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Vobasinyl-iboga bisindole alkaloids, potent acetylcholinesterase inhibitors from *Tabernaemontana divaricata* root

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Abstract

The roots of the Thai medicinal plant, *Tabernaemontana divaricata* (L.) R. Br. ex Roem. & Schult., were investigated for their content of acetylcholinesterase inhibitors. Bioassay-guided fractionation using the Ellman colorimetric method led to the isolation of two bisindole alkaloids, 19,20-dihydrotabernamine and 19,20-dihydroervahanine A. The compounds showed higher inhibitory activity on acetylcholinesterase in comparison with galanthamine, a well-known acetylcholinesterase inhibitor. The inhibitory activity of 19,20-dihydroervahanine A was proved to be specific, reversible and competitive. During the separation process, two inactive bisindole alkaloids, conodurine and tabernaelegantine A, were also isolated. The data suggest that the substitutions at the carbons 11′, 12′ and 16′ might affect the acetylcholinesterase inhibitory activity.

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Introduction

Alzheimer's disease is a progressive degenerative neurologic disorder resulting in impaired memory and behaviour. Epidemiological data indicate a potentially considerable increase in the prevalence of the disease over the next two decades (Johnson et al 2000). Most treatment strategies have been based on the cholinergic hypothesis, which postulates that memory impairments in patients suffering from this disease results from a deficit of cholinergic function in the brain. One of the most promising approaches for treating this disease is to enhance the acetylcholine level in the brain using acetylcholinesterase (AChE) inhibitors (Scarpini et al 2003). At present, AChE inhibitors are used in the treatment of Alzheimer's disease and also senile dementia, ataxia, myasthenia gravis and Parkinson's disease.

Tabernaemontana is a large genus belonging to the Apocynaceae family. There are about 100 species of *Tabernaemontana* widely distributed in tropical America, Africa, Asia and Australia (Leeuwenberg 1991). They are known for producing a wide variety of indole alkaloids of unusual structures, as well as novel bioactivity (van Beek et al 1984). *Tabernaemontana divaricata* (L.) R. Br. ex Roem. et Schult. (*Ervatamia coronaria* (Jacq.) Stapf., *E. divaricata* (L.) Burkill, *T. coronaria* (Jacq.) Willd) is a common garden plant in Southeast Asia and other tropical countries. It is also used as traditional medicine in many countries. In Thailand, the root of this plant is used as a neurotonic and analgesic (Taesotikul at al 1989). The plants have been reported to have various biological actions such as analgesic effect (Taesotikul at al 1989; Henriques et al 1996), cell proliferation inhibition (Kuo et al 1999), anti-inflammatory (Henriques et al 1996), and cytotoxic activity (Melo et al 1986). In a previous study we found that the alcoholic extract of *T. divaricata* roots contains a high AChE inhibitory activity (Ingkaninan et al 2003). We therefore aimed in this study at the isolation of AChE inhibitors from this plant.

Material and Methods

General experimental procedures

Optical rotation was measured with a Perkin Elmer 341 Polarimeter. The UV spectra were recorded on a Varian CARY 1E spectrometer. The ^{1}H, ^{13}C, DEPT

(distortionless enhancement by polarization transfer), ^1H-^1H COSY (correlated spectroscopy), NOESY (nuclear overhauser and exchange spectroscopy), HMQC (heteronuclear multiple quantum correlation experiment) and HMBC (heteronuclear multiple bond correlation experiment) NMR experiments were carried out using a Bruker av400 NMR spectrometer, operating at 400 MHz for proton and 100 MHz for carbon identification. The ESITOFMS (electrospray ionization time-of-flight mass spectrometry) spectra were obtained using a Micromass LCT mass spectrometer and the lock mass calibration was applied for the determination of the accurate masses.

Plant material

T. divaricata was collected from Phitsanulok, Thailand in July 2003. The voucher specimen (collection no. Changwijit 001) was deposited at a PBM herbarium, Faculty of Pharmaceutical Sciences, Mahidol University.

