New Xanthone Glycosides from *Comastoma pedunculatum*

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Abstract

Five new xanthone glycosides, comastomasides A–E (**1**–**5**), were isolated from aqueous ethanol extracts of the aerial portions of *Comastoma pedunculatum*. The structures of these compounds were elucidated by spectroscopic analysis methods. Compounds **1**–**5** were evaluated for their hepatoprotective activity and cytotoxicity against four human cancer cell lines by *in vitro* assays. Among them, compounds **3** and **5** exhibited potent hepatoprotective activity. However, none of the compounds displayed cytotoxic activity.

Key words

Comastoma pedunculatum · Gentianaceae · xanthone glycosides · hepatoprotective activity

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The herbs of *Comastoma pedunculatum* (Royle ex D. Don) Holub (Gentianaceae) have been used to treat hepatitis, liver fibrosis, and cholecystitis in traditional Tibetan medicine. These herbs have hepatoprotective, anti-inflammatory, antioxidant, and normalizing gallbladder properties. Phytochemical investigations of the *Comastoma* genus have uncovered a number of structurally diverse metabolites, such as xanthones, flavonoids, and oleanolic acid from *C. pulmonarium* [1] and *C. pedunculatum* [2]. Xanthones are the characteristic chemical constituents of the *Comastoma* genus. These compounds exhibit a wide range of biological properties including anticancer, antimicrobial, anti-inflammatory, anti-HIV, and antioxidant activities [3]. This report describes the isolation and characterization of five new xanthone glycosides from the aerial parts of *Comastoma pedunculatum* (**Fig. 1**). Spectroscopic methods were used to elucidate the structures of these new compounds. Compounds **1**–**5** were evaluated for hepatoprotective activity against D-galactosamine-induced toxicity and cytotoxicity in four human cancer cell lines.

Compound **1** was obtained as a yellow amorphous powder, and its molecular formula was determined to be $C_{36}H_{38}O_{18}$ by HR-ESI-MS. The UV spectrum for compound **1** displayed absorption maxima at 257, 273, 297, and 327 nm, indicating the presence of a xanthone skeleton [4]. IR absorption bands at 3457, 1650, 1606, 1564, and 1484 cm^{-1} indicated the presence of a hydroxyl group, conjugated carbonyl, aromatic ring, and C-C double bond, respectively. The ^{1}H-NMR of compound **1** (**Table 1**) showed a signal at δ_H 12.44 that corresponded to the proton of the phenolic hydroxyl, indicating that the phenolic proton was hydrogen bonded to the carbonyl group. In addition, there were two *ortho*-coupled aromatic proton signals at δ_H 7.37 and 6.64 and two *meta*-coupled aromatic proton signals at δ_H 6.77 and 6.65; these data were used to assign the tetrasubstituted xanthone moiety as an aglycone. Another group of aromatic proton signals corresponded to ABX-coupled aromatic protons at δ_H 7.14, 6.63, and 6.91. The pair of coupled olefinic protons at δ_H 7.33 and 6.28 with a coupling constant of J = 15.0 Hz confirmed that the configuration of the C-C double bond was *E*. These data were identical to the proton signals of the caffeic acid moiety. In addition, there were two anomeric proton signals which corresponded to sugars protons. The proton signal at δ_H 5.01 corresponded to the β-linkage of the sugar moiety in D-glucopyranose, and the other signal at δ_H 4.44 confirmed the presence of the β-linkage of the sugar moiety in D-xylopyranose. There were also 11 other sugar proton signals between 3.04 and 3.96 ppm. These data suggest that **1** is a xanthone glycoside. There were also three oxygenated methyl groups at δ_H 3.94, 3.88, and 3.74 present in compound **1**. Furthermore, the ^{13}C-NMR (**Table 2**) and DEPT spectra of compound **1** displayed thirty-six carbon signals corresponding to thirteen sp^2-hybridized quaternary carbons (two carbonyl, eight oxygenated, and three quaternary carbons), eighteen methine carbons (nine sp^2-hybridized and nine oxygenated sp^3-hybridized carbons), two sp^3-hybridized oxygenated methylene carbons, and three methoxy carbons. On acid hydrolysis of compound **1**, the sugar

