Characterization of Acetylcholinesterase Inhibitory Constituents from *Annona glabra* Assisted by HPLC Microfractionation

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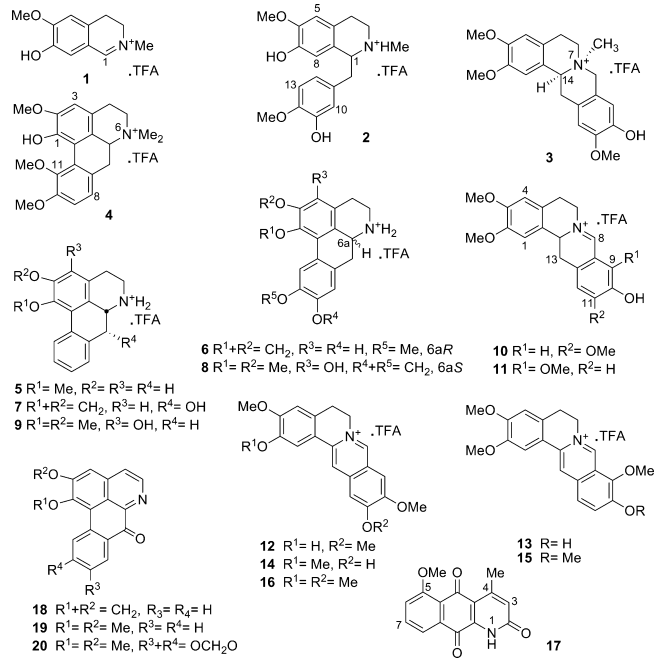
The active fraction of the EtOH extract of the stem of *Annona glabra* against acetylcholinesterase (AChE) was analyzed by combining HPLC microfractionation with a bioassay. The analytical-scale sample was fractionated by HPLC-DAD into 96-well microplates, which, after evaporation, were assayed against AChE. The active subfractions were scaled up by separation over semipreparative HPLC to give 20 compounds. Four of these, (7*S*,14*S*)-(−)-*N*-methyl-10-*O*-demethylxylopinine salt (**3**), *S*-(−)-7,8-didehydro-10-*O*-demethylxylopininium salt (**10**), *S*-(−)-7,8-didehydrocorydalminium salt (**11**), and 5-*O*-methylmarcanine D (**17**), were assigned as new natural products. In addition, compounds **10** and **11** represent the first natural occurrence of 7,8-didehydroprotoberberines. Compound **3**, pseudocolumbamine (**12**), palmatine (**15**), and pseudopalmatine (**16**) showed anti-AChE IC_{50} values of 8.4, 5.0, 0.4, and 1.8 μM, respectively.

One of the strategies for the treatment of Alzheimer's disease (AD) is based on the "cholinergic hypothesis", which suggests that the cause of memory impairment in AD patients is a deficit of cholinergic function in the hippocampal and cortical sections in the brain.[1−3] Inhibitors of acetylcholinesterase (AChE) can restore the level of acetylcholine in cholinergic synapses of the cerebral cortex,[4,5] leading to the improvement of cognitive function. Thus, AChE inhibitors are used as an effective clinical approach to treat AD. Up to now, only a few AChE inhibitors have been approved for such a purpose. However, the search for safer and more effective AChE inhibitors is still in great demand for the treatment of AD. Since natural products are versatile and often serve as drug leads for further development, a simple assay system has been applied to explore the potential of AChE inhibitors from higher plants. A preliminary study indicated the EtOH extract of the stem of *Annona glabra* L. (Annonaceae) to be active against AChE. Thus, this study was aimed to characterize the AChE inhibitors from the active fraction of this plant extract.

