HPLC-CD On-Line Coupling in Combination with HPLC-NMR and HPLC-MS/MS for the Determination of the Full Absolute Stereostructure of New Metabolites in Plant Extracts

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The applicability of on-line coupling of gradient eluted nonchiral reversed-phase HPLC to circular dichroism (CD) spectroscopy for the stereochemical analysis of crude plant extracts was investigated. The HPLC-system used was a PU-980i ternary gradient pump, equipped with a CD-995 circular dichroism chiral detector, simultaneously monitoring the UV and CD signal at one specific wavelength and even complete characteristic CD spectra in the stop-flow mode.

The constitution and relative configuration of two yet unknown natural products, the isoquinoline phylline and the naphthylisoquinoline alkaloid 5’-O-methyldioncopeltine A, were characterized.

Subsequent HPLC-CD experiments permitted the deduction of the absolute configurations of their CD data. Thus in combination with other analytical techniques, as NMR and MS, CD spectra permitted the complete structural elucidation of the two metabolites, without any isolation and sample purification prior to the coupling experiments.
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The applicability of on-line coupling of gradient eluted nonchiral reversed-phase high-pressure liquid chromatography (HPLC) to circular dichroism (CD) spectroscopy for the stereochemical analysis of crude plant extracts was investigated. A detector measuring simultaneously UV absorbances and CD effects at constant wavelengths in the same detection cell was used. The solvent system water/acetonitrile was found to be suited to give high-quality CD chromatograms as a function of the elution time and even complete characteristic CD spectra in the stop-flow mode. The method was applied to the tropical liana Habropetalum daunui (Dionoeophyllaceae). First, the extract solution was investigated by the combined application of HPLC–NMR and HPLC–ESI-MS/MS techniques. The constitution and relative configuration of two as yet unknown natural products thus detected, the isoquinoline phylline (5) and the neophylloquinoline alkaloid 5-O-methylxionocopelamine (6), were characterized. Subsequent HPLC–CD experiments permitted the deduction of the absolute configurations of these new metabolites by empirical analysis of their CD data. Thus, using identical sample solutions and chromatographic conditions (including the same columns and solvent gradient programs), the combination of NMR, MS, and CD data permitted the complete structural elucidation of the two new metabolites in the extract matrix solution, without any isolation and sample purification prior to the coupling experiments.

The isolation and purification of new natural products, e.g., from plant and marine sources, is often demanding and time-consuming. However, to enlarge the diversity of substance libraries, the search of new structural types for pharmaceutical applications is a rewarding goal even in the age of high-throughput screening of synthetic libraries. For this reason, there is an increasing demand for methods for rapidly identifying and characterizing known or new structures. The combination of liquid chromatography with detection methods such as NMR spectroscopy (HPLC–NMR) and tandem mass spectrometry (HPLC–MS/MS) has recently led to new strategies by which biological matrices, e.g., crude plant extracts, are screened to obtain as much information about known constituents as possible even with a minimum amount of material.1–7

By these techniques, even the discovery of new structures is possible, including the elucidation of the full constitution and, more recently,3 the relative configuration. But due to the intrinsic nonchiral character of NMR signals and mass fragments, no information concerning the absolute configuration is available unless by the use of chiral chromatographic phases, which requires prior possession of both—configurationally known—compounds! A method that allows the assignment of absolute configurations of compounds in an extract matrix would complete the structural elucidation without the necessity of isolation and purification. Widely used for the attribution of the absolute configuration is the application of circular dichroism (CD) spectroscopy, if experimental data from structurally related compounds are available or if structures fit into empirical CD rules.8 A most efficient alternative for the interpretation of CD spectra is their quantum chemical simulation.9 The application of CD spectroscopy, however, usually requires pure compounds. Although the on-line coupling of liquid chromatography with chiral detectors is well-known,10–14 applications are still rare, while in

