JOURNAL OF NATURAL PRODUCTS

Volume 69, Number 4 April 2006

Rapid Communications

Discovering New Natural Products Directly from Crude Extracts by HPLC-SPE-NMR: Chinane Diterpenes in *Harpagophytum procumbens*

Cailean Clarkson,[†] Dan Stærk,[†] Steen Honoré Hansen,[‡] Peter J. Smith,[§] and Jerzy W. Jaroszewski*[,†]

Department of Medicinal Chemistry and Department of Pharmaceutics and Analytical Chemistry, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen, Denmark, and Division of Pharmacology, Department of Medicine, University of Cape Town, K-45 OMB, Groote Schuur Hospital, Observatory 7925, Cape Town, South Africa

Received December 2, 2005

Abstract: HPLC-SPE-NMR experiments with crude extracts of *Harpagophytum procumbens* allowed the rapid identification of novel, unstable chinane-type tricyclic diterpenes (**1** and **2**), along with numerous other constituents. Dramatic sensitivity gains achieved with this novel hyphenated technique establish a new paradigm in natural products research: rapid and rigorous structure determination of multiple extract components, including minor components, without preparative-scale isolation.

Isolation of natural products is the main bottleneck in the natural products field, and tedious purifications are often performed with the main purpose of structure identification. In bioactivity-guided fractionation projects, the major problem is isolation of constituents that are, for various reasons, abandoned once their structures are elucidated. If the structures of extract constituents were known in advance, the isolation efforts could be focused on truly novel and interesting components, avoiding reisolation of known or trivial constituents and increasing productivity to a level of efficiency that is unknown in present-day natural products research.

Hyphenated techniques, in particular HPLC-NMR, have the potential of providing structural information directly from crude extracts.[1,2] However, the traditional direct HPLC-NMR methods, where the spectra are acquired with HPLC eluates, suffer from a number of limitations that restrict their use to favorable cases and to a few, major extract constituents or to prepurified fractions.[3] In this communication, we describe examples illustrating the ability of the HPLC-SPE-NMR technique[4] (high-performance liquid chromatography−solid-phase extraction−nuclear magnetic resonance) to provide high-quality 1D and 2D NMR data on numerous components, including minor constituents, directly from crude extracts.

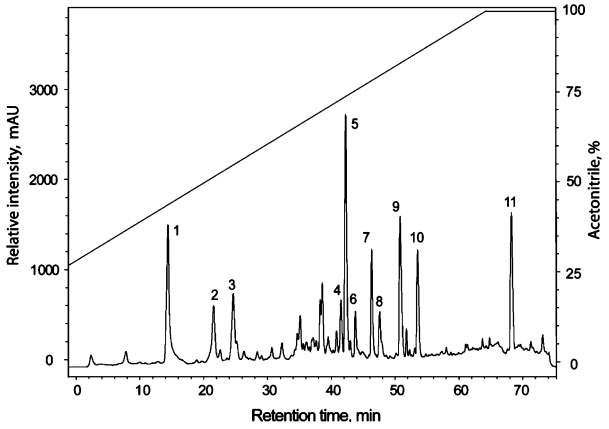


Figure 1. HPLC trace (230 nm) for the petroleum ether extract of *H. procumbens* roots. The peaks used for HPLC-SPE-NMR analysis are labeled 1−11.

Secondary roots of *Harpagophytum procumbens* (Burch.) DC. ex. Meissn (Pedaliaceae), also commonly known as Devil's claw, have gained popularity as a botanical dietary supplement or natural remedy for the treatment of inflammatory and rheumatic conditions.[5−7] Although polar constituents of the roots have been extensively investigated, little attention has been paid to the apolar fraction.[8−13] The HPLC trace (Figure 1) of a petroleum ether root extract showed the presence of numerous constituents, most of which were selected for on-line HPLC-SPE-NMR analysis. Following dilution of the HPLC eluate with water, the selected constituents were trapped on SPE cartridges filled with poly(divinylbenzene) sorbent. To maximize the amount of analytes available for subsequent NMR analysis, cumulative trappings of up to 10 injections were performed, and the analytes were eluted

* To whom correspondence should be addressed. Tel: (45) 35306372. Fax: (45) 35306040. E-mail: jj@dfuni.dk.
[†] Department of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences.
[‡] Department of Pharmaceutics and Analytical Chemistry, The Danish University of Pharmaceutical Sciences.
[§] Department of Medicine, University of Cape Town.

