A Dammarane Glycoside Derived from Ginsenoside Rb_3

Kejiang HE,[a] Yong LIU,[a,b] Yi YANG,[a] Peng LI,[a] and Ling YANG*,[a]

[a] *Laboratory of Pharmaceutical Resource Discovery, Dalian Institute of Chemical Physics, Chinese Academy of Sciences; 457 Zhong-shan Road, Dalian 116023, P.R. China: and* [b] *Graduate School of Chinese Academy of Sciences; P.R. China.*
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A dammarane glycoside, designated compound Mx (C-Mx), was isolated from the hydrolysate of 20(*S*)-protopanaxadiol type ginsenosides containing G-Rb_3 from *Panax notoginseng* leaves with crude snailase. Its chemical structure was elucidated to be 20-*O*-β-D-xylopyranosyl(1→6)-β-D-glucopyranosyl-20(*S*)-protopanaxadiol on the basis of spectral analysis. Its cytotoxicity against breast cancer cell line MCF-7 and effects on the sensitivity to doxocubicin of doxocubicin-resistant MCF-7 cells were also investigated. The new compound showed moderate cytotoxicity and partial reversal of doxocubicin resistance.

Key words ginsenoside Rb_3; compound Mx; structure; NMR data

Ginseng, the root of *Panax ginseng* C. A. MEYER, is one of the best-known Chinese traditional herbal medicines, which has been used as a tonic, sedative, antifatigue, or anti-gastric ulcer drug for thousands of years. Naturally occurring ginsenosides, isolated from ginseng, have been regarded as the principal components responsible for the pharmaceutical and biological activities of ginseng.[1] There are more than 30 different known naturally occurring ginsenosides, which can be classified into two groups according to their sapogenins with a dammarane skeleton, the protopanaxadiol and protopanaxatriol groups, except for ginsenoside R_0.

In most cases, Ginseng has been used as an orally administered crude drug. It has been reported that some naturally occurring ginsenosides abundant in ginseng, such as Rb_1, Rb_2 and Rg_1, are water soluble and their absorption from the intestines is very poor.[2,3] However, the intestinal bacterial metabolites of ginseng saponins can easily be absorbed from the intestine and are considered as primary active ingredients.[4,5] There are many studies showing that the intestinal bacterial metabolites[6] of 20(*S*)-protopanaxadiol-type (diol-type) ginsenosides, such as compound K (C-K), compound Y (C-Y) and ginsenoside Mc (G-Mc), exhibited excellent antitumor activities and were responsible for the main pharmacological activities of Ginseng.[7—10]

The previous studies on the intestinal bacterial metabolites of ginsenosides suggested that the pathway of ginsenoside metabolism by intestinal bacteria begins with the attack to sugar moieties at the position C-20 and ends with the attack to sugar moieties at the position C-3, and one of the major terminal products of this pathway is C-K.[6,11] However, we found a new kind of snailase with special properties, which may hydrolyze sugar moieties at the position C-3 of ginsenosides firstly. Thus, in order to clarify additional active components of Ginseng, we studied on other metabolites of ginsenosides, using the enzyme. As the results, a novel metabolite was isolated and characterized. In this paper, we report the preparation, structural elucidation and activity in reversing the doxocubicin, or adriamycin (ADM), resistance of the metabolite from ginsenoside Rb_3 with glycosidase hydrolysis.

Results and Discussion

The mixture of G-Rb_1, G-Rb_2, G-Rb_3, G-Rc and G-Rd, derived from the methanol extract of *Panax notoginseng* leaves, was digested with crude snailase to afford C-K and a less polar novel metabolite, named as compound Mx (C-Mx).

