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BIOSYNTHESIS OF ANTIBIOTICS OF THE VIRGINIAMYCIN FAMILY, 6. BIOSYNTHESIS OF VIRGINIAMYCIN S_1[1]

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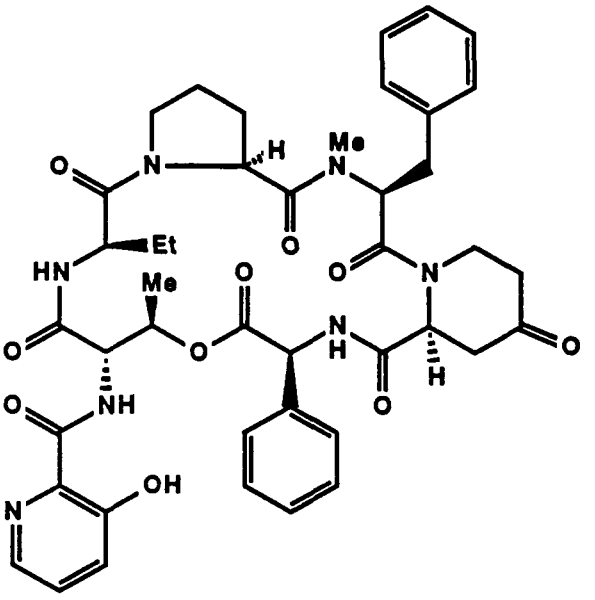
ABSTRACT.—The biosynthesis of virginiamycin S_1 [**1**] has been investigated by means of radiotracer techniques. As a part of this study, a new synthesis of (*R,S*)-4-oxopipecolic acid was devised, and a procedure for preparation and hplc analysis of amino acid *N*-benzoyl derivatives was developed. The antibiotic is shown to arise from the amino acids phenylalanine, threonine, methionine, lysine, and proline.

Virginiamycin S_1 [**1**] is a macrocyclic depsipeptide antibiotic produced by *Streptomyces virginiae* and a few other closely related *Streptomyces* species (2–4). It co-occurs with virginiamycin M_1, and mixtures of the two antibiotics show a marked synergism with respect to their activity against Gram-positive bacteria; this synergism is of considerable biochemical interest and has been the subject of extensive investigations (2). The virginiamycin antibiotics are extensively used in animal husbandry, both as growth promoters and for the treatment of certain specific infections, especially swine dysentery (2).

The structure of virginiamycin S_1 rests both on chemical degradations (4) and on an X-ray crystal structure (5), and its ^{1}H- and ^{13}C-nmr spectra have been assigned as part of a study of its conformation in solution (6). It is closely related to other macrocyclic peptidolactones of the virginiamycin family such as patricin B, mikamycin 1A, ostreogrycin B_1, and doricin; the structures and names of these antibiotics have been summarized by Crooy and DeNeys (7). Virginiamycin S_1 is also somewhat more distantly related to macrocyclic peptidolactones of other families such as pyridomycin (8) and viridogrisein



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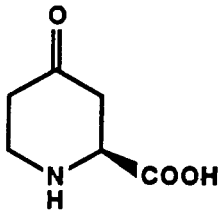
[1]For Part 5, see Reed and Kingston (1).

or etamycin (9,10). It contains the protein amino acids (*S*)-threonine and (*S*)-proline, but its major constituents are the less common amino acids (*R*)-2-aminobutanoic acid, (*S*)-*N*-methylphenylalanine, (*S*)-4-oxopipecolic acid [**2**], (*S*)-phenylglycine, and 3-hydroxypicolinic acid [**3**].



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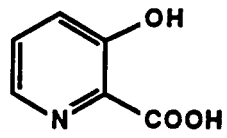

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The biosynthetic origins of some of these unusual amino acids have been investigated in other organisms. Thus L-α-phenylsarcosine [(*S*)-*N*-methylphenylglycine] has been shown to derive from phenylalanine in etamycin produced by *Streptomyces griseoviridus* (11), and 3-hydroxypicolinic acid has been shown to derive either from (*S*)-lysine in etamycin (10) or from three- and four-carbon precursors in pyridomycin (12). The origin of (*S*)-4-oxopipecolic acid has not previously been investigated, but several studies have shown that pipecolic acid is derived from lysine in such organisms as *Rattus* sp. (13–15), *Phaseolus* sp. (16,17), and *Neurospora crassa* (18,19) among others; a more complete list is given by Müller and Leistner (21). In all cases investigated except one (20), it was found that (*R*)-lysine served as the actual precursor to (*S*)-pipecolic acid (21–25). Other studies have indicated that 4-hydroxypipecolic acid is formed by hydroxylation of pipecolic acid (26–28). Finally, 2-aminobutanoic acid has been postulated to arise from transamination of 2-oxobutanoic acid derived from dehydration and deamination of threonine (29).

