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ORIGINAL PAPER

Cliniatines A–C, new Amaryllidaceae alkaloids from *Clivia miniata*, inhibiting Acetylcholinesterase

Yusuke Hirasawa[1] · Tomoko Tanaka[1] · Shiro Hirasawa[1] · Chin Piow Wong[2] · Nahoko Uchiyama[3] · Toshio Kaneda[1] · Yukihiro Goda[3] · Hiroshi Morita[1]

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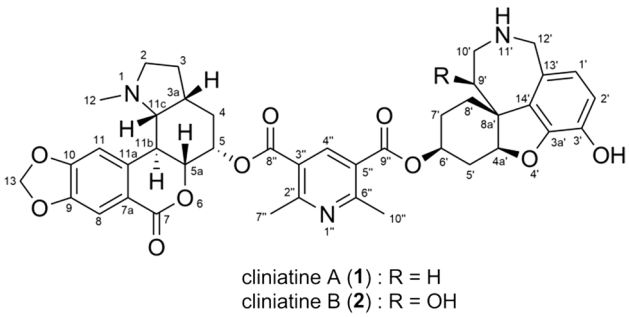
Abstract
Cliniatines A–C (**1**–**3**), three new Amaryllidaceae alkaloids, consisting of 2,6-dimetylpyridine and lycorine-type and/or galanthamine-type were isolated from *Clivia miniata* (Lindl.) Bosse. The structures and absolute configurations of **1**–**3** were elucidated based on spectroscopic data and chemical correlation. Cliniatines A–C showed moderate inhibitory activity against acetylcholinesterase.

Graphic abstract



Keywords Amaryllidaceae alkaloid · Cliniatine A · *Clivia miniata* · Acetylcholinesterase

Introduction

Clivia miniata (Lindl.) Bosse. is an evergreen plant that is distributed in southern Africa. The rhizome of the plant had been known as a remedy for snakebite, or associated pain. [1, 2]

Plants of the genus *Clivia* comprise about 4 species, [1] which were reported to be rich sources of Amaryllidaceae alkaloids such as lycorine [3], clivonine [4], clivacetine [5], clivimine [6], and galanthamine [7], among which galanthamine is used in the treatment of Alzheimer's disease due to its selective reversible and competitive inhibitory activity against Acetylcholinesterase (AChE). [8, 9] In our search for biogenetically interesting intermediates and new alkaloids with a novel skeleton from medicinal plants, [10–15] two new dimeric and a monomeric alkaloid with 2,6-dimethylpyridine unit, cliniatines A–C (**1**–**3**) were isolated from the whole plants of *C. miniata* (Fig. 1). Here we reported the isolation and structure elucidation of **1**–**3**, and its inhibitory activity against AChE.

✉ Yusuke Hirasawa
y-hirasawa@hoshi.ac.jp

✉ Hiroshi Morita
y-hirasawa@hoshi.ac.jp

[1] Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2-4-41 Shinagawa-ku, Tokyo 142-8501, Japan

[2] Tokiwa Phytochemical Co., Ltd, 158, Kinoko, Sakura-shi, Chiba 285-0801, Japan

[3] National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku Kawasaki, Kanagawa 210-9501, Japan

