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Cyclolignans from *Scyphocephalium ochocoa* via high-throughput natural product chemistry methods

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Abstract

Two 2,7′-cyclolignans, ocholignans A and B, were obtained as mass-limited samples from *Scyphocephalium ochocoa* via high-throughput natural products chemistry methods. The rapid structure elucidation of each compound was primarily facilitated by NMR data acquisition using a capillary-scale NMR probe, CapNMR™ probe. Ocholignan A was found to possess significant in vitro antibacterial activity against Gram-positive bacteria methicillin-resistant *Staphylococcus aureus* ATCC 33591 and *S. aureus* 78-13607A with a MIC of 16 μg/mL, respectively.

Keywords: *Scyphocephalium ochocoa*; Myristicaceae; High-throughput natural product chemistry; CapNMR™ probe; Miniaturization; Structure elucidation; Cyclolignans; Ocholignans; Antibacterial

1. Introduction

Previous publications (Eldridge et al., 2002; Cremin and Zeng, 2002) have documented our high-throughput purification methods applied to the production and analysis of the natural products libraries from plants. We have been using these methods to increase the rate of discovery of novel, biologically active compounds in various drug discovery collaborations. During the bioassay-guided fractionation of biologically active fractions, micrograms are used to locate activity, but one or more milligrams of a compound are needed for structure elucidation when using 3 or 5 mm conventional NMR probes. Therefore, increasing the NMR sensitivity is a crucial factor for the miniaturization of the structure elucidation and dereplication of the mass-limited samples. We have worked with both the MicroCryoProbe™ and CapNMR™, which are currently the two leading NMR technologies with the sensitivity enhancements that truly offer structure elucidation on microgram amounts of sample for natural products. Like most advanced technologies in scientific disciplines with numerous applications, each technology has their advantages and disadvantages. The MicroCryoProbe™ is better suited for samples with limited solubilities requiring 100 μL of solvent and performing ^{13}C and DEPT experiments with approximately 200 or more micrograms of material. The CapNMR™ has a higher filling factor resulting in fewer non-deuterated artifacts during acquisition, so it obtains better spectra when acquiring ^{1}H and COSY NMR spectra on approximately 5 to 10 μg of material. In our experience the MicroCryoProbe™ and CapNMR™ are equivalent when performing HSQC and HMBC experiments on 30 and 70 μg, respectively. The gain in sensitivity using the capillary-scale NMR probe

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comes from a reduced flow cell (5 μL total volume with 1.5 μL in active volume) and an Rf coil wound in a solenoid configuration. This probe is easier to shim, much less expensive to purchase and operate, and consumes approximately 50 μL of deuterated solvents for each sample without NMR tubes, thus reducing the total operating costs.

During a part of our program directed toward the discovery of novel antibacterial agents from plants (Hu et al., 2005), the natural products library constructed of the organic extract obtained from the leaves of the West African tree *Scyphocephalium ochocoa* Warb. (Myristicaceae) displayed a potent antibacterial activity against *Staphylococcus aureus*. So far, only fatty acids have been reported from *S. ochocoa* (Pamboutchivounda et al., 1992). The *Scyphocephalium* library was analyzed by parallel eight-channel HPLC–ELSD–MS after preparation as previously described (Eldridge et al., 2002; Cremin and Zeng, 2002). The compounds in the library were purified through semi-preparative HPLC systems. The structures of these two mass-limited compounds, ocholignan A (**1**) and B (**2**), were elucidated using the mass spectra and capillary NMR probe techniques.

2. Results and discussion

The lignans were located in the ethyl acetate (neat) flash fraction which was subjected to preparative HPLC C18 chromatography using 30–70% acetonitrile in water over 40 min, collecting fractions every minute. Compounds **1** and **2** (Fig. 1) resided in preparative HPLC fraction 15. The HPLC–ELSD–MS data acquired from fraction 15 contained compounds with molecular weights less than 500 Da that could readily be isolated using reversed-phase chromatography. The initial mobile phase gradient applied to isolating compounds **1** and **2** from fraction 15 was based on the elution profile observed during the preparative HPLC separation that afforded this fraction. An HPLC method was quickly developed which started at an isocratic gradient of 32% acetonitrile in water for 33.0 min, followed by a linear gradient of acetonitrile from 32% to 36% over 3.0 min. The final isocratic gradient of 36% acetonitrile in water was run for 5.0 min followed by a 95% acetonitrile in water gradient for 5.0 min. This afforded compounds **1** (70 μg, R_t = 31.5 min) and **2** (15 μg, R_t = 32.9 min).

