Alkaloids from the Roots of *Stemona aphylla*

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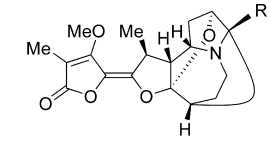
Three known compounds, stemofoline (**1**), (2′*S*)-hydroxystemofoline (**2**), and (11*Z*)-1′,2′-didehydrostemofoline (**3**), along with two new alkaloids, stemaphylline (**4**) and stemaphylline-*N*-oxide (**5**), have been isolated from a root extract of *Stemona aphylla*. The structures of these alkaloids were determined on the basis of their spectroscopic data. The analysis of the crude dichloromethane extract by GC-MS in the EIMS mode showed the presence of alkaloids **1**−**4**, the alkaloid **11**, and stilbostemin R (**12**). The crude dichloromethane extract and **4** were tested for their comparative biological activities. The results of their acetylcholinesterase (AChE) inhibitory activities showed that the crude extract had higher activity than that of **4**. The insecticidal properties of the crude extract and **4**, using a topical application, showed that **4** had an activity similar to the positive control, methomyl, whereas the crude extract had much lower activity. Their antimicrobial activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas auruginosa* ATCC 27853, and *Candida albicans* ATCC 90028 was weak (MIC 62.5−125 μg/mL, MBC 125−250 μg/mL, MFC 125 μg/mL) but much higher than that of the crude extract.

More than 100 *Stemona* alkaloids have been isolated from the *Stemona* genus. These alkaloids are structurally classified into eight different groups.[1] The pyrrolo[1,2-*a*]azepine nucleus is common to all compounds in six of these groups. More recently, the structures of *Stemona* alkaloids with a pyrido[1,2-*a*]azepine nucleus were discovered.[1−6] A miscellaneous group comprising a small number of alkaloids lacking these two basic nuclei has also been reported; these alkaloids however arise from the aforementioned alkaloids via oxidation or rearrangement processes.[1,7,8] The pure alkaloids derived from the extracts of the leaves and roots of *Stemona* species have insect toxicity, antifeedant, and repellent activities.[2−5,9−12] We report here the isolation of the known alkaloids **1**−**3** and isolation and structure determination of two new *Stemona* alkaloids, **4** and **5**, from the root extracts of *Stemona aphylla* (Stemonaceae) that were collected at Mae Hong Son, Thailand. No phytochemical work has been done on this *Stemona* species.



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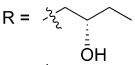

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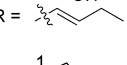

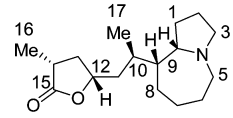

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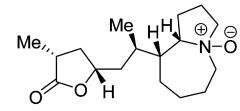

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Results and Discussion

A crude EtOH extract (25 g) of the roots of *S. aphylla* was partitioned between a mixture of MeOH/H_2O (1:1) and CH_2Cl_2. The CH_2Cl_2 fraction was evaporated to afford 3.5 g of crude extract. Successive purification of this material by column chromatography and TLC gave pure samples of stemofoline (**1**), (2′*S*)-hydroxystemofoline (**2**), (11*Z*)-1′,2′-didehydrostemofoline (**3**), stemaphylline (**4**), and stemaphylline-*N*-oxide (**5**). The former three known alkaloids were identified by comparison of their spectroscopic/spectrometric data (NMR and MS) with those reported.[1,3,7,8,10−12] Compounds **4** and **5** are new compounds; we have named them stemaphylline and stemaphylline-*N*-oxide, respectively, based on their botanical origin. Examination of the crude CH_2Cl_2 extract by TLC, ^{1}H NMR, and MS analyses showed the absence of compound **5**, indicating that this compound was most likely being produced via oxidation during the purification process.

