

Biologically Active Iridoids from *Hedyotis diffusa*

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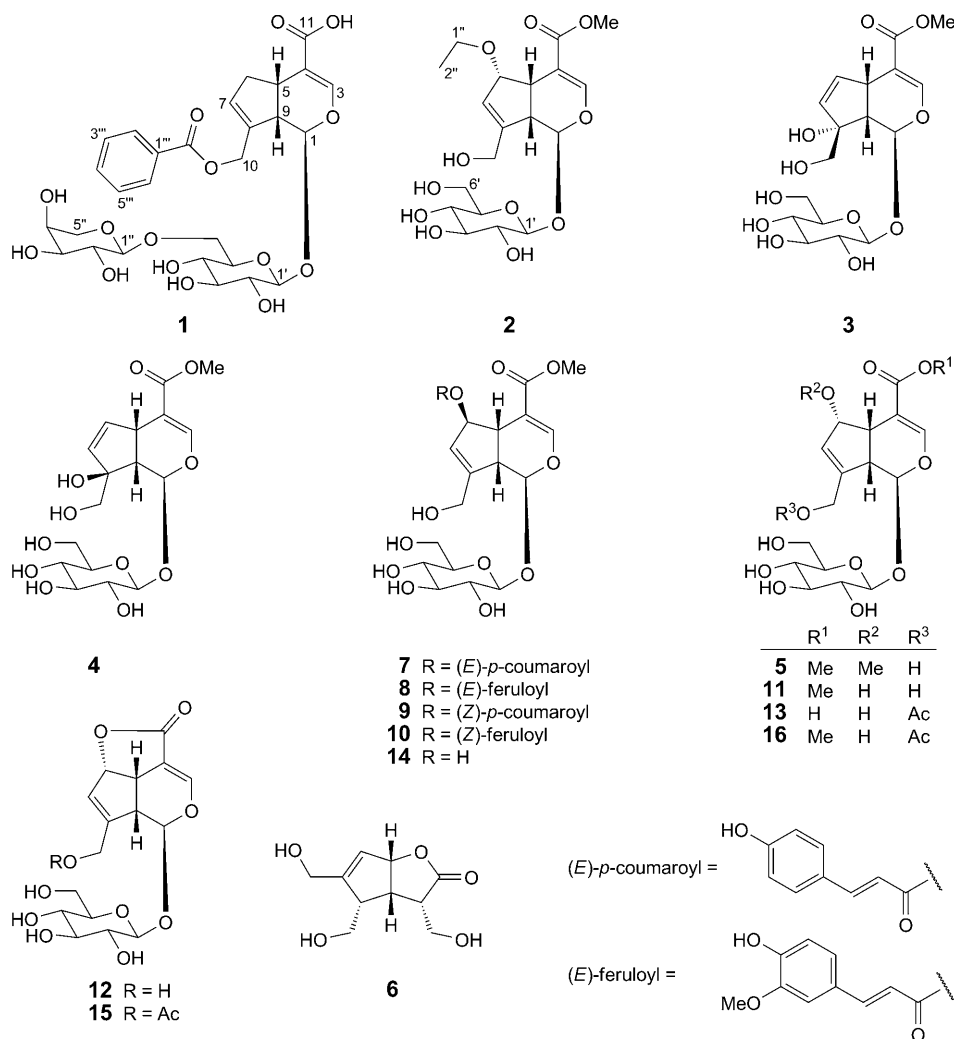
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Two new iridoids, 10-*O*-benzoyl-6'-*O*- α -L-arabino(1 $\rightarrow$ 6)- β -D-glucopyranosylgeniposidic acid (**1**), deacetyl-6-ethoxyasperulosidic acid methyl ester (**2**), along with 14 other known iridoids, were isolated from the whole plant 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, pelvisitis, 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 immunoregulation [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/H₂O extract of the whole plant of *H. diffusa* was chromatographed over macroporous resin HP-20 column, eluted with EtOH/H₂O in gradient. The 50% EtOH/H₂O 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.

