Synthesis of Labeled 3-Hydroxyproline and Biosynthesis of the Dehydroproline Moiety of Virginiamycin M_1

by

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(ABSTRACT)

(R,S)-[Carboxyl-^{14}C]-cis-3-hydroxyproline was synthesized from S-[carboxyl-^{14}C] proline. The oxygen functionality at the three position was obtained by acetylation of 1,2-dehydroproline methyl ester using lead tetraacetate. Reduction of the imine with sodium borohydride gave predominately (R,S)-[carboxyl-^{14}C]-cis-3-acetoxyproline which was hydrolyzed with hydrochloric acid and purified by ion-exchange chromatography and recrystallization.

In order to determine if cis-3-hydroxyproline is a precursor for the dehydroproline moiety of virginiamycin M_1, (R,S)-[carboxyl-^{14}C]-cis-3-hydroxyproline and S-[3,4-^{3}H] proline with a ^{3}H / ^{14}C of 9 were fed simultaneously to a virginiamycin producing strain of *Streptomyces*. The resulting antibiotic had a ^{3}H / ^{14}C ratio of 41.3. The proline portion of the antibiotic had a ratio of 19.9. Therefore, 45 % of the cis-3-hydroxyproline was incorporated, and cis-3-hydroxyproline is a precursor to the dehydroproline moiety.

Acknowledgements

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Special appreciation goes to my parents , who instilled a desire to learn and the patience not to give up, and also to my siblings , , and who were always there. To my various support groups and friends spread out all over campus, Blacksburg, and the country, THANKS. Also, a special thanks goes to for the use of his computer, even though 'I paid my dues.'

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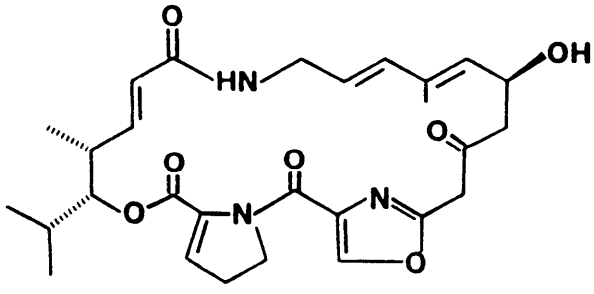
1.0 INTRODUCTION

Virginiamycin is an antibiotic which is used in animal husbandry as a feed additive. It is an effective performance promoter due to its ability to inhibit growth of intestinal flora by inhibition of protein synthesis. The antibiotic is also used for treatment of swine dysentery. The antibiotic is a synergistic mixture which is composed of A and B components. The A component is a macrocyclic lactone which contains a substituted aminodecanoic acid, a Δ^2-pyrroline ring, and an oxazole ring. The B component is characterized as a cyclic hexadepsipeptide. The presence of the Δ^2-pyrroline ring and the unusual oxazole ring in the A component virginiamycin M_1 (1) has sparked an interest in the biosynthesis of this compound.[1] This thesis will deal with the biosynthesis of the Δ^2-pyrroline ring.



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One possible pathway for formation of the Δ^2-pyrroline ring is dehydration of a 3-hydroxyproline intermediate. Trans-3-hydroxyproline was shown not to be a biosynthetic precursor to the 2,3-dehydroproline moiety.[2] Therefore, an efficient, convenient method for synthesis of ^{14}C-labeled cis-3-hydroxyproline was needed. Several variations of literature methods were investigated although to little avail. These attempts are outlined in Figure 1 even though only the methods involving the 3-acetoxy intermediate will be discussed in this thesis.

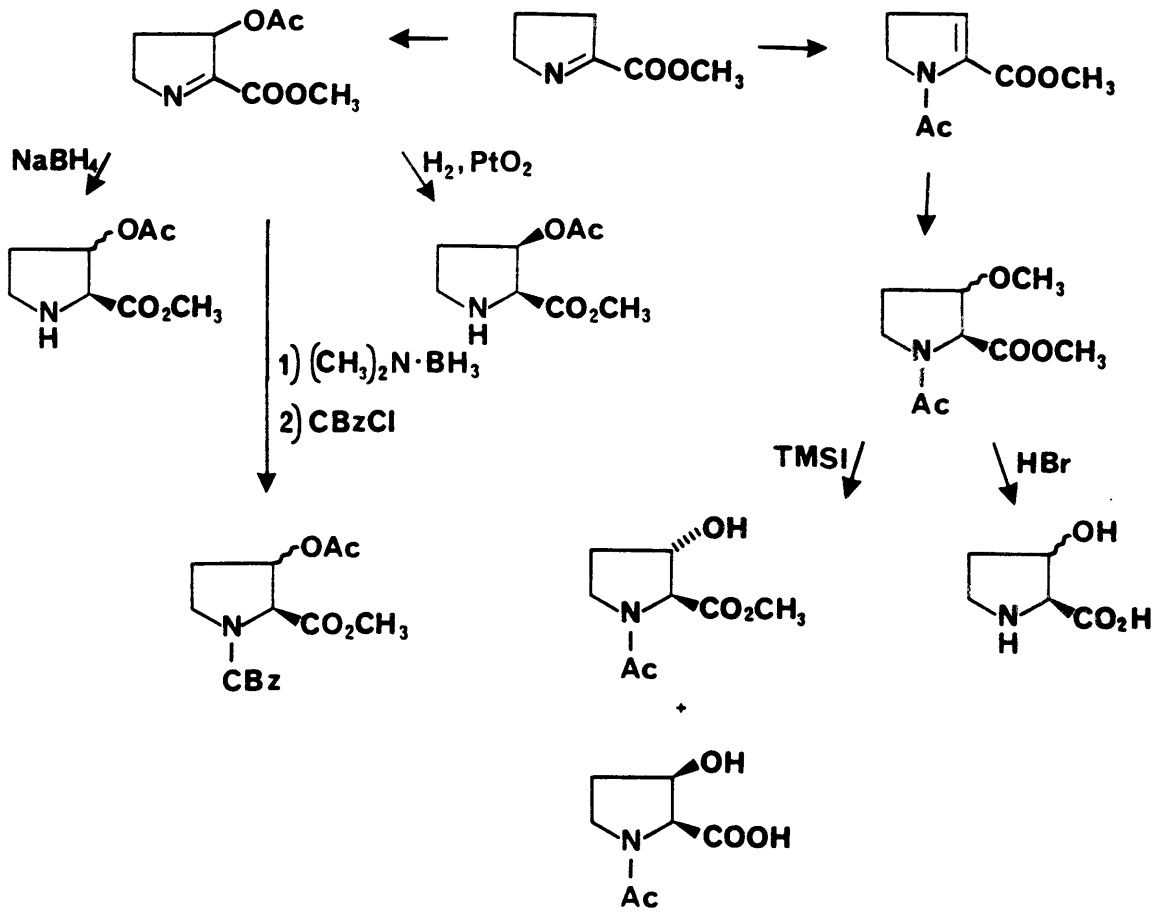


Figure 1. Cis-3-Hydroxyproline Synthetic Methods

2.0 LITERATURE REVIEW

2.1 3-HYDROXYPROLINE

2.1.1 Discovery and Formation

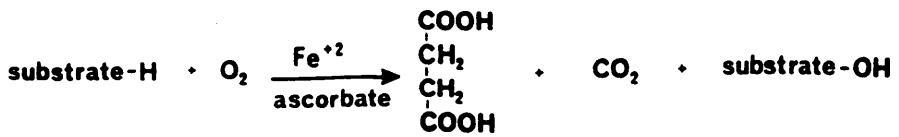
Partial reviews by Vickery[3] and by Mauger and Witkop[4] of the discovery and synthesis of 3-hydroxyproline may be found in Advances in Protein Chemistry and in Chemical Reviews, respectively. This review will briefly cover the discovery, formation, and the synthetic methods used for the synthesis of 3-hydroxyproline.

The first reported appearance of 3-hydroxyproline occurred at a meeting of the Federation of American Societies for Experimental Biology in 1961 by Ogle, Logan, and Arlinghaus. The imino acid was discovered in a peptide from enzymatic hydrolysates of collagen from bovine Achilles tendons which had the sequence Gly-X-Hypro.[5] [6] The imino acid comprises 0.26 % of bovine Achilles tendon collagen. Proof of the structure of 3-hydroxyproline for the unknown imino acid was obtained by elemental analysis of the compound and its derivatives. The derivatives were obtained by formation of an O-acetyl derivative, reduction to proline using hydrogen iodide and red

phosphorus, oxidation to β-alanine with $KMnO_4$, and comparison of the natural compound to the synthetic compound.[7] Later, evidence was given for accepting 3-hydroxyproline as an imino acid component of proteins.[8]

3-Hydroxyproline has also been discovered in several other sources besides bovine Achilles tendon collagen. The trans isomer of 3-hydroxyproline was isolated from acid hydrolysates of a Mediterranean sponge; the yield of 3-hydroxyproline was 62 mg from 64 g of dried sponge.[9] 3-Hydroxyproline has been found in two antibiotics, telomycin[10 11] and empedopeptin.[12] Acid hydrolysates of the antibiotic telomycin yielded two, cyclic imino acids which were determined to be trans-3-hydroxy-(S)-proline and cis-3- hydroxy-(S)-proline. The trans isomer was identical with the 3-hydroxyproline isolated from the Mediterranean sponge. 31 mg of the trans isomer was obtained from 1 g of telomycin, and 33 mg of the cis isomer was obtained from telomycin. Another source of trans-3-hydroxy-(S)-proline is the tropical legume *Delonix regia*. In contrast to the other sources, it occurs in the free amino acid pool of the seeds and vegetative tissues in an unbound form.[13] Basement membrane collagen has the unique characteristic of having a fairly large 3-hydroxyproline content. There are 6 - 25 3-hydroxyproline residues per 1000 residues for basement membrane collagen, while the α-1 chain of rat skin or calf skin interstitial collagen has one 3-hydroxyproline residue in the 1052 residues.[14 - 17] 3-Hydroxyproline can also be found in body collagen fractions of earthworm.[18]

In collagen 3-hydroxyproline is formed by the enzyme prolyl-3-hydroxylase. Prolyl-3-hydroxylase belongs to the enzyme group of 2-oxoglutarate dioxygenases which require $Fe,^{+2}$ 2-oxoglutarate, 0_2, and ascorbate.[19] 2-Oxoglutarate dioxygenase hydroxylates the substrate by decarboxylation of 2-oxoglutarate forming succinate and the hydroxylated product. One atom of the required O_2 molecule is incorporated into the succinate while the other atom is incorporated into the hydroxyl group of the substrate as shown below.[20]



Since 3-hydroxyproline has only been found in type I and type IV collagen in the sequence Gly-3Hyp-4Hyp-Gly, the substrate sequence for prolyl-3-hydroxylase is probably Gly-Pro-Pro-Gly or Gly-Pro-4Hyp-Gly. Although the hypothesis has not yet been proved, evidence indicates the required substrate sequence is Gly-Pro-4Hyp-Gly.[19 - 21] The prolyl-3-hydroxylase enzyme has not yet been isolated as a homogeneous protein, but it appears a to be a glycoprotein with a molecular weight of 160,000 when purified by gel filtration from chick embryos.[22] One must assume a similar prolyl-3-hydroxylase enzyme is used for formation of 3-hydroxyproline in sources of 3-hydroxyproline other than collagen.

2.1.2 Synthesis

There have been several syntheses of 3-hydroxyproline and its derivatives reported in the literature. A common method is the use of the 5-phthalimido-2-pentenoic acid intermediate which was made by the method of Baker et al.[23] Two versions of this synthesis are reported. The 5-phthalimido-2-pentenoic acid was converted either to methyl 2-chloro-3-methoxy-5-phthalimidovalerate[8 24 25] by esterification and methoxychlorination or to 2-bromo-3-methoxy-5-phthalimidopentanoic acid.[11 26] The phthalimido group was removed using base and cyclized to form 3-methoxyproline methyl ester or 3-methoxyproline, respectively. The methoxy group was hydrolyzed using constant boiling hydrobromic acid. Overall yields range from 30 - 40 % with a cis to trans ratio of 1:1. The two methods are outlined in Figure 2.

Witkop's group synthesized cis- and trans-3-hydroxyproline by two methods. The first method involves hydroboration of 3,4-dehydro-N-carbobenzyloxy proline methyl ester which gives 68 % of the trans compound plus 4-hydroxy- and allo-4-hydroxyproline.[9] Oxidation of the hydroxy group to the ketone followed by reduction of the ketone gives predominately cis-3-hydroxyproline.[27] The other method is a one-step condensation of aminomalonic acid with acrolein which avoids the inconvenient preparation of 3,4-dehydroproline[28] giving approximately

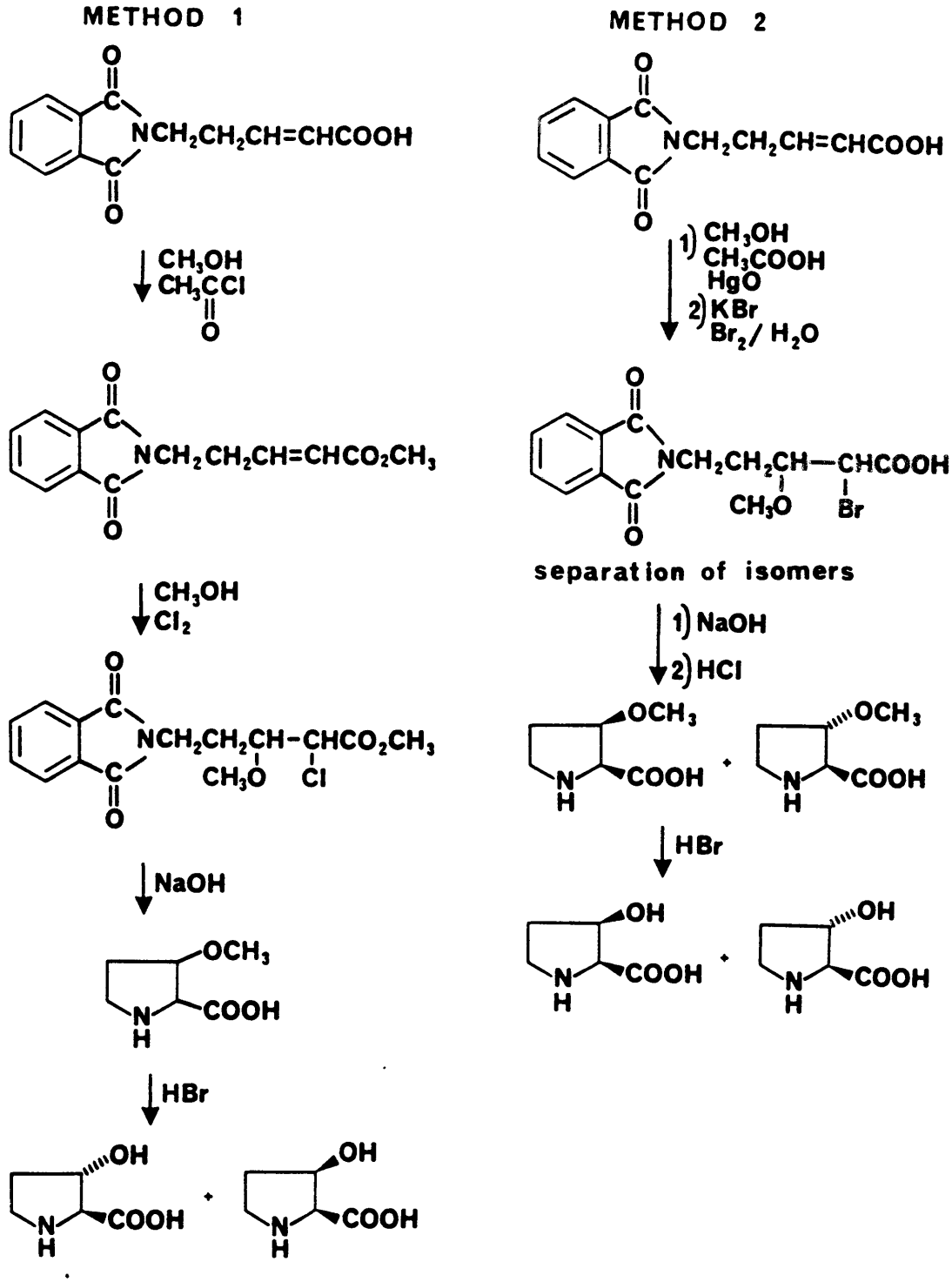


Figure 2. Synthesis using the 5-Phthalimido-2-Pentenoic Acid Intermediate

a 1:1 ratio of cis to trans product in a 40 % overall yield. The syntheses by Witkop are delineated in Figure 3.

