ANTHRACYCLINE METABOLITES FROM *Streptomyces violaceus* A262

I. ISOLATION OF ANTIBIOTIC-BLOCKED MUTANTS FROM *Streptomyces violaceus* A262

OSAMU JOHDO, TOMOYUKI ISHIKURA† and AKIHIRO YOSHIMOTO††

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

TOMIO TAKEUCHI

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

(Received for publication February 6, 1991)

Five mutant (or variant) strains producing new anthracycline antibiotics were derived from *Streptomyces violaceus* A262 by mutagenesis treatment. Strain SE1-625 showed a limited production of three known β-rhodomycinone diglycosides while the parent strain produced numerous unidentified β-rhodomycinone glycosides. Strain SU2-730 was an antibiotic-blocked mutant which produced only ε-rhodomycinone glycosides (named epelmycins). Strains SC-7 and SE2-2385 were variants which produced α-citromycinone glycosides (named yellamycins) and β-isorhodomycinone glycosides (named obelmycins), respectively. Strain SE2-2385-A1 produced α_2-rhodomycinone glycosides (named alldimycins). Glycosidation-less mutants which accumulated only aglycone were also obtained. Isolation of these mutants or variants and preliminary identification of their anthracycline products are described.

The anthracycline family of antibiotics is an important source for therapeutically useful antitumor agents. Adriamycin[1] (doxorubicin) is now the most useful drug for cancer treatment and has broad antitumor spectra with great therapeutic efficacy. Daunomycin[2] (daunorubicin) and aclacinomycin A[3] (aclarubicin) are also excellent therapeutic drugs for blood cancer treatment. However, further development of new anthracycline compounds is required to improve the therapeutic efficacy and reduce side effects such as cardiotoxicity and bone marrow supression.

In a continuing search for new anthracycline compounds of microbial origin, our recent attempt has been directed to the isolation of new analog-producing mutant or variant strains by mutation of known anthracycline producers. We tried the mutational derivation of such mutants from *Streptomyces violaceus* A262 and obtained some characteristic strains with a benefical anthracycline production. In this paper, we describe the isolation of antibiotic-blocked mutants and primary characterization of their anthracycline products. The details of new anthracycline antibiotics thus obtained will be described in separate papers[4~7].

Materials and Methods

Microbial Strains

S. violaceus A262 was a stock culture of our laboratory and was purified once by a technique of

Present address: † Showa Pharmacochemical Co., Ltd., 1-16-5 Kyobashi, Chuo-ku, Tokyo 104, Japan.
†† Faculty of Applied Biological Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-hiroshima 724, Japan.

monospore isolation. The purified strain, strain A262-4, was used as a parent one from which antibiotic-blocked mutants were artificially induced as described below. All strains were grown on YS agar medium (yeast extract 0.3%, soluble starch 1.0% and agar 1.5%, pH 7.2) and maintained at 5°C until use.

Media

YS agar medium as described above was used as the plating medium for mutant isolation. Seed cultures were grown in the medium containing soluble starch 0.5%, glucose 0.5%, soybean meal 1.0%, yeast extract 0.1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1% and NaCl 0.3% in tap water (pH 7.5) (seed medium). Flask medium used for the antibiotic production test contained soluble starch 4.0%, soybean meal 2.5%, yeast extract 0.2%, NaCl 0.25%, $CaCO_3$ 0.32%, $CuSO_4 \cdot 5H_2O$ 0.0007%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $MnSO_4 \cdot 4H_2O$ 0.0008% and $ZnSO_4 \cdot 7H_2O$ 0.0002% in tap water (pH 7.4). The medium used for large-scale fermentation was as follows: soluble starch 5.0%, soybean meal 3.0%, yeast extract 0.2%, NaCl 0.25%, $CaCO_3$ 0.32%, $CuSO_4 \cdot 5H_2O$ 0.0007%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $MnSO_4 \cdot 4H_2O$ 0.0008% and $ZnSO_4 \cdot 7H_2O$ 0.0002% in tap water (pH 7.5) (production medium).

