INTENSELY POTENT ANTHRACYCLINE ANTIBIOTIC OXAUNOMYCIN PRODUCED BY A BLOCKED MUTANT OF THE DAUNORUBICIN-PRODUCING MICROORGANISM

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A new potent anthracycline antibiotic oxaunomycin was isolated from the culture broth of a blocked mutant derived from a baumycin producer and was identified as 7-O-(α-L-daunosaminyl)-β-rhodomycinone. It exhibited about 100 times more strongly cytotoxic activity against leukemic L1210 cell culture than doxorubicin.

Search for the anthracycline metabolites produced by the blocked mutants or variants derived from anthracycline producers is thought to be an important approach to obtain new anthracyclines with improved anticancer activity. Recently we undertook the study of the blocked mutants obtained from a newly isolated baumycin[1]-producing strain and isolated several new blocked mutants which produced new anthracyclines. Among them, one strain was found to produce a unique anthracycline. In this paper, we report on this interesting anthracycline, designated as oxaunomycin.

Materials and Methods

Microorganisms

The oxaunomycin-producing strain OXA-1874 was obtained by successive mutagenic treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or UV light of baumycin-producing *Streptomyces* sp. D788 which was newly isolated from a soil sample. The strains were maintained on YS agar slant (yeast extract 0.35%, soluble starch 1.0% and agar 1.5%, pH 7.2) and stocked at 5°C.

Fermentation

A loopful of the slant culture was inoculated at a 500-ml Erlenmeyer flask containing 100 ml of the following seed medium; soluble starch 0.5%, glucose 0.5%, soybean meal 1.0%, yeast extract 0.1%, NaCl 0.1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, pH 7.4. Shake cultivation was carried out at 28°C for 2 days on a rotary shaker (220 rpm). The seed culture (1 ml) thus prepared was added to 500-ml Erlenmeyer flasks containing each 50 ml of fermentation medium which contained dry yeast 50 g, soluble starch 75 g, yeast extract 2 g, NaCl 2 g, $MgSO_4 \cdot 7H_2O$ 1 g, $CaCO_3$ 30 g in 1,000 ml of tap water, pH 8.0. The fermentation was carried out at 28°C for 8 days on a rotary shaker (220 rpm); 25 liters of the fermentation broth was prepared.

Microbial Growth

Microbial growth during fermentation was monitored by mycelial nucleic acid content which was

measured by the method of SCHNEIDER.[2]   In brief, 2 ml of the culture broth were added to 4 ml of water, mixed and centrifuged at 3,000 rpm for 15 minutes.   After removal of the supernatant fluid, the mycelial cake was rinsed with 4 ml of cold 10% TCA and finally suspended in 4 ml of 5% TCA. The suspension was heated at 98°C for 30 minutes, cooled and centrifuged.   The supernatant was diluted 50-fold with deionized water, and UV absorbance at 260 nm was measured.   The nucleic acid content was estimated from the UV absorption E300 at 260 nm, of a 1% nucleic acid solution.[3]

Antibiotic Assay

Oxaunomycin (**I**) in the fermentation broth and its yield and purity at the extraction and purification steps were determined either by thin-layer chromatography (TLC) or by high performance liquid chromatography (HPLC).   TLC was performed using Silica gel 60 F_{254} (E. Merck) in $CHCl_3$ - MeOH - formic acid (40 : 10 : 1).   The reddish anthracycline spots were quantified by scanning at 495 nm on a Shimadzu TLC scanner (model 930).   HPLC was performed on a Hitachi 655 liquid chromatographic apparatus with a reverse phase analytical YMC-packed column 312 (ODS) (6 × 15 mm) (Yamamura Chemical Lab.).   Acetonitrile - 0.01 M β-camphorsulfonic acid (pH 4.1) (40 : 60) was used as a mobile phase and run at a flow rate of 0.1 ml/minute.   The samples were dissolved in a mobile phase and 10 μl samples were injected.   Detection and quantification were done using a fluorescence detector (Fluospec-11A, ŌYŌ-Bunko Kiki Co.) at an excitation wavelength of 460 nm and at the maximum emission wavelength of 540 nm through an optical glass filter.

