New Anthracycline Metabolites Produced by the Aklavinone 11-Hydroxylase Gene in *Streptomyces galilaeus* ATCC 31133

HANG-SUB KIM†, YOUNG-SOO HONG, YOUNG-HO KIM,
OOK-JOON YOO† and JUNG-JOON LEE*

Microbial Chemistry Research Group, Korea Research Institute of Bioscience and Biotechnology, KIST, Yusung P. O. Box 115, Taejon 305-600, Korea
†Department of Biological Science, Korea Advanced Institute of Science and Technology, 371-1 Kusong-Dong, Yusung-Gu, Taejon 305-701, Korea

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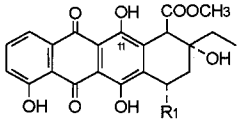
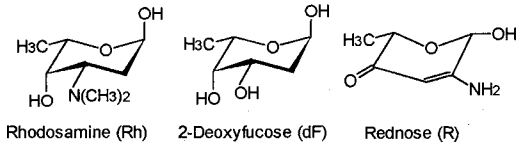
Transformation of *Streptomyces galilaeus* ATCC 31133 with the aklavinone 11-hydroxylase gene (*dnr*F) resulted in the production of many red pigments. The new metabolites were purified and their structures were determined as 11-hydroxylated aclacinomycin A, B and T by spectral analysis. This result indicated that the *dnr*F was stably expressed in the strain *S. galilaeus* ATCC 31133 to give rise to hybrid aclacinomycins. In addition, a new aclacinomycin analog named 11-hydroxyaclacinomycin X was isolated from the same culture. Its structure was elucidated as 2‴-amino-11-hydroxyaclacinomycin Y. It showed strong cytotoxicity against several human tumor cell lines, especially leukemia and melanoma cell lines.

The anthracycline antibiotics such as daunorubicin, doxorubicin and aclacinomycin are clinically and commercially important anticancer agents[1,2]. Their cardiotoxic nature limits their dose and long-term use[3]. This major drawback has led to an intensive search for new members of anthracycline either by a screening of blocked mutants[4~6] or by a hybrid biosynthetic approach[7,8]. In the course of study on the biosynthesis of doxorubicin, we previously reported the cloning and characterization of the aklavinone 11-hydroxylase gene (*dnr*F) from doxorubicin-producing *Streptomyces peucetius* subsp. *caesius* ATCC 27952[9]. Therefore *dnr*F should be an ideal probe for the application of hybrid biosynthesis in hosts such as *Streptomyces galilaeus* ATCC 31133, an aklavinone glycoside producer. Accordingly we transformed *S. galilaeus* ATCC 31133 with a plasmid pMC213 containing *dnr*F. Many reddish metabolites were purified from the culture of the transformant and their structures were identified as 11-hydroxyaclacinomycin A (**4**), 11-hydroxyaclacinomycin B (**2**) and 11-hydroxyaclacinomycin T (**3**). In addition, a new anthracyline 3‴-amino-11-hydroxyaclacinomycin Y was isolated and named 11-hydroxyaclacinomycin X (**1**). These results indicated not only that *dnr*F was stably expressed in *S. galilaeus* ATCC 31133 to introduce hydroxyl group at C-11 of aglycon but also that the host strain, *S. galilaeus* ATCC 31133 produced 2‴-aminoaclacinomycin Y (aclacinomycin X). The present communication describes the purification and structure determination of **1** and other known analogs produced by the transformant of *S. galilaeus* ATCC 31133 transformed with *dnr*F along with *in vitro* cytotoxicity of **1** against human tumor panels.

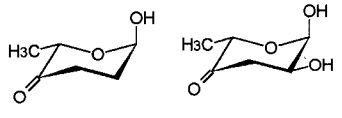
Fig. 1. Structures of 11-hydroxyaclacinomycins.



compounds	R1
11-Hydroxy aclacinomycin X (**1**)	Rh-dF-R
11-Hydroxy aclacinomycin B (**2**)	Rh-dF-CinB
11-Hydroxy aclacinomycin T (**3**)	Rh
11-Hydroxy aclacinomycin A (**4**)	Rh-dF-CinA



Rhodosamine (Rh) 2-Deoxyfucose (dF) Rednose (R)



Cinerulose A (CinA) Cinerulose B (CinB)

Materials and Methods

Microbial Strain

The aclacinomycin-producing *S. galilaeus* ATCC 31133 was purchased from the ATCC (Rockville MD, U.S.A.). The plasmid pMC213 which contained the *dnr*F encoding aklavinone 11-hydroxyase was obtained from our previous work[9]. Protoplast transformations of *S. galilaeus* ATCC 31133 with the plasmid pMC213 were carried out as described by HOPWOOD *et al.*[10]. The transformants were maintained in R2YE agar medium containing 25 μg/ml of thiostrepton (Sigma).

