Phenolic Constituents from *Brainea insignis*

Kou WANG,[a,b] Ming-Ming LI,[a,b] Xuan-Qin CHEN,[a,b] Li-Yan PENG,[a] Xiao CHENG,[a] Yan LI,[a] and Qin-Shi ZHAO*,[a]

[a] *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences; Kunming 650204, People's Republic of China: and* [b] *Graduate School of Chinese Academy of Sciences; Beijing 100049, People's Republic of China.*

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Phytochemical investigation on the methanol extract of *Brainea insignis* led to the isolation of 16 phenolic compounds, including an unusual flavanol coupled with phenylpropyl and shikimic acid units, brainicin (1), and a new flavonol acylglycosides brainoside B (2). The structures of new compounds were elucidated on the basis of extensive spectroscopic analysis. Compounds 1—5 were evaluated for their cytotoxic effects. However, none of the compounds showed significant cytotoxic activity against the HL-60, A549, SMMC-7721, MCF-7, SW480 cell lines ($IC_{50}>40\ \mu$M).

Key words *Brainea insignis*; flavonol; brainicin; brainoside B; brainoside C

As the only species of the genus *Brainea* (Blechnaceae), *Brainea insignis* (HOOK.) J. SMITH a kind of fern is widely distributed in south China. It is used as herbal medicine to treat cold, burn, bleeding wound, and ascariasis, *etc.*, as well as occasionally planted for ornament because of its beautiful shape.[1,2] Previous chemical investigations of *B. insignis* have been shown the presence of lignans,[3] flavonoids,[4] pyrone glucosides,[5] stilbene,[5] steroids,[5] and a series of volatile compounds.[6] As a part of our search for naturally occurring bioactive metabolites from ferns of China,[7—9] we investigated the chemical constituents of *B. insignis*, which led to the isolation of 16 phenolic compounds, including an unusual flavanol coupled with phenylpropyl and shikimic acid units, brainicin (**1**), and a new flavonol acylglycosides brainoside B (**2**). Herein, we describe the isolation and structure elucidation of new compounds.

Results and Discussion

Compound **1** was isolated as a brown amorphous solid. The molecular formula was established as $C_{31}H_{28}O_{14}$ by a quasimolecular ion peak at *m/z* 623.1406 $[M-H]^-$ in the negative HR-electrospray ionization (ESI)-MS analysis (Calcd for 623.1400), indicating 18 degrees of unsaturation. The IR spectrum showed absorption bands at 3426, 1710, 1627, 1606, 1527 and 1449 cm^{-1}, revealing the presence of hydroxyl, carbonyl and aromatic rings groups, respectively. The aromatic region of the ^{1}H-NMR spectrum of **1** showed the presence of three sets of aromatic protons (Table 1). One set corresponded to a tetrasubstituted aromatic ring with two *meta*-coupling protons and appeared at δ_H 5.88 (d, $J=2.0$ Hz, H-6) and 5.98 (d, $J=2.0$ Hz, H-8), and two sets corresponded to 3,4-dihydroxyphenyl groups at δ_H 6.82 (d, $J=1.5$ Hz, H-2′), 6.71 (d, $J=7.5$ Hz, H-5′), and 6.67 (dd, $J=7.5$, 1.5 Hz, H-6′), and at δ_H 6.77 (d, $J=2.0$ Hz, H-2″), 6.71 (d, $J=8.0$ Hz, H-5″), and 6.64 (dd, $J=8.0$, 2.0 Hz, H-6″), respectively. Resonances due to a coupling system at δ_H 5.14 (d, $J=5.0$ Hz, H-2), 3.97 (dd, $J=10.7$, 5.0 Hz, H-3), 3.05 (t, $J=10.8$ Hz, H-4), 2.61 (t, $J=10.6$ Hz, H-8″), and 4.81 (d, $J=10.0$ Hz, H-7″) suggested the presence of a –CH(O)–CH(O)–CH–CH–CH(O)– unit. This unit was confirmed by the ^{1}H–^{1}H correlation spectroscopy (COSY) experiment from the coupling of H-2/H-3, H-3/H-4, H-4/H-8″, and H-8″/H-7″. The additional signals at δ_H 6.67 (overlapped, H-2‴), 4.25 (s, br, H-3‴), 3.75 (dd, $J=7.0$, 4.0 Hz, H-4‴), 4.79 (dt, $J=7.0$, 5.0 Hz, H-5‴), 1.71 (d, br, $J=18.6$ Hz, H-6‴a), and 2.40 (dt, br, $J=18.6$, 2.4 Hz, H-6‴b) were suggested to be a shikimic acid moiety. The ^{13}C-NMR spectrum showed 31 carbon signals and supported the above assignments (Table 1). In combination with the observed heteronuclear multiple bond connectivity (HMBC) correlations (Fig. 1) from H-2 to C-1′, C-2′, and C-6′, from H-4 to C-4a and C-5, from H-7″ to C-1″, C-2″, and C-6″, from H-8″ to C-9″, and from H-2‴ to C-7‴, **1** was suspected to be composed of a flavan-3-ol unit, a phenylpropyl unit, and shikimic acid unit and to be formed by coupling of these units. The HMBC correlations of H-5‴ to C-9″, revealed the two oxygenated carbons C-5‴″ and C-9″ were connected with

