THESIS

SYNTHESIS AND BIOLOGICAL EVALUATION OF POTENTIAL DNA CROSS-LINKING AGENTS BASED ON PYRROLIZIDINE ALKALOIDS

Submitted by

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In partial fulfillment of the requirements

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WE HEREBY RECOMMEND THE THESIS PREPARED UNDER OUR SUPERVISION BY CHRISTI KOSOGOF ENTITLED "SYNTHESIS AND BIOLOGICAL ENVALUATION OF POTENTIAL DNA CROSS-LINKING AGENTS BASED ON PYRROLIZIDINE ALKALOIDS" BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Graduate Research Committee Members

ADVISOR

DEPARTMENT HEAD

ABSTRACT OF THESIS

SYNTHESIS AND BIOLOGICAL EVALUATION OF POTENTIAL DNA CROSS-LINKING AGENTS BASED ON PYRROLIZIDINE ALKALOIDS

DNA cross-linking agents represent one of the most potent categories of antitumor agents. Pyrrolizidine alkaloids are an abundant class of biologically active natural products. These alkaloids display high levels of DNA cross-linking ability, and could have potential use as antitumor agents. Unfortunately, the high levels of hepatotoxicity induced by these compounds negates their effectiveness as antitumor agents.

Modification of the chemical structure of the pyrrolizidine core could yield compounds with potent antitumor activity and reduced hepatotoxicity. The synthesis of a class of potential pyrrolizidine progenitors developed from monocrotaline is discussed. The synthesis of a potential water soluble photolabile protecting group is also described In addition, the biological evaluation of the synthesized pyrrolizidine progenitors was investigated.

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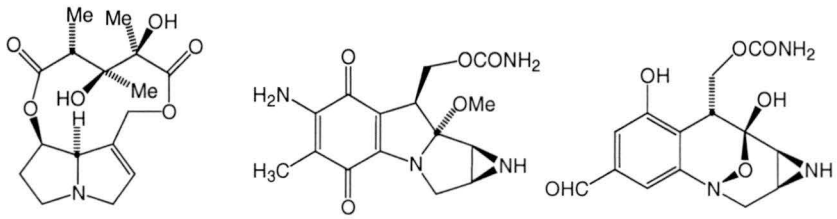
CHAPTER 1

1.1 DNA Cross-linking Agents

DNA interstrand cross-linking agents represent one of the most potent categories of antitumor antibiotics.[1] Interstrand cross-links in DNA are toxic to the cell because they shut down DNA replication, resulting in cell death. Among many compounds known for their DNA cross-linking capabilties, the ones of great interest in these laboratories include the mitomycins, the structurally related FR900482 class of antitumor antibiotics, and the pyrrolizidine alkaloids.[1]

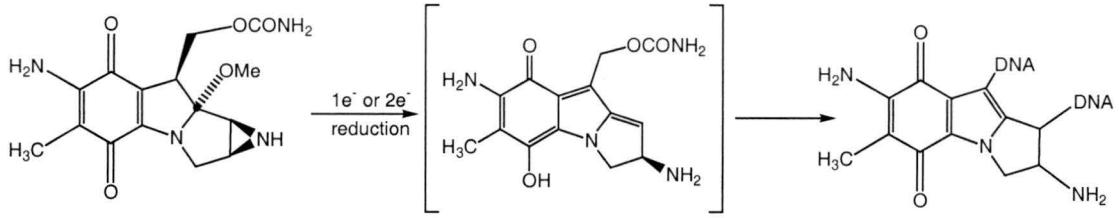


1, Monocrotaline **2**, Mitomycin C **3**, FR900482

Figure 1.1

1.2 Reductively Activated DNA Cross-Linkers

Mitomycin C (**2**), isolated in 1958 from *Streptomyces caespitosis* by the Kyowa Hakko corporation[1], remains one of the more widely used antitumor antibiotics in the treatment of various forms of cancer. Clinically, it is used for the treatment of breast, head, and neck tumors.[2c]



2, Mitomycin C **4** **5**

Scheme 1.1

Mitomycin C exerts its toxicity through the formation of DNA-DNA interstrand and DNA protein cross-links. It has also been shown to cause single strand breakage in DNA.[1] Mitomycin C is reduced *in vivo* by various flavoreductases[2], mimicked in a cell-free system by chemical reducing agents or purified reductases. A one- or two-electron reduction of mitomycin C results in a highly reactive pyrrolic intermediate (Scheme 1.1), capable of inducing DNA-DNA interstrand cross-link formation. It has been reported that the interstrand cross-linking occurs in the $^{5'}$CpG$^{3'}$ region in the minor groove of B-DNA[1]. A structural representation of such a cross-link is given in Figure 1.2. The sequence specificity observed for the mitosenes arises from a good geometrical fit of the two electrophilic centers of the mitosene (3.4 A) and the distances between the two cross-linked bases ($^{5'}$CpG$^{3'}$) of the DNA duplex (3.1 A). [1]

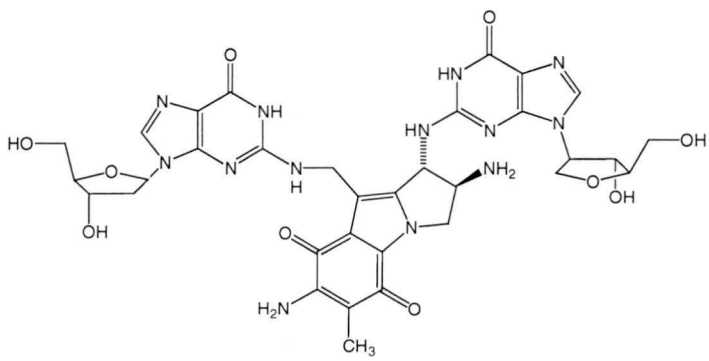


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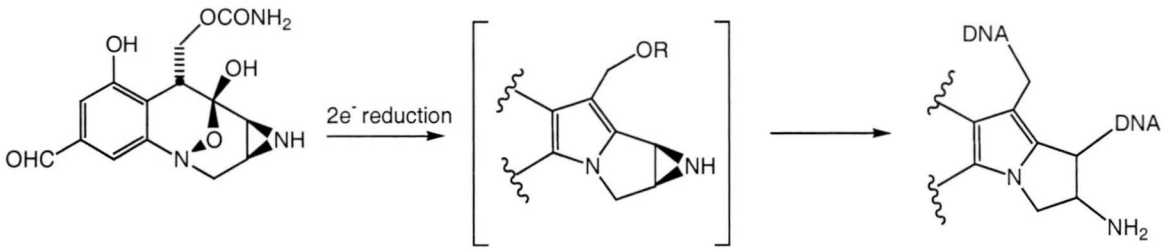
Figure 1.2

FR900482 (**3**) was obtained from the fermentation harvest of *Streptomyces sandaensis* at the Fujisawa Pharmaceutical Co. in Japan[16, 17]. In mitomycin C, it is the quinone moiety which is reductively labile. The FR900482 class of compounds contains a unique hydroxylamine hemiacetal bond, where the N-O bond is reductively labile.[35] FR900482 undergoes two electron reduction, which is followed by subsequent cyclization and dehydration which results in the proposed "mitosene"- type intermediate (**22**), capable of cross-linking DNA (Scheme 1.2).



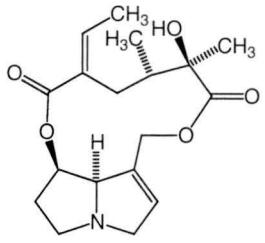
3, FR900482 **19** **20**

Scheme 1.2

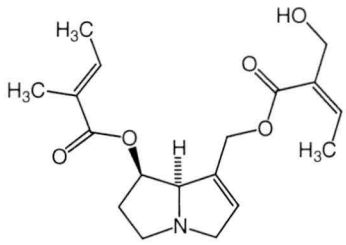
1.3 Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids contain a bicyclic ring structure similar to that of the mitomycins and are potent DNA cross-linkers. A wide spread use of these compounds as antitumor drugs has not occurred due to their high toxicity. Pyrrolizidine alkaloids comprise one of the most abundant classes of biologically active natural products. [4] The pyrrolizidine alkaloids consist of a dibasic "necine" ester and a pyrrolizidine diol "necine base". The pyrrolizidine alkaloids can be classified into five different categories (Figure 1.3).

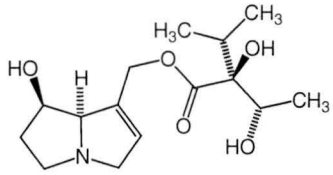
The senecionine type (**7**) contain greater than 100 structures. These alkaloids are abundant in the tribe Senicioneae, containing a branched C_{10}-necic acid biosynthetically derived from the C_5-carbon skeleton of isoluecine.



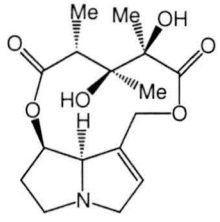
7, Senecionine



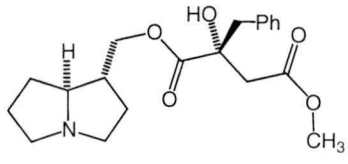
8, Triangularine



9, Lycopsamine



1, Monocrotaline



10, Phalaenopsine

Figure 1.3

The alkaloids belonging to the triangularine type (**8**) consist of some 50 structures found in the species of the Boraginaceae tribe and in the *Senecio* species. The triangularine type are characterized by 2 C_5-units, but exist as open chain analogs. The macrocyclic and open-chain diesters are never found together, but do occur in closely related species.

The third class of pyrrolizidine alkaloids belong to the lycopsamine type (**9**), with greater than 100 structures forming this group. This group of compounds is found in the tribe Eupatorieae and Boraginaceae. The lycopsamine type are characterized by a

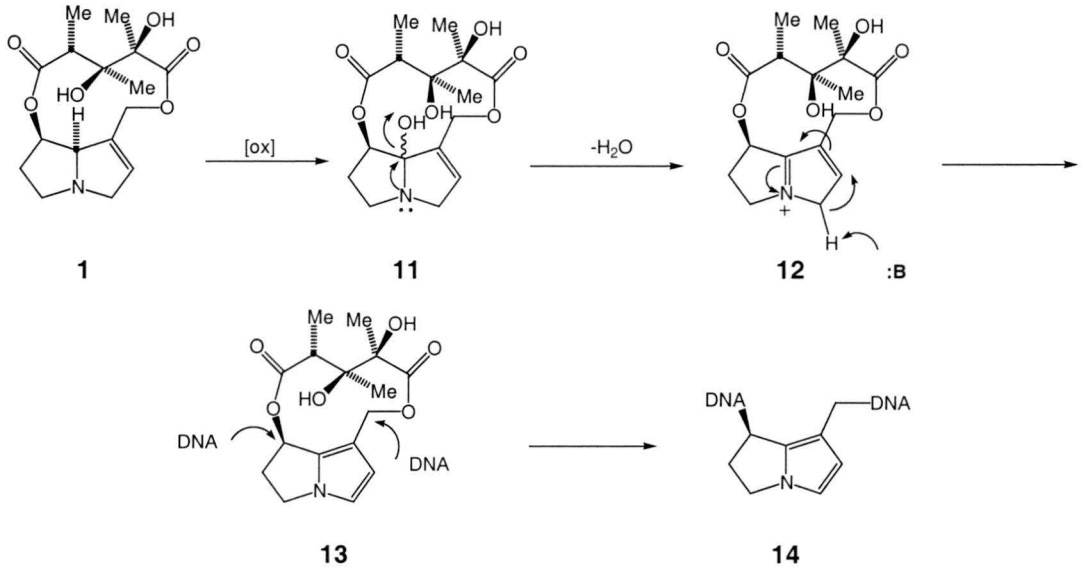
branched C_7-necic acid with the carbon skeleton of 2-isopropylbutyric acid. These compounds contain atleast one C_7-necic acid and may contain two.

The fourth class of pyrrolizidine alkaloids is the monocrotaline type (**1**), constiting of about 30 structures. These alkaloids are isolated from the genus *Crotalaria* and are characterized by the 11-membered macrocylic diester.

The last class of pyrrolizidine alkaloids is the phalaenopsine type (**10**), consisting of more than 20 structures. These alkaloids are from orchid pyrrolizidine alkaloids and do not contain a 1, 2-unsaturated necine base. These compounds are esterified with aryl-, aralkyl-, or alkylnecic acid fragments.

Pyrrolizidine alkaloids are known to be potent hepatotoxins and carcinogens, posing a threat to both humans and livestock. The pyrrolizidine alkaloids become toxic only when oxidized in the liver to the dehydroalkaloid form (Scheme 1.3). The pyrrolizidines are detoxified upon oxidation to the N-oxide form (Figure 1.4). These N-oxides are able to circulate in the blood, and must first be converted to the free base form before exerting any sort of toxicity.

Oxidation is accomplished by liver cytochrome P450 mixed-function oxidases.[5,6] The pyrrolizidine alkaloids containing the macrocyclic ester in which movement of the acid moeity is restricted, are metabolized more readily to the pyrrole. In contrast to the macrocyclic diester alakaloids, the open diester alkaloids have more steric hindrance at the C-8 position. This results in more N-oxide formation, making them less potent cross-linking agents. The dehydroalkaloid form **13** induces an irreversible, antimitotic effect on the liver cells resulting in necrosis. The dehydroalkaloid is highly reactive and therefore short-lived.

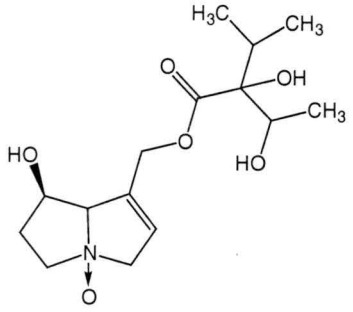


Scheme 1.3

On the molecular level, pyrrolizidine alkaloids exert their cytotoxicity through the formation of both DNA-DNA and DNA-protein cross-links.[7] When monocrotaline is oxidized to dehydromonocrotaline **13** (Scheme 1.2), this bifunctional electrophilic pyrrole reacts readily with nucleophilic molecules such as those in DNA and proteins. A fair amount of research has been conducted on the mechanism of biological action, however some controversy still exists. The pyrrolic compounds have been shown to cross-link synthetic duplex DNA (16 bp) containing the sequence 5'-d(CG).[8] This suggests that the pyrrolizidine alkaloids form interstrand DNA cross-links in a similar manner as the mitomycins. In addition to cross-link formation, dehydromonocrotaline has been found to alkylate the N7 of guanine in the major groove of DNA.[9] The same study also showed that the activated pyrroles are capable of undergoing polymerization which results in the formation of highly cross-linked DNA fragments, rather than one easily isolated DNA-DNA interstrand cross-link. Dehydromonocrotaline (**13**) is capable of

modifying DNA in several different manners *in vitro*, all of which could ultimately be responsible for its toxicity *in vivo*.

Several pyrrolizidine alkaloids were tested for their ability to inhibit tumor activity.[10] The pyrrolizidine alkaloids were investigated for their antitumor abilities because they behave as alkylating agents and some of the alkaloids, such as monocrotaline, displayed a high level of antitumor activity in various assays. One pyrrolizidine alkaloid derivative, indicine N-oxide **15** (Figure 1.4), was tested clinically as an antineoplastic agent.[11]



15, Indicine N-Oxide

Figure 1.4

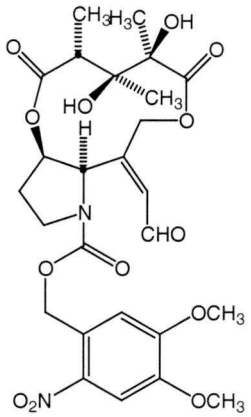
This compound was chosen as a potential candidate because it displayed reduced hepatotoxicity compared to other pyrrolizidine alkaloids. Indicine N-oxide (**15**) was used in phase I and II clinical trials in 1977 for acute leukemia. The resulting hepatotoxicity was more severe than initially expected, rendering it an impractical candidate.

1.4 Pyrrolizidine Progenitors

Analogs of pyrrolizidine alkaloids possess the potential to be useful antitumor agents, if their potent hepatotoxicity can be separated from their antitumor action.

Studies on the structure-activity relationships of these compounds have been performed.[12] A general trend among the pyrrolizidine alkaloids is that those containing the α,β-unsaturated 12-membered macrocyclic necic diester are more potent cross-linkers than the ones lacking the cyclic structure. Modification of the structure of the diester backbone of the pyrrolizidine alkaloids could potentially reduce the potent hepatotoxicity. Another approach to possibly avoiding the undesired toxicity could be accomplished by changing the mode of activation.

The oxidative mode of activation, occuring in the liver, results in undesired toxic side effects. Altering the mode of activation to a reductive mode should be manifested as lower hepatotoxicity. Alternatively, changing the mode of activation to a photoactivated mechanism, might also reduce the propensity for liver toxicity.



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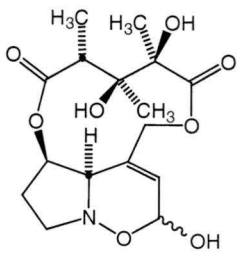

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Figure 1.5

Two different systems utilizing reductively and photolytically activated dehydropyrrolizidine progenitors were investigated in these labotatories by Dr. Jetze Tepe.[13,14] Both compounds were derived from monocrotaline (Figure 1.4). Monocrotaline, isolated from plants of the genus *Crotalaria*, displayed high levels of

activity against tumors in various assays.[10] The derivatives **16** and **17** employed different chemical "triggers" and upon activation resulted in the formation of dehydromonocrotaline.

1.4.1 Photoactivated Drugs and Potential Pro-Drugs

An important class of photoactivated DNA cross-linking agents includes the psoralens, such as 8-methoxypsoralen (Figure 1.6).[15] The psoralens are used to treat several diseases including psoriasis and T-cell lymphoma.

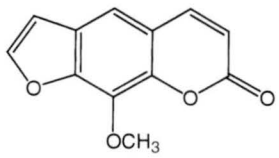

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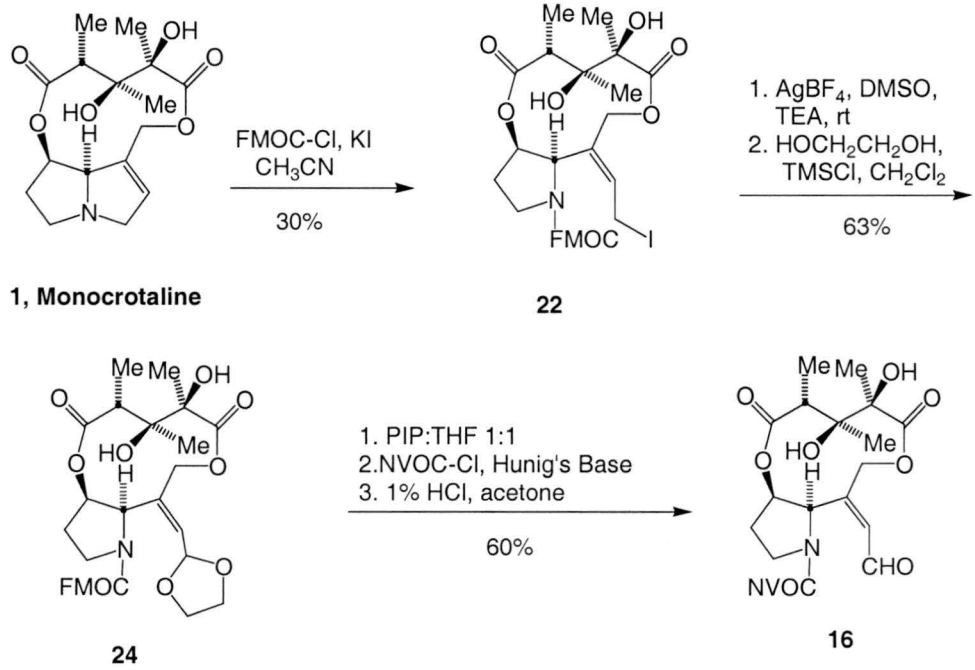
Figure 1.6

Photopheresis treatment involves the removal of lymphocytes from the patient, psoralen sensitization (exposure to 320-400 nm light), and the return of the cells to the patient. The treatment has been very successful against T-cell lymphoma as well as several autoimmune disorders.

Building on the concept of photopheresis therapy, the development of a photoactivated dehydromonocrotaline progenitor **16** utilized the 6-nitroveratryl-chloroformate protecting group to produce a masked pyrrolizidine alkaloid.

Using commercially available monocrotaline **1** (Scheme 1.4), the ring-opened allylic iodide **22** was synthesized in 30% yield with FMOC-Cl and KI in acetonitrile. Allylic oxidation followed by acetal protection of the α,β-unsaturated aldehyde gave the

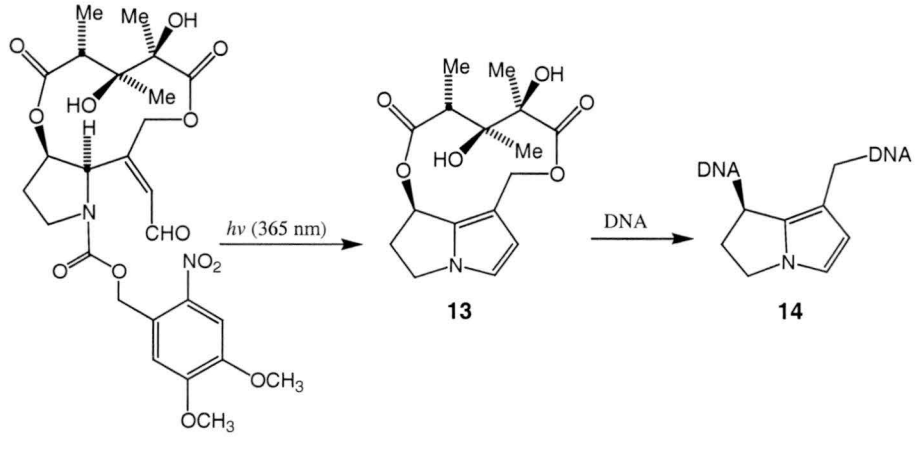
acetal **24** in 63% over two steps. Cleaveage of the FMOC-protecting group followed by NVOC-protection of the amine and acetal hydrolysis under acidic conditions gave the target aldehyde **16** in 60% yield over three steps.



1, Monocrotaline **22** **24** **16**

Scheme 1.4

Exposure to 365 nm light cleaves the NVOC group, and allows for subsequent cyclization and elimination of water to give dehydromonocrotaline (Scheme 1.5).