Fermentation

A loopful of the slant culture of transformant of *Streptomyces galilaeus* ATCC 31133 transformed with *dnr*F was used to inoculate a 500 ml baffled flask containing 100 ml of seed medium consisting of yeast extract 0.3% and soluble starch 1.0% (pH 7.2). The flasks were cultivated on a rotary shaker (210 rpm) for 4 days at 28°C. A 13-liter fermentor containing 10 liters of production medium consisting of glucose 1%, soluble starch 1.5%, soybean meal 3%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.3%, $CuSO_4 \cdot 5H_2O$ 0.007%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $MnSO_4 \cdot 4H_2O$ 0.0008% and $ZnSO_4 \cdot 7H_2O$ 0.0002% (pH 7.0) was inoculated with 300 ml of the seed culture. The fermentation was carried out at 28°C for 4 days for the isolation of **4** and for 7 days for the isolation of **1**, **2** and **3**. The concentration of thiostrepton in seed and main culture were 25 μg/ml and 10 μg/ml, respectively.

In Vitro Cytotoxicity

The cytotoxicity results of aclacinomycin antibiotic against human tumor cell line panels were obtained through the cooperation of Dr. V. L. NARAYANAN at the National Cancer Institute, U.S.A.

Spectroscopic Methods

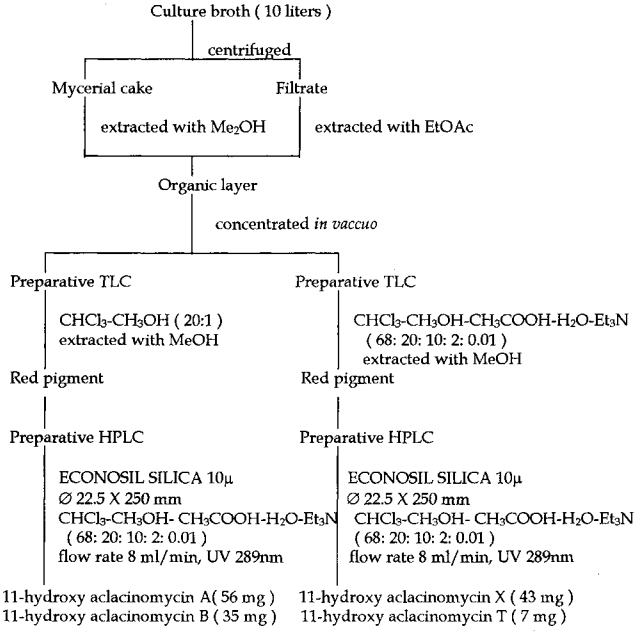
Melting points were determined without correction on a Electrothermal 9100. UV spectra were recorded on a Milton Roy 3000 spectronic array and IR spectra were measured with a Laser Precision Analytical RFX-65 FT-IR. Optical rotations were determined on a JASCO DIP-181 polarimeter. FAB-MS spectra were recorded on a JEOL HX-100 Spectrometer. The ^1H and ^{13}C NMR spectra were obtained in $CDCl_3$ solution on a Varian Unity 300 or 500 spectrometer. Chemical shifts are expressed in δ value with TMS as an internal reference and coupling constants (J) are given in Hz.

Results and Discussion

Isolation and Purification

11-Hydroxyaclacinomycins **1**, **2**, **3** and **4** were purified to microcrystalline powders by the procedure illustrated in Fig. 2. The culture broth was centrifuged to separate the mycelial cake from the supernatant. The pigments were extracted from the mycelium with acetone and from the supernatant with ethylacetate, separately. After evaporation of the solvent, the residues were combined and extracted with chloroform. The organic layer was concentrated to a small volume *in vacuo*. The red pigments were separated by preparative TLC (MERCK, Art. 5744) using chloroform-methanol (20:1) and $CHCl_3$-CH_3OH-CH_3COOH-H_2O-Et_3N (68:20:10:2:0.01). The red pigment bands were scraped off and extracted with methanol. The crude mixtures were further purified by HPLC using a ECONOSIL SILICA column (i.d. 22.5 × 250 mm, 10 μ) eluting with $CHCl_3$-MeOH-CH_3COOH-H_2O-Et_3N (68:20:10:2:0.01) to give **1** (43 mg), **2** (35 mg), **3** (7 mg) and **4** (56 mg).

