NMR Quantitation of Natural Products at the Nanomole Scale

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We describe a simple and accurate method for quantitation by solvent ^{13}C-satellites (QSCS) of very small amounts of natural products using microprobe NMR spectroscopy. The method takes advantage of integration of ^{13}C satellite peaks of deuterated solvents, in particular $CDCl_3$, that have favorable intensities for measurements of samples in NMR microcoils and microprobe tubes in the 1−200 nanomole range.

Recent developments in instrumentation have greatly increased the sensitivity of NMR measurements and have facilitated structural elucidation of natural products at the submilligram level. Microcoil probes[1] and narrow diameter tube microprobes[2] offer significant advantages for measurements of small samples of natural products.[3] Reduction of the sample volume by a factor *n* at a constant sample mass will increase sensitivity of NMR signals in proportion to *n*, at the same absolute concentration sensitivity or signal-to-noise (S/N). Microcoils typically have fill volumes of 2.5−5 μL, while 1 and 1.7 mm tube probes offer fill volumes of about 7 and 30 μL, respectively. In practice, microcoil and capillary probe designs optimized for small fill volumes sacrifice some S/N, yet the gain in mass sensitivity more than compensates for this loss. For example, a recently developed commercial 1.7 mm ^{1}H−^{13}C−^{15}N CPTCI cryoprobe operating at 600 MHz achieves a nominal S/N of 1000:1 for ^{1}H (ASTM standard) when compared to 9000:1 for a 5 mm TXI probe at the same field strength with sample fill volumes of 30 and 750 μL, respectively. Thus, the increase in mass sensitivity of a 1.7 mm cryoprobe compared to the 5 mm cryoprobe can be estimated as the product 1/9 × 750/30, or a factor of 2.7. Compared to a conventional “room-temperature” 5 mm probe at the same field strength, this represents >10-fold improvement in mass sensitivity. Higher gains in S/N have been realized with recent innovations in probe design, for example the 600 MHz 1 mm high-temperature superconducting (HTS) probe.[4]

Natural products structure elucidation at the submilligram level is most suited to microcoil and microtube NMR; however independent estimation of the amount of natural product undergoing measurement is not trivial. For example, 1 nanomole (nmol) of a compound of molecular mass 1000 is equivalent to 1 μg. Gravimetric assay of this amount on a standard analytical balance of nanomoles of material is impossible, as the precision of the instrument is typically limited to 0.1−0.01 μg. Accuracy suffers when the net must be calculated by subtraction of two large numbers: the gross mass and the tare mass of the sample container (often 1500−13000 mg). Submilligram samples may be weighed on a microbalance down to ~0.1 μg; however, transfers, especially from solutions, may be cumbersome and these instruments are not commonly available in natural products laboratories. UV−visible spectroscopy can be used for estimating concentrations, but only for compounds that contain known chromophores or where the extinction coefficient, ϵ, can be reasonably estimated. The NMR quantitation method known under the acronym ERETIC inserts an “electronic standard” into the FID in the form of a shaped rf signal of variable power that can be integrated along with the sample FID.[5] The method has considerable advantages, but it typically requires a three-channel spectrometer and licensed software. Moreover, the accuracy of ERETIC may suffer from high dynamic range problems encountered in nanomole samples. The use of ^{13}C-satellites for quantitative NMR (qNMR) has been exploited for validation of reference phytochemicals.[6,7] Here we describe quantitation by solvent ^{13}C satellite (QSCS), a simple method suitable for calculating the *absolute* amounts of natural product typically measured by NMR in the extremely small volumes of 1−1.7 mm tubes or microcoils. QSCS requires measurement of nothing more than the signals already present in a sample in a solution of $CDCl_3$ during normal NMR measurements. It can be used to obtain the mass for additional quantitative spectroscopic calculations (e.g., UV−vis molar absorptivity, ϵ, $[\alpha]_D$, and CD) and other applications in the elucidation of natural products at the “nanomole scale”.[8] The latter includes calculation of natural product yields in the nanomole range as demonstrated here for a new natural product, phorbaside F (**1**), isolated from the marine sponge *Phorbas* sp.