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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 1H -NMR spectrum of **1** (Table I), the H-atom signals at $\delta(H)$ 7.53 ($d, J = 0.9, 1\text{ H}$) and 5.20 ($d, J = 8.0, 1\text{ H}$) were characteristic for H-C(3) and H-C(1) of iridoids. In addition, another olefinic H-atom ($\delta(H)$ 5.98 (br. s, H-C(7))) and four olefinic C-atom signals at $\delta(C)$ 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 $\delta(H)$ 8.05 ($dd, J = 7.8, 1.3, \text{H-C}(2''', 6''')$), 7.60 ($tt, J = 7.4, 1.3, \text{H-C}(4''')$), and 7.48 ($t, J = 7.8, \text{H-C}(3''', 5''')$), together with the C-atom signals at $\delta(C)$ 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''') ($\delta(\text{H})$ 8.05)/C(7''') ($\delta(\text{C})$ 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 ^1H , ^1H -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. ^1H - and ^{13}C -NMR Data (400 MHz for ^1H , CD_3OD) of Compound **1**. δ in ppm, J in Hz. Arbitrary atom numbering.

	$\delta(\text{H})$	$\delta(\text{C})$
H–C(1)	5.20 (<i>d</i> , $J = 8.0$)	98.7
H–C(3)	7.53 (<i>d</i> , $J = 0.9$)	153.5
C(4)	–	112.4
H–C(5)	3.22 (<i>br. t</i> , $J = 7.7$)	36.8
CH ₂ (6)	2.89 (<i>dd</i> , $J = 16.6, 8.9$), 2.24 (<i>dd</i> , $J = 16.6, 8.9$)	40.0
H–C(7)	5.98 (<i>br. s</i>)	131.8
C(8)	–	139.8
H–C(9)	2.80 (<i>t</i> , $J = 8.0$)	47.4
CH ₂ (10)	5.08 (<i>br. d</i> , $J = 13.9$), 5.02 (<i>br. d</i> , $J = 14.0$)	64.4
C(11)	–	170.8
H–C(1')	4.72 (<i>d</i> , $J = 7.8$)	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 (<i>dd</i> , $J = 11.8, 1.9$), 3.66 (<i>dd</i> , $J = 11.9, 6.9$)	69.8
H–C(1'')	4.26 (<i>d</i> , $J = 6.8$)	105.4
H–C(2'')	3.51 (overlapped)	72.4
H–C(3'')	3.44 (overlapped)	74.2
H–C(4'')	3.73 (<i>dd</i> , $J = 3.2, 1.2$)	69.4
CH ₂ (5'')	3.76 (<i>dd</i> , $J = 12.2, 3.9$), 3.42 (<i>m</i>)	66.7
C(1''')	–	131.5
H–C(2''',6''')	8.05 (<i>dd</i> , $J = 7.8, 1.3$)	130.6
H–C(3''',5''')	7.48 (<i>t</i> , $J = 7.8$)	129.7
H–C(4''')	7.60 (<i>tt</i> , $J = 7.4, 1.3$)	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 ^1H - 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) ($\delta(\text{H})$ 5.08) and H_b–C(10) (5.02) with C(7''') ($\delta(\text{C})$ 167.8). The D-glucopyranosyl moiety is obviously attached to C(1) of the aglycone, due to the HMBC correlations H–C(1') ($\delta(\text{H})$ 4.72)/C(1) ($\delta(\text{C})$ 98.7), and H–C(1) ($\delta(\text{H})$ 5.20)/C(1') ($\delta(\text{C})$ 100.7). The L-arabinopyranosyl moiety is attached to C(6') of D-glucopyranosyl moiety, according to the HMBC correlations H–C(1'') ($\delta(\text{H})$ 4.26)/C(6') ($\delta(\text{C})$ 69.8) and of H_a–C(6') ($\delta(\text{H})$ 4.05)/C(1'') ($\delta(\text{C})$ 105.4). The β -configuration for D-glucopyranosyl unit and the α -configuration for L-arabinopyranosyl unit were

established due to the large $J(\text{H}-\text{C}(1'), \text{H}-\text{C}(2'))$ and $J(\text{H}-\text{C}(1''), \text{H}-\text{C}(2''))$ 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 $\rightarrow$ 6)- β -D-glucopyranosylgeniposidic acid.

