Identification of Major and Minor Constituents of *Harpagophytum procumbens* (Devil's Claw) Using HPLC-SPE-NMR and HPLC-ESIMS/APCIMS

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The HPLC-SPE-NMR technique, supported by HPLC-MS measurements, was used to determine structures of major as well as some minor constituents of ethanol and petroleum ether extracts of *Harpagophytum procumbens* (Devil's claw) roots. This method was also shown to be applicable for rapid and precise on-line identification of secondary metabolites present in commercial herbal products of *H. procumbens*. A total of 15 compounds (**1**−**14** and **17**) were identified from the ethanol and petroleum ether extracts, including a novel Diels−Alder dimer **14**. Optimization of the HPLC-SPE-NMR experiments included quantitative ^{1}H NMR measurements, determination of trapping and elution efficiency, effect of multiple trapping of analytes, use of various deuterated solvents for SPE cartridge elution, and effect of post-column dilution ratio of eluent with water. Linear accumulation of apolar and relatively polar analytes was demonstrated for at least 8−10 repeated trappings, resulting in greatly improved signal-to-noise ratios in NMR spectra and reduced acquisition times. Thus, the HPLC-SPE-NMR technique provides an efficient means of identification of multiple components of crude extracts. By allowing on-line generation of high-quality 2D NMR data without traditional purification of extract components, the HPLC-SPE-NMR methodology represents a paradigm shift in natural products research with respect to structure elucidation.

Harpagophytum procumbens (Burch.) DC. ex. Meissn (Pedaliaceae) is indigenous to the northwestern parts of Southern Africa, the Kalahari desert in Namibia, and the adjacent parts of Angola, Botswana, and Zimbabwe.[1] The Latin "harpago" in the genus name translates to hook, and the plant is commonly known as Devil's claw due to the appearance of its barbed fruit. Medicinal preparations of the secondary roots of *H. procumbens* are used by local populations to treat fevers, blood diseases, digestive disorders, arthritis, and rheumatism.[2,3] During the 20th century the plant was introduced into Europe and has since gained an international reputation as an anti-inflammatory and antirheumatic agent. In the past four decades, numerous in vitro, in vivo, and clinical studies have been conducted to assess the anti-inflammatory, antirheumatic, and analgesic effects of *H. procumbens*.[4−6] The results of these studies have been contradictory, making it difficult to draw conclusions on the effect of the preparations and the constituents responsible for the activity. Some of the observed discrepancies could be attributed to the lack of standardization of the *H. procumbens* extracts and insufficient chemical profiling of the plant material. Although phytochemical investigations and bioassay-guided fractionation of the roots have led to the identification of a number of secondary metabolites, mainly phenethyl glycosides and iridoids,[7−13] the characterized compounds have typically been limited to those present in sufficient amounts for isolation of milligram quantities, necessary for structural elucidation.

The aim of this study was to apply the newly developed hyphenated technique of high-performance liquid chromatography combined with solid-phase extraction and NMR spectroscopy[14,15] (HPLC-SPE-NMR) for characterization of constituents of *H. procumbens* extracts. HPLC-SPE-NMR has been successfully implemented in the dereplication of complex natural product mixtures.[16−19] The main advantages of introducing the SPE interface between the chromatography and NMR are (i) concentration of analytes from the HPLC eluent into a small-volume NMR flow-probe, (ii) possibility of increasing the amount of analytes by multiple trapping on the same SPE cartridge, and (iii) switch from nondeuterated HPLC solvents to deuterated NMR solvents. The combined effect is the ability to acquire high-quality 1D and 2D NMR spectra of a range of analytes without preparative-scale isolation. The present work demonstrates the usefulness of the HPLC-SPE-NMR technique for efficient structure elucidation of major as well as minor secondary metabolites of *H. procumbens* root extracts. HPLC-MS, either with ESI or with APCI, was used in parallel with HPLC-SPE-NMR in order to confirm the molecular mass of extract constituents. These techniques were also applied to the identification of constituents in two marketed *H. procumbens* products. In the course of this work, the optimization of SPE trapping and elution conditions was investigated. A preliminary account of a part of this study has been published already.[20]

Results and Discussion

The majority of the published investigations on *H. procumbens* constituents have been performed with H_2O or MeOH extracts of roots. Although these are common commercial forms, powdered root material is also available in international markets and has been used in clinical trials. In an attempt to obtain a comprehensive overview of the compounds present in *H. procumbens* roots, both EtOH and petroleum ether (PE) extracts were investigated in this study. While the EtOH extract accounted for about 13% of the plant material (dry weight), the PE extract corresponded to only 0.6%. Reversed-phase HPLC methods were developed for the separation of constituents after a mild precleaning of the extracts by SPE to remove waxy constituents. The criterion for HPLC method development was optimum separation of the analytes. This simplifies the threshold windows to be defined for analyte trapping in the subsequent HPLC-SPE-NMR experiments and facilitates higher column loadings without compromising chromatographic resolution. The developed HPLC methods allowed good resolution of the complex extracts, and the chromatograms revealed eight prominent peaks in the EtOH extract and numerous peaks in the PE extract. A total of 19 peaks were selected for automated HPLC-SPE-NMR

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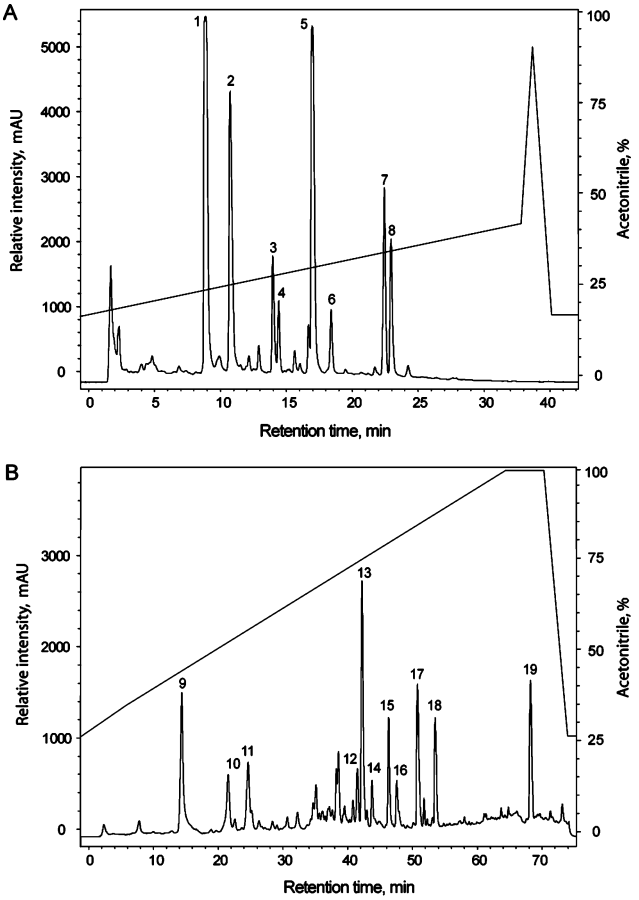


Figure 1. HPLC chromatograms monitored at 230 nm of EtOH (A) and petroleum ether (B) extracts of *H. procumbens* roots (150 × 4.6 mm i.d. C_{18} column; gradient elution profile is shown as percent of acetonitrile in H_2O). The peaks selected for the HPLC-SPE-NMR experiments are labeled 1−19.

analysis (Figure 1). The HPLC eluate was monitored at 230 nm, and thresholds defined at this wavelength were used to trigger analyte trapping on conditioned SPE cartridges filled with poly(divinylbenzene) stationary phase (HySphere GP phase). A total of 8−10 repeated trappings of the same peak, from subsequent chromatograms, were routinely performed after post-column eluent dilution with H_2O in a ratio of 2:5. The SPE cartridges were then dried with nitrogen gas, and the analytes were eluted with acetonitrile-d_3 directly into the NMR flow-probe (30 μL detection volume, total volume 60 μL).

