MAGNETIC RESONANCE IN CHEMISTRY
Magn. Reson. Chem. **36**, 601–607 (1998)

An ω_1-band-selective, ω_1-homonuclear decoupled ROESY experiment: application to the assignment of ^{1}H NMR spectra of difficult-to-assign peptide sequences

Andreas Kaerner and Dallas L. Rabenstein*

Department of Chemistry, University of California, Riverside, California 92521, USA

Received 18 December 1997; revised 6 March 1998; accepted 6 March 1998

ABSTRACT: Application of an ω_1-band-selective, ω_1-homonuclear decoupled ROESY (BASHD-ROESY) experiment to the assignment of ^{1}H NMR spectra of peptides is demonstrated. Band selection in the ω_1 dimension is achieved with the double pulsed field gradient spin echo (DPFGSE) technique; homonuclear decoupling in the ω_1 dimension is achieved by placing a non-selective 180° pulse together with the first half of the DPFGSE in the middle of the evolution period. Application of the BASHD-ROESY experiment is demonstrated with the complete assignment of the proton resonances of the synthetic 19 amino acid peptide *N*-Ac–Ala–Glu–Ala–Ala– Ala–Arg–Ala–Ala–Ala–Arg–Arg–Ala–Ala– Arg–Arg–Ala–Ala–Ala–Arg–NH_2. Critical to making the assignments was the significantly increased resolution in the C_αH–NH region of the ROESY spectrum measured with the BASHD-ROESY pulse sequence with band selection and homonuclear decoupling in the C_αH region. NOEs observed for the peptide indicate it has a helical secondary structure in solution. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{1}H NMR; ω_1-band-selective, ω_1-homonuclear decoupled ROESY; BASHD-ROESY; peptide sequences

INTRODUCTION

The standard strategy used to assign ^{1}H NMR spectra of peptides is first to assign the backbone amide NH resonances to amino acid type using NH connectivities to side-chain protons in 2D TOCSY spectra and then to establish the sequence of the amino acid residues in the peptide using NH_i–$C_\alpha H_i$ and $C_\alpha H_i$–NH_{i+1} connectivities obtained from 2D NOESY or ROESY spectra. This is a powerful procedure which, in principle, allows the assignment of ^{1}H NMR spectra of peptides of known sequence and the determination of the amino acid sequence of peptides of unknown sequence. In practice, however, ambiguities can be encountered in the sequential assignment step due to overlap of cross peaks, particularly if the peptide contains multiple residues of a given amino acid or if the C_αH–NH region of the NOESY or ROESY spectrum also contains long range NOE cross peaks. The overlap problem is generally due, in part at least, to low digital resolution in one or both dimensions.

In order to take advantage of the spectral resolution possible with high-field spectrometers, semi-selective or band-selective variants of the standard 2D experiments have been developed.[1–13] Such experiments are particularly useful when only a portion of the 2D spectrum is needed, e.g. the C_αH–NH region of the NOESY or ROESY spectrum for the sequential assignment step. The semi-selective experiments use soft- or band-selective pulses to select a desired spectral region in one or more dimensions. Resolution in semi-selective spectra can be increased further by homonuclear decoupling in the selected spectral region.[3,14] Homonuclear decoupling can be achieved in the evolution dimension by application of a semi-selective refocusing pulse together with a non-selective refocusing pulse in the center of the evolution period[3] and in the acquisition dimension by use of homonuclear shaped pulse decoupling in combination with the time-shared decoupling mode during data acquisition.[14]

Several schemes have been used to achieve band selection and homonuclear decoupling in the evolution dimension in semi-selective homonuclear 2D experiments.[7,11–13,15] Recently, an ω_1-band-selective, ω_1-homonuclear decoupled TOCSY (BASHD-TOCSY) experiment was reported[13] in which band selection and homonuclear decoupling were achieved in the evolution dimension by a band-selective double pulsed field gradient spin echo (DPFGSE) technique.[16] Selective excitation using the DPFGSE technique requires no phase cycling and yields pure phase band-selective TOCSY spectra.[13] Use of the DPFGSE technique in other homonuclear 2D experiments was also mentioned.[13]

We describe here the application of an ω_1-band-selective, ω_1-homonuclear decoupled ROESY (BASHD-ROESY) experiment to the assignment of peptide ^{1}H NMR spectra. Band selection is achieved using the DPFGSE technique. The utility of the BASHD-ROESY

* *Correspondence to*: D. L. Rabenstein, Department of Chemistry, University of California, Riverside, California 92521, USA.
Contract/grant sponsor: National Institutes of Health; *Contract/grant number*: GM37000; *Contract/grant number*: HL56588.
Contract/grant sponsor: University of California Biotechnology Research and Education Program.

