

AN ABSTRACT OF THE DISSERTATION OF

Chris R. Melville for the degree of Doctor of Philosophy in Chemistry presented on April 4, 1995.

Title: Secondary Metabolism in *Streptomyces murayamaensis*

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Abstract approved: _____

Professor Steven J. Gould

A multidisciplinary approach to unraveling the biosyntheses of secondary metabolites from the kinamycin producer *Streptomyces murayamaensis* is presented. The antineoplastic and antimicrobial kinamycins, originally believed to contain a cyanamide moiety, were determined to be diazo compounds by X-ray crystallographic analysis of **125**. This corrects the misinterpreted X-ray structures of **119** and **113**. The structure revision encompasses 19 natural products including **105**, **108**, **109**, **110**, and the subsequently isolated kinamycin J, **118**.

Synthetic studies to understand the transformations between the putative biosynthetic intermediates **11** and **127** were undertaken. A series of benz[a]anthracenes related to **11** were investigated, culminating in the preparations of **24** and **29**. During this work, unexpected reductive deoxygenations of the diols **49** and **53** were observed. A second series of compounds

prepared to mimic an anticipated biological ring contraction reaction culminated in the preparation of **84** and of **90**. An unexpected neighboring group assisted nitrile solvolysis affording either **79** or **80** (depending on the reaction conditions) was also observed.

The structure of the benzo[*b*]fluorene **126** was determined crystallographically, and a series of structurally similar compounds related to the kinamycins were prepared. Of these, **129** was found to be produced by *Streptomyces murayamaensis*, and was incorporated into **105**. Other potential biosynthetic intermediates including **121**, **132**, and **147a** were also prepared. Several compounds in this structural family failed to elicit NMR spectra, which was found to result from the the presence of diamagnetic species such as **149**. The synthetic work also revealed unanticipated addition and elimination processes that afforded compounds **162** and **163**.

Feeding experiments revealed the kinamycin co-metabolite **18** was generated by a complex biosynthetic sequence. A structurally related metabolite, **194**, was also discovered.

Additional structure work provided the structures of **174**, **191**, **175**, and of **213**. Feeding experiments revealed that **213** was generated by an unexpected biosynthesis involving a Krebs Cycle intermediate. Compound **214** was found to co-occur with - and **216** was incorporated into - **213**. The relevance of this to the biosynthesis of **220** is also discussed.

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Secondary Metabolism in *Streptomyces murayamaensis*

by

Chris R. Melville

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APPROVED:

Redacted for Privacy

Professor of Chemistry in charge of major

Redacted for Privacy

Chairman of the Department of Chemistry

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Dean of Graduate School

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List of Abbreviations

atm	atmosphere
br	broad
calcd	calculated
CFE	cell-free extract
CIMS	chemical ionization mass spectroscopy
d	day or doublet
dd	doublet of doublets
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DEPT	distortionless enhancement by polarizaton transfer
DIBAL-H	diisobutylaluminum hydride
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl hydrate
EIMS	electron ionization mass spectroscopy
FABMS	fast atom bombardment mass spectroscopy
HMBC	heteronuclear multiple-bond correlation
HMQC	heteronuclear multiple-quantum coherence
HPLC	high performance liquid chromatography
h	hour
HR-	high resolution, a prefix for mass spectroscopy
INADEQUATE	incredible natural abundance double-quantum transfer experiment
IR	infrared spectroscopy
KHMDS	potassium bis(trimethylsilyl)amide

List of Abbreviations Continued

LiHMDS	lithium bis(trimethylsilyl)amide
m	multiplet
MEM	2-methoxyethoxymethyl-
MEMCl	2-methoxyethoxymethyl chloride
mp	melting point
MPLC	medium pressure liquid chromatography
NMMO	4-methylmorpholine <i>N</i> -oxide
NMO	4-methylmorpholine
NMP	<i>N</i> -methylpyrrolidone
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
PC	polycarbonate or personal computer
q	quartet
rel	relative
rt	ambient (room) temperature
soln	solution
t	triplet
TBAF	tetrabutylammonium fluoride
THF	tetrahydrofuran
TMSE	2-trimethylsilylethyl-
TMSOTf	trimethylsilyl trifluoromethanesulfonate
TsOH	<i>para</i> -toluenesulfonic acid
UV/vis	ultraviolet and visible spectrum
vol	volume
wt	weight

Secondary Metabolism in *Streptomyces murayamaensis*

1. The Kinamycin Producer *Streptomyces murayamaensis*: Early Structure and Biosynthetic Investigations

Secondary metabolism, for the purpose of this treatise, is an anabolic process forming a low to medium molecular weight compound. It is distinguished from primary metabolism which is the set of processes that are essential for maintaining the structure, providing energy, or encoding information for a cell. The products of secondary metabolism are conveniently classified according to the primary metabolites which are their ultimate precursors. Compounds arising from the oligimerization of acetate and its subsequent tailoring are polyketides, and constitute a large and diverse group of compounds. Polyketides encompass many biologically active compounds, and their formation has been the object of periodic review.(1,2) Production of one such family of polyketides, the kinamycin antibiotics, has prompted the investigation of the series of metabolic reactions that together constitute their biosynthesis.

Traditional reasons for investigating biosynthesis have been to better understand what reactions are possible in biological systems, to discover new chemistry that can have general application in organic synthesis, and to gain insights into the structures and reactivity of specific groups of naturally occurring compounds. More recently, biosynthetic studies have gained

attention for their potential applications in improving processes for drug production(3), for providing access to industrial feedstock compounds(4), and for allowing the semi-rational design of new drug substances by genetic engineering.(5,6,7) Biosynthesis is a hybrid science which by its nature benefits from using a diverse array of techniques in its elucidation.(8) Within this framework, the remainder of this chapter will introduce the state of understanding of the kinamycin biosynthesis at the start of this dissertation work, arranged by the method of study.

Isolation, Structure, and Bioactivity of the Kinamycins

Omura and co-workers were the first to investigate the secondary metabolism of *S. murayamaensis*. From the culture broth of this organism they were able to isolate four antibiotic metabolites, kinamycins A, B, C, and D.(9,10) These metabolites differ in their degree and positions of acetylation. By a variety of derivatization and degradation reactions the relationships between the acylation patterns of each of these metabolites was determined.(11) Spectroscopic techniques provided partial structures, and the structure of a derivative, kinamycin C *para*-bromobenzoate, **1**, was determined by a single crystal X-ray diffraction study.(12) This provided the structures **2-5** for kinamycins A-D respectively.(13) These compounds contain two highly novel structural features, the benz[*b*]carbazole ring system, and the cyanamide substituent (N-cyano).

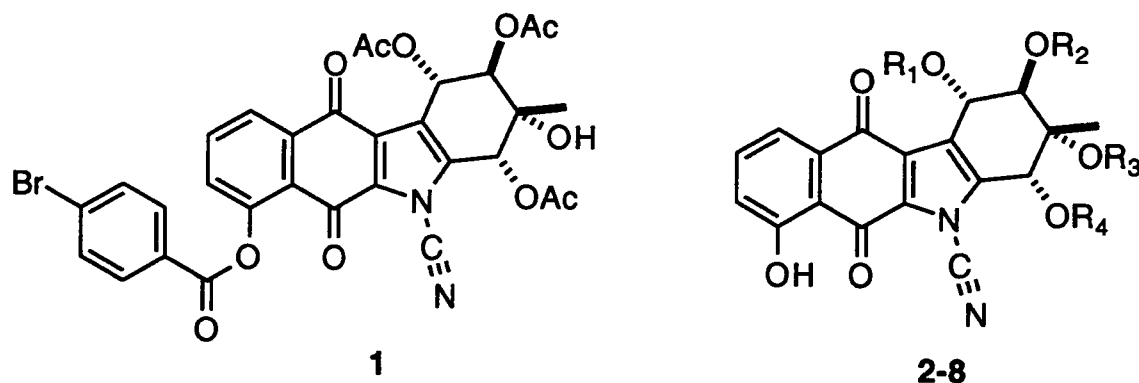


Figure 1.1. D ring substituents of 2-8

2	KA ^a	R ₁ =H	R ₂ =R ₃ =R ₄ =Ac	
3	KB	R ₁ =R ₂ =R ₄ =H	R ₃ =Ac	
4	KC	R ₃ =H	R ₁ =R ₂ =R ₄ =Ac	
5	KD	R ₁ =R ₃ =H	R ₂ =R ₄ =Ac	
6	K ^b		R ₁ =R ₂ =R ₄ =Ac	R ₃ =COiPr
7	K ^c	R ₃ =H	R ₁ =R ₂ =Ac	R ₄ =COiPr
8	A83016A	R ₃ =H	R ₁ =Ac	R ₂ =R ₄ =COiPr

^a Kinamycins A-D.

^b 3-O-Isobutyrylkinamycin C(14)

^c 4-Deacetyl-4-O-isobutyrylkinamycin C(14)

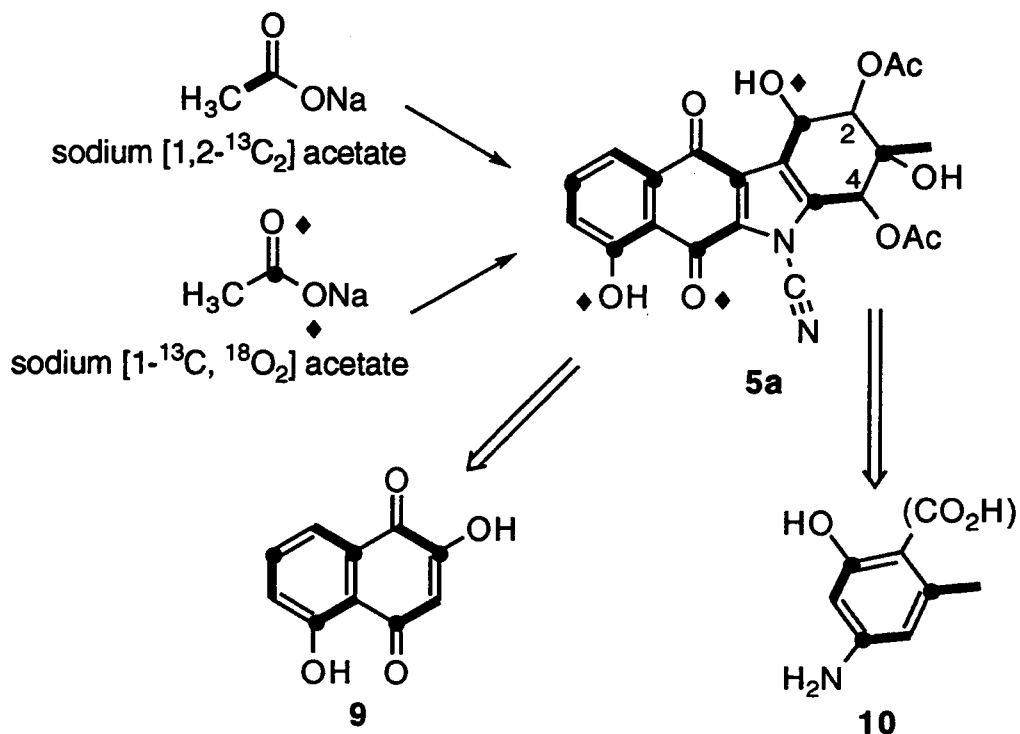
Two additional members of this group of antibiotics - 3-O-isobutyrylkinamycin C, **6**, and 4-deacetyl-4-O-isobutyrylkinamycin C, **7** - have been isolated from the fermentations of a *Sacharothrix* species, strain MI293-N4.(14) Another recent addition to this family is A83016A, **8**, isolated from an unidentified actinomycete.(15)

The kinamycins exhibit potent antibiotic activity against several Gram-positive bacteria. Additionally 4 and 5 are active against some Gram-negative bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, and *Shigella sonnei*. Kinamycin C, 4, additionally has antitumor activity against Ehrlich ascites carcinoma and sarcoma 180, manifested by increased survival times in mice.(10) The LD₅₀ for mice is ~30-40 mg/kg for these compounds. The isobutyryl substituted kinamycins 6 and 7 also showed *in vitro* cytotoxicity against L1210 (leukemia), and IMC carcinoma cultured cell lines.(14) Compounds 6 and 7 also inhibited growth of LX-1 (human lung carcinoma) and SC-6 (human stomach carcinoma) *in vitro*.

Primary Precursor Feeding Experiments

Biosynthetic studies on the formation of the kinamycins were initiated by both the Hornemann and Omura groups, but were not pursued.(16) The uniqueness of the kinamycin structures, and their biological activities, prompted our group's interest in studying this family of compounds. As a prerequisite to stable isotope feeding experiments to determine the primary metabolic precursors and their positions of incorporation, the ¹³C NMR spectrum of 5 was assigned with the use of a long-range heteronuclear COSY experiment.(17) Subsequent feeding experiments revealed the pattern of intact acetate subunit incorporation shown on structure 5a.(18,19) Due to the 5-

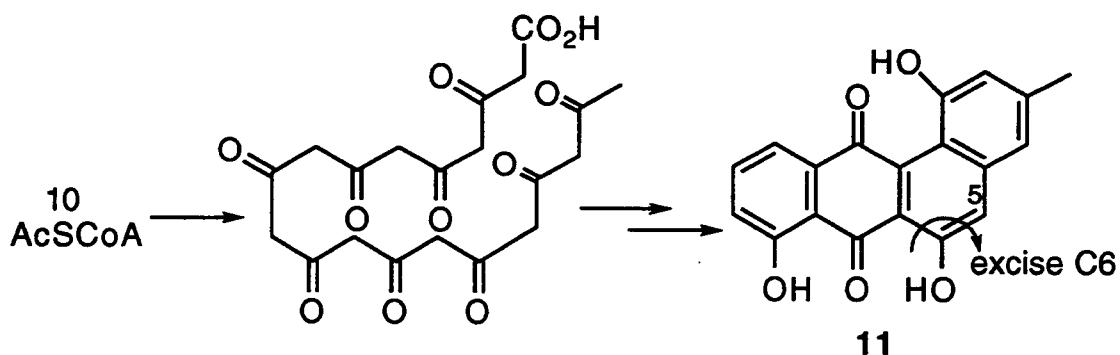
membered heterocyclic ring, a contiguous chain of intact acetate units cannot give rise to **5**. The reporting authors therefore proposed that **5** may arise from two polyketide derived subunits, for instance **9** and **10**. Incorporations of ^{18}O from feeding sodium $[1\text{-}^{18}\text{O}_2, 1\text{-}^{13}\text{C}]$ acetate showed that the oxygen atoms on carbons derived from C1 of acetate were acetate derived with the exception of the hydroxy at C3. A complementary $^{18}\text{O}_2$ feeding labeled all remaining oxygens. A feeding experiment with sodium $[2\text{-}^2\text{H}_3, 1\text{-}^{13}\text{C}]$ acetate showed no retention of deuterium at C2 or C4, consistent with an aromatic C ring precursor, which would be oxidized at C4 prior to loss of aromaticity.(18)



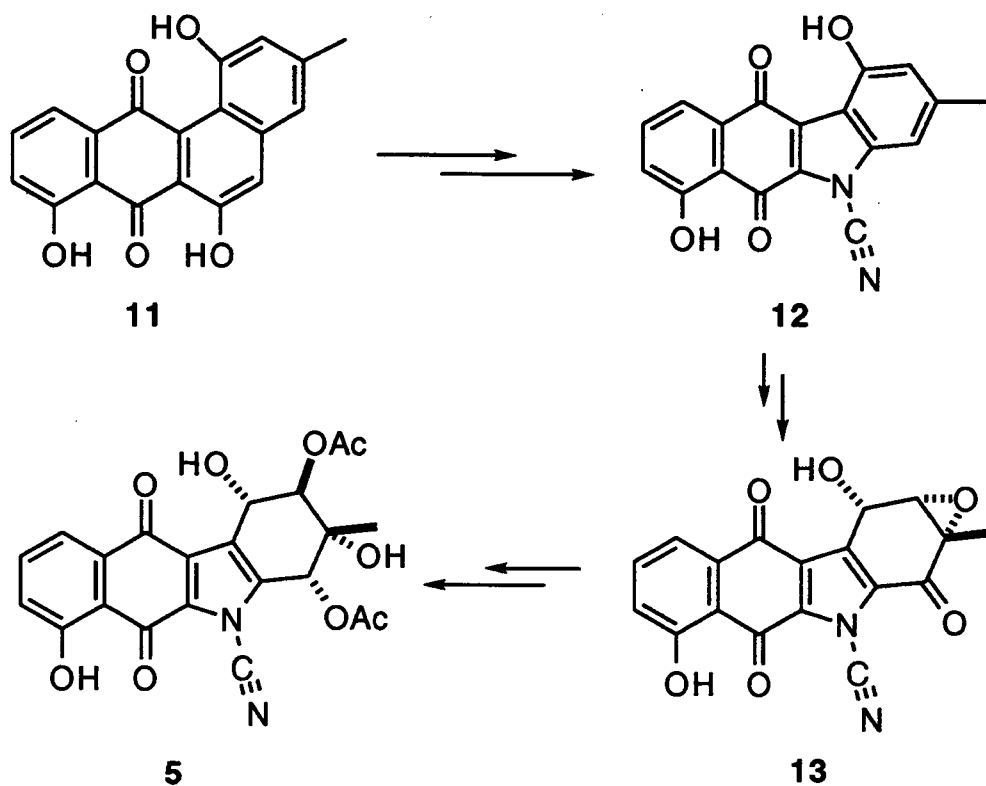
The cyanamide resonance, being slow to relax in the NMR and therefore not prominent, was not assigned in the initial biosynthetic work. However, in a subsequent feeding experiment with $(^{15}\text{NH}_4)_2\text{SO}_4$ as the sole nitrogen source, a doublet of doublets (δ 78.5, $J_{\text{CN}} = 21.2, 5.4$) was observed in the ^{13}C NMR spectrum of **5**.⁽²⁰⁾ Observation of two couplings to this carbon atom showed that it was proximal to both nitrogen atoms, identifying it as the cyanamide carbon. Subsequent acetate feeding experiments showed that this carbon was derived from C2 of acetate.

Isolation of Minor Metabolites

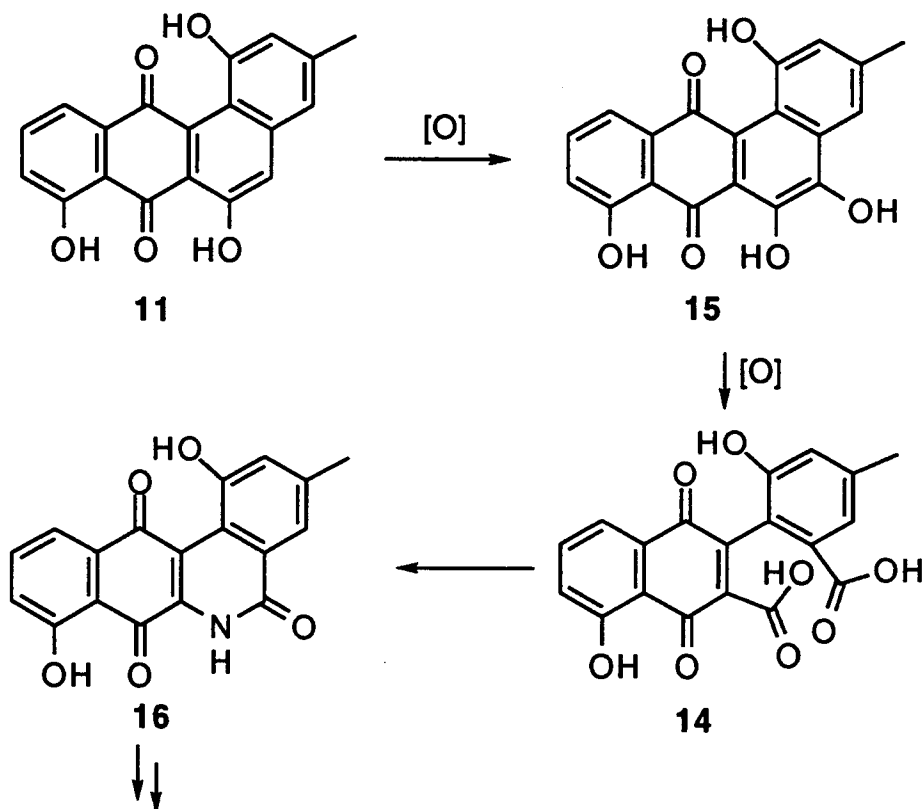
Isolation of minor colored metabolites from *S. murayamaensis* provided a known compound, dehydrorabelomycin **11**. Compound **11** arises from the simple folding and condensation of a linear decaketide precursor, with loss of the terminal carboxyl and oxidation to generate the quinone. Although structurally distinct from the kinamycins, **11** was recognized as a potential kinamycin biosynthetic intermediate by excision of C6, and transformation of C5 into the cyanamide moiety.⁽²¹⁾ By feeding deuterium labelled **11**, **5** was obtained in which a high level of deuterium from the precursor had been retained. This demonstrated that **11** was utilized in the biosynthesis of the kinamycins.

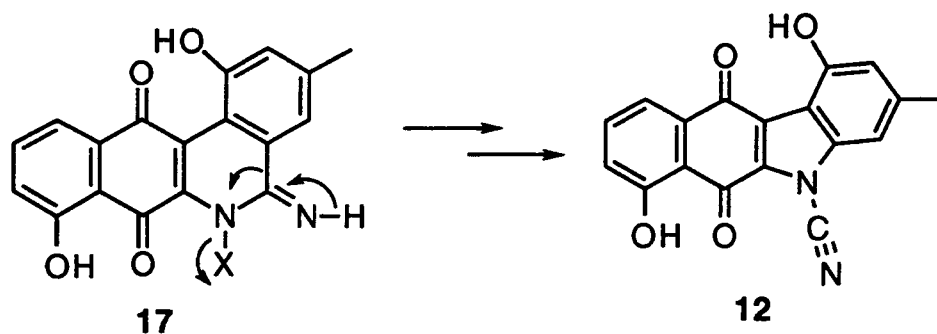


Also isolated were prekinamycin **12** and ketoanhydrokinamycin **13**, compounds with more obvious structural relationships to the kinamycins.(22) By arranging these compounds in order of increasing functionality, a plausible sequence of biosynthetic intermediates was obtained.

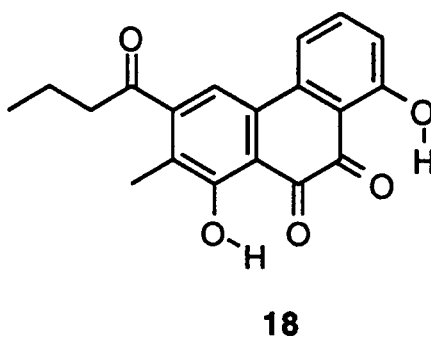


In order to rationalize the conversion of **11** to **12**, an oxidative cleavage of the C5-C6 bond of **11** was necessary. This was proposed to occur via the action of two sequential mono-oxygenase catalyzed oxidations to provide the diacid **14** via catechol **15**. Conjugate addition of ammonia to the naphthaquinone followed by decarboxylation and ring closure would then provide pyridone **16**, which would be transformed into the N-substituted amidine **17**. The amidine moiety of **17** would then undergo a transformation, with concomitant ring contraction, into the cyanamide moiety of **12**.(20,23)



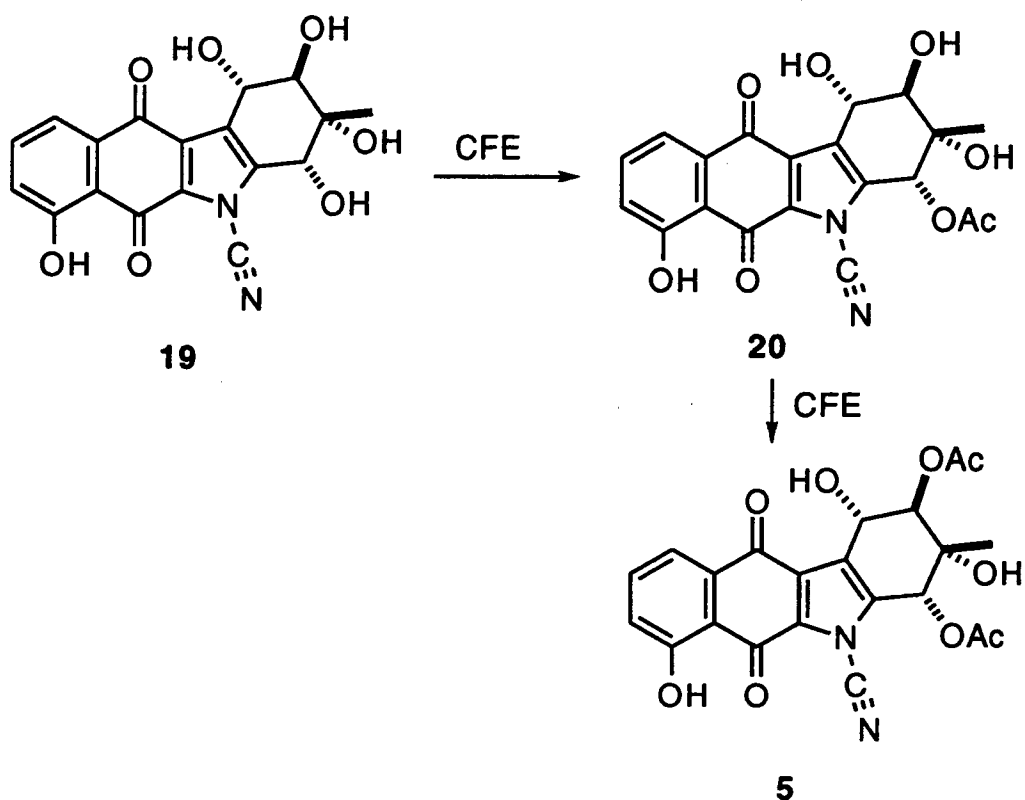


One additional metabolite, structurally unrelated to the kinamycins was also discovered in *S. murayamaensis* fermentation extracts.(24,25) Murayaquinone **18**, named to acknowledge its source organism, is also a polyketide metabolite. It has a low toxicity to mammals, and has been found to have antibiotic activity against *Mycoplasma galliseptica* and *Treponema hyodysenteriae*.(26)



Identification of an Acyl Transferase Activity, and Identification of Kinamycins E and F

Basic saponification of **5** removes the acyl groups, providing **19**.(13) Incubation of **19** and acetyl CoA in a cell-free preparation of *S. murayamaensis* generated an unidentified intermediate, and **5**. A more controlled saponification of **5** provided the two monoacetates, of which the minor component, **20**, was found to match the compound produced by the cell-free extract. Subsequent discovery of **20** in fermentation extracts, and an isotope trapping experiment with **19**, demonstrated that each was naturally produced by the organism.(22) Compounds **19** and **20** have since been named kinamycin F and kinamycin E to acknowledge their apparent involvement in the biosynthesis of **5**.



Subsequent work to elucidate the biosynthesis of the kinamycins has involved four areas of study. These are: 1. Synthetic work to mimic the chemistry of the proposed ring contraction, and to prepare potential biosynthetic intermediates for feeding experiments; 2. Isolation and structure elucidation of additional *S. murayamaensis* metabolites, and associated degradative and derivatization chemistry; 3. Identification and expression of the genetic material coding the *S. murayamaensis* biosynthetic pathway; and 4. Identification and isolation of enzymes involved in kinamycin biosynthesis. The remaining chapters of this work will detail a part of the recent work in the first two areas.

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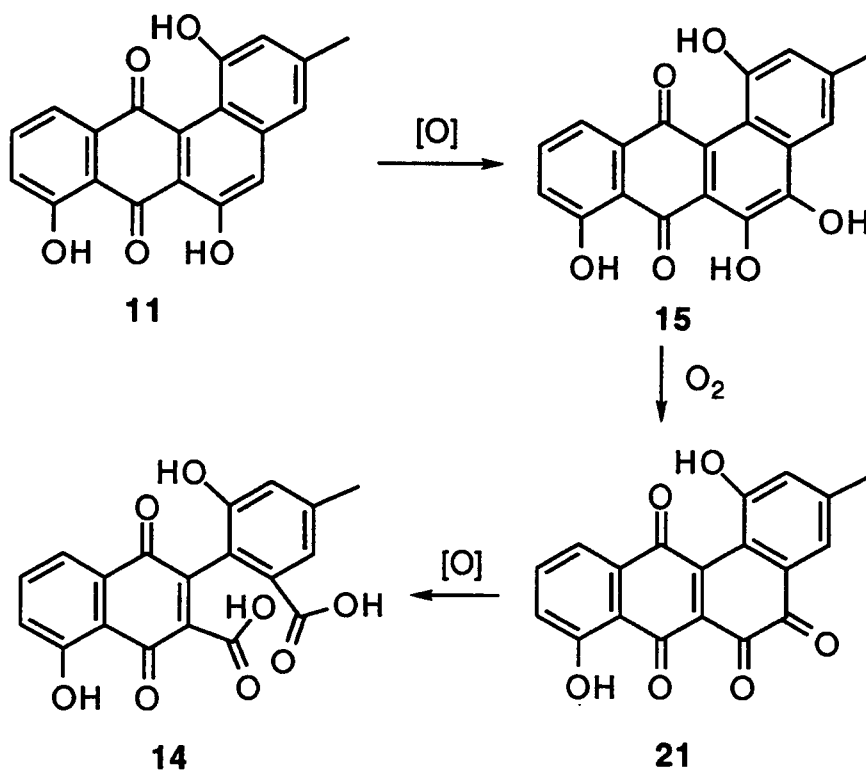
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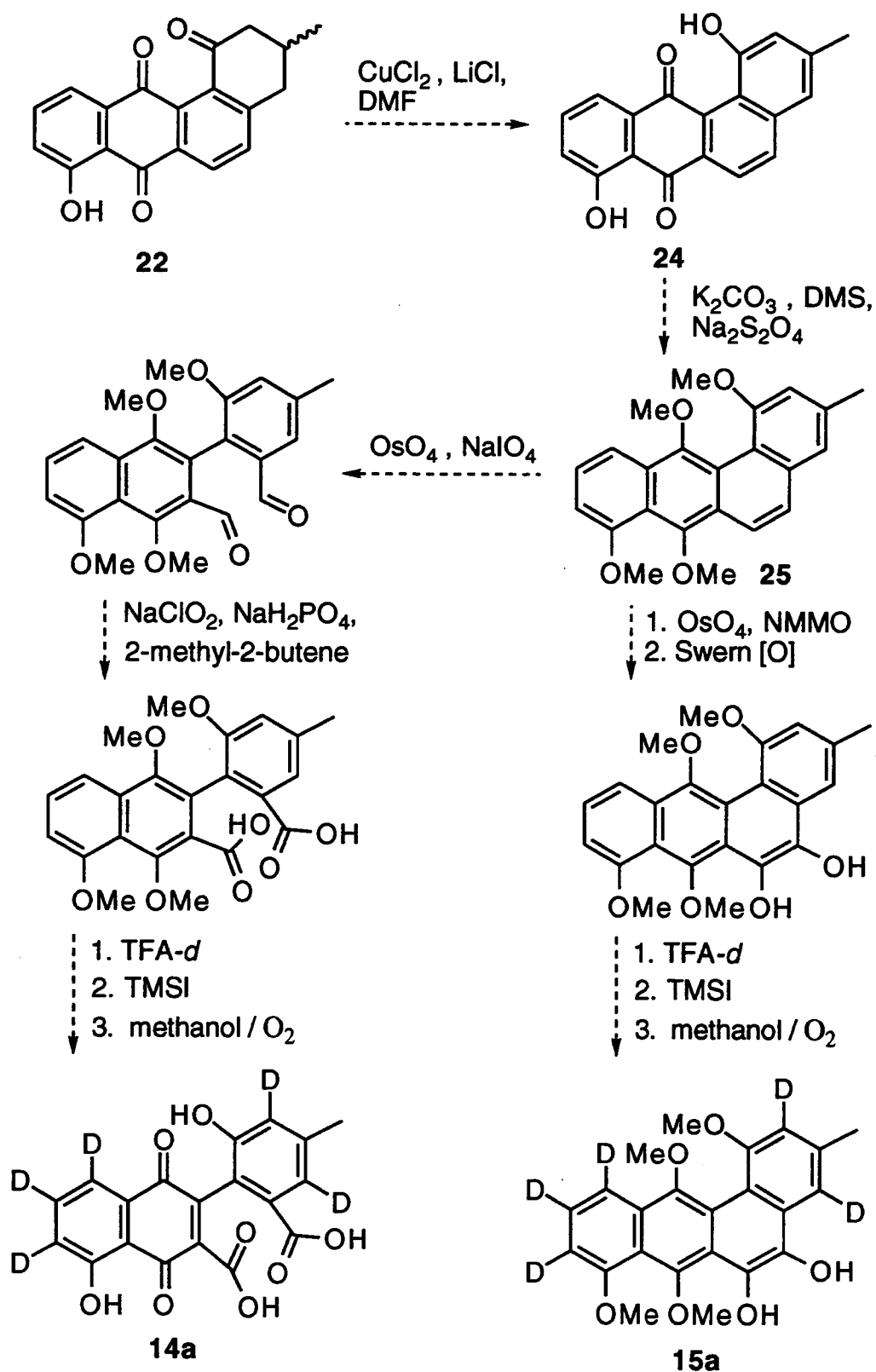
2. Benz[a]anthracenes

In the biosynthetic steps leading to **12**, dehydrorabelomycin **11**,(1) may be converted, via an intermediate oxirane, to **15** by a monooxygenase enzyme. Air oxidation of **15** could provide the *ortho*-quinone **21**, which could be the substrate for a second monooxygenase. The action of this second monooxygenase would cleave the C5-C6 bond, to give **14**. As part of the continuing effort to elucidate the biosynthesis of the kinamycin antibiotics, a synthesis of the proposed biosynthetic intermediates **15**, **21**, and **14**, was undertaken.



Ochromycinone(2,3) **22** was chosen as a strategic intermediate because its synthesis, and that of its methyl ether X-14881C, **23**,(39) (synonymous with rubiginone B2(4)) had been previously reported.(5,6,7) A synthesis of tetrangulol, **24**,(8) has also been reported. The method of Guingant and Barreto was chosen, but because this was published as a communication, some aspects of the synthesis required considerable experimentation to be worked out.

Scheme 2.1 shows the proposed route for conversion of **22** to the target compounds. This called first for a D ring aromatization, to provide tetrangulol.(9) A reductive alkylation(10,11) of the quinone moiety of **24** would then provide **25**. The angular (K-region) double bond of **25** was anticipated to have a lesser degree of aromatic stabilization relative to the naphthalene and benzene substructures that it connects. This can result in a useful selectivity for dihydroxylation(12,13) and oxidative cleavage(14) at this position. Oxidative tailoring of these products was expected to provide the presumed biosynthetic intermediates.



Scheme 2.1

The addition of acetylene was accomplished with lithium acetylide generated in ammonia, and added in THF, and was greatly accelerated by sonication. Use of the ethylenediamine complex resulted in a less clean reaction. Reactions using dimethyl formamide as solvent or with ethylmagnesium bromide, to deprotonate the acetylene, gave lower yields.

In the Favorskii-Shostakovskii addition of methanol to afford dieneone **28**, the selection of base was crucial to success.(16) Pyridine failed to catalyze the reaction, while the use of triethylamine, diisopropylethylamine, and dicyclohexylamine gave polymeric products. N-Methylmorpholine, however, gave a clean reaction, complete conversion, and gave exclusively the entgegen isomer. This product was stable on silica gel but needed to be stored in frozen benzene, as it decomposed in a day or two, neat at ambient temperature.

The literature preparation for boron triacetate(17) (a Lewis acid catalyst) provided a white microcrystalline material that showed partial melting over a wide range of temperatures. This product was not soluble in CH_2Cl_2 to any appreciable degree. A modified procedure where the catalyst was not exposed to air during preparation provided large clear crystals with a high solubility in CH_2Cl_2 , and this was the catalyst used for the Diels-Alder reactions.

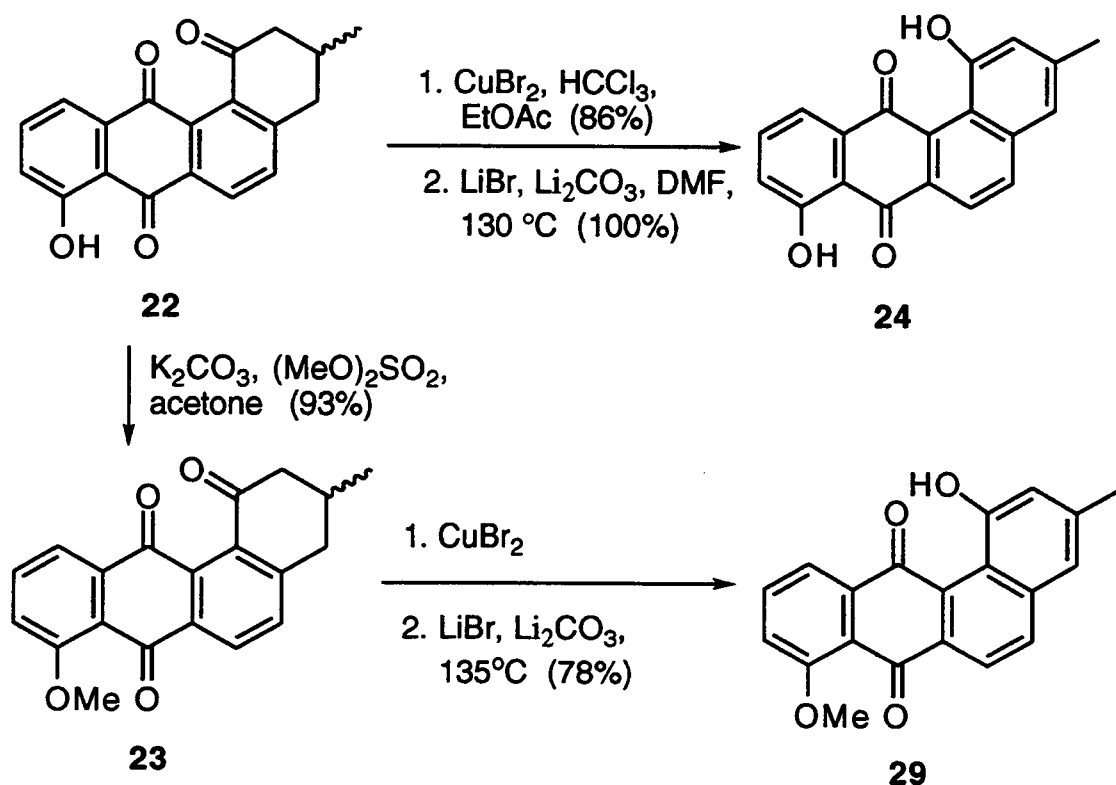
The Lewis acid catalyzed Diels-Alder reaction to provide the natural product **22** was troublesome. Both the diene **28** and

dienophile (juglone) were unstable in the presence of the catalyst. An uncatalyzed thermal Diels-Alder reaction at 65 °C rapidly decomposed the juglone giving only intractable products. Sonication without Lewis acid catalysis failed to induce reaction, and with added catalyst provided no improvement in yield or cleanliness. After considerable experimentation, it was found that simply adding the mixed starting materials to a solution of catalyst at 25 °C, gave 46% yield of **22**. The synthesis and experimental conditions are shown in Scheme 2.2.

Attempted single-step dehydrogenations of ochromycinone to the natural product **24** by treatment with cupric chloride or with dehydrogenation catalysts (palladium on carbon in methylnaphthalene, 180 °C; rhodium on alumina in methylnaphthalene, 180 °C; or palladium on carbon, in refluxing glacial acetic acid), and inert gas passing through the reaction mixture, gave no dehydrogenation. Also unsuccessful was an attempt to make the methyl enol ether by reaction with dimethyl sulfate and sodium hydride, which might have been more easily dehydrogenated. However, a convenient, and high yielding, two-step procedure involved bromination α to the ketone followed by elimination with lithium bromide to afford **24** in 82-87% yield.(18,19)

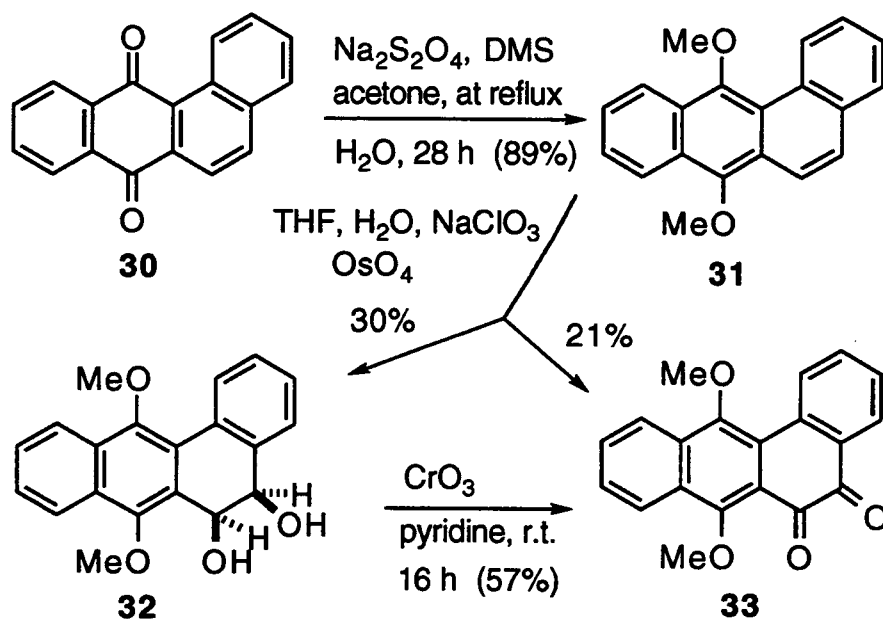
The natural product X-14881C, **23**, was prepared by treatment of **22** with silver oxide and methyl iodide in modest yield,(2) with numerous minor products. However, an alternative reaction

employing dimethyl sulfate and potassium carbonate cleanly gave **23** in 93% yield. The dehydrogenation procedure that was used to prepare **24** was now applied to **23** to prepare X-14881E, **29**. Spectral data for **29** were consistent with that for the natural product,(39) and for material prepared by dehydration of antibiotic MM 47755.(20) The original structure elucidation was based on the minimal data obtained from very small samples, so this work constituted the first total synthesis, and a verification of the assigned structure.



A Benz[a]anthracene Model System

Commercially available benzanthraquinone **30** was protected as its dimethyl ether **31**, and used as a model system for the oxidative chemistry. This system was osmylated in low yield using Yamamoto's method, 1,4-diazabicyclo[2.2.2]octane with potassium ferricyanide co-oxidant.(21) Treating the model system with the more reactive ruthenium tetroxide afforded a mixture from which the dihydrodiol **32**, and *ortho*-quinone **33** were isolated. Under no conditions were any tractable oxidation products observed for compound **30**.



The oxidation of **31** with permanganate under a variety of conditions gave no reaction. Similarly, treatment with ammonium

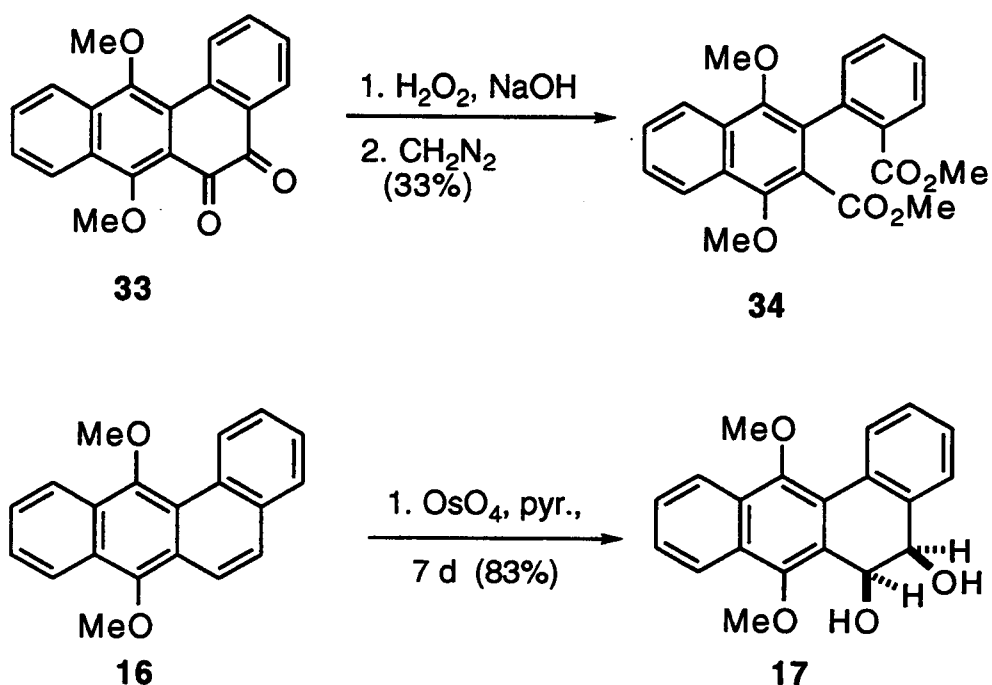
cerium(IV)nitrate, ammonium cerium(IV)sulfate, or peracetic acid gave only deprotection of the methyl ethers.

Compound **31** was reacted with osmium tetroxide,(12) or ruthenium tetroxide, and a variety of co-oxidants. Among the best osmylation procedures for the model system was sodium chlorate co-oxidant, with the reaction run at high concentration. The chlorate salt co-oxidants (barium,(22) strontium, silver, potassium, lithium, and sodium) were uniquely reactive compared with other co-oxidant systems. Additionally, each gave a somewhat different product mixture. A majority of osmylating conditions using other co-oxidants gave no reaction.

It was found that when heated at reflux the osmylation stopped after a few hours, presumably due to sublimation of the osmium tetroxide out of the reaction mixture. Similarly, after extended reactions - even without heating - the osmylations would stop if run in a flask with a septum stopper. The septum would become black, so presumably the osmium tetroxide was irreversibly reacting with the septum. A convenient way to overcome these problems, and to run the osmylations at a sufficient concentration to have an acceptable rate of reaction, was developed. The reactants were placed in a 1 dram vial with a magnetic spinbar, and dissolved in a small volume (50-250 μ L) of the osmium tetroxide containing solvent system. This was sealed with a Teflon lined cap, and heated by placing the entire vial inside a solvent filled flask fitted with a condenser for heating at

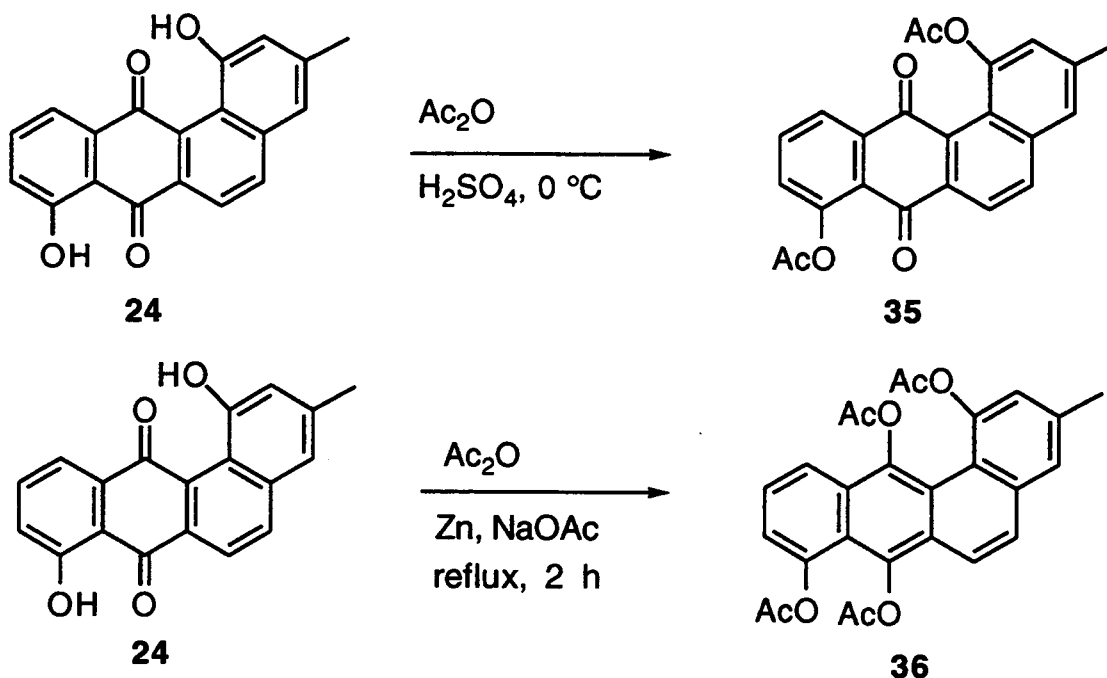
reflux. For comparing the various osmylating conditions, the reactions were heated with acetone (bp~56 °C), to a temperature just below the boiling point of the reaction co-solvent THF (bp~64 °C).

For comparing various oxidation conditions the ratios of oxidation products were obtained from the ^1H NMR spectra of the reaction mixtures. Subsequent to this determination, some of the product mixtures were treated with chromium trioxide in pyridine to provide a more easily separable mixture. This oxidizing system did not react with **30**, but converted **32** to the *ortho*-quinone, **33**. Compound **33** obtained from oxidations of the model system, was oxidatively cleaved to the diacid by alkaline hydrogen peroxide, and methylated *in situ* with diazomethane. This gave the corresponding diester **34**. No catalytic osmylation conditions were found where the yield of identifiable K region oxidation products significantly exceeded 50% of the expected total. An improved yield was realized by reaction of **31** with two theoretical equivalents osmium tetroxide in pyridine for 7 days, However, because there was no solvolysis of the intermediate osmate ester, the chemical selectivity for the dihydrodiol **32** was much improved.



Protecting Group and Redox Chemistry of Tetrangulol

Tetrangulol **24**, was protected as the diacetate **35**,⁽¹⁾ by the method reported for acetylation of **11**, and as the tetraacetate **36** by the reported procedure.⁽⁹⁾ Both of these compounds failed to react with *tert*-butyl hydroperoxide or *meta*-chloroperoxybenzoic acid, each in the presence of Wilkinson's catalyst in benzene at reflux. However, treatment of **36** with peracetic acid and Wilkinson's catalyst caused a rapid evolution of oxygen, and resulted in a complex mixture of products.

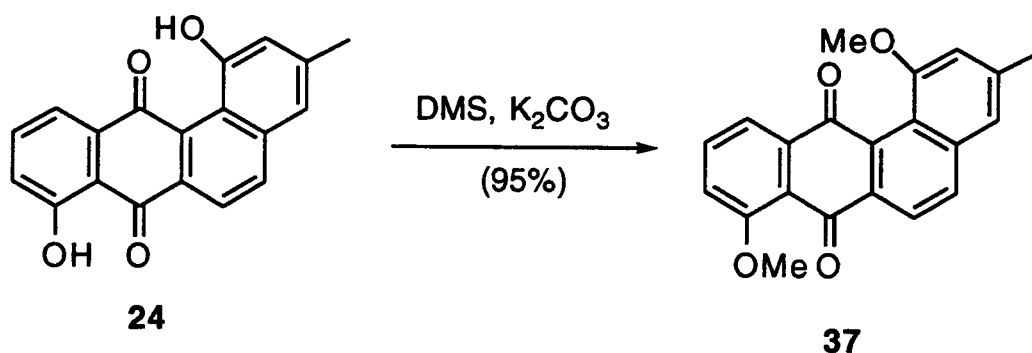


It was found, however, that **24** could not be protected as its reduced tetramethylether **25** by any of the literature methods,(23,10,11) or with several modifications of alkylating agents, phase transfer catalyst, or solvents. The method of Kelly(11) uniformly produced a mixture with large amounts of reaction byproducts not derived from tetrangulol. Attempted reduction with sodium borohydride failed to provide the hydroquinone. A catalytic hydrogenation in the presence of trifluoroacetic acid and diazomethane (a method developed by our former group member Dr. He) was also tried, but to no avail. Also attempted was catalytic hydrogenation with dimethoxy propane and toluenesulfonic acid to generate the diketal, but with this and the previous case the only new product was a red compound with a ^1H NMR spectrum consistent with the structure

of 5,6-dihydrotetrangulol, and was not further characterized. It was possible to protect tetrangulol using a two phase system using trimethylsilylethoxymethylenechloride which gave the tetra(SEM)ether in 48% yield.(24) This compound was somewhat labile on silica, and completely decomposed over two days. It was therefore apparently insufficiently stable for the desired sequence of steps.

It seemed at this time expedient to change substrates, so **35** was prepared by the method previously reported for the acetylation **11**.(1) This compound was then subjected to the conditions of Kelly(11) and afforded a complex mixture which was not pursued further. Attempted non-reductive methylation of tetrangulol under phase transfer conditions was a poor reaction; however, treatment with K_2CO_3 , dimethylsulfate, in acetone at reflux gave 7,12-O,O-dimethyltetrangulol, **37**, in 95% yield, a considerable improvement over the NaOH facilitated literature procedure.(9) Compound **37** was subjected to the known reductive alkylation conditions(23,10,11) without success. Similarly, **37** could not be reduced by two phase systems using sodium dithionite, or sodium thiosulfate at pH 7, 10, or 14. Other solvent systems for sodium dithionite were then explored, and it was found that this reagent was soluble in DMSO, and while insoluble in HMPA, it produces a light blue solution in that system. The later system reduces dimethyltetrangulol, but dimethylsulfate did not alkylate the resulting hydroquinone under

these conditions. When these conditions were duplicated but with added KOH four products were observed, and the desired tetramethyl ether **25** was isolated in 25% yield.(25) These conditions gave no reaction when methyl tosylate was substituted as the alkylating agent.

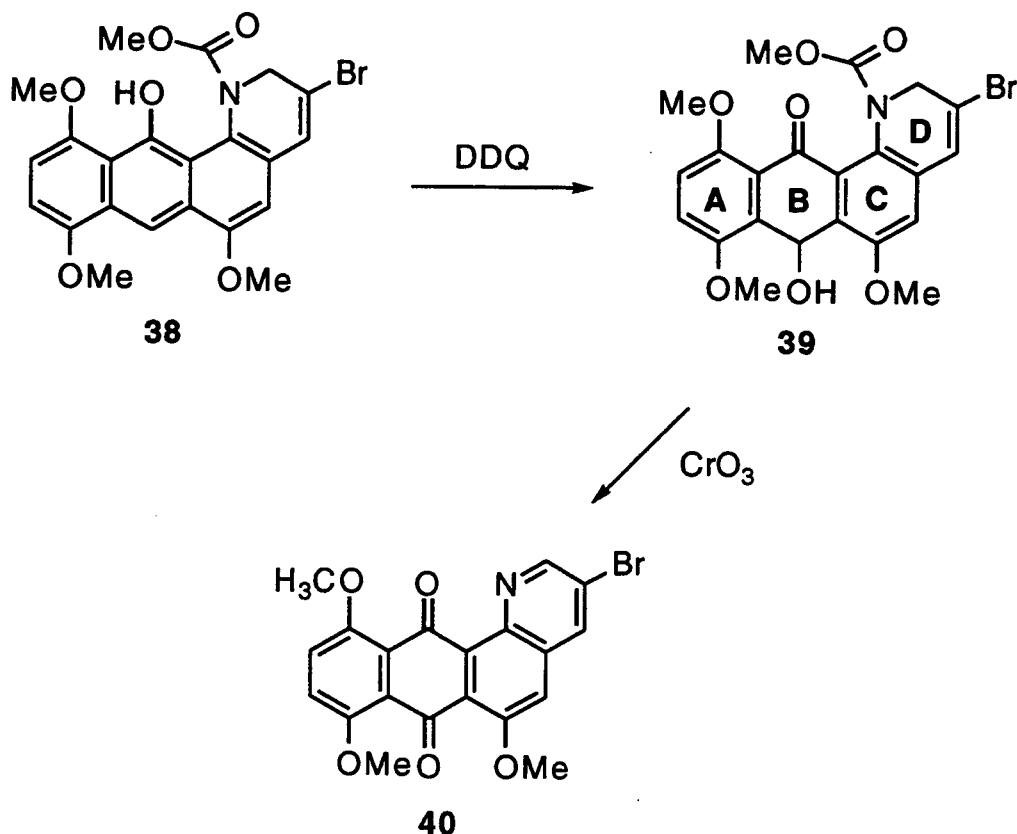


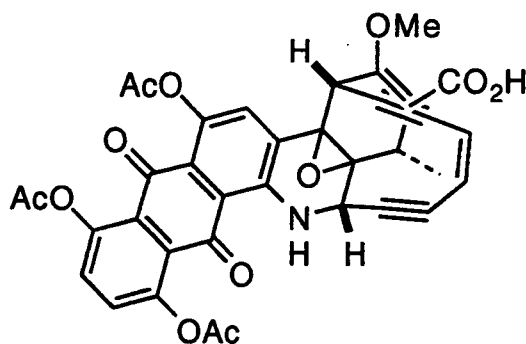
Several metal reducing systems were investigated with **37**. The first of these was potassium in THF, in which initially no reaction occurred. However, when this mixture was sonicated, a dark blue solution was generated, the color of which dissipated immediately when sonication was stopped. These conditions gave a complex mixture of products. Calcium in HMPA did not reduce **37**. Dimethylsulfate was found to be stable in THF solutions in contact with aluminum, calcium, and zinc. Of these only the zinc solution reduced **37**, but no alkylated products were isolable. Zinc metal in dioxane, by contrast, did not at first reduce **37** to its putative hydroquinone. However, addition of a trace of trifluoroacetic acid facilitated the reduction. Attempted

methylation of the hydroquinone with methyl triflate, methylfluorosulfate, or trimethyloxonium tetrafluoroborate with or without the addition of 18-crown-6 was unsuccessful. Similarly, **35** also failed to reductively methylate under similar conditions, and gave a complex mixture of products. In either case only a partial recovery of reactants was possible. A similar experiment using an HMPA/THF solvent system showed that after addition of solid KOH, blue solutions were generated from aluminum amalgam, and from powdered zinc. Surprisingly, aluminum amalgam did not significantly reduce **37**, although it reacted vigorously when placed in water. Also unsuccessful were the aluminum-nickel and zinc-nickel dissolving bimetal systems.

At this time a paper appeared that shed light on several aspects of this work. In the synthesis of the angular subunit of dynemicin A, Schreiber and co-workers experienced unexpected difficulty in oxidizing the phenol **38** to the desired hydroquinone. The product obtained was instead the semiquinone **39**, which could be oxidized only with difficulty to the quinone **40**. X-ray crystallography revealed the A and C rings of **39** deviated from planarity 16.9°, 26.2°, and 34.0° respectively for the three molecules in the unit cell. Additionally, the carbonyl had an average deviation of 29.7° from the plane of the B ring. This suggested that the planar hydroquinone was disfavored due to steric interactions between the C1 and C12 substituents. Also of interest was the X-ray structure of the anthraquinone **40** which

showed a 22.7° angle between the A and C rings, and an average deviation from planarity of 30.4° for the two B ring carbonyls. This provided an explanation for the failure of K-region oxidation attempts on the quinone, because the C and D rings are isolated from the A ring and so would be expected to show fully aromatic character. A similar 15° bowing was seen in the X-ray structure of dynemicin A triacetate, **41**, itself. To my knowledge the aforementioned are the only crystallographic studies on fully unsaturated benz[a]anthracenes, but it is probable that **11**, **29**, and **24** would show similar distortions from their ideal geometries.





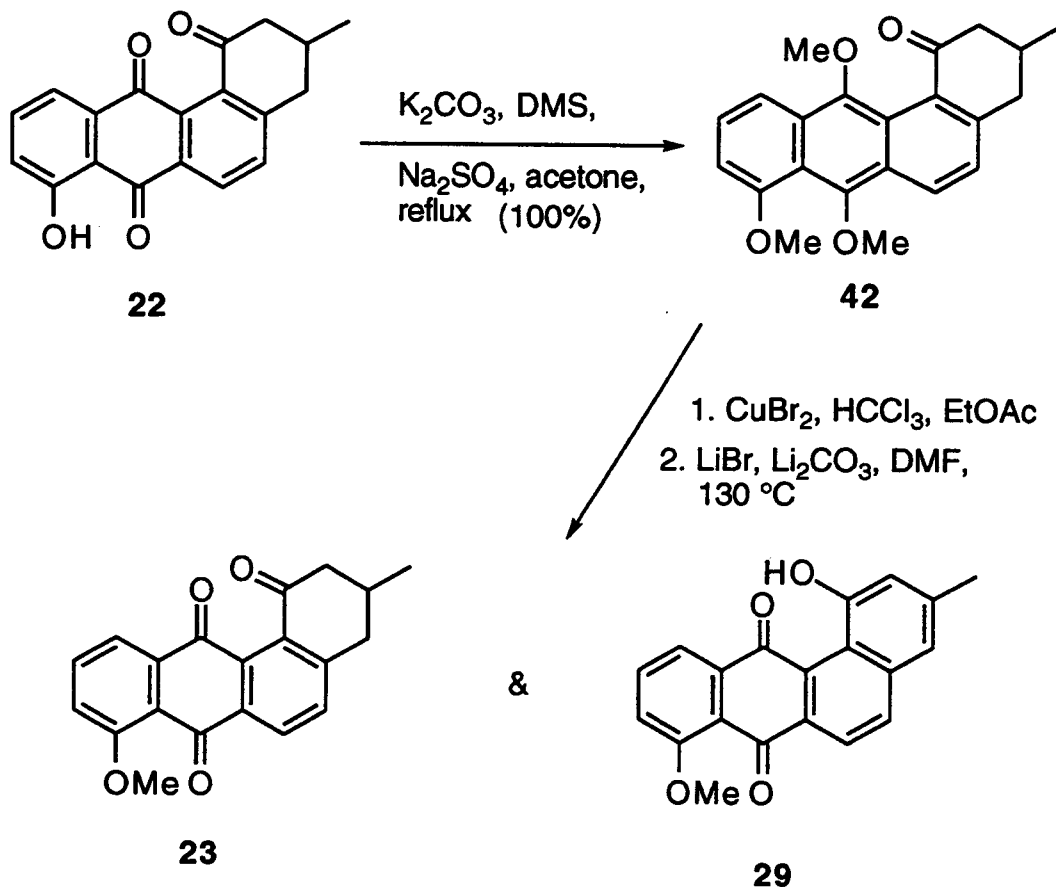
41

Protection and Dehydrogenation of Ochromycinone

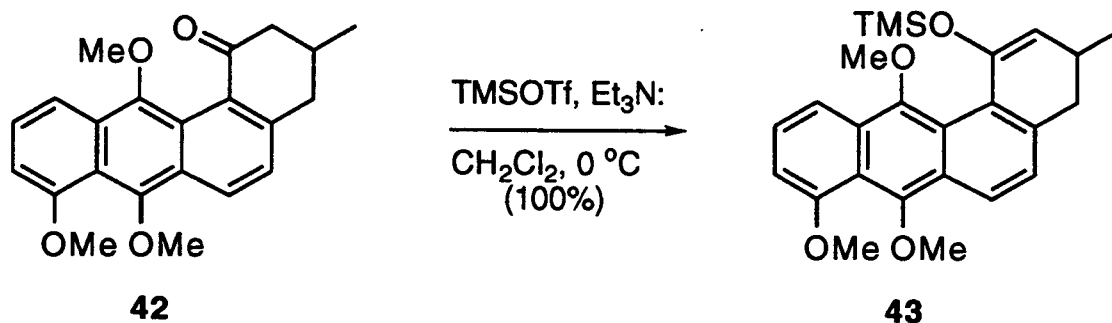
Although **24** could not be reductively methylated, presumably due to steric congestion at the hydroquinone oxidation level, a way to circumvent this problem was found. Because the D ring of ochromycinone, **22**, is not rigid, the substituents on C1 and C12 have a way to relieve some of their steric interaction. Consistent with this view, **22** was reductively methylated affording compound **42** in 100% yield.

Unfortunately, the aromatization conditions used previously^(19,18) caused the deprotection of tri-O-methylochromycinone **42** to X-14881C, **23**, and X-14881E, **29**. Attempted brominations with pyridinium bromide perbromide or N-bromosuccinimide similarly deprotected the quinone. Reaction of **42** with DDQ in methanol gave **23**, but in dioxane **29** was formed (both in poor yield). When **42** was treated with DDQ and TMSOTf, **29** (ca 10%) was formed. However, when the

trimethylsilylenolether of **42** was treated with DDQ no recognizable products were formed.



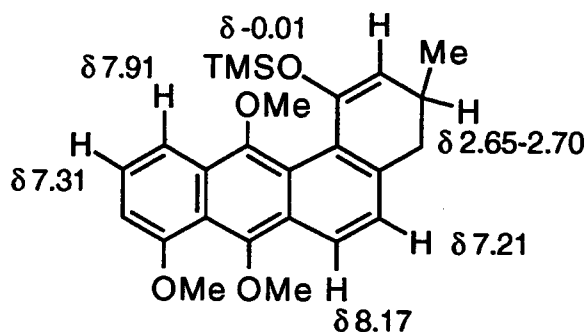
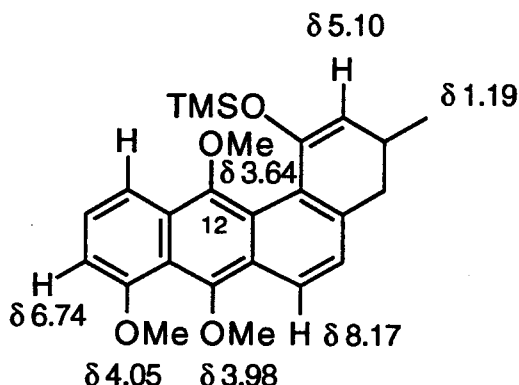
To circumvent the problems with bromination, the trimethylsilyl enolether **43** was prepared. The reaction was efficient, but had to be imaged by alumina TLC, as silica gel caused a rapid deprotection. Isolation also proved problematic, with gel filtration (1 inch support, CH_2Cl_2 , $R_f \sim 0.8$) typically causing some deprotection.

SupportRatio 20:19(by ¹H NMR)

florisil®	>95:5
alumina (neutral)	30:70
alumina (basic)	10:90
alumina (basic/wet w/ 5% MeOH in CH ₂ Cl ₂)	<5:95

By using a non-aqueous workup with filtration through florisil, spectroscopically pure material was obtained. In the ¹H NMR spectrum of **43**, an unusually high field resonance for one -OMe (δ 3.64 ppm) was observed. To ascertain if steric congestion was responsible for the anomalous shift, a nuclear Overhauser effect difference experiment was performed. This showed that indeed the upfield methyl resonance was the C12-OMe. Additionally enhancements served as a fortuitous proof of regiochemistry from the Diels-Alder cycloaddition, assigned the remaining -OMe resonances, and showed that the C12 -OMe and

the C2 enolether proton (δ 5.10 ppm) were remarkably close to each other.



Irradiated:

Observed:

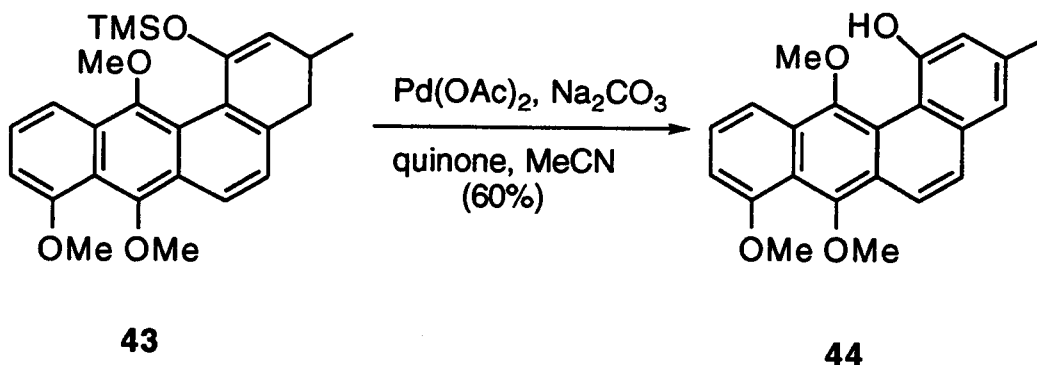
4.05	→	6.74 d (5.5%)
3.98	→	8.17 d (2.1%)
3.64	→	7.91 d (2.0%)
	→	5.10 d (0.4%)
	→	-0.01 s (2.3%)

Irradiated:

Observed:

8.17	→	7.21 d (10.6%)
	→	3.98 s (5.1%)
7.91	→	7.31 t (12.6%)
	→	3.64 s (3.5%)
5.10	→	2.67 m (3.5%)
	→	3.64 s (1.2%)
	→	1.19 d (3.7%)
	→	-0.01 s (7.7%)

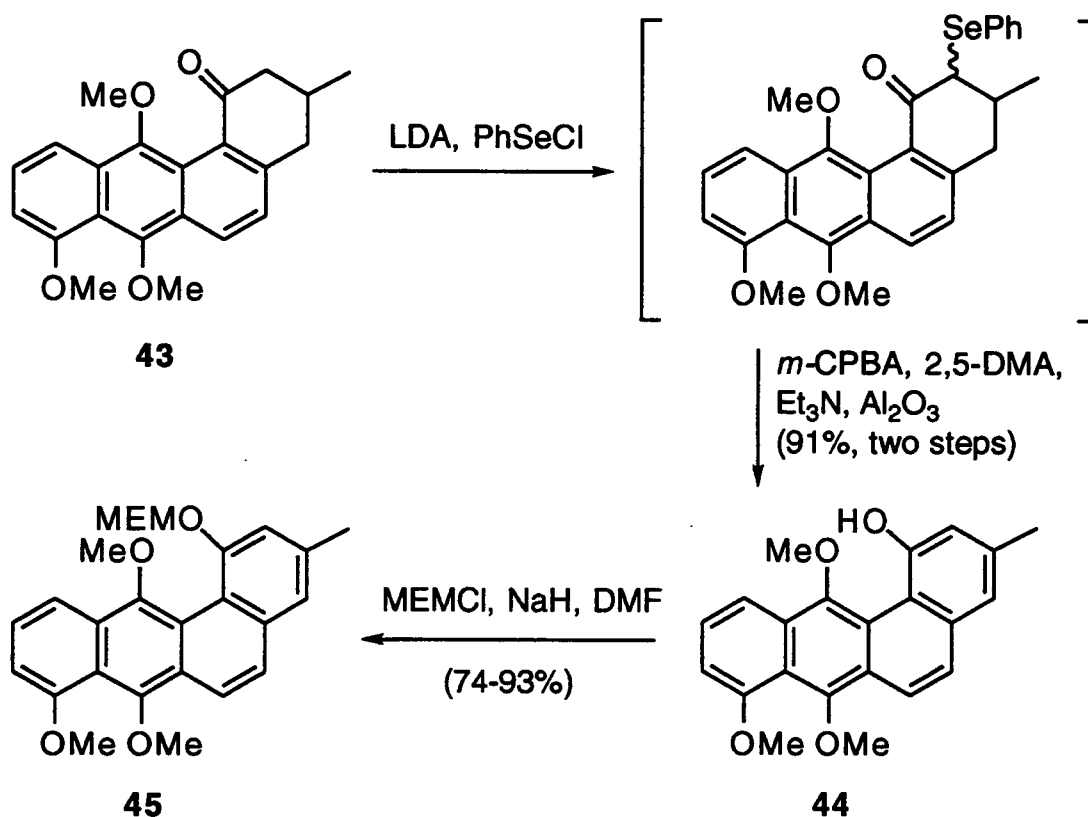
Silylenolether **43** reacted with DDQ(26) at room temperature, which gave mostly deprotected quinone **23**. Similarly, **43** reacted instantly with N-bromosuccinimide in THF to give a mixture of quinones that dehydrohalogenated to afford **29**. Finally, **43** was reacted with palladium acetate under Saegusa conditions(27) which provided a mixture containing desired phenol **44**. This reaction, however, gave a complex mixture of reaction byproducts, so an alternative route was investigated.



Phenyl selenyl chloride was found to efficiently selenate the lithium enolate of **43**. This was oxidatively eliminated to **44** in the presence of an aniline. The 2,5-dimethoxyaniline undergoes electrophilic aromatic selenation by the selenic acid byproduct of the reaction preferentially compared to **44**. This two step sequence gave a 91% overall yield, and large colorless crystals were obtained by recrystallisation from ethanol. Steric congestion between the substituents on C1 and C12 may cause this molecule to be twisted, and therefore chiral. This could readily be checked by determining the crystal structure of one of these compounds.

To perform the oxidative chemistry the C1 phenol had to be protected, so 2-(trimethylsilyl)ethoxymethyl chloride (SEMCl) was chosen first due to the greater stability usually observed for SEM derivatives over other methylenealkoxy protecting groups. Reaction in the presence of Hünig's Base and

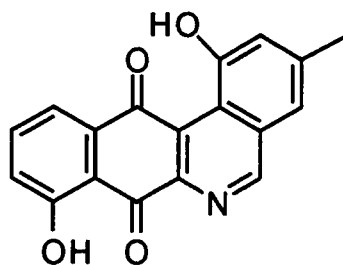
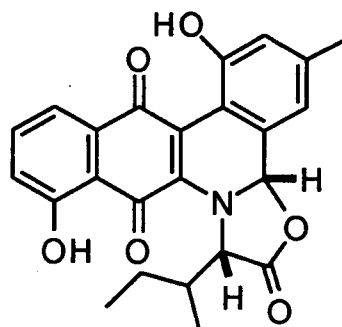
tetrabutylammonium iodide gave no reaction. To take advantage of the higher reactivity of the phenoxide, the reaction was repeated using sodium hydride in THF and gave clean conversion to a compound that fluoresces (turquoise) when exposed to long wave UV light. The remaining sodium hydride was quenched with methanol, and the product gel filtered through silica giving only recovered starting material. Taking this as an indication that SEM may be undergoing a steric relief accelerated solvolysis, benzylation was next tried. Under phase transfer conditions, 4-nitrobenzyl bromide gave no reaction. The reaction of 2,6-difluorobenzylbromide using silver borofluorate activation was similarly unsuccessful. Compound **44** could, however, be protected as its OMEM derivative, **45**, by reaction with MEMCl and sodium hydride in DMF.

