# Biosynthesis of Antibiotics of the Virginiamycin Family. 3. Biosynthesis of Virginiamycin $M_1$[1,2]

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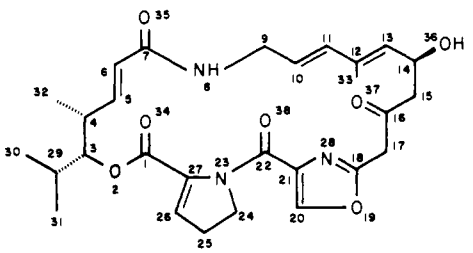
**Abstract:** The biosynthesis of the antibiotic virginiamycin $M_1$ (**1**) has been investigated with the aid of stable isotope techniques. The basic skeleton of the antibiotic is derived from valine, seven acetate units, glycine, serine, and proline, with the unusual oxazole ring being derived from serine. The methyl group at C-32 is derived from methionine, but the one at C-33 is derived by a new pathway involving decarboxylation of an acetate unit.

The virginiamycin family consists of antibiotics that are usually isolated as mixtures of synergistic compounds.[3-6] These antibiotics can be obtained from a number of different source microorganisms and have consequently been variously named as mikamycins, pristinamycins, streptogramins, ostreogrycins, or virginiamycins. Their nomenclature is thus somewhat confused, but the name virginiamycin has been adopted by Chemical Abstracts and a recent authoritative review,[6] and this name will thus be used in the paper.

The antibiotics in the virginiamycin family consist of a rather small number of compounds that belong to one of two distinct groups. The antibiotics of group A are polyunsaturated cyclic peptolides, which can be considered as highly modified depsipeptides.[7] The first of these to have its structure elucidated was ostreogrycin A,[8] which is identical with virginiamycin $M_1$ (**1**);[9]



**1**

the structure has since been confirmed by X-ray crystallography.[10] Very recently its absolute configuration has been confirmed by partial synthesis,[11] and its $^{13}C$ NMR spectrum has been assigned.[1] The antibiotics of group B are cyclic hexadepsipeptides of molecular weight about 800. The A and B group antibiotics are individually bacteriostatic, but in combination they show a marked synergism and are bacteriocidal against Gram-positive organisms.[12] The antibiotics are inhibitors of protein synthesis and act on the ribosomes: it has been shown that in vitro, type A compounds increase the affinity of ribosomes for type B compounds and the stability of the corresponding complexes against a competitive displacement by erythromycin. This effect may account for the synergistic action of type A and type B components in vivo.[13]

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

| precursor added | % incorporation, this work | % incorporation, ref 19 |
|---|---|---|
| sodium [2-$^{14}$C]acetate | 5.0 | 5.5, 3.4 |
| sodium [2-$^{14}$C]malonate | | 0.4 |
| DL-[2-$^{14}$C]mevalonolactone | *a* | 0.04 |
| L-[$^{14}CH_3$]methionine | 4.0 | 4.5, 5.7 |
| DL-[3-$^{14}$C]serine | 1.4 | |
| L-[3,4-$^3H_2$]proline | 5.0 | |
| [2-$^{14}$C]glycine | | 0.4, 0.8 |
| L-[U-$^{14}$C]alanine | | *a* |
| D-[U-$^{14}$C]glucose | 0.6 | |
| DL-[3,4-$^{14}C_2$]glutamic acid | | *a* |
| L-[1-$^{14}$C]leucine | | *a* |
| L-[U-$^{14}$C]valine | 15.2 | |

[a] No detectable incorporation.

Our interest has focused on the biosynthesis of **1**. The molecular structure, composed of a dehydroproline residue and an oxazole ring in a macrocyclic unsaturated system, is unique among natural products, with the exception of closely related antibiotics such as griseoviridin[14] and antibiotic A2315A.[15] Particular interest attaches to the oxazole ring, since this is a highly unusual structural feature of natural products, occurring elsewhere only in a few antibiotics such as berninamycin[16] and nocobactin NA[17] and in the alkaloid annuloline.[18]

In this communication we report results that reveal the origin of the oxazole ring and the macrocyclic ring of virginiamycin $M_1$ and that also uncover a new pathway for methylation of a carbon chain.

