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

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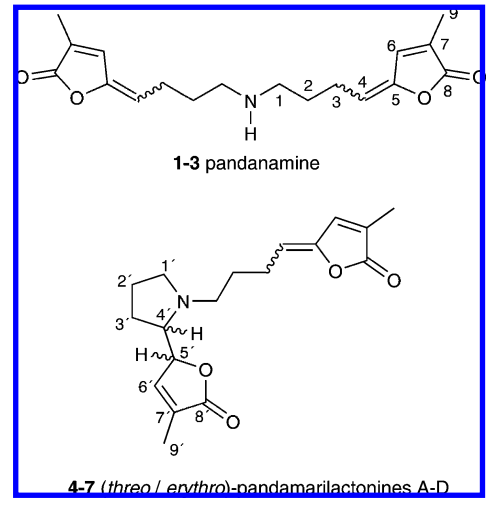
The isolation of (*Z*,*Z*)-pandanamine (**1**) and its corresponding isomers (*Z*,*E*)-pandanamine (**2**) and (*E*,*E*)-pandanamine (**3**) from *Stichoneuron calcicola* of the family Stemonaceae is of outstanding chemosystematic importance. This alkaloid was previously only known from the family Pandanaceae, 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 (**1**−**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 (**1**−**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 (**1**−**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.[1,2] 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 stichoneurine-, protostemonine-, and croomine-type alkaloids.[1] In a previous hypothesis they were speculated to be derived from a spermidine precursor linked with isoprene units.[3] 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 (**1**−**3**).[5]

The rediscovery of pandanamine (**1**−**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 (**1**−**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 (**1**−**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.[9]



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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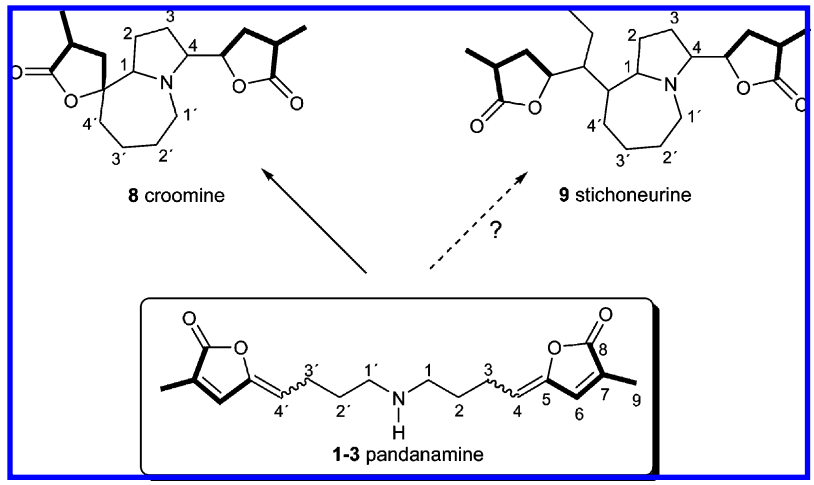

Figure 1. Possible structural relations between the *Stemona* alkaloids **1**−**3**, **8**, and **9**.