For the determination of antibiotic titers, to 1 ml of the fermentation broth were added 1 ml of 1 M citrate buffer (pH 3.8) and 2 ml of acetone.   After vigorous mixing and subsequent 1-hour standing, the mixture was centrifuged and the supernatant was subjected to the above chromatographic assay.

In Vitro Activity against Cultured L1210 Cells

Inhibitory effects of the products on cultured L1210 cells were examined as previously described.[4]

Total Acid Hydrolysis

I (100 mg) in 10 ml of 0.1 N HCl was heated in a water bath at 85°C for 30 minutes and extracted with $CHCl_3$.   The $CHCl_3$ layer was evaporated *in vacuo* to dryness and the red pigment residue was then purified by preparative TLC on Silica gel 60 PF_{254} (E. Merck) using $CHCl_3$ - MeOH (15 : 1) as solvent.

Alternatively, the aqueous phase was neutralized by addition of silver carbonate and a small amount of charcoal was added to remove a residual pigment.   The mixture was centrifuged and the supernatant was subjected to TLC using Silica gel 60 F_{254} with a solvent of BuOH - acetic acid - H_2O (4 : 1 : 1).   The sugar spots were detected by spraying with *p*-anisaldehyde - H_2SO_4 (each 5%) in 90% EtOH and then heating at 90°C.

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 EPI-GS spectrophotometer (KBr pellet).   ¹H and ¹³C NMR spectra were recorded with a Jeol GX-400 spectrometer at 400 MHz and 100 MHz, respectively.   Chemical shifts are expressed in δ values (ppm) with TMS as an internal reference and coupling constants are given in Hz (J).   CD spectra were measured in MeOH on a Jasco-J20 spectrometer.

Results

Isolation of the Oxaunomycin-producing Mutant

Streptomyces sp. D788, newly isolated from a soil sample, produced baumycins.[1]   It differed in its spore characteristics from two well-known baumycin producing strains *Streptomyces coeruleorubidus*[5] and *Streptomyces peucetius*.[6]   We first attempted to isolate a 4'-*O*-substitution-less mutant, *i.e.*, daunorubicin-producing mutant, by NTG treatment.   The mutant was designated as strain Gl-1 and did not produce

Fig. 1. Time course of antibiotic production in strain OXA-1874.

D788-1 production reached a maximum level of about 200 μg/ml 5 days after fermentation and its level then decreased to 150 μg/ml. The data were omitted for avoiding complication.
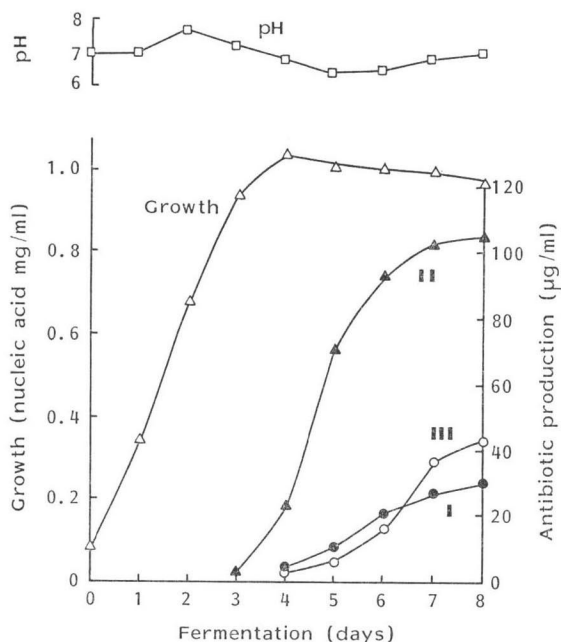

Table 1. Chromatographic behavior of oxauno-mycin and other products in the fermentation broth of strain OXA-1874.