Annona glabra, generally known as "pond-apple", is a tropical tree that is native to America and southern Asia and cultivated in southern Taiwan. It is commonly used as an insecticide and parasiticide.[6,7] Many bioactive constituents have been isolated from *A. glabra*, such as dioxoaporphines, oxoaporphines, aporphines, protoberberines, azaanthraquinones, amides, and steroids.[8,9] To accelerate the search for new anti-AChE compounds, the combination of HPLC microfractionation and a bioassay was utilized.[10] Samples were used on an analytical scale and fractionated by RP-HPLC monitored with DAD into a 96-well microplate, with the residue in each well assayed against AChE. This combination thus provides a delivery system and retention time, UV−vis absorption, and ESIMS data for each active compound, facilitating their further scaled-up separation and structural characterization. Altogether, 20 compounds were isolated from *A. glabra*, of which four (**3**, **10**, **11**, **17**) are new.

Results and Discussion

Since *A. glabra* has been reported to contain alkaloids, a general fractionation procedure for the alkaloids was adopted, with the EtOH extract divided into an acid−$CHCl_3$-soluble fraction (Fr. I), total free bases (Fr. II), and an acid-insoluble fraction (Fr. III). Among these, the acid−$CHCl_3$-soluble fraction was found to be the most active against AChE (69% inhibition at 100 μg/mL). This fraction was purified further via centrifugal partition chromatography (CPC), followed by Sephadex LH-20 and silica gel column chromatography. The active subfractions in an analytical scale were microfractionated by RP-HPLC into 96-well plates, and the residue in each well was assayed against AChE. Figure S1 (Supporting Information) shows the inhibitory effect of each HPLC peak and the corresponding well. The compounds in the active wells identified by this approach were scaled up via semipreparative HPLC using a similar delivery system to that for the analytical scale but with higher flow rate and manual collection. This led to the isolation of 20 compounds (**1**−**20**), of which **3**, **10**, **11**, and **17** are new.



Compound **3**, characterized as a trifluoroacetate (TFA) salt, gave the molecular formula $C_{21}H_{26}NO_4$, as deduced from the HRESIMS. Its ^{1}H NMR spectrum (Table 1) displayed signals for four aryl protons (H-1, H-4, H-9, and H-12), each as a singlet, and signal for three aryl methoxy groups, three aliphatic protons (H_2-13 and H-14) as an AMX system, and two aliphatic protons of the same methylene (H_2-8) as an AX system, all characteristic for a 2,3,10,11-tetraoxygenated protoberberine.[11,12] This spectrum also revealed a methyl singlet at δ 3.21, which, when combined with the even proton number of the molecular formula, suggested the presence

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Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Data for Compounds **3**, **10**, and **11** (δ in ppm, mult., J in Hz) in CD_3OD

	3		**10**		**11**	
position	^{1}H	^{13}C[a]	^{1}H	^{13}C[a]	^{1}H	^{13}C[a]
1	6.87 s	111.2 d	6.99 s	110.6 d	6.99 s	110.7 d
2		150.1 s		150.4 s		150.4 s
3		151.3 s		150.4 s		150.4 s
4	6.90 s	113.4 d	6.89 s	112.8 d	6.89 s	112.8 d
4a		121.6 s		126.6 s		126.5 s
5	3.24 m (α) 3.30 m (β)	24.1 t	3.06 br dd (3.1, 16.0) (α); 3.23 ddd (4.8, 13.5, 16.0) (β)	29.6 t	3.08 ddd (2.9, 4.1, 17.1) (α); 3.26 ddd (4.1, 12.2, 17.1) (β)	29.4 t
6	3.47 m (α) 3.85 m (β)	52.7 t	4.06 ddd (3.1, 12.4, 13.5) (α); 4.40 ddd (3.1, 4.8, 12.4) (β)	56.3 t	4.12, m (α) 4.58 ddd (2.9, 4.1, 12.8) (β)	57.0 t
8	4.55 d (15.5) (α) 4.78 d (15.5) (β)	65.1 t	8.97 s	166.8 d	9.36 s	164.8 d
8a		119.1 s		118.8 s		119.7 s
9	6.64 s	113.8 d	7.27 s	119.9 d		151.8 s
10		147.7 s		148.2 s		150.7 s
11		150.0 s		158.1 s	7.29 d (8.1)	127.9 d
12	6.78 s	120.0 d	7.17 s	112.2 d	7.08 d (8.1)	124.1 d
12a		121.7 s		134.7 s		130.5 s
13	3.47 m (α) 3.12 dd (10.7, 18.4) (β)	35.6 t	3.66 dd (5.6, 16.7) (α) 3.07 dd (16.4, 16.7) (β)	35.7 t	3.61 dd (5.5 16.7) (α) 2.99 dd (16.5, 16.7) (β)	35.4 t
14	4.73 m	67.5 d	5.33 dd (5.6, 16.4)	59.3 d	5.32 dd (5.5, 16.5)	59.8 d
14a		125.5 s		125.6 s		125.4 s
OMe-2	3.84 s	56.5 q	3.85 s	56.5 q	3.85 s	56.7 q
OMe-3	3.84 s	56.5 d	3.87 s	56.7 q	3.86 s	56.5 q
OMe-9					4.01 s	62.5 q
OMe-11	3.83 s	56.7 q	4.04 s	57.2 q		
N^+Me	3.21 s	50.1 q				

