

STEREOCHEMICAL ASPECTS OF VIRGINIAMYCIN BIOSYNTHESIS

BIOSYNTHESIS OF ANTIBIOTIC A33853

by

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(ABSTRACT)

The biochemical pathways for the formation of the unusual amino acids found in virginiamycin M₁ and A33853 were investigated.

Specifically tritiated and carbon 14 labeled serines were incorporated into virginiamycin M₁. (2S)-serine and (2S,3R)-[3-³H]serine were found to be precursors, thus giving evidence of stereochemical control in the formation of the oxazole moiety. This information allowed for postulation of a ring closure pathway. Stereochemical investigations were also carried out on the dehydroproline unit and it was shown that both (R) and (S) prolines were incorporated into the dehydroproline unit. (2S,3R)-[3-³H]proline was synthesized and upon incorporation lost the (3-³H) label as evidence of stereochemical control in the formation of the dehydroproline unit from a saturated precursor .

The basic biosynthetic origins of A33853 were investigated by feeding of D-[U-¹⁴C]glucose, sodium [U-¹⁴C]acetate, (S)-[U-¹⁴C]lysine,

(S)-[U-¹⁴C]aspartic acid, [carboxyl-¹⁴C]anthranilic acid, and (S)-[5-³H]tryptophan. D-[U-¹⁴C]glucose and (S)-[U-¹⁴C]lysine appeared to be the main precursors. ¹³C¹⁵N lysine was synthesized and used to examine the ring closure of the 3-hydroxypicolinic amide ring in virginiamycin S₁.

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I dedicate this dissertation to my parents and
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I thank the Kingston research group for all of their help and
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To: Mom and Dad

"Get wisdom, get understanding, forget it not...."

Proverbs 4:5

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CHAPTER I.

INTRODUCTION

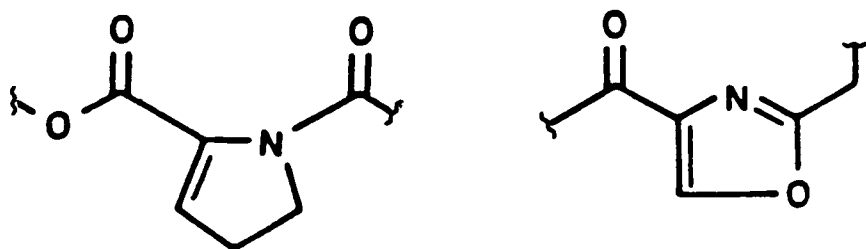
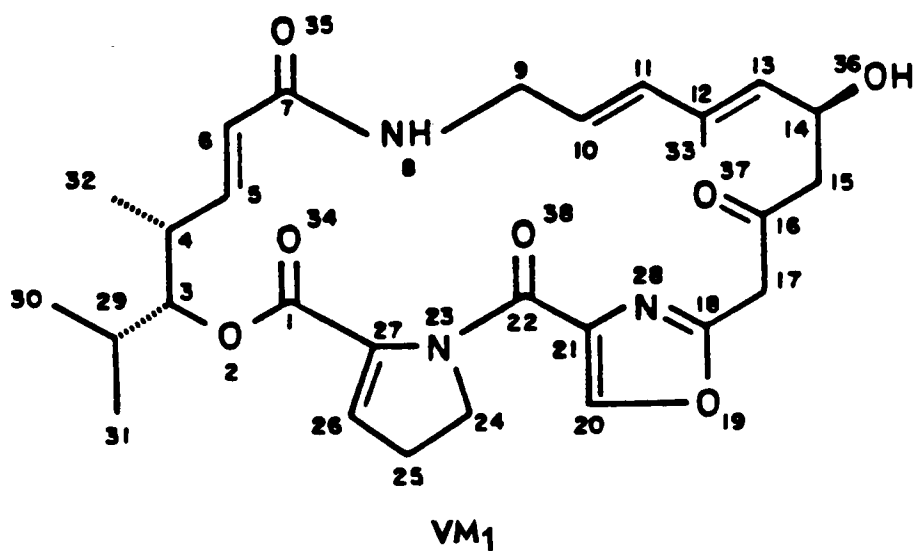
Virginiamycin M₁ (VM₁) is an unusual modified cyclic peptolide antibiotic first isolated in 1955 by Somer and Van Dijck.¹ Its structure was shown by chemical² and spectroscopic³⁻⁵ methods to be as pictured in Figure 1. It is particularly noteworthy that it contains two dehydroamino acid units, the unusual dehydroproline and oxazole components.

Virginiamycin M₁ is produced together with a cyclic peptidolactone antibiotic, virginiamycin S₁ (VS₁) by cultures of the organism Streptomyces virginiae. The two antibiotics, VM₁ and VS₁, show an unusual synergistic effect on their biological activity, and this effect has led to extensive investigations of their mechanism of action.⁶ The commercial importance of these antibiotics as animal feed additives and as potential antibiotics for other purposes has led to studies of their chemistry and biology and has been an important factor in biosynthetic studies by Kingston and his coworkers.⁷⁻¹⁰

The biosynthetic pathways proposed by Kingston, et al.⁷⁻¹⁰ for VM₁ are the foundations for this work and established the basic biosynthesis of the antibiotic. Some important stereochemical questions relating to the origin of the dehydroamino acid units were, however, not addressed in these studies, and it is these questions that form the basis for this work.

1. What is the role of stereochemical control in the formation of the oxazole moiety from serine? (Figure 2)

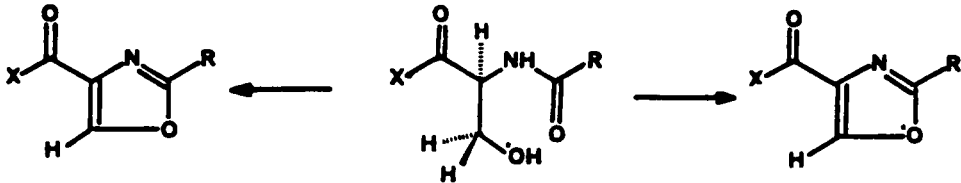
2. What stereochemical control is found in the formation of the dehydroproline unit from proline? (Figure 2)



Structure of Virginiamycin M₁ and Regions of Interest

Figure 1

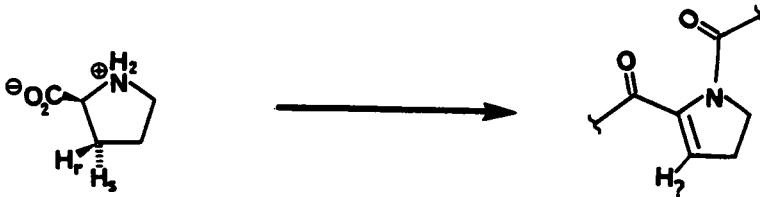
What is the origin of the oxygen atom of the oxazole ring?



What is the stereochemistry of serine incorporation?



What is the stereochemistry of proline incorporation?



Biosynthetic Questions and Possible Answers

Figure 2

CHAPTER II.

REVIEW OF THE LITERATURE

This chapter provides a synopsis of the literature covering the chemistry, mode of action, synthetic derivatives and biosynthetic studies of virginiamycin M₁. There have been several review articles covering the virginiamycin family of antibiotics, the most inclusive being those by Vazquez,^{11,12} Cocito^{6,13}, Tanaka¹⁴, and Biot.¹⁵ Therefore, this review is selective rather than exhaustive in its coverage, concentrating on aspects of the chemistry and biology of the antibiotics that are relevant to this dissertation.

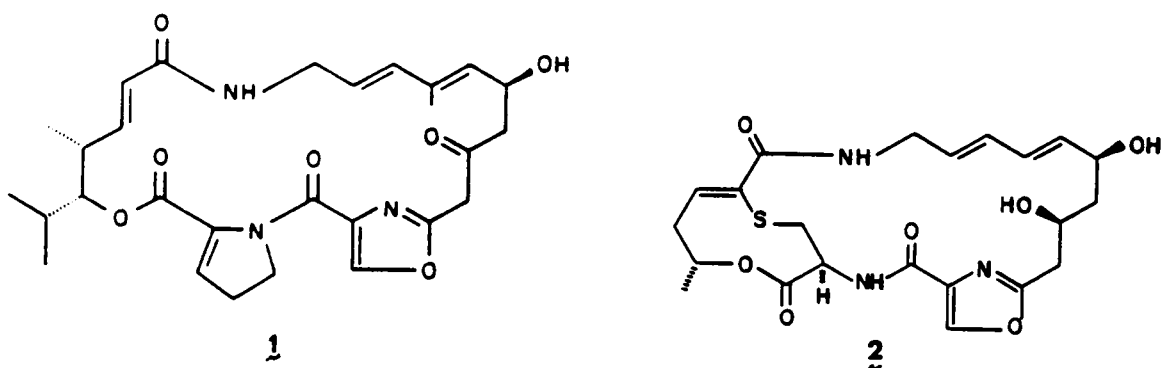
A. Chemistry

The virginiamycin antibiotics were isolated in the 1950's by several independent laboratories^{1,16,17} and were obtained as mixtures of the components known as virginiamycin M₁, M₂, S₁, S₂, S₃ and others. These antibiotics were important due to their individual bacteriostatic activity and synergistic activity against gram positive microorganisms, particularly those resistant to penicillin.¹⁸⁻²⁰

The virginiamycin family was divided into two groups known as the group A and B antibiotics. However, there was no common nomenclature until 1972 when the names virginiamycin M and virginiamycin S were suggested by Crooy and DeNeys²¹ as the standard. Figures 3 and 4 show the structures, alternative names and isolation references for the group A (VM) antibiotics and Figures 5 and 6 show the structures of the group B (VS) antibiotics.

Of the five type M antibiotics listed in Figures 3 and 4,

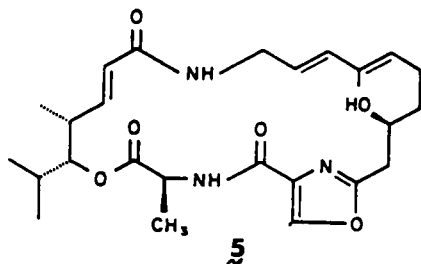
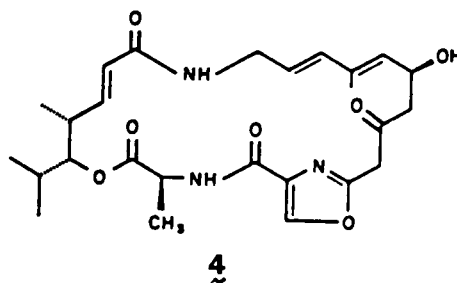
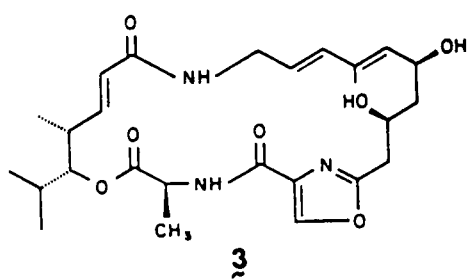
<u>Structure</u>	<u>Compound</u>	<u>Common Names</u>	<u>Isolation Reference</u>
<u>1</u>	Virginiamycin M ₁	vernAMYcin A, streptogramin A, ostreogrycin A, mikamycin A, pristinamycin 11A, synergistin A-1	26,70,2 22,71,17
<u>1</u>	Virginiamycin M ₂ dehydroproline moiety is saturated	ostreogrycin G, pristinamycin 11B	26,5,71
<u>2</u>	Griseoviridin		72,73



Structures of Virginiamycin M (group A) Antibiotic

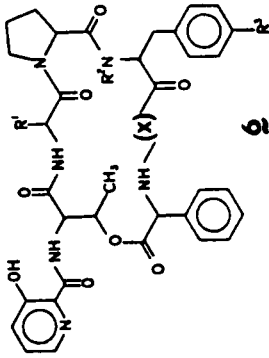
Figure 3

<u>Structure</u>	<u>Compound</u>	<u>Common Names</u>	<u>Isolation Reference</u>
3	A-2315A	CP-35763, madumycin 11 A-17002F	74-77
4	A-2315B	CP-36926, madumycin 1	74,75,77
5	A-17002C		77



Structures of Virginiamycin M (group A) Antibiotic

Figure 4

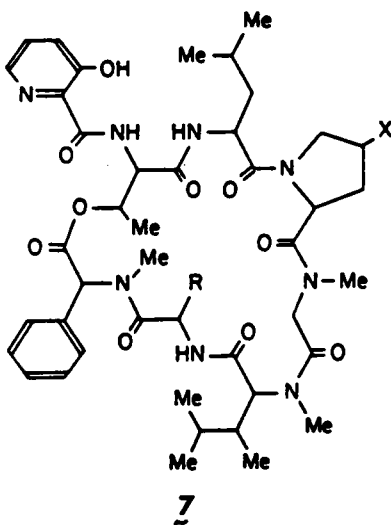


Antibiotic	Common Names	R ₁ —R ₂ —R ₃	X	Isolation Reference
Virginiamycin S ₁		C ₂ H ₅ CH ₃ H	4-oxopipelic acid	78
Virginiamycin S ₂		C ₂ H ₅ H H	4-hydroxypipelic acid	78
Virginiamycin S ₃		C ₂ H ₅ CH ₃ H	3-hydroxy-4-oxopipelic acid	78
Virginiamycin S ₄		CH ₃ CH ₃ H	4-oxopipelic acid	78
Vernamycin B _a	mitamycin 13, streptogramin B, pristinamycin 1A, ostreogrycin B	C ₂ H CH ₃ N(CH ₃) ₂	4-oxopipelic acid	79,80,81,71,82
Vernamycin B _b	ostreogrycin B ₂ , pristinamycin 1B	C ₂ H ₅ CH ₃ NHCH ₃	4-oxopipelic acid	79,82,71
Vernamycin B _c	ostreogrycin B ₁ , pristinamycin 1C	CH ₃ CH ₃ N(CH ₃) ₂	4-oxopipelic acid	79,82,71
Vernamycin B _d		CH ₃ CH ₃ NHCH ₃	4-oxopipelic acid	79
Vernamycin C	doricin	C ₂ H ₅ CH ₃ N(CH ₃) ₂	aspartic acid	83
Ostreogrycin B ₃		C ₂ H ₅ CH ₃ N(CH ₃) ₂	3-hydroxy-4-oxopipelic acid	84
Patricin A		C ₂ H ₅ CH ₃ H	proline	85
Patricin B		C ₂ H ₅ CH ₃ H	pipelic acid	85

Structure of Virginiamycin S (Group B) Antibiotic

Figure 5

<u>Antibiotic</u>	<u>Common Names</u>	<u>Structure</u>		<u>Isolation Reference</u>
		<u>R</u>	<u>X</u>	
Neoviridogrisein I		C ₂ H ₅	H	86
Neoviridogrisein II	etamycin B	CH ₃	H	87,88
Neoviridogrisein III		C ₂ H ₅	OH	
Viridogrisin	Etamycin, 6613, neovirigogrisein IV	CH ₃	OH	89,90,91
Neoviridogrisein-MP		CH ₃	CH ₃	92



Structure of Virginiaamycin S (Group B) Antibiotics

Figure 6

virginiamycin M₁ is the antibiotic of interest. Early characterization and hydrolysis work on VM₁ was carried out by Okabe²²⁻²⁵ supplying some of the structural information. The actual structure was elucidated by chemical²⁻⁴ and spectroscopic techniques such as ¹H-NMR and high resolution mass spectrometry.³⁻⁵ An X-ray crystal structure²⁶ confirmed the structure shown in Figure 1 and also proved the relative stereochemistry of the chiral centers to be as shown. Configurational and conformational studies have been carried out by Bycroft,²⁷ and a partial synthesis by Ganem showed the absolute configuration of carbons 3, 4 and 14 to be R, R and S respectfully.²⁸

B. Fermentation

Virginiamycin M₁ is produced by several different species of Streptomyces in complex media. Since the first production by S. virginiae in 1955, thousands of mutant organisms have been produced and the production of virginiamycin enhanced by 1000 fold.¹⁵ Virginiamycin M₁ and S₁ are co-produced by a fermentation process in industry, and the crude mixtures are used as feed additives. This production in large fermenters has been optimized by several techniques such as aeration, impeller size, temperature, growth time, etc.¹⁵

C. Mode of Action

As mentioned earlier, virginiamycin M₁ and S₁ are bacteriostatic individually and possess a synergistic effect when combined. They have been shown to be active against many different organisms.^{11,29-31}

The mode of action of the virginiamycin antibiotics has remained obscure although many attempts have been made to explain the anti-bacterial activity.^{11,32} However, recent advances have shed some light on the mechanisms of action.

The mechanism of action can be described by exploring its use as an animal feed additive in livestock. Researchers have explained the activity in the following ways:¹⁵

- 1) The drug produces changes in the composition and topographical distribution of intestinal flora by establishing a new equilibrium.
- 2) The drug produces a decrease in the microbial production of lactic acid, volatile fatty acids, ammonia, and amines, thereby reducing the toxic effect of their decomposition.
- 3) The drug optimizes the availability of glucose and amino acids thereby leading to a reduction in energy waste.
- 4) The drug causes a reduction in the rate of passage through the intestine accompanied by improved absorption, exhibiting an influence on the permeability of the intestinal mucosa.

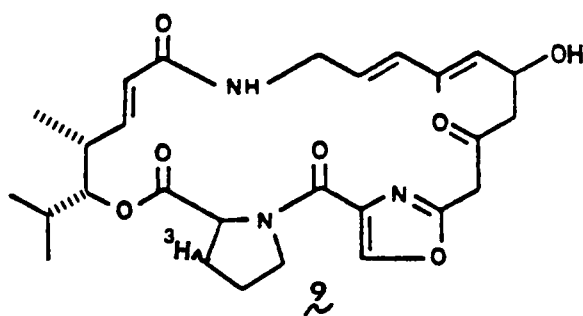
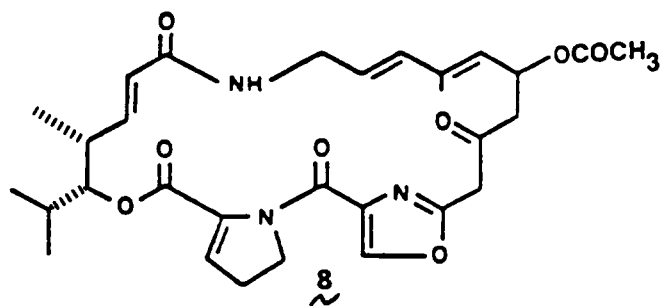
The mechanism of action can also be explained on a molecular basis. This molecular explanation has been under investigation since the late 50's, and has involved studies of the drug's effect on different organisms and studies on cell free systems.^{33,34}

The uptake of virginiamycin M_1 and S_1 by microbes has been shown to be by passive diffusion.^{35,36} The presence of K^+ , or NH_4^+ and Mg^{2+} were thought to be necessary^{34,37-40} for the action of VM_1 within the cells, but recent studies do not support this idea.^{51,52} Once inside the cell the antibiotics bind to the 50S⁴³⁻⁴⁵ and 70S⁴⁶ ribosomes.³⁷ Individually, this binding is reversible, but it is not reversible when both synergens are present.^{47,48} This irreversible binding results in a conformational change in the ribosome unit, thus preventing polypeptide chain elongation by interrupting the stabilizing interactions of the aminoacyl moiety of AA-tRNA with the substrate acceptor site of peptidyl transferase.^{43,32,49} Kinetic studies have been carried out on the binding of virginiamycin M_1 and S_1 using tritium labeled antibiotics. The results are still inconclusive as independent researchers have shown that VM_1 is used in catalytic amounts^{34,45} and stoichiometric amounts during the binding process.⁴⁶ However, it is apparent that the presence of both synergens shows increased anti-bacterial activity and that VM_1 increases the affinity of VS_1 for ribosomes.⁵⁰

D. Synthetic Modifications/Structure-Activity Relationship

There has been no complete synthesis of virginiamycin M_1 although partial syntheses of the oxazole portion and carbon skeleton have been carried out for virginiamycin M_2 ⁵¹ and griseoviridin.^{52,53} However, several derivatives of VM_1 and isotopically labeled VM_1 have been prepared and tested for biological activity.⁵⁵⁻⁶¹

An O-acylated virginiamycin derivative **8** (Figure 7) has been prepared both enzymatically⁵⁴ by a resistant strain of Staphylococcus



Dihydro Virginiamycin M₁ 9 and O-Acyl Virginiamycin M₁ 8

Figure 7

aureus^{55,56} and by chemical acylation with acetic anhydride.⁷ The presence of the C-14 acyl group diminished the anti-bacterial activity.⁵⁴

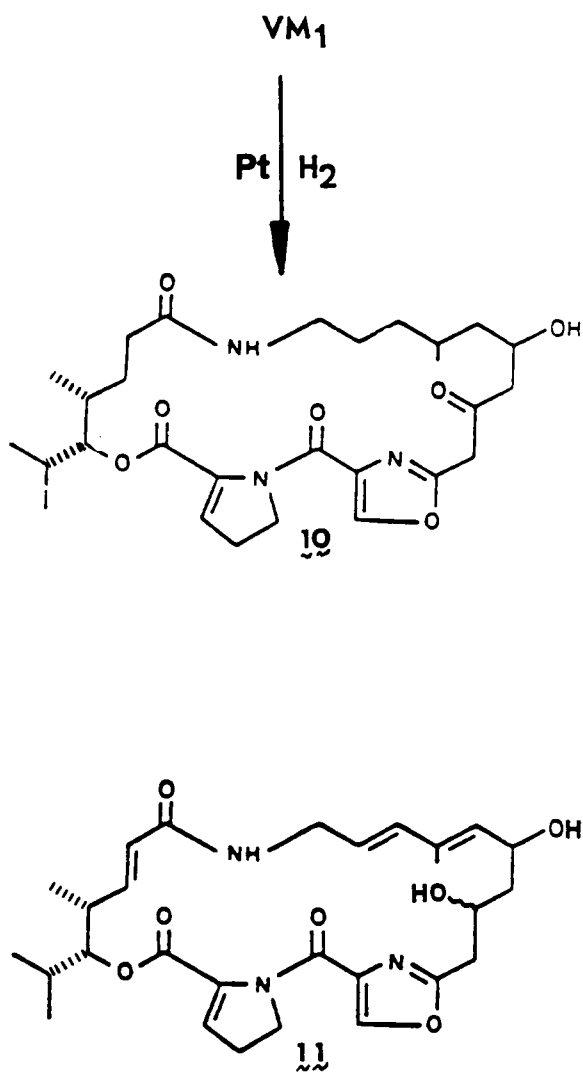
To study the binding of virginiamycin M₁ to ribosomal subunits, tritiated derivatives have been prepared by partial hydrogenation to the dihydro analog 9.^{57,58} (Figure 7) This dihydro analog 9 retains its biological activity.

Hydrogenation of VM₁ provides non-biologically active derivatives; complete hydrogenation of the ethylenic functions to the hexahydro derivative 10 causes a dramatic loss of biological activity (Figure 8).⁵⁹

Reduction has also been carried out with NaBH₄ to reduce the carbonyl at carbon 16 yielding the α and β diols of 11.⁶⁰ These alcohols have an increased affinity for bacterial ribosomes.

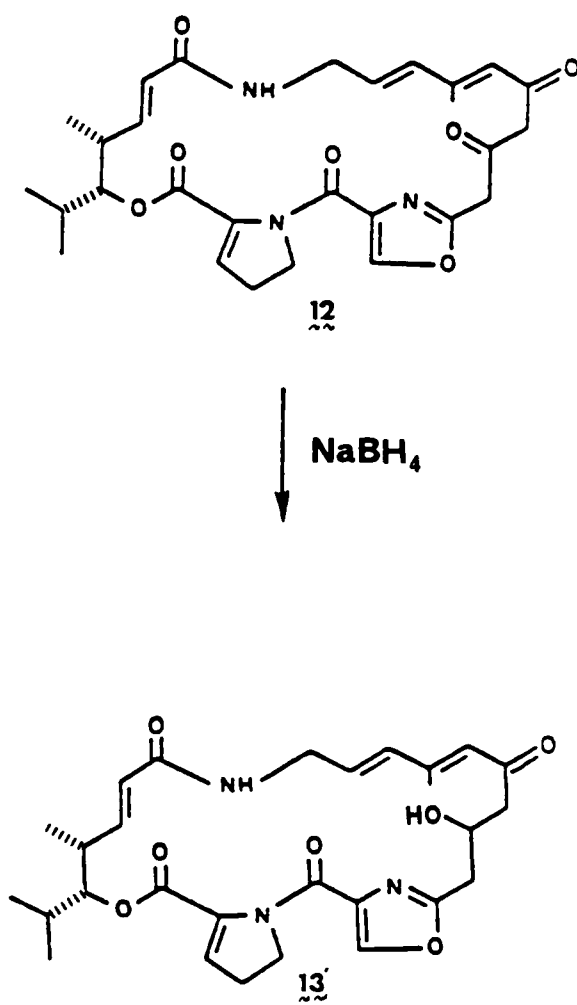
The allylic alcohol at carbon 14 of virginiamycin M₁ can be oxidized by 2,2 dichloro-5,6-dicyanobenzoquinone (DDQ) to give the diketone 12 (Scheme I) which is not biologically active.⁵⁴ Reduction of this diketone with NaBH₄ gave the keto-alcohol 13 which was also without antibiotic activity. (Scheme I) These results with the activity of the diol 11 confirm the importance of the allylic alcohol to the antibiotic activity of virginiamycin M₁.

Other derivatives are the Michael adducts from the treatment of VM₁ with alkylthio, alkylamino, and dialkylamino compounds in methanol, methylene chloride solution (Figure 9).⁶¹ These derivatives are anti-bacterial agents and synergistic but further details of their activity are unavailable.



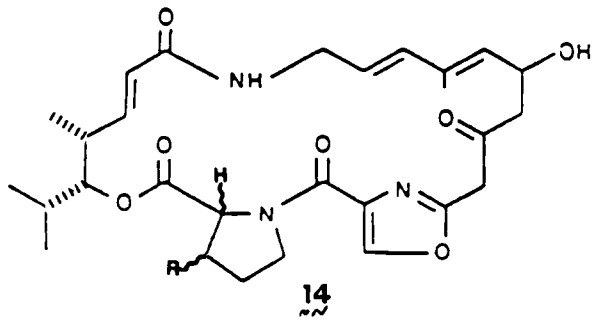
Partially Hydrogenated VM₁ Skeleton 10 and VM₁ α,β Diols 11

Figure 8

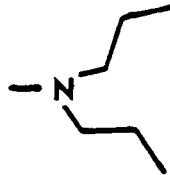
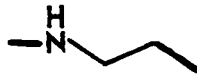


Reduction of Carbonyl Groups of Virginiamycin M_1 Diketone

Scheme I



R:



Products from Michael Addition to VM₁

Figure 9

E. Biosynthesis

The first biosynthetic studies were carried out by Roberfroid and Dumont in 1967; these workers showed that several carbon-14 labeled precursors were incorporated into VM₁, but they did not determine the location of the radiolabel in the resulting antibiotic⁶² (Table I). This work was repeated by Kingston and Kolpak and extended by the incorporation of additional precursors including proline, serine and valine.¹⁰ Table I summarizes these experiments.

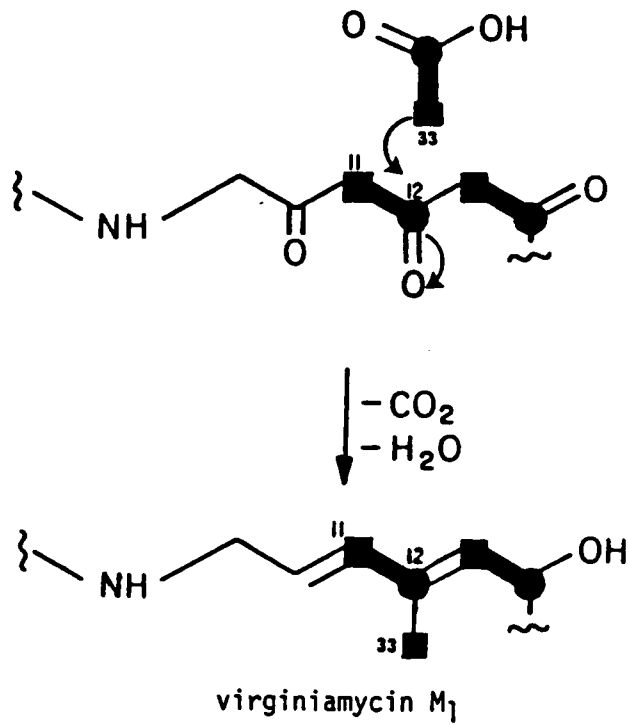
To determine the fate of the carbon 14 precursors within the VM₁ skeleton, incorporation of precursors labeled with stable isotopes were carried out using [1-¹³C]acetate, [2-¹³C]acetate, [1,2-¹³C₂]acetate, (S)-[methyl-¹³C]methionine and (RS)-[3-¹³C]serine.⁸ Analysis of the purified VM₁ from the feeding experiments by carbon-13 NMR gave a clear picture of the basic biosynthesis of this antibiotic.

Incorporation of [1-¹³C]acetate shows enrichments at carbons 5, 7, 12, 14, 16, and 18 are derived from the carbonyl carbon of acetate. The [2-¹³C]acetate experiment showed enrichment at carbons 4, 6, 11, 13, 15, 17, and 33 from the methyl carbon of acetate. The enrichment at carbon 33 suggested a novel pathway in the formation of the skeleton. This novel pathway was elucidated further by feeding [2-¹³C, ²H₃]acetate, [1,2-¹³C₂]acetate and (RS)-[3-¹³C]serine. The [1,2-¹³C₂]acetate feeding showed carbon-carbon couplings between carbons 6 and 7, 11 and 12, 13 and 14, 15 and 16, and 17 and 18 but none to carbon 33, while the (RS)-[3-¹³C]serine provided enrichment at carbons 4, 6, 11, 13, 15, 17, 20, 32, 31, and 33. This result proved serine to be the precursor to the oxazole ring as well as an active metabolite to other portions of VM₁,

Table I
 Incorporation of ^3H and ^{14}C Labeled Precursors into
 Virginiamycin M_1

Precursors	% Incorporation	
	<u>Kingston⁸</u>	<u>Dumont⁶²</u>
Sodium [2- ^{14}C]acetate	5.0	5.5, 3.4
Sodium [2- ^{14}C]malonate		0.4
(RS)-[2- ^{14}C]mevalonolactone	*	0.04
(S)-[$^{14}\text{CH}_3$]methionine	4.0	4.5, 5.7
(RS)-[3- ^{14}C]serine	1.4	
(S)-[3,4- $^3\text{H}_2$]proline	5.0	
[2- ^{14}C]glycine		0.4, 0.8
(S)-[U- ^{14}C]alanine		*
D-[U- ^{14}C]glucose	0.6	
(RS)-[3,4- $^{14}\text{C}_2$]glutamic acid		*
(S)-[1- ^{14}C]leucine		*
(S)-[U- ^{14}C]valine	15.2	

*Not detectable



Novel Formation of VM₁ Skeleton Methyl Functionality

Scheme II

probably by catabolism to acetyl Co-A by the pyruvate pathway, and confirmed the overall pathway to carbon 33 as shown in Scheme II.

Carbon-13 from (S)-[methyl-¹³C]methionine showed that carbon 32 arises from the methyl group of methionine, presumably by alkylation of the methylene group at carbon 4 with S-adenosylmethionine.

Further biosynthetic studies performed by Kingston and LeFevre⁸ involved incorporation of (RS)-[2-¹³C]valine, [1-¹³C]glycine, and (S)-[U-¹⁴C]proline.

The feeding of (RS)-[2-¹³C]valine supported the hypothesis that valine served as the starting unit for the biosynthesis at carbons 30,31 of the western portion of VM₁, as an enrichment of 16% was observed at carbon 3. Presumably, this occurs by transamination of valine to β-oxoisovaleric acid and then conversion to isobutyryl-coenzyme A by oxidative decarboxylation.

Incorporation of [1-¹³C]glycine led to enrichments at carbons 22 and 10. Enrichment at carbon 10 confirmed that nitrogen 8, carbon 9 and carbon 10 units were derived from glycine, while enrichment at carbon 22 can be explained by the conversion of glycine to serine by the tetrahydrofolate pathway and subsequent incorporation of serine as previously observed.

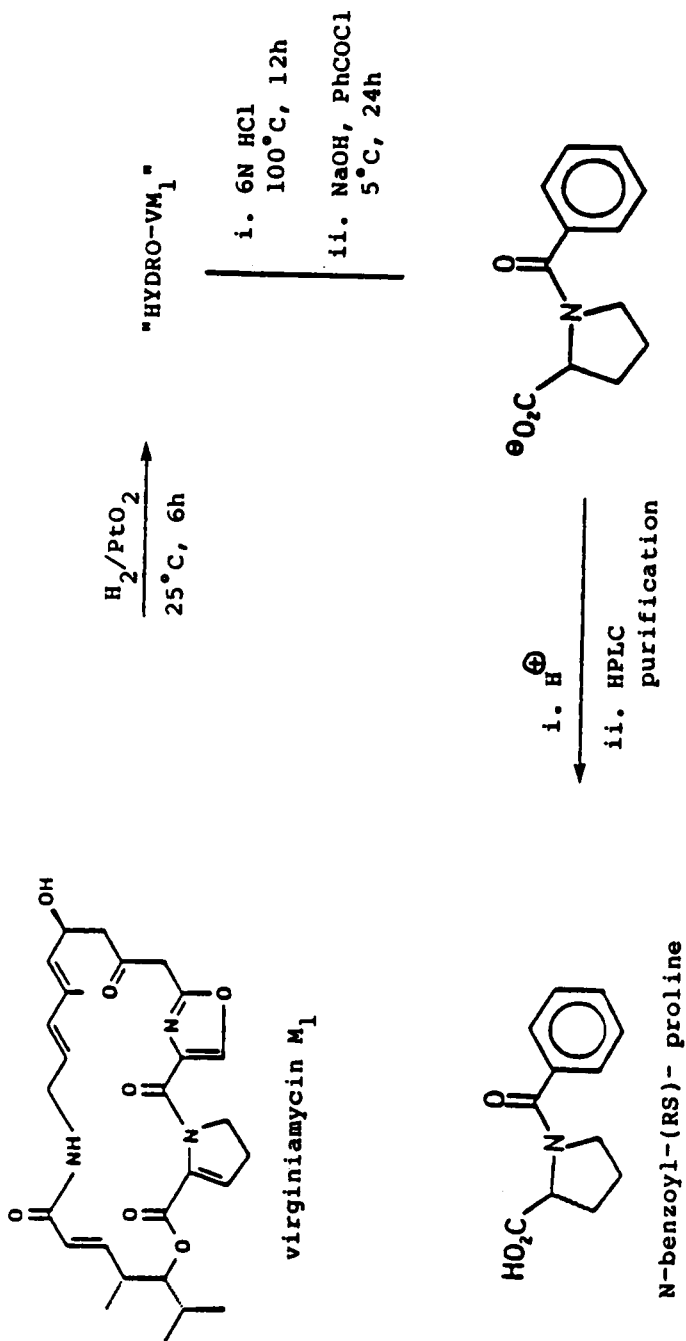
To examine the formation of the dehydroproline moiety, (2S)-[U-¹⁴C]proline was fed and the resulting antibiotic was hydrogenated and hydrolyzed to yield (RS)-proline, which was isolated as its N-benzoyl derivative (Scheme III).⁸ This proved that the dehydroproline moiety is formed from proline.

Both cis and trans-3-hydroxyproline were also tested as precursors, but neither one was found to be incorporated efficiently into VM₁; the result for cis-3-hydroxyproline was complicated by the presence of impurities in the labeled sample used. These data are given in Table II.

A result from the feeding of (S)-[3,4-³H₂]proline was useful as it provided the basis for the question of stereoselectivity in the formation of the dehydroproline moiety. Tritium NMR of the (2S)-[3,4-³H₂]proline precursor characterized the major stereoisomer to be the (2S,3S,4R) isomer 15 (Figure 10).⁶³ This isomer, when fed with (2S)-[U-¹⁴C]proline as an internal standard, had an initial ³H to ¹⁴C ratio of 6.8. The N-benzoylproline from the resulting VM₁ possessed a ³H to ¹⁴C ratio of 7.5, indicating no loss of tritium in the formation of the dehydroproline moiety.⁶⁴ Table II shows the results from this proline incorporation and Figure 11 is a synopsis of all biosynthetic results.

F. Objectives of This Work

The objectives of this work are to: determine the pathway for the formation of the oxazole ring and the dehydroproline ring in virginiamycin M₁. In light of these objectives a general review of the steps used in biosynthetic studies is in order. The steps are:



Isolation of the Dehydroproline Moiety as N-Benzoyl-(RS)-proline

Scheme III

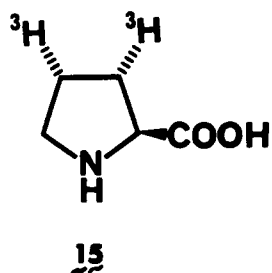
Table II

Incorporation of Labeled Prolines into Virginiamycin M₁

	³ H/ ¹³ C (precursor)	³ H/ ¹⁴ C N-benzoyl- (R,S)-proline	Incorp. &	³ H & retained	¹⁴ C & retained
(3S,4R,2S)-[3,4- ³ H ₂]-proline	6.8	7.5	---	100	---
(S)-[U- ¹⁴ C]proline					
(2S)-[U- ¹⁴ C]proline					
(S)-[3,4- ³ H ₂]proline	5.4	ND*	---	---	1.4
trans-3-hydroxy-(RS)-[U- ¹⁴ C]proline					
(S)-[3,4- ³ H ₂]proline	15.4	97.7	---	---	15.7
cis-3-methoxy-(RS)-[U- ¹⁴ C]proline + cis-3-hydroxy-(RS)-[U- ¹⁴ C]proline					

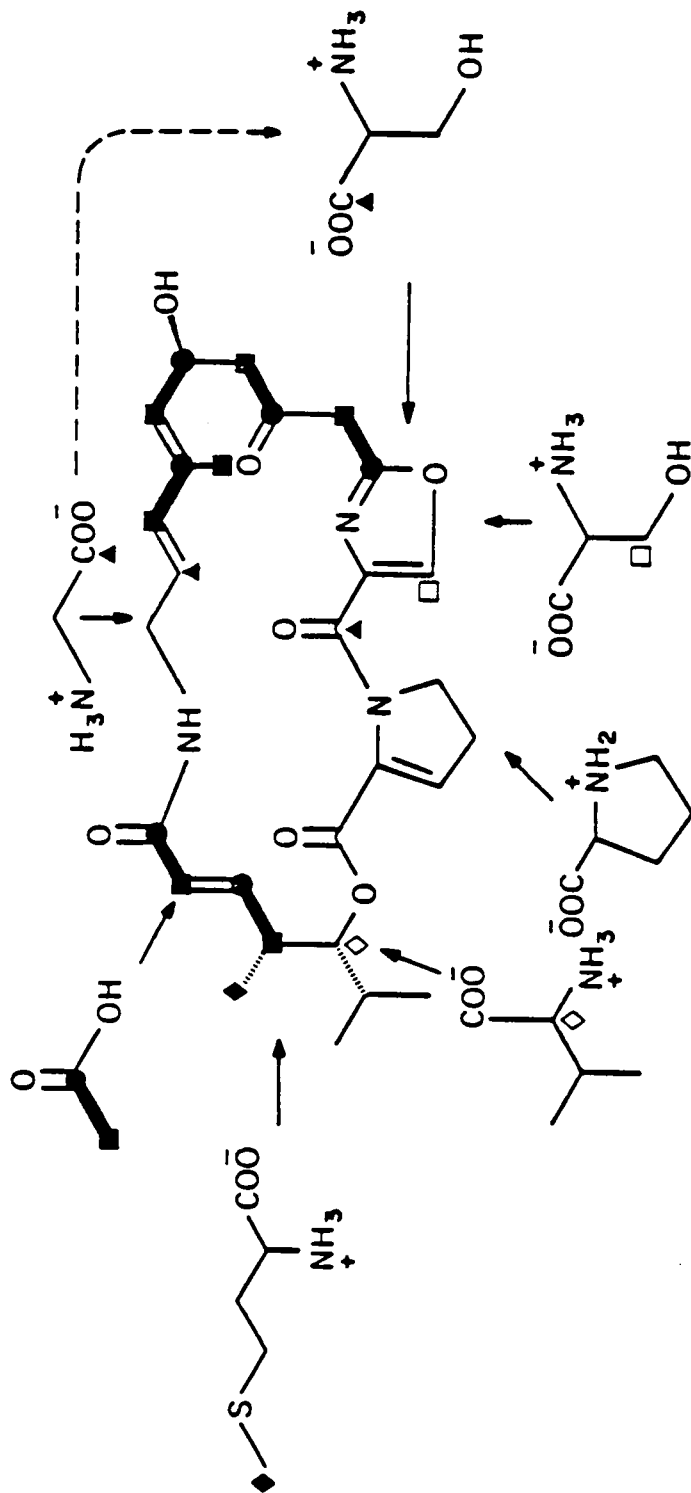
ND = not determined

* Ratio was >400 in isolated virginiamycin M₁



(2S,3S,4R)-[3,4-³H₂]Proline

Figure 10



Summary of Virginiaamycin M₁ Biosynthesis

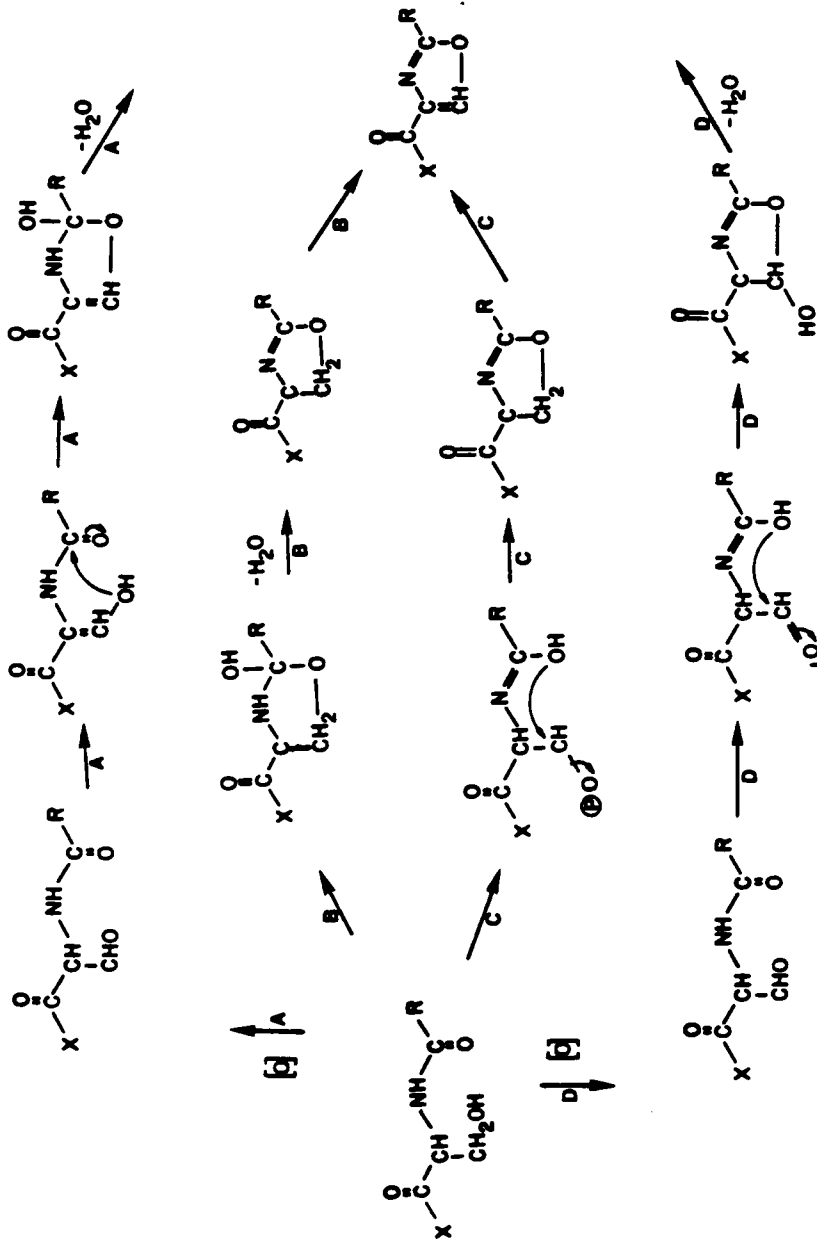
Figure 11

1. Growth of the producing microbe to obtain adequate production of metabolite.
2. Development of an isolation procedure for the metabolite.
3. Assignment of ^1H and ^{13}C NMR data for the metabolite.
4. Feeding appropriate isotopically labeled precursors.
5. Synthesis and incorporation of modified precursors.
6. Stereochemical studies.
7. Proposal of a plausible biosynthetic pathway.