16 **13** **14**

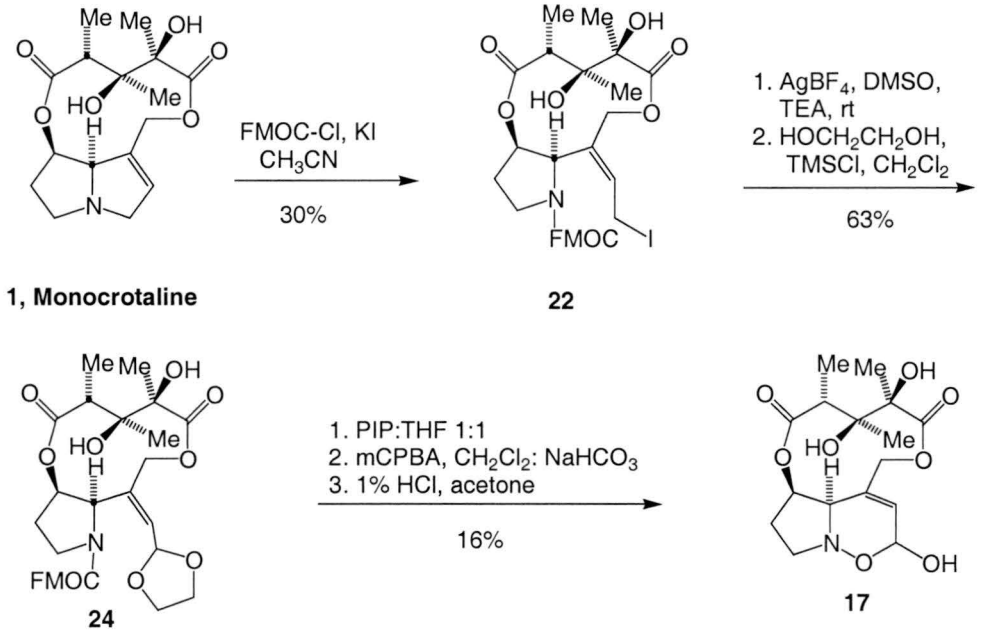
Scheme 1.5

In theory, a compound such as **16** could have potential clinical utility in the topical treatment of cancers, and introduce new possibilities for the development of other photoactivated prodrugs.

1.4.2 Reductively Activated Progenitors

In these laboratories, Dr. Tepe utilized the reductively labile hydroxylamine hemiacetal bond, found in the FR900482 class of compounds, as a trigger for another pyrrolizidine progenitor **17**.[14]

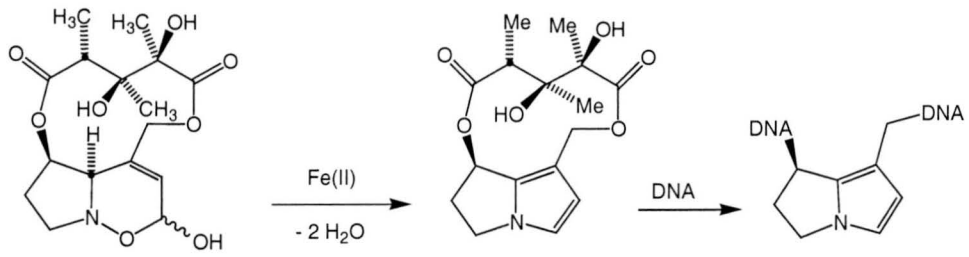
The hydroxylamine hemiacetal **17** was synthesized in a similar manner as **16** (Scheme 1.7). Deprotection of acetal **24** was followed by oxidation of the free amine to the hydroxyl amine with *m*-CPBA. The hydroxylamine was converted to the hydroxylamine hemiacetal **17** by hydrolysis of the acetal followed by cyclization.



1, Monocrotaline **22** **24** **17**

Scheme 1.7

The compound was easily reduced in the presence of Fe(II) to give the desired dehydromonocrotaline (Scheme 1.8). Once again, the mode of activation was altered, yet resulted in the same desired result (DNA cross-link formation).



17 13 14

Scheme 1.8

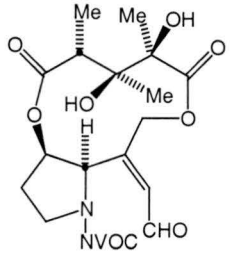
1.5 Conclusion

Ultimately, modification of the structure of the pyrrolizidine alkaloids provides the conceptual framework for the development of a new class of prodrugs. Modification of the mode of action *via* structural changes did not alter the DNA cross-linking ability of the compounds. These changes could potentially open the doorway to separating the severe hepatotoxicity the pyrrolizidines display from their potent antitumor activity.

CHAPTER 2

2.1 Synthesis of a Water Soluble Phototriggered Progenitor

While compound **16** proved to be a potent DNA cross-linker, it was minimally soluble in water. For a compound to have some clinical utility, water-solubility is key. While the compound need not be completely water-soluble, partial solubility would increase its ability to be administered intravenously. The synthesis of a more water soluble analog **21** was undertaken. Replacement of the diester backbone of the monocrotaline core with a dicarbamate moiety was investigated (Figure 2.1). In addition to enhancing the water solubility, the carbamate groups provide for good leaving groups in the covalent DNA cross-linking reactions, as illustrated by mitomycin C and the FR-class of antitumor antibiotics.[18, 19] Upon attack by a nucleophile (such as a guanine residue in DNA), if a poor leaving group is in place, it will not be displaced as easily by the entering nucleophile as if a good leaving group were in place.



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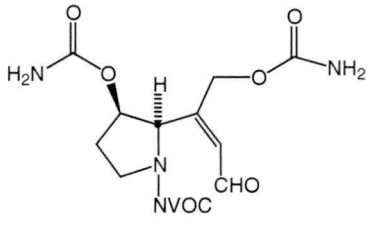

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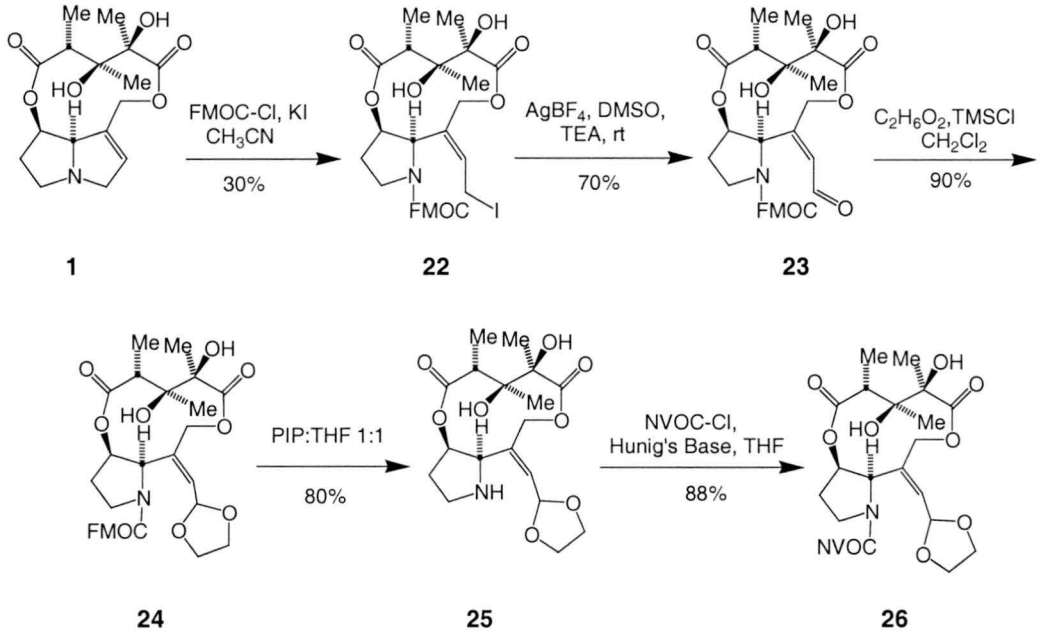
Figure 2. 1

Following the procedure described by Dr. Tepe[13], the synthesis began with commercially available monocrotaline. The ring-opening of the pyrrolizidine core (Scheme 2.1) was accomplished with 9-fluorenylmethylchloroformate (FMOC-Cl) in the presence of KI to give the desired minor product **21** in 30% yield. The major product of the reaction, is ring-opened with the iodide on the other carbon adjacent to the pyrrolizidine nitrogen. This compound can be recycled back to monocrotaline by cleavage of the FMOC-protecting group with piperidine. Oxidation of allylic iodide **22** to the α,β- unsaturated aldehyde **23** was accomplished with $AgBF_4$, DMSO, and triethylamine in 70% yield. The aldehyde was protected as the acetal **24** with ethylene glycol in 90% yield. Cleavage of the FMOC protecting group was accomplished with piperidine in 80% yield. Reprotection of the pyrrolidine nitrogen **25** using NVOC-Cl and Hunig's base gave **26** in 88% yield.



1 **22** **23**

24 **25** **26**

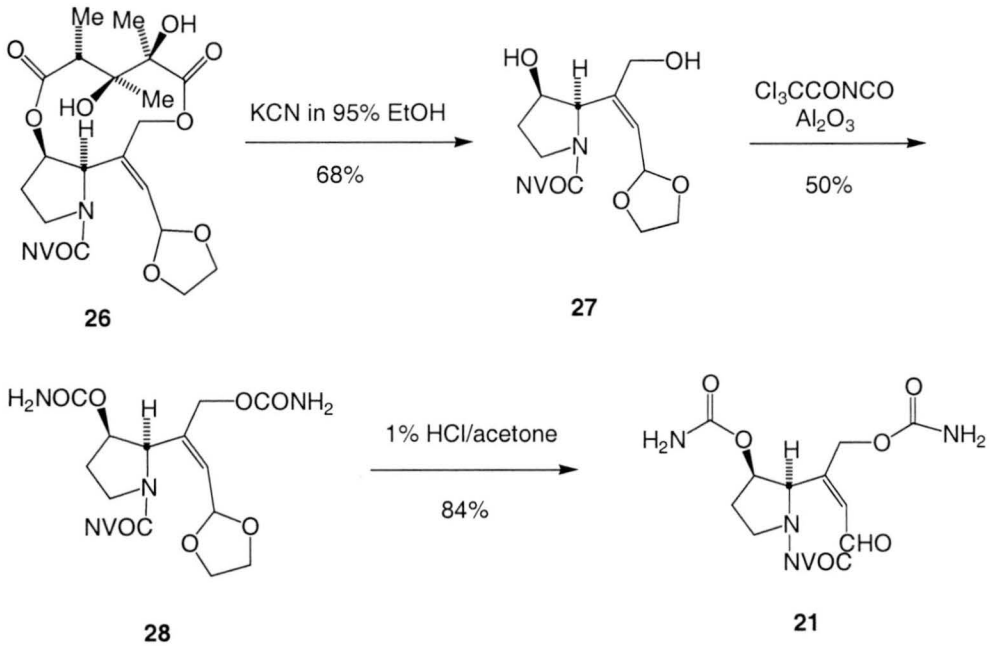
Scheme 2.1

Several attempts were made to cleave the macrocyclic diester backbone (Table 2.1). Tepe had previously reported cleavage of the diester backbone from dehydromonocrotaline could be accomplished with LiOH in H_2O and THF (unpublished data). The same conditions, when applied to this substrate, resulted in decomposition and no product formation. Various conditions were investigated, as described in Table 2.1. Presumably, decomposition was seen because of the cleavage of the NVOC-protecting group, resulting in undesired side product formation. The most successful method for ester hydrolosis was found to be KCN in 95% ethanol, reacting over a period of four days at room temperature. The desired diol **27** was produced in 68% yield.

Table 2.1 Conditions for Diester Cleavage

REAGENTS	CONDITIONS	RESULTS
LiOH: H_2O (2.0 eq)	1 h, RT	Decomposition
KCN in 95% EtOH	4 days, RT	68% yield
KCN in 95% EtOH	4 h, reflux	Decomposition
1 M HCl in MeOH	48 h, RT	No Reaction
1 M HCl in MeOH	5 h, reflux	Decomposition
1 M HCl in MeOH	6 h, 40°C	No. Iden. Prod.
$Ti(OiPr)_4$	8 h, RT	Trace Prod. Isol.

The carbamate moieties were installed using trichloroacetylisocyanate, followed by removal of the trichloroacetyl residues over neutral alumina.[20] The acetal **28** was hydrolyzed with 1% HCl in acetone to give the masked pyrrolizidine derivative **21** (Scheme 2.2).



Scheme 2.2

2.2 Synthesis of a phosphorylated NVOC-analog

Additional efforts to developing a more water soluble analog of the original progenitor included the synthesis of a phosphorylated analog of the NVOC-chloroformate **28** (Figure 2.2).

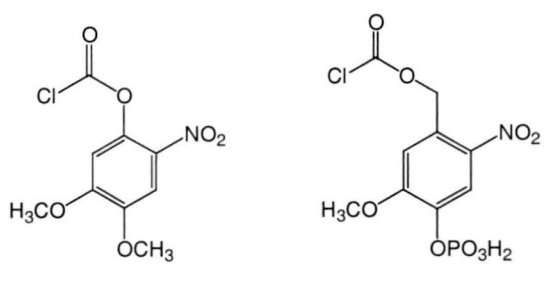
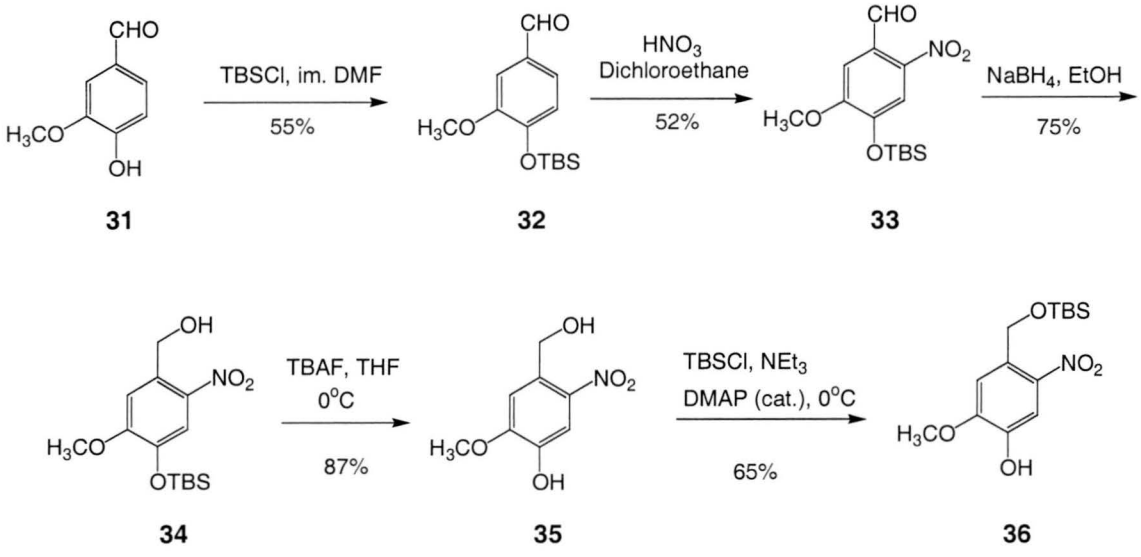


Figure 2.2

Starting with commercially available vanillin (**31**), TBS-protection of the free phenol was accomplished in 55% yield (Scheme 2.3). Subsequent *ortho*-nitration

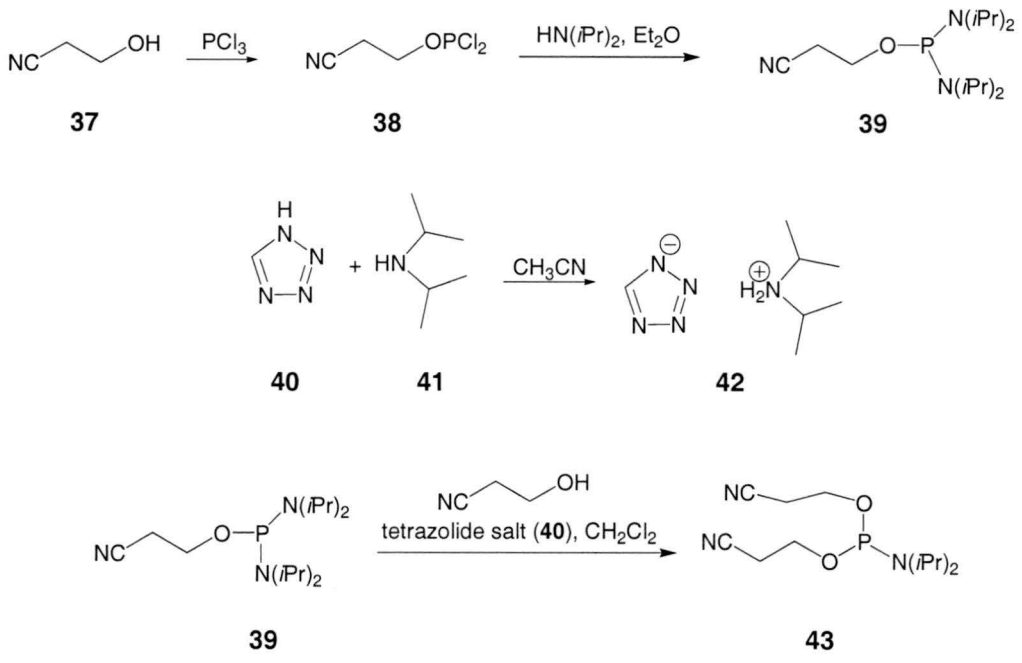
proceeded in 52% yield[21], followed by reduction of the benzaldehyde **33** with $NaBH_4$ in EtOH to give the primary alcohol **34** in 75% yield.



Scheme 2.3

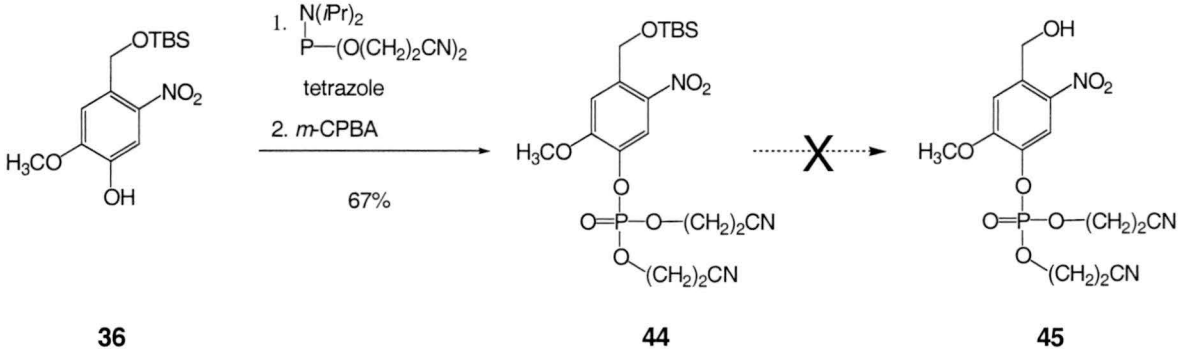
The TBS group of **34** was removed with TBAF in THF to give diol **35** in 87% yield. Initially, the TBS protecting group was chosen for selective protection of the primary hydroxyl group over the phenol. The protection proceeded in 65% yield using TBSCl, DMAP, and triethylamine.

The phosphoramidite **43**, used to couple to phenol **36**, was synthesized following the literature procedure (Scheme 2.4).[22] Commercially available 3-hydroxypropionitrile (**37**) was condensed with PCl_3 to give **38**. The dichlorophosphite **38** was subsequently coupled with diisopropylamine to give the phosphordiamidite **39**. Using freshly prepared tetrazolide salt **42**, it was coupled with 3-hydroxypropionitrile to give the desired phosphoramidite **43**. The phosphoramidite **43** was coupled to the free phenol **36** with tetrazole (**40**), and the resulting phosphite was oxidized with *m*-CPBA to give the desired phosphate **44** (Scheme 2.5).



Scheme 2.4

Problems were encountered when attempts were made to remove the TBS-protecting group from the primary alcohol **44**. The various conditions used are given in Table 2.2.

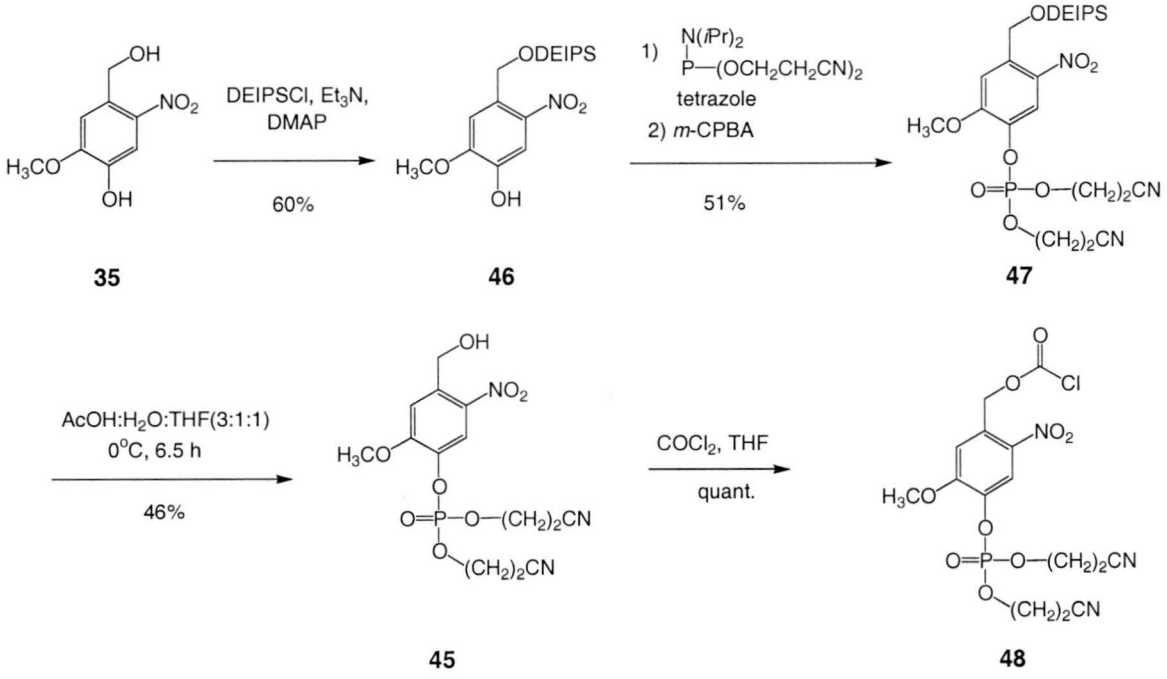


Scheme 2.5

Table 2.2 Conditions Used for Attempted TBS-group Removal from **44**

Reagents	Temperature	Time	Results
AcOH:THF:H_2O (3:1:1)	0°C to RT	12 h	No Reaction
AcOH:THF:H_2O (3:1:1)	30°C	8 h	No Reaction
AcOH:THF:H_2O (3:1:1)	50°C	5 h	Decomp.
HF: CH_3CN (5:95)	0°C	45 min.	Decomp.
HF: CH_3CN (5:95)	RT	3 h	No Iden. Prod.
TBAF: CH_3COOH (10:400)	0°C	0.5 h	No Iden. Prod.
1% HCl in EtOH	RT	2 h	No Iden. Prod.
Oxone in 50% Aqeous MeOH	RT	2 h	No Iden. Prod.
PPTS, EtOH	RT	3 h	No Iden. Prod.

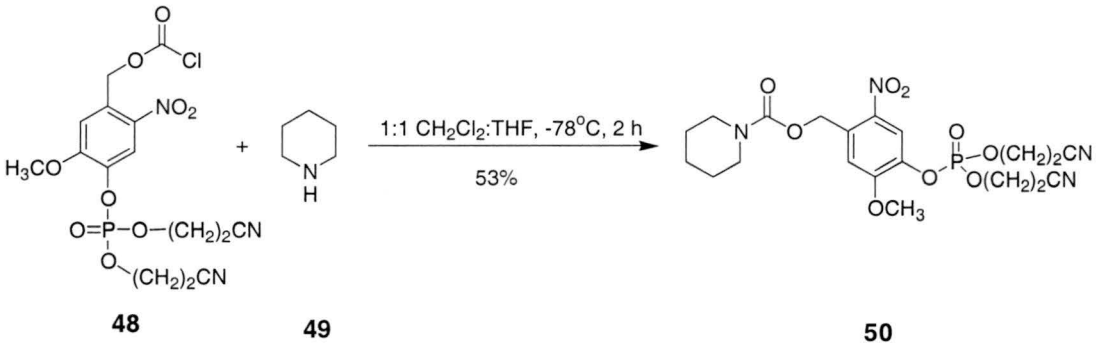
Presumably many of the difficulties encountered were a result of the cyanoethyl protecting groups on the phosphate group. Because these groups are labile under basic conditions, it was possible to only explore acidic methods to hydrolyze the TBS group. After several unsuccessful attempts at deprotection, a different silyl protecting group was employed.



