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Production of a New Hybrid Anthracycline 4-*O*-Methylepelmycin by Heterologous Expression of *dnrK* in Epelmycin-producing *Streptomyces violaceus*

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A new hybrid anthracycline antibiotic was produced by heterologous expression of *dnrK* encoding carminomycin 4-*O*-metyltransferase in an epelmycin-producing *Streptomyces violaceus*. pMK100 was constructed by insertion of *Steptomyces peucetius dnrK* gene in *Steptomyces*-expresion vector pIJ6021 and introduced to the epelmycin producer. The transformant produced a hybrid anthracycline antibiotic together with host epelmycins when cultured in antibiotic production medium in the presence of thiostrepton. The hybrid anthracycline was determined to be 7-*O*-L-rhodosaminyl-4-*O*-methyl-ε-rhodomycinone (4-*O*-methylepelmycin D). However, the attempts on production of hybrid 4-*O*-methylaclarubicin and 4-*O*-methyl-1-deoxyobelmycin by the transformants of aclarubicin and 1-deoxyobelmycin producers with pMK100 were unsuccessful.

Anthracycline family of antibiotics are important therapeutic agents for cancer chemotherapy. Daunorubicin (DNR)[1], doxorubicin (DOX)[2] and aclarubicin (ACR)[3] are commercially available natural anthracyclines, among which DOX had the widest spectrum of activity in human cancers. However, its cumulative cardiotoxicity was a dose-limiting side effect. New anthracyclines which have less cardiotoxicity and more improved therapeutic efficacy of antitumor activity are required to be developed. In a search for new natural anthracyclines, our efforts have been made on the isolation of a variety of biosynthetically blocked mutants and the microbial conversion of heterogeneous anthracyclines by antibiotic-negative mutants of ACR and DNR producers, and we have obtained many new anthracycline metabolites and hybrid anthracyclines[4~10]. Recently, genetic approach was made to preparation of new hybrid anthracyclines[11,12]. YLIHOUKO *et al.* reported the preparation of 10-hydroxy anthracyclines by introducing rhodomycin (RM)-producing *Streptomyces purpurascens* genes to a ACR-producing *Streptomyces galilaeus*[11]. KIM *et al.* also obtained 11-hydroxy ACRs by introducing a DNR-biosynthetic gene encoding 11-hydroxylase to ACR-producing *S. galilaeus* ATCC 31133[12]. We tried here the introduction of a DNR-biosynthetic gene *dnrK* encoding carminomycin (CM) 4-*O*-methyltransferase[13] to three kinds of anthracycline producers in aim at obtaining hybrid 4-*O*-methyl anthracyclines. In practice, pMK100 containing *dnrK* gene was constructed using a *Sreptomyces*-expression vector pIJ6021[14] and was introduced to the respective producers of ACRs[15], epelmycins (EPMs)[16] and 1-deoxyobelmycins (1-deoxyOBMs)[4]. As results, we found that the transformants of EPM producer with pMK100 coproduced a hybrid 4-*O*-methyl derivative. However, the other two transformants did not yield their 4-*O*-methyl derivatives. This paper deals with the production of 4-*O*-methylepelmycin D by heterologous expression of *dnrK* gene in EPM producer, its isolation and chemical identification.

Materials and Methods

Bacterial Strains and Plasmids

EPM-producing *S. violaceus* A262 strain SU2-730[16],

ACR-producing *S. galilaeus* MA144 strain 6U-21[15] and 1-deoxyOBM-producing *S. violaceus* A262 strain SC-7[4] were used as host strains. *S. peucetius* ATCC 29050 was purchased from American Type Culture Collection (Rockville, MD, USA) and used for isolation of *dnrK* gene. *Streptomyces lividans* TK24 was obtained from Dr. HOPWOOD (John Innes Institute, Norwich, UK). These *Strepromyces* strains were maintained on YGS agar slant (yeast extract 5 g, glucose 5 g, soluble starch 5 g and agar 15 g per liter tap water, pH 7.2). Thiostrepton-inducible *Streptomyces*-expression vector pIJ6021[14] was a gift from Dr. BIBB (John Innes Institute, Norwich, UK). *Escherichia coli* strain JM109 and plasmid pUC19 were laboratory stock and used for routine subcloning.

