Biosynthetic Studies on Virginiae Butanolide A, a Butyrolactone Autoregulator from *Streptomyces*. Part 2.[1] Preparation of Possible Biosynthetic Intermediates and Conversion Experiments in a Cell-free System

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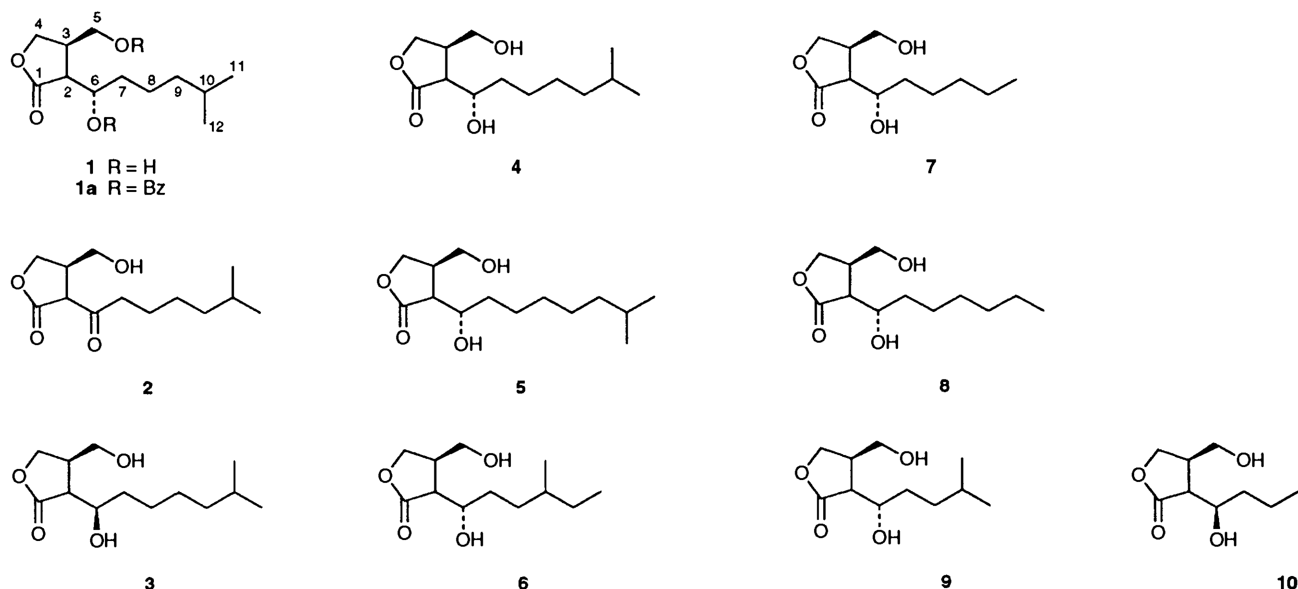
Virginiae butanolide A **1** is one of the virginiamycin-inducing factors and has a 2,3-disubstituted butanolide skeleton which is common to other signal molecules in *Streptomyces*. To investigate the biosynthetic pathway of **1**, possible intermediates have been synthesized. Conversion experiments with these intermediates in a cell-free system showed that the ester **11** was a biosynthetic intermediate of **1**.

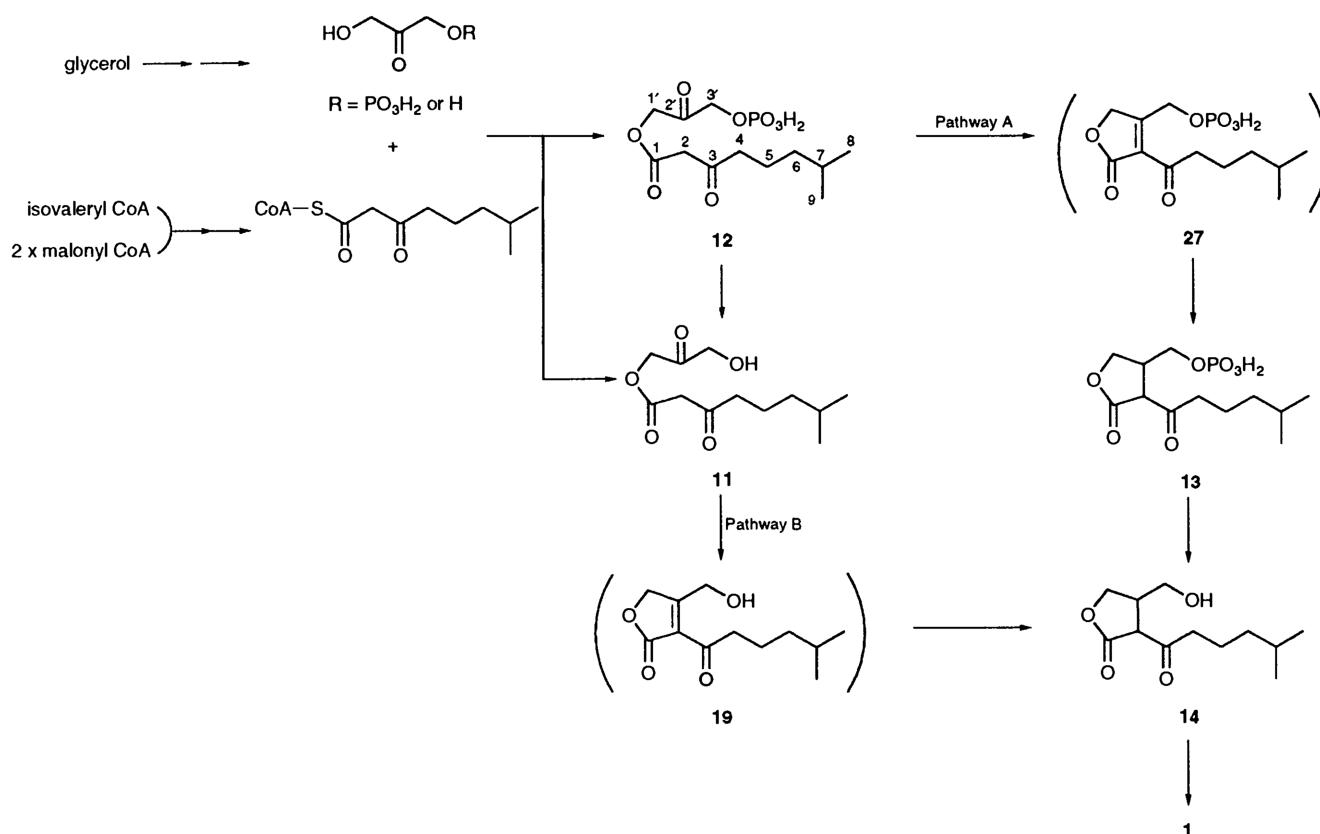
In *Streptomyces*, a number of signal molecules which regulate secondary metabolite production or cytodifferentiation are known. Virginiae butanolide (VB) A **1** was isolated as one such autoregulator from a culture broth of *Streptomyces virginiae*.[2] It induces the production of virginiamycin in the strain and has a unique 2,3-disubstituted butanolide skeleton which is common to other signal molecules in *Streptomyces*, such as A factor **2**,[3] factor 1 **3**,[4] Gräfe's factors **1**, **4** and **5**,[5] VB B-E **6**, **7**, **8** and **9**,[6,7] and IM-2 **10**.[8] With respect to the biosynthesis of this peculiar butyrolactone molecule, we first reported that the skeleton of **1** is formed from two molecules of acetic acid and one molecule each of isovaleric acid and glycerol,[9] and that the biosynthetic pathway of **1** involves coupling between a β-keto acid derivative and a dihydroxyacetone type C_3 unit from glycerol.[1] From these results, we proposed a plausible pathway for formation of the VB A molecule, which is shown in Scheme 1. In this route, a β-keto ester having the ketone group on its dihydroxyacetone moiety is the key intermediate and the butyrolactone skeleton is formed by intramolecular aldol condensation. Phosphate groups of hypothetical intermediates may be present if we assume that the acylation step to form the ester resembles that in glycerolipid biosynthesis.[10] In this paper, we describe the preparation of possible biosynthetic intermediates of **1** and conversion experiments with them in a cell-free system in order to clarify the biosynthetic pathway of **1**.

