THE STRUCTURE OF INDUCING FACTORS FOR VIRGINIAMYCIN PRODUCTION IN *STREPTOMYCES VIRGINIAE*

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Virginiamycin inducing factors (inducing material or inducing factor) of *Streptomyces virginiae* were isolated from the culture broth of this microbe and separated into three closely related compounds. They were named virginiae butanolides A, B and C and their structures were determined as 2-(1′-hydroxy-5′-methylhexyl)-3-(hydroxymethyl)butanolide (**6**), 2-(1′-hydroxy-4′-methylhexyl)-3-(hydroxymethyl)butanolide (**7**) and 2-(1′-hydroxyhexyl)-3-(hydroxymethyl)butanolide (**8**), respectively. Part of their stereochemistry was also determined. Racemic virginiae butanolide C was synthesized to confirm their structures.

During the studies on virginiamycin (staphylomycin) production with *Streptomyces virginiae*, YANAGIMOTO and TERUI pointed out the presence of a factor which induces the production of virginiamycin[1,2]. They denoted this factor as inducing material (IM) and attempted its isolation and structural elucidation[3]. They partially purified so called IM and showed that it is a δ- or γ-lactone with two hydroxyl groups. They also discovered that some simple plausible analogues of IM such as γ-nonalactone or γ-undecalactone have similar activity toward *Streptomyces virginiae* although their activity is much less than that of the natural factor. In this decade, some auto-regulators of actinomycetes, initially A-factor (**1**)[4,5] from *Streptomyces griseus* then factor I (**2**)[6], GRÄFE's factors from *S. bikiniensis* and *S. cyaneofuscatus* (**3**, **4** and **5**)[7], were discovered and their structures were almost unambiguously determined as shown in Fig. 1. On the other hand BEPPU *et al.* recently discovered B-factor from yeast extract, which induced rifamycin production in *Nocardia* sp.[8]. These findings stimulated us to carry out structural studies of virginiamycin inducers. In this report we describe the isolation and structure of these inducers which turned out to be plural. We named these structurally closely related factors as virginiae butanolides A, B and C, and the chemical synthesis of racemic virginae butanolide C was accomplished to confirm their structures.

Culture of *S. virginiae* for the Isolation of Inducing Factor

First Seed-culture

From the slant *S. virginiae* was inoculated into 500 ml of medium in Sakaguchi flasks and cultivated on a reciprocating shaker at 28°C for 24 hours.

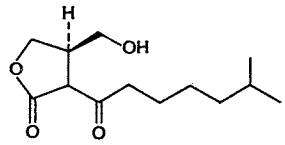
Second Seed-culture

First seed culture was inoculated into 30 liters of medium in a 50-liter fermentor and cultivated at 28°C, 280 rpm, aeration rate 1 vol/vol/minute, internal pressure 1 kg/cm^2 for 24 hours.

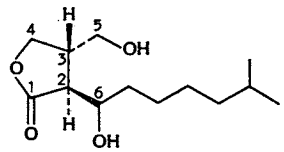
Culture for Production

Into 1,200 liters of medium in a 2,000-liter fermentor, the second seed-culture was inoculated and

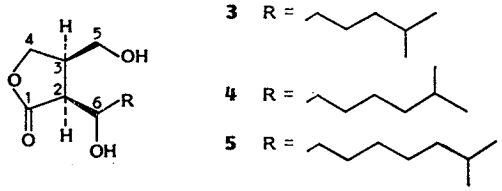
Fig. 1. The structure of auto-regulators isolated from *Streptomyces*.



A-factor (1)



Factor I (2)



3 R =

4 R =

5 R =

cultured under the same conditions as the second seed-culture for 24 hours.

Purification of Inducing Factor

Extraction from Fermented Broth

Fermented broth (1,150 liters) was adjusted to pH 5.7 with sulfuric acid and filtered with filter aid to give 1,280 liters of the filtrate. It was acidified to pH 3.0 with sulfuric acid and extracted with one third volume of ethyl acetate. The ethyl acetate layer (320 liters) was washed with one third volume of water and 315 liters of ethyl acetate solution was obtained. The evaporation of the solvent gave 318.8 g of residue.

Partition between *n*-Hexane and Methanol - Water

The ethyl acetate extract was dissolved in methanol - water (9 : 1) and extracted with *n*-hexane to remove non-polar substances such as wax.

Hydrolysis of Virginiamycin and Esters

To the methanol - water solution (3 liters) 40 g of potassium hydroxide was added and the solution was kept at room temperature overnight. Then the solution was refluxed for 1 hour. It was diluted with water and methanol was removed by evaporation. This mixture was acidified with HCl and extracted with ethyl acetate. The solvent layer was dried over anhydrous sodium sulfate and concentrated to give 166.6 g of residue.