Extraction and isolation

Flowers, leaves, stems and roots of *T. divaricata* were separated from the whole plants and dried at 55°C. The dried materials were ground and macerated with 95% ethanol twice (for 3 and 7 days). The ethanol extracts were evaporated under reduced pressure until dryness.

The ethanolic extract (229 g) obtained from the dried roots (2.5 kg) was dissolved in phosphate buffer (pH 3) and washed with EtOAc. The aqueous part was basidified with sodium bicarbonate solution until pH 10 and extracted with EtOAc. The organic layer was dried under reduced pressure to give the crude alkaloidal extract (19.3 g).

The crude alkaloidal extract was roughly separated by quick CC separation (13 × 6 cm) on silica gel 60 (300 g, Merck 1.09385.1000) using $CHCl_3$ with increasing proportions of MeOH to give 26 fractions (I–XXVI). **VIII** (fr. no. 15–16, $CHCl_3$–MeOH, 97:3, 2 × 300 mL), **IX** (fr. no. 17–19, $CHCl_3$–MeOH, 97:3, 3 × 300 mL) and **X** (fr. no. 20–23, $CHCl_3$–MeOH, 9:1, 4 × 300 mL), were further purified. **X** (1.6 g) was repeatedly chromatographed on a Sephadex LH-20 CC (2.5 × 110 cm) by elution with MeOH to give a yellowish gum (fr. no. 3–4, 2 × 50 mL, 259 mg), followed by silica gel CC separation (48 g, 3.5 × 15 cm) eluted with CH_2Cl_2–MeOH (gradient) to give 21 fractions. Fr 9 (fr. no 42–45 × 30 mL, 21 mg) was further subjected to Sephadex LH-20 CC (2.5 × 110 cm) and eluted with MeOH yielding compound **1** (Figure 1) (fr. 10–12, 3 × 40 mL, 7 mg). **IX** (2.1 g) was subjected to silica gel CC (97 g, 5 × 15 cm) eluted with CH_2Cl_2–MeOH (gradient) to give compound **3** (fr. no. 5–8, CH_2Cl_2–MeOH, 97:3, 4 × 30 mL, 10 mg). **VIII** (4.2 g) was repeatedly chromatographed on silica gel CC (174 g, 10 × 6 cm) using CH_2Cl_2–MeOH (gradient) as a mobile phase to give a light yellow mass (fr. 5–8, CH_2Cl_2–MeOH, 97:3, 4 × 300 mL, 3.6 g), followed by CC over silica gel (174 g, 10 × 6 cm) using CH_2Cl_2–MeOH (gradient) yielding a light yellow mass (fr. 5–8, CH_2Cl_2–MeOH, 97:3, 4 × 200 mL, 2.1 g). It was then subjected to silica gel CC (103 g, 5 × 15 cm) using CH_2Cl_2–MeOH (gradient) as a mobile phase resulting in 40 fractions from which compound **4** was obtained (fr. 15–17, CH_2Cl_2–MeOH, 98:2, 3 × 50 mL, 30 mg). Fractions 20–30 (CH_2Cl_2–MeOH, 98:2, 3 × 50 mL, 415 mg) were chromatographed on a Sephadex LH-20 CC (2.5 × 92 cm) eluted with MeOH to give fr. 19–20 (2 × 30 mL, 70 mg), followed by a separation on a Sephadex LH-20 CC (2.5 × 92 cm) eluted with MeOH yielding a whitish mass (fr. 3–9, 7 × 30 mL, 64 mg). The fractions obtained were subjected to a silica gel TLC plate (10 × 20 cm, 1 mm thickness). The fraction with an Rf value of 0.5 (25 mg) was obtained after the plate was developed with CH_2Cl_2–MeOH, 95:5. It was chromatographed on a Sephadex LH-20 CC (2.5 × 92 cm) eluted with MeOH to give 6 fractions. Frs 4–5 (2 × 30 mL, 19 mg) was repeatedly separated on a preparative TLC (10 × 20 cm, 1 mm thickness) using ethylacetate–hexane 1:1 saturated with ammonia as a mobile phase to yield **2** (Rf 0.5, 2.1 mg).

