly, metal-mediated activation of molecular oxygen resulting in the formation of hydroxyl radical or other reactive oxygen species which subsequently effects strand scission[62]. Secondly, generation of a carbon radical in a non-metal dependent manner that results in hydrogen abstraction from the ribose backbone which ultimately reacts with molecular oxygen resulting in strand scission[63]. Finally, generation of hydroxyl radical *via* photolytic means without metal participation[64].

1. Superoxide and Oxidative Damage to DNA

Reduction of ground-state oxygen results in the initial formation of superoxide followed by hydrogen peroxide, hydroxyl radical, and water. Many readily oxidizable

organic structures are capable of reducing molecular oxygen to superoxide. Substances such as thiols and semiquinone radical have shown such activity[65].

Scheme 37:

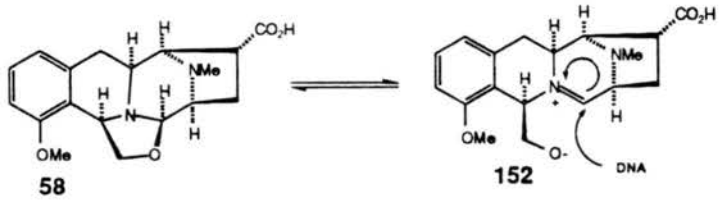


Lesko found that single strand scissions were produced in T7 DNA upon incubation with potassium superoxide as well as hydrogen peroxide[66]. Superoxide's ability to mediate DNA strand scission occurs by dismutation to hydrogen peroxide which can ultimately generate hydroxyl radical by means of a metal-catalyzed Haber-Weiss cycle and Fenton reaction[66]. Reduction of the metal ion occurs by oxidation of superoxide to molecular oxygen. Hydrogen peroxide is produced by dismutation of superoxide. Hydrogen peroxide, in the presence of a reduced metal species, can undergo Fenton chemistry resulting in the formation of the extremely reactive hydroxyl radical and hydroxide ion.

2. O_2-Dependent Cleavage of DNA by Quinocarcin

The pharmacological activity of quinocarcin, which is structurally similar to tetrazomine, has been the focus of considerable research. Quinocarcin has shown moderate activity against Gram-positive strains of bacteria and the citrate salt of quinocarcin, quinocarmycin citrate, has been active against several carcinomas[35a]. Thus, quinocarcin's mechanistic interaction with DNA has been of considerable interest.

Scheme 38:



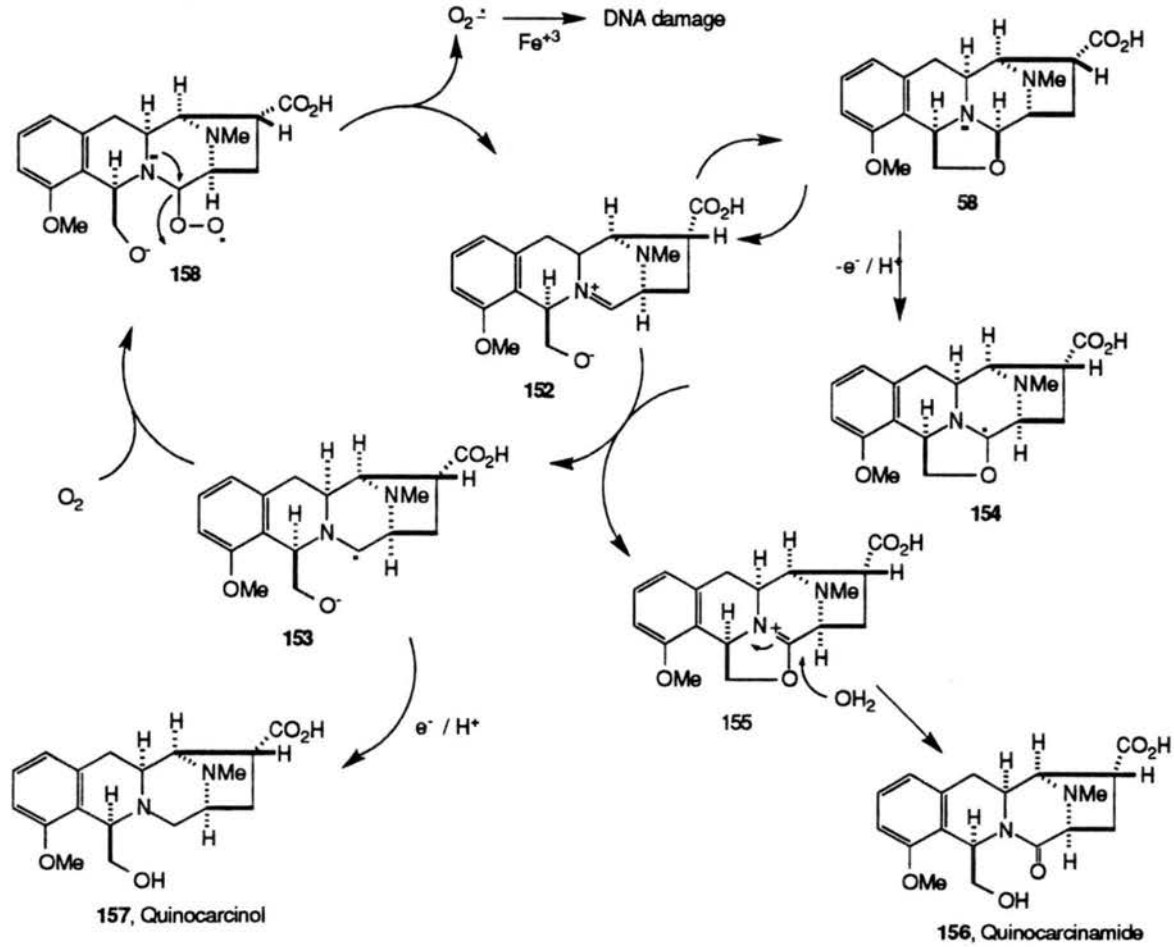
A DNA-alkylation mechanism has been invoked for quinocarcin's interaction with DNA based on molecular mechanic calculations[67]. Remers has proposed that quinocarcin alkylates DNA in the minor groove *via* the ring-opened oxazolidine iminium ion **152**. Using the representative DNA segment d(ATGCAT)2 it is believed that the iminium ion binds noncovalently in the minor groove followed by alkylation of the 2-amino group of guanine. Oxidative damage by quinocarcin has also been reported.

It was reported by Tomita that quinocarcin is capable of cleaving plasmid DNA in an O_2-dependent fashion[35a]. Free radical scavengers, superoxide dismutase, and catalase inhibited DNA cleavage suggesting that hydroxyl radical as well as superoxide might invoke DNA cleavage. It also was reported that quinocarcinol (**157**), which is the reduced form of the oxazolidine ring of quinocarcin, lacked antitumor activity and the ability to cleave plasmid DNA. The lack of biological activity exhibited by quinocarcinol suggested that the oxazolidine moiety of quinocarcin was involved in the oxidative degradation of DNA.

William's group has proposed a redox self-disproportionation mechanism of the oxazolidine moiety of quinocarcin resulting in the production of superoxide under aerobic conditions as shown in Scheme 39[68]. Superoxide can then produce hydroxyl radical in the presence of adventitious metals by Haber-Weiss/Fenton cycling. In essence, quinocarcin acts as its own reductant.

Single electron transfer from **58** with simultaneous loss from the oxazolidine nitrogen to tautomer **152** would furnish the radical anion **153** as well as the oxazolidinyl radical **154**. Radical **154** could then reduce another equivalent of **152** becoming oxazolidinium ion **155** which could then be hydrolyzed to quinocarcinamide (**156**). Radical anion **153** suffers a second electron transfer under anaerobic conditions with concomitant protonation yielding quinocarcinol **157**. However, under aerobic conditions **153** could react with O_2 to produce the peroxy radical anion **158** which can expel an equivalent of superoxide resulting in the regeneration of **152**.

Scheme 39 :



The proposed disproportionation mechanism was experimentally supported by the HPLC isolation of quinocarcinol (**157**) and quinocarcinamide (**156**) from a deoxygenated aqueous solution of quinocarcin. Further evidence supporting the intermediary of **156** was provided by conducting the disproportionation in 98% ^{18}O water. Analysis of product by mass spectrometry showed greater than 40% $^{18}O_2$ incorporation in **156**. To experimentally disprove the possibility that the peroxy radical anion **158** undergoes homolysis of the O-O bond furnishing hydroxyl radical and **156** directly the disproportionation was conducted under an $^{18}O_2$ atmosphere and no enhancement in the M+2 peak was observed when **156** was analyzed by mass spectrometry.

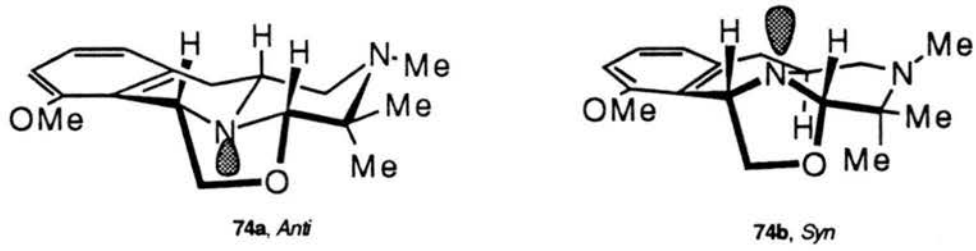
The rate of superoxide production by quinocarcin at various pH conditions has been measured by following the reduction of nitroblue tetrazolium (NBT)[65]. It was found that the rate of reduction of NBT by quinocarcin was pH dependent where the rate was greatest at higher pH's. Furthermore, it was found that when quinocarcin was allowed to react with plasmid DNA between pH 5 and 9 that optimal cleavage occurred between pH 8 and pH 9. These results suggested that the oxazolidine nitrogen must be in its unprotonated form during the redox cycle[68].

