Biologically Active Iridoids from *Hedyotis diffusa*

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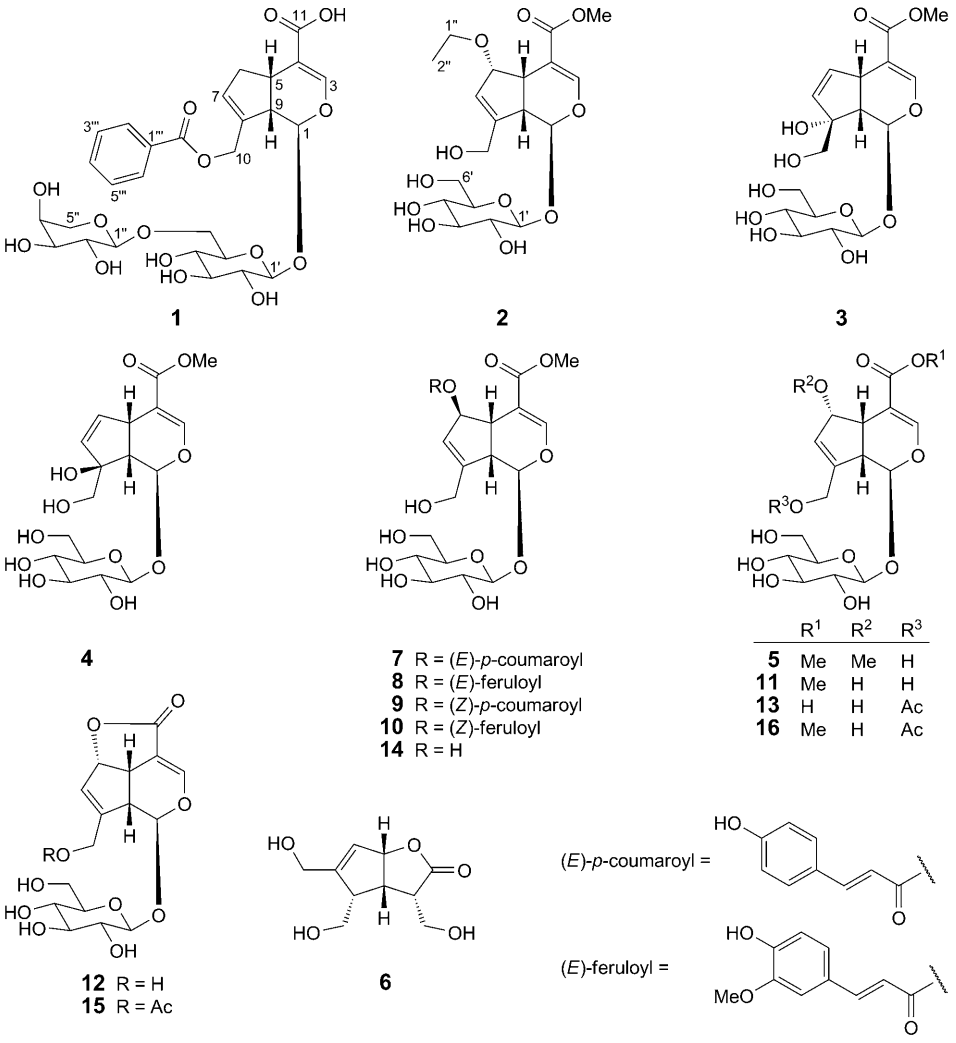
Two new iridoids, 10-*O*-benzoyl-6′-*O*-*α*-L-arabino(1 → 6)-*β*-D-glucopyranosylgeniposidic acid (**1**), deacetyl-6-ethoxyasperulosidic acid methyl ester (**2**), along with 14 other known iridoids, were isolated from the whole plant of *Hedyotis diffusa* WILLD. Their structures were determined on the basis of spectroscopic analysis. The short-term-memory-enhancement activities of compounds **1**, **7**–**9**, **11**, and **13** were evaluated on an A*β* transgenic drosophila model.