Blake et al. reported a synthesis of 3-hydroxyprolines from investigation of the Dieckmann condensation for preparation of 3-pyrrolidinones.[29] Either ethyl N-ethoxy carbonyl-N-(2-ethoxycarbonylethyl)-glycinate or N-ethoxycarbonyl-N-cyano-methyl-β-alanine ethyl ester was cyclized using potassium tert-butoxide in toluene. The 3-pyrrolidinone derivatives formed were reduced to the alcohol using sodium borohydride, and the protecting groups were hydrolyzed with barium hydroxide. The 3-hydroxyproline isomers were obtained in a 30 - 40 % overall yield and a 1:2 ratio of cis to trans isomers was obtained for either route due to the refluxing barium hydroxide step. The 3-hydroxyproline synthesis through the 3-pyrrolidinone intermediates is shown in Figure 4.

Vercellotti and Feil[30] report a synthesis similar to Blake's in which ethyl N-benzyloxycarbonyl-N-(2-ethoxycarbonylethyl)-glycinate undergoes the Dieckmann condensation using potassium t-butoxide in toluene. Cis-(R,S)-3-hydroxyproline is obtained in approximately a 15 % yield. Cyclization attempts using sodium hydride or n-butyl lithium as the base yielded predominately the 4-pyrrolidinone derivatives. This synthesis is also depicted in Figure 4.

Hausler synthesized a variety of prolines which were substituted at the three position.[31 32] Two of these methods were used to synthesize cis-3-hydroxyproline. One of the methods used was allylic bromination of 1,2-dehydroproline methyl ester. The bromine at the three position was then substituted with hydroxide ion, and the imine was reduced with sodium borohydride to give cis-3-hydroxyproline in approximately a 40% yield. The other method of synthesizing 3-hydroxyproline reported by Hausler also used the 1,2-dehydroproline intermediate. The oxygen functionality at the three position was obtained using lead tetraacetate in benzene giving approximately an 80 % crude yield which contains 10-13 mol % methylpyrrole-2-carboxylate. The 3-acetoxyimine was also reduced with $NaBH_4$ to the cis isomer. The overall yield for this synthetic method is 30 %. The methods used by Hausler are shown in Figure 5.

Ramaswamy prepared a ^{14}C labeled hydroxyproline in a 1-2 % yield from proline using peroxidation to give trans-3-hydroxy-proline as outlined in Figure 6.[33]

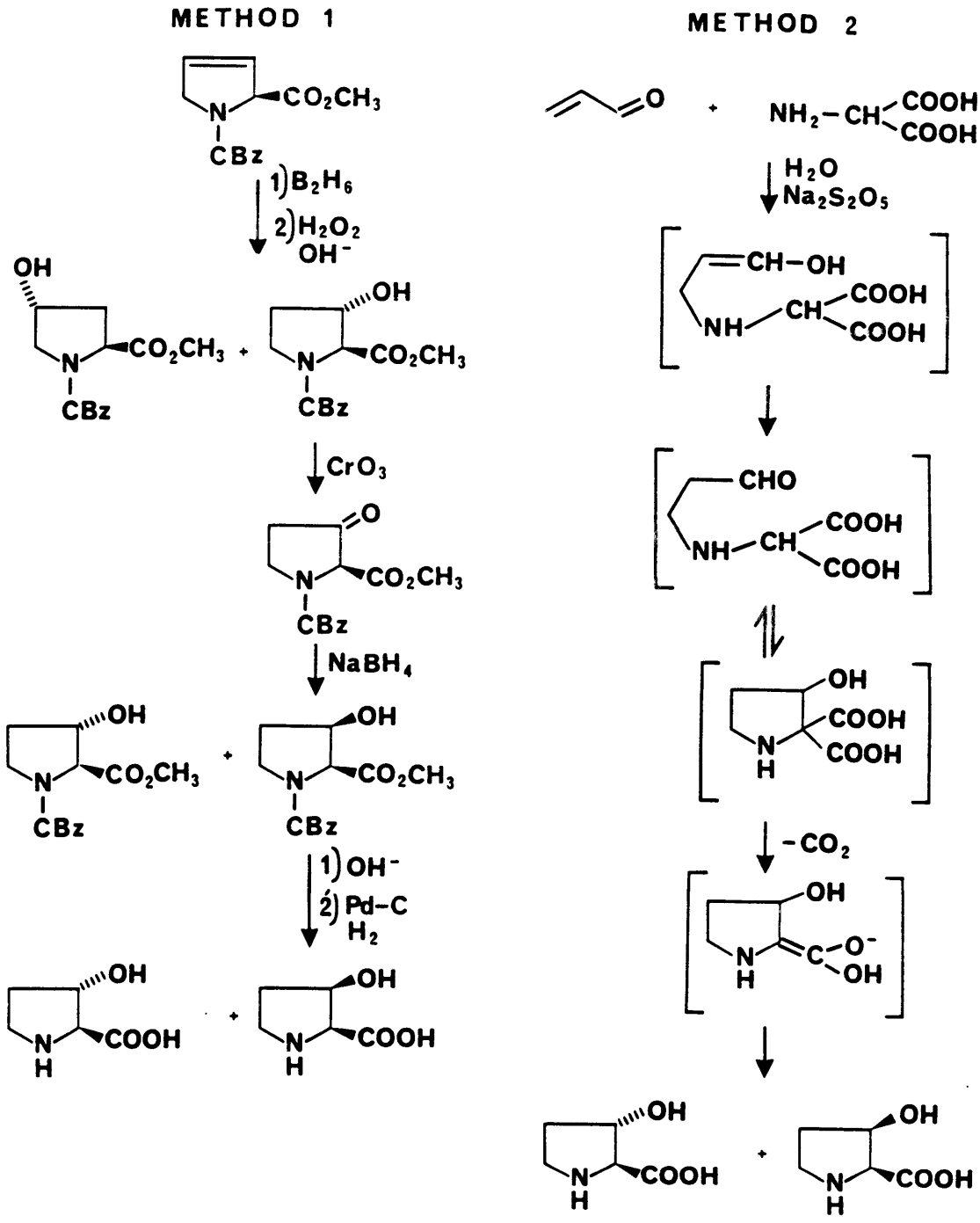


Figure 3. Syntheses by Witkop

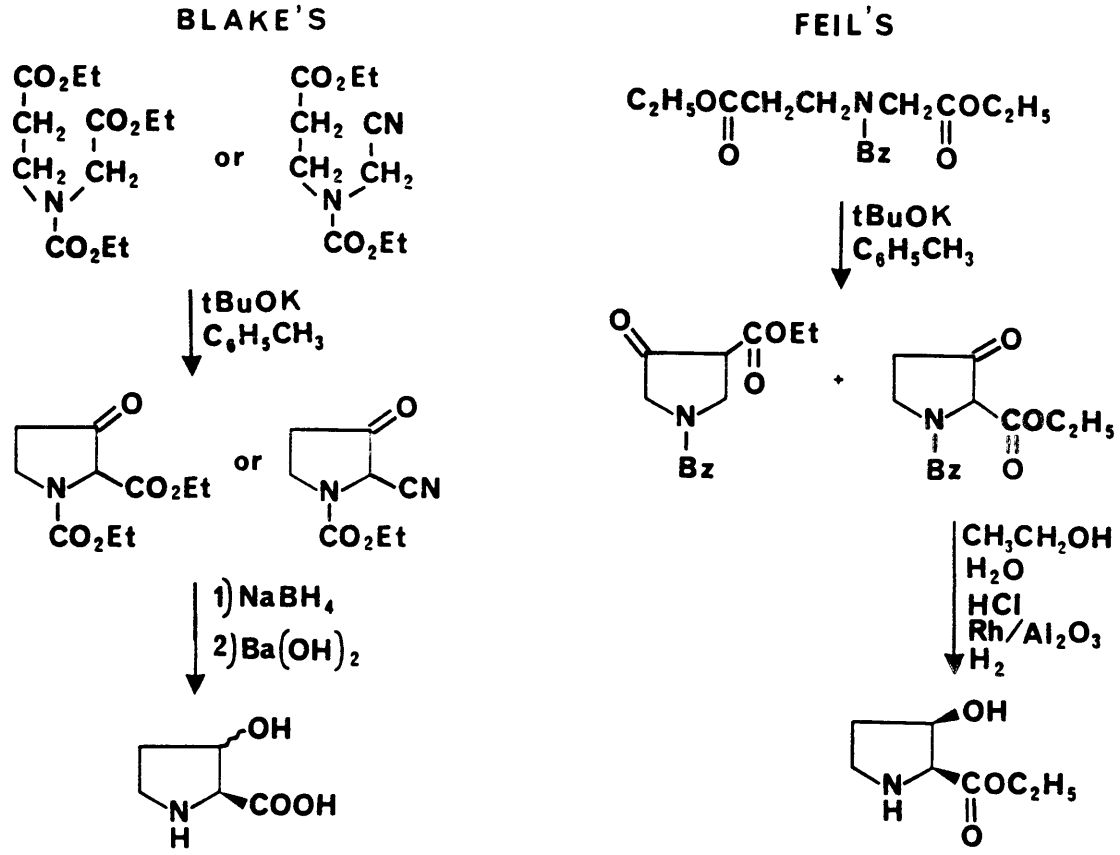


Figure 4. Syntheses with Pyrrolidinone Intermediates

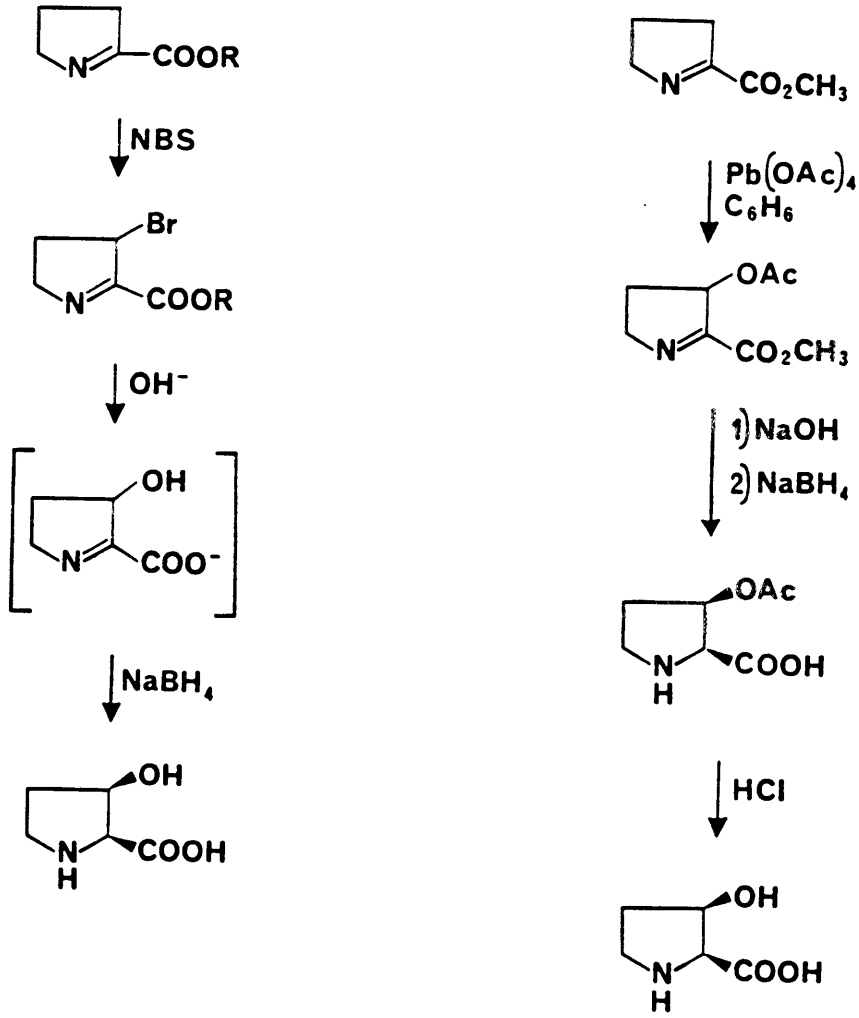


Figure 5. Syntheses by Hausler

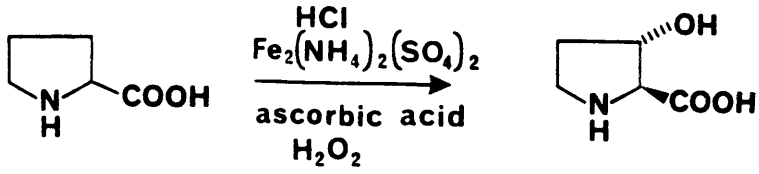


Figure 6. Synthesis by Ramaswamy

LeFevre[2] synthesized cis- and trans-3-hydroxyproline from N-acetyl-2,3-dehydroproline methyl ester which had been synthesized by the method of Hausler.[32] Michael addition of methoxide to the dehydroproline gave approximately a 4:1 ratio of trans to cis N-acetyl-3-methoxyproline methyl ester. Hydrolysis of the protecting groups with 48 % HBr gives a 3-hydroxyproline. However, cleavage of the methyl ether was incomplete for the cis compound. This synthesis is diagrammed in Figure 7.

Ewing et al.[33] used two methods for producing 3-hydroxyproline derivatives for use in the synthesis of (-) detoxinine. One method involved the use of 5-vinyl-2-isoxazoline. The isoxazoline was reduced with lithium aluminum hydride, and the amine group was protected with di-tert-butyl dicarbonate. The alkene was epoxidized with meta-chloroperbenzoic acid, and the epoxide was cyclized with boron trifluoride etherate giving a 40 - 45 % overall yield in a 1:1 ratio of cis to trans pyrrolidines. The other method used by Ewing was very similar to the method of Hausler which used lead tetraacetate to obtain the oxygen functionality at the three position. Ewing obtained better yields by using dichloromethane as the solvent instead of benzene. The resulting 1,2-dehydro-3-acetoxyproline methyl ester was reduced to the amine with dimethyl amine borane complex, and the amine was then protected using di-tert-butyl dicarbonate. The protected 3-hydroxyproline was obtained in a 65 - 74 % yielded from the 1,2-dehydroproline methyl ester in a cis to trans ratio of approximately 2:1. The methods of Ewing's group are delineated in Figure 8.

Two syntheses of 3-hydroxyproline derivatives which possibly could be varied and used to synthesize 3-hydroxyproline should also be included. DeShong et al. synthesized a 3-hydroxyproline derivative using a [3 + 2] thermal cycloaddition between a N-aryl aziridine and ethyl vinyl ether to obtain 3-ethoxy-N-p-methoxyphenyl proline methyl ester in a 42 % yield.[34] Evans and Weber synthesized 4-methyl-3-hydroxyproline by cyclization of methyl (2S,3S)-2-azido-3-hydroxy-4-methyl-4-pentenoate using dicyclohexylborane.[35] Use of acrolein instead of methacrolein in formation of the azido olefin could possibly lead to the formation of trans-3-hydroxyproline. These two synthetic methods are depicted in Figure 9.

