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Pyrrolo- and pyridoazepine alkaloids as chemical markers in *Stemona* species

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Abstract

Broad-based phytochemical investigations on 31 *Stemona* species and geographical provenances led to an overview concerning characteristic accumulation trends and the distribution of different *Stemona* alkaloids. Two major metabolic differences suggested a taxonomic segregation of the complex *Stemona tuberosa* group from the other species, and was supported by morphological characters. Whereas most of the *Stemona* species were characterised by protostemonine type alkaloids, the *S. tuberosa* group clearly deviated by accumulation trends towards tuberostemonine or croomine derived alkaloids belonging to two different skeletal types. Also of chemotaxonomic relevance was the structural divergence of protostemonine type alkaloids into pyrrolo- or pyridoazepine derivatives represented by stemofoline or oxystemokerrine, respectively, as major constituents. Their common occurrence in different provenances of *S. curtisii*, also deviating from the other species by various chromosome numbers, deserves special taxonomic attention. Species specific chemical markers were given by the unique accumulation of didehydrostemofoline (=asparagamine A) in *S. collinsae* and stemokerrine in *S. kerrii*. In contrast to previous reports, no bisdehydro derivatives with an aromatic pyrrole ring were detected supporting the hypothesis that these alkaloids are artifacts. A new stereoisomer of tuberostemonine was isolated and identified by spectroscopic methods.
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1. Introduction

Stemona alkaloids represent a typical chemical character of the small monocotyledonous family Stemonaceae, and so far are not detected in any other plant family. They are characterized by a pyrrolo[1,2-*a*]azepine core usually linked with two carbon chains mostly forming terminal lactone rings. Due to their structural complexity and instability almost all of the structures could only be determined by X-ray crystallographic analysis before 1980. Up to now about 100 derivatives have been described, mainly isolated from the tuberous roots of various species of the genus *Stemona*. By contrast, only four alkaloids are known so far from the two other genera of the family, *Croomia* and *Stichoneuron* (see Pilli et al., 2005; Greger, 2006; for new structures not cited herein Sastraruji et al., 2005, 2006; Jiang et al., 2006b; Lin et al., 2006). Based on our biosynthetic hypothesis and their various distribution in different species they were recently classified into three skeletal types: the stichoneurine-, protostemonine-, and croomine-type alkaloids. Considering the available data it became obvious that *Stemona* species fall into two groups, differentiated by the predominant accumulation of either protostemonine or stichoneurine derivatives, whereas alkaloids derived from croomine were found in both groups. However, as

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already pointed out in a recent review (Greger, 2006), there are some inconsistencies caused by not properly identified plant species. Although root extracts of *Stemona tuberosa* Lour., *S. japonica* (Bl.) Miq., and *S. sessilifolia* (Miq.) Miq. have long been used in traditional Chinese and Japanese medicine for the treatment of respiratory diseases and to prevent human and cattle parasites (Jiangsu New Medical College, 1977; Xu, 2000), the uncertainty of purchasing the proper plant material has already led to far-reaching confusions in the chemical and pharmaceutical literature. In addition, a remarkable variation of alkaloid profiles was observed in different geographical provenances of the two major species *S. tuberosa* (Schinnerl et al., 2005; Jiang et al., 2006b) and *S. curtisii* Hook. f. (Kaltenegger et al., 2003; Mungkornasawakul et al., 2004a), making chemotaxonomic conclusions within the genus rather difficult.

Based on properly identified and documented plant material a broad-based phytochemical investigation of various *Stemona* species was carried out in our laboratory to demonstrate their different biogenetic capacities. In this connection, alkaloid profiles were also compared from different geographical provenances of a species, as well as from different plant tissues. Since the correct taxonomic assignment of *Stemona* species in the literature is still encountering considerable difficulties, colour photographs are presented from eight species investigated to facilitate their recognition (Fig. 1). The present study reports on the distribution of characteristic *Stemona* alkaloids on the basis of UV-HPLC and TLC comparison, and attempts to determine species-specific accumulation trends, as well as structural affinities of the major alkaloids in order to contribute to a more natural grouping of species within the genus *Stemona*. The results from this study should also serve as a guide for exploiting these plants and their chemicals for further pharmaceutical development, pest control, and herbal uses. In addition, detailed NMR experiments were described for a new isomer of tuberostemonine to clarify its complicated stereochemistry.

2. Results and discussion

2.1. Distribution and structural affinities

Methanolic extracts from various *Stemona* species and geographical provenances were concentrated and partitioned between water and chloroform. The lipophilic fractions were shown to contain most of the alkaloids and were used for different chromatographic comparisons. Due to the lack of a characteristic chromophoric system stichoneurine- and croomine-type alkaloids were detected with Dragendorff's reagent on TLC (Fig. 1) (Schinnerl et al., 2005), whereas most of the protostemonine-type derivatives could be compared by UV-HPLC due to the presence of a conjugated dienone chromophore (Figs. 4–6). Major compounds were isolated by preparative MPLC and TLC, and identified by spectroscopic methods as described previously (Brem et al., 2002; Kaltenegger et al., 2003; Schinnerl et al., 2005), whereas minor derivatives, detected only in small amounts, were identified by co-chromatography with authentic samples.

Extracts from eight different collections (A–H in Fig. 1) were exclusively characterised by alkaloids lacking a typical chromophoric system. All were shown to be members of the taxonomically rather complex *S. tuberosa* group, most likely consisting of different species and/or varieties on the basis of different flower morphology (Duyfjes, 1993). They were collected in Vietnam, Indonesia, and different places in Thailand, as well as purchased in local markets (Table 1). Flowers could only be compared from the four collections B, D, F, and H, probably representing *S. phyllantha* Gagnep. (D, F) and *S. tuberosa* Lour. (H) after Duyfjes (1993), and a species probably related to *S. phyllantha* with similar, but smaller, flowers (B). Voucher specimens from the collections A and C were prepared from non-flowering samples only, whereas from E and G only underground parts were available for extraction. As reported recently, the roots of the *S. tuberosa* group are clearly bigger and thicker than those of the other species and are characterised by the presence of scattered fibers in the cortex and pith (Xu et al., 2006).

Altogether, six alkaloids (**1**–**6**) were detected with Dragendorff's reagent and could be sufficiently separated and isolated by preparative TLC (Fig. 1). They were shown to fall into two groups due to their different basic structures. Five collections (D–H) shared a common formation of tuberostemonine (**1**) with a stichoneurin skeleton (Fig. 1), whereas three (A–C) clearly deviated by an accumulation of croomine (**5**) representing a different structural type (Fig. 2) (Greger, 2006). This structural divergence has been reported previously (Schinnerl et al., 2005; Jiang et al., 2006a,b) and was underlined by accompanying alkaloids showing an additional accumulation of the stereoisomers of tuberostemonine, tuberostemonine A (**2**), and neotuberostemonine (**4**) in the first group, and a 6-hydroxylated derivative of croomine (**6**) in the latter. From the five collections of the first group, four (D–G) were uniformly characterised by an additional accumulation of tuberostemonine A (**2**), whereas one (H) clearly deviated by the formation of neotuberostemonine (**4**) and a new alkaloid identified as tuberostemonine N (**3**) in the course of this study. In contrast to the high R_f-value of tuberostemonine A (**2**), differing from tuberostemonine (**1**) only by a β-orientated H at position C-3, the unknown compound **3** could be clearly distinguished by its low R_f-value (Fig. 1). Detailed NMR analysis, including H/H-COSY, HMQC, and HMBC experiments led to the structure elucidation of a new stereoisomer, named tuberostemonine N (**3**) (see Section 2.3.).