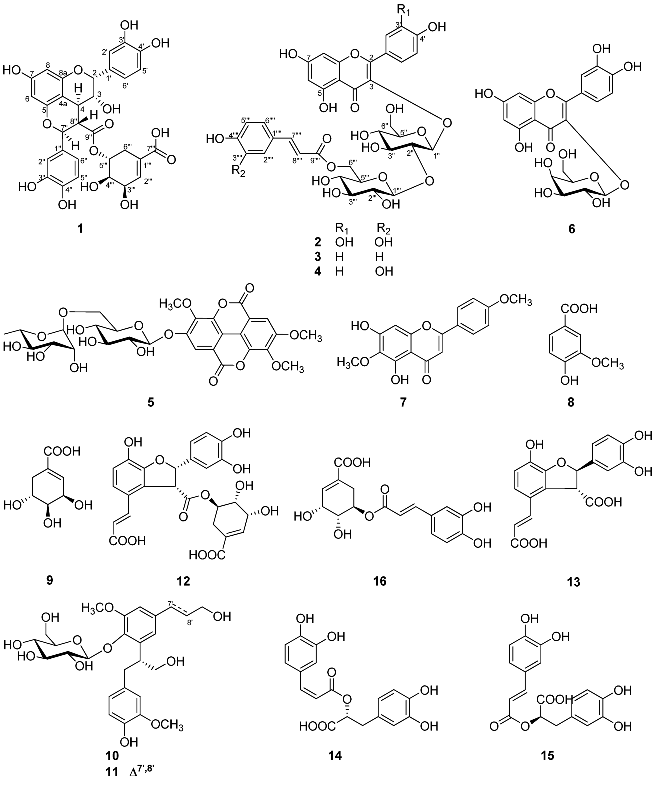

Chart 1.

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* To whom correspondence should be addressed. e-mail: qinshizhao@mail.kib.ac.cn

Table 1. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (100 MHz) Data of **1**—**3** in CD_3OD