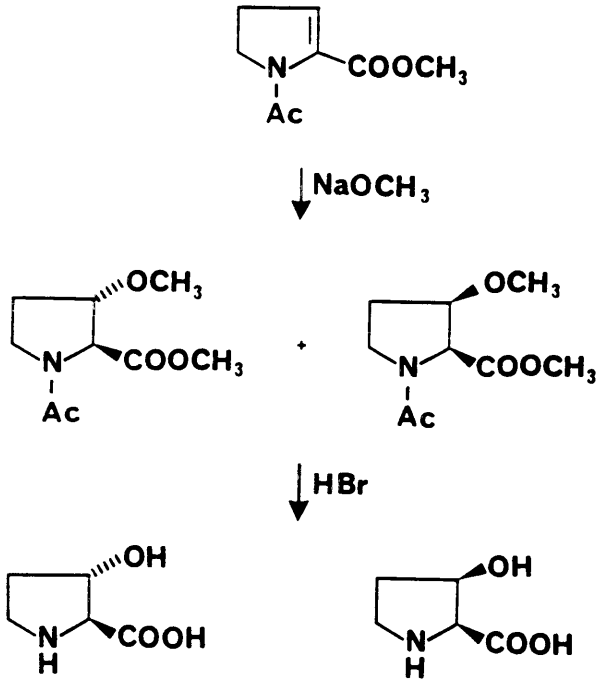


Figure 7. Synthesis by LeFevre

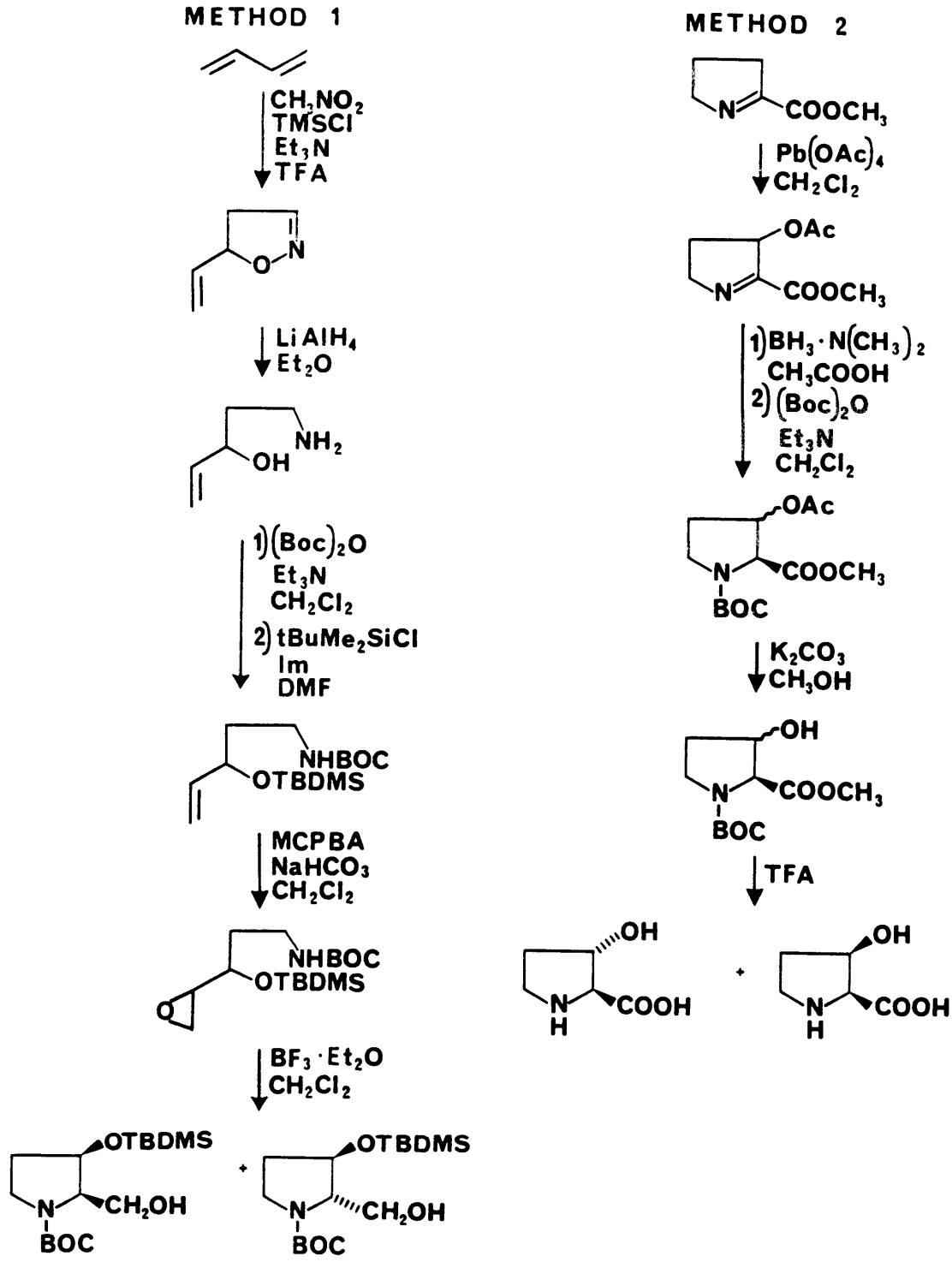


Figure 8. Syntheses by Ewing

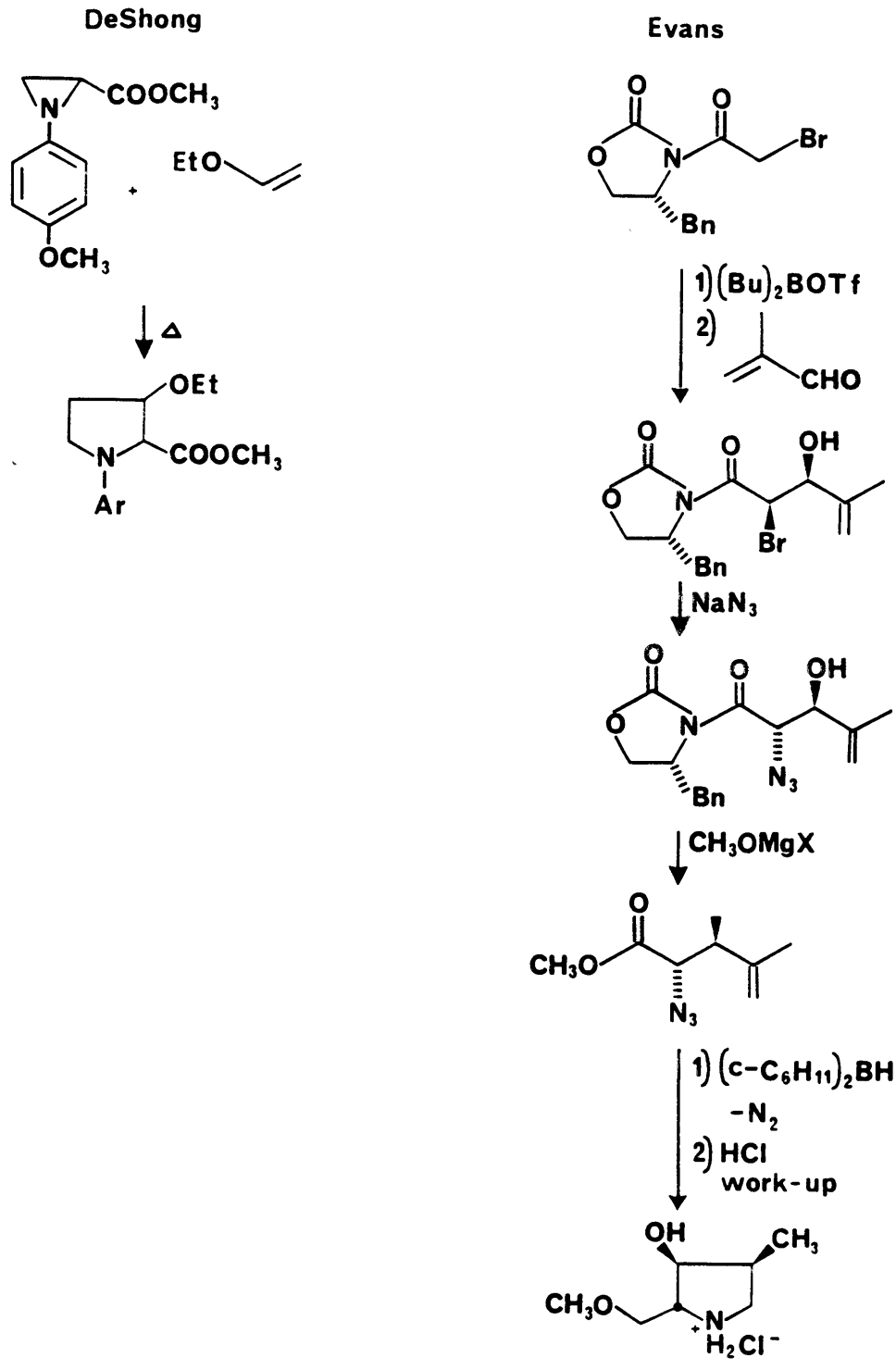


Figure 9. Syntheses of Derivatives

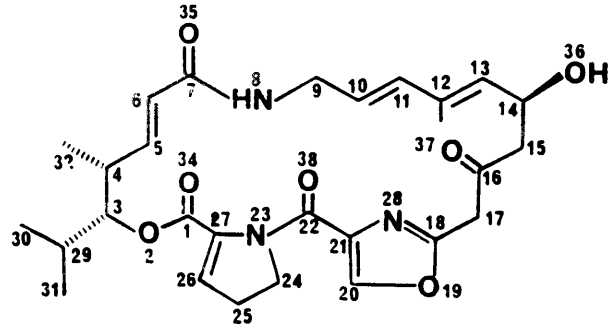
2.2 *VIRGINIAMYCIN M1*

2.2.1 History

Virginiamycin, a complex mixture of antibiotics, was isolated from a variety of bacterial species of the genus *Streptomyces*.[36-40] Because of the number of bacterial species which produce the antibiotic, it was isolated in several different laboratories and has been known under a variety of names such as ostreogrycin, mikamycin, synergistin, and pristinamycin among others. This ambiguity was clarified by Crooy and DeNeys by use of the name virginiamycin.[41] Chemical Abstracts have also adopted the name virginiamycin. Therefore virginiamycin will be the name used in this review. Several reviews of virginiamycin-like antibiotics[1 42-44] have been published.

The complex mixture of virginiamycin-like antibiotics is composed of two major components, designated as A and B. Group A virginiamycin-like antibiotics are characterized as macrocyclic lactones which contain a substituted aminodecanoic acid, a pyrrolidine ring, an unusual oxazole, system and have a molecular weight of approximately 525. The structure of virginiamycin M_1 (1) was determined by classical chemical degradation studies, nuclear magnetic resonance, and mass spectrometry in the mid-1960's.[45-47] Several years later the ^{13}C NMR spectrum for virginiamycin M_1 was assigned using ^{13}C labeled acetate feeding experiments.[48] Virginiamycin M_1 ($C_{28}H_{35}NO_3$), ostreogrycin A, mikamycin A, vernamycin A, synergistin A-1 (PA114), pristinamycin IIA, streptogramin A, and madumycin II have identical structures. Virginiamycin M_2 (2) has a structure almost identical to virginiamycin M_1, the difference being the pyrroline ring is saturated in the latter antibiotic. Virginiamycin M_2 has also been known as ostreogrycin G and pristinamycin IIB.[1 41-44] The structures of Virginiamycin M_1 and Virginiamycin M_2 and their respective numbering schemes are shown in Figure 10.

The B component of virginiamycin-like antibiotics is composed of several similar structures. Virginiamycin S, $C_{43}H_{49}N_7O_{10}$ (MW = 824), has been shown to be a depsipeptide and



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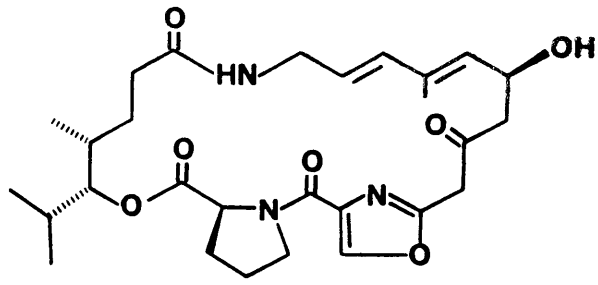

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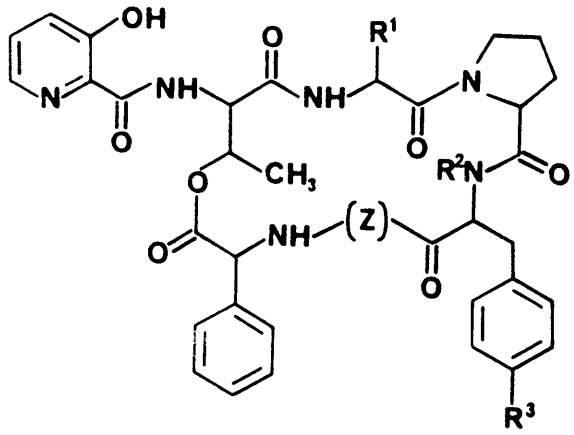
Figure 10. Virginiamycin M_1 and Virginiamycin M_2 Structures

similar in structure to mikamycin B, streptogramin B, PA113B1, vernamycins Bα, Bβ, Bγ, Bδ, and C, ostreogrycins B, B1, B2, and B3, pristinamycins IA,IB, and IC, virginiamycin S1, S2, S3, and S4, patricins A and B, and doricin.[1] [41-44] The basic structure of the B components of virginiamycin is shown in Figure 11.

Although virginiamycin has been shown to have some therapeutic uses for humans, it has been used predominantly as an performance promoter in animal husbandry.[44] The effectiveness of the antibiotic in animal husbandry is due to growth inhibition of gram-positive bacteria of the intestinal flora. Virginiamycin has been shown to promote growth and increase feed efficiency in poultry, swine, and cattle. The successful use of virginiamycin as a feed additive has been due to their auspicious biological properties which include : (i) extremely low toxicity; (ii) lack of accumulation in animal tissues; (iii) practically undetectable production of resistant mutants in intestinal flora; (iv) narrow spectrum, i.e. restricted inhibition of gram-positive microorganisms; (v) rare induction, if any, of episomal resistance carried by gram-negative plasmids; and (vi) biodegradability in cattle feces. Virginiamycin is also very successful for treating chronic and acute forms of swine dysentry, and its use as a therapeutic agent in veterinary medicine may increase in the future.[1] Tissue studies from the livers of turkeys, cattle, and rats fed ^{14}C-virginiamycin indicated virginiamycin is extensively metabolized and no single metabolite is a substantial portion of the total residue.[48]

Virginiamycin is an effective antibiotic due to its ability to inhibit protein synthesis. Two reviews, one by Cocito and the other by Cocito and Chinali, have thoroughly discussed the molecular mechanism of action of virginiamycin-like antibiotics.[1] [50] The antibiotics were found to be active against gram-positive bacteria and exhibit a synergistic effect. The individual components are bacteriastatic while mixtures of the two components are bacteriacidal.[1] Individually, virginiamycin M and S components inhibit protein synthesis, but together they show a 100-fold increase in their ability to inhibit protein synthesis.[51] A stoichiometric amount of type B virginiamycin-like components combined with a catalytic amount of type A components will interact with the ribosomes causing permanent inactivation, and thus protein inhibition.[52] The antibiotic binds to the ribosomes causing protein inhibition. Virginiamycin inactivates the ribosome by producing a stable conformational change which alters both the acceptor and donor



Antibiotic	R^1	R^2	R^3	(-Z-)
Virginiamycin S_1	C_2H_5	CH_3	H	4-oxopipecolic acid
Virginiamycin S_2	C_2H_5	H	H	4-hydroxypipecolic acid
Virginiamycin S_3	C_2H_5	CH_3	H	3-hydroxy-4-oxopipecolic acid
Virginiamycin S_4	CH_3	CH_3	H	4-oxopipecolic acid
Streptogramin B Mikamycin IA PA 114 B1 Pristinamycin IA Vernamycin Bα Ostreogrycin B	C_2H_5	CH_3	$N(CH_3)_2$	4-oxopipecolic acid
Pristinamycin IC Vernamycin Bγ Ostreogrycin B1	CH_3	CH_3	$N(CH_3)_2$	4-oxopipecolic acid
Pristinamycin IB Vernamycin Bβ Ostreogrycin B2	C_2H_5	CH_3	$NHCH_3$	4-oxopipecolic acid
Vernamycin Bδ	CH_3	CH_3	$NHCH_3$	4-oxopipecolic acid
Ostreogrycin B3	C_2H_5	CH_3	$N(CH_3)_2$	3-hydroxy-4-oxopipecolic acid
Vernamycin C Doricin	C_2H_5	CH_3	$N(CH_3)_2$	aspartic acid
Patricin A	C_2H_5	CH_3	H	proline
Patricin B	C_2H_5	CH_3	H	pipecolic acid

Figure 11. Group B Virginiamycin-like Structures

sites of peptidyl transferase on the ribosome.[53-55] Peptidyl transferase is the enzyme contained in the ribosome which is responsible for forming the peptide linkage.

2.2.2 Biosynthesis

The biosynthesis of virginiamycin M_1 has been of particular interest due to the presence of the unusual dehydroamino acids, a 2,3-dehydroproline and an oxazole ring. Feeding experiments using radioisotopes were used to deduce the major precursors of virginiamycin M_1. The results of Kingston et al.[56 57] and those of Roberfroid and Dumont[58] are shown in Table 1. These results indicate the antibiotic is biosynthesized from acetate and the amino acid precursors proline, glycine, serine, valine, and methionine. One would hypothesize the macrocyclic portion of the antibiotic is derived from acetate while proline gives rise to the dehydroproline residue, and glycine is the precursor for N-8, C-9, and C-10. Presumably S-adenosylmethionine, a well-known carbon and oxygen methylating agent from methionine, donates the carbons at positions 32 and 33. One expects valine to give rise to carbons 3, 29, 30, and 31, and literature precendence exists for serine as a possible precursor for the oxazole unit.[59] Incorporation of the ^{14}C labeled precursors at the expected places was verified using ^{13}C labeled precursors by Kingston's group.[56 60]

The formation of the macrocyclic portion of virginiamycin M_1 from acetate was confirmed using [1-^{13}C]- and [2-^{13}C] acetate. [1-^{13}C] acetate was found to be incorporated at carbons 5, 7, 12, 14, 16, and 18, therefore suggesting carbons 4, 6, 11, 13, 15, and 17 are from the methyl carbon of acetate. As anticipated, when [2-^{13}C] acetate was fed to virginiamycin, carbons 4, 6, 11, 13, 15, and 17 were observed by ^{13}C NMR to be derived from the methyl carbon of acetate. Unexpectedly, the methyl group of acetate was also observed to be the precursor for carbon 33 possibly suggesting a new pathway for methylation of carbon.