This dissertation discusses the last four steps (4-7) to examine the two biosynthetic questions outlined in Chapter I. Therefore, modified precursors were synthesized using both stable and radioactive isotopes to perform stereochemical investigations which provide information for plausible biosynthetic pathways.

The two biosynthetic questions addressed can be viewed as studies of dehydroamino acid formation. The origin of dehydroamino acids in natural products has been a subject of much speculation,^{66,67} as suggestions of tautomerization of an acylimino intermediate,⁶⁶ dehydration of hydroxy amino acids, and dehydrogenation have been made.⁶⁸

The first question studied was the nature and stereochemistry of formation of the oxazole ring. The stereochemical question was investigated by feeding isotopically labeled serines. The stereochemical information obtained allows one to postulate a pathway for formation of the unsaturated amino acid, thus helping to differentiate between the possible cyclization pathways



Hypothetical Pathways for Oxazole Ring Closure

Scheme IV

From previous biosynthetic studies by Kingston's^{9,10} group, an acyl serine unit was postulated as the immediate precursor of the oxazole ring. There are four theoretically possible pathways for oxazole ring formation from such a precursor, and these are shown in Scheme IV. Two pathways (A & B) involve the oxygen atom of serine acting as a nucleophile, with this oxygen being retained in the ring. The other two pathways involve the oxygen of the acyl group acting as a nucleophile, with this oxygen retained in the ring. Thus, addition to studies of the stereochemistry of cyclization, a study of the regiochemistry of cyclization was also attempted.

The stereochemistry of cyclization was studied with the aid of the following feeding experiments:

Experiment	Precursor	Objective
1	(S)-[G- ³ H]serine <hr/> (S)-[U- ¹⁴ C]serine	To determine if a total loss of ³ H occurs.
2	(S)-[G- ³ H]serine <hr/> (RS)-[carboxyl- ¹⁴ C]serine	To determine if (R)-serine is incorporated specifically.
3	(2S,3R)-[3- ³ H]serine <hr/> (S)-[U- ¹⁴ C]serine	To determine if the pro-R hydrogen is retained.
4	(2S,3S)-[3- ³ H]serine <hr/> (S)-[U- ¹⁴ C]serine	To determine if the pro-S hydrogen is retained.

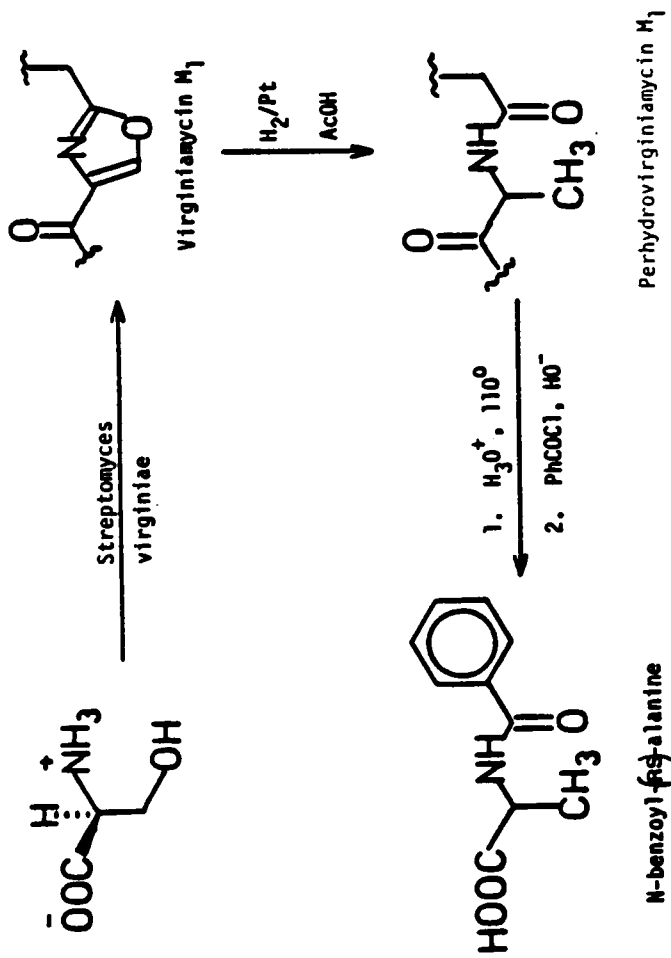
In each of the experiments a ³H to ¹⁴C ratio was determined for the precursor, thereby using one of the radioactive compounds as an internal standard.⁶⁸ The virginiamycin M₁ was purified from the fermentation medium, hydrogenated, and hydrolyzed, and the serine portion of the

oxazole moiety isolated as N-benzoylalanine (Scheme V). A ^3H to ^{14}C ratio was obtained for the isolated N-benzoylalanine and compared to the precursor ratio.

The second question investigated was the stereochemistry of formation of the dehydroproline ring. For this, earlier experiments by Kolpak and LeFevre showed that (S)-[U- ^{14}C]proline and (2S,3S,4R)-[3,4- $^3\text{H}_2$]proline were incorporated into virginiamycin M_1 .^{8,64} For the latter experiment all the tritium was retained, indicating stereospecificity in the formation of the dehydroproline moiety. To complete the stereochemical studies, (2S,3R)-[3- ^3H]proline was synthesized and the feeding experiments below were carried out.

Experiment	Precursor	Objective
1	(2R,3S,4R)-[3-4 $^3\text{H}_2$]proline <u>(RS)-[^{14}C-carboxyl]proline</u>	To determine if (2R)-proline is incorporated.
2	(2S,3R)-[3- ^3H]proline <u>(S)-[U-^{14}C]proline</u>	To determine if the pro-R hydrogen is retained.

As with the serine experiments, a ^3H to ^{14}C ratio was obtained for the proline precursors. The virginiamycin M_1 was purified, hydrogenated, and hydrolyzed, and the dehydroproline unit isolated as N-benzoylproline (Scheme III). The ^3H to ^{14}C ratio was determined for the N-benzoylproline and compared to the precursors ratio.



Isolation of the Serine Portion of the Oxazole

Ring as N-Benzoyl-(RS)-alanine

Scheme V

In summary, this portion of the dissertation describes stereochemical studies to provide details of dehydroamino acid formation in virginiamycin M₁.

CHAPTER III.

EXPERIMENTAL

A. General

Melting points were determined on a Kofler block and are uncorrected. Samples for liquid scintillation counting were evaporated in vacuo and dried in a vacuum desiccator for several hours to remove trace amounts of solvents that might cause quenching. (Evaporation in vacuo refers to solvent removal on a rotary evaporator at aspirator pressure and 25-45°C) Counting was carried out on Beckman LS 100 and LS 3800 liquid scintillation counters using a xylene emulsifier cocktail containing 0.3% PPO and 0.02% POPOP or a Beckman Ready Solv^R cocktail.

The Streptomyces virginiae were grown in baffled 250 ml wide mouth Erlenmeyer flask on a Lab-line orbit shaker or in a 2 liter Multigen fermenter fitted with a pH monitor.

¹⁴C and ³H-labeled compounds were obtained from ICN Chemical and Radioisotope Division, New England Nuclear, Research Products International, and from Amersham Corp. The (3R,2S)-[3-³H]serine and (3S,2S)-[3-³H]serine were obtained from Dr. Heinz G. Floss at Ohio State University. ⁶⁹ ¹³C labeled sodium cyanide (99% ¹³C enrichment) was obtained from Cambridge Isotope Laboratories and H₂¹⁸O (95-99% ¹⁸O enrichment) was obtained from the Mound Facility, Monsanto Research Corporation. SmithKline Animal Health Products supplied crude virginiamycin mixtures along with strains of S. virginiae PDT30 and 1830. Peanut oil cake was supplied by Southern of Rocky Mount, North Carolina.

B. Spectra

The ^{13}C -NMR spectra were taken at ambient temperature in either CDCl_3 , D_2O or d_6 -DMSO solutions using 5 mm spinning tubes on a Bruker WP-270-SY spectrometer operating at 67.3 MHz. For proton noise decoupled spectra (pnd), all protons were decoupled by gated heteronuclear decoupling and broad band irradiation. In order to observe ^{13}C - ^{18}O isotopic shifts in high resolution, the sweep widths were narrowed, usually to less than 10,000 Hz.

Proton NMR spectra were taken at ambient temperature in either CDCl_3 , D_2O or d_6 -DMSO solutions using an EM 390 90-MHz spectrometer, a Bruker WP-80-SY or a Bruker WP-270-SY, 270 MHz spectrometer. Chemical shifts are given in parts per million with reference to an internal tetramethylsilane (TMS) standard for protons and the ^{13}C signal of the solvent for ^{13}C spectra. The assignment of both ^1H and ^{13}C resonances were made based on common shift data unless otherwise referenced.

Infrared (IR) spectra were taken on a Perkin-Elmer 710B infrared spectrometer. Ultraviolet (UV) spectra were taken on a Perkin Elmer 530 UV-visible spectrometer.

Optical rotations were observed on a Perkin-Elmer 241 polarimeter.

Mass spectra were obtained on a Finnigan-MAT 112 mass spectrometer or a VG Analytical 7070E mass spectrometer.

C. Chromatography

High performance liquid chromatographic (HPLC) separations were performed on a system composed of a Waters Associates M6000A pump, a Valco six-port injection valve and a Waters Associate M441 UV detector

operating at 254 nm. The preparative reverse phase columns used were a Hibar column packed with LiChrosorb RP-8 (250 x 10 mm) and a Dynamax macro C-18 columns (300 x 10 mm). In the case of analytical columns a Waters RCM-100 module was used with Novapak C-8, (8 x 100 mm) or (5 x 100 mm) and Novapak C-18 (8 x 100 mm) or (5 x 100 mm) columns.

Thin-layer chromatography (TLC) was carried out on Merck silica gel 60F-254 (0.2 mm thickness).

Column chromatography was performed using Merck silica gel 60, (particle size 0.040-0.063 mm 230-400 mesh). Column sizes varied and are specified in the pertinent experimental sections.

Ion-exchange chromatography was performed using Dowex 50W resin in the sodium form. The strongly acidic cation exchange resin is a 200-400 dry mesh with 8% cross linkage.

Paper chromatography was performed using Whatman #1 paper in both ascending and descending modes.

D. Culture Conditions for Production of Virginiamycin M₁ and M₂

1. Slant Storage of Microbe

A strain of S. virginiae PDT 30 and S. virginiae 1830 (supplied by SmithKline and French) was maintained on potato dextrose agar (PDA) slants by J. W. LeFevre and J. W. Reed. These slants were prepared by boiling 200g of potatoes for 30 min. and filtering the extract through cheese cloth. Glucose (10g) and agar (20g) were added to the extract. Distilled water was added to raise the volume to 1000 ml, and the pH adjusted to 7.4 with 1N KOH. After sterilization by autoclave, the slants were inoculated with 0.2 ml of spore suspension from a S.

virginiae pellet and incubated for 6 days at 25-28°C.

2. Seed Cultures

To provide a population of the producing microbes cells, a seed culture medium, STA-2⁶⁴ was prepared with a spore suspension from a PDA slant. The seed medium was prepared as follows: 20g of corn steep solids (Sigma Chemical Co.) were added to 1000 ml of tap water. The pH was adjusted to 7.5 with 1N NaOH and 8g of peanut oil cake was added. The suspension was boiled for ~2 min and filtered to remove the peanut oil cake residue. To the filtrate was added 50g of dextrose, 0.01 g of $\text{MnSO}_2 \cdot 7\text{H}_2\text{O}$, and 5g of CaCO_3 . The medium was dispensed in 30 ml aliquots to 250 ml baffled wide mouth Erlenmeyer flask fitted with cotton plugs. The medium was sterilized in an autoclave at >15 psi for a minimum of 30 minutes. Each flask was inoculated with the spore suspension from one or two PDA slants and incubated at 25-27°C on a Lab-Line Orbit Environ-Shaker at 325 rpm for 48 hrs.

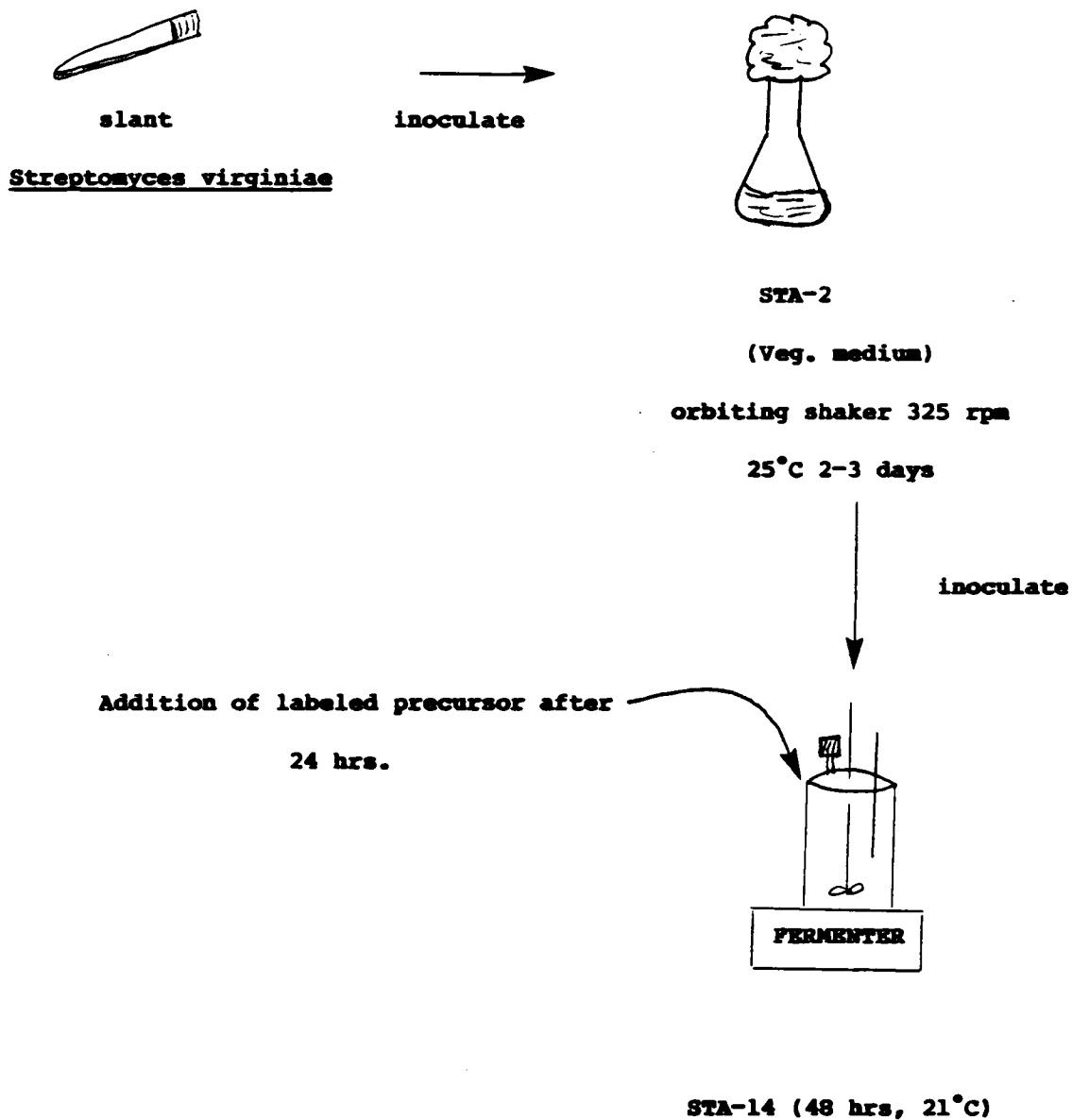
3. Virginiamycin M₁ and M₂ Production in Fermenter with STA-14 Medium

To maximize VM₁ production, the STA-14⁶⁴ production medium was used and the incubation carried out in a Multigen 2 liter fermenter. The STA-14 was prepared as follows: 20g of corn steep solids (Sigma Chemical Co.) and 5g of yeast autolyzate were added to 1000 ml of tap water. The pH was adjusted to 7.9 with NaOH if the pH was lower and with HCl if the pH was higher. Peanut oil cake (10g) was added and the suspension boiled for 2 minutes. To the filtrate was added 10g of linseed oil, 5g of dextrose, 25g of glycerol and 5g of CaCO_3 . The

solution was placed in the 2 liter glass fermentation vessel and sterilized at >15 psi for a minimum of 45 minutes. The medium cooled to room temperature and was fitted with a sterile pH electrode to monitor pH changes. (Post sterilization pH was 7.5-7.9). The fermenter was inoculated with 1 flask (30 ml) of STA-2 seed culture. The temperature was controlled at 21°C with a recirculating water bath cooled by a freeze dryer refrigeration unit. Air flow was maintained at 1-1.25 l/l/min. and an agitation rate of 400-500 rpm. The pH was adjusted to 7.0-7.5 throughout the fermentation time of 48 hours. The pH at the end of the fermentation varied from 6.5 to 7.5. Antifoam agents of linseed oil and Sigma's antifoam A emulsion were used as needed. Fermentation yielded 1-30 mg/liter of VM₁ after purification.

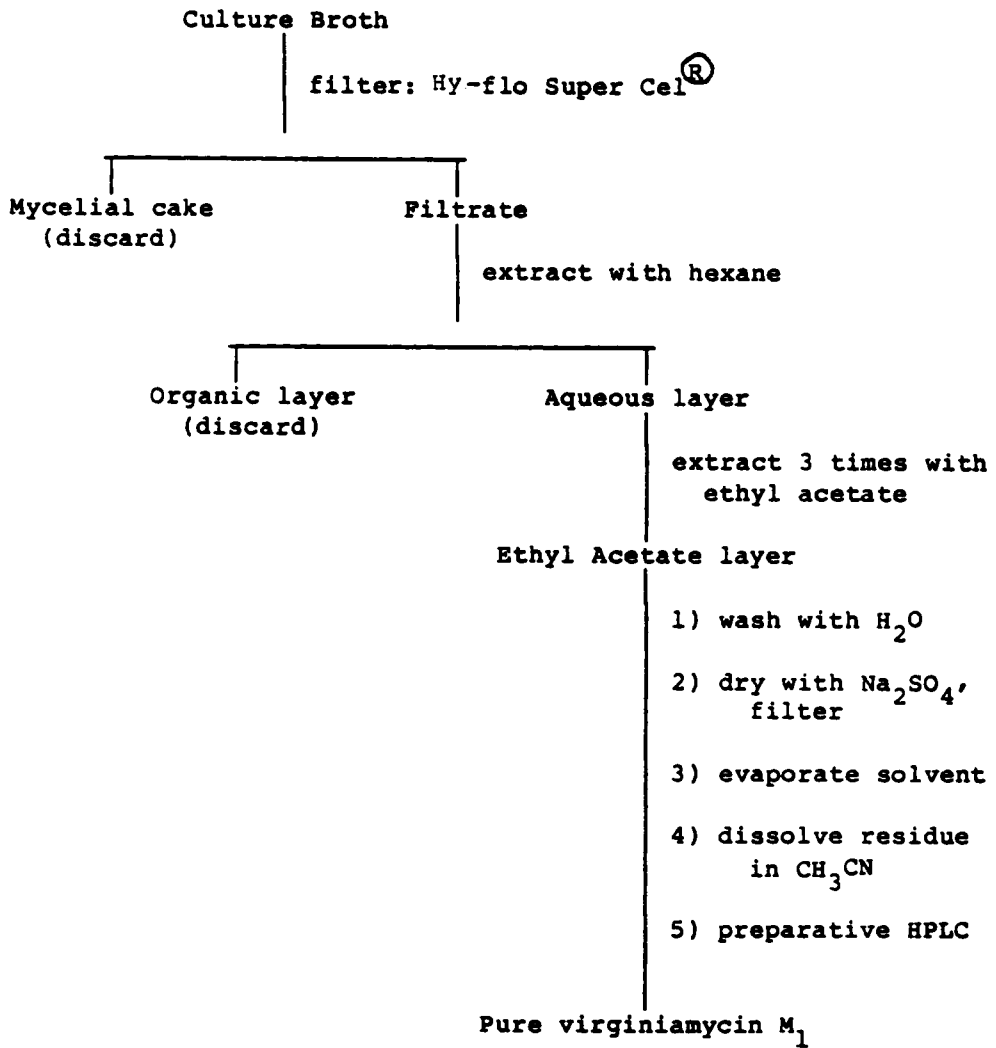
All ³H, ¹⁴C and ¹³C feeding experiments were carried out in the fermenter with addition of labeled precursors at 24 hours after inoculation with STA-2 seed culture. The labeled compounds were filtered through Millipore filters and all fermentation equipment, including air filters and electrodes, were sterile. All inoculations and precursor additions were performed using aseptic techniques (Scheme VI).

The virginiamycins were also produced by SmithKline-RIT Laboratories using a fermentation process. Labeled precursors were usually added early in the fermentation cycle, sometimes at the inoculation stage. The crude virginiamycins were isolated after filtration of the culture broth and extraction of the aqueous solution with isobutyl methyl ketone. The crude virginiamycin was precipitated by the addition of hexanes to yield a brown mixture of crude antibiotics.



Scheme of Virginiamycin M₁ Production and Feeding Experiments

Scheme VI



Isolation of Virginiamycin M₁

Scheme VII

E. Isolation of Antibiotics

1. Virginiamycin M₁

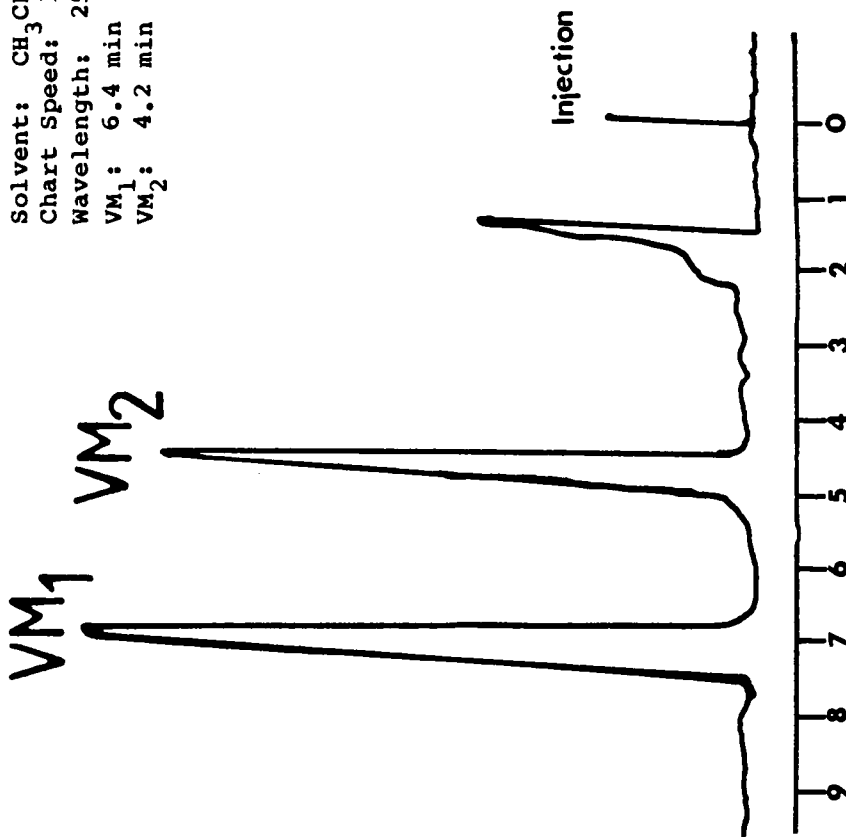
Virginiamycin M₁ is isolated from the STA-14 broth after 48 hours of growth. The procedure (Scheme VII) is as follows: the culture broth is filtered through Hy-flo-Super-Cel[®] to remove the mycelial bodies. The filtrate was then extracted two times with hexane (1/4 volume) and the hexane layer discarded. The aqueous layer was then extracted three times with ethyl acetate (1/2 volume). The combined extracts were washed with an equal volume of distilled water, dried with anhydrous Na₂SO₄, filtered and evaporated in vacuo to leave a yellow residue of crude virginiamycin M₁. The crude virginiamycin M₁ was then purified by HPLC using the equipment described in Section C. The HPLC solvent system was CH₃CN/H₂O 40/60 (vol/vol) with a flow rate of 6.0 ml/min.

Quantitation of the VM₁ was carried out by analytical HPLC using a solvent of CH₃CN-H₂O 40/60 with a flow rate of 2.0 ml/min. Figure 12 is a typical chromatography of crude virginiamycin M₁. Yields of pure virginiamycin ranged from 1-30 mg/liter of media. The virginiamycin M₁ was identified by chromatography against a standard and by comparison with published ¹H NMR data.² The purity of the antibiotic was routinely >99%.

2. Virginiamycin M₂

Virginiamycin M₂ was isolated during preparative HPLC of crude virginiamycin from the fermentation growth. The chromatography in Figure 12 shows the elution of virginiamycin M₂. Virginiamycin M₂ was identified by comparison with published ¹H NMR data.⁵ Yield 1-30 mg/liter. The purity of the antibiotic was routinely >99%.

Column: Novapak Radial-Pak RP-8
Flow Rate: 2 ml/min
Solvent: CH₃CN/H₂O 40/60
Chart Speed: 1 cm/min
Wavelength: 254 nm
VM₁: 6.4 min
VM₂: 4.2 min



HPLC Chromatography of Virginiamycin M₁ and Virginiamycin M₂

Figure 12

F. Incorporation of Precursors Containing Stable Isotopes into Virginiamycin M₁

1. General Procedure

One or two slants of S. virginiae PDT 30 were used to inoculate a vegetative medium (STA-2) as described earlier. After 48 hours at 25-27°C the STA-2 was used to inoculate one liter of STA-14 in a fermenter. After 24 hours of growth in the fermenter at 21-22°C, and agitation of 450 rpm and aeration of 1.75 l/l/min., 100-200 mg of the labeled compound were added to the fermenter using aseptic technique. Growth of the microbes was continued for an additional 24 hours and then the pure antibiotic was isolated as described earlier in Section E.

2. Incorporation of Sodium [1-¹³C, ¹⁸O₂]Acetate

One liter of STA-14 (production medium) in a fermenter was inoculated with STA-2 seed culture (35 ml). After 24 hours of growth, 0.245 g (3.91 mmol) of sodium [1-¹³C, ¹⁸O₂]acetate (prepared as described in section H.1) in 1 ml of sterile water was then added to the fermenter using aseptic techniques. The incubation was continued for an additional 24 hours with aeration of 1.5 l/l/min, agitation of 480-530 rpm and the pH adjusted manually to 6.8 every hour for the next six hours using 1N NaOH. The virginiamycin M₁ was isolated as in section E.1 to give 12 mg (2.6 mmol) of the antibiotic (dry weight). The ¹³C NMR spectra of the sample showed ¹³C enrichments as reported by LeFevre and Kolpak.⁸ However, decomposition occurred before adequate resolution was obtained due to the heat of the proton decoupler. The experiment was repeated by feeding 0.200 g (3.13 mmol) of sodium

[1-¹³C, ¹⁸O₂]acetate. Only 2 mg of VM₁ were obtained so the feeding experiment was repeated by SmithKline-RIT. Sodium [1-¹³C, ¹⁸O₂]acetate (0.150 g, 1.80 mmol) was sent to Dr. Biot of SmithKline-RIT in Belgium, who carried out fermentation under the conditions described previously in Section D3. A total of 0.408g of crude virginiamycin was returned.

The crude virginiamycin was semi-purified by flash chromatography using 6 grams of silica gel 60 in a 10 mm id. column. The column was dry packed and eluted with methylene chloride. The crude virginiamycin (0.408g) was dissolved in 10 ml of methylene chloride (black solution) and placed on the column. The column was eluted by a gradient process collecting 10-15 ml fractions using 100 ml CH₂Cl₂, 100 ml of 1% CH₃OH in CH₂Cl₂, 100 ml CH₂Cl₂, 100 ml of 2% CH₃OH in CH₂Cl₂, 100 ml of 4% CH₃OH in CH₂Cl₂ 100 ml of 6% CH₃OH in CH₂Cl₂ to elute the fractions. Fractions shown by TLC to contain virginiamycin M₁ and virginiamycin M₂ were further purified by preparative HPLC as described in Section C. virginiamycin M₁ (7 mg, 1.5 x 10⁻¹ mmol) and virginiamycin M₂ (5 mg, 1.3 x 10⁻¹ mmol) were obtained. The quantities were determined by analytical HPLC and by the dry weight of the solid compounds. The ¹³C-NMR spectra of the purified virginiamycin was obtained by Michael Kolpak at SmithKline and French Chemicals. The sample was also subjected to E.I. and C.I. mass spectrometry.

G. Incorporation of ³H and ¹⁴C Labeled Precursors into Virginiamycin

M₁

1. General Procedure

One or two slants of S. virginiae PDT 30 were used to inoculate a

vegetative medium (STA-2) as described earlier. After 48 hours at 25-27°C the STA-2 was used to inoculate one liter of STA-14 in a fermenter. After 24 hours of growth in the fermenter at 21-22°C, agitation of 450 rpm and aeration of 1.25 l/l/min., mixtures of ^{14}C and ^3H labeled compounds were added to the fermenter using aseptic techniques. Growth was continued for an additional 24 hours and then the pure antibiotics were isolated as described earlier.

The labeled precursors for the experiment were prepared by mixing ^3H compounds with ^{14}C compounds to obtain a ^3H to ^{14}C ratio between 3/1 and 10/1 as required. After mixing, approximately 10% of the mixture was taken and mixed with a known amount of unlabeled precursor. The mixtures were then recrystallized until constant specific activity ratios of ^3H to ^{14}C were obtained.

The purified virginiamycin M_1 from each experiment was subjected to hydrogenation over PtO_2 , 25°C for 24 hrs in acetic acid solution (Section H). After hydrogenation the residue was hydrolyzed in 6N HCl at 100-110°C in a sealed vial for 24 hrs. The hydrolyzate was then made alkaline to pH 10 and treated with excess benzoyl chloride dissolved in diethyl ether for maximum production of N-benzoylalanine. To maximize the formation of N-benzoylproline the concentration was 1N NaOH. The N-benzoyl derivatives were isolated by acidification of the alkaline mixture after extraction with diethyl ether. The acidic solutions were then extracted with methylene chloride. The organic layers were washed with distilled water, dried with anhydrous Na_2SO_4 , filtered and evaporated to dryness. The crude residue was then dissolved in CH_2Cl_2 and purified by

normal phase HPLC. The purified N-benzoylproline or N-benzoylalanine was then combined with the appropriate unlabeled compound and recrystallized to a constant ^3H to ^{14}C ratio.

Internal standards of ^{14}C and ^3H were used to determine efficiency unless otherwise stated. The samples were counted in tritium/carbon-14, tritium and carbon-14 only channels.

^3H to ^{14}C ratios were calculated as follows:

$$\frac{^3\text{H}}{^{14}\text{C}} = \frac{\text{dpm of } ^3\text{H}}{\text{dpm of } ^{14}\text{C}}$$

$$^3\text{H}_{\text{dpm}} = \left[\left(^3\text{H}_{\text{cpm}} - \text{bk } ^3\text{H}^3\text{H} \right) - \left(^{14}\text{C}_{\text{dpm}} \times \text{eff } ^{14}\text{C}, ^3\text{H} \right) \right] / \text{eff. } ^3\text{H}^3\text{H}$$

$$^{14}\text{C}_{\text{dpm}} = \left[\left(^{14}\text{C}_{\text{cpm}} - \text{bk } ^{14}\text{C}^{14}\text{C} \right) - \left(^3\text{H}_{\text{cpm}} \text{ } ^3\text{H}, ^{14}\text{C} \right) / \text{eff } ^3\text{H}^{14}\text{C} \right] / \text{eff. } ^{14}\text{C}^{14}\text{C}$$

$$\text{specific activity} = \frac{\text{number of } \mu \text{ Ci in sample}}{\text{number of mmol of sample}}$$

$$\% \text{ Specific Incorporation} = \frac{\text{specific activity of degradation product}}{\text{specific activity of precursor}} \times 100$$

$$\% \text{ Incorporation} = \frac{\text{radioactivity in product}}{\text{radioactivity feed}}$$

2. Preparation of Quench Curve for Serine and N-Benzoylalanine

A quench curve was prepared for evaluation of efficiency in counting ^3H and ^{14}C labeled N-benzoylalanine and serine. Known amounts of unlabeled benzoylalanine and serine were weighed out and dissolved in

Table III

Quench Curve Data for N-Benzoylalanine and Serine

Sample	Amount(mg)	¹⁴ C hexadecane/		$\frac{\text{Eff.}}{^3\text{H}}$	$\frac{\text{Eff.}}{^3\text{H} \text{ } ^{14}\text{C}_{1/2}}$	$\frac{\text{Eff.}}{^3\text{H}_{1/2} \text{ } ^{14}\text{C}}$	$\frac{\text{Eff.}}{^3\text{H} \text{ } ^{14}\text{C}}$
		³ H hexadecane	$\frac{\text{Eff.}}{^3\text{H} \text{ } ^{14}\text{C}_{1/2}}$				
N-benzoyl-(RS)-alanine	0			12.58	62.48	28.56	92.56
N-benzoyl-(RS)-alanine	24.81	33.33/0		14.92	59.47	33.35	93.67
N-benzoyl-(RS)-alanine	37.74	24.81/0		15.72	58.00	34.49	93.90
N-benzoyl-(RS)-alanine	60.79	27.41/0		16.06	53.10	34.22	91.12
N-benzoyl-(RS)-alanine	77.58	35.15/0		16.71	51.14	36.42	91.57
N-benzoyl-(RS)-alanine	102.46	31.83/0		17.65	49.82	38.91	91.34
N-benzoyl-(RS)-alanine	0	0/39.91		39.91	0.0	43.52	44.21
N-benzoyl-(RS)-alanine	25.48	0/35.93		40.50	0.0	41.90	42.50
N-benzoyl-(RS)-alanine	36.87	0/31.08		38.88	0.0	40.41	41.17
N-benzoyl-(RS)-alanine	62.48	0/34.76		37.36	0.0	37.91	38.07
N-benzoyl-(RS)-alanine	84.58	0.35.86		36.85	0.0	37.25	37.00
N-benzoyl-(RS)-alanine	94.27	0.34.17		36.37	0.0	36.12	36.02
(RS)-serine	0	34.41/0		-----	50.28	39.49	94.05
(RS)-serine	24.00	30.29/0		-----	48.43	38.22	92.21
(RS)-serine	55.25	26.10/0		-----	50.26	39.27	90.75
(RS)-serine	80.25	29.00/0		-----	52.13	40.76	95.67
(RS)-serine	145.25	37.39/0		-----	-----	-----	-----
(RS)-serine	0	0/24.89		-----	0	35.70	84.09
(RS)-serine	51.30	0/34.15		-----	0	36.09	35.87
(RS)-serine	75.00	0/27.81		-----	0	35.27	35.38
(RS)-serine	117.84	0/23.7		-----	0	35.25	35.36
(RS)-serine	172.6	0/21.5		-----	-----	-----	-----

scintillation cocktail. To these mixtures were added ^3H or ^{14}C standards of n-hexadecane as Table III. The samples were counted in a Beckman LS 100 scintillation counter using four channels which were ^3H only, ^{14}C only, ^3H and ^{14}C and ^3H , ^{14}C , ^{32}P . The samples were counted 3 times to a 2% sigma value, 2-20 minutes.

3. Incorporation of (S)-[G- ^3H]Serine and (S)-[U- ^{14}C]Serine into Virginiamycin M₁

A slant of S. virginiae was used to inoculate 50 ml of STA-2 seed culture. After 48 hours at 25-27°C, the 30 ml of seed culture was used to inoculate 1 liter of STA-14 in a fermenter at 21°C, 500 rpm, pH of 7.15 and aeration of 1.25 l/l/min. After 24 hours of growth, 2.6 ml of a mixture of (S)-[G- ^3H]serine (100 μCi , 1.9 Ci/mmol) and (S)-[U- ^{14}C]serine (10 μCi , 155 mCi/mmol) (^3H to ^{14}C ratio 10.83 for the crude mixture) in 3.0 of sterile water were syringed into the fermenter using aseptic techniques. A 0.3 ml aliquot (~10%) of the mixture was retained for determination of the precursor ^3H to ^{14}C ratio. The 10% aliquot of the labeled serines was added to 1.0g of unlabeled (S)-serine and recrystallized 3 times from ethanol and water to get a constant specific activity ratio of 6.02 ± 0.3 . A quench curve was used for determination of counting efficiency.

After 48 hours of growth, the fermentation was stopped and crude virginiamycin was isolated and purified as in Section E to give 8.4 mg (1.8×10^{-5} mol) of virginiamycin M₁ (purity >95% as determined by analytical HPLC) ^3H to ^{14}C ratio of 7.24. The isolated antibiotics

contained 1.80 μCi ^3H (specific activity 112.3 $\mu\text{Ci}/\text{mmol}$) and 0.25 μCi ^{14}C (specific activity 15.57 $\mu\text{Ci}/\text{mmol}$). The % incorporation was 1.8% and 2.5% for ^3H and ^{14}C , respectively, while the % specific incorporations were $5.91 \times 10^{-3}\%$ and $1.0 \times 10^{-2}\%$.

2.18 mg of the pure virginiamycin M_1 was mixed with 2.36 mg of PtO_2 in 0.8 ml of glacial acetic acid and hydrogenated at room temperature and atmospheric pressure for 24 hours. The catalyst was removed by centrifugal filtration and the "perhydro VM_1 " was hydrolyzed by heating at 100-110°C in 1.0 ml of 6N HCl for 24 hours. The pH of the hydrolysis residue was adjusted to 10 and treated with 40 equivalents of benzoyl chloride in 1 ml of diethyl ether at 0°C for 2 hours. Following work up as in section G 1, 308 μg of pure N-benzoylalanine was obtained by preparative HPLC. Unlabeled N-benzoylalanine (0.500 g, 2.59 mmol) was added to the isolated product and recrystallized from ethyl acetate-hexane. The isolated diluted N-benzoylalanine contained 1.88×10^{-3} μCi ^3H (specific activity 7.2×10^{-4} $\mu\text{Ci}/\text{mmol}$) and 6.07×10^{-4} μCi ^{14}C (specific activity 2.34×10^{-4} $\mu\text{Ci}/\text{mmol}$); based on these data and the known dilution by cold unlabeled N-benzoylalanine, the undiluted N-benzoylalanine had a specific activity of 0.94 $\mu\text{Ci}/\text{mmol}$ for ^3H and 0.32 $\mu\text{Ci}/\text{mmol}$ for ^{14}C , to give a final constant specific activity ratio of 2.95 ± 0.15 . The % incorporation for the 7.9×10^{-3} and 8.3×10^{-3} while the % specific incorporation was $5.2 \times 10^{-5}\%$ and $2.06 \times 10^{-4}\%$, respectively, for ^3H and ^{14}C . A quench curve was used for determination of counting efficiency.

4. Incorporation of (S)-[G-³H]Serine and (RS)-[1-¹⁴C]Serine into Virginiamycin M₁

A slant of S. virginiae was used to inoculate 30 ml of STA-2 seed culture. After 48 hours at 24-27°C, the 30 ml of seed culture was used to inoculate 1 liter of STA-14 in a fermenter at 21°C, 450 rpm, pH of 6.85 and aeration of 1.5 l/l/min. After 24 hours of growth, 2.3 ml of mixture of (S)-[G-³H]serine (100 µCi, 1.9 Ci/mmol) and (RS)-[1-¹⁴C]serine (10 µCi, 56.3 mCi/mmol) (³H to ¹⁴C ratio of 6.6) in 1 ml of sterile water was syringed into the fermenter using aseptic techniques. A 0.33 ml aliquot (~10%) of the mixture was retained for determination of the precursor ³H to ¹⁴C ratio.

The 10% aliquot of labeled serines was added to 1.0 g of (RS) serine and recrystallized 6 times from ethanol and water to get a constant ratio (³H to ¹⁴C) of 4.82±0.16. A quench curve was used for determination of counting efficiency.

After 48 hours of growth, the fermentation was stopped and the crude virginiamycin was isolated and purified as described in Section E to give 9 mg (1.7 x 10⁻² mmol) of virginiamycin M₁ ³H to ¹⁴C ratio of 9.33 (purity was >95% by analytical HPLC). The isolated antibiotic contained 0.0665 µCi ³H (specific activity 3.88 µCi/mmol) and 7.13 x 10⁻³ µCi ¹⁴C (specific activity 0.415 µCi/mmol). The % incorporation was 6.7 x 10⁻²% and 7.3 x 10⁻³% for ³H and ¹⁴C, respectively, while the % specific incorporation was 2.04 x 10⁻⁴% and 7.37 x 10⁻⁴%.

The pure virginiamycin M₁ (8.0 mg) was combined with 7.3 mg of PtO₂ in 1.5 ml of glacial acetic acid and hydrogenated at room

temperature and atmospheric pressure for 24 hours. The catalyst was removed by centrifugal filtration and the "perhydro VM₁" was hydrolyzed by heating at 100-110°C in 1.0 ml of 6N HCl for 24 hours. The hydrolysis products were evaporated in vacuo and the pH adjusted to 10 followed by treatment with 40 equivalents of benzoyl chloride in diethyl ether at 0°C for 2 hours. Following work up as in Section G 1, 616 ug (3.2×10^{-3} mmol) of pure N-benzoylalanine was obtained by preparative HPLC. N-benzoylalanine (500 mg, 2.59 mmol) was added to the isolated products and recrystallized from ethyl acetate-hexane. The isolated diluted N-benzoylalanine contained 1.37×10^{-3} $\mu\text{Ci } ^3\text{H}$ (specific activity 5.28×10^{-4} $\mu\text{Ci}/\text{mmol}$) and 3.95×10^{-4} $\mu\text{Ci } ^{14}\text{C}$ (specific activity 1.53×10^{-4} $\mu\text{Ci}/\text{mmol}$); based on these data and the known dilution by unlabeled N-benzoylalanine, the undiluted N-benzoylalanine had a specific activity of 0.43 $\mu\text{Ci}/\text{mmol}$ for ^3H and 0.124 $\mu\text{Ci}/\text{mmol}$ for ^{14}C , to give a constant ^3H to ^{14}C ratio of 3.46 ± 0.13 . The % incorporation was $1.37 \times 10^{-3}\%$ and $3.95 \times 10^{-3}\%$ for ^3H and ^{14}C , respectively, while the % specific incorporation was $2.26 \times 10^{-5}\%$ and $2.20 \times 10^{-4}\%$. A quench curve was used for determination of counting efficiency.