Results and Discussion

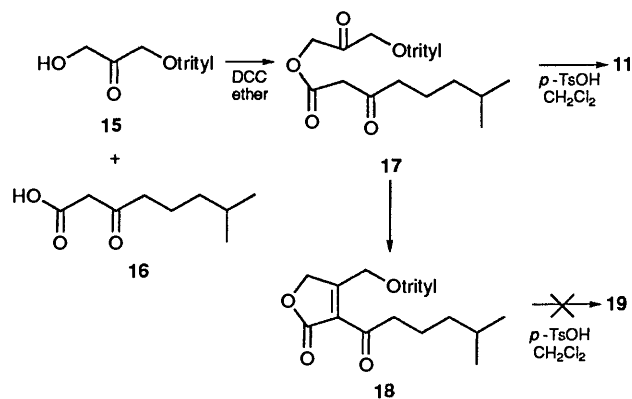
Preparation of Possible Biosynthetic Intermediates of Virginiae Butanolide A.—First, the β-keto ester **11** was prepared following Scheme 2. The trityl ether of dihydroxyacetone **15** was coupled with the β-keto acid **16** in the presence of dicyclohexylcarbodiimide. The pure ester **17** was obtained by purification with reverse-phase HPLC. In chromatography on a silica gel column, the ester was not eluted from the column and only the butenolide **18** was obtained, indicating that cyclization by intramolecular aldol condensation and subsequent dehydration occurred during the chromatography.[11] The treatment of **17** with molecular sieves in dichloromethane also afforded the butenolide **18**. Deprotection of the trityl group of **17** with toluene-*p*-sulfonic acid in dichloromethane afforded **11**. The ester **11** formed was not recovered from a silica gel column, as in the case of **17**. Moreover, **11** could not be purified by reverse-phase HPLC because of the instability of **11** in aqueous solution. Only rapid treatment of the reaction mixture on a Sep-Pak silica cartridge afforded pure **11**. To prepare the butenolide **19**, cleavage of the trityl group of **18** was also attempted, but this reaction gave no significant product. This result indicated that the butenolide **19** was too labile to be isolated.

The phosphate of the ester **11**, **12**, was next prepared following Scheme 3. The diazo compound **26** was synthesized





Scheme 1 Biosynthetic pathway of VB A



Scheme 2

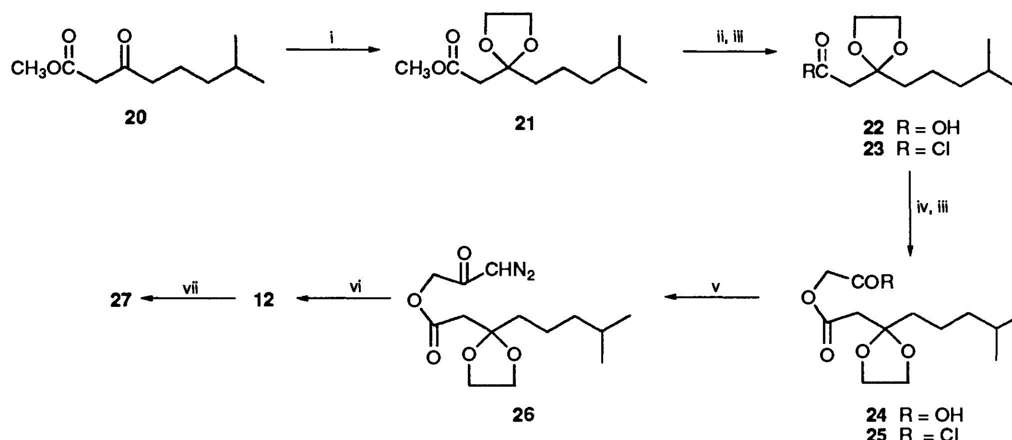
starting from the β-keto ester **20**, mainly following the method of Hajra *et al.*[12] By the reaction of **26** with phosphoric acid, the phosphoryl group was introduced and the ketal was deprotected to afford the phosphate **12**. Pure **12** was obtained by purification on reverse-phase HPLC under acidic conditions. Treatment of the phosphate **12** with saturated aqueous sodium hydrogen carbonate followed by acidification of the solution and subsequent extraction with ethyl acetate, gave the butenolide phosphate **27**.

