JOURNAL OF NATURAL PRODUCTS

Volume 61, Number 5 May 1998

Rapid Communications

Submicro Inverse-Detection Gradient NMR: A Powerful New Way of Conducting Structure Elucidation Studies with <0.05 μmol Samples

Gary E. Martin,*,† Jane E. Guido,† Russell H. Robins,† Maged H. M. Sharaf,‡ Paul L. Schiff, Jr.,§ and Albert N. Tackie⊥

Pharmacia & Upjohn, Pharmaceutical Development, Rapid Structure Characterization Group, Kalamazoo, Michigan 49001-0199, Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, Al-Azhar University, Nasr City, Cairo, Egypt, Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, and Centre for Scientific Research into Plant Medicine, Mampong-Akwapim, Ghana

Received March 16, 1998

Abstract: Quantities of material required for structural analysis were reduced substantially following the introduction of 3 mm microinverse and microdual NMR probes in 1992. We now report the first very low-level results obtainable with a new 1.7 mm submicro-inverse-detection gradient or SMIDG NMR probe. Using this technology at 600 MHz, it was possible to fully characterize an 8% impurity contained in a 0.55 μmol sample of cryptolepine (**1**) that had been standing in excess of 2 years since its initial isolation. The impurity was unequivocally identified as cryptolepinone (**2**) through the concerted interpretation of GHSQC, GHMBC, homonuclear TOCSY, and ROESY spectra in conjunction with APCI LC/MS and CID data acquired from a portion of the serial dilution solution used to prepare the NMR sample. Submicro-inverse-detection gradient probes offer the prospect of reducing still further the quantities of sample required for full characterization under favorable circumstances, making rare and potentially novel natural products amenable to structural determination. SMIDG NMR technology is equally applicable to a range of small samples requiring characterization such as isolated impurities from drug substances, isolates from drug degradation studies, and secondary metabolites.

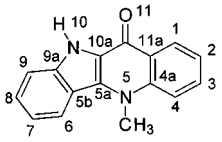
Micro-inverse-detection NMR probes and the capabilities that they afforded were first reported in 1992;[1] the performance characteristics of 3 mm microinverse and standard 5 mm probes were compared shortly thereafter.[2] In the intervening years, 3 mm microprobes have become widely used in the pharmaceutical industry and are now becoming more widely used in academic laboratories for the characterization of small samples of natural products and other rare samples. To the best of our knowledge, the lowest level full structural characterization reported in the literature using inverse-detected heteronuclear chemical shift correlation experiments in conjunction with 3 mm microprobes was that of ~0.25 μmol of cryptolepicarboline by several of the coauthors of the present work.[3] One of the problems inherent to the study of still smaller samples is the quadratic increase in acquisition times necessary to acquire even direct heteronuclear shift correlation data, as exemplified by previous attempts to characterize a ~0.1 μmol sample of Caribbean ciguatoxin.[4]

The first approach to dealing with the problem of acquiring heteronuclear shift correlation data for small samples was the development of specialized Shigemi NMR microcells in which the composition of the glass is matched to the dielectric properties of the solvent being used. Considerable time savings are possible, as has been demonstrated for Caribbean ciguatoxin.[4] Recalling that halving the sample volume, thereby doubling the effective working concentration, quarters the

* To whom inquiries should be addressed. Tel.: (616) 833-6283. E-mail: gary.e.martin@am.pnu.com.
† Pharmacia & Upjohn.
‡ Al-Azhar University.
§ University of Pittsburgh.
⊥ Centre for Scientific Research into Plant Medicine.

S0163-3864(98)00099-8 CCC: $15.00
Published on Web 05/22/1998

Table 1. ^{1}H and ^{13}C Resonance Assignments for Cryptolepinone (**2**) Determined in Situ as an 8% Impurity in a 0.55 μmol Sample of Cryptolepine Using Submicro-Inverse-Detection Gradient (SMIDG)-NMR Probe Capabilities Compared with Reported Assignments for Cryptolepinone (2) in Pure Form

