MICROBIAL CONVERSION OF ANTHRACYCLINONES TO CARMINOMYCINS BY A BLOCKED MUTANT OF *ACTINOMADURA ROSEOVIOLACEA*

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New anthracycline antibiotics, 1-hydroxy-11-deoxycarminomycin II and 11-deoxycarminomycin II were produced by a blocked mutant MuW1 of *Actinomadura roseoviolacea* from ε-pyrromycinone and aklavinone, respectively. We found that the enzyme catalyzing hydroxylation at the C-11 position was not lost but was down regulated in the strain MuW1.

Biosynthesis of anthracycline glycosides was extensively studied by OKI *et al.* They investigated the glycosidation steps of aclarubicins[1] and daunorubicin[2] in detail by the microbial conversion of anthracycline aglycones. Although structurally related to each other, carminomycin I[3] was only produced by *Actinomadura roseoviolacea*[4] and *Actinomadura carminata*, whereas daunorubicin was produced by *Streptomyces coeruleorubidus*, *Streptomyces peucetius* and some other *Streptomyces* strains. Thus, we were interested in the biosynthetic pathway of carminomycin I from the view point of producing organism-metabolite relationship.

During biosynthetic studies on the carminomycins, we isolated a blocked mutant MuW1 of *A. roseoviolacea* P40 which converted ε-rhodomycinone[5] to carminomycin II[3]. The parent strain produced mainly three anthracycline compounds: ε-Rhodomycinone, 7-deoxy-13-dihydrocarminomycinone[4] and carminomycin II. KOMIYAMA *et al.* described that daunorubicin and baumycins[6], structurally related to carminomycin II, were biosynthesized from aklavinone *via* ε-rhodomycinone in *S. coeruleorubidus*[7]. Therefore, MuW1 is useful for investigation of the glycosidation of anthracyclinones in *A. roseoviolacea*. We have studied the microbial conversion of anthracyclinones by MuW1, confirming the analogy between the biosynthetic pathway for the carminomycins and the baumycins. We wish to report herein the conversion of 6 anthracyclinones by MuW1, isolation of two new anthracyclines, 1-hydroxy-11-deoxycarminomycin II[8] and 11-deoxycarminomycin II, and mechanism of 11-hydroxylation by MuW1.

Materials and Methods

Isolation of Carminomycin-negative Mutants

Spore cells of *A. roseoviolacea* P40 from a 2-week old culture grown on YM agar (yeast extract 0.4%, malt extract 1%, glucose 0.4% and agar 2%, pH 7.0) were exposed to 1 mg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) in 0.2 M Tris-HCl (pH 8.5) with shaking at 37°C for 60 minutes. Spore cells were

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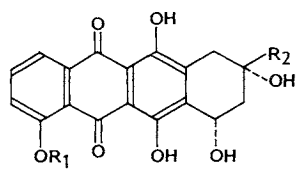
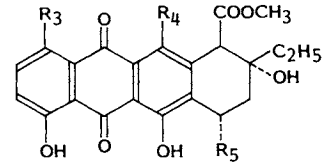
Fig. 1. Structures of anthracyclinones.





Carminomycinone	$R_1=H$	$R_2=COCH_3$	ε-Rhodomycinone	$R_3=H$	$R_4=R_5=OH$
13-Dihydrocarminomycinone	$R_1=H$	$R_2=CH(OH)CH_3$	ε-Pyrromycinone	$R_3=R_5=OH$	$R_4=H$
Daunomycinone	$R_1=CH_3$	$R_2=COCH_3$	Aklavinone	$R_3=R_4=H$	$R_5=OH$
			7-Deoxyaklavinone	$R_3=R_4=R_5=H$	

plated on YM agar after removing NTG, then about 500 colonies were isolated on YM agar and analyzed for the productivity of carminomycin II by cultivating them at 37°C for 4 days in 500-ml Erlenmeyer flasks containing 100 ml of KG medium: Glucose 2.5%, soybean meal 1.5%, yeast extract 0.2% and $CaCO_3$ 0.4%. Mutants which could not produce pigments were selected and then tested for their ability to produce carminomycin II upon addition of ε-rhodomycinone. As a result, a blocked mutant MuW1 which had the same physiological properties as the parent strain except for producing no anthracyclines was selected. MuW1 was used throughout this work.

Preparation of Aglycones

The following anthracyclinones were prepared by acid hydrolysis of the corresponding anthracyclines with 0.1 N HCl at 85°C for 30 minutes: Carminomycinone[3] and 13-dihydrocarminomycinone[9] from carminomycins, aklavinone from aclacinomycin A, and ε-pyrromycinone[10] from cinerubins[10]. ε-Rhodomycinone was isolated from the culture broth of *A. roseoviolacea* P40. 7-Deoxyaklavinone was prepared from aclacinomycin A by hydrogenolysis with H_2/Pd - $BaSO_4$. These anthracyclinones are shown in Fig. 1.