The preparation of 6-dehydro VB A phosphate **13** was carried out following Scheme 4. The butanolide **28** was converted into the diphenyl phosphate **29**, and hydrogenolysed to afford the phosphate **30**. The hydroxy groups of **30** were trimethyl silylated, and without purification of the silyl intermediate **31**, acylation with 5-methylhexanoyl chloride and subsequent treatment with water afforded the phosphate **13**.

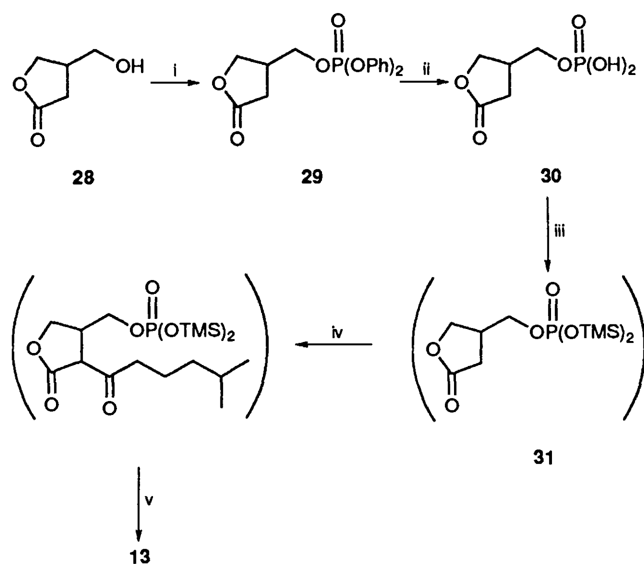
Conversion Experiments with Possible Biosynthetic Intermediates of VB A in a Cell-Free System.—*Streptomyces antibioticus*, which was found to be a high producer of VB A,[13] was used for the conversion experiments. *S. antibioticus* was cultivated in a 500 cm^3 Sakaguchi flask containing 100 cm^3 of medium on a reciprocating shaker. VB A production started at 24 h. Since cells cultivated for 28 h showed the highest 6-dehydro-VB A **14** to VB A transformation activity, cells were collected by filtration at that time and disrupted by sonication to give a cell-free system. After the conversion experiments with the cell-free system, VB A formed was converted into its dibenzoate, **1a**, and quantified by analysis using reverse-phase HPLC. Racemic VB D **8** was used as an internal standard.

Conversion experiments with 6-dehydro-VB A **14**, which has been proved to be a biosynthetic precursor of **1** in an *in vivo* system,[1] were carried out first. In the experiments, the enzyme solution of a phosphate buffer, which was roughly purified by ammonium sulfate precipitation, was used in the cell-free system. Table 1 shows the results obtained. 6-Dehydro-VB A was effectively converted into VB A in the presence of NADPH or NADH. It seemed that NADPH was mainly used in the reduction step of the 6-keto group of **14**, because **14** was more effectively converted into **1** in the presence of NADPH than NADH. Detection of a little conversion without the addition of co-factors suggested that the enzyme solution contained a small amount of endogenous NADPH or NADH.

Next, transformation experiments with the ester **11** were performed. The crude enzyme solution purified by ammonium sulfate precipiation was also used in these experiments. Since the ester **11** was unstable in aqueous solution, it was added to the solution five times at 30-min intervals. Table 2 shows the results obtained. The amount of VB A formed by the conversion of **11** was much smaller than that from 6-dehydro-VB A. The conversion from ester **11** to VB A was, however, clearly observed in the presence of both NADPH and NADH. Because the addition of NADPH, which was needed to convert **14** into **1**, was less effective for the conversion, NADH



Scheme 3 *Reagents:* i, $HO(CH_2)_2OH$, *p*-TsOH; ii, NaOH, $MeOH-H_2O$; iii, $(COCl)_2$; iv, $HOCH_2CO_2H$; v, CH_2N_2; vi, H_3PO_4; vi, sat'd. $NaHCO_3$, EtOAc extraction



Scheme 4 *Reagents:* i, $ClP(O)(OPh)_2$; ii, H_2-PtO_2; iii, MeC(:NTMS)O-TMS; iv, LDA, $ClCO(CH_2)_3CHMe_2$; v, H_2O

was preferentially used in the reduction step of the butenolide **19**, which was a putative intermediate during the biosynthetic process from ester **11** to 6-dehydro-VB A.

From our previous results obtained in an experiment with the feeding of [$^2H_5$]glycerol, it was speculated that the H_s on C-4 was lost mainly stereospecifically during the reduction step of the butenolide **19**.[1] To examine this reaction with a cell-free system, a conversion experiment with ester **11** in ^2H_2O solution was carried out. The cells of *S. antibioticus* were disrupted in phosphate buffer prepared with ^2H_2O. NADPH and NADH was added to the suspension and the mixture was used for the transformation experiments. 6-Dehydro-VB A was also converted into **1** in the same cell-free system. Table 3 shows the area of each ^1H signal observed in the ^1H NMR spectra of the resulting **1a**. The observed area of the 2-H signal was much reduced in both the conversion experiments due to the enolization of the β-keto system. Incorporation of deuterium on C-6 suggested that NADP^{2}H (NAD^{2}H) was produced in the reaction solution. In the conversion experiment with ester **11**, high incorporation of deuterium was observed on C-4. The observed area of both of 4-H_R and 4-H_S signals became less than half of the natural value, but the area of 4-H_R was a little larger than that of 4-H_S. This result suggested that the hydrogen atoms on C-4 was being rapidly exchanged for deuterium in the solution during the conversion process, and at that time 4-H_S was somewhat preferentially lost compared with 4-H_R. Exchange of the hydrogen atoms on C-1′ of ester **11** did not occur in ^2H_2O solution without the cell extracts (data not shown). Low incorporation of deuterium was observed on C-3 in the case of ester **11**, suggesting that the hydrogen on C-3 came from NADH in the reduction process.

