*Journal of Biochemical and Biophysical Methods*, 1 (1979) 135—143



# CROSS-PEPTIDE BOND $^{13}C$—$^{15}N$ COUPLING CONSTANTS BY $^{13}C$ AND $J$ CROSS-POLARIZATION $^{15}N$ NMR *

C. NIU [1], R.D. BERTRAND [2], H. SHINDO [1] and J.S. COHEN [1]

[1] *Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20014, and* [2] *Department of Natural Sciences, University of Michigan—Dearborn, Dearborn, MI 48128, U.S.A.*

(Received 4 December 1978; accepted 15 March 1979)

Comparative $^{13}C$—$^{15}N$ coupling constants are reported for the linear dipeptide tBoc-L-[U-$^{13}C$]Ala-[$^{15}N$]GlyOMe and the corresponding cyclic diketopiperazine, both in dimethylsulfoxide (DMSO) and, upon removal of the tBoc group, in water solutions. Spectra were obtained by $^{13}C$ NMR and by the first application of $J$ cross-polarization (JCP) $^{15}N$ NMR, which greatly reduces the time required to accumulate $^{15}N$ NMR spectra. In DMSO there was evidence for the formation of complexed species which were not present in water. The values obtained for the cross-peptide bond coupling constant $^{2}J_{^{13}C_\alpha - ^{15}N}$ were consistently less (by 2.2 Hz in DMSO, 4.3 Hz in water) for the cyclic than for the linear peptide, which may be related to the cross-peptide bond conformation. The $^{15}N$ resonance for the cyclic peptide was shifted only 2 ppm downfield from the linear peptide chemical shift value in both solvents.

Keywords: $^{13}C$ NMR; $^{15}N$ NMR; peptide bond; coupling constant.

## INTRODUCTION

The structural dependence of two-bond $^{13}C$—$^{15}N$ coupling constants has been described for a series of amides [1] and amines [2]. By contrast, three-bond couplings were either not observed or too small to be useful in conformational studies on peptides [3]. (For a recent review of $^{13}C$—$^{15}N$ coupling constants, see Wasylishen [4].)

We wished to test the utility of cross-peptide bond $^{13}C$—$^{15}N$ coupling constants as monitors of conformational effects. We used the simple stratagem of joining a $^{13}C$-labelled amino acid with an $^{15}N$-labelled amino acid by peptide synthesis. For comparison of two extreme cases we compared the

* Some of this work was presented at the American Society of Biological Chemists' meeting, Atlanta, June, 1978.

Abbreviations: DMSO, dimethylsulphoxide; tBoc, *t*-butyloxycarbonyl; JCP, *J* cross-polarization; FID, free induction decay; TLC, thin-layer chromatography; FT, Fourier transform.

linear (blocked) peptide which is in the trans conformation with the corresponding cyclic diketopiperazine which is in the cis form.

In addition to our use of the $^{13}C$ Fourier transform (FT) NMR technique, we also report the first application of the J cross-polarization (JCP) $^{15}N$ NMR technique [5]. This consists of the application of the double-resonance cross-polarization technique developed for solids, but applied in this case to liquids [6]. In liquids, spin—spin coupling (J) is responsible for the magnetization transfer which provides the name for the technique. Since the effective signal enhancement by this method is determined by the ratio of the gyromagnetic ratios of the nuclei irradiated (in this case $\gamma_H/\gamma_N = 9.9$), and since no negative NOE factors are produced in the method, this technique is especially appropriate for observation of $^{15}N$ NMR spectra. With the capability of avoiding the long repetition times normally associated with $^{15}N$ $T_1$ values, enhancements of up to 350 in time can be expected by JCP $^{15}N$ NMR over the standard technique [5]. With the application of both $^{13}C$ and $^{15}N$ NMR observation, consistent values of $^{13}C—^{15}N$ coupling constants were obtained.

## EXPERIMENTAL

### *Materials*

[$^{15}N$]Glycine (60%) was purchased from KOR Isotope, and L[U-$^{13}C$]-alanine (90%) was obtained from the Los Alamos Scientific Laboratory.

#### *HCl-glycine methyl ester*

0.5 g [$^{15}N$]glycine was suspended in dry methanol, and the solution was cooled to 0°C in an ice-water bath for 15 min. After dry hydrogen chloride had been bubbled into the solution for 10 min at 0°C, the methanol solution was stirred at room temperature for additional 60 min. After evaporating the solvent, crystals were formed, and dried with a vacuum pump overnight. However, TLC showed that the product was contaminated with approx. 10% unreacted glycine.