From the IR spectrum, C-Mx was assumed to contain a double bond (1640 cm^{-1}) and hydroxy group (3400 cm^{-1}). In the ^{1}H-NMR spectrum (400 MHz, Py-d_5) of C-Mx, one olefinic [δ 5.32 (1H, t, J=7.1 Hz)], two anomeric [δ 5.61 (1H, J=1.7 Hz), 5.10 (1H, d, J=7.8 Hz)], and eight singlet methyls [δ 1.69, 1.67, 1.67, 1.21, 1.01, 0.99, 0.92, 0.89 (each 3H, all s)] proton signals were observed. Comparative study on all these data and carbon signals (Table 2) with those of protopanaxadiol, protopanaxatriol and other ginsenosides suggested that C-Mx is a diol-type of ginsenoside.[12—14]

In the ^{13}C-NMR spectrum (100 MHz, Py-d_5), the chemical shift of two anomeric [δ 97.8 (C-1′), 105.0 (C-1″)] and other sugar moiety signals [δ 74.9 (C-2′), 78.9 (C-3′), 71.6 (C-4′), 76.9 (C-5′), 69.0 (C-6′), 74.3 (C-2″), 77.4 (C-3″), 70.7 (C-4″), 66.0 (C-5″)] revealed that the xylopyrnosyl moiety occurred as a terminal glycosyl, which formed a diglycosyl [D-xylopyranosyl(1→6)-β-D-glucopyranosyl] moiety in the C-Mx molecule. The chemical shift C-6′ (δ 69.0, downfield shift about 6 ppm relative to 6-hydroxyl free D-glucopyranosyl moiety) and C-1″ (δ 105.0) indicated that the terminal xylopyranosyl moiety was bound to the 6-hydroxy group of the innermost D-glucopyranosyl moiety. The linkage position of the D-glucopyranosyl moiety in C-Mx was determined to be 20-hydroxyl group according to the chemical shift of C-1′ (δ 97.8) and C-20 (δ 83.4, downfield shift about 10 ppm relative to protopanaxadiol).[11—13]

From the above results, C-Mx was assumed to correspond to a deglycosylated ginsenoside Rb_3 and characterized as 20-*O*-β-D-xylopyranosyl(1→6)-β-D-glucopyranosyl-20(*S*)-protopanaxadiol. This was supported by the ESI-MS spectral data of C-Mx (*m*/*z*: 754.4, 622.2 and 459.7) corresponding to molecular formula ($C_{41}H_{70}O_{12}$) and confirmed by comparing the NMR spectra of C-Mx with those of C-K, C-Y,[15] G-Mc[6] and G-Rb_3. The differences in NMR spectra among them are derived from the differences in their glycosyl moieties.

C-Mx was tested for its cytotoxicity against the human tumor cell line MCF-7 and its subline resistant to doxocubicin (ADM) MCF-7/ADM, and its effects on the sensitivity to ADM of MCF-7/ADM cells (Table 3). MCF-7/ADM cells were highly resistant to ADM when compared with MCF-7

* To whom correspondence should be addressed. e-mail: yling@dicp.ac.cn

cells, and the IC_{50} value of ADM in MCF-7/ADM cells was 120 fold that in MCF-7 cells. Both MCF-7 and MCF-7/ADM cells were sensitive to the toxicity of C-Mx to the same extent although a slight difference was observed. When a marginally toxic quantity of C-Mx was added to MCF-7/ADM cell growth medium, the 37.5 μM/ml IC_{50} of ADM was shifted to 7.61 μM/ml, and the MDR (multidrug resistance) ratio of C-Mx was 4.9 according to equation (1).

C-K, C-Y and G-Mc are the metabolites of G-Rb_1, G-Rb_2 and G-Rc produced by human intestinal bacteria.[10] C-Y and G-Mc differ from C-K in the presence of an additional sugar moiety, which is α-L-arabinopyranosyl for C-Y but α-L-arabinofuranosyl for G-Mc (Table 1). It has been reported that these metabolites have moderate cytotoxicity to[7,8] and partial reversal of multidrug resistance of human tumor cells.[9,10] This work disclosed a dammarane glycoside, C-Mx, derived from G-Rb_3, which is abundant in *Panax notoginseng* leaves, and the analogue of C-K has the similar bioactivity.