Table 1 Conversion experiments with 6-dehydro-VB A[a]

Expt.	6-Dehydro-VB A (μg)	NADH (μg)	NADPH (μg)	VB A formed (μg)[b]
1	100	100	0	1.5
2	100	100	0	1.8
3	100	0	100	2.6
4	100	0	100	2.8
5	100	0	0	0.5
6	100	0	0	0.5
7	0	0	0	Nd[c]
8	0	0	0	Nd

[a] Enzyme solution (1 cm^3) prepared by 80% ammonium sulfate precipitation was used. [b] Calibration from peak area of VB A dibenzoate and VB D dibenzoate (internal standard) on HPLC. [c] Not detected.

Table 4 shows the results of conversion experiments with the phosphates **12**, **27** and **13** as well as 6-dehydro-VB A and ester **11**. In these experiments, a buffer without phosphate was used to prepare the cell-free system to avoid its inhibitory effect against phosphatases. 6-Dehydro-VB A and the ester **11** were clearly converted into VB A, as previously observed (Tables 1 and 2). Significant conversion of the butanolide phosphate **13** into **1** was also observed, indicating that 6-dehydro-VB A was produced by the action of a non-specific or specific phosphatase in the conversion experiment with **13**. Transformation of the ester phosphate **12** into **1** was not effective, and conversion of the butenolide phosphate **27** was not detected under the tested conditions. It was not clear whether the ester phosphate **12** was directly transformed into the phosphate **13**, or first converted into the ester **11** by a phosphatase to produce **1**. A conversion experiment from **12** into **13** with a highly purified enzyme is necessary to confirm whether the pathway A in Scheme 1 is present or not.

The results obtained here proved that the biosynthetic pathway B from the ester **11** to VB A in Scheme 1 is present in *S. antibioticus*. A possible mechanism for the formation of the VB A skeleton from ester **11** is shown in Scheme 5. First, intramolecular aldol condensation occurs to form the butyrolactone skeleton. Dehydration gave the unsaturated butanolide intermediate as a keto–enol tautomeric mixture which upon subsequent reduction with NADH led to the 6-oxo skeleton.

Table 2 Conversion experiments with the ester **11** [a]

Expt.	Ester **11** (μg)	6-Dehydro-VB A (μg)	NADPH (μg)	NADH (μg)	VB A formed (μg)
1	100 × 5[b]	0	100	100	0.52
2	100 × 5	0	100	100	0.52
3	100 × 5	0	100	0	0.22
4	100 × 5	0	100	0	0.25
5	100 × 5	0	0	0	0.16
6	100 × 5	0	0	0	0.20
7	0	100	100	0	6.4
8	0	100	100	0	5.9
9	0	0	0	0	Nd[c]
10	0	0	0	0	Nd

[a] Enzyme solution (1 cm^3) prepared by 80% ammonium sulfate precipitation was used. The solution batch was different from that used in the experiments in Table 1. [b] Added five times at 30-min intervals. [c] Not detected.

Table 3 Area of ^1H signals in ^1H NMR spectra of VB A dibenzoate converted from 6-dehydro-VB A and the ester **11** in ^2H_2O solution

		Signal area [a]		
Proton	δ_H [b]	natural	from 6-dehydro-VB A	from ester **11**
2-H	2.88	1.00	0.27	0.28
3-H	3.17	0.97	0.97	0.72
4-H_R	4.54	1.03	0.97	0.42
4-H_S	4.22	1.00	1.00	0.32
5-H	4.45 4.42	2.08	2.08	1.84
6-H	5.59	1.00	0.47	0.60

[a] Based on the area of 11-H and 12-H as 6.0. [b] Signals do not overlap except for methylene protons on C-5.[1]

Finally, the 6-oxo group was reduced to a hydroxy group with a NADPH-dependent dehydrogenase to give the VB A molecule. Work to investigate the biosynthetic pathway before the formation of ester **11** is now in progress.

Experimental

General Methods.—^{13}C NMR spectra were recorded on a Bruker AM 600 spectrometer at 150 MHz, using $CDCl_3$ δ_C 77.0, CD_3CN δ_C 117.8 and CD_3OD δ_C 49.0 as internal references. ^1H NMR spectra were recorded at 600 MHz on a Bruker 600 or at 60 MHz on a Hitachi R-24B spectrometer, using TMS δ_H 0.0 as an internal reference. All *J* values are given in Hz. Mass spectra were obtained on a JEOL JMS-DX303 spectrometer. IR spectra were obtained with an Hitachi 215 or a JEOL JIR-AQS20M IR spectrometer.

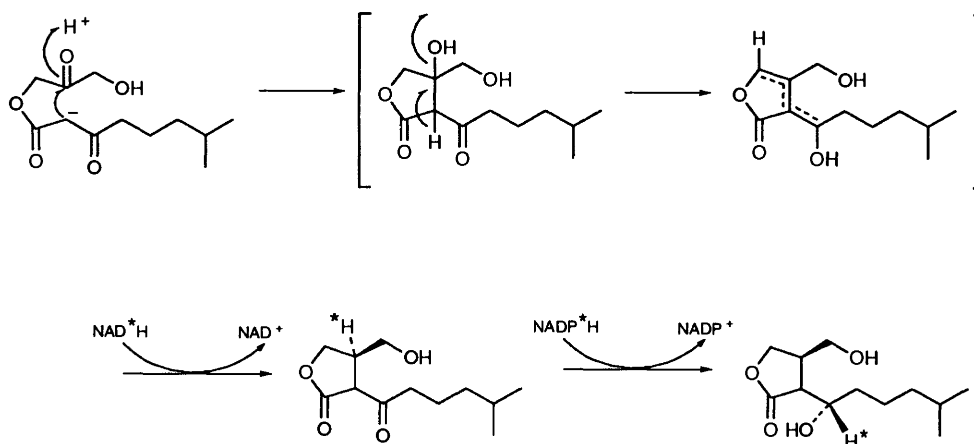
7-*Methyl*-1,3-*dioxooctyl* 2-*Oxo*-3-*trityloxypropyl Ether* **17**.—A mixture of hydroxymethyl trityloxymethyl ketone **15** (611 mg), 7-methyl-3-oxooctanoic acid **16** (196 mg), prepared by the method previously described,[1] and dicyclohexylcarbodiimide (DCC; 234 mg) was stirred in dry ether (5 cm^3) at 0 °C for 3.5 h to form a precipitate. This was filtered off, and the filtrate was purified by reverse-phase HPLC (column: Capcell-Pak C_{18}, 20 × 250 mm, Shiseido; mobile phase: gradient elution of 60–100% MeCN in water in 30 min; flow rate: 10 cm^3 min^{-1}). The peak having a retention time of 25.0 min afforded **17** as an oil (137 mg); *m/z* (CI–MS) 243 (M − trityl or trityl)$^+$, 225 (M − trityl − H_2O)$^+$; δ_H(600 MHz; $CDCl_3$) 7.2–7.5 (15 H, m, ph), 5.09 (2 H, s, 1'-H), 3.90 (2 H, s, 3'-H), 3.55 (2 H, s, 2-H), 2.59 (2 H, t, *J* 7.4, 4-H), 1.52–1.65 (3 H, m, 5-H and 7-H), 1.18 (2 H, m, 6-H) and 0.88 (6 H, d, *J* 6.6, 8-H and 9-H); λ_{max}(MeCN)/nm end absorption; ν_{max}/cm^{-1} 1740, 1720, 1655, 1620 and 1595.