The HREIMS (*m*/*z* 279.2207 $[M]^{\bullet+}$, calcd 279.2198) of **4** showed a molecular formula of $C_{17}H_{29}NO_2$. The EIMS showed a fragment ion at *m*/*z* 180 ($[M - C_5H_7O_2]^+$). The loss of $C_5H_7O_2$ is typical for an α-methyl-γ-butyrolactone moiety found often in *Stemona* alkaloids.[1,10,13] The ^{13}C/DEPT NMR spectrum displayed signals for two methyls, nine methylenes, five methines, and one quaternary carbon. The quaternary carbon signal at δ 179.6 and the methine signal at δ 78.4 were assigned to the carbonyl (C-15) and the oxymethine (C-12) carbons, respectively, of the γ-butyrolactone moiety. The ^{1}H NMR spectrum indicated the presence of two methyl groups that are attached to a methine at δ 1.26 (d, J = 7.0 Hz, 3H) and 0.98 (d, J = 6.5 Hz, 3H). The HMBC spectrum showed that C-12 (δ 78.4) correlated to H-13 (δ 1.48), H-11 (δ 2.00), and H-10 (δ 1.70); C-10 (δ 32.5) to H-9 (δ 1.62), H-8 (δ 1.58), and H-11 (δ 1.64); and C-9 (δ 45.9) to H-17 (δ 0.98). These data indicated that a 1-methyl-2-(3-methyl-2-oxo-tetrahydrofuran-5-yl) ethyl moiety was attached to C-9 of the pyrrolo[1,2-*a*]azepine nucleus (Figure 1). The 1D-NOESY correlation of Me-17 with H-12 suggested the β-orientation of H-12, while that of H-14 with H-12 suggested an α-orientation of Me-16. The full ^{1}H and ^{13}C NMR spectra assignments for **4** based on COSY, NOESY, HMBC, and

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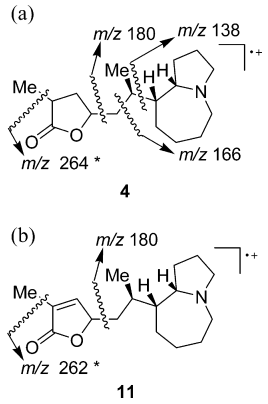


Figure 1. EIMS fragmentation patterns for compounds **4** and **11**.

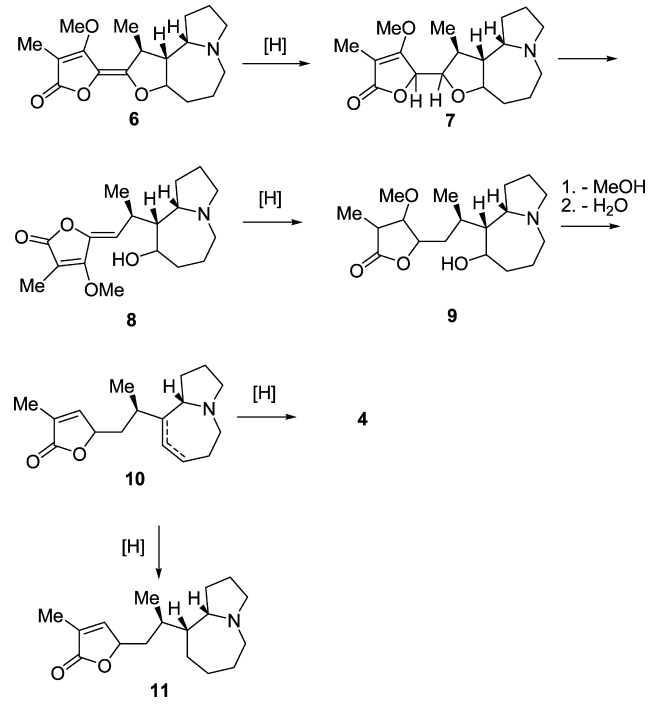
Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data for Compounds **4** and **5** in $CDCl_3$

	δ_H		δ_C	
position	**4**	**5**	**4**	**5**
1	1.79 (m)	2.06 (m)	28.1 (CH_2)	25.0 (CH_2)
		1.88 (m)		
2	1.72 (m)	2.22 (m)	23.8 (CH_2)	19.3 (CH_2)
		1.94 (m)		
3	3.01 (m)	3.57 (m)	54.3 (CH_2)	71.0 (CH_2)
	2.51 (m)			
5	2.94 (m)	3.33 (m)	52.3 (CH_2)	67.2 (CH_2)
	2.51 (m)			
6	1.50 (m)	2.34 (m)	25.9 (CH_2)	20.7 (CH_2)
		1.54 (m)		
7	1.80 (m)	1.66(m)	27.7 (CH_2)	25.2 (CH_2)
	1.33 (m)	1.52 (m)		
8	1.58 (m)	1.70 (m)	28.2 (CH_2)	25.4 (CH_2)
9	1.62 (m)	2.66 (m)	45.9 (CH)	35.8 (CH)
9a	2.93 (m)	4.65 (m)	64.8 (CH)	81.6 (CH)
10	1.70 (m)	1.80 (m)	32.5 (CH)	34.9 (CH)
11	2.00 (m)	1.70 (m)	39.5 (CH_2)	40.6 (CH_2)
	1.64 (m)	1.58 (m)		
12	4.39 (m)	3.52 (m)	78.4 (CH)	76.8 (CH)
13	2.51 (m)	2.55 (m)	37.8 (CH_2)	37.8 (CH_2)
	1.48 (m)	1.45,		
		appar q (12)		
14	2.61 (m)	2.61 (m)	35.8 (CH)	35.2 (CH)
15			179.6 (C)	179.8 (C)
16	1.26, d (7.0)	1.23, d (7.5)	15.0 (CH_3)	15.0 (CH_3)
17	0.98, d (6.5)	0.87, d (7.0)	19.2 (CH_3)	17.3 (CH_3)

