MICROBIAL GLYCOSIDATION OF SOME ANTHRACYCLINE ANTIBIOTICS BY AN ANTIBIOTIC-NEGATIVE MUTANT OF ACLARUBICIN PRODUCER

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Microbial conversion of anthracyclinone monosaccharides using aclarubicin-negative mutant of *Streptomyces galilaeus* was found to produce anthracyclinone disaccharides which had either rhodinose or 2-deoxyfucose as an additional sugar. By this conversion we obtained twelve new anthracyclines from seven anthracyclines which had rhodosamine, *N*-monomethyldaunosamine or daunosamine at C-7 as a glycosidic sugar. All products had a reduced cytotoxic activity in comparison with those of parent compounds. However, some of them showed a therapeutically improved antitumor effects against L1210 leukemia *in vivo*.

In continuing search for microbial origin of new anthracycline antibiotics, we have prepared lots of new hybrid anthracycline antibiotics by microbial glycosidation using antibiotic-negative mutant of anthracycline antibiotic baumycin-, aclarubicin- or daunorubicin-producing microorganisms[1~8], among which there were found some compounds with potent antitumor effect such as betaclamycin A (CG7)[3], 2-hydroxyaclacinomycin A[4], and trisarubicinol[5]. In these glycosidations we have used anthracyclinone aglycones, but not anthracycline glycosides, as substrate compounds. Further glycosidation of monosugar anthracyclines could be thus a useful mean for preparation of new anthracycline derivatives with improved antitumor activity. There have been few reports concerning derivation of anthracyclines by microbial conversion. Recently we reported 4-*O*-methylation of some monosugar anthracyclines by specific daunorubicin-negative mutant[9].

In this paper we tried microbial conversion of some monosugar anthracycline antibiotics using aclarubicin-negative mutant strain KE303 of *S. galilaeus*. Since the glycosidation of anthracyclinones with this mutant produce anthracyclinone trisaccharides which have the same sugar chain as that of aclarubicin. It was expected that the glycosidation would be expanded to monosugar anthracyclines to yield the corresponding anthracyclinone trisaccharides. However, it was proved that the glycosidation of monosugar anthracyclines resulted in not the corresponding trisaccharides, but new disaccharides 4′-substituted with either rhodinose or 2-deoxyfucose[10,11].

The antitumor activities of new disugar anthracyclines thus obtained were examined and evaluated

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in comparison with those of the parent monosugar anthracyclines.

Results

Bioconversion of Monoglycosidic Anthracyclines with Strain KE303

Strain KE303 was an aclarubicin-negative mutant which kept a glycosidation ability to produce aclarubicin from exogenously added aklavinone aglycone[2]. In preliminary bioconversion test with this strain we found that a pair of products (symbolized by **a** or **b**) occurred from each of the following monoglycosidic anthracyclines: oxaunomycin (7-*O*-daunosaminyl-β-rhodomycinone) (**1**)[12], D788-6 (7-*O*-daunosaminyl-ε-rhodomycinone) (**2**)[13], yellamycin A (7-*O*-rhodosaminyl-α-citromycinone) (**3**)[14], D788-5 (7-*O*-daunosaminyl-4-*O*-methyl-ε-rhodomycinone) (**4**)[15], 13-dihydrocarminomycin (7-*O*-daunosaminyl-13-dihydrocarminomycinone) (**5**)[16], betaclamycin T (7-*O*-rhodosaminyl-β-rhodomycinone) (**6**)[17], or LB-1 (7-*O*-(*N*-monomethyldaunosaminyl)-β-rhodomycinone) (**7**)[18]. All these pairs of products showed higher Rf values on TLC than their parental compounds, suggesting that further glycosidation occurred on the parent compounds. However, we observed that both doxorubicin and daunorubicin were converted into only 13-dihydro derivatives without any significant bioconversion.

Large scale bioconversion was carried out using a 10-liter jar fermentor containing 5 liters of conversion medium. Cultivation was done in the same manner as previously described[2]. Each substrate (**1**～**7**) gave a pair of products (**1a** and **1b**～**7a** and **7b**) on TLC. Chromatographic data of substrate anthracyclines and products are shown in Table 1. Rf values and retention times of the products of **5** or **6** were different from those of corresponding anthracyclinone trisaccharides (trisarubicinol or betaclamycin A), respectively, and that of **6b** was coincident with betaclamycin S (7-*O*-(2-deoxyfucosyl-rhodosaminyl)-β-rhodomycinone)[17]. These facts suggested that the anthracyclinone monosaccharides were converted into anthracyclinone disaccharides.