2-(5'-*Methylhexanoyl*)-3-*trityloxymethylbutenolide* **18**.—The mixture of **15** (321 mg), **16** (103 mg) and DCC (123 mg) was allowed to react under conditions identical with those described above. After the precipitate had been filtered off, the filtrate was chromatographed on a silica gel column using a solvent system of hexane–ethyl acetate (4.5:1). Crude **18** (114 mg) eluted from the column was purified by normal phase HPLC (column: Unisil Q100-10, 4.6 × 250 mm, Gasu,uro Kogyo; mobil phase: isocratic elution with hexane–ethyl acetate, 11:1, flow rate: 1 cm^3 min^{-1}). The peak having a retention time of 10.0 min afforded **18** as an oil (50 mg); *m/z* (CI–MS) 243 (trityl), 225 (M − trityl)$^+$ and 167; δ_H(600 MHz; $CDCl_3$) 7.2–7.5 (15 H, m, Ph), 5.12 (2 H, s, 4-H), 4.74 (2 H, s, 5-H), 2.88 (2 H, t, *J* 7.4, 7-H), 1.52–1.58 (3 H, m, 8-H and 10-H), 1.18 (2 H, m, 9-H) and 0.87 (6 H, d, *J* 6.4, 11-H and 12-H); δ_C(150 MHz; $CDCl_3$) 196.6 (C-6), 177.5 (C-1), 170.3 (C-3), 142.8 (Ph), 128.4 (Ph), 128.1 (Ph), 127.5 (Ph), 123.0 (C-2), 87.7 (CPh_3), 70.3 (C-4), 62.3 (C-5), 41.7 (C-7), 38.2 (C-9), 27.7 (C-10), 22.3 (C-11), 22.3 (C-12) and 20.9 (C-8); λ_{max}(MeCN/nm) end absorption; λ_{max}/cm^{-1} 1765, 1690 and 1630.

When **17** was treated with a mixture of molecular sieves 3A and 4A in CH_2Cl_2 for 4 h at room temperature, 25% of **17** was converted into **18**, deduced from the ^1H NMR spectrum of the reaction product.

3-*Hydroxy*-2-*oxopropyl* 7-*Methyl*-1,3-*dioxooctyl Ether* **11**.—A small amount of toluene-*p*-sulfonic acid was added to a solution of compound **17** (241 mg) in CH_2Cl_2 (5 cm^3) and stirred for 5 h at room temperature. The reaction mixture was concentrated and put on a Sep-Pak silica cartridge (Waters) pre-equilibrated with hexane–ethyl acetate (10:1). With the same solvent as eluent pure fractions were pooled and evaporated to afford **11** as oil (57 mg); half-life in 0.1 mol dm^{-3} phosphate buffer (pH 7.0) at 28 °C *ca.* 15 min; *m/z* (CI–MS) 245 (M + H)$^+$, 243 (M − H)$^+$, 227, 225, 183, 155, 129, 113 and 111; δ_H(600 MHz; $CDCl_3$) 4.84 (2 H, s, 1'-H), 4.42 (2 H, s, 3'-H), 3.59 (2 H, s, 2-H), 2.57 (2 H, t, *J* 7, 4-H), 1.5–1.65 (3 H, m, 5-H and 7-H), 1.18 (2 H, m, 6-H) and 0.90 (6 H, *J* 6, 8-H and 9-H); δ_C(150 MHz; $CDCl_3$) 203.7 (C-3 or C-2'), 202.2 (C-3 or C-2'), 166.4 (C-1), 66.6 (C-1' or C-3'), 66.3 (C-1' or C-3'), 48.6 (C-2), 43.4 (C-4), 38.2 (C-6), 27.8 (C-7), 22.4 (C-8), 22.4 (C-9) and 21.3 (C-5); λ_{max}(MeCN)/nm 248 (730); ν_{max}/cm^{-1} 1741 and 1720.

O-(3,3-*Ethylenedioxy*-7-*methyloctanoyl*)*glycolic Acid* **24**.—Methyl 3,3-ethylenedioxy-7-methyloctanoate **21** (1.99 g), was prepared by the reaction of methyl 3-oxo-7-methyloctanoate **20** (2.0 g) and ethyleneglycol (2.0 g) in the presence of toluene-*p*-sulfonic acid (catalytic amount). **21**: δ_H(60 MHz; $CDCl_3$) 3.92 [4 H, s, $O(CH_2)_2O$], 3.60 (3 H, s, OCH_3), 2.62 (2 H, s, 2-H), 1.1–2.0 (7 H, m) and 0.9 (6 H, d, *J* 6, 8-H and 9-H). Hydrolysis of **21** (1.99 g) with a solution of sodium hydroxide (0.33 g) in MeOH–H_2O (4:1; 30 cm^3) afforded 3,3-ethylenedioxy-7-methyloctanoic



Scheme 5 Possible mechanism of VB A skeleton formation from 3-hydroxy-2-oxopropyl 7-methyl-1,3-dioxooctyl ether **11**

Table 4 Conversion experiments with phosphates, **12**, **13** and **27**[a]

Expt.	Substrate[b]	VB A formed (μg)
1	12	0.52
2	12	0.44
3	11	3.8
4	11	4.5
5	27	Nd[c]
6	27	Nd
7	13	6.2
8	13	6.3
9	6-dehydro-VB A	22.9
10	6-dehydro-VB A	21.8
11	No[d]	Nd
12	No	Nd