The HPLC-SPE-NMR analysis of the EtOH extract (Figure 1) resulted in identification of seven compounds. The ^{1}H NMR spectrum of the compound eluted in the first peak (t_R = 9.2 min) showed signals of two aromatic rings, both with coupling patterns corresponding to a 1,2,4-trisubstituted benzene: δ 6.62 (H-6), 6.73 (H-5), and 6.76 (H-2) (J = 2.1 and 8.0 Hz), and δ 6.84 (H-5‴), 7.03 (H-6‴), and 7.10 (H-2‴) (J = 2.1 and 8.3 Hz). Characteristic resonances of a methyl group at δ 1.06 (d, J = 6.2 Hz, H-6″) and of an anomeric hydrogen at δ 5.12 (d, J = 1.6 Hz, H-1″) suggested the presence of a rhamnose residue; a second doublet corresponding to an anomeric hydrogen of a glucose residue was observed at δ 4.35 (J = 7.9 Hz, H-1). The assignment of all glucose resonances was possible from a TOCSY spectrum; the chemical shift of H-3′ (δ 3.79) and H-4′ (δ 4.85) confirmed the attachment point of the rhamnosyl and the caffeoyl residues, respectively.[22] A *trans*-coupled olefinic pair of doublets was observed at δ 6.27 and 7.59 (d, J = 15.9 Hz), which together with the aromatic hydrogen atom resonances indicated the presence of a caffeoyl moiety. An AA′BB′ pattern of an ethylene group was observed at δ 2.78 (2 H, apparent t, $J \approx$ 7.1 Hz), 3.70 (1 H, apparent dt, $J \approx$ 9.9 and 7.1 Hz), and 3.99 (1 H, apparent dt, $J \approx$ 9.9 and 7.1 Hz), corresponding to a glycosidically bound phenethyl unit.[21] All ^{13}C NMR signals corresponding to hydrogen-bearing carbon atoms were assigned on the basis of an HSQC experiment. From these NMR data the compound eluted as peak 1 was established to be acteoside (**1**), also commonly known as verbascoside. The observed spectra were in agreement with published data.[22,23]

The ^{1}H NMR spectrum obtained from peak 2 (t_R = 10.8 min) was similar to that of **1**, except that the caffeoyl group was attached to O-6′ of the glucose moiety rather than to O-4′. This was evident from the downfield shift of the H-6′ and C-6′ resonances ($\Delta\delta$ +0.77 and 0.84 for the two diastereotopic H-6′ hydrogens and +2.2 for C-6′) and the upfield shift of H-4′ ($\Delta\delta$ −1.45). Thus, the compound eluted as peak 2 was identified as the acteoside isomer **2**, isoacteoside. Good agreement of the observed ^{1}H and ^{13}C chemical shifts with those previously reported for isoacteoside[23] confirmed the identification.

Although peak 3 (t_R = 13.8 min) was very close to peak 4 (t_R = 14.3 min), the HPLC-SPE-NMR analysis of both peaks could be performed without difficulty. The ^{1}H NMR spectrum obtained with peak 3 was also similar to that of acteoside (**1**). However, a signal of an acetyl group was observed at δ 1.99, with the corresponding ^{13}C NMR signal in an HSQC spectrum at δ 20.7. Comparison of the ^{1}H NMR data with those of **1** revealed a downfield shift of the H-2′ signal ($\Delta\delta$ +1.5), indicating the attachment of the acetyl group to O-2′ of the glucose moiety. From the above results and comparison with literature data[24] the compound accounting for peak 3 was found to be 2′-*O*-acetylacteoside (**3**).

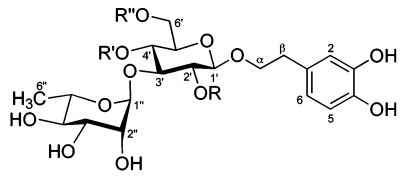
Analysis of the ^{1}H NMR spectrum obtained with peak 4 (t_R = 14.3 min) suggested the presence of an iridoid glycoside. The spectrum showed an AA′XX′ coupling pattern of a 1,4-disubstituted aromatic ring (δ 6.84, H-3″ and H-5″; δ 7.48, H-2″ and H-6″) and of two olefinic hydrogens at δ 6.28 and 7.59 (each d, J_{trans} = 15.9 Hz), which are indicative of a 4-coumaroyl group. An additional pair of olefinic hydrogens was observed at δ 4.93 and 6.39 (each d, J = 6.3 Hz, respectively H-4 and H-3), an ABX pattern at δ 1.90, 2.22, and 3.72 corresponding to a $-CH_2-CH-O-$ fragment with diastereotopic methylene hydrogens, two doublets at δ 2.83 and 6.20 (J = 1.3 Hz, respectively H-9 and H-1), a methyl group at δ 1.47 (s, H-10), an anomeric doublet at δ 4.58 (J = 7.9 Hz, H-1′), and the remaining signals of a glucose moiety in the region δ 3.15−3.80. Thus, the compound eluted in peak 4 was established to be 8-(4-coumaroyl)harpagide (**4**).[8]

The ^{1}H NMR spectrum obtained from the material eluted as peak 5 (t_R = 16.6 min) was almost identical to that of **3**, except for the signals of H-5′ and H-6′, which were shifted downfield ($\Delta\delta$ +0.18 for H-5′, and $\Delta\delta$ +0.54 and +0.57 for the two diastereotopic hydrogens attached to C-6′), and that of H-2′, which was shifted upfield ($\Delta\delta$ −1.44). This indicated that the acetyl group is linked to O-6′ and not to O-2′ as in **3**. The changed position of the acetyl group was confirmed by coupling between the carbonyl carbon (δ 171.4) and H-6 (δ 4.02 and 4.14) observed in an HMBC spectrum. Similarly, the attachment of the caffeoyl group, the rhamnosyl residue, and the 3,4-dihydroxyphenethyl group to O-4′, O-3′, and O-1′, respectively, was confirmed by the HMBC spectrum. Agreement of the observed ^{1}H and ^{13}C chemical shifts with those published[11] confirmed identification of the compound as 6′-*O*-acetylacteoside (**5**).

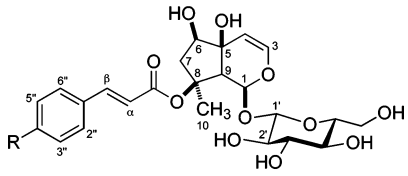
The ^{1}H NMR spectrum obtained with the minor peak 6 (t_R = 18.3 min) showed a mixture of at least two coeluting compounds. Although the spectrum suggested the presence of phenethyl glycosides, the structures of the individual components could not be elucidated due to the signal overlap and a relatively low signal-to-noise ratio.

The ^{1}H NMR spectrum of the compound eluted as peak 7 (t_R = 22.4 min) was similar to that of **4**, but the hydroxy group at C-4″ was missing, and the NMR data corresponded well to those reported

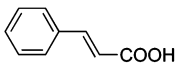
for harpagoside (**6**).[25] The 1D ^{1}H NMR and HSQC spectra of the compound eluted as peak 8 (t_R = 23.0 min) allowed its identification as 2′,6′-di-*O*-acetylacteoside (**7**).[12]



1 R = R" = H R' = caffeoyl
2 R = R' = H R" = caffeoyl
3 R = acetyl R' = caffeoyl R" = H
5 R = H R' = caffeoyl R" = acetyl
7 R = R" = acetyl R' = caffeoyl



4 R = OH
6 R = H



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Thus, seven compounds (**1**−**7**) were identified in the EtOH extract of *H. procumbens* roots. Acteoside (verbascoside, **1**),[9] isoacteoside (**2**),[9] 8-(4-coumaroyl)harpagide (**4**),[8] 6′-*O*-acetylacteoside (**5**),[11] harpagoside (**6**),[7] and 2′,6′-di-*O*-acetylacteoside (**7**)[12] are known for *H. procumbens*, but this is the first report of 2′-*O*-acetylacetoside (**3**) in this plant. Since all NMR spectra obtained in the HPLC-SPE-NMR mode were recorded using acetonitrile-d_3, the identification involved extensive interpretation of 2D NMR data in addition to comparison with reported chemical shift values, typically obtained using methanol-d_4 as solvent. The molecular masses of the identified compounds were confirmed by parallel HPLC-MS experiments using negative-mode electrospray ionization, which gave more abundant molecular ions than positive-mode spectra. The ESIMS spectra were very simple, showing molecular ions and practically no fragmentation. The observed $[M - H]^-$ ions for compounds **1**−**7** were *m*/*z* 623.3, 623.3, 665.4, 509.2, 665.4, 493.1, and 707.4, respectively.

