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Direct ^{13}C NMR Detection in HPLC Hyphenation Mode: Analysis of *Ganoderma lucidum* Terpenoids

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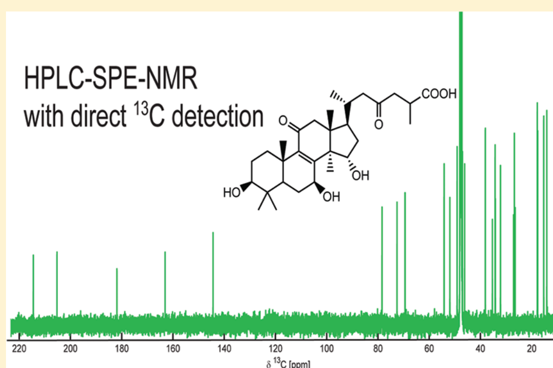
Supporting Information

ABSTRACT: Solid phase extraction (SPE) was introduced as a crucial step in the HPLC-SPE-NMR technique to enable online analyte enrichment from which proton-detected NMR experiments on submicrogram amounts from complex mixtures were possible. However, the significance of direct-detected ^{13}C NMR experiments is indubitable in simplifying structural elucidations. In the current study, we demonstrated direct ^{13}C NMR detection of triterpenoids from a *Ganoderma lucidum* extract in hyphenation mode. The combined advantage of a cryogenically cooled probe, miniaturization, and multiple trapping enabled the first reported application of HPLC-SPE-NMR analysis using direct-detected ^{13}C NMR spectra. HPLC column loading, accumulative SPE trappings, and the effect of different elution solvents were evaluated and optimized. A column loading of approximately 600 μg of a prefractionated triterpenoid mixture, six trappings, and an acquisition time of 13 h resulted in spectra with adequate signal-to-noise ratios to detect all C-13 signals.



In recent years, hyphenation of ^{1}H-detected NMR spectroscopy with high-performance liquid chromatography (HPLC) has emerged as a convenient and rapid tool for the analysis of complex mixtures, especially natural products.[1,2] In particular, the introduction of a solid-phase extraction (SPE) step as a means of automated recovery of analytes from the HPLC mobile phase for NMR analysis greatly extended the scope of the technique in terms of sensitivity, relative absence of interference from mobile phase components, and decoupling of the time scale of the HPLC separation from that of the NMR data acquisition. Thus, HPLC-SPE-NMR offers a combined advantage of analyte focusing, replacement of a nondeuterated HPLC mobile phase with a deuterated NMR solvent, and the possibility of performing time-consuming NMR experiments without compromising HPLC separation.[1,2]

In the field of natural product analysis, detection and assignment of ^{13}C resonances are often essential for unambiguous structure elucidation. These are routinely obtained from ^{1}H-detected one-bond (HSQC, HMQC) and multiple-bond (HMBC) two-dimensional ^{1}H,^{13}C-heteronuclear chemical shift correlation experiments.[3] However, although these ^{1}H-detected 2D heteronuclear NMR spectra in general offer a convenient means of measuring ^{13}C chemical shifts, detection of quaternary carbon atoms and even hydrogen-bearing carbon atoms can be problematic due to the dependence of coupling constants, coupling path geometry, and substituent patterns. This can necessitate acquisition of multiple 2D spectra with various mixing times,[4] contrasting the convenience of direct detection of carbon resonances by use of proton-decoupled ^{13}C NMR spectra, or edited ^{13}C NMR spectra such as experiments using the DEPT sequence. More importantly, limited resolution in the indirect dimension of 2D experiments often results in unresolved resonances of closely spaced signals, whereas the resolution in directly detected ^{13}C NMR spectra is limited only by the signal width.

Because the gyromagnetic ratio of ^{13}C is only one-fourth that of ^{1}H, and further because the natural abundance of ^{13}C is only 1.1% of the total carbon nuclei, the overall receptivity of ^{13}C is approximately 5700 times lower than that of ^{1}H at the same magnetic field strength.[5] Therefore, virtually all hyphenated NMR studies reported thus far use ^{1}H-detected experiments. In the present article we demonstrate that a combination of a miniaturized (30 μL) capillary NMR probe with a cryogenically cooled ^{13}C observe channel[6,7] and multiple trapping in the HPLC-SPE-NMR mode makes hyphenated ^{13}C NMR experiments feasible. The advantage of this technique is demonstrated by analysis of mixtures of terpenoids from *Ganoderma lucidum*, a mushroom widely used in traditional Chinese medicine.[8,9] Since *G. lucidum* triterpenoids differ by the placement of double bonds and oxygen atoms within a few well-conserved types of carbon skeletons, ^{13}C chemical shifts are particularly useful in their structure elucidation. Use of ^{1}H-detected 2D experiments is however hampered by the necessity of assigning often unresolved and overlapping peaks of diastereotopic methylene protons of the triterpenoid core. This study encompasses both model studies using pure

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compounds in order to characterize their behavior in the HPLC-SPE-NMR setup and analysis of a complex chromatographic fraction of a *G. lucidum* extract.