Introduction. – *Hedyotis diffusa* WILLD. (Rubiaceae), an annual herbaceous plant distributed in southern regions of China [1], is known as a folk medicine for the treatment of sphagitis, bronchitis, dysentery, mastitis, pneumonia, appendicitis, pelvitis, and some tumors [2]. In addition, the extract of this whole plant showed a series of bioactivities in the modern pharmacological investigations, such as anticancer [3–4], antimicrobial [5], anti-inflammatory [5], and immunoloregulation [6]. Previous phytochemical studies revealed the presence of iridoid glycosides [7][8], flavones [9], anthraquinones [10], and phenylpropanoids [11]. Here, we report the isolation and structural elucidation of two new iridoid glycosides, along with 14 known iridoids from this plant. Compounds **1**, **7**–**9**, **11**, and **13** showed potent activities in the short-term-memory-enhancement assay on an A*β* transgenic drosophila model.

Results and Discussion. – *Structure Elucidation.* The 60% EtOH/H_2O extract of the whole plant of *H. diffusa* was chromatographed over macroporous resin *HP-20* column, eluted with EtOH/H_2O in gradient. The 50% EtOH/H_2O eluted fraction, through further isolation by a series of silica gel, *ODS*, *Toyopearl HW-40*, *Sephadex LH-20* columns as well as preparative HPLC, yielded two new iridoid glycosides, **1** and **2**, and 14 known iridoids, **3**–**16**. To the best of our knowledge, compounds **3**–**6** were isolated from *Hedyotis* species for the first time.

[1]) These authors contributed equally to this work.



Compound **1** was obtained as a yellow amorphous powder. HR-ESI-MS gave a *quasi*-molecular-ion peak at *m*/*z* 609.1821 ($[M-H]^-$), corresponding to the molecular formula $C_{28}H_{34}O_{15}$. In the ^{1}H-NMR spectrum of **1** (*Table 1*), the H-atom signals at δ(H) 7.53 (*d*, $J = 0.9$, 1 H) and 5.20 (*d*, $J = 8.0$, 1 H) were characteristic for H–C(3) and H–C(1) of iridoids. In addition, another olefinic H-atom (δ(H) 5.98 (br. *s*, H–C(7)) and four olefinic C-atom signals at δ(C) 153.5 (C(3)), 112.4 (C(4)), 131.8 (C(7)), and 139.8 (C(8)) were assigned to the iridoid moiety. The H-Atom signals at δ(H) 8.05 (*dd*, $J = 7.8$, 1.3, H–C(2‴,6‴)), 7.60 (*tt*, $J = 7.4$, 1.3, H–C(4‴)), and 7.48 (*t*, $J = 7.8$, H–C(3‴,5‴)), together with the C-atom signals at δ(C) 131.5 (C(1‴)), 130.6 (C(2‴,6‴)), 129.7 (C(3‴,5‴)), and 134.3 (C(4‴)) indicated the presence of a monosubstituted benzene ring. Furthermore, the HMBC correlations observed for

H–C(2‴,6‴) (δ(H) 8.05)/C(7‴) (δ(C) 167.8) revealed the presence of a benzoyl (Bz) moiety. The ^{13}C-NMR spectrum of **1** exhibited 28 C-atom signals, with ten from the iridoid moiety and seven from the Bz group. Of the remaining eleven signals, six could be assigned to a glucopyranosyl moiety, and another set of five signals could be assigned to an arabinopyranosyl moiety on the basis of the analysis of the ^{1}H,^{1}H-COSY and TOCSY experiments. After acid hydrolysis and derivatization of **1** by the method of *Hara et al.* [12][13], GC Analysis revealed the presence of D-glucose and L-arabinose.

