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Phytochemical, Synthetic and Biological Studies on *Stemona* and *Stichoneuron* Plants and Alkaloids: A Personal Perspective

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Dedicated to the memory of Dr. Rosdayati Alino Ramli

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This report is an overview of our research on phytochemical, synthetic and biological studies of the *Stemona* and *Stichoneuron* species of plants.

Keywords: *Stemona*, *Stichoneuron*, AChE, P-glycoprotein, Insecticide, Biopesticide, Alkaloids.

This paper is a summary of the invited lecture presentation by Stephen Pyne at the ISNPF2016 conference at Tokushima in September 2016. Extracts of the roots of the *Stemona* species of plants 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 (Figure 1) [1]. Some of the pure alkaloids derived from these plants have significant antitussive activity in guinea pig after cough induction [1,2] as well as insect toxicity, anti-feedant and repellent activities [3,4].

Figure 1: *Stemona tuberosa* roots ("Non Tai Yak") for sale from a herbal medicine shop in Chiang Mai Provence. The sign indicates that the water extracts of the roots are useful for treating skin ailments (dermatitis and rashes) and can be used as an insecticide (photograph by S. Pyne).

The *Stemona* group of alkaloids includes more than 130 unique natural products which have been structurally classified into six

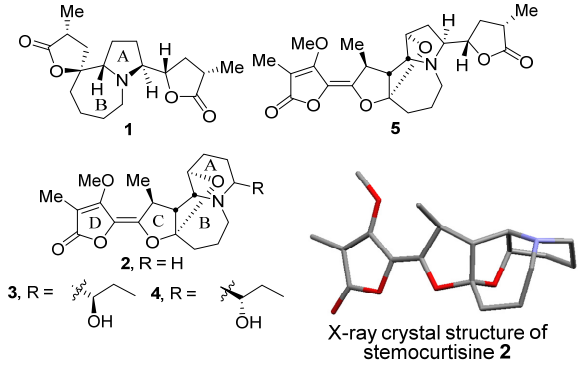


Figure 2: Structures of cromine **1**, the pyrido[1,2-*a*]azepine alkaloids, stemocurtisine **2**, oxystemokerrin **3** and stemocurtisinol **4**, and oxyprotostemonine **5** and X-ray structure of **2**.

different groups [1]. The pyrrolo[1,2-*a*]azepine (5,7-bicyclic A,B-ring system) nucleus is common to all compounds in five of these groups (croomine (**1**) for example in Figure 2). In 2003 we [3] and then Greger [4] reported the structures of *Stemona* alkaloids with a pyrido[1,2-*a*]azepine A,B-ring system (6,7-bicyclic A,B-ring system), including stemocurtisine **2**. These alkaloids now comprise the sixth structural group (the stemocurtisine group).

Certain *Stemona* alkaloids have oxytocin antagonistic effects [5], the ability to inhibit nitric oxide production [6], have P-glycoprotein (P-gp)-modulation effects [7] and are inhibitors of acetylcholinesterase AChE [8], a property associated with

insecticidal activity [9]. We have reported that the *Stemona* alkaloids stemofoline and 11(*Z*)-1',2'-didehydrostemofoline, and some of their synthetic derivatives, inhibit P-gp-mediated drug resistance in cancer cell lines [10] and are inhibitors of AChE [11]. This short review summarizes the results of our studies.

Stemona alkaloids are isolated from the roots of the *Stemona*ceae family of plants which comprise three genera: *Stemona* (the most common genus, found in SE Asia and the South Pacific); *Croomia* (found in SE North America and Japan); and *Stichoneuron* (found in Peninsula Malaysia and Thailand) [1]. In a 2002 collaboration with A. Prof. Araya Jatisatienr, Dr. Pitchaya Mungkornasawakul (Chiang Mai University (CMU), Thailand) and A. Prof. Alison Ung (then at University of Wollongong and now at University of Technology, Sydney) we isolated stemocurtisine **2** a new structural type of *Stemona* alkaloid, based upon the pyrido[1,2-*a*]azepine core, from *Stemona curtisii Hook. f.* collected from Trang Provence in Thailand [3]. Its structure was unequivocally determined by single-crystal X-ray analysis (Figure 2) [3].

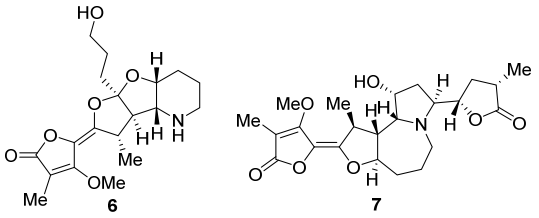


Figure 3: Structures of 6-hydroxy-5,6-*seco*-stemocurtisine **6** and 1-hydroxy-protostemonine **7**.