Isolation

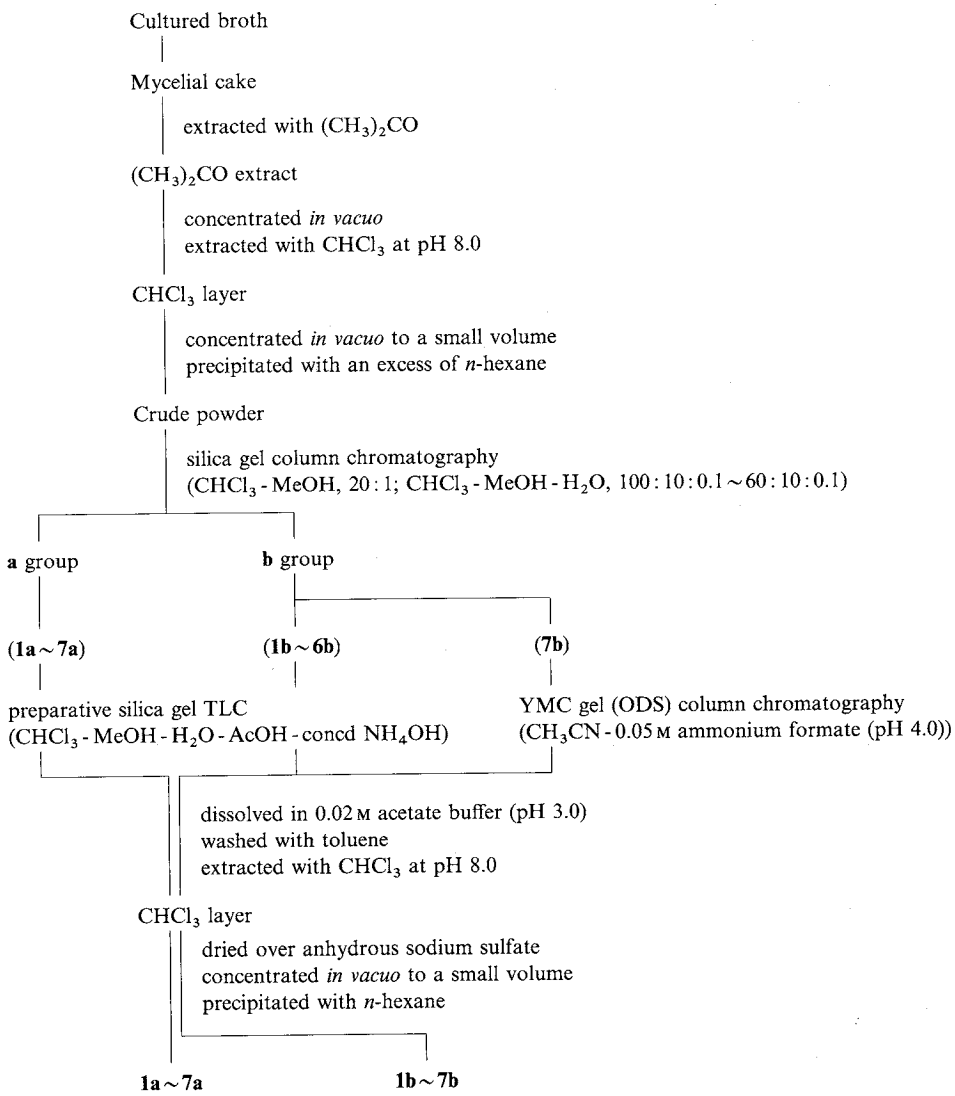
The general procedure for isolation of the products is shown in Fig. 1. The cultured broth (10～15 liters) was centrifuged and the products were extracted with 10 liters of acetone from the mycelial cake. The acetone extract was concentrated *in vacuo* to about 4 liters and washed with a half volume of $CHCl_3$ after the pH was adjusted to 2.0 with phosphoric acid. The products were extracted with 5 liters of $CHCl_3$

Table 1. Chromatographic data of the substrates and products.

Compound	TLC[a] Rf value	HPLC[b] Rt (minutes)	Compound	TLC[a] Rf value	HPLC[b] Rt (minutes)
1	0.21	4.27	**4b**	0.45	13.18
1a	0.46	9.72	**5**	0.15	7.48
1b	0.36	4.39	**5a**	0.36	17.84
2	0.30	19.41	**5b**	0.30	8.05
2a	0.53	59.09	**6**	0.23	4.92
2b	0.43	20.42	**6a**	0.49	10.42
3	0.19	3.95	**6b**	0.43	4.71
3a	0.45	7.41	**7**	0.21	4.61
3b	0.39	3.84	**7a**	0.45	9.99
4	0.32	12.92	**7b**	0.39	4.60
4a	0.54	33.74			

[a] $CHCl_3$-MeOH-H_2O-AcOH-concd NH_4OH (150:50:5:1:1).
[b] Column: YMC A312 (ODS); mobile phase: CH_3CN-H_2O (35:65, pH 2.0 with phosphoric acid); flow rate: 1.0 ml/minute.

Fig. 1. Isolation procedure for the products.



at pH 8.0. The organic layer was concentrated *in vacuo* to a small volume and an excess of *n*-hexane was added to it to precipitate a crude powder of the products. This crude powder was purified by some combinations of normal- or reverse-phase silica gel column chromatography, preparative TLC, or gel filtration column chromatography. The yield of **3a** was too poor to determine its structure.

Structural Elucidation

The structural elucidation of the products was mainly carried out by TLC analysis of aglycone and sugars obtained by acid hydrolysis, MS analysis, and ^1H and ^{13}C NMR analyses. The physico-chemical properties of the products are shown in Table 2. Concerning UV and IR spectra, significant difference was not observed between the products and parent compounds. Each molecular mass of **1a~7a** increased by 98 mass units in comparison with that of the corresponding parent compounds, respectively, and that of **1b~7b** by 114 mass units similarly.

Table 2. Physico-chemical properties of the products.

	1a	1b	2a	2b	3b
Appearance	Reddish brown powder	Reddish brown powder	Reddish orange powder	Reddish orange powder	Yellow powder
Molecular formula	$C_{32}H_{39}NO_{12}$	$C_{32}H_{39}NO_{13}$	$C_{34}H_{41}NO_{13}$	$C_{34}H_{41}NO_{14}$	$C_{34}H_{43}NO_{12}$
FAB-MS (*m/e*)	630 $(M+H)^+$	646 $(M+H)^+$	672 $(M+H)^+$	688 $(M+H)^+$	658 $(M+H)^+$
MP (°C, dec)	209~214	193~196	152~155	197~202	149~152
$[\alpha]_D^{20}$ (*c* 0.01, $CHCl_3$)	+9°	−40°	+400°	+67°	−71°
UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1\,cm}^{1\%}$)	203 (298), 235 (651), 254 (382), 293 (123), 495 (236), 527 (162), 592 (42)	204 (300), 235 (585), 254 (347), 293 (99), 495 (207), 529 (142), 588 (20)	204 (289), 235 (630), 254 (380), 294 (130), 493 (218), 527 (sh, 146)	204 (273), 235 (600), 254 (355), 294 (120), 493 (213), 526 (sh, 137)	204 (317), 231 (565), 258 (372), 290 (sh, 126), 436 (173)
IR *v* max (KBr) cm^{-1}	1590	1600	1725, 1600	1725, 1600	1625, 1605, 1580