Results and Discussion

The advent of micrcoil and microtube probes has allowed NMR measurements of extremely small amounts of compounds including natural products. During the course of investigations of submicromole samples of new natural products from *Phorbas* sp., we required a reliable way to quantitate the amount of sample in a solution of $CDCl_3$ (99.8% deuterium) during NMR measurements with a 1.7 mm Bruker 600 MHz microcryoprobe. Since our liquid handling regimen reduced the entire natural product sample to a solution in a constant volume of $CDCl_3$ (35 μL) and the range of sample sizes in our studies was 1−150 nmol, the dominant peaks observed in the ^{1}H NMR spectra were from residual $CHCl_3$ from the $CDCl_3$ (99.8% D). Excellent peak line-shape, high signal, and low artifact levels were observed near the base of the $CHCl_3$ signal at 1% signal height due, in part, to efficient vibration insulation of the 14 T cryomagnet employed in this study. Highly sensitive cryoprobes allow ready observation of signals for the ^{1}H−^{13}C couplet of $^{13}CHCl_3$ (J = 209 Hz), the so-called “^{13}C-satellites”, even after only one scan. Although the main $^{12}CHCl_3$ signal intensity is generally too large for accurate quantitation of nanomole samples due to dynamic range considerations, the $^{13}CHCl_3$ solvent satellite integrals are comparable in magnitude to those of typical methine CH signals in the natural product sample being measured. Thus, we were motivated to exploit the constant presence of the ^{13}C-satellite signals for nanomole-scale quantitation of the sample.

A series of volumetric solutions of cholesterol (**2**) in $CDCl_3$ (99.8% D) were prepared containing 1, 10, and 50 nmol/35 μL and measured at 600 MHz in a 1.7 mm cryo-microprobe under standard conditions (see Experimental Section) that included two preacquisition dummy scans. The FIDs were Fourier-transformed, and the resulting spectra phased, baseline-corrected, and integrated to give ratios of selected integrals A_{CH} for H-3 and H-6 to A_{sat}, which is defined in eq 1 as the sum integrals of the left-hand (A_L) and right-hand (A_R) components of the ^{1}H−^{13}C couplet of residual $^{13}CHCl_3$. The number of moles of

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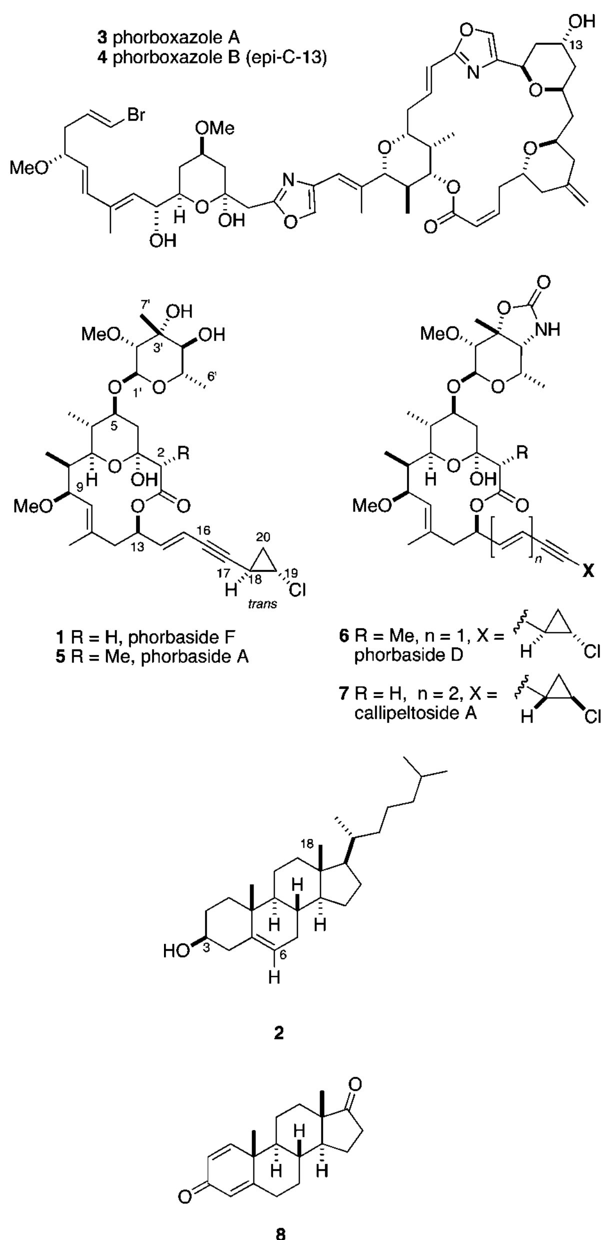
sample, *m*, is related to the ratio of signal integral A_{CH} to satellite integrals A_{sat} by the proportionality constant, *c* (eq 2), which may be obtained from the slope of a standard curve (Figure 1) of the measured integrals of the ^{13}C-satellites against the amount of standard in the *fixed volume* of sample solution.