[a] Multiplicities were obtained from DEPT experiments.

of a N^+Me group. The NOESY spectrum of **3** showed NOE relationships of OMe-2 (δ 3.84)/H-1 (δ 6.87)/H-14 (δ 4.73) and H-13α (δ 3.47); OMe-3 (δ 3.84)/H-4 (δ 6.90)/H-5α (δ 3.24); H-8α (δ 4.55)/H-9 (δ 6.64); and OMe-11 (δ 3.83)/H-12 (δ 6.78)/H-13α (δ 3.47), permitting the C-2, C-3, and C-11 positions to be assigned as methoxylated. The NOESY spectrum also indicated a NOE relationship between H-14 and N^+Me (δ 3.21), suggesting the *cis*-orientation for the ring junction. The CD spectrum of **3** showed a strong negative Cotton effect at 205 nm (H_2O), suggesting the typical 14*S*-configuration found among the protoberberine compounds.[13] These data when combined were used to establish compound **3** as (7*S*,14*S*)- *N*-methyl-10-*O*-demethylxylopinine salt.

Compounds **10** and **11**, again characterized as TFA salts, both gave the same molecular formula, $C_{20}H_{22}NO_4$, as deduced from HRESIMS. Their ^{1}H NMR spectra displayed signals for four aryl protons appearing as four singlets in **10** and two singlets and one AX system in **11**, a relative downfield shifted olefinic proton (s, δ 8.97 in **10** and δ 9.36 in **11**), three aryl methoxy group signals, an AMX system for three aliphatic protons (H_2-13 and H-14), and four aliphatic protons belonging to two methylene groups but lacking an AX system for H_2-8. These data suggested a common 7,8-didehydroprotoberberine skeleton for **10** and **11**, with **10** being 2,3,10,11-tetraoxygenated and **11** being 2,3,9,10-tetraoxygenated. The NOESY spectrum of **10** showed the correlations of OMe-2 (δ 3.85, s)/H-1 (δ 6.99, s)/H-14 (δ 5.33, dd) and H-13α (δ 3.66, dd); OMe-3 (δ 3.87, s)/H-4 (δ 6.89, s)/H-5α (δ 3.06, br dd); H-6β (δ 4.40, ddd)/H-8 (δ 8.97, s)/H-9 (δ 7.27, s); and OMe-11 (δ 4.04, s)/H-12 (δ 7.17, s)/H-13α (δ 3.66, dd), designating the OMe-2, OMe-3, and OMe-11 substitutions and an iminium function in **10** (Figure 1). The NOESY spectrum of **11** showed similar NOE relationships for the protons located in rings A−C, but different correlations between those protons in rings C and D. Thus, H-8 (δ 9.36, s) was correlated further to a methoxy group at δ 4.01 (s), aiding in the designations of the OMe-2, OMe-3, and OMe-9 substituents, and an iminium function in **11**. The CD spectra of **10** and **11** both showed a strong negative Cotton effect at 205 nm (H_2O), suggesting the 14*S*-configuration.[13] These data were used to establish compounds **10** and **11** as *S*-(−)-7,8-didehydro-10-*O*-demethylxylopininium salt and *S*-(−)-7,8-didehydrocorydalminium salt, respectively. The ^{1}H and ^{13}C NMR assignments for compounds **3**, **10**, and **11** (Table 1) were made from the analysis of 2D NMR spectra (COSY, NOESY, HMQC, and HMBC).