Biochemicals and Chemicals

Thiostrepton, kanamycin and ampicillin were obtained from Sigma Chemical (St.Louis, MO, USA). ε-Rhodomycinone (ε-RMN) and β-rhodomycinone (β-RMN) were prepared from mutant cultures of *S. violaceus* A262[4]. Aklavinone (AKN) and 4-*O*-methyl-ε-RMN were prepared from ACR[15] and compound D788-5 by acid hydrolysis, respectively. Compounds D788-5 and D788-6 were products of a blocked mutant 4L-660 of DNR producer[17]. Restriction enzymes and other molecular biology reagents were obtained from standard commercial sources and DNA primers from Sawady Technology (Tokyo).

Media and Cultural Condition

All *Streptomyces* strains were grown at 30°C in SS liquid medium [sucrose 200 g, soluble starch 10 g, Polypepton 4 g, yeast ext. 4 g, K_2HPO_4 4 g, KH_2PO_4 2 g, $MgSO_4 \cdot H_2O$ 0.5 g, glycine 5 g per liter distilled water, pH 7.0] for isolation of chromosomal DNA and preparation of protoplasts. R2YE medium[18] was used in transformation experiments. *E. coli* strain with or without plasmid was grown in Luria-Bertani medium and selected with ampicillin (50 μg/ml). Medium for antibiotic production was described previously[19].

Protoplast Preparation and Transformation

Streptomyces sp. protoplasts were prepared by a modification of the standard procedure[18]. The strains were shake-cultured in a 500-ml Sakaguchi flask containing 100 ml of SS medium and 8 g of glass beads (3 mm in diameter) for 3 days. The mycelial cells collected from one flask by centrifugation were washed twice with 30 ml of sterile 10% sucrose and resuspended in 20 ml of P buffer supplemented with egg lysozyme (3 mg/ml). After 1-hour incubation at 30°C, the resultant solution was filtered through a glass tube (20 mm in diameter) packed with glass wool (15 mm in length) and the protoplasts were collected from the filtrate by centrifugation at 3000 rpm for 10 minutes. The protoplasts washed with 20 ml of P buffer were suspended into 1 ml of P buffer to give a cell density of more than about 10^8/ml. Transformation was carried out according to the standard procedure[18] and transformants were selected by overlaying 2 ml of kanamycin layer (2 mg/ml).

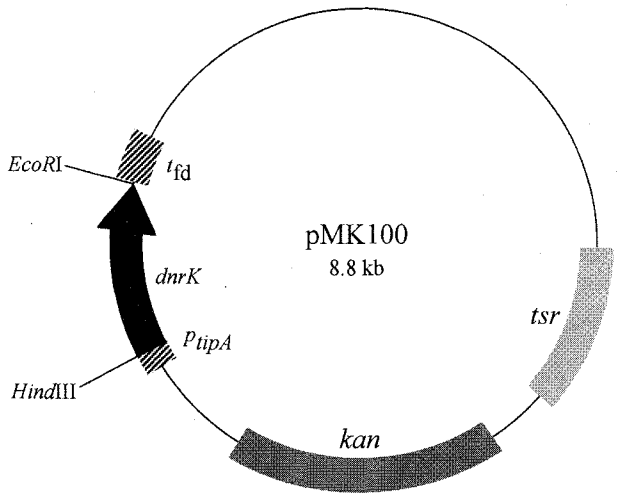
Construction of Expression Plasmid for *dnrK* Gene