## Results and Discussion

The biosynthesis of virginiamycin $M_1$ was studied in shake cultures of *Streptomyces virginiae* strain PDT 30 that were grown

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Table II. $^{13}C$ NMR Spectral Data for Virginiamycin $M_1$, Including Enrichments from Labeled Precursors

| carbon no. | $\delta_C$[a] | I labeled/I unlabeled[b] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | [1-$^{13}$C]Ac | [2-$^{13}$C]Ac | [3-$^{13}$C]Ser | [1-$^{13}$C]Gly | [2-$^{13}$C]Val | [Me-$^{13}$C]Met | $^1J_{C-C}$,[c] Hz |
| 1 | 160.9 | 1.1 | 0.8 | *e* | 1.1 | 0.8 | *e* | |
| 3 | 81.5 | 1.1 | 1.0 | 0.7 | 0.8 | *17.1* | 1.1 | |
| 4 | 37.6 | 1.3 | *4.6* | *1.8* | 1.0 | 0.8 | 1.1 | 42.7 |
| 5 | 143.1 | *3.0* | 0.8 | 0.6 | 0.9 | 0.8 | 1.1 | 42.0 |
| 6 | 125.3 | 1.0 | *4.2* | *1.7* | 1.4 | 1.0 | 0.8 | 64.9 |
| 7 | 167.6 | *2.0* | 0.8 | 0.4 | 0.6 | 1.0 | 0.8 | 65.2 |
| 9 | 40.5 | 1.2 | 1.1 | 0.9 | 1.0 | 1.0 | 1.3 | |
| 10 | 126.1 | 1.0 | 1.0 | 0.8 | *7.8* | 1.0 | 1.3 | |
| 11 | 133.7 | 1.0 | *3.7* | *1.7* | 0.8 | 1.0 | 1.1 | *g* |
| 12 | 134.7 | *1.9* | 0.7 | 0.4 | 0.5 | 1.0 | 0.5 | *g* |
| 33 | 12.7 | 1.0 | *4.1* | *3.0* | 0.6 | 0.8 | 1.4 | |
| 13 | 131.0 | 1.1 | *4.1* | *1.8* | 0.7 | 1.0 | 1.0 | 48.0 |
| 14 | 66.0 | *2.3* | 0.9 | 0.6 | 0.6 | 1.0 | 1.0 | 48.0 |
| 15 | 47.7 | 1.4 | *4.1* | *2.4* | 0.9 | 1.0 | 1.2 | 40.0 |
| 16 | 200.7 | *1.6* | 0.7 | 0.6 | 0.6 | 0.8 | 0.6 | 40.5 |
| 17 | 45.7 | 1.2 | *3.6* | *2.1* | 1.0 | 0.9 | 1.1 | 60.7 |
| 18 | 156.2 | *2.0* | 0.7 | 0.5 | 0.8 | 1.0 | 0.6 | 60.3 |
| 20 | 145.4 | 1.1 | 0.8 | *7.9* | 0.7 | 0.9 | 1.2 | |
| 21 | 136.1[d] | 0.7 | 1.2 | 0.7 | 0.9 | 0.9 | 0.6 | |
| 22 | 160.0 | 1.1 | 1.1 | *e* | *10.1* | 0.8 | *e* | |
| 24 | 50.5 | 1.3 | 1.0[f] | 1.0[f] | 1.0[f] | 1.0[f] | 1.0[f] | |
| 25 | 29.9 | 1.0[f] | 1.1 | 0.9 | 1.2 | 1.1 | 0.9 | |
| 26 | 122.7 | 1.0 | 1.2 | 0.9 | 1.1 | 1.0 | 1.0 | |
| 27 | 137.2[d] | 1.1 | 1.3 | 0.8 | 1.0 | 0.8 | 0.5 | |
| 29 | 30.1 | 1.3 | 1.4 | 0.9 | 1.1 | 1.2 | 0.9 | |
| 30 | 18.9 | 1.3 | 1.2 | *2.4* | 1.0 | 0.8 | 1.1 | |
| 31 | 19.6 | 0.9 | 1.1 | *2.2* | 1.1 | 0.8 | 1.2 | |
| 32 | 12.2 | 0.9 | 1.0 | *3.8* | 1.2 | 0.9 | *3.3* | |

[a] Chemical shifts are downfield from internal $Me_4Si$ in $CDCl_3$. [b] Intensity of each peak in the labeled antibiotic divided by that of the corresponding peak in the unlabeled antibiotic, normalized to give a ratio of 1.0 for an unenriched peak, either C-24 or C-25. [c] Carbon–carbon coupling observed when [1,2-$^{13}C_2$]acetate was incorporated into virginiamycin $M_1$. [d] These signals may be interchanged. [e] Signals for these carbons, which were of low intensity in the unlabeled antibiotic, were lost in the noise in the spectra of certain labeled compounds. [f] Peak selected as the unenriched standard for this spectrum. [g] Coupling between carbons 11 and 12 was observable, but the coupling was not first order, and the coupling constant was not determined.

in a complex medium. On the basis of time–course studies, sterile addition of labeled precursors 24 h after innoculation and harvest of the cultures 24 h later were chosen as standard conditions for feeding experiments. Virginiamycin $M_1$ was isolated by liquid–liquid partition followed by purification by preparative high-performance liquid chromatography (HPLC). For incorporation of radioactive precursors, the extent of incorporation was determined by counting the chromatographically homogeneous product. This procedure was checked against the classical technique of addition of authentic antibiotic and recrystallization to constant specific activity and was found to give results that were essentially identical. For incorporation of $^{13}C$-labeled precursors, the isolated antibiotic was observed directly by $^{13}C$ NMR spectroscopy.

