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Rapid Communications

Combination of LC−MS and LC−NMR as a Tool for the Structure Determination of Natural Products

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Abstract: Application of both LC−MS and LC−NMR to a partially purified extract of *Vernonia fastigiata* led to the direct identification of antibacterial sesquiterpene lactones **1**−**9** without isolation of individual compounds. The rapid structural analysis of both major and minor components of this class of compounds demonstrated the power of structure-guided screening as a complementary method to assay-guided screening.

Bioassay-guided natural products isolation often leads to already-known compounds of limited phytochemical or pharmacological interest. Hence, spectroscopic methods that would distinguish at an early stage novel rather than known or analogues of plant constituents would have considerable application. Thus, the combination of high-performance separation techniques with structurally informative spectroscopic methods such as MS and NMR could allow extracts to be screened not just for biological activity but at the same time for structural classes. LC−MS-coupled structure elucidation already plays a significant role in natural products isolation.[1] On the other hand, the direct combination of HPLC with NMR has achieved limited success due to the lack of sensitivity, the lack of general access to high-field NMR instruments, and the high price of deuterated solvents.

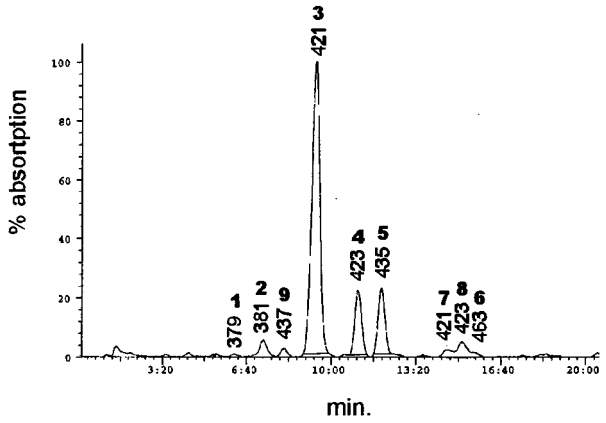

Figure 1. UV trace of the complete *Vernonia fastigiata* extract, MeOH/H_2O gradient (see text), with assigned quasi-molecular ions $[M + H]^+$.

Recently, this situation has changed as new solvent suppression techniques[2] have been introduced. Nevertheless, LC−NMR has mostly been applied to the study of metabolic pathways,[3] and the application to natural products isolation has been reported only in a few cases.[4,5] This may be due to loss of significant parts of proton spectra through solvent suppression. We report a strategy to avoid such problems and to obtain a complete set of all proton resonances.

Recently, we have described several new vernocistifolide-type sesquiterpene lactones from *Vernonia fastigiata* (Asteraceae).[6] In the present study, we describe the use of LC−MS and LC−NMR, which enabled us to rapidly assign the structures of major components of the previously described sesquiterpene lactones[6] and four additional minor components as well.

Ground *V. fastigiata* (whole plant) was extracted with ethyl acetate. Partitioning of the crude extract by rotational locular countercurrent chromatography (petroleum ether−ethyl acetate−methanol−water, 9:1:5:5) led to a fraction that showed significant activity against *Bacillus subtilis*. LC−MS[7] analysis (RP-18, 15% aceto-