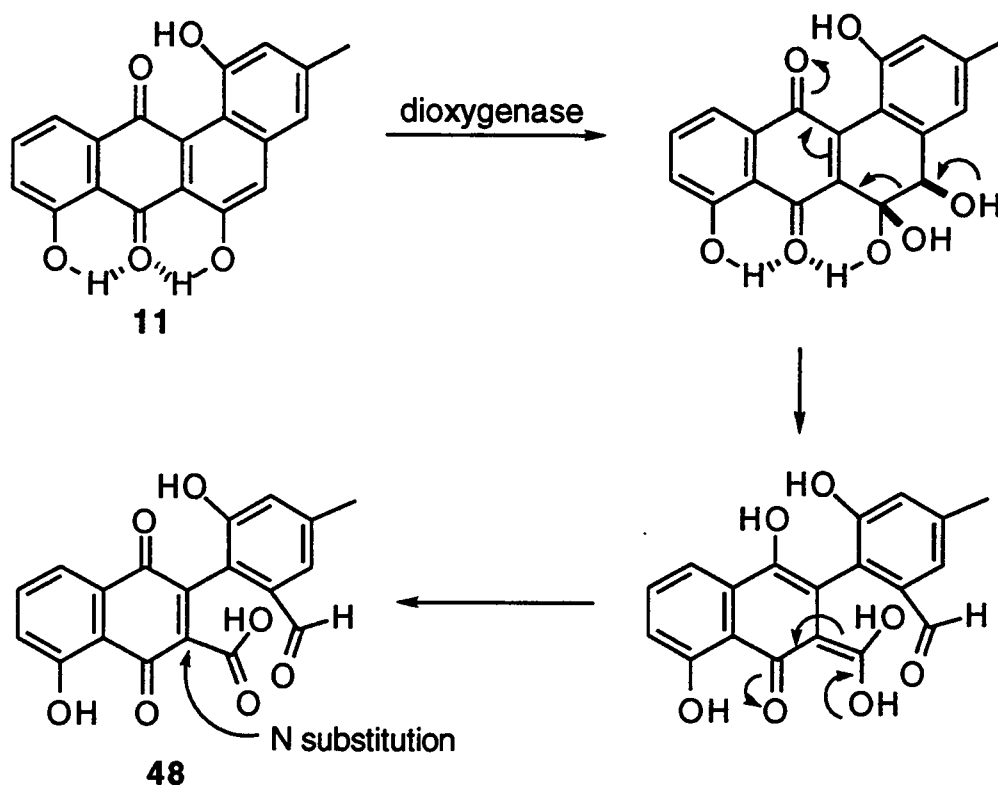


A Re-evaluation of the Synthetic Targets

Reports of the isolations of jadomycin A, **46**,⁽²⁸⁾ (and subsequently jadomycin B⁽²⁹⁾), phenanthroviridin, and its aglycone, **47**,^(30,31) stimulated a rethinking of the kinamycin biosynthesis. These compounds are expected to have been biosynthetically derived from **11**. However, the carbon atoms expected to have been derived from C5 of **11** are at the aldehyde rather than the carboxylic acid oxidation level. This suggested the possibility that rather than cleavage of the C ring of **11** by the action of two sequential oxidations introducing one oxygen each,

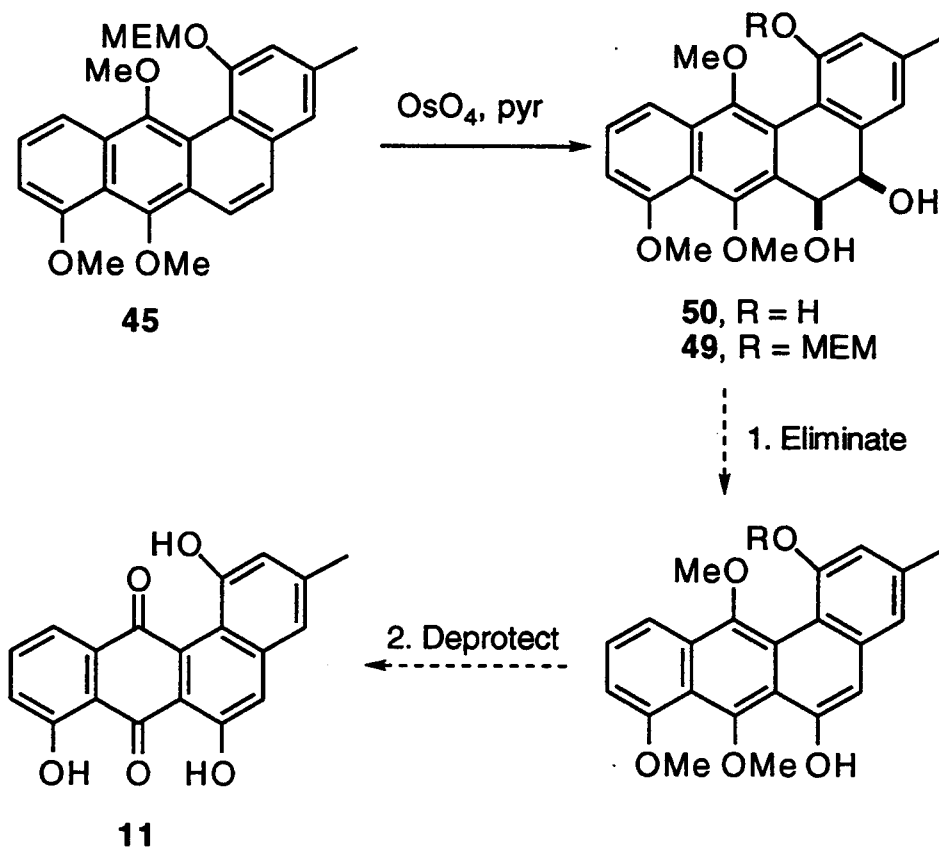
it may instead be cleaved by a dioxygenase,(32) in a single enzyme catalyzed step. A possible mechanism for such a cleavage beginning with the cis-dihydroxylated **11** is presented below. Consistent with this interpretation the newly favored hypothesis is for **11** to be oxidized to an acid aldehyde, rather than the catechol and diacid targets of this synthesis. For this reason, the targets of this work were changed to the synthesis of **11** itself, and the preparation of compounds to benefit the work on the biosynthesis of a related benz[a]anthraquinone from another *Streptomyces*.

**47****46**



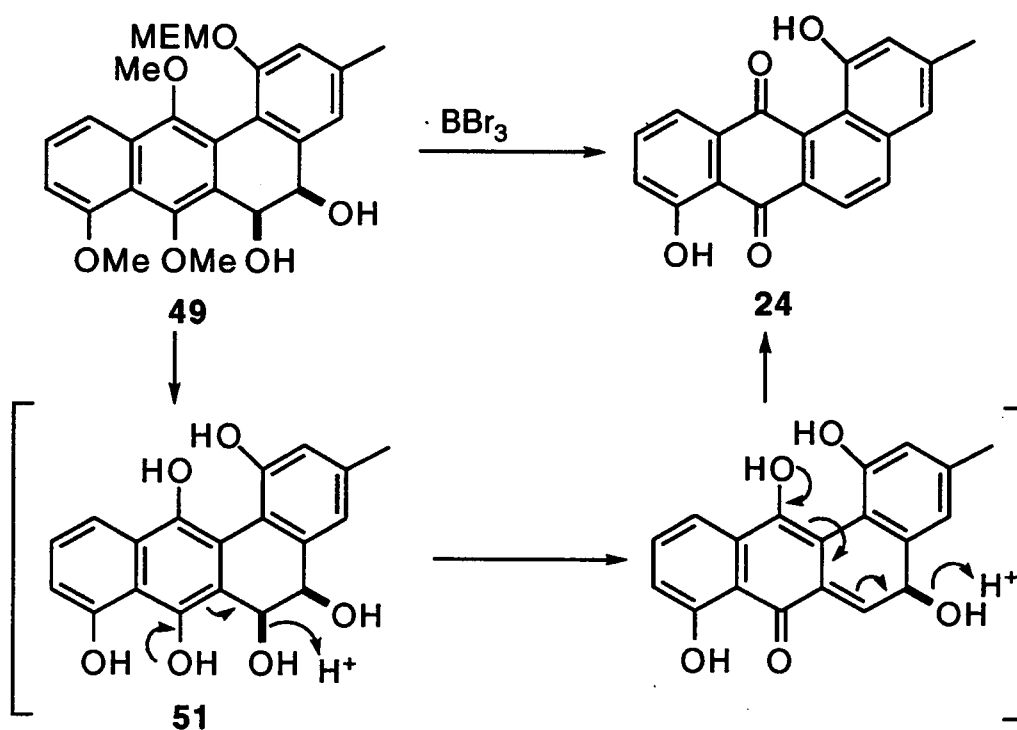
A synthesis of **11** from **45** requires an oxidation at C6. A C5-C6 epoxidation, if successful, would be expected to provide upon solvolysis the wrong regiochemistry of oxygen substitution,(33) affording an isomer of **11**. Therefore, a dihydroxylation would still be the desired means of introducing the oxygen. In the approach shown below, a subsequent elimination of the C5 hydroxyl would provide access to **11**. In the event, osmylation(13) of **45** gave two K region diols, the desired compound **49**, and the C1-deprotected phenol, compound **50**. Since the phenol **50** would not likely survive the osmylation conditions, it was probably formed during

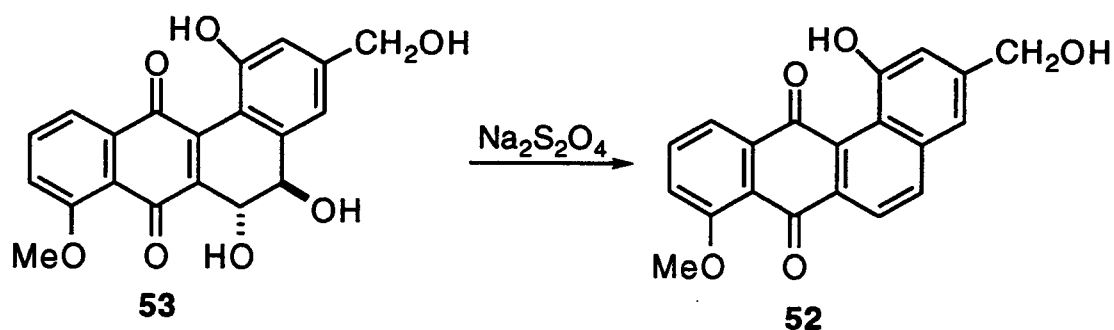
workup with aqueous bisulfite (pH ~4.0). A modified workup procedure would be expected to give an improved selectivity.



In an attempt to affect both the elimination and deprotection in a single synthetic step, compound **49** was treated with boron tribromide. Surprisingly, both K-region hydroxyl groups were eliminated, providing **24** as the only significant product. This was rationalized by a two step elimination from the hydroquinone **51**. This scenario is in essence a transfer of the oxidation of the K-region carbon atoms to the hydroquinone. Although unexpected,

this reaction had precedence in the reaction of antibiotic PD116740 - in hot, basic methanol - which gave 13-hydroxy-X-14881E, **52**.(34) No mechanistic rationalization was given for the latter reaction, so to test the possibility of a hydroquinone intermediate leading from PD116740, **53**, to **52**, the former was treated with sodium dithionite. This provided **52** without heating, or the addition of base, which lends credence to this mechanistic rationale.





Tetrangulol was deuterium-exchanged at C2 and C4 by electrophilic exchange in trifluoroacetic acid.⁽¹⁾ At 110-120 °C, 36 h, a 98% yield was obtained (97% ²H per position by EIMS), at 145-150 °C, 18 h, a 93% yield was obtained (99% ²H per position by ¹H NMR). Higher temperatures, or use of trifluoromethanesulfonic acid resulted in substantial decomposition of **24**. A former worker in our group, Dr. Cheng, found that deuteriated **24** was incorporated into **53**. Since compound **29** is produced by another *Streptomyces*, it can reasonably be anticipated that it is the next intermediate in the biosynthetic pathway leading to **53**, and may well be followed in the biosynthetic sequence by **52**.

Experimental

Materials and Methods. THF was distilled from sodium-benzophenone ketal, and acetonitrile was distilled from basic Al₂O₃ or was of HPLC grade. CH₂Cl₂ and toluene were distilled

from calcium hydride. Methanol for anhydrous reactions was first distilled from sodium, then from 4 Å molecular sieves. Ammonia, triethylamine, and N-methylmorpholine were distilled from sodium, trifluoroacetic acid from phosphorus pentoxide. Acetylene was purified by bubbling through concentrated sulfuric acid, then passing over KOH. *meta*-Chloroperoxybenzoic acid was washed, as a solution in Et₂O, with phosphate buffer (0.100 M, pH 7.0), dried over Na₂SO₄, and the solvent removed *in vacuo*. Lithium diisopropylamide was supplied as a 1.50 M solution of its THF complex in cyclohexane, purchased from Aldrich and used as received. All other chemicals were reagent or HPLC grade and used as received, with the exception of juglone which was twice recrystallized from hexane.

All reactions were monitored by thin-layer chromatography using 0.25 mm E. Merck silica gel plates. Flash chromatography was performed using silica gel-60 (particle size 0.040-0.063 mm) supplied by E. Merck. The purities of all products were $\geq 95\%$ based on ¹H NMR analysis. Sonication was accomplished with a Branson 2200 benchtop bath. All distillations save the Kugelrohr were performed under an argon atmosphere, and the Kugelrohr distillation was performed with a Büchi GKR-50 apparatus.

Removal of solvent refers to the use of a rotary evaporator at water aspirator pressures. Residual solvent was removed under high vacuum at less than 0.1 torr. Reaction flasks, and syringes were oven dried overnight at 120 °C, and cooled in a dessicator

over P₂O₅, or under a stream of argon. Melting points, unless otherwise specified, were measured using a Büchi melting point apparatus and are uncorrected. Elemental analyses were performed by Desert Analytics, Tucson, Arizona.

Carbon and proton nuclear magnetic resonance spectra were obtained using either a Bruker AC-300 or Bruker AM-400 spectrometer. Chemical shifts are reported in parts per million downfield from tetramethylsilane (0.00 ppm), using the δ scale, and the coupling constants are reported in Hertz. Electron impact mass spectra were determined using a Varian MAT311 spectrometer. Chemical ionization mass spectra were obtained with a Finnigan 4023 spectrometer, and fast atom bombardment mass spectra were determined with a Kratos MS-50 RFTC mass spectrometer. IR spectra were recorded on a Nicolet 5DXB FTIR spectrometer.

3-Ethoxy-5-methylcyclohex-2-en-1-one (26).(15) To 5-methyl-1,3-cyclohexanedione, **27**, (15.0 g) in benzene (78 mL) was added *para*-toluenesulfonic acid (0.654 g), and absolute ethanol (22 mL). To this was attached a Soxhlet extractor charged with Drierite brand calcium sulfate (36 g), and the mixture was heated at reflux for 28 h. At this time the vapor temperature had climbed to 78 °C, so the mixture was allowed to cool. 2,6-Di-*tert*-butyl-4-methylphenol (15 mg) was added and the mixture was washed sequentially with 10% sodium hydroxide in

saturated brine (4 x 30 mL), water (3 x 15 mL), and brine (1 x 15 mL). After drying over sodium sulfate, filtration using an ether rinse (40 mL), and evaporation of solvent gave a faintly yellow oil. Simple distillation gave 16.4 g 3-ethoxy-5-methylcyclohex-2-en-1-one (89%), as a transparent oil: bp 64-76 °C, 7 µm, lit. unreported; UV (EtOH) λ_{max} 248 (ϵ 16000) nm; IR (KBr) 2958.3, 2898.2, 2877.4, 1653.6, 1602.4, 1380.3, 1214.8; COSY CMI44; ^1H NMR (CDCl_3) δ 1.01 (d, 3H), 1.27 (t, 3H), 1.98 (m, 2H), 2.14 (m, 1H), 2.34 (m, 2H), 3.83 (ddd, 2H), 5.28 (s, 1H); ^{13}C NMR (CDCl_3) δ 199.7, 177.2, 102.2, 64.1, 45.0, 37.1, 28.7, 20.8, 14.0; LREIMS m/z (rel. intensity) 154.1 (M^+ , 56%), 139.1 (M^+ -methyl, 2%), 112.0 (M^+ -propene, 100%), 67.0 (112-ethanol, 7%).

3-Ethynyl-5-methylcyclohex-2-en-1-one.(35,36) Ammonia (300 mL) was distilled onto lithium wire (50 cm, 3.2 mm diameter, approx. 0.5 mol). The resulting blue solution was agitated with a Hirshberg stirrer under an acetylene atmosphere at -78 °C until the blue color had disappeared. Evaporation of the ammonia at rt over 48 h provided a white solid which was suspended in THF (ca 100 mL). 3-Ethoxy-5-methylcyclohex-2-en-1-one, **26**, (13.1 g) in THF (100 mL) was added over 2 h, while maintaining an acetylene atmosphere. Agitation was accomplished by sonication, and in 3 h total reaction time the bath temperature had risen to 45 °C, and the reaction appeared complete by TLC (20% ethyl acetate in hexane). The mixture was quenched by addition

of sulfuric acid (200 mL, 2.5 N), and the biphasic mixture extracted with dichloromethane (5 x 100 mL). Drying over Na₂SO₄-NaHCO₃ (10:1), addition of 2,6-di-*tert*-butyl-4-methylphenol (50 mg), and evaporation provided a brown oil which was distilled to give 5.67 g of 3-ethynyl-5-methylcyclohex-2-en-1-one (50%). In a separate experiment, an analytical sample was separated by chromatography (20% ethyl acetate in hexane, silica gel) rather than distillation. This gave a higher overall yield than did distillation: bp 61 °C, 5 µm, lit. unreported; IR (NaCl) 2871, 2094, 1662, 1589 cm⁻¹; UV (EtOH) λ_{max} 250 (ε 8500); ¹H NMR (CDCl₃) δ 6.28 (s, 2H), 3.56 (s, 1H), 2.52 (m, 1H), 2.19 (m, 3H), 1.10 (d, 3H); ¹³C NMR (CDCl₃) δ 199.0, 141.5, 133.6, 87.1, 82.5, 45.4, 38.2, 30.0, 20.9.

E,E-3-(2-Methoxyethenyl)-5-methylcyclohexenone (28).(16)

To a solution of 3-ethynyl-5-methylcyclohex-2-en-1-one (3.83 g, 28.5 mmol) in dry CH₂Cl₂ (285 mL) was added MeOH (46.2 mL, 36.5 g, 1.14 mol, 40 eq) followed by N-methylmorpholine (3.13 mL, 2.88 g, 28.5 mmol, 1 eq), and 2,6-di-*tert*-butyl-4-methylphenol (50 mg). The reaction mixture was protected from light, blanketed with argon, and stirred at 22 °C for 24 h at which time it had turned dark red. Washing with water (2 x 100 mL), and drying over Na₂SO₄ gave a solution which was loaded without concentration onto a silica gel column (4 x 10 cm) packed in CH₂Cl₂. Elution with CH₂Cl₂-EtOAc (19:1), and the removal of

solvent afforded 4.07 g E,E-3-(2-methoxyethenyl)-5-methylcyclohexenone, **28**, (86%) as a colorless oil: IR (KBr) 2956.9, 2875.8, 1617.4, 1301.9, 1234.7, 1158.4, 1131.0 cm^{-1} ; ^1H NMR (CDCl_3) δ 1.10 (d, 3H, $J = 6.4$), 2.06 (m, 2H), 2.20 (m, 1H), 2.46-2.56 (m, 2H), 3.71 (s, 3H), 5.64 (d, 1H, $J = 12.9$), 5.84 (d, 1H, $J = 1.6$), 7.04 (d, 1H, $J = 12.9$); ^{13}C NMR (CDCl_3) δ 199.7, 156.3, 154.3, 123.8, 106.9, 57.1, 45.7, 33.6, 29.8, 21.3; LREIMS m/z (rel. intensity) 166.1 (M^+ , 14%), 151.1 ($\text{M}^+ - \text{CH}_3$, 3%), 124.0 ($\text{M}^+ - \text{propene}$, 24%), 112.1 ($\text{M}^+ - \text{isobutyl}$, 16%), 67.0 (124-methylvinyl ether radical, 17%).

Boron triacetate.(17) Boric acid (1.66 g) and acetic anhydride (12.6 mL) were stirred for 1 h at 26 °C. The resulting mixture was heated, and became homogeneous before reaching reflux. After heating at reflux for 0.2 h, the volatile materials were removed *in vacuo*, and the resulting white solid was dissolved in anhydrous CH_2Cl_2 (~25 mL). This solution was transferred by cannula to a bottle with a septum closure, maintaining an atmosphere of argon, which gave the stock solution. The catalyst concentration (40.3 mg/mL) was found by removing the solvent from a sample of the stock solution (1.00 mL) *in vacuo*, and weighing the solid. This solution was kept in a desiccator at -20 °C: mp dec. ~85 °C, lit. unreported; ^1H NMR (CDCl_3) δ 2.23 (s, 3H); ^{13}C NMR (CDCl_3) δ 178.3, 22.5. IR and MS failed due to sample hydrolysis.

Ochromycinone (22).(5) A solution of E,E-3-(2-methoxyethenyl)-5-methylcyclohexenone, **28**, (4.07 g), and juglone (6.30 g) in CH₂Cl₂ (150 mL) was treated with a suspension of boron triacetate(17) (11.0 g, 58.5 mmol, 1.62 eq/juglone) in CH₂Cl₂ (60 mL) over 6 h. After an additional 20 h of stirring, MeOH (17.3 mL) was added, and the mixture was filtered (celite on sintered glass). The solid obtained from removal of the solvent *in vacuo* was loaded onto a column (5 x 7 cm, silica gel) packed in CH₂Cl₂, and eluted with CH₂Cl₂-acetone (1:1). This gave after concentration a black paste, which was further chromatographed (5 x 20 cm, silica gel), eluted with CH₂Cl₂, to give 2.45 g **22** after recrystallisation (EtOAc-hexanes). The mother liquor and mixed fractions were rechromatographed similarly to afford an additional 0.960 g after recrystallisation, for a total of 3.41 g **22** (46%) as yellow plates: mp 173.4-173.8 °C, lit. 152-153 °C, 168-169 °C(6); IR (KBr) 2956.2, 1703.4, 1635.1, 1452.4, 1363.2, 1280.2, 1217.4, cm⁻¹; ¹H NMR (CDCl₃) δ 12.29 (s, 1H), 8.28 (d, 2H, J = 8.0), 7.66-7.69 (m, 2H), 7.55 (d, 1H, J = 8.2), 7.25-7.28 (m, 1H), 2.97-3.07 (m, 2H), 2.53-2.73 (m, 2H), 2.45-2.49 (m, 1H), 1.21 (s, 3H); ¹³C NMR (CDCl₃) δ 199.2, 187.5, 183.0, 162.0, 150.4, 137.0, 136.6, 135.9, 135.0, 133.4, 133.0, 128.9, 123.6, 119.6, 115.4, 47.5, 38.4, 30.8, 21.5; LREIMS *m/z* (rel. intensity) 306.3 (M⁺, 64%), 278.3 (29%), 264.2 (100%).

2-Bromoochromycinone.(37,19) To **22** (1.36 g, 4.45 mmol), and cupric bromide (1.59 g, dried at rt *in vacuo*) was added a mixture of CHCl_3 and EtOAc (1:1, 150 mL, dried over CaSO_4). The mixture was heated at reflux under an atmosphere of argon until the black color of the cupric bromide turned to a light gray (4 h), filtered, and evaporated. Chromatography on a silica column (5 cm, 150 g silica) with toluene- CH_2Cl_2 (1:4) eluent (fractions 1-30, 20 mL each, from the beginning of the first colored band) yielded after solvent removal 1.29 g of 2-bromoochromycinone (75%) as a yellow solid (an inseparable 5:1 mixture of trans to cis diastereomers). Fractions 48-59 contained recovered ochromycinone, 0.174 g, so the yield corrected for conversion is 86%: mp 210.0-211.4 °C, decomposed to tetrangulol; IR (KBr) 1710.7, 1676.0, 1635.5, 1591.7, 1278.1 cm^{-1} ; (major isomer) ^1H NMR (CDCl_3) δ 12.25 (s, 1H), 8.29 (d, 1H, $J = 8.1$), 7.69-7.77 (m, 2H), 7.50 (d, 1H, $J = 8.2$), 7.31 (d, 1H, $J = 8.0$), 4.58 (d, 1H, $J = 2.4$), 2.98 (dd, 2H, $J = 8.0, 2.0$), 2.57 (m, 1H), 1.27 (d, 3H, $J = 6.4$); ^{13}C NMR (CDCl_3) δ 192.1, 187.2, 182.1, 161.9, 148.4, 137.1, 134.8, 133.9, 133.6, 133.0, 129.5, 129.3, 123.6, 119.6, 115.2, 59.0, 35.4, 34.4, 18.8; HRMS (EI) m/z (M^+) calculated ($\text{C}_{19}\text{H}_{13}\text{BrO}_4$) 383.9998, found 383.9997.

Tetrangulol (24). method a.(13) In a 1 dram vial with a Teflon lined cap a solution of OsO_4 (3.5 mg) in dry pyridine (35 μL) was added to a solution of **45** (5.1 mg) in pyridine (100 μL).

This was stirred for 24 h, then aqueous sodium bisulfate (0.5 mL, 10% wt/vol) was added, and the mixture was extracted with Et₂O (3 x 2 mL). Chromatography on a silica gel column (1 x 13 cm) developed with EtOAc-hexanes (4:1) provided 1.0 mg phenol **50** (22%), followed by 2.5 mg **49** (45%). A portion of **49** (1.8 mg) was dissolved in CH₂Cl₂ (0.2 mL), cooled in a dry ice-acetone bath, and BBr₃ (0.20 mL, 1.0 M soln in CH₂Cl₂) was added. The cooling bath was maintained for 4 h, and then the reaction mixture was stirred for 16 h at rt. MeOH (50 mL) was added, and the mixture was partitioned between H₂O and CH₂Cl₂. Drying the organic layer over Na₂SO₄, and evaporation gave 0.8 mg **24** (69%) as a brown solid.

Method b. A solution of 2-bromoochromycinone (1.23 g, 3.19 mmol) in DMF (40 mL) was added to a hot (135 °C), stirred suspension of LiBr (5.54 g), and Li₂CO₃ (4.72 g) in DMF (140 mL) over 0.25 h.^(18,38) The reaction was cooled to room temperature, filtered (celite on sintered glass), concentrated to about 50 mL, and diluted with toluene (150 mL). This solution was gel filtered (silica, 5 x 5 cm column) with additional toluene to complete transfer. Removal of the solvent *in vacuo* gave 1.00 g **24** (100%) as purple/brown needles. All spectral data were consistent with those reported:^(9,8) mp 198.2-200.8 °C, lit. 201-203 °C; IR (KBr) 3428.2, 2923.3, 1631.4, 1617.7, 1381.8, 1259.9, 787.9 cm⁻¹; UV (HPLC, R.T. 23.57 min) λ_{max} 226, 316, 430 nm; ¹H NMR (CDCl₃) δ 2.50 (s, 3H), 7.14 (s, 1H), 7.26 (s, 1H), 7.33

(dd, 1H, $J = 8.4, 0.9$), 7.69 (t, 1H, $J = 8.0$), 7.85 (dd, 1H, $J = 6.4, 0.8$), 8.12 (d, 1H, $J = 8.7$), 8.30 (d, 1H, $J = 8.5$), 11.28 (s, 1H), 12.26 (s, 1H); ^{13}C NMR (CDCl_3) δ 189.6, 187.8, 161.6, 155.2, 142.0, 139.1, 137.7,† 136.9, 134.7, 132.3, 124.7, 121.9, 121.2,† 120.2, 120.0, 114.5, 21.3; LREIMS m/z (rel. intensity) 304.3 (100%), 287.2 (5%), 189.2 (11%), 94.6 (12%); HREIMS m/z (M^+) calculated ($\text{C}_{19}\text{H}_{12}\text{O}_4$) 304.07355, found 304.07355.

† Signal was resolved into two lines with a 100 MHz magnetic field.

2,4- ^2H -Tetrangulol.(1) Trifluoroacetic anhydride (12.4 g), and deuterium oxide (1.34 g) were stirred together at 0 °C for 1 h to give trifluoroacetic acid- d . To **24** (50.3 mg) in a sealable tube was added the trifluoroacetic acid- d (2.00 mL), the suspension was frozen (dry ice-acetone), evacuated, and sealed. After heating in an oil bath (110-120 °C) for 1.5 d, the trifluoroacetic acid- d was removed *in vacuo*, and the remaining brown solid filtered through a silica gel plug (1 x 2.5 cm) eluting with CHCl_3 . Evaporation gave 49.4 mg of the title compound (98%) pure, as purple needles. ^2H NMR and EIMS showed 97% ^2H per exchanged position: mp 201.6-202.8 °C; IR (KBr) 1634.0, 1457.5, 1269.4, 1254.7 cm^{-1} ; ^1H NMR (CDCl_3) δ 2.50 (s, 3H), 7.33 (dd, 1H, $J = 8.4, 0.9$), 7.69 (t, 1H, $J = 8.0$), 7.85 (dd, 1H, $J = 6.4, 0.8$), 8.12 (d, 1H, $J = 8.7$), 8.28 (d, 1H, $J = 8.5$), 11.26 (s, 1H), 12.23 (s, 1H); ^2H NMR (61.4 MHz, dioxane- H_8) δ 7.12 (s, 1 ^2H), 7.31 (s, 1

^2H); ^{13}C NMR (CDCl_3) δ 189.5, 187.7, 161.6, 155.2, 141.8, 139.0, 137.5, 136.8, 134.7, 132.2, 124.7, 121.8, 121.2, 121.1 (t, J = 26.6), 120.1 (t, J = 23.5), 119.9, 114.5, 21.1; LREIMS m/z (rel. intensity) 306.3 (100%), 305.3 (6%), 278.3 (30%), 220.2 (45%); HREIMS m/z calculated for $(\text{C}_{19}\text{H}_{10}^2\text{H}_2\text{O}_4)$ 306.0861, found 306.0861.

X-14881C (23).(39,6) To **22** (36.0 mg) was added K_2CO_3 (184.6 mg), acetone (15.0 mL), and dimethyl sulfate (200 μL). Heating this suspension at reflux for 2 h was followed by addition of 10% sodium hydroxide in saturated brine (1.00 mL), and stirring was continued for 0.5 h at rt. After removal of the acetone *in vacuo*, the mixture was neutralized with solid NH_4Cl , and extracted with CH_2Cl_2 (2 x 20 mL). Silica gel chromatography (1.5 x 22 cm) eluted with EtOAc-hexane (60:40) gave 35.0 mg **23** (93%) as a yellow solid: mp 235.0-236.2 $^\circ\text{C}$, lit(39) 235 $^\circ\text{C}$ dec.; IR 1701.8, 1674.1, 1589.4, 1268.2 cm^{-1} ; UV (HPLC) λ_{max} 200 (sh), 266, 380 nm; ^1H NMR (CDCl_3) δ 8.26 (d, 1H, J = 6.0), 7.77 (d, 1H, J = 5.9), 7.71 (dd, 1H, J = 6.0, 6.0), 7.51 (d, 1H, J = 6.1), 7.30 (d, 1H, J = 6.1), 4.05 (s, 3H), 3.00-3.02 (m, 1H), 2.96-2.98 (m, 1H), 2.65-2.71 (m, 1H), 2.52-2.59 (m, 1H), 2.42-2.50 (m, 1H), 1.20 (d, 3H, J = 4.9); LREIMS m/z 320.3 (M^+ , 100%), 305.3 (M^+ -methyl, 18%), 164.2 (M^+ - $\text{C}_{11}\text{H}_8\text{O}$, 31%); HREIMS m/z calcd for $\text{C}_{20}\text{H}_{16}\text{O}_4$ 320.1049 (M^+), found 320.1048.

X-14881E (29).(19,37,38) To **23** (32.1 mg), and powdered cupric bromide (40.0 mg) was added EtOAc (10.0 mL) and CHCl₃ (10.0 mL). This mixture was heated at reflux for 3 h, a gas was evolved, and the color of the solids changed from black to light gray. Filtration through celite gave a yellow solution which was evaporated onto silica gel (0.8 g). The mixture was placed onto a silica gel column (1.5 x 19 cm), and eluted with EtOAc-CH₂Cl₂ (1:20), which gave after evaporation 34.1 mg mixed halogenated compounds (6:3:1 trans:cis:dibromo with respect to the C3 methyl by ¹H NMR) as a yellow solid, followed by 6.0 mg of recovered **23**. The mixed halogenation products were dissolved in DMF (2.0 mL), and added over 0.5 h to a 130-135 °C stirred suspension of LiBr (181.0 mg), and Li₂CO₃ (203 mg) in DMF (25.0 mL). Upon complete addition, this material was filtered through celite and the solvent was removed *in vacuo*. The resulting solid was chromatographed on a silica gel column (1.5 x 20 cm), eluted with CH₂Cl₂, which afforded 21.4 mg **29** (78%) as a brown solid: mp 206.5-209.5 °C, lit.(39,20) unreported; IR (KBr) 1660.0, 1574.6, 1295.0, 1268.5, 1174.5 cm⁻¹; UV (HPLC) λ_{max} 226, 310, 410 nm; ¹H NMR (CDCl₃) δ 11.16 (s, 1H), 8.17 (d, 1H, J = 8.7), 8.11 (d, 1H, J = 8.7), 7.93 (dd, 1H, J = 7.6, 0.8), 7.73 (dd, 1H, J = 7.8, 7.8), 7.35 (dd, 1H, J = 8.11, 0.7), 7.25 (s, 1H), 7.12 (d, 1H, J = 1.8), 4.07 (s, 3H), 2.48 (s, 3H); ¹³C NMR (CDCl₃) δ 190.6, 182.1, 159.4, 154.9, 141.0, 138.2, 137.5, 137.2, 136.6, 135.2, 130.5, 122.7, 121.0, 120.9, 119.7, 119.6, 119.0, 118.0, 56.5, 21.1; EIMS

m/z (relative intensity) 318.1 (M^+ , 100%), 189.1 (17%); HREIMS *m/z* calcd for $C_{20}H_{14}O_4$ 318.0892 (M^+), found 318.0892.

13-Hydroxy-X-14881E (52). $Na_2S_2O_4$ (5.1 mg) was dissolved in degassed H_2O (2.00 mL), and then added by syringe to a suspension of **53** (4.9 mg) in CH_2Cl_2 (5.00 mL). After 16 h, the layers were separated, and the CH_2Cl_2 layer was washed with H_2O (1.0 mL), then discarded. The combined aqueous extracts were acidified with HCl (1.0 mL, 1.00M) and stirred for 16h, open to the air. EtOAc (2 x 4.0 mL) extraction provided an organic layer that was dried over Na_2SO_4 , and evaporated *in vacuo* to afford 1.2 mg **52** (27%) as a yellow solid with a 1H NMR spectrum, and UV λ_{max} identical with those reported previously.(34)

7,12-Dimethoxybenz[*a*]anthracene (31).(11) Powdered sodium dithionite (3.75 g), **30** (3.06 g), and potassium carbonate (32.7 g) were combined in a 250 mL flask. Dimethyl sulfate (22.4 mL) was added, followed by acetone (80 mL), and the suspension was heated at reflux for 26 h. H_2O (2.0 mL) was added, and heating continued for an additional 2 h. The mixture was poured into H_2O , extracted with EtOAc, and the organic layer was dried over Na_2SO_4 . The solvent was removed *in vacuo*, and the resulting solid was recrystallised from EtOAc-hexanes which gave 3.05 g **31** (89%) as pale yellow prisms: mp 137.2-139.0 °C; IR (KBr), 1450.6, 1359.9, 1064.1, 998.3, 749.1 cm^{-1} ; 1H NMR

(CDCl₃) δ 9.66 (d, 1H, J = 8.5), 8.43 (dd, 1H, J = 3.0, 5.4), 8.32 (dd, 1H, J = 2.3, 7.3), 8.11 (d, 1H, J = 9.3), 7.79 (d, 1H, J = 7.6), 7.66 (dd, 1H, J = 8.1, 8.1), 7.56-7.60 (m, 4H), 4.06 (s, 3H), 3.94 (s, 3H); ¹³C NMR (CDCl₃) δ 151.1, 148.6, 132.6, 129.9, 128.4, 128.2, 127.6, 127.3, 127.0, 126.0, 125.9, 124.2, 123.1, 122.2, 121.0, 120.7, 63.1, 60.8 (two ¹³C resonances are coincidental) EIMS m/z (relative intensity) 288.2 (M⁺, 69%), 273.2 (100%), 258.1 (19%); HREIMS m/z calcd for C₂₀H₁₆O₂ 288.1150 (M⁺), found 288.1150.

General Procedure for Catalytic Osmylation of 7,12-Dimethoxybenz[*a*]anthracene (31).(12) In a 1 dram vial with a magnetic spinbar was placed **31** (100 mg), co-oxidant (1.1 eq), H₂O (0.5 mL), THF (0.5 mL), and OsO₄ [5.0 mg (100 μ L of a 5% solution in water)]. The vial was sealed with a Teflon lined cap, and was placed in a 50 mL round bottom flask containing acetone. After heating at the reflux temperature of acetone for 2.5 d, heating was discontinued. A saturated NaHSO₃ solution (1.0 mL) and celite (20 mg) were added, and stirring continued for an additional 1 h. The reaction mixture was diluted with brine, and extracted with EtOAc (20 mL). Drying over Na₂SO₄ and evaporation *in vacuo* gave the mixed oxidation products. For a representative example using NaClO₃ as the co-oxidant the ¹H NMR spectrum showed the following product composition: S.M. **31** (27%), dihydrodiol **32** (30%), *ortho*-quinone **33** (21%), and two

unidentified compounds comprising 22% combined. Separation of the two known oxidation products was accomplished by chromatography on silica gel, eluted with EtOAc-hexanes (3:2).

5,6-Dihydroxy-5,6-dihydro-7,12-

dimethoxybenz[a]anthracene (32). method b.(13) In a 1 dram vial with a Teflon lined cap, compound **31** (100.2 mg) was added to a solution of OsO₄ (90 mg) in pyridine (0.90 mL). This mixture was stirred for 7 d, then aqueous sodium bisulfate (1.0 mL, 10% wt/vol) was added, and the mixture was extracted with Et₂O (3 x 2.0 mL). After drying over Na₂SO₄, filtration, and removal of the solvent this gave 93.2 mg **32** (83%) as a white solid. White needles were obtained from CHCl₃-hexanes, R_f = 0.28 with EtOAc-hexanes (1:1): mp 192.0-192.5 °C; IR (NaCl) 3154.8-3594.1, 1352.4, 1069.4 cm⁻¹; ¹H NMR (CDCl₃) δ 8.57-8.60 (m, 1H), 8.21-8.25 (m, 1H), 8.09-8.13 (m, 1H), 7.78-7.81 (m, 1H), 7.53-7.60 (m, 2H), 5.35 (dd, 1H, J = 6.9, 3.4), 4.87 (dd, 1H, J = 9.4, 3.2), 4.06 (s, 3H), 3.72 (s, 3H), 2.76 (d, 1H, J = 9.7), 2.15 (d, 1H, J = 6.6); D₂O exchanged spectrum δ 8.57-8.60 (m, 1H), 8.21-8.25 (m, 1H), 8.09-8.13 (m, 1H), 7.78-7.81 (m, 1H), 7.53-7.60 (m, 2H), 5.33 (d, 1H, J = 3.4), 4.86 (d, 1H, J = 3.2); ¹³C NMR (CDCl₃) δ 150.9, 150.3, 137.6, 136.2, 130.4, 130.3, 128.4, 128.1, 127.1, 126.8, 125.8, 125.2, 125.1, 123.1, 122.6, 121.0, 71.0, 66.3, 63.9, 61.0; EIMS *m/z* (relative intensity) 322.0 (M⁺, 100%), 307.0 (M⁺-

Me, 29%), 233 (21%); HREIMS m/z calcd for $C_{20}H_{18}O_4$ 322.1205 (M^+), found 322.1205.

5,6-Diketo-7,12-dimethoxybenz[a]anthracene (33).

method **b**. To a solution of CrO_3 (8.0 mg) in pyridine (0.8 mL) was added **32** (11.5 mg). After stirring at rt for 18 h, the mixture was filtered with additional pyridine to complete the transfer. After removal of the pyridine *in vacuo*, chromatographic separation (1 x 15 cm, silica gel) eluted with EtOAc-hexanes (3:2) gave 6.5 mg **33** (57%). Red prisms were obtained from $CHCl_3$ -hexanes, with an R_f = 0.55 in EtOAc-hexane (1:1): mp 114.0-116.0 °C; IR (KBr) 1665.1, 1355.0, 1349.1, 1067.3, 992.3, 787.4 cm^{-1} ; 1H NMR ($CDCl_3$) δ 8.18 (d, 1H, J = 8.9), 8.35 (d, 1H, J = 8.1), 8.23 (d, 1H, J = 8.1), 8.13 (dd, 1H, J = 1.5, 7.8), 7.71-7.78 (m, 2H), 7.64 (ddd, 1H, J = 8.2, 7.2, 1.2), 7.47 (ddd, 1H, J = 7.8, 6.8, 1.00), 4.13 (s, 3H), 3.29 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 183.5, 181.3, 159.4, 151.2, 136.3, 135.7, 133.7, 130.7, 129.7, 129.4, 129.1, 128.8, 128.3, 125.0, 123.4, 121.6, 119.4, 63.8, 61.3; EIMS m/z (relative intensity) 318.3 (M^+ , 100%), 290.3 (18%), 261.2 (33%); HREIMS m/z calcd for $C_{20}H_{14}O_4$ 318.0892 (M^+), found 318.0892.

3-(2-Carboxymethylphenyl)-1,4-dimethoxy-2-naphthoic

Acid (34).(14) Compound **33** (6.5 mg) was dissolved in THF-30% hydrogen peroxide (1:1, 2.0 mL), and stirred at rt for 0.6 d. To this solution was added NaOH (1.0 mL, 1.00 M), and after an

additional 2 h, the mixture was diluted with ether, and extracted into bicarbonate. The bicarbonate solution was acidified with HCl (1.00 M, pH 2-3), re-extracted into ethyl acetate, and evaporated *in vacuo*. The resulting material reacted with ethereal diazomethane, which provided the crude diester which was chromatographed with EtOAc-hexanes (1:4) to afford 2.6 mg **34** (33%) as a gum: IR (KBr) 1732.5, 1354.8, 1294.3, 1070.7 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.15-8.18 (m, 2H), 8.05 (dd, 1H, $J = 1.3, 7.7$), 7.54-7.61 (m, 3H), 7.45-7.50 (ddd, 1H, $J = 1.6, 7.7, 7.7$), 7.39-7.42 (dd, 1H, $J = 1.3, 7.6$), 4.03 (s, 3H), 3.62 (s, 3H), 3.54 (s, 3H), 3.53 (s, 3H). ^{13}C NMR (CDCl_3) δ 167.5, 149.7, 148.8, 136.7, 131.9, 131.5, 131.1, 130.8, 130.3, 129.5, 128.9, 128.4, 127.9, 127.4, 126.6, 124.4, 123.0, 122.9, 63.7, 61.2, 52.0, 51.9; EIMS m/z (relative intensity) 380.4 (M^+ , 100%), 365.4 (24%), 291.2 (28%); HREIMS m/z calcd for $\text{C}_{22}\text{H}_{20}\text{O}_6$ 380.1260 (M^+), found 380.1256.

Tetrangulol Diacetate (35).(1) To **24** (43.7 mg) in Ac_2O (20.0 mL), cooled to 0-5 $^\circ\text{C}$, was added H_2SO_4 (two drops). At 2 h pyridine (100 μL) was added, and after an additional 0.5 h, the mixture was evaporated, then redissolved in EtOAc (20 mL). This solution was washed with brine (2 x 5.0 mL), dried over Na_2SO_4 , and evaporated *in vacuo*. Recrystallisation from EtOAc gave 53.6 mg **35** (96%) as a yellow solid: mp 179.9-183.2 $^\circ\text{C}$, lit.(9) 177-178 $^\circ\text{C}$; IR (KBr) 1772.4, 1668.6, 1365.9, 1280.6, 1198.1 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.22 (d, 1H, $J = 8.7$), 8.05 (d, 1H, $J = 8.7$), 8.02

(dd, 1H, $J = 1.1, 7.8$), 7.79 (dd, 1H, $J = 8.0, 8.0$), 7.59 (s, 1H), 7.39 (dd, 1H, $J = 1.3, 8.2$), 7.34 (s, 1H), 2.57 (s, 3H), 2.51 (s, 3H), 2.38 (s, 3H); ^{13}C NMR (CDCl_3) δ 184.9, 181.3, 169.5, 168.9, 149.5, 147.1, 140.1, 137.9, 137.8, 135.0, 134.2, 133.8, 133.2, 128.7, 125.9, 125.7, 124.4, 123.8, 122.3, 120.9, 21.5, 21.1, 21.1; EIMS m/z (rel. intensity) 388.4 (11%, M^+), 346.3 (60%), 304.3 (100%); HREIMS calcd for $\text{C}_{23}\text{H}_{16}\text{O}_6$ 388.0947, found 388.0946.

Tetrangulol Tetraacetate (36).(9) To 42.3 mg (0.139 mmol) **24**, powdered zinc (255.5 mg), and anhydrous sodium acetate (99.0 mg, fused) was added acetic anhydride (25.0 mL). After heating at reflux for 2 h, the mixture was diluted with toluene (25.0 mL), filtered, and evaporated *in vacuo*. Chromatography (1.5 x 22 cm, silica gel) of the resulting solid with EtOAc- CH_2Cl_2 (1:50) eluent, gave after evaporation 42.6 mg **36** (65%) as a yellow solid: mp 122.4-126.1 °C, lit. unreported; IR (KBr) 1789.7, 12784.1, 1383.2, 1194.5, 1154.5, 1021.0 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.02 (d, 1H, $J = 8.6$), 7.53-7.59 (m, 2H), 7.46 (d, 1H, $J = 9.4$), 7.45 (s, 1H), 7.25 (d, 1H, $J = 6.9$), 2.53 (s, 3H), 2.52 (s, 3H), 2.45 (s, 3H), 2.39 (s, 3H), 2.37 (s, 3H); ^{13}C NMR (CDCl_3) δ 169.4 (two resonances), 169.3, 168.5, 147.7, 145.3, 141.4, 139.0, 137.9, 134.5, 128.7, 128.0, 126.3, 125.5, 124.7, 123.1, 120.9, 120.4, 119.9, 119.6, 118.2, 21.6, 21.3 (two resonances), 20.9, 20.8; EIMS m/z (relative intensity) 474.0 (M^+ , 41%), 348.0 (70%) 321.0

(27%), 306.0 (100%), 304.0 (100%); HREIMS m/z calcd for $C_{27}H_{22}O_8$ 474.1315 (M^+), found 474.1315.

1,8-Di-O-methyltetrangulol (37).(11) To a suspension of **24** (36.1 mg) in acetone (15.0 mL) was added DMF (1.00 mL), and the mixture was heated until homogeneous. Dimethyl sulfate (100 μ L) followed by potassium carbonate (407 mg) were added, and the solution became black. After heating at reflux for 0.7 d the solution had turned yellow. Triethylamine (0.500 mL) was added and the reaction mixture was stirred at rt for an additional 0.5 h. The mixture was filtered, evaporated *in vacuo*, and purified by chromatography (1 x 13 cm, silica gel), eluting first with EtOAc- CH_2Cl_2 (1:50), then with EtOAc- CH_2Cl_2 (1:20). This gave after evaporation 38 mg **37** (95%) as a yellow solid: mp 191.2-193.3 $^{\circ}C$, lit.(9) 190-191 $^{\circ}C$; IR (KBr) 1661.2, 1615.9, 1468.3, 1283.0, 1260.3, 1007.7 cm^{-1} ; 1H NMR ($CDCl_3$) δ 8.21 (d, 1H, $J = 8.7$), 7.91 (d, 1H, $J = 8.5$), 7.66 (d, 2H, $J = 5.2$), 7.23-7.27 (m, 2H), 6.88 (s, 1H), 4.03 (s, 3H), 3.97 (s, 3H), 2.52 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 186.5, 182.1, 159.3, 156.9, 140.2, 139.3, 137.7, 135.1, 134.9, 133.8, 132.5, 122.6, 120.5, 120.0, 119.0, 118.4, 116.2, 111.1, 56.4, 56.0, 22.0; EIMS m/z (relative intensity) 332.0 (M^+ , 100%), 315.6 (49%); HREIMS m/z calcd for $C_{21}H_{16}O_4$ 332.1049 (M^+), found 332.1048.

7,8,12-Tri-O-methylochromycinone (42).(11)

Ochromycinone, **22**, (32.2 mg), potassium carbonate (372.4 mg), and dimethyl sulfate (0.40 mL) were suspended in acetone (15 mL), and heated at reflux. At 0.2 h the reaction had turned yellow-green, and when the green color had completely dissipated (2.5 h), the mixture was cooled in an H₂O-ice bath, and sodium dithionite (38.2 mg) was added. The reaction mixture was carefully purged with argon, and heated at reflux for an additional 20 h (the mixture turned dark red, then colorless). Filtration removed the solids, and after evaporation, water (15 mL) was added and the mixture was extracted with CH₂Cl₂ (4 x 6 mL). Drying the organic extract over Na₂SO₄, and evaporation *in vacuo* gave 39.9 mg of a yellow solid. This solid was chromatographed (1 x 15 cm, silica gel) with CH₂Cl₂ as eluent to afford after evaporation 36.7 mg **42** (100%): mp 204.0-205.°C; IR (KBr) 2929, 2837, 1683, 1666, 1069, 801 cm⁻¹; ¹H NMR (CDCl₃) δ 8.42 (d, 1H, J = 8.8), 7.98 (dd, 1H, J = 8.8, 0.9), 7.40 (dd, 1H, J = 8.8, 7.5), 7.18 (d, 1H, J = 8.9), 6.82 (d, 1H, J = 7.0), 4.06 (s, 3H), 3.99 (s, 3H), 3.73 (s, 3H), 3.02 (m, 2H), 2.74 (m, 1H), 2.53 (m, 2H), 1.23 (d, 3H, J = 6.2); DEPT CMIV31; COSY CMIV40; ¹³C NMR (CDCl₃) δ 197.4, 156.1, 149.1, 148.4, 145.4, 129.9, 129.4, 128.1, 125.9, 125.8, 125.6, 119.9, 118.5, 115.7, 104.2, 63.7, 61.2, 56.0, 47.5, 38.9, 30.8, 21.2; EIMS: *m/z* (rel intensity) 350.4 (34%, M⁺), 335.4 (69%), 182.4 (36%) 69.1 (100%); HREIMS calcd for

$C_{22}H_{22}O_4$ 350.1518, found 350.1518; Anal. Calcd. for $C_{22}H_{22}O_4$: C, 75.40%; H, 6.33%. Found: C, 75.64%, H 6.11%.

7,12-Dihydro-7,8,12-trimethyl-1-trimethylsilyl-ochromycinone (43). To a solution of **42** (42.9 mg) in CH_2Cl_2 (4.0 mL) was added triethylamine (40 μ L), followed by trimethylsilyltrifluoromethane sulfonate (36 μ L). After 1 h stirring at rt, the mixture was filtered through fluorisil (0.4 x 2 cm) with additional CH_2Cl_2 to complete recovery. Removal of the solvent *in vacuo* gave 51.8 mg pure **43** (100%) as a yellow glass: mp dec.~48 °C; IR (KBr) 2956.0, 2929.8, 1359.1, 1257.2, 1067.1, 728.8 cm^{-1} ; NOEDIFF see text; 1H NMR ($CDCl_3$) δ 8.17 (dd, 1H, J = 8.7, C6-H), 7.91 (d, 1H, J = 8.8, C11-H), 7.31 (dd, 1H, J = 8.1, 8.1, C10-H), 7.21 (d, 1H, J = 8.7, C5-H), 6.74 (d, 1H, J = 7.4, C9-H), 5.10 (d, 1H, J = 3.1, C2-H), 4.05 (s, 3H, C8-OMe), 3.98 (s, 3H, C7-OMe), 3.64 (s, 3H, C12-OMe), 2.78 (dd, 1H, J = 11.5, 15.7, C4-H), 2.64-2.70 (m, 1H, C3-H), 2.53 (dd, 1H, J = 15.6, 12.1, C4-H), 1.19 (d, 3H, J = 6.8, C3-Me), -0.01 (s, 9H, TMSO-C1); ^{13}C NMR ($CDCl_3$) δ 156.3, 150.3, 149.3, 148.1, 138.3, 128.4, 128.2, 126.8, 126.0, 125.7, 125.5, 124.9, 122.9, 120.6, 117.8, 115.4, 109.3, 103.6, 63.4, 62.4, 56.0, 47.6, 38.7, 28.5, 20.2, -.2 (three resonances); EIMS m/z (relative intensity) 350.0 (M^+ -TMS, 75%), 335.0 (M^+ -TMS-Me, 100%); EIMS m/z 422.1; HRCIMS m/z calcd for $C_{25}H_{30}O_4Si$ 422.1913 ($M+1$) $^+$, found 422.1911. Anal calcd C 71.05%, H 7.16%, found C 71.38%, H 6.77%.

7,8,12-Tri-O-methyltetrangulol (44). Method a.(40,27) In a 10 mL silanized flask was placed **43** (10.9 mg), benzoquinone (5.3 mg), palladium(II)acetate (9.6 mg) and powdered Na_2CO_3 (23.7 mg). MeCN (5.0 mL) was added, and the mixture heated at reflux for 6 h, then stirred at rt for 16 h. Toluene (3.0 mL) was added, the mixture was evaporated onto silica gel (~1 g), and placed onto a silica gel column (1 x 12 cm). Elution with EtOAc-hexanes (1:4) gave, after removal of the solvent, 5.8 mg impure **44**. This was rechromatographed in the same fashion and gave 5.3 mg **44** (60%).

Method b. Tri-O-methylochromycinone, **42**, (56.8 mg) was dissolved in THF (3.00 mL) and cooled in a dry ice-acetone bath. LDA (250 μL of a 1.5 M solution) was added, and the solution was stirred for 1.2 h. Phenylselenenyl chloride (105.1 mg) was added by cannula as a solution in THF (1.50+1.00 mL), and the red solution of the enolate became black. The reaction mixture was allowed to warm to rt overnight. NH_4Cl (50 μL , saturated aqueous solution) was added, and the solvent removed under a stream of air. After filtration through silica gel (1 cm in a Pasteur pipette) with 50% ethylacetate in hexanes, a silica gel column (1 x 12 cm), eluted with CH_2Cl_2 -hexanes (1:3) was used to remove the phenylselenenic acid byproduct. Continued elution with EtOAc- CH_2Cl_2 (1:9) provided the isomeric α -selenides. The long-wave UV fluorescent fractions were evaporated giving 87.8 mg crude α -selenides

which were dissolved in THF (3.00 mL) and CH₂Cl₂ (10.50 mL). This mixture was placed in a -20 to -30 °C cooling bath (dry ice-aq. CaCl₂), and *meta*-chloroperoxybenzoic acid (56.5 mg) was added, which resulted in the mixture turning orange. After 1.5 h, diethylamine (0.451 g), previously adsorbed onto neutral alumina (1.811 g), was added. After an additional 0.2 h of stirring, 2,5-dimethoxyaniline (31.4 mg) was added, and after 0.3 h the cooling bath was removed, and the reaction mixture was stirred for an additional 2 h at room temperature. Filtration through celite, followed by washings with HCl (1.00 M, 3 x 10 mL), sodium bicarbonate (saturated, 2 x 10 mL), and brine (1 x 10 mL) provided a crude organic extract. This material was dried over Na₂SO₄, and gave after evaporation 70.3 mg crude **44**. Chromatography on a silica gel column (1 x 16 cm) eluted with EtOAc-hexanes (1:9) gave 51.9 mg **44** (91%) as a colorless solid. An analytical sample was prepared by recrystallisation from ethanol: mp 206.0-206.5 °C; IR (KBr) 770, 794, 856, 889, 1044, 1558, 1612, 2832 cm⁻¹; ¹H NMR (CDCl₃) δ 10.20 (s, 1H), 8.05 (d, 1H, J = 9.2), 7.97 (dd, 1H, J = 8.1, 0.9), 7.52 (dd, 1H, J = 8.8, 7.7), 7.46 (d, 1H, J = 9.3), 7.19 (s, 1H), 7.11 (d, 1H, J = 1.5), 6.96 (d, 1H, J = 7.6), 4.10 (s, 3H), 4.01 (s, 3H), 3.79 (s, 3H), 2.51 (s, 3H); ¹H-¹H COSY: CMV44; ¹³C NMR (CDCl₃) δ 21.0, 56.2, 62.4, 63.4, 105.3, 114.5, 118.2, 118.5, 120.3, 120.4, 120.8, 125.1, 126.6, 127.9, 128.9, 135.1, 139.4, 145.4, 149.5, 155.2, 156.3 (one resonance coincidental); EIMS: *m/z* (rel intensity) 348.2 (M+,

100%), 333.2 (M-CH₃, 96%); HREIMS calcd for C₂₂H₂₀O₄ 348.1362, found 348.1360; Anal. Calcd. for C₂₂H₂₀O₄: C, 75.83; H, 5.79. Found: C, 75.55; H, 5.52.

1-(2-Methoxyethoxymethyl)-7,8,12-tri-O-methyltetrangulol

(45). Sodium hydride (102.6 mg 60 % dispersion in oil) was washed twice with dry toluene, and **44** (27.1 mg) was added as a solution in THF (2 x 2 mL + 1 mL) by cannula. Following 1 h stirring at 29 °C, 2-methoxyethoxymethyl chloride (15 µL) was added, which immediately dissipated the bright orange color. After 2.5 h, HOAc (200 µL) followed by hexanes (5.0 mL). The mixture was filtered, and the solvents were removed *in vacuo*. A silica gel column (1 x 15 cm) was prepared and loaded with EtOAc-hexanes (1:9), and developed with EtOAc-hexanes (1:3). After pooling the long-wave UV fluorescent fractions, and evaporation *in vacuo*, this gave 20.2 mg **45** (74%) as a light yellow solid. An analytical sample was recrystallized from cyclohexane: mp 92.0-98.5 °C; IR (NaCl) 748.2, 1053.1, 1259.2, 1551.2, 2904.2 cm⁻¹; ¹H NMR (CDCl₃) δ 8.06 (dd, 1H, J = 7.9, 0.9), 8.01 (d, 1H, J = 9.2), 7.44 (dd, 1H, J = 8.6, 7.7), 7.36 (d, 1H, J = 9.2), 7.27 (d, 1H, J = 1.3), 7.24 (s, 1H), 6.91 (d, 1H, J = 7.6), 5.31 (s, 2H), 4.08 (s, 3H), 3.99 (s, 3H), 3.34-3.87 (m, 2H), 3.51 (s, 3H), 3.46-3.49 (m, 2H), 3.34 (s, 3H), 2.52 (s, 3H); COSY CMV86; ¹³C NMR (CDCl₃) δ 156.2, 150.8, 147.1, 138.3, 134.5, 129.5, 126.2, 125.4, 125.0, 121.8, 121.0, 118.6, 117.7, 117.2, 115.7,

115.6, 105.1, 95.2, 71.5, 67.6, 63.3, 60.7, 58.8, 56.2, 21.4; EIMS *m/z* (relative intensity) 436.5 (M^+ , 100%), 421.5 (M^+ -Me, 5%), 348.4 (M^+ -MEM, 51%), 333.4 (M^+ -MEM-Me, 42%), 318.4 (M^+ -MEM-2Me, 28%); HREIMS *m/z* calcd for $C_{26}H_{28}O_6$ 436.1886 (M^+), found 436.1885.

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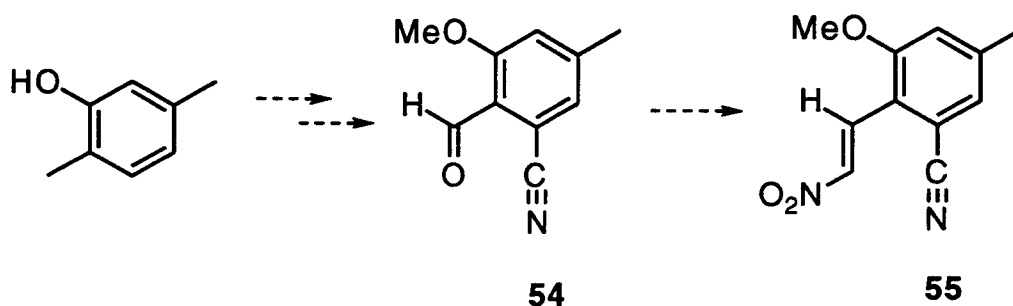
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25. Partial data for 1,7,8,12-tetramethyltetraangulol: ¹H NMR (CDCl₃) δ 8.11 (dd, 1H, J = 8.7, 0.8), 8.04 (d, 1H, J = 9.1), 7.44 (dd, 1H, J = 8.6, 7.7), 7.37 (d, 1H, J = 9.2), 7.18 (s, 1H), 6.93 (s, 1H), 6.19 (d, 1H, J = 9.5), 4.07 (s, 3H), 3.99 (s, 3H), 3.98 (s, 3H), 3.52 (s, 3H), 2.55 (s, 3H); ¹³C NMR (CDCl₃) δ 157.9, 156.2, 150.9, 147.0, 138.1, 134.5, 129.6, 126.2, 125.4, 125.1, 122.1, 119.6, 118.5, 117.4, 116.5, 115.7, 110.8, 105.1, 63.4, 60.4, 56.2, 56.1, 21.7.
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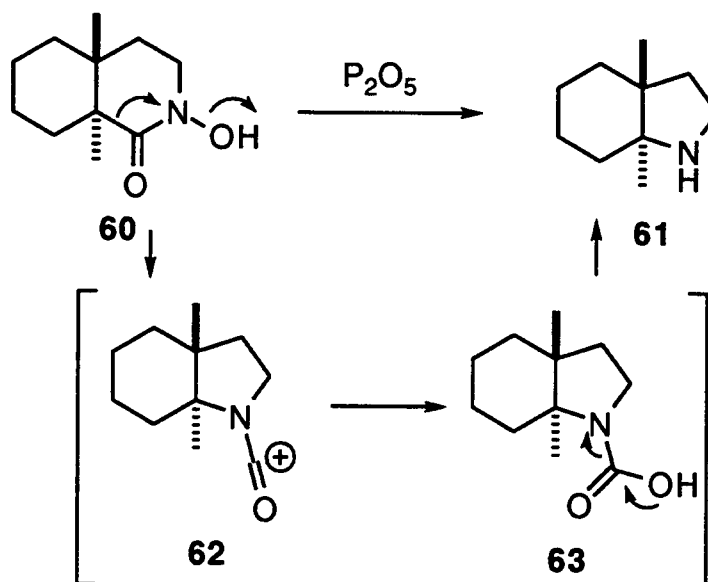
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3. An Attempted Biomimetic Ring Contraction

As was introduced in Chapter 1 (p. 9), the cyanamide moiety of the kinamycins may be formed by oxidative cleavage of the C ring of **11** to afford, after nitrogen insertion and reclosure of the C ring, an N-substituted amidine that could rearrange by an unprecedented, but chemically plausible ring contraction. This ring contraction would be the most novel step in the biosynthetic pathway, so an investigation was undertaken to see if this chemistry could be mimicked *in vitro*. The synthetic route chosen was to prepare aldehyde **54**, which would then undergo a Knoevenagel condensation with nitromethane to afford **55**. The resulting Michael acceptor **55** would then undergo a cyanophthalate annulation to provide **56**. Nitronitrile **56** would be reduced to the hydroxylamine **57**, with *in situ* ring closure(1) by addition to the nitrile, affording **58**. In the presence of acid, it was hoped that this would undergo the biomimetic rearrangement to **59**.

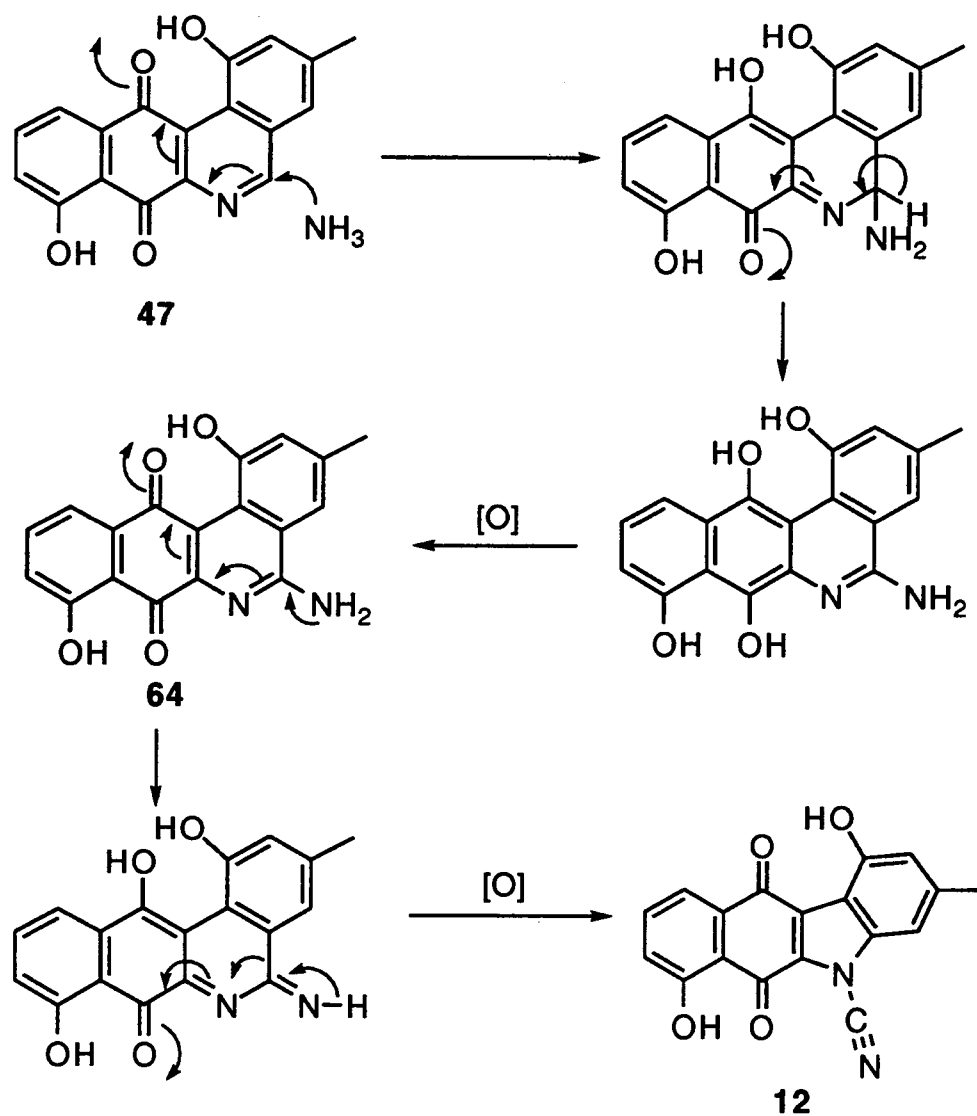


The Tiemann rearrangement of hydroxamic acids(2) is the reaction most similar to the proposed ring contraction. An illustrative example of this reaction is the rearrangement of **60**, with concomitant ring contraction, to provide **61**. This reaction can be rationalized as involving the rearrangement of an intermediate nitrene to provide **62**. Compound **62** then reacts with water to form the intermediate carbamic acid, **63**, which decarboxylates to **61**.



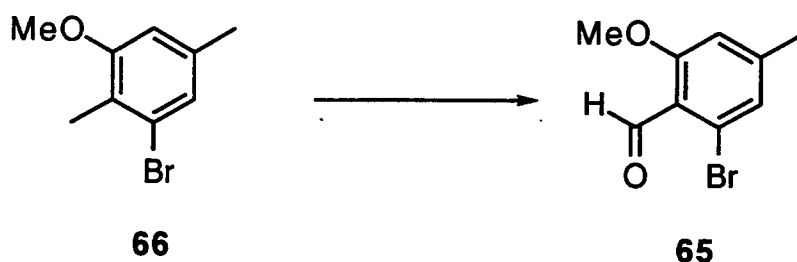
A short time after beginning this work, former coworker Dr. Gore proposed(3) a modification of the ring contraction mechanism. This modification involved a Michael-type 1,6-addition of ammonia to phenanthroviridin aglycone **47**, which after re-oxidation with oxygen, would give the aminopyridine **64**.

Rather than being dependent on nitrene formation to provide the driving force for the rearrangement reaction, the quinone was envisaged as driving the ring contraction as shown below. In this mechanism, the quinone was formally accepting the two hydrogen atoms from the amino group, and their associated electrons. Compound **47** has recently been detected in the extract of an *S. murayamaensis* fermentation.(4)



Preparation of Cyanoaldehyde 54

It was decided that the most expedient route to **54** would be to use compound **65**, first prepared by Dr. Gore.(3) The preparation of this compound from the corresponding bromoanisole **66** by persulfate oxidation was an unreliable and low yielding process (11-30%). A study to improve the preparation of **65** was therefore undertaken.



Direct oxidation of **66** with stoichiometric selenium dioxide and *tert*-butylhydroperoxide co-oxidant gave no reaction, even at elevated temperatures (CCl₄ at reflux). Similarly, reaction with *tert*-butylhydroperoxide in the presence of Wilkinson's Catalyst gave a 10% conversion of **66** to an unknown compound, with concomitant destruction of the catalyst evident by cessation of reaction, and the isolation of triphenylphosphine oxide. Reaction with dichlorodicyanoquinone(5) gave **65** (12%), recovered **66** (7%), and a large amount of white polymeric material.

It was, however, found that the light-initiated bromination of **66** with N-bromosuccinimide gave a reasonable selectivity for halogenation of the 2-methyl position. When 1.45 eq. N-bromosuccinimide was used, a selectivity of 3:2 (desired:dibrominated) was obtained, and with 1.00 eq. N-bromosuccinimide a slightly improved 6:1:1:1 (desired:dibrominated:5-isomer:starting material) ratio of products was obtained. Scale-up and MPLC separation of the reaction components gave **67** (72% isolated). A long-range HETCOSY experiment provided carbon assignments and showed coincidental resonances for C3 and C4, Table 3.1. Similarly, 3-bromo-2,5-bisbromomethylanisole, **68**, was prepared in 48% isolated yield.

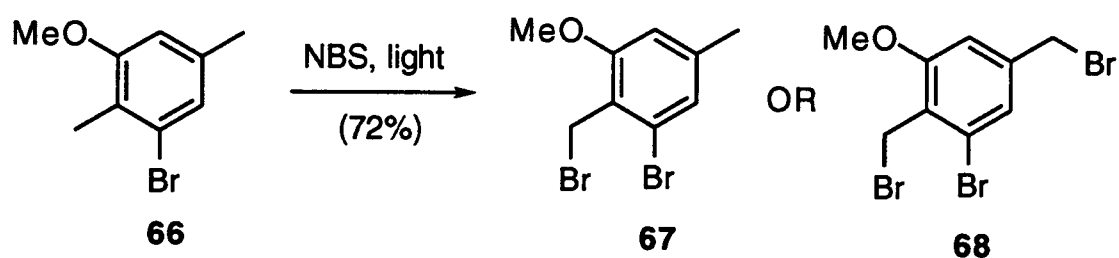


Table 3.1. Long Range HETCOSY of **67**

¹ H Shift ppm	One-Bond Corr.	Two-, and Three-Bond Corr. (F2 multiplicity)
2.31	21.4	141.2 (s) 111.0 (d) 125.6 (d)
3.87	56.1	158.3 (s)

Table 3.1. Continued

4.71	28.6	123.0 (s)
		125.6 (s)
		158.6 (s)
6.63	111.0	21.4 (d)
		123.0 (s)
		125.6 (d)
7.01	125.6	21.4 (d)
		111.0 (d)
		123.0 (s)

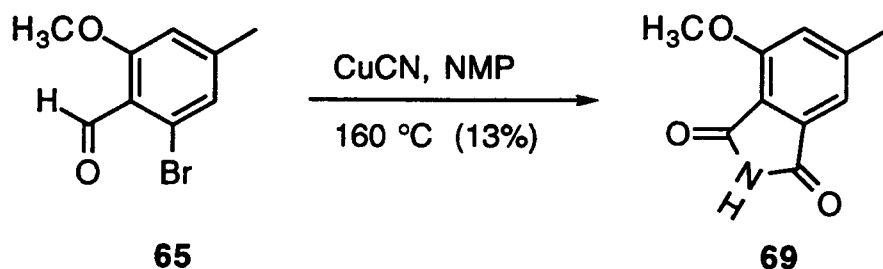
C3 and C4 coincidental at δ 125.6 ppm

Conversion of benzyl bromide **67** to **54** by treatment with silver nitrate in dimethylsulfoxide gave a complex mixture, and poor mass recovery. A similar reaction using silver fluoroborate was considerably cleaner, and gave a 50% yield. Although this may have been improved, hope for a better procedure, employing less expensive reagents, led to a survey of other methods.

Treatment of a mixture of the bromination products containing **67** with 2-nitropropane and sodium ethoxide gave the aldehyde **54** (54%, 36% from **66**), and small amounts of the isomeric aldehyde and dialdehyde corresponding to the minor bromination products. Also attempted was a bromide trifluoroacetolysis with trifluoroacetic acid. This reaction gave a complex mixture, which was not simplified by treatment with potassium carbonate in methanol. It is probable that Friedel-Crafts type trifluoroacylation of the aromatic ring was in

competition with the desired reaction. Owing to the poor solubility of the bromination products in water, attempted solvolysis with sodium hydroxide in aqueous THF gave no reaction. Using aqueous sodium hydroxide in dimethylformamide gave the alcohol, but with production of a large amount of an intractable polymer. To improve this, the hydrolysis was attempted with potassium hydroxide in ethanol, with and without THF as a co-solvent. At ambient temperature no reaction was observed, but when the former reaction was heated at reflux, a mixture of the alcohol and the corresponding ethyl ether were produced. Application of the method of Kornblum(6) was found to be the best method for preparing **65**, providing a 56% yield.

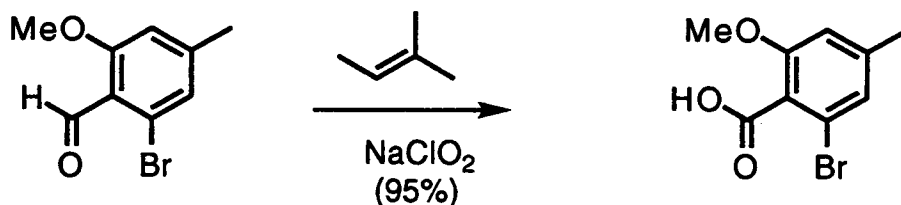
Rosenmund-von Braun cyanation of aldehyde **65** in dimethylformamide, or N-methylpyrrolidone gave complete consumption of the starting material. The major product, which was produced in 13% yield was the phthalimide **69**. The oxidation of the aldehyde carbon of **65** in this compound likely results from disproportionation. Attempted palladium-catalyzed cyanation using neutral alumina co-catalyst similarly failed, with consumption of the starting material. Reactions performed in dimethylsulfoxide, with or without 18-crown-6 catalysis, or with supported cuprous cyanide on charcoal were similarly unsuccessful.