Experimental

Melting points were determined on a WRS-1B digital melting point apparatus (Shanghai Precision Scientific Instrument Co. Ltd., China); The ^{1}H- and ^{13}C-NMR spectra were measured with a Varian Inova 300 Spectrometer (Talo Alto, CA, U.S.A.) and chemical shifts are expressed in δ from TMS as an internal standard; mass spectra were taken on a EIMS spectrometer (TSQ-7000, Finnigan); and IR spectra were recorded with a TENSOR 27 FT-IR spectrometer (Bruker, Germany).

The dry powder of *Panax notoginseng* leaves (1 kg, from Yunnan, China, deposited at a dry and adumbral place at room temperature) was extracted with methanol (31×3) at room temperature. The methanol extract (130 g) was successively purified on a macro-reticular resin (Amberlite XAD7, Sigma) column and then an anion-exchange resin (Amberlite IRA-900, Sigma) column to give 70% EtOH eluates that afforded the total saponins (70 g) after drying. The total saponins were dissolved in 10% NaOH aqueous solution (1 l) and extracted with water-saturated 1-butanol (300 ml×5). The aqueous layer was neutralized with 1 mol HCl solution and extracted with water-saturated 1-butanol (200 ml×3). The 1-butanol layer was taken and evaporated under vacuum to yield a diol-type of ginsenosides (45 g).

The diol-type of ginsenosides (5 g) was hydrolyzed with crude snailase (3 g, from Sigma) for 24 h at 40 °C in 300 ml of 0.01 mol phosphate-citrate buffer (pH 4.5) containing 10% EtOH. The precipitate thus formed was dissolved in water (30 ml) and the solution thus obtained was extracted with water-saturated 1-butanol (20 ml×3). The 1-butanol layer was evaporated under vacuum to yield the hydrolyzate (3 g). The hydrolyzate (3 g) was applied to a silica gel flash column (100 mm×25 mm i.d. (inner diameter)) eluted with EtOAc–1-BuOH–H_2O (10 : 1 : 1→5 : 1 : 1, upper layer) to afford C-K and a subfraction. The subfraction was evaporated to give the dammarane glycoside C-Mx (600 g).

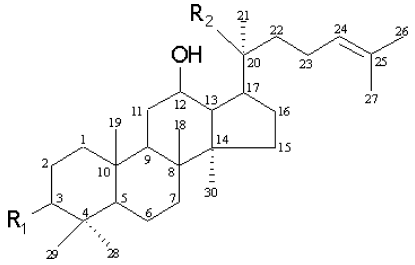
C-Mx: Amorphous powder (MeOH–EtOAc), mp 162.5—165 °C; $[\alpha]_D^{24}$ +12.6° (c=0.3, methanol); IR (KBr) ν_{max} cm^{-1}: 3400 (OH), 1640 (C=C); ESI-MS m/z (rel. int.): 777.4 (754.49 for $C_{41}H_{70}O_{12}$) $[M+Na]^+$ (100), 622.2 $[M-C_5H_8O_4]^+$ (5), 459.7 $[M-C_5H_8O_4-C_6H_7O_5]^+$ (4); ^{1}H-NMR (400 MHz, Py-d_5) δ: 3.39 (1H, t, J=10.5, 5.1 Hz, H-3), 0.80 (1H, d, J=11.0 Hz, H-5), 3.93 (1H, ddd-like, H-12), 5.32 (1H, t, J=7.1 Hz, H-24), 0.92 (3H, s, H-18), 0.89 (3H, s, H-19), 1.69 (3H, s, H-21), 1.67 (3H, s, H-26), 1.67 (3H, s, H-27), 1.21 (3H, s, H-28), 1.01 (3H, s, H-29), 0.99 (3H, s, H-30), 5.10 (1H, d, J=7.8 Hz, H-1′), 5.61 (1H, J=1.7 Hz, H-1″); ^{13}C-NMR (100 MHz, Py-d_5), see Table 2.