10.1021/np050504g CCC: $33.50
Published on Web 02/16/2006

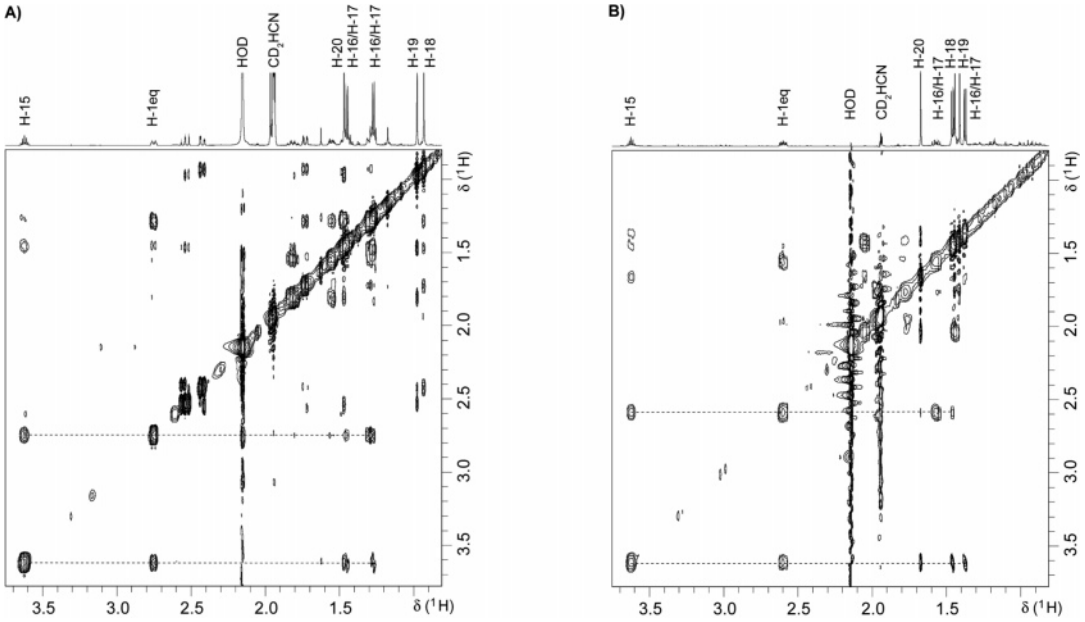
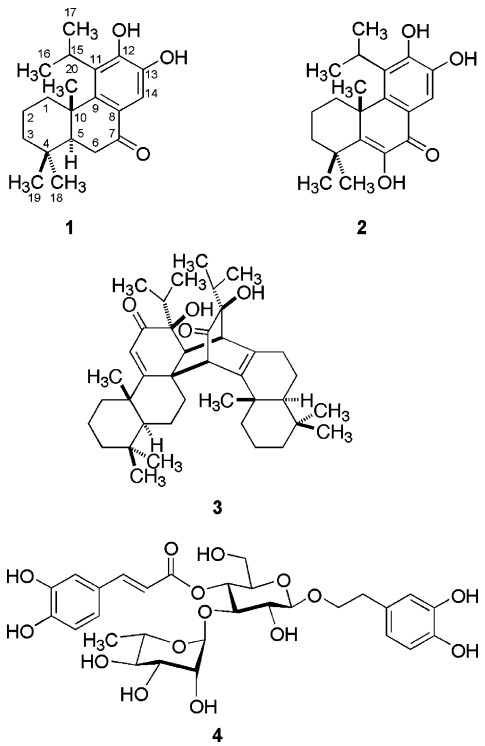