Failure of Rosenmund-von Braun cyanation reactions of **65** prompted the investigation of other substrates. Attempts to protect the aldehyde by dioxolane formation, either with Amberlyst 15 catalysis or more conventional *para*-toluenesulfonic acid catalysis, proved surprisingly troublesome. The product was labile to hydrolysis, and reverted to the aldehyde upon standing. Because of this lability it could not be purified on silica gel. This unexpected reactivity probably results from a combination of a release of steric congestion imposed by the bulky *ortho*-bromine, and by assistance of the *ortho*-methoxy group in stabilizing the charge generated by the initial ring opening. An attempted protection with catechol to give a potentially more stable acetal, gave no reaction.

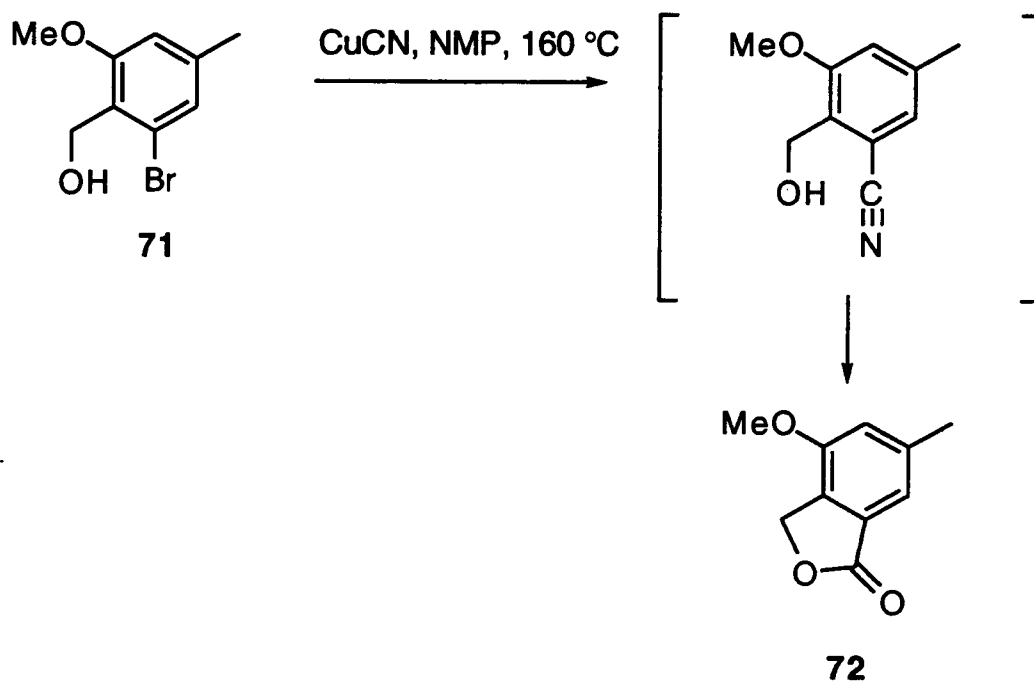
It was hoped that **65** could be protected by oxidation to the acid, and that the acid would assist the cyanation by coordinating the reactive copper intermediate.⁽⁷⁾ Regeneration of the aldehyde group would subsequently be accomplished by a Rosenmund Reduction of the corresponding acid halide. Sodium chlorite oxidation of **65** gave the benzoic acid **70** in 95% yield.

However, attempted cyanation gave primarily a product that appeared to be the des-bromo acid.

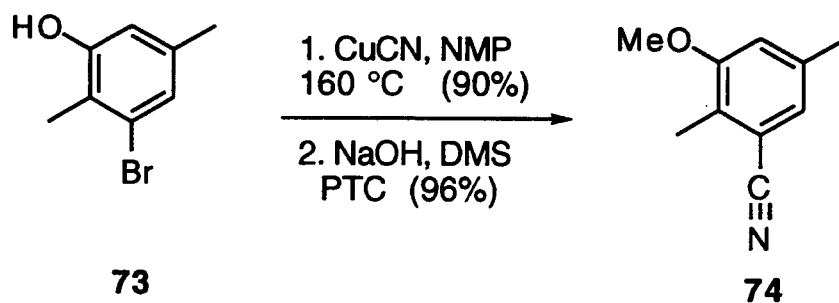


Since the acid **70** turned out to be a poor substrate for cyanation, the corresponding alcohol **71** was next targeted as a potential cyanation substrate. Application of literature conditions for the hydrolysis of benzyl bromides to their corresponding benzyl alcohols were uniformly low yielding, and accompanied by the formation of insoluble reaction by-products, when applied to **67**. The most plausible rationalization for this unexpected difficulty was that if the reactant was not completely solvated, a competing polymerization reaction took place. This polymerization may occur via a quinone methide intermediate resulting from the base-catalyzed 1,6-elimination of HBr. To avoid this problem, several alternative reaction conditions were explored. The first was to attempt solvolysis with potassium hydroxide in dimethylformamide without water co-solvent, but this also gave mostly polymeric material. Attempted silver activation of the bromine (silver nitrate with stoichiometric water in tetrahydrofuran), under sonicating conditions, gave no reaction.

Attempted solvolysis and oxidation using a chromic acid solution gave a vigorous reaction, but no product could be isolated. Hydrogen peroxide or tetra-*n*-butylammonium hydroxide (50% aqueous) gave homogeneous solutions in tetrahydrofuran, the former affording a complex mixture, the latter only recovered starting material. It was found, however, that hydrolysis with lithium hydroxide in dimethylsulfoxide gave the alcohol containing a small amount of the corresponding aldehyde (8-10%, ^1H NMR), in what may be a preparatively useful method. With the alcohol **71** in hand, its cyanation was next attempted. Charcoal supported cuprous cyanide gave a complex mixture; however, conditions utilizing N-methylpyrrolidone as the solvent produced the undesired lactone **72**. This compound, resulting from the alcoholysis-hydrolysis of the desired cyanobenzol, demonstrated that nucleophilic groups *ortho* to the nitrile were a liability.



Continuing the search for a suitable cyanation substrate, bromophenol **73** was found to cleanly react to give the cyanophenol in 90% yield. As expected, this was efficiently methylated providing cyanoanisole **74** in 96% yield.

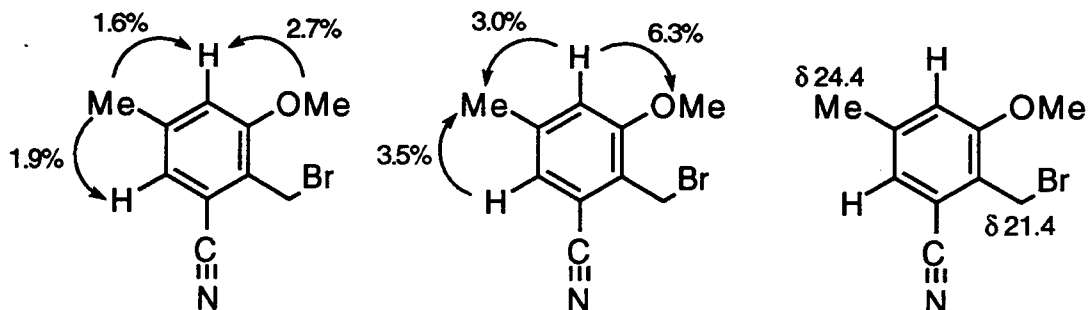


Remaining work on this project centered on conversion of cyanoanisole **74** to the cyanoaldehyde **54**. Attempted bromination of **74** with pyridinium bromide perbromide and light initiation gave poor selectivity, and poor conversion, to the desired benzyl bromide **75**. In attempting to improve the N-bromosuccinimide bromination selectivity, methyl formate was tried as the reaction solvent.(8) This led to mostly ring brominated products by ^1H NMR. The source of this problem was probably that commercially available anhydrous methyl formate (Sure-Seal,[®] Aldrich Chemical Co.) was used, which may have contained formic acid. Use of freshly distilled methyl formate may have alleviated this undesired reaction, as acid is known to catalyze aromatic bromination with N-bromosuccinimide. Recrystallized (white) N-bromosuccinimide gave no light initiated reaction, confirming the need to have elemental bromine present for free-radical chain initiation. Bromination selectivity in carbon tetrachloride and dichloromethane (also reputedly a more selective solvent)(8) were then compared, as was the effect of varying temperature, as summarized below. Reaction mixtures were inseparable on silica gel, but **75** was isolated using neutral alumina.

Table 3.2. Benzylic Bromination Selectivity

Solvent	Temperature	Selectivity (Aromatic ^1H)
CCl_4	reflux	63%
CCl_4	0-5 $^\circ\text{C}$	73%
CH_2Cl_2	reflux	70%
CH_2Cl_2	0-5 $^\circ\text{C}$	77%

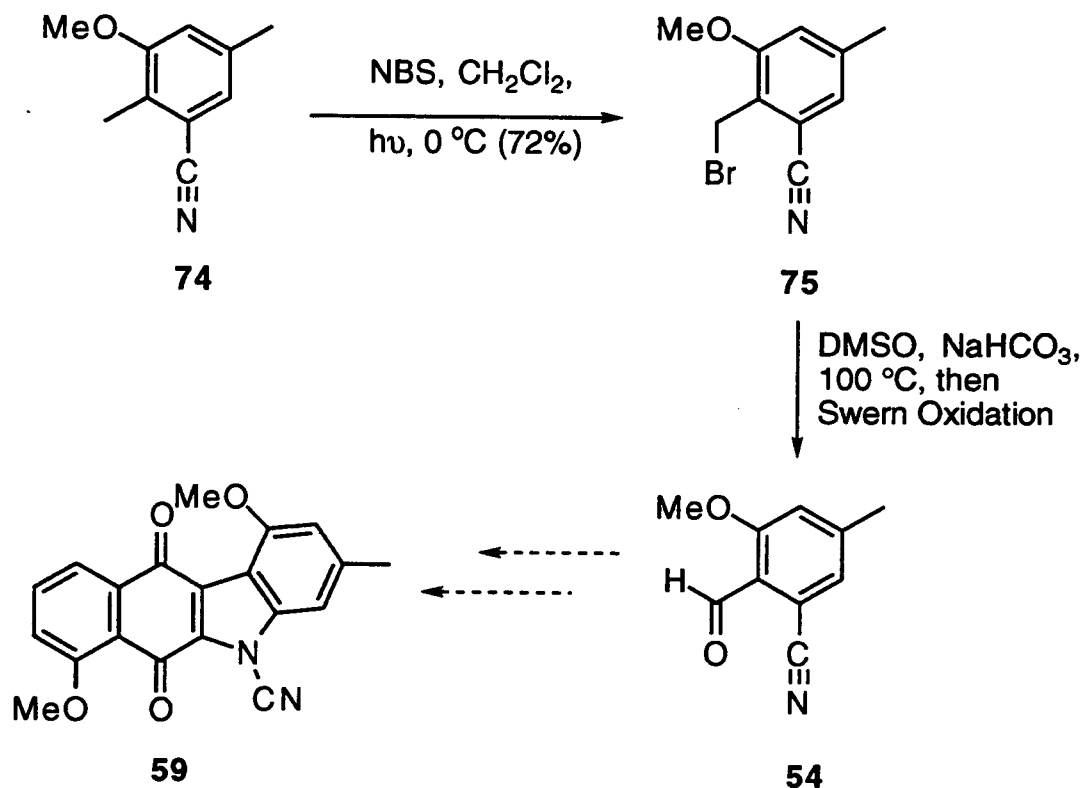
The regiochemistry of bromination was confirmed by observation of a nuclear Overhauser effect enhancement of the bromomethyl resonance when the -OMe signal was irradiated. Similarly, irradiation of the aryl methyl enhanced the signals from both of the aromatic protons. Assignment of the surprisingly similar methyl and bromomethyl ^{13}C NMR resonances was done with a DEPT-135 experiment.



Application of the low temperature benzylic bromination conditions to the cyanoanisole **74** gave the benzyl bromide **75** in 72% purified yield. Attempted solvolysis of **75** with a variety of

aqueous base systems failed to give any recognizable products. Similarly, Kornblum(6) oxidation conditions with an aqueous work-up gave none of the desired aldehyde **54**, in contrast to our previous experience in preparing the bromoaldehyde **65**. It was therefore anticipated, and later demonstrated, that the desired product, **54**, reacts with water to afford a mixture of uncharacterized products. A possible reason for this reactivity may be that electron withdrawal by the *ortho*-cyano group may favor the aldehyde existing in a hydrated structure when dissolved in hydroxylic solvents. This could cause neighboring group assisted solvolysis of the nitrile, leading to a variety of products. Compound **54** was prepared in 64% yield from a regioisomeric mixture of benzyl bromides by Kornblum conditions, omitting the aqueous work-up. Modifying the Kornblum conditions by performing the reaction at 0 °C was lower yielding, as was oxidation by sodium 2-nitropropanate. Therefore, the reaction time and reagent equivalents for cyanoaldehyde preparation via the Kornblum conditions(6) were carefully optimized. It was found that the heating time must be minimized, with the optimum time between five and seven minutes. Steven Ley oxidation(9) of the co-produced benzyl alcohol **76** failed; however, Swern oxidation(10,11) of the crude reaction mixture efficiently converted **76** to **54**. This reaction sequence provided **54** in 37% yield, over three steps. Two additional modifications in the experimental procedure were tried: first, acetone was used to elute the reaction mixture from a

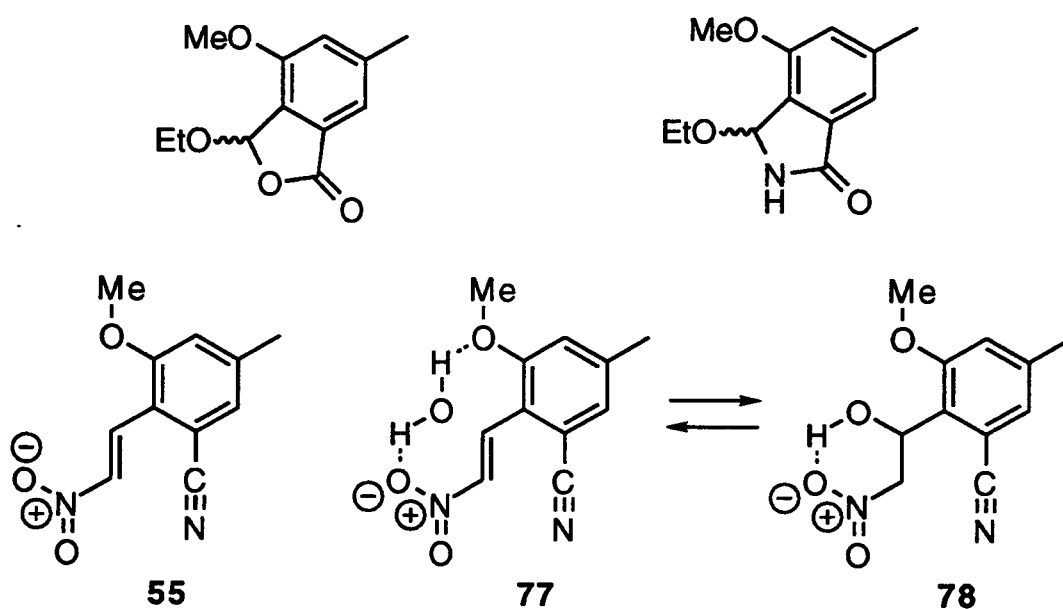
short silica gel column in the non-aqueous work-up to assure complete recovery of the cyanobenzyl alcohol, **76**, (**76** has not been identified on TLC); second, methanol was added to the completed Swern Oxidation at low temperature to quench any remaining sulfoxonium species present (to prevent alkylation of the aromatic ring). Neither modification gave an improvement in the yield. Flash chromatography was found to be preferable to MPLC for separation of the reaction mixture due to the comparatively low solubility of **54**. Final purification was best accomplished by recrystallization with an ethyl acetate-hexanes solvent system.



An Unexpected Olefination Product

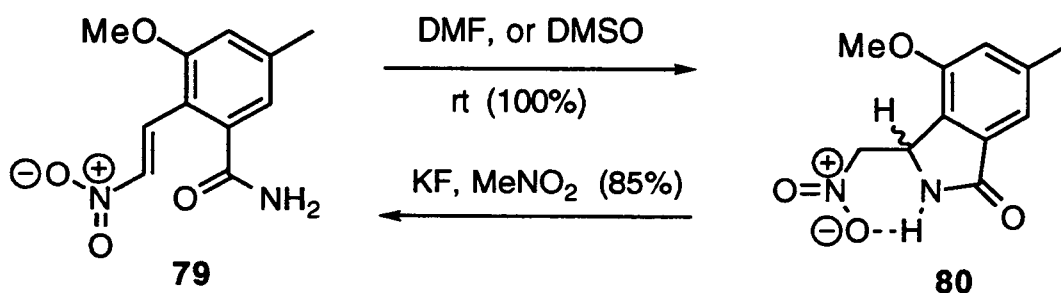
The next goal was to obtain the corresponding nitroolefin, **55**. When the conditions(12) successfully used in an analogous system(3) were used, addition of the solvent, ethanol, provided products that appeared to be the corresponding benzisofuranone and isoindole generated by neighboring group assisted solvolysis. Cyanoaldehyde **54** was found to be quite reactive, and since ethanol had been incorporated into the products of the initial reaction, it seemed expedient to avoid hydroxylic solvents. The few nitroolefination conditions not requiring alcoholic solvents (Barton(13,14), Al_2O_3 , (15) NH_4OAc , MeNH_4Cl)(16) gave intractable products with the exception of potassium fluoride catalysis.(17) Fluoride catalysis provided a product that had the downfield doublets in the ^1H NMR [δ 8.49 (d, $J = 13.5$ Hz), δ 8.10 (d, $J = 13.5$ Hz)] characteristic of a nitroolefin, but also had two singlets (δ 8.06 and δ 7.76 ppm) unaccounted for by the expected structure, **55**. As these two singlets were found to exchange with $^2\text{H}_2\text{O}$, the hydrated structure **77** was suspected.(18) A facile conversion, to a product with ^1H and ^{13}C NMR spectra consistent with the structure of the nitroaldol (Henri) product **78**, was observed in the in the NMR sample ($\text{DMSO}-d_6$). This conversion occurred with a half-life on the order of 1-2 hours, and precluded the acquisition of the ^{13}C NMR spectrum of the nitroolefin. The hydrated structure was consistent with this observed reactivity,

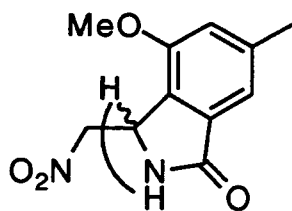
having a molecule of H_2O held in a position conducive to Michael-type addition to the nitroolefin by hydrogen bonding. To remove the suspected molecule of H_2O , dehydration with the toluene azeotrope, or by dry heating at $120\text{ }^\circ\text{C}$, was attempted. This gave no change in the ^1H NMR spectrum, and since such an association would be expected to involve only weak bonding, another alternative had to be found.



A more careful analysis of the IR spectrum of the nitroolefin revealed an amide I stretch at 1614 cm^{-1} , and an amide II stretch at 1601 cm^{-1} , providing the structure **79**. This product resulted from a formal addition of the H_2O lost in nitromethane condensation, to the nitrile. This conversion of the nitrile into an amide is likely to occur by neighboring group assisted hydroxyl

addition of an intermediate nitroaldol. Consistent with this contention, neither 3-cyano-2,5-dimethylanisole, nor benzonitrile, reacted under the KF-catalyzed nitroolefination conditions. The amide **79** was purified by chromatography on silica gel with 20% THF in chloroform, or by recrystallization from glýme. In solutions of dimethylformamide or dimethylsulfoxide, **79** was quantitatively converted to isoindole **80**. The isoindole failed to react under a number of potential dehydrating conditions, but was quantitatively converted back to the nitroolefin, **79**, by KF in nitromethane. The structure of the isoindole was secured by observed COSY and NOESY correlations between the NH and benzylic protons, excluding isomeric **81**. Furthermore, a DEPT experiment showed the ^{13}C NMR signal for the methylene α to the nitro group at 76.4 ppm, which might otherwise be obscured by CDCl_3 . Subsequently, HREIMS provided molecular formulas consistent with the structures of **79** and **80**. The amide **79** was not necessarily a synthetic dead end, as it could potentially be dehydrated to the desired nitrile, but other alternatives were pursued in preference.



**80**

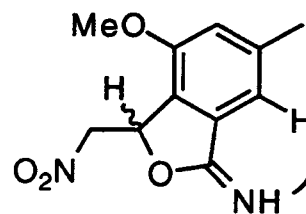
Experiment:

COSY

NOESY

Observed

Observed

**81**

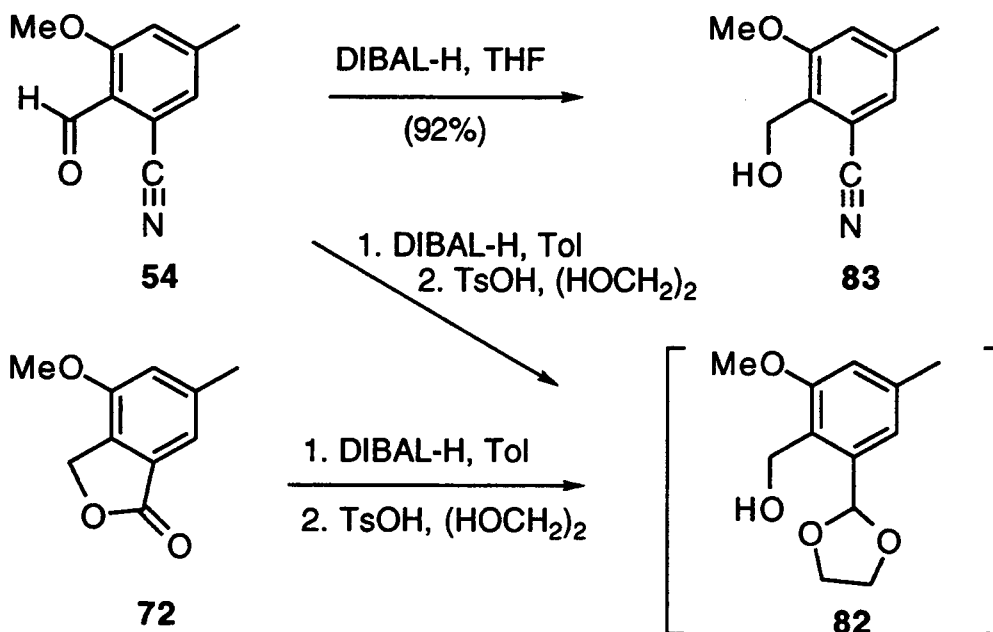
Not Observed

Not Observed

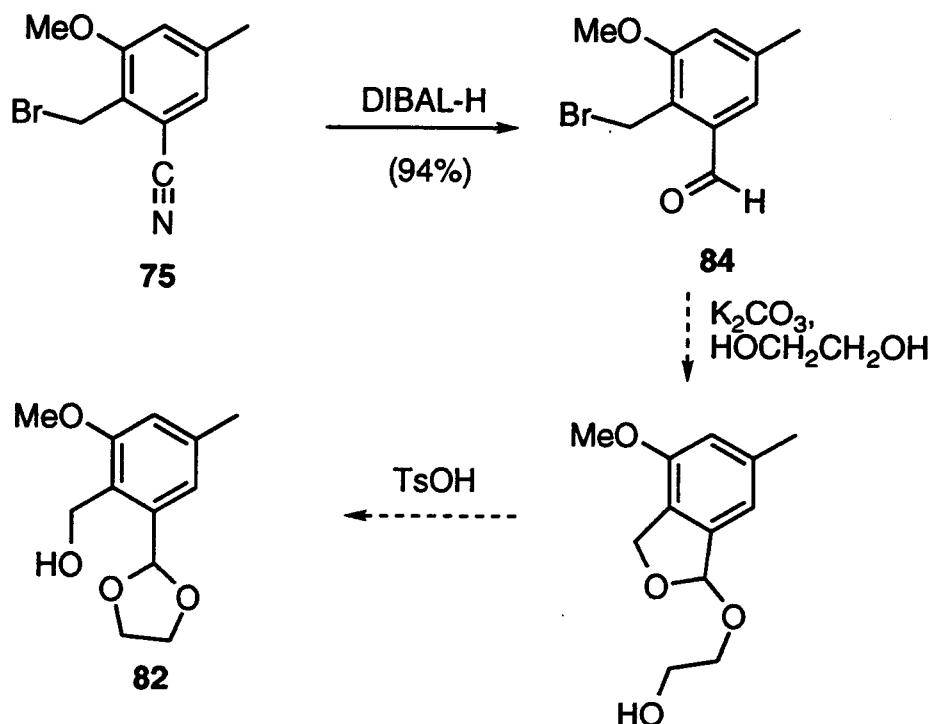
Acid-Aldehyde Intermediates

To make use of the experience gained in the biomimetic ring contraction project, a parallel approach to the acid-aldehyde proposed to be the ring cleavage product of dehydrorabelomycin was surveyed. The plan was to prepare the protected benzyl alcohol **82**, which would then be converted to the corresponding 2-trimethylsilylpropenoate. Cyanophthalide cyclization and deprotection would then be expected to provide the acid-aldehyde **48**.

H reduction of the benzisofuranone **72**, followed by dioxolane formation(20) also gave **82** in low yield.



The cyanobenzylbromide **75** was found to cleanly reduce(19,21) to the formylbenzylbromide **84** (94% yield). Formylbenzylbromide **84** was expected to provide access to an alcohol-aldehyde intermediate by neighboring group assisted solvolysis, as illustrated below. This approach was not pursued further.



Knoevenagel Condensations Affording the Cinnamate

Doebner-Knoevenagel conditions for the condensation of **54** and malonic acid gave a black mixture from which the desired cinnamate (propenoic acid) **85** was isolated in 40% yield.^(3,16) When this reaction was performed at room temperature for three days, a higher selectivity was observed. However, the resulting cinnamate was a stereoisomeric mixture, largely of the *cis* configuration. Since these acids were poorly separated by chromatography (1%HOAc/ 10%MeOH/ 89%CH₂Cl₂), it was considered of great advantage to have a clean and selective preparation. Toward this end it was found that performing the condensation in toluene rather than pyridine eliminated many

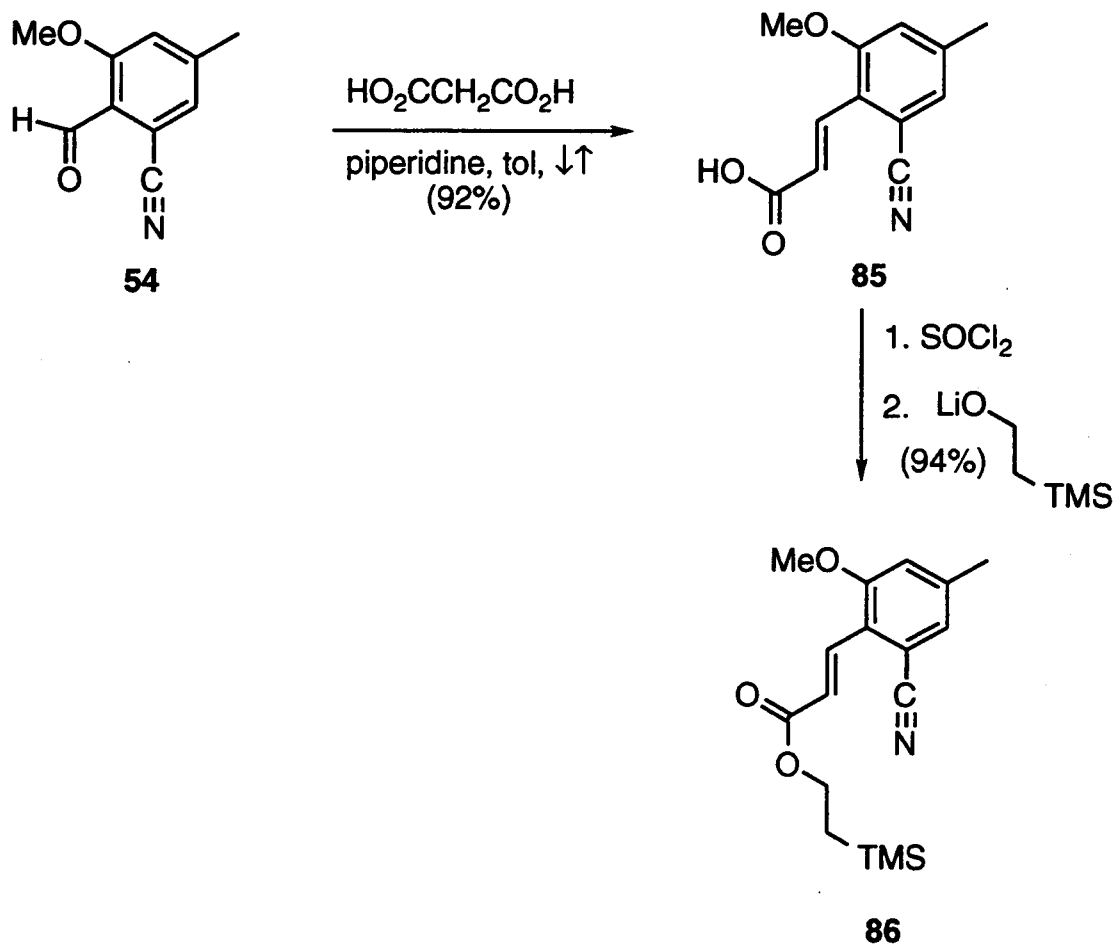
side reactions, and provided **85** in 92% yield.(22) When toluene was used as solvent the reaction mixture was no longer homogeneous, and appeared to occur in a molten phase. This suggests the reaction may also be amenable to microwave heating. Longer reaction times sharply reduced the yield.

Recrystallization of **85** from acetone worked well, but acetonitrile gave an even better mass recovery, probably because of its higher boiling point. Cinnamate **85**, however, has a limited thermal stability when being recrystallized, and recrystallizations from toluene-acetone or chloroform-acetone were not successful. Bicarbonate extraction followed by acidification and re-extraction did not significantly improve the purity of the reaction mixtures.

Esterification with 2-(Trimethylsilyl)ethanol

Consistent with Dr. Gore's earlier experience, treatment of the acid chloride generated from **85** by treatment with thionyl chloride with the lithium salt of 2-(trimethylsilyl)ethanol gave cyanoester **86**.(3) A non-aqueous work-up using silica gel filtration to adsorb the lithium alkoxide gave a 94% yield of **86** after further purification, but was experimentally difficult due poor solvent flow through the basic silica gel. Using a larger amount of silica gel did not alleviate this problem, but did result in a reduced yield, possibly due to saponification. It was,

however, found that reacting the lithium alkoxide with acetic anhydride left the mixture slightly acidic, and was a much more convenient, but lower yielding, way to work-up this reaction.



Cyanophthalide Annulations

Cyanoester **86** was reacted with the cyanophthalide using the conditions(3) Dr. Gore had applied to the bromoester. A 10% yield of the desired ester dione **87** was obtained, along with a

small quantity of recovered **86**, and many unidentified colorless products. A detailed analysis of varying reaction conditions on product formation was then undertaken. The reaction was analyzed by HPLC using the gradient in Table 3.3. The relative efficiency of each modification was determined by addition of anthracene as an internal standard to the cyanoester. Retention times and UV spectra of materials determined by injection of authentic samples are presented in Table 3.4. Use of the diode array detector has allowed incontrovertible assignment of peaks when variations in the retention times were observed.

Table 3.3. Modified HPLC Gradient Elution Protocol

Time:	0.1% HOAc in H ₂ O	0.1% HOAc in MeCN
Initial	89%	11%
12 min.	5%	95%
15 min.	5%	95%
22 min.	89%	11%
30 min.	89%	11%

reinject after 30 min.

To evaluate the effect of changing the counter-ion on the efficiency of the cyanophthalide addition reaction, lithium and potassium were compared, and lithium gave a more selective reaction. Addition of lithium bromide to the reaction mixture gave a lower yield of cyclization products. Using hexamethylphosphoramide(23) as co-solvent in tetrahydrofuran completely inhibited the reaction, even when the mixture was

heated at reflux. Quantification of the differences in these reactions was thwarted by difficulties in determining the isobestic point in the interconversion between the ester dione **87**, and its hydroquinone, so it was next necessary to find a reliable oxidative work up, both for preparative work, and for reaction analysis.

Table 3.4. Components of Cyanophthalide Addition Reactions

Compound:	Retention Time	λ max (nm)
cynoester 86	18.15 min.	226, 278, 334
cyanophthalide	10.25 min.	236, 296
anthracene	17.52 min.	
ester dione 87	16.14 min.	212, 298, 414
hydroquinone of 87	15.31 min.	222, 306, 354
acid dione	9.94 min.	212, 294, 408
amino dione 89	11.47 min.	208, 264, 292, 394

It was evident from analysis of the previously used conditions that the oxidation step (passing air through a solution of the reaction products in THF/ 1M NaOH) caused a 60-70% loss of **87**. Simply passing air through the basic reaction mixture provided no oxidation, so several inorganic co-oxidants were surveyed. Ammonium cerium(IV) nitrate, and potassium ferricyanide were examined under both basic and acidic conditions. These oxidants, and acidic hydrogen peroxide, converted the hydroquinone to quinone rapidly, although the ammonium cerium(IV) nitrate had the undesirable side effect of

oxidizing the anthracene internal standard to anthraquinone. Surprisingly, simply acidifying the mixture with aqueous HCl and exposure to air under normal work-up conditions afforded complete oxidation, and a higher product yield and starting material recovery than any of the other oxidants examined. A non-aqueous work-up with potassium dihydrogenphosphate failed, so the HCl protocol was optimized at two equivalents HCl (1M in water) for each equivalent of base.

In no reaction mixture thus far has unreacted cyanophthalide been detected. However, an experiment to ascertain if increasing the amount of cyanophthalide used would improve the reaction showed no dependence of product yield on the amount of cyanophthalide. Imagining that this might result from a reaction of the cyanophthalide anion prior to addition to the cyanoester, a deuteration was performed by adding $^2\text{H}_2\text{O}$ to see if the reaction could be run at an initially lower temperature. A low level of deuteration was observed, indicating that the cyanophthalide anion was not being readily generated under the reaction conditions used, Table 3.5. Failure to observe the cyanophthalide in the reaction mixtures may have been due to its low extinction coefficient at 254 nm, relative to the other reaction components. Increasing reaction times was found to decrease product yield.

Table 3.5. Deprotonation of Cyanophthalide

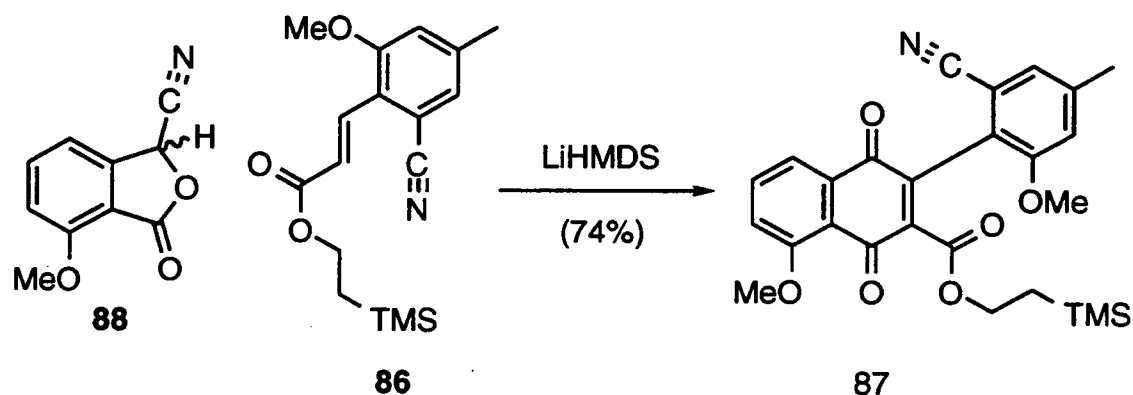
Base	Equiv.	Max. Temp.	Time	% ² H
LDA	1.10	-98 °C	1h	17%
LiHMDS	1.05	-78 °C	1h	33%
LiHMDS	1.05	0 °C	0.3h	62%
LiHMDS	1.05	rt	1h	77%

Ester dione **87** was poorly soluble in the TLC system (40% ethyl acetate in hexanes), but chromatographed well in 2% ethyl acetate in dichloromethane. Attempted recrystallization from chloroform-pentane, chloroform-ether, and ethyl acetate-pentane gave an oil like material, while a gum was obtained from dimethylformamide-water. Surprisingly, however, clear needles readily form in ethyl acetate-hexanes.

The cyanophthalide cycloaddition was performed on sufficient scale to provide 200 mg of the ester dione **87**. Prior formation of the cyanophthalide anion in toluene at room temperature proved successful, and drastically improved the previous yields. LHMDS was used in place of LDA, and **87** was isolated in 74% yield. At this yield, the modifications in generating the cyanophthalide anion, and modified oxidation, had each roughly tripled the efficiency of the overall reaction.

Summarizing what is known about this cyanophthalide addition: 1. product yield is independent of the amount of excess cyanophthalide **88** present; 2. lithium as the counterion of **88** without Lewis acid or co-solvent adducts gave the cleanest

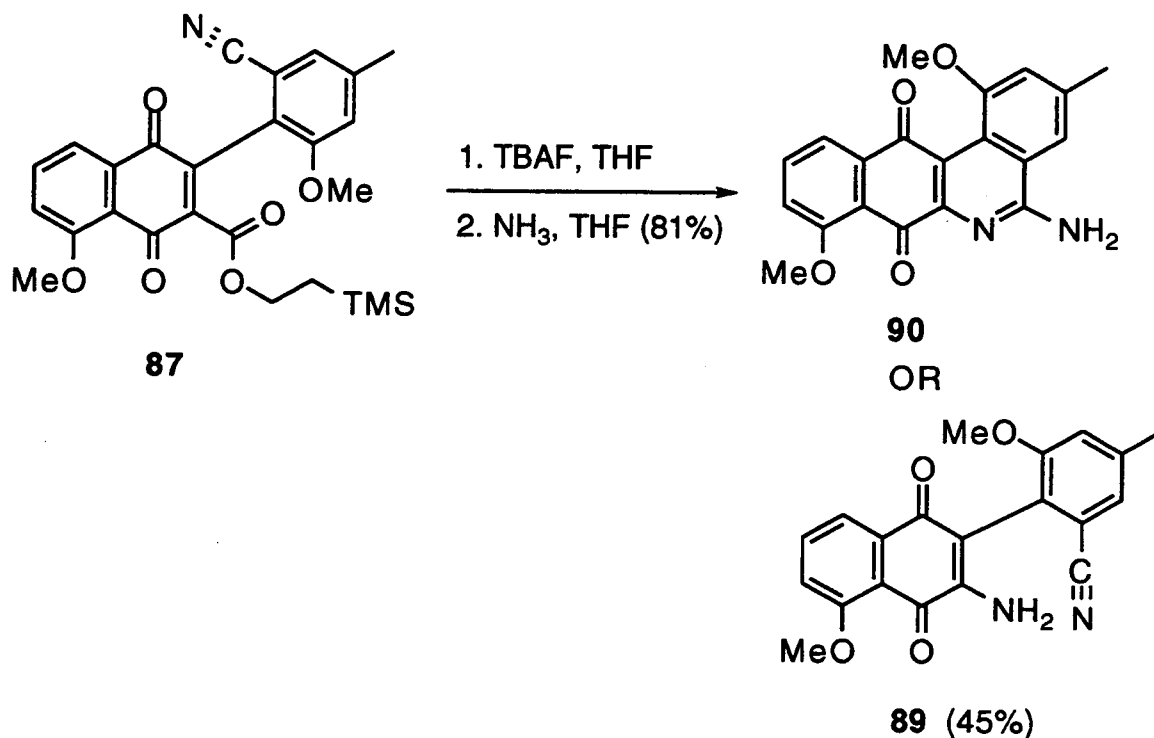
reaction; 3. conditions previously used did not completely generate the cyanophthalide anion prior to cyanoester addition; and 4. two equivalents HCl and exposure to air is the best work up and oxidation conditions found.



Reactions of the Ester Dione **87**

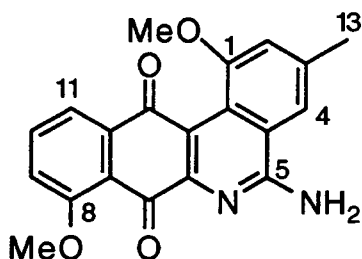
Reaction of the TMSE ester dione **87** with pyridinium polyhydrogen fluoride, or hydrogen fluoride in methanol gave no cleavage of the protecting group. Tetrabutylammonium fluoride on the other hand reacted rapidly in THF and gave a polar yellow compound that is presumably the corresponding acid (quantitative by HPLC, retention time 9.94 min., λ_{max} 212, 294, 408 nm). If air and ammonia were passed through a solution of the resulting acid, the amino dione **89** was produced in 45% yield, without accompanying amino pyridine **90**. However, when ammonia was passed through a solution of the acid in THF, the amino pyridine **90** was produced in 81% yield, accompanied by

the amino dione **89** in 10% yield. Thus, maintaining the hydroquinone oxidation level favored cyclization. Complete carbon assignments were determined for the amino pyridine **90** by HMBC and HMQC inverse correlation experiments to confirm its structure. Direct reaction of the TMSE ester dione with ammonia in methanol gave no identifiable products.



To induce **90** to undergo the anticipated ring contraction to give **59**, **90** was subjected to a variety of reaction conditions. These included basic, acidic, and free-radical generating conditions, and are outlined below. Dry heating of the amino pyridine **90** to 300 °C gave recovered starting material, as did

heating to 112 °C in dimethylsulfoxide. Treatment with trimethylaluminum(24) gave no reaction. Under most conditions the amino pyridine is bright red, and soluble only in polar aprotic solvents and acids (acetic); however, a second form of the aminopyridine has been observed which is less polar, orange, and is soluble in chloroform. This second form readily converted to the first, and in one experiment was obtained by heating the red form in dimethylsulfoxide to 153 °C. This transformation may be interconversion between the amino pyridine and amidine tautomers (or reduction to the hydroquinone). The nature of this transformation is not known, and was sporadically observed, suggesting it may require the absolute exclusion of oxygen, or basic or acidic impurities. The aminopyridine was unreactive in KOH-water-THF or potassium *tert*-butoxide-THF. However, when reacted with LDA in THF at -78 °C it gave unidentifiable products. Treatment with lead tetraacetate or *tert*-butyl hydroperoxide also gave no reaction. Similarly, deprotection with boron tribromide did not provide a ring contracted product.



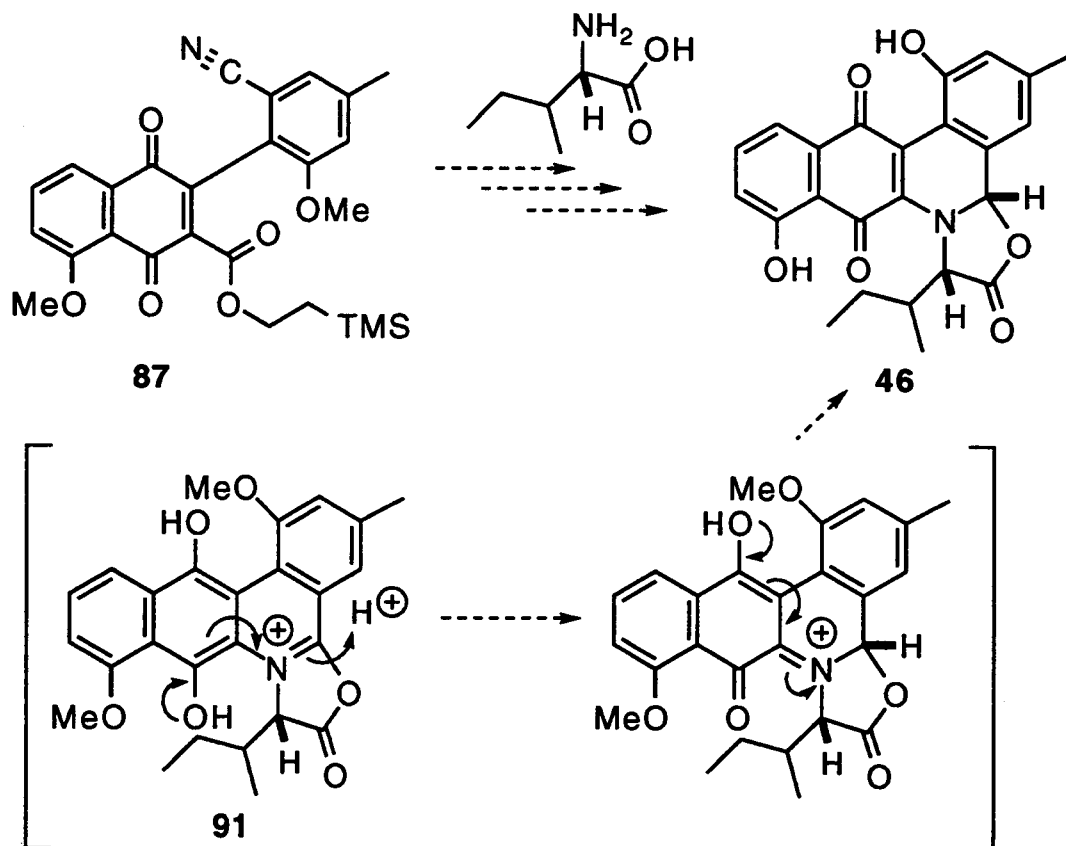
90

Table 3.6. NMR Assignments for the Aminopyridine **90**

Carbon #	¹³ C NMR	¹ H NMR
C1	156.4	
C2	114.7	7.23 (s, 1H)
C3	139.8	
C4	115.3	7.71 (s, 1H)
C4a	120.6	
C5	159.8	
C6a	146.6	
C7	180.4	
C7a	119.7	
C8	159.0	
C9	117.0	7.43 (d, 1H, J = 8.3 Hz)
C10	135.0	7.74 (dd, 1H, J = 8.1, 8.1 Hz)
C11	117.6	7.51 (dd, 1H, J = 7.6, 0.8 Hz)
C11a	137.8	
C12	182.4	
C12a	119.4	
C12b	122.1	
C13	21.7	2.49 (s, 3H)
C1-OMe	56.0	3.86 (s, 3H)
C8-OMe	56.3	3.92 (s, 3H)
C5-NH2		7.94 (s, 2H)

We had imagined that the proposed ring contraction would be facile. These experiments convinced us that this was not the case. This work has led to a re-evaluation of the plausibility of the ring contraction in general. A possible alternative use of **87** would be in a total synthesis of jadomycin **46**. Hydrogen transfer

from the hydroquinone of intermediate **91** may provide the necessary C ring reduction *in situ*.



Experimental

Materials and Methods. Michel-Miller columns were packed with Analtech Sorbent silica gel (10 μ m). Photobromination was accomplished with a 50-Watt GE-17912 halogen spot lamp shining up through an ice water bath in a pyrex container. Toluene was distilled from CaH₂. Nitromethane was distilled, and stored over 4 Å molecular sieves. Piperidine and pyridine

were stored over KOH, and filtered through Al₂O₃ (activity 1) before use. Otherwise, experiments were conducted as described in Chapter 2.

3-Bromo-2-bromomethyl-5-methylanisole (67).(8) To **66** (293 mg), and N-bromosuccinimide (243 mg) was added CH₂Cl₂ (10.0 mL). The mixture was cooled in an ice bath, purged with nitrogen, and a slow stream of nitrogen maintained while the mixture was illuminated by a halogen spotlight. At 2.0 h, illumination was ceased, toluene (10.0 mL) was added, and the CH₂Cl₂ was removed *in vacuo*. After storing at 4 °C overnight, filtration to remove the succinimide, and concentration gave mixed benzylbromides (410 mg). A filtered solution of this material in cyclohexane (2.00 mL) was applied to a Michel-Miller column (2.1 x 30 cm, 10 µm silica gel) eluted (3.0 mL/min) with EtOAc-hexanes (1:9, T_R~96 min). Fractions were analyzed by TLC, collected, and the solvent was removed to afford 278 mg of **67** (69%) as a white solid. An analytical sample was prepared by recrystallization from hexanes: mp 31.0-33.0 °C; IR (KBr) 2938.4, 1601.3, 1560.0, 1042.5 cm⁻¹; ¹H NMR (CDCl₃) δ 2.31 (s, 3H), 3.87 (s, 3H), 4.71 (s, 2H), 6.63 (s, 1H), 7.01 (s, 1H); ¹³C NMR (CDCl₃) δ 21.4, 28.6, 56.1, 111.0, 123.0, 125.6, 141.2, 158.3; EIMS *m/z* (rel intensity) 295.0 (M⁺⁺4, 15 %), 292.9 (M⁺⁺2, 52%), 290.9 (M⁺, 8%), 213.1 (M⁺⁺2-⁸¹Br, 100%); HREIMS calcd for C₉H₁₀⁷⁹Br₂O 291.9098, found 291.9099.

3-Bromo-2,5-bisbromomethylanisole (68).(8) To **66** (90.5 mg), and N-bromosuccinimide (150 mg) was added CH_2Cl_2 (10.0 mL). The mixture was cooled in an ice bath, purged with argon, and a slow stream of argon maintained while the mixture was illuminated from below by a 50 Watt halogen spotlight. At 1.0 h, illumination was ceased and the mixture was filtered through 1 cm of silica gel in a Pasteur pipet, with additional CHCl_3 to complete the transfer. Concentration of the eluent gave mixed benzylbromides (183.3 mg), which were dissolved in cyclohexane (1.0 mL), and applied to a Michel-Miller column (2.1 x 30 cm, 10 μm silica gel) eluted (2.5 ml/min) with EtOAc-hexanes (1:19, TR~82 min). Fractions were analyzed by TLC, and afforded after evaporation 75.6 mg **68** (48%) as a white solid. An analytical sample was prepared by recrystallization from hexanes: mp 126.5-127.0 $^\circ\text{C}$; IR (KBr) 1600.4, 1564.3, 1281.3, 1047.0, 669.0 cm^{-1} ; ^1H NMR (CDCl_3) δ 3.91 (s, 3H), 4.38 (s, 2H), 4.68 (s, 2H), 6.85 (d, 1H, $J = 1.0$), 7.21 (d, 1H, $J = 1.1$); ^{13}C NMR (CDCl_3) δ 27.7, 31.9, 56.3, 110.7, 125.4, 125.8, 126.2, 140.4, 158.5; EIMS m/z (rel intensity) 374.0 (M^{++4} , 17%), 293.0 ($\text{M}^{++4}-^{81}\text{Br}$, 100%), 212.1 ($\text{M}^{++4}-^{81}\text{Br}_2$, 28%); HREIMS calcd for $\text{C}_9\text{H}_9^{79}\text{Br}^{81}\text{Br}_2\text{O}$ 373.8163, found 373.8165.

3-Bromo-2-formyl-5-methylanisole (65).(6) To a solution of **67** (1.03 g) in DMSO (5.0 mL) was added NaHCO_3 (590 mg), and the resulting suspension was heated with a 115 $^\circ\text{C}$ oil bath. After 0.5 h of vigorous stirring, the mixture was cooled and partitioned

between H₂O (50 mL), and CH₂Cl₂ (75 mL). The aqueous layer was re-extracted with additional CH₂Cl₂ (20 mL), and the combined organic extracts were dried over Na₂SO₄. Evaporation gave a solid residue that was purified by chromatography (2.5 x 15 cm, silica gel) eluted with EtOAc-hexanes (1:4). Removal of the solvent *in vacuo* gave 449 mg **65** (56%) as a white solid, with spectral data conforming to that previously reported:(3) mp 79.5-81.5 °C, lit mp unreported.

4-Methoxy-6-methylphthalimide (69). To the bromoaldehyde **65** (13.2 mg) and cuprous cyanide (11 mg) was added dry DMF (2.0 mL). This mixture was heated at reflux for 1 d, then quenched with H₂O (1.0 mL) and NH₄OH (200 µL). CH₂Cl₂ (3 x 3.0 mL) extraction gave after evaporation an 8.2 mg residue which was chromatographed (0.4 x 4 cm, silica gel) with ETOAc-hexanes (2:3) to afford 1.4 mg **69** (13%) as the major product, a white solid: mp 227.5-231.5 °C; IR (NaCl) 3077.2, 1729.1, 752.7 cm⁻¹; ¹H NMR (CDCl₃) δ 7.37 (s, 1H), 7.03 (s, 1H), 4.02 (s, 3H), 2.51 (s, 3H); ¹³C NMR (CDCl₃) δ 167.8, 166.7, 156.8, 148.4, 134.9, 118.0, 116.6, 115.4, 56.3, 22.5; EIMS *m/z* (rel intensity), 191 (M⁺, 100%), 162 (41%); HREIMS calcd for C₁₀H₉NO₃ 191.0582, found 191.0583.

2-Bromo-6-methoxy-4-methylbenzoic acid (70).(25) Aldehyde **65** (44.0 mg) was dissolved in a mixture of *tert*-butanol (5.0 mL), and 2-methyl-2-butene (1.0 mL). To this was added

sodium chlorite (209 mg) and K_2HPO_4 (199 mg) in H_2O (2.0 mL) over 0.15 h, and the mixture was stirred for 24 h. The mixture was evaporated to near dryness, dissolved in H_2O (10 mL, with 1 drop saturated NaHCO_3), and washed with hexanes (2 x 5.0 mL). Acidification with HCl (1M aqueous, pH ~3), followed by extraction with CH_2Cl_2 (4 x 4.0 mL) gave after removal of the solvent 44.8 mg **70** (95%) as a white solid: mp 154.0-155.0 °C; IR (KBr) 2798-3068 (br), 2651.6, 2545.5, 1703.4, 1606.7, 1038.1, 825.1 cm^{-1} ; ^1H NMR (CDCl_3) δ 9.70 (s, 1H, exchanges with D_2O), 7.02 (s, 1H), 6.70 (s, 1H), 3.86 (s, 3H), 2.35 (s, 3H); ^{13}C NMR (CDCl_3) δ 171.4, 157.1, 142.5, 125.3, 121.9, 119.4, 110.9, 56.1, 21.5; EIMS m/z (rel intensity) 246.1 (M^{++2} , 94%), 244.1 (M^+ , 100%), 227.1 (M^+-OH , 17%), 199.1 ($\text{M}^+-\text{CO}_2\text{H}$, 41%); HREIMS calcd for $\text{C}_9\text{H}_9^{79}\text{BrO}_3$ 243.9735, found 243.9735 Anal. Calcd. for $\text{C}_9\text{H}_9\text{BrO}_3$: C, 44.11; H, 3.70; Found: C, 43.99; H, 3.48.

3-Bromo-2-hydroxymethyl-5-methylanisole (71). To a solution of the benzylbromide **67** (7.0 mg) in DMSO (200 μL) was added powdered LiOH (3.1 mg). After 24 stirring at rt, HCl (1.50 mL, 0.3M) was added, and the mixture was extracted with Et_2O (2 x 2.0 mL). Chromatographic purification (1 x 12 cm, silica gel) eluted with CH_2Cl_2 gave after removal of the solvent *in vacuo* 3.2 mg **71** (58%) as a white solid: mp 76.0-77.0 °C; IR (KBr) 3285.3, 1607.9, 1565.6, 1407.8, 1271.4, 1044.4, 1009.6 cm^{-1} ; ^1H NMR (CDCl_3) δ 7.01 (s, 1H), 6.65 (s, 1H), 4.83 (s, 2H), 3.85 (s, 3H),

2.32 (s, 3H); ^{13}C NMR (CDCl_3) δ 158.4, 140.3, 125.5, 125.3, 124.7, 110.7, 60.0, 55.7, 21.2; EIMS m/z (rel intensity) 232.1 (M^++2 , 92%), 230.1 (M^+ , 44%), 199.0 (64%), 151.1 (100%), 123.1 (91%), 104.1 (44%); HREIMS m/z calcd for $\text{C}_9\text{H}_{11}^{81}\text{BrO}_2$ 231.9922 (M^++2), found 231.9922, calcd for $\text{C}_9\text{H}_{11}^{79}\text{BrO}_2$ 229.9942 (M^+), found 229.9942.

4-Methoxy-6-methylbenzisofuran-1-one (72). In a 1-mL conical vial was placed the benzyl alcohol **71** (12.8 mg), cuprous cyanide (25.0 mg), and N-methylpyrrolidone (300 μL). This mixture was heated with a 160 $^\circ\text{C}$ oil bath for 6 h, cooled, quenched with ammonium hydroxide (0.50 mL), and extracted with CHCl_3 which gave after removal of the solvent 26.2 mg of the crude reaction mixture. Fractionation on a silica gel column (1 x 12 cm) eluted $\text{MeOH-CH}_2\text{Cl}_2$ (1:19) gave two fractions: 2.3 mg **72**, and 1.2 mg of a second compound that was converted to **72** upon standing (72 h as a solution in CHCl_3) (3.5 mg total, 36%): mp 132.5-134.8 $^\circ\text{C}$; IR (KBr) 1753.1 (unsaturated ester), 1112.2, 770.5 cm^{-1} ; ^1H NMR (CDCl_3) δ 7.30 (s, 1H), 6.91 (s, 1H), 5.23 (s, 2H), 3.90 (s, 3H), 2.46 (s, 3H); ^{13}C NMR (CDCl_3) δ 171.2, 153.8, 141.3, 132.3, 127.3, 117.2, 115.9, 68.0, 55.4, 21.6; EIMS m/z (rel intensity) 178.2 (47%), 149.1 (100%), 134.1 (2%), 121.1 (37%); HREIMS calcd for $\text{C}_{10}\text{H}_{10}\text{O}_3$ 178.0630, found 178.0630.

3-Cyano-2,5-dimethylphenol. To bromophenol **73** (100.0 mg) and cuprous cyanide (152.0 mg) in a 10-mL flask was added N-methylpyrrolidone (2.0 mL). This mixture was heated with a 145 °C oil bath for 14 h, quenched with HCl (1.0 mL, 1M), and extracted with Et₂O (3 x 2.0 mL). Drying over MgSO₄, and evaporation gave the crude material which was recrystallized from CHCl₃ and afforded 30.0 mg of the title compound as white, clustered needles. The mother liquor from the recrystallization was subsequently chromatographed with EtOAc-hexanes (1:4) to afford an additional 35.3 mg of the title compound (90%): mp 143.0-144.0 °C; IR (KBr) 3345.5, 2235.6, 1316.7, 1287.9, 846.2 cm⁻¹; ¹H NMR (CDCl₃) δ 6.98 (s, 1H), 6.86 (s, 1H), 5.96 (br s, 1H, exchanges with D₂O), 2.38 (s, 3H), 2.28 (s, 3H); ¹³C NMR (CDCl₃) δ 154.2, 137.6, 125.3, 125.2, 120.5, 118.3, 113.4, 20.7, 13.7; EIMS *m/z* (rel intensity) 147.1 (100%, M⁺), 132.1 (47%, M⁺-CH₃), 116.1 (3%, M⁺-OCH₃); HRMS calcd for C₉H₉NO: 147.0684, found 147.0684; Anal. Calcd. for C₉H₉NO: C, 73.44; H, 6.17; N, 9.52. Found: C, 73.31; H, 5.98; N, 9.27.

3-Cyano-2,5-dimethylanisole (74). In a 25-mL flask was placed the cyanophenol (52.6 mg), dimethyl sulfate (93.0 mg), and CH₂Cl₂ (1.50 mL), and the mixture was stirred until homogeneous. Aqueous NaOH (1.50 mL, 1M) was then added, followed by benzyltributylammonium chloride (11.6 mg). After 2 d of stirring, the layers were separated, and the aqueous layer extracted with CH₂Cl₂ (3 x 4.0 mL). The combined organic

extracts were dried over Na_2SO_4 , and gave upon evaporation 80.3 mg crude **74**. This was chromatographed (1 x 12 cm silica gel) with CH_2Cl_2 -hexanes (1:1) eluent, which afforded after removal of the solvent *in vacuo* 55.0 mg **74** (96%) as a white solid: mp 69.2-69.7 °C; IR (KBr) 2926.2, 2228.9, 1580.7, 1290.2, 842.4 cm^{-1} ; ^1H NMR (CDCl_3) δ 6.98 (s, 1H), 6.83 (s, 1H), 3.84 (s, 3H), 2.35 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (CDCl_3) δ 157.5, 137.4, 127.9, 124.0, 118.3, 115.2, 113.3, 55.5, 21.1, 13.8; MS m/z (rel intensity) 161.1 (M^+ , 100%), 146.1 ($\text{M}^+ - \text{CH}_3$, 54%), 130.1 ($\text{M}^+ - \text{OCH}_3$, 9%) 116.1 (13%); HRMS calcd for $\text{C}_{10}\text{H}_{11}\text{NO}$ 161.0841, found 161.0841; Anal. Calcd. for $\text{C}_{10}\text{H}_{11}\text{NO}$: C, 74.50; H, 6.88; N, 8.69. Found: C, 74.61; H, 6.69; N, 8.85.

3-Cyano-2-bromomethyl-5-methylanisole (75).(8) To **74** (149 mg) and N-bromosuccinimide (167 mg) was added CH_2Cl_2 (10.0 mL). The mixture was cooled in an ice-water bath, purged with argon, and a slow stream of argon maintained while the mixture was illuminated from below by a 50 Watt halogen spotlight. At 2.0 h, illumination was ceased and the mixture was filtered through 1 cm of silica gel in a Pasteur pipet, with additional CHCl_3 added to complete the transfer. Concentration gave mixed benzylbromides (260 mg), which were dissolved in EtOAc-cyclohexane (1:9, 2.00 mL) and applied to a Michel-Miller column (2.1 x 30 cm, 10 μm silica gel) eluted (15 mL/min) with EtOAc-hexanes (1:9, VR~200 mL). Fractions were analyzed by TLC, pooled, and evaporated *in vacuo* to afford 160 mg of **75**

(72%) as a white solid. An analytical sample was prepared by recrystallization from EtOAc-hexanes: mp 115.0-115.5 °C; IR (KBr) 2362.4, 1457.4, 1305.3, 1070.3, 772.4 cm^{-1} ; ^1H NMR (CDCl_3) δ 2.38 (s, 3H), 3.92 (s, 3H), 4.68 (s, 2H), 6.92 (s, 1H), 7.06 (s, 1H); ^{13}C NMR (CDCl_3) δ 21.5, 24.5, 56.0, 113.5, 116.4, 116.8, 125.1, 126.7, 141.2, 157.5; NOEDIFF, CMIV60; EIMS m/z (rel intensity) 240.9 (M^{++2} , 10 %), 238.9 (M^+), 160.0 (100%), 130.0 (61%); HREIMS calcd for $\text{C}_{10}\text{H}_{10}^{79}\text{BrNO}$ 238.9946 (M^+), found 238.9946; Anal. Calcd. for $\text{C}_{10}\text{H}_{10}\text{BrNO}$: C, 50.03; H, 4.20; N, 5.83; found: C, 50.00; H, 4.18; N 5.86.

3-Cyano-2-formyl-5-methylanisole (54).(6,10,11) Crude 3-cyano-2-bromomethyl-5-methylanisole **75** (2.55 g, from 10.62 mmol of the dimethyl anisole **74** as described above) and NaHCO_3 (8.990 g) in a 100-mL flask were heated with a 105 °C oil bath. DMSO (5.00 mL) was immediately added, and following 0.2 h vigorous stirring, the mixture was cooled and diluted with CH_2Cl_2 (75.0 mL). This mixture was filtered through a silica gel column (2.5 x 16 cm) eluted with EtOAc- CH_2Cl_2 (1:9). Concentration of the eluent, and evaporation of the DMSO (5 μm , 3d) gave a mixture of the aldehyde **54** and the corresponding the alcohol **76** which were added in CH_2Cl_2 (30 + 30 mL) to an activated DMSO complex prepared as follows. To a 500-mL flask containing CH_2Cl_2 (60 mL) in a dry ice-acetone bath at -75 °C was added oxaloyl chloride (1.02 mL) followed by DMSO (1.81 mL). After stirring 0.3 h, the aldehyde mixture was added over

0.2 h. Triethylamine (7.40 mL) was added after an additional 0.5 h, and the cooling bath was removed. Solvent removal *in vacuo* (5.0 μ m, 1 d) was followed by column chromatography (2.5 x 16 cm, silica gel) eluted with EtOAc-CH₂Cl₂ (1:49) which afforded 1.93 g of the aldehyde containing mixture. Solvent removal (5 μ m, overnight) was followed by a second identical chromatography affording 0.850 g **54** (46%, 3 steps) as a white solid: mp 155.0-155.8 °C; IR (KBr) 3090.7, 2226.9, 1683.3, 1603.2, 1299.3, 1205.2, 1079.1 cm⁻¹; ¹H NMR (CDCl₃) δ 10.43 (s, 1H), 7.17 (s, 1H), 7.07 (s, 1H), 3.98 (s, 3H), 2.46 (s, 3H); ¹³C NMR (CDCl₃) δ 186.6, 161.9, 147.1, 127.9, 122.8, 117.4, 116.8, 111.6, 56.1, 21.9; EIMS *m/z* (rel intensity) 175.1 (100 %), 158.1 (38%), 146.1 (48%), 133.1 (23%), 117.1 (40%), 90.1 (29%); HREIMS calcd for C₁₀H₉NO₂ 175.0633, found 175.0633.

2-(2-amido-6-methoxy-4-methylphenyl)-Nitroethene

(79).(17) Method a. In a 5-mL Teflon vial with septum and spinbar was placed the isoindole **80** (11.7 mg), KF (3.1 mg), and nitromethane (~0.50 mL). After stirring for 2 d, the mixture was diluted with toluene (2.0 mL), and the filtrate collected to afford 10.0 mg **79** (85%).

Method b. In a 5-mL Teflon vial with septum and spinbar was placed the cyanoaldehyde **54** (233.2 mg), KF (14.6 mg), and nitromethane (~2.10 mL). After stirring for 4 d, the mixture was filtered with additional toluene (2.0 mL) to afford 220.2 mg **79** (70%) as a yellow chalklike powder. A sample for combustion

analysis was recrystallized from glyme: mp 91.2-91.6 °C; IR (KBr) 3381.2, 3376.6, 1652.2, 1614.7, 1601.3, 1558.9, 1505.3, 1334.4, 1288.3, 969.1 cm^{-1} ; ^1H NMR ($\text{DMF-}d_7$) δ 8.49 (d, 1H, J = 13.5), 8.10 (d, 1H, J = 13.5), 8.06 (s, 1H, NH), 7.76 (s, 1H, NH), 7.19 (s, 1H), 7.05 (s, 1H), 4.07 (s, 3H), 2.42 (s, 3H); EIMS m/z (rel intensity) 236.0 (M^+ , 6%), 191.0 (12%), 190.0 (100%), 176.0 (49%), 147.0 (15%), 91.0 (14%); ^{13}C NMR unobtainable due to facile isomerization; HREIMS calcd. for $\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_4$ 236.0797, found 236.0797; Anal. Calcd. for $\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_4$: C, 55.93; H, 5.12; N, 11.86. Found: C, 55.64; H, 4.93; N, 11.71.

4-Methoxy-6-methyl-3-nitromethylisoindolin-1-one (80). In a 2-dram vial was placed the nitroolefin **79** (35.2 mg), and DMSO (1.50 mL). This mixture was stirred for 14 h, and the solvent was removed *in vacuo* (redissolution in CHCl_3 , its evaporation, and resubjecting the sample to vacuum, twice, was required to remove the DMSO completely) which afforded 35.1 mg **80** (100%) as a white solid: mp 92.0-93.2 °C; IR (KBr) 3183.0, 3072.0, 3033.8, 2888.6, 1714.8, 1553.3, 1361.1 cm^{-1} ; ^1H NMR (CDCl_3) δ 7.30 (s, 1H), 7.15 (s, 1H (NH)), 6.89 (s, 1H), 5.29 (dd, 1H, J = 14.4, 2.5), 5.21 (d, 1H, J = 10.2), 4.20 (dd, 1H, J = 14.6, 10.2), 3.92 (s, 3H), 2.46 (s, 3H); ^{13}C NMR (CDCl_3) δ 170.1, 154.4, 142.2, 133.8, 125.9, 116.7, 114.9, 76.4, 55.6, 52.1, 21.8; DEPT CMV175 76.4, methylene; ^1H - ^1H COSY 2.46-6.89, 2.46-7.30, 3.92-6.89, 4.20-5.21, 4.20-5.29, 5.21-5.29, 5.21-7.15 (NH to benzylic), 6.89-7.30; NOESY 2.46-6.89, 2.46-7.30, 3.92-6.89,

4.20-5.29, 5.21-7.15 (NH to benzylic); EIMS m/z (rel intensity) 236.1 (M^+ , %), 189.1 (86%), 176.1 (100%); HREIMS calcd. for $C_{11}H_{12}N_2O_4$ 236.0797, found 236.0797.

3-Cyano-2-hydroxymethyl-5-methylanisole (76).(19)

Cyanoaldehyde **54** (49.5 mg) in THF (5.00 mL) was cooled in a dry ice-acetone bath. Diisobutylaluminum hydride (0.700 mL, 1M soln in cyclohexane) was added, the mixture was stirred for 2.5 h, and was quenched by sequential addition of H_2O (20 μL) and glacial HOAc (100 μL). Removal of the cooling bath, addition of CH_2Cl_2 (5.00 mL), and stirring for 1h gave a turbid solution which was filtered. The solvent was removed, and the residue (52.6 mg) taken up in $CHCl_3$ and filtered again to afford after removal of the solvent 46.0 mg **76** (92%) as an amorphous white solid: mp 94.5-96.2 $^{\circ}C$; IR (KBr) 3350.6, 2229.0, 1757.8, 1607.5, 1464.8, 1330.5, 1087.6 cm^{-1} ; 1H NMR ($CDCl_3$) δ 7.04 (s, 1H), 6.93 (s, 1H), 4.83 (s, 2H), 3.90 (s, 3H), 2.38 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 158.0, 140.3, 129.3, 124.8, 117.4, 116.0, 112.9, 58.8, 55.8, 21.3; EIMS m/z (rel intensity) 177.0 (100%), 162.0 (61%), 148.0 (99%), 117.0 (36%); HREIMS calcd for $C_{10}H_{11}NO_2$ 177.0790, found 177.0790.

2-Bromomethyl-3-formyl-5-methylanisole (84).(21) A

solution of **75** (10.7 mg) in toluene (5.00 mL) was cooled in a dry ice-acetone bath. Diisobutylaluminum hydride (56 μL , 1M soln in cyclohexane) was added, and the mixture was stirred for 5.0 h.

Acetone (50 μ L), celite (45.2 mg), and HOAc (50 μ L) were sequentially added, the cooling bath was removed, and the mixture was diluted with CHCl_3 (5.0 mL). Overnight stirring, filtration, and evaporation of the filtrant *in vacuo* gave 10.2 mg **84** (94%) as a white solid: mp 91.4-93.5 $^{\circ}\text{C}$; ^1H NMR (CDCl_3) δ 10.22 (s, 1H), 7.25 (s, 1H), 6.96 (s, 1H), 5.05 (s, 2H), 3.94 (s, 3H), 2.43 (s, 3H); ^{13}C NMR (CDCl_3) δ 192.2, 157.8, 140.5, 133.9, 125.9, 124.7, 117.2, 56.2, 22.7, 21.6; EIMS m/z (rel intensity) 244.1 (M^{++2} , 5%), 242.1 (M^+ , 4%), 163.1 (100%); HREIMS m/z calcd for $\text{C}_{10}\text{H}_{11}\text{BrO}_2$ 241.9942 (M^+), found 241.9943.

3-(2-Cyano-6-methoxy-4-methylphenyl)-2-propenoic acid (85).(22) To a solution of **54** (344.6 mg) in toluene (50.0 mL) was added malonic acid (411.4 mg), followed by piperidine (50 μ L). The reaction vessel was fitted with a Dean-Stark Trap, and was heated in a 175 $^{\circ}\text{C}$ oil bath for a total of 2.5 h. The toluene was removed *in vacuo*, and the resulting solid was redissolved in EtOAc (50 mL), and washed with HCl (1M, 3 x 10 mL). The aqueous extracts were washed with EtOAc (2 x 10 mL), and the combined organic extracts were dried over Na_2SO_4 . Removal of the solvent *in vacuo* gave a white solid which was >95% **85** by ^1H NMR. Recrystallization from acetone gave 393.1 mg of pure **85** (92%) as white needles: mp 232.3-234.0 $^{\circ}\text{C}$; IR (KBr) 1689.5, 1621.5, 1598.2, 1317.4, 1308.4, 1215.1 cm^{-1} ; ^1H NMR ($\text{DMSO-}d_6$) δ 12.59 (s, 1H, exchangeable in D_2O), 7.72 (d, 1H, $J = 16.0$),

7.31 (s, 2H, two coincidental resonances), 6.78 (d, 1H, $J = 16.1$), 3.92 (s, 3H), 2.36 (s, 3H); ^{13}C NMR (DMSO- d_6) δ 167.4, 158.5, 142.5, 135.7, 126.0, 124.4, 121.6, 117.6 (perhaps two coincidental resonances), 112.3, 56.2, 20.8; EIMS m/z (rel intensity) 217.0 (100%), 170.0 (74%), 130.0 (36%), 103.0 (41%); HREIMS calcd for $\text{C}_{12}\text{H}_{11}\text{NO}_3$ 217.0740, found 217.0739.

