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Comprehensive Study of Alkaloids from *Crinum asiaticum* var. *sinicum* Assisted by HPLC-DAD-SPE-NMR

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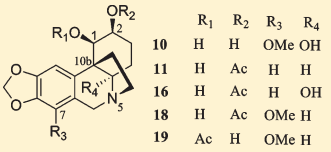
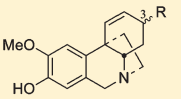
Supporting Information

ABSTRACT: A comprehensive study of the alkaloids present in the leaves of *Crinum asiaticum* var. *sinicum*, assisted by HPLC-SPE-NMR, led to the characterization of 21 compounds of similar polarity on an analytical scale. Thirteen of these were isolated for further structural confirmation. Seven are proved to be new, namely, (+)-siculine (**4**), 1-epijosephinine (**11**), 7-methoxycrinamabine (**10**), 2-*O*-acetylcrinamabine (**16**), 3-*O*-acetyl-8-*O*-demethylmaritidine (**17**), 2-*O*-acetylbulbisine (**18**), and 1-*O*-acetylbulbisine (**19**). In addition, dihydrovittatine (**6**) and 8-*O*-demethyloxomaritidine (**21**) were isolated for the first time from Nature, although they have been prepared previously as synthetic products. Their structures were established by spectroscopic analysis.



	R_1	R_2	R_3	R_4
10	H	H	OMe	OH
11	H	Ac	H	H
16	H	Ac	H	OH
18	H	Ac	OMe	H
19	Ac	H	OMe	H

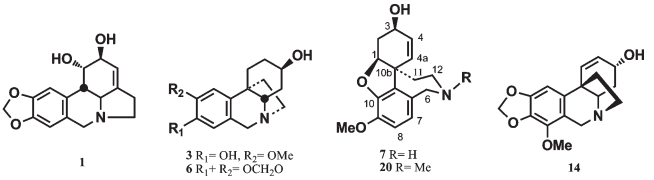


	R
4	α-OH
17	β-OAc
21	=O

The development of LC-NMR methods has allowed the analysis of natural products more efficiently in complicated plant extracts at the submilligram level.[1,2] Moreover, the availability of online solid-phase extraction (SPE) cartridges to be placed between HPLC and NMR instrumentations has enhanced significantly the sensitivity of this technique, making it a very useful tool in identifying metabolites, natural products, and drug impurities.[3,4] This tool has been applied successfully in characterizing natural products such as diarylbutane-type lignans of great structural similarity, present in *Phyllanthus urinaria*,[5] minor isoquinoline *N*-oxides from the leaves of *Neolitsea sericea* var. *aurata*,[6] and metabolites such as paracetamol[7] and dicentrine.[8] A preliminary HPLC analysis of the Amaryllidaceous alkaloids present in *Crinum asiaticum* L. var. *sinicum* (Amaryllidaceae) indicated that the number of alkaloids appearing in the HPLC chromatogram was much more than the four previously reported from the bulbs of the same plant.[9] These components, however, were almost nonresolvable by TLC analysis, and thus their separation would be not easy using general chromatographic methods. Alkaloids from related plants have been demonstrated to possess potent bioactivities, such as the antiviral narciclasine[10] and the antiacetylcholinesterase agent assoanine.[11] With our interest in exploring the potential of such alkaloids, the HPLC-DAD-SPE-NMR technique was applied in this study to facilitate and accelerate the structure characterization of this complex alkaloidal mixture. The following describes the outcome of this effort.

RESULTS AND DISCUSSION

The EtOH extract of the aerial parts of *C. asiaticum* var. *sinicum* was fractionated in a general manner to give non-phenolic and phenolic bases. During the alkalization process, the major component, lycorine (**1**), was precipitated out as a crystalline solid. Fractionation of the non-phenolic bases via centrifugal partition chromatography (CPC) yielded 12 fractions, with seven of them being rich in alkaloids and individually subjected to RP-18 HPLC analysis. The optimal HPLC solvent pairs for fractions I−III and V were found to be combinations of MeOH−H_2O, while those for fractions IV, VI, and VII were combinations of MeCN−H_2O, all containing 0.1% trifluoroacetic acid (TFA) (Figure 1 and Figure S1, Supporting Information). Using these HPLC conditions, 21 alkaloids (**1**−**21**) in total were separated and characterized from the nonphenolic bases using the HPLC-SPE-NMR (400 MHz) hyphenated technique (Figure 2, and Figures S2 and S3, Supporting Information). Following similar approaches, two alkaloids (**2** and **3**) were characterized from the phenolic bases (Figure S1, Supporting Information). For structure confirmation, 12 compounds (**2**, **4**−**6**, **8**, **11**, **13**, **14**, **16**−**19**) in addition to **1** were isolated by semipreparative RP-18 HPLC.

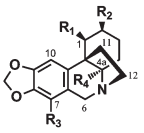


1

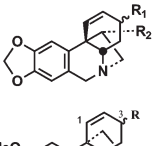
3 R_1= OH, R_2= OMe
6 R_1+ R_2= OCH_2O

7 R= H
20 R= Me

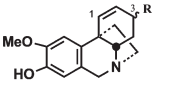
14



	R_1	R_2	R_3	R_4
5	OH	OH	H	H
8	OH	OH	OMe	H
10	OH	OH	OMe	OH
11	OH	OAc	H	H
15	OH	OH	H	OH
16	OH	OAc	H	OH
18	OH	OAc	OMe	H
19	OAc	OH	OMe	H



