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# Alkaloids from the roots and leaves of *Stichoneuron halabalensis* and their acetylcholinesterase inhibitory activities

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## Abstract

A study of the hitherto unreported *Stichoneuron halabalensis* Inthachub led to the characterization of the known compounds (+)- $\alpha$ -tocopherol and (R)-(+)- goniiothalamine; four known *Stemona* alkaloids, bisdehydroxystemoninine A (1), stemoninine (2), sessilistemonamine C (3) and sessilistemonamine A (4); and three new alkaloids, stichoneurine C (5), D (6) and E (7). The structures of these compounds were determined on the basis of their spectroscopic data. Alkaloid 7 showed significant inhibitory activity against electric eel acetylcholinesterase (AChE) (IC<sub>50</sub> 5.90±0.084  $\mu$ M), while goniiothalamine and compounds 1 and 2 showed significant inhibitory activities against human AChE (IC<sub>50</sub> 7.24±0.52, 5.52±0.13 and 3.74±0.09  $\mu$ M, respectively).

## Keywords

their, roots, acetylcholinesterase, inhibitory, activities, leaves, stichoneuron, halabalensis, alkaloids, CMMB

## Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

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# Alkaloids from the Roots and Leaves of *Stichoneuron halabalensis* Inthachub and their Acetylcholinesterase Inhibitory Activities

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A study of the hitherto unreported *Stichoneuron halabalensis* Inthachub led to the characterization of the known compounds (+)- $\alpha$ -tocopherol and (*R*)-(-)-goniothalamine; four known *Stemona* alkaloids, bisdehydroxystemoninine A (**1**), stemoninine (**2**), sessilistemonamine C (**3**) and sessilistemonamine A (**4**); and three new alkaloids, stichoneurine C (**5**), D (**6**) and E (**7**). The structures of these compounds were determined on the basis of their spectroscopic data. Alkaloid **7** showed significant inhibitory activities against electric eel acetylcholinesterase (AChE) ( $IC_{50}$  5.90 $\pm$ 0.084  $\mu$ M), while goniothalamine and compounds **1** and **2** showed significant inhibitory activities against human AChE ( $IC_{50}$  7.24 $\pm$ 0.52, 5.52 $\pm$ 0.13 and 3.74 $\pm$ 0.09  $\mu$ M, respectively).

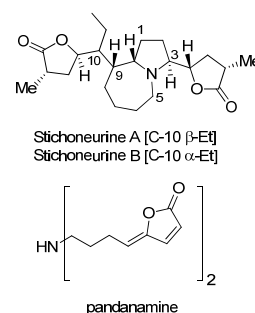
**Keywords:** *Stichoneuron halabalensis* Inthachub, *Stemona* alkaloids, Acetylcholinesterase.

Stemonaceae is a small monocotyledonous family comprising three genera, *Stemona*, *Croomia* and *Stichoneuron* [1]. Extracts of the roots of species from the largest genus, *Stemona*, have been used in traditional medicine to treat the symptoms of bronchitis, pertussis and tuberculosis and have been used as anti-parasitics on humans and animals [2]. Some of the pure alkaloids derived from the extracts of the leaves and roots of *Stemona* species have significant antitussive activity [3], as well as insect toxicity, antifeedant and repellent activities [4]. These last properties are most likely associated with the ability of these alkaloids to inhibit insect acetylcholinesterase [4, 5]. Others have shown oxytocin antagonism [6], nitric oxide inhibition [7] and the ability to inhibit P-glycoprotein in multi-drug-resistant cancer cell lines [8].