**Incorporation of Radioactive Precursors.** A series of feeding experiments with virginiamycin $M_1$ had been carried out earlier by Roberfroid and Dumont,[19] and Table I summarizes their work and our own results. These data indicate that the antibiotic is biosynthesized from the amino acids proline, glycine, serine, valine, and methionine and that the macrocyclic portion of the molecule derives from acetate. Although the isopropyl group might superficially appear to derive from mevalonate, incorporation of mevalonolactone was not detectable in our work, and another source of this group must be looked for.

**Incorporation of [1-$^{13}$C]-, [2-$^{13}$C]- and [1,2-$^{13}C_2$]Acetate.** We first investigated the incorporation of variously labeled sodium acetates into virginiamycin $M_1$, in order to establish the biosynthesis of the major part of the skeleton. Feeding experiments with sodium [1-$^{13}$C]acetate (Table II) indicated that C-5, -7, -12, -14, -16, and -18 were derived from the carboxyl carbon of this precursor, suggesting that the units consisting of C-4 to C-7 and C-11 to C-18 originated from acetate. In confirmation of this conclusion, a feeding experiment with sodium [2-$^{13}$C]acetate (Table II) indicated that C-4, -6, -11, -13, -15, and -17 originated from

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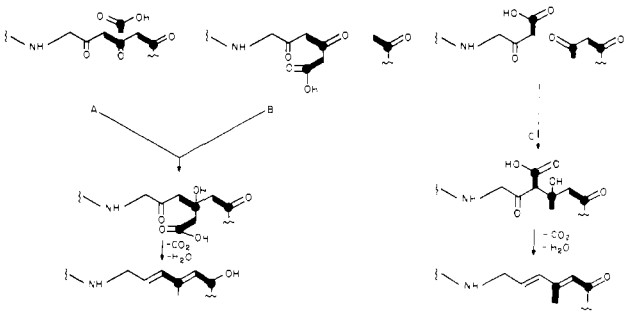

**Figure 1.** Possible pathways of incorporation of the C-33 methyl group into virginiamycin $M_1$.

the methyl carbon of acetate. Interestingly enough, this experiment indicated that the methyl group at C-33 also originated from the methyl group of the acetate precursor, suggesting that it arises by a novel pathway.

In considering ways in which the C-33 methyl group could arise, it appeared that three pathways were possible, represented schematically in Figure 1. In pathways B and C two polyketide chains couple via an aldol-type mechanism to yield a $\beta$-hydroxy acid which would undergo decarboxylation and elimination of water, together with reduction of the carbonyl groups, to yield the allylic alcohol system of virginiamycin $M_1$. In pathway A the same result is achieved by aldol condensation of an individual acetate unit (presumably as malonyl coenzyme A) to a preformed polyketide chain. Paths A and B would yield the same intermediate $\beta$-hydroxy acid, while pathway C would yield an isomeric hydroxy acid.

A distinction between paths A and B on the one hand and path C on the other was made by two independent methods. Incorporation of [2-$^{13}$C,$^2H_3$]acetate into virginiamycin $M_1$ would be expected to proceed with complete or partial retention of deuterium at the C-33 methyl group if pathway C were followed, since this

methyl group would be derived from the methyl group of a chain-starter acetate, and these units do not normally exchange hydrogen during polyketide biosynthesis.[20,21] On the other hand, formation of the methyl group by paths A or B would presumably involve malonyl coenzyme A as an intermediate, and extensive or complete loss of $^2H$ from the "malonate" positions would be expected by analogy with earlier results.[22] In the event, [2-$^{13}C$,$^2H_3$]acetate was incorporated into virginiamycin with an enrichment of 8 ± 3% at each of the carbons 4, 6, 11, 13, 15, 17, and 33[23] but with *no detectable incorporation of* $^2H$ as determined by $^2H$ NMR spectroscopy.[24] This result thus clearly favors incorporation by pathway A or B.

The second method used to distinguish the pathways was by incorporation of [1,2-$^{13}C_2$]acetate into virginiamycin $M_1$. The product showed couplings between the pairs of carbons 4 and 5, 6 and 7, 11 and 12, 13 and 14, 15 and 16, and 17 and 18, but *no coupling to carbon 33* (Table II). This result proves conclusively that pathway C cannot be operating and that the methyl group at C-33 is incorporated by pathway A or B.