Scheme 2.6

The diethylisopropylsilyl group was used in place of the *tert*-butyldimethylsilyl group (Scheme 2.6). Selective protection of the primary hydroxyl group over the phenol was accomplished in 60% yield. The free phenol **46** was then coupled to the phosphoramidite **42** in 50% yield to give the phosphate **47**. The DEIPS-group was cleaved in 46% yield with acetic acid, water and THF. The primary alcohol **45** was then converted to the chloroformate **48** quantitatively using phosgene. The cyanoethyl protecting groups were cleaved at a later stage, to avoid any difficulties arising from the anticipated water solubilty of the compound.

In order to determine if **30** could be cleaved in the same photolytic manner as the NVOC-chloroformate, it was first coupled to piperidine (**49**). Initial attempts at the coupling reaction utilized Hunig's base in THF. These conditions resulted in decomposition, presumably due to cleavage of one or both of the cyanoethyl protecting groups on the phosphate. To avoid undesired side products, piperidine and **48** were combined in a 1:1 CH_2Cl_2: THF mixture. After 2 h at –78°C, the desired coupled product **50** was obtained in 53% yield (Scheme 2.7).



48 **49** **50**

Scheme 2.7

The deprotection of the cyanoethyl protecting groups was more difficult than anticipated (Scheme 2.8). Cleavage was attempted under several basic conditions, as shown in Table 2.2. Exposure to triethylamine and *t*-butylamine resulted in cleavage of only one of the cyanoethyl groups. Reaction of **50** with NH_4OH resulted in decomposition. The cleavage was successfully accomplished using DBU and TMSCl in CH_2Cl_2 to give the desired product **51** in greater than 90% yield.

50 **51**

Scheme 2.8

Table 2.3 Conditions for cyanoethyl cleavage.

Reagents	**Conditions**	**Results**
Et_3N, CH_2Cl_2	25°C, 7 h	Monodeprotection
$tBuNH_2$, CH_2Cl_2	25°C, 8 h	Monodeprotection
NH_4OH	50°C, 3 h	Decomposition
DBU, TMSCl, CH_2Cl_2	25°C, 3 h	> 90% yield of **9**

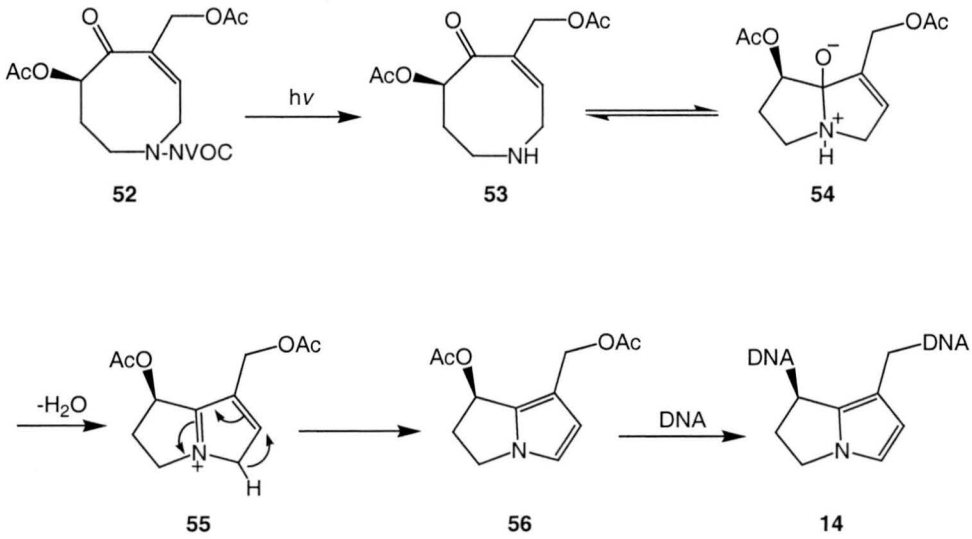
Once the deprotection was successful, the phosphate **51** was dissolved in D_2O and exposed to 365 nm light for 20 h. The experiment was monitored by ^1H NMR spectroscopy. The solution changed from a yellow to dark orange color, and the ^1H NMR indicated some type of decomposition. However, upon comparison of the NMR spectra to that of free piperidine in D_2O, the desired peaks were not observed. Other peaks aside

from that of the starting material began to appear in the NMR. If cleavage did occur, it may be possible that the products readily reacted with one another, therefore the desired peaks were not observed. It does not appear that the phosphate **51** is cleaved in a manner similar to the NVOC-protecting group.

It is possible that replacement of the electron-donating methoxy group with an electron-withdrawing group such as the phosphate altered the ability of the compound to be photolabile. Several other 2-nitrobenzyl groups do exist which have been shown to be photolabile.[36] Replacement of the methoxy at the 5'- position of the aromatic ring would provide possible insight to the electronics of this ring system. Further investigation of this system is warranted.

2.3 A photriggered azocine pyrrolizidine progenitor

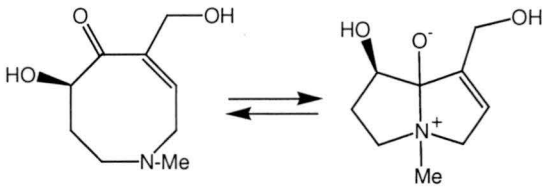
Based on the original findings reported in our laboratories by Dr. Tepe, the synthesis of an alternate pyrrolizidine progenitor **52**, which could also be photolytically cleaved (Scheme 2.9), was investigated.



Scheme 2.9

It was proposed that upon photolysis, the azocine ring would be deprotected to give the free amine (**53**), which would exist in equilibrium with its pyrrolizidine form. Subsequent loss of water would result in a compound with potential DNA cross-linking capabilities.

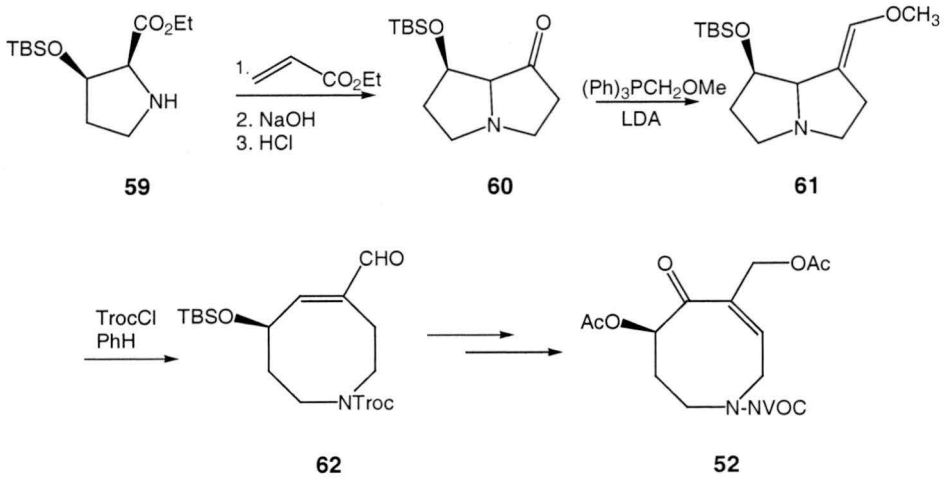
This "diacetate" derivative **52**, is a derivative of another known pyrrolizidine alkaloid, otonecine (**57**) (Figure 2.4). It was synthesized in 1983 by Yamada[23], and then in 1993 by Vedejs[24].



57, Otonecine **58**

Figure 2.4

It was originally envisioned that a derivative of 2-hydroxyproline **59** would easily be converted to the pyrrolizidine **60** through a Dieckmann-like cyclization (Scheme 2.10), as was reported by Koga, et al.[25]



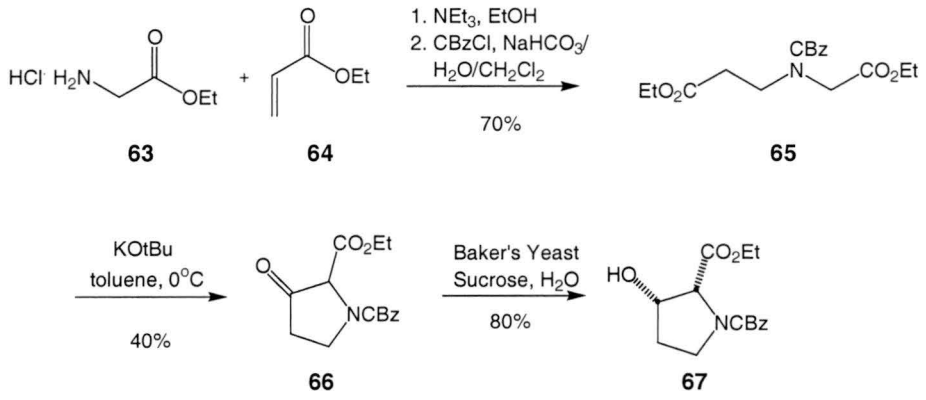
59 **60** **61**

62 **52**

Scheme 2.10

If the bicyclic compound **61** could be made, it could possibly be opened to the azocine **62**[26]. Further synthetic manipulations would then yield the target compound **52**.

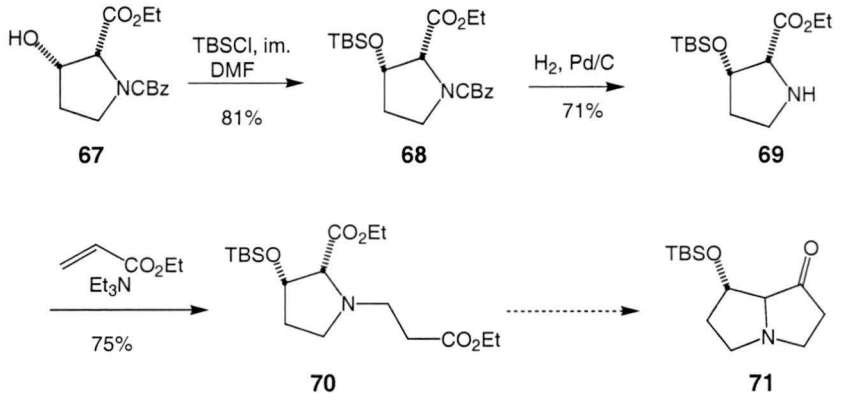
Synthesis of the hydroxyproline derivative **67** was accomplished following literature precedent (Scheme 2.11).[27] Glycine ethyl ester hydrochloride (**63**) was condensed with ethyl acrylate (**64**) to give the precursor **65**.



Scheme 2.11

Dieckmann cyclization using KOtBu was accomplished in 40%yield. Baker's yeast reduction of the ketone **66** resulted in the hydroxyproline derivative **67**.

Protection of the hydroxyl group of **67** with TBSCl, followed by removal of the CBz-protecting group gave compound **69**, in fair yields (Scheme 2.12). The free amine was then coupled with ethyl acrylate to give the precursor **68** of the bicyclic compound.



Scheme 2.12

Repeated attempts at the Dieckman cyclization (**70** to **71**) were made, at first using potassium *tert*-butoxide as the base, then using sodium ethoxide, as was reported in the original reference.[26] Each time, decomposition occurred after the attempted acid hydrolysis step. One reason for the decomposition might be due to elimination of the TBS-protected hydroxyl group. The TBS-group was removed from **70** with TBAF in THF, in poor yield, and another cyclization attempt on this deprotected compound was made using NaOEt. No recognizable product was isolated.

Many problems were encountered with this key cyclization step, therefore some alternatives were explored. The first option was a model study on the unsubstituted pyrrolizidinone **72**. A Wittig olefination at the carbonyl center had previously been reported by Kotchekov et al.[28] If the analog of **61** could be made from pyrrolizidinone, then it would have been possible to determine if the key, ring-opening step to give the desired azocine ring **62** was a viable option (Scheme 2.1).

Table 2.5. Various olefination attempts.

72 **73**

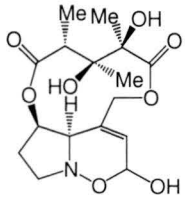
Reagents	Temperature	Time	Results
$Ph_3P^+CH_2OCH_3Br^-$	reflux	6 h	No Product
$Ph_3P^+CH_2OCH_3Br^-$	reflux	18 h	No Product
$CH_3OCH_2SiMe_3$, sec-BuLi, KH	-78°C to RT	3 h	No Product

Repeated attempts to react the carbonyl group of **72** with various olefinating agents (Table 2.5) were unsuccessful. It was reported by Murray et al.[29] that the carbonyl group of the unsubstituted pyrrolizidinone was unreactive to a number of reagents. In addition to this model study, alternative routes to achieve the desired pyrrolizidine **71** were also explored. In light of the difficulties encountered with the model study as well as the original problems encountered with making the desired pyrrolizidine, the proposed synthetic route was abandoned.

Certainly, other routes to the photoactivated azocine ring structure need to be investigated. A possible method to obtaining the desired azocine ring is that of ring closing metathesis. This methodology has been explored for the synthesis of eight-membered rings and could prove useful in this instance.

2.4 Synthesis of a derivative of the reductively activated progenitor

In developing a small library of analogs of these pyrrolizidine progenitors, the synthesis of a dicarbamate analog **76** of the hydroxylamine hemiacetal progenitor was proposed (Figure 2.5).



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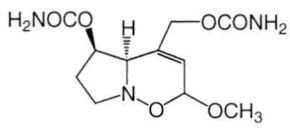
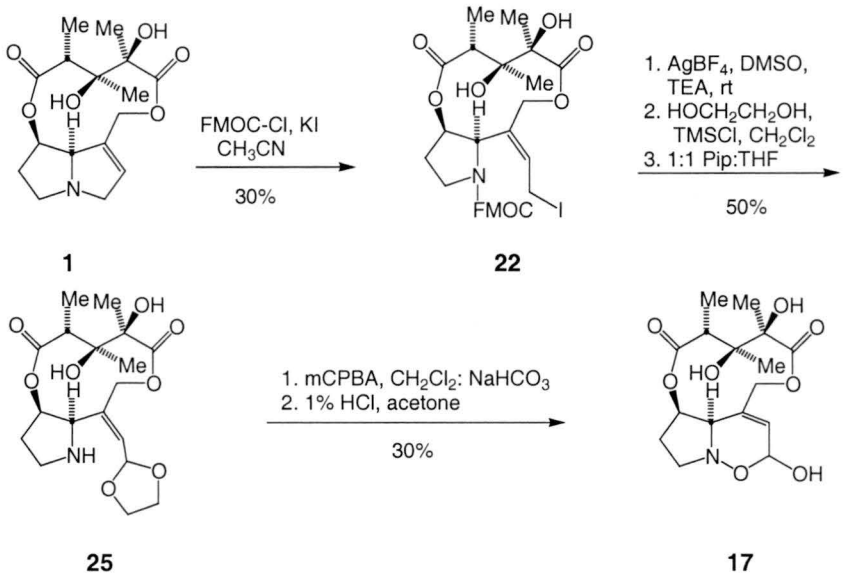


76

Figure 2.5

Following the procedure described by Tepe[14], the hydroxylamine hemiacetal **13** was synthesized from monocrotaline (Scheme 2.13). The acetal-protected amino compound **25** was synthesized as described previously. The amine was then oxidized using a slight excess of *m*-CPBA in a biphasic mixture of CH_2Cl_2 to give the hydroxylamine. Hydrolysis of the acetal and cyclization to give the hydroxyl amine hemiacetal **17** was accomplished with 1% HCl in acetone.

It is interesting to note that the oxidation of the amine to the hydroxylamine is a very difficult and inconsistent reaction. Several products are observed, the predominant one resulting from overoxidation ro give the nitrone. In addition to optimizing the reported conditions, other methods of oxidation were investigated. Use of urea-hydrogen peroxide with catalytic methyltrioxorhenium[30] showed some promise as a milder alternative. However, yields above 30% were never achieved, and so the procedure was not modified from the original.

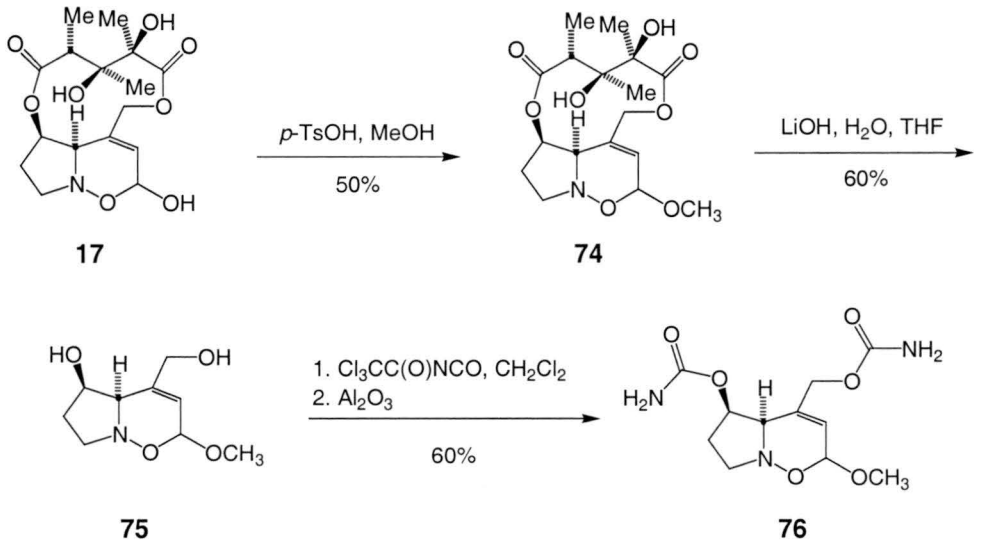


1 **22** **25** **17**

Scheme 2.13

Cleavage of the diester backbone of **17** was originally attempted with the free hydroxylamine hemi-acetal. Problems were encountered, probably because of the compound existing in equilibrium with the ring-opened free aldehyde. To avoid these complications, **17** was converted to the methyl hemiacetal **74** with *p*-TsOH in methanol (Scheme 2.14).

Initially, KCN in ethanol was used to cleave the diester, however, the compound proved to be unreactive after two days at room temperature under these conditions. Use of LiOH , H_2O, and THF proved to be successful, resulting in the diol **75** in about 60% yield. The dicarbamate **74** was then formed using trichloroacetylisocyanate in 64% yield.



17 74 75 76

Scheme 2.14

2.5 Conclusion

Two new derivatives of the original pyrrolizidine progenitors were successfully synthesized from monocrotaline. Alternatives to developing other phototriggered compounds were also explored. While a phosphorylated analog of the NVOC protecting group was synthesized, it was not photoactivated in the same manner as the NVOC-group. Synthesis of a phototriggered azocine ring structure was also attempted, but was abandoned due to the difficulties encountered.

CHAPTER 3

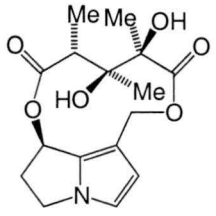
DNA-DNA interstrand cross-linking agents are very important to the treatment of many cancers. These agents are all activated differently. *In vitro*, it is possible to activate these compounds in a manner of different ways. Chemical reduction of compounds such as mitomycin C can be accomplished with Fe(II)-EDTA or various thiols.[31] Mitomycin C has been activated *in vitro* by a variety of one- and two-electron reducing enzymes. These enzymes include xanthine oxidase, xanthine dehydrogenase, DT-diaphorase, NADPH: cytochrome c reductase, and NADPH: cytochrome P450 reductase.[2, 32] While single enzymes have been isolated and studied with mitomycin C, it has been speculated that the interactions *in vivo* are much more complex.[32]

The DNA cross-linking abilities of the compounds studied was induced photolytically, chemically, and enzymatically. Alkaline agarose gel electrophoresis was used to visualize DNA-DNA cross-link formation.[34]

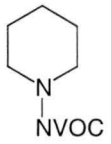
3.1 DNA Cross-linking studies of phototriggered dicarbamate 21

The DNA-DNA cross-linking ability of dicarbamate derivative **21** was investigated using linear pBR322 DNA by denaturing alkaline agarose gel electrophoresis. Compound **21** (various concentrations of a 10mM stock solution) and 0.5 μg DNA (EcoR1 linearized pBR322) in a DMSO-water solvent mixture (10 μL, 0.001%DMSO/H_2O) was exposed to 365 nm light at 23°C for 1 h, followed by

incubation at 37°C for 9 h. The crude reaction mixture was loaded onto a denaturing alkaline agarose gel and provided the results shown in Figure 3.2.



13



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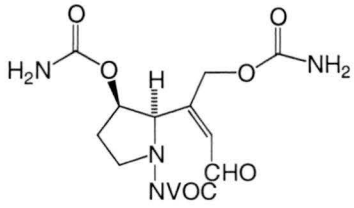


21

Figure 3.1

Lambda DNA BSTE II digest was employed as a molecular weight standard (lane 1). Control reactions were performed with NVOC-protected piperidine (10μM, 1μM, 100nM) to assure that the photocleaved side product, 6-nitroveratryl aldehyde does not induce cross-link formation. Dehydromonocrotaline (10 μM) was also used as a DNA cross-link standard.

As illustrated in Figure 3.2, incubation of compound **21** with the DNA duplex in the dark leads to no detectable cross-linked product (lanes 9, 11, 13). Incubation of compound **21** with linear pBR322 at 10 μM and 1 μM with exposure to 365 nm light for 1 hour results in DNA cross-link formation (lanes 10 and 12). As shown, only the reactions depicted in lanes 10 and 12 produced the interstrand DNA-DNA cross-link product similar to dehydromonocrotaline (lane 4).

The DNA cross-linking of compound **21** is comparable to the cross-linking ability of **16**.[13] The original photoactivated compound displayed DNA cross-linking at concentrations of 100 μM or more, and compound **21** displays cross-linking at 1μM and higher.