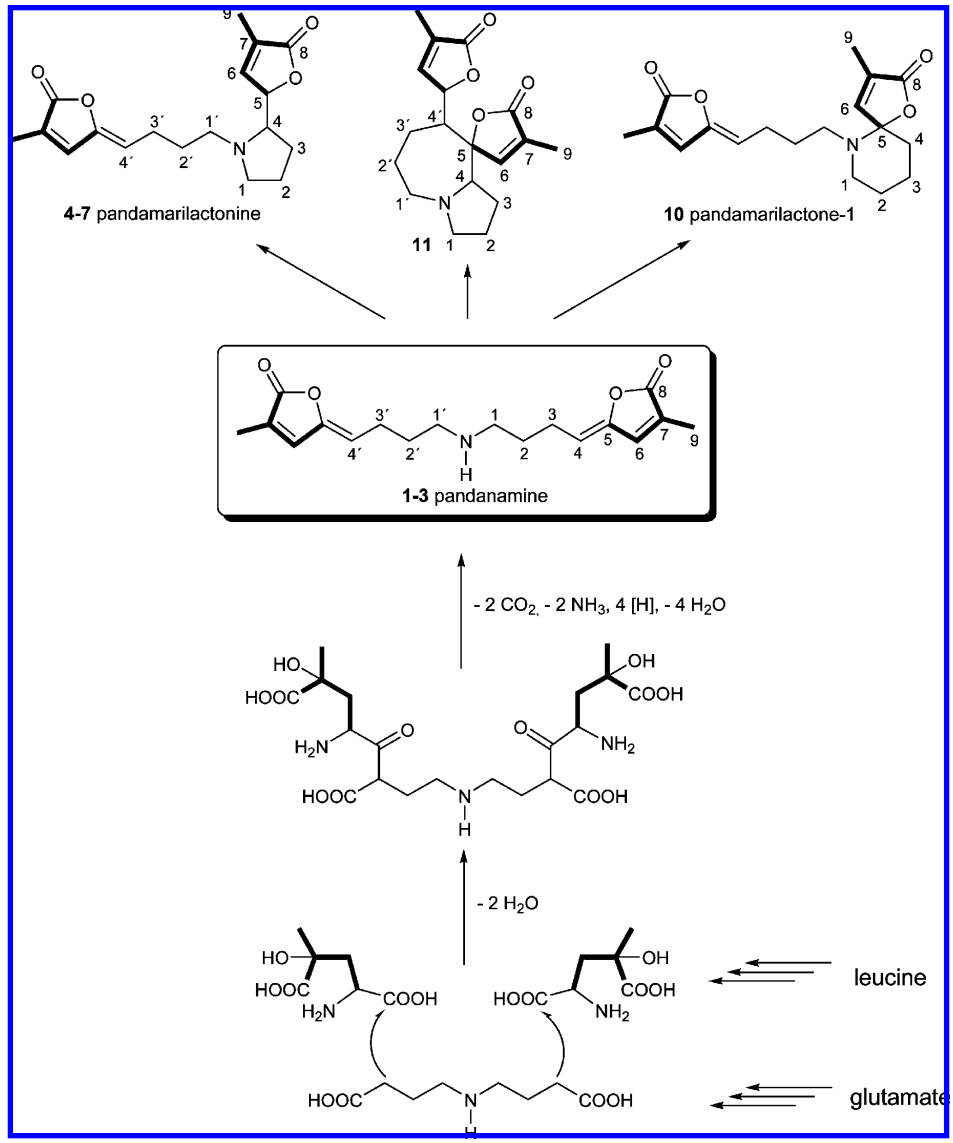

Figure 2. Hypothetical biosynthetic pathway of pandanamine (**1**−**3**) and the probable artificial cyclization to the pyrrolidine alkaloids **4**−**7**, **10**, and **11** (partly redrawn from ref 7).

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, CD_3OD) of the *Z*,*Z*-Pandanamine (**1**) and of the *E*,*E*-Pandanamine (**3**)

	Z,*Z*-pandanamine (**1**)		*E*,*E*-pandanamine (**3**)	
position	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/H_2O), 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 **1** 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 $C_{18}H_{23}O_4N$ 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 ^{13}C NMR recorded now for the first time in CD_3OD 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 $CDCl_3$ additional signals of amine protons were also detected. All other chemical shifts determined in $CDCl_3$ were consistent with data reported previously.[5,7,10,11] In both isomers, the methylene protons of position 1 showed coexistent $^1J_{C-H}$ 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 ^{13}C 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.[5] 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 pyrroloazepine core (**11**), where the second lactone ring was directly attached in a spiro system (Figure 2).[12] 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*-configurated 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-1 (**10**) and an unnamed pyrroloazepine derivative (**11**) both with a spiro system that strongly resembles some structures of *Stemona* alkaloids.[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 *Stemona* alkaloids 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 *Stemona* alkaloids. 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-1 (**10**) and pandamarilactonine A (**4**) are stable and not further racemized under the neutral conditions, but racemize at lower pH values.[14]

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 *Stemona tuberosa* 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 ^{14}C-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_1 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

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.[9] 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. ^{1}H and ^{13}C NMR spectra were measured at 298.15 K in $CDCl_3$ (>99.98% D) or CD_3OD (>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 $CHCl_3$ ($\delta_H = 7.26$) and $CDCl_3$ ($\delta_C = 77.36$) or to residual CHD_2OD ($\delta_H = 3.34$) and CD_3OD ($\delta_C = 49.86$). 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 H_2O 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*,*Z*-pandanamine (**1**). Purification of the latter on a Sephadex LH-20 column with MeOH led to 12 mg of a mixture of pandanamine isomeres (**1**−**3**) and 10% of various pyrrolidines, mainly consisting of pandamarilactonines A (**4**), B (**5**), C (**6**), and D (**7**), whereas the combined fractions 10−12 again afforded an inseparable mixture of cyclization products (4 mg).

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