Notes

ANTHRACYCLINE METABOLITES FROM *Streptomyces violaceus* A262

IV. NEW ANTHRACYCLINE YELLAMYCINS PRODUCED BY A VARIANT STRAIN SC-7

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During the course of a study of blocked mutants or variant strains derived from *Streptomyces violaceus* A262 a known producer of diglycosidic β-rhodomycins, we have isolated several which produce new anthracyclines.[1] One of these, strain SC-7 was found to produce α-citromycinone glycosides as minor components from which yellamycins were obtained by means of controlled hydrolysis.[2] This strain was deposited as FERM P-8720 in Fermentation Research Institute, Agency of Industrial Science and Technology.

In this paper, we describe the isolation, structural determination and biological activity of yellamycins A, B and C. Yellamycins are new α-citromycinone glycosides which possess L-rhodosamine at the C-7 and/or C-10 position.

The yellamycin-producing strain SC-7 was cultured using three 10-liter jar fermenters each containing 5 liters of a fermentation medium; soluble starch 50 g, soybean meal 30 g, yeast extract 2 g, NaCl 2 g, $CaCO_3$ 2 g, $CuSO_4 \cdot 5H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.0016 g, $ZnSO_4 \cdot 7H_2O$ 0.0032 g, $MnCl_2 \cdot 4H_2O$ 0.013 g per 1,000 ml of tap water, pH 7.0. Fermentation conditions employed were 2.5 liters/minute for aeration and 350 rpm for agitation. The fermentation was carried out at 28°C for 138 hours at which time the mycelial pigments were produced at approximately maximum level. About 15 liters of the culture broth (pH 8.6) was recovered for the isolation of purified yellamycins.

The culture broth adjusted to pH 1.0 with 6N HCl was heated (65°C) for 2 hours and centrifuged. The anthracycline products in the mycelial cake were extracted with a total of 8 liters of acetone. The acetone extract was evaporated *in vacuo* to about 2 liters. The concentrate was washed with 2 liters of $CHCl_3$ and then extracted with 4 liters of $CHCl_3$ after the pH was adjusted to 8.0 with 4N NaOH. On the other hand, the supernatant was washed with 2 liters of $CHCl_3$ and the anthracycline products were extracted with 5 liters of $CHCl_3$ after raising the pH to 8.0. The $CHCl_3$ extracts were combined and evaporated *in vacuo* to a small volume and an excess of *n*-hexane was added to precipitate the crude mixture of anthracycline products. This procedure provided about 5.8 g of yellowish orange crude powder, which consisted of two orange components, β-rhodomycin I and β-rhodomycin II (Rf values: 0.35 and 0.10, respectively) and three yellow components, yellamycins A, B and C (Rf values: 0.27, 0.08 and 0.22, respectively) after chromatography on Silica gel plate F_{254} (E. Merck) with $CHCl_3$-MeOH-H_2O-acetic acid-aq NH_3 (150:50:5:1:1).

The crude powder was chromatographed on a column (35 mm, i.d.) of silica gel (Wakogel C-200, 100 g) which was developed stepwise with $CHCl_3$-MeOH (500:1, 250:1, 100:1, 50:1 and 10:1) and $CHCl_3$-MeOH-H_2O (100:10:0.1, 80:10:0.1, 50:10:0.1 and 20:10:0.1) to elute sequentially β-rhodomycin I, yellamycin A, yellamycin C, β-rhodomycin II and yellamycin B. Each of the crude yellamycin preparations was evaporated to dryness and subjected to a second silica gel column (20 mm, i.d., 50 g) with solvent mixtures of $CHCl_3$-MeOH-aq NH_3 (100:8:0.05 and 100:10:0.1 for yellamycin A) or $CHCl_3$-MeOH-H_2O (100:10:0.1, 100:15:0.1 and 100:20:0.1 for yellamycin C, and 100:10:0.1, 100:12:0.1, 100:20:0.2, 100:30:0.2, 100:40:0.2, 100:50:0.5 and 100:100:1 for yellamycin B). Each yellamycin was further purified by preparative TLC. Yellamycin A was chromatographed using a solvent mixture of $CHCl_3$-MeOH-

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H_2O-acetic acid (40:10:0.4:0.2). Yellamycins B and C were chromatographed using a solvent mixture of $CHCl_3$-MeOH-formic acid (40:10:1 for C and 20:10:1 for B). The respective yellamycin band was scraped off the TLC plate and extracted with $CHCl_3$-MeOH (2:1). The extract was evaporated to dryness. Yellamycins B and C were chromatographed once again using a second solvent mixture of $CHCl_3$-MeOH-aq NH_3 (30:10:0.2). The antibiotics recovered were dissolved in 0.05 M acetate buffer (pH 3.0) and washed with toluene. The aqueous layer was adjusted to pH 8.0 by addition of 1 N NaOH and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried over Na_2SO_4 and concentrated. An excess of *n*-hexane was added to precipitate a yellow powder. Purified yellamycins A, B and C yielded 108 mg, 124 mg and 158 mg, respectively.