HSQC experiments are shown in Table 1, which indicated that **4** was an extensively reduced product of neostemonine (**6**) (Scheme 1). A possible mechanism for the conversion of **6** to **4** is shown in Scheme 1. Reduction of **6** to its dihydro derivative **7** followed by a ring-opening reaction would give **8**. Reduction of both double bonds of **8** and then elimination of methanol and water would give the diene **10**. Complete reduction of **10** would give **4** or partial reduction of **10** would provide **11**. Evidence for the presence of compound **11** from GC-MS analysis of the crude CH_2Cl_2 extract is provided in the latter half of this paper. Precedence for the chemistry shown in Scheme 1 for the conversion of compound **6** to **9** has been recently reported.[14]

The HRESI mass spectrum of **5** showed a protonated molecular ion $[M + H]^+$ at *m/z* 296.2231 (calcd for $C_{17}H_{30}NO_3$, 296.2226), in agreement with the molecular formula $C_{17}H_{29}NO_3$. The molecular formula indicated that **5** had one more oxygen atom than that of **4**. The full ^{1}H and ^{13} C NMR spectroscopic assignments for **5** based on 2D-NMR experiments are shown in Table 1 and indicated that **5** had the same skeleton as **4**. Comparison of the ^{13}C/DEPT NMR spectra of **5** with those of **4** showed that the main differences between them were the chemical shifts of the NMR signals for

Scheme 1



C-3, C-5, and C-9a. The chemical shifts of these carbons for **4** were δ 54.3, 52.3, and 64.8, respectively, whereas the chemical shifts in **5** were δ 71.0 (C-3), 67.2 (C-5), and 81.6 (C-9a), consistent with an *N*-oxide structure for compound **5**.[8] The structure of **5** was further confirmed by the HMBC correlations (see Supporting Information).

As a further structural proof, compound **5** was synthesized by oxidation of **4** with H_2O_2 and Na_2WO_4 in MeOH. The TLC mobility and ^{1}H NMR data of synthetic **5** were identical to those of natural **5**.

The crude CH_2Cl_2 extract was analyzed by GC-MS with the MS operating in the EIMS mode (see Supporting Information for GC-MS trace and EIMS details). Six peaks could be identified by their molecular ion peaks and EIMS fragmentation patterns. Four of these peaks corresponded to the isolated alkaloids **1**−**4**. The EIMS fragmentation pattern found for stemaphylline (**4**) is shown diagrammatically in Figure 1a. The EIMS of one other peak showed a molecular ion of two mass units less than that of **4** and a fragment ion at *m/z* 180, as found in **4**. Its MS fragmentation pattern was consistent with the structure **11**, a dehydro derivative of **4**, in which one degree of unsaturation resided in the γ-butyrolactone ring (Figure 1b). Our attempts to isolate alkaloid **11** in pure form by column chromatography were unsuccessful, and only a partially purified sample was obtained. Attempts to purify this compound resulted in further decomposition. The ^{1}H NMR spectrum of partially purified **11** indicated the presence of an α,β-unsaturated γ-lactone moiety. The signals for an olefinic proton at δ 7.07 (br s) and an allylic methyl at δ 1.94 (s) suggested the presence of an α-methyl-α,β-unsaturated γ-lactone moiety in its structure.[15] Moreover, the signal at δ 4.94 (dd, J = 1.3, 3.3 Hz, H-12) was consistent with an oxymethine proton of the lactone moiety. Examination of the crude CH_2Cl_2 extract by TLC indicated the presence of compound **11**, showing that it is a natural product. GC-MS analysis also identified the presence of the known natural product stibostemin R (**12**).[16]