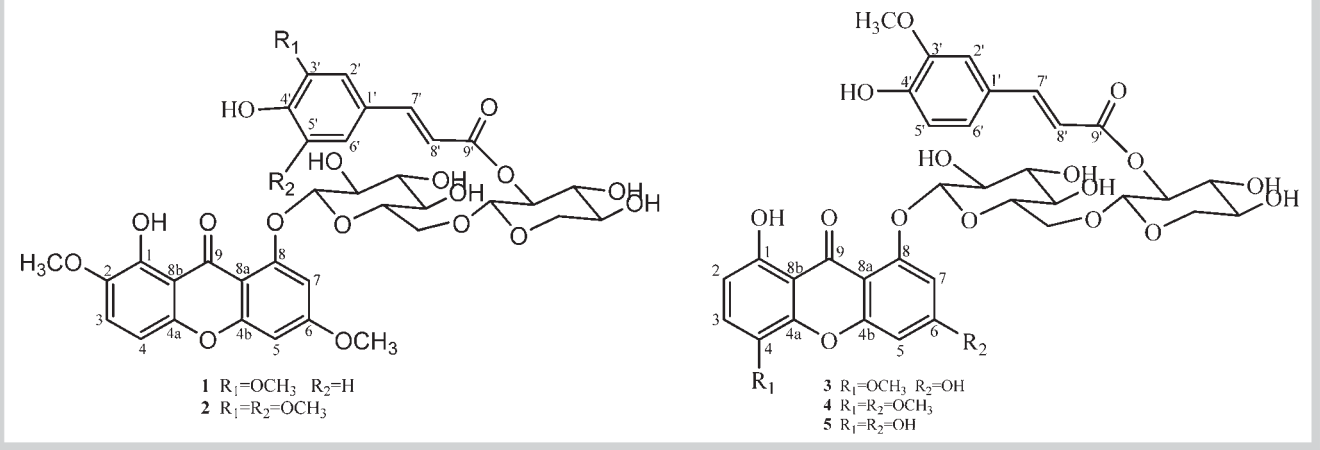

Fig. 1 Structures of compounds **1**–**5**.

Table 1 ^{1}H NMR spectra of compounds **1–5** (DMSO-d_6).

No.	1	2	3	4	5
1					
2			6.82, d (9.0)	6.84, d (9.0)	6.71, d (9.0)
3	7.37, d, (9.0)	7.35, d (8.0)	7.37, d (9.0)	7.41, d (9.0)	7.12, d (9.0)
4	6.64, d, (9.0)	6.63, d (8.0)			
4a					
4b					
5	6.77, br s	6.76, br s	6.31, br s	6.66, br s	6.20, br s
6					
7	6.65, br s	6.67, br s	6.45, br s	6.62, br s	6.46, br s
8					
8a					
8b					
9					
glc-1	5.01, d, (7.5)	5.00, d (7.0)	4.87, d (7.0)	5.02, d (8.0)	4.81, d (7.0)
glc-2	3.34, overlapping	3.32, overlapping	3.36, overlapping	3.34, overlapping	3.35, overlapping
glc-3	3.30, overlapping	3.32, overlapping	3.30, overlapping	3.30, overlapping	3.30, overlapping
glc-4	3.10, dd, (9.0, 9.0)	3.12, dd (9.0, 9.0)	3.16, m	3.10, m	3.16, m
glc-5	3.58, m	3.57, m	3.48, m	3.54, m	3.44, m
glc-6	3.96, br d (13.0)	3.96, br d (12.0)	3.93, br d (12.0)	3.96, br d (12.0)	3.94, br d (12.0)
	3.60, m	3.59, br s	3.62, dd (12.0, 6.0)	3.60, m	3.64, dd (12.0, 6.0)
xyl-1	4.44, d (8.0)	4.44, d (8.0)	4.47, d (8.0)	4.43, d (8.0)	4.51, d (8.0)
xyl-2	4.62, dd (9.0, 8.0)	4.63, dd (9.0, 8.0)	4.63, dd (9.0, 8.0)	4.62, dd (9.0, 8.0)	4.63, dd (9.0, 8.0)
xyl-3	3.30, overlapping	3.32, overlapping	3.34, overlapping	3.30, overlapping	3.35, overlapping
xyl-4	3.40, br s	3.39, overlapping	3.40, overlapping	3.40, br s	3.40, overlapping
xyl-5	3.76, overlapping	3.76, br s	3.70, overlapping	3.76, overlapping	3.76, overlapping
	3.04, dd (11.0, 11.0)	3.05, dd (11.0, 11.0)	3.07, dd (10.5, 10.5)	3.04, m	3.09, dd (10.5, 10.0)
1′					
2′	7.14, br s	6.85, br s	7.21, br s	7.15, br s	7.24, br s
3′					
4′					
5′	6.63, d (8.0)		6.70, d (8.0)	6.64, d, (8.5)	6.72, d (8.0)
6′	6.91, d (8.0)	6.85, br s	6.99, br d (8.0)	6.92, br d (8.5)	7.00, br d (8.0)
7′	7.33, d (15.0)	7.37, d (15.0)	7.45, d (16.0)	7.34, d, (16.0)	7.48, d (16.0)
8′	6.28, d (15.0)	6.34, d (15.0)	6.39, d (16.0)	6.28, d (16.0)	6.44, d (16.0)
9′					
OCH_3	3.94, s, (6-OCH_3)	3.94, s, (6-OCH_3)	3.80, s, (2-OCH_3)	3.92, s, (6-OCH_3)	3.78, s, (3′-OCH_3)
	3.88, s, (2-OCH_3)	3.86, s, (2-OCH_3)	3.76, s, (3′-OCH_3)	3.81, s, (4-OCH_3)	
	3.74, s, (3′-OCH_3)	3.74, s, (3′,5′-OCH_3)		3.74, s, (3′-OCH_3)	
OH	12.44, s, (1-OH)	12.43, s, (1-OH)	13.42, s, (1-OH)	13.25, s, (1-OH)	13.33, s, (1-OH)
			9.49, s, (4′-OH)	9.44, s, (4′-OH)	9.04, s, (4′-OH)