In connection with our studies on the biosynthesis of antibiotics of the virginiamycin family (1, 30–33), we have investigated the biosynthesis of virginiamycin S_1. In this paper we report the biosynthetic origin of each of the seven amino acid units of the antibiotic.

RESULTS AND DISCUSSION

Because virginiamycin S_1 is a macrocyclic peptidolactone, its individual amino acid components can be readily obtained by hydrolysis of the intact antibiotic. This fact indicated that the most efficient method of obtaining biosynthetic information would be by incorporation of radioactive potential precursors followed by hydrolysis of the labeled antibiotic, separation of the resulting amino acids, and counting of the isolated individual amino acids. The key feature of this scheme is clearly the separation of the individual amino acids of virginiamycin S_1.

Development of a separation procedure for the amino acids of virginiamycin S_1 required a supply of each amino acid. All of the amino acids except (*S*)-4-oxopipecolic acid [**2**] were available commercially. 4-Oxopipecolic acid itself has been prepared from 2-bromopyridine (27) and by hydrolysis of virginiamycin S_1 and ion-exchange separation of the resulting amino acid mixture (4). Although these procedures are adequate for the preparation of small quantities of **2**, we wished to devise a simpler preparation of this material.

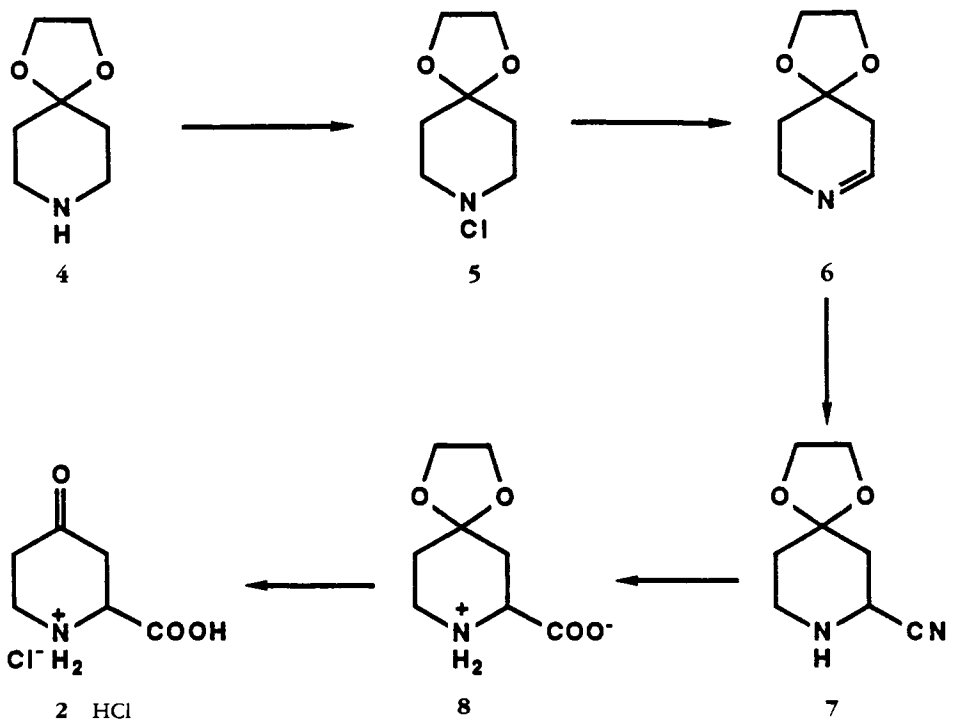
Treatment of the commercially available 4-piperidone ethylene ketal [**4**] with NCS followed by KO_2 and 18-crown-6 yielded the piperideine **6** via the *N*-chloro derivative **5** (34). Addition of HCN to **6** followed by hydrolysis of the nitrile under basic condi-

tions yielded the protected acid **8** in 35% overall yield. Removal of the ketal protecting group proved surprisingly difficult, but acid hydrolysis followed by ion-exchange chromatography yielded (*R,S*)-4-oxopipecolic acid [**2**] as the hydrochloride in modest but unoptimized yield (Scheme 1). The synthetic material had identical spectroscopic properties (^{1}H nmr) and tlc mobility with a sample obtained by hydrolysis of virginiamycin S_1. This procedure thus met our needs for 4-oxopipecolic acid [**2**]. [After the completion of this work, an improved synthesis of 4-oxopipecolic acid was published (35).]