■ RESULTS AND DISCUSSION

The hyphenated NMR system used in this work comprised a 600 MHz NMR magnet equipped with a cryogenically cooled probe head designed for tubes with an outer diameter of 1.7 mm (30 μL filling volume) encompassing cooled preamplifiers for both proton and carbon channels. Separations were performed on a C_{18} reversed-phase column (i.d. 4.6 mm), using a water–acetonitrile gradient with a small amount of formic acid to enhance ionization in the ESI interface of a hybrid quadrupole time-of-flight mass spectrometer. High-resolution mass spectra were acquired with an error typically less than 2 ppm. SPE trappings were performed on 10 × 2 mm cartridges (bed volume approximately 30 μL) filled with divinylbenzene-based resin (previously found to be the most efficient general purpose SPE phase[10]) using an automated SPE device. The cartridges were eluted into NMR tubes and subjected to NMR analyses by means of a robotic liquid handler and automated sample changer, respectively.

In HPLC-SPE-NMR, the amount of compound in the final detection cell, and hence the possibility to obtain NMR spectra with sufficient signal-to-noise ratios, is limited by the chromatographic separation system as well as by the SPE trapping and recovery processes. Critical parameters for the chromatographic separation are column capacity, the combined amount of analytes in each injection, and the heterogeneity of the sample. Adsorption of the compounds and elution from the stationary phase in the cartridges is governed by partition coefficients and by elution profiles. These parameters differ greatly for different compounds, solvents, and the SPE sorbent material used.[10,11] Moreover, the efficacy of multiple trapping has to be assessed, as it can vary depending on the water–organic solvent composition and the amount of already preloaded material on the SPE cartridge.[12−16]

To investigate suitable parameters for the analysis of terpenoids from *G. lucidum* and the overall performance of the current HPLC-SPE-NMR setup, a test mixture was prepared and analyzed. Three representative model compounds, lucidenic acid N (**1**),[17] ganoderic acid H (**2**),[18] and lucidenic acid E (**3**)[18] (Figure 1), were mixed and dissolved at concentrations of about 1.4 μg/μL (3.5, 2.3, and 2.3 mM for the three compounds, respectively). It was found that the chromatographic peak shapes were practically identical for increasing injection volumes in the range 2 to 25 μL, while injection volumes larger than 25 μL caused progressive fronting (data not shown). Injection volumes of 25 μL, which corresponded to a total triterpenoid amount of approximately 100 μg per injection, were therefore selected for further evaluations.

The solvent employed to elute compounds from the SPE cartridges has to fulfill several requirements. First and most importantly, the solvent has to effectively extract the compound from the solid phase in a small volume that matches the detection cell volume. Second, it should ideally have suitable NMR properties for studies of specific compounds in terms of solubility, viscosity, volatility, signal dispersion, position of solvent-specific signals, and possibly reactivity. In particular cases, it might be important to acquire spectra in a specific solvent to be able to use previously recorded reference data. In order to evaluate the efficiency of various eluting solvents,

