Combining HPLC-PDA-MS-SPE-NMR with Circular Dichroism for Complete Natural Product Characterization in Crude Extracts: Levorotatory Gossypol in *Thespesia danis*

Kennett Sprogøe, Dan Stærk, Hanne L. Ziegler, Thomas Høgh Jensen, Søren B. Holm-Møller, and Jerzy W. Jaroszewski*

Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark

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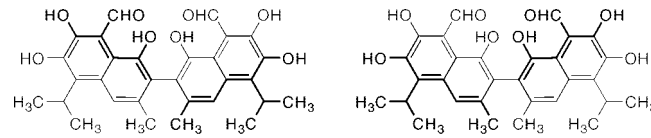
Abstract: Despite recent demonstration of the power of HPLC-PDA-MS-SPE-NMR (high-performance liquid chromatography−photodiode-array detection−mass spectrometry−solid-phase extraction−nuclear magnetic resonance) in structure determination of natural products directly from minute amounts of crude extracts, this technique leaves chirality of the compounds uncharacterized. In this work we demonstrate that postcolumn SPE is a useful method of analyte concentration and accumulation not only for NMR but also for CD (circular dichroism) spectroscopy. Thus, use of HPLC-PDA-MS-SPE-NMR in combination with CD allowed rapid detection of (*R*)-(−)-gossypol [(*R*)-**1**] in *Thespesia danis*, providing a very rare example of the predominance of the levorotatory enantiomer of gossypol. Enantioselectivity of the in vitro antiplasmodial activity of gossypol was also demonstrated; the IC_{50} value of (*R*)-**1** was 4.5 ± 0.2 μM, with the eudismic ratio of about 2.5. No gossypol was detected in *Gossypioides kirkii*.

Substitution of direct HPLC-NMR methods with HPLC-SPE-NMR, where an automated postcolumn solid-phase extraction (SPE) interface is used for analyte concentration and accumulation as well as for replacement of a nondeuterated HPLC solvent with a deuterated NMR solvent, greatly extended sensitivity and general utility of HPLC-NMR hyphenation.[1–3] The power of extended HPLC-PDA-MS-SPE-NMR hyphenation in discovery of *new* natural products directly from crude extracts has already been amply demonstrated.[2,4–10] Thus, combined use of homo- and heteronuclear ^{1}H-detected 2D NMR and MS data acquired with an integrated HPLC-PDA-MS-SPE-NMR system allows safe structure determination of even relatively complex natural products as efficiently as using traditional isolation schemes, but much faster. However, chirality of the compounds, an important property in relation to biological activity, is left unspecified by the HPLC-PDA-MS-SPE-NMR analysis. In this communication, we illustrate the concept of analyte separation, focusing, and accumulation by an automated SPE interface as a preparatory step not only for subsequent acquisition of NMR data but also of CD data in order to achieve complete natural product characterization directly from crude extracts. When implemented in an automated mode, the future HPLC-PDA-MS-SPE-NMR-CD system is envisioned as an effective natural product discovery tool.

Gossypol (**1**) is a polyphenolic aldehyde produced by cotton plants (*Gossypium* species).[11–14] It has also been found in a few other genera belonging to Gossypioideae.[15–18] Due to hindered rotation about the pivot bond between the two naphthyl moieties, axial dissymmetry of the molecule gives rise to the existence of (−)- and (+)-enantiomers, with the absolute configuration *R* and *S*, corresponding to the helical sense *M* and *P*, respectively.[19,20] While being a toxic contaminant of cottonseed products, gossypol exhibits a number of potentially useful biological activities, including cytotoxic activity.[13,14,21–26] Consequently, gossypol and its derivatives are currently being evaluated in phase I and II clinical trials as anticancer drugs.[27] Biological activities of gossypol exhibit pronounced enantioselectivity, (*R*)-**1** being the active enantiomer.[13,14] However, plants usually accumulate varying excess of the (*S*)-(+)-enantiomer,[17,28–30] and efforts to identify species that produce pure (*R*)-**1** for biopharmaceutical applications have thus far been unsuccessful. Only *Gossypium barbadense* and a few wild cotton varieties were found to produce (*R*)-**1**, but the enantiomeric excess never exceeded 30–35%,[17,31–34] and no (*R*)-**1**-producing plants outside the genus *Gossypium* have yet been identified.[17]