5. Incorporation of (2S,3R)-[3-³H]Serine and (S)-[U-¹⁴C]Serine into Virginiamycin M₁ and M₂

A slant of S. virginiae was used to inoculate 30 ml of STA-2 seed culture. After 48 hours at 25-27°C, the 30 ml of seed culture was used to inoculate 1 liter of STA-14 in a fermenter at 21°C, 480 rpm, pH of 7.08 and aeration of 1.25 l/l/min. After 24 hours of growth 10 μCi of

(2S,3R)-[3-³H]serine (specific activity 100 mCi/mmol) obtained from Dr. Heinz Floss⁶⁹ was mixed with 1.1 μ Ci of (S)-[U-¹⁴C]serine (specific activity 145 mCi/mmol) in 1.0 ml of sterile water and added to the fermenter. (³H to ¹⁴C ratio of 5.34). An aliquot (~10%) of the mixture was retained for determination of the precursor ³H to ¹⁴C ratio. The 10% aliquot of the labeled serines was added to 0.604g of unlabeled (S)-serine and recrystallized 5 times from ethanol and water to get a constant specific activity ratio of 2.42 \pm 0.07. Efficiency was determined by using internal standards, 18.63 mg (2.18×10^{-2} μ Ci) of ³H n-hexadecane and 5.75 mg (2.92×10^{-3} μ Ci) of ¹⁴C n-hexadecane.

After 48 hours of growth, the fermentation was stopped and the crude virginiamycin was isolated and purified as in Section E to give 4.2 mg (7.97×10^{-3} mmol) of virginiamycin M₁ (purity >95% by analytical HPLC). The ³H to ¹⁴C ratio of the Virginiamycin M₁ was 2.81. The isolated antibiotic contained 3.41×10^{-4} μ Ci of ³H (specific activity 4.26×10^{-2} μ Ci/mmol) and 1.21×10^{-4} μ Ci ¹⁴C (specific activity 1.5×10^{-2} μ Ci/mmol). The % incorporation was 3.1×10^{-3} % and 1.1×10^{-2} % for ³H and ¹⁴C, respectively, and the % specific incorporation was 4.26×10^{-5} % and 1.03×10^{-5} %.

Virginiamycin M₂ (6.7 mg, 1.28×10^{-2} mmol) was isolated from the same growth medium and had a ³H to ¹⁴C ratio of 2.35 (5.79×10^{-3} μ Ci of ³H; specific activity 0.456 μ Ci/mmol and 2.46×10^{-3} μ Ci of ¹⁴C; specific activity 0.194 μ Ci/mmol). The % incorporations were 5.78×10^{-2} % and 2.238×10^{-3} % for ³H and ¹⁴C while the % specific incorporation was 4.56×10^{-4} % and 1.34×10^{-4} % for ³H and ¹⁴C, respectively.

Virginiamycin M₁ (4.2 mg) was mixed with 3.77 mg of unlabeled virginiamycin M₁ and combined with 10.1 mg of PtO₂ in 5 ml of glacial acetic acid. The mixture was hydrogenated at room temperature and atmospheric pressure. The catalyst was removed by centrifugal filtration and the "perhydro VM₁" was hydrolyzed by heating to 100-110°C in 0.5 ml of 6 N HCl for 24 hours. The hydrolysis products were evaporated in vacuo and the pH adjusted to 10 followed by treatment with 40 equivalents of benzoyl chloride in diethyl ether at 0°C for 2 hours. Following work up as in section G1, 1.06 mg (5.49×10^{-3} mmol) of pure n-benzoylalanine was obtained by preparative HPLC. N-benzoylalanine (242 mg, 1.25 mmol) was added to the isolated product. The N-benzoylalanine was recrystallized 5 times from ethyl acetate hexane. Due to a lack of radioactivity the degradation was repeated using virginiamycin M₂.

Virginiamycin M₂ (4.7 mg) were combined with 6.63 mg of PtO₂ in 1 ml of glacial acetic acid. The mixture was hydrogenated at room temperature and atmospheric pressure. The catalyst was removed by centrifuge filtration and the "perhydro VM₂" was hydrolyzed by heating to 100-110°C in 7.0 ml of 6N HCl for 24 hours. The hydrolysis solvent was evaporated in vacuo and the pH adjusted to 10 with 1N NaOH followed by treatment with 40 equivalents of benzoyl chloride in 1.5 ml of diethyl ether for 2 hours at 0°C. Following work up as in section G,1. 0.239 mg (1.23×10^{-3} mmol) of pure N-benzoylalanine was obtained by preparative HPLC. The pure N-benzoylalanine was added to 100 mg (5.08×10^{-2} mmol) of unlabeled N-benzoylalanine and recrystallized in ethyl

acetate-hexane. The isolated diluted N-benzoylalanine contained 1.2669×10^{-4} $\mu\text{Ci } ^3\text{H}$ (specific activity 2.49×10^{-4} $\mu\text{Ci}/\text{mmol}$) and 7.94×10^{-5} $\mu\text{Ci } ^{14}\text{C}$ (specific activity 1.56×10^{-4} $\mu\text{Ci}/\text{mmol}$); based on these data and the known dilution by unlabeled N-benzoylalanine, the undiluted N-benzoylalanine had a specific activity of $0.104 \mu\text{Ci}/\text{mmol } ^3\text{H}$ and $6.54 \times 10^{-2} \mu\text{Ci}/\text{mmol}$ for ^{14}C to give a final constant activity ^3H to ^{14}C ratio of 1.60 ± 0.05 . The % incorporation was $1.15 \times 10^{-3}\%$ and $7.22 \times 10^{-3}\%$ for ^3H and ^{14}C , respectively, while the % specific incorporation was $1.04 \times 10^{-4}\%$ and $4.51 \times 10^{-5}\%$. Internal standards of 11.74 mg (1.37×10^{-2} μCi) of ^3H n-hexadecane and 16.50 mg (8.3×10^{-3} μCi) of ^{14}C n-hexadecane were used.

6. Incorporation of (2S,3S)-[^3H]Serine and (S)-[U- ^{14}C]Serine into Virginiamycin M₁ and M₂

A slant of S.virginiae was used to inoculate 30 ml of STA-2 seed culture. After 48 hours at 25-27°C, the 30 ml of seed culture was used to inoculate 1 liter of STA-14 in a fermenter at 21°C, 475 rpm, pH of 6.78 and aeration of 1.25 l/l/min. After 24 hours of growth 10 μCi of (2S,3S)-[3- ^3H]serine (specific activity 100 mCi/mmol) obtained from Dr. Heinz Floss⁶⁹ was mixed with 1.1 μCi of (S)-[U- ^{14}C]serine (specific activity 145 mCi/mmol) (^3H to ^{14}C ratio of 6.5 ± 2) in 1 ml of sterile water were syringed into the fermenter using aseptic techniques. A 0.1 ml aliquot (~10%) of the mixture was retained for determination of the precursor ^3H to ^{14}C ratio.

The 10% aliquot of the labeled serines was added to 0.554g of

(RS)-serine and recrystallized 6 times from ethanol and water to get a constant specific activity ^3H to ^{14}C ratio of 3.32 ± 0.03 . Efficiency was determined by using internal standards, 3.85 mg (8.5×10^{-3} μCi) of ^3H n-hexadecane and 4.81 mg (2.4×10^{-3} μCi) of the ^{14}C n-hexadecane.

After 48 hours of growth, the fermentation was stopped and the crude virginiamycin was isolated and purified as in Section G to give virginiamycin M_1 (15 mg, 2.86×10^{-2} mmol) (purity was >95% by analytical HPLC). The ^3H to ^{14}C ratio for the Virginiamycin M_1 was 2.74. The isolated antibiotic contained 2.15×10^{-2} μCi of ^3H (specific activity 0.754 $\mu\text{Ci}/\text{mmol}$ and 7.86×10^{-3} μCi ^{14}C (specific activity 0.275 $\mu\text{Ci}/\text{mmol}$). The % incorporation was 0.195% and 0.715% for ^3H and ^{14}C , respectively, while the % specific incorporation was 7.54×10^{-4} and 1.89×10^{-4} .

Virginiamycin M_2 (33 mg, 6.2×10^{-2} mmol) was isolated from the same growth. The ^3H to ^{14}C ratio for the Virginiamycin M_2 was 2.68. The isolated antibiotic contained 0.354 μCi of ^3H (specific activity 5.65 $\mu\text{Ci}/\text{mmol}$) and 0.131 μCi ^{14}C (specific activity 2.09 $\mu\text{Ci}/\text{mmol}$). The % incorporation was 3.54% and 11.9% for ^3H and ^{14}C , respectively, while the % specific incorporation was 5.65×10^{-3} and 1.44×10^{-3} .

Virginiamycin M_1 (10.5 mg) was mixed with 10.1 mg of PtO_2 in 5 ml of glacial acetic acid. The mixture was hydrogenated at room temperature and atmospheric pressure. The catalyst was removed by centrifugal filtration and the "perhydro VM_1 " was hydrolyzed by heating to 100-110°C in 5 ml of 6N HCl for 24 hours.

The hydrolysis products were evaporated in vacuo and the pH adjusted to 10 followed by treatment with 40 equivalents of benzoyl chloride in diethyl ether at 0°C for 2 hours. Following workup as in Section G1, 7.8 mg (3.95×10^{-2} mmol) of pure N-benzoylalanine was obtained by preparative HPLC. N-benzoylalanine (310.15 mg, 1.57 mmol) was added to the isolated product and recrystallized. The isolated diluted N-benzoylalanine contained 2.29×10^{-4} μCi of ^3H (specific activity 1.45×10^{-4} $\mu\text{Ci}/\text{mmol}$) and 6.0×10^{-4} μCi ^{14}C (specific activity 3.82×10^{-4} $\mu\text{Ci}/\text{mmol}$); based on these data and the known dilution by unlabeled N-benzoylalanine, the undiluted N-benzoylalanine had a specific activity of 5.19×10^{-3} $\mu\text{Ci}/\text{mmol}$ for ^3H and 1.56×10^{-2} $\mu\text{Ci}/\text{mmol}$ for ^{14}C , to give a constant ^3H to ^{14}C ratio of 0.38 ± 0.04 . The % incorporation was 2.29×10^{-3} and 5.4×10^{-2} for ^3H and ^{14}C , respectively, while the % specific incorporation was 5.99×10^{-6} and 1.08×10^{-5} . Internal standards of 6.62 mg (7.73×10^{-3} μCi) of ^3H n-hexadecane and 14.27 mg (7.18×10^{-3} μCi) of ^{14}C n-hexadecane were used.

7. Incorporation of (2S,3S,4R)-[3,4- $^3\text{H}_2$]Proline and (RS)-[carboxyl- ^{14}C]Proline into Virginiamycin M₁

A slant of S.virginiae was used to inoculate 30 ml of STA-2 seed culture. After 48 hours at 25-27°C, the 30 ml of seed culture was used to inoculate 1 liter of STA-14 in a fermenter at 21°C, 475 rpm, pH of 7.2 and aeration of 1.25 l/l/min. After 24 hours of growth, 0.1 ml of (2S,3S,4R)-[3,4- $^3\text{H}_2$]proline (100 μCi , 55.0 mCi/mmol) was mixed with 20 μl (10 μCi , 50.0 mCi/mmol) of (RS)-[carboxyl- ^{14}C]proline and added to

the fermenter. 10% of the mixture was retained for determination of the precursor ^3H to ^{14}C ratio.

The 10% aliquot of labeled prolines were mixed with 10 mg of unlabeled (RS)-proline in 1.0 ml of distilled water and 80 mg of NaOH was added to make the solution 1N in respect to NaOH. The reaction was worked up as in section G,1 and fractions of pure N-benzoylproline were collected for counting from preparative HPLC. The solvent was evaporated and the N-benzoylproline dried in a desiccator under vacuum for 1 hour. The sample was corrected to obtain a ^3H to ^{14}C ratio of 15.6 ± 0.7 . An external standard was used for determination of counting efficiency.

After 48 hours of growth, the fermentation was stopped and the crude virginiamycin was isolated and purified as in Section E to give 9.9 mg (1.89×10^{-2} mmol) of pure virginiamycin M_1 (purity >95% by analytical HPLC). The isolated antibiotic (^3H to ^{14}C ratio of 32.2) contained 0.167 μCi ^3H (specific activity 8.85 $\mu\text{Ci}/\text{mmol}$) and 5.19×10^{-3} μCi ^{14}C (specific activity 0.275 $\mu\text{Ci}/\text{mmol}$). The % incorporation was 0.17% and 0.05% for ^3H and ^{14}C , respectively, while the % specific incorporation was $1.6 \times 10^{-2}\%$ and $5.5 \times 10^{-4}\%$.

The pure virginiamycin M_1 (3.5 mg, 6.7×10^{-3} mmol) was mixed with 3.61 mg of PtO_2 in 0.64 ml of glacial acetic acid and hydrogenated at room temperature and atmospheric pressure for 24 hours. The catalyst was removed by centrifugal filtration and the "perhydro VM_1 " was hydrolyzed by heating at 100-110°C in 1.5 ml of 6N HCl for 24 hours. The hydrolysis solvent was removed in vacuo and the residue dissolved in

1 ml distilled water. NaOH (139 mg) was added to make the solution 1 N and the mixture was treated with 40 equivalents of benzoyl chloride in diethyl ether at 0°C for 24 hours. Following work up as in Section G1, aliquots of pure N-benzoylproline was collected from preparative HPLC, evaporated to dryness and dried in a vacuum desiccator for 1 hour. The sample was counted to obtain a ^3H to ^{14}C ratio of 17.7 ± 0.9 . The n-benzoyl proline (0.7 mg 1.33×10^{-3} mmol) was purified by HPLC and contained 4.28×10^{-2} $\mu\text{Ci } ^3\text{H}$ (specific activity 32.15 $\mu\text{Ci}/\text{mmol}$) and 2.42×10^{-2} $\mu\text{Ci } ^{14}\text{C}$ (specific activity 1.82 $\mu\text{Ci}/\text{mmol}$). The % incorporation was $4.28 \times 10^{-2}\%$ and $2.42 \times 10^{-2}\%$ for ^3H and ^{14}C , respectively, while the % specific incorporation was 0.058% and $3.64 \times 10^{-3}\%$. An external standard was used to determine counting efficiency.

8. Incorporation of (2S,3R)-[3- ^3H]Proline and (S)-[U- ^{14}C]Proline into Virginiamycin M_1 and M_2

14 μCi of (2S,3R)-[3- ^3H]proline (specific activity 744 $\mu\text{Ci}/\text{mmol}$) was synthesized as described later and mixed with 3 μCi (S)-[U- ^{14}C]proline (specific activity 265 $\mu\text{Ci}/\text{mmol}$). 10% of this mixture was kept for counting purposes. The remaining 90% was sent to SmithKline-RIT in Belgium.

The 10% mixture was added to 330 mg of (S)-proline and recrystallized from ethanol and water to a constant ^3H to ^{14}C ratio of 2.51 ± 0.12 .

A 2.3g extract was returned from SmithKline-RIT. The residue was purified on a flash column containing 100 g of silica gel 60 (200-400

mesh) which was eluted with 100 ml of CH_2Cl_2 , 100 ml of 1% MeOH in CH_2Cl_2 , 100 ml of 2% MeOH in CH_2Cl_2 and 200 ml of 4% MeOH in CH_2Cl_2 . Fractions containing 20-30 ml of eluent were collected. Fractions 10-14 contained VS_1 , fractions 17-35 contained VM_1 and VM_2 . The VM_1 and VM_2 were further purified by preparative HPLC to give 28 mg of VM_1 (3.72×10^{-3} $\mu\text{Ci } ^3\text{H}$, specific activity 0.069 $\mu\text{Ci}/\text{mmol}$, 9.06×10^{-3} $\mu\text{Ci } ^{14}\text{C}$, specific activity 0.169 $\mu\text{Ci}/\text{mmol}$) and 30 mg of VM_2 (3.06×10^{-3} $\mu\text{Ci } ^3\text{H}$, specific activity 0.054 $\mu\text{Ci}/\text{mmol}$, 1.59×10^{-3} $\mu\text{Ci } ^{14}\text{C}$, specific activity 0.028 $\mu\text{Ci}/\text{mmol}$). Aliquots of VM_1 and VM_2 were counted and corrected for quenching by using internal standards of ^{14}C n-hexadecane and ^3H n-hexadecane. The ^3H to ^{14}C ratios were 0.41 for VM_1 and 1.91 for VM_2 , respectively.

The % incorporation for VM_1 was .026% and 0.302% for ^3H and ^{14}C while the % specific incorporation was 9.27×10^{-3} and 6.38×10^{-2} %.

The % incorporation for VM_2 was 2.18×10^{-2} % and 5.30×10^{-2} % for ^3H and ^{14}C while the % specific incorporation was 7.25×10^{-3} % and 1.05×10^{-2} %.

The VM_2 (30 mg, 5.7×10^{-2} mmol) was dissolved in 1.5 ml of 6N HCl and heated under nitrogen in a sealed vial at 110°C for 16 hours. The HCl was removed in vacuo and the residue dissolved in 1.5 ml of 1N NaOH, followed by stirring 12 hours at 0°C with 1.5 ml of diethylether containing 40 eq. of benzoyl chloride. The ether layer was removed and the aqueous layer extracted with ether, acidified and extracted 3 times with equal volumes of EtOAc. After drying with anhydrous MgSO_4 , filtration and evaporation in vacuo to give the crude N-benzoylproline

6.21 mg (2.8×10^{-2} mmol). The N-benzoylproline was added to 159.7 mg of unlabeled N-benzoylproline and recrystallized to a constant ^3H to ^{14}C ratio of 1.99 ± 0.06 . The isolated diluted N-benzoylproline contained 3.98×10^{-4} $\mu\text{Ci } ^3\text{H}$ (specific activity 4.63×10^{-4} $\mu\text{Ci}/\text{mmol}$) and 1.99×10^{-4} $\mu\text{Ci } ^{14}\text{C}$ (specific activity 2.32×10^{-4} $\mu\text{Ci}/\text{mmol}$).

The undiluted N-benzoylproline would therefore have a specific activity for ^3H of 6.99×10^{-3} $\mu\text{Ci}/\text{mmol}$ and 1.39×10^{-2} $\mu\text{Ci}/\text{mmol}$ for ^{14}C . The % incorporation was 2.84×10^{-3} for ^3H and 9.95×10^{-3} while the % specific incorporation was 9.39×10^{-4} and 5.25×10^{-3} for ^3H and ^{14}C , respectively.

The VM_1 (25 mg) was dissolved in 10 ml of glacial acetic acid containing 20 mg of PtO_2 and hydrogenated for 12 hours at 1 atmosphere and room temperature. The catalyst was removed by filtration, the acetic acid evaporated and the "perhydro M_1 " hydrolyzed with 1.5 ml of 6N HCl at 110°C in a sealed vial under nitrogen for 16 hours. The HCl was removed in vacuo and the residue dissolved in 1.5 ml of 1N NaOH. The basic solution was stirred 12 hours with diethyl ether containing 40 eq benzoyl chloride at 0°C , after which the ether layer was discarded, the mixture extracted with ether, acidified and extracted 3 times with EtOAc. The organic layer was dried, filtered and evaporated to a crude residue of N-benzoylproline which was purified by HPLC to give 5.3 mg (2.4×10^{-2} mmol). The labeled N-benzoylproline was added to 300 mg of unlabeled N-benzoylproline and recrystallized from ethanol, petroleum ether. The isolated N-benzoylproline contained 1.2×10^{-5} $\mu\text{Ci } ^3\text{H}$ (specific activity 5.0×10^{-4} $\mu\text{Ci}/\text{mmol}$) and 1.12×10^{-4} $\mu\text{Ci } ^{14}\text{C}$

(specific activity 4.67×10^{-3} $\mu\text{Ci}/\text{mmol}$). The resulting ^3H to ^{14}C ratio was 0.1. The % incorporation was 8.57×10^{-5} and 3.73×10^{-3} for ^3H and ^{14}C while the % specific incorporation was 1.15×10^{-5} and 1.76×10^{-3} for ^3H and ^{14}C , respectively. Internal standards of ^3H -n-hexadecane and ^{14}C -n-hexadecane were used to calculate counting efficiencies.

H. Synthesis

1. Sodium [1- ^{13}C , $^{18}\text{O}_2$]Acetate 16⁹³

^{13}C enriched potassium cyanide (1.0g, 14.9 mmol) (99% ^{13}C) was dried at 170°C for 10 hours. The KCN was cooled in a desiccator and transferred to a dry 10 ml round bottom flask. Dry methanol 3.25 ml (distilled from CaH_2) and dry distilled methyl iodide (2.26 g, 16 mmol) were added under a nitrogen atmosphere. ^{18}O water 0.1 ml (95-99 atom % ^{18}O) was added and the mixture refluxed with stirring for 22 hours. The flask was then fitted with a distillation head and distilled to dryness. To the distillate in a 25 ml flask was added 0.81 g, 40.5 mmol of ^{18}O water and 16 mmol of potassium t-butoxide (16 mmol) prepared by dissolving potassium metal in dry t-butyl alcohol. The mixture was refluxed for 48 hours under a N_2 atmosphere. At the end of this time, the solvent was evaporated to leave a white shiny residue of potassium acetate. The potassium acetate was acidified by dissolving in approximately 10 ml of concentrated phosphoric acid. The resulting acetic acid was lyophilized and collected in a flask submerged in liquid nitrogen. The labeled acetic acid was then titrated with 1.09 N NaOH to form the sodium salt. Yield of sodium acetate was 0.444 g (5.4 mmol) by

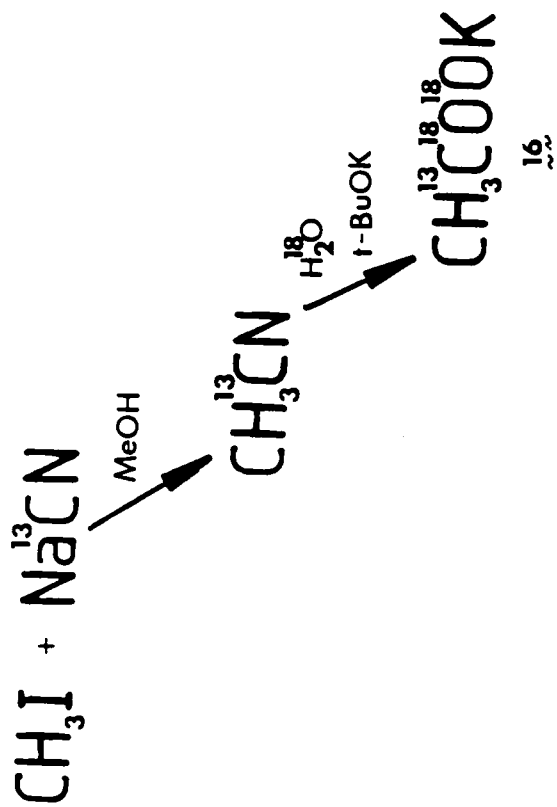
titration (0.5 g dry, 41% yield). An additional 150-200 mg of acetate was collected by repeating the lyophilization of the acidified potassium acetate (total yield 57%). Scheme VIII, Figure 13, Figure 14.

^1H NMR D_2O , 1.9 ppm doublet, ^{13}C NMR D_2O , 183.8(s), 26.0, (d) ppm. ^{13}C NMR expanded scale 3 singlets at 183.818 $^{13}\text{C}^{16}\text{O}_2$, 183.792 $^{13}\text{C}^{16}\text{O}^{18}\text{O}$, 183.766 $^{13}\text{C}^{18}\text{O}_2$.

Ratio of products by ^{13}C NMR were: 25:25:50.

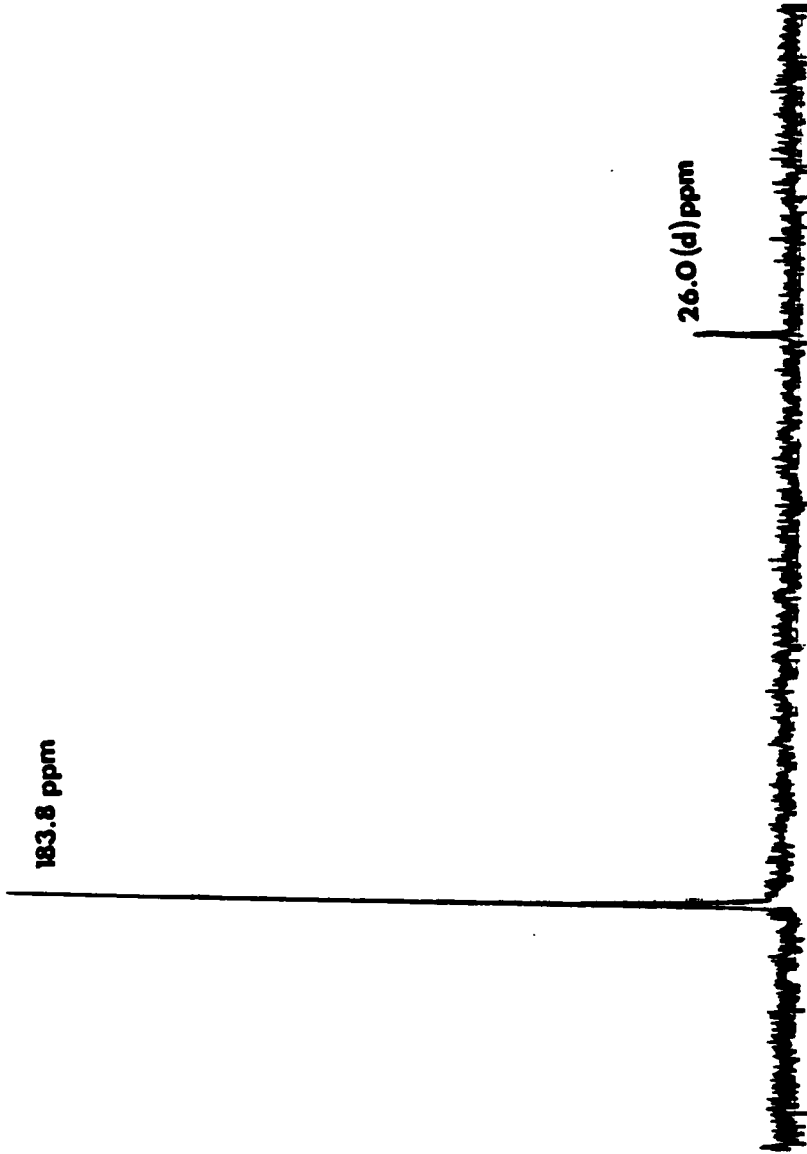
2. (S)-Proline Methyl Ester 17⁶⁴

Acetyl chloride (0.819g, 1.04×10^{-2} mol) was added to 50 ml of methanol in a 100 ml flask at 0-5°C. After stirring with a magnetic stirring bar for 15 minutes, 0.307g (2.6×10^{-3} mol) of (S) proline (Sigma) was added and refluxed for 3 hours. The solvent was removed in vacuo leaving the (S)-proline methyl ester hydrochloride. The salt was dissolved in dry CH_2Cl_2 and cooled in an ice bath to 0-5°C. Dry ammonia gas passed through a CaCl_2 drying tube was bubbled into the solution via a syringe for 3-5 minutes. A cloudy white precipitate of NH_4Cl formed and was removed by filtration. The filtrate was rinsed with dry CH_2Cl_2 to remove any ester adhering to the salt. The excess NH_3 was removed by evaporation using a rotary evaporator at aspirator pressure followed by evaporation of the CH_2Cl_2 to leave the clear pale-yellow liquid (S)-proline methyl ester 17 (0.267g, 2.07×10^{-3} mol) crude yield 77.2%. Purity 98% TLC, NMR.



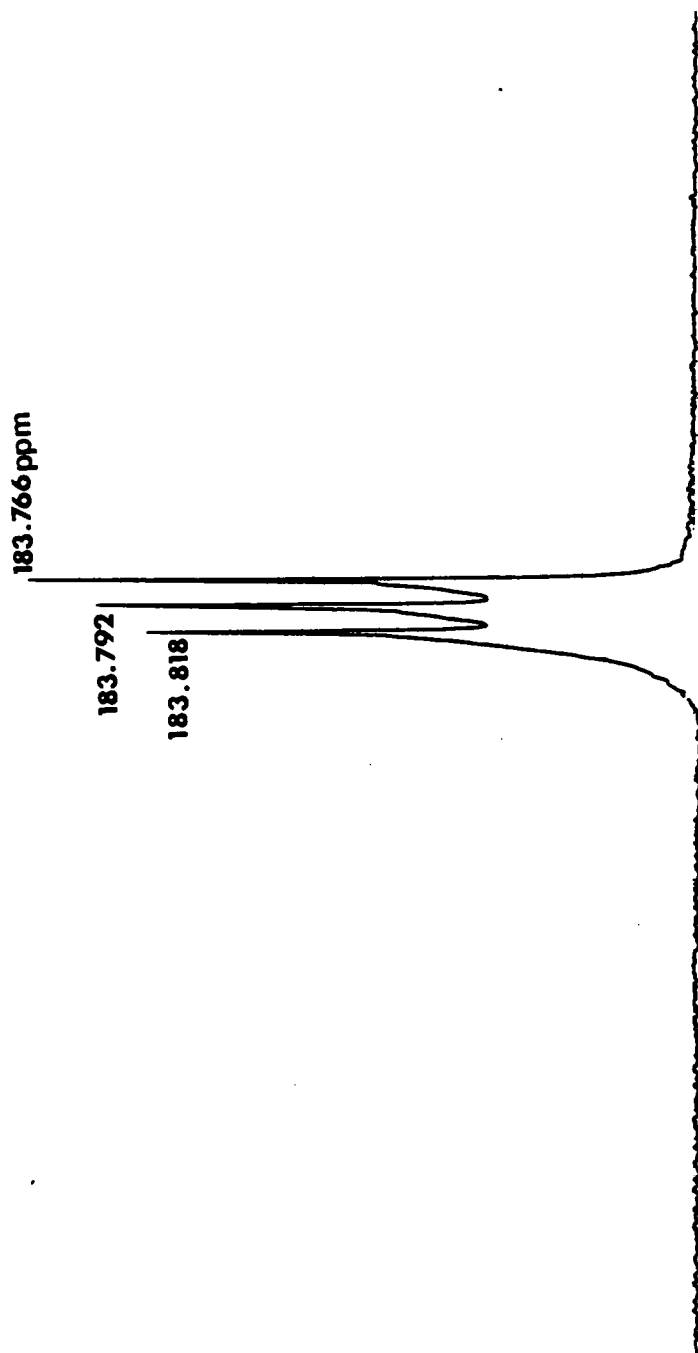
Synthesis of Potassium [1-¹³C-¹⁸O₂]Acetate

Scheme VIII



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

Figure 13



Expanded ^{13}C NMR Spectra of Carbonyl Region of Sodium $[1-^{13}\text{C}, 18\text{O}_2]\text{Acetate}$

Figure 14

^1H NMR: (CDCl_3) 1.85 (m, 2H, 4 α , β), 1.95 (m, 1H, 3 β), 2.11 (m, 1H, 3 α), 2.9 (m, 1H, 5 α), 3.1 (m, 1H, 5 β), 3.7 (s, 3H, OMe), 3.79 (t, 1H, 2 α) ppm

^{13}C NMR: (CDCl_3) 175.9 (carboxyl), 59.8 (C-2), 52.1 (C-5), 46.9 (OMe), 30.2 (C-3), 25.6 (C-4)

IR: (neat) 3410, 3005, 2940, 1735, 1485, 1394 cm^{-1}

3. 1,2-Dehydroproline Methyl Ester **18**^{64,94}

(S)-proline methyl ester (0.267g, 2.07×10^{-3} mol) in a 50 ml flask fitted with a stirring bar was dissolved in 25 ml of diethyl ether and cooled in a dry-ice- CCl_4 bath to -23°C . In total darkness, 0.255g (2.3×10^{-3} mol) of t-butyl hypochlorite was added via syringe over 20 minutes under a nitrogen atmosphere. The mixture was stirred under nitrogen for 30 minutes and was then treated with 0.209g (2.06×10^{-3} mol) of Et_3N . The reaction was monitored by TLC using solvent CH_2Cl_2 , CH_3OH 99/1 and was complete after 36 hours of stirring under a nitrogen atmosphere. The white precipitate of Et_3NHCl was removed by filtration and rinsed 3 times with dry diethyl ether. The ether layers were combined and the light brown solution was passed through a column containing 3 grams of Woelm-Alumina-Super 1 (Activity III). The ether was removed in vacuo to leave as a pale yellow oil of 1,2-dehydroproline methyl ester **18**. The product was distilled under vacuum to give 0.258g, (2.03×10^{-3} mol), 98% yield.

^1H NMR: (CDCl_3) 4.05 (t, 2H, 5 α , β), 3.81 (s, 3H, OMe), 2.78 (t, 2H, 3 α , β), 1.94 (q, 2H, 4 α , β) ppm

^{13}C NMR: (CDCl_3) 168.3 (carbonyl), 163.4 (C-2), 62.6 (C-5), 52.4 (C-3),
35.5 (C-4), 22.3 (OCH_3)

IR: (neat) 3005, 2375, 2940, 1742, 1655, 1485, 1378, 1341 cm^{-1}

4. **N-Carbobenzyloxy-2,3-dehydroproline Methyl Ester 19**

1,2 dehydroproline methyl ester (0.177g, 1.39×10^{-3} mol) were dissolved in 27 ml of dry CH_2Cl_2 in a 50 ml flask and cooled to -23°C in a dry ice CCl_4 bath. To the cold solution was added 0.168 ml (2.08×10^{-3} mol) of dry pyridine and 0.296 ml (2.07×10^{-3} mol) of benzylchloroformate. The mixture was stirred for 45 minutes at -23°C and then allowed to warm to room temperature. The mixture was then refluxed for 1.25 hours. After cooling the mixture was extracted twice with 50 ml of distilled H_2O and twice with 50 ml of brine. The CH_2Cl_2 was dried with anhydrous Na_2SO_4 , filtered and evaporated in vacuo to give an oil. (0.354g, 1.36×10^{-3} mol), 97.8% crude yield.

TLC: (EtOAc/Pet. ether) 2/1 $R_f=0.5$

^1H NMR: (CDCl_3) 7.3 (m, 5H, ArH), 5.81 (t, 1H, vinyl), 4.58 (s, 2H, $5\alpha, \beta$),
4.0 (s, 2H, $-\text{OCH}_2-$), 3.62 (s, 1.5H, OMe), 3.83 (s, 1.5H, OMe), 2.62
(d, t, 2H, $4\alpha, \beta$) ppm

IR: (neat) 2995, 1755, 1722, 1435, 1383, 1285 cm^{-1}

5. **N-Butyloxycarbonyl-2,3-dehydroproline Methyl Ester 20**

1,2-dehydroproline methyl ester 0.168g (1.32×10^{-3} mol) was dissolved in 20 ml of CH_2Cl_2 in a 50 ml flask. To the cooled solution (-23°C dry ice/ CCl_4) was added 2.0 eq of dry pyridine (2.62×10^{-3} mol)

and 20 eq of n-butyl chloroformate (2.64×10^{-2} mol) with stirring. The solution was stirred for 15 minutes at -23°C and allowed to warm to room temperature with the development of a slight pinkish color. The mixture was refluxed for 1 hour and the reaction quenched by extraction with equal volume of distilled H_2O twice and equal volume of brine twice. The CH_2Cl_2 was dried with anhydrous Na_2SO_4 , filtered and evaporated to give 0.272 g (1.19×10^{-3} mol) of a lightly colored clear oil. Crude yield 90.7%.

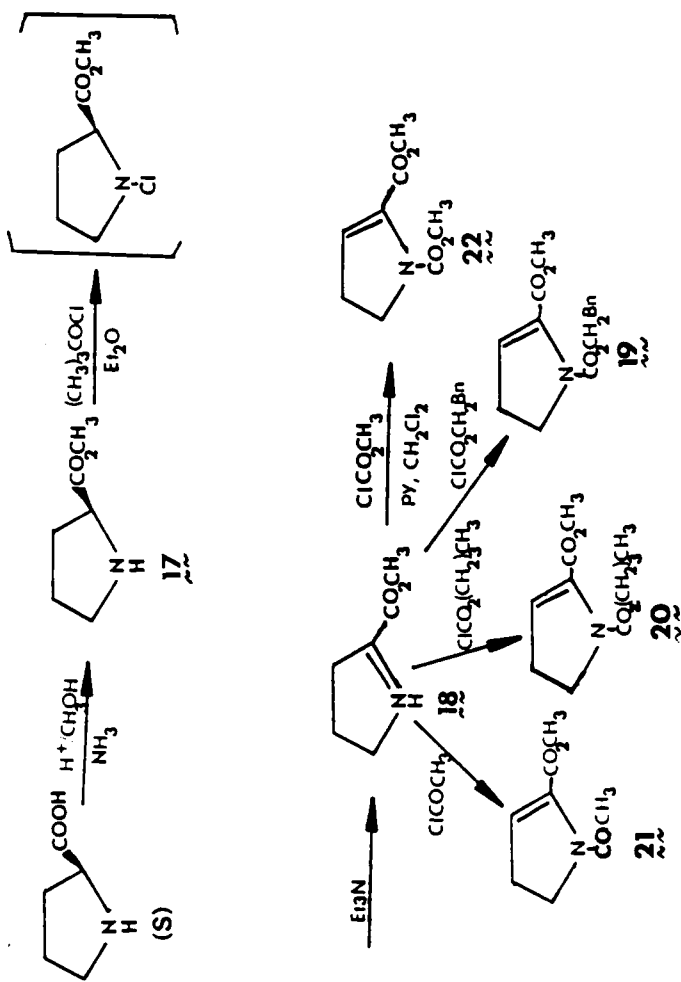
TLC: (EtOAc/Pet. ether) 2/1 $R_f=0.7$

^1H NMR: (CDCl_3) 5.81 (t, 1H, vinyl), 4.11 (t, 2H, $5\alpha, \beta$), 3.98 (t, 2H, $-\text{OCH}_2-$), 3.80 (s, 3H, OMe), 2.65 (d, t, 2H, $4\alpha, \beta$), 1.6 (m, 2H, $-\text{CH}_2-$), 1.38 (m, 2H, $-\text{CH}_2-$), 0.98 (t, 3H, $-\text{CH}_3$) ppm

IR: (neat) 2995, 1755, 1722, 1425, 1345 cm^{-1}

6. N-Acetyl-2,3-dehydroproline Methyl Ester **21** ^{64,94}

1,2-Dehydroproline methyl ester 0.999g (7.9×10^{-3} mol) in a 50 ml flask was dissolved in 25 ml of dry CH_2Cl_2 and cooled to -30°C . The cooled solution was treated with dry pyridine (8.2×10^{-3} mol) and acetyl chloride (8.2×10^{-3} mol) and stirred at -30°C for 30 minutes. The solution was warmed to room temperature and refluxed for 1.25 hours. After cooling the orange solution was extracted twice with distilled H_2O and twice with brine. (The orange color disappears.) The CH_2Cl_2 layer was dried with anhydrous Na_2SO_4 , filtered and evaporated in vacuo to give a colorless oil which crystallizes on cooling to -30°C . 0.890g (5.26×10^{-3} mol) 66.9% yield.



Synthesis of N-Protected-2,3-dehydroproline Methyl Ester

Scheme IX

TLC: (EtOAc/Pet. ether) 2/1, $R_f=0.4$

^1H NMR: (CDCl_3) 5.81 (t, 1H, vinyl), 4.05 (t, 2H, 5 α , β), 3.86 (s, 3H, OMe),
2.71 (t, d, 2H, 4 α , β), 2.09 (s, 3H, $-\text{CH}_3$) ppm

IR: (neat) 2995, 1745, 1662, 1440, 1280 cm^{-1}

7. **N-Carbomethoxy-2,3-Dehydroproline Methyl Ester 22**

1,2-Dehydroproline methyl ester 0.462g (3.6×10^{-3} mol) was dissolved in 50 ml of dry CH_2Cl_2 in a 100 ml flask and cooled to -23°C . The solution was treated with 0.435 g (5.5×10^{-3} mol) of dry pyridine and 0.519 g (5.5×10^{-3} mol) of methyl chloroformate at -28°C . The solution was stirred and allowed to warm to room temperature over 45 minutes followed by refluxing for 1.5 hours. After cooling, the reaction mixture was extracted with equal volumes of distilled H_2O twice and brine twice. The CH_2Cl_2 layer was dried with anhydrous Na_2SO_4 , filtered and evaporated in vacuo to give a clear colorless oil. (0.5445g, 2.94×10^{-3} mol). Purity >95% by NMR. Can be purified by column chromatography with ethyl acetate and petroleum ether. 80.5% yield.

TLC: (EtOAc/Pet. ether) 2/1 $R_f=0.34$

^1H NMR: (CDCl_3) 5.87 (t, 1H, vinyl), 3.97 (t, 2H, 5 α , β), 3.83 (s, 3H, OMe),
3.72 (s, 3H), 2.68 (t, d, 2H) ppm

IR: (neat) 2990, 1740 (broad 1720, 1760), 2570, 1458 cm^{-1}

8. **N-Acetyl-(2S,3S and 2R,3R)-[2,3- $^2\text{H}_2$]Proline Methyl Ester 23, 24**

N-Acetyl-2,3-dehydroproline methyl ester (0.0523g , 3.09×10^{-4}

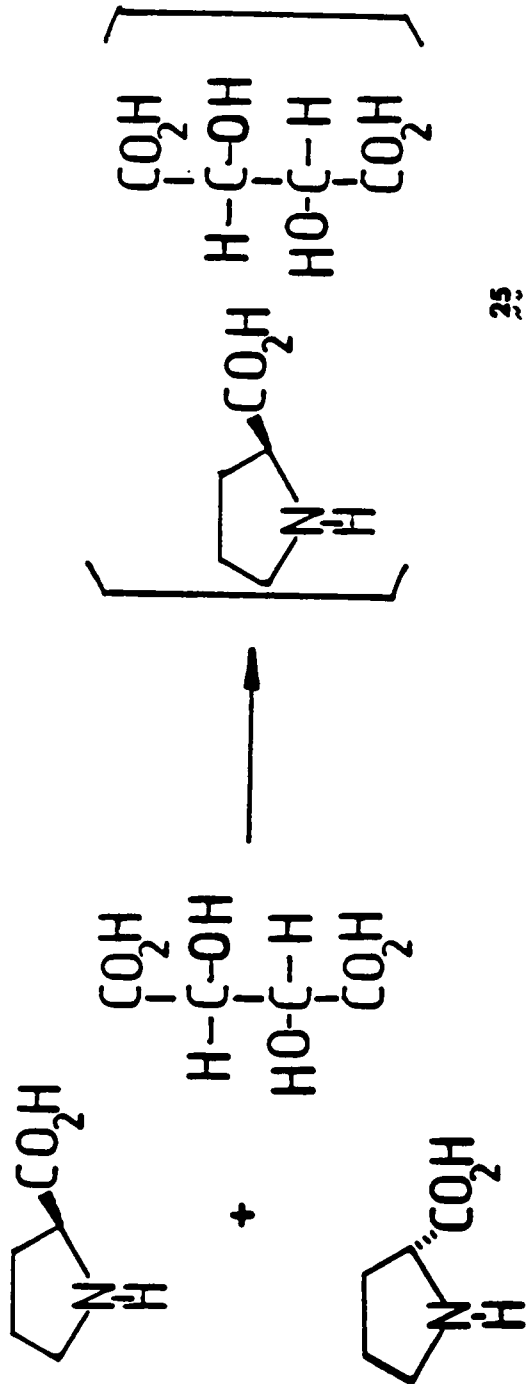
mol) was dissolved in ethyl acetate (5 ml) in a 10 ml flask with a stirring bar. 5% Pd on carbon (92.88 mg) was added as catalyst and the hydrogenation apparatus charged with deuterium gas. After 24 hours 3.6×10^{-4} mol of D_2 gas was taken up by the reaction. The catalyst was removed by filtration, and the solvent evaporated in vacuo to yield 0.0501g (2.9×10^{-4} mol) of N-acetyl-(2S,3S)-[2,3- 2H_2]proline methyl ester and N-acetyl-(2R,3R)-[2,3- 2H_2]proline methyl ester.