A choice between pathways A and B was made on the basis of a time–course study where DL-[3-$^{13}C$]serine was used as a delayed source of [2-$^{13}C$]acetate. The incorporation of DL-[3-$^{13}C$]serine into other portions of virginiamycin $M_1$ will be discussed in a later section, and for our immediate purposes it is sufficient to note that this compound can be converted into [2-$^{13}C$]acetyl coenzyme A via dehydration and deamination to pyruvate.[25] A feeding experiment with DL-[3-$^{13}C$]serine thus serves to produce, inter alia, the same products that would be produced by an experiment with [2-$^{13}C$]acetate but *with a built-in delay dependent on the rate of conversion of serine to acetyl-CoA*. As noted above, when virginiamycin $M_1$ was produced under standard conditions from [2-$^{13}C$]acetate, the C-33 methyl group was enriched in $^{13}C$ to approximately the same extent as all the other carbon atoms labeled by this precursor (average enrichment 2.95%, enrichment at C-33 3.0%). However, when DL-[3-$^{13}C$]serine was used as a precursor (Table II), the C-33 methyl group was enriched to a considerably greater extent than the other carbon atoms labeled from this precursor via acetyl-CoA. The average enrichment of the six carbons at C-4, -6, -11, -13, -15, and -17 was 0.8%, while that of the C-33 carbon was 1.9%. Biosynthesis of virginiamycin by pathway B requires that carbons 11 and 33 be linked together as part of an initial polyketide chain (or its biosynthetic equivalent) and thus demands that C-11 and C-33 should be enriched to approximately the same extent. Only pathway A, in which the C-33 methyl group is added to a preformed polyketide chain, accounts for the observation that the C-33 carbon is enriched to a significantly greater extent by the "delayed labeling" of acetyl-CoA derived from DL-[3-$^{13}C$]serine.

We thus conclude that pathway A represents, in schematic form, the pathway by which the C-33 methyl group is provided to virginiamycin $M_1$. It is of interest in this connection that the related antibiotic griseoviridin[14] does not have a methyl group corresponding to the C-33 methyl of virginiamycin $M_1$, suggesting that the enzyme(s) responsible for the reactions of pathway A is (are) lacking in *Streptomyces griseus*. Although methylation of activated methylene groups by methionine is a well-known biosynthetic process, methylation by the pathway described above has no obvious precedent and appears to be a newly recognized biosynthetic reaction. The nearest analogy is found in the formation of the methyl group of mevalonic acid by a pathway involving an aldol condensation between acetoacetyl-CoA and acetyl-CoA.

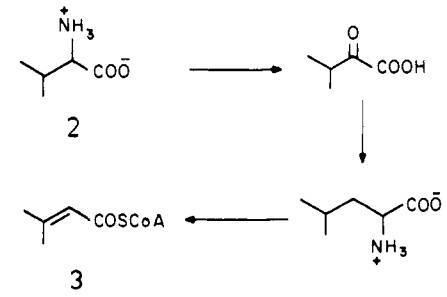


Figure 2. Biosynthetic conversion of valine to β-methylcrotonyl-CoA.

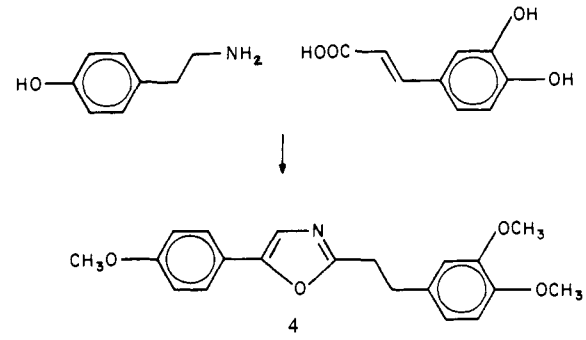


Figure 3. Biogenesis of the oxazole ring of annuloline.

**Incorporation of [2-$^{13}C$]Valine.** Since preliminary feeding experiments with L-[U-$^{14}C$]valine indicated efficient incorporation of this amino acid into virginiamycin $M_1$, we surmised that valine served as the precursor to carbons 3 and 29–31. Synthesis of DL-[2-$^{13}C$]valine was accomplished by the azlactone route starting with [2-$^{13}C$]glycine, and we were able to improve the published yield for this synthesis[26] by careful attention to experimental conditions. Incorporation of the labeled valine under standard conditions yielded antibiotic in which C-3 was enriched to the extent of 16%. This result indicates that valine provides the chain-starter unit for biosynthesis of the western portion of virginiamycin $M_1$, presumably by transamination to β-oxoisovaleric acid and then conversion to isobutyryl-CoA by oxidative decarboxylation.