Biological activity studies were performed on the crude CH_2Cl_2 extract and on the pure sample of **4**. These samples were screened by TLC bioautography for their acetylcholinesterase (AChE) inhibitory activities using physostigmine as the positive control[17] (see Supporting Information). Both samples exhibited low activities

when compared with the positive control. The crude extract showed a higher AChE inhibitory activity than that of **4**. This is most likely due to the higher activity associated with the more prominent compound in the crude extract, stemofoline.[18] The insecticidal activity of both samples was also determined by a topical application assay[19] against *Plutella xylostella*. The results showed compound **4** had insecticidal activity very similar (LC_{50} 1,824 μg/mL) to the positive control (methomyl, LC_{50} 1,840 μg/mL), while the crude extract showed lower insecticidal activity, with an LC_{50} of 13 766 μg/mL. The antimicrobial activities of the crude CH_2Cl_2 extract and compound **4** against two Gram-negative bacteria, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, and a Gram-positive bacterium, *Staphylococcus aureus* ATCC 25923, and the antifungal activity against *Candida albican*s ATCC 90028 were determined using the assay and procedures recommended in the literature (see Supporting Information).[20] Two antibiotic agents, gentamycin and amphotericin B, were used as the positive controls for the antibacterial activity and antifungal activity, respectively. The crude CH_2Cl_2 extract and **4** showed very weak activities (see Supporting Information).

In summary, two new alkaloids, stemaphylline (**4**) and stemaphylline-*N*-oxide (**5**), have been isolated from a root extract of *S. aphylla*, along with three known compounds, stemofoline (**1**), (2′*S*)-hydroxystemofoline (**2**), and (11*Z*)-1′,2′-didehydrostemofoline (**3**). The tentative structure of the new alkaloid **11** was based on GC-MS analysis. The AChE inhibitory, insecticidal, and antimicrobial activities of the crude CH_2Cl_2 extract and compound **4** were also determined.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a JASCO DIP-370 polarimeter. IR spectra were obtained on a Nicolet-AVATAR 360 FTIR spectrophotometer. ^{1}H (500 MHz), ^{13}C (125 MHz), and 2D NMR spectra were recorded on a Varian Unity INOVA 500 MHz spectrometer with a nanoprobe. High-resolution EIMS were recorded on a Fison/VG Autospec-TOF-oa mass spectrometer (70 eV). High-resolution ESIMS were obtained with a Micromass QTOF 2 mass spectrometer using a cone voltage of 30 V and polyethylene glycol (PEG) as an internal reference. TLC was performed on aluminum-backed Merck 60 GF_{254} silica gel, and bands were detected by UV light (λ 254 nm) and Dragendorff's reagent. Column chromatography was performed using Merck GF_{254} flash silica gel (40−63 μm). Physostigmine (eserine) and acetylcholinesterase (906 U/mg, from electric eel) were purchased from Sigma-Aldrich.

Plant Materials. The roots of *S. aphylla* were collected in Mae Hong Son, Thailand, in May 2008. A voucher specimen (number 029779) was deposited at the herbarium of the Department of Biology, Chiang Mai University. Plant material was identified by Mr. James F. Maxwell from the Department of Biology, Chiang Mai University.

Extraction and Isolation. The dry, ground roots of *S. aphylla* (1 kg) were extracted with 95% EtOH (3 × 800 mL) over 3 days at room temperature. The EtOH solution was evaporated to give a dark residue (130 g). A 25 g sample of this extract was partitioned between a mixture of MeOH/H_2O (1:1) (150 mL) and CH_2Cl_2 (200 mL) to afford a crude extract (3.5 g). A portion (2 g) of this material was chromatographed on silica gel (100 mL) using gradient elution from 100% CH_2Cl_2 to MeOH/CH_2Cl_2/conc aq ammonia (10:90:1) as eluent. On the basis of TLC analysis these fractions were pooled to give seven fractions. Fraction 2 (138.9 mg) was rechromatographed by preparative TLC (CH_2Cl_2/MeOH/conc aq ammonia, 98:2:1) to give 9.3 mg of pure (11*Z*)-1′,2′-didehydrostemofoline (**3**) and 89.7 mg of stemofoline (**1**). Fraction 3 (172.7 mg) was rechromatographed on silica gel using gradient elution from 100% EtOAc to MeOH/EtOAc/conc aq ammonia (10:90:1) as eluent to give 120.7 mg of pure stemofoline (**1**) and 29.5 mg of pure (2′*S*)-hydroxystemofoline (**2**), respectively. Fraction 6 (10.5 mg) was rechromatographed using gradient elution from 100% CH_2Cl_2 to MeOH/EtOAc/conc aq ammonia (10:90:1) as eluent to give 4 mg of a mixture containing compound **11**. Fraction 7 (51.8 mg) was rechromatographed using gradient elution from 100% CH_2Cl_2 to MeOH/CH_2Cl_2/conc aq ammonia (10:90:1) as eluent. Stemaphylline (**4**) (30.5 mg) and stemaphylline-*N*-oxide (**5**) (10.2 mg) were obtained. The ^{1}H and ^{13}C NMR data of **1**, **2**, and **3** were identical to those reported,[1,3,7,8,10,11] while those of **4** and **5** are shown in Table 1.

