Journal of the Chinese Chemical Society, 1989, *36*, 423–428

SEQUENCE DETERMINATION OF A TETRAPEPTIDE USING LONG-RANG HETERONUCLEAR SHIFT CORRELATION 2D NMR SPECTROSCOPY

FONG-KU SHI (石峰鵠), YING-CHIH LIN* (林英智) and KUNG-TSUNG WANG (王光燦)
Department of Chemistry, National Taiwan University, Taipei, Taiwan 10764 Republic of China

A tetrapeptide has been studied by means of NMR of ^{13}C in natural abundance. By the combined application of various two-dimensional $^{1}H/^{1}H$ and $^{13}C/^{1}H$ correlation techniques, the primary structure of the peptides could be unambiguously determined.

INTRODUCTION

The two-dimensional NMR method is a powerful experimental technique to explore the structure of biological molecules[1] which minimizes the necessity for chemical degradation in order to define the structure. The ^{13}C- ^{13}C connectivity pulse sequence[2] INADEQUATE provides an unambiguous method for total assignment of ^{13}C spectra of complex molecules. However because this experiment suffers from extremely low sensitivity, it is often impracticable. Long-range heteronuclear chemical shift correlation[3] offers an alternative to the INADEQUATE experiments that, being much more sensitive, is widely used for spectral peak assignments and structural elucidation. The basic sequence could employ the standard CH correlation sequence[4], with the delays Δ_1 and Δ_2 lengthened to facilitate long-range magnetization transfer; cf upper sequence of Figure 1. However, although the magnitude of the proton-carbon one-bond couplings of aliphatic carbons is about 130 Hz, couplings across two or more bonds occur with magnitudes from 0 to 25 Hz.[5] This condition has the disadvantage of a long duration of the pulse sequence. There is also considerable magnetization loss due to proton relaxation in both t_1 and the polarization delay Δ_1 and carbon relaxation in the refocusing delay Δ_2. The COLOC experiment[6] of Kessler et al modified the basic C-H correlation sequence by including the evolution of the Δ_1 delay and introducing 180° pulses which are incremented through a fixed ^{1}H evolution time, thus effecting broad-band decoupling in F_1; cf the lower sequence of Figure 1. Additional advantages result from refocusing magnetic inhomogeneities during t_1 and from the opportunity of an optimized setup of the experimental parameters via INEPT.[7]

The object of the present investigation is to use the 2D NMR spectroscopy to assign unambiguously the ^{13}C-resonances of the tetrapeptide Thr-Leu-Tyr-Tyr, including the quaternary carbonyl resonances which are important for the elucidation of the backbone conformation. So far, carbonyl resonances have been inaccessible and the proton-bearing carbons could not be unambiguously assigned on the basis of only literature data, which are not necessarily applicable to short peptide chains. The COLOC sequence is employed as the carbonyl resonances of the amino acids can be directly assigned via their two- and three-bond couplings to the α-H and β-H resonances respectively.

EXPERIMENTAL

The tetrapeptide Thr-Leu-Tyr-Tyr was synthesized by the solution method and characterized; details will be published elsewhere. NMR measurements of this tetrapeptide as a 1M-solution in DMSO-d_6 were carried out at ambient temperature (297 ±

Dedicated to Professor K.T. Wang on the occasion of his 60th birthday.