Three pathways which appear feasible for formation of the methyl group from acetate are represented in Figure 12. Two pathways involve an aldol-type condensation of two polyketide chains forming a β-hydroxy acid as exemplified in pathways B and C. Decarboxylation, elimination

Table 1. Radioisotope Precursors for Virginiamycin M_1

Precursor	% Incorporation
sodium [2-^{14}C]acetate	5.0,[56] 5.5, 3.4[58]
sodium [2-^{14}C]malonate	0.4[58]
(R,S)-[2-^{14}C]mevalonolactone	0.04[58]
(S)-[$^{14}CH_3$]methionine	4.0,[56] 4.5, 5.7[58]
(R,S)-[3-^{14}C]serine	1.4[56]
(S)-[3,4-$^{3}H_2$]proline	5.0[56]
[2-^{14}C]glycine	0.4, 0.8[58]
(S)-[U-^{14}C]alanine	★[58]
(R)-[U-^{14}C]glucose	0.6[56]
(R,S)-[3,4-$^{14}C_2$]glutamic acid	★[58]
(S)-[1-^{14}C]leucine	★[58]
(S)-[U-^{14}C]valine	15.2[56]

★ = no detectable incorporation

of water, and reduction of the carbonyl groups would then yield the allylic alcohol of virginiamycin M_1. In pathway A aldol condensation of a single acetate unit, presumably activated as malonyl conezyme A, with a polyketide chain would give the same result.

Paths A and B were distinguished from path C by two methods. The first method was the incorporation of [2-^{13}C,$^{2}H_3$] acetate. In pathway C the methyl group at carbon 33 was a chain starter unit which typically experiences no hydrogen exchange while in either pathway A or B substantial or complete loss of deuterium would be expected due to the malonyl coenzyme A intermediate. Carbons 4, 6, 11, 13, 15, and 17 were again found to be enriched, but no deuterium incorportion of was detected by ^{2}H NMR spectroscopy.

The second method used to eliminate pathway C as a possibility was incorporation of [1,2-^{13}C] acetate. The resulting virginiamycin M_1 exhibited coupling between the following carbon pairs: 4 and 5, 6 and 7, 11 and 12, 13 and 14, 15 and 16, and 17 and 18. However, no coupling to carbon 33 was found; therefore, the methyl at carbon 33 arose by pathway A or B.

Paths A and B were differentiated using (R,S)-[3-^{13}C] serine as a delayed source of [2-^{13}C] acetate since serine is converted to acetate via pyruvate. In contrast to the [2-^{13}C] acetate experiment, in which all carbons originating from the precursor were enriched to approximately the same extent, carbon 33 was enriched to a greater extent than the other carbons labeled by the [3-^{13}C] serine precursor. Since carbons 11 and 33 would be part of the same initial polyketide chain in pathway B, the two carbons should be enriched in similar proportions. Thus, pathway A with addition of the methyl group to a preformed polyketide chain accounted for the above results.

Feeding of [methyl-^{13}C] methionine resulted in virginiamycin M_1 with ^{13}C enrichment at carbon 32. The result, as expected, confirmed the alkylation at carbon 4 by S-adenosyl methionine.

(R,S)-[2-^{13}C] valine was synthesized and incorporated into the antibiotic. Carbon 3 was enriched to a large extent thereby showing valine is the precursor for carbons 3, 29, 30, and 31. Presumably valine served as the chain starter unit in the formation of isobutyryl-CoA. The conversion probably occurred via transamination to beta-oxoisovaleric acid and subsequent conversion to isobutyryl-CoA.

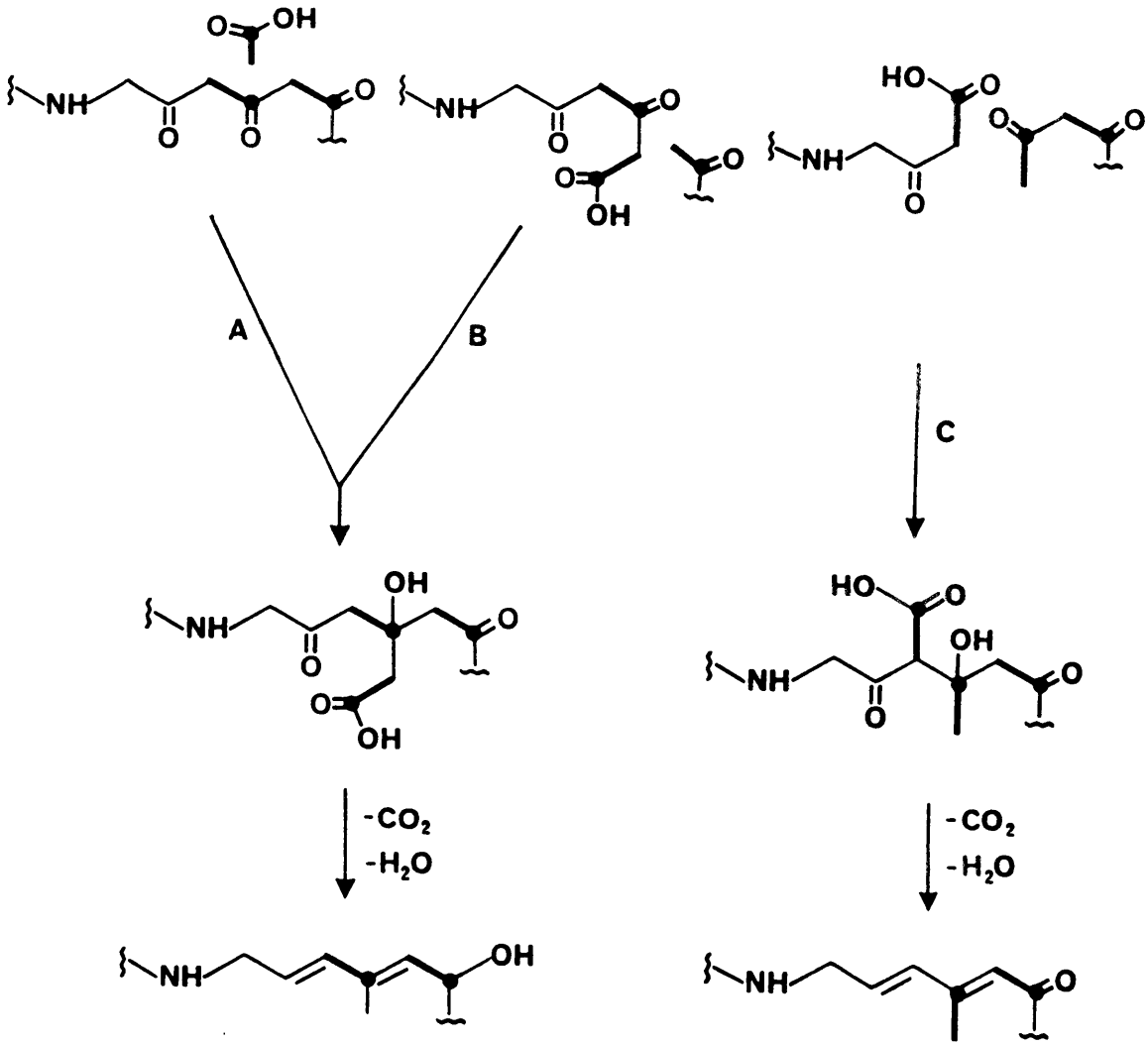


Figure 12. Pathways for Methylation from Acetate

Confirmation for glycine contributing N-8, C-9, and C-10 was obtained by a feeding experiment with [1-^{13}C] glycine. The resulting antibiotic showed enrichment of carbon 22 and carbon 10. This enrichment confirms N-8, C-9, and C-10 were derived from glycine. The enrichment at C-22 is easily explained by the known formation of serine from glycine.

Feeding of (R,S)-[3-^{13}C] serine, in addition to clarifying the methylation pathway for carbon 33, showed a 7.9 enrichment at carbon 20. This substantial enrichment confirmed serine was the precursor for the oxazole ring, presumably as an acylserine. The formation of the oxazole ring from serine was further explored using (2S,3R)-[3-^{3}H] serine and (2S,3S)-[3-^{3}H] serine.[60] Assuming the oxygen atom is derived from serine using the analogy of the thiazole ring from cysteine, the four major pathways which appear probable are depicted in Figure 13. Four incorpation experiments were performed and the results are given in Table 2. The first experiment proved tritium was not washed out using this method, thereby acting as a control. Preference for the (S)-serine isomer over racemic serine was demonstrated by the second experiment. By comparison of the (3R) and (3S) labeled ^{3}H serines in the third and fourth experiments, the 3-(pro-R) hydrogen was obviously incorporated. Of the four pathways, C and D allow for retention of the pro-R hydrogen. Since biosynthetic dehydrogenations have been shown to typically occur with overall syn stereochemistry,[61 62] formation of the oxazole ring through anti-dehydrogenation of the ring as in pathway D was improbable. Thus, the oxazole ring formation probably occurred by pathway C. Oxidation of serine to the aldehyde followed by enolization and cyclization of the aldehyde would give a hydroxy adduct which could easily dehydrate to the oxazole. In pathway C the stereochemistry of the hydrogen at C-3 is determined in the oxidation step.

Further work on the biosynthetic origin of the dehydroproline moiety of virginiamycin M_1 was performed by LeFevre[2] and also by Purvis.[63] Confirmation of (S)-proline as the precursor for the dehydroproline portion was obtained by feeding (S)-[U-^{14}C] proline to the antibiotic producing bacteria. Hydrogenation and hydrolysis of the resulting antibiotic yielded (R,S)-[U-^{14}C] proline which was derivatized with benzoyl chloride. The N-benzoyl proline contained 42.3% of the activity of the antibiotic. Therefore, one concluded (S)-proline was an effective precursor for the dehydroproline portion of the antibiotic.

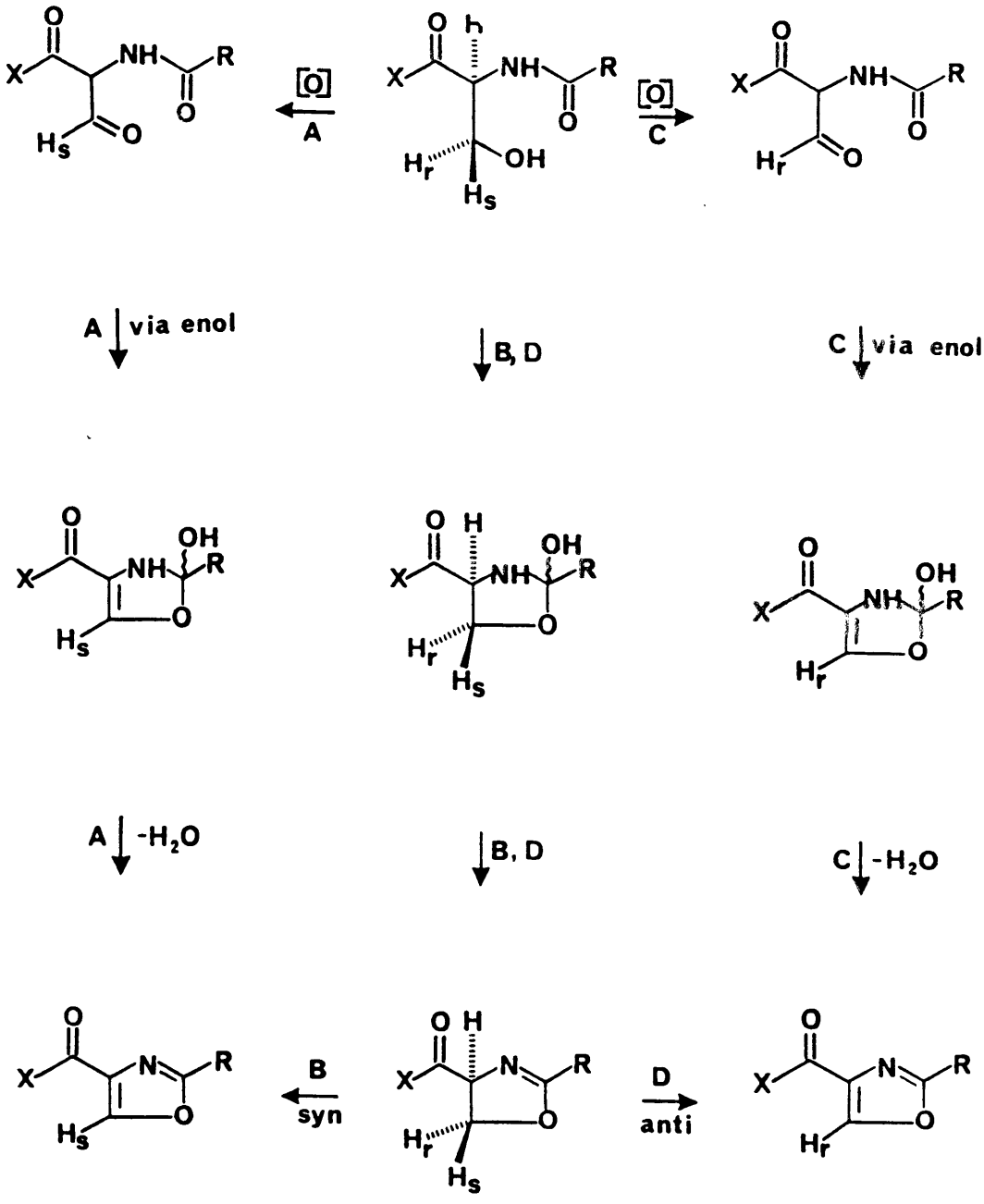


Figure 13. Pathways for Oxazole Formation

Table 2. Labeled Serine Precursors for the Oxazole Ring

Experiment	Precursor	$^{3}H/^{14}C$ Ratio of Precursor	$^{3}H/^{14}C$ Ratio of Precursor	% ^{3}H Retention
1	(2S)-[G-^{3}H]serine (2S)-[U-^{14}C]serine	6.02	2.95	49
2	(2S)-[G-^{3}H]serine (2R,S)-[carboxy-^{14}C]serine	4.81	3.46	72
3	(2S,3R)-[3-^{3}H]serine (2S)-[U-^{14}C]serine	2.42	1.60	66
4	(2S,3S)-[3-^{3}H]serine (2S)-[U-^{14}C]serine	3.32	0.38	11

To study the stereochemistry involved in the α,β-desaturation of the dehydroproline, (S)-[3,4-$^3H_2$]- and (S)-[1-^{14}C] proline were fed simultaneously to the antibiotic producing strain. The precursor ratio of $^3H/^{14}C$ was 7.2. The $^3H/^{14}C$ ratio of the N-benzoyl proline obtained from the virginiamycin M_1 was determined to be 7.5. From these results one may conclude the elimiantion is trans, since there is no loss of tritium. Confirmation of the trans-α,β-desaturation was aquired from a feeding experiment utilizing (2S)-cis-3-[^3H] proline and (S)-[U-^{14}C] proline with a precursor ratio of 2.51 $^3H/^{14}C$. The $^3H/^{14}C$ ratio of the derivatized proline from the virginiamycin M_1 was O.1, 4 % retention of the tritium.

In summary acetate was shown to be the precursor for the macrocyclic portion of the ring, carbons 4-7 and 11-18. Glycine was shown to serve as the precursor for N-8, C-9, and C-10 as well as the oxazole ring. Proline served as a precursor to the dehydroproline moiety while valine was shown to give rise to carbons 3, 29, 30, and 31. Methionine served as the precursor for the methyl group at carbon 32 while the carbon 33 methyl group arose from acetate by a novel pathway. These results are summarized in Figure 14.

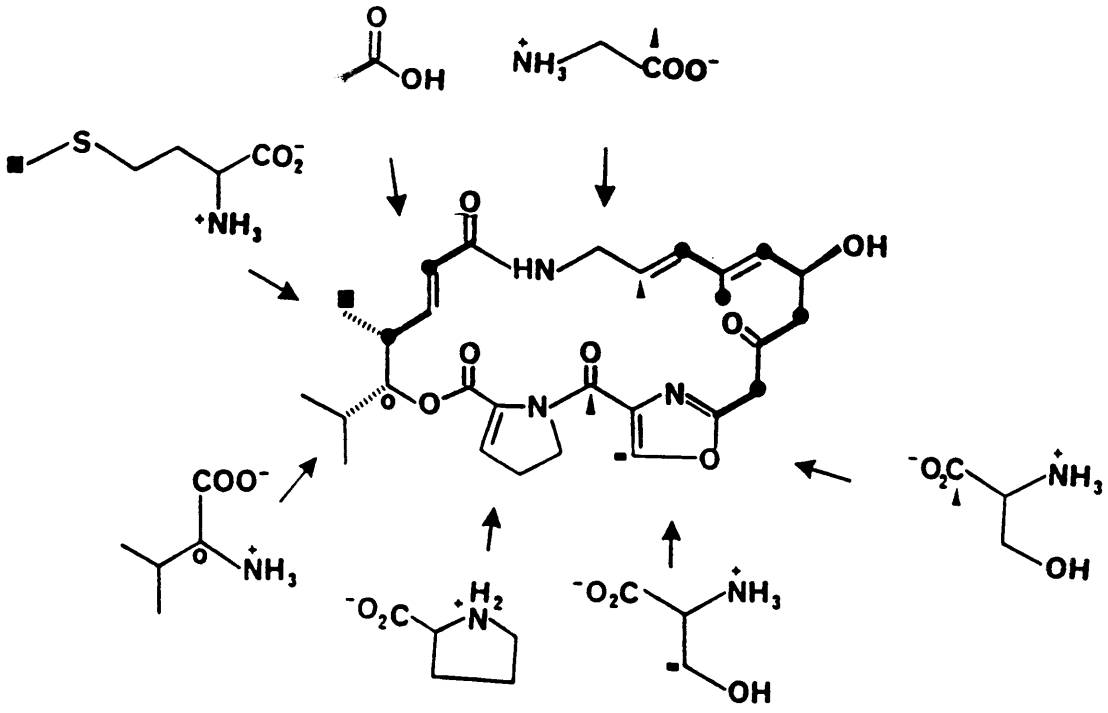


Figure 14. Biosynthetic Precursors for virginiamycin M_1

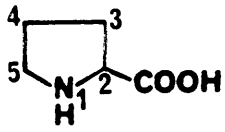
3.0 EXPERIMENTAL

3.1 GENERAL

^{14}C and ^{3}H labeled compounds were obtained from ICN Chemical and Radioisotope Division. Internal standards for counting were obtained from Amersham Corporation. Counting was performed in a Beckman LS 3800 liquid scintillation counter using either Beckman's Ready-Solv MP or National Diagnostic's Ecoscint cocktail.