Stemaphylline (4): pale yellow gum; $[\alpha]^{24}_D$ −36.7 (*c* 0.54, $CHCl_3$); IR (film) ν_{max} 2928, 1767, 1634, 1449, 1384, 1189, 1171, 998, 927 cm^{-1}; EIMS (see Table 2); HREIMS *m*/*z* 279.2207 $[M]^{\bullet+}$, calcd for $C_{17}H_{29}NO_2$ 279.2198.

Stemaphylline-*N*-oxide (5): yellow-brown gum; $[\alpha]^{24}_D$ −43.0 (*c* 0.32, $CHCl_3$); IR (film) ν_{max} 3385, 2965, 2934, 2873, 1761, 1460, 1190, 1176, 1001, 927, 748 cm^{-1}; HRESIMS *m*/*z* 296.2231 $[M + H]^{\bullet+}$, calcd for $C_{17}H_{30}NO_3$ 296.2226.

Oxidation of Stemaphylline. To a solution of stemaphylline (**4**) (10.2 mg) and Na_2WO_4 (3.6 mg) in MeOH (2 mL) at 0 °C was added dropwise H_2O_2 (approximately 0.2 mL).[21] The mixture was left to stir at room temperature for 5 h 15 min. To the reaction was added MnO_2 (approximately 5.0 mg), and a check was made using starch paper until no H_2O_2 remained. The mixture was then filtered through a small pad of Celite and washed with more MeOH. The solvent was removed under reduced pressure, and the crude product was purified using gradient elution from 100% CH_2Cl_2 to MeOH/CH_2Cl_2/conc aq ammonia (5:95:1) as eluent to give compound **5** as a yellow-brown gum (3.0 mg).

GC-MS. The GC-MS analyses were performed using a QP5050A GC-MS system (Shimadzu, Japan). The column used was a Rxi-5 ms fused-silica capillary column (30 m × 0.25 mm), coated with a 0.25 μm film (Restek, Bellefonte, PA.). Conditions applied were as follows: injection port temperature 280 °C, split ratio 1:15, helium as carrier gas, temperature program 80 °C (1 min) to 300 °C (15 min) at 6 °C/min, pressure program 27.1 kPa (1 min) to 100 kPa (15.2 min) at 2 kPa/min. Data were collected (over the mass range 35−450 Da) in the positive ion EI mode (70 eV). The ion source and interface temperature were set at 280 and 290 °C, respectively.

Bioautography Procedure. TLC bioautography was performed using the method described by Hostettmann et al.[17] TLC plates were prepared for bioautography by washing with acetone and were then thoroughly dried. The samples were applied to the plate in varying quantities and sprayed with AChE enzyme stock solution [prepared from acetylcholinesterase (906 U/mg) as described in the literature[17]]. The plates were incubated at 37 °C for 20 min and then sprayed with freshly prepared indicator solution (from 1-naphthylacetate and Fast Blue B salt prepared according to the literature[16]) to give the plate a purple coloration after 1−2 min. A white spot indicates inhibition of AChE by the sample.

Antimicrobial Activity Procedure. A series of dilutions of compounds in trypticase soy broth ranging from 500 to 1.95 μg/mL were tested against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 90028 using broth dilution techniques.[20] The solutions were incubated at 37 °C for 24 h. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of culture tube without visible growth of organisms.

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Supporting Information Available: Copies of the ^{1}H NMR spectra of alkaloids **4** and **5**, table of HMBC correlations for **4** and **5**, the GC-MS trace of the crude CH_2Cl_2 extract and EIMS details, and tables of the biological activities of the crude CH_2Cl_2 extract and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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