The molecular weight of compound **1** and its chemical formula of $C_{21}H_{26}O_6$ were deduced from the positive mode high-resolution electrospray ionization mass spectrum (HR-ESIMS), which resulted in an $[M + Na]^+$ ion peak (*m/z* = 397.1630; $C_{21}H_{26}O_6Na$ requires 397.1627). The ^1H NMR spectrum of **1** showed the presence of a 1,2,3,4,5-penta-substituted benzene ring [δ 6.52 (1H, *s*, H-6)], a 1,3,4,5-tetra-substituted benzene ring [δ 6.18 (1H, *brs*, H-2′), 6.04 (1H, *brs*, H-6′)] and three methoxy groups [δ 3.84 (3H, *s*), 3.73 (3H, *s*), 3.35 (3H, *s*)]. In addition to the correlations between H-2′ and H-6′, a proton spin system –CH_2CH(Me)CH(Me)CH– was clearly observed in the COSY spectrum of **1**, with couplings between H-7 [δ 2.69 (1H, *dd*), 2.36 (1H, *dd*)] and H-8 [δ 2.04 (1H, *m*)], between H-8 and H-9 [δ 0.87 (3H, *d*)], between H-8 and H-8′ [δ 1.84 (1H, *m*)], between H-8′ and H-9′ [δ 0.89 (3H, *d*)], as well as between H-8′ and H-7′ [δ 3.96 (1H, *brs*)].

Complete HSQC and HMBC 2D NMR spectroscopic data sets were acquired (Table 1). Limited amount of sample (70 μg) made it impossible to run a ^{13}C spectrum and a complete DEPT series of spectra for **1**. One-bond proton–carbon connectivities were determined by the HSQC NMR experiment, and ^2J

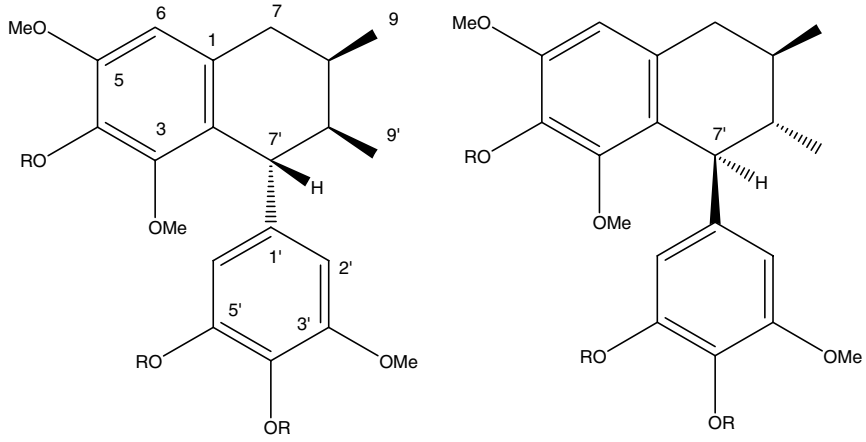

Fig. 1. Structures of ocholignans A (**1**) and B (**2**).

Table 1
^{1}H and ^{13}C NMR data for ocholignan A (**1**)