The *dnrK* encoding CM 4-*O*-methyltransferase[13] was isolated by PCR method. Total DNA of *S. peucetius* ATCC 2905 was used as a temperate for PCR. The primers DK1 and DK2 for polymerization were chosen to cover the whole sequence of *dnrK* gene with an artificially introduced restriction site as follow: DK1 (27 mer), 5′-CCCAAGCTTATGACAGCCGAACCGACG-3′ (including the start codon and *Hind*III site); DK2 (27 mer), 5′-CCGGAATTCTCAGGCGCCGGTGGCCGC-3′ (including the stop codon and *EcoR*I site). The PCR was performed in a ASTEC Program Temp. Control System PC700. The reaction cocktail (final 50 μl) contained about 100 ng of *S. peucetius* 29050 DNA, two primers DK1 and DK2 (10 pmol each), 2.5 mM dNTPs and TaKaRa LA Taq DNA polymerase (1.5 units). The PCR was done under the following condition: 94°C, 5 minutes for denaturation (1 cycle); 98°C, 20 seconds, 68°C, 30 seconds, 72°C, 1 minute for amplification (30 cycle); 72°C, 5 minutes for extension (1 cycle). The PCR product was digested with *Hind*III and *EcoR*I and purified by agarose gel electrophoresis. The 1.1 kb fragment thus obtained was ligated to pUC19 to give pDK10, which was used to transform *E. coli* JM109. To confirm that the PCR product is identical to *dnrK*, nucleotide sequences were analyzed by deoxy chain termination method. The verified and cloned 1.1-kb *Hind*III-*EcoR*I fragment from pDK10, including *dnrK*, was ligated to a vector pIJ6021 which was double digested with *Hind*III and *EcoR*I, and the products of ligation were submitted to transformation of *S. lividans* TK24. A transformant containing the 1.1-kb *Hind*III-*EcoR*I fragment was selected from the kanamycin-resistant colonies and the resultant plasmid was designated as pMK100 (Fig.1).

Antibiotic Production

The transformants with pMK100 were cultured in a 500-ml Sakaguchi flask containing 50 ml of seed medium: soybean meal 10 g, glucose 5 g, soluble starch 5 g, KH_2PO_4 1 g, NaCl 2 g and $MgSO_4 \cdot H_2O$ 0.5 g per liter tap water, pH 7.2. After cultivation for 3 days at 30°C, 1 ml of seed was

Fig. 1. Expression plasmid pMK100 constructed from pIJ6012 and *dnrK* gene encoding carminomycin 4-*O*-methyltransferase.



tsr; thiostrepton resistance gene, *kan*; kanamycin resistance gene, p_{tipA}; thiostrepton inducible promotor, t_{fd}; transcriptional terminator.

inoculated into twenty 500-ml Sakaguchi flasks containing 75 ml each of antibiotic production medium[20]. Kanamycin was added finally at 5 μg/ml to seed medium and production medium. Cultivation was carried out at 30°C for 7 days on a reciprocal shaker (130 rpm). Thiostrepton was added at final 5 μg/ml to the culture on day 2 in order to express the *dnrK* gene and cultivation was continued for further 5 days to give a maximum antibiotic yield. As control experiment, the transformants with pIJ6021 were also cultivated in the same manner.

TLC and HPLC Assays

Usually, 5 ml of culture broth was taken and mixed with 5 ml of acetone by Vortex. The mixture was extracted twice with 2 ml of $CHCl_3$. The $CHCl_3$ layer was pooled and concentrated to dryness, and the residual pigment was tested for whole antibiotics by TLC. The above extract was also subjected to acid hydrolysis by heating in 1 ml of 0.1 N HCl at 85°C for 30 minutes to release aglycone and sugar. The aglycone was extracted with 2 ml of $CHCl_3$ and determined by TLC and HPLC. For sugar analysis, a sample (about 1 mg) was acid hydrolyzed as described above and aglycone was removed by extraction with $CHCl_3$. The aqueous layer containing sugar constituents was neutralized by addition of solid silver carbonate with a small amount of charcoal and the insoluble materials were centrifuged off. The clear supernatant fluid was evaporated *in vacuo* to dryness and subjected to sugar analysis by TLC. Sugar mixture as reference (L-rhodosamine, L-deoxy-L-fucose and L-cinerulose A) were prepared by EPM-E by acid hydrolysis[16].

Analytical TLC was carried out using Wako silica gel plate $70F_{254}$ (Wako Pure Chemical Industries). Solvent systems were benzene - EtOAc - Me_2CO (4 : 4 : 1), $CHCl_3$ - MeOH - aq. NH_3 (8 : 1 : 0.5) and *n*-BuOH - AcOH - H_2O (4 : 1 : 1) for aglycone, whole antibiotic and sugar analyses, respectively. The sugar was detected by spraying TLC with 5% *p*-anisaldehyde-5% H_2SO_4 in 90% EtOH and heated at 90°C for 15 minutes. Preparative TLC was also carried out using silica gel plate PF_{254} (Merck Co.) for purification. Solvent system was the same as described above.