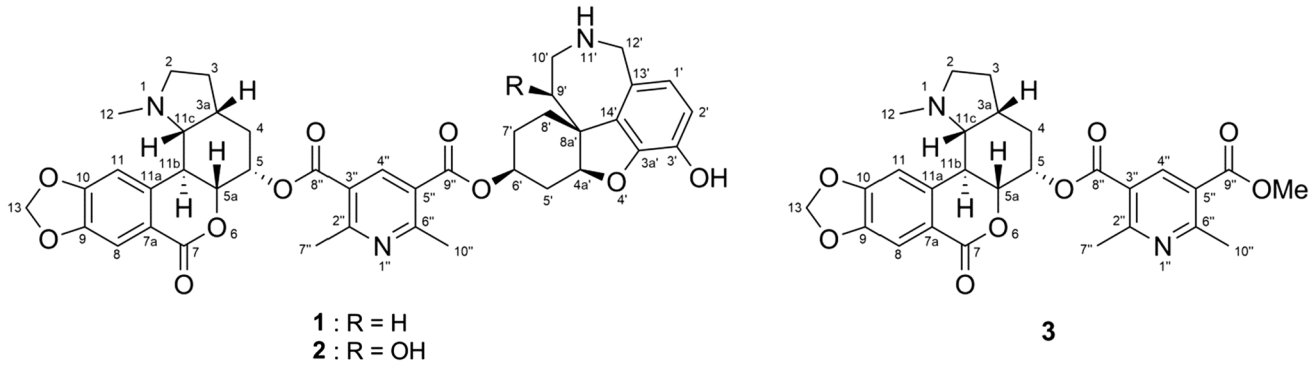


Fig. 1 Structures of **1**–**3**

Results and discussion

Cliniatine A (**1**) was shown to have the molecular formula $C_{41}H_{43}N_3O_{10}$ by HRESIMS [*m/z* 738.3027, $(M+H)^+$, $\Delta+0.0$ mmu]. The IR absorptions implied the presence of NH and/or OH (3340 cm^{-1}) and carbonyl (1720 cm^{-1}) functionalities. The analysis of ^1H and ^{13}C NMR data (Table 1) and HSQC spectrum of **1** revealed 41 carbon signals due to 7 sp^3 methines, 10 sp^3 methylenes, 3 methyls, 1 sp^3 quaternary carbon, 5 sp^2 methines, and 15 sp^2 quaternary carbons. Among them, 4 sp^3 methines (δ_C 69.4; δ_H 5.77, δ_C 78.9; δ_H 4.65, δ_C 88.9; δ_H 4.42, and δ_C 70.9; δ_H 5.38) and 1 sp^3 methylene (δ_C 104.3; δ_H 6.14 and 6.15) were attributed to those attached to an oxygen atom. Also, 1 sp^3 methine (δ_C 70.2; δ_H 4.23), 3 sp^3 methylenes (δ_C 56.2; δ_H 3.25 and 3.92, δ_C 46.8; δ_H 3.58 and 3.64, and δ_C 51.7; δ_H 4.26 and 4.39), and 1 methyl (δ_C 43.9; δ_H 3.23) were considered to be connected to the nitrogen atoms.

The gross structure of **1** was elucidated by analysis of 2D NMR data including the ^1H–^1H COSY, HMQC, and HMBC spectra in CD_3OD (Fig. 2).

The ^1H–^1H COSY spectra revealed the presence of four partial structures, **a** (C-2 ~ C5, C3a, C-5a, and C-11b ~ C-11c), **b** (C-4a′ and C-5′ ~ C-8′), **c** (C-9′ ~ C-10′), and **d** (C-1′ ~ C-2′), as shown in Fig. 2.

In unit A, the connectivity of C-2, C-11c, and C-12 through a nitrogen atom was revealed by the HMBC correlations of H_3-12/C-2 and C-11c. The HMBC cross-peaks of H_2-13/C-9 and C-10, H-11/C-7a and C-9, and H-8/C-9, C-10, and C-11a indicated the presence of the 1,3-benzodioxole (C-7a, C-8 ~ C-11, C-13, C-11a) moiety. In addition, the HMBC correlations of H-11/C-11b and H-8/C-7 indicated the linkage of C-11a ~ C-11b and C-7a ~ C-7, respectively. Finally, the connectivity between C-5a and C-7 through oxygen atom was deduced from its chemical shifts (CH-5a: δ_C 78.9; δ_H 4.65 and C-7: 164.9). Thus, unit A was assigned as clivonine [4].

In unit B, the presence of a cyclohexane ring (C-4a′, C-5′ ~ C-8′, and C-8a′) was elucidated by the HMBC correlations of H-5′b and H-8′/C-8a′ and H-4a′/C-8′. HMBC cross-peaks of H-9′/C-14′, H-10′/C-8a′, H-10′/C-12′, and H-12′/C-14′ indicated the presence of dehydroazepane ring (C-8a′, C-9′ ~ C-10′, C-12′ ~ C-14′, and *N*). Furthermore, the HMBC correlations of H-1′/C-3′ and C-14′ and H-2′/C-13′ and C-3a′ implied the presence of a phenol moiety (C-1′ ~ C-3′, C-3a′, and C-13′ ~ C-14′). Those HMBC correlations data and the chemical shift of C-3a′ (δ_C 147.4) and CH-4a′ (δ_C 88.9; δ_H 4.42) revealed that unit B was *O*-demethyl form of *N*-demethyllycoramine [16].