1H NMR: 4.39 (t, 0.5H, 2 α), 3.67 (s, 1.5H, -OCH₃), 3.62 (s, 1.5H, -OCH₃),
3.57 (m, 1H, 5 β), 3.42 (m, 1H, 5 α), 2.19 (m, 0.5H, 3 α),
2.09 (m, 3H, -CH₃), 1.98 (m, 3H, 3 β , 4 α , β) ppm

IR: 3002, 2300, 1765, 1722, 1460, 1400, 1210, 924 cm^{-1}

9. Resolution of (RS)-Proline by Tartaric Acid Complex

(RS)-proline (0.100g, 8.9×10^{-4} mol) and 0.0675 g (4.5×10^{-4} mol) of (S)-tartaric acid were mixed and dissolved in 0.1 ml of distilled H₂O. The mixture was allowed to stand at room temperature for 2 hours. After this time 1.5 ml of absolute ethanol was added to the mixture, along with a seed crystal of (S)-proline-(S)-tartaric acid complex and the solution refrigerated for 24 hours. The crystals of the (S-S) complex 25, Scheme X, were removed by filtration to give 0.055g (2.08×10^{-4} mol) (46% yield) of the complex. A second batch of crystals was collected. Total yield 55%. Optical purity was >99%.
 $[\alpha]_D^{25} = -24.82$ (C=1, H₂O), $[\alpha]_D^{25}$ lit. = -24.2^{95} (C=1, H₂O), mp 154-156°C, mp (lit.) 154.5°C



25

Formation of (S)-Proline-(S)-tartaric acid Complex from (RS)-Proline

Scheme X

10. (2S,3S)-[2,3-²H₂]Proline 26

(2R,3R)-[2,3-²H₂]Proline 27

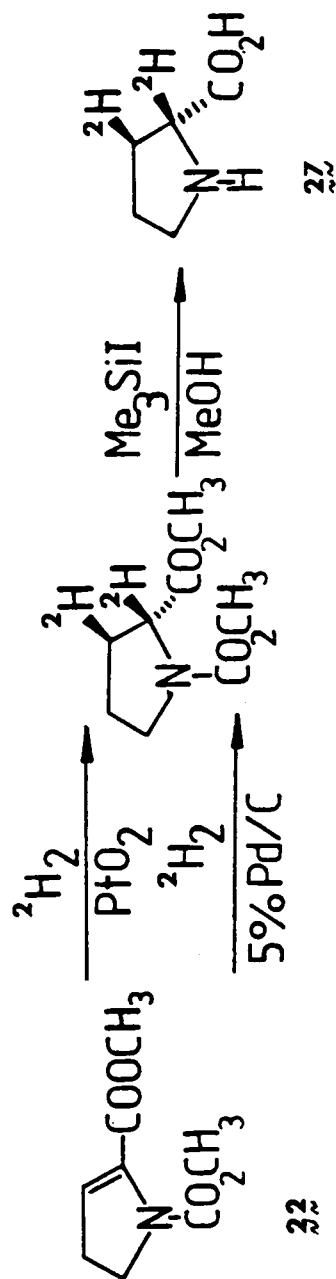
a) N-Carbomethoxy-2,3-dehydroproline methyl ester (0.100g, 5.4×10^{-4} mol) was dissolved in 10 ml of ethyl acetate containing 0.010g of PtO₂ which had been stirred 1 hour in a deuterium atmosphere. The hydrogenation was carried out in D₂ for 24 hours at room temperature and atmospheric pressure. The catalyst was removed by filtration and the solvent removed in vacuo. The yield appeared to be quantitative by TLC.

¹H NMR: (CDCl₃) 4.32 (t, 0.1H, 2α), 3.80 (d, 3H, -OCH₃), 3.71 (s, 1.5H, OCH₃), 3.69 (s, 1.5H, OCH₃), 3.50 (m, 1H, 5α), 3.40 (m, 1H, 5β), 2.20 (m, 0.1H, 3α), 1.92 (m, 1H, 3β), 1.90 (m, 2H, 4α, β) ppm. Shows 10% scrambling of label by NMR integrations.

IR: (neat) 3000, 2300, 1760, 1719, 1465, 1400, 1210, 924 cm⁻¹

b) The N-carbomethoxy proline methyl esters were deprotected by refluxing in 10 ml of dry CH₃CN containing 4 eq (0.712 g) of trimethyl silyl iodide for 20 hours followed by stirring with 20 ml of a ethanol-acetic acid 1:1 solution at 0°C for 20 minutes. The mixture was diluted with 10 ml of distilled H₂O and extracted 5 times with 10 ml of CH₂Cl₂. The aqueous layer was purified by ion exchange on a 10g column (10 mm x 30 cm) of Dowex 50X in the hydrogen form. The column was eluted with deionized H₂O until neutral to pH paper and the proline eluted with 4N NH₄OH. Ninhydrin positive fractions were evaporated to give 0.055g (4.78×10^{-4} mol) of product (89% yield). Scheme XI.

¹H NMR: (D₂O) 4.12 (t, 0.1H, 2α), 3.40 (m, 1H, 5α), 3.31 (m, 1H, 5β), 2.32 (m, 0.1H, 3α), 2.01 (m, 1H, 3β), 1.99 (m, 2H, 4α, β) ppm



PtO₂ ~10% label scrambling

5% Pd/C ~40-50% label scrambling

Synthesis of (2S,3S)-[2,3-²H₂] and (2R,3R)-[2,3-²H₂]proline by Deuteration of N-Carbomethoxy (2,3)-dehydroproline Methyl Ester by PtO₂ and Pd/C

Scheme XI

^{13}C NMR: 173.4 (carboxyl), 58.9 (C-2), 51.7 (C-5), 52.3 (C-5), 30.2 (C-3), 25.7 (C-4) ppm

IR: (KBr) 3450, 3000, 2940, 1750, 1480, 1395 cm^{-1}

11. (2S,3S)-[2,3- $^2\text{H}_2$]Proline 26

(2R,3R)-[2,3- $^2\text{H}_2$]Proline 27

a) N-Carbomethoxy-2,3-dehydroproline methyl ester (0.100g, 5.4×10^{-4} mol) was dissolved in 10 ml of ethyl acetate containing 0.050g of 5% Pd on carbon which had been stirred 1 hour in a deuterium atmosphere. The hydrogenation was carried out in D_2 for 24 hours at room temperature and atmospheric pressure. The catalyst was removed by filtration and the solvent removed in vacuo. The yield appeared to be quantitative by TLC.

^1H NMR: (CDCl_3) 4.32 (t, 0.5H, 2 α), 3.80 (d, 3H, $-\text{OCH}_3$), 3.71 (s, 1.5H, $-\text{OCH}_3$), 3.69 (s, 1.5H, $-\text{OCH}_3$), 3.50 (m, 1H, 5 α), 3.40 (m, 1H, 5 β), 2.20 (m, 0.5H, 3 α), 1.92 (m, 1H, 3 β), 1.90 (m, 2H, 4 α, β) ppm. Shows ~50% scrambling of label by NMR integrations.

IR: Same as previous sample.

b) The N-carbomethoxy proline methyl esters were deprotected by refluxing in 10 ml of dry CH_3CN containing 4 eq (0.712 g) of trimethyl silyl iodide for 20 hours followed by stirring with 20 ml of an ethanol-acetic acid 1:1 solution at 0°C for 20 minutes. The mixture was diluted with 10 ml of distilled H_2O and extracted 5 times with 10 ml of CH_2Cl_2 . The aqueous layer was purified by ion exchange on a 10g column (10mm x 30mm) of Dowex 50X in the hydrogen form. The column was eluted with deionized H_2O until neutral to pH paper and the proline eluted with 4N

NH_4OH . Ninhydrin positive fractions were evaporated to give 0.060 g (5.2×10^{-4} mol) of proline (96% yield).

^1H NMR (CDCl_3): 4.12 (t, 0.5H, 2 α), 3.40 (m, 1H, 3 α), 3.31 (m, 1H, 5 β), 2.32 (m, 0.5H, 3 α), 2.01 (m, 1H, 3 β), 1.99 (m, 2H, 4 α , β) ppm

12. (2S,3R)-[3- ^3H]Proline 33 and (2R,3S)-[3- ^3H]Proline 34

a) [2,3- $^3\text{H}_2$]Proline Methyl Ester 28

(S)-proline (0.303g, 2.63×10^{-3} mol) was added to 4 ml of a 0.1N HCl solution containing 3 mCi of (2S)-[2,3- $^3\text{H}_2$]proline in a 25 ml round bottom flask. The flask was evaporated in vacuo followed by evaporation at 0.1 torr for 1.5 hours to complete drying.

In another 20 ml dry round bottom flask 15 ml of dry MeOH was treated with 0.55 ml (3.0 eq) of acetyl chloride in an ice bath. After 10 minutes, the acidic methanol was transferred to the dry proline mixture and refluxed for 2.5 hours. The solvent was removed in vacuo to leave an oily proline methyl ester hydrochloride. After refrigeration overnight, the proline methyl ester hydrochloride was dissolved in dry CH_2Cl_2 cooled in an ice bath, and treated with anhydrous ammonia that was bubbled into the solution through a CaCl_2 trap. The resulting NH_4Cl was removed by filtration and the CH_2Cl_2 removed to give 0.263g (2.04×10^{-3} mol) of the labeled proline. 77.5% crude yield.

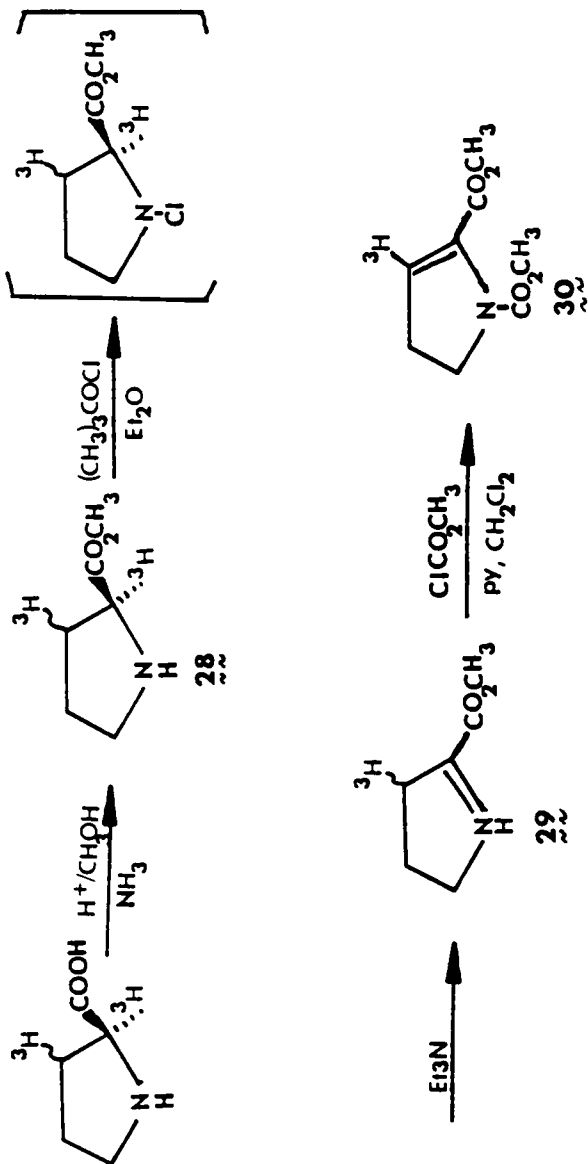
b) [3- ^3H]-1,2-Dehydroproline Methyl Ester 29

(2S)-[2,3- $^3\text{H}_2$]proline methyl ester (0.263g, 2.04×10^{-3} mol) was dissolved in 30 ml of Et_2O and cooled to -35°C . The cooled solution was treated with t-butylhypochlorite (0.240 ml, 2.1×10^{-3} mol) under N_2 in

total darkness over a 20 minute period. The mixture was stirred for an additional 45 minutes after which it was treated with dry Et_3N (300 ml, 2.2×10^{-3} mol). A white precipitate appeared in the flask and the reaction was allowed to stir at room temperature for 60 hours to complete the reaction. The Et_3NHCl was removed by filtration and the ether solution was passed through 0.250g of Woelm Alumina Super 1 (activity level III). Evaporation of the solvent left 0.271g (2.13×10^{-3} mol) of a brownish colored product. 97% crude yield.

c) N-Carbomethoxy-[3- ^3H]-2,3-dehydroproline Methyl Ester 30

The 0.271g (2.1×10^{-3} mol) of [3- ^3H]-1,2-dehydroproline methyl ester was dissolved in approximately 25 ml of dry CH_2Cl_2 and treated with 0.2452g (3.10×10^{-3} mol) of dry pyridine at -28°C under N_2 . After 15 minutes the solution was treated with methyl chloroformate (0.2955g, 3.14×10^{-3} mol) (Aldrich) and the mixture stirred for 45 minutes during which time it warmed to room temperature. The mixture was then refluxed for 2 hours, after which it was quenched by the addition of H_2O and extracted twice each with equal volumes of H_2O and brine. The CH_2Cl_2 layer was then dried with anhydrous Na_2SO_4 , filtered and evaporated in vacuo to give 0.234g of a dark brown oil. The oil was purified by flash chromatography using 3g of silica gel 60 and a solvent system of ethyl acetate hexane 1:2. The fractions containing the product were evaporated to yield 0.204g (1.10×10^{-3} mol) 42% yield from starting proline.



Synthesis of N-Carbomethoxy-[3-³H]-2,3-dehydroproline Methyl Ester

Scheme XII

d) N-Carbomethoxyl-(2R,3S and 2S,3R)-[3-³H]proline Methyl Ester
(31,32)

Compound 30 (0.204g, 1.10×10^{-3} mol) was dissolved in 5 ml of ethyl acetate containing 0.020g of PtO₂ and hydrogenated at room temperature and atmospheric pressure. After 20 hours an additional 0.010g of PtO₂ was added and the reaction allowed to continue for 12 more hours. The PtO₂ was removed by filtration and the ethyl acetate removed in vacuo to yield 0.176g (9.4×10^{-4} mol) of the product. 86% crude yield.

e) (2S,3R and 2R,3S)-[3-³H]Proline (33,34)

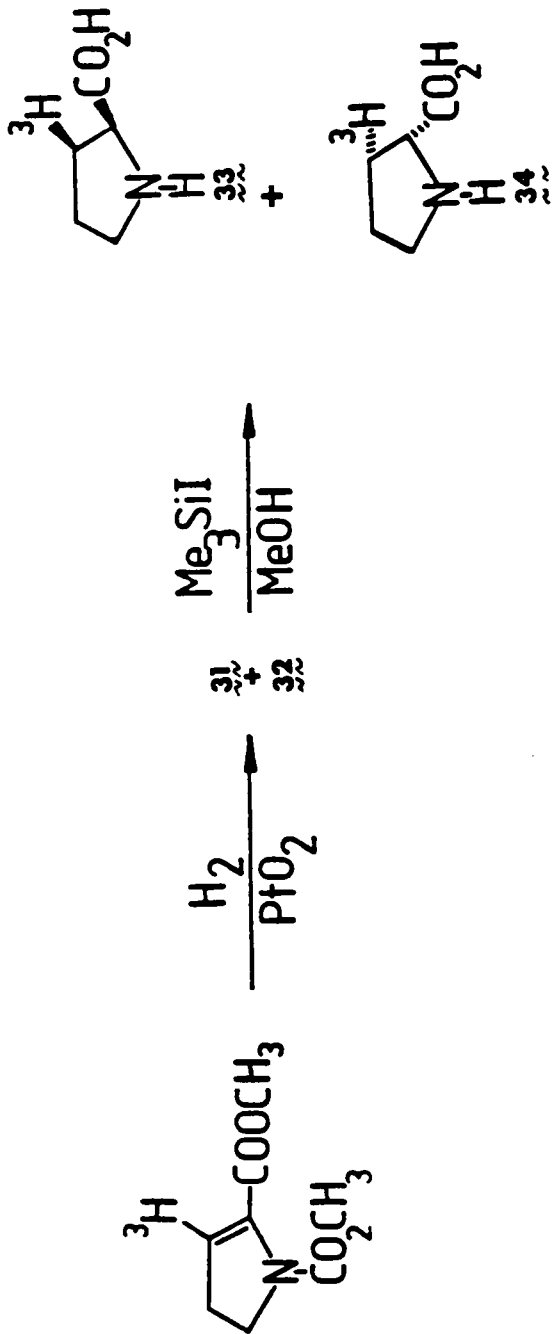
Compounds 31 and 32 were dissolved in 15 ml of dry acetonitrile under a nitrogen atmosphere and treated with 0.570 ml (4.1 eq, 3.8×10^{-3} mol) of trimethyl silyl iodide. The mixture was refluxed for 20 hours, then treated with 30 ml of an ethanol-acetic acid solution (1:1) at 0°C and stirred for 20 minutes. The mixture was further diluted with 10 ml of distilled H₂O and extracted 5 times with 10 ml of methylene chloride. The aqueous layer was then added to a column containing 10g of Dowex 50X ion exchange resin in the acid form. The column was eluted with deionized H₂O until the eluent was neutral to pH paper, and the proline was then eluted with 4N NH₄OH. All ninhydrin positive fractions were combined, treated with Norite and evaporated to dryness to yield 0.100g (8.69×10^{-4} mol, crude yield 94%) of proline as a light brown solid, identified by its ¹H NMR spectrum and its TLC (700 μCi). Radiochemical yield of ~23% based on [2,3-³H₂]proline, ~33% total yield from starting unlabeled proline.

f) Resolution of (2S,3R and 2R,3S)-[3-³H]Proline 33,34

The 0.100g (8.9×10^{-4} mol) of labeled prolines were mixed with 0.067 g (4.5×10^{-4} mol) of tartaric acid in 0.3 ml of distilled water and 3.0 ml of absolute ethanol and stirred for 2 hours. The mixture was seeded with a crystal of (S)-proline-(S)-tartaric acid complex. The mixture was refrigerated for 24 hours and the crystals collected by filtration. Several harvests of crystals were obtained to yield 0.045g (1.7×10^{-4} mol, 37% yield) of the complex. The crude mother liquor was frozen for further isolation. The (2S,3R)-[3-³H]proline was then isolated by ion exchange chromatography on a Dowex 50X column. The proline 34 was eluted with 0.1N HCl and evaporated to give pure product, determined by TLC with authentic (2S)-proline.

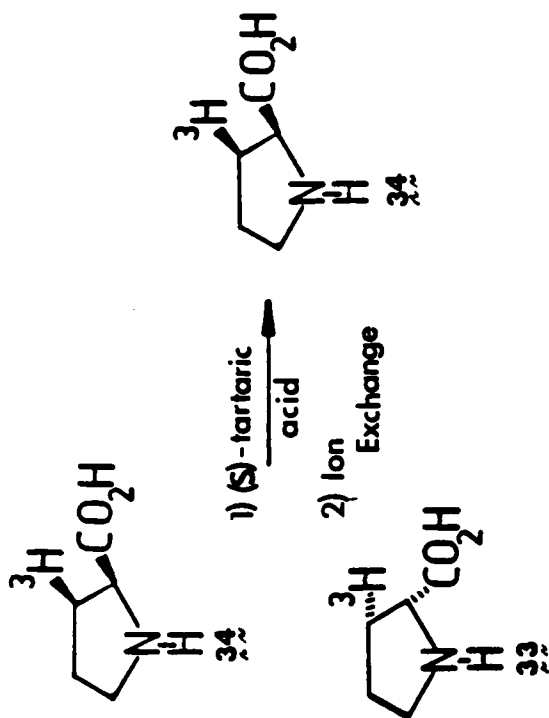
13. N-Benzoyl-(RS)-alanine from Virginiamycin M₁ and/or M₂ 35

Virginiamycin M₁ was dissolved in acetic acid (5-10 ml) and 1 equivalent of PtO₂ and a magnetic stirring bar were added. The solution was hydrogenated at 1 atmosphere pressure and room temperature for 24 hours. The catalyst was removed by filtration and evaporation of the solvent left an oily "perhydro M₁" which was heated to 105-110°C in 6N HCl for 24 hours in a sealed vial under N₂. The HCl solution was evaporated in vacuo and the residue dissolved in 1 ml of H₂O. The pH of the solution was adjusted to 10 with 1N NaOH using litmus paper. A heterogenous reaction was carried out as the aqueous solution was stirred with diethyl ether (1 ml) containing 40 eq of benzoyl chloride. The pH was continually adjusted to 10 while the reaction mixture was



Synthesis of (2R,3S)-[3-³H]Proline and (2S,3R)-[3-³H]Proline

Scheme XIII



Resolution of (2S,3R)-[3-³H]Proline from (2R,3S)-[3-³H]Proline

Scheme XIV

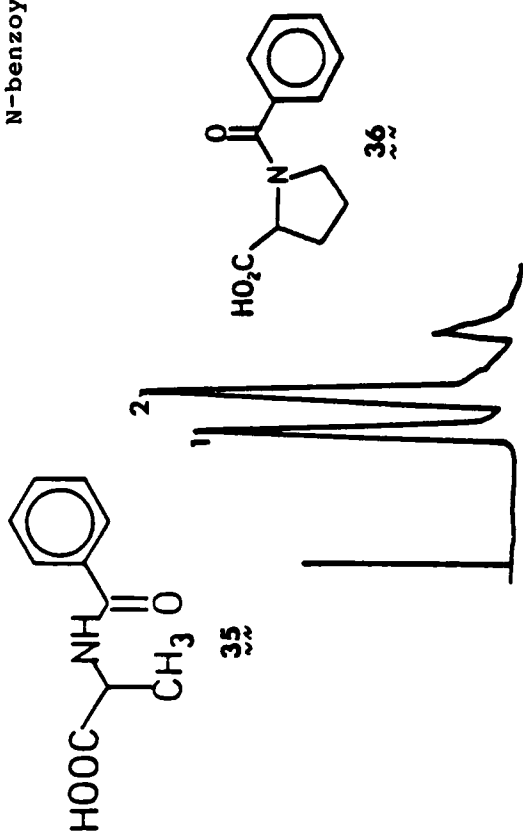
stirred for 2 hours at room temperature. The mixture was poured in a separatory funnel containing 10 ml of distilled H₂O. The ether layer was removed and the aqueous layer was extracted two times with 10 ml of diethyl ether. The aqueous solution was then acidified with concentrated HCl to a pH of 2.0 and extracted three times with equal volumes of ethyl acetate. The ethyl acetate was dried with anhydrous Na₂SO₄, filtered and evaporated to yield a crude solution of N-benzoyl amino acid. The N-benzoyl-(RS)-alanine was purified by HPLC using a reverse phase silica Novapak-8 column with a solvent system of CH₃CN/H₂O 22/77 (vol./vol.) (Figure 15).

14. N-Benzoyl-(RS)-proline from Virginiamycin M₁ and/or M₂ 36

Virginiamycin M₁ was dissolved in acetic acid (5-10 ml) and 1 equivalent of PtO₂ and a magnetic stirring bar was added. The solution was hydrogenated at 1 atmosphere and room temperature for 24 hours. The catalyst was removed by filtration and evaporation of the solvent left an oily "perhydro M₁" which was heated to 105-110°C in 6N HCl for 24 hours in a sealed vial under N₂. The HCl solution was evaporated in vacuo and the residue dissolved in 1 ml of H₂O. The solution was made 1N in NaOH and a heterogenous reaction carried out as the aqueous solution was stirred with 1 ml of ether containing 40 eq of benzoyl chloride. The mixture was stirred at 0°C for 24 hours and was then poured in a separatory funnel containing 10 ml of distilled H₂O. The ether layer was removed and the aqueous layer was extracted two times with 10 ml of diethyl ether. The aqueous solution was then acidified

with HCl to a pH of 2.0 and extracted three times with equal volumes of ethyl acetate. The ethyl acetate was dried with anhydrous Na_2SO_4 , filtered and evaporated to yield a crude solution of N-benzoyl amino acids. The N-benzoyl-(RS)-proline was purified by HPLC using a reverse phase Novapak-8 column with a solvent system of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 22/77. Figure 15.

Column: Nova-pak C-8
Flowrate: 1.0 ml/min.
Absorbance wavelength: 254 nm
Chart speed: 0.5 cm/min.
Retention time:
N-benzoyl-(RS)-alanine: 1.7 min.
N-benzoyl-(RS)-proline: 2.4 min.



HPLC of N-Benzoyl-(RS)-alanine 35 (1) and N-Benzoyl-(RS)-proline 36 (2)

Figure 15

CHAPTER IV.

A. RESULTS AND DISCUSSION

In Chapter II, Section F, the objectives of this work were summarized as:

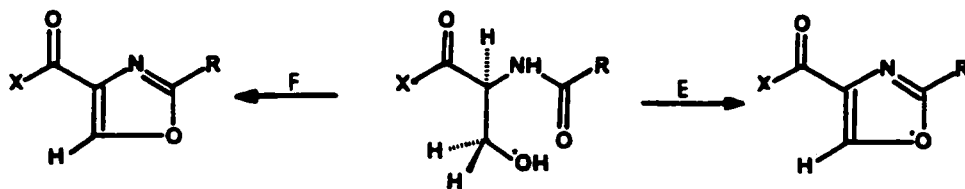
1. To determine the pathway for the formation of the oxazole ring.
2. To determine the pathway for the formation of the dehydroproline ring.

The following discussion represents the results of studies aimed at these objectives.

1. Incorporation of ^3H -Labeled Serines into the Oxazole Moiety of Virginiamycin M_1 and M_2

As mentioned in the introduction, the purpose in studying the stereochemistry of serine incorporation into VM_1 is to deduce the biosynthetic pathway giving rise to the unusual oxazole moiety. Previous work from Kingston's group has shown that the oxazole ring is formed from a serine unit, presumably by the cyclization of an acyl serine intermediate.⁹ This finding does not provide any information, however, on the mechanism of the cyclization process from the acyl serine intermediate to the oxazole ring.

Two basic pathways F and E for cyclization can be envisioned, in which the oxazole oxygen derives either from the serine unit or from the acyl group. (Scheme XV). Although a priori either pathway is possible, the fact that the thiazole ring is derived by cyclization of an acylcysteine unit^{86,96} makes pathway E (Scheme XV) in which the serine



Two Basic Pathways for Cyclization

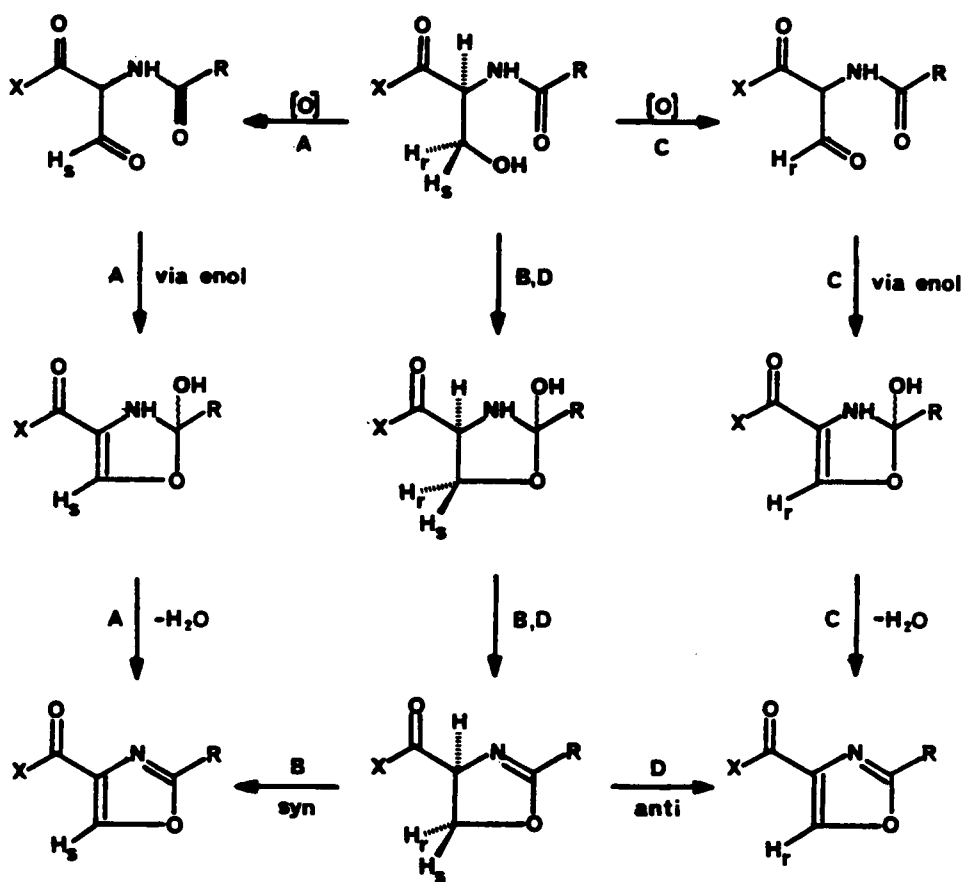
Scheme XV

oxygen is retained, the most probable pathway for this cyclization. Subsequent discussion is thus predicated on the assumption that cyclization is occurring by a pathway of this type.

With this assumption, the major hypothetical pathways for formation of the oxazole ring are shown in Scheme XVI. In pathways A and C, the oxidation of the acyl serine intermediate to an aldehyde is followed by enolization and cyclization to yield an adduct which dehydrates to the oxazole. In pathways B and D, cyclization and dehydration occur first and are followed by either syn or anti dehydrogenation to yield the oxazole.

We elected to distinguish between these mechanisms by the use of chirally labeled serines. These labeled serines have been prepared by several workers⁶⁹ and we obtained labeled material from Professor H. G. Floss of the Ohio State University. Incorporation studies were carried out in four experiments as indicated:

<u>Experiment</u>	<u>Presursor</u>	<u>Objective</u>
1	$(S)-[G-^3H]serine$ <hr/> $(S)-[U-^{14}C]serine$	Control experiment to demonstrate that the methods used do not result in complete loss of tritium.
2	$(S)-[G-^3H]serine$ <hr/> $(RS)-[carboxy-^{14}C]serine$	To determine if (R) serine is incorporated as well as (S) serine.



Hypothetical Pathways for Oxazole Formation

Scheme XVI

- | | | |
|---|--|---|
| 3 | <u>(2S,3R)-[3-³H]serine</u>
(S)-[U- ¹⁴ C]serine | To determine if the (3-pro-R) hydrogen is retained in the oxazole ring. |
| 4 | <u>(2S,3S)-[3-³H]serine</u>
(S)-[U- ¹⁴ C]serine | To determine if the (3-pro-S) hydrogen is retained in the oxazole ring. |

The feeding experiments were carried out by mixing the appropriate amounts of ³H and ¹⁴C-labeled serine to obtain a ³H to ¹⁴C ratio in the range 2-6. The ratio was determined by taking ten percent of the precursor mixture, adding it to a known amount of unlabeled serine and recrystallizing to a constant ³H to ¹⁴C ratio. The remaining ninety percent of the mixture was added to an STA-14 production medium containing Streptomyces virginiae after 24 hours of growth. The virginiamycin M₁ and M₂ was isolated and purified as described in the Experimental section.

Serine, as many other amino acids, is known as an active metabolite meaning that it can be incorporated into several parts of the virginiamycin skeleton by various metabolic conversions. Thus [3-¹³C]serine was found by Kingston et al. to be incorporated into acetate-derived portions of the antibiotic, presumably via conversion of serine to acetyl-CoA via pyruvate.⁸ It is thus essential to confirm that any observed incorporation of tritium from [3-³H]serine is in fact located in the oxazole ring. The isolated virginiamycin was therefore degraded by hydrogenation and acid hydrolysis and the serine portion of the oxazole was isolated as N-benzoylalanine. The literature hydrogenation

procedure² of PtO₂ in EtOH for 24 hours gave poor yields in our hands, but the process was improved by using PtO₂ and acetic acid for the hydrogenation. Table IV gives the various hydrogenation conditions and the yield for the overall process. The N-benzoylalanine isolated from the VM₁ was then mixed with unlabeled N-benzoylalanine and recrystallized to a constant ³H to ¹⁴C ratio.

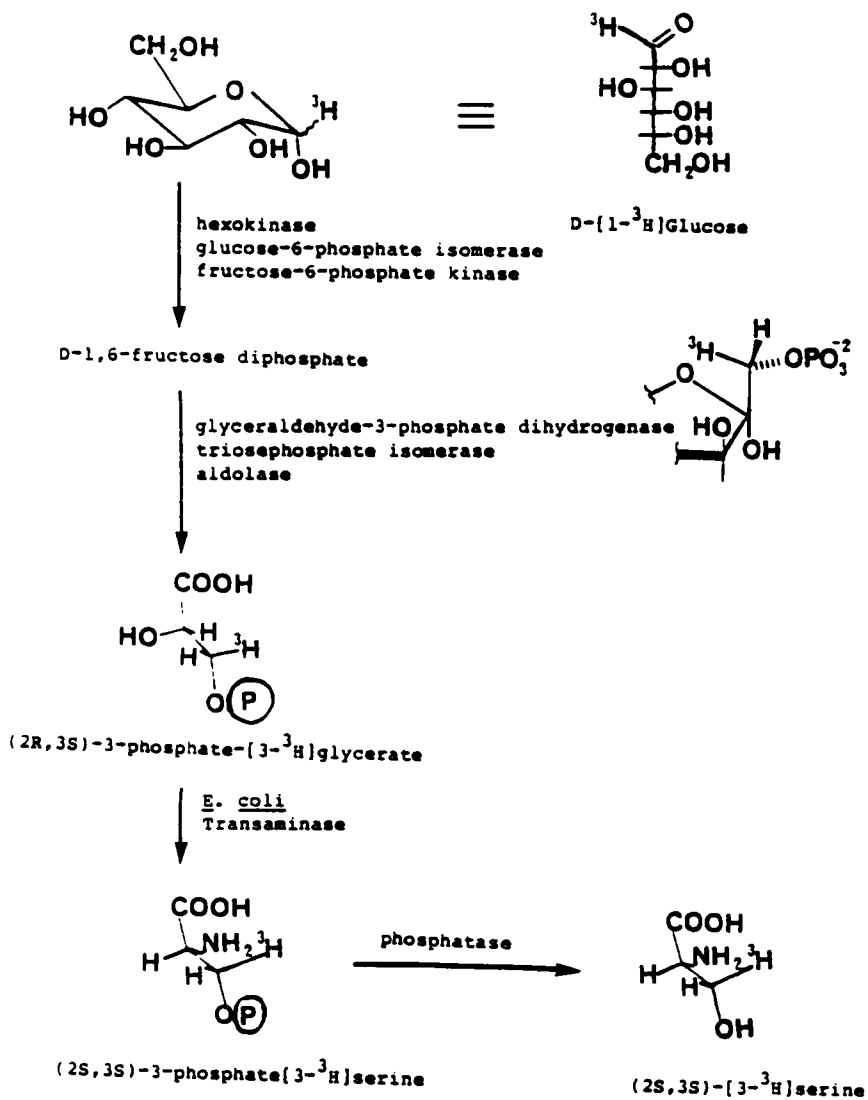
The (2S,3R)-[3-³H]serine and (2S,3S)-[3-³H]serine used in this work were supplied as previously noted by Prof. H. G. Floss of Ohio State University. The method of preparation was the enzymatic synthesis of (2S,3R)-[3-³H]serine and (2S,3S)-[3-³H]serine from D-[1-³H]mannose and D-[1-³H]glucose, respectively⁶⁹. Schemes XVII and XVIII outline these procedures.

The results of the experiments are shown in Table V. As one examines these results, there are several points which should be reviewed. First is the methodology of determining the ³H to ¹⁴C ratio. The mixtures of precursors were prepared by mixing the compounds using the apparent activities, followed by counting of the crude mixture's radioactivity to obtain a ³H to ¹⁴C ratio. The ratios were usually less than predicted due to errors in preparing the small aliquots of precursor. Also, the crude mixture ratios are crude ratios in themselves, used only to verify that an acceptable ratio of ³H to ¹⁴C labeled compounds had been prepared. Internal standards were not used to correct for quenching or solvent affects. The crude mixture ratios are crude approximations, not representing the true ³H to ¹⁴C ratios.

Table IV

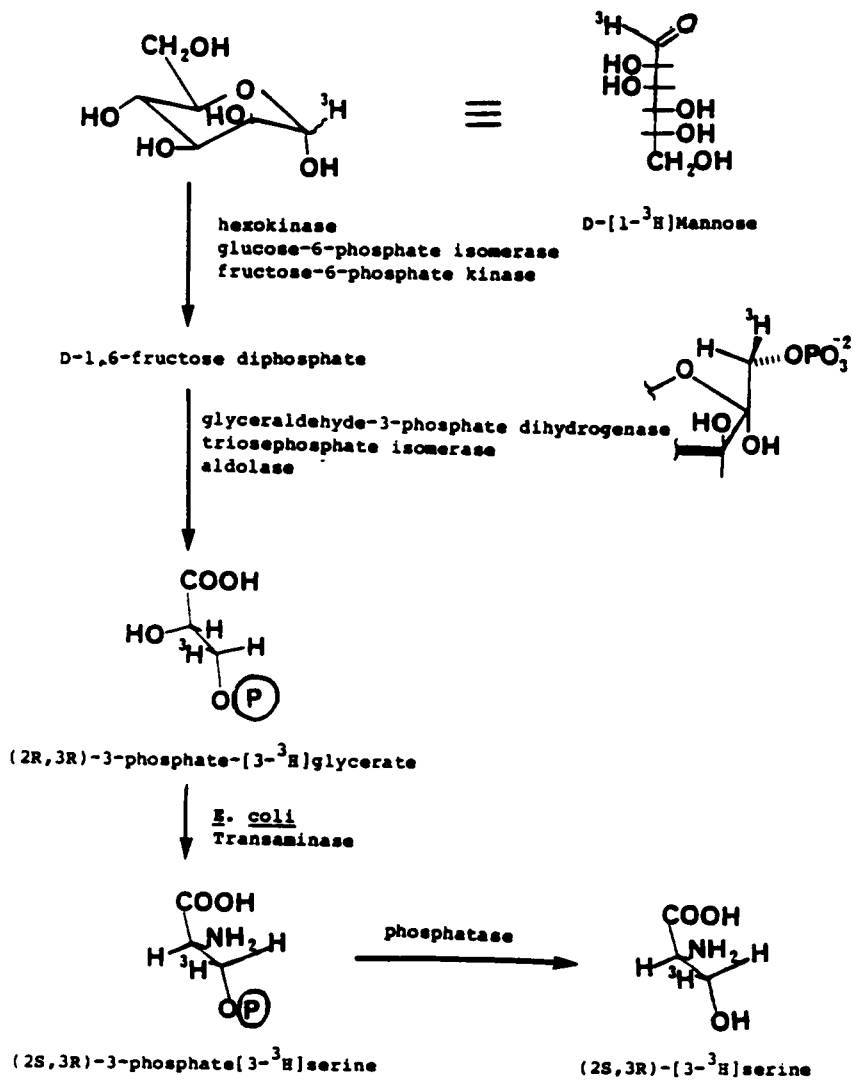
Yields of N-Benzoylalanine from Virginiamycin M₁
Hydrogenation, Hydrolysis and Benzoylation

<u>Substrate</u>	<u>Catalyst</u>	<u>Solvent</u>	<u>Yield N-benzoylalanine</u>
VM ₁	PtO ₂	Acetic Acid	25%
VM ₁	PtO ₂	95% EtOH	1%
VM ₁	5% Pd/C	95% EtOH	1-2%
Control experiment benzoylation yield			66%



Synthesis of (2S,3S)-[3-³H]Serine

Scheme XVII



Synthesis of (2S,3R)-[3-³H]Serine

Scheme XVIII

Table V

Incorporation of Labeled Serine into the Oxazole Portion of Virginiamycin

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	(S)-[6- ³ H]serine	(S)-[1- ¹⁴ C]serine	(S)-[6- ³ H]serine	(RS)-[carboxyl- ¹⁴ C]serine	(2S,3R)-[3- ³ H]serine	(2S)-[1- ¹⁴ C]serine	(2S,3S)-[3- ³ H]serine	(2S)-[1- ¹⁴ C]serine
specific activity	1.9 Ci/mmol	155 mCi/mmol	1.9 Ci/mmol	56.3 mCi/mmol	100 mCi/mmol	145 mCi/mmol	100 mCi/mmol	145 mCi/mmol
amounts mixed	100 μ Cl	10 μ Cl	100 μ Cl	10 μ Cl	10 μ Cl	1.1 μ Cl	10 μ Cl	1.1 μ Cl
³ H to ¹⁴ C ratio mixture	10.83	1	6.6	1	5.34	1	6.5	1
³ H to ¹⁴ C ratio after recrystallisation	6.02	1	4.82	1	2.42	1	3.32	1
³ H to ¹⁴ C ratio for VM ₁	7.24	1	9.33	1	2.81	1	2.74	1
specific activity of VM ₁	112.3 μ Cl/mmol	15.57 μ Cl/mmol	3.88 μ Cl/mmol	0.415 μ Cl/mmol	0.0426 μ Cl/mmol	0.0150 μ Cl/mmol	0.754 μ Cl/mmol	0.275 μ Cl/mmol
Z incorporation into VM ₁	1.8	2.5	6.7 x 10 ⁻²	7.3 x 10 ⁻³	3.1 x 10 ⁻³	1.1 x 10 ⁻²	0.195	0.715
Z specific incorporation VM ₁ (x10 ⁻³)	5.91	10.0	0.204	0.737	0.0426	0.0103	0.754	0.189
³ H to ¹⁴ C ratio VM ₂	NA	NA	NA	NA	2.35	1	2.68	1
specific activity VM ₂	NA	NA	NA	NA	0.456 μ Cl/mmol	0.194 μ Cl/mmol	5.65 μ Cl/mmol	2.09 μ Cl/mmol
Z incorporation VM ₂	NA	NA	NA	NA	5.78 x 10 ⁻²	.223 x 10 ⁻²	3.54	11.9
Z specific incorporation VM ₂ (x10 ⁻³)	NA	NA	NA	NA	0.456	0.134	5.65	1.44
³ H to ¹⁴ C ratio of NBA	2.95	1	3.46	1	1.60 ^e	1 ^f	0.38	1
specific activity NBA	0.94 μ Cl/mmol	0.32 μ Cl/mmol	0.43 μ Cl/mmol	0.124 μ Cl/mmol	0.104 μ Cl/mmol	0.065 μ Cl/mmol	0.00519 μ Cl/mmol	0.0156 μ Cl/mmol
Z incorporation NBA (x10 ⁻³)	7.9	8.3	1.37	3.95	1.15	7.22	2.29	54.0
Z specific incorporation NBA (x10 ⁻⁵)	5.2	20.6	2.26	22.0	10.4	4.51	0.599	1.08
Z ³ H retained	49Z	NA	72Z	1	66Z	1	11Z	1
theoretical ³ H to ¹⁴ C ratios	NA	NA	4.81 ^b	1	2.42 ^b	1	0 ^b	1
			0 ^c	0.5	0 ^f	1	3.32 ^f	1
			2.41 ^d	1	1.21 ^g	1	1.86 ^g	1

NOTES:

- NA - Not Applicable
a - based on NBA obtained from VM₂
b - if no (R)-serine incorporated
c - if no (S)-serine incorporated
d - if both (R)- and (S)-serine incorporated
e - if only (R)-serine incorporated
f - if only (S)-serine incorporated
g - if both (R)- and (S)-serine incorporated

To obtain the true value for the ^3H to ^{14}C ratio, an aliquot (10%) of the crude mixtures was added to unlabeled serine and recrystallized several times until a constant ^3H to ^{14}C ratio was obtained. In all of the experiments, the ^3H to ^{14}C ratio after recrystallization were less than the crude mixtures. These differences can be accounted for by possible quenching affects in the crude mixtures, by free ^3H in the form of ^3HCl or other impurities removed by recrystallization.