Figure 4: Enclosed field trials at CMU on Chinese cabbage using a biopesticide based on *S. curtisii* extract (left-hand side field) and control (right-hand side field). Photograph courtesy of A. Prof. Jatisatienr.

In the same year Greger [4] reported the isolation of **2,** and a new pyrido[1,2-*a*]azepine alkaloid, oxystemokerrin **3** (Figure 2). In 2004 we disclosed the structure of another pyrido[1,2-*a*]azepine alkaloid, stemocurtisinol **4** [12]. Greger [4] and shortly after our group [12] also published the isolation of oxyprotostemonine **5**, a pyrrolo[1,2-*a*]azepine alkaloid (Figure 2). Compounds **2**, **4** and **5** had significant larvicidal activity on malaria-carrying mosquito larvae (*Anopheles minimus* HO) [12]. Subsequent studies by our group of the extracts of *Stemona curtisii Hook. f.* from Trang Provence identified two other new natural products, 6-hydroxy-5,6-*seco*-stemocurtisine **6** [13], the only know *seco*-pyrido[1,2-*a*]azepine alkaloid to date, and 1-hydroxyprotostemonine **7** (Figure 3) [14]. A. Prof. Jatisatienr and Dr. Mungkornasawakul further developed the crude extracts of *Stemona curtisii* into a biopesticide formulation which showed promise in field trials as an anti-feedant on Chinese cabbage and other commercial vegetable crops (Figure 4).

In 2009 we reported the isolation and structure determination of two novel *Stemona* alkaloids, stemaphylline (**8**) and stemaphylline-*N*-oxide (**9**), from the root extracts of *Stemona aphylla* which was collected at Mae Hong Son, Thailand (Figure 5) [15]. The insecticidal activity of **8** was determined using a topical application assay against *Plutella xylostella*. Compound **8** had insecticidal activity very similar (LC_{50} 1,824 µg/mL) to the positive control (methomyl, LC_{50} 1,840 µg/mL).

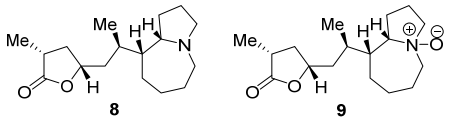


Figure 5: Structures of stemaphylline (**8**) and stemaphylline-*N*-oxide (**9**) isolated from the root extracts of *Stemona aphylla* (collected at Mae Hong Son, Thailand).

Our study of the root extracts of *S. aphylla*, collected in Lampang Province, Thailand, at a location different from that of our abovementioned study on this plant species resulted in the identification of a new stemofoline alkaloid. This was (2′*S*)-hydroxy-11(*S*),12(*R*)-dihydrostemofoline **12** which was isolated along with six new stemofurans and the known compounds stemofoline (**10**) and (2′*S*)-hydroxystemofoline (**11**) [Figure 6] [16]. (2′*S*)-Hydroxy-11(*S*),12(*R*)-dihydrostemofoline **12** was significantly less active as an AChE inhibitor than stemofoline (**10**) itself, perhaps due to the lack of rigidity of the C-11-C-12 double bond.

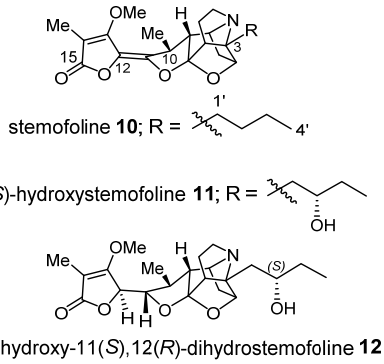
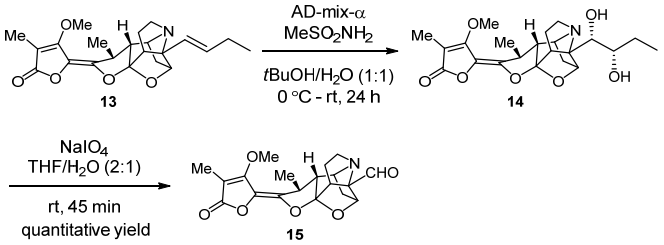


