MICROBIAL CONVERSION OF ε-PYRROMYCINONE AND ε-ISORHODOMYCINONE TO 1-HYDROXY-13-DIHYDRODAUNOMYCIN AND N-FORMYL-1-HYDROXY-13-DIHYDRODAUNOMYCIN AND THEIR BIOACTIVITIES

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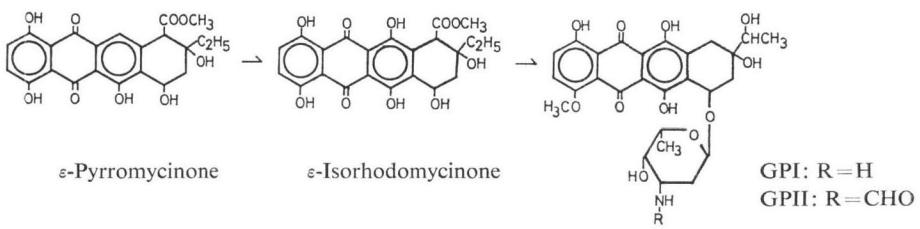
New anthracycline antibiotics, 1-hydroxy-13-dihydrodaunomycin and N-formyl-1-hydroxy-13-dihydrodaunomycin were biosynthesized by a blocked mutant of *Streptomyces coeruleorubidus* ME130-A4 from ε-pyrromycinone or ε-isorhodomycinone.

In studying the biosynthesis of new anthracycline antibiotic baumycins[1,2], structurally related to daunomycin, blocked mutants of *Streptomyces coeruleorubidus* ME130-A4 which required exogenous ε-rhodomycinone for baumycin production were isolated. It has been reported that ε-rhodomycinone exists as a non-glycosidic component in the fermentation broth of daunomycin producers such as *Streptomyces peucetius*[3] and *Streptomyces* PD Mycology No. J566[4] and a baumycin-producing strain ME130-A4[1]. We have confirmed that this aglycone (or its demethyl derivative) should be a biogenetic precursor of baumycin and daunomycin.[5] ^{14}C-Labeled aklavinone and ε-rhodomycinone were converted to baumycins[6]. This was also supported by isolation of ε-rhodomycinone-accumulating blocked mutants from baumycin- and daunomycin-producing strains (unpublished data). In contrast to ε-rhodomycinone, neither daunomycinone nor dihydrodaunomycinone was converted to baumycins and daunomycin[5].

In this paper we describe the conversion of ε-pyrromycinone (1-hydroxyaklavinone) and ε-isorhodomycinone (1,11-dihydroxyaklavinone) to novel anthracyclinone glycosides by a blocked mutant of *S. coeruleorubidus* ME130-A4, as shown in Fig. 1.

Fig. 1. Microbial conversion by a blocked mutant of *S. coeruleorubidus* ME130-A4.



Materials and Methods

Isolation of blocked mutants

Spores of *S. coeruleorubidus* ME130-A4 were collected from one-week culture grown on YS agar (0.3% yeast extract, 1% soluble starch and 1.5% agar, pH 7.2), suspended in 10 ml of sterilized physiological saline to give about 10^9 cells/ml and irradiated with ultraviolet light (98.5% killing rate). About 500 colonies were isolated on YS agar and tested for baumycin production by shaking the culture at 28°C for 5 days in a 500-ml Erlenmeyer flask containing 50 ml of the medium as described below. Mutants which were incapable of producing acetone-extractable reddish-brown pigments were selected and further tested for their ability to produce baumycins and related pigments in media to which ε-rhodomycinone was added. Thus, strains 1U-222 and 1U-479 were obtained as blocked mutants and the former was employed throughout the work in this paper.

Aglycones

ε-Pyrromycinone was obtained by acid hydrolysis of rhodirubins[7] with 0.1 N HCl at 85°C for 60 minutes, and ε-isorhodomycinone was isolated from the culture broth of a blocked mutant of *Actinomyces roseoviolaceus* ISP 5277[8] which accumulated mainly ε-rhodomycinone and ε-isorhodomycinone.