$$A_{sat} = A_L + A_R \quad (1)$$

$$(A_{CH}/A_{sat}) = cm \quad (2)$$

$$m = (A_{CH}/A_{sat})/c$$

From linear regressions of the standard curves, we obtained a proportionality constant of $c = 0.097$ nmol^{-1} ($R = 0.99972$) for the H-3 proton of **2** and similar value ($c = 0.099$ nmol^{-1}, $R = 0.9992$) for H-6. Therefore, under our standard conditions, *c* is approximately 0.10 nmol^{-1}. To put this another way, the presence of 10 nmol of sample in the tube will give ^{1}H sample CH integrals that will be approximately equal to the sum of the two ^{13}C-satellite integrals for residual $^{13}CHCl_3$ in NMR grade $CDCl_3$ of 99.8% D atom isotopic purity.[9]



Having optimized a simple method for measuring mole amounts of samples in 1.7 mm NMR tubes, we turned our attention to quantitation of a new natural product that was isolated in nanomole amounts from the marine sponge *Phorbas* sp. Previous results of our investigation of a single specimen of *Phorbas* sp. collected in Western Australia yielded two sets of different cytotoxic macrolides, phorboxazoles A (**3**) and B (**4**),[10] in 1996 and more recently the glycosidic macrolides phorbasides A−E[11] [e.g., phorbasides A (**5**) and D (**6**)]. Repurification of minor fractions yielded the new natural product phorbaside F (**1**), which was shown by HRESIMS (*m/z* 649.2753 [M + Na]$^+$, Δmmu = 0.40) to be a lower homologue of **5**.

Figure 3 shows the downfield region of the ^{1}H NMR spectrum of **1** (600 MHz, $CDCl_3$, 99.8% D) and the associated ^{13}C-satellite signals of residual solvent ($CHCl_3$). The integral ratio A_{CH}/A_{sat} measured for the downfield signals and averaged ($A_{CH}/A_{sat} = 1.16 \pm 0.05$) together with the proportionality constant *c* established above allowed determination of the amount of **1** present in the NMR tube to be 11.8 ± 0.6 nmol (7.7 μg).[12] Since the entire sample of **1** isolated from *Phorbas* sp. had been transferred to the 1.7 mm NMR tube, we calculated that the yield of phorbaside F from *Phorbas* sp. was 3×10^{-8} % dry weight. The complete structure of **1** was established from HRMS and 2D NMR experiments (COSY, HSQC, HMBC) performed on the entire sample and compared with **5**. HSQC of **1** revealed a characteristic AB pattern attributed to an isolated C-2 methylene group (δ 2.42, $J = 14.6$ Hz, 1H; δ 2.51, $J = 14.6$ Hz, 1H; δ_C 44.8 ppm, $CDCl_3$), consistent with the absence of the C-2 Me group of **5** (Table 1). The ^{13}C NMR chemical shift of C2 is similar to that of C-2 in callipeloside A (**7**)[13] (δ 2.50, d; 2.56, d, AB $J = 12.7$ Hz, 2H; δ_C 46, CD_3OD), a related chlorocyclopropane macrolide with an almost identical macrolide core. The remaining ^{1}H and ^{13}C NMR chemical shifts of **1** were essentially the same as those of **5**; therefore, we assigned the same relative configuration to both. The Cotton effect observed in the CD spectrum of **1** (λ_{max} 232, $\Delta\epsilon$ +13.5) was identical in sign and magnitude to that of **5**. Since we had earlier assigned the absolute configurations at C-13, C-18, and C-19 of phorbasides A (**5**) and B by quantitative analysis of the Cotton effects arising from $\pi-\pi^*$ transitions of the hyperconjugated ene-yne-chlorocyclopropane chromophores (λ_{max} 236 nm),[11,14] both **1** and **5** share the same absolute stereostructure.

The use of ^{13}C-satellite signals for quantitative NMR analysis (qNMR) has precedence. qNMR has been used for validation, certification, and quality control of reference phytochemicals.[6,7] A recent novel application by Claridge and co-workers[15] described a solution to an outstanding problem—quantitative measurement of diastereomeric very high diastereomeric ratios (dr's)—by integrating the minor diastereomer against the ^{13}C satellite of the major isomer, which enables measurements of dr's of up to 1000:1 (99.8% diastereomeric excess). In the present application, QSCS allows *absolute* quantitation of the mole amounts of sample contained within the NMR microtube.[8] By providing quantitation independent of gravimetric methods, QSCS facilitates further characterization of natural products at the nanomole scale by other important spectroscopic parameters that are mass- or mole-dependent, including specific rotation ($[\alpha]_D$), UV−visible absorbtivity (ϵ), and CD ($\Delta\epsilon$).

