CD_3OD) δ 175.09, 174.91, 132.75, 131.07, 129.22, 128.87, 128.25, 100.10, 74.90, 73.44, 71.70, 69.78, 67.17, 64.32, 54.32, 35.21, 34.99, 33.09, 30.82, 30.51, 30.36, 30.26, 28.21, 26.56, 26.44, 26.03, 23.74, 21.51, 14.68, 14.45; MS m/z 839 $(M + H)^+$; HRMS calcd for $C_{43}H_{76}NaO_{12}S$ $(M + H)^+$ 839.4958, found 839.4928.

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Biosynthesis of Virginiae Butanolide A, a Butyrolactone Autoregulator from *Streptomyces*

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Abstract: Virginiae butanolide A (VB A) (**3**) is one of the virginiamycin-inducing factors from *Streptomyces virginiae* and has a unique 2,3-disubstituted butanolide skeleton which is common to other signal molecules in *Streptomyces*. The biosynthesis of **3** in *Streptomyces antibioticus*, a high producer of **3**, was studied by experiments with labeled precursors. ^{13}C and ^{2}H NMR results as well as CI-MS analyses of dibenzoate samples indicated that the probable biosynthetic pathway to **3** involved coupling between a β-keto acid derivative and a C_3 unit from glycerol, such as dihydroxyacetone or a derivative.

Signal molecules which regulate secondary metabolite production or cytodifferentiation of *Streptomyces* have been known for nearly 20 years. The first one was A factor (**1**) found by Khokhlov et al., which induces formation of streptomycin, aerial mycelium, and spores in *Streptomyces griseus*.[1] After the discovery of A factor, Gräfe et al. isolated factor 1 (**2**) from the culture broth of *Streptomyces viridochromogenes* as an inducer of the formation of aerial mycelia and leukaemomycin in *S. griseus*.[2] Then anthracycline-inducing factors in *S. griseus* were found in the culture broth of *Streptomyces bikiniensis* and *Streptomyces cyaneofuscatus* by Gräfe et al.[3] They assigned these structures to what we will refer to as **3**, **4**, and **5**. Recently, we have reported the isolation and structural elucidation of virginiae butanolides (VB) A-E **3**, **6**, **7**, **8**, and **9**, which induce the production of virginiamycin in *Streptomyces virginiae*,[4] and more recently IM-2 (**10**), which induces the production of a blue pigment in *Streptomyces* sp. FRI-5.[5] All of these molecules have common structural features. They possess a 2,3-disubstituted butanolide skeleton but differ in the C-2 side chain containing functional groups, such as 6-hydroxy or 6-keto groups, and in the length or branching of the alkyl chain. A 2,3-cis or -trans configuration was once proposed for autoregulators which have a C-6 hydroxyl group, but recently the stereochemistry was revised as shown in Figure 1.[6] The absolute configurations of A factor and VB A, B, and C have been assigned to **1**, **3**, **6**, and **7** with their chiral synthesis being carried out by Mori et al.[7]

Table I. ^{13}C Abundances in **3a** Obtained from Feeding Experiments with ^{13}C-Labeled Precursors

carbon no.	δ_C	relative ^{13}C abundances[a]			
		[1-^{13}C]-acetate	[2-^{13}C]-acetate	[1-^{13}C]-isovalerate	[1,3-$^{13}C_2$]-glycerol
1	175.6	6.6	1.6	nd[b]	nd
2	46.4	0.8	10.7	0.8	4.5
3	36.4	0.8	1.0	0.8	nd
4	69.2	0.9	1.1	0.9	6.2
5	65.2	0.9	1.3	1.1	6.1
6	73.1	5.8	1.5	0.9	nd
7	33.0	0.9	9.3	0.9	3.7
8	23.2	0.9	0.9	7.3	nd
9	38.4	0.8	1.2	1.1	nd
10	27.7	1.0	1.0	1.0	nd
11	22.4	1.1	1.3	0.9	nd
12	22.4	0.9	1.2	0.7	nd

[a] Peak height ratio of ^{13}C enriched to natural abundance. [b] nd = Not detected.

This unique butanolide skeleton is hitherto known only in metabolites of *Streptomyces*. These molecules are efficient at extremely low concentrations, and specific receptor proteins are involved in the expression of their activity.[8] Biosynthetic studies on these signal molecules are very important as a new approach to understanding the mechanism of secondary metabolite production in *Streptomyces*. However, it has been very difficult to study the biosynthesis of these molecules because they are mainly produced in trace amounts in culture broths; e.g., only a few micrograms of VB A were obtained from 1 L of *S. virginiae* broth. Recently we found a strain of *Streptomyces antibioticus* which produces several milligrams of VB A per liter of culture broth,[9] and this finding has made it possible to elucidate the biosynthesis of **3** by feeding experiments with ^{13}C-labeled precursors. We have

(1) Kleiner, E. M.; Pliner, S. A.; Soifer, V. S.; Onoprienko, V. V.; Balashova, T. A.; Rosynov, B. V.; Khokhlov, A. S. *Bioorg. Khim.* **1976**, *2*, 1142-1147.
(2) Gräfe, U.; Schade, W.; Eritt, I.; Fleck, W. F.; Radics, L. *J. Antibiot.* **1982**, *35*, 1722-1723.
(3) Gräfe, U.; Reinhardt, G.; Schade, W.; Eritt, I.; Fleck, W. F.; Radics, L. *Biotechnol. Lett.* **1983**, *5*, 591-596.
(4) (a) Yamada, Y.; Sugamura, K.; Kondo, K.; Yanagimoto, M.; Okada, H. *J. Antibiot.* **1987**, *40*, 496-504. (b) Kondo, K.; Higuchi, Y.; Sakuda, S.; Nihira, T.; Yamada, Y. *Ibid.* **1989**, *42*, 1873-1876.
(5) Sato, K.; Nihira, T.; Sakuda, S.; Yanagimoto, M.; Yamada, Y. *J. Ferment. Bioeng.* **1989**, *68*, 170-173.
(6) Sakuda, S.; Yamada, Y. *Tetrahedron Lett.* **1991**, *32*, 1817-1820.
(7) (a) Mori, K. *Tetrahedron* **1983**, *39*, 3107-3109. (b) Mori, K.; Chiba, N. *Liebigs Ann. Chem.* **1990**, 31-37.
(8) (a) Kim, H. S.; Tada, H.; Nihira, T.; Yamada, Y. *J. Antibiot.* **1990**, *43*, 692-706. (b) Kim, H. S.; Nihira, T.; Tada, H.; Yamada, Y. *Ibid.* **1989**, *42*, 769-778. (c) Miyake, K.; Horinouchi, S.; Yoshida, M.; Chiba, N.; Mori, K.; Nogawa, N.; Morikawa, N.; Beppu, T. *J. Bacteriol.* **1989**, *171*, 4298-4302.
(9) Ohashi, H.; Zheng, Y.-H.; Nihira, T.; Yamada, Y. *J. Antibiot.* **1989**, *42*, 1191-1195.