moiety was determined to be composed of glucose and xylose by TLC [5]. NOESY experiments were used to further determine the substitution of the xanthone moiety and position of the three methoxy groups. The NOESY correlations between the following defined the linkage position of three methoxy groups and the 1,2,6,8-tetrasubstituted xanthone moiety: 6-OCH_3 and H-5, H-7; 2-OCH_3 and H-3, 1-OH; and 3′-OCH_3 and H-2′. To determine the linkage position of the xanthone, caffeic acid, D-glucose, and D-xylose moieties, 2D NMR experiments were performed. The protons and protonated carbons of compound **1** (**Tables 1** and **2**) were unambiguously assigned by heteronuclear single quantum coherence (HSQC). The heteronuclear multiple bond correlation (HMBC) spectrum helped to determine the structural moieties and connectivity of compound **1**. HMBC correlations (**Fig. 2**) from the anomeric proton signal of glucose (5.01) to C-8 (159.2) indicated that the glycosyl moiety was located at the C-8 position. The correlations from the anomeric proton signals of the xylosyl (4.44) to the C-6 position of the glycosyl (68.9) moiety indicated that the xylosyl moiety was located at the C-6 position of the glycosyl moiety. In addition, correlations from H-2 on the xylosyl (4.62) moiety to C-9′ (165.5), which is the *α,β*-unsaturated conjugated ester carbonyl of the caffeic acid moiety, confirmed that the caffeic acid moiety was linked to the C-2 position of the xylosyl moiety. The position of H-2 on the xylosyl (4.62) moiety was further supported by ^{1}H-^{1}H COSY correlations between H-1 (4.44) and H-2 (4.62) on this moiety. Ultimately, compound **1** was deduced to be 1-hydroxy-2,6-dimethoxy-8-*O*-[2-(4′-hydroxy-3′-methoxy-*E*-cinnamyl)-*β*-D-xylopyranosyl-(1–6)-*β*-D-glucopyranosyl]-xanthone and named comastomaside A.

The molecular formula of compound **2** was determined to be $C_{37}H_{40}O_{19}$ by HR-ESI-MS. The NMR spectrum of **2** (**Tables 1** and **2**) showed similarities to that of compound **1** with the exception of the signals corresponding to the aromatic ring of the caffeic acid moiety. One of the phenolic hydroxyl groups on the caffeic acid moiety in compound **1** was replaced by signals corresponding to a methoxy group in compound **2**. The NOESY correlations between 3′- and 5′-OCH_3 and H-2′, H-6′ defined the location of the methoxy group and further confirmed that the additional methoxy group was linked to C-5′ of the caffeic acid moiety in compound **2**. Other correlations were the same as those of

Table 2 ^{13}C NMR spectra of compounds **1**–**5** (DMSO-d_6).