HPLC was performed on Jasco TRI ROTAR-VI apparatus with a reverse phase of analytical column, ULTRON VX-ODS (4.6×250 mm) (Shinwa Chemical Industries Co. Ltd.). Solvent used as a mobile phase was 60% CH_3CN (pH 2.0 with H_3PO_4) and flow rate was 1.0 ml/minute. Samples were dissolved in the mobile phase and the 25 μl aliquotes were injected. Detection was conducted at 495 nm using a Jasco UV detector (UVIDEC-100-VI).

Biological Activity

Inhibitory effects of the products on growth and nucleic acids syntheses in murine L1210 leukemia cell culture were examined as previously described[20]. IC_{50} is expressed as a drug concentration required to inhibit by 50% control of the growth and nucleic acids syntheses of the cultured L1210 cells.

General

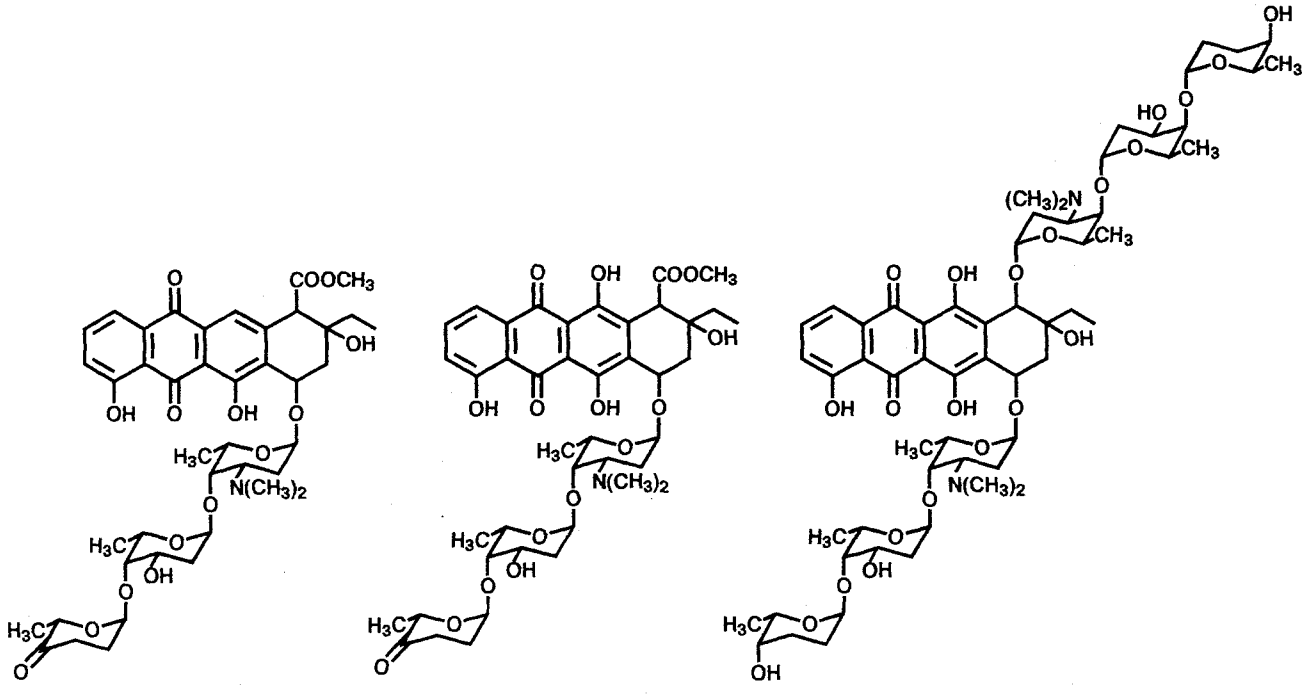
UV spectra were determined on a Shimadzu UV-160A spectrophotometer and IR spectrometer on a Jasco FT/IR-5300. ^1H and ^{13}C NMR were recorded with a Jeol GSX-400 spectrometer at 500 MHz and 125 MHz, respectively. Chemical shifts were expressed in δ value (ppm) with TMS as an internal reference and coupling constants are given in *J* (Hz). Mass spectra were recorded with Jeol SX102 Digital Polarimeter.