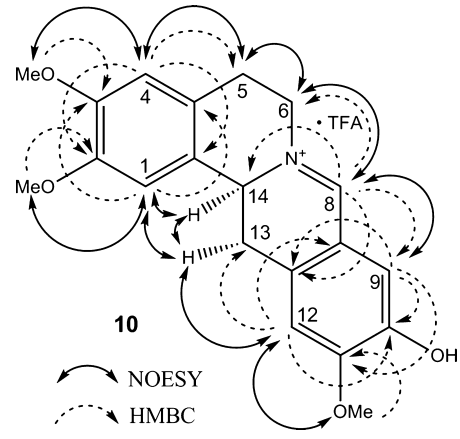

Figure 1. Key NOESY and HMBC correlations of **10**.

Up to the present time, only the natural 13,14-didehydroprotoberberines have been reported.[14] The occurrence of the 7,8-didehydroprotoberberines **10** and **11** in *A. glabra* represents the first report of such protoberberine analogues in nature, although some synthetic analogues of this type have been reported.[15,16]

Compound **17** gave the molecular formula $C_{15}H_{11}NO_4$, as deduced from HRESIMS, showing the quasi-molecular ion $[M + Na]^+$ at *m/z* 292.0595. The ^{1}H NMR spectrum of **17** ($CDCl_3$) exhibited signals for four aryl protons, three of which appear as an ABX-like system (δ 7.87, br d, J = 7.8 Hz; δ 7.78, dd, J = 7.8, 8.4 Hz; δ 7.31, br d, J = 8.4 Hz) and the other as a quartet (δ 6.61, J = 1.0 Hz), an arylmethyl group (δ 2.66, d, J = 1.0 Hz), and an arylmethoxy group (δ 4.05, s). These values are similar to the ^{1}H NMR data of marcanine A and marcanine D,[17] both 1-azaanthraquinones isolated from the same plant family (Annonaceae) as **17**. The coupling pattern of the ring C protons in **17** suggested the methoxy group to be located at either the C-5 or C-8 position. The NOESY spectrum was supportive of this in showing

Table 2. IC_{50} Values (μM) of Compounds **1**−**20** against Acetylcholinesterase

compound	IC_{50}	compound	IC_{50}	compound	IC_{50}
1	16.0 ± 0.6	**8**	76.9 ± 1.1	**15**	0.4 ± 0.0
2	72.2 ± 2.8	**9**	109.5 ± 2.5	**16**	1.8 ± 0.1
3	8.4 ± 0.5	**10**	153.9 ± 0.2	**17**	23.4 ± 0.4
4	>140.0	**11**	30.8 ± 0.8	**18**	>150.0
5	123.4 ± 2.0	**12**	5.0 ± 0.4	**19**	16.1 ± 1.5
6	>117.0	**13**	96.5 ± 0.9	**20**	>150.0
7	>126.0	**14**	97.6 ± 0.6	galanthamine	2.0 ± 0.1

NOE correlations of the OMe signal to the aryl proton at δ 7.31 (br d) and of the methyl group at δ 2.66 (Me-4) to the aryl proton at δ 6.61 (H-3). Since the proton signals in ring C of **17**, δ 7.31 (H-6) < δ 7.78 (H-7) < δ 7.87 (H-8), were found to be similar in chemical shifts and shift order to the corresponding signals in marcanine D (3-methoxy-5-hydroxy-4-methyl-1*H*-1-aza-2,9,10-anthracenetrione), δ 7.36 (H-6) < δ 7.62 (H-7) < δ 7.73 (H-8) ($CDCl_3$), but not to those in marcanine E (3-methoxy-8-hydroxy-*N*,4-dimethyl-1-aza-2,9,10-anthracenetrione), δ 7.26 (H-7) < δ 7.62 (H-5) < δ 7.77 (H-6) (acetone-d_6),[17] this methoxy group was designated as OMe-5. Accordingly, compound **17** was established as 5-*O*-methylmarcanine D. This compound was found not very stable, and the confirmation of the substitution at C-5 requires more material to afford decent HMBC data for such a purpose.