It is noteworthy that the previous isolations of **1**, **2**, and **4**−**7** from *H. procumbens* involved lengthy fractionation and purification procedures, which contrasts with the simplicity and efficiency of the HPLC-SPE-NMR technique used in this study. The quality of the ^{1}H NMR spectra obtained with this hyphenated technique matched that normally achieved using traditional techniques (milligram quantities in 5 mm NMR tubes) and is illustrated for the major peak 2 and the minor peak 4 in Figure 2.

The PE extract of *H. procumbens* was considerably more complex than the EtOH extract; for practical purposes, the 11 peaks selected for the HPLC-SPE-NMR analysis were numbered successively 9−19 after the eight peaks present in the chromatogram of the EtOH extract (Figure 1), and the identified compounds (**8**−**14**) are discussed in the order of increasing complexity and structural similarity. As the ionizability of compounds present in the PE extract was very poor in the ESI mode, the parallel HPLC-MS experiments were performed using APCI in negative mode, which yielded prominent $[M - H]^-$ ions.

The ^{1}H NMR spectrum of the compound eluted in peak 9 (t_R = 14.6 min) displayed the characteristic signals of cinnamic acid (**8**),

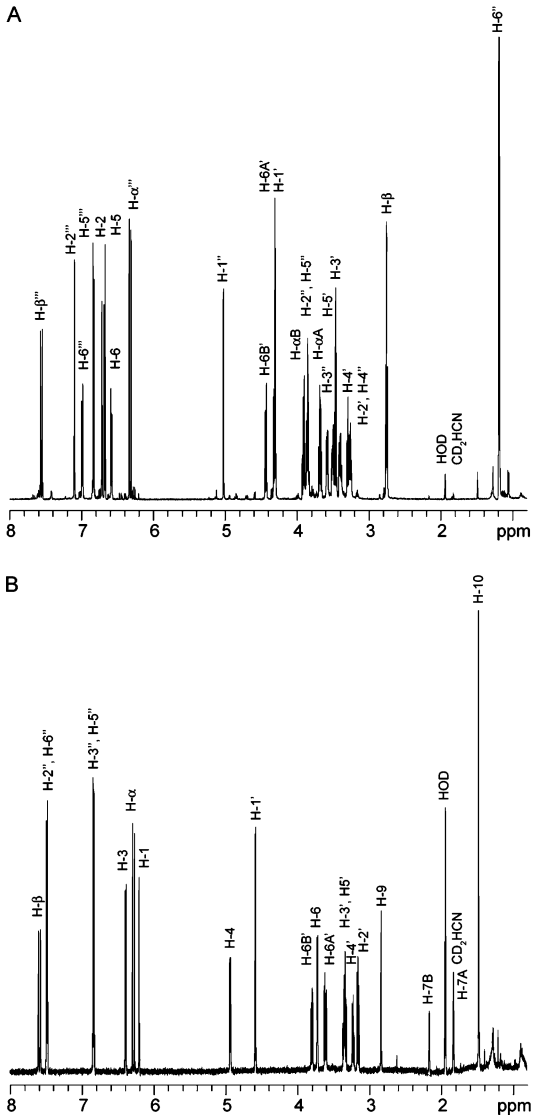
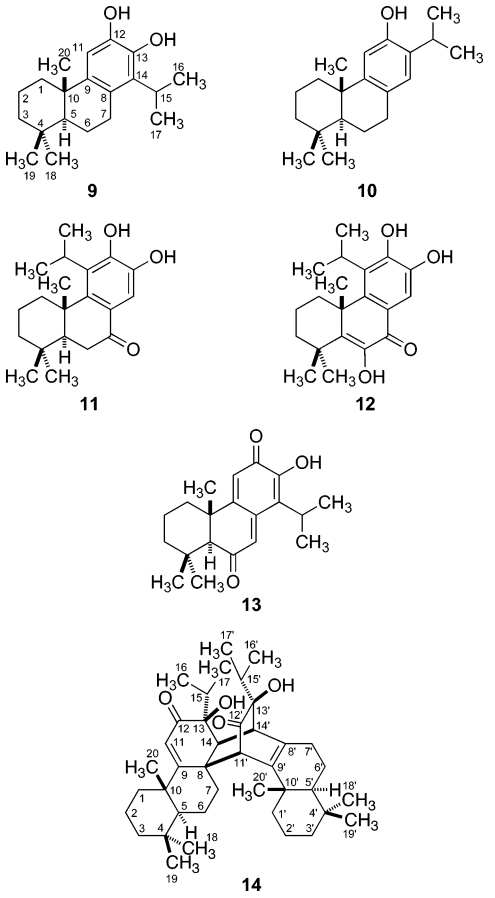


Figure 2. 600 MHz ^{1}H NMR spectra in acetonitrile-d_3 obtained in the HPLC-SPE-NMR mode of isoacteoside (**2**, peak 2) and 8-(4-coumaroyl)harpagide (**4**, peak 4). Spectra were recorded after eight SPE trappings, using 512 and 1024 transients, respectively.

whereas the remaining constituents identified in the PE extract were diterpenes. The ^{1}H NMR spectrum of the compound eluted as peak 17 (t_R = 50.9 min) showed signals characteristic for a tricyclic diterpene: three methyl singlets (δ 0.92, 0.95, and 1.15), signals of an isopropyl group (methyl doublets at δ 1.27 and 1.29, methine septet at δ 3.21; J = 7.1 Hz), and methylene resonances at δ 2.63, 2.83, and 1.15−1.95. A singlet at δ 6.63 suggested the presence of an aromatic, trisubstituted C-ring. The relatively deshielded resonance at δ 2.83 (dd, J = 16.7 and 6.5 Hz) was assigned to the benzylic H-7β, which showed geminal coupling to H-7α at δ 2.63 (ddd, J = 16.7, 11.7, and 6.5 Hz) and vicinal coupling to H-6β at δ 1.66 (J = 12.9, 11.7, and 6.5 Hz). In an HMBC spectrum, the singlet at δ 6.63 correlated with two quaternary carbon resonances at δ 125.6 and 142.8, whereas the carbon bearing the isopropyl group (δ 132.1), identified from correlation to the isopropyl methyl groups, correlated to a hydroxy group resonance at δ 5.87 and to H-7β. This confirmed the substitution pattern of the C-ring. The assignments of the methylene resonances were inferred from the COSY, HSQC, and HMBC spectra. On the basis of these data and the observed molecular ion in the APCIMS spectrum (*m*/*z* 301.3 $[M - H]^-$), the compound eluted in peak 17 could be identified as 8,11,13-totaratrien-12,13-diol (**9**), in agreement with previously reported NMR data.[26,27]



The ^{1}H NMR spectrum of the compound eluted in peak 18 (t_R = 53.6 min) resembled that of **9**, except for the presence of two singlets corresponding to two aromatic hydrogens (δ 6.64 and 6.79); the absence of ortho or meta coupling showed that these resonances correspond to H-11 and H-14 and that the compound is an abietane rather than a totarane. The exact assignment of these resonances and the position of the substituents in the C-ring (a hydroxy and an isopropyl group) were inferred from an HMBC spectrum after assignment of the signals of hydrogen-bearing carbon atoms using an HSQC experiment. In particular, H-11 (δ 6.64) correlated with C-10 (δ 38.0), C-12 (δ 152.5), C-13 (δ 132.8), and C-14 (δ 127.0), and H-14 (δ 6.79, also identifiable by signal broadening due to unresolved coupling to H-7) with C-7 (δ 30.2), C-9 (δ 148.9), C-12 and C-15 (δ 26.9), whereas C-14 correlated with H-7 (δ 2.71 and 2.80) and H-15 (δ 3.11). The oxygenated C-12 correlated with H-14 and H-15, and C-13 with H-15, H-16, and H-17 (δ 1.13). Agreement of the observed ^{1}H and ^{13}C NMR chemical shifts with literature values and APCIMS data (*m/z* 285.2 [M − H]$^-$) confirmed the compound to be 8,11,13-abietatrien-12-ol or ferruginol (**10**).[27,28]

^{1}H NMR analysis of compound **11** eluted in peak 13 (t_R = 42.4 min), the main peak in the chromatogram, and that of compound **12** eluted in the minor peak 14 has already been reported.[20] These diterpenes belong to the class of chinanes, encountered only twice prior to this work.[29,30]