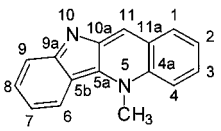


2

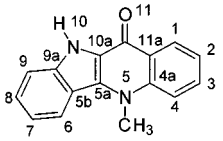
	previous study[a] (DMSO-d_6)[8,11]		present study (DMSO-d_6)	
position	δ^1H[a]	δ^{13}C	δ^1H	δ^{13}C
1	8.43	126.0	8.43	123.2
2	7.36	121.0	7.34	118.6
3	7.78	131.9	7.77	129.2
4	7.96	116.0	7.96	113.8
4a		141.0		140.2
N-5				
N-Me	4.35	36.3	4.35	*b*
5a		131.0		130.6
5b		116.5		116.5
6	8.38	123.0	8.38	120.6
7	7.19	119.8	7.19	117.2
8	7.48	127.0	7.46	125.4
9	7.58	113.0	7.56	111.8
9a		139.0		139.2
N-10H	11.95		*b*	
10a		124.0		*b*
11		167.4		*b*
11a		123.8		123.7

[a] ^{1}H- and ^{13}C NMR chemical shift assignments were established from a series of NOE difference spectra, an IDR−HMQC-TOCSY spectrum with a 24 ms mixing time, and a 10 Hz HMBC spectrum. The data were acquired for a sample consisting of 10 mg of **2** dissolved in 160 μL of DMSO-d_6. Experiments were performed on a Varian Unity 400 MHz spectrometer operating at 399.80 MHz for ^{1}H observation and equipped with a Nalorac Z·SPEC MID-400-3 micro-inverse probe. [b] Chemical shift not determined from the submicro-NMR data recorded.

acquisition time at constant s/n (signal-to-noise) ratio, the temporal benefits of further sample size reduction are obvious.[1,2,4] Generally, Shigemi NMR microcells make it feasible to reduce sample volumes from the 130−150 μL nominal sample volumes used in 3 mm micro-NMR tubes to the range of 70−80 μL. Reynolds and co-workers[5] have recently reported a comprehensive study of the benefits of using Shigemi NMR microcells in conjunction with modern micro-NMR probes. Aggressive use of a Shigemi cell, using volumes below 70 μL, however, currently requires that a portion of the glass top and bottom plugs must reside within the NMR probe coil volume, making shimming difficult and tedious. There has been little development directed toward the use of still smaller sample volumes with concomitantly higher working concentrations to help minimize heteronuclear shift correlation data acquisition times. Although the acquisition of ^{1}H NMR spectra has been reported using samples as small as 2 μL,[6] no heteronuclear shift correlation experiments have been reported using volumes lower than the ~70 μL attainable with Shigemi 3 mm micro-NMR cells. Hence, we recently reported the implementation of a SMIDG (submicro-inverse-detection gradient) NMR probe with a working sample volume of 20−30 μL.[7] Here, we have applied 1.7 mm SMIDG-NMR probe technology to the in situ trace level structural characterization of an 8% impurity contained in a 0.55 μmol sample of cryptolepine (**1**).



1



2

Our initial evaluation of the submicro-inverse-detection gradient (Nalorac SMIDG-600-1.7 mm) NMR probe utilized a 0.55 μmol sample of cryptolepine (**1**) prepared by serial dilution in DMSO-d_6. At that effective working concentration, GHSQC data were obtained for the aromatic region of this compound in 12 min and GHMBC data in 1.1 h. Interestingly, the proton spectrum of that sample showed the presence of an 8% impurity, corresponding to a calculated 0.04 μmol assuming the material to have a molecular weight comparable to cryptolepine. As a further test of the capabilities of the submicrogradient probe, the acquisition of the heteronuclear chemical shift correlation and mass spectral data necessary to characterize the impurity were undertaken.

After 6 h of GHSQC data acquisition, evidence of the impurity resonances could be discerned by examination of individual traces through F_2. After 16 h of accumulation, the eight protonated aromatic resonances of the impurity were clearly visible in the spectrum. The acquistion was terminated at 25.5 h, yielding the spectrum presented in Figure 1. The spectrum was acquired as 2048 × 32 (2 × 32 hypercomplex or States-TPPI increments) with 768 transients accumulated/t_1 increment.

