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Tropical Chemodiversity in Selected Angiosperm Families

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Abstract

The underlying hypothesis of this work is the expected correlation between phytochemical diversification, as expressed by the accumulation of secondary metabolites (SM), and systematic relationships in plant groups. Characteristic SM profiles and specific compounds may prove to be good chemical characters (=chemical markers) within plant families or orders. To verify this hypothesis, comparative phytochemical studies on alkaloids within different tropical plant families were carried out. We selected two families, the monocotyledonous Stemonaceae and some genera of the dicotyledonous Rubiaceae.

The major accumulation tendencies of alkaloids found in Stemonaceae comprise structural types showing a pyrrolo- or pyrido[1,2- α]azepine core structure, which can be grouped into the three main derivatives, the stichoneurines, the protostemonines and the croomines. Most of the investigated *Stemona* species accumulate protostemonine-type alkaloids. However, *Stemona tuberosa* and *S. phyllantha* can clearly be distinguished from the other studied *Stemona* species by the accumulation of stichoneurine-type alkaloids. This structural type of alkaloids occurs only in these both species. Croomine derivatives occur scattered in analyzed *Stemona* species. *Stichoneuron caudatum* and *S. halabalensis* accumulate stichoneurine-type alkaloids. By contrast, the recently described *Stichoneuron calcicola* accumulates the *Pandanus* alkaloid Pandanamine as the major alkaloid.

As for Rubiaceae, we focused on the presence of iridoids and tryptamine-iridoid alkaloids (= monoterpene-indole alkaloids) which proved to show characteristic major accumulation tendencies in the studied genera and species. *Ronabea emetica* and *R. latifolia* accumulate iridoids, whilst tryptamine-iridoid alkaloid derivatives are representative for *Palicourea acuminata*. A variation of this type of alkaloids could also be detected in the leaves of *Chassalia curviflora* var. *ophioxylodes* and also in the leaves of *Rudgea cornifolia*. The isolated and structurally elucidated alkaloids named Alstroline A and Rudgeifoline have two Secologanin glycoside moieties, in contrast to the Lagamboside, isolated from *P. acuminata*, which have one glycoside moiety directly linked to the nitrogen of the indole-ring.

Our results confirm that most of the isolated SM can be regarded as good chemical characters, supporting generic and specific delimitation in the studied groups.

Zusammenfassung

Die dieser Arbeit zugrundeliegende Hypothese ist der erwartete Zusammenhang zwischen phytochemischer Stoffausstattung und taxonomischer Verwandtschaft auf Familien- oder Gattungsebene. Das phytochemische Profil von einer Klasse von Sekundärmetaboliten umfasst sehr oft nur einige strukturell sehr ähnliche Derivate und ist zumeist charakteristisch für ein Taxon. Um diese Hypothese zu verifizieren wurden die Alkaloidprofile von Arten aus der paläotropisch verbreiteten Familie der Stemonaceae aus der Gruppe der Monokotyledonen sowie einige neotropische Arten der Rubiaceae aus der Gruppe der Dikotyledonen untersucht.

Die Pflanzenfamilie der Stemonaceae ist durch das Auftreten von speziellen Alkaloiden, den *Stemona* Alkaloiden, gekennzeichnet. Diese Stoffklasse umfasst Substanzen mit entweder einer Pyrrolo[1,2- α]- oder einer Pyrido[1,2- α]-azepin Struktur. Innerhalb der Gattung *Stemona* zeigen diese Alkaloide ein auffallendes Verbreitungsmuster, das sich auch in der Phylogenie widerspiegelt. Innerhalb der Gattung *Stichoneuron* stellt die Art *Stichoneuron calcicola* durch das Anreichern von Pandanamin einen Sonderfall dar, da die beiden anderen bisher untersuchten Arten *Stichoneuron caudatum* und *S. halabalensis* Alkaloide vom Stichoneurin-Typ akkumulieren. Das Pandanamin ist aus *Pandanus amaryllifolius* bekannt und das Auftreten in den Stemonaceae weist auf enge verwandtschaftliche Beziehungen zu der Familie der Pandanaceae hin.

Die Untersuchungen an verschiedenen neotropischen sowie paläotropischen Arten aus der *Psychotria* Verwandtschaft (Rubiaceae) zeigten einerseits die Tendenz Tryptamin-Iridoid Alkaloide, auch bekannt als Monoterpen-Indol Alkaloide (MIA), und andererseits Iridoide anzureichern. Die untersuchten Arten aus der Gattung *Ronabea*, *R. emetica* und *R. latifolia*, akkumulieren Iridoide als typische Sekundärstoffe. Im Gegensatz dazu weisen die anderen untersuchten Arten aus dieser Pflanzenfamilie Tryptamin-Iridoid Alkaloide als typische Sekundärstoffe auf. Innerhalb dieser Stoffklasse tritt jedoch eine Differenzierung im Verhältnis Tryptamin- zu Iridoidanteil von 1:1 bis 1:2 auf.

Die erhaltenen Resultate zeigen, dass einige der isolierten Sekundärstoffe als chemische Marker geeignet sind und die Resultate von phylogenetischen Studien weitgehend unterstützen.

Co-authorship statement

Chapter 2 (Paper 1): My contribution to the publication ***Pandanus Alkaloids in Stemonaceae: Finding of a Plausible Biogenetic Origin of Stemona Alkaloids***, published 2009 in *Journal of Natural Products*, was mainly the experimental work.

Chapter 3 (Paper 2): My contribution to the publication ***Structural Relationships of Stemona Alkaloids: Assessment of Species-Specific Accumulation Trends for Exploiting Their Biological Activities***, published 2011 in *Journal of Natural Products*, was to guide the first author through the experimental work. The writing of the manuscript was done in cooperation with the corresponding author.

Chapter 4 (Paper 3): My contribution to the publication ***Various types of tryptamine-iridoid alkaloids from Palicourea acuminata (= Psychotria acuminata)***, published 2012 in *Phytochemistry Letters*, was to instruct and help the co-authors (students) through their practical work.

Chapter 5 (Paper 4): My contribution to the publication ***Alstroines in Rubiaceae: Alstroine A from Chassalia curviflora var. ophioxyloides and a novel derivative, Rudgeifoline from Rudgea cornifolia***, published 2012 in *Phytochemistry Letters*, was to provide the theoretical background and to supervise the co-authors (students) through their practical work and to design the outlay of the manuscript.

Chapter 6 (Paper 5): My contribution to the publication ***Iridoids as chemical markers of false ipecac (Ronabea emetica), a previously confused medicinal plant***, published 2011 in *Journal of Ethnopharmacology*, was to provide the theoretical background and to supervise the student co-authors through their practical work and to design the outlay of the manuscript.

The structure elucidation of the isolated compounds, based on various NMR techniques, was done by Dr. Lothar Brecker and Susanne Felsinger, the mass spectrometry was performed by Dr. Eberhard Lorbeer and his team.

Aims and outline

The underlying hypothesis of this work is the postulated correlation between phytochemical diversity of plant species and their taxonomic relationship on the level of families and genera. Phytochemical Diversification is supposed to become evident by profiling and determination of accumulation tendencies of characteristic secondary metabolites (SM). Within investigated plant species the accumulations tendencies include quite often a set of structurally close related derivatives. To verify this hypothesis, various studies on different tropical plant families were carried out.

The accumulation tendencies of SM are important characters in plant systematics and chemotaxonomy because they partially reflect the genome. Some types of SM occur specifically in certain taxa (= chemical markers) and some occur erratically. The latter ones can be regarded as convergent traits, analogous to some morphological characters (Frohne and Jensen, 1998). The evaluation whether certain SMs are chemical markers or not depends largely on the level of the studied taxon. For example, the well-known alkaloid nicotine has been detected not only in *Nicotiana* species, but also in species of the genera *Juglans*, *Humulus*, *Prunus* etc. (Blaim, 1962), and therefore cannot be regarded as a chemical marker.

The major aim of this dissertation was to search for accumulation tendencies of selected types of SM in certain monocotyledonous and dicotyledonous species and, thus, to contribute to a better overview on the distribution pattern of these compounds. To achieve this aim, comparative studies on species and provenances of the monocotyledonous family Stemonaceae and on the dicotyledonous family Rubiaceae were carried out, in order to elucidate the accumulation trends of non-volatile compounds in these taxa.

By doing so, previous work (Schinnerl et al., 2007) was extended. The main focus in Stemonaceae was to elucidate the distribution pattern of alkaloids within the genus *Stemona*. Furthermore, the diversification of the alkaloid pattern of *Stichoneuron* species was of major interest, because *Stichoneuron calcicola* was newly discovered and described (Inthachub et al., 2009). *Stichoneuron caudatum* was the only investigated species within this genus before (Schinnerl et al., 2005). Recently, *S. halabalensis* has been studied from this genus (Ramli et al., 2013).

Within the Rubiaceae family, which contains several different tryptamine-iridoid alkaloids (= monoterpene-indole alkaloids), we investigated so far unstudied species of the *Psychotria* alliance, in order to elucidate accumulation tendencies of these alkaloids. It was expected that phytochemical diversification could be chemotaxonomically significant in this case.

This thesis is organized in seven chapters. Chapter 1 contains a short morphological description and an overview of relevant SMs of the studied species. Chapters 2 and 3 deal with *Stemona* alkaloids in *Stichoneuron* and *Stemona* species. Chapters 4-6 focus on iridoids and tryptamine-iridoid alkaloids in certain rubiaceous species. Finally, chapter 7 addresses the ecological relevance of these alkaloids. This aspect has not yet been fully dealt within the publications presented in the chapters 2-6.

Chapter 2: ***Pandanus* Alkaloids in Stemonaceae: Finding of a Plausible Biogenetic Origin of *Stemona* Alkaloids.** Pandanamine could be detected as the only alkaloid in *Stichoneuron calcicola*. This compound has already been described in *Pandanus* species (Takayama et al., 2001) and might be the precursor of the *Stemona* alkaloid Croomine in *Stemona* species. This finding supports the close relationship between Pandanaceae and Stemonaceae because the genetic background of involved enzymes leading to this substance is supposed to be very similar and may have evolved in an ancestor of both families.

Chapter 3, **Structural Relationships of *Stemona* Alkaloids: Assessment of Species-Specific Accumulation Trends for Exploiting Their Biological Activities.** *Stemona* alkaloids are only known from *Stemona* and *Stichoneuron* species. *Stemona* species are important ethnobotanical plants in SE-Asia due to the bioactive properties of many alkaloids. This study deals with the distribution pattern of various derivatives within Thailand's *Stemona* species.

The following papers (chapters 4-6) deal with the occurrence of the main secondary metabolites accumulated in the genera of the Rubiaceae. Within this family, the genus *Psychotria* is known as a source of tryptamine-iridoid alkaloids and/or dopamine-iridoid alkaloids, respectively (Bernhard et al., 2011). The latter alkaloids are typical for *Carapichea ipecacuanha*. The roots of this species have widely been used as a medical drug named Ipecac. During our studies we revealed that the roots of *Ronabea emetica*, which frequently have been confused with Ipecac, are accumulating iridoids instead of dopamine-iridoid

alkaloids. This is discussed in chapter 4 **Iridoids as chemical markers of false ipecac (*Ronabea emetica*), a previously confused medicinal plant**. The authors postulate that Iridoids are typical SMs of *Ronabea emetica*.

Species from the genus *Palicourea* are accumulating tryptamine-iridoid alkaloids. A new type, showing a glucose moiety directly linked to the nitrogen of the indole ring, is described in **Various types of tryptamine-iridoid alkaloids from *Palicourea acuminata* (= *Psychotria acuminata*)** (chapter 5). When investigating rarely studied species from smaller genera closely related to *Psychotria*, we discovered tryptamine-iridoid alkaloids with a ratio of 1:2 of tryptamine to iridoid moieties within *Chassalia* and *Rudgea* species. This outcome has been published in the paper: **Alstrostines in Rubiaceae: Alstrostine A from *Chassalia curviflora* var. *ophioxyloides* and a novel derivative, Rudgeifoline from *Rudgea cornifolia*** (chapter 6).

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General Introduction: *Plant secondary metabolites (SM)*

Secondary metabolites are organic compounds which play a significant role not only for the life of plants, but also for other organisms. These compounds are important in communication, e.g. for attracting animals for pollination or seed dispersal as well as for chemical defense and competition, e.g. allelochemicals. Wink (2008) estimated that more than 100.000 phytochemicals have been isolated and structurally characterized by various NMR and MS techniques. The vast numbers of SMs belong to a limited number of structural classes (e.g. alkaloids, terpenoids, polyphenols etc.). They are synthesized by several biosynthetic pathways which are often combined leading to that enormous structural diversity. The origin of SM is in many cases closely linked to attaining primary function, e.g. sesqui-, di- and triterpenes as phytohormones, flavonoids as development regulators, and the non-proteinogenic amino acid Canavanine as chemical defense and nitrogen source for the embryo (Hartmann, 2007). The occurrence of SM can be ubiquitous or limited to certain taxa, often depending on their basic backbone. Thus, flavonoids are common throughout the plant kingdom, whereas alkaloids show a scattered distribution pattern (Wink, 2008). In some cases SM can be used as chemical characters, but this heavily depends on the specific SM and also on the level of the specific taxon (Harborne and Baxter, 1993; Wink and Waterman, 1999). The chemical defense can either be constitutive or induced. Constitutive resistance requires energy and place for storage and is often linked to compounds based on a pure carbohydrate backbone (Sampedro et al., 2011). By contrast, the induced defense mechanism relies on a fast signaling system within the plant to switch on/off certain metabolic pathways in case of being attacked. Most of the plant secondary metabolites interact with other organisms e.g. insects or fungi on various levels. The interaction with insects is ranging from repellent over influencing the development of larvae to cause the immediate death of the herbivore (Baldwin, 1998; Ibanez et al., 2012).

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Chapter 1: studied Plants and their SM

1.1. Stemonaceae (Monocotyledons; order: Pandanales)

Various species of the genus *Stemona* are important in traditional medicine in China and some other countries from SE-Asia. The roots of *S. tuberosa* Lour., *S. phyllantha* Gagnep., *S. japonica* (Blume) Miq., *S. mairai* (H.Lév.) K.Krause, *S. sessilifolia* (Miq.) Miq., *S. collinsiae* Craib and *S. curtisii* Hook.f. are used either in traditional medicine against cough or against ectoparasites (But et al., 2012; Pilli and Ferreira De Oliveira, 2000; Stöger, 1999). The vernacular names of these drugs are Bai Bu in China and Non tai yak in Thailand.

The family Stemonaceae comprises three genera: *Stemona* Lour.(ca. 30 spp.), *Stichoneuron* Hook.f. (5 spp) and *Croomia* Torr. (6 spp.). Their major characters are perennial roots and tetramerous flowers. The leaves have conspicuous veinlets (Rudall and Bateman, 2006; Rudall et al., 2005). The species of this family are distributed in SE-Asia except of *Croomia*, occurring in southern Japan with 5 species and in SE of the United States with one species (Kadota, 2012; Kadota and Saito, 2010). The genus *Pentastemona* Steenis (2 spp.) with pentamerous flowers was placed in the monotypic family Pentastemonaceae (Duyfjes, 1991; Steenis, 1982) although DNA-based phylogenetic studies support the relationship to Stemonaceae (Caddick et al., 2002).

1.1.1 *Stemona* Lour.

Stemona is the largest genus within Stemonaceae and comprises approx. 30 species distributed in subtropical and tropical SE-Asia. The plants are perennial climbers, among them some species show an obvious seasonal development. The plants are deciduous and they survive with their storage roots during the hot season in subtropical areas. The shape of the roots differs among the specific species, ranging from approx. 5 cm in length and 1 cm in diameter (*S. kerrii* Craib, *S. burkillii* Prain) to 10-20 cm in length and 2-2.5 cm in diameter (*S. tuberosa*, *S. phyllantha*, (Fig. 1 and Fig. 2)) or 30-50 cm in length and 1-1.5 cm in diameter (*S. curtisii*). The color of the flowers varies from greenish/whitish (*S. collinsiae*) to pinkish (*S. burkillii*) and dark red (*S. tuberosa*, *S. curtisii*). The size of the tepals varies from 2 cm (*S. parviflora* C.H.Wright) to 7 cm in length (*S. phyllantha*). The stamens are elongated and

might be the site for releasing a decay scent. The pollinators are mainly flies and the fruits are capsules containing three or four seeds. Elaiosomes are attached to the seeds to attract ants for dispersal. *Stemona kerrii* is the only species with hairy leaves.

Phytochemistry: Roots are widely used for pharmaceutical purposes, which is due to the presence of *Stemona* alkaloids having a pyrido- or pyrrolo[1,2- α]nucleus (Fig. 5). Until now, approx. 130 derivatives have been described. Some of them exhibit distinct insecticidal properties (Brem et al., 2002; Kaltenegger et al., 2003), while others show antitussive characteristics (Chung et al., 2003; Hu et al., 2009; Lin et al., 2008a; Lin et al., 2008b; Xu et al., 2006; Xu et al., 2010; Zhou et al., 2009).

Apart from alkaloids, the roots are also accumulating stilbenoids with antifungal properties (Kostecki et al., 2004; Pacher et al., 2002; Zhang et al., 2008). Until now, approx. 68 derivatives belonging to four structural types have been described (Greger, 2012). Some of the derivatives solely occur after fungal infection or drought stress (Pacher, 2005).

Tocopherols have also been found in roots of *Stemona* species (Brem et al., 2004). The isolated derivatives differ in the substitution pattern of their aryl moiety. In addition to the mentioned compounds, some ubiquitously distributed chlorogenic acid derivatives (Ge et al., 2007) as well as benzoic acid and anthrachinone derivatives have been isolated so far (Tan et al., 2007).

Each of the three mentioned classes of compounds exhibits a distinct, but an uncorrelated distribution pattern within the genus *Stemona* (Brem et al., 2004; Greger, 2012; Schinnerl et al., 2007). Some chemical features appear to be of chemotaxonomic significance.

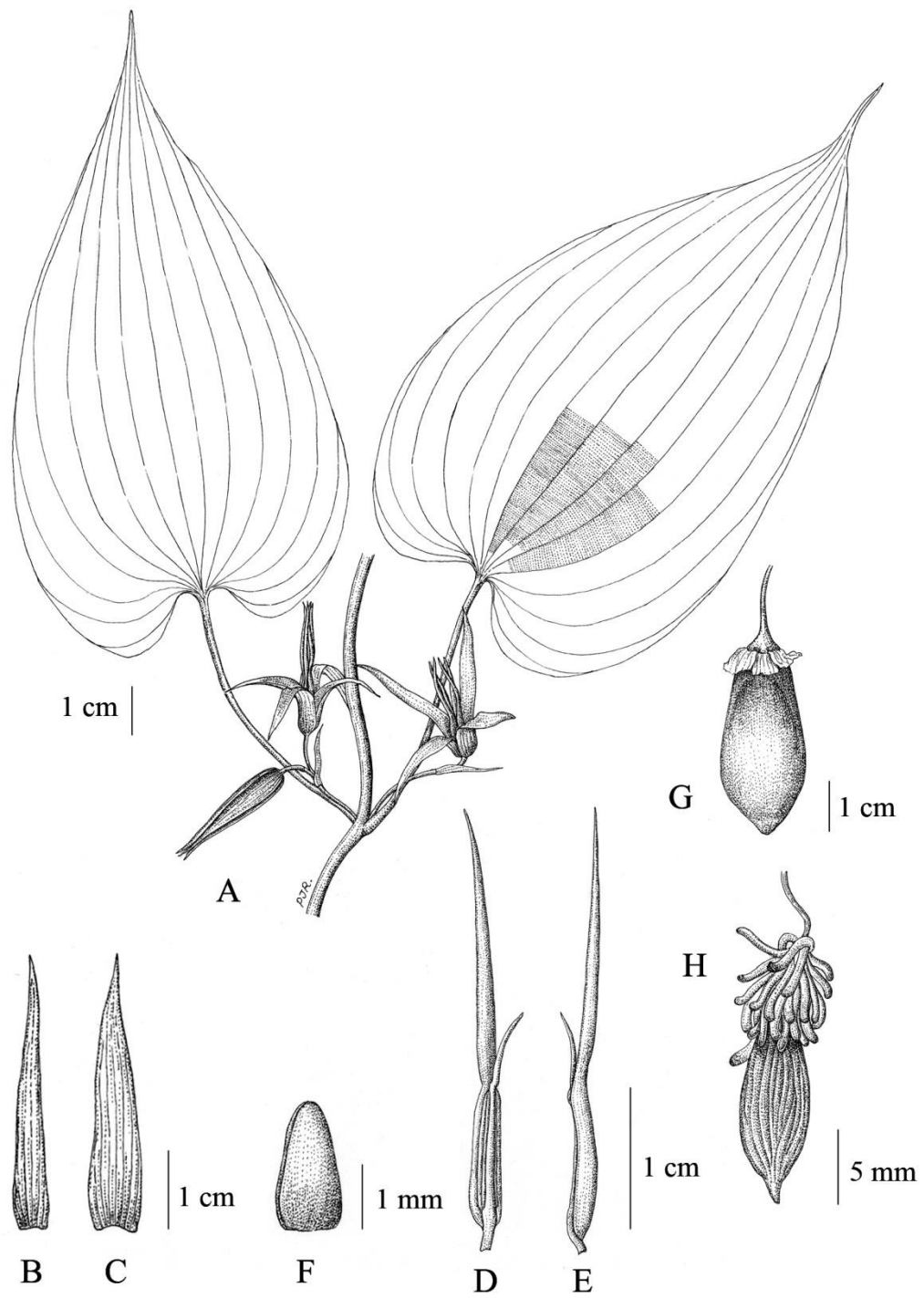


Fig. 1. Leaves and flowers from *S. tuberosa* (drawing from P. Inthachub).

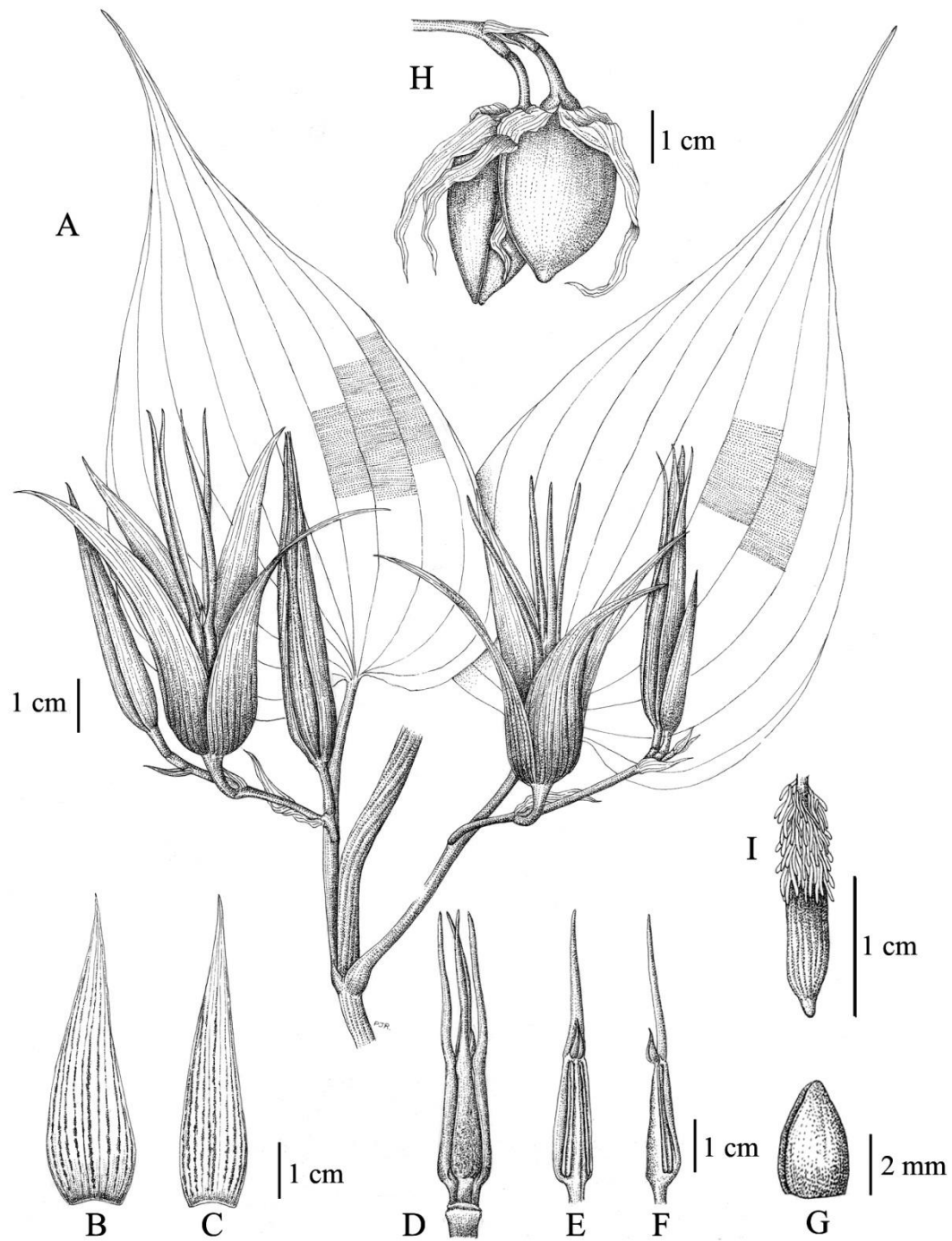


Fig. 2. Leaves and flowers from *S. phyllantha* (drawing from P. Inthachub).

1.1.2. *Stichoneuron* Hook.f.

This genus comprises five species of which *S. caudatum* Ridl., *S. calcicola* Inthachub and *S. halabalense* Inthachub have been phytochemically investigated. *Stichoneuron caudatum* (Fig. 3) is a herbaceous plant species growing in dense tropical forests on the Malayan peninsula. The flowers are tiny and have black hairy tepals of approx. 1 cm length. *Stichoneuron calcicola* (Fig. 4) has been collected from limestone rock, which is a completely different habitat as compared to that of *S. caudatum*. Until now, there is just little known about the ecological requirements and habitat preferences of this species.

Phytochemistry: The phytochemical analysis of *S. caudatum* revealed the presence of two alkaloids, named Stichoneurine A and B (Schinnerl et al., 2005) whereas *S. calcicola* yielded Pandanamine as the only alkaloid (Greger et al., 2009). Particular results are discussed within this thesis.