	R_1	R_2
9	α-OH	OH
12	β-OH	H
13	α-OH	H



	R
2	β-OH
4	α-OH
17	β-OAc
21	=O

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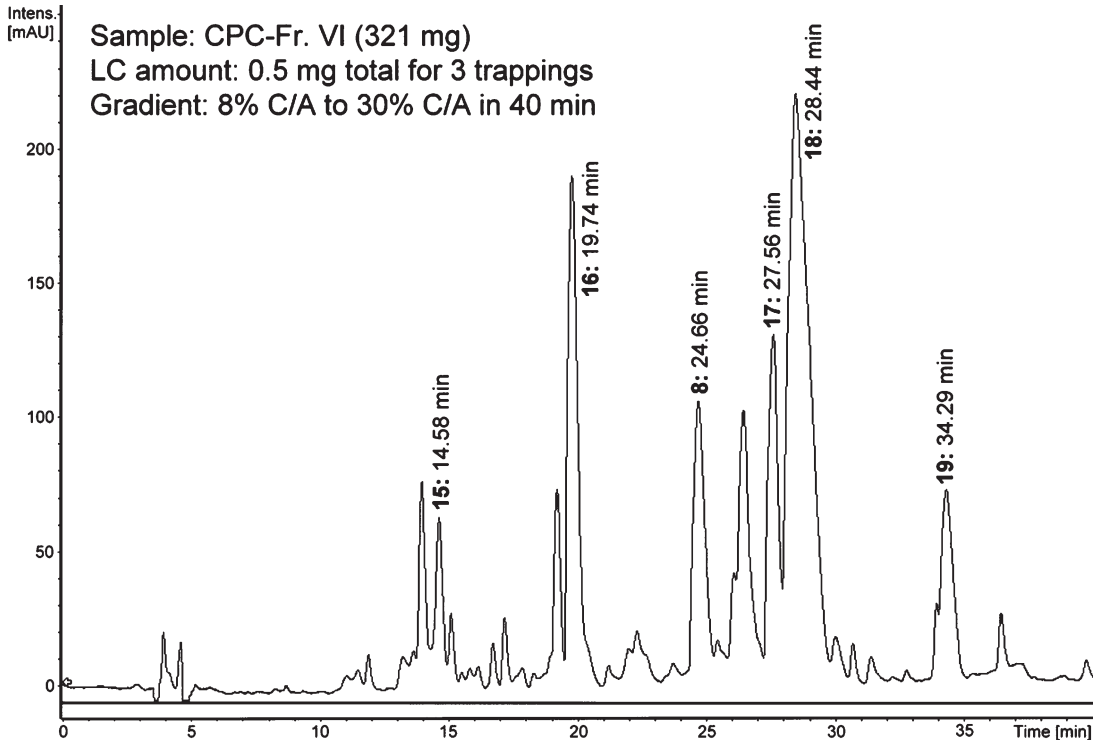

Figure 1. HPLC profiles of the CPC subfraction VI from *C. asiaticum* var. *sinicum* extracts, with the conditions in the Experimental Section. The peaks identified by the HPLC-DAD-SPE-NMR experiment are labeled in boldface.

Those compounds identified could be classified into four types of Amaryllidaceous alkaloids, i.e., the galanthan type (**1**), the crinan type with a β-ethano bridge (**5**, **8**, **10**, **11**, **14**−**16**, **18**, and **19**) or with an α-ethano bridge (**2**−**4**, **6**, **9**, **12**, **13**, **17**, and **21**), and the galanthamine type (**7** and **20**). According to this carbon skeleton grouping, the structure elucidation of these compounds is described as follows.

Galanthan-Type Alkaloid. Compound **1**, the major alkaloid component with a galanthan-type skeleton, was identified as lycorine by comparison of its spectroscopic data with those reported (^{1}H and ^{13}C NMR).[12]

***β*-Ethano Crinan-Type Alkaloids.** Compounds **5**, **8**, **10**, **11**, **14**−**16**, **18**, and **19**, isolated as their TFA salts, showed characteristic ^{1}H NMR signals of crinan-type alkaloids,[13−16] i.e., δ 5.92−5.94 (s) for 8-OC*H*$_2$O-9, 6.62−6.66 (s) for H-7, and 7.66 (s) for H-10 in **5**, **11**, **15**, and **16** and δ 3.73−3.82 (dd) for H-4a in **5**, **8**, **11**, **14**, **18**, and **19**, and an AX system for H$_2$-6 at δ 4.71−4.76 (α) and 4.21−4.34 (β), each as a doublet (Figure 2 and Figure S2, Supporting Information; Table 1 and Table S1, Supporting Information). These compounds contain a common β-ethano bridge attached to a tetrahydrophenanthridine moiety, as evidenced by their CD spectra, all displaying a negative Cotton effect (CE) around 290 nm and a positive CE around 245 nm.[17] Compounds **5** and **15** were identified as 4a-dehydroxycrinamabine and crinamabine, respectively, by comparison of their analytical data (^{1}H NMR, MS, and CD) with those reported.[13,14] Compound **11** had a molecular formula of $C_{18}H_{21}NO_5$, as deduced from HRESIMS, having one CH_2CO residue more than **5**. The ^{1}H NMR spectrum of **11**·TFA was similar to that of **5**·TFA (Figure S2, Supporting Information) except for the presence of an Ac-*Me* singlet (δ 2.14) and a downfield shifted H-2 (δ 5.29, brdd vs δ 4.05, m in **5**). Hence compound **11** was assigned as the 2-*O*-acetylated derivative of **5**. Thus, **11** was elucidated as the C-1 epimer of josephinine,[18] i.e., 1-epijosephinine. Likewise, compound **16** was established as 2-*O*-acetylated **15**, i.e., 2-*O*-acetylcrinamabine, based on the analyses of the HRESIMS, ^{1}H NMR (Table 1 and Table S1, Supporting Information), COSY, and NOESY data. The latter 2D NMR spectrum showed NOE relationships of H-7↔H$_2$-6 and H-10↔H-1↔H-2, allowing the ^{1}H NMR assignment for these protons and designating the *cis*-orientation for H-1 and H-2.

The ^{1}H NMR spectra of the TFA salts of compounds **8**, **10**, **14**, **18**, and **19** (Figure 2 and Figure S2, Supporting Information) shared a common signal for the MeO-7 group around δ 4.04, suggesting them to be 7-methoxycrinan-type alkaloids.[15,16] Of these, compounds **8** and **14** were identified as bulbisine (1-epideacetylbowdensine) and powelline, respectively, upon comparison of their spectroscopic data with those reported.[15,16] The ^{1}H NMR spectrum of **10**·TFA was similar to that of **15**·TFA (Figure 2 and Figure S2, Supporting Information) except for the presence of an additional MeO-7 signal (δ 4.04) and upfield shifted signals for H-10 (δ 7.37 vs δ 7.65, **15**) and H-6α (δ 4.45 vs δ 4.71, **15**), but lacking the H-7 singlet. Compound **10** gave a molecular formula of $C_{17}H_{21}NO_6$ as deduced from HRESIMS, having one CH_2O residue more than **15**, for which an additional MeO-7 group was demonstrated. These data when combined led to **10** being determined as 7-methoxycrinamabine. The ^{1}H NMR spectra of **18**·TFA (Table 1) and 1-epijosephinine·TFA (**11**·TFA) (Figure 2 and Figure S2, Supporting Information) revealed similar differences to those between compounds **10** and **15** (Table 1 and Table S1, Supporting Information). The molecular formula of **18**, $C_{19}H_{23}NO_6$, as deduced from HRESIMS, also had an additional CH_2O residue relative to compound **11**. These comparisons were used to establish **18** as 7-methoxy-1-epijosephinine or 2-*O*-acetylbulbisine. The structural assignment for **18** was also supported by the analysis of its