Proton and carbon spectra were taken at ambient temperature using a Bruker WP-270SY, 270.13 MHz, instrument. For solutions dissolved in $CDCl_3$, tetramethylsilane (TMS) was used as the internal standard. For deuterium oxide solutions, dioxane was used as the internal standard. Infrared (IR) spectra were taken on a Perkin-Elmer 710B infrared spectrometer.

For flash chromatography silica gel 60 (230-400 mesh) was used. The TLC plates used were aluminum backed silica gel 60 F_{254} with a layer thickness of 0.2 mm unless otherwise specified. The preparative TLC plates used were silica gel GF, 1000 microns, from Analtech. For NMR assignments the numbering scheme for proline is shown below.



^{13}C NMR assignments were made by comparison with published spectra for proline and proline derivatives.[64 65 66]

3.2 3-HYDROXYPROLINE SYNTHESIS

3.2.1 Proline Methyl Ester

HPLC grade methanol, 150 ml, was cooled between 0 and 5°C and 25 ml (3.5 eq.) of acetyl chloride was slowly syringed into the solution. The solution was stirred in the ice bath for 30 minutes and then stirred 15 minutes at room temperature. 11.51 g of proline (0.1000 mol) was added, and the solution was reflux for 2¼ hours. The solution was evaporated on the rotary evaporator leaving a slightly yellow oil. The proline methyl ester hydrochloride salt was suspended in 200 ml of dichloromethane and cooled in an ice bath. Anhydrous ammonia was bubbled through the mixture until the white NH_4Cl precipitate no longer formed. The salt was removed by vacuum filtration, and the solvent was evaporated leaving a 8.016 g (0.0621 mol, 62 % yield) of light yellow oil.

IR (neat) 1770, 1470, 1380, 1240, 1150 cm^{-1}

^1H NMR ($CDCl_3$) δ 3.77 (m, 1 H, C-2), 3.73 (s, 3 H, CH_3), 3.08 (m, 1 H), 2.91 (m, 1 H), 2.42 (s, 1 H), 2.13 (m, 1 H), 1.81 (m, 3 H)

^{13}C NMR ($CDCl_3$) δ 25.5 (C-4), 30.0 (C-3), 46.5 (C-5), 51.5 (OCH_3), 59.7 (C-2), 175.8 (CO)

3.2.2 1,2-Dehydroproline Methyl Ester

Proline methyl ester (8.016 g, 0.0621 mol) was dissolved in 30 ml of anhydrous diethyl ether. The ester solution and 7.1 ml of tert-butyl hypochlorite[67] were simultaneously added dropwise in the dark over a 25 minute period to 130 ml of anhydrous diethyl ether cooled in a dry ice / carbon tetrachloride ice bath. The solution was stirred in the ice bath for 30 minutes in the dark. Triethylamine (8.7 ml, 1 eq.) was added, at which point the lights were turned on, and the solution was allowed to react for 1½-2 days. The white $Et_3NH^+Cl^-$ precipitate was removed by vacuum filtration, and the solution was evaporated to 50 ml. The solution was then eluted through 25 g of Alumina-N-Super I of activity III using 200 ml of diethyl ether. The solution was evaporated and vacuum distilled from a 25 ml flask at 66°C and 1.2 mm Hg giving 5.443 g (0.0429 mol, 69 %) of 1,2-dehydroproline methyl ester.

IR (neat) 1740, 1660, 1460, 1380, 1340, 1280, 1110 cm^{-1}

^1H NMR ($CDCl_3$) δ 4.12 (m, 2 H, C-3), 3.89 (s, 3 H, CH_3), 2.01 (m, 2 H,C-4), 2.84 (m, 2 H,C-5)

^{13}C NMR ($CDCl_3$) δ 22.3 (C-4), 35.5 (C-3), 52.4 (OCH_3), 62.6 (C-5), 163, 168

3.2.3 1,2-Dehydro-3-Acetoxyproline Methyl Ester

1,2-dehydroproline methyl ester (1.947 g, 0.01533 mol) was dissolved in 30 ml of HPLC grade dichloromethane and transferred into a two-necked flask which had been fitted with a septum and a reflux condenser and had been flame-dried under nitrogen. The solution was cooled to 0°C using an ice bath with calcium chloride. Freshly recrystallized lead tetraacetate (7.499 g, 0.0169

mol, 1.1 eq.) was added in portions where the reflux condenser joined the flask by removing the apparatus from the water bath, drying the apparatus, adding the lead tetraacetate, and returning the reassembled flask to the bath. After completing the addition of the lead tetraacetate, the light brown-yellow solution was stirred in the ice bath for 15 minutes and then refluxed for 4 hours. The solution was vacuum filtered to remove the white lead oxide precipitate. The solution was evaporated to give 2.684 g of a reddish-brown oil.

IR (neat) 1760, 1640, 1460, 1380, 1240 cm^{-1}

^1H NMR ($CDCl_3$) δ 6.03 (m, 1 H, CO), 4.21 (m, 2 H, C- 5), 3.89 (s, 3 H, OCH_3), 2.46 (m, 1 H,C-4), 2.07 (s, 3 H, CH_3), 1.91 (m, 1 H, C-4)

^{13}C NMR ($CDCl_3$) δ 20.7 (C-4), 30.7 (CH_3), 52.6 (OCH_3), 60.6 (C-5), 77.2 (C- 3) 161, 165, 169

3.2.4 3-Acetoxy-N-Carbobenzyloxy Proline Methyl Ester

1,2-dehydro-3-acetoxyproline methyl ester (1.757 g, 9.50 mmol) was dissolved in 20 ml of acetic acid and cooled until the solution was partially frozen; borane-dimethylamine complex (0.8611 g, 1.54 eq.) was then added. The solution was stirred for 2 hours at room temperature. After the addition of 125 ml of water, and the acetic acid was azeotroped off using the rotary evaporator. The residue was dissolved in 50 ml of water, and 60 ml of ethyl acetate were added. The mixture was stirred vigorously at 0°C using a mechanical stirrer in a three-necked 250 ml flask. Potassium bicarbonate (5.7 g, 6 eq.) was added, and 1.7 ml (1.25 eq.) of benzyl chloroformate was added over a 15-20 minute period.[68] The solution was stirred vigorously in the ice bath for 3 hours. The reaction mixture was poured into a separatory funnel and acidified with 20 ml of 1 N HCl. The organic layer was kept and washed with 50 ml of water. The organic layer was dried with Na_2SO_4 and evaporated leaving 2.792 g (92 % crude yield). A column containing 84 g of Si gel was packed by the slurry method using hexane as the solvent and air pressure to settle the column. The crude mixture was dissolved in ethyl acetate and allowed to soak onto the column. Using flash

chromatography fractions 1-10 were collected using hexane:ethyl acetate (90:10) as the mobile phase, fractions 11-36 were collected using hexane:ethyl acetate (80:20) as the mobile phase, and fractions 37-44 were collected using hexane:ethyl acetate (70:30). By TLC fractions 28-39 were determined to contain the product. Evaporation of the fractions left 0.5514 g (0.00172 mol, 32 % yield) of a pale green oil. The mixture was further purified using normal phase HPLC with hexane:ethyl acetate (80:20) as the mobile phase. The flow rate was 8 ml/minute, the chart speed was 1 cm/minute, and absorbance was 0.2. The trans compound (0.1001 g) was obtained in a 3.4 % yield, and 0.349 g (11.5 %) of the cis compound was obtained. The presence of only two carbonyl carbons for the trans compound may be explained by two of the carbonyl carbons occurring at the same position.

Cis-3-acetoxy-N-carbobenzyloxy proline methyl ester

^{1}H NMR ($CDCl_3$) δ 7.31 (m, 5 H, C_6H_5), 5.48 and 5.43 (t, 1 H, J= 6.5 Hz, C-3), 5.15 (m, 2 H, CH_2), 4.64 and 4.61 (d, 1 H, J= 6.5 Hz, C-2), 3.73 and 3.56 (m, 2 H, C-5), 3.75 and 3.60 (s, 3 H, OCH_3), 2.17 (m, 2 H, C-4), 2.05 (s, 3 H, CH_3)

^{13}C NMR ($CDCl_3$) δ 20.46 (C-4), 28.89, 29.84 (CH_3), 44.00 (C-5), 51.87 (OCH_3), 60.99 (C- 2), 67.19 (CH_2) 72.71, 72.01(C-3), 127.77, 127.94, 128.36, 136.42 (C_6H_5), 154.55, 154.07, 169.21, 169.51

Trans-3-acetoxy-N-carbobenzyloxy proline methyl ester

^{1}H NMR ($CDCl_3$) δ 7.34 (m, 5 H, C_6H_5), 5.31 (broad s, 1 H, C-3), 5.15 (m, 2 H, CH_2), 4.56 and 4.38 (s, 1 H, C-2), 3.75 and 3.60 (m, 2 H, C-5), 3.77 and 3.63 (s, 3 H, OCH_3), 2.20 (m, 2 H, C-4), 2.07 (s, 3 H, CH_3) The two resonances for some of the carbons are probably due to the limited rotation of the carbonyl around the nitrogen bond as shown in the proton spectrum.

3.2.5 Cis- and Trans-3-Hydroxyproline (Hydrogenation Method)

1,2-dehydro-3-acetoxyproline methyl ester (185 mg, 1 mmol) was dissolved in 9.6 ml of 1 N HCl, and 30 mg (0.13 mmol) of platinum oxide was added. The mixture was hydrogenated for 24 hours, and an additional 11 mg (0.05 mmol) of platinum oxide was added in a small amount of hydrochloric acid. The reaction was ended, neutralized with sodium hydroxide and evaporated to dryness. The residue was dissolved in 5 ml 1 N NaOH and stirred in an ice bath between 0 and 10°C for 1 hour. The solution was rotary-evaporated to 1 ml and placed on a Dowex 2 strongly basic ion-exchange column (1.6 cm X 3 cm). The column was washed with 40 ml water and then eluted with 20 ml of 1 N acetic acid. A fraction which contained 2-3 ml of water just before the acetic acid solvent front and 7-8 ml of acetic acid after the solvent front was collected and evaporated to a light brown oil. The oil was dissolved in 3-4 ml of absolute ethanol and left to crystallize overnight. Off-white crystals (9.6 mg) were obtained which contained either trans-3-hydroxyproline or some other impurity by ^{1}H NMR. The NMR sample and mother liquor were added together and recrystallized from methanol/water giving 6 mg of cis-3-hydroxyproline with a small amount of trans contaminant by ^{1}H NMR. By NMR the mother liquor appeared to contain both the cis and trans compounds.

Cis-3-hydroxyproline

^{1}H NMR (D_2O) δ 4.51 (t, 1 H, J = 4 Hz, C-3), 3.94 (d, 1 H, J = 4 Hz, C-2), 3.33 (m, 2 H, C-5), 1.99 (m, 2 H,C-4)

Trans-3-hydroxyproline

^{1}H NMR (D_2O) δ 4.48 (broad s, 1 H, C-3), 3.87 (broad s, 1 H, C-2), 3.35 (m, 2 H, C-5), 1.99 (m, 2 H,C-4)

3.2.6 Cis- and Trans-3-Hydroxyproline (Sodium Borohydride Reduction)

1,2-dehydro-3-acetoxy proline methyl ester, 0.2988 g, was dissolved in 1 ml of water and cooled in an ice bath to 0°C. Aqueous sodium hydroxide (2.7 ml, 0.525 N, 1 eq.) was added, and the solution was stirred for a few minutes until the pH changed to approximately 8. Sodium borohydride (27 mg, 2 eq.) was added, and the solution was stirred in the ice bath for an additional 10 minutes. The precipitate which was insoluble in the aqueous solution was removed by filtration. The solution was evaporated to dryness. The residue was dissolved in 15 ml of 2 N HCl and refluxed for 2 hours. The brown solution was decolorized with Norite and evaporated almost to dryness leaving a brownish-yellow residue. The residue was dissolved in a few milliliters of water and allowed to soak onto a Dowex-50W column (1.6 X 8.5 cm) in the H^+ form. The column was rinsed with 20 ml of water and then eluted with 1 N NH_4OH. The ammonium hydroxide fraction was evaporated to dryness, the residue was dissolved in 10 ml of water, and the solution was decolorized with Norite. The decolorized solution was evaporated to dryness leaving 43.3 mg (20 %). ^1H NMR indicated 15-20 mole % of trans-3-hydroxyproline was present. The sample was recrystallized three times until no trans-3-hydroxyproline appeared to be present by ^1H NMR when compared to the published spectrum.[25]

3.2.7 Synthesis of ^{14}C-Labeled 3-Hydroxyproline

3.2.7.1 [Carboxyl-^{14}C] Proline Methyl Ester

Proline (0.230 g, 2 mmol) was weighed into a 10 ml round bottom flask, and 300 μCi of (R,S)-[carbonyl-^{14}C] proline (Specific Activity = 50 mCi/mmol) dissolved in 0.01 N hydrochloric acidwas added into the flask. A few milliliters of freshly opened HPLC grade methanol was added to the flask, and the solution was evaporated to dryness on the rotary evaporator. This procedure

was repeated 2 times in order to remove the hydrochloric acid solution in which the labeled proline had been dissolved. 10 ml of HPLC grade methanol was added into a two-necked, 50 ml, flame-dried flask and cooled in an ice bath. Acetyl chloride (1 ml, 7 eq.) was added to the flask and stirred in the ice bath for 30 minutes and at room temperature for 15 minutes. The proline was dissolved in HPLC grade methanol and added to the solution. The solution was then refluxed for 2 hours and transferred to a 50 ml Erlenmeyer flask. The pale yellow solution was evaporated to dryness. The oil was suspended in 20 ml dichloromethane and cooled in an ice bath. Anhydrous ammonia gas was bubbled through the solution, and the solution turned a brownish color. The brown NH_4Cl precipitate which formed was removed using vacuum filtration. The mother liquor in the 50 ml filter flask was colorless. The dichloromethane solution was evaporated in portions to an oil in a two-necked flask which had been weighed and fitted with a rubber septum on one neck. 0.248 g (1.923 mmol, 96 %) of the brown oil was obtained.

3.2.7.2 [Carboxyl-^{14}C] 1,2-Dehydro-Proline Methyl Ester

The proline methyl ester was dissolved in 10 ml of anhydrous diethyl ether under nitrogen. The solution was cooled to -23°C using a dry ice / carbon tetrachloride ice bath, and the lights were turned off. Tert-butyl hypochlorite (0.22 ml, 1.01 eq.) was added dropwise from a syringe over a 30-35 minute period. The solution was stirred in the ice bath for 30 minutes while the lights remained off. Triethyl amine (0.27 ml, 1.01 eq.) was syringed into the solution. The solution was left to sit for 61 hours with occasional stirring and flushing of the system with nitrogen. The yellow solution which contained a white precipitate was vacuum filtered, and the mother liquor was evaporated to a yellow-brown oil (0.210 g, 1.654 mmol, 86 % from the methyl ester, 83 % from proline).

3.2.7.3 [Carboxyl-^{14}C] 1,2-Dehydro-3-Acetoxyproline Methyl Ester

A two-necked flask was fitted with a condenser and flame-dried under nitrogen. The 1,2-dehydro-proline methyl ester from above was dissolved in HPLC grade dichloromethane and transferred to the flask using a cannula. The solution was cooled to 0°C using an ice bath containing calcium chloride. Lead tetraacetate (0.8144 g, 1.11 eq.) was added to the cold solution in small portions by removing the flask from the ice bath, drying the flask, and adding it at the condenser joint. After the addition was complete, the solution was stirred for 15 minutes at 0°C and then refluxed for 5 hours. The cooled solution was filtered into a 25 ml round bottom flask and evaporated to almost dryness. The lead oxide precipitate was removed by vacuum filtration. A few milliliters of HPLC grade dichloromethane and a few milliliters of heptane were added. The solution was evaporated leaving an oil, 191 mg. A TLC of the oil compared to the pure material developed in ethyl acetate and detected by UV light, showed both pyrrole and 1,2-dehydro-3-acetoxy proline methyl ester to be present. Liquid scintillation data of the two spots exhibited a significant amount of quenching which may explain the incorrect ratio of the pyrrole and proline (CPM in ^{3}H channel pyrrole = 25,000, proline = 100,000; CPM in ^{14}C channel pyrrole = 45,000, proline = 35,600). A ^{1}H NMR was run and approximately 13 mole % pyrrole appeared to be present in addition to 1,2-dehydro-3-acetoxyproline methyl ester.