No.	**1**[a]		**3**[b]
	δ_H (mult, *J* in Hz)	δ_c[c]	δ_H (mult, *J* in Hz)
1		128.1	
2		123.8	
3		148.2	
4		138.8	
5		148.8	
6	6.52 (1H, *s*)	107.9	6.48 (1H, *s*)
7	2.69 (1H, *dd*, *J* = 16.7, 5.6, α-H) 2.36 (1H, *dd*, *J* = 16.7, 11.5, β-H)	34.3	2.30–2.95 (2H, *m*)
8	2.04 (1H, *m*)	27.4	1.65–2.15 (*m*)
9	0.87 (3H, *d*, *J* = 7.3)	19.2	0.92 (3H, *d*, *J* = 6)
1′		132.4	
2′	6.18 (1H, *brs*)	105.4	6.22 (1H, *s*)
3′		148.5	
4′		139.8	
5′		140.5	
6′	6.04 (1H, *brs*)	110.8	6.22 (1H, *s*)
7′	3.96 (1H, *brs*)	48.3	4.00 (1H, *d*, *J* = 3)
8′	1.84 (1H, *m*)	42.0	1.65–2.15 (*m*)
9′	0.89 (3H, *d*, *J* = 7.5)	13.9	0.98 (3H, *d*, *J* = 7)
3-OMe	3.35 (3H, *s*)	60.4	3.38 (3H, *s*)
5-OMe	3.84 (3H, *s*)	56.7	3.88 (3H, *s*)
3′-OMe	3.73 (3H, *s*)	56.9	3.76 (3H, *s*)
4,4′,5′-OMe			3.82 (6H, *s*), 3.76 (3H, *s*)

[a] Recorded by using a CapNMR™ probe. Sample: 70 μg in 6.5 μL CD_3OD. Injection: 54 μg in 5 μL, and 16 μg in active volume (1.5 μL). Data acquisition for ^{1}H: Number of scans (NS) = 64, 5 min.; for ^{1}H–^{1}H COSY: NS = 4, 32 min.; for NOESY: NS = 16, mixing time of 300 ms, 120 min.; for HSQC: NS = 128, 128 increments, 5 h; for HMBC: NS = 200, 128 increments, 8 h acquisition time, HMBC long-range coupling delay optimized at 63 ms.

[b] Recorded in $CDCl_3$ (Dhal et al., 1994).

[c] Assignments were made by a combination of HR-ESIMS data, ^{1}H and 2D NMR (^{1}H–^{1}H COSY, HSQC, and HMBC) experiments.

and ^3J long range proton–carbon couplings were indicated through the HMBC NMR experiment (Fig. 2). Together with the HR-ESIMS, the chemical shifts of all the protons and carbons (sp^2, sp^3) could be assigned unambiguously (Table 1). By comparison of the chemical shifts and the coupling constants of protons of **1** with those of the synthetic compound, aryltetralin **3** (Dhal et al., 1994; Biftu et al., 1979) (Table 1), both **1** and **3** suggested having the same relative stereochemistry at C-8, C-7′ and C-8′ (Fig. 1). The relative stereochemistry of **1** was further supported on the basis of the analysis of the NOESY data. In this spectrum, H-8 at δ 2.04 showed NOE correlations with H-7α at δ 2.69. The proton of H-9′ at δ 0.89 showed correlations with H-7β at δ 2.36, while proton H-7′ at δ 3.96 showed correlations with both H-7β and H-9′. There were no NOE's found between H-7′ and H-8, although both show otherwise strong effects. Thus, the structure of **1** was deduced as (8β,7′α,8′β)-4,4′,5′-trihydroxy-3,5,3′-trimethoxy-2,7′-cyclolignan.

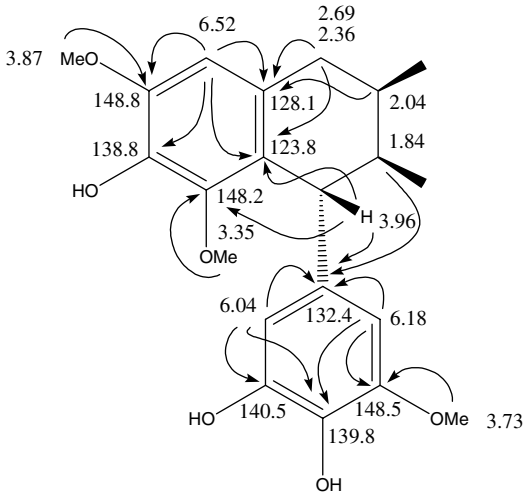

Fig. 2. Key HNBC correlations to assign the chemical shifts of the quaternary carbons of **1**.