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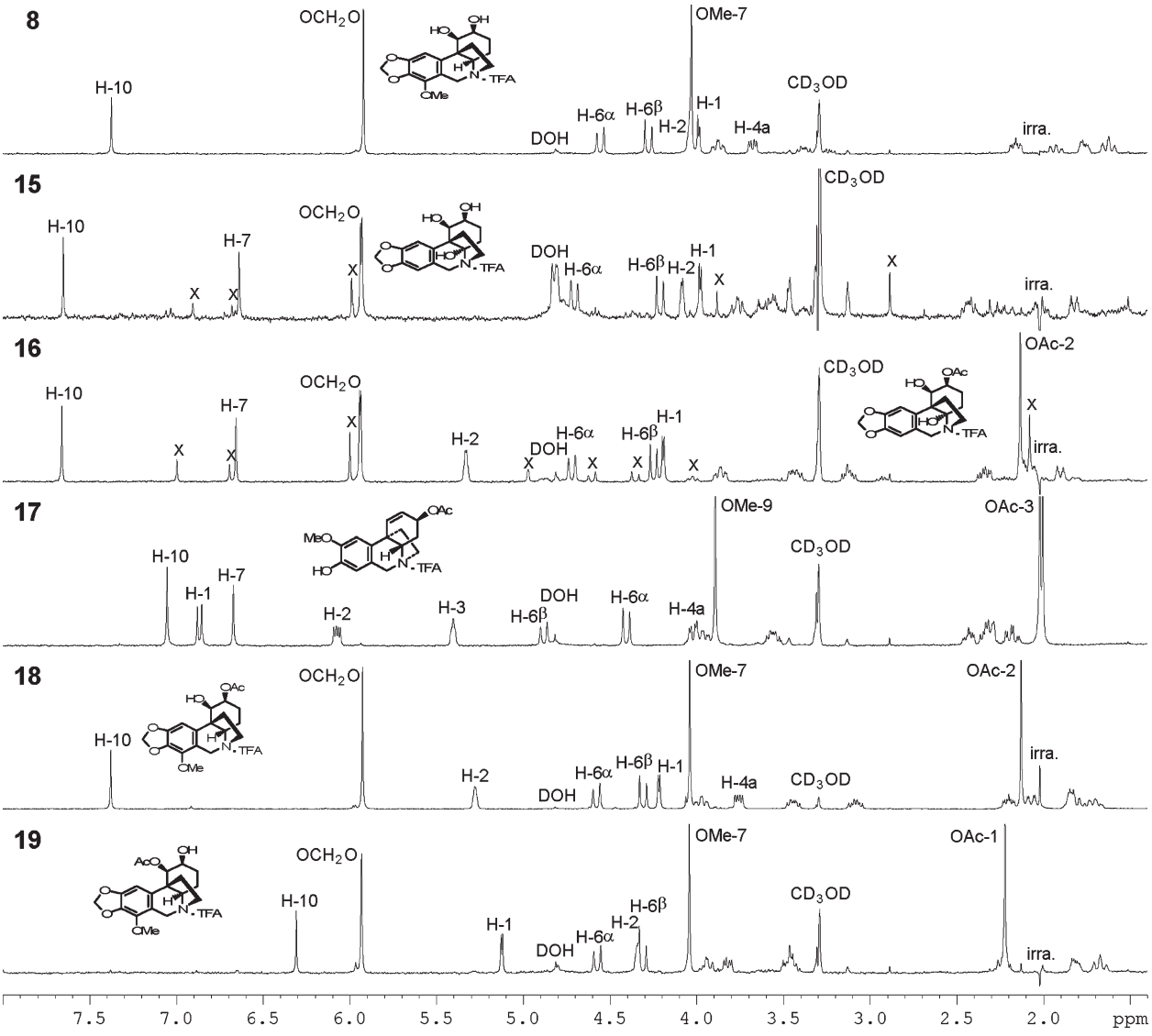

Figure 2. ^{1}H NMR spectra of crinan-type alkaloids, **8** and **15**−**19** (CD_3OD, 400 MHz), obtained from the HPLC-DAD-SPE-NMR for subfraction VI.

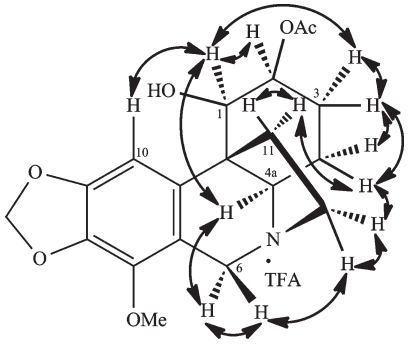

Figure 3. Key NOESY correlations for **18**·TFA.

NOESY spectrum (Figure 3), which displayed key correlations of H-10 (δ 7.38) to H-1α (δ 4.22) and H-11$_{endo}$ (δ 2.20). The ^{1}H NMR spectrum of **19**·TFA was similar to that of **8**·TFA (Figure 2) except for the presence of an additional Ac-*Me* singlet at δ 2.22, an upfield shifted H-10 (δ 6.31 vs 7.37, **8**), and a downfield shifted H-1 (δ 5.13 vs 3.99, **8**), all assigned from analysis of the COSY and NOESY spectra (see Experimental Section), indicating C-1 to be acetoxylated. This suggestion was supported by its HRESIMS, providing the molecular formula, $C_{19}H_{23}NO_6$, with a CH_2CO residue more than **8**. These data when combined helped establish **19** as 1-*O*-acetylbulbisine.

α-Ethano Crinan-Type Alkaloids. Compounds **2**−**4**, **6**, **9**, **12**, **13**, and **17** contain a common α-ethano-bridged crinan skeleton, as evidenced by their CD curves, all manifesting a negative CE around 247 nm and a positive CE around 290 nm.[17,19,20] Compounds **3**, **6**, **9**, **12**, and **13** were identified as maritinamine,[21] dihydrovittatine,[21] hamayne,[16,18] vittatine,[21,22] and epivittatine,[23] respectively, by comparison of their spectroscopic data [^{1}H NMR (Table 1 and Table S2, Supporting Information), MS, CD] to reported values for the corresponding compound. Compounds **2**, **4**, and **17** contain a common α-ethano-bridged Δ^1-8-hydroxy-9-methoxycrinan skeleton, as evidenced by the characteristic ^{1}H NMR spectroscopic data, $\delta_{MeO\text{-}9}$ ~3.90 (s), $\delta_{H\text{-}7}$ ~6.66 (s), $\delta_{H\text{-}6s}$ ~4.40 and 4.85 (Table 2 and Table S2, Supporting Information),[21,22] with the bathochromic shift in their UV absorptions under alkaline conditions due to the presence of a phenolic OH at the C-8 position. Compound **2** was

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Table 1. ^{1}H NMR Data (CD_3OD, δ/ppm, J/Hz) of the TFA Salts of 4, 6, 10, 11, 19, and 21 (400 MHz)[a]

