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Short communication

Acetogenic isoquinoline alkaloids CXXI.[1] Use of on-line high-performance liquid chromatography–nuclear magnetic resonance spectrometry coupling in phytochemical screening studies: rapid identification of metabolites in *Dioncophyllum thollonii*

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Abstract

Exemplarily for a root extract of the West African liana *Dioncophyllum thollonii* a strategy for the rapid identification of secondary metabolites in plant extracts is presented, based upon on-line coupling of high-performance liquid chromatography with nuclear magnetic resonance detection. For a first overview, an on-flow experiment was performed, which allowed the identification of the naphthylisoquinoline alkaloid dioncophylline A. A second alkaloid, 5′-*O*-demethyldioncophylline A, was identified by subsequent application of stop-flow two-dimensional HPLC–NMR ROESY experiments. In the case of coeluting compounds, the design of appropriate time-slice experiments allowed the characterization of the diastereomeric tetralones *trans*- and *cis*-isoshinanolone. Thus, through consequent use of all different experimental modes available in HPLC–NMR, a phytochemical screening of plant extracts can be realized in a fast and reliable way. The NMR detection allows the characterization of diastereomeric compounds even in the case of far-reaching chromatographic coelution.
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1. Introduction

Coupling methods combining powerful chromatographic separation and detection techniques (by mass spectrometry, infrared spectroscopy, or others) are well-established laboratory tools for the analysis of complex mixtures. In the last years the variety of such techniques has reached a new dimension by the introduction of nuclear magnetic resonance (NMR) spectrometers as detectors for liquid chromatography [2,3]. This on-line method links the highly flexible liquid chromatography separation with the enormous information content of the NMR experiment. Still, there are as yet only rather few fields of application and most of the work published so far focuses on the analysis of drug metabolism [4,5]. But also for the directed search for natural products in plant extracts,

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this technique seems to offer many advantages over the conventional off-line method. In the literature, only few examples of application of the high-performance liquid chromatography (HPLC)–NMR approach to plant screening studies have been described [6–12]. We have implemented this promising new tool for the rapid screening of tropical plants for secondary metabolites, especially for naphthylisoquinoline alkaloids. These naturally occurring biaryls, found in the small paleotropical families Ancistrocladaceae and Dioncophyllaceae [13] show remarkable biological properties, including antimalarial and anti-human immunodeficiency virus (HIV) activities [14,15]. In this contribution, we describe an HPLC–NMR based approach with complementary use of all experimental modes for the rapid identification of metabolites in plant extracts without any purification and thus without any time-consuming isolation step. As a rewarding example, we chose the West African plant species *Dioncophyllum thollonii* [16], a member of the small plant family Dioncophyllaceae, which consists of only three species. One of these, *Triphyophyllum peltatum*, is a rich source of naphthylisoquinoline alkaloids with an high amount of structural diversity [13,15]. Former phytochemical investigations on *D. thollonii* had demonstrated the occurrence of naphthylisoquinoline alkaloids, but due to inconsistencies in the structural elucidation, which is discussed in-depth in references [13,15,17], new investigations have become necessary.

2. Experimental

2.1. Chemicals

For chromatography and sample preparation analytical grade solvents and reagents were used. Deuterium oxide (99.9 atom%) was purchased by Deutero (Kastellaun, Germany). Acetonitrile was from Riedel-de Haën (Pestanal grade, Seelze, Germany) and trifluoroacetic acid (TFA, spectroscopic grade) was from Merck (Darmstadt, Germany). Membrane filters were delivered by Waters (Eschborn, Germany).

2.2. Analytical procedure

2.2.1. Sample preparation

Dioncophyllum thollonii (Dioncophyllaceae) was collected by one of us (A.M.L.) at Gabon in 1996. A voucher specimen (No. 1) has been deposited at Herb. Bringmann, University of Würzburg. Five grams of the dried and ground roots was extracted with a mixture of water (pH 2 with HCl)–methanol (8:2, v/v) in an ultrasonic bath at room temperature for 2 h. The extract solution was filtered and lyophilized. A 10-mg amount of this extract was redissolved in 1 ml ^2H_2O–CH_3CN (8:2, v/v) and filtered through a 0.2-μm membrane filter. The resulting solution was directly used for HPLC–NMR analysis.