(*R*)-**1** (*M*)-(−)-gossypol (*S*)-**1** (*P*)-(+)-gossypol

Extraction of aerial parts of *Thespesia danis* Oliv. (Malvaceae) with aqueous acetone[35] afforded crude extracts that were analyzed by HPLC and HPLC-PDA-MS-SPE-NMR. Prior to the extract analysis, evaluation of postcolumn SPE trapping and elution parameters was performed using an authentic sample of (*S*)-(+)-gossypol. Thus, aliquots of (*S*)-**1** (75 μg) were injected to a C_{18} column, postcolumn SPE trapping was performed with eight different stationary phases (see Experimental Section), and the resulting ^{1}H NMR spectra were assessed after desorption of the analyte into the NMR flow probe with acetonitrile-d_3. In accord with recent evaluation[36] of stationary phases for HPLC-SPE-NMR, the polymeric phase GP was the most efficient and was chosen for all subsequent experiments. Subsequently, elution of gossypol from the GP phase was evaluated with four different deuterated solvents (acetone-d_6, acetonitrile-d_3, chloroform-*d*, and methanol-d_4), using a ^{1}H NMR spectrum obtained after direct injection of a standard solution of (*S*)-**1** into the flow probe as a reference (see Experimental Section). Acetonitrile-d_3 proved to be the most efficient elution solvent; even though the elution profile of gossypol was relatively broad with considerable tailing, resulting in some of the analyte being left outside the 60 μL volume of the NMR cell (Figure 1), tens of micrograms of gossypol could be readily delivered to the NMR probe, furnishing high-quality ^{1}H NMR data in the HPLC-SPE-NMR mode.

After this initial evaluation of sorption, desorption, and push-volume conditions, optimized HPLC-PDA-MS-SPE-NMR experiments with plant extracts were performed. The extract of *T. danis* contained a peak of gossypol at t_R 10.8 min (Figure 2), as shown by UV, MS, and ^{1}H NMR spectra following cumulative SPE trappings. Since dilute solutions of gossypol were unstable, fresh extracts obtained by a simplified procedure (no evaporation) have been used in all quantitative analyses. From the intensity of HPLC peaks, the content of gossypol in *T. danis* could be determined as 0.19% of dry weight. In parallel experiments, cartridges cumulatively loaded with gossypol by automated HPLC-SPE trappings were manually eluted with 200 μL of acetonitrile into a 1 mm quartz cell, gossypol concentrations in the eluates were determined from UV spectra, and CD spectra of the solutions were determined. *T. danis* contained gossypol exhibiting a negative Cotton effect associated with the long-wavelength UV transition,[19,37,38] i.e., (*R*)-(−)-gossypol (Figure 3). From the intensity of the CD bands, the enantiomeric excess of (*R*)-**1** was determined as 44% [72:28 ratio between (*R*)-**1** and (*S*)-**1**].

* To whom correspondence should be addressed. Tel: (45) 35336372. Fax: (45) 35336040. E-mail: jj@farma.ku.dk.

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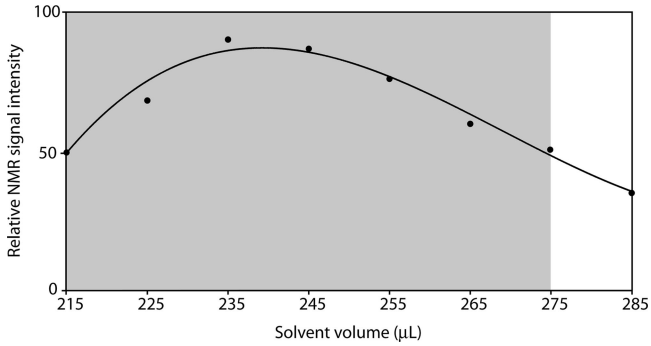

Figure 1. Elution profile of gossypol into the NMR probe from an SPE cartridge (GP phase, 10 × 2 mm i.d.) loaded with 75 μg of (+)-gossypol [(*S*)-**1**] by postcolumn trapping. Acetonitrile-d_3 was pushed through the cartridge in 10 μL steps, recording the intensity of the ^{1}H NMR signal for each step; 210 μL start volume corresponds to the amount of solvent necessary to establish a deuterium lock signal; shaded area represents the volume of the NMR cell.