Microbial Conversion of Anthracyclinones and Extraction of Conversion Products

Strain MuW1 was cultured at 37°C for 48 hours in a shaken 500-ml Erlenmeyer flask containing 100 ml of a seed medium (starch 1%, molasses 1%, Polypeptone 1% and meat extract 1%, pH 7.2). One ml of this culture was then inoculated into 500-ml Erlenmeyer flasks containing 100 ml of KG medium. After 24, 48, 72, 96 hours, anthracyclinones were separately added to the flasks and cultivation was continued for an additional 48 hours. The conversion products were extracted from the fermentation broth by vigorous mixing with 100 ml of $CHCl_3$ - MeOH (9 : 1) mixture and the organic layer was evaporated to dryness.

Conversion of 7-Deoxyaklavinone

In order to examine whether MuW1 had lost the enzyme catalyzing 11-hydroxylation, 7-deoxyaklavinone was used as the substrate for conversion experiments. By the same procedure described above, 7-deoxyaklavinone was incubated with MuW1 and extracted. After 48 hours cultivation of MuW1, 7-deoxyaklavinone (5 mg) was added to the fermentation broth. In the case of the parent strain, cerulenin (40 mg/100 ml) which inhibits aglycone biosynthesis was added at 0 hour and 7-deoxyaklavinone (5 mg) was added after 48 hours cultivation. The reaction rate of 11-hydroxylation was measured by the absorbance at 492 nm.

Conversion of 11-Deoxycarminomycin II

By the same procedure described above, cerulenin and 11-deoxycarminomycin II were added at 0 time and after 48 hours, respectively, to the culture broth of the parent strain.

TLC Identification of the Conversion Products

In order to examine the formation of carminomycin II, each conversion product was chromatographed on a silica gel plate before and after acid hydrolysis (0.1 N HCl, 85°C, 30 minutes), and chromatographic

behavior was compared with the metabolites produced by the parent strain.

General

UV and visible spectra were recorded using a Shimadzu UV-300 spectrophotometer. MS were measured on a Jeol DX-300 spectrometer. IR spectra were taken with a Jasco A-102 IR spectrophotometer. NMR spectra were obtained on a Jeol GX-400 spectrometer with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz.

Results and Discussion

Conversion of Six Anthracyclinones to Glycosides

As shown in Table 1, ε-rhodomycinone, carminomycinone and 13-dihydrocarminomycinone were all converted to carminomycin II, whereas ε-pyrromycinone and aklavinone were transformed to 1-hydroxy-11-deoxycarminomycin II and 11-deoxycarminomycin II, respectively. Aglycones with a methoxycarbonyl group (ε-rhodomycinone, ε-pyrromycinone and aklavinone) gave glycosides in 24 hours of cultivation, but carminomycinone and 13-dihydrocarminomycinone were converted to glycosides only after 72 hours cultivation.

7-Deoxyaklavinone with no hydroxy group to be glycosylated was also transformed to the corresponding 11-hydroxy derivative by MuW1 and the parent strain at different reaction rates (Table 2). 11-Deoxycarminomycin II was recovered with no change.

Conversion of ε-Pyrromycinone and Aklavinone

Addition of ε-pyrromycinone (300 mg) to the culture (10 liters) of the mutant strain MuW1 after 48 hours gave a new anthracycline glycoside (MG1) and modified aglycones (A1 and A2). After centrifuging the culture broth, the mycelial cake was extracted with 2 liters of acetone. The extract was concentrated to a small volume *in vacuo* and the residual aqueous solution was then extracted with 1 liter of $CHCl_3$ - MeOH (9 : 1). The solvent layer was evaporated to dryness *in vacuo*, and then the residue was subjected to silica gel column chromatography. The column was developed with $CHCl_3$ to give A1 (10 mg) and A2 (0.5 mg)

Table 1. Microbial conversion of six anthracyclinones.

Compounds	Conversion to glycosides				Converted products
	24*	48	72	96	
Aklavinone	+	+	+	+	11-Deoxycarminomycin II
ε-Rhodomycinone	+	+	+	+	Carminomycin II
ε-Pyrromycinone	+	+	+	+	1-Hydroxy-11-deoxycarminomycin II
Carminomycinone	−	−	+	+	Carminomycin II
13-Dihydrocarminomycinone	−	−	+	+	Carminomycin II
7-Deoxyaklavinone	−	−	−	−	7-Deoxy-ε-rhodomycinone

* Addition time (hours).