*19,20-Dihydrotabernamine (**1**)*
White solid; UV (MeOH) (log ϵ) 240 (4.52), 287 (4.22); ^1H NMR ($CDCl_3$) see Table 1; ^{13}C NMR (CD_3OD) δ: 173.5 (C-17), 143.2 (C-2′), 140.5 (C-11′), 139.1 (C-2), 138.0 (C-13), 136.8 (C-13′), 131.1 (C-8), 129.4 (C-8′), 122.0 (C-11), 119.7 (C-10′), 119.2 (C-10), 118.5 (C-9′), 118.2 (C-9), 111.0 (C-12), 110.2 (C-12′), 110.1 (C-7), 108.8 (C-7′), 60.8 (C-5), 59.3 (C-21′), 55.5 (C-5′), 50.8 (C-3′), 50.6 (CO_2CH_3), 48.3 (C-21), 46.8 (C-3), 44.9 (C-16), 44.4 (C-20), 43.6 (C-14), 43.5 (C-20′), 43.1 (NCH_3), 42.2 (C-16′), 35.9 (C-15), 35.3 (C-17′), 33.1 (C-15′), 28.7 (C-19′), 27.9 (C-14′), 26.7 (C-19), 21.7 (C-6′), 18.7 (C-6), 13.0 (C-18), 12.3 (C-18′); ES-MS m/z (rel. int.) 295 (5), 310 (30), 597 (3), 619 $[M+H]^+$ (100); $C_{40}H_{50}N_4O_2$.

*19,20-Dihydroervahanine A (**2**)*
White solid; ^1H NMR ($CDCl_3$) see Table 1; EIMS m/z (rel. int.) 363 (45), 494 (100), 676 (21), 705 $[M+H]^+$(2); $C_{42}H_{52}N_4O_4$.

*Conodurine (**3**)*
White solid; ^1H NMR ($CDCl_3$) see Table 1; ES-MS m/z (rel. int.) 338 (3), 353 (100), 705 $[M+H]^+$ (87); $C_{43}H_{52}N_4O_5$.

*Tabernaelegantine A (**4**)*
White solid; ^1H NMR ($CDCl_3$) see Table 1; EIMS m/z (rel. int.) 307 (22), 354 (100), 395 (85), 707 $[M+H]^+$(86); $C_{43}H_{54}N_4O_5$.

Cholinesterase activity determination

The assay for measuring AChE was performed as previously described (Ellman et al 1961; Ingkaninan et al 2003). The AChE used in the assay was from electric eel (type VI-S, EC 3.1.1.7; Sigma). Briefly, 125 μL of 3 mM 5,5′-dithiobis[2-nitrobenzoic acid], 25 μL of 1.5 mM acetylthiocholine iodide (Sigma), 50 μL of 50 mM Tris-HCl buffer, pH 8.0, and 25 μL of sample dissolved in buffer