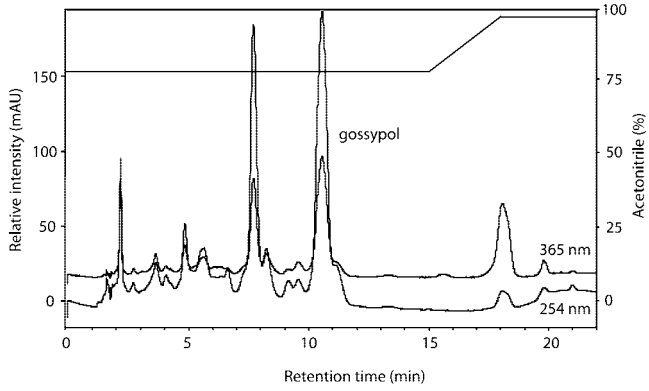

Figure 2. HPLC traces (254 and 365 nm) obtained with crude extracts of *T. danis* [150 × 4.6 mm i.d., Phenomenex Luna C_{18}(2) column, 3 μm, eluted at 0.8 mL/min with acetonitrile gradient in water (0.1% formic acid) as shown].

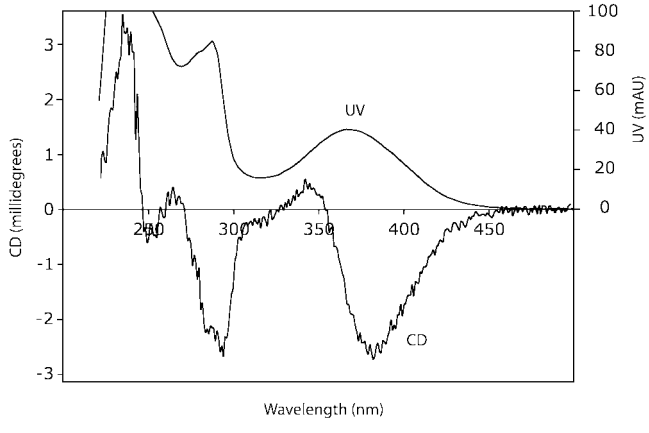

Figure 3. UV spectrum of gossypol in *T. danis* obtained with a PDA detector from a chromatogram similar to that shown in Figure 2, and CD spectrum obtained after four cumulative HPLC-SPE trappings of gossypol peak (GP phase, 10 × 2 mm i.d.) and elution of the SPE cartridge with 200 μL of acetonitrile.

The presence of levorotatory gossypol with a relatively high optical purity in *T. danis* is somewhat surprising, as another *Thespesia* species, *T. pupulnea*, consistently produces practically pure (*S*)-(+)-enantiomer.[17,38–42] This result shows that further search for natural sources of (*R*)-**1** should include all wildly growing plants that belong to gossypol-producing genera. Analysis of an extract of leaves of *Gossypioides kirkii* (Mast.) J. B. Hutch., another Gossypioideae species included in this work, failed to demonstrate any presence of gossypol, even though other studies indicated that *G. kirkii* is capable of producing gossypol of unknown enantiomeric composition.[43]

Gossypol and its derivatives have been reported to exhibit antiplasmodial activity,[44–46] but to our knowledge the antiplasmodial activity of gossypol enantiomers has not been evaluated. We found that similarly to other biological activities of gossypol, the antiplasmodial activity is highly enantioselective, with IC_{50} values of (*S*)-(+)-, (*R*)-(−)-, and (*RS*)-(±)-gossypol of 47.9 ± 2.1, 4.5 ± 0.2, and 12.0 ± 0.6 μM, respectively (*Plasmodium falciparum* strain 3D7). Because of the evidence that some compounds exhibit in vitro antiplasmodial effects due to their physical interaction with erythrocyte membrane, microscopically observable as stomatocyte or echinocyte formation,[47–49] we assessed membrane morphology of erythrocytes incubated with **1**. Racemic **1** and the pure enantiomers exhibited stomatocytogenic action at concentrations above 40 μM, but the transformation of discocytes to stomatocytes was practically complete (>90%) only at concentrations around 100 μM, with no appreciable differences between the stereoisomeric forms. No changes of the normal discocyte form of erythrocytes were observed at concentrations corresponding to the IC_{50} value of (*R*)-**1** or the racemate. This confirms that the antiplasmodial activity of (*R*)-**1** represents a genuine and stereoselective pharmacological activity, warranting further investigations.

