JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 695–700

Received December 20, 2005
Accepted February 9, 2006
DOI 10.1211/jpp.58.5.0015
ISSN 0022-3573

Acetylcholinesterase inhibitors from *Stephania venosa* tuber

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Abstract

Acetylcholinesterase (AChE) inhibitors have lately gained interest as potential drugs in the treatment of Alzheimer's disease. Three AChE inhibitors were isolated from tubers of a Thai medicinal plant, *Stephania venosa* (Bl) Spreng. They were identified as quaternary protoberberine alkaloids, stepharanine, cyclanoline and *N*-methyl stepholidine. They expressed inhibitory activity on AChE with IC50 values (concentration that caused 50% inhibition of activity) of 14.10 ± 0.81, 9.23 ± 3.47 and $31.30 \pm 3.67 \mu$M, respectively. The AChE inhibitory activity of these compounds was compared with those of the related compounds, palmatine, jatrorrhizine and berberine, as well as tertiary protoberberine alkaloids isolated from the same plant, stepholidine and corydalmine. The results suggest that the positive charge at the nitrogen of the tetrahydroisoquinoline portion, steric substitution at the nitrogen, planarity of the molecule or substitutions at C-2, -3, -9, and -10 affect the AChE inhibitory activity of protoberberine alkaloids.

Introduction

Acetylcholinesterase (AChE) inhibitors have gained interest due to their application in the treatment of Alzheimer's disease, a degenerative condition affecting memory, judgement and ability to reason (Scarpini et al 2003). Recently, some natural products have been reported to have inhibitory activity against AChE (Ingkaninan et al 2003; Mills et al 2004; Houghton & Howes 2005). These could be potential sources of leads for more potent and safe AChE inhibitors and the starting materials for such compounds. From a previous study, we found that a Thai medicinal plant, *Stephania venosa* (Bl) Spreng., possessed a high AChE inhibitory activity (Ingkaninan et al 2001). In this study, we therefore aimed to isolate and identify AChE inhibitors from this plant.

S. venosa, known in Thai as Sabuleud, belongs to the Menispermaceae family. It has been used as a traditional medicine in Thailand for a variety of indications including use as a nerve tonic. Some isoquinoline alkaloids, such as protoberberines, proaporphines and aporphines, have already been found in this plant (Guinaudeau et al 1981; Charles et al 1987; Pharadai et al 1987). Some biological activity of these compounds has been reported, such as their potential as anti-cancer and anti-malaria drugs (Likhitwitayawuid et al 1999). However, the compounds responsible for AChE inhibitory activity in this plant have never been studied before.

Materials and Methods

General experimental procedures

Optical rotation was measured on a Perkin Elmer 341 Polarimeter. UV spectra were recorded on a Varian CARY 1E spectrometer. ^{1}H, ^{13}C, DEPT (distortionless enhancement by polarization transfer), ^{1}H-^{1}H COSY (correlated spectroscopy), NOESY (nuclear overhauser and exchange spectroscopy), HMQC (heteronuclear multiple quantum correlation) and HMBC (heteronuclear multiple bond correlation) NMR

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Funding: Financial support from the National Science and Technology Development Agency and the International Foundation for Science is gratefully acknowledged.

experiments were carried out on a Bruker av400 NMR spectrometer, operating at 400 MHz for proton and 100 MHz for carbon. The MS spectra of all compounds except compound 3 were obtained as ESITOFMS (electrospray ionization time-of-flight mass spectrometry) using a Micromass LCT mass spectrometer, and the lock mass calibration was applied for the determination of accurate mass. The HREIMS (high-resolution electron impact mass spectometry) and LREIMS (low-resolution electron impact mas spectrometry) spectra of compound **3** were obtained using a MAT 95 XL mass spectrometer.

Plant material

S. venosa was collected from Phitsanulok, Thailand, in April 2002. A voucher specimen (Fansai0031) was deposited at PBM herbarium, Faculty of Pharmaceutical Sciences, Mahidol University.

