PRODUCTION OF NEW ANTHRACYCLINE ANTIBIOTIC BETACLAMYCIN B BY MICROBIAL CONVERSION WITH A SPECIFIC ACLACINOMYCIN-NEGATIVE MUTANT

AKIHIRO YOSHIMOTO,† OSAMU JOHDO, YOSHIO WATANABE, HIROSHI NISHIDA and ROKURO OKAMOTO

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

TOMIO TAKEUCHI

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

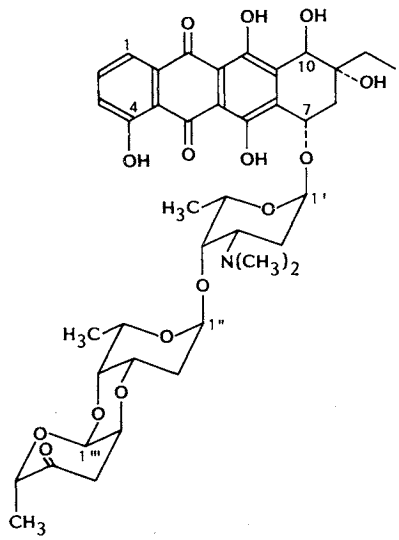
(Received for publication November 5, 1991)

The production of hybrid anthracycline antibiotics by biosynthetic conversion of natural and semisynthetic anthracyclinones using antibiotic-blocked mutants of anthracycline producers is now one of the important means for obtaining new anthracycline antibiotics with therapeutical efficacy. Betaclamycin A (BCM-A) was obtained by biosynthetic glycosidation of β-rhodomycinone (β-RMN) with a nonproducing mutant strain KE-303 of aclacinomycin (ACM)-producing *Streptomyces galilaeus* MA144-M1.[1,2] It has the same trisaccharide at C-7 on β-RMN as the glycosidic moiety of ACM-A and has been found to show good antitumor effects *in vivo* against several murine tumors (unpublished data). Since the parent strain MA144-M1 usually produces both ACM-A and ACM-B,[3] betaclamycin B (BCM-B) having B-type saccharide would be also an expected bioconversion product. However, we were unable to obtain BCM-B in the microbial conversion with strain KE-303.

In this paper we describe production of BCM-B by the selected biosynthetic glycosidation of β-RMN using a new ACM-nonproducing mutant strain 2HK-0134 which was derived from a mutant strain 7N-367 that produce large amounts of ACM-B.[4] BCM-B thus obtained exhibited higher antitumor activity *in vivo* against leukemic L1210 cell culture than BCM-A. The chemical structure of BCM-B which was finally established is shown in Fig. 1.

Microbial conversion with strain 2HK-0134 was carried out using a 3-liter jar fermenter containing 1.5 liters of medium: Soluble starch 5%, glucose 1%, soybean meal 2%, gluten meal 1%, yeast extract 0.1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1% and NaCl 0.3% in tap water, pH 7.4 (before autoclaving). The jar was seeded with 5% of the submerged culture. The fermentation was contorolled to a temperature of 30°C, with an agitation rate of 150 rpm and aeration of 1.5 liters/minute. After 48 hours 15 ml of β-RMN (75 mg) solution in MeOH was added to a jar fermenter and further 30-hour cultivation was allowed until complete conversion was achieved by TLC monitoring (TLC plate used: Silica gel 60 F_{254} (E. Merck); solvent used: $CHCl_3$ - MeOH - aqueous NH_3 (100 : 10 : 0.1)). The conversion broth from two fermentation runs was collected and centrifuged. The pigmented product was extracted from the mycelial cake with a total of 3 liters of acetone. The solvent extract was evaporated and extracted with 1 liter of $CHCl_3$. After evaporation of the $CHCl_3$ layer, the pigmented residue was dissolved in about 20 ml of $CHCl_3$ - MeOH (1 : 2) mixture, and put on a top of a Sephadex LH-20 column (20 × 400 mm), which was eluted with the same solvent mixture. Fractions were monitored by TLC on a Silica gel plate 60 F_{254} using a solvent of $CHCl_3$ - MeOH (15 : 1) and those containing the glycosidated products were pooled

Fig. 1. Structure of betaclamycin B.