2.3. Instrumentation

For HPLC–NMR analysis chromatographic separation was performed on a Symmetry C_{18} column (250×4.6 mm I.D., 5 μm) from Waters. The chromatographic system consisted of a Bruker LC22 pump and an UV detector from Bischoff working at 270 nm absorption. An isocratic solvent system with 40% acetonitrile and 60% ^2H_2O [containing 0.01% (v/v) TFA, pH 3] was used for all chromatographic separations. The flow-rate was set to 0.7 ml min^{-1} and the injection volume was 100 μl of the extract solution for the on-flow experiments and 50 μl for the stop-flow experiments. HPLC–NMR analysis was performed with a Bruker DMX 600 NMR spectrometer operating at 600.13 MHz ^1H frequency (Bruker, Rheinstetten, Germany) and controlled by the software system XWinNMR from Bruker. The outlet of the UV detector was connected to the flow-probe by a polyether ether ketone (PEEK) capillary via a BPSU interface (Bruker), controlling the experimental modes. The spectrometer was equipped with an inverse constructed ^1H,^{13}C flow probe with a 4-mm detection cell (Bruker).

The on-flow experiments were acquired using a one-dimensional (1D) NOESYPRESAT sequence [18] for double solvent suppression (**HO**^{2}H and **CH$_3$**CN) generating a pseudo-two-dimensional data set with a time axis and a frequency axis. With a sweep width of 12 kHz and 16 000 data points in F2, 16 scans were coadded per row with an acquisition

time of 0.340 s and a total scan duration of ca. 1 s per scan resulting in a time resolution of ca. 16 s per spectrum.

The one-dimensional time-slice ^{1}H HPLC–NMR spectra were obtained in the stop-flow mode with the following parameters: free induction decays (FIDs) were collected into 32 000 computer data points with a spectral width of 10 kHz. 90° pulses were used with an acquisition time of 1.683 s and the relaxation time delay was set to 2 s. Typically 72 scans were accumulated. Prior to Fourier transformation, an exponential apodization function was applied corresponding to a line broadening of 1.0 Hz. For calibration the residual signal of the acetonitrile was set to 2.0 ppm.

The phase-sensitive HPLC–NMR rotating frame Overhauser enhancement spectroscopy (ROESY) experiments were performed in the stop-flow mode using a sequence [19] with double presaturation during the relaxation time delay of 3 s. FIDs were collected with 200 t_1 increments of each 88 transients into 4000 computer data points. The spectral width was set to 10 kHz in both dimensions. The continuous wave (cw) spin lock pulse (strength of 2.3 kHz) was applied for 700 ms to build up the ROE interactions. With an acquisition time of 0.204 s a total acquisition time of 17 h resulted. The data were apodized with a shifted squared sine bell window function in both dimensions, and in F1 a linear prediction to 1000 data points with 100 coefficients was applied to enhance the resolution.

3. Results and discussion

HPLC–NMR allows the analysis of very complex mixtures even in the case of low chromatographic resolution, because of the unique specificity of the NMR detection [3]. Therefore, a quick and reliable analysis is achieved without any purification steps prior to the analysis. The sample was prepared by extracting the roots with a water–MeOH (pH 2 with HCl) solution at room temperature. To avoid complications caused by residual methanol impurities resulting in an intense NMR signal at 3.5 ppm, the extract was lyophilized and redissolved in $^{2}H_2O$–CH_3CN, which was also the solvent system used for the HPLC separation. The parameters of the chromatographic separation were also kept as simple as possible to allow a fast use without the necessity of optimization of the chromatography for each new experimental mode. Therefore, a simple isocratic separation protocol with acetonitrile–deuterium oxide (40:60, v/v) was used to screen the extract. The NMR detection outweighs the unfavorable separation results as can be seen in Fig. 1, which shows the on-flow contour plot of the resulting NMR chromatogram. In this experiment, the NMR data are collected continuously as the sample passes through the detection cell (see Fig. 1).