Mutagenesis Treatment

UV, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and ethylmethanesulfonate (EMS) were used as mutagens. Microbial strains were grown on YS agar at 28°C for 10 days, and the spore cells were collected and suspended in 0.9% saline, 0.1 M Tris-HCl buffer (pH 8.0) or 0.1 M phosphate buffer (pH 7.0) by mild sonication. UV treatment was conducted on a Petri dish (90 mm) containing 10 ml of a cell density of 5×10^8/ml of the spore suspension in the saline. The irradiation time was controlled to give a killing rate of over 95%. NTG treatment was carried out in a test tube containing 3 ml of a cell density of about 10^9/ml of the spore suspension in the Tris-HCl buffer. The NTG concentration and the contact time at room temperature were 1 mg/ml and about 45 minutes to give a killing rate of more than 80%. EMS treatment was done in a test tube containing 3 ml of a cell density of 5×10^8/ml of the spore suspension in the phosphate buffer. The EMS concentration and the contact time at room temperature were 5 mg/ml and about 25 hours to give a killing rate of about 90%. The spore suspensions thus treated were diluted with 0.9% saline, 0.1 ml of the aliquots was plated on YS agar and incubated at 28°C for 7 days. The colonies were tested for antibiotic production as described below.

Screening of Antibiotic-blocked Mutant

The colonies obtained above were inoculated in a small glass tube containing 3 ml of the seed medium and cultivated on a reciprocal shaker at 28°C for 2 days, and all of each culture was added to a 250-ml Erlenmeyer flask containing 30 ml of the flask medium. Cultivation was carried out at 30°C for 5 days on a rotary shaker (200 rpm). One ml of the culture broth was added to 1 ml of 0.6 N HCl in a test tube and the mixture was heated at 85°C in a water bath for 45 minutes till the antibiotics produced were completely hydrolyzed to aglycones, which were extracted with 0.3 ml of toluene by mixing on Touchmixer. After centrifugation, 10 μl of the solvent layer was spotted on Silica gel plate F_{254} (E. Merck), which was developed with a solvent mixture of $CHCl_3$-MeOH (15:1). The aglycones were discriminated by their colors and Rf values. In contrast, the whole antibiotics were extracted by vigorous mixing of 1 ml of the culture broth and 0.2 ml of toluene in a test tube on Touchmixer. After centrifugation, 10 μl of the solvent layer was subjected to TLC in the same manner described above, but the solvent used was $CHCl_3$-MeOH-concd NH_4OH (120:10:0.2). In some cases, 2D TLC was parformed to determine all anthracycline components of the mutant products. Solvent system were $CHCl_3$-MeOH (15:1) in the first direction and $CHCl_3$-MeOH-concd NH_4OH (120:10:0.2) in the second direction.

Fermentation and Product Isolation

A large scale fermentation was carried out using 100 Erlenmeyer flasks containing 50 ml each of the production medium. All seed cultures were grown at 28°C for 2 days in a 500-ml Erlenmeyer flask containing 100 ml of seed medium and inoculated 4% into a fermentation flask. The cultivation was carried out at 30°C for 5 days.

Concerning strains SU2-730, SE1-625 and SE2-2385, the culture broth was centrifuged and the

products were extracted with 3 liters of acetone from the mycelial pellet. The acetone extract was concentrated to a small volume and extracted with $CHCl_3$ after adjusting pH to 7.5 with 4 N NaOH. The $CHCl_3$ extract was evaporated and an excess of *n*-hexane was added to precipitate crude products. The precipitate was collected by centrifugation and dried *in vacuo*. Regarding strain SC-7 and SE2-2385-Al, the culture broth was acidified to pH 1.0 with 6 N HCl, heated at 65°C for 30 minutes and centrifuged after pH adjustment to 7.5 with 4 N NaOH. The products were extracted from mycelial pellet with acetone. The crude products were then isolated in the same manner as described above.

TLC

Each product (about 5 mg) in 0.5 ml of 0.1 N HCl was heated at 85°C for 30 minutes in a water bath. The pigment aglycone thus obtained was extracted with $CHCl_3$. The $CHCl_3$ layer was evaporated to dryness and the pigment residue was chromatographed on a Silica gel plate F_{254} using a solvent mixture of $CHCl_3$ - MeOH (15 : 1) for chromatographic determination. The reference aglycones ε-rhodomycinone (RMN) (red), ε-isorhodomycinone (isoRMN) (purple), α-citromycinone (CTN) (yellow), α_2-RMN (orange), β-RMN (red), β-isoRMN (purple) and γ-isoRMN (purple) showed Rf values of 0.79, 0.77, 0.29, 0.27, 0.45, 0.43 and 0.59, respectively. The aglycones were also determined by spot colors on the TLC.

Alternatively, the aqueous layer containing sugar components was neutralized by adding silver carbonate with a small amount of charcoal and centrifuged. The supernatant fluid was concentrated *in vacuo* and chromatographed on Silica gel plate F_{254} using a developing solvent of BuOH - acetic acid - H_2O (4 : 1 : 1). The sugar spots were detected by spraying with *p*-anisaldehyde - H_2SO_4 (each 5%) in 90% EtOH and heating at 90°C. Aclacinomycins A and B[3] were hydrolyzed in the same manner and the aqueous layers were used as a source of the reference sugars including L-rhodosamine (RN), 2-deoxy-L-fucose (dF), L-rhodinose (R), L-cinerulose A (CinA) and L-cinerulose B (CinB), which showed Rf values of 0.12, 0.56, 0.71, 0.82 and 0.80, respectively.

