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Short communication

Acetogenic isoquinoline alkaloids CXII[1]. Separation and identification of dimeric naphthylisoquinoline alkaloids by liquid chromatography coupled to electrospray ionization mass spectrometry

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Abstract

The atropodiastereomeric dimeric naphthylisoquinoline alkaloids, michellamines A (**1a**), B (**1b**) and C (**1c**), together with their monomers, korupensamines A (**2a**) and B (**2b**), were investigated using electrospray ionization tandem mass spectrometry coupled to liquid chromatography (LC–ESI-MS–MS). From the spectra obtained, characteristic product ions were chosen to monitor the chromatographic separation achieved on an RP-18 column. Under acidic conditions required for chromatographic analysis, the monomeric alkaloids **2a** and **2b** yielded protonated molecules $[M+H]^{+}$, while the dimers, the michellamines, exhibited doubly protonated $[M+2H]^{2+}$ molecules. In addition, the coeluting alkaloids **1b** and **2b** were identified unambiguously by means of tandem mass spectrometry. Thus, together with the retention times of the alkaloids, the product ion spectra allowed us the identification of michellamines in the presence of their presumed biogenetic monomeric precursors. Application of the HPLC–MS–MS method successfully proved the enzymatic formation of michellamine C (**1c**) by in vitro dimerization of korupensamine B (**2b**). © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Alkaloids; Naphthylisoquinolines; Michellamines; Korupensamines; Electrospray ionization

1. Introduction

Naphthylisoquinoline alkaloids have been demonstrated to exhibit remarkable biological activities, e.g., antimalarial properties [2,3]. They have likewise attracted attention as drugs against human immunodeficiency viruses, HIVs [4]. ‘‘Dimeric’’ (actually dehydrodimeric) naphthylisoquinolines such as michellamines A–C (**1a**–**c**) represent an entirely novel type of quateraryl alkaloids [5,6]. From a biosynthetic point of view, the michellamines are constitutionally symmetric dimers of 5,8′-coupled atropisomeric naphthylisoquinolines, the korupensamines A (**2a**) and B (**2b**), see Fig. 1.

Michellamines are exclusively found in the plant species *Ancistrocladus korupensis* [7], a liana growing in a small area at the Cameroonian/Nigerian border. For phytochemical and biosynthetic investigations, but also for clinical studies on the metabolism, a reliable analytical method for the identification of michellamines in the presence of their

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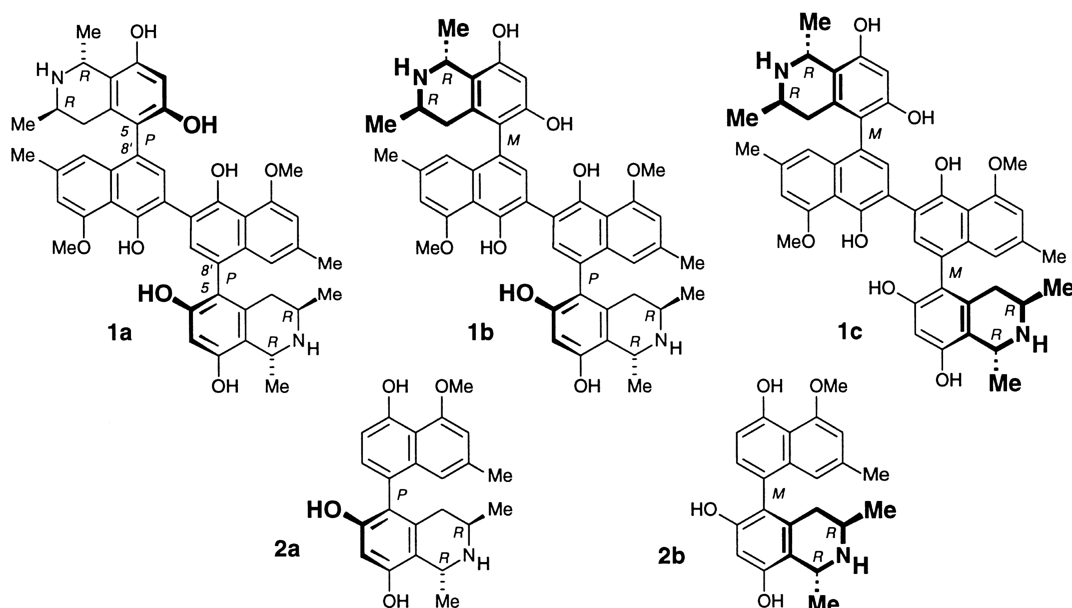