Column Chromatography on Amberlyst A-21

Amberlyst A-21 (500 g, Organo), an anion-exchanger, was packed in a column (76 mm) and treated with dilute HCl, dilute NaOH solution, water and methanol. Thirty g of the sample was placed on it. The column was eluted with methanol and fractions were assayed for activity. In the fractions of 0~900 ml (*ca.* 6 g) after void volume elution, there was strong activity.

The whole 166.6 g of sample was divided into four portions and each of them was treated with the anion-exchanger as described above to give in total 35.9 g of active oil.

Column Chromatography on Alumina

Alumina (Aluminum oxide 90, Merck Art. 1097, 700 g) was packed in a 55 mm-diameter column with ethyl acetate. Fractions eluted with ethyl acetate - methanol (8 : 2) from 600~800 ml contained active compound which gave 21.87 g of oil after the evaporation of solvent.

Preparative High Pressure Liquid Chromatography

The crude oil from the alumina column was extracted with *n*-hexane - 2-propanol (8 : 2) and the soluble part (16.6 g) in 400 ml of this solvent system was divided into 5 portions (each 80 ml) and injected on to the silica gel column (Prep PAK-500/SILICA) of a preparative HPLC (System 500, Waters) and eluted with *n*-hexane - 2-propanol (8 : 2) at a flow rate of 100 ml/minute and a pressure

of 20 atmosphere. Fractions of retention time from 10 to 15 minutes showed activity and the five runs of HPLC afforded 2.01 g of active oil. This oil was again applied to preparative HPLC under the same conditions and separated into smaller fractions. Fractions of retention time from 13 to 16 minutes gave 461 mg of active oil.

Purification on Octadecylsilyl (ODS) Column HPLC

Active oil (461 mg) was further purified repeatedly on an ODS column (Cosmosil 5 C18 Nakarai Chemical 10×250 mm, Jasco TRI ROTOR-V) in 10 mg portions eluting with methanol - water (50 : 50 for first step, 50 : 50 or 40 : 60 for second step, 40 : 60 for third step yielding virginiae butanolides A and C, 35 : 65 for fourth step yielding virginiae butanolides A and C). As there were three active peaks as shown in Fig. 2, we named them in order of increasing polarity (decreaing retention time) virginiae butanolides (VBs) A, B and C, respectively. By repeated chromatography with this HPLC system once for B and three times for A and C, each peak was purified to a single peak by refractive index (RI) and UV (210 nm) detector as shown in Fig. 3. Yield; virginiae butanolide A 0.823 mg, VB B 1.271 mg, VB C 1.210 mg.

The yield and activity at each step was shown in Table 1. The quantity of these factors in one ton of original fermented broth was estimated to be *ca.* 100 mg on the basis of these data. The specific activities of VBs A and C are almost the same and that of VB B is a little less. Purified VBs A, B and C were active at concentrations of ng/ml and these effective levels correspond with those of A-factor, other butanolide type factors and B-factor.

Fig. 2. The elution pattern of virginiae butanolides (VBs) A, B and C at the first HPLC purification step on ODS column.

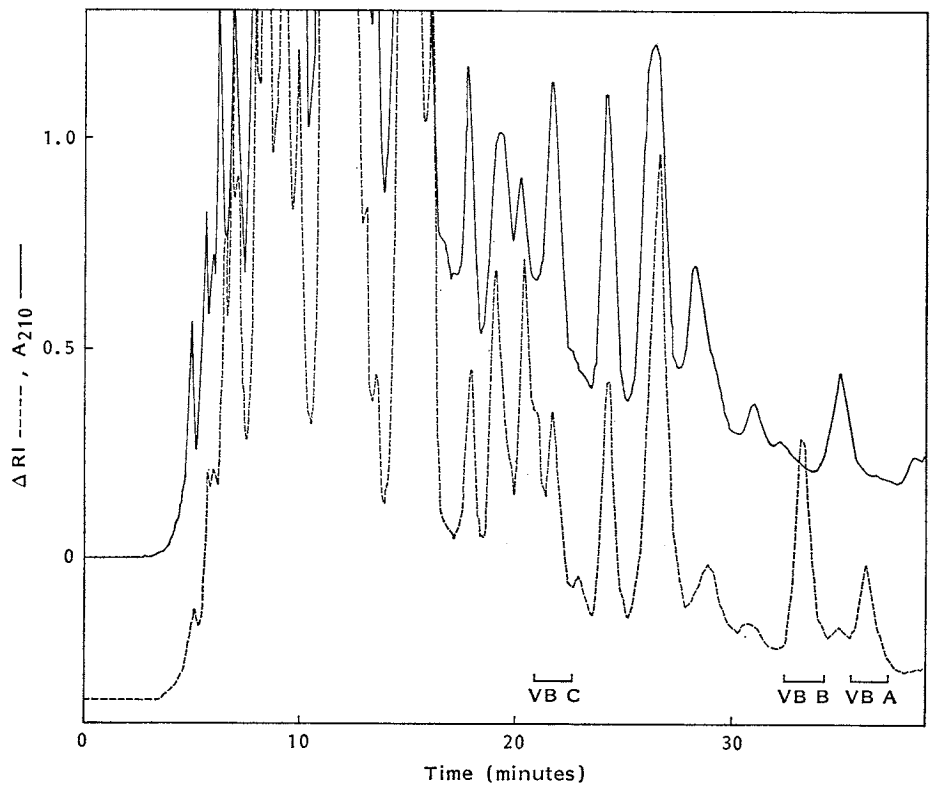


Fig. 3. The final purification of virginiae butanolides (VBs) A, B and C on ODS column with HPLC. The peak between arrows was collected.