Microbial conversion

Strain 1U-222 was grown at 28°C for 3 days on a rotary shaker (220 rpm) in a medium (100 ml/500-ml Erlenmeyer flask) consisting of 1.5% soluble starch, 1% glucose, 3% soybean meal, 0.1% yeast extract, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.3% NaCl, pH 7.4. One ml of the seed culture was added to a 500-ml Erlenmeyer flask containing 50 ml of the following medium: 4% sucrose, 2.5% soybean meal, 0.1% yeast extract, 0.25% NaCl, 0.3% $CaCO_3$, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.0005% $MnCl_2 \cdot 4H_2O$, 0.0005% $ZnSO_4 \cdot 7H_2O$, pH 7.4. After 90-hour cultivation at 28°C on a rotary shaker, 1 ml of the methanol solution of ε-pyrromycinone or ε-isorhodomycinone (1 mg/ml) was added to a flask and the cultivation was further continued for 48 hours.

Growth and nucleic acid biosynthesis of the cultured L1210 leukemia cells

L1210 leukemia cells grown in RPMI1640 medium containing 20% calf serum for 1 day at 37°C in a CO_2 incubator were collected by centrifugation at 1,000 rpm for 2 minutes. The collected cells were resuspended in a warmed fresh medium (8.7×10^5 cells/ml) and 0.8 ml of the cell suspension was incubated with 0.1 ml containing the test compound at various concentrations at 37°C for 15 minutes prior to the addition of 0.1 ml of ^{14}C-labeled uridine or thymidine (0.05 μCi/ml). After incubation for 60 minutes, 1.0 ml of cold 10% trichloroacetic acid (TCA) was added and centrifuged at 2,000 rpm for 5 minutes and the precipitate was washed twice with 5% TCA solution. The radioactivity of the acid-insoluble fraction was determined in BRAY's scintillator by an Aloka LSC-653 scintillation spectrometer. The cell growth in the presence of drugs at various concentrations was measured by counting the number of cells in duplicate tubes on day 2.

rec Assay for testing mutagenicity

Instead of the streak test method employed by KADA *et al.*,[9] a cup diffusion method was used for the quantitative assay of bacterial growth[10]. Commercial bouillon supplemented with 20 μg/ml of each L-tryptophan and L-arginine was used as the medium. Twenty ml of a basal layer containing 1.5% agar were solidified in a Petri dish (9 cm in diameter), and 4 ml of the melted top layer agar (1.5% Difco agar) containing either *rec*$^-$ (*Bacillus subtilis* M45T, *arg*$^-$) or *rec*$^+$ (*B. subtilis* H17A, *trp*$^-$) cells (one drop of overnight-shaking culture at 28°C to 50 ml of the top layer) were overlayed. After permitting the diffusion of the sample into the agar plate at 4°C for 2 hours, the plates were incubated at 28°C for 20 hours and the diameter of the growth-inhibition zone was measured.

General

Melting points (uncorrected) were determined on a Kofler hotstage microscope. UV spectra were determined on a Hitachi EPS-3T and IR spectra on a Hitachi EP1-G2 spectrophotometer (KBr pellets). NMR spectra were recorded on a Varian XL-100 spectrometer or a Hitachi R-24 spectrometer. Chemical shifts were expressed in values (ppm) with tetramethylsilane as an internal standard. Abbreviation:

s=singlet, d=doublet, t=triplet, q=quartet, bs=broad singlet and m=multiplet. The mass spectra were determined by a Hitachi RMU-6 mass spectrometer with a direct inlet system.

Thin-layer chromatography was carried out on silica gel plate (Kieselgel $60F_{254}$, E. Merck & Co.). Solvent systems were as follows: S1: chloroform - methanol (20: 1); S2: chloroform - methanol (10: 1); S3; chloroform - methanol - acetic acid (80: 20: 4); S4: benzene - acetone - formic acid (100: 10: 1).