[a] A cell extract suspension (4 cm^3) prepared by sonication was used. NADH (300 μg) and NADPH (300 μg) was added to the suspension. [b] Substrate (100 μg) was added at 30-min intervals (× 5). [c] Not detected. [d] No addition.

acid **22** (1.54 g); δ_H(60 MHz; $CDCl_3$) 10.85 (1 H, s, OH), 3.92 [4 H, s, $O(CH_2)_2O$], 2.65 (2 H, s, 2-H), 1.1–2.0 (7 H, m), 0.9 (6 H, d, *J* 6, 8-H and 9-H). 3,3-Ethylenedioxy-7-methyloctanoyl chloride **23** was prepared by the treatment of **22** with oxalyl chloride in dry benzene. **23**: ν_{max}/cm^{-1} 1810. The acid chloride **23** (1.67 g) was added dropwise to a solution of glycolic acid (0.67 g), dry pyridine (30 cm^3) and chloroform (12.5 cm^3) at −10 °C under an atmosphere of nitrogen, and the solution was stirred at −10 °C for 2 h and, subsequently, at room temperature for 4 h. A solution of MeOH–0.1 mol dm^{-3} HCl (1:1; 80 cm^3) was added to the reaction mixture and the solution was extracted with CH_2Cl_2. The water layer was extracted with CH_2Cl_2 again after adjusting its pH to 2.0. The CH_2Cl_2 solutions were combined, dried (Na_2SO_4) and evaporated to give crude **24**. Purification with a silica gel column using a solvent system of hexane–ethyl acetate–acetic acid (50:50:3) afforded **24** as a white powder (675 mg) after lyophilization. **24**: *m/z* (CI–MS) 275 (M + H)$^+$, 231, 217, 199 and 157; δ_H(600 MHz; $CDCl_3$) 5.00 (2 H, s, 1′-H), 3.96 [4 H, m, $O(CH_2)_2O$], 2.75 (2 H, s, 2-H), 1.73 (2 H, 5, *J* 8, 4-H), 1.51 (1 H, m, 7-H), 1.35 (2 H, m, 5-H), 1.15 (2 H, m, 6-H) and 0.85 (6 H, d, *J* 6.6, 8-H and 9-H); ν_{max}/cm^{-1} 1720, 1700 and 1590.

Diazoacetylmethyl (3,3-Ethylenedioxy-7-methyloctanoate) **26**.—*O*-(3,3-Ethylenedioxy-7-methyloctanoyl)glycoloyl chloride **25** was prepared by the reaction of **24** with oxalyl chloride in dry benzene. **25**: ν_{max}/cm^{-1} 1800 and 1720. A solution of **25** (360 mg) in ether (20 cm^3) was added dropwise to an ethereal solution of CH_2N_2 (large excess) at −5 °C under a nitrogen atmosphere, and the solution was stirred at −5 °C for 30 min and subsequently at room temperature for 1.5 h. Unchanged CH_2N_2 was removed with the ether by heating and flushing with nitrogen gas. The yellow residue obtained was purified on a silica gel column using a solvent system of hexane–ethyl acetate (3:1) to afford **26** as pale yellow oil (184 mg). **26**: *m/z* (CI–MS) 299 (M + H)$^+$, 271, 199 and 157; δ_H(600 MHz; $CDCl_3$) 5.72 (1 H, br s, 3′-H), 4.61 (2 H, s, 1′-H), 4.00 [4 H, m, $O(CH_2)_2O$], 2.74 (2 H, s, 2-H), 1.78 (2 H, t, *J* 8, 4-H), 1.54 (1 H, m, 7-H), 1.49 (2 H, m, 5-H), 1.18 (2 H, m, 6-H) and 0.86 (6 H, d, *J* 6.6, 8-H and 9-H); δ_C(150 MHz; $CDCl_3$) 168.4 (C-1), 109.5 (C-3), 66.5 (C-1′), 65.1 [$O(CH_2)O$], 42.4 (C-2, C-4 or C-6), 38.9 (C-2, C-4 or C-6), 38.0 (C-2, C-4 or C-6), 27.9 (C-7), 22.5 (C-8), 22.5 (C-9) and 21.3 (C-5) (signals of C-2′ and C-3′ were not clear); ν_{max}/cm^{-1} 2100, 1740 and 1640.

3-(7-Methyl-3-oxooctanoyloxy)-2-oxopropyl Phosphate **12**.—A solution of compound **26** (45 mg), 85% phosphoric acid (0.2 cm^3) and dioxane (3.8 cm^3) was heated at 70 °C for 1 h. The reaction product was directly purified by reverse-phase HPLC (column: Capcell-Pak C_{18}, 20 × 250 mm, Shiseido; mobile phase: gradient elution of MeCN 0–100% in 0.1% trifluoroacetic acid in 60 min; flow rate: 10 cm^3 min^{-1}). The eluate of the peak at a retention time of 30.8 min was collected and lyophilized to afford **12** as a white powder (13.4 mg); *m/z* (negative HRFAB–MS) 323.0958 (M − H)$^-$ (Calc. for $C_{12}H_{20}O_8P$: 323.0896); δ_H(600 MHz; CD_3CN) 4.89 (2 H, s, 1′-H), 4.66 (2 H, br d, $^3J_{HP}$ 8.4, 3′-H), 3.57 (2 H, s, 2-H), 2.55 (2 H, t, *J* 7.3, 4-H), 1.5–1.6 (3 H, m, 5-H and 7-H), 1.17 (2 H, m, 6-H) and 0.88 (6 H, d, *J* 6.6, 8-H and 9-H); δ_C(150 MHz; CD_3CN) 203.8 (C-3), 167.7 (C-1), 67.6 (C-1′), 49.2 (C-2), 43.6 (C-4), 38.9 (C-6), 28.5 (C-7), 22.7 (C-8), 22.7 (C-9) and 21.9 (C-5) (signals of C-2′ and C-3′ were not clear); λ_{max}/cm^{-1} 1746 and 1717; λ_{max}(MeCN)/nm 248.5 (750).