H-atom	**10**·TFA	**11**·TFA[b]	**19**·TFA[c]	**6**·TFA	**4**·TFA	**21**·TFA
H-1α	3.95 d (4.2)	4.24 d (4.4)	5.13 d (3.9)	2.25 ddd (2.7, 4.2, 13.7)	6.54 dd (1.9, 10.1)	7.89 d (10.4)
H-1*β*				2.11 ddd (5.6, 13.0, 13.7)		
H-2α	4.07 brdd (3.2, 6.6)	5.29 brdd (4.4, 5.9)	4.34 brdd (3.9, 5.8)	1.85 dddd (2.2, 4.4, 13.0, 13.7)	5.89 d (10.1)	6.21 d (10.4)
H-2*β*				1.88 m		
H-3α	2.01 m	1.71 dddd (1.7,5.9,12.2,14.2)	1.67 ddd (1.8, 13.2, 14.0)	4.21 m		
H-3*β*	2.01 m	2.08 brdd (2.9, 14.2)	2.02 m		4.43 ddd (1.9, 6.1, 8.1)	
H-4α	1.82 ddd (2.6, 3.0, 13.2)	1.85 m	2.01 m	2.12 m	1.82 brdd (11.5, 13.5)	2.95 dd (12.9, 16.3)
H-4*β*	2.43 ddd (7.6, 9.8, 13.2)	1.80 ddd (2.9, 10.4, 12.5)	1.81 dddd (3.2,12.5,13.2,13.9)	1.69 ddd (2.2, 12.1, 13.2)	2.37 ddd (3.5,6.1,11.5)	2.89 dd (6.7, 16.3)
H-4a		3.79 dd (6.1, 10.4)	3.82 dd (5.5, 12.5)	3.90 dd (5.9, 12.1)	4.01 dd (3.5, 13.5)	4.39 m
H-6α	4.45 d (15.6)	4.76 d (15.4)	4.57 d (16.0)	4.34 d (15.4)	4.38 d (15.4)	4.41 d (15.8)
H-6*β*	4.21 d (15.6)	4.34 d (15.4)	4.31 d (16.0)	4.79 d (15.4)	4.82[a]	4.81[a]
H-7		6.64 s		6.66 s	6.65 s	6.69 s
H-10	7.37 s	7.66 s	6.31 s	6.88 s	7.00 s	7.20 s
H-11*endo*	2.03 m	2.21 ddd (3.9, 9.5, 13.2)	2.23 m	2.60 ddd (7.1, 12.0, 12.0)	2.41 m	2.59 brt (7.7)
H-11*exo*	3.30[a]	3.09 ddd (6.9, 11.7, 13.2)	3.49 m	1.98 ddd (3.4, 8.9, 12.0)	2.41 m	2.59 brt (7.7)
H-12*endo*	3.32[a]	3.44 ddd (6.9, 9.5, 12.6)	3.44 ddd (7.3, 10.3, 12.9)	3.47 ddd (7.1, 8.9, 13.2)	3.55 ddd (7.6,8.3,12.5)	3.61 dt (7.7, 12.9)
H-12*exo*	3.74 brdd (11.5, 12.1)	3.97 ddd (3.9, 11.7, 12.6)	3.94 ddd (3.8, 11.0, 12.9)	3.89 m	4.01 m	4.11 dt (7.7, 12.9)
OCH_2O	5.92 s	5.94 s	5.93 s	5.96 d (1.2)		
MeO-7	4.04 s		4.04 s			
MeO-9					3.88 s	3.92 s

[a] Signals were overlapped with the residual proton signals of CD_3OD solvent. [b] $\delta_{2\text{-}O\text{-}Ac\text{-}Me}$ 2.14 (s). [c] $\delta_{1\text{-}O\text{-}Ac\text{-}Me}$ 2.22 (s).

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Table 2. ^{1}H NMR Data and HMBC Correlations[a] of 16 and 18 (600 MHz) and ^{13}C NMR Data of 11, 16, 18, and 19 (100 MHz) (CD_3OD)

	16·TFA			**18**·TFA			**19**·TFA	**11**·TFA
position	δ_H (multi) (J in Hz)	δ_C/m[b]	HMBC (corr C#)	δ_H (multi) (J in Hz)	δ_C/m[b]	HMBC (corr C#)	δ_C/m[b]	δ_C/m[b]
1	4.19 d (4.6)	70.8 d	2, 10a, 10b, 11, CO	4.21 d (4.4)	71.5 d	2, 10a, 10b, 11, CO	76.0 d	71.5 d
2	5.33 brdd (2.9, 7.2)	72.9 d	1	5.27 brdd (3.7, 6.2)	72.7 d	10b	66.0 d	72.7 d
3	2.08 m (α), 2.04 m (*β*)	25.6 t	4a	1.70 dddd (2.7, 3.0, 13.9, 14.7) (α)	26.1 t		28.3 t	26.1 t
				2.06 ddd (3.0, 6.2, 14.7) (*β*)				
4	1.94 ddd (3.1, 3.3, 13.4) (α)	26.3 t	3, 10b	1.88 m (α)	20.0 t		19.2 t	20.0 t
	2.34 ddd (4.6, 13.3, 13.4) (*β*)		3, 4a	1.80 dddd (2.7, 12.1, 12.5, 13.9) (*β*)		3, 4a		
4a		96.6 s		3.75 dd (5.1, 12.1)	69.1 d	4, 6, 10a, 11, 12	69.5 d	69.5 d
6	4.72 d (15.3) (α)	54.6 t	6a, 7, 10a, 12	4.58 d (15.9) (α)	56.9 t	6a, 7, 10a, 12	56.8 t	59.6 t
	4.24 d (15.3) (*β*)		4a, 6a, 7, 10a, 12	4.29 d (15.9) (*β*)		4a, 6a, 7, 10a, 12		
6a		120.6 s			110.8 s		111.0 s	119.3 s
7	6.65 s	107.0 d	6, 8, 9, 10a		141.2 s		141.6 s	107.3 d
8		148.1 s			135.5 s		135.6 s	148.6 s
9		149.2 s			151.4 s		151.5 s	149.3 s
10	7.66 s	108.3 d	6a, 8, 9, 10a, 10b	7.37 s	100.8 d	6a, 8, 9, 10a, 10b	98.6 d	107.0 d
10a		135.6 s			139.9 s		139.2 s	139.0 s
10b		51.6 s			50.3 s		—[c]	50.4 s
11	2.11 m (*endo*)	33.9 t	4a, 10a, 10b	2.20 ddd (4.0, 9.2, 13.2) (*endo*)	34.5 t	4a, 10a, 10b	35.6 t	34.7 t
	3.12 ddd (6.5, 11.5, 13.4) (*exo*)		1, 10a, 10b, 12	3.08 ddd (6.8, 11.9, 13.2) (*exo*)		1, 10a, 10b, 12		
12	3.42 ddd (6.5, 10.0, 13.4) (*endo*)	49.6 t	4a, 6, 11	3.42 ddd (6.8, 9.2, 12.8) (*endo*)	52.7 t	4a, 6, 11	53.0 t	52.6 t
	3.88 ddd (2.8, 11.5, 13.4) (*exo*)			3.98 ddd (4.0, 11.9, 12.8) (*exo*)		6		
MeO-7				4.03 s	60.0 q	7	60.0 q	
8-OCH_2O-9	5.93 d (1.0), 5.94 d (1.0)	102.7 t	8, 9	5.92 s	102.7 t	8, 9	102.9 t	102.9 t
Ac-CH_3-1							21.3 q	
Ac-CO-1							171.9 s	
Ac-CH_3-2	2.14 s	21.2 q	CO	2.13 s	21.2 q	CO		21.2 q
Ac-CO-2		172.1 s			172.2 s			172.2 s

