reduced in intensity and that the doublet of doublets at δ 1.97 and 1.58 (*pro-S* and *pro-R* H3) became simple doublets in the deuterated compound.

Incorporation of Deuterium at Position 3.[54] A solution of *N*-acetyl-D-neuraminic acid (0.50 g) in D_2O (10 mL) was adjusted to pD 11.6 (pH meter reading 11.2) by addition of 40% NaOD in D_2O. After 3.5 h at room temperature, the ^{1}NMR signal at δ 1.70 had disappeared, but the signal at δ 2.15 remained unchanged in intensity. Dowex 50x8-100 cation exchanger (H^+ form) was added, the suspension was stirred, the resin was filtered off and washed, and the filtrate and washings were lyophilized to yield a material which was used directly for production of methyl ([3-*pro-R*-^{2}H]-5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-nonulosyl chlorid)onate.

After 48 h at pD 11.6 both signals of the C3-bound protons of *N*-acetylneuraminic acid had disappeared, but no signs of decomposition were apparent, and the dideuterio compound was obtained on workup as above. The specifically *pro-S* deuterated compound was made by back-exchanging the dideuterated compound in water at pH 11.2 for 5 h. One proton (by NMR integration) could be incorporated without the *pro-S* proton signal becoming apparent.

Synthesis of Glycosides. Samples of *N*-acetylneuraminic acid were converted to the fully acetylated methyl ester by literature procedures[55] and thence to methyl 5-acetamido-3,5-dideoxy-β-*glycero*-*galacto*-nonulosyl chlorid)onate by reaction with acetyl chloride for 24 h at room temperature.[56] The glycosyl chlorides, used without purification, were converted to the various fully protected aryl neuraminides under the phase-transfer conditions of Rothermel and Faillard.[57] After chromatography on silica gel (ethyl acetate as eluant) fully protected glycosides were obtained in 50–60% yields from the chloride. Zemplén deacetylation[56] gave the aryl *N*-acetyl-α-neuraminide methyl esters (methyl(aryl 5-acetamido-3,5-dideoxy-α-*glycero*-*galacto*-2-nonulosid)onates), which were crystallized and characterized. Portions of the methyl esters were hydrolyzed[57] as required; the fully deprotected glycosides are unstable.

It was advantageous to keep all nitrophenyl glycosides in the dark.

Comparison of the 3-H proton resonances in unlabled *p*-nitrophenyl *N*-acetyl-α-neuraminide by ^{1}H NMR (400 Mz, CD_3OD; *pro-R* δ 1.89 (dd, apparent t, J = 12.0, 12.0 Hz), *pro-S* δ 2.97 (dd, J = 12.0, 3.0 Hz) with those of the deuterated analogues failed to reveal any back-exchange of deuterium under the alkaline conditions of the glycosidation reaction (0.1 M NaOH), in which production of glycoside by elimination to the acetylated 2,3-dehydroneuraminic acid methyl ester, followed by addition of *p*-nitrophenol, was a possibility.

Characterization Data of Samples of *p*-Nitrophenyl *N*-Acetyl-α-neuraminide. The compounds were repeatedly recrystallized from methanol/ether, between room temperature and −50 °C. Melting points are with decomposition (and hence particularly sensitive to trace impurities); rotations refer to 0.1% solutions in methanol: L, mp 134–136 °C, $[\alpha]^{25}_D$ −73.5°; D-[4-^{2}H], mp 134–136 °C, $[\alpha]^{25}_D$ +73.5°; D-[3-$^2H_2$], mp 134–136 °C, $[\alpha]^{25}_D$ +73.5°; D-[3-*pro-R*-^{2}H], mp 133.5–135.5 °C, $[\alpha]^{25}_D$ +74.5°; D-[3-*pro-S*-^{2}H], mp 134–137 °C, $[\alpha]^{25}_D$ +74.0°. Eschenfelder and Brossmer[46] report mp 113–115 °C, $[\alpha]^{25}_D$ +69° for the unlabeled compound in the D series.

Characterization Data of Methyl (Aryl 5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosid)onates. The methyl esters were recrystallized from ether/petroleum ether and the free acids from methanol/ether (neither with heating). Rotations refer to 0.1% solutions in methanol. *m-Nitrophenyl*: mp 101.1–102.4 °C; $[\alpha]^{25}_D$ +9.1°; MS $M^{\cdot+}$, 444. Anal. Calcd for $C_{26}H_{32}N_2O_{15}$: C, 50.98; H, 5.27. Found: C, 50.63; H, 5.19. Free acid mp 116–118 °C. *p-Cyanophenyl*: mp 105.4–106.4 °C, $[\alpha]^{25}_D$ +34.5°; MS $M^{\cdot+}$, 411. Anal. Calcd for $C_{27}H_{32}N_2O_{13}$: C, 53.87; H, 5.36. Found: C, 53.09; H, 5.46. Free acid mp 126–128 °C. 3,4-*Dichlorophenyl*: mp 102.4–103.9 °C, $[\alpha]^{25}_D$ +47°; MS $M^{\cdot+}$, 468. Anal. Calcd for $C_{26}H_{31}Cl_2N_2O_{13}$: C, 49.06; H, 4.91. Found: C, 48.99; H, 4.93. Free acid mp 134.5–136.5 °C. *m-Chlorophenyl*: mp 101.8–102.9 °C; $[\alpha]^{25}_D$ +7.5°; MS $M^{\cdot+}$, 433. Anal. Calcd for $C_{26}H_{32}ClNO_{13}$: C, 51.87; H, 5.36. Found: C, 51.78; H, 5.40. Free acid mp 114–115 °C. *p-Chlorophenyl*: mp 104.3–105.4 °C; $[\alpha]^{25}_D$ +54°; MS $M^{\cdot+}$, 433. Anal. Calcd for $C_{26}H_{32}ClNO_{13}$: C, 51.87; H, 5.36. Found: C, 51.65; H, 5.20. Free acid mp 124–126 °C. *Phenyl*: mp 104–107 °C (lit.[57] mp 102–106 °C); $[\alpha]^{25}_D$ +2.0°; MS $M^{\cdot+}$, 399. Anal. Calcd for $C_{26}H_{33}NO_{13}$: C, 55.02; H, 5.86. Found: C, 54.41; H, 5.88. Free acid mp 140–142 °C (lit.[55] mp 141–143 °C).

Acknowledgment. The support of this research by the National Institutes of Health (Grant GM 42469) is gratefully acknowledged.

(54) (a) Friebolin, H.; Schmidt, H.; Supp, M. *Tetrahedron Lett.* **1981**, *22*, 5171. (b) Roy, R.; Letellier, M.; Fenske, D.; Jarrell, H. C. *J. Chem. Soc., Chem. Commun.* **1990**, 378.

(55) (a) Paulsen, H.; Tietz, H. *Carbohydr. Res.* **1984**, *125*, 47. (b) Myers, R. W.; Lee, R. T.; Lee, Y. C.; Thomas, G. H.; Reynolds, L. W.; Jones, R. H. *Anal. Biochem.* **1980**, *101*, 166.

(56) Eschenfelder, V.; Brossmer, R. *Carbohydr. Res.* **1987**, *162*, 294.

(57) Rothermel, J.; Faillard, H. *Carbohydr. Res.* **1990**, *196*, 29.

(58) Venkatasubban, K. S.; Schowen, R. L. *CRC Crit. Rev. Biochem.* **1984**, *17*, 1.

(59) This effect was measured by direct comparison, rather than by the isotopic quasi-racemate method, which is precluded by the instability of the chiral products under the reaction conditions; it is therefore accurate to only about ±0.07.

(60) Elimination to give the 2,3-dehydroneuraminic acid derivatives is a common side reaction in glycosylations with protected *N*-acetylneuraminyl halides; for reviews, see: (a) Okatmoto, K.; Goto, T. *Tetrahedron* **1990**, *46*, 5835. (b) DeNinno, M. P. *Synthesis* **1991**, 583.

Biosynthesis of Acivicin. 3. Incorporation of Ornithine and N^δ-Hydroxyornithine[1]

Steven J. Gould* and Shyhchen Ju

Contribution from the Department of Chemistry, Oregon State University, Corvallis, Oregon 97331. *Received July 13, 1992*

Abstract: The biosynthesis of the antibiotics acivicin (**1**) and 4-hydroxyacivicin (**2**) has been studied in *Streptomyces sviceus*. Initial experiments identified ornithine (**11**) as the primary precursor, rather than glutamic acid or glutamine. Incorporation of [5-^{13}C,^{15}N]ornithine established the retention of the ω-amino nitrogen. Incorporation of [2,3,3-$^2H_3$]-, [3,3,4,4-$^2H_4$]-, [3*R*-^{2}H]-, and [3*S*-^{2}H]ornithines revealed the complete loss of H-2, replacement of the 3*S* hydrogen with the isoxazoline oxygen, and partial loss of the 3*R* hydrogen. Incorporation of $^{18}O_2$ revealed the derivation of the isoxazoline oxygen and the 4-hydroxyl group of **2** from molecular oxygen, although partial exchange of the former had occurred. N^δ-Hydroxyornithine (**18**), rather than either *erythro*- or *threo*-β-hydroxyornithine, was shown to be the first committed intermediate in the pathway. [^{14}C]-**1** was metabolized to [^{14}C]-**2**, but the reverse did not occur. A linear pathway from **11** through **18** to **1** and then to **2** is proposed to account for these findings.