Extraction and isolation

The tubers of *S. venosa* were cut into small pieces and dried at 55°C. The dried materials were ground and macerated with ethanol twice (for 3 and 7 days). The ethanol extracts were evaporated under reduced pressure until dryness. The ethanol extract (31 g) was roughly separated by vacuum column chromatography on 300 g silica gel using $CHCl_3$ with an increasing portion of MeOH to give 25 fractions. Fraction 19 (471 mg) was chromatographed on a silica gel column eluted with CH_2Cl_2–MeOH (85:15 v/v) to provide 20 fractions. Fraction 19-5 (120 mg) was further fractionated using a silica gel column with a mobile phase of CH_2Cl_2–MeOH (9:1 v/v). From 8 fractions obtained, fraction 19-5-5 (48 mg) and fraction 19-5-7 (21 mg) were separately chromatographed on Sephadex LH-20 columns eluted with MeOH, yielding compounds **1** (3 mg) and **2** (8 mg), respectively. Compound **3** was obtained from the separation of fraction 3 (2.5 g) using Sephadex LH-20 column chromatography eluted with MeOH. From 10 fractions obtained, fraction 3-1 (520 mg) was subjected to a Sephadex LH-20 column using MeOH as a mobile phase, providing 14 fractions. Fraction 3-1-6 (100 mg) was separated on a Sephadex LH-20 column using MeOH as a mobile phase, providing 7 fractions. Fraction 3-1-6-4 (40 mg) was subjected again to a Sephadex LH-20 column and eluted with MeOH, yielding 6 fractions. Fraction 3-1-6-4-2 (38 mg) was separated by silica gel column chromatography using CH_2Cl_2–MeOH (99:1) as a mobile phase. From 13 fractions obtained, fraction 3-1-6-4-2-4 (14 mg) was subjected to a Sephadex LH-20 column and eluted with MeOH to yield compound 3 (7 mg). Compound **4** (58 mg) was obtained from recrystallization of fraction 12. Compound **5** was obtained from the separation of fraction 2 (260 mg) using Sephadex LH-20 column chromatography eluted with MeOH. From 13 fractions obtained, fraction 2-4 (33 mg) was subjected to a silica gel column using CH_2Cl_2–MeOH (99:1) as a mobile phase, providing compound **5** (17 mg).

Stepharanine (**1**) was obtained as a reddish crystal. UV (MeOH) λmax (log ε): 212 (3.57), 280 (3.26), 352 (3.10). ^1H and ^{13}C NMR: see Tables 1 and 2. MS: m/z 324 $[M+H]^+$ $C_{19}H_{18}NO_4$.

Table 1 ^1H NMR data for isolated alkaloids (in $CDCl_3$ for **1**, **4** and **5**; in MeOD for **2** and **3**)

	1	**2**	**3**	**4**	**5**
1	7.52 s	6.71 s	6.72	6.74 s	6.72 s
4	7.01 s	6.87 s	6.87	6.67 s	6.61 s
5	3.24 brt $J = 6.2$ Hz 2H	3.26 m 2 H	3.25 m 2 H	ax 3.08 ddd $J = 16.8$, 11.1, 5.5 Hz	ax. 3.15 m
				eq 2.70 ddd $J = 16.8$, 3.6, 2.0 Hz	eq. 2.67 m
6	4.87 m 2H	ax 3.57 m	ax 3.57 m	ax 2.63 ddd $J = 11.1$, 9.6, 3.6 Hz	ax. 2.71 m
		eq 3.84 m	eq 3.83 m	eq 3.20 ddd $J = 9.6$, 5.5, 2.0 Hz	eq. 3.21 m
8	9.53 s	4.64 d $J = 16.1$ Hz	4.74 d $J = 16.2$ Hz	ax 3.51 d $J = 15.5$ Hz	ax 3.57 d $J = 15.3$ Hz
		4.84 d $J = 16.1$ Hz	4.85 d $J = 16.2$ Hz	eq 4.18 d $J = 15.5$ Hz	eq 4.22 d $J = 15.3$ Hz
11	7.72 d $J = 8.9$ Hz	6.95 d $J = 8.4$ Hz	6.87 d $J = 8.4$ Hz	6.73 d $J = 8.2$ Hz	6.76 d $J = 8.3$ Hz
12	7.82 d $J = 8.9$ Hz	6.70 d $J = 8.4$ Hz	6.84 d $J = 8.4$ Hz	6.79 d $J = 8.2$ Hz	6.79 d $J = 8.3$ Hz
13	8.53 (s)	ax 3.14 dd $J = 10.0$, 18.5 Hz	ax 3.10 dd $J = 10.2$, 18.4 Hz	ax 2.72 dd $J = 16.0$, 11.0 Hz	ax. 2.83 dd $J = 11.6$, 15.6 Hz
		eq 3.41 dd $J = 5.8$, 18.5 Hz	eq 3.40 dd $J = 5.8$, 18.4 Hz	eq 3.30 dd $J = 16.0$, 4.0 Hz	eq. 3.24 m
13a		4.69 dd $J = 5.8$, 10.0 Hz	4.69 dd $J = 6.1$, 10.1 Hz	3.50 m	3.60 m
$2OCH_3$					3.88 s 3H
$3OCH_3$	3.96 s 3H	3.87 s 3H	3.87 s 3H	3.82 s 3H	3.86 s 3H
$9OCH_3$	4.12 s 3H		3.88 s 3H	3.80 s 3H	3.80 s 3H
$10OCH_3$		3.86 s 3H			
NCH_3		3.28	3.27		

