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Gradient submicro inverse detection: rapid acquisition of inverse-detected heteronuclear chemical shift correlation data on submicromole quantities of material

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ABSTRACT: Micro NMR probes require substantially reduced quantities of sample for spectral characterization relative to traditional 5 mm probes. In addition, 3 mm micro probes have also made it feasible to routinely acquire inverse-detected heteronuclear shift correlation data on submicromole quantities. This paper reports the development and initial testing of a 1.7 mm submicro gradient inverse-detection probe. The further reductions in the sample volume required relative even to the combination of 3 mm micro probes and Shigemi micro NMR sample cells make it feasible to acquire heteronuclear shift correlation data on submicromole quantities of material in very short periods of time. Results obtained using a 0.55 μmol sample of the model alkaloid cryptolepine dissolved in 25 μl of solvent are described. Using submicro probe technology, GHSQC data were acquired on the 0.55 μmol sample of cryptolepine dissolved in 25 μl as rapidly as 12 min and GHMBC data were recorded in 1.1 h. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: gradient submicro NMR; gradient submicro probe; GHSQC; GHMBC; GHSQC-TOCSY; cryptolepine

INTRODUCTION

During the course of developing potential drug candidates, it is necessary to isolate and identify impurities and degradation products arising from synthesis and subjecting the potential drug molecule to various stress challenges according to regulatory mandate. Generally, anything present at levels >0.1% will eventually be isolated and characterized. During the initial phases of drug development, supplies of compounds being evaluated are generally available only in severely limited quantities, making it necessary to develop NMR methods and probe technologies for the characterization of their impurities or degradation products available only in submicromole quantities. A similar situation hampers the characterization of natural products. Many novel molecules with potentially interesting pharmacological properties are available only in very limited quantities from initial plant extracts.

Sample requirements for complete characterization were substantially reduced following the development of 3 mm micro NMR probes by the present authors in 1992.[1,2] For example, the earliest application of micro inverse-detected NMR used in the characterization of an impurity from drug substance was the identification of an impurity of the bisindole anticancer drug Navelbine made on slightly less than 0.3 μmol[3] with weekend data accumulations for HMBC long-range proton–carbon correlation data. Reductions in sample requirements derive from the substantial reduction in sample volume achieved on going from traditional 5 mm NMR sample tubes to 3 mm micro NMR tubes. Whereas the former generally require sample volumes of 500–500 μl, the latter, using a conventional NMR tube, use only 130–150 μl. Further reductions in sample volumes can be achieved in 3 mm format by resorting to Shigemi NMR micro cells. Using Shigemi tubes, in which the glass of the tube and a plunger are matched to the magnetic susceptibility of the NMR solvent being used (D_2O, $CDCl_3$ and DMSO-d_6 at present), sample volumes can be reduced to the range 70–80 μl under favorable circumstances. We have demonstrated the benefits of this approach in the acquisition of spectra on a *ca.* 0.1 μmol sample of Caribbean ciguatoxin.[4] More recently, Reynolds *et al.*[5] have reported a more detailed study of the benefits of using Shigemi NMR micro cells in conjunction with 3 mm micro probes.

We now report the results obtained with the first prototype of a new generation of NMR probe, a 1.7 mm gradient submicro inverse probe. This probe's optimal sample volume in a 1.7 mm NMR tube prepared from 1.7 mm precision capillary glass tubing is *ca.* 25–28 μl. The substantial reduction in sample volume relative even to that attainable with a Shigemi micro NMR cell offers considerable time savings for the acquisition of gradient inverse-detected heteronuclear chemical shift correlation data. Although it remains to be determined, it is also probable that the 1.7 mm probe format will still further reduce sample quantities also.

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EXPERIMENTAL

All experiments were performed using a Varian Inova 600 NMR spectrometer equipped with three r.f. channels and pulse field gradients and operating at a proton observation frequency of 599.75 MHz. The GHSQC, GHMBC and GHSQC-TOCSY pulses sequences used in this study were those from the standard Varian NMR pulse sequence library. The instrument was equipped either with a Nalorac Z·SPEC MIDTG-600-3 micro inverse-detection triple resonance gradient 3 mm probe or a prototype Nalorac Z·SPEC SMIDG-600-1.7 submicro inverse-detection gradient NMR probe.

