Two Novel *C*-Glycosides of Aureolic Acid Repress Transcription of the *MDR1* Gene

Motoyuki TAGASHIRA,*[,a] Takanori KITAGAWA,[a] Naoko NOZATO,[a] Seiji ISONISHI,[b] Aikou OKAMOTO,[b] Kazunori OCHIAI,[b] and Yasuyuki OHTAKE[a]

Foods and Pharmaceuticals Research and Development Laboratory, Asahi Breweries, Ltd.,[a] 1–1–21, Midori, Moriya-machi, Kitasohma-gun, Ibaraki 302–0106, Japan and Department of Obstetrics and Gynecology, The Jikei University School of Medicine,[b] 3–25–8, Nishi-shinbashi, Minato-ku, Tokyo 105–8461, Japan.
Received October 25, 1999; accepted December 8, 1999

In the search for compounds which repress *MDR1* gene expression, two novel aryl *C*-glycosides were isolated from a broth of *Streptomyces* sp. They had the characteristic structure of a dideoxy-carbohydrate (oliose or olivose) linked directly to chromomycinone, an aglycone of aureolic acids. Further investigation revealed that they were artifacts yielded from an aureolic acid, mithramycin. Acid and methanol were necessary to yield the *C*-glycosides. This reaction would contribute to the design of useful aryl *C*-glycosides.

Key words multidrug resistance; *C*-glycoside; aureolic acid; mithramycin; *MDR1* gene

Chemotherapeutic research on cancer has advanced, but the problem remains that tumor cells acquire resistance to antitumor drugs.[1)] Multidrug resistance (MDR) is one of the most serious problems preventing therapy. In recent years, several studies have revealed the molecular mechanism of MDR P-glycoprotein, a membrane protein encoded by the *MDR1* gene, removes exogenous organic substances such as anticancer drugs from the cells.[2)] Hence, it depletes intracellular drug concentrations with low selectivity and causes MDR. In most cases, a high level of *MDR1* expression was observed in MDR tumor cells.[3)]

Several drugs such as calcium antagonists (verapamil)[4)] and immunosuppressive agents (cyclosporin A, FK506)[5)] are reported to inhibit P-glycoprotein function and sensitize the MDR tumor cells. However, their original activities such as calcium antagonism or immunosuppression has prevented their therapeutic application. Their analogues with low-toxicity such as MS-209 and SDZ PSC 833, are still undergoing clinical trials.[6)]

We are researching *MDR1* gene expression inhibitors derived from microorganisms. From the broth of the *Streptomyces* species KS12571, two active compounds were isolated. Spectrometric studies elucidated their chemical structures as **1** and **2**. The compounds had quite characteristic chemical structures categorized in aryl *C*-glycosides: C-1″ of dideoxy carbohydrate (oliose or olivose) directly bonded to the C-5 of their aglycone, chromomycinone.[7)] High resolution (HR)-FAB-MS data showed that they had the same molecular formula ($C_{27}H_{34}O_{12}$), and the ^{1}H- and ^{13}C-NMR spectrum suggested they contained a chromomycinone moiety in their chemical structures (Table 1). ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence (HMBC) spectrum showed a correlation between the H-1″ of carbohydrate and C-5, C-6 and C-10a of aglycone (Table 2). Nuclear overhauser and exchange spectroscopy (NOESY) data also showed that the H-1″ of the carbohydrate correlated to the H-10 of the aglycone (Fig. 1). This result strongly suggested that the dideoxy carbohydrates directly bonded to their aglycone. From the NOESY spectrum, the carbohydrates of **1** and **2** were suggested to be oliose and olivose respectively (Fig. 1).

Their activity was tested using a *MDR1*-high expressing human small lung tumor cell line, SBC-3/ADM.[8)] Figure 2 shows that **1** and **2** (0.1—0.6 mg/ml) inhibited *MDR1* gene expression in a dose-dependent manner. They had little effect on that of β_2-microgroblin (β_2-m), used as a control. This result suggests that these compounds would be candidates for drugs against MDR in cancer chemotherapy.