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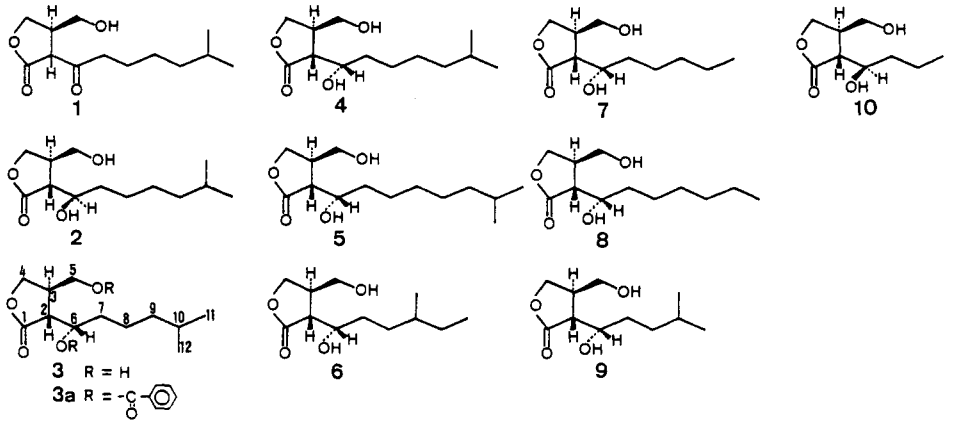


Figure 1. Structures of butyrolactone autoregulators from *Streptomyces*.

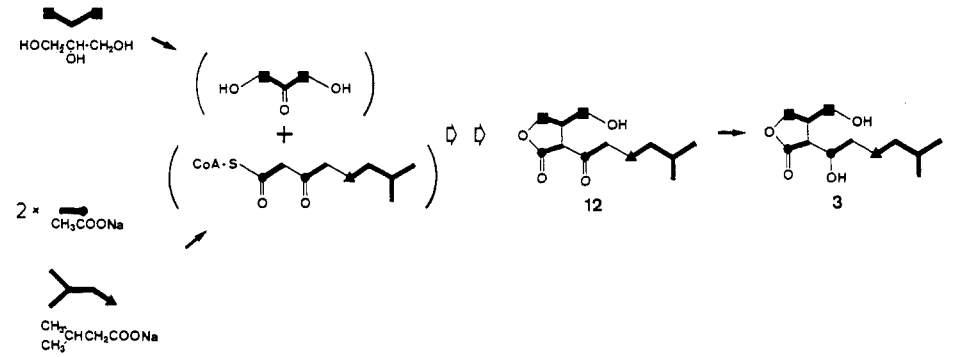
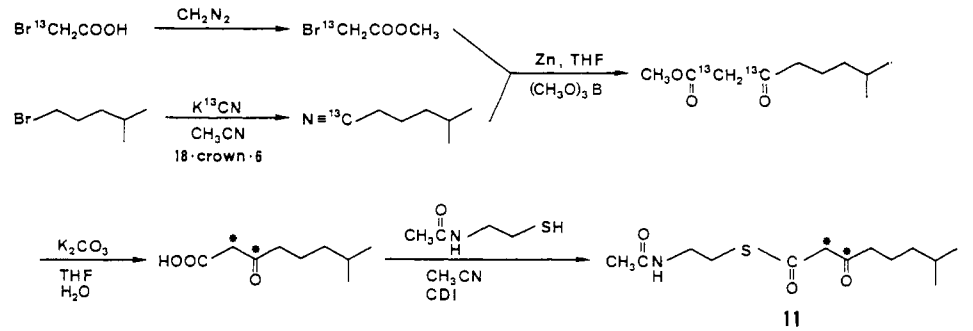


Figure 2. Possible biosynthetic pathway to VB A.

Scheme I



found that **3** is formed from two molecules of acetic acid and one molecule each of isovaleric acid and glycerol. We reported these results in a preliminary communication.[10] We now report a detailed description of these experiments, as well as the results of further feeding experiments, which indicate that the biosynthetic pathway of **3** involves coupling between a β-keto acid derivative and a dihydroxyacetone type C_3 unit from glycerol.

Results

S. antibioticus IFO 12838, which was found to be a high producer of VB A, was used throughout this study. *S. antibioticus* was cultivated in a 500-mL Sakaguchi flask containing 100 mL of medium on a reciprocating shaker. Before performing the incorporation experiments involving isotopically-labeled acetate, we investigated the effect of sodium acetate on the yield of **3**. Since the addition of acetate increased the yield of **3** by about 2-fold, sodium acetate was added twice to the culture at the 24th and 48th hours of cultivation. VB A production started at 24 h. After 96 h, VB A was isolated as its dibenzoate **3a** from the culture broth.

Since the structure of the carbon skeleton of VB A, especially that of C-2 side chain, suggested that acetate may be involved in its biosynthesis, incorporation experiments with labeled acetate were carried out first. A mixture of sodium [1-^{13}C]- or [2-^{13}C]acetate and unlabeled sodium acetate was administered to the culture twice. The ^{13}C NMR spectrum of the ^{13}C-labeled **3a** obtained showed enrichment at C-1 and C-6, and C-2 and C-7, respectively (Table I), indicating that each C_2 unit of C-1/C-2 and C-6/C-7 was derived from an acetate molecule.

In the next feeding experiment, sodium [1-^{13}C]isovalerate was administered to the culture. Since the addition of too much sodium isovalerate at one time caused strong growth inhibition, 2.5 mg of labeled isovalerate was added to 100 mL of the culture broth eight times at 2-h intervals. The **3a** obtained showed only one enriched peak at C-8 on its ^{13}C NMR spectrum (Table I), indicating that five carbons, C-8, -9, -10, -11, and -12, of the VB A molecule come from isovaleric acid.