No.	**1**		No.	**2**		**3**	
	δ_H	δ_C		δ_H	δ_C	δ_H	δ_C
2	5.14 (d, 5.0)	80.2 d	2		158.2 s		158.2 s
3	3.97 (dd, 10.7, 5.0)	71.3 d	3		135.1 s		135.0 s
4	3.05 (t, 10.8)	36.9 d	4		179.8 s		180.1 s
4a		98.1 s	4a		105.7 s		105.6 s
5		155.9 s	5		162.9 s		162.9 s
6	5.88 (d, 2.0)	96.1 d	6	6.17 (br s)	99.8 d	6.13 (br s)	99.9 d
7		159.4 s	7		165.6 s		165.6 s
8	5.98 (d, 2.0)	96.1 d	8	6.31 (br s)	94.7 d	6.19 (br s)	94.8 d
8a		155.7 s	8a		158.2 s		158.4 s
1′		130.9 s	1′		122.9 s		122.2 s
2′	6.82 (d, 1.5)	115.5 d	2′	7.69 (d, 2.0)	117.4 d	8.09 (d, 8.5)	132.7 d
3′		145.9 s	3′		145.9 s	6.87 (d, 8.5)	116.3 d
4′		145.7 s	4′		149.7 s		161.6 s
5′	6.71 (d, 7.5)	116.0 d	5′	6.91 (d, 8.4)	115.0 d	6.87 (d, 8.5)	116.3 d
6′	6.67 (dd, 7.5, 1.5)	120.3 d	6′	7.66 (dd, 8.4, 2.0)	123.7 d	8.09 (d, 8.5)	132.7 d
1″		130.7 s	1″	5.42 (d, 7.5)	100.9 d	5.12 (d, 7.5)	101.6 d
2″	6.77 (d, 2.0)	115.6 d	2″	3.76 (t, 8.8)	84.4 d	4.01 (t, 8.5)	82.7 d
3″		147.0 s	3″	3.51 (m)	77.7 d	3.48 (overlapped)	76.6 d
4″		146.2 s	4″	3.45 (m)	70.9 d	3.38 (m)	70.1 d
5″	6.71 (d, 8.0)	115.8 d	5″	3.64 (m)	77.7 d	3.57 (m)	76.1 d
6″	6.64 (dd, 8.0, 2.0)	120.5 d	6″	3.64 (m), 3.53 (m)	62.2 t	3.80 (m), 3.65 (m)	61.7 t
7″	4.81 (d, 10.0)	81.2 d	1‴	4.82 (d, 7.7)	105.8 d	4.74 (d, 7.4)	106.2 d
8″	2.61 (t, 10.6)	50.4 d	2‴	3.44 (overlapped)	76.0 d	3.43 (overlapped)	75.5 d
9″		173.9 s	3‴	3.22 (m)	78.2 d	3.13 (m)	77.7 d
1‴		130.4 s	4‴	3.43 (overlapped)	71.8 d	3.40 (m)	71.8 d
2‴	6.67 (overlapped)	138.4 d	5‴	3.70 (m)	75.7 d	3.65 (overlapped)	74.8 d
3‴	4.25 (br s)	67.0 d	6‴	4.44 (dd, 2.0, 12.0) 4.35 (dd, 6.0, 12.0)	64.6 t	4.44 (dd, 1.5, 11.8) 4.35 (dd, 6.3, 11.8)	64.6 t
4‴	3.75 (dd, 7.0, 4.0)	69.1 d	1⁗		127.4 s		126.7 s
5‴	4.79 (dt, 7.0, 5.0)	71.8 d	2⁗	6.90 (d. 1.6)	116.3 d	7.08 (d, 8.3)	130.8 d
6‴	1.71 (br d, 18.6) 2.40 (br dt, 18.6, 2.4)	28.0 t	3⁗		146.5 s	6.63 (d, 8.3)	116.6 d
7‴		170.0 s	4⁗		149.3 s		161.0 s
			5⁗	6.68 (d, 8.1)	116.3 d	6.63 (d, 8.3)	116.6 d
			6⁗	6.72 (dd, 1.6, 8.1)	122.8 d	7.08 (d, 8.3)	130.8 d
			7⁗	7.38 (d, 15.9)	147.0 d	7.33 (d, 15.9)	146.5 d
			8⁗	6.07 (d, 15.9)	114.5 d	6.01 (d, 15.9)	114.5 d
			9⁗		169.1 s		169.0 s

δ in ppm, J in Hz.

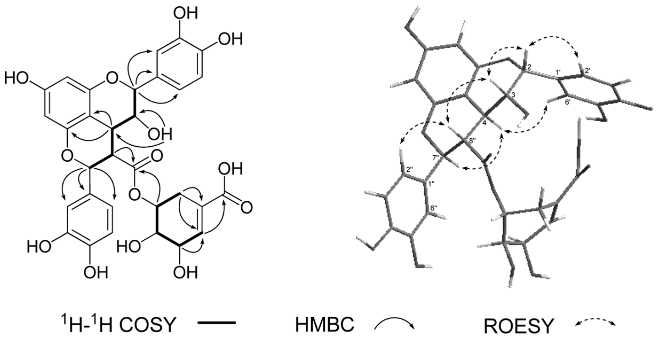

Fig. 1. Key COSY, HMBC (from H to C) and ROESY Correlations of **1**

a 5‴,9″-ester linkage. Moreover, NMR experiments were reperformed in DMSO-d_6, and crucial HMBC correlation from the hydrogen of 3-OH to C-3 and C-4 were observed, which suggested that an ether linkage should be between C-7″ and C-5 but not between C-7″ and C-3. Therefore, the structure of **1** was confirmed as shown in Fig. 1.