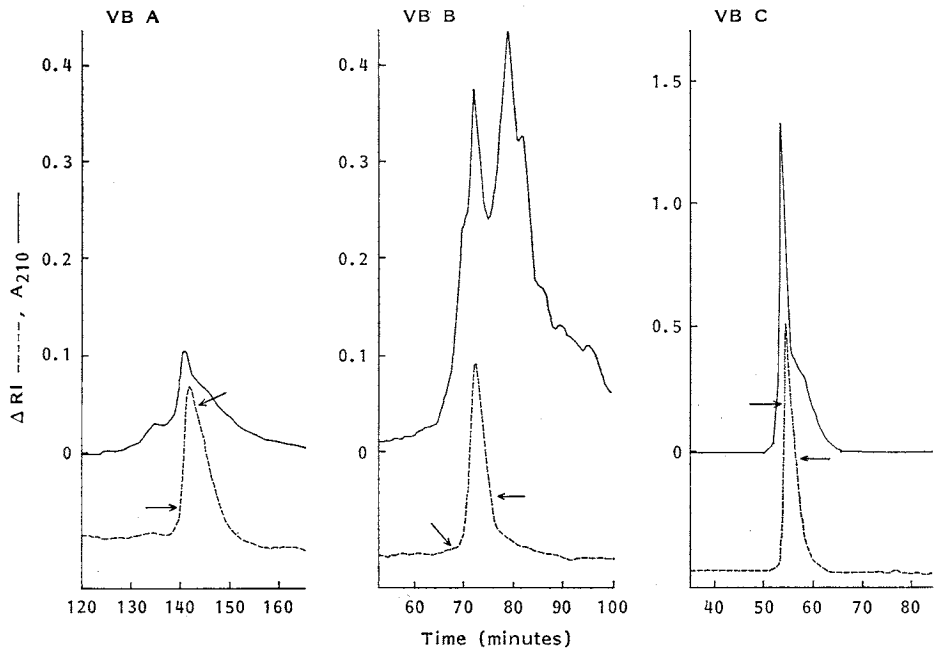


Table 1. The yield and activity of inducing factor at each purification step.

Step	Yield	Activity (U)	Specific activity (U/mg)
1. EtOAc extract	318 g	6×10^7	190
2. EtOAc extract after hydrolysis	167 g	4×10^7	240
3. Amberlyst A-21	35.9 g	3×10^7	840
4. Alumina column	21.9 g	$>3\times10^6$	—
5. *n*-Hexane - 2-PrOH soluble part	16.6 g	$>1\times10^7$	—
6. 1st System 500	2.01 g	1×10^7	5,000
7. 2nd System 500	461 mg	2×10^7	43,000
8. Final HPLC (ODS)			
VB A	0.823 mg	1.3×10^6	1.6×10^6
VB B	1.271 mg	1.3×10^5	1.0×10^5
VB C	1.210 mg	5×10^5	4.1×10^5

One unit was defined as the minimum amount of VBs per ml of the assay medium to induce virginiamycin production under the assay conditions described in Experimental.

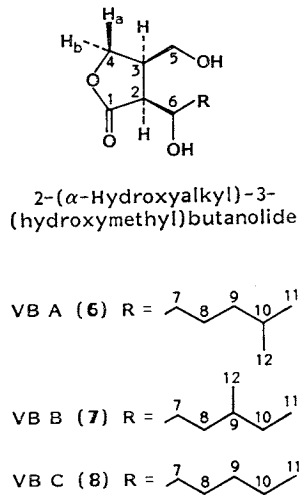
The Structure of Virginiae Butanolides

The structures of VBs A, B and C were assigned as **6**, **7** and **8**, respectively, as shown in Fig. 4 on the basis of their ^{1}H NMR and ^{13}C NMR data.

NMR spectra of VBs A, B and C clearly indicated the presence of 2,3-disubstituted γ-lactone (2-(α-hydroxyalkyl)-3-(hydroxymethyl)butanolide) moieties (Fig. 4) in common when they were compared with NMR spectra of GRÄFE's factors isolated from *S. bikiniensis* and *S. cyaneofuscatus* (**3**, **4** and **5**)[7]. The stereochemistry of the two substituents on the γ-lactone was determined to be *cis* in all VBs on the basis of the value of coupling constant 7.26 Hz between 2-H and 3-H which coincided with those

of GRÄFE's factors. GRÄFE *et al.* reached this conclusion by comparison with the larger coupling constant, $J_{2,3}$=9.4 Hz of factor I (**2**)[6] which they concluded to have the *trans* configuration. We also confirmed the *cis* configuration of VBs by the synthesis of *cis* and *trans* isomers of VB C. The stereochemistry of the hydroxyl group on C-6 is ambiguous at present. The coupling constants between 2-H and 6-H of all VB are around 3.6~3.7 Hz and those of GRÄFE's factors are also almost the same. This fact shows the configurations at C-6 of these factors are probably the same.