Several comments on residual solvent NMR signal intensities are pertinent to QSCS. Commercial $CDCl_3$ for NMR is manufactured to stringent specifications that meet quality control standards for purity and nominal atom % of deuterium content that also holds the residual $CHCl_3$ concentration at reasonably constant levels. For example, quality control measurements of batches of commercial $CDCl_3$ (nominally, 99.8% D) manufactured within a three-month period gave a range of deuterium content from 99.80 to 99.89% D (mean = 99.834%; standard

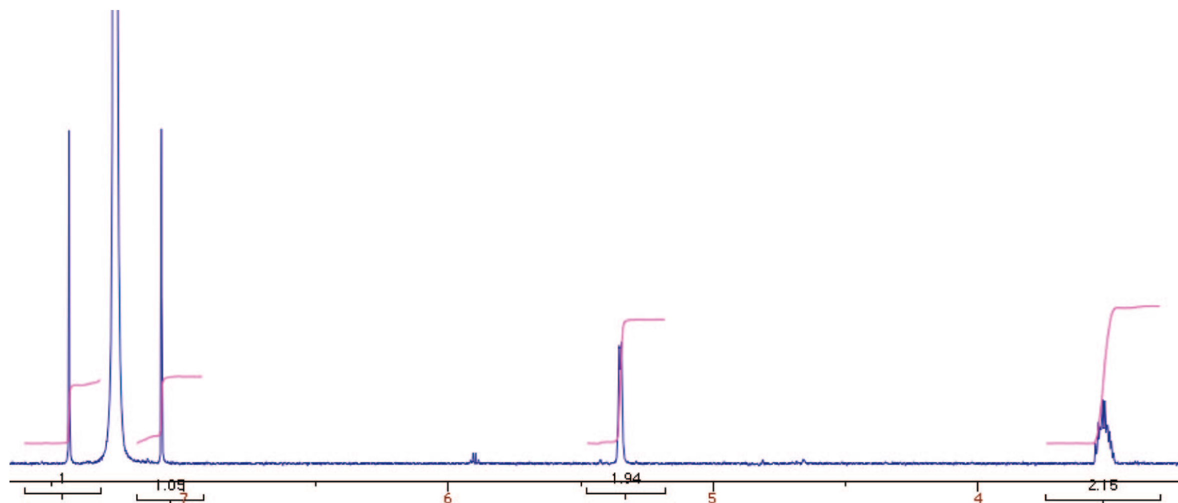


Figure 1. ^{1}H NMR spectrum of a solution of cholesterol (**2**), 10 nmol/35 μL of $CDCl_3$, 99.8% D (600 MHz) showing "^{13}C-satellites" from residual $^{13}CHCl_3$.

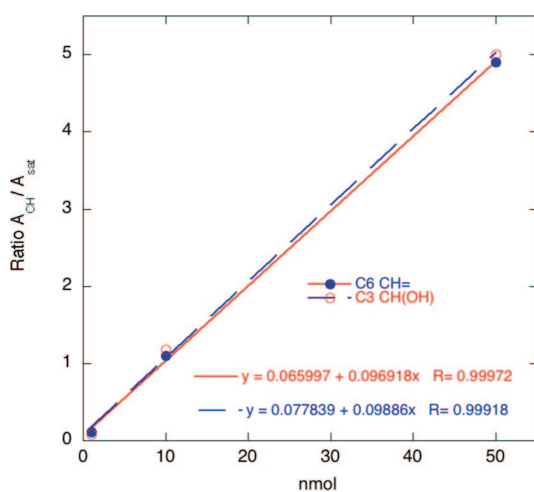


Figure 2. Standard curve of ^{1}H NMR integral ratio for methine signals, A_{CH}, of cholesterol (**2**) to residual $^{13}CHCl_3$ integral, A_{sat}, in $CDCl_3$ solvent (99.8% D). Linear regression gives the proportionality constant, $c = 0.097$ and 0.099 (nmol) for H-3 and H-6, respectively, from the slopes (see text). See Experimental Section for details of NMR acquisition parameters.

