ANTHRACYCLINE METABOLITES FROM BAUMYCIN-PRODUCING *Streptomyces* sp. D788

III. NEW ANTHRACYCLINE METABOLITES PRODUCED BY BLOCKED MUTANTS 4L-660 AND YDK-18

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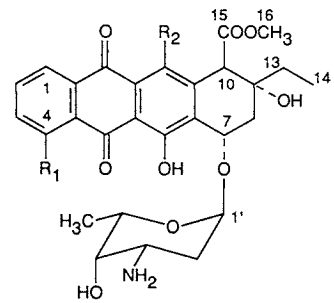
In a previous paper,[1] we described isolation of a variety of blocked mutants from baumycin-producing *Streptomyces* sp. D788 and identification of the biosynthetically blocked anthracycline metabolites produced by some of them. This paper deals with details of the anthracyclines produced by another two blocked mutants, strains 4L-660 and YDK-18. To well define the blocked mutants, all detectable anthracycline products accumulated in their culture broths were isolated, identified and quantified for their yields. Both the two mutants were found to produce only two anthracycline metabolites without any yield of their parental daunorubicin or 11-deoxydaunorubicin. Mutant 4L-660 produced a new metabolite, D788-5 (7-*O*-daunosaminyl-4-*O*-methyl-ε-rhodomycinone), and the known metabolite D788-6 (7-*O*-daunosaminyl-ε-rhodomycinone)[2] in comparable yields. Mutant YDK-18 produced two new metabolites D788-16 (7-*O*-daunosaminyl-4-*O*-methylaklavinone) and D788-17 (7-*O*-daunosaminylaklavinone).

The blocked mutant strains 4L-660 and YDK-18 were grown in 500-ml Erlenmeyer flasks containing 100 ml each of seed medium.[1] The cultivation was carried out at 28°C for 2 days on a rotary shaker (200 rpm). Fermentation was performed using sixty 500-ml Erlenmeyer flasks containing 50 ml each of production medium[1] using a 4% inoculum of the seed culture. The cultivation was carried out at 28°C for 6 days.

In order to examine the products and their yields, the culture broth was extracted by mixing with 10-vol acetone and the supernatant fluid was subjected to analytical HPLC as previously described[2] (solvent: 30% $CHCN_3$ (pH 2.0 with H_3PO_4)). The products of strain 4L-660 were found to be two whose retention times in HPLC were 9.09 minutes (**1a**, named D788-5) and 13.20 minutes (**1b**, named D788-6). The yield ratio of **1a** to **1b** was 1.30 based on the HPLC peak area (detection: 495 nm). Strain YDK-18 produced also two anthracyclines with a retention time of 7.73 minutes (**2a**, named D788-16) and 10.10 minutes (**2b**, named D788-17) in HPLC (detection: 420 nm). The yield ratio of **2a** to **2b** was 0.15 and a preferential production of **2b** was observed in this mutant. A few negligible peaks were also detected with both mutant extracts, but neither peaks of daunorubicin nor 11-deoxydaunorubicin were observed.

Each fermentation broth (about 3 liters) thus obtained was separated into supernatant fluid and mycelia by centrifugation. Products were extracted with 3 liters of acetone from the mycelial pellet and with 2 liters of $CHCl_3$ from the supernatant fluid. The acetone extract was evaporated to one third volume and extracted with $CHCl_3$. The $CHCl_3$ layer was combined with that from the supernatant extract and evaporated to about 200 ml followed by acidic extraction with dil H_3PO_4 (pH 2.0). The

Fig. 1. Structures of anthracycline metabolites produced by mutant strains 4L-660 and YDK-18.



Strain	Product (name)	R_1	R_2	Remarks
4L-660	**1a** (D788-5)	OCH_3	OH	New
	1b (D788-6)	OH	OH	Known[2]
YDK-18	**2a** (D788-16)	OCH_3	H	New
	2b (D788-17)	OH	H	New

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extract was washed with toluene and then extracted with $CHCl_3$ after pH adjustment to pH 8.0 with 4 N NaOH. The $CHCl_3$ layer was evaporated to a small volume and an excess of *n*-hexane was added to precipitate crude products (367 mg from 4L-660 culture; 473 mg from YDK-18 culture).

