PRODUCTION OF NEW ANTHRACYCLINE ANTIBIOTICS BY MICROBIAL 4-*O*-METHYLATION USING A SPECIFIC DAUNORUBICIN-NEGATIVE MUTANT

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Microbial 4-*O*-methylation using a specific daunorubicin-blocked, nonproducing mutant provided the new anthracycline antibiotics 4-*O*-methylbetaclamycin T, 4-*O*-methylyellamycin A and 4-*O*-methyl-13-hydroxyoxaunomycin, from which 4-*O*-methyloxaunomycin and 4-*O*-methyl-6-deoxyoxaunomycin were then prepared by further photochemical *N*-demethylation. Antitumor activities *in vitro* and *in vivo* against L1210 cells were compared with those of their 4-*O*-demethyl derivatives. It was found that all the 4-*O*-methyl derivatives had a markedly reduced cytotoxicity *in vitro* as compared with the 4-*O*-demethyl compounds. However, some of them were endowed with a significantly improved antitumor activity *in vivo*.

In the continuing search for new antitumor anthracycline antibiotics from biosynthetically blocked mutants and by means of microbial conversion using antibiotic-nonproducing mutants, we have recently obtained the unique anthracycline antibiotics, oxaunomycin (7-*O*-daunosaminyl-β-rhodomycinone)[1], 1-hydroxyoxaunomycin (7-*O*-daunosaminyl-β-isorhodomycinone)[2] and 6-deoxyoxaunomycin (7-*O*-daunosaminyl-α-citromycinone)[2]. These compounds had good antitumor activity against some murine tumors (unpublished data). However, they were too cytotoxic to be useful in therapy. We have observed in some instances that 4-*O*-methylation reduces the cytotoxic activity of anthracycline antibiotics without a decrease in any antitumor effect. Therefore, we attempted to obtain 4-*O*-methyl derivatives of the above compounds hoping to improve their antitumor effects by reducing their cytotoxicity.

In this paper, we describe the production of new 4-*O*-methyl derivatives[3] of the rhodomycin-group of antibiotics (oxaunomycin, betaclamycin T[4] and yellamycin A[5]) by selective 4-*O*-methylation using a specific daunorubicin-blocked, nonproducing mutant and by a subsequent photochemical *N*-demethylation. We thus obtained new 4-*O*-methyl anthracyclines as summarized in Table 1. Their antitumor activities *in vitro* and *in vivo* were also tested and evaluated in terms of the 4-*O*-methyl group.

Results

Microbial 4-*O*-Methylation Test

Microbial strain DKN-1 used for selective 4-*O*-methylation was a pigment-less nonproducing but

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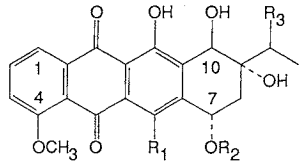
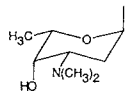
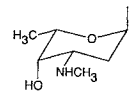
Table 1. Structures of MC-1 ~ MC-7.

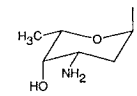


Compound	R_1	R_2	R_3
MC-1	OH	RN	H
MC-2	OH	mDN	H
MC-3	OH	DN	H
MC-4	H	RN	H
MC-5	H	mDN	H
MC-6	H	DN	H
MC-7	OH	DN	OH

RN: Rhodosamine mDN: *N*-Monomethyl-daunosamine DN: Daunosamine







biotransformable isolate of D788-5[6] (4-*O*-methyl-10-methoxycarbonyl-13-deoxocarminomycin)-accumulating mutant strain 58NR-58, which had been derived from daunorubicin-producing *Streptomyces* sp. D788, strain KL-58[7]. Strain 58NR-58 was damaged at the deesterification step of a methoxycarbonyl group of a presumptive precursor metabolite D788-6[8] (10-methoxycarbonyl-13-deoxocarminomycin), so that daunorubicin production was stopped before a metabolite D788-1[9] (10-carboxy-13-deoxocarminomycin). Preliminary bioconversion test with strain DKN-1 was undertaken on some aimed monoglycosidic anthracyclines: D788-6 (7-*O*-daunosaminyl-ε-rhodomycinone (RMN)), oxaunomycin, betaclamycin T (7-*O*-rhodosaminyl-β-RMN) and yellamycin A (7-*O*-rhodosaminyl-α-citromycinone (CTN)). Tests were performed by adding the substrate anthracyclines to the growing culture, after which the bioconversion products were extracted with an organic solvent and analyzed by TLC and HPLC. In all cases, the bioconversion products were homogeneous when assayed by TLC.

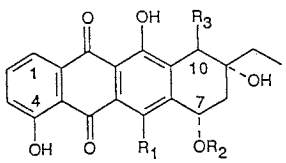
Our first concern was whether the bioconversion with strain DKN-1 was limited to 4-*O*-methylation. The selective 4-*O*-methylation was thus confirmed by the bioconversion of D788-6 to D788-5 as shown in Table 2. It was found that Rf values of the 4-*O*-methylated and unmethylated compounds were almost identical (0.28 for D788-5 and 0.27 for D788-6) although their retention times (Rts) in HPLC were greatly different (6.9 minutes for D788-5 and 9.6 minutes for D788-6; Rt ratio: 0.7 (6.9/9.6)). These chromatographic properties, unvaried Rf values in TLC and an Rt shift by a constant rate of about 0.7 in HPLC, would serve as evidence of a selective 4-*O*-methylation of the substrate without any modification. Table 2 summarized the results when four compounds including D788-6 were converted with strain DKN-1. 4-*O*-Methyl derivatives of betaclamycin T and yellamycin A as well as D788-6 were produced by this bioconversion. However, selective 4-*O*-methylation of oxaunomycin did not occur, based on chromatographic behavior.