Another alkaloid also lacking a conjugated system was detected in *S. parviflora* C.H. Wright originating from south China and in an unidentified species from northeast Thailand (HG 915). In accordance with previous communications, it was identified as parvistemonine (**7**) (Lin

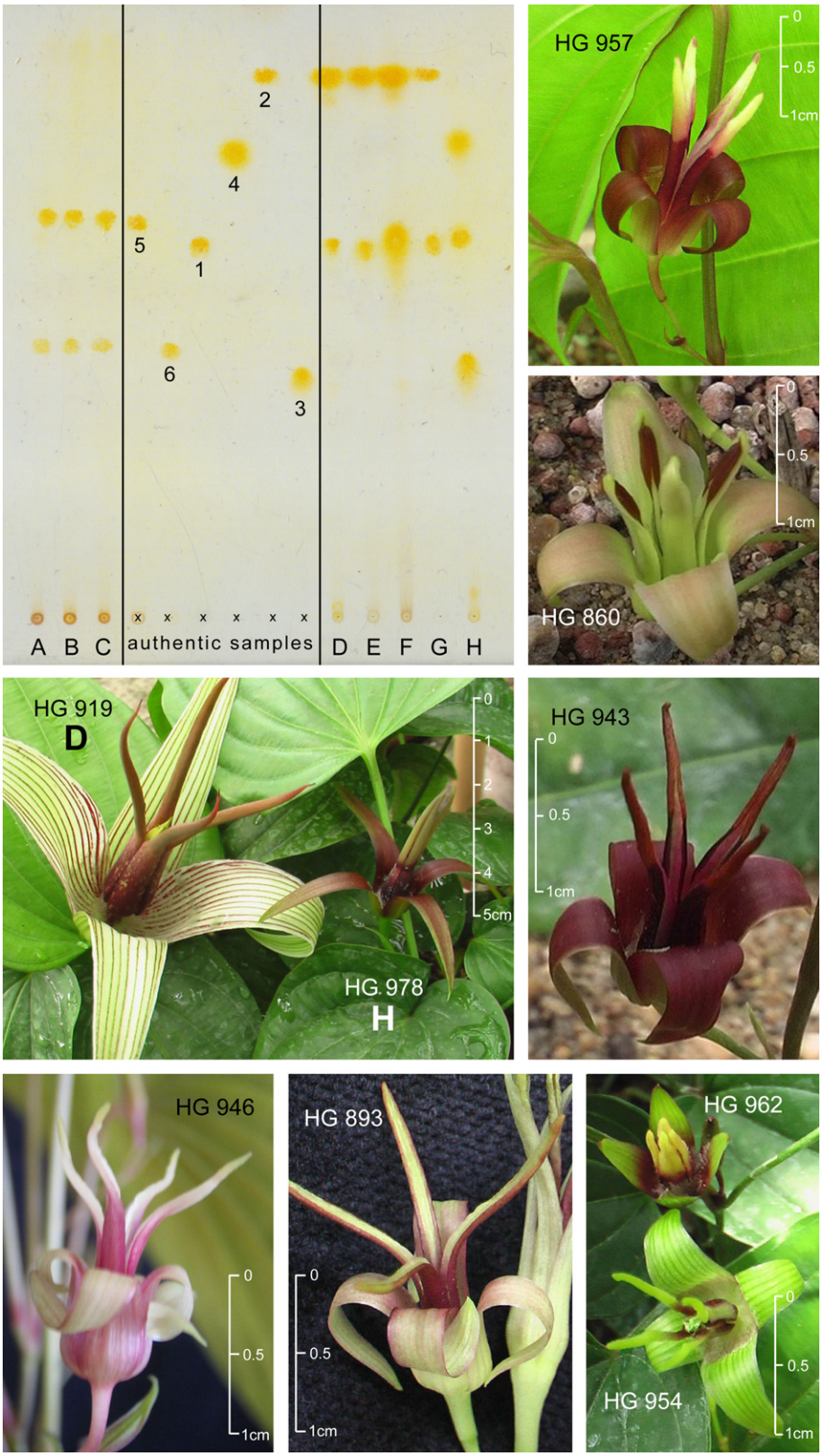

Fig. 1. TLC comparison of methanolic crude extracts of underground parts of eight members of the *S. tuberosa* group (A–H) with authentic samples sprayed with Dragendorff's reagent. A = HG 879, B = HG 918, C = HG 890, D = HG 919, E = HG 894, F = HG 851, G = HG 950, H = HG 978 (see Table 1). Solvent = CH_2Cl_2–EtOAc–MeOH–NH_4OH (50:45:5:1). Flower characters of *S. curtisii* (HG 957), *S. collinsae* (HG 860), *S.* cf. *phyllantha* (HG 919), *S.* cf. *tuberosa* s. str. (HG 978), unidentified spec. (HG 943), *S. burkillii* (HG 946), unidentified spec. (HG 893), *S. japonica* (HG 954), and *S. parviflora* (HG 962). Authentic samples: tuberostemonine (**1**), tuberostemonine A (**2**), tuberostemonine N (**3**), neotuberostemonine (**4**), croomine (**5**), 6-hydroxycroomine (**6**).

Table 1
Accumulation trends of pyrrolo- and pyridoazepine alkaloids in the underground parts of different *Stemona* species and provenances