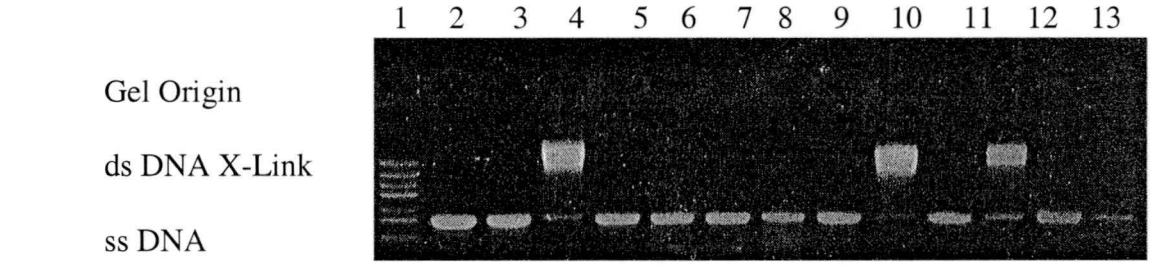


Figure 3.2 All dark (control) reactions were incubated at 37°C for 10 hours. The reactions exposed to UV radiation (1 hour) were incubated an additional 9 hours at 37°C. Lane (1) 0.5 μg lambda DNA BSTE II digest (molecular weight standard); lane (2) 0.5 μg pBR322 (control); lane (3) 0.5 μg pBR322 + hν, 1 hour (light control); lane (4) 0.5 μg pBR322 + 10 μM dehydromonocrotaline; lane (5) 0.5 μg pBR322 + 10 μM NVOC-piperidine (dark control); lane (6) 0.5 μg pBR322 + 10 μM NVOC-piperidine + hν, 1 hour (light control); lane (7) 0.5 μg pBR322 + 1 μM NVOC-piperidine + hν, 1 hour (light control); lane (8) 0.5 μg pBR322 + 100 nM NVOC-piperidine + hν, 1 hour (light control); lane (9) 0.5 μg pBR322 + 10 μM compound **21** (dark control); lane (10) 0.5 μg pBR322 + 10 μM compound **21**+ hν, 1 hour; lane (11) 0.5 μg pBR322 + 1 μM compound **21** (dark control); lane (12) 0.5 μg pBR322 +1 μM compound **21** + hν, 1 hour; lane (13) 0.5 μg pBR322 + 100 nM compound **21** (dark control); lane (14) 0.5 μg pBR322 + 100 nM compound **21** + hν, 1 hour.

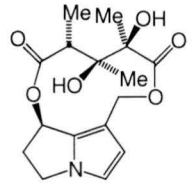
These studies suggest that replacement of the diester backbone with the dicarbamates does not affect the cross-linking potential of the pyrrolizidine compound, and demonstrates the viability of masked DNA-reactive pyrrolizidine progenitors that are capable of photochemical activation. Such agents hold promise as tools to further gain insight into the mechanism of DNA-DNA and DNA-protein cross-linking.

3.2 **Biochemical and Biological Studies of Dicarbamate 76**

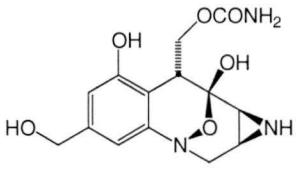
3.2.1 DNA Cross-linking Studies

In an attempt to determine the DNA cross-linking potential of the dicarbamate derivative **76**, several experiments were carried out. Initially, the compound (at various concentrations, Figure 3.4), in a DMSO/water mixture, was combined with 100 μM Fe(II) EDTA (pH = 8.0) and linearized pBR322 DNA. Dehydromonocrotaline (**13**) was

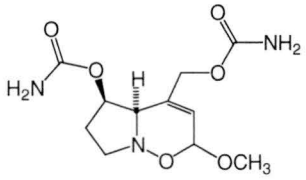
used as a cross-link standard, and FR66979 (**78**) was used as a standard for reducitve activation by Fe(II).



13, Dehydromonocrotaline



78, FR66979



76

Figure 3.3

After incubation at 37°C for 14 h, alkaline agarose gel electrophoresis was performed on the reactions. It can clearly be seen that 1 mM FR66979 is readily reduced in the presence of 100 μM Fe(II) EDTA, to produce a DNA cross-link (Figure 3.4, lane 5). Dicarbamate **76** displayed DNA cross-linking , but only at high concentrations (10 mM and 5 mM) (Figure 3.4, lanes 7, 8). Longer incubation times of 22 h showed no significant change in cross-link formation.

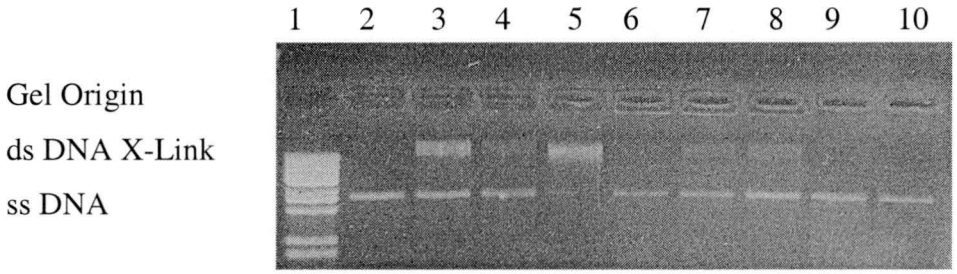


Figure 3.4. All reactions were incubated at 37°C for 14 hours. Lane (1) 0.5 μg lambda BSTE digest (molecular weight standard); lane (2) 0.5 μg pBR322 (control); lane (3) 0.5 μg pBR322 +10 μM dehydromonocrotaline; lane (4) 0.5 μg pBR322 + 1 mM FR66979; lane (5) 0.5 μg pBR322 + 1 mM FR66979 + 100 μM Fe(II) EDTA; lane (6) 0.5 μg pBR322 + 10 mM compound **76**; lane (7) 0.5 μg pBR322 + 10 mM compound **76** + 100 μM Fe(II)EDTA; lane (8) 0.5 μg pBR322 + 5 mM compound **76** + 100 μM Fe(II)EDTA; lane (9) 0.5 μg pBR322 + 1 mM compound **76** + 100 μM Fe(II)EDTA; lane (10) 0.5 μg pBR322 + 100 μM compound **76** + 100 μM Fe(II)EDTA.

It has been reported that thiols, such as dithiothreitol and β-mercaptoethanol, when combined with Fe(II) are more potent reducing agents than Fe(II) alone.[31] Reactions employing both thiols at a concentration of 1 mM , in combination with 100μM Fe(II)EDTA were employed. The results are shown in Figure 3.5.

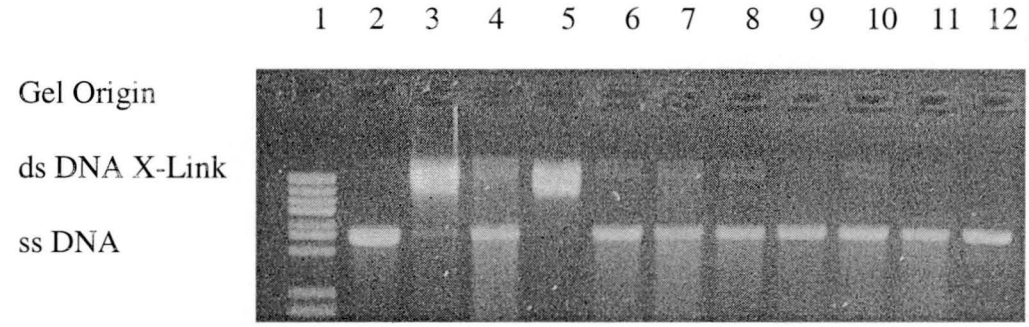
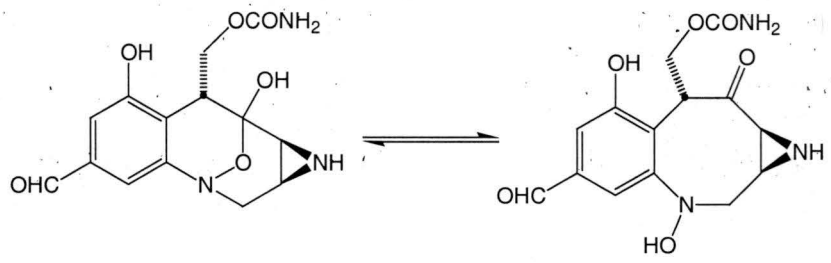


Figure 3.5 All reactions were incubated at 37°C for 14 hours. Lane (1) 0.5 μg lambda BSTE digest (molecular weight standard); lane (2) 0.5 μg pBR322 (control); lane (3) 0.5 μg pBR322 +10 μM dehydromonocrotaline; lane (4) 0.5 μg pBR322 + 1 mM FR66979; lane (5) 0.5 μg pBR322 + 1 mM FR66979 + 100 μM Fe(II) EDTA; lane (6) 0.5 μg pBR322 + 10 mM compound **76**; lane (7) 0.5 μg pBR322 + 5 mM compound **76** + 100 μM Fe(II)EDTA + 1 mM DTT; lane (8) 0.5 μg pBR322 + 5 mM compound **76** + 100 μM Fe(II)EDTA + 1 mM β-MET; lane (9) 0.5 μg pBR322 + 1 mM compound **76** + 100 μM Fe(II)EDTA + 1 mM DTT; lane (10) 0.5 μg pBR322 + 1 mM compound **76** + 100 μM Fe(II)EDTA + 1 mM β-MET. (11) 0.5 μg pBR322 + 100 μM compound **76** + 100 μM Fe(II)EDTA + 1 mM DTT. (12) 0.5 μg pBR322 + 100 μM compound **76** + 100 μM Fe(II)EDTA + 1 mM β-MET.

Addition of DTT (Figure 3.5, lane 7, 9, 11) and β-MET (Figure 3.5, lanes 8, 10, 12) did not result in additional cross-link formation. A possible explanation for the lack of cross-link formation might be due to the fact that the hydroxylamine methyl acetal bond has a much higher reduction potential than a normal hydroxylamine hemi-acetal bond. It has been reported that in FR900482, reduction of the compound occurs on the "ring-opened" isomer **79** (Figure 3.6).[33] It is thus reduction of the hydroxylamine bond which initiates the cascade of events, and not reduction of the hydroxylamine hemi-acetal

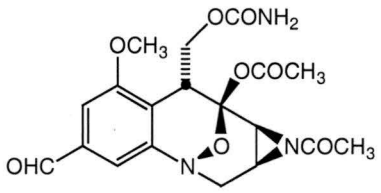
bond, according to this proposal. However, this is speculative and no experimental evidence has been reported to support this idea.



3, FR900482 **79**

Figure 3.6

In addition, FK317, another compound belonging to the FR900482 class of compounds, has been shown to require deacetylation of the hydroxylamine hemi-acetal -OH prior to reduction.[37] These observations would explain the low cross-link formation seen with compound **76**. The conversion of the hydroxylamine hemi-acetal to the methyl acetal prevents the compound from exisiting in a similar equilibrium, hindering it from undergoing a similar reduction.



78, FK317

Figure 3.7

In an attempt to initiate this "exchange" to the hydroxylamine hemiacetal, the reactions were performed in a pH = 6.0 sodium phosphate buffer and also in pH = 4.0 NaH_2PO_4 (Figure 3.8). Exchange of the methyl hemiacetal to the free hemi-acetal would

undoubtedly make the compound a more potent DNA cross-linking agent. It does appear, however, that compound **76** is a poor DNA cross-linking agent.

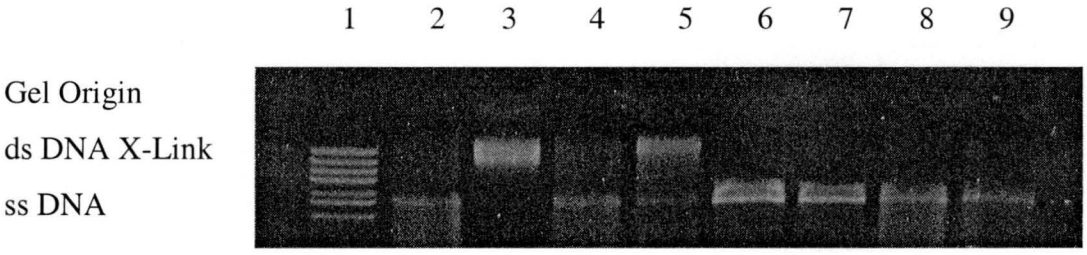


Figure 3.8 All reactions were incubated at 37°C for 22 hours. Lane (1) 0.5 μg lambda BSTE digest (molecular weight standard); lane (2) 0.5 μg pBR322 (control); lane (3) 0.5 μg pBR322 +10 μM dehydromonocrotaline; lane (4) 0.5 μg pBR322 + 1 mM FR66979; lane (5) 0.5 μg pBR322 + 1 mM FR66979 + 100 μM Fe(II) EDTA; lane (6) 0.5 μg pBR322 + 1 mM compound **76** + 50 mM sodium phosphate buffer (pH = 6.0); lane (7) 0.5 μg pBR322 + 1 mM compound **76** + 100 μM Fe(II)EDTA + 50 mM sodium phosphate buffer (pH = 6.0); lane (8) 0.5 μg pBR322 + 1 mM compound **76** + 50 mM NaH_2PO_4 (pH = 4.0); lane (9) 0.5 μg pBR322 + 1 mM compound **76** + 100 μM Fe(II)EDTA + 50 mM NaH_2PO_4 (pH = 4.0).

3.2.2 Antimicrobial Studies

The dicarbamate **76** was also tested for its ability to inhibit cell growth. A disc diffusion assay was performed. Five strains of bacteria were employed, and two different drug concentrations were tested. The results are given in Table 3.1. The Gram positive bacteria used were *Bacillus subtilis* and *Staphylococcus aureus*. The Gram-negative bacteria used in the assay were *Escerichia coli* and *Serratia marcesecens*, and the yeast used in the assay was *Saccharomyces cerevisiae*. In the antimicrobial assay performed, dicarbamate **76** showed no activity, with the cells being resistant to both concentrations tested. The parent compound **17** also showed no anitmicrobial actvity when tested by Dr. Tepe (unpublished data).

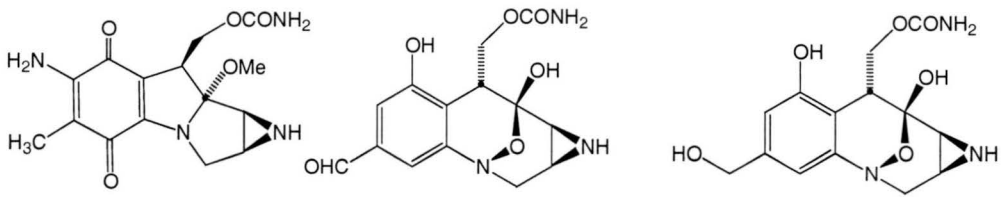
Table 3.1 Results of disc diffusion assay for **76**

Dilution	*B. subtilis*	*S. aureus*	*S.cerevisiae*	*E. coli*	*S. marcescens*
50 mM **74**	R	R	R	R	R
10 mM **74**	R	R	R	R	R
Streptomycin (10 μg)	-	-	-	9 mm	10 mm
Vancomycin (30 μg)	15 mm	10 mm	-	-	-
Nyastatin (100 μg)	-	-	8 mm	-	-

R= resistant; growth up to disc.

3.3 Enzymatic activation studies

Mitomycin C has been shown to be activated *in vitro* by various enzymes.[2,32] In addition, FK317 was also shown to be reduced by DT-Diaphorase.[37] In conjunction with Dr. Jetze Tepe, a DNA-cross-linking study of MC, FR900482, and compound **17** was performed (Figure 3.10).



2, Mitomycin C **3, FR900482** **78, FR66979**

Figure 3.9

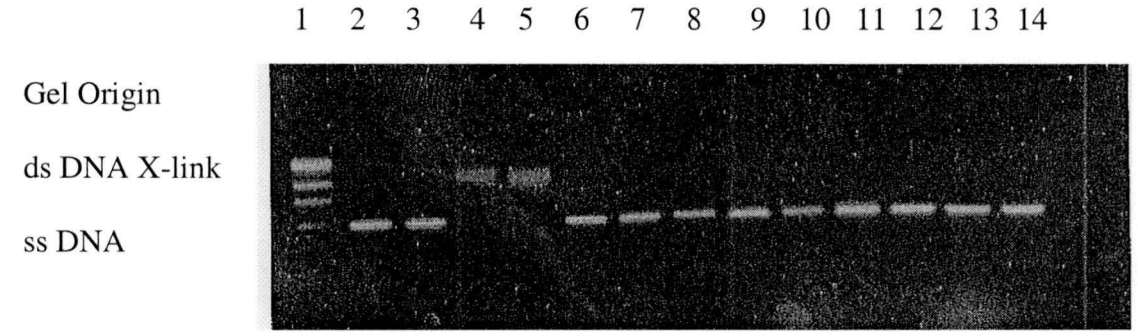


Figure 3.10 All samples were incubated at 25°C for 12 h. Lane (1): 0.5 μg Lambda Hind III digest (molecular weight standard); lane (2): 0.5 μg linear pBR322; lane (3): 0.5 μg pBR322 + 1 mM MC; lane (4): 0.5 μg pBR322 + 1 mM MC + 5 U FMN + 1 mM NADH; lane (5): 0.5 μg pBR322 + 1 mM MC + 5 U DIA + 1 mM NADH; lane (6): 0.5 μg pBR322 + 1 mM FR900482; lane (7): 0.5 μg pBR322 + 1 mM FR900482 + 5 U FMN + 1 mM NADH; lane (8): 0.5 μg pBR322 + 1 mM FR900482 + 5 U DIA + 1 mM NADH; lane (9): 0.5 μg pBR322 + 1 mM compound **17**; lane (10): 0.5 μg pBR322 + 1 mM compound **17** + 5 U FMN + 1 mM NADH; lane (11): 0.5 μg pBR322 + 1mM compound **17** + 5 U DIA + 1 mM NADH; lane (12): 0.5 μg pBR322 + 5 U DIA; lane (13): 0.5 μg pBR322 + 1 mM NADH; lane (14): 0.5 μg pBR322 + 5 U FMN.

Initially DT-diaphorase and FMN oxidoreductase known to induce mitomycin C activation, were used. The compounds were combined with linearized pBR322 DNA, the enzyme, and NADH, and they were incubated for 12 h at room temperature. Repeated attempts at activating FR900482 and **17** proved unsuccessful. The amounts of enzyme were varied, as well as concentration of the co-factor. Incubating the samples at 37° C for 12 h also had no effect on the activation of the compounds.

Another enzyme, NADPH: cytochrome P450 reductase, was also tried in the assay (Figure 3.11). FR66979 replaced FR900482 in the assay. Again, compound **17** showed no activation, but at high concentration FR66979 showed some DNA cross-link formation.

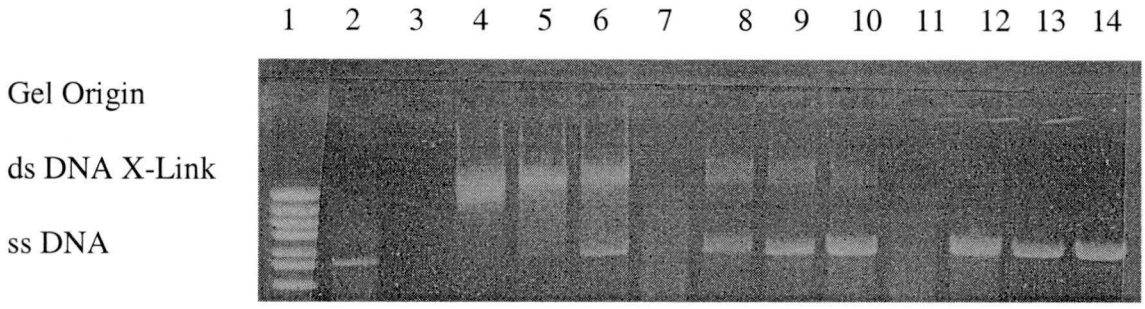


Figure 3.11 All reactions were incubated at 25°C for 12 h. Lane 1: 0.5 μg Lambda BSTE II digest (molecular weight standard); lane (2): 0.5 μg linear pBR322; lane (3): 0.5 μg pBR322 + 1 mM MC; lane (4): 0.5 μg pBR322 + 1 mM MC + 5 U FMN + 1 mM NADH; lane (5): 0.5 μg pBR322 + 1 mM MC + 5 U DIA + 1 mM NADH; lane (6): 0.5 μg pBR322 + 1 mM MC + 5 U cyt P450 + 1 mM NADPH; lane (7): 0.5 μg pBR322 + 20 mM FR66979; lane (8): 0.5 μg pBR322 + 20 mM FR66979 + 5 U FMN + 1 mM NADH +; lane (9): 0.5 μg pBR322 + 20 mM FR66979 + 5 U DIA + 1 mM NADH; lane (10): 0.5 μg pBR322 + 20 mM FR66979+ 5 U cyt P450 + 1 mM NADPH; lane (11): 0.5 μg pBR322 + 10 mM FR66979; lane (12): 0.5 μg pBR322 + 10 mM FR66979 + 5 U FMN + 1 mM NADH; lane (13): 0.5 μg pBR322 + 10 mM FR66979 + 5 U DIA 1 mM NADH; lane (14): 0.5 μg pBR322 + 10 mM + 5 U cyt P450 + 1 mM NADPH.

Continuing experiments were performed with MC and FR66979, however in order to obtain a fair amount of DNA cross-linking, a concentration of 20 mM of FR66979 was needed (Figure 3.11). At a concentration of 1 mM, FR66979 exhibits background cross-linking. Therefore at such high concentrations (20 mM), it is quite possible that a fair amount of the cross-linking visible is as a result of background. The validity of the experiment must then be questioned.

It is known that *in vivo*, a combination of enzymes allow for the reduction of MC, not simply one enzyme.[2,32] The reason for the lack of activation of the FR-compounds could be rationalized by this observation. Reduction probably requires the presence of several different reducing enzymes to fully activate FR900482 or FR66979. Because the quinone moiety is much more easily reduced than the hydroxylamine hemi-acetal bond,

only one enzyme may be needed *in vitro*. However, in the case of FR900482, the interaction of several enzymes is probably needed to reduce the bond.

3.4 DNA Subcloning Experiment

In conjunction with the Reeves' lab at Washington State University, a DNA subcloning experiment was performed. The FR900482 class of antitumor agents have been found to form a protein-DNA crosslink *in vivo* with the HMG I/Y oncoprotein.[38] This was the first report of covalent cross-linking of DNA to HMG I/Y, a minor groove binding oncoprotein, by FR900482. This experiment represents a potentail novel mechanism through which these compounds exert their antitumor activity.

A similar experiment was repeated using mitomycin C and FK317 in an attempt to determine if these compounds cross-link the same DNA sequence in the HMG I/Y oncoprtoein. The DNA cross-linking studies were carreid out in the Reeves' lab at Washington State University. The cross-linked DNA fragemnts were shipped to these laboratories and a DNA subcloning experiment was performed to amplify the isolated fragemts.

The DNA fragements were amplified using standard polymerase chain reaction protocol. The amplified DNA was ligated into the pGEM-T Easy vector system. The vectors were then transformed into JM 109 E. Coli cells and innoculated in LB broth. The plasmid was isolated from the JM 109 cells and digested with EcoR1. The results of the ligation were visualized using 1% agarose gel electrophoresis. Samples from lane 7, 10, and 11 (Figure 3.12) were sent for DNA sequencing. Upon sequencing, the desired DNA sequence was not present.