Fig. 4. The structure of virginiae butanolides (VBs) A, B and C.

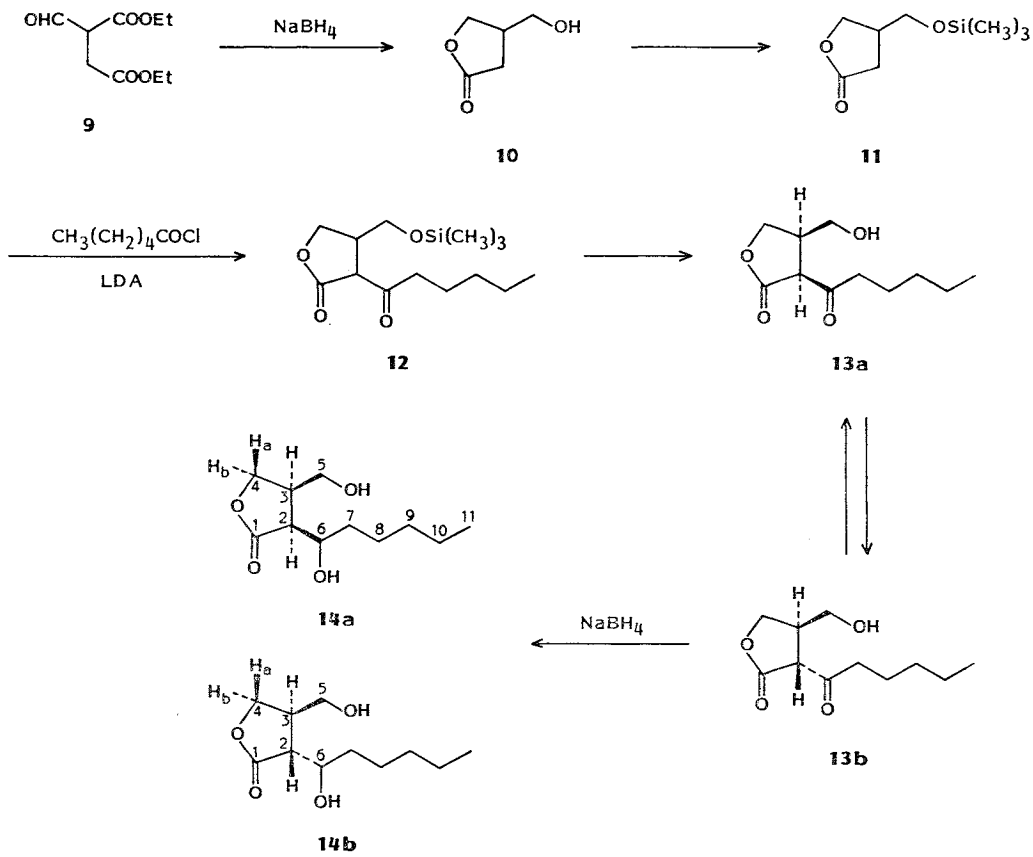


The structures of the side chains (R in Fig. 4) of VBs A, B and C were identified as 4-methylpentyl, 3-methylpentyl and pentyl, respectively. All of these ^{13}C NMR signals were assigned and ^{1}H NMR data also reasonably supported these structures. The structure of VB A coincided with one of GRÄFE's factors (**3**), which they could not separate from other closely related factors (**4** and **5**)[7]. CD spectra of VBs A, B and C are almost the same showing positive ellipticity around 215~217 nm. This fact suggests all of these VBs have the same absolute configuration. Since the alkaline treatment during the purification procedure did not decrease the activity, we presume the disubstituted butanolide ring system is rather resistant to alkaline hydrolysis which could lead to racemization and epimerization at C-3 by re-lactonization. The absolute configuration of these factors, including that of VB B side chain at C-9, remains for later studies.