Stemona species	Provenances	Stichoneurine-					Croomine-		Protostemonine-type alkaloids								
		1	**2**	**3**	**4**	**7**	**5**	**6**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
t u b e r o s a group																	
	HG 890, N-Thailand, Chiang Mai, Khun Chang Kien ca. 1000 m						●	○									
	HG 879, N-Vietnam, Hanoi, Ba Vi National Park ca. 800 m						●	○									
	HG 918, Indonesia, Bali, Tenganan, riverbank						●	○									
	HG 950[a], NW-Thailand, Tak, Doi Mueso ca. 800 m	●	●														
	HG 894[a], purchased in Phu Thok, originating from Ubon Ratchathani	●	●														
cf. phyllantha	HG 919, purchased at Chatuchak-Market, Bangkok	○	●														
	HG 851, SE-Thailand, Rayong, Khao Chamao, bamboo forest	●	●														
cf. tuberosa s.str.	HG 978, NE-Thailand, Uttaradit, Phu Soi Dao ca. 1200 m	○		●	○												
japonica	HG 954, China, Hong Kong, Chinese University								●								
sessilifolia	HG 953 China, Hong Kong, Chinese University								●								
parviflora	HG 962 China, Hong Kong, Chinese University					○			●	○							
burkillii	HG 946, N-Thailand, Lamphun, Doi Khun Tan ca. 800 m									●	○						
	HG 887, N-Thailand, Chiang Mai, Doi Suthep ca. 700 m									●	○						
cochinchinensis	HG 884, NE-Thailand, Nong Khai, Phu Thok, paddy field									●	○						
collinsae	HG 840, SE-Thailand, Chonburi, Khao Khieo									○	○	●					
	HG 955, E-Thailand, Nakhon Ratchasima, Pak Thong Chai, Sakaerat									○		●					
	HG 866, E-Thailand, Ubon Ratchathani, Dong Fa Huan									○	○	●					
kerrii	HG 888, N-Thailand, Chiang Mai, Mae Rim ca.700 m												○	○			●
	HG 892, NW-Thailand, Tak, Khao Chomphu ca. 600 m												○	○			●
curtisii	HG 911, S-Thailand, Krabi, Ban Tung Sung, Khlong Tom									●	○		○	○			
	HG 865, S-Thailand, Satun, Ko Lipe, near the beach									●	○		○				
	HG 917, S-Thailand, Narathiwat, rubber plantation									●			○	○		○	
	HG 957, S-Thailand, Chumphon, Hat Thung Wua Laen, beach														●	○	
	HG 920, Kaset Fair market Bangkok, originating from Chumphon												●	○	○	○	
	HG 899, S-Thailand, Narathiwat, near Pacho waterfall								●	○	○		○	○			
cf. pierrei	HG 910, E-Thailand, Surin, Sri Saket								○								
unidentified spec.	HG 943, E-Thailand, Nakhon Ratchasima, Ban Laem Tong								○				●	○			
	HG 893, N-Thailand, Sukhothai, foothill of Khao Luang												●	○			
	HG 915, NE-Thailand, Udon Thani, Nong Wua So, deciduous forest					○							○			●	
	HG 912, E-Thailand, Ubon Ratchathani, Pha Taem								○								
	HG 913, W-Thailand, Kanchanaburi, Sai Yok, Ban Pupong								○								

Major (●) and minor alkaloids (○) within the *S. tuberosa* group were correlated with the amounts of **1**, **3**, and **4** isolated from HG 978, and **1**, **2**, **5**, and **6** from HG 851 (Schinnerl et al., 2005). Alkaloids with characteristic UV absorptions were categorised as major compounds by their dominating peaks in the HPLC profiles shown in Figs. 4–6. The absolute concentration of these compounds were ranging from 0.5 (e.g. *S. parviflora*) to 10 mg/ml crude extract (e.g. *S. collinsae*) calculated on the basis of the peak area of stemofoline as standard.

[a] No voucher specimens available.

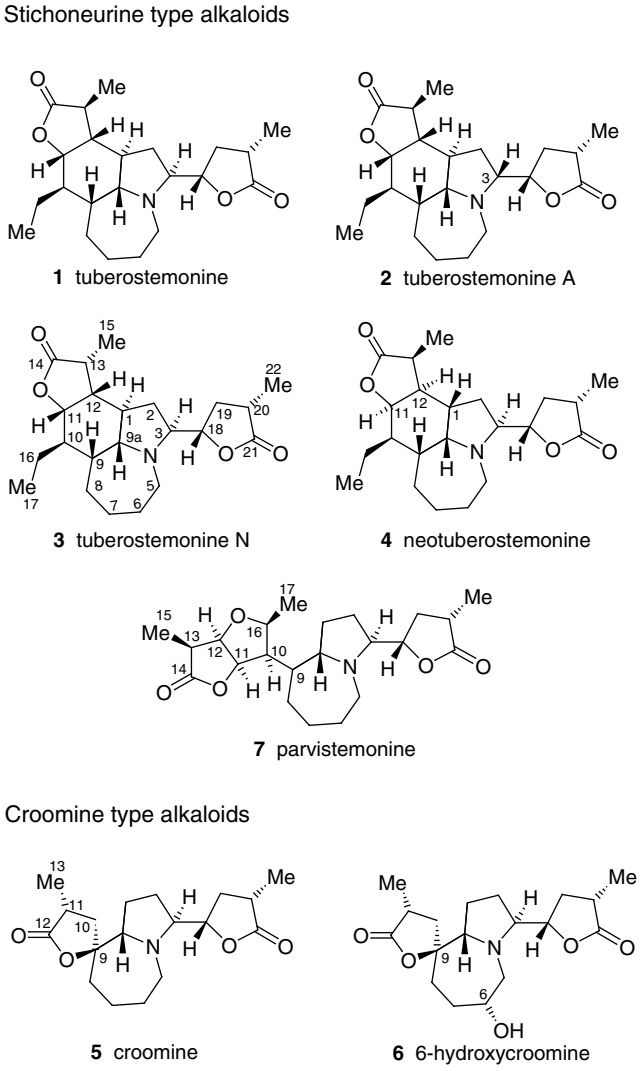

Fig. 2. Stichoneurine and croomine type alkaloids.

et al., 1990; Kaltenegger et al., 2003). Because of the presence of an ethyl group attached to C-10 it was recently also regarded as a stichoneurine type alkaloid (Fig. 2) (Greger, 2006).

Most of the *Stemona* species investigated in the present paper were characterised by protostemonine-type alkaloids with a dienone chromophore and hence could be detected under UV (Fig. 3). Protostemonine (**8**) itself dominated only in the underground parts of the three species *S. sessilifolia*, *S. japonica*, and *S. parviflora* originating from China, whereas the other species mostly showed a clear preponderance of derivatives closely related either to stemofoline (**9**–**11**) or oxystemokerrine (**12**–**16**) (Fig. 4). As shown in Fig. 3, stemofoline (**9**) represents a complex cage-type molecule most likely derived from protostemonine (**8**) by the formation of an oxygen bridge between C-2 and C-8 and an additional C–C linkage between C-3 and C-7. The butyl side chain attached to C-3 can be regarded as the result of hydrolysis of the methylated butyrolactone ring followed by decarboxylation (Kaltenegger et al., 2003). In contrast to stemofoline derivatives **9**–**11** characterised by a pyrroloazepine-based structure, the oxystemokerrine related alkaloids **12**–**16** clearly deviated by a pyridoazepine skeleton. Biosynthetically, the six-membered piperidine ring was thought to emerge from a five-membered pyrrolidine ring by ring cleavage between C-3 and N, and incorporation of C-18 from the butyl side chain (Fig. 3) (Kaltenegger et al., 2003).