	4a	4b	5a	5b	6a
Appearance	Reddish orange powder	Reddish orange powder	Reddish orange powder	Reddish orange powder	Reddish brown powder
Molecular formula	$C_{35}H_{43}NO_{13}$	$C_{35}H_{43}NO_{14}$	$C_{32}H_{39}NO_{12}$	$C_{32}H_{39}NO_{13}$	$C_{34}H_{43}NO_{12}$
FAB-MS (*m/e*)	686 $(M+H)^+$	702 $(M+H)^+$	630 $(M+H)^+$	646 $(M+H)^+$	658 $(M+H)^+$
MP (°C, dec)	158~162	200~204	152~155	164~168	158~162
$[\alpha]_D^{20}$ (*c* 0.01, $CHCl_3$)	+116°	+49°	+25°	−35°	−10°
UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1\,cm}^{1\%}$)	204 (292), 234 (582), 252 (319), 289 (126), 479 (172), 494 (465)	205 (292), 234 (591), 252 (324), 289 (124), 478 (175), 495 (169)	205 (263), 235 (544), 255 (429), 295 (116), 493 (228), 527 (157)	205 (263), 235 (539), 255 (425), 295 (116), 493 (225), 526 (155)	204 (281), 235 (607), 254 (352), 293 (116), 495 (221), 529 (148)
IR *v* max (KBr) cm^{-1}	1725, 1615, 1580	1725, 1615, 1580	1600	1600	1600

	6b	7a	7b
Appearance	Reddish brown powder	Reddish brown powder	Reddish brown powder
Molecular formula	$C_{34}H_{43}NO_{13}$	$C_{33}N_{41}NO_{12}$	$C_{33}H_{41}NO_{13}$
FAB-MS (*m/e*)	674 $(M+H)^+$	644 $(M+H)^+$	660 $(M+H)^+$
MP (°C, dec)	165~168	163~165	167~172
$[\alpha]_D^{20}$ (*c* 0.01, $CHCl_3$)	+10°	+51°	+49°
UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1\,cm}^{1\%}$)	204 (277), 235 (609), 254 (352), 293 (114), 495 (217), 529 (143)	206 (254), 235 (651), 254 (373), 293 (123), 495 (239), 529 (156)	206 (255), 235 (653), 255 (373), 293 (122), 495 (240), 529 (157)
IR *v* max (KBr) cm^{-1}	1600	1600	1600

Table 3. ^{1}H NMR data of the products.

Proton	1a	1b	2a	2b	3b	4a	4b
1-H	7.81 d (7.34)	7.86 d (7.34)	7.88 d (7.33)	7.87 d (7.34)	7.87 d (7.34)	8.02 d (7.32)	7.99 d (7.34)
2-H	7.69 t (7.34)	7.72 t (7.34)	7.71 t (8.06)	7.70 t (8.07)	7.71 t (8.07)	7.77 t (8.06)	7.74 t (8.07)
3-H	7.29 d (8.01)	7.31 d (8.80)	7.32 d (8.06)	7.31 d (8.07)	7.35 d (8.07)	7.38 d (8.42)	7.36 d (8.07)
4-OCH_3	—	—	—	—	—	4.08 s	4.06 s
6-H	—	—	—	—	7.80 s	—	—
7-H	5.10 d (2.20)	5.12 br s	5.23 d (2.20)	5.23 br s	5.01 br s	5.26 d (2.19)	5.25 br s
8-Ha	2.20 d (14.67)	2.19 d (14.67)	2.33 d (14.67)	2.32 d (15.41)	2.05~2.25	2.31 d (14.65)	2.29 d (14.67)
8-Hb	2.12 dd (14.67, 4.4)	2.14 dd (14.67, 3.67)	2.22 dd (14.67, 3.66)	2.22 dd (14.67, 4.40)		2.22 dd (15.02, 4.03)	2.22 dd (14.67, 3.66)
10-H	4.83 s	4.85 s	4.29 s	4.28 s	4.98 s	4.28 s	4.27 s
13-Ha	1.65~1.89	1.75~1.9 m (7.34)	1.78~1.88	1.84 m (6.60)	1.6~1.8	1.83 m (7.33)	1.83 m (7.34)
13-Hb			1.46 m (6.60)	1.44 m (6.60)		1.44 m (7.33)	1.44 m (7.34)
14-CH_3	1.10 t (7.33)	1.10 t (7.34)	1.11 t (6.60)	1.11 t (6.60)	1.13 t (7.34)	1.11 t (7.33)	1.11 t (7.33)
16-OCH_3	—	—	3.72 s	3.72 s	—	3.71 s	3.71 s
1'-H	5.45 br s	5.45 br s	5.49 br s	5.47 br s	5.36 d (2.93)	5.51 d (3.30)	5.49 br s
2'-Ha	1.67~1.88	1.7~1.8	1.7~1.9	1.7~1.8	1.75~1.95	1.75~1.90	1.72
2'-Hb							
3'-H	3.00 m	3.00 br t (8.80)	3.06 br d	3.04 br t	2.1	3.03 br d (9.89)	3.03 br t
4'-H	3.52 br s	3.51 br s	3.54 br s	3.51 br s	3.76 br s	3.53 br s	3.51 br s
5'-H	4.15 q (6.60)	4.15 q (6.61)	4.20 q (6.60)	4.12 q (5.87)	3.99 q (6.60)	4.11 q (6.59)	4.10 q (6.60)
6'-CH_3	1.29 d (6.60)	1.30 d (5.87)	1.26 d (6.87)	1.28 d (6.61)	1.30 d (6.60)	1.26 d (6.60)	1.26 d (5.87)
3'-$N(CH_3)_2$	—	—	—	—	2.17 s	—	—
1''-H	4.85 br s	4.95 d (2.93)	4.87 br s	4.97 br s	4.92 br t (2.93)	4.86 br s	4.96 br s
2''-Ha	2.0~2.1	1.9~2.05	2.0~2.1	2.06 br d (11.74)	2.05~2.2	1.9~2.05	2.02 dd (12.47, 4.40)
2''-Hb	1.7~1.8		1.7~1.8	1.90 br t (11.74)		1.6~1.8	1.9
3''-H	—	4.1	—	4.12	4.11 br d (11.00)	—	4.08
3''-Ha	2.05~2.15	—	2.05~2.15	—	—	2.0~2.15	—
3''-Hb	1.75~1.85	—	1.75~1.85	—	—	1.7~1.85	—
4''-H	3.65 br s	3.63 d (2.20)	3.65 br s	3.68 br s	3.64 br s	3.65 br s	3.65 br s
5''-H	4.13 q (6.60)	4.07 q (6.61)	4.13 q (6.60)	4.18 q (6.60)	4.54 q (6.60)	4.19 q (6.96)	4.13 q (6.61)
6''-CH_3	1.17 d (6.60)	1.25 d (6.61)	1.16 d (6.87)	1.25 d (6.60)	1.21 d (6.60)	1.16 d (6.59)	1.23 d (6.60)
Solvent	$CDCl_3$-CD_3OD (10:1)	$CDCl_3$-CD_3OD (6:1)	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$