Table 1. *^{1}H- and ^{13}C-NMR Data* (400 MHz for ^{1}H, CD_3OD) *of Compound* **1**. δ in ppm, *J* in Hz. Arbitrary atom numbering.

	δ(H)	δ(C)
H–C(1)	5.20 (*d*, *J* = 8.0)	98.7
H–C(3)	7.53 (*d*, *J* = 0.9)	153.5
C(4)	–	112.4
H–C(5)	3.22 (br. *t*, *J* = 7.7)	36.8
CH_2(6)	2.89 (*dd*, *J* = 16.6, 8.9), 2.24 (*dd*, *J* = 16.6, 8.9)	40.0
H–C(7)	5.98 (br. *s*)	131.8
C(8)	–	139.8
H–C(9)	2.80 (*t*, *J* = 8.0)	47.4
CH_2(10)	5.08 (br. *d*, *J* = 13.9), 5.02 (br. *d*, *J* = 14.0)	64.4
C(11)	–	170.8
H–C(1′)	4.72 (*d*, *J* = 7.8)	100.7
H–C(2′)	3.25 (overlapped)	74.8
H–C(3′)	3.37 (overlapped)	77.9
H–C(4′)	3.28 (overlapped)	71.8
H–C(5′)	3.49 (overlapped)	77.6
CH_2(6′)	4.05 (*dd*, *J* = 11.8, 1.9), 3.66 (*dd*, *J* = 11.9, 6.9)	69.8
H–C(1″)	4.26 (*d*, *J* = 6.8)	105.4
H–C(2″)	3.51 (overlapped)	72.4
H–C(3″)	3.44 (overlapped)	74.2
H–C(4″)	3.73 (*dd*, *J* = 3.2, 1.2)	69.4
CH_2(5″)	3.76 (*dd*, *J* = 12.2, 3.9), 3.42 (*m*)	66.7
C(1‴)	–	131.5
H–C(2‴,6‴)	8.05 (*dd*, *J* = 7.8, 1.3)	130.6
H–C(3‴,5‴)	7.48 (*t*, *J* = 7.8)	129.7
H–C(4‴)	7.60 (*tt*, *J* = 7.4, 1.3)	134.3
C(7‴)	–	167.8

Interpretation of the HSQC and HMBC spectra of **1** (*Fig. 1*) revealed the substitution pattern, and allowed the assignment of all ^{1}H- and ^{13}C-NMR signals (*Table 1*). The Bz group is located at C(10), due to the HMBC correlations of H_a–C(10) (δ(H) 5.08) and H_b–C(10) (5.02) with C(7‴) (δ(C) 167.8). The D-glucopyranosyl moiety is obviously attached to C(1) of the aglycone, due to the HMBC correlations H–C(1′) (δ(H) 4.72)/C(1) (δ(C) 98.7), and H–C(1) (δ(H) 5.20)/C(1′) (δ(C) 100.7). The L-arabinopyranosyl moiety is attached to C(6′) of D-glucopyranosyl moiety, according to the HMBC correlations H–C(1″) (δ(H) 4.26)/C(6′) (δ(C) 69.8) and of H_a–C(6′) (δ(H) 4.05)/C(1″) (δ(C) 105.4). The β-configuration for D-glucopyranosyl unit and the α-configuration for L-arabinopyranosyl unit were

established due to the large J(H–C(1′),H–C(2′)) and J(H–C(1″),H–C(2″)) coupling constants ($J = 7.8$ and $J = 6.8$ Hz). Therefore, the structure of compound **1** was deduced as 10-*O*-benzoyl-6′-*O*-α-L-arabino(1 → 6)-β-D-glucopyranosylgeniposidic acid.