Results

Antibiotic Production by Three Transformants with pMK100

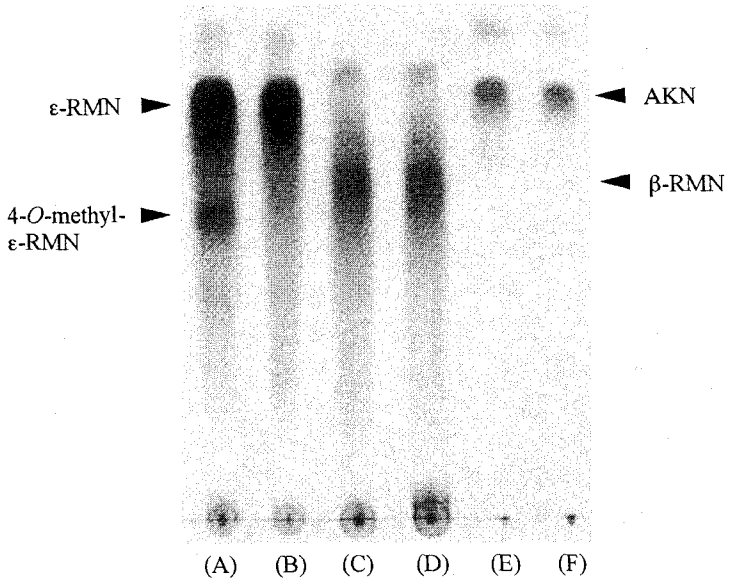
Vector pMK100 containing *dnrK* gene was prepared from a transformant *S. lividans* (pMK100) and introduced

Fig. 2. Major component produced by three kinds of anthracycline producers used in this experiment.



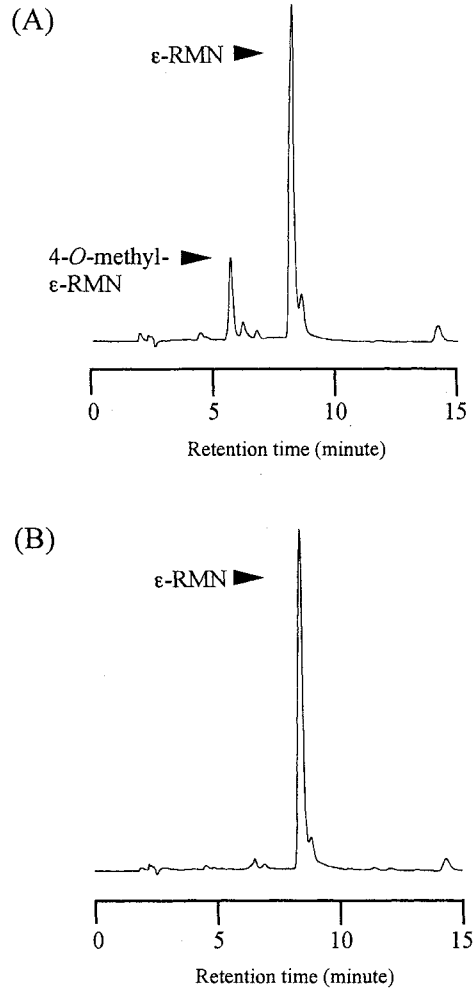
Aclarubicin Epelmycin E 1-Deoxyobelmycin D

Fig. 3. Anthracycline production of ACR, EPM and 1-deoxyOBM producers (strain 6U-21, SU2-730 and SC-7, respectively) which were transformed with pMK100 or pIJ6021.



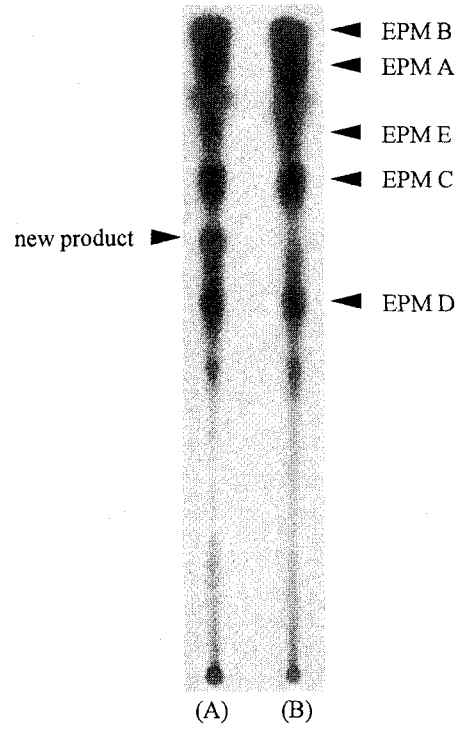
Aglycones after acid hydrolysis were analyzed by TLC: (A), SU2-730 (pMK100); (B), SU2-730 (pIJ6021); (C), SC-7 (pMK100); (D), SC-7 (pIJ6021); (E), 6U-21 (pMK100); (F), 6U-21 (pIJ6021).