The ESI mass spectrum of **2** showed a clear $[M + H]^+$ peak at *mlz* 375, suggesting it has a same molecular weight as **1**. Furthermore, the ^{1}H NMR spectroscopic data of **2** showed general features to those of **1**. The COSY NMR spectrum showed two separated proton spin systems from the 1,3,4,5-tetra-substituted benzene ring (H-2′ and H-6′) and the moiety of –CH_2-CH(Me)CH(Me)CH–. The chemical shift (δ 3.47) and the coupling constants (*J* = 7.7 Hz) of H-7′ in 2 is quite different from those of H-7′ [δ 3.96 (1H, *brs*)] in **1**, indicating **2** maintains a different stereochemistry at the C-7′ and C-8′ positions. Comparison with the literature did confirm this hypothesis. The relative stereochemistry at C-8, C-7 and C-8′ of **2** was similar to the synthetic compound aryltetralin **4** (Fig. 1) (Dhal et al., 1994; Krauss and Taylor, 1991; Biftu et al., 1979) (Table 2). The proposed stereochemistry of **2** was supported by its NOESY data. Clear effects were observed between H-7β at δ 2.52 and H-9 at δ 1.04, between H-7α at δ 2.61 and H-8 at δ 1.40, between H-8 and H-9′ at δ 1.02, as well as between H-9′ and H-7′ at δ 3.47. Therefore, the structure of **2** was elucidated as (8β,7′β,8′α)-4,4′,5′-trihydroxy-3,5,3′-trimethoxy-2,7′-cyclolignan.

Ocholignan A (**1**) was found to possess in vitro antibacterial activity against Gram-positive bacteria methicillin-resistant *S. aureus* ATCC 33591 and *S. aureus* 78-13607A with a MIC of 16 μ/mL, respectively. However, ocholignan B (**2**) was inactive in the same bioassays.

From extraction, isolation and purification to structure elucidation, active compound identification from natural products poses a real challenge in creating value for today's screening programs. Rapid structure elucidation on microgram quantities of confirmed active natural product compounds is a necessary requirement in realizing a true success for a high-throughput screening

Table 2
^{1}H NMR data for ocholignan B (**2**)

No.	δ (mult, J in Hz)	
	2[a]	**4**[b]
6	6.47 (1H, *s*)	6.41 (1H, *s*)
7	2.61 (1H, *dd*, J = 16.8, 3.2, α-H)	2.64 (1H, *dd*, J = 15, 4, α-H)
	2.52 (lH, *dd*, J = 16.8, 10.3, β-H)	2.55 (1H, dd, J = 15, 11.5, β-H)
8	1.40 (1H, *m*)	1.45 (1H, *m*)
9	1.04 (3H, *d*, J = 5.6)	1.03 (3H, *d*, J = 5.5)
2′	6.25 (1H, *d*, J = 1.6)	6.32 (1H, *s*)
6′	6.21 (1H, *d*, J = 1.6)	6.32 (1H, *s*)
7′	3.47 (1H, *d*, J = 7.7)	3.56 (1H, *d*, J = 9)
8′	1.34 (1H, *m*)	1.38 (1H, *m*)
9′	1.02 (3H, *d*, J = 5.7)	1.02 (3H, *d*, J = 5.5)
3-OMe	3.19 (3H, *s*)	3.08 (3H, *s*)
5-OMe	3.82 (3H, *s*)	3.84 (3H, *s*)
3′-OMe	3.76 (3H, *s*)	3.78 (3H, *s*)
4,4′,5′-OMe		3.80 (3H, *s*), 3.72 (3H, *s*), 3.78 (3H, *s*)

[a] Recorded by using a CapNMR™ probe. Sample: 15 μg in 6.5 μL CD_3OD. Injection: 12 μg in 5 μL, and 4 μg in active volume (1.5 μL). Data acquisition for ^{1}H: Number of scans (NS) = 64, 5 min.; for ^{1}H–^{1}H COSY: NS = 4, 32 min.; for NOESY: NS = 16, mixing time of 300 ms, 120 min.