Purification was carried out by preparative HPLC using a Waters M-600A liquid chromatographic apparatus with a reverse phase column, YMC-Pack S343 (ODS) (20 mm × 250 mm) (Yamamura Chemical Laboratories Co. Ltd.). Solvent was 50% CH_3CN in 10 mM camphorsulfonic acid (pH 4.2). The crude preparation was dissolved in the mobile phase and injected in 1.5-ml portions containing about 40 mg. The flow rate was 5.0 ml/minute and the eluate was collected in 5-ml fractions with monitoring at 254 nm using a UV detector (Model 440; Waters) and checked by the above analytical HPLC. A total of 5 runs were achieved for each sample. Fractions containing each compound were pooled and extracted with $CHCl_3$ at pH 8.0. The $CHCl_3$ layer was evaporated to dryness and the preparative HPLC was once repeated for complete purification. After $CHCl_3$ extraction and concentration *in vacuo*, the residual materials were dissolved in 0.1 M AcOH buffer (pH 3.0). After washing with $CHCl_3$ and adjustment to 8.5 with 1 N NaOH, the solution was extracted with $CHCl_3$. The $CHCl_3$ layer was washed with saturated saline, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The addition of an excess of *n*-hexane gave red powders of **1a** (23 mg) and **1b** (38 mg), and yellow powders of **2a** (9 mg) and **2b** (56 mg).

Physico-chemical properties of **1a**, **2a**, **2b** and their new aglycones are as follows:

1a (D788-5): Red powder; mp 159~161°C (dec); $[\alpha]_D^{20}$ +171° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 1730 (ester carbonyl), 1620 (carbonyl bonded to a *peri* hydroxyl), 1580, 1405, 1290, 1245, 1205, 1130, 1010, 990; UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1\ cm}^{1\%}$) 204 (372), 234 (747), 253 (415), 288 (162), 472 (220), 494 (sh, 211); FD-MS *m/z* 571 (M).

2a (D788-16): Yellow powder; mp 138~141°C (dec); $[\alpha]_D^{20}$ +106° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 1740 (ester carbonyl), 1680 (nonbonded carbonyl), 1640, 1595, 1450, 1390, 1300, 1255, 1130, 1020, 990; UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1\ cm}^{1\%}$) 204 (399), 229 (700), 258 (405), 418 (190); FD-MS *m/z* 556 $(M+H)^+$.

2b (D788-17): Yellow powder; mp 131~134°C (dec); $[\alpha]_D^{20}$ +192° (*c* 0.01, $CHCl_3$); IR (KBr) cm^{-1} 1735 (eater carbonyl), 1675 (nonbonded carbonyl),

Table 1. ^1H NMR chemical shift assignments for new compounds.

Proton	**1a** (D788-5)	**2a** (D788-16)	**2b** (D788-17)	D788-5 aglycone	D788-16 aglycone
Aglycone moiety					
1-H	7.99 d (8)	7.97 d (8)	7.81 d (8)	7.76 d (8.1)	7.98 d (6.6)
2-H	7.74 t (8)	7.76 t (8)	7.67 t (8)	7.60 t (8.1)	7.77 t (8.1)
3-H	7.36 d (8)	7.38 d (8)	7.29 d (8)	7.22 d (8.8)	7.39 d (8.8)
4-OCH_3	4.07 s	4.08 s	—	3.97 s	4.09 s
6-OH	—	—	—	13.74 s	13.71 s
7-H	5.25 br d	5.29 br s	5.26 br s	5.30 br t (3.0)	5.40 br s
8-Ha	2.33 d (15)	2.53 dd (15, 4)	2.52 dd (15, 4)	2.28 d (14.7)	2.55 dd (15.0, 4.0)
8-Hb	2.20 dd (15, 4)	2.33 d (15)	2.34 d (15)	2.21 dd (14.7, 5.0)	2.26 d (15.0)
10-H	4.26 s	4.10 s	4.11 s	4.21 s	4.08 s
11-H	—	7.63 s	7.67 s	—	7.67 s
11-OH	—	—	—	13.09 s	—
13-Ha	1.83 m (7)	1.75 m (7)	1.7~1.8	1.79 m (7.3)	1.72 m (7.3)
13-Hb	1.44 m (7)	1.51 m (7)	1.51 m (7)	1.53 m (7.3)	1.56 m (7.3)
14-CH_3	1.11 t (7)	1.09 t (7)	1.09 t (7)	1.15 t (7.3)	1.10 t (7.3)
16-CH_3	3.72 s	3.69 s	3.70 s	3.72 s	3.70 s
Daunosamine moiety					
1'-H	5.48 br d (4)	5.49 br d (4)	5.46 br d (3)	—	—
2'-Ha	1.77 m (14, 4)	1.74 m (13, 5)	1.66~1.81 m	—	—
2'-Hb	1.67 dd (14, 5)	1.66 dd (13, 5)		—	—
3'-H	3.09 br m	3.08 br	3.09 br d	—	—
4'-H	3.47 br s	3.44 br s	3.46 br s	—	—
5'-H	4.11 q (7)	4.12 q (7)	4.12 q (7)	—	—
6'-CH_3	1.33 d (7)	1.35 d (7)	1.35 d (7)	—	—