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

and evaporated to dryness. The resulting residue was then chromatographed by preparative TLC on Silica gel 60 PF_{254} (E. Merck) using a solvent system of $CHCl_3$ - MeOH (15 : 1). The main pigmented band corresponding to BCM-B was scraped off and eluted with $CHCl_3$ - MeOH (7 : 1) mixture. After evaporation the pigment residue was dissolved in 30 ml of 0.1 M acetate buffer (pH 3.2). This solution was washed with 20 ml of toluene, neutralized with sodium bicarbonate, and extracted with $CHCl_3$. The chloroform layer was dried over anhydrous sodium sulfate and evaporated to a small volume. An excess of *n*-hexane was added to precipitate BCM-B. Thus, BCM-B was obtained as a pure red powder in a yield of 35 mg. The purity of the BCM-B was an excess of 97% as determined by HPLC, which was performed on a Hitachi 655 liquid chromatographic apparatus with a reverse-phase analytical column, A312 (ODS) (6 × 150 mm) (Yamamura Chemical Laboratories, Co., Ltd.). Acetonitorile - 0.05 M ammonium formate (pH 4.0) (30 : 70) was used as a mobile phase and run at a flow rate of 1.0 ml/minute. The retention time was 7.24 minute. Physicochemical properties of BCM-B are as follows: MP 182 ~ 185°C; IR (KBr) cm^{-1} 3400, 2950, 1740, 1610, 1450, 1300, 1250, 1210, 1130, 1020; UV λ_{max} in 90% MeOH nm ($E^{1\%}_{1cm}$) 204 (332), 235 (589), 254 (344), 293 (115), 495 (208), 528 (sh, 140); λ_{max} in 90% MeOH - 0.01 N HCl nm ($E^{1\%}_{1cm}$) 206 (486), 235 (654), 254 (388), 291 (138), 495 (208), 528 (sh, 140); λ_{max} in 90% MeOH - 0.01 N NaOH nm ($E^{1\%}_{1cm}$) 207 (686), 240 (557), 298 (107), 550 (203), 592 (180); FD-MS *m/z* 784 $(M+H)^+$ (MW 783 for $C_{40}H_{49}NO_{15}$). Acid hydrolysis of BCM-B at 85°C for 30 minutes gave an aglycone and sugars. After recovery with $CHCl_3$ extraction, the aglycone was determined to be β-RMN by comparing its ^1H NMR and FD-MS with those of an authentic sample.[5] The remaining sugar components in the solution were determined according to the qualitative TLC analysis as previously described[6] and were found to be L-rhodosamine, 2-deoxy-L-fucose and cinerulose B. Its sugar components were same as those of ACM-B based on a comparative TLC sugar analysis. Methanolysis of BCM-B according to the method

Table 1. Chemical shift-assignments of ^1H NMR spectrum of BCM-B.

Assignment	δ ppm BCM-B	δ ppm BCM-A[a]	Remarks
1-H	7.86 d	7.84 dd	Aglycone
2-H	7.71 t	7.70 t	moiety
3-H	7.32 d	7.28 dd	
7-H	5.12 br s	5.13 b	
8-Ha	2.19 d	2.4~ m	
8-Hb	2.09 dd	2.4~ m	
10-H	4.90 s	4.90 br s	
13-Ha	1.84 g	1.7~ m	
13-Hb	1.77 q	1.7~ m	
14-CH_3	1.12 t	1.11 t	
1′-H	5.47 s	5.50 br	Rhodosamine
2′-CH_2	1.82 br d	1.9~ m	moiety
3′-H	2.07 br t	—	
3′-$N(CH_3)_2$	2.14 s	2.17 s	
4′-H	3.77 s	3.75 br s	
5′-H	4.02 q	4.03 q	
6′-H	1.29 d	1.29 d	
1″-H	5.12 br s	5.04 br	2-Deoxyfucose
2″-Ha	2.47 dt	1.9~ m	moiety
2″-Hb	1.93 dd	1.9~ m	
3″-H	4.33 m	3.5~ m	
4″-H	4.02 br s	3.67 br s	
5″-H	4.66 q	4.56 q	
6″-H	1.23 d	1.16 d	
1‴-H	5.19 d	5.07 t	Cinerulose
2‴-CH_2	4.37 q	2.0~ m	moiety
3‴-CH_2	2.58 d	2.4~ m	
5‴-H	4.79 q	4.50 q	
6‴-CH_3	1.36 d	1.33 d	

The spectra were measured in $CDCl_3$ at 400 MHz using TMS as an internal reference.

[a] Data cited from ref 1, measured with a 100 MHz spectrometer.

Table 2. Chemical shift assignments of ^{13}C NMR spectrum of BCM-B.

Carbon	δ ppm BCM-B	δ ppm β-RMN	Carbon	δ ppm BCM-B
1	119.70	119.8	1′	101.55
2	137.12	137.5	2′	29.28
3	124.96	124.9	3′	61.50
4	162.75	162.6	3′-$N(CH_3)_2$	43.20
4a	116.10	116.4	4′	74.19
5	190.85	191.1	5′	68.34
5a	112.16	112.1	6′	17.87
6	156.87	156.5	1″	99.09
6a	135.12	137.3	2″	27.00
7	70.44	62.2	3″	67.32
8	32.93	33.8	4″	66.94
9	71.81	73.0	5″	65.29
10	66.73	65.9	6″	16.03
10a	138.67	138.3	1‴	91.55
11	157.29	156.5	2‴	63.03
11a	111.53	111.8	3‴	39.75
12	186.30	186.5	4‴	208.18
13	30.48	30.6	5‴	77.94
14	6.60	6.3	6‴	16.19

The spectra were measured in $CDCl_3$ at 100 MHz using TMS as an internal reference.