The observation that C-3 is the *only* carbon enriched in $^{13}C$ from valine eliminates from consideration a possible alternate pathway for the origin of the methyl group at the C-33 position. It might be argued that this unit could arise from β-methylcrotonyl-CoA (**3**), which is a known intermediate in primary metabolism of amino acids, and can be formed (albeit by a rather roundabout route) from acetyl-CoA.[25] If this were the case, however, then valine (**2**) should be an effective precursor of the C-11, -12, -13, -14, and -33 carbons of virginiamycin $M_1$ via conversion to α-oxoisovaleric acid and thence to leucine and thence to β-methylcrotonyl-CoA (Figure 2).[26] This putative pathway would result in enrichment of C-13 where [2-$^{13}C$]valine is used as a precursor, and the lack of any detectable enrichment at this carbon (Table II) clearly eliminates β-methylcrotonyl-CoA as a significant intermediate in the biosynthesis of virginiamycin $M_1$.

**Incorporation of [*methyl*-$^{13}C$]Methionine.** Feeding [*methyl*-$^{13}C$]methionine under standard conditions yielded virginiamycin $M_1$ in which the only carbon enriched in $^{13}C$ was the C-32 methyl group. This result is consistent with alkylation of a methylene group at C-4 with *S*-adenosylmethionine.

**Incorporation of DL-[3-$^{13}C$]Serine.** The origin of dehydro amino acid units in natural products has been the ojbect of speculation for a number of years, and various theories have been proposed for their formation.[27,28] In the particular case of the oxazole ring

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(22) McInnes, A. G.; Walter, J. A.; Wright, J. L. C. *Tetrahedron Lett.* **1979**, 3245–3248.
(23) The error in this measurement is due to the very low intensity of resonances due to the unlabeled carbons in this preparation.
(24) The $^2H$ NMR spectrum was observed under conditions such that an enrichment 5% as great as the $^{13}C$ enrichment would have been readily detectable.
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(27) Bycroft, B. W. *Nature (London)* **1969**, *224*, 595–597.

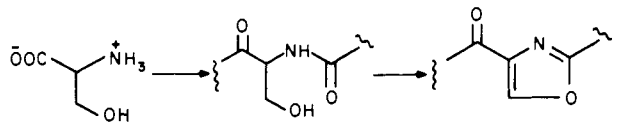

**Figure 4.** Biogenesis of the oxazole ring of virginiamycin $M_1$ from serine.

system, which can be visualized as a modified dehydroalanine or dehydroserine, the only experimental evidence bearing on its formation comes from studies on the alkaloid annuloline[29] (**4**) and the antibiotic berninamycin.[30] In the former case the alkaloid was shown to derive from phenylalanine and tyrosine, and tyramine and caffeic acid were shown to be intermediates as shown in Figure 3. This pathway would correspond to the formation of the oxazole ring from alanine in virginiamycin $M_1$. In the case of berninamycin, it was shown that L-[U-$^{14}$C]threonine was incorporated into the antibiotic, but degradation to establish that this amino acid was indeed the precursor of the oxazole units was not carried out.[30] Formation of the 5-methyloxazoles of berninamycin from threonine would correspond to the formation of the oxazole of virginiamycin $M_1$ from serine.

In the light of these reports, it became of interest to establish unambiguously the origin of the oxazole unit of virginiamycin $M_1$. Feeding experiments with L-[U-$^{14}$C]alanine and DL-[3-$^{14}$C]serine indicated that only the latter was incorporated into the antibiotic (Table I), and hence a sample of DL-[3-$^{13}$C]serine was obtained and fed to cultures of *S. virginiae*.[31] The isolated antibiotic showed enrichment of several carbon atoms in $^{13}$C. The most dramatic enrichment of almost 7% was observed for C-20, indicating conclusively that the oxazole ring was formed from serine, presumably by a pathway such as that shown in Figure 4. The exact details of this pathway, and in particular the interesting question of whether the oxygen atom of the oxazole ring derives from the serine hydroxyl group or the carbonyl group, are at present unknown, although analogy with the case of the thiazole ring system would suggest that the former possibility is the most likely.

In addition to the strong enrichment found for C-20, C-32 was enriched to the extent of 2.7%, while C-4, -6, -11, -13, -15, -17, -30, -31, and -33 were enriched in the range 0.6–1.9%. The extensive labeling observed for C-32 is consistent with the transfer of the C-3 of serine to methionine via $N^5$-methyltetrahydrofolate,[25] while the labeling of C-4, -6, -11, -13, -15, -17, and -33 is in agreement with their formation from [2-$^{13}$C]acetyl-CoA derived from L-[3-$^{13}$C]serine via pyruvate as discussed earlier. Finally, the labeling of C-30 and -31 is consistent with their formation from valine, which is also biosynthesized from pyruvate.[25]

**Incorporation of [1-$^{13}$C]Glycine, L-[U-$^{14}$C]Proline, and L-[3,4-$^3H_2$]Proline.** The remaining two nitrogen atoms of virginiamycin $M_1$ were considered to be derived from the amino acids proline and glycine. These considerations were confirmed by the observations that both L-[3,4-$^3H_2$]proline and [2-$^{14}$C]glycine were incorporated into the antibiotic (Table I).