[a] In the HMBC experiment, J_{H-C} was set at 8 Hz. [b] Multiplicities were obtained from DEPT experiments. [c] Overlapped with CD_3OD signals.

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Table 3. ^{1}H NMR Data and HMBC Correlations[a] of 17 (600 MHz) and ^{13}C NMR Data of 4, 6, and 17 (100 MHz) (CD_3OD)

	17·TFA			4·TFA	6·TFA
position	δ_H (multi) (*J*/ Hz)	δ_C/m[b]	HMBC (corr C#)	δ_C/m[b]	δ_C/m[b]
1	6.86 d (10.1)	132.0 d	3, 4a, 10a, 10b	126.3 d	28.0 t
2	6.07 dd (5.2, 10.1)	125.6 d	3, 4, 10b, CO	133.8 d	31.6 t
3	5.40 ddd (1.7, 4.3, 5.2)	65.9 d	1, 2	66.6 d	65.3 d
4	2.17 ddd (4.3, 13.4, 13.6) (α)	28.1 t	3, 4a, 10b	32.4 t	23.0 t
	2.31 m (*β*)		2, 3, 4a		
4a	4.02 dd (4.3, 13.4)	66.3 d	4, 6, 10a, 11, 12	68.6 d	66.5 d
6	4.40 d (15.3) (α)	59.7 t	4a, 6a, 7, 10a, 12	59.6 t	59.5 t
	4.88 d (15.3) (*β*)		6a, 7, 10a, 12		
6a		118.7 s		118.5 s	119.3 s
7	6.67 s	115.0 d	6, 8, 9, 10a	114.9 d	107.5 d
8		147.8 s		147.6 s	148.7 s
9		149.2 s		149.2 s	149.6 s
10	7.05 s	107.6 d	6a, 8, 9, 10a, 10b	107.6 d	104.7 d
10a		133.0 s		133.6 s	139.0 s
10b		46.0 s		46.2 s	44.6 s
11	2.43 ddd (4.1, 9.1, 12.7) (*endo*)	41.8 t	4a, 10a, 10b	42.7 t	35.5 t
	2.34 brdd (7.1, 11.9) (*exo*)		4a, 10a, 10b, 12		
12	3.55 ddd (7.1, 9.1, 13.1) (*endo*)	53.8 t	6	53.5 t	52.9 t
	3.98 ddd (4.1, 11.9, 13.1) (*exo*)				
8-OCH_2O-9					102.9 t
MeO-9	3.89 s	56.7 q	9	56.6 q	
Ac-CH_3-3	2.00 s	20.9 q	CO		
Ac-CO-3		171.8 s			

[a] In the HMBC experiment, J_{H-C} was set at 8 Hz. [b] Multiplicities were obtained from DEPT experiments.

identified as (+)-8-*O*-demethylmaritidine by comparison of the ^{1}H NMR data (Table S2, Supporting Information) and CD curve with those reported.[21,22] Compound **4** was assigned a molecular formula of $C_{16}H_{19}NO_3$, as deduced from HRESIMS. The ^{1}H NMR data of **4**·TFA (Table 1) and the known (−)-siciline (**4a**)[21] were very similar. Their CD curves, however, were almost mirror images to one another, indicating them to be enantiomers. Since the CD data designated the ethano bridge in **4** to be α-oriented, as indicated above, this bridge in (−)-siciline (**4a**) should be *β*-oriented, leading to the revision of the previous α-oriented assignment.[21] Accordingly, compound **4** was designated as (4a*S*,5*R*,10b*R*)-(+)-siciline. Compound **17** gave a molecular formula of $C_{18}H_{21}NO_4$, as deduced from its HRESIMS, having one more CH_2CO residue than **2**. The ^{1}H NMR spectrum of **17**·TFA (Figure 2, Table 3) was similar to that of **2**·TFA (Figure S3 and Table S2, Supporting Information) except for a significantly downfield shifted H-3 (δ 5.04, dt vs 4.38, m, **2**), verified from the COSY and NOESY spectra (see Experimental Section). This proton (H-3) was located pseudoequatorially, i.e., the α-position, from the analysis of its coupling constants (dt, J = 3.0, 4.4 Hz). Accordingly, compound **17** was established as 3-*O*-acetyl-8-*O*-demethylmaritidine.

Compound **21** was assigned a molecular formula of $C_{16}H_{17}NO_3$, as deduced from HRESIMS, two hydrogen atoms less than **2** or **4**. The ^{1}H NMR spectrum of **21**·TFA (Figure S3, Supporting Information, and Table 1) was similar to that of **2**·TFA (Figure S3 and Table S2, Supporting Information) except for the presence of an AX system for H-1 (δ 7.89) and H-2 (δ 6.21) (J_{AX} = 10.4 Hz), both being assigned from the analyses of COSY and NOESY spectra (see Experimental Section). The chemical shifts and coupling constant in this AX system and the MS data indicated that **21** contains an α,*β*-unsaturated carbonyl function.[24,25] The CD curve of **21**·TFA exhibited a positive exciton chirality at 298/270 nm, arising from the enone and aryl chromophore, reflecting the fact that **21** contains an α-ethano bridge based on a chemical model study. Hence compound **21** was established as 8-*O*-demethyloxomaritidine.[26−28]

Galanthamine-Type Alkaloids. Compounds **7** and **20** belong to the galanthamine-type alkaloids, for which the CD curves exhibit two positive Cotton effects around 290 and 242 nm. They were identified as norgalanthamine (**7**) and galanthamine (**20**), respectively, by comparison of their physical data to those reported for the corresponding compound.[29−31]

