Xanthones from *Gentianella amarella* ssp. *acuta* with Acetylcholinesterase and Monoamine Oxidase Inhibitory Activities

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Two new xanthone glycosides, corymbiferin 3-*O*-β-D-glucopyranoside (**1**) and swertiabisxanthone-I 8′-*O*-β-D-glucopyranoside (**2**), were isolated from *Gentianella amarella* ssp. *acuta*, along with eight known xanthones: triptexanthoside C, veratriloside, corymbiferin 1-*O*-glucoside, swertianolin, norswertianolin, swertiabisxanthone-I, bellidin, and bellidifolin, four of them identified for the first time in *G. amarella* ssp. *acuta*. The isolation was conducted mainly by centrifugal partition chromatography, and the structures of the isolated compounds were established on the basis of spectrometric data including 2D NMR and mass spectrometry. Xanthones were weakly active against acetylcholinesterase (AChE), except triptexanthoside C, which inhibited AChE with an IC_{50} of 13.8 ± 1.6 μM. Some compounds were active against monoamine oxidases (MAO): bellidin and bellidifolin showed interesting inhibitory activity of MAO A, while swertianolin, the 8-*O*-glucopyranoside form of bellidifolin, gave 93.6% inhibition of MAO B activity at 10^{-5} M.

Gentianella amarella ssp. *acuta* (Michx.) J.M.Gillett is a species of the family Gentianaceae traditionally used as folk medicine in Mongolia to treat fever and disorders of the gallbladder.[1] There has only been one phytochemical study on this gentian, referring to xanthones with 1,3,5,8- and 1,3,4,5,8-substitution patterns.[2] In previous work, it has been observed that xanthones from *G. campestris* inhibited acetylcholinesterase (AChE) activity.[3] The close taxonomic links between *G. campestris* and the Mongolian *G. amarella* ssp. *acuta* led to an investigation of the latter for new anti-AChE compounds, using a bioautographic assay.[4] On TLC, the methanolic extract of *G. amarella* ssp. *acuta* clearly exhibited several inhibition zones.

The methanolic extract of *G. amarella* was first submitted to liquid–liquid fractionation to give four phases of increasing polarity. From the *n*-BuOH phase, two new xanthone glycosides were isolated: corymbiferin 3-*O*-β-D-glucopyranoside (**1**) and swertiabisxanthone-I 8′-*O*-β-D-glucopyranoside (**2**). Five other known glycosides were obtained: triptexanthoside C (**3**),[5] veratriloside (**4**),[6] corymbiferin 1-*O*-glucoside (**5**),[7] swertianolin (**6**), and norswertianolin (**7**).[8] Three xanthone aglycones were also obtained from the nonpolar phases: swertiabisxanthone-I (**8**),[9] bellidin (**9**), and bellidifolin (**10**).[10]

Compound **1** was obtained as a pale yellow powder. Its molecular formula was deduced from the HRESIMS data to be $C_{21}H_{22}O_{12}$, on the basis of the formate adduct at *m/z* 511.1080 $[M + HCOO]^-$ (calcd for 511.1088). The APCI-MS spectrum exhibited, in addition to a pseudomolecular ion at *m/z* 467 $[M + H]^+$, a fragment at *m/z* 305 $[M + H - 162]^+$ indicating the loss of a hexose moiety; the enzymatic hydrolysis of **1** confirmed this sugar to be β-D-glucose. The UV spectrum was characteristic of xanthones.[11] The ^{1}H NMR spectrum of **1** showed signals for two methoxy groups (δ_H 3.86, 3.91) and three aromatic protons, two doublets *o*-coupled ($J = 8.8$ Hz), and a singlet (δ_H 6.68) (Table 1). The spectrum also exhibited two singlets characteristic of hydrogen-bonded hydroxy groups, which permitted assignment of hydroxylation at C-1 and C-8 (δ_H 11.18, 11.64). The singlet at δ_H 6.68 was assigned to H-2 due its long-range coupling correlations with C-1 (δ_C 156.9) and C-8b (104.0). It also correlated with two other carbons at δ_C 158.3 and 129.2 (Figure 1). The carbon at δ_C 158.3 was linked to the glucose moiety, as observed through the HMBC correlation with the anomeric proton (δ_H 5.12). In addition, ^{1}H−^{13}C NMR correlations indicated that the carbon at δ_C 129.2 carried one of the two methoxy groups (δ_C 60.9/δ_H 3.86). This chemical shift at δ_C 60.9 was typical for a methoxy group substituted *ortho* to two oxygen atoms.[12] Thus, this implied that the methoxy group had to be at C-4 (δ_C 129.2), while the glucosyl moiety was connected at C-3 (δ_C 158.3). The two doublets at δ_H 6.75 and 7.50 correlated with C-8 (δ_C 153.1) and were consequently assigned to H-7 and H-6, respectively. The chemical shifts of the corresponding carbons (δ_C 109.1 and 121.7) were in agreement with literature values for ring B *O*-substituted at C-5 and C-8.[12] Carbon 5 (δ_C 140.0) was actually substituted by

