ANTHRACYCLINE METABOLITES FROM *Streptomyces violaceus* A262

V. NEW ANTHRACYCLINE ALLDIMYCIN A: A MINOR COMPONENT ISOLATED FROM OBELMYCIN BEER

OSAMU JOHDO, HIROSHI TONE, ROKURO OKAMOTO and AKIHIRO YOSHIMOTO†

Central Research Laboratories, Mercian Corporation, 4-9-1 Johnan, Fujisawa 251, Japan

HIROSHI NAGANAWA, TSUTOMU SAWA and TOMIO TAKEUCHI

Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication February 6, 1991)

During the course of the study of blocked mutants or variant strains obtained from *Streptomyces violaceus* A262 which produced known diglycosidic β-rhodomycins, we isolated several blocked mutant or variant strains which produce new anthracyclines.[1] One of them, strain SE2-2385-A1 was found to produce α_2-rhodomycinone glycosides as minor components together with β-isorhodomycinone glycosides (obelmycins).[2] We obtained α_2-rhodomycinone glycosides, designated as alldimycins A, B and C, by means of controlled hydrolysis. Alldimycin A was identified as 7-*O*-(α-L-rhodosaminyl)-α_2-rhodomycinone. Alldimycins B and C were identical to alpharubicins II and I,[3] respectively.

In this paper, we describe the isolation, structural determination and biological activity of alldimycins. Since structural determination about alpharubicin I (alldimycin C) and alpharubicin II (alldimycin B) have not been reported in detail, we also mentioned to the structural determination of them.

The alldimycin-producing strain SE2-2385-A1 was isolated from *S. violaceus* A262 as previously described[1] and maintained on YS agar slant (yeast extract 0.3%, soluble starch 1.0% and agar 1.5%, pH 7.2) by successive cultivation at 28°C and stocked at 5°C.

Seed culture was aerobically grown at 28°C for 48 hours in a 500-ml Erlenmeyer flask containing 100 ml of a following medium; soluble starch 0.5%, glucose 0.5%, soybean meal 1.0%, yeast extract 0.1%, NaCl 0.1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, pH 7.4. It was added with an inoculum size of 5% to three 30-liter jar fermenters containing each 15 liters of fermentation medium which contained soluble starch 50 g, soybean meal 30 g, yeast extract 2 g, NaCl 2 g, $CaCO_3$ 2 g, $CuSO_4 \cdot 5H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.0016 g, $ZnSO_4 \cdot 7H_2O$ 0.0032 g, $MnCl_2 \cdot 4H_2O$ 0.013 g in 1,000 ml of tap water, pH 7.0. Fermentation conditions were 15 liters/minute for aeration and 300 rpm for agitation. The cultivation was carried out at 28°C for 130 hours when the mycelial orange pigments occurred at approximately maximum level. About 45 liters of the culture broth was prepared for the isolation of purified alldimycins.

The fermentation broth (45 liters) was heated at 60°C for 3 hours after being adjusted to pH 1.0 with 6 N HCl and centrifuged. The supernatant was adjusted to pH 2.5 and subjected to the adsorption chromatography on a column of Diaion HP-20 (i.d. 6 × 100 cm). The anthracycline products were eluted with acetone-acidic water (pH 2.0) (80:20) after washed with 6 liters of acidic water (pH 2.5), and reddish purple fractions were collected. The eluate was evaporated *in vacuo* to remove acetone and the products were extracted with 2.5 liters of $CHCl_3$ after being adjusted to pH 8.0 with 4 N NaOH. The $CHCl_3$ extracts were evaporated *in vacuo* to a small volume and an excess of *n*-hexane was added to precipitate the crude mixture of anthracycline products. This isolation procedure provided about 10.3 g of reddish purple crude powder which involved alldimycins, obelmycin A[2] and β-isorhodomycin II.[4]

The crude powder was chromatographed on a column (50 mm, i.d.) of silica gel (Wakogel C-200, 600 g) which was washed with $CHCl_3$ and developed with a solvent mixture of $CHCl_3$-MeOH-H_2O-acetic acid-aq NH_3 (150:50:5:1:1) to elute obelmycin A, alldimycin A, alldimycin C, alldimycin B and β-isorhodomycin II in this order. Fractions containing each alldimycin were separately pooled, washed with equal volume of saturated aqueous $NaHCO_3$ and evaporated *in vacuo* to dryness. Each alldimycin was further purified by preparative TLC on Silica gel PF_{254} (E. Merck) using a solvent mixture of $CHCl_3$-MeOH-formic acid (4:1:0.1).