Acivicin (AT-125) (**1**)[2,3] and 4-hydroxyacivicin (**2**),[4] antibiotics produced by *Streptomyces sviceus*, are members of a small group of metabolites containing an isoxazolidine ring at various levels of oxidation. Acivicin has potent anticancer activity[5] and has found

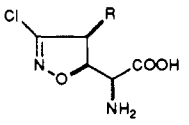
0002-7863/92/1514-10166$03.00/0

Table I. Summary of Feeding Experiments

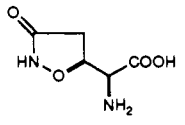
amino acid	DPM fed (×10⁶)	mg fed	mg produced	% inc (compd)	position: % enrich (compd)
DL-[1-^{14}C]glutamic acid	32.0	NA	NA	<0.001 (**1**) <0.001 (**2**)	NA
L-[1-^{14}C]glutamine	34.0	NA	NA	<0.02 (**1**) <0.02 (**2**)	NA
DL-[2-^{14}C]ornithine	20.8	NA	NA	0.46 (**1**) 2.28 (**2**)	NA
DL-[5-^{13}C,5-^{15}N]ornithine + DL-[5-^{15}C]ornithine	29.2	38.8	9 (**1a**) 29 (**2a**)	0.28 (**1a**) 2.64 (**2a**)	C-5: 1.26 (**1a**); J_{CN} = 2.7 Hz C-5: 4.16 (**2a**); J_{CN} = 2.7 Hz
DL-[2,3,3-$^2H_3$]ornithine[a] + DL-[5-^{14}C]ornithine	26.7	88.9	12 (**1b**) 27 (**2b**)	0.21 (**1b**) 1.30 (**2b**)	H-3[b] (**1b**) H-3: 1.0[c] (**2b**)
DL-[3*S*-^{2}H]ornithine[d] + DL-[5-^{14}C]ornithine	39.0	90.0	21 (**1c**) 62 (**2c**)	0.29 (**1c**) 1.32 (**2c**)	H-3[e] (**1c**) H-3: 0.14[f] (**2c**)
DL-[3*R*-^{2}H]ornithine[g] + DL-[5-^{14}C]ornithine	37.0	91.0	22 (**1d**) 73 (**2d**)	0.29 (**1d**) 1.49 (**2d**)	H-3[h] (**1d**) H-3: 1.0[i] (**2d**)
DL-[3,3,4,4-$^2H_4$]ornithine[j] + DL-[5-^{14}C]ornithine	70.1	75.0	15 (**1e**) 64 (**2e**)	0.47 (**1e**) 0.61 (**2e**)	H-3: 1.0;[k] H-4 1.1 (**1e**) H-3: 0.4;[l] H-4: 0.4 (**2e**)
DL-*N*⁶-hydroxy[3,3,4,4-$^2H_4$]ornithine[m]	NA	114.9	32 (**1f**) 88 (**2g**)	2.0[n] (**1f**) 3.9[n] (**2g**)	H-3: 4.3;[o] H-4: 5.8 (**1f**) H-3: 3.3;[p] H-4: 4.4 (**2g**)

[a] 100% ^{2}H at H-2, 76% ^{2}H at H-3. [b] Signal:noise too poor for accuracy; 1.6% expected [μmol produced − (μmol fed × % incorp × precursor enrichment)]. [c] 2.7% expected. [d] 85% 3*S*-^{2}H, 15% 3*R*-^{2}H. [e] Signal:noise too poor for accuracy; 0.15% from the 3*R* label expected. [f] 0.31% from the 3*R* label expected. [g] 70% 3-^{2}H, 30% 3*R*-^{2}H. [h] Signal:noise too poor for accuracy; 1.2% from the 3*R* label expected. [i] 2.1% from the 3*R* label expected. [j] 100% ^{2}H at H-3, 44% ^{2}H at H-4. [k] 2.5% expected. [l] 0.9% expected on the basis of one hydrogen replaced by oxygen and one hydrogen retained. [m] 100% ^{2}H at H-3, 10% ^{2}H at H-4. [n] Based on observed ^{2}H enrichment at H-4. [o] 6.1% expected on the basis of the H-4 enrichment. [p] Assuming the same 30% ^{2}H loss observed for **1f**.

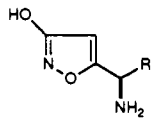
use as an important tool for studying xenobiotic metabolism involving glutathione.[6] Three subgroups can be readily discerned: **1**, **2**, tricholomic acid (**3**),[7,8] ibotenic acid (**4**),[9-11] and muscimol (**5**)[12] comprise one. D-Cycloserine (**6**)[13-15] and lactivicin (**7**)[16,17] comprise a second, while a series of isoxazolin-5-ones (**8**)[18] comprises a third subgroup.



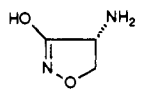
1, R = H
2, R = OH



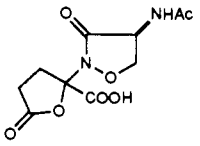
3



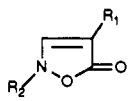
4, R = COOH
5, R = H



6

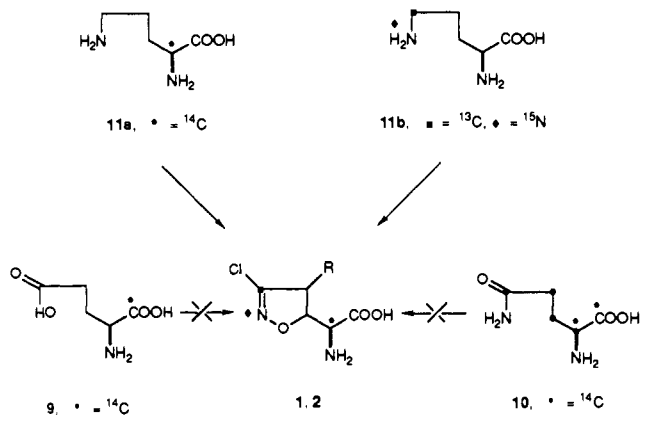


7



8

Scheme I



11a, • = ^{14}C

11b, ■ = ^{13}C, ♦ = ^{15}N

9, • = ^{14}C

1, 2

10, • = ^{14}C

Evidence was obtained[13] for serine as the source of the carbon skeleton in the biosynthesis of **6**. More recently[19-21] it has been demonstrated that in *S. garyphalus* **6** is derived from *N*-hydroxyurea (the source of the heterocyclic nitrogen and presumably of the oxygen, too) and *O*-acetyl-L-serine. Two serine racemases have been isolated from this organism.[20,21] The origins of the side chains (R_2) have been established[22] for two of the compounds **8**, but no work on the origin of the heterocycle has been reported.

Our interest in this group of metabolites first focused on the apparent oxidative chemistry at C-3 of the presumed amino acid precursor. Numerous examples of α-amino acids modified at C-3,[23-31] as well as of β-amino acids,[32-35] have been shown to be