Product	TLC	HPLC
	Rf value	Retention time (minutes)
Oxaunomycin (I)	0.24	4.2
13-Deoxocarminomycin (II)	0.30	14.32
13-Dihydrocarminomycin (III)	0.12	5.40
D788-1 (10-carboxy-13-deoxocarminomycin)	0.18	3.24

The details are described in Materials and Methods.

baumycins and accumulated daunorubicin in the culture broth. Moreover, a water-soluble, new anthracycline D788-1 was also produced. This anthracycline was identified as 10-carboxy-13-deoxocarminomycin.[7] The further mutation of this strain with NTG provided a double blocked mutant strain RPM-5 which lacked the daunoru-bicin productivity and accumulated 13-deoxocar-minomycin (II)[8] and 13-dihydrocarminomycin (III)[9] in the cultured broth. The D788-1 pro-ductivity was unchanged. Detailed TLC analy-sis of anthracycline products indicated that the cultured broth of the RPM-5 strain contained some new anthracyclines in minor amounts, among which only one product, designated as oxaunomycin (I), exhibited a significantly strong growth-inhibitory activity against Bacillus subtilis by bioautographic assay. Therefore, much effort was made to improve the production of I by this strain. A variant strain with 10 or more times enriched productivity of I, OXA-1874, was obtained by examined about 3,000 colonies randomly obtained after a two step-mutagenic treatment of the strain RPM-5.

Production of I

The time course of production of I and other anthracyclines by strain OXA-1874 is shown in Fig. 1. The product assay was carried out by TLC. The Rf values of the anthracyclines produced by the strain OXA-1874 are shown in Table 1. The mycelial growth measured by the nucleic acid content reached nearly a plateau after 3 day-fermentation, and at which time, production of D788-1 began. Its production reached the maximum 2 days later (200 μg/ml). On the other hand, I and other two anthracyclines were detectable after 4 or more days. The final yields of I, II and III in the broth were 34, 105 and 42 μg/ml, respectively.

Isolation and Purification of I

The fermentation broth (25 liters) containing 34 μg/ml of I was filtered and the anthracyclines were extracted from mycelial cake with a total of 10 liters of acetone. The acetone extract was evapo-rated in vacuo to about 3 liters. The concentrate was extracted with 1 liter of $CHCl_3$ after being

Fig. 2.   IR spectrum of oxaunomycin.
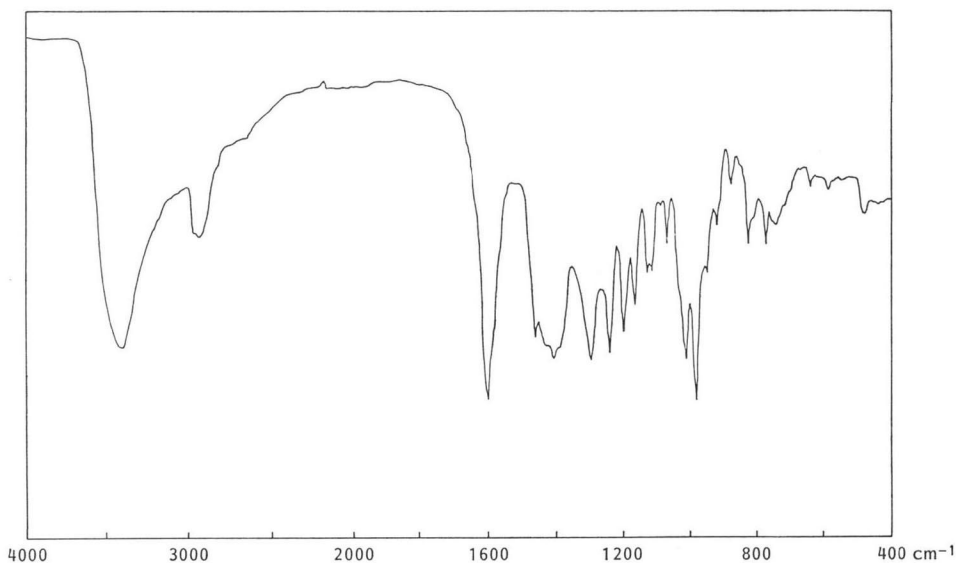
acidified to pH 3.8 with 1 N H_2SO_4, and then it was neutralized and extracted 3 times, each with 1 liter of $CHCl_3$ (the $CHCl_3$ extract did not contain the major product D788-1 since it was unextractable with $CHCl_3$). The combined $CHCl_3$ extracts were evaporated *in vacuo* to a small volume and an excess of *n*-hexane was added to precipitate the crude mixture of anthracycline products. This yielded about 4.2 g of reddish crude powder.