The present work represents a proof-of-concept of an extended hyphenation system involving UV, MS, ^{1}H NMR, and CD spectroscopy and employing an SPE interface. Although HPLC-CD is increasingly popular for characterization of optically active analytes,[50–54] and a combination of HPLC-CD with direct HPLC-NMR methods for natural product characterization has already been exploited,[55–62] hyphenation of CD with HPLC-SPE offers numerous advantages, similar to those of replacing HPLC-NMR with HPLC-SPE-NMR. Thus, the SPE interface decouples the HPLC separation from the CD data acquisition on the time scale, offers increased analyte concentrations due to analyte focusing and accumulation during the SPE step, and enables recording of CD spectra in a well-defined solvent used for elution of the SPE cartridge, rather than in a mixed HPLC mobile phase under stopped-flow conditions, as practiced previously.[55–62] Thus, a future integrated HPLC-PDA-MS-SPE-NMR-CD system will offer a convenient way of obtaining chirality data along with comprehensive natural product characterization by NMR methods in natural product discovery programs.

Experimental Section

General Experimental Procedures. UV spectra were recorded at ambient temperature on a Spectronic Unicam Helios Gamma spectrophotometer using a 1 mm quartz cell. CD spectra were recorded at 20 °C on an Olis DSM 10 CD spectrophotometer equipped with a Quantum Systems temperature control module, using a 1 mm quartz cell (integration time 5–10 s, 1 data point per nm). The UV and CD spectra were recorded with freshly prepared solutions in acetonitrile. The HPLC-PDA-MS-SPE-NMR system consisted of an Agilent 1100 series chromatograph (quaternary pump, autosampler, column oven, PDA detector), a flow splitter (5:95), a Bruker Esquire LC ion-trap mass spectrometer with electrospray-ionization source, a Knauer K100 Wellchrom postcolumn solvent delivery pump, a Spark Holland Prospekt 2 SPE unit, and a Bruker Avance 600 spectrometer (^{1}H frequency 600.13 MHz) equipped with a ^{1}H{^{13}C} flow probe (30 μL active volume, 60 μL total volume), operated at 25 °C. 1D ^{1}H NMR spectra were acquired either using NOESYPRESAT pulse sequence for dual presaturation of solvent resonances (H_2O and CD_2HCN) during the mixing time (100 ms) and relaxation delay (2.4 s) or without solvent-peak suppression; typically, 256–512 transients were accumulated. Mass spectra were acquired in positive- as well as negative-ion mode. Water was purified by deionization and 0.22 μm membrane filtration (Millipore). HPLC grade solvents from commercial suppliers were used for

all operations. Samples of racemic and (*S*)-**1** were available from earlier studies at this laboratory;[17] (*R*)-**1** was obtained as described elsewhere.[63]

Plant Material. Twigs (with leaves and flowers) of *Thespesia danis* Oliv. and leaves of *Gossypoides kirkii* (Mast.) J. B. Hutch. were collected on April 1 and March 31, 2001, respectively, in Shimba Hills National Park, Kwale District, Kenya. The plants were authenticated by Mr. Simon G. Mathenge, Department of Botany, University of Nairobi. The material was air-dried immediately after the collection and kept in paper bags until use. Voucher specimens (DFHJJ38 and DFHJJ39, respectively) were deposited in Herbarium C (Botanical Museum, University of Copenhagen, Denmark).

Sample Preparation. Finely ground plant material (5 g) was extracted with 50 mL of acetone–water (7:3) for 24 h at ambient temperature. The extracts were filtered, concentrated, and lyophilized to give 517 mg of crude extract of *T. danis* or 580 mg of crude extract of *G. kirkii*. The residues were reconstituted with acetonitrile (1 mL/50 mg) and centrifuged to remove insoluble particles. In an alternative, simplified procedure, samples of plant material of *T. danis* (0.5 g) were extracted with 4–5 mL of acetone–water (7:3) for 3 h at room temperature with 3 × 5 min sonification periods (at the beginning, in the middle, and toward the end of the extraction period), the mixtures were centrifuged, and the extracts were used directly.