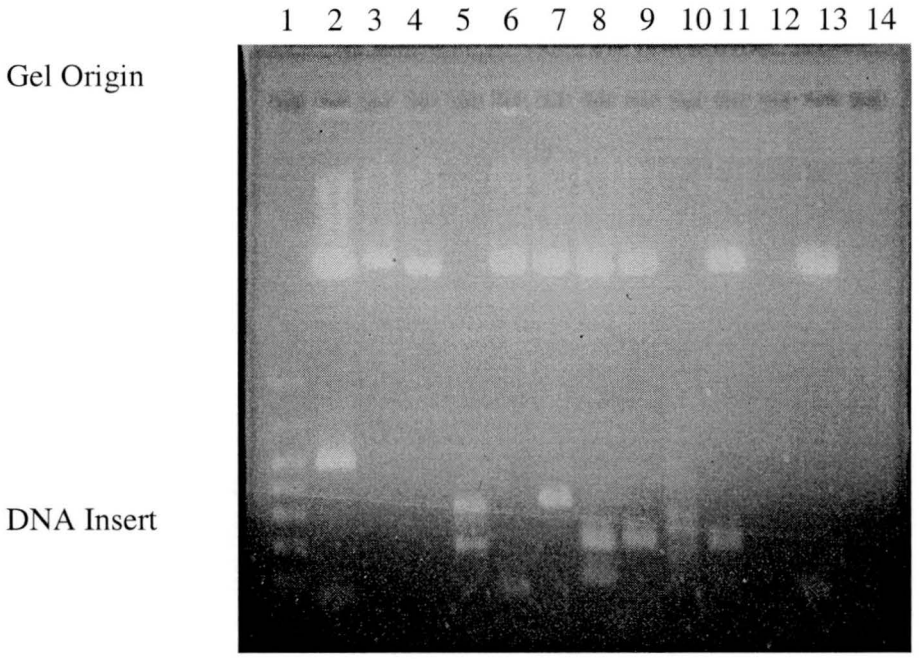


Figure 3.12 Lane 1: 1kB DNA ladder; Lane 2, 3: pGEM-T Easy positive DNA insert control ; Lane 4, 5: pGEM-T Easy negative controls; Lane 6-9: Mitomycin C inserts; Lane 10-13: FK317 inserts.

3.5 Conclusion

The DNA cross-linking potential of several compounds was investigated using denaturing alkaline agarose gel electrophoresis. Upon irradiation with 365 nm light, compound **21** proved to be a potent DNA cross-linker. The dicarbamate **74** proved to be a poor cross-linking compound, when activated with various reducing agents, at different pH levels. Enzymatically activating mitomycin C proved facile. Other compounds (FR900482, compound **17**) capable of undergoing chemical reduction *in vitro*, were unreactive to enzymatic reduction, presumably due to the relative stability of the reductively labile hydroxylamine hemiacetal bond.

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CHAPTER 4

4.1 General Considerations for Chemical Reactions

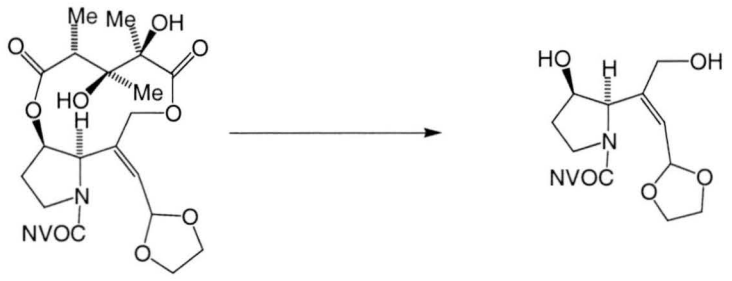
Unless otherwise noted, materials were obtained from commercially available sources and used without further purification. Diethyl ether and tetrahydrofuran were distilled from sodium benzophenone ketyl under an Ar atmosphere. Methylene chloride and triethylamine were distilled from calcium hydride under an Ar atmosphere.

All reactions involving hydroscopic substances were conducted in flame dried glassware under an Ar atmosphere.

Nuclear magnetic resonance (NMR) spectra were obtained using a Varian Mercury or Inova spectrometer. NMR chemical shifts are given in ppm relative to internal $CHCl_3$, TMS. DMSO, or methanol. Proton (^1H NMR) are tabulated in the following order: number of protons, multiplicity (s = singlet; d = doublet; t = triplet; q = quartet; and m = multiplet), and coupling constant(s) in hertz. When appropriate the multiplicity of a signal is denoted as "br" in indicate that the signal was broad.

Infrared spectra were obtained on a Nicolet Avatar 320 FT-IR as thin films from CH_2Cl_2.

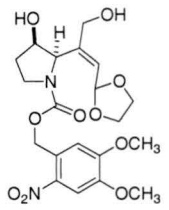
4.2 Specific Procedures



26 **27**

2-(2-[1,3] Dioxolan-2-yl-hydroxymethyl-vinyl)-3-hydroxypyrrolidine-1-carboxylic acid 4, 5-dimethoxy-2-nitrobenzylester (27).

To a solution of compound **26** (20 mg, 0.032 mmol, 1.0 equiv) in freshly prepared 95% EtOH (0.5 mL) was added KCN (4 mg, 0.064 mmol, 2.0 equiv). The reaction was kept from light and stirred at room temperature for 4 days after which the starting material was gone by TLC analysis (EtOAc as solvent). The EtOH was removed *in vacuo* and the residue was taken up in 3 mL of EtOAc and washed with brine (1 mL). The brine was then back extracted with EtOAc (2 x 2 mL) and the combined organics were dried over Na_2SO_4, concentrated and purified on silica gel (EtOAC as eluant) to give 10 mg of **27** as a pale yellow foam (68% yield). TLC (10:1 CH_2Cl_2: MeOH) R_f = 0.5 (UV and anisaldehyde). ^{1}H NMR (300 MHz)($CDCl_3$): δ 2.05-2.1 (2 H, m), 2.78-2.81 (1H, d), 4.5-4.6 (2 H, m), 4.01 (6H, s), 4.3-4.38 (2 H, m), 4.5-4.59 (1 H, m), 4.8 (1 H, s), 5.38-5.4 (1H, s), 5.5-5.7 (2H, d), 5.9 (1 H, s), 7.04 (1H, s), 7.7 (1 H, s). ^{13} C NMR (300 MHz)($CDCl_3$): 14.84, 21.70, 32.92, 33.51, 45.46, 56.99, 61.01, 62.01, 64.71, 65.02, 72.78, 73.54, 99.92, 108.7, 110.69, 11.79, 126.07, 127.62, 128.62, 153.84. IR (NaCl, neat): 1065, 1111, 1221, 1278, 1327, 1362, 1415, 1523, 1581, 1701, 2893, 3427 cm^{-1}. HRMS (FAB) calc. for $C_{20}H_{26}N_2O_{10}$ (MH^+) 455.1665; found 455.1653.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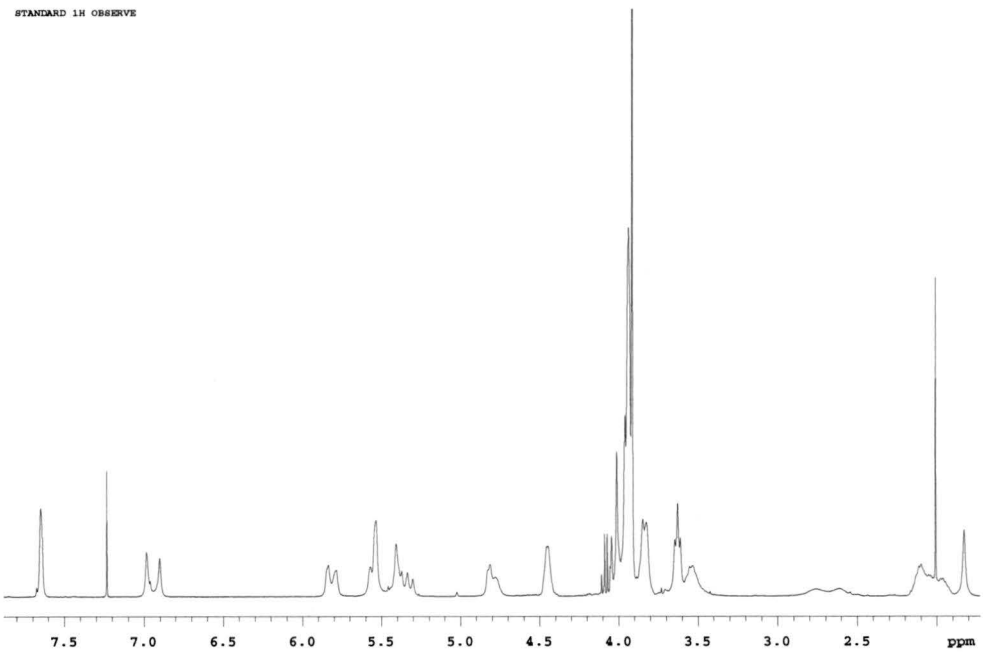
OCH3
O2N
OCH3

27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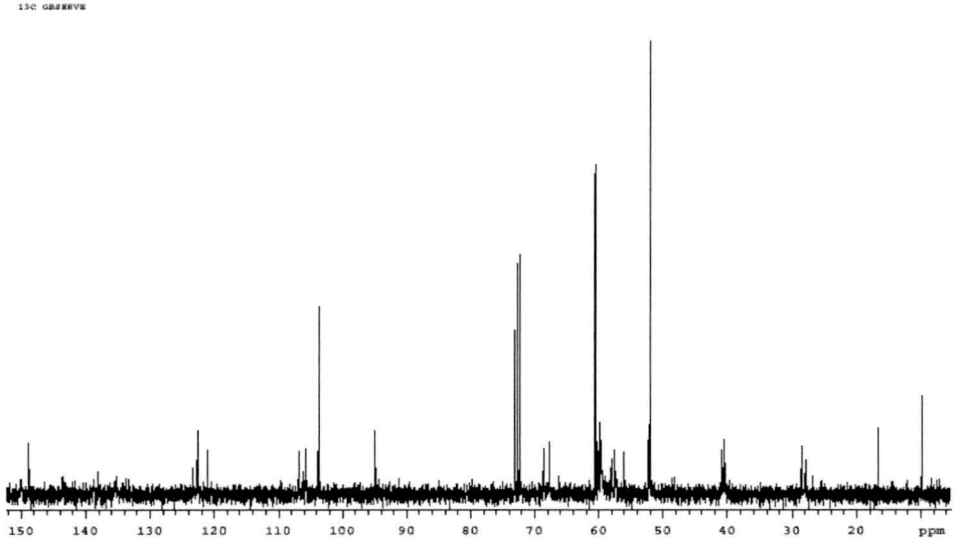
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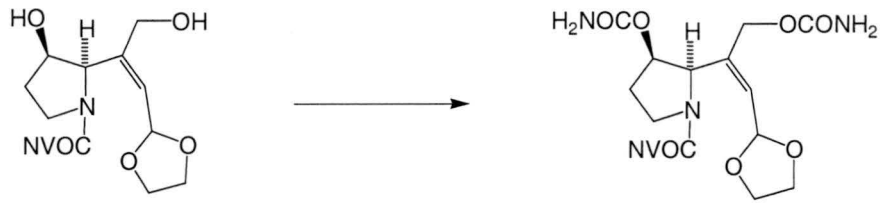
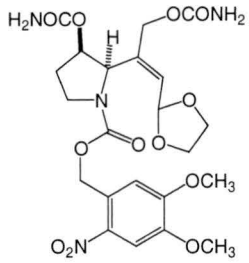


27 **28**

3-Carbamoyloxy-2-(1-carbamoyloxymethyl-2-[1,3]-dioxalan-2-yl-vinyl)-pyrrolidine-1-carboxylic acid 4,5-dimethoxy-2-nitrobenzylester (28).

The diol **27** (26 mg, 0.057 mmol, 1.0 equiv.) was dissolved in 1 mL of CH_2Cl_2 and cooled to 0°C. Trichloroacetylisocyanate (0.016 mL, 0.126 mmol, 2.5 equiv) was then added to the solution. The reaction stirred overnight at room temperature, after which the solution was loaded on to a plug of neutral alumina. After 2 h, the plug was flushed with 10:1 CH_2Cl_2: MeOH. The fractions were concentrated and further purified on silica gel using 2% MeOH in CH_2Cl_2 to give 19 mg of **28** as a yellow foam (61% yield). TLC (10:1 CH_2Cl_2: MeOH) R_f = 0.4 (UV and anisaldehyde). ^{1}H NMR (300 MHz)(DMSO): δ 1.86-1.94 (1 H, m), 2.05-2.14 (1 H, m), 3.39-3.44 (1 H, m), 3.66-3.72 (1 H, m), 3.74 (1 H, d, J=2.7 Hz), 3.80 (1 H, d, J= 1.2 Hz), 3.88 (3 H, s), 3.92 (3 H, s), 4.35 (2 H, d, J=14.4 Hz), 4.54 (2 H, d, J=14.7 Hz), 4.94 (1 H, d, J= 6.3 Hz), 5.28-5.25 (1 H, m), 5.37 (2 H, d, J= 1.5 Hz), 5.51 (2 H, d, J= 2.1 Hz), 6.13 (2 H, br s), 6.29 (2 H, br s), 7.12 (1 H, s), 7.67 (1 H, s). ^{13} C NMR (300 MHz)($CDCl_3$): 31.46, 32.10, 45.42, 45.97, 56.60, 61.09, 64.61, 64.99, 65.21, 75.52, 99.51, 108.40, 111,21, 112.79, 127.49, 140.47, 148.50, 153.51, 153.51, 153.52, 156.27, 156.56, 163.76. IR(NaCl, neat): 1064, 1277, 1327, 1523, 1711, 3357 cm^{-1}. HRMS (FAB) calc. for $C_{22}H_{28}N_4O_{12}$ (MH^+) 541.1782; found 541.1784.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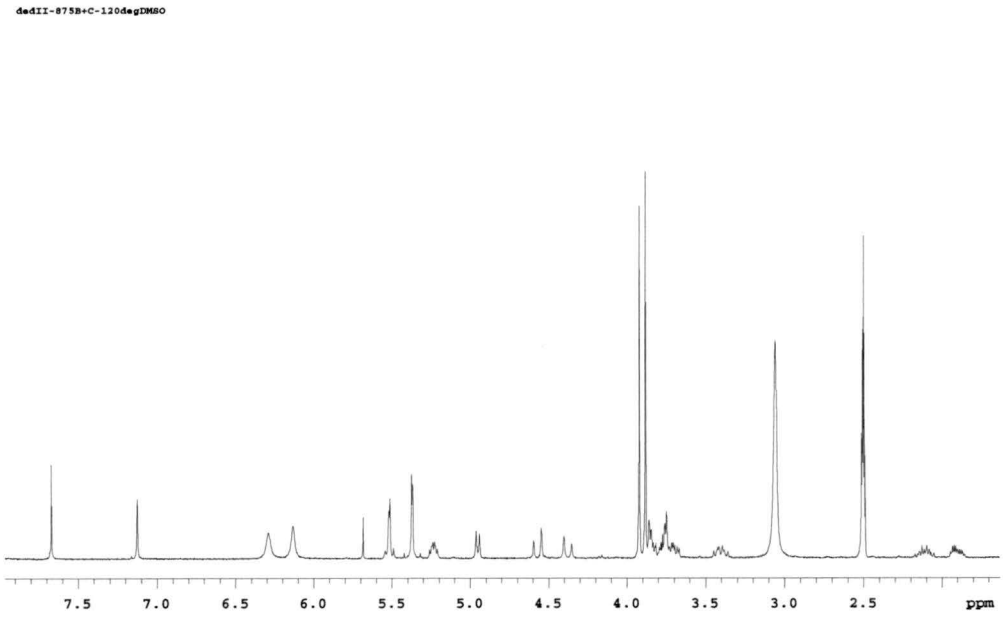
H
N
O
O
O
O
O
OCH3
O2N
OCH3

28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3.0
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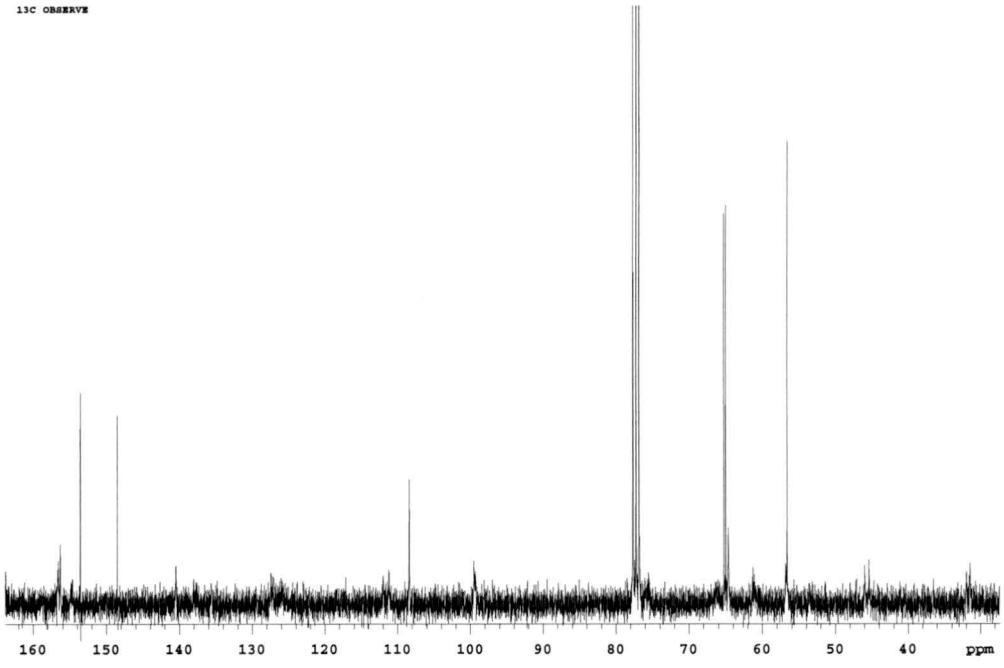
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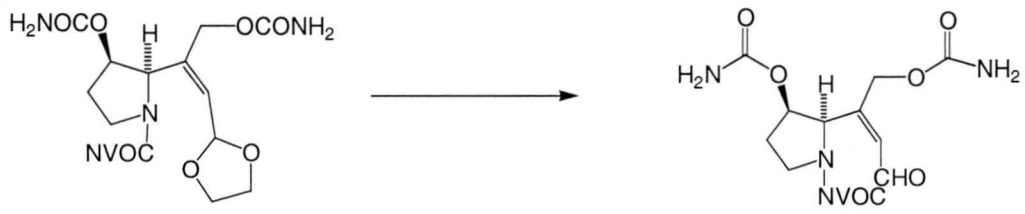
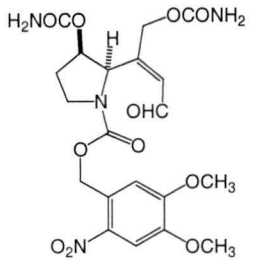
ppm



28 **21**

3-Carbamoyloxy-2-(1-carbamoyloxymethyl-3-oxo-propenyl)-pyrrolidine-1-carboxylic acid 4,5-dimethoxy-2-nitrobenzyl ester (21).

The dicarbamate **28** (18 mg, 0.033 mmol, 1.0 equiv) was dissolved in 0.35 mL of reagent grade acetone. To the solution was added 0.15 mL of 1% HCl (v/v). The reaction stirred for 1.7 h after which starting material had disappeared by TLC. The acetone was removed *in vacuo* and the residue was taken up in 3 mL of $CHCl_3$. The resulting solution was washed with saturated $NaHCO_3$ solution and the aqueous layer was then backwashed with $CHCl_3$. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified on silica gel (1%MeOH in CH_2Cl_2) to give 13 mg of **21** as a pale yellow foam (78% yield). TLC (10:1 CH_2Cl_2: MeOH) R_f = 0.35 (UV and anisaldehyde). ^{1}H NMR (300 MHz)(D_6- DMSO, 80°C): δ 1.9-2.0 (m, 1H), 2.2-2.3 (m, 1H), 3.5-3.59 (m, 1 H), 3.75-3.8 (m, 1 H), 3.89 (s, 6 H), 4.61-4.7 (m, 2 H), 5.39-5.4 (m, 2 H), 5.59-6.01 (d, 1 H), 6.29 (s, 1 H), 6.48 (s, 1 H), 7.11 (s, 1 H), 7.68 (s, 1 H), 10.0 (d, 1 H). ^{13}C NMR (300 MHz)($CDCl_3$): δ 14.40, 19.32, 21.26, 31.56, 45.71, 53.64, 56.65, 60.61, 64.42, 108.58, 111.21, 112.34, 124.07, 126.91, 140.52, 149.26, 153.49, 155.33, 155.80, 189.64. IR (NaCl, neat): 3363, 1712, 1582, 1523, 1392, 1328, 1277, 1220, 1112, 1065 cm^{-1}. HRMS (FAB) calc. for $C_{20}H_{24}N_4O_{11}$ (MH^+) 497.1519; found 497.1530.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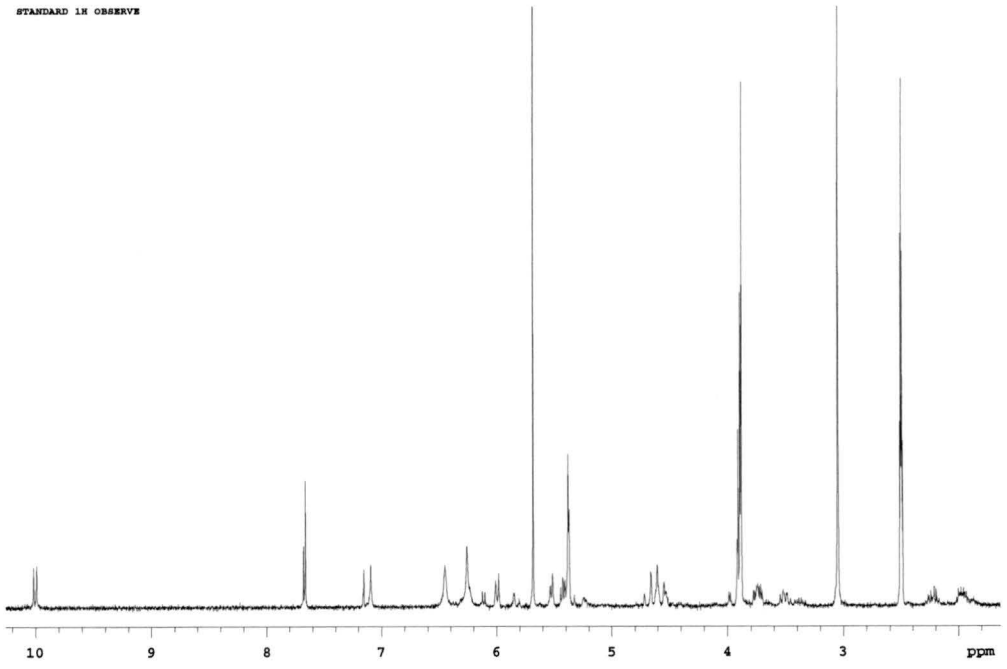
N
OHC
O
O
OCH3
O2N
OCH3

21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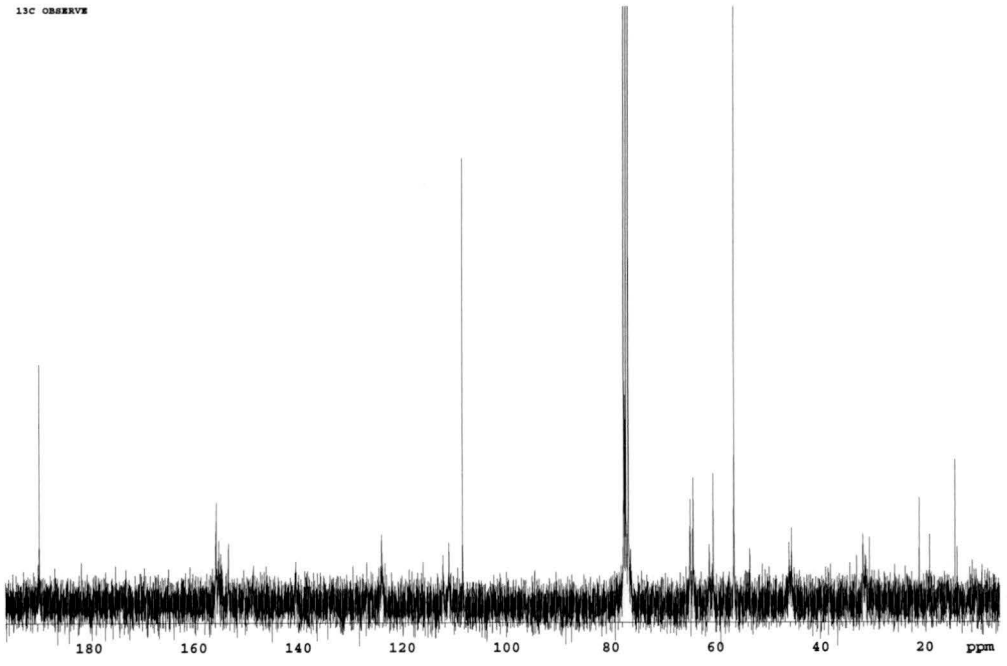
ppm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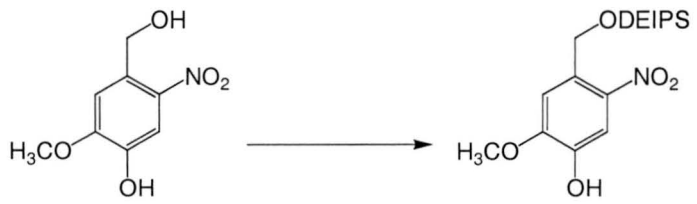
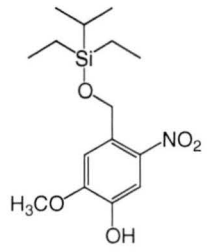
20
ppm



35 **46**

4-Hydroxy-5-methoxy-2-nitro-(diethylisopropylsilyl)benzyl alcohol (46).