We tried to investigate the mechanism of biosynthesis of the *C*-glycosides, and further research revealed that the compounds were artifacts yielded from an aureolic acid, mithramycin (**3**).[9)] At first, from the broth of KS12571, the peak of mithramycin (Fig. 3a) was fractionated by HPLC with HCl conditions. In the concentration process, the rotary vacuum evaporator N-2NR (Tokyo Rika Kikai Co.) equipped with a digital water bath SB-651 (Tokyo Rika Kikai Co.) was used. The water bath temperature was fixed at 40 °C. We collected the fraction (about 800—1000 ml) containing mithramycin in a 2 l glass flask. When the solvent was concentrated to about 100—150 ml, the fraction was moved into a smaller glass flask (300 ml). At that point, we washed the 2 l flask with MeOH (10—30 ml) and added the wash solvent to the concentrated sample. Then, the solvent was dried by the same evaporator to give a pale yellow-green residue (about 180 mg). Finally, 100 mg of the residue was dissolved in MeOH (10 ml), filtered by a Cosmonice Filter S (pore size, 0.5 μm; Nacalai Tesque Ltd.), and subjected to preparative HPLC again (Fig. 3c). The *C*-glycosides were then isolated (yields from 180 mg of residue: **1**, 8 mg; **2**, 14 mg). Without HCl in HPLC conditions, mithramycin was detected again by HPLC (Fig. 3b, 3d). Besides, without MeOH in the concentration process, mithramycin was degraded, but no *C*-glycosides were obtained. In conclusion, as far as we tested, without HCl or MeOH, **1** and **2** were not isolated from the fraction. We also checked that **1** and **2** could be obtained from authentic mithramycin purchased commercially (Sigma-Aldrich). The mechanism of this reaction has not been elucidated, but the chemical structure of mithramycin would suggest that its 6-*O*-glycoside (carbohydrate A in Chart 1) was converted into a 5-*C*-glycoside when it was hydrolyzed.

In recent years, interest in aryl *C*-glycosides has been growing because of their various biological functions, such

* To whom correspondence should be addressed.

Table 1. ^{1}H- and ^{13}C-NMR Data of Isolated Aryl *C*-Glycosides in CD_3OD

Part	Position	1		2	
		δ_C	δ_H	δ_C	δ_H
Aglycone[a)]	1	204.2		204.2	
	2	72.7	4.48 (1H, d, *J*=11.3 Hz)	72.7	4.48 (1H, d, *J*=11.2 Hz)
	3	43.9	2.81 (1H, m)	43.9	2.77 (1H, m)
	4	27.8	2.74 (1H, m)	27.8	2.69 (1H, m)
			3.01 (1H, m)		3.02 (1H, m)
	5	109.8		108.6	
	6	159.8		159.4	
	7	110.8		110.8	
	8	156.0		156.0	
	9	164.4		164.6	
	10	111.4	6.96 (1H, s)	111.4	6.93 (1H, s)
	4a	136.6		136.8	
	8a	106.4		106.6	
	9a	107.0		107.1	
	10a	134.9		134.9	
	1′	82.8	4.91 (1H, m)	82.8	4.91 (1H, m)
	2′	212.4		212.3	
	3′	79.1	4.24 (1H, d, *J*=2.7 Hz)	79.1	4.24 (1H, d, *J*=2.7 Hz)
	4′	68.3	4.28 (1H, dq, *J*=2.7, 6.4 Hz)	68.3	4.28 (1H, dq, *J*=2.7, 6.4 Hz)
	5′	18.7	1.28 (3H, d, *J*=6.4 Hz)	18.6	1.28 (3H, d, *J*=6.4 Hz)
	OMe1′	58.6	3.48 (3H, s)	58.6	3.48 (3H, s)
	6′	7.1	2.16 (3H, s)	7.1	2.15 (3H, s)
Carbohydrate	1″	74.7	5.31 (1H, m)	39.0	2.12 (1H, m)
	2″	32.8	2.16 (1H, m)	39.0	2.12 (1H, m)
			1.82 (1H, m)		1.81 (1H, m)
	3″	69.3	3.95 (1H, m)	71.8	3.82 (1H, m)
	4″	70.2	3.68 (1H, m)	77.7	3.15 (1H, dd, *J*=9.1, 9.3 Hz)
	5″	75.6	3.78 (1H, dq, *J*=6.4, 1.9 Hz)	77.6	3.59 (1H, dq, *J*=6.4, 9.1 Hz)
	6″	16.8	1.36 (3H, d, *J*=6.4 Hz)	17.6	1.45 (3H, d, *J*=6.4 Hz)

a) NMR data of chromomycinone were previously reported.[7)]