General

MP's were determined on a Kofler hotstage microscope. UV spectra were determined on a Hitachi EPS 3T and IR spectra (KBr pellet) on a Hitachi EPI-GS spectrophotometer. ^{13}C NMR spectra were recorded with a Jeol GX-400 spectrometer at 100 MHz. Chemical shifts were expressed in δ values (ppm) with TMS as an internal reference. Mass spectra were recorded with a Hitachi M-80H spectrometer. Specific rotations were determined on a Jasco DIP-181 Digital Polarimeter.

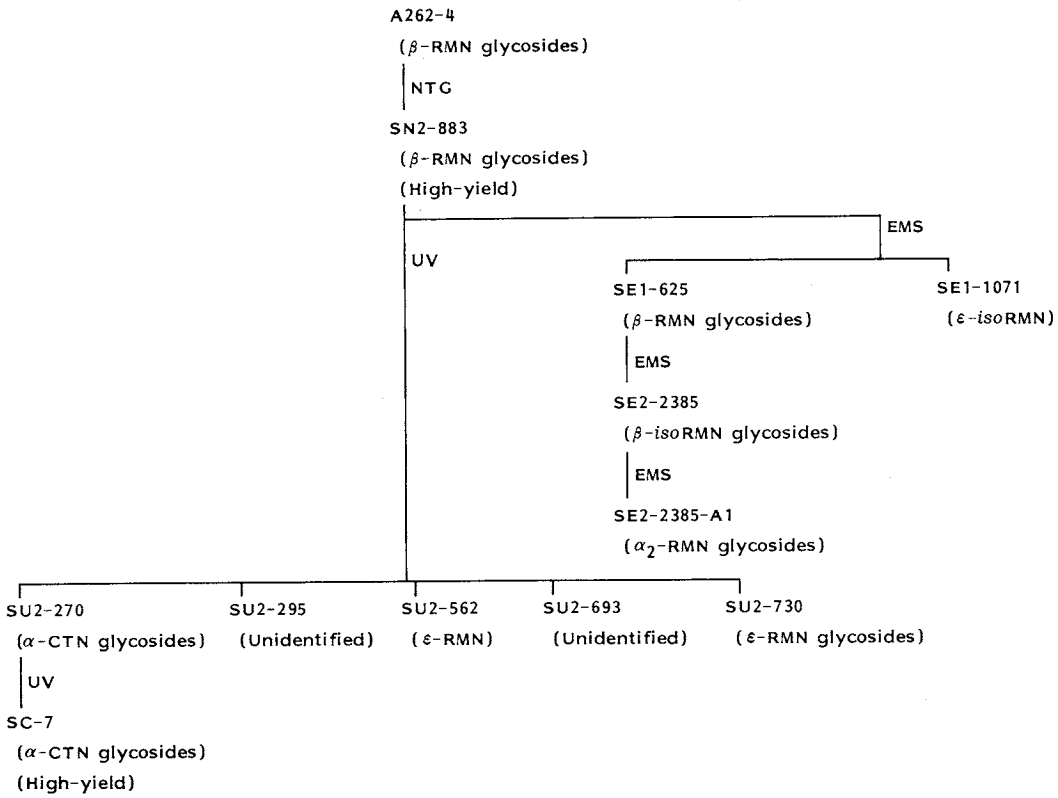
Results

Isolation of Antibiotic-blocked Mutants or Variants

By 2D TLC of the toluene extract from the culture broth, *S. violaceus* A262-4 (parent strain) was found to produce more than 15 anthracycline components containing A262-1, A262-2 and A262-3, which were identified as products of SE1-625. TLC analysis of the hydrolyzed products also revealed the presence of β-RMN as a main aglycone, suggesting that the parent strain produced numerous components of β-RMN glycosides with a variety of sugar chains.

Therefore, we mutated *S. violaceus* A262-4 to yield mutant or variant strains which produced new anthracycline analogs other than the parent products or only a limited number of components. Fig. 1 shows the scheme of mutational derivation from the parent strain. Strain SN2-883 was selected as a strain with increased potency of parent antibiotic production. During seven steps of mutagenesis treatment about 6,000 microbial isolates were tested for the mutant screening based on a TLC check of the constituent aglycone, and seven characteristic strains were derived from strain SN2-883 by treatment with either EMS or UV. Strain SE1-625 showed a limited production of only three anthracycline components when the whole toluene extract was examined by TLC. The aglycone of these products was also parent β-RMN (red) by TLC analysis after complete hydrolysis. The aglycones of products of strains

Fig. 1. Scheme of the mutant selection in *Streptomyces violaceus* A262.