Total acid hydrolysis of anthracyclines was performed with 0.1 N HCl at 85°C for 30 minutes. After extraction of aglycone with chloroform, the aqueous layer was neutralized with $AgCO_3$ and the precipitate was centrifuged off. The supernatant was concentrated and chromatographed on silica gel plate with a solvent of 1-butanol - acetic acid - water (4: 1: 1) and then the sugar residues were detected with *p*-anisaldehyde[11] and ninhydrin reagents.

Results and Discussion

Isolation and Purification of Conversion Products

Conversion of ε-Pyrromycinone

ε-Pyrromycinone (600 mg) was added to the growing culture (30 liters) of mutant strain 1U-222 of *S. coeruleorubidus* ME130-A4 and converted to new anthracyclinone glycosides: GPI, II and III, and the structurally modified aglycone. After separation of the mycelium, the crude pigments were obtained separately from the mycelium and filtrate by extraction with 10 liters of acetone and with 8 liters of chloroform, respectively. The acetone extract from mycelium containing glycosidic products (GP) I and III and aglycones was extracted with 2 liters of chloroform after concentration and was subjected to chromatography on a Sephadex LH-20 column (5×68 cm) with a mixture of chloroform - methanol (1: 2). Two pigment bands separated: the first band contained GPI and III, and the second was a mixture of aglycones consisted of ε-pyrromycinone and its structurally modified aglycone. The glycosidic components were thereafter rechromatographed on a Sephadex LH-20 column (4×68 cm) using a mixture of toluene - methanol (3: 1). The active eluate was evaporated to dryness and the residue was dissolved in 50 ml of toluene followed by three successive extractions with 60 ml of 0.1 M acetate buffer (pH 3.1). GPIII (5 mg) was extracted with 200 ml of chloroform from the acidic aqueous layer after adjusting pH to 7.0. The GPI remaining in the aqueous layer was extracted with 100 ml of 1-butanol, evaporated to dryness, dissolved in 60 ml of 0.02 M acetate buffer (pH 5.0) and applied to a CM-cellulose column (1.5×5 cm) equilibrated with the same buffer. After washing with the buffer, GPI was eluted with 0.02 N HCl, adjusted to pH 7.0 with 1 N NaOH and re-extracted with 1-butanol. Reddish pure GPI (23.5 mg) was obtained after further repetition of the chromatography on Sephadex LH-20 column (2×60 cm) with methanol. Besides GPI and GPIII, the second pigment band containing ε-pyrromycinone and the structurally modified aglycone, which was obtained by the first Sephadex LH-20 column chromatography of mycelial extract, was further purified by preparative thin-layer chromatography on a silica gel plate with a benzene - acetone - formic acid (100: 10: 1) solvent system and subsequently by rechromatography on Sephadex LH-20 column (3.5×40 cm) with methanol. About 80 mg of the structurally modified aglycone were obtained.

On the other hand, GPII was extracted with chloroform from the cultural filtrate and purified by Sephadex LH-20 column (5×68 cm) chromatography with toluene - methanol (3: 1). The active eluate was evaporated to dryness, and the residue was dissolved in 30 ml of 0.1 M acetate buffer (pH 3.1). After removing insoluble materials by centrifugation and adjusting the pH to 6.5, the aqueous solution was extracted three times with 60 ml of chloroform. The extract was subjected to preparative silica gel thin-layer chromatography. GPII band eluted with chloroform - methanol mixture (5: 1) was further chro-

matographed on Sephadex LH-20 column (1.8×42 cm) with methanol. Pure GPII (16.2 mg) was obtained.

Conversion of ε-Isorhodomycinone

ε-Isorhodomycinone (800 mg) was also converted by the mutant strain 1U-222 to GPI (31.6 mg), II (23.8 mg) and III (trace) by similar procedures to those used for ε-pyrromycinone. The modified aglycone described above was identified as ε-isorhodomycinone.