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the field of phytochemical analysis virtually no work in this direction has been done at all. In the past, detectors based on optical rotation dispersion (ORD) have mainly been used for this purpose.\textsuperscript{11,12} Because of the operation principle, which is based on refractive index differences between right and left circularly polarized light, there are some limitations which include low sensitivity and the lack of compatibility with gradient elution. Circular dichroism, which is based on the absorption difference between right and left circularly polarized light, is much more specific than ORD because CD effects show up only in the presence of a chromophore. This gives a higher intrinsic stability and sensitivity and is compatible with gradient elution. In the literature, most applications of HPLC–CD coupling have focused on the determination of the elution order of the enantiomers of given chiral compounds (e.g., amino acids or drugs) on chiral stationary phases using normal-phase chromatographic solvent conditions.\textsuperscript{13–15} The use of reversed-phase systems with nonchiral columns and CD detection has been reported only for the analysis of model mixtures of small proteins.\textsuperscript{16} No applications of HPLC–CD for the profiling of crude extracts with standard C-18 stationary phases operating under reversed-phase conditions have been reported so far. In this paper, we describe the first application of HPLC–CD on-line coupling to the phytochemical analysis of natural products in plant extracts, specifically for natural and synthetic compounds 1–7 (see Figure 1). Moreover, we present a strategy for the complete structural elucidation, including the absolute configuration, of new alkaloids by the complementary use of the "HPLC–NMR/HPLC–MS/HPLC–CD triad", exemplarily for the tropical liana *Habropetalum dawei*, from which two novel alkaloids, phylline (6) and 5'-O-methylidioncopoline A (6) (see Figure 1), were characterized.

**EXPERIMENTAL SECTION**

**Samples and General Procedures.** Material of the tropical liana *H. dawei* (Dioncophyllaceae) was collected in Sierra Leone in 1997. The extract of 1 g of the dried and ground stem material (15 mL of water (pH 2)/acetonitrile, 9:1, v/v) was centrifuged and directly used for further HPLC–MS/MS and HPLC–CD experiments. For HPLC–NMR, 5 mL of this extract was lyophilized and redissolved in 1 mL of D$_2$O/CH$_3$CN (9:1, v/v). After syringe filtration through a 0.22-µm filter (Waters, Eschborn, Germany), the solution was directly applied to the HPLC–NMR analysis. The related liana, *Triphymylum petiolaris* (Dioncophyllaceae), was present from a previous collection.\textsuperscript{17} The root extract of this plant was prepared as described earlier.\textsuperscript{20} The alkaloids and their synthetic congeners (see Figure 1) were isolated or synthesized as formerly described.\textsuperscript{18–24} Reference solutions of the following compounds, dioncopoline A (1), dioncopoline A (2), N-methylidioncopoline A (3a), N-methyl-7-epi dioncopoline A (3b), (1R,3S)-8-hydroxy-1,3-dimethyltetrahydroisoquinoline ("phylline"), (5R)-8-formyl-8-hydroxy-6-methoxy-1,3-dimethyltetrahydroisoquinoline (7), and its 15,3S-epi (ent-7), were prepared by dissolving 1 mg of the pure compound in 1 mL of water/ acetonitrile (82:8, v/v). The experimental details of the ruthenium-catalyzed oxidative degradation procedure have been described in detail elsewhere.\textsuperscript{25}

**General HPLC Parameters.** All chromatographic separations were performed using Symmetry C-18 columns from Waters (5 µm, 250 × 4.6 mm i.d. for HPLC–CD and HPLC–NMR and 150 × 2.0 mm i.d. for HPLC–MS). As mobile phases (A) acetonitrile (quality Pestican, Kieselgel, Germany), (B) deionized water (Milli-Q quality, Millipore, Eschborn, Germany), and (C) deuterium oxide (99.9 atom %, obtained from Deubner GmbH, Germany) were used. (B) and (C) were acidified to pH 3 with trifluoroacetic acid (TFA, Merck, Darmstadt, Germany).