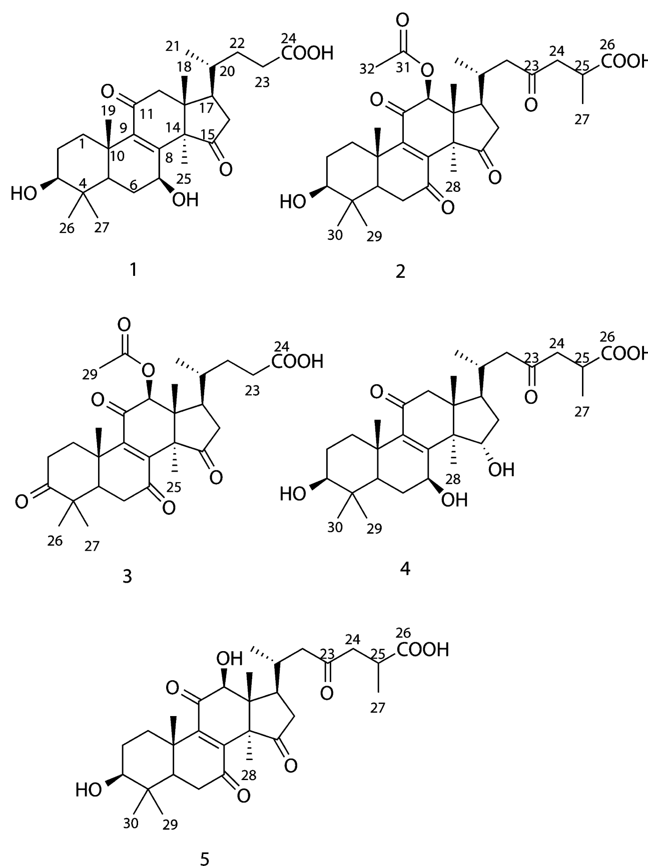

Figure 1. Triterpenoids from *Ganoderma lucidum*. Lucidenic acid N (**1**), ganoderic acid H (**2**), and lucidenic acid E (**3**) were used as model compounds to evaluate the HPLC-SPE-NMR setup. Ganoderic acids C_2 (**4**) and C_6 (**5**) were found as minor components in an extract and analyzed using direct-detected ^{13}C NMR spectra.

single injections of the above-described test mixture were followed by chromatographic separation, trapping, and elution with either methanol-d_4, acetonitrile-d_3, or chloroform-*d*. These solvents represent a (relatively) polar protic, a polar aprotic, and a nonpolar solvent, respectively, with acetonitrile-d_3 often used as a default solvent in HPLC-SPE-NMR. The signal-to-noise ratios of methyl group singlets in the resulting ^{1}H NMR spectra are shown in Table 1. While line shapes in methanol-d_4 and acetonitrile-d_3 were similar (half-width 1.4−1.6 Hz), the signals in chloroform-*d* were considerably broader (2.0−2.6 Hz). Therefore, in addition to signal-to-noise ratios, peak areas calculated from fitted pure Lorentzians are also presented. It is concluded that all three solvents are suitable for transferring *G.*

Table 1. Comparison of Eluting Efficiency of Different Solvents in HPLC-SPE-NMR Experiments with Compounds 1−3[a]

	methanol-d_4		acetonitrile-d_3		chloroform-*d*	
compound	SNR	peak area	SNR	peak area	SNR	peak area
1	9200	1600	11 200	1600	5700	1600
2	5800	900	6300	1000	4800	1100
3	4600	900	4700	800	3600	1000

[a]For each solvent and compound, signal-to-noise ratios (SNR) and peak areas (in arbitrary units) of the methyl singlet at highest field in the ^{1}H NMR spectra (64 scans). The values are rounded to the nearest hundred.

lucidum triterpenoids from the currently used SPE cartridges to NMR tubes. Methanol-d_4 was selected for further experiments because of better signal dispersion and more favorable positions of residual solvent signals. The spectra acquired in chloroform-*d* also exhibited broad undesired background signals in the aliphatic range (data not shown). This might be due to minor amounts of residues extracted from the cartridge material and tubing during the elution process.

To further increase the amount of compounds submitted to NMR analysis, it is possible to perform multiple trappings on the SPE cartridges.[12−16] To evaluate the efficiency of this procedure, a series of chromatographic separations and cumulative trappings were performed on the test mixture with methanol-d_4 as the solvent in the elution step. Signal-to-noise ratios in the ^{1}H NMR spectra were evaluated after one and up to six consecutive injections and trappings of the three separated compounds. This corresponded to 30−240 μg of the compounds in the test mixture. The signal-to-noise ratios versus number of injections of the triterpenoid mixture showed excellent linearity, with R^2-values > 0.97 without any noticeable leveling off (Figure 2). Thus, multiple trapping is concluded to be an effective way of increasing the amount of pure metabolites for analysis.

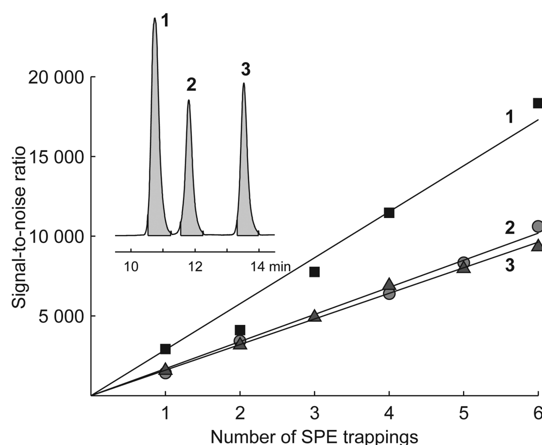

Figure 2. Increase of signal-to-noise ratios in ^{1}H NMR spectra obtained in multiple-trapping HPLC-SPE-NMR experiments with **1**−**3** (25 μL injections corresponding to 40, 33, and 30 μg or 87, 58, and 58 nmol of **1**, **2**, and **3** per injection, respectively). Inset shows UV trace (254 nm) for a single (25 μL) injection of the test mixture. Shaded ranges in the chromatogram represent the time intervals for the trappings.