Figure 2. NOESY spectra (600 MHz, 600 ms) of **1** (A) and **2** (B) recorded with crude *H. procumbens* root extract (peaks 5 and 6, respectively) in the HPLC-SPE-NMR mode (10 trappings).

to a flow-probe with acetonitrile-d_3 for NMR data acquisition. The whole HPLC-SPE-NMR process was performed automatically under software control.[4]

The NMR analysis revealed that most of the HPLC peaks contain known abietane- or totarane-type diterpenes (data not shown), similar to those previously isolated.[10] However, ^{1}H NMR spectra acquired with the major peak 5 (t_R = 42.4 min) and the minor peak 6 (t_R = 43.9 min) showed unexpected features. Both compounds exhibited three methyl group singlets characteristic of tricyclic diterpenes, a single hydrogen resonance around δ 7.4 attributable to an aromatic ring, a deshielded isopropyl methine resonance at δ 3.62, and a deshielded resonance of H-1eq ($\delta \geq$ 2.6), as assigned from COSY experiments. The chemical shifts of H-1eq appear to be higher than in abietanes and totaranes having an aromatic C-ring that is unsubstituted at C-11, but lower than in derivatives oxygenated at C-11.[14−17] HMBC spectra acquired in the HPLC-SPE-NMR mode showed that both compounds contain a carbonyl group at δ 198.4 for peak 5 and at δ 180.4 for peak 6. The carbonyl position at C-7 was in the former case immediately apparent from the fact that the neighboring deshielded methylene resonances formed the AB part of an ABX spin system composed of H-5 and H-6. In the case of peak 6, no resonances attributable to H-5 and H-6 were observed, and the downfield shift of methyl singlets of H-18, H-19, and H-20 (δ 1.41, 1.44, and 1.67), as compared to those for peak 5 (δ 0.93, 0.98, and 1.47), suggested a substantial conformational change of the decalin ring system; an additional, enolized carbonyl group at C-6 can account for these spectroscopic features.[16,17] HMBC and COSY spectra were used to establish the structure of the A- and B-rings. The structures **1** and **2** of the compounds eluted as peaks 5 and 6, respectively, were finally established using NOESY spectra recorded in the HPLC-SPE-NMR mode. These showed a NOE between H-1eq and the isopropyl hydrogen H-15 (Figure 2); the close proximity of these nuclei resulted in cross-peak intensities comparable to those observed for geminal hydrogen pairs in the A- and B-rings. Further NOEs, including those from H-15 to H-20 and from H-16/H-17 to OH, were observed. The novel structures **1** and **2** are in agreement with negative-mode APCIMS data (*m*/*z* 315 and 329, respectively; $[M - H]^-$) obtained in parallel HPLC-MS experiments.

The structures of compounds **1** and **2**, rigorously determined from 2D NMR data obtained in the HPLC-SPE-NMR mode, are surprising, as they bear the isopropyl group at C-11 in contrast to the common diterpenes with abietane and totarane skeletons, having the isopropyl group at C-13 and C-14, respectively. Only two instances of this diterpene skeleton, named chinane, have been reported so far, one with an alicyclic and the other with a degraded C-ring.[18,19]



1 **2** **3** **4**

We believe that the significance of the present finding of **1** and **2** in *H. procumbens* is twofold. First, it is demonstrated that rapid and complete structural determination of relatively complex natural products with the aid of demanding 2D NMR experiments can be achieved directly from crude extracts, even for a minor constituent such as **2** (peak 6). This ability is further illustrated by analysis of peak 11, found to contain **3**. Despite the complexity of this Diels−Alder dimer,[20] a HMBC spectrum (Figure 3) obtained in the HPLC-SPE-NMR mode contained the full network of cross-peaks, allowing

