DISTRIBUTION OF VIRGINIAE BUTANOLIDES IN ANTIBIOTIC-PRODUCING ACTINOMYCETES, AND IDENTIFICATION OF THE INDUCING FACTOR FROM *STREPTOMYCES ANTIBIOTICUS* AS VIRGINIAE BUTANOLIDE A

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In the course of screening for physiologically active compounds, we recently isolated inducing factors for virginiamycin production from *Streptomyces virginiae*[1]. The compounds, named virginiae butanolides A, B and C (VB-A, -B and -C), are all 2,3-disubstituted γ-butyrolactones. Except B-factor and pamamycin, the structure of substituted γ-butyrolactone is common to inducing factors from *Streptomyces*, such as A-factor from *Streptomyces griseus*[2,3], and factors from *Streptomyces bikiniensis* and *Streptomyces cyaneofuscatus*[4]. Therefore, we suspect that VB-like compounds may function generally as intra- or intercellular signal molecules, especially in antibiotic-producing species. In this paper, we report the distribution of VBs in several species of antibiotic-producing *Streptomyces*, and the isolation and structure of a inducing factor from one of the strains, *Streptomyces antibioticus*.

We selected 11 species of actinomycetes, one of *Bacillus* and one of *Cephalosporium*. Culture medium for these strains except for *Cephalosporium acremonium* contains soybean meal 1%, corn steep liquor 1%, potato starch 1%, NaCl 0.5%, K_2HPO_4 0.2% and $MgSO_4$ 0.05%. The medium described by DEMAIN *et al.*[5] was used for *C. acremonium*.

For seed culture preparation, 20 ml of the medium in 100-ml Erlenmeyer flasks was inoculated from slants, and incubated at 28°C for 3 days on a reciprocating shaker (120 spm). Main cultivation was performed by inoculating 3-ml portions of the seed culture into 100 ml of the medium in 500-ml Sakaguchi flasks, followed by incubation for 3 days at 28°C on a reciprocating shaker (120 spm). The culture broth (80 ml) was adjusted to pH 2.0 and extracted with a 3-fold volume of EtOAc. The solvent layer was evaporated to dryness, redissolved in 2 ml of EtOAc, and the inducing activity was measured as described[6,7] using inducing-factor dependent production of virginiamycin by *S. virginiae*.

Among 13 species tested, 4 species (*S. antibioticus*, *S. griseus*, *S. lincolnensis* and *Bacillus brevis*) produced factors capable of stimulating virginiamycin production by *S. virginiae* (Table 1). Although the number tested is limited, 3 among 11 species of *Streptomyces*