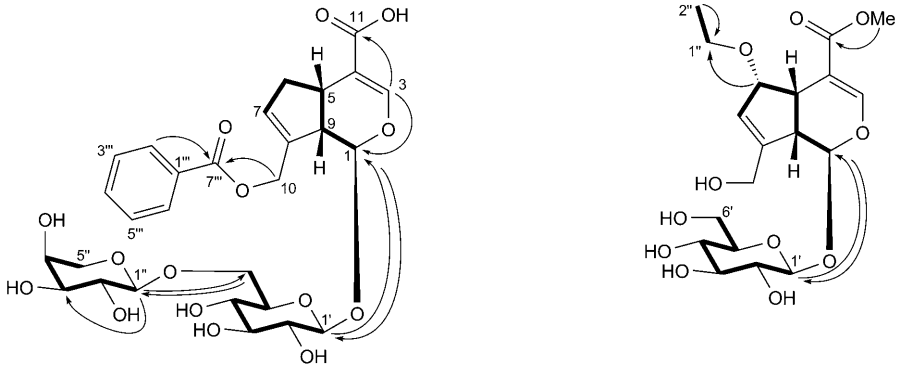


Fig. 1. *Structures and significant HMBC* (H → C) *and COSY* (—) *correlations of* **1** *and* **2**

Compound **2** was obtained as a yellowish-brown amorphous powder with a molecular formula of $C_{19}H_{28}O_{11}$. The ^{1}H- and ^{13}C-NMR data of **2** were identical to those of deacetylasperulosidic acid methyl ester [14], except for additional signals, *i.e.*, at δ(H) 3.49 (*dd*, $J = 13.0, 7.0$, H_a–C(1″)), 3.38 (*dd*, $J = 12.8, 6.7$, H_b–C(1″)), 1.04 (*t*, $J =$ 7.0, Me(2″)), and at δ(C) 66.1 (C(1″)), 15.9 (C(2″)), arising from an EtO group in **2**.

Interpretation of the HSQC and HMBC spectra of **2** (*Fig. 1*) allowed the assignment of all ^{1}H- and ^{13}C-NMR signals (*Table 2*). The EtO group is located at C(6), due to the HMBC correlations between H–C(6) (δ(H) 4.46) and C(1″) (δ(C) 66.1). Furthermore, the linkage could be confirmed by the significant obvious low-field shift of C(6) (δ(C) 83.3) of **2** compared to that of deacetylasperulosidic acid methyl ester (δ(C) 75.5). The relative configuration of H–C(6) was established as β from the correlation H–C(6)/H–C(9) in the ROESY experiment. The H-atom signals of H–C(6) (δ(H) 4.46 (*d*, $J = 7.8$)) further confirmed the above inference, which is consistent with the values found for 6α-butoxygeniposide [15]. The presence of D-glucose was revealed by acid hydrolysis, derivatization, and GC analysis. The β-configuration for D-glucopyranosyl was established due to its large J(H–C(1′), H–C(2′)) coupling constant ($J = 7.8$ Hz). Thus, **2** was deduced as deacetyl-6-ethoxy-asperulosidic acid methyl ester.

To find out whether compound **2** was an artefact of isolation, dried plant material (12 g) was refluxed twice with CH_2Cl_2 (2 × 250 ml) for 2 h each time. After evaporation under reduced pressure, the residue and compound **2** were analyzed by HPLC, respectively, eluted with 30% MeOH/H_2O. The absence of peak of compound **2** (t_R 13.8 min) in the HPLC chromatogram of CH_2Cl_2 extract indicated that compound **2** is an artefact.

In addition, 14 known iridoids were identified as monotropein methyl ester (galioside; **3**) [14], gardenoside (**4**) [16], 6-*O*-methyldeacetylasperulosidic acid methyl ester (**5**) [17], 4-epiborreriagenin (**6**) [18], (*E*)-6-*O*-*p*-coumaroylscandoside methyl

Table 2. *^{1}H- and ^{13}C-NMR Data* (400 MHz for ^{1}H, CD_3OD) *of Compound* **2**. δ in ppm, J in Hz. Arbitrary atom numbering.