Table 1. NMR Spectroscopic Data (500 MHz, DMSO-d_6) for Corymbiferin 3-*O*-β-D-Glucopyranoside (**1**)

position	δ_C	δ_H (*J* in Hz)
1	156.9	
2	99.2	6.68, s
3	158.3	
4	129.2	
4a	151.3	
4b	147.2	
5	140.0	
6	121.7	7.50, d (8.8)
7	109.1	6.75, d (8.8)
8	153.1	
8a	108.5	
8b	104.0	
9	183.1	
OCH_3-4	60.9	3.86, s
OCH_3-5	57.2	3.91, s
1′	99.9	5.12, d (7.3)
2′	73.1	3.31, m
3′	76.6	3.30, m
4′	69.5	3.20, m
5′	77.2	3.47, m
6′	60.5	3.70, 3.48, m

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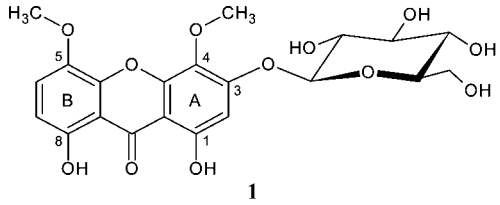

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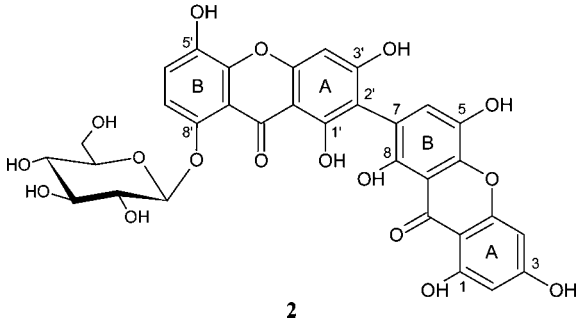

2

the second methoxy group (δ_C 57.2/δ_H 3.91), as indicated by $^1H-^{13}C$ correlations. Compound **1** was thus identified as corymbiferin 3-*O*-β-D-glucopyranoside. All ^1H chemical shifts were identical to those of corymbiferin,[13] except for H-2, which was downfield shifted by 0.35 ppm due to the substitution of the glucosyl moiety at C-3.

Compound **2** was obtained as an apricot-colored powder. Its UV spectrum was characteristic of xanthones and strikingly similar to that of bellidin, which is a 1,3,5,8-tetrahydroxyxanthone. The HRMS exhibited a pseudomolecular ion peak at *m/z* 679.0891 [M − H]$^-$, corresponding to the molecular formula $C_{32}H_{24}O_{17}$ (calcd for 679.0877). As for compound **1**, the APCI MS spectrum indicated the loss of a hexosyl moiety characterized by a fragment at *m/z* 519 [M + H − 162]$^+$. The enzymatic hydrolysis proved this moiety to be β-D-glucose. These data indicated that **2** could be a dimeric xanthone consisting of two tetraoxygenated units linked by a carbon−carbon bond, with one of the xanthone moieties being glycosylated. The ^1H NMR spectrum exhibited six aromatic signals, in addition to the chemical shifts corresponding to the glucosyl moiety: two *m*-coupled protons on ring A (δ_H 6.16, 6.36, J = 1.4 Hz), two *o*-doublet protons on ring B (δ_H 7.13, 7.24, J = 9.3 Hz), and two singlets at δ_H 6.45 and 7.15 (Table 2). The HSQC correlations gave more information about the two singlets: the chemical shift of the carbon attached to the proton at δ_H 6.45 was characteristic of C-2 or C-4 (δ_C 94.4), and the shift value of the carbon carrying the proton at δ_H 7.15 was typical of C-6 substitution (δ_C 126.7). These observations were consistent with the hypothesis of two linked 1,3,5,8-xanthone units. The observed HMBC correlations showed that glucose was attached at C-8 (δ_C 151.0) on ring B, where the proton pair δ_H 7.13/7.24 was situated (Figure 2). All the different $^1H-^{13}C$ NMR correlations corresponded to the structure of a bisxanthone consisting of two 1,3,5,8-tetraoxygenated xanthones connected by a 7–2′ linkage, with a glucosyl moiety at C-8′. The chemical shifts for the aglycone carbons and protons were in good agreement with the values obtained for swertiabisxanthone-I.[9]

