concentrated and chromatographed (silica gel, 10% ethyl acetate in hexanes followed by 10% methanol in ethyl acetate) to provide 1.224 g (58%) of the spiroketal **22** and 0.760 g (36%) of recovered pyrone **24**. The pyrone **24** was resubjected to trifluoroacetic acid in benzene as before to produce an additional 440 mg of spiroketal **22**, giving a total of 2.373 g (81%) of spiroketal **22** from the acetal **21**.

**8(*R*)-Ethyl-4(*S*)-hydroxy-1,7-dioxaspiro[5.5]undec-2-ene (25) and 8(*R*)-Ethyl-4(*R*)-hydroxy-1,7-dioxaspiro[5.5]undec-2-ene (26).** A solution of 85 mg (0.432 mmol) of spiroketal **22** and 177 mg (0.475 mmol) of $CeCl_3 \cdot 7H_2O$ in 15 mL of methanol was cooled to −78 °C and 18 mg (0.475 mmol) of sodium borohydride was added. The bath was removed and the reaction mixture was allowed to warm to room temperature. The reaction was quenched with aqueous $NH_4Cl$ and diluted with ether and the biphase was stirred for 16 h. The ether layer was then separated and dried over $MgSO_4$. Concentration gave a quantitative yield of a 2.4:1 mixture of two labile allylic alcohols **25**:**26**. Flash chromatography (ethyl acetate/hexanes, 1:9) gave 17.4 mg (20%) of the α-alcohol **26** and 40.6 mg (48%) of alcohol **25**. Allylic alcohol **25**: IR (film) 3380, 1655 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.88 (3 H, t, $J$ = 7.5 Hz, $CH_2CH_3$), 1.07–1.92 (8 H, band), 2.16 (1 H, ddd, $J$ = 1 Hz, 6 Hz, 12 Hz, $HCOHCH_{eq}H$), 3.36 (1 H, t, $J$ = 10.5 Hz, $OCH_2$), 3.61 (1 H, -OCH*H*, m), 4.49 (1 H, m, C*H*OH), 4.89 (1 H, ddd, $J$ = 1 Hz, 1 Hz, 6 Hz, CH=C*H*CHOH), 6.25 (1 H, dd, $J$ = 1 Hz, 6 Hz, -OC*H*=CH); $[\alpha]^{22}_D$ = −192.88° ($CHCl_3$, $c$ = 1.53). Anal. Calcd for $C_{11}H_{18}O_3$: C, 66.64; H, 9.15. Found: C, 66.20; H, 9.15.

Allylic alcohol **26**: IR (film) 3380, 1655 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.88 (3 H, t, $J$ = 7.5 Hz, $CH_2CH_3$), 1.16–1.83 (7 H, band), 1.90 (1 H, dd, $J$ = 10 Hz, 6 Hz, $HCOHCH_{eq}H$), 2.13 (1 H, ddd, $J$ = 2 Hz, 15 Hz, 2 Hz, $CHOHCH_2$), 3.37 (1 H, dd, $J$ = 12 Hz, 4 Hz, $OCH_2$), 3.61 (1 H, -OCH*H*, m), 3.95 (1 H, m, C*H*OH), 5.15 (1 H, ddd, $J$ = 1.5 Hz, 2 Hz, 6.5 Hz, CH=C*H*CHOH), 6.31 (1 H, d, $J$ = 6.5 Hz, -OC*H*=CH).

**Silyl Ether 27.** To a solution of 38.3 mg (0.192 mmol) of allylic alcohol **25** in 2 mL of dry DMF was added 19.6 mg (0.287 mmol) of imidazole and 40 μL (0.287 mmol) of triethylamine. After stirring for 5 min, 40 μL (0.287 mmol) of (bromomethyldimethyl)silyl chloride was added, and the reaction mixture was stirred for 3 h. The reaction was then quenched with saturated $NaHCO_3$ and diluted with ether. The ether layer was washed with brine, dried over $MgSO_4$, concentrated, and chromatographed (ethyl acetate/hexanes, 1:1) to yield 62.2 mg (93%) of silyl ether **27** as a labile colorless oil, which was homogeneous (≥95%) by $^1H$ NMR: $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.26 (2 H, AB, $J_{AB}$ = 10.5 Hz, $BrCH_2Si$), 0.29 (6 H, $Si(CH_3)_2$), 0.88 (3 H, t, 7.5 Hz, $CH_2CH_3$), 1.39–1.90 (7 H, band), 1.75 (1 H, dd, $J$ = 9 Hz, 12 Hz, $CH(OSiR_3)CH_2$), 2.07 (1 H, ddd, $J$ = 1 Hz, 6 Hz, 12 Hz, $CHOSiR_3CH_2$), 3.50 (2 H, m, $OCH_2$), 4.59 (1 H, m, C*H*OSi$R_3$), 4.80 (1 H, ddd, $J$ = 1 Hz, 1 Hz, 6 Hz, OCH=C*H*), 6.22 (1 H, dd, $J$ = 1 Hz, 6 Hz, OC*H*=CH).