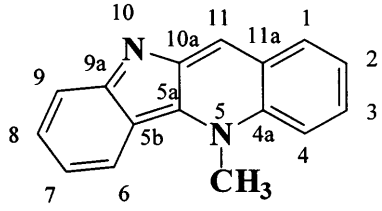
Pulses were calibrated for both probes using standard methods. The MIDTG-600-3 probe employed a 7.3 μs 90° ^{1}H pulse (tpwr = 48, max. = 63) and a 90° ^{13}C pulse of 12 μsec (dpwr = 58, max. = 63). A 90 μs 90° ^{13}C pulse applied at a power level of 41, affording a decoupling field for GARP decoupling of 12 800 Hz, was used for all X-band decoupling on the MIDTG-600-3 probe. The SMIDG-600-1.7 probe employed a 6.6 μs 90° ^{1}H pulse (tpwr = 47, max. = 63) and a 90° ^{13}C pulse of 11.6 μs (dpwr = 57, max. = 63). A 90 μs 90° ^{13}C pulse applied at a power level of 40, affording a decoupling field for GARP decoupling of 12 346 Hz, was used for all X-band decoupling on the SMIDG-600-1.7 probe.

The proton reference spectrum shown in Fig. 1, and the segment of the aromatic region shown in Fig. 3 were acquired as a single trace using 24 000 data points to digitize a spectral width of 4806 Hz. The data were acquired as a single transient following a 90° pulse. No water suppression was employed, nor was any weighting applied to the FID prior to Fourier transformation. The segment of the proton reference spectrum plotted above the contour plots shown in Figs 2, 4, 5 and 6 was acquired in a fashion identical with that shown in Fig. 1 except that a total of 256 transients were accumulated. The minor impurity in the spectrum was present at a level of 8%, which based on the molecular weight of the impurity determined by liquid chromatography–mass spectrometry corresponds to *ca.* 0.04 μmol.

A proton spectral width of 4806 Hz was used for F_2 in all cases. The GHSQC and GHSQC-TOCSY experiments performed employed an F_1 spectral width of 4524 Hz; the GHMBC experiment used an F_1 spectral width of 9050 Hz. The GHSQC and GHSQC-TOCSY experiments performed were digitized in F_2 using 2048 points, giving an acquisition time of 213 ms; the GHMBC spectrum was digitized in F_2 using 4096 points giving an acquisition time of 426 ms. The levels of digitization in F_1 and the numbers of transients accumulated per t_1 increment are given in the figure captions.

Gradient versions of all of the 2D experiments performed in this study were used. In the case of the GHSQC experiment, for example, the square gradients used to encode and decode magnetization were applied in a ratio of *ca.* 2:1. The actual gradient strengths used were 10:5.02 G cm^{-1}, and were applied for 2.0 and 1.0 ms, respectively. For the GHMBC experiment, gradient ratios of 2:2:1 were used; the applied gradient strengths were 10:10:5.02 G cm^{-1} and the gradient durations were 1.0 ms for all three gradients. In addition to the gradient used to encode/decode magnetization, a gradient–90°(^{1}H)–gradient was used to randomize magnetization prior to the beginning of the pulse sequence. A TANGO–gradient combination was used to suppress protons directly bound to ^{12}C.

The model alkaloid used in the studies reported was cryptolepine (**1**) isolated from *Cryptolepis sanguinolenta*.[6] NMR samples containing 0.55 μmol of **1** were prepared by serial dilution in an argon glove-box. Solutions were prepared in 1 ml volumetric flasks in 99.992% DMSO-d_6 (Isotec) using solvent supplied in 0.6 ml ampoules. Samples were transferred to NMR tubes using a 50 or 250 μl Hamilton gas-tight syringe and flexible PTFE needles. The 1.7 mm NMR tubes used in this study were prepared in the laboratory from Wilmad 1.7 mm o.d. precision glass capillary tubing. Wilmad 3 mm 327-PP tubes were used for the 3 mm samples; the sample volumes were 25 and 150 μl, respectively. All samples were prepared under a dry argon atmosphere. The 1.7 mm tubes were prepared at a length of 5 in and 'capped' by coring *ca.* 6–8 layers of Parafilm several times and then overwrapping with Parafilm prior to removal from the glove-box. The 3 mm tubes were capped and Parafilmed prior to removal from the glove-box. After removal, tubes were sealed using a micro torch. As used for this study, the 1.7 mm tubes were sealed at a length of 3.5–4 in and were inserted into the probe using a chuck-type sample holder. Standard 3 mm NMR turbines were used with the sealed 3 mm NMR tubes. None of the samples were spun.



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Two-dimensional data for the GHSQC and IDR-GHSQC-TOCSY spectra were processed using Gaussian multiplication prior to both Fourier transformations matched to the acquisition times in each frequency domain. The GHSQC data were processed with zero-filling to 2048 and 256 points prior to the first and second transformations, respectively. No linear prediction was employed with the GHSQC data shown in the present study although it can be readily employed even with the scant 16 files in F_1 accumulated.

The flexibility of using linear prediction affords the investigator the obvious trade-off choices between the numbers of transients and the numbers of increments in F_1 that can be selected for a given overall acquisition

time.[7] When used with submicro NMR data, we have typically linear predicted to a total of $n_i + 3n_i$ files in F_1. Linear prediction was modestly used to extend the 32 files acquired in F_1 of the IDR-GHSQC-TOCSY data to 96 files followed by zero-filling to 256 points prior to the second Fourier transformation.

