BMS-181184, A NEW PRADIMICIN DERIVATIVE

SCREENING, TAXONOMY, DIRECTED BIOSYNTHESIS, ISOLATION AND CHARACTERIZATION

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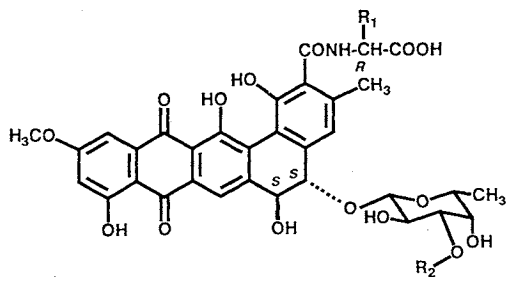
BMS-181184 is a new semisynthetic pradimicin derivative with a broad-spectrum antifungal activity. In a search for actinomycetes producing BMS-181184, 4 strains of *Actinomadura* sp. isolated from soil samples were found to produce the antibiotic under conditions of directed biosynthesis. Among them, *Actinomadura* sp. AB1236 proved most useful in the production of BMS-181184 when fermented in a medium containing D-serine and D-cycloserine. A minor product isolated from the broth of strain AB1236 was identified as the dexylosyl analog of BMS-181184, which was also obtained by acid hydrolysis of BMS-181184.

The pradimicins[1~3] and benanomicins[4,5] belong to a novel family of dihydrobenzo[*a*]naphthacenequinone antibiotics with a broad-spectrum antifungal activity. BMS-181184 is a novel derivative of pradimicin FA-2 with demonstrable *in vivo* efficacy in mice against pathogenic fungi and excellent water-solubility[6,7]. However, as this compound had to be semisynthesized from pradimicin FA-2, it was highly desirable to develop an alternative and direct process suitable for mass production of BMS-181184. Since pradimicins FA-1 and FA-2, D-serine analogs of pradimicins A and C, respectively, were produced by a process of directed biosynthesis with exogenously added D-serine[3], production of BMS-181184 by a microorganism should be feasible, provided that a proper strain is used. For this purpose, a screening system was designed and run in a search for benanomicin A-producing actinomycetes from soil samples. This paper describes the screening system, taxonomy of the producers, directed biosynthesis of BMS-181184 and the structure of the minor component, BMS-181532 (Fig. 1).

Fig. 1. Structures of BMS-181184, BMS-181532 and benanomicin A.



	R_1	R_2
BMS-181184	CH_2OH	
BMS-181532	CH_2OH	H
Benanomicin A	CH_3	

Materials and Methods

General

Melting points were taken on a Yanagimoto

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micro melting point apparatus and are uncorrected. IR and UV spectra were measured on a Jasco IR-810 spectrometer (KBr pellet) and a Jasco UVIDEC-610C spectrometer, respectively. ^{1}H NMR spectra (400 MHz) were recorded on a JEOL JMN-GX 400 spectrometer in DMSO. FAB-MS spectra were obtained with a JEOL JMS-AX505H spectrometer. HPLC was run with a Yokogawa LC-100.

Screening

The activity of each broth supernatant against *Candida albicans* A9540 was tested by the hole plate assay method on yeast nitrogen base-glucose agar plates, pH 7. The lowest concentration detectable by this assay was 6.3 μg/ml for benanomicin A and BMS-181184. Broths showing activity were collected by centrifugation at 10,000 rpm for 10 minutes, diluted 10-fold with DMSO, and filtered (Gelman Sciences Japan, Ltd., Ekicrodisc 13CR, pore size: 0.45 μm). The filtrates were analyzed by HPLC on YMC ODS column (Yamamura Chemical Lab.) using acetonitrile - 0.02 M phosphate buffer, pH 3.5 (25 : 75), at a flow rate of 1 ml/minute with 460 nm detection and by TLC on silica gel thin layer plates (Kiesel gel 60 F_{254} 0.25 mm; E. Merck). The developing solvent systems used for TLC were *n*-butanol - acetic acid - water (2 : 1 : 1, BW-14) and methyl acetate - *n*-propanol - 28% aq ammonia (45 : 105 : 60, S-114).

Production of antibiotics in each broth supernatant was estimated from optical density at 500 nm. An aliquot was diluted ($\times$ 20 or $\times$ 40) with a solution consisting of 0.02 N NaOH - MeOH (1 : 1) and the optical densities at 500 nm and 600 nm were read. Titer was calculated by the following equation using pradimicin A as reference: Titer (μg/ml) = $(A_{500} - A_{600})/180 \times$ dilution factor $\times$ 100.