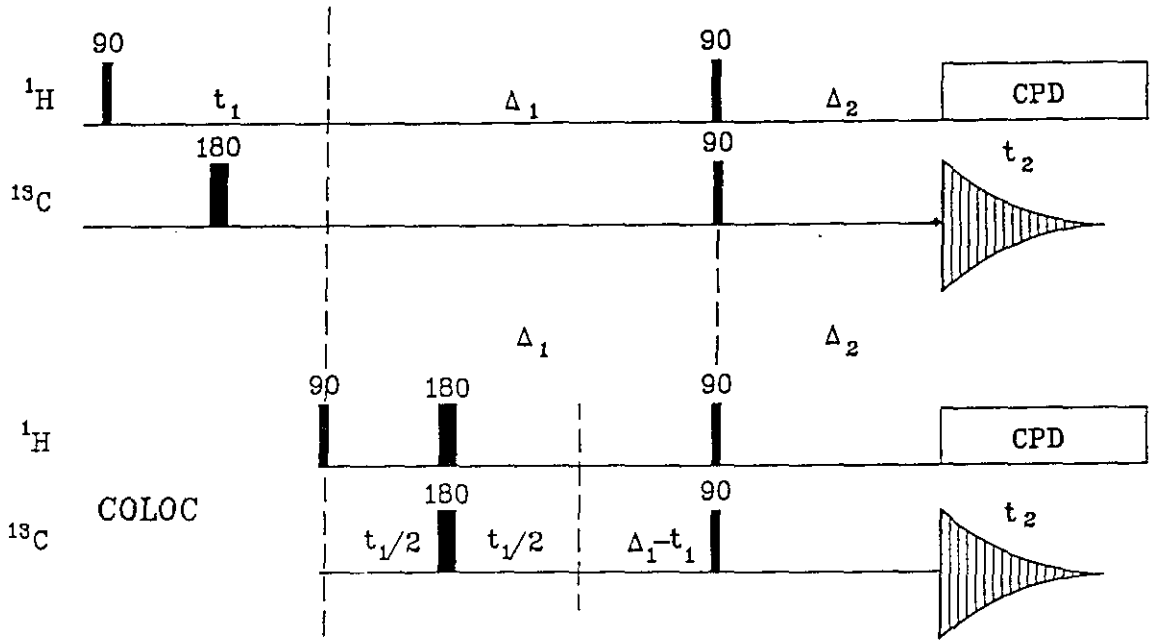


Fig. 1 Pulse sequences used for the conventional CH correlation (upper) and long-range CH correlation (lower) 2D NMR spectra.

1°K) on a Bruker AM 300 NMR spectrometer equipped with a 5-mm $^{13}C/^1H$ dual probe, operating in the Fourier-transform mode at 300 MHz for protons and 75.5 MHz for ^{13}C. The 90° proton (decoupler) and carbon pulse widths were 10.6 and 5.8 μs respectively. The decoupler pulse width was calibrated using the DEPT sequence.[8] Data collection and processing were controlled through an Aspect 3000 computer using the 1986 Bruker DISNMR software. ^1H-and ^{13}C- chemical shifts were determined relative to the residual proton (2.49 ppm)/carbon (29.5 ppm) resonances of the solvent.

One-dimensional proton spectra were recorded using a sweep width of 3300 Hz and 16 K data points. Thirty-two scans were accumulated and an exponential multiplication (line broadening factor 0.2 Hz) prior to Fourier transformation was applied. The one dimensional ^{13}C spectra were recorded in the range between −10 and 250 ppm (800 scans) using a sweep width 19630 Hz and 32 K data points. The NOE was generated during a 1s relaxation delay and broadband decoupling was performed during the acquisition time. All decoupling was done in the composite pulse decoupling mode[9] to prevent any unwanted dielectric heating. Prior to the Fourier transformation, the FID was multiplied by a gaussian function (line broadening factor −1.0 Hz, gaussian multiplication factor 0.1). This procedure proved to be superior to the more common exponential multiplication in the sense that it provided a good compromise between resolution and sensitivity.

The general parameters of the various two-dimensional NMR experiments are summarized in Table 1. Again, they can be divided into two groups, one dealing with the assignments of protonated carbons and the other allowing the quaternary carbons to be assigned. In the first category belongs the heteronuclear shift correlation experiment,[4] abbreviated XHCORR as it uses the polarization transfer from the proton to the carbon nuclei. In

Table 1 Summary of the experimental parameters used in the 2D NMR techniques for the tetrapeptide.