Finally, direct-detected ^{13}C NMR spectra of **1**−**3** were acquired using the tubes that received the eluate after six cumulative trappings. After approximately 6.5 h of acquisition for each sample, 86 of the total 88 expected signals are clearly observed (Figure 3, Figures S-1 and S-2 in the Supporting Information). Spectra were calculated from the free induction signal (FID) both by standard Fourier transform (FT) and by the maximum entropy method (MaxEnt). The latter method has been shown to suppress noise while keeping the line widths narrow, thus improving the signal-to-noise ratio.[19] This does not imply a higher sensitivity but rather a nonlinear scaling of the signals observed in the Fourier transformed data, thus producing spectra with signals that are more easily detected by the eye.[20,21] The two remaining signals that still were not clearly observed, one weak signal (compound **1**, C-8) and one absent signal (**2**, C-3), could easily be confirmed by using proton-detected HSQC and HMBC experiments.

Analysis of a *G. lucidum* extract was performed to validate the performance of the protocol exemplified by the analysis of the synthetic test mixture. A defatted methanol extract of wall-broken spores was fractionated using vacuum liquid chromatography. The early eluting fractions contained triterpenes, while the later contained phenolic glycosides as determined from HRMS and ^{1}H NMR spectra (data not shown). The pooled triterpenoid fractions were further fractionated using preparative RP-HPLC, and an early eluting minor fraction with a few chromatographic peaks was selected for further analysis for this study (Figure 4). On the basis of the relative intensities of the UV signal at 254 nm and total ion chromatogram, this fraction contained less abundant terpenoids. Hence, the successful acquisition of ^{13}C NMR spectra demonstrated for this fraction indicated that analysis of more abundant compounds is also possible.

An initial analysis using a column loading comparable to the one in the test mixture and with eight trappings gave ^{13}C NMR spectra with considerably lower signal-to-noise ratio than those obtained previously (data not shown). A nearly 6-fold increase in the amount of injected material, with six trappings where each injection (80 μL) corresponded to approximately 560 μg, was necessary to achieve the expected signal-to-noise ratios. There are three possible explanations for this. First, the number of constituents in the test mixture was three, whereas the real sample fraction contained at least six major compounds with four of them co-eluting. Peaks a and c (Figure 4) corresponded to mixtures of two co-eluting compounds as determined from HRMS and ^{1}H NMR data. Second, unlike the test mixture, the real sample fraction contained minor background components that contributed to the total amount of column loading but not to the NMR signals of trapped compounds. The third explanation is related to the lower chromatographic resolution; threshold value based trapping of unresolved peaks will inevitably yield less compound than fully resolved peaks. Unsymmetrical broadening of the peak due to overloading will also contribute to a lower yield. In addition to the increased column loading, the number of scans for the following direct-detected ^{13}C NMR experiments was doubled compared to the study of the test mixture to make sure the spectra would be of sufficient quality for detection of all C-13 signals.

The acquired direct-detected ^{13}C NMR spectra for the two single-component peaks b and d are shown in Figure 5 (and in Figures S-3 and S-4 in the Supporting Information). All signals were of sufficient signal-to-noise ratios without using MaxEnt processing and assigned in the structure elucidation process based on combinations of 1D and 2D NMR experiments (DQF-COSY, HSQC, and HMBC). The structures of the compounds in peaks b and d were found to be ganoderic acids C_2 (**4**) and C_6 (**5**), respectively (Figure 1).[22]

The advantage of using direct-detected ^{13}C NMR spectra in the assignments was evident from the high chemical shift dispersion, making it possible to differentiate between signals in the crowded regions at high field, one example being the two methylene carbons at positions 22 and 23 in compound **1**, which differ by 0.08 ppm (12 Hz). Guided by the small difference, the assignment of the diastereotopic protons was simplified. Another example is compound **3**, with four methyl signals (in positions 21, 25, 27, and 29) within 0.32 ppm (48 Hz).