No.	1	2	3	4	5
1	153.6	153.6	150.4	150.3	148.9
2	139.2	139.2	104.5	104.7	104.5
3	119.7	119.6	120.0	120.5	120.0
4	108.7	108.7	142.3	142.4	139.7
4a	143.9	143.9	147.9	147.9	147.8
4b	158.6	158.7	159.1	158.9	158.9
5	94.9	95.0	97.7	94.8	97.0
6	165.8	165.8	168.3	165.8	165.4
7	99.1	99.1	101.6	98.5	101.6
8	159.2	159.2	159.9	159.3	159.5
8a	105.3	105.3	102.6	104.6	103.8
8b	108.9	108.9	108.3	108.5	108.2
9	180.5	180.5	180.4	181.1	180.4
glc-1	100.7	100.9	102.0	100.6	101.2
glc-2	73.1	73.1	73.3	73.1	73.1
glc-3	76.3	76.3	76.0	76.3	75.8
glc-4	70.0	70.0	69.4	70.0	69.1
glc-5	75.6	75.6	76.0	75.6	75.6
glc-6	68.9	69.0	68.0	69.0	67.7
xyl-1	101.7	101.8	101.6	101.7	101.6
xyl-2	73.3	73.3	73.5	73.3	73.1
xyl-3	74.5	74.6	74.4	74.5	74.0
xyl-4	69.8	69.8	69.8	69.8	69.4
xyl-5	65.8	65.8	65.8	65.8	65.5
Cinnamyl					
1′	125.5	124.4	125.8	125.6	125.5
2′	110.7	105.9	111.0	110.8	110.6
3′	147.9	148.0	148.2	148.2	147.6
4′	149.3	138.3	149.3	149.3	146.9
5′	115.3	148.0	115.5	115.4	115.1
6′	122.9	105.9	123.0	123.0	122.8
7′	144.7	145.1	144.9	144.7	144.6
8′	114.5	114.9	114.9	114.6	114.6
9′	165.5	165.6	165.7	165.5	165.4
OCH_3					
2-	56.7	56.6			
4-			55.7	55.6	
6-	56.3	56.3		56.2	
3′-	55.6	56.0	56.6	56.6	55.3
5′-		56.0			

compound **1**. HMBC (**Fig. 3**) and ^1H-^1H COSY correlations confirmed that the linkage positions of the glycosyl, xylosyl, and caffeic acid moieties were identical to those in compound **1**. Thus, the structure of compound **2** was deduced to be 1-hydroxy-2, 6-dimethoxy-8-*O*-[2-(4′-hydroxy-3′,5′-dimethoxy-*E*-cinnamyl)-*β*-D-xylopyranosyl-(1–6)-*β*-D-glucopyranosyl]-xanthone and named comastomaside B.

The molecular formula of compound **3** was determined to be $C_{35}H_{36}O_{18}$ by HR-ESI-MS. The NMR spectrum of compound **3** (**Tables 1** and **2**) was similar to that of compound **1** with the exception of the substitution of the xanthone nucleus and a missing methoxy group (**Fig. 4**). NOESY correlations between the following determined the locations of the two methoxy groups and the 1,4,6,8-tetrasubstituted xanthone moiety in compound **3**: 4-OCH_3 and H-3; and 3′-OCH_3 and H-2′. Thus, the structure of compound **3** was deduced to be 1,6-dihydroxy-4-methoxy-8-*O*-[2-(4′-hydroxy-3′-methoxy-*E*-cinnamyl)-*β*-D-xylopyranosyl-(1–6)-*β*-D-glucopyranosyl]-xanthone and named comastomaside C.

The molecular formula of compound **4** was determined to be $C_{36}H_{38}O_{18}$ by HR-ESI-MS. The NMR spectrum of compound **4** (**Tables 1** and **2**) showed similarities to that of compound **3**. With the exception of the signals corresponding to the aromatic ring moiety of the xanthone nucleus, one of the phenolic hydroxyl groups on the aromatic ring in compound **3** was replaced by a signal corresponding to a methoxy group in compound **4**. The NOESY correlations between 6-OCH_3 and H-5, H-7 determined the location of the additional methoxy group and further confirmed that the methoxy group was linked to C-6 of the xanthone nucleus. Thus, the structure of compound **4** was deduced to be 1-hydroxy-4,6-dimethoxy-8-*O*-[2-(4′-hydroxy-3′-methoxy-*E*-cinnamyl)-*β*-D-xylopyranosyl-(1–6)-*β*-D-glucopyranosyl]-xanthone and named comastomaside D.