#### *tBoc[U-$^{13}C$]AlaOH*

This was synthesized by the method of Schnabel [7]. The yield was 95%.

#### *tBoc[U-$^{13}C$]Ala-[$^{15}N$]GlyOMe*

This was prepared by the mixed anhydride method. The compound could not be cyrstallized; nonetheless, TLC showed that a single spot on silica gel plate with $CHCl_3/CH_3OH$ (93 : 7). The yield was 85%. To remove the tBoc group from this product it was dissolved in ethyl acetate and dry HCl was bubbled for 10 min at 0°C, then stirred at room temperature for 15 min and the solvent was evaporated.

*Cyclo(L-[U-$^{13}$C]Ala-[$^{15}$N]Gly)*

This was prepared by the method of Nitecki et al. [8]. The cyclic peptide was ninhydrin-negative but gave a positive result with chlorine reagent; TLC showed a single spot with $CHCl_3/CH_3OH$/acetic acid (85 : 10 : 5) and ethanol/water/aqueous ammonia (7 : 3 : 1) as solvents. The yield was 40%.

*NMR methods*

$^{15}$N NMR spectra were obtained using the JCP pulse technique [6] as well as by the conventional pulse FT technique. This JCP method involves applying a $\pi/2$ pulse at the proton frequency followed by a 90° phase-shifted spin-locking pulse. RF radiation is simultaneously applied to the $^{15}$N spins during the spin-locking pulse. The magnitudes of the RF field strengths are adjusted so that $\gamma B_1^{^{15}N} = \gamma B_1^{^{1}H}$. During observation of the FID's, noise decoupling was applied to the protons. The spectrometer used in these experiments operated at 2.3 tesla, with an observing $^{15}$N frequency of 10.13 MHz with $\gamma B_1$ = 3 kHz. Samples of approx. 80 mg in 0.5 ml were contained in 5-mm NMR tubes. Optimum or near-optimum cross-polarization times were used in obtaining the $^{15}$N NMR spectra. These were determined from equations given earlier [6] and measured $^{15}$N—$^{1}$H one-bond J couplings. Chemical shifts were determined relative to an external sample of a saturated solution of $^{15}$N-enriched ammonium chloride in acidified water. $^{15}$N NMR spectra were also obtained at 27.3 MHz by the conventional probe FT NMR method with a partially home-built spectrometer equipped with a Bruker magnet operating at 6.4 tesla and a Nicolet 1180 computer. Proton noise decoupling was used and 10 000—14 000 pulses were accumulated with a 3 s recycle time.

$^{13}$C NMR spectra were also obtained using the same superconducting-magnet spectrometer at 67.9 MHz. Resolution was 32 768 real points in 15 kHz, with a recycle-time of 2.16 s and a $\pi$ pulse of 20 $\mu$s. Either 500 or 1000 transients were collected with no exponential filtering. Samples from the JCP experiments were diluted to 1.3 ml and placed in 10-mm NMR tubes, containing an anti-vortex device.

## RESULTS AND DISCUSSION

Several attempts have been made to determine the potential utility of $^{15}$N—$^{13}$C coupling constants for conformational studies of peptides [1—4]. In all cases the $^{15}$N and $^{13}$C nuclei considered were within the same amino acid residue. We have measured cross-peptide bond coupling constants by using $^{13}$C- and $^{15}$N-enriched amino acids coupled by standard peptide synthesis methods. In order to ascertain if conformational effects could cause changes in the values of the cross-peptide $^{15}$N—$^{13}$C coupling constants, we compared the linear dipeptide and cyclic diketopiperazines in two solvents.

The values of the coupling constants determined are shown in Table 1. Only one- and two-bond couplings were observed, no three-bond couplings could be resolved. The $^{15}$N NMR spectra obtained by the JCP method for

TABLE 1

DIPEPTIDE $^{13}C$—$^{15}N$ COUPLING CONSTANTS

| Compound [a] | Solvent | NMR method | Coupling constant (Hz) [b] | | | |
|---|---|---|---|---|---|---|
| | | | $^{2}J_{^{13}C_{\alpha}-^{15}N}$ | $J_{^{13}C_{0}-^{15}N}$ | $J_{^{13}C_{0}-^{13}C_{\alpha}}$ | $J_{^{13}C_{\alpha}-^{13}C_{\beta}}$ |
| Cyclo(Ala-Gly) | DMSO | JCP $^{15}N$ | 6.8 | 15.0 | — | — |
| | | $^{13}C$ | 6.8 | 14.9 | 51.0 | 36.6 |
| | $H_2O/^{2}H_2O$ (4 : 1) | $^{13}C$ | 5.0 | 16.4 | 51.3 | 35.4 |
| tBoc-Ala-GlyOMe | DMSO | JCP $^{15}N$ | 9.0 | 14.4 | — | — |
| | | $^{13}C$ | 8.9 | 14.2 | 53.0 | 35.5 |
| Ala-GlyOMe | $H_2O/^{2}H_2O$ (4 : 1) | $^{13}C$ | 9.3 | 16.6 | 52.5 | 33.9 |

