ISOLATION OF NEW MINOR BENANOMICINS

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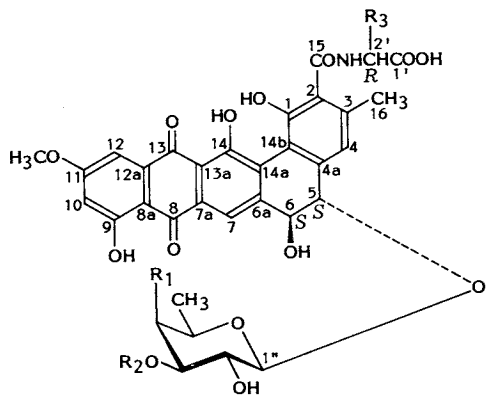
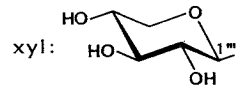
Four minor benanomicins, dexylosylbenanomicins A and B, 2'-demethylbenanomicin A and 7-methoxybenanomicinone have been isolated from the culture filtrate of *Actinomadura* sp. MH193-16F4. Their structures were confirmed by spectral analyses. Dexylosylbenanomicins A and B were derived chemically from benanomicins A and B, respectively.

New antibiotics of the benzonaphthacenequinone family, benanomicins A and B, produced by *Actinomadura* sp. MH193-16F4 showed excellent *in vitro* and *in vivo* activities against a wide range of fungi including *Candida*, *Cryptococcus* and *Aspergillus*,[1~4] and inhibited the infection of T-cells with human immunodeficiency virus.[5] The biosynthetic studies showed that the aglycone of benanomicins was derived from a dodecaketide, methionine and alanine.[6]

We have found that the benanomicin-producing strain produces some minor antibiotics in addition to benanomicins A and B. In this paper, the isolation and characterization of four minor antibiotics, dexylosylbenanomicins A and B, 2'-demethylbenanomicin A and 7-methoxybenanomicinone are reported. Dexylosylbenanomicin B had already been prepared by acid hydrolysis of benanomicin B.[1,2] The other three antibiotics are new.





	R_1	R_2	R_3
Benanomicin A (A)	OH	xyl	CH_3
Benanomicin B (B)	NH_2	xyl	CH_3
Dexylosylbenanomicin A (A-DX)	OH	H	CH_3
Dexylosylbenanomicin B (B-DX)	NH_2	H	CH_3
2'-Demethylbenanomicin A (A-DM)	OH	xyl	H

Isolation

Antibiotics in the culture filtrate of the strain MH193-16F4 were analyzed by HPLC using a reverse-phase ion-pair column and a UV detection method at 280 nm. As shown in Fig. 1, in addition to benanomicins A and B (Rt's 24.6 and 8.2 minutes, respectively), several minor antibiotics including dexylosylbenanomicin A, 2'-demethylbenanomicin A, dexylosylbenanomicin B and 7-methoxybenanomicinone (Rt's 22.8, 16.9, 8.7 and 6.8 minutes, respectively) were detected.

Antibiotics in the culture filtrate were adsorbed on a column of Diaion HP-20 (Mitsubishi Chemical Ind.) and eluted with aq MeOH and MeOH to separate the benanomicin A fraction from the benanomicin B fraction.[1] The benanomicin A fraction contained also dexylosylbenanomicin A and 2'-demethylbenanomicin A. It was purified by reverse-phase column chromatography and by precipitation in an acidic solution, and each antibiotic was isolated in a pure state. The benanomicin B fraction contained dexylosylbenanomicin B and 7-methoxybenanomicinone and was purified by repetition of Sephadex LH-20 (Pharmacia Fine Chemicals) column chromatography to isolate each antibiotic in a pure state.

Structures

Structures of dexylosylbenanomicin A, 2'-demethylbenanomicin A and 7-methoxybenanomicinone were determined by comparison of their ^{1}H and ^{13}C NMR spectra with those of benanomicin A, as shown in Tables 1 and 2. The *trans*-diequatorial relation of 5-H and 6-H (5*S*,6*S* or 5*R*,6*R*) in 7-methoxybenanomicinone was shown by the coupling constants ($^{3}J_{5\text{-H,C-4}}$, $^{3}J_{5\text{-H,C-6a}}$, $^{3}J_{5\text{-H,C-14b}}$, $^{3}J_{6\text{-H,C-4a}}$, $^{3}J_{6\text{-H,C-7}}$ and $^{3}J_{6\text{-H,C-14a}}$) of long range selective proton decoupling (LSPD) experiment (Fig. 2) and $J_{5,6}$ value of ^{1}H NMR (Table 1).