The Synthesis of VB C

In order to confirm the structure of VBs we synthesized racemic VB C mainly following MORI's route[9] which was developed for the synthesis of A-factor. In Scheme 1 the synthetic path is shown. We found that diethyl formylsuccinate (**9**)[10] was reduced to 3-(hydroxymethyl)butanolide (**10**) in one step when we used more than 2 equivalents of $NaBH_4$. This unusual reduction of an ester group is presumably due to the neighboring effect of borohydride attached to the reduced hydroxymethyl group in the intermediate. The hydroxyl group of **10** was protected as its trimethylsilyl ether and acylated with lithium diisopropylamide (LDA) and hexanoyl chloride to give **12** which gave A-factor type compound (**13**) after deprotection. The ketone (**13**) should be a mixture of two tautomers (**13a** and **13b**) and the thermodynamically stable *trans* isomer (**13b**) is presumed to be the major. The ketone (**13**) was reduced with $NaBH_4$ and the resultant isomers (**14a** and **14b**) were separated by HPLC. The ratio of **14a** : **14b** was 2 : 5 and on the basis of the logic mentioned above we assigned the minor one (**14a**) as the *cis* isomer and the major one (**14b**) as the *trans* isomer. Actually ^{1}H NMR and ^{13}C NMR spectra of **14a** were completely identical with those of VB C. As the coupling constants between 2-H and 6-H of the synthesized VB C and the natural one are almost the same value (3.96 Hz and 3.63 Hz, respectively), the configurations at C-6 of synthetic VB C and of the natural one are probably identical. On the other hand the NMR spectra of the *trans* isomer (**14b**) bear resemblance to

Scheme 1. The synthetic route of racemic virginiae butanolide C and its stereoisomer.



those of factor I showing coupling constants, $J_{2,3}$=9.24 Hz and $J_{2,6}$=4.62 Hz. These coupling constants indicate that the two substituents of **14b** was *trans* and the configuration at C-6 is identical with that of factor I.

The activity of synthetic VB C (**14a**) was 1.6×10^5 U/mg, about one third the value of the natural optically active material and that of the *trans* isomer (**14b**) was 8×10^3 U/mg (about 1/200 of natural VB C). As synthetic 6-deoxy VB C and the ketone (**13**) showed very poor activity (about 1/40 of natural VB C), the 2-(α-hydroxyalkyl)-3-(hydroxymethyl)butanolide moiety as well as its *cis* configuration are essential for the inducing activity of VBs.

Experimental

Microorganisms

Streptomyces virginiae of YANAGIMOTO and TERUI was used throughout this study. This strain was kept on oatmeal agar slants. *Bacillus subtilis* PCI 219 was an assay organism for virginiamycin production.

Culture Media

Medium for Assay and First Seed-culture: Bacto-casitone (Difco) 7.5 g, yeast extract (Difco) 7.5 g, glycerol 15.5 g, NaCl 2.5 g, distilled water 1 liter, pH 6.5.

Medium for Second Seed-culture and Production Culture: Bacto-casitone and yeast extract in

the above medium were replaced with Polypepton (Daigo Eiyo Kagaku, Japan) and yeast extract (Takeda Chemical Industries Ltd., Japan), respectively.

Assay of the Inducing Factor

The assay procedure was based on YANAGIMOTO's method[2,3].

a) Preculture: *S. virginiae* was inoculated from a slant into a 100-ml conical flask which contained 20 ml of medium and incubated on a reciprocating shaker at 27°C, 120 strokes/minute for 36 hours. The cells were harvested by centrifugation at 6,000×*g* for 10 minutes. To the cell mass 20 ml of fresh medium was added to make inoculum.

b) Main Culture: Seventy ml of medium in 500-ml conical flask was inoculated with 2.1 ml of above cell suspension and incubated on a reciprocating shaker at 27°C, 120 strokes/minute for 8 hours. The cells were harvested by centrifugation at 8,000×*g* for 10 minutes. The cells were suspended in 35 ml of fresh medium.

c) Assay Culture: Five ml of above cell suspension was added to 5 ml of fresh medium in 24 mm-test tubes with samples of appropriately diluted inducing factors. These test tubes were incubated on a reciprocating shaker at 27°C, 120 strokes/minute for 4 hours. The cells were separated in a 10-ml cone tube by centrifugation at 1,500×*g* for 10 minutes. The cell growth was estimated by packed volume and the production of virginiamycin in the supernatant was determined by cylinder-plate method with *B. subtilis*. When the diameter of the inhibition zone was more than 13 mm, we defined the activity of inducing factors in that supernatant as more than 1 unit/ml and determined its approximate value by the dilution method. As a positive reference, we used γ-nonalactone at concentration of 20 μg/ml to confirm the results from unknown samples.

Analytical Methods

^{1}H NMR spectra were obtained with Hitachi R-24B(60 MHz), Brucker AM 360 (360 MHz) and Jeol GX270 (270 MHz). ^{13}C NMR spectra were obtained with Brucker AM 360 (90 MHz) or Jeol GX270 (68 MHz). IR spectra were obtained with Hitachi 215 IR spectrometer. CD curves were recorded on Jasco J-20A. Mass spectra were obtained with JMS-O1SC.