deviation = ±0.021, $n = 30$).[16] Assuming the isotopic natural abundance of ^{13}C in $CDCl_3$ is invariant, the slope of the curves in Figure 1 should only change slightly between different solvent batches if measured under the same conditions. Intramolecular spin−lattice relaxation dominates nuclear spin magnetization recovery in bulk $^{12}CHCl_3$. Chloroform exhibits very long relaxation times (T_1 and T_2), as may be expected for inefficient spin−lattice relaxation of a small, rapidly tumbling molecule with only one H atom. Dinesh and Rogers measured longitudinal spin−lattice relaxation times in carefully purified, oxygen-free $^{12}CHCl_3$ and obtained a value of $T_1 = 86 \pm 2$ s at 25.0 °C.[17] Under normal usage, $CDCl_3$ will show greater longitudinal spin relaxation, governed by an apparent T_1* that differs from T_1 due to the presence of additional relaxation pathways, in particular paramagnetic relaxation induced by the presence of dissolved oxygen. To our best knowledge, the T_1's of dilute solutions of $^{13}CHCl_3$ in bulk $^{12}CHCl_3$ at *natural abundance* have not been reported; however, it was expected to be similar to $^{12}CHCl_3$ given the low efficiency of intramolecular dipolar relaxation from ^{1}H to the low-γ nucleus ^{13}C. In fact, measurements of the apparent T_1*'s of residual $^{13}CHCl_3$ and $^{12}CHCl_3$ in atmosphere-equilibrated "$CDCl_3$" NMR solvent (600 MHz, 25 °C) by inversion recovery experiments gave almost equal values (T_1* = 7.3 and 6.9 s, respectively), both significantly shorter than that of pure oxygen-free $CHCl_3$. The reported T_1's of CH and CH_2 protons in 1-dehydrotestosterone (**8**), a steroid comparable in size and shape to **2**, are much shorter (e.g., CH_2's, $T_1 = 1.0-1.2$ s; CH's 1.3−1.7 s)[18] than those of residual $CHCl_3$ molecules. Consequently, partial saturation of the solvent residue ^{13}C-satellites upon repetitive rf pulse NMR experiments may be greater in $CHCl_3$ than those of molecules of similar or greater size than **2** and give disproportionately lower integrals.[19]

Changes in intensity of the ^{13}C-satellite signals of $CHCl_3$ may also be expected with variation of experimental parameters such as the relaxation delay, d1, the presence of paramagnetic materials (particularly, triplet molecular oxygen), and the temperature.[18] Partial saturation of residual solvent is established largely by the first two dummy scans in the pulse sequence. and changes in d1 may influence $A_{CH}/A_{sat.}$. In addition, differential recovery of magnetization of the sample signal during d1 and the acquisition time may further alter the value of $A_{CH}/A_{sat.}$. This should be more pronounced for molecules with T_1's largely different from **2**. On the other hand, the relatively large number of scans used for 1D spectra may obviate these factors if the system is under dynamic saturation conditions. In order to address these issues experimentally, we varied the relaxation delay from d1 = 1 s to d1 = 1.5 s and found A_{CH}/A_{sat} decreased by only about 9% for A_{CH}/A_{sat} for H-3 and H-6 with 10 nmol of **2** and 20% when d1 = 2 s (see Table S1, Supporting Information), consistent with a larger relative increase of A_{sat} compared to A_{CH}. Nevertheless, the linearity of the standard curve (Figure 2) shows that the spin−lattice relaxation pathways for sample and residual $CHCl_3$ protons are constant over the range of concentrations of **2** that were tested (0.0285−1.42 mM). Consequently, the ratio A_{CH}/A_{sat} and the constant c can be reliably used for quantitation of samples if measurements are made under the same experimental conditions, particularly d1, used for calibration with **2**. The total number of scans acquired for each sample of **2** was >400. For smaller molecules, with longer T_1's or data sets acquired with fewer scans, it may be preferable to use more dummy scans ($n = 8-16$). Nevertheless, it should be stressed that application of solvent ^{13}C-satellite integration for *natural products quantitation should always be preceded by calibration with a standard molecule* displaying fast relaxation (short T_1's) and well-resolved integrals for each solvent batch.

Other experimental factors can also influence c. These include field strength, nature of the NMR solvent, probe fill factor, and, of course, atom % deuterium content of the solvent that