It was important that the true ^3H to ^{14}C ratio for serine be determined as the biosynthetic pathway would be selective for serine and any impurities would cause the crude ratios to be in error (larger than they should be). Alternatively, it can be argued that feeding the crude mixtures without purification would cause errors in the resulting product ratios; however, the specificity of the biosynthetic pathway for serine would prevent the incorporation of impurities. An alternative approach would have been to purify the precursors through HPLC, but it is also limiting.

A second point of concern is the low % incorporations and % specific incorporations. The incorporation of amino acids such as serine and proline into a secondary metabolite, i.e. virginiamycin, is dependent on the amino acid pool concentration within the organism as well as the free amino acid in the production medium. Therefore, the feeding of a radioactive precursor involves only a few micromoles of precursor while there may be several grams of unlabeled precursor representing several hundred or thousands of millimoles of precursor resulting in very small % specific incorporations. This is why the use

of the ^3H to ^{14}C ratio technique of Spenser, et. al.⁹⁷ is important, by using their technique the % incorporations and % specific incorporation values which can vary from production run to production run making the interpretations difficult, are not the determining factors.

Thirdly, the values of the ^3H to ^{14}C ratios for the virginiamycin M_1 and M_2 are not representative of the pathways being studied and cannot be considered as evidence to support or dismiss a closure pathway for the acylserine unit. The reason for their difference from the n-benzoylalanine and invalidity is that the precursor has been shown by Kingston, et. al.⁸ to be incorporated in several locations within the virginiamycin skeleton. Therefore, to study the origin of the oxazole and dehydroproline units the antibiotic must be degraded and these portions of the compounds isolated.

The first experiment had a precursor ^3H to ^{14}C ratio of 6.02 and a product ratio of 2.95, indicating a retention of 49% of the tritium. This experiment would have been expected to have a retention of 33% of the tritium if the precursor (2S)-[G- ^3H]serine were completely randomly labeled, and the observed retention thus suggests that the precursor was not randomly labeled. (This label could vary from 50 to 25% depending on the method and/or materials used in the synthesis.) Nevertheless, the retention of 49% clearly indicates that washout of tritium does not occur at the number 3 carbon.

An alternative explanation for the 49% retention of tritium would be an isotope affect due to the ^{14}C carbons in the 2 and 3 position of

(2S)-[U-¹⁴C]serine where ¹⁴C-H bonds are being broken and the carbons are also changing hybridization from sp³---sp². The K_{12C} to K_{14C} could range from 1.01 to 1.5. This would give an artificially high ³H to ¹⁴C ratio.

The second experiment had a precursor ³H to ¹⁴C ratio of 4.81 and a product ratio of 3.46, with an apparent tritium retention of 72%.

The theoretical incorporation of these labeled serines can be calculated under three different assumptions. In case A, it is assumed that only (S)-serine is incorporated. In case B, it is assumed that only (R)-serine is incorporated, and in case C, it is assumed that both (R) and (S) serine are incorporated equally. The predicted ratio for each case is indicated below, assuming incorporation of 49% of the ³H label from (S)-[G-³H]serine as previously determined.

$$\frac{(S)-[G-^3H]serine(4.81 \mu Ci)}{0.5 \mu Ci (R)[carboxyl-^{14}C]serine + 0.5 \mu Ci (S)[carboxyl-^{14}C]serine} = \frac{^3H}{^{14}C}$$

Let x = (S)-[G-³H]serine
 y = (R)-[carboxyl-¹⁴C]serine
 z = (S)-[carboxyl-¹⁴C]serine

Case A	$\frac{4.81x}{(0.5y + 0.5z)}$	if y=0 no (R)-serine incorporated	$\frac{(4.81x)49/100}{0.5z} = 4.81$
Case B	$\frac{4.81x}{(0.5y + 0.5z)}$	if x=0 y=0 no (S)-serine incorporated	$\frac{0}{0.5y} = 0$
Case C	$\frac{4.81x}{(0.5y + 0.5z)}$	if both (R) and (S) are incorporated	$\frac{(4.81x)49/100}{(0.5y + 0.5z)} = 2.41$

The result from the experiment of 3.46 lies between the predictions of cases A and C. The most likely explanation of this result is that only (S)-serine is incorporated into VM₁ as in case A. The fact the ³H retained is not exactly twice the value of experiment 1 may be due to the (S)-[U-¹⁴C]serine not being completely homogeneous or the amounts of (RS)-[carboxyl-¹⁴C]serine not being exactly 50:50. Alternatively, the result could indicate some minor utilization of (R)-serine in the pathway.

Based on the ³H to ¹⁴C ratio it would appear that the above results are valid; however, when one examines the % specific incorporation ratios for the ³H and ¹⁴C in experiments 1 and 2 the ratios are 0.25 and 0.10, respectively. Thus suggesting that (R,S)-[carboxyl ¹⁴C]serine was incorporated 2.5 times better than the (S)-[U-¹⁴C]serine. If the ³H to ¹⁴C ratio had agreed with the specific incorporation by giving the results of case C the interpretation would lead to both being equally incorporated.

In contrast to case C conclusions, the % specific incorporation ratio leads one to suggest that a ¹⁴C isotope affect was occurring in experiment 1 leading to the large % retention of ³H (larger ³H to ¹⁴C ratio). Experiment 2 would not possess this isotope affect thereby decreasing the ratio from 4.81 (case A) due to a larger incorporation of ¹⁴C. The best explanation of these results appears to be that (S)-serine is the major precursor and a minor amount of (R)-serine is incorporated.

In experiment three, the serine precursor ³H to ¹⁴C ratio was 2.42

and the ^3H to ^{14}C ratio of the product was 1.60 with 66% of the tritium retained. The theoretical results would be retention of 0% for complete loss of the (pro-R) hydrogen, 50% for a stereorandom process, and 100% corresponding to complete retention of the (pro-R) hydrogen. This result, thus, suggests that the (pro-R) hydrogen is partially retained during the biosynthetic process.

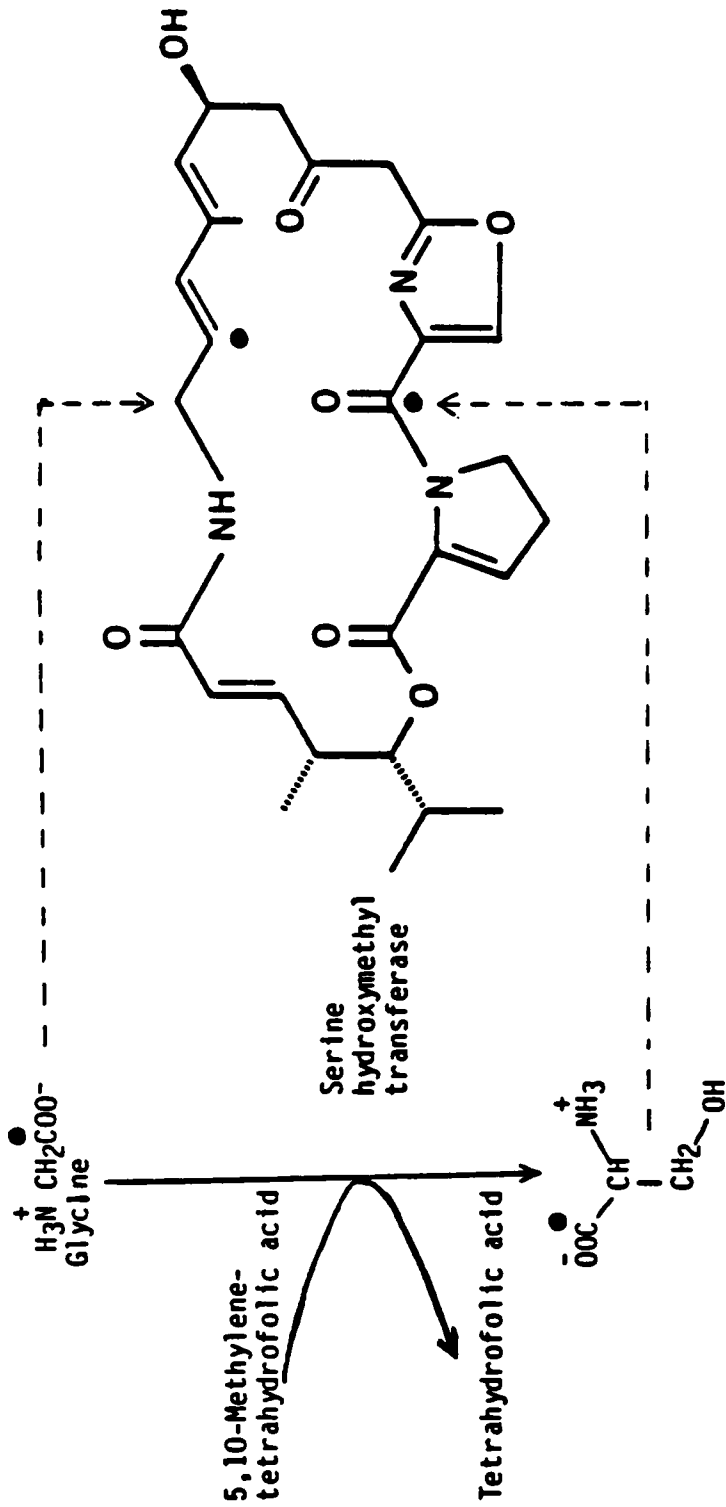
In the above result, the ^3H to ^{14}C ratio for the oxazole ring portion of virginiamycin skeleton was obtained by degradation of virginiamycin M_2 due to poor yields from degradation of VM_1 and low incorporations. The poor yield and low incorporation resulted in non detectable radioactivity for ^3H and ^{14}C .

Experiment four complements the result of the previous experiment with a precursor ^3H to ^{14}C ratio of 3.32 and a product ratio of 0.38. The theoretical complementary results would be 100% for complete retention of the (pro-S) hydrogen, 50% for a stereorandom process, and 0% corresponding to complete loss of the (pro-S) hydrogen. The retention of 11% of the tritium label shows that the (pro-S) hydrogen is lost during the incorporation. The fact that the retention of tritium in experiments three and four are not 100% and 0% respectively can be explained by the known stereochemical randomization accompanying the competing but reversible side reaction of the transfer of the hydroxymethyl group of serine to tetrahydrofolate by serine transhydroxymethylase⁹⁸ (Scheme XIX). In addition, a secondary isotope effect may also be a factor; such an isotope effect would be of the order 1.2-1.3 for a reaction involving the conversion of an sp^3 into an

sp^2 hybridized carbon⁹⁹ (Scheme XX). This isotope effect would cause a reduced incorporation of the labeled (pro-R) hydrogen (Scheme XX).

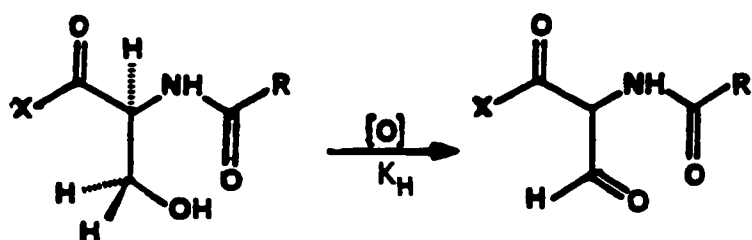
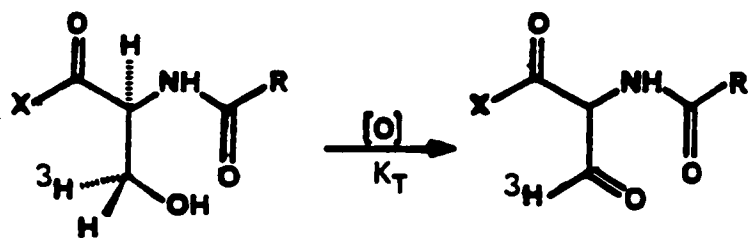
Two basic facts are obtained from the four experiments: that only (2S)-serine is incorporated into the oxazole moiety over (2R)-serine and that the 3-(pro-R) hydrogen is retained. These stereochemical results allow the postulation of a biosynthetic pathway. First, only pathways involving (S)-serine and retaining the 3-(pro-R) hydrogen are considered as plausible explanations so pathways A and B in Scheme XXI are incorrect. The possibilities are pathways C and D. Pathway D involves cyclization dehydration, followed by an anti-dehydrogenation process while C is an oxidation-enolization followed by dehydration (Scheme XXII). Literature examples of dehydrogenation process reveal that the reaction normally occurs by a syn process, as in the biosynthesis of cryptochinuline,¹⁰⁰ mycelianamide,¹⁰¹ elaiomycin¹⁰² and the side chain dehydrogenation of n-benzyl-oxycarbonyl-L-tryptophan¹⁰³ (Figure 16). This suggests (but does not require) that the oxazole ring formation does not occur via anti dehydrogenation, thereby eliminating pathway D. Pathway C thus converges as the most reasonable one on the basis of the available evidence.

The stereochemistry of the proposed oxidation of pathway C is unknown. In the case of oxidation by alcohol dehydrogenase, ethanol binding occurs such that the 1-(pro-R) hydrogen is transferred¹⁰⁴. In contrast, the conversion of (2R,3R)[2,3-³H₂]cysteine into benzylpenicillin, which may proceed via a thioaldehyde intermediate, proceeds with retention of the tritium label¹⁰⁵ (Figure 17). If this



Serine
Interconversion of Serine and Glycine by the Tetrahydrofolic Acid Cycle

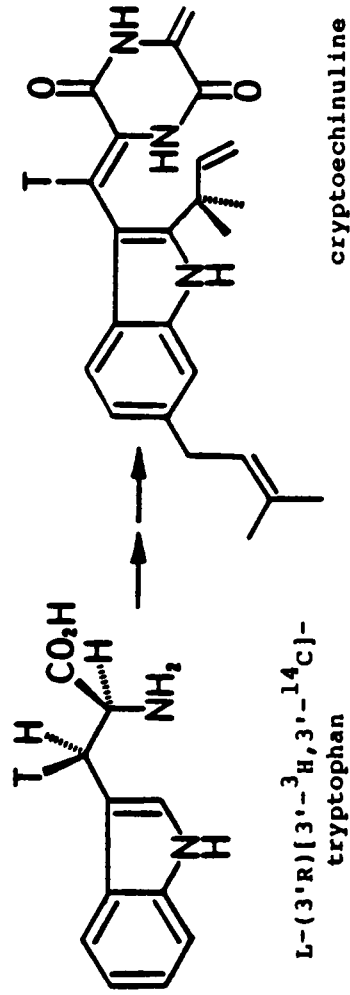
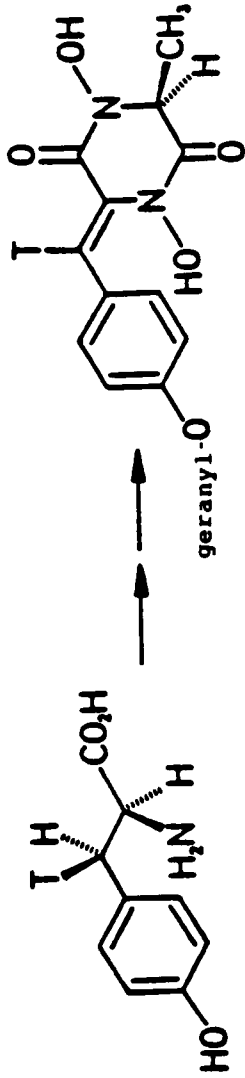
Scheme XIX



$$\frac{K_\text{H}}{K_\text{T}} = 1.2 \text{ to } 1.3$$

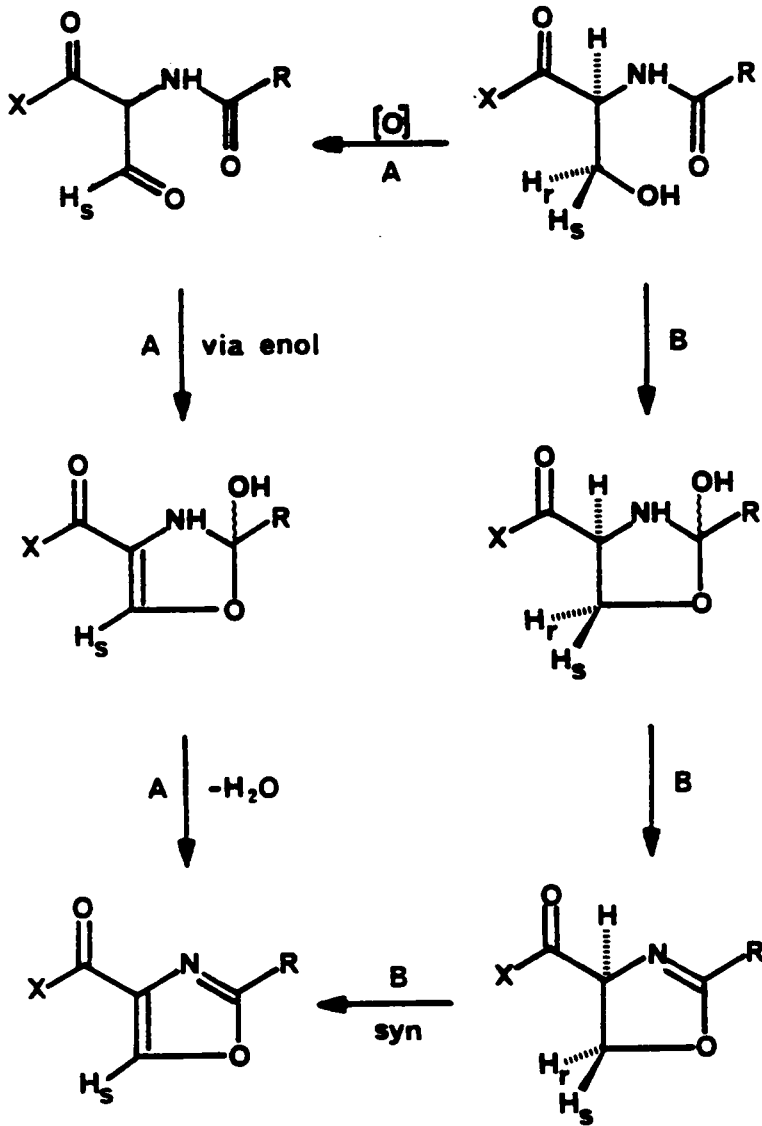
Secondary Isotope Effect in Formation of the Oxazole Ring

Scheme XX



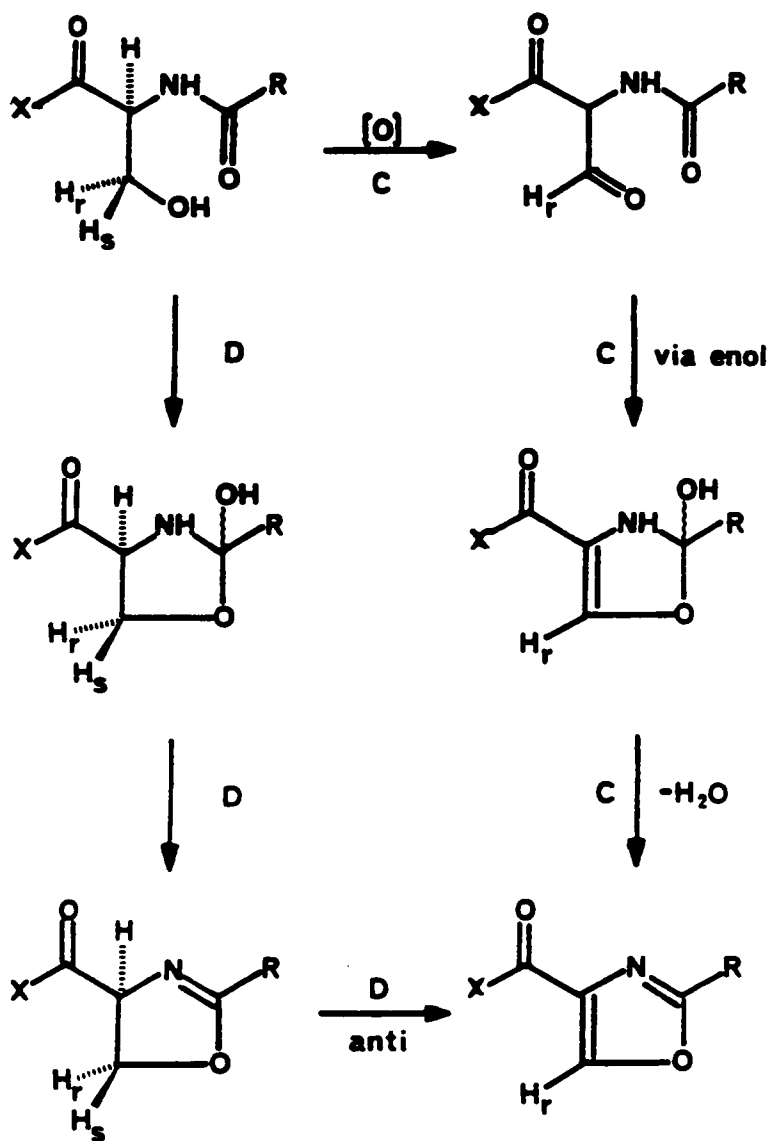
Examples of Syn Dehydrogenation Processes

Figure 16



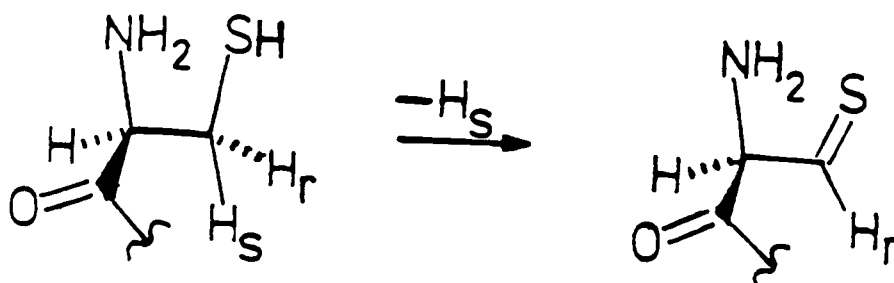
Pathways Retaining the (pro-S) Hydrogen

Scheme XXI



Pathways Retaining the (pro-R) Hydrogen

Scheme XXII



The Benzyl Penicillin Thioaldehyde Intermediate

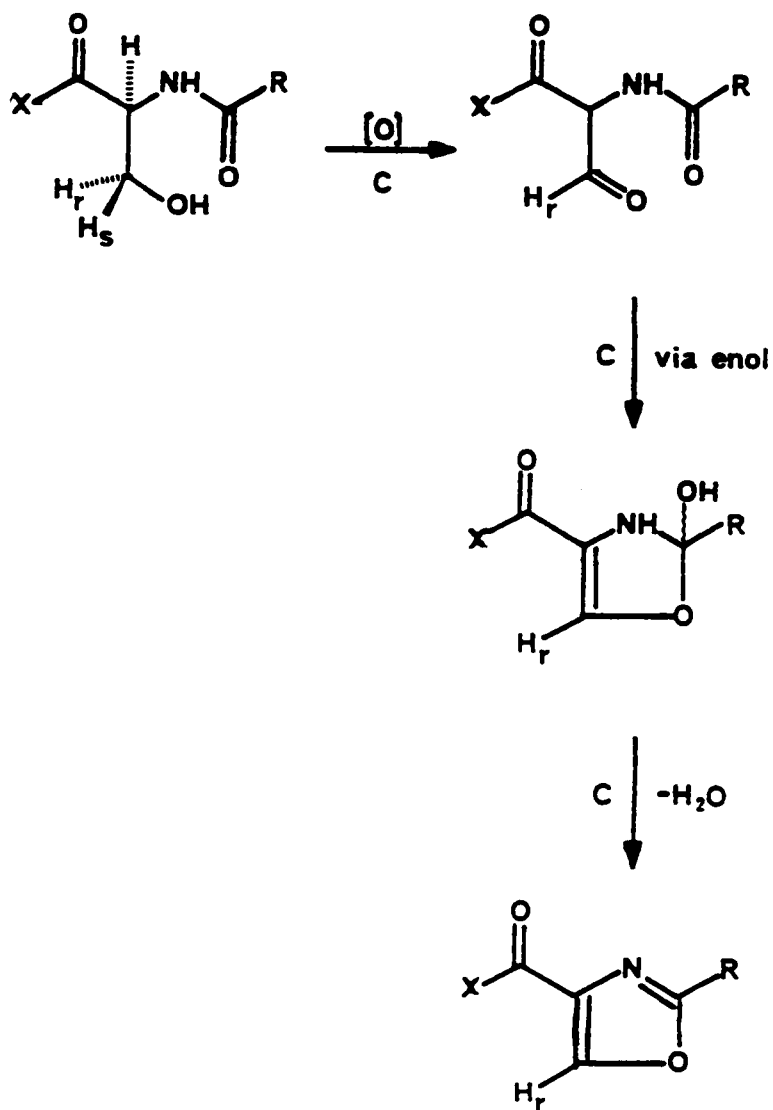
Figure 17

latter stereochemistry is followed in the present case, then the oxidation of serine to its aldehyde proceeds with retention of the 3- (pro-R) hydrogen, and pathway C is the correct one for the formation of the oxazole ring (Scheme XXIII).

As indicated earlier, the interpretation given above is only meaningful on the assumption that the oxygen of the oxazole ring derives from the serine unit. Although there is clear precedent for this assumption in the formation of the thiazole ring system from cysteine in other antibiotics^{86,96} it was, nevertheless, desirable to obtain independent evidence for this ring closure pathway. Our attempts to do this are described in the next section.

2. Incorporation of Sodium [1-¹³C, ¹⁸O₂]Acetate

As mentioned above, the purpose of incorporating sodium [1-¹³C, ¹⁸O₂]acetate into virginiamycin M₁ was to examine the origin of the oxygen atom in the oxazole ring. There are four possible pathways for the formation of this ring (Scheme IV). Pathways A and B are based on the oxygen atom of serine being the nucleophile, and pathways C and D are based on the oxygen atom of acetate being the nucleophile. LeFevre's incorporation study of sodium [1-¹³C]acetate into VM₁ showed an incorporation as in figure 18.⁸ It appeared that feeding sodium [1-¹³C, ¹⁸O₂]acetate would show this same ¹³C enrichment together with an isotopic shift in the NMR for ¹³C-labeled carbons attached to an oxygen-18 atom. The actual reason for the isotopic shift is unknown except that the oxygen-18 changes the electron density around the carbon



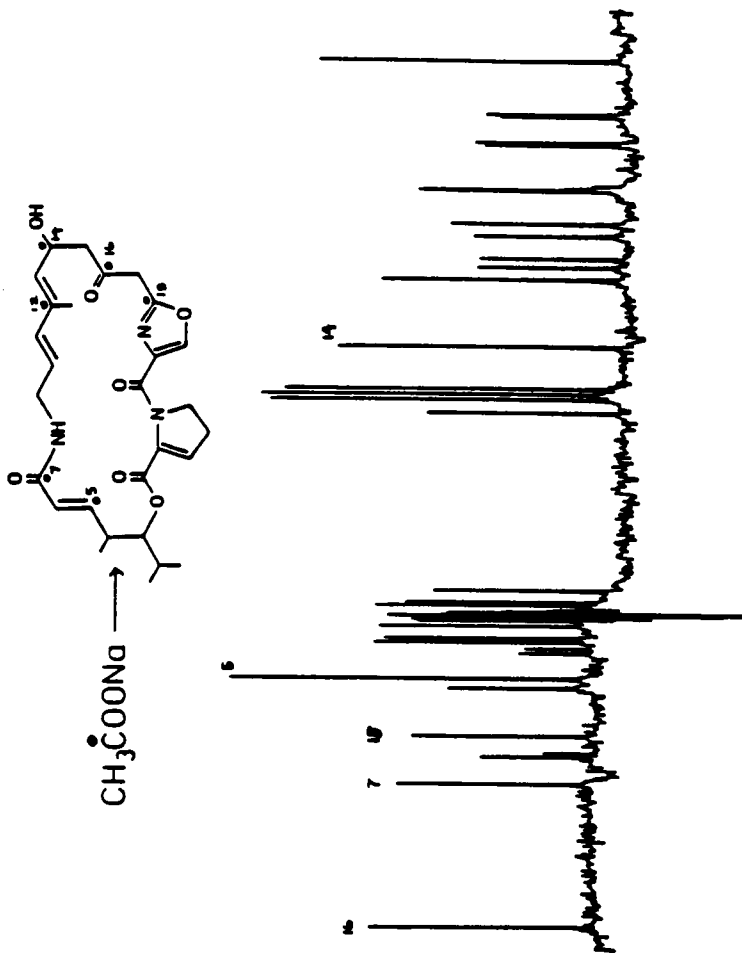
Proposed Pathway for Oxazole Formation

Scheme XXIII

nucleus, with the size of the shift varying with the type of carbon oxygen bond. Literature examples of carbon-oxygen isotopic shift are shown in Table VI. Thus, the origin of four of the oxygen atoms (those at C-7, C-14, C-16 and the oxazole oxygen at C-18) could be examined by this technique.

Sodium [$1\text{-}^{13}\text{C}, ^{18}\text{O}_2$]acetate¹⁰⁶ was prepared by refluxing Na^{13}CN in dry methanol with dry methyl iodide. The ^{13}C acetonitrile was isolated by distillation and hydrolyzed by treatment with 1.0N potassium t-butoxide in butanol with H_2^{18}O . The sodium acetate was isolated after purification by lyophization and titration with 1N NaOH. Its ^1H NMR spectrum in D_2O showed a doublet at 1.9 ppm, and its ^{13}C NMR spectrum showed a doublet at 26.0 ppm and three signals at 183.818, 187.792 and 183.766 ppm corresponding to signals for $^{13}\text{C}^{16}\text{O}_2$, $^{13}\text{C}^{16}\text{O}^{18}\text{O}$, $^{13}\text{C}^{18}\text{O}_2$ (Figure 14). Mass spectrometric analysis of the acetate was not possible without conversion to the free acid which could cause ^{18}O exchange; therefore, the ratio of the labeled compounds was determined by ^{13}C NMR to be 50% $\text{CH}_3^{13}\text{C}^{18}\text{O}_2\text{Na}$, 25% $\text{CH}_3^{13}\text{C}^{18}\text{O}^{16}\text{ONa}$, 25% $\text{CH}_3^{13}\text{C}^{16}\text{O}_2\text{Na}$.

The labeled acetate was fed to Streptomyces virginiae two times in batch of 245 mg and 200 mg and the virginiamycin M_1 isolated and purified as described in Chapter 3, Sections G and E. Approximately 12.0 mg of pure virginiamycin were isolated and examined by ^{13}C NMR. The same ^{13}C enrichments were observed as in LeFevre's sodium [$1\text{-}^{13}\text{C}$]acetate experiment. However, the spectroscopic resolution was insufficient to detect the presence of any isotopic shift, and extended scanning of the sample in an increased resolution mode caused



^{13}C NMR Spectra from Incorporation of [^{13}C]Acetate 8

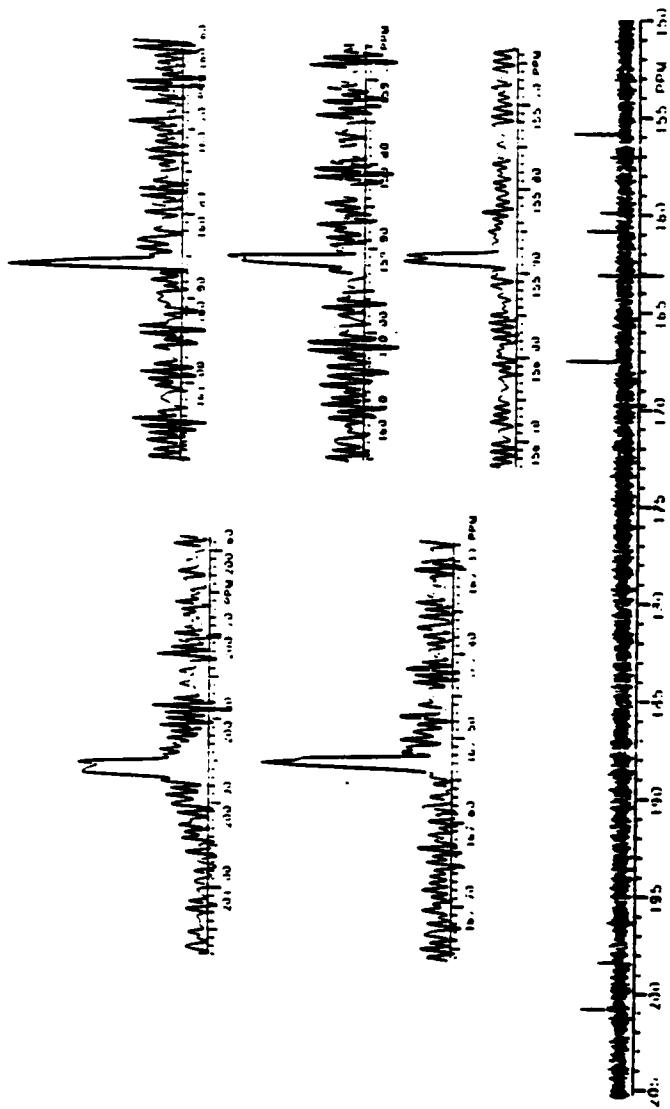
Figure 18

decomposition of the sample due to decoupler heat over a long period of time.

An additional feeding experiment was carried out by SmithKline-RIT in an attempt to produce larger quantities of the antibiotic. However, isolation of VM₁ from the crude virginiamycin complex yielded only 7 mg of pure antibiotic. The ¹³C NMR spectrum of the sample was obtained by Mike Kolpak at SmithKline and French. The spectra showed a low incorporation of the acetate and no peaks or shoulders which could be isotopic shifts (Figure 19).

Mass spectrometry was carried out on pure unlabeled virginiamycin M₁ (Figure 20). The peak at m/z 221 was believed due to the ion of interest formed by fragmentation of the antibiotic. Therefore, it was possible that mass spectrometric analysis of VM₁ from the incorporation study would show ¹³C and ¹⁸O enrichments if present. Mass spectrometric analysis of the labeled VM₁ showed an M-18 peak with an estimated 2% incorporation of carbon 13 and a large M+2 peak possibly meaning some ¹⁸O was incorporated. However, repeated attempts to run the spectrum under various conditions failed to yield reproducible peaks at m/z 221. High resolution measurements on the weak ion at m/z 221 also failed to yield interpretable results.

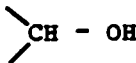
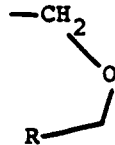
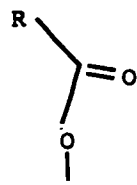
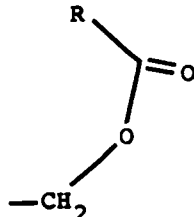
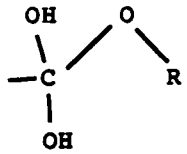
Although these attempts to determine the origin of the oxazole oxygen atom failed for technical reasons, the most likely pathway of ring closure is one in which the serine oxygen is retained, by analogy with the formation of the thiazole system from cysteine as discussed earlier. It is thus suggested that pathway C of Scheme XXIII is the



¹³C-NMR Spectra from Sodium [1-¹³C, 18 O₂]acetate Incorporation

Figure 19

Table VI
Isotopic Shifts of ^{13}C Resonances by ^{18}O

<u>Compound</u>	<u>Carbon-Oxygen Bond</u>	<u>Isotopic Shift</u> (ppm/Hz at ~50 MHz)		<u>Ref.</u>
Nargenicin A ₁		0.02 ppm	1.26 Hz	93
Monensin A		0.02 ppm	1.26 Hz	106
Nargenicin A ₁		0.04 ppm (C=O)	2.51 Hz	93
Nargenicin A ₁		0.03 ppm (CH ₂)	1.89 Hz	93
Monensin A		0.03 ppm	1.89 Hz	106

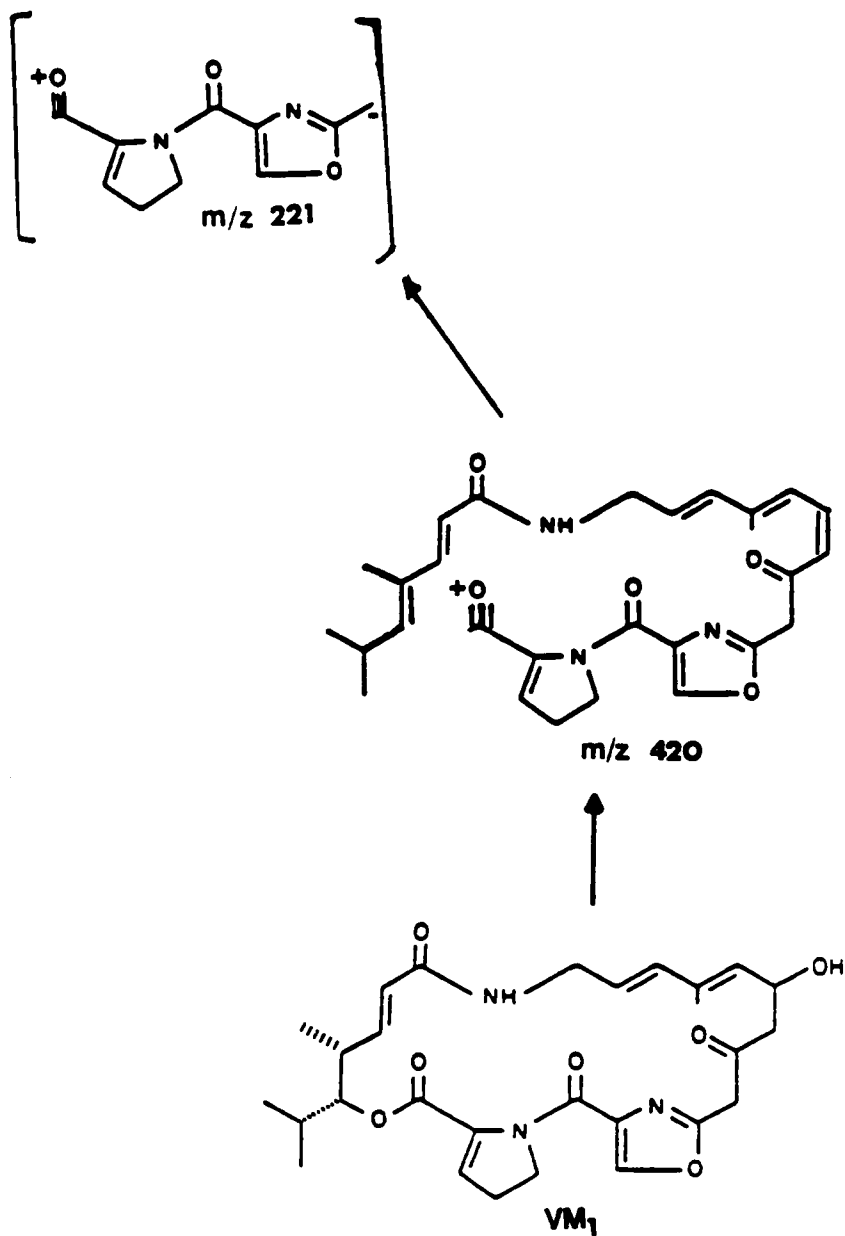
most probable pathway of formation of the oxazole ring.

3. Incorporation of Labeled Prolines into the Dehydroproline Residue of Virginiamycin M₁.

Dehydroamino acid residues have been of interest due to the numerous dehydropeptides that have been isolated, most of which like virginiamycin are cyclopeptides or cyclodepsipeptides that possess antibiotic activity. Their origin has been the focus of much speculation with the hypothesis of their formation including tautomerization of an acylimine intermediate,⁶⁶ elimination of hydroxy and thio amino acids, and dehydrogenation⁶⁷ (Scheme XXIV).

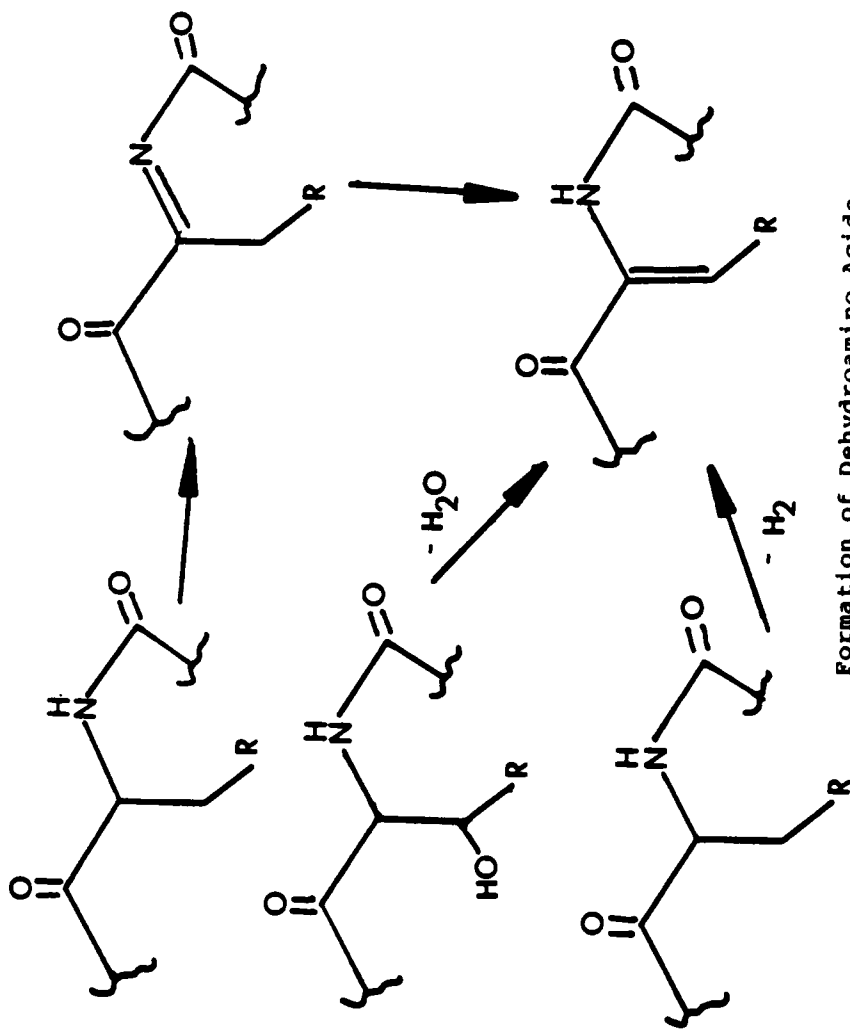
In virginiamycin M₁ the formation of the dehydroproline residue can be viewed as occurring through one of two basic types of mechanisms, dehydration of hydroxylated intermediates or direct dehydrogenation (Scheme XXV).