**HPLC–CD Experiments.** Chromatographic separation was performed using a PU-980i ternary gradient pump (Jasco Deutschland, Gross-Umstadt, Germany), a Rheodyne 7725i injection valve (20-µL loop), and a Jasco CD-95 circular dichroism chiral

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\textsuperscript{22} Brügmann, G.; Brüning, M.; Tschöck, F. Synthesis 1999, 523–558.
\textsuperscript{25} Brügmann, G.; God, R.; Schäffer, M. Phytochemistry 1996, 43, 1493–1493.
detector (Hg–Xe lamp, cell path length 25 mm), simultaneously monitoring the CD and UV signals at one specific wavelength (range 220–420 nm). The baseline-corrected CD spectra were obtained in the off-flow HPLC–CD technique under isocratic conditions (C-18, CH₃CN–H₂O 1:1, pH 3 with TFA). (b) Measured in ethanol with a conventional stand-alone spectrometer (Optograph CD 5, John Veen, France).

**Figure 2.** CD spectra of dioneophylline A (1). (a) Acquired by the stop-flow HPLC–CD technique under isocratic conditions (C-18, CH₃CN–H₂O 1:1, pH 3 with TFA). (b) Measured in ethanol with a conventional stand-alone spectrometer (Optograph CD 5, John Veen, France).

**Figure 3.** Simultaneous UV (absorbance at 302 nm) and CD (CD effect at 302 nm) detected HPLC chromatograms obtained by injection of a root extract of *T. peltatum*. For details, refer to Experimental Section.

HPLC–ESI–MS/MS Experiments. The HPLC system used was an Applied Biosystems dual-syringe pump model 140B (Bai, Bensheim, Germany) equipped with a Rheodyne valve fitted with a 5 µL loop. The separation of the extract solution was carried out with a flow of 200 µL/min. A nonlinear solvent gradient was programmed starting at 5% A and 95% B to 80% A within 30 min. A 5 µL aliquot of extract solution was injected. ESI–MS was performed on a Finnigan triple-stage quadrupole (Q1Q2Q3) TSQ 7000 (Bremen, Germany) equipped with a pneumatically assisted electrospray interface. Data acquisition and postprocessing were done on a Personal DECstation 5000/33 (Digital Equipment, Unterfoehring, Germany) with ICIS 8.1 software. Nitrogen served both as sheath (60 psi) and auxiliary gas (10 units), argon as collision gas. The spray capillary voltage was 3 kV and the temperature of the heated inlet capillary 220 °C. Positive ions were detected by scanning from 150 to 500 m/z with a total scan duration of 1.0 s for a single spectrum. For HPLC–MS/MS analysis, product ions were detected by selecting the protonated precursors [M + H]+ in Q1, performing CID in the collision cell Q2, and detecting the product ions in Q3. MS/MS experiments were performed at a collision gas pressure of 1.8 mTorr and collision energies ranging from 15 to 35 eV (quadrupole framed reference) scanning a mass range from 20 to 400 µ with a total scan duration of 5.0 s. The mass spectra were measured in the centroid mode and referenced to 100% intensity of the base peak.

HPLC–NMR Experiments. The chromatographic separation system consisted of a Bruker LC–220 pump, a Rheodyne valve with a 100 µL loop, and a Biscoff UV detector operating at 270 nm. The outlet of the UV detector was connected to the flow probe via a PEEK capillary (0.25 mm i.d.) and a BPSU (Bruker peak sampling unit) interface from Bruker. The interface and the HPLC system were controlled by a PC using the software packages BPSU-12 and Chemstar from Bruker.

For the HPLC–NMR on-flow experiments, a isocratic composition of 49% A and 51% C at a flow rate of 0.7 mL/min was used, and 100 µL of the extract solution was injected. For the stop-flow experiments, a nonlinear gradient starting at 5% A to
80% A was programmed. At a flow rate of 1 mL/min, 50 μL of the extract was injected.

All HPLC–NMR data were acquired with a Bruker DMX 600 spectrometer (Rheinstetten, Germany) working at 600.13 MHz H frequency and controlled by the XWinNMR software system. The spectrometer was equipped with an inverse 1H, 13C flow probe (cell width 4 mm). To suppress the intense solvent signals (CD3CN and the residual HOD in D2O), a modification of the NOESY PRESAT pulse sequence was applied.