Bioconversion products of betaclamycin T, yellamycin A and oxaunomycin were named MC-1, MC-4 and MC-7, respectively, and structurally identified below.

Table 2. Bioconversion of anthracycline glycosides by strain DKN-1.

Substrate	Structure[a]			TLC[c] Rf value	HPLC		Product	TLC Rf value	HPLC		Conversion rate[f] (%, mol/mol)
	R_1	R_2	R_3		mp[d]	Retention time (minutes)			Retention time (minutes)	Rt_p/Rt_s[e]	
D788-6	OH	DN[b]	$COOCH_3$	0.27	A	9.6	D788-5	0.28	6.9	0.72	21.6 (100)
Oxaunomycin	OH	DN	OH	0.18	B	7.4	MC-7	0.10	3.7	0.53	10.0 (100)
Betaclamycin T	OH	RN[b]	OH	0.23	B	8.7	MC-1	0.25	6.1	0.70	16.6 (74)
Yellamycin A	H	RN	OH	0.17	B	7.7	MC-4	0.19	5.4	0.70	14.9 (79)

[a]



[b] DN: Daunosamine, RN: rhodosamine.
[c] $CHCl_3$ - MeOH - H_2O - AcOH - concd NH_4OH (150 : 50 : 5 : 1 : 1).
[d] mp: Mobile phase (described in the text).
[e] Ratio of retention times (retention $time_{product}$/retention $time_{substrate}$).
[f] Values in parentheses indicate a ratio of the main product to total products detected by HPLC. Yellamycin A and its products were detected at 436 nm and others at 495 nm.

Isolation and Identification of MC-1, MC-4 and MC-7

To determine the structures of MC-1, MC-4 and MC-7 a large scale microbial conversion of betaclamycin T (425 mg), yellamycin A (102 mg) or oxaunomycin (425 mg) was carried out with the growing culture of the mutant strain DKN-1. The fermentation broth was filtered and the anthracycline products were extracted from the mycelial cake with 0.7 volume of acetone. The acetone extract was concentrated *in vacuo* to about 0.25 volume. To the concentrate, 2 volumes of H_2O were added, adjusted to pH 2.0 with phosphoric acid and charged onto a column of Diaion HP-20 (42 mm i.d. × 300 mm, Mitsubishi Chemical Ind.) packed in acidic water (pH 2.0). After washing with 2 liters of dil phosphoric acid (pH 2.0) and 1 liter of 40% aq MeOH (pH 2.0), the products were eluted with 80% aq MeOH (pH 2.0). Main fractions containing the objective compound were collected, diluted with 0.3 volume of water and adjusted to pH 8.0 with 4 N NaOH. The anthracycline pigments were then extracted with $CHCl_3$ and the extract was evaporated *in vacuo* to dryness. Yields of crude MC-1, MC-4 and MC-7 were 265 mg, 72 mg and 121 mg, respectively.

The crude MC-1 was purified by column chromatography on reverse phase silica gel (150 ml, YMC gel 60 Å) which was developed stepwise with acetonitrile - water (15 : 85 ~ 20 : 80, pH 2.0, H_3PO_4). The fractions containing MC-1 were adjusted to pH 8.0 with 1 N NaOH, and the pigment was extracted with $CHCl_3$ and reextracted with 1% AcOH. After washing with toluene the aqueous solution was adjusted to pH 8.0 with 1 N NaOH and extracted with $CHCl_3$. After washing with H_2O and drying over Na_2SO_4, the $CHCl_3$ layer was concentrated *in vacuo* to a small volume, and an excess of *n*-hexane was added to precipitate 173 mg of pure MC-1 as an orange powder.

The crude MC-4 was purified by preparative HPLC (column: CAPCELL PAC C18 SG120, 5 μm, 30 mm i.d. × 250 mm, Shiseido, mobile phase: acetonitrile - water (25 : 75, adjusted to pH 2.0 with

Table 3. Physico-chemical properties of MC-1, MC-4 and MC-7.

	MC-1	MC-4	MC-7
Molecular formula	$C_{29}H_{35}NO_{10}$	$C_{29}H_{35}NO_9$	$C_{27}H_{31}NO_{11}$
MS (*m/z*)	557 $(M)^+$ (EI-MS)	542 $(M+H)^+$ (FAB-MS)	545 $(M)^+$ (FD-MS)
Melting point	182~186°C (dec)	128~131°C (dec)	187~189°C (dec)
$[\alpha]_D^{23}$ (*c* 0.02, $CHCl_3$)	+317°	+128°	+162°
UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1cm}^{1\%}$)	205 (408), 234 (792), 251 (444), 288 (168), 481 (249), 497 (245), 530 (sh, 130)	229 (688), 258 (440), 413 (198)	204 (334), 234 (703), 252 (369), 289 (141), 481 (210), 497 (205), 531 (sh, 106)
IR (KBr) cm^{-1}	3400, 2920, 1610, 1580	3400, 2910, 1660, 1625, 1580	3400, 2930, 1610, 1580

H_3PO_4), flow rate: 5 ml/minute, detection: UV at 254 nm). From the fractions containing MC-4, 42 mg of pure MC-4 was obtained as a yellow powder after acidic extraction and hexane precipitation as described above.