For structure confirmation, 12 compounds (**2**, **4**−**6**, **8**, **11**, **13**, **14**, and **16**−**19**) in addition to lycorine (**1**) were isolated by RP-18 semipreparative HPLC. Compound **19**, however, was partially converted to **18** via the trans-acetylation mechanism during condensation of the separated eluate, containing 0.1% trifluoroacetic acid. The ^{1}H and ^{13}C NMR data of these isolated compounds (Tables 2, 3, and Table S4, Supporting Information) were completely assigned on the basis of 1D and 2D NMR spectroscopic analysis. Of these, the HMBC spectra of **16**·TFA and **18**·TFA (Table 2), showing the correlation of $\delta_{H\text{-}2}/\delta_{Ac\text{-}CO}$, as in **16**, δ 5.33/δ 172.1, and that of **17** (Table 3), showing the correlation of δ 5.40 (H-3)/δ 171.8 (Ac-CO), confirmed the location of the respective acetoxy group. This 2D NMR spectrum of **16** also showed the correlations of the hydroxylated quaternary carbon (δ 96.6) to H_2-3 (δ 2.04 and 2.08), H-4*β* (δ 2.34), H-6*β* (δ 4.24), and H-12$_{endo}$ (δ 3.42), permitting the C-4a signal

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assignment and supporting the inference that C-4a in **16** is hydroxylated. The presence of MeO-7 in **18** was also supported by this spectrum by showing the correlation of δ 141.2 (C-7)/δ 4.03 (MeO-7), 4.58 (H-6α), and 4.29 (H-6β). Although compound **19** was obtained as a 1:1 mixture with **18**, the ^{13}C NMR data of **19**·TFA were inferred by elimination of those of **18**·TFA from the spectrum of the mixture and assigned by comparison to those of **18**·TFA.

In this study, 21 alkaloids have been characterized from the EtOH extract of the leaves of *C. asiaticum* L. var. *sinicum*. Among these, seven were found to be new chemical entities, i.e., (4a*S*,5*R*,10b*R*)-(+)-siculine (**4**), 7-methoxycrinamabine (**10**), 1-epijosephinine (**11**), 2-*O*-acetylcrinamabine (**16**), 3-*O*-acetyl-8-*O*-demethylmaritidine (**17**), 2-*O*-acetylbulbisine (**18**), and 1-*O*-acetylbulbisine (**19**). Dihydrovittatine (**6**) and 8-*O*-demethyloxomaritidine (**21**) have been isolated as natural products for the first time, although they had been prepared as synthetic products.[26−28,32] The known galanthamine (**20**) is used clinically in enhancing cognition for patients with mild dementia.[33,34]

It should be addressed that CPC fractionation[35] of the non-phenolic alkaloids in this study is very helpful to facilitate the subsequent optimization of HPLC conditions for separation, which is critical to achieve successful application of HPLC-SPE-NMR analysis. The amounts of each fraction (Frs. I−VII) from CPC, taken for HPLC-SPE-NMR analysis, were around 0.5 mg, equivalent to at most 10 g of dried leaves theoretically. A previous study has reported the isolation of four alkaloids, i.e., lycorine (**1**), crinine, crinisine, and powelline (**14**), from the bulbs of the same plant.[9] Considering the fact that 21 alkaloids with similar structures were characterized in this study using this small amount of analyte, it is obvious that HPLC-SPE-NMR, combined with other methods (e.g., ESIMS, CD), is useful for a thorough study of natural constituents from a complex mixture.

The ^{1}H NMR data of the TFA salts of known alkaloids, obtained from analysis of online ^{1}H NMR spectra and 2D NMR data (COSY and NOESY for **8**, **9**, **12**, and **13**), are listed in Tables S1−S3 (Supporting Information). These data should be helpful for the identification of other Amaryllidaceous alkaloids using a similar approach. The addition of TFA to the solvent system MeOH−H_2O or CH_3CN−H_2O was found to improve peak separation of these alkaloids in the reversed-phase HPLC, although it will cause protonation of the basic nitrogen atom, resulting in the downfield shifted signals for those adjacent protons as footnoted in Tables S1−S3 (Supporting Information). It should be noted, however, that trans-esterification was observed during condensation of the TFA-containing eluate of the separated **19** from RP-semipreparative HPLC, of which half was converted to **18**, and detected by HPLC analysis.

EXPERIMENTAL SECTION

General Experimental Procedures. UV (MeOH): U-2001 Hitachi double-beam spectrophotometer for compounds separated by semipreparative HPLC. CD (MeOH): JASCO J-720 spectropolarimeter. Off-line NMR spectra: Bruker AV-400 and AV III-600, the latter equipped with an SEI ^{13}C−^{1}H probehead for 2D spectra (methanol-d_4, δ_H 3.30 and δ_C 49.0 ppm). HPLC-DAD (diode array detector)-SPE-NMR (400 MHz): Agilent 1100 liquid chromatograph (Waldbronn, Germany) equipped with a photodiode array detector (Bruker DAD, Bruker, Rheinstetten, Germany), a Knauer K120 HPLC pump (makeup pump), followed by a Prospekt 2 automated solid-phase extraction unit (Spark Holland, Emmen, Holland), containing 192 HySphere resin GP cartridges (10 × 2 mm, 10−12 μm), a Bruker nitrogen separator, and a Bruker AV-400 NMR spectrometer equipped with a 30 μL inverse NMR probe. ESIMS: Bruker Daltonics Esquire 2000. HRESIMS: micrOTOF orthogonal ESI-TOF mass spectrometer (Bruker Daltonik). CPC: Sanki LLN type, equipped with 6 cartridges, delivery system $CHCl_3$−MeOH−0.5% $HOAc_{(aq)}$ (5:5:3), flow rate 3 mL/min, rotation speed 700 rpm, and 14 mL per fraction.

Plant Material. The aerial parts of *C. asiaticum* L. var. *sinicum* were collected in May 2004 in the Pin-Lin area, Taipei County, Taiwan. The specimen obtained (NTU05012004) was authenticated by Professor Chung-Fu Hsieh, Department of Life Science, National Taiwan University (NTU), and was stored in the herbarium of the School of Pharmacy, NTU.

Sample Preparation. The dried, powdered leaves (2.84 kg) were percolated with 95% EtOH (10 L × 5) at room temperature. Evaporation under reduced pressure gave an EtOH extract (1.02 kg), which was stirred vigorously with 2% $HOAc_{(aq)}$ (0.5 L × 3). The combined acidic solutions were extracted with EtOAc (1.2 L × 3) to give an EtOAc-soluble fraction (13.63 g). The aqueous layer was adjusted to pH 9 with 25% ammonia−water, and the precipitate (**1**, 13.70 g) was collected by filtration. The filtrate was extracted with $CHCl_3$ (1.2 L × 3) to give the free bases (10.94 g), which were partitioned between CH_2Cl_2 (0.2 L) and 1% $NaOH_{(aq)}$ (0.15 L × 2). The CH_2Cl_2 layer was dried over anhydrous Na_2SO_4 and condensed to give non-phenolic bases (5.13 g). The 1% NaOH layer was adjusted to pH 9 by 20% $NH_4Cl_{(aq)}$, followed by extraction with $CHCl_3$ (0.2 L × 3) to give phenolic bases (4.27 g) upon condensation. Most of the non-phenolic bases (4.54 g) were fractionated by CPC using the upper and lower layers of the delivery system indicated above as the mobile and stationary phases, respectively, to give 12 fractions (Frs. I−XII). Frs. I−VII were subjected to HPLC analysis, each at a concentration of 10 mg per 0.3 mL of MeOH.