The starting benzyl alcohol **35** (200 mg, 1.01 mmol, 1.0 eq) was dissolved in dry CH_2Cl_2 (10 mL) under Ar, then cooled to -10°C. Triethylamine (280 μL, 2.02 mmol, 2.0 eq) was added followed by 2, 6-dimethylaminopyridine (5 mg, cat.). Then DEIPSCl (0.14 mL, 1.2 mmol, 1.2 eq) was slowly added. After 2h some product formation was visible. Water (5 mL) was slowly added and the reaction warmed to room temperature over 4 h. The aqueous layer was extracted with EtOAc (3 x 5 mL). The combined organic layers were dried over sodium sulfate and concentrated. The crude reaction product was purified via flash chromatography on silica gel (4:1 hexanes:ethyl acetate) to give 660 mg (64% yield) of **46** as a yellow solid. TLC (3.5:1 Hexanes:EtOAc) R_f = 0.3 (UV). ^{1}H NMR (300 MHz)($CDCl_3$): δ 7.825 (s, 1 H), 7.534 (s, 1 H), 5.157 (s, 2 H), 4.047 (s, 3 H), 1.107-1.033 (m, 13 H), 0.811-0.758 (q, J= 7.5 Hz, 7.8 Hz, 4 H). ^{13}C NMR (300 MHz)($CDCl_3$): δ 146.7, 139.32, 128.77, 106.786, 104.02, 95.471, 57.70, 51.83, 8.836, 2.675, 0.821. IR (NaCl, Neat): 3435, 2956, 2877, 1571, 1520, 1463, 1329, 1289, 1217, 1151, 1068, 1012, 972, 904, 731 cm^{-1}. HRMS (FAB) calc. for $C_{15}H_{25}NO_5Si$ (MH^+) 328.1580; found 328.1579.



46

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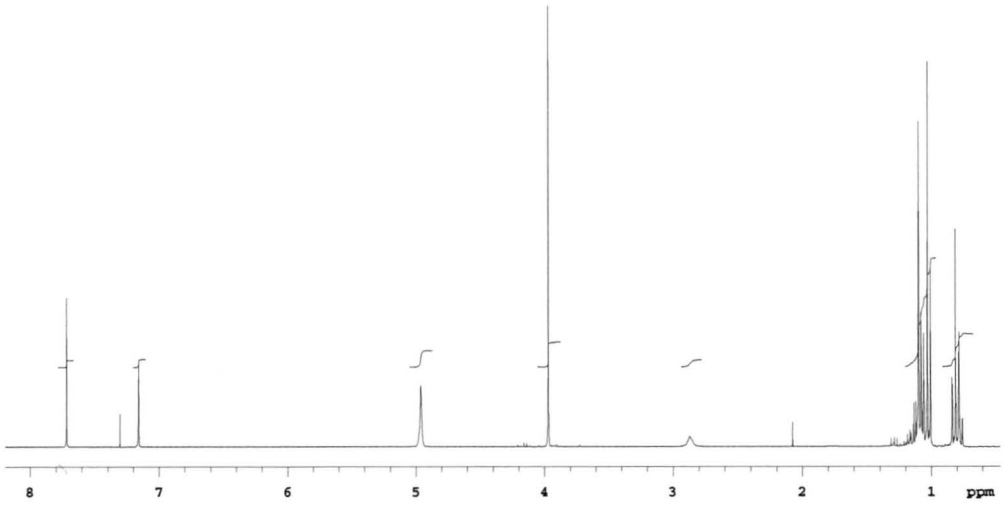
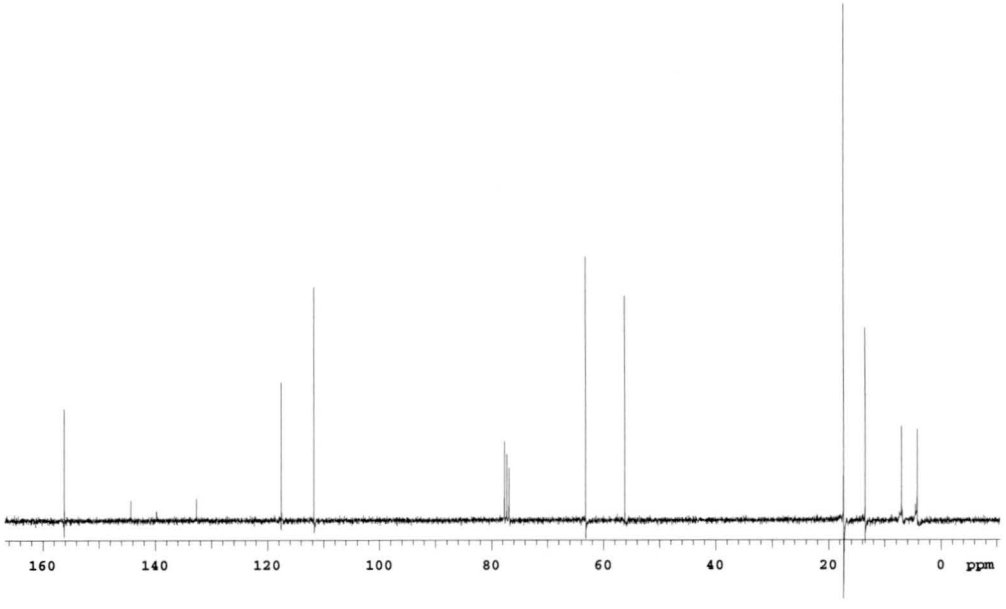
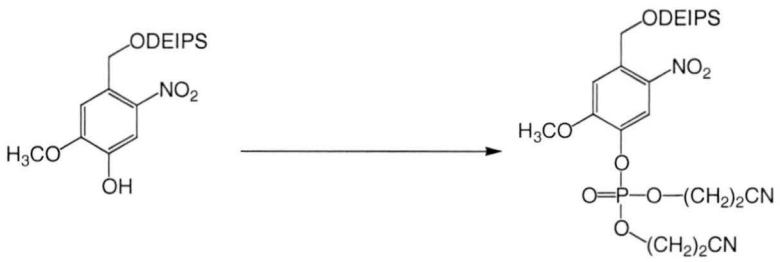


13C OBSERVE

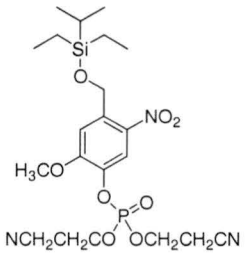




46 **47**

Phosphoric acid bis-(2-cyanoethyl)ester4-diethyl-isopropyl-silanyloxymethyl)2-methoxy-5-nitrophenyl ester (47).

The phenol **46** (50 mg, 0.153 mmol, 1.0 equiv) and the phosphate (55 mg, 0.229 mmol, 1.5 equiv.) were combined in a flask, purged with argon and immediately taken up in 3 mL dry THF. Tetrazole (27 mg, 0.383 mmol, 2.5 equiv) was then added to the solution. After stirring for 1 h at room temperature, the solution was cooled to –78°C and mCPBA (132 mg, 0.383 mmol, 2.5 equiv) was added. After stirring for 5 min. it was warmed to room temperature. After 20 min. at room temp., a solution of $NaHSO_3$ in saturated $NaHCO_3$ (5 mL) was added. After vigorous stirring for 10 minutes, the aqueous was extracted with EtOAc (3 x 7 mL), the combined organics were backwashed with brine, dried over Na_2SO_4, and concentrated. The crude mixture was purified on silica gel (3: Hex:EtOAc, followed by EtOAC to elute product) to give 40 mg of **47** as a yellow oil (51 % yield). TLC (EtOAc) R_f = 0.4 (UV). ^1H NMR (300 MHz)($CDCl_3$): δ 0.76-0.81 (3H, t, J=7.5 Hz), 1.04-1.10 (12H, m), 2.86-2.91 (3H, t, J=5.7 Hz), 4.06 (3H, s), 4.51-4.46 (4H, m), 5.18 (2H, s), 7.68 (1H, s), 8.16 (1H, s). ^{13}C NMR (300 MHz)($CDCl_3$): δ 3.92, 7.72, 13.41, 17.9, 20.2, 57.2, 62.7, 63.7, 100.5, 111.4, 116.6, 119.2, 137.2, 138.5, 140.2, 155.8. IR (NaCl, neat): 3419, 1586, 1521, 1332, 1286, 1069, 1005, 944, 855 cm^{-1}. HRMS (FAB) calc. for $C_{21}H_{32}N_3O_8PSi$ (MH^+) 514.1774; found 514.1764.



47

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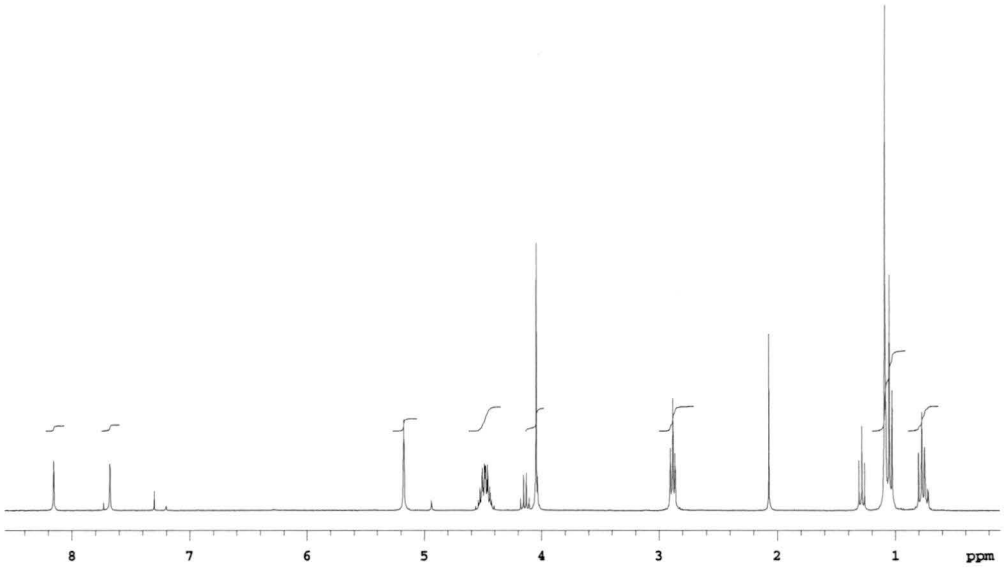

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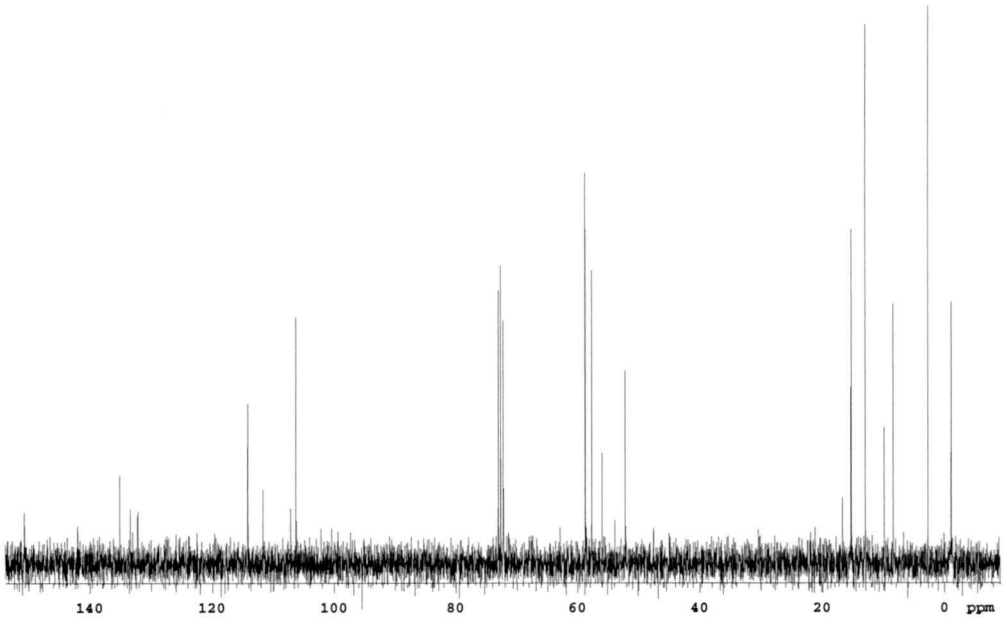
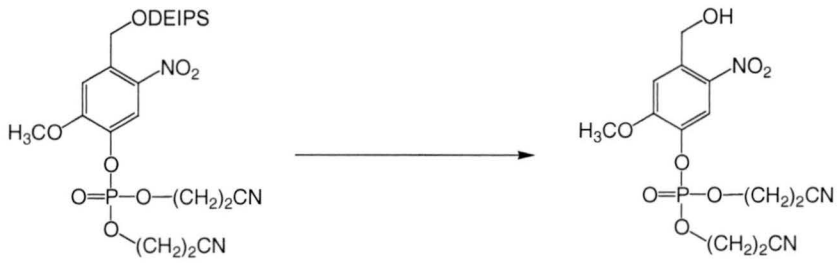
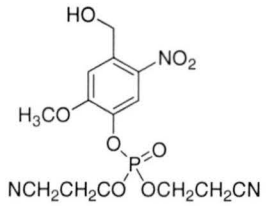




47 45

Phosphoric acid bis-(2-cyanoethyl)ester 4-hydroxymethyl-2-methoxy-5-nitrophenyl ester (45).

The phosphate **47** (18 mg, 0.035 mmol) was dissolved in THF (0.15 mL) and cooled to 0°C, after which 0.2 mL of AcOH:H_2O (3:1) was added. The reaction warmed to room temperature and was stopped after 6. 5 h. It was diluted with a large excess of EtOAc (35 mL), washed with saturated $NaHCO_3$ (2 x 10 mL), and brine. The organic layer was dried over Na_2SO_4, concentrated and purified on silica gel (EtOAc as eluant) to give 6 mg of **45** as a clear oil (46% yield). TLC (2% MeOH/EtOAc) R_f = 0.3 (UV). ^1H NMR (300 MHz)($CDCl_3$): 2.86-2.91 (4 H, t, J=5.4 Hz), 4.09 (3H, s), 4.46-4.52 (4H, m), 5.09 (2 H, d, J=6.3 Hz), 7.30 (1H, s), 8.16 (1H, s). ^{13}C NMR (300 MHz)(CD_3OD): 20.64, 57.77, 62.53, 65.73, 112.64, 118.61, 120.14, 138.57, 140.25, 141.12, 157.03. IR (NaCl, neat): 3583, 2923, 1726, 1519, 1465, 1283, 1260, 1069 cm^{-1}. HRMS (FAB) calc. for $C_{14}H_{16}N_3O_8P$ (MH^+) 386.0753; found 386.0752.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P
O
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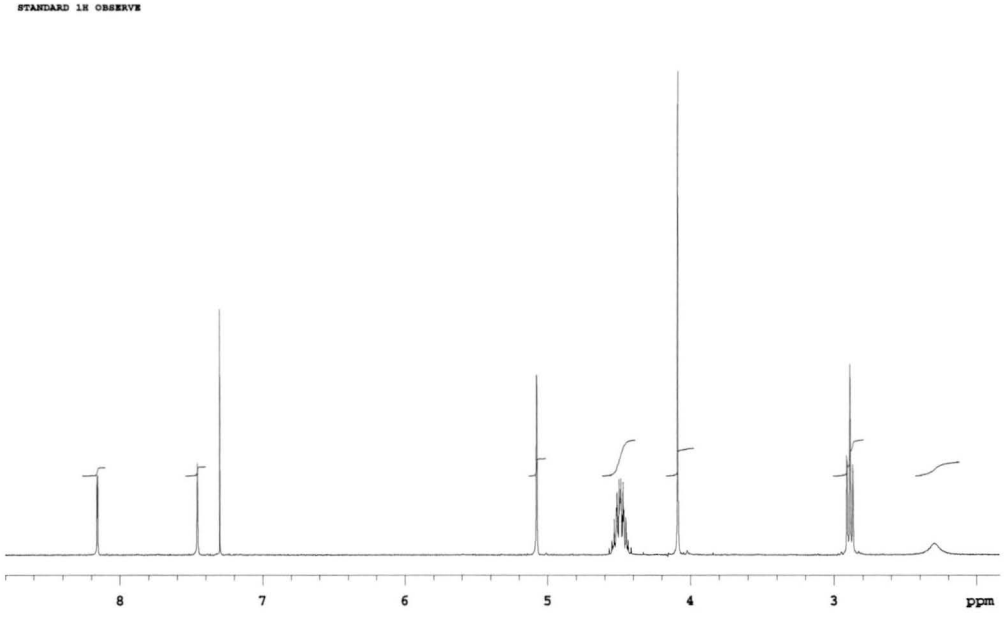
45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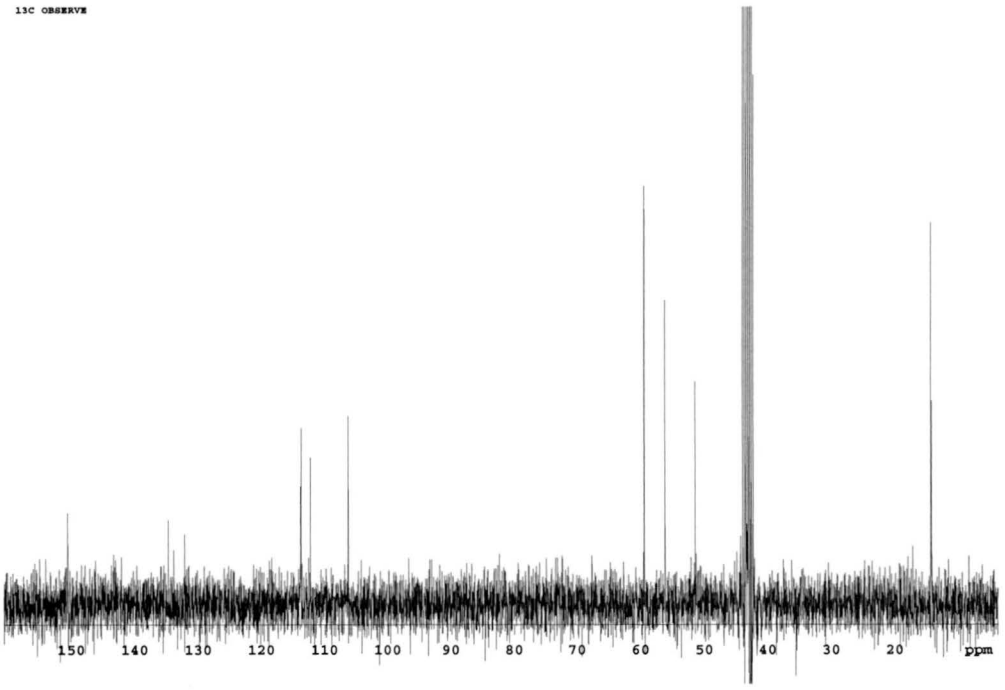
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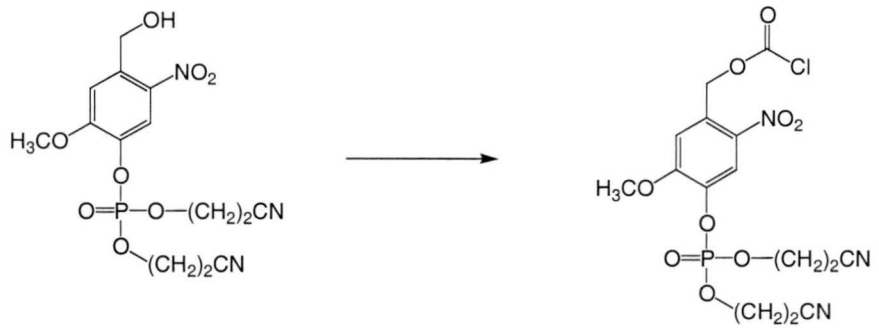
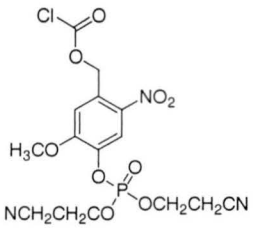
ppm



45 **48**

Phosphoric acid bis-(2-cyanoethyl)ester 4-hydroxymethylchloroformate-2-methoxy-5-nitrophenyl ester (48).