Fig. 2. Isolation and purification of 11-hydroxyaclacinomycin X and its analogues from *Streptomyces galilaeus* ATCC 31133/pMC213.



Physico-chemical Properties

The new anthracycline compound **1**, obtained as red powder, was optically active $[\alpha]_D^{20}$ +56.0 (c 0.13, $CHCl_3$). It was soluble in chloroform, ethylacetate and methanol and insoluble in hexane and water. Other 11-hydroxy anthracycline compounds **2**, **3**, and **4** were also red. Their physico-chemical properties and ^1H NMR spectral data were summarized in Tables 1 and 2.

Structure Determination

The UV spectrum of **1** exhibited absorption maxima in methanol around 234, 254, 284 and 492 nm and were

Table 1. Physico-chemical properties of **1**, **2**, **3**, and **4**.

		1	2	3	4
Appearence		Red powder	Red powder	Red powder	Red powder
MP (°C)		184-187	175-178	145-147	160-162
$[\alpha]_D^{20}$		+ 56.0	-127.3	+35.6	-20.4
		(c 0.13, $CHCl_3$)	(c 0.15, MeOH)	(c 0.1, MeOH)	(c 0.15, MeOH)
FAB-MS(m/z)		841 $(M+H)^+$	826 $(M+H)^+$	585 $(M+H)^+$	828 $(M+H)^+$
Molecular formula		$C_{42}H_{52}N_2O_{16}$	$C_{42}H_{51}NO_{16}$	$C_{30}H_{35}NO_{11}$	$C_{42}H_{53}NO_{16}$
UV λ^{MeOH}_{max} (log ε)		234 (4.55)	234 (4.69)	234 (4.45)	234(4.52)
		254 (4.37)	254 (4.48)	254 (4.21)	252(4.30)
		284 (4.33)	284 (4.00)	292 (3.75)	294(3.84)
		492 (4.08)	494 (4.24)	492 (3.99)	491(4.06)
IR υ^{KBr}_{max} cm^{-1}		3444, 1731,	3486, 1733,	3475, 1731,	3442, 1732,
		1602, 1008	1604, 1010	1604, 1027,	1604, 1020
				987	
Solubility	Soluble	$CHCl_3$, MeOH	$CHCl_3$, MeOH	$CHCl_3$, MeOH	$CHCl_3$, MeOH
		EtOAc	EtOAc	EtOAc	EtOAc
	Insoluble	C_6H_{12}, H_2O	C_6H_{12}, H_2O	C_6H_{12}, H_2O	C_6H_{12}, H_2O
Rf value [a]		0.65	0.95	0.74	0.93

[a] The values were obtained on silica gel TLC.
Solvent system: $CHCl_3$ - CH_3OH - CH_3COOH - H_2O - Et_3N = 68 : 20 : 10 : 2 : 0.01

Table 2. ^1H NMR chemical shifts of **2**, **3** and **4**.

proton	2 (J, Hz)	3 (J, Hz)	4 (J, Hz)
1-H	7.79 d (7.8)	7.87 dd (8.0, 0.9)	7.84 d (7.7)
2-H	7.65 t (7.8)	7.71 t (8.0)	7.68 t (7.7)
3-H	7.25 d (7.8)	7.31 dd (8.0, 0.9)	7.28 d (7.7)
7-H	5.20 br	5.24 br dd	5.22 br s
16-OCH_3	3.70 s	3.71 s	3.69 s
1'-H	5.48 br s	5.56 br d	5.50 br s
1''-H	5.10 br s	-	5.03 br d
1'''-H	5.18 d (3.3)	-	5.05 t (6.5)