Studies performed in the William's group showed that the exclusion of oxygen, as well as the addition of iron chelators, superoxide dismutase, and catalase inhibited plasmid DNA cleavage, but the addition of hydrogen peroxide stimulated the reaction. These results supported the dependency of DNA cleavage on superoxide production and the likelihood of Fenton-chemistry being responsible for DNA scission.

The William's research group has also shown that the generation of superoxide by quinocarcin analogs is stereoelectronically controlled[37]. The quinocarcin analogs **74a** and **74b** were prepared as discussed earlier. The analog **74a** is such that all three methines are *syn* and analog **74b** has inverted stereochemistry at C-6'a (based on tetrazomine numbering). Analog **74a** positions the nitrogen lone pair *trans*-antiperiplanar to the

oxazolidine methine placing the piperazine ring in a chair-like conformation. Analog **74b**

Figure 11:



74a, *Anti* **74b**, *Syn*

positions the nitrogen lone pair *trans*-antiperiplanar to the oxazolidine C-O bond favoring nitrogen pyramidal inversion. These conformations were confirmed by x-ray crystal structure.

Since neither compound **74a** nor **74b** was soluble in aqueous buffer solutions, experiments were conducted with the citrate or hydrochloride salts. The DNA cleavage ability of the citrate salt of analog **74a** was far greater than that of the citrate salt of analog **74b** in studies conducted with plasmid DNA. As with quinocarcin, analog **74a** showed reduced cleavage with the addition of superoxide dismutase. Furthermore, only the hydrochloride salt of **74a** generated superoxide. These results clearly showed that the ability of quinocarcin analogs **74a** and **74b** to produce superoxide and effect DNA cleavage are influenced by stereoelectronic factors. Only when the oxazolidine ring exists in a *trans*-antiperiplanar confirmation does superoxide production occur, leading ultimately to DNA scission.

3. Superoxide Production and Plasmid DNA Cleavage by Tetrazomine

Given the broad biological activity of tetrazomine and its similarity in structure to quinocarcin studies were initiated in the William's group to investigate if tetrazomine might be capable of producing superoxide. It was shown that tetrazomine has similar aerobic

behavior to that of quinocarcin[38, 69]. The rate of reduction of nitroblue tetrazolium[65] by tetrazomine was spectrophotometrically measured at varied pH levels and it was found that superoxide production was maximal at pH 8 and that the reduction was completely inhibited by the addition of superoxide dismutase as tabulated in Table 6. These results verified that tetrazomine was capable of superoxide production.

Table 6:

Rates of Superoxide Production: Measurements by Reduction of Nitroblue Tetrazolium (NBT)

entry	substrate	pH	rate (sec^{-1} x10^{-9})
1	1.0 mM tetrazomine	6	2.46
2	1.0 mM tetrazomine	7	10.6
3	1.0 mM tetrazomine	8	17.5
4	1.0 mM quinocarcin	8	2.18
5	1.0 mM tetrazomine + 10µg/mL SOD	8	0.0
6	20 mM phosphate buffer (control)	8	0.0

When tetrazomine was allowed to react with supercoiled plasmid DNA at pH values ranging from 5 to 9 under aerobic conditions it was found that the reaction was pH dependent[38, 69]. The greatest cleavage was observed at pH values greater 7. Cleavage was measured by the S value which is equal to the average number of single hits per DNA molecule. These results suggested that if tetrazomine shared the same mechanism with quinocarcin for the production of superoxide, that the oxazolidine nitrogen must be in its unprotonated form during the redox cycle. It was suggested that the slight differences in pH versus nicking yields of quinocarcin and tetrazomine was likely a result of slight differences in the pKa's of the oxazolidine nitrogens.

Table 7:

Cleavage of Supercoiled Plasmid DNA (pUC 19).

entry	conditions	[tetrazomine or quinocarcin]	S
1	20 mM phos. pH 5	1.0 mM tetrazomine	0.9
2	20 mM phos. pH 6	1.0 mM tetrazomine	4.4
3	20 mM phos. pH 7	1.0 mM tetrazomine	9.0
4	20 mM phos. pH 8	1.0 mM tetrazomine	10.0
5	20 mM phos. pH 9	1.0 mM tetrazomine	10.0
6	20 mM phos. pH 8	1.0 mM quinocarcin	9.7
7	20 mM phos. pH 8	0.01 mM tetrazomine	0.0
8	20 mM phos. pH 8	0.1 mM tetrazomine	0.4

The cleavage of plasmid DNA by tetrazomine in the presence of several additives was also analyzed[38, 69]. Partial elimination of oxygen, the addition of superoxide dismutase, and to a lesser extent the addition of catalase inhibited DNA cleavage. The addition of picolinic acid[69], which is known to be a scavenger of hydroxyl radical, also resulted in inhibition of DNA cleavage. The addition of hydrogen peroxide had a stimulatory effect on the cleavage reaction. These results offered further evidence that tetrazomine cleaved DNA by the production of superoxide which could undergo reaction to form the reactive hydroxyl radical.

Furthermore, the addition of Fe(III) to the reactions conducted by the William's group had no stimulatory effect, but the addition of the iron chelating agent desferal did significantly inhibit DNA cleavage by tetrazomine. Desferal is known to form a hexacoordinate complex with Fe(II) and uncouples the oxidation of Fe(II) from the formation of hydroxyl radical. It is recognized that higher oxidation states of metals such as iron and copper can participate in Fenton reactions[69]. The rate limiting step of the

Haber-Weiss/Fenton reaction, 76 M^{-1} s^{-1}, is the reduction of hydrogen peroxide by Fe(II)[65]. This is considerably faster (10^4-10^5 times faster) than the rate of formation of superoxide measured for tetrazomine. The kinetic data suggested that DNA cleavage by tetrazomine was metal-dependent and the low concentrations of adventitious Fe(III) present in solution was in excess of that required for Fenton-mediated cleavage of DNA. These studies suggested that the rate limiting step in the cleavage of DNA is the slow production of superoxide by tetrazomine which in the presence of metal ions can generate hydroxyl radical.

B. RESULTS AND DISCUSSION

Given the similarities in structure it was of interest whether quinocarcin and tetrazomine shared similar mechanisms for superoxide production. If so, tetrazomine would be the second member of this newly recognized class of simple heterocyclic compounds which is capable of reducing molecular oxygen based on the inherent intermolecular redox chemistry of the compound itself.

Tetrazomine's interaction with synthetic DNA was of interest. Such studies would provide insight into whether tetrazomine's oxidative cleavage of DNA was non-specific or if some specificity was witnessed.

1. Disproportionation of Tetrazomine

To investigate the mechanism of superoxide production the disproportionation of tetrazomine was examined endeavoring to isolate and characterize any newly formed products. Tetrazomine was dissolved in a carefully deoxygenated phosphate buffer (pH = 8) and allowed to stand at room temperature. Initial disproportionation studies conducted at

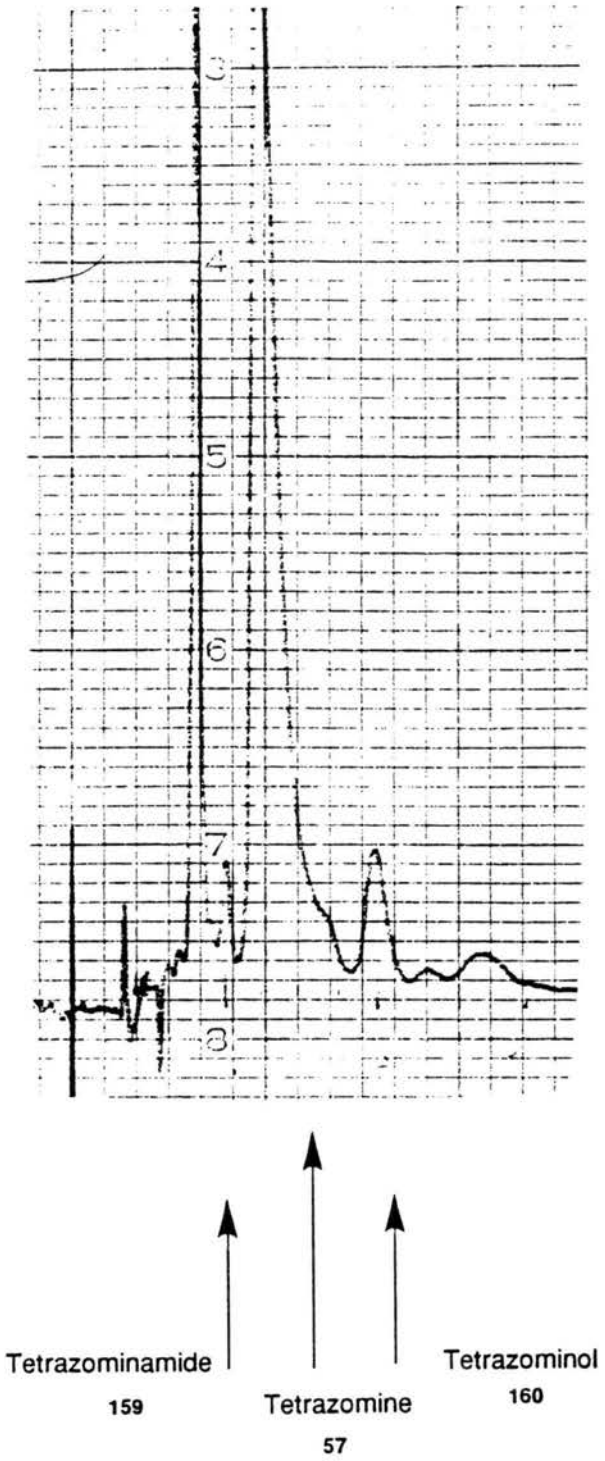
room temperature and analyzed after 10 days by HPLC yielded a complex mixture of products. Tetrazomine was completely consumed in the disproportionation.