Assuming the impurity to be an oxidative degradation product of cryptolepine (**1**) was logical given that the sample had been stored for over 2 years since its isolation at 99+% chromatographic purity. A 5 μL aliquot of the serial dilution from which the NMR sample was prepared, which could have easily come from the NMR tube after completion of the NMR data acquisition, was diluted in 1 mL (200×) of mobile phase A (MPA, comprised of water/acetonitrile (95:5) containing 0.1% TFA) for the APCI LC/MS analysis via a 25 μL loop injection. The chromatographic system was composed of an LDC Constametric 4100 series gradient solvent delivery system operating at a flow rate of 2.0 mL/min. Mobile phase B (MPB) was composed of water/acetonitrile (5:95) containing 0.01% TFA. The separation of cryptolepine (**1**) from the 8% impurity was achieved using a Zorbax Eclipse XDB C_8 (5 cm × 4.6 mm) reversed-phase HPLC column and a linear gradient from 0% to 100% MPB in 5 min. Mass spectra were recorded at unit mass resolution via repetitive scanning from 150 to 300 Da every second using a Finnigan TSQ-7000 mass spectrometer. Product ion spectra of cryptolepine (**1**) and the 8% impurity, ultimately identified as cryptolepinone (**2**, mol wt 248 Da), were obtained by CID (collision induced dissociation) in the second quadrupole (E_{lab} = 55 eV), using argon collision gas at an indicated pressure of 1.5 Torr. Product ion spectra were recorded between 10 and 250 Da. The product ion MS of cryptolepinone (**2**) is shown in Figure 2.