Table 3. (Continued)

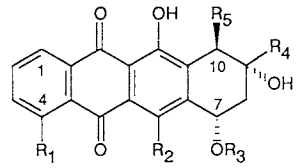
Proton	5a	5b	6a	6b	7a	7b
1-H	7.82 d (8.07)	7.86 d (7.33)	7.90 d (7.34)	7.85 d (6.60)	7.78 d (7.34)	7.87 d (7.34)
2-H	7.69 t (8.07)	7.71 t (8.07)	7.73 t (8.07)	7.71 t (8.07)	7.68 t (7.33)	7.72 t (8.07)
3-H	7.28 d (8.07)	7.30 d (8.07)	7.33 d (9.54)	7.31 d (6.60)	7.28 d (8.81)	7.32 d (8.80)
7-H	5.20 d (1.47)	5.23 br s	5.15 d (2.20)	5.13 br s	5.09 d (2.20)	5.13 br s
8-Ha	2.54 d (14.67)	2.53 d (14.67)	2.15	2.1～2.25	2.20 d (14.67)	2.20 d (14.67)
8-Hb	1.7～1.85	1.75～1.85	2.10 dd (16.14, 2.93)		2.13 dd (14.67, 3.67)	2.14 dd (14.67, 3.67)
10-H	—	—	4.92 s	4.87 s	4.82 s	4.86 s
10-Ha	3.14 d (19.07)	3.15 d (19.07)	—	—	—	—
10-Hb	2.60 d (19.07)	2.64 d (19.07)	—	—	—	—
13-H	3.67 q (6.60)	3.68 q (6.60)	—	—	—	—
13-Ha	—	—	1.65～1.9	1.7～1.9	1.7～1.9	1.75～1.9
13-Hb	—	—				
14-CH_3	1.32 d (6.60)	1.33 d (6.60)	1.11 t (7.33)	1.11 t (7.34)	1.10 t (7.34)	1.11 t (7.33)
1′-H	5.47 d (2.94)	5.47 br s	5.53 d (3.67)	5.50 br s	5.46 d (3.67)	5.46 d (2.94)
2′-Ha	1.7～1.9	1.7～1.8	1.7～2.0	1.8～1.9	1.90 dd (12.47, 3.67)	1.8～1.9
2′-Hb					1.7～1.85	1.71 td (12.47, 3.67)
3′-H	3.04 br d (9.7)	3.02 br t (8.06)	2.1～2.2	2.1	2.72 br d (11.00)	2.67 br d (11.01)
4′-H	3.53 br s	3.52 br s	3.80 br s	3.79 br s	3.76 br s	3.73 br s
5′-H	4.14 q (5.87)	4.15 q (6.60)	4.03 q (6.60)	4.03 q (6.60)	4.14 q (6.60)	4.11 q (6.61)
6′-CH_3	1.28 d (5.86)	1.30 d (5.87)	1.29 d (6.60)	1.30 d (5.87)	1.30 d (6.61)	1.31 d (6.60)
3′-$NHCH_3$	—	—	—	—	2.38 s	2.36 s
3′-$N(CH_3)_2$	—	—	2.20 s	2.19 s	—	—
1″-H	4.85 br s	4.95 d (2.20)	4.93 br s	5.03 d (2.93)	4.86 br s	4.97 d (2.94)
2″-Ha	1.95～2.1	1.9～2.05	1.7～2.0	2.0～2.1	2.0～2.15	2.02 dd (12.47, 4.40)
2″-Hb	1.65～1.8			1.8～1.9	1.7～1.85	1.94 td (12.47, 3.67)
3″-H	—	4.05	—	4.10 m	—	4.08
3″-Ha	1.95～2.15	—	1.7～2.0	—	2.0～2.15	—
3″-Hb	1.7～1.85	—		—	1.7～1.85	—
4″-H	3.65 br s	3.64 d (2.20)	3.58 br s	3.60 d (2.20)	3.65 br s	3.63 d (2.93)
5″-H	4.13 q (5.87)	4.07 q (6.60)	4.53 q (6.60)	4.45 q (6.60)	4.09 q (6.60)	4.05 q (6.60)
6″-CH_3	1.17 d (6.60)	1.25 d (6.60)	1.26 d (6.60)	1.20 d (6.60)	1.18 d (6.61)	1.25 d (6.60)
Solvent	$CDCl_3$-CD_3OD (20:1)	$CDCl_3$	$CDCl_3$	$CDCl_3$-CD_3OD (20:1)	$CDCl_3$-CD_3OD (10:1)	$CDCl_3$-CD_3OD (10:1)

Table 4. ^{13}C NMR data of the products.