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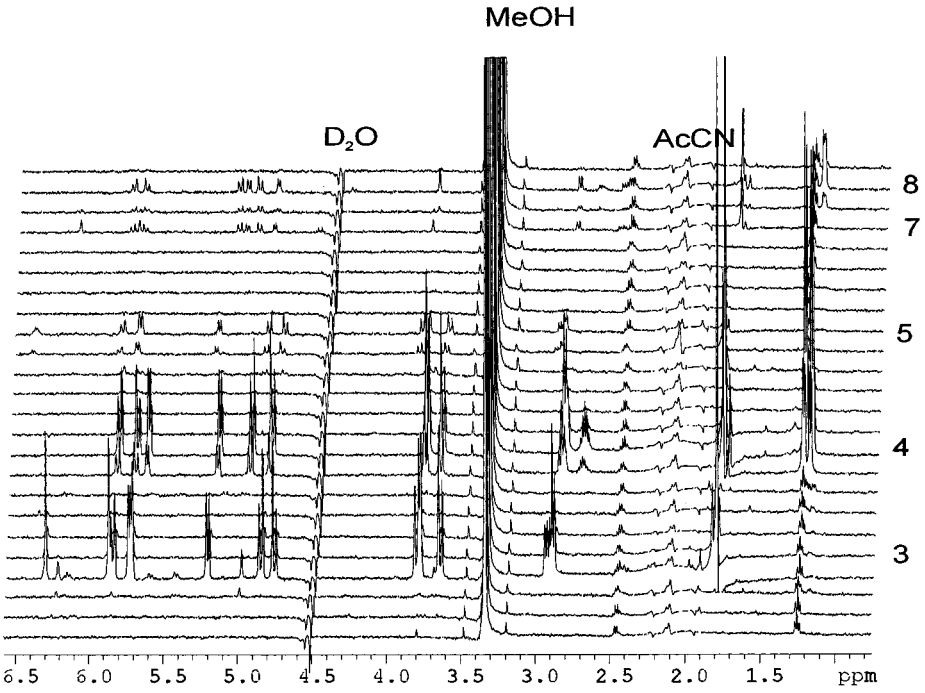
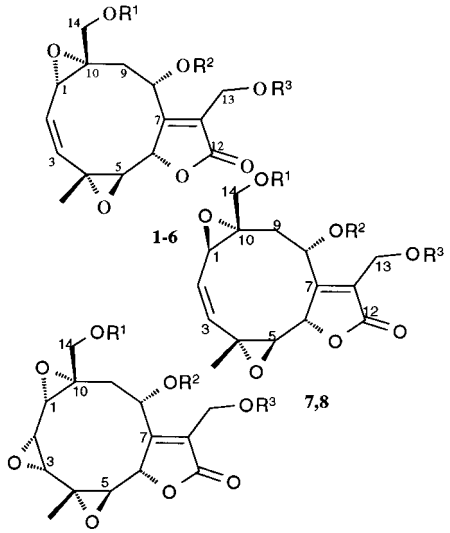


Figure 2. Part of the LC−NMR data (less polar fraction) in MeCN/D_2O.

Table 1. Analysis of Compounds **1**−**9** by APCI-LC−MS[a]



compd	*m/z* $[M + H]^+$	R^1	R^2 [b]	R^3
1	379	H	Methac	H
2	381	H	*i*-Bu	H
3	421	H	Methac	Ac
4	423	H	*i*-Bu	Ac
5	435	H	Ang	Ac
6	463	Ac	Methac	Ac
7	421	H	Methac	Ac
8	423	H	*i*-Bu	Ac
9	437	H	Methac	Ac

[a] Solvents used: MeCN/H_2O 15−40% for 55 min, 40−100% for 10 min. [b] Methac = methacroyl; *i*-Bu = isobutyroyl; Ang = angeloyl.

nitrile → 40% acetonitrile for 30 min → 100% acetonitrile for 30 min, flow 0.7 mL/min) of this mixture revealed a series of closely related compounds identified by their quasimolecular ions $[M + H]^+$ (Table 1). The corresponding CID spectra exhibited fragmentation patterns typical for the degradation of the vernocistifolide sesquiterpene lactone skeletons[6] with three modified hydroxyl groups (Table 1). Angelic, isobutyric, methacrylic, and acetic acid functional groups were identified by their corresponding acyloxy ions. From close inspection of the data, it became obvious that we were dealing with two sets of compounds possessing the same $[M]^+$ and mass fragmentation patterns but different chromatographic behavior (compounds **3**/**7** and **4**/**8**, respectively, Figure 1, Table 1).

For the LC−NMR[8] study of the minor components the crude fraction was separated by HPLC (RP-18, 15% acetonitrile → 40% acetonitrile for 30 min → 100% acetonitrile for 30 min, flow 1 mL/min) into fractions more polar and less polar than prevernocistifolide 8-*O*-methacrylate (**3**), which we already had identified as the main peak in the chromatogram.[6] This peak accounted for roughly 70% of the whole sample.

The less polar fraction was investigated by LC−NMR using the on-flow mode (700 μg injection, RP-18, 15% acetonitrile → 40% acetonitrile for 30 min → 100% acetonitrile for 30 min, flow 0.9 mL/min). A part of the on-flow run is shown in Figure 2. The results of this on-flow run support the findings of our LC−MS studies: Two different skeletons with the same degree of saturation (C-2/C-3) could be confirmed. Different ester groups were easily identified by their corresponding resonances either in the olefinic and/or alkyl region of the proton spectra (Table 2).