(1) Preliminary accounts of portions of this work have appeared previously: (a) Ju, S.; Palaniswamy, V. A.; Yorgey, P.; Gould, S. J. *J. Am. Chem. Soc.* **1986**, *108*, 6429. (b) Gould, S. J.; Ju, S. *J. Am. Chem. Soc.* **1989**, *111*, 2329.
(2) Martin, D. G.; Duchamp, D. J.; Chidester, C. *Tetrahedron Lett.* **1973**, 2549.
(3) Hanka, L. J.; Gerpheide, P. R.; Martin, D. G.; Belter, P. A.; Coleman, T. A.; Meyer, H. F. *Antimicrob. Agents Chemother.* **1975**, *7*, 807.
(4) Martin, D. G.; Chidester, C. G.; Mizak, S. A.; Duchamp, D. J.; Baczynskyj, L.; Krueger, W. C.; Wunk, R. J.; Menlman, P. A. *J. Antibiot.* **1975**, *28*, 91.
(5) Martin, D. G.; Hanka, L. J.; Neil, G. L. *Cancer Chemother Rep., Part I* **1974**, *58*, 935.
(6) Shasteen, C. S.; Curthoys, N. P.; Reed, D. G. *Biochem. Biophys. Res. Commun.* **1983**, *112*, 564.
(7) Takemoto, T.; Nakajima, T. *J. Pharm. Soc. Jpn.* **1964**, *84*, 1183.
(8) Takemoto, T.; Nakajima, T. *J. Pharm. Soc. Jpn.* **1964**, *84*, 1230.
(9) Onda, M.; Fukushima, H. *Chem. Pharm. Bull.* **1964**, *12*, 751.
(10) Eugster, C. H.; Muller, C. F. R.; Good, R. *Tetrahedron Lett.* **1965**, 1813.
(11) Wieland, T. *Science* **1968**, *159*, 946.
(12) Bowden, K.; Drysdale, A. C. *Tetrahedron Lett.* **1965**, 727.
(13) Harned, R. L.; Hidy, P. H. *Antibiot. Chemother.* **1955**, *5*, 204.
(14) Cuckler, A. C.; Frost, B. M. *Antibiot. Chemother.* **1955**, *5*, 183.
(15) Stammer, C. H.; Wilson, A. N. *J. Am. Chem. Soc.* **1955**, *77*, 2344.
(16) Harada, S.; Tsubotani, S.; Hida, T.; Ono, H.; Okazaki, H. *Tetrahedron Lett.* **1986**, *27*, 6229.
(17) Harada, S.; Tsubotani, S.; Hida, T.; Koyama, K.; Kondo, M.; Ono, H. *Tetrahedron* **1988**, *44*, 6589.
(18) (a) Lambein, F.; Kuo, Y. H.; Parijs, R. V. *Heterocycles* **1976**, *4*, 567. (b) Ikegami, F.; Tajima, Y.; Ohmiya, S.; Lambein, F.; Murakoshi, I. *Chem. Pharm. Bull.* **1984**, *32*, 2450.
(19) Tanaka, N.; Saschikata, K. J. *J. Gen. Appl. Microbiol.* **1963**, *9*, 409.
(20) Svensson, M. L.; Gatenbeck, S. *Arch. Microbiol.* **1981**, *129*, 210.
(21) Svensson, M. L.; Gatenbeck, S. *Arch. Microbiol.* **1982**, *131*, 129.
(22) Murakoshi, I.; Kato, F.; Haginiwa, J.; Fowden, L. *Chem. Pharm. Bull.* **1973**, *21*, 918.
(23) McDonald, J. C.; Slater, G. P. *Can. J. Biochem.* **1975**, *53*, 475.
(24) Kirby, G. W.; Narayanaswami, S. *J. Chem. Soc., Perkin Trans. I* **1976**, 1564.
(25) Marchelli, R.; Dossena, A.; Casnati, G. *J. Chem. Soc., Chem. Commun.* **1975**, 779.

derived from common α-amino acids. In our program to further study this chemistry, we have investigated the pathway leading to **1** and **2**.[1]

Results and Discussion

Fermentation and Isolation. A freeze-dried culture of *S. sviceus*, obtained from The Upjohn Company, was initially transferred to agar slants and incubated until sporulation. These slants were then stored at 4 °C. However, antibiotic production in liquid culture was found to degenerate when broths were inoculated with spores stored in this manner. Stabilization was subsequently effected when the organism was maintained as spores on sterile soil. After an initial incubation at 26 °C for 2 weeks, such soil cultures could be stored for up to 5 years at 4 °C without losing potency.

Liquid seed cultures, inoculated with a loopful of soil culture, were incubated at 28 °C in an incubator shaker for 69 h, and then a portion was used to inoculate 200-mL production broths in 1-L wide-mouth, baffled Erlenmeyer flasks. These were then incubated at 32 °C in the incubator shaker. Acivicin bioactivity against *Bacillus subtilis* UC-902 first appeared in the broth at 48 h and peaked at 120 h. The ferment was then centrifuged, and the supernate was worked up through a series of ion-exchange and flash chromatographies. One liter of ferment typically yielded 6 mg of pure **1** and 12 mg of pure **2**.

Initial ^{14}C Feedings. We anticipated that one of the common five-carbon amino acids—glutamic acid (**9**), glutamine (**10**), or possibly ornithine (**11**)—would be the primary precursor (Scheme I). Initially, DL-[1-^{14}C]-**9** and L[U-^{14}C]-**10** were each fed to separate 200-mL production broths at 48, 72, and 96 h after inoculation, and each fermentation was continued for a total of 120 h. These six experiments were worked up in standard fashion after approximately 25 mg each of **1** and **2** were added to each broth as carriers. In none of these experiments were the recovered metabolites radioactive (Table I). When DL-[2-^{14}C]ornithine (**11a**) was fed at 48 h, however, subsequent workup at 120 h yielded radioactive **1** and **2** (Table I).

[5-^{13}C,5-^{15}N]Ornithine. In order to determine whether **11** was the direct primary precursor, a sample double-labeled with ^{13}C and ^{15}N was selected.[1a] [5-^{13}C,5-^{15}N]Ornithine (**11b**), previously synthesized and used by us,[36,37] was mixed with **11a** and fed in equal portions to five 200-mL production broths at 48 h after inoculation. These were worked up as usual. The 100.6-MHz ^{13}C NMR spectrum of each metabolite, **1a** and **2a**, in D_2O exhibited a spin-coupled doublet (J_{CN} = 2.7 Hz) for C-5 (152.6 and 154.7 ppm, respectively). In each case, the lower-field satellite was superimposed on the natural abundance singlet (1.4-Hz upfield isotope shift). The measured level of ^{13}C enrichment for **1a** and **2a** was 1.3% and 4.2%, respectively, and by normalizing signals to the natural abundance spectra it was clear that the original ^{13}C-^{15}N bond had remained intact (Scheme I).

Deuterated Ornithines. In order to probe the chemistry beyond ornithine, a series of deuterated ornithines was prepared and fed and the derived metabolites were analyzed in deuterium-depleted water by ^2H NMR at 61.4 MHz. We have previously used [2,3,3-$^2H_3$]-α-amino acids as general probes for secondary metabolism.[29,33] In the present case, [2,3,3-$^2H_3$]ornithine (**11c**) was prepared by exchange of **11** in D_2O in the presence of pyridoxal and $Al_2(SO_4)_3$.[38] The deuterium enrichment obtained was 100% and 76% at H-2 and H-3, respectively.

A portion of **11c** (88.9 mg) was mixed with DL-[5-^{14}C]ornithine (**11d**) (12.0 μCi) and fed in equal portions to ten 200-mL production broths. This experiment afforded 3.6 mg of **1b** and 27.0 mg of **2b**. ^2H NMR analysis of **2b** showed, in addition to resonances for residual HOD (δ 4.93) and *tert*-butyl alcohol added for deuterium quantitation and chemical shift reference (δ 1.27), a signal for deuterium at C-3 (δ 5.19). Similarly, the spectrum of **1b** showed deuterium at C-3 (δ 5.73), although the signal-to-noise ratio was much poorer due to the small sample size.

From the spectrum of **2b**, an enrichment of 1.0% at C-3 was measured. However, from the specific radioactivity of **2b**, an enrichment of 2.7% was anticipated. Thus, approximately 62% of the deuterium at the remaining C-3 hydrogen had apparently been lost, and only 38% was retained. No deuterium was detectable at C-2 of **2b**, even though the signal-to-noise ratio would have allowed detection of 2% retention relative to C-3. Much of the deuterium label had been similarly lost from C-3 of **1b**, although, due to the poor signal-to-noise ratio, the amount could not be as accurately estimated.