superimposable on that of 11-hydroxyaclacinomycin A with the exception of a new absoption band at 284 nm which did not shift on addition of either dilute base or dilute acid. It meant that characteristic UV chromophore might be located in sugar residues. The IR spectrum (KBr) showed the presence of ester carbonyl (1731 cm^{-1}) and chelated carbonyl (1602 cm^{-1}) and the absence of non chelated carbonyl absorption indicating that the aglycone would be ε-rhodomycinone rather than aklavinone[11]. The molecular formula of **1** was determined to be $C_{42}H_{52}N_2O_{16}$ (MW 840) by HRFAB-MS ($M+H^+$: *m/z* Found 841.3502; Calcd 841.3395). The FAB-MS spectrum confirmed this elemental formula exhibiting ions at *m/z* 841 $(M+H)^+$, 714 and 586 corresponding to the molecular ion and cleavages at the C-1''' and C-1'' anomeric carbons, respectively. The structure of **1** was elucidated from the ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, ^1H-^{13}C COSY and HMBC spectral analysis. The ^1H NMR spectrum of **1** showed four methyl groups in the upfield region and five proton signals in the anomeric region (Table 3). Three signals appeared at δ 5.51, 5.05, and 5.23 were assigned as anomeric protons attached at the C-1', C-1'' and C-1''' and one signal at δ 5.20 was determined as the proton at C-7. The chemical shifts of these four protons were similar to those of 11-hydroxyaclacinomycin A[4,12]. In addition, one characteristic sharp singlet integrating for one proton was observed at δ 5.34. This signal was correlated to a sp^2 carbon signal at δ 95.3 in the DEPT and HETEROCOSY spectra. The chemical shift of this carbon is characteristic of the α-carbon of an α,β-unsaturated carbonyl group. The carbonyl carbon of the α,β-unsaturated ketone appeared at δ 194.1 and the β carbon appeared at δ 159.5 as a quaternary carbon. Together with the presence of the extra nitrogen atom revealed by HRFAB-MS spectra, these chemical shifts suggest that the third sugar would have a β-enamino ketone moiety[11]. In fact, the placement of the NH_2 group at C-2''' was fully consistent with all the spectral

Table 3. ^{1}H and ^{13}C NMR chemical shifts of **1** in $CDCl_3$.

Position	δc(ppm)	δH(ppm)	Assignment	Position	δc(ppm)	δH(ppm)	Assignment
1	119.6	7.81 (d, *J*=7.8 Hz)	=CH	16	52.4	3.68 (s)	OCH_3
2	137.1	7.66 (t, *J*=7.8 Hz)	=CH	1'	101.4	5.51 (br d)	CH(O)
3	124.8	7.25 (d, *J*=7.8 Hz)	=CH	2'	28.7	1.25 (m)	CH_2
4	162.5	13.46 (br)	=C(OH)	3'	61.7	1.99 (m)	CH(N)
4a	115.9		=C	4'	73.6	3.78 (br. s)	CH(O)
5	190.7		C=O	5'	68.2	4.01 (q, *J*=6.4 Hz)	CH(O)
5a	111.5		=C	6'	17.9	1.27 (d, *J*=6.4 Hz)	CH_3
6	156.7	12.82 (br)	=C(OH)	NMe2	42.9	2.30 (s)	$N(CH_3)_2$
6a	135.9		=C	1"	99.3	5.05 (br s)	CH(O)
7	70.8	5.20 (br)	CH(O)	2"	34.1	2.18 (m)	CH_2
8	33.4	2.44 (m), 2.32 (m)	CH_2	3"	65.4	4.17 (m)	CH(OH)
9	71.2	5.43 (s)	C(OH)	4"	83.0	3.86 (br s)	CH(O)
10	52.0	4.24 (s)	CH	5"	67.0	4.62 (q, *J*=6.1 Hz)	CH(O)
10a	135.3		=C	6"	17.2	1.21 (d, *J*=6.1 Hz)	CH_3
11	156.9	12.07 (br)	=C(OH)	1"'	96.4	5.23 (s)	CH(O)
11a	111.2		=C	2"'	159.5		$=C(NH_2)$
12	186.1		C=O	3"'	95.3	5.34 (s)	=CH
12a	133.3		=C	4"'	194.1		C=O
13	32.3	1.84 (m), 1.44(m)	CH_2	5"'	72.0	4.52 (q, *J*=7.0 Hz)	CH(O)
14	6.8	1.09 (t, *J*=7.2 Hz)	CH_3	6"'	15.9	1.37 (d, *J*=7.0 Hz)	CH_3
15	171.2		C=O				