Table 2. HMBC Correlation between ^{1}H and ^{13}C of Compound **2**

^{1}H	⟶	^{13}C
2-H		1-C
3-H		2-C
4-Ha		2-C, 3-C, 4a-C, 9a-C, 10-C
4-Hb		3-C, 4a-C
10-H		4-C, 5-C, 8a-C, 9a-C, 10a-C
1′-H		2-C, 3-C, 4-C, 2′-C, OMe1′-C
3′-H		2′-C, 4′-C
4′-H		5′-C
5′-Me		3′-C, 4′-C
OMe1′		1′-C
6′-Me		5-C, 6-C, 7-C, 8-C, 8a-C
1″-H		5-C, 6-C, 10a-C
2″-Ha		3″-C
2″-Hb		1″-C, 3″-C
3″-H		ND
4″-H		3″-C, 5″-C
5″-H		ND
6″-Me		5″-C

ND: Not detected.

as antibacterial activity,[10)] protection against radiation,[11)] inhibition of platelet aggregation,[12)] and antitumor activities.[13)] Therefore, it is worth investigating **1** and **2** for further activities. *C*-Glycosides also have some advantages over *O*-glycosides in terms of chemical stability. In fact, in the present study we showed that **1** and **2** were not degraded under acidic HPLC conditions while mithramycin, an *O*-glycoside com-

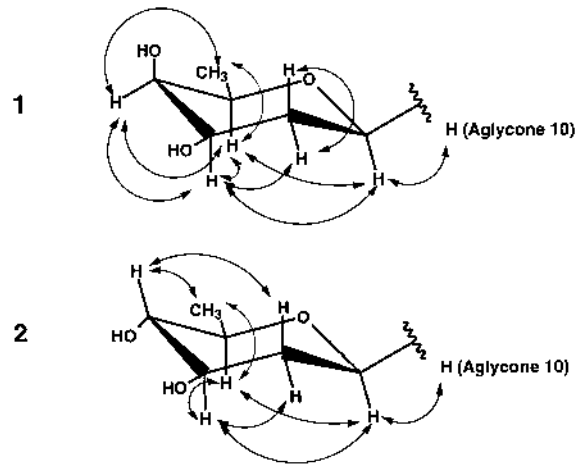

Fig. 1. NOE Interactions Observed in the NOESY Spectra of the Carbohydrate Moiety of **1** and **2**

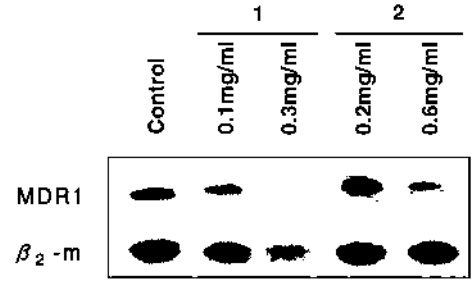

Fig. 2. Repression of MDR1 Gene Expression by **1** and **2**
Control denotes the results of experiments without drugs.