**Talaromycin A (1).** A solution of 62.2 mg (0.178 mmol) of silyl ether **27** and 4 mL of degassed benzene was heated to reflux, whereupon a solution of 60 μL (0.213 mmol) of $Bu_3SnH$ and 8.6 mg (0.053 mmol) of AIBN in 1 mL of benzene was added by syringe pump over 2 h. The reaction was heated at reflux for an additional 5 h and then concentrated in vacuo to provide the crude silacycle **28**, which was not characterized and was used without further purification. The crude silacycle from above was added to a mixture of 0.2 mL of 30% $H_2O_2$, 30 mg of $Na_2CO_3$, 2 mL of methanol, and 2 mL of THF. The mixture was heated at reflux for 12 h, cooled to room temperature, diluted with water, 10% $NaHSO_3$, and 10% $NaHCO_3$. This mixture was then placed in a continuous extractor and extracted with ether for 4 h. Concentration of the ether layer and flash chromatography (ethyl acetate/hexanes 1:9; then 100% ethyl acetate) gave 34.1 mg (84%) of (−)-talaromycin A (**1**) which gave spectral data identical with that reported by Lynn:[2] IR (film) 3381 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 400 MHz) δ 0.92 (3 H, t, $J$ = 7.7 Hz, $CH_2CH_3$), 1.11–1.25 (2 H, m), 1.37–1.75 (5 H, band), 1.75 (1 H, B of ABX, $J$ = 13, 16.8 Hz, $-CH(OH)CH_{eq}CH_{ax}$), 1.93 (1 H, A of ABX, $J$ = 5, 13 Hz, $-CH(OH)CH_{eq}CH_{ax}$), 2.18 (1 H, m), 3.22 (1 H, B of ABX, $J$ = 10, 10 Hz, $-OCH_{eq}CH_{ax}$-CHEt), 3.55 (1 H, A of ABX, $J$ = 10, 5, 2 Hz, $-OCH_{eq}CH_{ax}CH$Et), 3.62 (1 H, B of ABX, $J$ = 11.8, 1.7, -OC*H*H-CH($CH_2OH$)-), 3.78 (1 H, A of ABX, $J$ = 11.8, 3.4, -OC*H*H-CH($CH_2OH$)-), 3.83 (1 H, B of ABX, $J$ = 5, 10 Hz, -C*H*HOH), 4.24 (1 H, A of ABX, $J$ = 8.4, 10 Hz, -C*H*HOH), 4.44 (1 H, ddd, $J$ = 11.8, 5, 5 Hz, C*H*OH); $[\alpha]^{22}_D$ = −105.7° ($c$ = 0.505, $CHCl_3$); lit.[10] $[\alpha]^{20}_D$ = −110.2° (c = 0.83, $CHCl_3$).

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**Registry No.** **1**, 83720-10-9; **2**, 112320-61-3; **9**, 118418-77-2; **10**, 118418-78-3; **11**, 118418-79-4; **12**, 118418-80-7; **13**, 118490-61-2; **14**, 118490-63-4; **15**, 89790-38-5; **16**, 89790-39-6; **17**, 111456-66-7; **18**, 118418-81-8; **19**, 118490-62-3; **20**, 118418-83-0; **21**, 118418-84-1; **22**, 118418-86-3; **23**, 118418-85-2; **24**, 118418-87-4; **25**, 118418-88-5; **26**, 118490-64-5; **27**, 118418-89-6; **28**, 118418-90-9; $BrCH_2SiMe_2Cl$, 16532-02-8; (*R*)-$HOCH_2CH(Et)CH_2CH_2COOH$, 118418-82-9; MeOCH=CHC≡CH, 2798-73-4.

# Biosynthesis of Antibiotics of the Virginiamycin Family. 7. Stereo- and Regiochemical Studies on the Formation of the 3-Hydroxypicolinic Acid and Pipecolic Acid Units[1]

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Details of the biosynthesis of two components of virginiamycin $S_1$ (**1**) derived from (*S*)-lysine have been studied in *Streptomyces virginiae* by incorporation of lysines labeled with stable isotopes. Both the (*S*)-4-oxopipecolic acid (**2**) and 3-hydroxypicolinic acid (**3**) portions incorporate (*RS*)-[6-$^{13}$C,6-$^{15}$N]lysine (**11**) with retention of the labeled nitrogen. Thus, the cyclization of lysine in both cases occurs with the loss of the α-nitrogen and retention of the ε-nitrogen. In addition, the 3-hydroxypicolinic acid unit incorporates deuterium from (2*RS*,5*R*)-[5-$^2$H]lysine (**20b**) but not from (2*RS*,5*S*)-[5-$^2$H]lysine (**20a**). The 5-*pro*-*R* hydrogen of lysine is thus retained in the biogenesis of 3-hydroxypicolinic acid.

In the previous paper in this series,[1] we described our studies on the basic biosynthetic pathways leading to the cyclic peptidolactone antibiotic virginiamycin $S_1$ (**1**). A key finding to emerge from this work was that the amino

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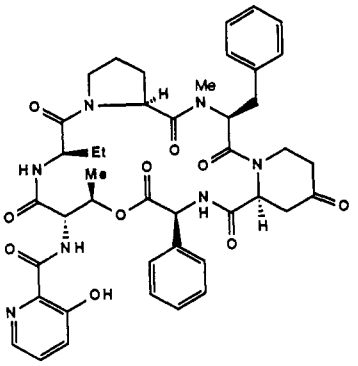

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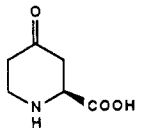
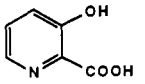
acids 4-oxopipecolic acid (**2**) and 3-hydroxypicolinic acid (**3**) were both derived from a lysine precursor. In this paper we report the results of our studies designed to elucidate further details of the pathways leading to both these amino acids.