The structural studies on virginiamycin S_1 were carried out by hydrolysis of the antibiotic and by ion-exchange separation of the resulting amino acids (4). However, this method is slow and somewhat tedious, and is not well suited to the small-scale work required for biosynthetic investigations with radioactive precursors. Although ion-exchange methods are routinely used for analysis of protein amino acids, the isolation and quantification of non-protein amino acids requires additional studies that are not always compatible with the routine use of commercial amino acid analyzers. For these reasons we elected to develop an hplc method for the separation of the amino acids derived from virginiamycin S_1. Such a separation demanded that the amino acids be derivatized with a suitable derivatizing reagent.

The selection of an appropriate derivative for hplc of the amino acids from virginiamycin S_1 is complicated by the fact that three of them are secondary amino acids and thus will not form derivatives with *o*-phthalaldehyde (36,37). After investigating dansyl derivatives (38,39), phenylthiohydantoin derivatives (40–44) and *N*-2,4-dinitrophenyl derivatives, we selected *N*-benzoyl derivatives for a detailed investigation.

All of the amino acids derived from virginiamycin S_1 except 3-hydroxypicolinic acid readily formed *N*-benzoyl derivatives when reacted with benzoyl chloride under Schotten-Baumann conditions, although the derivative from 4-oxopipecolic acid was



SCHEME 1

only obtained in reduced yield as an oil, presumably because of competing reactions of the ketone part of the molecule under the basic conditions of the reaction. The failure of 3-hydroxypicolinic acid to form a derivative was expected, since any *O*-benzoyl derivative formed would be hydrolyzed under the reaction conditions. This was acceptable, because 3-hydroxypicolinic acid could be detected on hplc by its uv absorption or by its fluorescence, and because it could be separated chemically from the *N*-benzoyl derivatives prior to chromatography.

The separation of the six *N*-benzoylamino acids on one hplc system proved to be a challenging task. A number of normal and reversed-phase systems were tried, and various ion-pairing systems were also used, but no simple isocratic or gradient solvent sys-