Table 2 ^{13}C NMR data for isolated alkaloids (in $CDCl_3$ for **1**, **4** and **5**; in MeOD for **2** and **3**)

C	1	2	3	4	5
1	112.9	114.3	114.3	113.1	108.8
2	148.1	147.3	147.4	146.0	147.8
3	151.1	149.9	150.0	147.8	147.7
4	112.0	113.1	113.1	112.5	111.6
4a	128.1	120.3	120.2	126.3	126.7
5	27.9	24.1	24.1	29.3	29.1
6	57.3	53.7	53.5	52.9	51.7
8	144.1	60.8	60.8	54.9	54.0
8a	121.5	114.2	121.9	128.8	127.1
9	143.5	144.5	145.4	145.0	143.5
10	154.3	147.5	150.1	148.8	146.8
11	134.5	113.1	118.8	116.4	114.6
12	124.7	119.8	125.0	125.4	124.7
12a	134.0	123.0	121.0	127.3	127.9
13	121.1	34.7	34.8	36.6	36.2
13a	138.2	67.1	67.1	60.7	59.6
13b	124.6	125.4	125.4	130.8	129.6
$2OCH_3$					56.3
$3OCH_3$	56.6	56.6	56.5	56.3	56.0
$9OCH_3$	61.8		56.5		60.7
$10OCH_3$		56.5		60.4	
NCH_3		50.9	50.8		

Cyclanoline (**2**) was obtained as a slightly yellow powder. UV (MeOH) λmax (log ε): 212 (4.24), 285 (3.59). ^1H and ^{13}C NMR: see Tables 1 and 2. MS: m/z 343 $[M+H]^+$ $C_{20}H_{24}NO_4$.

N-Methyl stepholidine (**3**) (2,10-dihydroxy-3,9-dimethoxy-7-methyl-5,8,13,13a-tetrahydro-6*H*-dibenzo[a,g]quinolizinium) was obtained as a slightly brown powder. mp: 194°C. $[\alpha]^{30}_D$: −19.8° (*c* 0.004, MeOH). UV (MeOH) λmax (log ε): 213 (4.74), 284 (4.25). FT-IR ν max 2925, 1607, 1507, 1439, 1360, 1284, 1114, 1082, 876, 753 cm^{-1}. ^1H and ^{13}C NMR: see Tables 1 and 2. LREIMS 1.6 KV: m/z (rel. int.) 341 (58), 313 (21), 284 (22), 267 (80), 239 (30), 61 (100). HREIMS: m/z obs. 342.1663. $C_{20}H_{24}NO_4$ requires 342.1705.

Stepholidine (**4**) was obtained as a slightly brown powder. ^1H and ^{13}C NMR: see Tables 1 and 2. MS m/z 328 $[M+H]^+$; $C_{19}H_{21}NO_4$.