Compound **1** is a simple quaternary isoquinoline, isolated as a TFA salt, and was identified as pycnarrhine by comparison of its spectroscopic data with those reported.[18] Compounds **2** and **4**−**9** are free bases and were isolated as trifluoroacetic acid (TFAH) salts. They were identified as (+)-reticuline (**2**),[19] *S*-(+)-*N*-methylcorydine (**4**),[20] *R*-(−)-asimilobine (**5**),[21] *R*-(−)-actinodaphnine (**6**),[22] *R*-(−)-norushinsunine (**7**),[23] *S*-(+)-3-hydroxynornantenine (**8**),[24]and *R*-(−)-3-hydroxynornuciferine (**9**)[25] by their characteristic ^{1}H NMR and MS data and the respective optical properties. Compounds **12**−**16** are berberine-type alkaloids and were isolated as TFA salts. They were identified as pseudocolumbamine (**12**),[26] dehydrocorydalmine (**13**),[27] dehydrocorytenchine (**14**),[28] palmatine (**15**),[29] and pseudopalmatine (**16**)[30] by comparison of their ^{1}H NMR and MS data to the reported data in the literature. Compounds **18**−**20** are oxoaporphines and were identified as liriodenine,[8] lysicamine,[8] and oxonantenine,[31] respectively. Among these known compounds, **1**, **4**, **6**−**9**, **12**, **14**−**16**, and **20** were isolated for the first time from *A. glabra*.

The IC_{50} values of these isolated compounds against acetylcholinesterase were measured and are shown in Table 2. The results indicate that the berberine-type compounds, inclusive of palmatine (**15**), pseudopalmatine (**16**), pseudocolumbamine (**12**), and compound **3**, exhibited IC_{50} values of 0.4, 1.8, 5.0, and 8.4 μM, respectively. It is noted that the 2,3,9,10-tetrasubstituted berberines and 7,8-didehydro-protoberberines showed better activity than the corresponding 2,3,10,11-tetrasubstituted isomers, e.g., **15** > **16**, and **11** > **10**. In addition, for berberines possessing the same substitution pattern, those with less phenolic functions and more methoxy groups were found to have more potent inhibitory activity (Table 2). As for the remaining compounds, only the simple isoquinoline, **3**, the azaanthraquinone, **17**, and the oxoaporphine, **19**, showed some activity, with IC_{50} values around 20 μM.

Previous studies have demonstrated the protoberberines, epiberberine, palmatine, pseudodehydrocorydaline, berberine, and pseudocoptisine to be active against mouse cortex AChE, with IC_{50} values less than ca. 10 μM.[32,33] However, the IC_{50} value of palmatine (**15**) in this study against the AChE type VI-S from electric eel is less than in previous reports (IC_{50} 0.51 μM,[34] 5.8 μM,[32] and 10.4 μM,[33] and the positive controls, eserine 0.02 μM,[34] tacrine 0.2 μM,[32] 0.17 μM[35]) and may be attributable to assay conditions, such as the concentrations of enzyme and substrate and the source of enzyme.

The present study has shown that HPLC microfractionation and a bioassay combined approach not only speed up the recognition of bioactive compounds from a plant extract but also reduce the sample size used for experimentation. Although the HPLC-AChE assay cannot offer precise IC_{50} values ascribable to the small amounts of separated bioactive ingredients, the advantage of this approach, in separating and detecting active compounds simultaneously, is efficient for screening plant extracts. In addition, it provides an easy follow-up isolation and purification process, facilitating a larger scale separation and subsequent structure elucidation and the IC_{50} determinations of the purified bioactive compounds.