The relative configuration of C-2 and C-3 in **1** was concluded to be of the epicatechin type from the characteristic feature of the H-2 resonance in the ^{1}H-NMR spectrum: δ_H 5.14 (d, J=5.0 Hz).[10,11] And the C-2 aryl substituent was suggested to be the equatorial orientation according to the thermodynamically favoured conformation existing in natural flavanones.[12,13] The 2,3-*cis* configuration was determined by a small H-2/H-3 coupling constant of 5.0 Hz. The spatial relationships were deduced from the nuclear Overhauser enhancement spectroscopy (NOESY) spectra. The correlations observed for H-4/H-2′ (-6′), H-3/H-8″, H-8″/H-2″ (-6″), and H-4/H-7″ indicated that H-2, H-3, and H-8 were cofacial, while H-4 and H-7″ were on opposite sides of the molecule. The large coupling constants $J_{3,4}$=10.7 Hz, $J_{4,8''}$=10.6 Hz, and $J_{7'',8''}$=10.0 Hz were also in agreement with the *trans*-orientations of H-3/H-4, H-4/H-8″, and H-8″/H-7″. On the basis of the above discussion and referring to published epicatechin derivatives,[10—12,14] the structure of **1** was finally assigned and named brainicin.

Compound **2** was obtained as a yellow gummy solid, and assigned the molecular formula $C_{36}H_{36}O_{20}$ by the negative HR-ESI-MS analysis ([M−H]$^-$ at m/z 787.1706, Calcd for 787.1721). The UV and IR spectra of **2** showed absorption bands at 210, 268, 303 and 374 nm, and 3427 br, 1689, 1649, 1630, 1608, 1501, and 1444 cm^{-1}, respectively, which are indicative of the presence of aromatic rings, carbonyl and hydroxyl groups.

The anomeric signals at δ_C 100.9 (d) and 105.8 (d), as well as the other characteristic signals of the ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra (Table 1), displayed the presence of two glycopyranosyl units. Besides, the spectra showed 15 carbon signals for 3-*O*-glycosylated quercetin[15] and 9 carbon signals for a caffeoyl moiety.[16] They were further supported by ^{1}H-NMR spectrum: two broad singlets at δ_H 6.17 and 6.31, typical of the H-6 and H-8 protons of ring A of a flavonoid unit, one set aromatic ABX system protons corresponded to 3,4-dihydroxyphenyl group of ring B [δ_H 7.69 (d, J=2.0 Hz, H-2′), 7.66 (dd, J=2.0, 8.4 Hz, H-6′), and 6.91 (d, J=8.4 Hz, H-5′)], and a set protons of the caffeoyl moiety at δ_H 7.38 (d, J=15.9 Hz, H-7′′′′), 6.90 (d, J=1.6 Hz, H-2′′′′) , 6.72 (dd, J=1.6, 8.1 Hz, H-6′′′′), 6.68 (d, J=8.1 Hz, H-5′′′′), and 6.07 (d, J=15.9 Hz, H-8′′′′). In addition, the double bond was suggested as *trans*-due to the coupling constant. Acidic hydrolysis of **2** gave D-glucose as the sole sugar moiety, which was determined to have a β configuration on the basis of the large coupling constants of the anomeric protons [δ_H 5.42 (J=7.5 Hz) and 4.82 (J=7.7 Hz)]. Assignment of glucosidic protons system was achieved by analysis of ^{1}H–^{1}H COSY and heteronuclear single quantum coherence (HSQC) experiments.

Connectivities of glucosyl, quercetin, and caffeoyl moieties were confirmed by the HMBC experiments, in which correlations of H-1′′ (δ_H 5.42) with C-3 (δ_C 135.1), H-1′′′ (δ_H 4.82) with C-2′′ (δ_C 84.4), and H-6′′′ (δ_H 4.44, 4.35) with C=O (δ_C 169.1) were observed. On the basis of all these results, the structure of compound **2** was established as quercetin 3-*O*-β-D-glucopyranosyl (1→2)-[(6′′′-*O*-*trans*-caffeoyl)-β-D-glucopyranoside], named brainoside B (Fig. 2).