Table 2. Conversion of 7-deoxyaklavinone by MuW1 and parent strain.

	Rate of 11-hydroxylation (%)				
	8 hours	16 hours	24 hours	32 hours	48 hours
Parent	100	100	100	100	100
MuW1	0	50	70	70	100

in pure forms, and subsequent elution with $CHCl_3$-MeOH (10:1) gave a crude sample of MG1 (5 mg). Further purification was achieved by TSK HW-40 column chromatography (50×2 cm i.d.) with MeOH-AcOH (100:1) to give pure MG1 (3 mg).

Aklavinone (300 mg) added to the culture (10 liters) of mutant strain MuW1 after 48 hours cultivation was converted to a new anthracycline glycoside MG2 (2 mg) which was purified by similar procedures just described for ε-pyrromycinone.

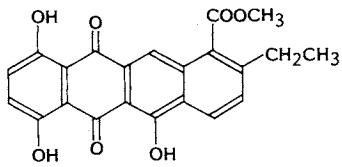
Identification of A1 and A2

A1 and A2 proved to be identical with η-pyrromycinone[11] and ζ-isorhodomycinone[12], respectively (Fig. 2), by UV, mass, ^1H NMR spectral analysis.

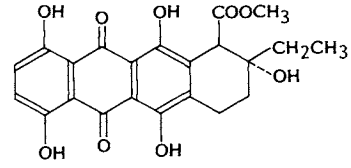
Structural Elucidation of MG1 and MG2

The physico-chemical properties of MG1 and MG2 are listed in Table 3. The molecular formula

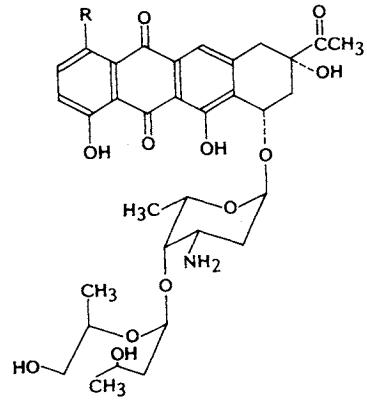
Fig. 2. Structures of converted products.



η-Pyrromycinone



ζ-Isorhodomycinone



11-Deoxycarminomycin II R=H
1-Hydroxy-11-deoxycarminomycin II R=OH

Table 3. Physico-chemical properties of MG1 and MG2.

	MG1	MG2
Appearance	Red powder	Red brown powder
MP (°C)	210~212	105~110
$[\alpha]_D^{25}$	+20° (*c* 0.05, $CHCl_3$)	+78° (*c* 0.1, MeOH)
UV λ_{max}^{MeOH} nm (ε)	235 (45,300), 259 (24,700), 294 (10,200), 492 (14,200)	226 (18,200), 257 (11,300), 286 (4,600), 428 (5,400)
MW (*m/z*)	682 $(M+Na)^+$ (FD-MS)	644 $(M+1)^+$ (FAB-MS)
Molecular formula	$C_{33}H_{41}NO_{13}$	$C_{33}H_{41}NO_{12}$
IR cm^{-1}(KBr)	1710, 1600, 1010	1710, 1610, 1010

FD-MS: Field desorption MS.
FAB-MS: Fast atom bombardment MS.

$C_{33}H_{41}NO_{13}$ of MG1 corresponds to that of carminomycin II. The ^1H NMR spectrum of MG1 (Fig. 3) was superimposable on that of carminomycin II except for aromatic protons. Thus the ^1H NMR spectrum of carminomycin II showed the signals assignable to ε-rhodomycinone at δ 7.16 (d, J=8.0 Hz, 3-H), 7.61 (dd,. J=8.0 and 8.0 Hz, 2-H) and 7.68 (d, J=8.0 Hz, 1-H), whereas MG1 showed proton signals corresponding to ε-pyrromycinone at δ 7.30 (2H, s, 2-H and 3-H) and 7.65 (s, 11-H). These results indicate that MG1 is 1-hydroxy-11-deoxycarminomycin II (Fig. 2).

The UV spectral data for MG2 are identical to those of aclarubicin. Acid hydrolysis of MG2 with 0.1 N HCl at 85°C for 30 minutes yielded 4-*O*-demethyl-11-deoxydaunomycinone[13] as the aglycone. The ^1H NMR spectrum of MG2 (Fig. 4) was identical to that of carminomycin II except for a new signal

Fig. 3. ^1H NMR spectrum of 1-hydroxy-11-deoxycarminomycin II (400 MHz in $CDCl_3$).