A slice corresponding to the retention time of 15.5 min revealed a ^{1}H NMR spectrum that was assigned to the well-known naphthylisoquinoline alkaloid dioncophylline A (**1**)[2], known from the plant *Triphyophyllum peltatum*, which belongs to the same plant family as *D. thollonii*, the Dioncophyllaceae [13]. The identity (of course except for the *absolute* configuration) was confirmed by injection of a reference solution of **1** (50 μg on column) under identical experimental conditions. Both spectra displayed identical spin systems and chemical shifts when compared to each other. A second slice at a retention time of 19.5 min gave a spectrum with a signal pattern very similar to that of **1** (see Fig. 1). Analysis of the spin systems and chemical shifts revealed a constitution identical to **1** except for the presence of only one single methoxy group. The pattern of the aromatic protons with a pseudo-triplet at 7.22 ppm and the chemical shifts of the aliphatic protons at position C-4 (2.8–3.2 ppm) suggested a 7,1′-coupling position of the naphthalene and isoquinoline moiety. For a deduction of the exact position of the OMe group in this alkaloid **2** (4′-C or 5′-C on the naphthalene part), the information content of the simple proton spectrum was not sufficient. This problem was solved by performing a stop-flow HPLC–NMR experiment in combination with nuclear Overhauser effect (NOE) spectroscopy. This experiment correlates spins that are spatially close to each other. We have recently introduced a variant of the two-dimensional ROESY experiment with selective presaturation for HPLC–NMR in the stop-flow mode [19]. Since in the stop-flow mode

[2]Or to *ent*-**1**, since NMR spectroscopy does not provide any information on the absolute configuration.

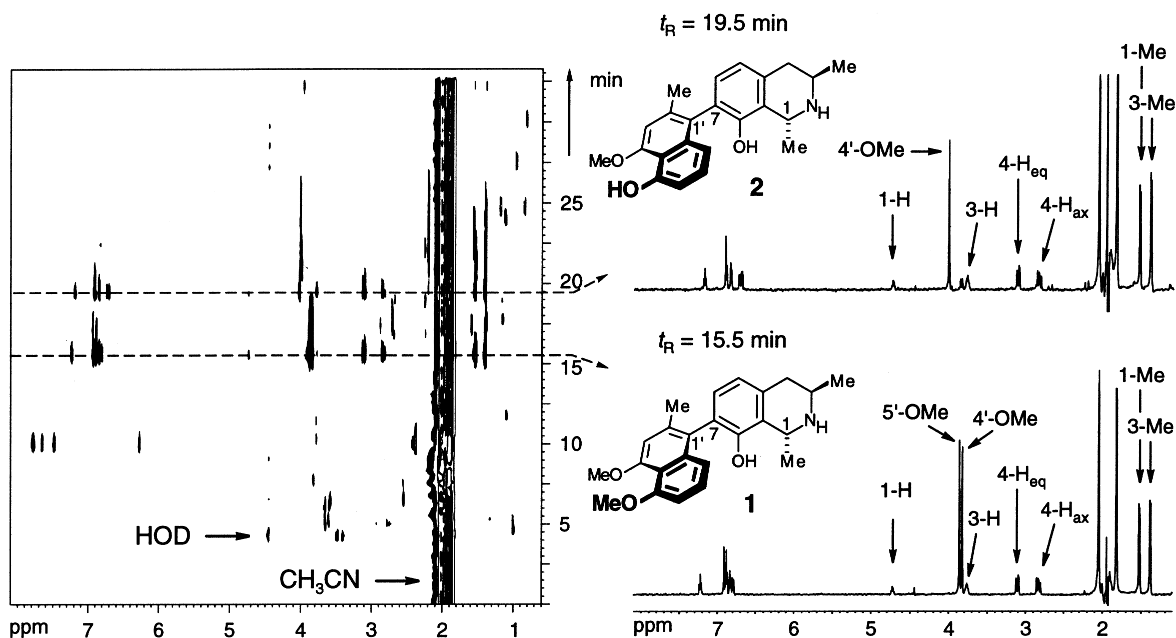

Fig. 1. HPLC–NMR (600 MHz) on-flow experiment (left), performed with 100 μl (corresponding to 1 mg solid sample) of a root extract of *Dioncophyllum thollonii*. Evaluation of the slice (right) corresponding to t_R = 15.5 min revealed the presence of dioncophylline A (**1**). The occurrence of 5′-*O*-demethyldioncophylline A (**2**, t_R = 19.5 min) was proven by further HPLC–NMR experiments (see Fig. 2).

each of the individual components are captured in the probe, the chromatography is stopped by cutting off the solvent flow. Subsequently NMR data collection is started. This mode allows to decide individually how many scans might be necessary to achieve an acceptable signal-to-noise ratio. Besides the detection of minor compounds in the sub-milligram range, this mode is well suited to apply two-dimensional NMR correlation experiments. Fig. 2 presents a section of such a HPLC–NMR ROESY spectrum obtained for the alkaloid with the retention time of 19.5 min.