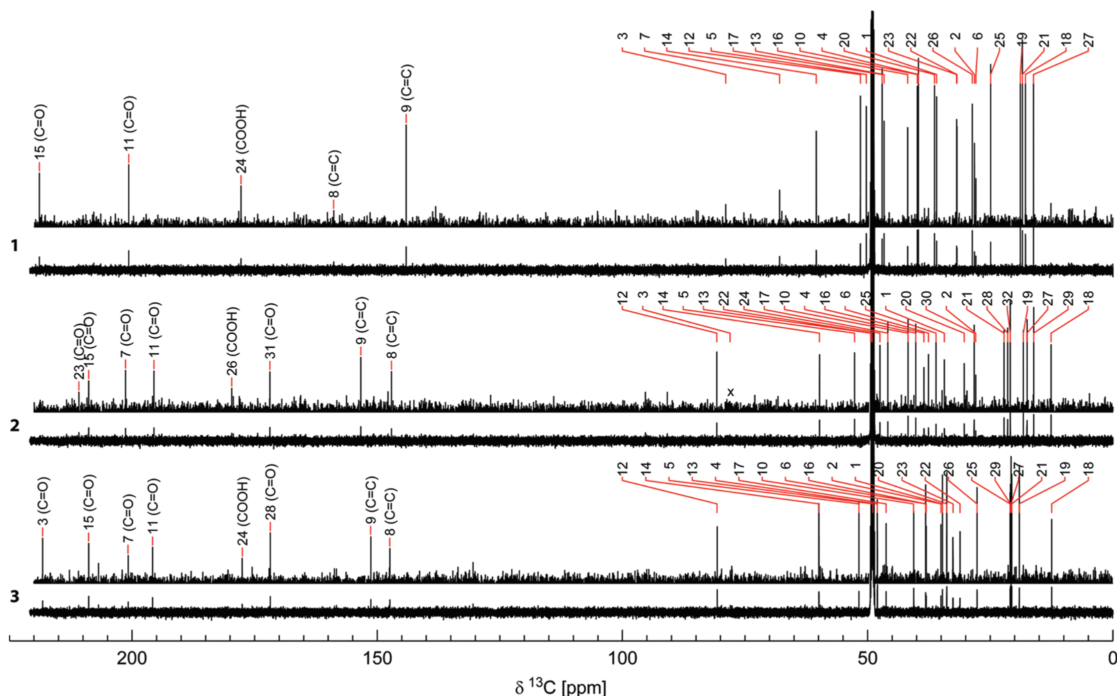

Figure 3. ^{13}C NMR spectra of **1**−**3** calculated using the maximum entropy method (upper) and Fourier transformed (lower spectra). The spectra were acquired following six trappings and elution in methanol-d_4. The spectra are scaled to the same noise evaluated for the range 100−120 ppm. One missing signal, C-3 in compound **2**, is marked with "x".

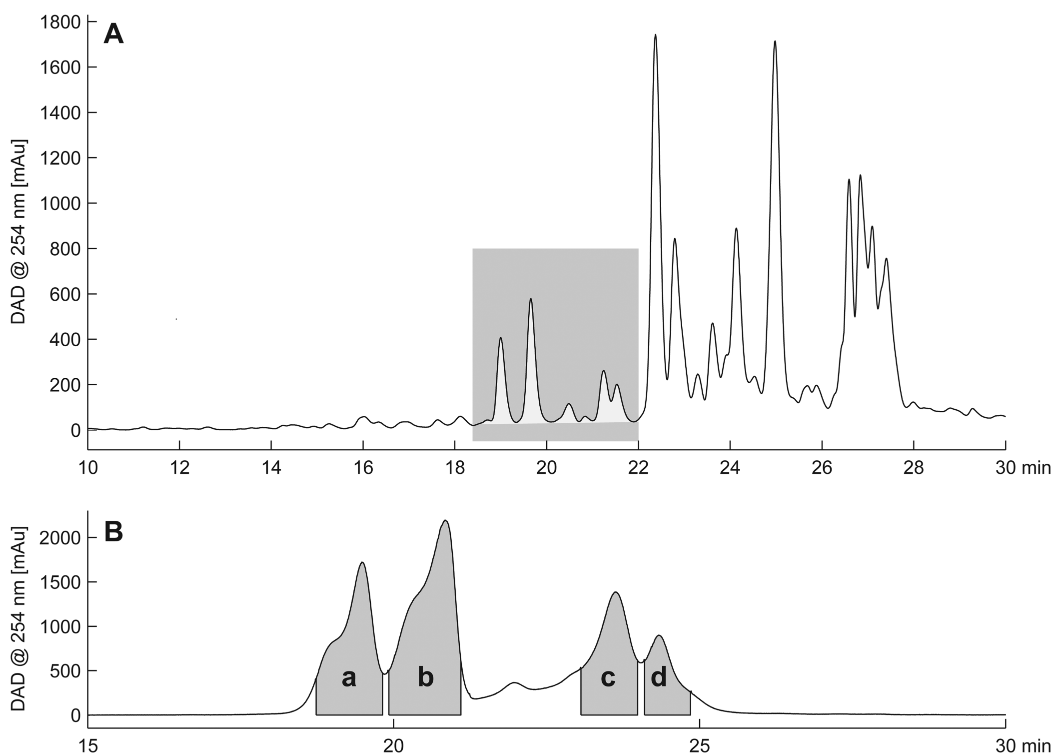

Figure 4. Crude triterpenoid mixture from *Ganoderma lucidum* spores analyzed by RP-HPLC with UV detection at 254 nm (A). The shaded range indicates a fraction submitted to HPLC-SPE-NMR analysis using overloading of the column (approximately 560 μg in 80 μL) (B). The four peaks, a−d, were trapped for further analysis by NMR.