CCC 0749-1581/98/080601–07 $17.50

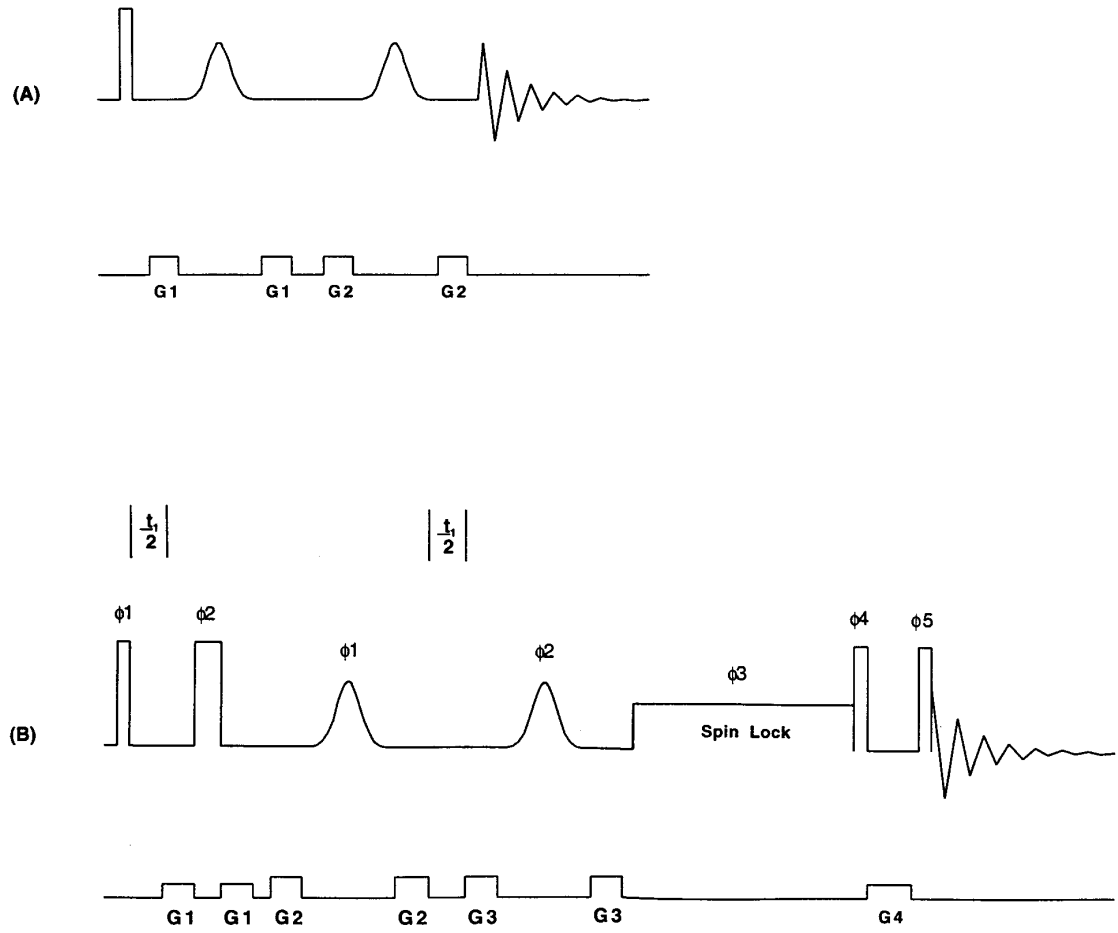

Figure 1. Pulse sequences for (A) the one-dimensional semi-selective experiment with band selection by the DPFGSE and (B) the ω_1-band-selective, ω_1-homonuclear decoupled (BASHD) ROESY experiment. Gradient strengths of 8 and 11 G cm^{-1} were used for G1 and G2 in the 1D experiment; the duration of the gradient pulses was 6.5 and 14 ms for the amide NH and $C_\alpha H$ regions, respectively. In the BASHD-ROESY pulse sequence, the narrow and wide rectangular pulses are $\pi/2$ and π pulses, respectively. The band-selective Gaussian pulses are π pulses. The phase cycling is $\phi1 = (x, -x)_4$, $\phi2 = (x, -x, -x, x)_2$, $\phi3 = (y, -y)_4$, $\phi4 = (x)_4(-x)_4$ and $\phi5 = (x)_8$. Gradient strengths of 2, 8, 11 and 10 G cm^{-1} were used for G1, G2, G3 and G4, respectively; the duration of the gradient pulses was 500 μs for G1 and 1 ms for G2, G3 and G4. A 500 μs recovery period was used after each gradient pulse.

experiment in peptide NMR is demonstrated with the complete assignment of the ^{1}H NMR spectrum of the synthetic heparin-binding 19mer peptide *N*-Ac–Ala–Glu–Ala–Ala–Ala– Arg–Ala–Ala–Ala–Arg–Arg– Ala–Ala–Arg–Arg–Ala–Ala–Ala–Arg–NH_2.[17] Assignment of the proton resonances of this peptide is particularly challenging owing to the presence of six arginine and 12 alanine residues.

EXPERIMENTAL

The peptide was dissolved in H_2O–D_2O (9:1) at a concentration of 5 mM and the pH was adjusted to 3.0. The pH was measured directly in the NMR tube using an Ingold combination ultramicroelectrode. The 300 μl sample solution was contained in an NMR tube obtained from Shigemi. Sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4 was added as a chemical shift reference.

^{1}H NMR spectra were recorded at 500 MHz and 5 °C on a Varian Unity Inova 500 spectrometer equipped with waveform generators, a Performa *X*, *Y*, *Z* gradient module and a $^1H\{^{13}C, ^{15}N\}$ triple resonance, *X*, *Y*, *Z* triple axis pulsed field gradient probe. The band-selective pulses used in the BASHD-ROESY and BASHD-TOCSY experiments were Gaussian cascade Q3 pulses[15] phase modulated to shift the center of excitation 1690 or −400 Hz to the NH or $C_\alpha H$ regions, respectively. The duration of the band-selective pulses was 6.5 and 14 ms, with exciting bandwidths of 600 Hz for the NH region and 225 Hz for the $C_\alpha H$ region. An F_1 spectral window of 550 Hz was used for band selection in the backbone amide NH region and 175 Hz for band selection in the $C_\alpha H$ region. The F_1 spectral window was set to 5700 Hz for the non-selective ROESY experiment. In all cases, the spectral window in the F_2 dimension was set to 5700 Hz. All 2D spectra were measured in the phase-sensitive mode by the hypercomplex method of States *et al.*[18] A cw spin-lock of *ca.* 4 kHz effective field strength and a mixing time of 125 ms were used for dipolar magnetization transfer in the BASHD-ROESY and non-selective ROESY experiments. A windowed MLEV-17 spin lock