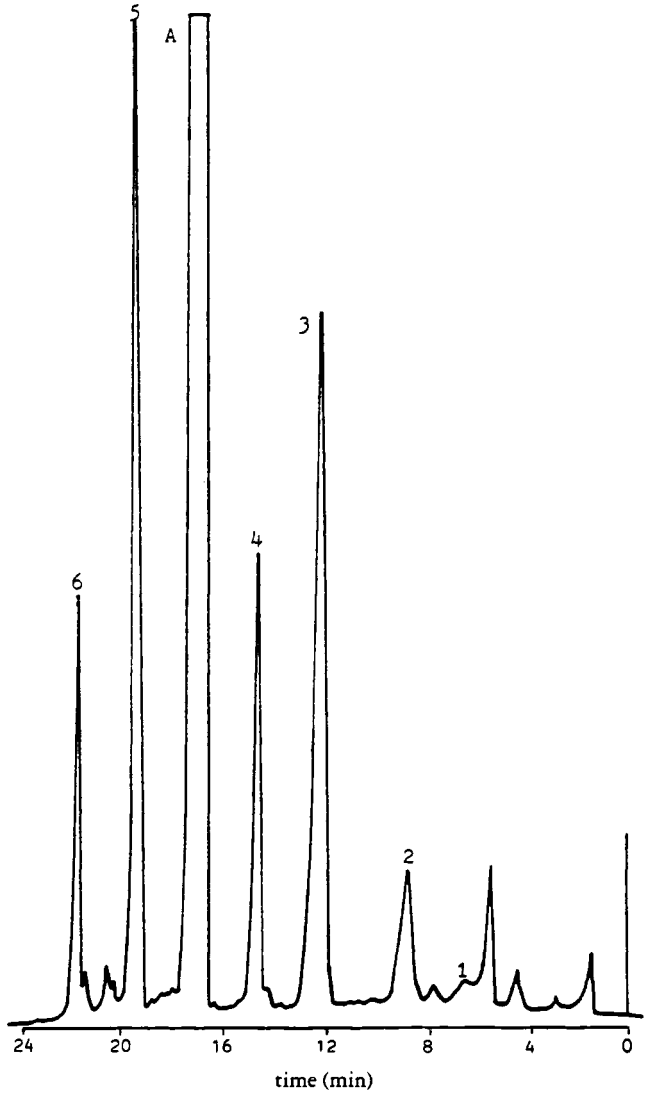


FIGURE 1. Separation of *N*-benzoyl derivatives of amino acids derived from virginiamycin S_1; for conditions see Experimental section. 1 = (*S*)-4-oxopipecolic acid; 2 = (*S*)-threonine; 3 = (*S*)-proline; 4 = (*R*)-2-aminobutanoic acid; 5 = (*S*)-phenylglycine; 6 = (*S*)-*N*-methylphenylalanine; A = benzoic acid. The *N*-benzoyl derivative of the ethylene ketal of (*S*)-4-oxopipecolic acid elutes between peaks 4 and A in this system.

tem gave satisfactory results. A solution was finally devised by means of a simplified version of the four-solvent optimization routine developed by Glajch *et al.* (45). In this system various mixtures of the three modifier solvents MeOH, MeCN, and THF in the base solvent of H_2O are evaluated for their chromatographic properties with a reversed-phase column, and the optimum solvent combination is developed by application of a modified simplex optimization procedure. The use of this approach yielded an effective separation of five of the six *N*-benzoylamino acids with the system described in the Experimental section; a typical chromatogram with this system is shown in Figure 1. In our system MeCN is unnecessary, and HCO_2H is added to control the pH and suppress ionization.

The one amino acid that is not clearly resolved by this system is the *N*-benzoyl derivative of 4-oxopipecolic acid, a problem that was exacerbated by the poor yield of derivative obtained from this acid. Our synthetic approach to 4-oxopipecolic acid provided the solution to this problem, however, when it was realized that this acid readily forms its ethylene ketal derivative **8** in the presence of aqueous acid and ethylene glycol. The unusual stability of the ketal is presumably associated with the positive charge on the ring in the protonated or even in the neutral zwitterionic form of the acid. In any event, treatment of the acid hydrolysate of virginiamycin S_1 with ethylene glycol and concentration of the resulting solution, followed by *N*-benzoylation as before, yielded the *N*-benzoyl derivative of the ketal amino acid **8**, and this derivative was well separated from the other *N*-benzoyl derivatives by our hplc system.

With an appropriate analytical system in hand, we then turned our attention to the possible biosynthetic precursors of the various amino acid units of virginiamycin S_1. *S. virginiae* strain 1830 was used in this work; it produced 6–20 mg of virginiamycin S_1 per liter when grown on a complex medium at 21–23°. We tested a number of possible precursors to virginiamycin S_1 in this system, and the results from this work are shown in Tables 1 and 2.

TABLE 1. Incorporation of radioactivity into virginiamycin S_1 [**1**] in cultures of *Streptomyces virginiae* administered labeled substrates.

Precursor		Virginiamycin S_1		
Compound administered	Ci/mol	mg/liter	Ci/mol	Spec. inc.[a]
(*S*)-[U-^{14}C]phenylalanine . . .	450	15.4	0.086	0.019
(*S*)-[U-^{14}C]threonine	200	19.5	0.036	0.018
(*S*)-[Me-^{14}C]methionine	46	5.9	0.011	0.024
(*S*)-[U-^{14}C]lysine	300	6.5	0.020	0.0067
(*S*)-[U-^{14}C]proline	240	5.8	0.014	0.0059
(*S*)-[U-^{14}C]alanine	174	13.3	0.003	0.0016
(*S*)-[U-^{14}C]aspartic acid	184	3.6	0.0011	0.00059

[a]Specific incorporation = specific activity of virginiamycin $S_1 \times 100$/specific activity of precursor.