When aliquots of the disproportionation were checked at daily intervals by HPLC at room temperature a complex mixture was still observed, even though unreacted tetrazomine still remained. It was not clear whether tetrazomine itself was producing additional products not seen in the quinocarcin disproportionation, or if the disproportionation was occurring in an analogous manner and the oxidized and reduced disproportionation products were then undergoing further reaction. Irregardless, it was clear that some functionality inherent to the tetrazomine molecule was resulting in the formation of additional products. Given the small amount of tetrazomine in our possession and the myriad of products formed it was not possible to characterize all of the adducts formed.

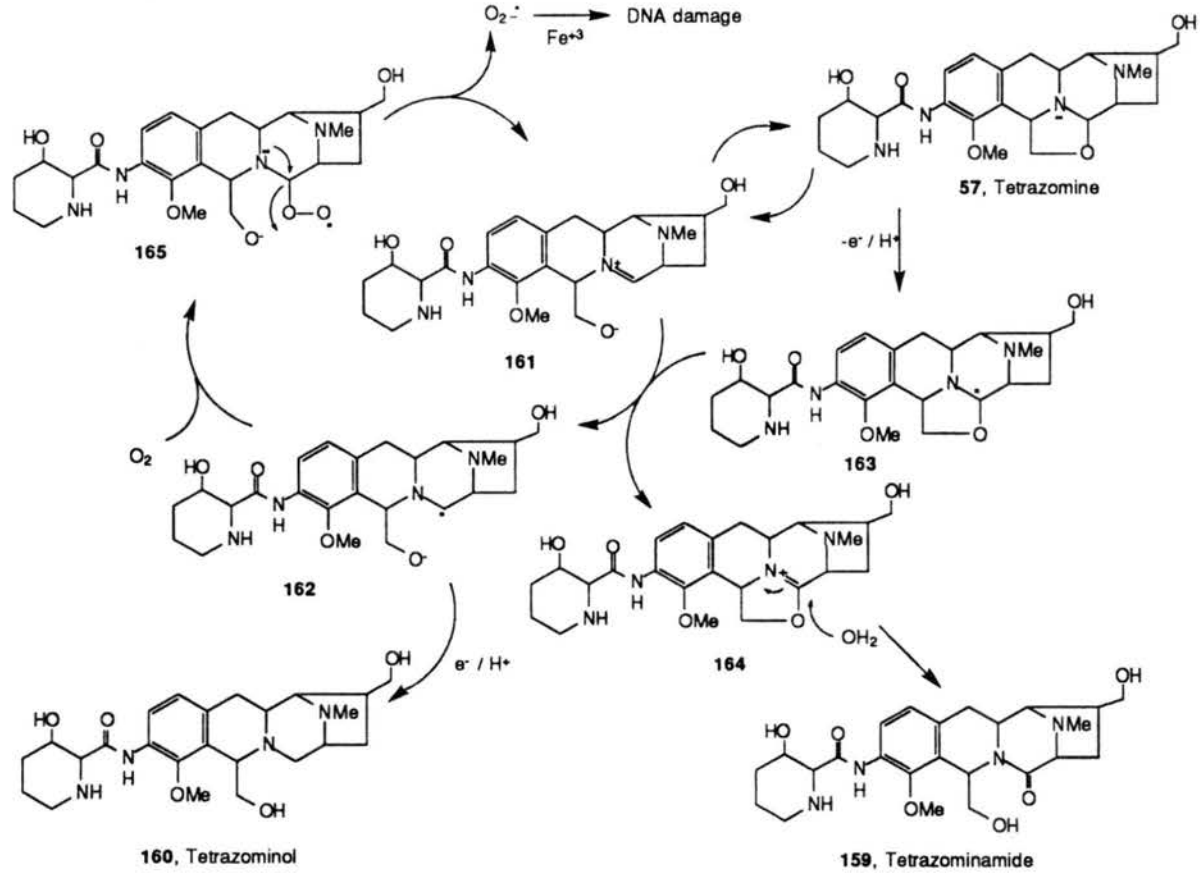
In an effort to try to slow the disproportion reaction down and reduce additional transformations that might be occurring the disproportionation was conducted at a lower temperature. When the temperature of the disproportionation was decreased to 4°C the disproportionation proceeded at a much slower rate yielding 3 major peaks as well as unreacted tetrazomine after 14 days. Approximately 20% conversion of tetrazomine to new products was observed during this time period. The disproportionation was stopped and the individual peaks isolated. The peak eluting just before tetrazomine in Figure 12 was isolated and identified by mass spectrometry as the oxidized product, hereafter referred to as tetrazonamide (**159**) . The slower eluting peak was identified as the reduced product, hereafter referred to as tetrazominol (**160**) The fastest eluting peak was an unidentifiable decomposition product.

Based on the identification of the analogous anaerobic products to that of quinocarcin and similar aerobic behavior the same Cannizzaro-driven reduction of molecular oxygen mechanism as that of quinocarcin is proposed where tetrazomine serves as its own reductant. Therefore, as suggested in Scheme 40, single electron transfer from

Figure 12: Disproportionation of Tetrazomine (4°C)



Scheme 40:

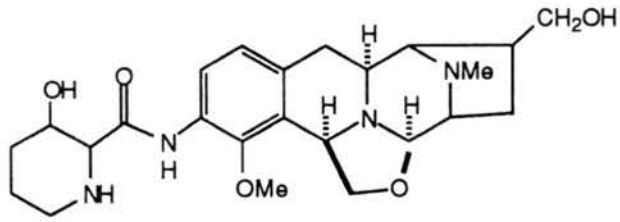


57 with concomitant proton loss from the oxazolidine nitrogen to the ring-opened tautomer **161** would furnish radical anion **162** and the oxazolidinyl radical **163.** Radical **163** should be capable of reducing a second equivalent of **161** ultimately becoming oxazolidinium ion **164** which would hydrolyze to tetrazominamide **159**. Under anaerobic conditions, radical anion **162** subsequently suffers a second electron transfer (presumably from **57** or **163**) with concomitant protonation resulting in tetrazominol **160**. Under aerobic conditions, radical anion **162** can react with molecular oxygen to produce peroxy radical anion **165** which, with nitrogen participation, expels one molar equivalent of superoxide, regenerating **161.**

The common mechanism for the production of superoxide by tetrazomine and quinocarcin suggests that the relative stereochemical configuration of the oxazolidine

moiety is the same as that of quinocarcin. This is supported by the requirement that the

Figure 13:



57, Tetrazomine

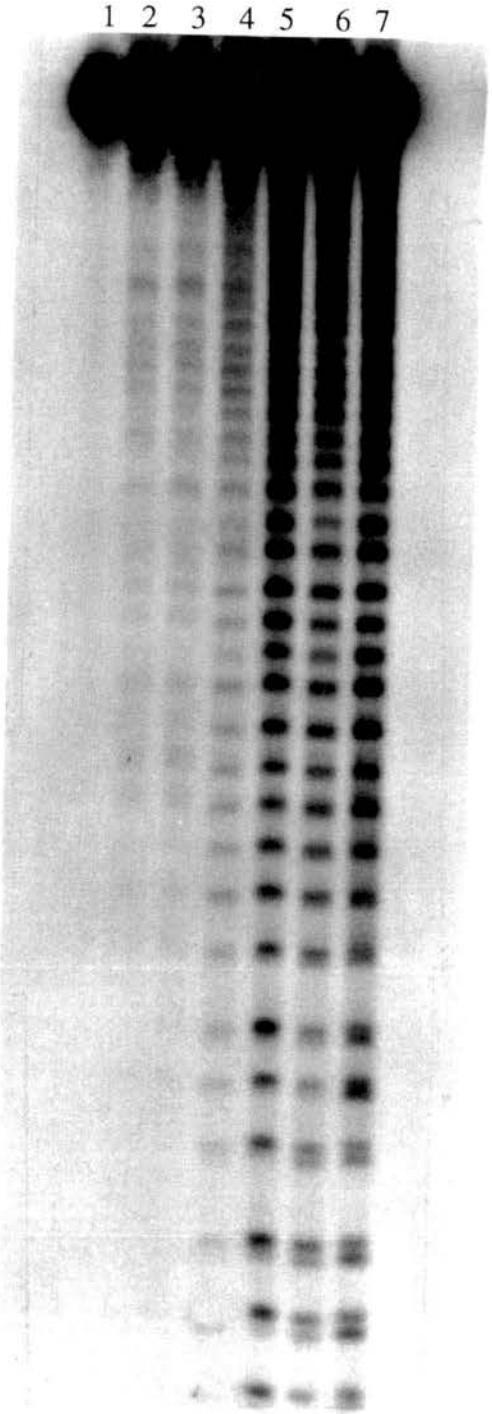
nitrogen lone pair be *trans*-antiperiplanar to the adjacent oxazolidinyl methine for redox chemistry to occur in the quinocarcin analogs[37]. Thus, the identification of an analogous disproportionation reaction to that of quinocarcin has provided the first evidence of the relative stereochemical configuration of tetrazomine as shown in Figure 13.

2. Reaction of Tetrazomine with a 5'-^{32}P-labeled Synthetic 45-mer for High-Resolution Polyacrylamide Gel Electrophoresis

Analysis of the reaction of tetrazomine with a small synthetic oligionucleotide by high-resolution polyacrylamide gel electrophoresis in collaboration with Mark Flanagan[38] provided evidence for oxidative damage without specificity. The annealment of a 45 base pair 5'-^{32}P end-labeled synthetic oligionucleotide to its complement followed by reaction with tetrazomine (0.1, 1.0, 10.0 mmol) without the addition of additional reducing agents resulted in non-sequence specific cleavage. Cleavage occurred at each nucleotide and each band appeared as a doublet when analyzed by 20% polyacrylamide gel electrophoresis as shown in Figure 14. DNA cleavage increased with increasing concentration of tetrazomine. Furthermore, tetrazomine showed greater DNA nicking than quinocarcin at comparable concentrations as evidenced by the comparison of lanes 5 and 6 in Figure 14.