Careful analysis of all cross peaks unambiguously proved the suggested constitution of **2** and the position of the OMe group to be located at 4′-C in the naphthalene part. Recently, an alkaloid of the same constitution and relative configuration at the isoquinoline moiety, 5′-*O*-demethyldioncophylline A (**2**), was isolated from *Triphyophyllum peltatum* [20], demonstrating the close phytochemical relationship between this species and *D. thollonii*. But compared to *T. peltatum*, *D. thollonii* contains noticeably lower alkaloid quantities.

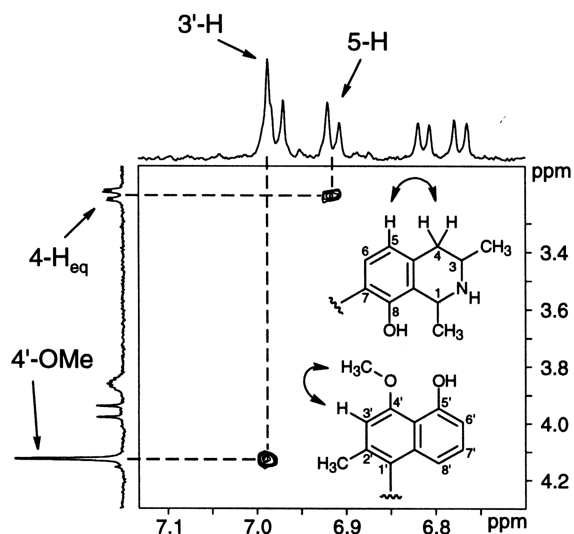

Fig. 2. Section of a stop-flow two-dimensional HPLC–NMR ROESY experiment, performed with the elution peak at t_R = 19.5 min. The cross peak between the aromatic methoxy protons and the 3′-H aromatic singlet determines the position of the methoxy function to be at C-4′.

Additionally, stop-flow HPLC–NMR allows the analysis of coeluting chromatographic peaks in the so-called time-slice experiment. In this mode, for which to our knowledge no concrete application to natural products has so far been described in the literature, the whole chromatographic peak is cut into slices of stopping points for NMR data acquisition. After chosen intervals, e.g., every 10 s, the pump is halted and a ^{1}H NMR spectrum is acquired. Two chromatographically unresolved compounds with different concentrations and slightly different retention times can be distinguished easily with this HPLC–NMR strategy by comparison of all proton spectra acquired at different parts of the observable elution peak. In the extract of *D. thollonii*, a peak eluting at 27.5 min was localized that consisted of two structurally very similar compounds, the diastereomeric tetralones, *trans*- and *cis*-isoshinanolone (**3a** and **3b**), which are biosynthetically close related to naphthylisoquinoline alkaloids [13,21]. Both metabolites were identified by time-slice analysis of the peak with the retention time of 27.5 min (Fig. 3).

