NOTES

Isolation of New Anthracyclines 10-*O*-Rhodosaminyl β-Rhodomycinone and β-Isorhodomycinone from Mild-acid Treated Culture of Obelmycin-producing *Streptomyces violaceus*

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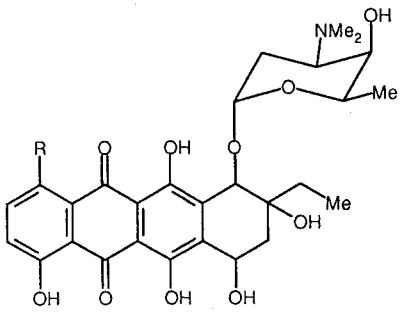
Our search for new anthracycline analogs by mutation of rhodomycin-producing *Streptomyces violaceus* A262 has provided several unique blocked mutants[1] which produced β-isorhodomycinone (β-iso-RMN) glycosides (obelmycins A~G),[2] α-citromycinone (α-CMN) glycosides (yellamycins A~C),[3] α_2-rhodomycinone (α_2-RMN) glycosides (alldimycins A~C),[4] and ε-rhodomycinone (ε-RMN) glycosides (epelmycins A~E),[5] respectively. The compounds thus obtained were mostly either 7-*O*-glycosyl or 7,10-*O*-glycosyl anthracycline compounds, but only a few 10-*O*-glycosyl derivatives (yellamycin C and alldimycin C) were obtained. There have been no other reports of natural 10-*O*-glycosyl anthracyclines, although 10-*O*-glycosyl-7-deoxy analogs (γ-rhodomycinone glycosides) have been produced by catalytically reductive 7-deglycosylation.[6] The reason for the rarity of natural 10-*O*-glycosyl anthracyclines seems to be that they are not direct fermentation products.

Since the 10-*O*-glycosyl anthracyclines are rare compounds and their antitumor activities have not been intrinsically evaluated, we were interested in 10-*O*-rhodosaminyl β-RMN and β-iso RMN. We have found that 10-*O*-glycosyl bond is slightly more stable to acid hydrolysis than 7-*O*-glycosyl bond when 7,10-*O*-diglycosyl anthracyclines were subjected to acid hydrolysis. Therefore, our attempt was depended on direct acid treatment of culture broth of obelmycin-producing *Streptomyces violaceus* strain SE2-2385 to obtain the 10-*O*-glycosyl anthracyclines. This paper deals with isolation of 10-*O*-rhodosaminyl β-RMN and β-iso-RMN (named A262-6 and obelmycin H, respectively), elucidation of their structure and assessment of their *in vitro* cytotoxicities against cultured murine leukemic L1210 cells compared with related anthracyclines.

The obelmycin-producing strain SE2-2385 was isolated as previously described.[1] A seed culture was grown aerobically at 28°C for 48 hours in 500-ml Erlenmeyer flasks containing 100 ml of the 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. This culture was employed as inoculum (3%) for two 30-liter jar fermentors each containing 15 liters of the following medium: soluble starch 40 g, soybean meal 25 g, yeast extract 1 g, NaCl 2.5 g, $CaCO_3$ 3 g, $CuSO_4 \cdot 5H_2O$ 0.001 g, $FeSO_4 \cdot 7H_2O$ 0.00016 g, $ZnSO_4 \cdot 7H_2O$ 0.00032 g, $MnCl_2 \cdot 4H_2O$ 0.0013 g per 1000 ml of tap water, pH 7.0. Conditions used were 15 liters/minute for aeration and 300 rpm for agitation. Cultivation was carried out at 28°C for 138 hours at which time the mycelial purple pigments were produced at maximum levels.

The culture broth (30 liters) adjusted to pH 1.0 with concd hydrochloric acid was heated at 65°C for 2 hours and filtered. The filtrate was adjusted to pH 2.5 and subjected to the adsorption chromatography on a column of Diaion HP-20 (100 cm × 6 cm i.d., Mitsubishi Chemical Ind.). The products were eluted with acetone - acidic water (pH 2.0) (80 : 20) after washing with 6 liters of acidic water (pH 2.5), and pigmented fractions were collected. This eluate was evaporated *in vacuo* to remove acetone and products were extracted with $CHCl_3$ after

Fig. 1. Structures of A262-6 and obelmycin H.