Fig. 1. HPLC analysis of benanomicins.

(A) Benanomicins in the culture filtrate. (B) Mixture of B and B-DX.

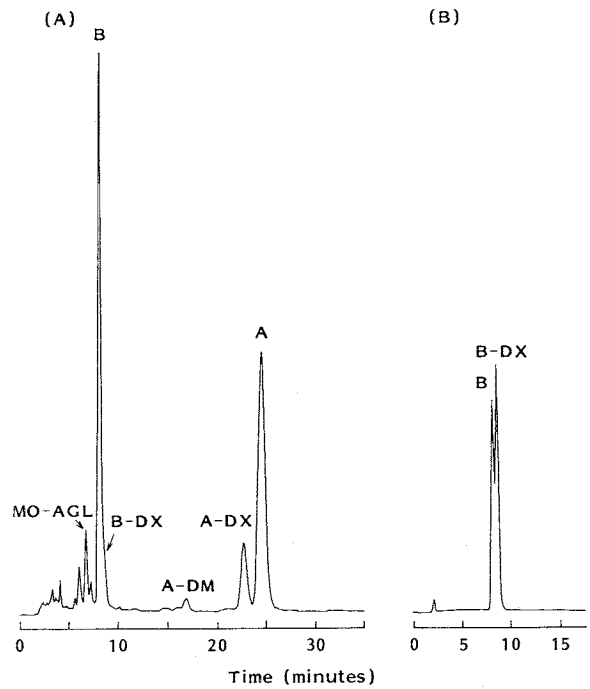


Table 1. ^{1}H NMR data of benanomicin components.

Proton	A[1]		A-DX		A-DM		MO-AGL	
	δ	J (Hz)	δ	J (Hz)	δ	J (Hz)	δ	J (Hz)
1-OH	8.65[a]	br	8.55[a]	br			8.63[a]	br
4-H	7.21	br s	7.21	br s	7.20	br s	6.83	br s
5-H	4.53	d (10.2)	4.47	d (10.2)	4.51	d (10.2)	4.54	d (3.1)
6-H	4.57	br d (10.2)	4.56	br d (10.2)	4.61	br d (10.2)	5.06	d (3.1)
7-H	8.05	s	8.09	s	8.08	s	—	
7-OCH_3	—		—		—		3.93	s
9-OH	12.77	s	12.82	s	12.82	s	13.25	s
10-H	6.86	d (2.3)	6.93	d (2.7)	6.94	d (2.3)	6.89	d (2.5)
11-OCH_3	3.92	s	3.95	s	3.97	s	3.93	s
12-H	7.24	d (2.3)	7.30	d (2.7)	7.31	d (2.3)	7.26	d (2.5)
14-OH	13.69[a]	br	13.80[a]	br	13.79[a]	br	14.67[a]	br
16-H_3	2.34	s	2.33	s	2.36	s	2.34	s
1′-OH	12.47[a]	br	12.50[a]	br	12.52[a]	br		
2′-H	4.43	dq (7.4, 7.0)	4.42	dq (7.4, 7.0)	3.93	d (5.9)	4.44	dq (7.2, 7.0)
2′-NH	8.45	d (7.0)	8.47	d (7.0)	8.48	t (5.9)	8.48	d (7.0)
3′-H_3	1.35	d (7.4)	1.34	d (7.4)	—		1.39	d (7.2)
1″-H	4.65	d (7.8)	4.54	d (7.4)	4.64	d (8.2)		
2″-H	3.74	br	3.53	br	3.71	br		
3″-H	3.56	dd (9.8, 2.7)	3.39	dd (9.4, 3.1)	3.54	dd (9.8, 2.7)		
4″-H	3.63	br s	3.45	br d (3.1)	3.61	br s		
5″-H	3.62	br q (6.3)	3.56	br q (6.6)	3.60	br q (6.6)		
6″-H	1.14	d (6.3)	1.13	d (6.6)	1.12	d (6.6)		
1‴-H	4.43	d (7.0)			4.42	d (7.0)		
2‴-H	3.13	dd (8.6, 7.0)			3.11	dd (9.0, 7.0)		
3‴-H	3.17	dd (8.6, 8.6)			3.15	dd (9.0, 8.2)		
4‴-H	3.32	ddd (10.6, 8.6, 5.1)			3.31	ddd (10.2, 8.2, 5.1)		
5‴-H_{ax}	3.09	dd (10.9, 10.6)			3.07	dd (11.3, 10.2)		
5‴-H_{eq}	3.71	dd (10.9, 5.1)			3.71	dd (11.3, 5.1)		