[b] Recorded in $CDCl_3$ (Dhal et al., 1994).

program. With the utilization of the CapNMR™ probe, new opportunities have been created to take the sample requirements down to the microgram scale, allowing structure elucidation without the need to recollect and scale up, thus accelerating the process. Full NMR data set acquisitions on single digit microgram scale are now possible. However, a time constraint still exists in working with large numbers of active pure and semi pure natural product isolates, creating additional roadblocks in working with compounds from natural sources. New methodologies will still need to be exploited in order to accelerate the process further. Implementing intelligent structure elucidation software could provide a new tool to speed the structure elucidation process. Routine high-resolution mass spectra, providing accurate mass on all samples could also facilitate the process. What we have clearly demonstrated in this paper is a tremendous step toward the rapid isolation and structure elucidation of active compounds from a natural product mixture, thus reinvigorating the field of natural products into high-throughput screening regimes.

3. Experimental

3.1. General experimental procedures

Automated flash chromatography separations were performed on 50 g silica gel flash columns (International Sorbent Technology Ltd., Mid Glamorgan, UK) using a Flash Master II automated chromatographic system (Jones Chromatography Inc., Lakewood, CO) using a step gradient of (1) hexane–EtOAc, 3:1 (2) hexane–EtOAc, 1:1 (3) EtOAc, (4) EtOAc–MeOH, 3:1 (5) EtOAc–MeOH, 1:1. Flash Fraction 1 (hexane:EtOAc, 3:1) was extremely lipophilic ($\log P > 5$), and was discarded. One gram of organic extracts was loaded for each silica gel flash column. The flow rate was set up as 30 mL/min, and the eluting time for step 1 was 13 min (total 390 mL solvent collected), and step 2 through step 5 each was 10 min (each 300 mL solvent collected). Preparative HPLC separations were performed on Betasil C_{18} columns (20 × 100 mm, 5 mm, Keystone Scientific Inc., Bellefonte, PA). A parallel four-channel preparative HPLC system was assembled and consisted of 4 Beckman System Gold 126 gradient HPLC pumps (Beckman Coulter Inc., Fullerton, CA) with system controllers and four-way solvent delivery modules, 4 Beckman System Gold 166 single wavelength UV detectors with preparative flow cells, a Gilson 215/849 multiple probe autosampler (Gilson Inc., Middleton, WI), and 4 Gilson 204 fraction collectors. The fractions were separated into 40 fractions (20 mL/min, 1 min per collection per tube) using the parallel four-channel preparative HPLC system. A different 40 min gradient was applied to each flash fraction for adequate separation: 60–85% CH_3CN in H_2O for Flash Fraction 2 (hexane:EtOAc, 1:1), 30–70% CH_3CN in H_2O for Flash Fraction 3 (EtOAc, neat), and 5–40% acetonitrile in water for Flash Fractions 4 (EtOAc: MeOH, 70:30) and 5 (EtOAc:MeOH, 1:1). The system was controlled by Beckman 32 Karat chromatography software.

A Mega 1200 evaporator (Genevac Technologies, Suffolk, UK) was used to remove solvents from the preparative HPLC fractions. The preparative HPLC fractions were transferred from tubes to 96-deep-well plates by a MultiProbe II liquid handling system (Perkin Elmer Corporation, Boston, MA). A Genevac HT-12 evaporator was used to remove solvents from the 96-well plates. A parallel eight-channel HPLC–ELSD–MS system was assembled and consisted of a LCT time-of-flight mass spectrometer with an eight-way MUX electrospray interface (Micromass Ltd, Manchester, UK), a Waters 600E Multisolvent Delivery System (Wasters Corporation, Milford, MA) to pump solvents through an eight-way manifold which splits the flow to 8 HPLC columns (4.6 × 50 mm, 3 mm, Keystone Betasil C-18), a Gilson 215/889 multiple probe autosampler, and 8 Alltech 500 ELSD detectors (Alltech Associates Inc., Deerfield, IL). HPLC chromatographic conditions of CH_3CN–H_2O (1:19) for the first 1.0 min, a linear gradient of CH_3CN–H_2O from (1:19 to 19:1) in 8.0 min, followed by CH_3CN–H_2O (95:5) for 1.0 min. After each analysis the column was equilibrated in CH_3CN–H_2O

(1:19) for 2.5 min. The system was controlled by Waters MassLynx software version 4.0. The instrument was operated in both positive and negative electrospray modes with the capillary voltage set to 3.1 kV in positive electrospray mode and 2.7 kV in negative electrospray mode. Desolvation temperatures of 200 °C with a source temperature of 120 °C were used for all experiments. The nitrogen desolvation and nebulizer gas flow rates were set to 1200 and 6 L/h, respectively. The sample cone voltage was set to 25 V with extraction cone voltages set at 1.0 V for positive mode and 2.0 V for negative mode. The Rf lens voltage was set to 300 V. Data analysis was performed using the Waters OpenLynx software followed by Extractor, a customized software package developed for Sequoia Sciences by Koch Associates, La Jolla, CA.