Parameter	Unit	COSY	XHCORR	COLOC
Sweep width in F_2	Hz	2857	12195	6024
Sweep width in F_1	Hz	1428	1229	1425
Matrix size				
before zero filling		128x2K	128x2K	128x2K
after zero filling		1Kx2K	1Kx2K	1Kx2K
Evolution time				
Initial value	μs	3	3	3
Increment	ms	0.35	0.20	0.175
Number of scans		32	128	256
Acquisition time	s	0.358	0.084	0.17
Relaxation delay	s	2.27	1.5	2.27
Other delay	ms		Δ_1=3.45	Δ_1=34.2
			Δ_2=1.72	Δ_2=30
Window function for 2D FT		S/S[a]	G/G	G/G

[a]G: Gaussian multiplication
S: Sine bell
Q: sine-bell square.

the second category belongs the COLOC experiment[6] which provides shift correlation information between unprotonated carbons and nearby protons via long-range couplings. This experiment records the F_1 domain in the H-H decoupled mode thus producing only singlets for every proton involved in the C/H correlation pattern. As outlined above for the one-dimensional carbon spectra, sensitivity versus resolution was also of concern in the two-dimensional work. The window functions (sine bell and sine bell square) with different phase shift were carefully checked to find a good compromise.

RESULTS AND DISCUSSION

Even though the resonances of peptides are better resolved in D_2O, the exchange of NH protons prohibits the sequence determination in pure D_2O. Chemical shifts of peptides for NH, α-CH and β-CH generally appear in the range of 8-9, 3.9–4.8 and 1.4–4.2 ppm respectively.[10] The first step of the assignment procedure is a H, H-COSY spectrum of the peptide in DMSO. For the spectrum obtained from the regular pulse sequence RD - 90° - t_1 – 90° – t_2,[11] the diagonal signals are commonly much more intense than the cross peaks of interest. The problem was partially alleviated by a modified sequence. In order to decrease the intensities of the diagonal peak, we used the COSY-45° pulse sequence[12] to obtain the homonuclear correlation spectrum, because magnetization is transferred mainly into connected transitions if the second pulse in a COSY experiment is smaller than 90°. The contour plot of the COSY-45 spectrum is shown in Fig. 2(A). In the tetrapeptide, crosspeaks for Thr are clearly visible at the intersection of the chemical shifts 3.88, 3.39 and 3.88, 1.60 ppm. That no cross peak between these and any of an NH is observed indicates that Thr is the N-terminus of the peptide. The spin systems of the amino acids Leu and two Tyr were similarly identified. The Leu and one Tyr show overlapping spin systems in α-protons. The β- and γ-resonances of the Leu residue also appear at similar chemical shift values. Fortunately, all expected cross peaks including those of the β-γ coupling are visible in this plot. These spectra show the complete J-connectivity within each amino acid residue without any decoupling being necessary.

In the 2D heteronuclear shift correlation spectrum, some resonances have been severely attenuated with respect to the 1D spectrum. Nevertheless, many ^{13}C resonances can be easily assigned by comparing the contour plot with the chemical shifts of the protons, see Fig. 2(B). For example, all four C_α signals are readily distinguished, and it is clear that the solvent carbon signal at 39.5 ppm is completely suppressed. The resonance of the β- carbon of the Thr residue appears at lower field than that of the α-carbon because of the presence of the OH group. Even with the small chemical shift difference of