Corydalmine (**5**) was obtained as a slightly brown powder. UV (MeOH) λmax (log ε) 216 (4.12), 282 (3.80). ^1H and ^{13}C NMR: see Tables 1 and 2. MS m/z 342 $[M+H]^+$; $C_{20}H_{23}NO_4$.

For reference standards, galanthamine was obtained from Sigma (USA) and berberine, jatrorrhizine and palmatine were isolated from the stem of *Coscinium bluemeanum* Miers (Menispermaceae) from a previous experiment (Kaewpradub et al 2005).

Microplate assay for AChE activity determination

AChE activity was assayed using a microplate as previously described (Ellman et al 1961; Ingkaninan et al 2003). The AChE used in the assay was from electric eel (type VI-S lyophilized powder; 480 U (mg solid)$^{-1}$, 530 U (mg protein)$^{-1}$; Sigma). Briefly, 125 μL of 3 mM 5,5′-dithiobis(2-nitrobenzoic acid), 25 μL of 1.5 mM acetylthiocholine iodide (Sigma), and 50 μL of 50 mM Tris-HCl buffer pH 8.0 and 25 μL of sample dissolved in buffer 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 reactions rates were measured. Enzyme activity was calculated as a percentage of the velocity of the 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. Stock solutions of samples in Tris-HCl buffer containing not more than 10% MeOH were diluted serially with Tris-HCl buffer to obtain 8 or 9 different concentrations. The IC50 value, corresponding to the inhibitor concentration that caused 50% inhibitory activity, was analysed using the software package Prism (Graph Pad Inc, San Diego, USA).

Thin-layer chromatography (TLC) assay for detection of AChE inhibitors

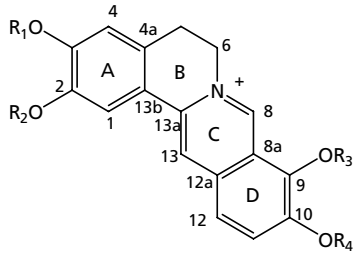
The TLC assay for detection of AChE inhibitors was modified from the method used by Rhee et al (2001). A 2.5-mm silica gel plate F254 no. 5554 (Merck, Darmstadt, Germany) was used as a stationary phase. Samples (3 μL) dissolved in methanol at a concentration of 5 mg mL^{-1} were applied to the plate. After the plate had been developed, it was dried at room temperature and then sprayed with 30 mM acetylthiocholine iodide followed by 20 mM 5,5′-dithiobis[2-nitrobenzoic acid]. The plate was dried at room temperature for 45 min, then sprayed with 10.17 U mL^{-1} AChE. After 20 min, the plate was observed under visible light. A positive spot, indicating AChE inhibitor, was a colourless spot on a yellow background.

Statistical analysis

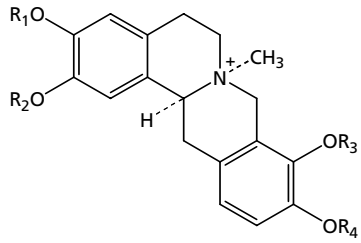
The effects of the various compounds on the IC50 were statistically evaluated using the Kruskal–Wallis test. Individual differences were then assessed using a post-hoc test. In all cases $P < 0.05$ denoted significance.

Results and Discussion

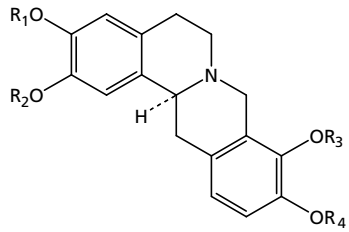
Fractionation of the *S. venosa* alcoholic extract guided by using a microplate and a TLC assay for detection of AChE inhibitory activity provided five acetylcholinesterase inhibitors, **1**–**5** (Figure 1). The chemical structures of the compounds isolated were elucidated on the basis of spectroscopic evidence. Compound **1** was obtained as a reddish crystal, with the molecular ion corresponding to $C_{19}H_{18}NO_4$. The ^1H and ^{13}C NMR spectra indicated the presence of three aromatic rings, two methoxy groups, two hydroxy groups and two CH_2 groups. A pair of