The remaining of unit C was elucidated as 2,6-dimethylpyridine with two ester functionalities by chemical shifts of literature [17] and HMBC correlations. The HMBC correlation of H-5/C-8″ and the chemical shift characteristic of CH-6′ (δ_C 70.9; δ_H 5.38) established the connection between C-5 and C-8″ also C-6′ and C-9″ through an ester bond, respectively.

Thus, the gross structure of cliniatine A was assigned as **1**.

The relative stereochemistry of **1** was elucidated by NOESY correlations and ^3J coupling constants.

In unit A, the NOESY correlation of H-3a/H-11c suggested that H-3a and H-11c were oriented to the same side (Fig. 3). In addition, the coupling constants of 3JH-11b/H-11c (12.6 Hz) and $^3J_{H\text{-}11b/H\text{-}5a}$ (12.3 Hz) indicated the anti-relationship of H-11b/H-11c and H-11b/H-5a, respectively. Furthermore, the *gauche*-relationship between H-5 and H-5a was elucidated by the ^3J coupling constant of H-5/H-5a (2.8 Hz). Thus, the relative configurations of unit A were the same as those of clivonine [4].

On the other hand, in unit B (Fig. 4), the coupling pattern of both H-4a′ and H-6′ was broad singlet. This indicated that both H-4a′ and H-6′ oriented equatorially in the cyclohexane ring (C-4a′, C-5′ ~ C-8′, and C-8a′). Then the NOESY correlation of H-4a′/H-9′a suggested that H-4a′ and C-9′ were oriented to the same side. Thus, the relative configuration

Table 1 ^{1}H and ^{13}C NMR data of cliniatines A (**1**) and B (**2**)

No.	**1** (TFA salt) in CD_3OD		**2** (TFA salt) in CD_3OD	
	δ_H	δ_C	δ_H	δ_C
2	3.25 (1H, m)	56.2	3.21 (1H, m)	56.2
	3.92 (1H, m)		3.90 (1H, m)	
3	2.22 (1H, m)	29.1	2.32 (1H, m)	29.2
	2.36 (1H, m)		2.41 (1H, m)	
3a	3.08 (1H, m)	34.6	3.09 (1H, m)	34.7
4	2.25 (1H, m)	27.2	2.25 (1H, m)	27.3
	2.41 (1H, brd, 16.2)		2.45 (1H, brd, 15.6)	
5	5.77 (1H, ddd, 3.0, 3.0, 2.8)	69.4	5.78 (1H, ddd, 3.0, 2.4, 2.4)	69.3
5a	4.65 (1H, dd, 12.3, 2.8)	78.9	4.65 (1H, dd, 12.6, 3.0)	78.9
7		164.9		165.0
7a		120.2		120.2
8	7.44 (1H, s)	110.9	7.45 (1H, s)	110.9
9		149.4		149.4
10		154.6		154.6
11	6.78 (1H, s)	105.9	6.80 (1H, s)	105.9
11a		136.8		136.9
11b	3.58 (1H, dd, 12.6, 12.3)	36.2	3.57 (1H, dd, 12.6, 12.6)	36.2
11c	4.23 (1H, m)	70.2	4.22 (1H, m)	70.2
12	3.23 (3H, s)	43.9	3.21 (3H, s)	44.0
13	6.14 (1H, s)	104.3	6.15 (1H, s)	104.3
	6.15 (1H, s)		6.16 (1H, s)	
1′	6.71 (1H, d, 8.4)	123.4	6.71 (1H, s)	123.8
2′	6.68 (1H, d, 8.4)	116.9	6.71 (1H, s)	117.3
3′		144.2		144.2
3a′		147.4		148.3
4a′	4.42 (1H, brs)	88.9	4.80 (1H, brs)	89.0
5′	2.20 (1H, m)	29.6	2.38 (1H, m)	32.2
	2.65 (1H, brd, 16.1)		2.57 (1H, brd, 16.2)	
6′	5.38 (1H, m)	70.9	5.41 (1H, m)	71.5
7′	1.87 (1H, m)	25.6	1.52 (1H, m)	25.4
	1.96 (1H, m)		2.24 (1H, m)	
8′	1.75 (1H, m)	25.1	1.80 (1H, m)	22.2
	1.93 (1H, m)		2.08 (1H, dd, 12.0, 12.0)	
8a′		47.9		53.4
9′a	2.03 (1H, brdd, 14.1, 12.6)	33.1	4.10 (1H, dd, 10.8, 3.0)	72.8
9′b	2.29 (1H, brd, 14.1)			
10′	3.58 (1H, m)	46.8	3.40 (1H, m)	51.3
	3.64 (1H, m)		3.53 (1H, m)	
12′	4.26 (1H, d, 15.0)	51.7	4.20 (1H, d, 14.1)	49.9
	4.39 (1H, d, 15.0)		4.32 (1H, d, 14.1)	
13′		121.3		122.2
14′		137.0		131.8
2″		163.1		163.2
3″		124.8		124.5
4″	8.67 (1H, s)	142.1	8.67 (1H, s)	141.9
5″		125.2		125.0
6″		163.5		163.7
7″	2.83 (3H, s)	24.2	2.84 (3H, s)	24.4
8″		165.7		165.5

