3-Hydroxymethylglutaryl Flavonol Glycosides from a Mongolian and Tibetan Medicine, *Oxytropis racemosa*

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Three new flavonol 3-*O*-glycosides, rhamnetin 3-*O*-[(*S*)-3-hydroxy-3-methyl-glutaroyl(1→6)]-β-D-glucopyranoside (1), rhamnocitrin 3-*O*-[(*S*)-3-hydroxy-3-methylglutaroyl(1→6)]-β-D-glucopyranoside (2), and isorhamnetin 3-*O*-[(*S*)-3-hydroxy-3-methylglutaroyl(1→6)]-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranoside (3), along with 13 known compounds, were isolated from *Oxytropis racemosa* Turcz. Their structures were deduced by means of spectroscopic methods and chemical evidence. 2 and 6 showed cytotoxic activities against HCT-8 (IC_{50} 6.38 μM) and A549 (IC_{50} 5.20 μM), respectively.

Key words *Oxytropis racemosa*; Fabaceae; 3-hydroxy-3-methylglutaroyl; flavonol glycoside; cytotoxic activity

The *Oxytropis* genus (Fabaceae) includes more than 350 species, and previous investigation revealed that their main constituents are flavonoids, triterpenoids and alkaloids.[1] Some species of *Oxytropis* in Inner Mongolia, Xinjiang, Qinghai and Tibet of China are used in folk medicine, such as *O. racemosa* Turcz, *O. myriophylla* (Pall) DC., *O. glabra* DC., *O. falcate* Bge, *O. chiliophylla* Royle, *O. oxyphylla* DC., *O. kansuensis* Bunge, *O. melanocalyx* Bge, and *O. leptophylla* (Pall.) DC.[2] As an important Mongolian and Tibetan medicine, *O. racemosa* has been applied to children's indigestion for its promoting digestion and invigorating the spleen properties.[3,4] However, studies on the chemical constituents and bioactivities have not been reported so far. In a search for biologically active compounds, a chemical study on the whole plant of *O. racemosa* revealed three new flavonol glycosides (**1**—**3**) bearing a 3-hydroxy-3-methylglutaric acid (HMG) moiety, along with 13 known compounds. Cytotoxic activities of compounds **1**—**12** were evaluated against different cell lines HCT-8, A549, Bel-7402, BGC-823, and A2780 using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

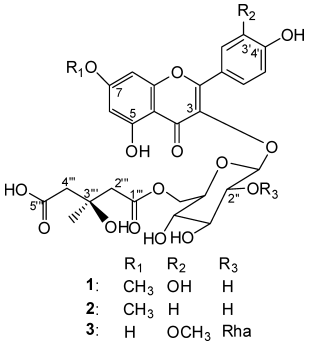

Fig. 1. Structures of Compounds **1**—**3**

Results and Discussion

The EtOH extract (1095 g) of the whole plant of *O. racemosa* was suspended in H_2O, and then sequentially partitioned with petroleum ether, EtOAc, and *n*-BuOH. The *n*-BuOH fraction (300 g) was subjected to separation using various column chromatographic techniques to afford 16 compounds, three of which are new acylated flavonol glycosides.

Compound **1** was isolated as a yellow powder. It showed a UV spectrum typical of flavonoids with absorption bands at 256, 266 (sh), and 354 nm. The high resolution-electrospray ionization-mass spectrum (HR-ESI-MS) (negative-ion mode) analysis led to a pseudomolecular ion peak at *m*/*z* 621.1459 $[M-H]^-$ (Calcd 621.1456), which indicated the molecular formula $C_{28}H_{30}O_{16}$. Its ESI-MS (positive-ion mode) displayed a pseudomolecular ion at *m*/*z* 645 $[M+Na]^+$, and fragment ions at *m*/*z* 329 $[hexose+144+Na]^+$, and 185 $[hexose+Na]^+$ were observed in the MS^n spectra, indicating the presence of a hexosyl (162 u) and an unassigned moiety (144 u).