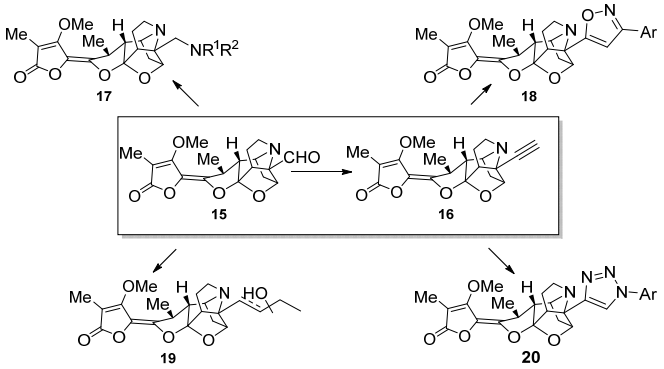
Figure 6: Structures of stemofoline (**10**) and (2′*S*)-hydroxystemofoline (**11**) and (2′*S*)-hydroxy-11(*S*),12(*R*)-dihydrostemofoline (**12**) isolated from the root extracts of *Stemona aphylla* (collected in Lampang Province, Thailand).

In 2008 Kwankamol Sastraruji, a graduate from CMU, joined my laboratory as a PhD student. She brought with her extracts of an unidentified *Stemona sp.* rich in 11(*Z*)-1',2'-didehydrostemofoline **13**. From 935 g of the dry root we could isolate 6 g of 11(*Z*)-1',2'-didehydrostemofoline **13** after purification by column chromatography. This compound served as a starting material to prepare a library of derivatives and analogues around the aldehyde **15** (Scheme 1) and the alkyne derivative **16** (Scheme 2) using, reductive amination (**15** → **17**), organometallic addition reactions (**15** → **19**), Wittig-type reactions (**15** → **19**) and "click" chemistry (**16** → **18** and **20**). A number of natural *Stemona* alkaloids and their derivatives were made from aldehyde **15**, some of the synthesized compounds are shown in Table 1 [11(b)] and Table 2 [10].

These derivatives were screened as inhibitors against AChE and P-gp. There is current interest in AChE inhibitors for the treatment



Scheme 1: Synthesis of aldehyde **15** from 11(*Z*)-1',2'-didehydrostemofoline **13**.



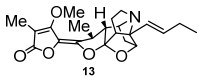
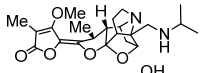
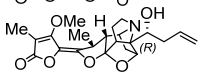
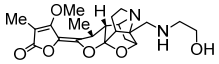
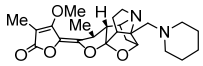
Scheme 2: Synthesis of a library of compounds (**17**-**20**) from aldehyde **15** and alkyne **16**.

of Alzheimer's disease (AD) [17]. These inhibitors act by increasing the acetylcholine concentration in the brain which helps improve cognitive, behavioural and functional impairments. Reversible AChE inhibitors, for example the alkaloid galanthamine (Reminyl), have been used in the treatment of patients with AD to alleviate the symptoms associated with this disease [18]. Unfortunately, these inhibitors are not a cure for AD and do not stop progression of the disease. Current drug development strategies involve targeting microtubule-associated τ-protein, metal ion dyshomeostasis and the various β-amyloid (Aβ) pathological mechanisms of this disease [18,19]. The finding that AChE colocalizes with Aβ and promotes and accelerates Aβ aggregation [20-22] has renewed an intense interest in AChE inhibitors, including dual binding AChE inhibitors [23] and those that can be activated by AChE [24] and have Aβ–anti-aggregating action. The identification of new AChE inhibitors is therefore of importance for new applications in both agriculture and medicine.

Unfortunately the AChE inhibitory activities of our compounds were at best modest with IC_{50} values > 12 μM against electric eel (ee) AChE and IC_{50} values > 19 μM against human (h) AChE, Table 1, entry 3, far less than that of galanthamine (entry 1) with IC_{50} values of 0.9 and 0.6 μM, respectively in our in-house assay. The most potent compound being a synthetic amine derivative, the structure of this compound is shown in Table 1 (entry 3) [11(b)].

The semi-synthesis of the *Stemona alkaloids*, (3′*R*)-stemofolenol (**21**), (3′*S*)-stemofolenol (**22**), methylstemofoline (**23**) and (3′*S*)-hydroxystemofoline (**24**), and the unnatural analogues (11*E*)-methylstemofoline and (3′*R*)-hydroxystemofoline, was achieved by Morwenna Baird starting from the aldehyde **15** [11 (a)]. This synthesis allowed for the first time access to diastereomerically enriched samples of alkaloids **21** and **22** and the assignment of their absolute configurations at C-3′. These compounds were obtained in sufficient quantities to allow for their biological testing. These compounds were screened by TLC bioautography for their AChE inhibitory activities using the method of Hostettmann [25] and

Table 1: Acetylcholinesterase inhibitory activity of stemofoline derivatives.