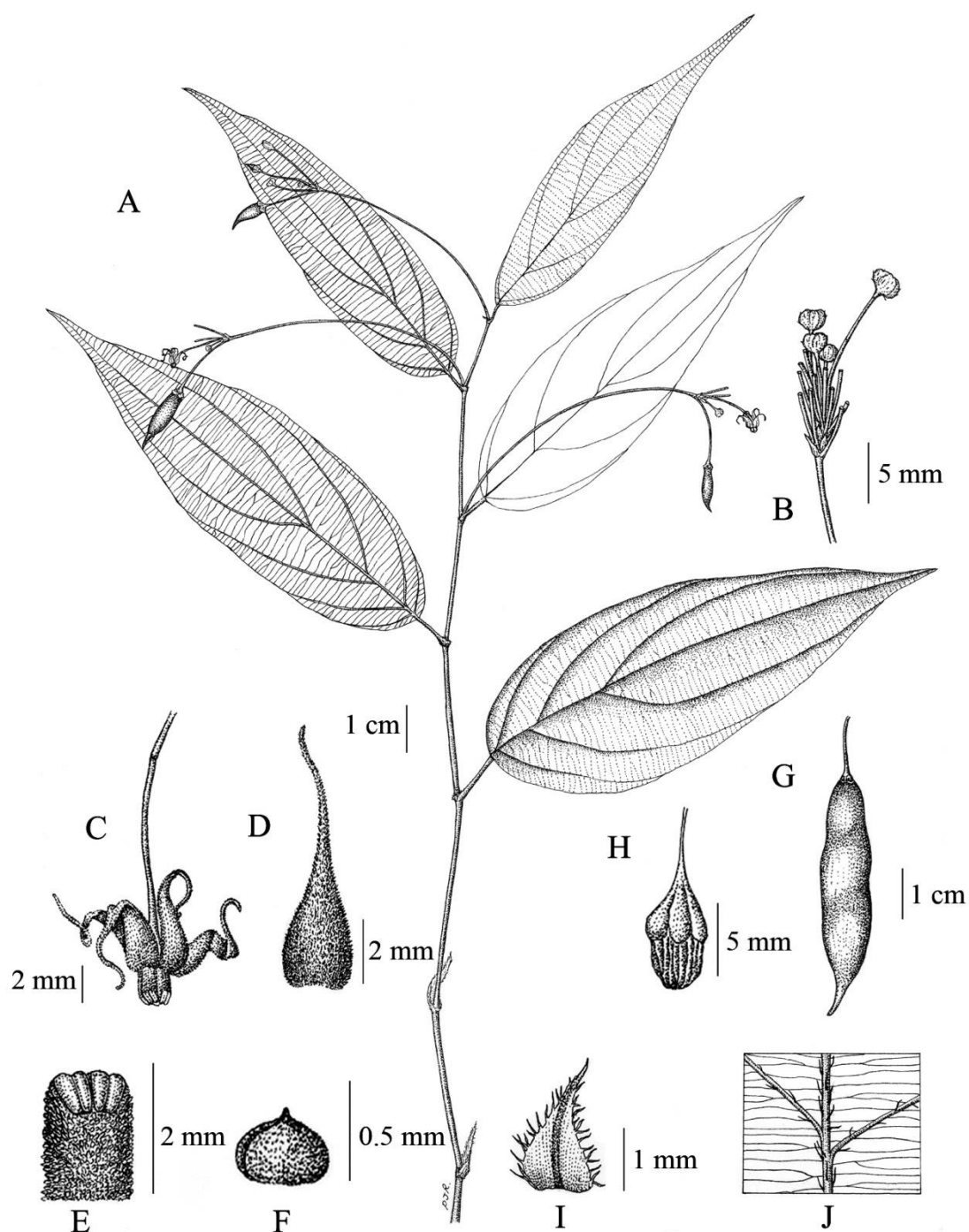


Fig. 3. Habitus of *Stichoneuron caudatum* (drawing from P. Inthachub).

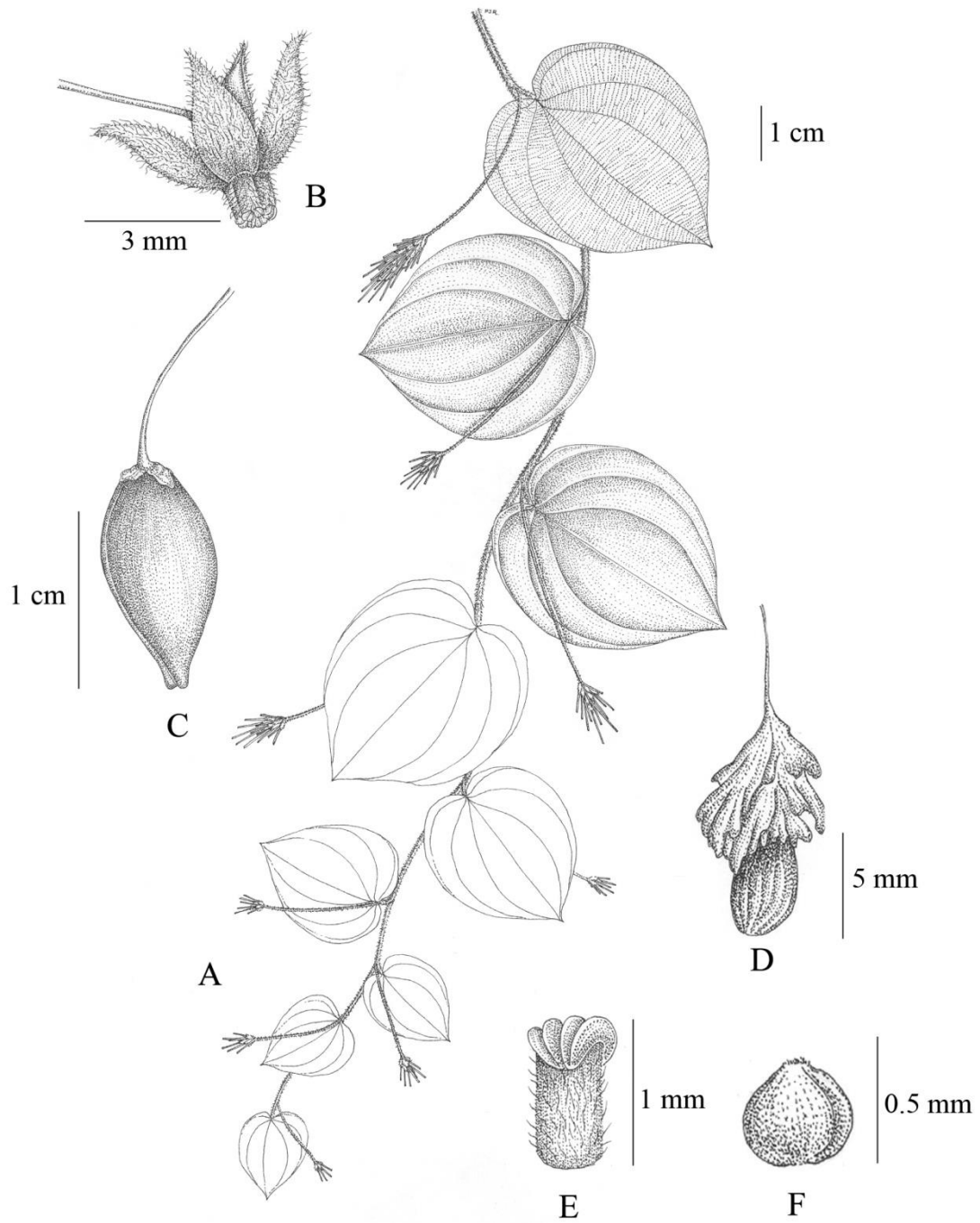


Fig. 4. Habitus of *Stichoneuron calcicola* (drawing from P. Inthachub).

1.2. *Stemona* alkaloids and their presumed biosynthesis

This specific type of alkaloids is restricted to the small monocotyledonous family of Stemonaceae (order Pandanales). More than 130 compounds showing a pyrrolo[1,2- α]azepine (**1**) or a pyrido[1,2- α]azepine (**2**) core structure (Fig. 5) have been described since 1934. Based upon structural diversity, either three (Greger, 2006) or eight subclasses (Pilli et al., 2005, 2010) have been defined.

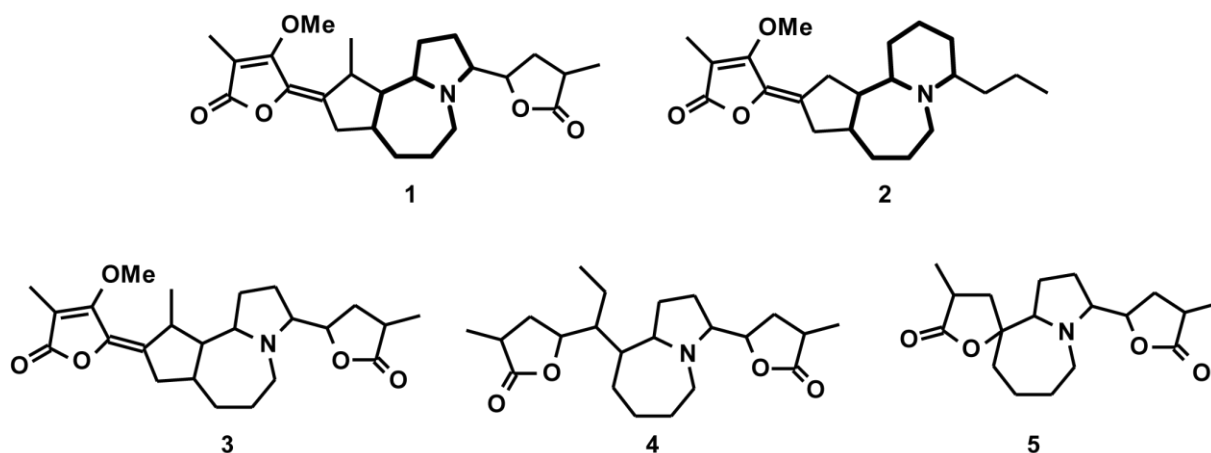


Fig. 5. Pyrrolo[1,2- α]azepine (**1**) and Pyrido[1,2- α]azepine core structure (**2**) (bold lines) and the main types of *Stemona* alkaloids according to Greger (2006). Protostemonine (**3**), Stichoneurine (**4**) and Croomine (**5**).

1.2.1. Proposed biosynthesis of the pyrrolo[1,2- α]azepine core structure

Seeger et al. (2004) postulated the formation of the core structure starting from ornithine. Various steps lead to an iminium ion (Fig. 6) which might be the central part of the core structure. The subsequent addition of a geranyl and a prenyl unit to the iminium ion leads to an intermediate structure. Further oxidation steps are leading to the formation of the rings A-E of Protostemonine (**3**) (Fig. 7). The enzyme Homospermidine synthase appears to play an important role during these biosynthetic steps, catalyzing the NAD^+ -dependent transfer of an aminobutyl group of Spermidine to Putrescine to form Homospermidine (Ober and Hartmann, 1999).

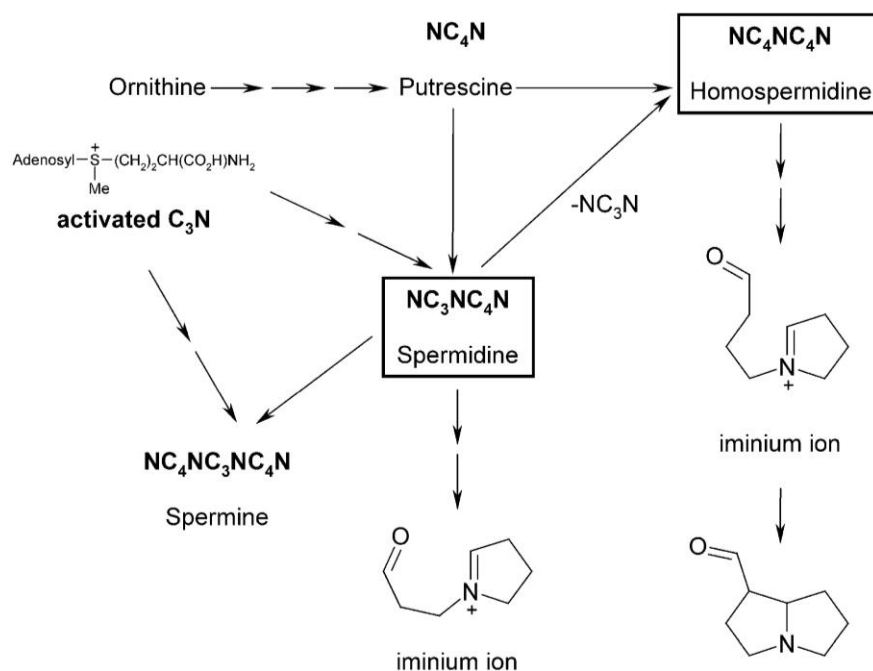


Fig. 6. Formation of the Iminium ion as part of the core structure of *Stemona*- and pyrrolizidine-alkaloids. Modified from Seger et al. (2004).

The Iminium ion (Fig. 6) is also the precursor of the Necic acid which forms the backbone of the pyrrolizidine alkaloids, being widely distributed within the plant families Asteraceae, Fabaceae and Boraginaceae. However, to reach the pyrrolo[1,2- α]azepine core, incorporation of two more carbons is necessary. Both carbons might come from a prenyl- or a geranyl-moiety (Fig. 7). The structural similarities of the core structures lead to the assumption that *Stemona* alkaloids and pyrrolizidine alkaloids share an early evolutionary origin.

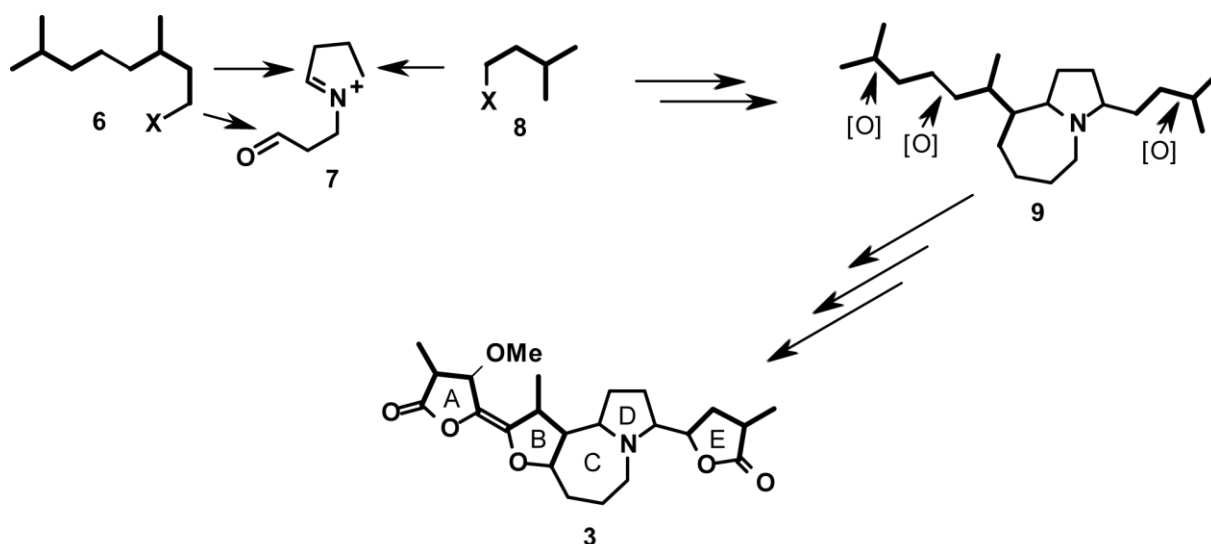


Fig. 7. Formation of the rings A-E of pyrido[1,2- α]azepines by addition of a geranyl- and a prenyl-unit to the Iminium ion, according to Seger et al. (2004). Activated geranyl-moiety (6), Iminium ion (7), activated prenyl-unit (8), postulated intermediate (9) and Protostemonine (3).

Following Greger (2006), Protostemonine (3), Stichoneurine (4) and Croomine (5) are basic core structures (Fig. 5), from which most of the other *Stemona* alkaloids might be derived from these structures. Biosynthetic relationships with alkaloids of the Pandanaceae have also been suggested (Nonato et al., 2008). However, respective biosynthetic steps and their enzymatic background have not been elucidated yet. Structural relationships are evident and suggest biosynthetic routes which are commented shortly. Thus, Stichoneurine A and B, isolated from *Stichoneuron caudatum* (Schinnerl et al., 2005), might be the precursors of Tuberostemonine and its derivatives (Greger, 2006). On the other hand, Croomine might be derived from Pandanamine (chapter 2). So far nothing is known about any precursors of Protostemonine (3). Seger et al. (2004) suggests the formation of the pyrrolo[1,2- α]azepine core by an Iminium ion together with an activated geranyl- and prenyl-unit, respectively (Fig. 7).

1.2.2. Formation of Pyrido[1,2- α]azepines

The formation of pyrido[1,2- α]azepine derivatives has been postulated by Kaltenecker et al. (2003). This starts from Protostemonine, ring cleavage of the lactone ring

E and decarboxylation is a feasible step which is followed by a subsequent incorporation of the carbon-18 from the aliphatic side chain into the pyrroling place (Fig. 8).

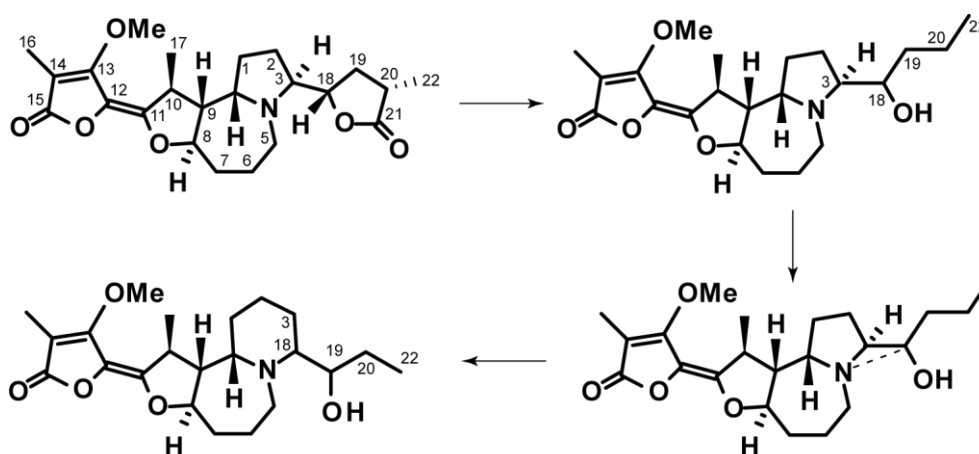


Fig. 8. Proposed biosynthesis connections between pyrrolo[1,2- α]azepine and pyrido[1,2- α]azepines, modified from Kaltenegger et al. (2003).

An alternative pathway leading to the pyrido[1,2- α]azepines has been proposed by Mungkornasawakul et al. (2005) (Fig. 9A). This suggestion is based on the already known biosynthesis of Conhydrine (**14**) (Fig. 9B) according to Leete (1964). However, these reaction steps do not explain the biosynthesis of the more frequent occurring pyrrolo[1,2- α]azepines. Furthermore, the non-amino acid derived building blocks suggest that the pyrido[1,2- α]azepines are pseudoalkaloids. Pseudoalkaloids are generally based either on a diterpene- or a steroidal-core structure and have been found extensively in Ranunculaceae, Solanaceae and Apocynaceae (Roberts and Wink, 1998). The occurrence of pyrido[1,2- α]azepine derivatives in *S. kerrii* and *S. curtisii* (Kaltenegger et al., 2003; Schinnerl et al., 2007) leads to the assumption, that this structural type could be derived from a specific biosynthetic pathway, which might not be expressed in other *Stemona* species.

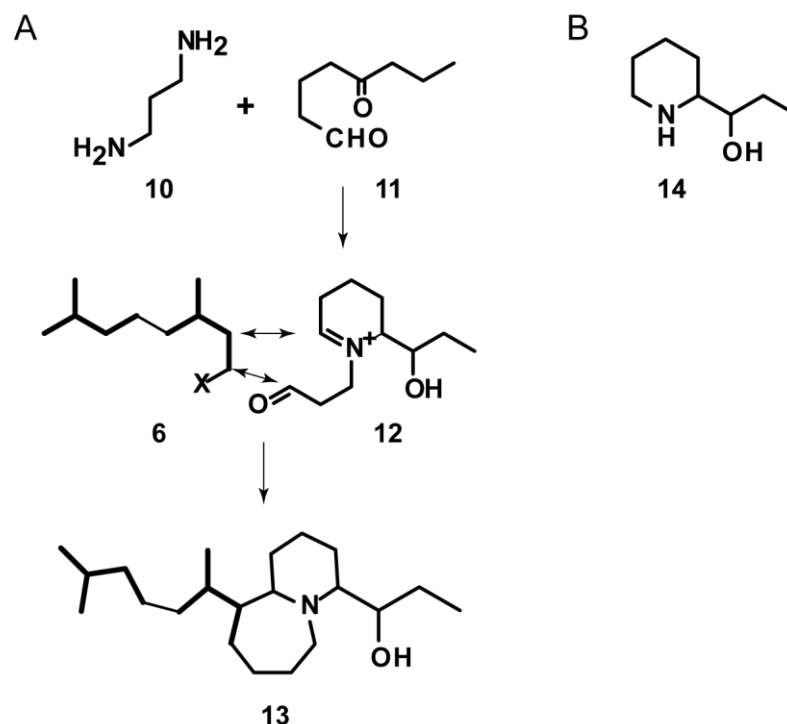


Fig. 9. A: Alternative biosynthesis leading to the pyrido[1,2- α]azepine core, modified from Mungkornasawakul et al. (2005). 1,4-Diaminopropane (**10**), acetat-derived polyketid derivative (precursor of Conhydrine) (**11**), adduct of 1 and 2 (**12**), activated geranyl-moiety (**6**), alkaloid with a pyrido[1,2- α]azepine core (**13**). **B:** Structure of Conhydrine (**14**).

The occurrence of the typical *Pandanus* alkaloid Pandanamine (**15**) (Fig. 10) in *Stichoneuron calcicola* of Stemonaceae (Chapter 2) suggests biosynthetic relationships between these two types of alkaloids. Pandanamine has been described from *Pandanus amaryllifolius*, and it is a main precursor of most of the other *Pandanus* alkaloids (Takayama et al., 2001). It might be more widespread but its detection is hampered due to the fact that Pandanamine (**15**) is probably being unstable under acid conditions, which leads to formation of the already described pandamarilactonines (Fig. 10) (Salim et al., 2004).

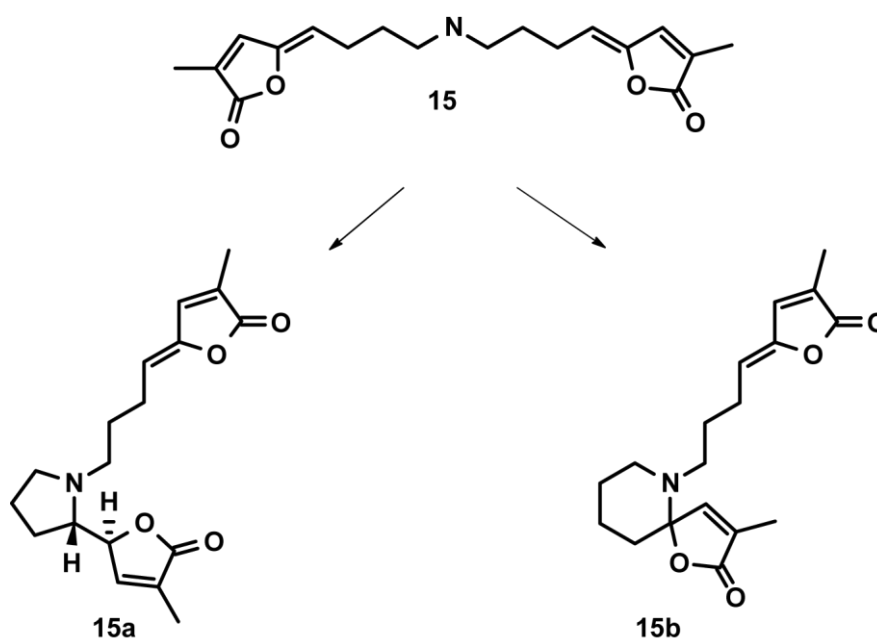


Fig. 10. Formation of *Pandanus* alkaloids from Pandanamine. 6-E/Z Pandanamine (**15**), Pandamarilactonines (**15a**) and Pandamarilactones (**15b**). Modified from Takayama et al. (2001) and Salim et al. (2004).

1.3. Rubiaceae (Dicotyledons; order: Gentianales)

Rubiaceae (coffee family) is one of the largest plant families containing more than 13000 species. Despite the species richness in this family, it is considered as being monophyletic, with the three known subfamilies Cinchonoideae, Rubioideae and Ixoroideae (Bremer, 1996; Bremer and Manen, 2000). In the study of Robrecht and Manen (2006) that is based on anatomy, morphology as well as on flower biology, only two subfamilies, Cinchonoideae and Rubioideae, are recognized. Taxa of this family are distributed worldwide and show a remarkable variation in their appearance. Species from the tropics and subtropics are mostly trees or lianas, whereas species from the temperate zones are predominantly herbs. Due to the species richness of this family only the investigated species are briefly described within the following sub-items.

1.3.1. *Ronabea emetica* (L.f.) A.Rich.Aubl.

Within the tribe Lasiantheae, the genus *Ronabea* which comprises three species, is closely related to *Psychotria* L. Morphological characters and molecular phylogenetic studies support segregation from *Psychotria* (Taylor, 2004). In the past, the roots of this taxon have often been intermixed and confused with the important emetic drug ipecac (*Carapichea ipecacuana*) because of the superficial similarities of the roots.

Phytochemistry: *R. emetica* is a source of circular miniproteins (Gruber et al., 2008). On the level of secondary metabolites, it accumulates iridoid compounds (Berger et al., 2011). The results of this study dealing also with the confusion about ipecac sources are discussed within this thesis (chapter 4).

1.3.2. *Palicourea acuminata* Aubl.(Benth.) Borhidi

The genus *Palicourea* Aubl. is one of the most speciose genera within the *Psychotria* alliance and contains more than 300 species (Borhidi, 2011; Taylor et al., 2010). They are either shrubs or small trees, growing at higher altitudes in wet habitats. Most of the species have long tubular pentamerous colorful flowers and colored inflorescence axes. Hummingbirds are known as pollinators (Taylor, 1997).