Inspection of Table 1 indicates that the specific incorporations of all the precursors tested are very low. This result contrasts with the finding in the case of etamycin (11), where the specific incorporations were excellent, and can be attributed to the fact that etamycin was grown in a defined medium with glucose and potassium nitrate as carbon and nitrogen sources, whereas virginiamycin S_1 was necessarily grown in a complex medium, leading to dilution of the radioactive precursors. In spite of this somewhat disappointing result, it was nevertheless possible to compare the relative incorporations

TABLE 2. Distribution of radioactivity in the hydrolysis products of virginiamycin S_1 [1] isolated from cultures of *Streptomyces virginiae* administered labeled substrates.[a,b]

Compound administered	Thr	AmBu	Pro	*N*-MePhe	4-Oxpip	Phgly	3-Hypic
(*S*)-[U-^{14}C]phenylalanine	0.2	0.1	0.0	44.9	0.5	39.7	0.3[c]
(*S*)-[U-^{14}C]threonine	96.2	14.5	0.2	0.2	1.4	0.7	1.1[c]
(*S*)-[Me-^{14}C]methionine	2.3	0.6	0.2	88.4	1.7[c]	0.2	1.9[c]
(*S*)-[U-^{14}C]lysine	2.4	0.6	0.4	0.6	13.7	0.0	50.7[c]
(*S*)-[U-^{14}C]proline	1.8	1.0	69.1	0.6	1.4	0.4	5.2[c]
(*S*)-[U-^{14}C]alanine	14.3	0.0	5.8	8.1	0.0	7.9	n.d.[d]
(*S*)-[U-^{14}C]aspartic acid	25.2	3.1	16.4	0.4	9.1	0.4	43.7[c]

[a]Expressed as percentage of the specific activity of the original antibiotic in each amino acid; numbers do not add to 100% because of errors in determination of the specific activity of some samples. Thr = (*S*)-threonine; AmBu = (*R*)-2-aminobutanoic acid; Pro = (*S*)-proline; *N*-MePhe = (*S*)-methylphenylalanine; 4-Oxpip = (*S*)-4-oxopipecolic acid; Phgly = (*S*)-phenylglycine; 3-Hypic = 3-hydroxypicolinic acid.

[b]Analysis carried out on *N*-benzoylamino acids unless otherwise stated.

[c]Analysis carried out on free amino acid, isolated as described in the Experimental section.

[d]n.d. = not determined.

of the various precursors, and it is noteworthy that the first five amino acids in Table 1 are incorporated significantly better than alanine and aspartic acid. When this evidence is taken in conjunction with the fact that these latter two amino acids give rise to a fairly randomly labeled antibiotic (Table 2), we can conclude that (*S*)-alanine and (*S*)-aspartic acid merely serve as general precursors through involvement in amino acid metabolism.

The remaining amino acids tested are all incorporated to a greater extent, and the distribution of their label within virginiamycin S_1 (Table 2) can largely be rationalized on the basis of known metabolic pathways for amino acids. Thus radioactivity from (*S*)-[U-^{14}C]phenylalanine appeared either in (*S*)-*N*-methylphenylalanine or in (*S*)-phenylglycine. These results are expected based on our study of the incorporation of ^{13}C-labeled phenylalanine and its conversion to phenylglycine in virginiamycin S_1 (1). Other workers have also demonstrated the conversion of phenylalanine derivatives to phenylglycine derivatives (11, 46–48).

A similar result was obtained from the study with (*S*)-[U-^{14}C]threonine, which was incorporated largely into (*S*)-threonine and to a lesser extent into (*R*)-2-aminobutanoic acid. A pathway from threonine to 2-aminobutanoic acid via 2-oxobutanoic acid has been proposed (29), and this appears to be the major pathway leading to (*R*)-2-aminobutanoic acid in *S. virginiae*, although the involvement of peptide-bound dehydrobutyrine is not excluded.

Incorporation of (*S*)-[methyl-^{14}C]methionine gave the expected labeling of (*S*)-*N*-methylphenylalanine, with no significant incorporation into any of the other amino acids. Also as expected, (*S*)-[U-^{14}C]proline was incorporated specifically into the proline residue of virginiamycin S_1.

The origin of the (*S*)-4-oxopipecolic acid unit was of considerable interest, since it is unique to this class of antibiotic. As noted previously, pipecolic acid itself arises from lysine, and 4-hydroxypipecolic acid [which replaces 4-oxopipecolic acid in virginiamycin S_2 (49) and S_5 (50)] is formed by hydroxylation of pipecolic acid. However, a pathway involving condensation of aspartic acid with a two-carbon unit would also yield 4-oxopipecolic acid; such a pathway has been observed in the biosynthesis of the pyridine derivative 3-hydroxypicolinic acid in the antibiotic pyridomycin (12).