3.2.7.4 [Carboxyl-^{14}C] 3-Hydroxyproline

The crude 1,2-dehydro-3-acetoxy proline methyl ester (191 mg) was cooled in an ice bath and 1.8 ml of 0.502 N NaOH (1 eq. excluding the pyrrole) was added. The solution was stirred for a few minutes in the ice bath. Sodium borohydride (17.8 mg, 2.1 eq.) was added, and the solution was stirred for 10 minutes in the ice bath. The pH was adjusted to 6 using 24 % HBr. The solution was filtered and evaporated to almost dryness. The residue was dissolved in 25 ml of 2 N HCl and refluxed for 2 hours. The brown solution was evaporated to 10 ml and decolorized with Norite,

and evaporated to dryness leaving a yellow precipitate. The precipitate was dissolved in a minimum amount of water and allowed to soak onto a Dowex 50W ion-exchange column (1.6 cm ID X 8 cm) in the H^+ form. The column was rinsed with distilled water. The column was then eluted with 1 N NH_4OH. A fraction of 125 ml of the ammonium hydroxide eluate was collected and evaporated to approximately 20 ml. The solution was decolorized with Norite and evaporated to almost dryness leaving a whitish residue. The residue was dissolved in a few milliliters of water, transferred to a 1 dram vial, and evaporated to dryness under a nitrogen stream. The residue was crystallized from water and ethanol giving 10.13 mg (0.077 mmol, 3.9 % crude yield).

3.2.7.5 Purification of [Carboxyl-^{14}C] Cis-3-Hydroxyproline

The residue was dissolved in 5 drops of deionized water and approximately 1.5 ml of absolute ethanol was added. The solution was stirred and seeded with a few milligrams of pure, unlabeled cis-3-hydroxyproline. The solution was left to sit until crystals formed. The crystals were removed from the mother liquor by centrifugal filtration. This recrystallization procedure was repeated two times. A TLC of trans-3-hydroxyproline, the three mother liquors, the sample, and cis-3-hydroxyproline were spotted on Whatman analytical $MKC_{18}F$ reverse-phase plates and developed in ethanol:1 % acetic acid (7:3). The spots were detected by ninhydrin and indicated the sample was pure. The Rf for standard trans-3-hydroxyproline was 0.88, and the Rf for standard cis-3-hydroxyproline was 0.73. The 3-hydroxyproline standards, mother liquors, and the hydroxyproline sample were again spotted on the reverse phase plates and developed in ethanol:1 % acetic acid. The plates were left to dry overnight before performing radioautography. In the dark the TLC plates were put in a cassette with X-ray film and left to develop for 3 days at -70°C. In the dark the X-ray film was gently stirred in developer for 5 minutes and rinsed for 30 seconds in tap water. The film was gently stirred in fixer for 5 minutes and rinsed for 30 seconds in tap water. The excess water was removed by passing the film through a squeezer, and the film was allowed to dry overnight. By comparison of the TLC spots when detected with ninhydrin and the

dark spots on the X-ray film, the [carboxyl-^{14}C]-cis-3-hydroxyproline sample was determined to be pure and to have a Rf identical to that of authentic cis-3-hydroxyproline. The sample contained 2.73 μCi of activity (0.91 % radiochemical yield).

3.3 VIRGINIAMYCIN M1 BIOSYNTHESIS

3.3.1 Sample Preparation

The pure [carboxyl-^{14}C] cis-3-hydroxyproline sample was evaporated to dryness for 3 hours under a nitrogen stream to remove all traces of solvent. The sample was dissolved in 1 ml of water and a 2 μl aliquot was taken to obtain the activity of the sample. The sample was determined to contain 2.50 μCi. [3,4-^{3}H] proline (43.9 mg, 22 μCi), which had been recrystallized from methanol:acetone to a constant specific activity of 57.8 μCi&divslmmol, was added to the hydroxyproline sample. The combined hydroxyproline and proline sample was dissolved in 2 ml of water and a 2 μl aliquot was taken to determine the ^{3}H / ^{14}C ratio. The ratio was 9.0 (&sup3H DPM = 56574, ^{14}C DPM = 6273).

3.3.2 Incorporation of [1-^{14}C] Cis-3-Hydroxyproline and S-[3,4-^{3}H] Proline in Virginiamycin

Streptomyces virginiae strain 5266 was grown for 72 hours in the vegetative medium and transferred in the production stage to 10 flasks containing 40 ml/flask. The precursor solution, which consisted of a [1-^{14}C]-3-hydroxyproline and [^{3}H]-proline (0.0025 mCi, 0.022 mCi, respec-

tively) mixture, was solubilized in 5 ml of distilled water, filter - sterilized (Millipore, 0.22 μm) and added after 24 hours (500 μl/flask). The virginiamycins were extracted after 3 days production time by the procedure described below. The broth was acidified with 20 % H_2SO_4 to pH 4.7 and extracted three times with methyl-isobutyl ketone. The combined organic extracts were concentrated in vacuo to dryness, and the residue was taken up in 1500 ml of acetonitrile. The acetonitrile solution was washed twice with 150 ml n-hexane, and evaporated to dryness in vacuo. The residue was taken up in 4 ml of chloroform. Virginiamycin was precipitated by the addition of 40 ml of n-hexane and recovered by filtration. The final dried powder (VM-^{14}C-Hyp ^{3}H-Pro lot 15.01.88) contained 42 mg M_2, 19 mg M_1, 5 mg M_3, 3 mg S_4, and 15 mg S_1 by HPLC.

3.3.3 Purification of Crude Virginiamycin

The crude virginiamycin sample was partially purified by flash chromatography as described below. A column (1.6 cm X 20 cm) was packed with 8 g of silica gel 60 by the slurry method using chloroform. The sample was dissolved in methanol and pipetted on the top of the column. Fractions 1-5 of 20 ml were eluted using chloroform:methanol (99:1). Fractions 6-10 of 20 ml each were eluted using chloroform:methanol (97:3). Fractions 11-15 were eluted using chloroform:methanol (95:5) as the eluate. TLC of the fractions using chloroform:methanol (9:1). as the mobile phase, UV detection, and comparison with standards indicated virginiamycin S, virginiamycin M_1, and virginiamycin M_2 were all present in fractions 1-4 while only virginiamycin M_1 and virginiamycin M_2 were present in fractions 5-13. Fractions 5-13 were added together, evaporated, dissolved in the acetonitrile and further purified with preparative HPLC. The conditions were as follows: Dynamax, Macro, 1 X 25 cm, C-18 column, a flow rate of 6 ml/minute, 0.5 absorbance, a chart speed of 0.5 cm/minute, and a mobile phase of acetonitrile:water (40:60). Fractions 1-4 were partially purified using preparative TLC plates with chloroform:methanol (90:10) as the mobile phase and then purified by HPLC using conditions identical to those stated above. A typical separation identifying the fractions collected is shown in Figure 15. From the

purification steps 6.57 mg of virginiamycin M_1 was obtained, 5.08 mg of virginiamycin M_2, and 7.83 mg of virginiamycin S.

The individual antibiotics were dissolved in 100 μl of acetonitrile and 100 μl of methanol. A 20 μl aliquot of each antibiotic was removed, dissolved in 0.5 ml of water, and counted to determine the activity. For virginiamycin S the specific activity was 3.69 μCi/mmol, and the ^3H incorporation was 0.305 %. Virginiamycin M_2 was determined to have a specific activity of 3.81 μCi/mmol, the ^3H incorporation was 1.38 %, and the $^3H/^{14}C$ ratio was found to be 80.8. Virginiamycin M_1 was found to have a specific activity of 0.0636 μCi/mmol for ^{14}C and 2.63 μCi/mmol for ^3H. For ^{14}C the % incorporation was 0.0312, and for ^3H the % incorporation was 0.433. The ^3H / ^{14}C ratio was 41.3.

3.3.4 $^3H/^{14}C$ Ratio of the Proline Moiety of Virginiamycin M_1

The virginiamycin sample was hydrogenated for 17 hours in 1 ml of acetic acid using 5.9 mg of platinum oxide (1 mg catalyst/1 mg antibiotic). The solution was filtered, and evaporated to dryness. The residue was transferred to a Reacti-Therm vial with 1 ml of 6 N HCl. The solution was heated at 101°C for 18 hours. The hydrolysate was made basic to litmus by addition of a 45 % sodium hydroxide solution. Diethyl ether (1 ml) and benzoyl chloride (20 μl) were added to the solution which was stirred vigorously for 33 hours. The aqueous layer was removed, and the ether layer was discarded. The aqueous layer was acidified with 6 N HCl and extracted 2 times with ethyl acetate. The combined extracts were evaporated to dryness, dissolved in a small amount of methanol, and decolorized by centrifugal filtration through Norite. A small amount of cold N-benzoyl proline was added. The precipitate was dissolved in a few drops of absolute ethanol, and petroleum ether was added until the solution turned cloudy. A drop of ethanol was added, the solution turned clear, and more petroleum ether was added until the solution again turned cloudy. The solution was left to crystallize a few hours. The crystals were separated from the mother liquor

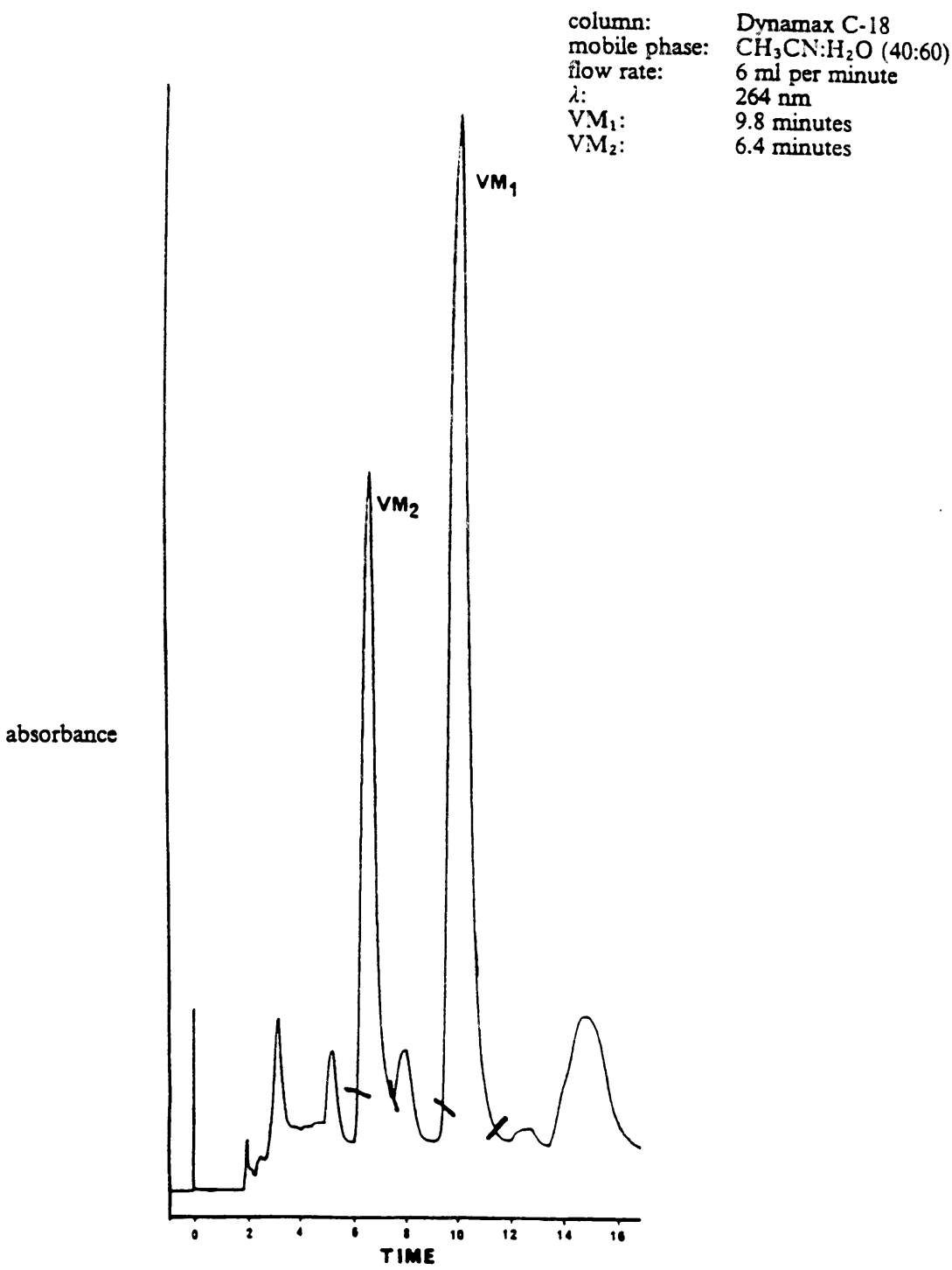


Figure 15. Chromatogram of Virginiamycin M_1 and Virginiamycin M_2

by centrifugal filtration. The crystals were dissolved in 50 μl of methanol, and a 30 μl aliquot was removed. The ^{3}H / ^{14}C ratio was 20.4 (^{3}H DPM= 1791, ^{14} C DPM= 88).

The remaining sample was evaporated to dryness. A small amount of cold N-benzoyl proline was added, and the sample was recrystallized from ethanol and petroleum ether as before. The crystals were dissolved in methanol and water and counted giving a ^{3}H / ^{14}C ratio of 19.3 (^{3}H DPM= 366, ^{14} C DPM= 19). The average of the ratios is 19.9.

4.0 RESULTS AND DISCUSSION

4.1 3-HYDROXYPROLINE SYNTHESIS

Several methods for an efficient synthesis of cis-3-hydroxyproline were investigated. The methods involved cleavage of a 3-methoxy ether intermediate[2] or reduction of 1,2-dehydro-3-acetoxy proline methyl ester. The literature procedure of Hausler[32] with a solvent variation when acetylating with lead tetraacetate was the synthetic method chosen for the ^{14}C labeled synthesis.

Several problems were prevalent with the 3-methoxy-N-acetyl proline methyl ester intermediate. The major problem is a trans to cis ratio of 4:1 when the intermediate is formed by Michael addition of methoxide to 2,3-dehydro-N-acetyl proline methyl ester. Cleavage of cis-3-methoxy-N-acetyl proline methyl ester by refluxing with constant boiling hydrobromic acid had previously been shown to give incomplete cleavage of the ether. Also, less vigorous conditions would be preferable for hydrolysis of the methyl ether.

Hydrolysis of the methyl ether using trimethyl silyl iodide was the next method explored for synthesis of 3-hydroxyproline. On a NMR scale the method looked quite promising for hydrolysis of the trans compound. Hydrolysis of the cis-3-methoxy-ether when explored on the

NMR scale required an extended length of time for completion of the reaction (155 hours). When trying to scale up the reaction for the trans compound, the results obtained on the NMR scale were never reproducible. Thus, 3-methoxy-N-acetyl proline methyl ester as an intermediate in the synthesis of 3-hydroxyproline was eliminated as a viable method.

Instead of Michael addition of methoxide, acetylation of 1,2-dehydroproline methyl ester using lead tetraacetate in dichloromethane was used to obtained the oxygen functionality at the three position. The first method utilized derivatization of the amine with benzyl chloroformate. The advantage of this derivation step is the cis and trans isomers could be separated using high-performance liquid chromatography instead of the laborious ion-exchange technique which does not always give complete separation of the isomers. This synthetic scheme was rejected due to the extremely poor yields for the protection step, approximately 10 %. The reason for the repeatedly low yields is unknown, but steric hindrance is a possible hypothesis.

The second method examined was hydrogenation of 1,2-dehydroproline-3-acetoxyproline methyl ester. Theoretically, hydrogenation of 1,2-dehydro-3-acetoxy proline methyl ester should give only the cis isomer by syn addition of the hydrogen molecule across the double bond since the hydrogen molecule should be directed to attack the proline molecule on the side opposite of the 3-acetoxy group. Cis-3-hydroxyproline was indeed the predominant isomer formed; however, this reaction was not a feasible method for synthesizing cis-3-hydroxyproline either since the yields were very low. Poisoning of the catalyst by amines during reduction by hydrogenation is well known and presumably occurred in this case.[69]

The method finally chosen for synthesis of ^{14}C labeled 3-hydroxyproline was the literature method of Hausler[32] using the solvent variation of Ewing.[34] Although Hausler reported the cis isomer as the only product, 10-20 % of the trans isomer was determined to be present by ^{1}H NMR. Recrystallization of the mixture from ethanol/water seeded with pure cis-3-hydroxyproline eliminated any trace of the trans isomer. Yields of this synthetic method were very modest but sufficient for the biosynthetic experiment. This synthesis is outlined in Figure 16.