In the ^{1}H-NMR spectrum, the occurrence of an ABM spin system [δ 7.56 (1H, d, J=2.0 Hz), 6.83 (1H, d, J=8.5 Hz), and 7.54 (1H, dd, J=8.5, 2.0 Hz)] characterized an *ortho*-disubstituted B ring of the flavonol aglycone. Signals at δ 6.36 (1H, d, J=2.0 Hz) and 6.67 (1H, d, J=2.0 Hz) have been attributed to the aromatic protons H-6 and H-8 of the A-ring, respectively. Additionally, ^{1}H-NMR spectrum also exhibited a singlet for a methoxy group at δ 3.85 (3H, s). In the ^{13}C-NMR spectrum, the signals for the aglycone were in good agreement with those of rhamnocitrin, and full identification of the aglycone was finally achieved by heteronuclear multiple bond connectivity (HMBC) spectrum, in which the correlation from the methoxy protons to C-7 could be observed. A glucosyl moiety could be assumed from the signals between δ 3.00 and 5.42 in the ^{1}H-NMR spectrum together with a set of characteristic signals at δ 100.6, 73.9, 76.1, 68.7, 74.2, and 63.1 in the ^{13}C-NMR spectrum. This was later confirmed by acid hydrolysis of **1** followed by TLC analysis of the water-soluble sugar fraction and comparison with reference sugars. Furthermore, the coupling constant (J=7.0 Hz) of the anomeric proton of the glucosyl moiety demonstrated that it has β-anomeric configuration, and the D configuration for glucose was assumed on the basis of the fact that glucose encountered among the plant glucosides in natural products was mostly D-form. The remaining signals in the ^{1}H-NMR

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spectrum indicated the presence of two methylenes [δ 2.38 (1H, d, J=14.0 Hz, H-2‴a), 2.27 (1H, d, J=14.0 Hz, H-2‴b), and 2.28 (2H, s, H-4‴)] and a *C*-methyl group [δ 1.02 (3H, s, 3‴-CH_3)] in the unassigned moiety. The remaining signals in the ^{13}C-NMR spectrum included four upfield carbons at δ 27.0 (3‴-CH_3), 45.13 (C-4‴), 45.06 (C-2‴), and 68.7 (C-3‴) as well as two carbonyl carbons at δ 170.1 (C-1‴) and 172.3 (C-5‴). Taking into account the lost fragment (-144) in ESI-MS, the moiety was then deduced as a 3-hydroxy-3-methylglutaroyl (HMG) moiety. Further confirmation was derived by HMBC correlations from H_2-2‴ to C-1‴, C-3‴, C-4‴ and 3‴-CH_3, from H_2-4‴ to C-2‴, C-3‴, C-5‴ and 3‴-$\underline{C}H_3$, and from 3‴-C$\underline{H}_3$ to C-1‴, C-3‴ and C-4‴. *S*-configuration for the C-3‴ stereogenic carbon of HMG was assumed because naturally occurring HMG esters are formed *via* the acylation of the hydroxy group with (*S*)-HMG-CoA.[5,6] The HMBC correlation from anomeric proton to C-3 indicated that sugar chain was located at C-3 of the agylcone. The downfield chemical shifts of H_2-6″ [δ 4.16 (d, J=12.0 Hz, H-6″a) and 3.91 (dd, J=12.0, 7.0 Hz, H-6″b)] suggested acylation of the glucose unit at C-6″, and this was confirmed by an HMBC correlation from H_2-6″ to C-5‴. Thus, **1** was identified as rhamnetin 3-*O*-[(*S*)-3-hydroxy-3-methylglutaroyl(1→6)]-β-D-glucopyranoside.

Compound **2** was obtained as a yellow powder and also showed a UV spectrum typical of flavonoids with absorption bands at 266 and 348 nm. Its HR-ESI-MS (negative-ion mode) data exhibited a pseudomolecular ion at *m*/*z* 605.1504 $[M-H]^-$ (Calcd 605.1501), consistent with a molecular formula of $C_{28}H_{30}O_{15}$. Its ESI-MS^n gave a pseudomolecular at *m*/*z* 629 $[M+Na]^+$ as well as two fragment ions at *m*/*z* 329 $[glc+HMG+Na]^+$, and 185 $[glc+Na]^+$, suggesting that it differed from **1** by an absent hydroxy group on the aglycone. Comparison of the ^{1}H-NMR data of **2** and **1** showed that the ABM spin system was replaced by an AA′BB′ spin system [δ 8.02 (2H, d, J=9.0 Hz) and 6.88 (2H, d, J=9.0 Hz)]. Further analyses of the ^{1}H- and ^{13}C-NMR data (see Table 1) led to the identification of the aglycone as rhamnocitrin, which was supported by an HMBC correlation from the methoxy at δ 3.85 (3H, s) to C-7 of the aglycone. Signals derived from a 3-hydroxy-3-methylglutaroyl(1→6)-β-glucopyranosyl moiety were also observed in the NMR spectra of **2**. The presence of the glucosyl moiety was confirmed by acid hydrolysis. In addition, the HMBC spectrum showed long-range correlations of C-3 with H-1″, and C-1‴ with H_2-6″. Thus, **2** was identified as rhamnocitrin-3-*O*-[(*S*)-3-hydroxy-3-methyl-glu-