2



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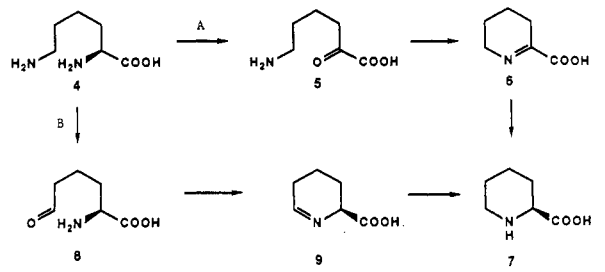
(*S*)-4-Oxopipecolic acid (**2**) is an unusual amino acid that to date has been detected only in virginiamycin $S_1$ and some related antibiotics.[4] The reduced amino acids 4-hydroxypipecolic acid and pipecolic acid are more common, however. (*S*)-Pipecolic acid is widely distributed in nature,[5] whereas (2*S*,4*S*)-4-hydroxypipecolic acid has been isolated from various plant species.[6]

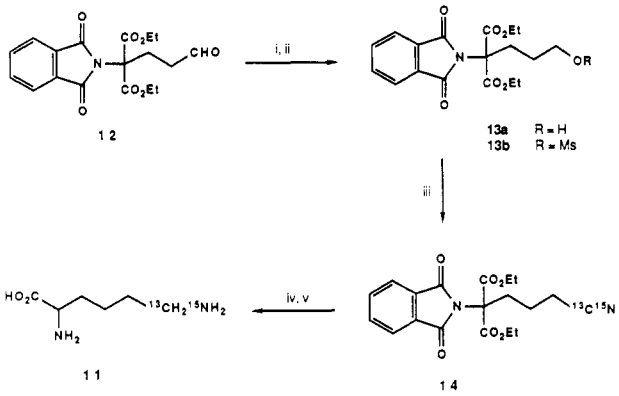
The formation of pipecolic acid in various organisms has been shown to occur from (*R*)-lysine with retention of the $\epsilon$-amino nitrogen atom;[7] a report that its formation in *Acacia* sp. occurs with loss of the $\epsilon$-amino nitrogen[8] has not been substantiated. These results indicate that cyclization occurs by pathway A of Scheme I (**4** → **5** → **6** → **7**) rather than by the theoretically possible pathway B (**4** → **8** → **9** → **7**). The biosynthesis of 4-hydroxypipecolic acid has been studied in *Acacia* sp.;[9] unlike 5-hydroxypipecolic acid, which arises from 5-hydroxylysine,[10] the 4-hydroxy acid is formed by hydroxylation of pipecolic acid. Indeed, 4-hydroxylysine is rarely observed in nature, occurring only as a component of antibiotics such as cerexin.[11]

The amino acid 3-hydroxypicolinic acid (**3**) is also an uncommon natural product, although picolinic acid and its isomer nicotinic acid are relatively common. The bio-

**Scheme I**



**Scheme II**[a]



[a] (i) 9-BBN; (ii) MsCl, $Et_3N$; (iii) $Na^{13}C^{15}N$, DMSO; (iv) $H_2$, $PtO_2$; (v) $H_3O^+$.

synthesis of 3-hydroxypicolinic acid has been studied in the antibiotics pyridomycin[12] and etamycin.[13] In pyridomycin **3** originates from (*S*)-aspartic acid and glycerol or pyruvate by a mechanism that could be similar to that for the biogenesis of picolinic acid. In etamycin, on the other hand, (*S*)-lysine is the precursor and (*S*)-aspartic acid is not incorporated to any significant extent. In a recent paper,[14] it was shown that 5-hydroxylysine (mixed isomers[15]) and 5-hydroxypipecolic acid (obtained from *Phoenix dactylifera* (dates) and thus of the 2*S*,5*R* configuration (**10**)[15]) both reduced the incorporation of (*S*)-[U-$^{14}$C]lysine into etamycin. This result suggests that both hydroxyamino acids serve as precursors of 3-hydroxypicolinic acid in this antibiotic.

This paper describes the results of experiments designed to confirm the regiochemistry of the cyclization of lysine to form 4-oxopipecolic acid and 3-hydroxypicolinic acid and to uncover the cryptic stereochemistry of the desaturation of lysine to 3-hydroxypicolinic acid.

## Results and Discussion

**Regiochemistry of Lysine Cyclization.** Our approach to this question was by the synthesis of (*RS*)-[6-$^{13}$C,6-$^{15}$N]lysine (**11**) and its incorporation into virginiamycin $S_1$. If cyclization of lysine to 4-oxopipecolic acid and/or 3-hydroxypicolinic acid occurs by a pathway analogous to pathway A (Scheme I), then both labeled atoms will be retained in the product amino acid. Cyclization by pathway B would of course result in loss of the $^{15}$N label but retention of the $^{13}$C label. The use of a doubly labeled precursor gave us the option of using either $^{13}$C NMR or mass spectrometry to determine the outcome of the in-

(1) For paper 6 in the series, see: Molinero, A. A.; Kingston, D. G. I.; Reed, J. W. *J. Nat. Prod.*, in press.
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**Table I. Relative Abundance for Ions for the Base Peak of Butyl *N*-(Trifluoroacetyl)-4-oxopipecolate from Virginiamycin $S_1$ Derived from (2*RS*)-[6-$^{13}$C,6-$^{15}$N]Lysine (11)**

| *m/z* | unlabeled standard | labeled sample | corrected labeled[a] | % |
|---|---|---|---|---|
| 194 | 100.0 | 100.0 | 100.0 | 93 |
| 195 | 10.5 | 11.5 | 1.0 | 1 |
| 196 | 1.0 | 7.2 | 6.2 | 6 |

[a] Calculated by subtracting the abundances of the ions at *m/z* 195 and 196 in the standard from those in the labeled sample.