Further purification was carried out on preparative HPLC (Waters, Prep. LP/System 500) with a normal phase (Waters, Prep. PAK™-500/Silica Column). The above crude powder dissolved in 1 liter of $CHCl_3$ was injected into the HPLC apparatus and elution was performed with 1 liter of $CHCl_3$ and then with 3 liters of $CHCl_3$ - MeOH - H_2O - trimethylamine (680: 120: 50: 0.05). Fractions of 150 ml were collected and anlayzed by TLC. **II**, **I** and **III** were separately eluted in this order. The fractions containing **I** were pooled (about 1.5 liters) and 500 ml of water was added. After adjusting the pH to 8.0 by addition of 4 N NaOH and then vigorous mixing, the $CHCl_3$ layer was taken and extracted twice with 400 ml of 0.1 M acetate buffer (pH 3.0). The aqueous layer was washed twice with 300 ml of toluene and then extracted twice with 300 ml of $CHCl_3$ after adjusting to pH 8.0 by addition of 4 N NaOH. The final $CHCl_3$ layer was concentrated and an excess of *n*-hexane was added to precipitate **I**. This gave a deep red powder of **I** of a 99.0% purity in a yield of 0.42 g. The recovery of **I** from the fermentation broth was about 50%.
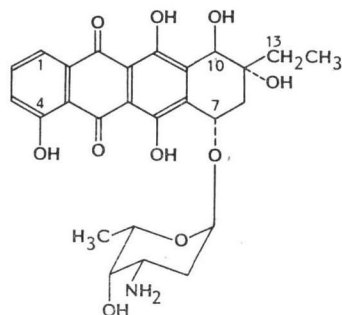
Structural Determination of I

The physico-chemical properties were as follows: MP 185~187°C (dec); $[\alpha]_D^{23}$ +192° (*c* 0.0198, $CHCl_3$); IR λ_{max}^{KBr} cm^{-1} 1600 (C=O quinone), 1400, 1290, 1240, 1200 (phenolic OH), 1160, 1130, 1110 (Fig. 2); UV $\lambda_{max}^{90\% MeOH}$ nm (E$_{1cm}^{1\%}$) 205 (380), 235 (945), 254 (503), 293 (164), 367 (74), 494 (305); $\lambda_{max}^{0.01N HCl-90\%MeOH}$ nm (E$_{1cm}^{1\%}$) 206 (385), 235 (952), 255 (508), 293 (166), 367 (76), 495 (310), 528 (sh, 200); $\lambda_{max}^{0.01N NaOH-90\%MeOH}$ nm (E$_{1cm}^{1\%}$) 209 (671), 240 (984), 296 (151), 563 (284); ^{1}H NMR (400 MHz, CDCl$_3$ - CD$_3$OD) δ 7.89 (1H, dd, J=8.0, 1.5 Hz, 1-H), 7.73 (1H, t, J=8.0 Hz, 2-H), 7.33 (1H, dd, J=8.0, 1.5 Hz, 3-H), 5.15 (1H, dd, J=4.0, 2.0 Hz, 7-H), 4.87 (1H, s, 10-H), 2.23 (1H, dd, J=15.0, 2.0 Hz,

Table 2. ^{13}C NMR chemical shifts of oxaunomycin.