Physicochemical Properties and Structures of Conversion Products GPI, II and the Structurally Modified Aglycone

Total acid hydrolysis of GPI, II and III gave a new anthracyclinone, and daunosamine which was identified by thin-layer chromatographic comparison with daunosamine obtained from authentic daunomycin (Rf 0.32 with S4). The physicochemical properties of GPI and II, their aglycones and a modified aglycone were listed in Table 1, and their structures were determined as follows (Fig. 1).

Aglycone

GPI (31 mg) was hydrolyzed with 0.1 N HCl (50 ml) at 85°C for 60 minutes and purified by preparative thin-layer chromatography using a chloroform - methanol (20: 1) mixture and by Sephadex LH-20 column chromatography (2.0×40 cm) with methanol. Pure red crystalline aglycone (13.7 mg) was obtained from benzene.

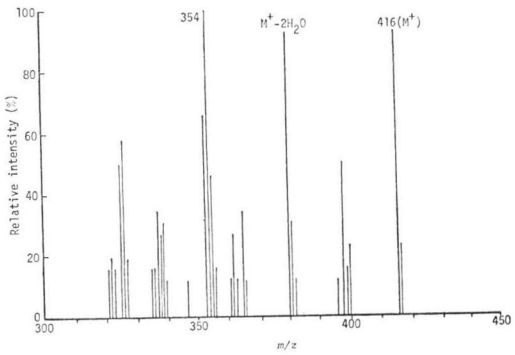
The molecular formula $C_{21}H_{16}O_9$ of the aglycone from GPI was established by elemental analysis and the mass spectrum (M^+ 416) as shown in Fig. 2, which suggested the structure containing an additional oxygen atom to 13-dihydrodaunomycinone. The IR spectrum resembled that of 13-dihydrodaunomycinone with a chelated carbonyl at 1590 cm^{-1}. The absence of absorption at 1740 cm^{-1}

Table 1. Physicochemical properties of conversion products.

Conversion products		GPI	GPII	Aglycone from GPI (1-hydroxy-13-dihydro-daunomycinone)	Modified aglycone (ε-isorhodo-mycinone)
Nature		Red powder	Red powder	Red powder	Deep red powder
m.p. (°C)		188~190	179~180	216~219	219~227
MW (MS)		—	—	416	444
Molecular formula		$C_{27}H_{31}NO_{11}$	$C_{28}H_{31}NO_{12}$	$C_{21}H_{20}O_9$	$C_{22}H_{20}O_{10}$
Anal. Found (%)		C 59.14, H 5.72 N 2.68, O 32.11	C 58.78, H 5.46 N 2.59, O 33.1	C 60.92, H 4.90 O 34.18	C 59.39, H 4.51 O 36.10
Calcd. (%)		C 59.45, H 5.69 N 2.57, O 32.29	C 58.64, H 5.41 N 2.44, O 33.51	C 60.77, H 4.82 O 34.41	C 59.46, H 4.50 O 36.03
λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)		240 (720), 285 (123), 520 (239), 543 (216), 600 (40)	240 (760), 285 (123), 520 (258), 546 (241), 600 (61)	240 (1132), 285 (190), 520 (402), 543 (343),	239 (1150), 296 (193), 488 (313), 510 (414), 520 (443)
cm^{-1} (KBr)		3400, 1590, 1260, 1200, 1020, 980	3400, 1678, 1590, 1270, 1200, 1020, 980	3400, 1590, 1290, 1265	3455, 1730, 1590, 1280, 1255
Rf	S1	—	—	0.27	0.79
	S2	0.00	0.19	0.43	0.98
	S3	0.10	0.54	—	—
	S4	—	—	0.02	0.34