R=H A262-6
R=OH Obelmycin H

the pH was adjusted to 8.0. The $CHCl_3$ layer was evaporated to a small volume *in vacuo*. An excess of *n*-hexane was added to precipitate 5.8 g of crude powder of anthracycline products. The powder was chromatographed on a silica gel column which was developed stepwise with $CHCl_3$ - MeOH - H_2O - AcOH - concd NH_4OH (200 : 50 : 2 : 0.5 : 0.5 and 150 : 50 : 5 : 1 : 1). The eluate was analyzed by TLC on Silica gel 60 F_{254} (E. Merck) using a developing solvent of $CHCl_3$ - MeOH - H_2O - AcOH - concd NH_4OH (120 : 50 : 5 : 1 : 1), and the fractions containing A262-6 and obelmycin H (OBM-H) were collected. The products mixture was further purified by preparative HPLC (column: CAPCELL PAK C18 SG120, 5 μm, 250 mm × 30 mm i.d.,

Table 1. Physico-chemical properties of A262-6 and obelmycin H.

	A262-6	Obelmycin H
Appearance	Orange powder	Purple powder
Molecular formula	$C_{28}H_{33}NO_{10}$	$C_{28}H_{33}NO_{11}$
HRFAB-MS (*m/z*)	544.2195	560.2124
	($(M+H)^+$, Δ +1.3 mmu)	($(M+H)^+$, Δ -0.8 mmu)
MP (℃, dec)	138～140	173～177
$[\alpha]_D^{20}$ ($CHCl_3$)	+560° (*c* 0.01)	-768° (*c* 0.02)
UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$)	235 (727), 254 (416)	240 (842), 297 (134)
	294 (138), 496 (257)	521 (292), 549 (272)
IR ν_{max} (KBr) cm^{-1}	1599	1589
RT (min) [a]	14.6	15.2

[a] Column: YMC A312 (ODS), 150 mm × 4.6 mm i. d.; mobile phase: water-acetonitrile (75:25, pH 2.0 with H_3PO_4); flow rate: 1.0 ml/minute. β-Rhodomycin I and OBM-A had retention times at 16.9 and 18.1 minutes, respectively, in the same condition.

Table 2. ^1H NMR data for A262-6 and obelmycin H.

Proton	A262-6	Obelmycin H
1-H	7.89 d (6.60)	-
2-H	7.72 t (8.07)	7.31 s
3-H	7.31 d (7.33)	7.31 s
7-H	5.24 d (5.14)	5.21 dd (5.14, 1.47)
8-Ha	2.29 dd (14.67, 5.14)	2.24 dd (14.67, 5.14)
8-Hb	2.14 d (14.67)	2.14 d (14.67)
10-H	5.00 s	4.98 s
13-Ha	1.85 m (7.34)	1.83 m (7.33)
13-Hb	1.74 m (7.34)	1.7~1.8
14-CH_3	1.12 t (7.34)	1.11 t (7.34)
1'-H	5.45 d (3.67)	5.44 d (2.94)
2'-Ha	1.70 td (12.47, 3.67)	1.7~1.8
2'-Hb	1.55 dd (12.47, 5.13)	1.56 dd (12.47, 5.14)
3'-H	2.2	2.35 ddd (12.48, 2.20)
4'-H	3.66 br s	3.70 br s
5'-H	3.90 q (6.60)	3.92 q (6.60)
6'-CH_3	1.35 d (6.60)	1.33 d (6.60)
3'-$N(CH_3)_2$	2.21 s	2.26 s
Solvent	$CDCl_3$	$CDCl_3$-CD_3OD (20:1)

Spectra were measured at 400 MHz. Chemical shifts are expressed in δ values (ppm) from internal TMS. Coupling constants in parentheses are given in *J* (Hz).

Table 3. ^{13}C NMR data for A262-6 and obelmycin H.

Carbon	A262-6	Obelmycin H
1	119.84	157.67
2	137.31	129.58*
3	124.83	129.41*
4	162.73	157.67
4a	115.96	112.64*
5	191.00	189.25*
5a	112.07*	112.19*
6	155.94	155.99
6a	138.26	138.34
7	62.62	62.17
8	34.18	33.91
9	71.98	71.93
10	70.56	70.56
10a	137.42	136.45
11	157.77	156.72
11a	112.01*	112.00
12	186.06	189.11*
12a	133.54	112.61*
13	30.83	30.70
14	6.51	6.30
1'	97.11	96.90
2'	29.24	28.67
3'	59.73	59.94
4'	66.18	66.21
5'	66.59	66.83
6'	17.21	16.91
3'-NMe_2	42.01	41.83
Solvent	$CDCl_3$	$CDCl_3$-CD_3OD (20:1)

Spectra were measured at 100 MHz. Chemical shifts are expressed in δ values (ppm) from internal TMS.