2-Propanoic acid, 3-(2-cyano-6-methoxy-4-methylphenyl)-2-(trimethylsilyl)ethyl ester (86).(3) A solution of 2-trimethylsilylethanol (0.500 mL) in THF (3.00 mL) was cooled in a dry ice-acetone bath, and *n*-butyllithium (2.66 mL, 1.18M) was added by syringe. This mixture was allowed to warm to rt over 1 h, then was rechilled with the dry ice-acetone bath. In a second vessel thionyl chloride (2.00 mL) was added to the propanoic acid **85** (100.9 mg), and the mixture was stirred for 1.5 h, then diluted with CCl_4 (3.00 mL). After an additional 0.5 h of stirring at rt, the volatile components were removed *in vacuo*, and the resulting acid chloride redissolved in THF (4.00 mL). The acid chloride was added to the preformed alkoxide with additional THF (2.00 mL) to complete the transfer. After 1 h, the cooling bath was removed, and the reaction mixture was stirred for an additional 2 h at rt. The volatile components were removed, the mixture was redissolved in CH_2Cl_2 (20 mL), and silica gel (2.0 g) was added. Filtration, with additional CH_2Cl_2 (10 mL) to complete the transfer, gave after evaporation of the filtrate 157.0 mg crude **86**. Chromatography, with CH_2Cl_2 as eluent (2 x 18

cm, silica gel), gave after removal of the solvent 138.3 mg **86** (94%) as a white solid: mp 149.9-150.2 °C; IR (KBr) 2952.2, 2230.0, 1629.7, 1309.4, 1289.2, 1158.4, 840.1 cm^{-1} ; ^1H NMR (CDCl_3) δ 7.92 (d, 1H, $J = 16.2$), 7.12 (s, 1H), 6.95 (s, 1H), 6.92 (d, 1H, $J = 16.2$), 4.31 (m, 2H), 3.92 (s, 3H), 2.40 (s, 3H), 1.08 (m, 2H), 0.07 (s, 9H); ^{13}C NMR (CDCl_3) δ 167.1, 158.7, 141.5, 136.0, 126.1, 124.5, 123.2, 117.6, 116.2, 113.5, 62.8, 55.7, 21.4, 17.3, -1.55 (three coincidental resonances); EIMS m/z (relative intensity) 317.2 (M^+ , 16%), 274.2 (24%), 230.2 (68%), 200.1 (90%), 73.1 (100%); HREIMS m/z calcd for $\text{C}_{17}\text{H}_{23}\text{NO}_3\text{Si}$ 317.1447 (M^+), found 317.1447. Anal. Calcd. for $\text{C}_{17}\text{H}_{23}\text{NO}_3\text{Si}$ C, 64.32; H, 7.30; N, 4.41. Found: C, 64.20; H, 7.17; N, 4.39.

2-Naphthalenecarboxylic acid, 3-(2-cyano-6-methoxy-4-methylphenyl)-1,4-dihydro-8-methoxy-1,4-dioxo-, 2-(trimethylsilyl)ethyl ester (87).(3) The cyanophthalide **88** was dissolved in dry toluene (3.00 mL) by briefly heating. A solution of lithium hexamethyldisilylamide in hexanes (0.680 mL, 1.0M) was added, and the mixture was stirred at rt for 1 h. This solution was then cooled in a dry ice-acetone bath, and diluted with THF (12.0 mL). To the resulting suspension of the cyanophthalate anion was added **86** (187.0 mg) as a solution in THF (6.00 + 6.00 mL), and the cooling bath was maintained for 4 h. After allowing the reaction to gradually warm to rt, stirring was continued until the color of the solution had changed from orange to green (22-24 h). At 1 d, HCl (1.50 mL, 1.0M) was added, and after stirring

briefly the mixture was extracted with CH_2Cl_2 (20 mL). Next, the resulting organic layer was dried over Na_2SO_4 , then evaporated *in vacuo* onto silica gel (3.0 g). After standing open to the air for 1 d, the sample was loaded onto a column (2.5 x 20 cm, silica gel) and eluted with $\text{EtOAc-CH}_2\text{Cl}_2$ (1:19) to afford after evaporation 207.0 mg **87** (74%) as a yellow solid: mp 91.5-92.8 °C; IR (KBr) 2230.5, 1735.8, 1666.2, 1275.7, 1114.9 cm^{-1} ; UV λ_{max} see text; ^1H NMR (CDCl_3) δ 7.77 (dd, 1H, $J = 7.7, 1.3$ Hz), 7.71 (dd, 1H, $J = 8.2, 8.2$), 7.35 (dd, 1H, $J = 8.2, 1.4$), 7.13 (s, 1H), 6.97 (s, 1H), 4.13-4.19 (m, 2H), 4.02 (s, 3H), 3.76 (s, 3H), 2.42 (s, 3H), 0.82-0.93 (m, 2H), -0.01 (s, 9H); ^{13}C NMR (CDCl_3) δ 182.9, 180.2, 163.4, 159.9, 157.0, 143.4, 141.8, 138.3, 135.3, 133.6, 124.7, 121.8, 119.7, 119.4, 118.3, 116.8, 116.2, 113.4, 64.2, 56.5, 56.0, 21.5, 17.2, -1.73 (three coincidental resonances); EIMS m/z (relative intensity) 477.3 (M^+ , 5%), 449.2 (21%), 434.2 (39%), 390.2 (69%), 73.1 (100%); HREIMS m/z calcd for $\text{C}_{26}\text{H}_{22}\text{NO}_6\text{Si}$ 477.1608 (M^+), found 477.1607. Anal. Calcd. for $\text{C}_{26}\text{H}_{22}\text{NO}_6\text{Si}$ C, 65.39; H, 5.70; N, 2.93; Found C, 65.70; H, 5.64; N, 2.89.

2-Amino-3-(2-cyano-6-methoxy-4-methylphenyl)-1,4-dihydro-8-methoxy-1,4-dioxonaphthalene (89).(3) To a solution of **87** (14.1 mg) in THF (5.0 mL) at 0-5 °C was added tetrabutylammonium fluoride [60 μL , 1.0M soln. in THF (10% H_2O)]. After removing the cooling bath, and allowing the dark brown solution to stir at rt for 1 h, two steel needles were

sequentially introduced, carrying gentle streams of air and anhydrous ammonia respectively, into the THF solution. The flow of gas was maintained for approximately 2 h, until all the solvent had evaporated. The mixture was partitioned between CH₂Cl₂ (5.0 mL), EtOAc (5.0 mL), and H₂O (5.0 mL), and the organic layer was evaporated *in vacuo*. Suspension of the resulting brown solid in MeOH-CH₂Cl₂ (1:9) and chromatography (1 x 12 cm, silica gel) in this same system provided 4.6 mg of the aminodione **89** (45%) as a yellow solid. Bright yellow needles were obtained by recrystallisation from DMF-H₂O, or a microcrystalline precipitate was obtained by recrystallization from EtOAc: mp 289.6-290.2 °C; IR (KBr) 3448.0, 2182.5, 1725.7, 1705.5, 1283.0, 1057.6 cm⁻¹; ¹H NMR (DMSO-*d*₆) δ 7.84 (d, 1H, J = 7.6), 7.19 (dd, 1H, J = 7.9, 7.9), 7.20 (d, 1H, J = 8.1), 7.18 (s, 1H), 7.03 (s, 1H), 5.12 (s, 2H, exchangeable), 4.05 (s, 3H), 3.78 (s, 3H), 2.42 (s, 3H); ¹³C NMR (DMSO-*d*₆) δ 180.2, 179.3, 160.0, 157.3, 146.9, 141.0, 135.9, 135.6, 125.6, 122.2, 119.6, 118.3, 117.5, 116.7, 116.1, 115.2, 108.9, 56.4, 56.0, 21.4; EIMS *m/z* (rel intensity) 348.0 (38%), 317.0 (100%), 300.0 (8%); HREIMS calcd for C₂₀H₁₆N₂O₄ 348.1110, found 348.1109.

5-Amino-6-aza-1,8-dimethoxybenz[*a*]anthracene-7,12-dione (90).(3) To a solution of the esterdione **87** (68.1 mg) in THF (10.0 mL) was added tetrabutylammonium fluoride [285 μL, 1M soln. in THF (10% H₂O)] under an atmosphere of argon. The light yellow color of the solution immediately changed to black,

and at 1 h the solvent was removed *in vacuo*. The remaining viscid liquid was resuspended in CH₂Cl₂ (10.0 mL). Ammonia gas was passed through this suspension for 2 h with periodic addition of CH₂Cl₂ to maintain an approximately constant solvent volume. A red precipitate was formed, and at 23 h was filtered to give 34.8 mg of **90**. The liquid portion was evaporated to dryness, and applied to a column (1 x 12 cm, silica gel) which was eluted with MeOH-CH₂Cl₂ (1:19). Fractions 4-7 (5 mL each) contained the compounds of interest. These fractions were evaporated, resuspended in CH₂Cl₂ (1.00 mL) and filtered to give after evaporation red and yellow solids from the filtrate and filtrant, respectively. Both portions were then washed with water (2 x 1.00 mL) to remove the remaining tetrabutylammonium fluoride, and dried *in vacuo*, which afforded an additional 5.6 mg of the aminopyridine **90** (40.4 mg total, 81%), and 5.2 mg of the aminodione **89** (10%). Data for **90**: mp >300 °C; IR (KBr) 3425.3, 1647.8, 1584.5, 1504.1, 1286.4, 1125.0 cm⁻¹; ¹H NMR (DMSO-*d*₆) δ 7.94 (s, 2H), 7.74 (dd, 1H, J = 8.1, 8.1 Hz), 7.71 (s, 1H), 7.51 (dd, 1H, J = 7.6, 0.8 Hz), 7.43 (d, 1H, J = 8.3 Hz), 7.23 (s, 1H), 3.92 (s, 3H), 3.86 (s, 3H), 2.49 (s, 3H); ¹³C NMR (DMSO-*d*₆) δ 182.4, 180.3, 159.8, 159.0, 156.4, 146.6, 139.8, 137.8, 135.0, 122.1, 120.6, 119.7, 119.4, 117.6, 117.0, 115.3, 114.7, 56.3, 56.0, 21.7; HMBC correlations (DMSO-*d*₆) 7.94-120.6, 7.74-159.0, 7.74-137.8, 7.74-117.0 (2 bond), 7.71-159.8, 7.71-122.1, 7.71-114.7, 7.71-21.7, 7.51-182.4, 7.51-119.7, 7.51-117.0, 7.43-119.7, 7.43-117.6, 7.23-156.4 (2 bond), 7.23-122.1, 7.23-

115.3, 7.23-21.7, 3.92-159.0, 3.86-156.4, 2.49-139.8 (2 bond), 2.49-115.3, 2.49-114.7; EIMS: m/z (rel intensity) 348.1 (M^+ , 79%), 331.0 (100%), 317.0 (9%), 305.0 (10%), 301.0 (22%), 290.0 (11%); HREIMS calcd for $C_{20}H_{16}N_2O_4$ 348.1110, found 348.1110.

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4. Reactivity and Structures of the Kinamycins

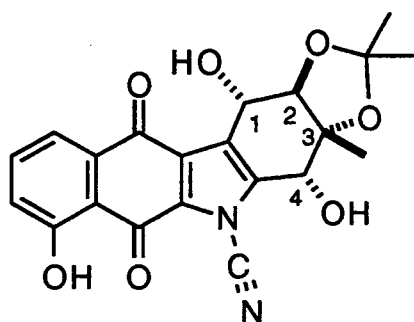
The kinamycin antibiotics had been assigned the benzo[*b*]carbazole skeleton, a structure unique among naturally occurring compounds.(1,2) Our group and others have subsequently isolated several additional members of this family of compounds, and assigned their structures based on analogy to the original structure elucidation.(3,4,5) Due to the relative scarcity of protons for NMR analysis inherent in this ring system, several key aspects of these structures were originally elucidated by a single crystal diffraction study of kinamycin C *para*-bromobenzoate, 1.(1) In this study, Furusaki and Matsui experienced trouble with crystal stability, and achieved a final R factor of 8.9%. Several crystals were analyzed, and partial data sets were obtained before each crystal in turn crumbled. The low R factor obtained can be due to intrinsic instability of the crystal when exposed to X-irradiation, as proposed by the authors, or may have been due to the choice of benzene as the recrystallization solvent. The unit cell contained four molecules of benzene, and four molecules of the kinamycin derivative. The reported instability was likely caused by a slight warming of the crystal by X-radiation, volatilizing the benzene present, thereby disrupting the crystal lattice. Benzene was also certainly detracting from the quality of the data set because it did not

occupy a well defined position in the unit cell, so its individual atoms could not be resolved.

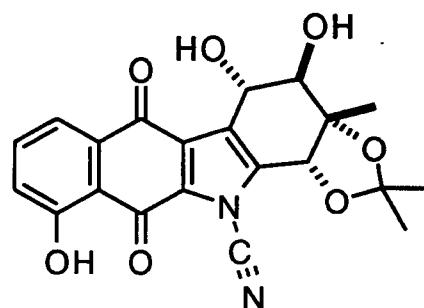
Partial structures of the kinamycins were determined prior to crystallographic analysis, but four elements rely on the crystallographic work. The 6,6,5,6 ring system, the C3 regiochemistry, the absolute stereochemistry, and the nature and attachment of the cyanamide functionality. The following analysis suggested that none of these features, apart from the assignment of the ring system, was beyond question in the previous study. The reasoning for concern about each point is briefly described below.

C3 Regiochemistry

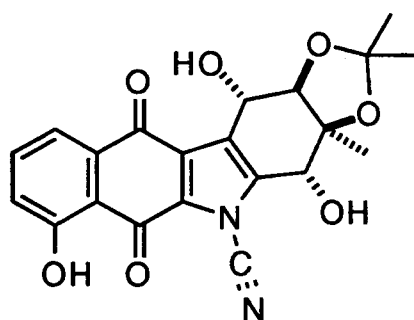
Bearing no protons, the C3 regiochemistry cannot be ascertained directly from coupling data. The crystallographic data assigned the trans C2-O/C3-O configuration. A 2D nuclear Overhauser effect (NOESY) experiment on kinamycin A83016 from an unidentified actinomycete recently gave supporting evidence for the previously reported regiochemistry.⁽⁵⁾ In a study of desacetyl kinamycin (KF), an acetonide was formed between the C2 and C3 oxygens, rather than the C3 and C4 oxygens, which appear to be sterically similar and cis.⁽²⁾



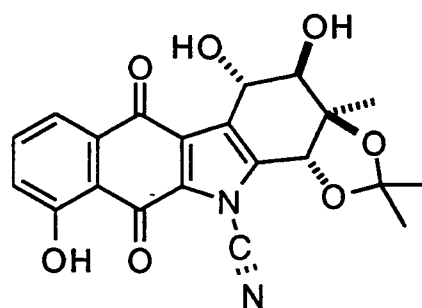
KF-2,3-acetonide



KF-3,4-acetonide



iso KF-2,3-acetonide



iso KF-3,4-acetonide

Figure 4.1. Possible Kinamycin F Acetonides

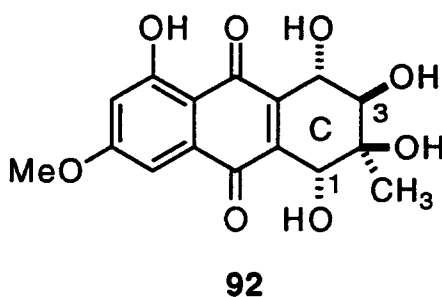
Molecular modeling of the competing acetonides using the MM+ force field in Autodesk Inc.'s HyperChem™, on an Iris Indigo workstation, suggested a different outcome. Energies given in Table 4.1 are sums of steric, torsional, and dipolar interactions for each compound. Each value was the lowest obtained for four minimizations with an intervening 3 ps heating cycle with 0.01 ps intervals as follows: 100 to 500 °K over 1 ps; equilibrate at 500 °K for 1 ps; 500 °K to 0 °K over 1 ps, then reminimize. KF is kinamycin F, and iso KF is its epimer at C3. Axial and equatorial designations are for the C2 and C3 hydroxyl groups.

Table 4.1. Modeling of Possible Acetonides

compound:	diequatorial:	diaxial:
KF	39.705 kcal/mol	40.763 kcal/mol
iso KF	39.624 kcal/mol	39.402 kcal/mol

compound:	2,3 acetonide:	3,4 acetonide:
KF	49.204 kcal/mol	44.988 kcal/mol
iso KF	50.974 kcal/mol	55.511 kcal/mol

Doubt about the C3 regiochemistry was reinforced by the recognition that the alternative regiochemistry was present in altersolanol A, **92**. A labelling study with sodium [2-²H₃, 1-¹³C]acetate(6) has shown that **92** is derived from the regular folding of a single polyketide chain. Furthermore, deuterium was retained at C1, but not C3, which suggested that an aromatic intermediate may have been involved in the biosynthesis.



Absolute Configuration

Absolute stereochemistry determination by the heavy atom method was used for the kinamycin C *para*-bromobenzoate

structure. Furusaki and Matsui did not however report the R factor differences used to determine the absolute stereochemistry, as would be customary,(7) or mention the process of their determination.(1) Since their methodology was unknown, an independent assessment of the validity of their finding was not possible.

A complementary determination of the absolute stereochemistry could be done by optical or NMR methods with a suitable derivative. Alternatively, incorporation of a new stereogenic center of known configuration into a suitable crystal would allow an unambiguous confirmation by X-ray crystallography. No confirmation of the reported stereochemistry had been reported.

Cyanamide Substituent

^{13}C NMR resonances for the cyanamide moiety in the kinamycins have been a paradox since they were first identified.(3) N-cyanamide ^{13}C NMR resonances determined for several model systems have ranged from 103.6 to 119.4 ppm,(3,8) whereas the resonance for kinamycin D was at 78.5 ppm (83.7 ppm for prekinamycin diacetate).(3,4) Similarly, the IR absorbances of the proposed cyanamide moiety have varied from model systems which appear from 2237 to 2245 cm^{-1} ,(8) occurring instead at 2155 cm^{-1} for kinamycin D (2162 cm^{-1} for prekinamycin).(4,9)

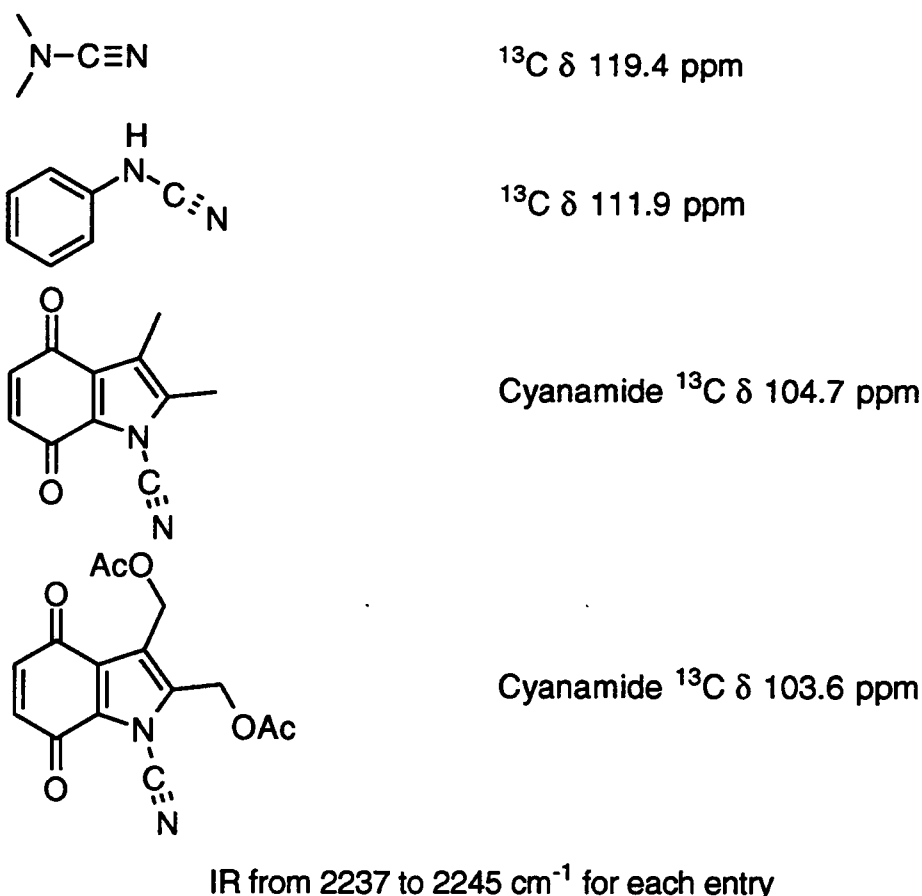


Figure 4.2. Spectral Data for Model Cyanamides

An inverse detected long-range ^1H - ^{13}C NMR correlation experiment(10) was performed on **93**.(4) Correlations expected for the A and D rings of **93** were readily observed. Examination of the row in the transformed spectrum corresponding to the carbon atom assigned as the cyanamide (δ 83.7) showed a clear correlation to the aromatic proton at C4 (δ 6.84), which was improbable in the assigned structure. This correlation, although

observed in the spectra of previous *S. murayamaensis* metabolites, had in the past been overlooked due to its proximity to the solvent resonance for CHCl_3 (δ 77.0). Similarly, a fragmentation of **93** (374, M^+-28) had previously been observed by EIMS. This observed peak could be rationalized as a $[\text{M}+2\text{H}-\text{CN}]^+$ fragment. Enlisting the aid of Brian Arbogast, FABMS spectra of **93** were determined in 3-nitrobenzyl alcohol matrix, and gave an exact mass corresponding to the fragment in question calculated for $\text{C}_{22}\text{H}_{17}\text{O}_6$ 377.1025 $[\text{M}+3\text{H}-2\text{N}]^+$, found 377.1026, consistent with loss of N_2 , not CN. A similar fragmentation was also observed for **12** itself, calculated for $\text{C}_{18}\text{H}_{12}\text{O}_4$ 292.0736 $[\text{M}+2\text{H}-\text{N}_2]^+$, found 292.0737. The molecular formula for the molecular ions of **93** and **12** were also confirmed.

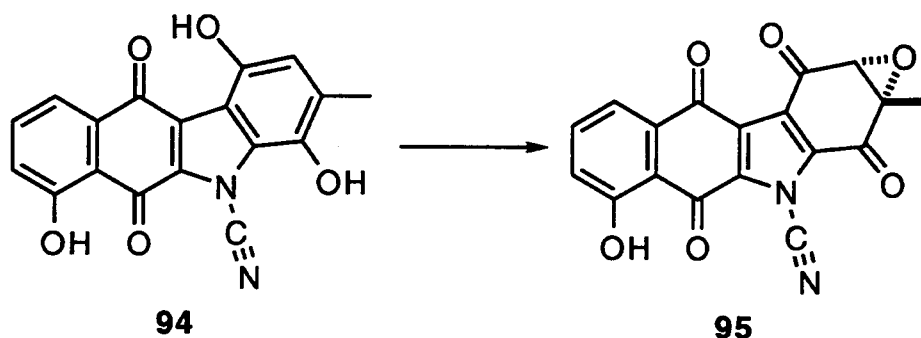
Concurrently with this work Echavarren and Tamayo reported the total synthesis of **12**, the structure proposed for prekinamycin.(11) The ^1H NMR of their synthetic **12**, and that of prekinamycin were not identical, albeit the structure of the synthetic **12** has also been called into question.(12) Dr. Tamayo has, since joining our group, used her experience in transition metal catalysis, and in working with quinones related to **12**, to reinvestigate the structure of prekinamycin.

Disagreement between the spectral properties of several model systems, and experimental uncertainties reported by the original authors have prompted reanalysis of the original kinamycin structures. To this end, a variety of kinamycin derivatives were prepared to gain insights into the reactivity of

the kinamycins, to prepare potential biosynthetic intermediates, and to obtain a chiral derivative for analysis by single crystal X-ray diffraction.

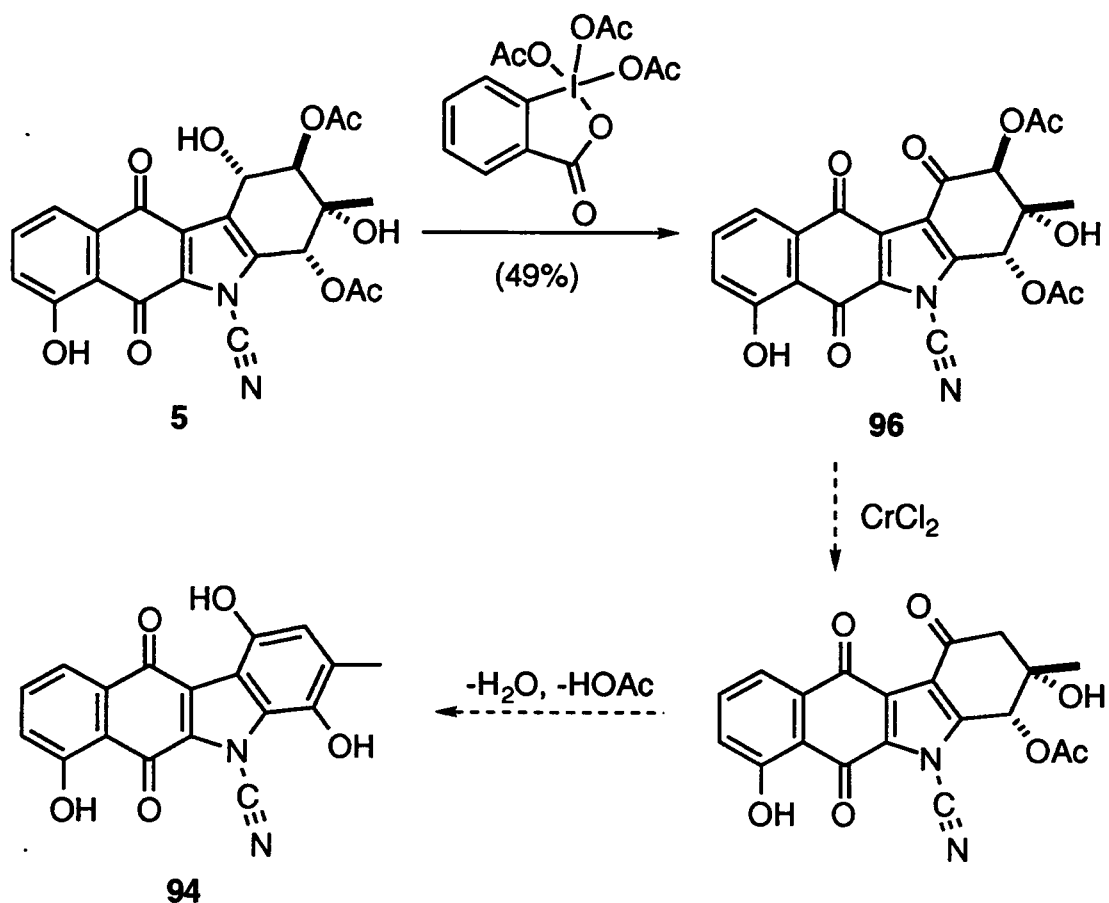
C1 Oxidations

Dr. Seaton isolated ketoanhydrokinamycin, **13**, and prekinamycin, **12**,(4) both anticipated to be intermediates in the kinamycin biosynthetic pathway. The biological conversion of **12** to **13** may involve an oxidation to the hydroquinone **94**, followed by a hydroquinone epoxidase reaction affording the epoxydione **95**. This type of epoxidation has precedence in the 2,5-dihydroxyacetanilide epoxidases from *Streptomyces* LL-C10037 and *Streptomyces* MPP 3051.(13) An enzymatic reduction of **95** would then give **13**.

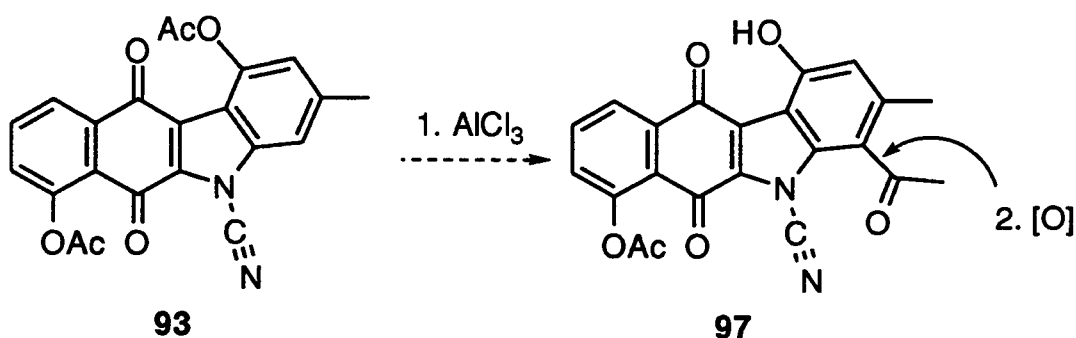


Kinamycin D, **5**, was oxidized to kinamycin D-1-one, **96**, by treatment with the Dess-Martin Periodinane.(14-16) HPLC

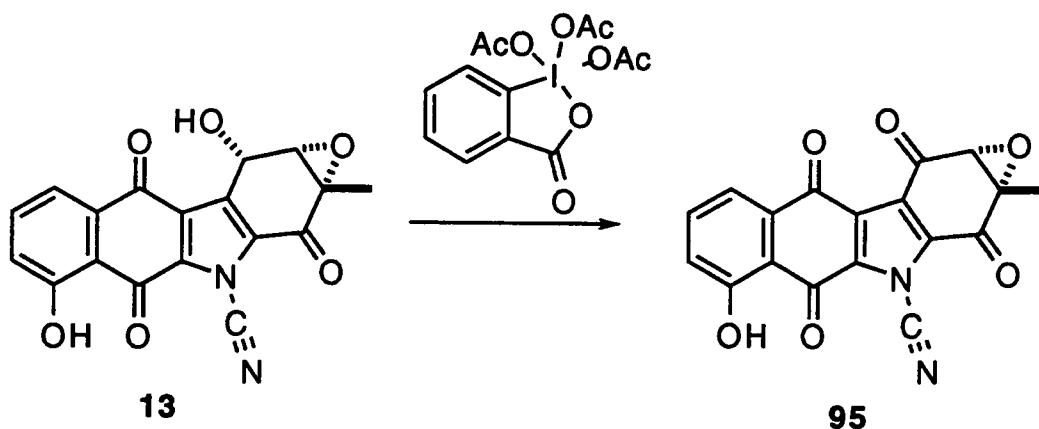
analysis indicated that the reaction proceeded cleanly, but the yield suffered from an inefficient isolation. The product was partly bound to silica gel during purification as was evident from the brightly colored columns, and modest mass recovery from chromatography. If **96** can be deoxygenated α to the newly formed carbonyl, dehydration could provide the potential biosynthetic intermediate, hydroquinone **94**. In a single attempt, deoxygenation with SmI_2 (17) was unsuccessful, but other deoxygenation conditions, like treatment with CrCl_2 (18) or zinc(19) may yet provide **94**.



An alternative access to **94** may be via prekinamycin diacetate, **93**. A Fries rearrangement(20) would be expected to occur preferentially on the more electron-rich D ring to provide **97**. A subsequent Dakin oxidation (a special case of the Baeyer-Villiger oxidation) or similar oxidation(21) would provide the desired C4 oxygen, and likely deprotect concomitantly to afford **94**.



The oxidation of **13**(14-16) generated epoxydione **95** as the sole product, which was expected to be biosynthetically derived from **94**, and the biosynthetic precursor of **13**. This was performed on 0.3 mg, providing a product with the expected ^1H NMR signals [^1H NMR (CDCl_3 , ignoring periodinane derived resonances) δ 11.82 (s, 1H), 8.29 (d, 1H, $J = 7.8$), 7.93 (dd, 1H, $J = 7.7, 7.7$), 7.23 (d, 1H, $J = 7.5$, partly obscured by CHCl_3), 3.85 (s, 1H, α to oxirane), 1.70 (s, 3H)], but mass loss during HPLC purification has prevented full characterization of **95**.

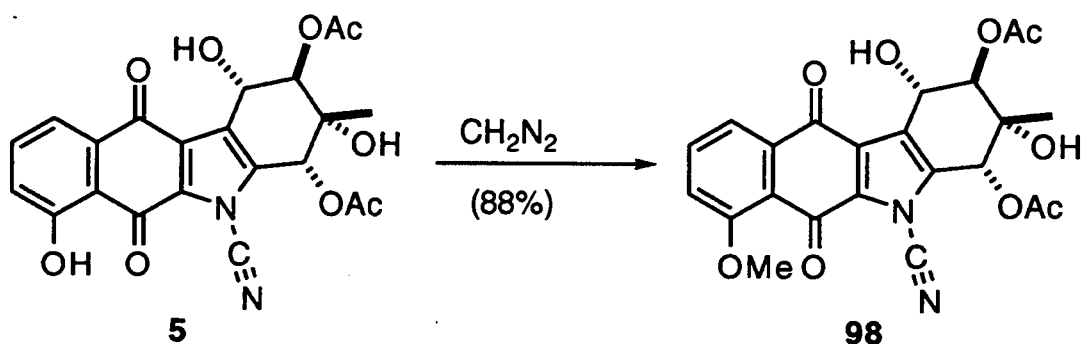


Methylation

A solution of **5** was found to readily react with diazomethane to provide **98**. This reaction was analogous to the methylation of **4** described by Omura and co-workers.(2)

Compound **98** may be useful in two ways. As will be introduced in succeeding chapters, two O-methylated aromatic compounds have been identified from *S. murayamaensis*. This suggested the possibility that a promiscuous methyltransferase activity may be their common source, and suggested the possibility that an O-methyl derivative of the major metabolite, **5**, may also be accumulated. Secondly, if compound **98** retains its antibiotic activity, this could provide access to a radiolabelled kinamycin for a mode of action study. It has been proposed that the kinamycins are reductively converted to their metabolically active form, and some possible alkylating agents that could be derived from a reductive activation have been postulated.(22-24) Exposure of a sensitive organism to radiolabelled **98**, followed by

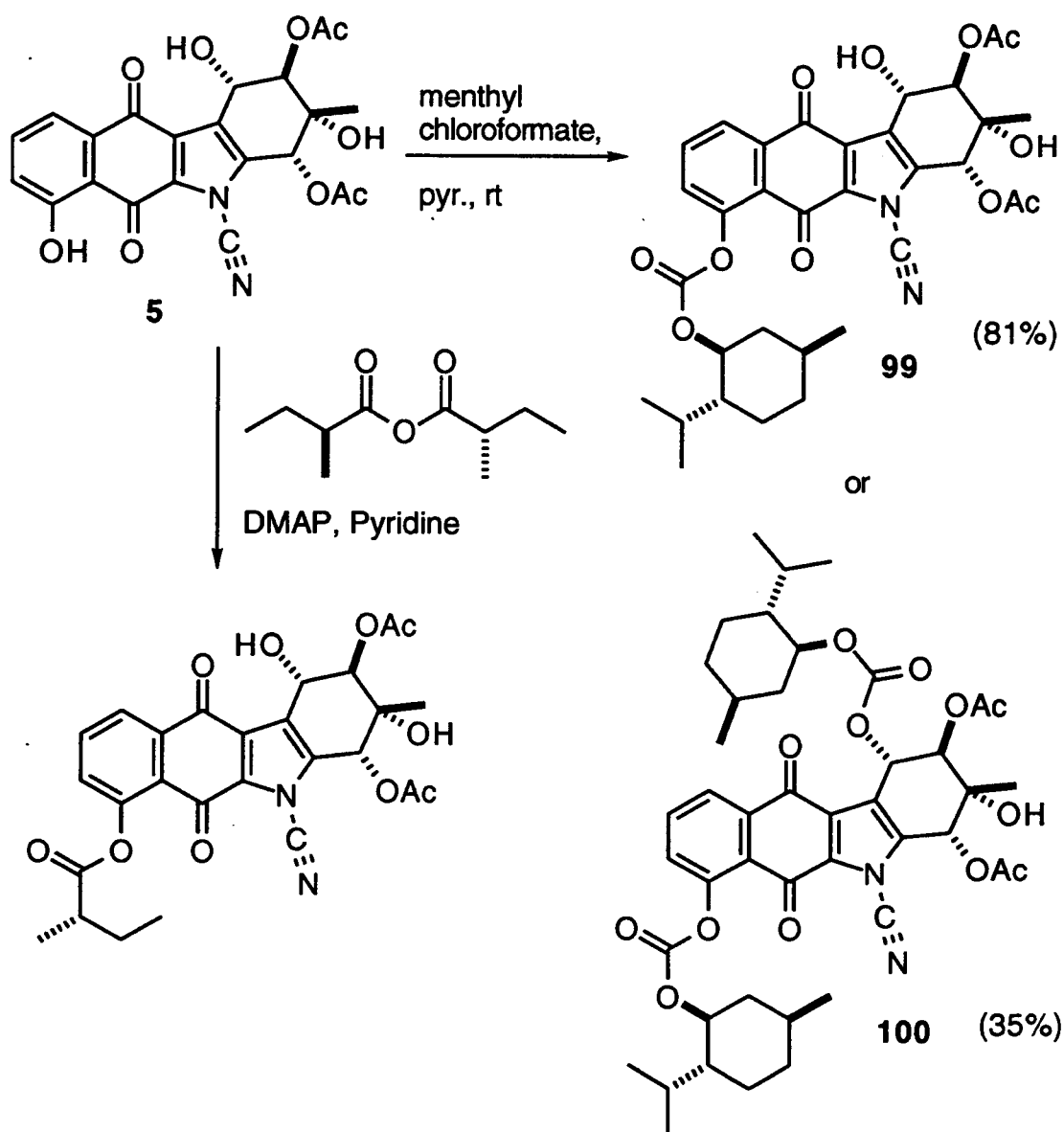
fractionation of the major cell components, may identify the cellular target at a gross level (re: soluble protein, structural protein, cell wall debris, RNA, DNA, etc.) by making it radioactive.



Base Catalyzed Acylation

Kinamycin D failed to react with sterically demanding acylating agents. These included: O-acetyl mandelic acid with dicyclohexylcarbodiimide, DMAP;(25) camphorsulfonyl chloride;(26) camphanic chloride with pyridine; and methylbenzylisocyanate(27,28) neat, with pyridine, or with DMAP in DMF. Kinamycin F, prepared from kinamycin D by solvolysis(2,4) of the two acetoxy substituents, also failed to react with methylbenzylisocyanate in CH_2Cl_2 . Each of these reaction conditions caused decomposition of the starting materials. However, reaction with (S)-(+)-2-methylbutyric anhydride in pyridine gave an acylation of the phenolic hydroxyl in 22% yield. Similarly, reaction with menthyl chloroformate in pyridine provided the monomenthylate, **99**, in 81% yield. If the latter

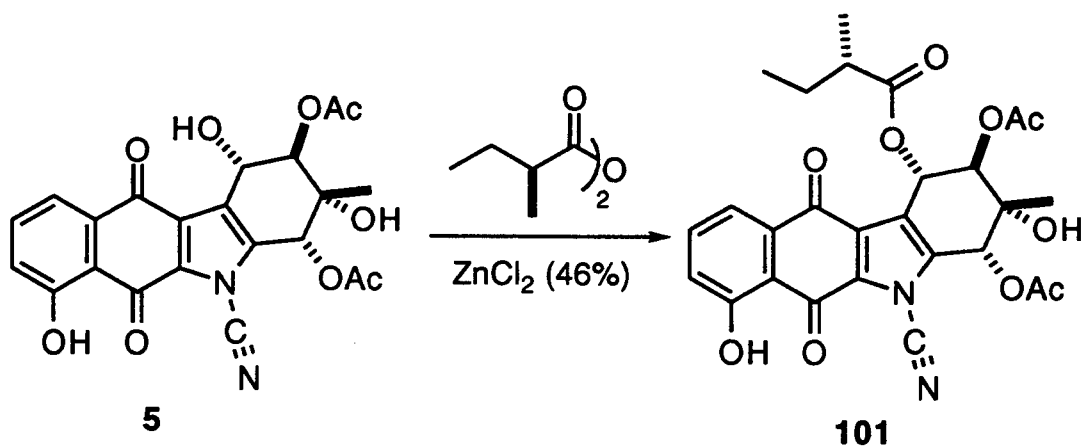
reaction was allowed to progress further, the 1,7-O-bismenthylcarbonate, **100**, was also produced in 35% yield (89% combined yield). Reaction between kinamycin D and menthylchloroformate in THF without catalysis gave only recovered starting material. Of these compounds, only the bismenthylate was crystalline, but was of a higher molecular weight than was desired for X-ray crystallographic analysis.



When **5** was reacted at low temperature with KHMDS and methylbenzyl isocyanate, no TLC-mobile components remained in the reaction mixture. Similar reactions using juglone as a model system, and other derivatization reagents, including methyl iodide, also resulted in decomposition.

Acid Catalyzed Acylation

Reaction of kinamycin D with (S)-(+)-2-methylbutyric anhydride in the presence of catalytic TsOH gave no reaction in THF, and in trichloroethane it resulted in decomposition of the starting material. Co-catalysis with TsOH and DMAP gave intractable mixtures. Reactions of (S)-methylbutyric anhydride or camphorsulfonyl chloride with catalysis by anhydrous cobalt(II)chloride gave no reaction. Similarly, treatment of **5** with (S)-methylbutyric anhydride under conditions of zinc(II)chloride catalysis in THF gave no reaction. By contrast, when **5** was reacted with the anhydride and zinc(II)chloride without this co-solvent, 1-O-(S)-(2-methylbutyryl)kinamycin D, **101**, was produced in 37-46% yield. This reaction was also found to progress in CH₂Cl₂, so the lack of reactivity observed for THF was likely the result of solvent co-ordination competing with anhydride activation. Camphorsulfonyl chloride was still unreactive under these conditions. Compound **101** provided crystals from ethyl acetate/pentane, or from DMF/water suitable for X-ray crystallographic analysis.

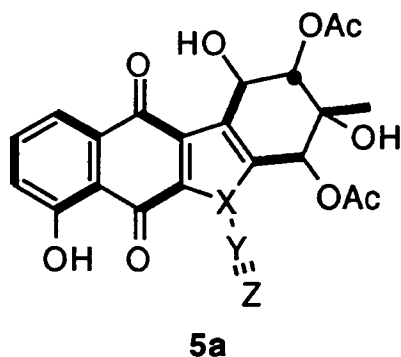


X-ray Crystallography

Data sets were collected on two crystals of 1-O-(S)-(2-methylbutyryl)kinamycin D. In both cases the non-hydrogen atoms were readily found by the direct methods program SHELXS. The first crystal, however, provided a smaller number of observed reflections than would ideally have been desired, and gave non-positive temperature factors for some atoms when anisotropically refined. The second crystal provided a solution that refined uneventfully, and was of sufficient quality to convincingly define the structure. Absolute stereochemistry, and the regiochemistry of the C3 position were found to be consistent with the previous reports.

The identity of the cyanamide-like moiety was evaluated by parallel solution of the various possible structural permutations. Only the three possible orientations of the CN₂ group were considered due to prior biosynthetic studies which secured

certain features of the structure, summarized below. To obtain structural information about the locations of the nitrogen atoms, Dr. Seaton had fed $(^{15}\text{NH}_4)_2\text{SO}_4$ to *S. murayamaensis* as the sole nitrogen source.(3) The ^{15}N NMR of the kinamycin D thus obtained showed two doublets ($J_{\text{NN}}=3.4$ Hz) at δ 344.5 and 241.6 ppm relative to H^{15}NO_3 (362.0 ppm). Similarly, the ^{13}C NMR showed a doublet of doublets at δ 78.5 ($J_{\text{CN}}=21.2$ and 5.4 Hz) suggesting that the two nitrogens are in the vicinity of this carbon, and of one another. Furthermore, Dr. Sato had established carbon-carbon connectivities of the A, B, and D rings by ^{13}C NMR analysis of labelled kinamycin D, **5a**, derived from feeding sodium $[1,2-^{13}\text{C}_2]\text{acetate}$. This precluded nitrogen substitution within these rings, leaving as candidate substituents the cyanamide, the C-substituted-diazonium, and the N-substituted-isonitrile.



For both crystals, and at all stages of structural refinement the diazo substituted 1-O-(S)-(2-methylbutyryl)kinamycin D gave a lower R factor than did the cyanamide or N-isonitrile substituents.

Table 4.2 gives the values obtained for the comparisons for the initial structure solution.

Table 4.2. Initial Structure Solutions of 1-(2-O-(S)-Methylbutyryl)kinamycin D

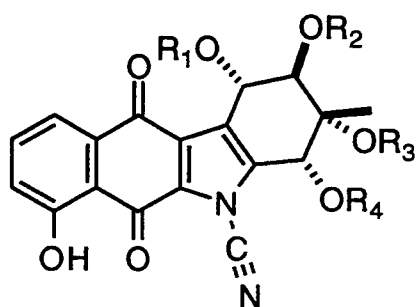
C Ring Group	R ^a	R _w	Shift/Error	GOF ^b
Cyanamide	5.92	6.32	0.114	1.84
C-Diazonium	5.27	5.54	0.117	1.62
N-Isonitrile	6.05	6.47	0.350	1.88

a. All solutions were for a complete data set collected to 55° in 2θ (2577 observations, 352 variables). b. Goodness of fit.

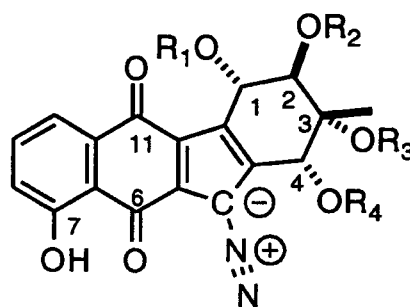
At Dr. Keszler's suggestion, these three possibilities were solved a second time using a DIFABS absorption correction after isotropic convergence. He also showed how the temperature factor varies with atomic scattering factor, revealing how to identify anomalous assignments. A diazo substituent gave the best solution with $R = 5.18\%$ and $R_w = 5.63\%$. Furthermore, in the cyanamide solution, the temperature factor for the putative cyanamide carbon was anomalously low (2.7) compared with the other non-hydrogen atoms (3.3 to 3.8 for the A, B, C, and D ring carbons, with 3.3 the next lowest overall temperature factor, C11a).

These results demonstrated that the kinamycin family of antibiotics, formerly thought to be the benzo[*b*]carbazoles **2-8**, **19**, **20** are in fact the benzo[*b*]fluorenes **102-110**. This revision also

applies to the ketoanhydrokinamycin 11. Two co-metabolites of the kinamycins with aromatic D rings have somewhat different spectroscopic characteristics, but in concurrent work were also demonstrated to be diazo rather than cyanamide substituted.(29)



2-8, 19, 20



102-110

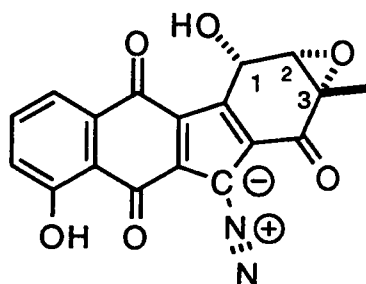
Figure 4.3. D ring substituents of 2-8, 19, 20, and 102-110

2	102	KA ^a	R ₁ =H	R ₂ =R ₃ =R ₄ =Ac
3	103	KB	R ₁ =R ₂ =R ₄ =H	R ₃ =Ac
4	104	KC	R ₃ =H	R ₁ =R ₂ =R ₄ =Ac
5	105	KD	R ₁ =R ₃ =H	R ₂ =R ₄ =Ac
20	106	KE	R ₁ =R ₂ =R ₃ =H	R ₄ =Ac
19	107	KF	R ₁ =R ₂ =R ₃ =R ₄ =H	
6	108	K ^b		R ₁ =R ₂ =R ₄ =Ac R ₃ =COiPr
7	109	K ^c	R ₃ =H	R ₁ =R ₂ =Ac R ₄ =COiPr
8	110	A83016A	R ₃ =H	R ₁ =Ac R ₂ =R ₄ =COiPr

^a Kinamycins A-F.

^b 3-O-Isobutyrylkinamycin C(30)

^c 4-Deacetyl-4-O-isobutyrylkinamycin C(30)



111

Bond length data from the structure solution shows that the diazo substituent is best described as a triple bonded N_2 unit attached by a sp^2 C-N single bond, and it exists largely in its charge separated tautomer. This accounts for the unusual stability of the kinamycin diazo substituent, as the five membered ring has six electrons, and so is formally aromatic. The strongly electron withdrawing quinone moiety would also be expected to stabilize a diazo substituent. The D ring oxygen substituents at C2 and C3 are equatorially disposed, with the C4 oxygen substituent showing more axial character than the C1 substituent. The quinone carbonyls are distorted from the ring plane by a steric interaction with the C1 substituent.

Crystallographic data for the final solution structure is contained in Tables 4.3, 4.8, and 4.9. Comparisons of the final solutions of the cyanamide, isonitrile, and diazo structures are presented in Tables 4.4, 4.5, 4.6, and 4.7. An ORTEP presentation of the solution molecular structure is presented in Figure 4.1.

Table 4.3. Crystal and Collection Data for 1-O-(2-(S)-(Methylbutyryl)kinamycin D

Empirical Formula	C ₂₇ H ₂₆ N ₂ O ₁₀
Formula Weight	538.51
Melting Point	>300° C
Crystal Dimensions (mm)	0.40 x 0.35 x 0.25
Crystal System	orthorhombic
Crystal Color	red
Habit	prism
No. Reflections Used for Unit Cell Determination (2 θ range)	15 (20.05-27.50°)
Lattice Parameters:	
a =	12.408 (3) Å
b =	30.427 (3) Å
c =	6.734 (3) Å
V =	2542 (2) Å ³
Space Group	P2 ₁ 2 ₁ 2 ₁ (#19)
Z value	4
D _{calc}	1.407 g/cm ³
Radiation	Mo K α
Temperature	23 \pm 1 °C
Scan Type	ω -2 θ
Scan Rate	8.0°/min (in omega)
Scan Width	(1.50 + 0.30 tan θ)°
2 θ _{max}	55.00
sin θ / λ	0.5947 Å ⁻¹
Counting Time (background:peak)	2:1
No. of Reflections Measured	
Total:	6241
Unique:	5886 (R _{int} = .029)
Observed:	2577
R (R _w)	0.052 (0.056)
Goodness of Fit Indicator	1.64

Table 4.3. Continued

Corrections (DIFABS, Lorentz and polarization effects):	Transmission Factors	0.62 to 1.31
Shift/Error (max) in Final LS Cycle		0.015
Max. Peak in Final Diff. Map		8.61 e-/Å ³
Min. Peak in Final Diff. Map		-0.22 e-/Å ³
Absolute Configuration Determination		yes

Table 4.4. 1-O-(2-(S)-Methylbutyryl)kinamycin D Structure Solutions^c

C Ring Group	R ^a	R _w	Shift/Error	GOF ^b
Diazonium	5.18	5.63	0.0150	1.637
Cyanamide	5.69	6.35	0.0177	1.845
N-Isonitrile	5.73	6.41	0.1782	1.864

a. All solutions are for complete data set collected to 55° in 2θ (2577 observations, 352 variables). b. Goodness of fit. c. A DIFABS absorption correction was applied.

Table 4.5. Behavior of Shift/Error (max.)

C Ring Group	Anisotropic Convergence	Final LS	Final LS+1
Diazonium	0.0150	0.0039	0.0038
Cyanamide	0.0177	0.0053	0.0053
N-Isonitrile	0.1782	0.0595	0.0216

Table 4.6. Number of Poorly Fitting Reflections

C Ring Group	delF/sigF>5.0	>10.0	>20.0
Diazonium	13	0	0
Cyanamide	23	2	0
N-Isonitrile	28	2	0

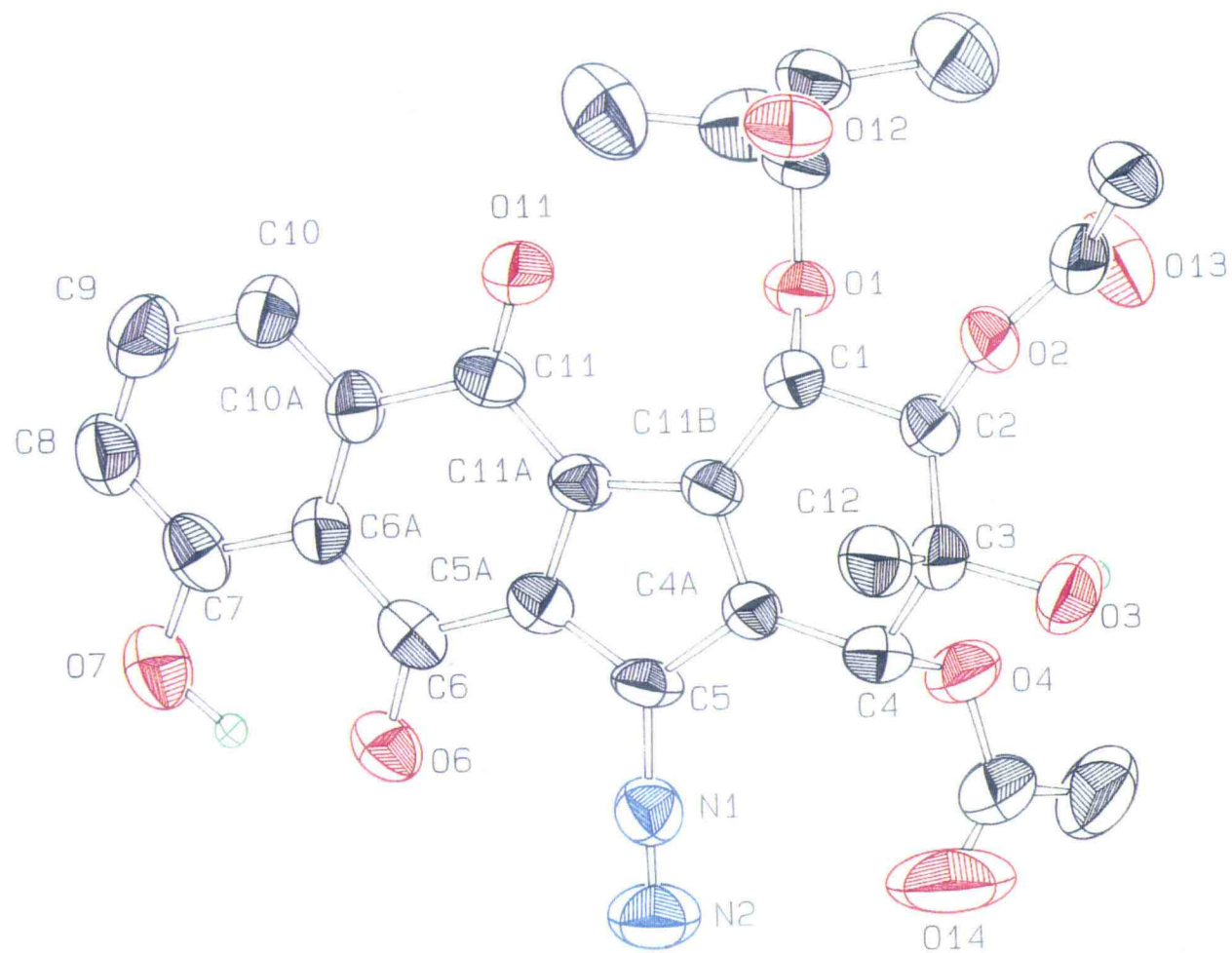


Figure 4.4. ORTEP Representation of 1-O-(2-(S)-Methylbutyryl)kinamycin D

Table 4.7. B Factors

C Ring Group	Atom X	Atom Y	Atom Z
Diazonium	3.790	4.146	6.237
Cyanamide	5.996	2.677	6.277
N-Isonitrile	5.990	4.225	4.417

Smallest B factor in final solution 3.256 (C11a).

Table 4.8. Fourier Peak Heights

Largest synthesis Fourier peak 8.612 ($e^-/\text{\AA}^3$)

Largest difference Fourier peak 0.211 (2.5%)

Largest negative peak -0.220

Table 4.9. Diazonium Bond Lengths and Angle

C-N 1.354 Å

N-N 1.114 Å

C-N-N angle 178.0

Kinamycin Structure Revision: Ramifications and Perspective

X-ray crystallography and total synthesis have long been regarded as the final proofs of structure. Therefore, revision of molecular structures determined by X-ray crystallography is an uncommon event,(31,32) occurring with a frequency similar to revision of structures confirmed by total synthesis.(33-37) Interchanging of heteroatoms, or missassigned absolute stereochemistry(7) are the most common features of a crystal structure which require revision, but Hamilton and others have developed tests to evaluate the probable correctness of a particular structure solution, or pair of possible solutions.(38,39)

While in the kinamycin family of compounds two incorrect X-ray crystal structures (see below) were revised, it would be a misinterpretation of this work to underrate the much greater statistical significance of a crystal structure solution compared to a structure obtained by NMR, or other spectroscopic methods.

Both the diazo carbanion, and cyanamide sp^3 nitrogen atom have three single bonds and a lone pair of electrons. Similarly, the positively charged diazo nitrogen, and the cyanamide carbon each have the same bonding environment and total electron count, so both the diazonium, and cyanamide groups are isoelectronic. For this reason mechanisms rationalized involving the cyanamide moiety may be directly applied to the newly revealed diazo substituted structures.

Several other naturally occurring diazo substituted compounds have been previously reported. These include diazonorleucine,(40,41) azaserine,(42,43) FR900840,(44) and LL-DO5139 β . A recent addition to this group of compounds that was reported at the time of this work was lagunamycin.(45)

IR and NMR data for model diazo substituted compounds are in good agreement with that of the kinamycins.(46-49) This structural revision not only allows the spurious data in the literature to be accounted for, but provides an opportunity to resolve a couple of inconsistencies in the literature. Specifically, two numbering systems have been in use for the kinamycins, one of which is consistent with the Chemical Abstracts Service numbering system and is exemplified on structure 105. This

system was preceded by the numbering of the stealthins, and of kinafluorenone, and so would be the the best choice to use in the future.

Shortly after completion of this work a group from Taiwan reported a subspecies of *S. chattanoogensis* that produces **105** along with six previously unreported kinamycins, **113-118**.^(50,51) Their producing organism differs from *S. murayamaensis* in its sporulating structure, spore adornment, melanin production, and carbon source utilization. They have elucidated the first kinamycin structures reduced at C4 that contain an oxirane. This supports the prediction that the C4 carbonyl of **111** is reduced prior to oxirane opening.⁽⁵²⁾ As their report was submitted prior to publication of our structure revision, these compounds have also been assigned cyanamide structures. The authors obtained an X-ray crystal structure of FL-120B, **114**, with the C ring apendage assigned as a cyanamide. No experimental details were given, but an accompaniing ORTEP plot suggests that there is a problem with the temperature factors in the atoms assigned as the cyanamide. It also appears that the oxirane forces C1 into the plane of the C ring, relieving the C1-O/C11-O steric interaction. This allows the quinone carbonyls of **114** to lie in the aromatic plane, in contrast to 1-O-(S)-(2-methylbutyryl)kinamycin D.

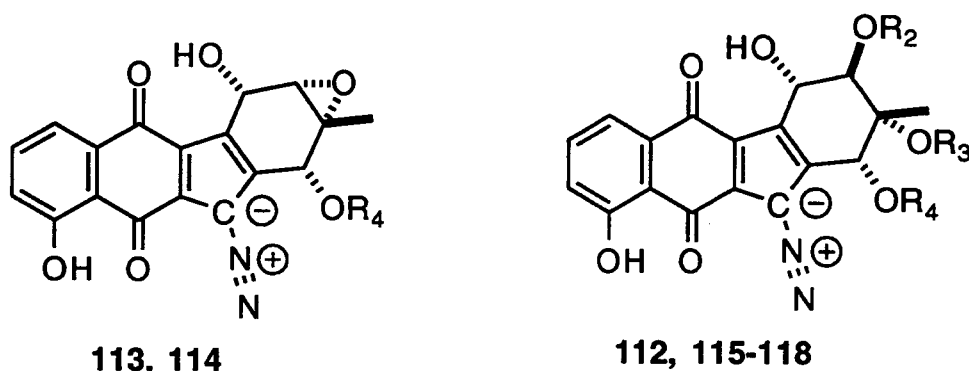


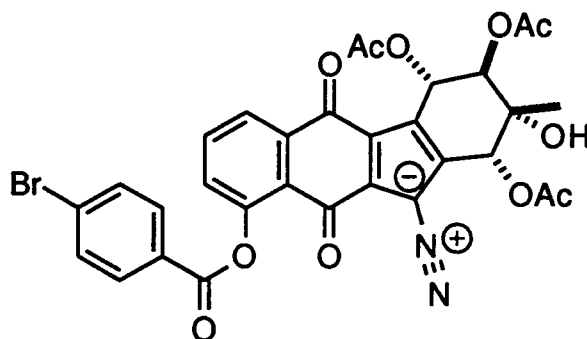
Figure 4.5. D ring substituents of **113**, **114**, and **112**, **115-118**

118	kinamycin J	$R_1=R_2=R_3=R_4=Ac$		
112	FL-120A	$R_2=R_4=Ac$	$R_3=COiPr$	
113	FL-120B	$R_4=Ac$		
114	FL-120B'		$R_4=COiPr$	
115	FL-120C	$R_3=H$	$R_2=Ac$	$R_4=COiPr$
116	FL-120C'	$R_3=H$	$R_2=Ac$	$R_4=COEt$
117	FL-120D'	$R_2=R_3=H$	$R_4=COiPr$	

Recently, a new kinamycin, **118**, was isolated from *S. murayamaensis*. Its structure elucidation is presented in Chapter 6. Considering all the structures reported to date, this structure revision includes 17 naturally occurring members of the kinamycin family, obtained from a variety of producing organisms, and two related metabolites from *S. murayamaensis*. Also revised are the corresponding kinamycin derivatives described in this chapter, and in previous degradative work.(2)

As a result of our communication on the revised structures, Chemical Abstracts Service has changed the indexed structures for all the *S. murayamaensis* metabolites formerly assigned in the

Registry File as cyanamide substituted to diazo substituted. They have also indexed the structures of the FL-120 family of compounds as diazo rather than cyanamide substituted, in contrast to the articles that report these structures. However, since the FL-120 compounds, and others like 111, were not specifically named in our structure revision, there is no reference for these compounds that connects them to the structure revision. For this reason, a complete literature search of one of these compounds would only produce references with the incorrect structures. Furthermore, the Chemical Abstract Services correction of the structures was not quite complete in scope. The structures of the naturally occurring compounds 3-O-Isobutyrylkinamycin C (120901-48-6), 4-Deacetyl-4-O-isobutyrylkinamycin C (120901-49-7),(30), and A83016A (142383-42-4),(5) are still listed as cyanamide substituted. Additionally, several synthetic derivatives of the kinamycins are still indexed as cyanamide substituted, including the *para*-bromobenzoate of kinamycin C, 119, (37592-73-7) used in the original X-ray structure determination. Other non-specific kinamycin derivatives still indexed as cyanamide substituted include those with the following registry numbers; 35303-09-4, 35303-10-7, and 35303-11-8. One means to rectify this situation would be to tabulate all the kinamycin related structures in a communication, prompting Chemical Abstract Services to index a reference to their revised structures.



119

Subsequent to the structure work reported here, a variety of reactions to selectively react the diazo group were performed. Among these, the method of Griess(53) was applied to kinamycin D to obtain a kinamycin derivative with a proton substituted for the C5 diazo group. Thus, when **105** was heated in EtOH at reflux, a new non-polar red compound was obtained. Elemental analysis and IR spectra showed that no diazo groups were present. The NMR spectra showed 42 carbon lines, and 34 hydrogen resonances, which were confirmed by HRFABMS which afforded the formula $C_{42}H_{35}O_{11}$ ($M+1$)⁺. Further analysis of the 1H NMR showed that two molecules of EtOH had been incorporated into the product, and only one acetate remained. The data is consistent with a dimer of **KD** in which one of the former kinamycin D rings became aromatic, but a specific structure was not determined.

It was hoped that photolysis of the diazo group would generate a carbene that would insert into the OH bond of an

alcohol solvent. With this in mind, an EtOH solution of **105** was exposed to sunlight. This produced a new product with ^1H NMR resonances consistent with the product of the expected insertion reaction. The similar polarity of the new compound with the unreacted **105** remaining from the reaction, and its limited stability on silica gel, prevented the isolation of the presumed insertion product.

Experimental

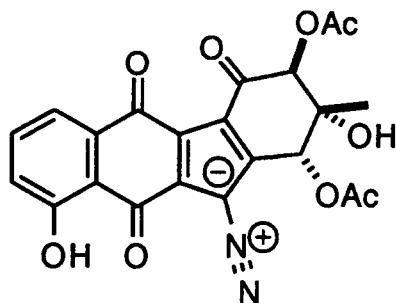
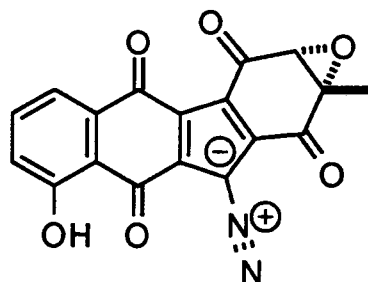
Materials and Methods. Kinamycin D was obtained by fermentation, and purified by silica gel chromatography and recrystallization in EtOAc. Pyridine was dried over KOH and filtered through basic Al_2O_3 (activity 1) before use. ZnCl_2 was dried *in vacuo* with heat from a Bunsen burner until a stable vacuum of 0.1 torr was achieved. The resulting solid was ground to a fine powder, the heating repeated, and the resulting powder stored under argon until used. Otherwise, experiments were conducted as described in Chapter 2.

Kinamycin D-1-one (120).(14,15) To kinamycin D (57.2 mg, 0.126 mmol) and the Dess-Martin Periodinane (165.1 mg, 0.389 mmol) was added CH_2Cl_2 (10.0 mL), and the mixture was stirred for 1 h. The reaction mixture filtrate was twice chromatographed with ethyl acetate on silica gel (1 x 12 cm) which afforded 27.7 mg of **120** (49%) as an orange solid: mp > 345 °C; UV (CH_2Cl_2) λ_{max}

(ϵ) 200 (sh, 4080), 221 (4250), 233 (sh, 3240), 246 (sh, 2890), 283 (2700), 366 (614), 439 (681) nm; IR (KBr pellet) 2172.3, 1740.0, 1703.1, 1624.9, 1458.4, 1229.2 cm^{-1} ; ^1H NMR (CDCl_3) δ 11.70 (s, 1H), 7.63 (dd, 1H, $J = 1.5, 7.6$), 7.58 (app. t, 1H, $J = 8.0$), 7.16 (dd, 1H, $J = 8.2, 1.3$), 6.07 (s, 1H), 5.74 (s, 1H), 3.26 (s, 1H, exchangeable), 2.31 (s, 3H), 2.27 (s, 3H), 1.31 (s, 3H); ^{13}C NMR (CDCl_3) δ 184.0, 182.7, 176.1, 171.4, 171.1, 161.7, 139.4, 136.9, 134.5, 132.2, 129.5, 124.9, 123.5, 120.4, 114.8, 79.8 (cyanamide), 78.0, 74.7, 70.5, 20.8, 20.7, 19.1; FABMS: m/z 453.0 ($\text{M}+1$) $^+$, 323.0, 154.1; HRFABMS (positive mode) calcd for $\text{C}_{22}\text{H}_{17}\text{N}_2\text{O}_9$ 453.0934 ($\text{M}+1$) $^+$, found 453.0934.

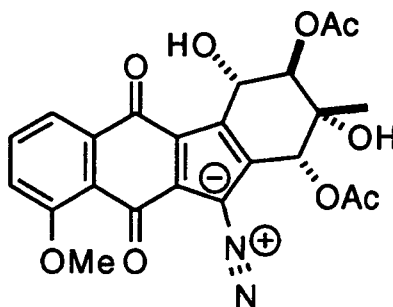
5-Diazo-7-hydroxy-2,3-oxy-3-methylbenzo[*b*]fluorene-1,4,6,11-tetraone (121).(14,15) To the ketoanhydrokinamycin **111** (0.3 mg) and the Dess-Martin Periodinane (19.4 mg) was added CH_2Cl_2 (0.50 mL), and the mixture was stirred for 5 h. After a brief sonication, and continued stirring for 12 h, complete consumption of the starting material was evident by HPLC analysis. The reaction mixture was filtered, and was twice chromatographed on LH-20 [0.7 x 50 cm, CH_2Cl_2 -MeOH (1:1)]. Refiltration in CHCl_3 gave after evaporation 1.6 mg **121** as an orange solid. HPLC analysis showed the desired compound as 64% of the chromatogram monitored at 254 nm, with the remaining peaks being periodinane derived: mp undetermined; UV (HPLC, 16.19 min) λ_{max} 234, 300, 324, 390, 432 nm; ^1H NMR (CDCl_3 , ignoring periodinane derived resonances) δ 11.82 (s,

1H), 8.29 (d, 1H, $J = 7.8$), 7.93 (dd, 1H, $J = 7.7, 7.7$), 7.23 (d, 1H, $J = 7.5$, partly obscured by CHCl_3), 3.85 (s, 1H), 1.70 (s, 3H).

**120****121**

8-O-Methylkinamycin D (122).(2) A solution of **105** (22.0 mg, 0.0483 mmol) in CH_2Cl_2 (2.50 mL) was chilled in an ice-water bath, and CH_2N_2 was added as a solution in Et_2O (15 mL, approx. 0.5 M). After stirring 2 h, HOAc (0.100 mL) was added, dissipating the yellow color, and the volatile materials were removed under a stream of air. Filtration of the CHCl_3 soluble portion of the sample, and recrystallization from CHCl_3 -pentane gave 19.9 mg of **122** (88%) as a red-brown solid: mp $> 310\text{ }^\circ\text{C}$ (darkens at $163\text{--}165\text{ }^\circ\text{C}$); IR 766.9, 1238.83, 1652.4, 1745.6, 2153.4, cm^{-1} ; ^1H NMR (CDCl_3) δ 7.84 (d, 1H, $J = 7.8\text{ Hz}$), 7.67 (dd, 1H, $J = 8.0, 7.8\text{ Hz}$), 7.31 (d, 1H, $J = 8.1\text{ Hz}$), 5.60 (d, 1H, $J = 5.8\text{ Hz}$), 5.03 (s, 1H), 5.44 (s, 1H), 4.79 (d, 1H, $J = 5.9\text{ Hz}$), 4.03 (s, 3H), 2.23 (s, 3H), 2.16 (s, 3H), 1.23 (s, 3H); ^{13}C NMR (CDCl_3) δ 181.0, 178.6, 172.2, 171.1, 160.5, 136.2, 136.1, 135.0, 130.7, 126.9, 126.4, 120.3, 118.2, 78.1, 75.9, 73.8, 71.3, 67.3, 56.5,

21.2, 20.9, 18.3; FABMS (positive mode) m/z 469.1 ($M+1$)⁺, 460.1, 451.1, 321.1; HRFABMS m/z calcd for C₂₃H₂₁N₂O₉ 469.1247 ($M+1$)⁺, found 469.1246.



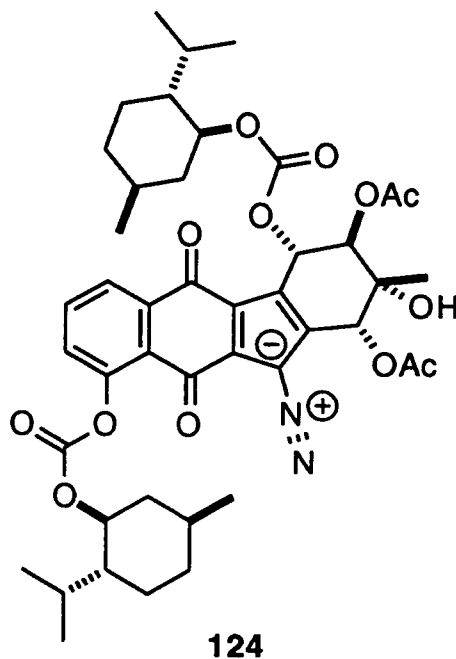
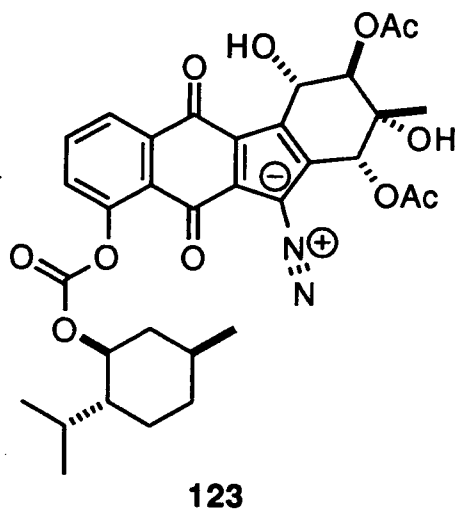
122

7-O-Menthylcarbonate kinamycin D (123). Kinamycin D (6.0 mg, 0.0132 mmol) in a 1-dram vial was dissolved in pyridine (0.400 mL), and (-)-menthyl chloroformate (20 μ L, 0.093 mmol) added. After 24 h stirring, evaporation *in vacuo* gave a mixture that was applied to a Pasteur pipette column (silica gel), and eluted with ethyl acetate:hexane (1:4). Fractions 3-5 (1.5 mL each) gave after evaporation 6.8 mg **123** (81%) as a yellow solid: mp 145.5-155.0 $^{\circ}$ C; IR (KBr) 2956.9, 2932.3, 2152.1, 1758.2, 1700.7, 1649.1, 1308.4, 1220.6 cm^{-1} ; ^1H NMR (CDCl_3) δ 8.18 (dd, 1H, J = 7.7, 1.1), 7.73 (dd, 1H, J = 8.0, 8.0), 7.44 (dd, 1H, J = 8.1, 1.3), 5.58 (d, 1H, J = 8.0), 5.42 (s, 1H), 5.41 (s, 1H), 4.80 (dd, 1H, J = 8.0, 1.8), 4.63 (dt, 1H, J = 10.9, 4.4), 2.87 (s, 1H), 2.26-2.32 (m, 2H), 2.26 (s, 3H), 2.18 (s, 3H), 1.69-1.78 (m, 2H), 1.48-1.58 (m, 4H), 1.21-1.30 (m, 1H), 1.21 (s, 3H), 1.05-1.15 (m, 1H),

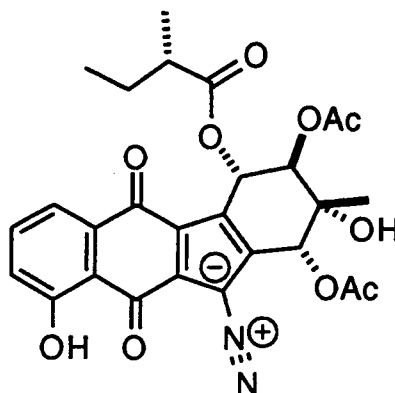
0.98 (d, 3H, J = 7.0) 0.96 (d, 3H, J = 6.5), 0.90 (d, 3H, J = 6.9); ^{13}C NMR (CDCl_3) δ 180.3, 177.7, 172.3, 171.2, 152.5, 150.5, 135.6, 134.7, 134.6, 131.3, 129.3, 127.5, 127.1, 126.0, 124.7, 80.3, 78.4, 75.8, 73.7, 71.3, 67.3, 46.9, 40.6, 34.0, 31.4, 26.0, 23.3, 22.0, 21.2, 20.9, 20.7, 18.2, 16.3; ESIMS m/z 1273.4 (2M+1)⁺, 654.1 (M+NH₄)⁺, 637.1 (M+1)⁺; HRFABMS (positive mode) m/z calcd for C₃₃H₃₇N₂O₁₁ 637.2397 (M+1)⁺, found 637.2398.