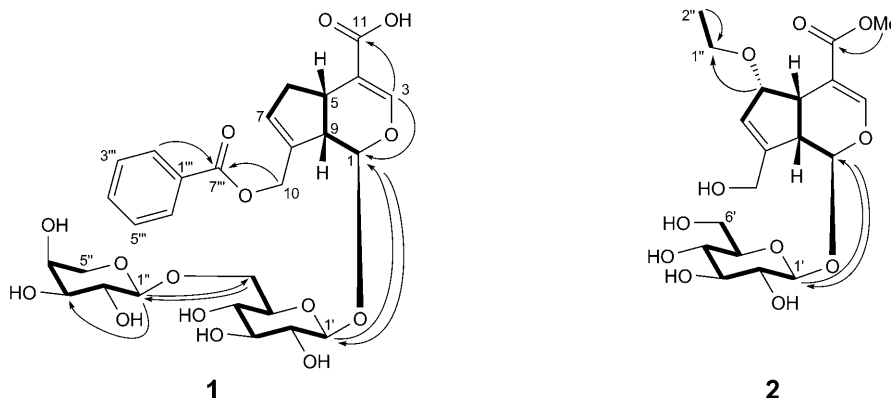


Fig. 1. Structures and significant HMBC ($\text{H} \rightarrow \text{C}$) and COSY ($\text{H} \rightarrow \text{H}$) correlations of **1** and **2**

Compound **2** was obtained as a yellowish-brown amorphous powder with a molecular formula of $\text{C}_{19}\text{H}_{28}\text{O}_{11}$. The ^1H - and ^{13}C -NMR data of **2** were identical to those of deacetylasperulosidic acid methyl ester [14], except for additional signals, *i.e.*, at $\delta(\text{H})$ 3.49 (*dd*, $J = 13.0, 7.0$, $\text{H}_a-\text{C}(1'')$), 3.38 (*dd*, $J = 12.8, 6.7$, $\text{H}_b-\text{C}(1'')$), 1.04 (*t*, $J = 7.0$, $\text{Me}(2'')$), and at $\delta(\text{C})$ 66.1 ($\text{C}(1'')$), 15.9 ($\text{C}(2'')$), arising from an EtO group in **2**.

Interpretation of the HSQC and HMBC spectra of **2** (Fig. 1) allowed the assignment of all ^1H - and ^{13}C -NMR signals (Table 2). The EtO group is located at C(6), due to the HMBC correlations between $\text{H}-\text{C}(6)$ ($\delta(\text{H})$ 4.46) and $\text{C}(1'')$ ($\delta(\text{C})$ 66.1). Furthermore, the linkage could be confirmed by the significant obvious low-field shift of C(6) ($\delta(\text{C})$ 83.3) of **2** compared to that of deacetylasperulosidic acid methyl ester ($\delta(\text{C})$ 75.5). The relative configuration of $\text{H}-\text{C}(6)$ was established as β from the correlation $\text{H}-\text{C}(6)/\text{H}-\text{C}(9)$ in the ROESY experiment. The H-atom signals of $\text{H}-\text{C}(6)$ ($\delta(\text{H})$ 4.46 (*d*, $J = 7.8$)) further confirmed the above inference, which is consistent with the values found for 6 α -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 $J(\text{H}-\text{C}(1'), \text{H}-\text{C}(2'))$ coupling constant ($J = 7.8$ Hz). Thus, **2** was deduced as deacetyl-6-ethoxyasperulosidic 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% $\text{MeOH}/\text{H}_2\text{O}$. 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. ^1H - and ^{13}C -NMR Data (400 MHz for ^1H , CD_3OD) of Compound **2**. δ in ppm, J in Hz. Arbitrary atom numbering.

	$\delta(\text{H})$	$\delta(\text{C})$
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 $\text{A}\beta_{42}$ transgenic fly AD model. The activities were indicated by the performance index (*PI*), as shown in Fig. 2.

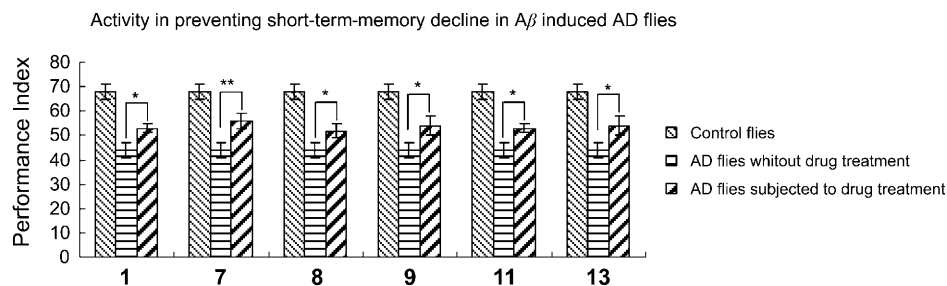


Fig. 2. Performance index (*PI*) of AD flies fed with compounds. $N = 8$ for all groups. Each value is the mean $\pm$ SE (SE refers to standard error), *: $P < 0.05$, and **: $P < 0.01$, compared with AD flies without drug group.