† Present address: Faculty of Applied Biological Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-hiroshima 724, Japan.

Each alldimycin band on the TLC plate was scraped and extracted with $CHCl_3$-MeOH (7:1). The extract was washed with equal volume of saturated aqueous $NaHCO_3$ and evapolated to dryness. The antibiotic recovered was dissolved in 20 ml of 0.1 M acetate buffer (pH 3.5) and washed with 10 ml of toluene twice. The aqueous layer was adjusted to pH 7.5 by addition of saturated aqueous $NaHCO_3$ and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with saturated aqueous NaCl and concentrated to a small volume after being dried over Na_2SO_4. An excess of *n*-hexane was added to precipitate orange powder. Purified alldimycins A, B and C yielded 185 mg, 149 mg and 171 mg, respectively. The physico-chemical properties of alldimycins are as follows:

Alldimycin A: MP 132~134°C (dec); $[\alpha]_D^{20}$ +210° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2950, 1600 (hydrogen bonded carbonyl), 1450, 1290, 1220, 1160, 1030, 1010, 990; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 203 (334), 235 (777), 258 (404), 292 (sh, 138), 492 (250); SI-MS *m/z* 544 ($(M+H)^+$, corresponding to the molecular formula $C_{28}H_{33}NO_{10}$).

Alldimycin B: MP 134~137°C (dec); $[\alpha]_D^{20}$ +233° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2920, 1590 (hydrogen bonded carbonyl), 1445, 1290, 1220, 1155, 1120, 1015, 980; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 203 (273), 234 (671), 258 (344), 293 (sh, 118), 494 (213); FAB-MS *m/z* 701 ($(M+H)^+$, corresponding to the molecular formula $C_{36}H_{48}N_2O_{12}$).

Alldimycin C: MP 135~138°C (dec); $[\alpha]_D^{20}$ +195° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2920, 1590 (hydrogen bonded carbonyl), 1445,1285, 1215, 1150, 1020; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 202 (312), 234 (741), 257 (393), 290 (sh, 141), 493 (241); FAB-MS *m/z* 544 ($(M+H)^+$, corresponding to the molecular formula $C_{28}H_{33}NO_{10}$).

The structural determination of alldimycins was mainly carried out by MS, ^1H and ^{13}C NMR analyses and TLC analysis of the sugar components obtained from acid hydrolysis.

Acid hydrolysis (1 mg/ml, 0.1 N HCl, 85°C, 30

Table 1. ^1H NMR chemical shifts of alldimycins and α_2-rhodomycinone.

Proton	Alldimycin A	Alldimycin B	Alldimycin C	α_2-Rhodomycinone	α_2-Rhodomycinone
2-H	7.33 d (9.0)*	7.25 s	7.32 d (9.0)*	7.34 d (9.5)*	7.37 d (9.5)*
3-H	7.30 d (9.0)*	7.25 s	7.28 d (9.0)*	7.31 d (9.5)*	7.34 d (9.5)*
6-H	7.81 s	7.85 s	8.05 s	7.98 s	7.82 s
7-H	4.94 t (3.5)	4.97 dd (5.9, 4.4)	4.87 d (6.0)	4.84 d (2.9)	4.79 br
8-Ha	]2.2	2.50 dd (14.7, 6.6)	2.41 dd (15.0, 6.0)	2.21 dd (14.7, 5.1)	2.17 dd (13.9, 5.9)
8-Hb		2.08 dd (14.7, 3.7)	2.09 d (15.0)	2.12 d (14.7)	1.81 d (13.2)
10-H	4.96 s	5.11 s	5.03 s	4.87 s	4.67 s
13-Ha	1.90 m (7.5)	]1.75~1.85 m	1.87 m (7.5)	1.86 q (6.6)	1.69 m (7.3)
13-Hb	1.74 m (7.5)		1.81 m (7.5)	1.78 q (6.6)	1.65 m (7.3)
14-CH_3	1.13 t (7.5)	1.12 t (7.3)	1.11 t (7.5)	1.10 t (7.3)	0.98 t (7.3)
1'-H	5.36 d (3.0)	5.34 d (3.7)	5.36 d (4.0)	—	—
2'-Ha	]1.8	2.00 dd (12.5, 3.7)	1.68 dt (13.0, 4.0)	—	—
2'-Hb		1.88 dt (12.5, 3.7)	1.52 dd (13.0, 5.0)	—	—
3'-H	2.31 m (12.0, 6.0, 3.0)	2.42 m	2.24 m (13.0, 5.0, 3.0)	—	—
4'-H	3.72 br s	3.72 br s	3.66 br s	—	—
5'-H	4.03 q (7.0)	4.04 q (6.6)	3.90 q (7.0)	—	—
6'-CH_3	1.42 d (7.0)	1.40 d (6.6)	1.38 d (7.0)	—	—
3'-$N(CH_3)_2$	2.23 s	2.29 s	2.20 s	—	—
1''-H	—	5.20 d (3.7)	—	—	—
2''-Ha	—	1.68 dt (13.2, 3.7)	—	—	—
2''-Hb	—	1.53 dd (12.5, 4.4)	—	—	—
3''-H	—	2.25	—	—	—
4''-H	—	3.68 br s	—	—	—
5''-H	—	3.94 q (6.6)	—	—	—
6''-CH_3	—	1.39 d (6.6)	—	—	—
3''-$N(CH_3)_2$	—	2.22 s	—	—	—
Solvent	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$-CD_3OD (5:1)	DMSO-d_6