11c **1b, 2b**

11e **1c, 2c**

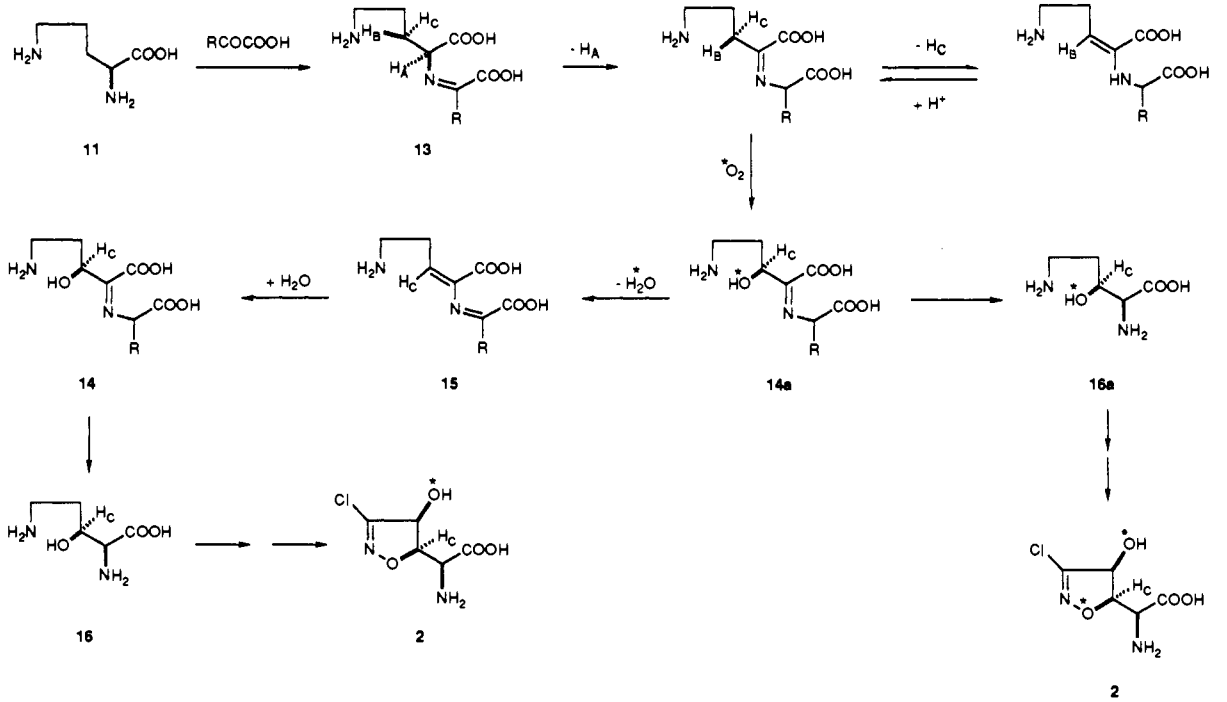
11f **1d, 2d**

11g **1e, 2e**

The stereochemistry of oxygenation at C-3 was next investigated. [3*R*-^2H]- and [3*S*-^2H]ornithines (**11e** and **11f**, respectively) had previously been synthesized in our laboratory via Midland reductions of a deuterated aldehyde,[39] yielding the two expected chirally deuterated alcohols. The camphanates of these were analyzed in the presence of Eu(fod)$_3$ to establish the enantiomeric purities. Thus, the intermediate leading to **11e** was found to be 70% enantiomerically pure, while this value was 85% for the intermediate leading to **11f**. Each was mixed with **11d** and fed to five 200-mL ferments.

(26) Allen, C. M., Jr. *J. Am. Chem. Soc.* **1973**, *95*, 2386.
(27) Adams, E.; Frank, L. *Ann. Rev. Biochem.* **1980**, *49*, 1005–1061, and references cited therein.
(28) Sheehan, J. C.; Mania, D.; Nakamura, S.; Stock, J. A.; Maeda, K. *J. Am. Chem. Soc.* **1968**, *90*, 462.
(29) Martinkus, K. J.; Tann, C.-H.; Gould, S. *J. Tetrahedron* **1983**, *39*, 3493.
(30) Gould, S. J.; Minott, D. A. *J. Org. Chem.* **1992**, *57*, 5214.
(31) Townsend, C. A.; Ho, M.-f. *J. Am. Chem. Soc.* **1985**, *107*, 1065.
(32) Kurylo-Borowska, Z.; Abramsky, T. *Biochem. Biophys. Acta* **1972**, *264*, 1.
(33) Thiruvengadam, T. K.; Gould, S. J.; Aberhart, D. J.; Lin, H.-J. *J. Am. Chem. Soc.* **1983**, *105*, 5470.
(34) Prabhakaran, P. C.; Woo, N.-T.; Yorgey, P. S.; Gould, S. J. *J. Am. Chem. Soc.* **1988**, *110*, 5785.
(35) Ju, S.; Palaniswamy, V. A.; Yorgey, P.; Gould, S. J. *J. Am. Chem. Soc.* **1986**, *108*, 6429.
(36) Wityak, J.; Palaniswamy, V. A.; Gould, S. J. *J. Labeled Compds. Radiopharm.* **1985**, *22*, 1155.
(37) Gould, S. J.; Palaniswamy, V. A.; Zmijewski, M. J. *J. C. S. Chem. Commun.* **1985**, *18*, 1261.
(38) Lemaster, D. M.; Richards, F. M. *J. Labelled Comp. Radiopharm.* **1982**, *19*, 639.
(39) Prabhakaran, P. C.; Gould, S. J.; Orr, G. R.; Coward, J. K. *J. Am. Chem. Soc.* **1988**, *110*, 5779.

Scheme II



Workup of the **11e** feeding yielded 11.1 mg of **1c** and 18.0 mg of **2c**. The ^2H NMR spectra again showed resonances at δ 5.78 (**1c**) and 5.19 (**2c**). Again, comparison of the measured deuterium enrichment for **2c** (1.4%) with that calculated (2.1%) on the basis of specific radioactivity for the *pro*-3*R* position indicated an apparent 32% loss of deuterium.

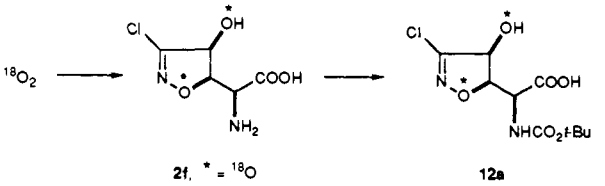
Workup of the **11f** feeding afforded 4.2 mg of **1d** and 17.6 mg of **2d**. The ^2H NMR spectra of these samples revealed the loss of the *pro*-3*S* hydrogen and clearly revealed that the introduction of oxygen had occurred with overall retention of configuration. The ^2H NMR spectrum of each metabolite also contained a small resonance for deuterium at H-3, due to partial retention of the small amount of [3*R*-^2H]ornithine that had been in the material that had been fed. Again, calculations based on the radioactivity indicated a 54% loss of this label.

The repeatedly low levels of deuterium at C-3 were put onto firmer ground when [3,3,4,4-$^2H_4$]ornithine[40] (**11g**) was fed. This experiment provided 2.4 mg of **1e** and 23.5 mg of **2e**. The ^2H NMR spectra of these samples contained resonances for H-4 at δ 3.4 and 5.35, respectively, as well as for H-3. Now the calculated (based on ^{14}C) and observed deuterium enrichments at H-4 were in agreement (loss of 50% of the original deuterium for **2e** due to hydroxylation), while the enrichments at H-3 were again low (63% and 53% lost, respectively).

$^{18}O_2$. With overall retention of configuration at C-3 established by the results from **11e** and **11f**, a fermentation in the presence of $^{18}O_2$ was carried out to establish the source(s) of the oxygen at C-3 of both metabolites and at C-4 of **2**. The closed, manostated system previously used[41] was again employed. However, in trial fermentations with $^{16}O_2$, little or no production of **1** or **2** was observed. Fortunately, it was found that addition of a small amount of $NaHCO_3$[42] to the fermentation broth reestablished production in this system.

Two 200-mL broths were connected in series. $^{16}O_2$ (100%) was used for the first and last 45 h, and $^{18}O_2$ (50%) was used in between. Over the period of 45–120 h, a total of 3.39 L of O_2 was consumed. Workup yielded 12.7 mg of **2f**.

^{13}C NMR analysis of **2f** revealed two resonances in a 1:1 ratio for C-4, one at δ 77.2 for unenriched metabolite, and a second shifted 1.2 Hz upfield for metabolite with a ^{13}C–^{18}O bond. However, no isotope-shifted resonance could be observed for C-3. Suspicious of such a result, we next converted **2f** to the *tert*-butoxycarbonyl (*t*-BOC) derivative **12a** and analyzed the derivative by positive ion–fast atom bombardment (FAB) mass spectrometry.



2f, * = ^{18}O **12a**

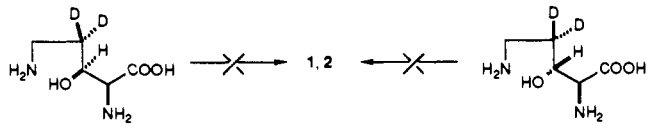
Comparison of this mass spectrum with that of unlabeled **12** prepared from authentic **2** revealed a composition of 60% [$^{16}O_2$]-**12**, 31% [$^{16}O^{18}O$]-**10**, and 9% [$^{18}O_2$]**12**. Thus, ^{18}O was also present at C-3, but the enrichment was only one-third that at C-4 and the small ^{13}C–^{18}O resonance in the ^{13}C NMR spectrum apparently had been lost in the base of the major peak.