The molecular formula of compound **5** was determined to be $C_{34}H_{34}O_{18}$ by HR-ESI-MS. Its NMR spectrum (**Tables 1** and **2**) showed similarities to that of compound **3** with the exception of the signals corresponding to the aromatic ring of the xanthone nucleus. The two phenolic hydroxyl groups on the aromatic ring in compound **3** were replaced by signals corresponding to two methoxy groups in compound **5**. The enhanced NOE between 3′-OCH_3 and H-2′ confirmed that the methoxy group was connected at the C-3′ position of the cinnamyl moiety. In addition, hydroxyl groups were confirmed to be connected to C-4 and C-5 of the xanthone nucleus. Thus, the structure of compound **5** was de-

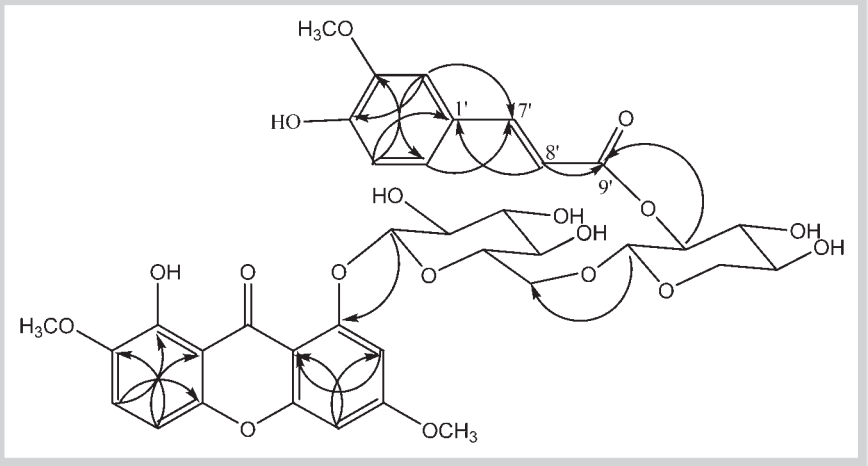

Fig. 2 Main HMBC (H → C) correlations of compound **1**.

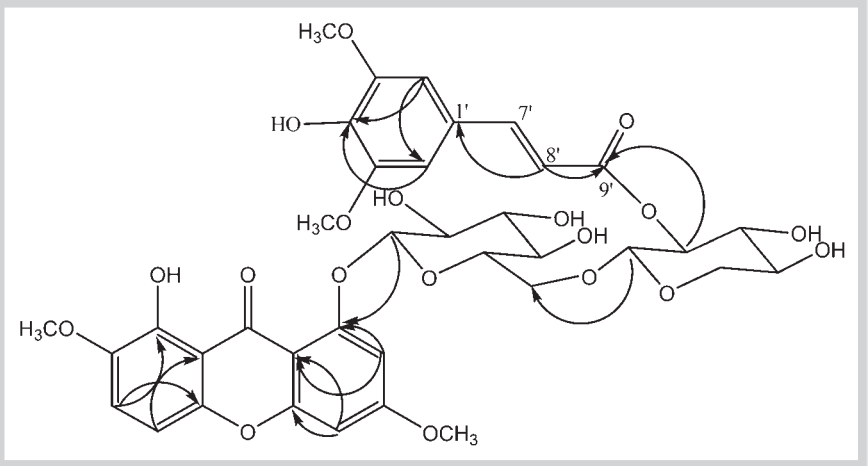

Fig. 3 Main HMBC (H → C) correlations of compound **2**.