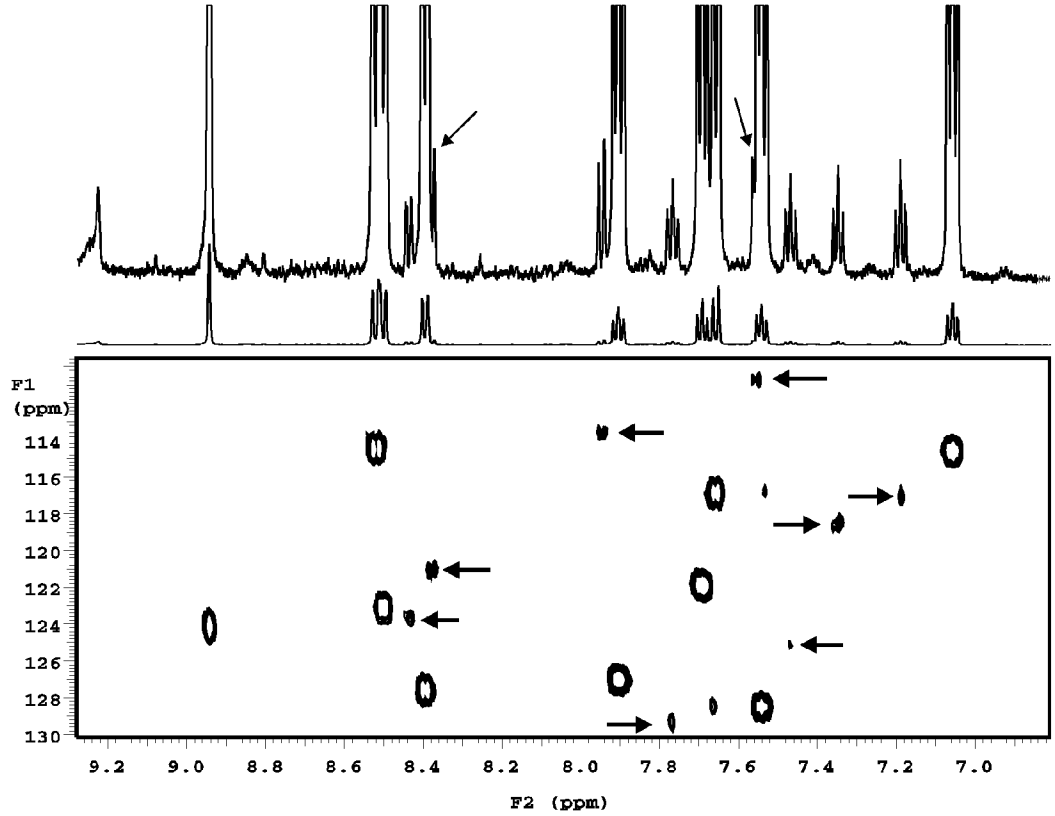


Figure 1. Top trace: ^{1}H NMR reference spectrum of a 0.55 μmol sample of cryptolepine (**1**) dissolved in 25 μL of 99.992% DMSO-d_6 acquired in 256 transients using a Nalorac SMIDG-600-1.7 submicro inverse-detection gradient NMR probe in a Varian INOVA 600 spectrometer. The spectrum is plotted with the main resonances of cryptolepine (**1**) off-scale and truncated to show detail in the spectrum of the 8% impurity contained in the sample. Partially obscured peaks from the impurity are denoted by arrows. Middle trace: ^{1}H NMR spectrum identical to the top trace except plotted to keep the cryptolepine (**1**) resonances on-scale. Bottom trace: contour plot of the GHSQC spectrum acquired on the sample in 25.5 h. The data were recorded as 2048 × 32 (2 × 32 hypercomplex or States-TPPI files in t_1 to digitize the 30 ppm F_1 spectral window). Resonances arising from the 8% impurity are denoted by arrows in the contour plot. Note the absence of a resonance corresponding to the impurity peak resonating at ~9.22 ppm, suggesting that this resonance is probably the indole-N(10)H resonance of **2** rather than a resonance comparable to the H-11 resonance of cryptolepine (**1**).

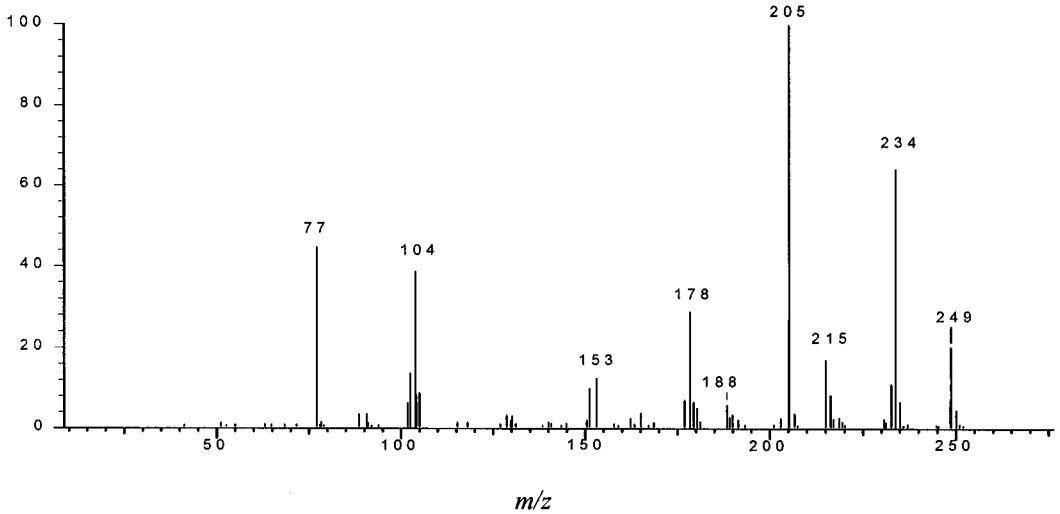


Figure 2. APCI/LC/MS product ion mass spectrum of the 8% impurity contained in the SMIDG-NMR sample of cryptolepine (**1**). The spectrum was obtained by CID (collisionally induced dissociation) in the second quadrupole of a Finnigan TSQ-7000 mass spectrometer using argon as a collision gas at an indicated pressure of 1.5 Torr. The mass spectrum is consistent with the structure of cryptolepinone (**2**).

The 8% impurity was tentatively identified as cryptolepinone (**2**) on the basis of the ^{1}H and GHSQC NMR spectra, the mass spectral data, and comparison with data contained in the literature.[8−11] The capability of the Nalorac SMIDG-600-1.7 NMR probe to record very low-level GHMBC spectra, necessary to elucidate the impurity structure, was further evaluated. The 8 Hz optimized GHMBC spectrum recorded for the impurity in 56.5 h is shown in Figure 3.