Complications in structural elucidation as a result of limited resolution in the indirect dimension of inverse-detected 2D experiments can also be circumvented by having a direct-detected ^{13}C experiment as a guide for the interpretation. This is exemplified in **4**, where HMBC cross-peaks from H-1a/H-5, H-1b, H-19, H-29, and H-30 could be interpreted as correlations to a single ^{13}C signal at 39.7 ppm (Figure 6). With the direct-detected ^{13}C NMR spectrum as a guide in the F1 dimension and partial sums of each of the cross-peaks in the HMBC spectrum, the correct number of different correlations

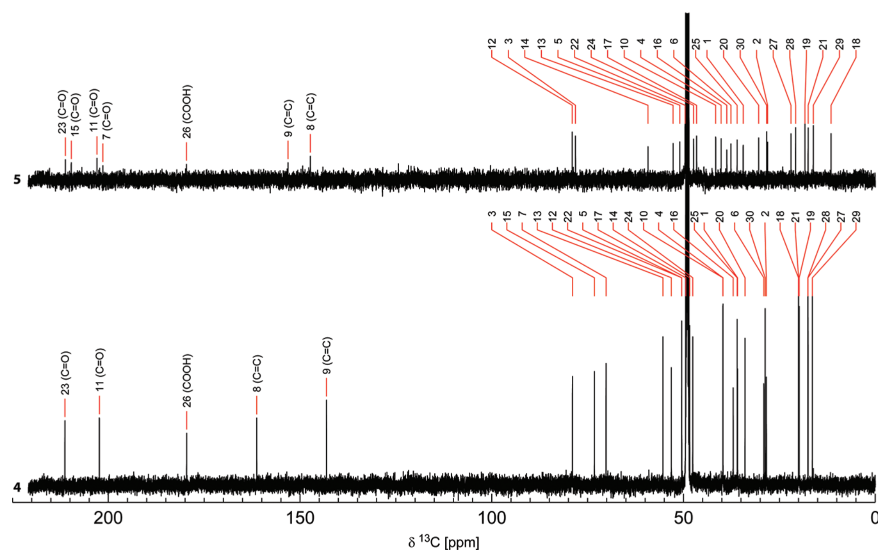

Figure 5. Direct-detected ^{13}C NMR spectra of ganoderic acids C_2 (**4**) and C_6 (**5**) acquired on trapped and eluted peaks b and d, respectively.

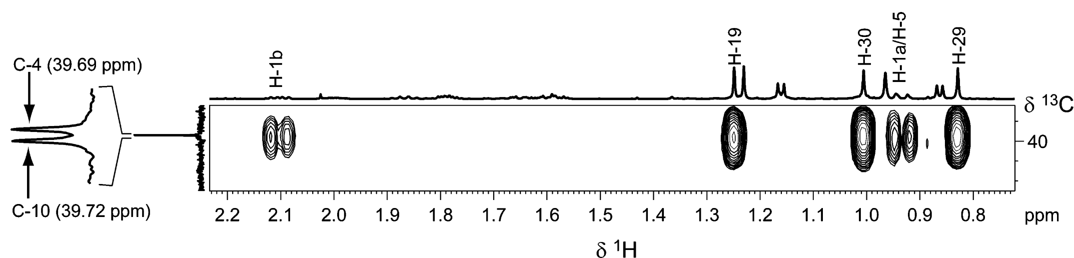

Figure 6. Detail of the HMBC spectrum of **4** with the direct-detected ^{13}C spectrum plotted to the left along the F1 axis including an expansion showing the two separate signals from C-4 and C-10. The 1D ^{1}H NMR spectrum is plotted above with the relevant assignments marked.

could accurately be assigned. H-1b and H-19 show $^2J_{H,C}$ correlations to C-10 at 39.72 ppm, while the H-29 and H-28 methyl groups show the corresponding correlations to C-4 at 39.69 ppm. C-4 and C-10 are closely spaced not only on the chemical shift axis ($\Delta\delta$ 0.03 ppm) but also in the actual structure (two bonds apart), which might confuse the constraints of a structure elucidation solely based on the information from inverse-detected 2D experiments.