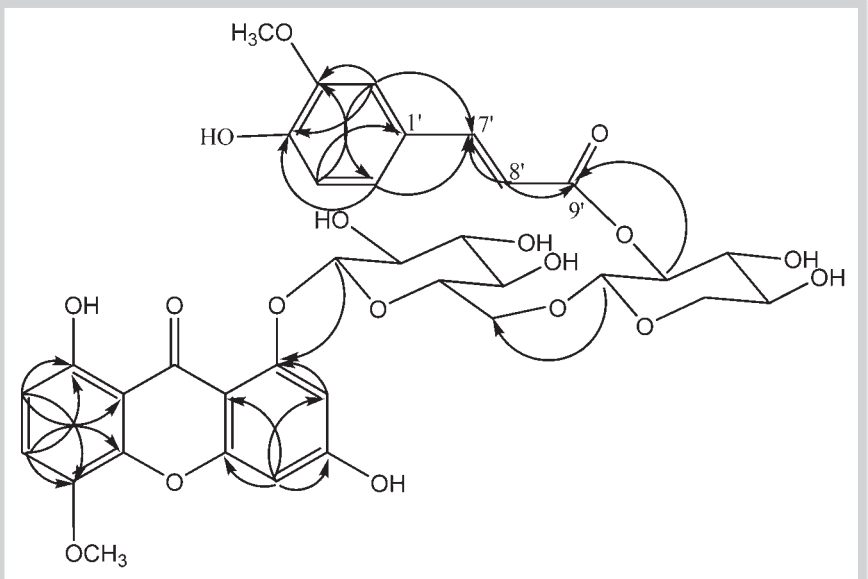

Fig. 4 Main HMBC (H → C) correlations of compound **3**.

duced to be 1,4,6-trihydroxy-8-*O*-[2-(4′-hydroxy-3′-methoxy-*E*-cinnamyl)-*β*-D-xylopyranosyl-(1–6)-*β*-D-glucopyranosyl]-xanthone and named comastomaside E.

Compounds **1**–**5** were evaluated for hepatoprotective activity against D-galactosamine-induced toxicity in WBF344 cells. At a concentration of 10^{-5} M, compounds **3** and **5** showed potent hepatoprotective activities (**Table 3**), whereas the other com-

Table 3 Hepatoprotective activity of compounds **1–5** against D-galactosamine-induced toxicity in WB-F344 cells[a].

Compound	Cell survival rate (% of normal)	Inhibition (% of control)
Normal	100.23 ± 0.52	
Control	24.14 ± 0.16	
Bicyclol[b]	37.12 ± 0.22**	17.10
1	29.33 ± 0.26	6.58
2	29.19 ± 0.26	6.58
3	35.12 ± 0.33*	14.47
4	29.06 ± 0.19	6.58
5	33.20 ± 0.20*	11.84

[a] Results are expressed as means ± SD (n = 3; for normal and control, n = 6); * $p < 0.05$, ** $p < 0.01$. Compounds were tested at 1×10^{-5} M; [b] Positive control substance bicyclol

pounds tested exhibited no significant activity at a concentration of 10^{-5} M. Compounds **1–5** were also evaluated for cytotoxicity against four human cancer cell lines (HCT-8, Bel-7402, BGC-823, and A2780) and determined to be inactive ($IC_{50} > 10$ µM).

Materials and Methods

The aerial parts of *C. pedunculatum* were collected in the County of Gonghe, Qinghai province, China, in August 2006. The plant material was identified by Prof. Lin Pengcheng. A voucher specimen (HMH200608A) was deposited in the College of Life and Environmental Science, Minzu University of China.

Optical rotations were measured using a PE Model 343 polarimeter. IR spectra were recorded on a Nicolet 5700 FT-IR spectrophotometer. NMR spectra were collected on Varian Inova-500 and Bruker AV-500 spectrometers in DMSO, and solvent peaks were used as references. Mass spectra were obtained on a Mass Agilent 1100 Series LC-MSD-Trap-SL spectrometer (ESI-MS) and 6210 ESI-TOF spectrometer (HR-ESI-MS).