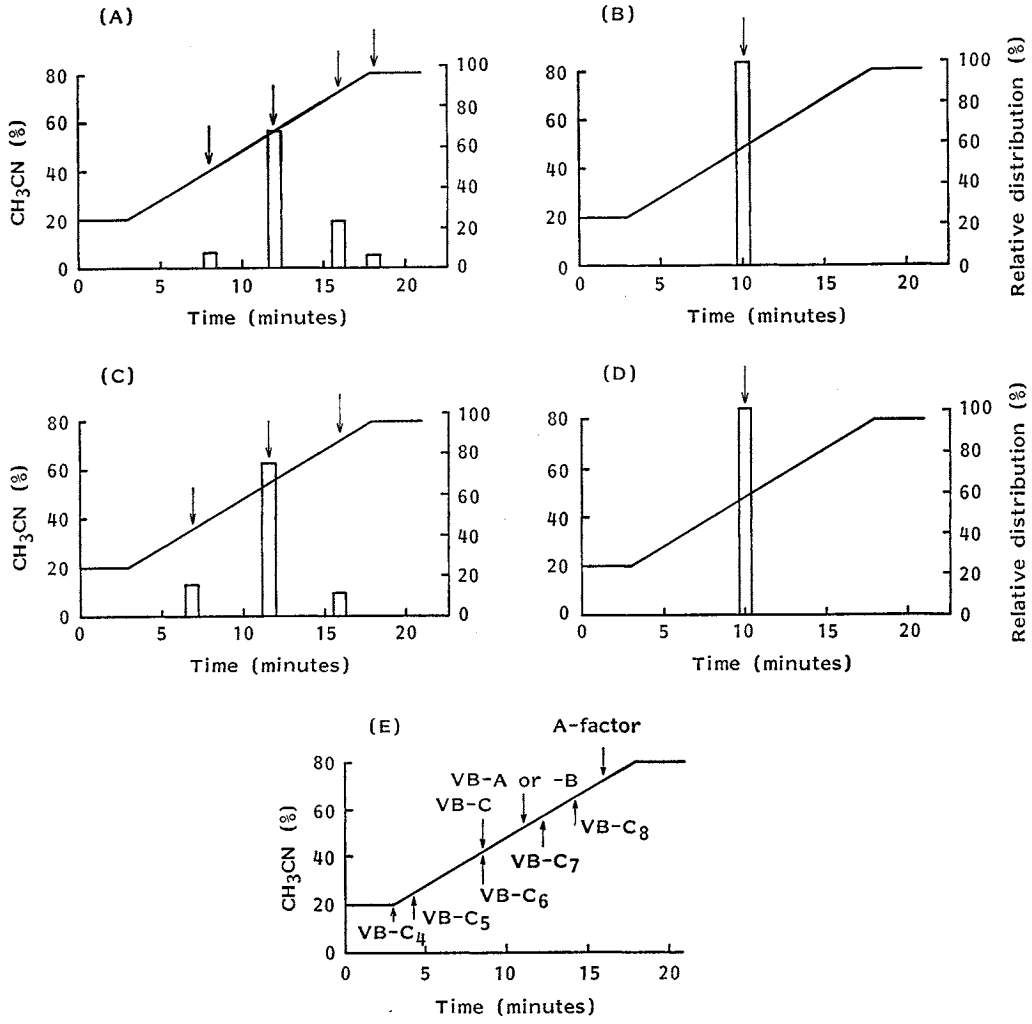
Table 1. Production of natural inducing factors by several species of actinomycetes.

Strain	Antibiotic	Inducing activity (U/ml)	Antibiotic production
Streptomyces lincolnensis IFO 13054	Lincomycin	4	+
*S. antibioticus*I FO 12838	Actinomycin	100	+
S. griseus IFO 3430	Streptomycin	0.5	+
S. lactamdurans IFO 13305	Cephamycin	—	—
S. kanamyceticus IFO 13414	Kanamycin	—	+
S. aureofaciens IFO 12843	Tetracycline	—	+
S. graminofaciens IFO 13455	Virginiamycin	—	+
S. ostreogriseus IFO 13423	Virginiamycin	—	+
S. caespitosus IFO 13128	Mitomycin	—	+
Saccharopolyspora erythraea IFO 13426	Erythromycin	—	+
Bacillus brevis IFO 3331	Gramicidin S	0.2	—
Amycolatopsis mediterranei IFO 13415	Rifamycin	—	+
Cephalosporium acremonium CW-19	Cephalosporin C	—	+

Experimental conditions are described in the text.

Fig. 1. Separation of inducing factors from several *Streptomyces* species and *Bacillus brevis* on reverse phase HPLC.

(A) *Streptomyces griseus*, (B) *Streptomyces lincolnensis*, (C) *Streptomyces antibioticus*, (D) *B. brevis*, (E) synthetic and natural VBs.



HPLC was performed on a C_{18} column (Cosmosil 5C18, 4.6×100 mm) using a linear gradient of CH_3CN from 20 to 80% in 0.1% TFA at a flow rate of 0.7 ml/minute. Sample dissolved in 20% CH_3CN - 0.1% TFA was injected, and eluent was collected for every minute for VB activity measurement. For synthetic VB-C analongues[10], they are *cis* analogues and the n in VB-C_n indicates the chain length at C-2.