In order to clarify the origin of the remaining three carbons, C-3, -4, and -5, the incorporation of [1,3-$^{13}C_2$]glycerol was carried out. In this case, potato starch was used as the carbon source for the cultivation in place of glycerol to avoid dilution of isotopically-labeled glycerol. Accordingly, the yield of **3** significantly decreased. The ^{13}C NMR spectrum of **3a** clearly showed enriched peaks at C-4 and C-5 (Table I) as we had expected. The C-2 and C-7 signals on the spectrum were also enriched due to the metabolism of [1,3-$^{13}C_2$]glycerol to [2-^{13}C]acetic acid and in-

(10) Sakuda, S.; Higashi, A.; Nihira, T.; Yamada, Y. *J. Am. Chem. Soc.* **1990**, *112*, 898-899.

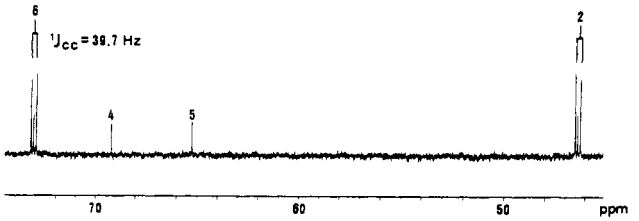


Figure 3. ^{13}C NMR spectrum of **3a** derived from [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoic acid *N*-acetylcysteamine thioester (150 MHz, 0.37 mg in 0.6 mL of $CDCl_3$, 20043 transients).

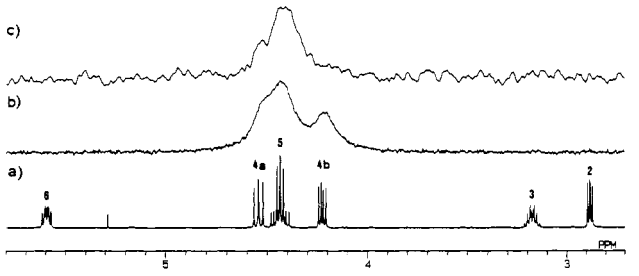
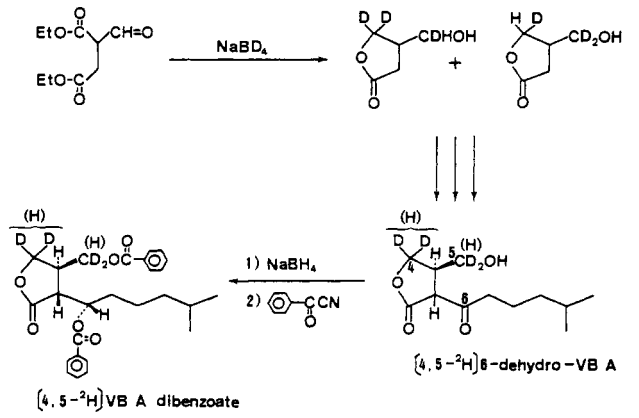


Figure 4. (a) ^1H NMR spectrum of natural **3a** (400 MHz, 6.8 mg in 0.6 mL of $CDCl_3$, 16 transients). (b) ^2H NMR spectrum of synthetic [4,5-^{2}H]VB A dibenzoate (61 MHz, 20 mg in 0.6 mL of $CHCl_3$, 64 transients). (c) ^2H NMR spectrum of **3a** derived from [$^2H_5$]glycerol (61 MHz, 15 mg in 0.6 mL of $CHCl_3$, 3210 transients).

Scheme II



corporation of it into **3**. Unfortunately, under the experimental conditions used for ^{13}C NMR, an expected two-bond coupling between C-4 and C-5 could not be observed because of its small value.[11] Thus, the incorporation of the intact glycerol molecule into **3** was not verifiable by the NMR spectrum. However, the CI-MS spectrum of labeled **3a** indicated that the di-^{13}C-labeled molecule increased by 5.3%; this was deduced from the relative intensities of ion peaks around the pseudomolecular mass range on the spectra of labeled and natural **3a**. Since the only possible site of incorporation of the intact glycerol molecule is the C-3, -4, and -5 moiety and the increased ratio of dilabeled molecule calibrated by the MS spectrum was approximately consistent with the incorporation percent estimated by NMR of C-4 (4.5%) or C-5 (4.4%),[12] we concluded that the glycerol molecule was incorporated into the C-3, -4, and -5 moiety of **3** in intact form.

From the above results, it became clear that the VB A molecule is an assembly of two acetate, one isovalerate, and one glycerol molecule. The most probable pathway for these molecules is shown in Figure 2. In order to verify this pathway, the incorporation

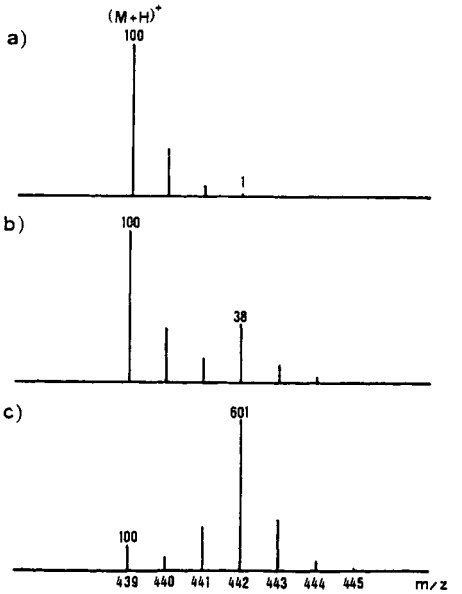


Figure 5. CI mass spectra of **3a**: (a) natural abundance; (b) and (c) derived from 1.0 and 10 mg of [4,5-^{2}H]-6-dehydro-VB A, respectively.

of [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoic acid *N*-acetylcysteamine thioester **11** was undertaken. This thioester is a mimic of β-keto acyl CoA, which is the plausible key precursor of **3**. The labeled thioester **11** was prepared following Scheme I, in which a modified Blaise reaction[13] was used to prepare the ^{13}C dilabeled β-keto acid methyl ester by coupling between methyl [2-^{13}C]bromoacetate and [1-^{13}C]-5-methylhexanenitrile. Incorporation of the β-keto acid itself and its methyl ester was unsuccessful, but thioester **11** was incorporated into **3** successfully.[14] The relevant region of the ^{13}C NMR spectrum of the resulting **3a** is shown in Figure 3. In the spectrum, the enriched C-2 and C-6 signals coupled to each other with a coupling constant of 39.7 Hz. This fact indicated that the β-keto acid moiety of **11** was incorporated in an intact form without cleavage of the C–C bond between C-2 and C-3.