Spectra were measured at 400 MHz. Chemical shifts are expressed by δ (ppm) (J=Hz) from internal TMS. Similar values asterisked may be interchanged.

minutes) followed by silica gel TLC analysis[5,6] revealed that the sugar moiety of alldimycins consisted of rhodosamine by a direct comparison with authentic samples (sourced from aclacinomycin A[5]), while the aglycone moiety of alldimycins was identified as α_2-rhodomycinone by a direct comparison with an authentic sample (sourced from CG11[7]) using a solvent mixture of $CHCl_3$-MeOH (20:1). The sugar was purified by Dowex 50W ion exchange resin and silica gel column chromatography with $CHCl_3$-MeOH (10:1~3:1). Rhodosamine was determined to be L since its hydrochloride showed $[\alpha]_D$ value of $-45.6°$ (c 0.1, H_2O) (literature 8: $-48.2°$, H_2O). The aglycone was purified by preparative TLC on Silica gel PF_{254} (E. Merck) using a solvent mixture of $CHCl_3$-MeOH (15:1) and its identity was confirmed by its UV, mass (m/z 387 $(M+H)^+$) and ^1H and ^{13}C NMR spectra. On partial acid hydrolysis (1 mg/ml, 0.1 N HCl, room temperature, 1 hour) alldimycin B gave alldimycins A and C almost equally. This fact and their mass spectral data indicated that alldimycin B have two molecules of L-rhodosamine and alldimycins A and C have one molecule of L-rhodosamine.

The chemical shift assignments of ^1H and ^{13}C NMR spectra of alldimycins were carried out by means of pulse technique, DEPT, ^1H-^1H and ^1H-^{13}C COSY and are shown in Tables 1 and 2, respectively. On ^{13}C NMR the chemical shifts of C-7 of alldimycin A and C-10 of alldimycin C (73.80 and 69.92 ppm, respectively) distinctly shifted lower field comparing with those of α_2-rhodomycinone.

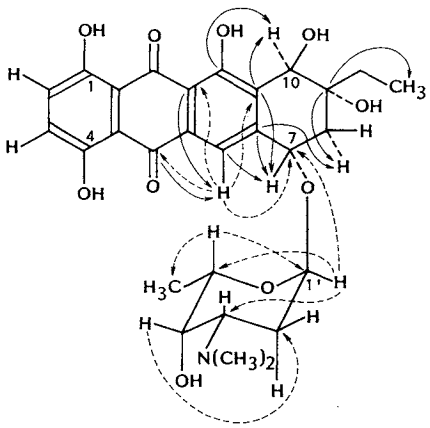
Table 2. ^{13}C NMR chemical shifts of alldimycins and α_2-rhodomycinone.

Carbon	Alldimycin A	Alldimycin B	Alldimycin C	α_2-Rhodomycinone
1	158.52*	158.20*	158.39*	157.06*
2	130.33*	129.91*	130.01*	129.42
3	129.80*	129.71*	129.62*	129.42
4	158.04*	157.80*	157.88*	156.36*
4a	112.55*	112.37*	112.68*	112.47*
5	185.79	185.70	185.94	185.88
5a	132.32	132.54	132.95	133.85
6	120.71	119.01	121.10	119.51
6a	143.31	147.17	149.15	148.86
7	73.80	73.98	67.18	65.49
8	33.72	36.44	36.19	36.01
9	72.20	72.08	73.49	72.22
10	66.96	70.56	69.92	64.58
10a	133.74	131.79	130.90	131.03
11	162.16	161.88	161.89	160.90
11a	115.38	114.82	114.84	113.72
12	190.67	190.33	190.70	190.01
12a	112.35*	112.22*	112.55*	112.38*
13	30.48	31.08	31.16	30.56
14	6.63	6.68	6.32	6.6
1′	98.91	99.92	96.39	—
2′	28.97	28.99	29.28	—
3′	59.53	59.52	59.68	—
4′	65.95	65.97	66.19	—
5′	66.66	66.39	66.58	—
6′	17.08	17.03	17.28	—
3′-$N(CH_3)_2$	42.00	42.00	42.03	—
1″	—	96.02	—	—
2″	—	29.07	—	—
3″	—	59.68	—	—
4″	—	66.14	—	—
5″	—	66.52	—	—
6″	—	17.20	—	—
3″-$N(CH_3)_2$	—	42.00	—	—
Solvent	$CDCl_3$	$CDCl_3$	$CDCl_3$	DMSO-d_6