Experimental Section

General Experimental Procedures. Optical rotations were recorded using a JASCO DIP-370 polarimeter, and CD spectra were measured in a JASCO-720 spectropolarimeter. UV spectra were measured in MeOH using a Hitachi 150-20 double-beam spectrophotometer. ^{1}H, ^{13}C, and 2D NMR spectra were obtained on a Bruker AV400 and a Bruker AV600 spectrometer (methanol-d_4, δ_H 3.30 and δ_C 49.0 ppm) using standard pulse programs. TLC analyses were carried out on silica gel plates (KG_{60}-F254, Merck). Centrifugal partition chromatography was carried out on a Sanki CPC instrument (model LLB-M, 110 mL). Analytical and semipreparative HPLC were performed on an Agilent 1100 HPLC system, coupled with a diode array detector (G1315A) and an automated fraction collector (Gilson FC 204, Middleton, WI). Two HPLC delivery programs were applied, program A: MeOH−0.1% TFA_{aq} 5% to 36% in 20 min, 36% to 64% in 60 min, 64% to 95% in 15 min, all linear gradient; program B: MeOH−H_2O (55:45) for 90 min. HPLC analysis was performed on an RP-18 column (Phenomenex Prodigy ODS-3, 250 × 4.6 mm, 5 μm), eluted by delivery system A or B, sampling 10 μL, flow rate 0.5 mL/min, and monitored at UV 254, 280, 365, and 400 nm. Semipreparative HPLC was performed on an RP-18 column (Phenomenex Prodigy ODS-3, 250 × 10 mm, 5 μm). MS data were measured on an Esquire 2000 ion trap mass spectrometer (Bruker Daltonik) with an electrospray ion source, and the HRESIMS data were measured on a micrOTOF orthogonal ESI-TOF mass spectrometer (Bruker, Daltonik, Bremen, Germany). A SPECTRAmax PLUS microplate spectrophotometer (Molecular Devices) was used for visualization of the enzyme activity assay.

Plant Material. The stems of *Annona glabra* were collected at Taipei Botanical Garden, Taipei, Taiwan, in March 2006. A voucher specimen (NTUSP200603) was authenticated by Mr. Lu, Sheng-You, Associate Researcher, Institute of Taiwan Forestry Research Institute, Taipei, Taiwan, and was deposited in the herbarium of this institute.

Extraction and Isolation. An EtOH extract (817 g) of the stem wood (12 kg) of *A. glabra* was triturated with 3% acetic acid (2 L × 2) and filtered. The filtrate was partitioned against $CHCl_3$ (1 L × 3). The combined organic layers, after drying over Na_2SO_4, were evaporated under reduced pressure to give a residue (Fr. I, 3.34 g). The aqueous layer was basified with 25% NH_4OH to pH 9.0 and filtered to give a precipitate (Fr. Ip, 7.8 g). The filtrate was partitioned against $CHCl_3$ (1 L × 3). The combined $CHCl_3$ layers after drying over Na_2SO_4 were evaporated under reduced pressure to give the total free bases (Fr. II, 2.87 g). Fr. I (3.30 g) was fractionated using CPC, using the lower and upper layers of $CHCl_3$−MeOH−H_2O (10:10:5) as the stationary and mobile phases, respectively, to give five subfractions. Fr. I-4 (631 mg) was subjected to Sephadex LH-20 column chromatography (high 90 cm, o.d. 1.1 cm; MeOH−$CHCl_3$, 7:3) to give five subfractions. Fr. I-4-4 (129 mg) was chromatographed on a silica gel column (5.0 g, 230−400 mesh), eluted with 0.8−2% MeOH−$CHCl_3$, saturated with 25% NH_4OH, to give three further subfractions (Fr. I-4-4-1−3), combined on the basis of TLC examination. Subfr. 2 (33.3 mg) was recrystallized with $CHCl_3$ to give crystalline **18** (7.3 mg). The mother liquor was evaporated to give a residue (Fr. I-4-4-2m, 25.2 mg).