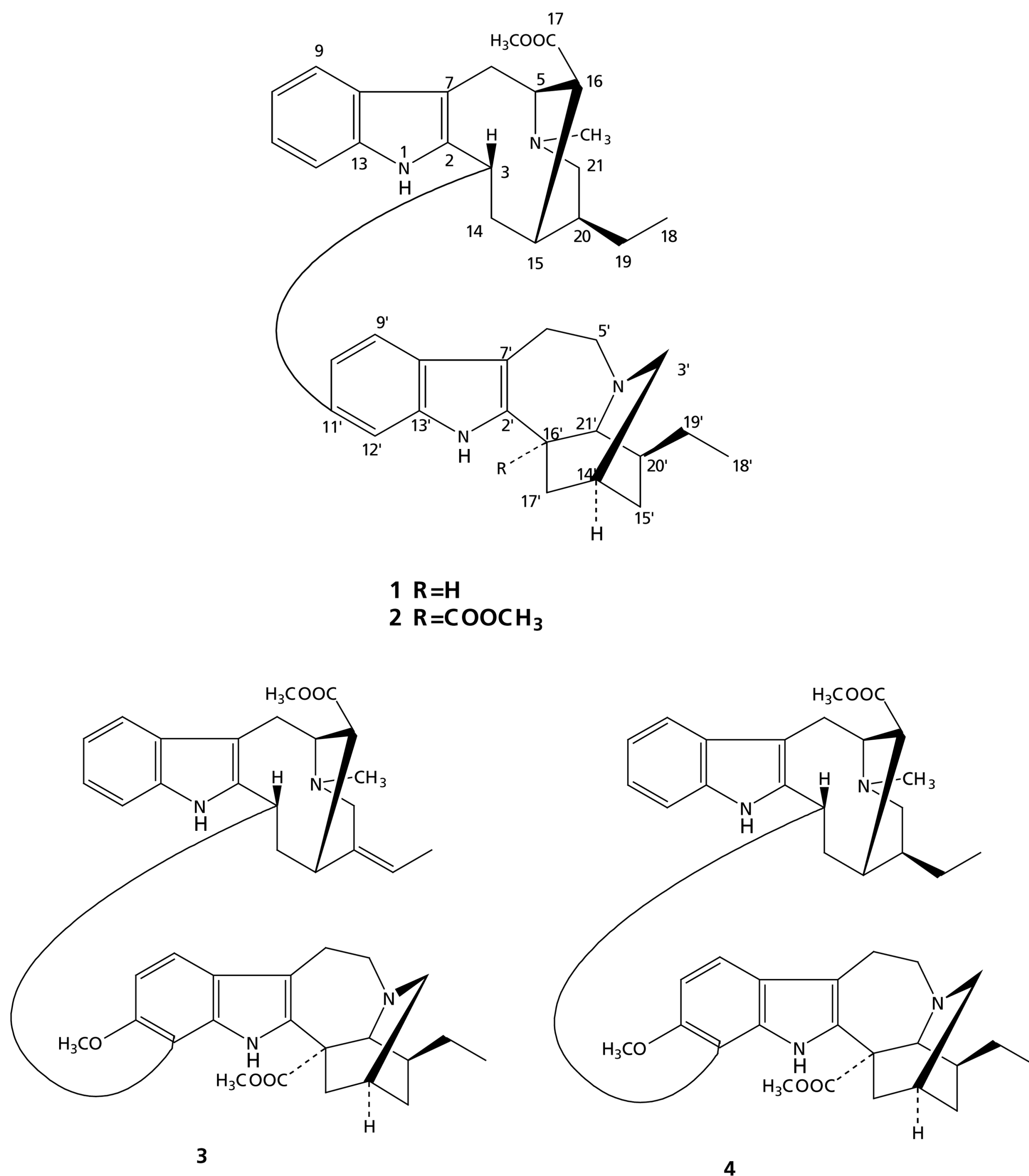

Figure 1 Structures of **1**–**4** isolated from *Tabernaemontana divaricata* roots.

containing not more than 10% methanol were added to the wells followed by 25 μL of 0.28 U mL^{-1} AChE. The microplate was then read at 405 nm every 5 s for 2 min by a CERES UV 900C microplate reader (Bio-Tek instrument, USA). The velocities of the reactions were measured. Enzyme activity was calculated as a percentage of the velocity of sample compared with that of the blank. Inhibitory activity was calculated from one-hundred percentage subtracted by the percentage of enzyme activity. Every experiment was done in triplicate. Sample solutions were prepared in Tris-HCl buffer containing not more than 10% MeOH. The IC50 (concentration required for half-maximal AChE inhibition), K_m and V_{max} values were analysed using the software package Prism (Graph Pad Inc, San Diego, CA, USA). The assay for butyrylcholine esterase (BuChE) determination was