One-dimensional FIDs were obtained collecting rows with 16 accumulated FIDs into 8K computer data points with a spectral width of 12 kHz. Ninety degree pulses were used with a presaturation time of 0.5 s and an acquisition time of 0.340 s resulting in a time resolution of one spectrum per 16 s. Prior to Fourier transformation, an exponential apodization function was applied to the FID corresponding to a line broadening of 1 Hz.

One-dimensional stop-flow spectra were acquired with a solvent suppression irradiation time of 2 s before applying the first 90° pulse. FIDs were collected into 32K data points with a spectral width of 10 kHz and 1.638 s acquisition time. Typically, 64 scans were accumulated. Prior to Fourier transformation, an exponential apodization function was applied to the FID corresponding to a line broadening of 1.0 Hz.

The phase-sensitive TOCSY and ROESY experiments were carried out in the stop-flow mode with experimental parameters identical to those described previously.

RESULTS AND DISCUSSION

Reversed-Phase HPLC–CD Coupling. CD detection coupled with HPLC is usually carried out with chiral phases and normal-phase solvent systems such as hexane or ethyl acetate in order to determine the elution order of enantiomers. For the chiral characterization of compounds in complex mixtures of biological origin, these experimental conditions are not sufficient. One goal of this study was to investigate the practicability of HPLC–CD on-line coupling in the reversed-phase mode. As a test system, we chose naphthyisoquinoline alkaloids, biologically active natural products with axial chirality and centrosymmetry shown by the CD curves of 3a and 3b. The elucidation of their absolute configuration is based mainly on a ruthenium-catalyzed degradation procedure for the stereogenic centers and on nonempirical quantum chemical CD methods for the axial configuration. Likewise, HPLC procedures for the analysis of this type of alkaloid have been elaborated. The solvents water (acified with TFA to pH 3) and acetonitrile are transparent down to 190 nm, which was proven by recording a baseline UV spectrum with a conventional off-line CD spectrometer. To test the effect of the chromatographic reversed-phase system on the CD spectra under HPLC–CD coupling conditions, a reference solution of 1 was prepared, of which 1 μg was injected under isocratic conditions. Due to the necessity of gradient elution for complex mixtures, which results in the fact that the solvent composition is a function of the elution time, tR, tR should have a nonnegligible influence on the CD spectra. To avoid such effects, we measured all reference CD spectra with the theoretically calculated eluent composition that would be present in the detector cell if the optimized gradient was used, i.e., ~1:1 in the case of 1. The HPLC–CD system used consists of a conventional pump and a novel detector that allows simultaneous recording of UV and CD signal at constant wavelength. It is also possible to detect the CD response as a function of the wavelength on-line (scan range 220–420 nm). This, however, takes ~15 s and requires broad elution peaks and hence poor chromatographic resolution if two or more data points are desired. Therefore we chose a simple stop-flow approach, mechanically separating the solvent flow from the column and the detector by manually opening the waste valve at the pump as soon as the desired peak had been trapped in the detection cell.


Then, a CD spectrum was acquired using the scan rate with the best resolution. In Figure 2 the corresponding CD spectrum is compared with a conventional spectrum acquired in ethanol.

Both spectra show positive ellipticities with two distinct peaks between 210 and 280 nm and a negative one in the region of 296–330 nm. The maxima of the peaks is slightly shifted (by ~3 nm), which may be explained by solvent effects. This example demonstrates that the spectrum quality (shown without mathematical curve smoothing) achieved under the HPLC–CD conditions described above is acceptable for empirical comparisons. The negative ellipticity at 302 nm was further used to monitor the separation of a crude root extract of _T. pallidum_, which contains 1 as the main secondary metabolite. Figure 3 shows the resulting UV- and CD-detected chromatograms, obtained by injection of the extract with gradient elution.