Processing of the GHMBC data differed from that used for the GHSQC and IDR-GHSQC-TOCSY experiments in that the data were treated with sine-bell weighting prior to the first transformation followed by zero-filling to 256 points and cosine multiplication prior to the second transformation. Again, linear prediction can be used to advantage with GHMBC data, although it was not applied to the data shown in this study.

RESULTS AND DISCUSSION

The ^{1}H reference spectrum of a 0.55 μmol sample of cryptolepine (**1**) in 25 μl of DMSO-d_6 recorded following a single 90° pulse is shown in Fig. 1. No water suppression was employed. The detail in the aromatic region of the spectrum is shown in the proton reference spectra flanking plots of the heteronuclear shift correlation spectra discussed and presented below.

Characterization of impurities and degradation products of pharmaceuticals and potentially novel natural products is generally based on the acquisition of heteronuclear shift correlation data. Obviously, homonuclear detection experiments such as COSY and homonuclear TOCSY can be acquired with ease given the single transient signal-to-noise shown in the proton reference spectrum presented in Fig. 1. In the past, heteronuclear shift correlation data on low-level samples have generally been acquired without using gradients to avoid the signal losses inherent to the gradient experiments. Indeed, as a rule of thumb, we have generally resorted to a non-gradient HMQC[8] or HSQC[9] rather than their gradient versions of these experiments[10–12] when the sample quantities are below *ca.* 1 μmol, although GHMBC[10] has been used routinely since the problems associated with t_1 noise at low levels far outweigh losses of signal due to the use of gradients. Investigators, of course, have recourse to the sensitivity-enhanced heteronuclear shift correlation methods[13] and the gradient enhanced version as developed by Kay *et al.*,[14] although we have tended not to use them in our laboratories because of the length of the pulse sequences. Nevertheless, we were interested in evaluating the performance characteristics of the SMIDG-600-1.7 probe and elected to use gradient experiments exclusively for our evaluation with a sample at the *ca.* 0.5 μmol level.

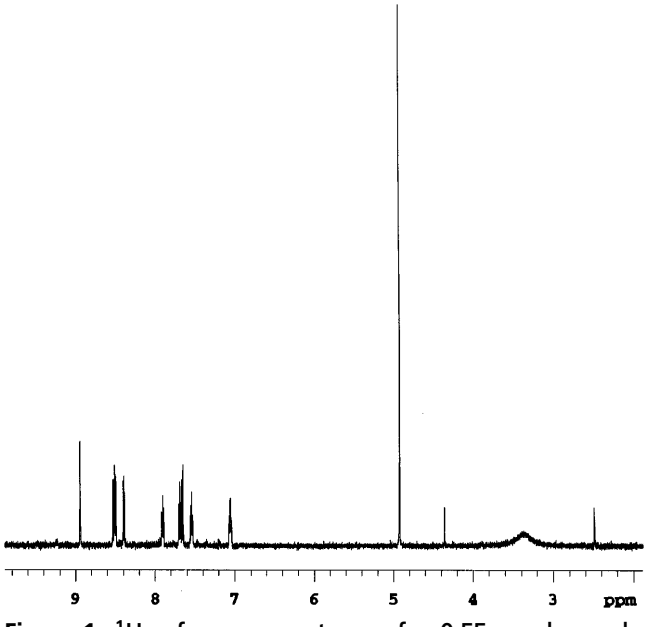

Figure 1. ^{1}H reference spectrum of a 0.55 μmol sample of cryptolepine (**1**) dissolved in 25 μl of 99.992% DMSO-d_6 acquired in a single transient following a 90° pulse in a Nalorac SMIDG-600-1.7 submicro indirect detection gradient inverse probe installed in a Varian Inova 600 spectrometer. No water suppression or weighting was employed.

As a performance benchmark, an identical 0.55 μmol sample was prepared in a 150 μl volume in a standard 3 mm probe, and a GHSQC spectrum was acquired using the Nalorac MIDTG-600-3 probe. The data (not shown) were acquired in 3 h and gave a signal-to-noise (S/N) ratio in the projection through F_2 of *ca.* 30:1. Using the Nalorac SMIDG-600-1.7 probe, an initial experiment was set up to run for 24 min. The acquisition parameters were those specified above; the F_1 frequency domain was digitized using 32 hypercomplex files (2 × 32) to afford a phase-sensitive presentation in both frequency domains with 16 transients accumulated per t_1 increment. Based on the quality of these data, the number of files used to digitize F_1 was halved, affording the high-quality spectrum shown in Fig. 2 in 12 min.