Phytochemistry: Tryptamine-Iridoid alkaloids are common within *Palicourea* species, some of them bearing a loganin-glycoside moiety instead of Secologanin. Croceaine A and B have been isolated from *Palicourea crocea* (Sw.) Roem. & Schult. (Düsman et al., 2004). The occurrence of Psychollatine, which has already been isolated from *Psychotria umbellata* Vell. (Kerber et al., 2008), and the *N*-oxide, showing a β -carboline moiety named as 3,4-dihydro-1-(1- β -D-glucopyranosyloxy-1,4a,5,7a-tetrahydro-4-methoxycarbonylcyclopenta[c]pyran-7-yl)- β -carboline-*N*₂-oxide, have been reported from *P. crocea* (Narine and Maxwell, 2009). Alkaloids with a β -carboline structure have also been isolated from *P. adusta* Standley (Valverde et al., 1999). From *P. coriacea* (Cham.) K.Schum., Strictosidin derivatives and a tetrahydro and β -carboline trisaccharide have been isolated (do Nascimento et al., 2006; do Nascimento et al., 2008). Furthermore, cinnamic acid derivatives have been detected in the leaves of this species (da Silva et al., 2008).

1.3.3. *Chassalia curviflora* Thwaites var. *ophioxyloides* (Wall.) Deb & B.Krishna

The genus *Chassalia* consists of approx. 40 species. Most of the species are small trees, shrubs or climbers distributed in the Palaeotropics. Members of this genus differ conspicuously, e.g. concerning the shape of the flowers, floral buds and pyrenes, compared to the other species of Psychotrieae (Piesschaert et al., 1999, 2001).

Phytochemistry: Studies dealing with secondary metabolites are rare, but some of the *Chassalia* species proved to be a source of macrocyclic peptides, also known as cyclotids (Gruber et al., 2008; Gustafson et al., 1994; Gustafson et al., 2000). The methanolic extract of *Chassalia kolly* Schumach. exhibits some positive effect against human pathogenic

bacteria e.g. *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and some other strains (Onocha and Ali, 2010; Raja et al., 2011) and also against the insect *Rhizopertha dominica*. Additionally, some growth inhibition of the crude methanolic extract against *Lemna minor* has been shown (Onocha and Ali, 2010).

1.3.4. *Rudgea cornifolia* (Kunth) Standl.

The genus *Rudgea* comprises more than 120 species in the Neotropics from Mexico to Argentina with two centers of distribution: one in the northwestern part of South America, and the other one in the eastern part of SE Brazil (Zappi, 2003). Species of this genus grow either as shrubs or as small trees.

Phytochemistry: Several triterpenes and saponins have been isolated so far from *Rudgea virburnoides* (Cham.) Benth., a tree species from the cerrado of Brazil (Young et al., 1998). The occurrence of alkaloids, identified by the indole chromophor, has been reported from *R. heurckii* Müll.Arg., *R. jasminoides* (Cham.) Müll.Arg. and *R. recurva* Müll.Arg., respectively (Lopes et al., 2004).

1.4. Tryptamine-Iridoid Alkaloids and Iridoids

Tryptamine-iridoid alkaloids, also known as monoterpene-indole alkaloids, are widely distributed within several genera of Rubiaceae such as *Psychotria* (Achenbach et al., 1995; Lopes et al., 2004), *Palicourea* (Berger et al., 2012), and Apocynaceae such as *Catharanthus* (Armstrong, 1968; Shimizu and Uchimarui, 1958; Svoboda et al., 1963), and *Alstonia* (Cai et al., 2011). The well-known biosynthesis leads to the key intermediate strictosidine (Fig. 11), which contains a tryptamine- and a secologanin-glycoside moiety. Strictosidine is modified in further steps to the other types of tryptamine-iridoid alkaloids (El-Sayed and Verpoorte, 2007; O'Connor and Maresh, 2006; Guirimand et al., 2011). Most of these compounds (Fig. 12) have bioactive properties and therefore the species, which contain these compounds, are widely used for ethnobotanical and medicinal purposes (Schultes, 1985; Schultes and Raffauf, 1990).

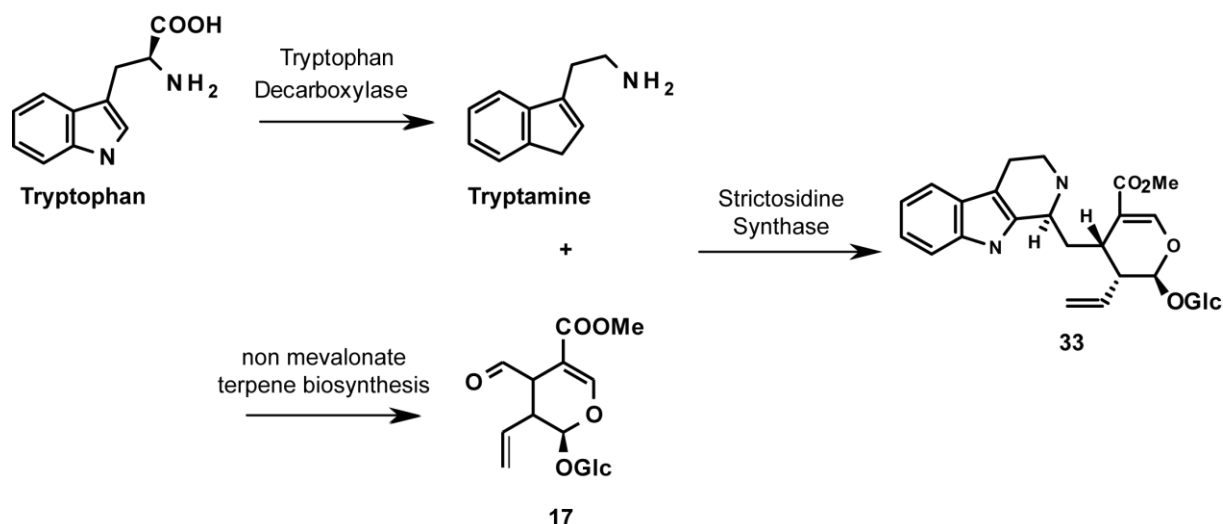


Fig. 11. Biosynthesis of Strictosidine. Redrawn from O'Connor and Maresh, 2006. Secologanin (**17**) and Strictosidine (**33**).

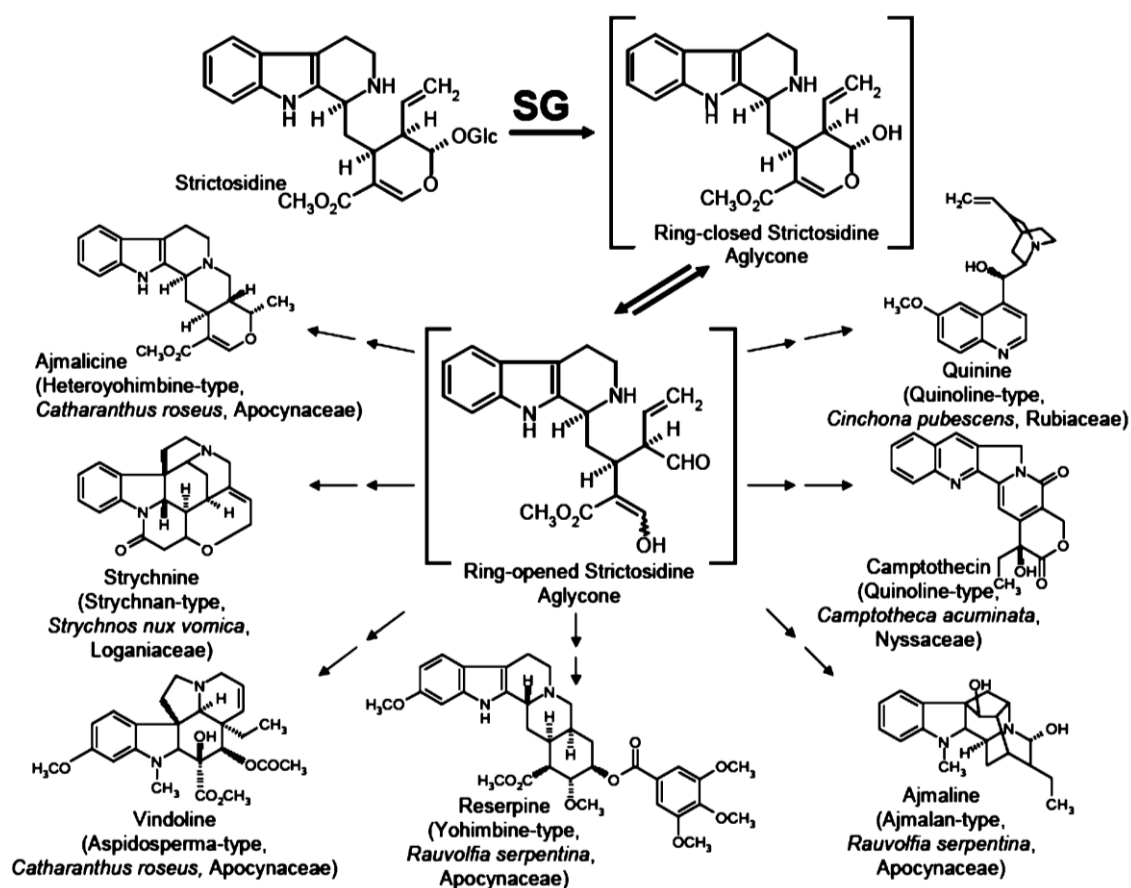


Fig. 12. Various tryptamine-iridoid alkaloids derived from Strictosidine, according to Barleben et al. (2007).

Iridoids as constituents of Rubiaceae

These monoterpenoids are widely distributed within the orders Gentianales, Ericales, Cornales, Lamiales and some others (Sampaio-Santos and Kaplan, 2001; Wink, 2003). The first isolated derivatives Iridodial and Iridomyrmecin have been originated from the anal glands of the ant genus *Iridomyrmex*. Ants use these compounds as defense chemicals (Cavill et al., 1982). Some iridoids taste extremely bitter and have various pharmacological properties (Dinda et al., 2011). The bitter taste might be causal to protection against herbivory. Iridoids occur in plants either as a methylcyclopenta[c]pyrane core structure like Loganin (**16**) or in the open form, just possessing a pyrane core like in Secologanin (**17**) (Fig. 13). In consequence of their high reactivity, they are often linked with glucose as a means of detoxification in plants, thus preventing damages to cell structures. Both substances are characteristic for the genus *Gentiana* (Gentianaceae) e.g. (Garcia and Chulia, 1986; Rodriguez et al., 1998). In Rubiaceae and Apocynaceae species, Iridoids often occur either as single structures or as iridoid moieties, linked to tryptamine units, forming the tryptamine-iridoid alkaloids (Armstrong, 1968; Cai et al., 2011; Kerber et al., 2008; Van De Santos et al., 2001). Common iridoids in various rubiaceous species are Asperuloside (**18**) and its derivative Deacetylasperuloside (**19**) (Fig. 13) (Lopes et al., 2004; Gonzalez and Dieck, 1996).

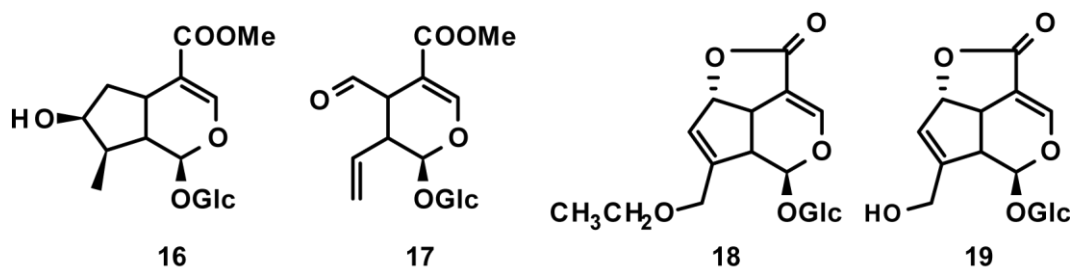


Fig. 13. Chemical structures of common Iridoids in Rubiaceae. Loganin (**16**), Secologanin (**17**), Asperuloside (**18**) and Deacetylasperuloside (**19**).

Biosynthesis of Iridoids

The building blocks of iridoids are the hemiterpenes Dimethylallylpyrophosphate (DMAPP) and Isopentenylpyrophosphate (IPP) which are derived from the deoxyxylulose 5-phosphate pathway (Rohmer, 1999; Schwarz, 1994) now designated as 2-C-methyl-D-

erythritol-4-phosphate (MEP) pathway (Rodríguez-Concepción and Boronat, 2002). The Iridoids as such are built by a head-tail linkage of DMAPP and IPP (Fig. 14).

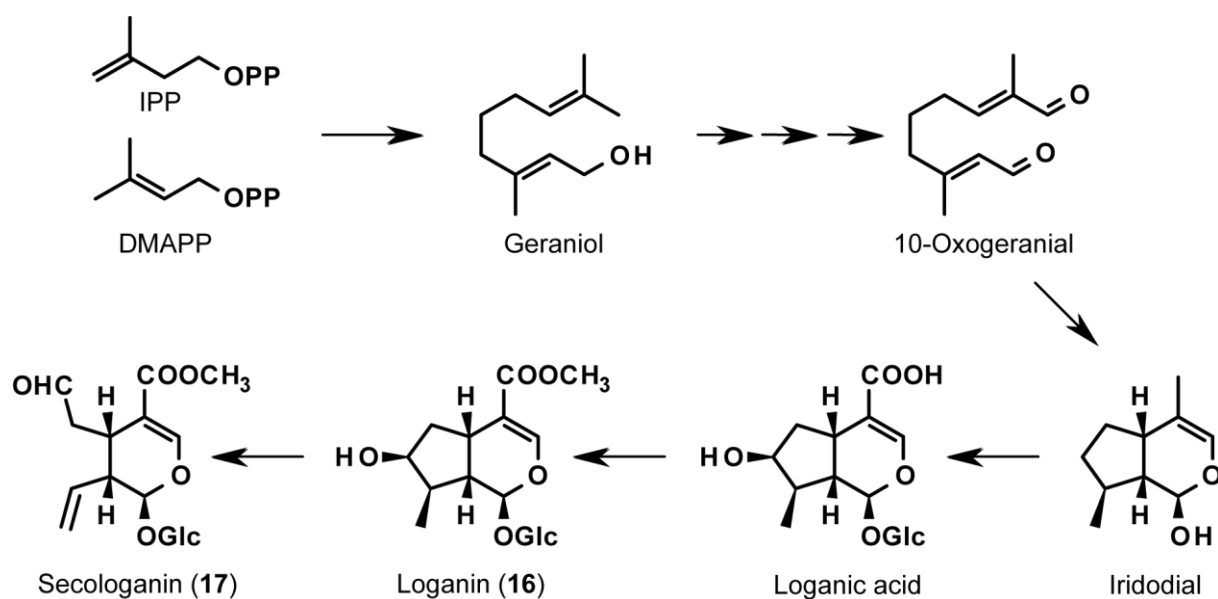


Fig. 14. Formation of Iridoids from IPP and DMAPP. Modified from Oudin et al. (2007).

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Chapter 2: *Pandanus Alkaloids in Stemonaceae: Finding of a Plausible Biogenetic Origin of Stemona Alkaloids*

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Pandanus Alkaloids in Stemonaceae: Finding of a Plausible Biogenetic Origin of Stemona Alkaloids

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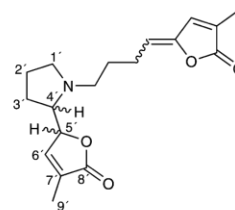
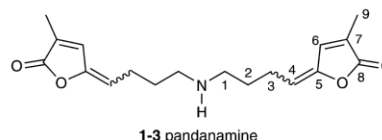
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The isolation of (Z,Z)-pandanamine (1) and its corresponding isomers (Z,E)-pandanamine (2) and (E,E)-pandanamine (3) from *Stichoneuron calcicola* of the family Stemonaceae is of outstanding chemosystematic importance. This alkaloid was previously only known from the family Pandanaceae, where it was accompanied by a series of pyrrolidines, collectively called Pandanus alkaloids. The pyrrolidines pandamarilactonines A (4), B (5), C (6), and D (7) were also detected in the present study, most likely representing artificial cyclization products of pandanamine (3) formed by acidic conditions during chromatographic separation on silica gel. Similar structures were found in various *Stemona* alkaloids, suggesting a close relationship between the two plant families. Structurally, pandanamine (3) can be regarded as a direct precursor of croomine (8), originally isolated from *Croomia*, a genus closely related to *Stichoneuron* but later also found in various *Stemona* species. The co-occurrence of pandanamine (3), croomine (8), and stichoneurin (9) in the family Stemonaceae represents a sound argument for a new interpretation of the biogenetic origin of *Stemona* alkaloids and at the same time substantiates the removal of the family from the order Dioscoreales and its inclusion into Pandanales, as already suggested by DNA sequencing.

The accumulation of *Stemona* alkaloids has been shown to constitute a unique chemical feature of the small monocotyledonous family Stemonaceae, not detected so far in any other plant family. The chemical structure of this alkaloidal class is characterized by a pyrrolo[1,2-a]azepine core usually linked with two carbon chains forming mostly terminal lactone rings.² Because of the wide use of *Stemona* roots as bioinsecticides as well as in traditional Chinese medicine, for the treatment of respiratory diseases and to prevent parasites, many investigations have already been carried out to determine the structural diversity and biological activity of *Stemona* alkaloids.¹ However, in spite of this increasing interest little is known about their biosynthetic origin. Up to now about 100 derivatives have been described, mainly isolated from the tuberous roots of the genus *Stemona* whereas only four are known so far from the two other genera of the family, *Stichoneuron* and *Croomia*. On the basis of their various distribution and structural similarities they were classified recently into three skeletal types: the stichoneurin-, protostemonine-, and croomine-type alkaloids. In a previous hypothesis they were speculated to be derived from a spermidine precursor linked with isoprene units. However, no adequate precursors were found so far in broad-based comparisons within the family.²

In the course of our comparative screening we collected recently a rare and hitherto undescribed representative of the family Stemonaceae. As a result of the characteristic flower and fruit morphology, this species was identified tentatively as a member of the genus *Stichoneuron*. Its chemical profile clearly deviated from *Stichoneuron caudatum* Ridley, the only species of the genus chemically investigated so far. Whereas *S. caudatum* was characterized by the occurrence of the pyrroloazepine-type alkaloid stichoneurin (9) (Figure 1),⁴ the presently investigated species accumulated a symmetrical secondary amine, for which the structure turned out to be identical with the already known pandanamine (3).⁵

The rediscovery of pandanamine (3) in a member of the family Stemonaceae, later newly described as *Stichoneuron calcicola* Inthachub,⁶ may be considered to be of outstanding chemosystematic interest, since this compound is only known so far from *Pandanus amaryllifolius* Roxb. of the family Pandanaceae. Here it was shown to be the precursor of a series of pyrrolidine-type alkaloids,⁷ from which pandamarilactonines A (4), B (5), C (6), and D (7) were also detected in *S. calcicola* (Figure 2). Moreover, pandanamine (3) can also be regarded as a precursor of croomine (8) isolated for the first time from the genus *Croomia* (Figure 1),⁸ which is regarded to be closely related to *Stichoneuron*.^{6,9} Later, croomine (8) and structurally closely related alkaloids were also shown to be widespread in different *Stemona* species.^{1,2} The co-occurrence of pandanamine (3), croomine (8), and stichoneurin (9) in the family Stemonaceae represents a sound argument for a new interpretation of the biogenetic origin of *Stemona* alkaloids. Furthermore, these findings substantiate the removal of the family Stemonaceae from the order Dioscoreales and their inclusion into Pandanales, as already suggested by DNA sequencing.



S. calcicola differed clearly from *S. caudatum* by its decumbent habit and specialized ecology. Whereas the latter was collected in

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† Deceased February 10, 2009. This paper is dedicated to his memory. His enthusiasm, insights, and unique perspective were an inspiration to many, and his presence is greatly missed.

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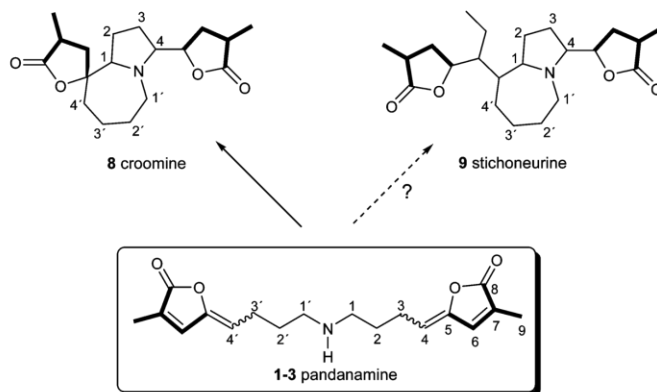


Figure 1. Possible structural relations between the tropane alkaloids 1- 3, 8, and 9.

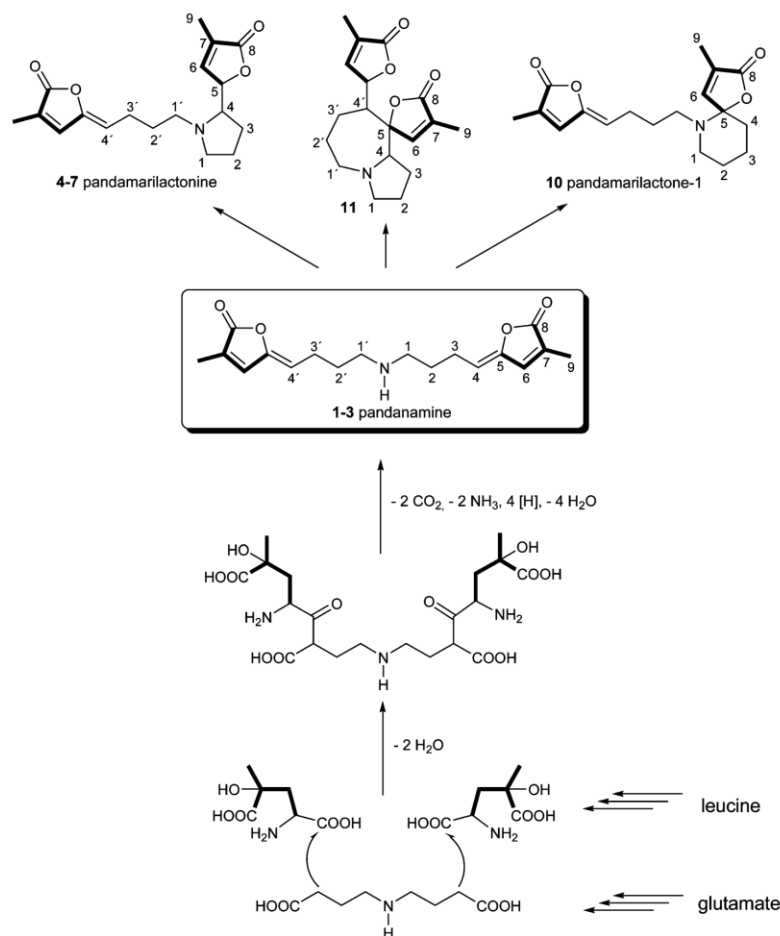


Figure 2. Hypothetical biosynthetic pathway of pandanamine (3) and the probable artificial cyclization to the pyrrolidine alkaloids 7, 10, and 11 (partly redrawn from ref 7).

humid habitats along sandy riverbanks in equatorial rainforests near the Thai-Malay border;⁴ *S. calcicola* was found hanging on open vertical limestone rocks in Klong Phanom National Park near Surathani in south Thailand. The roots and rhizomes were hidden

in small fissures of very hard limestone, and hence, only small amounts were available for extraction and HPLC-UV comparison. However, a larger quantity of aerial parts (leaves and stems) could be used for preparative analysis, showing a nearly identical HPLC

Table 1. NMR Spectroscopic Data (400 MHz, CD₃OD) of the Z,Z-Pandanamine (1) and of the E,E-Pandanamine (3)

position	Z,Z-pandanamine (1)		E,E-pandanamine (3)	
	δ_H , mult.	δ_C , mult.	δ_H	δ_C
1	3.04, m	48.4, CH ₂	3.04	48.4
2	1.88, m	26.7, CH ₂	1.88	26.7
3	2.46, m	24.1, CH ₂	2.41	24.3
4	5.33, m	112.7, CH	5.66	112.6
5		150.9, qC		151.0
6	7.28, s	139.5, CH	7.67	135.6
7		130.7, qC		132.0
8		172.5, qC		172.5
9	1.96, s	10.3, CH ₃	1.97	10.6

profile. The methanolic extract was concentrated and directly used for comparison. The chromatographic profile was characterized by only one dominant peak with UV absorption at 275 nm (MeOH/H₂O), accompanied by some small peaks showing the same maximum. Corresponding results were obtained by parallel TLC comparison sprayed with Dragendorff's reagent, confirming the presence of only one major alkaloid. After preliminary fractionation on a silica gel column the pure compounds were obtained by Sephadex LH-20 separation. Further chromatographic purification resulted in the formation of additional compounds consistent with the lability of the major alkaloid due to the acid conditions used in HPLC and TLC.¹²

The predominant compound was identified by NMR spectroscopy and mass spectrometry. HRMS proved the molecular formula to be C₁₈H₂₃O₄N by the [M + H]⁺ peak (m/z 318.1707 g/mol), being in agreement with its calculated mass of m/z 318.1705 g/mol. However, ¹H and ¹³C NMR recorded now for the first time in CD₃OD showed two similar sets of nine carbon atoms and 11 carbon-bonded protons each (Table 1). Their integral ratio was about 5:1 and indicated the presence of two isomeric forms, which represented together 90% of the material in the sample. In CDCl₃ additional signals of amine protons were also detected. All other chemical shifts determined in CDCl₃ were consistent with data reported previously.^{5,7,10,11} In both isomers, the methylene protons of position 1 showed coexistent ²J_{C-H} couplings and ³J_{C-H} couplings to the respective N-bonded carbon atoms. The predominant compound was hence indicated to be a secondary amine. Further information on the structure of the alkyl chains was proven by DQF-COSY and TOCSY, and the lactone skeletons were identified by ²J_{C-H} and ³J_{C-H} couplings of the methine protons in positions 4 and 6 as well as of the methyl protons in position 9. In the NOESY spectrum, a strong NOE between H-4 and H-6 indicated the major isomer to be Z-pandanamine (1). The minor isomer had the corresponding E-configuration. The two double bonds in the molecule are separated and do not influence each other. Hence, there is no evidence for an exclusive presence of E,E- or Z,Z-isomers. The natural statistic distribution of Z,Z-pandanamine (1), Z,E-pandanamine (2), and E,E-pandanamine (3) is therefore 69%, 28%, and 3%, respectively. The residual tenth of the material comprised about half a dozen cyclization products, which all showed intact lactone rings but also possessed various cyclic tertiary amines. Four of those were identified as pandamarilactonines A (4) and C (6), as well as B (5) and D (7) by indicating ¹H and ¹³C NMR shifts of their geminal C-H groups.^{11,12} Samples of further chromatographically treated fractions did not show any pandanamine (1-3), but contained a mixture of other cyclization products.