data. This assignment was confirmed by HMBC spectral analysis. In the HMBC spectrum, 1'''-H (δ 5.23) correlated with C-2''' (δ 159.5) and 3'''-H (δ 5.34) correlated with C-4''' (δ 194.1), C-3''' (δ 95.3) and C-2''' (δ 159.5). To date, the sugar residues containing an α,β-unsaturated ketone moiety in the anthracyclines were known as L-aculose and rednose which were found in aclacinomycin Y[2] and in rudolphomycin[11], respectively. The UV and ^{1}H and ^{13}C NMR spectral data indicated that **1** would have rednose in the sugar residue. The chemical shift assignments of the ^{13}C NMR spectrum of **1** were similar to those of 11-hydroxyaclacinomycin A with the exception of the third sugar residue at C-1''', 2''', 3''' and C-4'''[12]. The assignment of the carbons of the sugar residues was made by comparison with the spectral data of rudolphomycin. From all these findings, the structure of this new anthracycline compound was determined as 7-O-[O-α-L-rednosyl-(1→4)-2-deoxy-O-α-L-fucosyl-(1→4)-α-L-rhodosaminyl]-ε-rhodomycinone which was named 11-hydroxyaclacinomycin X. Other 11-hydroxy anthracycline compounds, **2**, **3**, and **4** were also isolated and their structures elucidated. They showed molecular ion peaks at *m/z* 826 $(M+H)^+$, 586 $(M+H)^+$ and 828$(M+H)^+$ in the FAB-MS spectrum, respectively. The UV spectra of the compounds showed very similar absorption maxima around 234, 254, 292 and 492 nm, indicating the presence of another hydroxyl group in the molecule which is causing a bathochromic shift from 430 nm to 492 nm (data not shown). ^{1}H NMR (Table 2) and ^{13}C NMR spectral analysis (data not shown) further indicated that these compounds were ε-rhodomycinone glylcosides, according to the absence of a singlet aromatic proton (H-11) and the characteristic downfield shift of C-11 signal resulting from the hydroxylation at C-11. Comparisons of their physico-chemical properties and ^{1}H and ^{13}C NMR spectral data with those of epelmycins indicated that **2** was epelmycin B, **3** was epelmycin D and **4** was epelmycin E, which were previously isolated from the culture broth of a blocked mutant of β-rhodomycin-producing *Streptomyces violaceus* A262[4].

In Vitro Antitumor Activity

The *in vitro* cytotoxicity of **1** for various types of human tumor cell lines were tested by the Developmental Therapeutic Program of National Cancer Institute, U.S.A. in order to evaluate the drug's selectivity for

Table 4. *In vitro* test results for **1** against human cancer cell lines.

Panel	Cell line	log_{10}TGI (M)	Panel	Cell line	log_{10}TGI (M)
Leukemia	CCRF-CEM	-7.90	Melanoma	M14	-7.57
	HL-60(TB)	<-8.00		SK-MEL-2	<-8.00
	K-562	-7.63		SK-MEL-28	-7.69
	MOLT-4	<-8.00		SK-MEL-5	<-8.00
	RPMI-8226	<-8.00		UACC-257	-7.62
	SR	-7.95		UACC-62	<-8.00
Non-Small Cell	EKVX	-7.01	Ovarian Cancer	OVCAR-3	-6.82
Lung Cancer	HOP-92	-5.78		OVCAR-5	-6.02
	NCI-H226	<-8.00		OVCAR-8	-6.58
	NCI-H23	-7.44	Renal Cancer	786-0	-6.83
	NCI-H322M	-6.23		CAKI-1	<-8.00
Colon Cancer	COLO205	-7.89		SN12C	-5.95
	HCC-2998	<-8.00		TK-10	-7.11
	HCT-116	>-4.00		UO-31	-7.56
	HCT-15	-5.93	Prostate Cancer	PC-3	-7.13
	KM12	-6.70	Breast Cancer	MCF7	-5.85
	SW-620	-7.07		MDA-MB-231	-6.53
CNS Cancer	SF-268	-6.52		HS578T	-5.83
	SF-295	-6.85		MDA-N	-7.92
	SF-539	-7.65		T-47D	-5.56
	SNB-75	-6.88	MG-MID		-7.05
	U251	-5.97	Delta		0.95
Melanoma	LOXIMVI	-6.21	Range		4.00
	MALME-3M	<-8.00			

particular tumor types[13]. As shown in Table 4. **1** showed similar, very strong cytotoxic activities against human tumor cell lines. The average log_{10} TGI of **1** was -7.05. **1** showed very active and selective cytotoxicities against certain cell lines of leukemia (HL-60, MOLT-4 and RPMI-8226), non-small cell lung cancer (NCI-H226), colon cancer (COLO 205 and HCC-2998), melanoma (MALME-3M, SK-MEL-2, SK-MEL-5 and UACC-62) and renal cancer (CAKI-1). These remarkably potent cytotoxicities and the high sensitivity of certain human cancer cells suggest that this compound should be further evaluated for future application in cancer chemotherapy.

Acknowledgments

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