(ester carbonyl) excluded the presence of a carbomethoxyl group at C-10. The PMR spectrum (100 MHz, $CDCl_3$) revealed one methyl (δ 1.32, d, J=6Hz, CH_3-14) coupled with one methine (δ 3.7~3.9, m, H-13), two methylenes (δ 2~3.3, m, CH_2-8, CH_2-10), an aromatic methoxyl (δ 4.03, OCH_3-4), a methine (δ 5.25, m, CH-7) coupled with a methylene, two aromatic protons (δ 7.32, d, J=9Hz and δ 7.44, d, J=9Hz, CH-2 and CH-3) and three hydrogen bonded phenolic hydroxyls (δ 12.56, δ 12.69 and δ 14.12). In the PMR spectrum of ε-pyrromycinone having two hydroxyl groups at C-1 and C-4, two aromatic vicinal protons at C-2 and C-3 appear as a singlet peak at δ 7.18,[12] but that of GPI aglycone shows two splitting doublet peaks with a big coupling constant (J=9Hz) for two aromatic vicinal protons at C-2 and C-3. This indicates the presence of different functional groups, *i.e.*, hydroxyl at C-1 and methoxyl at C-4, in the GPI aglycone. Thus the aglycone was determined to be 1-hydroxy-13-dihydrodaunomycinone.

Fig. 2. Mass spectrum of 1-hydroxy-13-dihydrodaunomycinone.



GPI

The molecular formula obtained by the elemental analysis was $C_{27}H_{31}NO_{11}$ corresponding to that of daunosaminyl 1-hydroxy-13-dihydrodaunomycinone. The PMR spectrum (Fig. 3) showed the signals assigned to 1-hydroxy-13-dihydrodaunomycinone with additional daunosamine as a sugar residue linked at C-7: δ 5.5 (H-1′, bs), δ 1.82~2.0 (CH_2-2′, m), δ 4.2 (H-3′, m), δ 3.73 (H-4′, bs), δ 4.2 (H-5′, overlapped with H-3′ and OH-4′) and δ 1.3 (CH_3-6′, d, J=6Hz), and thus GPI was identified as 1-hydroxy-13-dihydrodaunomycin (Fig. 1).

GPII

The PMR spectrum (Fig. 4) of GPII ($C_{28}H_{31}NO_{12}$) was superimposable on that of GPI except for

Fig. 3. PMR spectrum of 1-hydroxy-13-dihydrodaunomycin (100 MHz in 1: 1, $CDCl_3$ - CD_3OD).