Pandanamine (1-3) was first isolated as a natural product from fresh leaves of *Pandanus amaryllifolius*, the fragrant screw pine, well known in southeast Asia as a flavoring agent. The structure was already known from an intermediate previously prepared for the synthesis of the pyrrolidine alkaloids pandamarilactonines A (4) and B (5).⁷ Both pyrrolidines were also isolated from *P. amaryllifolius* together with the previously described pandamarilactone-1 (10) (Figure 2),¹³ using silica gel chromatography of

the alkaloid fraction obtained by conventional acid-base extraction in EtOH.⁷ Later, an N-oxide of pandamarilactonine was also isolated from an Indonesian collection together with a related derivative with a pyrrolazepine core (1), where the second lactone ring was directly attached in a spiro system (Figure 2).² However, the ¹H NMR data suggested that the extract compositions had drastically changed by acid base treatment as well as during chromatographic separations, leading to the pyrrolidine alkaloids mentioned above. In fact, a comparative study of alkaloids isolated by two different extraction methods clearly showed that a careful solvent partitioning method led only to Z,Z-pandanamine (1) as the major alkaloid accompanied by small amounts of 2 and 3, carrying one or two E-configured double bonds, whereas the conventional acid-base extraction gave rise to the formation of the isomeric pyrrolidine alkaloids pandamarilactonines A (4), B (5), C (6), and D (7).¹²

The sensitivity of pandanamine (1-3) to acidic conditions leads to different cyclization products such as pandamarilactone-10 and an unnamed pyrrolazepine derivative (1) both with a spiro system that strongly resembles some structures of Stemonalalkaloids.¹ In particular the structure of croomine (8) could be directly derived from pandanamine (1-3) by cyclization processes (Figure 1). Moreover, the formation of artifacts of Stemonalalkaloids during extraction and/or fractionation has also been suggested for a number of derivatives in a recent review,¹ confirming the supposed relationship between Pandanus and Stemonalalkaloids. The earlier reported small or negligible enantiomeric excesses of isolated pandamarilactone-1 (10) and pandamarilactonines A (4) support this suggestion.^{7,13,14} Takayama et al.¹⁴ speculated that the pyrrolidine 4 represents an enzymatically formed natural product with moderate optical purity, which changed to a racemate after acid treatment. This initial enantiomeric excess may be caused by enzyme-catalyzed formation with ineffective chiral induction. However, a more likely reason is a chiral induction by the complex chiral matrix present in plant materials. Such a matrix presumably causes optical transfer of the supramolecular chirality into the products during chemical reactions, which are not enzymatically catalyzed.¹⁵ The reactions occur either in the living plants or during isolation procedures. The two products pandamarilactone-10 and pandamarilactonine A (4) are stable and not further racemized under the neutral conditions, but racemize at lower pH values.

However, no explanation could be obtained for the formation of stichoneurine (9) isolated from *S. caudatum*.⁴ It deviates from croomine (8) by four additional carbon atoms forming a characteristic free ethyl group typical for tuberostemonine derivatives of the complex Stemonal group (Figure 1).^{1,2} A hypothetical biosynthetic route of pandanamine (1) itself has already been proposed.^{7,10} According to this, the characteristic terminal vinyl lactone rings could be derived from 4-hydroxy-4-methylglutamic acid (Figure 2). Since this amino acid was already known to accumulate in another Pandanus species, *P. tectorius* Parkinson ex Du Roi (syn.: *P. Veitchii* Mast.),¹⁶ its role as a biosynthetic building block appeared plausible. In a previous study with ¹³C-labeled precursors it was shown that methylation at C-4 of glutamic acid was unlikely to arise directly by an expected addition of a C unit from methionine. Instead, leucine was shown to be mainly converted into 4-methylglutamic acid.¹⁷ As shown in Figure 2, a C-4-N-C-4-dicarboxylic acid, probably derived from two glutamic acid units, may be assumed as a symmetrical central building block, where the two 4-hydroxy-4-methylglutamic acid units are attached.^{7,10}

The formation of pandanamine (1-3) in the Stemonaceae not only offers insight into the possible biogenetic origin of a unique class of alkaloids but also represents an important characteristic for chemosystematic considerations. Morphological similarities, particularly the leaf shape, tuberous roots, and twining habit, lead to expected affinities between the Stemonaceae and the Dioscoreaceae, and this has been reflected by the previous inclusion of both

Notes

families into the order Dioscoreales.¹⁸ However, a detailed phylogenetic analysis of morphological and macromolecular data (including plastid *rbcl*, *atpB*, and 18S rDNA) suggested a removal of the Stemonaceae to the order Pandanales. The discovery of a rare type of alkaloid in both the Pandanaceae and the Stemonaceae underlines the recently proposed relationship between these two families and demonstrates impressively the value of combined character analyses for phylogenetic conclusions.

Experimental Section

General Experimental Procedures. ¹H and ¹³C NMR spectra were measured at 298.15 K in CDCl₃ (>99.98% D) or CD₃OD (>99.95% D). Spectra were performed on a Bruker Avance DRX-400 or DRX-600 NMR spectrometer at 400.13 MHz (100.61 MHz) or 600.13 MHz (150.90 MHz), respectively. Chemical shifts were referenced to residual CHCl₃ (δ_H) 7.26 and CDCl₃ (δ_C) 77.36 or to residual CHD₃OD (δ_H) 3.34 and CD₃OD (δ_C) 49.86. All chemical shifts are given in ppm. Assignments of proton resonances were confirmed by two-dimensional homonuclear (DQF-COSY, TOCSY, NOESY) and heteronuclear (HSQC, HMBC) spectroscopy. Mass spectra were measured on a Micro mass, trio 200 Fisons Instruments mass spectrometer. High-resolution mass spectra (HRMS) were performed with a Finnigan MAT 8230 instrument with a resolution of 10000. HPLC was performed on an Agilent 1100 Series system with Hypersil BDS 4.6 mm × 250 mm, 5 μm, using MeOH/0.01 M ammonium acetate in H₂O as eluent. The fractions were detected by UV-DAD at 280 nm.

Plant Material. *Stichoneuron calcicola* was collected in Klong Phanom National Park, near Surathani in South Thailand in February 2006. Voucher specimens were deposited at the Herbarium of the Institute of Botany, University of Vienna (WU, voucher accession number HG 1026).

Extraction and Isolation. Dried leaves and stems (100 g) were extracted with MeOH at room temperature for 3 days, filtered, and concentrated (3.5 g). A portion (500 mg) was roughly separated on a silica gel column (Merck silica gel 60, 0.2–0.5 mm) with petroleum ether and acetone mixtures of increasing polarity and finally with MeOH. Alkaloid-containing fractions, detected by TLC sprayed with Dragendorff's reagent, were eluted with 50% petroleum ether in acetone (fractions 10–12), leading to a complex mixture of various pyrrolidine alkaloids, and with 20% MeOH in acetone (fractions 25 and 26),

affording contaminated Z-pandanamine 1). Purification of the latter on a Sephadex LH-20 column with MeOH led to 12 mg of a mixture of pandanamine isomers 1–3 and 10% of various pyrrolidines, mainly consisting of pandamarilactonines A–D, B (5), C (6), and D (7), whereas the combined fractions 1012 again afforded an inseparable mixture of cyclization products (4 mg).

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Chapter 3: *Structural Relationships of Stemona Alkaloids: Assessment of Species-Specific Accumulation Trends for Exploiting Their Biological Activities*

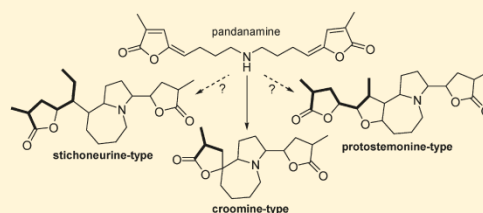
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ABSTRACT: On the basis of a comparison of 42 *Stemona* samples, representing eight different species collected and cultivated in Thailand, species-specific accumulation trends of *Stemona* alkaloids were analyzed. An overview was achieved by comparative HPLC analyses of methanolic crude extracts of underground parts coupled with diode array or evaporative light scattering detectors. All major compounds were isolated and their structures elucidated by NMR and MS analyses. Protostemonine- and stichoneurine-type derivatives dominated, from which the latter characterize *S. tuberosa* and *S. phyllantha* accumulating species-specific isomers of tuberostemonine (3). The widespread *S. curtisii* and *S. collinsiae* clearly deviate by protostemonine-type derivatives dominated by stemofoline (10) and/or dihydrostemofoline (11). Further diversification within this structural type results from a mutual accumulation of derivatives with a pyrrolo- or pyridoazepine nucleus, leading to chemical variability in *S. curtisii* and *S. aphylla*.



Stemona alkaloids represent a unique chemical character of the small monocotyledonous family Stemonaceae consisting of the three genera, *Stemona*, *Stichoneuron*, and *Croomia*, comprising about 30 species. On the basis of more recent taxonomic treatments for the flora of Thailand the genus *Stemona* is represented by 11¹ and *Stichoneuron* by five species.² More than 130 alkaloids are known from *Stemona*,³ whereas only two were reported from *Stichoneuron*, isolated as various isomers.^{4,5} Generally, the highest concentration was found in the underground parts of *Stemona* species, consisting of tuberous roots and rhizomes.⁶ With respect to biosynthetic considerations and their various distribution, they were classified into three skeletal types.^{6,7} On the basis of purely chemical aspects an alternative classification into eight groups was suggested, where the name of the structurally simplest derivative was used for group denominations.³

A wide range of practical applications has led to an increasing interest in this class of alkaloids. Important activities were summarized in a previous review.⁷ In the meantime further investigations supported their antitussive activity^{8–13} and insect toxic properties.^{14–16} Moreover, oxytocin antagonism,¹⁷ nitric oxide inhibition,¹⁸ and increasing chemosensitivity via P-glycoprotein-mediated multi-drug resistance¹⁹ revealed further pharmacological activities. Basically, most effects can be attributed to either tuberostemonine (3) or stemofoline (10) derivatives, representing two different skeletal types.⁷ As shown in a previous overview, this

chemical divergence also suggests a clear taxonomic segregation within the genus *Stemona*,⁶ later confirmed by morphological characters^{17,20} and microsatellite markers.²¹ Further progress in exploiting the various biological activities is greatly hampered by using not properly identified and documented plant material or, even more, by purchasing mixtures of underground parts from local markets or medical plant suppliers originating from different localities or species.⁷ Moreover, with respect to chemical instability of some *Stemona* alkaloids, the formation of artifacts may be expected, especially after extensive acid–base treatment.^{5,7}

In the course of identifying the genetic diversity of 68 *Stemona* samples, collected from different geographical localities in Thailand, corresponding plant material was cultivated under field conditions in the Maize and Sorghum Research Centre “Rai Suwan” of Kasetsart University near Nakhon Ratchasima in east Thailand.²¹ Besides morphological and anatomical studies,²⁰ this comprehensive collection also served for a broad-based phytochemical comparison to establish species-specific accumulation trends. Additional collections from natural habitats are included to complete this survey. Taking into account the chemical instability of some alkaloids and the difficulties in species delimitations, the present paper should provide a basis

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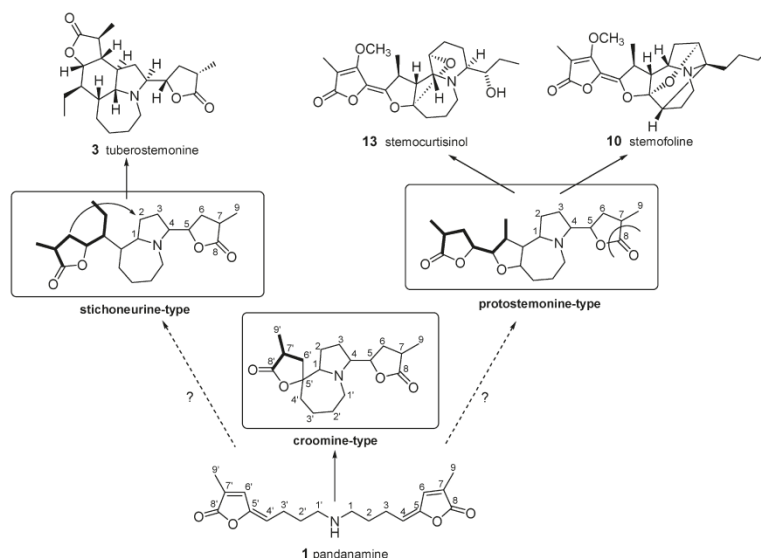


Figure 1. Proposed biosynthetic pathways of *Stemonia* alkaloids.

for exploiting these compounds for further pharmaceutical development and pest control.

RESULTS AND DISCUSSION

Dried and powdered underground parts of 42 *Stemonia* samples were extracted with methanol. According to a recent taxonomic revision,¹ they represent eight different species and two collections with uncertain taxonomic positions. In order to get an overview on characteristic alkaloid profiles, comparative HPLC analyses of the crude extracts were carried out coupled with diode array (DAD) or evaporative light scattering (ELSD). This method was validated to be sensitive and accurate to detect various types of *Stemonia* alkaloids both with and without characteristic chromophoric groups.²² Characteristic major compounds were isolated, and their structures elucidated by NMR and MS analyses. On the basis of these findings two clear-cut biosynthetic trends can be distinguished, either toward protostemonine- or stichoneurine-type derivatives (Figure 1). In contrast to reports on many Chinese collections^{9,11,22–26} the croomine-type was represented only by croomine (2) itself in one sample of *S. tuberosa* Lour. (HG 890) collected in north Thailand (Figure 4).⁶

Because of far-reaching morphological similarities between the genera *Croomia* and *Stichoneuron*, Duyfjes recently pointed to a possible amalgamation of both.² So far only two species of *Stichoneuron*^{4,5} and one of *Croomia*²⁷ have been subjected to chemical analysis. Of special chemotaxonomic importance was our recent discovery of the secondary amine pandanamine (1) in the rare *Stichoneuron calcicola* Inthachub, which was regarded as a plausible biogenetic precursor of *Stemonia* alkaloids (Figure 1).⁵ Interestingly, protostemonine-type alkaloids, otherwise representing the greatest diversity in the genus *Stemonia*, could not be

detected in the two genera. Instead, the isomers stichoneurines A and B were isolated from *Stichoneuron caudatum* Ridley,⁴ and croomine (2) was isolated from *Croomia heterosepala* Okuyama.²⁷ Whereas the latter can structurally be directly derived from pandanamine (1), no explanation can be given for the formation of the stichoneurine- and protostemonine-type skeletons containing four additional carbon atoms (Figure 1).

In the present survey alkaloids with a stichoneurine-type skeleton were represented mainly by tuberostemonine (3) together with three isomers (4–6) showing a species-specific distribution. The formation of tuberostemonine A (4) appears to be typical for *S. phyllantha* Gagnep., whereas tuberostemonine N (5) together with neotuberostemonine (6) characterize *S. tuberosa* Lour. (Figures 2 and 4). Though this confirms our previous results,⁶ it is in contrast to many phytochemical investigations in China, where in addition to isomers of tuberostemonine (3) a number of stemonine (7) derivatives have been isolated (Figure 2).^{10,24,26,28} Moreover, a series of oxidative modification products of tuberostemonine (3)⁷ has been reported that could not be detected in the present study.

Most of the *Stemonia* species in Thailand are characterized by alkaloids derived from a protostemonine-type skeleton (Figure 3). On the basis of the present survey protostemonine (8) itself plays a subsidiary role, mostly occurring in only small amounts. Major accumulation trends lead either to stemofoline (10) or stemocurtisinol (13) derivatives (Figure 1, Table 1). Both share an acyclic side chain and an oxygen bridge linking the pyrrolidine (10–12) or pyridazepine rings (13, 14, 16), respectively. As outlined previously,²⁹ the formation of the six-membered piperidine ring can be explained by opening the five-membered pyrrolidine ring and incorporating C-18 of the butyl side chain (Figure 3). Stemofoline (10) represents a complex cage-type molecule with an oxygen bridge between C-2 and C-8 and an

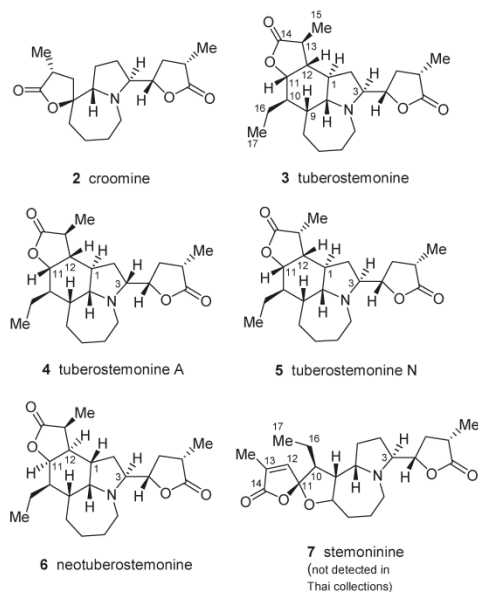


Figure 2. Alkaloids with a croomine (2)- or stichoneurine-type skeleton (3–7) detected by ELSD.

additional C–C linkage between C-3 and C-7. It is a major compound in many *Stemona* species, but has not been detected in members of the *S. tuberosa* group characterized by stichoneurine-type alkaloids (Figures 2 and 4). In some species stemofoline (10) was shown to co-occur with the pyridoazepine derivatives 13 and 15 and small amounts of oxyprotostemonine (9) (Table 1). In this case an infraspecific variation was observed, especially in *S. curtisii* Hook.f., a prominent species of south and southwest Thailand. From 11 different collections five were characterized by 10 as the major compound, two by additional small amounts of 13 and 15, and four deviate by a preponderance of 13 and 15 (Table 1). These findings are in good agreement with our previous results, where six different collections of *S. curtisii* showed various accumulation trends either toward 10 or the pyridoazepine derivatives 13–15.⁶ However, it is interesting to note that in contrast to previous findings no oxystemokerrine N-oxide (16) was detected in the present analysis.

Unlike in *S. curtisii*, a far-reaching chemical uniformity was found in 11 different collections of *S. collinsiae* Craib, another well-known and widespread species of Thailand. All samples were characterized by a clear preponderance of didehydrostemofoline (11) accompanied by only small amounts of stemofoline (10) (Table 1). In spite of similarities with *S. collinsiae*, two collections from the provinces Lopburi and Nakhon Ratchasima in northeast Thailand were separated because of some morphological divergencies. In comparison to all other collections, characterized by yellowish white tepals and mostly erect habit, they deviate by pinkish tepals and a twining habit. Moreover, their stamens appear somewhat narrower and longer than those of *S. collinsiae*. This slight divergency is also underlined by a different chemical profile,

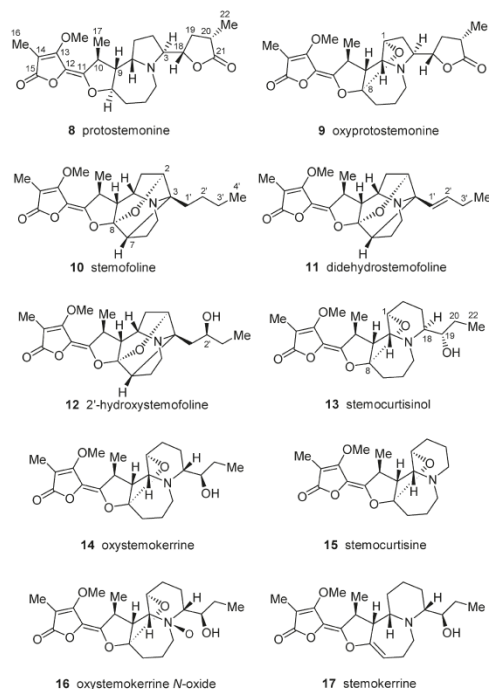


Figure 3. Pyrrolo- (8–12) and pyridoazepine alkaloids (13–17) with a protostemonine-type skeleton detected by DAD.

consisting of nearly equal amounts of stemofoline (10) and didehydrostemofoline (11) (Table 1).

S. cochinchinensis Gagnep. can be readily distinguished from other *Stemona* species by its characteristic stamens, showing an elongated appendage of the connective narrower than the anther.¹ The HPLC profile in the present study suggests an infraspecific chemical variability. Whereas previous findings from the Nong Khai Province in northeast Thailand (HG 884) were characterized by a predominance of stemofoline (10) accompanied by small amounts of 2'-hydroxystemofoline (12),⁶ the present results show a reduced alkaloid pattern represented by small amounts of protostemonine (8). Stemofoline (10) was previously also reported as the major compound for three different samples of *S. burkillii* D. Prain together with its 2'-hydroxy derivative 12 and small amounts of closely related derivatives.^{6,30} They were collected in the mountains of the provinces Lamphun and Chiang Mai in north Thailand (HG 946, HG 887).⁶ However, in the present study no plant material was available for reinvestigation.

S. aphylla Craib and the recently described *S. involuta* Inthachub appear to be closely interrelated and are mainly distributed in the dry and open habitats in north, northeast, and east Thailand.¹ Differences are mainly based on stamen morphology as well as on size and color of the flowers. The chemical profiles of *S. aphylla*, collected in different localities, show some variability. In two samples, originating from the provinces Mahasarakham and Ubon Ratchathani in northeast Thailand, the alkaloid formation

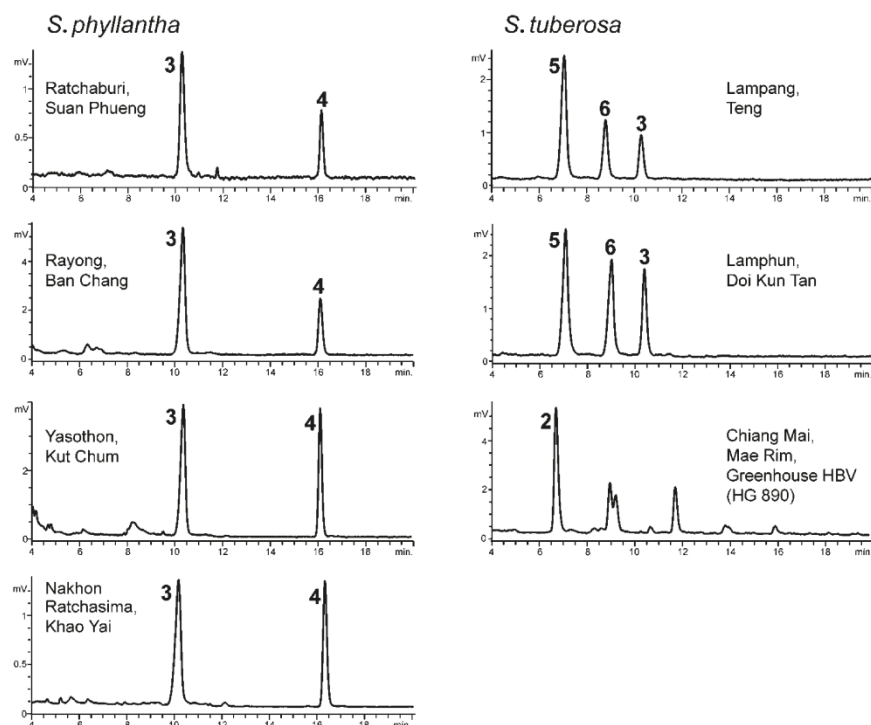


Figure 4. HPLC-ELSD comparison of methanolic crude extracts of underground parts of *S. phyllantha* accumulating tuberostemonine (3) and tuberostemonine A (4), and *S. tuberosa* accumulating tuberostemonine (3), tuberostemonine N (5), neotuberostemonine (6), and croomine (2).

is strongly reduced, showing only small amounts of protostemonine (8) (Table 1). In addition, both of the corresponding HPLC profiles are characterized by an accumulation of stilbenoids (Figure 5).³¹ A similar chemical trend has been reported for two other collections from Ubon Ratchathani and Kanchanaburi, previously published as unidentified species HG 912 and HG 913.⁶ In order to gain more insight into the chemical variability of *S. aphylla*, extracts of two further collections, originating from the provinces of Udon Thani (HG 915) and Sukhothai (HG 893), were reinvestigated. Both have been published previously as unidentified species.⁶ They are characterized by pyridozepines, either by stemocurtisine (15) and oxystemokerrine (14) (HG 915) or by oxystemokerrine (14) together with its *N*-oxide (16) (HG 893), respectively (Figure 5 A). More detailed comparative analyses between extracts of different individuals from the same locality near Sukhothai (HG 893) show different accumulation trends toward either the pyridozepines 14 and 16 or a series of stilbenoids (Figure 5 B, individuals 1–4).³¹ This vicariance between alkaloid and stilbenoid accumulation was observed in the protostemonine (8)-containing collections mentioned before. The closely related *S. involuta* was not analyzed in the present study, but results from our previous investigation can be used for comparison. In this case the corresponding collection has been published as unidentified species HG 943.⁶ It originated from “*locus classicus*” near Nakhon Ratchasima and was later used

as specimen for the description of the new species.¹ Its HPLC profile was characterized by oxystemokerrine (14) as the major compound together with its *N*-oxide 16 and small amounts of protostemonine (8).⁶

S. rupestris Inthachub represents another newly described species endemic to northeast Thailand. It is closely related to *S. pierrei* Gagnep., but differs by longer tepals and shorter appendages of the thecae.¹ Accordingly, its chemical profile is similar to that of *S. pierrei*, which has been published previously.^{6,32} It shows also a strongly reduced alkaloid formation, consisting only of traces of protostemonine (8), but is accompanied by a prominent pattern of stilbenoids. However, on the basis of comparative HPLC-DAD analyses, the stilbenoids differ from those of *S. aphylla* most likely representing dihydrophenanthrenes (stemanthrenes) as reported for *S. pierrei*.³²

Two different samples of *S. kerrii* Craib, collected in the mountains of the provinces of Lamphun and Chiang Mai in north Thailand, exhibited closely related HPLC profiles. In accordance with previous findings^{6,29} they are characterized by stemokerrine (17) as the major compound accompanied by small amounts of oxystemokerrine (14) and oxyprotostemonine (9). This confirms a recent taxonomic statement that *S. kerrii* represents a rather homogeneous species.¹ However, in contrast to previous results no oxystemokerrine *N*-oxide (16) was detected.^{6,29}

Table 1. Distribution of Protostemonine-Type Alkaloids in Thailand^a

geographical distribution	pyrrolizapines					pyridoazepines				
	8	9	10	11	12	13	14	15	17	
<i>S. curtisii</i>										
Surat Thani, Kanchanadit			●							
Krabi, Ao Luek			●							
Krabi, Muang			●							
Songkhla, Na Tha Wi			●							
Songkhla, Hat Yai			●							
Trang, Ratsada			●			+	+		○	
Phattalung, Muang			●			+	+		+	
Prachuap Khiri Khan, Bang Saphan Noi	+						○		●	
Prachuap Khiri Khan, Sam Roi Yot	+						+		●	
Prachuap Khiri Khan, Thap Sakae	+	○					○		●	
Nakhon Si Thammarat, Thung Song	○								●	
<i>S. collinsiae</i>										
Chonburi, Si Racha	+		●							
Saraburi, Kaeng Khoi	+		●							
Nakhon Nayok, Pak Phli	+		●							
Prachinburi, Kabin Buri	+		●							
Nakhon Ratchasima, Wang Nam Khieo	+		●							
Nakhon Ratchasima, Tup Lan	+		●							
Nakhon Ratchasima, Khao Yai	+		●							
Phitsanulok, Phu Hin Rong Khla	+		●							
Loei, Phu Krahueng	+		●							
Ubon Ratchathani, Mueang	+		●							
Ubon Ratchathani, Si Mueang Mai	+		●							
unidentified species										
Nakhon Ratchasima, Pak Chong			●	○						
Lopburi, Chai Badan			●	●						
<i>S. aphylla</i>										
Maharakam, Na Dun	+									
Ubon Ratchathani, Khong Chiam	+									
Udon Thani, Nong Wu So (HG915)							○	●		
Sukhothai, Khiri Mat (HG893)							●			
<i>S. rupestris</i>										
Yasothon, Loeng Nok Tha	+									
<i>S. cochinchinensis</i>										
Surin, Rattanaburi	+									
<i>S. kerrii</i>										
Chiang Mai, Mae Rim		○					○		●	
Lamphun, Doi Khun Tan							+		●	

^a Symbols correspond to peak sizes of HPLC-DAD profiles (compare Figure 5). ● = major compound, ○ = minor compound, + = traces.