The dehydration mechanism involves the possibility of isomeric hydroxyproline intermediates (plus their stereoisomers), that could dehydrate to leave a dehydroproline intermediate which would then isomerize to the 2,3 derivative (Figure 21). The involvement of some of these intermediates was examined by LeFevre, who fed (2S,4S)-4-hydroxyproline, (2S,3R + 2R,3S)-[U-¹⁴C]-3-hydroxyproline (cis-3-hydroxyproline) and (2S,3S + 2R,3R)-[U-¹⁴C]-3-hydroxyproline (trans-3-hydroxyproline) and found no incorporation of these compounds.⁶⁴ Therefore, the intermediacy of hydroxyproline intermediates as direct precursors is unlikely, although LeFevre's results do not exclude the



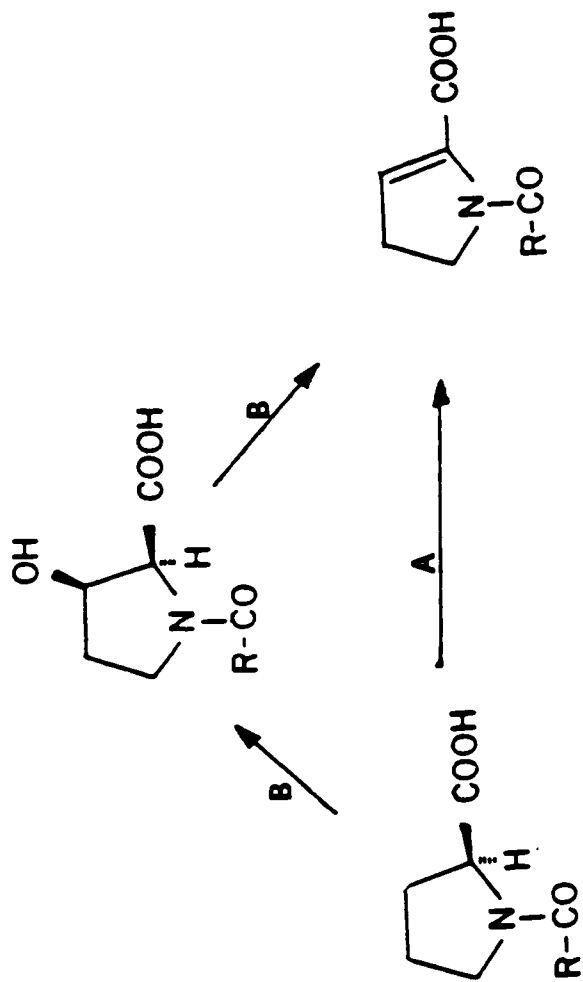
Mass Spectrometry Fragments of Virginiamycin M₁

Figure 20



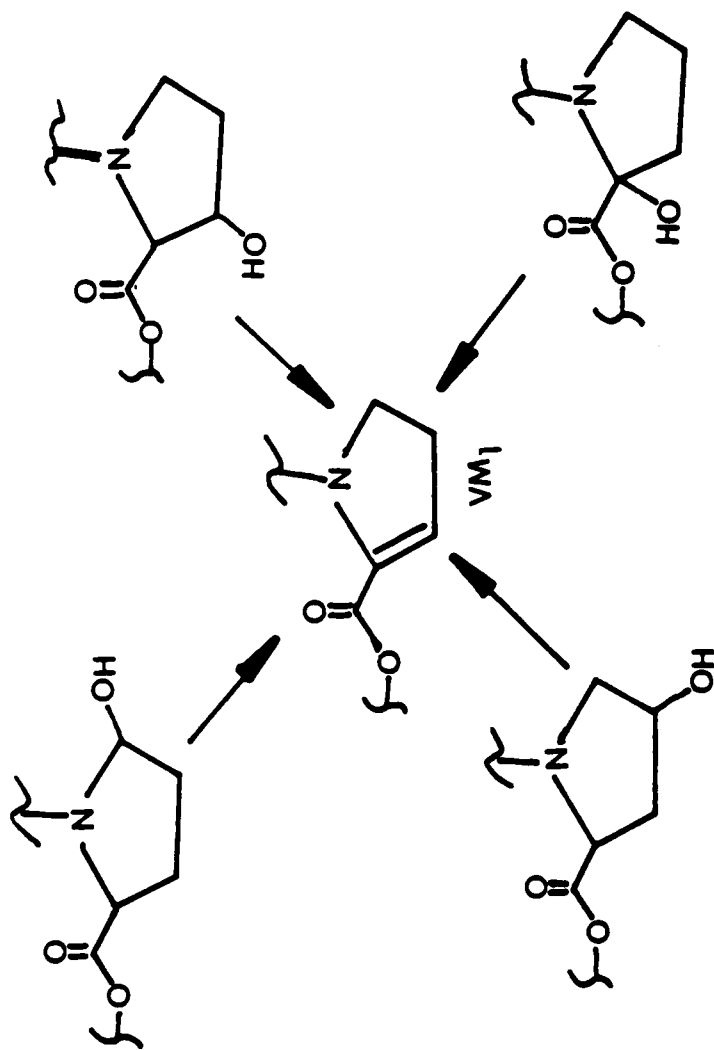
Formation of Dehydroamino Acids

Scheme XXIV



Two Basic Mechanism Leading to Dehydroproline Moiety

Scheme XXV



Possible Hydroxy Proline Precursors

Figure 21

involvement of a hydroxyproline as part of an advanced biosynthetic intermediate.

The only previous result that bears on the stereochemistry of formation of the dehydroproline system is a study by LeFevre⁶⁴, who showed that the tritium label of [3,4-³H₂]proline is retained on incorporation into virginiamycin M₁. A ³H NMR study of the precursor showed that the proline was actually a mixture of tritiated prolines whose major component was (2S,3S,4R)-[3,4-³H₂]proline.⁶³ This suggested that stereochemical control could be present in the formation of the dehydroproline residue and that the stereochemistry of the process could be used to postulate a pathway for dehydroproline formation.

The first goal of the current work was to determine if both (R) and (S) proline are incorporated into virginiamycin M₁ by the experiment below.

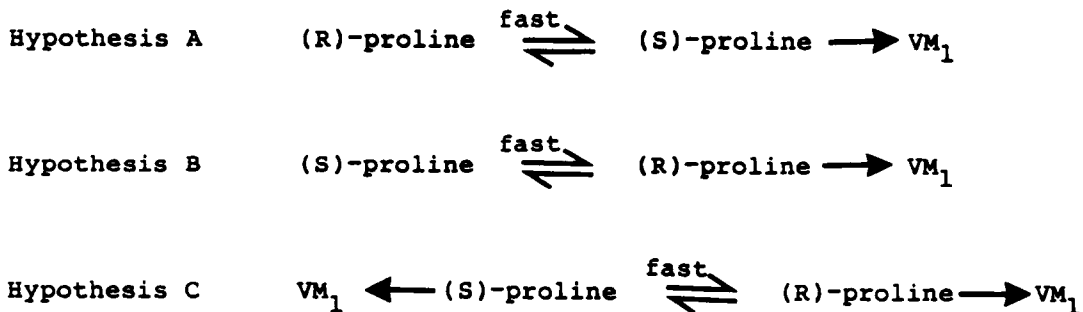
<u>Experiment</u>	<u>Objective</u>
(2S,3S,4R)-[3,4- ³ H ₂]proline	To determine if (R) and (S) proline are incorporated.
(RS)-[carboxyl- ¹⁴ C]proline	

Using the techniques of Spenser⁹⁷ and co-workers a mixture of (2S,3S,4R)-[3,4-³H₂]proline and (RS)-[carboxyl-¹⁴C]proline was prepared with a ³H to ¹⁴C ratio of 15.6. The mixture was fed to S. virginiae 1830 and the virginiamycin M₁ isolated as in the Experimental Section.

The virginiamycin M₁ isolated from this experiment had a ³H to ¹⁴C ratio of 32.2 and no virginiamycin M₂ was found in the production medium. It is not feasible or meaningful to comment on this ratio

since no ^{13}C labeling experiments have been carried out on virginiamycin M_1 to distinguish all incorporation positions within the virginiamycin skeleton, although the majority of the proline was shown by Kingston, et. al.⁹ to reside in the dehydroproline unit. It would also appear that some quenching was occurring in this VM_1 sample; however, the VM_1 must be degraded so the VM_1 ratio was not considered important. The antibiotic was hydrogenated, hydrolyzed and derivatized to yield N-benzoylproline with a ^3H to ^{14}C ratio of 17.7. Theoretically, three results were possible. A doubling of the ratio would indicate that only (S)-proline is incorporated. An unchanged ratio would indicate incorporation of both (R) and (S) proline equally. A ratio of zero (while retaining ^{14}C labeling of the antibiotic) would indicate incorporation of only (R)-proline. In this case the ratio was at 17.7, close to the precursor ratio of 15.6 and thus indicating incorporation of both (R) and (S) proline to the same extent.

With the fact that both (R) and (S) proline are incorporated to the same extent, three hypotheses can be proposed for the incorporation of proline as shown:



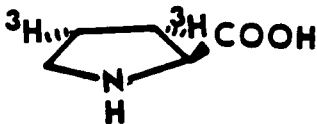
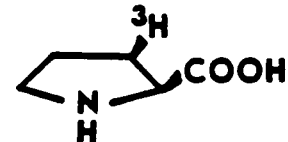
Under hypothesis A, incorporation occurs via (S)-proline, with a rapid racemization occurring as a side reaction to convert (S)-proline to (R) proline. Under hypothesis B, incorporation occurs through (R)-proline, with rapid racemization converting natural (S)-proline into (R)-proline. Under hypothesis C, two independent pathways for incorporation occur from (R) and (S), respectively. These possibilities are depicted schematically in Scheme XXVI.

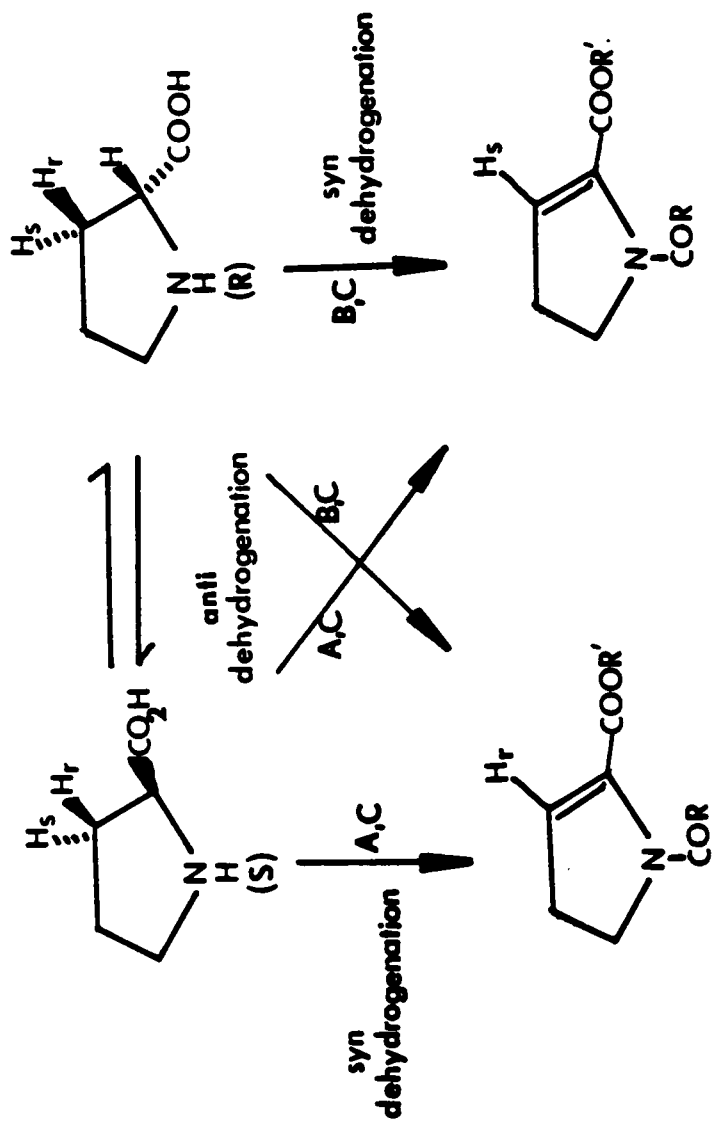
Examination of the stereochemistry of hydrogen loss at C-3 of proline provides a way to distinguish between these pathways. The theoretical possibilities are summarized in Table VII for the incorporation of both (2S,3S,4R)-[3,4-³H₂]proline (as carried out by LeFevre) and (2S,3R)-[3-³H]proline, where the terms syn and anti refer to the overall stereochemistry of the dehydrogenation process. The key conclusion from this analysis is that the two different precursor molecules give clearly different incorporation results, depending on the pathways actually used. It may be noted, however, that this analysis fails to differentiate between, for example, an anti dehydrogenation by pathway A and a syn dehydrogenation by pathway B; such a differentiation must be made on other grounds.

The precursor (2S,3R)-[3-³H]proline was not available commercially, and was thus prepared by a stereospecific synthesis. Literature methods for the preparation of labeled prolines by the displacement of p-toluene sulfonyl groups with lithium aluminum tritide were explored, but these proved to be lengthy and of uncertain yield. Various other routes were investigated before the improved route described below was selected.

Table VII

Theoretical Experiment Results from Feeding Labeled Prolines

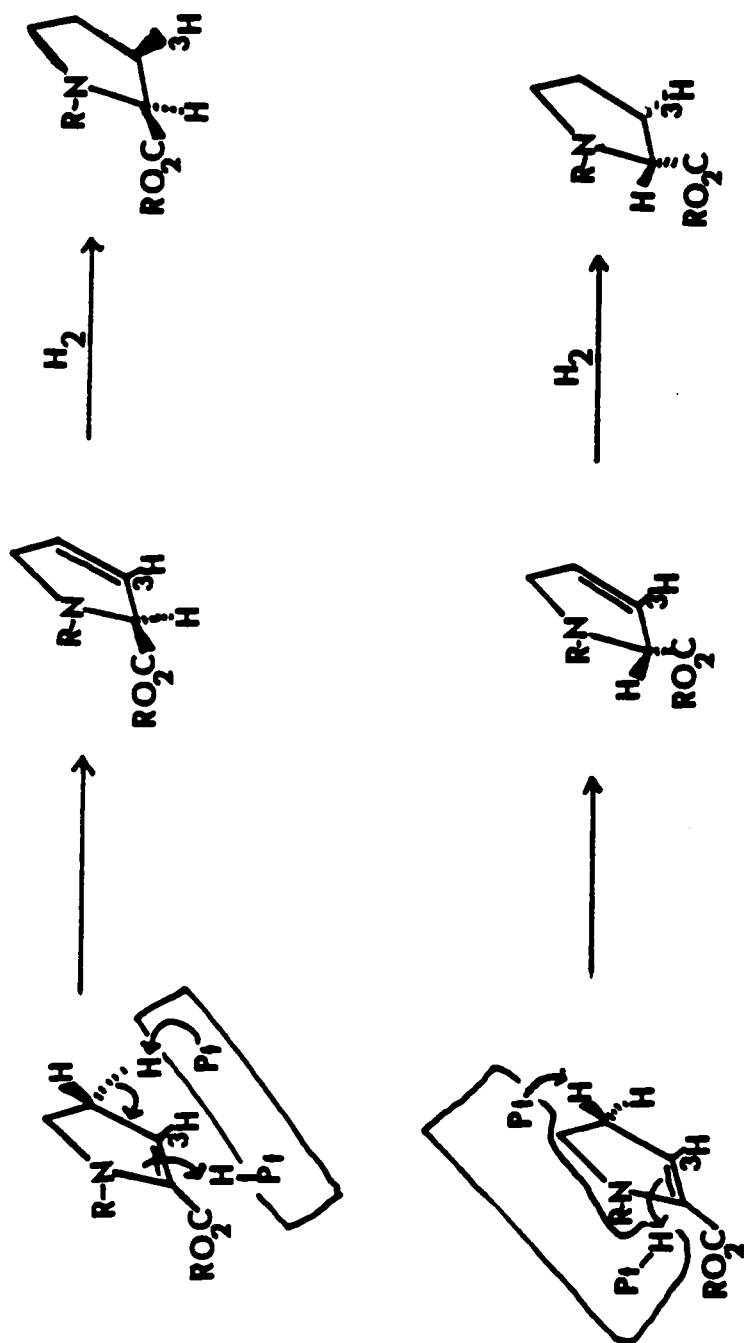
precursor	% retention of ^3H label					
	pathway A		pathway B		pathway C	
	syn	anti	syn	anti	syn	anti
	50	100	100	50	75	75
	100	0	0	100	50	50



Hypothetical Pathways for Dehydroproline Moiety Formation

Scheme XXVI

The starting material for the synthesis was commercially available (S)-[2,3-³H₂]proline. This was received in 1N HCl, and the solution was evaporated to dryness to give the hydrochloride salt and was added to unlabeled (S)-proline. Under anhydrous conditions and a nitrogen atmosphere the proline methyl ester was formed by refluxing in methanolic HCl. Evaporation in vacuo left the (2S)-[2,3-³H₂]proline methyl ester hydrochloride **28** as a colorless oil. The proline methyl ester **28** was liberated by dissolving in anhydrous methylene chloride followed by treatment with dry ammonia, filtration and evaporation in vacuo to a colorless oil. [3-³H]-1,2-dehydro proline methyl ester **29** was prepared by treatment of **28** with t-butylhypochlorite^{94,107} and dry triethylamine for 30 minutes. Compound **29** was then converted to its N-carbomethoxy derivative by treatment with methylchloroformate and pyridine. The yield was 0.204 g (crude yield 51.9%) of N-carbomethoxy [3-³H]-2,3-dehydroproline methyl ester **30**. This process removed all tritium at carbon 2 while retaining that on carbon 3, and enabled the stereochemistry at C-3 to be set by hydrogenation. The hydrogenation process was examined by hydrogenation of unlabeled N-carbomethoxy-2,3-dehydroproline methyl ester using deuterium and three different catalysts. The use of a homogenous catalyst¹⁰⁸ gave no reduction product. The use of Pd/C gave a cis product with a scrambling of 40-50% of the label, presumably by a vinyl rearrangement (Scheme XXVII).^{109,110} The use of PtO₂ gave a cis product with only 5-10% scrambling of the label. These results were as expected since the order Pd>Rh>Ru>Ir>Pt



Vinyl Rearrangement During Hydrogenation

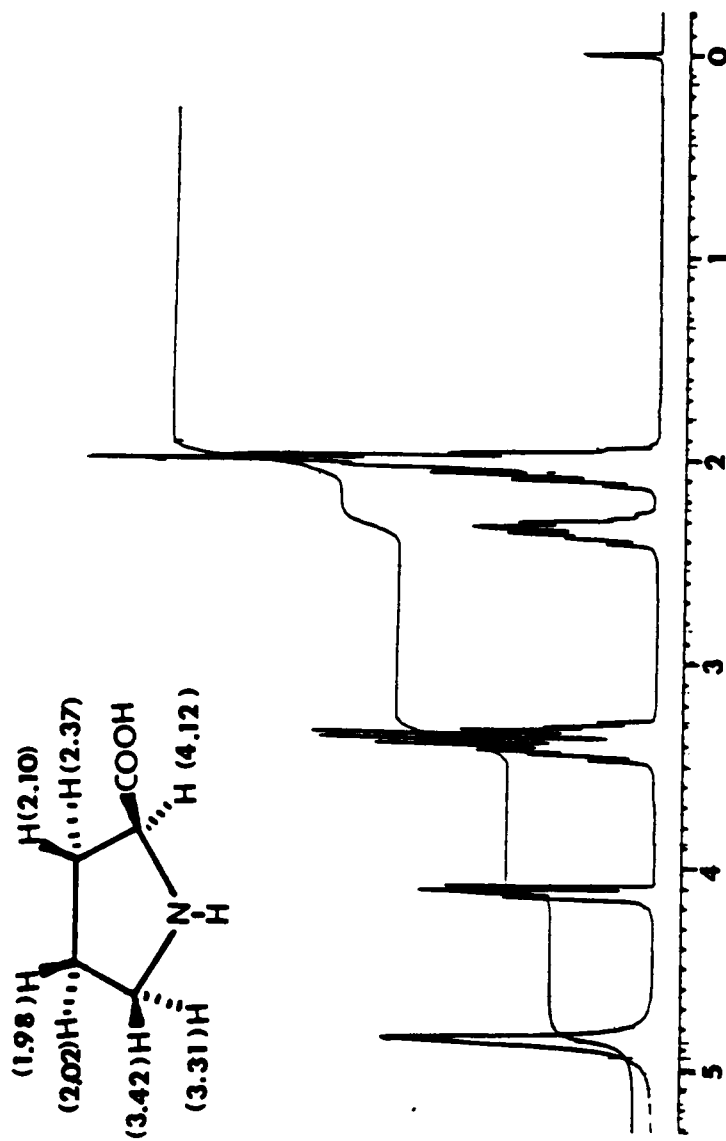
Scheme XXVII

has been observed for promotion of the vinyl rearrangement.¹⁰⁹ The position of the deuterium within the compound was determined by ¹H NMR of the protected and unprotected proline; the NMR assignment was as shown in Figure 22¹¹⁰.

Although one would desire no scrambling of the label, a 5-10% scrambling is not detrimental to the assignment of the stereochemistry. It has been shown that the mechanism of the vinyl rearrangement occurs as in Scheme XXV where the hydrogen migration occurs on the same face of the molecule.¹⁰⁹ Thus the resulting products are N-carbomethoxyl (2S,3R)-[3-³H]proline methyl ester and N-carbomethoxyl (2R,3S)-[3-³H]proline methyl ester, the same products as the unrearranged case.

The stereoisomers **33** and **34** were then decarbomethoxylated by treatment with trimethylsilyl iodide¹¹² to yield a crude mixture of (2S,3R)-[3-³H]proline and (2R,3S)-[3-³H]proline. The proline was purified by ion exchange chromatography on a Dowex 50X ion exchange column. The ninhydrin positive fractions were combined and evaporated in vacuo to give a mixture of (2S,3R)-[3-³H]proline and (2R,3S)-[3-³H]proline (**33** and **34**) as a light tan crystalline product in 94% crude yield. Total yield to this point is 33% based on initial proline.

The stereoisomers were separated by preferential recrystallization of the (S)-proline-(S)-tartaric acid complex.⁹⁵ To the mixture of **33** and **34** (67 mg) 0.5 eq of tartaric acid was added and the mixture dissolved in a minimum amount of distilled water and stirred for two hours. The solution was diluted with absolute ethanol until a 1 to 15



¹H NMR Assignment for Proline

Figure 22

ratio of water to ethanol was obtained. A seed crystal of the (S)-proline-(S)-tartrate complex was added and the mixture cooled in a freezer overnight. Crystals of the (2S,3R)-[3-³H]proline tartrate complex were harvested through filtration, but no physical data were taken of the complex. To support the idea that resolution had taken place a mixture of 100 mg of unlabeled (RS)-proline was treated in identical fashion with a 46% yield and optical purity of 99% of the pure proline-tartrate complex. The (2S,3R)-[3-³H]proline was isolated by ion exchange chromatography.

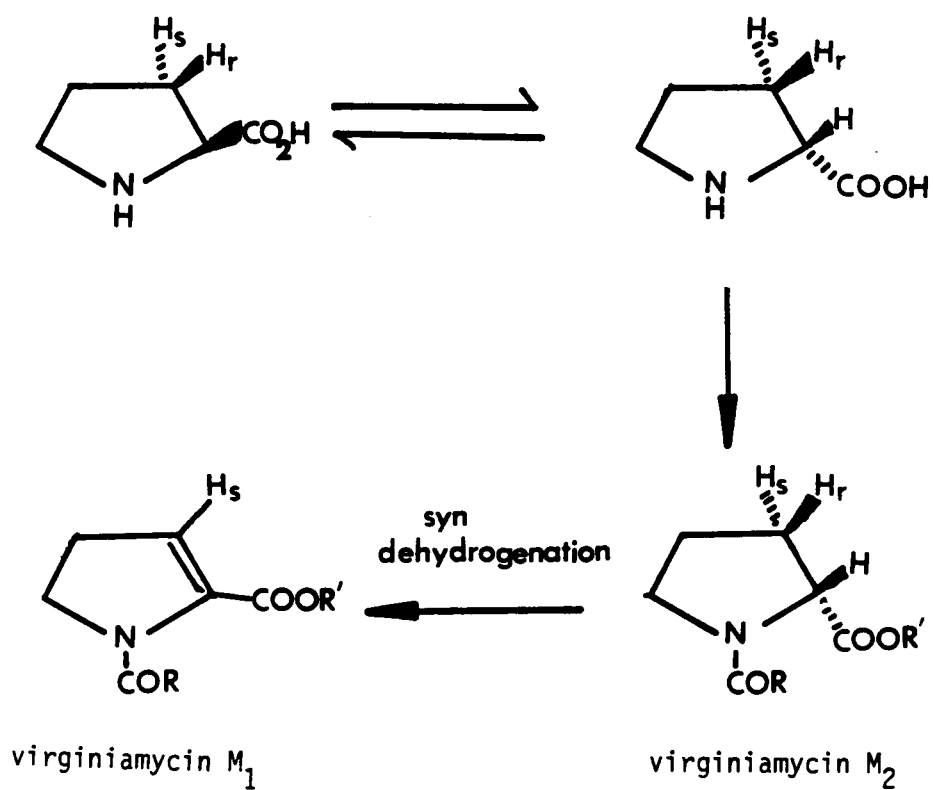
The purified (2S,3R)-[3-³H]proline was mixed with 3 μ Ci of [U-¹⁴C]proline and the mixture recrystallized to a constant ³H to ¹⁴C ratio of 2.51 \pm 0.12. The proline was fed to streptomyces virginiae at SmithKline-RIT (Brussels) and the impure virginiamycins isolated from the production medium were purified initially by flash chromatography using 1% methanol in chloroform as eluent to yield 100 mg of a crude mixture of VM₁ and VM₂. Further purification by reverse phase preparative HPLC yielded 28 mg of VM₁ and 30 mg of VM₂. These samples had ³H to ¹⁴C ratio of 0.41 and 1.91, respectively, indicating the retention of ³H in the VM₂. However, the ratio are not valid representations of the dehydroproline unit since all incorporation locations are known for the virginiamycin skeleton. The VM₁ was hydrogenated (PtO₂, acetic acid), and the resulting "perhydro VM₁" was hydrolysed and benzoylated. The resulting N-benzoylproline was isolated by normal phase HPLC and recrystallized to a constant ³H to ¹⁴C ratio of 0.10.

The VM₂ was also hydrolyzed and derivatized as described above to

give N-benzoylproline. The purified product was recrystallized to give a ^3H to ^{14}C ratio of 1.99 ± 0.06 . The deviation of this result from the possible (expected) 2.51 is not obvious and eludes explanation aside from the formation of some reversible product or intermediate.

Several major conclusions can be drawn from these data (Table VIII). In the first place, the fact that (2S,3R)-[3- ^3H]proline loses essentially all of its ^3H -label on incorporation into VM₁ corroborates the earlier work of LeFevre and indicates clearly that the conversion of proline to dehydroproline is, indeed, a stereospecific process. The small amount of tritium retained may be due to a small amount of label scrambling in the synthesis of the purchased starting material or a small impurity of (2R,3S)-[3- ^3H]proline.

In the second place, the stereochemistry of the proline to dehydroproline may be defined as either an overall anti elimination of hydrogen from (S)-proline (hypothesis A) or an overall syn elimination of hydrogen from (R)-proline (hypothesis B). In deciding between these hypotheses, it may be noted that virginiamycin M₂, which contains a proline residue in place of the dehydroproline unit of VM₁, has the (R) stereochemistry of proline.⁵ This fact suggests, although it does not prove, that the pathway to VM₁ consists of the steps (S)-proline ---> (R)-proline ---> VM₂ ---> VM₁ (Scheme XXVIII). A further argument in support of this pathway is the fact that the proline racemase function present in the S. virginiae cultures would serve no purpose unless it were required for the conversion of (S)-proline to (R)-proline for incorporation into VM₂.



Proposed Pathway for Dehydroproline Formation

Scheme XXVIII

Table VIII
Summary of Proline Stereochemical Study

	Experiment 1				Experiment 2			
	(2S,3R,3R)-[3,4- ³ H ₂]proline	[carboxyl- ¹⁴ C]proline	(2S,3R,4R)-[3,4- ³ H ₂]proline	(S)-[U- ¹⁴ C]proline	(2S,3R)-[3- ³ H ₂]proline	(S)-[U- ¹⁴ C]proline		
precursors								
specific activity	55.0 uCi/mmol	50.0 uCi/mmol			744 uCi/mmol	265 uCi/mmol		
uCi mixed	100 uCi ³ H	10 uCi ¹⁴ C	a	a	14 uCi ³ H	3 uCi ¹⁴ C		
³ H to ¹⁴ C mixed	15.6	1	6.8	1	2.51	1		
³ H to ¹⁴ C VM ₁	32.2	1	a	a	0.41	1		
specific activity VM ₁	8.85 uCi/mmol	0.275 uCi/mmol	a	a	0.069 uCi/mmol	0.169 uCi/mmol		
% incorporation VM ₁	0.17%	0.05%	a	a	0.026%	0.302%		
% specific incorporation (x10 ⁻³)	16	.55	a	a	9.27	63.8		
³ H to ¹⁴ C VM ₂	NA	NA	a	a	1.91	1		
specific activity VM ₂	NA	NA	a	a	0.054 uCi/mmol	0.028 uCi/mmol		
% incorporation VM ₂	NA	NA	a	a	2.18x10 ⁻²	5.30x10 ⁻²		
% specific incorporation VM ₂ (x10 ⁻³)	NA	NA	a	a	7.25	10.5		
³ H to ¹⁴ C ratio NBP (VM ₁)	17.7	1	7.5	1	0.1	1		
specific activity NBP (VM ₁)(x10 ⁻³)	32.1 uCi/mmol	1.82 uCi/mmol	a	a	5.0x10 ⁻² uCi/mmol	4.67 uCi/mmol		
% incorporation NBP (VM ₁)	4.28	2.42	a	a	8.57x10 ⁻⁵	3.73x10 ⁻³		
% specific incorporation NBP (VM ₁)(x10 ⁻³)	0.58	3.64	a	a	1.15x10 ⁻²	1.76		
³ H to ¹⁴ C ratio NBP (VM ₂)	NA	NA	a	a	1.99	1		
specific activity NBP (VM ₂)(x10 ⁻³)	NA	NA	a	a	6.99 uCi/mmol	1.39 uCi/mmol		
% incorporation NBP (VM ₂)	NA	NA	a	a	2.84x10 ⁻³	9.95x10 ⁻³		
% specific incorporation NBP (VM ₂)(x10 ⁻³)	NA	NA	a	a	0.939	5.25		
Theoretical ratios	31.2 ^b	1	---	---	0 ^e	1		
	15.6 ^c	1	---	---				
	0 ^d	1	---	---	2.51 ^f	1		

a: Not determined by LeFevre

b: If only (S) proline is incorporated

c: If both (R) and (S) proline are incorporated

d: If only (R) proline is incorporated

e: If (3R) H is lost

f: If both (3R) and (3S) H are lost

If these arguments are accepted, then the overall stereochemistry of hydrogen loss from proline in the formation of VM₁ is by a syn process (Scheme XXVIII). This finding is consistent with the examples given earlier of syn dehydrogenations.

A final and very important conclusion to emerge from these results is that virginiamycin M₂ cannot be formed by reduction of virginiamycin M₁. Prior to this work the possibility existed that VM₁ was formed by some independent pathway and then reduced to VM₂, in accordance with the proposal that D-amino acids derive from dehydroamino acids.⁶⁶ However, the finding that VM₂ retains the ³H label while VM₁ loses it shows that VM₂ cannot be formed from VM₁. Instead, as discussed above, the most reasonable conclusion is that VM₁ is formed by dehydrogenation of VM₂.

4. Summary

The stereochemistry of the formation of the oxazole ring and the dehydroproline unit of virginiamycin M₁ has been studied with the help of stereospecifically labeled precursors.

In the case of the biosynthesis of the oxazole ring from serine, the results indicated that reaction most probably proceeds from serine with loss of the (pro-S) hydrogen (Scheme XXIII). An attempt to determine the origin of the oxygen atom of the oxazole ring failed due to technical problems during the crucial experiments.

Studies of the incorporation of labeled prolines into virginiamycin M₁ indicated that VM₂ is not formed from VM₁, but rather that VM₁ is most probably produced by a syn dehydrogenation reaction on VM₂.

CHAPTER V.

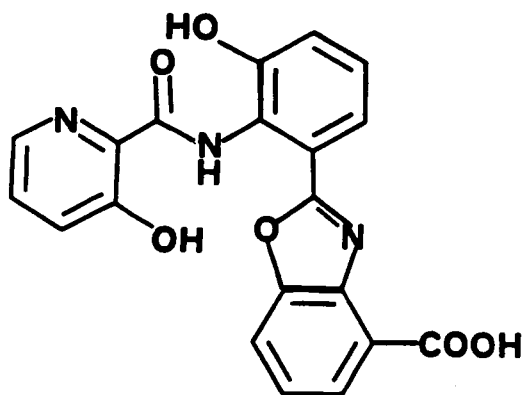
INTRODUCTION TO THE BIOSYNTHESIS OF A33853

This portion of the dissertation covers basic biosynthetic studies concerning the origin of antibiotic A33853 using isotopically labeled precursors and studies of the formation of the 3-hydroxypicolinic amide moiety from lysine.

Antibiotic A33853 37 was first isolated in 1979 from the fermentation of Streptomyces NRRL 12067 and was shown to have the structure in Figure 23.¹¹² The secondary metabolite is an unusual compound composed of a 3-hydroxypicolinic acid moiety 38 and a phenyl benzoxazole moiety which appears to be a condensation product of two molecules of 3-hydroxyanthranilic acid 39 (Figure 24). It is these aromatic amino acids that fuel the interest in the biosynthesis of A33853.

As with other secondary metabolites the purpose for the formation of A33853 by the microbe is not known, but information regarding its biosynthesis is useful as follows:

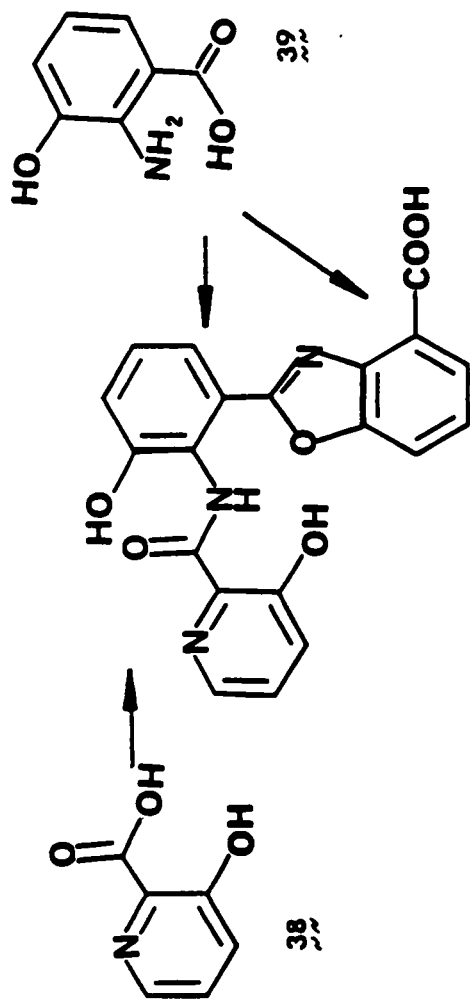
- A. Gives the basic knowledge of how the cell assembles secondary metabolites.
- B. Gives biosynthetic information that may serve as a model for organic synthesis.
- C. Provides details for understanding enzyme mechanisms.



37

Structure of A33853

Figure 23



Aromatic Amino Acid Components of A33853

Figure 24

In Chapter I of this dissertation, seven basic steps for studying biosynthesis were listed. In this portion these seven steps were used in the following ways to study the origin of A33853.

1. Growth of the producing microbe A33853 is produced by Streptomyces NRRL 12067 growing first on a modified Bennett Slant, then a CSSXII vegetative medium for cell growth, followed by growth in SCO production medium. A growth curve was prepared to examine the appropriate time to harvest the microbes.

2. Isolation of the metabolite. A33853 was isolated from the growth medium by extraction followed by preparative TLC or HPLC.

3. Assignment of the ^1H NMR and ^{13}C NMR spectra. The ^{13}C NMR spectra was partially assigned by comparison to the spectra of model compounds and by using the INEPT pulse sequence.

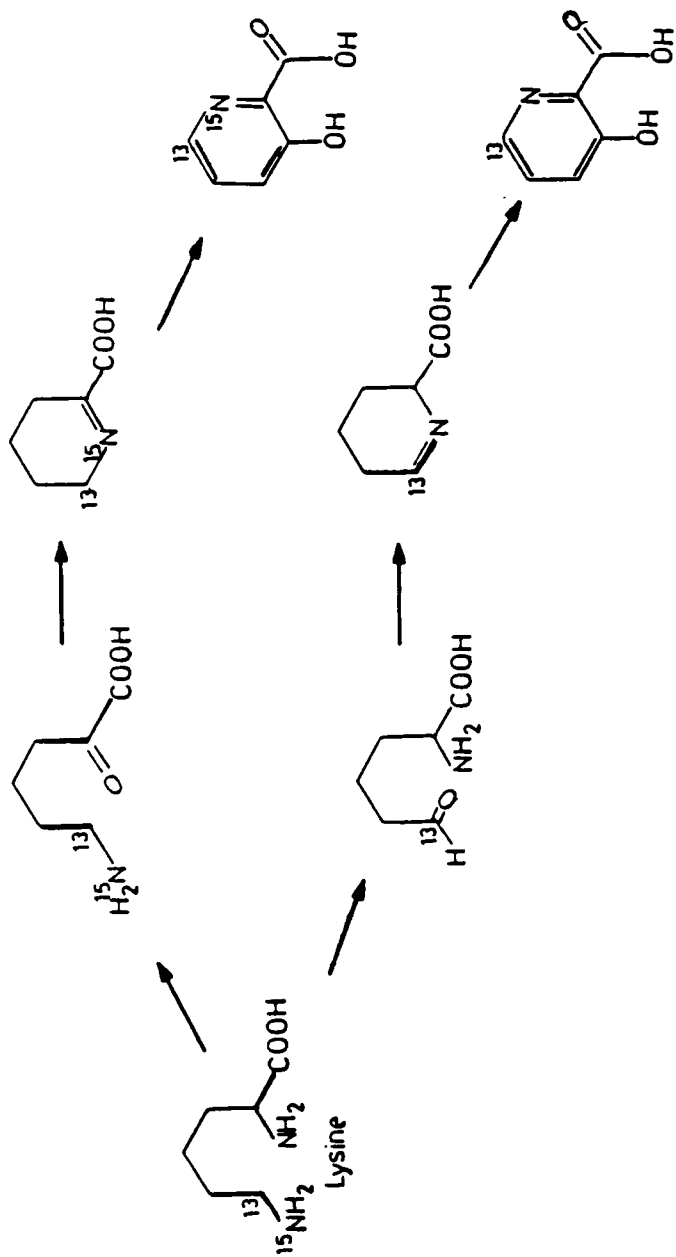
4. Appropriate ^{14}C and ^3H labeled precursors were fed to the microbes and the A33853 harvested and examined for radioactivity. The precursors fed were D-[U- ^{14}C]glucose, (S)-[U- ^{14}C]lysine, sodium [U- ^{14}C]acetate, (S)-[U- ^{14}C]aspartic acid, [carboxyl- ^{14}C]anthranilic acid and [5- ^3H]tryptophan.

5. Synthesis and incorporation of modified precursors. Carbon-13 labeled precursors of acetate and glucose were fed, but insufficient production of the antibiotic limited ^{13}C NMR detection. A doubly labeled precursor was synthesized, (RS)-[6- ^{13}C ,6- ^{15}N]lysine. The lysine was fed to streptomyces virginiae to investigate the formation of the picolinic acid ring in the antibiotic virginiamycin S₁ (Scheme 29). The picolinic acid moiety formed was isolated and its isotope content determined by mass spectrometry.

6. No stereochemical studies were carried out on the biosynthesis of A33853.

7. Based on results of the experiments outlined above biosynthetic pathways have been postulated for the origin of A33853 and the ring closure of 3-hydroxypicolinic amide. These pathways are discussed in Chapter 8.

In summary, the origin of A33853 was examined by feeding labeled precursors. ^{13}C ^{15}N lysine was synthesized and incorporated into VS₁ and possible biosynthetic pathways were postulated.



Proposed Pathways for Cyclization of Lysine to 3 Hydroxypicolinic Acid

Scheme XXIX

CHAPTER VI.

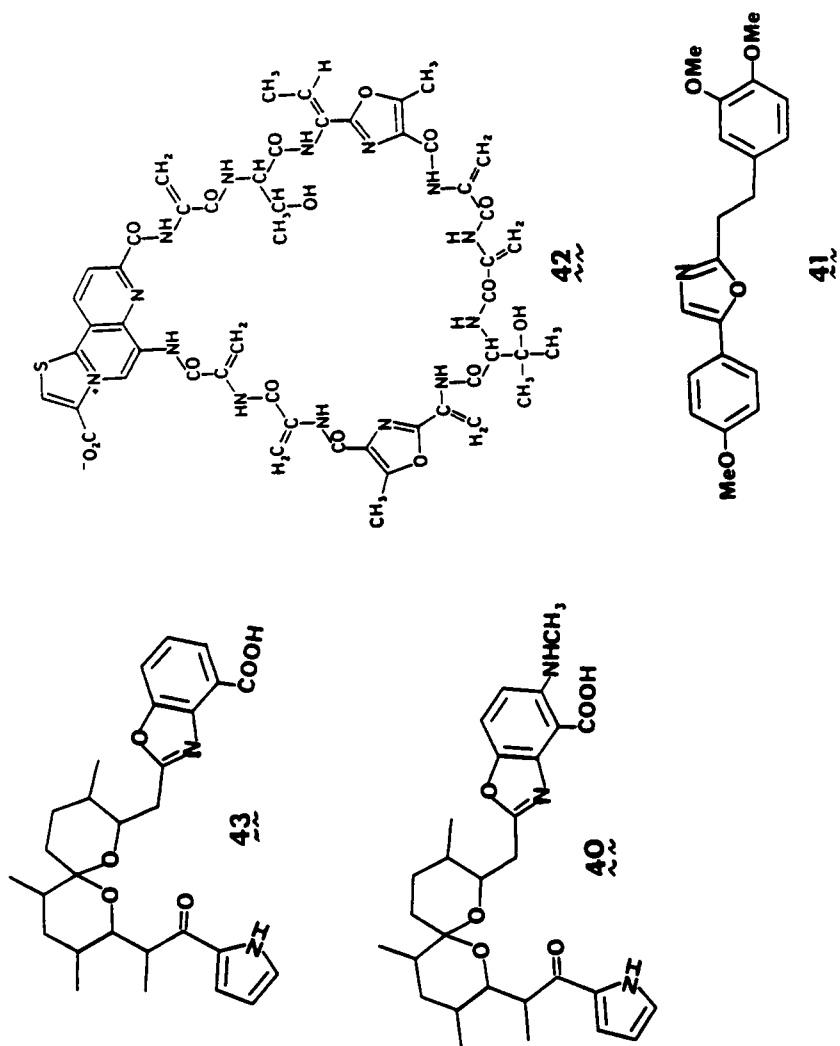
REVIEW OF LITERATURE

A. Chemistry

Antibiotic A33853 was first isolated in 1979 by the Eli Lilly Company. The Chemical Abstract's nomenclature for A33853 is 2-[3-hydroxy-2-[[3-hydroxy-2-pyridinyl] carbonyl] amino] phenyl]-4-benzoxazole carboxylic acid.¹¹² As evident in the name, A33853 is a member of a novel class of compounds containing an oxazole ring such as virginiamycin M₁, A23187^{40, 113} annuloline^{41, 114} and berninamycin^{42. 115} Closer relatives containing a benzoxazole ring are the ionophore antibiotics cezomycin^{43 116} and X148854.¹¹⁷ Figure 25 shows the structures of these rare compounds.