The crude MC-7 was purified by preparative TLC (Silica gel 60 PF_{254}, E. Merck) which was developed with $CHCl_3$-MeOH-H_2O-AcOH-concd NH_4OH (110:50:5:1:1). The MC-7 bands were scraped from the TLC plates and MC-7 was extracted with $CHCl_3$-MeOH (5:1). To the extract, the same volume of H_2O was added, and the aqueous layer was separated and washed with $CHCl_3$ and toluene. The pigment was extracted with $CHCl_3$ after the pH was raised to 8.0. The organic layer was concentrated *in vacuo* to a small volume and an excess of *n*-hexane was added to precipitate 11 mg of pure MC-7 as an orange powder.

The structural determination of MC-1, MC-4 and MC-7 was carried out by MS, ^1H and ^{13}C NMR analyses and TLC analysis of the sugar components obtained by acid hydrolysis.

The physico-chemical properties of MC-1, MC-4 and MC-7 are shown in Table 3. The UV and visible light absorption spectra of MC-1 and MC-7 were comparatively similar to those of their parent compounds, while those of MC-4 were obviously different. MC-4 exhibited a maximum absorption at 413 nm which shifted by 12 nm in comparison with that (425 nm) of yellamycin A[5]. This conspicuous shift seemed to be a result of 4-*O*-methylation as seen with 11-deoxydoxorubicin and its related compounds[10], and their 4-*O*-demethyl derivatives[11]. The IR absorptions related to the quinone carbonyl groups of MC-1, MC-4 and MC-7 were different from those of their parent compounds. Two maximum absorptions at 1580 cm^{-1} and 1610 cm^{-1} related to hydrogen bonded quinone carbonyl groups were observed with MC-1 and MC-7 although only one maximum absorption at 1600 cm^{-1} with betaclamycin T and oxaunomycin. One additional maximum absorption at 1660 cm^{-1} observed in MC-4 indicated a presence of a nonhydrogen bonded quinone carbonyl group. These results also suggested a modification of the hydroxyl group at C-4.

On total acid hydrolysis, MC-1, MC-4 and MC-7 were hydrolyzed as previously described[12] and gave an orange aglycone, a yellow aglycone and an orange aglycone, respectively. The Rf values of the aglycones on TLC using $CHCl_3$-MeOH (20:1) were 0.33, 0.19 and 0.11, respectively, and were different from those (0.35, 0.26 and 0.35, respectively) of their parental aglycones, β-rhodomycinone, α-citromycinone and β-rhodomycinone. The Rts of the aglycones obtained by HPLC analysis (system C) were 5.61, 4.75 and 3.76 minutes, respectively, and were also different from those (8.42, 7.65 and 8.42,

Table 4. ^{1}H NMR chemical shifts of MC-1, MC-4 and MC-7.

Proton	MC-1	MC-4	MC-7
1-H	7.99 dd (7.7, 1.1)[a]	7.96 d (7.3)	7.98 d (8.1)
2-H	7.78 t (8.1)	7.75 t (8.1)	7.78 t (8.1)
3-H	7.39 d (7.7)	7.38 d (8.1)	7.39 d (8.8)
4-OCH_3	4.09 s	4.06 s	4.07 s
6-H	—	7.71 s	—
7-H	5.15 br d (2.2)	4.91 br t (2.9)	5.20 br d (2.2)
8-Ha	2.24 d (15.0)	2.15~2.3	2.54 d (15.4)
8-Hb	2.13 dd (15.0, 4.0)		2.19 dd (15.4, 3.7)
9-OH	4.04 br s	—	—
10-H	4.89 s	4.95 s	4.94 s
13-Ha	1.85 q (7.3)	1.89 q (7.3)	—
13-Hb	1.75~1.8	1.72 q (7.3)	—
13-H	—	—	4.01 q (6.6)
14-CH_3	1.12 t (7.3)	1.12 t (7.3)	1.38 d (6.6)
6-OH	13.82 br s	—	—
11-OH	13.37 br	—	—
1'-H	5.52 br s	5.35 br s	5.49 br d (2.9)
2'-Ha	1.75~1.8	1.76~1.82	1.85 ddd (13.2, 3.7)
2'-Hb			1.73 dd (13.2, 4.4)
3'-H	2.2	2.3	3.09 br d (11.0)
3'-$N(CH_3)_2$	2.20 s	2.21 s	—
4'-H	3.70 br s	3.70 br s	3.56 br s
5'-H	4.1	4.03 q (6.6)	4.11 q (6.6)
6'-CH_3	1.40 d (6.6)	1.40 d (6.6)	1.32 d (6.6)

[a] Coupling constants are indicated in parenthesis.
Solvent: MC-1 and MC-4; $CDCl_3$, MC-7; $CDCl_3$-CD_3OD (10:1).

respectively) of their parental aglycones. The sugar components were analyzed by direct comparison with authentic samples derived from aclarubicin, MA144 L1[13] and daunorubicin on TLC. The Rf values (0.14) of the sugars of MC-1 and MC-4 were the same as that of L-rhodosamine, and that (0.35) of the sugar of MC-7 was the same as that of L-daunosamine.