The aerial parts of *C. pedunculatum* were dried in the shade and chipped. The plant material (5.0 kg) was extracted with 70% aqueous ethanol (3 × 40 L) under reflux for 1 hour. The solution was filtered, evaporated *in vacuum* and successively partitioned with petroleum ether, ethyl acetate, and *n*-butanol (1 : 1, v/v). The *n*-butanol (230 g) fraction was subsequently purified on an AB-8 porous polymer resin (10 L; column, 10 × 150 cm) and eluted with EtOH-H_2O gradients to yield 4 fractions [HBT (20% EtOH, 80 L), HBF (40% EtOH, 80 L), HBS (60% EtOH, 60 L), and HBN (95%EtOH, 40 L)]. The HBF fraction (13.8 g) was applied to CC [silica gel (mesh, 45–75 µm, 150 g); column, 3 × 120 cm] and eluted with solvent gradients of $CHCl_3$-MeOH (1 : 0 to 8 : 2; 10 L) to afford fractions A–H. Fraction D (2095 mg) was further purified by CC [ODS (mesh, 50 µm, 100 g); column, 3 × 90 cm] and eluted with a MeOH-H_2O gradient (2: 8 to 6 : 4; 6.0 L) to yield subfractions D_1-D_5. Subfraction D_2 (1236 mg) was purified by CC [Sephadex LH-20 (50 g); column, 1.5 × 60 cm] and eluted with $CHCl_3$-MeOH (1 : 1) to afford a yellow solid (647 mg), which was purified by preparative HPLC [Allsphere C_{18} column, (250 × 10 mm, 5 µm); 2.5 L of MeOH-H_2O (40 : 60); 2.0 mL/min flow rate] to obtain compounds **1** (9 mg), **2** (7 mg), and **4** (20 mg). Fraction E (864 mg) was further purified by CC [ODS (mesh, 50 µm; 50 g); column, 3 × 60 cm] and eluted with a MeOH-H_2O gradient (2 : 8 to 6 : 4; 3.0 L) to obtain subfractions E_1-E_5. Subfraction E_2 (450 mg) was purified by CC [Sephadex LH-20 (50 g); column, 1.5 × 60 cm] eluted with $CHCl_3$-MeOH (1 : 1), to obtain a yellow solid (257 mg) which was purified by preparative HPLC [Allsphere C_{18} column, (250 × 10 mm, 5 µm); 2.5 L of MeOH-H_2O (35 : 65); 2.0 mL/min] to obtain compounds **3** (10 mg) and **5** (9 mg).

Isolates: *Comastomaside A* (**1**): yellow amorphous solid (94.3% purity), m. p. 284–285 °C, $[\alpha]_D^{20}$ −144.2 (*c* 0.10, DMSO). UV (DMSO) λ_{max}(log ε): 257 (4.41), 273 (4.28), 297 (4.21), 324 (4.38) nm; NMR data, see **Tables 1** and **2**; ESI-MS (pos): 781 $[M + Na]^+$; HR-ESI-MS: 781.1967 $[M + Na]^+$ (calcd. for $C_{36}H_{38}O_{18}Na$, 781.1950).

Comastomaside B (**2**): yellow amorphous solid (98.2% purity), m. p. 270–272 °C, $[\alpha]_D^{20}$ −33.1 (*c* 0.10, DMSO). UV (DMSO) λ_{max} (log ε): 257 (4.29), 269 (4.30), 318 (4.26) nm; NMR data, see **Tables 1** and **2**; ESI-MS (pos): 811 $[M + Na]^+$, HR-ESI-MS: 811.2067 $[M + Na]^+$ (calcd. for $C_{37}H_{40}O_{19}Na$, 811.2056).

Comastomaside C (**3**): yellow amorphous solid (99.9% purity), m. p. 260–262 °C, $[\alpha]_D^{20}$ −54.6 (*c* 0.10, DMSO). UV (DMSO) λ_{max} (log ε): 257 (4.38), 268 (4.30), 299 (4.24), 324 (4.39) nm; NMR data, see **Tables 1** and **2**; ESI-MS (pos): 767 $[M + Na]^+$, HR-ESI-MS: 767.1789 $[M + Na]^+$ (calcd. for $C_{35}H_{36}O_{18}Na$, 767.1794).

Comastomaside D (**4**): yellow amorphous solid (98.2% purity), m. p. 277–279 °C, $[\alpha]_D^{20}$ −55.5 (*c* 0.10, DMSO). UV (DMSO) λ_{max} (log ε): 257 (4.46), 272 (4.40), 295 (4.18), 356 (4.49) nm; NMR data, see **Tables 1** and **2**; ESI-MS (pos): 781 $[M + Na]^+$, HR-ESI-MS: 781.1936 $[M + Na]^+$ (calcd. for $C_{36}H_{38}O_{18}Na$, 781.1950).