Taxonomy

The media and procedures used for taxonomy of the strain were those described by SHIRLING and GOTTLIEB[8] and by WAKSMAN[9]. Agar media described by ARAI[10] were also used. Each strain was incubated at 37°C for 2 to 4 weeks. Color determination was made by comparing the culture with color chips according to the Manual of Color Names (Japan Color Enterprise Co., Ltd., 1987). Temperature range for growth was determined on yeast - starch agar medium using a temperature gradient incubator TN-3 (Toyo Kagaku Sangyo Co., Ltd.). The susceptibility test was carried out using antibiotic disks (Tridisk, Eiken Chemical Co., Ltd.) which were placed onto the surface of yeast - glucose - malt agar (yeast extract 0.1%, glucose 1%, malt extract 0.1%, $CaCl_2 \cdot 2H_2O$ 0.05% and agar 1.5%) seeded with strain AB1236 (4% inoculum) and the plates incubated at 37°C. Diaminopimelic acid and sugars in whole cell hydrolysate were analyzed by the method described by BECKER *et al.*[11] and LECHEVALIER[12] using thin layer chromatography sheets as described by STANECK and ROBERTS[13]. Phospholipid and mycolate compositions were determined by the methods of LECHEVALIER *et al.*[14] and MINNIKIN *et al.*[15], respectively. Menaquinone samples were prepared by the procedure of COLLINS *et al.*[16] and analyzed with a mass spectrometer. The composition of cellular fatty acids was quantitatively determined by the method of SUZUKI *et al.*[17] after esterification with 5% methanolic HCl overnight at 50°C. The GC contents of the DNAs isolated by the previously reported procedure[18] were determined by HPLC[19,20].

Acid Hydrolysis of BMS-181184

A solution of BMS-181184 (510 mg) in DMSO (10 ml), dioxane (34 ml) and 1 N HCl (44 ml) was heated at 50°C for 7.5 days. The reaction mixture was cooled, adjusted to pH 7.0 with 6 N NaOH, and the solvent was removed *in vacuo*. The aqueous residue was adjusted to pH 2.5 with 1 N HCl and the resulting solid was collected by filtration and washed with 0.001 N HCl. The solid was suspended in water (10 ml), the pH was adjusted to 7.0, and the resulting solution was lyophilized to give a mixture (425 mg) containing unreacted BMS-181184 and dexylosyl BMS-181184. This mixture was chromatographed on a column of YMC gel, ODS A60 (5 liters) using acetonitrile - 0.02 M phosphate, pH 7.0 (13 : 87) as eluent, to give, after desalting and lyophilization, dexylosyl BMS-181184 (44 mg): MP $>$ 180°C (dec).

Antifungal Activity

The antifungal spectrum was determined by the 2-fold agar dilution assay on yeast morphology agar (Difco Laboratories) containing 1/15 M phosphate buffer, pH 7.0, with inocula of fungi adjusted to 10^4 cells/5-μl spot. MICs were determined after 40 hours of incubation at 28°C (incubation period of 60 hours and inocula of 10^5 cells/5-μl spot for *Trichophyton mentagrophytes* 4329).

Results

Screening

The screening system designed to search for strains producing BMS-181184 consisted of 4 steps: (1) Isolation of actinomycetes, mainly *Actinomadura*, (2) primary selection of strains based on the production of diffusible red pigments in glycerol-, glucose- and soluble starch-based agar media, (3) secondary selection based on the production of benanomicin A, and (4) final selection by the production of BMS-181184 under D-serine-fed fermentation conditions. As shown in Table 1, 15 actinomycetes producing red pigments and/or reverse growth were isolated from 140 soil samples collected in various countries. A loopful of each actinomycete was inoculated as a patch and incubated at 37°C for 10 days on 3 agar plates; glucose-yeast-Soytone agar (glucose 1%, yeast extract 0.05%, Soytone (Difco Laboratories) 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01% and agar 1.5%), glycerol-yeast-Soytone agar (glycerol 1%, yeast extract 0.05%, Soytone 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01% and agar 1.5%) and starch-yeast-Soytone agar (soluble starch 1%, yeast extract 0.05%, Soytone 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01% and agar 1.5%). Pradimicin A-producing strains, *Actinomadura hibisca* P157-2 and Q-278-4[21], and *Actinomadura verrucosospora* subsp. *neohibisca* R103-3[22] were also tested for comparison. As shown in Table 2, 4 strains (AB1236, AB1367, AB3811 and AB4095) produced diffusible bright red pigments on the 3 media tested and 2 strains (AA6712 and AB0534) produced pigments on glucose- and soluble starch-based media. These 6 strains were then tested for antibiotic production by incubating them at 32°C for 10 days in 2 nutrient liquid media; one containing glucose 2%, defatted soybean meal (Esusan mi-to, Ajinomoto Co., Ltd.) 1.5%, $CoCl_2 \cdot 6H_2O$ 0.0001%, KH_2PO_4 0.1125% and K_2HPO_4 0.0025%, and the other containing the ingredients described above except glycerol 2% instead of glucose, distributed

Table 1. Collection sites of fifteen actinomycetes producing red pigment.