HPLC Conditions Used in HPLC-SPE-NMR. HPLC separation was carried out on a Phenomenex Prodigy ODS3 100 Å (250 × 4.6 mm, 5 μm) column, delivered by a linear gradient of 0.1% (v/v) TFA containing water (solvent A), methanol (solvent B), or acetonitrile (solvent C) with a flow rate of 0.7 mL/min and detected at 280 nm. The chromatographic profiles of Frs. I−VII from CPC fractionation, including the sample amount and the delivery system, are shown in Figure 1 and Figure S1 (Supporting Information).

SPE-NMR (400 MHz) Procedure. After the HPLC separation, post-column water was added to the eluate at a flow rate of 1.8 mL/min for Frs. I−III and V, 1.6 mL/min for Fr. IV, and 1.5 mL/min for Frs. VI and VII, and each compound peak was then passed through a HySphere resin GP cartridge to undergo solid-phase extraction. This process was repeated three times, injecting 5 μL of sample per run. The compound-loaded cartridges were then flushed with dry nitrogen, supplied by a nitrogen separator, to remove the residual solvents. Each trapped analyte in a resin GP cartridge was eluted into the NMR probehead by CD_3OD (265 μL). The ^{1}H NMR spectrum of each separated compound was recorded using a multiple solvent suppression pulse program for residual proton and water signals in the CD_3OD (δ 3.30, 4.80) and the residual CH_3CN (δ 2.02). Shaped low-power rf pulse and CW decoupling on the F2 channel for the decoupling of the ^{13}C satellites were utilized. All spectra were measured at 298 K, and the ^{1}H chemical shift was referenced to a residual signal of CD_2HOD at δ 3.30. A total of 256 to 1024 scans for each measurement were accumulated into 16K data points with a sweep width of 8000 Hz. 2D NMR spectra were recorded using standard pulse programs (COSY, NOESY, HSQC, and HMBC), and the correlation maps consisted of 2048 × 256 data points per spectrum.

CD Measurement. After the NMR spectra were acquired, each sample was collected and concentrated under reduced pressure. The sample residue was dissolved in MeOH (4 mL) for CD measurement. The step resolution was set at 0.1 nm with a scan speed of 50 nm/min.

The response and sensitivity were set at 0.25 s and 200 mdeg, respectively.

HPLC-DAD-MS. An aliquot (5%) of each separated eluate from HPLC was introduced into the Esquire 2000 ion trap mass spectrometer via an Upchurch Scientific graduated microsplitter valve. MS data were acquired in a scan range between 50 and 650 Da with an electrospray interface under positive-ionization conditions. The spray voltage was set at 4.0 kV with the nitrogen dry temperature at 325 °C. The nebulizer gas pressure was set at 28 psi with a dry gas flow of 9 L/min. The trap drive and capillary exit voltage were set at 30−34 (arbitrary units) and 101−106 V, respectively.

Semipreparative HPLC Separation. Fractions II, V, and VI were further separated by a semipreparative HPLC: Phenomenex Prodigy ODS3 100 Å (250 × 10 mm, 5 μm) column, delivery system composed of solvents A−C, all containing 0.1% TFA (v/v), flow rate 3.4 mL/min, and detection at 280 nm. The concentrations of each fraction were prepared as 40−50 mg/mL, and the injection volumes were 40−60 μL/run. Under these conditions and using a linear gradient of A/B (82:18) to A/B (50:50) in 30 min, Fr. II (25 mg) gave compounds **2** (7.8 mg, t_R: 10.52 min), **4** (5.1 mg, t_R: 11.91 min), **5** (2.4 mg, t_R: 13.65 min), and **6** (1.8 mg, t_R: 19.90 min). Under the general conditions indicated above and using a linear gradient of A/B/C (95:3:2) to A/B/C (33:65:2) in 60 min, Fr. V (44 mg) yielded compounds **11** (10.5 mg, t_R: 33.54 min), **13** (8.9 mg, t_R: 41.11 min), **8** (3.4 mg, t_R: 44.09 min), **18** (3.6 mg, t_R: 46.49 min), and **14** (6.5 mg, t_R: 47.97 min). With the same conditions and using the linear gradient of A/C (92:8) to A/C (70:30) in 40 min, Fr. VI (41 mg) afforded compounds **16** (2.6 mg, t_R: 20.25 min), **8** (3.3 mg, t_R: 25.06 min), **17** (1.9 mg, t_R: 27.89 min), **18** (17.4 mg, t_R: 28.60 min), and **19** (2.9 mg, t_R: 34.80 min).

(4a*S*,5*R*,10b*R*)-(+)-Siculine·TFA (4·TFA): $[\alpha]_D^{23}$ +56.9 (*c* 0.51, MeOH); UV (MeOH) λ_{max} nm (log ε) 288 (3.45), 227 (3.63, sh), (MeOH+NaOH) λ_{max} 296 nm; CD (*c* 2.58 × 10^{-4} M, MeOH) $[\theta]_{314}$ 0°, $[\theta]_{288}$ +3240°, $[\theta]_{263}$ 0°, $[\theta]_{246}$ −950°, $[\theta]_{232}$ +5740°, $[\theta]_{226}$ +4250°; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 3; (+)HRESIMS *m*/*z* 274.1437 $[M + H]^+$, calcd for $C_{16}H_{20}NO_3$, 274.1443.

Dihydrovittatine·TFA (6·TFA): $[\alpha]_D^{23}$ +11.1 (*c* 0.18, MeOH); UV (MeOH) λ_{max} nm (log ε) 291 (3.56), 234 (3.49); CD (*c* 2.79 × 10^{-4} M, MeOH) $[\theta]_{312}$ 0°, $[\theta]_{289}$ +2240°, $[\theta]_{269}$ 0°, $[\theta]_{245}$ −4380°, $[\theta]_{229}$ 0°, $[\theta]_{222}$ +1600°, $[\theta]_{215}$ −120°; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 3; (+)HRESIMS *m*/*z* 274.1435 $[M + H]^+$, calcd for $C_{16}H_{20}NO_3$, 274.1443.