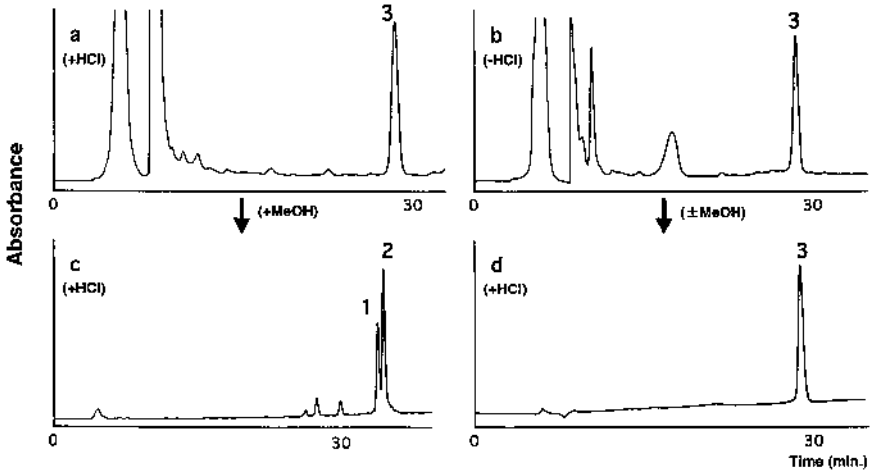

Fig. 3. HPLC Chromatogram in Isolating **1** and **2**

a) Chromatogram of the broth of KS12571 (with HCl conditions); HPLC conditions: system, Shimadzu LC-8A system; column, TSK-Gel ODS-80Ts (55 mm i.d.×30 cm, Tosoh Ltd.); flow rate, 80 ml/min; mobile phase, linear gradient from A to B in 30 min (solvent A, 30% CH_3CN containing 0.1% HCl; B, 45% CH_3CN containing 0.1% HCl); detection, UV 220 nm; sample concentration, 30 mg/ml (MeOH); injection volume, 10 ml. b) Chromatogram of the broth of KS12571 (without HCl conditions); HPLC conditions: mobile phase, linear gradient from A to B in 35 min (solvent A, 25% CH_3CN; B, 60% CH_3CN); other conditions were as for chromatogram (a). c) Chromatogram of the fractionated peak 3 in (a); HPLC conditions: column, Shim-pack PREP-ODS (H) column (20 mm i.d.×25 cm, Shimadzu Corp.); flow rate, 10 ml/min; mobile phase, linear gradient from A to B in 40 min (solvent A, 25% CH_3CN containing 0.1% HCl; B, 45% CH_3CN containing 0.1% HCl); other conditions were as for chromatogram (a). d) Chromatogram of the fractionated peak 3 in (b); HPLC conditions were as for chromatogram (a).

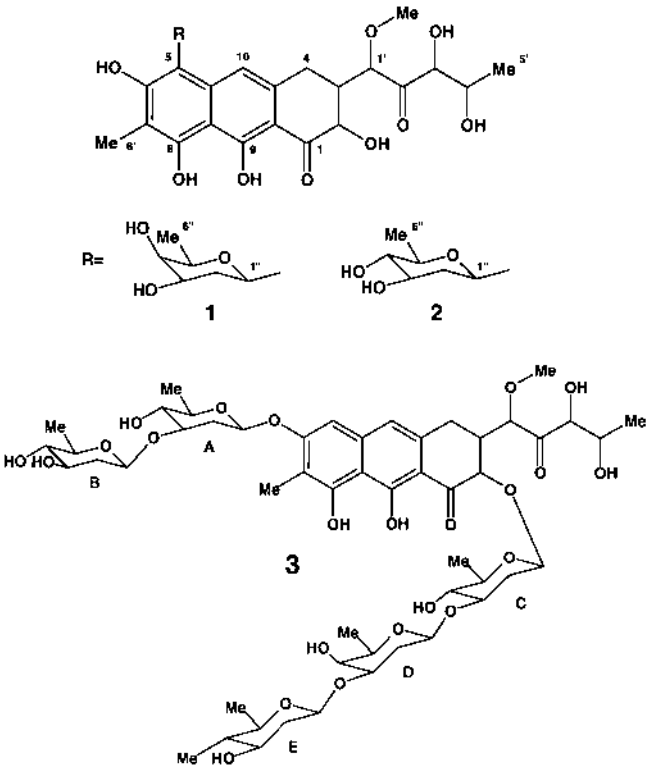

Chart 1

pound, was easily hydrolyzed.