Due to solvent suppression, significant parts of the ^1H NMR spectra (range 2.0 ± 0.125 ppm, acetonitrile signal, and ~4.5 ppm, water signal,[9] Figure 3, lower trace) could not be analyzed. In order to get information about the suppressed signals, the LC−NMR spectra were run again in a methanol/water gradient (700 μg injection, RP-18, 15% methanol → 40% methanol for 30 min → 100% methanol for 30 min, flow 0.9 mL/min). Comparison of the resulting spectra with the spectra

Table 2. HPLC−^{1}H NMR (500 MHz) Spectral Data of Compounds **1**−**9** in MeCN/D_2O (MeOH/D_2O)

proton(s)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	3.68 s	3.68 bs	3.75 bs	3.62 s	3.69 bs	3.63 bs	3.82 bs	3.80 bs	3.54 bs
2	5.71 d	5.72 d	5.80 d	5.75 d	5.76 d	5.75 d	5.83 d	5.82 d	3.80 d
3	5.62 d	5.61 d	5.71 d	5.61 d	5.63 d	5.64 d	5.77 d	5.76 d	3.59 d
5	2.76 d	2.74 d	2.78 d	2.76 d	2.78 d	2.78 d	2.84 d	2.82 d	3.21 d
6	5.10 d	5.03 d	5.16 d	5.08 d	5.09 d	5.05 d	4.93 d	4.95 d	5.09 d
8	5.54 d	5.45 d	5.62 d	5.54 d	5.62 d	5.61 d	5.18 d	5.16 d	5.4 d
9_a	2.81 dd	2.76 dd	2.80 dd	2.80 dd	2.80 dd	2.90 dd	2.64 dd	2.39 dd	2.76 dd
9_b	1.65 d	1.64 d	1.84 d	1.65 d	1.65 d	1.71 d	(1.82 d)	(1.85 d)	1.64 d
13_a	4.27 d	4.30 d	4.78 d	4.85 d	4.79 d	4.74 d	5.00 d	5.00 d	4.30 d
13_b	4.16 d	4.23 d	4.64 d	4.73 d	4.68 d	4.66 d	5.20 d	5.18 d	4.23 d
14_a	3.58 d	3.52 d	3.59 d	3.58 d	3.56 d	4.18 d	4.18 d	4.19 d	3.58 d
14_b	3.68 d	3.68 d	3.70 d	3.69 d	3.75 d	3.99 d	4.02 d	4.00 d	3.67 d
15	1.65 s	1.66 s	1.72 s	1.64 s	1.70 s	1.72 s	1.79 s	1.79 s	1.63
OAc			(2.07 s)	(2.12 s)	(2.07 s)	(2.1 s)	(2.08 s)	(2.1 s)	(2.1s)
OAc						(2.04 s)			
OCOR	5.73 bs	2.64 h	5.79 s	2.71 h	6.32 m	5.78 bs	5.81 bs	2.70 h	5.80 s
	6.20 bs	1.08 d	6.22 s	1.06 d	(2.05 m)	6.20 bs	6.21 bs	1.21 bs	6.22 s
	(2.0 s)	1.22 d	(2.0 s)	1.09 d	(1.97 m)	(2.0 s)	(2.0 s)	1.24 bs	(2.0 s)

J (Hz)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1,2									2.2
2,3	12.5	12.6	12.5	12.5	12.5	12.4	12.5	12.5	4.5
5,6	8.6	8.8	8.8	8.8	8.6	8.8	8.8	8.8	8.8
$8,9_a$	8.3	8.5	8.2	8.3	8.8	9.2	8.8	8.8	8.3
9a,9b	15.6	15.5	15.6	15.5	15.8	15.4	15.2	15.2	15.6
13a,13b	12.8	12.8	12.7	12.6	12.2	12.7	12.6	12.7	12.6
14a,14b	12.3	12.3	12.3	12.3	12.2	12.2	12.3	12.3	12.4
1′, 2′, 3′		7.0		7.0				7.0	

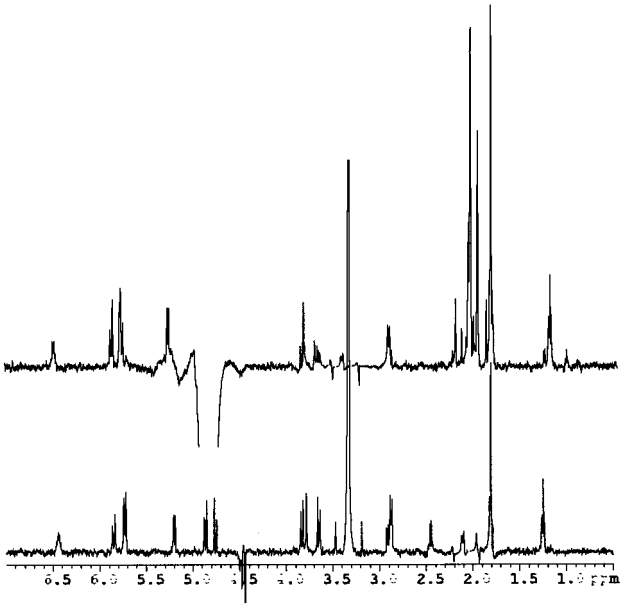


Figure 3. Comparison of the ^{1}H NMR spectra of **5** in MeOH/D_2O (upper trace) and MeCN/D_2O (lower trace).