(27%) were found to produce inducing factors. HARA and BEPPU[8] reported that 15% of actinomycetes produce A-factor. On the other hand, GRITT *et al.*[9] studied the distribution of inducing factors and reported a value of 26.3%. Therefore, it can be concluded that as many as 25% of actinomycetes produce either A-factor, GRÄFE's factors or VBs as signal molecules.

It is noteworthy that virginiamycin producing strains, *i.e.*, *Streptomyces aureofaciens* and *Streptomyces ostreogriseus*, did not produce inducing factors, suggesting that the ability to produce inducing factors is independent of the ability to produce particular type of antibiotic. A similar conclusion was also drawn by GRITT *et al.*[9]. Because our assay strain *S. virginiae* responds

toward *cis* VB-type compounds at a sensitivity of 100~1,000-fold more than *trans* or A-factor type compounds[10], the inducing activity detected appears to represent *cis* VB-type factors. However, we were able to detect inducing activity, although at a level of 0.5 U/ml only, in *S. griseus* which is a A-factor producer. To confirm that *S. griseus* produces inducing factors in addition to A-factor, we separated active compounds from *S. griseus* by reverse phase HPLC using a 20 to 80% CH_3CN gradient elution (Fig. 1). Besides A-factor, which eluted at 16 minutes under our HPLC conditions, inducing factors from *S. griseus* were separated into at least 3 components (retention times 8, 12 and 18 minutes, respectively). These factors represented 6, 67 and 5%, respectively, of total activity; thus the second compound appears to be a major VB-type factor of *S. griseus*. Similarly, *S. antibioticus* was found to produce at least 3 compounds (15, 75 and 10%, respectively, of total activity), and in both *S. lincolnensis* and *B. brevis* one compound was detected. The position of elution of synthetic *cis* VB-C analogs having different C-2 side chains are also shown in the figure, and comparison of their elution times suggest that some of the factors (2 factors from *S. griseus* and one factor from *S. antibioticus*) behave similarly to VB-A, -B or -C. Other factors showed slightly different elution times but actually possessed VB activity; thus, they may represent VB-like compounds with minor modification on the substituents.

To confirm that the detected factors are actually the VB-type compounds, we purified the major inducing factor from *S. antibioticus*. For that purpose, we first selected high producing strains by single colony isolation. One strain, termed

Table 2. Effect of carbon sources on the production of inducing factors by *Streptomyces antibioticus* NF-18.

Carbon sources (30 g/liter)	Final pH	Growth (ml-wet mycelia/liter)	Inducing activity (U/ml-broth)
Glucose	7.0	9.3	375
Xylose	7.0	6.3	200
Arabinose	7.0	5.0	250
Fructose	7.0	5.8	250
Galactose	7.0	7.5	125
Lactose	7.0	3.8	300
Mannitol	7.0	8.4	125
Maltose	7.0	6.5	300
Sucrose	7.0	6.3	300
Dextrin	7.0	14.0	375
Starch	7.0	30.0	375
Soluble starch	7.0	20.0	375
Na-acetate	7.0	22.5	300
Malt extract	7.0	5.8	400
Glycerol	7.0	28.8	1,562
Corn steep liquor	7.0	10.0	1,562
Na-propionate	7.0	6.0	500
Na-succinate	7.0	9.0	1,250
Na-succinate +hexanoic acid (0.5 g/liter)	8.0	6.4	500
Na-succinate+oleic acid (0.5 g/liter) +stearic acid (0.5 g/liter) +linoleic acid (0.5 g/liter)	6.5	2.5	250
Na-succinate +*iso*-valeric acid (0.5 g/liter)	8.0	6.4	500
Corn steep liquor+soybean	7.0	57.5	1,562