Strain	Collection site
AA6712	Lima, Peru
AA8507	Mirazgaon, Maharashtra state, India
AA8689	Davao, Mindanao Island, Philippines
AA8966	Kagoshima city, Kagoshima prefecture, Japan
AB0534	Rizal, Ruzon Island, Philippines
AB1236	Shinjuku, Tokyo, Japan
AB1350	Chichibu city, Saitama prefecture, Japan
AB1367	Chichibu city, Saitama prefecture, Japan
AB2801	Rome, Italy
AB2837	Naples, Italy
AB2957	Naples, Italy
AB3054	Rome, Italy
AB3107	Matalom, Leyte Island, Philippines
AB3811	Yokohama city, Kanagawa prefecture, Japan
AB4095	Kamakura city, Kanagawa prefecture, Japan

Table 2. Production of diffusible red pigments on three agar plates.

Strain	Carbon source			Color of diffusible pigment
	Glycerol	Glucose	Soluble starch	
P157-2[a]	+	++	−	Bright red (37)
Q278-4[a]	±	++	+	Bright red (37)
R103-3[b]	+	++	++	Bright red (37)
AA6712	±	++	++	Bright red (37)
AA8507	−	−	−	
AA8689	−	−	−	
AA8966	−	−	−	
AB0534	±	++	++	Bright red (37)
AB1236	++	+	++	Bright red (37)
AB1350	−	−	−	
AB1367	++	+	++	Bright red (37)
AB2801	−	−	−	
AB2837	−	−	−	
AB2957	−	−	+	
AB3054	−	−	−	
AB3107	−	−	−	
AB3811	++	+	++	Bright red (37)
AB4095	++	++	++	Bright red (37)

[a] Strains P157-2 and Q278-4: *Actinomadura hibisca*[18].
[b] Strain R103-3: *Actinomadura verrucosospora* subsp. *neohibisca*[19].
−; No production, ±; doubtful, +; low production, ++; high production.
Color number was in accordance with the Manual of Color Names (Japan Color Enterprise Co., Ltd).

Table 3. Production of pradimicins and benanomicins by 6 red pigment-producing strains.

Strain	Carbon source	Inhibition zone against *C. albicans* (mm)	TLC Rf BW-12	TLC Rf S-114	HPLC retention time (minutes)	Titer (μg/ml) Pradimicin A	Benanomicins A	Benanomicins B
AA6712	Glucose	12	0.4	0.3	8.2	56		
	Glycerol	11	0.38	0.3	8.2	104		
AB0534	Glucose	12	0.41	0.31	8.1	171		
	Glycerol	11	0.39	0.29	8.2	213		
AB1236	Glucose	12	0.5	0.2	7.4, 25.8,		130	67
	Glycerol	19	0.51	0.19	7.4, 25.9		415	654
AB1367	Glucose	12	0.5	0.2	7.3, 25.7		124	57
	Glycerol	13	0.48	0.2	7.6, 26.1		94	166
AB3811	Glucose	13	0.51	0.21	7.5, 26.0		219	115
	Glycerol	17	0.5	0.22	7.5, 26.1		228	338
AB4095	Glucose	13	0.53	0.19	7.5, 26.1		199	110
	Glycerol	15	0.5	0.21	7.6, 26.1		196	248

Medium: Carbon source 2%, defatted soybean meal 1.5%, $CoCl_2 \cdot 6H_2O$ 0.005%, K_2HPO_4 0.0025% and KH_2PO_4 0.1125%.
Fermentation: 28°C for 10 days.

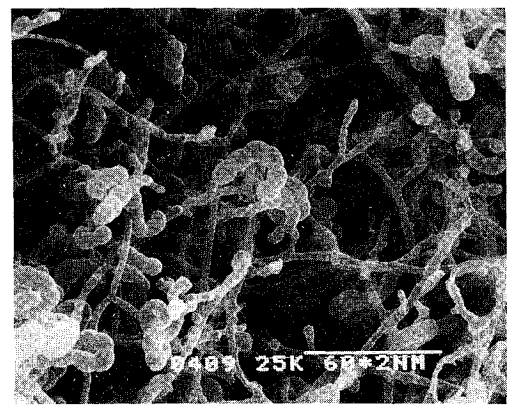
in 100-ml volumes in 500-ml Erlenmeyer flasks with rotary shaking (200 rpm). As summarized in Table 3, 4 strains (AB1236, AB1367, AB3811 and AB4095) produced compounds identified as benanomicins A and B based on their physico-chemical properties in comparison with those reported in the literature[4,23].