At the retention time _t_r = 28.2 min_, typical for 1 within the applied gradient program, the expected negative CD effect was observed. From former HPLC–NMR investigations on this extract it is known that a second alkaloid, 2, elutes at _t_r = 23.5 min_. Compound 2 shows a CD behavior similar to that of 1 because of the close structural relationship. Interestingly, the CD chromatogram not only gives chiral information but also exhibits a remarkably higher resolution when compared with the corresponding UV chromatogram due to the different signs of the CD signals. The chromatographic CD profile of the corresponding UV-detected peak at _t_r = 28.2 min_ demonstrates that at least three different chiral compounds form this absorption peak with a positive CD sign in the front followed by two peaks with negative sign, of which the most intense one corresponds to 1. Thus, the chromatographic resolution achieved can be visualized by CD detection even if the absorbance monitors only one single peak.

**Application of the Method to a New Plant Species.** Recently, it was demonstrated that the application of HPLC–NMR and HPLC–electrospray-MS/MS to the phytochemical analysis of plant extracts is a powerful tool for the discovery of new naphthylisoquinoline alkaloids.[2] We will demonstrate in the following that by combination of HPLC–NMR, HPLC–MS/MS, and HPLC–CD data, it is possible to fully characterize new metabolites in crude extracts without time-consuming isolation procedures by using identical chromatographic conditions for all of the three coupling techniques. As an example, we chose a stem extract of _H. daueri_, of which only a few grams of plant material were available. Thus, conventional isolation procedures, which require significantly higher quantities, were not possible. Former classical investigations revealed the occurrence of the
teratolone 4,26 but no alkaloids have so far been reported. For a first overview, an HPLC-NMR on-flow experiment was performed (see Figure 4).

Analysis of the spin systems and chemical shifts suggested the presence of 4 and, in addition, the occurrence of 1 and two atropodiastereomeric N-methyl derivatives, 3a and 3b, which have been long known from the West African liana Ancistrocladus abbreviatus (Ancistrocladaceae).27 In addition, 3a and 3b have recently been discovered in Dicenophyllum balteum (Dictyonophyllum), by HPLC-NMR analysis; their heretofore unknown occurrence in H. daewi is now established by HPLC-ESIMS/MS experiments with gradient elution. Figure 5 shows the corresponding chromatogram obtained in the full-scan MS mode.

The alkaloids 1, 3a, and 3b, and the tetraolone 4 were identified by their protonated molecular ions [M + H]+ with m/z 378 for 1, m/z 392 for 3a and 3b, and m/z 193 for 4. The elution order of the diastereomeric alkaloids 3a and 3b was determined by spiking the extract with reference solutions and testing for coelution. Their identity was further proven by product ion experiments (data not shown). The protonated molecule ions were isolated in the first quadrupole and fragmented by argon atoms under low collision energy conditions (15–35 eV). The spectra were compared with reference spectra obtained under identical experimental conditions. Both coupling experiments, HPLC-NMR and HPLC-MS/MS, allow the unambiguous identification of diastereomeric compounds because of different retention behavior in chromatography and slightly different chemical NMR shifts. However, these techniques fail if enantiomers have to be distinguished. In principle it is likewise possible that the enantiomeric form of 4 (i.e., ent-1, with S,S, instead of R,R,P configuration) is present in H. daewi. For this reason, HPLC-CD coupling experiments were performed on the extract. With the stop-flow strategy, it was possible to obtain a CD spectrum from the peak corresponding to the elution of the compound with the constitution and relative configuration of 1 (see Figure 6).

The CD spectrum of that peak was found to be identical to the reference spectrum (see Figure 2 top), thus proving the occurrence of 1 rather than ent-1 in H. daewi. This example demonstrates that the combined use of HPLC coupled to NMR, MS/MS, and CD is able to characterize chiral metabolites of known structure rapidly without any isolation step.