The location of the glycine unit in virginiamycin $M_1$ was determined by a feeding experiment with [1-$^{13}$C]glycine, which resulted in production of antibiotic in which C-22 and C-10 were both significantly enriched in $^{13}$C (Table II). Enrichment at C-10 confirms that the N-8, C-9, and C-10 unit derives from glycine, while enrichment at C-22 is expected from the known incorporation of serine and the known formation of L-serine from glycine by the action of $N^5$,$N^{10}$-methylenetetrahydrofolate and serine hydroxymethyltransferase.[25]

The formation of the dehydroproline moiety of virginiamycin $M_1$ from L-proline was confirmed by a feeding experiment with L-[U-$^{14}$C]proline, in which the antibiotic obtained was subjected to hydrogenation and hydrolysis to yield DL-proline, isolated as its *N*-benzoyl derivative. Approximately half of the activity of the antibiotic was found in the resulting *N*-benzoylproline, indicating that proline (presumably as its L isomer) is the precursor of the dehydroproline moiety of the antibiotic. The activity in virginiamycin $M_1$ that was not found in the dehydroproline moiety presumably arose by catabolism of proline (possibly via glycine[25]) and subsequent incorporation into the antibiotic.

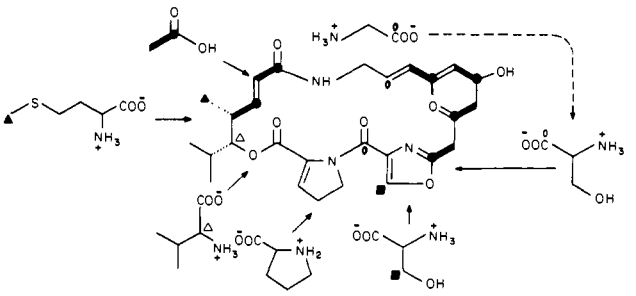

**Figure 5.** Biogenetic origin of the major units of virginiamycin $M_1$.

**Incorporation of [U-$^{13}$C]Glucose.** Glucose metabolism by the glycolytic pathway provides the major source of acetyl-CoA for incorporation into polyketides, but it also provides pathways to valine and serine via pyruvate and 3-phosphoglycerate, respectively. Since both valine and serine are precursors to portions of virginiamycin $M_1$, it was of interest to determine whether glucose metabolism provided a significant source of these amino acids under the conditions of antibiotic biosynthesis. In the event, incorporation of [U-$^{13}$C]glucose into virginiamycin $M_1$ proceeded with enrichment only of the carbons labeled by [1,2-$^{13}C_2$]acetate. Incorporation into the carbons labeled by valine and serine was not detectable at the rather low enrichment levels observed (about 0.2%), indicating that the route from glucose to acetyl-CoA constitutes the major pathway for glucose incorporation into virginiamycin $M_1$.

## Conclusions

The major pathways from simple precursors to virginiamycin $M_1$ have been elucidated by this work and are summarized in Figure 5. The incorporation of serine into the oxazole ring system represents the first rigorously confirmed demonstration of the formation of an oxazole ring from a $\beta$-hydroxy amino acid. The formation of the C-33 methyl group from acetate represents a new mechanism for biosynthesis of C-methyl groups in secondary metabolites and is an alternate route to the pathways via propionate that have been established for other metabolites such as the macrolides.[32] It will be interesting to discover whether compounds such as brevetoxin[33] are formed by this new pathway from acetate.

## Experimental Section

**General.** Melting points were determined on a Kofler block and are uncorrected. $^{13}$C NMR spectra were taken on a JEOL FX-200 spectrometer at 46 kG. All spectra were proton noise decoupled (pnd) and were obtained at ambient temperature in 10-mm tubes. The typical pulse width was 7.5 $\mu$s. Thin-layer chromatography (TLC) was carried out on Merck silica gel PF254 adsorbent. HPLC was performed on an apparatus consisting of a Waters Associates M6000A pump, a Valco six-port injection valve, and a Pharmacia UV monitor operating at 254 nm. The columns used were EM Hibar columns packed with LiChrosorb RP-8, 250 × 4.6 mm (analytical) and 250 × 10 mm (preparative). Radioactive samples were counted in a Beckman LS 100 liquid scintillation counter in an emulsifier cocktail containing 0.3% PPO and 0.02% POPOP.[34] Evaporation in vacuo refers to solvent removal on a rotary evaporator at aspirator pressure and 25–45 °C.