Taxonomy

Strain AB1236 grew better on organic media than on inorganic media at temperatures between 20 and 40°C and formed a branched vegetative mycelium. The color of mature aerial mycelia was white to grayish white on both yeast-starch agar and yeast-starch-malt agar (YSM, soluble starch 1%, yeast extract 0.1%, malt extract 0.1%, $CaCl_2 \cdot 2H_2O$ 0.05% and agar 1.5%). Examination under light and scanning electron microscopes revealed that the top of short sporophores had 2 to 8 spores per chain. The shape of the spores were subglobose ($0.8 \sim 1.0 \times 1.0 \sim 1.2$ μm in size) and their surface was smooth (Fig. 2). These spores were not motile. The color of vegetative mycelia and diffusible pigments on organic agar media ranged from soft pink to dark red. The color changed from soft orange to strong yellowish orange by the addition of 0.1 N HCl. The cultural, physiological characteristics of strain AB1236 and utilization of carbon sources are summarized in Tables 4, 5 and 6, respectively.

Fig. 2. Scanning electron mircrograph of *Actinomadura* sp. AB1236.

Bar represents 5.6 μm.



AB1236 grown on YSM agar for 3 weeks at 28°C, showing short spore chains with smooth spores.

Strain AB1236 contained *meso*-diaminopimeric acid, madurose, ribose, mannose, glucose and galactose. Thus, strain AB1236 has a cell wall belonging to type III-B. Mycolic acids were not detected. Phospholipid analysis showed that the cell wall had the type PI pattern containing diphosphatidylinositol

Table 4. Cultural characteristics of strain AB1236.

Medium	Growth	Reverse	Aerial mycelium	Diffusible pigment
Sucrose-nitrate agar (Waksman med. No. 1)	Yellowish white (393)	Yellowish white (393)	None	None
Glycerol-nitrate agar (Waksman med. No. 14)	Grayish red (60), good	Grayish red (60)	None	Pinkish white (391)
Glucose-asparagine agar (Waksman med. No. 2)	Yellowish white (393), good	Yellowish white (393)	None	None
Yeast ext.-malt ext. agar (ISP med. No. 2)	Dark red (57), good	Dark red (57)	Grayish white (390) to pinkish white (391)	Dark red (58)
Oat meal agar (ISP med. No. 3)	Soft pink (25), good	Soft pink (26)	White (388), cottony	Soft pink (26)
Inorganic salts-starch agar (ISP med. No. 4)	Yellowish white (393), good	Yellowish white (393) to pinkish white (391)	None	None
Glycerol-asparagine agar (ISP med. No. 5)	Dark red (58), good	Dark red (58)	None	Soft pink (25)
Tyrosine agar (ISP med. No. 7)	Dark red (58), good	Dark red (58)	None	Soft pink (25)
Nutrient agar (Waksman med. No. 14)	Yellowish white (393), poor	Yellowish white (393)	None	None
Yeast starch agar	Dark grayish red (61), good	Dark grayish red (61)	White (388) to grayish white (390), cottony	Deep yellowish red (53)
BENNETT's agar	Dark red (57), good	Dark red (58)	Grayish white (390), scant	Deep pink (22)

Table 5. Physiological characteristics of strain AB1236.

Test	Results
Starch hydrolysis (ISP med. No. 4)	+
Nitrate reduction (Difco, nitrate broth)	−
10% skimmed milk (Difco, 10% skimmed milk)	
Coagulation	+
Peptonization	±
Cellulose decomposition (sucrose-nitrate solution with a strip of paper as the sole carbon source)	−
Gelatin liquefaction	No growth
Melanine formation	
On ISP med. No. 7	−
Temperature range for growth (°C)	20~41
Optimum temperature (°C) (on yeast-starch agar)	30.5~35.5
pH range for growth (on Trypticase soy broth, BBL)	6~8
Optimum pH (on Trypticase soy broth, BBL)	7
Antibiotic susceptibility	
Resistant: Fosfomycin (50 μg) and polymixin B (300 U)	
Sensitive: Ampicillin (20 U), cephalexin (10 μg), chloramphenicol (10 μg), erythromycin (2 μg), josamycin (2 μg), lincomycin (2 μg), kanamycin (5 μg), gentamicin (5 μg), tobramycin (5 μg), nalidixic acid (2 μg), norfloxacin (2 μg) and colistin (50 U)	

−; Negative, ±; doubtful, +; positive.

mannoside, phosphatidylinositol and diphosphatidylglycerol. Quantitative analysis of the menaquinones revealed that the components were 47% MK-9 (H_8), 35% MK-9 (H_6), 10% MK-9 (H_4) and 8% MK-9 (H_{10}). The whole-cell fatty acids were consisted of 49% 14-methylpentadecanoic acid (*i*-16:0), 13%

14-methylhexadecanoic acid (*a*-17:0) and 8% 10-methylheptadecanoic acid (10Me-17:0), and other minor fatty acids. The GC content of the strain was 67.9 mol%.