The isolated xanthones were evaluated *in vitro* for their ability to inhibit acetylcholinesterase (AChE) and monoamine oxidase (MAO) B, two enzymes implicated in aging-related neurodegenerative diseases such as Alzheimer's disease. The enzymatic inhibition rates are shown in Table 3. Most of the xanthones showed a weak inhibitory activity against AChE, triptexanthoside C being the most active. Additional enzymatic experiments were performed with this compound in order to determine its IC_{50} value (13.8 ± 1.6 μM), a low value compared to the inhibition power of, for example, galanthamine (0.35 ± 0.02 μM). Some compounds exhibited interesting activity against MAO B, particularly corymbiferin 1-*O*-glucoside and swertianolin, with 70.5% and 93.6% of inhibition, respectively, when tested at 10^{-5} M. The effect against monoamine oxidase A was also studied. The majority of xanthones were weakly active against MAO A, except bellidin and bellidifolin, which inhibited the enzyme by 90.5% and 98.9%, respectively, when tested at 10^{-5} M.

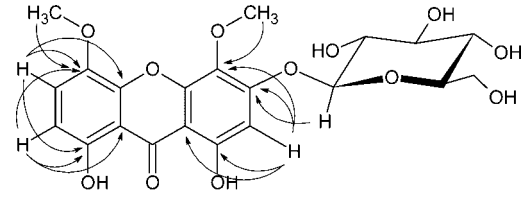

Figure 1. Key gHMBC correlations of compound **1**.

To date, no bisxanthones have been reported in *Gentianella* species. In this work, two minor dimeric xanthones have been isolated from *Gentianella amarella* ssp. *acuta*, swertiabisxanthone-I and its 8′-*O*-glucoside. The latter is a new compound.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Perkin-Elmer 241 MC polarimeter. UV spectra were measured in MeOH using a Perkin-Elmer Lambda 20 spectrometer. 1D and 2D NMR spectra (^1H, ^{13}C, gDQF-COSY, gHSQC, and gHMBC) were recorded on a Varian Unity Inova 500 MHz spectrometer, using DMSO-d_6 as solvent. The HPLC/APCI-MS data were obtained using an HP 1100 system coupled with a Finnigan MAT LCQ spectrometer. ESI-TOF-MS data were recorded on a Waters Micromass LCT-Premier mass spectrometer. HPLC-UV was performed on an HP 1100 instrument equipped with a photodiode array detector and a Nova-Pak RP-18 column (4 μm, 150 × 3.9 mm i.d., Waters). An H_2O−MeCN gradient containing 0.1% formic acid (95–5 to 70–30 in 20 min, then 70–30 to 0–100 in 10 min) was applied at a flow rate of 1.0 mL/min. Preparative centrifugal partition chromatography was performed on a Quattro CCC Mk 5 LabPrep 1000 instrument at a flow rate of 3.0 mL/min. The total volume of the four coils was 904 mL, and the rotation speed was 870 rpm. Semipreparative fractionations were performed on a Dynamic Extractions Mini Centrifuge instrument at a flow rate of 1.0 mL/min. The volume of the coil was 17 mL, and the rotation speed was 2000 rpm. Elution was monitored at 254 nm with a Knauer UV–visible detector and a Tarkan model 600 integrator. Fractions were collected with a LKB Bromma 2070 Ultrorac II fraction collector. Enzymatic hydrolysis was performed by dissolving glycoside xanthones (1 mg) in 1 mL of acetate buffer pH 5.0 with 1 mg of β-D-glucosidase (Sigma). After incubation at 37 °C for 24 h, the mixture was extracted with EtOAc and the completion of hydrolysis was checked by TLC (hexane−EtOAc, 1:1, v/v).