of *ca.* 9.5 kHz field strength and a mixing time of 120 ms was used in the BASHD-TOCSY experiment. Pulsed field gradients were applied in the *Z*-direction. Data were processed with zero-filling to 4K points in the F_1 dimension and, unless noted otherwise, a 90° shifted sine-bell squared apodization was applied to both dimensions to drive the FID to zero at the last point. A frequency shift corresponding to the difference between the transmitter offset and the center of the excitation window was applied to the t_1 interferograms prior to Fourier transformation for the band-selective experiments. No baseline correction, linear prediction or drift correction was applied. One-dimensional spectra were acquired with 32K points and a 5700 Hz spectral width and no weighting functions were applied. The water resonance was suppressed in the measurement of all one- and two-dimensional spectra using a selective saturation pulse during the relaxation delay.

RESULTS AND DISCUSSION

The pulse sequence for band selection in 1D ^{1}H NMR with the DPFGSE is shown in Fig. 1(A);[13,16] the pulse sequence for the BASHD-ROESY experiment is given in Fig. 1(B). The BASHD-ROESY pulse sequence is a modification of the pulse sequences used in the non-selective ROESY and the BASHD-TOCSY experiments.[13,19,20] Band selection in the evolution dimension in the BASHD-ROESY experiment is achieved with the DPFGSE; homonuclear decoupling in the evolution dimension is achieved by placing the non-selective 180° pulse together with the first half of the DPFGSE in the middle of the evolution period.[13] A gradient *Z*-filter was added after the spin lock for improved suppression of the water resonance.[21,22]

The full one-dimensional ^{1}H NMR spectrum of the peptide is shown in Fig. 2. Also shown are band-selective DPFGSE spectra of (B) the $C_\alpha H$ and (C) the amide backbone NH regions measured with pulse sequence A in Fig. 1. The phase and vertical scale used for the selective excitation spectra were the same as for the full spectrum. The DPFGSE provides band-selective spectra free of phase imperfections regardless of the quality of the inversion pulse; since the same inversion pulse is used for each gradient echo, phase imperfections are compensated for at the end of the second gradient echo.[13,16] Signal amplitude will be reduced owing to T_2 relaxation during the echoes, but this is typically not significant for peptides.

Because of the multiple alanine and arginine residues in the test peptide, there is extensive resonance overlap in the one-dimensional spectrum. The $C_\alpha H$ resonances