The underground parts of *S. burkillii* D. Prain, *S. cochinchinensis* Gagnep., and *S. collinsae* Craib showed a clear preponderance of stemofoline derivatives **9**–**11**, whereas those of *S. kerrii* Craib and some collections of *S. curtisii* Hook.f. mainly accumulated alkaloids closely related to oxystemokerrine (**12**–**16**) (Fig. 4). Comparison of different geographical provenances of *S. burkillii*, *S. collinsae*, and *S. kerrii* displayed uniform chemical capacities, whereas those of *S. curtisii* exhibited remarkable infraspecific variability (Table 1; Fig. 4). *S. burkillii* from two different localities in northern Thailand uniformly accumulated stemofoline (**9**) as major alkaloid, along with small amounts of 2′-hydroxystemofoline (**10**). These results were also confirmed by a previous report on *S. burkillii*, where, in addition to the alkaloids **9** and **10**, two, closely-related derivatives were also isolated in small amounts (Mungkornasawakul et al., 2004b). A similar chemical profile was also found in *S. cochinchinensis*. However, in this case, no infraspecific variation could be postulated because only plants from one location in northeast Thailand were available for comparison. Three collections of *S. collinsae* originating from southeast and east Thailand (Table 1) were also following this biosynthetic trait towards stemofoline formation, but they could be clearly distinguished from the former species by a preponderance of the didehydro derivative **11**. As shown in Fig. 4, stemofoline (**9**) could not be separated from **11** by the standard eluent used for HPLC comparison. However, flattening of the time gradient led to a partial separation of both alkaloids (Fig. 4). Originally, **11** was isolated from a plant erronously identified as *Asparagus racemosus* Willd. and named asparagamine A (Sekine et al., 1995), but was later shown to be the major alkaloid of *S. collinsae*, and renamed as didehydrostemofoline (**11**) (Jiwajinda et al., 2001; Brem et al., 2002; Seger et al., 2004). Different provenances of *S. kerrii*, collected in north and northwest Thailand, could be clearly distinguished by an accumulation of the pyridoazepine type alkaloids **12**, **13**, and especially **16**, as previously reported (Kaltenegger et al., 2003). Stemokerrine (**16**) differed from the other related derivatives by lacking the characteristic ether bridge between C-1 and C-8 and the additional formation of a double bond between C-8 and C-9 obviously representing a species specific chemical trend (Fig. 4).

In contrast to the more species specific chemical profiles discussed above, six different provenances of *S. curtisii* originating from southern Thailand deviated by an infraspecific variability with accumulation trends both to stemofoline (**9**)- and/or oxystemokerrine (**12**)-related alkaloids, as well as to protostemonine (**8**). As shown in Fig. 4,

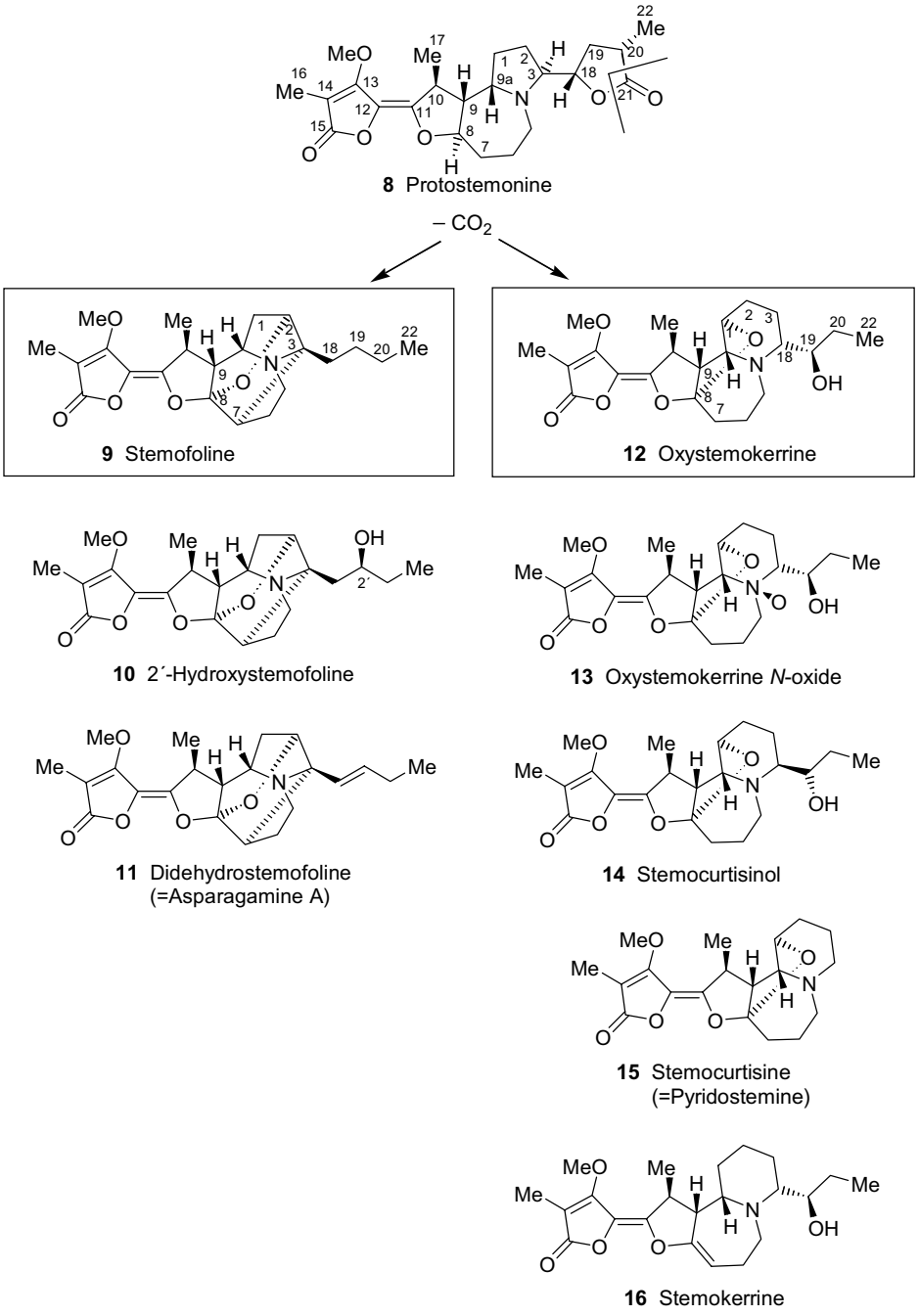

Fig. 3. Oxystemokerrine and stemofoline related alkaloids representing major accumulation trends most likely derived from protostemonine.