Entry	Compound	IC_{50} values μM (R^2)	
		eeAChE	hAChE
1	galanthamine	0.902±0.04 (0.9953)	0.597±0.07 (0.9877)
2		19.20±0.26 (0.8749)	24.98±0.13 (0.9714)
3		12.94±0.08 (0.9883)	19.93±0.17 (0.9455)
4		302.3±0.29 (0.9245)	41.17±0.22 (0.9114)
5		52.45±0.14 (0.9668)	37.49±0.16 (0.9540)
6		77.19±0.22 (0.9274)	28.72±0.19 (0.9210)

eserine as a reference compound. Compound **13** showed the highest inhibitory activity of AChE with a minimum inhibitory quantity of 5 ng, followed by compound (3'*S*)-**24** at 10 ng. The (3'*R*)-diastereomer of **24** was less active than (3'*S*)-**24** with a minimum inhibitory quantity of 100 ng [11(a)].

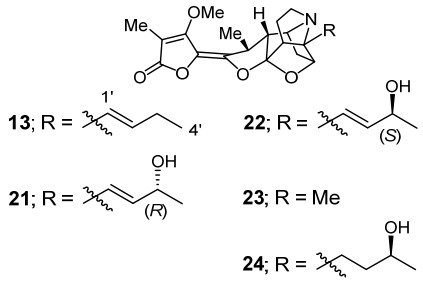


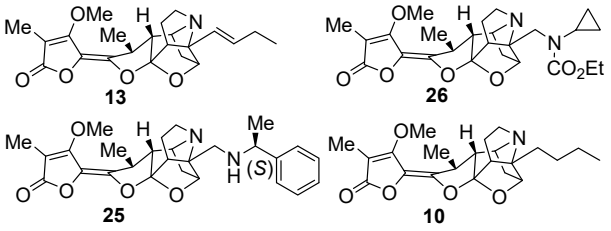
Figure 7: Structures of (3′*R*)-stemofolenol (**21**), (3′*S*)-stemofolenol (**22**), methylstemofoline (**23**) and (3′*S*)-hydroxystemofoline (**24**) prepared by semi-synthesis from **13**.

In collaboration with A. Prof. Pornngarm Limtrakul (Biochemistry, CMU) a number of *Stemona* alkaloids and their synthetic derivatives have been studied for their abilities to inhibit P-gp-mediated drug resistance in cancer cell lines [10]. P-glycoprotein is responsible for the efflux of chemotherapeutic drugs from cancer cells and its overexpression is one of the mechanisms of drug resistance. We have identified several compounds that reverse multi-drug resistance (MDR) to important clinically used drugs (e.g. vinblastine and doxorubicin) in drug-resistance human cervical carcinoma (KB-V1) and human leukemic (K562/Adr) cell lines that overexpress P-gp. It was also demonstrated that these compounds inhibit P-gp function. As shown in Table 2, the IC_{50} for vinblastine against a drug-sensitive human cervical carcinoma (KB-3-1) is 0.61 nM but against a drug-resistance human cervical carcinoma (KB-V1 (MDR)) vinblastine is 1000 fold less potent (IC_{50} = 0.6 μM). However in the presence of 11(*Z*)-1',2'-didehydrostemofoline **13** and stemofoline **10** and their synthetic analogues (**25** and **26**) the IC_{50} of vinblastine is significantly enhanced (3-7 fold) against KB-V1 (MDR (Table 2 (a)). The most effective compound was stemofoline **10** which enhanced the potency of vinblastine by 7 fold [10]. In the case of drug resistance human leukemic (K562/Adr (MDR)), we found that the most effective compound was the synthetic analogue **25** which enhanced the potency of doxorubicin by 7 fold followed by stemofoline **10** which enhanced the potency of doxorubicin by 4 fold (Table 2 (b)) [10].

Table 2: Effect of stemofoline analogues on treatment of MDR cervical cancer (KB) cell lines with vinblastine (a) and MDR leukemic (K562/Adr) cell line with doxorubicin (b).