[a] Ala was uniformly labelled approx. 90% $^{13}C$. Gly was approx. 60% $^{15}N$.

[b] Errors are $< \pm 0.2$.

the two peptides shown in Figs. 1 and 2 were readily analyzed to give two coupling constants. The $^{13}C$ spectra of the same samples were naturally more complex due to the presence of [U-$^{13}C$]Ala, but were readily analyzed for the various $^{15}N—^{13}C$ and $^{13}C—^{13}C$ coupling constants (Fig. 3). The consistency of the $^{15}N—^{13}C$ coupling constants obtained by both methods is quite striking, and helps to confirm the coupling patterns shown.

While the one-bond $J_{^{13}C_0—^{15}N}$ coupling constant showed little variation the two-bond $^2J_{^{13}C_\alpha—^{15}N}$ showed a significant change between the linear and cyclic dipeptides in both solvent systems. Although it is quite likely that these differences, of 2.2 Hz in DMSO and 4.3 Hz in water solutions, represent conformational effects, other considerations must be taken into account. Thus, it is clear by a comparison of the spectra of the peptides in DMSO and water that some form of complexation is occurring in DMSO giving rise to extra peaks and more complex spectra (Fig. 4). While these extra peaks remain sharp for the cyclic peptide they are broadened for the blocked linear peptides (Figs. 2 and 4). In addition, from the $^{13}C$ chemical shifts in DMSO compared to those in water (Table 2) it is clear that different modes of complexation with DMSO are occurring in each case. Thus, the carbonyl-$^{13}C$ resonance, relative to the same compound in water is shifted downfield by approx. 3 ppm for the cyclic peptide and upfield by approx. 1.5 ppm in the linear case. It should be noted that in order to dissolve the linear peptide in aqueous solvent it was necessary to deblock the amino group by removing the tBoc blocking group. This too may have an effect on

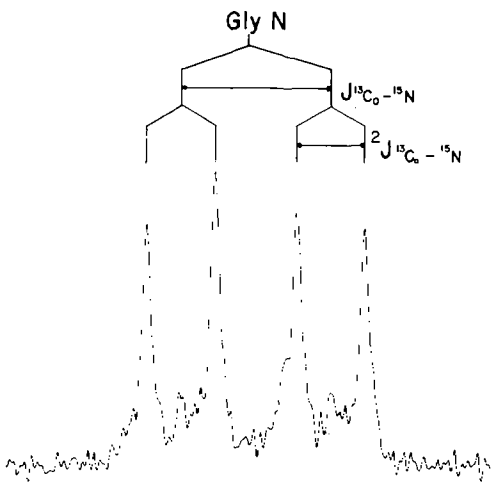


Fig. 1. JCP $^{15}N$ NMR spectrum of cyclo([U-$^{13}C$]Ala-[$^{15}N$]Gly) in DMSO. Shown in a 48.3 Hz portion of a 500 Hz spectrum obtained by Fourier transformation of 1412 signal-averaged FID's digitized into 8192 points. The FID's were produced using the JCP pulse sequence [6] with a 10 ms cross-polarization time, repeated every 9.19 s. Chemical shift values are given in Table 2.

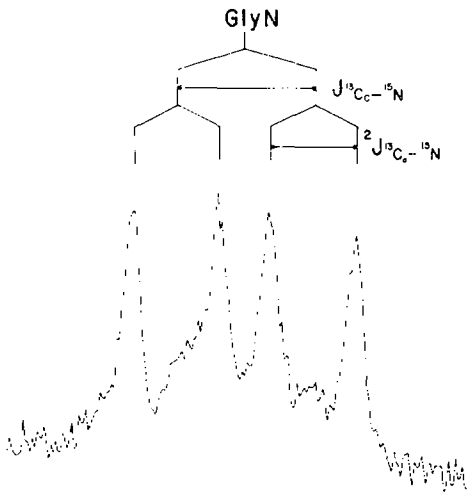


Fig. 2. JCP $^{15}N$ NMR spectrum of tBoc-[U-$^{13}$C]Ala-[$^{15}$N]GlyOMe in DMSO, Shown is a 44.2 Hz portion of a 500 Hz spectrum obtained by Fourier transformation of 256 signal-averaged FID's digitized into 8192 points. A 0.2 Hz exponential line broadening was applied before transformation. The FID's were produced by using the JCP pulse sequence [6] with an 11 ms cross-polarization time, repeated every 9.19 s.