Carbon	1a	1b	2a	2b	3b	4a	4b	5a	5b	6a	6b	7a	7b
1	119.50	119.45	119.64	119.64	119.55	119.79	119.72	119.43	119.43	119.70	119.58	119.48	119.54
2	136.91	136.91	137.07	137.07	136.96	135.71	135.70	136.82	136.84	137.13	137.02	136.86	136.99
3	124.62	124.55	124.83	124.83	125.25	118.39	118.37	124.51	124.48	124.95	124.72	124.60	124.66
4	162.22	162.22	162.69	162.65	162.93	161.06	161.00	162.20	162.17	162.75	162.34	162.13	162.28
4a	115.83	115.84	116.10	116.07	116.10	120.93	120.80	115.88	115.95	116.14	115.90	115.73	115.90
5	190.53	190.62	190.83	190.80	187.56	187.15	187.04	190.33	190.48	190.91	190.69	190.39	190.68
5a	111.87	111.89	111.63	111.61	132.05	112.20	112.13	110.94*	111.03*	112.19	112.00	111.78	112.00
6	156.33	156.26	156.88	156.86	120.55	155.78	155.69	156.44*	156.37*	156.86	156.45	156.28	156.32
6a	134.70	134.63	135.93	135.86	143.14	136.18	136.07	134.61*	134.53*	135.08	134.85	134.64	134.63
7	70.58	70.54	70.87	70.90	73.87	70.94	70.87	69.67	69.74	70.40	70.37	70.65	70.67
8	32.41	32.34	33.42	33.39	33.74	33.47	33.46	33.99	33.86	32.97	32.45*	32.36	32.45
9	71.87	71.93	71.18	71.18	72.13	71.22	71.18	71.68	71.64	71.80	71.90	71.92	71.93
10	65.77	65.63	52.00	52.05	66.18	51.89	51.84	32.69	32.74	66.70	66.06	65.66	65.77
10a	138.59	138.54	135.35	135.35	134.00	133.24	133.18	137.88	137.82	138.59	138.51	138.48	138.47
11	156.99	157.01	156.88	156.86	162.34	156.69	156.61	156.79*	156.74*	157.27	156.45	156.97	156.97
11a	111.29	111.31	111.26	111.25	115.36	111.55	111.49	110.08*	110.16*	111.55	111.38	111.20	111.38
12	186.00	186.30	186.25	186.19	187.84	186.68	186.60	186.00	186.14	186.40	186.14	185.89	186.14
12a	133.22	133.25	133.47	133.43	133.08	135.55	135.43	133.27*	133.35*	133.42	133.27	133.11	133.29
13	30.04	29.93	32.30	32.28	30.39	32.28	32.25	73.05	72.90	30.48	30.19	29.98	30.04
14	6.26	6.13	6.79	6.79	6.65	6.77	6.74	16.61	16.47	6.62	6.39	6.24	6.51
15	—	—	171.42	171.40	—	171.64	171.57	—	—	—	—	—	—
16	—	—	52.43	52.43	—	52.33	52.31	—	—	—	—	—	—
4-OMe	—	—	—	—	—	56.66	56.61	—	—	—	—	—	—
1′	101.43	101.29	101.84	101.77	99.36	101.74	101.52	100.63	100.83	101.59	101.39	101.45	101.47
2′	33.86	33.69	34.15	34.25	29.49	34.12	33.90	33.71	33.73	29.71	29.03	30.99	31.12
3′	46.29	46.22	46.77	46.71	61.38	46.66	46.59	46.35	46.29	61.67	61.50	54.03	54.14
4′	81.59	81.92	81.56	81.89	74.21	81.45	81.54	81.39	81.87	73.88	73.84	77.11	77.53
5′	68.02	67.93*	68.22	68.14	68.49	68.12	67.98*	68.11*	68.09	68.58	68.42	67.95*	68.02
6′	17.09	16.98	17.40	17.43	17.92	17.38	17.35	17.14	17.05	17.93	17.74	17.24	17.31
3′-NHMe	—	—	—	—	—	—	—	—	—	—	—	32.12	32.30
3′-NMe_2	—	—	—	—	43.30	—	—	—	—	43.23	43.08	—	—
1″	100.19	100.81	100.05	100.49	99.15	100.00	100.52	100.15	100.54	98.63	98.99	99.97	100.63
2″	23.91	32.34	23.91	33.03	33.03	23.89	32.83	23.93	32.43	23.76	32.53*	23.93	32.34
3″	25.39	65.13	25.68	65.65	65.90	25.65	65.48	25.45	65.17	25.79	65.45	25.28	65.15
4″	66.76	70.54	67.30	71.09	71.52	67.27	70.95	66.87	70.58	66.70	71.11	66.59	70.54
5″	67.75	67.07*	67.56	66.82	67.10	67.54	66.96*	67.77*	67.09	67.68	66.24	67.89*	67.11
6″	16.63	16.49	17.05	16.83	16.66	17.01	16.80	16.74	16.61	17.06	16.54	16.58	16.67
Solvent	C-M[a] (10:1)	C-M (6:1)	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$	C-M (20:1)	$CDCl_3$	$CDCl_3$	C-M (20:1)	C-M (10:1)	C-M (10:1)

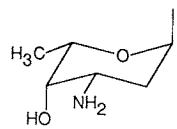
Similar values asterisked may be interchanged. [a] C-M: $CDCl_3$-CD_3OD.