Next, the incorporation of [$^2H_5$]glycerol was attempted to estimate the species of the three-carbon unit derived from glycerol in the biosynthetic pathway. To obtain a large enough amount of labeled **3** for the ^2H NMR measurement, cultivation on a 25-L scale using 250 500-mL flasks was carried out. The ^2H NMR spectrum of the **3a** obtained is shown in Figure 4c. When this spectrum was compared with that of chemically-synthesized authentic [4,5-^{2}H]VB A dibenzoate (Figure 4b) prepared following Scheme II[4] and the ^1H NMR spectrum of **3a** (Figure 4a) derived from natural **3**, no ^2H signal was observed on C-3 and one ^2H signal was clearly detected on C-4. On the other hand, a ^2H signal was observed on C-5. Thus, during the incorporation of [$^2H_5$]-glycerol, the ^2H on C-2 of the glycerol molecule was lost and one of the ^2H atoms on C-1 or C-3, which corresponds to the *pro-S* hydrogen of glycerol, was also lost mainly stereospecifically.

Finally, in order to verify the reduction step from 6-dehydro-VB A **12** to **3**, incorporation of synthetic [4,5-^{2}H]-6-dehydro-VB A was performed. Labeled **12** is a mixture of four compounds at about the same molar ratio in which each three of the four protons on C-4 and C-5 together are replaced by ^2H according to its synthetic strategy (Scheme II). Figure 5b,c shows the CI-MS spectra around the pseudomolecular ion of **3a**, obtained by the feeding experiments with different amounts of labeled **12**. Compared to the spectrum of natural **3a** (Figure 5a), which shows the $(M + H)^+$ ion at m/z 439, the relative abundance of the 3 mass units larger ion at m/z 442 clearly increased according to

(11) Marshall, J. L. In *Methods in Stereochemical Analysis*; Marchand, A. P., Ed.; Verlag Chemie International: Deerfield Beach, FL, 1983; Vol. 2, pp 193–195.

(12) These values were deduced from the enrichment percent of peaks and the contamination percent (23%) of [1-^{13}C]glycerol.

(13) (a) Cason, J.; Rinehart, K. L., Jr.; Thornton, S. D., Jr. *J. Org. Chem.* **1953**, *18*, 1594–1600. (b) Rathke, M. W.; Lindert, A. *Ibid.* **1970**, *35*, 3966–3967.

(14) Analogous incorporation experiments of a doubly labeled acid *N*-acetylcysteamine thioester have been reported in the following. (a) Yue, S.; Duncan, J. S.; Yamamoto, Y.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1987**, *109*, 1253–1255. (b) Cane, D. E.; Yang, C.-C. *Ibid.* **1987**, *109*, 1255–1257. (c) Cane, D. E.; Ott, W. R. *Ibid.* **1988**, *110*, 4840–4841.

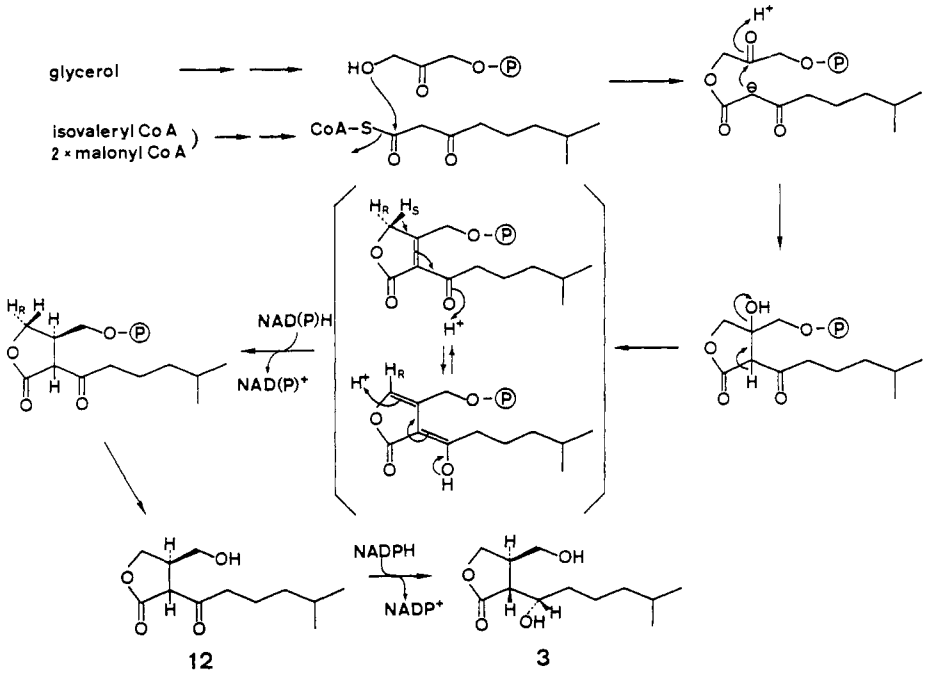


Figure 6. Plausible reaction mechanism of VB A skeleton formation.

the amount of precursor added. Furthermore, **3a** showed the same CD spectra as that of natural **3a** in spite of the racemic nature of labeled **12**, indicating the presence of an enzyme stereospecifically reducing the 6-oxo group to the 6-hydroxy group.

Discussion

The results presented here establish the basic building blocks of virginiae butanolide A and afford proof of its biosynthetic pathway as shown in Figure 2. We believe that the pathway is common to all species of *Streptomyces* for the biosynthesis of butyrolactone autoregulators. In this pathway, β-keto acyl CoA is thought to be the key precursor, which couples with a C_3 unit from the glycerol molecule. The results obtained from incorporation experiments with labeled acetate and isovalerate strongly suggested that the β-keto acyl CoA on VB A is synthesized from isovaleryl CoA as a starter and two malonyl CoA's derived from two acetate molecules, as in the polyketide biosynthesis. Thus, the variety of starter molecules and the number of malonyl CoA molecules may determine the length and branching of the C-2 side chains among the autoregulators. For example, in A factor (**1**) biosynthesis, isobutyryl CoA would be the starter and three malonyl CoA's would be involved. The loss of the proton on C-2 of the glycerol molecule during its incorporation into VB A, which was clarified by the feeding of [$^2H_5$]glycerol, suggests that a C_3 unit having a C-2 oxo group, such as dihydroxyacetone or its derivatives, may be the precursor which forms a C–C bond with the C-2 of the β-keto acyl moiety in the aldol sense.