Strain AB1236 has morphological and cultural properties as well as chemotaxonomic properties that are consistent with those of the genus *Actinomadura* Lechevalier and Lechevalier, 1970[24], and with the definition of this genus proposed by KROPPENSTEDT *et al.*[25]. Thus, strain AB1236 was identified as a species of *Actinomadura*. From the published descriptions of the known species of *Actinomadura*, *Actinomadura spadix* Nonomura and Ohara, 1971[26] appeared to be the only one that had a resemblance to strain AB1236. However, direct comparison between strain AB1236 and *A. spadix* JCM 3146^T revealed that strain AB1236 differs from strain JCM 3146^T in utilization of inositol, color of growth and diffusible pigment, B-vitamins requirement, and DNA-DNA homology (^{32}P-labeled DNA from strain AB1236 vs. DNA from strain JCM 3146^T:

Table 6. Utilization of carbon sources by strain AB1236.

Carbon source	Utilization
D-Glucose	+
L-Arabinose	+
D-Xylose	+
Inositol	+
Mannitol	+
D-Fructose	+
L-Rhamnose	+
Sucrose	+
Raffinose	+

+; Positive. (ISP 9 medium, 37°C for 3 weeks).

Table 7. Characteristics of three benanomicin-producing strains.

Characteristic	Strain AB1367	Strain AB3811	Strain AB4095
Sporulation of aerial mycelium	Short spore chains	Short spore chains	Short spore chains
Spore surface	Smooth	Smooth	Smooth
Aerial mass color	White	White	Pinkish white
Diffusible pigment	Red pH indicator	Red pH indicator	Red pH indicator
Melanin formation (ISP-7)	Positive	Doubtful	Positive
Starch hydrolysis	Positive	Positive	Positive
Carbon source utilization:			
D-Glucose	+	+	+
L-Arabinose	+	+	+
D-Xylose	−	−	−
Inositol	−	−	−
D-Mannitol	+	+	+
D-Fructose	−	+	+
L-Rhamnose	±	±	±
Sucrose	++	++	++
Raffinose	+	+	+
Temp. range for growth	18~38°C	15~41°C	14~39°C
Characteristic amino acid	*meso*-DAP	*meso*-DAP	*meso*-DAP
Characteristic sugar	Madurose	Madurose	Madurose
Menaquinone			
MK-9 (H_4)	4%	5%	5%
MK-9 (H_6)	26	28	29
MK-9 (H_8)	64	61	56
MK-9 (H_{10})	6	6	9
Major fatty acid			
i-16:0	35	40	43
a-17:0	30	19	17
10Me-17:0	12	14	14
Phospholipid type	PI	PI	PI

21%, and ^{32}P-labeled DNA from strain JCM 3146^T vs. DNA from strain. AB1236: 28%) when tested by the method described by MIYADOH *et al.*[27].

The taxonomy of the 3 other strains (AB1367, AB3811 and AB4095) were carried out comparatively by the procedures mentioned above and the taxonomical characteristics of these strains are summarized in Table 7. For the reasons described above, all these strains were identified as the same species belonging to the genus *Actinomadura*.

Actinomadura sp. AB1236 has been deposited with the American Type Culture Collection (ATCC) under accession number of ATCC 55208.

Directed Biosynthesis of BMS-181184

The 4 benanomicin A-producing strains were grown at 37°C for 2 weeks on YSM agar slants. The seed culture was incubated at 32°C for 5 days in a medium composed of glucose 0.5%, soluble starch 2%, yeast extract 0.2%, NZ-case 0.3%, Fish Meal Extract D30X (Banyu Eiyou K. K.) 0.5% and $CaCO_3$ 0.3% (pH was adjusted to 7.0 before autoclaving), distributed in 100-ml volumes in 500-ml Erlenmeyer flasks on a rotary shaker (200 rpm). Five-ml of the seed were used as inocula to start directed fermentation as well as production of the antibiotics in 100-ml volumes in 500-ml Erlenmeyer flasks.

Table 8. D-Serine supplemented fermentation on the benanomicin-producing strains.