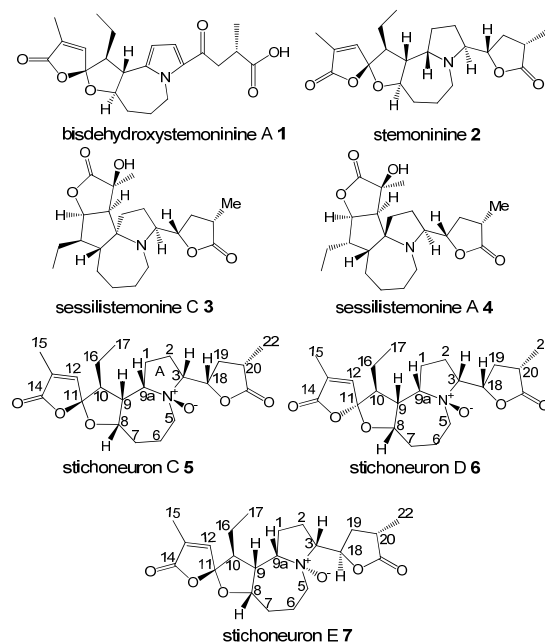
The *Stemona* group of alkaloids includes more than 130 unique natural products which have been structurally classified into seven different structural groups by Pilli [2a,c] and three skeletal types by Greger [2b,d]. The pyrrolo[1,2-*a*]azepine nucleus is common to most compounds in these groups, however, several with a pyrido[1,2-*a*]azepine system [9] and two with a pyrido[1,2-*a*]azonine [10] and one with an indolizidine nucleus [11] have been reported. In contrast to *Stemona* species, only a few studies have been made on species of the genera *Croomia* and *Stichoneuron* [2]. Four species of *Stichoneuron* have been documented from Peninsular Thailand and Malaysia; these are *S. bognerianum*, *S. calcicola*, *S. caudatum* and *S. halabalensis* [1]. A study of the root extracts of *S. caudatum* from south Thailand resulted in the isolation and identification of stichoneurines A and B (Figure 1) [12], which can be classified structurally as stichoneurine-type *Stemona* alkaloids. A similar analysis of the root extracts of *S. calcicola* from south Thailand surprisingly led to the isolation of geometric isomers of pandanamine (Figure 1) [13], which suggested a close relationship between the families Pandanaceae and Stemonaceae.

In this paper we describe our phytochemical studies of the root and leaf extracts of *Stichoneuron halabalensis* Inthachub collected from plants growing in the east part of Peninsular Malaysia. Successive purifications of the crude ethanol extract of the roots by column chromatography and preparative TLC gave pure samples of the known compounds, (+)- $\alpha$ -tocopherol [14] (4.5 mg), (*R*)-(-)-

**Table 1.**  $^{13}C$  NMR (125 Hz) and  $^1H$  NMR (500 MHz) spectral data for compounds **7**, **8** and **9** in  $CDCl_3$  solution.



**Figure 1:** Alkaloids previously isolated from *S. calcicola* and *S. caudatum*.



**Figure 2.** Structures of alkaloids **1**–**7**.

goniothalamine [15] (7.2 mg), bisdehydroxystemoninine A [16] (**1**, 6.2 mg), stemoninine [17] (**2**, 41.5 mg), sessilistemonamine C [18]