In the event, incorporation of (*S*)-[U-^{14}C]aspartic acid into virginiamycin S_1 proceeded only to a limited extent, and this limited incorporation was distributed over several amino acid residues. Incorporation of (*S*)-[U-^{14}C]lysine, on the other hand, proceeded more efficiently, and the label was located primarily in the 4-oxopipecolic acid

and 3-hydroxypicolinic acid residues. We may thus conclude that 4-oxopipecolic acid is formed from lysine. Our results do not distinguish between (*S*)-lysine and (*R*)-lysine as the precursor, since they are interconvertible (25), but it is probable that (*R*)-lysine serves as the actual precursor by analogy with previous work (21–25). The slight incorporation of aspartic acid observed can best be explained by its conversion to lysine via the diaminopimelate pathway (51).

The final amino acid of interest is the nonprotein acid 3-hydroxypicolinic acid. The biosynthesis of this amino acid has been studied previously in the antibiotics pyridomycin (12) and etamycin (11). As noted above, in pyridomycin it is formed from aspartic acid and glycerol or pyruvate, while in etamycin it derives from (*S*)-lysine (11). Further studies have suggested that 5-hydroxylysine and 5-hydroxypipecolic acid are also precursors (52). In virginiamycin S_1, 3-hydroxypicolinic acid appears to be formed essentially exclusively from lysine; as noted previously, the small incorporation observed from aspartic acid may be explained by the conversion of aspartic acid into lysine, and our results do not distinguish between (*R*)- and (*S*)-lysine as the precursor.

The results described above thus show that virginiamycin S_1 is formed from the amino acids phenylalanine, threonine, methionine, lysine, and proline, with both phenylalanine and lysine being the sources of two different amino acid units of the antibiotic.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Incubations were carried out on a Lab Line Orbit Environ-Shaker. Hplc was carried out on an apparatus consisting of a Waters Associates M6000A pump, a Tracor Model 980A solvent programmer, a Valco six-port injection valve, and a Waters 440 absorbance detector operating at 254 nm or a Waters 420-C fluorescence detector. Columns used were 4.6 × 25 cm (analytical) or 10 mm × 25 cm (preparative), with packing materials as specified. Radioactivity was measured by liquid scintillation on a Beckman LS-100 liquid scintillation counter with a toluene-based scintillation cocktail (53). All radioactive chemicals were purchased from ICN Chemical and Radioisotope Division, New England Nuclear, or Amersham.

2-CYANO-4-PIPERIDONE ETHYLENE KETAL [**7**].—Piperideine **6**, prepared from 4-piperidone ethylene ketal [**4**] (5.15 g) as previously described (34), was added in 200 ml 0.5 N HCl to a solution of KCN (11.4 g, 0.175 mmol) in 100 ml of H_2O at 0° and pH 7 over 0.5 h. The reaction mixture was then adjusted to pH 5 with KOH pellets, and stirring was continued for 7 h, with warming to room temperature. The mixture was cooled in an ice-H_2O bath, and KOH pellets were added to adjust the solution to pH 9. The mixture was extracted with EtOAc (3 × 200 ml). The combined extracts were dried over $MgSO_4$. Evaporation yielded a pale yellow oil, which after flash chromatography [$CHCl_3$-MeOH (9:1)] afforded 2.75 g of product (47% overall yield). ^{1}H nmr ($CDCl_3$) δ 1.72 (t, 2H, J = 5.6 Hz), 1.78 (br s, 1H), 1.96 (m, 2H), 2.93 (m, 2H), 3.16 (m, 1H), 4.01 (m, 5H); ^{13}C nmr (DMSO) δ 34.9, 37.1, 41.4, 44.4, 63.6, 63.8, 105.4, 120.6; ir 2220 cm^{-1}; ms *m/z* (rel. int.) $[M]^+$ 168 (63), 141 (21), 126 (10), 99 (32), 96 (8), 87 (34), 86 (100).