The ^{1}H NMR spectrum obtained from peak 15 (t_R = 46.5 min) was also characteristic of a tricyclic diterpene and exhibited a pair of doublets at δ 6.35 and 6.58, with a coupling constant (J = 1.5 Hz) indicative of a long-range coupling along a zigzag path. The chemical shift of the isopropyl methine hydrogen H-15 was δ 3.12, substantially lower than in **11** and **12**.[20] Other key features were a deshielding of H-5 (δ 2.57, $\Delta\delta$ +0.84 relative to **11**[20]), the absence of B-ring methylene resonances, and a hydroxy group resonance at δ 7.48. The HMBC spectrum showed the presence of a carbonyl group at δ 200.6 correlating with H-5, as well as correlations from C-5 (δ 62.2) to the neighboring methyl groups (δ 1.12, 1.22, 1.23)

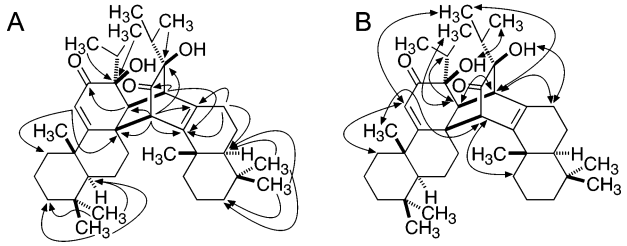


Figure 3. (A) Selected correlations observed in the HMBC spectrum of **14** (H → C). (B) Selected NOE correlations observed in a NOESY spectrum of **14**. Both spectra were recorded in the HPLC-SPE-NMR mode with peak 19 (10 trappings).

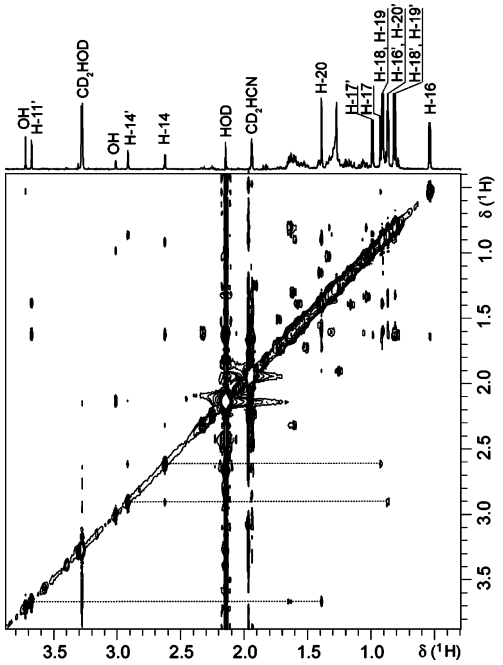


Figure 4. NOESY spectrum (600 MHz, 600 ms) of **14** recorded in the HPLC-SPE-NMR mode using a crude petroleum ether extract of *H. procumbens* roots (10 trappings, total acquisition time 14 h).

and to a resonance at δ 6.58, assigned to H-7. Correlations from H-15 to C-14 (δ 127.1) and from the latter to H-7 and OH confirmed the position of the isopropyl group at C-14. From the APCIMS data (*m/z* 313.3 [M − H]$^-$) and agreement of the observed ^{1}H and ^{13}C chemical shifts with literature data, the compound was confirmed to be 13-hydroxytotara-7,9,13-trien-6,12-dione or maytenoquinone (**13**).[26,31]

The ^{1}H NMR spectrum of the material eluted as peak 19 (t_R = 68.2 min) suggested a bis-diterpene structure. This was evident from the presence of two spin systems corresponding to two isopropyl groups (δ 0.54, 0.92, and 1.65, J_{vic} = 6.7 Hz; δ 0.87, 0.99, and 1.62, J_{vic} = 6.8 Hz) and six methyl singlets [δ 0.90 (H-18), 0.91 (H-19), and 1.39 (H-20), and δ 0.81 (H-19′), 0.82 (H-18′), and 0.87 (H-20′)]. A pair of doublets at δ 2.62 and 2.91 (J = 2.5 Hz) was assigned to H-14 and H-14′, respectively, and the singlets at δ 5.99 and 3.67 to H-11 and H-11′, respectively. HMBC experiments showed the presence of two carbonyl groups (δ 202.6, C-12, and δ 211.2, C-12′) and two quaternary, oxygenated carbons (δ 79.4, C-13, and δ 76.3, C-13′). The HMBC spectrum contained a network of connectivities (Figure 3) enabling assignment of all ^{1}H and ^{13}C NMR resonances for each portion of the molecule individually and identification of the compound as the Diels−Alder dimer **14**. The relative configuration of the carbon atoms at the Diels−Alder junction was unequivocally inferred from a NOESY experiment (Figures 3 and 4). The observed ^{1}H and ^{13}C NMR data for **14** are listed in Table 1. The bis-diterpene structure was

Table 1. ^{1}H and ^{13}C NMR Data for **14** Obtained in the HPLC-SPE-NMR Mode from a Petroleum Ether Extract of *H. procumbens* Roots and Comparison with Literature Data for Maytenone (**16**)

	^{1}H NMR[a]		^{13}C NMR	
position	**14**, CD_3CN[b]	**16**, $CDCl_3$[c,d]	**14**, CD_3CN[e]	**16**, $CDCl_3$[c,f]
1	1.92 m	1.97	40.7	37.0
	1.26 m	1.40		
2	1.62−1.51 m	1.67	19.1	18.8
		1.60		
3	1.40 m	1.49	41.8	41.8
	1.17 m	1.12		
4			34.6	34.0
5	1.06 m	1.75 (11.6, 8.0)	54.2	43.0
6	1.62−1.51 m	2.08	19.1	16.4
		1.50		
7	2.14 m	1.91	30.7	30.9
	1.96 m	1.98		
8			48.9	48.4
9			169.5	172.6
10			42.6	42.0
11	5.99 s	6.15	123.8	120.0
12			202.6	200.6
13			79.4	78.7
14	2.62 d (2.5)	2.56 (2.4)	47.1	43.3
15	1.65 m	1.50	37.9	37.0
16	0.54 d (6.7)	0.94 (6.3)	16.6	15.4
17	0.92 d (6.7)	0.53 (6.7)	15.7	16.2
18	0.90 s	0.95	33.5	32.9
19	0.91 s	0.95	22.3	21.1
20	1.39 s	1.0	22.3	26.5
1′	1.60 m	1.30	42.6	36.2
	2.33 m	1.08		
2′	1.62−1.51 m	1.53	19.1	18.4
		1.46		
3′	1.34 m	1.39	42.1	41.6
	1.04 m	1.15		
4′			*g*	33.1
5′	0.79 m	1.05 (12.5, 1.9)	53.3	49.2
6′	1.43−1.30 m	1.69	19.1	18.6
		1.34		
7′	2.14 m	2.09	30.7	29.4
	1.96 m	2.00		
8′			141.2	138.6
9′			140.0	138.5
10′			37.4	37.3
11′	3.67 s	3.46	60.1	57.8
12′			211.2	213.4
13′			76.6	76.1
14′	2.91 d (2.5)	3.11 (2.5)	45.8	45.0
15′	1.62 m	1.60	34.9	34.6
16′	0.99 d (6.8)	0.97 (6.9)	17.4	17.0
17′	0.87 d (6.8)	0.96 (7.4)	18.7	17.6
18′	0.82 s	0.89	33.5	33.0
19′	0.81 s	0.76	21.6	21.3
20′	0.87 s	0.76	18.8	20.8

[a] Multiplicity of signals is given as follows: s, singlet; d, doublet; m, multiplet; coupling constants (apparent splittings) are given in parentheses as numerical values in Hz. [b]Relative to the residual CD_2HCN signal set to δ 1.94. [c]Data from ref 36, where the previously described maytenone (**16**) is designated as celastroidine B. [d]Relative to the residual $CHCl_3$ signal set to δ 7.24. [e]^{13}C NMR chemical shifts obtained from HMQC and HMBC experiments, relative to the nitrile group resonance of the solvent set to δ 118.26. [f]Relative to the solvent signal set to δ 77.0. [g]HMBC peaks not observed due to overlap with other resonances.

corroborated by the molecular ion observed in the APCIMS spectrum (*m/z* 603.5 $[M - H]^-$).