	δ(H)	δ(C)
H–C(1)	5.01 (*d*, *J* = 8.8)	101.7
H–C(3)	7.60 (*d*, *J* = 1.2)	154.9
C(4)	–	108.5
H–C(5)	3.07 (*td*, *J* = 6.7, 1.4)	42.1
H–C(6)	4.46 (*d*, *J* = 7.8)	83.3
H–C(7)	6.11 (*d*, *J* = 1.8)	128.3
C(8)	–	152.1
H–C(9)	2.53 (*t*, *J* = 8.2)	46.0
CH_2(10)	4.45 (*d*, *J* = 14.8), 4.19 (*d*, *J* = 15.7)	61.7
C(11)	–	169.5
H–C(1′)	4.71 (*d*, *J* = 7.8)	100.7
H–C(2′)	3.23 (*t*, *J* = 8.9)	75.0
H–C(3′)	3.40–3.34 (*m*)	78.3
H–C(4′)	3.40–3.34 (*m*)	71.4
H–C(5′)	3.40–3.34 (*m*)	77.9
CH_2(6′)	3.81 (*dd*, *J* = 12.1, 2.1), 3.66 (*dd*, *J* = 12.1, 5.2)	62.6
MeO	3.73 (*s*)	51.8
CH_2(1″)	3.49 (*dd*, *J* = 13.0, 7.0), 3.38 (*dd*, *J* = 12.8, 6.7)	66.1
Me(2″)	1.04 (*t*, *J* = 7.0)	15.9

ester (**7**) [19], (*E*)-6-*O*-feruloylscandoside methyl ester (**8**) [19], (*Z*)-6-*O*-*p*-coumaroylscandoside methyl ester (**9**) [7], (*Z*)-6-*O*-feruloylscandoside methyl ester (**10**) [7], deacetylasperulosidic acid methyl ester (**11**) [14], deacetylasperuloside (**12**) [20], asperulosidic acid (**13**) [21], scandoside methyl ester (**14**) [14], asperuloside (**15**), and [22], asperulosidic acid methyl ester (**16**) [22] by direct comparison with authentic samples, and/or by comparison of various spectral and chemical data with those reported in the literature.

Biological Studies. Compounds **1**, **7**–**9**, **11**, and **13** were evaluated for the short-term-memory-enhancement activities by using the human Aβ42 transgenic fly AD model. The activities were indicated by the performance index (*PI*), as shown in *Fig. 2*.

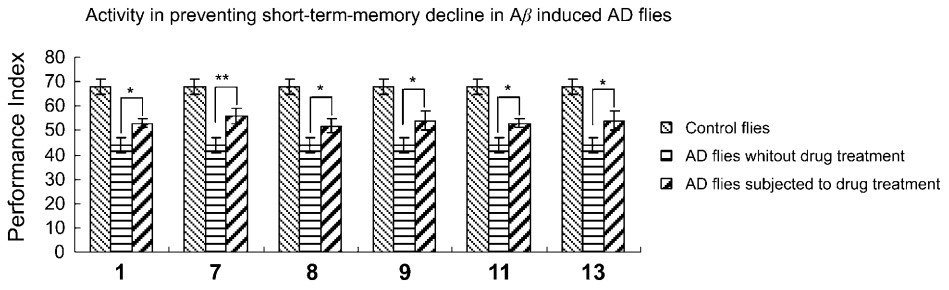


Fig. 2. *Performance index* (*PI*) *of AD flies fed with compounds. N* = 8 for all groups. Each value is the mean ± SE (SE refers to standard error), *: $P < 0.05$, and **: $P < 0.01$, compared with AD flies without drug group.

All six compounds showed potent activities, with compound **7** being the most active. Combined with our recent research on *Gardenia jasminoides* [23], this suggests that the iridoid glycosides could improve the short-term-memory capacity of Aβ42 transgenic flies, and might have a potential antagonism effect against *Alzheimer*'s disease.