The present survey of 42 different *Stemona* samples collected in Thailand confirms to a large extent our previous findings based on 39 collections.⁶ It shows again a clear chemical segregation of *S. tuberosa* and *S. phyllantha*, exclusively characterized by tuberostemonine derivatives with a stichoneurine-type skeleton (3–6) (Figures 2, 4). The reinvestigation of *S. tuberosa*, originating from the mountains near Chiang Mai in north Thailand (HG 890),^{4,6} cultivated in the Botanical Garden of the University of Vienna, shows again a preponderance of croomine (2). This skeletal type, also classified as tuberostemospironine type,²³ appears to be

more widespread in samples of *S. tuberosa* originating from the mountains of south China^{9,11,22–26} and Vietnam,⁶ but was also found in *S. phyllantha* from Indonesia, originally published as *ex aff. S. tuberosa* (HG 918).⁶

On the basis of previous reports the well-known antitussive properties of *Stemona* extracts were thought to be restricted to stichoneurine- and croomine-type alkaloids of the *S. tuberosa* group.^{7,9–11,33} However, recent findings also exhibited significant activities in derivatives with a protostemonine-type skeleton isolated from the Chinese *S. sessilifolia* (Miq.) Miq.¹³ More detailed experiments with the citric acid-induced guinea pig cough model combined with electrical stimulation of the superior laryngeal nerve showed that the stichoneurine-type derivatives tuberostemonine (3), neotuberostemonine (6), and stemonine (7) target the peripheral cough reflex pathway, whereas croomine (2) acts on central sites. The latter also showed central depressant effects, which may partly account for the side effects of some *Stemona* extracts, leading to nausea, vomiting, delirium, and headache.¹²

The strong insecticidal activity of stemofoline (10) was first detected in the leaf extract of *S. japonica*.³⁴ This was later confirmed by more detailed electrophysiological in vitro tests on the insect nicotinic acetylcholine receptor and by in vivo screenings against relevant agricultural insect pests.¹⁵ Stemofoline (10) represents the major compound in the underground parts of many collections of *S. curtisii* (Table 1). It was also reported to dominate in *S. burkillii*^{6,19,30} and in one collection of *S. cochinchinensis*.^{6,29} In *S. burkillii* it was shown to increase the chemosensitivity via P-glycoprotein-mediated multidrug resistance.¹⁹ Moreover, in *S. javanica* (Kunth) Engl., collected in east Java, Indonesia, it was reported to inhibit nitric oxide production (NO) in a mouse macrophage-like cell line stimulated by lipopolysaccharide.¹⁸ The closely related didydrostemofoline (11) also showed pronounced insect toxic effects against larvae of the two well-known pest insects *Plutella xylostella*³⁵ and *Spodoptera littoralis*.³⁶ This compound, previously named asparagamine A, also exhibited an oxytocin antagonistic effect by delaying parturition in Wistar rats.¹⁷ As shown in Table 1 didydrostemofoline (11) represents a typical chemical marker of *S. collinsiae*, where it accumulated in all collections and was also found in two probably closely related, but as yet unidentified samples. Stemofoline derivatives with their high insecticidal activity already inspired as lead structure the development of synthetic cyanotropans, a novel class of commercial insecticides interacting at the nicotinic acetylcholine receptor.³⁷

Apart from accumulation trends toward alkaloids with different skeletal types (Figure 1) the present survey repeatedly shows a general reduction of alkaloid formation, mainly in *S. rupestris* and *S. cochinchinensis*, but also in some collections of *S. aphylla* (Figure 5; Table 1; ref 6), originating from periodically dry and hot habitats. In this case only small amounts of protostemonine (8) were detected accompanied by an accumulation of stilbenoids. It is tempting to interpret this metabolic trend as a loss of enzymatic activity, and protostemonine (8) as the remaining biosynthetic precursor, usually leading to the formation of stemofoline (10) or stemocurtisinol (13) derivatives (Figure 1).

To establish species-specific accumulation trends, only compounds occurring in higher concentration were considered for the present comparison. Inspection of literature data showed an additional number of minor constituents that were detected only in extracts obtained from larger quantities of plant material. They were shown to represent mostly closely related derivatives of the

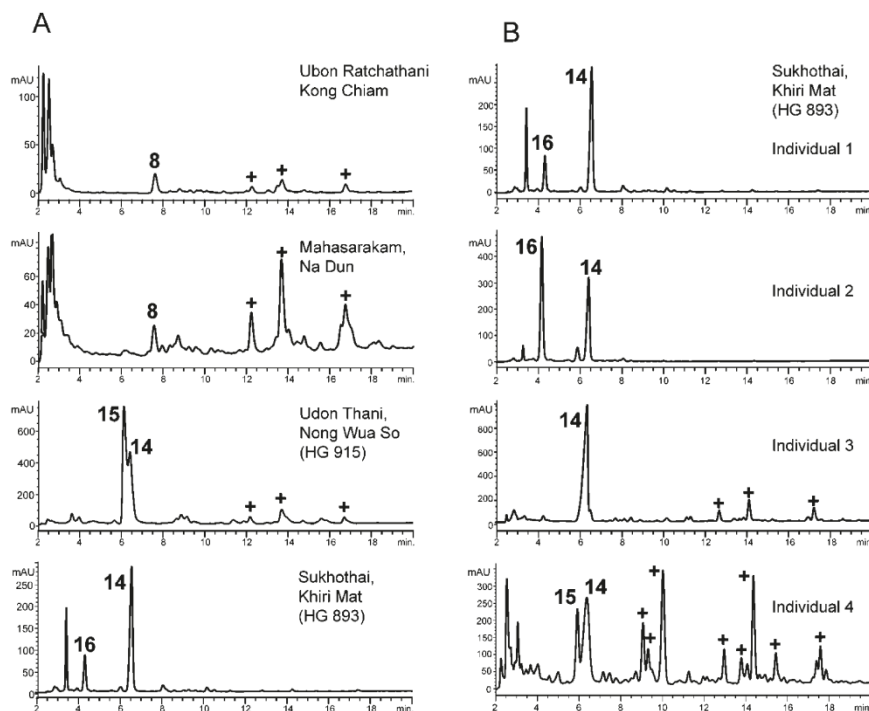


Figure 5. HPLC-DAD comparison of methanolic crude extracts of underground parts of *S. aphylla* demonstrating chemical variability between different localities (A) and interindividual differences within the same population (B). + = stilbenoids, UV detection at 280 nm.

major compounds with the same basic structure. This has been exemplified in extracts with dominating stemokerrine (17)³⁸ or stemofoline (10) alkaloids.^{15,30,39,40} Moreover, the formation of artifacts should also be taken into consideration. For example the 11E-isomer of didehydrostemofoline (11) is most likely formed by photoisomerization.^{35,39} In a previous review it has been speculated that the frequently published bisdehydro derivatives of all three skeletal types with a stable aromatic pyrrole system possibly represent artifacts.⁷ This hypothesis seems to be confirmed by the present comprehensive survey, where no accumulation of these compounds was detected. That possibly also applies to a series of oxidative modification products of tuberostemonine (3) reported for Chinese collections of *S. tuberosa*.^{7,9,26} The absence of *N*-oxides, formerly frequently found in different species,^{6,38,39} might be explained by different ways of sample preparation. Whereas in the present study the underground parts were rapidly dried at 50 °C, ground, and stored in a gastight plastic container, the plant material of previous investigations, indicated by HG numbers (Figures 4 and 5; Table 1), has been slowly dried as a whole at room temperature and was immediately extracted after grinding.⁶

In conclusion, only derivatives of the stichoneurine or proto-stemonine skeletal type play a prominent role in Thai *Stemona* species. Within the stichoneurine group it is interesting to note that no stemoninine (7) derivatives were detected, which otherwise

were frequently reported as dominating compounds in some Chinese collections. On the other hand, tuberostemonine A (4), frequently found in Thailand, was not detected so far in China.^{9–11,22,24–26} This isomer was originally identified in the roots of *S. sessilifolia*, obtained from Mikuni and Co., Tokyo, which was most likely confused with *S. tuberosa*.⁴¹ Regarding the protostemonine group, it should be pointed out that no maistemone derivatives with a spiro system were detected in the present study, but were frequently accumulated as major compounds in collections from China.^{7,25} Remarkable differences were also observed in the distribution of the biologically highly active stemofoline (10). Although originally isolated from the leaf extract of *S. japonica*, it represents a typical and widespread chemical character of the underground parts of various *Stemona* species in Thailand, but is only rarely reported so far for Chinese collections.

EXPERIMENTAL SECTION

General Experimental Procedures. HPLC: Agilent 1100 series, UV diode array detection at 280 nm. ELSD: Agilent 1260 infinity, temp 40 °C, nitrogen flow 3.6 L/min. Hypersil BDS-C₁₈ column (4.6 mm i.d. × 25 cm, 5 μm), mobile phases: (A) 10 mM NH₄OAc in H₂O and (B) MeOH. Gradient elution was used from 55% to 90% B in A for 19 min, 90% B in A to 100% B for 1 min, and 100% B for 10 min; flow rate was

1 mL/min and injection volume 10 μ L. The separation of stemofoline (10) from dihydrostemofoline (11) was achieved by changing the mobile phase A to 1 mM NH_4OAc in H_2O . TLC was performed on silica gel 60 F₂₅₄ plates (0.2 mm, Merck) with $\text{CH}_2\text{Cl}_2/\text{EtOAc}/\text{MeOH}/\text{NH}_4\text{OH}$ (70:25:5:1) for protostemonine- and $\text{CH}_2\text{Cl}_2/\text{EtOAc}/\text{MeOH}/\text{NH}_4\text{OH}$ (50:45:5:1) for stichoneurine-type alkaloids, respectively, and sprayed with Dragendorff's reagent. The concentration of samples subjected to HPLC and TLC was 10 mg/mL for crude extracts and 0.2 mg/mL for pure compounds.

NMR and MS: The compounds were dissolved in 99.98% CDCl_3 or in 99.8% methanol- d_4 (ca. 5 mg in 0.7 mL) and transferred into 5 mm NMR sample tubes (Promochem, Wesel, Germany), respectively. Spectra were recorded by the Bruker Topspin 1.3 software on a Bruker DRX-600 AVANCE spectrometer (Bruker, Rheinstetten, Germany) at 600.13 MHz (^1H) or 150.90 MHz (^{13}C) at 298.0 ± 0.1 K. For 1D spectra 64k data points were measured using a 1.0 s relaxation delay. Fourier transformation led to spectra with a range of 8000 Hz (^1H) and 32000 Hz (^{13}C). 2D COSY, TOCSY, NOESY, HMQC, and HMBC spectra were measured by 128 to 256 experiments with 1024 to 2048 data points each. Linear forward prediction to 4k and 512 data points in the f_1 and f_2 dimension, respectively, sinusoidal multiplication in both dimensions, and Fourier transformation produce 2D spectra with a range of ca. 6000 and 32 000 Hz for ^1H and ^{13}C NMR, respectively. In methanol- d_4 small amounts of methanol- d_1 were used as internal standard for ^1H (δ_{H} 3.340) and methanol- d_4 for ^{13}C (δ_{C} 49.86) NMR measurements. In CDCl_3 residual CHCl_3 (δ_{H} 7.240) and CDCl_3 (δ_{C} 77.02) were used as internal standards for ^1H and ^{13}C NMR measurements, respectively. Structure determination: Combined SPININFO and CSEARCH databases running under the CSEARCH program have been used for spectral similarity searches and prediction of ^{13}C NMR spectra.^{42–44} Mass spectra were recorded on a 900S (Finnigan MAT) by direct infusion ESI in positive and negative mode, respectively.

Plant Material. Forty-two samples representing *S. aphylla*, *S. cochinchinensis*, *S. collinsiae*, *S. curtisii*, *S. kerrii*, *S. phyllantha*, *S. ruprestis*, *S. tuberosa*, and two unidentified samples were collected from various localities in Thailand (Table 1, Figures 4 and 5). Voucher specimens are deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, and those with HG numbers at the Herbarium of the Institute of Botany, University of Vienna. To facilitate the recognition of different species, pictures of voucher specimens and living plants are presented at <http://www.phytochemie.botanik.univie.ac.at/herbarium/> and <http://herbarium.univie.ac.at/database/search.php>. Moreover, photographs of relevant Thai representatives have been published previously.^{6,29}

Extraction and Isolation. Underground parts were dried in an oven at 50 $^{\circ}\text{C}$, ground, and passed through a sieve (60 mesh). Each sample was stored separately in an airtight plastic container. Collections with HG numbers were slowly dried at room temperature as a whole and immediately extracted after grinding.²⁹ For HPLC-DAD or -ELSD analyses ca. 10 g of each sample was extracted with MeOH and partitioned between CHCl_3 and H_2O , and the lipophilic layer was used for comparison (Figures 4 and 5). For preparative isolation the CHCl_3 fractions were roughly separated by CC (Merck silica gel 60, 0.2–0.5) with solvent mixtures of petroleum ether, EtOAc, and MeOH or petroleum ether, CHCl_3 , and MeOH with increasing polarity. The alkaloid-containing fractions, monitored by TLC sprayed with Dragendorff's reagent, were pooled and further separated by MPLC (Merck silica gel 60, 40–60 μm) with mixtures of EtOAc and MeOH as described previously.²⁹ Final purification was achieved by isocratic CC using Sephadex LH-20 with MeOH.

Dried and ground underground parts of *S. tuberosa* (HG 890, 36 g) gave a CHCl_3 fraction (197 mg) affording purified croomine (2) (55 mg). *S. phyllantha* (Ratchaburi, Suan Phueng, 120 g) gave a CHCl_3 fraction (127 g)

affording tuberostemonine (3) (152 mg) and tuberostemonine A (4) (270 mg). *S. tuberosa* (Lamphun, Doi Kun Tan, 80 g) gave a CHCl_3 fraction (2.04 g) affording tuberostemonine N (5) (90 mg) and neotuberostemonine (6) (98 mg). *S. kerrii* (Chiang Mai, Mae Rim, 100 g) gave a CHCl_3 fraction (510 mg) affording oxyprotostemonine (9) (5.3 mg), oxystemokerrine (14) (5.4 mg), and stemokerrine (17) (17.4 mg). *S. curtisii* (Songkhla, Na Tha Wi, 300 g) gave a CHCl_3 fraction (2.5 g) affording stemofoline (10) (49 mg). *S. collinsiae* (Nakhon Ratchasima, Wang Nam Kiao, 400 g) gave a CHCl_3 fraction (2.2 g) affording dihydrostemofoline (11) (121 mg). *S. curtisii* (Prachuap Khiri Khan, Bang Saphan Noi, 200 g) gave a CHCl_3 fraction (1.13 g) affording stemocurtisinol (13) (40 mg) and stemocurtisine (15) (78.5 mg). Protostemonine (8) was not isolated in the present study. An authentic sample, originating from our previous work on *S. pierrei*,³² was used for chromatographic comparison.

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Chapter 4: *Iridoids as chemical markers of false ipecac (Ronabea emetica), a previously confused medicinal plant*

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Iridoids as chemical markers of false ipecac (*Ronabea emetica*), a previously confused medicinal plant

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ABSTRACT

Ethnopharmacological relevance: Several roots or rhizomes of rubiaceous species are reportedly used as the emetic and antiamoebic drug ipecac. True ipecac (*Carapichea ipecacuanha*) is chemically well characterized, in contrast to striated or false ipecac derived from the rhizomes of *Ronabea emetica* (syn. *Psychotria emetica*). Besides its previous use as substitute of ipecac, the latter species is applied in traditional medicine of Panama and fruits of its relative *Ronabea latifolia* are reported as curare additives from Colombia.

Materials and methods: Compounds of *Ronabea emetica* were isolated using standard chromatographic techniques, and structurally characterized by NMR spectroscopy and mass spectrometry. Organ specific distribution in *Ronabea emetica* as well as in *Ronabea latifolia* was further assessed by comparative HPLC analysis.

Results: Four iridoid-glucosides, asperuloside (1), 6 α -hydroxygeniposide (2), deacetylasperulosidic acid (3) and asperulosidic acid (4) were extracted from leaves of *Ronabea emetica*. Rhizomes, used in traditional medicine, were dominated by 3. HPLC profiles of *Ronabea latifolia* were largely corresponding. These results contrast to the general tendency of producing emetine-type and indole alkaloids in species of *Psychotria* and closely related genera and merit chemotaxonomic significance, characterizing the newly delimited genus *Ronabea*.

Conclusions: The aim of the work was to resolve the historic problem of adulteration of ipecac by establishing the chemical profile of *Ronabea emetica*, the false ipecac, as one of its less known sources. The paper demonstrates that different sources of ipecac can be distinguished by their phytochemistry, thus contributing to identifying adulterations of true ipecac.

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1. Introduction

Ronabea Aubl. (Rubiaceae) is a small neotropical genus comprising three species: *Ronabea emetica* (L. f.) A. Rich., *Ronabea insanae* (J. H. Kirkbr.) C. M. Taylor, and *Ronabea latifolia* Aubl. (Taylor, 2004). Based on macromolecular data (Piesschaert et al., 2000) and re-evaluation of morphological characters (Piesschaert, 2001), these species, previously included in the genus *Psychotria* L. of the tribe Psychotrieae, are now classified as a separate genus *Ronabea* Aubl., member of the tribe Lasiantheae (Taylor, 2004).

Ronabea emetica (syn. *Psychotria emetica* L. f., Fig. 1), was early regarded as being a source of the emetic drug ipecac (Linné, 1782; Humboldt and Bonpland, 1813). After the discovery of ipecacuanha by Piso and Markgraf (1648), its true botanical identity

remained unclear until Gomes (1801) discovered three rubiaceous species used as different kinds of ipecac. According to his studies, three sources of ipecacuanha are distinguished: white ipecac from *Richardsonia brasiliensis* (Gomes) Hayne, striated or false ipecac from *Ronabea emetica* and annulated or true ipecac derived from *Carapichea ipecacuanha* (Brot.) L. Andersson.

Most commercial ipecac was collected in the wild, and several similar looking species have long been confused and intermixed with true ipecac (e.g. Flückiger, 1891; Tschirch, 1923). Therefore, the active principle of the drug remained unclear until Pelletier and Magendie (1817) reported the occurrence of emetine in material they addressed to *Ronabea emetica*, but after re-examination of their original collection, Tschirch (1923) clarified that through confusing both species, *Carapichea ipecacuanha* was studied instead. Later, Tschirch and Lüdtké (1888) as well as Flückiger (1891) could not detect emetine in authentic material of *Ronabea emetica*, and its chemical components remained unclear.

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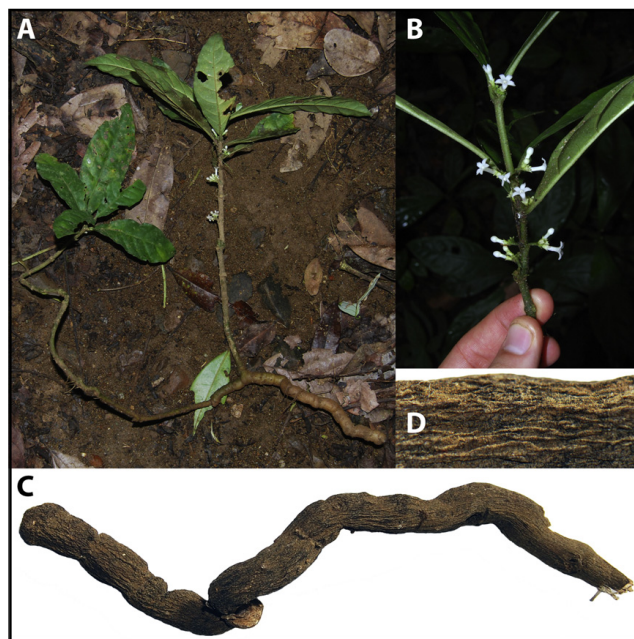


Fig. 1. *Ronabea emetica*, whole plant (A, note the creeping rhizome), flowering branch (B), dried rhizome (C, the part applied in traditional medicine), bark detail of a dried rhizome (D, note the longitudinally striated bark distinctive for false ipecac).

Whilst *Carapichea ipecacuanha* became the only source of commercial ipecac, *Ronabea emetica* is additionally applied in traditional medicine of the Teribes, an indigenous group of Bocas del Torro province, Panama. They use a decoction of the rhizomes against chicken pox and fever, indications clearly differing from those against which true ipecac is applied (Gupta et al., 2005). Karapanás and Witotos, two northwestern Amazonian tribes, use the fruits of the related species *Ronabea latifolia* for their reported toxicity as additive in curare preparation (Schultes and Raffauf, 1990).

Previously, only cyclotides, circular disulfide-rich miniproteins, were detected in *Ronabea emetica* during a broad scale screening of Rubiaceae (Gruber et al., 2008). In order to contribute to a better understanding of the current and historic usage of *Ronabea emetica* and the phytochemical composition of drugs labeled ipecac, extracts of leaves, stems and rhizomes of *Ronabea emetica* were analyzed. Its compounds, their structure and their occurrence in different organs of the plant were elucidated and compared with the profiles obtained from *Ronabea latifolia*.

2. Materials and methods

2.1. Plant material

Leaves, stems and rhizomes of *Ronabea emetica* were collected alongside the main trail from Los Patos to Sirena Ranger Station in Corcovado National Park, leaves and stems of *Ronabea latifolia* were collected near La Gamba Field Station in the “Rainforest of the Austrians”, Puntarenas, Costa Rica. Collection and export of plant material was kindly permitted by the Costa Rican Ministry of Ambient and Energy under 191-2009-SINAC. Voucher specimens were deposited at

the Herbarium of the Institute of Botany, University of Vienna (WU, voucher accession numbers: *Ronabea emetica* 0046606-0046610, *Ronabea latifolia* 0060915-0060918), digitised images can be viewed at <http://herbarium.univie.ac.at/database/search.php>. Radix Ipecacuanhae (*Carapichea ipecacuanha*) was kindly provided by the Department of Pharmacognosy, University of Vienna, Austria.

2.2. Extraction and isolation

For HPLC-UV analysis, small quantities of powdered leaves, stems and rhizomes of *Ronabea emetica*, fruits, leaves and stems of *Ronabea latifolia* as well as Radix Ipecacuanhae were extracted with MeOH and directly used for chromatographic comparison. For isolation, leaves of *Ronabea emetica* were selected because of highest amount of available material. Air dried and ground leaves (12.5 g) were extracted twice for three days each with MeOH at room temperature. The combined and filtered crude extract was concentrated under vacuum to give 2.1 g residue, which was partitioned between CHCl₃, EtOAc and H₂O, respectively. 850 mg of the aqueous phase was roughly separated by column chromatography (CC, Merck silica gel 60, 0.2–0.5 mm) with solvent mixtures of PE, EtOAc and MeOH with increasing polarity. Fractions eluted with EtOAc/MeOH (85:15), containing compound **1**, were combined (165 mg) and purified by isocratic methanolic CC with Sephadex LH-20 to give 110 mg pure **1**. The more polar fractions were combined (340 mg) and further separated by CC (Merck silica gel 60, 40–63 µm) eluted with CHCl₃/MeOH (4:1) to afford 32 mg **1** and a mixture of **2**, **3** and **4**, which were subsequently separated by preparative TLC using CHCl₃/MeOH (4:1) to yield 10 mg **2**, as well as the organic phase of BuOH/H₂O/acetic acid (5:4:1) to give 18 mg

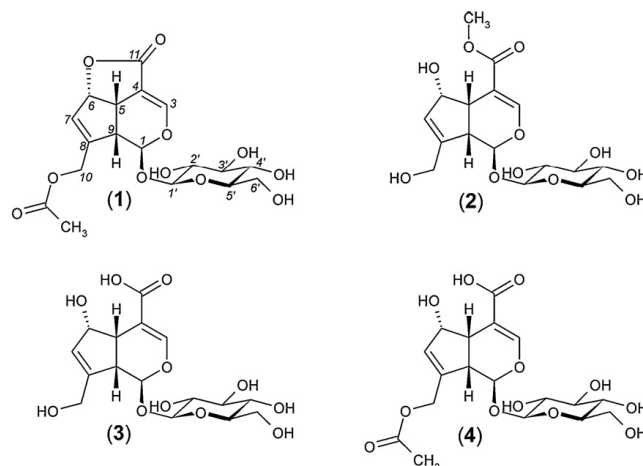


Fig. 2. Structures of four iridoid-glucosides, isolated from leaves of *Ronabea emetica*: asperuloside (1), 6 α -hydroxygeniposide (2), deacetylasperulosidic acid (3) and asperulosidic acid (4).

3 and **3 mg 4**. All separations were monitored by HPLC-UV and TLC, respectively.

2.3. Experimental

Analytical TLC was performed on silica gel 60 F254 plates (Merck company), developed with CHCl₃/MeOH/H₂O (80:20:1), detected under UV at 254 and 366 nm and subsequently sprayed with anisaldehyde reagent. The HPLC measurements were performed on Agilent 1100 series with acetonitrile (A) and an aqueous buffer (B) containing 15 mM *ortho*-phosphoric acid and 1.5 mM tetrabutylammonium hydroxide with a linear gradient 0–15 min: 15–60% A in B, followed by 5 min 100% A at a flow rate of 0.8 mL/min. The measurements were performed on a Hypersil BDS C18 column (250 mm $\times$ 4.6 mm, 5 μ m particle size) with UV diode array detection at 230 nm. Concentration of samples was adjusted to 5 mg/mL.