Position	7			8			9		
	$\delta_C$ (DEPT)	$\delta_H$ (mult., $J$ (Hz), assign.)	HMBC	$\delta_C$ (DEPT)	$\delta_H$ (mult., $J$ (Hz), assign.)	HMBC	$\delta_C$ (DEPT)	$\delta_H$ (mult., $J$ (Hz), assign.)	HMBC
1	34.9 (CH <sub>2</sub> )	2.18 (q, $J = 6.5$ ) $\alpha$	2, 3	33.2 (CH <sub>2</sub> )	2.31-2.22 (m) $\beta$	2, 3	34.4 (CH <sub>2</sub> )	2.23-2.19 (m) $\beta$	5
2	24.1 (CH <sub>2</sub> )	1.75 (ddd, $J = 5.2, 6.5, 6.5$ ) $\beta$	-	24.1 (CH <sub>2</sub> )	1.59-1.52 (m) $\alpha$	-	24.0 (CH <sub>2</sub> )	1.78-1.72 (m) $\alpha$	-
		2.44 (dddd, $J = 6.5, 6.5, 6.5$ ) $\beta$	3		2.50-2.42 (m) $\beta$	3		2.14-2.04 (m) $\beta$	1, 3
3	75.5 (CH)	2.03-1.93 (m) $\alpha$	-	75.5 (CH)	2.03-1.92 (m) $\alpha$	-	76.1 (CH)	2.47-2.39 (m) $\alpha$	-
		3.57 (ddd, $J = 6.5, 6.5, 12.0$ )	2, 1, 18		3.57 (overlap)	2, 18		3.70 (ddd, $J = 5.5, 7.5, 13.6$ )	19, 2b, 5b, 18
5	67.7 (CH <sub>2</sub> )	3.89-3.81 (m) $\beta$	2, 6	68.1 (CH <sub>2</sub> )	3.87-3.75 (overlap) $\beta$	-	66.5 (CH <sub>2</sub> )	4.01-3.93 (m) $\alpha$	-
		3.77-3.68 (m) $\alpha$	-		3.77-3.68 (overlap) $\alpha$	-		4.15-4.06 (m) $\beta$	-
6	21.3 (CH <sub>2</sub> )	2.03-1.86 (m)	7	21.0 (CH <sub>2</sub> )	1.92-1.88 (m)	-	20.8 (CH <sub>2</sub> )	2.06-2.02 (m) $\alpha$	5
		2.03-1.86 (m)	-		1.92-1.88 (m)	-		1.94-1.88 (m) $\beta$	-
7	34.9 (CH <sub>2</sub> )	2.23-2.15 (m) $\beta$	-	33.5 (CH <sub>2</sub> )	2.31-2.22 (m) $\beta$	-	24.2 (CH <sub>2</sub> )	2.30-2.22 (m) $\beta$	-
		1.75-1.60 (m) $\alpha$	-		2.31-2.22 (m) $\alpha$	-		1.89-1.79 (m) $\alpha$	-
8	85.8 (CH)	4.43 (dt, $J = 5.2, 5.2, 13.7$ )	10	86.8 (CH)	4.37 (ddd, $J = 5.0, 8.0, 11.0$ )	7	84.1 (CH)	4.85-4.77 (dt, $J = 6.0, 12.5, 18.5$ )	6, 10, 2, 9, 9a
9	48.7 (CH)	2.95 (ddd, $J = 4.9, 10.8, 13.7$ )	1, 7	50.7 (CH)	2.59 (ddd, $J = 4.5, 10, 12.5$ )	1, 7	48.1 (CH)	3.03 (ddd, $J = 4.5, 9.8, 13.6$ )	1, 10
9a	78.8 (CH)	3.99 (dt, $J = 3.4, 10.8, 10.8$ )	1	78.7 (CH)	4.13 (ddd, $J = 3.5, 7.5, 7.5$ )	-	78.5 (CH)	4.06-3.98 (m)	9, 1
10	51.5 (CH)	1.92 (ddd, $J = 4.9, 6.3, 12.6$ )	16, 17	53.0 (CH)	2.21-2.10 (m)	16, 17	51.3 (CH)	1.98-1.89 (m)	17, 16, 9
11	112.4 (C)	-	10, 12, 16	113.7 (C)	-	-	112.2 (C)	-	16, 10, 9a, 12
12	143.7 (CH)	6.61 (d, $J = 1.3$ )	15	142.5 (CH)	6.78 (d, $J = 1.3$ )	15	143.5 (CH)	6.62 (d, $J = 1.3$ )	15
13	134.7 (C)	-	15	134.1 (C)	-	15	134.5 (C)	-	10, 12
14	171.1 (C)	-	15	171.3 (C)	-	15	170.9 (C)	-	15, 12
15	10.7 (CH <sub>2</sub> )	1.95 (d, $J = 1.3$ )	-	10.8 (CH <sub>2</sub> )	1.95 (overlap)	-	10.6 (CH <sub>2</sub> )	1.94 (d, $J = 1.2$ )	12
16	19.5 (CH <sub>2</sub> )	1.51-1.39 (m)	10, 17	19.5 (CH <sub>2</sub> )	1.60-1.57 (m)	17	19.2 (CH <sub>2</sub> )	1.54-1.47 (m)	15, 17
		1.51-1.39 (m)	-		1.60-1.57 (m)	-		1.44-1.37 (m)	-
17	13.0 (CH <sub>3</sub> )	0.85 (t, $J = 7.5$ )	16	12.2 (CH <sub>3</sub> )	0.95 (t, $J = 7.5$ )	16	12.8 (CH <sub>3</sub> )	0.85 (d, $J = 7.5$ )	16, 15
18	73.5 (CH)	5.42 (dt, $J = 6.5, 6.5, 12.1$ )	19, 3	73.3 (CH)	5.42 (overlap)	19, 3	72.9 (CH)	5.36-5.26 (m)	19b, 3
19	35.4 (CH <sub>2</sub> )	2.51 (ddd, $J = 6.5, 12.0, 12.5$ ) $\beta$	20	35.4 (CH <sub>2</sub> )	2.51 (overlap) $\beta$	20	35.2 (CH <sub>2</sub> )	2.59-2.53 (m) $\beta$	22
		1.77-1.60 (m) $\alpha$	-		1.77-1.60 (overlap) $\alpha$	-		1.72-1.61 (m) $\alpha$	-
20	35.3 (CH)	2.68 (ddd, $J = 7.0, 12.0, 12.5$ )	19	35.2 (CH)	2.68 (overlap)	19	34.8 (CH)	2.76-2.67 (m)	20, 19, 22
21	178.3 (C)	-	19, 20	178.3 (C)	-	19, 20	177.9 (C)	-	22, 20, 19
22	14.7 (CH <sub>3</sub> )	1.27 (d, $J = 7.0$ )	19, 20	14.7 (CH <sub>3</sub> )	1.27 (overlap)	19, 20	14.5 (CH <sub>3</sub> )	1.27 (d, $J = 6.8$ )	20, 19,