Comastomaside E (**5**): yellow amorphous solid (93.7% purity, HPLC); m. p. 281–284 °C, $[\alpha]_D^{20}$ −59.1 (*c* 0.10, MeOH). UV (MeOH) λ_{max} (log ε): 204 (4.65), 240 (4.57), 269 (4.44), 316 (4.49) nm; NMR data, see **Tables 1** and **2**; ESI-MS (pos): 753 $[M + Na]^+$, HR-ESI-MS: 753.1642 $[M + Na]^+$ (calcd. for $C_{34}H_{34}O_{18}Na$, 753.1637).

Acid hydrolysis TLC [5]: Compounds **1, 2, 3, 4**, and **5** were dissolved in ethanol separately to a concentration of 0.5 mg per mL. The solutions were applied separately at approximately 1 cm from the bottom edge of the HPTLC silica gel plate (10 × 10 cm), and the plate was hydrolyzed in an airtight container with hydrogen chloride vapor for 20 min in a 50–60 °C water bath. Then, D-glucose and D-xylose were dissolved in ethanol separately, and the solutions were also applied separately to the base of the plate. Glacial acetic acid (1 mL) was added to 9 mL of the lower layer containing a mixture of chloroform-methanol-water (30 : 12 : 4), which was used as the developing solvent. The plate was saturated for 20 min and then developed for 9 cm using an ascending technique. The plate was subsequently soaked in a coloring reagent consisting of 0.93 g of aniline and 1.66 g of *o*-phthalic acid in 100 mL of *n*-butanol, and then heated at 100 °C for 15 min until colored spots were visible. TLC analysis showed the presence of glucose and xylose, which are the sugars present in compounds **1–5**.

Hepatoprotective activities against D-galactosamine-induced cytotoxicity in WB-F344 cells: The hepatoprotective activities of compounds **1-5** were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay in WB-F344 cells. Each cell suspension of 1×10^4 cells in 200 µL of Dulbecco's modified Eagle's medium containing fetal calf serum (3%), penicillin (100 units/mL), and streptomycin (100 µg/mL) was placed in a 96-well microplate and precultured for 24 h at 37 °C under a 5% CO_2 atmosphere. Fresh medium (200 µL) containing the positive control bicyclol (purity > 99%, Beijing Union Pharmaceutical Factory) or test samples were added, and the cells were cultured for 1 h. The cultured cells were

exposed to 40 mM D-galactosamine for 24 h. The cytotoxic effects of test samples were simultaneously measured in the absence of D-galactosamine. The medium was replaced and contained 0.5 mg/mL MTT. After 3.5 h incubation, the medium was removed, and 150 µL of DMSO was added to dissolve formazan crystals. The optical density (OD) of the formazan solution was measured at 492 nm using a microplate reader. Inhibition (%) was determined using the following formula:

$$\text{Inhibition (\%)} = [(OD_{sample} - OD_{control})/(OD_{normal} - OD_{control})] \times 100$$

Assessment of the inhibitory activity of compounds 1–5 against several human cancer cell lines: Human colon cancer (HCT-8), hepatoma (Bel-7402), stomach cancer (BGC-823), ovarian cancer (A2780), and epithelial WISH cell lines (Susan Hayflick Wistar Institute) were obtained from the American Type Culture Collection (ATCC). Cells were maintained in RRMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

HCT-8, Bel-7402, BGC-823, A2780 cells, and human epithelial WISH cells were seeded in 96-well microtiter plates at a concentration of 1200 cells/well. After 24 h, compounds **1**–**5** were added to cells. 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. MTT assay results were monitored at 570 nm using an MK 3 Wellscan (Labsystem DROGON) plate reader. All compounds were dissolved in 100% DMSO to yield a final DMSO concentration of 0.1% in each well and tested at five different concentrations. The concentration of each compound was tested in three parallel wells. IC_{50} values were calculated using Microsoft Excel software.

Statistical analysis: The Student's t-test for unpaired observations between control and test samples was performed to identify significant differences, and p values less than 0.05 were considered to be significantly different.

Supporting information

UV, IR, MS, ^{1}H NMR, and ^{13}C NMR and 2D NMR correlation spectra of compounds **1**–**5** are available as Supporting Information.

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Conflict of Interest

The corresponding authors declare that this manuscript is submitted on behalf of all authors. The authors do not have any conflicts of interest. The copyright belongs to the publisher upon acceptance of the manuscript.

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