We propose a hypothetical reaction mechanism for the formation of the VB A skeleton from glycerol and β-keto acid thioester as shown in Figure 6. Glycerol is oxidized to a dihydroxyacetone derivative such as dihydroxyacetone 3-phosphate via glycero-3-phosphate, which is then acylated with the β-keto acyl CoA by an acyltransferase such as dihydroxyacetone-phosphate acyltransferase[15] to form the β-keto ester. The ketone group of the dihydroxyacetone moiety of the β-keto ester undergoes an intramolecular aldol condensation on the C-2 methylene of the β-keto acid moiety to afford the butyrolactone skeleton. The feasibility of this cyclization has recently been suggested by synthesis of an analogous β-keto ester by Rickards.[16] After dehydration, enol and double bond formation with stereospecific loss of H_S on C-4 and reduction leads to the C-6 oxo skeleton. Finally, the C-6 oxo group is reduced to a hydroxyl group by an alcohol dehydrogenase to give the VB A molecule. Reduction enzymes of the C-6 keto group are probably classified into two groups, depending on the orientation of the hydroxyl group produced on C-6. Stereospecific loss of the deuterium on C-3 from [$^2H_5$]glycerol is also possible by the action of aldolase, which exchanges the *pro-S* hydrogen of dihydroxyacetone 3-phosphate.

Since the acylation of a glycerol derivative such as dihydroxyacetone 3-phosphate or glycero-3-phosphate is well-known in the biosynthesis of glycerolipids, the above hypothetical mechanism adopts the acylation step to form the β-keto ester before an aldol condensation. However, the alternative order of reactions, i.e., first the C–C bond formation between two components and second the lactonization, is also possible.

Recently, the last reduction step has been confirmed by the transformation of **12** to **3** with cell-free extracts of *S. antibioticus* in the presence of NADPH.[17] Further work to prove the biosynthetic mechanism is now in progress with the cell-free system.

Experimental Section

General Methods. ^{13}C NMR spectra were recorded on a Bruker AM 600 spectrometer at 150 MHz and 300 K with power-gated broad-band proton decoupling (sweep width = 38 462 Hz, 128 K data points, pulse width = 43°, acquisition time = 1.704 s). ^1H NMR spectra were recorded at 600 MHz and 300 K on a Bruker AM 600 or at 400 MHz on a JEOL JNM-GSX-400 spectrometer. $CDCl_3$ δ_C 77.0 and TMS δ_H 0.0 were used as internal references for $CDCl_3$ solutions. ^2H NMR spectra were recorded at 61 MHz on a JEOL JNM-GSX-400 spectrometer in an unlocked mode with ^1H broad-band decoupling (pulse width = 45°, acquisition time = 4.096 s). Natural abundance $CDCl_3$ δ_D 7.25 was used as an internal standard. Infrared spectra were obtained with a Hitachi 215 IR spectrometer. Mass spectra were recorded on a JEOL JMS-DX303 spectrometer, and CD spectra were obtained on a JASCO J-20A spectropolarimeter.

Culture. *S. antibioticus* IFO 12838 was obtained from The Institute for Fermentation, Osaka, Japan. One strain of the organism, which was selected as a high producer of VB A by single-cell isolation,[9] was used in this study. Spores of the strain maintained on oatmeal agar slants were inoculated into 100 mL of the medium for preculture, which consisted of 1% soybean meal, 1% potato starch, 1% corn steep liquor, 0.5% NaCl, 0.05% $MgSO_4$, and 0.2% K_2HPO_4 in a 500-mL Erlenmeyer flask. The flask was incubated at 28 °C and 150 rpm on a rotary shaker for 72 h. Three milliliters of this culture was transferred to 100 mL of the production medium, consisting of 0.75% Bacto-casitone, 0.75% yeast extract, 1.5% glycerol, and 0.25% NaCl (pH 6.5) in a 500-mL Sakaguchi flask.

(15) Bosch, H. V. D. *Ann. Rev. Biochem.* **1974**, *43*, 243–277.
(16) Rickards, R. W. *Proc. Asian Symp. Med. Plants Spices, 6th* **1989**, 91–100, and personal communication.
(17) Tanaka, S.; Sakuda, S.; Yamada, Y. Unpublished data.

Incubation was carried out on a reciprocating shaker at 28 °C and 120 rpm for 96 h. Sodium acetate (100 mg) dissolved in 1 mL of the production medium was added twice at the 24th and 48th hours of the cultivation.