Table 1 (continued)

No.	**1** (TFA salt) in CD_3OD		**2** (TFA salt) in CD_3OD	
	δ_H	δ_C	δ_H	δ_C
9″		166.9		165.7
10″	2.81 (3H, s)	25.0	2.81 (3H, s)	25.3

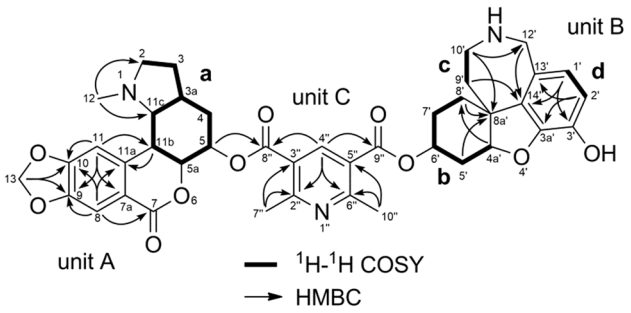


Fig. 2 Selected 2D NMR correlations for cliniatine A (**1**)

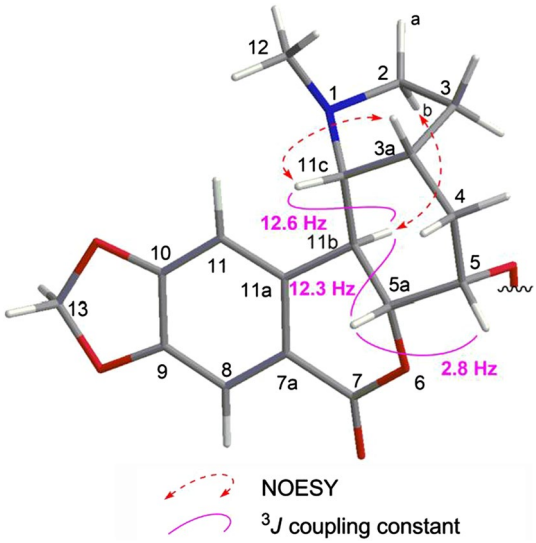


Fig. 3 Selected NOESY correlations and ^{3} *J* coupling constants for unit A of cliniatine A (**1**)

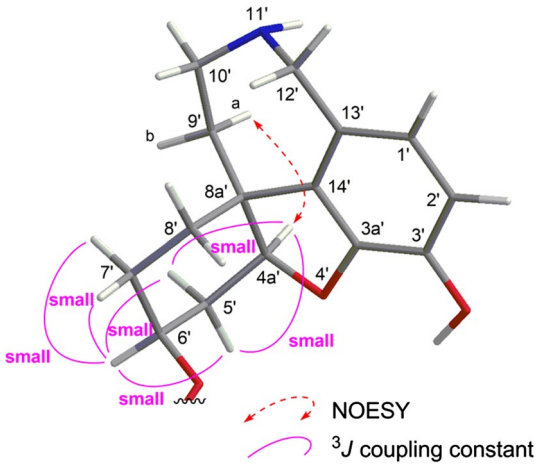


Fig. 4 Selected NOESY correlations and ^{3} *J* coupling constants for unit B of cliniatine A (**1**)

of unit B was identified to be the same as *O*-demethyl form of *N*-demethyllycoramine [16].