The physico-chemical properties of yellamycins are as follows:

Yellamycin A: Yellow powder; mp 127~130°C (dec); $[\alpha]_D^{23}$ +108° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2940, 1625, 1605 (hydrogen bonded carbonyl), 1580, 1460, 1370, 1330, 1260, 1205, 1030, 1020, 980, 790; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 204 (403), 231 (743), 258 (492), 290 (sh, 168), 435 (230); FD-MS *m/z* 527 (M^+).

Yellamycin B: Yellow powder; mp 134~137°C (dec); $[\alpha]_D^{23}$ +149° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2950, 1620, 1610 (hydrogen bonded carbonyl), 1580, 1460, 1370, 1330, 1260, 1220, 1120, 1020, 980, 900, 800; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 205 (325), 231 (578), 258 (371), 293 (sh, 124), 436 (176); FD-MS *m/z* 684 (M^+).

Yellamycin C: Yellow powder; mp 174~175°C (dec); $[\alpha]_D^{23}$ +185° (*c* 0.02, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2950, 1630, 1610 (hydrogen bonded carbonyl), 1580, 1460, 1380, 1330, 1270, 1220, 1120, 1020, 990, 910, 800; UV $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 205 (372), 231 (730), 258 (451), 291 (sh, 151), 435 (216); FD-MS *m/z* 527 (M^+).

On total acid hydrolysis (0.1 N HCl, 85°C, 30 minutes) followed by TLC analysis,[3,4] yellamycins gave a yellow aglycone and one type of sugar.

Table 1. ^1H NMR chemical shifts of yellamycins and α-citromycinone.

Proton	Yellamycin A	Yellamycin B	Yellamycin C	α-Citromycinone
1-H	7.81 d (8.0)	7.83 d (8.0)	7.81 d (7.5)	7.84 d (8.0)
2-H	7.68 t (8.0)	7.69 t (8.0)	7.68 t (7.5)	7.71 t (8.0)
3-H	7.32 d (8.0)	7.31 d (8.0)	7.30 d (7.5)	7.33 d (8.0)
6-H	7.75 s	7.84 s	8.00 s	7.94 s
7-H	4.92 t (4.0)	4.96 dd (6.0, 4.0)	4.86 dd (6.0, 2.0)	4.83 b
8-Ha	2.2	2.49 dd (17.0, 6.0)	2.38 dd (15.0, 6.0)	2.21 dd (16.0, 4.0)
8-Hb		2.07 dd (17.0, 4.0)	2.02 br d (15.0)	2.12 br d (16.0)
10-H	4.95 s	5.10 s	5.03 s	4.86 s
13-Ha	1.89 q (7.5)	1.83 q (7.5)	1.81 q (7.5)	1.85 q (8.0)
13-Hb	1.73 q (7.5)	1.78 q (7.5)	1.80 q (7.5)	1.80 q (8.0)
14-CH_3	1.13 t (7.5)	1.12 t (7.5)	1.09 t (7.5)	1.10 t (8.0)
1'-H	5.35 br s	5.34 d (4.0)	5.34 d (4.0)	—
2'-Ha	1.8	1.95 dt (16.0, 5.0)	1.69 dt (16.0, 4.0)	—
2'-Hb		1.88 d (16.0, 4.0)	1.54 dd (16.0, 5.0)	—
3'-H	2.3 m	2.38 m (16.0, 5.0, 3.0)	2.29 m (16.0, 5.0, 3.0)	—
4'-H	3.71 br s	3.71 br s	3.68 br s	—
5'-H	4.02 q (7.0)	4.03 q (7.0)	3.94 q (7.0)	—
6'-CH_3	1.42 d (7.0)	1.40 d (7.0)	1.34 d (7.0)	—
3'-$N(CH_3)_2$	2.22 s	2.27 s	2.22 s	—
1''-H	—	5.25 d (4.0)	—	—
2''-Ha	—	1.68 dt (16.0, 4.0)	—	—
2''-Hb	—	1.54 dd (16.0, 5.0)	—	—
3''-H	—	2.25	—	—
4''-H	—	3.67 br s	—	—
5''-H	—	3.93 q (7.0)	—	—
6''-CH_3	—	1.38 d (7.0)	—	—
3''-$N(CH_3)_2$	—	2.22 s	—	—
Solvent	$CDCl_3$	$CDCl_3$	$CDCl_3$-CD_3OD (10:1)	$CDCl_3$-CD_3OD (10:1)

Spectra were measured at 400 MHz. Chemical shifts are expressed by δ (ppm) (*J*=Hz) from internal TMS.