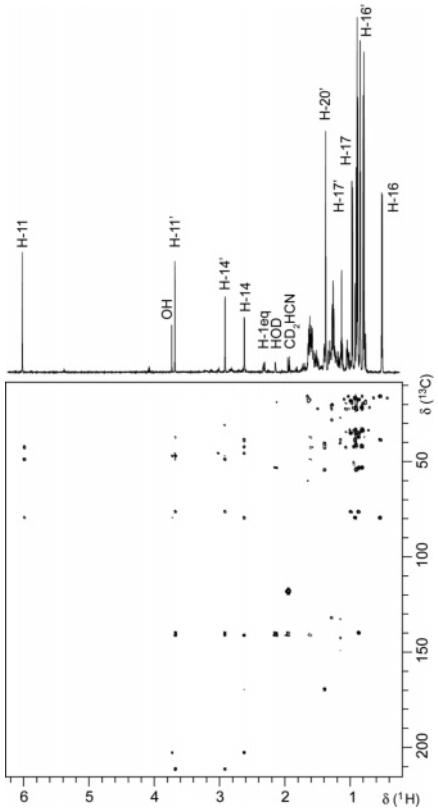


Figure 3. HMBC spectrum of **3** (600 MHz) recorded with crude *H. procumbens* root extract (peak 11) in the HPLC-SPE-NMR mode (eight trappings); ^{1}H resonances in the region 1.9−2.3 ppm are affected by solvent suppression.

firm identification. Second, we believe that the chinane skeleton present in **1** and **2** may be more common than currently recognized. There are reported structures of diterpenes, which were assumed to have the abietane or totarane skeleton without considering other positions of the isopropyl group. For example, a compound isolated from the neem tree and named margosone was formulated as 12,13-dihydroxytotara-8,11,13-trien-7-one.[21] However, synthetic 12,13-dihydroxytotara-8,11,13-trien-7-one exhibited ^{1}H and ^{13}C NMR chemical shifts different from those reported for margosone,[22] and we now propose that the latter may have the chinane structure similar to **1**. Although biosynthesis of the diterpene skeleton with an isopropyl group at C-11 was originally proposed to involve a rearrangement of ferruginol (8,11,13-abietatrien-12-ol),[23,24] the chinane system may be viewed as a regular head-to-tail diterpene that may be formed more directly from some other precursor formed by cyclization of appropriately folded geranylgeranyl diphosphate, i.e., possibly without involvement of an aromatic abietane-type precursor.

Because of the novelty of **1** and **2**, we attempted their preparative isolation in order to determine optical rotations. However, the compounds turned out to be unstable, resulting in loss of material, and NMR spectra of the isolates showed complex mixtures. The degradation is presumably due to oxidation, which parallels the behavior of other diterpenoid catechols[25] and is possibly accelerated by steric strain caused by the position of the isopropyl group.[23] On the other hand, in the HPLC-SPE-NMR experiments the SPE cartridges are stored under nitrogen, and clean NMR spectra were obtained for **1** and **2** after trapping from the crude extract. We therefore conclude that **1** and **2** might not have been discovered if classical isolation schemes were used instead of the HPLC-SPE-NMR technique.

One on the major advantages of the HPLC-SPE-NMR technique, exploited in this work, is the ability to obtain NMR data from all recognizable HPLC peaks without potential distortion of the

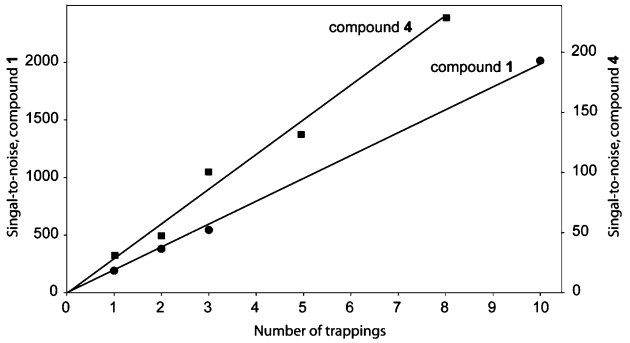


Figure 4. Improvement of signal-to-noise ratio in ^{1}H NMR spectra (600 MHz) of compound **1** (signal region, 0.86−1.00 ppm; noise region, 8.00−10.6 ppm) and compound **4** (signal region, 7.40−7.70 ppm; noise region, 8.50−10.00 ppm) in HPLC-SPE-NMR experiments, after repeated injections of crude petroleum ether *H. procumbens* root extract (25 μL, 40 mg/mL) or isolated **4** (10 μL, 5 mg/mL), respectively.