Fig. 1. Dimeric naphthylisoquinoline alkaloids, michellamines A (**1a**), B (**1b**), and C (**1c**), and their putative biosynthetic precursors, korupensamines A (**2a**) and B (**2b**).

monomers in complex matrices is necessary. Established approaches rely on high-performance liquid chromatography (HPLC) with UV detection or micellar electrokinetic chromatography (MEKC) coupled with fluorescence detection [8–10]. But due to the problem of coelution of some of the monomers and dimers, a detection method is required that allows the unambiguous identification of the alkaloids in biological systems. For the efficient analysis of minor compounds in complex matrices, HPLC–tandem mass spectrometry (MS–MS) [11,12] is a powerful analytical method with numerous potential applications. Especially in combination with soft ionization techniques such as electrospray ionization (ESI) [13], HPLC–MS–MS provides versatile approaches to the reliable identification of bioactive natural products [14]. Here we report on a method based on HPLC–ESI-MS–MS for the characterization and identification of various atropisomeric michellamines and their monomeric precursors, the korupensamines. Application of the developed procedure unambiguously proved the formation of michellamine C (**1c**) by enzymatic in vitro dimerization of korupensamine B (**2b**), without isomerization at the axis [15].

2. Experimental

2.1. Chemicals

For chromatography and sample preparation, analytical grade solvents and reagents were used. Water was purified by the Milli-Q system (Millipore, Bedford, MA, USA). Acetonitrile (Pestanal grade) was obtained from Riedel de Haën, (Seelze, Germany) and trifluoroacetic acid (TFA) (spectroscopic grade) from Merck (Darmstadt, Germany). Membrane filters were supplied from Waters (Eschborn, Germany). Michellamines A, B, and C (**1a**–**c**) as well as korupensamines A and B (**2a**,**b**) were all prepared by synthesis and/or by isolation [16,17].

2.2. Analytical procedure

2.2.1. Sample preparation

Each alkaloid was dissolved in acetonitrile–water (2:8, v/v) and diluted to 20 ng μl^{-1} solutions. For HPLC–MS–MS analysis, 50 μl of each of the reference solutions were mixed together. The samples were filtered through membrane filters of pore

size 0.2 μm, and 5 μl of the solutions were subjected to HPLC–ESI-MS–MS analysis.

The experimental details of the enzymatic dimerization experiments have been described elsewhere [15]. The reaction mixture was filtered through a membrane filter of pore size 0.2 μm, and 5 μl of the filtrate were injected for HPLC–MS–MS analysis without further sample treatment.

2.3. Instrumentation

For HPLC–ESI-MS–MS, chromatographic separation was performed on a Symmetry C_{18} column (150×2.1 mm I.D., 5 μm, Waters) with a binary gradient delivered by an Applied Biosystems 140b pump. Solvent A was 0.01% (v/v) TFA in water, solvent B was acetonitrile. HPLC was programmed as follows: t=0 min 15% solvent B, 8 min 23% B, 14 min 30% B, 16 min 40% B, 19 min 40% B. The flow-rate was set to 0.2 ml min^{-1} and the injection volume was 5 μl. HPLC–ESI-MS–MS analysis was performed with a triple stage quadrupole TSQ 7000 mass spectrometer equipped with an ESI interface (Finnigan MAT, Bremen, Germany), and a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) with ICIS 8.1 software (Finnigan MAT). Nitrogen served both as sheath and auxiliary gas, argon as collision gas.