A few Diels−Alder dimers similar to **14** have previously been reported. The first is maytenone, isolated[32,33] from *Maytenus dispermus* (Celastraceae) and identified by crystallographic analysis of the alcohol formed by reduction of the carbonyl group at C-12′.[34] Although the compound (reported[33] mp 198 °C, $[\alpha]^{18}_D$ +115) was unambiguously identified by the X-ray analysis of its reduction product, no NMR data were reported. Later, two oxygenated

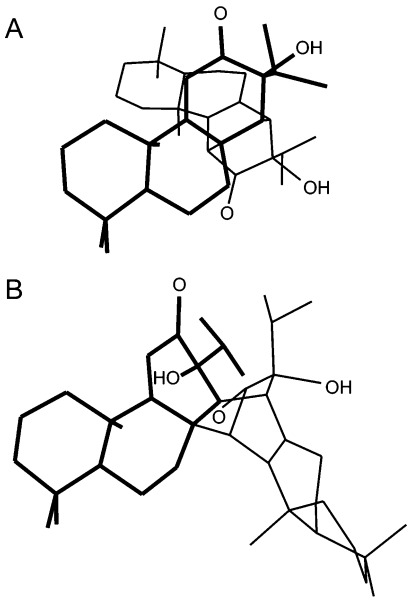
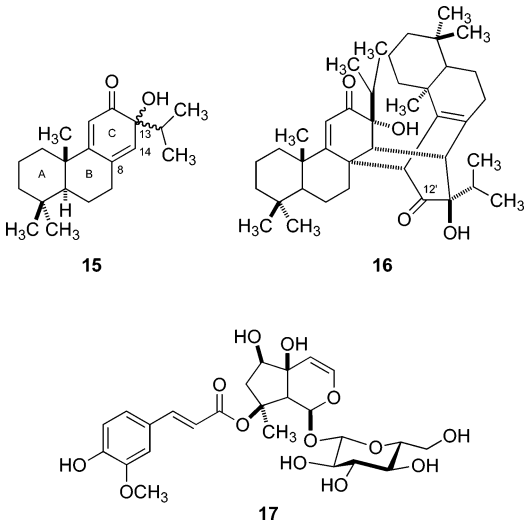


Figure 5. Molecular model of compound **16** (A) obtained using published X-ray coordinates[36] and a constructed molecular model of **14** (B) obtained by MM2 force-field energy optimization. The dienophile portion of the molecule is indicated in bold.

derivatives with a maytenone skeleton were isolated from *Hugonia castaneifolia* (Linaceae).[35] More recently, a Diels−Alder dimer named celastroidine B (reported[36] mp 189−190 °C, $[\alpha]_D$ +55.5) was isolated from *Hippocratea celastroides* (Hippocrateaceae). Its structure was determined by ^{1}H and ^{13}C NMR spectroscopy and confirmed by X-ray analysis.[36] Such adducts are believed to be formed by Diels−Alder dimerization of a diterpene precursor with the general structure **15**, in which the C-8−C-9 bond in one molecule serves as a dienophile for the C-ring diene system of another molecule. In principle, either face of the dienophile can approach the diene system of another molecule either from above (β-face) or from below (α-face) and can do so in two possible orientations, which may be termed as a syn or an anti orientation (with the A-rings oriented in the same or in the opposite directions, respectively). This would give eight possible dimers for each configuration at C-13 in **15**, although formation of some of the adducts is expected to be sterically prohibitive. Even though the work on celastroidine B does not cite the previous studies of maytenone,[36] the two compounds are in fact identical, as shown by comparison of their atomic coordinates deposited at the Cambridge Crystallographic Data Center (structure codes BUYHIM[34] and EBEJAW[36]). Thus, both *Maytenus dispermus* and *Hippocratea celastroides* produce maytenone, the structure of which can be described as the α-diene/α-dienophile/syn dimer (**16**) of compound **15** with the *R** configuration at C-13. On the other hand, neither the observed NOEs (Figure 4) nor the ^{13}C NMR data (Table 1) of the Diels−Alder dimer present in *H. procumbens* (**14**) are compatible with maytenone. Thus, compound **14** is the α-diene/β-dienophile/syn Diels−Alder dimer of compound **15** with the *S** configuration at C-13 (Figure 5). Several instances of other terpenoid Diels−Alder dimers have been reported.[36−40] We also note that the nature of a Diels−Alder dimer isolated from *Buddleja globosa* (Buddlejaceae), and claimed to be maytenone,[41] is subject to confirmation because of the absence of comparative spectroscopic data.

The HPLC-SPE-NMR and HPLC-MS experiments performed with peaks 10−12 and 16 showed the presence of multiple coeluting constituents. Although these were diterpenoids, as shown by the observation of methyl and isopropyl ^{1}H resonances, the compounds were present in very small amounts, and full structural elucidation was not conducted. While compounds **9** and **10** have previously been isolated from the PE extract of *H. procumbens* in 0.03% and



0.02% yields, respectively (by dry weight of the root material),[10] this is the first report of **11**−**14** from this source. Considering the yield of the crude PE extract (0.6%), it can be concluded that the diterpenes are minor constituents of *H. procumbens* roots. In order to estimate the content of polar constituents present in the EtOH extract, quantitative HPLC measurements using authentic[42] acteoside (**1**) as an external standard were performed. From a calibration curve, the amount of **1** present in the dry EtOH extract (Figure 1) was established to be 15.8 ± 0.2%, corresponding to the content in the dried plant material of about 2%. Since **2**, **3**, **5**, and **7** have practically identical chromophores as those present in **1**, the content of these constituents in the EtOH extract could be estimated as 5, 2, 10, and 3%, respectively. Thus, *H. procumbens* roots are very rich in these phenethyl derivatives.

In extending the above HPLC-SPE-NMR studies of *H. procumbens* extracts, two commercial products, one from the Danish market and another from the U.S. market, were analyzed. Both products were capsules apparently containing powdered root material. According to the HPLC-DAD profiles of EtOH extracts (Figure 6), the commercial products contained compounds **1**, **2**, **4**, **5**, and **6**, but lacked compounds **3** and **7**. Interestingly, the extracts of the commercial products were remarkably similar (Figure 6). Both commercial products contained a relatively high concentration of a compound eluting at t_R = 15.1 min. Although this compound is detectable in the original extract (Figure 1), it was not selected for the HPLC-SPE-NMR analysis described above due to very low peak intensity. In order to determine the structure of this compound, the HPLC-SPE-NMR analysis was performed using the commercial product from the U.S. market. The ^{1}H NMR and HSQC spectra obtained were almost identical to those of **4**, except for the presence of a methoxy group at the C-3″ position of the coumaroyl moiety. Comparison with literature data[25] confirmed the compound to be 8-feruloylharpagide (**17**), previously identified in *H. procumbens* roots.[11] From the intensity of the chromatographic peaks and ^{1}H NMR signal intensities, it could be roughly estimated that the content of **17** in the extracts of the commercial products (Figure 6) and in the original root extract (Figure 1) is 1% and 0.25%, respectively. These results emphasize that the HPLC-SPE-NMR technique has the necessary sensitivity to provide detailed structural information on minor constitiuents of commercial herbal products. Furthermore, the use of standards is not necessary for identification and quantification of constituents.