Isolation of Virginiae Butanolide A Dibenzoate. VB A was isolated as its dibenzoate from the culture broth by the following procedure. Typically, the mycelium was removed from the broth (10 × 100 mL) by centrifugation (6000 g, 10 min), and the supernatant fluid was adsorbed on a charcoal (200 mL, Charcoal Activated, Wako Pure Chemical Ind.) column. After being washed with 600 mL of H_2O, the column was eluted successively with 1 L each of 10% MeOH, 25% MeOH, 50% MeOH, and 100% MeOH. VB A was eluted with 100% MeOH, and the solution was concentrated in vacuo to about 5 mL and brought to 200 mL with H_2O. Ten milliliters of the solution was applied to a SEP-PAK C_{18} cartridge (Waters) preequilibrated with H_2O and eluted successively with 5 mL each of H_2O, 10% CH_3CN, 20% CH_3CN, 50% CH_3CN, and 100% CH_3CN. This operation was repeated 20 times, and all of the 50% CH_3CN eluates were combined and concentrated. After being lyophilized, the residue (ca. 50 mg) was benzoylated with benzoyl cyanide (500 mg) and tri-*n*-butylamine (50 μL) in dry CH_3CN (1 mL) for 10 min. The reaction was stopped by adding 200 mL of H_2O and then treated on a SEP-PAK C_{18} cartridge in the same manner as mentioned above. VB A dibenzoate was eluted with 100% CH_3CN, and the fraction was finally purified by HPLC on a Cosmosil $5C_{18}$ column (8 × 250mm, Nacalai Tesque) using a mobile phase of CH_3CN–H_2O (80:20). With a flow rate of 1.5 mL/min, the peak having the retention time of 20.0 min afforded ca. 1 mg of VB A dibenzoate (**3a**) as a colorless oil: CI-MS *m/z* 439 $(M + H)^+$, 317, 123; HRMS-EI *m/z* 316.1686 $(M - C_6H_5CO_2H)^+$ (calcd for $C_{19}H_{24}O_4$ 316.1675); CD (CH_3CN) $\Delta\epsilon_{223}$ = −2.4; ^1H NMR δ ($CDCl_3$, 600 MHz) 8.00 (2 H, m, Ph), 7.57 (1 H, m, Ph), 7.45 (2 H, m, Ph), 5.59 (1 H, m, H-6), 4.54 (1 H, dd, $J_{4a,3}$ = 8.1 Hz, $J_{4a,4b}$ = 9.4 Hz, H-4a), 4.45 (1 H, dd, $J_{5a,5b}$ = 11.3 Hz, $J_{5a,3}$ = 5.5 Hz, H-5a), 4.42 (1 H, dd, $J_{5b,3}$ = 6.1 Hz, H-5b), 4.22 (1 H, dd, $J_{4b,3}$ = 5.3 Hz, H-4b), 3.17 (1 H, m, H-3), 2.88 (1 H, dd, $J_{2,3}$ = 5.6 Hz, $J_{2,6}$ = 4.8 Hz, H-2), 1.91 (1 H, m, H-7a), 1.79 (1 H, m, H-7b), 1.45 (2 H, m, H-10), 1.41 (1 H, m, H-8a), 1.34 (1 H, m, H-8b), 1.17 (2 H, m, H-9), 0.8 (3 H, d, *J* = 6.6 Hz, H-11), 0.8 (3 H, d, *J* = 6.6, H-12); ^{13}C NMR δ ($CDCl_3$, 150 MHz) 175.6 (C-1), 166.2 (C=O), 165.5 (C=O), 133.5 (Ph), 133.3 (Ph), 129.6 (Ph), 129.6 (Ph), 129.6 (Ph), 129.6 (Ph), 129.2 (Ph), 129.2 (Ph), 128.6 (Ph), 128.6 (Ph), 128.5 (Ph), 128.5 (Ph), 73.1 (C-6), 69.2 (C-4), 65.2 (C-5), 46.4 (C-2), 38.4 (C-9), 36.4 (C-3), 33.0 (C-7), 27.7 (C-10), 23.2 (C-8), 22.4 (C-11), 22.4 (C-12). Each signal was unambiguously assigned by DEPT, COSY, and C–H COSY experiments.

[1-^{13}C]Isovaleric Acid. Isobutyl bromide (3.12 g) was added to a solution of 25 mL of dry CH_3CN containing 1.0 g of $K^{13}CN$ (99 atom % ^{13}C, Sigma), 0.4 g of 18-crown-6, and 1.0 g of KI. The reaction mixture was refluxed for 9 h and filtered. The filtrate was distilled at atmospheric pressure at 150 °C, yielding a mixture of isovaleronitrile and CH_3CN. Then 100 mL of concentrated HCl was added, and the solution was refluxed for 5 h. The reaction solution was extracted with ethyl acetate, and the acidic fraction obtained was dried over anhydrous Na_2SO_4 and concentrated in vacuo to give 100 mg of [1-^{13}C]isovaleric acid (the overall yield was very low (6.5%) because of steric hindrance of methyl groups in the substitution reaction between isobutyl bromide and KCN): ^1H NMR δ ($CDCl_3$, 600 MHz) 2.33 (2 H, t, J_{CH} = 6.9 Hz, H-2), 2.11 (1 H, m, H-3), 0.99 (6 H, d, *J* = 6.7 Hz, H-4 and -5); ^{13}C NMR δ ($CDCl_3$, 150 MHz) 179.2 (C-1), 43.1 (d, $^1J_{CC}$ = 54.9 Hz, C-2), 25.5 (d, $^2J_{CC}$ = 1.7 Hz, C-3), 22.4 (C-4 or -5), 22.3 (C-4 or -5).

[1,3-$^{13}C_2$]Glycerol. This was synthesized from $K^{13}CN$ (99 atom % ^{13}C, Sigma) and sodium [1-^{13}C]acetate (99.4 atom % ^{13}C, MSD Isotopes) via bromoacetic acid and diethyl malonate as described by Floss et al.[18] Since unlabeled acetic acid was contaminated during the course of the reaction from [1-^{13}C]acetic acid to [1-^{13}C]bromoacetic acid, the obtained [1,3-$^{13}C_2$]glycerol contained 77 atom % ^{13}C at C-1 and 99 atom % ^{13}C at C-3.

[1-^{13}C]-5-Methylhexanenitrile. This was prepared from 1-bromo-4-methylpentane and $K^{13}CN$ (99 atom % ^{13}C, Sigma) in 88.5% yield by the same method used to prepare [1-^{13}C]isovaleronitrile: EI-MS *m/z* 113 $(M + H)^+$, 111 $(M - H)^+$, 97 $(M - CH_3)^+$; IR (film) 2940, 2850, 2240, 1465, 1415, 1385, 1365 cm^{-1}; ^1H NMR δ ($CDCl_3$, 600 MHz) 2.29 (2 H, dt, $^2J_{CH}$ = 9 Hz, H-2), 1.62 (2 H, m), 1.55 (1 H, m), 1.30 (2 H, m), 0.87 (6 H, d, *J* = 5.5 Hz); ^{13}C NMR δ ($CDCl_3$, 150 MHz) 37.8 (d, $^3J_{CC}$ = 3.2 Hz, C-4), 27.4 (C-5), 23.3 (d, $^2J_{CC}$ = 2.7 Hz, C-3), 21.6 (C-6 and -7), 17.3 (d, $^1J_{CC}$ = 55.8 Hz, C-2).