$^{14}$C- and $^3$H-labeled compounds were obtained from ICN Chemical and Radioisotope Division and from Amersham Corp. $^{13}$C-Labeled compounds were obtained from KOR Isotopes. L-[$^{13}CH_3$]Methionine was prepared by the method of Mani et al.[35] Strains of *S. virginiae* PDT

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30 and 1830 and samples of crude mixed virginiamycins were generous gifts from Dr. C. J. DiCuollo of SmithKline Animal Health Products. Corn steep liquor was supplied by A. E. Staley Manufacturing Co., Decatur, IL, and peanut oil meal was obtained from Southern of Rocky Mount, NC.

**Culture Conditions.** *S. virginiae* was maintained on agar slants prepared from the extract of 200 g of boiled potatoes, glucose (10 g), agar (20 g), and water (to 1000 mL); the pH was adjusted to 7.4 with KOH before sterilization. Slants were incubated 6 days at 25–28 °C after inoculation. Seed cultures were prepared by inoculating 30 mL of medium, containing 2% corn-steep solids, 0.8% peanut oil cake, 5% glucose, 0.5% $CaCO_3$, and 0.001% $MnSO_4$, with spores from an agar slant. The culture, contained in a baffled 250-mL Erlenmeyer flask, was incubated at 25 °C on a Lab-Line Orbit Environ-shaker at 325 rpm for 48 h. Production medium, consisting of 2% corn-steep solids, 0.5% yeast autolyzate, 1% peanut oil cake, 0.5% $CaCO_3$, 1% linseed oil, 0.5% glucose, and 2.5% glycerol, was inoculated 3% v/v with vegetative inoculum from seed cultures and incubated at 20–22 °C for 48 h at 325 rpm. For $^{14}C$ feedings 180 mL of medium distributed between six 250-mL Erlenmeyer flasks was used, while for each $^{13}C$ feeding 750 mL of medium distributed between twenty-five Erlenmeyer flasks was used. Precursors were added in a sterile manner through disposable Millipore filters at the onset of virginiamycin $M_1$ production approximately 24 h after inoculation with seed culture.

**Isolation.** The cultures were filtered through Hyflo Super-Cel and the aqueous filtrate was extracted with hexane to remove oils. The residual aqueous layer was then extracted 3 times with ethyl acetate. Evaporation of the combined dried ethyl acetate extracts in vacuo yielded a residue which was dissolved in acetonitrile and purified by preparative HPLC, using the preparative column described, the solvent system $CH_3CN$–$H_2O$, 40:60, and a flow rate of 6.0 mL/min. The virginiamycin $M_1$ peak had a $k'$ value of 4.2 and an elution volume (peak maximum) of 66 mL. The isolated antibiotic was checked by analytical HPLC on the analytical column described with the same solvent system and a flow rate of 2.0 mL/min. The virginiamycin $M_1$ peak had a $k'$ value of 4.5 and an elution volume (peak maximum) of 11 mL, and the purity of the isolated antibiotic was routinely >99% based on HPLC.

**[2-$^{13}C$]Hippuric Acid.** [2-$^{13}C$]Glycine (266 mg) was dissolved in 0.5 mL of distilled water in a 10-mL Erlenmeyer flask equipped with a magnetic stirrer. Benzoyl chloride (1.0 mL) was then added in 0.1-mL portions alternately with a solution of sodium hydroxide (1 g) in distilled water (5.0 mL) over a period of 1 h in such a way that the solution's pH was maintained in the range 7–10 and its temperature in the range 23–25 °C. After addition of the benzoyl chloride was complete, stirring was continued for 1.5–2 h with occasional monitoring of temperature and pH to ensure that they remained within the specified limits. The reaction mixture, which was colorless or very slightly yellow, was then acidified with concentrated hydrochloric acid, yielding a precipitate of hippuric acid and benzoic acid. The reaction mixture was allowed to stand 1 day and was then treated with 5 mL of ice water and filtered. The residue was air-dried and washed with five 1-mL portions of ether to remove benzoic acid. It was then dissolved in a mixture of acetone and ethanol (1:1), and the solution was filtered into a 250-mL round-bottomed flask and evaporated to dryness in vacuo. The white product (574 mg, 90%) was homogeneous on TLC with ethanol as a solvent.

