MICROBIAL CONVERSION OF ANTHRACYCLINE ANTIBIOTICS

II. CHARACTERIZATION OF THE MICROBIAL CONVERSION PRODUCTS OF AURAMYCINONE BY *STREPTOMYCES COERULEORUBIDUS* ATCC 31276

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Auramycinone was subjected to microbial conversion by *Streptomyces coeruleorubidus* ATCC 31276, a producer of baumycins. As a result, auramycinone was converted to 11-hydroxyauramycinone and 9-methyl-10-hydroxydaunomycin (feudomycin D). The results implicate auramycinone as a presumptive intermediate in the biosynthesis of 9-methyl-10-hydroxydaunomycin by *S. coeruleorubidus*.

Daunomycin[1] is one of the most important drugs used in cancer chemotherapy. Its biosynthetic pathway was elucidated by YOSHIMOTO *et al.*[2] from an analysis of the structures of anthracyclines produced during microbial conversion of a presumptive intermediate, aklavinone, by the pigment-negative mutant of *Streptomyces coeruleorubidus* ME130-A4 (ATCC 31276). *S. coeruleorubidus* ATCC 31276 is reported to produce baumycins[4] which are 4′-substituted daunomycins. Aklavinone was first converted to ε-rhodomycinone (11-hydroxyaklavinone), followed by 10-decarbomethoxylation and further β-oxidation of the ethyl moiety at C-9.

Recently, we discovered a new anthracyclinone, auramycinone, as the aglycone of auramycins[3]. Its structure differs from aklavinone in having a methyl group in the C-9 side chain. This paper describes the result of studies on microbial conversion of auramycinone by *S. coeruleorubidus* ATCC 31276 and characterization of the transformation products.

Results and Discussion

S. coeruleorubidus ATCC 31276 was used throughout the present studies. Auramycinone was added to a 3-day culture and incubation was continued. Changes in the anthracycline components present during the incubation were followed by TLC (toluene - methanol, 9: 1). After 24 hours, the yellow color due to auramycinone changed to red. The red product, designated red pigment I, had a similar Rf value to that of auramycinone on TLC plates developed with toluene - methanol (9: 1). However, red pigment I disappeared at 48-hour incubation and a new component, designated red pigment II, was observed at Rf 0.09.

Red pigment I was isolated and characterized from 24-hour cultures. The change in color from yellow to red suggested substitution of a hydroxyl group for an aromatic proton in the anthraquinone. The UV and visible spectrum of red pigment I resembled that of 1-hydroxyauramycinone[5]. The molecular weight (414) and molecular formulae ($C_{21}H_{18}O_9$) confirmed that one oxygen atom had been added to auramycinone ($C_{21}H_{18}O_8$). In the ^1H NMR spectrum, the signal for the aromatic proton at C-11 of auramycinone was replaced by a singlet at δ 13.4. These results show red pigment I to be 11-hydroxy-

auramycinone. The conversion of auramycinone to 11-hydroxyauramycinone by *S. coeruleorubidus* is analogous to the conversion of aklavinone to ε-rhodomycinone.

Red pigment II, which appeared after 48-hour incubation, was isolated. TLC with the solvent system chloroform - methanol - acetic acid (20: 5: 1) gave an Rf value of 0.23, which was slightly lower than that of daunomycin (Rf 0.32). On hydrolysis in 0.1 N hydrochloric acid, red pigment II liberated L-daunosamine and the aglycone, which was isolated as red needles. This product showed a different Rf value, 0.25, on TLC from that of 11-hydroxyauramycinone (Rf 0.35) with the toluene - methanol (9: 1) solvent system. The molecular formulae was determined to be $C_{20}H_{18}O_8$ by high resolution mass spectrometry. In the ^1H NMR spectrum a signal for a methyl group, observed as a singlet at δ 1.32, indicated that the side chain of auramycinone at C-9 remained unchanged. A signal for methyl protons in the $COOCH_3$ group at C-10 disappeared and a new signal was generated at δ 5.56. This single-proton doublet exchanged with D_2O and was coupled with a proton at C-10, indicating that the methoxycarbonyl group of 11-hydroxyauramycinone had been changed to a hydroxyl group. A singlet (3H) at δ 4.00 was assigned to the OCH_3 group at C-4. On the basis of these results, the structure of the aglycone was 9-methyl-10-hydroxydaunomycinone. Among the known anthracyclinones, feudomycinone D[6], which was isolated as a minor component from the hydrolysate of a crude extract from a mutant strain of *S. coeruleorubidus* ME130-A4, corresponds to the aglycone of red pigment II. The identity of these compounds was confirmed by a comparison of their ^1H NMR spectra (Table 1).