4-(5′-Methylhexanoyl)-5-oxo-2,5-dihydro-3-furylmethyl Phosphate **27**.—A solution of compound **26** (88 mg), 85% phosphoric acid (0.2 cm^3) and dioxane (3.8 cm^3) was heated at 70 °C for 1 h and then diluted with water and extracted with ethyl acetate. The organic layer was extracted with saturated aq. $NaHCO_3$ after which the water layer was acidified (pH 2.0) and again extracted with ethyl acetate. The organic layer obtained was dried (Na_2SO_4) and concentrated and the residue was purified by reverse-phase HPLC (column: Daisopak SP-120-5-ODS-B, 4.6 × 250 mm, Daiso; mobile phase: gradient elution of MeCN 30–100% in 0.1% trifluoroacetic acid in 15 min; flow: 1 cm^3 min^{-1}). The eluate of the peak at a retention time of 9.8 min was collected and lyophilized to give **27** (7.9 mg). Compound **12** was not obtained from the acidic fraction by this procedure. **27**: negative *m/z* (HRFAB–MS) 305.0716 (M − H)$^-$

(Calc. for $C_{12}H_{18}O_7P$: 305.0790); δ_H(600 MHz; CD_3CN) 5.32 (2 H, d, $^3J_{HP}$ 7.5, 5-H), 5.03 (2 H, s, 4-H), 2.87 (2 H, t, J 7.3, 7-H), 1.5–1.6 (3 H, m, 8-H and 10-H), 1.2 (2 H, m, 9-H) and 0.88 (6 H, d, J 6.7, 11-H and 12-H).

5-Oxotetrahydrofuran-3-ylmethyl Phosphate **30**.—A mixture of 3-hydroxymethylbutanolide **28** (1.0 g), dimethylaminopyridine (1.32 g), diphenyl phosphorochloridate (2 cm^3) and dry pyridine (5 cm^3) was stirred at room temperature for 1 h and then diluted with water (100 cm^3) and extracted with CH_2Cl_2 (2 × 100 cm^3). After being washed with 1 mol dm^{-3} HCl (2 × 60 cm^3) and water (60 cm^3), the CH_2Cl_2 solution was dried (Na_2SO_4) and concentrated. The residue was purified on a silica gel column (hexane–ethyl acetate, 1:1) to afford **29** (2.3 g); m/z (EI-MS) 348 $(M)^+$; δ_H(600 MHz; $CDCl_3$) 7.2–7.4 (10 H, m, Ph), 4.34 (1 H, dd, J 7.7, 9.5, 4a-H), 4.24 (2 H, m, 5-H), 4.09 (1 H, dd, J 9.5, 5.7, 4b-H), 2.93 (1 H, m, 3-H), 2.60 (1 H, dd, J 9.2, 17.9, 2a-H) and 2.33 (1 H, dd, J 6.4, 17.9, 2b-H); λ_{max}/cm^{-1} 1770 and 1585. Hydrogenolysis of **29** (2.3 g) with PtO_2 in methanol gave **30** (1.3 g); m/z (negative FAB–MS) 195 $(M - H)^-$; δ_H(600 MHz; CD_3OD) 4.45 (1 H, dd, J 7.9, 9.2, 4a-H), 4.22 (1 H, dd, J 5.4, 9.2, 4b-H), 4.00 (2 H, m, 5-H), 2.94 (1 H, m, 3-H), 2.69 (1 H, dd, J 9.3, 17.8, 2a-H) and 2.43 (1 H, dd, J 6.3, 17.8, 2b-H); λ_{max}/cm^{-1} 1760.

4-(5'-Methylhexanoyl)-5-oxotetrahydrofuran-3-ylmethyl Phosphate **13**.—A solution of lithium diisopropylamine was prepared in tetrahydrofuran (100 cm^3) from diisopropylamine (5.1 cm^3) and butyllithium (1.436 mol dm^{-3} hexane solution; 25 cm^3) at −78 °C. To this solution, a solution of the bis(trimethylsilyl) ether **31**, prepared by the reaction of the phosphate **30** (650 mg), *N,O*-bis(trimethylsilyl)acetamide (2.6 cm^3) and *N,N*-diisopropylethylamine (2 cm^3) at room temperature for 1 h, was added dropwise. The reaction mixture was stirred for 1 h after which 5-methylhexanoyl chloride (1.47 g) was added dropwise to it at −78 °C; it was then stored at the same temperature for 1 h and then allowed to rise to 0 °C. The reaction mixture was poured into cooled 1 mol dm^{-3} aq. HCl (100 cm^3) and extracted with ethyl acetate (100 cm^3 × 3). The ethyl acetate solution was extracted with saturated aq. $NaHCO_3$ (150 cm^3 × 3). After the pH of the aqueous solution had been adjusted to 2, it was extracted with ethyl acetate (200 cm^3 × 3) again. The ethyl acetate solution was dried (Na_2SO_4) and concentrated and the residue was purified by reverse-phase HPLC under the same conditions as those described for the purification of the phosphate **12**. The eluate of the peak at a retention time of 31.4 min was collected and lyophilized to afford **13** as oil (43 mg); m/z (negative HRFAB–MS) 307.0979 $(M - H)^-$ (Calc. for $C_{12}H_{20}O_7P$: 307.0947); δ_H(600 MHz; CD_3OD) 4.45 (1 H, m, 4a-H), 4.18 (1 H, m, 4b-H), 4.02 (2 H, m, 5-H), 3.32 (1 H, m, 3-H), 2.90 (1 H, m, 7a-H), 2.66 (1 H, m, 7b-H), 1.50–1.65 (3 H, m, 8-H and 10-H), 1.20 (2 H, m, 9-H) and 0.90 (6 H, d, J 6.7, 11-H and 12-H); δ_C(150 MHz; CD_3OD) 204.4 (C-6), 174.3 (C-1), 70.1 (C-4), 66.6 (d, $^2J_{CP}$ 5.1, C-5), 43.6 (C-7), 39.5 (C-2), 39.5 (C-3), 39.4 (C-9), 29.0 (C-10), 22.8 (C-11), 22.8 (C-12) and 22.1 (C-8); λ_{max}(MeCN)/nm 256 (75); ν_{max}/cm^{-1} 1760 and 1720.