Cliniatine B {**2**, [α] -28 (*c* 1.0, MeOH) showed the pseudomolecular ion peak at *m/z* 776 $(M+Na)^+$ and the molecular formula, $C_{41}H_{43}N_3O_{11}$, was established by HRESIMS [*m/z* 776.2797, $(M+Na)^+$, Δ + 0.2 mmu]. The IR absorptions implied the presence of NH and/or OH (3350 cm^{-1}) and carbonyl (1720 cm^{-1}) functionalities. The ^{1}H and ^{13}C NMR (Table 2) spectra suggested that **2** had a similar structure as that of **1**, except for the presence of a hydroxy group at C-9′ (δ_C 72.8; δ_H 4.10). The α-configuration of H-9′ was elucidated by NOESY correlation of H-4a′/H-9′ (Fig. 5).

The HRESIMS data [*m/z* 509.1925, $(M+H)^+$, Δ + 0.1 mmu] of cliniatine C (**3**) established the molecular formula to be $C_{27}H_{28}N_2O_8$, which was smaller than cliniatine A (**1**) by a $C_{14}H_{15}NO_2$ unit. The ^{1}H and ^{13}C NMR data (Table 3) of **3** were analogous to those of **1**, although ^{1}H and ^{13}C signals of *N*-demethyllycoramine moiety observed for **1** were absent for **3**.

Detailed analyses of 2D NMR spectra of **3** indicated that cliniatine C possessed methyl ester instead of *N*-demethyllycoramine moiety of **1** (Fig. 6). The relative stereochemistry of clivonine moiety of **3** was the same as **1**.

The CD spectra (Fig. 7) of **1**–**3** showed a similar CD curve to that of clivonine [18] [λ_{max} 233 (Δε + 16.9), 252 (− 1.46), 269 (+ 0.90), and 314 (− 2.65) nm]. The absolute stereochemistry of clivonine has been established by chemical correlation [19–24]. Therefore, the absolute configurations of clivonine moiety of **1**–**3** were assigned as 3a*R*,5*S*,5a*R*,11b*S*,11c*R*.

To elucidate the absolute configurations of *N*-demethyllycoramine moiety, **2** was hydrolyzed by $LiAlH_4$ condition. However, the reaction gave a complex mixture.

Therefore, the absolute configuration was elucidated by comparing the CD data of *N*-demethyllycoramine, which was isolated in this study, and lycoramine [7] synthesized from commercially available (–)–galanthamine. The CD spectrum of *N*-demethyllycoramine in MeOH showed a similar CD curve to that of lycoramine (see supporting

Table 2 ^{1}H and ^{13}C NMR data of cliniatine C (**3**)

No	**3** (free base) in $CDCl_3$	
	δ_H	δ_C
2	2.66 (1H, m), 3.37 (1H, m)	52.6
3	1.94 (1H, m), 2.16 (1H, m)	30.5
3a	2.66 (1H, m)	32.6
4	2.01 (1H, m), 2.48 (1H, brd, 16.2)	27.5
5	5.63 (1H, ddd, 3.0, 3.0, 2.9)	70.0
5a	4.29 (1H, dd, 12.2, 2.9)	78.7
7		164.1
7a		118.7
8	7.50 (1H, s)	109.4
9		146.9
10		152.7
11	7.79 (1H, s)	107.1
11a		139.9
11b	3.32 (1H, dd, 12.2, 9.3)	34.7
11c	3.04 (1H, dd, 9.3, 7.2)	69.5
12	2.59 (3H, s)	45.0
13	6.04 (1H, m), 6.06 (1H, m)	101.9
2′		162.9
3′		122.6
4′	8.67 (1H, s)	140.9
5′		122.6
6′		162.9
7′	2.87 (3H, s)	25.2
8′		164.7
9′		166.0
10′	2.85 (3H, s)	25.0
11′	3.88 (3H, s)	52.4