Semi-preparative HPLC isolations performed on a single channel Beckman HPLC system consisting of a Beckman 168 diode array UV detector, Sedex 75 ELSD detector (Sedere, France), and Foxy Jr. fraction collector (Isco, Inc., Lincoln, NE). A splitter was used to split the flow in 10:90 to ELSD and fraction collector, respectively. NMR data for the structure elucidation of compounds were acquired utilizing a Bruker Avance 600 MHz NMR system (Bruker, Rheinstetten, Germany) and a 5 μL capillary microcoil NMR flow probe with 1.5 μL active volume (Magnetic Resonance Microsensors, Savoy, IL). The probe was operated at a temperature of 293 K. Pulse widths were 5.5 μs at a power of 23 dB for the ^{1}H spectra. Pure compounds were dissolved into 6.5 μL CD_3OD and loaded manually into the microcoil NMR flow probe.

3.2. Plant material

The leaves of *S. ochocoa* were collected from Gabon in April 2000. Plant samples were dried on site, then shipped to Sequoia Sciences. They were identified by John Stone (Missouri Botanical Garden Herbarium, St. Louis, MO). A voucher specimen (No. 535) was deposited at the Herbarium of the Missouri Botanical Garden.

3.3. Extraction and isolation

Dried leaves (99 g) were extracted with EtOH:EtOAc (50:50) followed by H_2O:MeOH (30:70), to obtain 3.7 g and 11.2 g dry extracts, respectively. As previously described, the EtOAc flash fraction (120 mg) was generated, with 50 mg of same fractionated by preparative C18 HPLC from CH_3CN–H_2O (3:7 to 7:3) collecting 40 one minute fractions. The isolation of individual compounds from preparative HPLC fraction 15 was performed using semi-preparative Keystone BetaMax Neutral C18 (8 × 250 mm ID, 5 mm) as described above.

3.3.1. Ocholignan A (*1*)

For ^{1}H and ^{13}C NMR spectroscopic data, see Table 1. ESIMS *m/z* 373 $[M - H]^-$, 397 $[M + Na]^+$, 771 $[2M + Na]^+$. HR-ESIMS *m/z* 397.1630 $[M + Na]^+$ (calcd for $C_{21}H_{26}O_6Na$, 397.1627).

3.3.2. Ocholignan B (*2*)

For ^{1}H NMR spectroscopic data, see Table 2. ESIMS *m/z* 375 $[M + H]^+$, 397 $[M + Na]^+$, 771 $[2M + Na]^+$.

3.4. Antibacterial assay

Compounds **1** and **2** were bioassayed for their in vitro antibacterial activity against Gram-positive bacteria methicillin-resistant *S. aureus* ATCC 33591 and *S. aureus* 78-13607A, a clinical isolate from France isolated from a patient with a documented bloodstream infection. The assay was performed at the Jones Group/JMI Laboratories (North Liberty, Iowa). Organisms were inoculated into broth microdilution panels at concentrations suggested by the National Committee for Clinical Laboratory Standard methods. Vancomycin was used as a control and tested concurrently. Vancomycin had a MIC of 1 μg/mL. Ethanol controls were used to determine the significance of antimicrobial effect at the highest test concentration. Cation-adjusted Mueller–Hinton broth was used as the growth medium. Isolates were incubated overnight to 24 h at 35 °C in ambient air. Panels were read manually and no visual growth of organisms was recorded as the endpoint MIC. Ocholignan A (**1**) was found to possess significant in vitro antibacterial activity against Gram-positive bacteria methicillin-resistant *S. aureus* ATCC 33591 and *S. aureus* 78-13607A with a MIC of 16 μg/mL, respectively. But ocholignan B (**2**) was inactive in the same bioassays.

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