2.4. NMR spectroscopy

For NMR spectroscopic measurements the compounds were dissolved in 99.8% CD₃OD (~5 mg in 0.7 mL) and transferred into 5 mm NMR sample tubes (Promochem, Wesel, Germany). Spectra were measured at 298.1 K on a Bruker DRX-400 AVANCE spectrometer (Bruker, Rheinstetten, Germany) at 400.13 MHz (¹H) and 100.61 MHz (¹³C). For ¹H, ¹³C, COSY, TOCSY, NOESY, HMQC, and HMBC, the standard pulse programs of Topspin 1.3 have been applied to record spectra with a range of 7200 Hz (¹H) and 30,000 Hz (¹³C). CHD₂OD was used as internal standard for ¹H (δ_{H} 3.34) and CD₃OD for ¹³C (δ_{C} 49.0) spectra.

2.5. Mass spectrometry

Mass Spectra were recorded on a 900S (Finnigan MAT) by direct infusion electrospray ionization in positive and negative mode, respectively.

3. Results

Four iridoid-glucosides, asperuloside (**1**), 6 α -hydroxygeniposide (**2**), deacetylasperulosidic acid (**3**) and asperulosidic acid (**4**)

were extracted from leaves of *Ronabea emetica* (Fig. 2). Structure determination of the isolated compounds was performed by NMR spectroscopy and mass spectrometry. The NMR chemical shifts, coupling constants, and NOEs allowed identification of the joint carbon skeleton and the β -glucoside, present in all compounds. Furthermore, the presence of methyl ester (compound **2**), acetyl groups (compounds **1** and **4**) and lactone ring (compound **1**) were identified by indicative signals in NMR spectra (Table 1) and corresponding molecular masses determined by mass spectrometry. Similarly, the relative configuration of all chiral centres has been deduced from the data and is, in accordance with literature, identical as shown in Fig. 2. The structures were furthermore confirmed by searching for spectral similarity in the combined CSEARCH and SPECINFO database system (Schütz et al., 1997; Seger et al., 1997; Robien, 2009). The hits of these searches are in excellent agreement with the direct analysis from NMR and MS spectra. The compounds **1**, **2**, **3** and **4** have previously been isolated from other sources and have hence been fully analyzed in the past, e.g. they have been described by Li et al. (2006), Ono et al. (2007), Kamiya et al. (2008) and Demirezer et al. (2006), respectively. All analytical data determined for the substances isolated from false ipecac (*Ronabea emetica*) are in full agreement with the data previously reported.

Comparison of HPLC profiles between the species *Ronabea emetica* and *Ronabea latifolia* revealed almost identical accumulation tendencies for all major compounds present in corresponding organs (Fig. 3). Leaf extracts were dominated by the presence of **1**, whereas the stems or rhizomes clearly deviated by accumulation of **3** as major compound. Additionally, HPLC profiles of fruits from *Ronabea latifolia* were characterized by equal amounts of **1** and **3**. When compared with true ipecac *Carapichea ipecacuanha*, as well as with ipecac alkaloids isolated from the related plant *Carapichea affinis* (Standl.) L. Andersson in a previous study (Bernhard et al., 2011), no similarities in HPLC profiles and substances could be observed.

4. Discussion

The present work is the first detailed phytochemical study of the genus *Ronabea* and two of its species, reporting the occurrence of

Table 1

¹H and ¹³C chemical shifts (δ, ppm) as well as ¹H multiplicities and coupling constants (Hz) of the substances **1**, **2**, **3** and **4** isolated from false ipecac (*Ronabea emetica*). Indication of the atoms is made in correspondence to the numbering given in Fig. 2. All four samples were measured in CD₃OD at 400 MHz and 100 MHz, respectively.

Atom nr.	Asperuloside (1)		6α-Hydroxygeniposide (2)		Deacetylasperulosidic acid (3)		Asperulosidic acid (4)	
	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C
1	5.99 (d, 9.1)	99.4 (d)	5.09 (d, 8.6)	101.4 (d)	5.12 (d, 8.9)	101.7 (d)	5.06 (d, 8.7)	99.0 (d)
3	7.31 (d, 0.8)	149.8 (d)	7.69 (s, br)	155.7 (d)	7.62 (d, 1.0)	155.8 (d)	7.64 (d, 1.3)	152.5 (d)
4	–	105.7 (s)	–	108.1 (s)	–	108.3 (s)	–	107.9 (s)
5	3.62 (dd, 7.0, 5.7)	37.0 (d)	3.05 (dd, 6.9, 5.8)	42.5 (d)	3.03 (ddd, 7.2, 5.5, 1.0)	42.9 (d)	3.05 (ddd, 7.0, 5.3, 1.3)	40.7 (d)
6	5.58 (br, m)	85.0 (d)	4.81 (d, br, 5.2)	75.7 (d)	4.78 (dd, 5.5, 1.7)	74.9 (d)	3.30 (dd, 5.3, 2.1)	73.2 (d)
7	5.76 (d, 1.2)	128.2 (d)	6.05 (s, br)	130.3 (d)	6.02 (d, 1.7)	130.1 (d)	6.01 (d, 2.1)	132.3 (d)
8	–	144.3 (s)	–	151.2 (s)	–	150.9 (s)	–	142.4 (s)
9	3.34 (pt, 7.5)	44.3 (d)	2.61 (dd, 8.6, 6.9)	45.9 (d)	2.58 (dd, 8.9, 7.2)	46.2 (d)	2.69 (pt, 7.4)	44.7 (d)
10	4.85/4.70 (2xd, 15.0)	61.2 (t)	4.47/4.24 (2xd, 15.1)	61.7 (t)	4.48/4.20 (2xd, 15.4)	62.9 (t)	5.01/4.81 (2xd, 15.1)	62.0 (t)
11	–	169.9 (s)	–	169.8 (s)	–	170.9 (s)	–	168.6 (s)
Ac (CH ₃)	2.10 (s)	21.1 (q)	–	–	–	–	2.11 (s)	20.9 (q)
Ac (C=O)	–	170.9 (s)	–	–	–	–	–	170.1 (s)
CH ₃	–	–	3.77 (s)	51.3 (q)	–	–	–	–
1'	4.72 (d, 8.0)	100.4 (d)	4.74 (d, 7.8)	100.3 (d)	4.72 (d, 7.9)	100.6 (d)	4.73 (d, 8.0)	100.1 (d)
2'	3.23 (dd, 8.0, 7.9)	74.3 (d)	3.28 (dd, 7.9, 7.8)	75.1 (d)	3.29 (dd, 8.0, 7.9)	75.1 (d)	3.28 (dd, 8.0, 7.8)	73.2 (d)
3'	3.30 (m)	78.1 (d)	3.43 (m)	78.0 (d)	3.46 (m)	78.4 (d)	3.43 (m)	77.0 (d)
4'	3.26 (m)	71.9 (d)	3.24 (m)	71.9 (d)	~3.25 (m)	71.6 (d)	~3.25 (m)	70.3 (d)
5'	3.37 (m)	78.1 (d)	3.26 (m)	78.4 (d)	~3.25 (m)	78.2 (d)	~3.25 (m)	77.6 (d)
6'	4.00/3.85 (2xm)	62.0 (d)	3.89/3.66 (2xm)	62.7 (t)	3.85/3.60 (2xm)	61.9 (t)	3.80/3.60 (2xm)	61.2 (t)

iridoid glucosides. As shown in Fig. 3, deacetylasperulosidic acid (**3**) was found to be the major compound in the rhizomes of *Ronabea emetica*, the organ used as a drug, whereas asperuloside (**1**), the main compound in the leaves, was only present in small amounts. 6α-hydroxygeniposide (**2**) and asperulosidic acid (**4**) were found as minor constituents in all studied organs.

4.1. Confusion of ipecac sources

Early studies report the use of striated ipecac, derived from *Ronabea emetica*, as an emetic agent. Together with other kinds of ipecac, it was collected by indigenous people in surrounding forests and sold for medical usage and export (e.g. Gomes, 1801; Humboldt

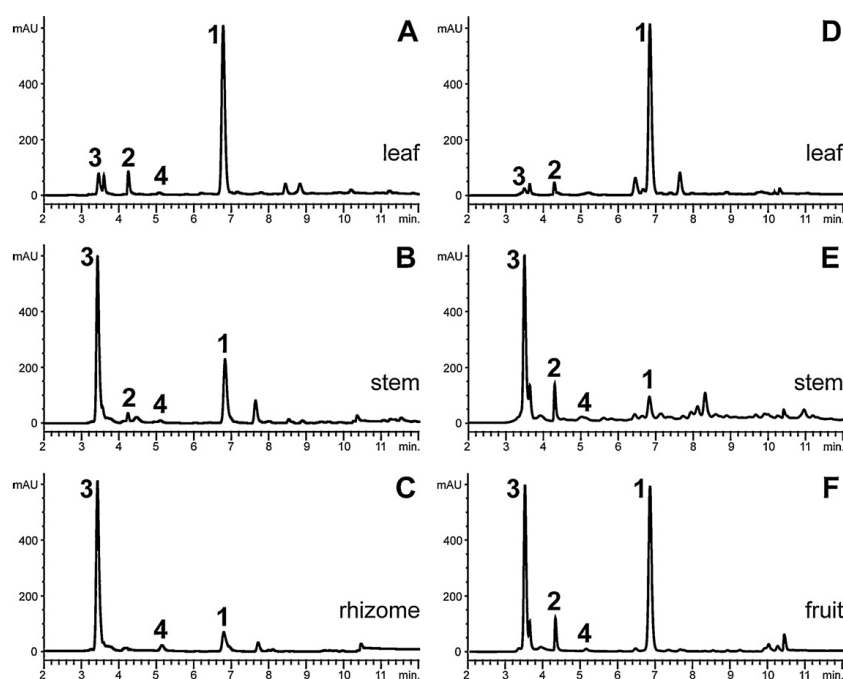


Fig. 3. HPLC-chromatograms of crude methanolic extracts of false ipecac, *Ronabea emetica* (A: leaf, B: stem and C: rhizome) and *Ronabea latifolia* (D: leaf, E: stem and F: fruit). Both species contain the iridoid-glucosides asperuloside (**1**), 6α-hydroxygeniposide (**2**), deacetylasperulosidic acid (**3**) and asperulosidic acid (**4**). Rhizomes of *Ronabea emetica* are applied in traditional medicine, fruits of *Ronabea latifolia* as curare additive.

and Bonpland, 1813). Later, the species was found to be devoid of the alkaloid emetine; it was merely considered as an adulteration of true ipecac, and its export and usage declined considerably (Tschirch and Lüdtke, 1888; Flückiger, 1891).

Considering observations of ipecac-like usage of *Ronabea emetica* such as those made by Humboldt and Bonpland (1813) during their journey through Latin America, active principles similar to those of true ipecac should be expected. In contrast to true ipecac containing so-called emetine-type or ipecac alkaloids (monoterpene isoquinoline alkaloids, e.g. Itoh et al., 1991) as well as to other *Psychotria* and *Palicourea* Aubl. species characterized by indole alkaloid accumulation (e.g. Porto et al., 2009; Berger, A., in prep.), our samples of the genus *Ronabea* showed presence of iridoid glucosides only. This phytochemical differentiation is challenging early reports of its applicability as ipecac source, and reasons for historic confusion are rather speculative at this point. It may be that both species bare emetic and/or antiamebic effects due to their chemistry, or that *Ronabea emetica* and its iridoid compounds are devoid of emetine like effects and have been collected and used as an adulteration only. Collecting of false ipecac might have happened accidentally caused by its similar appearance or as “commercial cheating” maximising yields when supply of true ipecac collected from the forests was limited. In that way, knowledge and usage of the similar looking *Ronabea emetica* may have spread and is likely to have given rise to assuming different origins of active ipecac, when in fact only one species bares proven activity. Considering the remarkable differences in phytochemistry between both kinds of ipecac, and as long as it remains unclear whether *Ronabea emetica* and its iridoid compounds bare emetic and antiamebic activity, the rhizomes of this species should be considered as adulteration as long as its properties have not been ascertained.

A similar case of confusing name and origin of a medicinal plant has led to the description of *Cinchona officinalis* L. as the source of quinine containing bark, but the species, as currently circumscribed, was later found never to contain more than traces of quinine, whilst other species, *Cinchona calisaya* Wedd. and *Cinchona pubescens* Vahl were shown to accumulate significant amounts of quinine (Andersson, 1997). Another case of confusing drugs due to their similar appearance is found in *Stemona* Lour. (Stemnaceae). In traditional medicine of South East Asia, roots of several species have been recommended for treating respiratory diseases and enteric helminthes. In Thailand, it is sold on local markets under the vernacular name “Non Tai Yak”. Due to their similarities, roots of other families such as *Asparagus* L. (Asparagaceae) or *Clitoria* L. (Fabaceae) are equally named and sometimes intermixed on purpose or accidentally (Brem et al., 2002). These examples illustrate the importance of comparative studies in related species and even across families, to guarantee that the expected bioactivity is reflected by the chemical composition.

4.2. Usage and bioactivity

Iridoids show a broad spectrum of bioactivity (Dinda et al., 2007, 2009), however only scarce reports exist, which could explain its ipecac-like usage as emetic or antiamebic drug. To our knowledge, emetic activity of iridoids has not been reported yet and so far, only iridoid derivatives linked with cinnamic acid were found exhibiting antiamebic activity. They have been found in leaves of *Morinda morindoides* (Baker) Milne-Redh. (Rubiaceae), a species used for the treatment of parasitic diseases in the traditional medicine of the Democratic Republic of Congo. Both its crude extracts as well as five isolated iridoids displayed significant *in vitro* activity against *Entamoeba histolytica*, against which true ipecac was excessively used (Cimanga et al., 2006). Several iridoids display antibacterial and antiviral activity (Dinda et al., 2007, 2009), which could be related to usage of the rhizomes in treating chicken pox and fever in

traditional medicine of the Teribes (Gupta et al., 2005). Furthermore, asperuloside (1) and deacetylasperulosidic acid (3), the main compounds present in leaves and rhizomes of *Ronabea emetica*, both possess antirheumatic activity (Li et al., 2006).

Fruits of *Ronabea latifolia* are reported as toxic and used by the Karapanás and Witotos, two northwestern Amazonian tribes, as additive in their curare preparation (Schultes and Raffauf, 1990). Toxicity of iridoid containing plants is scarcely studied. Besides other compounds, four iridoids have been isolated from *Chiococca alba* (L.) Hitchc. (Rubiaceae) (Carbonezi et al., 1999). Its ethanolic extracts exhibit moderate parental toxicity in a mouse model (Gazda et al., 2006).

Although largely untested for a broader spectrum of activity, it could be assumed that the iridoids found in the present study contribute to pharmacological effects and thus explain the current use of the species in ethnomedicine. Concerning historic reports on ipecac-like usage indicating emetic as well as antiamebic activity, its suitability in treating those diseases remains to be studied in appropriate model systems.

4.3. Chemosystematic significance

Chemosystematic interpretation of secondary metabolite profiles may allow prediction of accumulation tendencies to be expected in yet unstudied, but closely related species, thus facilitating search for bioactive compounds. From the systematic point of view, chemical diversification within the heterogeneous *Psychotria* and segregate genera (e.g. Andersson, 2002; Bernhard et al., 2011), and within different sources of ipecac is remarkable. Based on a broad survey of HPLC-profiles of more than 50 species of *Psychotria* and relatives, *Ronabea emetica* and *Ronabea latifolia* clearly deviated by the lack of alkaloid accumulation characterizing several species of *Psychotria* (e.g. Porto et al., 2009; Berger, A., in prep.). By contrast, the two studied species of the segregate *Ronabea* are characterized by accumulation of iridoid glucosides only. The occurrence of various iridoid glucosides in the genus *Lasianthus* (Takeda et al., 2002, 2004; Li et al., 2006), and detection of the same group of compounds in two *Ronabea* spp. confirms the close relationship between these two genera and their assignment to the same tribe. Iridoids are generally widespread within Rubiaceae, but lack of alkaloids is a feature that appears to be a significant chemical character for the genus *Ronabea* and the tribe Lasiantheae in contrast to alkaloid rich *Psychotria* and *Palicourea*. These observations are in line with macromolecular data and the current taxonomic concept (Bremer and Manen, 2000; Taylor, 2004; Robbrecht and Manen, 2006).

5. Conclusion

The current study demonstrates that different sources of ipecac can be distinguished by their phytochemistry. It reports the occurrence of four iridoid glucosides in *Ronabea emetica*, the source of false ipecac, where their sole presence in its rhizomes might be a useful tool for checking adulterations of true ipecac, in which they do not occur. The results question if *Ronabea emetica*, lacking ipecac alkaloids, could be used as an emetic and antiamebic drug or if reports of ipecac like activity are the result of constant confusion about its source plants. However, some correlations exist between the accumulation of iridoids, their reported bioactivity, and the use of *Ronabea emetica* in ethnomedicine of Panama. It is strongly suggested to test both, the plant material and its iridoid compounds for activities that might explain the use of *Ronabea* in traditional medicine.

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Chapter 5: *Various types of tryptamine-iridoid alkaloids from Palicourea acuminata (= Psychotria acuminata)*

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Various types of tryptamine-iridoid alkaloids from *Palicourea acuminata* (= *Psychotria acuminata*, Rubiaceae)

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ABSTRACT

The novel tryptamine-iridoid alkaloid lagamboside was isolated from the methanolic leaf extracts of *Palicourea acuminata* (Benth.) Borhidi (= *Psychotria acuminata* Benth.) collected in Costa Rica. Its structure was determined by extensive NMR experiments and is characterized by an unusual *N*-glycosylation and an iridoid moiety closely related to the less common vallesiachotamine. The co-occurrence with the rare bahienoside, containing two iridoid moieties, is of chemotaxonomic interest.

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1. Introduction

As a result of our current comparative HPLC-UV analyses of certain species of the large *Psychotria* s.l. complex (Rubiaceae) collected in Costa Rica, four major chemical trends were established. Besides the recently published accumulation of dopamine-iridoid alkaloids (Bernhard et al., 2011) and iridoid glycosides (Berger et al., 2011), HPLC-profiles were mostly characterized either by dominating peaks with an indole chromophore or a complex mixture of insufficiently separated compounds with simple UV spectra. Preliminary NMR analyses of the latter let expect a series of mono- or oligomeric catechins. It might be of chemotaxonomic significance that alkaloids of the pyrrolidinindoline or quinoline group, known from other *Psychotria* species (e.g. Verotta et al., 1999; Jannic et al., 1999), were not detected in the present survey. Detailed studies with species showing indole chromophores led to the isolation of the tryptamine-iridoid alkaloids strictosidinic acid (1) from *Psychotria buchtienii* (H.J.P. Winkl.) Standl. (Plagg, 2012), previously often erroneously identified as *Psychotria officinalis* (Aubl.) Raeusch. ex Sandwith, and its methylester strictosidine (2) from *Palicourea elata* (Sw.) Borhidi (= *Psychotria elata* (Sw.) Hammel) (Fasshuber, 2011) as major compounds. The closely related *N*-methyl

derivative palicoside (3) (Morita et al., 1989) was shown to dominate in *Psychotria tsakiana* C.M. Taylor, identified as *Psychotria racemosa* Rich. (Fasshuber, 2011; Plagg, 2012), whereas *Psychotria suerrensii* Donn. Sm. deviated by the formation of the two β -carbolines lyalosidic acid and ophirine A (Aimi et al., 1985; Fasshuber, 2011). A more complex HPLC-UV profile was found in five collections of *Psychotria acuminata* Benth., a species which has not been investigated so far. In a recent nomenclatural revision a number of *Psychotria* species occurring in Mexico have been transferred to the genus *Palicourea*, where *P. acuminata* was renamed as *Palicourea acuminata* (Benth.) Borhidi (Borhidi, 2011). The present paper reports on the isolation and identification of four alkaloids from the leaves and stem bark of *P. acuminata*. Whereas 4–6 were shown to be known compounds, 7 represented an as yet unknown tryptamine-iridoid alkaloid characterized by an unusual *N*-glycosylation. Its structure was elucidated by extensive NMR experiments.

2. Results and discussion

The air dried leaves, stem and root bark of five different collections of *P. acuminata*, originating from Costa Rica, were extracted separately with MeOH at room temperature. On the basis of HPLC-UV comparison the profiles of the leaf extracts of all collections were shown to be nearly identical characterized by two main peaks, whereas those of the stem and root bark deviated by an additional dominating peak. The two main compounds of the leaf extracts were separated by CC and their structures identified

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by NMR and MS analyses. The rare bahienoside B (**4**) (Paul et al., 2003; Xin et al., 2011), characterized by an indole chromophore, was shown to be the major compound accompanied by desoxycordifoline (**6**) (Brown and Warambwa, 1978) with a more complex UV spectrum indicative for the higher unsaturated β -carboline. Beyond that an unknown compound (**7**) was isolated, which showed a different UV spectrum. The stem and root bark additionally accumulated significant amounts of 5 α -carboxystrictosidine (**5**) (De Silva et al., 1971; Ferrari et al., 1986), representing the third major peak in the HPLC-profile characterized by an indole chromophore.

The unknown compound **7** was obtained as an amorphous solid and showed a simple UV spectrum with two maxima at 226 and 292 nm (MeOH). It was analyzed by high resolution electrospray ionization mass spectroscopy (HR ESI MS) in positive ionization mode. The resulting $[M+Na]^+$ showed m/z 537.2212 being in good accordance with calculated molecular mass of $C_{27}H_{34}N_2O_8Na$ (m/z 537.2207). The corresponding molecular formula of **7** is hence $C_{27}H_{34}N_2O_8$. A ^{13}C NMR spectrum confirmed the presence of 27 carbon atoms. The tricyclic tetrahydro- β -carboline skeleton, also present in compounds **1–5** (Fig. 1), was identified by very similar ^{13}C and ^1H chemical shift as well as by according scalar couplings. However, $^2J_{H-C}$ and $^3J_{H-C}$ couplings as well as NOEs of nuclei in positions 2, 3, and 5 indicated an additional six membered ring to be annulated at positions 3 and 4. In particular, there are $^3J_{C-H}$

couplings between C-2 and H-14a/H-14b as well as between C-14 and H-2. Additionally, both C-3 and C-5 show $^3J_{C-H}$ couplings to H-17 and vice versa. These scalar couplings are supported by reasonable dipolar interactions between H-17 and H-5a/H-5b as well as by weak NOEs from H-17 to H-3 and H-14b. The resulting substituted tetracyclic system for compound **7** is shown in Fig. 1. The carboxylic acid methyl ester group and the 1-(hydroxymethyl)-2-propen-1-yl moieties, bound in positions 16 and 15, respectively, were also identified and located by scalar long range heteronuclear couplings. Both configurations of chiral centers in positions 3 and 14 were assumed to be in *S* configuration with respect to those in compound **1** (Patthy-Lukáts et al., 1997). Based on this assumption and the original configuration of the secologanin moiety NOEs indicate position 20 to be in *R* configuration. Assuming a hydrogen bridge mediated interaction between the hydroxyl functionality at position 21 and the carboxylic group at position 22, a conformation with spatial closeness of these two functionalities is very likely preferred. In such conformation H-20 is located spatial close to H-15 and H-14a and hence shows reasonable NOEs. Additionally there is only a very weak dipolar interaction to H-14b. Such interactions are only possible with *R* configuration of position 20. All NMR spectroscopic data of this aglycon moiety were in good agreement with those of an analogue synthesized compound (Brown et al., 2000), which had not been identified as part of a natural product at that time.

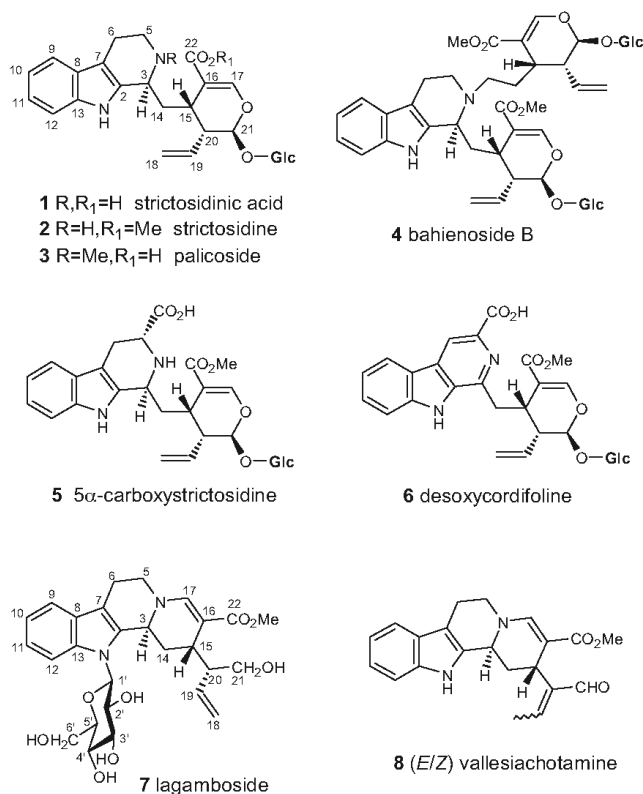


Fig. 1. Structures of various tryptamine-iridoid alkaloids.

Our isolated compound **7**, however, additionally carries a β -D-glucoside bound to the nitrogen atom in position 1. This saccharide moiety was identified by typical large coupling constants between the axial protons. The chemical shifts were influenced by the spatial close aromatic system and were not directly comparable with those of free β -D-Glc. ^1H and ^{13}C NMR shifts as well as coupling constants are in good agreement with those of β -D-glucopyranosyl vincosamide (Henriques et al., 2004). In particular, the similarity of the two anomeric $^3J_{\text{H-H}}$ coupling, 9.1 Hz and 8.9 Hz, respectively, indicate the β -conformation of both glucosides. The ^{13}C chemical shift observed for C-1' in NMR ($\delta\text{C} = 87.6$ ppm) spectra also supports this finding. All spectroscopic data of compound **7** are listed in Table 1. This rare *N*-glycosylation is known so far only from *N*, β -D-glucopyranosyl vincosamide isolated as major component from *Psychotria leiocarpa* Cham. & Schltld. (Henriques et al., 2004). However, the aglycone of compound **7** clearly deviated and was identical to that of glabratine, a 9-O-glycoside from *Uncaria glabrata* DC. (Arbain et al., 1993). The new alkaloid was designated as lagamboside (**7**) referring to the place of collection near the village of La Gamba in Costa Rica.