Spectrometry of Virginiae Butanolide A (**6**): ^{1}H NMR (360 MHz, $CDCl_3$) δ 4.42 (1H, dd, 4-H_a, $J_{4a,4b}$=9.04 Hz, $J_{4a,3}$=8.76 Hz), 4.13 (1H, m, 6-H), 4.10 (1H, dd, 4-H_b, $J_{4b,4a}$=9.0 Hz, $J_{4b,3}$=6.88 Hz), 3.74 (2H, m, 5-H_2), 2.86 (1H, m, 3-H), 2.57 (1H, dd, 2-H, $J_{2,3}$=7.40 Hz, $J_{2,6}$=3.77 Hz), 1.55 (4H, m, 7-H_2, 8-H_2), 1.34 (1H, m, 10-H), 0.88 (6H, d, 11-H_3, 12-H_3, $J_{10,11}$=6.59 Hz); ^{13}C NMR (91 MHz, by insensitive nuclear enhanced polarization transfer, $CDCl_3$) δ 70.87 (C-6), 69.27 (C-4), 63.38 (C-5), 48.06 (C-2), 38.68 (C-9), 38.14 (C-3), 35.09 (C-7), 27.87 (C-10), 23.55 (C-8), 22.55, 22.46 (C-11, C-12); Electron impact mass spectra (EI-MS) *m/z* 181, 145, 116, 85.

Spectrometry of Virginiae Butanolide B (**7**): ^{1}H NMR (270 MHz, $CDCl_3$) δ 4.40 (1H, dd, 4-H_a, $J_{4a,4b}$=8.91 Hz, $J_{4a,3}$=8.56 Hz), 4.10 (1H, m, 6-H), 4.10 (1H, dd, 4-H_b, $J_{4b,4a}$=8.91 Hz, $J_{4b,3}$=6.60 Hz), 3.73 (2H, m, 5-H_2), 2.85 (1H, m, 3-H), 2.52 (1H, dd, 2-H, $J_{2,3}$=7.26 Hz, $J_{2,6}$=3.63 Hz), 1.61~1.11 (7H, m, 7-H_2, 8-H_2, 9-H, 10-H_2), 0.89 (3H, d, 12-H_3, $J_{12,9}$=6.59 Hz), 0.88 (3H, t, 11-H_3, $J_{11,10}$=6.93 Hz); ^{13}C NMR (68 MHz, $CDCl_3$) δ 178.46 (COO), 71.22 (C-6), 69.42 (C-4), 63.27 (C-5), 48.06 (C-2), 38.13 (C-3), 34.39 (C-9), 32.68 (C-7), 32.45 (C-8), 29.32 (C-10), 19.23 (C-12), 11.32 (C-11); EI-MS *m/z* 181, 145, 116, 85.

Spectrometry of Virginiae Butanolide C (**8**): ^{1}H NMR (270 MHz, $CDCl_3$) δ 4.42 (1H, dd, 4-H_a, $J_{4a,4b}$=8.91 Hz, $J_{4a,3}$=8.58 Hz), 4.14 (1H, m, 6-H), 4.10 (1H, dd, 4-H_b, $J_{4b,4a}$=8.91 Hz, $J_{4b,3}$=6.93 Hz), 3.74 (2H, m, 5-H_2), 2.87 (1H, m, 3-H), 2.57 (1H, dd, 2-H, $J_{2,3}$=7.26 Hz, $J_{2,6}$=3.63 Hz), 1.64~1.32 (6H, m, 7-H_2, 8-H_2, 9-H_2), 1.32 (2H, m, 10-H_2), 0.90 (3H, t, 11-H_3, $J_{11,10}$=6.27 Hz); ^{13}C NMR (68 MHz, $CDCl_3$) δ 178.39 (COO), 70.89 (C-6), 69.33 (C-4), 63.41 (C-5), 48.07 (C-2), 38.16 (C-3), 34.83 (C-7), 31.60 (C-9), 25.49 (C-8), 22.57 (C-10), 13.98 (C-11); EI-MS *m/z* 167, 145, 116, 102, 85.

CD Spectra: Virginiae butanolides A, B and C showed positive ellipticities around 215~218 nm in methanol at 22°C in CD spectra.

Synthesis of Racemic Virginiae Butanolide C and its Stereoisomer

1) 3-(Hydroxymethyl)butanolide (**10**): To a solution of diethyl formylsuccinate (**9**, 49.61 g) in

200 ml of ethanol $NaBH_4$ (9.28 g) was added portionwise with stirring and ice-cooling. After the addition the reaction mixture was stirred for 2 hours at room temp. Diluted HCl (4 N) was added to the mixture and the precipitate was removed by filtration. The filtrate was concentrated under vacuum and the residue was extracted with ethyl acetate. The extract was dried over anhydrous sodium sulfate and concentrated to give 30.83 g of oil. The oil was dissolved in 120 ml of methanol and 40 ml of water and 15 g of potassium carbonate was added portionwise to it. Then the solution was refluxed for 2 hours. The reaction mixture was acidified with 4 N HCl and concentrated. The residue was extracted with ethyl acetate and dried over anhydrous sodium sulfate. The evaporation of the solvent gave crude 20.79 g of lactone (**10**). The crude product was adsorbed on 200 g of silica gel column in CH_2Cl_2 and eluted with CH_2Cl_2 - MeOH (9 : 1) and 14.46 g of oil was obtained. This oil was distilled in vacuum and 6.42 g of almost pure 3-(hydroxymethyl)butanolide (**10**) (150°C/5 mmHg) was obtained. Its ^1H NMR and IR spectra were identical with those of MORI *et al.* ^1H NMR (60 MHz, $CDCl_3$) δ 4.6~4.0 (2H, m, 4-H_2), 3.61 (2H, d, 5-H_2), 3.0~2.3 (3H, m, 2-H_2, 3-H); IR (film) cm^{-1} 3400, 1770.