SU2-730 and SU2-270 were found to be ε-RMN (red) and α-CTN (yellow), respectively. Strain SE2-2385 formed a deep purple color of colony and β-isoRMN (purple) and γ-isoRMN (purple) were detected as constituent aglycones. Strains SU2-693 and SU2-295 produced new yellow and orange compounds, but their aglycones have not been identified. Whereas, strain SC-7 was then improved to have an increased productivity of α-CTN glycosides. Further mutation of strain SE2-2385 resulted in a variant strain SE2-2385-A1 which accumulated α_2-RMN glycosides (orange) together with β-isoRMN glycosides. On the other hand, pigment-less mutants and glycosidation-less mutants were often detected. Strains SU2-562 and SE1-1071 were picked up as the later type of mutants and accumulated ε-RMN and ε-isoRMN in the culture broth, respectively.

Identification of Strain SE1-625 Products

Strain SE1-625 varied to produce only three anthracycline components designated as A262-1, A262-2 and A262-3. These were also major products in parental strains A262-4 and SN2-837 although they produced other numerous anthracycline components at the same time. A262-1, A262-2 and A262-3 were isolated and purified from the culture broth of strain SE1-625, and were identified as follows.

The crude products recovered from 5 liters of culture broth were subjected to adsorption chromatography on a silica gel column (32 mm i.d., Wakogel C-200, 30 g). Solvent systems were $CHCl_3$-MeOH (20:1) and $CHCl_3$-MeOH-concd NH_4OH (100:7:0.1) and were used in this order. Fractions containing each of three components (named A262-1, A262-2 and A262-3 in the elution order) were pooled and evaporated to dryness. The each residue was chromatographed on preparative Silica gel

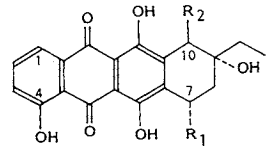
Table 1. ^{13}C NMR chemical shifts of A262-1, A262-2, A262-3, A262-A and A262-B.

Carbon	A262-1	A262-2	A262-3	A262-A	A262-B	Remarks
1	119.67	119.70	119.70	119.28	119.37	Aglycone moiety
2	137.04	137.03	137.06	136.75	136.83	
3	124.64	124.65	124.63	124.18	124.32	
4	162.53	162.53	162.54	162.30	162.55	
4a	115.99	116.00	116.01	115.83	116.17	
5	190.72	190.74	190.74	190.53	190.90	
5a	111.97	111.97	112.00	110.45	110.74	
6	157.12	157.10	157.05	156.28	156.41	
6a	136.45	136.46	136.40	137.08	137.11	
7	70.89	70.93	70.94	20.95	21.04	
8	32.94	32.95	32.95	26.78	27.08	
9	71.73	71.72	71.73	71.64	71.88	
10	70.33	70.34	70.34	70.20	70.34	
10a	138.28	138.20	138.16	140.99	140.99	
11	157.65	157.62	157.59	158.14	158.26	
11a	111.67	111.70	111.71	110.05	110.31	
12	185.92	185.94	185.93	185.32	185.70	
12a	133.46	133.47	133.47	133.46	133.79	
13	30.66	30.66	30.64	30.75	30.81	
14	6.61	6.60	6.60	6.61	6.63	
1′	101.96	101.95	101.91	—	—	Sugar moeity
2′	29.32	29.31	29.23	—	—	attached to C-7
3′	61.44	61.37	61.32	—	—	
4′	73.80	74.09	74.19	—	—	
5′	68.51	68.29	68.29	—	—	
6′	17.82	17.77	17.77	—	—	
3′-$N(CH_3)_2$	43.24	43.26	43.24	—	—	
1″	98.60	99.49	99.44	—	—	
2″	24.53	34.35	34.32	—	—	
3″	24.68	65.59	65.51	—	—	
4″	75.30	83.68	83.65	—	—	
5″	66.65	66.65	66.79	—	—	
6″	17.11	16.92	16.92	—	—	
1‴	99.51	100.30	100.30	—	—	
2‴	23.60	23.92	23.90	—	—	
3‴	25.96	25.47	25.47	—	—	
4‴	66.81	67.15	67.15	—	—	
5‴	67.49	68.06	68.06	—	—	
6‴	17.11	17.10	17.09	—	—	
1′	97.30	97.31	97.22	96.86	97.11	Sugar moeity
2′	29.76	29.73	29.64	29.47	29.71	attached to C-10
3′	61.53	61.41	61.35	61.33	61.54	
4′	74.11	74.27	74.25	74.29	74.19	
5′	68.68	68.70	68.47	68.48	68.80	
6′	18.04	18.03	18.01	18.02	18.13	
3′-$N(CH_3)_2$	43.24	43.21	43.18	43.22	43.33	
1″	98.60	98.57	99.39	99.35	98.67	
2″	24.53	24.51	34.21	34.17	24.57	
3″	24.77	24.77	65.57	65.46	24.74	
4″	75.30	75.29	83.54	83.47	75.38	
5″	66.76	66.79	66.86	66.78	66.82	
6″	17.11	16.97	16.97	16.92	17.12	
1‴	99.46	99.44	100.24	100.19	99.48	
2‴	23.60	23.60	23.90	23.87	23.67	
3‴	25.96	25.95	25.47	25.41	26.02	
4‴	66.81	66.85	67.48	67.09	67.56	
5‴	67.49	67.48	68.00	67.98	66.68	
6‴	17.11	17.10	16.97	16.87	17.12	