The chemical shift assignments of ^{1}H and ^{13}C NMR spectra of MC-1, MC-4 and MC-7 were carried out by means of DEPT, ^{1}H-^{1}H and ^{1}H-^{13}C COSY, and the correlation *via* long range coupling (COLOC) and ^{1}H detected heteronuclear multiple-bond connectivity (HMBC) experiments and are shown in Tables 4 and 5, respectively.

In ^{1}H NMR spectra of MC-1 and MC-4, the chemical shifts were almost identical to those of their parent compounds, betaclamycin T and yellamycin A, respectively, except for one additional singlet (δ 4.1) which indicated a presence of a methoxyl group. The long range connectivity between the proton at δ 4.1 and C-4 in MC-1 and MC-4 revealed that the methoxyl group is linked to the C-4 position as shown in Fig. 1. Similarly HMBC analysis of MC-7 revealed that the hydroxyl group at C-4 of oxaunomycin was methylated, while the signals for the side chain at C-9 of MC-7 was entirely different from those of oxaunomycin. Correlated signals were observed between δ 4.01 (quartet, 13-H) and δ 1.38 (doublet, 14-CH_3) and the molecular ion appeared at 30 mass units upper than the corresponding ion of oxaunomycin, indicating that C-13 of MC-7 was oxidized to possess a hydroxyl group substituent.

From all these findings, the structures of MC-1, MC-4 and MC-7 were determined to 4-*O*-methylbetaclamycin T, 4-*O*-methylyellamycin A and 4-*O*-methyl-13-hydroxyoxaunomycin, respectively. The ^{13}C NMR spectra of each also supported these structures.

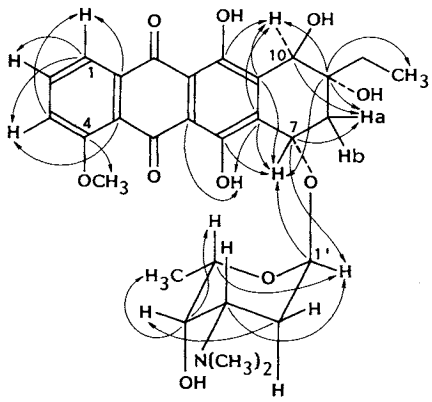
Table 5. ^{13}C NMR chemical shifts of MC-1, MC-4 and MC-7.

Carbon	MC-1	MC-4	MC-7	Carbon	MC-1	MC-4	MC-7
1	119.81	119.54	119.65	11	156.20	161.44	155.97*
2	135.84	135.27	135.81	11a	112.00	114.84	111.97*
3	118.51	118.72	118.50	12	186.72	188.34	186.60
4	161.12	160.63	160.98	12a	135.84	135.19	134.96
4a	120.83	121.37	120.60	13	30.41	30.22	70.73
5	187.14	181.28	187.02	14	6.60	6.61	16.38
5a	112.45	133.78	112.48*	4-OMe	56.69	56.57	56.50
6	156.64	120.29	155.60*	1′	101.37	99.39	100.61
6a	135.42	143.42	135.25	2′	28.60	28.92	31.37
7	71.03	74.42	70.03	3′	59.66	59.46	46.46
8	32.96	33.84	28.68	4′	65.97	65.90	68.77
9	71.96	72.26	72.78	5′	66.48	66.45	67.45
10	66.57	67.09	66.47	6′	17.01	17.02	16.06
10a	136.56	131.77	135.96	3′-NMe_2	41.95	41.94	—

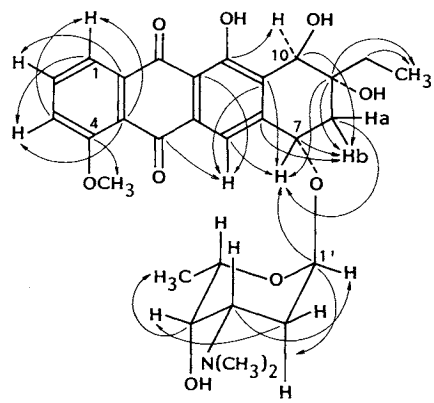
Similar values asterisked may be interchanged.
Solvent: MC-1 and MC-4; $CDCl_3$, MC-7; $CDCl_3$-CD_3OD (10:1).

Fig. 1. COLOC experiments of MC-1 and MC-4.

The arrows indicate ^1H-^{13}C long range couplings.



MC-1



MC-4

N-Demethyl Derivatives of MC-1 and MC-4

Using the photochemical treatment described previously[14], *N*-monodemethyl and *N*-didemethyl derivatives of MC-1 and MC-4 were prepared. The sample was dissolved in $CHCl_3$ and exposed to a light to afford the two derivatives. The products were purified by preparative TLC. *N*-Demethyl derivatives, MC-2 and MC-3 were obtained from MC-1, and similarly MC-5 and MC-6 from MC-4. Their structures were identified by TLC analysis after acid hydrolysis. Aglycones of MC-2 and MC-3, and MC-5 and MC-6 were the same as those of MC-1 and MC-4 (4-*O*-methyl-β-RMN and 4-*O*-methyl-α-CTN). With respect to the sugar residue, their Rf values were compared with the authentic samples described above, and *N*-monodemethyl rhodosamine (Rf value: 0.26) was detected in MC-2 and MC-5, and *N*-didemethylrhodosamine (daunosamine) (Rf value: 0.35) in MC-3 and MC-6.