The alcohol **45** (50 mg, 0.129 mmol) was dissolved in 3 mL of dry THF and then 1 mL of phosgene in toluene (1.9 M) was added. The reaction stirred overnight at room temperature and then the solvent was evaporated by was bubbling argon through the solution then out into 1M NaOH solution to quench the excess phosgene. The reaction proceeded in quantitative yield to give 65 mg of **48** as a yellow oil. ^{1}H NMR (300 MHz)($CDCl_3$): δ 2.89 (4 H, t, J = 6 Hz, 5.7 Hz), 4.09 (3 H, s), 4.46-4.51 (4 H, m), 5.78 (2 H, s), 7.20 (1 H, s), 8.21 (1 H, d, J= 1.5 Hz). ^{13}C NMR (300 MHz)($CDCl_3$): δ 20.3, 26.2, 57.6, 63.9, 69.8, 100.5, 112.3, 116.7, 120.1, 128.7, 129.5, 130.5, 138.9, 139.6, 150.8, 155.9. IR (NaCl, neat): 3445, 2973, 2256, 1753, 1613, 1588, 1524, 1467, 1335, 1290, 1200, 1056, 1006 cm^{-1}.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O
P
O
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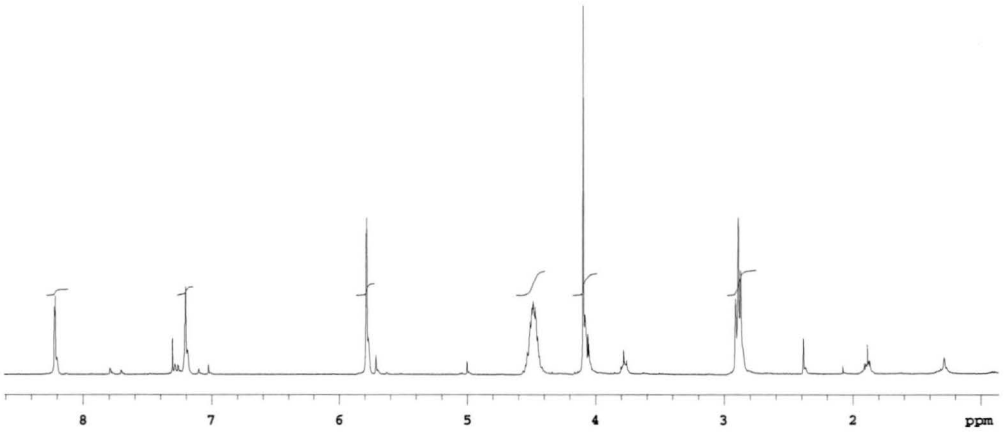
OCH2CH2CN

48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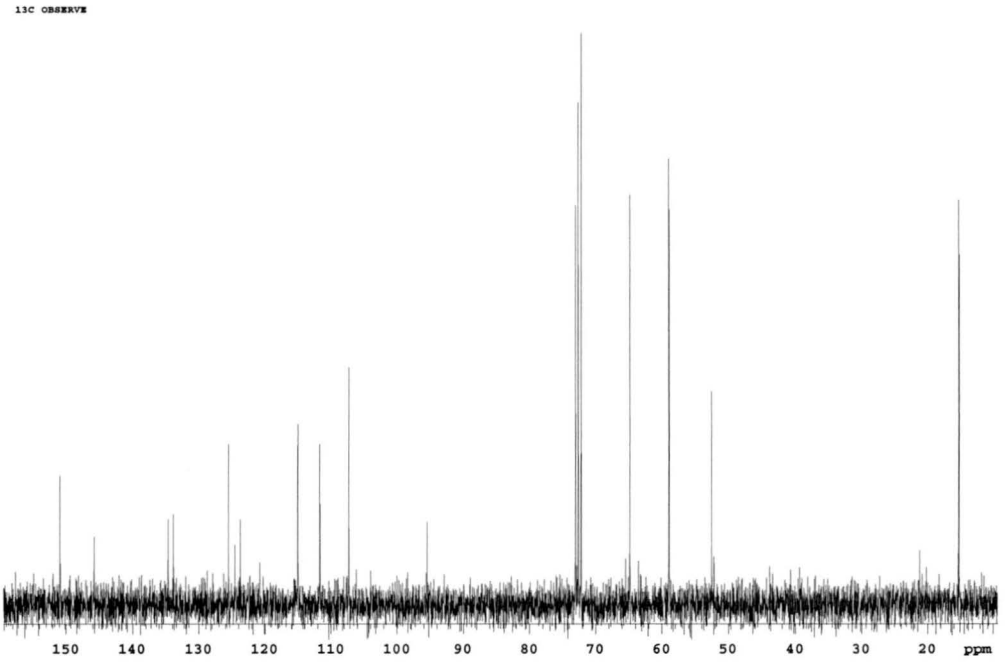
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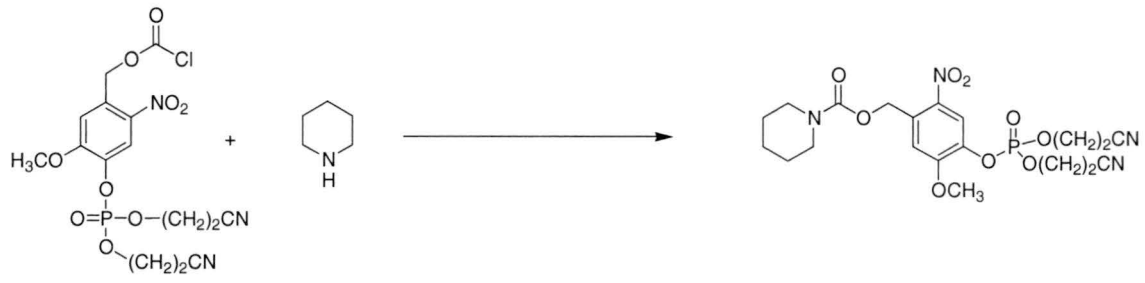
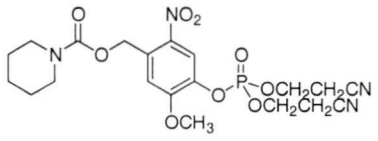
ppm



48 **49** **50**

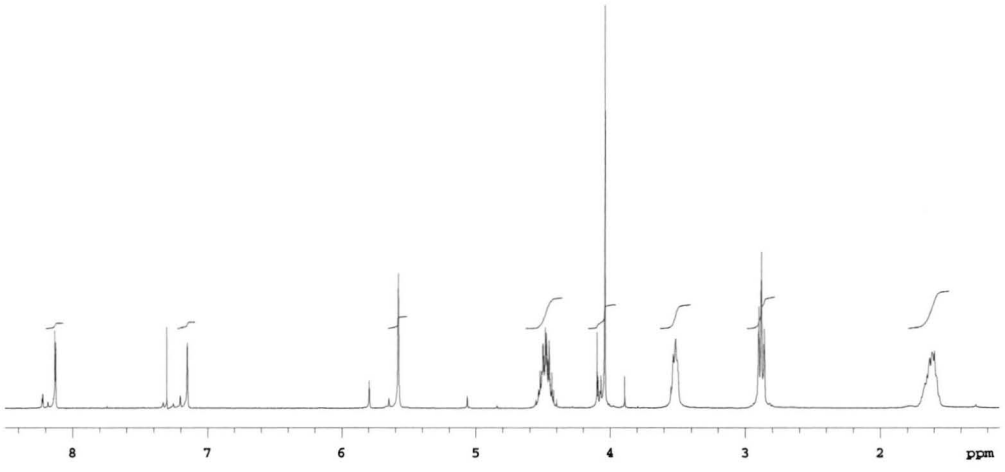
Piperidine-1-carboxylic acid 4[bis-(2-cyanoethoxy)-phosphoryloxy]-5-methoxy-2-nitrobenzy ester (50).

The chloroformate **48** (60 mg, 0.136 mmol, 1.5 equiv) was dissolved in 750 μL of dry THF under Ar. An additional 750 μL of dry CH_2Cl_2 was added. The solution was cooled to –78°C, after which piperidine (9μL, 0.908 mmol, 1.0 equiv.) was added. The solution stirred for 2 h at –78°C, after which the solvent was removed *in vacuo*. The crude reaction mixture was purified via silica gel chromatography (3% MeOH in CH_2Cl_2) to give the coupled product **50** (27 mg, 0.054 mmol) in 60% yield. TLC (3% MeOH/CH_2Cl_2) R_f = 0.3 (UV). ^{1}H NMR (D_2O, 400 MHz) δ : 1.58-1.66 (m, 6 H), 2.85-2.90 (t, 4 H, J= 10.8 Hz, 9.2 Hz), 3.49-3.53 (t, 4 H, J=5.6 Hz, 7.6 Hz), 4.04 (s, 3 H), 4.43-4.52 (m, 4 H), 5.57 (s, 2 H), 7.15 (s, 1 H), 8.12 (d, 1 H, J=2 Hz). ^{13}C NMR δ (D_2O, 400 MHz) : 19.86, 19.97, 24.53, 45.27, 56.82, 63.37, 63.72, 111.75, 116.25, 119.17, 119.64, 134.29, 154.42, 154.91. IR (NaCl, neat): 2922, 2359, 1698, 1523, 1288, 1085 cm^{-1}. HRMS (FAB) calc. for $C_{20}H_{25}N_4O_9P$ (MH^+) 455.1665; found 455.1653.

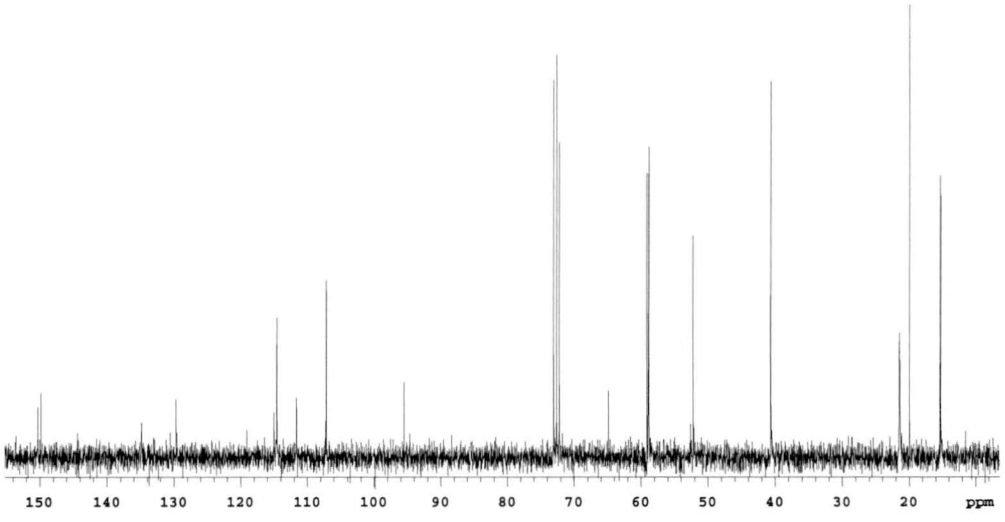


50

STANDARD 1H OBSERVE



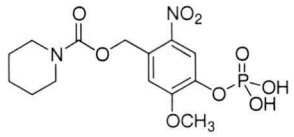
13C OBSERVE



50 51

Piperidine-1-carboxylic acid 5-methoxy-2-nitro-4-phosphonoxybenzyl ester (51).

The starting compound **50** (18 mg, 0.363 mmol, 1.0 equiv). was dissolved in 500 μL dry CH_2Cl_2 under Ar at room tempertature. TMSCl (9 μL, 0.726 mmol, 2.0 equiv.) was then added, followed by DBU (13.5 μL, 0.0908 mmol, 2.5 equiv.). After 3 h, 2 mL of DI H_2O was added followed by 0.5 mL of 3M NaOH. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (3x 3mL). The aqueous layer was then acidified with 1 M HCl to bring the pH= 7, it was frozen, and lyophilized to give the desired product **51** in quantitative yield. TLC (10:1 CH_2Cl_2: MeOH) R_f = 0.1 (UV). ^1H NMR δ (D_2O, 400 MHz) : 1.57-1.64 (dd, 6 H, J= 4.5 Hz, 6.6 Hz, 6.6 Hz), 3.48 (bs, 4 H), 4.00 (s 3 H), 5.47 (s, 2 H), 7.14 (s 1H), 8.13 (d, 1 H, 1.5 Hz). ^{13}C NMR δ (D_2O, 400 MHz) : 23.77, 25.53, 45.29, 56.61, 64.56, 111.66, 118.62, 131.59, 155.92, 155.97, 156.25, 174.99. IR (NaCl, neat): 3396, 1709, 1579, 1530, 1446, 1323, 1282, 1216 cm^{-1}. HRMS (FAB) calc. for $C_{14}H_{19}N_2O_9P$ (MNa^+) 413.0725; found 413.0715.



51

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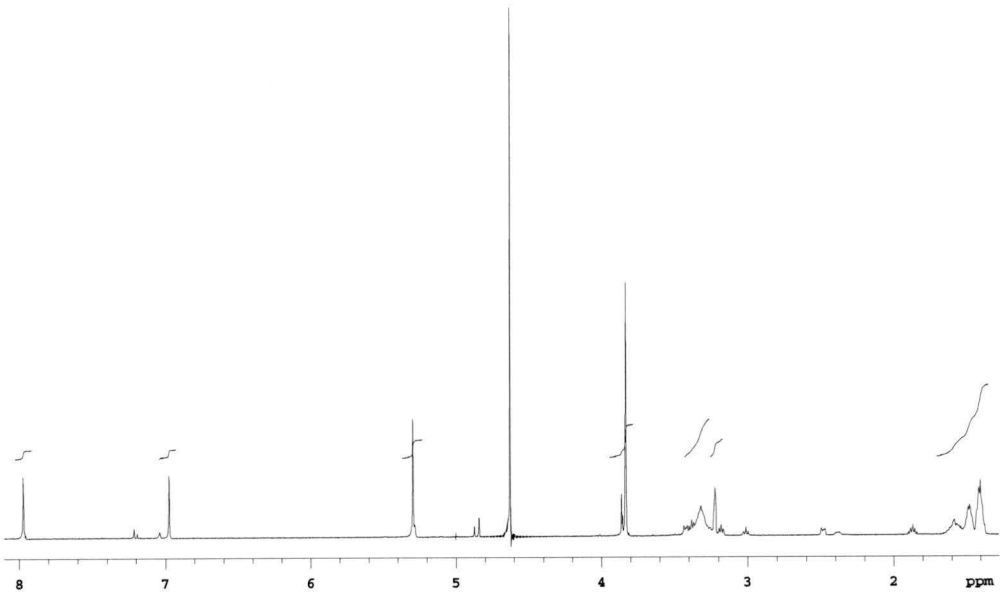

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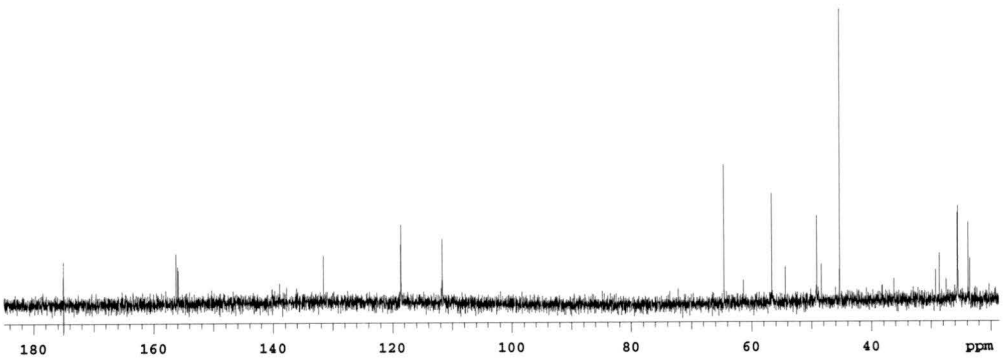
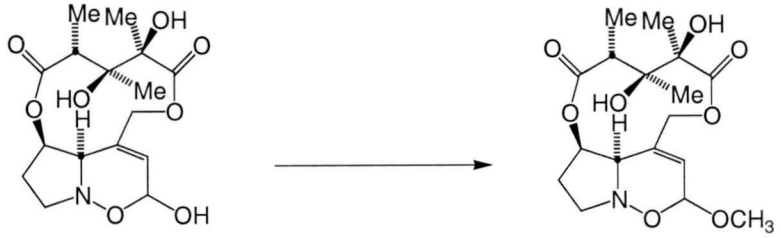


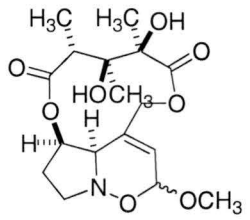


17 74

5,6-Dihydroxy-15-methoxy-5,6,7-trimethyl-3,9,14-trioxa-13-aza-tricyclo [8.6.1.0]heptadec-1(16)-ene-4,8-dione (74).

The hydroxylamine hemiacetal **17** (12 mg, 0.0337 mmol, 1.0 equiv) was dissolved in 1 mL of MeOH. To the solution, an excess of p-TsOH (10 mg) was added. The reaction stirred at room temperature for 7 h, after which the solvent was removed in vacuo. The residue was taken up in EtOAc (5mL) and washed with saturated $NaHCO_3$ solution (5 mL). The aqueous layer was back-extracted with EtOAc (2 x 5 mL). The combined organic layers were dried over Na_2SO_4, concentrated, and purified via silica gel chromatography using 5% MeOH in CH_2Cl_2 to give 4.5 mg of **74** as a clear oil (50% yield). TLC (5% MeOH/CH_2Cl_2) R_f = 0.4 (anisaldehyde). ^1H NMR ($CDCl_3$, 300 MHz) : δ 1.25 (d, 3H, J=6.9 Hz), 1.36 (s, 3H), 1.44 (s, 3 H), 2.14-2.27 (m, 1H), 2.62-2.72 (m, 1 H), 3.03-3.14 (m, 1H), 3.56 (s, 3H), 3.59 (d, 1H, J=9 Hz), 3.71 (d, 1H, J=6.6Hz), ~~5.20 (s, 2H),~~ 6.26 (d, 1H, J=4 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz): δ 13.39, 17.65, 21.95, 34.46, 44.73, 55.43, 55.86, 66.24, 68.67, 75.33, 78.59, 92.25, 96.79, 129.81, 142.86, 173.50, 174.04. IR (NaCl, neat) : 972, 1060, 1113, 1188, 1340, 1732, 3465 cm^{-1}. HRMS (FAB) calc. for $C_{17}H_{25}NO_8$ (MH^+) 372.1658; found 372.1655.

Missing ^1H : 4.95-5.00 (1H, m) ; 4.93 (1H, s) ; 4.71-4.80 (q, 1H, J=11.6, 16, 4.6Hz)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O
HO
CH3
O
O
H
H
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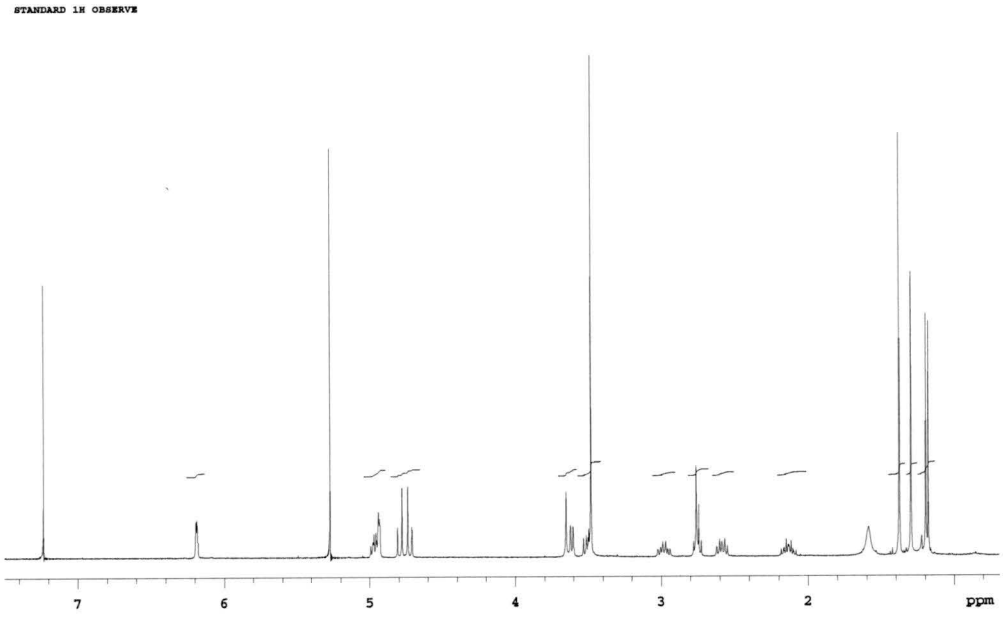
O
OCH3

74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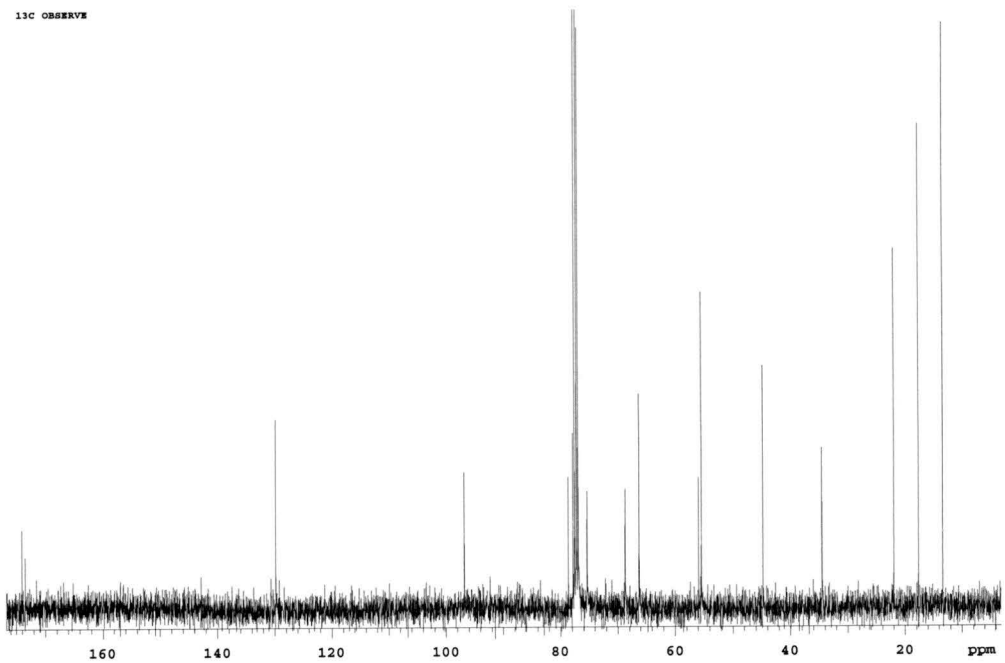
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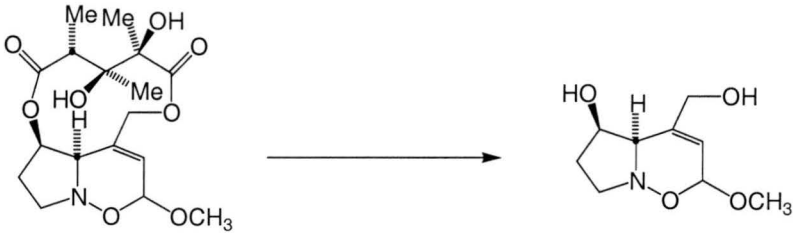
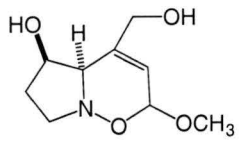


74 75

4-Hydroxymethyl-2-methoxy-4a, 5, 6, 7-tetrahydro-2*H*-pyrrolo[1,2b][1,2]oxazin-5-ol (75).