Cultivation was performed with 100-ml portion of medium containing (per liter) carbon source 30 g, sodium nitrate 20 g, 1 M potassium phosphate buffer (pH 7.0) 30 ml, NaCl 5 g, yeast extract 0.1 g, $CaCO_3$ 2 g, $CaCl_2 \cdot 6H_2O$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 20 mg, $ZnSO_4 \cdot 7H_2O$ 10 mg, $MnSO_4 \cdot H_2O$ 10 mg, $CuSO_4 \cdot 5H_2O$ 10 mg and $Na_2MoO_4 \cdot 2H_2O$ 10 mg in 500-ml Sakaguchi flasks, and incubated at 28°C on a reciprocating shaker for 96 hours.

Table 3. Effect of nitrogen sources on the production of inducing factors by *Streptomyces antibioticus* NF-18.

Nitrogen source (20 g/liter)	Final pH	Growth (ml-wet mycelia/liter)	Inducing activity (U/ml-broth)
$NaNO_3$	8.0	10.0	1,250
NH_4Cl	7.5	15.0	1,250
$(NH_4)_2SO_4$	7.5	8.0	1,250
L-Asparagine	9.0	8.0	1,250
L-Glutamic acid	5.0	10.0	1,250
Casamino acid	9.0	60.0	750
Peptone	9.0	50.0	1,562
Tryptone	9.0	80.0	1,250
Yeast extract	9.0	130.0	1,562
Meat extract	9.0	40.0	1,250
Soybean powder	8.5	80.0	2,087
Soybean powder+0.5% choline-Cl	8.0	70.0	1,250
L-Leucine	8.0	12.0	1,562
Urea	8.0	8.0	1,787

Cultivation conditions are almost identical to those described in Table 2 except that sodium succinate (30 g/liter) and the indicated nitrogen source (20 g/liter) were used.

Table 4. Combined effect of carbon and nitrogen sources on the production of inducing factors by *Streptomyces antibioticus* NF-18.

Carbon source	Production of inducing factors (U/ml-broth)			
	Yeast extract	Soybean powder	L-Leucine	Urea
Sodium succinate	1,562 (9.0)	2,080 (8.5)	1,562 (8.0)	1,787 (8.0)
Glycerol	3,757 (7.2)	3,125 (7.0)	2,500 (6.0)	3,125 (7.5)
Corn steep liquor	3,125 (9.0)	2,500 (6.0)	2,500 (8.0)	2,500 (9.0)
Sodium succinate +glycerol +corn steep liquor[a]	5,000 (8.5)	—	—	—

Cultivation was performed with a 100-ml portion of medium containing the indicated carbon (30 g/liter) or nitrogen (20 g/liter) source in a 500-ml Sakaguchi flask for 96 hours at 28°C. The pH at the end of cultivation is indicated in parentheses; the initial pH was adjusted to 7.0. Other experimental conditions are identical to those described in Table 2.

[a] Sodium succinate (20 g/liter)+glycerol (20 g/liter)+corn steep liquor (10 g/liter) was used as carbon source.

NF-18, was found as the highest producer. After choosing optimal carbon and nitrogen sources (Tables 2, 3 and 4), strain NF-18 produced higher than 5,000 U/ml-broth of the inducing factor. Culture broth (6 liters) was extracted with 9 liters of EtOAc under acidic conditions: Removal of the solvent gave 1.8×10^7 U (2,340 mg). The residue was dissolved in 10% MeOH, applied to a active charcoal column (20 g), and eluted successively with 200 ml each of 10% MeOH, 80% MeOH, MeOH, 10% EtOAc-MeOH, 20% EtOAc-MeOH and EtOAc. The major activity eluted by MeOH and 10% EtOAc-MeOH was combined (7.0×10^6 U, 153 mg), evaporated, and further purified by reverse phase middle pressure chromatography. This activity was eluted by 40% and 60% CH_3CN in 0.1% TFA (5.0×10^6 U, 14.5 mg). The active fractions were pooled, evaporated and purified further by reverse phase HPLC using 30% CH_3CN in 0.1% TFA as mobile phase yielding 2.3 mg (4.0×10^6 U) of a pure compound. NMR spectral analysis clearly indicated that the compound is identical with VB-A isolated previously from *S. virginiae*[1].