**Table II. Relative Abundance of Ions for the Base Peak of Butyl 3-Hydroxypicolinate from Virginiamycin $S_1$ Derived from (2*RS*)-[6-$^{13}$C,6-$^{15}$N]Lysine (11)**

| *m/z* | unlabeled standard | labeled sample | corrected labeled[a] | % |
|---|---|---|---|---|
| 95 | 100 | 100 | 100 | 91 |
| 96 | 8.8 | 12.3 | 3.5 | 3 |
| 97 | 0.8 | 7.0 | 6.2 | 6 |

[a] See footnote *a*, Table I.

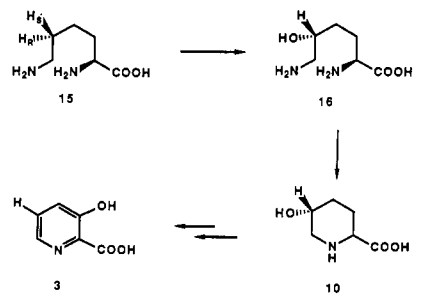
corporation and provided the added bonus of giving the potential for an independent confirmation of the incorporation of intact lysine into the antibiotic.

The labeled lysine 11 was prepared from the aldehyde 12 via the mesylate **13b** and the nitrile 14 in a pathway similar to that used by Townsend and his colleagues for the synthesis of labeled ornithine[16] (Scheme II). Incorporation of 11 into virginiamycin $S_1$ gave an antibiotic whose $^{13}$C NMR spectrum showed strongly enhanced signals at 36.8 ppm for the C-6 atom of 4-oxopipecolic acid and at 139.6 ppm due to the C-6 atom of 3-hydroxypicolinic acid.[17] The signal at 36.8 ppm appeared as a poorly resolved doublet ($^1J_{CN}$ = 8.7 Hz); the peak for unlabeled material was concealed under the center of the signal. This result clearly indicates that the $\epsilon$-amino nitrogen is retained in the cyclization of 4-oxopipecolic acid and thus that cyclization occurs by a type A pathway (Scheme I). This result was confirmed by GC/MS analysis of the *N*-trifluoroacetyl butyl esters of the amino acids obtained by acid hydrolysis of the labeled antibiotic. The data obtained (Table I) for the relative abundances of the $(M - COOC_4H_9)^+$ ion from the 4-oxopipecolic acid derivative show that this ion (and thus its precursor amino acid) consists of 1% singly labeled species and 6% doubly labeled species.

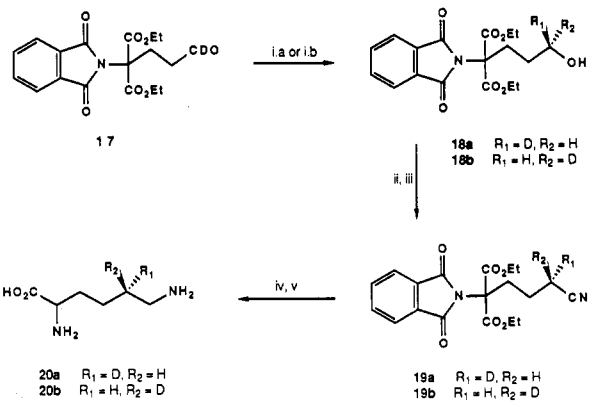
The peak at 139.6 ppm in the $^{13}$C NMR spectrum of the labeled antibiotic did not show any observable splitting. However, $^1J_{CN}$ values are very small in aromatic systems,[18] and thus this apparently negative result is not meaningful. Analysis of the GC/MS of butyl 3-hydroxypicolinate (Table II) did however indicate that *both* labeled atoms of 11 are retained in the 3-hydroxypicolinic acid unit. The retention of the $\epsilon$-amino nitrogen atom leads to the conclusion that this amino acid also is formed by a pathway of type A (Scheme I).

**Stereochemistry of Formation of 3-Hydroxypicolinic Acid.** If it is accepted that 5-hydroxylysine and (2*S*,5*R*)-5-hydroxypipecolic acid are indeed precursors of 3-hydroxypicolinic acid,[14,15] then it is possible to propose

**Scheme III**




**Scheme IV**[a]




[a] (i.a) (−)-$\alpha$-pinene-9-BBN; (i.b) (+)-$\alpha$-pinene-9-BBN; (ii) MsCl, $Et_3N$; (iii) KCN, DMSO; (iv) $H_2$, $PtO_2$; (v) $H_3O^+$.

**Table III. Relative Abundance of Ions for the Base Peak of Butyl 3-Hydroxypicolinate from Virginiamycin $S_1$ Labeled with (2*RS*,5*R*)-[5-$^2H_1$]Lysine and (2*RS*,5*S*)-[5-$^2H_1$]Lysine**

| precursor | *m/z* | unlabeled standard | labeled sample | corrected labeled[a] | % |
|---|---|---|---|---|---|
| 5*R* | 95 | 100 | 100 | 100 | 95 |
| | 96 | 8.8 | 14.1 | 5.3 | 5 |
| 5*S* | 95 | 100 | 100 | 100 | 100 |
| | 96 | 8.8 | 7.9 | (−0.9) | 0 |

[a] See footnote *a*, Table I.

the pathways of Scheme III for the formation of the latter acid. This scheme implies that lysine is hydroxylated to (2*S*,5*R*)-5-hydroxylysine (**16**), which then cyclizes by a type A pathway to (2*S*,5*R*)-5-hydroxypipecolic acid (**10**), and this in turn is converted to 3-hydroxypicolinic acid by steps that must include oxidation at C-3 and dehydration. Although the presence of a lysyl hydroxylase (protocollagen lysine dioxygenase) has not been established in *S. virginiae*, the related organism *S. griseoviridus* does contain a proline hydroxylase that is similar to that of animal origin,[15] and the presence of a proline hydroxylase in *S. virginiae* is implied by recent studies on the formation of the dehydroproline residue of virginiamycin $M_1$.[14] Since animals have both prolyl hydroxylase and lysyl hydroxylase enzymes, it is certainly possible that *S. virginiae* contains a lysyl hydroxylase.