These results indicated that MC-2, MC-3, MC-5 and MC-6 were *N*-monodemethyl-MC-1,

Table 6. Inhibitory activities of MC-1 ~ MC-7 and their 4-*O*-demethyl compounds on growth and nucleic acid synthesis of cultured L1210 leukemia cells.

Compound	IC_{50} (μg/ml)			DNA/RNA
	Growth	DNA synthesis	RNA synthesis	
MC-1	0.033	0.80	0.20	4.0
MC-2	0.14	2.10	0.75	2.8
MC-3	0.036	0.75	2.30	0.3
MC-4	1.05	>5	>5	—
MC-5	5.13	>5	>5	—
MC-6	1.41	>5	>5	—
MC-7	0.25	>5	>5	—
BCM-T (4-*O*-Demethyl-MC-1)	0.01	0.21	0.06	3.5
LB-1 (4-*O*-Demethyl-MC-2)	0.006	0.38	0.26	1.5
OXM (4-*O*-Demethyl-MC-3)	0.0003	0.29	0.68	0.4
YLM-A (4-*O*-Demethyl-MC-4)	0.007	0.28	0.23	1.2
LCS-1 (4-*O*-Demethyl-MC-5)	0.26	0.84	0.77	1.1
LCS-2 (4-*O*-Demethyl-MC-6)	0.006	0.55	4.00	0.1

In the inhibition test for nucleic acid synthesis, cultured L1210 leukemia cells (8×10^5 cells/ml) were exposed for 60 minutes to the drugs supplemented with ^{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, cultured L1210 leukemia cells (5×10^4 cells/ml) were exposed for 48 hours to the drugs and the viable cells were counted by coulter counter. IC_{50} is expressed as a drug concentration required to inhibit by a 50% of control growth, and DNA and RNA syntheses of cultured L1210 cells.

Abbreviations: BCM-T, betaclamycin T; OXM, oxaunomycin; YLM-A, yellamycin A.

N-didemethyl-MC-1, *N*-monodemethyl-MC-4 and *N*-didemethyl-MC-4, respectively. Mass and NMR data described in experimental part confirmed their structures.

Antitumor Activity *in vitro* and *in vivo*

The antitumor activities *in vitro* of MC-1 ~ MC-7 against cultured L1210 cells were examined and the results are shown in Table 6 in comparison with those of their 4-*O*-demethyl derivatives. The cytotoxicities of MC-1 ~ MC-6 were obviously reduced. Especially, the 4-*O*-methyl derivatives, MC-4 ~ MC-6, obtained from yellamycin A illustrate this remarkably reduced cytotoxicity. Their IC_{50} values are more than 1.0 μg/ml while that of yellamycin A is 0.007 μg/ml. The cytotoxicities of *N*-didemethyl compounds, MC-3 and MC-6, were almost the same as those of MC-1 and MC-4, respectively, while those of *N*-monodemethyl compounds, MC-2 and MC-5, were reduced as seen with *N*-monodemethyl derivatives of some rhodosamine-containing anthracyclines[14]. The cytotoxicity of MC-7 (13-hydroxy-MC-3) was 6.9 times weaker than that of MC-3. MC-3 inhibited DNA synthesis more strongly than RNA synthesis as seen with oxaunomycin[1].

Antitumor effects *in vivo* of MC-1 ~ MC-3 were tested in mice bearing leukemia L1210 by daily ip administration from day 1 to 10. MC-1 ~ MC-3 had a maximum antitumor activity of 231%, 176% and 183% (T/C) at an optimum dose of 5.0, 1.25 and 0.63 mg/kg/day, respectively. Under the same conditions betaclamycin T, LB-1 and oxaunomycin had a T/C value of 182%, 170% and 204% at an optimum dose of 0.63, 0.06 and 0.20 mg/kg/day, respectively.

Discussion

By microbial 4-*O*-methylation using a specific daunorubicin-blocked, nonproducing mutant and by

further photochemical *N*-demethylation, we could obtain six new 4-*O*-methyl anthracyclines including our targets, 4-*O*-methyloxaunomycin (MC-3) and 4-*O*-methyl-6-deoxyoxaunomycin (MC-6). In spite of much efforts we failed at chemical 4-*O*-methylation of oxaunomycin. However, more recently 4-*O*-methyloxaunomycin was synthesized from β-RMN by KOLAR *et al.*[15]. Microbial 4-*O*-methylation of anthracyclines is a very good technique, although limited by substrate specificity. In the bioconversion test with strain DKN-1 of four monoglycosidic anthracyclines which contained ε-RMN, β-RMN or α-CTN as a constituent aglycone, we discovered that the 4-*O*-methylation occurred in all cases. However, it was found that only oxaunomycin underwent an additional modification, 13-oxidation, to yield 13-hydroxy-4-*O*-methyloxaunomycin (MC-7). Since the 13-oxidation, an important biosynthetic reaction toward daunorubicin, did not take place on betaclamycin T (*N*-dimethyloxaunomycin) and 10-methoxycarbonyl anthracycline (D788-6), it is possible that hydroxylation is affected at least by a sugar residue or a functional group at C-10.