Spectral data of the compound: ^1H NMR

(270 MHz, $CDCl_3$) δ 4.42 (1H, dd, 4-H_a, $J_{4a,4b}$= 9.03 Hz, $J_{4a,3}$=8.67 Hz), 4.13 (1H, m, 6-H), 4.10 (1H, dd, 4-H_b, $J_{4b,4a}$=9.0 Hz, $J_{4b,3}$=6.89 Hz), 3.74 (2H, m, 5-H_2), 2.86 (1H, m, 3-H), 2.57 (1H, dd, 2-H, $J_{2,3}$=7.3 Hz, $J_{2,6}$=3.67 Hz), 1.56 (4H, m, 7-H_2, 8-H_2), 1.34 (1H, m, 10-H), 1.23 (2H, m, 9-H), 0.88 (6H, d, J=6.7 Hz, 11-H_3, 12-H_3); ^{13}C NMR (68 MHz, $CDCl_3$) δ 178.36 (C-1), 70.84 (C-6), 69.35 (C-4), 63.33 (C-5), 48.07 (C-2), 38.67 (C-9), 38.10 (C-3), 35.05 (C-7), 27.88 (C-10), 23.58 (C-8), 22.56, 22.51 (C-11, C-12); Chemical ionization mass spectra (CI-MS) *m/z* 231 (M+1), 213 (M$-H_2O$+1); Fourier transformation (FT)-IR (film) cm^{-1} 3419, 2955, 2935, 1753, 1690, 1679.

The stereochemistry of the two substituents on the γ-lactone was determined to be *cis* on the basis of the coupling constant (7.3 Hz) between 2-H and 3-H, which is in good agreement with that of VB-A (7.4 Hz). CD spectrum of the compound showed the similar positive ellipticity at 215 nm (θ +2,210), indicating that the compound and VB-A share the same absolute configuration. Purification and structural elucidation of the minor components having inducing activity from *S. antibioticus* are under way.

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References

1) YAMADA, Y.; K. SUGAMURA, K. KONDO, M. YANAGIMOTO & H. OKADA: The structure of inducing factors for virginiamycin production in *Streptomyces virginiae*. J. Antibiotics 40: 496~504, 1987
2) 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
3) MORI, K.: Revision of the absolute configuration of A-factor. Tetrahedron 39: 3107~3109, 1983
4) 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
5) DEMAIN, A. L.; J. NEWKIRK & D. HENDLIN: Effect of methionine, norleucine, and lysine derivatives on cephalosporin C formation in chemically defined media. J. Bacteriol. 85: 339~344, 1963
6) 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
7) YANAGIMOTO, M.; Y. YAMADA & G. TERUI: Extraction and purification of inducing material produced in staphylomycin fermentation. Hakko Kogaku Kaishi (Japanese) 57: 6~14, 1979
8) HARA, O. & T. BEPPU: Mutants blocked in streptomycin production in *Streptomyces griseus* — the role of A-factor. J. Antibiotics 35: 349~358, 1982
9) ERITT, I.; U. GRÄFE & W. F. FLECK: Inducers of both cytodifferentiation and anthracycline biosynthesis of *Streptomyces griseus* and their occurrence in actinomycetes and other microorganisms. Z. Allg. Mikrobiol. 24: 3~12, 1984
10) NIHIRA, T.; Y. SHIMIZU, H. S. KIM & Y. YAMADA: Structure-activity relationships of virginiae butanolide C, an inducer of virginiamycin production in *Streptomyces virginiae*. J. Antibiotics 41: 1828~1837, 1988