**Complete Structural Elucidation of New Natural Products.** Evaluation of the MS data revealed two chromatographic peaks in Figure 5, denoted A (m/z 178 [M + H]+) and B (m/z 394 [M + H]+), which displayed fragmentation pathways typical of tetrahydroisquinolines.28 For example, both unknown compounds undergo a retro-Diels–Alder fragmentation (neutral loss of 43 u) in the nitrogen-containing heterocycle, resulting in [M + H - C4H5N] ions.29 Both peaks were intensively analyzed by means of HPLC-MS/MS and HPLC-NMR. In the following, the determination of the constitution and relative configuration is briefly discussed. Peak A showed a molecular mass of 177 u. Careful analysis of the mass spectrum and the NMR spectrum suggested the occurrence of a naphtthaleae-free isouquinoline with the gross structure as shown in Figure 7a. From the chemical shifts of the aliphatic protons, a relative trans configuration of the methyl groups was deduced.30

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From former synthetic work, a reference sample of enantiomerically pure 5 with 1R,2R configuration was available. In Figure 7, the mass spectrum of peak A is compared with that of 5. Both spectra show identical fragmentation patterns and fragment intensities. Finally, the identity of peak A with the gross structure of 5 was proven by co-injection of extract and reference solution with HPLC–ESI-MS/MS detection, giving a perfect coelution. Thus, the new alkaloid should possess the same constitution and relative trans configuration as the reference 5, an intermediate in the total synthesis of naphthylisoquinoline alkaloids. The last information still required at this point, was the absolute configuration at the stereogenic centers.

Peak B was intensively studied by stop-flow HPLC–NMR. The concentration was high enough to perform two-dimensional HPLC–NMR experiments. In Figure 8, all experiments including TOCSY and ROESY experiments are summarized.

Analysis of the spin systems and chemical shifts with consideration of the cross-peak network in TOCSY and ROESY suggested a structure similar to that of the known alkaloid 2, but with an additional methyl group at the C-5′ oxygen. Like 2, it has a relative trans configuration at the isoquinoline moiety. As is the case for 5, this alkaloid, 5′-O-methylisolocryptopine A (6), has not yet been described as a natural product in the literature. Compare, however, the alkaloid "O-methyltriptopholine", which has been reported to have the same constitution as 6; no information was acquired, however, on its relative or absolute configuration, nor is any comparison material available any more, so that 6 has to be considered and treated as a new compound.

To deduce the absolute configurations of the two new natural products, the HPLC–CD coupling technique was applied. For this purpose, the same extract that had been used for the HPLC–NMR and HPLC–MS/MS experiments was separated under identical chromatographic conditions. Due to the relatively high concentration of 6 (corresponding to peak B), it was possible to acquire a complete CD spectrum of 6 in the stop-flow mode. In Figure 9, the CD spectrum of 6 is compared with the spectra of reference compounds 2 and 3b, which are constitutionally closely related and therefore appropriate for empirical CD comparisions.

Alkaloid 6 shows CD ellipticities identical to those of the reference compound 2. As expected, the CD spectrum of 3b exhibits a mirror image-like behavior. Since the effect of the additional O-methyl group on the chromophore should be negligible, this indicates an M configuration at the biaryl axis of 6 according to the absolute configuration of 2. The CD spectra of naphthylisoquinoline alkaloids are dominated by the effect of the π systems connected by the biaryl axis. Up to now it has not been possible to separate the contributions of the single stereoelements, so that the absolute configuration at the stereocenters are not deducible from the HPLC–CD data. Therefore, the whole extract was subjected to the well-established ruthenium-catalyzed oxidative degradation procedure with subsequent GC–MSD analysis of the chiral degradation products. The exclusive (>99%) formation of (S)-anhydrohyoscine acid (data not shown) clearly revealed that only tetrahydroisoquinoline substructures with an R configuration at C-3 were present in the extract mixture. This is also in agreement with the observation that all the naphthylisoquinoline alkaloids that have so far been isolated from Dioncophyllum plants are 3R-configured. 2,27 From the known relative trans configuration of compound B at the isoquinoline moiety, finally the full stereostructure of 6, with R,R,R configuration, is deducible.