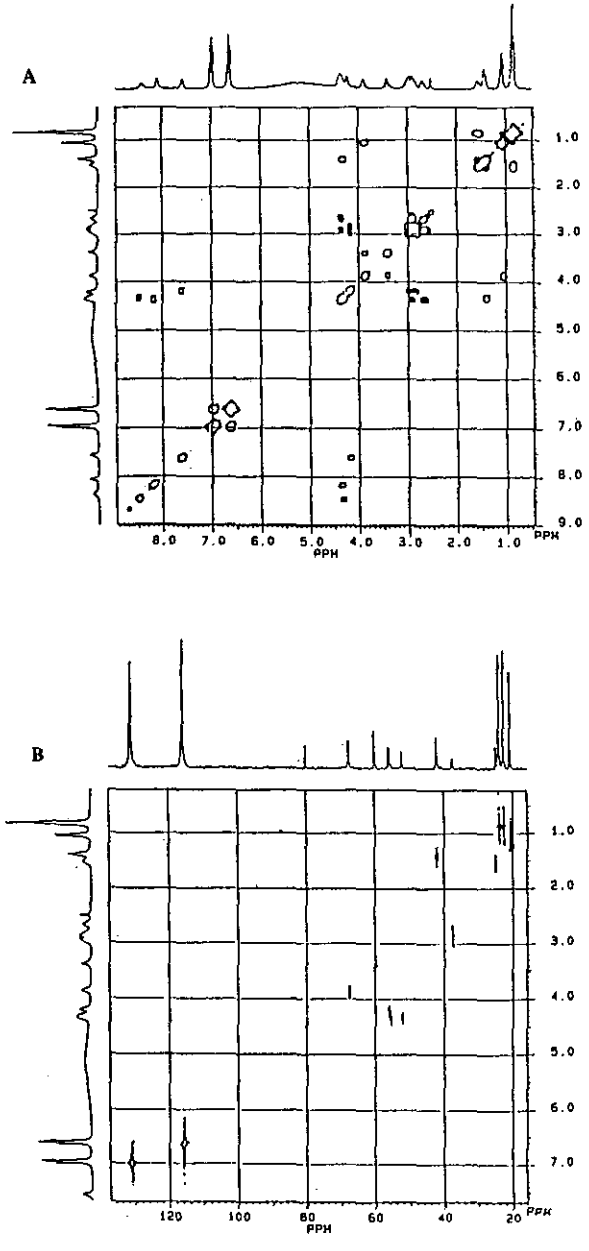


Fig. 2 Contour plot of the ^{1}H-^{1}H COSY (A) and ^{1}H-^{13}C correlation (B) spectra of the tetrapeptide.

these protons, we were able to assign three methyl groups from Leu and Thr. In some cases, when the directly attached protons have the same chemical shift, no decision might be possible on the basis of the correlation spectrum alone. Consequently, one can apply a heteronuclear relayed coherence transfer (RELAY) experiment[13] to solve the problem. Fortunately in our case, all proton resonances are well resolved. The careful analysis of the $^{13}C/^{1}H$ 2D experiment led to the complete, unambiguous assignment of all proton-bearing carbon resonances, summarized in Table 3.

Table 2 ^{1}H *chemical shifts of the tetrapeptide in DMSO at ambient temperature.*

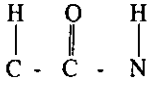
Residue	NH	α-H	β-H	others
	Tetrapeptide Thr-Leu-Tyr-Tyr			
Thr[1]	- - - -	3.39	3.88	1.60
Leu[2]	8.39	4.32	1.40	1.53, 0.84
Tyr[3]	8.07	4.35	2.67, 2.88	6.97, 6.62
Tyr[4]	7.56	4.20	2.88, 2.94	6.97, 6.62

The observation and assignment of the quaternary carbons is not as straightforward as for the protonated ^{13}C because of the long spin-lattice relaxation times, the absence of NOE sensitivity enhancements and the impracticability of polarization transfer techniques. Assigning the quaternary resonances on the basis of literature data alone is unwise because of the short peptide under investigation. We therefore applied a COLOC experiment to our sample. The COLOC technique is advantageous

Table 3 ^{13}C chemical shifts of the tetrapeptide in DMSO at ambient temperature.