Analytical-Scale HPLC Microfractionation. Fr. I-2 (10 μL, conc. 20 mg/mL), Fr. I-4-2 (10 μL, conc 10 mg/mL), and Fr. I-4-4-2m (10 μL, 5 mg/mL) were microfractionated by an RP-18 HPLC column, eluted by the delivery program A for Fr. I-2 and Fr. I-4-2 or the delivery program B for Fr. I-4-4-2m, and other conditions as indicated above, into a 96-well plate via a Gilson 204 robotic fraction collector, each well collected at 0.63 min, i.e., 315 μL per well. Galanthamine, the positive control for AChE inhibitory assay, in 10% MeOH (final conc 0.04, 0.4, 4.0 μM), was added to the well at the initiation and the end of the chromatography. The AChE inhibitory assay was followed after

evaporation of the solvent in microplates at 45 °C with a SpeedVac concentrator (ThermoSavant, Holbrook, NY).

Separation of Compounds 1−20 by Semipreparative HPLC. Fr. I-2 (77 mg) was subjected to semipreparative HPLC with a flow rate of 2.5 mL/min, eluted by delivery program A to give nine compounds (**1**, 0.1 mg; **2**, 2.6 mg; **3**, 0.9 mg; **4**, 2.5 mg; **5**, 10.4 mg; **6**, 0.5 mg; **7**, 10.0 mg; **8**, 1.8 mg; **9**, 1.1 mg). Separated with the same semipreparative HPLC system, Fr. I-4-2 (13.5 mg) yielded seven compounds (**10**, 0.8 mg; **11**, 1.4 mg; **12**, 0.6 mg; **13**, 0.9 mg; **14**, 0.1 mg; **15**, 0.6 mg; **16**, 2.2 mg). Fr. I-4-4-2m (10.1 mg) was subjected to semipreparative HPLC with a flow rate of 2.5 mL/min, eluted by delivery program B, to give four compounds (**17**, 0.3 mg; **18**, 1.3 mg; **19**, 0.6 mg; **20**, 0.8 mg).

The retention times of these isolated compounds using the analytical RP-18 HPLC were as follows: 16.4 min (**1**), 28.4 min (**2**), 29.2 min (**3**), 30.4 min (**4**), 37.7 min (**5**), 40.6 min (**6**), 41.4 min (**7**), 51.9 min (**8**), 54.2 min (**9**), 39.0 min (**10**), 40.8 min (**11**), 45.2 min (**12**), 45.7 min (**13**), 48.6 min (**14**), 50.1 min (**15**), 50.7 min (**16**), eluted by delivery program A; 17.6 min (**17**), 35.6 min (**18**), 57.2 min (**19**), and 62.1 min (**20**), eluted by delivery program B.

(7*S*,14*S*)-(−)-7-Methyl-10-*O*-demethylxylopinine trifluoroacetate (3): $[\alpha]^{25}_{D}$ −88.9 (*c* 0.09, MeOH); UV λ_{max} (MeOH) (log ε) 229 (4.47), 284 (4.19) nm; CD (H_2O, *c* 2.13 × 10^{-4} M) $\Delta\varepsilon_{283}$ +0.11, $\Delta\varepsilon_{228}$ −2.73, $\Delta\varepsilon_{224}$ +0.89 (max), $\Delta\varepsilon_{223}$ +0.72 (sh), $\Delta\varepsilon_{206}$ −14.85; ^{1}H and ^{13}C NMR data, see Table 2; (+)ESIMS *m*/*z* 356 (100, [M − TFA]$^+$); (+)HRESIMS *m*/*z* 356.1856 [M − TFA]$^+$ (calcd for $C_{21}H_{26}NO_4$, 356.1856).