Spectra were measured in $CDCl_3$ at 400 MHz and chemical shifts are expressed by δ (ppm) (J=Hz) from internal TMS.

Table 2. ^{13}C NMR chemical shift assignments for new compounds.

Carbon	**1a** (D788-5)	**2a** (D788-16)	**2b** (D788-17)	**1a** aglycone	**2a** aglycone
Aglycone moiety					
1	119.72	120.25	120.14	119.45	120.32
2	135.62	135.93	137.33	135.63	136.11
3	118.40	118.33	124.79	118.22	118.29
4	161.05	160.98	162.57	160.78	161.00
4a	120.92	120.73	115.75	120.16	120.53
5	187.03	188.68	192.65	186.51	188.80
5a	112.18	115.77	114.64	111.88	115.79
6	155.77	162.17	162.08	155.62	161.14
6a	136.17	131.34	132.89	137.61	131.83
7	71.10	71.20	71.04	62.44	62.75
8	33.46	33.88	33.75	34.21	34.66
9	71.24	71.71	71.66	71.29	71.55
10	51.95	57.03	57.12	51.38	56.72
10a	133.27	141.08	142.67	132.63	141.03
11	156.62	119.55	120.95	155.47	120.03
11a	111.52	132.13	131.24	111.07	132.82
12	186.59	182.30	181.26	186.05	182.21
12a	135.50	135.71	133.47	134.96	135.69
13	32.25[a]	32.13	32.09	32.52	32.32
14	6.74	6.66	6.63	6.74	6.72
15	171.52	171.49	171.26	171.46	171.51
16	52.26	52.38	52.48	52.26	52.42
4-OCH_3	56.63	56.68	—	56.43	56.55
Daunosamine moiety					
1′	101.47	101.48	101.49	—	—
2′	32.31[a]	32.53	32.47	—	—
3′	46.31	46.26	46.24	—	—
4′	70.41[b]	70.75[c]	70.59[d]	—	—
5′	67.00[b]	66.94[c]	66.98[d]	—	—
6′	16.87	16.92	16.89	—	—

Spectra were measured in $CDCl_3$ at 100 MHz and chemical shifts are expressed by δ (ppm) from internal TMS. Values marked by [a, b, c] and [d] may be interchanged.

1629, 1385, 1290, 1240, 1215, 1010, 985; UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1\,cm}^{1\%}$) 229 (786), 259 (469), 289 (186), 432 (239); FAB-MS *m/z* 542 $(M+H)^+$.

1a aglycone: Red powder; mp 218~221°C (dec); $[\alpha]_D^{20}$ +80° (*c* 0.01, $CHCl_3$); IR (KBr) cm^{-1} 1720 (ester carbonyl), 1610 (carbonyl bonded to a *peri* hydroxyl), 1570, 1430, 1280, 1240, 1200, 1120, 1060, 1040, 985; UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1\,cm}^{1\%}$) 233 (983), 252 (548), 289 (208), 478 (297), 494 (291); FAB-MS *m/z* 442 (M).

2a aglycone: Yellow powder; mp 104~108°C; $[\alpha]_D^{20}$ +90° (*c* 0.01, $CHCl_3$); IR (KBr) cm^{-1} 1730 (ester carbonyl), 1670 (nonbonded carbonyl), 1630, 1580, 1440, 1380, 1285, 1250, 1180, 1120, 1050, 1020, 980; UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1\,cm}^{1\%}$) 228 (916), 258 (524), 419 (242); FAB-MS *m/z* 427 $(M+H)^+$.