HPLC Separations. All HPLC separations were performed at 40 °C using a 150 × 4.6 mm i.d. Phenomenex Luna $C_{18}(2)$ column (3 μm, 100 Å) with a guard column. A binary eluent, delivered at 0.8 mL/min, was obtained by mixing solvent A [0.1% formic acid in water−acetonitrile (95:5)] and solvent B) [0.1% formic acid in acetonitrile−water (95:5)] as follows: 0 min, 80% B; 15 min, 80% B; 18 min, 100% B; 22 min, 100% B. The chromatography was monitored at 254 and 365 nm.

HPLC-PDA-MS-SPE-NMR Experiments. The postcolumn eluate flow (0.8 mL/min) was diluted with water (at 2 mL/min) prior to analyte trapping on 10 × 2 mm i.d. SPE cartridges (Spark Holland). For initial evaluation, 75 μg of (*S*)-**1** (10 μL of acetonitrile solution) was injected to the HPLC column for trapping on the following SPE cartridges: CN (cyanopropyldimethylsilyl silica, 8 μm), C_2 (ethyldimethylsilyl silica, 8 μm), C_8 (octyldimethylsilyl silica, 8 μm), C_8(EC) (octyldimethylsilyl silica, end-capped, 8 μm), C_{18} (octadecyldimethylsilyl silica, 8 μm), C_{18}(HD) (octadecyldimethylsilyl silica, high-density, 7 μm), Resin GP [general purpose resin, poly(divinylbenzene), 5–15 μm], and Resin SH (strongly hydrophobic resin, polystyrene and divinylbenzene copolymer, 15–25 μm). Absorbance thresholds at 254 nm were used to trigger the trappings, and the trapped analyte was eluted from dried cartridges (nitrogen gas stream, 45 min) with acetonitrile-d_3 for acquisition of ^{1}H NMR data. For determination of elution efficiency, GP phase cartridges were loaded with (*S*)-**1** as above and eluted with acetone-d_6, acetonitrile-d_3, chloroform-*d*, or methanol-d_4. Elution profiles were determined by pushing the deuterated solvents through the loaded cartridges in 10 μL steps and recording the ^{1}H NMR spectrum for each step. Reference spectra were obtained by filling the probe with a standard solution of (*S*)-**1** (1 mg/mL, corresponding to 30 μg in the active volume of the probe). Final analyses of *T. danis* extracts were performed after 4–8 cumulative trappings on GP cartridges, each time injecting 40 μL of a crude extract solution. ^{1}H NMR (HPLC-SPE-NMR mode; no presaturation of solvent peaks; 600 MHz, acetonitrile-d_3): δ 14.94 (s, 2H, chelated OH), 11.19 (s, 2H, CHO), 7.82 (s, 2H, aromatic), 7.10 (br s, 2H, OH), 6.74 (br s, 2H, OH), 4.00 (septet, J = 6.8 Hz, 2H, isopropyl CH), 2.06 (s, 6H, aromatic CH_3), 1.53 (doublet, 12H, J = 6.8 Hz, isopropyl CH_3). ESI MS: m/z 517.3 $[M - H]^-$ in negative-ion mode; m/z 541.3 $[M + Na]^+$ in positive-ion mode.

HPLC-SPE Combined with CD. HPLC separations and SPE trappings were performed as described above for HPLC-PDA-MS-SPE-NMR experiments. After 4–8 cumulative trappings, the dried cartridges were eluted with 200 μL of acetonitrile into a 1 mm quartz cell using an SPE hand-clamp (Spark Holland). The UV spectrum of the solution was recorded and the concentration of gossypol calculated (ϵ_{367} = 17 200 L mol^{-1} cm^{-1} in acetonitrile[38]). Subsequently, a CD spectrum of the solution was recorded and the enantiomeric excess of (*R*)-**1** calculated ($\Delta\epsilon_{378} = \pm 14.0$ L mol^{-1} cm^{-1} for pure enantiomers in acetonitrile[38]).

Antiplasmodial Assay. The assay was performed as detailed elsewhere,[48] using chloroquine-sensitive *Plasmodium falciparum* strain 3D7. The IC_{50} values were determined in triplicate using 8 concentrations of the test compound (each in duplicate) for determination of growth inhibition curves. Chloroquine diphosphate (IC_{50} 0.048 ± 0.013 μM) was used as a positive control. Light-microscopic evaluation of erythrocyte membrane changes was performed as previously described.[48]

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