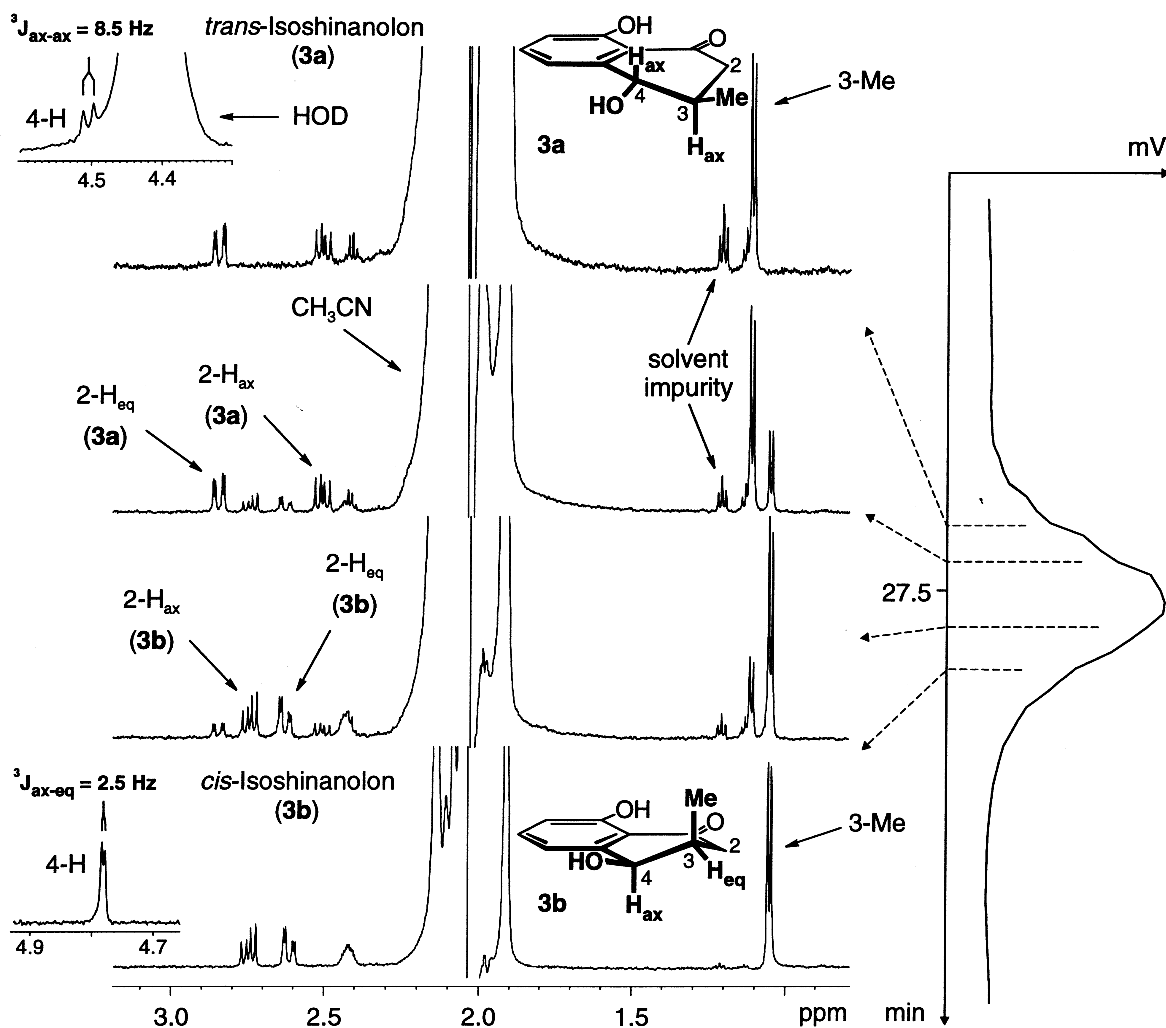

Fig. 3. HPLC–NMR time-slice analysis of the chromatographic peak (schematically sketched on the right) at t_R=27.5 min. The critical sections of the ^{1}H spectra, acquired in 10 s intervals, are shown. Comparison of the spectra monitors the coelution of two diastereomeric compounds (**3a** and **3b**), which are clearly identified by their characteristic ^{3}J coupling constants between 3-H and 4-H.

The assignment of the relative configuration is based on the typical coupling constants of the vicinal protons of 3-H and 4-H. In the case of *trans*-isoshinanolone (**3a**), this constant is 8.5 Hz indicating an axial–axial relationship, while in *cis*-isoshinanolone (**3b**) an axial–equatorial relationship with a coupling constant of 2.5 Hz is observed (Fig. 3). Both diastereomeric compounds have the same molecular mass, thus the characterization of them is not possible with other coupling methods like HPLC–MS or HPLC–diode array detection as they display identical UV and mass spectra. This is an impressive example of the successful application of the time-slice experiment to the distinction of two coeluting diastereomeric compounds directly from an extract without any purification step and clearly demonstrate the potential of HPLC–NMR for characterization of isomolecular mass compounds even in the case of low chromatographic resolution. Besides this, from a phytochemical point of view, the occurrence of *trans*- and *cis*-isoshinanolone (**3a**, **3b**) in *D. thollonii* was proved for the first time [13].

Acknowledgements

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