Positive ions were detected by scanning from 150 to 800 u with a total scan duration of 1.0 s for a single spectrum. MS–MS experiments were performed at a collision gas pressure of 2.5 mTorr Ar (1 Torr=133.322 Pa) and a collision energy of 25 eV for korupensamines and 20 eV for michellamines scanning a mass range from 20 to 400 u with a total scan duration of 3.0 s. For HPLC–MS–MS analysis of alkaloid mixtures product ions were detected by scanning alternately from precursor ion m/z 379.4 to m/z 380.4 with a scan duration of 3.0 s for one experiment.

3. Results and discussion

Initial experiments revealed the electrospray process to ionize effectively monomeric naphthylisoquinoline alkaloids such as korupensamines A (**2a**) and B (**2b**). In the positive mode, exclusively protonated molecules $[M+H]^+$ at m/z 380.4 (100% rel. abundance) were obtained from each of the references (see Fig. 2A), whereas the atropisomeric dimeric alkaloids, michellamines A (**1a**), B (**1b**) and C (**1c**), yielded mainly doubly protonated molecules $[M+2H]^{2+}$ at m/z 379.4 (100% rel. abundance) besides the monoprotonated species $[M+H]^+$ at m/z 757.4 (1% rel. abundance, see Fig. 2B). Identification of the doubly protonated molecule $[M+2H]^{2+}$

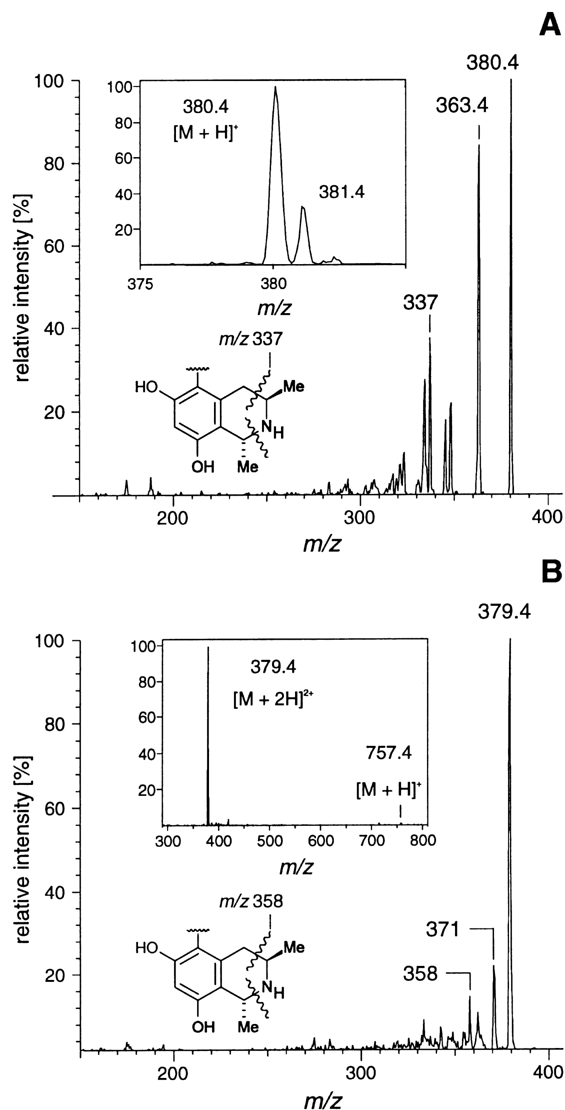

Fig. 2. Product ion spectra of (A) **2b** (25 eV, 2.5 mTorr Ar) precursor ion m/z 380.4 $[M+H]^+$ and (B) **1c** (20 eV, 2.5 mTorr Ar) precursor ion m/z 379.4 $[M+2H]^{2+}$.

was deduced from the characteristic difference of 0.5 u between monoisotopic [[^{12}C]michellamine+2H]$^{2+}$ and [[^{13}C]michellamine+2H]$^{2+}$. Furthermore, product ion spectra confirmed identity of doubly protonated michellamines as will be discussed in detail below.