Shiseido; mobile phase: acetonitrile-water (75:25, adjusted to pH 2.0 with phosphoric acid); flow rate: 5 ml/minute; detection: UV at 254 nm). Fractions containing A262-6 and OBM-H, monitored by HPLC analysis (see Table 1), were pooled separately and washed with $CHCl_3$. The product was extracted with $CHCl_3$ after raising the pH to 8.0, and the organic layer was dried over anhydrous sodium sulfate and concentrated to a small volume. To the concentrate, an excess of *n*-hexane was added to precipitate the respective products. This purification procedure gave 7 mg and 5 mg of A262-6 and OMB-H, respectively.

The physico-chemical properties of A262-6 and OBM-H are shown in Table 1. The molecular formulas of A262-6 and OBM-H were determined to be $C_{28}H_{33}NO_{10}$ and $C_{28}H_{33}NO_{11}$, respectively, by high resolution FAB-MS analysis. The UV and visible light absorption spectra in 90% MeOH solution exhibited characteristic peaks due to their orange and purple colors, respectively. The infrared absorption spectra (KBr) indicated the presence of a hydrogen bonded quinone carbonyl (1590~1600 cm^{-1}) which is characteristic of anthracyclines. Qualitative analysis of their aglycone and sugar components were carried out as previously described.[2] The aglycones of A262-6 and OBM-H were found to be β-RMN and β-iso-RMN, respectively, by direct comparison with authentic samples on TLC using $CHCl_3$-MeOH (20:1) (A262-6: orange, Rf value 0.27; OBM-H: purple, Rf value 0.26). Their sugar components were found to be rhodosamine by direct comparison with an authentic sample prepared from rhodomycin I.[1] These results indicated A262-6 and OBM-H consisted of β-RMN and rhodosamine, and β-iso-RMN and rhodosamine, respectively. The chemical shift assignments of ^1H and ^{13}C NMR spectra of A262-6 and OBM-H were carried out by means of DEPT, ^1H-^1H and ^1H-^{13}C correlation spectroscopy, and HMBC, and are shown in Tables 2 and 3, respectively. On ^1H NMR analysis, the spectra of A262-6 and OBM-H were similar to those of β-rhodomycin I (7-*O*-rhodosaminyl β-RMN) and obelmycin A (OBM-A; 7-*O*-rhodosaminyl β-iso-RMN),[2] respectively. ^{13}C NMR chemical shifts of A262-6 and OBM-H were superimposable on those of β rhodomycin I and OBM-A, respectively, except for those of their C-1', C-7, and C-10. The chemical shifts of C-1' shifted by about 4 ppm up-field in comparison with those of β-rhodomycin I and OBM-A, which indicated that the sugar links at C-10 but not at C-7. The sugar linkage site was confirmed by HMBC experiments. Long range coupling was detected between H-1' and C-10 in both compounds. A small coupling constant (~3.7 Hz) of the anomeric proton was also observed with both compounds, indicating that the configuration of the glycosidic bond are both α. From all these findings, the structures of A262-6 and OBM-H were determined as shown in Fig. 1.

The cytotoxic activity of A262-6, OBM-H, and their related compounds *in vitro* against cultured L1210 leukemia cells was examined according to the method previously described,[7] and the results are shown in Table 4. A262-6 and OBM-H exhibited poor cytotoxic activity in comparison with their corresponding 7-*O*-rhodosaminyl analogs, β-rhodomycin I and OBM-A. We found that the cytotoxicities of other 10-*O*-glycosyl anthracyclines, yellamycin C (10-*O*-rhodosaminyl-α-CMN) and

Table 4. Inhibitory activity of A262-6, obelmycin H (OBM-H), and related compounds on growth of cultured L1210 leukemia cells.

Compound	Structure		IC50
	Aglycone	Sugar[a]	(μ g/ml)
A262-6	β-RMN	RN (10)	0.27
OBM-H	β-isoRMN	RN (10)	0.33
β-RM-I[b]	β-RMN	RN (7)	0.01
OBM-A[b]	β-isoRMN	RN (7)	0.001

Cultured L1210 leukemia cells (5×10^4 cells/ml) were exposed for 48 hours to the drugs and viable cells were counted by coulter counter. IC50 is expressed as a drug concentration required to inhibit by 50% control growth of cultured L1210 cells.

[a] RN: Rhodosamine; values in parenthses indicate a sugar linkage site.

[b] Data sited from reference 3.

alldimycin C (10-*O*-rhodosaminyl-α_2-RMN), were more than 20 times weaker compared with those of the corresponding 7-*O*-glycosyl analogs, yellamycin A and alldimycin A, respectively.[3,4] These and previous results show the importance of 7-*O*-glycosylation for biological activity.

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