(**3**, 4.2 mg) and sessilistemonine A [**18**] (**4**, 2.5 mg), and an inseparable mixture (7:3) of two new alkaloids, stichoneurine C and D (**5** and **6**, 13.3 mg). Further investigation of the leaf extract gave a new alkaloid, stichoneurine E (**7**, 12.5 mg) (Figure 2).

The HRMS analysis of the mixture of **5** and **6** and that of **7** (ESI,  $m/z$   $[M+H]^+$ , 406.2218 and 406.2217, calcd 406.2230) showed all three compounds had the molecular formula C<sub>22</sub>H<sub>32</sub>NO<sub>6</sub>, indicating their isomeric structural relationship and that they had one more oxygen than **2**. This structural relationship was further supported from their <sup>13</sup>C NMR/DEPT, HSQC and HMBC spectra, which indicated that **2** and **5–7** had the same number of carbons and carbon types. The full <sup>1</sup>H and <sup>13</sup>C NMR spectral assignments for **5–7**, based on extensive NOESY/ROESY, HSQC and HMBC experiments, are shown in Table 1. In the <sup>13</sup>C NMR spectrum of the mixture of **5** and **6** a complete twofold set of signals with the intensity ratio of 7:3 was observed. In the <sup>1</sup>H NMR spectrum, the majority of resonances for **6** could also be observed, with eight overlapping with those of **5**. Due to the clear intensity differences of the <sup>13</sup>C NMR resonances for components **5** and **6** and by the use of 2D methods (COSY, HMQC, HMBC, and NOESY) it was possible to assign all <sup>13</sup>C NMR resonances of both isomers. Both were characterized by the presence of 3 methyl groups, 7 methylenes, 7 methines, 2 olefinic and 2 quaternary carbons. For compound **5**, the <sup>1</sup>H NMR signals at 0.85 (t,  $J = 7.0$  Hz), 1.27 (d,  $J = 7.0$  Hz) and 1.95 (d,  $J = 1.3$  Hz) ppm were assigned to the methyl groups C-17, C-22 and C-15, respectively. The olefinic proton resonance at 6.61 ppm (apparent d,  $J = 1.3$  Hz) was assigned to H-12. The <sup>13</sup>C NMR signals at 178.3 and 171.1 ppm indicated 2 lactone carbonyls, while the signal at 112.4 ppm indicated a ketal group such as that present in **2**. The analysis of the 1D and 2D NMR spectra of **5** led to the full assignment of the carbon and proton signals (Table 1). The signals resonating at 178.3 (C-21) and 171.1 ppm (C-14) correlated with those of H-19 and H-22, and with H-12, and H-15, respectively in the HMBC spectrum. The quaternary carbon signal at 112.4 ppm (C-11) showed correlations with H-8, H-10, H-12 and H-16, thus indicating the position of the ketal moiety at C-11 and the ethyl group at C-10. The <sup>1</sup>H and <sup>13</sup>C NMR signals of **6** were similar or sometimes the same as those of **5** (Table 2). A comparison of the <sup>13</sup>C/DEPT NMR spectra of **5** and **6** with that of **2** [10] showed that the main differences between them were the <sup>13</sup>C NMR chemical

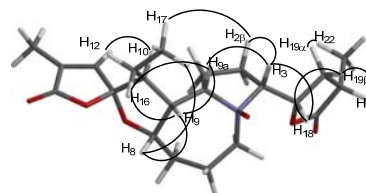


Figure 3: Spartan '10 generated AM1 structure of **5** showing key NOESY cross-peaks.