The S/N ratio of the spectrum shown in Fig. 2 is probably best evaluated by examination of the projection of the data through the F_2 frequency domain. The F_2 projection is presented in Fig. 3. The GHSQC results shown in Fig. 2, despite the short 12 min duration of the experiment, gave an S/N ratio slightly greater than 20:1 using a 200 Hz region between the two multiplets furthest upfield to define the noise level in the spectrum.

The level of performance reflected in the data presented in Figs 2 and 3 is certainly adequate for the task of most structure elucidation efforts. Undeniably, acquisition times will generally, of necessity, be longer than that in the present case. The F_1 spectral window is reasonably restricted and hence readily digitized. However, even for more complicated molecules, the judicious choice of several smaller F_1 windows can readily keep acquisition times very manageable even at the 0.5 μmol level.

When dealing with a total unknown, long-range heteronuclear shift correlation data will generally be required if the structure is to be fully elucidated. In contrast, in a well studied family of compounds, it may be possible to make the structural assignment from direct heteronuclear correlation spectra supplemented by

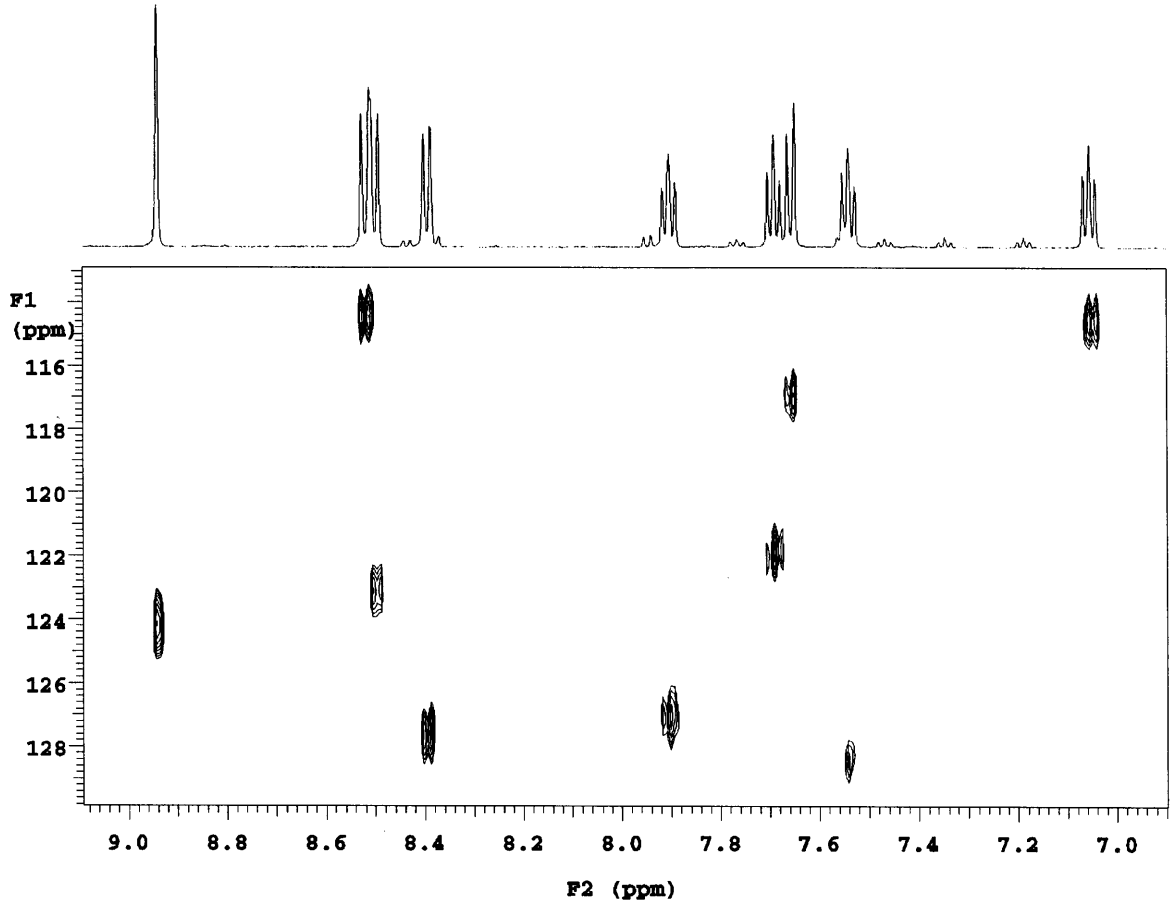

Figure 2. GHSQC spectrum of a 0.55 μmol sample of cryptolepine (**1**) dissolved in 25 μl of 99.992% DMSO-d_6 acquired as 2048 × 16 hypercomplex files (2 × 16) with 16 transients per t_1 increment in a Nalorac SMIDG-600-1.7 submicro indirect detection gradient inverse probe installed in a Varian Inova 600 spectrometer. The data were acquired in 12 min and were processed to 1024 × 128 points by zero-filling in the second frequency domain.