Carbon	δ ppm*	Carbon	δ ppm*
1	119.38	11	(156.98)
2	137.01	11a	(111.49)
3	124.71	12	186.27
4	162.40	12a	133.41
5	190.83	13	30.13
5a	(112.11)	14	6.32
6	(156.40)	1'	101.40
6a	134.77	2'	32.48
7	70.75	3'	46.21
8	32.94	4'	(67.52)
9	71.94	5'	(70.38)
10	66.09	6'	16.64
10a	138.56		

* Measured in CDCl$_3$ - CD$_3$OD (10 : 1).
Values in parenthesis are interchangeable.

Fig. 4. Structure of oxaunomycin.

Fig. 3. CD curve of oxaunomycin (in MeOH, 9.7×10^{-5} M).

8-H$_B$), 2.13 (1H, dd, $J=15.0$, 4.0 Hz, 8-H$_A$), 1.7 (2H, m, 13-CH$_2$), 1.11 (3H, t, $J=8.0$ Hz, 14-CH$_3$) for aglycone moiety and δ 5.45 (1H, d, $J=3.0$ Hz, 1'-H), 4.13 (1H, q, $J=6.0$ Hz, 5'-H), 3.47 (1H, br s, 4'-H), 1.7 (2H, m, 2'-CH$_2$), 1.33 (3H, d, $J=6.0$ Hz, 6'-CH$_3$) for daunosamine moiety; FD-MS m/z 516 (M+H)$^+$.
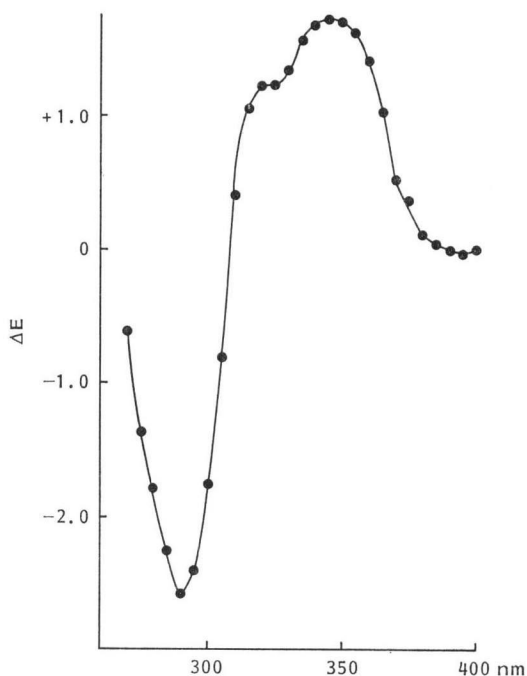
On acid hydrolysis **I** yielded a red aglycone and one sugar component. The aglycone was found to be β-rhodomycinone by direct comparison with an authentic sample on TLC. The identity was established by comparsion of the UV/Vis, mass (m/z 386 M$^+$) and ^{1}H NMR spectra with those of β-rhodomycinone. The sugar was identified as daunosamine[10] by TLC comparison with an authentic sample, which gave a sky-blue spot with p-anisaldehyde reagent on TLC (Rf value 0.32). Moreover, these data indicated that **I** consisted of one molecule of β-rhodomycinone linked to one of daunosamine. The molecular formula of C$_{26}$H$_{29}$O$_{10}$N was determined by mass fragmentation (a molecular ion peak m/z 516 (M+H)$^+$ in the FD-MS spectrum).

The chemical-shift assignment of ^{13}C NMR spectrum of **I** are shown in Table 2. All the signals for the 26 carbones of **I** were observed in the ^{13}C NMR spectrum, which included the signals belonging to β-rhodomycinone moiety and daunosamine moiety.[7] The ^{1}H NMR spectrum also revealed the corresponding proton signals.