A: Benanomicin A, A-DX: dexylosylbenanomicin A, A-DM: 2′-demethylbenanomicin A, MO-AGL: 7-methoxybenanomicinone.

δ: ppm from TMS in DMSO-d_6 at 40°C.

[a] Tentative assignment.

The stereochemistry of the alanine moiety in dexylosylbenanomicin A or 7-methoxybenanomicinone was confirmed as the *R*-configuration by HPTLC pre-coated plate CHIR (E. Merck) of its hydrolysis product. The presence of the glycine moiety in 2′-demethylbenanomicin A was also confirmed by the acid hydrolysis.

Dexylosylbenanomicin A was derived from benanomicin A by periodate oxidation and reductive cleavage, and the stereochemistry of this material was identical with that of the same compound isolated from the fermentation. Dexylosylbenanomicin B was also identical with the dexylosyl product obtained by acid hydrolysis of benanomicin B,[1,2] in all respects.

Recently, Bristol-Myers scientists isolated pradimicins D and E as the glycine analogues of pradimicins A and C.[7] As their structures are 2′-demethyl-*N*-methyl and 2′-demethyl derivatives of benanomicin B, respectively, four minor benanomicins described in this paper are recognized as new microbial products.

Biological Activities

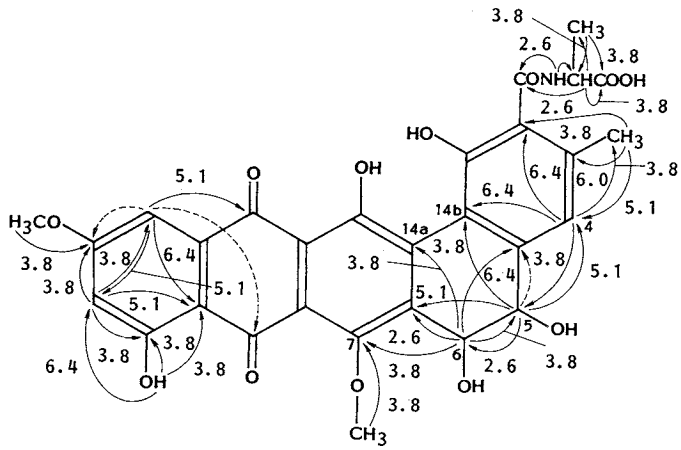
The antifungal activity of dexylosylbenanomicins A and B was similar or greater than that of benanomicins A and B against *Candida*, *Saccharomyces* and *Cryptococcus*, but these compounds had

Table 2. ^{13}C NMR data of benanomicin components.

