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# 3′-*R/S*-Hydroxyvoacamine, a potent acetylcholinesterase inhibitor from *Tabernaemontana divaricata*

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ABSTRACT

Guided by the acetylcholinesterase inhibiting activity, the bisindole alkaloid 3′-*R/S*-hydroxyvoacamine was isolated from a stem extract of *Tabernaemontana divaricata*, a plant used in Thailand in traditional rejuvenation remedies for improving the memory. The structure of the alkaloid was elucidated by extensive use of NMR spectroscopy and the complete assignment of the $^{1}H$ and $^{13}C$ NMR spectra is reported. The alkaloid acted as a non-competitive inhibitor against AChE with an $IC_{50}$ value of 7.00 ± 1.99 μM. An HPLC method was developed for the quantitative analysis of the AChE inhibitor. It suggested that there was 12.4% (w/w) of 3′-*R/S*-hydroxyvoacamine in the alkaloid enriched fraction of *T. divaricata* stem.



## Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder with a remarkably pure impairment of cognitive function (Selkoe 2002). Increased levels of cholinesterase enzymes have been found in post-mortem brain samples of AD patients which led to the hypothesis that the cognitive decline in AD patients is related to progressive cholinergic degeneration (Farfara et al., 2008; Snyder et al., 2001). No treatment is available to cure or delay the disease, but for symptomatic treatment cholinesterase inhibitors are indicated (Schelterns and Feldman 2003). During the past decade, some inhibitors of acetylcholinesterase (AChE) have been clinically evaluated (Mercier et al., 2007; Lefèvre et al., 2008). Most of them are synthetic compounds which reveal some extent of toxicity in prolonged use (Johnson et al., 2000; Wentrup et al., 2008; Winblad et al., 2007). Consequently, there is still a great demand for new drug candidates of AD treatment especially from natural sources with less toxicity.

*Tabernaemontana divaricata* (L.) R. Br. Ex Roem. & Schult is a common garden plant in Southeast Asia and other tropical countries. It has been used in Thai traditional rejuvenation remedies for improving memory. In Africa and Continental Asia the plant has been used as a central nervous system stimulant (Chattipakorn et al., 2007). *T. divaricata* belongs to the *Apocynaceae* family. The major constituents of *T. divaricata* are indole alkaloids (Van Beek et al., 1984; Pratchayasakul et al., 2008). It was reported that alcoholic extracts from both root and stem of *T. divaricata* exhibited a very high inhibitory activity against AChE (Ingkaninan et al., 2003). Two vobasinyl-iboga bisindole alkaloids *i.e.* 19,20-dihydrotabernamine and 19,20 dihydroervahanine A were isolated from *T. divaricata* roots and the AChE inhibitory activity of them was higher than that of the standard inhibitor, galantamine (Ingkaninan et al., 2006). The *in vivo* study of *T. divaricata* root extract showed that the extract acted as a reversibly selective AChE inhibitor and enhanced neuronal activity (Chattipakorn et al., 2007). Moreover, the subchronic oral administration of *T. divaricata* extract remarkably improved cognitive deficits induced by the Aβ25–35 peptides which are presumed to be one of the causes of AD (Nakdook et al., 2010). No acute or subchronic toxicity was found in the mice treated with *T. divaricata* extracts (Nakdook et al., 2010; Henriques et al., 1996). Therefore, *T. divaricata* might be a potential candidate for the treatment of AD. Since roots are less abundant comparing to stems, in this study, we explored the possibility of using stem extract of *T. divaricata* as AChE inhibitor.

## Materials and methods

### *Chemicals and reagents*

AChE from human erythrocytes E.C. 3.1.1.7, 5,5′-dithiobis (2-nitrobenzoic acid), acetylthiocholine iodide (ATCI), tris(hydroxymethyl) aminomethane hydrochloride and ammonia

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solution were purchased from Sigma–Aldrich (St. Louis, MO, USA). All organic solvents used in the studies were AR grade. Methanol, ethanol and orthophosphoric acid were purchased from Merck (Darmstadt, Germany). Dichloromethane, diethylether, sodium bicarbonate and disodium hydrogen phosphate were purchased from Fisher Chemicals (Loughborough, UK). Ethyl acetate were purchased from Labscan (Dublin, Ireland). Other chemicals and reagents were of the highest grade available.

*Plant material*

The stems of *T. divaricata* were collected near Chiang Mai, Thailand in June 2010. The voucher specimen (collection no. 0010115) was deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Chiang Mai University.

*Preparation of stem extract*

The dried powder of *T. divaricata* stems (3.36 kg). was macerated with 95% (v/v) ethanol for 3 days. After filtration, the filtrate was collected and the residue was macerated again with 95% (v/v) ethanol for 3 days. Finally, the total filtrate was evaporated under reduced pressure until dryness to give the stem extract (123.30 g).

*Alkaloid enriched fraction extraction*

The stem extract (123.30 g) was dissolved in phosphate buffer (pH 3) and washed with ethyl acetate (EtOAc). The aqueous part was adjusted with sodium bicarbonate solution until pH 10 and extracted with EtOAc. The organic layer was dried under reduced pressure to give the alkaloid enriched fraction (27.43 g).