Table 1 ^{1}H NMR spectral data of **1**, **2**, **3** and **4** (400 MHz)

	1 (in CD_3OD)	2 (in $CDCl_3$)	3 (in $CDCl_3$)	4 (in $CDCl_3$)
3	4.51 dd (2.0, 12.5)	4.60 brd (9.3)	5.33 dd (3.3, 13.1)	5.28 dd (3.2, 11.9)
5	3.92 t (7.9)	3.98 m	4.18 m	4.16 m
6	3.13 dd (8.0, 14.5)	3.00 m	3.46 m	3.38 m
	3.41 dd (10.5, 14.2)	3.41 m		
9	7.48 dd (6.3, 3.0)	7.52 brd (7.5)	7.66 brd (7.8)	7.62 brd (7.6)
10	6.96 m	7.05 m	7.13 ddd (1.1, 6.8, 7.5)	7.12 ddd (1.1, 6.9, 7.5)
11	6.96 m	7.05 m	7.07 ddd (1.1, 6.8, 7.7)	7.07 ddd (1.1, 6.9, 7.7)
12	7.05 dd (6.3, 3.0)	6.93 brd (8.0)	7.02 brd (7.7)	7.01 brd (7.8)
14	1.95 m	2.02 m	1.91 ddd (3.4, 7.0, 15.3)	1.95 m
	2.77 m	2.75 m	2.64 m	2.73 m
15	2.60 m	2.67 m	3.84 m	2.67 m
16	2.77 m	2.67 m	3.01 m	2.67 m
18	0.94 t (7.2)	0.95 t (7.3)	1.68 dd (1.4, 6.7)	0.96 t (7.4)
19	1.50 m	1.50 m	5.37 q (6.8)	1.51 m
	1.60 m			
20	1.32 m	1.35 m		1.38 m
21	2.45 m	2.50 m		2.45 m
	3.05 m	3.17 m	3.26 m	3.04 m
C17-$COOCH_3$	2.45 br s	2.44 s	2.51 s	2.47 s
NH		7.67 s	7.70 s	7.66 s
N CH_3	2.45 brs	2.44 s	2.69 brs	2.67 brs
3′	2.86 dt (1.0, 9.3)	2.46 m	2.46 d (8.4)	2.45 d (8.5)
	3.02 m	2.66 m	2.66 m	2.67 m
5′	3.02 m	3.02 m	2.89 m	2.98 m
	3.26 m	3.17 m	3.08 m	3.18 m
6′	2.60 m	2.92 m	2.85 m	2.91 m
	3.27 m	3.10 m	3.14 m	2.98 m
9′	7.25 d (8.1)	7.38 d (8.3)	7.25 d (8.6)	7.26 d (8.6)
10′	6.82 dd (1.1, 8.1)	7.25 m	6.83 d (8.6)	6.83 d (8.6)
12′	6.96 br s	7.05 br s		
14′	1.77 br s	1.72 m	1.51 m	1.50 m
15′	1.16 br d (10.1)	1.11 m	0.89 m	0.89 m
	1.81 m	1.72 m	1.51 m	1.45 m
16′	2.96 br d (11.8)			
17′	1.55 m	0.88 m	0.71 brd (12.6)	0.67 brd (12.9)
	2.03 br t (12.6)	1.77 m	1.77brd (13.8)	1.76 brd (13.7)
18′	0.91 t (7.0)	0.88 t (7.3)	0.81 t (7.4)	0.81 t (7.4)
19′	1.44 m	1.50 m	1.35 m	1.50 m
	1.55 m		1.51 m	
20′	1.98 m	1.10 m	1.12 quintet (7.4)	1.11 quintet (7.7)
21′	2.77 m	3.52 brs	3.37 brs	3.30 brs
C11-OCH_3			3.98 s	3.97 s
C16′-$COOCH_3$		3.68 s	3.69 s	3.70 s
N′H		7.44 s	7.57 s	7.65 s

similar to the AChE assay as described above except that horse BuChE (EC 3.1.1.3) and butyrylthiocholine chloride (Sigma) were used as the enzyme and substrate.