Table 1. ^{1}H- and ^{13}C-NMR Spectroscopic Data of Compounds **1**, **2**, and **3** in DMSO-d_6

Position	**1**		**2**		**3**	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
2		156.8		156.8		156.3
3		133.3		133.2		132.4
4		177.4		177.5		177.1
5		160.9		160.9		161.2
6	6.36 d (2.0)	97.8	6.36 d (1.5)	97.9	6.19 d (1.5)	98.7
7		165.1		165.1		164.1
8	6.67 d (2.0)	92.1	6.70 d (1.5)	92.3	6.42 d (1.5)	93.7
9		156.2		156.3		156.2
10		104.9		104.9		104.0
1′		120.9		120.6		120.9
2′	7.56 d (2.0)	116.2	8.02 d (9.0)	130.8	7.83 d (2.0)	115.2
3′		144.8	6.88 d (9.0)	115.1		146.8
4′		148.6		160.1		149.4
5′	6.83 d (8.5)	115.1	6.88 d (9.0)	115.1	6.89 d (8.5)	113.1
6′	7.54 dd (8.5, 2.0)	121.5	8.02 d (9.0)	130.8	7.54 dd (8.5, 2.0)	122.1
7-OCH_3	3.85 s	56.0	3.85 s	56.0		
3′-OCH_3					3.85 s	55.7
5-OH	12.60 s		12.55 s		12.60 s	
1″	5.42 d (7.0)	100.6	5.38 d (7.5)	100.8	5.67 d (7.0)	98.3
2″	3.26 m	73.9	3.23 m	74.0	3.47 m	77.6
3″	3.26 m	76.1	3.22 m	76.1	3.45 m	76.8
4″	3.11 m	68.7	3.10 m	70.0	3.10 m	70.5
5″	3.32 m	74.2	3.23 m	74.0	3.37 m	74.0
6″	4.16 d (12.0)	63.1	4.13 d (12.0)	63.1	4.12 d (12.0)	62.9
	3.91 dd (12.0, 7.0)		3.90 dd (12.0, 7.0)		3.96 dd (12.0,7.0)	
1‴		170.1		170.1		170.0
2‴	2.38 d (14.0)	45.06	2.38 d (14.0)	45.1	2.38 d (14.0)	45.2
	2.27 d (14.0)		2.27 d (14.0)		2.28 d (14.0)	
3‴		68.7		68.7		68.6
4‴	2.28 s	45.13	2.28 s	45.2	2.23 s	45.0
5‴		172.3		172.3		172.4
3‴-CH_3	1.02 s	27.0	1.02 s	27.0	0.97 s	27.1
1⁗					5.06 s	100.6
2⁗					3.46 m	70.3
3⁗					3.74 m	70.5
4⁗					3.12 m	71.7
5⁗					3.72 m	68.3
6⁗					0.71 d (6.5)	17.1

taroyl(1→6)]-β-D-glucopyranoside.

The UV spectrum of compound **3** was nearly identical to that of **1**, which suggested that **3** was a quercetin derivative, too. Its HR-ESI-MS (positive-ion mode) data indicated a pseudomolecular ion at *m/z* 791.1988 $[M+Na]^+$ (Calcd 791.2011) corresponding to a molecular formula of $C_{34}H_{40}O_{20}$. The ESI-MS (positive-ion mode) data showed a pseudomolecular ion at *m/z* 791 $[M+Na]^+$ and a fragment ion at *m/z* 645 $[M-rha+Na]^+$, indicating a terminal rhamnosyl. Acid hydrolysis gave two sugars, identified as rhamnose and glucose by TLC. In the ^{1}H-NMR spectrum, five aromatic signals corresponding to H-6 [δ 6.19 (1H, d, J=1.5 Hz)] and H-8 [δ 6.42 (1H, d, J=1.5 Hz)] on the A ring, and to an ABM spin system [δ 7.83 (1H, d, J=2.0 Hz), 6.89 (1H, d, J=8.5 Hz), and 7.54 (1H, dd, J=8.5, 2.0 Hz)] on the B ring of the aglycone were found. A methoxy group signal at δ 3.85 (3H, s) could also be observed. On the basis of ^{1}H- and ^{13}C-NMR analyses, the aglycone was identified as isorhamnetin, and this was confirmed by a correlation between the methoxy group and C-3′ in the HMBC spectrum. The ^{1}H- and ^{13}C-NMR also showed signals for rhamnosyl, glucosyl, and HMG moieties. An HMBC correlation from H-1″ to C-3 suggested the attachment of the glucosyl moiety to C-3 of the aglycone. HMBC correlations of C-2″ with H-1‴, and C-1‴ with H_2-6″ indicated the rhamnosyl and HMG moieties were located at C-2″ and C-6″, respectively, of the glucosyl moiety. The common L configuration for α-rhamnose was assumed. Thus, the structure of **3** was established as isorhamnetin 3-*O*-[(*S*)-3-hydroxy-3-methylglutaroyl(1→6)]-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.