Compound **3** was initially determinated from immature leaves of *Brassica rapa* L. ssp. *chinensis* L. (HANELT.) by ROCHFORT and co-workers, but its spectroscopic data were lacking.[17] In this paper, compound **3** was isolated as a yellow gummy solid and named brainoside C. The ^{1}H- and ^{13}C-NMR data of **3** were showed in Table 1.

The structures of the known compounds **4**—**16** isolated were identified as camsibriside A,[18] 3,3′,4-tri-*O*-methyl-4′-*O*-rutinosylellagic acid,[19] hyperin,[20] pectolinarigenin,[21] vanillic acid,[22] shikimic acid,[23] icariside E$_3$,[24] icariside E$_5$,[25] brainic acid,[3] 7-epiblechnic acid,[3] (*Z*)-rosmarinic acid,[26] rosmarinic acid,[27,28] and 5-*O*-caffeoylshikimic acid,[29] respectively, by comparison of their spectroscopic data with literature values.

Compounds **1**—**5** were evaluated for their cytotoxicity against five human cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as reported previously.[30] However, none of the compounds showed significant cytotoxic activity against the HL-60, A549, SMMC-7721, MCF-7, SW480 cell lines (IC$_{50}$> 40 μM).

Experimental

General Experimental Procedures Melting point was obtained on an X-4 micro melting point apparatus. Optical rotations were measured on a Horiba SEPA-300 polarimeter. IR spectra were obtained with a Tensor 27 FT-IR spectrometer with KBr pellets. UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were acquired with a Bruker AV-400 and DRX-500 spectrometers in CD$_3$OD at room temperature (δ in ppm, J in Hz). ESI-MS (including HR-ESI-MS) and FAB-MS were carried out on API QSTAR Pulsar i and VG Autospec-3000 mass spectrometers, respectively. Silica gel (200—300 mesh), Silica gel H (Qingdao Marine Chemical Ltd., China), LiChroprep RP-18 silica gel (40—63 μm, Merck, Dramstadt, Germany), and Sephadex LH-20 (Amersham Pharmacia biotech, Sweden) were used for column chromatography. Preparative and semipreparative HPLC were performed on Shimadzu LC-8A preparative liquid chromatograph with a Shimadzu PRC-ODS (K) column and Agilent 1100 liquid chromatograph with a Zorbax SB-C$_{18}$ (9.4 mm×25 cm) column, respectively. Fractions were monitored by TLC and spots were visualized by heating silica gel plates immersed with 15% H$_2$SO$_4$ in ethanol. Solvents were distilled prior to use.

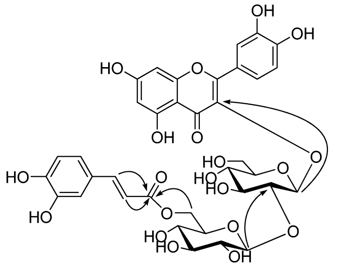

Fig. 2. Key HMBC (from H to C) Correlations of **2**

Plant Material The aerial parts of *B. insignis* were collected in Jinping County, Yunnan Province, PRC, in July 2007 and identified by professor Xiao Cheng of Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 200707F03) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation The dried and powdered plant materials (4.5 kg) were extracted with methanol (15.0 l×3, each 2 d) at room temperature. After evaporation of the solvent *in vacuo*, the concentrate was suspended into H$_2$O and partitioned successively with petroleum ether (PE) and ethyl acetate (EtOAc). The EtOAc extract (150 g) was subjected to column chromotography (CC) over silica gel (100—200 mesh) and eluted with gradient PE–Me$_2$CO (1 : 0 to 0 : 1) to give five fractions 1—5. Fraction 2 was further subjected to column chromatograph over silica gel to obtain three subfractions 2.1—2.3. Subfraction 2.3 was further purified by silica gel CC and Sephadex LH-20 (MeOH) to yield **6** (5 g) and **7** (10 mg). Fraction 3 was eluted with CHCl$_3$–MeOH (20 : 1 to 8 : 2) over silica gel CC to afford four subfractions 3.1—3.4. Subfraction 3.2 and 3.3 were separated repeatedly on silica gel and RP-18 (MeOH–H$_2$O gradient elution of increasing concentration) to obtain **5** (22 mg), **8** (7 mg), and **9** (116 mg). Fraction 4 was subjected to silica gel CC eluting with EtOAc–MeOH (9 : 1 to 6 : 4) to yield five subfractions 4.1—4.5. Subfractions 4.1 and 4.2 were chromatographed on silica gel and finally purified by semipreparative HPLC using MeOH–H$_2$O (30 : 70) and MeOH–H$_2$O (32 : 68, plus 0.5% formic acid) as eluents respectively, to afford **10** (7 mg), **11** (7 mg), **13** (3.5 mg), **14** (2.7 mg), and **15** (21 mg). Subfraction 4.4 was further separated by silica gel CC to give **4** (2 g). Subsequently, preparative HPLC (MeOH–H$_2$O, 30 : 70, plus 0.5% formic acid for subfraction 4.3 and MeOH–H$_2$O, 33 : 67, plus 0.5% formic acid for subfraction 4.5) was used to purify **1** (21 mg), **12** (155 mg), **16** (11 mg), **2** (14 mg), and **3** (35 mg) from subfractions 4.3 and 4.5.