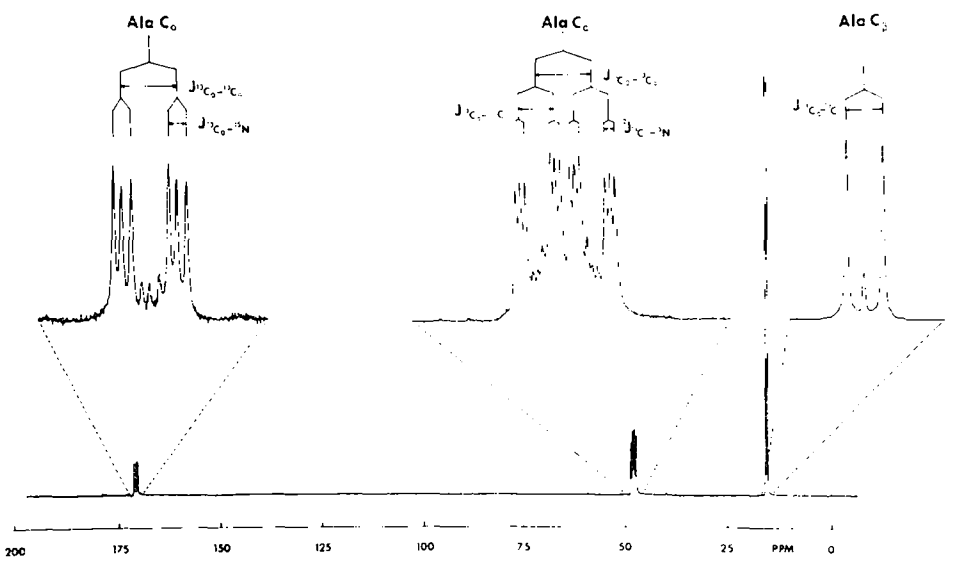


Fig. 3. $^{13}$C NMR spectrum of [U-$^{13}$C]Ala-[$^{15}$N]GlyOMe in water obtained at 67.9 MHz. The insets are expansions of the resonances for each $^{13}$C-enriched atom showing the splitting pattern.

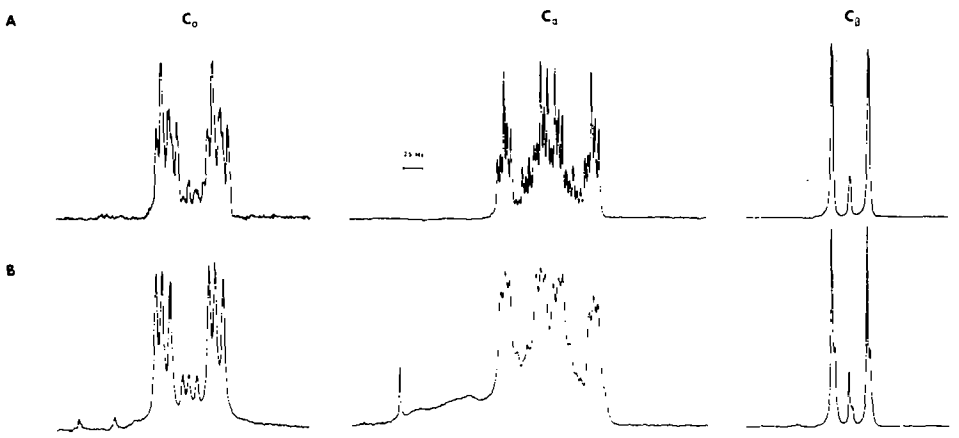


Fig. 4. Differences in the $^{13}C$ resonances for the cyclic (upper) and linear blocked (lower) peptides in DMSO at 67.9 MHz. These should also be compared to the appropriate $^{13}C$ resonances in Fig. 3.