Methyl [2,3-$^{13}C_2$]-3-Oxo-7-methyloctanoate. Zinc powder was washed successively with 2% HCl, H_2O, ethanol, acetone, and anhydrous ether and dried at 100 °C for 15 min under reduced pressure for activation just before use. Methyl [2-^{13}C]bromoacetate (1.74 g), which was prepared from [2-^{13}C]bromoacetic acid (99 atom % ^{13}C, Sigma) and CH_2N_2, was added dropwise to a mixture of 3 g of zinc powder, 3.16 g of [1-^{13}C]-5-methylhexanenitrile, 5 mL of dry tetrahydrofuran, and 5 mL of trimethyl borate at room temperature, and the reaction mixture was refluxed for 45 min under N_2 gas. After cooling, 10 mL of ether, 5 mL of glycerol, and 4 mL of ammonium hydroxide were added to the mixture, and it was transferred to a separatory funnel. The organic layer was washed with H_2O carefully until it became clear and was then dried over anhydrous Na_2SO_4 and concentrated to obtain a brown oil. The oil was dissolved in 15 mL of tetrahydrofuran and mixed with 4 mL of 12 N H_2SO_4. The solution was stirred for 3 h at room temperature and extracted with ethyl acetate. After the solution was washed with saturated $NaHCO_3$ and H_2O, the organic layer was dried and concentrated. The obtained oil was purified on a silica gel (Silica gel 60, Merck Art. 7734) column with a hexane–ethyl acetate (20:1) solvent to afford 0.92 g of methyl [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoate as an oil in 43.3% yield: EI-MS *m/z* 188 (M^+), 168; IR (film) 2960, 2870, 1760–1710, 1660, 1640, 1250 cm^{-1}; ^1H NMR δ ($CDCl_3$, 600 MHz) 3.70 (3 H, s, OCH_3), 3.41 (2 H, dd, $^1J_{CH}$ = 130.1 Hz, $^2J_{CH}$ = 6.1 Hz, H-2), 2.48 (2 H, q, *J* = 7.4 Hz, $^2J_{CH}$ = 7.4 Hz, H-4), 1.56 (1 H, m, H-7), 1.50 (2 H, m, H-5), 1.14 (2 H, m, H-6), 0.84 (6 H, d, *J* = 6.5 Hz, H-8 and -9); ^{13}C NMR δ ($CDCl_3$, 150 MHz) 202.7 (d, $^1J_{CC}$ = 37.0, C-3), 51.0 (d, C-2). Unlabeled methyl 3-oxo-7-methyloctanoate: ^{13}C NMR 202.7, 167.6, 52.2, 51.0, 43.2, 38.1, 27.7, 22.4, 22.4, 21.3.

[2,3-$^{13}C_2$]-3-Oxo-7-methyloctanoic Acid. A solution of 0.90 g of [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoic acid, 0.40 g of K_2CO_3, 35 mL of tetrahydrofuran, and 30 mL of H_2O was stirred for 120 h at room temperature. After the tetrahydrofuran was removed by evaporation, the reaction solution was extracted with ethyl acetate. The ester (0.26 g) was recovered from the neutral fraction, and the acidic fraction afforded 0.24 g of [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoic acid as a white plate in 28.8% yield: mp 58–60 °C; FABMS *m/z* 175 $(M + H)^+$; IR (Nujol) 1735, 1715, 1385 cm^{-1}; ^1H NMR δ ($CDCl_3$, 600 MHz) 3.48 (2 H, dd, $^1J_{CH}$ = 128.4 Hz, $^2J_{CH}$ = 5.5 Hz, H-2), 2.53 (2 H, q, *J* = 7.4 Hz, $^2J_{CH}$ = 7.4 Hz, H-4), 1.60 (1 H, m, H-7), 1.53 (2 H, m, H-5), 1.17 (2 H, m, H-6), 0.87 (6 H, d, *J* = 6.5 Hz, H-8 and -9); ^{13}C NMR δ ($CDCl_3$, 150 MHz) 204.7 (d, $^1J_{CC}$ = 37.3 Hz, C-3), 47.7 (d, C-2). Unlabeled 3-oxo-7-methyloctanoic acid: ^{13}C NMR 204.7, 171.0, 47.7, 43.5, 38.1, 27.8, 22.4, 22.4, 21.3.

[2,3-$^{13}C_2$]-3-Oxo-7-methyloctanoic Acid *N*-Acetylcysteamine Thioester 11. 1,1′-Carbonyldiimidazole (0.25 g) was added to a solution of 0.22 g of [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoic acid and 20 mL of dry CH_3CN at −5 °C. After the mixture was stirred for 1.5 h at below 0 °C, 0.18 g of *N*-acetylcysteamine was added and the solution was further stirred for 1 h at the same temperature. After the CH_3CN was removed by evaporation, the residue was partitioned between H_2O and ethyl acetate. The organic layer was dried, concentrated, and purified on a silica gel column with ethyl acetate as the solvent to give 77.5 mg of [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoic acid *N*-acetylcysteamine thioester as a white plate in 22.3% yield: mp 46–48 °C; FABMS *m/z* 276 $(M + H)^+$; IR (Nujol) 3300, 3100, 1715, 1700, 1660, 1640, 1570, 1385, 1375, 1300, 1210 cm^{-1}; ^1H NMR δ ($CDCl_3$, 600 MHz) 5.87 (1 H, br s, NH), 3.68 (2 H, dd, $^1J_{CH}$ = 131.0 Hz, $^2J_{CH}$ = 6.0 Hz, H-2), 3.42 (2 H, m, CH_2N), 3.05 (2 H, m, SCH_2), 2.50 (2 H, q, *J* = 7.4 Hz, $^2J_{CH}$ = 7.4 Hz, H-4), 1.97 (3 H, s, CH_3C=O), 1.56 (1 H, m, H-7), 1.52 (2 H, m, H-5), 1.16 (2 H, m, H-6), 0.87 (6 H, d, *J* = 6.9 Hz, H-8 and -9); ^{13}C NMR δ ($CDCl_3$, 150 MHz) 202.3 (d, $^1J_{CC}$ = 35.9 Hz, C-3), 57.2 (d, C-2). Unlabeled thioester: ^{13}C NMR 202.3, 177.6, 170.4, 57.2, 43.6, 39.1, 38.1, 29.2, 27.8, 23.1, 22.4, 22.4, 21.2.

[4,5-^2H]-6-Dehydro-VB A and [4,5-^2H]VB A Dibenzoate. These were synthesized following the same route used for VB A (Scheme II).[4] The only difference was that $NaBD_4$ (98 atom % ^2H, Aldrich Chemical Co.) was used instead of $NaBH_4$ at the reduction step from diethyl formylsuccinate to 3-(hydroxymethyl)butanolide. By the reaction, [4a,4b,5a-$^2H_3$]-, [4a,4b,5b-$^2H_3$]-, [4a,5a,5b-$^2H_3$]-, and [4b,5a,5b-$^2H_3$]-3-(hydroxymethyl)butanolide were mainly obtained at about the same molar ratio. [4,5-^2H]VB A dibenzoate: CI-MS *m/z* (rel intensity as that of *m/z* 442 is 100) 439 $(M + H)^+$ (<0.26), 440 $(M + H + 1)^+$ (1.6), 441 $(M + H + 2)^+$ (22.7), 442 $(M + H + 3)^+$ (100); ^1H NMR δ ($CDCl_3$, 600 MHz) 4.54 (0.25 H, br d, *J* = 8.1 Hz, H-4a), 4.45 (0.25 H, br d, *J* = 5.5 Hz, H-5a), 4.42 (0.25 H, br d, *J* = 6.1 Hz, H-5b), 4.22 (0.25 H, br d, *J* = 5.3 Hz, H-4b).