Spectra were measured in $CDCl_3$. Chemical shifts are expressed by δ (ppm) from internal TMS.

plate PF_{254} using a solvent mixture of $CHCl_3$-MeOH-concd NH_4OH (120:10:0.3). Main pigmented silica gel bands containing each component were scraped off and eluted with $CHCl_3$-MeOH (8:1). After evaporation to dryness, the residue was dissolved in 1% acetic acid (pH 3.0) and the solution was washed with an equal volume of toluene. The aqueous layer was adjusted to pH 7.5 with 4 N NaOH and extracted with $CHCl_3$. After washing with water and drying over anhydrous Na_2SO_4 the extract was evaporated to a small volume and an excess of *n*-hexane was added to precipitate the products. Thus, pure A262-1, A262-2 and A262-3 were obtained with yields of 110 mg, 102 mg and 94 mg, respectively.

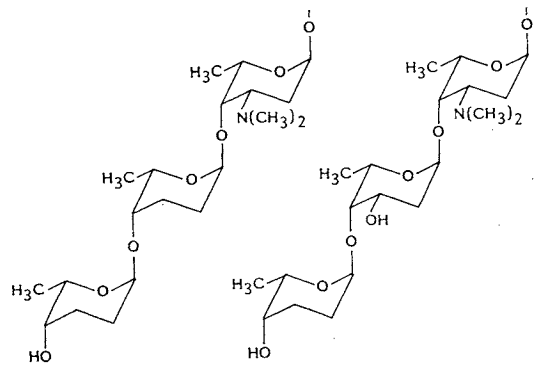
Fig. 2. Structures of A262-1, A262-2 and A262-3.



A262	R_1	R_2
1	RN-R-R	RN-R-R
2	RN-dF-R	RN-R-R
3	RN-dF-R	RN-dF-R

RN: Rhodosamine, dF: 2-deoxyfucose, R: rhodinose.

RN-R-R: RN-dF-R:



The structural determination of these compounds was carried out based on spectrochemical analyses of the hydrogenated products, MS and ^1H and ^{13}C NMR. ^{13}C NMR of A262-1, A262-2, A262-3 and their hydrogenated compounds (A262-A and A262-B) are shown in Table 1. A262-A, identified as cosmomycin A[8] was obtained as a result of hydrogenolysis of A262-1 or A262-2 and A262-B identified as cosmomycin B[8] was obtained from A262-3 similarly. Thus, A262-1, A262-2 and A262-3 were identical to A447 C[9], cosmomycins C and D[10], respectively, as illustrated in Fig. 2.

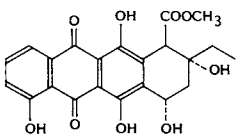
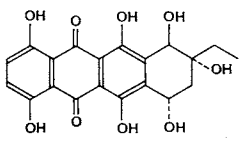
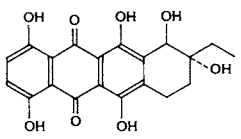
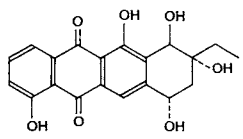
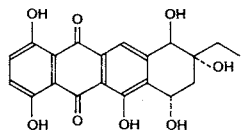
Preliminary Identification of the Mutant Products

For identification of the constituent aglycone of the mutant product, the crude preparation from each mutant culture was hydrolyzed in 0.1 N HCl by heating at 85°C for 30 minutes and the aglycone was extracted with $CHCl_3$. The extract was evaporated to dryness and the residue was purified by preparative TLC on Silica gel plate PF_{254} with a solvent mixture of $CHCl_3$-MeOH (20:1). The physico-chemical properties of the pure aglycones thus obtained are summarized in Table 2, where it is evidenced that the aglycone of mutant products are β-RMN for strain SE1-625, β-isoRMN and γ-isoRMN for strain SE2-2385, α_2-RMN for strain SE2-2385-Al, ε-RMN for strain SU2-730 and α-CTN for strain SC-7.