2) 3-(Trimethylsilyloxymethyl)butanolide (**11**): 3-(Hydroxymethyl)butanolide (**10**, 6.2 g) was dissolved in dry pyridine (6 ml) and 6 ml of hexamethyldisilazane was added with ice-cooling and then 6 ml of trimethylsilyl chloride was added dropwise. After the addition the reaction flask was tightly sealed and stirred for 2 hours. Benzene - *n*-hexane (1 : 1) was added to the reaction mixture and the precipitate was removed by filtration with Celite. The filtrate was concentrated and the residue was distilled under vacuum. Yield 5.21 g, 80°C/5 mmHg. ^1H NMR (60 MHz, $CDCl_3$) δ 4.5~4.1 (2H, m, 4-H_2), 3.6 (2H, d, 5-H_2), 3.0~2.3 (3H, m, 2-H_2, 3-H), 0.14 (9H, s, $Si(CH_3)_3$); IR (film) cm^{-1} 1780.

3) 2-Hexanoyl-3-(hydroxymethyl)butanolide (**13**): LDA was prepared in 80 ml of tetrahydrofuran from 3.61 ml of diisopropylamine and *n*-butyl lithium (1.05 M *n*-hexane solution, 25.70 ml) at −78°C. To this solution 2.20 g of trimethylsilyl ether (**11**) in THF was added dropwise. After the addition the reaction mixture was stirred for 1.2 hours at −78°C. Then 1.64 ml of hexanoyl chloride was added dropwise at −78°C and the reaction mixture was kept at −78°C for 1 hour. The temperature of the reaction mixture was then allowed to rise to 0°C. The reaction mixture was poured into 100 ml of ice-water and 15 ml of acetic acid and extracted with CH_2Cl_2. The dichloromethane layer was concentrated and the residue was dissolved in 200 ml of CH_2Cl_2 again. It was washed with saturated sodium bicarbonate solution and water and dried over anhydrous sodium sulfate. The evaporation of the solvent gave crude acylated trimethylsilyl ether (**12**, 3.05 g). Trimethylsilyl ether (**12**) was refluxed in 40 ml of ethanol and 10 ml of water for 30 minutes to remove the trimethylsilyl group. The reaction mixture was concentrated and resulting crude oil (2.51 g) was purified on silica gel (20 g) column developed with *n*-hexane - ether (4 : 1) and eluted with *n*-hexane - ether (1 : 1). Yield of acylated lactone (**13**), 0.67 g. ^1H NMR (60 MHz, $CDCl_3$) δ 4.6~3.9 (2H, m, 4-H_2), 3.5~3.8 (2H, dd, 5-H_2), 3.4~2.9 (2H, dt, $COCH_2$), 2.0~1.0 (6H, m, CH_2), 0.9 (3H, t, CH_3); IR (film) cm^{-1} 3450, 1770, 1720.

4) 2-(1′-Hydroxyhexyl)-3-(hydroxymethyl)butanolide (**14**): Acyl butanolide (**13**, 200 mg, 0.93 mmol) was dissolved in 20 ml of methanol and 13.4 mg of $NaBH_4$ (1.5 equiv) was added at −5°C. After addition of $NaBH_4$, the reaction mixture was kept at room temp for 1 hour. 4 N HCl was added to it and the precipitate was removed by filtration. The filtrate was concentrated and the residue was extracted with ethyl acetate. The extract gave 175.28 mg of product after drying over anhydrous sodium sulfate and evaporation of solvent. This oil is the mixture of *cis* isomer (**14a**) and *trans* isomer (**14b**) (1 : 2.5). ^1H NMR (60 MHz, $CDCl_3$) δ 4.6~3.9 (3H, m, 4-H_2, 6-H), 3.8~3.6 (2H, dd, 5-H_2), 3.0~2.3 (2H, m, 2-H, 3-H), 2.0~1.1 (8H, m, 4×CH_2), 0.9 (3H, t, CH_3).

5) Separation of *cis* (**14a**) and *trans* (**14b**) Isomer: These isomers were separated by reverse phase HPLC with a pre-column (5×50 mm, Nucleosil 7 C18) and a column of ODS (10×250 mm Cosmosil 5 C18) being eluted with MeOH - H_2O (55 : 45) at flow rate of 1.8 ml/minute, pressure 280 kg/cm^2. Peaks were detected by RI and UV (210 nm). In this HPLC system *cis* isomer **14a** and *trans* isomer **14b** showed retention times of 16.0 minutes and 17.5 minutes, respectively and the ratio **14a**: **14b** estimated by peak area was 2 : 5. By repeated fractionation of the mixture of stereoisomers

obtained by reduction of 670 mg of ketone (**13**), 43.265 mg of **14a** and 89.095 mg of **14b** were obtained in pure form.