In addition, 13 known compounds were elucidated as uracil,[7] isorhamnetin 3-*O*-α-arabinopyranoside,[8] rhamnetin 3-*O*-β-glucopyranoside (**4**),[9] rhamnocitrin 3-*O*-β-glucopyranoside (**5**),[10] adenosine,[11] quercetin 3-*O*-α-arabinopyranoside,[8,12] isorhamnetin 3-*O*-β-glucopyranoside (**6**),[13] genistein 4′-*O*-β-glucopyranoside (**7**),[14] dihydrokaempferol 4′-*O*-β-glucopyranoside (**8**),[15] acacetin 7-*O*-β-rutinoside (**9**),[16] isorhamnetin 3-*O*-β-rutinoside (**10**),[17,18] quercetin 3-*O*-β-glucopyranoside (**11**),[19] and rutin (**12**),[19] by spectroscopic analysis.

Cytotoxic activities of compounds **1**—**12** were evaluated against HCT-8, A549, Bel-7402, BGC-823 and A2780 using the MTT method. Among the tested compounds, **2** and **6** were the most potent, with IC_{50} values of 6.38 μM against HCT-8 and 5.20 μM against A549, respectively, and the other compounds exhibited IC_{50} values >10 μM.

Experimental

General Experimental Procedures The optical rotations were measured on a Jasco P-2000 polarimeter. The UV spectra were scanned by a Jasco V650 spectrophotometer. IR spectra were recorded on an IMPACT 400 (KBr) spectrometer. ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz), and HMBC spectra were run on INOVA 500 MHz spectrometer. HR-ESI-MS were performed on a Finnigan LTQ FT mass spectrometer. The ESI mass spectra were recorded on an Agilent 1100 series LC/MSD time-of-flight (TOF) from Agilent Technologies, U.S.A. Column chromatography was performed with Macroporous resin (Diaion HP-20, Mitsubishi Chemical Corp., Tokyo, Japan), Rp-18 (50 μm, YMC, Kyoto, Japan), and silica gel (100—200, 200—300 mesh, Qingdao Marine Chemical Inc., Qingdao, People's Republic of China). Preparative HPLC was carried out on a Shimadzu LC-6AD instrument with an SPD-20A detector, using a YMC-Pack ODS-A column (250 mm×20 mm, 5 μm). Precoated silica gel GF-254 plates (Yantai Jiangyou Silica Gel Exploitation Company, China) were used for analytical TLC.

Plant Material *O. racemosa* was collected from Inner Mongolia autonomous region of People's Republic of China in August 2007. The plant material was identified by Associate Professor Lin Ma (Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College). A voucher specimen (ID-21970) has been deposited in the Herbarium of the Department of Medicinal plants, Institute of Materia Media, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, P. R. China.

Extraction and Isolation The dried whole plant of *O. racemosa* (10 kg) was exhaustively extracted with 95% EtOH under reflux for 3 h. The EtOH extract was concentrated under reduced pressure to give a residue (1095 g), which was suspended in H_2O (7500 ml) with the suspension sequentially extracted with petroleum ether (3×5000 ml), EtOAc (3×5000 ml), and *n*-BuOH (3×5000 ml). The *n*-BuOH extract was evaporated *in vacuo* to give a residue (300 g), which was subjected to silica gel CC eluting with a MeOH–$CHCl_3$ gradient to afford 8 fractions (A—H). Fraction D (2 g) was applied to an ODS column eluted with a MeOH–H_2O step gradient (in ratio 1 : 1) to yield **2** (98 mg). Fraction G (50 g) was subjected to macroporous resin with an EtOH–H_2O step gradient (in ratio 3 : 7) to afford subfraction G-3 (1 g), which was purified by reversed-phase preparative HPLC, using a mobile phase of MeOH–H_2O (in ratio 1 : 1), to give **1** (140 mg). Fraction H (50 g) was subjected to macroporous resin with an EtOH–H_2O step gradient (in ratio 1 : 3) to afford subfraction H-3 (1 g), which was then chromatographed by an ODS column eluted with MeOH–H_2O (in ratio 2 : 3) to give **3** (190 mg).