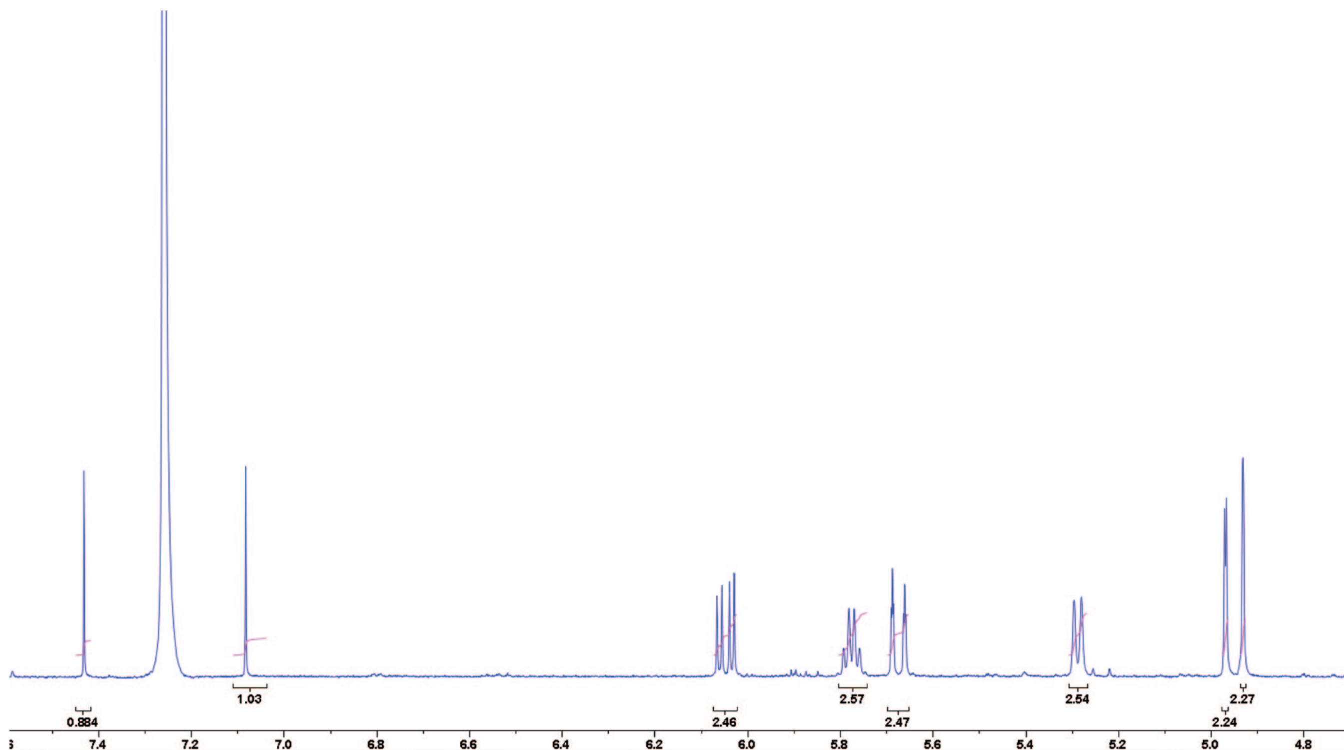


Figure 3. ^{1}H NMR spectrum of a solution of phorbaside F (**1**) (600 MHz, 7.7 μg, $CDCl_3$, 99.8% D) showing the downfield vinyl proton region and residual solvent "^{13}C-satellite" signals.

determines the levels of residual ^{1}H isotopomers. To some extent, less than optimal sample volumes in the NMR tube may also affect c. Microcoil probes should suffer less from this effect because coil edge-effects are eliminated by "infinitely" constant dielectric composition in the axial direction of the capillary.[20] Calibration should be needed only once for particular combinations of spectrometer, probe, temperature, and solvent batch and should give confidence for nanomole-level quantitation of natural products when NMR spectra of samples are recorded under similar conditions. Solvent overlap with sample signals must be considered when appropriate. The NMR ^{13}C-satellite quantitation method can be applied to compounds with vinyl or aromatic signals present in the region δ 7−8 ppm provided that at least one of the two halves of the satellite ^{13}C−^{1}H doublet is clearly observed and can be integrated. If only one of A_L or A_R can be accurately integrated, a proportionality constant of $2c$ should be used for calculating the number of nmol. For natural products with limited solubility in $CDCl_3$, the method may be extended to other NMR solvents after appropriate external calibration with a standard (e.g., Figure 1). The ^{1}H−^{13}C couplets of residual nondeuterated isotopomers in common NMR solvents (e.g., CD_3OD, $(CD_3)_2SO$, $(CD_3)_2CO$, CD_3CN, CD_3COOH) have smaller $^{1}J_{CH}$ values than $CHCl_3$ (e.g., d_6-acetone CD_2HCOCD_3, J_{CH} = 126 Hz; CHD_2OD, J_{CH} = 139 Hz). Observation of the ^{13}C-satellites of multiply deuterated solvents may be more difficult than with $CDCl_3$ due to additional splitting by ^{13}C−^{2}H scalar coupling, resulting in lower peak heights and occurrence of the satellite signals in midfield regions of the ^{1}H NMR spectrum.