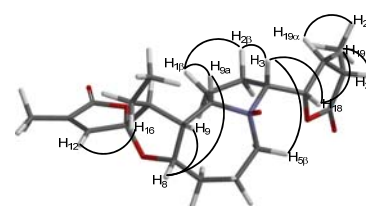


Figure 4: Spartan '10 generated AM1 structure of **6** showing key NOESY cross-peaks.

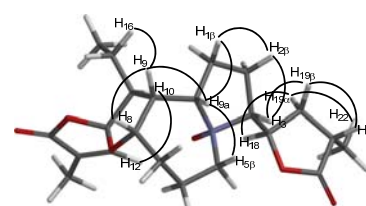


Figure 5: Spartan '10 generated AM1 structure of **7** showing key NOESY cross-peaks.

shifts of the signals for C-3, C-5, and C-9a. The chemical shifts of these carbons for **2** were at 63.4, 45.6 and 58.3 ppm, respectively, whereas the chemical shifts for these carbons for **5** {75.5 (C-3), 67.7 (C-5), and 78.7 (C-9a) ppm} and **6** {75.5 (C-3), 68.1 (C-5), and 78.7 (C-9a) ppm} were significantly downfield and consistent with an N-oxide structure [19]. The assignment of the relative configurations of **5** and **6** were made from NOESY and molecular modeling experiments (Figures 3 and 4). In compound **5**, there were strong NOE correlations between H-3 and H-18, as well as between H-8, H-9 and H-9a, which indicated their  $\beta$  configurations. The NOE correlations between H-10 and H-12 in **5** indicated that the configuration of C-11 was the same as that of **2**. These correlations indicated that **5** was an isomer of stemoninine (**2**) at C-3 and C-8. Most of these NOE correlations were also observed in the NOESY spectrum of **6**. The difference was that the correlation between H-10

and H-12 was now absent and a correlation between H-12 and H-16 was now observed.

The relative stereochemistry assigned to **7** was based on NOESY and molecular modeling experiments (Figure 5). NOESY correlations were observed between H-3 and H-19 $\alpha$  and between H-8 and H-9. However, no correlation was observed between H-3 and H-18, which indicated an *anti*-relationship between these protons. NOESY experiments also showed a *syn*-relationship between H-10 and H-12, similar to that found in compound **5**. Thus, compound **7** is an isomer of **5** at C-3 and C-18.

$\alpha$ -Tocopherol, goniotalamin, and compounds **1–4** and **7** were tested as inhibitors of electric eel and human acetylcholinesterase (AChE). Only alkaloid **7** showed significant inhibitory activities against electric eel AChE with an IC<sub>50</sub> value of 5.90 $\pm$ 0.08  $\mu$ M (Table 2). Goniotalamin and compounds **1** and **2** showed significant inhibitory activities against human AChE (IC<sub>50</sub> 7.24 $\pm$ 0.52, 5.52 $\pm$ 0.13, 3.74 $\pm$ 0.09  $\mu$ M, respectively), while  $\alpha$ -tocopherol and compounds **3**, **4** and **7** were less active. All compounds were less active than the positive control, galanthamine.

**Table 2:** AChE inhibitory activities of isolated compounds.

Compound	IC <sub>50</sub> values $\mu$ M (R <sup>2</sup> )	
	eeAChE	hAChE
(+)- $\alpha$ -tocopherol	>100	27.82 $\pm$ 0.04 (0.94)
(R)-(+)-goniotalamin	>100	7.24 $\pm$ 0.52 (0.91)
<b>1</b>	>100	5.52 $\pm$ 0.13 (0.94)
<b>2</b>	>100	3.74 $\pm$ 0.09 (0.91)
<b>3</b>	>100 [18]	>100 [18]
<b>4</b>	68.8 $\pm$ 9.5 [18]	68.80 $\pm$ 9.5 [18]
<b>7</b>	5.90 $\pm$ 0.08 (0.99)	34.63 $\pm$ 0.81 (0.98)
Ethanol roots extract	>100	32.94 $\pm$ 0.88 $\mu$ mol <sup>-1</sup> (0.90)
Galanthamine	0.82 $\pm$ 0.25 (0.97)	0.54 $\pm$ 1.25 (0.96)

## Experimental

**General experimental procedures:** All column chromatography (CC) was performed on flash silica gel (0.040-0.063 mm) using gradient elution.

**Plant material:** *S. halabalensis* Inthachub (Stemonaceae) was collected at Endau-Rompin National Park, Pahang, Malaysia in April 2010. A voucher specimen [number UKM29953] was identified by Prof. Latiff Mohammad and deposited at the Department of Biology, National University of Malaysia.