In total acid hydrolysis (0.1 N HCl, 85°C, 30 minutes) followed by TLC on Silica gel plate F_{254} (Merck Co.) (solvent: benzene-EtOAc-AcOH (4:4:1)), **1a** and **2a** gave new aglycones (Rf value: both 0.39), while **1b** and **2b** gave known aglycones (Rf value: both 0.53), ε-rhodomycinone (ε-RMN) and aklavinone (AKN), respectively, which were identified by mass spectrum and ^1H or ^{13}C NMR spectra.[3,4] The sugar component of these compounds was identical to daunosamine by comparison with an authentic sample (Rf value: 0.25; spot color: sky blue) on TLC carried out according to the previously described method.[2] **1b** and **2b** were thus 7-*O*-daunosaminyl-ε-rhodomycinone (D788-6) and 7-*O*-daunosaminylaklavinone (D788-17), respectively. **1b** has been isolated as one of minor components from the culture broth of mutant RPM-5.[2]

^1H and ^{13}C NMR spectra of the new compounds are shown in Tables 1 and 2, respectively. Chemical shifts were mainly assigned by HMBC experiments. **1a** aglycone and **2a** aglycone were identified as

Table 3. Biological activity *in vitro* against L1210 cell culture.

Compound	IC_{50} (μg/ml)			
	Growth	DNA synthesis	RNA synthesis	DNA/RNA
1a (D788-5)	0.50	1.40	0.32	4.4
1b (D788-6)	0.25	2.60	1.30	2.0
2a (D788-16)	1.20	2.60	1.70	1.5
2b (D788-17)	0.91	3.27	1.49	2.2
Daunorubicin	0.02	0.42	0.16	2.6

In the inhibition test for nucleic acid synthesis, L1210 cell culture (8×10^5 cells/ml) with supplemented ^{14}C-labeled uridine or thymidine (0.05 μCi/ml) was exposed to the indicated compound for 60 minutes and the incorporation of the radioisotopes into acid insoluble material was measured. For the growth inhibition test, the compounds were contacted with L1210 cell cultures (5×10^4 cells/ml) for 48 hours and the viable cells were counted by coulter counter.

IC_{50} is expressed as a concentration required to inhibit 50% control of the growth, and DNA and RNA syntheses of L1210 cell culture.

4-*O*-methyl-ε-rhodomycinone and 4-*O*-methylaklavinone, respectively. In ^{1}H and ^{13}C NMR spectra, their chemical-shift assignments were superimposable with those of ε-RMN[3] and AKN,[4] respectively, except for the chemical shifts assigned for 4-*O*-methyl. The same was seen between **1a** and **1b**, and **2a** and **2b**. The chemical shifts assigned for their sugar moiety accorded with those of daunosamine in daunorubicin.[2] ^{13}C NMR chemical shifts of C-7 of the glycosides shifted to a lower field compared with those of the aglycone showing that the glycosidic bond was at C-7, and the small coupling constants ($J<2.9$ Hz) of the anomeric protons also indicated that the configuration was α.

The molecular formulas of the new compounds given by ^{1}H and ^{13}C NMR were established by a high resolution FAB-MS: **1a**, $C_{29}H_{33}NO_{11}$ (*m/z* 572.2136, $(M+H)^+$, $\Delta+0.4$ mmu); **2a**, $C_{29}H_{33}NO_{10}$ (*m/z* 556.2195 $(M+H)^+$, $\Delta+1.2$ mmu); **2b**, $C_{28}H_{31}NO_{10}$ (*m/z* 542.2008 $(M+H)^+$, $\Delta-1.8$ mmu). These results prove that **1a** (D788-5) is 7-*O*-daunosaminyl-4-*O*-methyl-ε-rhodomycinone, and that **2a** (D788-16) and **2b** (D788-17) are 7-*O*-daunosaminyl-4-*O*-methylaklavinone and 7-*O*-daunosaminylaklavinone, resepectively, as illustrated in Fig. 1.

Antitumor activities of the four anthracycline metabolites **1a**, **1b**, **2a** and **2b** were evaluated by examining their inhibitory effects on cell growth and nucleic acid synthesis of murine leukemia L1210 cell culture according to the method previously described.[2] Daunorubicin was used as a reference drug. The results are shown in Table 3. New compounds **1a**, **2a** and **2b** as well as **1b** were very weakly active against L1210 cell culture. Their cell growth inhibitory activity were 1/10 to 1/20 less than that of daunorubicin. When the antitumor effect *in vivo* was examined on mice ip implanted with L1210 cells under daily ip administration on day 1 to 10, **1a** showed a significant antitumor effect of about 160% (T/C) at an optimum dose of 20 mg/kg. However, the antitumor effects of **1b**, **2a** and **2b** were less than 120% (T/C) at their optimum doses.

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