If this logic is correct, then hydroxylation of lysine by a mechanism involving *retention* of stereochemistry would yield a 5-hydroxylysine and ultimately a 3-hydroxypicolinic acid in which the 5-*pro*-*S* proton is retained. Conversely, hydroxylation by a mechanism involving *inversion* of stereochemistry would lead to a 3-hydroxypicolinic acid in which the 5-*pro*-*R* proton is retained.

Stereospecifically labeled [5-$^2H_1$]lysines **18a** and **18b** were prepared by the pathway of Scheme IV. Incorporation of the labeled lysines into virginiamycin $S_1$ followed by hydrolysis of the isolated antibiotic and preparation of

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the *N*-trifluoroacetyl butyl esters of the resulting amino acids gave a mixture of amino acid esters that was analyzed by GC/MS. The results of the analysis are shown in Table III and indicate clearly that the deuterium atom from (5*R*)-[5-$^2H_1$]lysine (**20b**) is *retained* in the formation of 3-hydroxypicolinic acid, while that from the corresponding (5*S*)-[5-$^2H_1$]lysine (**20a**) is lost.

The implications of this finding for the overall mechanism of formation of 3-hydroxypicolinic acid follow from our earlier discussion. If the biosynthesis proceeds through the pathway of Scheme III, then hydroxylation of lysine must occur with *inversion* of stereochemistry at C-5 for the deuterium label from (5*R*)-[5-$^2H_1$]lysine (**10b**) to be retained in 3-hydroxypicolinic acid (**3**). The stereochemistry of the formation of 5-hydroxylysine has not been established, but the related hydroxylation of proline to 4-hydroxyproline is known to proceed with *retention* of stereochemistry at C-4.[20] If lysine hydroxylase has a similar stereoselectivity, as would be expected from its known similarity to proline hydroxylase,[21] then the stereochemistry observed in *S. virginiae* is the opposite of that predicted from Scheme III.

At the present time, therefore, all that can be said with certainty is that the formation of 3-hydroxypicolinic acid from lysine in *S. virginiae* is stereoselective and that it does involve retention of the 5*R* proton. The possible intermediacy of 5-hydroxylysine and 5-hydroxypipecolic acid and the stereochemistry of the hydroxylation of lysine if 5-hydroxylysine is an intermediate are questions that will require further work to resolve.

## Experimental Section

**General.** Melting points were determined on a Kofler block and were uncorrected.

For analytical thin-layer chromatography (TLC), Merck silica gel 60 F254 on aluminum, 0.2-mm thickness, was used. For preparative scale TLC, Analtech silica gel GF plates (500 μm) were used. For column chromatography (flash), Merck silica gel 60 (230–400 mesh) was used.

Nuclear magnetic resonance (NMR) spectra were determined on a Bruker WP-270 or a Bruker WP-200 spectrometer. Mass spectra were obtained on a Finnigan-MAT 112 mass spectrometer or a VG-Analytical 7070E mass spectrometer. The gas chromatographs used in GC/MS analysis were a Varian 2100 (coupled to the Finnigan instrument) or a Hewlett-Packard 5790A (coupled to the VG instrument). Infrared (IR) spectra were determined on a Perkin-Elmer 710B or a Perkin-Elmer 283B infrared spectrophotometer.

**4,4-Dicarbethoxy-4-phthalimidobutanal (12).** To a solution of diethyl phthalimidomalonate (6.11 g, 20 mmol) in benzene (30 mL) was added 0.057 g of sodium methoxide (1.1 mmol), and the resulting yellow solution was cooled to 0 °C in an ice–water bath. A solution of freshly distilled acrolein (1.5 mL, 22 mmol) in benzene (5 mL) was added dropwise over 0.5 h with stirring under an $N_2$ atmosphere. After addition was complete, stirring was continued for 1 h as the reaction mixture was allowed to warm to room temperature, and the reaction was quenched with 4 drops of acetic acid. Evaporation of the benzene left a straw-colored oil, which was purified by flash chromatography (ethyl acetate/hexane, 3:7) to yield the aldehyde **12** as a colorless oil: $R_f$ (ethyl acetate/hexane, 6:4) 0.31; $^1H$ NMR δ 1.29 (t, 6 H, $J$ = 7.1 Hz), 2.72 (m, 2 H), 2.86 (m, 2 H), 4.32 (q, 2 H, $J$ = 7.1 Hz), 4.33 (q, 2 H, $J$ = 7.1 Hz), 7.81 (m, 4 H), 9.71 (t, 1 H, $J$ = 0.9 Hz); $^{13}C$ NMR δ 13.6, 25.3, 39.0, 62.8, 66.8, 123.4, 134.4, 165.8, 167.2, 200.5.