For isolation of the glycosidic components, the crude preparation from each mutant culture was subjected to preparative TLC on Silica gel plate PF_{254}. The solvent systems used were $CHCl_3$-MeOH-concd NH_4OH (80:10:1) for strain SE2-2385 products, $CHCl_3$-MeOH-HCOOH (40:10:1) for strain SE2-2385-A1 products, $CHCl_3$-MeOH-concd NH_4OH (120:10:0.2) for strain SU2-730 products and $CHCl_3$-MeOH-H_2O-CH_3COOH-concd NH_4OH (150:50:5:1:1) for SC-7 products. Silica gel bands containing each anthracycline component were scraped off and extracted with a solvent mixture of $CHCl_3$-MeOH (5:1). The extract was evaporated to dryness and hydrolyzed with 0.1 N

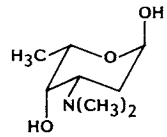
Table 2. Physico-chemical properties of aglycones of mutant products.

	Aglycone				
	SU2-730	SE2-2385		SC-7	SE2-2385-A1
		1	2		
Molecular formula	$C_{22}H_{20}O_9$	$C_{20}H_{18}O_9$	$C_{20}H_{18}O_8$	$C_{20}H_{18}O_7$	$C_{20}H_{18}O_8$
MS (*m/z*)	428 M^+	402 M^+	387 $(M+H)^+$	371 $(M+H)^+$	387 $(M+H)^+$
MP (°C, dec)	215~217	232~235	231~234	195~198	196~198
$[\alpha]_D^{23}$ ($CHCl_3$)	+160° (*c* 0.011)	−98° (*c* 0.002)	−300° (*c* 0.002)	+189° (*c* 0.009)	+63° (*c* 0.01)
UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1\,cm}^{1\%}$)	234 (1,095), 254 (647), 292 (216), 492 (391)	240 (1,184), 296 (193), 490 (273), 522 (466), 548 (444), 560 (473)	241 (761), 297 (147), 488 (219), 520 (360), 546 (326), 558 (362)	230 (970), 257 (641), 287 (sh, 233), 435 (291)	234 (1,165), 257 (573), 290 (202), 490 (366)
IR ν_{max} (KBr) cm^{-1}	3400, 2920, 1725, 1600, 1430, 1285, 1240, 1195, 1160	3400, 2920, 1590, 1450, 1300, 1190, 1070, 1030, 980, 790	3400, 2920, 1575, 1445, 1270, 1190, 1020, 790	3380, 2910, 1665, 1610, 1570, 1460, 1440, 1380, 1270, 1010, 750	3400, 2920, 1590, 1445, 1280, 1220, 1155, 1030, 1000, 780
Rf value ($CHCl_3$-MeOH (15:1))	0.79	0.45	0.59	0.29	0.27
^{13}C NMR[a]:					
C-1	119.4	157.4	(158.6)	121.3	(157.1)
C-2	137.7	129.1	(130.2)	137.0	129.4
C-3	124.6	129.1	(130.1)	125.0	129.4
C-4	162.1	157.4	(157.6)	162.7	(156.4)
C-4a	115.6	112.7	114.2	116.4	(112.5)
C-5	190.3	189.3	(189.9)	188.0	185.9
C-5a	111.1	111.8	111.0	133.2	133.9

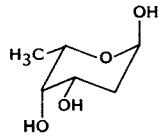
C-6	(155.9)	156.1	156.7	120.0	119.5
C-6a	134.4	137.0	140.5	147.0	148.9
C-7	61.8	61.7	21.7	67.5	65.5
C-8	34.2	33.3	26.8	34.5	36.0
C-9	71.5	72.6	71.4	73.6	72.2
C-10	52.3	65.4	65.1	66.0	64.6
C-10a	136.9	137.5	140.7	132.4	131.0
C-11	(156.2)	156.7	157.6	162.3	160.9
C-11a	110.9	111.6	110.7	115.0	113.7
C-12	185.7	189.3	(189.6)	187.9	190.0
C-12a	133.0	112.7	114.1	133.5	(112.4)
C-13	32.4	30.2	31.9	30.7	30.6
C-14	6.5	5.9	7.8	6.3	6.6
C-15	171.3	—	—	—	—
C-16	51.5	—	—	—	—
Solvent	$CDCl_3$-CD_3OD (10:1)	$CDCl_3$-CD_3OD (5:1)	DMSO	$CDCl_3$-CD_3OD (10:1)	DMSO
Aglycone identified					
	ε-Rhodomycinone	β-Isorhodomycinone	γ-Isorhodomycinone	α-Citromycinone	α_2-Rhodomycinone

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

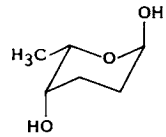
Table 3. Preliminary identification of the products of the blocked mutant or variant strains.