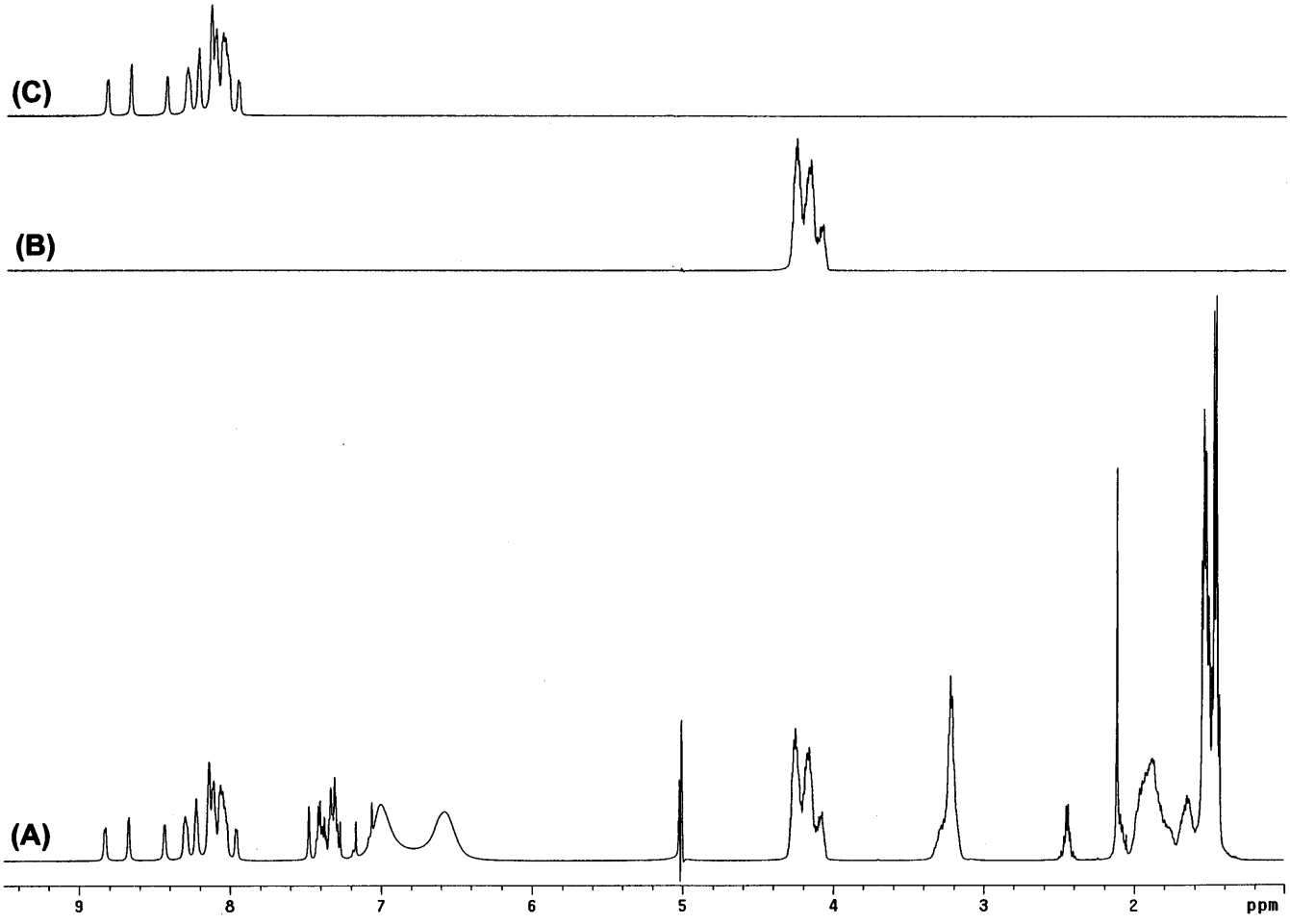

Figure 2. (A) The full one-dimensional spectrum of the peptide and band-selective spectra of (B) the $C_\alpha H$ and (C) the backbone amide NH regions measured with pulse sequence A in Fig. 1.

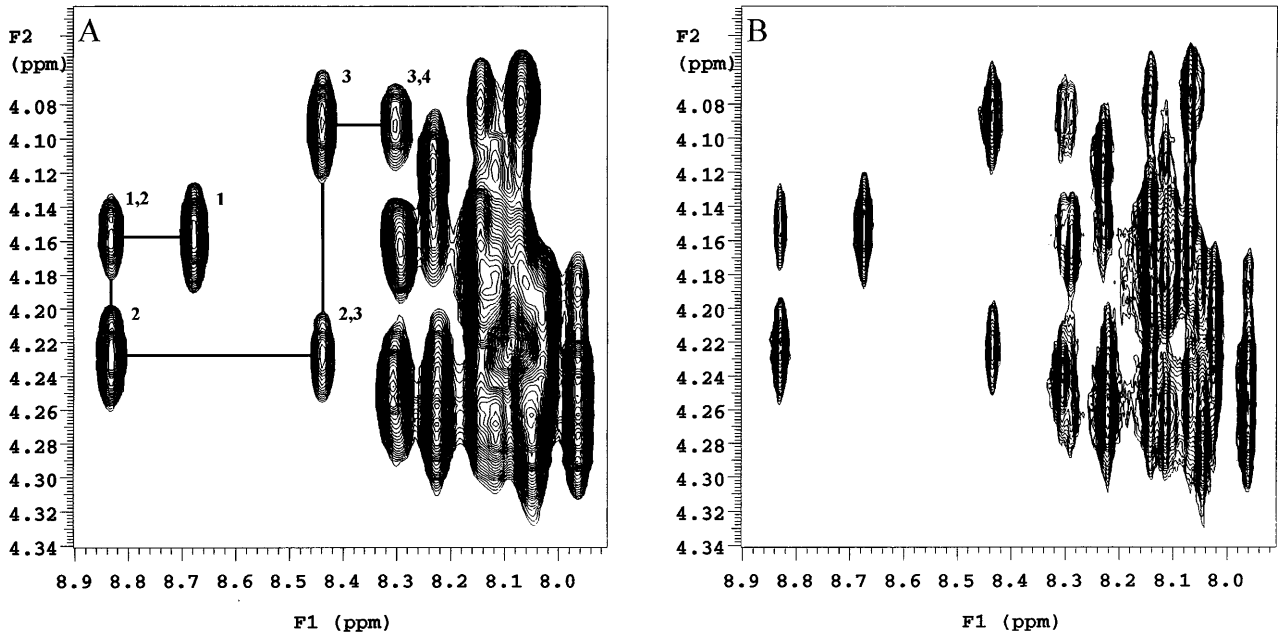

Figure 3. The $C_\alpha H(F_2)$–$NH(F_1)$ regions of (A) the full ROESY spectrum and (B) the BASHD-ROESY spectrum measured with ω_1-band selection and ω_1-homonuclear decoupling of the NH region. For the full ROESY spectrum, 512 × 4K complex data points were acquired in t_1 and t_2, respectively, a spectral width of 5700 Hz was used in both dimensions and 16 scans were co-added. A 90° shifted squared sine-bell apodization was applied in both dimensions, and the indirectly detected dimension was zero filled to 4K. The total acquisition time was *ca.* 8 h. For the BASHD-ROESY spectrum, 256 × 4K complex points were acquired in t_1 and t_2 for spectral widths of 550 Hz in F_1 and 5700 Hz in F_2, and 16 transients were co-added. ω_1-Band selection was achieved with the DPFGSE using 6.5 ms Q3 Gaussian cascade inversion pulses. The total acquisition time was *ca.* 2 h. A 90° shifted sine-bell apodization was applied in both dimensions. For ease of comparison with spectra in Fig. 4, the F_1/F_2 axes are interchanged from the usual format.

are confined to a 175 Hz (0.35 ppm) region and all but three of the backbone amide NH resonances fall in a 200 Hz region. Nevertheless, it has been possible to do a complete assignment of the ^{1}H NMR spectrum with the resolution which can be achieved in ω_1-band-selective, ω_1-homonuclear decoupled 2D TOCSY and ROESY spectra. In the first step of the assignment, the backbone amide resonances were assigned to alanine, arginine or