**Ethyl 2-Carbethoxy-2-phthalimido-5-hydroxypentanoate (13a).** Sodium borohydride (0.470 g, 12.1 mmol) was added in portions over 2 h to aldehyde **12** (4.0 g, 12 mmol) in ether (30 mL) and water (5 mL) with vigorous stirring at room temperature. Addition of water (35 mL) followed by separation of the ether layer and extraction of the aqueous layer with additional ether yielded crude alcohol. Flash chromatography (ethyl acetate/hexane, 1:1) afforded the alcohol **13a** as a colorless oil (2.6 g, 60%): $R_f$ (ethyl acetate/hexane, 1:1) 0.5; $^1H$ NMR δ 1.24 (t, 6 H), 1.66 (m, 2 H), 2.55 (t, 2 H), 3.62 (t, 2 H), 4.25 (m, 4 H), 7.80 (m, 4 H); $^{13}C$ NMR δ 13.77, 27.6, 29.6, 62.3, 72.6, 65.2, 67.7, 123.4, 131.5, 134.3, 166.3, 167.3; IR 3400, 2981, 1778, 1769, 1715 $cm^{-1}$.

**Ethyl 2-Carbethoxy-2-phthalimido-5-[(methylsulfonyl)oxy]pentanoate (13b).** A solution of methanesulfonyl chloride (2.83 g, 24.8 mmol) in dichloromethane (20 mL) was added dropwise to a stirred solution of the alcohol **13a** (4.5 g, 12.4 mmol) in dichloromethane (50 mL) containing triethylamine (2.87 g, 24.8 mmol) at 0 °C. After complete addition, the mixture was stirred for 2 h at room temperature and then concentrated in vacuo. The yellowish residue was treated with ethyl acetate/hexane (6:4) to precipitate triethylammonium chloride, and the supernatant was filtered off, evaporated in vacuo, and purified by flash chromatography to give a colorless oil (4.84 g, 11 mmol, 89%): $R_f$ (ethyl acetate/hexane, 1:1) 0.75; $^1H$ NMR δ 1.25 (t, 6 H, $J$ = 7.1 Hz), 1.90 (m, 2 H), 2.59 (m, 2 H), 2.99 (s, 3 H), 4.25 (m, 6 H), 7.8 (m, 4 H); $^{13}C$ NMR δ 13.7, 24.2, 29.3, 37.2, 62.7, 67.1, 69.4, 123.5, 131.2, 134.4, 165.4, 167.2; IR 3020, 2980, 1741, 1360, 960, 920 $cm^{-1}$.

**Ethyl 2-Carbethoxy-2-phthalimido-5-[$^{13}C$,$^{15}N$]cyanopentanoate (14).** Mesylate **13b** (2.5 g, 5.68 mmol) in dry dimethyl sulfoxide (10 mL) was treated with NaCN (0.034 g, 0.67 mmol) and heated at 47 °C under argon. After 5 h a portion of a labeled sodium cyanide $Na^{13}C^{15}N$ (90% $^{13}C$, 99% $^{15}N$) (0.174 g, 3.41 mmol) was added, and a third portion (0.034 g, 0.67 mmol) after 15 h. After a final 3 h at 47 °C, the mixture was poured into water (10 mL) and extracted three times with ethyl acetate. The ethyl acetate extracts were washed with brine, dried ($Na_2SO_4$), filtered, and evaporated to yield an oil that was purified by chromatography (ethyl acetate/hexane, 4:6) to yield 0.802 g (2.15 mmol, 45% based on cyanide) of colorless crystals, mp 89 °C: $^1H$ NMR δ 1.30 (t, 6 H, $J$ = 7.1 Hz), 1.82 (m, 2 H), 2.38 (m, 2 H), 2.61 (m, 2 H), 4.30 (q, 4 H), $J$ = 7.1 Hz), 7.82 (m, 4 H); $^{13}C$ NMR δ 13.7, 16.6, 20.7, 32.2, 62.8, 67.0, 118.9 (d, $J_{CN}$ = 14.5 Hz), 123.5, 131.2, 134.4, 165.9, 167.2; the signal at 118.9 ppm was much more intense than the other signals; IR 3020, 2980, 2200, 1800, 1779, 1740, 1385 $cm^{-1}$. Elemental analysis was performed on an unlabeled sample prepared by a similar method except that potassium cyanide was used in excess in place of sodium cyanide. Anal. Calcd for $C_{19}H_{20}N_2O_6$: C, 61.29; H, 5.41; N, 7.52. Found: C, 61.49; H, 5.36; N, 7.56.

**(2*RS*)-[6-$^{13}C$,6-$^{15}N$]Lysine Dihydrochloride (11).** The nitrile **14** (0.80 g, 2.13 mmol) in 20 mL of glacial acetic acid/hydrochloric acid (1:1) containing platinum oxide (0.25 g, 1.1 mmol) was hydrogenated at 60 psi for 24 h. The platinum oxide was removed by filtration, the solvents were evaporated in vacuo, and the crude residue was hydrolyzed with 6 N hydrochloric acid at 115 °C for 15 h. The hydrolysate was treated with water and evaporated to dryness, and the residue was dissolved in water (20 mL), extracted with ether, and treated with activated carbon. After filtration and evaporation, the residue was recrystallized from ethanol to yield 0.15 g (0.83 mmol, 39%) of labeled lysine **11**. The isolated product gave one spot on TLC ($R_f$ 0.20, 1-propanol/$NH_4OH$, 7:3): $^1H$ NMR ($D_2O$, DSS) δ 1.45 (m, 2 H), 1.69 (m, 2 H), 1.90 (m, 2 H), 3.00 (dt, 1.5 H [$^{13}C$ labeled sample], $J$ = 106, 7.5 Hz), 3.01 (t, 0.5 H [unlabeled sample], $J$ = 7.5 Hz), 3.74 (t, 1 H, $J$ = 6.0 Hz); $^{13}C$ NMR ($D_2O$, $p$-dioxane = 66.5) δ 21.2, 25.9, 29.5, 38.9 (d), 54.4, 173.1; the peak at δ 38.9, corresponding to C-6,[22] was much more intense than the other signals.