The co-occurrence of the rare bahienoside B (**4**) and 5 α -carboxystrictosidine (**5**) together with lagamboside (**7**) in *P. acuminata* is of chemotaxonomic interest and let expect a relationship to *P. bahiensis* DC. showing a similar chemical composition (Paul et al., 2003). Regarding the biosynthesis of tryptamine-iridoid alkaloids, also classified as monoterpene indole alkaloids, strictosidine (**2**) is the key intermediate formed by condensation of tryptamine with an iridoid moiety catalyzed by strictosidine synthase. In the next important step strictosidine

(**2**) is deglycosylated by strictosidine- β -glucosidase converting it to a reactive hemiacetal which opens to a dialdehyde intermediate (Gerasimenko et al., 2002; Pasquali et al., 2006; O'Connor and Mares, 2006). In the case of the new lagamboside (**7**) this dialdehyde most likely reacted at C-17 with the secondary amine at position 4 similar to the formation of the less common (*E/Z*) vallesiachotamine (**8**) (Shen et al., 1998; O'Connor and Mares, 2006) which has also been isolated from *P. bahiensis* (Paul et al., 2003). Considering the reorganization of the former *Psychotria* complex inferred from ITS and rbcL sequence data (Nepokroeff et al., 1999), the common trend towards tryptamine-iridoid alkaloid accumulation shown in the literature (e.g. De Santos et al., 2001; Lopes et al., 2004) and in the present findings characterizes species of the subgenus *Heteropsychotria* Steyerf. and *Palicourea* (Taylor, 1997), and has not been detected so far in the subgenus *Psychotria*. This supports a taxonomic splitting and suggests the incorporation of *Psychotria* subgenus *Heteropsychotria* into the genus *Palicourea*.

3. Experimental

3.1. General procedures

HPLC analyses were performed on Agilent 1100 series using a Hypersil BDS-C18 column (250 mm $\times$ 4.6 mm, 5 μm particle size) at a flow rate of 1.0 mL/min. MeOH (A) and an aqueous solution containing 10 mM ammonium acetate (B) with a gradient from 40% to 100% A for 15 min followed by 100% A for 10 min was used as eluent. The injection volume was 10 μL , the UV diode array

Table 1

NMR spectroscopic data of lagamboside (**7**). The indication of nuclei is made in accordance to numbering given in Fig. 1.

Position	δH^a	$^2J_{\text{H-H}}$ (COSY) ^c	Selected NOEs (NOESY) ^c	δC^b	Selected $^2J_{\text{C-H}}$ (HMBC) ^c
2	—	—	—	136.0, s	H-6ab, H-14ab
3	4.93, 1H, m	H-14ab	H-1', H-14b	50.2, d	H-17
5a	3.79, 1H, m	H5b, H6ab	H-17	53.0, t	H-17
5b	3.53, 1H, m	H5a, H6ab	H-17	—	—
6a	2.82, 1H, m	H5ab, H6b	H-5a	23.4, t	n.d.
6b	2.80, 1H, m	H5ab, H6a	H-5b	—	—
7	—	—	—	111.3, s	H-5ab, H-9
8	—	—	—	129.7, s	H-6ab, H-10, H-12
9	7.46, 1H, d, 7.9	H-10	H-10	119.0, d	H-11
10	7.08, 1H, dd, 7.9, 7.4	H-9, H-11	H-9, H-11	121.0, d	H-12
11	7.15, 1H, dd, 7.5, 7.4	H-10, H12	H-10, H12	122.7, d	H-9
12	7.64, 1H, d, 7.5	H-11	H-3, H-11	114.6, d	H-10
13	—	—	—	136.0, s	H-1', H-9, H-11
14a	3.21, 1H, m	H-3, H-14b, H-15	H-15, H-20	35.1, t	H-20
14b	1.43, 1H, ddd	H-3, H-14a, H-15	H-3	—	—
15	3.01, 1H, m	H-14ab, H-20	H-14a, H-20	33.7, d	H-17
16	—	—	—	95.3, s	H-14ab, H-15
17	7.81, 1H, s	—	H-5ab	149.3, d	H-3, H-5ab
18a	5.20, 1H, dd, 16.8, 1.5	H-18b, H-19	H-21ab	116.9, t	H-20
18b	5.13, 1H, dd, 6.7, 1.5	H-18a, H-19	H-19	—	—
19	5.82, 1H, ddd, 16.8, 7.4, 6.7	H-18ab, H-20	H-15, H-18b	140.8, d	H-15, H-21ab
20	2.46, 1H, m	H-15, H-18, H-21ab	H-14a, H-15	55.2, d	H-14
21a	3.70, 1H, m	H-20, H-21b	H18a, H-20	65.4, t	n.d.
21b	3.60, 1H, m	H-20, H-21a	H18a, H-20	—	—
22	—	—	—	171.8, s	Me, H-17
Me	3.69, 3H, s	—	n.d.	51.2, q	n.d.
1'	5.28, 1H, d, 9.1	H-2'	H-3, H-3', H5'	87.6, d	H-3', H-5'
2'	4.53, 1H, dd, 9.1, 8.5	H-1', H-3'	H-1', H-4'	72.4, d	H-4'
3'	3.62, 1H, dd, 9.0, 8.5	H-2', H-4'	H-1', H-5'	79.4, d	H-1', H-5'
4'	3.53, 1H, dd, 8.5, 8.5	H-3', H-5'	H-2', H-6'ab	71.8, d	H-2', H-6ab'
5'	3.52, 1H, m	H-4', H-6ab'	H-1' H-3', H-6'ab	81.2, d	H-1', H-3'
6'a	3.92, 1H, dd, 12.3, 8.5	H-5', H-6b'	H-4', H-5', H-6b'	63.0, t	—
6'b	3.65, 1H, dd, 12.3, 5.5	H-5', H-6a'	H-4', H-5', H-6a'	—	—

n.d., not detected.

^a Chemical shift [ppm], integral, multiplicity, coupling constants [Hz].

^b Chemical shift [ppm], multiplicity.

^c Intensity of cross-peaks is not considered.

signal has been set to 230 nm with a reference wavelength at 360 nm.

For NMR spectroscopy the substances were solved in 99.8% CD₃OD (~3 mg in 0.7 mL) and transferred into 5 mm NMR sample tubes (Promochem, Wesel, Germany). A Bruker DRX-400 AVANCE spectrometer (Bruker, Rheinstetten, Germany) was applied to record the spectra at 400.13 MHz (¹H) or 100.61 MHz (¹³C) using the Topspin 1.2 software. Measurement temperature was 298.1 ± 0.1 K. For 1D spectra 32k data points were recorded and Fourier transformed to spectra with a range of 4800 Hz (¹H) and 20,000 Hz (¹³C), respectively. Common 2D spectra (COSY, TOCSY, NOESY, HSQC, and HMBC) were measured by 256 experiments with 1024 data points each. Linear forward prediction, sinusoidal multiplication, and Fourier transformation led to 2D spectra with a range of 4800 Hz and 22,000 Hz for ¹H and ¹³C, respectively. CHD₂OD was used as internal standard for ¹H (δH: 3.340 ppm) and CD₃OD for ¹³C (δC: 49.00 ppm) spectra. Compounds **4**, **5**, and **6** were identified by combined CSEARCH and SPECINFO databases running under the CSEARCH-program, which were used for spectral similarity searches and prediction of ¹³C NMR spectra (Bremser et al., 1981; Robien, 2009). All spectroscopic data of the identified bahienoside B (**4**) (Paul et al., 2003; Xin et al., 2011), 5α-carboxystrictosidine (**5**) (De Silva et al., 1971; Ferrari et al., 1986), and desoxycordifoline (**6**) (Brown & Warambwa, 1978) are in excellent agreement with the earlier published data.

Mass spectra were recorded on a high resolution time-of-flight (HR-TOF) mass spectrometer (maXis, Bruker Daltonics) by direct infusion electrospray ionization (ESI) in positive ionization mode. TOF MS measurements have been performed within the selected mass range of *m/z* 100–2500. ESI was made by capillary voltage of 4 kV to maintain a (capillary) current between 30 and 50 nA. Nitrogen temperature was maintained at 180 °C using a flow rate of 4.0 L min⁻¹ and the N₂ nebulizer gas pressure at 0.3 bar.

3.2. Plant material

Five provenances of *P. acuminata* were collected in the “Rainforest of the Austrians” near Golfito, SW-Costa Rica, in July 2008. Voucher specimens (HG 240708-02, -14, -15; HG 200708-01, -09), identified as *P. acuminata* by H. Greger, were deposited at the Institute of Botany, University of Vienna (WU) <http://herbarium.univie.ac.at/database/search.php> and Museo Nacional de Costa Rica in San José (CR).

3.3. Extraction and isolation

The leaves were air dried, ground (75 g), and extracted two times for three days each with MeOH at room temp. The combined and filtered crude extract was concentrated under vacuum to give 13.5 g residue, which was partitioned between CHCl₃, EtOAc and H₂O, respectively. 800 mg of the aqueous phase was roughly separated by CC (Merck silica gel 60, 0.2–0.5 mm) with solvent mixtures of PE, EtOAc and MeOH with increasing polarity. The fraction eluted with EtOAc/MeOH (70:30) (190 mg) was further separated with MeOH on Sephadex LH-20 to give 61 mg **4** and 49 mg **6**. 780 mg of the EtOAc-phase was roughly separated by CC (Merck silica gel 60, 0.2–0.5 mm) with solvent mixtures of PE, EtOAc and MeOH with increasing polarity. Fractions eluted with EtOAc were combined (51 mg) and purified with MeOH on Sephadex LH-20 to give 5.2 mg **7**. Air dried and ground stem bark (16.5 g) was extracted with MeOH (1.9 g) and partitioned as described before. 600 mg of the aqueous phase was roughly separated by CC with solvent mixtures of EtOAc and MeOH with increasing polarity. Fractions eluted with EtOAc/MeOH (60:40)

were combined (115 mg) and further separated with MeOH on Sephadex LH-20 to yield 21 mg **5**.

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Chapter 6: *Alstrostines in Rubiaceae: Alstrostine A from Chassalia curviflora var. ophioxyloides and a novel derivative, Rudgeifoline from Rudgea cornifolia*

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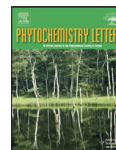
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Alstrostines in Rubiaceae: Alstrostine A from *Chassalia curviflora* var. *ophioxylodes* and a novel derivative, rudgeifoline from *Rudgea cornifolia*

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ABSTRACT

Monoterpenoid indole alkaloids have frequently been isolated from the species-rich *Psychotria* alliance (Rubiaceae), a complex group including several tribes and genera. In our aim of understanding the chemical diversification within this remarkably heterogeneous group, members of two genera of the tribe Palicoureae have been studied. Alstrostine A was isolated from *Chassalia curviflora* var. *ophioxylodes*, and a novel derivative, rudgeifoline from *Rudgea cornifolia*, respectively. Alstrostines, an unusual class of alkaloids comprising one tryptamine and two iridoid units, have recently been discovered in *Alstonia rostrata* (Apocynaceae). The presence of alstrostines in two rubiaceous species is remarkable but not unexpected as both families share similar biosynthetic pathways and are capable of synthesizing related alkaloids.

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1. Introduction

Tryptamine-iridoid alkaloids also classified as monoterpenoid indole alkaloids (MIA), are mainly distributed in two families of the order Gentianales, i.e. in Apocynaceae (dogbane family) and in Rubiaceae (coffee family) (O'Connor and Maresch, 2006). The species-rich *Psychotria* alliance (Rubiaceae), including several genera and well over 2000 species (Nepokroeff et al., 1999; Robbrecht and Manen, 2006), is especially rich in MIA (for example Berger et al., 2012; Lopes et al., 2004; Porto et al., 2009). Within this remarkably heterogeneous group, the two large genera *Palicourea* and *Psychotria* have been subject of extensive phytochemical investigations, whereas smaller or lesser known genera are lacking equal attention. In our ongoing effort of filling those gaps, the phytochemistry of two segregate genera of *Psychotria* has already been surveyed (Berger et al., 2011; Bernhard et al., 2011).

The genera *Chassalia* and *Rudgea* are closely related according to DNA phylogenetic data (Nepokroeff et al., 1999; Robbrecht and Manen, 2006) and are currently included in the tribe Palicoureae (Robbrecht and Manen, 2006; but see Bremer and Eriksson, 2009). The genus *Chassalia* includes more than 110 species with paleotropical distribution (Goevarts et al., 2012) whereas the genus *Rudgea* is neotropically distributed and consists of up to 130

species (Goevarts et al., 2012; Zappi, 2003). Previous studies on the phytochemistry of both genera have been quite limited. In *Rudgea jasmynoides* (Cham.) Müll. Arg. and *R. viburnoides* (Cham.) Benth., anthraquinone, naphthohydroquinone and triterpene constituents have been reported (Lopes et al., 1999; Oliveira et al., 2007; Young et al., 1998). Some species from the genus *Chassalia* are known for their ability to synthesize cyclotides, macrocyclic and cystine knotted peptides (Gruber et al., 2008). Flavonoids have been identified in several Mauritian endemic *Chassalia* species (Soo-brattee et al., 2005). Benzoic acid- and benzoquinone derivatives have additionally been isolated from *C. curviflora* (Wall.) Thwaites (Wang and Zhou, 1999).

MIA are generally regarded as being derived from a strictosidine synthase catalyzed Pictet–Spengler condensation of the amino acid derivative tryptamine and the iridoid secologanin in a 1:1 ratio to yield the first MIA, strictosidine (O'Connor and Maresch, 2006). Alstrostines are MIA which have recently been described from *Alstonia rostrata* (Apocynaceae) and were unknown so far in Rubiaceae (Cai et al., 2011). Their different tryptamine to secologanin ratio of 1:2 and their pyrrolidinoindoline core indicate a divergent biosynthetic route apart from strictosidine. Although a technical reaction scheme was proposed, the biosynthetic steps leading to alstrostines are still unrevealed (Cai et al., 2011).

In our aim of further understanding the chemical diversification within the *Psychotria* alliance, two species of the tribe Palicoureae, *Chassalia curviflora* var. *ophioxylodes* (Wall.) Deb & B. Krishna and *Rudgea cornifolia* (Kunth) Standl., have been studied for their alkaloid constituents.

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2. Results and discussion

2.1. Structure elucidation

Compound **1**, isolated from *C. curviflora* var. *ophioxylodes* had a protonated molecular ion $[M+H]^+$ with m/z 917.3544, determined by HR-ESI-MS in a positive ionization mode. It corresponded to the molecular formula $C_{44}H_{56}N_2O_{19}$ with a calc. m/z 917.3550 for the $[M+H]^+$. An attached proton test ^{13}C NMR spectrum confirmed the presence of 44 carbon atoms with 29 methine and methyl groups as well as 15 methylene groups and quaternary carbon atoms. 2D NMR spectra indicated the presence of a tetracyclic system, which was based on an indole skeleton. A tetrahydro-pyrrolo ring was annulated to this skeleton at later carbon atoms C-2 and C-7, which was indicated from the HMBC by $^3J_{H-C}$ couplings between C-5 and H-2, C-6 and H-2, as well as between C-8 and H-6. Chemical shift and multiplicity of C-7 further indicated a hydroxyl group to be bound to this position. $^2J_{H-C}$ and $^3J_{H-C}$ couplings from C-13 to H-3' and C-3 to H-5ab as well as NOEs between H-12 and H-3' indicated both nitrogen atoms in later positions 1 and 4 to be part of an additional annulated monounsaturated six-membered ring. The double bond located between C-3' and C-14' was identified by the according chemical proton and carbon shifts. This ring derived from C-3' and C-14' of one secologanine moiety bound to N-1 and from C-3 of a second secologanine moiety at N-4. $^2J_{H-C}$ and $^3J_{H-C}$ couplings from H-15 to C-3 and C-14 and from H-15' to C-3' and C-14', respectively, confirmed this finding. Chemical shifts and couplings of all further atoms indicated the presence of the two secologanine moieties with two α,β -unsaturated carboxylic methyl esters. Relative stereochemistry in positions 15, 20, and 21 as well as of the anomeric position in glycosides were determined by those NOEs and $^3J_{H-H}$ coupling constants, which are typically found in structurally related strictosidine. The absolute configuration of strictosidine (Patthy-Lukáts et al., 1997) was taken as basis. The resulting structure shown in Fig. 1 was identical to those of the recently described alstrostine A (Cai et al., 2011). A list of all NMR spectroscopic data is given in Table 1 and selected indicative long range couplings as well as an NOE are shown in Fig. 1.

Compound **2**, isolated from *R. cornifolia* had a deprotonated molecular ion $[M-H]^-$ with m/z 901.3326, determined by HR-ESI-MS in a negative ionization mode. It corresponded to the molecular formula $C_{43}H_{54}N_2O_{19}$ with a calc. m/z 901.3248 for $[M-H]^-$. NMR spectra indicated the structure to be very similar to those of alstrostine A. The lack of the methyl group at the carboxyl group in position 22 and a distinct up-field shift of H-17 indicated the

presence of a free carboxylic group at position 22. However, the protons of the methyl group in ester position 22' still showed the long range coupling to C-22', which indicated this carboxylic group to be methylated. This new compound of the alstrostine family is named rudgeifoline with respect to its source, a species of the genus *Rudgea*. The structure is shown in Fig. 1 and a list of the NMR spectroscopic data is given in Table 1.

2.2. Chemosystematic significance

Alkaloids with different tryptamine to secologanine ratios have previously been reported from the *Psychotria* alliance: Pyrrolidinoindolines can be defined by a 1:0 ratio (Gueritte-Voegelein et al., 1992; Hart et al., 1974; Takayama et al., 2004), strictosidine and related compounds have a 1:1 ratio (Lopes et al., 2004; Porto et al., 2009), and bahienosides show a 1:2 ratio (Paul et al., 2003). The latter compounds with a 1:2 tryptamine to secologanine ratio are not uncommon within the Rubiaceae: bahienosides, neonaucleosides, and hirsutaside D, for example isolated from *Psychotria bahiensis* DC. *Neonauclea sessilifolia* (Roxb.) Merr., and *Uncaria hirsuta* Havil., respectively, comprise a tricyclic skeleton. The bahienosides consist of the typical 2,3,4,9-tetrahydro-1H-pyrido[3,4-*b*]indole skeleton, also present in strictosidine, and carry two secologanine moieties bound to positions 3 and 4, respectively (Paul et al., 2003). Neonaucleoside A and its epimer regarding position 3 are identical with the bahienosides A and B. Neonaucleoside C has a 9H-pyrido[3,4-*b*]indole skeleton, also present in lyalosidic acid. It carries one secologanine moiety linked to position 3, while a second secologanine bound to position 6 comprises a newly formed lactone ring between the carboxylic function and the alcohol in position 7'' (Itoh et al., 2003). The recently described hirsutaside D is structurally related to neonaucleoside C, but does not form the lactone ring due to lack of the hydroxyl function in position 7'' (Xin et al., 2011). However, alstrostines differ distinctly, as no tetracyclic ring system is formed in all these compounds.

The presence of alstrostines in the two rubiaceaceous species *C. curviflora* and *R. cornifolia* is notable, because the ability to form its unusual tetracyclic MIA alkaloid structure was previously known only from *A. rostrata* (Apocynaceae), where these compounds have been discovered. Nevertheless, the joint occurrence of a new group of alkaloids in Apocynaceae and Rubiaceae is not unexpected. Both families generally share similar biosynthetic pathways and are capable of synthesizing related alkaloids (O'Connor and Maresh, 2006).

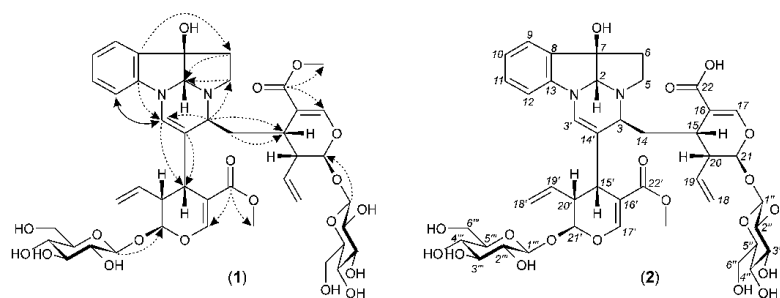


Fig. 1. Structures of alstrostine A (**1**) isolated from *C. curviflora* var. *ophioxylodes* and of the new alstrostine derivative rudgeifoline (**2**) extracted from *R. cornifolia*. Numbering is indicated for compound **2**, while in compound **1** a couple of selected, indicative long range C–H couplings (dotted arrows, showing from carbon to proton) and NOEs (solid double arrows) indicate the NMR based correlations found in both compounds.

Table 1

NMR chemical data of alstroisine A (1) and rudgeifoline (2). ¹H NMR chemical shifts [ppm] are amended with integral, multiplicity, and detectable coupling constants [Hz]. For ¹³C NMR chemical shifts [ppm] and the multiplicities are given. Indications of the positions were made in accordance to Fig. 1.

Position	Alstroisine A (1)		Rudgeifoline (2)	
	δ ¹ H	δ ¹³ C	δ ¹ H	δ ¹³ C
2	4.88 (1H, s)	81.6, d	4.89 (1H, s)	81.2, d
3	3.30 (1H, m)	56.0, d	3.27 (1H, m)	55.7, d
5	2.81 (1H, m)	50.8, t	2.83 (1H, m)	50.7, t
	2.58 (1H, m)		2.61 (1H, m)	
6	2.27 (1H, m)	43.4, t	2.27 (1H, m)	43.5, t
	1.96 (1H, m)		1.97 (1H, m)	
7	–	89.0, s	–	89.5, s
8	–	135.6, s	–	135.6, s
9	7.24 (1H, d, 6.9)	124.8, d	7.22 (1H, d, 7.0)	124.6, d
10	6.78 (1H, dd, 6.9, 6.9)	121.2, d	6.76 (1H, dd, 7.0, 7.0)	120.7, d
11	7.11 (1H, dd, 6.9, 6.6)	130.6, d	7.09 (1H, dd, 7.0, 6.8)	130.1, d
12	6.65 (1H, d, 6.6)	108.4, d	6.62 (1H, dd, 6.8)	107.9, d
13	–	149.3, s	–	149.3, s
14	2.05 (1H, m)	32.9, t	2.05 (1H, m)	32.9, t
	1.97 (1H, m)		1.88 (1H, m)	
15	3.30 (1H, m)	28.6, d	3.34 (1H, m)	27.1, d
16	–	112.4, s	–	n.d.
17	7.43 (1H, d, 1.5)	152.8, d	6.87 (1H, s)	151.9, d
18	5.31 (1H, dd, 17.0, 1.5)	119.9, t	5.27 (1H, dd, 16.0, 1.5)	119.3, t
	5.27 (1H, dd, 9.9, 1.5)		5.24 (1H, dd, 10.2, 1.5)	
19	5.80 (1H, ddd, 17.0, 9.9, 9.7)	136.0, d	5.83 (1H, ddd, 16.0, 10.2, 10.1)	136.2, d
20	2.71 (1H, m)	45.3, d	2.69 (1H, m)	44.7, d
21	5.54 (1H, d, 4.4)	98.4, d	5.32 (1H, d, 2.8)	97.5, d
22	–	169.4, s	–	n.d.
22-O-CH ₃	3.69 (3H, s)	51.7, q	–	–
3'	6.29 (1H, s)	126.0, d	6.22 (1H, s)	125.6, d
14'	–	115.0, s	–	n.d.
15'	3.26 (1H, m)	37.6, d	3.29 (1H, m)	37.2, d
16'	–	110.5, s	–	110.5, s
17'	7.57 (1H, d, 1.0)	153.8, d	7.53 (1H, d, 1.0)	152.6, d
18'	5.16 (1H, dd, 17.0, 1.5)	118.9, t	5.14 (1H, dd, 18.0, 1.5)	118.6, t
	5.11 (1H, dd, 9.6, 1.5)		5.10 (1H, dd, 9.9, 1.5)	
19'	5.64 (1H, ddd, 17.0, 9.6, 9.5)	136.8, d	5.64 (1H, ddd, 18.0, 9.9, 9.6)	136.6, d
20'	2.52 (1H, m)	47.0, d	2.51 (1H, m)	46.9, d
21'	5.47 (1H, d, 8.1)	97.9, d	5.47 (1H, d, 8.1)	97.6, d
22'	–	168.9, s	–	168.7, s
22-O-CH ₃	3.65 (3H, s)	51.7, q	3.65 (3H, s)	51.7, q
1''	4.67 (1H, d, 7.9)	100.0, d	4.64 (1H, d, 7.9)	99.2, d
2''	3.22 (1H, dd, 8.0, 7.9)	74.6, d	3.28 (1H, dd, 8.0, 7.9)	74.3, d
3''	3.37 (1H, m)	78.0, d	3.36 (1H, m)	77.5, d
4''	3.30 (1H, m)	71.4, d	3.30 (1H, m)	71.3, d
5''	3.31 (1H, m)	78.4, d	3.32 (1H, m)	78.4, d
6''	3.89 (1H, dd, 12.2, 1.9)	62.7, t	3.89 (1H, m)	62.5, t
	3.67 (1H, m)		3.67 (1H, m)	
1'''	4.62 (1H, d, 7.9)	100.9, d	4.63 (1H, d, 7.9)	100.7, d
2'''	3.15 (1H, dd, 7.9, 7.9)	74.5, d	3.17 (1H, dd, 7.9, 7.9)	74.5, d
3'''	3.36 (1H, m)	78.1, d	3.33 (1H, m)	78.1, d
4'''	3.34 (1H, m)	70.9, d	3.34 (1H, m)	70.7, d
5'''	3.07 (1H, ddd, 9.5, 4.3, 2.4)	77.9, d	3.08 (1H, ddd, 9.3, 4.4, 2.0)	77.7, d
6'''	3.47 (1H, dd, 12.5, 4.3)	61.7, t	3.48 (1H, dd, 12.5, 4.3)	61.6, t
	3.24 (1H, dd, 12.5, 9.5)		3.25 (1H, m)	

n.d.: not determined due to low signal to noise ratio.

3. Conclusion

Alstroisines contain a pyrrolidinoindoline core linked with two secologanin moieties in a tetracyclic system. They are structurally related to pyrrolidinoindoline alkaloids bare of an iridoid skeleton. Strictosidine and bahienosides with a 1:1 and 1:2 tryptamine to secologanin ratio, respectively, differ in the structure of the tricyclic core. Within *Psychotria*, little is known concerning the phylogenetic relationships of species assigned to these different groups of alkaloids. However the genera *Chassalia* and *Rudgea* are closely related which is underlined by the occurrence of rare alstroisine derivatives, previously unknown in rubiaceae species. Together with *Carapichea*, *Geophila*, *Margaritopsis*, and *Notopleura*, these genera form a clade showing a sister group relationship to *Psychotria* subg. *Heteropsychotria* and *Palicourea*, respectively (Nepokroeff et al., 1999; Robbrecht and Manen, 2006). While

species of *Psychotria* subg. *Heteropsychotria* and *Palicourea* are largely characterized by accumulation of strictosidine type alkaloids (Lopes et al., 2004; Porto et al., 2009), little is known concerning the other genera within the tribe Palicoureeae. Considering the report of dopamine derived isoquinoline alkaloids in *Carapichea* (Bernhard et al., 2011), the present work on *Chassalia* and *Rudgea* hypothesizes that segregate genera of *Psychotria* are chemically distinct and a source of new and unexpected compounds.