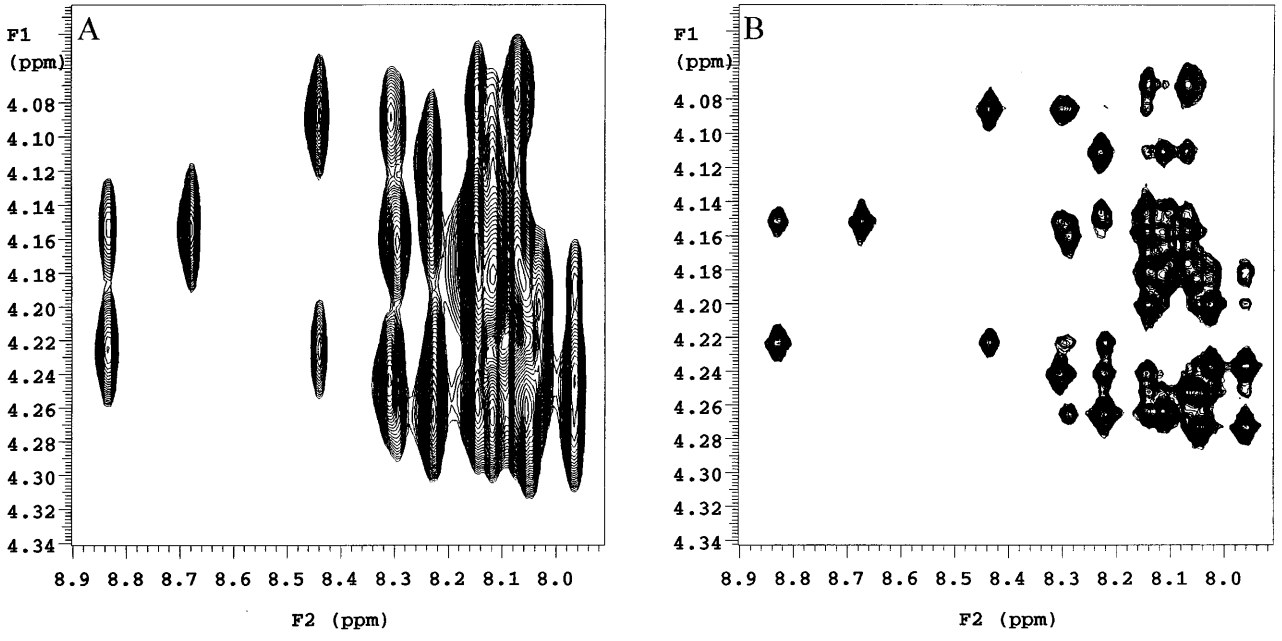

Figure 4. The $C_\alpha H(F_1)$–$NH(F_2)$ regions of (A) the full ROESY spectrum and (B) the BASHD-ROESY spectrum measured with ω_1-band-selection and ω_1-homonuclear decoupling of the $C_\alpha H$ region. Measurement conditions as in the legend to Fig. 3, except the following for the BASHD-ROESY spectrum: 128 × 4K complex points were acquired in t_1 and t_2, the spectral width was 175 Hz in F_1 and 5700 Hz in F_2. The Q3 Gaussian cascade inversion pulses were of 14 ms duration.