**Ethyl [5-$^2H_1$]-2-Carbethoxy-2-phthalimido-5-hydroxypentanoate.** Sodium borodeuteride (0.767 g, 18.3 mmol) was added in small portions over 3 h to a vigorously stirred mixture of aldehyde **12** in ether (85 mL) and water (5 mL). The mixture was stirred for an additional 0.5 h, then for another 15 min following the addition of 35 mL of water. The aqueous layer was separated from the ether layer and subsequently extracted with additional ether (5 × 50 mL). The combined ether layers were

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dried over $Na_2SO_4$ and evaporated in vacuo. Flash chromatography (ethyl acetate/hexane, 1:1) afforded the labeled alcohol as a colorless oil (3.43 g, 53%): $^1H$ NMR ($CDCl_3$) $\delta$ 1.29 (t, 6 H, $J$ = 7.1 Hz), 1.69 (m, 2 H), 2.58 (m, 2 H), 3.66 (br m, 2 H), 4.30 (q, 2 H, $J$ = 7.1 Hz), 4.31 (q, 2 H, $J$ = 7.1 Hz), 7.81 (m, 4 H); $^{13}C$ NMR ($CDCl_3$) $\delta$ 13.77, 27.5, 29.5, 61.8 (t, $J$ = 21.4 Hz), 62.5, 67.6, 123.4, 131.3, 134.3, 166.2, 167.3.

**[1-$^2H_1$]-4,4-Dicarbethoxy-4-phthalimidobutanal (17).** To a mixture of pyridinium chlorochromate (7.30 g, 3.9 mmol) and sodium acetate (1.39 g, 16.9 mmol) in dry $CH_2Cl_2$ (45 mL) was added labeled alcohol in dry $CH_2Cl_2$. An additional 30 mL of $CH_2Cl_2$ was used to rinse the flask that had contained the alcohol. The dark brown mixture was stirred at room temperature under $N_2$ for 4 h. Ether (100 mL) was added to the mixture, and stirring was continued for 5 min. The mixture was filtered through a layer of Florisil (3.5 × 6.5 cm) topped with a 1-cm layer of Hyflo Supercel. Ether (100 mL) and $CH_2Cl_2$ (100 mL) were added to the residue remaining in the flask and filtered. The filtrate was evaporated to give a pale yellow oil, which was purified by flash chromatography (ethyl acetate/hexane, 3:7) to provide the aldehyde (4.33 g, 73%) as a colorless oil. $^1H$ NMR (identical with that of the unlabeled aldehyde) showed the aldehyde to be approximately 88–90% labeled. $^{13}C$ NMR ($CDCl_3$): $\delta$ 199.9 (t, $J$ = 26 Hz), otherwise identical with the unlabeled aldehyde.

**Ethyl (5*S*)-[5-$^2H_1$]-2-Carbethoxy-2-phthalimido-5-hydroxypentanoate (18b).** A solution of (+)-$\alpha$-pinene (3.69 g, 27.1 mmol)[23] in 49 mL of 0.5 M 9-BBN in THF (24.5 mmol) was heated at reflux with stirring under an $N_2$ atmosphere for 4 h.[24] The mixture was cooled and stored under $N_2$ overnight. Labeled aldehyde **17** was dissolved in 25 mL of dry THF and transferred by means of a cannula to the $\alpha$-pinene–9-BBN mixture. The reaction mixture was stirred under $N_2$ for 8 h; then it was placed on a silica gel column (5.5 × 15 cm) and eluted with ether. The fractions containing the alcohol were combined, and the solvent was removed in vacuo. The alcohol was purified further by flash chromatography (ethyl acetate/petroleum ether, 1:1 then 6:4). The chirally deuterated alcohol (3.78 g) was obtained in 84% yield: $^1H$ NMR ($CDCl_3$) $\delta$ 3.63 (br t, 1 H); $^{13}C$ NMR ($CDCl_3$) $\delta$ 61.8 (t, $J$ = 21.4 Hz). Other resonances were identical with those of the unlabeled alcohol.

**Ethyl (5*R*)-[5-$^2H_1$]-2-Carbethoxy-2-phthalimido-5-cyanopentanoate (19b).** The alcohol **18b** was converted to the mesylate as previously described. The mesylate was displaced with cyanide as previously described except that potassium cyanide was used in a 2-fold excess and added in one portion. The overall yield for both steps was 61%. The nitrile **19b** had mp 89 °C: $^1H$ NMR $\delta$ 1.30 (t, 6 H, $J$ = 7.1 Hz), 1.82 (m, 2 H), 2.38 (m, 1 H), 2.61 (m, 2 H), 4.31 (q, 2 H, $J$ = 7.1 Hz), 4.32 (q, 2 H, $J$ = 7.1 Hz), 7.82 (m, 4 H); mass spectrum (CI, isobutane), $m/z$ 374 ($(M + H)^+$, 100), 373 (15).