***S*-(−)-7,8-Didehydro-10-*O*-demethylxylopinine trifluoroacetate (10):** $[\alpha]^{24}_{D}$ −66 (*c* 0.03, MeOH); UV λ_{max} (MeOH) (log ε) 252 (3.43), 314 (3.21), 365 (2.96) nm; CD (H_2O, *c* 2.21 × 10^{-4} M) $\Delta\varepsilon_{364}$ −0.08, $\Delta\varepsilon_{316}$ +2.08 (max), $\Delta\varepsilon_{280}$ +0.52, $\Delta\varepsilon_{250}$ +1.48, $\Delta\varepsilon_{231}$ −2.48, $\Delta\varepsilon_{218}$ +0.36, $\Delta\varepsilon_{205}$ −8.28; ^{1}H and ^{13}C NMR data, see Table 2; NOESY and HMBC, see Figure 1; (+)ESIMS *m*/*z* 340.1 (100, [M − TFA]$^+$); (+)HRESIMS *m*/*z* 340.1552 [M − TFA]$^+$ (calcd for $C_{20}H_{22}NO_4$, 340.1543).

***S*-(−)-7,8-Didehydrocorydalmine trifluoroacetate (11):** $[\alpha]^{24}_{D}$ −100 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 282 (3.13), 307 (3.71) nm; CD (H_2O, *c* 2.21 × 10^{-4} M) $\Delta\varepsilon_{341}$ 0, $\Delta\varepsilon_{307}$ +2.53 (max), $\Delta\varepsilon_{277}$ +0.40, $\Delta\varepsilon_{244}$ −0.07, $\Delta\varepsilon_{224}$ −4.38, $\Delta\varepsilon_{218}$ −3.97 (sh), $\Delta\varepsilon_{205}$ −17.61; ^{1}H and ^{13}C NMR data, see Table 2; (+)ESIMS *m*/*z* 340.1 (100, [M − TFA]$^+$); (+)HRESIMS *m*/*z* 340.1551 [M − TFA]$^+$ (calcd for $C_{20}H_{22}NO_4$, 340.1543).

5-*O*-Methylmarcanine D (17): UV λ_{max} (MeOH) (log ε) 260 (3.84), 296 (3.75), 406 (3.29) nm; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 2.66 (1H, d, J = 1.0 Hz, Me-4), 4.05 (3H, s, OMe-5), 6.61 (H, q, J = 1.0 Hz, H-3), 7.31 (1H, d, J = 8.4 Hz, H-6), 7.78 (1H, dd, J = 7.8, 8.4 Hz, H-7), 7.87 (1H, d, J = 7.8 Hz, H-8); (+)ESIMS *m*/*z* 270 (100, [M + H]$^+$); (+)HRESIMS *m*/*z* 292.0595 [M + Na]$^+$ (calcd for $C_{15}H_{11}NO_4$+Na, 292.0580).

Acetylcholinesterase Inhibitory Assay. The inhibitory assay against AChE was based on Ellman's reagent and was modified to measure the activity in a 96-well plate.[10,35] In the 96-well plate, three regents, including 10 μL of 10% $MeOH_{aq}$ or sample in 10%$MeOH_{aq}$, 13 μL of 2.5 mM acetylthiocholine iodide (ATCI) in water, and 64 μL of 3 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in Tris-HCl containing 0.1 M NaCl, were added into each well. The reaction was started when 13 μL of 0.22 U/mL AChE (EC 3.1.1.7, type VI-S from electric eel, Sigma in 50 mM Tris-HCl containing 0.1% bovine serum albumin) was added to the 96-well plate. Due to the spontaneous hydrolysis of the substrate, yellow products were generated, and their absorbance was measured at 405 nm for 10 min with 2 min intervals on a microplate spectrophotometer. Compared to the absorbance value (A) of the blank ($A_{control}$), the inhibitory percentage against AChE (%) could be calculated for the test sample (A_{sample}) with an equation: Inhibition (%) = [1 − (A_{sample}/$A_{control}$)] × 100. Galanthamine was used as a positive control.

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Supporting Information Available: Analytical-scale HPLC microfractionation combined with AChE assay (S1); ^{1}H, ^{13}C, and 2D NMR spectra of **3**, **10**, **11**, and **17** (S2−S18); and UV, $[\alpha]^{25}_{D}$, CD, ^{1}H NMR, and ESIMS data of **1**, **2**, **4**−**9**, **12**−**16**, and **18**−**20** (S19). These materials are available free of charge via the Internet at http://pubs.acs.org.

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