The human breast cancer cell line MCF-7, and its resistant subline MCF-7/ADM were used as the target cells in the cytotoxicity assay. Exponentially growing cells were prepared and maintained at 37 °C in RPMI 1640 containing 10% fetal bovine serum, 0.5% DMSO and gentamycin sulfate (80 μ/ml). C-Mx or ADM was first dissolved in PBS containing 0.5% DMSO at a suitable concentration and further diluted with the growth medium. For drug exposure experiments, tumor cells (1×10^5) were inoculated into 96-well plates containing serial 1 : 2 dilutions of C-Mx or ADM in the presence or absence of C-Mx at a marginally toxic concentration (6 μM, growth inhibition less than 10%) in a final volume of 200 μl of growth medium and incubated at 37 °C in a humidified atmosphere of 5% CO_2 air for 44 h. After incubating, 10 μl of MTT solution (5 mg/ml) was added to each well and incubated for further 4 h. At the end of the incubation, the growth medium was removed and replaced with 100 μl of DMSO (at room temperature). After agitating on a vortex for 5 min, the absorbance was determined at 550 nm and 620 nm as reference on a Bio-Rad (model 550) microplate reader to calculate 50% inhibition concentration (IC_{50}). The MDR ratio was obtained using the following equation:

$$\text{MDR ratio} = IC_{50\,ADM}/(IC_{50\,ADM+C\text{-}Mx}) \quad (1)$$

DMSO, ADM and MTT were purchased from Sigma Chemical Co.

Table 1. Structure of Diol-Type Ginsenosides and Their Metabolites



Compounds	R_1	R_2
G-Rb_1	O-Glc$^{2\text{-}1}$Glc	O-Glc$^{6\text{-}1}$Glc
G-Rb_2	O-Glc$^{2\text{-}1}$Glc	O-Glc$^{6\text{-}1}$Arap
G-Rb_3	O-Glc$^{2\text{-}1}$Glc	O-Glc$^{6\text{-}1}$Xyl
G-Rc	O-Glc$^{2\text{-}1}$Glc	O-Glc$^{6\text{-}1}$Araf
G-Rd	O-Glc$^{2\text{-}1}$Glc	O-Glc
C-K	OH	O-Glc
C-Y	OH	O-Glc$^{6\text{-}1}$Arap
C-Mx	OH	O-Glc$^{6\text{-}1}$Xyl
G-Mc	OH	O-Glc$^{6\text{-}1}$Araf

Glc, β-D-glucopyranosyl; Arap, α-L-arabinopyranosyl; Xyl, β-D-xylopyranosyl; Araf, α-D-arabinofuranosyl.

Table 2. ^{13}C-NMR Chemical Shifts of C-Mx (1) and G-Rb_3 (2)[a]

C	1	2	3[b]	C	1	2	3
Aglycon moiety				28	28.0	28.1	28.7
1	39.3	39.5	39.5	29	16.5	16.4	16.4
2	28.0	26.6	28.3	30	17.4	17.5	17.5
3	78.1	89.0	78.2	3-sugar moieties			
4	39.5	39.6	39.5	1′		105.0	
5	56.6	56.5	56.5	2′		83.4	
6	18.3	18.4	18.8	3′		77.9	
7	35.2	35.3	35.2	4′		71. 6	
8	40.1	39.8	40.2	5′		78.7	
9	50.1	50.2	50.4	6′		62.8	
10	36.9	37.0	37.4	1″		105.7	
11	30.8	30.7	30.8	2″		76.8	
12	70.2	70.3	70.3	3″		78.2	
13	49.4	49.3	49.5	4″		71.6	
14	51.5	51.4	51.5	5″		78.1	
15	30.8	30.6	30.9	6″		63.0	
16	26.6	26.7	26.7	20-sugar moieties			
17	51.7	51.6	51.8	1′	97.8	97.9	98.1
18	16.2	16.3	16.3	2′	74.9	75.0	75.1
19	16.2	16.0	16.1	3′	78.9	79.0	79.2
20	83.4	83.5	83.2	4′	71.6	71.6	72.2
21	22.3	22.4	22.4	5′	76.9	76.4	76.5
22	36.1	36.1	36.2	6′	69.0	69.0	68.5
23	23.2	23.1	23.2	1″	105.0	105.1	110.1
24	125.8	125.8	126.1	2″	74.3	74.1	83.5
25	130.9	130.8	131.0	3″	77.4	77.2	79.0
26	25.7	25.6	25.8	4″	70.7	70.5	86.3
27	17.6	17.7	17.9	5″	66.0	65.9	62.8