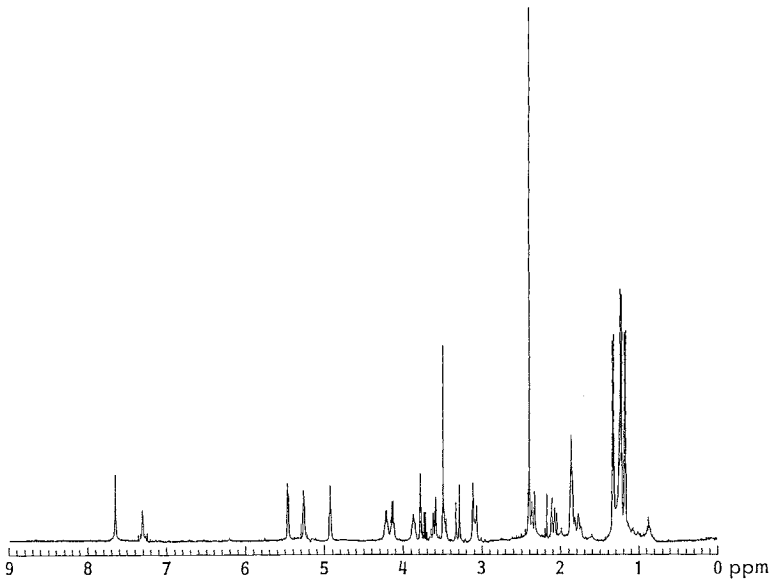
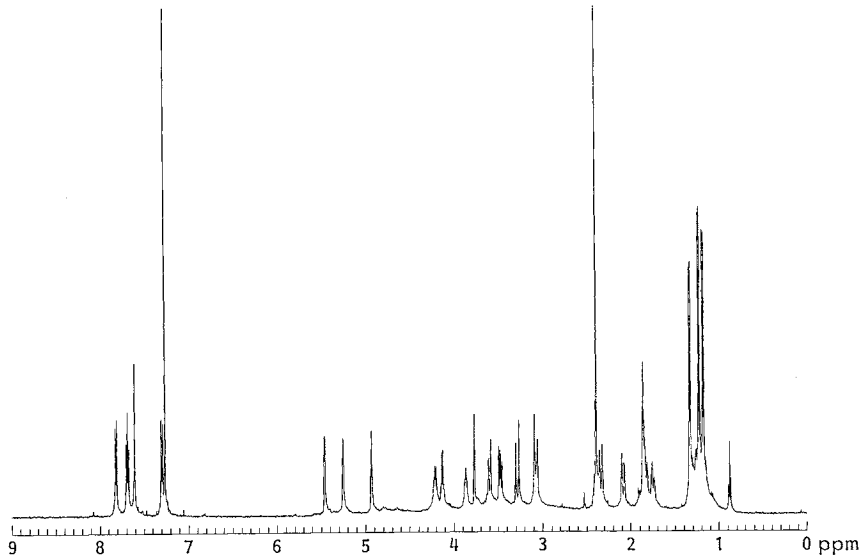


Fig. 4. ^1H NMR spectrum of 11-deoxycarminomycin II (500 MHz in $CDCl_3$).



at δ 7.60 (s, 11-H). In agreement with this, the molecular weight of MG2 differed from that of carminomycin II by 16, corresponding to replacement of the hydroxyl group of carminomycin II with a hydrogen atom in MG2. These results show that MG2 is 11-deoxycarminomycin II (Fig. 2). We had already obtained the same compound from the cultured broth of a blocked mutant Ru7062 of *A. roseoviolacea*[14].

The result of microbial conversion of anthracyclinones by *A. roseoviolacea* was the same as that by *S. coeruleorubidus* except for 11-hydroxylation. KOMIYAMA *et al.* reported that 11-hydroxylation did not take place in the case of glycosylated aglycones[6] in *S. coeruleorubidus*, and we obtained the same result with *A. roseoviolacea*. Therefore, the glycosidation takes place predominantly in MuW1, whose enzyme activity catalyzing hydroxylation at the C-11 position is lower than in the parent strain. These results show that the biosynthetic pathway from aklavinone to carminomycin II in *A. roseoviolacea*, especially the substrate specificity of 11-hydroxylation enzyme, is very similar to that from aklavinone to baumycins in *S. coeruleorubidus*.

We have previously obtained a blocked mutant Ru7062 which had completely lost the enzyme catalyzing 11-hydroxylation. On the other hand, as shown in Table 2, weak 11-hydroxylation enzymatic activity was observed with MuW1. These results would suggest that two separate and distinct enzymes catalyzing 11-hydroxylation existed in *A. roseoviolacea*. Probably it seems that mutant Ru7062 has completely lost two enzyme activities by double mutations and MuW1 has lost only one of those enzyme activities (the major one).

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