4. Experimental

4.1. General experimental procedures

HPLC analyses were performed on Agilent 1100 series employing a Hypersil BDS-C18 column (250 mm × 4.6 mm, 5 μm particle

size) with methanol (MeOH) (B) and an aqueous buffer containing 10 mM ammonium acetate (A) from 40% B till 100% B in 15 min, 100% B was kept for 10 min. The UV diode array signal was set to 230 nm with a reference wavelength at 360 nm. The injection volume was 10 μ L and the flow rate 1.0 mL/min. TLC analyses was done on silica gel 60 F254 plates, thickness 0.2 mm (Merck) developed with CHCl₃/MeOH/H₂O 80:20:1. Silica gel 60 plates with 0.5 mm thickness were used for preparative TLC. The stationary phases for CC and MPLC were either Sephadex LH20 (GE Healthcare) or silica gel 60 (Merck) with 0.2–0.5 mm or 40–63 μ m for the MPLC column, respectively. Specord 205 UV-Photometer (Analytik Jena) was employed for working out the molar extinction coefficient.

4.2. NMR spectroscopy

For NMR spectroscopy each compound was dissolved in methanol-*d*₄ (~3.0 mg in 0.7 mL) and transferred into 5 mm high precision NMR sample tubes. Residual CH₃OD was used as internal standard for ¹H (δ_{H} 3.34) and CD₃OD for ¹³C (δ_{C} 49.0) measurements. All spectra were measured on a Bruker DRX-600 at 600.13 MHz (¹H) or 150.61 MHz (¹³C) and performed using the Topspin 1.3 software. Measurement temperature was 298 K $\pm$ 0.05 K. 1D spectra were recorded by acquisition of 32k data points and after zero filling to 64k data points and Fourier transformation spectra were performed with a range of 7200 Hz (¹H) and 32,000 Hz (¹³C), respectively. To determine the 2D COSY, TOCSY, NOESY, HMQC, and HMBC spectra 256 experiments with 2048 data points each were recorded and Fourier transformed to 2D-spectra with a range of 6000 Hz and 32,000 Hz for ¹H and ¹³C, respectively.

4.3. Mass spectrometry

Mass spectra were recorded on a high resolution time-of-flight (HR-TOF) mass spectrometer (maXis, Bruker Daltonics) by direct infusion electrospray ionization (ESI) in positive ionization mode (mass accuracy $\pm$ 5 ppm) as well as in negative mode (mass accuracy $\pm$ 10 ppm). TOF MS measurements have been performed within the selected mass range of *m/z* 100–2500. ESI was made by capillary voltage of 4 kV to maintain a (capillary) current between 30–50 nA. Nitrogen temperature was maintained at 180 °C using a flow rate of 4.0 L min⁻¹ and the N₂ nebulizer gas pressure at 0.3 bar.

4.4. Plant material

Two accessions of *C. curviflora* (Wall.) Thwaites var. *ophioxylodes* (Wall.) Deb. & B. Krishna were collected: HG 1073 near the Trong Nong Waterfall in the Nam Tok Pliu Nationalpark, Chanthaburi province, Thailand and AB 3107111 near Pong Duet Hot Springs, Chiang Mai province, Thailand. *R. cornifolia* (Kunth) Standl. (AB 21021001) was collected in 'Regenwald der Österreicher' near La Gamba, Piedras Blancas National Park, Puntarenas province, Costa Rica. Species were identified by one of the authors, voucher specimens have been deposited at the Herbarium of the University of Vienna (WU). Database accession numbers are WU-0063824 (*R. cornifolia*, Dupl. in MO) and WU-0045614, 0045615 and 0045616 as well as WU-0064562 and 0064563 (*C. curviflora* var. *ophioxylodes*), respectively. Images of digitized specimens are accessible under their corresponding numbers at <http://herbarium.univie.ac.at/database/search.php>.

4.5. Extraction and isolation

C. curviflora var. *ophioxylodes* – small quantities of powdered leaves of both accessions were extracted with MeOH and used for

HPLC-UV comparison. Both chromatograms were found almost identical. For isolation, air dried leaves (19 g) of *C. curviflora* var. *ophioxylodes* accession HG 1073 were powdered and extracted with MeOH (500 mL, 3 $\times$ 2 d), filtered and concentrated under reduced pressure. The remaining viscous extract was suspended in H₂O and extracted subsequently with CHCl₃, EtOAc and *n*-BuOH. The EtOAc and BuOH phases were pooled (450 mg) and subjected to CC with silica gel 60 (0.2–0.5 mm) as stationary phase using a mobile phase of various mixtures of petrol ether, EtOAc and MeOH with increasing polarity. Fractions containing compound **1** were combined (40 mg) and further purified by CC over Sephadex LH20 with MeOH as eluent. Final purification by preparative TLC (CHCl₃/EtOAc/MeOH 50:30:20) afforded 6.4 mg of **1**. Alstroline A (**1**): λ nm (log ϵ): 212 (4.68), 234 (4.49), 278 (4.32) and 314 (sh) (4.21) in MeOH.

R. cornifolia – for isolation, air dried leaves (21 g) of *R. cornifolia* were extracted (1.3 g) and partitioned as mentioned above. The *n*-BuOH phase (160 mg) was subjected to column chromatography (CC) using Sephadex LH20 as stationary phase eluted with MeOH. Fractions containing compound **2** were combined according to their HPLC profiles (50 mg) and further purified by medium pressure chromatography (MPLC) over a silica gel 60 column with MeOH as an eluent to give 3.5 mg of pure **2**. Rudgeifoline (**2**): λ nm (log ϵ): 210 (4.4), 230 (4.22), 278 (4.03) and 314 (sh) (3.92) in MeOH.

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Chapter 7: Ecological relevance of *Stemona*- and Tryptamine-Iridoid-Alkaloids

7.1. *Stemona* Alkaloids

Various feeding experiments, performed according to Kubo (1991) and Srivastava and Proksch (1991), of isolated *Stemona* alkaloids revealed either toxic or repellant effects against herbivorous insect pests. Especially the [1,2- α]-pyrroloazepine type alkaloids Stemofoline (**20**), which occur in various *Stemona* species (Irie et al., 1970; Kaltenegger, 2003; Schinnerl et al., 2007), and 1,2-Didehydrostemofoline (**21**) (Fig. 15), occurring in *S. collinsiae* (Brem et al., 2002; Jiwajinda et al., 2001; Seger et al., 2004), showed a high toxicity and also a high growth inhibition rate against neonate larvae of *Spodoptera littoralis* Boisduval (Egyptian cotton leafworm) (Brem et al., 2002; Kaltenegger et al., 2003; Kongkiatpaiboon et al., 2013a). Tang et al. (2008) postulated that **20** and some of its derivatives act as neurotoxins. Both compounds act as agonists for nicotinic acetylcholine receptors and, thus, cause the immediate death of the insects. This mode of action has been assessed in *in vitro* experiments on isolated neurons from the moth *Heliothis virescens* (tobacco budworm) (Tang et al., 2008). Furthermore, Sastraruji et al. (2010) and Kongkiatpaiboon et al. (2013b) described an inhibitory effect on acetylcholine-esterase of **20** and **21**. The more polar derivative 2'-Hydroxystemofoline (**22**) (Fig. 15) showed less toxicity against *S. littoralis* (Kaltenegger et al., 2003). This result indicates that the toxicity of these compounds depends on the polarity of these substances. Kaltenegger (2003) and Tang et al. (2008) ascribed the toxic level to the cageous core structure of these molecules. In fact, both structural characters, the core structure as well as the substitution pattern in the butyl side chain (Fig. 15), are involved in their properties against insects.

The [1,2- α]pyridoazepine type alkaloids Oxystemokerrine (**23**) and Stemocurtisinol (**24**) (Fig. 15), occurring in *S. kerrii* and *S. curtisii* (Kaltenegger et al., 2003; Schinnerl et al., 2007), caused a much lower mortality and also a lower growth inhibition rate than the [1,2- α]pyrroloazepines against *S. littoralis* (Kaltenegger et al., 2003; Kongkiatpaiboon et al., 2013a). The effects of **23** and **24** are comparable to that of Tuberostemonine (**25**) and its derivative Neotuberostemonine (**26**) (Fig. 15). Both **25** and **26** occur in *S. tuberosa* and *S. phyllantha* (Greger, 2006; Jiang et al., 2006; Schinnerl et al., 2007). Both types of alkaloids

are weakening the insect larvae and promote paralysis without acute toxicity (Brem et al., 2002; Kaltenecker et al., 2003; Kongkiatpaiboon et al., 2013a). In conclusion of the mentioned results, Stemofoline and its derivatives are effective natural insecticides, whereas pyrido[1,2- α]azepine- and Tuberostemonine-derivatives show repellent effects against *S. littoralis* (Brem et al., 2002; Kaltenecker et al., 2003).

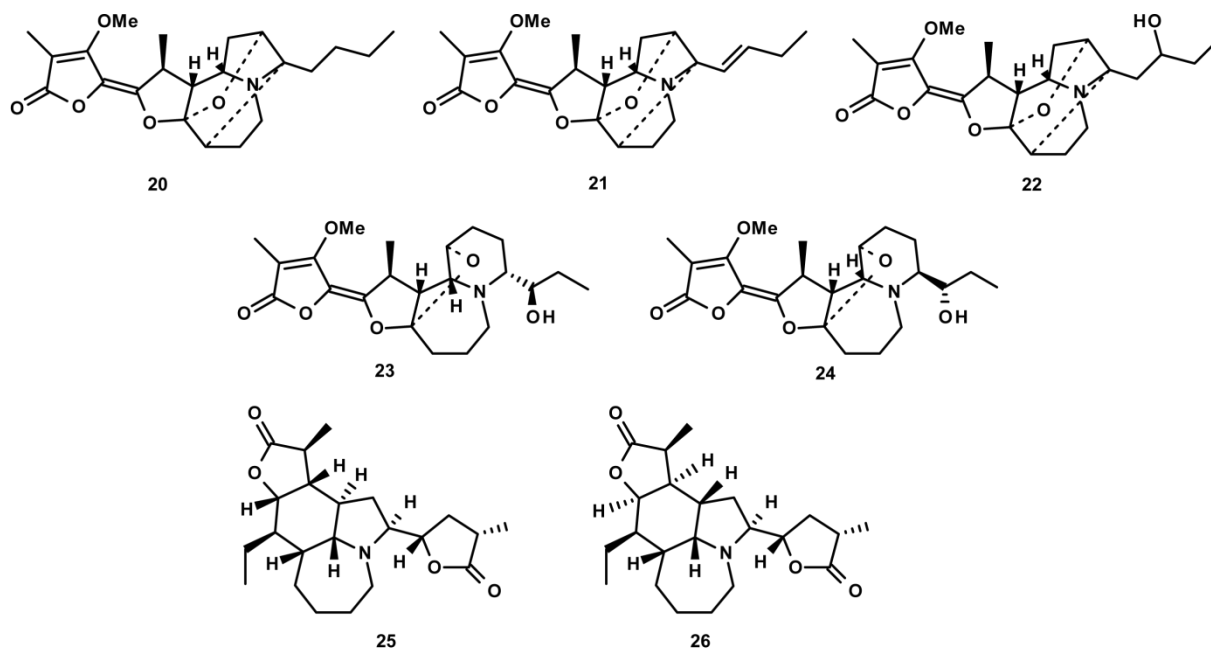


Fig. 15. Chemical structures of Stemofoline-, Oxystemokerrine- and Tuberostemonine-derivatives. Stemofoline (**20**), 1,2-Didehydrostemofoline (**21**), 2'-Hydroxystemofoline (**22**), Oxystemokerrine (**23**), Stemocurtisinol (**24**), Tuberostemonine (**25**) and Neotuberostemonine (**26**).

The role of the occasionally occurring *N*-oxide derivatives (Fig. 16) is still unknown. They were isolated from different parts of various *Stemona* species. The pyrido[1,2- α]azepine-*N*-oxide derivatives Oxystemokerrine-*N*-oxide (**27**) and Methoxystemokerrine-*N*-oxide (**28**) were isolated from roots of *S. kerrii* Craib (Kaltenecker et al., 2003), the pyrrolo[1,2- α]azepines Stemaphylline-*N*-oxide (**29**) from the roots of *S. aphylla* Craib (Mungkornasawakul et al., 2009) and 1,2-Didehydrostemofoline-*N*-oxide (**30**) was obtained from the roots of an unidentified *Stemona* species (Sastraruji et al., 2005) and also isolated from the leaves of *S. collinsiae* (Schinnerl, unpubl.). The Tuberostemonine derivative *N*-Oxytuberostemonine (**31**) was isolated from *S. tuberosa* (Lin and Fu, 1999). These charged derivatives are much better soluble in aqueous systems than the non-oxygenated alkaloids

and hence perfectly modified for translocation within the different organs of the plant. The transfer into cells and/or vacuoles might be performed similarly to the incorporation of charged pyrrolizidine alkaloid *N*-oxides like Senecionine-*N*-oxide (**30**) (Fig. 16) (Ehmke et al., 1988). Sequestration tests of the labeled pyrrolizidine alkaloid Senecionine and its *N*-oxide on the moth *Cretonotos transiens* (Lepidoptera, Arctiidae) and the grasshopper *Zonocerus variegatus* (Orthoptera, Pyrgomorphidae) revealed, that both insect species modify Senecionine by the enzyme Monooxygenase and store it as *N*-oxide derivative. This is in contrast to the non-sequestering insect *S. littoralis*. Larvae of this species defecated Senecionine and its *N*-oxide with high efficacy (Lindigkeit et al., 1997). The authors of this study assume that the *N*-oxide derivative will be reduced in the gut and the lipophilic alkaloid will be passively absorbed in the haemolymph and subsequently defecated. Comparable studies with *Stemona* alkaloids have not been performed yet.

However, the occurrence of toxic and repellent alkaloids in *Stemona* species together with the seasonal growth period of these species in monsoon influenced subtropical areas protect these plants from excessive herbivory by insects.

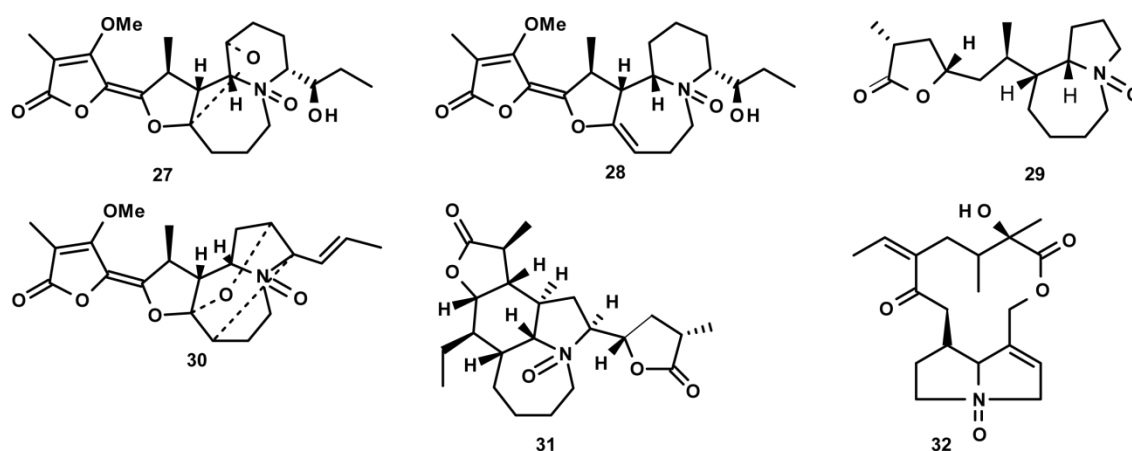


Fig. 16. Chemical structures of *Stemona* Alkaloid-*N*-oxides. Oxystemokerrine-*N*-oxide (**27**), Methoxystemokerrine-*N*-oxide (**28**), Stemaphylline-*N*-oxide (**29**), 1,2-Didehydrostemofoline-*N*-oxide (**30**), *N*-oxytuberostemonine (**31**) and Senecionine-*N*-oxide (**32**).

7.2. Tryptamine-Iridoid Alkaloids and Iridoids

Feeding experiments on neonate larvae from *S. littoralis* with pure Strictosidine (**33**) and Alstroline A (**34**) (Fig. 17), isolated from *Psychotria elata* (Fasshuber, 2011) and

Chassalia curviflora var. *ophioxyloides* (Schinnerl et al., 2012), respectively, did not show any exceed mortality (Berger, 2013; Kainradl, 2013). This is in accordance with the results from Luijendijk et al. (1996) and Matsuura and Fett-Neto (2013). A tentative leaf disc choice test performed according to Wada and Munakata (1968), carried out on 2nd instar larvae of *S. littoralis* of **33**, revealed a movement disorder after consumption but no mortality. In another study (Cardoso et al. 2004), this compound did not show any inhibition of acetylcholine-esterase. The bioactive effect of tryptamine-iridoid alkaloids against insects might be strongly connected to the glycoside moiety attached to the Secologanin unit. The removal of the sugar moiety by a glucosidase induces several unspecific conformation changes leading to various aglyca (Fasshuber, 2011) which might have anti-insecticidal properties either in plant material or in the digestive tract of larvae.

The benefits of alkaloids for plants, except as antiherbivoral agents, are still insufficiently studied. Paranhos et al. (2009) demonstrated that the tryptamine-iridoid alkaloid Psychollatine (**36**) (Fig. 17), isolated from *Psychotria umbellata* Thonn., plays a role in quenching oxygen radicals and might therefore be enriched in leaves. This radical scavenging effect has also been assessed for the more hydrophilic Psychollatine derivative Brachycerine (**34**) (do Nascimento et al., 2007) and *N*, β -D-glucopyranosyl Vincosamide (**35**) from *Psychotria leiocarpa* (Matsuura and Fett-Neto, 2013). Furthermore, the biosynthesis of **34** is induced by UV radiation (Gregianini et al., 2004), which is an important fact in tropical regions during bright sunlight. Studies on the content of **34** during the development of *Psychotria brachyceras* seedlings showed an increased amount of this alkaloid. These experiments also proved that its production is influenced strongly by Jasmonic acid but not by Salicylic acid (Gregianini et al., 2004). The function as radical scavenger may also be true for other tryptamine-iridoid alkaloids because of their structural similarities with a Secologanin- instead of a Loganin-moiety. However, tentative DPPH assays from crude methanolic extracts of various rubicaeous species, which contain tryptamine-secologanin alkaloid derivatives, revealed only weak activities in contrast to that of Catechin containing taxa (Berger, unpubl.). This radical scavenging effect of **36** and **37** is in particular remarkable because functional structural features, which have been described for flavonoids (Williams et al., 2004), are absent.

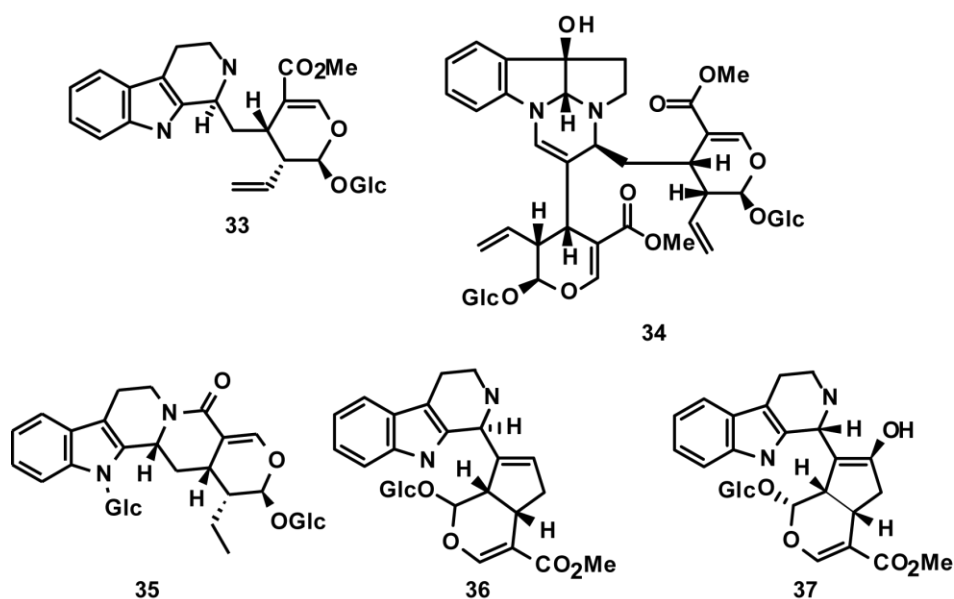


Fig. 17. Chemical structures of Tryptamine-Iridoid Alkaloids. Stictosidine (**33**), Alstroline A (**34**), *N*, β -D-glucopyranosyl vincosamide (**35**), Psychollatine (**36**) and Brachycerin (**37**).

Iridoids are highly reactive secondary metabolites possessing many biological and pharmaceutical properties (Ghisalberti, 1998). Maguro et al. (2011) reported some insecticidal activities from iridoids occurring in *Ajuga* (Lamiaceae) against the 2nd instar larvae from *Aedes aegypti* (the mosquito vector of dengue). Pungitori et al. (2004) revealed the allelochemical and as well as the inhibitory effect of Catalpol (**38**) (Fig. 18) and some of its derivatives on DNA polymerase. The bioactive potential of these compounds might be strongly linked to the formation of either pyridine monoterpene alkaloids under acid conditions or via β -glucosidase, in presence of Ammonium (Frederiksen and Stermitz, 1996). Hattori et al. (1990) and Kawata et al. (1991) reported the transformation of Aucubin (**39**) and Geniposide (**40**), respectively, to such alkaloids (**41** and **42**) by human intestinal bacteria (Fig. 18). Geniposide (**40**) also plays a role in the formation of pigments, during the drying process of plant material from *Gardenia* species (Lee et al., 1998).

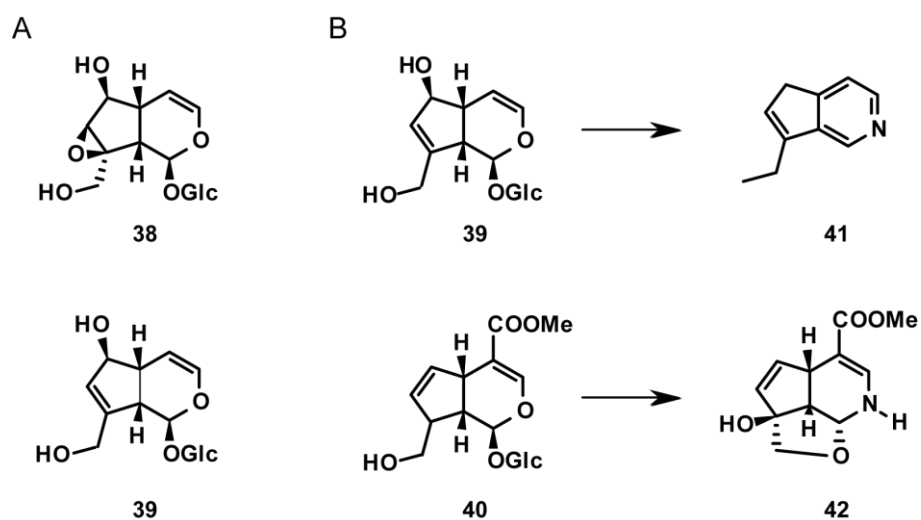


Fig. 18. Chemical structures of bioactive Iridoids (A) and the transformation into pyridine monoterpene alkaloids by bacteria (B). Modified from Ghisalberti (1998). Catalpol (**38**), Aucubin (**39**), Geniposide (**40**), Aucubinine A (**41**) (Hattori et al., 1991) and Gardenine (**42**) (Kawata et al., 1991).

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Conclusion and Outlook

This thesis was set up to challenge the hypothesis of concerted evolution of SM with other characters in the investigated plant families. The results show, that certain correlations between current phylogenies (Keeratinijakal, 2005; Bremer and Manen, 2000) and the investigated class of compounds exist, albeit with some constraints. It is concluded that some specific SM merit the status of chemical characters. This is exemplified by the restricted occurrence of the highly insecticidal alkaloid 1,2-Didehydrostemofoline and Tuberostemonine A in *Stemona collinsiae* and *S. phyllantha*, respectively (Kongkiatpaiboon et al., 2011). Within genera closely related to *Psychotria*, it can be stated that the presence or absence of Strictosidine or its derivatives correlates well with the current phylogeny (Berger, 2013).

The function of SMs is independent of their role as chemical characters. On the one hand, the biological function of most of the compounds is still unrevealed, and on the other hand, bioassays are sometimes a bit arbitrary in the chosen test organisms. Hence, the results do not always explain the functional significance of the tested SM for the plants.

Concluding the results of this work, the studied plant species and provenances showed a distinct pattern of SMs, based on their relationship and biogenetic capacities which is a result of the evolutionary history. Specific SM proved to have the potential of being chemical markers. In order to avoid misleading interpretations, character stability must be checked across the same taxonomic level. Homologous plant parts should be used at comparable ontogenetic stage of development. As the latter could be considered in parts only, extended studies are suggested in future work.

Further work should be done within Stemonaceae and also within Pandanaceae species to reveal the biosynthetic origin of the exceptional *Stemona* alkaloids. Some species of Stemonaceae are still lacking phytochemical investigations. This work should be combined with molecular phylogenetic analysis and also with studies on the proteomics level to elucidate the biogenesis of these special alkaloids. Within the *Psychotria* alliance there are many species and genera lacking phytochemical studies. Due to the fact of both, species richness and abundance in tropical rainforests, phytochemical studies in combination with ecological investigations would improve our understanding of the ecological importance of

SMs for plants. In general, these data can bring valuable contributions to improve the systematic classifications and, furthermore, improve our knowledge of the biological function of phytochemical diversification.

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List of Publications

Karoline Peter, Johann Schinnerl, Susanne Felsinger, Lothar Brecker, Rudolf Bauer, Heimo Breiteneder, Riurong Xu, Yan Ma. A novel concept for detoxification: Complexation between aconitine and liquiritin in a Chinese herbal formula ('Sini Tang'). *Journal of Ethnopharmacology* (2013), 149, 562-569.

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