Brainicin (**1**): Brown amorphous powder. mp 207—209 °C. $[\alpha]_D^{23.9}$ +69.8 (c=0.29, MeOH). UV λ_{max} (MeOH) nm (log ε): 291.8 (4.19), 216.4 (4.74), 197.8 (4.52). IR (KBr) cm^{-1}: 3426, 2925, 1710, 1627, 1606, 1527, 1449, 1383, 1285, 1147, 1114, 1059. ^{1}H- and ^{13}C-NMR: see Table 1. ESI-MS (neg.) *m/z*: 623 [M−H]$^-$, 1247 [2M−H]$^-$. HR-ESI-MS (neg.) *m/z*: 623.1406 (Calcd for $C_{31}H_{27}O_{14}^-$, 623.1400).

Brainoside B (**2**): Yellow gummy solid. $[\alpha]_D^{25.8}$ −67.2 (c=0.12, MeOH). UV λ_{max} (MeOH) nm (log ε): 374 (4.55), 303 (4.34), 268 (4.56), 210.0 (4.82). IR (KBr) cm^{-1}: 3427, 2923, 1689, 1649, 1630, 1608, 1501, 1444, 1272, 1169, 1076. ^{1}H- and ^{13}C-NMR: see Table 1. HR-ESI-MS (neg.) *m/z*: 787.1706 (Calcd for $C_{36}H_{35}O_{20}^{£≠}$, 787.1721).

Brainoside C (**3**): Yellow gummy solid. $[\alpha]_D^{25.8}$ −36.2 (c=0.25, MeOH). UV λ_{max} (MeOH) nm (log ε): 315 (4.47), 268 (4.36), 206 (4.55), 196 (4.42). IR (KBr) cm^{-1}: 3427, 2921, 1688, 1655, 1606, 1512, 1444, 1360, 1278, 1261, 1172, 1077. ^{1}H- and ^{13}C-NMR: see Table 1. HR-ESI-MS (neg.) *m/z*: 755.1840 (Calcd for $C_{36}H_{35}O_{18}^-$, 755.1823).

Acidic Hydrolysis of Compound 2 Compound **2** (6 mg) was hydrolyzed with 2 M HCl–dioxane (1 : 1, 4 ml) under reflux for 6 h. The reaction mixture was extracted with $CHCl_3$ five times (4 ml×5). The aqueous layer was neutralized with 2 M $NaHCO_3$ then dried to give a monosaccharide mixture. Then, a solution of the sugar mixture in pyridine (2 ml) was added to L-cysteine methyl ester hydrochloride (about 1.5 mg) and kept at 60 °C for 1 h. Next, trimethylsilylimidazole (about 1.5 ml) was added to the reaction mixture in ice water and kept at 60 °C for 30 min. The mixture was subjected to GC analysis, run on a Shimadzu GC-14C gas chromatograph equipped with a 30 m×0.32 mm i.d. 30QC2/AC-5 quartz capillary column and an H_2 flame ionization detector with the following conditions: column temperature, 180—280 °C; programmed increase, 3 °C/min; carrier gas, N_2 (1 ml/min); injector and detector temperature, 250 °C; injection volume, 4 μl; and split ratio, 1/50. The configuration of D-glucose for compound **2** was determined by comparison of the retention time of the corresponding derivative with that of standard D-glucose, giving a peak at 18.926 min.

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