Fig. 4. HPLC analysis of anthracycline products of transformants SU2-730 (pMK100) (A) and SU2-730 (pIJ6021) (B).



Aglycones after acid hydrolysis were analyzed.

Fig. 5. TLC analysis of crude anthracycline extract from culture broths of transformants SU2-730 (pMK100) (A) and SU2-730 (pIJ6021) (B).



to respective protoplasts of EPM producer (strains SU2-730), 1-deoxyOBM producer (strain SC-7) and ACR-producer (strain 6U-21) according to the standard procedure. Transformants SU2-730 (pMK100), SC-7 (pMK100) and 6U-21 (pMK100) were selected by overlaying 2 ml of 2 mg/ml of kanamycin soft agar. pIJ6021 was also introduced to these three host strains to give transformants SU2-730 (pIJ6021), SC-7 (pIJ6021) and 6U-21 (pIJ6021) in the same manner. Antibiotic production by three pairs of the transformants were tested.

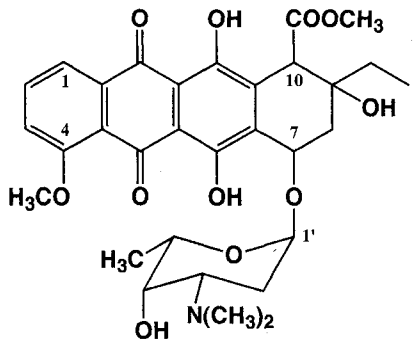
ACRs, EMPs and 1-deoxyOBMs are structurally aklavinone (AKN) glycosides, ε-RMN glycosides and β-RMN diglycerides, respectively, and their major components are shown in Fig. 2. Host strain 6U-21 produced two major ACR components A and B. Strain SU2-730 produced five EPM components and strain SC-7 did 1-deoxyOBMs of more than 7 components. All these components differed in the sugar chain of a trisaccharide moiety from one another. In order to see easy the effect of introducing *dnrK* gene to these anthracycline producers, we examined by TLC 4-*O*-methyl derivatives of AKN, ε-RMN and β-RMN which would occur from their possible products by mild acid hydrolysis. These 4-*O*-methyl aglycones are distinguishable from the corresponding non 4-*O*-methyl aglycones by TLC. The results are shown in Fig. 3 where the occurrence of a new spot different from that of parental aglycone was observed with SU2-730 (pMK100) but not with two other transformants SC-7 (pMK100) and 6U-21 (pMK100). HPLC also confirmed the presence of the new aglycone which was corresponding to 4-*O*-methyl-ε-RMN in the retention time (Fig. 4). We thus concluded that heterologous expression of *dnrK* gene led to the production of hybrid anthracycline antibiotic in EPM producer but not in ACR and 1-deoxyOBM producers. When whole antibiotics were extracted from

both the cultures of SU2-730 (pMK100) and SU2-730 (pIJ6021) and analyzed by TLC, it was found that the former coproduced a new spot of antibiotic with five spots of parental EPMs A~E (Fig. 5). Two dimensional TLC following by treatment with acid hydrolysis demonstrated that only a new aglycone occurred from the new spot of compound while ε-RMN occurred from all spots of EPMs. The yield of the new compound was about 10% of a total of the antibiotics produced when enumerated by the peak area at 495 nm in HPLC.