obtained in the acetonitrile/water gradient displayed a nice overlap of both proton spectra, as shown in Figure 3 for compound **5**. Hence, the combination of both spectra allowed the assignment of all proton resonances of the corresponding compounds in just two LC−NMR runs. Finally, we examined the more polar fraction by stopped-flow techniques, where compounds **1**, **2**, and **9** were found to be present in μg quantities. The structures could be unambiguously assigned by ^{1}H NMR. Furthermore, COSY spectra (WETGCOSY[10]) of components **2** and **3** were recorded under stopped-flow conditions in order to confirm the structures of these compounds.

The crucial stereochemistry of the 1,10-epoxy functions was assigned on the basis of the chemical shifts and coupling constants of H-$9_{a,b}$ and H-8. The major components **3** and **4** showed proton resonances at 2.8 (dd), 1.84, 1.65 (d, H-9_a, H-9_b), and ~5.6 ppm (d, H-8), whereas in the less polar components **7** and **8**, possessing the same molecular mass as **3** and **4**, respectively (Figure 1), the signals for H-8 and H-9 have shifted to 2.64 (**7**), 2.39 (**8**) (dd, H-9_a), 5.18 (**7**), and 5.16 (**8**) ppm (d, H-8), respectively. Complete stereochemical information was obtained from coupling patterns (H-8, H-$9_{a,b}$; H-1, H-2, H-3; H-5, H-6, Table 2) and NOESY experiments, (H-6 → H-15; H-6 → H-8; H-6 → H-15, Figure 4). Compound **3** was examined by 2D-NOESY (WETNOESY[10]), which furnished the same spectral information as the off-line spectra[6] (Figure 4). The NOE's of one or two protons often give enough information to clarify stereochemical features of the respective compounds. Thus, we also have examined the 1D-selective-NOESY on compound **3**, implemented as a pulsed-field gradient spectrum[11] (Figure 5). This method has the advantage that the recording of the spectra takes much less time than a 2D version and enables the spectroscopist to do a rapid analysis of all crucial protons. It is furthermore noteworthy that due to the selective excitation technique this type of spectroscopy needs no solvent suppression.

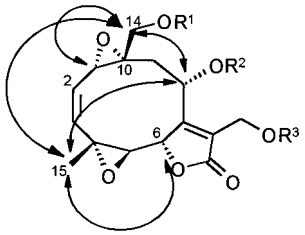


Figure 4. Observed nuclear Overhauser enhancements in compound **3**.

Our results show clearly that the combination of LC−

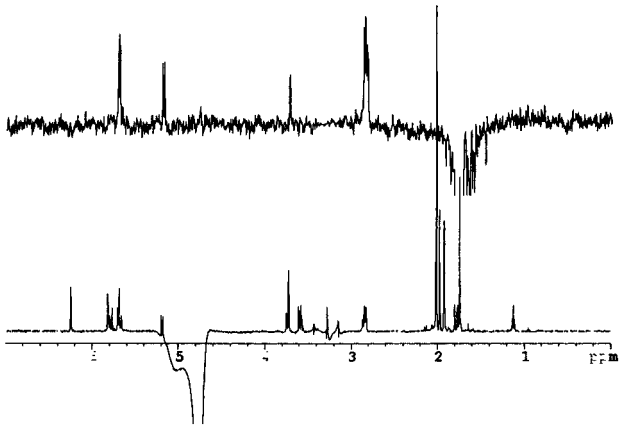


Figure 5. DPFGNOE spectrum of compound **3** in MeOH/D_2O.

MS and LC−NMR techniques makes structural information of even complex mixtures rapidly accessible. LC−MS gives not only structural information but can be used for cross-referencing under different chromatographic conditions as well. With such newly developed NMR techniques a rapid structure elucidation process can be performed. Even complicated structures with highly demanding stereochemical features can now be studied with small amounts of samples, using the complementary methods of LC−MS and LC−NMR.

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(7) LC−MS spectra were recorded on a Finnigan TSQ 700 in the APCI mode.
(8) LC−NMR spectra were recorded on a Varian Unity Inova 500 MHz spectrometer equipped with a PFG-probe having a flow cell of 65 μL active volume.
(9) The resonance of water changes with respect to the referenced acetonitrile signal.
(10) Standard Varian Pulse Library.
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