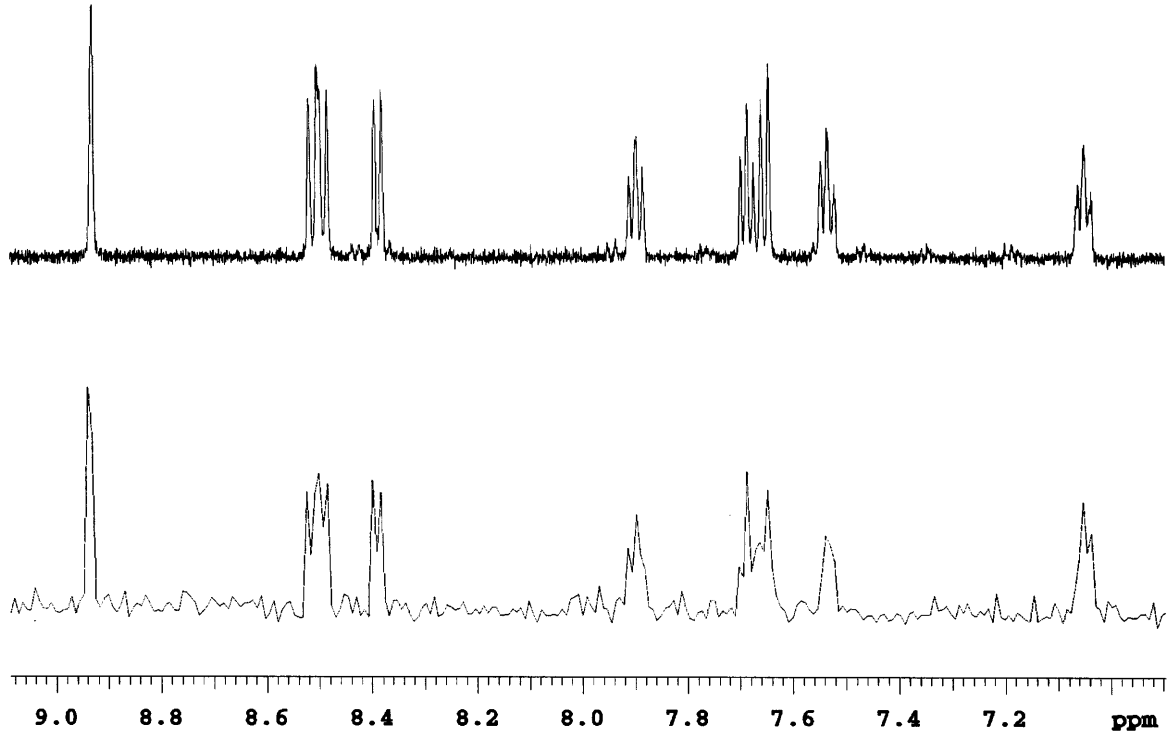

Figure 3. Comparison plots of the F_2 projection of the GHSQC spectrum shown in Fig. 2 (bottom trace) and the aromatic region of the proton reference spectrum shown in Fig. 1 (top trace). The S/N ratio in the projection was slightly greater than 20 : 1 for the 12 min data accumulation. The region of the spectrum selected to define 'noise' was a 200 Hz region between the two upfield multiplets containing the significant noise spikes in that region of the spectrum.

homonuclear shift correlation data. In any case, we were interested in the performance of the submicro probe at the 0.5 μmol level for the acquisition of GHMBC spectra. A long-range proton–carbon shift correlation spectrum of **1** was acquired using the GHMBC sequence optimized for 10 Hz. The spectral width in F_1 was doubled to accommodate the downfield shifted C-9a resonance at 160.0 ppm. Hence the number of t_1 increments was correspondingly increased to 64. As an initial attempt, a GHMBC spectrum was acquired using only 16 transients per t_1 increment since the sensitivity of the GHMBC experiment is generally accepted to be between a factor of 4 and 8 lower than the direct correlation experiments. The number of transients chosen thus gave an accumulation time for the GHMBC data of 1.1 h. The resulting full spectrum is shown in Fig. 4 and an expansion of the aromatic region of the molecule is shown in Fig. 5.

Again, despite the relatively short 1.1 h data accumulation for the 0.55 μmol sample of **1** being studied, the data were of more than adequate quality for structure elucidation purposes. Assessing performance of the experiment by the S/N ratio of the projection through F_2, the 1.1 h data accumulation gave an S/N ratio in the F_2 projection of slightly greater than 30:1.