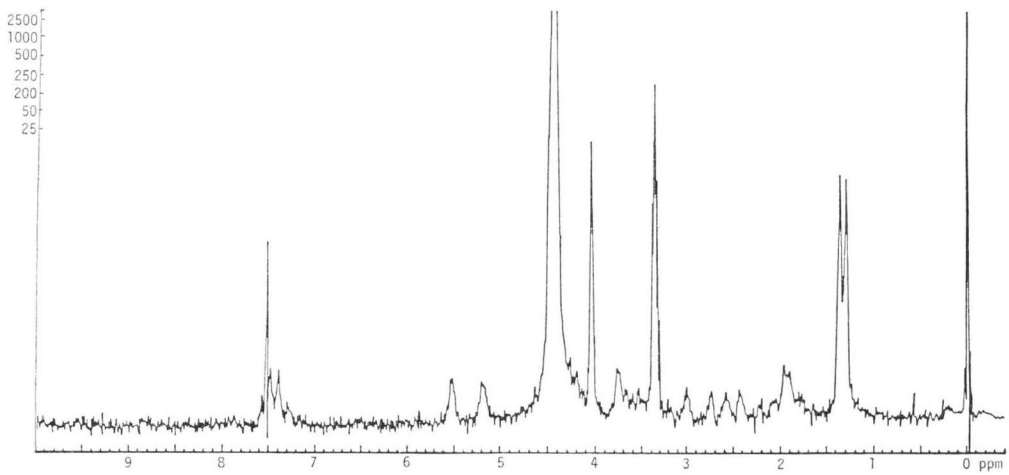
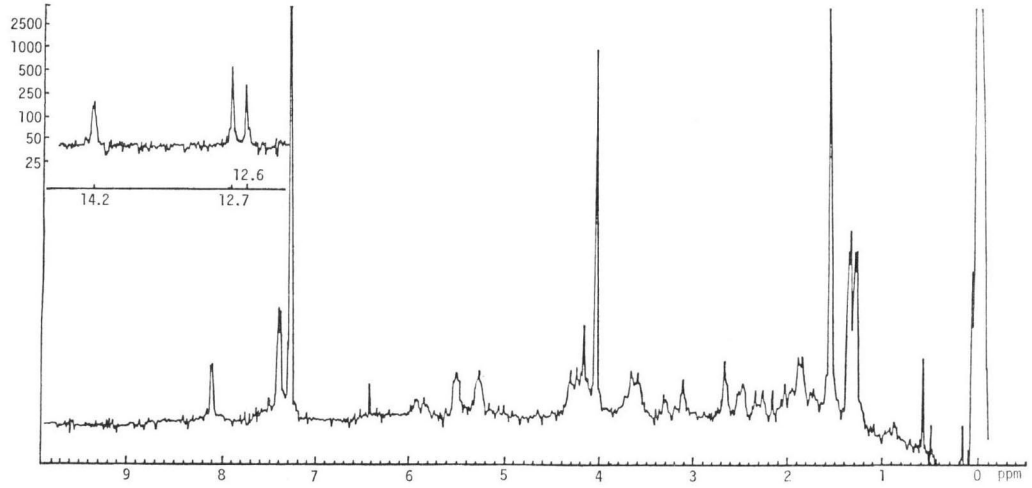


Fig. 4. PMR spectrum of N-formyl-1-hydroxy-13-dihydrodaunomycin (100 MHz in $CDCl_3$).



additional signals at δ 5.9 (NH-3′, broad doublet, J=8Hz) and δ 8.1 (CHO, d, slightly broad singlet) which were assigned to the N-formyl group. The Cl-MS spectrum showed a fragment ion peak at m/z 176 (relative intensity, 13.3%) corresponding to N-formyldaunosamine ($C_7H_{13}NO_4$) and the base peak at m/z 158 ($C_7H_{13}NO_4$–O_1). The IR spectrum also showed the presence of an N-formyl group at 1678 cm^{-1}. From these results, the structure of GPII was proposed to be N-formyl-1-hydroxy-13-dihydrodaunomycin.

GPIII

GPIII has not been identified yet because of poor yields (m.p. 142~146°C). However, qualitative TLC analysis after acid hydrolysis revealed that at least GPIII contained 1-hydroxy-13-dihydrodaunomycinone and daunosamine. The Rf value of GPIII (0.33) was larger than that of GPI (0.10) in the S3 solvent system. Since baumycins A1 and B1, the main products of the parent strain, have the structure of derivatives of daunomycin, GPIII was suggested to be a baumycin-type analog of GPI.

Structurally Modified Aglycone (ε-Isorhodomycinone)

The molecular formula $C_{22}H_{20}O_{16}$ (MW 444) was established by the elemental analysis and the mass spectrum (M^+ 444). The PMR spectrum showed one ethyl (δ 1.14, t, CH_3-14 and δ 1.67, q, J= 7 Hz, CH_3-13), one methylene (δ 2.28, m, CH_2-8) coupled with one methine (δ 5.34, m, CH-7), two hydroxyl (δ 3.48, d, OH-7 and δ 3.79, s, OH-9), one methoxyl (δ 3.73, s, OCH_3-16), one methine (δ 4.28, s, CH-10), two aromatic protons (δ 7.32, CH-2 and CH-3) in singlet peak, and four hydrogen bonded phenolic hydroxyls (δ 12.32, δ 12.34, δ 12.82 and δ 13.60). These signals were assigned to ε-isorhodomycinone. The IR spectrum indicated the presence of chelated carbonyl (1590 cm^{-1}), and ester carbonyl (1730 cm^{-1}) and ether carbonyl (1280 cm^{-1}), which indicated carbomethoxyl group at C-10. UV and visible spectra and melting point of the structurally modified aglycone (m.p. 219~227°C) also agreed with those of ε-isorhodomycinone (m.p. 227~229°C) in the literature.[13] The identity was further confirmed by cochromatography on silica gel thin-layer with an authentic sample (Rf 0.79 and 0.34, in S1 and S4 systems, respectively).