A33853 is produced by a fermentation process using Streptomyces sp. NRRL 12068. The growth conditions include storage of the producing microbe on a Bennett's modified agar followed by transfer to a complex CSSXII vegetative medium and finally to a SCO production medium.¹¹⁸ Growth curve data indicate that maximum production of the antibiotic is found after 72 hours in the SCO medium.¹¹⁸

Physical and chemical characterization¹¹² has been obtained for A33853 and its tetraacetyl derivative. The tetraacetyl derivative was prepared by dissolving A33853 in pyridine and acetic anhydride. Proton NMR and carbon NMR have been recorded for both compounds but no assignments have been made. The actual structure elucidation was carried out by an X-ray crystal structure on the tetraacetyl derivative. The physical chemical data are recorded in Table IX. A33853 is a very



Compounds Containing Oxazole Rings

Figure 25

Table IX

Chemical Data of A33853 and Tetraacetyl A33853

A33853 (C₂₀H₁₃O₆)		Tetraacetyl A33853 (C₂₈H₂₁N₃O₁₀)	
m/z:	391	m/z:	559
mp:	310-315°C	mp:	184-189°C
UV maxλ:	neutral/acidic dioxane 250, 313, 322 nm 254, 310, 360, 400 nm	UV maxλ:	270, 320 nm neutral, acidic 254, 310, 365, 400 basic
IR:	3338, 3280, 3080, 2990, 1760, 1646, 1595, 1583, 1534, 1457, 1449, 1422, 1339, 1312, 1298, 1254, 1227, 1186, 1170, 1133, 1120, 1100, 1050, 990, 961, 912, 893, 867, 848, 756, 739, 720, 710, 691, 675, 654, 622, 593, 579, 531, 509, 473, 443, 375 cm ⁻¹	IR:	3600-3200, 3060, 2910, 1951, 1810, 1774, 1728, 1714, 1690, 1601, 1589, 1549, 1471, 1452, 1441, 1418, 1369, 1350, 1322, 1301, 1282, 1271, 1261, 1254, 1215, 1197, 1163, 1150, 1128, 1092, 1059, 1003, 981, 955, 916, 910, 873, 849, 820, 800, 792, 760, 752, 729, 690, 670, 655, 330, 310 cm ⁻¹
¹ H NMR: (ppm)	8:32 (1.0:4.0), 7.90 (1.0:7.8), 7.81 (1.0:7.8), 7.71 (1.5, 7.5), 7.60 (4.5:8.0) 7.46 (1.0, 8.0) 7.42 (8.0:8.0), 7.26 (1.5:8.0) in DMS, TMS	¹ H NMR: (ppm)	8:26 (1.5:4.4), 8.24-- 8.08 (1.0, 7.8), 7.86 (1.1, 8.1), 7.58 (7.8, 7.8), 7.50 (1.3, 8.0), 7.50 (1.3, 8.0), 7.50 (1.3, 8.0), 7.46 (8.0, 8.0), 7.33 (4.5; 8.2) CDCl ₃
¹³ C NMR: (ppm)	167.5, 165.8, 162.6, 157.2, 153.2, 150.4, 140.3, 140.1, 131.2, 129.3, 127.8, 126.5, 125.9, 124.9, 123.5, 122.9, 122.5, 120.6, 119.7, 114.5, DMSO		

interesting compound in respect to biological activity. It has in vitro activity against several organisms including polio type III, herpes type I, rhino virus, Salmonella typhosa, etc., but no in vivo activity was detected.¹¹⁸ The tetraacetyl compound possesses less activity than A33853. The lack of in vivo activity and problems in obtaining physical data are the result of very low solubility of A33853 in water and common organic solvents.

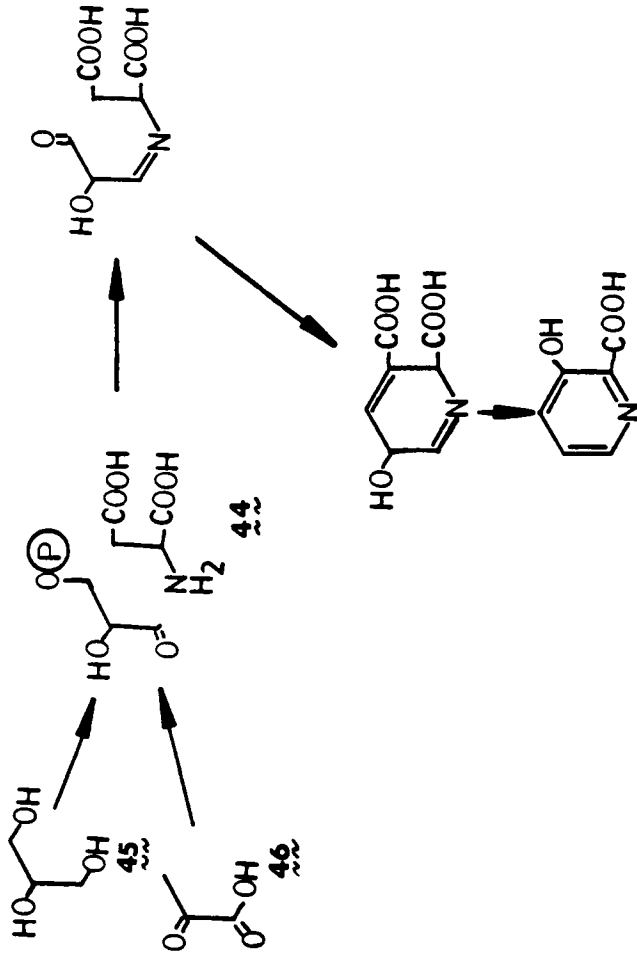
B. Biosynthesis

No biosynthetic studies have been performed on A33858. However, as mentioned in the introduction, A33853 can be viewed as 3-hydroxypicolinic acid connected through a peptide bond to the phenyl benzoxazole moiety, which may be derived from two molecules of 3-hydroxyanthranilic acid. The biosynthesis of both 3-hydroxypicolinic acid and 3-hydroxyanthranilic acid have been studied individually as well as in other natural products.¹¹⁹⁻¹³⁰

The biosynthesis of 3-hydroxypicolinic acid by Streptomyces has been described in 2 different pathways.^{119,120} The first is from the combination of aspartic acid 44, glycerol 45 and or sodium pyruvate 46. Ogawara, et al.¹¹⁹ found that the 3-hydroxypicolinic acid unit of pyridomycin arose from (S)-[U-¹⁴C]aspartic acid, [1-¹⁴C]glycerol and sodium [2-¹⁴C]pyruvate with incorporations of 0.38%, 0.95% and 0.83% respectively (Scheme XXX).

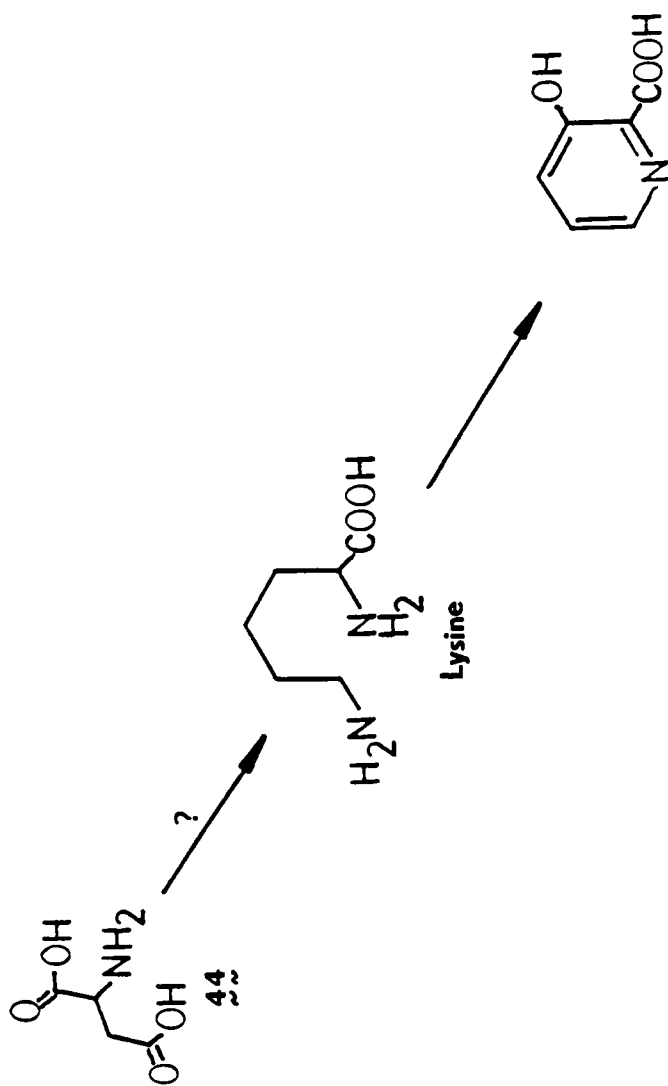
The second pathway is the lysine pathway reported by Hook and Vining.¹²⁰ They fed (S)-[U-¹⁴C]lysine to Streptomyces griseoviridus ATCC 04955 and recorded a 30% incorporation into etamycin with 95.9% of the radioactivity being in the 3-hydroxypicolinic acid residue. A similar result has been reported by Kingston, Molinero and Reed for the incorporation of (S)-[U-¹⁴C]lysine into the hydroxypicolinic acid portion of virginiamycin S₁.¹²¹ They also reported a small incorporation of aspartic acid (Scheme XXXI).

No details of the formation of 3-hydroxypicolinic acid from lysine have been published. However, lysine has been studied as a precursor to



Biosynthesis of 3 Hydroxypicolonic Acid From Aspartic Acid, Glycerol and/or Pyruvate

Scheme xxx



Formation of 3 Hydroxypicolinic Acid From Lysine

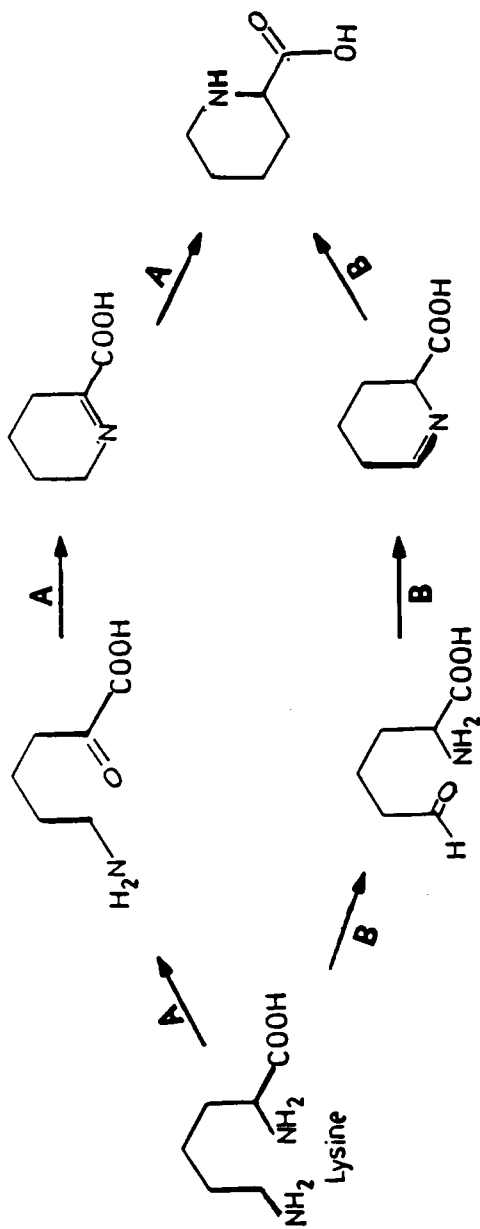
Scheme XXXI

the pipercolic acid nuclei found in higher plant and animal systems. Two pathways of interest have been proposed for the formation of pipercolic acid, one by Gupta and Spenser in Sedum acre and Phaseolus vulgaris¹²², the second by Fowdeh, in Acaracia Phyllodia¹²³. (Scheme XXXII). The results of Gupta and Spenser were in favor of pathway A, while Fowden found evidence for Pathway B. Both groups used the ³H to ¹⁴C ratio of precursors and products as their key data. These pathways are of interest as possible pathways for the formation of 3-hydroxypicolinic acid in A33853.

Other pathways have been reported for the formation of the pyridine ring in higher plants and animals by Leete.¹²⁴ These include the tryptophan-3-hydroxyanthranilic acid pathway,^{125,126} and the mevalonate pathway. The usual metabolites from the pathways are nicotine, niacin, and picolinic acid; none except those mentioned earlier have been shown to give rise to 3-hydroxypicolinic acid. Therefore, the details of these pathways will not be reviewed at this time.

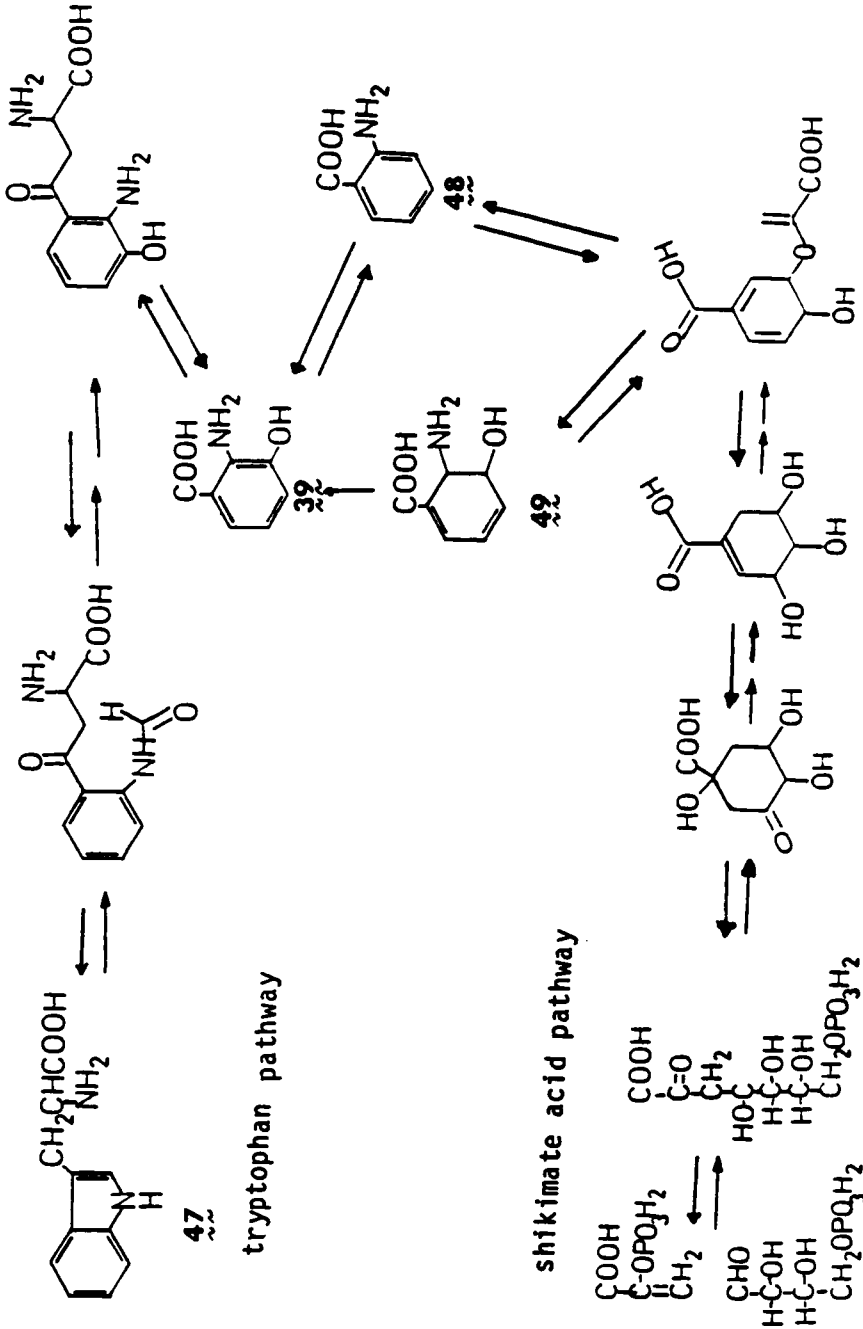
3-Hydroxyanthranilic acid has been shown to arise by the pathways of Scheme XXXIII. The pathways described are interconnected by 3-hydroxyanthranilic acid as it is an intermediate compound in the metabolism of glucose through the shikimate acid pathway to tryptophan and is the major product in the catabolism of tryptophan 47.¹²⁷ 3-Hydroxyanthranilic acid has also been documented as arising from the direct hydroxylation of anthranilic acid 48 without going through the shikimate pathway. It has also been observed that a shikimate pathway may not include anthranilic acid, as McCormick, et.al. have isolated

trans-2,3-dihydro-3-hydroxyanthranilic acid **49** from a strain of Streptomyces aureofaciens.¹²⁸ This compound is inactive as a precursor to anthranilic acid, presumably because its stereochemistry is incorrect for the E₂-anti elimination step.



Literature Pathways for Lysine to Pipecolic Acid

Scheme XXXII



Pathways of 3-Hydroxyanthranilic Acid Biosynthesis

Scheme XXXIII

CHAPTER VII.

EXPERIMENTAL

A. General

Melting points were determined on a Kofler block and are uncorrected. Samples for liquid scintillation counting were evaporated in vacuo and dried in a vacuum desiccator for several hours to remove trace amounts of solvent that might cause quenching. (Evaporation in vacuo refers to solvent removal on a rotary evaporator at aspirator pressure and 25-45°C). Counting was carried out on a Beckman LS-100 or a LS 3800 liquid scintillation counter using a xylene emulsifier cocktail containing 0.3% PPO and 0.02% POPOP or a Beckman Ready-Solv[®] scintillation cocktail.

The microbes were grown in baffled 250 ml wide mouth Erlenmyer flask on a Lab-Line orbit shaker or in a 2 liter Multigen fermenter fitted with a pH monitor.

Carbon and tritium labeled compounds were obtained from ICN Chemical and Radioisotopes Division and from Amersham Corporation. ^{13}C ^{15}N was obtained from Prochem, BOC Limited.

Eli Lilly Company supplied pure samples of A33853 and its tetraacetyl derivative.

B. Spectra

The ^{13}C NMR spectra were taken at ambient temperature in either CDCl_3 , D_2O or d_6 -DMSO solutions using a 5 mm or 10 mm spinning tube on a Bruker WP-270-SY and/or a Jeol FX-200 spectrometer operating at 67.3

MHz and 49.8 MHz respectively. For proton noise decoupled (pnd) spectra all protons were decoupled by gated heteronuclear decoupling and broadband irradiation.

Proton NMR spectra were taken at ambient temperature in either CDCl_3 , D_2O , or d_6 -DMSO solutions using a Varian EM-390 90 MHz spectrometer, a Bruker WP-801-SY 90 MHz spectrometer or a Bruker WP-270-SY 270 MHz spectrometer. Chemical shifts are given in parts per million with reference to internal tetramethylsilane (TMS) for proton and the ^{13}C signal of the solvent for the ^{13}C spectra.

The Insensitive Nuclei Enhancement Polarization Transfer (INEPT) pulse sequence used was ^1H : $90^\circ(\text{V})-\tau-180^\circ(\times)-\tau-90^\circ(\times)-\Delta-[\text{H}]$ and $^{13}\text{C}(\text{obsd})$: $180^\circ(\times)-\tau-90^\circ(\text{Y})-\Delta-180^\circ(\times)-\Delta$, where $\Delta = \frac{1}{2}J$, $\frac{1}{4}J$ or $\frac{3}{4}J$ and $\tau = \frac{1}{2}J$. The value $\Delta = \frac{3}{4}J$ was used for methene carbons.

Infrared (IR) spectra were taken on a Perkin-Elmer 710B infrared spectrometer. Ultraviolet (UV) spectra were taken on a Perkin-Elmer 330 UV-visible spectrometer.

Mass spectra were obtained using a Finnigan MAT 112 mass spectrometer or a VG Analytical 7070E mass spectrometer.

C. Chromatography

High-performance liquid chromatography (HPLC) separations were carried out on a system consisting of a Waters Associates M6000A pump, a Valco six-port injection valve and a Waters Associates M441 UV detector operating at 254 nm. The normal phase columns used were Whatman Partisil-PAC and Alltech- NH_2 .

Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F-254 adsorbent (0.2 mm thickness).

Column chromatography was performed using Merck silica gel 60 particle size 0.040-0.063 mm (230-400 mesh). Column sizes varied and are specified in the pertinent experimental sections.

D. Production of A33853

1. Slant Storage

Antibiotic A33853 producing microbes Streptomyces NRRL 12068 were obtained as pellets from Eli Lilly Co. The microbes were maintained on modified Bennett Agar slants composed of dextrin 8.0 g/l, yeast extract 1.0 g/l, enzyme hydrolyzed casein 2.0 g/l, KCl 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, agar 20 g/l and deionized water. The pre-sterilization pH was 7.0. The slants were sterilized at a pressure > 15 psi for a minimum of 30 minutes.

The sterile slants were inoculated with a pellet of the producing microbe that had been suspended in sterile H_2O . The slants were then incubated at 37°C for 7-10 days. Growth of the microbe appeared as grayish-white colonies.

2. Seed Culture

To provide a producing population of the microbes, one to two slants prepared as above were used to inoculate a complex CSSXII vegetative growth medium. The CSSXII medium was composed of glucose 15.0 g/l, soybean grits 15.0 g/l, corn steep liquor 10.0 g/l, NaCl 5.0 g/l, $CaCO_3$ 2.0 g/l and tap water. The pre-sterilization pH was adjusted to 6.5 using 1N NaOH. The medium was dispensed in 55 ml aliquots to 250

ml baffled wide mouth Erlenmeyer flasks fitted with cotton plugs.

After sterilization at a pressure > 15 psi for a minimum of 30 minutes one of the flasks was inoculated using one or two of the Bennett slants. The inoculated flask was then incubated at 30°C on an orbit shaker at 250 rpm for 48 hours or until heavy cell growth appeared.

3. A33853 Production Medium

To produce A33853 a 1% inoculum of the CSSXII medium was transferred to sterile SCO medium composed of corn dextrin type III 70.0 g/l cornmeal 5.0 g/l, O.M. peptone 10.0 g/l, $MgSO_4 \cdot 7H_2O$ 2.0 g/l, $CaCO_3$ 2.0 g/l and tap water. The pH was adjusted to 6.6 and the medium dispensed in 55 ml aliquots to 250 ml baffled wide mouth Erlenmeyer flasks fitted with cotton plugs. The medium was sterilized at pressure >15 psi for a minimum of 25 minutes. After cooling, the flasks containing the SCO medium were inoculated with a 1% inoculum (0.5 ml of inoculated CSSXII medium) from a single flask of CSSXII. The medium was then incubated at 30°C for 60 hours on an orbit shaker operating at 250 rpm.

E. Isolation of A33853

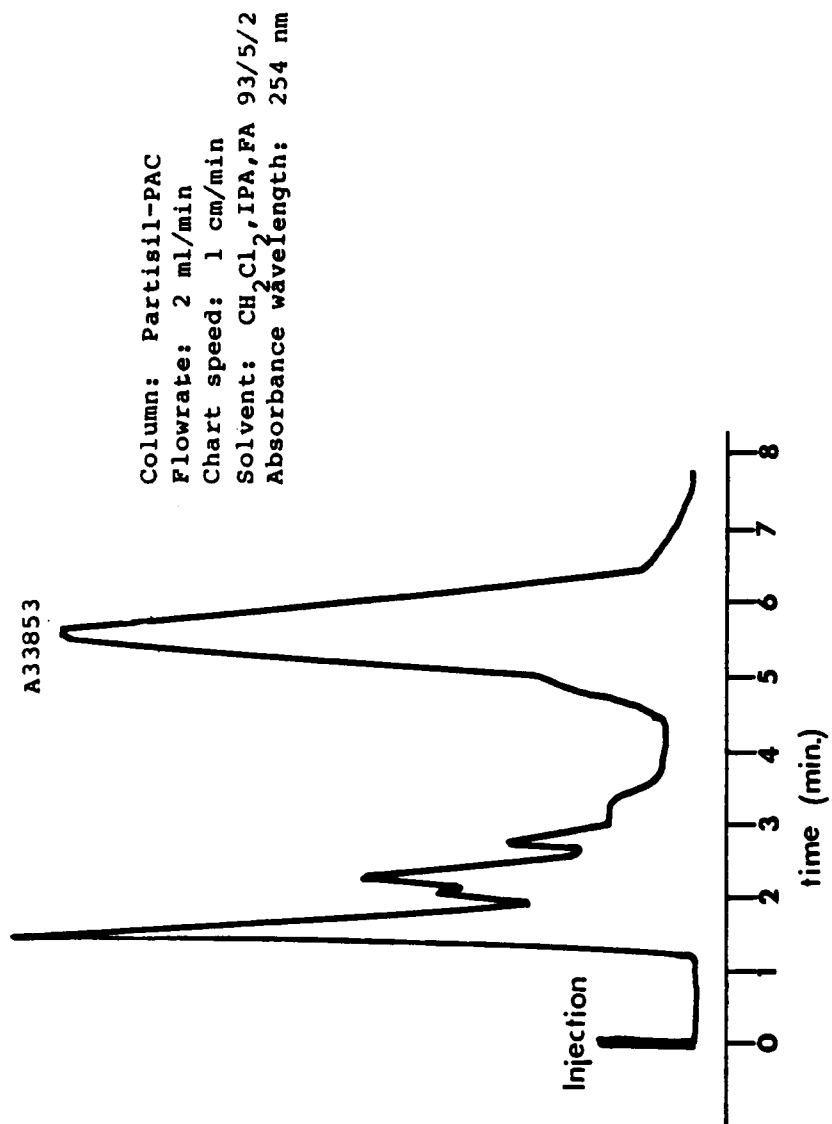
1. Crude Material

After the appropriate growth period the SCO production medium was filtered through Hy-flo-Super-Cel^R to remove mycelial material. The pH of the filtrates (usually 7.0-8.0) was adjusted to 7.0 and extracted two times with a half volume of chloroform or methylene chloride. The extractions were repeated at pH's of 3.0, 7.0 and 9.0.

All extracts were combined, washed with distilled water (10% volume) and dried with anhydrous MgSO_4 . The MgSO_4 was removed by filtration, and the extraction solvent was evaporated in vacuo to about 5 ml, to yield a yellow solution of crude A33853. The antibiotic appeared at R_f 0.4 on TLC using methylene chloride/ethanol 96/4. For preparative purposes the band visible as A33853 was scraped from the plate and the antibiotic eluted with 95% ethanol. The ethanol was removed in vacuo and the purified antibiotic dissolved in chloroform and filtered to remove any dissolved silica gel. The chloroform was removed in vacuo and the A33853 was quantitated by analytical HPLC. The analytical HPLC was carried out on a Partasil-PAC or Alltech-NH₂ column (5mm x 250mm) with a solvent system of CH_2Cl_2 , isopropanol, and formic acid in 93/5/2. The flowrate was 2.0 ml/min (Figure 25).

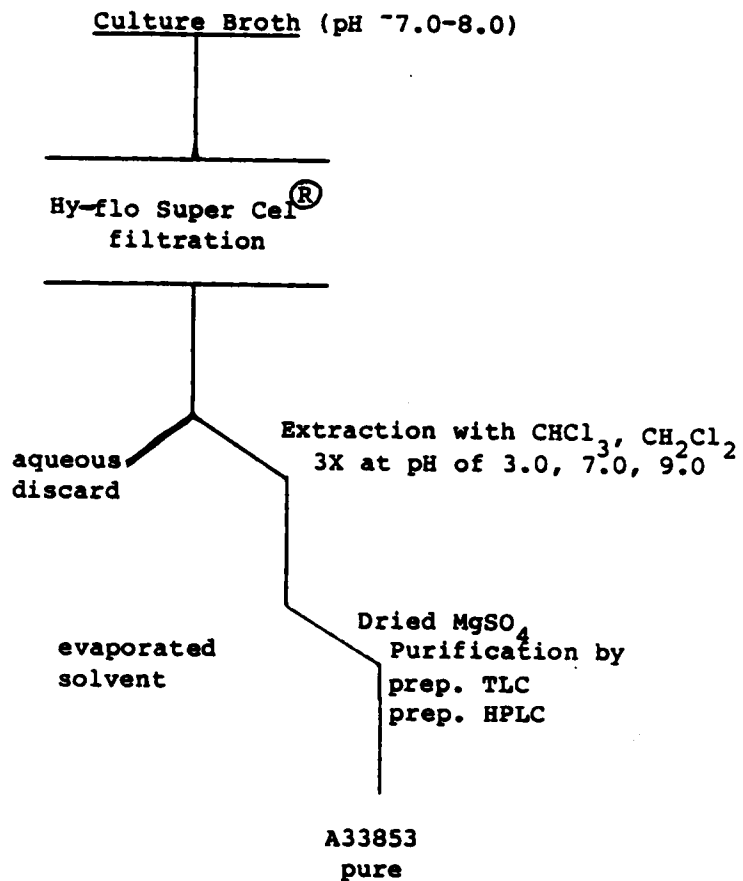
F. A33853 Production Curve

A slant of Streptomyces NRRL 12068 was used to inoculate one flask of CSSXII vegetative medium which was incubated at 30°C and 275 rpm for 48 hours. A 1.1% (vol.) inoculum of the CSSXII medium was added to each of 24 baffled flasks containing SCO production medium. The flasks were incubated at 30°C on an orbit shaker at 275 rpm. After 24 hours of growth, 4 flasks were harvested by filtration through Hy-Flo-Super-Cel^R and purified as in section E. Every 12 hours thereafter 4 flasks were harvested and purified as in section E. The A33853 was quantitated using analytical HPLC. Column: Partasil-PAC, flow rate 20 ml/min., recorder 1 cm/min. and solvent: CH_2Cl_2 , $\text{CH}_3\text{CH}(\text{OH})\text{CH}_3$, CH_3COOH 93/5/2. The production was found to be 4.8 mg/liter, 7.8 mg/liter, 8.6



Chromatograph of A33853

Figure 26



Isolation of A33853

Scheme XXXIV

mg/liter, 25 mg/liter, 7.9 mg/liter and 4.6 mg/liter for hours of 24, 36, 48, 60, 72 and 84, respectively. These data are depicted in Figure 27.

G. Incorporation of Radioactive Precursors into A33853

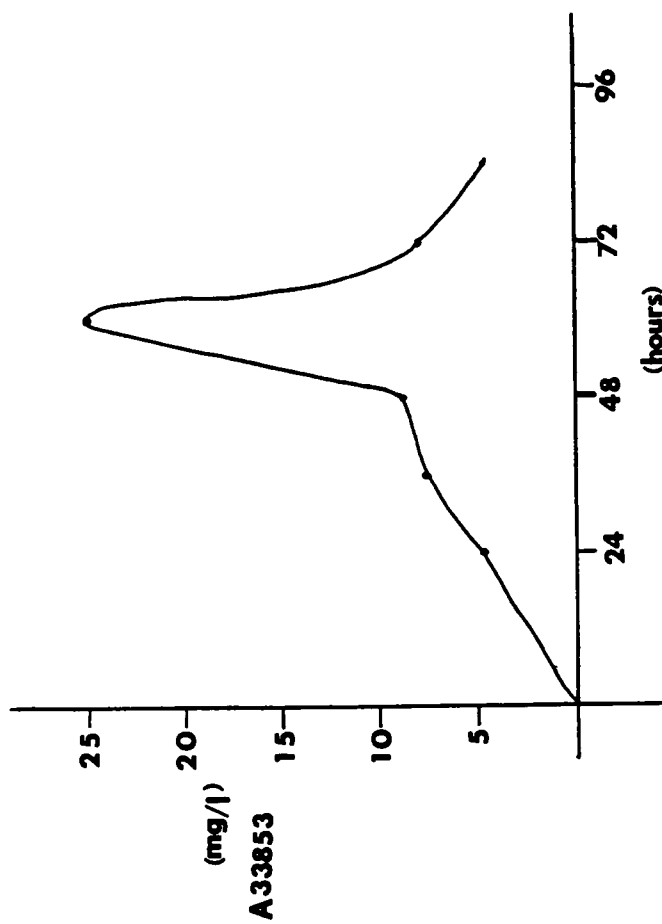
1. General

The antibiotic A33853 was produced as described in Chapter 8, section D. A slant of Streptomyces NRRL 12068 was used to inoculate a CSSXII vegative medium for 2 days. The CSSXII medium was then used to inoculate 25 flasks of the SCO production media. At growth times of 24, 36 and 48 hours, carbon-14 and/or tritium labeled precursors were added to eight of the inoculated production media using aseptic technique of filtration. After a growth time of 60 hours the sets of flasks injected with the labeled precursors were harvested and purified as described in section E. Each sample was then counted on a scintillation counter and the percent incorporation and percent specific incorporation was calculated as follows.

$$\% \text{ incorporation} = \frac{\mu\text{Ci in A33853}}{\mu\text{Ci of precursor fed to microbes}} \times 100$$

$$\% \text{ specific incorporation} = \frac{\text{specific activity of A33853 isolated}}{\text{specific activity of precursor}}$$

A sample of all precursors was counted before feeding to microbes. Internal standards were used only as noted in the Experimental.



A33853 Production Curve

Figure 27

2. Incorporation of D-[U-¹⁴C]Glucose into A33853

A slant of Streptomyces NRRL 12068 was used to inoculate one flask of CSSXII vegetative medium which was incubated at 30°C on an orbit shaker at 275 rpm for 48 hours. Twenty-five flasks of SCO medium were then inoculated from the CSSXII media and incubated at 30° and 275 rpm. After 24 hours of growth 2 µCi (0.5 ml) of D-[U-¹⁴C]glucose (specific activity 3.002 µCi/mmol) were fed to each of four flasks for a total of 8 µCi. A 5 µl aliquot of the glucose was taken for counting as a standard. After 36 hours of growth, 4 more of the flasks were fed D-[U-¹⁴C]glucose in the same way for a total of 8 µCi. After 48 hours of growth 4 additional flasks of microbes were fed D-[U-¹⁴C]glucose as above for a total of 8 µCi. The fermentation was stopped after 60 hours of growth and the flask that had D-[U-¹⁴C] glucose added were combined in 3 sets of 4 corresponding to the time of feeding. Each set of flask was individually filtered, extracted and purified as in Section E. The samples from feeding of 24 hours, 36 hours and 48 hours were analyzed by analytical HPLC with fraction collection followed by scintillation counting. HPLC conditions, solvent methylene chloride, isopropyl alcohol, formic acid 93/5/2, column Partasil PAC, and flow rate 2.0 ml/min. Figure 26 represents a chromatograph. After collection, the fractions were evaporated in vacuo; dried in a desiccator; dissolved in scintillation cocktail and counted for 2-5 minutes with an efficiency of 96%.

Results of incorporation of D-[U-¹⁴C]glucose:

<u>Sample</u>	<u>Amount</u> (mg/ml)	<u>cpm/mg</u>	<u>%</u> <u>Incorporation</u>	<u>($\mu\text{Ci}/\mu\text{mol} \times 10^{-3}$)</u> <u>Specific Activity</u>	<u>%</u> <u>Specific</u> <u>Incorp.</u>
24 hrs	1.7x10 ⁻²	41,176	0.56	7.5	0.25
36 hrs	1.3x10 ⁻²	3,911	0.05	0.7	0.02
48 hrs	3.6x10 ⁻³	14,909	0.07	2.7	0.09

This experiment was carried out a second time, with the data for the second run shown below:

<u>Sample</u>	<u>Amount</u> (mg/ml)	<u>cpm/mg</u>	<u>%</u> <u>Incorporation</u>	<u>($\mu\text{Ci}/\mu\text{mol} \times 10^{-3}$)</u> <u>Specific Activity</u>	<u>%</u> <u>Specific</u> <u>Incorp.</u>
24 hrs	2.3x10 ⁻²	11,569	0.51	2.1	0.07
36 hrs	1.2x10 ⁻²	16,399	0.39	3.0	0.10
48 hrs	2.5x10 ⁻³	37,500	0.18	6.9	0.23

3. Incorporation of Sodium [U-¹⁴C]Acetate into A33853

A slant of Streptomyces NRRL 12068 was used to inoculate one flask of CSSXII vegetative medium which was incubated at 30°C on an orbit shaker at 275 rpm for 48 hours. Twenty-five flasks of SCO medium were then inoculated from the CSSXII media and incubated at 30° and 275 rpm. After 24 hours of growth 6 of the flasks were fed ~2 μCi each (0.33 ml) of [U-¹⁴C]acetate (specific activity 11.1 mCi/mmol) each for a total of 12.5 μCi . A 5 μl aliquot of the acetate was taken for counting as a standard. After 36 hours of growth 6 more of the flasks were fed [U-¹⁴C]acetate in the same way for a total of 12.6 μCi . After 48 hours of growth 6 additional flasks of microbes were fed [U-¹⁴C]acetate as above for a total of 12.6 μCi . The fermentation was stopped after 60 hours of

growth and the flasks that had [U-¹⁴C]acetate added were combined in 3 sets of 6 corresponding to the time of feeding. Each set of flasks was individually filtered, extracted and purified as in Section E. The samples from feeding of 24 hours, 36 hours and 48 hours were analyzed by analytical HPLC with fraction collection followed by scintillation counting. HPLC conditions were as previously described. After collection the fractions were evaporated in vacuo, dried in a desiccator, dissolved in scintillation cocktail and counted for 2-5 minutes with an efficiency of 96%.

Results of sodium [U-¹⁴C]acetate incorporation:

<u>time of feeding</u>	<u>amount mg/ml</u> A33853	<u>cpm/mg</u> A33853	<u>%</u> <u>Incorporation</u>	<u>($\mu\text{Ci}/\mu\text{mol} \times 10^{-3}$)</u> <u>Specific Activity</u>	<u>% Spec.</u> <u>Incorp.</u>
24 hrs.	1.4×10^{-2}	4832	0.07	0.9	0.008
36 hrs.	0.7×10^{-2}	9678	0.07	1.8	0.016
48 hrs.	1.3×10^{-2}	5700	0.07	1.0	0.009

This experiment was repeated with the results shown below:

<u>time of feeding</u>	<u>amount mg/ml</u> A33853	<u>cpm/mg</u> A33853	<u>%</u> <u>Incorporation</u>	<u>($\mu\text{Ci}/\mu\text{mol} \times 10^{-3}$)</u> <u>Specific Activity</u>	<u>% Spec.</u> <u>Incorp.</u>
12 hrs.	4.2×10^{-3}	7119	0.003	1.3	0.012
24 hrs.	6.7×10^{-3}	1390	0.001	0.3	0.003
36 hrs.	2.5×10^{-3}	7840	0.002	1.4	0.013
48 hrs.	1.8×10^{-3}	4916	0.001	0.9	0.008

4. Incorporation of (S)-[U-¹⁴C]Lysine into A33853

A slant of Streptomyces NRRL 12068 was used to inoculate one flask of CSSXII vegetative medium which was incubated at 30°C on an orbit shaker at 275 rpm for 48 hours. Twelve flasks of SCO medium were then

inoculated from the CSSXII media and incubated at 30° and 275 rpm. After 24 hours of growth 4 of the flasks were fed 0.75 µCi of (S)-[U-¹⁴C]lysine (specific activity 0.44 µCi/mmol) each for a total of 3 µCi. A 5 µl aliquot of the lysine was taken for counting as a standard. After 36 hours of growth 4 more of the flasks were fed (S)-[U-¹⁴C]lysine in the same way for a total of 3 µCi. After 48 hours of growth 3 additional flasks of microbes were fed (S)-[U-¹⁴C]lysine as above for a total of 3 µCi. The fermentation was stopped after 60 hours of growth and the flasks that had (S)-[U-¹⁴C]lysine added were combined in 3 sets of 4 corresponding to the time of feeding. Each set of flasks was individually filtered, extracted and purified as in Section E. The samples from feedings of 24 hours, 36 hours and 48 hours were analyzed by analytical HPLC as previously described with fraction collection followed by scintillation counting. After collection the fractions were evaporated in vacuo, dried in a desiccator, dissolved in scintillation cocktail and counted for 2-5 minutes with an efficiency of 96%.

Results of (S)-[U-¹⁴C]lysine incorporation:

<u>time of feeding</u>	<u>amount mg/ml A33853</u>	<u>dpm/mg A33853</u>	<u>% Incorporation</u>	<u>(µCi/umol x 10⁻³) Specific Activity</u>	<u>% Spec. Incorp.</u>
24 hrs.	1.1 x 10 ⁻²	27907	1.15	5.2	1.18
36 hrs.	2.5 x 10 ⁻²	8035	0.72	1.5	0.34
48 hrs.	1.4 x 10 ⁻²	12167	0.87	2.3	0.79

5. Incorporation of [Carboxyl-¹⁴C]Anthranilic Acid into A33853

A slant of Streptomyces NRRL 12068 was used to inoculate one flask

of CSSXII vegetative medium which was incubated at 30°C on an orbit shaker at 275 rpm for 48 hours. Fifteen flasks of SCO medium were then inoculated from the CSSXII medium and incubated at 30° and 275 rpm. After 24 hours of growth four of the flasks were fed 5 µCi of [carboxyl-¹⁴C]anthranilic acid (specific activity 46 mCi/mmol) each for a total of 20 µCi. A 5 µl aliquot of the anthranilic acid was taken for counting as a standard. After 36 hours of growth 4 more of the flasks were fed [carboxyl-¹⁴C]anthranilic acid in the same way for a total of 20 µCi. After 48 hours of growth 3 additional flasks of microbes were fed [carboxyl-¹⁴C]anthranilic acid as above for a total of 15 µCi. The fermentation was stopped after 60 hours of growth and the flasks that had [carboxyl-¹⁴C]anthranilic acid added were combined in 3 sets corresponding to the time of feeding. Each set of flasks was individually filtered, extracted and purified as in section E. The samples from feedings of 24 hours, 36 hours and 48 hours were analyzed by analytical HPLC as previously described with fraction collection followed by scintillation counting. After collection the fractions were evaporated in vacuo, dried in a desiccator, dissolved in scintillation cocktail and counted for 2-5 minutes with an efficiency of 96%.