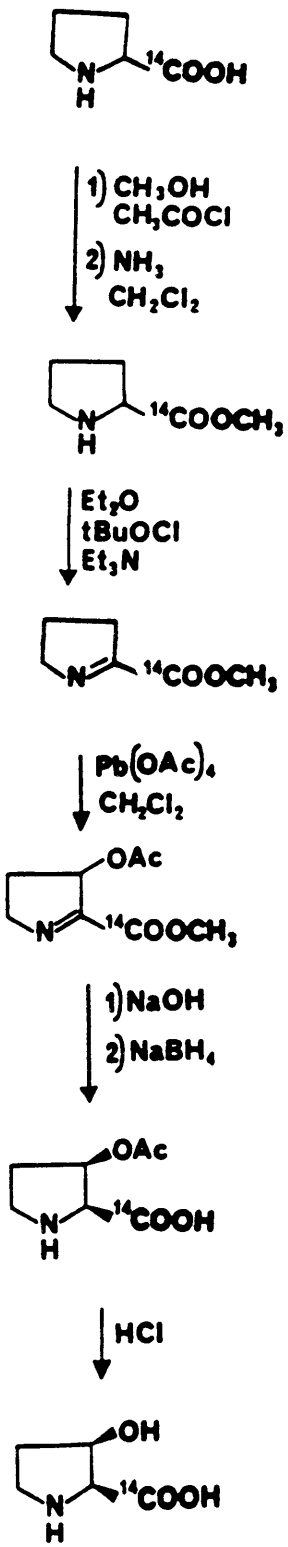


Figure 16. ^{14}C Labeled Synthesis

4.2 *VIRGINIAMYCIN M_1 BIOSYNTHESIS*

α,β-dehydroamino acids have been identified as components of fungal metabolites, and the metabolites frequently exhibit antibiotic properties. However, the antibiotic properties have not yet been associated with the presence of the dehydroamino acid. Several biosynthetic pathways are possible for the formation of dehydroamino acids. Elimination of α- and β-mercapto-α-amino acids, elimination of α-keto acids and their aminals, or elimination of α- and β-hydroxy-α-amino acids are all possible. Another feasible pathway is dehydrogenation.[70] These pathways are summarized in Figure 17.

Elimination of a β-hydroxy-α-amino acid has been studied as a possible precursor to the dehydroproline moiety in virginiamycin M_1. A previous study utilizing ^{14}C labeled trans-3-hydroxyproline showed no incorporation of trans-3-hydroxyproline into virginiamycin M_1.[2] In this study ^{14}C labeled (R,S)-[carboxyl-^{14}C]-cis-3-hydroxyproline was fed as a possible precursor to the dehydroproline moiety. Approximately 50 % of the cis-3-hydroxyproline was incorporated into the dehydroproline portion of virginiamycin M_1, presumably only the (S) enantiomer. From this result one may conclude dehydration of a β-hydroxy-α-amino acid is a feasible biosynthetic pathway to dehydroamino acids and may be an intermediate in the formation of other dehydroamino acids.

Studies employing ^3H labeled prolines were also undertaken in order to assist with delineation of the biosynthesis of the dehydroproline portion of virginiamycin M_1. Feeding of (S)-[3,4-$^3H_2$] showed complete retention of tritium thereby indicating an anti elimination.[2] Both (R) and (S) proline have been shown to be a precursor to the dehydroproline moiety. Also, (S)-[3-^3H] proline was fed to the antibiotic producing organism and showed complete loss of the tritium label. These results are summarized in Figure 18.

Since both (R) and (S) proline are incorporated a racemase for proline must be present in the system. One might hypothesize 3-hydroxyproline racemizes as easily as proline. In this case (S)-cis-3-hydroxyproline would form (R)-trans-3-hydroxyproline and visa versa. Also,

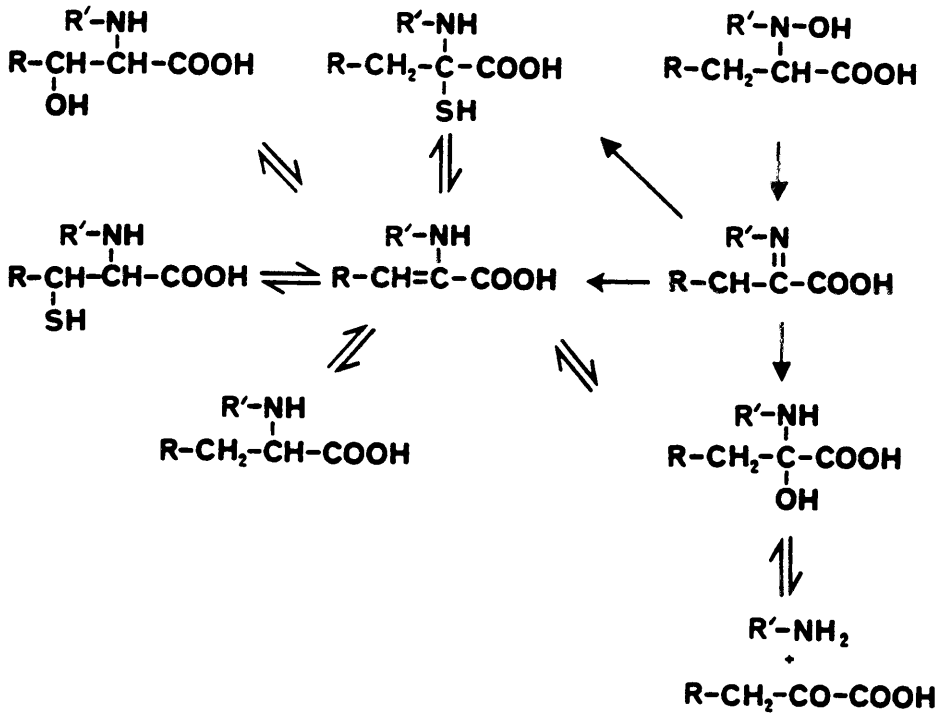


Figure 17. Biosynthetic Pathways of Dehydroamino Acids

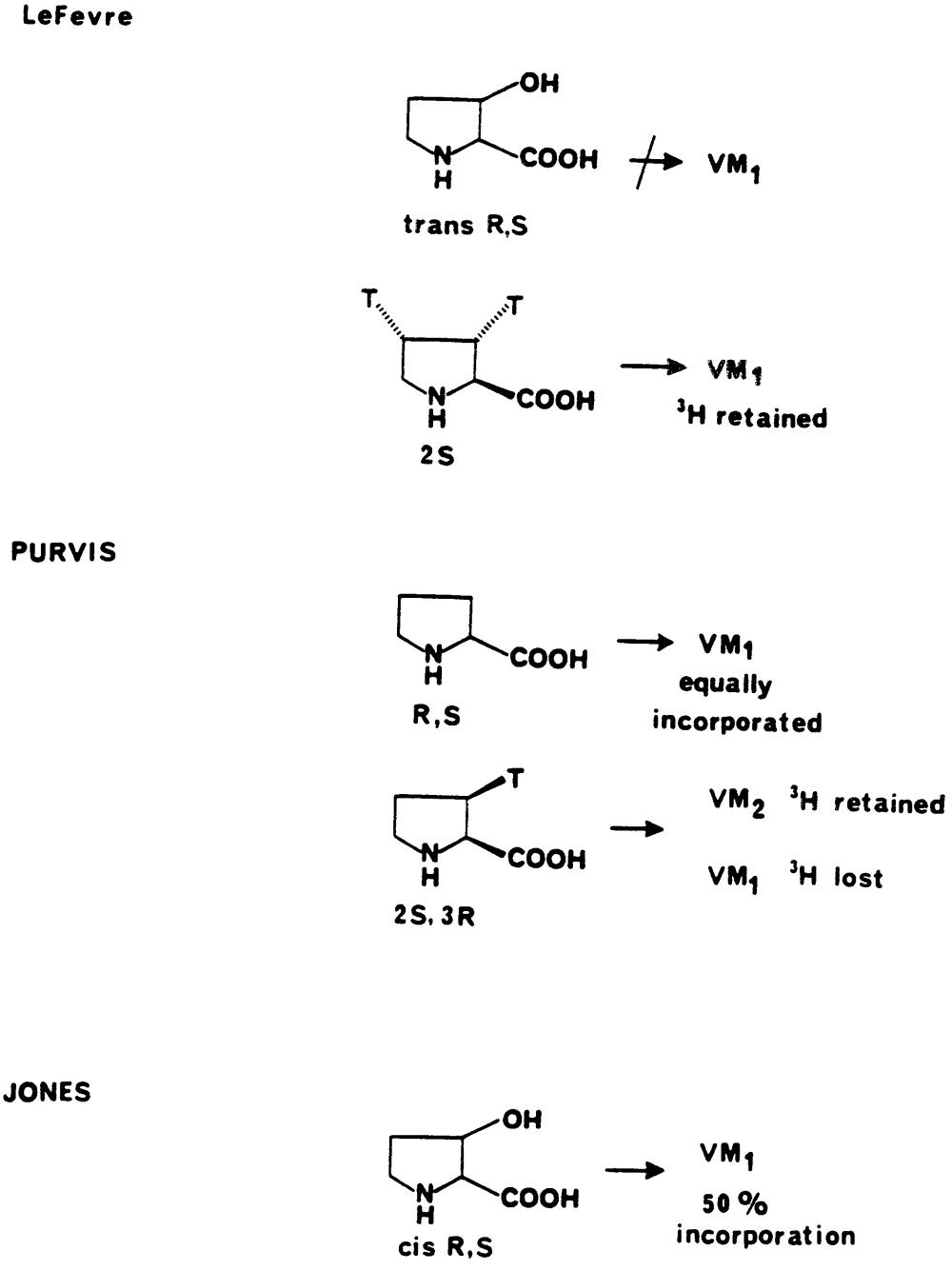


Figure 18. Possible Precursors for the Dehydroproline Moiety

(R)-cis-3-hydroxyproline would be racemized to (S)-trans-3-hydroxyproline and again visa versa. This possibility can excluded since free (R,S)-trans-3-hydroxyproline was not incorporated into the dehydroproline portion of virginiamycin M_1. The racemization process is demonstrated in Figure 19.

In order to elucidate the possible pathways for formation of virginiamycin M_1, the hydroxylations of (S)-cis-[3-$^3H_1$] proline were considered. Both the (R) and (S) isomers were hydroxylated assuming formation of either cis- or trans-3-hydroxyproline. Also, both retention and inversion of stereochemistry during hydroxylation were considered as options. These possibilities are illustrated in Figure 20 and 21. Assuming retention of configuration either a cis hydroxylation of (S) proline followed by an anti elimination to the dehydroproline or a trans hydroxylation of (R)-proline followed by a syn elimination is a viable option. However, since (R,S)-trans-3-hydroxyproline was demonstrated not to be a precursor to virginiamycin M_1, that possibility can be eliminated. When one assumes inversion of stereochemistry for the hydroxylation process, a trans hydroxylation of (S)-proline followed by a syn elimination and cis hydroxylation of (R)proline followed by an anti elimination give loss of the tritium as shown. Again, a trans hydroxylation is not a possible pathway since no trans-3-hydroxyproline was incorporated. Thus, either (S)-proline is incorporated with retention of stereochemistry for the hydroxylation process or (R)-proline is the precursor and the stereochemistry is inverted during the hydroxylation.

From the above results two pathways are possible for formation of virginiamycin M_1. The proline moiety present in virginiamycin M_2 is of the (R) configuration. Therefore, if virginiamycin M_1 is derived from virginiamycin M_2, (R)-cis-3-hydroxyproline serves as the precursor to the dehydroproline portion of virginiamycin M_1, and the stereochemistry of the hydroxylation is inversion. Since hydroxylations in both plants and animals are usually known to occur with retention of stereochemistry,[71] [72] [73] this pathway is suspect. If (S)-cis-3-hydroxyproline serves as the precursor to the dehydroproline moiety, then two separate routes must be present, one to virginiamycin M_1 and one to virginiamycin M_2. This pathway appears inefficient since two completely different sets of enzymes would be required for the two very similar antibiotics.