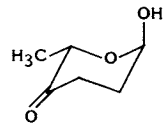
Rhodosamine (RN)



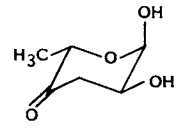
2-Deoxyfucose (dF)



Rhodinose (R)



Cinerulose A (CinA)



Cinerulose B (CinB)

Mutant strain	Product	TLC (Rf value)		Component	
		Solvent 1[a]	Solvent 2[b]	Aglycone	Sugar
SU2-730	Epelmycin A	0.78	0.83	ε-Rhodomycinone	RN, R, CinA
	Epelmycin B	0.79	0.85	ε-Rhodomycinone	RN, dF, CinB
	Epelmycin C	0.35	0.62	ε-Rhodomycinone	RN, dF
	Epelmycin D	0.44	0.46	ε-Rhodomycinone	RN
	Epelmycin E	0.76	0.82	ε-Rhodomycinone	RN, dF, CinA
SE2-2385	Obelmycin A	0.25	0.33	β-Isorhodomycinone	RN
	Obelmycin B	0.54	0.69	β-Isorhodomycinone	RN, R
	Obelmycin C	0.46	0.67	β-Isorhodomycinone	RN, dF, R
	Obelmycin D	0.39	0.65	β-Isorhodomycinone	RN, dF, R
	Obelmycin E	0.58	0.68	γ-Isorhodomycinone	RN, R
	Obelmycin F	0.49	0.68	γ-Isorhodomycinone	RN, dF, R
	Obelmycin G	0.34	0.34	γ-Isorhodomycinone	RN
SC-7	Yellamycin A	0.31	0.27	α-Citromycinone	RN
	Yellamycin B	0.25	0.08	α-Citromycinone	RN
	Yellamycin C	0.30	0.22	α-Citromycinone	RN
SE2-2385-A1	Alldimycin A	0.23	0.23	α_2-Rhodomycinone	RN
	Alldimycin B	0.17	0.05	α_2-Rhodomycinone	RN
	Alldimycin C	0.22	0.21	α_2-Rhodomycinone	RN

[a] $CHCl_3$-MeOH-aq NH_3 (80:10:1). [b] $CHCl_3$-MeOH-H_2O-AcOH-aq NH_3 (150:50:5:1:1).

HCl followed by TLC analysis for aglycone and sugar. New or main anthracycline compounds produced by the mutant strains are summarized in Table 3 with the data on their constitutive aglycone and sugars.

In conclusion, five components of ε-RMN glycosides (named epelmycins A~E) were isolated from the culture broth of strain SU2-730. Four components of β-isoRMN glycosides (named obelmycins A~D) and three components of γ-isoRMN glycosides (named obelmycins E~G) were isolated from the culture broth of strain SE2-2385. Strain SC-7 showed coproduction of α-CTN glycosides with β-RMN glycosides (mainly A262 compounds). Three components of α-CTN glycosides (named yellamycins A~C) were isolated from the culture broth after mild acid hydrolysis. Strain SE2-2385-A1 varied in additional production of α_2-RMN glycosides from obelmycin producer SE2-2385. Three components of α_2-RMN glycosides (named alldimycins A~C) were also isolated from the culture broth after mild acid hydrolysis.

Sugar analysis by TLC proved that the sugar composition of the mutant products were much the same as those of parent products. Thus, the aminosugar was rhodosamine and the neutral sugars were 2-deoxyfucose, rhodinose and cineruloses A and B. Eventually, all the mutants were found to have genetic alteration in the biosynthesis of the aglycone moiety on their anthracycline production.