Correlations from the *N*-methyl region of the spec-

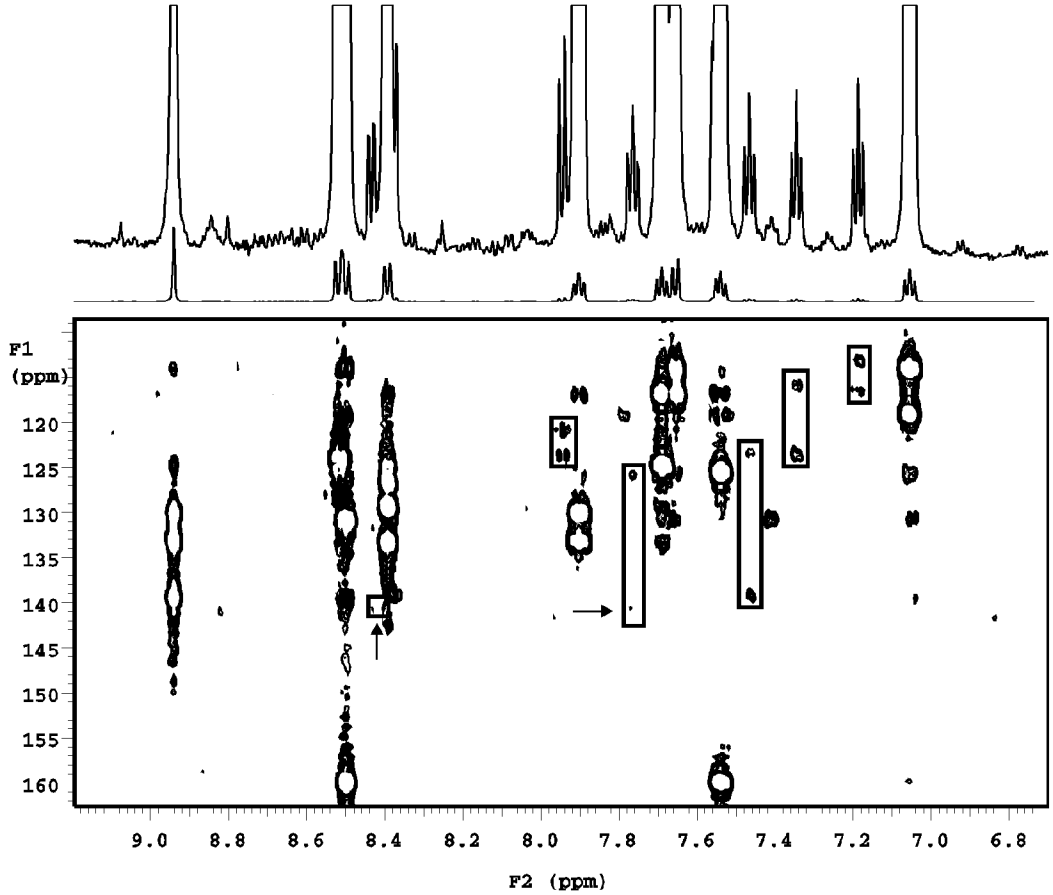


Figure 3. Top trace: ^{1}H NMR reference spectrum of a 0.55 μmol sample of cryptolepine (**1**) dissolved in 25 μL of 99.992% DMSO-d_6 acquired in 256 transients using a Nalorac SMIDG-600-1.7 submicro inverse-detection gradient NMR probe in a Varian INOVA 600 spectrometer. The spectrum is plotted with the main resonances of cryptolepine off-scale and truncated to show detail in the spectrum of the 8% impurity contained in the sample. Partially obscured peaks from the impurity are denoted by arrows. Middle trace: ^{1}H NMR spectrum identical to the top trace except plotted to keep the cryptolepine resonances on-scale. Bottom trace: contour plot of the GHMBC spectrum acquired in 56.5 h as 4096 × 64 (2 × 64 hypercomplex or States-TPPI files used to digitize the 70 ppm F_1 spectral width); 832 transients were accumulated/t_1 increment. Responses arising from the 8% cryptolepinone impurity contained in the sample are boxed to group them vertically beneath the proton from which they arise. Responses denoted by arrows were verified by examining F_2 traces plotted from the spectral data matrix.

trum are not plotted. Correlations arising from the *N*-methyl of cryptolepinone (**2**), however, which resonates at 4.35 ppm, were visible even in data processed and plotted after 1.1 h. Long-range couplings were observed from the *N*-methyl to the C-4a and C-5a quaternary carbons that flank the nitrogen, resonating at 140.2 and 130.6 ppm, respectively (reported assignments for these resonances were 141.0 and 131.0 ppm).[8,11] Sufficient correlations were extracted from the GHMBC spectrum shown in Figure 3 to make unequivocal assignments for every carbon resonance in the spectrum with the exception of the C-10a quaternary and C-11 carbonyl carbon resonances, to which no correlations were observed in the GHMBC spectrum. For those resonances assigned, there was general agreement (Table 1) between the data from the present study (albeit with the shifts in the present study upfield of those reported for the pure alkaloid) and those previously reported for this compound.[8−11] It is probable that the upfield shift of the resonances due to cryptolepinone (**2**) in the present study may be a function of the very large difference in the concentration of the alkaloid in this study relative to the concentration employed when spectra of the pure alkaloid were recorded.