Residue	C_α	C_β	others	$C_{C=0}$
	Tetrapeptide Thr-Leu-Tyr-Tyr			
Thr[1]	59.9	67.4	3.88	160.8
Leu[2]	52.1	41.8	22.5, 23.9, 24.6	172.3
Tyr[3]	55.5	37.3	115.7, 129.0	171.2
			131.0, 156.6	
Tyr[4]	56.0	37.3	115.7, 129.0	174.7
			131.0, 156.6	

for the assignment of signals of both nitrogen and quaternary carbon. This experiment provides C/H shift correlation via long-range couplings with H-H decoupling in the F_1 domain for improved sensitivity. Specifically, the magnetization transfer is directed via $^3J_{H\text{-}H}$ couplings and $^1J_{C\text{-}H}$ couplings successively to the adjacent carbon leading to the detection of the next neighbors of a carbon. The H-H decoupling yields a homonuclear "decoupled" proton spectrum from the projection of COLOC onto F_1. The carbonyl region of the resulting two-dimensional contour plot of such an experiment, carried out with the parameter set COLOC in Table 1, is shown in Fig. 3(A).



$^2J_{HCC}$ and $^2J_{HNC}$

The assignment of the carbonyl signals is possible if one considers that cross peaks are observed at the proton positions of the NH of the adjacent amino acid and of the α-CH proton of a particular amino acid. Hence the coupling to the carbonyl carbon provides the spectroscopic link between adjacent amino acid residues, and sequence analysis of peptides is possible if the α- proton and NH proton signals are resolved. Only the carbonyl group at the lowest field (174.7 ppm) shows no coupling to an NH proton, but is coupled to both α- and β protons of Tyr. This resonance is assigned to the carbonyl group of the acid terminus of the peptide. The NH (7.56 ppm) of this terminal Tyr and α- CH (4.35 ppm) of the second Tyr are both coupled to the carbonyl resonance at 171.2 ppm, see Fig. 3(B). This condition yields the connectivity Tyr-Tyr as the C-terminus of the peptide. By means of this principle, the carbonyl groups of Leu and Thr are easy to assign because of their distinct cross peaks in the NH and $C_\alpha H$ region (connectivity Try-Leu and Leu-Thr). This procedure allowed a complete

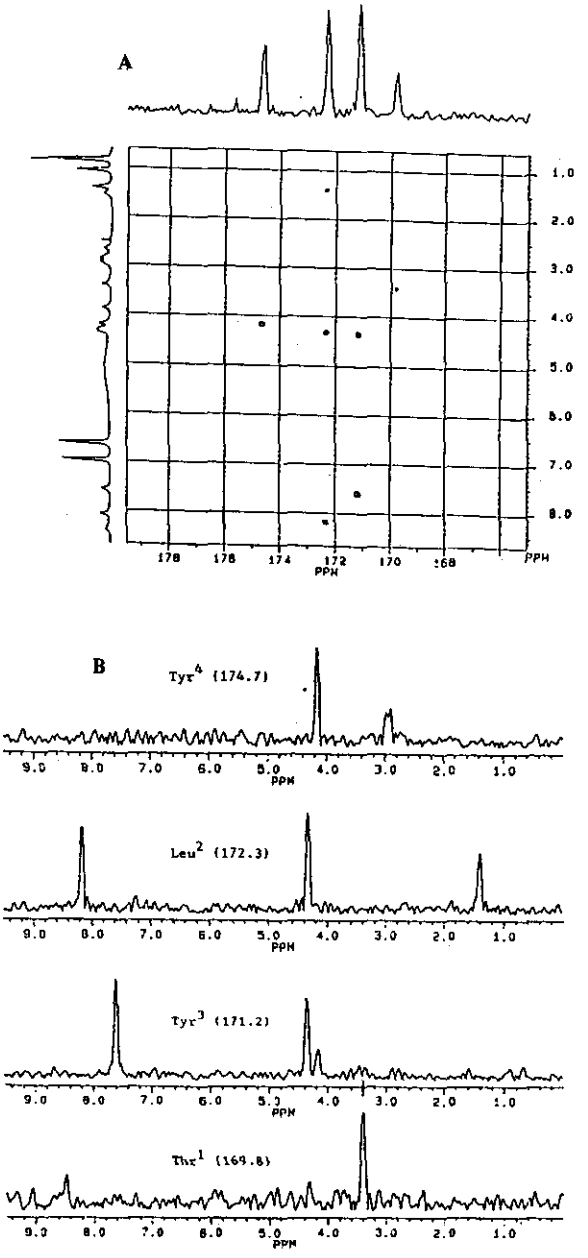


Fig. 3 Contour plot of the long range ^1H-^{13}C correlation spectra of the tetrapeptide (A) and its cross-section (B). Only the carbonyl region is shown.

sequencing of the tetrapeptide. The complete sequence thus obtained from these fragments is Thr-Leu-Tyr-Tyr.