As predicted, it was proved that 4-*O*-methyl derivatives of six anthracyclines all have the diminished cytotoxicity *in vitro* against L1210 cell culture in comparison with the corresponding unmethylated compounds. MC-7 with a hydroxyl group at C-13 was also less active than MC-3 without the hydroxyl group. On the other hand, it was shown that 4-*O*-methyl derivatives, MC-1, MC-2 and MC-3 exhibited a significantly improved antitumor activity *in vivo* as compared with their corresponding unmethylated compounds. MC-1 and MC-2 showed more potent antitumor effects than their unmethylated derivatives, and MC-3 was improved by a broader range of effective dose than 4-*O*-demethyl-MC-3. However, MC-4, MC-5 and MC-6, derived from yellamycin A, had no antitumor effect *in vivo* because of drastic decrease in the cytotoxicity.

Experimental

General

MP's were determined on a Kofler hot stage microscope. UV spectra were determined on a Hitachi EPS 3T and IR spectra (KBr pellet) on a Hitachi EPI-GS spectrophotometer. ^1H and ^{13}C NMR spectra were recorded with a JEOL JNM-GSX400 spectrometer at 400 MHz and 100 MHz, respectively. Chemical shifts were expressed by δ values (ppm) from internal TMS and coupling constants were given in J (Hz). Mass spectra were recorded with a Hitachi M-80H spectrometer. Specific rotations were determined on a JASCO DIP-181 Digital Polarimeter. TLC was performed on Silica gel 60 F_{254} (E. Merck).

Microorganism

The D788-5-negative mutant strain DKN-1 was obtained from *Streptomyces* sp. D788 as previously described[7], cultivated at 28°C on YS agar slant (yeast extract 0.3%, soluble starch 1.0% and agar 1.5%, pH 7.2) and then stored at 5°C. This strain was deposited as FERM P-10643 in Fermentation Research Institute, Agency of Industrial Science and Technology.

Microbial Conversion

Seed culture was grown aerobically at 28°C for 72 hours in 500-ml Erlenmeyer flasks 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, and was used as inoculum (2% v/v) for conversion medium. Conversion medium contained soluble starch 50 g, maltose 30 g, dry yeast 20 g, cotton seed meal 20 g, fish meal 10 g, yeast extract 2 g, NaCl 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, $CaCO_3$ 2 g, $CuSO_4 \cdot 5H_2O$ 0.003 g, $FeSO_4 \cdot 7H_2O$ 0.0005 g, $ZnSO_4 \cdot 7H_2O$ 0.0008 g, $MnCl_2 \cdot 4H_2O$ 0.003 g in 1,000 ml of tap water, pH 7.0.

Preliminary microbial conversion of some anthracycline glycosides was examined with 250-ml Erlenmeyer flasks each containing 30 ml of conversion medium. The cultivation was carried out at 28°C on a rotary shaker (210 rpm). After 72 hours, a methanolic solution of a substrate (2 mg/ml) was added to each flask to give a final concentration of 30 μg/ml and the cultivation was further continued for 48 hours to accomplish the conversion. To 2 ml of the culture broth was added 2 ml of acetone, 0.1 ml of 1 M Tris-HCl buffer (pH 8.5) and the products were extracted with 2 ml of $CHCl_3$. The extract was evaporated to dryness and analyzed by TLC and HPLC (system A and B: described below).

A large scale microbial conversion was performed with 500-ml Erlenmeyer flask containing 50 ml of

conversion medium and 0.5 ml of a methanolic solution of a substrate (5 mg/ml) was added to each flask.

HPLC Analysis

Conversion rate and purity of the products, and the aglycones generated by acid hydrolysis were analyzed by HPLC using a Shimadzu HPLC system consisting of LC-6A pump, SPD-6AV detector and Chromatopak C-R3A integrator with a reverse-phase column, YMC-A312 (ODS) (Yamamura Chemical Laboratories Co., LTD.). Acetonitrile - 0.05 M ammonium formate (pH 4.0) (system A: 38 : 62, system B: 30 : 70) and acetonitrile - water (45 : 55; adjusted to pH 2.0 with H_3PO_4) (system C) were used as the mobile phase and run at a flow rate of 1.0 ml/minute. Samples were dissolved in the mobile phase and 10 μl samples were injected. UV absorbance was monitored at 254 nm and visible light absorbance at 436 nm or 495 nm.

Biological Activity

In vitro cytotoxicity and inhibition of DNA and RNA syntheses in cells of murine L1210 leukemia were assayed according to the method previously described[16].

4-*O*-Methyl-β-rhodomycinone

MC-1 (20 mg) was dissolved in 20 ml of 0.1 N HCl and heated at 85°C for 30 minutes in a water bath. The aglycone was extracted with $CHCl_3$ and then purified by preparative TLC on silica gel plates (PF_{254}, E. Merck) using a developing solvent of $CHCl_3$ - MeOH (20 : 1). One major band was scraped from the plates and extracted with $CHCl_3$ - MeOH (10 : 1). The eluate was washed with H_2O, dried over Na_2SO_4, concentrated to a small volume and precipitated with an excess of *n*-hexane to give an orange powder (11 mg); mp 208~210°C (dec); $[\alpha]_D^{23}$ +37° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2900, 1615, 1580; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 205 (381), 233 (774), 251 (449), 288 (167), 481 (237), 496 (241); FAB-MS *m/z* 401 ($(M+H)^+$, $C_{21}H_{20}O_8$); ^{13}C NMR ($CDCl_3$ - CD_3OD, 20 : 1) δ 187.36 (C-5), 186.87 (C-12), 161.19 (C-4), 156.14 (C-11), 155.54 (C-6), 137.07 (C-10a), 135.99 (C-2 and C-12a), 135.59 (C-6a), 120.88 (C-4a), 119.90 (C-1), 118.63 (C-3), 112.75 (C-5a), 112.01 (C-11a), 72.35 (C-9), 66.11 (C-10), 62.42 (C-7), 56.75 (OCH_3), 33.61 (C-8), 30.49 (C-13), 6.47 (C-14).