Care should be taken with the handling and storage of enolizable NMR solvents (e.g., d_6-acetone) that may undergo deuterium exchange with adventitious H_2O and alter the residual protonated isotopomer content.[21] This will be of special concern for NMR measurements of samples that are appreciably basic or acidic, although under neutral conditions exchange should be negligible. In the most prudent approach, the application of QSCS in other solvents will take into consideration the foregoing

Table 1. ^{1}H and ^{13}C NMR Data of Phorbaside F (**1**) ($CDCl_3$, 600 MHz)

#	^{1}H, δ (m, J (Hz)	^{13}C,[a] δ
1		171.8
2	2.42 (d, 14.6)	44.8
	2.51 (d, 14.6)	
3		97.0
4	1.35 (m)	42.5
	2.22 (dd, 12.3, 4.5)	
5	3.69 (td, 10.8, 4.5)	79.3
6	1.46 (m)	38.7
7	3.64 (m)	74.9
8	2.19 (m)	37.0
9	3.79 (dd, 9.5, 2.6)	79.9
10	5.29 (d, 9.5)	127.9
11		132.4
12	2.26 (d, 7.3)	46.7
13	5.77 (dd, 16.0, 6.4)	71.2
14	6.05 (dd, 16.0, 6.4)	140.1
15	5.67 (d, 16.0)	111.7
16		ND[b]
17		ND[b]
18	1.77 (m)	ND[b]
19	3.17 (ddd, 9.0, 6.6, 3.0)	36.9
20	1.27 (m)	18.0[c]
21	1.72 (s)	16.4
22	0.98 (d, 7.4)	6.6
23	0.92 (d, 5.8)	12.6
9-OMe	3.23 (s)	55.4
OH	2.9 (s)	
1′	4.93 (s)	98.9
2′	3.12 (s)	84.8
3′		72.3
4′	3.35 (d, 9.3)	76.8
5′	3.65 (m)	67.2
6′	1.25 (d, 5.7)	18.0[c]
7′	1.31 (s)	18.0[c]
3′-OH	4.97 (s)	
2′-OMe	3.46 (s)	59.2

[a] δ inferred from indirect ^{13}C detection experiments, HSQC, and HMBC. [b] Not detected. [c] Interchangeable.

issues on a case-by-case basis. Finally, it should be noted that the precision of QSCS will be no better than that of electronic integration of signals in NMR spectroscopy, typically 5−10%, although this may be improved by averaging values of A_{CH}/A_{sat} over several integral measurements.

In conclusion, we have illustrated here a very simple method for absolute quantitative estimation of nanomole amounts of natural products by comparative measurements of integrals of ^{13}C-satellites of NMR solvent in a microprobe. The method is illustrated for samples dissolved in $CDCl_3$, including structure elucidation and the quantitation of a new natural product, phorbaside F (**1**) from *Phorbas* sp. Quantitation by solvent ^{13}C-satellite (QSCS) should be applicable to samples in other NMR solvents that give rise to measurable ^{13}C-satellite signals that are well separated from the major ^{12}C isotopomer signal and the sample signals. QSCS expands the window of discovery and characterization of new natural products, especially those that can be procured only in vanishingly small amounts.[8]

Experimental Section

General Experimental Procedures. ^{1}H NMR spectra were recorded on a Bruker DRX spectrometer equipped with an Avance III console and a CPTCI 1.7 mm probe operating at 599.82 MHz. Measurements of T_1 were conducted using a standard inversion recovery pulse sequence (180°−τ−90°) using a 5 mm CPTCI probe, and the data processed using the TOPSPIN T1/T2 module. The same batch of NMR-grade solvent $CDCl_3$ (99.8% D, Cambridge Isotopes, Inc.) was used for all measurements. HPLC-grade solvents (CH_3OH, CH_3CN) were redistilled prior to use in the final stages of HPLC purification of natural product and sample transfers. Other general procedures are reported elsewhere.[22]

NMR Measurements. Sample Preparation. Samples of **1** and other natural products for NMR measurements were prepared by coevaporation with $CDCl_3$ (99.8% D) and dried under a stream of N_2 immediately prior to transfer to 1.7 mm NMR tubes. Samples were dissolved in $CDCl_3$ (99.8% D) and the solutions centrifuged before transferring to NMR tubes using a Hamilton gastight syringe fitted with an extra long, narrow gauge needle (26G, 10.8 cm). Standard solutions of cholesterol (Aldrich, 99%) in $CDCl_3$ were made by serial dilutions of a stock solution (2.85 mM) prepared by dissolving accurately weighed samples of **2** (±0.01 mg) in the calculated volumes of $CDCl_3$. Final concentrations were 0.0285, 0.285, and 1.42 mM, corresponding to 1, 10, and 50 nmol in 35 μL. Aliquots (35 μL) of each standard solution were transferred to 1.7 mm NMR tubes (Bruker Biospin) and their ^{1}H NMR spectra recorded as described below.