	R_1	R_2	R_3	R_4
Stepharanine (**1**)	Me	H	Me	H
Palmatine	Me	Me	Me	Me
Jatrorrhizine	H	Me	Me	Me
Berberine	-CH_2-		Me	Me



	R_1	R_2	R_3	R_4
Cyclanoline (**2**)	Me	H	H	Me
N-methyl stepholidine (**3**)	Me	H	Me	H



	R_1	R_2	R_3	R_4
Stepholidine (**4**)	Me	H	Me	H
Corydalmine (**5**)	Me	Me	Me	H

Figure 1 Structures of the protoberberine alkaloids in this study.

doublets and four singlets at the aromatic region of the ^{1}H NMR spectra suggested the presence of two ortho protons on one of the aromatic rings and four isolated protons in the other two aromatic rings. The aliphatic region of the ^{1}H NMR spectra displayed a pair of signals that belonged to adjacent methylene protons, confirmed by ^{1}H-^{1}H COSY. These data indicated that **1** was a protoberberine alkaloid with two methoxy and two hydroxy substitutions. With the assistance of 2D NMR, the position of the substitute groups were elucidated and all H and C signals were assigned (Tables 1 and 2). This analysis indicated that this alkaloid was stepharanine. Stepharanine was firstly found in *S. glabra* (Doskotch et al 1967) and the isolation was further reported in the literature (Bhakuni et al 1983; Nishiyama et al 2004). However, the complete assignments, especially at the unprotonated carbons and methoxyl groups, have not been reported before. In this study, the complete assignments were obtained using HMQC, HMBC and NOESY.

The molecular ion of compound **2** corresponded to $C_{20}H_{24}NO_4$. The similarity of its ^{1}H and ^{13}C NMR spectra to that of **1** suggested a closely related structure with a tetrahydroprotoberberine skeleton. The presence of two *ortho* protons on one of the aromatic rings, two isolated protons in the other aromatic ring, two methoxy groups and two hydroxy groups was still observed with the additional *N*-methyl group at δ 3.28. The signal at δ 3.56 (2H) was assigned as H-5, whereas the signals at 3.57 (1H) and 3.84 (1H) were assigned as geminal protons at position 6. Moreover, signals of a pair of methylene protons and an isolated CH-CH_2 moiety were found in the aliphatic region. The large coupling constant (16.1 Hz) of a pair of doublets at δ 4.64 and δ 4.84 suggested the existence of geminal protons, which was confirmed by HMQC. This deshield, isolated methylene group is a typical characteristic of the berberine bridge (C-8) of the protoberberine alkaloids (Blanchfield et al 2003). The signals of an isolated CH-CH_2 moiety proved to belong to C-13a and C-13. 2D NMR experiments led to the complete assignment of protons and carbons, including the substitutions of **2** (Tables 1 and 2). This compound was finally elucidated as a quaternary protoberberine alkaloid, cyclanoline. The 7S, 13aS configuration was confirmed by a NOESY experiment. *N*-Methyl protons showed NOE correlation with H-13a. Moreover, the NOE correlation between 6-H at δ 3.76 and *N*-methyl protons suggested the axial position of 6-H. The 13-H signal at δ 3.14 showed a large coupling constant (10.0 Hz) with the signal of 13a-H indicating that this proton was at axial position. Cyclanoline has also been found in *S. elegan* (Singh et al 1981) and *S. cepharantha* (Tanahashi et al 2000).

Compound **3** showed the same molecular ion as that of **2**, indicating the possibility that they were isomers. Very similar ^{1}H and ^{13}C NMR spectra were obtained. However, HMBC of **3** showed a long-range coupling of the proton from the methoxy group at δ 3.88 with C-9, while the methoxy proton at δ 3.86 of **2** had long-range correlation with C-10. Therefore, it is concluded that **3** is *N*-methyl stepholidine. The configuration of **3** was assigned in the same way as **2**. This compound was previously reported by Huang et al (1984) but the ^{1}H and ^{13}C NMR assignments are reported here for the first time (Tables 1 and 2).