Table 5. Structures of the substrates and products.

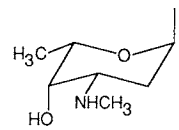


Compound[a]	R_1	R_2	R_3	R_4	R_5
1	OH	OH	DN	CH_2CH_3	OH
1a	OH	OH	DN-R	CH_2CH_3	OH
1b	OH	OH	DN-dF	CH_2CH_3	OH
2	OH	OH	DN	CH_2CH_3	$COOCH_3$
2a	OH	OH	DN-R	CH_2CH_3	$COOCH_3$
2b	OH	OH	DN-dF	CH_2CH_3	$COOCH_3$
3	OH	H	RN	CH_2CH_3	OH
3b	OH	H	RN-dF	CH_2CH_3	OH
4	OCH_3	OH	DN	CH_2CH_3	$COOCH_3$
4a	OCH_3	OH	DN-R	CH_2CH_3	$COOCH_3$
4b	OCH_3	OH	DN-dF	CH_2CH_3	$COOCH_3$
5	OH	OH	DN	$CH(OH)CH_3$	H
5a	OH	OH	DN-R	$CH(OH)CH_3$	H
5b	OH	OH	DN-dF	$CH(OH)CH_3$	H
6	OH	OH	RN	CH_2CH_3	OH
6a	OH	OH	RN-R	CH_2CH_3	OH
6b	OH	OH	RN-dF	CH_2CH_3	OH
7	OH	OH	mDN	CH_2CH_3	OH
7a	OH	OH	mDN-R	CH_2CH_3	OH
7b	OH	OH	mDN-dF	CH_2CH_3	OH

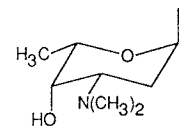
[a] **1**~**7**: Substrates; **1a**~**7a** and **1b**~**7b**: products.



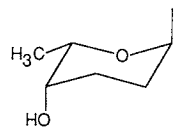
DN: Daunosamine



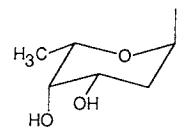
mDN: *N*-Methyldaunosamine



RN: Rhodosamine



R: Rhodinose



dF: 2-Deoxyfucose

On acid hydrolysis followed by TLC analysis, it was evidenced that the pairs of products obtained from a substrate anthracycline had the same aglycone as the substrate and contained either rhodinose or 2-deoxyfucose in addition to parental aminosugar. Products **1a**~**7a** gave rhodinose (Rf value: 0.72) as an additional sugar and compounds **1b**~**7b** gave 2-deoxyfucose (Rf value: 0.59), indicating that the products are anthracyclinone disaccharides which were glycosidated with further rhodinose or 2-deoxyfucose. It was supported by two anomeric protons detected in ^1H NMR spectra of the products (Table 3).

In ^{13}C NMR analysis (Table 4), the chemical shifts of the products were superimposable on those of

Table 6. Inhibitory activities of the products and their parent compounds on growth and nucleic acid synthesis of cultured L1210 leukemia cells.

Compound	IC_{50} (μg/ml)			DNA/RNA
	Growth	DNA synthesis	RNA synthesis	
Oxaunomycin (**1**)	0.0003	0.29	0.68	0.4
CG15-A (**1a**)	0.002	1.2	3.0	0.4
CG15-B (**1b**)	0.04	4.6	2.5	1.8
D788-6 (**2**)	0.25	2.6	1.4	1.9
CG17-A (**2a**)	0.50	3.3	1.1	3.0
CG17-B (**2b**)	1.4	>10	2.3	—
Yellamycin A (**3**)	0.007	0.28	0.23	1.2
CG18-B (**3b**)	0.16	2.6	2.3	1.1
D788-5 (**4**)	0.50	1.4	0.32	4.4
CG19-A (**4a**)	1.03	4.8	1.3	3.7
CG19-B (**4b**)	2.4	>10	>10	—
13-Dihydrocarminomycin (**5**)	0.06	1.2	1.2	1.0
CG20-A (**5a**)	0.17	1.2	1.2	1.0
CG20-B (**5b**)	1.5	>10	>5	—
Betaclamycin T (**6**)	0.01	0.21	0.06	3.5
CG21-A (**6a**)	0.01	0.38	0.18	2.1
CG21-B (**6b**)	0.01	0.36	0.13	2.8
LB-1 (**7**)	0.006	0.38	0.26	1.5
CG22-A (**7a**)	0.01	0.87	1.4	0.6
CG22-B (**7b**)	0.21	>5	>10	—

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 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, 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.

parent compounds except for one more glycosidic sugar moiety. The additional sugars of the products were determined to link at C-4′ since the chemical shifts of C-4′ of the products shifted to lower field (10~12 ppm) in comparison with those of the parent compounds. The chemical shifts of the additional sugars were identical to those of rhodinose residue of MA144 N1[19] for products **1a**~**7a** and those of 2-deoxyfucose residue of MA144 S1[19] for products **1b**~**7b**. The small coupling constants (~3.67 Hz) of the anomeric protons indicated the glycosidic bonds are all α.

From all these findings, the structures of the products were determined as shown in Table 5.