**Extraction and isolation:** The dry ground root (199.2 g) and leaves (248.5 g) of *S. halabalensis* were extracted separately with 95% EtOH (4 x 2.5 L) over 4 days at rt. The ethanolic extracts were evaporated to give a dark brown (4.4 g) and a dark green residue (20.2 g), respectively. The root extract was chromatographed on silica gel (200 mL) using gradient elution from *n*-hexane/EtOAc (9:1 to 0:10) to EtOAc/MeOH (10:0 to 8:2). A total of 1.5 L eluent was collected in test tubes of 20 mL. These fractions were pooled on the basis of TLC analysis to give non-alkaloid fraction A (180.0 mg) and the alkaloid fractions B (209.0 mg) and C (1.5 g). Separation of fraction A by CC (light petrol/EtOAc, 8:2) gave (+)- $\alpha$ -tocopherol (4.5 mg) and fraction A2 (30 mg). Further purification of fraction A2 by preparative TLC gave (R)-(+)-goniotalamin (7.2

mg). Fraction B was purified by CC (CH<sub>2</sub>Cl<sub>2</sub>/EtOAc, 1:1) to give bisdehydrostemoninine A (**1**, 6.2 mg) and stemoninine (**2**, 41.5 mg). Separation of fraction C by CC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 98:2) then preparative TLC (light petrol/EtOAc, 8:2) gave fractions C1 (66.7 mg) and C2 (180 mg). A portion of fraction C1 (12.4 mg) was subjected to preparative TLC (CH<sub>2</sub>Cl<sub>2</sub>/EtOAc, 7:3), which gave sessilistemoamine C (**3**, 1.0 mg). This technique was repeated for fraction C2 (32 mg) to gain more of compound **3** (3.2 mg). Further purification by preparative TLC (CH<sub>2</sub>Cl<sub>2</sub>/EtOAc, 1:1) of fraction C3 (30 mg) gave sessilistemoamine A (**4**, 2.5 mg). A mixture of compounds **5** and **6** (13.3 mg) was isolated from fraction C3 (30 mg) by using repeated (x 2) preparative TLC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 9.5:0.5). The ethanol extract of the leaves of *S. halabalensis* was subjected to reverse phase CC using MeOH/H<sub>2</sub>O (7.5:2.5) to remove chlorophyll and gave an alkaloid fraction B (953.0 mg). Separation of fraction B by CC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 95:5) gave fraction B1 (120 mg). Further purification by CC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 95:5) gave stemoninine (**2**, mg) and stichoneurine E (**7**, 12.5 mg).

### Stichoneurine C and D (**5** and **6**)

R<sub>f</sub> 0.13 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 95:5).

yellow gum.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

HRMS (ESI) *m/z* [M+H]<sup>+</sup> 406.2218, calcd for C<sub>22</sub>H<sub>32</sub>NO<sub>6</sub>, 406.2230).

### Stichoneurine E (**7**)

R<sub>f</sub>: 0.08 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 95:5).

brown gum.

[ $\alpha$ ]<sub>D</sub><sup>23</sup>: -43.4 (c 0.33, CHCl<sub>3</sub>).

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

HRMS (ESI) *m/z* [M+H]<sup>+</sup> 406.2217, calcd for C<sub>22</sub>H<sub>32</sub>NO<sub>6</sub>, 406.2230). NMR data are in Table 1.

**AChE inhibitory assays:** AChE inhibitory assays were performed according to Sastraruji *et al.* [5]. The final concentrations of compounds that were used were 300, 150, 75, 37.5, 18.75, 9.375, and 4.6875  $\mu$ M. The multi well plate was placed directly into the microplate reader, which was thermostated at 25°C. The absorbances were read using a POLARstar Omega microplate thermostated spectrophotometer (Offenburg, Germany) at 412 nm. Enzyme activity was calculated as a percentage compared with an assay using a buffer without any inhibitor. The AChE inhibitory data were analysed with the software package GraphPad Prism (Graph Pad Inc., San Diego, USA). IC<sub>50</sub> values are means  $\pm$ SD of 3 individual determinations, each performed in triplicate.

**Statistical analysis:** The results were presented as means  $\pm$  standard deviation from triplicate samples of 3 independent experiments. Differences between the means were analyzed by t-test: two sample assuming unequal variances. Results are expressed according to significance: *P* < 0.05.

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