chromatographic process by stopping the mobile phase flow, necessary in direct HPLC-NMR experiments.[3] Advantage was also taken of multiple SPE trappings, which is another key feature of the HPLC-SPE-NMR experiments, resulting in greatly improved signal-to-noise ratios in NMR spectra.[4] As shown in Figure 4, a linear increase of signal-to-noise ratio was observed in 10 repeated trappings of compound **1** (peak 5), leading to a 10-fold performance improvement of the NMR experiments recorded in the HPLC-SPE-NMR mode as compared to direct[3] HPLC-NMR experiments. Arguably, the reversed-phase SPE trapping mechanism would not work with very polar analytes.[4] However, we have shown that for acteoside (**4**), a rather polar constituent of the ethanolic extract of *H. procumbens* also analyzed in the course of this study (data not shown), the multiple trapping works equally well, as indicated in Figure 4. Previously, we have reported that the HPLC-SPE-NMR technique is also applicable to alkaloids, which are eluted from HPLC columns as charged species.[26] These results demonstrate the broad scope of applicability of the HPLC-SPE-NMR technique for natural products. The need for preparative isolation of milligram quantities of natural products for the sake of structure elucidation may soon be history.[27,28]

HPLC-SPE-NMR analysis of the other constituents present in the petroleum ether extract (Figure 1), as well as the analysis of an ethanol extract of *H. procumbens* roots, is deferred to a forthcoming full account of this study.

Experimental Section

General Experimental Procedures. The experimental setup consisted of a Bruker Avance 600 MHz NMR spectrometer equipped with a 30 μL flow-probe, a Spark Holland Prospekt II SPE unit, and a HPLC chromatograph configured similarly as previously described.[29,30] Poly(divinylbenzene) (GP phase) SPE cartridges, 2 × 10 mm i.d., from Spark Holland were used for analyte trapping. HPLC separations were performed on a 150 × 4.6 mm i.d. C_{18}(2) Phenomenex Luna column (3 μm) eluted with an acetonitrile gradient in water at 0.8 mL/min; for SPE trappings, postcolumn water addition at 2 mL/min was used. The cartridges were dried with nitrogen gas and eluted with acetonitrile-d_3. 1D ^{1}H NMR spectra were recorded with solvent peak presaturation (water and acetonitrile) using 1D NOESY pulse sequence. 2D NOESY spectra were recorded with mixing times of 300 and 600 ms. Gradient-selected HMBC experiments were optimized for $J_{C,H}$ = 7 Hz. HPLC-APCIMS experiments were performed using an HPLC-MS instrument equipped with an Agilent G 1978A dual ESI and APCI mode ion source.

Plant Material. Secondary roots of *Harpagophytum procumbens* (Burch.) DC. ex. Meissn (Pedaliaceae) were collected in the Western Cape, South Africa. A voucher specimen (BOL110816) was deposited in the Bolus Herbarium (Department of Botany, University of Cape Town, South Africa).

Extract Preparation. Dried and pulverized root material was repeatedly extracted by maceration with petroleum ether or ethanol at room temperature and processed as previously described.[29,30]

Preparative Isolation of 1. The petroleum ether extract (600 mg, corresponding to 100 g of *H. procumbens* root) was repeatedly purified by preparative HPLC [250 × 20 mm C_{18}(2) Phenomenex Luna column, 5 μm, acetonitrile gradients in water, 20 mL/min] to give 5.8 mg (0.006%) of an isolate containing about 30% **1**.