That L-daunosamine was attached at the C-7 position was shown by comparing the ^{13}C NMR chemical shift of C-7 in the aglycone and in red pigment II (Table 2). Thus the structure of red pigment II was determined to be 9-methyl-10-hydroxydaunomycin. This glycoside was briefly referred to by MATSUZAWA *et al.*[7] as feudomycin D, but its physico-chemical properties were not reported.

The results indicate that 9-methyl-10-hydroxydaunomycin is biosynthesized from auramycinone through 11-hydroxyauramycinone by *S. coeruleorubidus* ATCC 31276, as illustrated in Fig. 1. Neither 11-hydroxyauramycinone glycoside nor 9-methyl-10-hydroxydaunomycinone was detected as a biosynthetic intermediate.

Table 1. Comparison of ^1H NMR chemical shifts of the aglycone of red pigment II and feudomycinone D.

Aglycone of red pigment II (DMSO-d_6)			Feudomycinone D* (DMSO-d_6)		
δ (ppm)	H	Assignment	δ (ppm)	H	Assignment
1.32	3H (s)	H-13	1.30	3H (s)	H-13
1.8~	2H (m)	H-8	1.7~	2H (m)	H-8
2.2			2.2		
4.00	3H (s)	OMe	3.90	3H (s)	OMe
4.57	1H (m)	H-10	4.50	1H (d)	H-10
4.80	1H (m)	H-7	4.75~	2H (m)	H-7
5.00	1H (s)	OH-9**	5.10		OH-7
5.27	1H (s)	OH-7**	5.20	1H (s)	OH-9
5.56	1H (d)	OH-10	5.48	1H (d)	OH-10
7.67	1H (dd)	H-3	7.5	1H (dd)	H-3
7.90	2H (m)	H-1, H-2	7.8	2H (m)	H-1, H-2

* Data cited from Ref. 6.

** Assigments for OH at C-7 and C-9 are as described in the Table, despite the difference from those of feudomycinone D.

Fig. 1. Proposed biosynthetic pathway for 9-methyl-10-hydroxydaunomycin.

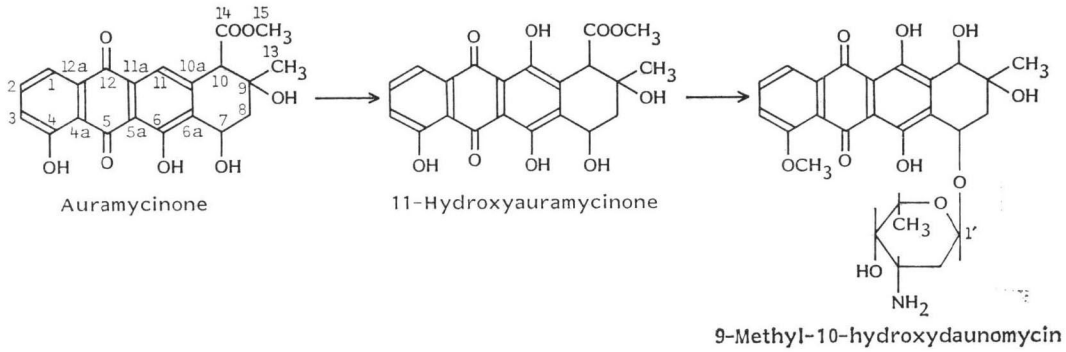

Table 2. ^{13}C NMR chemical shift assignments (ppm).