In order to estimate the analyte trapping and elution efficiency in the HPLC-SPE-NMR experiments, quantitative ^{1}H NMR measurements were performed using acteoside (**1**) as a reference. Thus, the flow-probe used for the HPLC-SPE-NMR experiments was filled with a standard solution of **1** in acetonitrile-d_3 (1.67 mg/mL or 2.67 μM solution, corresponding to 50 μg of **1** in the active

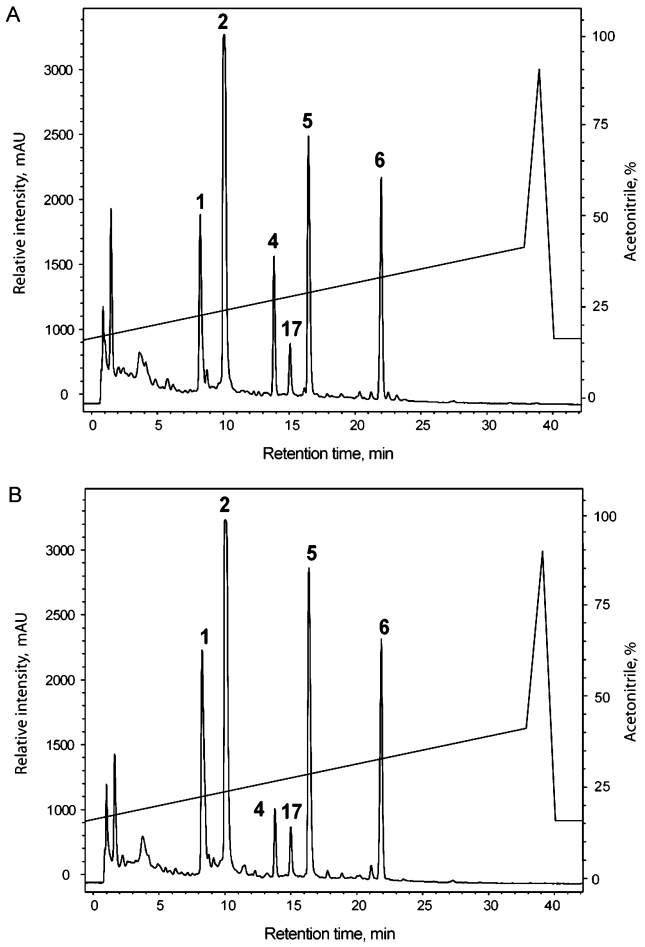


Figure 6. HPLC chromatograms (230 nm) of the EtOH extracts of the commercial *H. procumbens* health products (150 × 4.6 mm i.d. C_{18} column; gradient elution profile is shown as percent of acetonitrile in H_2O). (A) A product from the U.S. market; (B) a product from the Danish market. Identified compounds **1**, **2**, **4**−**6**, and **17** are indicated above the respective peaks.

volume of the probe of 30 μL), and longitudinal (T_1) relaxation times were determined using the inversion recovery method. The longest relaxation time was T_1 = 1.8 s (for H-β of the caffeoyl moiety), and quantitative ^{1}H NMR spectra of the sample were acquired using 90° pulses and interpulse intervals of 15.4 s, corresponding to 8.5 × T_1. Under these conditions, the signal-to-noise ratio for the H-β resonance was determined (S/N = 130) and compared with that measured in a ^{1}H NMR spectrum recorded under identical conditions in the HPLC-SPE-NMR mode following injection of 50 μg of **1** into the HPLC column. Ideally, the entire amount of **1** injected should be recovered in the NMR probe. Possible losses could be due to incomplete elution from the HPLC column, inefficient SPE trapping or desorption, and imperfect centering of the sample in the NMR probe. Thus, the maximum signal-to-noise ratio obtainable in the latter experiment is half of that obtained in the initial experiment with direct sample injection (S/N = 65), because now the sample (50 μg) occupies the whole NMR cell volume, which is equal to 60 μL. The signal-to-noise ratios were determined in a series of such HPLC-SPE-NMR experiments using various eluting solvents (Table 2).

It can be seen that methanol-d_4 has a tendency to give a higher signal-to-noise ratio than the standard eluting solvent, acetonitrile-d_3; that is, the cartridge elution with methanol-d_4 appears to be more effective than with acetonitrile-d_3. No improvement in the signal-to-noise ratio was observed when the elution flow-rate of acetonitrile-d_3 was reduced from the normal 220 μL/min to 110 μL/min. Similarly, no improvement was observed when the elution

Table 2. Comparison of Signal-to-Noise Ratios Obtained in HPLC-SPE-NMR Experiments Using Different Eluting Solvents and Multiple Trappings[a]

eluting solvent[b]	compound **1**[c]	compound **11**[d]
acetonitrile-d_3	28.1 ± 3.1	81.0 ± 2.9
methanol-d_4	41.4 ± 4.9	82.5 ± 6.1
chloroform-*d*	0	96.1 ± 7.9
acetonitrile-d_3	31.2	185.2[e]
acetonitrile-d_3 (2 trappings)	47.4	383.0[e]
acetonitrile-d_3 (3 trappings)	100.7	545.3[e]
acetonitrile-d_3 (5 trappings)	131.8	n.d.
acetonitrile-d_3 (8 trappings)	229.2	n.d.
acetonitrile-d_3 (10 trappings)	n.d.	2014.5[e]

[a] Acteoside (**1**) standard (50 μg), or crude *H. procumbens* petroleum ether extract (1 mg), injected into the HPLC column; signal-to-noise ratios for selected resonances in fully relaxed ^{1}H NMR spectra (16 transients) are reported (average ± 1/2 difference from two independent determinations); n.d.: not determined. [b]Single SPE trapping unless otherwise stated. [c]Signal region 7.40−7.70 ppm (H-β), noise region 8.50−10.00. The signal-to-noise ratio obtained after filling the NMR probe with a solution of **1** (1.67 mg/mL), determined under identical acquisition conditions, was 129.4. [d]Signal region 0.86−1.00 ppm (H-18 and H-19), noise region 8.00−10.60. [e]These experiments were performed with a PE extract enriched in **11** by preparative SPE.

flow was stopped for a few minutes after introduction of the first 30 μL to the SPE cartidge. This suggests that desorption kinetics does not limit the amount of the analyte recovered in the NMR probe. As expected, no elution of **1** was observed when chloroform-*d* was used as the eluting solvent (Table 2). The observed eluting efficiency of different solvents (Table 2) is in accord with the polar nature of **1**. It can be noted that the highest signal-to-noise ratio obtained in these experiments (with methanol-d_4, Table 2) is about 64% of the theoretical value mentioned above. This estimate is similar to that obtained in a model study by Sandvoss et al.[43] The major loss of sensitivity in the HPLC-SPE-NMR experiment is due to the fact that the active volume of the NMR cell is only 50% of the total volume, which calls for an improvement of the cell design. It can be calculated that using the same hardware and the same HPLC peak of **1** (peak-width at base 320 μL, Figure 1), the maximum analyte recovery in the sensitive volume of the 30 μL NMR flow-cell achievable under ideal conditions in a direct HPLC-NMR experiment[14] is 14%, assuming that the analyte distribution within the HPLC elution volume is Gaussian and the center of the peak is successfully placed in the middle of the flow-cell. The loss of sensitivity in direct HPLC-NMR experiments, i.e., in the absence of the analyte focusing effect of the SPE interface, will be more severe for broader peaks, but can be counteracted by the use of larger NMR flow-cells, albeit at the expense of reduced mass-sensitivity. Previous comparison of loop-collection mode[14] and the HPLC-SPE-NMR mode using iridoid glucosides demonstrated the improvement in the latter case by a factor of 2.[13]

When the above cartridge elution experiments were repeated with the apolar compound **11** using injection of the PE extract of *H. procumbens* to the HPLC column, the best eluting solvent was chloroform-*d* (Table 2), as expected. However, the signal-to-noise ratios obtained using acetonitrile-d_3 or methanol-d_4 were only slightly lower than that for chloroform-*d*. The results in Table 2 demonstrate that the commonly used acetonitrile-d_3 is a good compromise if the same elution solvent for nonpolar and relatively polar analytes has to be used.

In addition to the cartridge elution efficiency, the trapping efficiency of **1** was investigated at a lower ratio of post-column eluent dilution with H_2O, following injection of 50 μg of **1** to the HPLC column. No drop in signal intensity was observed when the post-column dilution ratio was changed from the normal ratio 2:5 to 4:5. This shows that the dilution ratio of 2:5 was appropriate for **1**, and since **1** is the most polar analyte in the EtOH extract (Figure 1) and least likely to interact with the SPE sorbent, this dilution ratio can be assumed to be adequate for the remaining compounds in this extract (Figure 1). Since adequate trapping of even the most apolar compound (**14**) was observed, it can be assumed that the post-column dilution ratio is sufficient to counteract the higher eluting power of the HPLC eluent toward the end of gradient HPLC runs.