Rhamnetin 3-*O*-[(*S*)-3-Hydroxy-3-methylglutaroyl(1→6)]-β-D-glucopyranoside (**1**): Yellow powder. $[\alpha]_D^{20}$ −66.9 (*c*=0.20, MeOH). UV λ_{max} (MeOH) nm: 256, 266 (sh), 354. IR (KBr) cm^{-1}: 3365, 2978, 1717, 1656, 1594, 1498, 1444, 1345, 1297, 1211, 1164, 1119, 1085, 1009, 956, 935, 806. ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (DMSO-d_6) data, see Table 1. (+)-ESI-MS *m/z* 645 $[M+Na]^+$, MS^2 [645] *m/z* 329 $[glc+HMG+Na]^+$, MS^3 [645→329] *m/z* 185 $[glc+Na]^+$. (−)-HR-ESI-MS *m/z* 621.1459 $[M-H]^-$ (Calcd 621.1456).

Rhamnocitrin 3-*O*-[(*S*)-3-Hydroxy-3-methylglutaroyl(1→6)]-β-D-glucopyranoside (**2**): Yellow powder. $[\alpha]_D^{20}$ −1.4 (*c*=0.12, MeOH). UV λ_{max} (MeOH) nm: 266, 348. IR (KBr) cm^{-1}: 3328, 2945, 1716, 1656, 1593, 1498, 1443, 1346, 1284, 1213, 1170, 1065, 1007, 969, 841, 806. ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (DMSO-d_6) data, see Table 1. (+)-ESI-MS *m/z* 629 $[M+Na]^+$, MS^2 [645] *m/z* 329 $[glc+HMG+Na]^+$, MS^3 [645→329] *m/z* 185 $[glc+Na]^+$. (−)-HR-ESI-MS *m/z* 605.1504 $[M-H]^-$ (Calcd 605.1501).

Isorhamnetin 3-*O*-[(*S*)-3-hydroxy-3-methylglutaroyl(1→6)]-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranoside (**3**): Yellow powder. $[\alpha]_D^{20}$ −65.8 (*c*=0.15, MeOH). UV λ_{max} (MeOH) nm: 256, 266 (sh), 354. IR (KBr) cm^{-1}: 3360, 2931, 1711, 1658, 1603, 1512, 1494, 1457, 1427, 1347, 1282, 1200, 1124, 1052, 1028, 921, 804. ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (DMSO-d_6) data, see Table 1. (+)-ESI-MS *m/z* 791 $[M+Na]^+$, MS^2 [791] *m/z* 645 $[M-rha+Na]^+$, 475 $[glc+rha+HMG+Na]^+$, MS^3 [791→475] *m/z* 329 $[glc+HMG+Na]^+$, 185 $[glc+Na]^+$. (+)-HR-ESI-MS *m/z* 791.1988 $[M+Na]^+$ (Calcd 791.2011).

Acid Hydrolysis of 1, 2 and 3 Compounds **1**, **2** and **3** (5 mg each) were refluxed separately in 2 N HCl (1.5 ml) on a water bath for 1.5 h. After extraction with EtOAc, the water layer was neutralized with a saturated solution of $NaHCO_3$. All the three samples were analyzed on TLC by comparison with standard sugars ($CHCl_3$/MeOH/HOAc/H_2O, 14 : 7 : 2 : 1, detection by spraying with an EtOH/H_2SO_4, 90 : 10 solution and heating).

Cytotoxicity Assay Cytotoxicity against Human lung adenocarcinoma (A549), human stomach cancer (BGC-823), human ovarian cancer (A2780), human hepatoma (Bel-7402), and human colon cancer (HCT-8) cell lines was measured. The cells were seeded in 96-well microtiter plates at 1200 cells/well and preincubated for 24 h to allow cell attachment. Then the tested compounds were added to the cultures. After 96 h of drug treatment, cell viability was determined by measuring the metabolic conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into purple formazan crystals by active cells.[20,21] After 4 h reincubation at 37 °C, 100 μl of DMSO was added to dissolve the precipitate of reduced MTT. Microplates were agitated on a rotation platform at room temperature for 15 min, and the absorbance of the reaction mixtures was determined at 570 nm with a multiwell scanning spectrophotometer.

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