The carbohydrate structure of aureolic acid is thought to be important because it regulates its binding to DNA. Indeed, an NMR study showed that mithramycin and chromomycin, which differ only in carbohydrate structure, had different binding regions for DNA and different anti-tumor activities.[14] Therefore, several attempts have been made to create more useful aureolic acid analogues by the modification of their carbohydrate structures.[15] Further study of this reaction would contribute to the creation of novel useful aryl *C*-glycoside analogues, not only of the aureolic acids, but of other aryl bioactive compounds.[16]

Also, we are now investigating the *MDR1* repressing activity of mithramycin and other aureolic acids. The results will be described in another paper.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Spectroscopic measurements were done with the following instruments: Hitachi U-2000 spectrometer (UV), Shimadzu FTIR-8700 (IR), JEOL JMS-700 (MS), Bruker DRX-500 (500 MHz-NMR), JASCO DIP-360 (optical rotation).

Bacterial Strain The *Streptomyces* sp., named KS12571, was isolated from a soil sample collected in Ibaraki Prefecture, Japan by a method previously described.[17] Identification of the species was performed by Japan Food Research Laboratories.

Repression of the MDR1 Gene Expression SBC-3/ADM tumor cells were cultured for 48 h in RPMI1640 medium (Gibco BRL.) containing 2 μl doxorubicin (Sigma-Aldrich). The medium was replaced with RPMI1640 medium containing 0.1—0.6 mg/ml **1** or **2**. The cells were cultured for 48 h and the mRNA was collected and tested. The cultivation was done at 37 °C under a humidified 5% CO_2 atmosphere. SBC-3/ADM tumor cells were kindly provided by Dr. N. Saijo (National Cancer Center-Japan).

RNA Preparation, Reverse Transcription, and cDNA-PCR Total cellular RNA was isolated using a Trizol kit (Life Technologies, Inc.). Total RNA(3 μg) was used for reverse transcription and cDNA-PCR, using an RNA PCR kit (AMV) Ver.2.1 (Takara). Primers *MDR1*-F (5′-cgc cat tgc acg tgc cct gg) and *MDR1*-R (5′-cct ttc tct ttc atg agt tc) were used to amplify *MDR1*. Primers β_2-m-F (acc ccc act gaa aaa gat ga) and β_2-m-R (atc ttc aaa cct cca tga tg) were used to amplify β_2-m. β_2-m was used as a control to check the integrity of RNA preparations and accuracy of the dilutions, as well as to demonstrate that the effect of mithramycin on *MDR1* expression in SBC-3/ADM tumor cells was not due to nonspecific decreases in transcription. PCR was performed for 15 and 20 cycles on the dilution of cDNA.

Spectroscopic Data **1**: mp 121—124 °C (dec.). HR-FAB-MS *m/z*: 551.2136 (Calcd for $C_{27}H_{35}O_{12}$ $(M+H)^+$ 551.2128). $[\alpha]_D^{22}$ +91.3° (c=0.1, MeOH). IR (KBr) cm^{-1}: 1629, 1265, 1188, 1094, 1063. UV λ_{max} (MeOH) nm (log ε): 420 (3.92), 326 (3.81), 282 (4.58), 233 (4.40), λ_{min} (MeOH) nm (log ε): 358 (3.56), 318 (3.77), 251 (4.14), 219 (4.31).

2: mp 127—131 °C (dec.). HR-FAB-MS *m/z*: 551.2141 (Calcd for $C_{27}H_{35}O_{12}$ $(M+H)^+$ 551.2128). $[\alpha]_D^{22}$: +92.0° (c=0.1, MeOH). IR (KBr) cm^{-1}: 1628, 1261, 1186, 1070. UV λ_{max} (MeOH) nm (log ε): 419 (3.94),

324 (3.76), 282 (4.59), 233 (4.40), λ_{min} (MeOH) nm (log ε), 353 (3.46), 317 (3.72), 252 (4.13), 219 (4.31).

3: The mithramycin isolated from KS12571 was consistent with the authentic compound purchased from Sigma-Aldrich. mp 180—183 °C (lit. 180—183 °C), $[\alpha]_D^{22}$: −44.9° (c=0.1, MeOH), the authentic: mp 180—183 °C, $[\alpha]_D^{22}$: −44.3° (c=0.1, MeOH).

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