1,7-O-Bismenthylcarbonate kinamycin D (124). To pyridine (3.00 mL) containing kinamycin D (38.4 mg, 0.0845 mmol) was added (-)-menthyl chloroformate (72 μL), and the mixture was stirred for 2 d. Additional (-)-menthyl chloroformate (200 μL) was added, and at 3 additional h, methanol (250 μL), and toluene (0.5 mL) were added and the mixture evaporated *in vacuo*. Silica gel chromatography (1.5 x 18 cm, ethyl acetate-CH₂Cl₂ (1:1) eluent) gave fractions (5 mL each) 4-7 (212.3 mg mixed fractions) and 8-15 containing after evaporation 28.8 mg 7-O-menthylcarbonate kinamycin D, **123** (53%). The mixed fractions were rechromatographed (1.5 x 20 cm, silica gel, 5% ethyl acetate in CH₂Cl₂ eluent) which gave fractions (5 mL each) 9-14 containing after evaporation 24.4 mg **124** (35%) as a yellow solid. Data for the title compound: mp 175.5-177.2 °C; IR (KBr) 2956.6, 2148.7, 1755.6, 1710.1, 1263.3, 1224.5 cm⁻¹; ^1H NMR (CDCl_3) δ 8.09 (dd, 1H, J = 8.1, 1.3), 7.69 (dd, 1H, J = 8.1, 8.1), 7.39 (d, 1H, J = 8.1), 6.06 (d, 1H, J = 7.4), 5.69 (d, 1H, J = 7.6), 5.45 (s, 1H), 4.71

(dt, 1H, $J = 10.9, 4.4$), 4.62 (dt, 1H, $J = 10.9, 4.4$), 2.78 (s, 1H), 2.42-2.50 (m, 1H), 2.23-2.42 (m, 2H), 2.18 (s, 3H), 2.17 (s, 3H), 1.93-2.00 (m, 1H), 1.66-1.74 (m, 5H), 1.49-1.65 (m, 4H), 1.43-1.47 (m, 1H), 1.08-1.18 (m, 4H), 1.26 (s, 3H), 1.01 (d, 3H, $J = 6.4$), 0.97 (d, 3H, $J = 7.3$), 0.95 (d, 3H, $J = 6.7$), 0.90 (d, 3H, $J = 7.1$), 0.89 (d, 3H, $J = 6.9$), 0.83 (d, 3H, $J = 7.0$); ^{13}C NMR (CDCl_3) δ 178.2, 177.5, 171.7, 171.0, 154.6, 152.5, 150.2, 136.1, 134.6, 134.2, 129.2, 128.4, 127.8, 125.7, 125.4, 124.5, 80.2, 78.6, 77.7, 75.4, 73.6, 71.7, 70.9, 47.1, 46.9, 40.6, 34.2, 34.1, 31.4, 26.3, 26.1, 23.5, 23.4, 22.1, 21.9, 20.9, 20.8, 20.7, 20.6, 18.4, 16.4, 16.4; ESIMS m/z 857.4 ($\text{M}+\text{K}$) $^+$, 836.4 ($\text{M}+\text{NH}_4$) $^+$, 819.4 ($\text{M}+1$) $^+$, 637.0 ($\text{M}+1-\text{C}_{11}\text{H}_{18}\text{O}_2$) $^+$; HRFABMS (positive mode) m/z calcd for loss of one menthylate $\text{C}_{33}\text{H}_{37}\text{N}_2\text{O}_{11}$ 637.2397 ($\text{M}+1-\text{C}_{11}\text{H}_{18}\text{O}_2$) $^+$, found 637.2398.



1-O-(S)-Methylbutyrylkinamycin D (125). To powdered zinc(II)chloride (95.2 mg) was added (S)-(+)-2-methylbutyric anhydride (52 μ L) followed by CH_2Cl_2 (1.0 mL). After stirring for 0.5 h, **105** (15.0 mg, 0.330 mmol) was added as a solution in CH_2Cl_2 (2.0 mL), generating a red precipitate. At 1 h, nearly all the red precipitate had re-dissolved, and the mixture was diluted with CH_2Cl_2 (4.0 mL), washed with H_2O (3 x 4.0 mL), and dried over Na_2SO_4 . Chromatography (1 x 18 cm, silica gel) eluted with EtOAc-hexanes (2:5) afforded 8.2 mg (46%) of **125** as an orange solid: mp > 300 $^\circ\text{C}$; IR 3418.4, 2133.4, 1752.9, 1710.4, 1237.2, 1227.1, 1153.1, 1074.8 cm^{-1} ; ^1H NMR (CDCl_3) δ 12.10 (d, 1H, J = 2.0), 7.64 (dd, 1H, J = 7.3, 1.1), 7.54 (dd, 1H, J = 8.2, 7.6), 7.18 (dd, 1H, J = 8.0, 1.1), 6.29 (d, 1H, J = 7.4), 5.60 (d, 1H, J = 7.4), 5.51 (s, 1H), 2.69 (s, 1H, exch.), 2.37 (app. q, 1H, J = 6.9), 2.22 (s, 3H), 2.17 (s, 3H), 1.71 (dq, 1H, J = 7.1, 6.9), 1.45 (dq, 1H, J = 7.5, 6.8), 1.27 (s, 3H), 1.22 (d, 3H, J = 6.7), 0.90 (t, 3H, J = 7.5); ^{13}C NMR (CDCl_3) δ 184.1, 177.8, 175.4, 171.9, 171.0, 162.0, 136.2, 134.3, 132.5, 130.1, 129.2, 126.9, 123.7, 119.8, 115.6, 75.5, 73.6, 71.0, 70.6, 67.4, 40.9, 26.6, 26.5, 21.0, 18.5, 16.4, 11.5; EIMS m/z (relative intensity) 538.7 (M^+ , 22%), 454.6 (14%), 324.4 (100%), 306.3 (58%); HREIMS m/z calcd for $\text{C}_{27}\text{H}_{26}\text{N}_2\text{O}_{10}$ 538.1587 (M^+), found 538.1586.



125

X-ray Crystallography of 125. A red prism of 0.40 x 0.35 x 0.25 mm was secured on a glass fiber mount, and the intensity data were collected at 23 °C on a Rigaku AFC6R single crystal diffractometer. A 12-kW rotating anode generator with a graphite incident beam monochromator provided Mo K α radiation. The unit cell parameters (orthorhombic) were determined from 15 reflections in the range of $20.05 < 2\theta < 27.50^\circ$. The intensities of three standard reflections, measured every 300 reflections throughout the data collection, were constant and served to demonstrate the stability of the crystal. The molecular structure was solved and refined in the space group $P2_12_12_1$ (#19), $Z = 4$, with the TEXSAN(54) crystallographic software package. The positions of all non-hydrogen atoms were determined with the direct method program SHELXS based on 2577 observed reflections ($2\theta_{\text{max}} = 55.0^\circ$). Hydrogen atoms were placed in calculated positions. A DIFABS(55) empirical absorption correction was applied (transmission factors 0.62 to 1.31), and

equivalent reflections were averaged. Final cycle anisotropic refinement of non-hydrogen atoms gave an R value of 0.052 ($R_w = 0.056$) with a p -factor of 0.03.

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5. Benz[*b*]fluorenones and Fluorenes

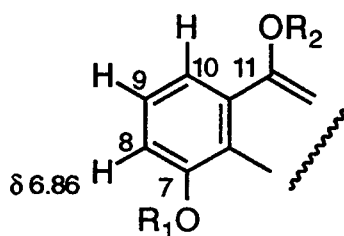
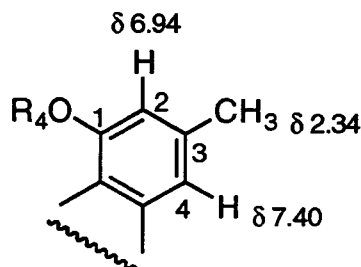
Our group's first experience with the benz[*b*]fluorene ring system was the crystallographic and NMR spectroscopic elucidation of the structure of kinafluorenone **126**. Subsequent crystallographic determination that the kinamycins are also benz[*b*]fluorenes led to a synthetic investigation of the chemistry believed to occur in the conversion of dehydrorabelomycin **11** to prekinamycin **127**.

Formylation of bromoacid **128** had been demonstrated. This would be expected to provide access to the acid aldehyde product of the putative dioxygenase cleavage of dehydrorabelomycin **11**. Kinobscurinone **129** was prepared and, remarkably, was found to be an NMR silent species, a characteristic also reported for the structurally related stealthins A **130** and B **131**, p. 183. Compound **129** was shown to be utilized in the biosynthesis of the kinamycins, and to be produced in fermentations of *S. murayamaensis*. A fluorenone reducing activity was detected in this organism, which may have relevance to the biosynthetic pathway.

Nitrogen containing fluorenes were prepared in order to access potential biosynthetic intermediates, including **132**. In the course of this work an unusual addition elimination reaction was observed that provided **133**. This material then underwent an unexpected loss of N₂ to provide **134**.

Kinafluorenone Triacetate

As part of her work studying the survivors of mutagenesis of *S. murayamaensis* that do not accumulate the kinamycins, Dr. Cone observed a new metabolite that was produced as the major product of mutant MC1. Initial isolation work proved difficult, so the material was acetylated and the resulting triacetate purified. NMR experiments yielded one and three bond H-C correlations that allowed the determination of part structures **135** and **136**. Also observed were three acetates and one O-methyl group initially believed to be a methyl ester.

**135****136**

Nuclear Overhauser effect experiments then provided enhancements consistent with these part structures, and also provided correlations from each of two of the acetoxy groups to the protons at C2 (3.6%) and C10 (2.5%), respectively. This demonstrated that R₂ and R₄ were acetyl substituents. An unexpected enhancement of the proton at C8 that was observed

from irradiation of the O-methyl group demonstrated that R₁ was methyl substituted since an ester would have too much rotational freedom for a strong enhancement.

A crystal of this compound was grown from ether-chloroform, and examined by single crystal X-ray diffraction. A solution was obtained from the direct methods program SHELXS in the TEXAN(1) structure solution software package, Figure 5.1, with the structure **137**. This revealed the benz[*b*]fluorene ring system, and answered all regiochemical questions. The solution molecular formula of C₂₃H₂₀O₈ was confirmed by high resolution fast atom bombardment mass spectroscopy. Kinafluorenone, **126**, most likely arises from the condensation of the acid-aldehyde intermediate **48** proposed to be involved in the kinamycin biosynthetic pathway. This would cyclize, and be oxidized as proposed for the kinamycins. In the case of **126**, the resulting fluorenone **138** would then be O-methylated, Scheme 5.1. This suggests that the mutation responsible for the lack of kinamycin production, and accumulation of **126** in this mutant, occurs in a gene involved in generation of the diazo group.

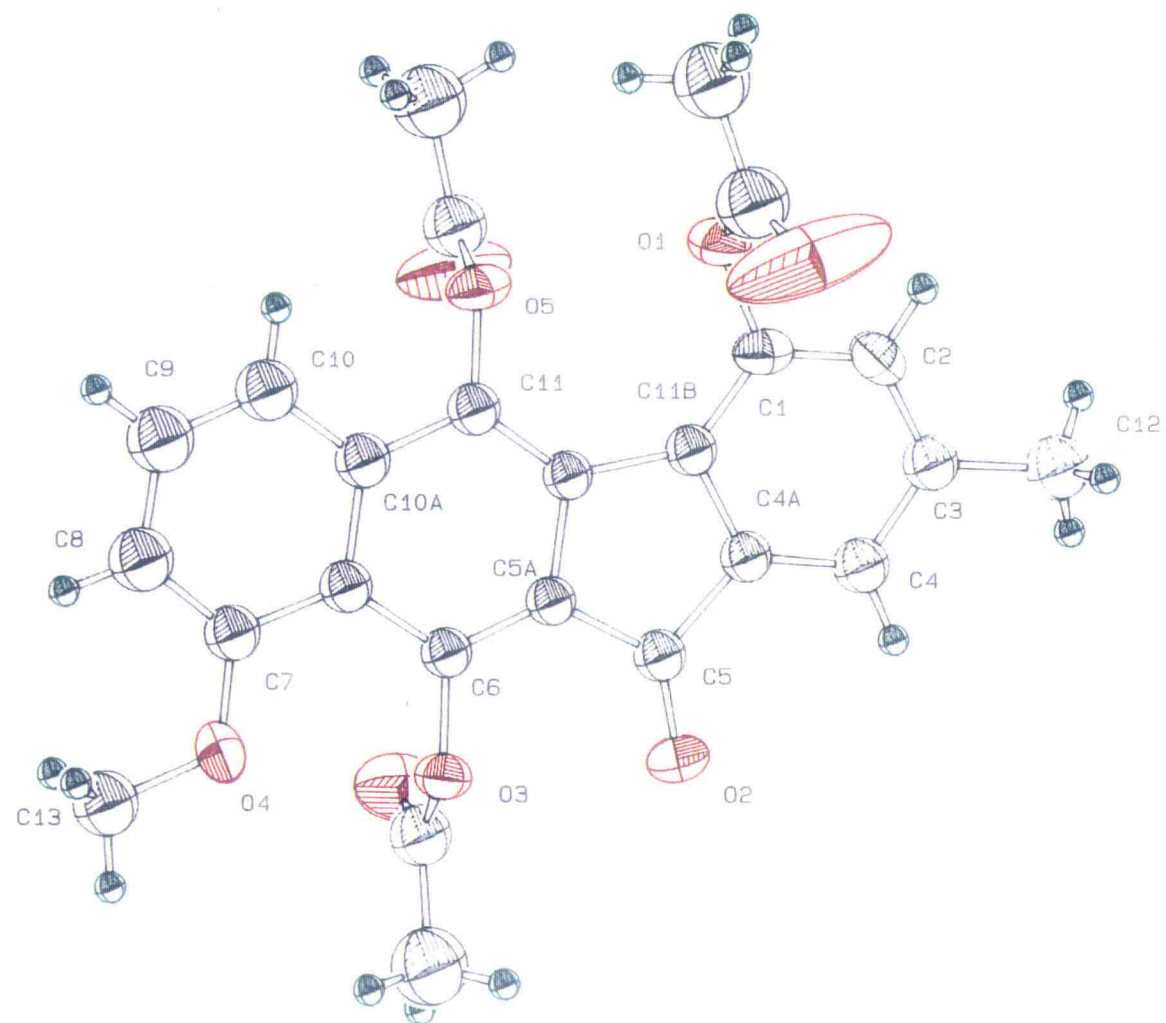
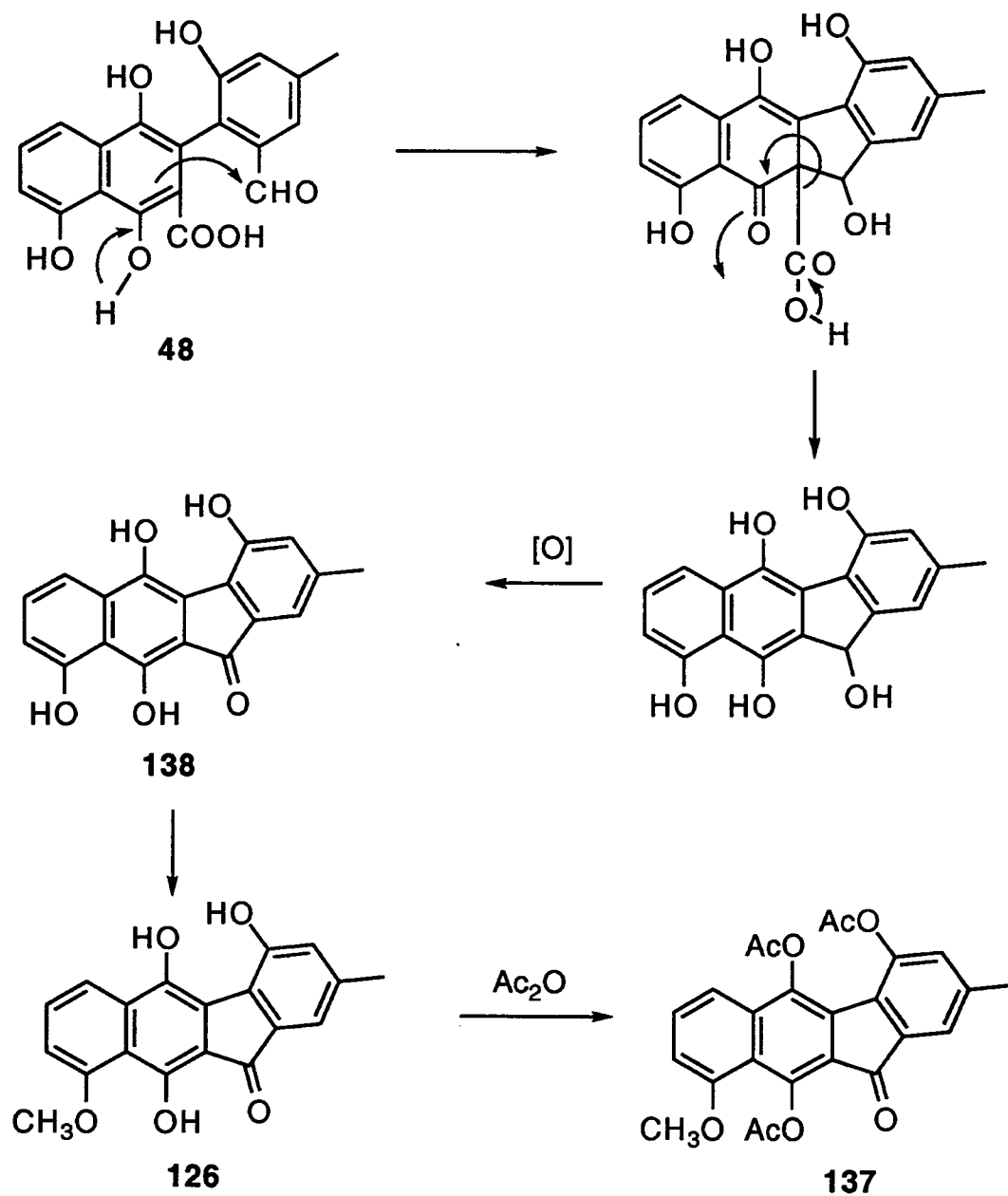


Figure 5.1. ORTEP Representation of Kinafluorenone Triacetate, **137**

Table 5.1. Crystal and Collection Data for **137**

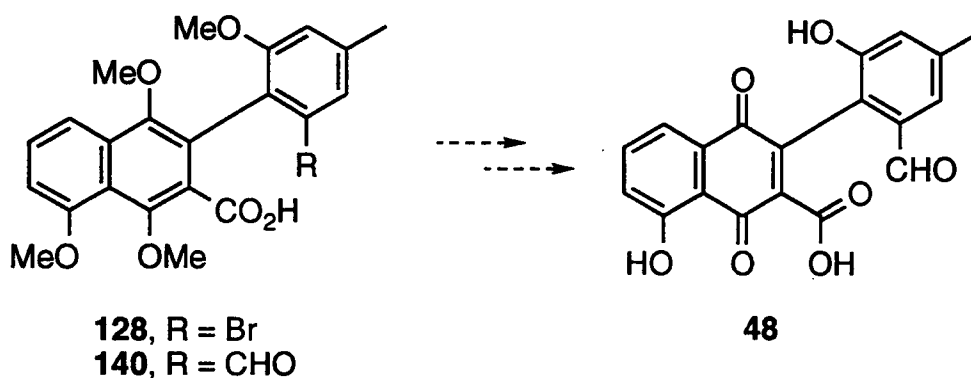
Empirical Formula	C ₂₅ H ₂₀ O ₈
Formula Weight	448.43
Crystal Dimensions (mm)	0.20 x 0.20 x 0.40
Crystal System	triclinic
Crystal Color	yellow
Habit	needle
No. Reflections Used for Unit	
Cell Determination (2 θ range)	19 (26.1<2 θ <30.30°)
Lattice Parameters:	
a = 10.758 (2) Å	α = 90.93 °
b = 11.202 (1) Å	β = 110.32 °
c = 9.487 (2) Å	γ = 89.09 °
V = 1071.9 (4) Å ³	
Space Group	P-1
Z value	2
D _{calcd}	1.389 g/cm ³
Radiation	Mo K α
Temperature	23 \pm 1 °C
Scan Type	ω -2 θ
Scan Rate	16.0°/min (in omega)
Scan Width	(1.70 + 0.30 tan θ)°
2 θ _{max}	55.00
Counting Time (background:peak)	2:1
No. of Reflections Measured	Total: 4370
Unique:	4103 (R _{int} = .041)
Observed:	2239
R (R _w)	0.055 (0.066)
σ in Observation of Unit Weight	1.96



Scheme 5.1

Kinobscurinone, Synthesis and Characterization

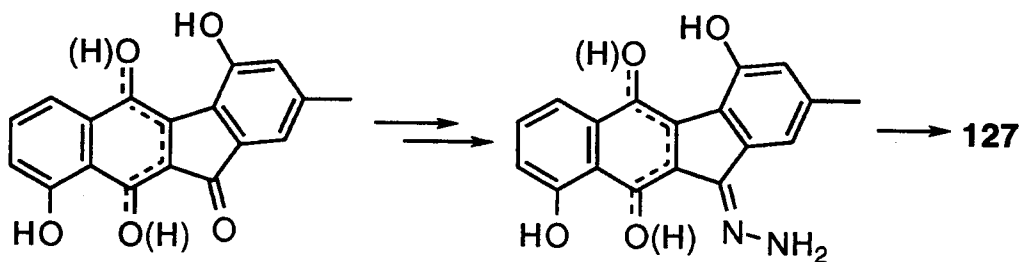
The protected bromoacid, **128**, was prepared by former group member Dr. Gore, who used it to successfully synthesize phenanthroviridine aglycone, and other compounds of interest to our group.(2,3) In order to prepare the acid aldehyde **48**, it was hoped that the acid function of **128** could be protected from nucleophilic addition by forming its carboxylate, allowing the bromoacid to be formylated with DMF under metal halogen exchange conditions.(4)



Prior formation of the lithium carboxylate gave principally the cyclized tetramethoxy fluorenone **139** in 56% yield, accompanied by the des bromo acid and starting materials. Potassium carboxylate formation, or in situ lithium carboxylate formation(5-7) gave a new acidic, 2,4-dinitrophenyl hydrazine active product that was not readily separable, but gave a molecular ion

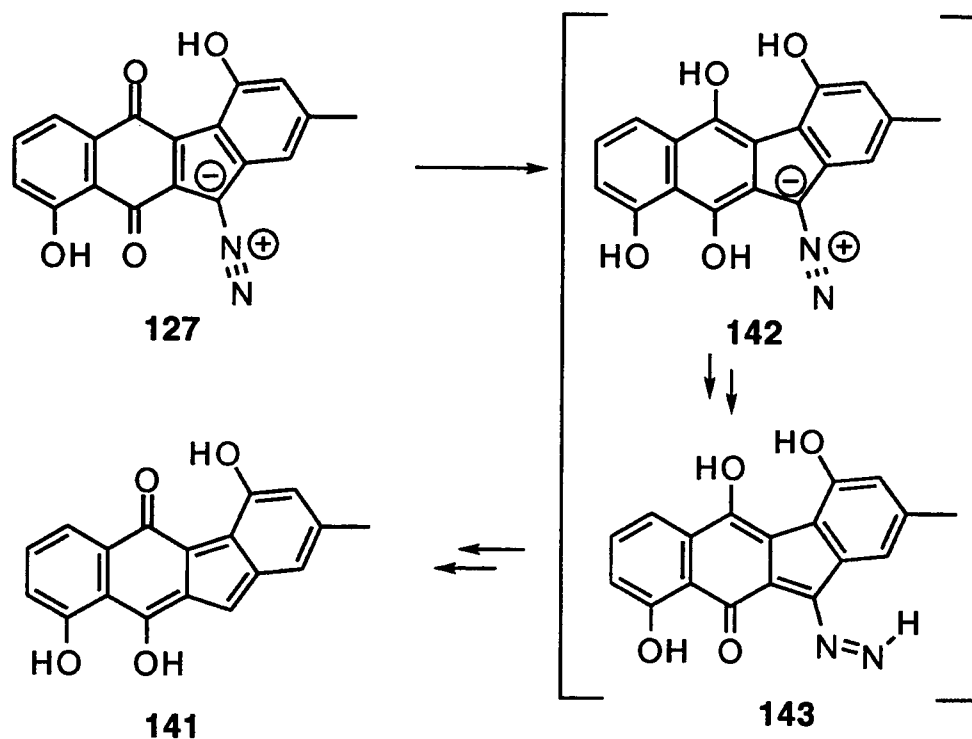
consistent with the tetra-O-methylacid-aldehyde **140**, [410.0 (33%, M⁺), 382.0 (100%, M⁺-CO), 366.0 (34%, M⁺-CO₂); HREIMS calcd for C₂₃H₂₂O₇ 410.1365, found 410.1365]. A portion of this material was deprotected with boron tribromide to provide two products - expected to be forms of **48** - to serve as UV/vis HPLC references for future natural product or enzymology work (retention time 10.46 min λ_{max} 210, 280, 426 nm; retention time 12.23 min λ_{max} 206, 282, 424 nm).

The isolation of the triacetate of kinafluorenone demonstrated the formation of a benzfluorenone from a putative acid-aldehyde intermediate; therefore, a synthetic approach to determine the nature of the early benzfluorene intermediates was undertaken. The first biosynthetic explanation conceived for the formation of diazonium group of prekinamycin was via a benzfluorenone. By analogy to laboratory chemistry, a desmethyl kinafluorenone, or its corresponding quinone, could be converted to a hydrazone (by one or several steps), and that oxidized to prekinamycin **127**.



A possible alternative to a benzfluorenone intermediate was suggested from structural analysis of a red compound accumulated by the wild type *S. murayamaensis*, and isolated by former group member Dr. Gore. Subsequently, Dr. Tamayo prepared this compound by partial synthesis from the reaction of prekinamycin with $\text{Rh}(\text{OAc})_2$ in MeOH at reflux, and elucidated its structure as **141**. A carbene mechanism has been proposed for this conversion based on analogy to reactions of diazoketones with $\text{Rh}(\text{OAc})_2$. However, for most quinones the hydroquinone oxidation level is readily accessible, and some compounds with which we have worked appear to spontaneously reduce in the absence of O_2 (aminopyridine **90**, and murayaquinone **18**). This suggested the possibility of **141** being generated by a spontaneous reaction of the prekinamycin hydroquinone **142** (Scheme 5.2). The hydroquinone could directly lose N_2 by a Griess reaction.⁽⁸⁾ Alternatively, a [1,5]hydride shift followed by a proton transfer could provide aryldiazine **143**, which would then eliminate N_2 with net reduction.⁽⁹⁾ This possibility was tested by treating prekinamycin with sodium dithionite, which afforded **141** as the major product by HPLC. Similarly, Dr. Cone found that treatment of prekinamycin with dithiothreitol yielded **141**, and Dr. Tamayo found that the addition of $\text{Rh}(\text{OAc})_2$ was not necessary under the conditions of the original reaction for the conversion to **141**. This reductive conversion probably accounts for the occurrence of **141** in *S. murayamaensis* fermentations.

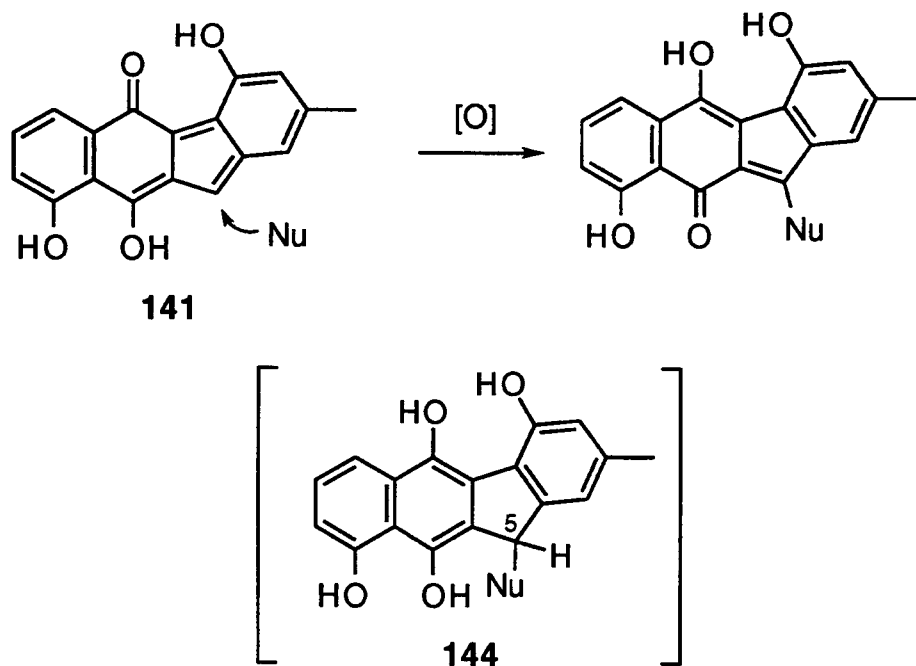
Thus **141** may be formed by a nonspecific reduction process, rather than by a substrate specific enzyme-catalyzed reaction.



Scheme 5.2

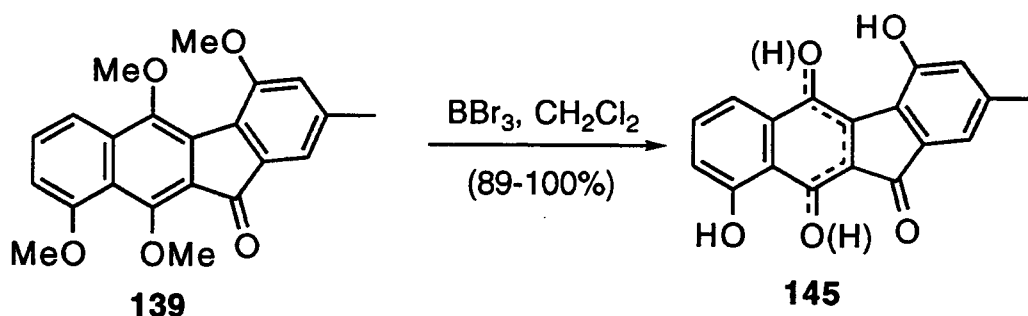
After the structure of **141** was determined, an alternative to the involvement of **129** in the formation of intermediates leading to prekinamycin was proposed. This would involve the addition of nucleophiles to C5 of **141**, leading, after oxidation and tautomerization, to the expected intermediates. This sequence of events was unlikely by analogy to the known reactivity of naphthoquinones(10), which do not typically undergo

intermolecular 1,4 addition, but rather undergo 1,4 addition-elimination reactions. Analogy would therefore suggest that a better leaving group than protium would be required for substitution to take place. Additionally, the first formed intermediate does not have a ready way to tautomerize the C5 (structure **144**) hydrogen as in addition to a quinone, but would first have to be oxidized to the quinone allowing ample opportunity for reversion. This alternative biogenetic hypothesis however did provide a pair of testable alternatives, which have been addressed by feeding experiments described below.



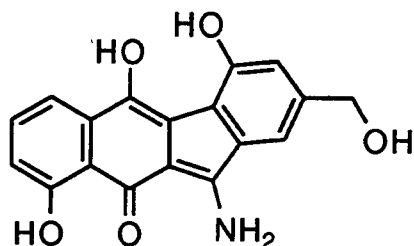
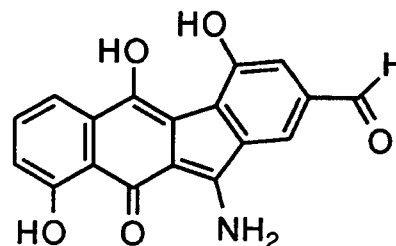
Protected fluorenone **139** was found to be cleanly deprotected(11) upon treatment with BBr_3 , and this afforded a

sparingly soluble, intensely purple-red compound, expected to be some permutation of structure **145**. HPLC analysis showed formation of a single product. However, ^1H and ^{13}C NMR with up to 30 mg in the NMR tube showed no signals from the expected product.



Seto and coworkers recently reported the *Streptomyces* metabolites stealthins A **130** and B **131**,⁽¹²⁾ which are so named for their lack of observable NMR signals. These workers suggested that the NMR spectra were unobserved due to severe line broadening resulting from rapid interconversion of the many possible tautomeric forms of these compounds. This would give a time averaged spectrum with indistinct signals. If their assertion is correct then by changing the proton donating, or hydrogen bonding, ability of the medium the equilibrium may be shifted to favor one tautomeric contributor. Varying the solvents (including use of TFA-*d* and pyridine) did not provide a NMR spectrum of our deprotection product. Since changing solvents

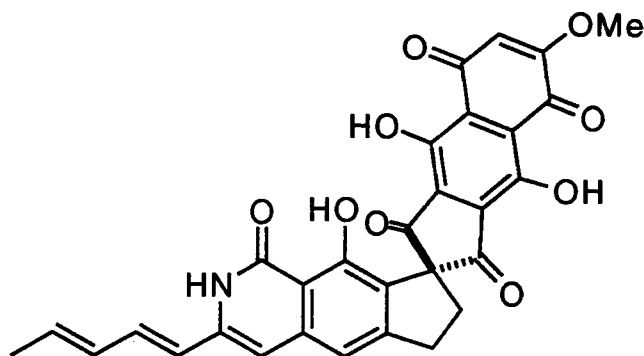
had no effect, and no sample signal, even a very broad one was observed, the rapid tautomerization explanation was relegated to the highly improbable.

**130****131**

The NMR experiment uses an externally applied magnetic field to establish an unequal distribution of NMR active nuclei between two energy levels. In order to observe an NMR spectrum, this population distribution is disturbed by application of a radio frequency at the precessional frequency of the nuclei. Since obtaining an NMR spectrum relies on establishing a non-equilibrium energy level distribution, it is reasonable to assume that the reason that no signals are observed is that the NMR active nuclei in the sample return to a steady state energy distribution faster than the instrument can collect data. This rapid relaxation can be caused by the presence of additional magnetic fields interacting with the sample nuclei, such as those arising from the unshared electrons in free radicals and magnetically active metals.

To address the possibility of magnetically active metal contaminants, or the possibility that the deprotection product is a siderophore, solutions of the deprotection product were treated with Chelex® Resin, or washed with aqueous ethylenediaminetetraacetic acid. These NMR samples were then prepared using acid washed glassware but still no useful spectra were obtained.

Another example of a molecule in which part of the structure was unobserved in the NMR spectra is fredericamycin A, **146**.⁽¹³⁾ In this case elucidation of the structure was done with single crystal X-ray diffraction (and the originally reported molecular formula revised). Subsequently, Misra and co-workers found that addition of a trace of trifluoroacetic acid to the NMR sample made the naphthylquinone region visible (this is not the case with our compounds). These authors suggested the possibility that a radical was formed by air oxidation, causing a rapid relaxation of the nuclei in the naphthylquinone fragment and accounting for the lack of observed NMR spectra. They accounted for the ability to observe a spectrum after addition of TFA as resulting from the quenching of a nucleophilic radical in the sample. They also found simply excluding oxygen from the sample allowed for the observation of normal spectra, which was not the case with our compound.



146

Lack of observed NMR spectra in structurally disparate polycyclic quinone compounds may turn out to be a more general phenomenon than one would at first expect. Another lab has shared with us an anecdotal instance of an NMR silent naphthaquinone compound,(14) and a former coworker, Dr. Cheng, was unable to observe the NMR signals of the naphthaquinone portion of PD116198 at one stage of his work. An unknown purple co-metabolite of PD116198 isolated by our former co-worker Mrs. Halley also gave no NMR signals in CDCl_3 , for a sample of roughly 0.4 mg (the degree of purity, and the origin of the sample are uncertain). Dehydrorabelomycin, **11**, a compound which our group has obtained in sizable quantities, and worked with over a considerable time, is sparingly soluble in CDCl_3 , making it difficult to obtain good quality ^{13}C NMR spectra in this solvent. To improve solubility, former workers have prepared the corresponding triacetate, which has been fully

characterized.(15) Dehydrorabelomycin is, however, freely soluble in DMSO-*d*6. In exploring conditions to analyze **11** derived from an isotope feeding experiment, an unlabelled sample of **11** was analyzed by NMR in DMSO-*d*6. Only solvent and water lines were observed. When the DMSO-*d*6 was removed, this sample gave a normal ^1H NMR in CDCl_3 .

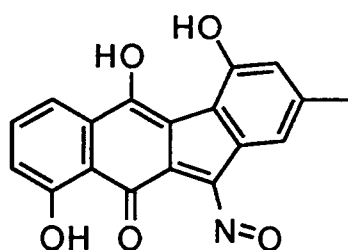
Electron spin resonance, ESR (synonymous with electron paramagnetic resonance, EPR), allows the detection of unpaired electrons in solid and solution samples. To determine if a radical was the cause of the lack of an observed NMR spectrum of the deprotection product, an ESR spectrum of **145** was attempted. A single, narrow signal was observed, both for the solid, and for a solution of **145** in tetrahydrofuran. Two additional compounds whose preparations are described later in this chapter; 5-nitrosokinobscurinone, **147a**, and 12-deoxystealthin A, **132**, were also shown to have ESR spectra, as were compounds **11** and **126**. No ESR signal was seen for Mrs. Halley's purple compound, nor was any signal obtained from the solvents, sample tubes, or instrument cavity under the experimental conditions used.

A 'g' value in ESR is analogous to chemical shift in NMR, and is related to the chemical environment of the unpaired electron. The stable free radical DPPH was used as an external standard for determining the g value for each sample. On the instrument used, the experimentally determined g value for DPPH was

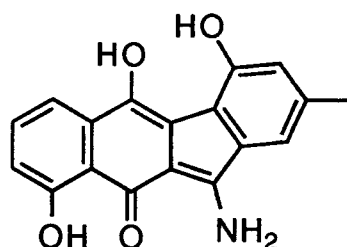
2.0061, compared to a literature value of 2.0036, so each g value reported here has had 0.0025 subtracted from the experimentally determined number. A free electron has a theoretical g value of 2.0023. Table 5.2 contains the g values determined for each sample, in the solid state.

Table 5.2. Samples Exhibiting ESR Spectra, and Their Experimental g Values

Compound	g Value
dehydrorabelomycin, 11	2.0072
kinafluorenone, 126	2.0068
kinobscurinone, 129	2.0052
5-nitrosokinobscurinone, 147a	2.0029
12-deoxystealthin A, 132	2.0067
DPPH	2.0036
free electron	2.0023



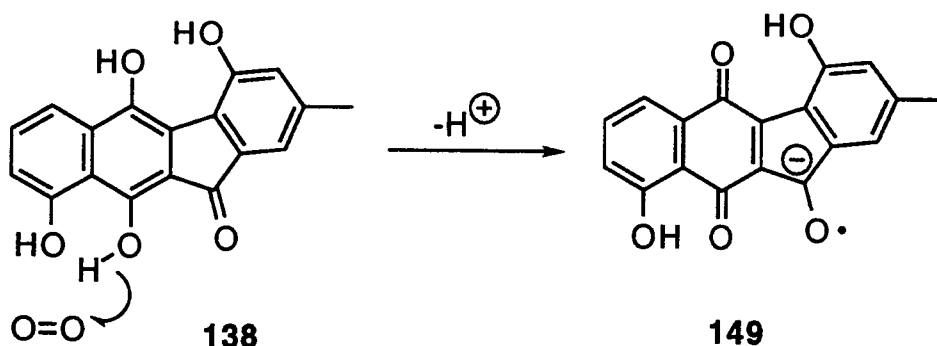
147a



132

The distance between the peaks in the derivative plot (distance between the inflection points in the absorption spectrum) of solid **145** was ~16 Gauss. In a tetrahydrofuran solution the signal width was ~3.5 Gauss. All of the other solid

samples showed a width similar to **145**. Attempted solution spectra of **147a** and **132** were of a poor quality. Observation of a single resonance, with no splitting, for **145** indicates that the observed radical is not sampling the electronic environments of the proton substituted carbon atoms. In some instances, however, dynamic processes can cause otherwise split ESR resonances to appear as single lines.(16,17) The structure **149** is consistent with this observation, and can be envisaged as arising from **138** by transfer of one electron to oxygen.

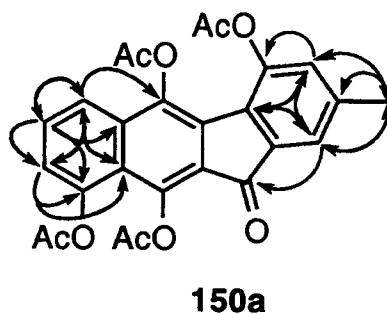
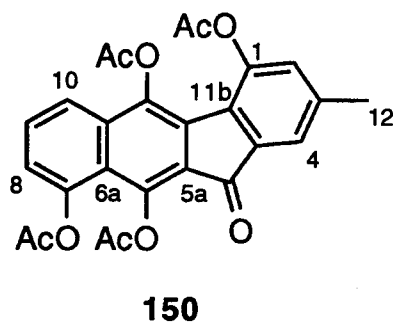


To determine if the radical **149** was the major constituent, or a minor contributor to the mass of the sample, a quantification of the spins (unshared electrons) in the spectrum was performed.(18) Known masses of both DPPH and **145** were diluted with to identical volumes with tetrahydrofuran, and their ESR spectra were obtained. The purities of both analytes were confirmed by HPLC analysis. The areas of the peaks were obtained by digital integration of the derivative plots, and cutting

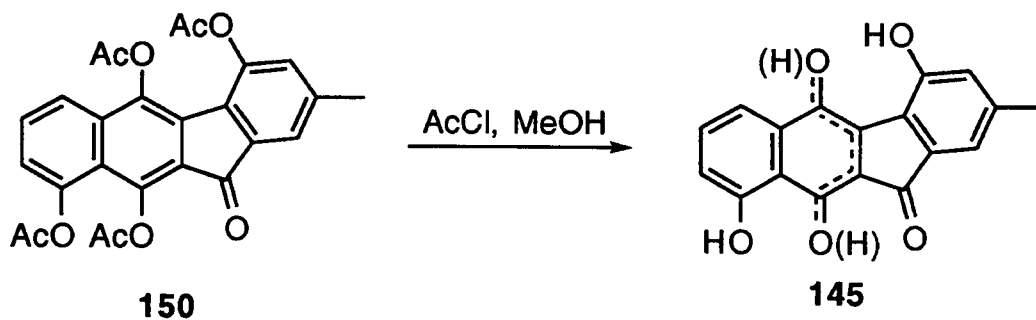
out and weighing the resulting curves. These were compared for two separate dilutions and gave an intensity of signal for **149** which accounted for 4.9% and 6.6% of the mass of the samples of **145** respectively. This showed that **149** is a minor component of **145**, that is present to the extent of several percent. This left the identity of the major form of **145** to be determined in an alternative manner, described below.

A variety of reactions were performed to try to: 1. obtain a characterizable derivative of **145**, 2. attempt conversion to **141**, and 3. to determine the oxidation level of **145**. No reaction was observed with conditions expected to provide an acetonide, dioxolane, or phenyl boronate. Per-bromination and 2,4-dinitrophenyl hydrazone forming conditions resulted in complex mixtures. A variety of alkylating conditions with ethyl iodide gave no identifiable products. Acetylation with acetyl chloride in pyridine gave poor conversion to a new compound, while acetylation with acetic anhydride in pyridine(19) gave poor conversion to a different compound. The latter reaction was used to provide the yellow tetraacetate **150** in 19% yield. Full characterization, including inverse detected long-range ^1H - ^{13}C NMR correlations,(20) **150a**, established the regiochemistry of acetylation shown. The observed color change upon derivatization has been observed in conversions of tetrangulol (purple) to its di-O-methyl and diacetate derivatives (yellow), or in dissolving tetrangulol or juglone (red) in acetic anhydride,

which provided yellow solutions presumably by disrupting hydrogen bonding.

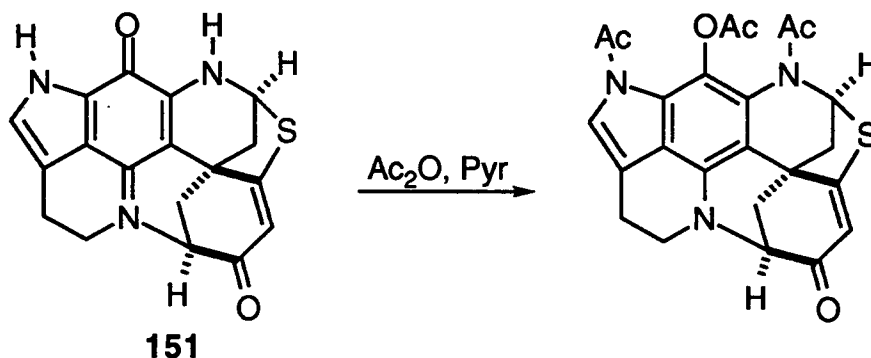


The danger in characterizing a derivative is the possibility that additional reactions have occurred, so that the derivative differs in an unexpected way from its precursor. To establish that compound **145** had not undergone further reactions, conditions were found to remove the acetates. While Et₃N in water was insufficiently reactive and K₂CO₃ in methanol caused rapid decomposition, methanolic HCl (generated *in situ*) cleanly regenerated the deprotection product.

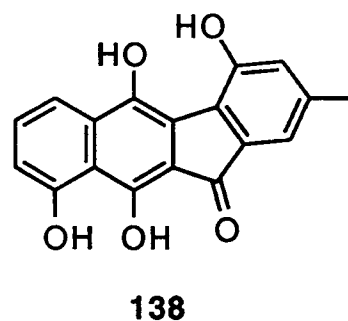
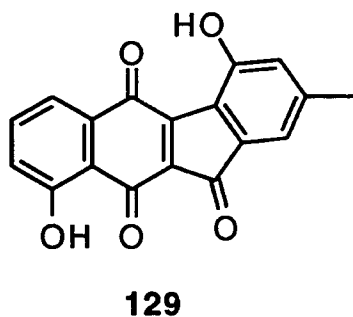


Formation of **150**, low and high resolution MS of **145**, and the conditions of several deprotection and derivatization attempts suggested that the deprotection product was at the hydroquinone oxidation level. To further establish the oxidation level of **145**, some additional reactions were attempted. Subjecting compound **145** to hydrogenation, or sodium borohydride reduction, conditions did not result in a loss of color, and provided only recovered starting material. Nitrous oxide oxidation(21) resulted only in disappearance of the starting material. There was, however, one troubling piece of data. When the deprotection product was treated with hydroxylamine a new compound was formed with a nearly identical UV/vis spectrum, and low and high resolution MS consistent with the quinone oxidation level. Subsequently, Dr. Cone found that treating the deprotection product with sodium dithionite gave a new peak by HPLC, and it displayed a UV/vis spectrum similar to that of kinafluorenone. While direct characterization of kinafluorenone is still lacking, it is assumed to exist at the hydroquinone oxidation level. This apparent reduction is preceded by a similar reaction of the iminoquinone discorhabdin D, **151**, which has been found to reduce upon treatment with acetic anhydride in pyridine.(22) In any case, since stable derivatives of both **129**, and **126** have been obtained at the hydroquinone oxidation level, it can reasonably be inferred that reductive acetylation,(23) or

reductive alkylation(24-26) procedures would be the best ways to derivatize unknown metabolites of this group.

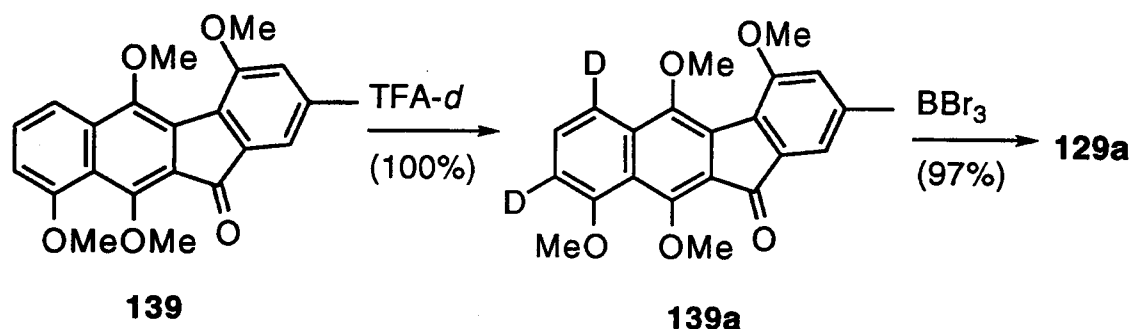


Based on the observed reaction of **145** with dithionite and the chemical precedence for reductive acetylation, our working hypothesis is that the structure of **145** is that of quinone **129**, rather than hydroquinone **138**. To indicate both the lack of observed NMR spectra, and its role in the kinamycin biosynthetic pathway (below), **129** has been named kinobscurinone.



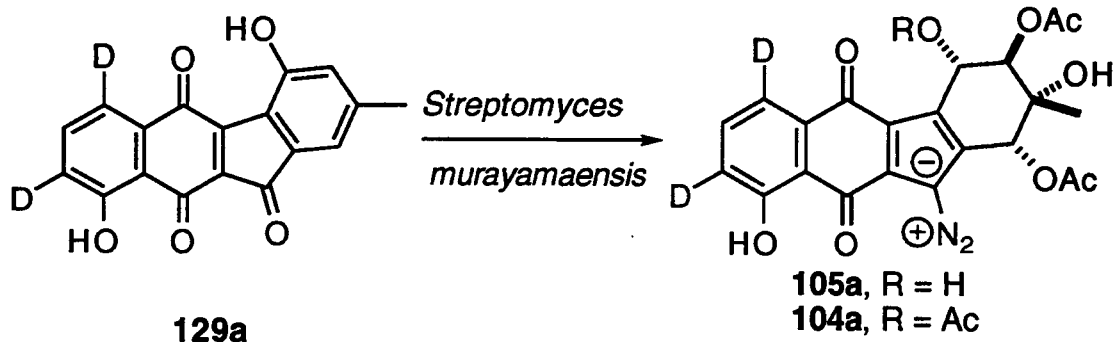
Kinobscurinone Feeding and Detection

Kinobscurinone **129** was prepared labelled with deuterium to test its intermediacy in kinamycin biosynthesis. Compound **139** was treated with trifluoroacetic acid-*d* at ambient temperature according to the procedure previously used for labeling dehydrorabelomycin,(27) **11**, and tetrangulol, **24**.(28) This provided a quantitative yield of **139a** specifically labelled at H-8 and H-10. The A ring resonances at δ 7.77 and 6.89 were absent in the ^1H NMR, and 96% exchange per position was established by EIMS [m/z 366 (100%, M^{++2}), 365 (8%, M^{++1})]. This **139a** was treated with boron tribromide to provide labelled **129a**.



Compound **129a** was added to actively growing cultures of *S. murayamaensis* in glycerol-asparagine medium(29) as a solution in DMSO. Additions were made in four pulses at 3 hour intervals from 12 to 21 hours after inoculation of the production medium. These additions initially suppressed kinamycin production, which

usually begins between 12 and 14 hours. Indeed at 24 hours, no kinamycins were evident in aliquots taken from the cultures. By 36 hours however, kinamycins A **102**, C **104**, and D **105** had been produced along with one new member of the kinamycin family which is described elsewhere. Kinamycins A and D are the normal products in this medium. Chromatographic purification provided kinamycins C and D, 63.4 mg, as a 1:1 mixture. Further separation was deemed unnecessary since both compounds have nearly identical ^1H NMR chemical shifts for their aromatic resonances(30,31) ($\text{H-8 } \Delta \delta = 0.10 \text{ ppm}$, $\text{H-10 } \Delta \delta = 0.09 \text{ ppm}$). In previous feeding experiments using deuterated acetates, it had been found that the resonances for H-8 (δ 7.23) and H-10 (δ 7.71) were not resolved in the ^2H NMR spectra of kinamycin D **105**.(31) Using natural abundance CDHCl_2 at δ 5.32 as chemical shift reference and for deuterium quantitation, a broad resonance centered at δ 7.5 was observed. The deuterium enrichment was calculated to be 0.18% per labelled site. HPLC analysis of the fermentation monitored at 254 nm showed that 65 mg of **104** and **105** were produced, which therefore corresponded to a 0.22% incorporation of **129a**.



A fermentation was performed to determine the timing of kinamycin production. Under the conditions subsequently used for the feeding, a peak with the HPLC retention time and UV/vis spectrum of **129** was observed. Coinjection with authentic **129** gave symmetrical enhancement of the height of this peak without altering the UV/vis spectrum. This demonstrated that the organism has the ability to produce **129**. Compound **129** was present in the extracts of aliquots taken from 12 to 24 hours, with kinamycin D production beginning between 12 and 14 hours. Compound **129** was present in the extract at a concentration that gave a peak area (absorbance at 254 nm) similar to that of the minor metabolites **11** and **18**. At 36 hours kinamycin D production had peaked and no **129** remained.

Since *in vivo* redox chemistry may alter the oxidation level of any quinone intermediates we might choose to feed, we cannot discern the oxidation level of **145** that is incorporated into **105**. Furthermore, chemical oxidation of the hydroquinone of **138** could occur in work-up of the fermentation, so observation of **129**

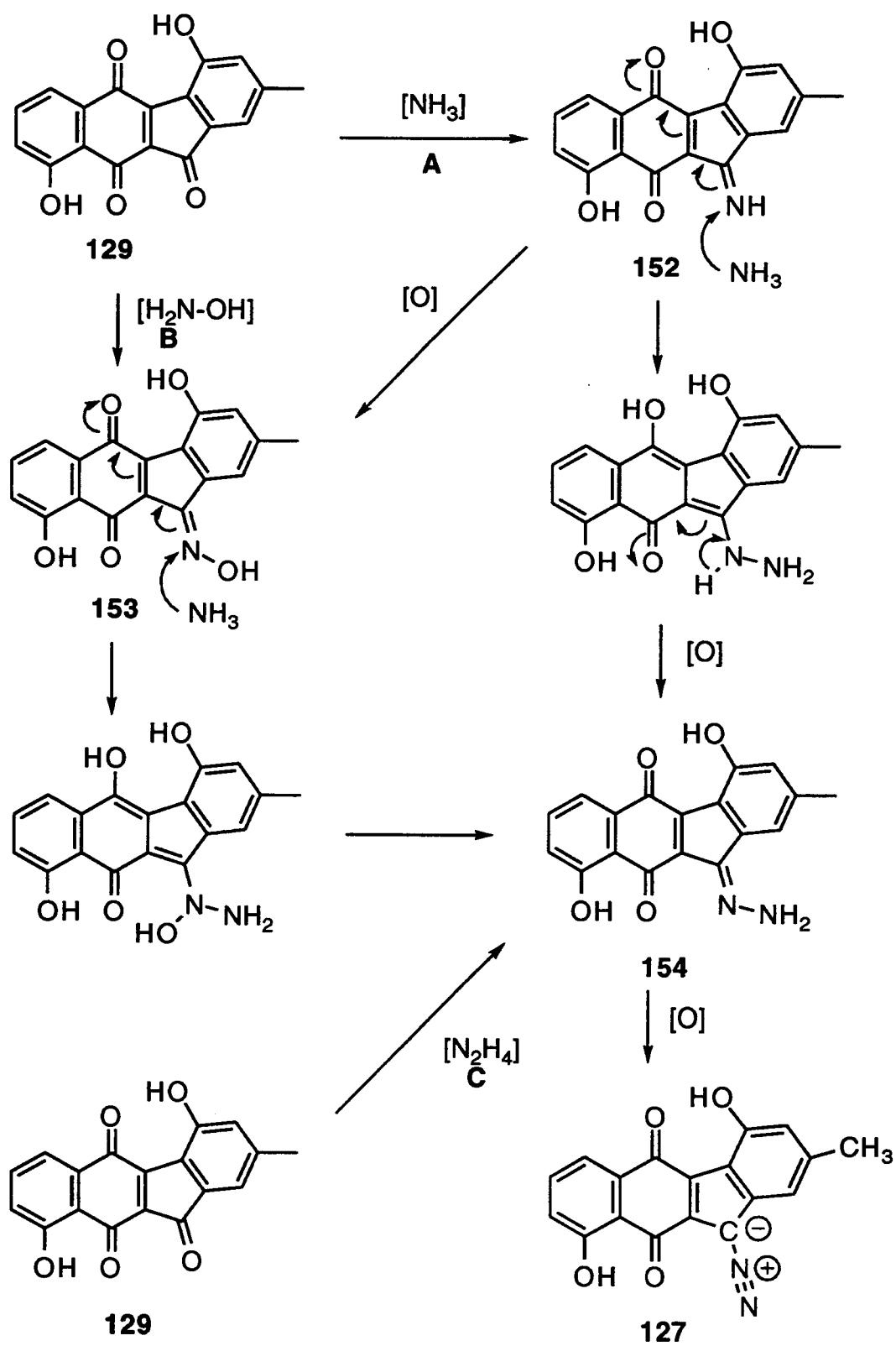
does not establish the oxidation level of the endogenous **145**. This leaves determination of the oxidation level of **145** used in subsequent enzyme mediated reactions for future cell free work.

Production and incorporation of **129**, or any other, advanced intermediate are strong evidence in support of a compound being a true biosynthetic intermediate. There is, however, the possibility that an observed and incorporated compound may not be representative of the route by which the majority of the metabolic product arises. A final criterion, kinetic competence, re: that the enzymes responsible for the conversion of a suspected intermediate be present at an activity sufficient to account for the amount of product produced, has not been demonstrated. This last criterion requires cell free work to quantify the enzymatic activities present, and so is rarely met.

Possible Nitrogen Containing Precursors to Prekinamycin

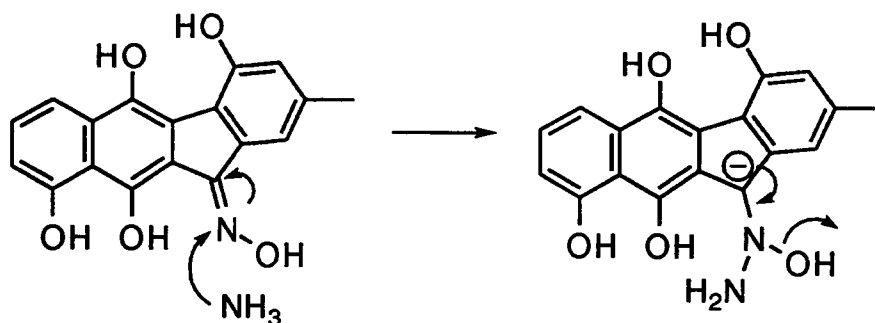
Since it now appears that kinobscurinone **129**, at some oxidation level, is an intermediate in kinamycin biosynthesis, and prekinamycin is expected to be an intermediate, the chemistry that could occur between **129** and **127** will now be considered. Outlined below (Scheme 5.3) are three possibilities differing in their oxidation level of nitrogen. While it is convenient to represent these processes at one oxidation level, this is by no means intended to suggest that other oxidation levels or

tautomers are any less viable alternatives. Routes A and B provide dehydro-12-deoxy stealthin A derivatives **152** and **153**. These may undergo nucleophilic addition followed by oxidation, or an addition elimination process to afford a hydrazone (hydrazine) intermediate such as **154**. Compound **154** would then undergo an enzymatic oxidation directly to **127**. Use of the quinone as a 2-proton 4-electron pump as was suggested for conversion of 5-aminophenanthroviridin aglycone to the cyanamide structure previously reported for **127** could also be applied in this case to effect the same oxidation. Alternatively, **154** could be formed directly from **129** by addition of hydrazine or its equivalent, as represented by route C.

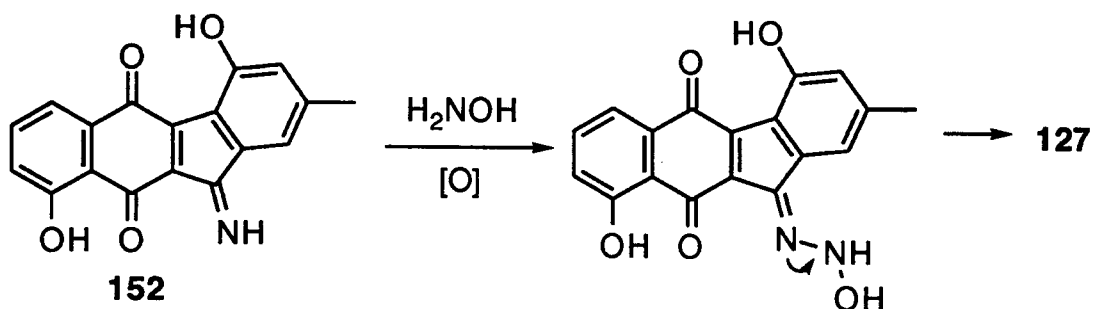


Scheme 5.3

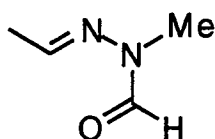
Addition-elimination reactions could make use of the stability of a cyclopentadienyl anion to facilitate addition-elimination reactions, were they to occur at the hydroquinone oxidation level.



Also, oxidized nitrogen species may be utilized rather than oxidation after assembly. A single example of this is represented below. The imine moiety of compound **152** may be stable to solvolysis, because the quaternization of the imine carbon would lead to a loss of aromatic stabilization. This rationale may also account for the solvolytic stability of many iminoquinones.



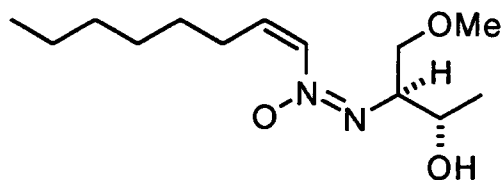
Schiff's base formation with a nitrogen from some source is probably the first step. Hydrazine is an intermediate in the fixation of nitrogen,(32) and monomethyl hydrazine is found in species of *Helvella*, apparently coming from the solvolysis of gyromytrin, **155**.(33)

**155**

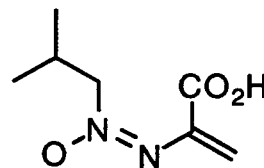
Although introduction of an N₂ unit is the most conceptually direct route to the diazo group, the N-N bond in biosyntheses reported so far have shown that the two nitrogens are introduced from separate organic nitrogen sources. Examples so far reported are the biosyntheses of elaiomycin **156**, in which [1-¹³C, ¹⁵N]-*n*-octylamine was found to be incorporated with retention of the C-N bond. A similar strategy was used for investigating the synthesis of valanimycin **157**, in which both [2-¹³C, ¹⁵N]-DL-alanine and [1-¹³C, ¹⁵N]isobutylamine were incorporated with intact C-N bonds.

In this latter case, [1-¹³C, ¹⁵N]-isobutylhydroxylamine was also found to be incorporated at a remarkable 48%. While in the case of prekinamycin only one nitrogen is carbon substituted,

making these precedents less than ideal, a working hypothesis is that the two nitrogens are introduced stepwise.

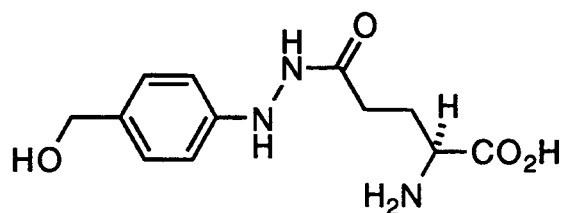


156

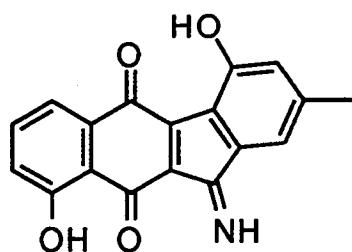
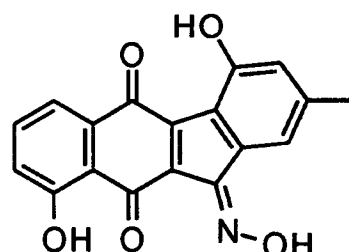


157

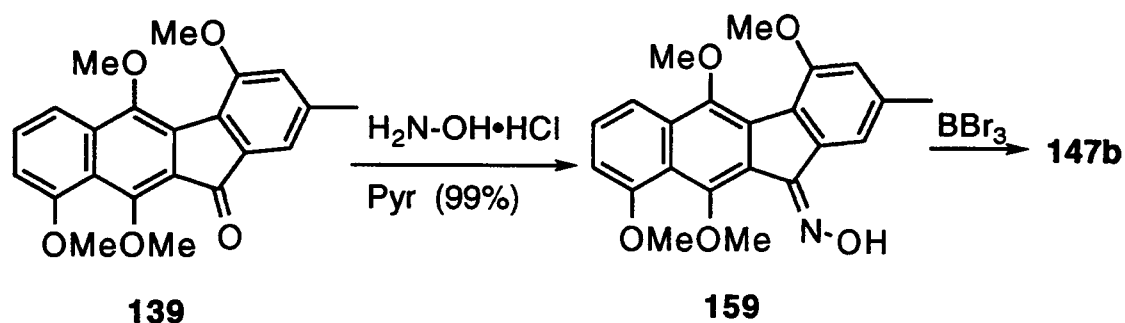
Agaricus bisporus has also been found to convert 4-hydroxymethyl phenylhydrazine to 4-hydroxymethyl benzene diazonium ion. In this same species, glutamine has been found to contribute both nitrogens in the biosynthesis of agaritine **158**.⁽³³⁾ Both of these conversions have been demonstrated in cell free systems. Horseradish peroxidase has been found to convert chloroanilines to the corresponding azo benzene dimers, via an intermediate hydroxylamine or nitroso compound. The biosynthesis of a N-nitrosourea, streptozotocin, has also been investigated.⁽³⁴⁾ This work was done exclusively with radioisotopes, so the origins of the nitrogens was inferred from that of the carbons, and is consistent with these other systems.

**158**

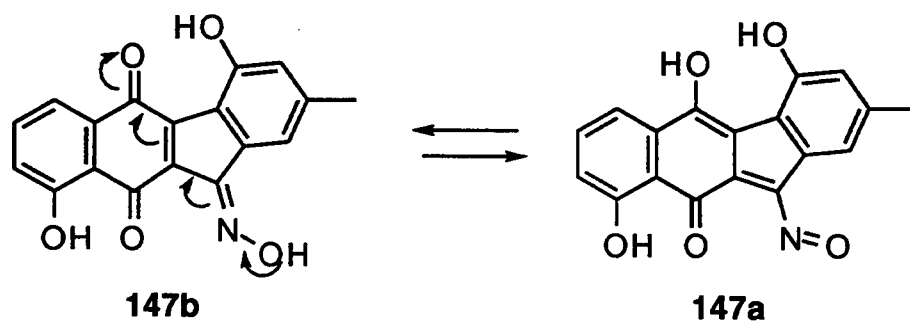
Recognition of these possible intermediates provided the opportunity to test the chemical facility with which they could be formed *in vitro*. With this objective, kinobscurinone **129** (λ_{max} 224, 252, 272, 477, and 578 nm) was reacted with ammonium hydroxide in methanolic dichloromethane, and provided a new compound with a nearly identical UV/vis spectrum (λ_{max} 220, 248, 266, 322, and 560 nm), but eluting 1.61 min earlier than **129**. Similarly, **129** was found to react with hydroxylamine hydrochloride under conditions expected to provide an oxime and gave **147b** (retention time 19.51 min, λ_{max} 212, 254, 282, 470, and 554 nm) in 69% yield. Neither of these compounds provided ^1H or ^{13}C NMR spectra under standard acquisition conditions, but the latter gave a satisfactory HREIMS (calcd for $\text{C}_{18}\text{H}_{11}\text{NO}_5$ 321.0637, found 321.0636).

**152****147b**

These results suggested that the proposed chemistry was facile, it was clear at this point that without the benefit of NMR spectra, the regiochemistry of the expected additions could not be determined. A general strategy to establish the nature of the observed reaction was therefore developed. If the same compound could be obtained by two different routes, one of which having no ambiguity about the position of the new heteroatom, this would demonstrate the nature and position of the observed reaction. To this end **139** was treated with hydroxylamine hydrochloride in pyridine, and **159** was obtained as a stereoisomeric mixture of oximes. The stereoisomers could be separated chromatographically, but this was somewhat difficult due to their low solubility. Additionally, once separated, the major product reverted to a stereoisomeric mixture over a few hours at ambient temperature.(35)

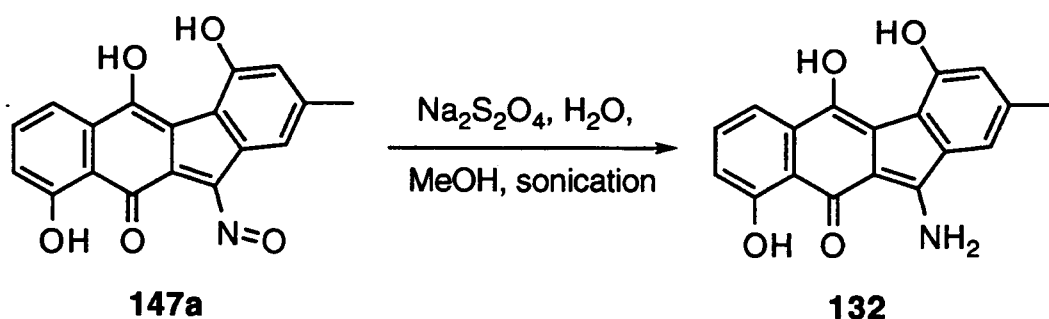


Deprotection of the oxime mixture **159** with BBr_3 gave a single product in 78% yield, identical with **147b**. Observation of a single product from the stereoisomeric mixture used for this reaction can be the result of a rapid interconversion of oxime stereoisomers, or existence of the product in only one isomeric form. Either of these possibilities suggest that nitroso compound **147a** is likely to be the predominant product structure, or a significant tautomeric contributor.



Work with another series of nitroso compounds described in a later chapter revealed that their reduction to amines with

sodium dithionite is very facile. Adding an aqueous solution of $\text{Na}_2\text{S}_2\text{O}_4$ to a methanolic solution of **147a**, and sonicating briefly gave a new red compound as the only product. The UV/vis spectrum of this new compound (212, 275, sh 328, sh 342, sh 470, 505, 537) and that of **130** (p. 183) were identical in both neutral, and basic conditions. A molecular ion was observed at 306.0 corresponding to the hydroquinone oxidation level. This indicated that the expected reaction occurred, providing 12-deoxystealthin A **132**. Dr. Cone subsequently identified **132** as a metabolite in the kinafluorenone-producing *S. murayamaensis* mutant MC1.



Determination of the Primary Nitrogen Source

As has been presented above, the two nitrogen atoms of the diazonium group can be envisaged originating either from glutamine or pyridoxamine phosphate, with the former being preceded by the work on agaratine.(33) These represent two somewhat independent nitrogen pools in the organism.(36) As

such, selective labelling of one of the two pools may directly provide the answer as to which is being used. Presented below is an experimental rationale, and work addressing this question.

Bacteria assimilate ammonia either by reductive condensation with α -keto acids, or by reaction with mixed carboxyl-phosphoric anhydrides to provide amides. In the former, glutamate dehydrogenase, or for some bacteria alanine dehydrogenase, are the enzyme activities involved.(36) For amide formation the ATP dependent glutamine synthetase activity is the enzyme activity involved. In any particular combination of bacteria and fermentation condition, one of these activities would be expected to account for the majority of the metabolic flux. If the glutamine synthetase pathway is operating, then ammonia in the fermentation would drive the glutamate synthase activity, which moves the amide nitrogen into the α -amino acid/pyridoxamine pool. It would therefore be desirable, not knowing which ammonia assimilation pathway would operate, to have a fermentation medium without an ammonia source.

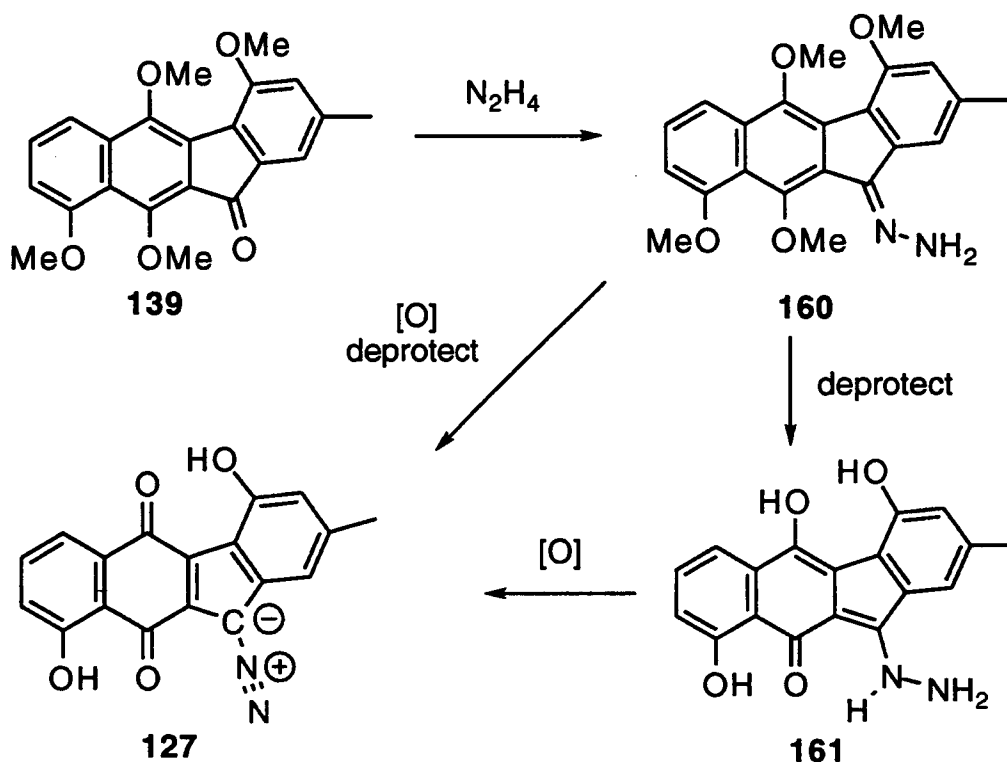
S. murayamaensis produces the kinamycins in a variety of media. However, of the defined media we have used to provide the kinamycins all have contained either an ammonium salt, or the amino acid asparagine. Asparagine catabolism involves an asparaginase activity to give aspartic acid and ammonia, and so is also an ammonia source. To find a medium without this complication, the asparagine in the glycerol asparagine medium

was replaced with equal weights of other amino acids. To the alanine containing medium so derived, was added asparagine and sodium acetate under typical kinamycin feeding conditions. A normal **105** and **102** producing fermentation was observed demonstrating the suitability of this system.