Spectrometry of **14a**: ^{1}H NMR (270 MHz, $CDCl_3$) δ 4.42 (1H, dd, 4-H_a, $J_{4a,4b}$=8.91 Hz, $J_{4a,3}$= 8.58 Hz), 4.15 (1H, dd, 4-H_b, $J_{4b,4a}$=8.91 Hz, $J_{4b,3}$=6.60 Hz), 4.10~4.15 (1H, m, 6-H), 3.72 (2H, m, 5-H_2), 2.86 (1H, sextet, 3-H), 2.56 (1H, q, 2-H, $J_{2,3}$=7.26 Hz, $J_{2,6}$=3.96 Hz), 1.4~1.6 (2H, m, CH_2), 1.2~1.4 (6H, m, 3×CH_2), 0.95 (3H, t, CH_3); ^{13}C NMR (68 MHz, $CDCl_3$) δ 178.40 (COO), 70.88 (C-6), 69.41 (C-4), 63.40 (C-5), 48.14 (C-2), 38.17 (C-3), 34.85 (C-7), 31.60 (C-9), 25.49 (C-8), 22.57 (C-10), 13.97 (C-11).

Spectrometry of **14b**: ^{1}H NMR (270 MHz, $CDCl_3$) δ 4.41 (1H, dd, 4-H_a, $J_{4a,4b}$=8.91 Hz, $J_{4a,3}$= 8.25 Hz), 4.00 (1H, dd, 4-H_b, $J_{4b,4a}$=8.9 Hz, $J_{4b,3}$=8.58 Hz), 4.0~4.1 (1H, m, 6-H), 3.72 (2H, octet, 5-H_2), 2.87 (1H, m, 3-H), 2.64 (1H, q, 2-H, $J_{2,3}$=9.24 Hz, $J_{2,6}$=4.62 Hz), 1.27~1.4 (6H, m, 3×CH_2), 1.45~1.65 (2H, m, CH_2), 0.9 (3H, t, CH_3); ^{13}C NMR (68 MHz, $CDCl_3$) δ 177.17 (COO), 70.86 (C-6), 68.35 (C-4), 62.93 (C-5), 48.95 (C-2), 40.10 (C-3), 33.90 (C-7), 31.53 (C-9), 25.41 (C-8), 22.47 (C-10), 13.89 (C-11).

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References

1) YANAGIMOTO, M. & G. TERUI: Physiological studies on staphylomycin production. J. Ferment. Technol. 49: 604~610, 1971
2) YANAGIMOTO, M. & G. TERUI: Physiological studies on staphylomycin production II, formation of a substance effective in inducing staphylomycin production. J. Ferment. Technol. 49: 611~618, 1971
3) YANAGIMOTO, M.; Y. YAMADA & G. TERUI: Extraction and purification of inducing material produced in staphylomycin fermentation. Hakko Kogaku Kaishi 57: 6~14, 1979
4) KLEINER, E. M.; S. A. PLINER, V. S. SOIFER, V. V. ONOPRIENKO, T. A. BALASHOVA, B. V. ROSYNOV & A. S. KHOKHLOV: The structure of A-factor-bioregulator from *Streptomyces griseus*. Bioorg. Chem. 2: 1142~1147, 1976
5) MORI, K.: Revision of the absolute configuration of A-factor. Tetrahedron 39: 3107~3109, 1983
6) GRÄFE, U.; W. SCHADE, I. ERITT, W. F. FLECK & L. RADICS: A new inducer of anthracycline biosynthesis from *Streptomyces viridochromogenes*. J. Antibiotics 35: 1722~1723, 1982
7) GRÄFE, U.; G. REINHARDT, W. SCHADE, I. ERITT, W. F. FLECK & L. RADICS: Interspecific inducers of cytodifferenciation and anthracycline biosynthesis from *Streptomyces bikiniensis* and *S. cyaneofuscatus*. Biotechnol. Lett. 5: 591~596, 1983
8) KAWAGUCHI, T.; T. ASAHI, T. SATOH, T. UOZUMI & T. BEPPU: B-Factor, an essential regulatory substance inducing the production of rifamycin in a *Nocardia* sp. J. Antibiotics 37: 1587~1595, 1984
9) MORI, K.: Synthesis and absolute configuration of A-factor, the inducer of streptomycin biosynthesis in inactive mutants of *Streptomyces griseus*. Tetrahedron Lett. 22: 3431~3432, 1981
10) TOCANNE, J. & C. ASSELINEAU: Preparation of optically active paraconic acid and correlation of its configuration. Bull. Soc. Chim. Fr. 1965: 3346~3348, 1965