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Experimental Part

General. Column chromatography (CC): *Diaion HP-20* (*Mitsubishi-Chemical*, Japan), silica gel (SiO_2; 100–200 mesh, *Qingdao Marine Chemical Ltd.*, China), octadecylsilanized (ODS) SiO_2 (*YMC Ltd.*, Japan), *Toyopearl HW-40* (*TOSOH Co.*, Japan), and *Sephadex LH-20* (*Amersham Pharmacia Biotech*, Sweden) columns. TLC: SiO_2 GF_{254} (*Yantai Chemical Inst.*, China) plates and visualized by spraying with conc. H_2SO_4/vanillin soln., followed by heating. Optical rotations: *JASCO P-1020* digital polarimeter. UV Spectra: *JASCO V-550* UV/VIS spectrometer; λ_{max} (log ε) in nm. IR Spectra: *JASCO FT/IR-480* plus spectrometer; $\tilde{\nu}$ in cm^{-1}. 1D and 2D NMR spectra: *Bruker AV-400* spectrometer at r.t.; CD_3OD solns.; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI-MS: *Finnigan LCQ Advantage MAX* mass spectrometer; in *m*/*z* (rel. %). HR-ESI-MS: *Micromass Q-TOF* mass spectrometer; in *m*/*z*.

Plant Material. The plant material was purchased from the *Qingping Market of Traditional Chinese Medicine*, Guangdong Province, China, in July 2008, and was identified as the whole plant of *H. diffusa* by Prof. *Danyan Zhang*, Guangzhou University of Traditional Chinese Medicine. A voucher specimen (20080731 HEDI) was deposited with the Institute of Traditional Chinese Medicine & Natural Products, College of Pharmacy, Jinan University, Guangzhou, China.

Extraction and Isolation. The whole plant (4.4 kg) of *H. diffusa* was refluxed twice with 60% aq. EtOH (2 × 40 l) for 2 h each time. After filtration, the filtrate was concentrated under reduced pressure. The concentrated soln. was suspended in H_2O, centrifuged, and passed through a macroporous resin *Diaion HP-20* column, successively eluted with H_2O, and EtOH/H_2O 1:1 and 95:5, to afford 381.1, 102.5, 68.4 g of extracts, resp. The EtOH/H_2O 1:1 eluate (72.5 g) was passed through a SiO_2 column, eluted with a stepwise gradient mixture of $CHCl_3$/MeOH 100:0, 95:5, 9:1, 8:2, 7:3, and 5:5, and finally with MeOH alone, to give ten fractions, *Frs. 1–10*. *Fr. 5* (1.5 g; eluted with $CHCl_3$/MeOH 9:1) was fractionated on *HW-40* CC, eluted with MeOH/H_2O in gradient, to yield four subfractions, *Frs. 5.1–5.4*. *Fr. 5.2* (eluted with 30% MeOH/H_2O) afforded compounds **2** (25.6 mg), **5** (17.3 mg), **6** (15.1 mg), **15** (529.4 mg), and **16** (66.2 mg) after purification by prep. ODS HPLC with 27% MeOH/H_2O as mobile phase. *Fr. 6* (8.2 g; eluted with $CHCl_3$/MeOH 8:2) was further fractionated by CC (ODS; MeOH/H_2O in gradient) to yield eight subfractions, *Frs. 6.1–6.8*. *Fr. 6.5* (eluted with 50% MeOH/H_2O) was subjected to CC (*HW-40*; MeOH/H_2O in gradient) to yield five subfractions, *Frs. 6.5.1–6.5.5*. *Subfr. 6.5.4* (eluted with 60% MeOH/H_2O) yielded compound **7** (4.8 g). *Subfr. 6.5.3* (eluted with 40% MeOH/H_2O) was purified by prep. HPLC (ODS; 45% MeOH/H_2O) to yield compounds **8** (211.9 mg), **9** (88.4 mg), and **10** (16.2 mg). *Fr. 7* (5.8 g; eluted with $CHCl_3$/MeOH 8:2) was fractionated by CC (ODS; MeOH/H_2O in gradient) to yield nine fractions, *Frs. 7.1–7.9*. *Fr. 7.2* (eluted with 30% MeOH/H_2O) was subjected to CC (*HW-40*; gradient MeOH/H_2O) to yield seven subfractions, *Frs. 7.2.1–7.2.7*. Compounds **3** (31.2 mg), **4** (75.6 mg), **11** (563.8 mg), **12** (188.7 mg), and **14** (94.3 mg) were obtained from *Fr. 7.2.5* (eluted with 30% MeOH/H_2O) after purification by prep. HPLC (ODS; 15% MeOH/H_2O); compound **13** (526.6 mg) was obtained from *Fr. 7.3* (eluted with 30% MeOH/H_2O) after purification by CC (*Sephadex LH-20*; with 50% MeOH/H_2O; and compound **1** (262.0 mg) was obtained from *Fr. 7.7* (eluted with 50% MeOH/H_2O) after purification by CC (*HW-40*; 40% MeOH/H_2O.