three collections from Krabi (HG 911), Satun (HG 865), and Narathiwat (HG 917) were characterised by a predominance of stemofoline (**9**); but oxystemokerrine (**12**) and oxystemokerrine *N*-oxide (**13**) co-occurred in varying amounts. In contrast, the two samples originating from Chumphon (HG 957, HG 920; Table 1) exclusively accumulated oxystemokerrine (**12**), together with the closely related pyridoazepine derivatives **13**, **14**, and **15** (Fig. 4). The latter findings were in agreement with a previous report on *S. curtisii* collected in Trang province in southern Thailand, where stemocurtisine (=pyridostemine) (**15**) was also isolated as the major component along with stemocurtisinol (**14**), a stereoisomer of oxystemokerrine (**12**) (Mungkornasawakul et al., 2003, 2004a). With respect to the HPLC profiles of the different provenances of *S. curtisii* shown in Fig. 4, the predominance of protostemonine (**8**) in collections from Pacho near Narathiwat (HG 899) was surprising. However, in this case, only roots were investigated without rhizomes. In order to make the result comparable with those of other collections, consisting of roots and rhizomes as a whole, a small portion of rhizomes obtained from three individual plants was additionally ana-

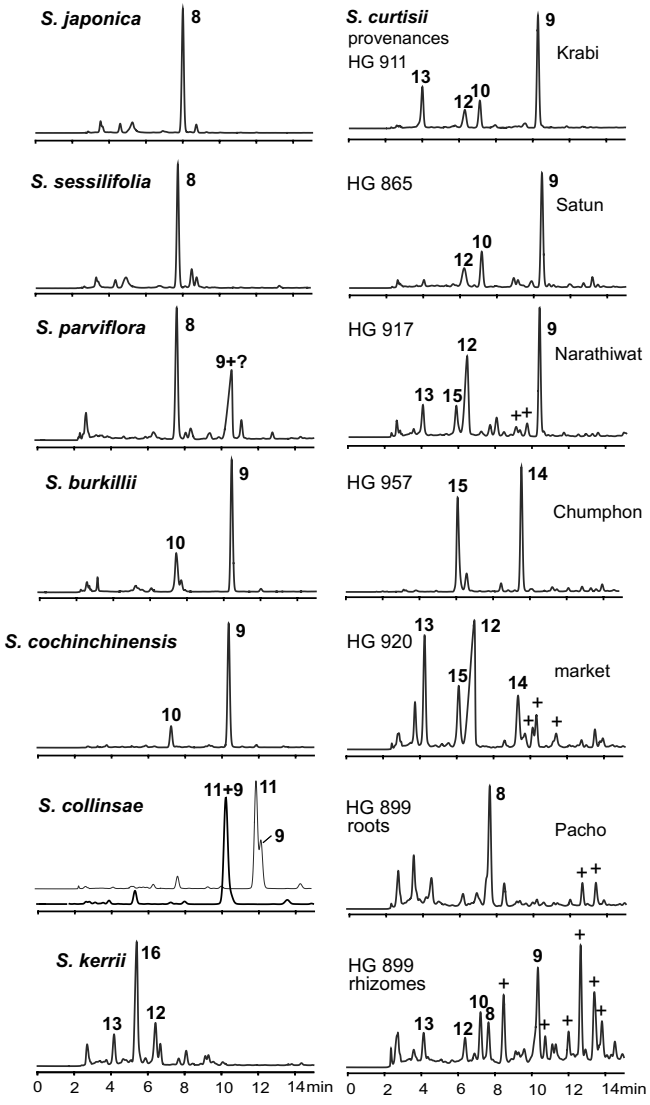

Fig. 4. UV-HPLC comparison of methanolic crude extracts of underground parts of eight *Stemona* species also demonstrating infraspecific chemical variability of different provenances of *S. curtisii* as well as tissue specific chemical profiles. Beside the standard eluent in *S. collinsae* flattening of the time gradient led to a partial separation of **9** and **11**. + = stilbenoids. UV diode array detection at 280 nm.

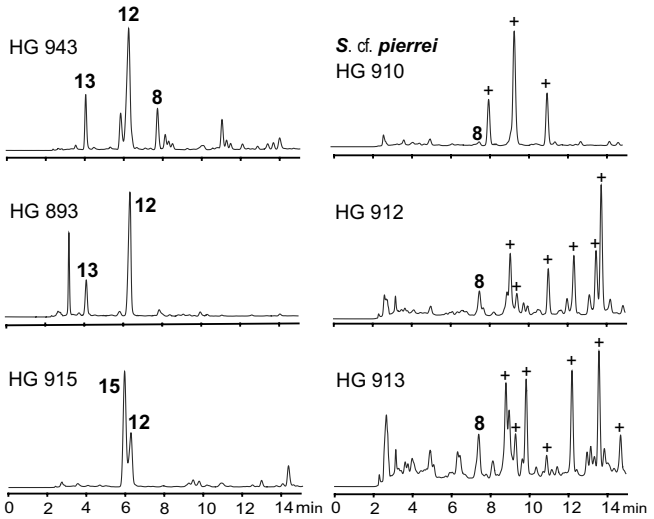

Fig. 5. UV-HPLC comparison of methanolic crude extracts of underground parts of *S.* cf. *pierrei* and four collections most likely representing as yet undescribed species. + = stilbenoids. UV diode array detection at 280 nm.

lysed. Interestingly, in this case, a preponderance of stemofoline (**9**) and 2′-hydroxystemofoline (**10**) was also found, together with only small amounts of protostemonine (**8**) accompanied by traces of oxystemokerrine (**12**) and oxystemokerrine *N*-oxide (**13**), indicating an overall biosynthetic capacity for the formation of different protostemonine-type alkaloids.

In order to provide a comprehensive overview regarding the different accumulation trends within the genus *Stemona*, species were also used for comparison whose taxonomic position could not be definitely determined in the present study (Fig. 5). Whereas collection HG 910, originating from dry habitats in east Thailand, was tentatively published as *S.* cf. *pierrei* Gagnep. previously (Kostecki et al., 2004), the other collections most likely represent as yet undescribed species. Compared to the results discussed above (Fig. 4), neither stemofoline (**9**) nor related alkaloids could be detected. Instead, the collections HG 943, HG 893, and HG 915, originating from rather dry habitats in east, north, and north east Thailand (Table 1), respectively, shared common accumulation trends towards oxystemokerrine (**12**) and its related derivatives **13** and **15**. Moreover, in *S.* cf. *pierrei* and the two collections HG 912 and HG 913 a general reduction of alkaloid accumulation was observed leading to only small amounts of protostemonine (**8**) accompanied by a series of stilbenoids (Fig. 5) (Kostecki et al., 2004). The two latter samples were collected in geographically so distant provinces such as Ubon Rachathani in east, and Kanchanaburi in west Thailand (Table 1).