Low-energy collision induced dissociation (CID) of korupensamines yielded characteristic product ion spectra as shown in Fig. 2A. The most abundant product ion at *m/z* 363.4 (84% rel. abundance) apparently resulted from the loss of ammonia (−17 u) [18]. A further characteristic fragmentation was the neutral loss of the C_2H_5N moiety (−43 u) due to retro-Diels–Alder fragmentation as described for the structurally related tetrahydro-β-carbolines [19], resulting in a product ion at *m/z* 337 (37% rel. abundance, see Fig. 2A). The atropisomeric alkaloids **2a** and **2b**, however, were found to deliver identical spectra.

For the investigation of the dimeric alkaloids **1a**, **1b** and **1c**, the doubly protonated precursor ions [M+2H]$^{2+}$ at *m/z* 379.4 were chosen. CID experiments of the doubly protonated [M+2H]$^{2+}$ at 20 eV, compared to 25 eV as required for the fragmentation of the korupensamines, revealed product ion spectra characteristic for michellamines. Two major reaction pathways of the precursor *m/z* 379.4 were observed: (a) loss of a fragment with formally 8.5 u, which can be explained by the neutral loss of an ammonia unit per doubly charged molecule yielding a product ion [M+2H−NH_3]$^{2+}$ at *m/z* 371 (22% rel. abundance) and (b) loss of 21.5 u due to a retro-Diels–Alder fragmentation per dication (see Fig. 2B) resulting in the product ion [M+2H−C_2H_5N]$^{2+}$ at *m/z* 358 (15% rel. abundance).

Based on the initial experiments described above, the following HPLC–MS–MS experiment was developed for the analysis of mixtures of korupensamines and michellamines: naphthylisoquinoline alkaloids were identified by retention times, protonated molecules [M+H]$^{+}$ for korupensamines and [M+2H]$^{2+}$ for michellamines, and by characteristic product ions formed at specific collision energies (see Fig. 3).

Under the chromatographic conditions selected in the reconstructed total ion chromatogram (Fig. 3D), the monomeric alkaloid korupensamine B (**2b**) and the heterodimer michellamine B (**1b**) were found to coelute. Only by application of MS–MS, a reliable identification of the two korupensamines **2a** and **2b** by their protonated molecules [M+H]$^{+}$ at *m/z* 380.4 (Fig. 3C), and all of the three michellamines, **1a**–**1c**, by their protonated molecules [M+2H]$^{2+}$ at *m/z* 379.4 and their fragments [M+2H−C_2H_5N]$^{2+}$ at