Finally, in many instances, particularly when dealing with natural products or drugs derived from them, there will be regions of the proton spectrum that are too congested for proton–proton connectivity networks to be successfully traced through them. In such cases, it is highly desirable to be able to resort to a coupled experiment such as GHXQC-TOCSY (where X = M for the HMQC-based experiment or X = S for the HSQC-based variant) capable of sorting proton–proton connectivity information using the generally much greater dispersion of ^{13}C. Since in most cases there is seldom overlap or a high degree of congestion in the ^{13}C spectrum, such an approach is almost invariably successful in establishing unequivocally the proton–proton connectivity network. It was for precisely this reason that we resorted to the use of IDR-HMQC-TOCSY[15] in the total assignment of the proton and carbon spectra of the marine toxin brevetoxin-3.[16] The IDR (inverted direct response)-GHSQC-TOCSY[17–19] spectrum of **1**

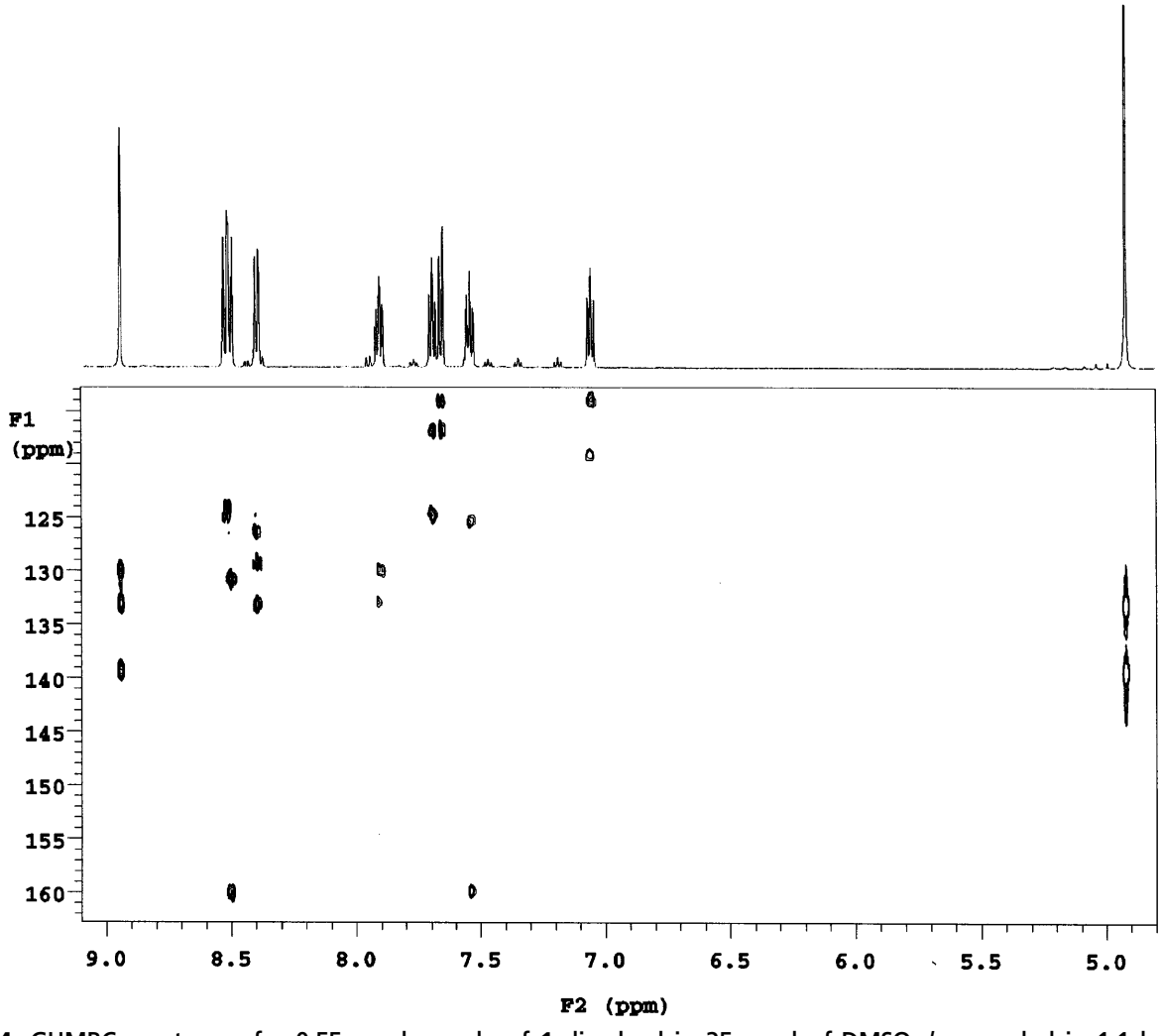

Figure 4. GHMBC spectrum of a 0.55 μmol sample of **1** dissolved in 25 μmol of DMSO-d_6 recorded in 1.1 h using a Nalorac SMIDG-600-1.7 probe. The data were acquired as 4096 × 64 (2 × 64) hypercomplex files with 16 transients accumulated per t_1 increment and were processed with zero-filling to 2048 × 128 points. The long-range coupling delay was optimized for 10 Hz (50 ms).