The tetrapeptide studied is relatively simple.

The non-degradable sequencing technique of long-range heteronuclear 2D NMR spectroscopy relies on the well resolved resonances of α-CH and NH peaks. As the number of peptides increases, one can expect more overlapping resonances; then other complimentary techniques[14] may become necessary.

ACKNOWLEDGMENT

This research has been supported by the National Science Council of Republic of China.

Received January 26, 1989

Key Word Index– 2D NMR; Oligopeptide; C,H Correlation.

REFERENCES

1. Bax, A. *Two dimensional Nuclear Magnetic Resonance in Liquids* Delft University Press: delft, **1984**. (b) Wuthrich, K. *NMR of proteins and Nucleic Acids* Wiley- Interscience, New York, **1986**.
2. (a) Bax, A.; Freeman, R.; Kempsell, S. P. *J. Amer. Chem. Soc.* **1980** *102*, 4849. (b) Bodenhausen, G.; Vold, R. C.; Vold, R. R. *J. Magn. Reson.* **1980** *37*, 93. (c) Bolte, P.; Klessinger, M.; Wilhelm, K. *Angew. Chem.* **1984**, *96*, 149.
3. Hull, W. E. in *Two Bimensional NMR Spectroscopy* Croasmum, W. R.; Carlson, R. M. K. Eds,; VCH Publisher, New York **1987** *p. 199.*
4. (a) Freeman, R.; Morris, G. A. *J. Chem. Soc. Chem. Commuun.* **1978**, 684. (b) Muller, L.; Ernst, R. R. *Mol. Phys.* **1979**, *38*, 963.
5. (a) Levy, G. C.; Lichter, R. L.; Nelson, G. L. *"Carbon-13 Nuclear Magnetic Resonance Spectroscopy." 2nd.* Ed. **1980**. (b) Levy, G. C. *"Topics in Carbon-13 NMR Spectroscopy."* Vol. 4, Wiley-Interscience, New York **1984**.
6. Kessler, H.; Griesnga, C.; Zarbock, J.; Loosli, H. R. *J. Magn. Reson.*, **1984**, *57*, 331.
7. Burum, D.P.; Ernst, R. R. *J. Magn. Reson.* **1980**, *39*, 163.
8. Doddrell, D. M., Pegg, D. T.; Bendall, M. R. *J. Magn. Reson.* **1982**, *48*, 323.
9. Levitt, M. H. *Progress in NMR Spectrscopy* **1986**, *18*, 61.
10. Gross, K. H.; Kalbitzer, H. R. *J. Magn. Reson.* **1988**, *76*, 87.
11. (a) Jeener, J. Ampere International Summer School, Basko, Polji, Yugoslavia. **1971**. (b) Kumar, A.; Wagner, G.; Ernst, R. R.; Wuthrich, K. *Biochem. Biophys. Res. Commun.* **1980** 96, 1156. (c) Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976**, *64*, 2229.
12. Bax, A.; Freeman, R. *J. Magn. Reson.*, **1981** *44*, 542.
13. (a) Kessler, H.; Bernd, M.; Kogler, H.; Zarbock, J.; Sorensen, O. W.; Bodenhausen, G.; Ernst, R. R. *J. Amer. Chem. Soc.* **1983**, *105*, 6944. (b) Bolton, P. H. *J. Magn. Reson.*, **1982**, *48*, 336.
14. Ernst, R. R.; Bodenhausen, G.; Wokaun, A. *Principles of Nuclear Magnetic Resonance in One and Two Dimensions* Clarendon Press, Oxford, **1987**.