In summary, direct ^{13}C detection was demonstrated for both an artificial test mixture and a triterpene-containing fraction from an extract of *G. lucidum*. As far as we are aware, this is the first time direct-detected ^{13}C NMR spectra have been demonstrated in HPLC-SPE-NMR hyphenated mode in the analysis of a real extract. It was achieved by high column loading on an analytical scale HPLC-system, multiple trappings, and a highly sensitive miniaturized 1.7 mm cryogenically cooled probe head with an integrated cooled carbon channel preamplifier. This study provides the experimental envelope in terms of loading, number of trappings, elution solvent, acquisition times, quality, and signal-to-noise ratios of the resulting spectra.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Compounds **1**−**3** were from Planta Analytica LLC (Danbury, CT, USA). Wall-broken spores of *G. lucidum* were from Wan Yuen Tong Medicine Co., Ltd. (Hong Kong). Methanol-d_4 was from Sigma-Aldrich (St. Louis, MO, USA); acetonitrile-d_3 and chloroform-*d* were from Eurisotop (Saclay, France) (min. 99.8 atom %). Formic acid was from Merck (Darmstadt, Germany). Ethanol (96%), HPLC grade acetonitrile, chloroform, and methanol were from Sigma-Aldrich. Petroleum ether (40−65 °C), *n*-hexane, and silica 60 (15−40 μm) were from VWR International S.A.S. (Fontenay-sous-Bois, France), POSH S.A. (Gliwice, Poland), and Merck KGaA (Germany), respectively. Water was deionized and membrane-filtered (0.22 μm) on a Millipore system (Billerica, MA, USA). HPLC chromatography was performed using binary gradient mixtures of acetonitrile−water, 5:95 (solvent A), and acetonitrile−water, 95:5 (solvent B), both with 0.1% formic acid.

HPLC-DAD/MS-SPE. HPLC separations were performed with an Agilent 1100 system consisting of a degasser, a quaternary pump, an autosampler, a column oven, a diode array detector (DAD), and a 4.6 × 150 mm Phenomenex C_{18}(2) Luna (5 μm) (Phenomenex, Inc., Torrance, CA, USA). The column was operated at 25 °C, and the mobile phase was delivered at 0.5 mL/min. The flow from the column was split by a T-piece connected to capillaries of different length and i.d. (10 cm/0.05 μm, 100 cm/0.5 μm), directing <1% of the column outflow to a micrOTOF-Q mass spectrometer equipped with an electrospray ionization (ESI) interface (Bruker Daltonik GmbH, Bremen, Germany). Mass spectra were acquired in positive-ion mode, using a drying temperature of 200 °C, a nebulizer pressure of 2.0 bar, and a drying gas flow of 7 L/min. The rest of the mobile phase flow was directed to the DAD detector and then, after diluting with 1 mL/min of water by means of a Knauer Smartline Pump 100 (Herbert Knauer GmbH, Berlin, Germany), to a Prospect 2 SPE-unit (Spark Holland B.V., Emmen, The Netherlands). Selected chromatographic peaks were trapped on SPE cartridges (Hysphere GP-phase, 10 × 2 mm i.d., from Spark Holland), preconditioned with 500 μL of acetonitrile followed by 500 μL of water. After trapping, the cartridges were dried with a stream of nitrogen gas for 35 min and eluted with deuterated solvents directly to 1.7 mm o.d. NMR tubes (Bruker Biospin, Karlsruhe, Germany) by means of a Gilson 215 liquid handler (Gilson, Inc., Middleton, WI, USA). The tubes were filled with 30 μL of solvent and sealed with plastic balls as per manufacturer

instructions. Separation, mass spectrometry, trapping, and elution were controlled with Hystar software (version 3.2, Bruker Biospin).

NMR Spectroscopy. NMR experiments were performed with a 600 MHz Avance III system equipped with a Samplejet sample changer and a cryogenically cooled gradient inverse triple-resonance 1.7 mm TCI probe head (Bruker Biospin) optimized for ^1H and ^{13}C observation. Bruker standard pulse sequences were used throughout this study. Acquisition and processing of NMR data were performed using Topspin (version 3.0, Bruker Biospin) and IconNMR (version 4.2, Bruker Biospin) for controlling the automation. One-dimensional ^1H NMR spectra were acquired in automation (temperature equilibration to 300 K, optimization of lock parameters, gradient shimming, and setting of receiver gain) with 30° pulses, interpulse intervals of 3.66 s, and 64k data points zero-filled to 128k and multiplied with an exponential function corresponding to line-broadening of 0.3 Hz prior to Fourier transform. ^1H NMR spectra line widths, heights, and areas were evaluated by fitting a pure Lorentzian line shape to the methyl singlets resonating at the highest field (δ 0.81−0.90) using Matlab (ver. R2010b, Mathworks Inc., MA, USA). Signal-to-noise ratios (64 scans) were determined using noise region δ 10.1−11.1. One-dimensional ^{13}C NMR spectra were acquired in automation with 30° pulses, 64k data points, an acquisition time of 0.9 s, a spectral width of 36 kHz, and an additional relaxation delay of 2.0 s. Protons were decoupled during acquisition using waltz16 composite pulse sequence. Linear back prediction was used to correct the first 22 complex data points of ^{13}C FIDs before zero-filling to 128k data points and application of Traficante window function with a line-broadening factor of 1.0 Hz.[23] Spectra calculation according to the maximum entropy method, MaxEnt,[19−21] was performed in Topspin using a symmetrical Lorentzian parametric point spread function with a total half-height width of 2.0 Hz. Spectra restricted to positive signals were calculated in two separate regions to avoid the large solvent signal (0−48.4 and 49.8−220 ppm, respectively). The noise factor was evaluated automatically by the program. Two-dimensional homo- and heteronuclear experiments were acquired with 1024 or 2048 data points in the direct dimension and 512 (DQF-COSY), 256 (multiplicity edited HSQC), and 128 (HMBC) in the indirect dimension with spectral widths determined from the corresponding one-dimensional experiments. The HMBC and HSQC experiments were optimized for $^nJ_{H,C}$ = 8 Hz and $^1J_{H,C}$ = 145 Hz, respectively. HMBC and HSQC spectra were processed to 2k × 1k data matrices, using linear prediction (32 coefficients) in F1 after application of a shifted sine-bell window function in F1 and F2.