Carbon	A[1]		A-DX		A-DM		MO-AGL		Carbon	A[1]		A-DX		A-DM		MO-AGL	
	δ	m	δ	m	δ	m	δ	m		δ	m	δ	m	δ	m	δ	m
C-1	151.1	s	151.0	s	151.2	s	151.8[a]	s	C-13a	115.5	s	115.4	s	115.5	s	115.3[b]	s
C-2	127.5	s	127.4	s	127.0	s	127.7	s	C-14	156.8	s	156.8	s	156.9	s	155.3[a]	s
C-3	137.4	s	137.3	s	137.3	s	137.2	s	C-14a	125.6	s	125.6	s	125.6	s	130.8	s
C-4	118.6	d	118.5	d	118.5	d	123.7	d	C-14b	113.7	s	113.6	s	113.6	s	115.3	s
C-4a	138.1	s	138.2	s	138.2	s	139.6	s	C-15	166.9	s	166.9	s	167.6	s	167.1	s
C-5	81.7	d	81.7	d	81.3	d	71.1	d	C-16	19.1	q	19.1	q	19.2	q	18.7	q
C-6	71.9	d	71.9	d	71.8	d	63.7	d	C-1′	173.9	s	173.9	s	171.0	s	174.0	s
C-6a	147.7	s	147.9	s	147.8	s	144.1	s	C-2′	47.6	d	47.6	d	40.8	t	47.7	d
C-7	115.4	d	115.4	d	115.5	d	153.6	s	C-3′	16.9	q	16.8	q	—		16.9	q
7-OCH_3	—		—		—		62.5	q	C-1″	104.4	d	105.2	d	104.3	d		
C-7a	131.3	s	131.3	s	131.3	s	121.7[b]	s	C-2″	70.1	d	71.2	d	70.0	d		
C-8	184.9	s	184.9	s	185.0	s	185.2	s	C-3″	83.0	d	73.5	d	83.0	d		
C-8a	110.0	s	110.0	s	110.1	s	110.9	s	C-4″	70.3	d	71.0	d	70.3	d		
C-9	164.7	s	164.7	s	164.7	s	164.5	s	C-5″	70.1	d	70.3	d	70.0	d		
C-10	106.8	d	106.8	d	106.9	d	107.3	d	C-6″	16.3	q	16.5	q	16.3	q		
C-11	165.9	s	165.9	s	166.0	s	165.3	s	C-1‴	105.2	d			105.2	d		
11-OCH_3	56.3	q	56.3	q	56.4	q	56.2	q	C-2‴	73.6	d			73.6	d		
C-12	107.5	d	107.6	d	107.6	d	106.3	d	C-3‴	76.0	d			76.0	d		
C-12a	134.2	s	134.2	s	134.4	s	133.6	s	C-4‴	69.4	d			69.4	d		
C-13	187.3	s	187.4	s	187.5	s	187.9	s	C-5‴	65.6	t			65.6	t		

A: Benomicin A, A-DX: dexylosylbenanomicin A, A-DM: 2′-demethylbenanomicin A, MO-AGL: 7-methoxybenanomicinone.
δ: ppm from TMS in DMSO-d_6 at 40°C.
m: Multiplicity.
[a,b] Tentative assignment.

Fig. 2. Long range ^1H-^{13}C COSY and LSPD experiments of 7-methoxybenanomicinone (MO-AGL).



Coupling constants (Hz) are beside the solid arrows. Dotted arrows indicate the low coupling constant ($J=ca.$ 1 Hz) and/or NOE.

reduced activity against *Aspergillus*.[8] 2′-Demethylbenanomicin A showed similar antifungal activity to that of benanomicin A. 7-Methoxybenanomicinone exihibited little or no antifungal activity,[8] but inhibited the activity of α-glucosidase (EC 3.2.1.20, type 1 from baker's yeast, Sigma Chemical Co.) with an IC_{50} of 60 μg/ml.

Experimental

General

MP's were determined with a Yanaco MP-S3 micro melting point apparatus and were uncorrected. MS were measured on a Hitachi M-80B mass spectrometer. ^1H and ^{13}C NMR spectra of samples in DMSO-d_6 at 40°C were recorded on Jeol JNM-GX400 and Jeol JNM-GSX400 spectrometers. The ^1H and ^{13}C NMR spectra of benanomicin A and new compounds are summarized in Tables 1 and 2.

HPLC Analysis of Antibiotics in Culture Filtrate

A sample solution (5 μl) was applied to a Shimadzu HPLC system consisting of Shimadzu LC-6A pump, Shimadzu SPD-2A UV detector and Chromatopack R-C1B integrator. The reversed phase Cosmosil C18 column (4.6 i.d. × 150 mm, particle size 5 μm, Nacalai Tesque Inc.) and pre-column (4.6 i.d. × 50 mm, particle size 10 μm) were used and operated at 40°C and a flow rate of 1.0 ml/minute, using a 1 : 1 mixture of 0.5% KH_2PO_4 aq solution (pH 5.8) and MeOH as a mobile phase. UV absorbance was monitored at 280 nm.