β-Hydroxyornithine. The data so far obtained could be explained if a Schiff base such as **13**, derived either from an α-keto acid such as pyruvate or α-ketobutyrate, as shown, or from pyridoxal phosphate (not shown), were hydroxylated at C-3 (**14**). Retention of configuration would be consistent with known biological hydroxylations at an aliphatic carbon.[43–45] Reversible elimination of water (**14a** → **15** → **14**) could account for the lower ^{18}O enrichment observed at the ring oxygen, as shown in Scheme II. Thus, *erythro*-β-hydroxyornithine **16** seemed a likely biosynthetic intermediate.

erythro-[4,4-$^2H_2$]-(2*S*,3*S*)-3-Hydroxyornithine (**16b**) (50 mg) and *threo*-[4,4-$^2H_2$]-(2*S*,3*R*)-3-hydroxyornithine (**17a**) (52 mg), previously prepared from the protected L-[4,4-$^2H_2$]vinylglycine,[46,47] were each fed to *S. sviceus*. Acivicin and 4-hydroxyacivicin were isolated in standard fashion. However, in no case did the ^2H NMR spectrum show any deuterium enrichment. Normal incorporation

(40) The synthesis of this ornithine will be described elsewhere: Gould, S.; Wityak, J., unpublished work.
(41) Erickson, W. R.; Gould, S. J. *J. Am. Chem. Soc.* **1985**, *107*, 5831.
(42) Stadtman, E. R.; Smith, L. D. *J. Bacteriol.* **1972**, *110*, 758.
(43) Battersby, A. R.; Staunton, J.; Wiltshire, H. R.; Bircher, B. J.; Fuganti, C. *J. Chem. Soc., Perkin Trans. I* **1975**, 1162.
(44) Quigely, F. R.; Floss, H. G. *J. Org. Chem.* **1981**, *46*, 464.
(45) Pang, C.-P.; White, R. L.; Abraham, E. P.; Crout, D. H. G.; Lutstorf, M.; Morgan, P. J.; Derome, A. E. *J. Biol. Chem.* **1984**, *222*, 777.
(46) Gould, S. J.; Wityak, J. *J. Org. Chem.* **1987**, *52*, 2179.
(47) Krol, W. J.; Mao, S.-s.; Steele, D. L.; Townsend, C. A. *J. Org. Chem.* **1991**, *56*, 728.

of **11d** that had been co-fed was obtained in both cases (11.2×10^6 dpm fed: 0.5% and 5.6% incorporation into **1** and **2**, respectively), indicating that the fermentations had behaved normally.

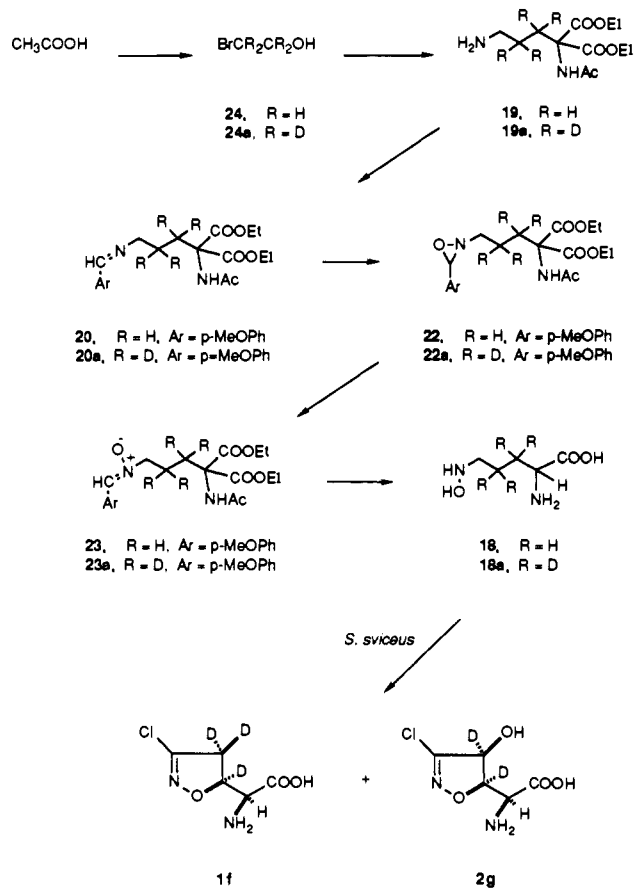


16b **17a**

Having chosen not to include a radioactive label in the synthesis of **16b** and **17a**, it was not possible to directly determine whether either could be taken up by *S. sviceus*. However, we next examined whether either isomer was produced by this organism. DL-[5-^{14}C]ornithine (**11d**) was fed to a growing culture. The fermentation was terminated some hours later, the culture was divided in two portions, the cells were disrupted by sonication, and a quantity of authentic **16** or **17** was added to each and then reisolated. Any endogenous β-hydroxyornithine biosynthesized de novo should have been radioactive and would have been trapped by the carrier material added. Rigorous purification by repeated recrystallization afforded only samples of **16** and **17** that were devoid of radioactivity. A series of such fermentations in the presence of **11d** was carried out, stopping the fermentations at 3, 6, or 15 h after the feeding. However, all yielded the same negative results.

N^{δ}-Hydroxyornithine. Although free *N*-hydroxyamino acids have not been found in living cells, they have been obtained from naturally occurring hydroxamic acids after chemical hydrolysis. Examples are N^{ϵ}-hydroxylysine[48-51] and N^{δ}-hydroxyornithine (**18**).[52-63] The siderophore ferrichrome has been shown to be derived from **18** in *Ustilago sphaeroglua*.[56] We now tested **18** as the first committed intermediate in the biosynthesis of **1** and **2**. Three syntheses of **18** were available.[64-66] However, difficulties were encountered when attempting to introduce isotope labels at the necessary positions. We have developed a new synthesis, as shown in Scheme III, that allowed the convenient introduction of deuterium labels at C-3 and C-4. Diethyl(aminopropyl)-acetamidomalonate hydrochloride (**19**) was prepared from the nitrile, as previously described.[36] Although this hygroscopic material could not be converted directly to Schiff base **20** with *p*-methoxybenzaldehyde (**21**) in dichloromethane, chloroform, or ethanol, the desired product was obtained in benzene/triethylamine with azeotropic removal of water. The imine was oxidized without purification to the oxaziridine **22** with monoperphthalic acid[67] and

Scheme III



rearranged to the nitrone **23** by silica gel chromatography. The yield from **19** was 32%. Hydrolysis of the nitrone with 6 N HCl directly afforded **18** in 62% yield.

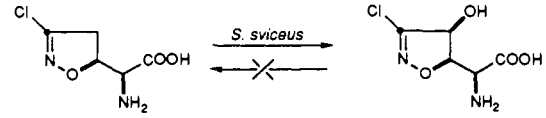
When [2-$^2H_3$]acetic acid was brominated, esterified, and reduced with $LiAlD_4$, [1,1,2,2-$^2H_4$]bromoethanol (**24a**) was obtained, and this was readily converted to **19a** in three steps.[36] In the event, cyanide displacement in CH_3OH led to extensive hydrogen exchange so that **19a** was 100% deuterated at C-3 but only 10% deuterated at C-4 (calculated by ^1H NMR integration); nonetheless, this proved sufficient for our needs.

Deuterium-labeled **18a** (57.46 mg, label distribution as in **19a** above) was divided and fed to ten production broths (200 mL each in 1-L Erlenmeyer flasks) 48 h after inoculation with a seed culture of *S. sviceus*, and an equal amount was fed 12 h later. After a total of 120 h, the broths were worked up in standard fashion to yield 8.0 mg of pure **1f** and 43.1 mg of pure **2g** (Scheme III). Each sample in deuterium-depleted water was then analyzed by ^2H NMR, and both spectra revealed excellent enrichments at C-3 and C-4. The H-3 and H-4 resonances of **1f** (δ 5.2 and 3.4, respectively) were well-resolved and allowed measurement of their individual enrichments (4.3% and 5.8%, respectively). On the basis of the H-4 enrichment, a 2.0% incorporation of **18a** was calculated, and on the basis of the deuterium enrichments of the **18a** fed, it was clear that, in addition to the deuterium that had been replaced by oxygen, 30% of the remaining deuterium at C-3 had been lost. This loss is consistent with that observed previously with **11c**, **11e**, **11f**, and **11g**. The H-3 and H-4 resonances of **2g** at δ 5.2 and 5.3, respectively, could not be resolved in the ^2H NMR spectrum due to the broad line widths; however, if the same relative loss of deuterium from C-3 is assumed, then the enrichments for H-4 and H-3 are 4.4% and 3.3% respectively, and a 3.9% incorporation (based on H-4) of **18a** was obtained.