Perhaps the major advantage of the HPLC-SPE-NMR technique is the possibility of analyte accumulation by repeated trapping of the same chromatographic peak from multiple injections.[15] In order to investigate the performance of this approach in the *H. procumbens* case, repeated trappings of **1** (from multiple injections of the standard sample) and **11** (from multiple injections of the PE extract) were performed, and the cartridges were eluted with acetonitrile-d_3 (Table 2). A linear increase in the amount of analyte on the SPE cartridges was observed for the entire range of the accumulations tested (up to eight injections of **1** and up to 10 injections of **11**), as expressed by linear improvement in the signal-to-noise ratios in the resulting ^{1}H NMR spectra (correlation coefficient 0.982 and 0.998 for **1** and **11**, respectively; Table 2).[20] From the above-described quantitative measurements, we conclude that analyte amounts exceeding 100 μg can easily be achieved inside the active probe volume, making NMR data accumulation as easy and efficient as in traditional NMR work with milligram quantities in NMR tubes.

In conclusion, this work demonstrates that the HPLC-SPE-NMR technique is a very efficient tool for the structure elucidation of constituents of *H. procumbens* roots without involving classical preparative-scale isolation schemes. Excellent-quality 1D and 2D NMR spectra could be routinely obtained, with the main sensitivity gain being achieved by analyte accumulation via multiple trapping. For the first time, multiple trapping was demonstrated to be a very effective means of sensitivity improvement for a rather polar analyte such as **1**. The technique was also applied to two commercial preparations of *H. procumbens*, allowing rapid authentication and structure determination of constituents.

It is noteworthy that the present study, representing de novo structure elucidation of 15 constituents of *H. procumbens*, has been performed over a period of a few months. This represents extractions, numerous optimizations and repetitions of HPLC-SPE-NMR experiments with both extracts, auxiliary experiments involving quantitative studies of trapping and elution efficiency, attempts of preparative isolation of unstable extract constituents,[20] and comparative studies of two commercial products, while the HPLC-SPE-NMR equipment has been shared with other members of our research group working on different projects. In terms of structures of *H. procumbens* constituents, the results of this study nevertheless represent an amount of knowledge that is equivalent to a major part of what has been achieved by numerous research groups over the period of the last four decades.[7−13,25,44−50] The amount of data that the HPLC-SPE-NMR technique can generate in days and weeks is equivalent to what has previously required months. Further progress is expected from improvements of HPLC-SPE-NMR protocols and computer-aided spectra interpretation.

We believe that the productivity enhancements brought about by the envisaged proliferation of hyphenated NMR methods, mainly HPLC-SPE-NMR, represent a paradigm shift with respect to how natural products research can and should be approached regarding structure elucidation. While isolation and purification of natural products will remain to be a fundamental requirement in many areas, notably in relation to their pharmacological activity, prior knowledge of extract constituents can be regarded as highly valuable when testing for biological activity. In many other cases, for example in projects related to biotechnology, ecology, or toxicology, an exact knowledge of the structure of extract constituents will be sufficient by itself. Notably, we have demonstrated that the HPLC-SPE-NMR technique enables structure elucidation of novel and complex natural products directly from extracts as rigorously as normally achieved by isolation followed by standard NMR analysis. While there are limits to the sensitivity of the HPLC-SPE-NMR technique, they

can be counteracted in part by technology developments,[14,15] and in part by studying enriched fractions, rather than crude extracts as in the present work. Although the HPLC-SPE-NMR analyses described in the present work leave the absolute chirality of the compounds undetermined, this can be, if necessary, circumvented by extended hyphenation involving circular dichroism.[14,15]

Experimental Section

Chemicals. Deuterated solvents (99.8 atom % of deuterium) were obtained from Cambridge Isotope Laboratories. Authentic acteoside (**1**) was originally isolated from *Retzia capensis*.[42] Solvents were analytical or HPLC grade and were used as received. Water was purified by deionization and 0.22 μm membrane filtration (Millipore).

Plant Material. Secondary roots of *Harpagophytum procumbens* (Burch.) DC. ex. Meissn (Pedaliaceae) were collected toward the end of July 2001 from 3-year-old plants in the Gouda region in the Western Cape, South Africa. The plant was cultivated under controlled conditions for commercial purposes by the Grassroots Natural Products Group (Gouda). A voucher specimen (BOL110816) was deposited in the Bolus Herbarium (Department of Botany, University of Cape Town, South Africa). Two commercial products of *H. procumbens* (capsules containing a powder appearing to be micronized root material) were purchased from drugstores, one in Los Angeles, CA, and the other in Copenhagen, Denmark.

Sample Preparation. Dried and powdered root material (500 g) was exhaustively extracted with 7 × 1 L of 96% EtOH, each time for about 24 h at room temperature. The combined extracts were concentrated in vacuo below 40 °C, and any residual solvent was removed on a freeze-dryer, yielding 63.0 g (13%) of extract. A similar procedure was followed for another 500 g of root material using 8 × 1 L of petroleum ether (PE), to give 3.0 g (0.6%) of nonpolar extract. The crude extracts were passed through C_{18} SPE cartridges (8.5 × 2.0 cm i.d., 5 g of the sorbent, Varian BondElut) as previously described.[17] For analysis of the commercial products of *H. procumbens*, 12 capsules were emptied to give 5 g of material from the product purchased in Denmark and 8 g of material from that purchased in the U.S. The material was extracted once with 75 mL of EtOH for 24 h at room temperature, to yield 88 mg (2%) and 414 mg (5%) of the extract, respectively. Solutions for the HPLC and HPLC-SPE-NMR analyses contained 40 mg/mL of the extract in H_2O−acetonitrile (70:30) and were centrifuged at 18000*g* for 5 min to remove any particulate matter. For the HPLC calibration curve, a solution containing 40 mg/mL of authentic acteoside (**1**) was made up in H_2O−acetonitrile (70:30) and diluted with the same solvent to give the following concentrations: 5.0, 4.0, 3.0, 2.5, 1.3, 0.6, 0.3, and 0.15 mg/mL. A solution for testing trapping and elution efficiency in HPLC-SPE-NMR experiments contained 5 mg/mL of authentic **1** in H_2O−acetonitrile (70:30). A reference standard for quantitative ^{1}H NMR spectra contained 1.67 mg/mL of **1** in acetonitrile-d_3.

HPLC Separations. Optimization of HPLC separations was carried out at 40 °C on a 150 × 4.6 mm i.d. Luna C_{18}(2) Phenomenex column (3 μm, 100 Å) using a Shimadzu HPLC system configured as previously described[15] and operated with Shimadzu Class-VP ver. 6.10 software. The chromatograms were monitored at 230 and 280 nm, and the extracts were separated using mixtures of H_2O (A) and acetonitrile (B) at 0.8 mL/min. Typical injection volumes were 25 μL for extract solutions and 10 μL for the standard solution of **1**. The linear gradient elution program for the EtOH extract was as follows: 0 min, 17% B; 35 min, 42% B; 37 min, 90% B; 40 min, 17% B, followed by a 10 min conditioning period. The following gradient profile was used for the PE extract: 0 min, 26% B; 5 min, 34% B; 64 min, 100% B; 70 min, 100% B; 73 min, 26% B, and 10 min conditioning. For the HPLC calibration curve for **1**, isocratic elution with H_2O−acetonitrile (70:30) at 0.8 mL/min was used. Peak integrals for each analyte concentration were determined in triplicate, and averaged values were used for construction of the calibration curve ($r^2 = 0.99$) and for the determination of concentration of **1** in the EtOH extracts. The same column and identical separation conditions were used for the quantitative HPLC determinations, the HPLC-SPE-NMR experiments, and the HPLC-MS experiments.

HPLC-SPE-NMR and NMR Experiments. The HPLC system was an Agilent 1100 system consisting of a quaternary solvent delivery pump equipped with a degasser, a column oven operated at 40 °C, an autosampler, and a diode array detector. A Knauer K100 Wellchrom makeup pump (flow rate 2.0 mL/min) diluted the post-column eluent flow with water before peak trapping using a Prospekt II SPE unit (Spark Holland). HySphere GP polymer resin [poly(divinylbenzene)] SPE cartridges (10 × 2 mm i.d.) from Spark Holland were used for trappings based on UV-absorption thresholds defined at 230 nm. The SPE device was coupled to a Bruker Avance 600 MHz spectrometer equipped with a 30 μL (active volume) ^{1}H{^{13}C} flow-probe. For structure determination, a total of eight or 10 trappings were performed for each chromatographic peak. The cartridges were dried with a stream of nitrogen for 30 min, and the analytes were eluted with 275 μL of acetonitrile-d_3 or another deuterated solvent at 220 mL/min into the NMR probe for data acquisition. HPLC separations, SPE peak trapping, and analyte transfer to the NMR spectrometer were controlled by Bruker HyStar ver. 2.3 software, while NMR acquisition and processing were performed using Bruker XwinNMR ver. 3.1 software. The 1D and 2D NMR experiments were performed as previously described.[17,18] Longitudinal relaxation times (T_1) for **1** in acetonitrile-d_3 were determined using the inversion recovery method with 16 different relaxation delays in the range 0.01−60 s and fitting the data into a three-parameter equation.