The antibiotics in the culture filtrate[1] (1 ml) were adsorbed on a small column of Diaion HP-20 (3 ml). After being washed with H_2O (10 ml), the column was eluted with 50% aq Me_2CO (6 ml). The eluate was concentrated to dryness and the residue was dissolved in H_2O (1 ml). Separation of the antibiotics in the solution by HPLC is shown in Fig. 1A. Benanomicin B and dexylosylbenanomicin B (each 100 μg/ml) were clearly separated as shown in Fig. 1B.

Crude Precipitates of Antibiotics from Culture Filtrate[1]

The antibiotics in the filtrate (250 liters, pH 6.0) which was obtained by fermentation of *Actinomadura* sp. MH193-16F4 in a 570-liter fermenter[1] were adsorbed on a column of Diaion HP-20 (15 liters). After the column was washed with H_2O (100 liters) and 50% aq MeOH (45 liters), the antibiotics were eluted with 70% aq MeOH (45 liters) and then MeOH (90 liters). The eluate was divided into three fractions; I (benanomicin A fraction, 53 liters), II (benanomicin A fraction, 38 liters) and III (benanomicin B fraction, 27 liters). Each fraction was concentrated to 3.0, 2.0 and 1.5 liters and adjusted to pH 3.5 to give crude precipitates; I (152 g), II (81 g) and III (99 g), respectively.

Isolation of Dexylosylbenanomicin A

Crude precipitate I (150 g) described above, was dissolved in DMF (600 ml) and kept in a H_2O-saturated desiccator at room temperature for 3 days to obtain a crystalline precipitate (29 g) of the DMF solvate containing mostly benanomicin A. The mother liquour of the solvate was concentrated to obtain a brownish crude powder (130 g). A solution of the crude powder (5 g) in H_2O (200 ml) adjusted to pH 9 with 1 N NaOH was re-adjusted to pH 2.5 with 1 N HCl to yield a precipitate. The precipitate was dissolved in H_2O (100 ml) adjusted to pH 7 and purified by reverse-phase column chromatography (1.0 liter, Cosmosil 75C_{18}-OPN, Nacalai Tesque, Inc.) developed with 1% MeOH. The eluate which showed Rt 22.7 minutes by HPLC, was adjusted to pH 3.5 with 1 N HCl and a reddish precipitate of pure dexylosylbenanomicin A (345 mg) was obtained. MP >200°C, FD-MS m/z 696 $(M+1)^+$. Pure benanomicin A (2.1 g) was also obtained from the eluate with 5% MeOH (Rt 24.5 minutes).

Isolation of 2'-Demethylbenanomicin A

The DMF solvate (1 g) described above, was dissolved in H_2O (40 ml) adjusted to pH 7.5 with 1 N NaOH and purified by reverse-phase column chromatography (1.0 liter, Cosmosil 75C_{18}-OPN) developed with 5% MeOH. From the eluate showing Rt 24.5 minutes by HPLC, pure benanomicin A (850 mg) was obtained. The eluate showing Rt 17 minutes by HPLC was concentrated to yield a reddish powder. The powder was purified by preparative HPLC (column: YMC-Pack D-ODS-5, 20 i.d. × 250 mm, Yamamura Chemical Research, mobile phase: 0.5% KH_2PO_4-MeOH (1 : 1), flow rate: 10 ml/minutes, temperature: 40°C), and by column chromatography on Diaion HP-20 (10 ml) developed with 50% aq Me_2CO. The purified eluate was concentrated and adjusted to pH 3.5 to obtain a reddish precipitate of pure 2'-demethylbenanomicin A (5.3 mg). MP >200°C.

Isolation of 7-Methoxybenanomicinone

Crude precipitate III (1 g) described above, was dissolved in DMF (40 ml) and chromatographed on a column of Sephadex LH-20 (1.0 liter) developed with DMF. The eluate was collected in 6-ml fractions. Fractions 64~72 were combined and concentrated to obtain a crude powder (657 mg). The powder was dissolved in a mixture of MeOH (100 ml) and 1 N HCl (1 ml), and the solution was concentrated to dryness. A solution of the residue in DMSO (3 ml) was added dropwise into chloroform (200 ml) and then stirred for 20 minutes. Pure benanomicin B hydrochloride (565 mg) which showed Rt 8.2 minutes by HPLC, was precipitated. The filtrate was concentrated to 5 ml and chromatographed on a column of Sephadex LH-20 (600 ml) which was developed with MeOH. The eluate showing Rt 6.8 minutes by HPLC was concentrated to obtain a reddish powder of pure 7-methoxybenanomicinone (38.5 mg). MP>220°C, FD-MS *m/z* 579 (M^+).