Since [amide ^{15}N]glutamine is commercially available, this can be fed to provide **105**. On a small scale, this can be analyzed by observing the FABMS, which can be expected to show 0, 1, or 2 incorporated nitrogens by enhancement of the corresponding molecular ions. The maximum enrichment that could be obtained by complete mixing of the nitrogen pools can be determined from the molar ratios of α amino versus glutamine amide nitrogens in the fermentation. Complementary experiments with ^{15}N ammonium and an [amino ^{15}N] alanine could also be inexpensively performed. In any case of 0 or 2 incorporated nitrogen atoms the position of incorporation is unambiguous. However in the case of any precursor labeling only one nitrogen, a larger scale fermentation would need to be done to obtain sufficient material, at a high enough level of enrichment to detect the ^{15}N by ^{15}N or ^{13}C NMR.(37) In the latter case, sensitivity may be improved by co-feeding of sodium [2- ^{13}C]acetate. Position assignments would be made by comparing chemical shifts or coupling constants with known diazonium substituted compounds.

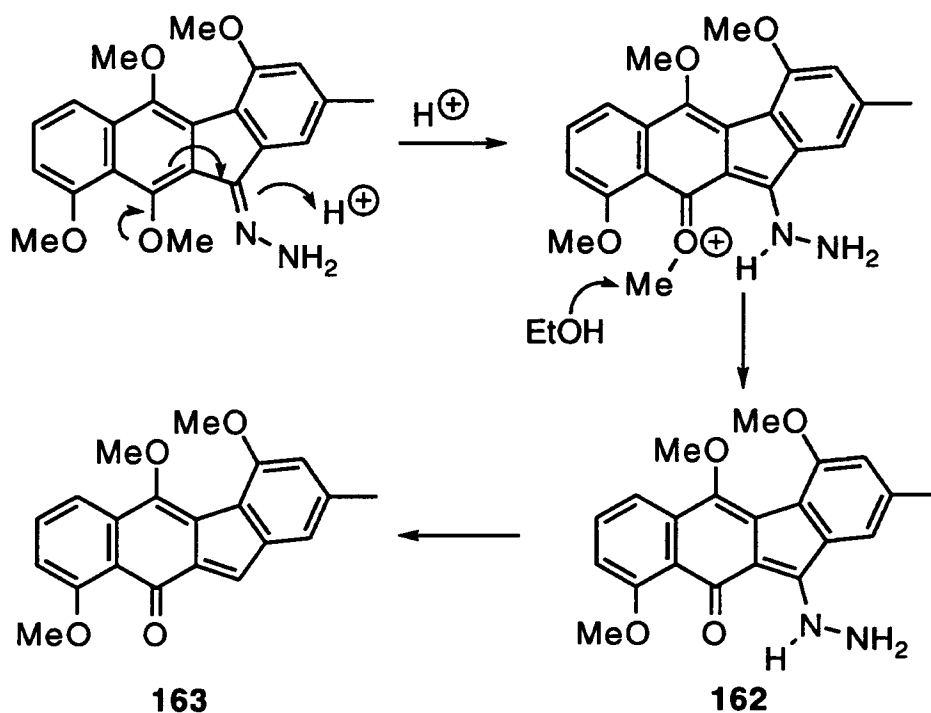
Other Benz[*b*]fluorene Chemistry

Preparation of the hydrazone **160** was next attempted. It was hoped that **160** could be deprotected to the hydrazine **161**, and that would spontaneously, or by chemical oxidation provide prekinamycin **127**. Alternatively, the protected hydrazone could be converted to the diazofluorene, and then deprotected to **127**, Scheme 5.4. Hydrazine **161** may also be a biosynthetic intermediate, so synthesis of this compound in a labelled form was a secondary objective.

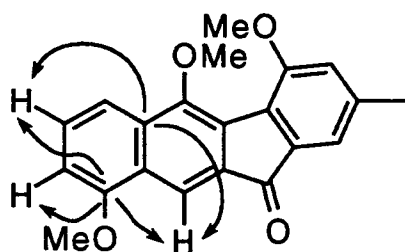
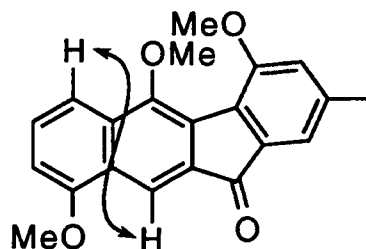


Scheme 5.4

Reaction of **139** with anhydrous hydrazine efficiently produced a hydrazine addition product that unexpectedly had retained only three of the methoxy groups. Since a hydrogen bonded NH was observed in the ^1H NMR, structure **162** was assigned. Formation of this compound can be rationalized by formation of the expected hydrazone followed by protonation of the hydrazone sp^2 nitrogen followed by solvolytic cleavage of its oxonium tautomer. Oxidation of this compound with ammonium cerium(IV)nitrate,(3) or with silver oxide,(38) provided the product of nitrogen elimination rather than the expected quinone or diazo compounds. Compound **163** would be expected to deprotect to provide **141**, however this proved unsuccessful.

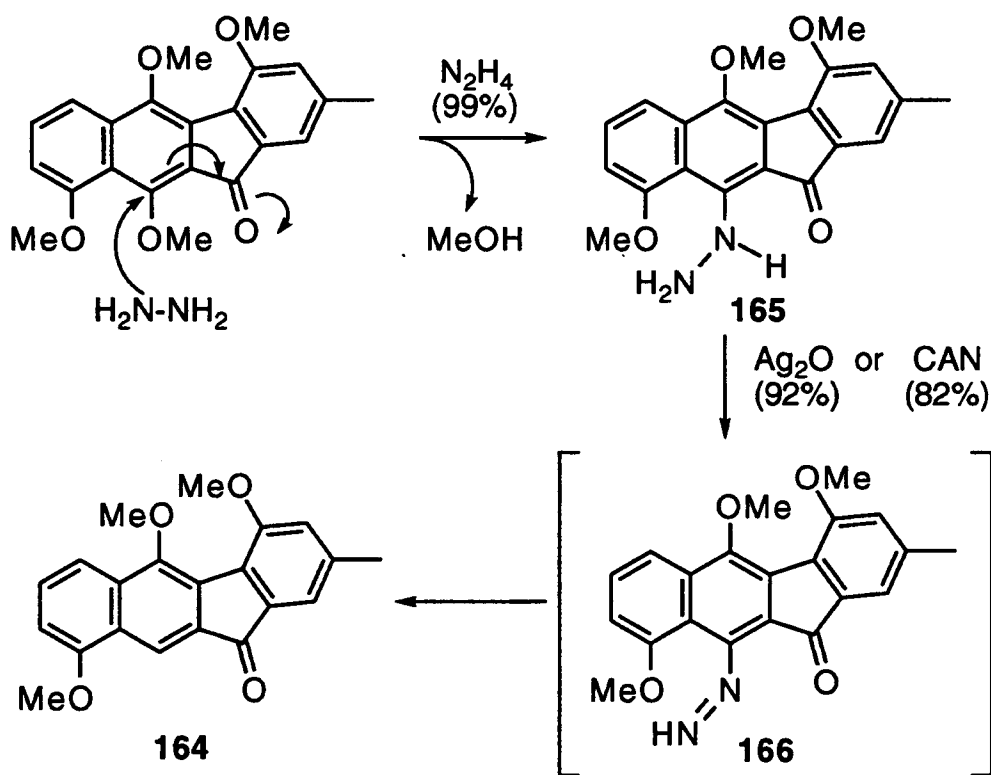


While in the process of compiling the data for these compounds, it was noticed that the carbonyl of the nitrogen eliminated product was more downfield (δ 193.0 ppm) than would be expected for structure **163**. Carbonyls in five-membered rings, however, are more downfield than those in six-membered rings due to the decrease in the C-C-C bond angle. The possibility of the alternative structure **164** was therefore checked using the inverse detected NMR experiments HMQC and HMBC. The long range correlations that are important for defining the regiochemistry are shown on structure **164a**. Additionally, a ^1H - ^1H COSY experiment showed the correlation shown on structure **164b**, further supporting the revised structure assignment.

**164a****164b**

Formation of **164** shows that under the conditions of this reaction, an addition-elimination reaction is favored over hydrazone formation, providing hydrazone **165**. When subjected to oxidizing conditions, a loss of N_2 occurred, presumably via the intermediacy of a quinone monoimine (oxonium ion), which

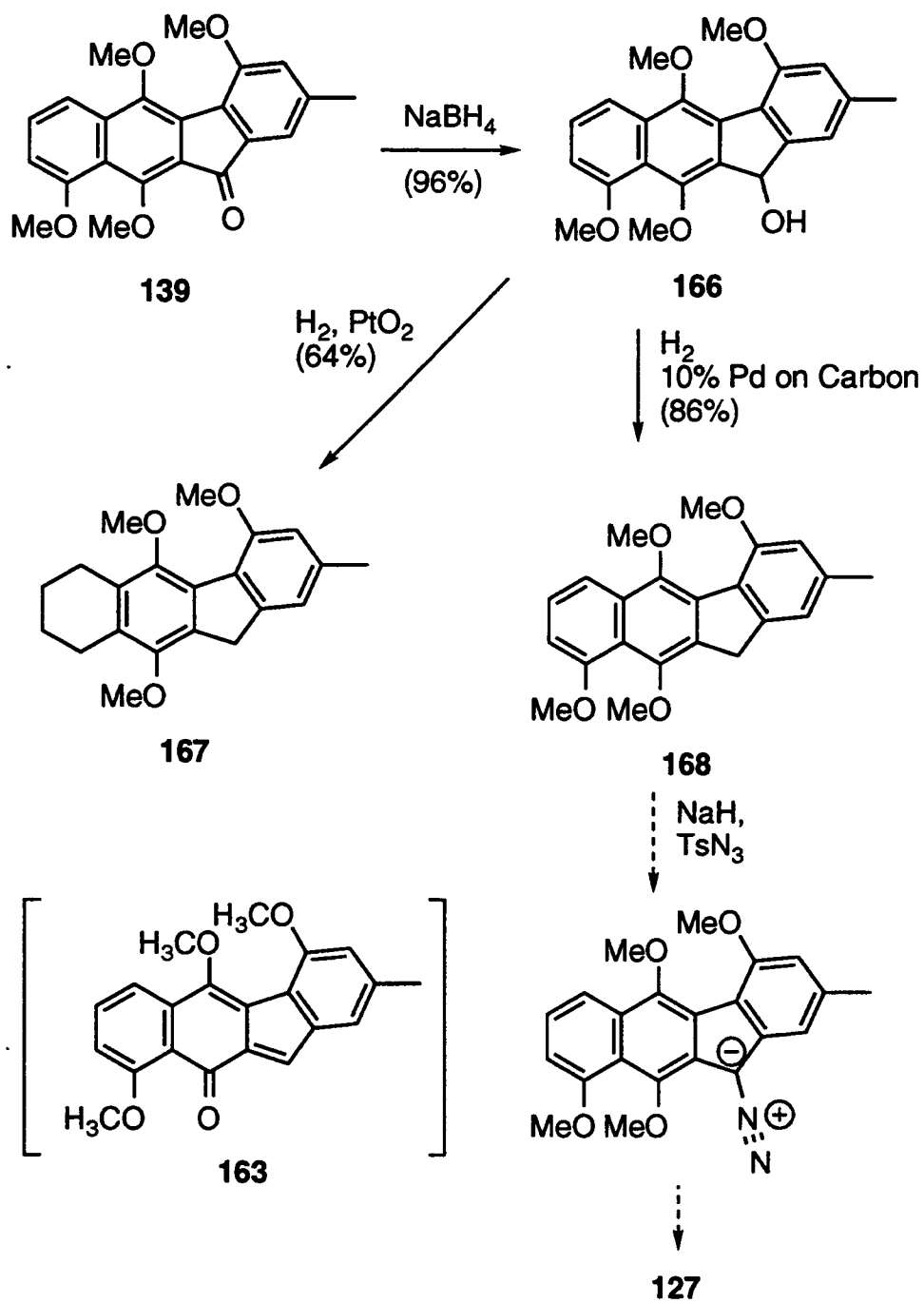
tautomerized to the aryldiazine **166**.⁽⁹⁾ Elimination of the resulting aryldiazine could then afford **164**, Scheme 5.5. Observation of this reaction lends credence to the proposed sequence of events leading from prekinamycin to **141**. The desired hydrazone should be accessible via acid-catalyzed hydrazone formation.



Scheme 5.5

Borohydride reduction of **139** has provided the fluorenol, **166**, which was over-reduced to the cyclohexyl fluorene **167** by

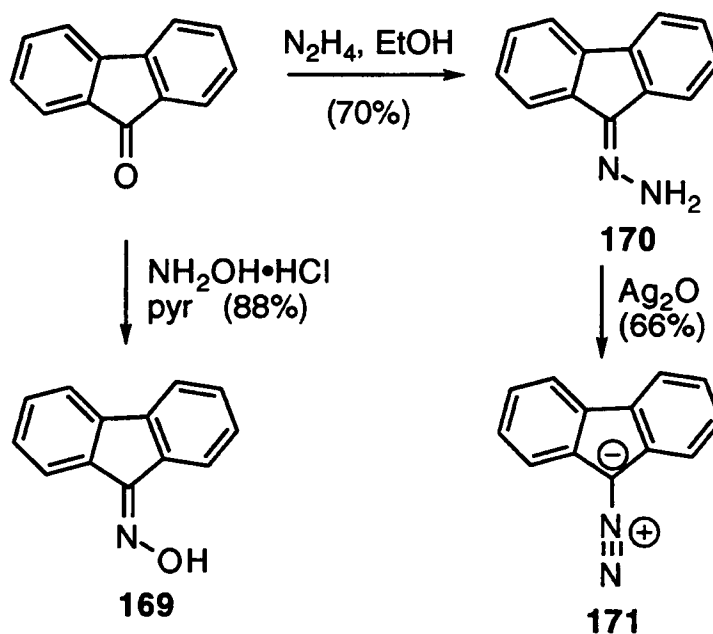
hydrogenation with platinum oxide. Hydrogenation over palladium on carbon, however, gave the fluorene **168**.⁽³⁹⁾ Deprotonation of **168**, and reaction with tosyl azide may provide a ready access to prekinamycin, **127**, Scheme 5.6. The fluorenol **166** was also reacted with trifluoroacetic acid to give a new red compound, which is expected to be the corresponding tri-O-methylfluorene, **163**, which may provide access to **141**. Analogously to the reaction of 1,8-di-O-methyltetrangulol, **37**, free radical bromination of the fluorenone **139** with N-bromosuccinimide occurs at the C3 methyl, and may provide access to compounds oxidized at that position.



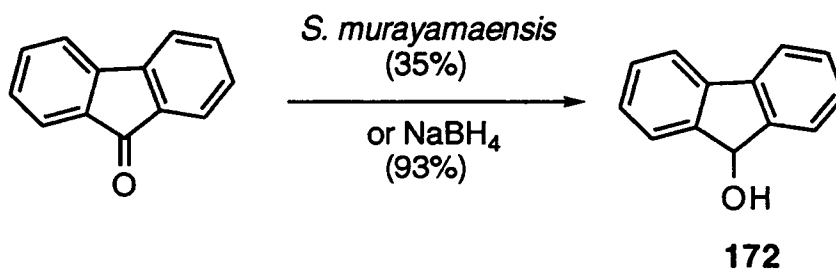
Scheme 5.6

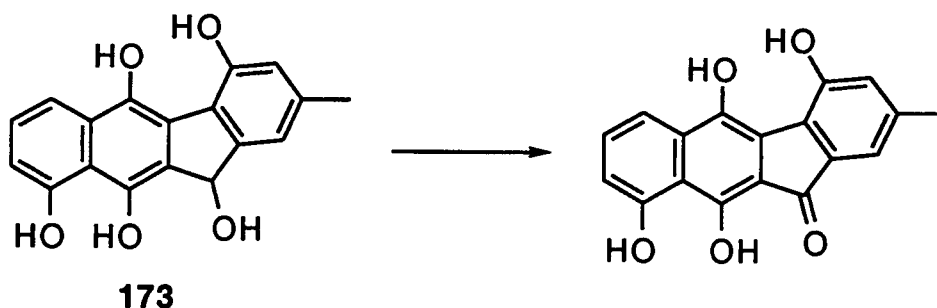
9-Fluorene Derivatives

Concurrently with the above work, a series of model reactions were performed. Beginning with 9-fluorenone, literature procedures were used to give the oxime **169** and hydrazone **170**. The hydrazone **170** was further converted to 9-diazofluorene, **171**.⁽³⁸⁾ These reactions were used to select conditions for the same reactions performed on the benzfluorene series. Additionally, since this series mimics the expected sequence of biosynthetic intermediates, although not the expected tautomers, it was hoped that one or more of these compounds could serve as a fortuitous substrate for the kinamycin biosynthetic enzymes. Compounds **169**, **170**, and **171** were fed at 15 to 19 mg per 400 mL broth to growing cultures of *S. murayamaensis*, but at this level of addition, no secondary metabolism was observed.



The 9-fluorenone itself however was similarly fed, and was mostly converted to a new product not matching those expected. A simple reduction was suspected, so 9-fluorenol was prepared chemically and found to match the fermentation product. Subsequently, **172** was isolated from the fermentation in 35% purified yield. This suggested the possibility that the observed reduction is the reverse reaction catalyzed by the enzyme that is expected to oxidize the fluorenyl addition product **173**.





Experimental

Materials and Methods. THF was distilled from Na/benzophenone ketyl, CH_2Cl_2 from CaH, and pyridine from KOH. Reactions were performed under an atmosphere of argon in oven dried glassware. Chromatography was performed using Kieselgel 60 (Merck, particle size 0.040-0.063 mm) and distilled solvents. Fermentation media were prepared with deionized water (Millipore Milli Q System), and with glucose added as a 40% wt/vol solution to the other ingredients after autoclaving. Sonication was accomplished with a Heat Systems Inc. model W-225R sonicator.

X-ray Crystallography of Kinafluorenonetriacetate (137). Crystallographic data on a needle of dimensions 0.2 x 0.2 x 0.4 mm, mounted on a glass fiber, was collected at 23°C on a Rigaku AFC6R single crystal diffractometer with graphite monochromated Mo $\text{K}\alpha$ radiation from a 12 kW rotating anode

generator. The P-1 space group, and unit cell parameters (triclinic, $Z = 2$) were determined from 19 reflections in the range $26.13 < 2\theta < 30.27^\circ$. Three reflections measured every 300 reflections throughout data collection demonstrated crystal stability.

The structure was solved and refined with the use of the TEXSAN(1) crystallographic software package, and the positions of all non-hydrogen atoms determined with the direct methods program SHELXS based on 2239 observed reflections ($2\theta_{\max} = 55.0^\circ$). No absorption correction was applied, and all hydrogens were placed in calculated positions. Final cycle anisotropic refinement of C and O atoms gave an R value of 0.055 with a p -factor of 0.03.

Diode Array HPLC. Samples were analyzed using a Waters 600E gradient HPLC equipped with Waters 990+ diode array detector. Separation was accomplished on a Waters NovaPak C₁₈ radial compression column (0.8 x 10 cm, 5 μ m beads) using a gradient of 5 to 95% acetonitrile in water over a period of 20 minutes at 1.5 mL/min. Both solvents contained 0.1% HOAc. Absorption spectra were obtained over a wavelength range of 200-650 nm with a 2 nm interval. A library of UV/vis spectra of the kinamycins, and all other characterized metabolites of *S. murayamaensis*, constructed under the same chromatographic conditions, was used for reference.

Culture and Feeding Conditions. *S. murayamaensis* was maintained at 4 °C on Petri plates composed of 1.25% glucose, 0.05% L-asparagine, 0.05% K₂HPO₄, 0.02% MgSO₄·7H₂O 0.01% FeSO₄·7H₂O, and 1.5% agar. A glycerol-soybean flour seed medium(29) (100 mL broth in a 500 mL foam plugged Erlenmeyer flask) was inoculated with actively growing agar plugs, and maintained at 27 °C, 280 rpm, for 48 h. This was used to inoculate four glycerol-asparagine(29) production flasks (400 mL broth in each 2L flask) at 5% inoculum. Synthetic kinobscurinone (31.2 mg) was dissolved in DMSO (3.20 mL) and added to the production flasks (0.200 mL per addition per flask) at 12, 15, 18, and 21 h after inoculation. An experiment to determine the timing of kinamycin accumulation was performed with one production flask, and additions of DMSO as above.

Time Course and Observation of Kinobscurinone. A sample of whole broth (10.0 mL) was mixed with EtOAc (10.0 mL), acidified with HCl (1.00 mL), and briefly sonicated. Filtration through celite, evaporation *in vacuo*, and redissolution in CH₂Cl₂-MeOH (10:1, 1.00 mL) provided samples for HPLC analysis. Kinamycin production was found to begin between 12 and 14 h, and to reach a maximum between 24 and 36 h after inoculation. A peak with identical HPLC retention time and UV/visible spectrum with kinobscurinone was observed in

samples from 12 to 24 h. Co-injection of the extracts with authentic kinobscurinone provided a single peak of unchanged UV/visible spectrum.

Isolation of Kinamycins C and D. At 36 h, the combined broth was adjusted to pH = 2.8 with HCl (1M), and sonicated (10 min, 90% duty cycle), and the whole broth filtered through celite. The cell mass was sequentially extracted with acetone (400 mL), and EtOAc (2 x 300 mL) and concentrated to approximately 100 mL. This concentrate was extracted with EtOAc (3 x 100 mL) and combined with extracts from the broth (4 x 250 mL). Drying over Na₂SO₄ and concentration gave 820 mg crude extract, with a kinamycin C and D content of 65.2 mg calculated by monitoring the HPLC trace at 254 nm. Extraction of the crude solids with CHCl₃ (2 x 20 mL) gave after evaporation 550.3 mg, which was loaded on a silica gel column (5 x 15 cm) eluted with EtOAc. Pooled kinamycin containing fractions (90.0 mg) were loaded on a second column (5 x 15 cm) eluted with EtOAc/hexanes (7:3) which gave nonpolar kinamycins (10.0 mg), followed by 63.4 mg of kinamycins C and D. This chemically pure sample of kinamycins was found to contain deuterium by ²H NMR, but was re-separated to exclude the possibility of a signal from deuterated impurity. A silica gel column (3.5 x 19 cm) was prepared and loaded in 1% MeOH in CH₂Cl₂. Elution with 5% MeOH in CH₂Cl₂ gave upon evaporation 55.1 mg kinamycins C and D.

²H NMR of Kinamycin C and D. A solution of 47.6 mg kinamycins C and D in CH₂Cl₂ (0.345 g) in a 5 mm tube was prepared. This was acquired under standard ²H NMR conditions: SF 61.423; O1 21350; SI 16K; TD 4K; SW 1433; PW 13.5; NS 38662, TE 297. Integration of aromatic deuterium compared with that of the natural abundance solvent line indicated 8.5 times natural abundance, and an incorporation of 0.13% (also 0.13% enrichment per site).

Kinobscurinone (129). a.(11) A solution of 1,6,7,11-tetramethylkinobscurinone **139** (23.7 mg, 0.0650 mmol) in CH₂Cl₂ (3.25 mL) was cooled to -78 °C and treated with a solution of boron tribromide (0.650 mL, 1 M in hexanes, 0.650 mmol) over 0.1 h. The resulting black suspension was allowed to warm to rt over 14 h, then MeOH (0.200 mL) was added and stirring continued for 24 h. Filtration and washing sequentially with HCl (5.00 mL, 1.00 M) and water (5.00 mL) was followed by drying *in vacuo*. The filtrate was dissolved in THF (11 mL) and refiltered, affording after concentration 17.8 mg (89%) of **129** as a purple solid. HPLC analysis showed a single product peak: mp >300 °C; IR 2960, 2923, 2854, 1717, 1614, 1283, 1079 cm⁻¹; UV λ_{max} 580, 488, 272, 252, 224 nm; EIMS *m/z* (relative intensity) 308.0 (100%), 307.0 (66%); ESIMS *m/z* (relative intensity) 309.0 (8%), 317.2 (100%); positive CIMS *m/z* (relative intensity) 309.1

(18%), 323.1 (100%, OMe substituted); HRCIMS m/z calcd for $C_{18}H_{13}O_5$ 309.0763, found 309.0791.

b. To a solution of the 1,6,7,11-tetraacetylkinobscurinone **150** (0.25mg) in MeOH (0.100 mL) was added acetyl chloride (0.020 mL). HPLC analysis showed kinobscurinone as the only significant constituent after 8 h of stirring.

1,6,7,11-Tetraacetyldihydrokinobscurinone (150).(19) To a solution of kinobscurinone **129** (12.6 mg) in DMF (1.50 mL) was added pyridine (1.50 mL) followed by acetic anhydride (0.75 mL). After stirring 24 h at rt, solvents were removed *in vacuo*. Chromatography on silica gel (1 x 12 cm) eluted with EtOAc/hexanes (1:1) gave after evaporation 3.7 mg **150** (19%) as pale yellow needles: mp 222.6-224.1 °C; IR 1773.7, 1711.8, 1190.8, 1141.2, 1017.5 cm^{-1} ; UV λ_{max} 420, 290, 214 nm; 1H NMR ($CDCl_3$) δ 7.82 (dd, 1H, J = 8.5, 1.1 Hz, C10-H), 7.59 (dd, 1H, J = 8.5, 7.7 Hz, C9-H), 7.48 (q, 1H, J = 0.7 Hz, C4-H), 7.18 (dd, 1H, J = 7.7 1.1 Hz, C8-H), 7.01 (q, 1H, J = 0.7 Hz, C2-H), 2.55 (s, 3H), 2.54 (s, 3H), 2.46 (s, 3H), 2.42 (s, 3H), 2.41 (s, 3H, C12-H); ^{13}C NMR ($CDCl_3$) δ 188.45 (C5), 169.24 (Ac), 168.66 (Ac), 168.31 (Ac), 167.78 (Ac), 148.36 (C7), 145.75 (C1), 142.83, 142.44 (C3), 138.55, 137.97 (C11), 134.84 (C10a), 131.71 (C11b), 130.62 (C2), 130.00 (C9), 128.63, 123.27 (C4), 122.95 (C6a), 122.69 (C8), 120.92 (C10), 21.07 (4 acetate Me), 20.88 (C12); EIMS m/z (relative intensity) 476.0 (M^+ , 3%), 434.0 (10%), 392.0 (24%),

350.0 (29%), 308.0 (100%); HREIMS m/z calcd for $C_{26}H_{20}O_9$ 476.1107 (M^+), found 476.1106.

[8,10- 2H_2] 1,6,7,11-Tetramethyldihydrokinobscurinone (139a).(27) 1,6,7,11-Tetramethylkinobscurinone (38.2 mg, 0.101 mmol) was dissolved in trifluoroacetic acid- d (2.02 g, 17.6 mmol, 173 eq.) and stirred at ambient temperature under an atmosphere of nitrogen for 2 d. Removal of the trifluoroacetic acid *in vacuo* gave 38.4 mg (100%) of pure **139a** as a yellow solid. 1H NMR and EIMS showed 96% 2H per exchanged position: mp 108.2-110.4 °C; 1H NMR ($CDCl_3$) δ 2.39 (s, 3H), 3.88 (s, 3H), 3.98 (s, 3H), 4.01 (s, 3H), 4.03 (s, 3H), 6.92 (s, 1H), 7.22 (s, 1H), 7.47 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.42, 56.15, 56.35, 62.44, 63.12, 107.90 (t, J = 22.9 Hz), 115.84 (t, J = 23.4 Hz), 117.15, 118.95, 121.48, 122.79, 126.76, 127.75, 129.68, 137.19, 138.80, 141.30, 146.40, 155.04, 155.23, 159.34, 190.43; EIMS m/z (relative intensity) 366.2 ($[M+2]^+$, 100%), 365.2 ($[M+1]^+$, 8%), 351.2 (43%), 337.2 (34%); HREIMS calcd for $C_{22}H_{18}^2H_2O_5$ 366.1436, found 366.1437.

[8,10- 2H_2]-Kinobscurinone (129a).(11) A solution of [8,10- 2H_2] 1,6,7,11-tetramethylkinobscurinone **139a** (37.9 mg, 0.104 mmol) in CH_2Cl_2 (5.00 mL) was cooled to -78 °C and treated with a solution of boron tribromide (1.04 mL, 1 M in hexanes, 1.04 mmol) over 0.1 h. The resulting black suspension was allowed to

warm to rt over 14 h, then MeOH (0.250 mL) was added and stirring continued for 1 h. HCl (2.00 mL, 0.5M) was next added, and the resulting suspension repeatedly extracted (MeOH/CH₂Cl₂ = 1:9) until the extracts were colorless. Concentration gave 31.2 mg (97%) of **129a** as a purple solid. HPLC analysis showed a single product peak.

5-Nitrosokinobscurine (147a). a. From kinobscurinone:(40) To a solution of kinobscurinone **129** (15.3 mg, 0.0500 mmol) in pyridine (0.250 mL) was added hydroxylamine hydrochloride (84.1 mg), and the mixture stirred at rt 1 d. After evaporation of solvent *in vacuo* and redissolution in MeOH-CH₂Cl₂ (1:10, 25.0 mL), the mixture was washed with HCl (1.0 M, 3 x 5.0 mL). Removal of solvents gave 12.5 mg (78%) of the nitroso compound **147a** as a purple solid: mp >300 °C; IR (KBr) 3233.3, 1628.3, 1586.4, 1456.8, 1379.2, 1270.5, 1040.7 cm⁻¹; UV λ_{max} 210, 254, 284, 380, 468, 555 nm; ¹H and ¹³C NMR unobserved; EIMS m/z (relative intensity) 321.0 (M⁺, 78%), 306.0 (100%), 278.0 (29%); HREIMS calcd for C₁₈H₁₁NO₅ 321.0637, found 321.0636.

b. From 1,6,7,11-Tetramethyl-5-kinafluorenone oxime **159**.(11) A THF solution of **159** (15.4 mg) was evaporated *in vacuo*, and the resulting solid suspended in CH₂Cl₂ (2.00 mL), and cooled to -78 °C. Boron tribromide (1M in CH₂Cl₂, 0.820 mL) was added dropwise over 0.2 h, and the resulting black

solution allowed to slowly warm to rt. After 14 h, the flask was packed in ice, MeOH (200 μ L) was added, and stirring continued for 1 d. Collection of the solid precipitate by filtration gave 9.1 mg (69%) of **147a** as a purple solid.

4,5,9,10-Tetramethoxy-2-methyl-11H-benzo[b]fluoren-11-one oxime (159).(40) To a solution of the tetramethylkinafluorenone **139** (7.9 mg, 0.022 mmol) in ethanol (2.20 mL) and pyridine (2.20 mL) was added hydroxylamine hydrochloride (8.1 mg). After stirring at rt 3 d, evaporation gave an orange gum that was suspended in H₂O and extracted with EtOAc (5 x 5 mL). Drying over Na₂SO₄, followed by evaporation gave 8.3 mg (99%) of a stereoisomeric mixture of oximes as a yellow solid. In a separate experiment, isolation of the major isomer by flash chromatography (40% EtOAc in hexanes) gave an analytical sample that reverted to a mixture on standing in DMSO:(35) mp 231.1-233.6 °C; IR (KBr) 1602.0, 1577.7, 1368.9, 1339.9, 1270.2, 1059.1, 978.4 cm⁻¹; ¹H NMR (DMSO-*d*₆) δ 12.40 (s, 1H), 8.14 (s, 1H), 7.72 (d, 1H, *J* = 7.9), 7.45 (dd, 1H, *J* = 8.1, 8.1), 7.05 (s, 1H), 7.01 (d, 1H, *J* = 7.5), 3.95 (s, 3H), 3.93 (s, 3H), 3.78 (s, 3H), 3.75 (s, 3H), 2.50 (s, 3H); ¹³C NMR (DMSO-*d*₆, major isomer) δ 162.3, 159.8, 156.0, 154.4, 151.0, 145.6, 138.9, 138.2, 133.8, 132.6, 131.8, 129.3, 127.3, 125.6, 120.6, 120.4, 112.6; EIMS *m/z* (relative intensity) 378.3 (100%), 364.3 (52%),

334.3 (22%), 304.3 (9%); HREIMS calcd for $C_{22}H_{21}NO_5$ 379.1420, found 379.1419.

10-Hydrazo-4,5,9-trimethoxy-2-methyl-11H-benzo[b]fluoren-11-one (165). The tetramethylfluorenone **139** (21.1 mg, 0.0579 mmol) and absolute EtOH (5.00 mL) were heated at reflux until homogeneous. Anhydrous hydrazine (0.500 mL) was added, at 0.2 h the heating bath was removed, and upon cooling the solvent removed *in vacuo*. Flash chromatography (1 x 12 cm silica, 5% MeOH in CH_2Cl_2 eluent) afforded 20.9 mg (99%) of the hydrazine **165** as an orange solid: mp 172.0-173.5 °C; IR (KBr) 3403.5, 2931.4, 1649.7, 1601.3, 1360.7, 1052.6 cm^{-1} ; 1H NMR ($CDCl_3$) δ 9.05 (br s, 1H, exch.), 7.74 (dd, 1H, $J = 8.2, 0.9$), 7.45 (dd, 1H, $J = 8.2, 8.2$), 7.20 (s, 1H), 6.85-6.87 (m, 2H), 5.05 (br s, 2H, exch.), 4.01 (s, 3H), 4.00 (s, 3H), 3.82 (s, 3H), 2.41 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 188.7, 159.2, 154.8, 151.8, 142.4, 140.8, 140.0, 136.2, 129.8, 127.8, 125.4, 117.3, 116.8, 116.6, 116.3, 110.6, 107.8, 62.8, 56.2, 56.0, 21.5; EIMS m/z (rel intensity) 364.3 (M^+ , 40%), 349.3 (100%), 334.2 (93%), 320.2 (17%); HREIMS calcd for $C_{21}H_{20}N_2O_4$ 364.1423, found 364.1423.

4,5,9-Trimethoxy-2-methyl-11H-benzo[b]fluoren-11-one (164). **a.** A solution of ammonium cerium nitrate (1.46 mL, 0.055 M soln in H_2O) was added dropwise to a solution of the

fluorenehydrazine **165** (13.3 mg) in DMF (1.92 mL) while maintaining the mixture at 0 °C. After removal of the cooling bath and 1 h stirring, water (3.00 mL) was added and the mixture extracted with CHCl₃ (6 x 2.00 mL, emulsions broken by centrifugation). Washing the organic extracts with brine (2.00 mL) and evaporation gave a solid that was redissolved in CHCl₃ (2.00 mL) and filtered to remove remaining NaCl. Evaporation *in vacuo* gave 10.0 mg (82%) of **164** as a yellow solid: mp 193.0-193.2 °C; IR (KBr) 2926.4, 2841.0, 1702.4, 1610.0, 1364.9, 1314.2, 1265.8, 1059.3 cm⁻¹; UV (HPLC) λ_{max} 230, 270, 382, 452 nm; ¹H NMR (CDCl₃) δ 8.46 (s, 1H), 7.76 (d, 1H, J = 8.6), 7.47 (dd, 1H, J = 8.1, 8.1), 7.26 (s, 1H, coincidental with CHCl₃), 6.95 (s, 1H), 6.83 (d, 1H, J = 7.8), 4.04 (s, 3H), 3.99 (s, 3H), 3.94 (s, 3H), 2.41 (s, 3H); ¹³C NMR (CDCl₃) δ 193.0, 158.0, 155.2, 150.1, 141.3, 138.3, 135.0, 133.0, 129.4, 128.4, 127.4, 126.2, 119.5, 117.6, 117.0, 115.6, 105.7, 63.4, 56.2, 55.6, 21.4; EIMS *m/z* (rel intensity) 334.0 (M⁺, 100%), 319.0 (21%), 289.0 (22%); HREIMS calcd for C₂₁H₁₈O₄ 334.1205, found 334.1205.

b. A suspension of sodium sulfate (121 mg) and silver(I)oxide (31.2 mg) in THF (5.00 mL) was heated at reflux. To this was added the hydrazine **165** (21.2 mg) in THF (2.00 mL) by cannula over 0.1 h. After 0.2 h the reaction was allowed to cool, and was then filtered and evaporated to give 17.9 mg (92%) of pure **164** as a yellow solid.

11-Hydroxy-4,5,9,10-tetramethoxy-2-methyl-11H-benzo[*b*]fluorene (166). To a suspension of 1,6,7,11-tetramethyldihydrokinobscurinone, **139**, (10.1 mg) in MeOH (3.00 mL) was added sodium borohydride (10.0 mg). The yellow color of the fluorenone was dissipated in 0.1 h, and after 1 h stirring at rt, acetone (0.200 mL) was added to quench the remaining sodium borohydride. Removal of the solvents *in vacuo* gave a white solid that was suspended in HCl (1 M, 1.00 mL), and extracted with CH₂Cl₂ (3 x 3 mL). Drying over Na₂SO₄ and evaporation gave 9.7 mg (96%) of **166** as a white solid: mp 92.6-93.9 °C; IR (KBr) 2930.8, 2837.9, 1702.3, 1602.7, 1357.0, 1058.2 cm⁻¹; ¹H NMR (CDCl₃) δ 7.89 (dd, 1H, J = 8.0, 0.9), 7.41 (dd, 1H, J = 8.1, 8.1), 7.14 (s, 3H), 6.89 (d, 1H, J = 7.6), 6.80 (s, 1H), 5.94 (s, 1H), 4.04 (s, 3H), 4.02 (s, 3H), 4.01 (s, 3H), 3.90 (s, 3H), 3.22 (br s, 1H, exchange), 2.44 (s, 3H); EIMS m/z (rel intensity) 366.2 (63%, M⁺), 349.2 (100%), 335.2 (60%); HREIMS calcd for C₂₂H₂₂O₅ 366.1467, found 366.1467.

Cyclohexyl[*e*]-1,6,9-trimethoxy-3-methylfluorene (167). To a solution of 11-Hydroxy-4,5,9,10-tetramethoxy-2-methyl-11H-benzo[*b*]fluorene **166** (7.3 mg) in EtOH (3.00 mL) was added platinum oxide (2.2 mg), and the mixture stirred at rt under 1 atm H₂ gas for 28 h. Filtration through a paper plug and evaporation gave 7.1 mg crude **167**. This was chromatographed with CH₂Cl₂ (1 x 12 cm, silica gel) to afford 4.1 mg (64%) of pure **167** as

colorless prisms: mp 155.4-156.5 °C; IR (KBr) 2934.1, 1460.5, 1323.2, 1262.5 cm⁻¹; ¹H NMR (CDCl₃) δ 6.97 (s, 1H), 6.73 (s, 1H), 3.98 (s, 3H), 3.86 (s, 2H), 3.83 (s, 3H), 3.72 (s, 3H), 2.86 (m, 2H), 2.78 (m, 2H), 2.42 (s, 3H), 1.75-1.81 (m, 4H); ¹³C NMR (CDCl₃) δ 154.6, 150.3, 149.1, 145.6, 138.0, 132.6, 131.6, 131.5, 129.5, 126.2, 118.3, 111.1, 61.4, 59.4, 55.9, 34.7, 24.0, 23.7, 22.8, 22.5, 21.5; EIMS *m/z* (rel intensity) 324.3 (M⁺, 100%), 309.2 (19%), 293.2 (21%); HREIMS calcd for C₂₁H₂₄O₃ 324.1725, found 324.1725.

Feeding of 9-Fluorenone to *S. murayamaensis*. A 3% glycerol asparagine production medium (400 mL broth in 2 L production flasks) was inoculated (5% v/v) with 48h glycerol soybean seed medium prepared as before. 9-Fluorenone (18.8 mg) was dissolved in DMSO (1.00 mL), and added in four aliquots 12, 15, 18, and 21 h after inoculation. At 36 h the whole broth was acidified with HCl (1M, 15.0 mL), and sonicated. Celite (50 mL) was added and the broth stirred with EtOAc (200 mL), then filtered and the organic layer dried over Na₂SO₄ and evaporated. This was twice chromatographed with 5% EtOAc in CH₂Cl₂ eluent (1 x 12 cm, silica), which afforded 9.7 mg fluorenol with aliphatic contaminants. This was recrystallized from EtOAc/pentane to give 6.5 mg (35 %) **172**, identical to that prepared below.

9-Fluoreinol 172. 9-Fluorenone (71.3 mg) was dissolved in MeOH (10.0 mL) and sodium borohydride (37.9 mg) was added. At 0.2 h, H₂O (40 mL) was added and after brief stirring the precipitate was filtered and washed with H₂O to provide after drying in vacuo 67.0 mg (93%) of pure **172** as a white solid: mp 153.2-154.8 °C, lit 156 °C(41); ¹H NMR (CDCl₃) δ 7.65 (dd, 4H, J = 7.4, 2.0), 7.39 (ddd, 2H, J = 7.6, 7.6, 1.4), 7.32 (ddd, 2H, J = 7.5, 7.5, 0.8), 5.59 (d, 1H, J = 9.9, singlet when D₂O exchanged), 1.86 (d, 1H, J = 10.1); ¹³C NMR (CDCl₃) δ 145.6, 140.0, 129.1, 127.8, 125.1, 120.0, 75.3.

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6. Additional Benz[a]anthraquinone Derived Natural Product Structures

As part of a continuing effort to identify intermediates in the biosynthesis of the kinamycins, the structures of four new *Streptomyces* metabolites were determined. *Streptomyces phaeochromogenes* strain WP3668, and the wild-type *S. murayamaensis* each produce a benz[a]anthraquinone that has undergone oxidative modification. An *S. murayamaensis* mutant blocked in the production of the kinamycins accumulates a dibenzo[b,f]chrysene, and a kinamycin with a new acylation pattern was isolated from the wild-type organism. A unifying feature of these structures is that they all co-occur with, and are probably derived from, dehydrorabelomycin, 11.

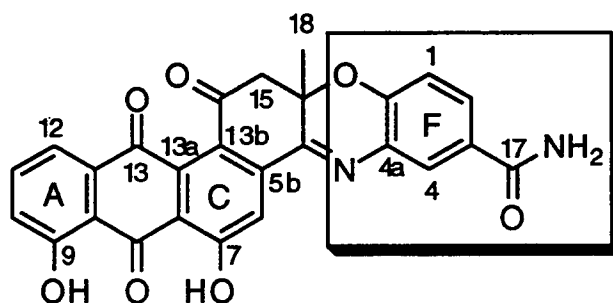
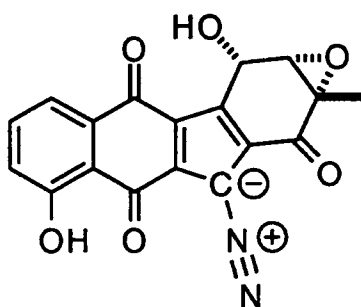
Although none of these structures are particularly complex by modern standards, only a quite limited quantity of each was obtained. Additionally, in one case a remarkably poor solubility rendered useless standard ^{13}C NMR based structure elucidation techniques. In some cases the minute amounts of material available for the structure elucidation served to illustrate the considerable gains that have been made in the sensitivity of NMR experiments through the use of inverse detection, and microprobe technologies.

Murayaanthraquinone

As part of her work isolating and determining the structures of metabolites accumulated by *S. murayamaensis* mutant MC2, Mrs. Hassan purified a new orange-yellow compound. Since the UV/vis spectrum of the new metabolite closely resembled that of ketoanhydrokinamycin **111**, a possible intermediate in the kinamycin biosynthetic pathway was at first suspected. From the ^1H NMR spectrum, two AMX spin systems were identified corresponding to protons 1,2,3- and 1,2,4-substituted on two aromatic rings. During further NMR work at elevated temperature in $\text{DMSO-}d_6$, the sample crystallized. To obtain larger crystals, Mrs. Hassan heated the NMR sample in near-boiling water, and allowed it to cool in a water-filled Dewar over 48 h, obtaining clustered orange needles.

X-ray diffraction analysis of a single triclinic needle was performed to a maximum $\sin\theta/\lambda = 0.5436 \text{ \AA}^{-1}$. Using 1336 observed reflections, the space group P-1 (#2), and the direct methods program SHELXS (TEXAN crystallographic software package)(1) the positions of a dibenzo[*b,f*]chrysene substructure, along with a molecule of DMSO were determined. Severe D ring distortion was resolved by identifying an angular methyl substituent at position 15a. Abnormally low temperature factors for positions 5 and 16 indicated the presence of heteroatoms at these positions. The identities of the heteroatoms at positions 5, 16, and on the three atom group appended at position 3 were

determined by iterative replacement of carbon, nitrogen, and oxygen. Retaining the substitution pattern that gave the smallest crystallographic residuals provided the solution structure **174**. Hydrogen atoms were placed in calculated positions, and a DIFABS(2) absorption correction (transmission factors 0.81 to 1.28) was applied. Anisotropic refinement of all non-hydrogen atoms gave $R = 0.041$ and $R_w = 0.044$. Crystallographic data is summarized in Table 6.1. Successful refinement in the centrosymmetric space group P-1 (#2) demonstrates that the crystal contains a racemic mixture of **174**.

**174****111**

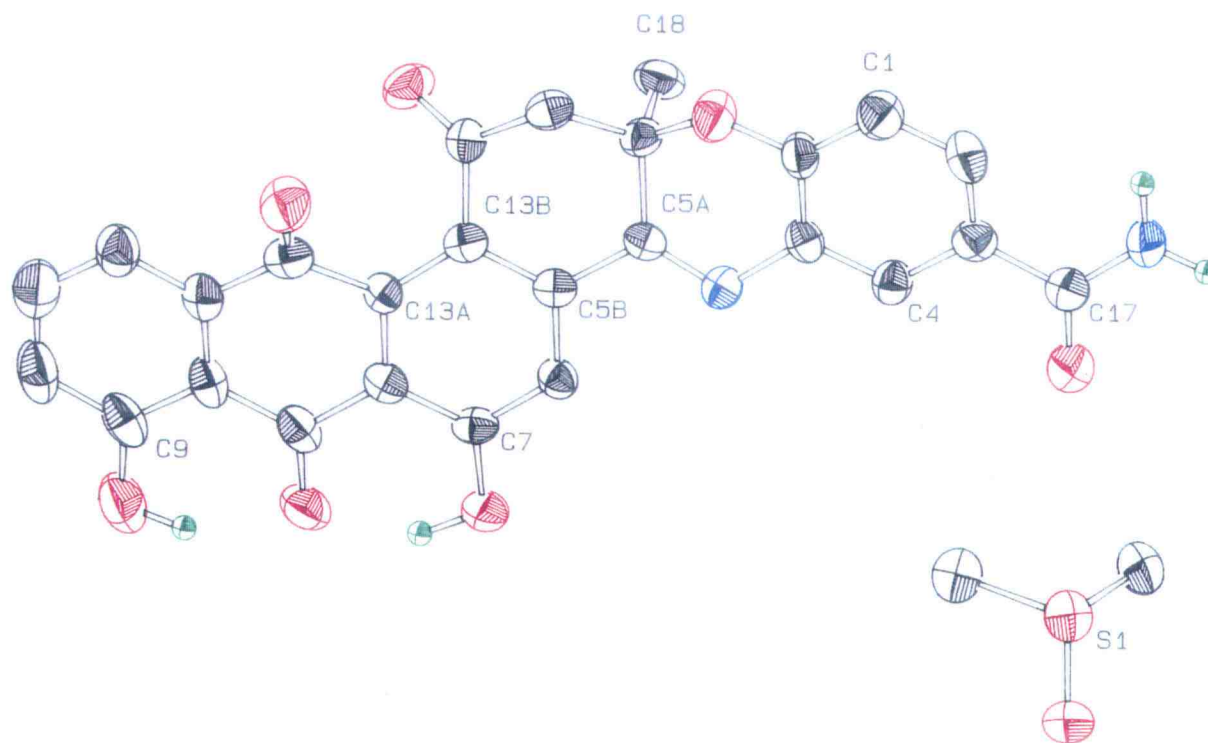


Figure 6.1. ORTEP Representation of Murayaanthraquinone, 174

Table 6.1. Crystal and Collection Data for **174**, $C_{26}H_{16}N_2O_7$

Empirical Formula	$C_{26}H_{16}N_2O_7 \cdot C_2H_6OS$
Formula Weight	546.55
Crystal Dimensions (mm)	0.32 x 0.08 x 0.06
Crystal System	triclinic
Crystal Color	orange
Habit	needle
No. Reflections Used for Unit Cell Determination (2θ range)	18 (20.05 - 26.14°)
Lattice Parameters:	
a =	9.799 (3) Å
b =	16.243 (4) Å
c =	8.000 (2) Å
α =	$102.13(2)^\circ$
β =	$94.63(2)^\circ$
γ =	$97.94(2)^\circ$
V =	1225 (1) Å ³
Space Group	P-1 (#2)
Z value	2
D _{calc}	1.482 g/cm ³
Radiation	Mo K α
Temperature	$23 \pm 1^\circ\text{C}$
Scan Type	ω -2 θ
Scan Rate	8.0°/min (in omega)
Scan Width	$(1.50 + 0.30 \tan \theta)^\circ$
$2\theta_{\text{max}}$	49.90°
$\sin \theta / \lambda_{\text{max}}$	0.5436 Å ⁻¹
Counting Time (background:peak)	2:1
No. of Reflections Measured	
Total:	5788
Unique:	2969 ($R_{\text{int}} = 0.073$)
Observed:	1336

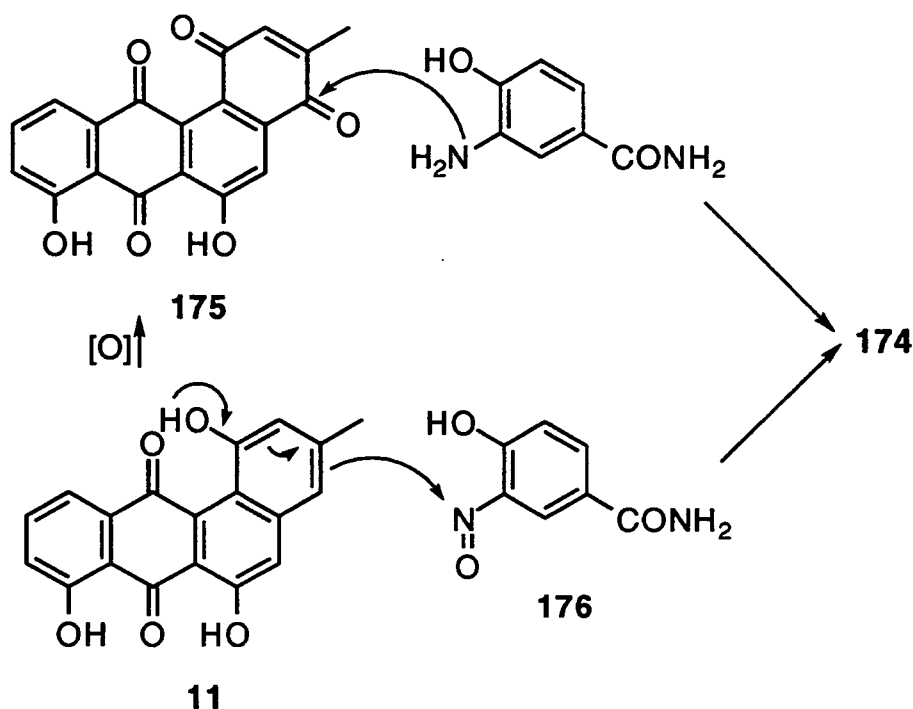
Table 6.1. Continued

R	0.041
R _w	0.044
Goodness of Fit Indicator	1.29
Corrections:	
DIFABS, Lorentz and polarization effects:	
Transmission Factors	0.81 to 1.28
Friedel Mates Averaged	Yes
Shift/Error (max) in Final LS Cycle	0.0266
Max. Peak in Final Diff. Map	16.664 e ⁻ /Å ³
Max. Unassigned Peak in Final Diff. Map	0.166 e ⁻ /Å ³
Min. Peak in Final Diff. Map	-0.174 e ⁻ /Å ³
F(000)	568

High resolution positive ion FABMS confirmed the formula C₂₆H₁₇N₂O₇ (M+H⁺, *m/z* 469.1030, calcd 469.1031). A ¹³C NMR spectrum could not be obtained due to poor sample solubility. Mrs. Hassan overcame this problem by preparing a ¹³C-enriched sample (increasing the concentration of ¹³C atoms in the NMR sample) by feeding [U-¹³C₆]-D-glucose to MC2. With the enriched sample, and using a variety of inverse detected NMR experiments, she was able to identify and assign the chemical shifts of all but four carbon atoms.

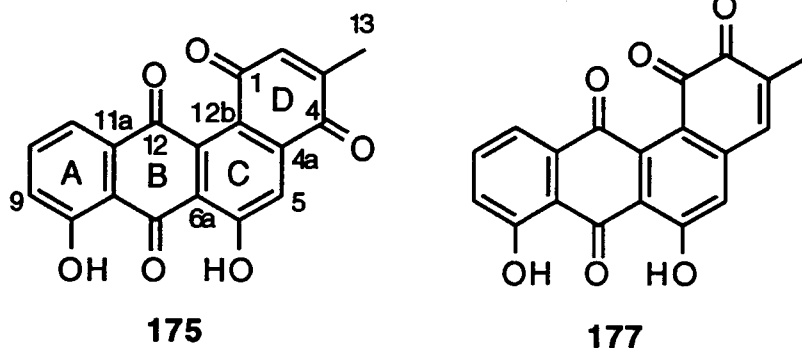
Examination of the structure of **174** revealed that imbedded within its dibenzo[*b,f*]chrysene ring system was the benz[*a*]anthraquinone substructure, appended with a C₇ fragment (boxed). This analysis suggests a probable biogenesis in which either electrophilic aromatic substitution of **11**, or Shiff base

formation with its oxidation product **175**, would provide an intermediate that could ring close to **174**. Since the crystal contained a racemic mixture of **174**, either the ring closure step was not enzyme mediated, or the initially stereochemically pure **174** was subsequently racemized by reversible opening and reclosure of the E ring, perhaps during recrystallization. Both **11** and **176** are produced by mutant MC2. Identification of the C₇ substructure represents the first natural occurrence of the 3-amino-4-hydroxy regioisomer of a benzoic acid substructure. Identification of the amide, and the regiochemistry of heteroatom substitution of the C₇ subunit has directed the structural analysis of five additional compounds with this substructure isolated from *S. murayamaensis* and its mutants (see Chapter 8).



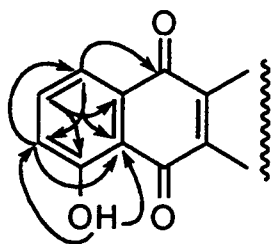
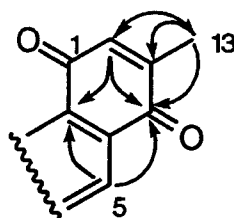
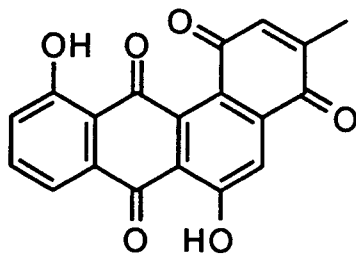
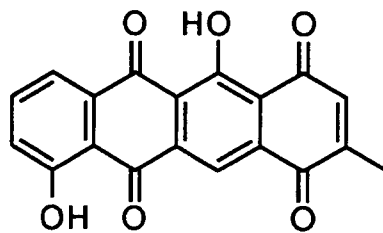
Antibiotic PD116744

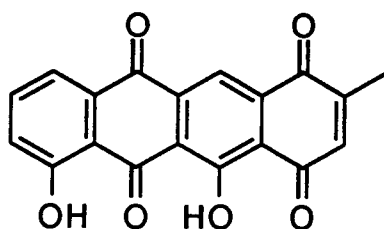
French and co-workers at Warner-Lambert Company(3) obtained an orange compound from *Streptomyces phaeochromogenes* WP3668. They determined that the structure of this new metabolite was either **175** or **177**, and gave our group an opportunity to help them distinguish these possibilities. The aforementioned possible involvement of **175** in the biosynthesis of **174** provided a renewed impetus to investigate this structure.



Initial NMR studies in CDCl_3 were complicated by overlapping of the C10-H and C11-H signals in the ^1H NMR. These were resolved by changing the solvent to CD_2Cl_2 , and a sample of 0.5 mg was analyzed using our newly acquired Nalorac microprobe. Using the inverse detected INVD2D(4) Bruker pulse program, one-bond C-H connectivity was determined. Similarly, the HMBC(5) spectrum provided three-bond C-H correlations, along with one two-bond correlation from C3 to C13-H. Three

correlations [δ 183.7 (C4) to δ 6.95 ppm (C2-H), δ 7.84 (C5-H), and δ 2.17 ppm (C13-H)] served to define the *para*-quinone regioisomer, and its orientation relative to the C ring. These initial experiments provided part structures **178** and **179**, with the long range couplings shown. While this addressed our original question, assigning the structure at this point would require taking the connectivity of these fragments for granted. Four possible structures, **175** and **180-182**, could be envisaged by varying the orientation of the BC ring fusion and the relative position (*ortho* or *para*) of the remaining phenol to the aromatic singlet at C5.

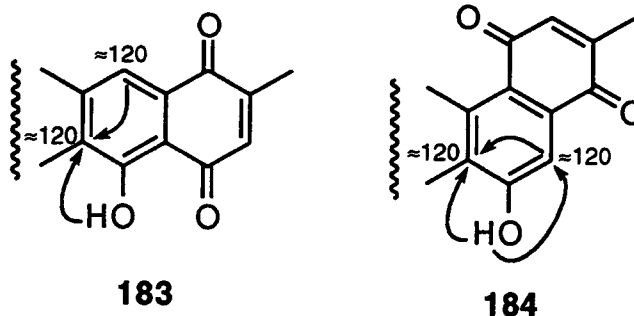
**178****179****180****181**

**182**

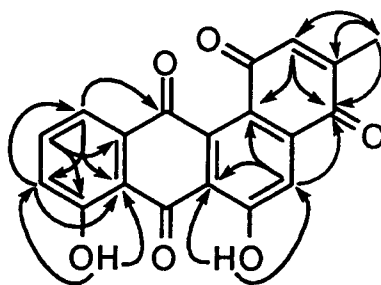
Intramolecular hydrogen bonding between a carbonyl and a *peri* substituted phenol has been observed to deshield the ^{13}C NMR carbonyl resonance by 3-6 ppm.(6-8) The four carbonyl resonances in the ^{13}C NMR appear at δ 182.7, δ 182.9, δ 183.7, and δ 192.9 ppm. Observation of the carbonyl chemical shift of 192.9 indicated that it was twice deshielded by hydrogen bonding relative to the other carbonyl resonances, precluding structures **180** and **181**.

Using normal acquisition parameters, the resolution of two dimensional NMR experiments was insufficient to distinguish two carbonyl resonances at: δ 182.7 and 182.9 ppm; two protonated and one quaternary aromatic carbons at δ 120.1, 120.2, and 120.7 ppm; and two signals at δ 138.7 and 138.8 ppm. Long-range correlations were observed in the HMBC spectrum from the phenol at δ 12.46 ppm and the aromatic singlet at δ 7.84 ppm to approximately δ 120 ppm. Additionally, from the one-bond correlation experiment the proton at δ 7.84 ppm was known to be attached to a carbon at approximately δ 120 ppm. Therefore it was known that one or both of the carbons *ortho* to the phenol

must be at approximately δ 120 ppm, consistent with both part structures **183** and **184**. A DEPT-135 spectrum showed two methine carbons at δ 120.2 and 120.1 ppm, and that a δ 120.7 ppm carbon was not proton bearing.



To distinguish these possibilities the HMBC spectrum was re-acquired at considerably higher resolution by reducing the F1 window to the region from δ 149 to δ 112 ppm. This showed that the phenolic proton at δ 12.46 ppm had three-bond correlations to both a methine carbon at δ 120.1 ppm (from INVD2D below), and a quaternary carbon at 120.7 ppm. This excludes the linear structure, **182**, and provides structure **175a** with all observed long range correlations. Similarly, a high resolution HMBC of the carbonyl region gave δ 182.9 ppm for C12, and a high resolution INVD2D experiment showed the chemical shift of C11 was δ 120.2 ppm. NMR chemical shift assignments are provided in Table 6.2, and are complete with the exception that δ 139.4 ppm and δ 140.0 ppm were not correlated to any proton, and so could not be distinguished.

**175a****Table 6.2. NMR Spectral Assignments for PD116744, 175**

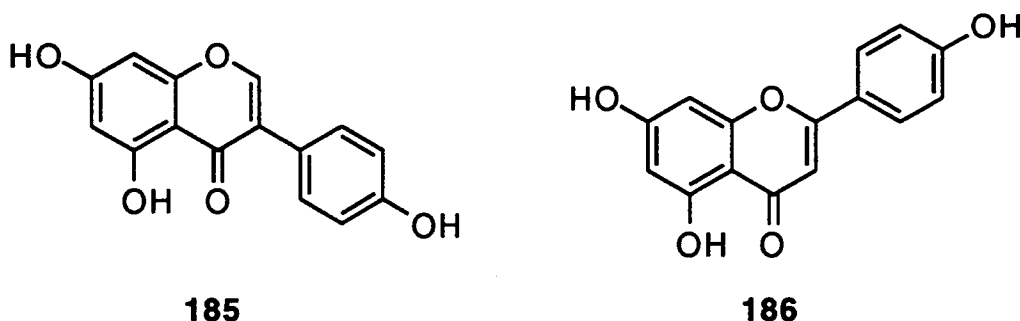
<u>Position</u>	<u>¹H NMR of Proton on CX</u>	<u>¹³C NMR</u>
C1		182.7
C2	6.95 (q, 1H, J=1.5)	138.8
C3		146.7
C4		183.7
C4a		139.4 or 140.0
C5	7.84 (s, 1H)	120.1
C6 (C6 OH)	12.47 (s, 1H, exch.)	164.9
C6a		120.7
C7		192.9
C7a		115.6
C8 (C8 OH)	11.51 (s, 1H, exch.)	162.7
C9	7.32 (dd, 1H, J=8.4, 1.1)	124.4
C10	7.77 (dd, 1H, J=8.4, 7.5)	138.7
C11	7.68 (dd, 1H, J=7.4, 0.8)	120.2
C11a		136.3
C12		182.9
C12a		139.4 or 140.0
C12b		127.7
C13	2.17 (d, 1H, J=1.3)	16.1

Murayaanthraquinone B

Fermentation of the wild type *S. murayamaensis* in a soy-cornmeal medium(9) was undertaken in an attempt to produce ketoanhydrokinamycin, **111**. Due to senescence of the stock culture, poor production was observed. However, HPLC analysis showed several unidentified products, the three most prominent being 11.11 λ 260, 11.47 λ 302, and 12.97 λ 260 (retention time in minutes and most distinctive UV maximum). The crude organic extract was partitioned between hexanes and acetonitrile, and the hexane-soluble portion discarded. Purification of the remaining material on Sephadex LH-20 provided four peaks by monitoring the UV absorption at 260 nm. The fourth peak provided a pure colorless compound (12.97 λ 260). The third peak was chromatographed again in the same fashion, and although only one peak was apparent in the UV absorbance trace, the first fraction corresponding to that peak was yellow, and the remainder colorless. These fractions were separated by color providing approximately 0.1 mg of a minor yellow constituent (18.02 λ 428), and 2.0 mg of the still impure colorless compound (11.11 λ 260). A similar experiment growing the wild-type organism on oat meal-trace metals medium(10) with added polyethylene glycol produced 17.46 λ 262 as a new major metabolite, but this was not pursued.

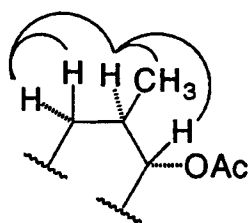
Structure elucidation of 12.97 λ 260 using inverse detected NMR to obtain one-, three-, and many two-bond proton-carbon

correlations quickly provided the structure of genestein **185**. Comparison of the spectra of **185** and the other colorless compound revealed that the only significant difference was that the δ 8.31 ppm singlet in the isoflavone **185** was shifted to δ 6.90 ppm. This suggested the structure of apigenin, **186**, for 11.11 λ 260, and these structures were confirmed by comparison with reported chemical shifts.(11) Since the 4'-O-glycoside of **185**, genistin, is known from both corn and soy, these two compounds are probably media derived artifacts, as has been demonstrated in fermentations of another *Streptomyces*.(12)



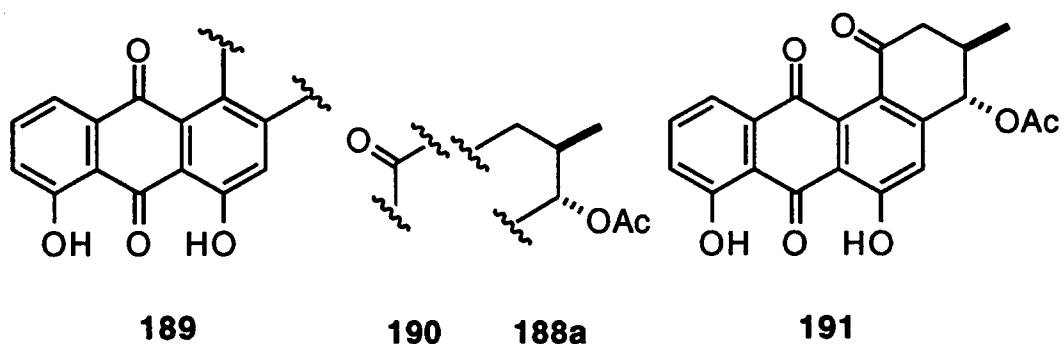
HPLC analysis with diode array detection revealed that 18.02 λ 428 had a UV/vis spectrum similar to that of ochromycinone **22**.(13-15) Since *S. murayamaensis* produces **11**, its possible biosynthetic precursor rabelomycin, **187**, was suspected. ^1H NMR analysis of 18.02 λ 428 revealed a tri-substituted aromatic ring, an isolated aromatic proton, and two hydrogen bonded phenols, but the spectral data did not match that of rabelomycin.(16) Also present were an aliphatic methyl

doublet at δ 1.12 ppm, and a methyl at δ 2.22 ppm. COSY analysis of the remaining aliphatic protons revealed the methine proton at δ 2.35 ppm was responsible for the observed coupling in the methyl at δ 1.12 ppm. The δ 2.35 ppm methine was also coupled to a diastereotopic methylene (δ 2.55 and δ 3.15 ppm), and another methine at δ 5.75 ppm. The δ 5.75 ppm chemical shift was consistent with acetoxy substitution at this position, accounting for the δ 2.22 ppm methyl resonance. A 7.7 Hz coupling between the protons at δ 5.75 ppm and δ 2.35 ppm was also observed. This coupling suggested that the angle between these protons was large, so the methyl and acetoxy substituents attached to adjacent carbon atoms were *trans* to each other. These correlations are shown on partial structure **188**. The expected correlations for the tri-substituted aromatic ring were also observed. HRCIMS provided a molecular formula of $C_{21}H_{16}O_7$ ($[M+1]^+$ m/z 381.0972, calcd 381.0974).

**188**

At this point four possible permutations of partial structures **189**, **190**, and **188a** consistent with the UV/vis and 1H NMR data

were considered. Observation of a small (0.9 Hz) coupling between the oxygen substituted methine at δ 5.75 ppm, and the aromatic singlet at δ 7.10 ppm was evidence of the proximity of these protons. This provided the orientation of **188a**, and therefore the structure **191**. Assigning **191** the name murayaanthraquinone B indicates its source organism, and structure type.



Several compounds with similar D ring substitution patterns have been reported. Most closely related are rubiginones A₂ and C₂(17,18) and fujianmycins A and B.(19) Table 6.3 compares the ¹H NMR data of these compounds with **191**.

Biosynthetic studies on the antibiotic PD116740(20,15) have shown that there are two subgroups of benz[a]anthraquinone natural products differing in whether they retain an oxygen at C6 or have that oxygen removed at a prearomatic stage. Compound **191** is the first example from the C6 oxygenated

benz[a]anthraquinone subclass which is oxy substituted at C4.(21)

Biogenetic analysis of **191** reveals that it may be derived from PD116744, **175**, by two reductions, or from 3-deoxyrabelomycin,(22) **192**, by an oxidation. The co-occurrence of **11** in this organism suggests that in either case the D ring was formerly aromatic. Whether **22**, **192**, the rubiginones, or the fujianmycins are derived from precursors with an aromatic, or aliphatic D ring has not been investigated. This could however be discerned by feeding sodium [2,2,2- $^2\text{H}_3$, 1- ^{13}C]acetate to determine the retention of deuterium atoms at C2 and C4.

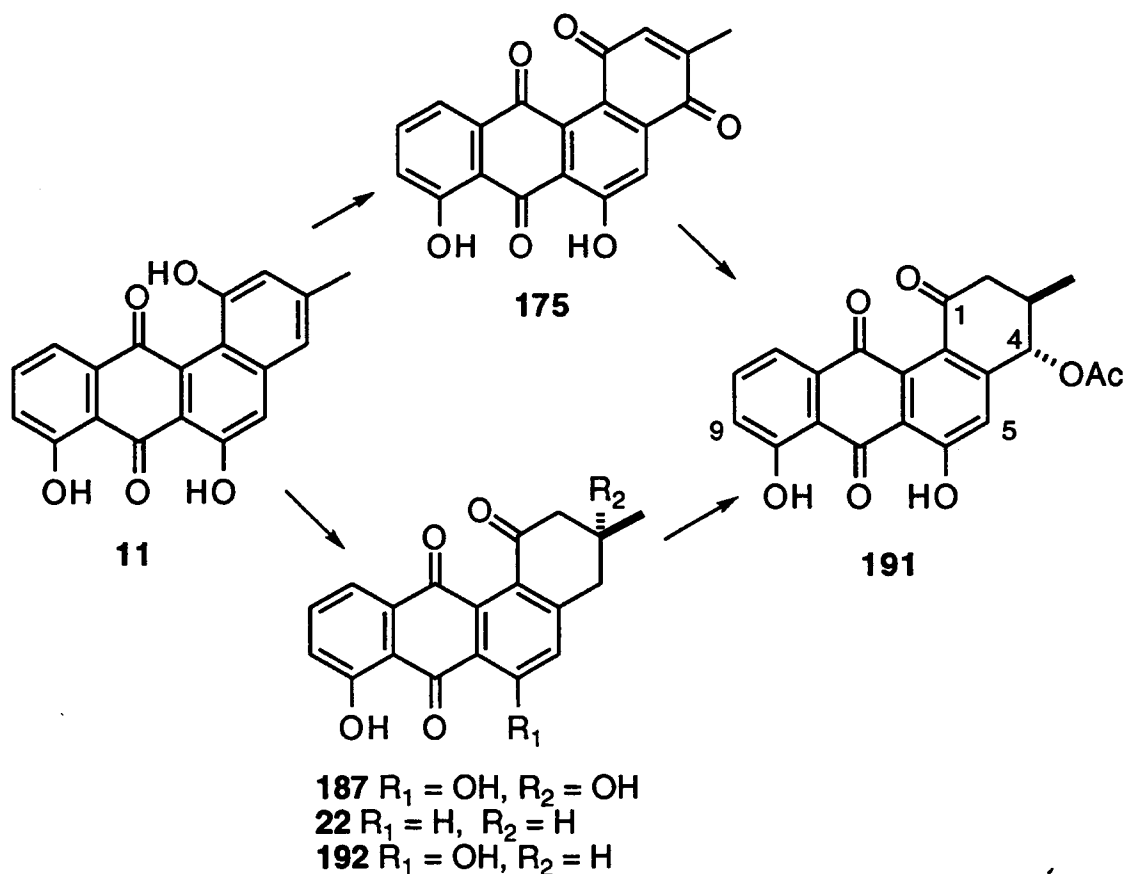
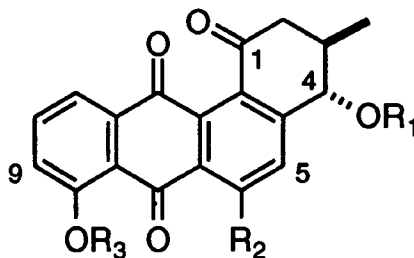


Table 6.3. ^1H NMR Data for **191**, Rubiginones, and Fujianmycins

Compound:	18.02λ432, 191	Rubiginone C ₂ in DMSO- <i>d</i> ₆	Rubiginone A ₂ in DMSO- <i>d</i> ₆	Fujianmycin B CDCl ₃	Fujianmycin A CDCl ₃
Solvent:	CDCl ₃	CO ⁱ Pr, H, Me	H, H, Me	H, H, Me	H, H, H
R1, R2, R3	Ac, OH, H				
Proton(s) at:	Shift (J1, J2 Hz)				
C2α	3.14 (m)	2.98 (16, 5.8)	2.91 (16, 5.5)	3.09 (16.4, 5.6)	3.14 (16.4, 6.1)
C2β	2.47-2.65* (m)	2.69 (16, 10)	2.53 (16, 11)	2.56 (16.4, 10.7)	2.58 (16.4, 10.3)
C3	2.47-2.65* (m)	2.53 (m)	2.2 (m)	2.35 (m)	2.35 (m)
C3Me	1.12 (6.4)	1.04 (7)	1.14 (6.6)	1.26 (6.6)	1.26 (d)
C4	5.76 (7.7, 0.9)	5.88 (8.1)	4.4 (9.1)	4.50 (9.2)	4.51 (9.3)
R1	2.22	2.73 (m, α)			
C5	7.10 (0.9)	7.57 (8.1)	8.02 (8.4)	8.00 (8.2)	8.09 (8.3)
R2	11.65* (OH)	8.23 (8.1)	8.22 (8.4)	8.34 (8.2)	8.41 (8.3)
R3	12.37*	3.95	3.95	4.05	12.30
C9	7.69 (3.4)	7.58 (8, 1)	7.56	7.31 (7.9, 1.5)	7.29* (CHCl ₃)
C10	7.29 (6.1, 3.3)	7.85 (8.1, 8)	7.84 (8.5, 7.7)	7.71 (7.8)	7.68* (m)
C11	7.70 (6.1)	7.70 (8.1, 1)	7.58	7.77 (7.7, 1.6)	7.68* (m)

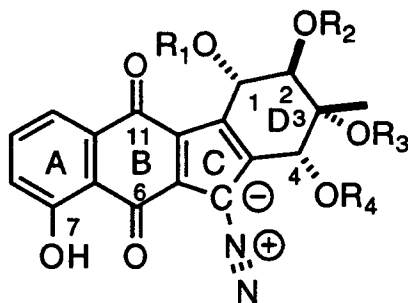
* These assignments can be interchanged.