Biological Activity

The inhibitory activity of the products on the growth and nucleic acid synthesis of cultured murine leukemic L1210 cells were shown in Table 6. The cytotoxic activity of the products except for **6a** and **6b** were reduced in comparison with those of their parent compounds, and **1b**, **2b**, **4b**, **5b**, and **7b** were less active than **1a**, **2a**, **4a**, **5a**, and **7a**, respectively. **6a** and **6b** were as cytotoxic as **6**. The inhibitory activity of the products on nucleic acid synthesis were also reduced or similar to those of the parent compounds. The IC_{50} ratio of DNA and RNA (DNA/RNA) had no relation to the structure of the products. Antitumor effects *in vivo* of the products were tested in mice bearing L1210 leukemia by daily administration from day 1 to 10. **1a**, **1b**, **3b**, **5a**, and **5b** had a maximum antitumor activity of 213, 183, 213, 226, and 277% (T/C) at an optimum dose of 0.16, 0.08, 1.25, 1.0, and 4.0 mg/kg/day, respectively. Under the same

conditions, **1** and **3** had a T/C of 200 and 169% at an optimum dose of 0.06 and 0.39 mg/kg/day, respectively.

Discussion

We have reported that various anthracyclinones were converted into the corresponding trisaccharides which contained the same sugar moiety (cinerulosyl-2-deoxyfucosyl-rhodosaminyl trisaccharide: RN-dF-C) as that of aclarubicin when they were fed to the growing culture of aclarubicin-negative mutant strain KE303. Hybrid anthracyclines having the same trisaccharide residue, such as betaclamycin A (CG7), CG5, CG12, and trisarubicinol, were thus obtained by bioconversion of β-rhodomycinone, ε-rhodomycinone, α-citromycinone, and 13-dihydrocarminomycinone, respectively[2,7]. In this study, however, we found that the bioconversion of anthracyclinone monosaccharides with strain KE303 gave no corresponding trisaccharides but a pair of anthracyclinone disaccharides in which one had a rhodinose and the other a 2-deoxyfucose as an additional sugar. These results suggest that the presence of an aminosugar residue affects the biosynthetic formation of the trisaccharide residue on the aglycone. A sort of the attached aminosugar unaffected the disaccharide formation and glycosidation pattern. Doxorubicin and daunorubicin were not glycosidated but reduced to their 13-dihydro derivatives. It is very interesting since the same was seen with the conversion of their aglycones (doxorubicinone and daunorubicinone)[2], suggesting that both a methoxyl group at C-4 and oxygen at C-13 inhibited biosynthetic glycosidation.

Substitution at C-4′ with rhodinose or 2-deoxyfucose, except for product **6**, caused a reduced cytotoxic activity against cultured L1210 cells. The cytotoxic activities of **1a**～**5a**, and **7a** were decreased 2～7 fold and those of **1b**～**5b**, and **7b** were decreased 5～133 fold as compared with those of parental monosaccharides. However, it was found that the 4′-substitution with a natural sugar gave a therapeutically improved effect by increased antitumor activity and diminished cytotoxicity.

Experimental

General

MP's were determined on a Kofler hot stage microscope and uncorrected. UV spectra were recorded on a Hitachi EPS 3T and IR spectra (KBr pellet) on a Hitachi EPI-GS Spectrophotometer. ^1H and ^{13}C NMR were recorded with a JEOL JNM-GSX400 spectrometer at 400 MHz and 100 MHz, respectively. Chemical shifts were expressed in δ values (ppm) with TMS as an internal standard and coupling constants were given in J (Hz). Mass spectra were recorded with a Hitachi M-80H or JEOL JMS-SX102A spectrometer. Specific rotations were recorded on a Jasco DIP-181 digital polarimeter. TLC analyses were performed on Silica gel 60 F_{254} (E. Merck).

Microorganism

The aclarubicin-negative mutant strain KE303 of *S. galilaeus* MA144-M1 was obtained as previously described[2], grown 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.

Microbial Conversion

A seed culture was grown aerobically 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. This culture was employed as inoculum (2%, v/v) to a 10-liter jar fermentor containing 5 liters of conversion medium. Conversion medium consisted of soluble starch 20 g, glucose 10 g, soybean meal 30 g, NaCl 3 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, yeast extract 1 g, $CuSO_4 \cdot 5H_2O$ 0.0056 g, $FeSO_4 \cdot 7H_2O$ 0.0008 g, $MnCl_2 \cdot 4H_2O$ 0.0064 g, $ZnSO_4 \cdot 2H_2O$ 0.0016 g in 1,000 ml of tap water, pH 7.0. Conditions used were 2.5 liters/minute for aeration and 300 rpm for agitation. Cultivation was carried out at 28°C. After 24 hours, a methanol solution of a substrate (5 mg/ml) was added to the growing culture to give a final concentration of 50 μg/ml and the cultivation was further continued for 66 hours.

Antibiotic Assay

For the detection of the products by microbial conversion, to 2 ml of the culture broth was added equal volume of acetone and 0.1 ml of 1 M Tris-HCl buffer (pH 8.0). After agitation, products were extracted with 2 ml of $CHCl_3$ and the organic layer was evaporated to dryness. The residual anthracycline pigments were analyzed by TLC and HPLC. TLC was performed using a developing solvent of $CHCl_3$ - MeOH - AcOH - concd NH_4OH (150 : 50 : 5 : 1 : 1). HPLC was performed on 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 - H_2O (35 : 65) (adjusted to pH 2.0 with phosphoric acid) was used as a mobile phase and run at at flow rate of 1.0 ml/minute. Samples were dissolved in the mobile phase and 10 μl of sample solution was injected. UV absorbance was monitored at 254 nm.