Statistical analysis

The effects of the various samples on AChE represented as % inhibition and IC50 values were statistically evaluated using the Kruskal–Wallis test. Individual differences were then assessed using a post-hoc test. In all cases $P < 0.05$ denoted significance. Each experiment was done in triplicate.

Results and Discussion

The different parts of *T. divaricata* were tested for their AChE inhibitory activity using the Ellman colorimetric method (Ellman et al 1961) (Table 2). It became clear that a high activity was found in stem and root extracts while leaf and flower extracts gave lower activity. The IC50 values of the stem and root extracts on AChE were 2.92 ± 0.48 and $4.81 \pm 0.55\,mg\,L^{-1}$, respectively.

The bioassay-guided fractionation of *T. divaricata* roots provided two AChE inhibitors, **1** and **2**. The UV spectrum of **1** showed the characteristic of an indole

Table 2 Acetylcholinesterase inhibitory activity of the ethanolic extracts from different parts of *T. divaricata* at a concentration of 0.1 mg mL^{-1}

Extract source	% Inhibition
Flowers	51.81 ± 0.87
Leaves	41.78 ± 0.72
Stems	94.72 ± 2.09
Roots	99.72 ± 0.26

The experiments were done in triplicate. The values are expressed as average ± standard deviation.

chromophore with λ_{max} of 240 and 287 nm. MS indicated a molecular weight of 618. The ^{1}H NMR and ^{13}C NMR spectra were assigned using COSY, HMQC and HMBC techniques (Table 1). They revealed a bisindole structure composed of an unsubstituted vobasinyl unit and an iboga unit. The NOESY experiment suggested 16 S, 20 S and 20′ S configurations. The structure of **1** was therefore concluded to be 19,20-dihydrotabernamine. Although this compound has previously been found in the root bark of *T.* coffeoides (van Beek et al 1984), the complete ^{1}H and ^{13}C NMR assignments of this molecule are reported here for the first time. The ^{1}H NMR spectrum of **2** was similar to that of **1**, except for the presence of the methyl protons at δ 3.69 ppm. The structure of **2** was elucidated as 19,20-dihydroervahanine A, which was a carbomethoxy derivative of **1**. The ^{1}H NMR and MS data were in agreement with the report of Henriques et al (1996).

Determination of the AChE inhibitory activity using a microplate assay revealed that 19,20-dihydrotabernamine and 19,20-dihydroervahanine A inhibited the AChE activity in a dose-dependent manner. The concentrations required for half-maximal AChE inhibition (IC50 values) of 19,20-dihydrotabernamine and 19,20-dihydroervahanine A were 227 ± 154 and 71 ± 13 nM, respectively, which were significantly lower than that of the well-known AChE inhibitor galanthamine (594 ± 140 nM) (Table 3). These results indicate that 19,20-dihydrotabernamine and 19,20-dihydroervahanine A are approximately 2-fold and 8-fold more potent than galanthamine. The fact that 19,20-dihydroervahanine A is more potent than 19,20-dihydrotabernamine suggests that a carbomethoxy substitution at C16′ increases the AChE inhibitory activity.

Table 3 IC50 values on acetylcholinesterase of the alkaloids isolated from *T. divaricata* in comparison with galanthamine

Compounds	IC50 (nM)
1	227 ± 154
2	71 ± 13
3	More than 10^{5}
4	More than 10^{5}
Galanthamine	594 ± 140

The values are the averages ± standard deviations from triplicate experiments.