To complete the initial evaluation of the performance of the Nalorac SMIDG-600-1.7 submicro-NMR probe for very low-level samples, a ROESY spectrum with a mixing time of 600 ms was also acquired in an effort to observe the ROE correlations between the cryptolepinone (**2**) *N*-methyl group and the flanking H-4 and H-6 protons of the two four-spin systems, which resonated at 7.96 and 8.38 ppm, respectively. Remarkably, as shown in Figure 4, these correlations were readily observed after only 45 min of data accumulation. Data acquisition was terminated at 1.5 h after accumulating 8 transients/t_1 increment; no effort was made to observe ROE correlations between the vicinal proton resonances of the 8% cryptolepinone (**2**) impurity. ROESY correlations between the vicinal protons of the cryptolepinone (**2**) impurity should, however, be observable without much difficulty since vicinal connectivities for the impurity protons were successfully established from an 80 ms homonuclear TOCSY spectrum (not shown), which was acquired in 3 h.

In conclusion, we have demonstrated that under favorable circumstances full 2D-NMR structural characterization is now possible using samples of less than 0.05 μmol of material. This represents a 1000-fold reduction in sample size from the ~50 μmol samples required for full structural characterization 15 years ago when it was necessary to rely on X-nucleus detection and conventional 5 mm probe technology. In the