The appearance of each band as a doublet is indicative of the 3'-phosphate and 3'-phosphoglycolate ends resulting from non-selective Fenton-mediated cleavage. The slower

Figure 14: Reactions of Tetrazomine with 5'-^{32}P-Labeled Synthetic 45-mer High Resolution Polyacrylamide Gel



Lane 1, 5', ^{32}P labeled, duplex 45mer

2, DNA (control)

3, DNA + 0.1mM tetrazomine

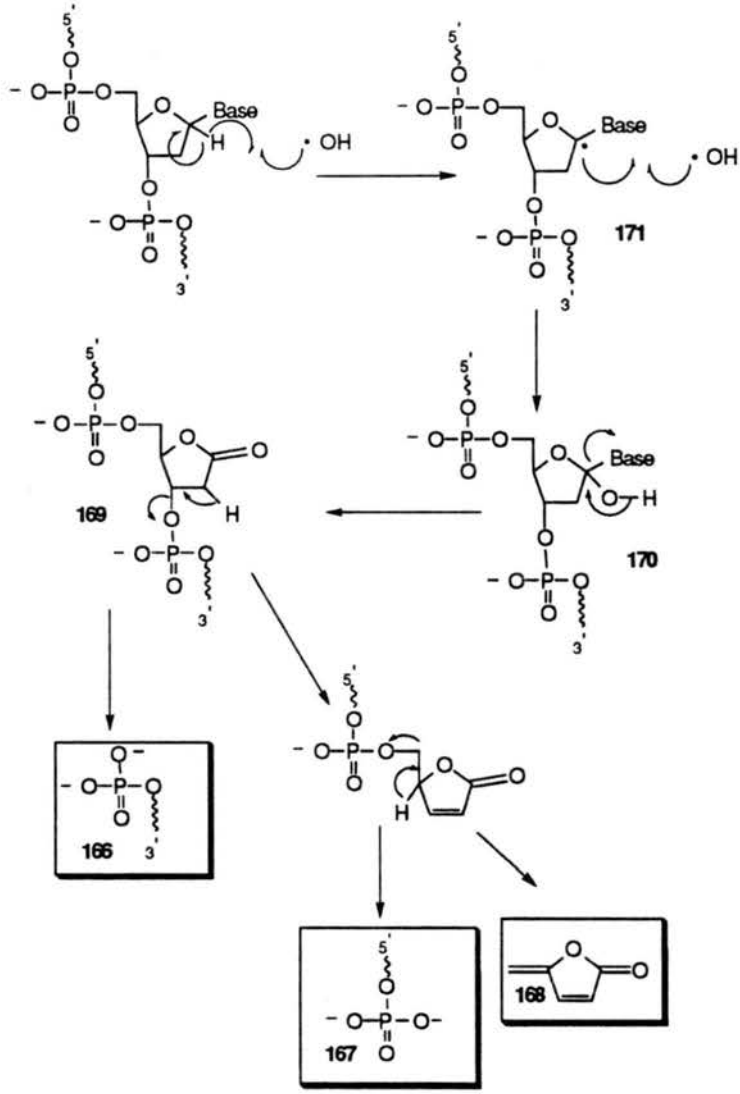
4, DNA + 1.0mM tetrazomine

5, DNA + 10mM tetrazomine

6, DNA + 10mM quinocarcin

7, DNA + 1.0mM Fe^{II}/EDTA

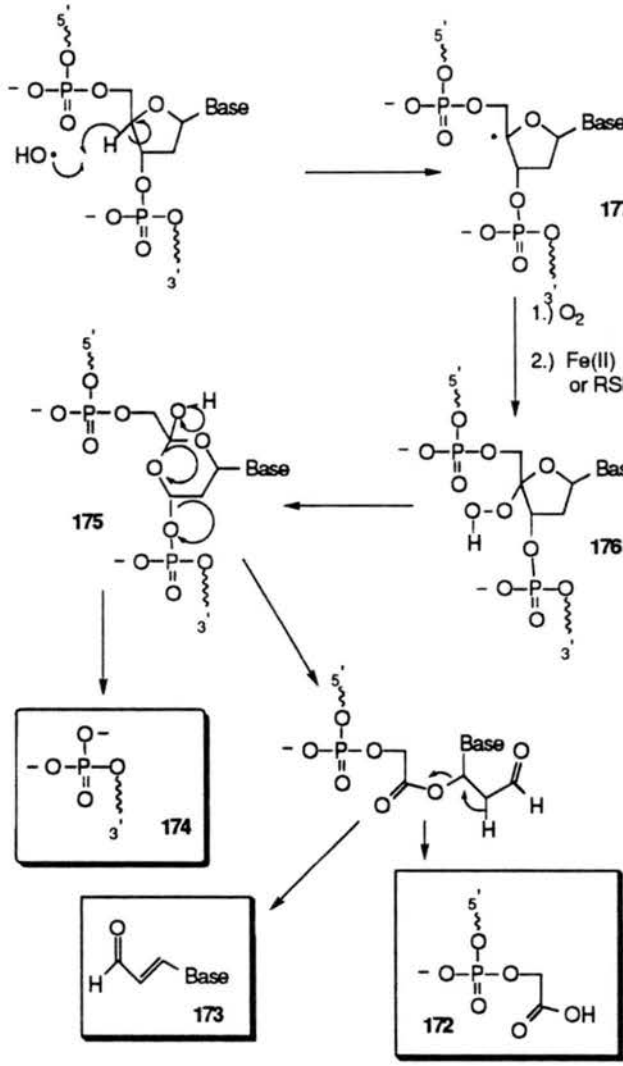
Scheme 41:



migrating band being the 3'-phosphate and the faster migrating being the 3'-phosphoglycolate.

Formation of the 3'-phosphate is proposed to result from C-H abstraction at C1' of the deoxyribose sugar[62]. Radical species **171** can couple with hydroxyl radical and the hemiacetal **170** eliminates the base forming the lactone **169**. The unsaturated lactone **168** is generated by elimination of the 3'-phosphate **166** followed by elimination of the ^{32}P-labeled- 5'-phosphate **167**.

Scheme 42:



The 3'-phosphoglycolate product can be rationalized by hydrogen abstraction at C4'-of the deoxyribose sugar. Oxygen then adds to radical **177** to form a peroxy radical which is subsequently reduced to the peroxide **176**. The peroxide can then undergo a Criegee-type rearrangement furnishing **175**. Ring-opening followed by elimination of **174** yields the radio-labeled 3'-phosphoglycolate **172** and the base-propenal **173**.

It was earlier observed in studies conducted in the William's group that attenuated cleavage was observed at higher tetrazomine concentrations (10 mM) which was not

consistent with linear superoxide production observed by NBT reduction[38]. However, when the solutions were well oxygenated prior to the introduction of tetrazomine a more linear rate of DNA damage was observed as evidenced by Figure 14. It is possible that when the solutions were not well oxygenated that the source of oxygen was exhausted by high concentrations of tetrazomine and that the hydroxyl radical produced was not in close enough proximity to the DNA to produce oxidative damage.

Likewise, it is possible that at higher concentrations of tetrazomine the drug might associate with the minor groove thus blocking potential reaction sites. Studies conducted by Mark Flanagan[38,69] have shown that when a synthetic 5'-^{32}P-labeled synthetic oligionucleotide was incubated with tetrazomine for 5 hours and then exposed to Fe/EDTA a marked decrease in nicking was observed by high-resolution polyacrylamide gel electrophoresis in comparison to DNA which was not first treated with tetrazomine. When the order of reagents is reversed (first treated with Fe/EDTA followed by tetrazomine) significantly more cleavage is observed. Virtually no 3'-phosphoglycolate product was observed in reactions in which the DNA exposed to Fe/EDTA had first been incubated with tetrazomine.

It is reasonable to assume that tetrazomine is capable of interacting with the minor groove of DNA in the same fashion as Remer's proposal for quinocarcin *via* the ring-opened oxazolidine iminium ion. Furthermore, it was shown by Flanagan using scanning densitometry[38, 69] that the ratio of 3'-phosphate vs. 3'-phosphoglycolate for Figure 14 was 8:2 for 10 mM tetrazomine, 6:4 for 10 mM quinocarcin, and 4:6 for 1.0 mM Fe/EDTA. These results suggest that tetrazomine may protect DNA from 4'-hydrogen abstraction which ultimately leads to the 3'-phosphoglycolate product.

3. Conclusion

Tetrazomine shares a common mechanism for the reduction of oxygen to superoxide as that of quinocarcin. In both cases a redox self-disproportionation of the oxazolidine moiety results in the reduction of molecular oxygen to generate superoxide which can ultimately produce the highly reactive hydroxyl radical. Thus, tetrazomine is the second compound identified to induce oxidative damage to DNA by this novel mechanism. Furthermore, the identification of this mechanism provides the first experimental evidence for the relative stereochemical configuration of tetrazomine.

It has also been shown that tetrazomine is capable of cleaving synthetic DNA in a non-specific manner at every base pair. Thus, this behavior is characteristic of the generation of hydroxyl radical as evidenced by the Fe/EDTA reaction.

C. Experimental

Disproportionation of Tetrazomine and Analysis of Products

A 10mM solution of **57** was prepared with natural tetrazomine provided by the Yamanouchi Pharmaceutical Co. in 20mM phosphate buffer (pH 8.0). The solution was then deoxygenated by freeze-thaw purging with nitrogen 5 times. A sample was analyzed by HPLC (resolve pack C18 column; 10mM sodium sulfate, 3% acetonitrile, 0.05% acetic acid, 0.002% 1-pentanesulfonate sodium salt (isocratic), and detected by UV at 254nm which revealed a single peak at 6 minutes corresponding to **57**. The solution was then aged at 4°C under anaerobic conditions with aliquots being taken periodically (every third day for two weeks) and analyzed by HPLC for tetrazomine and the formation of new products. Approximately 20% conversion of tetrazomine to new products was observed. These breakdown products were isolated (by HPLC) and desalted by triturating with methanol. Following filtration, the methanol solutions were concentrated under reduced

pressure, re-dissolved in ddH_2O, passed through a column of Dowex 1 (Cl^- form) and lyophilized. One product was identified as the reduced form of tetrazomine; tetrazominol **160**, with a retention time of 9 minutes MS m/e (M+), 460.26960 (Calcd for $C_{24}H_{36}N_4O_5$ 460.2696). Another product isolated from the disproportionation by HPLC with a retention time of 5 minutes was identified as the oxidized form of tetrazomine; tetrazominamide **159**. MS m/e (M+), 475.2578 (Calcd for $C_{24}H_{35}N_4O_6$ 475.258).