12,13-Dihydroxychina-8,11,13-trien-7-one (1): ^{1}H NMR (HPLC-SPE-NMR mode, 600 MHz, CD_3CN) δ 0.93 (3H, s, H-18), 0.98 (3H, s, H-19), 1.27 and 1.45 (3H, d, J = 6.9 Hz, H-16 and H-17), 1.29 (2H, m, H-1ax and H-3ax), 1.47 (3H, s, H-20), 1.47 (1H, m, H-3eq), 1.55 (1H, dp, J = 14.1 and 3.8 Hz, H-2eq), 1.73 (1H, dd, J = 14.8 and 2.3 Hz, H-5), 1.81 (1H, qt, J = 13.5 and 3.5 Hz, H-2ax), 2.42 (1H, dd, J = 16.5 and 2.3 Hz, H-6eq), 2.54 (1H, dd, J = 16.5 and 14.7 Hz, H-6ax), 2.75 (1H, dt, J = 13.5 and 3.7 Hz, H-1eq), 3.62 (1H, sp, J = 6.9 Hz, H-15), 6.77 (1H, br s, 12-OH), 7.34 (1H, s, H-14), 7.36 (1H, br s, 13-OH); ^{13}C NMR (from HSQC and HMBC spectra acquired in HPLC-SPE-NMR mode, 125 MHz, CD_3CN) δ 18.3 (C-20), 19.4 and 20.2 (C-16 and C-17), 19.8 (C-2), 22.0 (C-19), 30.6 (C-15), 33.2 (C-18), 34.1 (C-4), 35.1 (C-6), 39.1 (C-1), 41.4 (C-3), 42.0 (C-10), 52.4 (C-5), 110.8 (C-14), 125.4 (C-8), 133.1 (C-11), 142.9 (C-13), 147.9 (C-9), 151.3 (C-12), 198.4 (C-7).

6,12,13-Trihydroxychina-5,8,11,13-tetraen-7-one (2): ^{1}H NMR (HPLC-SPE-NMR mode, 600 MHz, CD_3CN) δ 1.37 and 1.46 (3H, d, J = 7.0 Hz, H-16 and H-17), 1.41 (3H, s, H-19), 1.44 (3H, s, H-18), 1.44 (1H, m, H-3A), 1.67 (3H, s, H-20), 1.57 (1H, dt, J = 14.3 and 8.7 Hz, H-1ax), 2.07 (1H, m, H-3B), 2.60 (1H, ddd, J = 14.3, 9.4, and 4.0 Hz, H-1eq), 3.62 (1H, sp, d, J = 7.0 Hz, H-15), 6.92 (1H, br s, 12-OH), 7.10 (1H, br s, 6-OH), 7.47 (1H, s, H-14), 7.57 (1H, br s, 13-OH); ^{13}C NMR (from HSQC and HMBC spectra acquired in HPLC-SPE-NMR mode, CD_3CN) δ 19.6 (C-16 and C-17), 26.7 (C-20), 27.4 and 28.5 (C-18 and C-19), 30.4 (C-15), 32.0 (C-1), 36.3 (C-3), 37.1 (C-4), 42.6 (C-10), 109.9 (C-14), 132.5 (C-11), 145.1 (C-13), 145.3 (C-5), 147.0 (C-6), 147.3 (C-9), 151.7 (C-12), 180.4 (C-7), C-2 and C-8 not identified.

Acknowledgment. NMR and MS equipment used in this work was purchased via grants from "Apotekerfonden af 1991" (Copenhagen) and "Augustinus Fonden" (Copenhagen), respectively. A postdoctoral fellowship to C.C. from the Drug Research Academy, The Danish University of Pharmaceutical Sciences, is gratefully acknowledged.