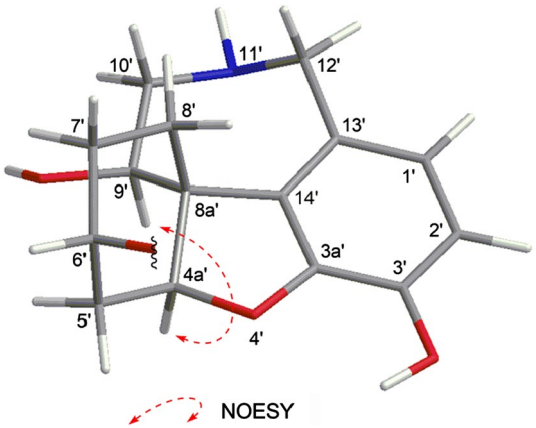


Fig. 5 Selected NOESY correlation for cliniatine B (**3**)

information S22). Considering that *N*-demethyllycoramine and cliniatines A and B those were isolated from

Table 3 Inhibitory activity against Acetylcholinesterase (IC_{50} in μM)

	1	**2**	**3**	Lycorine	Galanthamine
AChE	5.7	68.8	> 100	> 100	4.69

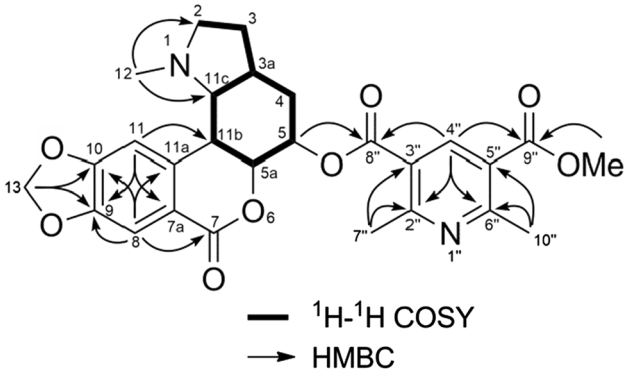


Fig. 6 Selected 2D NMR correlations for cliniatine C (**3**)

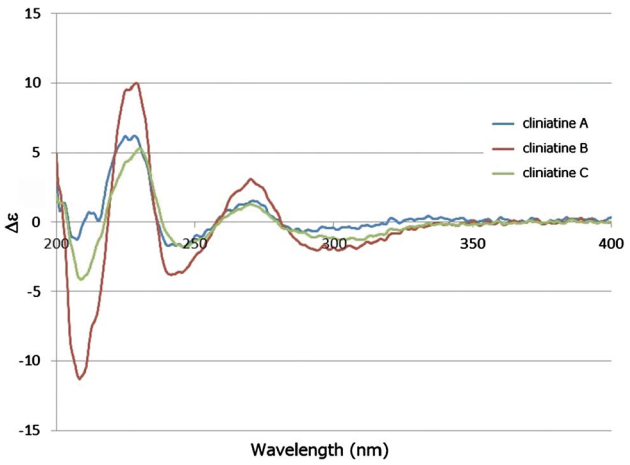


Fig. 7 CD spectra of cliniatines A–C (**1**–**3**) in MeOH

this study having similar structures, the absolute configuration of **1** and **2** were considered as 4a′*S*,6′*S*,8a′*S* and 4a′*S*,6′*S*,8a′*S*,9′*R*, respectively.

A plausible biogenetic pathway for cliniatines A–C is proposed as shown in Fig. 8. It was considered that both galanthamine and clivonine moieties of cliniatine A were generated from nobelladine, and 2,6-dimethylpyridine-3,5-dicarboxylate binding to them was biosynthesized from four acetic acid, ammonia, and formaldehyde. [17]

The isolated compounds were tested for inhibitory activity against AChE. [25] As can be seen in Table 3, **1** inhibited AChE with an IC_{50} value of 5.7 μM, which was comparable to that of galanthamine (Table 3).