^{32}P High-Resolution Polyacrylamide Gel Electrophoresis

To a solution of the 45 base pair synthetic deoxynucleotide (100 pmol) in 74 μL of ddH_2O was added 20 μL of polynucleotide kinase buffer, 4 μL (40 units) of T4 polynucleotide kinase and 1 μL of γ-^{32}P ATP. The reaction was incubated for 1 hour at 37°C and 10 minutes at 70°C. The reaction was loaded onto a 2 mL column of Sephadex G-50 and eluted with TE buffer (10 mM, 1 mM EDTA, pH 8). The first radioactive fraction was collected and precipitated with EtOH/3M NaOAc (pH 5.2), and dried. Annealing was performed by mixing equimolar amounts of the 5' ^{32}P-end-labeled strand and the complimentary strand in ddH_20 to a final concentration of 2 pmol/mL; each was heated in 65°C for 30 minutes and slowly cooled to 0°C. Reactions were made up by additions at 0°C of appropriate amounts of stock solutions to 3 μL of labeled DNA (5pmol). The total volume of the reactions were brought to 16 μL with ddH_2O and enough 80 mM phosphate buffer (pH 8), which was oxygenated prior to use by bubbling oxygen through the solution for 2 minutes, such to achieve a final concentration of 20 mM phosphate. Each reaction was incubated at 37°C for 8 hours after which 1μL of 3 M NaOAc (pH 5.2) and 60 μL of EtOH were added and the resulting solutions cooled at -70°C for 10 minutes. Each tube was centrifuged at 14K for 10 minutes at 4°C, the supernatants discarded, and the DNA pellet dried under reduced pressure. To each dried pellet of DNA, was added 10 μL of loading buffer

(formamide, 10 mM EDTA, pH 8, 0.025% xylenecyanol FF and 0.025% bromophenol blue) and heated to 90°C for 5 minutes, placed on ice and loaded onto a 20% denaturing (urea) polyacrylamide gel and run for 6 hours at 1300 V. Band visualization was achieved *via* autoradiography.

Chapter 4

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Appendix

Publications

Reprinted from Biochemistry, 1994, 33.

O_2-Dependent Cleavage of DNA by Tetrazomine[†]

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Received December 13, 1993; Revised Manuscript Received January 24, 1994[•]

ABSTRACT: A mechanism for the reduction of molecular oxygen that results in the O_2-dependent cleavage of both single-stranded and double-stranded DNA by the antitumor antibiotic tetrazomine (**1**) is presented. The results are discussed in the context of a redox self-disproportionation of the oxazolidine moiety of tetrazomine. Comparisons are made to the structurally analogous natural product quinocarcin (**2**) in which, like tetrazomine, the oxazolidine moiety is invoked in redox chemistry, which ultimately results in the reduction of molecular oxygen to superoxide.

It is now widely recognized (Fisher & Aristoff, 1988; Remers, 1985) that a variety of clinically significant antitumor antibiotics can mediate oxygen-dependent cleavage of the ribose phosphate backbone of cellular DNA and RNA. An enormous structural array of interesting natural products and semisynthetic and totally synthetic substances mediates oxidative strand scission of nucleic acids through three main families of reactions: (1) metal-mediated activation of O_2, ultimately producing hydroxyl radical or other reactive oxygen species (Stubbe & Kozarich, 1987; Walling, 1975); (2) non-metal-dependent generation of reactive carbon radicals (Myers, 1987; Nicolaou et al., 1988; Magnus et al., 1988; Hawley et al., 1989) that mediate CH abstraction from the deoxyribose backbone (the resulting deoxyribosyl radical subsequently reacts with molecular oxygen, culminating in strand scission); and (3) photolytic production of hydroxyl radical (Saito et al., 1990; Zafirou & Bonneau, 1987), which does not require metal participation for the DNA cleavage event. A rich array of chemistry can be found in the metal-dependent family of DNA-damaging agents. Many readily oxidizable organic substances are capable of reducing molecular oxygen, resulting in the production of superoxide, such as semiquinone radical anions, thiols (Misra, 1974), and ascorbate, among others. Superoxide is well-documented (Lesko & Lorentzen, 1980) to mediate DNA strand breakage via dismutation to hydrogen peroxide followed by Fenton chemistry [in the presence of Fe(III)], generating the highly reactive hydroxyl radical.

Tetrazomine (**1**) is a natural secondary metabolite that was recently isolated from *Saccharothrix mutabilis* subsp. *chichijimaensisi* by Yamanouchi Pharmaceutical Co. (Japan) (Suzuki et al., 1991) and is the most recent member of the quinocarcin (**2**)/naphthyridinomycin/saframycin class of antitumor agents. Tetrazomine has been shown to display broad antimicrobial activity against both Gram-positive and Gram-negative bacteria *in vitro*. Tetrazomine also displays promising *in vitro* antitumor activity (Suzuki et al., 1991) against lymphoid leukemia L1210 and P388 leukemia at 0.0427 and 0.014 μg/mL, respectively. This substance also displayed antitumor activity against P388 leukemia *in vivo*.

Our interest in this substance stems from structural similarities between tetrazomine and the antitumor antibiotic quinocarcin (**2**), which has been the subject of considerable investigation (Williams et al., 1991, 1992). Like quinocarcin, tetrazomine (**1**) is capable of cleaving both synthetic oligonucleotides and plasmid DNA in an O_2-dependent manner in the absence of external reducing agents such as dithiothreitol (DTT); (2) is not stimulated by the addition of metal ions [Fe(II), Fe(III)]; (3) is inhibited by free radical scavengers such as picolinic acid (Sheu et al., 1990); and (4) is inhibited by superoxide dismutase (SOD[1]) and catalase. We have recently obtained experimental evidence, presented herein, that tetrazomine undergoes a redox self-disproportionation reaction similar to that observed for quinocarcin, which may be coupled to the capacity of this substance to affect the production of superoxide in the presence of molecular oxygen and results, at least in part, in Fenton-mediated lesions in DNA: a proposed mechanism for this process is presented in Figure 2. The rate at which tetrazomine produces superoxide has been carefully examined by following the reduction of nitroblue tetrazolium (NBT) spectrophotometrically under various conditions. It was found that, in addition to being completely inhibited by SOD, these experiments closely paralleled trends observed for the nicking of supercoiled plasmid DNA by tetrazomine.

MATERIALS AND METHODS

Disproportionation of Tetrazomine and Analysis of Products. A 10 mM solution of **1** was prepared in 20 mM phosphate buffer (pH 8.0), which was then deoxygenated by freeze–thaw purging with nitrogen. A sample was then analyzed by HPLC (resolve pack C18 column; 10 mM sodium sulfate, 3% acetonitrile, 0.05% acetic acid, and 0.002% 1-pentanesulfonate sodium salt (isocratic); detected by UV at 254 nm), which revealed only a peak at 5 min corresponding to **1**. The solution was then aged at 4 °C under anaerobic conditions, with aliquots being taken periodically (every third day for 2 weeks), and analyzed by HPLC for tetrazomine and the formation of new products. Approximately 20% conversion of tetrazomine to new products was observed after 2 weeks. These breakdown products were isolated (by HPLC) and desalted by trituration with methanol. Following filtration, the methanol solutions were concentrated under reduced pressure, redissolved in ddH_2O, passed through a column of Dowex 1 (Cl^- form), and lyophilized. One product was identified as the reduced form of tetrazomine, which henceforth will be referred to as tetrazominol (**4**). Authentic tetrazominol was obtained from

[†] This research was supported by the National Institutes of Health (Grant No. CA 43969).

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[•] Abstract published in *Advance ACS Abstracts*, March 1, 1994.

[1] Abbreviations: SOD, superoxide dismutase; NBT, nitroblue tetrazolium; TE, 100 mM Tris base/10 mM EDTA; EDTA, ethylenediaminetetraacetic acid; ddH_2O, double deionized water.

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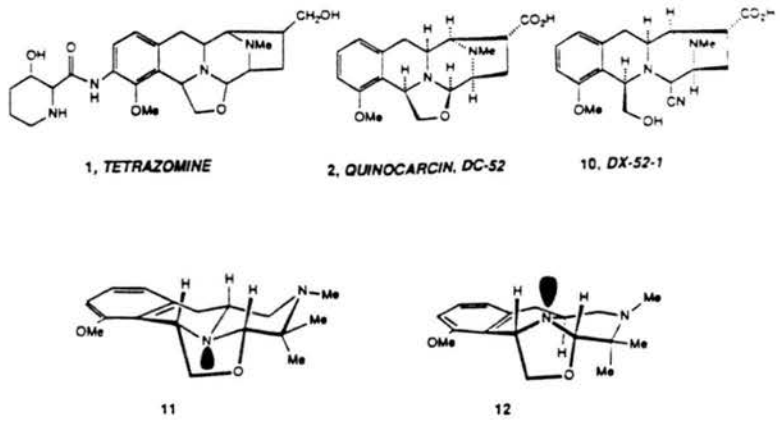


FIGURE 1: Tetrazomine and structural analogs.