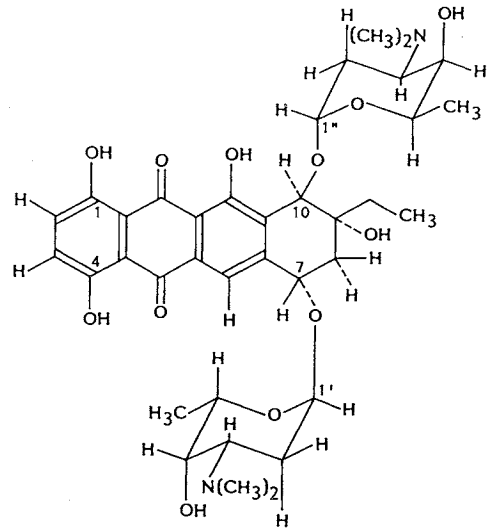
Spectra were measured at 100 MHz. Chemical shifts are expressed by δ (ppm) from internal TMS. Similar values asterisked may be interchanged.

Fig. 1. Structures of alldimycins.

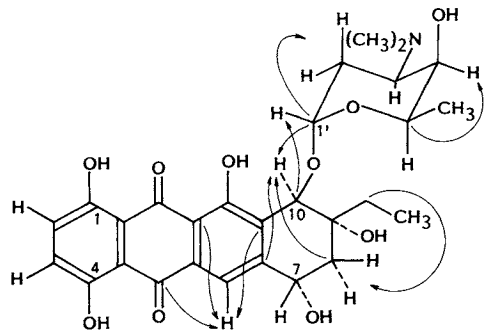
The solid-line arrows and dashed-line arrows indicate ^{1}H-^{13}C long range couplings detected by COLOC and HMBC, respectively.



Alldimycin A



Alldimycin B



Alldimycin C

These down-field shifts are attributable to the sugar linkage at C-7 for alldimycin A and C-10 for alldimycin C, respectively. The positions of sugar linkage of alldimycins A and C were confirmed by the correlation *via* long range coupling (COLOC) and ^{1}H detected heteronuclear multiple-bond connectivity (HMBC) experiments (Fig. 1). A long range coupling of 1′-H (5.36 ppm) with C-7 (73.80 ppm) was observed in alldimycin A and, those of C-1′ (96.39 ppm) with the proton at C-10 (5.03 ppm) and C-10 (69.92 ppm) with the proton at C-1′ (5.36 ppm) was observed in alldimycin C. On ^{1}H NMR the small coupling constants ($J = \sim 4$ Hz) of all anomeric protons in alldimycins indicate that the configurations of the glycosidic bonds are all α.

All these findings show that alldimycins A, B and C are 7-*O*-(α-L-rhodosaminyl)-α_2-rhodomycinone, 7,10-*O*-di-(α-L-rhodosaminyl)-α_2-rhodomycinone and 10-*O*-(α-L-rhodosaminly)-α_2-rhodomycinone, respectively, as illustrated in Fig. 1.

In vitro cytotoxicity and inhibition of DNA and RNA syntheses against the cell culture of murine leukemia L1210 were assayed according to the method as previously described.[9] The cytotoxic activities of alldimycins and other anthracyclines against leukemic L1210 cell culture were examined under continuous exposure and shown in Table 3. Alldimycin A had less cytotoxic activity than related anthracyclines, 6-hydroxyalldimycin A (obelmycin A),[2] 1-deoxy-6-hydroxyalldimycin A (β-rhodomycin I) and 1-deoxyalldimycin A (yellamycin A),[10] and representative anthracyclines, doxorubicin and

Table 3. Inhibitory activity of alldimycins and other anthracyclines on the growth and nucleic acid synthesis of murine leukemic L1210 cell culture.