Strain	Carbon source	Titer (μg/ml)					
		Pradimicin	BMS-		Benanomicins		
		FA-2	181184	181532	A	B	DXBNM
AB1236	Glucose	82	50	7	107	32	+
	Glycerol	339	151	63	339	163	38
AB1367	Glucose	21	42	6	−	5	−
	Glycerol	6	12	−	+	3	−
AB3811	Glucose	20	13	+	33	58	+
	Glycerol	15	10	+	66	87	+
AB4095	Glucose	50	44	+	100	113	+
	Glycerol	36	27	+	78	135	14

Basal medium: Carbon source 2%, defatted soybean meal 1.5%, $CoCl_2 \cdot 6H_2O$ 0.005%, K_2HPO_4 0.0025%, KH_2PO_4 0.1125% and D-serine 0.2%.
Fermentation: 28°C for 10 days.
DXBNM: Dexylosylbenanomicin A.

Table 9. Effect of D-cycloserine on the production of BMS-181184 by *Actinomadura* sp. AB1236.

D-Cycloserine (μg/ml)	Titer (μg/ml)					
	Pradimicin	BMS-		Benanomicins		
	FA-2	181184	181532	A	B	DXBNM
20	396	410	42	198	127	+
10	463	465	64	192	144	+
5	429	398	17	247	181	+
0	323	132	84	313	156	36

Medium: Glycerol 2%, deffated soybean meal 1.5%, $CoCl_2 \cdot 6H_2O$ 0.005%, K_2HPO_4 0.0025%, KH_2PO_4 0.1125% and D-serine 0.2%.
Fermentation: 28°C for 10 days.

As the amino acid side chain of BMS-181184 is D-serine, the 4 candidate strains were tested for the production of BMS-181184 in 2 media containing D-serine. As shown in Table 8, strain AB1236 was best producer, giving the highest titer of BMS-181184. In order to optimize the production of BMS-181184 while reducing that of benanomicin A, D-cycloserine, an inhibitor of alanine racemase, was added to the production medium. As shown in Table 9, addition of D-cycloserine at concentrations of up to 10 μg/ml improved the production of BMS-181184.

Production and Isolation of BMS-181184

The following procedure is an example of the routine production and isolation of the antibiotic. A production medium consisting of glucose 2%, Pharmamedia 1%, KH_2PO_4 0.1%, D-serine 0.2% and D-cycloserine (final concentration: 10 μg/ml, added after autoclaving). Five-ml inocula of strain AB1236 were transferred into three hundred 500-ml Erlenmeyer flasks, and the broths were fermented at 28°C for 11 days on a rotary shaker (200 rpm). The whole broth was acidified to pH 2.0 using 6 N HCl and centrifuged. The product in the supernatant (27 liters) was adsorbed on 3.8 liters of Diaion HP-20, washed successively with 80% acetone (8 liters) and a mixture of acetone - 0.01 N HCl (60 : 40, 12 liters), and eluted with a mixture of acetone - 0.01 N NaOH (60 : 40, 12 liters). The eluate was concentrated and lyophilized to yield 16.1 g of crude solid. Part (2 g) of the solid was chromatographed on a column of YMC gel, ODS A60 (Yamamura Chemical Lab., 10 liters) using acetonitrile - 0.02 M phosphate buffer, pH 7.0 (12.5 : 87.5) as eluent, and the eluates were monitored by HPLC (column: Cosmosil 5C18AR, mobile phase: acetonitrile - 0.02 M phosphate buffer, pH 3.5 (27 : 73), flow rate: 1.0 ml/minute, UV: 254 nm). Fractions containing the major product were concentrated, desalted on Diaion HP-20 and lyophilized to give BMS-181184 (700 mg). Part (50 mg) of the lyophile was dissolved in H_2O and acidified with 0.1 N HCl. The resulting solid was washed successively with H_2O and acetone, and dried at 60°C under vacuum for 24 hours to yield dark red solid (36 mg): MP >218°C (dec); *Anal* Calcd for $C_{39}H_{41}NO_{20} \cdot 2H_2O$: C 53.24, H 5.16, N 1.59, Found: C 53.34, H. 5.05, N 1.49.

Physico-chemical and spectroscopic properties of BMS-181184 obtained by the fermentation were identical with those of BMS-181184 derived from pradimicin FA-2[6].

Fractions containing the minor product were similarly worked up to yield BMS-181532 (62 mg): MP >180°C (dec). The UV spectrum of BMS-181532 in MeOH - 0.02 N NaOH (1 : 1) exhibited λ_{max} nm (ε) 213 (31,500), 242 (30,100), 320 (13,600) and 498 (12,600). The HRFAB-MS indicated a MW of 711

Table 10. *In vitro* antifungal activity of BMS-181532.