CG15-A (**1a**) and CG15-B (**1b**)

1 (500 mg) was added to 10 liters of the growing culture of strain KE303 and 522 mg of the crude powder containing **1a** and **1b** was prepared. The crude powder was dissolved in $CHCl_3$, applied to a column of silica gel (Wakogel C-200), and eluted with $CHCl_3$ - MeOH (20 : 1) and $CHCl_3$ - MeOH - H_2O (100 : 10 : 0.1, 80 : 10 : 0.1, and 60 : 10 : 0.1). Each of **1a** and **1b** was purified by silica gel TLC (Silica gel 60 PF_{254}, E. Merck) using $CHCl_3$ - MeOH - H_2O (50 : 10 : 0.1) as a developing solvent. The objective band was scraped from the TLC plate and extracted with $CHCl_3$ - MeOH (7 : 1). The extract was further purified by column chromatography on Toyopearl HW-40 (Toyo Soda MFG. Co., Ltd.). The product was eluted with $CHCl_3$ - MeOH (1 : 2) and concentrated *in vacuo* to dryness. The residual pigment was dissolved in 0.02 M acetate buffer (pH 3.5), washed with toluene and extracted with $CHCl_3$ at pH 8.0. $CHCl_3$ layer was washed with H_2O, dried over anhydrous sodium sulfate and concentrated *in vacuo* to a small volume. To the concentrate, an excess of *n*-hexane was added to precipitate an reddish brown powder. This procedure yielded 41 mg of **1a** and 32 mg of **1b**.

CG17-A (**2a**) and CG17-B (**2b**)

2 (250 mg) was added to 5 liters of the growing culture of strain KE303 and 275 mg of the crude powder containing **2a** and **2b** was prepared. The crude powder was dissolved in $CHCl_3$, applied to a column of silica gel (Wakogel C-200), and eluted with $CHCl_3$ - MeOH (20 : 1) and $CHCl_3$ - MeOH - H_2O (100 : 10 : 0.1 and 70 : 10 : 0.1). Each of **2a** and **2b** was further purified by silica gel TLC (Silica gel 60 PF_{254}, E. Merck) using $CHCl_3$ - MeOH - H_2O - AcOH - concd NH_4OH (120 : 50 : 5 : 1 : 1) as a developing solvent. The objective band was scraped from the TLC plate and extracted with $CHCl_3$ - MeOH (5 : 1). After being concentrated, the extract was dissolved in 0.02 M acetate buffer (pH 3.5), washed with toluene and extracted with $CHCl_3$ at pH 8.0. $CHCl_3$ layer was washed with H_2O, dried over anhydrous sodium sulfate and concentrated *in vacuo* to a small volume. To the concentrate, an excess of *n*-hexane was added to precipitate an reddish orange powder of the product. This procedure yielded 12 mg of **2a** and 34 mg of **2b**.

CG18-B (**3b**)

3 (250 mg) was added to 5 liters of the growing culture of strain KE303 and 270 mg of the crude powder containing **3a** and **3b** was prepared. The crude powder was purified in the same way as described above (**2a** and **2b**) to yield a small quantity of **3a** and 15 mg of **3b** as yellow powders.

CG19-A (**4a**) and CG19-B (**4b**)

4 (500 mg) was added to 10 liters of the growing culture of strain KE303 and 392 mg of the crude powder containing **4a** and **4b** was prepared. The crude powder was purified in the same way as described above (**2a** and **2b**) to yield 27 mg of **4a** and 52 mg of **4b** as reddish orange powders.

CG20-A (**5a**) and CG20-B (**5b**)

5 (500 mg) was added to 10 liters of the growing culture of strain KE303 and 287 mg of the crude powder containing **5a** and **5b** was prepared. The crude powder was purified in the same way as described above (**2a** and **2b**) to yield 35 mg of **5a** and 28 mg of **5b** as reddish orange powders.

CG21-A (**6a**) and CG21-B (**6b**)

6 (250 mg) was added to 5 liters of the growing culture of strain KE303 and 321 mg of the crude powder containing **6a** and **6b** was prepared. The crude powder was purified in the same way as described above (**2a** and **2b**) to yield 6 mg of **6a** and 16 mg of **6b** as reddish brown powders.

CG22-A (**7a**) and CG22-B (**7b**)

7 (250 mg) was added to 5 liters of the growing culture of strain KE303 and 456 mg of the crude powder containing **7a** and **7b** was prepared. The crude powder was purified by silica gel chromatography in the same way as described above (**2a** and **2b**). **7a** was purified by silica gel TLC using $CHCl_3$-MeOH-H_2O-AcOH-concd NH_4OH (120:50:5:1:1). **7b** was purified by column chromatography on reverse phase silica gel (50 ml, YMC gel 60 Å) which was developed with acetonitrile-0.05 M ammonium formate (pH 4.0) (15:85~20:80). This procedure yielded 6 mg of **7a** and 16 mg of **7b** as reddish brown powders.

Qualitative Determination of Aglycone and Sugars by TLC

The product (1 mg) was dissolved in 1 ml of 0.1 N HCl and heated at 85°C for 30 minutes in a water bath. The aglycone, thus obtained, was extracted with $CHCl_3$. The $CHCl_3$ layer was evaporated to dryness *in vacuo* and the pigment residue was then subjected to TLC using a developing solvent of $CHCl_3$-MeOH (20:1).

Alternatively, the aqueous layer containing sugar components was neutralized by addition of silver carbonate with a small amount of charcoal and centrifuged. The supernatant fluid was concentrated *in vacuo* and subjected to TLC using a developing solvent of BuOH-AcOH-H_2O (4:1:1). Sugars were detected by spraying with *p*-anisaldehyde-H_2SO_4 (each 5%) in 90% EtOH and heating at 90°C. Aclarubicin, MA144 N1[19], MA144 L1[19], and daunorubicin were also hydrolyzed under the same condition and the aqueous layers were used as a source of authentic sugars including L-rhodosamine, *N*-monomethyl-L-daunosamine, L-daunosamine, 2-deoxy-L-fucose, L-rhodinose, and L-cinerulose A; Rf values of them were 0.13, 0.24, 0.32, 0.59, 0.72, and 0.80, respectively.

Biological Activity

In vitro cytotoxicity and inhibition of DNA and RNA syntheses in cells of murine leukemia L1210 were assayed according to the method previously described[1].

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