a) All spectra recorded in pyridine-d_5. Chemical shifts in ppm relative to internal TMS. The spectra were recorded at 100 MHz. *b*) Data on ginsenoside Mc from Hasegawa (1996).

Table 3. IC_{50} values of C-Mx and ADM

Compound	IC_{50} (μM/ml)	
	MCF-7/ADM	MCF-7
C-Mx	58.2±8.57	48.5±7.27
ADM	37.5±7.01	0.31±0.09
ADM+C-Mx[a)]	7.61±1.47	0.29±0.07

a) IC_{50} value of ADM in the presence of C-Mx 6 μM.

(St. Louis, MO, U.S.A.). The results are summarized in Table 3.

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References

1) Shibata S., Tanaka O., Shoji J., Saito H., "Economic and Medicinal Plant Research," Vol. 1, Academic Press, London, 1985, p. 217.
2) Odani T., Tanizawa H., Takino Y., *Chem. Pharm. Bull.*, **31**, 292—298 (1983).
3) Odani T., Tanizawa H., Takino Y., *Chem. Pharm. Bull.*, **31**, 1059—1066 (1983).
4) Karikura M., Miyase T., Tanizawa H., Taniyama T., Takino Y., *Chem. Pharm. Bull.*, **39**, 2357—2361 (1991).
5) Akao T., Kida Kanaoka M., Hattori M., Kobashi K., *J. Pharm. Pharmacol.*, **50**, 1155—1160 (1998).
6) Hasegawa H., Sung J. H., Matsumiya S., Uchiyama M., *Planta Med.*, **62**, 453—457 (1996).
7) Lee S. J., Sung J. H., Lee S. J., Moon C. K., Lee B. H., *Cancer Lett.*, **144**, 39—43 (1999).
8) Atopkina L. N., Malinovskaya G. V., Elyakov G. B., Uvarova N. I., Woerdenbag H. J., Koulman A., Pras N., Potier P., *Planta Med.*, **65**, 30—34 (1999).
9) Hasegawa H., Sung J. H., Matsumiya S., Uchiyama M., Inouye Y., Kasai R., Yamasaki K., *Planta Med.*, **61**, 409—413 (1995).
10) Hasegawa H., Sung J. H., Matsumiya S., Uchiyama M., Huh J. D., U.S. Patent 5919770 (1999).
11) Bae E. A., Han M. J., Choo M. K., Park S. Y., Kim D. H., *Biol. Pharm. Bull.*, **25**, 58—63 (2002).
12) Asakawa J., Kasai R., Yamasaki K., Tanaka O., *Tetrahedron*, **33**, 1935—1939 (1977).
13) Kasai R., Souzu M., Asakawa J., Tanaka O., *Tetrahedron Lett.*, **2**, 175—178 (1977).
14) Kasai R., Okihara M., Asakawa J., Mizutani K., Tanaka O., *Tetrahedron*, **35**, 1427—1432 (1979).
15) Karikuma M., Miyase T., *Chem. Pharm. Bull.*, **38**, 2859—2861 (1990).