The methoxy compound **74** (5 mg, 0.0135 mmmol, 1.0 equiv) was dissolved in 0.5 mL THF followed by addition of 0.1 mL of H_2O. The $LiOH{\cdot}H_2O$ (3 mg, 0.0808 mmol, 6.0 equiv) was added. The reaction appeared complete after 6 h at room temp. The solvent was removed *in vacuo* and the residue was taken up in a 1:1 mixture of CH_2Cl_2: MeOH and flashed through a short plug of silica gel using 10:1 CH_2Cl_2: MeOH to give **75** as a clear oil (1 mg, 0.004 mmol) in 40% yield. TLC (10:1 CH_2Cl_2: MeOH) R_f = 0.2 (anisaldehyde). ^{1}H NMR (400 MHz)($CDCl_3$): δ 2.01 (1H, s), 2.16-2.22 (2 H, m), 2.40-2.46 (1H, m), 3.08-3.11 (1 H, m), 3.43 (1 H, d, J= 6 Hz), 3.48 (3 H, s), 3.52-3.58 (1 H, q, J=2.4, 2.8, 2.8, 2.4), 4.23 (2 H, d, J= 3.6 Hz), 4.45 (1 H, s), 4.87 (1 H, s), 5.97-5.98 (1 H, d, J=3.6 Hz). ^{13}C NMR(300 MHz)($CDCl_3$): 29.70, 36.34, 55.54, 64.81, 69.08, 73.71, 97.08, 122.41, 137.14. IR(NaCl, neat): 968, 1054, 1118, 1338, 1685, 3373 cm^{-1}. HRMS (FAB) calc. for $C_9H_{15}NO_4$ (MH^+) 202.1079; found 202.1075.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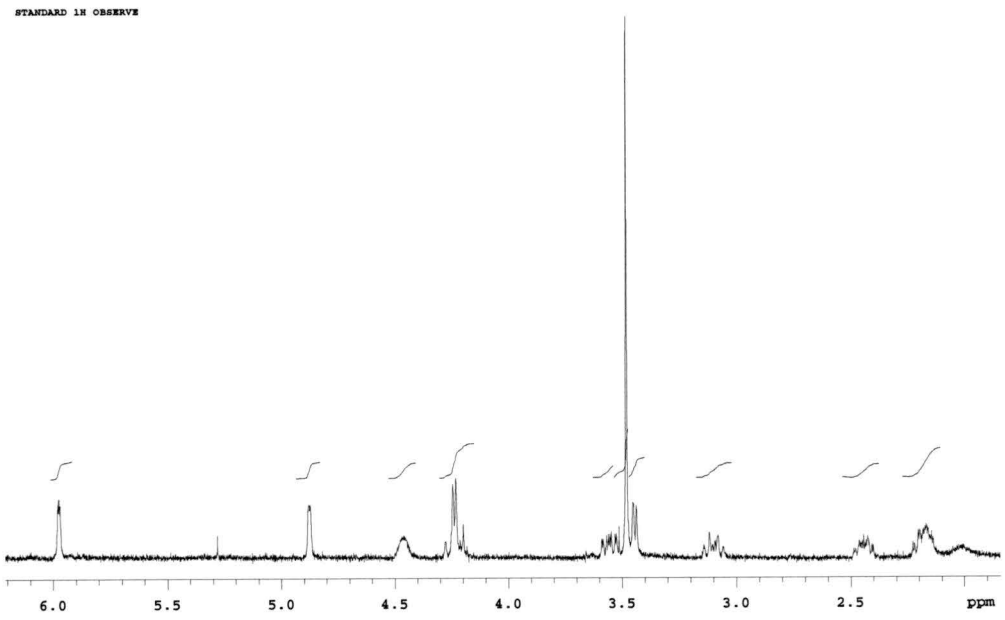
N
O
OCH3

75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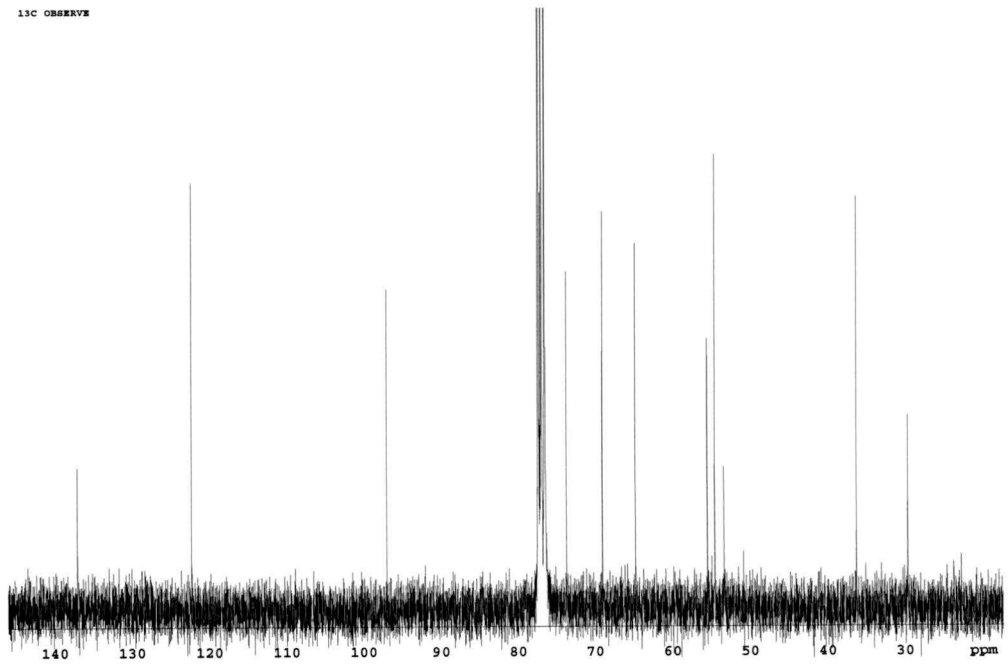
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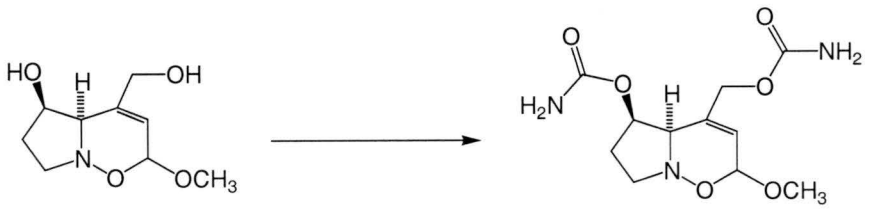
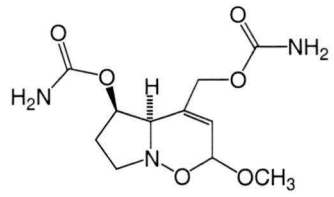


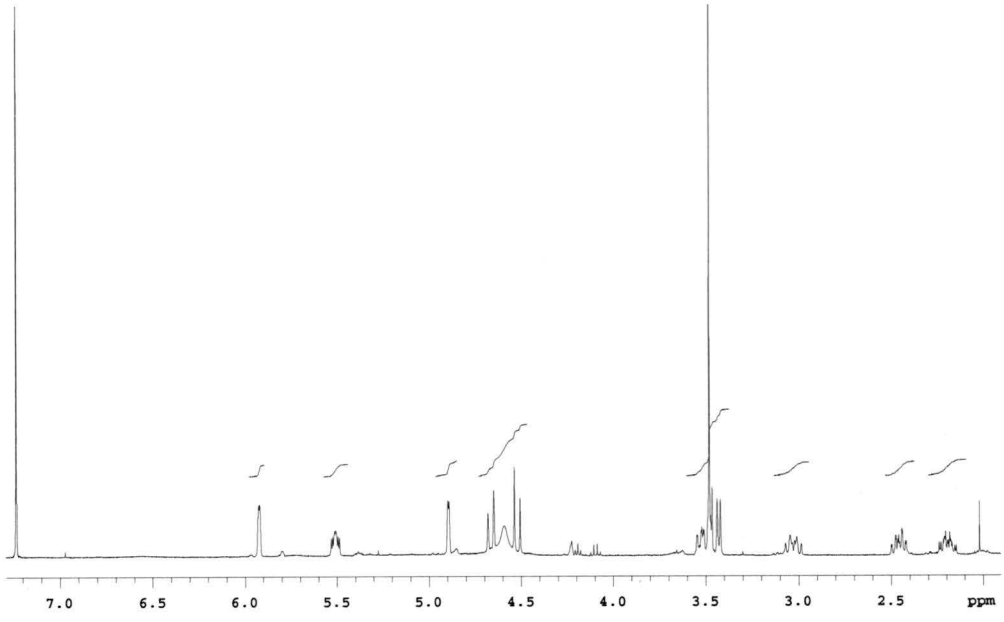
75 76

Carbamic acid 4-carbamoyloxymethyl-2-methoxy-4α, 5, 6, 7-tetrahydro-2H-pyrrolo[1,2-β][1,2]oxazi-5-yl ester (76).

The diol **75** (1.4 mg, 0.00696 mmol, 1.0 equiv) was dissolved in 0.5 mL CH_2Cl_2 and cooled to 0°C. The trichloroacetyl isocyanate (2 μL, 0.0174 mmol, 2.5 equiv) was added. The reaction stirred for 2 h (0°C to room temp.). The solvent was removed *in vacuo* and the residue was taken up in CH_2Cl_2 and loaded onto a plug of neutral alumina. After 0.5 h, the column was flushed with 10:1 CH_2Cl_2: MeOH to give 0.5 mg of **76** as a clear oil. TLC (10:1 CH_2Cl_2: MeOH) R_f = 0.15 (anisaldehyde) ^{1}H NMR (400 MHz)($CDCl_3$) : δ 2.14-2.23 (1 H, m), 2.41-2.50 (1 H, m), 2.98-3.07 (1 H, m), 3.42-3.43 (1 H, d, J= 6.4 Hz), 3.48 (3 H, s), 3.51-3.54 (1 H, m), 4.5 (1 H, d, J= 12.4 Hz), ~~4.59 (1 H, s)~~, 4.64 (1 H, d, J= 12.8 Hz), 4.89 (1 H, d, J=2.8 Hz), 5.48-5.32 (1 H, m), 5.92 (1 H, m, J= 2.0 Hz). ^{13} C NMR(300 MHz)($CDCl_3$): δ 29.92, 34.29, 55.67, 66.65, 67.50, 74.60, 96.98, 123.93, 132.13, 156.52, 156.92. IR (NaCl, neat): 968, 1054, 1120, 1180, 1333, 1401, 1608, 1710, 2953, 3353 cm^{-1}. HRMS (FAB) calc. for $C_{11}H_{17}N_3O_6$ (MH^+) 288.1195; found 288.1192.



75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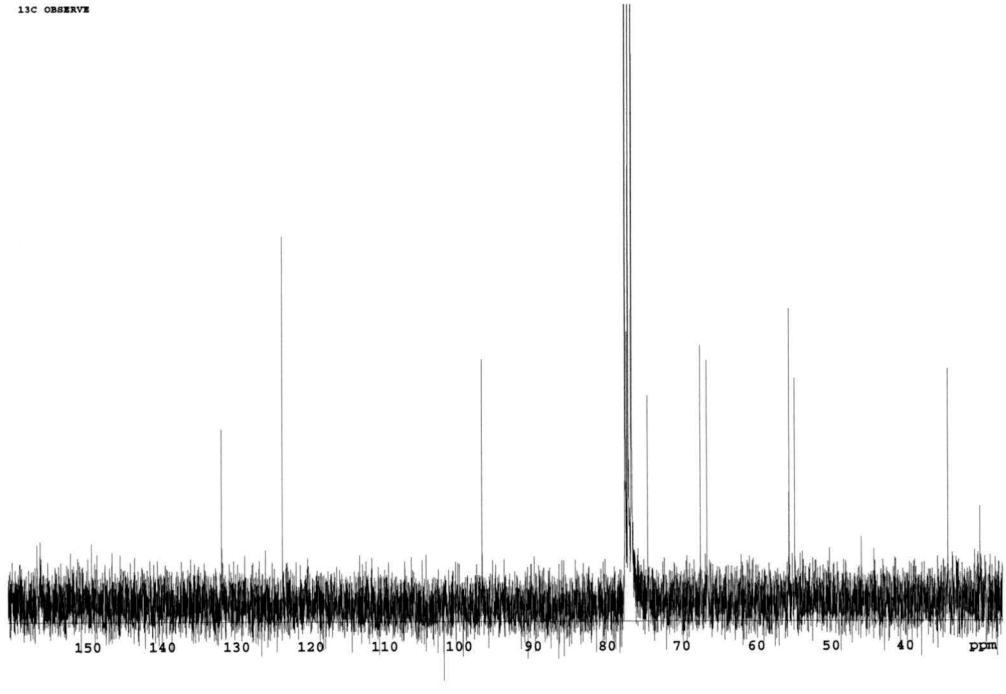
13C OBSERVE
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4.3 General Considerations for Biology Experiments

Monocrotaline was purchased from the Aldrich Chemical Company. Dehydromonocrotaline was prepared from monocrotaline according to the procedure described by Tepe.[13] FR900482 was provided by the Fujisawa Pharmacuetical Co., Ltd, Japan. FR66979 was synthesized according to the Fujisawa patent Kokai 61-10590. Plasmid DNA substrate pBR322 and EcoR1 enzyme and buffer were obtained from Roche Molecular Biochemicals. All drug stock solutions were made up to 50 mM in DMSO immediately prior to use unless otherwise noted. Mercaptoethanol (from Kodak) and dithiothreitol (from Gibco BRL) stock solutions were made using distilled deionized water immediately prior to use. Fe(II)EDTA solutions were made with distilled deionized water and filter sterilized immediately prior to use. All photoreactions were conducted in a rayonet with 365 nm light bulbs.

4.3.1 General procedure for linearization of plasmid pBR322 by EcoR1

To supercoiled pBR322 (120 μL, 30 μg) was added sterile H_2O (50 μL), EcoR1 (10 μL), and 10X EcoR1 buffer (20 μL). The reaction was incubated for 1.3 hour at 37°C. Addition of 3M NaOAc solution (66 μL, pH = 5.2) and ethanol (798 μL) followed by cooling to –80°C for 20 minutes resulted in DNA precipitation. The mixture was centrifuged at the highest setting (14G) for 15 min at 4°C. The ethanol solution was decanted off and the resultant pellet was dried *in vacuo*. The DNA was resuspended in sterile TE buffer (60 μL, pH = 7.4). The linearized pBR322 was quantitated by UV

analysis as described by P.N. Borer.[1] The conversion factor for this DNA plasmid was determined to be ODN_{260} = 1 for 50 μg/mL DNA.

4.3.2 General procedure for alkaline gel electrophoresis.

Gels (1.2 % agarose) were cast using 50 mM NaCl, 2 mM EDTA (pH = 8.0) in which to melt and dissolve the necessary quantity of agarose. All gels were 50 mL in volume, requiring 0.6 g agarose into 50 mL NaCl/EDTA solution. Upon microwave dissolving of agarose, gels were poured and allowed to solidify for 30 minutes at room temperature. The gel was soaked in a freshly prepared alkaline running buffer (40 mM NaOH + 1mM EDTA) for 1 hour. The basic running buffer was then discarded, and new buffer was added (2 mm above the wells), and the comb was removed. To the samples (10 μL) was added 3 μL alkaline loading dye (50 mM NaOH, 1 mM EDTA, 2.5 % (w/v) Ficoll, 0.25% (w/v) bromocresol blue). To each well was loaded 7.5-10 μL of sample and electrophoresis was carried out at 60 Volts for 2.5-3 hours, which resulted in a travelling distance of about 4 cm from the well. The gel was neutralized in 3 changes of 100 mM Tris (pH = 7.5) over the course of 45 minutes. The gel was subsequently stained with an ethidium bromide solution (200 μL of a 10 mg/mL ethidium bromide solution in 1L 100 mM Tris pH = 7.5 solution) for 20 minutes. The gel was then destained (to remove background staining) in 500 mL of 50mM NH_4Oac + 10 mM β-mercaptoethanol for 30 minutes. Gels were visualized on a UV transilluminator and photographed using Polaroid Black + White film #667.

4.3.3 General procedure for Disc Diffusion Assay

All agar and broth solutions were autoclaved at 121°C at 15 lb./in^2 pressure for 20 minutes prior to use. Samples of *B. subtilis* (G+), *S. aureus* (G+), and *E. coli* (G-) were streaked onto nutrient agar plates (prepared from 11.5 g nutrient agar in 500 mL DD H_2O) and were incubated overnight at 37°C. A sample of *S. marcescens* (G-) was streaked onto a nutrient agar plate and incubated at 25°C overnight. The *S. cerevisiae* was streaked onto a yeast morphology plate (prepared from 1.5 g malt extract + 2.5 g peptone + 5 g glucose + 7.5 g Bacto agar in 500 mL H_2O) and incubated at 25°C overnight. One colony from each plate (gram positive and gram negative bacteria) was swabbed with the wooden end of a sterile cotton swab and dissolved in 5 mL of nutrient broth (prepared from 4 g nutrient broth in 500 mL H_2O) and incubated at 37°C with shaking (x rpm) for 4 h or until the solution appeared cloudy. The same procedure was applied to the yeast, substituting yeast morphology broth for the nutrient broth. The bacteria were swabbed with the cotton end of a sterile cotton swab. Excess broth was removed from the tip, and Muellar Hinton II agar plates were streaked with the cells to give complete coverage. After 5 minutes, the paper discs were applied (compound **76** at 50 mM and 10 mM) with sterile forceps. Vancomycin (30 μg) was loaded as a control for the Gram positive bacteria. Streoptomycin (10 μg) was loaded as the control for Gram negative bacteria. Nyastatin (100 μg) was loaded as the control for yeast. The agar plates containing *B. subtilis*, *S. aureus*, and *E. coli* were incubated at 37°C and those containing *S. marcescens* and *S. cerevisiae* were incubated at 25°C for 17 h. The diameter of the zone of inhibition of cell growth around the applied paper disc was used for the determination of drug activity.

4.3.4 General Procedure for PCR amplification

All PCR buffers, enzymes, and solutions were obtained from Promega Corporation. To a sterile PCR tube was added 8 μL $MgCl_2$ (25 mM in water), 5 μL 10X reaction buffer (without $MgCl_2$), 2.5 μL PCR nucleotide mix (containing 500 μL of each NTP), 1 μL upstream primer (2.5 mM), 1 μL downstream primer (2.5 mM), 1 μL plasmid DNA (approx. 1.5 μg /μL) and 32 μL sterile H_2O. The reactions are heat started and after 5 minutes the Taq polymerase (0.5 μL) is added to give a total reaction volume of 50 μL . A drop of mineral oil is added on top to prevent evaporation and run the PCR with the following settings.

Cycle 1: 94°C for 15 minutes; Cycle 2: 94°C for 30 seconds; Cycle 3: 50°C for 30 seconds; Cycle 4: 70°C for 45 seconds; Cycle 5: Return to 2 for 30 X; Cycle 6: 70°C for 15 minutes; Cycle 7: 11°C until reaction is stopped. The product is run on a 1% agarose non-denaturing gel.

4.3.5 PCR Product Purification

The PCR products of the IL-2 and IL-2a receptor primers were first purified using a "wizard PCR purification" kit from Promega using the procedure recommended by Promega. Briefly, 100μL of the "direct purification buffer" was added to the eppendorf containing the dried PCR product. Add 1.0 mL of the Resin (supplied in the Promega kit) and vortex briefly 3X over a 1 minute period. Attach the column to a 5 mL sterile syringe

and pipet the resin solution into the syringe. Slowly push the solution into the column and collect the solution in a 15 mL collection tube. Rinse the column with 2 mL 80% isopropanol/water mixture. The column was transferred into an eppendorf and was centrifuged for 2 minutes at 10,000 XG. The column was transferred to a new sterile eppendorf and 50 μL sterile water was added. After 1 minute the column was centrifuged for 20 seconds at 10,000 XG. The 50 μL collected in the eppendorf contained the pure PCR product. The purity of the product was tested on a 1% agarose gel.

4.3.6 General Procedure for 1% non-denaturing agarose gel

Add 0.5 grams of agarose (high melting) to 50 mL 1% TAE buffer. (from 10% TAE stock: 48 grams Tris base, 11 mL glacial acetic acid, 20 mL 0.5 M EDTA at Ph 8, dilute to a 1 L solution). Pre-stain the gel with 1 μL ethidium bromide stock solution (10 mg/mL). Soak the gel for 30 min in 1X TEA buffer, load the samples (Lane 1: 1 μL 1 Kb DNA ladder from New England Biolabs and 2 μL agarose dye, lane 2: 7 μL PCR product and 2 μL agarose dye). Run the gel at 90 V for 1.5 hours. The quantity of DNA was estimated by comparison to the intensity of the PCR product band relative to the 1 Kb ladder. The band in the 1Kb ladder are 23130, 9416, 6557, 4361, 2322, 2027, 507 per total 48,380 base pairs of 1μg DNA).

4.3.7 Ligation of PCR product into the pGEM-T Easy Vector System I.

The PCR product was ligated in the pGEM-T Easy vector plasmid from Promega following the protocol of the manufacturer. Sample L1 contained 5 uL of the 2x rapid

ligation buffer, 1 uL of pGEM-T Easy vector, 3 uL of the FK317 PCR product, and 1 uL of T4 DNA ligase. Sample L2 contained 5 uL of the 2x rapid ligation buffer, 1 uL of pGEM-T Easy vector, 3 uL of the MC PCR product, and 1 uL of T4 DNA ligase. Sample L3 contained 5 uL of the 2x rapid ligation buffer, 1 uL of pGEM-T Easy vector, 2 uL of the control DNA, 1 uL of T4 DNA ligase, and 1 uL of sterile water. Sample L4 contained 5 uL of the 2x rapid ligation buffer, 1 uL of pGEM-T Easy vector, 1uL of T4 DNA ligase, and 3 uL sterile water. Samples were mixed by pipetting and incubated at room temperature for 1h.

4.3.8 Transformation of Vectors L1-L4 in JM 109 Cells.

Each of the four samples (2 uL) was transferred to a new sterile eppendorf on ice. The JM 109 E. Coli cells were thawed on ice, and then transferred (50 uL) to the eppendorfs containing the vector. The suspensions were carefully mixed and kept on ice for 20 minutes. The samples were then heat-shocked for 45 seconds in a 42°C water bath, then placed back on ice for 2 minutes. To the suspensions was added 950 uL of SOC solution, and set in the shaker for 1.5 hours at 37°C. From each solution, 100 uL was streaked on LB agar plates containing x-gal, IPTG, and ampicillin. The plates were incubated overnight at 37°C.

4.3.9 Innoculation of JM 109 cells.

One white colony was isolated by dipping the wooden end of a sterile cotton swab on the colony and suspending the tip in LB broth. The innoculate was shaken at 250 rpm overnight at 37°C.

4.3.10 Isolation of Plasmid from JM 109 Cells.

After incubation of white colonies in LB broth, 1.5 mL was transferred into a sterile eppendorf. The suspension was centrifuged for 2 minutes at the #10 setting of the minicentrifuge. The supernatant was discarded and the pellet was resuspended in 200 uL of GTE buffer. The lysis buffer (300 uL) was carefully added. The tube was inverted 5x to mix the solutions, and then incubated for 5 minutes at room temperature. To the solution was added 300 uL of 3M potassium acetate solution (sterilized through filtration, pH=4.8), and it was incubated on ice for 5 minutes. The resulting suspension was centrifuged for 10 minutes at the highest setting, and the pellet was discarded. The supernatant was transferred to a new eppendorf, after which 1 uL of RNAse was added. The solution was incubated for 20 minutes at 37°C. It was then extracted with chloroform (2X) and precipitated with an equal volume of isopropanol. The resulting mixture was centrifuged for 15 minutes at the highest setting. The isopropanol was decanted off and the precipitate was dried by evaporation. The pellet was resuspended in 50 uL of sterile water.

4.3.11 Plasmid Digest.

To 5 uL of each of the plasmid samples was added 1 uL of EcoR1 buffer, 1 uL EcoR1, and 3 uL of water. The mixtures were incubated at 37°C for one hour. The samples were mixed with 2 uL of 6% agarose loading dye, and run on a 1.2%agarose gel. The samples containing inserts were sent for sequencing.