Hydroxylation of Acivicin. A priori, either **1** and **2** could be derived by separate subpathways branching by early hydroxylation

(48) Gibson, F.; Magrath, D. *Biochim. Biophys. Acta* **1969**, *192*, 175.
(49) Miller, M. J.; Maurer, P. J. *J. Am. Chem. Soc.* **1982**, *104*, 3069.
(50) Snow, T. A. *Bacteriol. Rev.* **1970**, *34*, 99.
(51) Snow, G. A.; Ratledge, C. *Biochem. J.* **1974**, *139*, 407.
(52) Sayer, J. M.; Emery, T. F. *Biochemistry* **1968**, *7*, 3734.
(53) Emery, T. F. *Biochemistry* **1965**, *4*, 1410.
(54) Atkin, C. L.; Neiland, R. B. *J. Bacteriol.* **1970**, *103*, 722.
(55) Diekmann, H. *Arch. Microbiol.* **1970**, *73*, 65.
(56) Emery, T. F. *Biochemistry* **1966**, *5*, 3694.
(57) Diekmann, H. *Angew. Chem.* **1968**, *7*, 551.
(58) Turkova, O.; Mike, O. *Experientia* **1963**, *19*, 633.
(59) Turkova, O.; Mike, O.; Sorm, F. *Collect. Czech. Chem. Commun.* **1964**, *29*, 280.
(60) Schierlein, W.; Dur, A. *Helv. Chim. Acta* **1963**, *46*, 1907.
(61) Schierlein, W.; Diekmann, H. *Helv. Chim. Acta* **1970**, *53*, 2035.
(62) Galles, T. L.; Jalal, M. A. *J. Org. Chem.* **1985**, *50*, 5642.
(63) Hossain, M. B.; Jalal, M. A. *J. Am. Chem. Soc.* **1987**, *109*, 4948.
(64) Takashima, I. T.; Ohmori, M.; Kurita, H. *Bull. Chem. Soc. Jpn.* **1972**, *45*, 1461.
(65) Maehr, H.; Leach, M. *J. Org. Chem.* **1974**, *39*, 1161.
(66) Nieland, R. B.; Rogers, S. *Biochemistry* **1963**, *2*, 6.
(67) Polonski, J.; Chimiak, A. *Bull. Acad. Pol. Sci. Ser. Sci. Chim.* **1979**, *27*, 459.
(68) Gould, S. J.; Lee, J.; Wityak, J. *Bioorg. Chem.* **1991**, *19*, 333.

at C-4, or they could be derived from a linear pathway in which **1** is oxidized to **2**. To distinguish between these, a sample of L-[5-^{14}C]acivicin (**1g**), obtained biosynthetically, was fed to *S. sviceus* at 76, 88, and 100 h after inoculation of the production broth. After workup and purification of **2h** by repeated re-



1g, * = ^{14}C 2h

crystallization, it was established that 15% of **1g** had been converted to **2h**. As one would thus expect, a similar experiment in which L-4-hydroxy[5-^{14}C]acivicin (**2h**) was fed yielded only nonradioactive **1**.

The results presented reveal that a linear pathway from ornithine (**11**) via N^{δ}-hydroxyornithine (**18**) leads to acivicin (**1**), which is then hydroxylated at C-4 to yield **2**. This is the first time the involvement of **18** in a nonsiderophore biosynthesis has been demonstrated. The isoxazolidine oxygen of **1** and **2**, as well as the hydroxyl oxygen of **2**, is derived from molecular oxygen. Exchange of oxygen in some form at C-3 apparently takes place, since the enrichment in the isoxazolidine is only one-third that of the $^{18}O_2$ used, while the enrichment at C-4 is undiluted. During the conversion of **11**, one hydrogen at C-3 is replaced by oxygen with retention of configuration, while the second hydrogen is only partially retained. Furthermore, the hydrogen originally at C-2 of ornithine is completely lost from the final products.

A number of plausible pathways for generating the isoxazoline ring of these metabolites can be envisioned. These include acyclic 3-hydroxy or 2,3-didehydro intermediates, as well as isoxazolidine, isoxazoline, or isoxazolidinone intermediates. Given this complexity and the need to account for the oxygen and hydrogen exchange observed during the feeding experiments, it would be premature to speculate on the actual pathway. However, it will in any event be critical to establish whether the N^{δ}-hydroxyl oxygen of **18** becomes the isoxazoline oxygen. These studies will be reported in due course.

Experimental Section

General. Chemical shifts for ^{2}H spectra are relative to the natural abundance deuterium resonance of *t*-BuOH (δ 1.27). Radioactivity measurements were carried out using a Beckman Model LS 7800 Liquid Scintillation Counter with automatic quench correction and external standarization to yield disintegrations per minute. Elemental analyses were performed at MicAnal (Tucson, AZ).

Materials. $^{18}O_2$ gas (50% enriched) was purchased from Cambridge Isotope Laboratories; ethanol-d_1 (99.5 atom % D), lithium aluminum deuteride (98 atom % D), and acetic acid-d_4 (99.5 atom % D) were purchased from Aldrich Chemical Company, as were deuterium oxide (99.8 atom % D) and deuterium-depleted water (natural abundance × 0.0046%).

***erythro*-β-Hydroxy-L-[4,4-$^2H_2$]ornithine (16b).** The title compound was synthesized in 78% yield from the protected [4,4-$^2H_2$]isoxazolidinylglycine (**16a** in ref 68): mp 230–232 °C; ^{1}H NMR (D_2O 400 MHz) δ 4.25 (1 H, d, J = 3.6 Hz), 3.87 (1 H, d, J = 3.6 Hz), 3.18 (2 H, AB q, J = 12 Hz).

***threo*-β-Hydroxy-L-[4,4-$^2H_2$]ornithine (17a).** The title compound was synthesized in 100% yield from the protected [4,4-$^2H_2$]isoxazolidinylglycine (**17a** in ref 68): mp 122.5–123 °C; ^{1}H NMR (D_2O, 400 MHz) δ 4.22 (1 H, d, J = 5.2 Hz), 3.72 (1 H, d, J = 5.2 Hz), 3.23 (2 H, AB q, J = 10 Hz), 2.08 (0.18 H, m), 1.97 (1 H, m).

2-Bromo[1,1,2,2-$^2H_4$]ethanol (24a). Bromoacetic acid-d_3 was prepared[69] from HOAc-d_4 (10.24 g, 0.16 mol), red phosphorus (0.2 g, 6 mmol), and Br_2 (9.37 mL, 0.182 mol) in 91% yield (20.67 g) after Kugelrohr distillation. A portion of this (20.60 g, 0.145 mol) was treated with concentrated H_2SO_4 (34.9 μL) and EtOH-d_1 (13.7 mL, 0.23 mol) in benzene (50 mL) at reflux for 4 h while H_2O was removed with a Dean–Stark trap. Additional ethanol-d_1 (2.7 mL, 46.6 mmol) was added, and the reaction was cooled to room temperature after an additional 0.5 h. Workup and concentration in vacuo gave 21.82 g (89%) of ethyl bromo[2,2-$^2H_2$]acetate: ^{1}H NMR ($CDCl_3$, 80 MHz) δ 4.23 (2 H, q, J = 7 Hz), 1.25 (3 H, t, J = 7 Hz). This was added to a mixture of $LiAlD_4$ (5.6 g, 0.13 mol) and $AlCl_3$ (17.04 g, 0.1305 mol) in dry ether (150 mL) at −78 °C. After 2 h at −78 °C, the excess hydride was destroyed by the sequential addition of EtOAc (22 mL) and water (23 mL). Workup afforded 7.72 g (49%) of **24a**: ^{1}H NMR ($CDCl_3$, 80 MHz) δ 2.5 (s).