Isolation and Purification of the New Hybrid Product

The culture broth (1.5 liter) of SU2-730 (pMK100) was prepared by cultivation using Sakaguchi flasks and centrifuged. The antibiotic products were extracted with 500 ml of acetone from mycelial cake. The acetone extract was evaporated to about 200 ml and extracted twice with 200 ml of $CHCl_3$ after adjustment of pH to 8.5. The under layers were pooled and evaporated to a small volume. An excess of *n*-hexane was added to precipitate the crude products (415 mg). This crude powder was chromatographed on a silica gel column (30 mm in diameter, Wakogel C-200, 100 g), which was eluted with solvent systems of $CHCl_3$-MeOH (100 : 1~100 : 30). The eluate was fractionated every 10 ml and monitored by TLC. The new compound was eluted when the column was developed with $CHCl_3$-MeOH (100 : 10). The fractions were pooled and evaporated to dryness, followed by further purification on preparative TLC. Single and major pigmented band was scrapped off and eluted with $CHCl_3$-MeOH (5 : 1). The extract was evaporated to dryness and dissolved in 5 ml of 1% acetic acid. The solution was washed with 5 ml of toluene by Vortex. The aqueous layer was taken, adjusted to pH 7.5 with 4 N NaOH and extracted with 5 ml of $CHCl_3$. After washed with distilled water, the $CHCl_3$ layer was dried over anhydrous Na_2SO_4 and concentrated to a small volume and an excess of *n*-hexane was added to precipitate pure orange compound. The yield was 4.6 mg.

Fig. 6. Chemical structure of hybrid 4-*O*-methylepelmycin D produced by a transformant SU2-730 (pMK100).



Structural Determination

Since it was anticipated that the new compound was 4-*O*-methyl derivative of either one of EPMs, we determined the aglycone and sugar of the new compound by TLC after acid

Table 1. Physico-chemical properties of new product.

Appearance	Orange powder
Molecular formula	$C_{31}H_{37}NO_{11}$
FAB-MS (*m/z*)	
Found	600.2449 $(M+H)^+$
Calcd	600.2445
$[\alpha]^{20}$ (c 0.02, $CHCl_3$)	+181°
$UV\lambda^{90\%\ MeOH}$ nm ($E^{1\%}$)	234(682), 251(375), 290(143)
IR (KBr) ν cm^{-1}	1720 (ester carbonyl)
	1610 (carbonyl bonded to *peri* hydroxyl)
Rf value (TLC) *	0.58

* Silica gel plate $70F_{254}$ (Wako): $CHCl_3$-MeOH-aq. NH_3 (8 : 1 : 0.5)

Table 2. ^{1}H and ^{13}C NMR chemical shift assignments of new compound (in $CDCl_3$).

	^{13}C		^{1}H
position	ppm (mult)	position	ppm (mult, J(Hz))
1	119.8	1-H	8.03 dd (7.8, 0.9)
2	135.8	2-H	7.78 dd (8.4, 7.8)
3	118.4	3-H	7.39 brd (8.4)
4	161.1		
4-OCH_3	56.7	4-OCH_3	4.09 s
4a	121.0		
5	187.3		
5a	111.6		
6	156.8		
6a	136.3		
7	71.3	7-H	5.27 dd (4.2, 1.7)
8	33.4	8-Ha	2.36 brd (15.0)
		8-Hb	2.24 dd (15.0, 4.2)
9	71.4	9-OH	4.48 s
10	52.0	10-H	4.27 d (1.0)
10a	133.3		
11	155.8		
11a	112.2		
12	186.7		
12a	135.6		
13	32.3	13-Ha	1.84 dq (14.3, 7.3)
		13-Hb	1.46 dq (14.3, 7.3)
14	6.7	14-CH_3	1.13 t (7.3)
15	171.5		
16	52.4	16-OCH_3	3.71 s
1'	101.6	1'-H	5.54 brs
2'	28.5	2'-CH_2	1.80 m
3'	59.8	3'-H	2.24 m
4'	65.9	4'-H	3.70 brs
5'	66.5	5'-H	4.04 q (6.5)
6'	17.0	6'-CH_3	1.38 d (6.5)
3'-$N(CH_3)_2$	41.9	3'-$N(CH_3)_2$	2.23 s

hydrolysis. TLC showed that the aglycone was identical to 4-*O*-methyl-ε-RMN by their complete comigration at the same Rf value of 0.41. Sugar analysis by TLC provided that the new compound contained only a L-rhodosamine which gave a sky blue color of spot with *p*-anisaldehyde reagent at Rf value of 0.12[16]. These results suggested the identity of the new compound as 4-*O*-methyl EPM-D (7-*O*-L-rhodosaminyl-4-*O*-methyl-ε-RMN). This was demonstrated by ^{1}H and ^{13}C NMR and FAB-MS analyses.