Chemical differences between the underground and aerial parts of five different species are shown in Fig. 6. Generally, the underground parts, including the roots and rhizomes, were characterised by much higher quantities of alkaloids than the aerial ones, including the leaves and stems. Hence, for optical reasons, the peak intensities of aerial parts were scaled up in Fig. 6 by factors indicated in the corresponding profiles. In accordance with previous studies, the leaves and stems of *S. japonica* mainly accumulated stemofoline (**9**), whereas the underground parts clearly differed by large amounts of protostemonine (**8**) (Irie et al., 1970a,b). A similar difference was also observed in the probably related *S. parviflora*. However, the presence of stemofoline (**9**) in the underground parts could not be unambiguously determined chromatographically in the present study, but it has already been published previously for that species co-occurring with its optical antipode parvistemoninine (Lin et al., 1991; Xu et al., 1991). Very few qualitative differences between the two different plant parts were detected in *S. cochinchinensis* and *S. collinsae*,

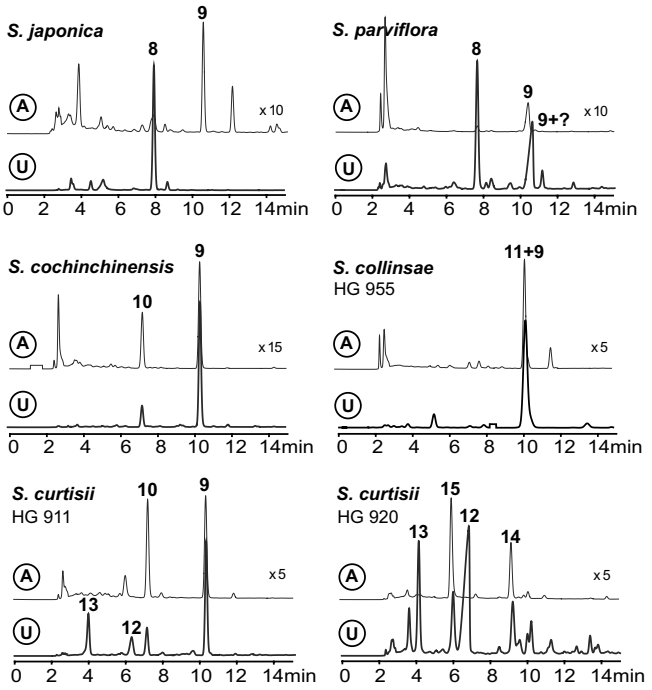

Fig. 6. UV-HPLC comparison of methanolic crude extracts of aerial "A" and underground parts "U" of five different species displaying much higher quantities of alkaloids in the latter. For optical reasons the peak intensities of the aerial parts "A" were scaled up by factors indicated in the corresponding profiles. UV diode array detection at 280 nm.

showing a clear preference towards stemofoline (**9**) or didehydrostemofoline (**11**) accumulation, respectively. A reduction of chemical diversity was observed in the aerial parts of the two provenances of *S. curtisii* HG 911 and HG 920 showing only the two pyrroloazepines **9** and **10** in the former, and the two pyridoazepines **14** and **15** in the latter. The same alkaloids were also detected in the underground parts of both collections together with oxystemokerrine (**12**) and its *N*-oxide (**13**) (Fig. 6). Summarising, it can be stated that both plant parts were characterised by the same skeletal type of alkaloids, mostly showing only quantitative differences, sometimes with only slight modifications of the substitution pattern of the basic skeleton.

2.2. Chemotaxonomic conclusions

With respect to the chemical data already published for *Stemona* species, the present results were surprising and generated new insights into the occurrence and distribution of the major alkaloids within the genus. Due to the broad application of *S. tuberosa*, *S. sessilifolia*, and *S. japonica* in Traditional Chinese medicine, tuberostemonine (**1**) and protostemonine (**8**) were known as the most prominent representatives of this class of alkaloids. In fact, in the first review in 1973, only seven derivatives were described with a defined structure, all closely related to these two alkaloids (Götz and Strunz, 1973). However, based on the present overview the formation of tuberostemonine (**1**) and its closely related stichoneurine-type derivatives with a saturated tricyclic pyrrolobenzazepine nucleus was restricted to the *S. tuberosa* group, and an accumulation of protostemonine (**8**) itself was only observed in the Chinese species *S. japonica*, *S. sessilifolia*, and *S. parviflora*, and in the roots of one provenance of *S. curtisii* (Fig. 4). Instead, a prevalent occurrence of stemofoline (**9**) and oxystemokerrine (**12**), together with closely related derivatives, has now been ascertained for many *Stemona* species (Figs. 4 and 5).

In accordance with more detailed comparative HPLC/MS studies within *S. tuberosa*, *S. japonica*, and *S. sessilifolia* (Jiang et al., 2006a; Zhou et al., 2006) no protostemonine-type alkaloids were detected in the samples of the *S. tuberosa* group, whereas protostemonine itself (**8**) was the major component only in the underground parts of *S. japonica*, *S. sessilifolia*, and the probably related *S. parviflora* (Fig. 4). Furthermore, the previously reported chemical variability within the *S. tuberosa* group (Schinnerl et al., 2005; Jiang et al., 2006b; Zhou et al., 2006) was confirmed by the determination of different accumulation trends either towards the croomine- (A–C) or stichoneurine-type alkaloids (D–H in Fig. 1). Moreover, the formation of different isomers of tuberostemonine (**1**) appeared to be at least partly in accord with floral characters. Collection HG 919 (D) with big yellowish tepals up to 7 cm and the peduncle of the one-flowered inflorescence frequently fused with the petiole was consistent with the description of *S. phyllantha* by Gagnepain (1934). Similar, but mostly 2–3 flowers in an axillary inflorescence were observed in HG 851 (F). Both collections were characterised by an additional accumulation of tuberostemonine A (**2**). This biogenetic trend was also found in the two samples E and G (Fig. 1), where no voucher specimens were available (Table 1). Hence, the deviating chemical profile of HG 978 (H), characterised by the two stereoisomers neotuberostemonine (**4**) and tuberostemonine N (**3**), deserve special chemosystematic interest. With tepals up to only 3 cm, inside purple or brown-red and green towards the tips (Fig. 1), this collection matched the description of *S. tuberosa* Lour. (Duyfjes, 1993). No correlations between the chemical and floral characters could be deduced from the croomine-accumulating samples (A–C), because only one voucher specimen from an Indonesian collection (B) was available with flowers. In this case, the yellowish flowers resembled *S. phyllantha*, but differed by smaller tepals up to only 3.5 cm. For completeness it should be pointed out that no stemoninine could be detected in the present study. Stemoninine represents another stichoneurine-type alkaloid with a spiro system and was detected as major component in some Chinese provenances of *S. tuberosa* by reversed-phase HPLC of 13 samples linked with evaporative light-scattering detection (ELSD) (Jiang et al., 2006b, and recently also by Lin et al., 2006). On the other hand, no tuberostemonine A (**2**) could be detected in these investigations which otherwise was shown now to be widespread in different Thai provenances (Fig. 1). Furthermore, it is also interesting to note that no bisdehydro derivatives with a stable aromatic pyrrole system could be found, neither with

HPLC/ELSD nor with TLC/Dragendorff detection. With respect to previous reports on the frequent occurrence of bisdehydro derivatives these findings support the hypothesis that these alkaloids may be regarded as artifacts only produced during storage, extraction, and/or fractionation (Greger, 2006).