4-*O*-Methyl-α-citromycinone

MC-4 (20 mg) was dissolved in 20 ml of 0.1 N HCl and heated at 85°C for 30 minutes in a water bath and the aglycone was purified in the same manner as 4-*O*-methyl-β-rhodomycinone to give a yellow powder (8 mg); mp 203~205°C (dec); $[\alpha]_D^{23}$ +46° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2910, 1660, 1625, 1580; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 228 (607), 257 (371), 413 (165); FAB-MS *m/z* 385 ($(M+H)^+$, $C_{21}H_{20}O_7$); ^{13}C NMR ($CDCl_3$ - CD_3OD, 20 : 1) δ 188.23 (C-12), 181.55 (C-5), 161.19 (C-11), 160.58 (C-4), 146.60 (C-6a), 135.27 (C-2), 135.23 (C-12a), 133.92 (C-5a), 130.81 (C-10a), 121.35 (C-4a), 120.99 (C-6), 119.49 (C-1), 118.65 (C-3), 114.40 (C-11a), 73.24 (C-9), 67.29 (C-10), 66.07 (C-7), 56.48 (OCH_3), 34.36 (C-8), 30.55 (C-13), 6.22 (C-14).

4-*O*-Methyl-13-hydroxy-β-rhodomycinone

MC-7 (8 mg) was dissolved in 8 ml of 0.1 N HCl and heated at 85°C for 3 hours in a water bath. The aglycone was extracted with $CHCl_3$ and then purified by preparative TLC on a silica gel plate (PF_{254}, E. Merck) using a developing solvent of $CHCl_3$ - MeOH (10 : 1). One major band was scraped from the plates and extracted with $CHCl_3$ - MeOH (5 : 1). The eluate was washed with H_2O, dried over Na_2SO_4, evaporated and crystallized from $CHCl_3$ - MeOH (5 : 1) to give an orange crystalline powder (3 mg); mp 245~248°C (dec); $[\alpha]_D^{23}$ −42° (*c* 0.005, $CHCl_3$); IR (KBr) cm^{-1} 3370, 2905, 1610, 1580; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 233 (1304), 251 (706), 287 (261), 480 (386), 496 (385); FAB-MS *m/z* 439 ($(M+Na)^+$, $C_{21}H_{20}O_9$); ^{13}C NMR ($CDCl_3$ - CD_3OD, 10 : 1) δ 187.08 (C-5), 186.55 (C-12), 160.93 (C-4), 155.76[a] (C-11), 155.46[a] (C-6), 135.79 (C-2 and C-10a), 135.42 (C-6a), 135.25 (C-12a), 120.53 (C-4a), 119.58 (C-1), 118.45 (C-3), 112.50[b] (C-5a), 111.73[b] (C-11a), 73.15 (C-9), 70.88 (C-13), 66.03 (C-10), 61.72 (C-7), 56.39 (OCH_3), 29.46 (C-8), 15.84 (C-14). (Assignments marked a and b may be interchanged.)

7-*O*-(*N*-Monomethyldaunosaminyl)-β-rhodomycinone (MC-2) and 7-*O*-Daunosaminyl-β-rhodomycinone (MC-3)