The physico-chemical properties are shown in Table 1. Molecular formula was determined to be $C_{31}H_{37}NO_{11}$ by high resolution FAB-MS. The UV and visible light absorption spectra in 90% MeOH exhibited characteristic peak due to the anthracycline orange pigment. The chemical shift assignments for ^{1}H and ^{13}C NMR spectra are shown in Table 2. These NMR spectra were similar to those of compound D788-5[17], except for the presence of an additional *N*-methyl signals [δ_H 2.23 (6H, s) and δ_C 41.9 ($CH_3\times2$)]. In the HMBC experiment, the *N*-methyl signal at 2.23 showed a long-range correlation to C-3' (δ_C 59.8) in

Table 3. Antitumor activity *in vitro* of 4-*O*-methyl EMP-D and some other related anthracyclines against murine leukemic L_{1210} cell culture.

Compound	IC_{50}(μg/ml)			Index
	Growth	DNA synthesis	RNA synthesis	DNA/RNA
4-*O*-methylEPM-D	0.03	0.70	0.18	3.9
EPM-D	0.01	0.84	0.21	4.0
Compound D788-5[a)]	0.50	1.40	0.65	2.2
Compound D-788-6[b)]	0.25	2.60	1.30	2.0
DNR	0.02	0.40	0.17	2.4

a) *N,N*-didemethyl-4-*O*-methylEPM-D
b) *N,N*-didemethyl-4-*O*-demethylEPM-D

the HMBC spectrum, indicating that the new compound was an *N,N*-dimethyl derivative of compound D788-5. Thus, the structure of the new compound was determined to be 4-*O*-methyl EPM-D (7-*O*-L-rhodosaminyl-4-*O*-methyl-ε-RMN).

Biological Activity

The biological activities of 4-*O*-methyl EPM-D, EPM-D and their *N,N*-didemethyl analogs (D788-5 and D788-6)[17] against leukemic L1210 cell culture were tested for the inhibition of growth and nucleic acids syntheses under the continuous exposure and are shown in Table 3. 4-*O*-Methyl EPM-D was somewhat less active in the growth inhibition, but more active in the inhibition of RNA and DNA syntheses, than EPM-D. It was much more active concerning both the activities than its *N,N*-didemethyl analogs, compound D788-5 which was produced by a blocked mutant of DNR producer[17]. As results, it was found that 4-*O*-methyl EPM-D was comparable to DNR in the biological activities *in vitro* against L1210 cell culture.

Discussion

Several group described the gene engineering of polyketide synthesis including anthracycline aglycone and the production of possible hybrid ploketide[21~23]. There are only a few reports concerning the production of hybrid whole anthracyclines by the use of heterologous genes. KIM *et al.*[12] and YLIHOUKO *et al.*[11] reported the production of hybrid anthracylines (11-hydroxy ACRs and 11-deoxy RMs) by the introduction of certain respective biosynthetic genes of DNR and RM producers to a ACR producer. We tried the application of carminomycin 4-*O*-methyltrasnferase gene (*dnrK*) on preparation of hybrid 4-*O*-methyl derivatives by heterologous expression of pMK100 carring *dnrK* gene in the respective producers of ACR, EPM and 1-deoxyOBM, and found that only the transformant SU2-730 (pMK100) produced a new antibiotic among three ones. The new compound was 4-*O*-methyl EPM-D (7-*O*-L-rhodosaminyl-4-*O*-methyl-ε-RMN). Strain SU2-730 produces usually five components of EPM, A~E (Fig. 3), among which only EMP-D is monosaccharide (L-rhodosamine) of ε-RMN while the other four analogs have either a trisacchraide or disaccharide as the sugar moiety. This fact suggests that *dnrK* enzyme acts only on a monosaccharide type of anthracycline but not the trisaccharide or disaccharide type. This is also a reason why the transformants 6U-21 (pMK100) and SC-7 (pMK100) could not produce hybrid 4-*O*-methyl derivatives since all of their products are the trisaccharide type which are unavailable substrate for *dnrK* enzyme. We have investigated the microbial 4-*O*-methylation of some important anthracyclines using a specific DNR-nonproducing mutant strain DKN-1 and obtained respective 4-*O*-methyl derivatives from D788-6, oxaunomycin, betaclamycin T and yellamycin A[8], all of

which are the monosaccharide-type of anthracyclines. These findings show that there is the possibility of an extensive use of pMK100 to direct production of 4-*O*-methyl anthracycline when strain improvements are achieved to accumulate mainly such a monosaccharide-type of anthracyclines.

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