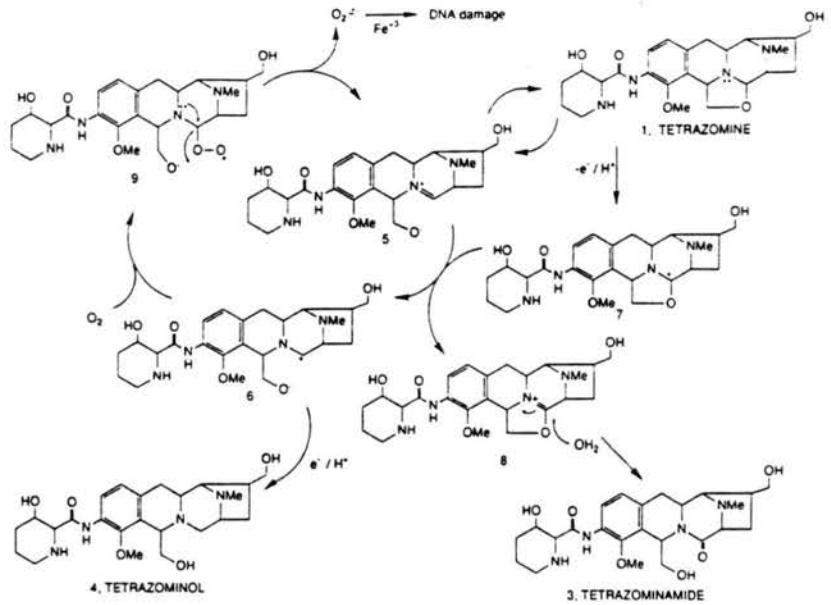


FIGURE 2: Proposed disproportionation of tetrazomine: under anaerobic conditions, resulting in the formation of **3** and **4**; under aerobic conditions, resulting in the production of superoxide.

tetrazomine by reacting 10 mg of the pure drug with 10 equiv of $NaBH_4$ (Baker) in 3 mL of methanol (0 °C) for 3 h. The sample of tetrazominol obtained following isolation by preparative thin-layer chromatography (silica; 4:1 CH_2Cl_2/methanol, $R_f = 0.2$) had the exact same HPLC retention time (8 min) and molecular weight by mass spectrometry as **4** collected from the disproportionation of **1** described above: ^1H NMR (300 MHz) (D_2O) δ (HOD) 1.63–1.94 (5H, m), 2.24 (1H, t, J = 11.4 Hz), 2.48 (1H, d, J = 14.1 Hz), 2.57 (1H, q, J = 11.5 Hz), 2.79–2.89 (5H, m), 2.96 (1H, d, J = 16.3 Hz), 3.00 (1H, d, J = 12.9 Hz), 3.18 (1H, br s), 3.36 (1H, d, J = 13.8 Hz), 3.53–3.68 (5H, m), 3.60 (3H, s), 3.84–3.90 (2H, m), 4.17 (1H, d, J = 1.8 Hz), 4.56 (1H, s), 6.89 (1H, d, J = 8.2 Hz), 7.33 (1H, d, J = 8.2 Hz). MS m/e (M^+): 460.26960 (calcd for $C_{24}H_{36}N_4O_5$, 460.2696). Another product isolated from the disproportionation by HPLC (retention time, 4 min) was identified as the oxidized form of tetrazomine, which henceforth will be referred to as tetrazominamide (**3**): MS m/e (M^+) 475.2578 (calcd for $C_{24}H_{35}N_4O_6$, 475.258). The other material formed was an unidentifiable decomposition product. We were not able to obtain a sufficient quantity of tetrazomine from Yamonouchi Co. to rigorously isolate and characterize this material.

Reductions of Nitroblue Tetrazolium by Tetrazomine. Each reaction was performed in triplicate by adding an appropriate amount of tetrazomine stock solution (20 mM) to an aerated solution of nitroblue tetrazolium (0.12 mM) in 20 mM phosphate buffer (at the indicated pH) containing 1% Triton X-100 detergent with the final volume brought to 750 μL with ddH_2O. The optical absorbance was measured at 25 °C over a 30-min period at 500 nm (Varian DMS 80 UV/vis spectrophotometer), and the ΔOD was the average slope for the linear OD change over the reaction time. The rates for superoxide production were calculated by assuming that $[O_2]$ does not appreciably change over this time period and is in excess (zero order in oxygen). The rates (reported in Table 1) were calculated from the ΔOD measurements and based

Table 1: Rates of Superoxide Production: Measurements by Reduction of Nitroblue Tetrazolium (NBT)

entry	substrate	pH	rate ($\mu s^{-1} \times 10^{-9}$)
1	1.0 mM tetrazomine	6	2.46
2	1.0 mM tetrazomine	7	10.6
3	1.0 mM tetrazomine	8	17.5
4	1.0 mM quinocarcin	8	1.1
5	1.0 mM tetrazomine + 10 μg/mL SOD	8	0.0
6	1.0 mM DX-52-1	8	0.0
7	1.0 mM **11**	8	0.41
8	1.0 mM **12**	8	0.0
9	20 mM phosphate buffer (control)	8	0.0

on a molar extinction coefficient (ϵ_0) of 12 200 for the formazan product of NBT at 500 nm.

Preparation and Purification of Supercoiled Plasmid DNA (pUC 19). To 40 μL of electrocompetent cells (*E. coli*, MC 1061) was added 1 μL (0.35 μg) of pUC 19 plasmid DNA (New England Biolabs), and the mixture was agitated and aged in an ice bath for 1 min. The mixture was then transferred to an ice-cooled, 0.1-cm electroporation cuvette, and the material was pulsed (Bio-Rad gene pulser) at 25 μF, 200 Ω, and 1.1 kV. The cells were then quickly transferred to 1 mL of sterile SOC broth (20 mM glucose, 20 g of bacto-tryptone (Difco), 5 g of bacto-yeast (Difco), 1 g of $MgCl_2$, 0.5 g of NaCl, and 186 mg KCl to 1.0 L) and incubated at 37 °C, with agitation (200 rpm), for 1 h. The mixture was transferred to a sterile Eppendorf tube and centrifuged (14 000 rpm, 5 min, room temperature). The supernatant was discarded, and the cells were resuspended in 400 μL of sterile SOC broth. Dilutions were made to 10^2, 10^4, 10^6, 10^8 (400 μL each), and 100 μL of each plated onto LB agar plates containing 30 μg/mL ampicillin (in duplicate). The plates were inverted and incubated at 37 °C for 18 h, at which time colonies were counted and a yield of 10^{10} transformants was assessed. A resistant colony was then grown in 500 mL of sterile LB broth (10 g of bacto-tryptone, 5 g of bacto-yeast extract, and 10 g of NaCl to 1.0 L) containing 30 μg/mL ampicillin at 37 °C for 18 h. The cells were pelleted by centrifugation (5000 rpm, 10 min, 4 °C). Plasmid DNA (pUC 19) was then isolated following the Promega Magic Maxi-prep kit procedure. The supercoiled plasmid was further purified by low-melt agarose gel electrophoresis (1.2%). The band corresponding to supercoiled plasmid (visualized by 0.4 μg/mL ethidium bromide using a hand-held UV light) was cut from the gel and melted at 75 °C. To this was added 4 vol of 37 °C TE, and the mixture was aged at 37 °C for 1 h. To this solution was then added 1 vol of Tris-equilibrated phenol (pH 8) (room temperature), the tube was mixed gently by rocking, and the layers were separated by centrifugation (4000 rpm, 15 min, room temperature). The aqueous layer (top) was removed and extracted with 2 × 2 vol of butanol (centrifugation was necessary for the separation of layers). To the aqueous layer (bottom) were added 3 vol of ethanol and 50 μL of 3 M NaOAc, pH 5.2, and the solution was mixed gently, aged at −70 °C for 10 min, and centrifuged (20 000 rpm, 30 min, 4 °C). The supernatant was discarded and the pellet resuspended in 200 μL of sterile deionized water. Supercoiled plasmid DNA (pU 19) was recovered in 90% yield from the low-melt gel.

Cleavage of Supercoiled Plasmid DNA (pUC 19). DNA nicking reaction mixtures were made up by the addition at 0 °C of appropriate amounts of reagent stock solutions to a stock solution of supercoiled plasmid DNA (pUC 19), prepared as described above and containing 0.15 μg of DNA per reaction (23 μM base-pair concentration). The total volumes of the reaction mixtures were brought to 10 μL each with distilled, deionized water when necessary, and the reaction mixtures were incubated at 37 °C for 2 h in tightly capped plastic Eppendorf tubes. Stock solutions for experiments including DNA were prepared using distilled, deionized water and commercially available reagents: sodium phosphate, monobasic, EM Science; sodium phosphate, dibasic and 30% hydrogen peroxide, Malinckrodt; superoxide dismutase and beef liver catalase (suspension in water), Boehringer Mannheim Biochemical. Desferal was the generous gift of Ciba-Geigy Co. The cleavage of plasmid DNA was detected by loading the reactions onto 1.2% agarose gels containing 0.4 μg/mL ethidium bromide and running for 2 h at 55 V. The electrophoresis gels were immediately visualized on a UV transilluminator and photographed using black and white instant films (Polaroid T667). The measurements of the relative intensities of DNA bands were performed on the photographs using a Dell System 325 computer and Technology Resources Inc. image processing software. The film used to photograph the gels was confirmed to have a linear response to the range of DNA quantities used. The mean number of single-strand scissions (S) per supercoiled DNA substrate was calculated using the Poisson distribution (Hertzberg & Dervan, 1984), where the value S for the DNA control represents the amount of nicked open circular DNA present in the starting plasmid, and was subtracted from the S values calculated for the individual cleavage reactions. When only forms I (supercoiled) and II (nicked open circular) are present, the equation simplifies to $S = -\ln f_I$, where f_I is the fraction of form I molecules from the densitometry data. In those cases where form III (linear) DNA is present, S is calculated from $f_I + f_{II} = [1 - S(2h + 1)/2L]^{S/2}$, where h is the distance between hits on opposite strands to produce a linear molecule (16 base pairs) and L is the total number of base pairs in pUC 19 (2686 base pairs).