C	Red pigment II ($CDCl_3$)	Aglycone of red pigment II ($CDCl_3$)	C	Red pigment II ($CDCl_3$)	Aglycone of red pigment II ($CDCl_3$)
1	120.2	118.2	10a	(136.5)	(135.3)
2	138.0	136.8	7	72.4	63.0
3	120.5	118.8	8	36.3	35.6
4	162.6	161.3	9	71.3	70.6
6	(157.5)*	(156.4)	10	68.8	68.6
11	(157.5)	(155.8)	13	26.5	25.9
5	(187.5)	(187.2)	OMe	57.1	56.8
12	(188.5)	(187.9)	1′	101.7	
4a	121.6	119.9	2′	30.8	
12a	137.3	136.1	3′	46.4	
5a	(113.6)	(113.7)	4′	68.1	
11a	(113.0)	(113.0)	5′	68.0	
6a	(136.6)	(135.9)	Me-5′	17.0	

* Similar values in parenthesis may be interchanged.

Oki *et al.*[6] reported the discovery of feudomycin D (L-daunosaminyl feudomycinone D) from the cultured broth of a mutant strain of *S. coeruleorubidus* ME130-A4. Considering these results together with ours, it is likely that *S. coeruleorubidus* can produce auramycinone and also convert it to feudomycins. This activity parallels the formation of aklavinone and its conversion to daunomycin by this organism.

Experimental

Microbial Conversion

Spores scraped from an agar slant of *S. coeruleorubidus* ATCC 31276 were transferred to a 500-ml Erlenmeyer flask containing 100 ml of sterilized medium consisting of 1% soluble starch, 1% glucose, 3% soybean meal, 0.1% yeast extract (Daigo Eiyo-Kagaku Co.), 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.3% NaCl. The flask was incubated at 27°C for 72 hours on a rotary shaker (180 rpm). Five ml of the vegetative culture was transferred to 100 ml of conversion medium consisting of 4% sucrose, 2.5% soybean meal, 0.1% yeast extract, 0.25% NaCl and 0.3% $CaCO_3$. After three days incubation at 27°C with agitation at 180 rpm, 5 mg of auramycinone dissolved in 1 ml of methanol was added and the incubation continued for one or two days at 27°C. The culture was then harvested and analyzed.

Thin-layer Chromatography

Silica gel F_{254} plates (Merck Co.) were used. The solvent systems used to analyze the conversion products were toluene - methanol (9: 1) and chloroform - methanol - acetic acid (20: 5: 1). For sugar analysis, the plate was developed with 1-butanol - acetic acid - H_2O (4: 1: 1), sprayed with 5% *p*-anisaldehyde and 5% sulfuric acid in ethanol, and heated at 90°C for color development.

Isolation of Red Pigment I

Two liters of a culture which had been supplemented with 100 mg of auramycinone and incubated for a further 24 hours was extracted with 3 liters of chloroform - methanol (1: 1). The chloroform layer was separated and concentrated *in vacuo* to a small volume (*ca.* 1 ml). The concentrate was subjected to TLC (toluene - methanol, 9: 1), and the band which corresponded to red pigment I was scraped off and extracted with chloroform - methanol (9: 1). A total of 23.5 mg of red pigment I was isolated as a red powder.