(*R,S*)-4-OXOPIPECOLIC ACID ETHYLENE KETAL [**8**].—A mixture of **7** (0.166 g, 0.99 mmol), $Ba(OH)_2 \cdot 8H_2O$ (0.176 g, 0.56 mmol), and H_2O (2 ml) was heated at 95° for 2.5 h. After the addition of more H_2O (2 ml), dry ice was added to the reaction mixture, with the temperature maintained at 90–95°. The mixture was stirred for 15 min, then filtered. H_2O (2 ml) was added to the residue, which was heated for 10 min, then filtered. The combined filtrates were heated with Norite and filtered. The filtrate was evaporated to give a colorless crystalline residue (0.14 g, 74%). The product was recrystallized from iPrOH with a trace of H_2O and Et_2O. Mp 246°; ^{1}H nmr (D_2O) δ 1.78–1.64 (m, 3H), 2.09 (m, 1H), 2.93 (m, 1H), 3.29 (dt, 1H), 3.56 (dd, 1H), 3.84 (m, 4H); ^{13}C nmr (D_2O) δ 30.7, 34.8, 41.1, 57.3, 64.6, 104.9, 172.8; ir (KBr) 1600 cm^{-1}.

(*R,S*)-4-OXOPIPECOLIC ACID [**2**] HYDROCHLORIDE.—(*R,S*)-4-Oxopipecolic acid ethylene ketal hydrochloride (0.16 g, 0.72 mmol) was heated in 5 ml of 2 N HCl at 85° for 15 h. The mixture was neutralized with 6 N NaOH, then placed on a Dowex 2 column (HO^-, 2 × 14 cm). The column was washed with H_2O (100 ml) to remove the sodium ions, then eluted with 1 N aqueous HOAc. The fractions containing 4-oxopipecolic acid [tlc, *n*-PrOH-concentrated NH_4OH (7:3)] were combined along with 1 ml of 6 N HCl and evaporated to dryness to afford 38 mg (30%) of yellowish product, which showed one spot on

tlc and co-chromatographed with authentic material (R_f = 0.4). ^{1}H nmr (D_2O) δ 1.68–1.88 (m, 3H), 2.21 (m, 1H), 3.00 (dt, 1H), 3.29 (dt, 1H), 3.89 (dd, 1H).

CULTURE CONDITIONS.—The organism used in this work, *S. virginiae* strain 1830, was obtained from SmithKline Animal Health Products, West Chester, Pennsylvania. It was maintained on soluble starch agar slants with this composition: soluble starch 10 g, $(NH_4)_2SO_4$ 2 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, NaCl 1 g, $CaCO_3$ 3 g, agar 20 g, H_2O to 1 liter. Newly inoculated slants were incubated at 28° for 7 days and stored at 4° until needed. A vegetative inoculum was prepared by transferring mycelium and spores from a slant to a 250-ml baffled Erlenmeyer flask containing 30 ml of vegetative medium. This medium was prepared by suspending corn steep solids (20 g) in H_2O (1000 ml) and adjusting the pH of the suspension to 7.5 with NaOH. Peanut oil cake (8 g) was then added, and the mixture was boiled for 2 min and filtered through cheesecloth. $CaCO_3$ (5 g), $MnSO_4$ (0.01 g), and glucose (50 g) were added and the mixture was sterilized. The vegetative inoculum was incubated at 25–26° on a rotary shaker at 330 rpm for 48 h, and 1-ml portions were then inoculated into 30-ml portions of fermentation medium in baffled 250-ml Erlenmeyer flasks. The medium was prepared by adding corn steep solids (20 g) and yeast autolysate (5 g) to H_2O (1000 ml), raising the pH to 7.5, and adding peanut oil cake (10 g). The mixture was boiled for 2 min, filtered through cheesecloth, treated with glycerol (25 g), glucose (5 g), linseed oil (10 g), and $CaCO_3$ (5 g), and finally sterilized. Incubation was carried out at 21–23° on a rotary shaker at 330 rpm for 48 h.

ISOLATION OF VIRGINIAMYCIN S_1 [**1**].—Culture filtrates (about 750 ml) were extracted twice with one-third volume of hexane and then three times with a half volume of EtOAc. The EtOAc extracts were combined, washed once with H_2O, dried (Na_2SO_4), and evaporated. The resulting residue was dissolved in 5 ml of MeCN and subjected to preparative hplc on a LiChrosorb RP-8 10 μm column (1.25 μm) with MeCN-H_2O (46:54) and a flow rate of 6 ml/min. Virginiamycin S_1 eluted at about 15.5 min under these conditions, after virginiamycin M_1. The isolated antibiotic had identical hplc properties with an authentic sample prepared by crystallization of chromatographically homogeneous material isolated from crude mixed virginiamycins.