These compounds were purified according to the procedure previously described.[5] The aglycone was identified as α-citromycinone from its UV, MS (*m/z* 371 $(M+H)^+$) and ^1H and ^{13}C NMR spectra, while the sugars were found to be L-rhodosamine by a direct comparison with authentic samples (derived from aclarubicin) and its $[\alpha]_D$ value of $-45.8°$ (hydrochloride, *c* 0.1, H_2O) (literature 6: $-48.2°$, H_2O). From the mass spectral data, yellamycin B has two residues of L-rhodosamine, whereas yellamycins A and C each possess one residue of L-rhodosamine.

The chemical shift assignments of ^1H and ^{13}C NMR spectra of yellamycins were carried out by means of pulse technique, DEPT, ^1H-^1H and ^1H-^{13}C COSY, and are shown in Tables 1 and 2, respectively. On ^{13}C NMR the chemical shifts of C-7 of yellamycin A and C-10 of yellamycin C (73.7 and 70.2 ppm, respectively) shifted down approximately 4~6 ppm in comparison with those of α-citromycinone (67.5 and 66.0 ppm, respectively). These down-field shifts are attributable to the sugar linkage at C-7 for yellamycin A and C-10 for yellamycin C, respectively. Similarly yellamycin B has sugars at both C-7 and C-10. The position of the sugar linkage in yellamycin A and C was confirmed by the correlation *via* long range coupling (COLOC) experiment wherein a long range coupl-

Table 2. ^{13}C NMR chemical shifts of yellamycins and α-citromycinone.

Carbon	Yellamycin A	Yellamycin B	Yellamycin C	α-Citromycinone
1	119.50	119.51	119.29	121.3
2	136.88	136.87	136.64	137.0
3	125.23	124.95	124.64	125.0
4	162.84	162.73	162.30	162.7
4a	116.00	115.98	115.87	116.4
5	187.35	187.55[a]	187.42	188.0
5a	131.93	132.38	132.28	133.2
6	120.46	118.89	120.29	120.0
6a	142.98	146.67	149.09	147.0
7	73.74	74.02	66.78[a]	67.5
8	33.74	36.41	36.25	34.5
9	72.16	72.16	72.45	73.6
10	66.94	70.97	70.16	66.0
10a	134.00	132.15	131.25	132.4
11	162.26	162.17	161.49	162.3
11a	115.27	115.01	114.53	115.0
12	187.69	187.52[a]	187.42	187.9
12a	132.97	133.15	133.09	133.5
13	30.41	31.04	30.97	30.7
14	6.60	6.67	6.21	6.3
1′	98.81	99.81	96.18	—
2′	28.95	29.06	28.71	—
3′	59.47	59.53	58.80	—
4′	65.92	66.01	66.44	—
5′	66.59	66.44	66.62[a]	—
6′	17.05	17.06	16.85	—
3′-$N(CH_3)_2$	41.98	42.02	41.82	—
1″	—	96.32	—	—
2″	—	29.16	—	—
3″	—	59.71	—	—
4″	—	66.20	—	—
5″	—	66.57	—	—
6″	—	17.22	—	—
3″-$N(CH_3)_2$	—	42.02	—	—
Solvent	$CDCl_3$	$CDCl_3$	$CDCl_3$-CD_3OD[b]	$CDCl_3$-CD_3OD[b]

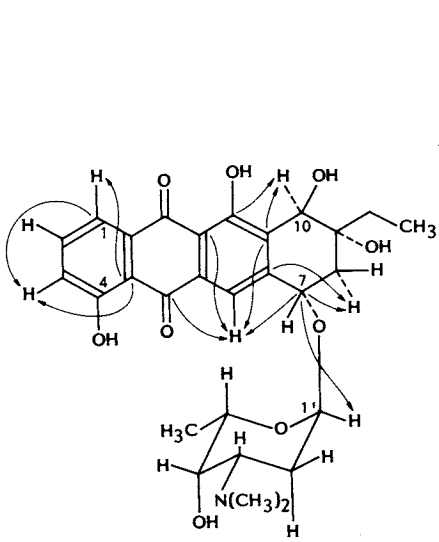
Spectra were measured at 100 MHz. Chemical shifts are expressed by δ (ppm) from internal TMS.

[a] Similar values may be interchanged.