Conversion Experiments with 6-Dehydro-VB A and the Ester **11**.—*S. antibioticus* IFO 12838 was cultured by the method previously described.[1] After 28 h of cultivation, cells were collected by centrifugation (6000 *g*, 10 min) and stored at −80 °C before use. Cells (wet, 30 g) were disrupted in 0.1 mol dm^{-3} phosphate buffer (pH 7.1; 100 cm^3) by sonication and the suspension was centrifuged (5000 *g*, 10 min). The supernatant obtained was mixed with ammonium sulfate to give 80% saturation. After the solution had been kept at 4 °C for 1 h, the precipitate was collected by centrifugation (5000 *g*, 10 min) and dissolved in 0.1 mol dm^{-3} phosphate buffer (pH 7.1; 20 cm^3). It was then dialysed against the same buffer overnight and the dialysate was concentrated by ultrafiltration (UK-10, Toyo Roshi) to 12 cm^3. This solution was used as the enzyme solution. Conversion experiments were performed in a reaction tube (1.5 cm^3 Eppendorf tube) containing the enzyme solution (1.0 cm^3). Co-factor (NADPH or NADH) and the substrate (6-dehydro-VB A or the ester **11**) was dissolved in water and MeCN, respectively, at a concentration of 10 mg cm^{-3}, and the solution was added to the reaction tube. Co-factor or 6-dehydro-VB A was added to the tube once at the beginning of the incubation. The ester **11** was added to the reaction mixture at 30-min intervals (×5) starting from the beginning of the incubation. Incubation was carried out at 28 °C and 150 rpm on a rotary shaker for 2 h. After incubation, racemic VB D (5 µg or 10 µg) was added to the reaction mixture as an internal standard. The mixture was then applied to a charcol (Charcoal Activated, Wako Pure Chemical Ind.; 2 cm^3) column. After being washed with water (10 cm^3), the column was eluted successively with 10 cm^3 each of 10%, 25%, 50% and 100% MeOH. VBs were eluted with 100% MeOH, and the solution was concentrated and brought to 5 cm^3 with water. This solution was applied to a SEP-PAK C_{18} cartridge (Waters) preequilibrated with water and eluted successively with 5 cm^3 each of water, 10%, 20%, 50% and 100% MeCN. The 50% eluate was concentrated and lyophilized. The residue was benzoylated with benzoyl cyanide (*ca.* 5 mg) and tributylamine (5 × 10^{-3} cm^3) in dry MeCN (0.1 cm^3) for 10 min. Water (5 cm^3) was added to the reaction mixture which was then treated on a SEP-PAK C_{18} cartridge in the same manner as mentioned above. Dibenzoates of VBs were eluted with 100% MeCN, and the fraction was analysed by reversed-phase HPLC (column: Wakosil-II $5C_{18}$-HG, 4.6 × 250 mm, Wako Pure Chemical Ind.; mobile phase: gradient elution of 50–100% MeCN in water in 50 min; flow rate: 1 cm^3 min^{-1}). The retention times of VB A dibenzoate and VB D dibenzoate were 30.5 and 31.5 min, respectively. The amount of VB A produced by the conversion experiment was calibrated from the area ratio of the peaks of VB A dibenzoate and VB D dibenzoate.

Conversion Experiments in ^2H_2O Solution.—Cells (wet; 30 g) obtained by the same procedure as described above were disrupted by sonication in a 0.1 mol dm^{-3} phosphate buffer (pH 7.1; 100 cm^3) of ^2H_2O, which was prepared by adding the powder of Na_2HPO_4 and NaH_2PO_4 to ^2H_2O (99.75%, Wako Pure Chemical Ind.) NADH (10 mg) and NADPH (10 mg) were added to the suspension and the mixture was distributed into 100 reaction tubes; 20 tubes were used for the conversion of 6-dehydro-VB A and 80 for the ester **11**. 6-Dehydro-VB A (500 µg) was added to each tube once at the beginning of the incubation. The ester **11** (100 µg) was added to each tube at 30-min intervals (×5) starting from the beginning of the incubation. After incubation at 28 °C and 150 rpm for 15 h, the 20 tubes and 80 tubes, respectively, were pooled. The reaction mixture of 6-dehydro-VB A was adsorbed on a charcoal column (60 cm^3). After being washed with water (180 cm^3), the column was eluted successively with 300 cm^3 each of 10%, 25%, 50% and 100% MeOH. The eluates of 50% and 100% MeOH were combined and concentrated. After being lyophilized, the residue was benzoylated with benzoyl cyanide (240 mg) and tributylamine (0.2 cm^3) in dry MeCN (2 cm^3). The reaction solution was then purified by preparative HPLC (column: Capcell-Pak C_{18}, 20 × 250 mm, Shiseido; gradient elution of 50–100% MeCN in water in 90 min; flow rate: 10 cm^3 min^{-1}). The peak having a retention time of 44 min afforded VB A dibenzoate (11.0 mg) converted from 6-dehydro-VB A. The reaction mixture of the ester **11** was purified and benzoylated by the same procedure on a scale four times as large. Final

purification with HPLC afforded VB A dibenzoate (6.8 mg) converted from the ester **11**.

Conversion Experiments with the Phosphates **12**, **13** *and* **27**.— Cells (wet; 12 g) were disrupted by sonication in 25 mmol dm^{-3} *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer (40 cm^3), and the suspensions were used as the cell extracts. Conversion experiments were carried out in the reaction mixture containing the cell extracts (4 cm^3), NADPH (300 μg), NADH (300 μg) and a substrate in a 50 cm^3 SUMILON tube. Substrates were dissolved in MeCN at the concentration of 10 mg cm^{-3}. Each substrate (100 μg) was added to the reaction tube at 30-min intervals (×5) starting from the beginning of incubation. Incubation was performed at 28 °C and 150 rpm on a rotary shaker for 2.5 h. After incubation, the reaction mixture was treated by the same procedure as described above to give the VB A dibenzoate sample. Since in this case some peaks overlapping those of VB dibenzoates were observed on reverse-phase HPLC, the sample was further purified by a normal-phase HPLC (column– Unisil Q100-10, 4.6 × 250 mm, Gasukuro Kogyo; mobile phase: isocratic elution with hexane–propan-2-ol, 9:1; flow rate: 1.0 cm^3 min^{-1}). The eluate having a retention time of 3.5–7.8 min was collected and the solution was analysed by reverse-phase HPLC.

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