Administration of Labeled Compounds to *S. antibioticus*. Labeled acetate, isovalerate, or 6-dehydro-VB A was dissolved in the production medium. Labeled glycerol was dissolved in the modified production medium which contains 1.5% potato starch instead of glycerol for labeled glycerol. Labeled β-keto acid, methyl ester, and thioester were dissolved in ethanol. The solutions of medium and precursor were autoclaved

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before administration, but the ethanol solution was directly added to the culture. Less than 1 mL of medium and 0.1 mL of ethanol solution were used for administration into each flask at one time.

In feeding experiments with labeled acetate, a mixture of 50 mg of sodium [1-^{13}C]- or [2-^{13}C]acetate (99 atom % ^{13}C, Sigma) and 50 mg of unlabeled sodium acetate was added twice to each 500-mL flask containing 100 mL of the medium at the 24th and 48th hours of cultivation. After workup, this afforded 1.87 and 188 mg of **3a** from 15 × 100 mL broths in the experiments with sodium [1-^{13}C]- and [2-^{13}C]-acetate, respectively. For labeled isovalerate, 2.5 mg of sodium [1-^{13}C]isovalerate was fed to the culture four times (each 2-h interval from 24 to 30 h of cultivation) and again four times (from 48 to 54 h of cultivation); 0.33 mg of **3a** was obtained from 5 × 100 mL broths.

In feeding experiments with ^{13}C- and ^{2}H-labeled glycerol, the modified production medium was used for cultivation. Fifty milligrams of [1,3-$^{13}C_2$]glycerol was added twice to the culture at the 24th and 48th hours of cultivation, and 0.062 mg of **3a** was obtained from 5 × 100 mL broths. CI-MS of this labeled **3a**: m/z (rel intensity as that of m/z 439 $(M + H)^+$ is 100) 439 (100), 440 (38.7), 441 (14.6); that of natural **3a** 439 (100), 440 (30.2), 441 (5.9). From the MS data, the relative abundance of non-, mono-, and di-^{13}C-labeled molecules in the labeled **3a** was calibrated as 100:8.5:6.1,[19] indicating that the mono- and dilabeled forms increased by 7.4% and 5.3%, respectively. In the feeding experiment with [$^{2}H_5$]glycerol (98 atom % ^{2}H, CEA), 2.5 mg of the labeled glycerol was fed to the culture eight times in a manner similar to that of labeled isovalerate, and 15 mg of **3a** was obtained from 250 × 100 mL broths.

Doubly labeled β-keto acid or its derivative was fed to the culture eight times in the same manner as above. After workup, this afforded 0.18, 0.56, and 0.37 mg of **3a** from 5 × 100, 10 × 100, and 8 × 100 mL broths in feeding experiments in which each flask received at one time 1.25 mg of [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoic acid, 1.1 mg of methyl [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoate, and 1.2 mg of [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoic acid *N*-acetylcysteamine thioester, respectively.

In feeding experiments with [4,5-^{2}H]-6-dehydro-VB A, 1.0 or 10 mg of the labeled compound was added to the culture eight times as above, and 0.48 and 2.84 mg of **3a** were obtained from each 2 × 100 mL broth, respectively.

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Registry No. 3, 109215-47-6; **3a**, 135304-75-5; [4,5-^{2}H]-**3a**, 137570-41-3; **11**, 137570-40-2; **12**, 122922-47-8; glycerol, 56-81-5; isovaleric acid, 503-74-2; [1-^{13}C]isovaleric acid, 87994-84-1; [1,3-$^{13}C_2$]glycerol, 102088-01-7; [1-^{13}C]-5-methylhexanenitrile, 137570-37-7; methyl [2,3-$^{13}C_2$]-3-oxo-7-methyl loctanoate, 137570-38-8; [2,3-$^{13}C_2$]-3-oxo-7-methyloctanoic acid, 137570-39-9.

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A Corrected Structure for Pyrrolosine

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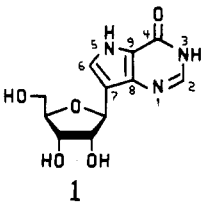
Abstract: Pyrrolosine, a C-nucleoside recently isolated from *Streptomyces albus*, was assigned structure **1** by Ikegami and co-workers largely on the basis of single-crystal X-ray diffraction. This is also the structure previously assigned to the synthetic C-nucleoside, 9-deazainosine. Because the physical and biological properties of the two compounds differ, Ikegami and co-workers suggested that the structure of 9-deazainosine should be reinvestigated. However, it is shown in this paper that the structure they proposed for pyrrolosine is incorrect. Furthermore, on the basis of the reported physical properties, it is shown that pyrrolosine is actually the known furo[3,2-*d*]pyrimidine C-nucleoside adenosine analogue **2**. This conclusion, which is more consistent with the types of biological activity reported for pyrrolosine, has now been confirmed by reanalysis of the X-ray data published by Ikegami and co-workers. All bond angles and distances in the reinterpreted model are consistent with structure **2** for pyrrolosine. The model also accounts more adequately for the hydrogen atoms of the base and the methanol of solvation and for all potential hydrogen bond donors. After the appropriate changes were made, the crystallographic *R* factor decreased from the reported value of 0.065 to 0.039. X-ray crystallographic data as well as additional UV and NMR studies have been used to reaffirm that 9-deazainosine is indeed the pyrrolo[3,2-*d*]pyrimidin-4-one **1**.

Pyrrolosine, a new inhibitor of starfish RNA synthesis, was isolated recently from the culture broth of *Streptomyces albus* by Ikegami and co-workers.[1] Based principally on their interpretation of single-crystal X-ray diffraction data, these investigators concluded that the structure of pyrrolosine is 1,5-dihydro-7-β-D-ribofuranosyl-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (**1**). This is also the structure assigned to 9-deazainosine, a synthetic C-nucleoside that we originally reported in 1980.[2] Since a comparison of the physical and biological properties revealed that the two substances are different, Ikegami and co-workers suggested that the chemical structure of our "reputed" 9-deazainosine should be promptly reinvestigated. In fact the structure of 9-deazainosine already rests solidly on an unambiguous synthetic route,[2,3] on



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convincing spectroscopic evidence,[2,3] and on a substantial body of chemical transformations.[4] However, in view of the important

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