Compound	IC_{50} (μg/ml)			DNA/ RNA
	Growth	DNA synthesis	RNA synthesis	
Alldimycin A	0.05	0.92	0.47	2.0
Alldimycin B	1.0	7.50	1.30	5.8
Alldimycin C	1.0	3.2	3.00	1.1
Yellamycin A	0.007	0.28	0.23	1.2
β-Rhodomycin I	0.01	0.21	0.06	3.5
Obelmycin A	0.001	0.58	0.14	4.1
Aclarubicin	0.01	0.65	0.085	7.6
Doxorubicin	0.02	1.40	0.55	2.5

In the inhibition test for nucleic acid synthesis, the drugs were exposed for 60 minutes to L1210 cell culture (8×10^5 cells/ml) with supplemented ^{14}C-labeled uridine or thymidine (0.05 μCi/ml), and the incorporation of the radioisotopes into acid insoluble material was measured. For the growth inhibition test, the drugs were exposed for 48 hours to L1210 cell culture (5×10^4 cells/ml) and the viable cells were counted by coulter counter.

IC_{50} is expressed as a drug concentration required to inhibit by a 50% control of the growth, and DNA and RNA syntheses of cultured L1210 cells.

aclarubicin. Alldimycin A inhibited RNA synthesis approximately twice more strongly than DNA synthesis like doxorubicin. Alldimycins B and C scarcely exhibited inhibitory activity on the growth and nucleic acid synthesis. It is the first case that a 7,10-diglycosidic anthracycline dose not exhibit cytotoxicity. What only alldimycin A remaines biological activity is considered that 7-*O*-sugar linkage plays an important function as seen with yellamycins.[10]

References

1) JOHDO, O.; T. ISHIKURA A. YOSHIMOTO & T. TAKEUCHI: Anthracycline metabolites from *Streptomyces violaceus* A262. I. Isolation of antibiotic-blocked mutants from *Streptomyces violaceus* A262. J. Antibiotics 44: 1110~1120, 1991

2) JOHDO, O.; Y. WATANABE, T. ISHIKURA, A. YOSHIMOTO, H. NAGANAWA, T. Sawa & T. TAKEUCHI: Anthracycline metabolites from *Streptomyces violaceus* A262. III. New anthracycline obelmycins produced by a variant strain SE2-2385. J. Antibiotics 44: 1130~1140, 1991

3) FLECK, W. F.; B. SCHLEGEL & W. IHN: Structure-activity relationship of rhodomycin-type anthracycline antibiotics. Stud. Biophys. 104: 215~218, 1984

4) BROCKMANN, H. & P. PATT: Iso-rhodomycin A, ein neues Antibioticum aus Actinomyceten, XXXII. Chem. Ber. 88: 1455~1468, 1955

5) OKI, T.; I. KITAMURA, Y. MATSUZAWA, N. SHIBAMOTO, T. OGASAWARA, A. YOSHIMOTO, T. INUI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Antitumor anthracycline antibiotics, aclacinomycin A and analogues. II. Structural determination. J. Antibiotics 32: 801~819, 1979

6) MATSUZAWA, Y.; A. YOSHIMOTO, T. OKI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Biosynthesis of anthracycline antibiotics by *Streptomyces galilaeus*. II. Structure of new anthracycline antibiotics obtained by microbial glycosidation and biological activity. J. Antibiotics 33: 1341~1347, 1980

7) YOSHIMOTO, A.; O. JOHDO, Y. TAKATSUKI, T. ISHIKURA, T. SAWA, T. TAKEUCHI & H. UMEZAWA: New anthracycline antibiotics obtained by microbial glycosidation of β-isorhodomycinone and α_2-rhodomycinone. J. Antibiotics 37: 935~938, 1984

8) BROCKMANN, H.; E. SPOHLER & T. WAEHNELDT: Rhodosamin, Isolierung, Konstitution und Konfiguration. Chem. Ber. 96: 2925~2936, 1963

9) YOSHIMOTO, A.; Y. MATSUZAWA, T. OKI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Microbial conversion of ε-pyrromycinone and ε-isorhodomycinone to 1-hydroxy-13-dihydrodaunomycin and N-formyl-1-hydroxy-13-dihydrodaunomycin and their bioactivities. J. Antibiotics 33: 1150~1157, 1980

10) JOHDO, O.; H. TONE, R. OKAMOTO, A. YOSHIMOTO, H. NAGANAWA, T. SAWA & T. TAKEUCHI: Anthracycline metabolites from *Streptomyces violaceus* A262. IV. New anthracycline yellamycins produced by a variant strain SC-7. J. Antibiotics 44: 1155~1159, 1991