*10-*O*-Benzoyl-6′-*O*-α-*L*-arabino(1 → 6)-β-*D*-glucopyranosylgeniposidic Acid* (= *(1*S*,4a*S*,7a*S*)-7-[(Benzoyloxy)methyl]-1,4a,5,7a-tetrahydro-1-(6-*O*-α-*L*-arabinopyranosyl-β-*D*-glucopyranosyloxy)cyclo-*

penta[c]pyran-4-carboxylic Acid; **1**). Yellow amorphous solid. $[\alpha]_D^{18} = +0.8$ ($c = 0.5$, MeOH). UV (MeOH): 230.8 (3.82). IR (KBr): 3330, 1707, 1277, 1075, 716. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz): see *Table 1*. ESI-MS (pos.): 1243 ($[2M + Na]^+$), 633 ($[M + Na]^+$). ESI-MS (neg.): 609 ($[M - H]^-$). HR-ESI-MS (neg.): 609.1821 ($[M - H]^-$).

Deacetyl-6-ethoxyasperulosidic Acid Methyl Ester (= *Methyl (1S,4aS,5S,7aS)-5-Ethoxy-1-(β-*D*-glucopyranosyloxy)-1,4a,5,7a-tetrahydro-7-(hydroxymethyl)cyclopenta[c]pyran-4-carboxylate*; **2**). Yellowish-brown amorphous solid. $[\alpha]_D^{18} = +13.5$ ($c = 0.5$, MeOH). UV (MeOH): 237.2 (3.42). IR (KBr): 3381, 2925, 1703, 1632, 1439, 1384, 1158. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz): see *Table 2*. ESI-MS (pos.): 887 ($[2M + Na]^+$), 455 ($[M + Na]^+$). HR-ESI-MS (neg.): 431.1574 ($[M - H]^-$).

Acid Hydrolysis. Acid hydrolysis of **1** and **2** was performed according to the method of *Hara et al.* [12][13] to determine the absolute configuration of the monosaccharide. After hydrolysis with 1M HCl for 2 h at 80° and then further derivatization, the derivatives of **1** were analyzed by GC [13]. Two peaks were observed at t_R 15.43 (L-Ara) and 29.61 min (D-Glc), while the peaks of the mixed standard monosaccharide derivatives were recorded at t_R 15.81 (L-Ara), 19.63 (L-Rha), 29.67 (D-Glc), 32.21 (L-Glc), and 32.88 min (D-Gal). In addition, the peak of derivatives of **2** was observed at t_R 30.16 min (D-Glc).

Determination of Short-Term-Memory-Enhancement Activity. The bioassays were performed by using the method of *Yu et al.* [23]. The activities of compounds **1**, **7**–**9**, **11**, and **13** were indicated by the performance index (*PI*; see *Fig. 2*).

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