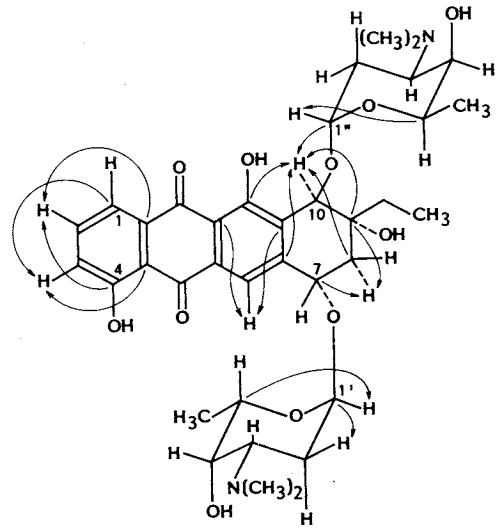
[b] 10:1.

Fig. 1. Structures of yellamycins.

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



Yellamycin A



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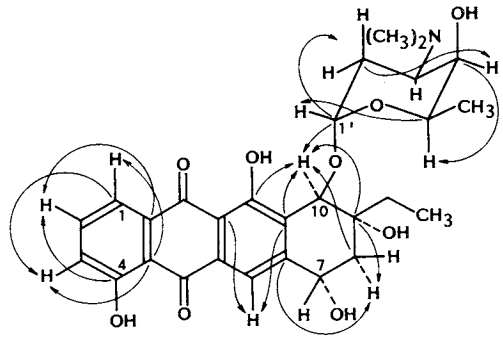

Yellamycin C

ing of C-7 (73.74 ppm) with the proton at C-1′ (5.35 ppm) was observed in yellamycin A and a long range coupling of C-1′ (96.18 ppm) with the proton at C-10 (5.03 ppm) was seen in yellamycin C (Fig. 1). Furthermore, the site of the sugar linkage in yellamycin C was confirmed by ^{1}H NMR analysis of its acetylated derivative which was determined as 4,7,11,4′-*O*-tetraacetylyellamycin C (*m/z* 695 $(M+H)^{+}$). A multiplet signal of H-7 in the tetraacetate was observed at 6.15 ppm instead of the signal at 4.86 ppm. On ^{1}H NMR the small coupling constants ($J=\sim 4$ Hz) of all anomeric protons in yellamycins indicate the configurations of the glycosidic bonds are all α.

These findings indicate that yellamycins A, B and C are 7-*O*-(α-L-rhodosaminyl)-α-citromycinone ($C_{28}H_{33}NO_9$), 7,10-*O*-di-(α-L-rhodosaminyl)-α-citromycinone ($C_{36}H_{48}N_2O_{11}$) and 10-*O*-(α-L-rhodosaminyl)-α-citromycinone ($C_{28}H_{33}NO_9$), respectively as illustrated in Fig. 1.

In vitro cytotoxicity and inhibition of DNA and RNA syntheses against the cell culture of murine L1210 leukemia were assayed according to the method as previously described.[7] The data are shown in Table 3. Yellamycin A had more potent cytotoxic activity than typical anthracycline antibiotics, aclarubicin and doxorubicin, or 6-hydroxyyellamycin A (β-rhodomycin I), and exhibited an almost equal inhibition of DNA and RNA synthesis. Yellamycin B exhibited little cytotoxicity whereas 6-hydroxyyellamycin B (β-rhodomycin II) had potent cytotoxic activity. Yellamycin C was also

Table 3. Inhibitory activity of yellamycins and other anthracyclines on the growth and nucleic acid synthesis of murine leukemic L1210 cell culture.

Compound	IC_{50} (μg/ml)			DNA/RNA
	Growth	DNA synthesis	RNA synthesis	
Yellamycin A	0.007	0.28	0.23	1.2
Yellamycin B	>1.0	6.00	0.55	10.9
Yellamycin C	>1.0	5.00	3.90	1.3
β-Rhodomycin I	0.01	0.21	0.06	3.5
β-Rhodomycin II	0.02	2.50	1.19	2.1
Aclarubicin	0.01	0.65	0.085	7.6
Doxorubicin	0.02	1.40	0.55	2.5

In the inhibition test for nucleic acid synthesis, the drugs were exposed for 60 minutes to L1210 cell culture (8×10^5 cells/ml) 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, the drugs were exposed for 48 hours to L1210 cell culture (5×10^4 cells/ml) and the viable cells were counted by coulter counter.

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

less active against L1210 cell culture as seen with CG11 and CG12[8,9] having sugar chain at C-10. This fact suggests that a sugar linkage only at C-7 plays an important function with respect to the biological action exhibited by α-citromycinone glycosides. The antitumor effect *in vivo* of yellamycin A on mice bearing leukemia L1210 was tested by daily ip administration from day 1 to 10. Yellamycin A had a maximum antitumor activity of 169% (T/C) at an optimum dose of 3.9 μg/mouse/day. Under the same conditions aclarubicin and doxorubicin had activities of 180 and 290% at an optimum dose of 75 and 25 μg/mouse/day.

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