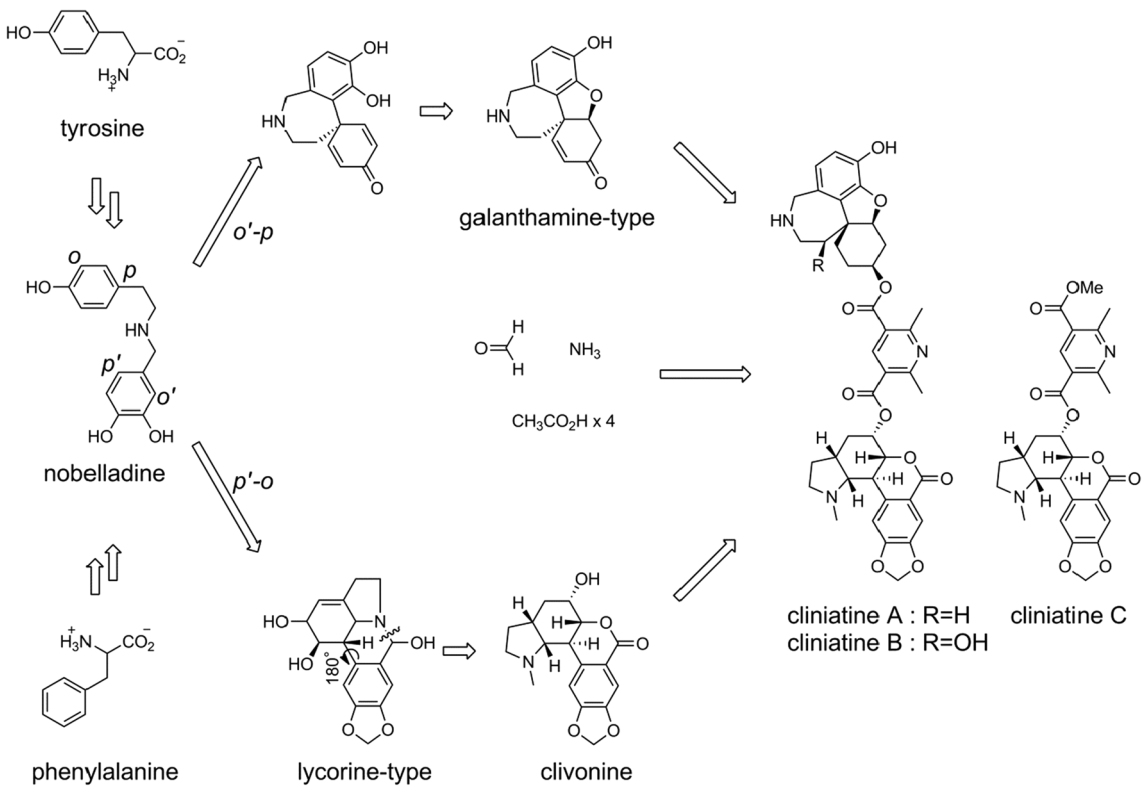


Fig. 8 Plausible biogenetic pathway for cliniatines A–C (**1**–**3**)

Experimental section

General Experimental Procedures. Optical rotations were measured with a JASCO P-1030 polarimeter. IR spectra were obtained by a JASCO FT/IR-230 using Zn/Se cell. CD spectra were obtained using a JASCO J-820 spectropolarimeter. 1D and 2D spectra were recorded on a JEOL ECZ600 and Bruker AV400 spectrometer. Chemical shifts (ppm) were referenced to the residual solvent peaks (δ_H 7.26 and δ_C 77.0 for $CDCl_3$ and δ_H 3.31 and δ_C 49.0 for CD_3OD). Positive mode ESITOFMS was obtained on a Waters Xevo G2-XS QTof LC/MS spectrometer using a sample dissolved in MeOH. Column chromatography was performed using silica gel (230–400 mesh; Merck KGaA, Darmstadt, Germany), amino silica gel (NH-DM1020; Fuji Silysia Chemical Ltd., Aichi, Japan), and ODS HPLC (CAPCELL PAK C_{18} MG II; Shiseido, Tokyo, Japan).

Material. Clivia miniata was collected at Akita, Japan in 2015. The botanical identification was made by Dr. Yusuke Hirasawa, Faculty of Pharmaceutical Sciences, Hoshi University. The voucher specimen (Herbarium No. HS31) was deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Hoshi University, Tokyo, Japan.

Extraction and isolation. The whole plants of *Clivia miniata* (481 g) collected in Akita were extracted with MeOH at rt, and the extract (93 g) was partitioned between EtOAc and 3% tartaric acid. Water-soluble materials, which were adjusted at pH 10 with sat. Na_2CO_3 aq., were extracted with $CHCl_3$. $CHCl_3$-soluble materials (1050 mg) were subjected to an amino SiO_2 column (hexane/EtOAc, 1:0 → 0:1, and then $CHCl_3$/MeOH, 1:0 → 0:1) to give nine fractions (I–IX).