**(2*RS*,5*R*)-[5-$^2H_1$]Lysine Dihydrochloride (20b).** (*R*)-Nitrile **19b** (0.434 g, 1.16 mmol) in 5 mL of acetic acid and 2 mL of concentrated HCl was hydrogenated over $PtO_2$ (0.162 g, 0.71 mmol). After 5.5 h an additional portion of $PtO_2$ (0.162 g, 0.71 mmol) was added, and the mixture was stirred under $H_2$ for an additional 18 h. The catalyst was removed by filtration, and the filtrate was evaporated to give a yellow oil. Thin-layer chromatography showed no starting material to be present. The crude product was heated in 5 mL of 6 N HCl at 115 °C for 15 h. An equal portion of water was added, and the mixture was evaporated at reduced pressure. An additional portion of water was added and also evaporated to remove any excess acid. The solid residue was dissolved in 20 mL of water, and the mixture was extracted with ether (2 × 20 mL) to remove the phthalic acid and then treated with activated carbon. After evaporation of the water, the residue was recrystallized from ethanol–ether to yield 0.063 g (25%) of the labeled lysine dihydrochloride, which on TLC co-chromatographed with authentic lysine ($R_f$ = 0.20, 1-propanol/$NH_4OH$, 7:3); $^1H$ NMR ($D_2O$, DSS) $\delta$ 1.45 (m, 2 H), 1.69 (m, 1 H), 1.89 (m, 2 H), 3.00 (d, 2 H, $J$ = 7.5 Hz), 3.74, (t, 1 H, $J$ = 6.0 Hz); $^{13}C$ NMR ($D_2O$, $p$-dioxane = 66.5) $\delta$ 173.1, 54.4, 38.9, 29.5, (t, $J$ = 19.5 Hz), 21.2.

**(2*RS*,5*S*)-[5-$^2H_1$]Lysine Dihydrochloride (20a).** This amino acid was prepared from the aldehyde **17** as described above with the substitution of (−)-$\alpha$-pinene–9-BBN (81% optical purity[25]) in place of (+)-$\alpha$-pinene–9-BBN in the reduction step.

**Culture Conditions.** General conditions for the production of virginiamycin $S_1$ were as previously described.[26] *S. virginiae* 5722 was grown in a vegetative medium and transferred to a production medium (40 mL in each of 29 250-mL Erlenmeyer flasks) after 2 days. After 24 h, 200 $\mu$L of a solution of 65 mg of labeled lysine and 100 mg of L-threonine in 6 mL of distilled water, sterilized by passing through a Sartorius membrane (0.22 $\mu$m), was added to each flask.

After 3 days, the broth was acidified to pH 4.8 with 10% $H_2SO_4$ and then extracted three times with methyl isobutyl ketone. The organic extracts were combined and evaporated to dryness. The residue was dissolved in acetonitrile; the solution was washed twice with *n*-hexane and evaporated to dryness. The new residue was dissolved in 4 mL of chloroform. Crude virginiamycin (672 mg) was precipitated by the addition of 40 mL of *n*-hexane and then filtered. HPLC analysis indicated that the crude material contained approximately 156 mg of virginiamycin $M_2$, 80 mg of virginiamycin $M_1$, 16 mg of virginiamycin $M_3$,[27] and 41 mg of virginiamycin $S_1$.

Virginiamycin $S_1$ was isolated from the mixture as previously described.[26]

**Analysis of Amino Acids by GC/MS.** Virginiamycin $S_1$ to be analyzed by GC/MS was hydrolyzed in 1 mL of 6 N HCl at 105 °C for 24 h. *N*-Trifluoroacetyl amino acid butyl esters of the virginiamycin $S_1$ amino acids were prepared by evaporating the residue to dryness and dissolving it in dry 3–4 N HCl in 1-butanol (prepared either by bubbling dry HCl through dry 1-butanol or by adding acetyl chloride to 1-butanol) and heating the solution at 110 °C for 3 h (15 h in some cases). The excess reagent was evaporated under a stream of $N_2$; then 1 mL of dichloromethane and 1 mL of trifluoroacetic anhydride were added. The reaction mixture was allowed to stand at room temperature for 24 h and was then evaporated to dryness under a stream of $N_2$. The residue was dissolved in a minimal amount of dichloromethane for analysis by GC/MS.

Derivatives of unlabeled amino acids (25–40 mg of each amino acid of interest) for use as standards were prepared in the same way. The derivative of 3-hydroxypicolinic acid was prepared on a larger scale: 3-hydroxypicolinic acid (110 mg, 0.79 mmol) in 2 mL of 4 N HCl in dry 1-butanol was heated overnight at 110 °C. There appeared to be considerable starting material remaining at the end of this period. The mixture was evaporated to dryness, and the residue was dissolved in 2 mL of distilled water and brought to pH 6 with NaOH. The product (28 mg, 15% yield) was isolated by extraction of the aqueous mixture with an equal volume of ethyl acetate, evaporation to dryness, and then flash chromatography (5 g of silica gel; ethyl acetate/hexane, 6:4). Butyl 3-hydroxypicolinate: $^1H$ NMR ($CDCl_3$) $\delta$ 0.98 (t, 3 H, $J$ = 7.4 Hz), 1.48 (m, 2 H), 1.86 (m, 2 H), 4.78 (t, 2 H, $J$ = 6.9 Hz), 7.40 (m, 2 H), 8.30 (dd, 1 *H*, = 4.0 Hz, $J$ = 4.0 Hz), 10.80 (s, 1 H).

For the virginiamycin $S_1$ produced from a labeled lysine, a portion of the antibiotic was hydrolyzed and derivatized as described above. The separation of the proline and 3-hydroxypicolinic acid derivatives was not complete on our GC system, so a preliminary purification by preparative TLC (ethyl acetate/hexane, 1:1) was carried out. Analysis of the resulting mixture was performed by GC/MS on the VG7070E instrument. The isotopic composition of the major fragment ion of the derivative of interest was determined on an average of 4–10 spectra; the data are recorded in Tables I–III.

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