(a)	IC_{50} / Fold reversing activity
Vinblastine Treatment	
KB-3-1 (drug-sensitive)	0.61 ± 0.01 nM / 1.00 ± 0.00
KB-V1 (MDR)	0.60 ± 0.05 μM / 1.00 ± 0.00
Stemofoline derivative	
KB-V1 + 5 μM of **13**	0.11 ± 0.04 μM / 5.76 ± 1.92
KB-V1 + 5 μM of **25**	0.15 ± 0.04 μM / 4.33 ± 1.28
KB-V1 + 5 μM of **26**	0.18 ± 0.05 μM / 3.59 ± 1.12
KB-V1 + 5 μM of **10**	0.09 ± 0.01 μM / 7.03 ± 1.28

(b)	IC_{50}/Fold reversing activity
Doxorubicin treatment	
K562 (drug-sensitive)	0.45 ± 0.01μM / 1.00 ± 0.00
K562/Adr (MDR)	17.33 ± 1.15 μM / 1.00 ± 0.00
Stemofoline derivatives	
K562/Adr + 5 μM of **13**	4.70 ± 0.26 μM / 3.70 ± 0.44
K562/Adr + 5 μM of **25**	2.27 ± 0.49 μM / 7.97 ± 2.31
K562/Adr + 5 μM of **26**	7.57 ± 0.40 μM / 2.29 ± 0.10
K562/Adr + 5 μM of **10**	4.47 ± 0.55 μM / 3.94 ± 0.71



While many phytochemical studies have been conducted on *Stemona* plant species relatively little is known about the phytochemicals of the *Stichoneuron* species. In 2005 Greger reported the isolation of an inseparable 60:40 mixture of two new alkaloids stichoneurines A and B (Figure 8) from *Stichoneuron caudatum* collected in Thailand [26]. In 2011, Rosdayati Alino Ramli (MSc with A. Prof. Jalifah Latip, Universiti Kebangsaan, Malaysia) joined my research group as a PhD student. She isolated and structurally characterized four new *Stemona* alkaloids from *Stichoneuron caudatam* growing in Peninsula Malaysia, these were stichoneurines F and G (Figure 8) having a novel tetrahydrofuran ring and sessillistemoamines E and F (structures not shown) [27]. From the extracts of *Stichoneuron halabalensis*, also growing in Peninsula Malaysia, three new alkaloids, stichoneurines C −E were isolated (Figure 8) [28]. One can imagine that all seven stichoneurine alkaloids could be biosynthetically related through different redox pathways.

We are also interested in the total synthesis of *Stemona* alkaloids [29,30]. Our recent strategy for the synthesis of stemocurtisine **2** (Figure 2) was based on the premise that the synthetically challenging bridging ketal moiety of this compound could be realized via a photochemically induced oxidative cyclization reaction of the tricyclic alcohol **29** (Scheme 3). We proposed that this reaction would proceed via H-atom abstraction at C-3a by the generated C-11 alkoxy radical. However, irradiation of a solution of racemic **29**, I_2 and $PhI(OAc)_2$ led only to the aldehyde product **30**,

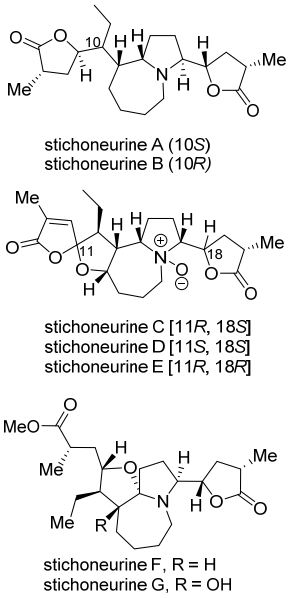
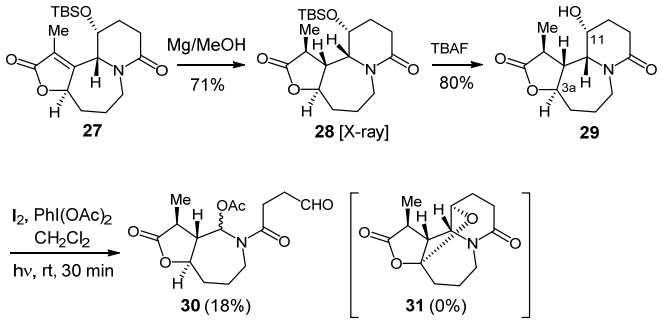


Figure 8: Structures of stichoneurines A-G isolated from *Stichoneuron sp*.



Scheme 3: Attempted synthesis of **31** having the A-B-C ring system and the cyclic ketal structure of stemocurtisine **2**.

formed by oxidative cleavage of the A-ring [29]. None of the desired product **31** was isolated.

In conclusion, the *Stemona* and *Stichoneuron* species of plants have and continue to produce alkaloids having novel chemical structures and biological activities. The root extracts, and their associated alkaloids and their synthetic analogues show potential applications in agriculture as anti-feedants and insecticides and in medicine as AChE and P-gp inhibitors. New biotechnology methods of producing these alkaloids in larger quantities are required to progress this area further.

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