For the determination of the absolute configuration of peak A, the application of a different strategy became necessary because the concentration in the extract solution (combined with low ellipticities due to the absent naphthyl chromophore) was too low to scan a complete CD spectrum in the stop-flow mode. Therefore, it was decided to acquire CD spectra of reference compounds with known absolute configuration and look for a CD ellipticity
maximum at a distinct wavelength that is characteristic of the absolute configuration. Then, an HPLC–CD experiment performed at this wavelength would allow a sensitive detection of the CD effect of the isomeric compound corresponding to peak A. As reference structures, the isomeric 5, 7, and ent-7, which were available from total synthetic work, were chosen. The corresponding CD spectra, acquired under stopped-flow conditions with a solvent composition according to the actual composition at the elution time of peak A, are shown in Figure 10. The spectrum of 7 (not shown) is the mirror image of the one of ent-7 and thus similar to the one of 5.

Evaluation of the course of ellipticities along the wavelength axis revealed a maximum CD effect at 266 nm. For the reference 5 with R, R configuration, a positive CD peak was observed (Figure 10 top). Thus, the enantiomeric S, S form, which was not available as a reference, should exhibit a negative effect at this wavelength. This is demonstrated by the CD spectrum of the structurally related S, S configured compound ent-7, which shows a negative CD ellipticity at 266 nm. Consequently, an HPLC–CD experiment with the extract solution and UV/CD detection at 266 nm was performed. The corresponding chromatograms are shown in Figure 11.

The CD chromatogram (bottom) displays a positive sign for peak A, thus unambiguously establishing R, R configuration for this alkaloid. The structure of this new natural product from *H. dawei* is fully identical to that of the known synthetic intermediate 5. Being exactly the heterocyclic half of dioncophylline A (1) as well as dioncophyllines B–D (structures not shown), 5 was named phylline. From a biosynthetic point of view, 5 is presumably a biogenetic precursor to all these dioncophyllines A–D and is the smallest known Diconophyllaceae alkaloid, and thus the "parent" acetogenenic isooquinoline alkaloid. The NMR signals of the two new alkaloids, 5 (peak A) and 6 (peak B), and their assignments are available as Supporting Information.

The complete structural elucidation of 6 and 5 demonstrates that the HPLC–CD technique allows the deduction of absolute configurations in a simple way from the crude extract if a CD spectrum can be measured in the stop-flow mode or if the characteristic CD sign at a distinct wavelength is known, followed by empirical comparison with the CD behavior of structurally related compounds with known absolute configurations. Since significant solvent UV peaks are not observable in the CD chromatogram, this selectivity of CD detection allows one to simplify the chromatographic profile because only chiral compounds that give CD signals are monitored.

**CONCLUSIONS**

It has been demonstrated that the coupling of HPLC with CD detection gives valuable information on single compounds in...
mixtures. In contrast to the chromatography with chiral phases in the normal-phase mode mainly applied to date, it is possible to use a reversed-phase system. This enables one to analyze samples with a complex composition that need a highly efficient chromatographic separation technique. It was shown that even crude plant extracts may be screened by this coupling method. Through a complementary combination of data derived by HPLC–NMR, HPLC–ESI-MS/MS, and HPLC–CD experiments, where identical chromatographic conditions allow a rapid and simple analysis, the complete structure of novel secondary plant metabolites is accessible directly from the extract matrix without any isolation step. In future studies, the method will be further optimized by systematic investigations on solvent effects and detection limits. This can be accompanied by theoretical studies on the effect of the aqueous solvent compositions to the CD spectra, aiming at the elucidation of absolute configurations by comparison of experimental CD spectra of new compounds with calculated spectra in a nonempirical way. This would open the way to the determination of the configuration of new, unprecedented structure types by HPLC–CD. In addition, CD detection in HPLC analysis can be helpful any time if samples with low chemical purity have to be investigated, including the rapid and reliable characterization of metabolites in body fluids, the screening of complex compound libraries, and the analysis of the secondary structure of small proteins in protein mixtures.

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SUPPORTING INFORMATION AVAILABLE

A table with the NMR assignments of 5 and 6 is available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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