Loading, Trapping, and Elution of 1−3. The test mixture was prepared by dissolving **1** (2.4 mg), **2** (2.0 mg), and **3** (1.8 mg) in methanol (1.5 mL), to give solutions of 3.5, 2.3, and 2.3 mM of **1**−**3**, respectively. The chromatography was performed using the following gradient profile: 0 min, 100% A, 0% B; 0.1 min, 70% A, 30% B; 10 min, 30% A, 70% B; 20 min, 0% A, 100% B; 25 min, 0% A, 100% B; 30 min, 100% A, 0% B, and 5 min conditioning with 100% A and 0% B. To evaluate SPE elution efficiency as a function of eluting solvent (methanol-d_4, acetonitrile-d_3, or chloroform-d), single 25 μL injections of the test mixture (40, 33, and 30 μg or 87, 58, and 58 nmol of **1**, **2** and **3**, respectively) were performed, and the analytes detected and trapped based on the UV absorption at 254 nm. Multiple trappings were evaluated by a series of one to six cumulative injections of the test mixture (trappings n = 3 for **2** and n = 5 for **3** failed due to instrumental malfunction).

Extraction and Fractionation of *Ganoderma* Triterpenoids. A 10.0 g sample of *G. lucidum* spores was extracted with 96% ethanol (3 × 500 mL). Each extraction was performed for 3 h at room temperature with 5 min sonication every hour. The crude extract was defatted by partitioning between 95% aqueous methanol and petroleum ether. A 1.4 g amount of an oily residue was obtained after concentration and lyophilization of the aqueous methanol phase. Then 1.0 g was coated onto 3 g of silica gel powder and applied on a 2.5 × 4 cm packed silica gel column. The elution was carried out using 30 mL each of hexane, hexane−chloroform (50:50), chloroform−methanol (98:2, 95:5, 93:7, 90:10, 80:20, 78:22, 75:25, 73:27, 70:30, 60:40, 58:42, 55:45, 53:47, 50:50, 30:70), and neat methanol followed by 50 mL of methanol−water (80:20 and 50:50) to afford 20 fractions. Fractions were analyzed by HPLC and pooled accordingly to yield four fractions. Subsequent concentration under reduced pressure and lyophilization yielded 193, 104, 177, and 170 mg, respectively. The first fraction (containing triterpenoids) was further fractionated using a Shimadzu CMD-20A system equipped with a dual-solvent pump, a diode array detector, an autosampler, and a fraction collector. The chromatography was performed with a 21.2 × 250 mm Phenomenex Luna(2) C_{18} (5 μm) (Phenomenex, Inc., Torrance, CA, USA) at room temperature using a 20 mL/min flow with the following gradient profile: 0 min, 90% A, 10% B; 40 min, 50% A, 50% B; 45 min, 5% A, 95% B; 50 min, 5% A, 95% B; 53 min, 90% A, 10% B, and 5 min conditioning with 90% A and 10% B. The 120 mg was separated in two runs. A fraction (A1) was manually collected from 27 to 32 min. A 7 mg amount was obtained after concentration under reduced pressure and lyophilization.

HPLC-SPE-NMR Analysis of *Ganoderma* Triterpenoids. Fraction A1 was dissolved in methanol (7 mg/mL). HPLC separations (each 80 μL, approximately 560 μg) were performed using a binary solvent gradient (0 min, 0% B; 0.1 min, 15% B; 45 min, 70% B; 50 min, 100% B; 55 min, 100% B; 60 min, 0% B, and 5 min conditioning with 0% B) with automatic trapping based on UV-absorption threshold levels at 254 nm. Six accumulated trappings of selected chromatographic peaks were performed, and the SPE cartridges were eluted with methanol-d_4.

■ ASSOCIATED CONTENT

Supporting Information

^1H and ^{13}C chemical shift data of **1**−**5** and expansions of Figures 3 and 5 are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.
[†]Deceased, October 2011.

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