Test organism	MIC (μg/ml)			
	BMS-181532	BMS-181184	Amphotericin B	Ketoconazole
Saccharomyces cerevisiae ATCC 9763	1.6	1.6	0.8	100
Candida albicans A9540	3.1	3.1	0.8	25
C. albicans ATCC 38247	3.1	3.1	0.8	6.3
C. albicans ATCC 32354	3.1	3.1	0.8	50
C. albicans Juntendo 83-2-14	1.6	3.1	0.8	25
Candida tropicalis Kitasato 85-8	1.6	12.5	0.8	100
C. tropicalis IFO 10241	6.3	12.4	1.6	50
Cryptococcus neoformans D49	1.6	3.1	0.8	6.3
C. neoformans IAM 4514	3.1	1.6	0.8	6.3
Aspergillus fumigatus IAM 2034	6.3	3.1	0.8	3.1
Trichophyton mentagrophytes 4329	6.3	3.1	0.8	0.8

(observed (M+H) at *m/z* 712.1871), 132 mass units less than BMS-181184 (*m/z* 844 $(M+H)^+$), implying that BMS-181532 lacks a xylose moiety. The ^1H NMR spectrum of BMS-181532 in DMSO-d_6 (400 MHz) was consistent with the dexylosyl analog of BMS-181184; δ 1.10 (3H, d, $J=6.4$ Hz, 5'-CH_3), 2.33 (3H, s, 3-CH_3), 3.33~3.39 (1H, m, 3'-H), 3.42 (1H, d-like, $J=3.0$ Hz, 4'-H), 3.48~3.53 (1H, m, 2'-H), 3.54 (1H, q-like, $J=6.4$ Hz, 5'-H), 3.74 (2H, d-q, $J=11.1$, 5.1 Hz, 17-CH_2), 3.95 (3H, s, 11-OCH_3), 4.46 (1H, t, $J=5.1$ Hz, 17-H), 4.47 (1H, 5-H), 4.54 (1H, d, $J=7.7$ Hz, 1'-H), 4.56 (1H, d, $J=11.1$ Hz, 6-H), 6.92 (1H, d, $J=2.6$ Hz, 10-H), 7.18 (1H, s, 4-H), 7.28 (1H, d, $J=2.6$ Hz, 12-H), 8.04 (1H, s, 7-H).

The identity of BMS-181532 with the dexylosyl analog of BMS-181184 was established by comparison with the product obtained by acid hydrolysis of BMS-181184.

Antifungal Activity

The antifungal activity of BMS-181532 was determined and compared with those of BMS-181184, amphotericin B and ketoconazole. As shown in Table 10, BMS-181532 had a broad spectrum of activity with potency superior to ketoconazole. The activity against a polyene-resistant strain of *Candida albicans* (ATCC 38247) indicates that there is no cross resistance between BMS-181532 and amphotericin B.

Discussion

BMS-181184 is a semisynthetic derivative of pradimicin FA-2 with promising antifungal activity *in vitro* and *in vivo*. The purpose of this study was to establish a direct process amenable to large scale preparation of BMS-181184. The results presented in this paper demonstrated that directed biosynthesis of the target molecule was feasible, supporting the hypothesis that D-alanine acts as a direct precursor for the amino acid side chain.

Selection of the soil microorganisms appeared to be the key to the success. We have focused on screening actinomycetes, especially members of the genus *Actinomadura*, because the pradimicins and benanomicins have been reported to be produced by microorganisms belonging to the single genus *Actinomadura*. Indeed it is important to note that although the soil samples were collected in various countries, the organisms producing benanomicins were those isolated in Japan and taxonomically assignable to the genus *Actinomadura*.

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References

1) OKI, T.; M. KONISHI, K. TOMATSU, K. TOMITA, K. SAITOH, M. TSUNAKAWA, M. NISHIO, T. MIYAKI & H. KAWAGUCHI: Pradimicin, a novel class of potent antifungal antibiotics. J. Antibiotics 41: 1701~1704, 1988
2) SAWADA, Y.; M. NISHIO, H. YAMAMOTO, M. HATORI, T. MIYAKI, M. KONISHI & T. OKI: New antifungal antibiotics, pradimicins D and E. Glycine analogs of pradimicins A and C. J. Antibiotics 43: 771~777, 1990
3) SAWADA, Y.; M. HATORI, H. YAMAMOTO, M. NISHIO, T. MIYAKI & T. OKI: New antifungal antibiotics pradimicins FA-1 and FA-2: D-Serine analogs of pradimicins A and C. J. Atibiotics 43: 1223~1229, 1990
4) TAKEUCHI, T.; T. HARA, H. NAGANAWA, M. OKADA, M. HAMADA, H. UMEZAWA, S. GOMI, M. SEZAKI & S. KONDO: New antifungal antibiotics, benanomicins A and B from an *Actinomycete*. J. Antibiotics 41: 807~811, 1988
5) KONDO, S.; S. GOMI, K. UOTANI, S. INOUYE & T. TAKEUCHI: Isolation of new minor benanomicins. J. Antibiotics 44: 123~129, 1991
6) KAMACHI, H.; S. IIMURA, S. OKUYAMA, H. HOSHI, S. TAMURA, M. SHINODA, K. SAITOH, M. KONISHI & T. OKI: Synthesis and antifungal activities of pradimicin derivatives, modification at C4'-position. J. Antibiotics 45: 1518~1525, 1992