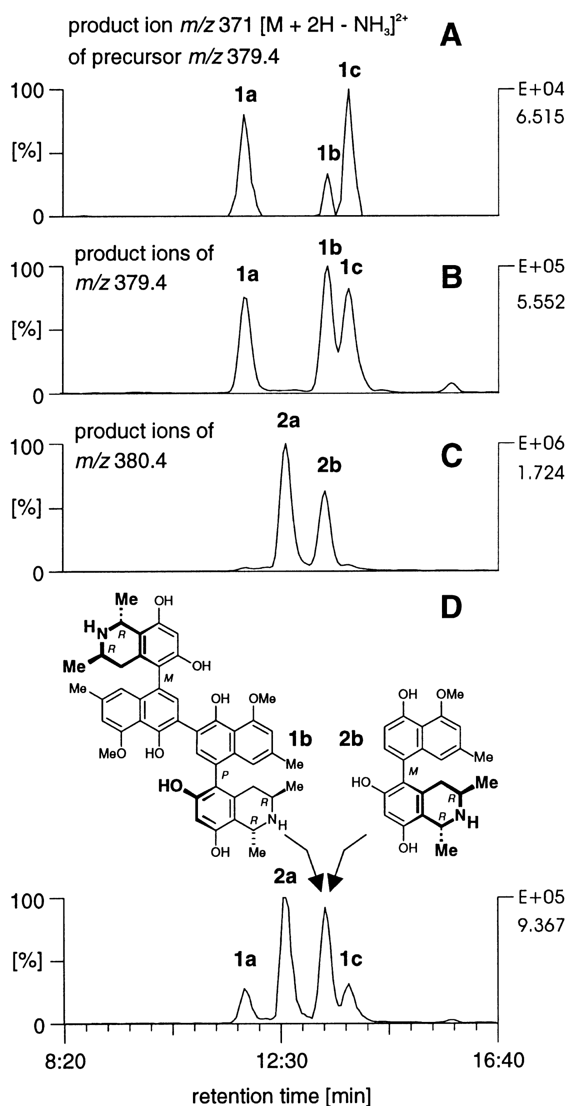

Fig. 3. HPLC–MS–MS chromatogram of a mixture of **1a**–**2b**. (A) Mass chromatogram of product ion *m/z* 371 indicating michellamines; (B) mass chromatogram of product ions derived from *m/z* 379.4 [M+2H]$^{2+}$; (C) mass chromatogram of product ions derived from *m/z* 380.4 [M+H]$^{+}$; (D) reconstructed total ion chromatogram.

m/z 358, respectively (Fig. 3B, Fig. 3A), in the presence of each other, was achieved.

The developed analytical method has meanwhile been applied to study the enzymatic dimerization of korupensamines to give michellamines, presumably the final step in the biosynthesis of these dimeric naphthylisoquinoline alkaloids [15]. Reaction mixtures containing e.g., substrate **2b** and a peroxidase purified from leaves of *Ancistrocladus heyneanus* were analyzed by means of HPLC–MS–MS. Only by the method presented here, korupensamine B (**2b**) was shown to be dehydrodimerized enzymatically to michellamine C (**1c**), exclusively, without formation of michellamine B (**1b**), which might have resulted from a possible atropisomerization via the intermediate phenoxy radical. The formation of michellamine B (**2b**) was excluded by the LC–ESI-MS–MS technique presented (see Fig. 4).

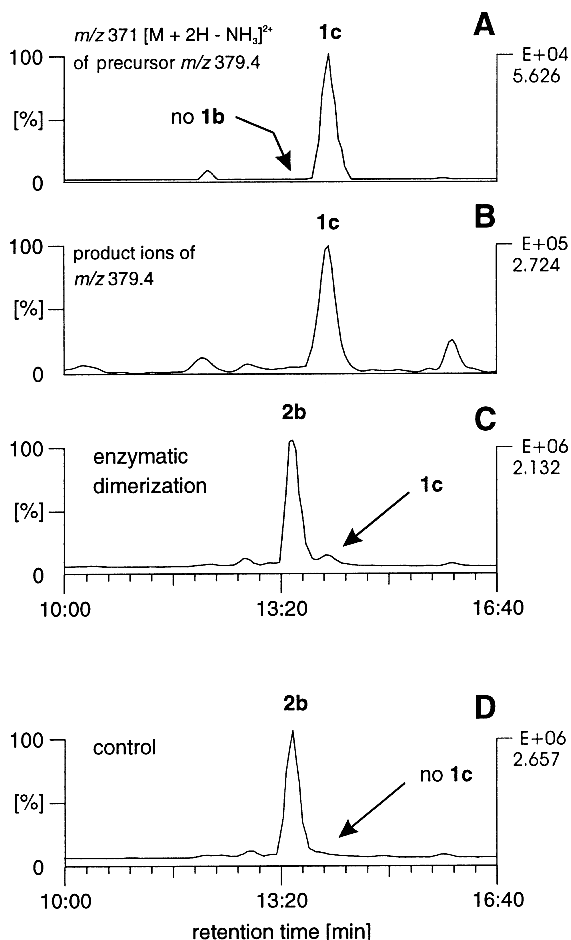

Fig. 4. HPLC–MS–MS chromatogram of a reaction mixture of korupensamine B (**2b**), H_2O_2 and a peroxidase isolated from leaves of *Ancistrocladus heyneanus* [15]. (A) Mass chromatogram of product ion m/z 371 (michellamine C, **1c**); (B) mass chromatogram of product ions derived from m/z 379.4 $[M+2H]^{2+}$ (**2b**); (C) reconstructed total ion chromatogram; (D) reconstructed total ion chromatogram of a control reaction mixture of **2b** without enzyme extract.

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