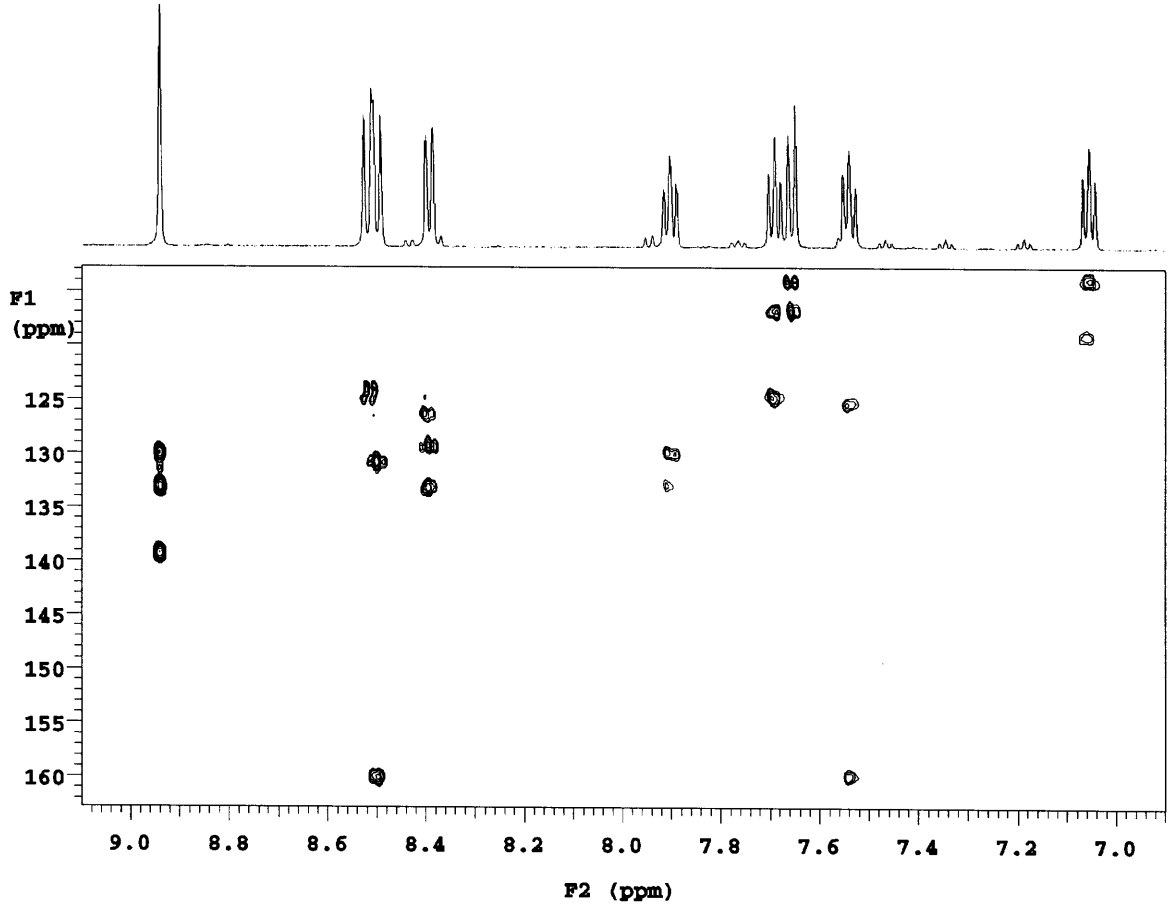

Figure 5. Expansion of the aromatic region of the GHMBC spectrum shown in Fig. 4.

recorded with a 24 ms mixing time is shown in Fig. 6. The data were again acquired using 32 (2 × 32) hypercomplex files to digitize F_1 since the resolution requirements are identical with the GHSQC direct correlation experiments.

Although the sensitivity of the GHSQC-TOCSY experiment is generally regarded as being comparable to that of the GHMBC experiment, a higher S/N ratio was desirable in the present case to allow the partially overlapped direct and relayed correlations near 8.5/114 ppm. Electing not to invert the direct response decreases the time required to achieve comparable S/N ratios.