Comparison of ^{13}C NMR spectra of **I** and β-rhodomycinone[7] showed that the chemical shifts assigned to β-rhodomycinone and its corresponding moiety in **I** were nearly identical, except for those of C-7 which were 70.8 ppm for **I** and 65.9 ppm for β-rhodomycinone. This 4.9 ppm downfield shift is attributable to a 7-O-sugar linkage as seen with many anthracyclines.[11] This fact indicates that the

Table 3. Inhibitory activities of oxaunomycin and some 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	
Oxaunomycin (I)	0.0003	0.29	0.68	0.4
13-Deoxocarminomycin (II)	0.03	1.30	1.30	1.0
13-Dihydrocarminomycin (III)	0.10	2.50	2.00	1.3
D788-1 (10-carboxy-13-deoxo-carminomycin)	0.025	>5.0	>5.0	—
Betaclamycin T	0.003	0.44	0.044	10.0
Doxorubicin	0.02	1.40	0.55	2.5
Daunorubicin	0.02	0.42	0.16	2.6

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 viable cells were counted by coulter counter.
IC$_{50}$ is expressed as drug concentration required to inhibit by a 50% control of the growth, and DNA and RNA synthesis of cultured L1210 cells.

sugar daunosamine is attached to C-7. The additional evidence for this came from the findings that the chemical shifts (91.0~96.6 ppm) of the sugar C-1′ attached C-10 always shift to upfield as compared with those (100.3~102.2 ppm) bonded to C-7.[11~13] The sugar linkage of I proved to be α-configuration since the coupling constant of an anomeric proton (H-1′) is small (3.0 Hz).

The CD spectrum of the aglycone of I was identical with that of β-rhodomycinone which has the configuration of 7(S), 9(R) and 10(R),[14] and was also similar to that of I (Fig. 3). All these findings indicate that I is 7-O-(α-L-daunosaminyl)-β-rhodomycinone as shown in Fig. 4.

Biological Activity

The activity of I and other related anthracyclines against cultured L1210 cells was examined under continuous exposure and the results are shown in Table 3. I was found to have a strikingly potent cytotoxic activity. The IC$_{50}$ value was 0.0003 μg/ml. I was about 100 times active than doxorubicin and daunorubicin, and was about 10 times stronger than betaclamycin T,[15] the N,N-dimethyl derivative of I (Table 3). Other anthracyclines II, III and D788-1 were also far less active than I.

When the synthesis of nucleic acid in the L1210 cell culture was measured, I showed a 50% inhibition of RNA and DNA synthesis at 0.68 and 0.29 μg/ml, respectively. It should be noticed that I inhibited DNA synthesis more strongly than RNA synthesis (Table 3). There have been no other anthracyclines which showed a preferential inhibition of DNA synthesis at lower concentration than those inhibiting RNA synthesis.[16]

Antitumor effect of I on mice bearing leukemia L1210 was tested by daily ip administration on day 1 to 10. I exhibited a maximum effect of 280% (T/C) at an optimal dose of 1.25 μg/mouse/day.

Discussion

On the basis of studies on the blocked mutants derived from a daunorubicin producer and the bioconversion of precursors to daunorubicin, we have demonstrated that daunorubicin is biosyn-

thesized by the glycosidation of a key precursor aglycone (ε-rhodomycinone) and the subsequent modifications, such as 10-decarbomethoxylation, 13-oxidation and 4-O-methylation of the aglycone moiety.[17]

It is obvious that **I** is not an intermediate of daunorubicin biosynthesis, but a by-product which is secondarily formed from the compound accumulated by a specific blockage of the biogenetic pathway to daunorubicin. The **I**-producing strain OXA-1874 was a variant, with improved **I** productivity, of a blocked mutant which lacked the biosynthetic step of 4-O-methylation so that it accumulated **II** and **III** in the culture broth.

It is of great interest that **I** has a very strong cytotoxic activity against leukemic L1210 cells. Our recent study on the structure-activity relationships among anthracycline derivatives has shown that the derivatives with an N,N-dimethylated sugar (rhodosamine) are always about 10 times more active than those with N-non-methylated sugar (daunosamine).[16] **I** was, however, *vice versa* 10 times more active than its N,N-dimethylated derivative, betaclamycin T.[15] **I** also exhibited a strong antitumor effect on L1210 cell *in vivo*.

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