Discussion

We have isolated producers of unique new rhodomycin analogs by mutational derivation of *S.*

violaceus A262 which produced a number of 7,10-diglycosidic β-RMN glycosides, among which the major compounds were named A262-1, A262-2 and A262-3. It was found that a variant strain SE1-625 produced only these three compounds specifically. Therefore, the compounds were isolated from the culture broth of SE1-625, purified and identified. A262-1, A262-2 and A262-3 were known 7,10-diglycosidic β-RMN glycosides which were identified as A447 C[9], cosmomycins C and D[10], respectively. It is noticed that the terminal sugar of all these compounds is rhodinose. We have reported an extracellular oxidoreductase which catalyzes C-4 dehydrogenation from the terminal rhodinose to cinerulose A[11]. This played an important role in the production of glycosidic derivatives as seen with aclacinomycins. There is a possibility that the production of limited components by the strain SE1-625 is due to the loss of the enzyme activity. Strain SU2-730 seems to have a genetic loss of 10-demethoxycarbonylation on the biosynthetic step from ε-RMN to β-RMN. It is very interesting that all the aglycone-accumulating blocked mutants isolated were producers of not β-RMN but 10-methoxycarbonyl aglycone such as SU2-562 and SE1-1071. This suggests that the biosynthetic conversion from ε-RMN to β-RMN occurs at a glycoside level. We have evidenced that the biosynthesis of daunorubicin in *S. coeruleorubidus* also take place *via* intermediate ε-RMN glycosides[12].

Strain SE2-2385 showed coproduction of abundant β-isoRMN (1-hydroxy-β-RMN) glycosides (obelmycins). Therefore, it is supposed that strain SE2-2385 has the enhanced enzyme activity on the C-1 oxidation for the biosynthesis of β-isoRMN glycosides. Strains SE2-2385-A1 and SC-7 also varied to coproduce α_2-RMN glycosides (alldimycins) and α-CTN glycosides (yellamycins), respectively. Since the anthracycline aglycone formed *via* polyketide has an oxygen at C-6[13], these compounds may be yielded from β-isoRMN glycosides and β-RMN glycosides by the enzymatic 6-deoxygenation. If this is true, it is considered that the enzyme activity is expressed strongly in strains SE2-2385-A1 and SC-7.

Thus, we isolated five biosynthetic mutants from β-rhodomycin-producing *S. violaceus* A262 and obtained thirteen new anthracycline antibiotics. The structural identifications of epelmycins, obelmycins, yellamycins and alldimycins and their biological activities will be reported in further papers in details[4~7].

References

1) ARCAMONE, F. G.; G. CASSINELLA, G. FANTINI, A. GREIN, P. OREZZI, C. POL & C. SALLA: Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from *S. peucetius var. caesius*. Biotechnol. Bioeng. 11: 1101~1110, 1969
2) DIMARCO, A.; M. GAETANI, P. OREZZI, B. M. SCARPINATO, R. SILVESTRINI, M. SOLDATI, T. DASDIA & L. VALENTINI: 'Daunomycin', a new antibiotic of the rhodomycin group. Nature 201: 706~707, 1964
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) 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
5) 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
6) 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
7) JOHDO, O.; H. TONE, R. OKAMOTO, A. YOSHIMOTO, H. NAGANAWA, T. SAWA & T. TAKEUCHI: Anthracycline metabolites from *Streptomyces violaceus* A262. V. New anthracycline alldimycin A: A minor component isolated from obelmycin beer. J. Antibiotics 44: 1160~1164, 1991
8) ANDO, T.; K. HIRAYAMA, R. TAKAHASHI, I. HORINO, Y. ETOH, H. MORIOKA, H. SHIBAI & A. MURAI: The structures of anthracycline antibiotics, cosmomycin A and B. Agric. Biol. Chem. 49: 1207~1209, 1985
9) SHIMOSAKA, A.; Y. HAYAKAWA, M. NAKAGAWA, K. FURIHATA, H. SETO & N. ŌTAKE: Isolation of new anthracycline antibiotics, A447 C and D. J. Antibiotics 40: 116~121, 1987
10) ANDO, T.; K. HIRAYAMA, R. TAKAHASHI, I. HORINO, Y. ETOH, H. MORIOKA, H. SHIBAI & A. MURAI: Cosmomycin D, a new anthracycline antibiotic. Agric. Biol. Chem. 49: 259~262, 1985
11) YOSHIMOTO, A.; T. OGASAWARA, I. KITAMURA, T. OKI, T. INUI, T. TAKEUCHI & H. UMEZAWA: Enzymatic conversion

of aclacinomycin A to Y by a specific oxidoreductase in *Streptomyces*. J. Antibiotics 32: 472~481, 1979

12) YOSHIMOTO, A.; T. OKI & H. UMEZAWA: Biosynthesis of daunomycinone from aklavinone and ε-rhodomycinone. J. Antibiotics 33: 1199~1201, 1980

13) OLLIS, W. D. & I. O. SUTHERLAND: The incorporation of propionate in the biosynthesis of ε-pyrromycinone (rutilantinone). Proc. Chem. Soc. (London) 347: 34~38, 1960