Red pigment I: mp 177°C; $[\alpha]_D$ +60° (*c* 0.1, $CHCl_3$); ν_{max}^{KBr} cm^{-1} 1730, 1700, 1600; ^1H NMR ($CDCl_3$) δ in ppm 1.46 (3H, s, H-13), 1.7~2.2 (2H, m, H-8), 3.49 (1H, bs, OH-7), 3.73 (3H, s, H-15), 4.0 (1H, s, OH-9), 4.21 (1H, s, H-10), 5.31 (1H, m, H-7), 7.30 (1H, d, H-3), 7.69 (1H, t, H-2), 7.85 (1H, d, H-1), 12.06 (1H, s, OH-4), 12.88 (1H, s, OH-6), 13.40 (1H, s, OH-11); λ_{max}^{MeOH} ($E_{1cm}^{1\%}$) 234 (950), 252 (605), 295 (193), 494 (300), 528 (276), 576 nm (136); $\lambda_{max}^{0.1N\ NaOH\text{-}MeOH}$($E_{1cm}^{1\%}$) 242 (1,050), 285 (230), 558 nm (390); MS *m/z* 414 (M^+);

Anal. Calcd. for $C_{21}H_{18}O_9$: C 60.89, H 4.38, O 34.73.
Found: C 60.78, H 4.33, O 34.89.

Isolation of Red Pigment II

Two liters of a culture which was incubated for 48 hours after addition of 100 mg of auramycinone was extracted with 3 liters of chloroform - methanol (1: 1). The chloroform layer was separated and concentrated *in vacuo* to a small volume (*ca.* 1 ml). The concentrate was fractionated by TLC (chloroform - methanol - acetic acid, 20: 5: 1) and the band which corresponded to red pigment II was scraped off and extracted with chloroform - methanol (7: 3). A total of 12.3 mg of red pigment II was isolated as a red powder.

Red pigment II: mp 121°C; ν_{max}^{KBr} 1610, 1518; λ_{max}^{MeOH} ($E_{1cm}^{1\%}$) 234.5 (320), 253 (220), 290 (58), 480 (87), 497 (100), 532 (64), 576 nm (20); $\lambda_{max}^{0.1N\ NaOH\text{-}MeOH}$($E_{1cm}^{1\%}$) 237 (225), 248 (20), 290 (sh 35), 560 (90), 590 nm (85); MW 515;

Anal. Calcd. for $C_{26}H_{29}O_{10}N$: C 60.58, H 5.63, O 31.07, N 2.72.
Found: C 60.48, H 5.71, O 31.20, N 2.61.

Hydrolysis of Red Pigment II

Red pigment II (50 mg) was heated in 10 ml of 0.1 N hydrochloric acid at 90°C for 60 minutes. A red precipitate (20 mg) was recovered and crystallized from chloroform to give the aglycone of red pigment II (16 mg) as red needles.

Aglycone of red pigment II: mp 123~127°C; $[\alpha]_D$ +190° (*c* 0.1, MeOH); ν_{max}^{KBr} 1610, 1580; λ_{max}^{MeOH} ($E_{1cm}^{1\%}$) 234.5 (424), 253 (292), 290 (84), 480 (116), 497 (128), 532 (88), 576 nm (30); $\lambda_{max}^{0.1N\ NaOH\text{-}MeOH}$($E_{1cm}^{1\%}$) 237 (300), 248 (316), 290 (sh 64), 560 (128), 590 nm (120); ^1H NMR (DMSO-d_6) δ in ppm 1.32 (3H, s, H-13), 1.8~2.2 (2H, m, H-8), 4.00 (3H, s, OMe), 4.57 (1H, m, H-10), 4.80 (1H, m, H-7), 5.00 (1H, s, OH-9), 5.27 (1H, s, OH-7), 5.56 (1H, d, OH-10), 7.67 (1H, dd, H-3), 7.9 (2H, m, H-1, H-2); MS *m/z* 386 (M^+);

Anal. Calcd. for $C_{20}H_{18}O_8$: C 62.18, H 4.66, O 33.16.
Found: C 63.10, H 4.52, O 32.38.

The hydrochloric solution was neutralized and its sugar composition was examined by TLC. One spot was detected with sky blue to grayish blue color at Rf 0.24. This sugar was identified to be L-daunosamine by direct comparison of its $[\alpha]_D$ (−57°, *c* 0.1 in H_2O), melting point (165°C) and ^1H NMR spectrum with those of an authentic sample obtained by acid hydrolysis of daunomycin.

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