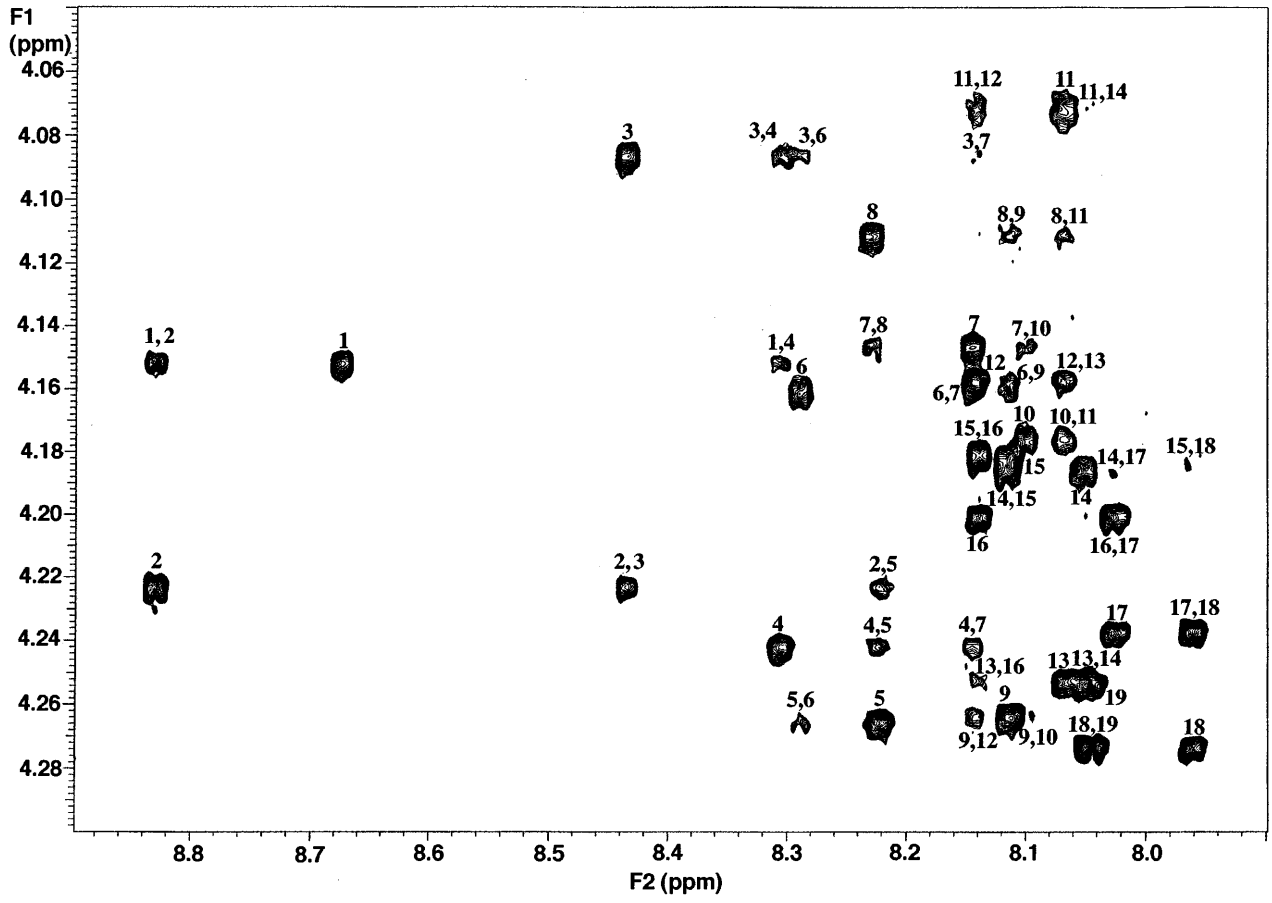

Figure 5. The $C_\alpha H(F_1)$–$NH(F_2)$ region of the BASHD-ROESY spectrum measured with ω_1-band-selection and ω_1-homonuclear decoupling of the $C_\alpha H$ region [Fig. 4(B)]. A 60° shifted sine-bell apodization function was applied in both the F_1 and F_2 dimensions. Some of the weaker cross peaks are not observed in this spectrum owing to the scaling factor used.

glutamic acid using cross-peak patterns in BASHD-TOCSY spectra (data not shown) measured with band selection and homonuclear decoupling of the $C_\alpha H$ and backbone amide NH regions. The sequential assignment of the specific amino acids was then determined using BASHD-ROESY spectra. However, to demonstrate the greatly increased resolution possible with the BASHD-ROESY experiment, we shall first consider spectra obtained from the non-selective ROESY experiment.

Figure 3(A) shows the $C_\alpha H(F_2)$–$NH(F_1)$ region of the non-selective ROESY spectrum. Measurement and data processing conditions are given in the figure legend. Using information from the BASHD-TOCSY spectrum, assignments can be made starting with the amide NH resonance of Ala^1 through the NH resonance of Ala^4, as indicated in Fig. 3(A). However, extensive overlap prevents the sequential assignment of resonances beyond the NH resonance of Ala^4. Overlap in the F_1(NH) dimension is due in part to the width of the multiplets for the NH protons and to low digital resolution. Digital resolution in the F_1 dimension prior to zero filling was 11.1 Hz per point; digital resolution in the F_2 dimension was 2.8 Hz per point. The effect of low digital resolution in the F_1 dimension is even more apparent in Fig. 4(A), which shows the $C_\alpha H(F_1)$–$NH(F_2)$ region from the same non-selective ROESY spectrum.

Resolution in the $C_\alpha H$–NH region of the ROESY spectrum was increased by using the BASHD-ROESY experiment with ω_1-band selection and ω_1-homonuclear decoupling in either the backbone amide NH or the $C_\alpha H$ regions. Figure 3(B) shows the $C_\alpha H(F_2)$–$NH(F_1)$ region of the BASHD-ROESY spectrum measured with ω_1-band selection and ω_1-homonuclear decoupling in the NH region; Fig. 4(B) shows the $C_\alpha H(F_1)$–$NH(F_2)$ region of the spectrum measured with ω_1-band selection and ω_1-homonuclear decoupling in the $C_\alpha H$ region. Digital resolution in the F_1 dimension in the spectra in Figs 3(B) and 4(B) prior to zero filling was 2.1 and 1.4 Hz per point, respectively. The increased digital resolution in the F_1 dimension together with the collapse of the NH multiplet structure in Fig. 3(B) and the $C_\alpha H$ multiplet structure in Fig. 4(B) results in significant increases in spectral resolution. A comparison of the spectra in Figs 3(B) and 4(B) indicates that the resolution in the BASHD-ROESY spectrum measured with band selection in the $C_\alpha H$ region is better owing to collapse of the broader $C_\alpha H$ multiplets. Also, the signal-to-noise ratio of the $C_\alpha H$ band-selective spectrum is higher than in the NH band-selective spectrum. However, the two BASHD-ROESY spectra tend

Table 1. Chemical shift data for the peptide *N*-Ac–Ala–Glu–Ala–Ala–Ala– Arg–Ala–Ala–Ala–Arg–Arg–Ala–Ala–Arg–Arg– Ala–Ala–Ala–Arg–NH_2 [a]