Results of [carboxyl-¹⁴C]anthranilic acid incorporation:

<u>time of feeding</u>	<u>amount mg/ml</u> A33853	<u>cpm/mg</u> A33853	<u>%</u> <u>Incorporation</u>	<u>(uCi/umol x 10⁻³)</u> <u>Specific Activity</u>	<u>% Spec.</u> <u>Incorp.</u>
24 hrs.	1.7 x 10 ⁻³	98738	0.17	17.52	0.038
36 hrs.	2.7 x 10 ⁻³	32279	0.038	5.72	0.012
48 hrs.	----	----	----	----	----

This experiment was carried out three times but poor production of A33853 yielded only these results.

6. Incorporation of (S)-[U-¹⁴C]Aspartic Acid into A33853

A slant of Streptomyces NRRL 12068 was used to inoculate one flask of CSSXII vegetative medium which was incubated at 30°C on an orbit shaker at 275 rpm for 48 hours. Twenty flasks of CSSXII medium were then inoculated from the SCO media and incubated at 30° and 275 rpm. After 24 hours of growth, 5 of the flasks were fed 1 µCi (0.5 ml) of (S)-[U-¹⁴C]aspartic acid (specific activity 14.72 µCi/mmol) each for a total of 5 µCi. A 5 µl aliquot of the aspartic acid was taken for counting as a standard. After 36 hours of growth, 5 more of the flasks were fed (S)-[U-¹⁴C]aspartic acid in the same way for a total of 5 µCi. After 48 hours of growth, 5 additional flasks of microbes were fed (S)-[U-¹⁴C]aspartic acid as above for a total of 5 µCi. The fermentation was stopped after 60 hours of growth and the flasks that had (S)-[U-¹⁴C]aspartic acid added were combined in 3 sets of 4 corresponding to the time of feeding. Each set of flasks was individually filtered, extracted and purified as in section E. The samples from feedings of 24 hours, 36 hours and 48 hours were analyzed by analytical HPLC as previously described with fraction collection followed by scintillation counting. After collection, the fractions were evaporated in vacuo, dried in a desiccator, dissolved in scintillation cocktail and counted for 2-5 minutes with an efficiency of 96%.
Results of (S)-[U-¹⁴C]aspartic acid incorporation:

<u>time of feeding</u>	<u>amount mg/ml A33853</u>	<u>cpm/mg A33853</u>	<u>% Incorporation</u>	<u>(uCi/umol x 10⁻³) Specific Activity</u>	<u>% Spec. Incorp.</u>
24 hrs.	3.7×10^{-2}	50	<1	0.14	0.001
36 hrs.	----	----	----	----	----
48 hrs.	4.5×10^{-2}	82	<1	0.28	0.002

7. Incorporation of (S)-[5-³H]Tryptophan into A33853

A slant of Streptomyces NRRL 12068 was used to inoculate one flask of CSSXII vegetative medium which was incubated at 30°C on an orbiting shaker at 275 rpm for 48 hours. Twenty-five flasks of SCO medium were then inoculated from the CSSXII media and incubated at 30° and 275 rpm. After 24 hours of growth 5 of the flasks were fed 1 µCi (0.5 ml) of (S)-[5-³H]tryptophan (specific activity 24.4 Ci/mmol) each for a total of 5 µCi. A 5 µl aliquot of the tryptophan was taken for counting as a standard. After 36 hours of growth 5 more of the flasks were fed (S)-[5-³H]tryptophan in the same way for a total of 5 µCi. After 48 hours of growth 5 additional flasks of microbes were fed (S)-[5-³H]tryptophan as above for a total of 5 µCi. The fermentation was stopped after 60 hours of growth and the flask that had (S)-[5-³H]tryptophan added were combined in 3 sets of 5 corresponding to the time of feeding. Each set was individually filtered, extracted and purified as in Section E. The samples from feedings of 24 hours, 36 hours and 48 hours were analyzed by analytical HPLC as described previously with fraction collection followed by scintillation counting. After collection the fractions were evaporated in vacuo, dried in a desiccator, dissolved in scintillation cocktail and counted for 2-5 minutes with an efficiency of 96%.

Results:

<u>time of feeding</u>	<u>amount mg/ml A33853</u>	<u>cpm/mg A33853</u>	<u>% Incorp.</u>	<u>(uCi/umol x 10⁻³) Specific Activity</u>	<u>% Spec. Incorp.</u>
24 hrs.	1.8x10 ⁻⁵	73814	0.002	13.1	5.36x10 ⁻⁵
36 hrs.	4.7x10 ⁻⁵	51310	0.004	9.11	3.73x10 ⁻⁵
48 hrs.	4.8x10 ⁻⁵	37300	0.003	6.63	2.71x10 ⁻⁵

H. Synthesis

1. γ,γ -Dicarbethoxy- γ -phthalimidobutyraldehyde 50^{131}

A benzene solution (10 ml) of acrolein (1.5 ml, 22 mmol) was added dropwise over a 10-20 minute period to a stirred solution of diethyl phthalimidomalonate (6.41 g, 22 mmol) in benzene containing sodium methoxide (50 mg, 0.9 mmol) at 19°C. After addition of the acrolein the mixture was stirred at room temperature (25-27°C) for 2 hours. At the end of this time a few drops of glacial acetic acid were added until the yellow solution became colorless. The solution was then filtered and evaporated in vacuo to give a yellow tinted oil 8.4 g. The aldehyde was purified by flash chromatography using 200 g of 60-230 mesh silica gel and a solvent system of ethyl acetate:hexane 3:7 to give a colorless oil. TLC: ethyl acetate:hexane 50:50 0.64 (3.97 g, 11 mmol).

IR ν_{\max} : (CHCl₃) 3020, 2980, 2940, 2900, 1780, 1710, 1370, 1140 cm⁻¹

¹H NMR: (CDCl₃) 1.29 (6H,t,J=7.2 Hz), 2.79 (4H,m,CH₂CH₂), 4.31 (4H,q, J=7.2 Hz), 7.81 (4H,m,ArH), 9.70 (1H,t,J=1.0 Hz, -CHO) ppm

¹³C NMR: (CDCl₃) 200.23 (CHO), 167.17 (CO₂ET), 165.93 (C=O), 134.36 (Ar), 131.31 (Ar), 123.43 (Ar), 66.86 (C-2), 62.71 (OCH₂), 39.19 (C-3), 25.36 (C-4), 13.64 (-CH₃) ppm

2. 2-Carbethoxy-2-phthalimido-5-hydroxyvalerate ¹³¹ 51

γ,γ -Dicarbethoxy- γ -phthalimidobutyraldehyde (4.0 g, 12 mmol), dissolved in a mixture of ether:water (6:1), was vigorously stirred at room temperature. Sodium borohydride (0.470 g, 12.1 mmol) was added in portions over 2 hours. A few drops of acetic acid were added to destroy the excess hydride. The aqueous layer was extracted with ether (5 x 60 ml). The extracts were dried over anhydrous Na_2SO_4 and evaporated in vacuo. The oil was purified by flash chromatography (silica gel 230-400 mesh, 200 g, ethyl acetate:hexane 6:4) to afford a colorless oil. (2.6 g, 7.18 mmol) 59% yield. TLC: ethyl acetate:hexane 50:50, $R_f=0.5$.

IR ν_{max} : (neat) 3400, 2981, 1778, 1715, 1769 cm^{-1}

^1H NMR: (CDCl_3) 7.80 (4H,m,ArH), 4.25 (4H,m,-O- CH_2 -), 3.62 (2H,t), 2.55 (2H,t), 1.66 (2H,m), 1.24 (6H,t), ppm

^{13}C NMR: (CDCl_3) 167.32 (CO_2Et), 166.33 (C=O), 134.29 (Ar), 131.46 (Ar), 123.45 (Ar), 67.69 (C-2), 62.58 (-O CH_2 -), 62.31 (C-5), 29.57 (C-4), 27.62 (C-3), 13.77 (CH_3) ppm

3. Ethyl 2-Carbethoxy-2-phthalimido-5-methanesulfonyloxyvalerate 52

A solution of methanesulfonyl chloride (2.83 g, 24.8 mmol) in methylene chloride (20 ml) was added dropwise to a stirred solution of ethyl-2-carbethoxy-2-phthalimido-5-hydroxyvalerate (4.5 g, 12.4 mmol) in methylene chloride (50 ml) containing triethylamine (2.87 g, 24.8 mol) at 0°. After complete addition, the mixture was stirred at room temperature for 2 hours and then concentrated in vacuo. The yellowish residue was then treated with an ethyl acetate:hexane (6:4) solution to

precipitate the triethylammonium salt. After filtration and washing of the filtrate with the same solvent the combined filtrates were concentrated in vacuo and purified by column chromatography (silica gel 230-400 mesh, 200 g, ethyl acetate hexane 4:6 followed by 1:1 mixture) to give a colorless oil (4.84 g, 11.0 mmol 89%). TLC: ethyl acetate:hexane 50:50, $R_f = .75$.

IR ν_{\max} : (neat) 3020, 2980, 1741, 1360, 1769, 960, 920 cm^{-1}

^1H NMR: (CDCl_3) 7.8 (m, 4H, Ar), 4.25 (m, 6H, C-4), 2.99 (s, 3H, Ms), 2.59 (m, 2H), 1.90 (m, 2H), 1.25 (m, 6H) ppm

^{13}C NMR: (CDCl_3) 167.17 (CO_2Et), 165.91 (C=O), 134.40 (Ar), 131.23 (Ar), 123.48 (Ar), 69.42 (C-5), 67.07 (C-2), 62.69 (-O- CH_2 -), 37.24 (C-3), 29.41 (C-4), 24.33 (Ms), 13.69 (- CH_3) ppm

4. Ethyl 2-Carbethoxy-2-phthalimido-5- ^{13}C , ^{15}N]cyanovalerate 53

Ethyl-2-carbethoxy-2-phthalimido-5-methanesulfonyl oxyvalerate (2.5 g, 5.68 mmol) was dissolved in dry dimethyl sulfoxide (10 ml) in a 50 ml two neck flask. The solution was heated to 47°C and treated with potassium cyanide (0.034 g, 0.52 mmol) while being stirred under argon. After 5 hours, the solution was treated with potassium $^{13}\text{C}^{15}\text{N}$ cyanide (90% ^{13}C , 99% ^{15}N) (0.174 g, 2.6 mmol). After an additional 15 hours, the solution was treated with potassium cyanide (0.034 g, 0.52 mmol) and stirred 3 more hours. The reaction mixture was poured into 10 ml of distilled water and then extracted 3 times with equal volumes of ethyl acetate. The ethyl acetate extracts were washed with brine, dried with Na_2SO_4 (anhydrous), filtered and evaporated in vacuo to leave a crude

oil which upon purification by column chromatography (230-400 mesh ethyl acetate:hexane, 4:6 yielded 0.8023 g, (2.15 mmol) of a crystalline product. The yield was 58.9% based on total KCN. The yield was 81.6% based on labeled cyanide. The enrichment of $^{13}\text{C}^{15}\text{N}$ was 83.9% based on mass spectrometry data of the m/z 374 peak and unlabeled m/z 372.

IR: (KBr) 3020, 2980, 2200, 1800, 1779, 1740, 1385 cm^{-1}

^1H NMR: (CDCl_3) 7.82 (4H, dm, Ar), 4.30 (4H, dd, (OCH_2^-), 2.59 (2H, m), 2.38 (2H, m), 1.79 (2H, m), 1.24 (6H, t, CH_3) ppm

^{13}C NMR: (CDCl_3) 167.17 (CO_2Et), 165.84 (C=O), 134.43 (Ar), 131.23 (Ar), 123.50 (Ar), 119.03, 118.80

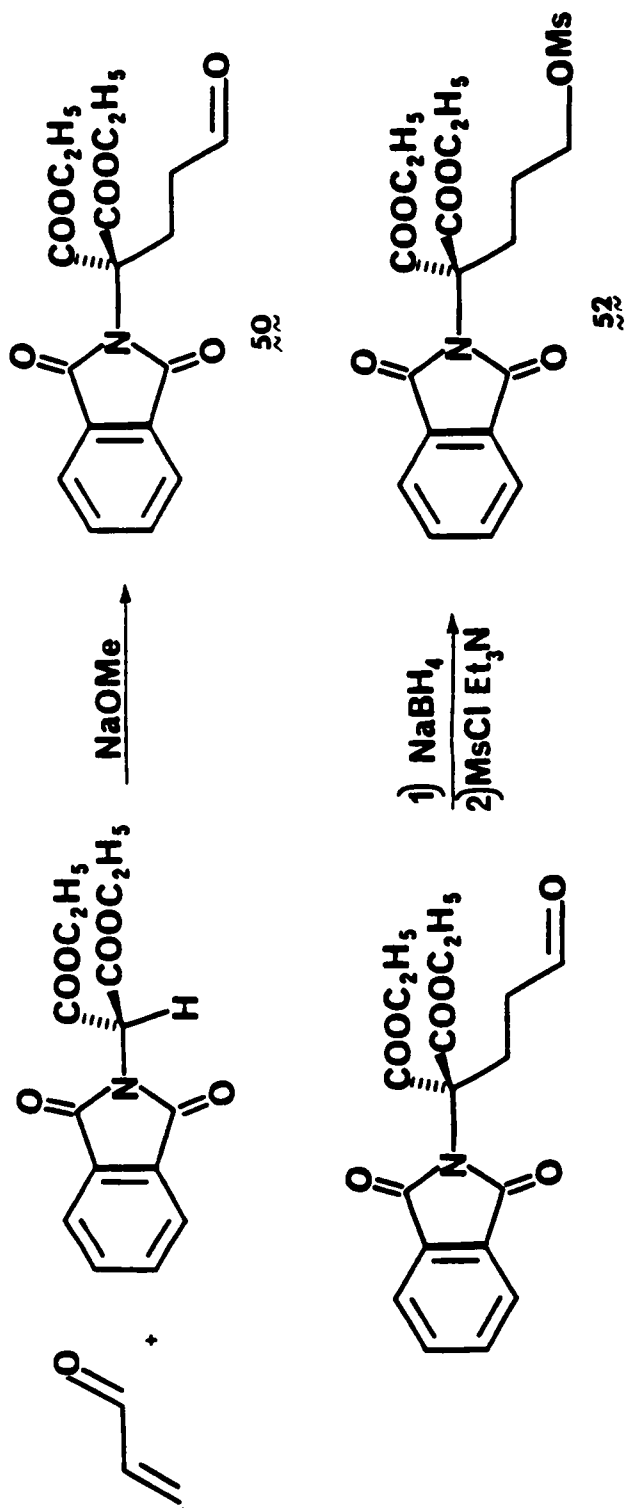
$J_{\text{CN}}=14.5$ Hz C=N), 67.05 (C-2), 62.78 ($-\text{O}-\text{CH}_2^-$), 32.22 (C-5), 20.75 (C-4), 17.45 (C-3), 16.60 ($-\text{CH}_3$), 13.71 ($-\text{CH}_3$) ppm

5. (RS)-[6- ^{13}C , 6- ^{15}N]Lysine Hydrochloride 54

Ethyl-2-carbethoxy-2-phthalimido-5[^{13}C , ^{15}N] cyanovalerate (0.8023 g, 2.15 mmol) was dissolved in 20 ml of a 50% solution of glacial acetic acid in concentrated HCl containing PtO_2 (0.250 g, 1.1 mmol). The mixture was hydrogenated in a Parr hydrogenator at room temperature and 60 psi for 24 hours. The PtO_2 was removed by filtration and the acid evaporated in vacuo. The crude residue was hydrolyzed in 6N HCl for 24 hours followed by neutralization with 1N NaOH and evaporation of the H_2O to give the crude lysine. The lysine was purified by ion exchange chromatography using Dowex 50X resin. 0.150 g (0.83 mmol, 38%) of product was obtained. By NMR, the amount of labeled lysine was estimated to be 76%.

^1H NMR: (D_2O) 3.74 (1H,t,CH), 2.90 (2H,m,- CH_2^-), 1.72 (2H,m,- CH_2^-),
1.49 (2H,m,- CH_2^-), 1.24 (2H,m,- CH_2^-) ppm

^{13}C NMR: (D_2O) 179.2 (C-1,s), 54.4 (C-2,s), 38.9 (C-6,d), 29.7 (C-3,s),
26.4 (C-5,s), 26.2 (C-4,s) ppm



Synthesis of Ethyl-2-carboxy-2-phthalimido-5-methanesulfonylvalerate

Scheme XXXV

CHAPTER VIII.

RESULTS AND DISCUSSION

A. ^{13}C NMR Assignment

The first test in carrying out a biosynthetic study with the use of stable isotopes is the assignment of the ^{13}C NMR spectrum of the target molecule. The ^1H NMR resonances which can often be used to assist in making these assignments were between 7.2-8.4 ppm in A33853, and were thus of little value in making the ^{13}C assignment. No attempt was made to assign these resonances.

As with the proton spectra, the carbon spectra was difficult to assign due to the many aromatic resonances from 114 to 168 ppm. The decoupled spectrum gave the resonances in Table X. An INEPT (Induced Nuclear Enhance Polarization Transfer) ^{13}C spectra showed the peaks at 140.0, 129.3, 125.1, 127.7, 126.3, 124.8, 120.5, 119.4, 114.5 ppm to be due to methine carbons and the remaining peaks due to quarternary carbons (Table X).

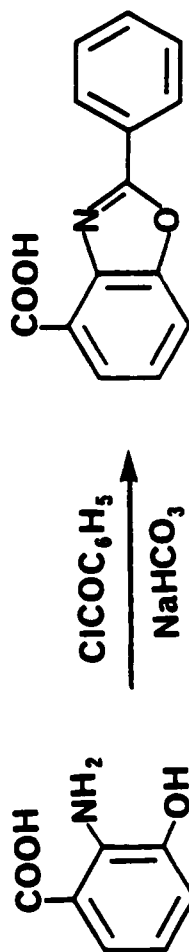
Model compounds were used to help make the assignments. They included 3-hydroxypicolinic amide, 3-hydroxypicolinic acid, 3-hydroxy anthranilic acid, picolinic acid, benzoxazole, phenyl benzoxazole, A23187¹¹³, and cezomycin¹¹⁷. Their structures and ^{13}C NMR assignments are shown in Figure 28.

Attempts at acid and base hydrolysis of A33853 failed to provide any isolatable model compounds. Synthesis of 2-benzylbenzoxazole carboxylic acid as a model compound also failed as the 3-benzoyloxy anthranilic acid would not condense (Scheme XXXVII).

Table X

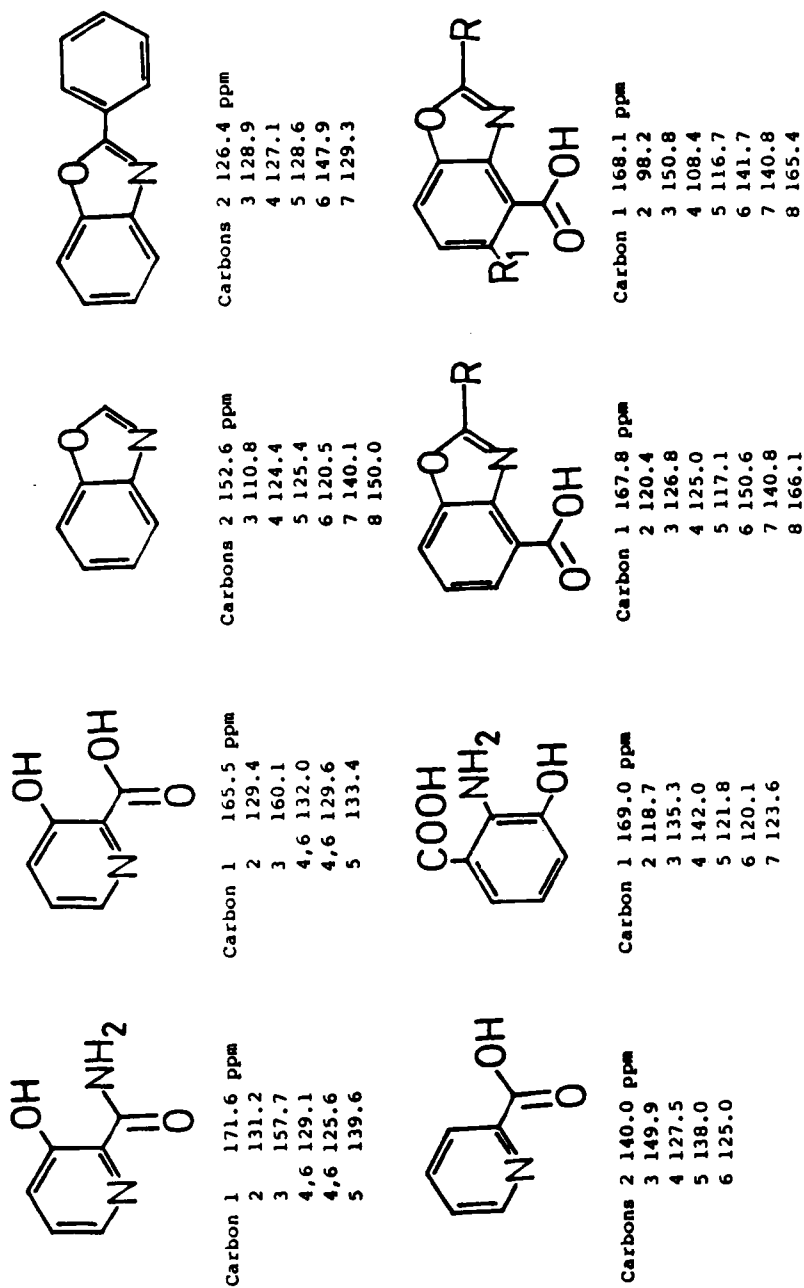
¹³C NMR Data From Decoupled and INEPT Spectra

<u>literature</u> ¹³ C nmr	<u>experimental</u> ¹³ C d ₆ -DMSO	<u>Identified as methines</u> <u>by INEPT</u>
167.5 ppms	167.5 ppms	
165.8	165.7	
162.6	162.6	
157.2	157.2	
153.2	153.2	
150.4	150.4	
140.3	140.6	
140.1	140.0	140.0
131.2	131.4	
129.3	129.2	129.3
127.8	127.7	127.7
126.5	126.4	126.3
125.9	125.8	125.1
124.9	124.9	124.8
123.5	123.6	
122.9	122.9	
122.5	122.5	
120.6	120.5	120.5
119.7	119.6	119.4
114.5	114.5	114.5



Attempted Synthesis of 2-Phenylbenzoxazole Carboxylic Acid

Scheme XXXVII



Model Compounds for ^{13}C NMR Assignment of A33853

Figure 28

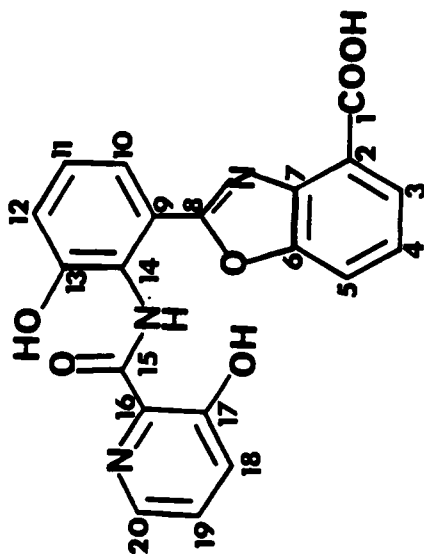
¹³C NMR of A33853

ppm

- 167.5
- 165.8
- 162.6
- 157.2
- 153.2
- 150.4
- 140.3
- 140.1
- 131.2
- 129.3
- 127.8
- 126.5
- 125.9
- 124.9
- 123.5
- 122.9
- 122.5
- 120.6
- 119.7
- 114.5

Tentative
Assignments

- C-15
- { C-1
- { C-8
- C-17
- { C-13
- { C-6
- { C-7
- C-19
- C-16
- C-18
- { C-12
- { C-11
- C-20
- C-4
- { C-14
- { C-9
- { C-2
- C-10
- { C-3
- { C-5



{ may be interchanged

¹³C NMR Assignment to A33853

Figure 29

Consideration of the ^{13}C NMR spectra of A33853 and the model compounds led to the assignment shown in Figure 29.

B. Production Curve

A production curve was prepared to determine the growth time for maximum production of A33853. This was done by harvesting the microbes after 36, 48, 60, 72 and 84 hours of growth and analysis of the A33853 content. Maximum production was found to occur at 60 hours and the amount appears to decrease after 70 hours (Figure 27). The reduction in A33853 is probably due to feedback inhibition followed by utilization or degradation of A33853 by other pathways (i.e. tryptophan).

C. Incorporation of ^{14}C and ^3H Precursors

Several ^{14}C and ^3H precursors were fed to the producing microbes. These included sodium [$\text{U-}^{14}\text{C}$]acetate, [carboxyl- ^{14}C]anthranilic acid, D-[$\text{U-}^{14}\text{C}$]glucose, (S)-[5- ^3H]tryptophan, (S)-[$\text{U-}^{14}\text{C}$]aspartic acid, and (S)-[$\text{U-}^{14}\text{C}$]lysine. The precursors were chosen so as to represent various pathways for the production of A33853. Sodium [$\text{U-}^{14}\text{C}$]acetate would represent a polyketide pathway; D-[$\text{U-}^{14}\text{C}$]glucose a polyketide pathway and shikimate acid pathway; tryptophan and anthranilic acid would be through metabolism-catabolism pathways, while lysine, aspartic acid and pyruvate would represent pathways that give rise to the pyridine nucleus of 3-hydroxypicolinic acid.

The precursors were fed at 24 hr., 36 hr., and 40 hours of growth to determine if they had higher incorporation into A33853 rather than to

other cellular pathways (i.e. cell wall production). The feeding results are shown in Table XI.

1. Sodium [U-¹⁴C]Acetate

Two feeding experiments were carried out with A33853 and each time the precursors were added at 24, 36 and 48 hours. The microbes were harvested after 60 hours of growth and the A33853 isolated and purified as in Chapter 7, section C. The maximum incorporation occurred with precursor addition after 36 hours of growth at a level of incorporation of 0.07% with a specific incorporation of 0.016%. These results are interpreted as meaning that a polyketide pathway using sodium acetate, is not a major contributor to A33853 production (Figure 30).

2. (S)-[U-¹⁴C]Aspartic acid

One feeding experiment was carried out with aspartic acid and precursor addition occurred at 24, 36 and 48 hours. The microbes were harvested after 60 hours of growth. The specific incorporations were 0.001% for 24 hours and 0.002% for 48 hours. No A33853 was isolated from the 36 hour feeding. The results are interpreted to mean that aspartic acid is not directly incorporated into A33853. This result could be due to metabolism of aspartic acid into other cellular functions. The result is slightly surprising in that aspartic acid is involved in lysine biosynthesis and has been postulated as a possible precursor to pyridine rings (Schemes XXX and XXI).

The results of the aspartic acid and lysine incorporation studies are similar to other published results in that when lysine is

incorporated to a significant level aspartic acid incorporation is poor.¹²¹ This is as suggested by the postulated two mechanisms, one in which lysine forms the picolinic acid nucleus, and the second in which aspartic acid and pyruvate form the nucleus without the incorporation of lysine (Schemes XXX, XXXI).

3. [Carboxyl-¹⁴C]Anthranilic Acid

Anthranilic acid was fed at 24, 36 and 48 hours. Maximum incorporation occurred at 24 hours with 0.17 percent incorporation and 0.038 percent specific incorporation. The incorporation is very low and suggests that anthranilic acid is not a direct precursor to A33853 and, more precisely, not a precursor to the 3-hydroxyanthranilic acid moiety found in A33853. Other reasons for the poor incorporation would be failure of anthranilic acid to transport across the cellular membranes and/or utilization in other cellular functions.

4. (S)-[5-³H]Tryptophan

(S)-[5-³H]tryptophan was fed at 24, 36 and 40 hours. The basis for this experiment was the work by David¹¹⁶ in which he postulated tryptophan to be the precursor to the 2-alkyl benzoxazole of cezomycin 43. Another basis is that the catabolism of tryptophan gives a 3-hydroxyanthranilic acid intermediate (Scheme XXXIII). The resulting maximum percent incorporation is 0.004 and 5.36×10^{-5} percent specific incorporation at 24 hours. The tryptophan was not incorporated into A33853 or was used in other cellular functions.

This result is more important as one realizes that tryptophan can give rise to both the 3-hydroxyanthranilic acid and 3-hydroxypicolinic acid.¹³² A large incorporation of tryptophan would not only suggest that tryptophan is a precursor but that the purpose of A33853 biosynthesis might be as a control pathway for storage of the amino acid or for growth in a tryptophan enriched environment.

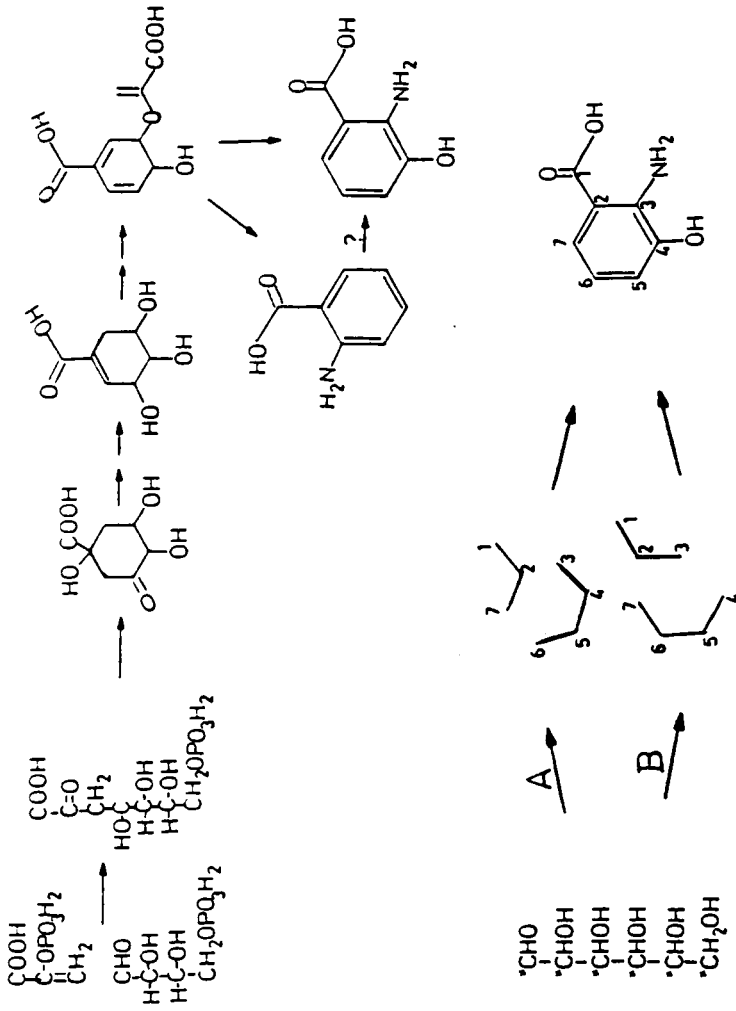
5. D-[U-¹⁴C]Glucose

D-[U-¹⁴C]glucose was fed at hours 24, 36 and 40 as a possible carbon source for A33853. Maximum incorporation occurred at 24 hours with a 0.250 percent specific incorporation in the first run and at 48 hours with 0.23 percent incorporation in the second feeding experiment. These incorporations are significantly larger than any other carbon source fed that would give rise to the 3-hydroxyanthranilic portion of A33853.

Interpretation of this result leads to the postulation of D-glucose as the major carbon source for A33853. Zmijewski,^{129,130} studying the biosynthesis of A23187, found glucose as a precursor to the aromatic ring (Scheme XXXVIII). This type of pathway (shikimate acid type) is probably occurring in A33853.

6. (S)-[U-¹⁴C]Lysine

(S)-[U-¹⁴C]lysine was fed at 24, 36 and 48 hours. The maximum percent incorporation and percent specific incorporation occurs at 24 hours and is at least an order of magnitude larger than any other precursor. The lysine is believed to be incorporated into the 3-



[U-¹³C]Glucose as a Precursor to A23187 via Shikimate Acid Pathway

Scheme XXXVIII

Table XI

Summary of Labeled Precursor Incorporations into A33853

<u>Precursor</u>	<u>Feeding Time</u> hours	<u>mg/ml</u> <u>A33853</u>	<u>cpm/mg</u> <u>A33853</u>	<u>%</u> <u>Incorp.</u>	<u>uCi/μmole</u> <u>Specific</u> <u>Activity</u> <u>(x10⁻³)</u>	<u>% Specific</u> <u>Incorp.</u>	
[U- ¹⁴ C]sodium acetate	24	1.4x10 ⁻²	4839	0.07	0.9	0.008	
	36	0.7x10 ⁻²	9678	0.07	1.8	0.016	
	48	1.3x10 ⁻²	5700	0.07	1.0	0.009	
	12	4.2x10 ⁻³	7119	0.003	1.3	0.012	
	24	6.7x10 ⁻³	1390	0.001	0.3	0.003	
	36	2.5x10 ⁻³	7840	0.002	1.4	0.013	
	48	1.8x10 ⁻³	4910	0.001	0.9	0.008	
	D-[U- ¹⁴ C]glucose	24	1.7x10 ⁻²	41176	0.56	7.5	0.250
		36	1.3x10 ⁻²	3911	0.05	0.7	0.020
	48	3.6x10 ⁻³	14909	0.07	2.7	0.090	
	24	2.3x10 ⁻²	11569	0.51	2.1	0.07	
	36	1.2x10 ⁻²	16399	0.39	3.0	0.10	
	48	2.5x10 ⁻³	37500	0.18	6.9	0.23	
(S)-[U- ¹⁴ C] aspartic acid	24	3.7x10 ⁻²	50	<1	0.14	0.001	
	36	---	---	---	---	---	
	48	4.5x10 ⁻²	82	<1	0.28	0.002	
[carboxyl- ¹⁴ C]-anthranilic acid	24	1.7x10 ⁻³	98738	0.17	17.52	0.038	
	36	2.7x10 ⁻³	32279	0.038	5.72	0.012	
	48	---	---	---	---	---	
(S)-[U- ¹⁴ C]lysine	24	1.1x10 ⁻²	27907	1.15	5.2	1.18	
	36	2.5x10 ⁻²	8035	0.72	1.5	0.340	
	48	1.4x10 ⁻²	12167	0.87	2.3	0.790	
(S)-[³ H]tryptophan	24	1.8x10 ⁻⁵	73814	.002	13.1	5.36x10 ⁻⁵	
	36	4.7x10 ⁻⁵	51310	.004	9.11	3.73x10 ⁻⁵	
	48	4.8x10 ⁻⁵	37300	.003	6.63	2.71x10 ⁻⁵	

hydroxypicolinic amide moiety of A33853 such as described by Hook and Vining into etamycin¹²⁰ and Reed into virginiamycin S₁.¹²¹ It is interesting to note that lysine is incorporated while aspartic acid incorporation is zero or very small, although aspartic acid is a precursor of lysine.

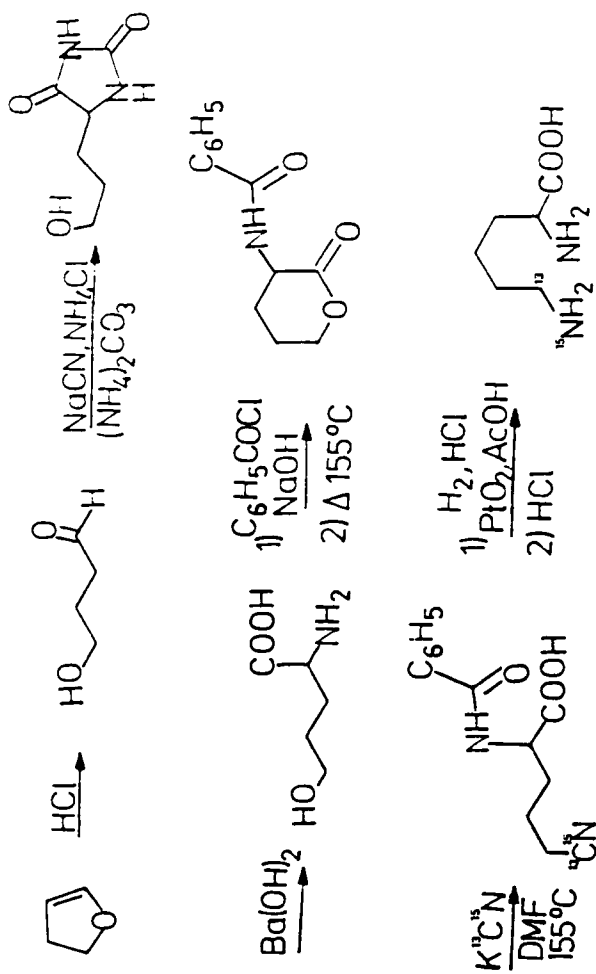
D. Incorporation of ¹³C-Labeled Precursors

1. Incorporation of Sodium [1,2-¹³C₂]Acetate, D-[U-¹³C]Glucose and (RS)-[6-¹³C,6-¹⁵N]Lysine

Feeding experiments were carried out repeatedly with sodium [1,2-¹³C₂]acetate and D-[U-¹³C]glucose in order to determine the position of glucose in the biosynthesis and to help in assigning carbon-13 resonances by feeding sodium [1,2-¹³C₂]acetate. Both experiments failed because of inadequate production.

A third experiment involving (RS)-[6-¹³C,6-¹⁵N]lysine was planned in which the labeled lysine would be fed to prove that the lysine is incorporated into the 3-hydroxypicolinic amide moiety and to postulate the mode of ring closure for the ring from lysine. The previous carbon-13 experiments suggested that the experiment might fail due to inadequate production. Therefore, the synthesized (RS)-[6-¹³C,6-¹⁵N]lysine was given to J. Reed for incorporation into the 3-hydroxypicolinic acid found in virginiamycin S₁.¹²¹

Several synthetic approaches were attempted in producing the (RS)-[6-¹³C,6-¹⁵N]lysine. Scheme XXXIX outlines one of the approaches that failed. The actual synthetic strategy was to add the ¹³C¹⁵N label as late as possible in the synthesis and to use a "sandwich" technique



Attempted Synthesis of (RS)-[6-¹³C, 6-¹⁵N] Lysine

Scheme XXXIX

to obtain maximum yield.

Acrolein underwent a Michael addition with N-phthalimido diethyl malonate in benzene and a catalytic amount of sodium methoxide to give product 50. The γ aldehyde 50 was then reduced with sodium borohydride and purified to yield the resulting alcohol. Mesylation in methylene chloride yields the γ -mesylate 52.¹³¹ The mesylate underwent cyanide displacement by a sandwich technique in which 2 equivalents of mesylate were stirred with 0.25 equivalents of KCN in DMSO at 50°C for 5 hours, with 1 equivalent of K¹³C¹⁵N (90% ¹³C, 99% ¹⁵N) for 15 hours followed by 3 hours with 0.25 equivalents of KCN. The resulting product ethyl-2-carbethoxy-2-phthalimido-5[¹³C¹⁵N]cyanovalerate 53 was found to be 83.9% labeled. Hydrogenation with 0.5 equivalent PtO₂ in conc. HCl and acetic acid 50:50 followed by filtration and hydrolysis gave (RS)-[6-¹³C,6-¹⁵N]lysine hydrochloride 54.

Ninety milligrams of the labeled lysine were sent to SmithKline Laboratories in Belgium where it was fed to Streptomyces virginiae and the virginiamycin isolated and returned to Josephine Reed. The virginiamycin S₁ was purified and examined by ¹³C NMR. The ¹³C NMR showed ¹³C enrichment in the 3-hydroxypicolinic acid portion and the 4-oxopiperic acid portion. The ¹³C¹⁵N coupling constants in the pyridine ring were too small (~1-3 Hz) to be detected so the virginiamycin S₁ was hydrolyzed in 6N HCl and examined by mass spectrometry.

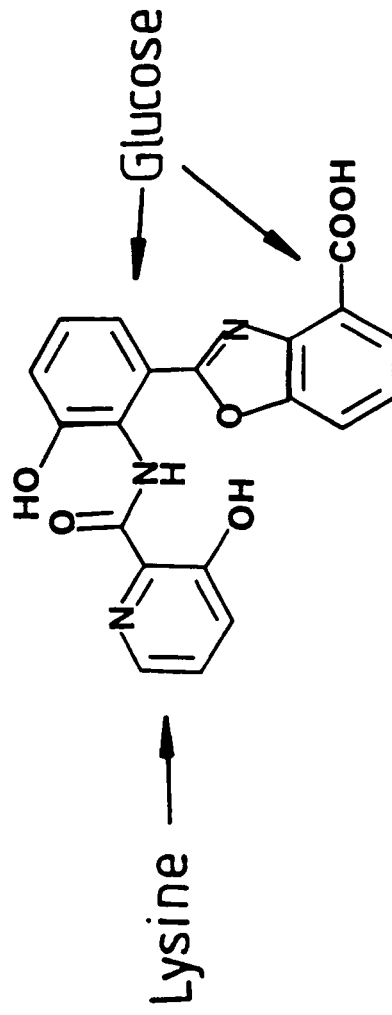
The results showed both carbon 13 and nitrogen 15 enrichments in the 3-hydroxypicolinic acid, thereby providing evidence that lysine is a precursor and that the ring closure occurs via a pathway postulated by

Gupta and Spenser for pipercolic acid. This pathway, Scheme XXXX, postulates a deamination of the α -amino group followed by ring closure, dehydration and aromatization of the resulting ring. This same type of pathway is believed to occur in A33853.

E. Summary

The biosynthesis of A33853 was studied by feeding a series of radioactive precursors. It was suggested that D-glucose and (S)-lysine were the two major pathways (Figure 31) giving rise to A33853 and that maximum product occurs after 60 hours of growth. A partial carbon-13 assignment was made using model compounds. (RS)-[6-¹³C,6-¹⁵N]lysine was synthesized and used to study the formation of 3-hydroxypicolinic acid from lysine in virginiamycin S₁ by Josephine Reed.

Future work on A33853 should involve increasing the antibiotic yield through microbiology techniques so that ¹³C labeling experiment can be carried out. Although the synthesis of model compounds and hydrolysis of A33853 were attempted, a selective degradation procedure would also be useful for studying ¹³C-incorporations.



Basic Precursors in A33853 Biosynthesis

Figure 31

CHAPTER IX.

CONCLUSIONS

Part A of this dissertation was concerned with the stereochemistry of virginiamycin M_1 biosynthesis. The following significant findings were made:

- 1) Oxazole ring formation involves incorporation of (S)-serine and loss of the (pro-S) hydrogen from carbon-3, possibly by oxidation followed by enolization as in pathway C, Scheme XXIII.
- 2) (R) and (S) prolines are both incorporated into virginiamycin M_1 .
- 3) Formation of the dehydroproline moiety occurs with loss of the 3-(pro-R) hydrogen, possibly by the pathway (S)-proline \rightarrow (R)-proline \rightarrow virginiamycin M_2 \rightarrow virginiamycin M_1 . This pathway would involve a syn dehydrogenation of virginiamycin M_2 to virginiamycin M_1 (Scheme XXVIII).
- 4) The first evidence that virginiamycin M_2 is possibly a precursor to virginiamycin M_1 .
- 5) The first stereoselectivity shown in the formation of dehydroamino acids.

Part B was concerned with the basic biosynthesis of A33853. The major conclusions were that D-glucose and (S)-lysine are the major precursors to this antibiotic. In addition, (RS)-[6- ^{13}C ,6- ^{15}N]lysine was synthesized to study the ring closure of 3-hydroxypicolinic acid, and a tentative ^{13}C NMR spectrum assignment was proposed.

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