Ethyl 2-Acetamido-2-carbethoxy-5-amino[3,3,4,4-$^2H_4$]pentanoate (19a). A solution of **24a** (7.72 g, 0.0598 mol) and NaCN (3.23 g, 0.066 mol) in MeOH (20 mL) and H_2O (20 mL) was refluxed overnight. After the solution was concentrated to 5 mL, the remaining water was azeotropically distilled with EtOH, and the residue was extracted with EtOAc (100 mL). Workup yielded an oil that was Kugelrohr distilled (4 mm, 80 °C), giving the desired nitrile in 75% yield (3.36 g): ^{1}H NMR ($CDCl_3$, 80 MHz), δ 2.5 (s). The nitrile (3.36 g, 0.0047 mol) in dry pyridine (35 mL) and *p*-toluenesulfonyl chloride (11.83 g, 0.06 mol) yielded 7.66 g (79.7%) of the tosylate: mp 62–63 °C; IR ($CHCl_3$) 2260, 1407 cm^{-1}; ^{1}H NMR ($CDCl_3$, 80 MHz) δ 7.75 (2 H, m), 7.25 (2 H, m), 2.75 (1.8 H, t, J = 6.5 Hz), 2.46 (3 H, s). The ^{1}H NMR spectrum revealed complete deuteration at C-3 (δ 4.25, 2 H, t, J = 6.5 Hz in an unlabeled sample) but only 10% deuteration at C-2 (δ 2.75). The tosylate (7.66 g, 33.4 mmol) was added to diethyl acetamindomalonate (7.53 g, 34.7 mmol) in liquid NH_3 (100 mL) at −78 °C. After 2 h, the NH_3 was evaporated, and the resulting residue was crystallized from CH_2Cl_2/*n*-hexane, giving 7.0 g (76.4%) of the nitrile **19a**: mp 92–93.5 °C (lit.[70] mp 94 °C); IR ($CHCl_3$) 3400, 2250, 1740, 1664 cm^{-1}; ^{1}H NMR ($CDCl_3$, 80 MHz) δ 6.73 (1 H, br s), 4.28 (4 H, q, J = 7.1 Hz), 2.7 (2 H, m), 2.25 (2 H, m), 2.07 (3 H, s), 1.27 (6 H, t, J = 7.1 Hz). The H-3 resonances (δ 2.7) was completely absent. The nitrile (3.21 g, 11.88 mmol) and $PtO_2 \cdot H_2O$ (0.485 g, 1.98 mmol) in absolute EtOH (90 mL) and concentrated HCl (6 mL) were hydrogenated at atmospheric pressure and room temperature for 5 h. After filtration and washing of the catalyst with EtOH, the combined filtrate was concentrated in vacuo, giving a hydroscopic residue (3.71 g).

Diethyl (γ-(*N*-(*p*-methoxybenzylidene)amino)propyl)acetamidomalonate *N*-Oxide (23). To a suspension of unlabeled (aminopropyl)-malonate (3.7 g, 13.65 mmol) in benzene (96 mL) were added anisaldehyde (**21**) (2.18 mL, 17.91 mmol) and Et_3N (33 mL, 238.8 mmol). These were allowed to stir for 4 h at 110 °C. Using a Dean–Stark trap, the water was then removed by azeotropic distillation. After evaporation of the benzene, the residue was placed on a high vacuum pump to remove trace amounts of Et_3N. This crude Schiff base was added to a solution of monoperoxyphthalic acid magnesium salt (8.17 g, 16.53 mmol) in dry ether (40 mL) and absolute EtOH (20 mL) at 0 °C and stirred for 3 h. The mixture was then kept in a refrigerator overnight. The precipitated phthalic acid was filtered and washed with $CHCl_3$, and the solvent was removed at 30 °C under vacuum. To the residue was added $CHCl_3$ (40 mL), and the remaining phthalic acid was removed after an additional 30 min of cooling. The filtrate was then evaporated onto flash grade silica gel (30 g) and placed on a column of the same material (4 × 28 cm). Elution with $CHCl_3/CH_3OH$ (9:1) yielded the desired product, contaminated with **21**. This was purified by PLC (silica gel, developed with EtOAc), eluting with $CHCl_3/CH_3OH$ (9:1) to give 1.5 g (32%) of **23**: mp 149–149.5 °C; IR ($CHCl_3$) 3400, 3040, 1738, 1671, 1602 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.25 (2 H, d, J = 7 Hz), 7.35 (1 H, s), 6.95 (2 H, d, J = 7.0 Hz), 6.85 (1 H, s), 4.25 (4 H, q, J = 7.1 Hz), 3.9 (2 H, t, J = 7.2 Hz), 3.8 (3 H, s), 2.4 (2 H, m), 2.05 (3 H, s), 1.85 (2 H, m), 1.25 (6 H, t, J = 7.0 Hz); ^{13}C NMR ($CDCl_3$) δ 169.31, 167.74, 161.17, 134.44, 130.66, 123.28, 113.84, 66.06, 65.98, 62.73, 55.33, 29.37, 23.06, 22.17, 13.94; EIMS (70 ev), m/z (relative intensity) 408 (M^+, 48.9), 335 (100). Anal. Calcd. for $C_{20}H_{28}O_7N_2$: C, 58.82; H, 6.86; N, 6.86. Found: C, 58.84; H, 6.92; N, 6.80.

Ethyl (γ-(*N*-(*p*-methoxybenzylidene)[3,3,4,4-$^2H_4$]amino)propyl)acetamidomalonate *N*-Oxide (23a). Deuterated (aminopropyl)malonate (**19a**) (3.8 g, 13.67 mmol) suspended in benzene (100 mL) was treated with **21** (2.26 mL, 8.56 mmol) and Et_3N (33 mL, 238.8 mmol). The crude product was oxidized with monoperoxyphthalic acid magnesium salt and worked up as described above. Workup and purification gave 1.53 g (32%) of **23a**. The ^{1}H NMR spectrum ($CDCl_3$) was identical to that of **23** except for the lack of the H-3 resonance (δ 2.4) and a 10% decrease in the H-4 resonance (δ 1.85).

N^{δ}-Hydroxy-DL-[3,3,4,4-$^2H_4$]ornithine (18a). A solution of nitrone **23a** (1 g, 2.54 mmol) in 6 N HCl[65] (200 mL) was heated in an oil bath at 130 °C for 7 h, and the solution was then concentrated to dryness under reduced pressure. The residue was neutralized to pH 4.5 and chromatographed on Dowex 50Wx4 (H^+) (200–400 mesh, 3 × 20 cm), which was first washed with water until neutral and then eluted with 1.5 N HCl. This gave 0.35 g (61%) of **18a** as a white glossy solid which could not be recrystallized because of its very high hygroscopic nature: ^{1}H NMR (D_2O, 80 MHz) δ 4.25 (1 H, s), 3.50 (2 H, t, J = 7 Hz), 2.0 (1.8 H, br t, J = 8 Hz).

(69) Ward, C. F. *J. Chem. Soc.* **1922**, *121*, 1161.

(70) Moe, A.; Warner, D. T. *J. Am. Chem. Soc.* **1948**, *70*, 2763.

Biosynthetic Studies. Maintenance of *S. sviceus*. Screw-cap tubes (25- × 150-mm) were half filled with sandy loam soil and sterilized by autoclaving on three separate occassions, with 2-day incubation periods at 25 °C in between. Spores from *S. sviceus*, grown on a slant of Hickey–Tresner agar,[71] were suspended in saline (5 mL, 0.9% NaCl containing 0.01% Tween 80). Aliquots (0.5 mL) were transferred to the tubes of sterile soil, incubated at 26 °C for 2 weeks, and then stored at 4 °C.

Production of Acivicin and 4-Hydroxyacivicin. Sterile seed broth (glucose 0.5 g, peptone 0.5 g, yeast extract 0.13 g in 50 mL Milli-Q water in a 250-mL Erlenmeyer flask) was inoculated with *S. sviceus* spores from the soil culture using a sterile inoculating loop. The seed culture was incubated at 28 °C and 270 rpm in a gyrotatory shaker for 69 h, at which time it showed dense growth. Using a sterile pipet, 5 mL of this seed culture (2.5% v/v) was transferred to 200 mL of sterile production broth (cerelose 0.4 g, whole yeast 0.5 g, Kaysoy 200C (Archer Daniels Midland Co., Decatur, IL) 4.0 g, corn starch 2.0 g, NH_4Cl 1.0 g, and lard oil 1.0 mL, in tap water) prepared in a 1-L baffled flask, and this was then incubated at 32 °C and 250 rpm in the incubator shaker. The production was found to be maximum at 120 h.

Feeding Protocol. DL-[1-^{14}C]Glutamic acid and L-[U-^{14}C]glutamine were fed to three separate broths at 48, 72, and 96 h, while the labeled ornithines and β-hydroxyornithines were each fed to individual broths at 48 h after inoculation. Labeled *N*-hydroxyornithine was fed in two pulses, at 48 and 60 h, after inoculation of the broth. Labeled acivicin and hydroxyacivicin were fed in three pulses, at 76, 88, and 100 h. All the fermentations were carried out using baffled flasks. Aqueous solutions of the precursors were slowly injected in a sterile manner into the production broths at the appropriate time by filtration through a Gelman membrane filter (product No. 4192, size 0.2 μm).