* Partially overlapping resonances.



Kinamycin J

A wild type *S. murayamaensis* fermentation in glycerol asparagine(10) production medium was harvested and extracted according to established protocols. HPLC analysis of the combined extracts with diode array UV/vis detection revealed a prominent new metabolite. An in-house database of *S. murayamaensis* derived natural products showed an exact match of the spectrum of this new product with that of kinamycin C, **104**, but since its relative retention time did not match, it was identified as a new kinamycin. Since small differences in the UV/vis spectra of the kinamycins are observed with varying acylation patterns on the D rings, this suggested that the oxygen atoms at C1 and C4 were acyl-substituted, as in **104**. Furthermore, the known kinamycins can be divided into a nonpolar group, and a more polar group based on their chromatographic behavior on silica gel, or reversed phase HPLC. The substitution of the C3 tertiary alcohol is the determining factor, with acylation of the other alcohols having only a minor influence on the apparent polarity. The new kinamycin had an HPLC retention time consistent with C3 acylation. The three kinamycins which are known to be accumulated by our putative wild type strain are all 2,4-diacylated, and kinamycin B **103** (like the ketoanhydrokinamycin) is no longer observed. For these reasons a kinamycin tetraacylated on the D ring was anticipated.



102-107

D ring substituents of 102-107

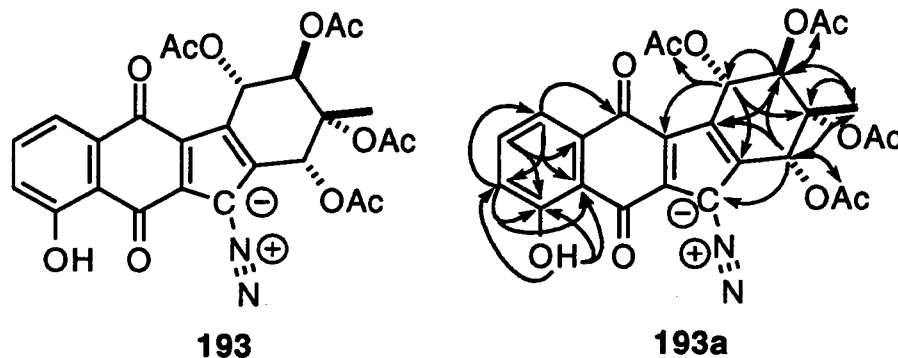
102	KA ^a	R ₁ =H	R ₂ =R ₃ =R ₄ =Ac
103	KB	R ₁ =R ₂ =R ₄ =H	R ₃ =Ac
104	KC	R ₃ =H	R ₁ =R ₂ =R ₄ =Ac
105	KD	R ₁ =R ₃ =H	R ₂ =R ₄ =Ac
106	KE	R ₁ =R ₂ =R ₃ =H	R ₄ =Ac
107	KF	R ₁ =R ₂ =R ₃ =R ₄ =H	

^a Kinamycin A, etc.

Isolation was accomplished by silica gel chromatography and recrystallization, and afforded 2.7 mg of an orange solid. An IR absorption peak at 2153 cm⁻¹ confirmed the presence of a diazo group. ¹H NMR showed three aromatic protons in an AMX spin system, and a hydrogen bonded phenol at 12.13 ppm consistent with a kinamycin A ring. Two doublets were observed at 6.21 and 5.87 ppm (*J* = 6.8 Hz), and a singlet at 6.56 ppm with chemical shifts consistent with acylation at C1 (δ 6.21 ppm), C2 (δ 5.87 ppm), and C4 (δ 6.56 ppm). Between δ 2.03 and 2.16 ppm four acetate methyl groups were observed, and a methyl singlet appeared at δ 1.59 ppm. The C19 methyl chemical shift (δ 1.59 ppm), being greater than δ 1.50 ppm, indicated(23) that it was

adjacent to an acetoxy substituent. Similarly, the presence of the hydrogen bonded phenol demonstrated that the remaining acetate was substituted on the C3 oxygen.

All one-bond carbon-hydrogen correlations were obtained by use of the inverse detected INVD2D(4) Bruker pulse sequence, while two- and three-bond correlations were obtained with the HMBC(5) experiment. These experiments, using our newly acquired Nalorac microprobe, established the expected A and D ring substitution patterns, and provided unambiguous carbon chemical shift assignments for these two rings. The diazo carbon (δ 77.6 ppm) was identified by its three-bond correlation to the proton at C4 (δ 6.56 ppm). Similarly, correlations from each of the three D-ring methine protons to the acetoxy carbonyl carbons allowed the assignment of the latter. C11a (δ 128.9 ppm) was assigned by a correlation to C1 (δ 6.21 ppm). Within the A-ring, the two-, and two three-bond couplings to the hydrogen-bonded phenol, along with all expected three-bond couplings provided the carbon assignments. In addition, a three-bond coupling from the proton at C10 (δ 7.66 ppm) to C11 (δ 187.2 ppm) provided the carbonyl assignment. The proton and carbon assignments of the new kinamycin, **193**, and **105** are compared in Table 6.4, and the long-range correlations used in their assignment are shown on structure **193a**. All other data, including the HRCIMS calcd for $C_{26}H_{22}N_2O_{11}$ 539.1302 [(M+1)⁺], found 539.1302, were consistent with the structure of **193**.

Table 6.4. NMR Spectral Data of **193** and **105** Compared

Position	<u>Data for 193.</u>		<u>Data for 105.(8)</u>	
	<u>¹H</u>	<u>¹³C</u>	<u>¹H</u>	<u>¹³C</u>
C7 OH	12.13 (s, 1H)		12.13 (s, 1H)	
C1	6.21 (d, 1H, J=6.8)	68.0	4.87 (d, 1H, J=8)	67.3
C2	5.87 (d, 1H, J=6.8)	73.3	5.59 (d, 1H, J=8)	75.7
C3		81.0		73.7
C4	6.56 (s, 1H)	66.9	5.48 (s, 1H)	71.3
C4a		126.0		127.8
C5		77.6		78.5
C5a		132.5		132.8
C6		184.1		183.6
C6a		115.6		115.6
C7		162.0		162.4
C8	7.18 (dd, 1H, J=8.4, 1.2)	123.8	7.22 (d, 1H, J=8)	124.3
C9	7.55 (dd, 1H, J=7.6, 8.2)	136.2	7.58 (t, 1H, J=8)	136.3
C10	7.66 (dd, 1H, J=7.6, 1.2)	119.9	7.68 (d, 1H, J=8)	120.3
C10a		134.2		133.8
C11		178.2		180.8
C11a		128.9		129.0
C11b		129.4		132.1
C12	1.59 (s, 3H)	16.2	1.22 (s, 3H)	18.3

Table 6.4. Continued

		¹³ C Me	CO		¹³ C Me	CO
C1 OAc†	2.16 (s, 3H)	21.8	170.4			
C2 OAc†	2.10 (s, 3H)	20.7	170.1	2.19 (s, 3H)	20.9	171.2
C3 OAc†	2.03 (s, 3H)	20.7	169.8			
C4 OAc†	2.14 (s, 3H)	21.0	170.8	2.26 (s, 3H)	21.2	172.3

† All acetoxy assignments are arbitrary except for **193** carbonyls

Observation of this new acylation pattern suggests a possible rationale for the pattern of kinamycin acylation under our present production conditions. Two alternative metabolisms occur, either **105** and **102** are produced, or **104**, **193**, **105**, and **102** are produced together. This can be rationalized by considering the conversion of **107** to **103** as a branch point, with the enzyme activities leading to the other acylation products being insensitive to whether C3 is acetoxy- or hydroxy-substituted.

Groups that have isolated new antibiotics related to the kinamycins from other organisms(24,25,9,26) have opted to not use the sequential lettering system used for the *S. murayamaensis* metabolites (kinamycins A-F) to name their compounds. Dmitrienko and co-workers, however, have assigned the names kinamycin G and kinamycin H(27,28) to 3-O-isobutyrylkinamycin C, and 4-desacetyl-4-O-isobutyrylkinamycin C(24) respectively. Subsequently, another group(29) has adopted this convention. Chemical abstract service however has not indexed these assignments as alternate names, so there

remains some ambiguity as to the acceptance of these designations. In deference to Dmitrienko and co-workers the name using the next unclaimed letter, the name kinamycin J is proposed for **193**.

Experimental

Materials and Methods. Media were prepared from deionized water, and reagent grade chemicals. Dichloromethane and ethyl acetate were distilled, and acetone was of chemically pure grade. Kieselgel 60 (230-400 mesh) silica gel was used for flash chromatography. Medium pressure liquid chromatography (MPLC) was performed on a LiChroprep Si60 column (EM Science), with a 40-63 μm bead size. Melting point determination utilized a Kofler hot-stage microscope. An IBM 9420 UV/vis spectrophotometer was used to determine quantitative absorption spectra. Optical rotations were measured using a Perkin-Elmer 243 polarimeter at ambient temperature using a 0.9998 decimeter cell with 1 mL capacity. Infrared spectra were recorded with a Nicolet 5DXB FT-IR spectrometer.

HPLC. A Waters 600E gradient elution HPLC with a Waters NovaPak C18 radial compression column (0.8 x 10 cm, 5 μm particles) was used for analysis of all broth extracts, and isolates. A linear gradient with a flow rate of 1.50 mL/min from 5 to 95% acetonitrile in water over 20 min, with each solvent containing

0.1% acetic acid effected separation. A Waters 990+ photodiode array detector provided absorbance spectra from 200 to 650 nm with 2 nm resolution of all analyte peaks, and allowed comparisons with an in house database of *S. murayamaensis* metabolites.

X-ray Crystallography of 174. A crystal of 0.32 x 0.08 x 0.06 mm was secured on a glass fiber mount, and the data were collected at 23°C on a Rigaku AFC6R single crystal diffractometer with graphite-monochromated Mo K α radiation from a 12-kW rotating anode generator. The unit cell parameters (triclinic) were determined from 18 reflections in the range of 20.05 <2 θ < 26.14. Three standard reflections measured every 300 reflections throughout data collection demonstrated crystal stability. The structure was solved and refined in the space group P-1 (#2), Z = 2, with the TEXSAN(1) crystallographic software package. The positions of all non-hydrogen atoms were determined with the direct method program SHELXS based on 1336 observed reflections (2 θ_{max} = 49.9°). Hydrogen atoms were placed in calculated positions. A DIFABS(2) empirical absorption correction was applied, and equivalent reflections were averaged. Final cycle anisotropic refinement of non-hydrogen atoms gave an R value of 0.041 (R_w = 0.044) with a p-factor of 0.03.

NMR Experiments for PD116744 (175). All experiments were performed with samples in CD₂Cl₂ on a Bruker AM 400

spectrometer with a Nalorac microprobe. The pulse programs cited were used without modification. High resolution HMBC spectra were obtained using the Bruker pulse program INVDR2LP.AUR, and the following acquisition parameters: D1 2.0; S1 0H; P1 7.00; D2 0.00330; P2 14.00; RD 0.0; PW 0.0; D4 0.050; P3 4.8; D0 3 μ s; NE 64; ND0 2; MC2 M; SI2 2048 W; SI1 256 W; NS 384; WDW2,1 Q; SSB2,1 2. One-bond C-H correlation experiments were obtained with the INVD2D.AUR Bruker pulse program, and the following acquisition parameters: D1 2.0; S1 0H; P1 7.00; D2 0.00330; P2 14.00; RD 0.0; PW 0.0; D4 0.050; P3 4.8; D0 3 μ s; NE 64; ND0 2; MC2 M; SI2 2048 W; SI1 256 W; NS 384; WDW2,1 Q; SSB2,1 2.

Fermentation and Isolation of 191. A Kinako soybean-glucose seed culture(10) (50 mL in a 250 mL foam plugged Erlenmeyer flask) was inoculated with Hornemanns agar plugs of the actively growing wild-type *S. murayamaensis*, and incubated on a rotary shaker at 27 °C, 280 rpm for 96 h. Soy-cornmeal production medium(9) (400 mL in a 2 L foam plugged flask) was inoculated with 5% of its volume of the seed medium, and cultured under the same conditions for 48 h. The whole broth was diluted with an equal volume of EtOAc, adjusted to pH 3.0, sonicated (70% duty cycle, 5 min.), and filtered through celite. After separating the resulting layers, the aqueous solution was re-extracted with EtOAc (2 x 200 mL). Drying the combined organic

layers over Na_2SO_4 and evaporation gave 69 mg of a viscous yellow liquid.

This material was dissolved in acetonitrile (5 mL) and washed with hexanes (3 x 5 mL). Removal of the acetonitrile *in vacuo* provided a material that was loaded onto a Sephadex LH-20 column (0.7 x 50 cm) prepared and eluted in MeOH. Monitoring the UV absorption at 260 nm revealed four resolved peaks in the UV absorbance trace. Evaporation of the fractions corresponding to the fourth peak provided 1.8 mg pure **185** as colorless needles. The third peak, 2.6 mg, was chromatographed again in the same fashion, and was separated into yellow, and UV absorbing colorless fractions. Evaporation of these fractions provided approximately 0.1 mg of **191** as a yellow film, and 2.0 mg of partly purified **186** as a white solid. For **191**: UV/vis (HPLC) λ_{max} 228, 266, 428 nm. ^1H NMR assignments for **191** are found in table 6.3; CIMS m/z 383 ($[\text{M}+3]^+$), 381 ($[\text{M}+1]^+$), 367, 309; HRCIMS m/z calcd for $\text{C}_{21}\text{H}_{17}\text{O}_7$ 381.0974 ($[\text{M}+1]^+$), found 381.0972, calcd for $\text{C}_{21}\text{H}_{19}\text{O}_7$ 383.1131 ($[\text{M}+3]^+$), found 383.1129.

COSY of Murayaanthraquinone B (191). A Bruker AM 300 spectrometer was used with CDCl_3 solvent. The pulse program cited was used without modification. The spectrum was obtained using the Bruker pulse program COSY.AUR, and the following acquisition parameters: D1 1.0; P1 7.50; RD 0.0; PW 0.0; D4 0.050; P3 3.8; D0 3 μs ; NE 256; ND0 1; MC2 M; SI2 1024 W; SI1

512 W; NS 104; WDW2,1 S; SSB2,1 4. The matrix was squared by zero filling, and symmetrized to reduce noise.

Fermentation and Extraction of *S. murayamaensis* for 193.

Kinako soybean-glucose seed cultures(10) (50 mL in each 250 mL foam stoppered Erlenmeyer flask) were inoculated from agar plugs of the actively growing wild-type organism, and incubated on a rotary shaker at 27 °C, 280 rpm for 48 h. Glycerol-asparagine(10) production media (400 mL in each of four 2 L foam stoppered flasks) were inoculated with 5% of their volume of seed medium. After 36 h under the same conditions, the whole broth was adjusted to pH 2.8 with HCl (1.00 M), sonicated (10 min, 90% duty cycle), and filtered through celite. The cell mass was sequentially extracted with acetone (400 mL) and ethyl acetate (2 x 300 mL), and concentrated to approximately 100 mL. This concentrate was extracted with EtOAc (3 x 100 mL). The broth was extracted with EtOAc (4 x 250 mL), the organic extracts were combined and dried over Na₂SO₄, and concentration gave 820 mg of a dark brown gum. Extraction of the crude solids with CHCl₃ (2 x 20 mL) gave, after filtration and removal of solvent, 550.3 mg of a viscous brown liquid.

Isolation of 193. The crude extract above was loaded on a silica gel column (5 x 15 cm) eluted with EtOAc. Nonpolar kinamycins (10.7 mg) were collected followed by mixed kinamycin containing fractions (90.0 mg). The latter were loaded on a

second column (5 x 15 cm) eluted with EtOAc/hexanes (7:3) which gave additional nonpolar kinamycins (10.0 mg). The combined nonpolar kinamycins (20.7 mg) were loaded onto an MPLC column (1.5 x 24 cm) equilibrated and eluted with EtOAc-CH₂Cl₂ (1:19) at a flow rate of 3.2 mL/min. Fractions were analyzed by HPLC. Elution gave **193** (5.2 mg) followed by mixed fractions (2.4 mg), and **102** (4.5 mg). The former was recrystallized from CHCl₃-pentane to afford 2.7 mg of pure **193** as an orange solid: mp 129.6-130.2 °C; $[\alpha]_D^{23}$ -84.2° (c = 2.00, CHCl₃); IR (KBr) 3445.8, 2153.2, 1748.4, 1623.3, 1460.2, 1372.7, 1232.9, 1080.5, 1042.6 cm⁻¹; UV/vis (MeOH) λ_{\max} (ε) 209 (99,700), 242 (19,600), 274 (10,700), 392 (5,360), 440 (5,790) nm; UV/vis (MeOH with 1 mg/L NaOH) λ_{\max} (ε) 200 (sh, 69,100), 238 (14,200), 273 (12,800), 317 (sh, 6,320), 382 (6,320), 544 (6,220) nm; ¹H NMR (CDCl₃) δ 12.13 (s, 1H), 7.66 (dd, 1H, J = 7.6, 1.2), 7.55 (dd, 1H, J = 8.2, 7.6), 7.18 (dd, 1H, J = 8.4, 1.2), 6.56 (s, 1H), 6.21 (d, 1H, J = 6.8), 5.87 (d, 1H, J = 6.8), 2.16 (s, 3H), 2.14 (s, 3H), 2.10 (s, 3H), 2.03 (s, 3H), 1.59 (s, 3H); ¹³C NMR (CDCl₃) δ 184.0, 178.2, 170.7, 170.4, 170.1, 169.8, 162.0, 136.2, 134.2, 132.6, 129.4, 128.9, 126.0, 123.8, 119.9, 115.6, 81.0, 77.51, 73.3, 68.0, 66.9, 21.8, 20.9, 20.7 (2 lines), 16.2; CIMS *m/z* (relative intensity) 539 ([M+1]⁺, 31%), 479 (26%), 419 (18%), 393 (38%), 335 (100%), 277 (37%); HRCIMS *m/z* calcd for C₂₆H₂₃N₂O₁₁ 539.1302 ([M+1]⁺), found 539.1302.

NMR Experiments for Kinamycin J (193). All experiments were performed on a Bruker AM 400 spectrometer with a Nalorac microprobe, and the sample in CDCl₃. The pulse programs cited were used without modification. HMBC spectra were obtained using the Bruker pulse program INVDR2LP.AUR, and the following acquisition parameters: D1 2.0; S1 0H; P1 7.00; D2 0.00330; P2 14.00; RD 0.0; PW 0.0; D4 0.050; P3 4.8; D0 3 μs; NE 128; ND0 2; MC2 M; SI2 2048 W; SI1 512 W; NS 160; WDW2,1 Q; SSB2,1 2. One-bond C-H correlation experiments were obtained with the INVD2D.AUR Bruker pulse program used the following acquisition parameters: D1 2.0; S1 0H; P1 7.00; D2 0.00330; P2 14.00; RD 0.0; PW 0.0; D4 0.050; P3 4.8; D0 3 μs; NE 128; ND0 2; MC2 M; SI2 2048 W; SI1 512 W; NS 160; WDW2,1 Q; SSB2,1 2.

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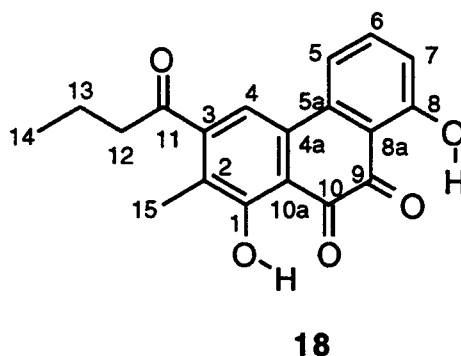
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7. Murayaquinone Pathway: Structural and Biosynthetic Studies

As part of the ongoing studies of the secondary metabolism of *S. murayamaensis*, there has been a continued effort to determine the structures of additional colored fermentation products. The principal focus of this isolation work has been to obtain compounds related to the kinamycins. Indeed, much of the present understanding of kinamycin biosynthesis is due to the discovery of minor metabolites which directed the biogenetic analysis of the pathway.(1-6) Analysis of minor metabolites has also led to the discovery of murayaquinone, **18**, by Dr. Sato.(7) Compound **18** has antibacterial activity against *Mycoplasma galliseptica*, and *Treponema hyodysenteriae*, and a low oral toxicity in mice.(8,9)



The present discussion will report the structure of murayalactone, **194**, produced by a UV-generated mutant of *S.*

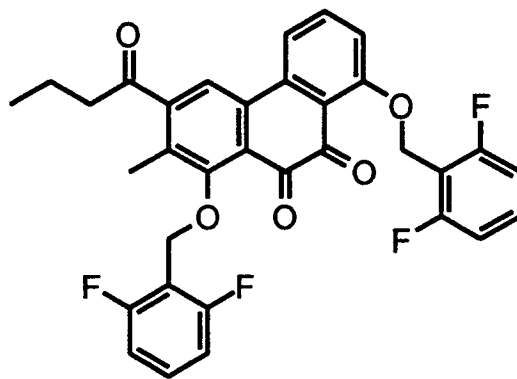
murayamaensis. Generation and characterization of mutants blocked in the biosynthesis of the kinamycins has been described.(4-6) Compound **194** is structurally related to and probably derived from **18**. Accumulation of **194** in the wild type organism has also been demonstrated. Additionally, the biosynthetic sequence leading to **18** has been investigated by stable isotope feeding experiments. It had been anticipated that this compound is the product of a second decaketide pathway operating in this organism. Indeed, these studies are consistent with the derivation of **18** from a decaketide, but with extensive tailoring of the initial condensation product. A possible biosynthetic sequence consistent with the observed labeling is also presented.

Murayalactone Isolation

An *S. murayamaensis* mutant, MC3, selected from the survivors of ultraviolet irradiation as has been previously described does not produce the kinamycins, but does produce **18**.(7) Examination of extracts from fermentation in a glycerol-ammonium sulfate medium or a 7% farina cereal medium(5) showed two blue spots by thin layer chromatography (TLC). The glycerol-ammonium sulfate medium was chosen for isolation of the new metabolites. Although the growth of the organism in this medium is sparse, extraction of the colored metabolites proved easier than with the semi-solid farina medium. The two blue

spots on TLC (BB1 and BB2) were separable from each other on silica gel using 2% ethyl acetate in dichloromethane, or with 3% ethyl acetate in chloroform. The production of BB1 was greatest, giving 3-4 mg (adjusted for impurities observed) from ~15 L broth. Partial purification showed the less polar of these spots to be a mixture of two components, BB1a and BB1b, with ^1H NMR spectra closely related to **18**. The similarity in the NMR spectrum with murayaquinone suggested the possibility that these compounds were artifacts (coordination compounds, quinhydrones, or some type of addition compound) derived from the much more abundant **18**. Further, two-dimensional TLC demonstrated that BB1 slowly converted to murayaquinone on silica gel. However, attempts to generate BB1 by reduction, or by adding metal salts to murayaquinone failed. Similarly, BB1 and BB2 were found to be stable to treatment with hydrogen peroxide or ethylenediamine tetraacetic acid. A third blue compound (B3) precipitated from the crude extract (ethyl acetate) of the 2% farina medium when dried over magnesium sulfate. It was insoluble in water, acetone, and chloroform, but was soluble in dimethylformamide. This compound converted to murayaquinone when spotted onto silica gel TLC plates. In a search for conditions to provide crystalline derivatives to aid in the isolation and characterization of the unknown blue compounds, a variety of model reactions were undertaken with **18**. Of these the most promising was phase transfer catalyzed benzylation with 2,6-difluorobenzyl bromide which gave a 73% yield of the dibenzylated derivative, **195**.

Large needles were obtained by crystallization from ethanol, but **18** is also very easily crystallized. A triacetate of undetermined structure was also prepared.



195

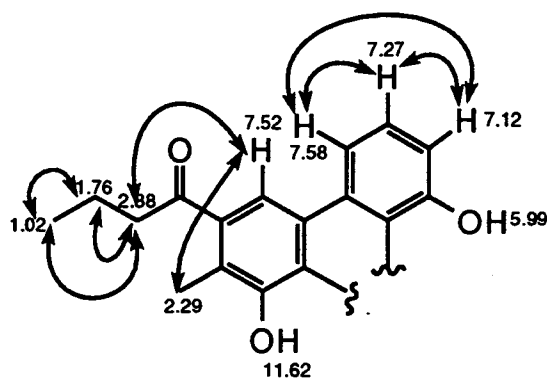
During further purification of the more polar blue compound, BB2, using medium pressure liquid chromatography, a colorless UV-absorbing fraction was collected. This fraction contained a compound that was nearly pure except for a small amount of an oily material that was easily removed by recrystallisation, affording 1.8 mg of a pure white powder.

The ^1H NMR spectrum of the colorless compound revealed a functional array that was also closely related to **18**: an *n*-propyl moiety attached to an sp^2 carbon, an aryl methyl, an isolated aromatic proton, and three contiguous aromatic protons. However, whereas **18** has two hydrogen bonded phenols (12.27 and 12.73 ppm) the colorless compound showed one hydrogen

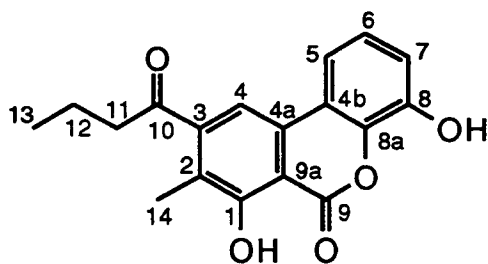
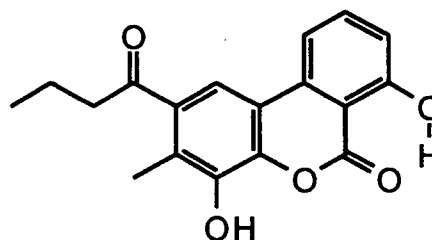
bonded phenol at 11.62 ppm, and one additional exchangeable proton at 5.99 ppm.

Electron impact mass spectroscopy (EIMS) showed a molecular ion at m/z 312.09970 which corresponded to a molecular formula of $C_{18}H_{16}O_5$. This differed from the formula of **18** by one carbon less. The low resolution MS also contained an m/z 284.0 ion (loss of ethylene by McLafferty Rearrangement) and an ion at m/z 269.0 (base peak) attributed to loss of a propyl radical. An m/z 241.0 ion corresponded to loss of a *n*-oxobutyl radical, presumably via a tropylium ion, which along with the above fragments supported the presence of a butyryl side chain.

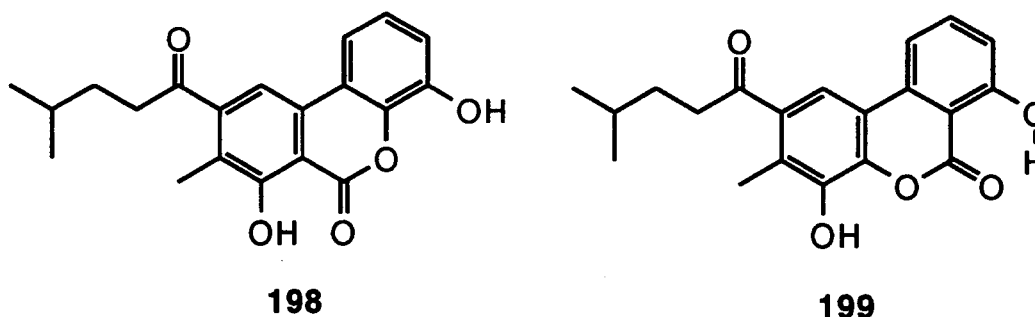
A 1H - 1H COSY45 spectrum of the colorless compound showed all of the expected vicinal couplings expected for **196**. Additionally, a four-bond coupling between the doublets at 7.12 and 7.58 ppm, and a five-bond coupling between the singlets at 2.29 and 7.52 ppm were observed. A long-range 1H - 1H COSY experiment provided two additional couplings, one from 1.02 to 2.88, and one from 2.88 to 7.52 (five-bond) which served to further define the environment of the A ring of **196**. No coupling was observed for the protons attached to oxygen.

**196**

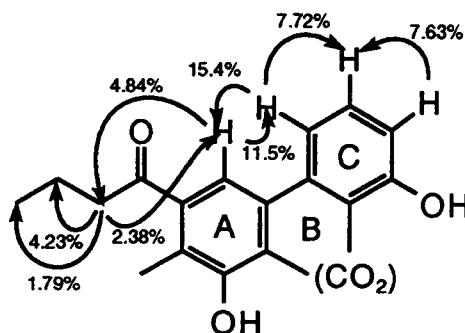
Murayaquinone, **18**, is purple (pink on TLC) and has ^{13}C carbonyl resonances at 182.6 and 182.2 ppm due to the *ortho*-quinone moiety. The new metabolite lacked the quinone resonances in the ^{13}C NMR spectrum and, additionally, had one fewer carbon atoms in its molecular formula. These points, in conjunction with the previously noted ^1H NMR data, suggested that in place of the C_2O_2 unit of the *ortho*-quinone the new metabolite had a CO_2 unit. Two regiochemical possibilities, **194** and **197**, were consistent with having one hydrogen-bonded phenol in a less deshielded environment than in **18**, and one non-hydrogen bonded phenol.

**194****197**

A pair of closely related compounds, **198** and **199**, have been reported from the chemical oxidation of pilquinone.⁽¹⁰⁾ Comparison of the ultraviolet absorbance maxima of **198** (λ max (nm), ϵ : 232, 33,500; 264, 26,100; 285 sh, 14,000; 355 sh, 9,980), **199** (230, 40,800; 268, 17,000; 343 sh, 10,800; 355, 7,900), and the new metabolite (200 sh, 23,600; 228, 22,700; 246, 16,000; 266, 10,400; 284 sh, 7,820; 359, 5,390) indicated their structural similarity, but failed to distinguish **194** from **197**.



One dimensional difference nuclear Overhauser effect (nOe) experiments were used to probe the through space distance separating the observed resonances. Signal enhancements, shown on structure **200**, confirmed the relationship between the A and C rings (demonstrated the proximity of the aromatic protons at δ 7.52 and δ 7.58), and the enhancement between the δ 7.52 singlet, and the triplet at δ 2.88 confirmed the coupling seen in the long-range COSY spectrum.



200

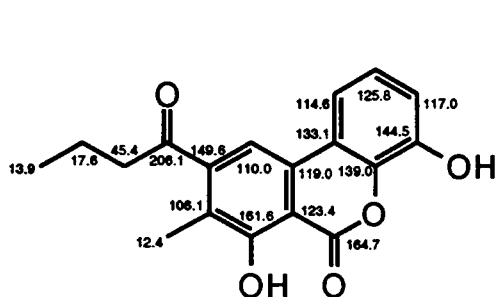
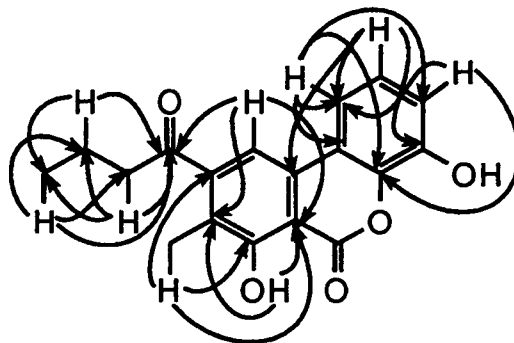
It had been hoped that enhancements of the phenols would be seen from irradiation of the aryl methyl at δ 2.31 and the aryl proton at δ 7.12. With these assigned, and knowing that the more deshielded phenolic proton would be the one proximal to the lactone carbonyl, the regiochemistry of the lactone could have been inferred. Unexpectedly, irradiation of the δ 7.12 doublet in CDCl₃ gave enhancement to not one, but both phenols in these experiments (not shown). In DMSO-*d*₆ the phenol at δ 5.99 ppm was shifted to δ 10.36 ppm. When, in DMSO-*d*₆, the peak at δ 7.12 was irradiated, the water and DMSO peaks also showed strong enhancements. Irradiation of the δ 2.31 methyl on the other hand showed no enhancements. Similarly, the two dimensional nOe (NOESY) experiment in DMSO-*d*₆ showed correlations between both phenols and the doublet at 7.12 ppm.

These enhancements are of resonances corresponding to positions too far apart for a true nOe to be observed, or in the case of the solvent lines are intermolecular. These were not the

kind of enhancements that had been hoped for. However, as it turned out they encoded valuable structural information. Enhancement of the signals for exchangeable protons and solvent by irradiation of the 7.12 ppm doublet suggested that the adjacent phenolic proton was being enhanced, and exchanging rapidly with the medium. By contrast, the lack of dipolar coupling between the methyl at δ 2.29 and its neighboring phenol was manifested by a lack of observed enhancement of exchangeable proton or solvent resonances. These results, taken together, positioned the rotationally fixed, and hence hydrogen bonded, phenol on the A ring. This demonstrated that the colorless isolate has structure **194** not **197**.

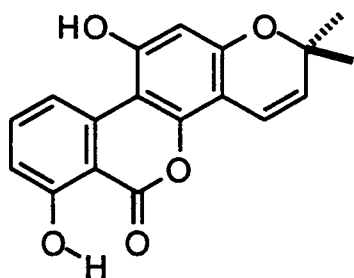
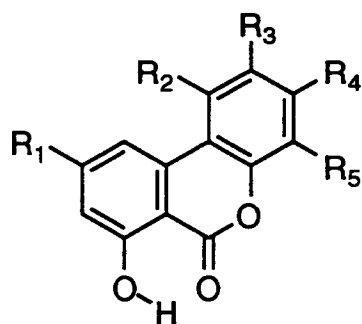
In support of structure **194**, the ^{13}C NMR was assigned. An HMQC(11) experiment provided all the one bond ^1H - ^{13}C connectivities. Long-range ^1H - ^{13}C correlations were detected with the HMBC experiment.(12) All HMBC correlations are shown on structure **194a**. In addition to 2- and 3-bond correlations, three four-bond couplings were also observed.(13,14) These allowed direct assignment of all carbons except the lactone carbonyl, which was then assigned by default to 164.7 ppm, **194b**. Of critical importance were assignments for C-8a and C-9a. Three-bond correlations to 139.0 ppm from H-5 (7.58 ppm) and H-7 (7.12 ppm) identified C-8a, while a three-bond correlation from H-4 (7.52 ppm) and a four-bond correlation from the aryl methyl (2.29 ppm) to 123.4 ppm identified C-9a. These assignments are consistent with the structure **194** in which the lactone ether

oxygen is placed at C-8a, rather than **197**. The chemical shifts for C-8 and C-8a are also consistent with calculated values.(15)

**194b****194a**

Recognizing that **194** could arise from a chemical Baeyer-Villiger type reaction, as in the formation of **198**, extracts of the mutant organism grown in three different media were analyzed by HPLC with photo diode array detection. Each showed a peak with the identical retention time and UV/vis absorbance spectrum of **194**. Co-injection with authentic **194** in each case gave symmetrical enhancement of the peak, and an unchanged spectrum. Thus, **194** appears to be a naturally produced compound. Extracts from wild type *S. murayamaensis* were next examined. Of six extracts from fermentation in glycerol-ammonium sulfate, 2% glucose-0.5% peptone,(16) or galactose-asparagine(17) two contained neither **18** nor **194**, four contained **18**, and two of the latter also contained **194**. The other two extracts containing **18** also contained an unknown metabolite that obscured the region of the chromatogram where **194** would elute.

Several compounds with the dibenzo- α -pyrone ring system of **194** have been reported: the plant products sarolactone **201**, from *Hypericum japonicum*,⁽¹⁸⁾ sabilactone, **202**, from *Sabina vulgaris*.⁽¹⁹⁾ and autumnariol, **203**, and autumnariniol, **204**, from the bulbs of *Eucomis autumnalis*,^(20,21) and the mycotoxins alternariol, **205**, and alternariol monomethyl ether, **206**, from *Alternaria tenuis*⁽²²⁾ and *A. dauci*,⁽²³⁾ and altenuisol, **207**, from *A. tenuis*⁽²⁴⁾ and *A. alternata*.⁽²⁵⁾ The present study is the first report of this ring system from kingdom Monera.

**201**

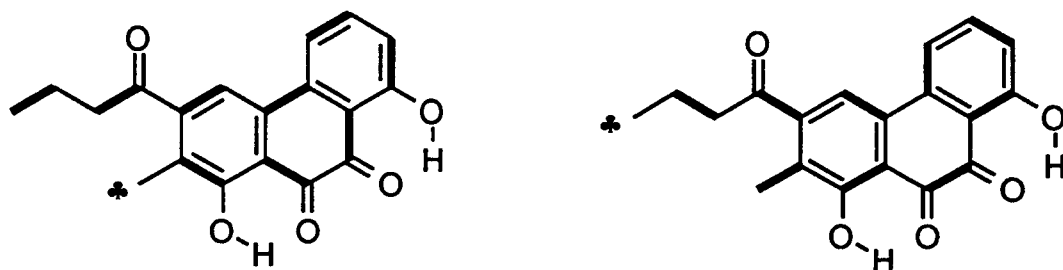
- 202** $R_1=R_3=OMe$ $R_2=R_4=H$ $R_5=Me$
203 $R_1=R_3=R_5=H$ $R_2=Me$ $R_4=OH$
204 $R_1=R_3=H$ $R_2=Me$ $R_4=OH$ $R_5=OMe$
205 $R_1=R_4=OH$ $R_2=Me$ $R_3=R_5=H$
206 $R_1=OMe$ $R_2=Me$ $R_3=R_5=H$ $R_4=OH$
207 $R_1=R_3=OH$ $R_2=R_5=H$ $R_4=OMe$

Studies of the biosynthesis of **205** and **206** have been consistent with simple formation from a heptaketide intermediate.⁽²⁶⁻³⁰⁾ By contrast, the co-occurrence of **18** suggested that **194** was derived by tailoring a decaketide to yield **18**, followed by oxidative CO excision via decarboxylation of a

diacid. More will be said about the observed labeling, and possible tailoring of the intermediates below. This last step would presumably require a monooxygenase activity cleaving the K region C-C bond, which is the same chemical and regiochemical selectivity proposed to precede the ring contraction step in the biosynthesis of the kinamycin antibiotics, also produced by *S. murayamaensis*.(31,32,4) Recognition of this second example of loss of a K region carbon in this organism serves to illustrate how an understanding of the biosynthesis can show the biochemical relationships between apparently dissimilar compounds.

Rationale for Biosynthetic Feeding Experiments

Murayaquinone can be envisaged to arise from either of two possible regular polyketide labeling patterns. It was recognized that feeding sodium [1,2- $^{13}\text{C}_2$]acetate would determine which of the two possibilities was occurring. Since **18** has 19 carbon atoms, one of the two methyl groups (C14 or C15) was expected to have lost its coupling partner by decarboxylation. This is shown below, where ♣ indicates the carbon atom, derived from C2, that had lost its coupling partner. NMR analysis of the resulting labeling pattern using the INADEQUATE (^{13}C - ^{13}C coupling) experiment would then allow the determination of the last added acetate residue (by its lack of a coupling partner).



In the biosynthesis of macrolides and polyethers, the expected sequence of reactions is that the reductive tailoring of each added acetate residue occurs immediately after its addition to the growing polyketide chain. This mode of assembly is called processive, and is analogous to the sequence of events in fatty acid biosynthesis. By contrast the prototypical assembly motif for the aromatic polyketides is that the complete polyketide chain is formed with carbonyl substituents at each alternating carbon, with reductions and cyclizations taking place subsequently.(33)

In light of this, one of the rationale for doing the above experiment was to establish whether the butyryl side chain contained the acetate starter unit (the first residue from which the growing polyketide chain is extended). If so, there would be the possibility that a preassembled butyrate subunit may be the actual starter unit used by the organism. Alternatively, reduction of position C13 to the methylene may be occurring in a processive fashion, combining characteristics of both the aromatic and nonaromatic assembly motifs. The proposal was to then feed an intact, specifically labeled butyrate unit. If incorporated, this would indicate that butyrate was either an intermediate, or the

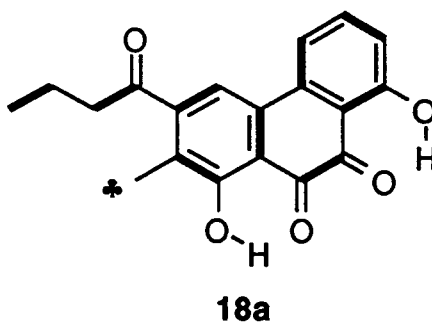
starter unit for chain elongation. While these two possibilities could not be distinguished, it would exclude the third possibility of late reduction of a fully assembled polyketide intermediate (late in this regard could be either before or after the ring-forming condensations).

Sodium [1,2- $^{13}\text{C}_2$]Acetate Feeding

A model feeding study was performed to establish a reasonable set of conditions for feeding the labeled precursors. Beginning with a 48-hour old seed medium in Kinako soy bean meal-glucose medium(2), four permutations of timing of precursor addition, and medium were compared by adding unlabeled sodium acetate along with 10 μCi of sodium [1- ^{14}C]acetate. The radioactivity was added to determine the level of precursor incorporated into the murayaquinone produced. The highest production was seen in the glycerol ammonium sulfate medium(2) with additions made at 12, 15, 18, 21, and 24 h after inoculation with 5% v/v seed medium. A simplified isolation involving extraction, silica gel filtration, and recrystallization from chloroform-pentane afforded 160 mg of pure **18** from 400 mL of broth. While such a high level of production was not observed in the subsequent experiments, this does serve to illustrate how amenable this system is to feeding experiments.

Feeding sodium [1,2- $^{13}\text{C}_2$]acetate gave 31.9 mg **18a**. The C15 methyl group appeared as a singlet (except for a small

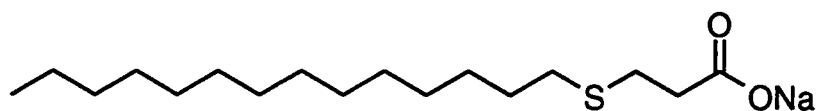
interunit coupling) indicating loss of its coupling partner, defining the orientation of polyketide assembly. However, much to our surprise, an irregular labeling pattern was observed for the aromatic rings. As part of this, the two o-quinone carbonyl carbons were found to be derived from the same acetate unit. Because the difference in the chemical shifts of these two carbon atoms is less than the resolution of the experiment this correlation appears as a single spot lying along the folded diagonal, Figure 7.1.



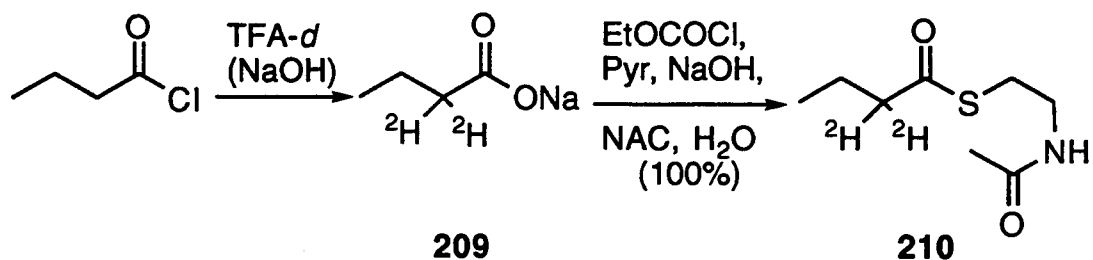
Labeled Butyrate Feedings

A variety of recent studies have demonstrated the incorporation of short chain fatty acids,(34,35) or of chain elongation intermediates(36-39) in polyketide biosynthesis. In a majority of these studies, successful incorporation required the use of both an inhibitor of fatty acid β -oxidation(40) to inhibit catabolism(41) of the intermediate, and use of N-acetyl cysteamine thioester derivatives to mimic a putative pantothenic acid arm(42-45) in the polyketide synthase enzyme complex.

Since the sodium [1,2- $^{13}\text{C}_2$]acetate feeding experiment revealed that the butyryl side chain was comprised of two intact acetate residues, the labeling pattern was consistent with the possibility of a butyrate precursor or intermediate. To address this question a variety of butyrate feedings were attempted. Vederas and co-workers ranked the effectiveness of various inhibitors,(36) with 4-thiostearic acid (3-(tetradecylthio)propanoic acid) **208** being one of the most effective. This was prepared by a modification of the procedure reported for similar compound. Feeding this along with the inexpensive and commercially available labeled butyric acid derivative, ethyl 4,4,4-trifluorobutyrate, however gave no incorporation of trifluorobutyrate into murayaquinone.

**208**

Deuterium labeled butyric acid **209** was next prepared by reaction of butyryl chloride with anhydrous trifluoroacetic acid-*d*. This gave 83% exchange per position of the two protons α to the carboxyl. Feeding **209** with or without added inhibitor also gave negative results. This deuterated butyric acid was therefore converted to its N-acetyl cysteamine thioester **210**, via a mixed anhydride.(34) Feeding **210** along with **208** on two occasions failed to provide a detectable level of deuterium incorporation, which lessens the likelihood that a previously formed butyrate is an intermediate.



Sodium [1- ^{13}C , $^{18}\text{O}_2$]Acetate Feeding

As the orientations of the acetate-derived subunits defined in the feeding experiment with doubly ^{13}C -labeled acetate were unknown, and would be necessary to distinguish among various

possible biosynthetic schemes, it was deemed desirable to feed a singly-labeled sodium acetate. Since three of the murayaquinone oxygen atoms could reasonably be expected to be derived from acetate, sodium $[1-^{13}\text{C},^{18}\text{O}_2]\text{acetate}$ was selected so that this additional information could be obtained by observing upfield chemical shifts corresponding to carbons to which ^{18}O were attached. This was fed in an uneventful experiment that produced 45.5 mg **18b**, calculated from the area of an HPLC peak representing a known volume of broth, monitored at 254 nm. Of this, 42.9 mg was isolated in a pure state.

An average enrichment per site of 17.4% above natural abundance was determined by comparison of the enriched and unenriched line heights for a pair of methyls, methylenes, methines, quaternary oxygen bearing carbons, and carbonyl resonances for both a labeled and unlabeled sample under identical spectral acquisition conditions. Since the level of ^{13}C enrichment was so high, the ^{18}O isotope shifts could not be observed, so a sample composed of 5.1 mg unlabeled **18** and 2.2 mg labeled **18b** was prepared and analyzed. This revealed isotope-induced shifts for C10 at δ 205.1 and δ C8 at 166.1, Figure 7.2. The ^{13}C -enriched *ortho*-quinone carbon at 182.2 ppm did not show an isotope shift, although that oxygen is also presumably acetate derived. A carbonyl that is *alpha*-substituted with an electron withdrawing group would be expected to have a greater contribution to its structure from the ketone hydrate. Therefore, the ^{18}O at this position had most likely been diluted to

below the limit of detection by exchange with water, either in the production medium or during work-up and isolation.

An unexpected and fortuitous observation in the ^{13}C NMR of the $[1\text{-}^{13}\text{C}, \text{}^{18}\text{O}_2]\text{acetate}$ derived **18b** was that the enriched resonances at 114.6 and 182.2 ppm were each straddled by small doublets, indicating ^{13}C - ^{13}C coupling. An INADEQUATE spectrum of this sample (Figure 7.3) showed a coupling between these two carbons, corresponding to an interunit coupling arising from C1 to C1 attachment of two acetate residues. This fortuitous coupling, observable because of the very high enrichment, allowed assignment of the *ortho*-quinone carbonyl resonances, C9 (182.6 ppm) and C10 (182.2 ppm), which being remote from proton bearing carbons could not be determined in any other way. More surprisingly, this orientation shows that neither end of this K region C_2 unit can be attached to the same carbons that would neighbor it in an initially formed polyketide chain! This result excluded both possible interpretations we had imagined to account for the intact subunit labeling pattern, and indicate a much greater degree of post assembly modification than was anticipated. The orientation of intact acetate subunits, and the positions of ^{18}O labeling are shown in Figure 7.4.

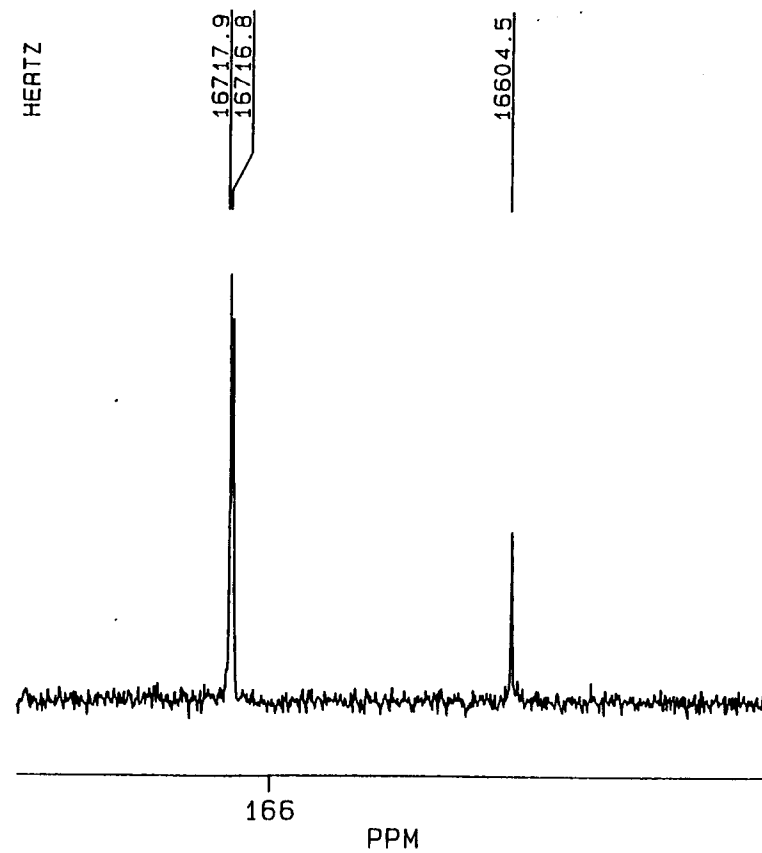
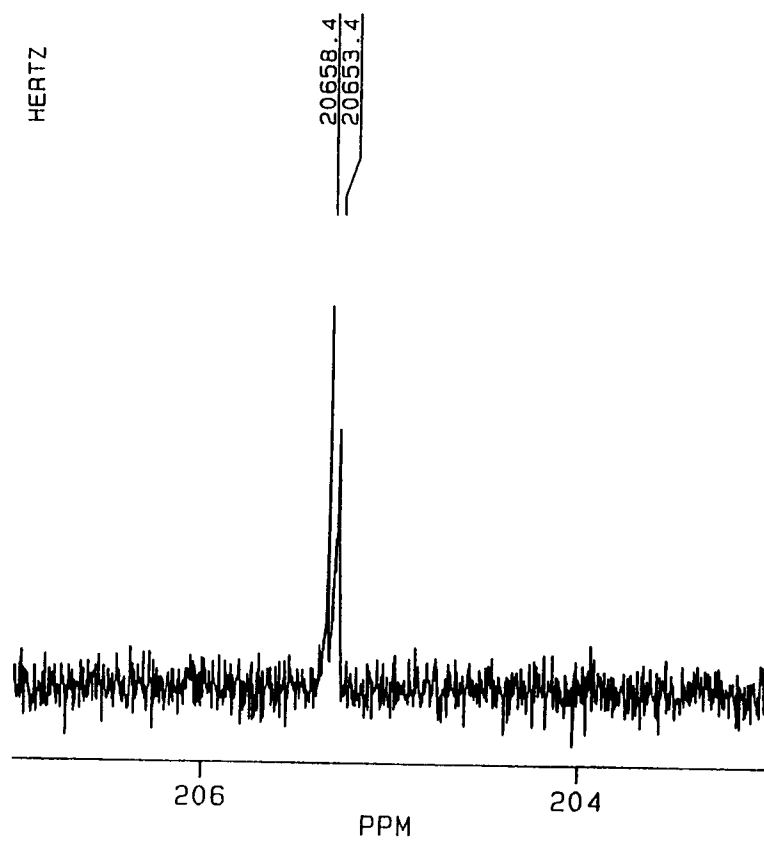


Figure 7.2. ^{18}O Isotope Shifts Observed by ^{13}C NMR

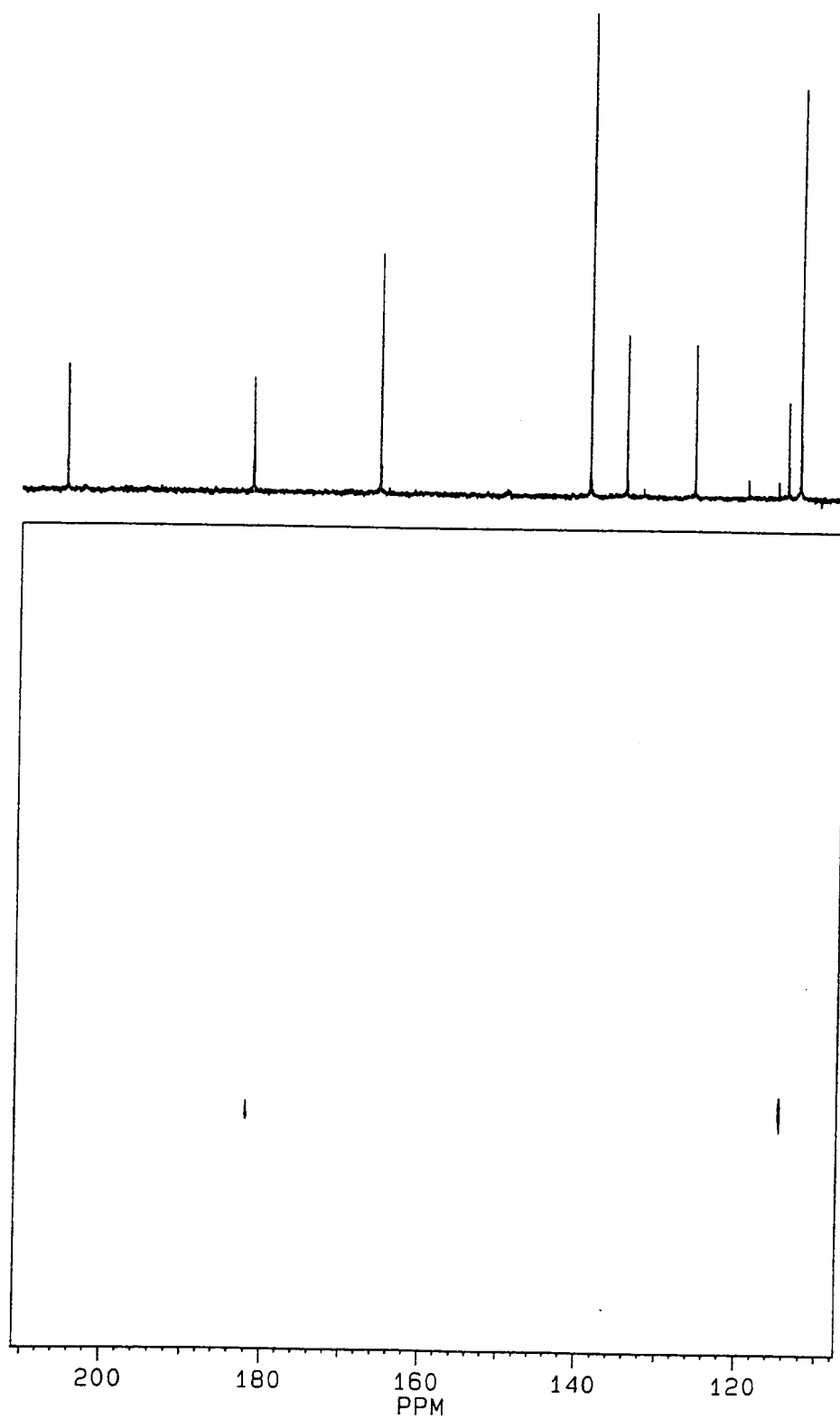


Figure 7.3. Inadequate Spectrum of $[1-^{13}\text{C}, ^{18}\text{O}_2]\text{acetate}$ derived **18**

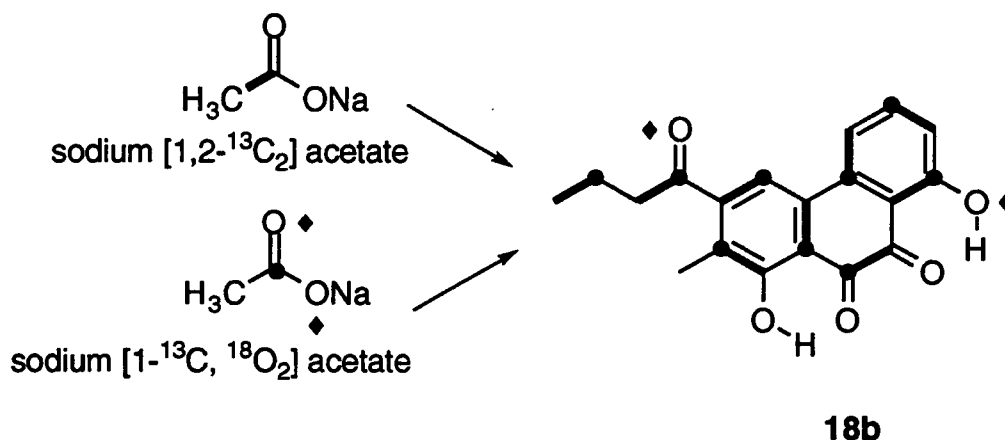


Figure 7.4. Observed ^{13}C and ^{18}O Labeling Pattern

Interpretation of the Observed Labeling

The observed labeling pattern differs from that which could be accounted for by a simple polyketide assembly and cyclization model in several ways, which are summarized in Figure 7.5. A late stage reduction of C13 to a methylene may also be part of this tailoring, but in the absence of positive evidence, this is excluded from the remaining discussion. The most novel and interesting aspect of this labeling is the connection of carbon atoms C8a and C9, both derived from C2 of acetate.

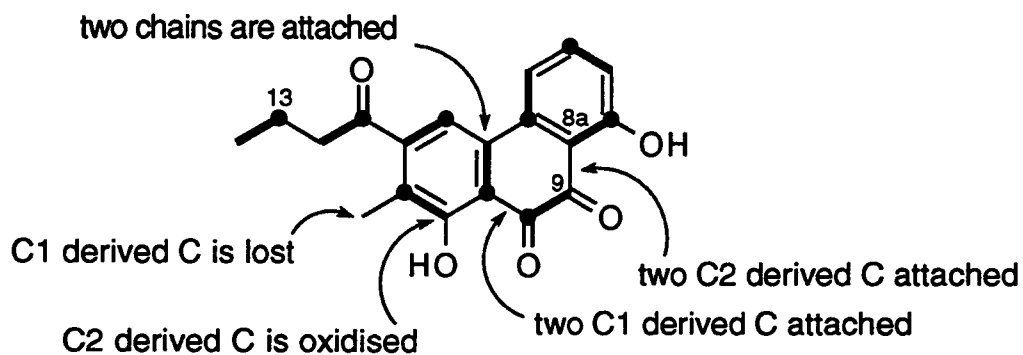


Figure 7.5. Ramifications of the Observed Labelings on the Biosynthesis of **18**

While the variety of possible processes by which these connectivities and modifications is no doubt large, some possibilities are summarized in Figure 7.6. An additional, albeit unlikely alternative would be that the structure is derived from two or more preformed polyketides which were subsequently linked. While condensation of multiple preformed polyketide subunits has been proposed in many systems, alternate explanations are typically found.(46)

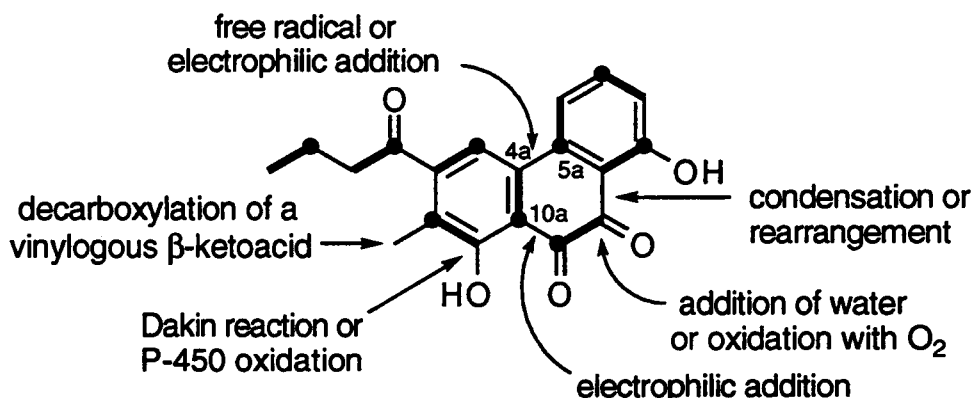


Figure 7.6. Possible Mechanisms to Account for the Observed Labelings of **18**

The large number of putative transformations required to convert a regular polyketide chain to the rearranged skeleton of **18** suggests that any hypothetical sequence of transformations that we would propose has very little chance of being entirely correct. However, with this necessary disclaimer aside, one possible sequence is outlined in Figure 7.7. The usefulness of this proposal is that it identifies possible reaction types that may be targeted by future biosynthetic work with inhibitors.

Key features of the biosynthesis in Figure 7.7 are introduction of the oxygen at C1 by a biological Baeyer-Villiger oxidation,(47,50) with a Fries-type rearrangement(48) of the acyloxy carbon to what becomes C10a. The C5a to C4a bond formation is rationalized as occurring by a radical addition, or electrophilic aromatic substitution. Subsequent ring opening of the resultant spirocycle, and reclosure provides the C8a to C9a bond. This affords a 1,8-conjugated dione which may add H_2O to

give the oxygen at C9, or be reduced. Oxidation of the resulting catechol or phenol would then provide the o-quinone moiety.

Experimental

General. Media were prepared from deionized water, and reagent grade chemicals. Dichloromethane and ethyl acetate were distilled, and acetone was of chemically pure grade. Kieselgel 60 (230-400 mesh) silica gel was used for flash chromatography, and 10 μ m Analtech Sorbent silica gel was used for medium pressure chromatography. NMR shifts of **194** are reported for dichloromethane- d_2 although some experiments were run in chloroform- d and these gave slightly different chemical shifts. Melting point determination utilized a Kofler hot-stage microscope. Isotopically labeled sodium acetates were obtained from Cambridge Isotope Laboratories Inc. Otherwise experiments were performed as indicated in Chapter 2.

Fermentations for Isolation Work. A soy-glucose seed medium,(2) inoculated with frozen seed medium (1.0 mL) was incubated for 5.5 days at 27 °C and 280 rpm. Glycerol-ammonium sulfate medium consisting of 3% glycerol, 0.3% NH_4Cl , 0.2% CaCO_3 , 0.1% $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.01% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (400 mL/2 L flask), was inoculated with the seed culture (10% v/v), and cultures were similarly grown for 5.5 days, 27 °C, 280 rpm.

Isolation of Murayalactone, 194. Filtration of the whole broth (nine flasks) through celite on cotton denim cloth gave a gray cell mass that was sequentially extracted with acetone (200 mL) and ethyl acetate (200 mL). After drying with Na_2SO_4 and concentration, the crude residue (0.91 g) was washed with hexane to remove oily materials, dissolved in dichloromethane, filtered and concentrated to afford 0.44 g.

This was chromatographed on a flash column (2.5 x 15 cm, packed in dichloromethane) eluted with 2% ethyl acetate in dichloromethane. Fractions of 20 mL each were collected with fractions 12-30 containing primarily **18** (112 mg, total purified production of **18** is ca. 23 mg/L) and mixed fractions 31-37 containing **18**, **194**, and the two blue compounds (49.3 mg). A second column (1 x 11 cm) prepared as above separated this mixture into six mL fractions in which the mixed blue compounds and **194** (fr. 6, and 11-15, 16.8 mg) were retained and applied to a similar column (1 x 12 cm) eluted with 3% ethyl acetate in

chloroform. This gave the less polar blue compound (fr. 7-8, 4.2 mg, impure), and a mixture of the blue compounds and **194** (fr. 9, 2.8 mg), followed by the more polar blue compound (fr. 10-11, 4.6 mg). The mixed fraction was combined with similarly prepared material from an additional 6.8 L of fermentation in glycerol-ammonium sulfate and with material obtained from fermentation in 3.6 L farina broth. This gave 8.2 mg of mixed metabolites containing **194** and the more polar blue compound. An MPLC column (2 x 30 cm) was loaded with the 8.2 mg sample and eluted with 30% ethyl acetate in hexane at 12 mL/min, 80-100 psi. UV detection at 300 nm showed a peak centered at 4.6 min that was collected in 25 mL solvent. Evaporation gave 2.7 mg **194**, which was recrystallized from chloroform by addition of pentane to give pure **194** (1.8 mg) as a colorless powder: mp 158.8-161.5°C; IR (KBr pellet) 3546.4, 3443.2, 2962.6, 2930.0, 1698.5, 1676.4, 1195.3, 1126.6, 781.1 cm^{-1} ; UV (MeCN) λ_{max} (ϵ) 200 sh (23,600); 228 (22,700); 246 (16,000); 266 (10,400); 284 sh (7,820); 359 (5,390); ^1H NMR (CD_2Cl_2 , 300 MHz) δ 11.62 (s, 1 H), 7.58 (dd, 1H, $J = 8.1, 1.2$ Hz), 7.52 (s, 1H), 7.27 (app. t, 1H, $J = 8.1$ Hz), 7.12 (dd, 1H, $J = 8.0, 0.9$ Hz), 5.99 (s, 1H), 2.88 (t, 2H, $J = 7.2$ Hz), 2.29 (s, 3H), 1.76 (app. q, 2H, $J = 7.3$ Hz), 1.02 (t, 3H, $J = 7.4$ Hz); ^{13}C NMR (CD_2Cl_2 , 75 MHz) δ 206.1 (C-10), 164.7 (C-9), 161.6 (C-1), 149.6 (C-3), 144.5 (C-8), 139.0 (C-8a), 133.1 (C-4b), 125.8 (C-6), 123.4 (C-9a), 119.0 (C-4a), 117.0 (C-7), 114.6 (C-5), 110.1 (C-4), 106.1 (C-2), 45.4 (C-11), 17.6 (C-12), 13.9 (C-13), 12.4 (C-14); EIMS: m/z (rel intensity) 312.0 (55%), 284.0 (1%), 269.0 (100%),

241.0 (17%) 128.0 (6%); HRMS calcd for $C_{18}H_{16}O_5$ 312.0998, found 312.0997 (dev. 0.2 ppm).

Limited data were also obtained for the partially purified blue compounds: Bblue 1 was found to consist of two components similar to **18** and **194**: Bblue 1a 1H NMR ($CDCl_3$, 300 MHz) δ : 13.70 (s, 1H), 12.20 (s, 1H), 9.62 (s, 1H), 7.59 (app. t, 1H), 7.52 (d, 1H), 7.31 (s, 1H), 6.96 (d, 1H), 2.80 (t, 2H), 2.25 (s, 3H), 1.79 (sept., 2H), 1.03 (t, 3H); and Bblue 1b 1H NMR ($CDCl_3$, 300 MHz) δ : 11.05 (s, 1H), 7.74 (app. t, 1H), 7.69 (d, 1H), 7.55 (s, 1H), 7.09 (d, 1H), 6.10 (s, 1H), 2.95 (t, 2H), 2.45 (s, 3H), 1.78 (sept., 2H), 1.03 (t, 3H); HPLC retention time (UV/vis maxima): 19.57 min (230, 260, 340 nm); 20.83 min (222, 300, 606 nm). Bblue 2 1H NMR ($CDCl_3$, 300 MHz) δ : 13.71 (s, 1H), 12.70 (s, 1H), 11.60 (s, 1H), 10.05 (s, 1H), 2.90 (t, 2H), 2.35 (s, 3H), 1.79 (sept., 2H), 1.05 (t, 3H), where the aromatic region was obscured by impurities and unintelligible; HPLC retention time (UV/vis maxima): 21.09 min (222, 284, 390, 508 nm).

Diodearray HPLC for Murayalactone Analysis. Fermentation samples (10 mL, including mycelia) were mixed with an equal volume of ethyl acetate, and immediately frozen until analyzed. After thawing, the organic layer was concentrated to near dryness, redissolved in dichloromethane (1.00 mL), and 10 μ L aliquots analyzed. A Waters 600E gradient elution HPLC with a Waters NovaPak C18 radial compression column (0.8 x 10 cm, 5 μ m beads) was used for analysis of all broth extracts and isolates. A

linear gradient with a flow rate of 1.50 mL/min from 5 to 95% acetonitrile in water over 20 min, with each solvent containing 0.1% acetic acid effected separation. A Waters 990+ photodiode array detector provided absorbance spectra from 200 to 650 nm with 2 nm resolution of all analyte peaks, and allowed comparisons with an in house database of *S. murayamaensis* metabolites.

Structural NMR Experiments. All experiments were performed in CD₂Cl₂ solvent on a Bruker AM 300 spectrometer except where otherwise noted. A 90° ¹H pulse corresponds to 15.5 μs, and the Bruker pulse programs cited were used without modification. Long Range COSY: COSYLR.AUR; D1 1.50; P1 11.00; D0 3 μs; D2 0.080; P3 11.00; RD 0.0; PW 0.0; ND0 2; MC2 M; NS 64; NE 256; SW2 2702.7; SW1 675.7; SI2 1024W; SI1 512W; WDW2,1 S; SSB2,1 0. HMQC (CDCl₃): BIRDDP9.AUR; D1 2.0; S1 0H; P1 15.50; D2 0.003125; P2 31.00; P4 8.60; D4 0.450; P3 4.30; D0 3 μs; D5 135 μs; D6 2 μs; P0 5.00; P8 100.0 μs (5 Watt soft pulse, ¹³C); L2 36; NE 128; ND0 4; MC2 W; SI2 2048W; SI1 512W; SW2 2336.4; SW1 6443.3; NS 144; WDW2,1 Q; SSB2,1 2. HMBC (CDCl₃): INVDR2LP.AUR; D1 2.0; S1 0H; P1 15.50; D2 0.00330; P2 31.00; RD 0.0; PW 0.0; D4 0.050; P3 5.00; D0 3 μs; NE 128; ND0 2; MC2 M; SI2 2048W; SI1 512W; SW2 3448.3; SW1 7987.2; NS 112; WDW2,1 Q; SSB2,1 2.

1,8-Di-O-(2,6-difluorobenzyl)murayaquinone (195). To a solution of murayaquinone (16.9 mg) and α -bromo-2,6-difluorotoluene (115.5 mg) in CH_2Cl_2 (5.00 mL) was added benzyltributylammonium chloride (36.5 mg) in NaOH (2.00 mL, 1.00 M). After 5 h vigorous stirring the mixture had turned from purple to yellow, the CH_2Cl_2 layer was removed with the aid of additional CH_2Cl_2 (5.00 mL), and evaporated. Chromatographic purification of the yellow component on a silica gel column (1 x 14 cm) eluted with EtOAc-hexanes (3:7) gave 21.8 mg (73%) of pure **195**: mp 208.4-211.0 °C; IR (KBr) 2965.6, 1629.5, 1472.1, 1272.0, 1056.2 cm^{-1} ; ^1H NMR (CDCl_3) δ 7.68 (s, 1H), 7.65 (dd, 1H, $J = 8.2$); 7.55 (d, 1H, $J = 7.8$), 7.26-7.39 (m, 2H), 7.16 (d, 1H, $J = 8.1$), 6.86-6.97 (m, 4H), 5.28 (s, 2H), 5.17 (s, 2H), 2.83 (t, 2H, $J = 7.2$), 2.11 (s, 3H), 1.73 (q, 2H, $J = 7.3$), 1.00 (t, 3H, $J = 7.6$); ^{13}C NMR (CDCl_3) δ 205.2, 182.3, 181.0, 163.5 (d), 160.6, 160.1 (d), 159.6, 147.6, 137.9, 136.3, 135.9, 132.0, 131.0 (m), 124.2, 120.0, 118.5, 117.4, 114.5, 112.8 (m), 111.3 (m), 63.9, 59.2, 44.7, 17.3, 15.8, 13.6, 12.7; EIMS m/z (rel. abundance) 576.5 (M^+ , 3%), 451.0 (12%), 127.0 (100%); HREIMS calcd. for $\text{C}_{33}\text{H}_{24}\text{F}_4\text{O}_5$ 576.1560, found 576.1560.

Fermentation and Isolation for Biosynthetic Feeding Experiments. A Kinako soybean-glucose seed culture(2) (50 mL in a 250 mL foam-stoppered Erlenmeyer flask) was inoculated from agar plates and incubated on a rotary shaker at 27 °C, 280 rpm for 48 h. Production media (400 mL in each 2 L foam-

stoppered flask) were inoculated with 5% of their volume of seed medium. Wild type *S. murayamaensis* was grown on glycerol-asparagine medium, and mutant MC3,(49) on glycerol-ammonium sulfate medium.(2) Labeled materials were introduced in pulses via a 2 micron syringe filter. The whole broth was diluted with an equal volume of EtOAc, and the aqueous layer adjusted to pH 2-3. After sonication and filtration, the aqueous layer was re-extracted with EtOAc (2 x 200 mL), and after drying the combined organic solutions over Na₂SO₄, evaporation *in vacuo* gave crude **18**.

Purification was accomplished by chromatography of the CHCl₃ soluble portion of the crude extract on silica gel (1.5 x 20 cm) prepared and eluted with MeOH:EtOAc:CH₂Cl₂ (1:2:97). Evaporation gave **18** along with oily materials which were separated by recrystallization from CHCl₃-pentane to afford pure **18**.

At harvest, a sample of whole broth (10.0 mL) was mixed with EtOAc (10.0 mL), acidified with HCl (1.00 mL), and sonicated. Filtration through celite, with additional added EtOAc to complete transfer, evaporation *in vacuo*, and redissolution in CH₂Cl₂-MeOH (10:1, 1.00 mL) provided samples for HPLC analysis. Production was calculated from the area of the murayaquinone peak monitored at 254 nm, compared to the area from injection of a known quantity of **18**.

Sodium [1,2-¹³C₂]Acetate Feeding. To one 2 L flask was added a solution of sodium [1,2-¹³C₂]acetate (248.6 mg), sodium

[1- ^{14}C]acetate (2.00 μCi), and unlabeled sodium acetate (251.1 mg) in H_2O (10.0 mL). Additions were made 12, 15, 18, 21, and 24 h after inoculation, 2.00 mL per addition. Harvesting at 36 h gave 107.5 mg organic extract, which gave 57.3 mg after chromatography, and a recrystallized yield of 31.9 mg **18**. HPLC analysis provided an estimated production of 33.4 mg **18**.