Residue	NH amide	αH	βH	γH	δH	εH	Others
Ala^1	8.673	4.153	1.444				*N*-Ac 2.114
Glu^2	8.829	4.224	2.079	2.423			
Ala^3	8.436	4.087	1.466				
Ala^4	8.305	4.242	1.494				
Ala^5	8.221	4.266	1.544				
Arg^6	8.288	4.161	(a) 1.876	1.630	(a) 3.184	7.411	
			(b) 1.982		(b) 3.276		
Ala^7	8.145	4.147	1.529				
Ala^8	8.229	4.112	1.534				
Ala^9	8.114	4.264	1.544				
Arg^{10}	8.095	4.178	(a) 1.854	1.637	(a) 3.205	7.375	
			(b) 1.953		(b) 3.248		
Arg^{11}	8.069	4.073	(a) 1.866	1.579	(a) 3.193	7.422	
			(b) 1.976		(b) 3.300		
Ala^{12}	8.142	4.158	1.514				
Ala^{13}	8.062	4.254	1.516				
Arg^{14}	8.051	4.186	(a) 1.813	1.680	(a) 3.204	7.335	
			(b) 1.927		(b) 3.231		
Arg^{15}	8.116	4.183	(a) 1.782	1.664	(a) 3.205	7.330	
			(b) 1.903		(b) 3.248		
Ala^{16}	8.140	4.202	1.466				
Ala^{17}	8.026	4.238	1.466				
Ala^{18}	7.963	4.274	1.464				
Arg^{19}	8.046	4.254	(a) 1.834	(a) 1.686	(a) 3.215	7.301	NH_2 (a) 7.313
			(b) 1.913	(b) 1.738	(b) 3.242		(b) 7.482

[a] 5 mM peptide; pH = 3.0; 5 °C.

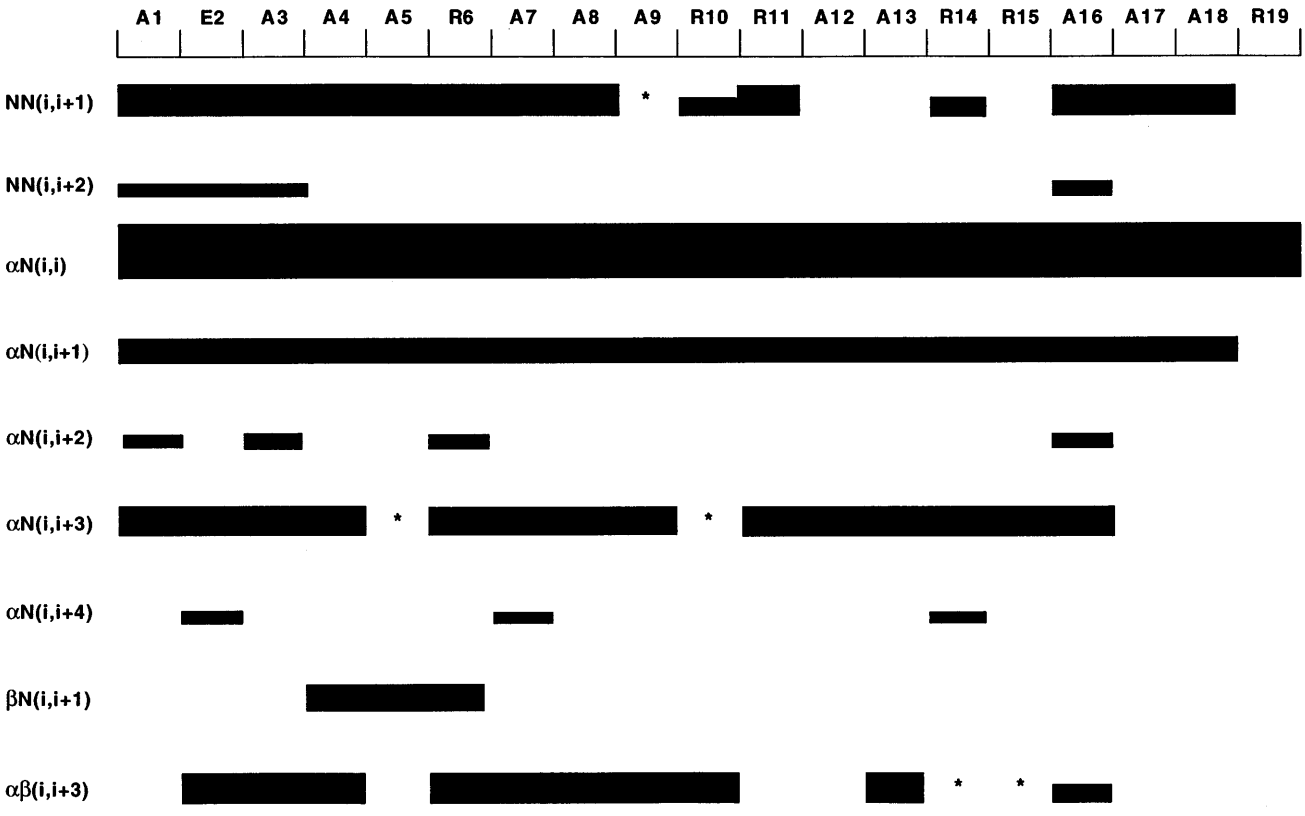

Figure 6. Plots of NOEs obtained from BASHD-ROESY spectra measured using the conditions given in the captions to Figs 4 and 5. The asterisks indicate resonance overlap. The width of the lines indicates the relative magnitude of the NOEs.

to be complementary. For example, with the increased resolution in the F_1(NH) dimension in Fig. 3(B), it is evident there are two cross peaks in the 8.3 ppm (F_1)–4.10 ppm (F_2) region, whereas with the increased resolution in the F_1 ($C_\alpha H$) dimension in Fig. 4B, three cross peaks are apparent in the 4.22–4.26 ppm (F_1)–8.22 ppm (F_2) region.