Purification of Acivicin and 4-Hydroxyacivicin. Production broth (2 L) from ten flasks harvested after 120 h of incubation was centrifuged at 9000*g* for 20 min to remove the solid materials. The pellets were washed with a minimum amount of water, and the washings were combined, adjusted to pH ~7.8 using 2 N HCl, and passed through a cation exchange column (Dowex 50x8, H^+, 100–200 mesh, 5 × 47 cm). Impurities were removed with water (1500 mL), and acivicin and 4-hydroxyacivicin were eluted from the column using 2.5 N NH_4OH. Fractions containing acivicin (determined by a ninhydrin test and TLC analysis) were combined and rotoevaporated at ambient temperature to 50 mL. The concentrate was then adjusted to pH 7 with 2 N HCl and passed through an anion exchange column (AG3-X4A, 100–200 mesh, 3 × 12 cm). The column was washed with deionized water (1500 mL), 50% CH_3OH (2 L), and 90% MeOH (2.5 L). The column was finally eluted with methanol–H_2O–glacial acetic acid (90:10:3 v/v). Fractions from the last treatment, containing acivicin and 4-hydroxyacivicin, were combined, rotoevaporated at 45 °C to remove the solvent, and then lyophilized. The residues from these fractions were redissolved in water (10 mL) and evaporated onto a minimum quantity of flash grade silica gel (0.4 g). This was then added to the top of a silica gel column (3 × 22 cm) and eluted with methyl ethyl ketone–acetone–water (65:20:15). Pure acivicin and 4-hydroxyacivicin were each obtained as white crystals from the appropriate fractions by recrystallization from MeOH–H_2O. Radioactive samples were repeatedly recrystallized until constant specific activity was obtained (±3%).

DL-[2-^{14}C]Ornithine (11a). DL-[2-^{14}C]Ornithine (20.75 × 10^6 dpm) was fed to one 200-mL broth. Authentic **1** (5.0 mg) was added before ion-exchange chromatography, while 25.3 mg of **1** and 25.1 mg of **2** were added to the respective biosynthetic samples prior to recrystallization. Radiochemically pure **1** (7.67 × 10^4 dpm/mmol) and **2** (26.9 × 10^5 dpm/mmol) were obtained, indicating an approximate incorporation of 0.2% and 2.0%, respectively, based on incorporation of both isomers.

DL-[5-^{13}C,5-^{15}N]Ornithine (11b). 4-Hydroxyacivicin (**2a**) and acivicin (**1a**) were obtained from feeding DL-[5-^{13}C,5-^{15}N]ornithine (**11b**) (38.8 mg, 0.23 mmol) and DL-[5-^{14}C]ornithine (29.20 × 10^6 dpm) to five 200-mL broths. Workup yielded **1a** (6.0 mg, 0.034 mmol, 1.6 × 10^6 dpm/mmol, 0.28% incorporation) and **2a** (11.9 mg, 0.06 mmol, 5.1 × 10^6 dpm/mmol, 2.64% incorporation).

The ^{13}C NMR spectrum of **2a** showed a 4.2% enrichment for C-5 (154.7 ppm, J_{CN} = 2.7 Hz overlapping the natural abundance peak), and the spectrum of **1a** showed a 1.3% enrichment for C-5 (152.6 ppm, J_{CN} = 2.7 Hz overlapping the natural abundance peak). The spin-coupled doublets were clearly revealed by substraction of the relevant natural abundance ^{13}C NMR spectrum.

L-[5-^{14}C]Acivicin (1g). After workup through chromatography, 4-hydroxyacivicin (**2h**) (15.2 mg) was obtained from feeding L-[5-^{14}C]-acivicin (**1g**) (4.4 × 10^4 dpm) in three pulses, at 76, 88, and 100 h. Authentic 4-hydroxyacivicin (5.2 mg) was added, and the mixture was recrystallized from CH_3OH–H_2O to constant specific activity: 5.10 × 10^4 dpm/mmol, 15.3% incorporation.

Oxygen-18 Fermentation. A seed culture of *S. sviceus* was used to inoculate two production broths (200 mL each in 1-L baffled flasks equipped with ball joints). $NaHCO_3$ (0.1 g) in 10 mL of water was sterilized by micropore filtration and added to each production broth as a CO_2 source. Air was circulated through the closed system[41] at the rate of 2 L/min, while the fermentation flasks were shaken at 32 °C (250 rpm). A buret was filled with $^{18}O_2$ at 45 h, refilled periodically until the 75th hour, and thereafter refilled with $^{16}O_2$. A total of 3.39 L of $^{18}O_2$ had been consumed. Workup afforded 4-hydroxyacivicin (**2f**) (12.7 mg): ^{13}C NMR (100.6 MHz, D_2O) δ 170.2 (C-1), 154.7 (C-5), 81.7 (C-3), 77.2 (C-4), 53.1 (C-2). In addition to the ^{13}C–^{16}O resonance for C-4 (77.2 ppm), an upfield ^{13}C–^{18}O resonance (isotope shift 1.2 Hz) was observed (46% ^{18}O enrichment).

***N*-(*tert*-Butyloxycarbonyl)-4-hydroxyacivicin (12).** 4-Hydroxyacivicin (**2**) (10 mg, 0.05 mmol) in 50% aqueous dioxane (200 μL) at room temperature was treated with triethylamine (17.77 μL, 0.128 mmol) followed by BOC-ON (25.32 mg, 0.103 mmol). H_2O (10 mL) was added the next day, and the mixture was extracted with EtOAc (50 mL). The aqueous phase was adjusted to pH 3.82 and extracted with additional EtOAc. The extracts were dried and concentrated to give 15 mg (55%) of the desired product as an oil: ^{1}H NMR ($CDCl_3$) δ 7.8 (2 H, br), 5.6 (1 H, d, J = 3.5 Hz), 5.1 (1 H, br), 4.5 (1 H, m), 1.4 (10 H, s); ^{13}C ($CDCl_3$) δ 172.61, 156.37, 152.15, 84.22, 81.63, 79.51, 52.46, 28.20; FABMS (glycerol, positive ion), *m/e* (relative intensity) 293 (100), 295 (32.7); high-resolution FAB mass spectrum calcd for $C_{10}H_{15}N_2O_3Cl$, 293.05402, found, 293.05166.

Oxygen-18-labeled *N*-(*tert*-Butyloxycarbonyl)-4-hydroxyacivicin (12a). The above procedure was used to convert [^{18}O]-4-hydroxyacivicin (**2f**) (10.1 mg, 0.056 mmol) into 16 mg (51%) of [^{18}O]*N*-(*tert*-butyloxycarbonyl)-4-hydroxyacivicin: FABMS (negative ion), *m/e* (adjusted relative intensity) 293 (M − 1, 100), 295 (51.9), 297 (7.5), 299 (2.6).

Protocol for *erythro*- and *threo*-β-Hydroxy-L-ornithine Trapping Experiments. A small production ferment (100 mL) was harvested after the desired duration. The broth was centrifuged, and the supernate was decanted. The pellet was resuspended in water (100 mL) and sonicated (Heat-Systems Ultrasonic, output control 6, 90% duty cycle) for 5 min, with cooling, to break the cells. The supernate was contrifuged at 19800*g*; the combined supernates were then lyophilized, the residue was dissolved in 50 mL of distilled H_2O, and 5 mg of *threo*- or *erythro*-β-hydroxy-L-ornithine was added. After adjustment to pH 4.3, the solution was loaded onto a cation ion-exchange column (Dowex 50Wx4, H^+, 100–200 mesh, 2 × 15 cm). The column was washed with deionized water and then eluted with a 0–2 N HCl gradient (500 mL total volume) at a flow rate of 1.5–2.0 mL/min. Cellulose PLC developed with BuOH:H_2O:AcOH (3:1:1 v/v) was used to further purify this material. The nearly pure *threo*- or *erythro*-β-hydroxyornithine was diluted with an additional 30–40 mg of authentic compound, and the mixture was recrystallized repeatedly, ultimately yielding background levels of radioactivity.

L-4-Hydroxy[5-^{14}C]acivicin (2h). L-4-Hydroxyl[5-^{14}C]acivicin (**2h**) (3.6 × 10^5 dpm) was fed to a 200-mL fermentation broth at 76, 88, and 100 h. This was worked up as usual. There was no radioactivity in the acivicin obtained after the silica gel column chromatography.

Acknowledgment. Dr. David Martin and Shirley Gerpheide of The Upjohn Co. are thanked for providing samples of acivicin and 4-hydroxyacivicin, slants of *S. sviceus*, and the design of the baffled flask used in fermentations. Dr. John Wityak is thanked for preparing the sample of [3,3,4,4-$^2H_4$]ornithine used in this study. Dr. P. C. Prabhakaran and Rodger Kohnert are thanked for obtaining the ^{2}H NMR spectra. The work was supported by a grant from Public Health Service GM 32110 to S.J.G. NMR spectra were obtained on a Bruker AM 400 spectrometer purchased in part by grants from the National Science Foundation (CHE-8216190) and from the M. J. Murdock Charitable Trust to Oregon State University.

(71) Dextrin 10.0 g, beef extract 1.0 g, yeast extract 1.0 g, NZ Amine A 2.0 g, $CoCl_2$ 0.02 g, and agar 17.5 g, in 1 L of distilled H_2O at pH 7.5.