7) OKI, T.; M. KAKUSHIMA, M. HIRANO, A. TAKAHASHI, A. OHTA, S. MASUYOSHI, M. HATORI & H. KAMEI: *In vitro* and *in vivo* antifungal activities of BMS-181184. J. Antibiotics 45: 1512~1517, 1992
8) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
9) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. II. Classification, Identification and Description of Genera and Species. pp. 328~334, The Williams & Wilkins Co., 1961
10) ARAI, T. (*Ed.*): Culture Media for Actinomycetes. The Society for Actinomycetes Japan, 1975
11) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
12) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968
13) STANECK, J. L. & G. D. ROBERTS: Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl. Microbiol. 28: 226~231, 1974
14) LECHEVALIER, M. P.; C. DEBIEVRE & H. LECHEVALIER: Chemotaxonomy of aerobic actinomycetes: Phospholipid composition. Biochem. Syst. Ecol. 5: 249~260, 1977
15) MINNIKIN, D. E.; L. ALSHAMAONY & M. GOODFELLOW: Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of whole-organism methanolysates. J. Gen. Microbiol. 88: 200~204, 1975
16) COLLINS, M. D.; H. N. SHAH & D. E. MINNIKIN: A note on the separation of natural mixtures of bacterial menaquinones using reverse-phase thin-layer chromatography. J. Appl. Bacteriol. 48: 277~282, 1980
17) SUZUKI, K. & K. KOMAGATA: Taxonomic significance of cellular fatty acid composition in some coryneform bacteria. Int. J. Syst. Bacteriol. 33: 188~200, 1983
18) FURUMAI, T.; M. SUDOH, S. SAWAIRI & H. B. MARUYAMA: DNA homology studies in *Streptomyces* using S1 nuclease. J. Antibiotics 37: 641~645, 1984
19) TAMAOKA, J. & K. KOMAGATA: Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol. Lett. 25: 125~128, 1984
20) KANEKO, T.; K. KATOH, M. FUJIMOTO, M. KUMAGAI, J. TAMAOKA & Y. KATAYAMA-FUJIMURA: Determination of the nucleotide composition of a deoxyribonucleic acid by high-performance liquid chromatography of its enzymatic hydrolysate: a review. J. Microbiol. Methods 4: 229~240, 1986
21) TOMITA, K.; M. NISHIO, K. SAITOH, H. YAMAMOTO, Y. HOSHINO, H. OHKUMA, M. KONISHI, T. MIYAKI & T. OKI: Pradimicins A, B and C: New antifungal antibiotics. I. Taxonomy, production, isolation and physico-chemical properties. J. Antibiotics 43: 755~762, 1990
22) SAITOH, K.; Y. SAWADA, K. TOMITA, T. TSUNO, M. HATORI, & T. OKI: Pradimicins L and FL: New pradimicin congeners from *Actinomadura verrucosospora* subsp. *neohibisca*. J. Antibiotics 46 (3): 1993, in press
23) GOMI, S.; M. SEZAKI, S. KONDO, T. HARA, H. NAGANAWA & T. TAKEUCHI: The structures of new antifungal antibiotics, benanomicins A and B. J. Antibiotics 41: 1019~1028, 1988
24) LECHEVALIER, H. A. & M. P. LECHEVALIER: A critical evaluation of the genera of aerobic actinomycetes. *In* The Actinomycetales. *Ed.*, H. PRAUSER, pp. 393~405, VEB Gustav Fischer Verlag, Jena, 1970
25) KROPPENSTEDT, R. M.; E. STACKEBRANDT & M. GOODFELLOW: Taxonomic revision of the actinomycete genera *Actinomadura* and *Microtetraspora*. System. Appl. Microbiol. 13: 148~160, 1990
26) NONOMURA, H. & Y. OHARA: Distribution of actinomycetes in soil. XI. Some new species of the genus *Actinomadura* Lechevalier *et al.* J. Ferment. Technol. 49: 904~912, 1971
27) MIYADOH, S.; H. ANZAI, S. AMANO & T. SHOMURA: *Actinomadura malachitica* and *Microtetraspora viridis* are synonyms and should be transferred as *Actinomadura viridis* comb. nov. Int. J. Syst. Bacteriol. 39: 152~158, 1989