^{13}C - ^{13}C Coupling Experiments for **18.** All experiments were performed on a Bruker AM 400 spectrometer in CDCl_3 . The pulse program cited was used without modification. INADEQUATE spectra were obtained using the Bruker pulse program INAD2D.AUR, and the following acquisition parameters: D1 3.5; P9 90; S1 15H; D3 0.003; S2 15H; P1 8.4; D2 0.0114; P2 16.8; RD 0.0; PW 0.0; D0 3 μs ; NE 196; ND0 1; MC2 M; SI2 2048 W; SI1 512 W; NS 64 x 2; WDW2,1 Q; SSB2,1 2.

Sodium [1- ^{13}C , $^{18}\text{O}_2$]Acetate Feeding. To two 2 L flasks were added a solution of sodium [1- ^{13}C , $^{18}\text{O}_2$]acetate (750 mg) and sodium [1- ^{14}C]acetate (3.00 μCi) in H_2O (100 mL). Additions were made 14, 17, 20, 23, and 27 h after inoculation, 10.0 mL per addition. Harvesting at 36 h gave 214.1 mg organic extract, chromatography of which gave fractions containing dehydrorabelomycin followed by those containing **18** (71.2 mg), which after recrystallisation yielded 22.6 mg pure **11**, and 42.9 mg pure **18** respectively. Further elution with acetone provided fractions from which kinobscurinone (3.1 mg), and

murayaanthraquinone (0.2 mg) spontaneously precipitated in a pure form after the fractions had been left open to the air for approximately 18h. HPLC analysis provided an estimated production of 45.5 mg **18**. An average enriched site was found to have 18.5% ^{13}C (17.4% above natural abundance), calculated by comparison of ^{13}C resonance line heights of labeled and unlabeled samples.

2,2- ^2H -Butyric Acid Sodium Salt (209). Trifluoroacetic acid-*d* (10.0 mL) was added to n-butyrylchloride (0.515 g) and the mixture was heated at reflux for 2 d. Evaporation *in vacuo* was followed by dissolving three times in CHCl_3 (10.0 mL) and re-evaporation to remove residual trifluoroacetic acid. NaOH (1.00 M) was added to pH 8.0. Lyophilization then gave 578 mg of **209** (92%) as a white powder: mp 245.1-149.2 °C, lit. 250-253 °C; EIMS *m/z* (rel. intensity) 89.1 (100%), 88.1 (43.8%), 87.1 (5%); HREIMS calcd. for $\text{C}_4\text{H}_5^2\text{H}_2\text{NaO}_2$ 89.0572, found 89.0572.

[2- $^2\text{H}_2$]Butanethioic acid S-[2-(acetylamino)ethyl]ester (210).(34) To a THF (35.0 mL) solution of **209** (158.0 mg, previously converted to the acid by treatment with H_2SO_4) cooled to 5 °C was added triethylamine (245 μL) followed by ethylchloroformate (168 μL). After stirring at rt 1 h, the mixture was re-cooled, and to it was added an aqueous solution of N-acetylcysteamine (477.0 mg) in H_2O (25.0 mL), previously cooled to 5 °C and adjusted to pH 7.8 with NaOH. Following removal of

the cooling bath, the pH of the reaction mixture was adjusted to 8.0 three times over 2 h, then the mixture was acidified to pH 3.4, saturated with NaCl, and the resulting biphasic mixture extracted with Et₂O (3 x 60 mL). Washing the ether layer successively with NaHCO₃ (30 mL) and brine (30 mL), drying over Na₂SO₄, and evaporation gave 366.8 mg of **210** contaminated with N-acetylcysteamine. Chromatography on silica gel (1.5 x 25 cm) with acetone:CH₂Cl₂ (1:6) gave after evaporation 339 mg (100%) of **210** as a low melting white solid: ¹H NMR consistent with that reported for the unlabeled compound; UV λ_{max} 234 nm (HPLC); EIMS 191.1 ([M+2]⁺, 3%), 45.1 (100%).

3-(tetradecylthio)propanoic acid (208). To mercaptopropionic acid (20.34 g) in a 500 mL flask was added a solution of NaOH (26.75 g) in MeOH (300 mL). Upon cooling to rt tetradecylbromide (30.0 mL) was added and the mixture stirred 0.5 d. The mixture was poured into H₂O (600 mL) and heated until homogeneous, then cooled to 4 °C and the resulting white powder redissolved in boiling H₂O (350 mL). Addition of acetone, and cooling gave a gel which was acidified with HCl (pH 3.0) and filtered to afford 45.96 g (80%) pure **208** as a white powder: ¹H NMR (CDCl₃) δ 2.78 (t, 2H, J = 7.1), 2.66 (t, 2H, J = 7.1), 2.53 (t, 2H, J = 7.2), 1.58 (tt, 2H, J = 7.6), 1.37 (m, 2H), 1.26 (m, 22H), 0.88 (t, 3H, J = 6.4); ¹³C NMR (CDCl₃) δ 178.16, 34.71, 32.22, 31.90, 29.67, 29.65, 29.63, 29.58, 29.53 (2 lines), 29.50, 29.33, 29.20, 28.85, 26.60, 22.66, 14.07.

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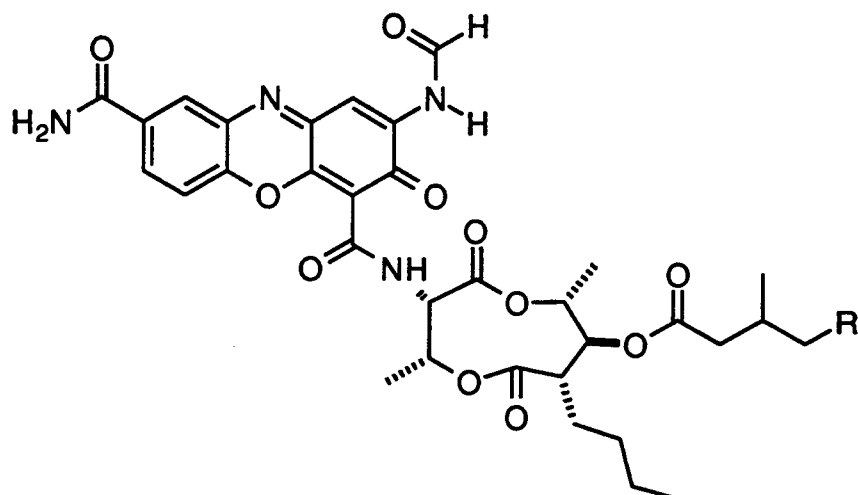
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8. Structure and Biosynthesis of Sodium tris(4-hydroxy-3-nitrosobenzamide) Ferrate (1-)

S. murayamaensis has been found to produce derivatives of 3-amino-4-hydroxybenzamide. This substructure was first elucidated in the murayaanthraquinone **174** crystal structure, and subsequently has been determined to be a part of the structures of B1, B2, 4-hydroxy-3-nitrosobenzamide, and its ferrous chelate. The isolation and structure determination of B1 **211** and B2 **212** has been done by our co-workers Awatef Hassan, and Dr. Carney. Murayaanthraquinone, B1, and B2 are metabolites of *S. murayamaensis* mutant MC2. 4-Hydroxy-3-nitrosobenzamide **176**, and its ferrous chelate **213**, are produced both by the wild type organism, and by a variety of mutant strains. These latter compounds will be referred to as the red and green metabolites, respectively, in discussing the structure elucidation process.

Biosynthetic investigations of the 2-amino-3-hydroxy-, 2-amino-6-hydroxy-,⁽¹⁾ and 3-amino-5-hydroxybenzoic acid^(2,3) regioisomers have shown they are derived from the shikimic acid pathway. Shikimic acid is the prototypical precursor for C₇N substructures, and is derived from the condensation of one C₃ (phosphoenolpyruvate) subunit,



211, R = H

212, R = Me

and one C₄ (erythrose-4-phosphate) subunit. Feeding experiments to determine the primary precursors of **213** have revealed that it is indeed biosynthesized by condensation of one C₃, and one C₄ subunit, but in a surprisingly different manner than members of the shikimate family of C₇N compounds. Two probable post aromatization intermediates in the biosynthesis of **213** have also been identified.

Isolation and Structure of 213

In the course of a feeding experiment to investigate the intermediacy of kinobscurinone in the biosynthesis of the kinamycin antibiotics,(4) a green water soluble metabolite was observed. After extracting the less polar kinamycins with EtOAc,

the aqueous layer was re-extracted with n-BuOH to afford 320 mg of a bright green solid. Purification on Sephadex LH-20 provided 35 mg of pure metabolite.

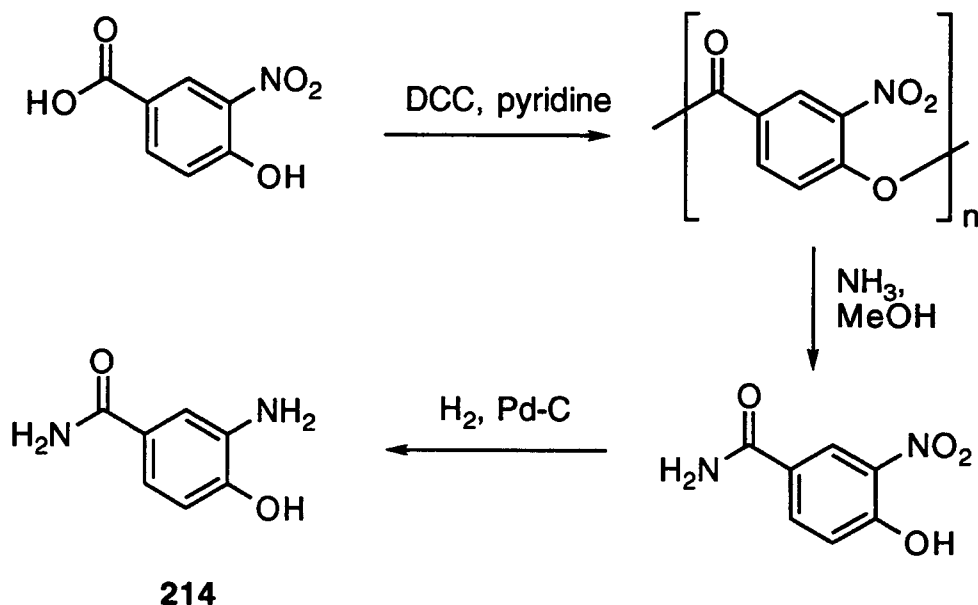
It was subsequently found that the green compound was consistently produced in fermentations of *S. murayamaensis* mutants MC3 and MC11, from which it is readily isolated. Generation and characterization of mutants blocked in the biosynthesis of the kinamycins has been described.(5-7) Using glycerol asparagine(8) or glycerol ammonium sulfate(9) media, the green compound is almost exclusively found in the mycelium in MC3, which has been observed to turn bright green when not obscured by **18** production. In similar fermentations of MC11, however, the green pigment is distributed between the cells and broth as is seen in the wild type organism. Both of these mutants express variable metabolite production. The color of the seed medium at inoculation may indicate what metabolism will predominate. For MC3, a 48 h old seed medium that is olive-brown to dark gray, or green-brown with red droplets gave good production of murayaquinone and the green metabolite. If the seed is however more the color of milk chocolate, kinobscurinone is the major metabolite. For the MC11 mutant, a red-brown seed medium is conducive to good production of both pathways, but in one instance a dark brown seed culture gave none of the green metabolite in the production culture. The green metabolite accumulates in the fermentation medium after all, or the majority of **18** production has subsided.

Broad resonances in the ^1H NMR of the green metabolite centered at 7.85 and 7.10 ppm indicated the presence of an amide. This was confirmed by observation of amide N-H stretches in the IR (3426 and 3387 cm^{-1}), and amide I and amide II bands at 1656 and 1599 cm^{-1} respectively. HMQC(10) and HMBC(11) experiments provided all expected correlations, and served to define a 3,4-disubstituted benzamide substructure. Dr. Cone had previously isolated a red metabolite from a fermentation system found by Dr. Gore. This compound showed the same 3,4-disubstituted aromatic part structure, and exhibited a pH dependent solubility suggestive of a carboxylic acid. This data led Dr. Carney to suggest that the red metabolite was the *ortho*-quinone tautomer of 3-hydroxy-4-nitrosobenzoic acid. However, the oxidative preparation of both possible regioisomers of the nitroso acids from 4-amino-3-hydroxybenzoic acid and 3-amino-4-hydroxybenzoic acid, respectively, gave compounds with different NMR spectra than the natural product.

The green metabolite however differed from the red metabolite in that it was more chemically stable, and its solubility showed no dependence on pH. Furthermore, the UV/vis spectrum of the green metabolite was unaltered by addition of acid or base. These results indicated that the green compound had no acidic protons, suggesting a cyclic substituent, *ortho*-quinone, or chelate structure. Additionally, determination of the structure of murayaanthraquinone demonstrated that the organism could produce a benzamide with a nitrogen substituent at C3 and an

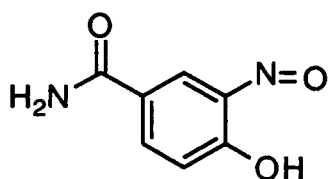
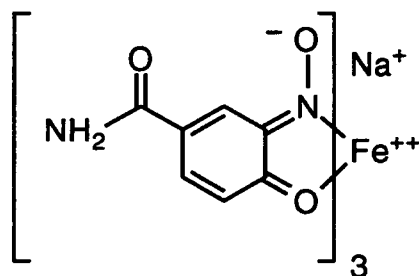
oxygen substituent at C4, which directed further work on these structures, as it has for the chromophore of B1 and B2.

An authentic sample of 3-amino-4-hydroxybenzamide **214** was synthesized as shown below. It was hoped this could provide access to any permutation of the nitrogen oxidation level, in order to determine the structure of the green metabolite. Concurrently with this work, however, Dr. Cone catalytically reduced the red compound, giving **214**. Subsequently, the green metabolite was found to reduce to **214** under the same conditions, albeit much more slowly. A much more facile reduction of the red, and green metabolites was accomplished with sodium dithionite.



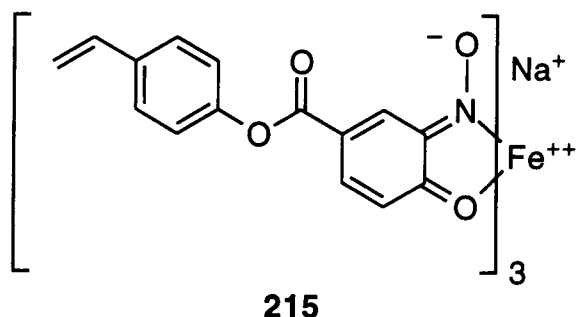
Electron microprobe analysis of the green compound detected the presence of a stoichiometric quantity iron, to the exclusion of other transition metals. This explained the presence

of a FABMS (negative mode) 551 $[\text{Fe}(\text{C}_7\text{H}_5\text{N}_2\text{O}_3)_3]^-$ peak. Dr. Cone prepared the red metabolite by nitrosation of 4-hydroxybenzamide, via a copper complex. This copper complex was also found to rapidly reduce with $\text{Na}_2\text{S}_2\text{O}_4$ to afford **214**. Dr. Cone found that treating a sample of the red metabolite with FeSO_4 in water gave a compound with the UV/vis spectrum and retention time of the green compound. This led to the structures **176** and **213** for the red and green metabolites, respectively.

**176****213**

Another iron containing *Streptomyces* metabolite, ferroverdin **215**, has been reported.(12,13) The structure of this ferrous chelate was determined crystallographically,(14) and it has a UV/vis spectrum very similar to that of **213**. This X-ray structure shows that the iron is in an octahedral coordination environment. This explains an unusual phenomena observed in the chromatographic separation of **213** on LH-20. Two green bands were resolved by the column, but were found to have identical ^1H and ^{13}C NMR spectra. These may be diastereomers varying in the orientation of one bidentate ligand about the iron, which are

however, in rapid equilibrium in the more co-ordinating DMSO-*d*6 NMR solvent. An alternative explanation would be two forms of **213** varying in the alkali earth cation.



Consistently co-occurring with **213** was a polar brown compound that was more mobile on LH-20. The ^1H NMR spectrum of this brown compound suggested it was another monocyclic aromatic compound. By feeding 3-amino-4-hydroxybenzoic acid to the wild type organism, a new compound 10.88 λ 432 (named for retention time in min. and most distinctive UV maximum in nm) was produced in substantial quantities. The UV/vis spectrum of 10.88 λ 432 was very similar to that of B1 and B2. This compound was similarly produced by feeding 3-amino-4-hydroxybenzoic acid to the MC3 mutant, but with a much reduced background metabolism. The same feeding to mutant MC11 provided 10.88 λ 432 along with similar compounds, the most abundant of which was 10.33 λ 340. Both 10.88 λ 432 and 10.33 λ 340 were isolated from a feeding experiment, described below, by chromatography on LH-20 in THF based solvent

systems. Due to the nature of the feeding, these were too highly deuterated to be appropriate for structure elucidation.

Peaks at the retention times for each of these compounds are consistently observed in fermentations of the wild type and mutant organisms. Both of these compounds are dimers (14 carbon atoms by ^{13}C NMR, and assuming all carbons present were observed) of the compound fed. These compounds do not show the pH dependent solubility expected for carboxylic acids, suggesting they may arise from conversion of the added acid to its amide via an adenylate.⁽¹⁵⁾ The UV/vis spectrum of 10.88 λ 432, observed by diode array detected HPLC, closely resembles that of B1 and B2. As with generation of the chromophore of **211** and **212**, this compound is probably formed by a phenoxazine synthase catalyzed condensation reaction.⁽¹⁶⁾

Acetate Feeding Experiments

Feeding sodium [1,2- $^{13}\text{C}_2$]acetate to a culture of the producing organism gave after purification 8.1 mg **213a**. An estimated production of 12.6 mg of **213a** was calculated from the area of an HPLC peak representing a known volume of broth, and monitored at 254 nm. Analysis of **213a** obtained from this experiment with the INADSYM Bruker pulse program revealed two highly enriched, coupled pairs of carbons, δ 167.5 (C7) to δ 119.6 (C1) ppm and δ 137.5 (C6) to δ 120.8 (C5) ppm, Figure 8.1. Interestingly, by comparing the height of the central lines of C7

and C5 with those of C3 and C4 in the ^{13}C NMR spectra of enriched and unenriched samples of **213**, the non-coupled resonances corresponding to C7 and C5 were also found to be ^{13}C enriched by an average of 4.1% above natural abundance. This enrichment is inferred for C1 and C6, but could not be directly measured due to diminution of the center lines by interunit coupling, note 1D ^{13}C NMR in Figure 8.2. Using the more sensitive INAD2D and COSYX Bruker pulse programs an additional ^{13}C - ^{13}C coupling was observed between δ 179.9 (C4) and δ 158.7 (C3) ppm, Figure 8.3 and Figure 8.4. The remaining carbon, at δ 110.2 (C2) ppm, was found to have a low enrichment, 0.5% above natural abundance, compared to the center lines at δ 179.9 (C4) and δ 158.7 (C3) ppm (measured enrichments were 1.53% ^{13}C compared to δ 179.9, and 1.64% ^{13}C compared to δ 158.7, and necessarily ignoring any enrichment of C3 and C4 that would arise from multiple passes through the tricarboxylic acid cycle). This level of signal enhancement is below the generally accepted confidence limit of a doubling in line height. These three carbon atoms show a very different level of enrichment than the remaining four. They are labeled in a manner consistent with an acetate label entering the C_3 pool via the tricarboxylic acid cycle, and loss of one carbon atom by the action of phosphoenolpyruvate carboxykinase as shown in Figure 8.5, with ♣ indicating positions that may arise from C2 of acetate. Therefore, this C_3 unit is probably

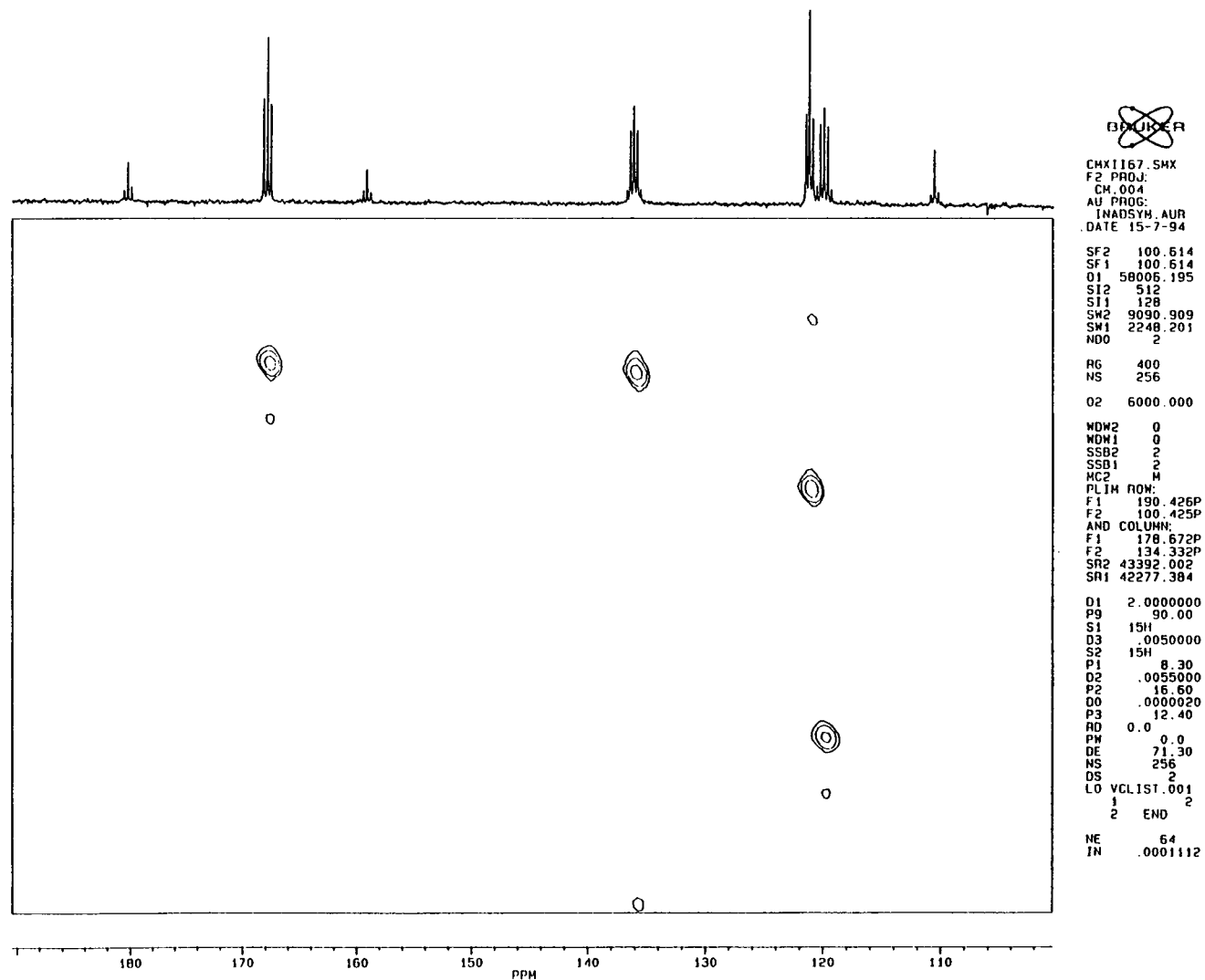


Figure 8.1. INADSYM Spectrum of 213a

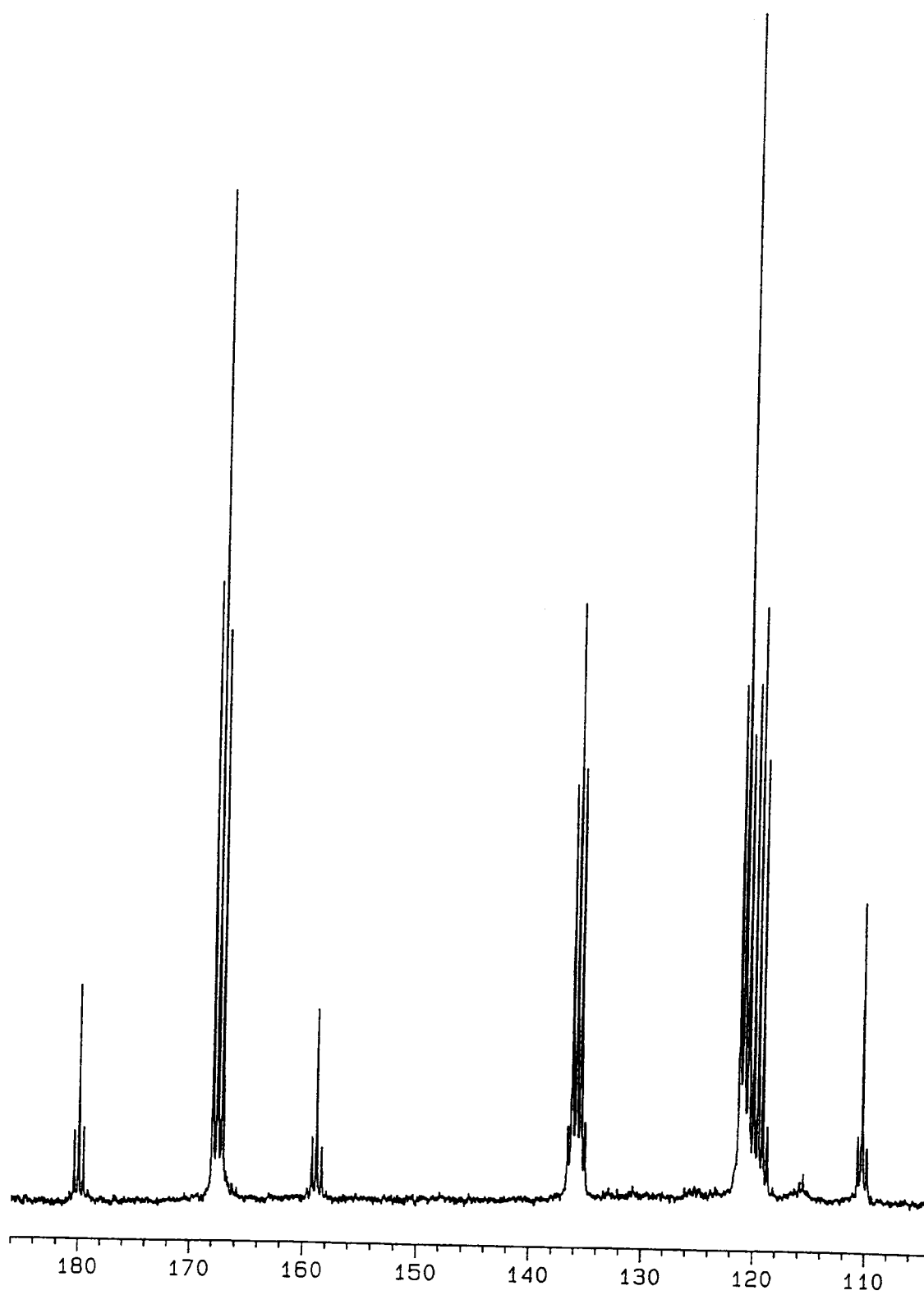


Figure 8.2. ^{13}C NMR Spectrum of **213a**

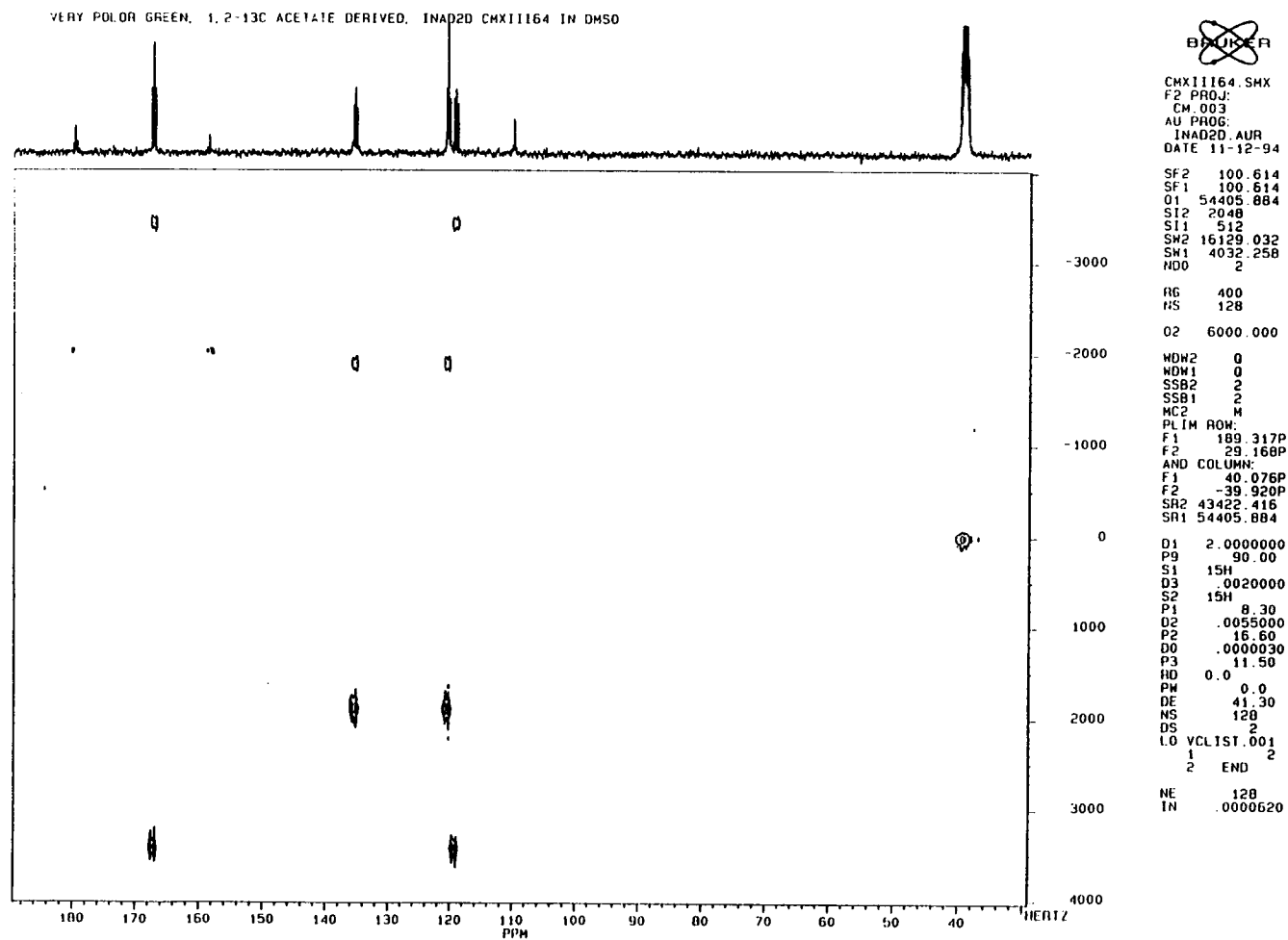


Figure 8.3. INAD2D Spectrum of 213a

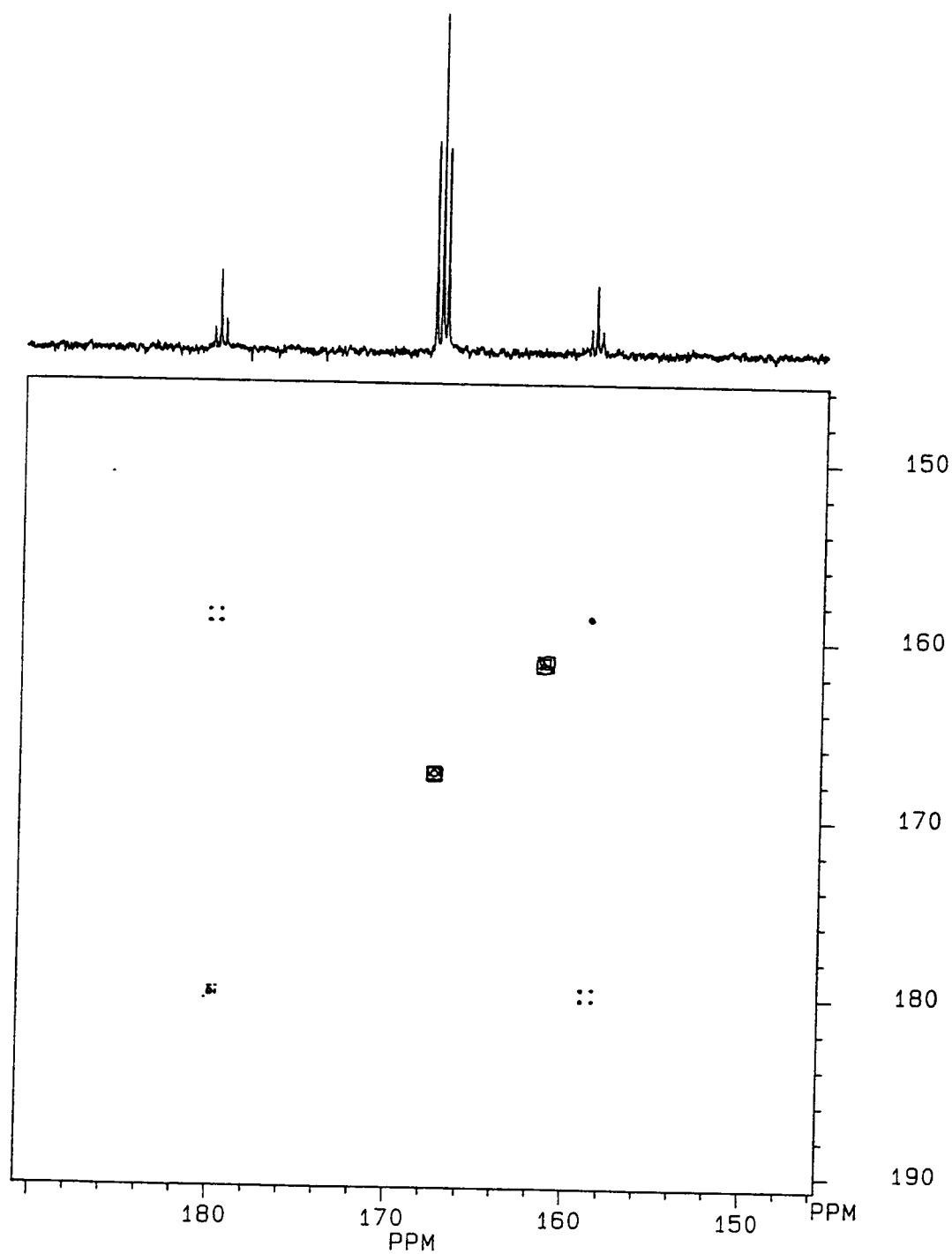


Figure 8.4. COSYX Spectrum of **213a**

derived from one of the glycolysis intermediates from dihydroxyacetone phosphate to pyruvate (triose pool). This labeling pattern is presented on structure **213a**.

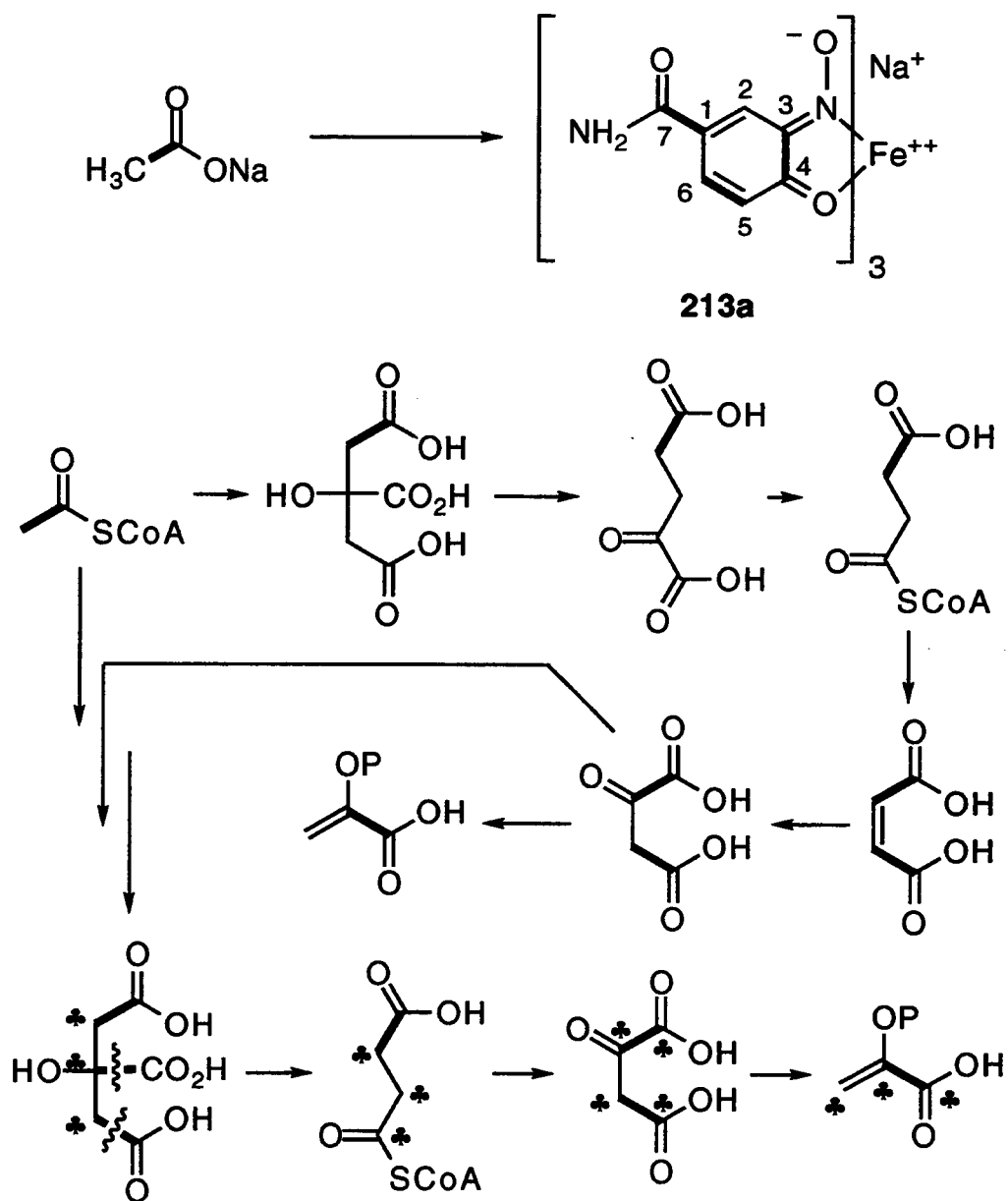
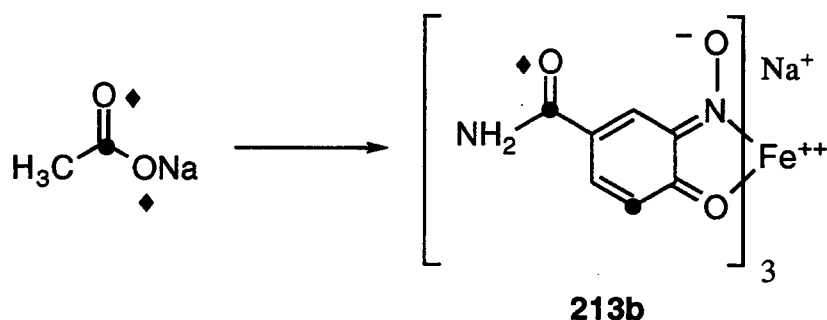


Figure 8.5. Acetate Labeling of Selected Tricarboxylic Acid Cycle Intermediates

Feeding sodium $[1-^{13}\text{C},^{18}\text{O}_2]\text{acetate}$ to a culture of the producing organism gave after purification 16.7 mg of **213b**. An estimated production of 27.1 mg of **213b** was calculated as described above. ^{13}C NMR analysis showed the carboxyl carbon was enriched and showed an ^{18}O -derived isotope shift of 3.2 Hz. Surprisingly however, the carbon atom at C5, rather than C6 was also enriched. This was the key piece of information that showed the C7-C1-C6-C5 subunit is derived not from direct incorporation of two molecules of acetate, but from acetate through the intermediacy of a preformed C_4 unit, once again the product of the tricarboxylic acid cycle. An average enrichment per site of 10.0% above natural abundance was calculated by comparison of ^{13}C line heights in enriched, and unenriched samples. The line height of the ^{18}O -shifted carbon resonance was 16% that of the enriched carbon line. The pattern of acetate labeling for the C_4 -derived portion is shown on structure **213b**. No enrichment was observed for the C_3 subunit.



Possible Post Aromatization Precursors

The nitroso group of **176** can be envisaged as arising either from the corresponding amino acid **216** by oxidation, or the nitro acid **217** by reduction. In order to test the former possibility, a deuterium-exchanged sample of **216a** was fed to a culture of *S. murayamaensis* mutant MC11 and the derived **213c** isolated. ^2H NMR analysis of **213c** showed a strong resonance centered at δ 7.5. Comparison of the integral of this resonance with that of the solvent signal yielded a 28.5% enrichment at H-2, corresponding to a 3.2% incorporation of the **216a** fed. The presence and location of the deuterium label in **213c** was also readily apparent from the ^{13}C NMR spectrum, which showed additional signals due to ^2H -induced β -isotope shifts for the C-1 and C-3 resonances (0.05 ppm and 0.09 ppm, respectively), as shown in Figure 8.6.

A variety of commercially available C_7 compounds were compared with fermentation extracts to see if any matching compounds could be identified. While no perfect matches were found, both 3-hydroxybenzoic acid **218** and 3,4-dihydroxybenzoic acid **219** had very similar (but not identical) UV/vis spectra with compounds in the spectral library.

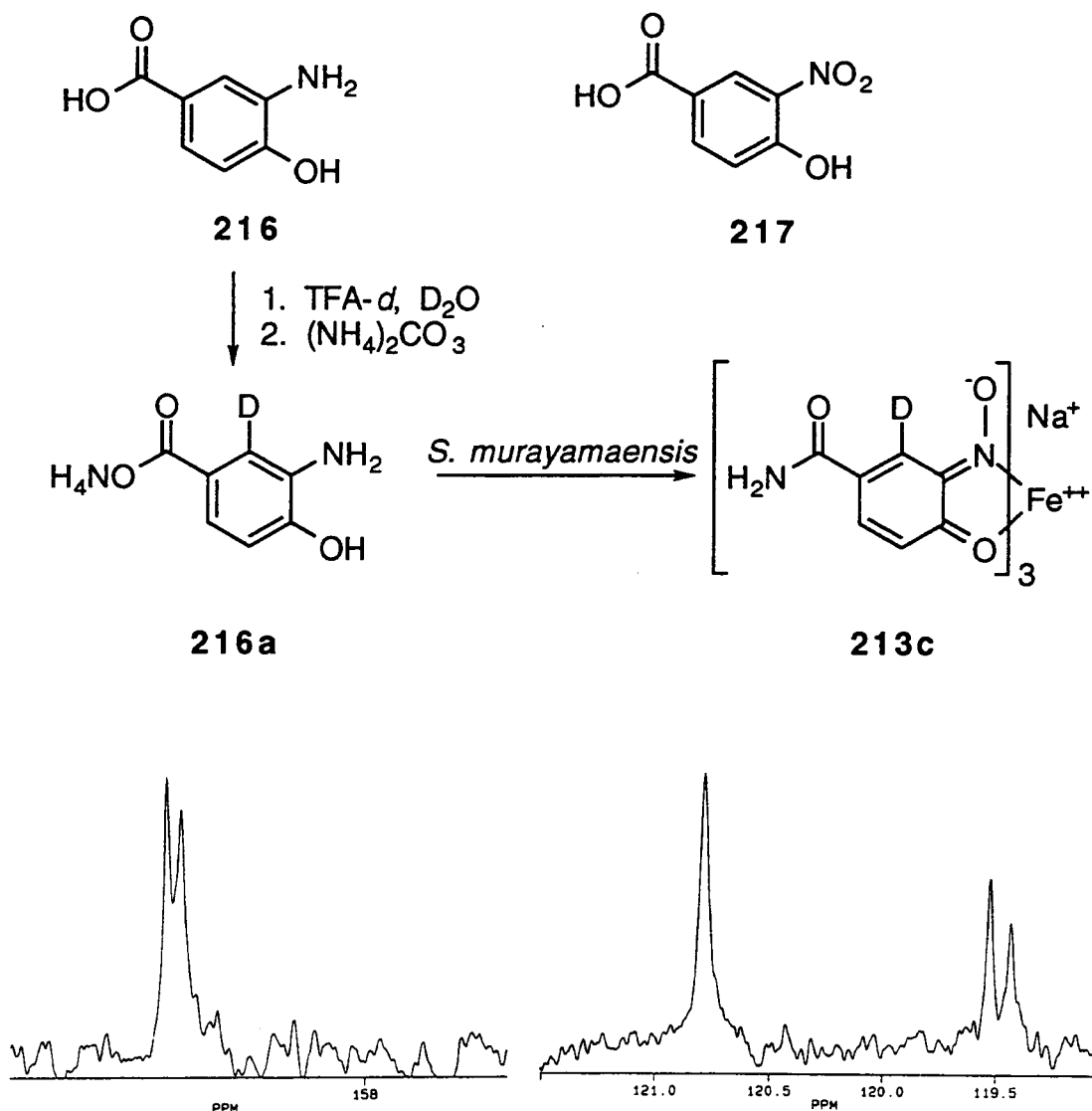
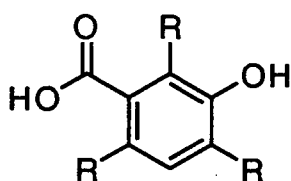


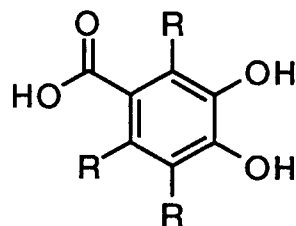
Figure 8.6. Partial ¹³C NMR spectrum of **213c** showing resonances for C-5 (δ 120.8), C-1 (δ 119.6), and C-3 (δ 158.7)

It may be that the fermentation-derived HPLC peaks are the corresponding benzamides of **218** and **219**. Either of these could be envisaged as a precursor to acid **216** via the intermediacy the

corresponding *ortho*-quinone. Neither **218** or **219** accumulated in the feeding experiment fermentations. Hence, a biosynthesis of **213** that does not involve these intermediates is presented below. The deuterium labelled compounds **218a** and **219a** were however prepared, and may be fed at a future time with the expectation that they will not be incorporated.



218 R = H
218a R = ^2H



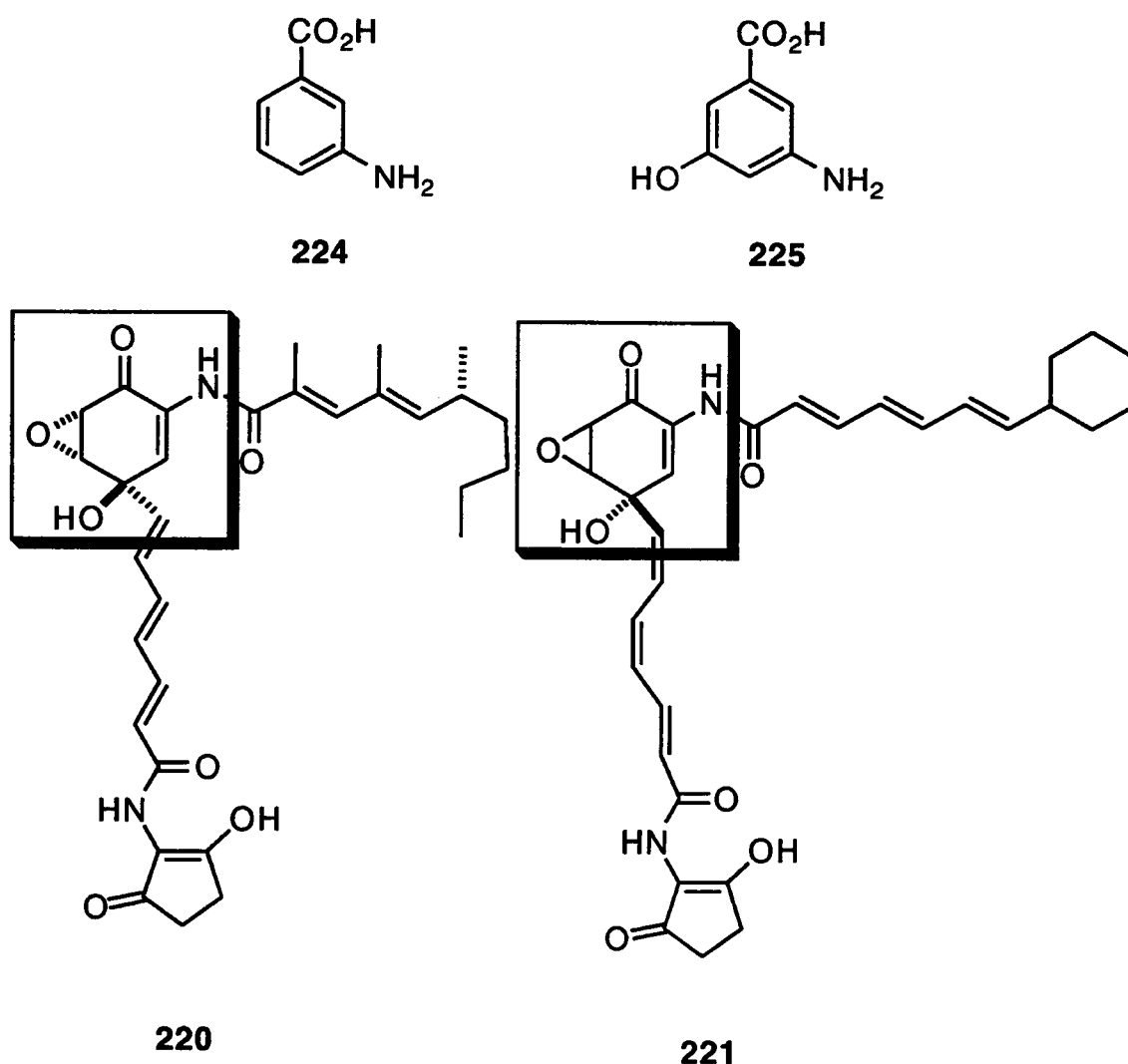
219 R = H
219a R = ^2H

HPLC analysis of fermentation extracts typically showed the presence of the amide **214**. In one instance this was produced as the major metabolite rather than **213**, and was compared with the authentic synthetic material by coinjection under modified HPLC conditions. This compound was partially purified by passing the broth through a strongly acidic cation exchange resin, and eluting with ammonium acetate.

Biosynthesis

Floss and co-workers(17) have proposed that a nonaromatic *meta*-C₇N unit is a novel polyketide starter unit in their

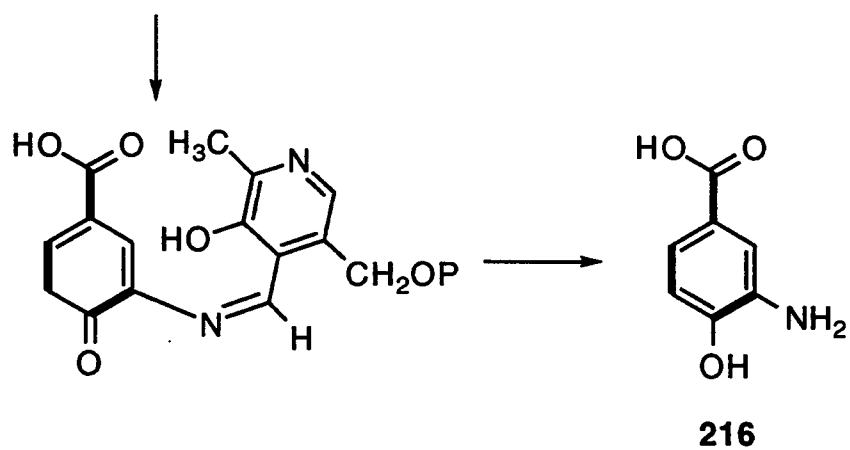
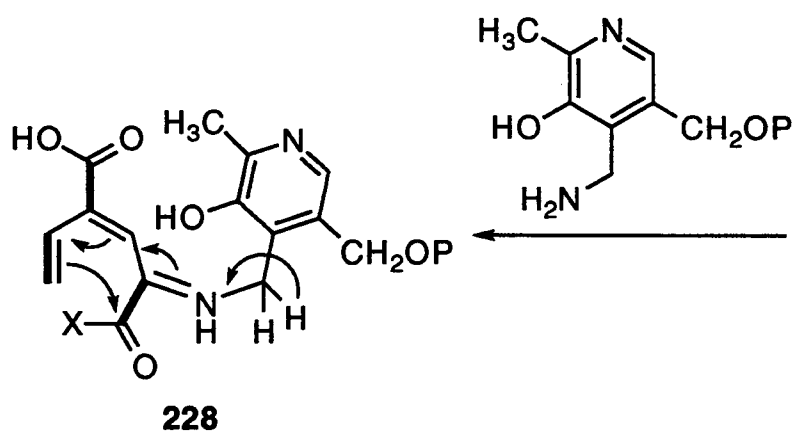
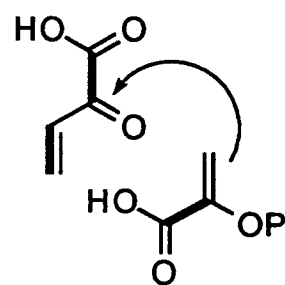
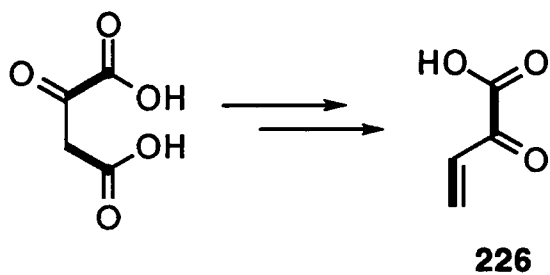
biosynthetic studies of manumycin **220**, and asukamycin **221**. These two compounds share a labeling pattern that is consistent with that presented above for **213**. However, they were not able to observe any labeling of the C₃ derived subunit from acetate. These workers rationalized their feeding results by proposing that the *meta*-C₇N subunit is derived from the condensation of dihydroxyacetone **222** and succinylCoA **223**, as shown, leading to **220** and **221** without the involvement of any aromatic intermediates. Thiericke and co-workers(17,18) fed [*carboxy*-¹³C]-**224** and [*carboxy*-¹³C]-**225**, compounds involved in the biosynthesis several *meta*-C₇N subunits, and observed no incorporation. With the manumycin producing organism, new metabolites, containing the substructures of the aromatic compounds fed, were obtained by directed biosynthesis. Since we now know that **216a** successfully incorporates into **213c**, we can predict that **216a** is also involved in the biosynthesis of **220** and **221**. This would account for the directed biosynthetic results of incorporating aromatic subunits into **220**, but producing metabolites not having a sufficiently close structure to undergo the subsequent oxidative transformations. It can now reasonably be surmised that the oxidative modification of the *meta*-C₇N subunit in **220** and **221** occurs after the polyketide assembly steps, in contrast to the original proposal.

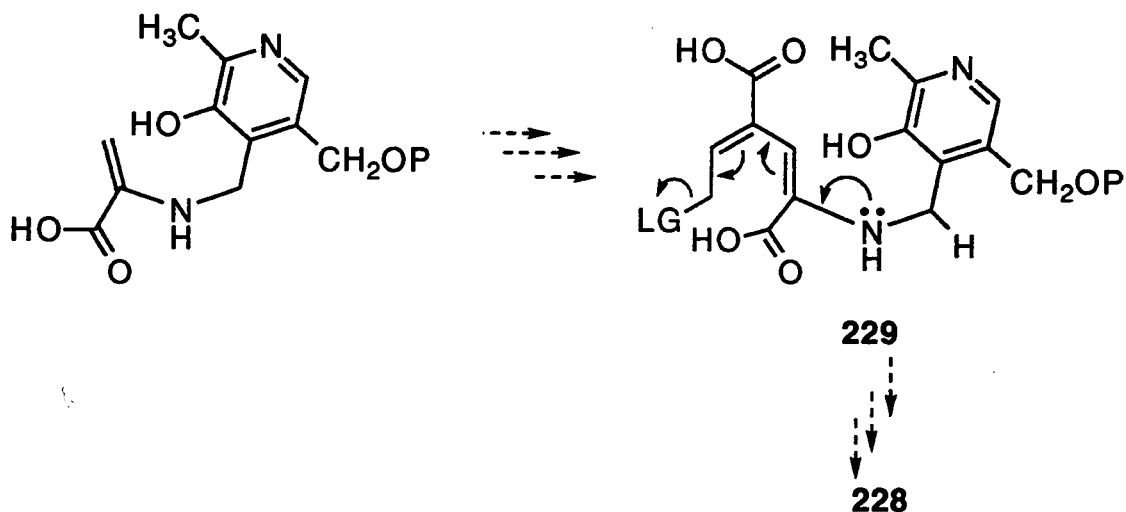


Our labeling shows a symmetrical disposition of intact acetate residues, and of uncoupled enrichment, in the C₄ derived subunit. This is inconsistent with *in vivo* utilization of the dissymmetric **223**, but would be consistent with the pathway branching from a tricarboxylic acid pathway intermediate from succinate through oxaloacetate. Similarly, the proposal for using dihydroxyacetone is inconsistent with our observed labeling because of the observed regiospecificity of incorporation of both

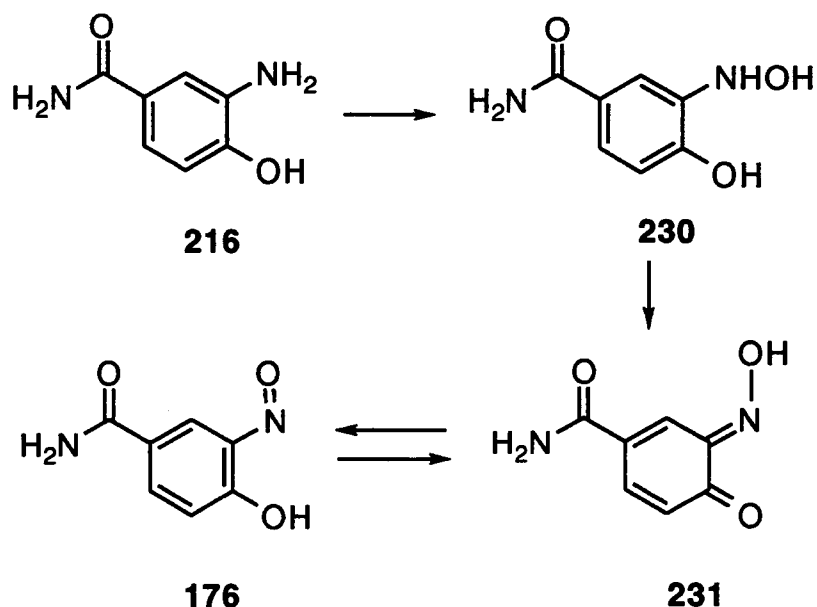
Although any members of the aforementioned C₃ and C₄ pools could be the primary precursor to its respective subunit, it is necessary to account for: the two newly formed C-C bonds; for deoxygenation of C5; and for introduction of nitrogen at C3, in any biosynthetic sequence. A very different, and succinct explanation of the observed labeling would be prior reduction of oxaloacetate to compound **226**,^(19,20) or its formation from homoserine or methionine, and its condensation to afford **227**. One way to convert **227** to **216** would be formation of the Shift's Base **228** with pyridoxylamine phosphate, and ring closure accompanied by tautomerism to give **216**.

An alternative to the direct involvement of a member of the triose pool would be to use the enamine formed from the reaction of alanine and pyridoxal phosphate as the nucleophilic moiety. The resulting enamine could condense with an α -keto acid that possesses a γ leaving group. Examples of the possible condensation partners would be the α -keto acids corresponding to the amino acids homoserine or methionine. Loss of water and tautomerism may then form an intermediate, **229**, which upon undergoing a 1,6 elimination would provide a molecule of the form of **228**, obviating the need to involve preformed vinylglyoxylate, **226**. Alternative permutations of the above mechanistic components would also potentially be equally plausible.





This scenario would involve **216** as the first aromatic intermediate. Since **216** was incorporated, and **214** was observed in the broth, the next step would likely be amide formation via a mixed anhydride.⁽¹⁵⁾ Conversion of the amino group to a nitroso likely involves prior conversion to the hydroxylamine **230**, followed by oxidation to the *ortho*-quinone monooxime **231**. However, the addition of water to an *ortho*-quinone monoimine is a possible alternative.



Experimental

General. Media were prepared from deionised water, and reagent grade chemicals. Ethyl acetate was distilled before use, and all other solvents were of reagent grade. Isotopically labeled sodium acetates were obtained from Cambridge Isotope Laboratories, Inc. An IBM 9420 UV/vis spectrophotometer was used to determine quantitative absorption spectra. Compounds fed were dissolved in H₂O, and introduced in equal aliquots through a 2 μ m syringe filter. Otherwise experiments were performed as described in Chapter 2.

HPLC Analysis of 3-Amino-4-hydroxybenzamide (214). A Waters NovaPak C₁₈ radial compression column (0.8 x 10 cm, 5 μ m bead size) was eluted at a flow rate of 1.5 mL/min with Milli-Q water containing acetic acid (0.1 %). Absorption spectra were

obtained using a Waters 990+ diode-array detector with 2 nm resolution over a wavelength range of 200-650 nm.

Fermentation for 213. A Kinako soybean-glucose seed culture(8) (50 mL in a 250 mL foam stoppered Erlenmeyer flask) was inoculated with agar plugs of actively growing mycelium, and incubated on a rotary shaker at 27 °C, 280 rpm for 48 h. Production media (400 mL in each 2 L foam stoppered flask) were inoculated with 5% of their volume of seed medium. Wild type *S. murayamaensis* was grown on glycerol-asparagine medium, and mutants MC3,(9) and MC11 on glycerol-ammonium sulfate medium.(8)

Isolation of 213 from the Wild Type *S. murayamaensis*. At 36 h, 1.6 L whole broth was adjusted to pH 2.8, sonicated, and filtered through celite. Extraction of the broth with EtOAc (4 x 250 mL) removed nonpolar metabolites, and a second celite filtration removed precipitated proteins. The broth was then extracted with n-butanol (4 x 200 mL), and the organic extracts were each sequentially washed with H₂O (100 mL), then evaporated to dryness. The resulting solids (320 mg) were sequentially washed with CHCl₃ and EtOAc (2 x 10 mL each) to give 140 mg crude **213**. This was applied to a Sephadex LH-20 column (2.5 x 15 cm) prepared and eluted in MeOH. A brown band eluted followed by the green compound, which was collected and evaporated to give 46 mg. Rechromatography in the same manner, but on a column prepared and eluted with 5% H₂O in MeOH, gave 35 mg pure **213**:

mp >300 °C; IR (KBr) 3426.4, 3387.9, 1655.9, 1599.4, 1076.3 cm^{-1} ; UV/vis λ_{max} (ϵ , FW 551, MeOH) 203 (33,600), 272 (24,400), 292 (21,400), 434 (5,300), 688 (5,700) nm; ^1H NMR (DMSO-*d*₆) δ 7.93 (dd, 1H, J = 9.1, 2.2), 7.85 (br s, 1H), 7.53 (d, 1H, J = 2.1), 7.10 (br s, 1H), 6.97 (d, 1H, J = 9.2); ^{13}C NMR (DMSO-*d*₆) δ 179.9 (C4), 167.5 (C7), 158.7 (C3), 135.7 (C6), 120.8 (C5), 119.6 (C1), 110.2 (C2); CIMS (negative mode) 386 $[\text{Fe}(\text{C}_7\text{H}_5\text{N}_2\text{O}_3)_2]^-$; FABMS (negative mode) 551 $[\text{Fe}(\text{C}_7\text{H}_5\text{N}_2\text{O}_3)_3]^-$, (positive mode) 575 $[\text{Fe}(\text{C}_7\text{H}_5\text{N}_2\text{O}_3)_3 \cdot \text{Na}^+\text{H}^+]$.

Isolation of 213 from Mutants MC3 and MC11. The whole broth was diluted with an equal volume of EtOAc. Sonication, filtration, and partition of the resulting layers gave an aqueous solution, which was adjusted to pH 2-3 and re-extracted with EtOAc (2 x 200 mL). Extraction of the resulting aqueous layer with *n*-BuOH (2 x 200 mL), gave crude **213** which was chromatographed on a 2.5 x 15 cm LH-20 column prepared and eluted with 5% H₂O in MeOH. Each production flask (400 mL in a 2 L Erlenmeyer) provided 7.1 to 9.2 mg pure **213**.

Incorporation of 3-amino-2-²H-4-hydroxybenzoic acid (216a). A solution of **216a** (206.4 mg) in H₂O (10 mL) was introduced to two 400 mL cultures of *S. murayamaensis* mutant MC11. Additions were made 12, 15, 18, 21, and 24 h after inoculation, and the culture harvested at 36 h. Extraction gave 72 mg crude **213c** which, after purification on Sephadex LH-20, gave 11.2 mg of **213c**. ²H quantitation by comparison with the natural

abundance DMSO resonance in the ^2H NMR spectrum (61 MHz, 32890 scans, natural abundance DMSO reference and quantification) showed an enrichment of 28.5%. This corresponded to a 3.4% incorporation based on a calculated production of 18.4 mg **213c** determined by HPLC monitoring at 254 nm. Signals arising from 0.09 and 0.05 ppm β isotope effects in the ^{13}C NMR were observed for resonances corresponding to C2 (119.6) and C4 (158.7).

Incorporation of Sodium [1,2- $^{13}\text{C}_2$]Acetate. Sodium [1,2- $^{13}\text{C}_2$]acetate (102.2 mg) and sodium [1- ^{14}C]acetate (3.00 μCi) dissolved in H_2O (3.00 mL) were introduced to a 400 mL culture of MC3 in equal portions 14, 20, and 26 h after inoculation. Extraction by the above protocol gave a murayaquinone-containing extract (225 mg), an **176** containing extract (146 mg), and crude **213a** (42 mg). LH-20 purification of the latter gave 8.1 mg of **213a**. An estimated production of 12.6 mg calculated by analysis of the area of peak corresponding to **213a** in the HPLC trace monitored at 254 nm.

Incorporation of Sodium [1- ^{13}C , $^{18}\text{O}_2$]Acetate. Sodium [1- ^{13}C , $^{18}\text{O}_2$]acetate (154.2 mg) and sodium [1- ^{14}C]acetate (3.00 μCi) dissolved in H_2O (50.0 mL) were introduced by a 2 μm syringe filter to two 400 mL culture broths of MC3 in equal portions 14, 17, 20, 23, and 27 h after inoculation. Extraction by the above protocol gave a murayaquinone-containing extract (214.1 mg), and crude **213b** (154.2 mg). LH-20 purification of the

latter gave 16.7 mg of **213b**. An estimated production of 27.1 mg calculated by analysis of the area of peak corresponding to **213b** in the HPLC trace monitored at 254 nm.

¹³C-¹³C Coupling Experiments for 213a. All experiments were performed on a Bruker AM 400 spectrometer in DMSO-*d*₆. The pulse programs cited were used without modification. Symmetrical INADEQUATE spectra were obtained using the Bruker pulse program INADSYM.AUR, and the following acquisition parameters: D1 2.0; P9 90; S1 15H; D3 0.005; S2 15H; P1 8.3; D2 0.00550; P2 16.6; RD 0.0; PW 0.0; D0 3 μs; NE 64; ND0 2; MC2 M; SI2 512 W; SI1 128 W; NS 256 x 2; WDW2,1 Q; SSB2,1 2. INADEQUATE spectra were obtained using the Bruker pulse program INAD2D.AUR, and the following acquisition parameters: D1 2.0; P9 90; S1 15H; D3 0.002; S2 15H; P1 8.3; D2 0.00550; P2 16.6; RD 0.0; PW 0.0; D0 3 μs; NE 128; ND0 2; MC2 M; SI2 2048 W; SI1 512 W; NS 128 x 2; WDW2,1 Q; SSB2,1 2. COSYX spectra were obtained using the Bruker pulse program COSYX.AUR, and the following acquisition parameters: D1 1.0; P9 90; S1 15H; D3 0.002; S2 15H; P1 8.3; D2 0.00550; P2 8.3; RD 0.0; PW 0.0; D0 3 μs; NE 128; ND0 1; MC2 M; SI2 2048 W; SI1 1024 W; NS 736; WDW2,1 S; SSB2,1 0.

4-Hydroxy-3-nitrobenzamide.(21) 4-Hydroxy-3-nitrobenzoic acid (3.46 g, 18.9 mmol) was dissolved in DMF (27.0 mL) and cooled in an ice water bath. Pyridine (3.10 mL, 38.3 mmol), followed by dicyclohexylcarbodiimide (3.97g, 19.2 mmol) were

added and the suspension allowed to warm to rt overnight. Dilution with Et₂O (50 mL) and filtration with an EtOH rinse gave poly-4-hydroxy-3-nitrobenzoic acid (3.40g) as a white powder. A portion of this material (1.25 g) was added to methanol (100 mL) which had been pre-saturated with ammonia gas.(22) NH₃ gas was passed through the suspension for 0.1 h and after 36 h stirring the solvent was evaporated under a stream of air. The resulting solid was recrystallized from EtOH (110 mL) gave the ammonium salt as a yellow solid, which was dissolved in HCl (100 mL, 0.5 M) and extracted into EtOAc (3 x 50 mL) to provide after evaporation 2.34 g of 3-nitro-4-hydroxybenzamide (68%) as a beige solid: mp 184.2-185.8 °C, lit. 185.5-187.5 °C; IR 1672.8, 1614.8, 1530.8, 1402.3, 1262.8 cm⁻¹; ¹H NMR (DMSO-*d*₆) δ 8.42 (d, 1H, J = 2.2), 8.02 (dd, 1H, J = 8.7, 2.2), 7.33 (br. t, 1H, J = 10), 7.16 (d, 1H, J = 8.7), 6.99 (br. t, 1H, J = 10); ¹³C NMR (NH₄ salt, DMSO-*d*₆) δ 167.99, 167.40, 136.00, 132.55, 127.27, 125.68, 114.12; EIMS *m/z* (rel intensity) 182.1 (0.3%), 166.1 (1.5%), 100.1 (12%), 84.1 (100%); HREIMS calcd for C₇H₆N₂O₄ 182.0328, found 182.0328.

3-Amino-4-hydroxybenzamide (214). A. To a MeOH (2.00 mL) solution of **213** (1.4 mg) was added 10% palladium on carbon (0.6 mg) and the mixture stirred under an atmosphere of hydrogen for 12 h. HPLC analysis of the colorless solution showed **214** as the only product.

B. A solution of **213** (8.1 mg) in MeOH-H₂O (19:1) was evaporated to near dryness, then diluted with H₂O (2.00 mL) to

give a dark green solution. A freshly prepared dilute solution of $\text{Na}_2\text{S}_2\text{O}_4$ in H_2O was added dropwise over 0.2 h with stirring. Addition was stopped when the mixture turned colorless, and the H_2O was lyophilized. The resulting solids were sonicated in EtOH (10.0 mL), and filtered through celite which gave, after evaporation of the filtrate, 4.5 mg of **214** (67%) as a brown solid.

C. To a MeOH (10.0 mL) solution of the 3-nitro-4-hydroxybenzamide (0.853 g, 4.68 mmol) was added 10% palladium on carbon (3.2 mg). After 1 h stirring under an atmosphere of H_2 , the sample was filtered, and evaporated to give 705.4 mg **214** (99%) as a tan solid: mp 167.2-170.0 °C; IR (KBr) 3360.3, 1654.9, 1584.9, 1403.3, 1294.7 cm^{-1} ; ^1H NMR ($\text{DMSO}-d_6$) δ 7.54 (br. s, 1H), 7.14 (d, 1H, $J = 2.1$), 6.98 (dd, 1H, $J = 8.2, 2.1$), 6.90 (br. s, 1H), 6.64 (d, 1H, $J = 8.2$), 4.68 (br. s, 2H), 3.41 (br. s, 1H); ^{13}C NMR ($\text{DMSO}-d_6$) δ 168.59, 146.93, 136.11, 125.73, 116.56, 114.08, 113.31; EIMS m/z (rel abundance) 152.1 (M^+ , 96%), 136.0 (82%), 108.0 (29%), 84.0 (100%); HREIMS calcd for $\text{C}_7\text{H}_8\text{N}_2\text{O}_2$ 152.0586, found 152.0586.

Ammonium-3-amino-2- ^2H -4-hydroxybenzoate (216a). 3-Amino-4-hydroxybenzoic acid (526 mg, 3.43 mmol) was dissolved in trifluoroacetic acid- d (10.0g) and deuterium oxide (10.0g). This solution was heated at reflux for 1 d, then diluted with Et_2O (20.0 mL), filtered and the solid was rinsed with Et_2O . The purple trifluoroacetate salt thus obtained was dissolved in ammonium bicarbonate (25 mL, 1M, pH 8.5) and lyophilized to give 585 mg **216a** (100%), 73% ^2H substituted at position 2, as a brown solid:

^1H NMR (trifluoroacetate, $\text{DMSO-}d_6$) δ 7.67 (s, 1H, partially exchanged), 7.58 (d, 1H, $J = 8.2$), 6.95 (d, 1H, $J = 8.4$); EIMS m/z (rel intensity) 154.1 (100%, M^++1), 153.1 (37%), 137.1 (25%); HREIMS calcd for $\text{C}_7\text{H}_6^2\text{HNO}_3$ 154.0489, found 154.0489.

2,4,6- ^2H -3-Hydroxybenzoic acid (218a). To **218** (1.07 g) was added trifluoroacetic acid- d (20.2 g). This was heated at reflux for 48h, the TFA- d was removed by distillation, and the deuterium atoms substituted on oxygen were removed by twice evaporating from MeOH. This gave 1.10 g (100%) of **218a**, 79% exchanged per position, as a white solid: EIMS m/z (rel intensity) 141.0 ($[\text{M}+3]^+$, 100%), 140.0 (85%), 139 (19%).

2,5,6- ^2H -Protocatechuic acid (219a). To **219** (1.03 g) was added trifluoroacetic acid- d (20.0 g). This was heated at reflux for 48h, the TFA- d was removed by distillation, and the deuterium atoms substituted on oxygen were removed by twice evaporating from MeOH. This gave 1.03 g (99%) of **219a**, 94% exchanged at positions 2 and 5, 24% exchanged at position 6, as a brown solid: EIMS m/z (rel intensity) 157.0 ($[\text{M}+3]^+$, 80%), 156.0 (100%), 155.0 (63%), 154.0 19%).

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