Compounds **4** and **5** were elucidated as tertiary tetrahydroprotoberberine alkaloids since their ^{1}H and ^{13}C NMR spectra showed the same pattern as compounds **2** and **3** without the presence of the N-methyl group. The complete assignments of H and C signals of **4** and **5** are shown in Tables 1 and 2. They are unambiguously identified as stepholidine and corydalmine, respectively. Stepholidine was found in *S. glabra* (Bhakuni et al 1983),

Table 3 IC50 of protoberberine alkaloids and galanthamine on acetylcholinesterase evaluated by a microplate assay

Compounds	IC50 (μM)
Stepharanine (**1**)	14.10 ± 0.81
Cyclanoline (**2**)	9.23 ± 3.47
N-Methyl stepholidine (**3**)	31.30 ± 3.67
Stepholidine (**4**)	>100
Corydalmine (**5**)	100.09 ± 45.02
Palmatine	0.26 ± 0.03
Jatrorrhizine	0.93 ± 0.43
Berberine	0.58 ± 0.29
Galanthamine	0.59 ± 0.10

Values are averages $\pm$ s.d. from three experiments.

while corydalmine was found in *S. bancroftii* (Blanchfield et al 2003). The complete ^{1}H and ^{13}C NMR assignments of corydalmine were reported by Blanchfield et al (2003), and were in agreement with our findings.

The AChE inhibitory activity of the five protoberberine alkaloids isolated are shown in Table 3. The well-known AChE inhibitor galanthamine was used as a positive standard. We also tested the AChE inhibitory activity of the quaternary protoberberine alkaloids palmatine and berberine, which had been previously reported as AChE inhibitors (Ulrichova et al 1983; Schmeller et al 1997). In addition, the activity of a related quaternary protoberberine alkaloid, jatrorrhizine, against AChE was evaluated. This is the first time that the AChE inhibitory activity of this compound has been reported.

The results showed that the AChE inhibitory activity of the tertiary protoberberine alkaloids stepholidine (**4**) and corydalmine (**5**) was lower than that of the quaternary protoberberine alkaloids tested. The tertiary protoberberine **4** had significantly less potency compared with the quaternary protoberberines with the same substitutions, *N*-methyl stepholidine (**3**) and stepharanine (**1**). Moreover, aromatization at ring C and the absence of a methyl group at the nitrogen improved the AChE activity of **1** about 2 fold in comparison with **3**. This information suggests that a positive charge at the nitrogen of the tetrahydroisoquinoline portion, steric substitution at the nitrogen or the planarity of molecule may influence the AChE inhibitory activity.

The tested molecules have 4 substitutions at positions 2, 3, 9 and 10, which can be hydroxy or methoxy groups. These substitutions may be responsible for the binding pocket or they may be the parts that can interact with enzymes as proton donor or proton acceptor. When comparing the quaternary protoberberine **1** with palmatine, which has different substitutions at C-2 and C-10, the inhibitory activity against AChE of palmatine was approximately 50-fold higher, indicating the significant influence of methoxy groups at C-2 and C-10. Changing the methoxy group at C-3 in palmatine to a hydroxy group in jatrorrhizine caused a reduction in activity of about 3 fold. This confirms the importance of substitutions of the protoberberine ring. It should be emphasized that palmatine, jatrorrhizine and berberine showed AChE inhibitory activity in the same range as that of galanthamine. Study of the structure–activity relationship of this group might lead to the discovery of more active AChE inhibitors. One of the points to consider for the further development of such compounds is the permeation ability of the quaternary alkaloids through the blood–brain barrier (BBB). Using BBB cell culture techniques may result in easy identification of suitable lead compounds against Alzheimer's disease.

Conclusion

From the investigation of AChE inhibitors from *S. venosa* tubers, five protoberberine alkaloids were isolated and identified. The quaternary protobeberine alkaloids stepharanine, cyclanoline and *N*-methyl stepholidine showed moderate inhibitory activity against AChE, while the tertiary protobeberine alkaloids stepholidine and corydalmine were considered as inactive. It is possible that the quaternarization, steric substitution at the nitrogen atom, planarity of the molecule and substitutions at C-2, 3, 9, 10 affect the AChE inhibitory activity of protoberberine alkaloids. This group of compounds could be an interesting lead for acetylcholinesterase inhibitors.

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