NMR Parameters. FIDs were acquired at room temperature (25 °C) under steady state conditions on nonspinning samples using a standard one-pulse acquisition experiment with a nonselective pulse corresponding to a pulse width of 3.3 μs, or 30° tip angle (15 dB attenuation of 100 W pulse power), with the following parameters: two dummy pulses to achieve steady state, followed by acquisition of 240−480 FIDs of 32K complex points and an acquisition time of 2.6564 s (12 335.526 Hz spectral width). FIDs were processed under identical conditions using iNMR software (Mestre Laboratories, Spain) as follows: Fourier transformation with exponential apodization (line broadening = 0.50 Hz), followed by manual phase correction and autobaseline correction (fifth-order polynomial fit). The signals of the left and right components (A_L and A_R, respectively) of the ^{1}H−^{13}C couplet of the residual solvent signal $^{13}CHCl_3$ were integrated and summed to give A_{sat}, taking care to exclude the large central $^{12}CHCl_3$ signal. The integral, A_{CH}, for each of the ^{1}H NMR signals of cholesterol (**2**) H-3 (δ 3.52, m, 1H), H-6 (δ 5.35, bs, 1H), and the methyl group H-18 (δ 0.67, 3H) were measured and the A_{CH}/A_{sat} ratios plotted against the number of nmol of sample (Figure 1 and Supporting Information).

Isolation and Characterization of Phorbaside F (1). The previously described CCl_4-soluble fraction (93-054-B-1, 350 mg)[10a,11] from a MeOH extract of *Phorbas* sp. (93-054) was separated by flash chromatography on a prepacked cartridge (silica, Analogix RS-12, 12 g, 2 × 7.5 cm) using a stepped gradient of hexane−ethyl acetate mixtures (0−100%) to yield seven fractions. Fraction 5 (93-054-B1-5, 12.5 mg) was further purified twice by reversed-phase HPLC (phenylhexyl column, 250 × 10 mm, 90:10 MeOH−H_2O, then phenylhexyl column, 250 × 4.6 mm, 60:40 CH_3CN−H_2O, HPLC grade, redistilled) to afford a pure UV-active fraction, 93-054-B1-12 (t_R = 12.0 min), which was collected in a 1.8 mL vial and dried under a stream of N_2. The residual solvent was removed by coevaporation from $CDCl_3$, and the sample of pure **1** (7.7 μg, 2 × 10^{-8} % dry weight) was redissolved in $CDCl_3$ (99.8% D, 35 μL) and transferred to a 1.7 mm NMR tube using a gastight syringe as described under "Sample Preparation": UV (MeOH), λ_{max} 237 nm, ε 12 000; CD (MeOH) Δε 232 (+13.5), 241 (+12.5); ^{1}H NMR (600 MHz, $CDCl_3$, 99.8% D), see Table 1; HRESIMS *m/z* 649.2753 [M + Na]$^+$, calcd 649.2749 for $C_{32}H_{47}ClO_{10}Na$. 2D NMR spectra (COSY, HSQC, HMBC, 600 MHz, 1.7 mm CPTCI microprobe) were acquired using standard Bruker software. Total NMR time required for acquisition of 2D NMR data was about 36 h; see Supporting Information for 2D NMR spectra.

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Supporting Information Available: Calculations of moles of $^{13}CHCl_3$ in commercial $CDCl_3$ (99.8% D), HSQC spectra for **1**, and a table of dependence of A_{CH}/A_{sat} upon relaxation delay, d1, for **2** in $CDCl_3$. This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) Nevertheless, in our case we found that the $^{13}CHCl_3$ satellite and cholesterol integrals were similar. The calculated content of $CHCl_3$ in 35 μL of $CDCl_3$ (99.8% D) is 9.6 nmol (see Supporting Information). Assuming equal ^{1}H NMR signal responses for $CHCl_3$ and

sample, the ratio of $^{13}CHCl_3$:**2** integrals should be approximately 1 for a solution containing 10 nmol of **2** in 35 μL of $CDCl_3$. This is, in fact, close to the observed ratio A_{CH}/A_{sat} (Figure 1), a result that may be more a fortuitous coincidence than accurate accounting of all factors responsible for signal intensity.

(20) While $CDCl_3$ is a common NMR solvent used in natural products research, a practical limitation to its use in microcoils with very small fill volumes (2.5−5 μL) is a relatively "weak" deuterium resonance that results in poor field-lock stability. The larger fill volume in 1.7 mm tube microprobes obviates this problem, but substitution of $CDCl_3$ with "stronger" solvents (e.g. d_6-benzene or CD_2Cl_2) may also alleviate lock issues.

(21) Other simple experimental precautions should be taken. For example, the common habit of storing $CDCl_3$ over anhydrous K_2CO_3 should be avoided to prevent erosion of deuterium content and increase in $CHCl_3$ levels as a result of base-catalyzed exchange with adventitious H_2O.

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