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 A β 42 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 (SiO₂; 100–200 mesh, *Qingdao Marine Chemical Ltd.*, China), octadecylsilylanized (ODS) SiO₂ (*YMC Ltd.*, Japan), *Toyopearl HW-40* (*TOSOH Co.*, Japan), and *Sephadex LH-20* (*Amersham Pharmacia Biotech*, Sweden) columns. TLC: SiO₂ *GF₂₅₄* (*Yantai Chemical Inst.*, China) plates and visualized by spraying with conc. H₂SO₄/vanillin soln., followed by heating. Optical rotations: *JASCO P-1020* digital polarimeter. UV Spectra: *JASCO V-550* UV/VIS spectrometer; $\lambda_{\max}$ (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.; CD₃OD solns.; δ in ppm rel. to Me₄Si 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 $\times$ 40 l) for 2 h each time. After filtration, the filtrate was concentrated under reduced pressure. The concentrated soln. was suspended in H₂O, centrifuged, and passed through a macroporous resin *Diaion HP-20* column, successively eluted with H₂O, and EtOH/H₂O 1 : 1 and 95 : 5, to afford 381.1, 102.5, 68.4 g of extracts, resp. The EtOH/H₂O 1 : 1 eluate (72.5 g) was passed through a SiO₂ column, eluted with a stepwise gradient mixture of CHCl₃/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 CHCl₃/MeOH 9 : 1) was fractionated on *HW-40* CC, eluted with MeOH/H₂O in gradient, to yield four subfractions, *Frs. 5.1–5.4*. *Fr. 5.2* (eluted with 30% MeOH/H₂O) 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/H₂O as mobile phase. *Fr. 6* (8.2 g; eluted with CHCl₃/MeOH 8 : 2) was further fractionated by CC (ODS; MeOH/H₂O in gradient) to yield eight subfractions, *Frs. 6.1–6.8*. *Fr. 6.5* (eluted with 50% MeOH/H₂O) was subjected to CC (*HW-40*; MeOH/H₂O in gradient) to yield five subfractions, *Frs. 6.5.1–6.5.5*. *Subfr. 6.5.4* (eluted with 60% MeOH/H₂O) yielded compound **7** (4.8 g). *Subfr. 6.5.3* (eluted with 40% MeOH/H₂O) was purified by prep. HPLC (ODS; 45% MeOH/H₂O) to yield compounds **8** (211.9 mg), **9** (88.4 mg), and **10** (16.2 mg). *Fr. 7* (5.8 g; eluted with CHCl₃/MeOH 8 : 2) was fractionated by CC (ODS; MeOH/H₂O in gradient) to yield nine fractions, *Frs. 7.1–7.9*. *Fr. 7.2* (eluted with 30% MeOH/H₂O) was subjected to CC (*HW-40*; gradient MeOH/H₂O) 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/H₂O) after purification by prep. HPLC (ODS; 15% MeOH/H₂O); compound **13** (526.6 mg) was obtained from *Fr. 7.3* (eluted with 30% MeOH/H₂O) after purification by CC (*Sephadex LH-20*; with 50% MeOH/H₂O); and compound **1** (262.0 mg) was obtained from *Fr. 7.7* (eluted with 50% MeOH/H₂O) after purification by CC (*HW-40*; 40% MeOH/H₂O).

10-O-Benzoyl-6'-O- α -L-arabino(1 $\rightarrow$ 6)- β -D-glucopyranosylgeniposidic Acid (= (1S,4aS,7aS)-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. $[\alpha]_D^{25} = +0.8$ ($c = 0.5$, MeOH). UV (MeOH): 230.8 (3.82). IR (KBr): 3330, 1707, 1277, 1075, 716. ^1H - (400 MHz) and ^{13}C -NMR (100 MHz): see Table 1. ESI-MS (pos.): 1243 ($[2M + \text{Na}]^+$), 633 ($[M + \text{Na}]^+$). ESI-MS (neg.): 609 ($[M - \text{H}]^-$). HR-ESI-MS (neg.): 609.1821 ($[M - \text{H}]^-$).

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. $[\alpha]_D^{25} = +13.5$ ($c = 0.5$, MeOH). UV (MeOH): 237.2 (3.42). IR (KBr): 3381, 2925, 1703, 1632, 1439, 1384, 1158. ^1H -NMR (400 MHz) and ^{13}C -NMR (100 MHz): see Table 2. ESI-MS (pos.): 887 ($[2M + \text{Na}]^+$), 455 ($[M + \text{Na}]^+$). HR-ESI-MS (neg.): 431.1574 ($[M - \text{H}]^-$).

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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