HPLC-MS Experiments. The HPLC-MS system used consisted of a Hewlett-Packard 1100 series chromatograph equipped with a quaternary pump, a degasser, a column oven, an autosampler, a diode array detector operated at 230 and 280 nm, and a single quadrupole mass-selective detector equipped with an Agilent G 1978A multimode ion source spray-chamber for simultaneous ESI and APCI, operated at default settings. Data were collected using the LC/MSD ChemStation software, version B.01.03.

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References and Notes

(1) Stewart, K. M.; Cole, D. J. *Ethnopharmacol.* **2005**, *100*, 225−236.
(2) Watt, J. M.; Breyer-Brandwijk, M. G. *The Medicinal and Poisonous Plants of Southern Africa;* E. & S. Livingstone Ltd. Publishers: London, 1962; p 830.
(3) Van Wyk, B. E.; van Oudtshoorn, B.; Gericke, N. *Medicinal Plants of South Africa;* Briza Publishers: Pretoria, 1997; p 144.
(4) Wegener, T. *HerbalGram* **2000**, *50*, 47−54.
(5) Barnes, J.; Anderson, L. A.; Phillipson, J. D. *Herbal Medicines*, 2nd ed.*;* Pharmaceutical Press: London, 2002; pp 174−180.
(6) Chrubasik, S.; Conradt, C.; Roufogalis, B. D. *Phytother. Res.* **2004**, *18*, 187−189.
(7) Lichti, H.; von Wartburg, A. *Helv. Chim. Acta* **1966**, *49*, 1552−1556.
(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) Jaroszewski, J. W. *Planta Med.* **2005**, *71*, 691−700.
(15) Jaroszewski, J. W. *Planta Med.* **2005**, *71*, 795−802.
(16) Exarchou, V.; Godejohann, M.; Van Beek, T. A.; Gerothanassis, I. P.; Vervoort, J. *Anal. Chem.* **2003**, *75*, 6288−6294.
(17) Clarkson, C.; Stærk, D.; Hansen, S. H.; Jaroszewski, J. W. *Anal. Chem.* **2005**, *77*, 273−283.
(18) Lambert, M.; Stærk, D.; Hansen, S. H.; Sairafianpour, M.; Jaroszewski, J. W. *J. Nat. Prod.* **2005**, *68*, 1500−1509.

(19) 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.
(20) Clarkson, C.; Stærk, D.; Hansen, S. H.; Smith, P. J.; Jaroszewski, J. W. *J. Nat. Prod.* **2006**, *69*, 527−530.
(21) Cooper, R.; Solomon, P. H.; Kubo, I.; Nakanishi, K.; Shoolery, J. N.; Occolowitz, J. L. *J. Am. Chem. Soc.* **1980**, *102*, 7955−7956.
(22) Wu, J.; Huang, J.; Xiao, Q.; Zhang, S.; Xiao, Z.; Li, Q.; Long, L.; Huang, L. *Magn. Reson. Chem.* **2004**, *42*, 659−662.
(23) Miyase, T.; Koizumi, A.; Ueno, A.; Noro, T.; Kuroyanagi, M.; Fukushima, S.; Akiyama, Y.; Takemoto, T. *Chem. Pharm. Bull.* **1982**, *30*, 2732−2737.
(24) Kobayashi, H.; Karasawa, H.; Miyase, T.; Fukushima, S. *Chem. Pharm. Bull.* **1984**, *32*, 3880−3885.
(25) Li, Y.; Jiang, S.; Gao, W.; Zhu, D. *Phytochemistry* **1999**, *50*, 101−104.
(26) Burnell, R. H.; Jean, M.; Marceau, S. *Can. J. Chem.* **1988**, *66*, 227−230.
(27) Evans, G. B.; Furneaux, R. H. *Bioorg. Med. Chem.* **2000**, *8*, 1653−1662.
(28) Harrison, L. J.; Asakawa, Y. *Phytochemistry* **1987**, *26*, 1211−1212.
(29) Fang, J.-M.; Lee, C.-K.; Cheng, Y.-S. *Phytochemistry* **1993**, *33*, 1169−1172.
(30) Lee, C.-K.; Cheng, Y. -S. *J. Nat. Prod.* **2001**, *64*, 511−514.
(31) Martin, J. D. *Tetrahedron* **1973**, *29*, 2553−2559.
(32) Grant, P. K.; Johnson, A. W. *J. Chem. Soc.* **1957**, 4079−4089.
(33) Johnson, A. W.; King, T. J.; Martin, R. J. *J. Chem. Soc.* **1961**, 4420−4425.
(34) Falshaw, P. C.; King, T. J. *J. Chem. Soc., Perkin Trans. 1* **1983**, 1749−1752.
(35) Mdee, L. K.; Waibel, R.; Nkunya, M. H. H.; Jonker, S. A.; Achenbach, H. *Phytochemistry* **1998**, *49*, 1107−1113.
(36) Jiménez-Estrada, M.; Reyes-Chilpa, R.; Hernández-Ortega, S.; Cristobal-Telésforo, E.; Torres-Colin, L.; Jankowski, C. K.; Aumelas, A.; Van Calsteren, M. R. *Can. J. Chem.* **2000**, *78*, 248−254.
(37) Alvarenga, N. L.; Ferro, E. A.; Ravelo, A. G.; Kennedy, M. L.; Maestro, M. A.; González, A. G. *Tetrahedron* **2000**, *56*, 3771−3774.
(38) Jankowski, C. K.; Savoie, A.; Lesage, D.; Boivin, J.; Leclair, G.; Diaz, E. T.; Reyes-Chilpa, R.; Jimenez-Estrada, M.; Barrios, H. *Polish J. Chem.* **2005**, *79*, 429−440.
(39) Stocking, E. M.; Williams, R.M. *Angew. Chem., Int. Ed.* **2003**, *42*, 3078−3115.
(40) Onan, K. D.; Kelley, C. J.; Patarapanich, C.; Leary, J. D.; Aladesanmi, A. J. *J. Chem. Soc., Chem. Commun.* **1985**, 121−122.
(41) Menash, A. Y.; Houghton, P. J.; Bloomfield, S.; Vlietnick, A.; Berghe, D. V. *J. Nat. Prod.* **2000**, *63*, 1210−1213.
(42) Damtoft, S.; Franzyk, H.; Jensen, S. R.; Nielsen, B. J. *Phytochemistry* **1993**, *34*, 239−243.
(43) Sandvoss, M.; Bardsley, B.; Beck, T. L.; Lee-Smith, E.; North, S. E.; Moore, P. J.; Edwards, A. J.; Smith, R. J. *Magn. Reson. Chem.* **2005**, *43*, 762−770.
(44) Czygan, F. C.; Kruger, A.; Schier, W.; Volk, O. *Deutsch. Apoth. Ztg.* **1977**, *117*, 1431−1434.
(45) Ziller, K.; Franz, G. *Planta Med.* **1979**, *37*, 340−348.
(46) Eich, J; Schmidt, M; Betti, G. *Pharm. Pharmacol. Lett.* **1998**, *8*, 75−78.
(47) Levielle, G.; Wilson, G. *Plant Cells Rep.* **2002**, *21*, 220−225.
(48) Guillerault, L.; Ollivier, E.; Elias, R.; Balansard, G. *J. Liq. Chrom.* **1994**, *17*, 2951−2960.
(49) Sticher, O.; Meier, B. D. *Deutsch. Apoth. Ztg.* **1980**, *120*, 1592−1594.
(50) Tunmann, P.; Bauersfeld, H. J. *Arch. Pharm.* **1975**, *308*, 655−657.

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