Comparing the different distributions of protostemonine-type alkaloids shown in Figs. 4–6 some species-specific accumulation trends became apparent. The pyrroloazepine alkaloid stemofoline (**9**), originally isolated from the aerial parts of *S. japonica* (Irie et al., 1970b), was shown to be a typical chemical marker for some species occurring both in the aerial and underground parts (Fig. 6). Of special chemosystematic significance, however, was the restricted occurrence of its didehydro derivative **11**. Although its formation could be regarded as a simple dehydration step from the widespread 2′-hydroxystemofoline (**10**), it was found so far only in *S. collinsae* (Jiwajinda et al., 2001; Brem et al., 2002; Seger et al., 2004). More recently, stemofoline (**9**) and closely related alkaloids were also reported for an unidentified *Stemona* species. Because of the large quantities of plant material involved, six new stemofoline related alkaloids could be additionally isolated as minor constituents together with substantial amounts of the known ones **9**–**11** (Sastraruji et al., 2005). Based on the previous results and an inspection of the corresponding plant photographs, available in the supporting information, it can now be stated that this plant also represented *S. collinsae*. The report on tuberostemonine derivatives in *S. collinsae* collected in Vietnam was obviously based on a confusion with *S. tuberosa* (Pham et al., 2002; H.D. Pham, pers. commun.).

The accumulation of the pyridoazepine alkaloid stemokerrine (**16**) in *S. kerrii* represented another important chemical character not detected so far in other species. Even though two provenances of *S. curtisii* (Fig. 4) and three unidentified species (Fig. 5) were also characterised by an accumulation of related pyridoazepine alkaloids, all derivatives **12**–**15** clearly deviated by the formation of an ether bridge between C-1 and C-8 (Fig. 3). With respect to the two different accumulation trends towards pyrrolo-**9**–**11** or pyridoazepine-alkaloids **12**–**16** (Fig. 3), which clearly characterised different *Stemona* species, their common occurrence in *S. curtisii* merits taxonomic interest. In this connection it should be pointed out that *S. curtisii* also deviated by its chromosome number $2n = 13$–16, whereas *S. tuberosa*, *S. japonica*, *S. sessilifolia*, *S. kerrii*, *S. collinsae*, and the unidentified species HG 915 were shown to have the same chromosome number $2n = 14$ (Oginuma et al., 2001; Hartl and Kiehn, 2004).

The lack of accumulation of protostemonine-type alkaloids, as well as phenylbenzofuran stilbenoids, in the *S. tuberosa* group (Pacher et al., 2002; Pacher, 2005), which otherwise both represented typical chemical features of a number of *Stemona* species, suggested a splitting of the genus into two subgroups. This taxonomic segregation of the *S. tuberosa* group could be also supported by its common accumulation of dehydro-δ-tocopherol (Brem et al., 2004) and some morphological and anatomical characters recently described for the roots (Xu et al., 2006). Whereas the formation of the stichoneurine-type alkaloid parvistemonine (**7**) in *S. parviflora* and HG 915, which were otherwise characterised by an accumulation of protostemonine-type alkaloids (Figs. 4 and 5), might be interpreted as a rudimentary side path, the accumulation of protostemonine (**8**) as the major alkaloid in the underground parts of the Chinese species *S. japonica*, *S. sessilifolia*, and *S. parviflora* possibly represents less advanced biosynthetic evolution.

2.3. Structure elucidation of tuberostemonine N (*3*)

All of the spectral data of the new alkaloid **3** were in favor of a stereoisomer of tuberostemonine (**1**). EI-MS showed a small 3% molecular peak of $m/z = 375$ for $C_{22}H_{33}NO_4$ and the typical 100% base peak m/z 276 for M^+-99 after loss of the lactone ring attached at C-3. FAB-MS showed the expected $M^+ + 1$ peak at m/z 376. The ^1H, and especially the ^{13}C NMR spectrum were also similar to tuberostemonine (**1**). The assignments of the ^1H and ^{13}C resonances were accomplished by means of H/H-COSY, HMQC and HMBC experiments. Analysis of the NOESY data was used to assign the stereochemistry of alkaloid **3**. Since this was the crucial point of structure elucidation it should be discussed in some detail: A network of β-protons 11, 12, 13, 16 (17), 9, 9a, 2β, 5β, 18, 19β, and 20 was obtained on the basis of characteristic NOEs 11 → 12, 13, 16, 17; 12 → 11, 13, 2β, 9a; 13 → 11, 12; 9 → 9a, 5β, 16, 17; 9a → 9, 2β, 12, 16; 2β → 9a, 12, 18; 18 → 2β, 19β, 20; 19β → 18, 20. An α-proton network was obtained for protons 1, 15, 2α, 5α, 3, 19α, 22, and 10 which was derived from the NOE correlations 15 → 1, 2α; 1 → 2α, 3, 15; 3 → 1, 2α, 5α, 19α; 19α → 22. Since the absolute stereochemistry is unknown, α and β could still be reversed. However, assuming that the unknown alkaloid is closely related to tuberostemonine (**1**), most configurations should agree with the basic alkaloid. Indeed, only the α-position of the methyl group C-15 differed from that of tuberostemonine (**1**), unambiguously characterized by the strong NOE effects of this methyl group with H-1α and H-2α. The β-position of the corresponding H-13 agreed fully with characteristic NOEs of 13 to 11 and 12. It should be mentioned that two "unexpected" NOEs 11 → 10 and 9 → 10 appear, in addition to 11 → 16 and 9 → 16. This could be explained by a pseudoequatorial position for H-10 in a chair-like 6-ring. The NOEs of the necessarily pseudoaxial orientated ethyl group 16 with the opposite axial protons 9a and 12 also agreed very well with this geometry. Inspection of a model showed that an equatorial ethyl group would force the sterically strained 6-ring into an unfavourable boat form. The 2D spectra were measured in $CDCl_3$ as well as in MeOH-d_4 in order to allow a more reliable interpretation in the case of overlapping proton resonances. Generally, the overlap was less severe in MeOH-d_4.

3. Experimental

3.1. General

NMR: Bruker DRX400 WB ($CDCl_3$ ^1H δ 7.26; 13C δ 77.0). MS: Finigan MAT 900 S. IR: Perkin-Elmer 16PC FT-IR. Optical rotation: Perkin Elmer Polarimeter 241. HPLC: Agilent 1100, UV diode array detection at 280 nm, column Hypersil BDS-C18 250 × 4.6 mm, 5 μm, mobile phase gradient MeOH 55–100% in 0.01 mol/l ammonium acetate in H_2O, flow rate 1 ml/min.