*Isolation, activity determination and identification of AChE inhibitor*

*AChE inhibitor isolation*

A part of the alkaloid enriched fraction (200 mg) was fractionated on a Sephadex LH-20® column (2 cm diameter and 150 cm length) using 100% MeOH as the mobile phase. 10 ml fractions were collected and evaporated on 50 °C water bath. White crystals were obtained in fractions 13–15. They were washed with cold methanol to obtained 5.0 mg of **1**. The purity of isolated compound was determined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

*Structure elucidation*

The $^{1}H$, $^{13}C$, DEPT, $^{1}H-^{1}H$ COSY, NOESY, HMQC and HMBC NMR experiments were carried out using a Bruker Avance 400 NMR spectrometer (Bruker, Germany), operating at 400 MHz for $^{1}H$ and 100 MHz for $^{13}C$. The electrospray ionization time-of-flight mass spectrometry (ESITOFMS) spectra were obtained using a Micromass LCT mass spectrometer (Water, USA).

3′-*R*/*S*-Hydroxyvoacamine [**1**] – $C_{43}H_{52}N_4O_6$; $[M-H]^-$ 719; UV $\lambda_{max}$ (EtOH) nm 200, 224, 295; ms *m/z* (%) 719 (10), 704 (99), 660 (29), 637 (23), 394 (90), 353 (100), 337 (98), 321 (37), 307 (39), 280 (42); $^{1}H$ and $^{13}C$ NMR is shown in Table 1.

**Table 1**
The $V_{max}$ and $K_m$ values of a human AChE-catalyzed reaction and the reaction in the presence of 3′-*R*/*S*-hydroxyvoacamine (**1**).

| Parameters | Human AChE-catalyzed reaction | |
|---|---|---|
| | Without 1 | With 1 |
| $V_{max}$ | 157.1 | 44.27 |
| $K_m$ | 1433 | 1586 |

*Anticholinesterase activity determination*

The AChE activity was evaluated by means of Ellman's method introduced by Ellman et al. (1961) with some modification. Human AChE was used and ATCI was employed as an enzyme substrate of the reaction. Briefly, 50 μl of 50 mM Tris–HCl buffer, 25 μl of 1.5 mM ATCI, 125 μl of 3 mM DTNB, pH 8.0, and 25 μl of sample dissolved in buffer containing not more than 10% methanol were added to each well of the microplate, followed by 25 μl of 0.25 U/ml AChE. The microplate was then read at 415 nm every 7 s for 2 min after 1 min of moderate shaking. The mean velocity of reactions was recorded. All of the experiments were done in triplicate. The cholinesterase inhibitory activity was then calculated using the following equation; $E = [1 - (V_s/V_b)] \times 100$, where $V_s$ is the mean reaction rate in the presence of a certain concentration of sample and $V_b$ is the mean reaction rate in absence of the sample. $IC_{50}$ values were statistically evaluated using the graphpad/prism program (Graphpad software, USA). The inhibitory effects of the samples on AChE were represented as $IC_{50}$ values. To evaluate the enzyme kinetics, the concentration of sample was fixed at 5 μg/ml while the concentration of ATCI was varied from 0.05 to 100 mM. The initial rate of a reaction ($V$) was recorded and plotted in the graph according to the Michaelis–Menten and Lineweaver–Burk equations. $V_{max}$ and $K_m$ were then determined using Prism (Graphpad software, USA).

*Fingerprints of AChE inhibitor in the alkaloid enriched extract of T. divaricata stems*

*TLC analysis*

The TLC plate was developed in dichloromethane: methanol (9:1) or diethylether:dichloromethane:methanol (8:1.5:0.5). Then, it was observed in daylight, 254 nm and 366 nm. After that, it was sprayed with Dragendorff's reagent and then observed again in daylight. The alkaloid components were seen as orange spots on the yellow background. To determine the bioactivity on TLC, the method of Rhee et al. (2001) was used. After the TLC plate was developed with the mobile phase, it was sprayed with 30 mM ATCI, 8 mM 2,2′-dinitrobenzoic acid (DTNB) and AChE, respectively. The plate was then observed in daylight. The positive result which referred to the inhibitor was observed as a white spot on the yellow background. All the TLC experiments were done in triplicate and two different mobile phase systems were used to confirm the purity of isolated compound.

*HPLC analysis*

HPLC analysis was performed using an HP1100 system and a Photodiode array detector (PDA) set at 295 nm (Hewlett-Packard, Palo Alto, CA). A reversed phase column Inertsil® ODS-2 (250 mm × 4.6 mm i.d., 5 mm, GL Sciences Inc., Japan) was connected with a guard column (12.5 mm × 4.6 mm i.d., 5 mm, Agilent, USA). A mixture of methanol and phosphate buffer solution (pH 7.4) in the ratio of 86:14 was used to elute samples at ambient temperature at a flow rate of 1 ml/min and 20 μl of sample was injected. Samples and mobile phases were filtrated through a 0.45 mm Millipore filter, type GV (Millipore, Bedford, MA) prior to HPLC injection. Peak purity was determined using a PDA.

The HPLC method of validation for 3′-*R*/*S*-hydroxyvoacamine analysis was performed as followings. The limit of detection (LOD) and limit of quantitation (LOQ) of 3′-*R*/*S*-hydroxyvoacamine were determined by serial dilution of the compound until the signal to noise ratio (S/N) was 3:1 and 10:1 for LOD and LOQ, respectively. For the calibration curve of 3′-*R*/*S*-hydroxyvoacamine, six solutions in different concentrations were prepared. All solutions were injected twice. Three calibration curves were done and the average AUC of each concentration was plotted. For the intraday precision, six concentrations of 3′-*R*/*S*-hydroxyvoacamine (2.5, 5, 10, 20, 40 and 80 μg/ml) were prepared in triplicate. Each sample