The $C_\alpha H$–NH region was assigned completely using the BASHD-ROESY spectrum obtained by apodization of the $C_\alpha H$ band-selective time-domain data with a 60° shifted sine-bell weighting function in both the F_1 and F_2 dimensions. The $C_\alpha H$–NH region of the resolution-enhanced BASHD-ROESY spectrum with cross peaks identified is shown in Fig. 5. The chemical shifts of the other carbon-bonded protons were then determined from cross-sections taken from BASHD-TOCSY spectra measured with band selection in the NH and $C_\alpha H$ regions. Resonance assignments are reported in Table 1.

As indicated in Fig. 5, resolution in the BASHD-ROESY spectrum is sufficient also to detect weaker, longer range NOEs due to the secondary structure of the peptide, e.g. the numerous $C_\alpha H_i$–NH_{i+3} NOEs identified in Fig. 5. The observed NOEs are summarized in Fig. 6. The sequential NH_i–NH_{i+1} NOEs, the relative intensities of the NH_i–$C_\alpha H_i$ and NH_i–$C_\alpha H_{i+1}$ NOEs and the longer range NH_i–$C_\alpha H_{i+2}$ and NH_i–$C_\alpha H_{i+3}$ NOEs taken together indicate that the peptide adopts an helical secondary structure in solution.[23,24] This result is consistent with circular dichroism data for the peptide.[17]

CONCLUSIONS

The results presented here demonstrate the dramatically increased resolution in 2D ROESY spectra measured with the BASHD-ROESY pulse sequence and the application of the BASHD-ROESY experiment to the complete assignment of difficult-to-assign ^{1}H NMR spectra of peptides. Resolution can be improved in the $C_\alpha H$–NH region by measuring BASHD-ROESY spectra with ω_1-band selection and ω_1-homonuclear decoupling in either the $C_\alpha H$ or the NH region. However, in the example presented here, the greatest increase in resolution is achieved with ω_1-band selection and ω_1-homonuclear decoupling in the $C_\alpha H$ region owing to the greater width of the multiplets on the $C_\alpha H$ axis. The results presented here suggest that the BASHD-TOCSY and BASHD-ROESY experiments should significantly reduce the need for peptides labelled with ^{15}N and/or ^{13}C in peptide NMR.

Acknowledgements

This research was supported in part by National Institutes of Health Grants GM37000 and HL56588 and by a grant from the University of California Biotechnology Research and Education Program. Funding for the Varian UnityInova 500 spectrometer was provided in part by NSF-ARI Grant 9601831. The authors thank H. Huang for synthesis of the peptide and V. V. Krishnamurthy for his assistance with the band-selective experiments.

REFERENCES

1. R. Brüschweiler, J. C. Madsen, C. Griesinger, O. W. Sørensen and R. R. Ernst, *J. Magn. Reson.* **73**, 380 (1987).
2. J. Cavanagh, J. P. Waltho and J. Keeler, *J. Magn. Reson.* **74**, 386 (1987).
3. R. Brüschweiler, C. Griesinger, O. W. Sørensen and R. R. Ernst, *J. Magn. Reson.* **78**, 178 (1988).
4. H. Kessler, U. Anders, G. Gemmecker and S. Steuernagel, *J. Magn. Reson.* **85**, 1 (1989).
5. G. Otting, L. P. M. Orbons and K. Wüthrich, *J. Magn. Reson.* **89**, 423 (1990).
6. L. Emsley, P. Huber and G. Bodenhausen, *Angew. Chem., Int. Ed. Engl.* **29**, 517 (1990).
7. H. Kessler, S. Mronga and G. Gemmecker, *Magn. Reson. Chem.* **29**, 527 (1991).
8. L. Emsley, *Methods Enzymol.* **239**, 207 (1994).
9. C. Dalvit, *Magn. Reson. Chem.* **33**, 570 (1995).
10. E. Kupce and R. Freeman, *J. Magn. Reson. A* **112**, 134 (1995).
11. C. Roumestand, P. Mutzenhardt, C. Deley and D. Canet, *Magn. Reson. Chem.* **34**, 807 (1996).
12. V. V. Krishnamurthy, *J. Magn. Reson. B* **113**, 46 (1996).
13. V. V. Krishnamurthy, *Magn. Reson. Chem.* **35**, 9 (1997).
14. A. Hammarström and G. Otting, *J. Am. Chem. Soc.* **116**, 8847 (1994).
15. L. Emsley and G. Bodenhausen, *J. Magn. Reson.* **97**, 135 (1992).
16. K. Stott, J. Stonehouse, J. Keeler, T.-L. Hwang and A. J. Shaka, *J. Am. Chem. Soc.* **117**, 4199 (1995).
17. D. S. Ferran, M. Sobel and R. B. Harris, *Biochemistry* **31**, 5010 (1992).
18. D. J. States, R. A. Haberkorn and D. J. Ruben, *J. Magn. Reson.* **48**, 286 (1982).
19. A. A. Bothner-By, R. L. Stephens, J.-M. Lee, C. D. Warren and R. W. Jeanloz, *J. Am. Chem. Soc.* **106**, 811 (1984).
20. A. Bax and D. G. Davis, *J. Magn. Reson.* **63**, 207 (1985).
21. O. W. Sørensen, M. Rance and R. R. Ernst, *J. Magn. Reson.* **56**, 527 (1984).
22. M. Rance, *J. Magn. Reson.* **74**, 557 (1987).
23. K. Wüthrich, *NMR of Proteins and Nucleic Acids.* Wiley–Interscience, New York, (1986).
24. I. L. Barsukov and L.-Y. Lian, in *NMR of Macromolecules. A Practical Approach*, edited by G. C. K. Roberts, pp. 315–357. Oxford University Press, New York (1993).