CONCLUSIONS

Micro NMR probes have had a profound impact on the sample size necessary for the elucidation of many classes of chemical structures, in particular impurities and degradants of drug substances in the pharmaceutical industry and in the characterization of novel natural product structures. Using NMR micro probes, many research groups have generally become adept at total characterization over a weekend of samples in the range 0.5–1.0 μmol. With the development of the prototype SMIDG-600-1.7 gradient submicro inverse detection probe described in this paper, data acquisition time for the complete characterization of a sample in the range of 0.5 μmol have been demonstrated to be reduced to less than a working day under ideal circumstances or, we would assume, no worse than overnight in most cases. In addition, the significant reduction in sample volume to 25 μl for the 1.7 mm submicro probe from the normal working range of 130–150 μl in a standard 3 mm tube or the 70–80 μl which is possible using a Shigemi NMR micro cell holds the promise of being able to push the minimum workable sample size below the 0.07 μmol contained in our original paper on the development of the first 3 mm micro inverse probe.[1] We are at present working to evaluate the performance of the submicro probe below the 0.1 μmol level, and the results of these studies will for the basis for a future paper.[20]

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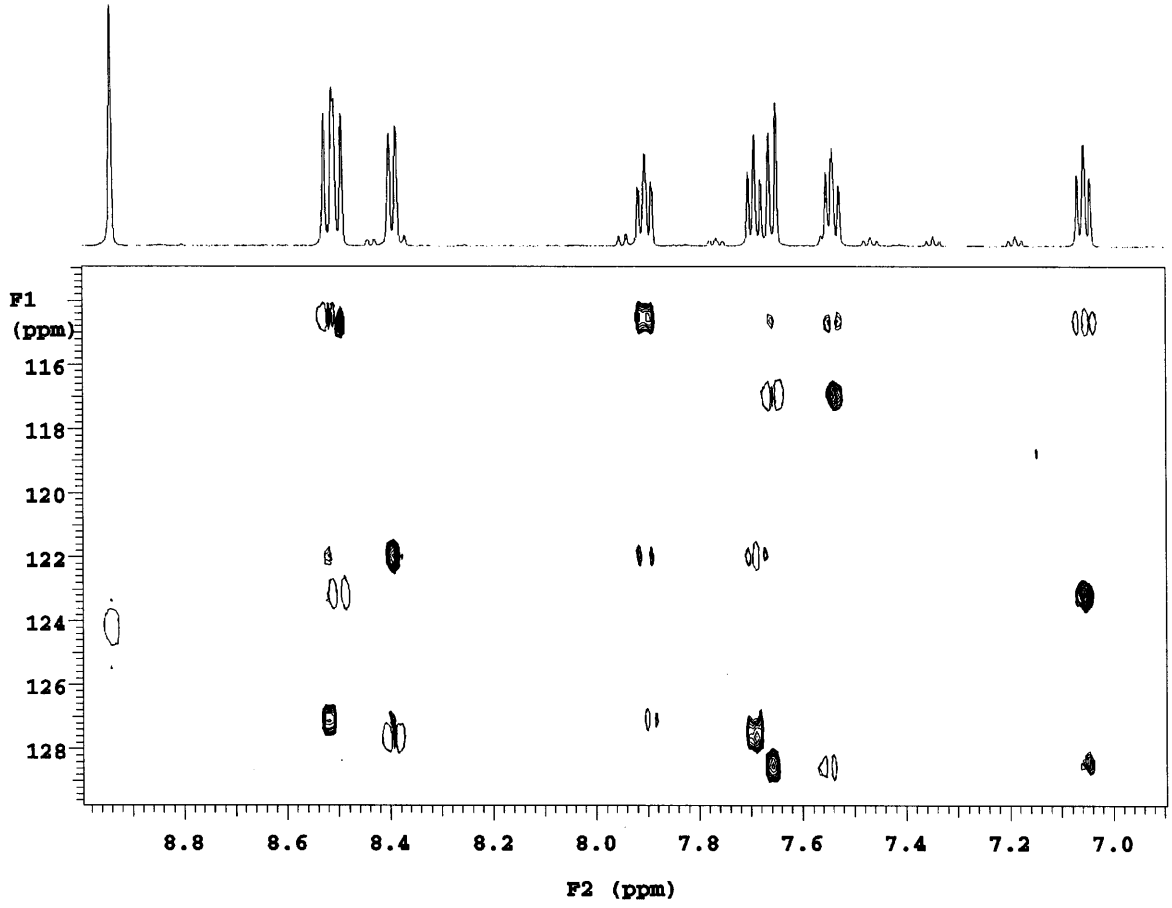

Figure 6. IDR-GHSQC-TOCSY spectrum of a 0.55 μmol sample of **1** dissolved in 25 μl of DMSO-d_6. The data were acquired in 3 h as 2048 × 32 (2 × 32) hypercomplex files, accumulating 128 transients per t_1 increment. The mixing time used was 24 ms. Direct correlations have negative phase in this experiment and are presented as open contours (e.g. the 11-H direct response at 8.94/124.2 ppm). Relayed responses have positive phase and are shown as closed black contours (e.g. the 7-H relayed response at 7.05/123.2 ppm).

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