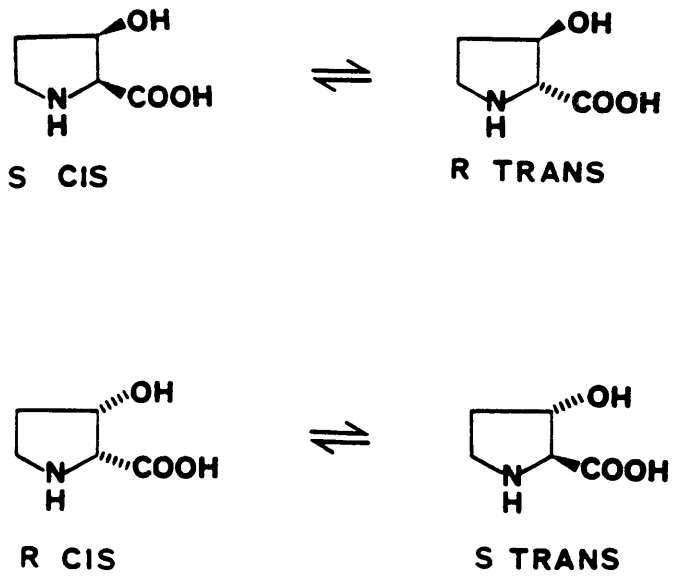


Figure 19. Racemization of 3-Hydroxyproline

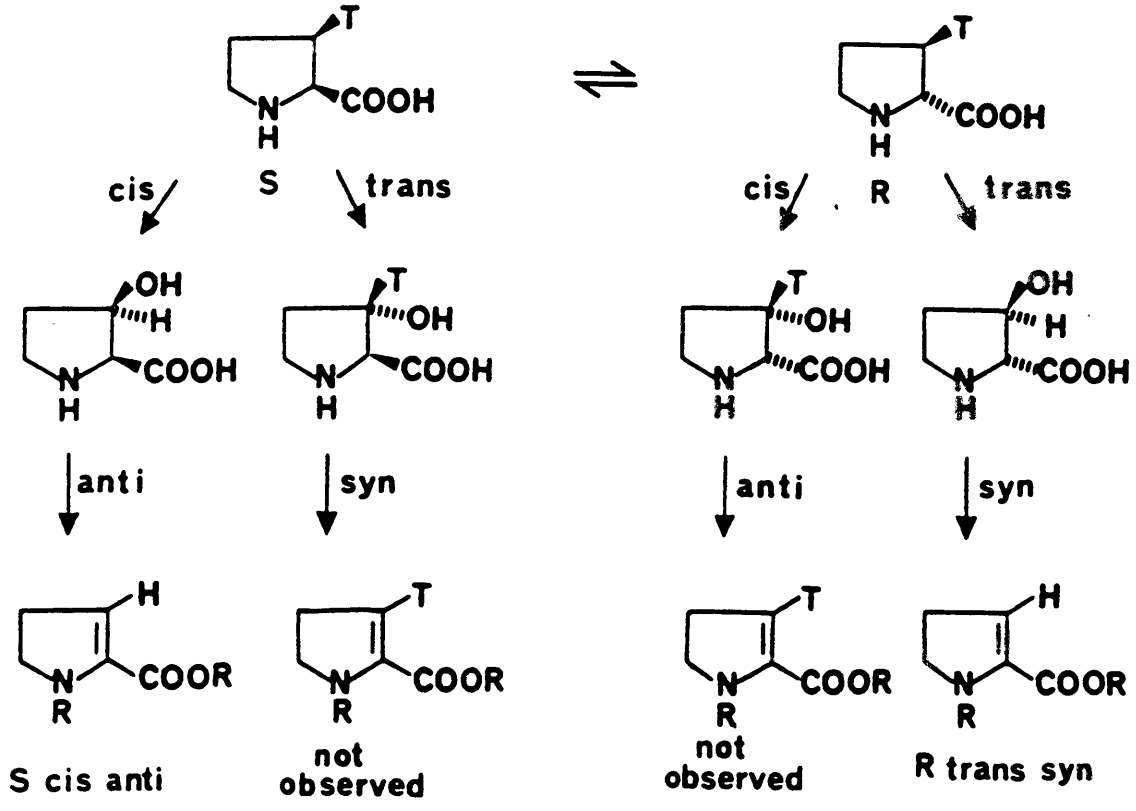


Figure 20. Hydroxylation with Retention

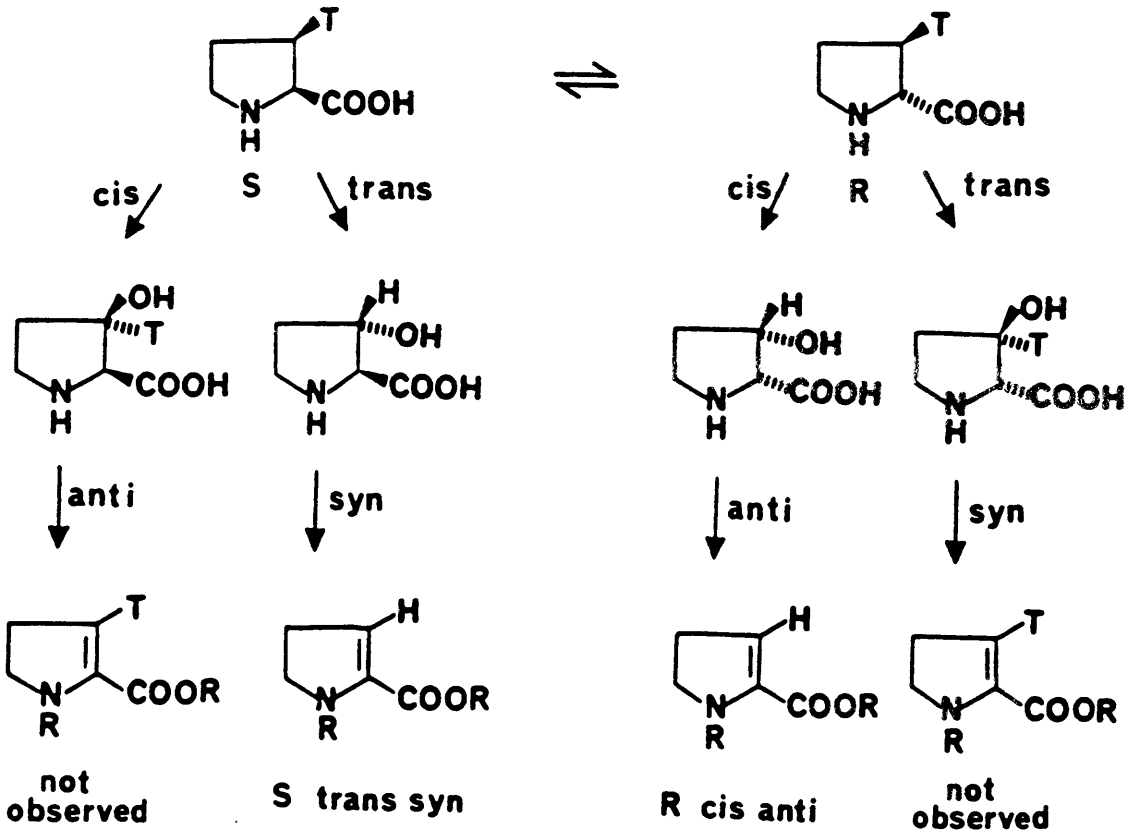


Figure 21. Hydroxylation with Inversion

Another possible pathway for the biosynthesis of virginiamycin M_1 is retention of stereochemistry during the hydroxylation followed by inversion of the stereochemistry at a later point. This pathway is supported by Katz in a biosynthetic study of etamycin, an antibiotic which is similar to virginiamycin S.[74] Etamycin contains (R)-cis-4-hydroxyproline. However, cis-4-hydroxy-(R)-proline was shown not to be a precursor to this portion of the antibiotic while both (S)-proline and (S)-trans-4-hydroxyproline were demonstrated to be precursors to the cis-4-hydroxy-(R)-proline in etamycin. From these results, Katz postulates a (S)-proline is hydroxylated to free (S)-trans-4-hydroxyproline which is epimerized as an activated, enzyme-bound hydroxyimino acid similar to racemization of phenylalanine in gramacidin S and tyrocidine biosynthesis.[75] By analogy (S)-proline would be hydroxylated to free (S)-cis-3-hydroxyproline followed by epimerization of the hydroxyimino acid in an activated, enzyme-bound form to (R)-trans-3-hydroxyproline. The activated form of the epimerized hydroxyimino acid would be incorporated into a hydroxylated form of virginiamycin M_2 which would then eliminate to form virginiamycin M_1.

In summary three pathways are possible for the formation of virginiamycin M_1 as exemplified in Figure 22. In the first pathway two routes exist, one to virginiamycin M_1 and another to virginiamycin M_2. This pathway could be tested by feeding labeled virginiamycin M_2 to the antibiotic producing bacteria and determining if virginiamycin M_1 contained the label. If virginiamycin M_1 is formed from virginiamycin M_2, the resulting virginiamycin M_1 should contain the label. In the second pathway virginiamycin M_2 is hydroxylated with inversion of stereochemistry to form a hydroxylated virginiamycin M_2 at which point free (R)-cis-3-hydroxyproline could also be incorporated into the hydroxylated virginiamycin M_2. The third pathway proposed is hydroxylation of (S)-proline to (S)-cis-3-hydroxyproline followed by epimerization of an activated, enzyme-bound form of the hydroxyimino acid. The activated, enzyme-bound imino acid would be incorporated into the hydroxylated virginiamycin M_2 and then elimination would yield virginiamycin M_1.

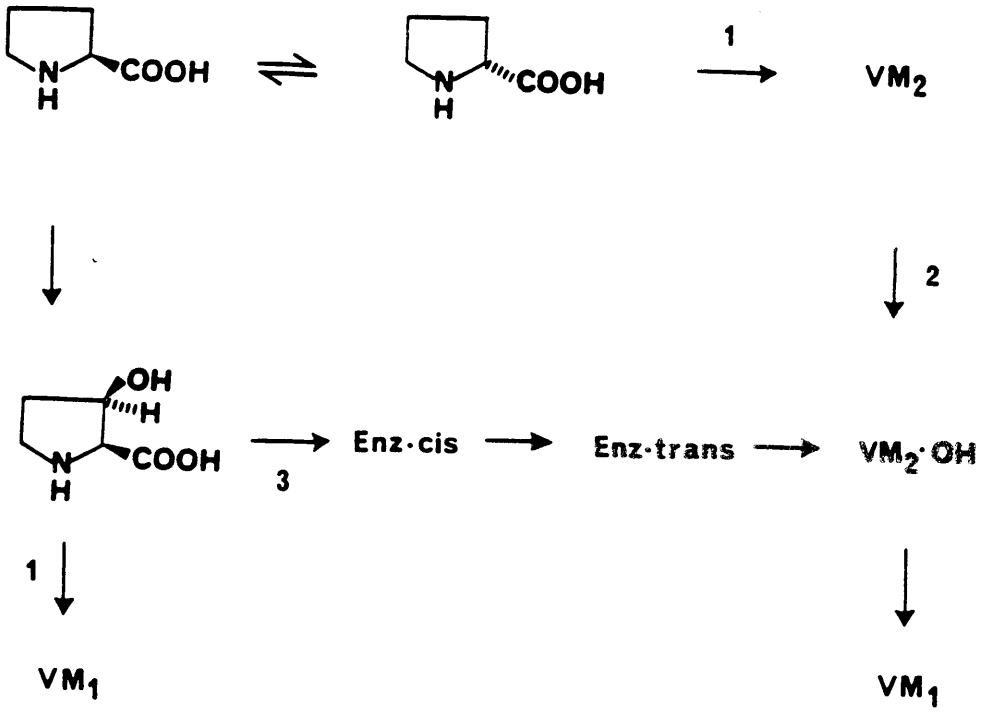


Figure 22. Possible Pathways for Virginiamycin M_1 Dehydroproline Moiety Biosynthesis

REFERENCES

1. Cocito, C. *Microbiol. Rev.* **1979** June 145-198.
2. LeFevre, J. W. Ph. D. Dissertation, VPI & SU, Blacksburg, VA **1983.**
3. Vickery, H. B. *Adv. Prot. Chem.* **1972** *26* 81-171.
4. Mauger, A. B.; B. Witkop *Chem Reviews* **1966** *66* 47-86.
5. Ogle, J. D.; M. A. Logan ; R. B. Arlinghaus *Fed. of Am. Soc. for Exp. Biol.* **1961** *20* 1.
6. Ogle, J. D.; R. B. Arlinghaus; M. A. Logan *Arch. Biochem. Biophys.* **1961** *94* 85-93.
7. Ogle, J. D.; R. B. Arlinghaus; M. A. Logan *J. Biol. Chem.* **1962** *237* 3667-3673.
8. Wolff, J. S. III; M. A. Logan; J. D. Ogle *Fed. Proc.* **1966** *25* 862-3.
9. Irreverre, F.; K. Morita; A. V. Robertson; B. Witkop *Biochem. Biophys. Res. Commun.* **1962** *8* 453-455.
10. Irreverre, F.; K. Morita; S. Ishii; B. Witkop *Biochem. Biophys. Res. Commun.* **1962** *9* 69-71.
11. Sheehan, J. C.; J. G. Whitney *J. Am. Chem. Soc.* **1962** *84* 3980.
12. Sugawara, K.; K. Numata; M. Konishi; H. Kawaguchi *J. Antibiot.* **1984** *XXXVII* (No. 9) 958-964.
13. Sung, M.; L. Fowden *Phytochemistry* **1968** *7* 2061-2063.
14. Kefalides, N. A.; B. Denduchis *Biochem.* **1969** *68* 4613-4621.
15. Denduchis, B.; N. A. Kedalides; A. Bezkorovainy *Arch. Biochem. Biophys.* **1970** *138* 582-589.
16. Man, M.; E. Adams Biochem. *Biophys. Res. Commun.* **1975** *66* 9-16.

17. Gryder, R. M.; M. Laman; E. Adams *J. Biol. Chem.* **1975** *250* 2470-2474.

18. Fujimoto, D.; E. Adams *Biochim. Biophys. Acta* **1965** *107* 232-246.

19. Risteli, J.; K. Tryggvason; K. Kivirikko *Eur. J. Biochem.* **1977** *73* 485-92.

20. Kivirikko, K. I.; R. Myllyla *Methods Enzymol.* **1982** *82* (Pt. A) 245-304.

21. Tryggvason, K.; J. Risteli; K. Kivirikko *Biochem. Biophys. Res. Commun.* **1977** *76* 275-281.

22. Tryggvason, K.; K. Majamaa; J. Risteli; K. Kivirikko *Biochem. J.* **1979** *183* 303-307.

23. Baker, R.; R. E. Schaub; M. V. Querry; J. H. Williams *J. Org. Chem.* **1952** *17* 77-96.

24. Tschesche, R.; E. U. KaussMann; H Fehlhaber *Chem. Ber.* **1972** *105* 3094-3105.

25. Wolff, J. S. III; J. D. Ogle; M. A. Logan *J. Biol. Chem.* **1966** *241* 1300-1307.

26. Sheehan, J. C.; J. G. Whitney *J. Am. Chem. Soc.* **1963** *85* 3863-3865.

27. Irreverre, F. ; K. Morita; A. V. Robertson; B. Witkop *J. Am. Chem. Soc.* **1963** *85* 2824-2831.

28. Morita, K.; F. Irreverre; F. Sakiyama; B. Witkop *J. Am. Chem. Soc.* **1963** *85* 2832-2834.

29. Blake, J.; C. D. Willson; H. Papoport *J. Am. Chem. Soc.* **1964** *86* 5293-5299.

30. Feil, P. D.; J. R. Vercellotti *Carbohydrate Res.* **1973** *31* 311-322.

31. Hausler, J.; U. Schmidt *Liebigs Ann. Chem.* **1979** 1881-1889.

32. Hausler, J. *Liebigs Ann. Chem.* **1981** 1073-1088.

33. Ramaswamy, S. G. *J. Labelled Compd. Radiopharm.* **1983** *XX* (No. 2) 233-242.

34. Ewing, W. R.; B. D. Harris; K. L. Bhat; M. M. Joullie *Tetrahedron* **1986** *42* 2421-2428.

35. DeShong, P.; D. A. Kell; D. R. Sidler *J. Org. Chem* **1985** *50* 2309-2315.

36. Evans, D. A.; A. E. Weber *J. Am. Chem. Soc.* **1987** *109* 7150-7157.

37. Charney, J.; W. P. Fisher; C. Curran; R. A. Machlowitz; A. A. Tytell *Antibiot. Chemother.* **1953** *3* 1283-1286.

38. Arai, M.; S. Nakamura; Y. Sakagami; K. Fukuhara; H. Yonehara *J. Antibiot.* **1956** Ser. A *9* 193.

39. Preud'homme, J.; P. Tarridec; A. Belloc *Bull. Soc. Chim. Fr.* **1968** *2* 585-591.

40. Celmer, W. D.; B. A. Sobin *Antibiot. Annu.* **1955-1956** 437-441.

41. De Somer, P.; P. Van Dijck *Antibiot. Chemother.* **1955** *5* 632-639.

42. Croy, P.; DeNeys R. *J. Antibiotics* **1972** *XXV (6)* 371-372.

43. Vazquez, D. *Antibiotics* **1975** *3* 521-534.

44. Tanaka, N. *Antibiotics* **1975** *3* 387-497.

45. Biot, A. M. *Drugs Pharm. Sci.* **1984** *22 (Biotechnol. Ind. Antigiot.)* 695-720.

46. Delpierre, G. R.; F. W. Eastwood; G. E. Gream; D. G. I. Kingston; P. S. Sarin; L. Todd; D. H. Williams *J. Chem. Soc. C* **1966** 1653-1669.

47. Kingston, D. G. I.; L. Todd; D. H. Williams *J. Chem. Soc. C* **1966** 1669-1676.

48. LeFevre, J. W.; T. E. Glass; M. X. Kolpak; D. G. I. Kingston *J. Nat. Prod.* **1983** *46* 475-480.

49. Gottschall, D. W.; C. Gombatz; R. Wang *J Agric. Food Chem.* **1987** *35* 900-904.

50. Cocito, C.; G. Chinali *J. Antimicrob. Chemother.* **1985** *16* Suppl. A 35-52.

51. Cocito, C. *J. Gen. Microbiol.* **1969** *57* 179-194.

52. Parfait, R.; M. Di Giambattista; C. Cocito *Biochim. Biophys. Acta* **1981** *654* 236-241.

53. Moureau, P.; M. Di Giambattista; C. Cocito *Biochim. Biophys. Acta* **1983** *739* 164-172.

54. Chinali, G.; P. Moureau; C. G. Cocito *Eur. J. Biochem.* **1981** *118* 577-583.

55. Chinali, G.; P. Moureau; C. G. Cocito *J. Biol. Chem* **1984** *259* 9563-9568.

56. Kingston, D. G. I.; M. X. Kolpak; J. W. LeFevre; I. Borup-Grochtman *J. Am. Chem. Soc.* **1983** *105* 5106-5110.

57. Kingston, D. G. I.; M. Kolpak *J. Am. Chem. Soc.* **1980** *102* 5964-5966.

58. Roberfroid, M.; P. Dumont *Ind. Chem. Belge* **1967** *32 (Spec. No., Part 3)* 1-3.

59. Pearce, C. J.; K. L. Rinehart, Jr. *J. Am. Chem. Soc.* **1979** *101* 5069-5070.

60. Purvis, M. B.; D. G. I. Kingston; N. Fujii; H. G. Floss *J. Chem. Soc., Chem. Commun.* **1987** *4* 302-303.

61. Kirby, G. W.; S. Narayanaswami *J. Chem. Soc., Chem. Commun.* **1973** 322-323.

62. Parry, R. J.; S. P. Rao; J. Mueller *J. Am. Chem Soc.* **1982** *104* 339-340.

63. Purvis, M. B. Ph. D. Dissertation, VPI & SU, Blacksburg, VA **1988.**

64. Thomas, W. A.; M. A. Williams *J. Chem. Soc., Chem. Commun.* **1972** 994.

65. Dorman, D. E.; F. A. Bovey *J. Org. Chem.* **1973** *38* 2379-2382.

66. Voelter, W.; S. Fuchs; R. H. Seuffer; K. Zech *Monatsh. Chem.* **1974** *105* 1110-1135.

67. Baumgarten, H. E. *Org. Synth.* **1973** *Coll. Vol. V* 184 Wiley and Sons, Inc. NY

68. Ito,A; R. Takahashi; Y. Baba *Chem. Pharm. Bull.* **1975** *23* 3081-3087.

69. Hudlicky, M. *Reductions in Organic Chemistry* **1984** M. Hudlicky/Ellis Horwood Limited Chichester, England

70. Schmidt, U.; J. Hausler; E. Ohler; H. Poisel *Prog. Chem. Org. Nat. Prod.* **1979** *37* 251-327.

71. Jones, D. F. *J. Chem. Soc (C)* **1968** 2827-2833.

72. Varma, K. R.; E. Caspi *Phytochemistry* **1970** *9* 1539-1543.

73. Heinz, E.; A. P. Tulloch; J. F. T. Spencer *J. Biol. Chem.* **1969** *244* 882-888.

74. Katz, E.; F. Kamal; K. Mason *J. Biol. Chem.* **1979** *254* 6684-6690.

75. Lipmann, F.; W. Gevers; H. Kleinkauf; R. Roskowski *Adv. Enzymol.* **1971** *35* 1-34.

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