RADIOTRACER EXPERIMENTS.—Cultures were prepared and grown as described above. Aliquots (0.5 ml) of a sterilized aqueous solution containing 2 μCi of the chosen precursor were added to each of 10 flasks 8 h after inoculation of the fermentation medium. The cultures were then incubated a further 40 h before harvesting and isolation of the virginiamycin S_1 as described above. A portion of the antibiotic was used to determine the specific incorporation of each precursor.

LOCATION OF RADIOACTIVITY IN VIRGINIAMYCIN S_1 [**1**].—The remaining antibiotic (90%) was hydrolyzed with 6 N HCl (1 ml) in a sealed vial at 110° for 24 h. After hydrolysis, the resulting solution was made 1 N in NaOH by the addition of solid NaOH (0.28 g) and then treated with a solution of benzoyl chloride (1.1 equivalents) in Et_2O (1 ml). The heterogeneous solution was stirred vigorously at room temperature for 24 h, and the aqueous layer was then removed with a pipet and acidified with 6 N HCl. The suspension was extracted with EtOAc (2 × 1 ml) and the extract evaporated.

The *N*-benzoylamino acids from the above procedure were subjected to hplc on an analytical LiChrosorb RP-8 10 μm column with a gradient solvent system. Solvent A consisted of H_2O-MeOH-THF-HCO_2H (85:11.5:2.5:1), and solvent B had the ratio 58:40:1:1. The gradient used, with a flow rate of 2 ml/min, was 100% A (4 min), 100% A to 100% B at 10%/min, followed by 100% B (10 min). The individual *N*-benzoylamino acids were collected, and the solutions were evaporated to dryness and then dissolved in MeOH (1 ml) and liquid scintillation cocktail (10 ml) for counting.

3-Hydroxypicolinic acid remained in the aqueous layer during this procedure. It was isolated by hplc of a portion of the aqueous layer on an analytical LiChrosorb RP-18 10 μm column, with elution with H_2O at 2 ml/min and detection by fluorescence; the amino acid eluted at 5.0 min under these conditions, and its solution was evaporated and subjected to counting as described above. An authentic sample of 3-hydroxypicolinic acid was prepared by diazotization of 3-aminopicolinic acid (54), itself prepared from quinoline imide (55) derived from quinolinic acid (56). (3-Hydroxypicolinic acid has since become available from Aldrich Chemical Company.)

(*S*)-*N*-BENZOYL-4-OXOPIPECOLIC ACID.—A sample of crude virginiamycin S_1 (2 g) in 6 N HCl (150 ml) was heated in a sealed container at 105° for 24 h. The solution was made 1 N in NaOH by the addition of 6 N NaOH and was then treated with benzoyl chloride (2.9 m) in Et_2O (100 ml) with vigorous stirring at room temperature for 36 h. The Et_2O layer was removed, the aqueous layer acidified, and the resulting suspension extracted with EtOAc (3 × 75 ml). The combined EtOAc fractions were evaporated and subjected to hplc on a preparative Partisil PAC column (1 × 50 cm) with elution by $CHCl_3$-iPrOH-HOAc (85:12:3). A total of 24 mg (4%) (*S*)-*N*-benzoyl-4-oxopipecolic acid was obtained as an oil which was shown to be >97% pure by analytical hplc; all attempts to induce the oil to crystallize failed. The hplc

characteristics of the *N*-benzoyl derivative were identical with those of a small sample prepared by benzoylation of (*S*)-4-oxopipecolic acid received from Dr. J.W. Clark-Lewis (57).

ISOLATION OF (*S*)-4-OXOPIPECOLIC ACID.—A sample of virginiamycin S_1 (4 mg) biosynthesized from L-[Me-^{14}C]methionine or L-[U-^{14}C]lysine was hydrolyzed as described previously. The evaporated crude hydrolysate was placed on a column of Dowex-50W ion-exchange resin, 400 mesh, 1×44 cm, and eluted with 1 N HCl with collection of 1-ml fractions. (*S*)-4-Oxopipecolic acid eluted in fractions 56–63, and it was shown to be homogeneous by tlc on Si gel with elution by H_2O–*n*-PrOH (30:70).

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