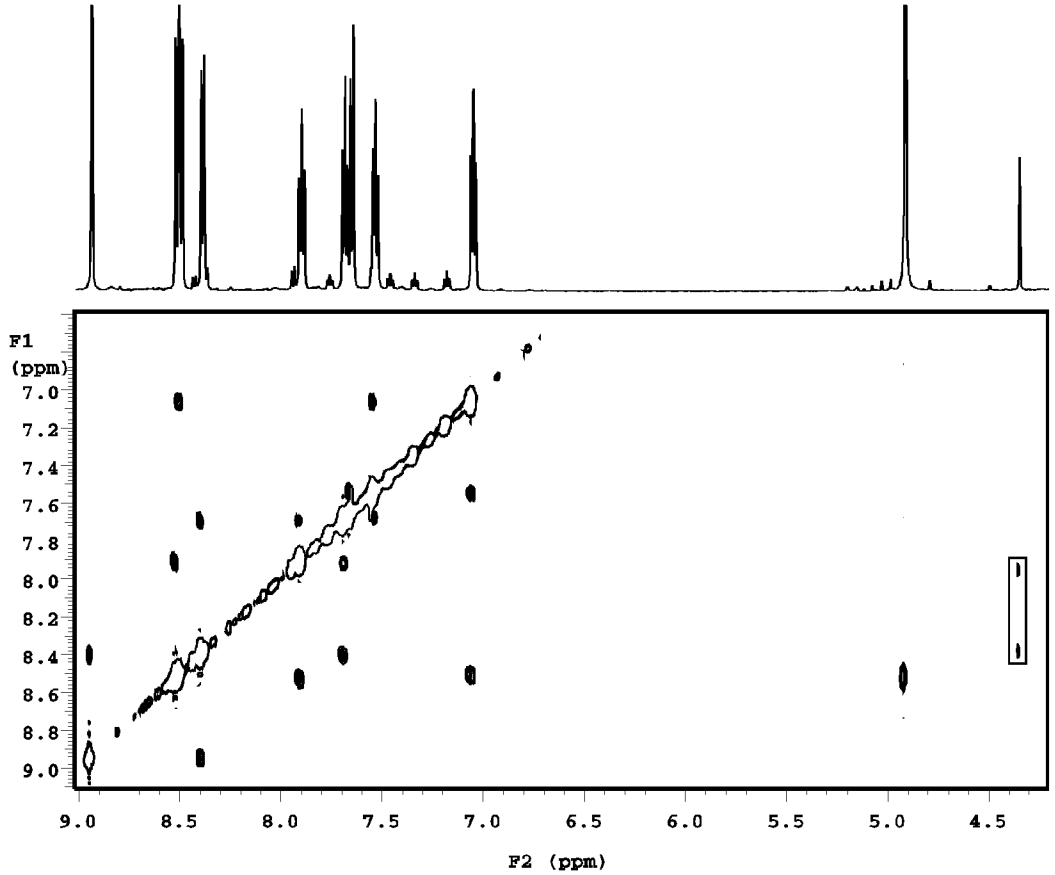


Figure 4. ROESY spectrum of a 0.55 μmol sample of cryptolepine (**1**) containing an 8% (0.04 μmol) cryptolepinone (**2**) impurity. Data were acquired as 2048 × 128 (2 × 128 hypercomplex or States-TPPI files) accumulating 8 transients/t_1 increment giving a total acquisition time of 1.5 h. Data presented were plotted after four transients/t_1 increments. Correlations are observed from the *N*-methyl group of cryptolepinone, which resonates at 4.35 ppm, to the flanking H-4 and H-6 resonances resonating at 7.96 and 8.38 ppm, respectively, denoted by the box along the right edge of the contour plot. The mixing time, 600 ms, was established from the average proton T_1 relaxation time, which was 1.2 s. The interpulse delay was set to 1.8 s.

present case, the characterization was made at this level despite the presence of a major component present in nearly a 12-fold excess. Further work remains to be done to fully assess the capabilities offered by the 1.7 mm submicro-inverse-detection gradient (or SMIDG)-NMR probe used in this study. These efforts will form the basis of future reports.

Acknowledgment. The authors would like to acknowledge Ron Crouch and Toby Zens of the Nalorac Corporation, Martinez, CA, who designed and built the prototype SMIDG-600-1.7 NMR probe used in this study, and for engaging in numerous helpful discussions regarding probe performance optimization during the course of this research. The authors also wish to acknowledge the assistance of Kenneth Visscher in maintaining the Varian INOVA 600 NMR spectrometer used in this study.

References and Notes

(1) Crouch, R. C.; Martin, G. E. *J. Nat. Prod.* **1992**, *55*, 1343−1347.
(2) Crouch, R. C.; Martin, G. E. *Magn. Reson. Chem.* **1992**, *30*, S66−70.
(3) Sharaf, M. H. M.; Schiff, P. L., Jr.; Tackie, A. N.; Phoebe, C. H., Jr.; Howard, L.; Meyers, C.; Hadden, C. E.; Wrenn, S. K.; Davis, A. O.; Andrews, C. W.; Minick, D.; Johnson, R. L.; Shockcor, J. P.; Crouch, R. C.; Martin, G. E. *Magn. Reson. Chem.* **1995**, *33*, 767−778.
(4) Crouch, R. C.; Martin, G. E.; Musser, S. M.; Grenade, H. R.; Dickey, R. W. *Tetrahedron Lett.* **1995**, *36*, 6827−6830.
(5) Reynolds, W. F.; Yu, M.; Enriquez, R. G. *Magn. Reson. Chem.* **1997**, *35*, 614−618.
(6) Olson, D. L.; Peck, T. L.; Webb, A. G.; Magin, R. L.; Sweedler, J. V. *Science* **1995**, *270*, 1967−1970.
(7) Martin, G. E.; Crouch, R. C.; Zens, A. P. *Magn. Reson. Chem.* **1998**, *36*, in press.
(8) Sharaf, M. H. M. Alkaloids of *Cryptolepis sanguinolenta* (Lindl.) Schlechter (Asclepiadaceae). Ph.D. Dissertation, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA, 1993, pp 177−192.
(9) Paulo, A.; Gomes, E. T.; Houghton, P. J. *J. Nat. Prod.* **1995**, *58*, 1485−1491.
(10) Cooper, M. M.; Lovell, J. M.; Joule, J. A. *Tetrahedron Lett.* **1996**, *37*, 4283−4286.
(11) Sharaf, M. H. M.; Schiff, P. L., Jr.; Tackie, A. N.; Martin, G. E. *J. Heterocycl. Chem.* **1998**, *35*, in press.

NP980099B