The fraction VIII was further separated by a SiO_2 column ($CHCl_3$/MeOH, 4:1 → 0:1) and, then ODS HPLC (25% CH_3CN aq/0.1% TFA) to afford cliniatines A (**1**, 2.7 mg, 0.0006%), B (**2**, 8.2 mg, 0.002%), C (**3**, 3.3 mg, 0.0007%), and lycorine (10.6 mg, 0.002%). The fraction VI was subjected to a SiO_2 column to give clivimine (280 mg, 0.06%) and *N*-demethyllycoramine (5.6 mg, 0.001%). The fraction II was purified by SiO_2 column ($CHCl_3$/MeOH, 10:1 → 0:1) to afford clivonine (30.5 mg, 0.006%).

Cliniatine A (1): colorless amorphous solid; [α] − 30 (*c* 1.0, MeOH); IR (Zn-Se) λ_{max} 3340, 2920, 1720, 1260, and 1030 cm^{-1}; UV (MeOH) λ_{max} 306 (log ε 3.57), 273 (3.84), 227 (4.41), and 206 (4.41) nm; CD (MeOH) λ_{max} 225 ($\Delta\varepsilon$ + 6.18), 240 (− 1.73), 271 (+ 1.50), 289 (− 0.70) nm; ESIMS *m/z* 738 $(M+H)^+$; HRESIMS *m/z* 738.3027 [calcd for $C_{41}H_{44}N_3O_{10}$ $(M+H)^+$].

Cliniatine B (2): colorless amorphous solid; [α] − 28 (*c* 1.0, MeOH); IR (Zn-Se) λ_{max} 3350, 2960, 2930, 1720, and 1560 cm^{-1}; UV (MeOH) λ_{max} 306 (log ε 3.49), 273 (3.74), and 205 (4.62) nm; CD (MeOH) λ_{max} 208 ($\Delta\varepsilon$ − 11.27),

229 (+10.00), 241 (−3.79), 270 (+3.09), 294 (−2.03) nm; ESIMS *m/z* 776 $(M+Na)^+$; HRESIMS *m/z* 776.2797 [calcd for $C_{41}H_{43}N_3O_{11}Na$ $(M+Na)^+$].

Cliniatine C (3): pale yellow amorphous solid; [α] +4 (*c* 1.0, MeOH); IR (Zn–Se) λ_{max} 3440, 2950, 1710, 1670, and 1030 cm^{-1}; UV (MeOH) λ_{max} 306 (log ε 3.44), 273 (3.57), 228 (4.13), and 204 (4.24) nm; CD (MeOH) λ_{max} 209 ($\Delta\varepsilon$ −4.13), 230 (+5.30), 246 (−1.83), 270 (+1.26), 293 (−1.11) nm; ESIMS *m/z* 509 $(M+H)^+$; HRESIMS *m/z* 509.1925 [calcd for $C_{27}H_{29}N_2O_8$ $(M+H)^+$].

Hydrolysis of cliniatine B (2). To a solution of **2** (0.5 mg) in THF (100 µl) was added $LiAlH_4$. The mixture was allowed to stand at 80 °C for 12 h, and then concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ and washed with Na_2CO_3 aq. After evaporation of solvent, complex mixture was obtained.

Hydrogenation of galanthamine. Galanthamine (20.7 mg) was dissolved in MeOH and hydrogenated over 10% Pd/C (17 mg) for 19 h. The catalyst was removed by filtration, and the filtrate was evaporated to give a residue, which was separated by SiO_2 ($CHCl_3$/MeOH, 10:1 → 0:1) to give lycoramine (7.0 mg) as an colorless amorphous solid; ^1H NMR (400 MHz, $CDCl_3$) δ 6.56 (1H, d, 8.4), 6.61 (1H, d, 8.4), 4.33 (1H, t, 3.2), 4.05 (1H, m), 3.95 (1H, d, 15.0), 3.82 (3H, s), 3.58 (1H, d, 15.1), 3.17 (1H, dd, 13.4, 13.4), 3.00 (1H, m), 2.45 (1H, brd, 16.0), 2.33 (3H, s); ESIMS *m/z* 290.

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