3.2. Plant material

Thirty-one collections representing *S. burkillii* (HG 946, HG 887), *S. cochinchinensis* (HG 884), *S. collinsae* (HG 840, HG 955, HG 866), *S. curtisii* (HG 911, HG 865, HG 917, HG 957, HG 920, HG 899), *S. japonica* (HG 954), *S. kerrii* (HG 888, HG 891), *S. parviflora* (HG 962), *S.* cf. *pierrei* (HG 910), *S. sessilifolia* (HG 953), and the *S. tuberosa* group (HG 890, HG 879, HG 918, HG 950, HG 894, HG 919, HG 851, HG 978), as well as the unidentified species HG 893, HG 912, HG 913, HG 915, and HG 943 were collected in China, Indonesia, Vietnam, and various localities of Thailand (Table 1). Voucher specimens were deposited at the Herbarium of the Institute of Botany, University of Vienna (WU).

3.3. Extraction and chromatography

Short-dried underground (including roots and rhizomes) and aerial parts (including leaves and stems) were ground separately and extracted twice with MeOH at room temp for one week. Crude extracts were filtered and concentrated. The aqueous residues were extracted with $CHCl_3$ and the concentrated $CHCl_3$ fractions dissolved in MeOH were used for comparative UV-HPLC and TLC, respectively. For preparative isolation, lipophilic crude extracts were roughly separated by column chromatography (Merck Silica gel 60, 0.2–0.5) with solvent mixtures of hexane, EtOAc, and MeOH. Further separation was achieved by MPLC with mixtures of MeOH in EtOAc as mobile phase as described previously (Kaltenegger et al., 2003).

Alkaloids **1**, **3**, and **4**, were isolated from the underground parts of *S. tuberosa* (HG 978) (53 g). The $CHCl_3$ fraction (200 mg) was roughly separated by column chromatography. Based on TLC with CH_2Cl_2/EtOAc/MeOH/NH_4OH (70:25:5:1) all fractions with positive Dragendorff's reaction were combined according to different R_f values. Fractions containing the main alkaloid at R_f 0.35 were further separated with CH_2Cl_2/EtOAc/MeOH (70:25:5) to afford **3** (10 mg), and those with R_fs at 0.45 and 0.55 with 100% MeOH to afford **1** (7 mg) and **4** (5 mg), respectively.

3.4. Tuberostemonine N (*3*)

Colorless amorphous powder; $[\alpha]_D^{20} = -24°$ ($CHCl_3$, $c = 0.5$). IR υ^{CCl_4} cm^{-1} 2924 m, 1781 s, 1456 m, 1379 w, 1174 m, 1158 m, 1018 m, 985 m, 924 w, 914 w. EI-MS (70 eV) $m/z = 375$ (3%, M^+), 276 (100, $M^+ - C_5H_7O_2$), remaining peaks <5%. ^1H NMR ($CDCl_3$) $\delta = 4.20$ (*dd*, 1H, $J = 4.3$ and 1.8 Hz, 11-H), 4.19 (*ddd*, 1H, $J = 10.6$, 8.1, and 5.3 Hz, 18-H), 3.31 (br, *dd*, 1H, $J = 15.1$ and 6.3 Hz, 5α-H), 3.21 (*ddd*, 1H, $J = 10.5$, 8.1, and 6.3 Hz, 3-H), 3.12 (*dd*, 1H, $J = 11.9$ and 3.8 Hz, 9a-H), 2.81 (*dq*, 1H, $J = 7.3$, 7.3 Hz, 13-H), 2.79 (*ddd*, 1H, $J = 15.1$, 6.3, 4.8 Hz, 5β-H), 2.62 (*m*, 1H, 20-H), 2.36 (*ddd*, 1H, $J = 12.5$, 8.4, 5.4 Hz, 19β-H), 2.26 (*ddd*, 1H, $J = 10.2$, 7.3, 4.3 Hz, 12-H), 2.21 (*ddd*, 1H, $J = 11.8$, 6.3, 5.6 Hz, 2α-H), 2.01 (br, *d*, 1H, $J = 12.0$ Hz, 9-H), 1.98 (br, *t*, 1H, $J = 7.3$ Hz, 10-H), 1.77 (*m*, 1H, 8α or β), 1.76 (*m*, 1H, 7α or β), 1.75 (*m*, 1H, 1-H), 1.60 (*m*, 1H, 8α or β), 1.54 (*m*, 1H, 19α − H), 1.49 (*m*, 1H, 6α or β), 1.42 (*m*, 2H, $16H_2$), 1.33 (*m*, 1H, 6α or β), 1.272 (*d*, 3H, $J = 7.3$ Hz, 15-H_3), 1.270 (*d*, 3H, $J = 7.1$ Hz, 22-H_3), 1.19 (*m*, 1H, 7α or β), 1.09 (*m*, 1H, 2β-H), 1.00 (*t*, 3H, $J = 7.3$ Hz, 17-H_3). For the analysis of the coupling constants the ^1H NMR in CD_3OD was used as well.

^1H NMR (CD_3OD, reference 3.30 ppm) $\delta = 4.30$ (11-H), 4.24 (18-H), 3.32 (5α-H), 3.20 (3-H), 3.08 (9a-H), 2.95 (13-H), 2.82 (5β-H), 2.71 (20-H), 2.42 (19β-H), 2.38 (12-H), 2.27 (2α-H), 2.06 (9-H), 1.98 (10-H), 1.85 (*m*, 1H, 1-H), 1.83 (*m*, 1H, 8α or β), 1.78 (*m*, 1H, 7α or β), 1.60 (*m*, 1H, 8α or β), 1.56 (*m*, 1H, 19α-H), 1.53 (*m*, 2H, 6α-β), 1.46 (16-H_2), 1.24 (15-H_3), 1.23 (7α or β), 1.21 (22-H_3), 1.15 (2β-H), 1.03 (17-H_3).

^{13}C NMR ($CDCl_3$) $\delta = 179.4$ (*s*, C-21), 178.7 (*s*, C-14), 83.4 (*d*, C-18), 81.7 (*d*, C-11), 65.2 (*d*, C-3), 64.2 (*d*, C-9a), 50.7 (*t*, C-5), 46.9 (*d*, C-10), 45.2 (*d*, C-12), 41.5 (*d*, C-13), 40.1 (*d*, C-9), 35.2 (*d*, C-1), 35.2 (*d*, C-20), 34.2 (*t*, C-19), 33.7 (*t*, C-2), 32.8 (*t*, C-8), 29.8 (*t*, C-7), 26.9 (*t*, C-6), 25.9 (*t*, C-16), 15.0 (*q*, C-22), 13.1 (*q*, C-17), 11.9 (*q*, C-15).

^{13}C NMR (CD_3OD, reference 49.0 ppm) $\delta = 181.9$ (C-21), 181.4 (C-14), 84.9 (C-18), 83.2 (C-11), 67.6 (C-3), 65.7 (C-9a), 52.7 (C-5), 49.9 (C-10), 46.6 (C-12), 42.7 (C-13), 41.1 (C-9), 36.3 (C-20), 36.0 (C-1), 35.4 (C-19), 34.7 (C-2), 33.9 (C-8), 29.7 (C-7), 27.1 (C-6), 26.8 (C-16), 15.0 (C-22), 13.4 (C-17), 12.0 (C-15).

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