Inhibition of AChE by 19,20-dihydroervahanine A was independent of incubation time (up to 60 min, data not shown). This result suggests that 19,20-dihydroervahanine A inhibited AChE reversibly. The kinetic analysis of the AChE inhibitory activity of 19,20-dihydroervahanine A is shown in Figure 2. The K_m and V_{max} values were calculated from nonlinear regression using the software package Prism. Upon the addition of 19,20-dihydroervahanine A (1.0 μM), the V_{max} value of AChE against ATCI was relatively unchanged (from 138 to 105 dmA min^{-1}). In contrast, the K_m value was significantly increased (from 848 to 1599 μM). These results indicate that 19,20-dihydroervahanine A inhibits AChE in a competitive manner. The selectivity of 19,20-dihydroervahanine A for AchE, as opposed to BuChE, was also tested. 19,20-Dihydroervahanine A inhibited BuChE less than 30% at the concentration of 0.10 mM. Therefore, it can be concluded that 19,20-dihydroervahanine A has high selectivity on AChE.

During the course of separation, other two compounds, **3** and **4**, were also isolated. The spectroscopic data, especially 1D and 2D NMR, indicated that the compounds were related to 19,20-dihydroervahanine A. The NMR spectra of both compounds showed the presence of a carbomethoxy group at C-16′ and a methoxyl group at C11′. The HMBC data suggested that the vobasinyl unit connected to the iboga unit at C3 and C12′. The structures of **3** and **4** were elucidated as bisindole alkaloids, conodurine and tabernaelegantine A, respectively. The two compounds have previously been isolated from other species of *Tabernaemontana* (van Beek et al 1984; Cordell et al 1989; Kam & Sim 2003). Interestingly, they showed no activity on AChE (Table 3). This indicates that the substitutions at C11′ and C12′ might have a great effect on AChE inhibitory activity.

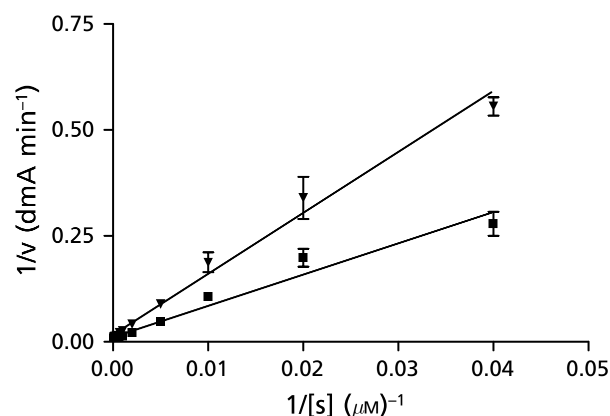

Figure 2 Lineweaver–Burk plots of AChE activity over a range of substrate concentrations (25 μM to 10 mM) in the absence (squares) or presence (triangles) of **2** (1.0 μM). The points are averages of one typical experiment done in triplicate. The standard deviations are presented as error bars.

The very mild anticholinesterase activity of some indole alkaloids from the Apocynaceae family, such as akuammicine, cimicine and yohimbine, has been previously observed. Recently, Andrade et al (2005) reported that four iboga-indole alkaloids isolated from *Tabernaemontana austratis* Miers (i.e. coronaridine, voacangine, voacangine hydroxyindolenine and rupicoline) showed AChE inhibitory activity, which was qualitatively detected via TLC assay using modified Ellman's method. However, the activity of the bisindole alkaloids against AChE is reported for the first time in our study.

Conclusions

Four vobasinyl-iboga bisindole alkaloids were isolated from *T. divaricata*. The AChE inhibitory activity of 19,20-dihydrotabernamine and 19,20 dihydroervahanine A was higher than that of the standard inhibitor, galanthamine, while their related compounds, conodurine and tabernaelegantine A, showed no activity. It is likely that the substitutions at carbons 11′, 12′ and 16′ affect the activity dramatically. The structure–activity relationship of this type of alkaloids on AChE should be extensively studied to understand the pharmacological activity of the pharmacophores of this group of compounds.

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