N-Demethylated compounds of MC-1 (MC-2 and MC-3) were photochemically obtained as described below. MC-1 (108 mg) was dissolved in 108 ml of $CHCl_3$ and exposed to a mercury lamp (UVL-400HA, Riko Science Industry; distance: 10 cm) at 24°C for 7 hours, when production of MC-2 and MC-3 reached to a maximum (assayed by silica gel TLC ($CHCl_3$-MeOH-H_2O-AcOH, 30:10:0.4:0.2)). The products were purified by preparative TLC (Silica gel 60 PF_{254}, E. Merck) using $CHCl_3$-MeOH-H_2O-AcOH (25:10:0.4:0.2) as a solvent. The MC-2 band (Rf: 0.35) and MC-3 band (Rf: 0.20) were scraped from the plates and extracted with $CHCl_3$-MeOH (5:1), respectively. Each of them was further purified by the repeated preparative TLC. Each compound extracted was evaporated, dissolved in 1% AcOH and washed with toluene. After raising the pH to 8.0, the pigment was extracted with $CHCl_3$. The extract was washed with satd NaCl solution, dried over Na_2SO_4, and concentrated to a small volume. To the concentrate, an excess of *n*-hexane was added to precipitate an orange powder. This procedure gave 21.2 mg of MC-2 and 27.1 mg of MC-3, respectively. MC-2: mp 150~153°C (dec); $[\alpha]_D^{23}$ +247° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2920, 1610, 1580; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 205 (385), 233 (777), 251 (437), 288 (163), 480 (244), 496 (242), 529 (sh, 128); EI-MS *m/z* 543 (M^+, $C_{28}H_{33}NO_{10}$); ^1H NMR ($CDCl_3$-CD_3OD, 20:1) δ 2.70 (1H, m, 3′-H), 2.33 (3H, s, 3′-$NHCH_3$); ^{13}C NMR ($CDCl_3$-CD_3OD, 20:1) δ 187.19 (C-5), 186.78 (C-12), 161.05 (C-4), 155.90 (C-11 and C-6), 136.50 (C-10a), 135.82 (C-2), 135.41 (C-12a), 135.06 (C-6a), 120.81 (C-4a), 119.74 (C-1), 118.50 (C-3), 112.50 (C-5a), 112.05 (C-11a), 101.27 (C-1′), 71.95 (C-9), 70.96 (C-7), 66.91 (C-5′), 66.66 (C-4′), 66.18 (C-10), 56.60 (OCH_3), 54.06 (C-3′), 32.65 (C-8), 32.09 (3′-$NHCH_3$), 30.20 (C-13), 29.75 (C-2′), 16.77 (C-6′), 6.42 (C-14). MC-3: mp 162~165°C (dec): $[\alpha]_D^{23}$ +205° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2920, 1610, 1580; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 205 (350), 233 (721), 251 (401), 288 (151), 481 (225), 496 (223), 530 (sh, 119); EI-MS *m/z* 529 (M^+, $C_{27}H_{31}NO_{10}$); ^1H NMR ($CDCl_3$-CD_3OD, 10:1) δ 2.95 (1H, m, 3′-H); ^{13}C NMR ($CDCl_3$-CD_3OD, 10:1) δ 187.11 (C-5), 186.64 (C-12), 160.97 (C-4), 156.04 (C-6), 155.95 (C-11), 136.52 (C-10a), 135.77 (C-2), 135.32 (C-12a), 135.00 (C-6a), 120.67 (C-4a), 119.65 (C-1), 118.45 (C-3), 112.40 (C-5a), 111.91 (C-11a), 101.26 (C-1′), 72.04 (C-9), 70.78 (C-7), 70.09 (C-4′), 67.48 (C-5′), 65.89 (C-10), 56.48 (OCH_3), 46.17 (C-3′), 32.39 (C-8), 32.81 (C-2′), 30.02 (C-13), 16.53 (C-6′), 6.25 (C-14).

7-*O*-(*N*-Monomethyldaunosaminyl)-α-citromycinone (MC-5) and 7-*O*-Daunosaminyl-α-citromycinone (MC-6)

N-Demethylated compounds of MC-4 (MC-5 and MC-6) were also obtained photochemically. MC-4 (30 mg) was dissolved in 30 ml of $CHCl_3$ and exposed to the light for 7 hours. The reaction mixture was evaporated and purified by preparative HPLC (mobile phase: acetonitrile-water, 20:80~25:75, adjusted to pH 2.0 with H_3PO_4; other conditions were the same as those used for MC-4). Fractions of MC-5 and MC-6 were pooled separately and extracted with $CHCl_3$ after raising the pH to 8.0. The extract was washed with satd NaCl solution, dried over Na_2SO_4, and concentrated to a small volume. To the concentrate, an excess of *n*-hexane was added to precipitate a yellow powder. MC-5 and MC-6 were obtained with yields of 10.4 mg and 5.7 mg, respectively. MC-5: mp 123~126°C (dec); $[\alpha]_D^{23}$ +96° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2920, 1660, 1630, 1580; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 229 (676), 257 (430), 413 (194); FAB-MS *m/z* 528 ($(M+H)^+$, $C_{28}H_{33}NO_9$); ^1H NMR ($CDCl_3$-CD_3OD, 20:1) δ 2.83 (1H, m, 3′-H), 2.38 (3H, s, 3′-$NHCH_3$); ^{13}C NMR ($CDCl_3$-CD_3OD, 20:1) δ 188.46 (C-12), 181.34 (C-5), 161.47 (C-11), 160.70 (C-4), 143.23 (C-6a), 135.29 (C-2 and C-12a), 133.86 (C-5a), 131.87 (C-10a), 121.51 (C-4a), 120.37 (C-6), 119.59 (C-1), 118.79 (C-3), 114.96 (C-11a), 98.95 (C-1′), 74.18 (C-7), 72.25 (C-9), 67.29 (C-5′), 66.62 (C-10), 66.22 (C-4′), 56.60 (OCH_3), 54.14 (C-3′), 33.94 (C-8), 32.50 (3′-$NHCH_3$), 30.23 (C-13), 29.69 (C-2′), 17.03 (C-6′), 6.62 (C-14). MC-6: mp 143~146°C (dec); $[\alpha]_D^{23}$ +131° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2910, 1660, 1630, 1580; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 229 (703), 258 (447), 413 (201); FAB-MS *m/z* 514 ($(M+H)^+$, $C_{27}H_{31}NO_9$); ^1H NMR ($CDCl_3$) δ 3.19 (1H, m, 3′-H); ^{13}C NMR ($CDCl_3$) δ 188.49 (C-12), 181.40 (C-5), 161.50 (C-11), 160.72 (C-4), 143.09 (C-6a), 135.31 (C-2 and C-12a), 133.85 (C-5a), 131.95 (C-10a), 121.52 (C-4a), 120.41 (C-6), 119.62 (C-1), 118.80 (C-3), 115.01 (C-11a), 98.74 (C-1′), 73.83 (C-7), 72.22 (C-9), 70.63 (C-4′), 67.32 (C-5′), 67.03 (C-10), 56.62 (OCH_3), 46.10 (C-3′), 33.92 (C-8), 32.63 (C-2′), 30.27 (C-13), 16.97 (C-6′), 6.62 (C-14).

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