Plant Material. Whole plants of *Gentianella amarella* ssp. *acuta* (Michx.) J.M.Gillett (Gentianaceae) were collected in Mongolia in 1993 in the vicinity of Ulaanbaatar and identified by Dr. Sanchir (Herbarium of the Botanical Institute, Ulaanbaatar, Republic of Mongolia). A voucher specimen (no. 93013) is deposited at the Laboratory of Pharmacognosy and Phytochemistry, University of Geneva, Switzerland.

Extraction and Isolation. Air-dried powdered plants (287 g) were extracted successively with CH_2Cl_2 (3 × 1.5 L, 24 h) and MeOH (3 × 1.5 L, 24 h) at room temperature, to give respectively 14.3 and 44.0 g of extract. A part of the methanolic extract (35 g) was dissolved in 200 mL of H_2O and then extracted successively at room temperature with Et_2O (3 × 100 mL), EtOAc (3 × 100 mL), and *n*-BuOH (3 × 100 mL) to give respectively 4.76, 2.39, and 7.56 g of dry extracts. The aqueous phase contained 20.16 g of residue.

The *n*-BuOH fraction (600 mg) was subjected to CPC with the ternary biphasic system $CHCl_3$−MeOH−H_2O (45:30:25 v/v), using the organic phase as first mobile phase. This separation was repeated in such a manner that a total of 3 g of *n*-BuOH phase was fractioned. Each separation afforded nine fractions with the organic phase and three more fractions in the reverse mode; similar fractions were combined after HPLC examination to provide 12 fractions (Fr. 1–12). Fr. 4 (30.9 mg) was separated by CPC with $CHCl_3$−MeOH−H_2O (45:22:33 v/v) to give **3** (7.6 mg), **4** (0.8 mg), and a third fraction that was submitted to a further CPC with cyclohexane−EtOAc−MeOH-H_2O (1:5:1:5 v/v)

Table 2. NMR Spectroscopic Data (500 MHz, DMSO-d_6) for Swertiabisxanthone-I 8′-*O*-β-D-Glucopyranoside (**2**)

position	δ_C	δ_H (J in Hz)	position	δ_C	δ_H (J in Hz)	position	δ_C	δ_H (J in Hz)
1	162.2		1′	160.5		1″	103.4	4.75, d (7.8)
2	98.6	6.16, d (1.4)	2′	113.2		2″	73.5	3.35, m
3	167.8		3′	167.2		3″	76.0	3.28, m
4	94.4	6.36, d (1.4)	4′	95.0	6.45, s	4″	69.8	3.18, m
4a	157.5		4a′	155.6		5″	77.4	3.31, m
4b	142.8		4b′	144.8		6″a	60.9	3.74, d (11.2)
5	137.8		5′	137.4		6″b		3.50, dd (11.5, 5.6)
6	126.7	7.15, s	6′	122.7	7.24, d (9.3)			
7			7′	112.7	7.13, d (8.8)			
8	149.5		8′	151.0				
8a	107.2		8a′	107.3				
8b	101.8		8b′	100.9				
9	181.1		9′	184.1				

to afford **3** (0.9 mg) and **1** (0.3 mg). Fr. 5 (50.0 mg) was separated with the same CPC system as Fr. 4 to give **3** (1.4 mg), **4** (7.6 mg), and a third fraction; the latter was submitted to a further CPC with cyclohexane−EtOAc−MeOH−H_2O (1:3:1:3 v/v) to yield **3** (0.5 mg), **1** (0.8 mg), and **4** (0.1 mg). Compound **4** (3.8 mg) crystallized from Fr. 6 (20.4 mg); the remaining fraction after removal of crystals was applied to a Sephadex LH-20 column eluted with MeOH to afford three subfractions. Fraction 6-2 gave 1.2 mg of **1** after a CPC separation with cyclohexane−EtOAc−MeOH-H_2O (5:5:3:3 v/v). Compound **5** was obtained as crystals from Fr. 7 (26.0 mg). Fr. 8 submitted to a CPC with cyclohexane−EtOAc−MeOH-H_2O (1:5:1:5 v/v) afforded 9.0 mg of **6**. Fr. 10 (1.5 g) was fractionated by Sephadex LH-20 column chromatography using MeOH as solvent to afford five subfractions; fraction 10-3 contained compound **7** (23.4 mg), and fraction 10-5 was subjected to further chromatography on Sephadex LH-20 to yield 4.1 mg of compound **2**.