7-Methoxycrinamabine·TFA (10·TFA): UV (MeOH) λ_{max} 284 nm; CD (MeOH) (CE) 286 (−), 247 (+) nm; ^{1}H NMR, see Table 1; (+)HRESIMS *m*/*z* 336.1451 $[M + H]^+$, calcd for $C_{17}H_{22}NO_6$, 336.1447.

1-Epijosephinine·TFA (11·TFA): $[\alpha]_D^{23}$ +7.6 (*c* 1.05, MeOH); UV (MeOH) λ_{max} nm (log ε) 292 (3.50), 232 (3.50); CD (*c* 2.36 × 10^{-4} M, MeOH) $[\theta]_{305}$ 0°, $[\theta]_{295}$ −1280°, $[\theta]_{259}$ 0°, $[\theta]_{243}$ +1780°, $[\theta]_{232}$ 0°, $[\theta]_{223}$ −1960°, $[\theta]_{219}$ −890°; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; (+)HRESIMS *m*/*z* 332.1493 $[M + H]^+$, calcd for $C_{18}H_{22}NO_5$, 332.1498.

2-*O*-Acetylcrinamabine·TFA (16·TFA): $[\alpha]_D^{24}$ +15.4 (*c* 0.26, MeOH); UV (MeOH) λ_{max} nm (log ε) 292 (3.53), 233 (3.52); CD (*c* 2.26 × 10^{-4} M, MeOH) $[\theta]_{305}$ 0°, $[\theta]_{296}$ −870°, $[\theta]_{257}$ 0°, $[\theta]_{239}$ +3150°, $[\theta]_{219}$ 0°; ^{1}H, ^{13}C NMR and HMBC, see Table 2; COSY data H-1 ↔ H-2 ↔ H_2-3 ↔ H_2-4, H-6α ↔ H-6*β*, H_2-11 ↔ H_2-12; NOESY data H-10 ↔ H-1 ↔ H-2, H-4α ↔ H-4*β*, H-6α ↔ H-6*β* ↔ H-7, H_{endo}-11 ↔ H_{exo}-11, H_{endo}-12 ↔ H_{exo}-12; (+)HRESIMS *m*/*z* 348.1461 $[M + H]^+$, calcd for $C_{18}H_{22}NO_6$, 348.1442.

3-*O*-Acetyl-8-*O*-demethylmaritidine·TFA (17·TFA): $[\alpha]_D^{24}$ −10.5 (*c* 0.19, MeOH); UV (MeOH) λ_{max} nm (log ε) 287 (3.43), 230 (3.63, sh), (MeOH+NaOH) λ_{max} 302 nm; CD (*c* 1.17 × 10^{-4} M, MeOH) $[\theta]_{308}$ 0°, $[\theta]_{289}$ +4810°, $[\theta]_{256}$ 0°, $[\theta]_{233}$ +4620°, $[\theta]_{225}$ +1680°; ^{1}H, ^{13}C NMR and HMBC, see Table 3; COSY data H-1 ↔ H-2 ↔ H-3 ↔ H_2-4 ↔ H-4a, H-6α ↔ H-6*β*, H_2-11 ↔ H_2-12; NOESY data MeO-9 ↔ H-10 ↔ H-1 ↔ H-2 ↔ H-3 ↔ H-4α ↔ H-4*β*, H-7 ↔ H-6α ↔ H_{endo}-12 ↔ H_{exo}-12, H_{endo}-11 ↔ H_{exo}-11; (+)HRESIMS *m*/*z* 316.1579 $[M + H]^+$, calcd for $C_{18}H_{22}NO_4$, 316.1543.

2-*O*-Acetylbulbisine·TFA (18·TFA): $[\alpha]_D^{23}$ +11.1 (*c* 0.36, MeOH); UV (MeOH) λ_{max} nm (log ε) 275 (3.41), 236 (3.78, sh); CD (*c* 2.27 × 10^{-4} M, MeOH) $[\theta]_{302}$ 0°, $[\theta]_{286}$ +700°, $[\theta]_{262}$ 0°, $[\theta]_{241}$ +1100°, $[\theta]_{233}$ 0°, $[\theta]_{214}$ −11 330°; ^{1}H, ^{13}C NMR and HMBC, see Table 2; COSY data H-1 ↔ H-2 ↔ H_2-3 ↔ H_2-4 ↔ H-4a, H-6α ↔ H-6*β*, H_2-11 ↔ H_2-12; NOESY, see Figure 3; (+)HRESIMS *m*/*z* 362.1581 $[M + H]^+$, calcd for $C_{19}H_{24}NO_6$, 362.1598.

1-*O*-Acetylbulbisine·TFA (19·TFA): UV (MeOH) λ_{max} 285 nm; CD (MeOH) (CE) 286 (−), 248 (+) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; COSY data H-1 ↔ H-2 ↔ H_2-3, H_2-4 ↔ H-4a, H-6α ↔ H-6*β*, H_2-11 ↔ H_2-12; NOESY data H-10 ↔ H-1 ↔ H-2, H_2-3 ↔ H_2-4, H-6α ↔ H-6*β*, H_{endo}-11 ↔ H_{exo}-11, H_{endo}-12 ↔ H_{exo}-12; (+)HRESIMS *m*/*z* 362.1581 $[M + H]^+$, calcd for $C_{19}H_{24}NO_6$, 362.1598.

8-*O*-Demethyloxomaritidine·TFA (21·TFA): UV (MeOH) λ_{max} 286 nm, (MeOH+NaOH) λ_{max} 297 nm; CD (MeOH) (CE) 298 (+), 270 (−), 245 (+), 230 (+) nm; ^{1}H NMR, see Table 1; COSY data H-1 ↔ H-2, H_2-4 ↔ H4a, H-6α ↔ H-6*β*, H_2-11 ↔ H_2-12; NOESY data MeO-9 ↔ H-10 ↔ H-1, H-6α ↔ H-6*β*, H_{endo}-12 ↔ H_{exo}-12; (+)HRESIMS *m*/*z* 272.1277 $[M + H]^+$, calcd for $C_{16}H_{18}NO_3$, 272.1281.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. ^{1}H NMR data of known alkaloids (**2**, **3**, **5**, **7**−**9**, **12**−**15**, **20**) as TFA salts (Tables S1−S3), ^{13}C NMR data of the TFA salts of **2**, **5**, **8**, **13**, and **14** (Table S4), HPLC profiles of CPC subfrs. I−V and VII and the phenolic bases (Figure S1), and 1D and 2D spectra of new compounds (Figures S2−S14). This information is available free of charge via the Internet at http://pubs.acs.org.

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■ DEDICATION

Dedicated to Dr. Koji Nakanishi of Columbia University for his pioneering work on bioactive natural products.

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