References and Notes

(1) Hostettmann, K.; Wolfender, J.-L.; Terreaux, C. *Pharm. Biol. Suppl.* **2001**, *39*, 18−32.
(2) Wolfender, J.-L.; Ndjoko, K.; Hostettmann, K. *Phytochem. Anal.* **2001**, *12*, 2−22.
(3) Jaroszewski, J. W. *Planta Med*. **2005**, *71*, 691−700.
(4) Jaroszewski, J. W. *Planta Med*. **2005**, *71*, 795−802.
(5) Stewart, K. M.; Cole, D. *J. Ethnopharmacol*. **2005**, *100*, 225−236.
(6) Van Wyk, B. E.; van Oudtshoorn, B.; Gericke, N. *Medicinal Plants of South Africa*; Briza Publishers: Pretoria, 1997; p 144.
(7) Barnes, J.; Anderson, L. A.; Phillipson, J. D. *Herbal Medicines*, 2nd ed.; Pharmaceutical Press: London, 2002; pp 174−180.
(8) Kikuchi, T.; Matsuda, S.; Kubo, Y.; Namba, T. *Chem. Pharm. Bull*. **1983**, *31*, 2296−2301.
(9) Burger, J. F. W.; Brandt, E. V.; Ferreira, D. *Phytochemistry* **1987**, *26*, 1453−1457.
(10) Clarkson, C.; Campbell, W. E.; Smith, P. J. *Planta Med*. **2003**, *69*, 720−724.
(11) Boje, K.; Lechtenberg, M.; Nahrstedt, A. *Planta Med*. **2003**, *69*, 820−825.
(12) Munkombwe, N. M. *Phytochemistry* **2003**, *62*, 1231−1234.
(13) Seger, C.; Godejohann, M.; Tseng, L. H.; Spraul, M.; Girtler, A.; Sturm, S.; Stuppner, H. *Anal. Chem*. **2005**, *77*, 878−885.
(14) Evans, G. B.; Furneaux, R. H. *Bioorg. Med. Chem*. **2000**, *8*, 1653−1662.
(15) Evans, G. B.; Furneaux, R. H.; Gravestock, M. B.; Lynch, G. P.; Scott, G. K. *Bioorg. Med. Chem*. **1999**, *7*, 1953−1964.
(16) Evans, G. B.; Furneaux, R. H.; Gainsford, G. J.; Murphy, M. P. *Bioorg. Med. Chem*. **2000**, *8*, 1663−1675.
(17) Rodríguez, B. *Magn. Reson. Chem*. **2003**, *41*, 741−746.
(18) Fang, J.-M.; Lee, C.-K.; Cheng, Y.-S. *Phytochemistry* **1993**, *33*, 1169−1172.
(19) Lee, C.-K.; Cheng, Y.-S. *J. Nat. Prod*. **2001**, *64*, 511−514.
(20) Falshaw, P. C.; King, T. J. *J. Chem. Soc., Perkin Trans. 1* **1983**, 1749−1752.
(21) Ara, I.; Siddiqui, B. S.; Faizi, S.; Siddiqui, S. *Planta Med*. **1990**, *56*, 84−86.
(22) Bendall, J. G.; Cambie, R. C.; Rutledge, P. S.; Woodgate, P. D. *Aust. J. Chem*. **1993**, *46*, 1825−1843.
(23) Wenkert, E.; Jackson, B. G. *J. Am. Chem. Soc*. **1958**, *80*, 211−217.
(24) Bendall, J. G.; Cambie, R. C. *Aust. J. Chem*. **1995**, *48*, 883−917.
(25) Burnell, R. H.; Jean, M.; Marceau, S. *Can. J. Chem*. **1988**, *66*, 227−230.
(26) Jaroszewski, J. W. In *Magnetic Resonance in Food Science, the Multivariate Challenge*; Engelsen, S. B., Belton, P. S., Jacobsen, H. J., Eds; The Royal Society of Chemistry: London, 2005; pp 39−46.
(27) Hamburger, M. *Planta Med*. **2005**, *71*, 689−690.
(28) Gronquist, M.; Meinwald, J.; Eisner, T.; Schroeder, F. C. *J. Am. Chem. Soc*. **2005**, *127*, 10810−10811.
(29) Clarkson, C.; Stærk, D.; Hansen, S. H.; Jaroszewski, J. W. *Anal. Chem*. **2005**, *77*, 3547−3553.
(30) Lambert, M.; Stærk, D.; Hansen, S. H.; Sairafianpour, M.; Jaroszewski, J. W. *J. Nat. Prod*. **2005**, *68*, 1500−1509.

NP050504G