**4-Isopropylidene-2-phenyl-2-[4-$^{13}C$]oxazolin-5-one.** Dry [2-$^{13}C$]hippuric acid (574 mg) in a 250-mL round-bottomed flask equipped with a magnetic stirring bar was treated with 942 mg (3.58 equiv) of freshly fused sodium acetate and 85 mL of dry acetone. The suspension was stirred for 5 min at room temperature and then treated with acetic anhydride (0.78 mL, 2.9 equiv). The resulting suspension was then gently heated under reflux for 20 h in an apparatus equipped with a drying tube; an initial white voluminous suspension became pale yellow by the end of this time. The acetone was removed in vacuo, and the residue treated with 150 mL of ice water and allowed to stand for 20 min. The almost white solid was then removed by filtration through a fritted glass filter, washed with several small volumes of cold water, and allowed to air-dry. The solid from the filter, together with traces left behind in the flask, was then dissolved in methylene chloride (50 mL) and transferred to a separatory funnel. The solution was shaken vigorously with 10% aqueous sodium carbonate solution (20 mL), and the alkaline aqueous extract was washed with methylene chloride. The combined methylene chloride solutions were dried ($Na_2SO_4$), filtered, and evaporated to yield the oxazolinone as an off-white solid, mp 99–100 °C (534 mg, 83%). The product was homogeneous on TLC with hexane–ethanol, 9:1, as the solvent.

**DL-[2-$^{13}C$]Valine.** The oxazolinone (534 mg) was placed in a 100-mL round-bottomed flask, and red phosphorus (950 mg) and acetic anhydride (6.0 mL) were added. A total of 6.0 mL of 47% hydriodic acid was then slowly added, and the mixture was heated under reflux for 20 h. The cooled mixture was filtered through a fritted glass filter, and the flask and filter were washed with water and 70% ethanol. The combined filtrate and washings were then evaporated to dryness on a rotary evaporator, taking care to remove the last traces of acetic acid. The residue was dissolved in 70% ethanol (50 mL) and the solution allowed to stand overnight. A fine crystalline precipitate that formed was filtered off and washed with 70% ethanol, and the combined clear brown solution of filtrate and washings (75 mL) was passed slowly through a column prepared from 35 mL of Amberlite IR-120 ion-exchange resin in the acid form. The column was washed with about 200 mL of 70% ethanol until the eluate became colorless and then with 200 mL of distilled water. The product was eluted with 200 mL of 2 N ammonium hydroxide solution followed by 200 mL of wash water. Evaporation of the eluate to dryness yielded DL-[2-$^{13}C$]valine as a white powder, homogeneous on TLC with 80% ethanol as the solvent: yield 275 mg (88.5% from oxazolinone, 66% from glycine).

**Incorporation of L-[U-$^{14}C$]Proline.** *S. virginiae* strain 1830 was used to inoculate a vegetative medium as described earlier, and after incubation at 25–26 °C for 48 h the resulting inoculum was used to inoculate 300 mL of production medium distributed between ten 250-mL Erlenmeyer flasks. Incubation was carried out at 22–23 °C for 8 h, and each flask was then treated with 2 $\mu$Ci of [U-$^{14}C$]-L-proline (ICN lot number 914272). After a further 40-h incubation, the antibiotic was isolated and purified as described. The virginiamycin $M_1$ had a specific activity of $2.33 \times 10^4$ $\mu$Ci/mol.

A portion of the antibiotic (2.17 mg, $4.13 \times 10^{-6}$ mol) was subjected to hydrogenation over platinum oxide (25 °C, 7 h, atmospheric pressure), and the resulting hydrogenated material was hydrolyzed with 6 N hydrochloric acid (1 mL) in a sealed vial for 12 h at 105 °C. The hydrolysate was made 1 N in NaOH by the addition of solid NaOH (0.2, g) and ether (1 mL) and benzoyl chloride (2.5 $\mu$L, $7.15 \times 10^{-5}$ mol) added. The heterogeneous solution was stirred at 0 °C for 24 h, and the aqueous layer was separated and acidified with 6 N hydrochloric acid. The resulting solution was extracted 2 times with 1 mL of ethyl acetate; the ethyl acetate layers were combined and evaporated to dryness.

The ethyl acetate extract was purified by HPLC on a LiChrosorb RP-8 column with the solvent system $CH_3CN$–$H_2O$–formic acid, 22:77:1. The peak corresponding to *N*-benzoylproline was collected, and the amount of material was determined by HPLC comparison of an aliquot with a standard solution of *N*-benzoylproline. The specific activity of the *N*-benzoylproline was $9.87 \times 10^3$ $\mu$Ci/mol.

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**Registry No. I**, 21411-53-0; proline, 147-85-3; glycine, 56-40-6; serine, 56-45-1; valine, 72-18-4; methionine, 63-68-3; [2-$^{13}C$]hippuric acid, 83303-30-4; 4-isopropylidene-2-phenyl-2-[4-$^{13}C$]oxazolin-5-one, 86023-98-5; DL-[2-$^{13}C$]valine, 86116-63-4.

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