One gram of the Et_2O extract was separated by centrifugal partition chromatography using the biphasic solvent system cyclohexane−EtOAc−MeOH−H_2O (5:5:3:3 v/v) and the aqueous phase as first mobile phase to give four fractions (Fr. 1–4). Fr. 2 was purified on Sephadex LH-20 to give compound **8** (8.1 mg), Fr. 3 led after crystallization from cyclohexane−EtOAc to **9** (280 mg), and Fr. 4 gave crystallized xanthone **10** (115 mg).

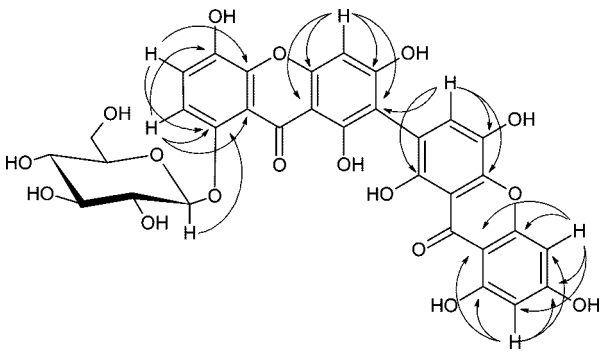

Figure 2. Key gHMBC correlations of compound **2**.

Table 3. Inhibition of Acetylcholinesterase (AChE) and Monoamine Oxidases (MAO) A and B by Isolated Xanthones and Reference Inhibitors[a]

	enzyme		
compound	AChE	MAO A	MAO B
1	17.6 ± 1.8	40.2 ± 2.6	47.8 ± 2.2
2	12.3 ± 2.9	21.4 ± 4.9	39.1 ± 1.2
triptexantoside C	43.7 ± 3.3	13.4 ± 2.7	38.8 ± 3.2
veratriloside	28.2 ± 2.5	19.4 ± 2.6	56.0 ± 1.6
corymbiferin 1-*O*-Glc	1.5 ± 1.2	15.0 ± 2.6	70.5 ± 1.0
swertianolin	9.8 ± 3.9	43.8 ± 1.2	93.6 ± 0.2
norswertianolin	4.4 ± 4.4	12.2 ± 1.4	28.9 ± 0.8
swertiabisxanthone-I	20.9 ± 3.3	14.8 ± 2.3	41.1 ± 4.6
bellidin	17.5 ± 5.7	90.5 ± 0.5	59.0 ± 13.0
bellidifolin	21.9 ± 6.2	98.9 ± 0.0	65.2 ± 5.0
galanthamine	96.82 ± 0.04		
pargyline		60.0 ± 1.8	98.2 ± 0.1

[a] Compounds tested at 10^{-5} M. Values given in % ± standard deviation (mean of 2–12 experiments).

Corymbiferin 3-*O*-β-D-glucopyranoside (1): pale yellow, amorphous powder; $[\alpha]_D^{30}$ +66.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 (sh 3.81), 258 (3.97), 276 (sh 3.73), 346 (3.51); ^{1}H and ^{13}C NMR data, see Table 1; positive APCI-MS (rel int) *m/z* 467 $[M + H]^+$ (100), 305 $[M + H - 162]^+$ (16); negative ESI-TOF-MS *m/z* 511.1080 $[M + HCOO]^-$ (calcd for $C_{22}H_{23}O_{14}$, 511.1088).

Swertiabisxanthone-I 8′-*O*-β-D-glucopyranoside (2): apricot-colored, amorphous powder; $[\alpha]_D^{30}$ −32.5 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 226 (sh 4.12), 255 (4.16), 280 (4.08), 334 (3.97); ^{1}H and ^{13}C NMR data, see Table 2; positive APCI-MS (rel int) *m/z* 681 $[M + H]^+$ (100), 663 $[M + H - H_2O]^+$ (10), 561 (9), 519 $[M + H - Glc]^+$ (27), 513 (12); negative ESI-TOF-MS *m/z* 679.0891 $[M - H]^-$ (calcd for $C_{32}H_{23}O_{17}$, 679.0877).

Bioassays. The TLC bioautographic assay used in this study was described by Marston et al.[4] The solutions were applied to TLC in varying dilutions, and plates were developed with a mixture of $CHCl_3$−MeOH−H_2O (20:8:1 v/v). The microplate inhibition assay was based on a modified Ellman's method.[14] IC_{50} values were determined for compounds that inhibited AChE by at least 40% when tested at 10^{-5} M. Galanthamine was used as reference compound for both assays. Inhibition of monoamine oxidases was measured as described previously, pargyline being used as reference compound.[15]

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