Microbial conversion of [9-^{14}C] and [16-^{14}C] labeled aklavinone by strain 1U-222 demonstrated that aklavinone is the biosynthetic precursor of daunomycinone glycosides[6], in which hydroxylation at C-11

Table 2. Biological properties of GPI, II and daunomycin.

		GPI*	GPII*	Daunomycin
rec Assay for mutagenicity**	Antimicrobial activity (μg/ml) on			
	B. subtilis M 45 T (*rec*$^-$)	19	100	9
	B. subtilis H 17 A (*rec*$^+$)	82	100	38
	rec Potency index (*rec*$^+$/*rec*$^-$)	4.7	—	4.2
50% Inhibition concentration (μg/ml) for L 1210 cultured cells:	Cell growth on day 2	0.023	0.5	0.036
	^{14}C-Uridine incorporation	0.59	10	0.18
	^{14}C-Thymidine incorporation	1.8	10	0.3
	Inhibition ratio (DNA/RNA)	3.1	—	1.7

* GPI; 1-Hydroxy-13-dihydrodaunomycin, GPII: N-Formyl-1-hydroxy-13-dihydrodaunomycin
** Concentration(μg/ml) that gives a 15-mm growth inhibition zone.

and subsequent decarbomethoxylation at C-10 of aklavinone occur to form daunomycinone *via* ε-rhodomycinone at the first step of glycosidation. The modified aglycone (ε-isorhodomycinone) was thus first produced by hydroxylation at C-11 of ε-pyrromycinone and then new anthracyclinone glycosides, 1-hydroxy-13-dihydrodaunomycin and N-formyl-1-hydroxy-13-dihydrodaunomycin, were biosynthesized by the mutant strain 1U-222 through glycosidation of ε-isorhodomycinone as well as the biosynthesis from ε-rhodomycinone to daunomycin and baumycins in *S. coeruleorubidus* ME130-A4[5].

Biological Properties of GPI and GPII

GPI and GPII were tested for the antimicrobial activity against *rec*$^+$ and *rec*$^-$ strains of *B. subtilis* and the inhibitory effects on growth and nucleic acid biosynthesis of cultured leukemic L1210 cells in comparison with daunomycin, as shown in Table 2.

The potency index for mutagenicity of the compounds was expressed by the *rec*$^+$/*rec*$^-$ ratio of concentration (μg/ml) which produced a 15-mm growth inhibition zone against *rec*$^+$ and *rec*$^-$ strains. GPI showed weaker antimicrobial activity against *B. subtilis* than daunomycin, but almost the same index was observed as daunomycin had on the *rec* assay system. GPI inhibited 50% of ^{14}C-uridine incorporation into cold TCA insoluble fraction of L1210 cells at the concentration of 0.59 μg/ml, while this was less effective in inhibiting ^{14}C-thymidine uptake: 50% inhibition at 1.8 μg/ml. Inhibitory effects of GPI on nucleic acid biosynthesis were weaker than those of daunomycin, but this antibiotic showed stronger inhibition on the cell growth. GPII which is N-formyl GPI was less active on bacterial and mammalian cells, and 50% inhibition concentration for the growth of L1210 cells required approximately 20 times that of GPI.

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