Isolation of Dexylosylbenanomicin B

From the Sephadex LH-20 chromatography of crude precipitate III described above, fractions 75~80 were combined and concentrated to obtain a crude powder of dexylosylbenanomicin B DMF solvate (68.5 mg). A solution of the crude powder in H_2O adjusted to pH 9.0 with 1 N NaOH was re-adjusted to pH 5.5 with 1 N HCl to give a precipitate. The precipitate was purified by column chromatography on Sephadex LH-20 (700 ml) which was developed with 80% aq MeOH. The eluate showing Rt 8.7 minutes by HPLC was concentrated to yield a reddish powder of pure dexylosylbenanomicin B hydrochloride (52.3 mg). It was identical with the hydrolysis product derived from benanomicin B.[1,2]

Synthesis of Dexylosylbenanomicin A

Sodium metaperiodate (2.71 g) was added to a solution of benanomicin A (1.05 g) in 0.02 N NaOH (127 ml) and the solution was stirred at room temperature for 40 minutes. After addition of ethylene glycol (0.8 ml), the solution was stirred for a further 15 minutes and passed through a column of Diaion HP-20 (800 ml). After being washed with H_2O (2 liters), the column was eluted with 40% aq Me_2CO and the eluate was collected in 100-ml fractions. Fractions 7~12 were combined and concentrated to 20 ml. The concentrate was adjusted to pH 2.5 with 0.1 N HCl to obtain a reddish precipitate of the diformyl compound (905 mg). To a solution of this compound (200 mg) in 0.01 N NaOH (25 ml), $NaBH_4$ (15 mg) was added and the solution was stirred at room temperature for 20 minutes. After being adjusted to pH 3.0 with 0.1 N HCl and stirred for 30 minutes, the solution was re-adjusted to pH 7.5 with 0.1 N NaOH and passed through a column of Diaion HP-20 (100 ml). After being washed with H_2O (200 ml), the column was eluted with 30% aq Me_2CO and the eluate was collected in 5-ml fractions. Fractions 12~28 were combined and concentrated to give a reddish powder (159 mg). The powder was dissolved in DMSO (2 ml) and chromatographed on a Sephadex LH-20 column (1.0 liter) developed with MeOH. The eluate was collected in 7-ml fractions and fractions 52~64 were combined and concentrated to 5 ml. To the concentrate, 0.01 N HCl (5 ml) was added to yield a reddish precipitate of dexylosylbenanomicin A (52.6 mg) which was identical with material isolated directly from the fermentation.

Determination of D-Alanine in Dexylosylbenanomicin A and 7-Methoxybenanomicinone

Dexylosylbenanomicin A (5 mg) in 6 N HCl (20 ml) was heated at 110°C in a sealed tube for 15 hours. The reaction mixture was filtered and the filtrate was concentrated to dryness. An aq solution (0.5 ml) of the residue was passed through a column of Diaion HP-20 (1 ml) and the column was washed with H_2O (1.5 ml). The effluent and washings were combined and charged on a column of Diaion PK-208 (H^+, 1 ml, Mitsubishi Chemical Ind.). After the column was washed with H_2O (3 ml), an amino acid was eluted with 0.5 N NH_4OH (2 ml). The eluate was concentrated to dryness and the residue was dissolved in H_2O (0.1 ml). The 5 μl portion of the solution was applied on HPTLC pre-coated plate CHIR (E. Merck, Art. No. 14285) developed with a 1:1:4 mixture of MeOH - H_2O - MeCN. The amino acid, Rf 0.46, detected with ninhydrin reagent, was identical with D-alanine. L-Alanine showed an Rf 0.51 in this system.

By similar treatment of 7-methoxybenanomicinone, D-alanine was also detected.

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