Labeling (5′-^{32}P) and Reactions of Synthetic 45-mer for High-Resolution Polyacrylamide Gel Electrophoresis. To a solution of the synthetic deoxyoligonucleotide (100 pmol) in 74 μL of ddH_2O were added 20 μL of polynucleotide kinase buffer, 4 μL (40 units) of T4 polynucleotide kinase (New England Biolabs), and 1 μL (10 μCi) of [γ-^{32}P]ATP (DuPont). The reaction was incubated for 60 min at 37 °C and for 10 min at 70 °C. The solution was loaded onto a 2-mL column of Sephadex G-50 and eluted with TE buffer. The first radioactive fraction was collected and precipitated with ethanol/3 M NaOAc (pH 5.2) and dried. Annealing was performed by mixing equimolar amounts of the 5′-^{32}P-end-labeled strand and the complementary strand in ddH_2O to a final concentration of 2 pmol/μL; each was heated to 65 °C for 30 min and slowly cooled to 0 °C. Reactions were made up by the additions at 0 °C of appropriate amounts of stock solutions to 3 μL each of labeled DNA (5 pmol). The total volumes of the reactions were brought to 16 μL each with ddH_2O and enough 80 mM phosphate buffer (pH 8) so as to achieve final concentrations of 20 mM phosphate. Each reaction was incubated at 37 °C for 5 h (none of the reactions contained any additional reducing agents, such as DTT). To the reaction mixtures were then added 1 μL of 3 M NaOAc (pH 5.2) and 60 μL of ethanol, and the resulting solutions were aged at −70 °C for 10 min. Each tube was centrifuged at 14 000 rpm for 10 min at 4 °C, the supernatants were discarded, and the DNA pellets were dried under reduced pressure. To each dried pellet of DNA was added 10 μL of loading buffer (formamide, 10 mM EDTA (pH 8), 0.025%

xylenecyanol FF, and 0.025% bromophenol blue), and the sample was then heated to 90 °C for 5 min, placed on ice, and immediately loaded (20 000 cpm/lane as measured using a Packard 1500 liquid scintillation counter) onto a 20% denaturing (urea) polyacrylamide gel and run for 6 h at 1300 V. The bands were visualized by autoradiography.

RESULTS

Disproportionation of Tetrazomine. Natural tetrazomine, obtained from Yamanouchi Pharmaceutical Co., Ltd., was allowed to stand in carefully deoxygenated phosphate buffer (20 mM, pH 8) at 4 °C, with new products being produced. The identification of the anaerobic redox products **3** and **4** supports the proposal that tetrazomine serves as its own reductant, since no other reducing agents were present. Therefore, on the basis of the analogous chemical structure, the observation of anaerobic disproportionation products, and the analogous aerobic behavior (i.e., superoxide production) that tetrazomine shares with quinocarcin, we propose the same Cannizzaro-driven reduction of molecular oxygen for tetrazomine. Therefore, as suggested in Figure 2, single-electron transfer from **1** with concomitant proton loss from the oxazolidine nitrogen to the ring-opened tautomer **5** would furnish radical anion **6** and the oxazolidinyl radical **7**. Radical **7** should be capable of reducing a second equivalent of **5**, ultimately becoming oxazolidinium ion **8**, which would hydrolyze to tetrazominamide (**3**). Under anaerobic conditions, radical anion **6** subsequently suffers a second electron transfer (presumably from **1** or **7**) with concomitant protonation, resulting in tetrazominol (**4**). Under aerobic conditions, radical anion **6** can react with molecular oxygen to produce peroxy radical anion **9** which, with nitrogen participation, expels 1 molar equiv of superoxide, regenerating **5**.

Reductions of Nitroblue Tetrazolium by Tetrazomine. The rate of superoxide production was carefully followed by reduction of NBT (Misra, 1974; Tsou, 1956) under various pH conditions, and it was found that these reactions are completely inhibited by SOD, thus supporting the role of superoxide in this mechanism; the results are collected in Table 1. The reduction of NBT by tetrazomine is pH-dependent, exhibiting an increased rate of reduction as the pH is raised (Table 1, entries 1–3). This behavior directly parallels the pH dependency for the plasmid DNA cleavage reactions described below. Entries 6–8 of Table 1 support the notion that the oxazolidine moiety of tetrazomine must be "intact" and in the quinocarcin configuration, exhibited by synthetic analog **11**, in order for the production of superoxide to proceed.

Cleavage of Supercoiled Plasmid DNA (pUC 19) by Tetrazomine. In order to examine the interaction of tetrazomine with DNA, the pure antibiotic (0.01–10 mM) was allowed to react with supercoiled plasmid DNA (pUC 19) in phosphate buffer (20 mM) between pH 5 and 9 at 37 °C for 2 h in the presence of air. Salient experimental results are collected in Tables 2 and 3. Tetrazomine displayed nicking of the DNA at a 0.1 mM concentration (Table 2, entry 8) at pH 8 without the addition of any external reductants. The reaction was found to be pH-dependent; optimal cleavage occurred at or above pH 7. At lower pH values (below pH 6), nicking was observed, but to a lesser extent (Table 2, entries 1–5). This is consistent with the obligate participation of the *unprotonated* oxazolidine nitrogen atom in the redox cycle. The slightly different pH versus nicking yield (S) profiles for tetrazomine and quinocarcin, illustrated in Figure 3, could therefore be explained by small differences in the pK_a's for the oxazolidine nitrogens. In other words, a slightly higher pH medium is necessary for deprotonation of the quinocarcin oxazolidine nitrogen, which subsequently turns on the redox cycle. Also supporting this notion is the fact that the NBT reductions for tetrazomine and quinocarcin exhibit the same overall trends.

Table 2: Cleavage of Supercoiled Plasmid DNA (pUC 19)

entry	conditions	[tetrazomine or quinocarcin]	S
1	20 mM phosphate, pH 5	1.0 mM tetrazomine	0.9
2	20 mM phosphate, pH 6	1.0 mM tetrazomine	4.4
3	20 mM phosphate pH 7	1.0 mM tetrazomine	9.0
4	20 mM phosphate, pH 8	1.0 mM tetrazomine	10.0
5	20 mM phosphate, pH 9	1.0 mM tetrazomine	10.0
6	20 mM phosphate, pH 8	1.0 mM quinocarcin	9.7
7	20 mM phosphate, pH 8	0.01 mM tetrazomine	0.0
8	20 mM phosphate, pH 8	0.1 mM tetrazomine	0.4

Table 3: Effects of Additives on Plasmid DNA Cleavage

entry	conditions[a]	[tetrazomine] (mM)	% inhibn	% enhancemt
1	0.1 mM $Fe^{II}SO_4$	1.0	0	0
2	0.1 mM $Fe^{III}NH_4SO_4$	1.0	5	
3	0.1 mM desferal	1.0	0	0
4	1.0 mM desferal	1.0	37	
5	10 mM desferal	1.0	94	
6	deoxygenated	1.0	80	
7	0.1 mM H_2O_2	0.1		68
8	0.1 mM H_2O_2	1.0		29
9	1.0 mM picolinic acid	1.0	28	
10	10 mM picolinic acid	1.0	71	
11	10 μg/mL catalase	1.0	55	
12	100 μg/mL catalase	1.0	54	
13	10 μg/mL SOD	1.0	94	

[a] All reactions were run in 20 mM phosphate buffer (pH 8.0).

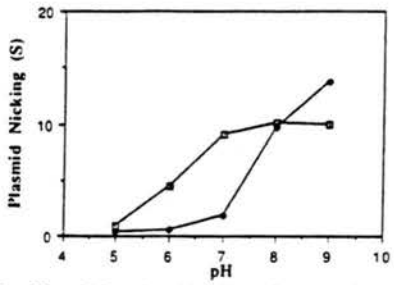


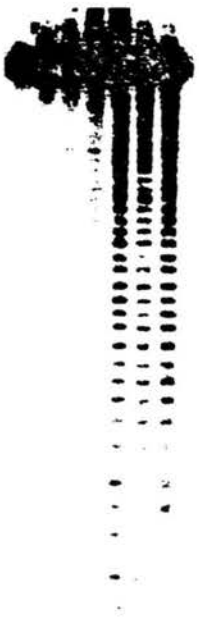
FIGURE 3: pH trends for plasmid nicking of tetrazomine (□) versus quinocarcin (◆). All reactions contained 0.15 μg of supercoiled plasmid DNA (pUC 19) and 1 mM drug and were run in 20 mM phosphate buffer at the indicated pH's (2 h, 37 °C). Values for S were calculated from the Poisson distribution, on the basis of the ratios of forms I (supercoiled), II (nicked open circular), and III (linear) of the DNA from scanning densitometry data of agarose electrophoresis gels following reactions.

Complete removal of oxygen from these experiments during the many manipulations is very difficult; however, partial exclusion of oxygen significantly inhibited this reaction, as expected (Table 3, entry 6). Superoxide dismutase also greatly inhibited DNA cleavage (Table 3, entry 13), which is consistent with both the capacity of tetrazomine to generate superoxide and the corresponding DNA cleavage event to be exclusively superoxide-dependent. Catalase inhibits the reaction, but not as potently as SOD (Table 3, entries 11 and 12). Addition of hydrogen peroxide to tetrazomine/DNA reactions had a potent stimulatory effect on DNA cleavage over control reactions containing hydrogen peroxide at the same concentrations (Table 3, entries 7 and 8). Taken together, the above

results point strongly to Fenton-type chemistry being responsible for the scission of DNA.