Table 3. Antitumor activity *in vitro* and *in vivo* of BCM-B against L1210 leukemia.

Antitumor test	BCM-B	BCM-A	Doxorubicin
1) L1210 cell culture			
IC_{50} value (μg/ml):			
Cell growth	0.005	0.005	0.02
RNA synthesis	0.54	0.46	1.40
DNA synthesis	0.25	0.12	0.55
RNA/DNA	2.2	3.8	2.5
2) L1210-bearing mice[a]			
T/C%	240	210	—
Optimum dose (mg/kg/day) on day 1 to 10	1.25	2.5	—

[a] Treatment: ip-ip.

described previously[5] gave β-rhodomycin-1[7] and a methyl disaccharide, which coincided with that released from ACM-B by methanolysis and determined by ^{1}H NMR.[5] Therefore, the sugar moiety of BCM-B was identical to that of ACM-B as shown in Fig. 1.

The chemical shifts and assignments of ^{1}H NMR and ^{13}C NMR spectra of BCM-B are shown in Tables 1 and 2, respectively, in comparison with those of BCM-A and β-RMN. In ^{1}H NMR the chemical shifts were superimposable between BCM-A and BCM-B except for 2‴-H which shifted about 2 ppm down as compared that of BCM-A. This was due to an etheral bonding between 2-deoxy-L-fucose and L-cinerulose B. ^{13}C NMR confirmed the 40 carbons for a molecular formula of $C_{40}H_{49}NO_{15}$. The chemical shift of C-7 differed significantly between BCM-B and β-RMN, indicating that the glycosidic linkage was on C-7.

The antitumor activity of BCM-B is shown in Table 3. BCM-B was very active as was BCM-A against leukemic L1210 cell culture when the bioactivities were assayed as previously described.[8] IC_{50} value (μg/ml) against the cell growth was 0.005 which was 40 times more active than doxorubicin (0.02). The IC_{50} ratio of RNA synthesis to DNA synthesis was 2.2 and also lower than that of doxorubicin (2.5) and BCA-A (3.8). Thus, BCM-B exhibited a greater effect on the inhibition of DNA synthesis than these agents. Antitumor activity *in vivo* of BCM-B against L1210 in mice was 240% (T/C%) with an optimum dose of 1.25 mg/kg/day on day 1 to 10 under ip-ip treatment. BCM-B was superior to BCM-A in this model.

References

1) OKI, T.; A. YOSHIMOTO, Y. MATSUZAWA, T. TAKEUCHI & H. UMEZAWA: Biosynthesis of anthracycline antibiotics by *Streptomyces galilaeus*. I. Glycosidation of various anthracyclinones by an aclacinomycin-negative mutant and biosynthesis of aclacinomycins from aklavinone. J. Antibiotics 33: 1331～1340, 1980

2) YOSHIMOTO, A.; Y. MATSUZAWA, T. ISHIKURA, T. SAWA, T. TAKEUCHI & H. UMEZAWA: New anthracycline derivatives from betaclamycin A. J. Antibiotics 37: 920～922, 1984

3) OKI, T.; Y. MATSUZAWA, A. YOSHIMOTO, K. NUMATA, I. KITAMURA, S. HORI, A. TAKAMATSU, H. UMEZAWA, M. ISHIZUKA, H. NAGANAWA, H. SUDA, M. HAMADA & T. TAKEUCHI: New antitumor antibiotics, aclacinomycins A and B. J. Antibiotics 28: 830～834, 1975

4) YOSHIMOTO, A.; Y. MATSUZAWA, T. OKI, T. TAKEUCHI & H. UMEZAWA: New anthracycline metabolites from mutant strains of *Streptomyces galilaeus* MA144-M1. I. Isolation and characterization of various blocked mutants. J. Antibiotics 34: 951～958, 1981

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) JOHDO, O.; Y. WATANABE, T. ISHIKURA, A. YOSHIMOTO, H. NAGANAWA, T. SAWA & T. TAKEUCHI: Anthracycline metabolites from *Streptomyces violaceus* A262. II. New anthracycline epelmycins produced by a blocked mutant strain SU2-730. J. Antibiotics 44: 1121～1129, 1991

7) BROCKMANN, H. & E. SPOHLER: Zur Konstitution des Rhodomycin A. Naturwissenschaften 48: 716～721, 1961

8) 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