Tomita previously reported that the addition of metal ions such as Fe(III) had no stimulatory effect on the ability of quinocarcin to cleave DNA; we have corroborated this finding with tetrazomine, and the results appear in Table 3 (entries 1 and 2). The addition of the potent iron chelator desferal did, however, exhibit significant inhibition of DNA cleavage, particularly at high concentrations (Table III, entries 3–5). Desferal is known to have a high affinity for Fe(III) (log k_f = 30.7), forming a hexacoordinate complex that excludes iron-associated water and uncouples the oxidation of Fe(II) from the formation hydroxyl radical (Fenton reaction). The participation of higher oxidation states of iron and copper in Fenton reactions and related CH oxidation chemistry is now well-recognized (Saito et al., 1990; Zafirou & Bonneau, 1987). Picolinic acid is known (Sheu, 1990) to be a very potent scavenger of hydroxyl radical and inhibitor of the Fe(II)/Fe(III) redox couple. Addition of picolinic acid to reaction mixtures of DNA and tetrazomine at 1 and 10 mM showed 28% and 71% inhibition, respectively. The rate of formation of superoxide by tetrazomine (see Table 1) is extremely slow (10^4–10^5 times slower) relative to the rate-limiting step of the Haber-Weiss/Fenton reaction, which is 76 M^{-1} s^{-1} (Lesko, 1980) for the reduction of hydrogen peroxide by Fe(II). Thus, our data support a hypothesis wherein the limiting reagent in the DNA cleavage mediated by tetrazomine and quinocarcin is the slow production of superoxide. These results also support the notion that adventitious metal in these reaction mixtures in a number of possible oxidation states can be activated by the slow release of superoxide and cause Fenton-related damage to DNA. Thus, these results indicate that DNA cleavage is indeed metal-dependent and that the low concentration of adventitious Fe(III) present is already in excess of that required to effect Fenton-mediated cleavage of DNA; the addition of excess iron therefore would not be expected to have any additive effect. Thus, our data again support the hypothesis wherein the limiting reagent in the DNA cleavage mediated by quinocarcin or tetrazomine is the slow production of superoxide.

Reactions of Tetrazomine with 5′-^{32}P-Labeled Synthetic 45-mer for High-Resolution Polyacrylamide Gel Electrophoresis. Further evidence for a non-DNA-associated oxidant was obtained from analysis of the reaction of tetrazomine with a small synthetic oligonucleotide by high-resolution polyacrylamide gel electrophoresis. A synthetic 45-base-pair oligonucleotide, end-labeled with ^{32}P and annealed to its complement, was reacted with tetrazomine (0.1, 1.0, and 10 mM without any additional reducing agents) at 37 °C for 5 h (20 mM phosphate buffer, pH 8), resulting in non-sequence-specific cleavage at every nucleotide as evidenced by denaturing 20% polyacrylamide gel electrophoresis (Figure 4, lanes 3–5). Furthermore, every cleavage band appeared as a doublet, which is characteristic of the 3′-phosphate and 3′-phosphoglycolate ends resulting from nonselective Fenton-mediated cleavage (Tullius & Dombroski, 1985; Hertzberg & Dervan, 1984). We observed a similar cleavage pattern from incubating the duplex with 10 mM quinocarcin and with 1.0 mM $FeSO_4$/EDTA under aerobic conditions (Figure 4, lanes 6 and 7, respectively). Initially, we found that attenuated cleavage was observed at higher tetrazomine concentrations (10 mM) and was inconsistent with the results obtained by NBT reduction, which shows linear superoxide production over the same concentration range. One explanation for this observation was that in the presence of limited oxygen and high

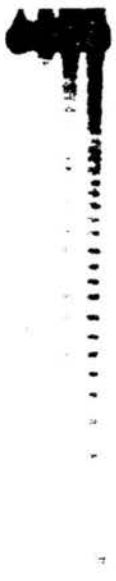


1 2 3 4 5 6 7

FIGURE 4: Lane 1: 5′-^{32}P-labeled duplex 45-mer. Lane 2: DNA (control). Lane 3: DNA + 0.1 mM tetrazomine. Lane 4: DNA + 1.0 mM tetrazomine. Lane 5: DNA + 10 mM tetrazomine. Lane 6: DNA + 10 mM quinocarcin. Lane 7: DNA + 1.0 mM Fe^{II}/EDTA. There was no significant evidence for any sequence specificity to the cleavage of this substrate (sequence along the gel is therefore superfluous and has been deleted).

tetrazomine concentrations a large portion of the hydroxyl radical produced was not in close proximity to the DNA, therefore exhausting the oxygen source before significant damage to DNA could occur. When reaction mixtures are saturated with oxygen, however, a more linear rate of DNA damage with respect to tetrazomine concentration is observed, as illustrated in Figure 4. An additional factor for this observation could be that tetrazomine, noncovalently or covalently associated within the minor groove of DNA, may protect the DNA from its own oxidative damaging potential. It has been proposed through molecular modeling (Hill et al., 1988) that quinocarcin docks in the minor groove and may alkylate DNA through the ring-opened iminium form (which would be analogous to **5**). The effect of tetrazomine associated to DNA in a similar manner might, therefore, be to protect the ribose phosphate backbone from oxidative damage by blocking sites in the minor groove. Evidence for the latter comes from experiments that show an increased resistance to Fe/EDTA cleavage for DNA that has first been incubated with tetrazomine (10 mM) for 5 h followed by ethanol precipitation (Figure 5, lane 3). However, when the order of reagents is reversed (i.e., treated with Fe/EDTA followed by 10 mM tetrazomine), significantly more cleavage is observed consistently (Figure 5, lane 4).

Another noteworthy observation is the change in relative ratios of the two 3′-base-pair products (phosphate versus phosphoglycolate) observed when comparing tetrazomine to quinocarcin and Fe/EDTA (Figures 4 and 6). By scanning densitometry, the relative ratios for phosphate to phosphoglycolate are 8:2 for tetrazomine, 6:4 for quinocarcin, and 4:6 for Fe/EDTA (lanes 5–7, respectively). Although speculative,



1 2 3 4

FIGURE 5: Lane 1: 5′-^{32}P-labeled duplex 45-mer. Lane 2: DNA (control). Lane 3: DNA + 10 mM tetrazomine followed by 1.0 mM Fe^{II}/EDTA. Lane 4: DNA + 1.0 mM Fe^{II}/EDTA followed by 10 mM tetrazomine. All reactions were carried out in phosphate buffer (pH 8) for 5 h with ethanol precipitations carried out between subsequent reactions.

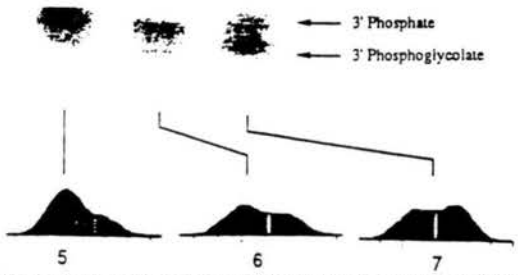


FIGURE 6: Representative base-pair cleavages from Figure 4 (lanes 5–7). Ratios of 3′-phosphate to 3′-phosphoglycolate by scanning densitometry are 8:2, 6:4, and 4:6 for tetrazomine (5), quinocarcin (6), and Fe/EDTA (7), respectively.

these results point to this protective behavior that these compounds exhibit, at least as far as abstraction of the 4′-hydrogen, which ultimately leads to the 3′-phosphoglycolate product, is concerned. This is again supported by the results illustrated in Figure 5, which show virtually no 3′-phosphoglycolate formation upon the treatment of DNA with Fe/EDTA that has first been incubated with tetrazomine.

DISCUSSION

Superoxide production by quinocarcin was previously reported (Tomita et al., 1984), and a mechanistic explanation has now been offered (Williams et al., 1992). Structural determination for tetrazomine (Sato et al., 1991) has revealed a ring system similar to that of quinocarcin, although little is currently known about the stereochemistry of this compound. On the basis of the analogous aerobic behavior that tetrazomine shares with quinocarcin, we now propose the same relative stereochemical configuration of quinocarcin for tetrazomine (with respect to the oxazolidine moiety). This assertion is based on studies (Williams et al., 1991) involving synthetic analogs **11** and **12** in Figure 1 (three-dimensional structures based on X-ray analysis), which suggest the quinocarcin oxazolidine configuration to be necessary for this redox mechanism to operate. When **11**, which has the same configuration as quinocarcin (*syn*), is allowed to stand in phosphate buffer (pH 8) under aerobic conditions at room temperature, it spontaneously produces superoxide (Table 1, entry 7). Under the same conditions, **12**, which is in the *anti* (non-quinocarcin) configuration, does not generate superoxide (Table 1, entry 8). Therefore, since tetrazomine readily produces superoxide under these conditions, this suggests that it possesses the same relative stereochemical configuration illustrated by **11** and quinocarcin, where the nitrogen lone pair is *trans*-antiperiplanar to the adjacent oxazolidinyl methine. This appears to be a requirement for redox chemistry to proceed on the basis of our observations.

The capacity of many antitumor antibiotics to cause oxidative damage to DNA in cancerous tissues is typically inseparable from the nonspecific damage inflicted on healthy cells by these reduced oxygen species and is widely recognized to be associated with the undesirable host toxicity of most antitumor drugs. The recognition of new mechanisms for the production of such reactive oxygen species and the chemical means to attenuate this reactivity without compromising other modes of action often displayed by such substances, such as nucleic acid alkylation, intercalation, and DNA polymerase inhibition, will be essential to designing more specific and efficacious cancer chemotherapeutic agents.

In summary, we have presented another example of a previously unrecognized reaction for the reduction of molecular oxygen by a simple heterocyclic ring system. This reduction is driven by the inherent intermolecular redox chemistry of the drug itself, requiring no exogenous reductants. Chemical means to attenuate the ability of this class of antitumor drugs to produce reactive oxidants (**11** versus **12**, **10**, stabilizers such as citric acid, stimulators such as DTT and hydrogen peroxide) may contribute to possible approaches to designing more selective and less toxic cancer chemotherapeutic agents. Studies aimed at elucidating the details of the expected covalent interactions of this class of compounds with nucleic acid targets are underway. In addition, further mechanistic studies employing synthetic analogs of these compounds are underway in these laboratories.

ACKNOWLEDGMENT

We are grateful to Yamanouchi Pharmaceutical Co. for providing the generous gift of natural tetrazomine. Quinocarcin used in this study was obtained as the generous gift of Kyowa Hakko Kogyo Co. High-resolution mass spectral data was supplied by the Midwest Center for Mass Spectrometry, with partial support by the National Science Foundation, Biology Division (Grant No. DIR9017262).

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