the various chemical shifts, although it is not likely to affect greatly the carbonyl-$^{13}C$ chemical shift. It should be noted that the formation of hydrogen-bonded peptide—DMSO complexes has been described previously [9]. Thus, the difference in the $^2J_{^{13}C_\alpha-^{15}N}$ coupling constants might be ascribed to the apparent differences in complexation of DMSO between the cyclic and linear peptides. On the other hand, the results in aqueous solution, with no evidence of complexation or other species formed, give an even larger difference for the $^2J_{^{13}C_\alpha-^{15}N}$ values. At the present time the precise origin of the electronic changes in the $^{13}C-^{15}N$ bonds giving rise to these differences is not known. It should be noted, however, that the angle subtended by the $N-C_0-C_\alpha$ atoms is generally considered to be 120° [10]. The values of the

TABLE 2

CHEMICAL SHIFT VALUES OF SYNTHETIC PEPTIDES

| Compound | Solvent | Chemical shift (ppm) [a] | | | |
|---|---|---|---|---|---|
| | | $^{13}C_\alpha$ | $^{13}C_\beta$ | $^{13}C_\sigma$ | $^{15}N$ |
| Cyclo(Ala-Gly) | DMSO | 48.46 | 17.51 | 167.73 | 80.6 |
| | $H_2O$ | 49.5 | 17.73 | 170.65 | — |
| tBoc-Ala-GlyOMe | DMSO | 48.33 | 17.02 | 172.23 | 78.5 |
| Ala-GlyOMe | $H_2O$ | 48.36 | 15.64 | 170.67 | — |

[a] $^{13}C$ chemical shifts are in ppm downfield from internal $^{13}CH_3CN$. $^{15}N$ chemical shifts were obtained using the JCP technique and are in ppm downfield from external $^{15}NH_4Cl$.

$^{13}C—^{13}C$ coupling constants obtained were consistent with those reported previously [11].

Apart from the coupling constants described above $^{15}N$ chemical shifts were also measured. The $^{15}N$ NMR spectra obtained by the JCP method on the small peptides were accumulated approximately an order of magnitude more quickly than by the conventional pulse FT NMR method, for approximately the same S/N. However, the differences in field strength and sample size preclude a more exact comparison of total accumulation time in each case. Similarly, although absolute values of chemical shifts cannot be compared from the results of the two methods, due to the different magnet configurations, different bulk susceptibilities of the samples and different standards used, differences in chemical shifts are reasonably comparable. The $^{15}N$ resonance of the cyclic peptide was shifted only 2.1 ppm downfield from the position of the linear peptide resonance in DMSO (Table 2). At the higher frequency a consistent difference of 2.0 ppm was also found for the two peptides in water at pH 7.0, with the tBoc group removed from the linear peptide to confer solubility. This difference is quite small compared to other $^{15}N$ chemical shift effects which have been reported [12,13] *.

## SIMPLIFIED DESCRIPTION OF THE METHOD AND ITS APPLICATIONS

The JCP $^{15}N$ NMR technique enabled us to measure $^{15}N$ spectra of small peptides very readily. Comparison of cross-peptide $^{2}J_{^{13}C_{\alpha}-^{15}N}$ for $^{13}C$- and $^{15}N$-enriched linear and cyclic dipeptides in DMSO and water revealed a small but consistent difference which may reflect the cross-peptide bond conformation.

## ACKNOWLEDGEMENTS

The JCP $^{15}N$ NMR spectra were obtained while one of us (R.D.B.) was on sabbatical leave at the Naval Research Laboratory. The assistance of W.B. Moniz, A.N. Garroway and G.C. Chingas is gratefully acknowledged.

## REFERENCES

1 Lichter, R.L., Fehder, C.G., Patton, P.H. and Combes, J. (1974) Chem. Commun. 114—115

---

* The $^{15}N$ resonance of the synthetic amino-terminal peptide of ribonuclease, containing 15 amino acids with $^{15}N$ enrichment at Ala-6, in $H_2O$ at pH 2 was shifted downfield by approx. 15 ppm compared to the linear dipeptide and an $^{15}N$-enriched tripeptide. However, no significant difference (< 0.7 ppm) was observed at both $^{15}N$ NMR frequencies between the free peptide and its active complexes with ribonuclease S-protein ([$N^{15}$]-Ala-6 [1—15]RNAase S′; for preparative details see ref. 14). Since the backbone at Ala-6 is known to be in an α-helical conformation in the complex, this is contrary to the conclusion that $^{15}N$ chemical shifts are sensitive to H-bonding effects [13].

2 Berger, S. and Roberts, J.D. (1974) J. Am. Chem. Soc. 96, 6757—6758
3 Blasi, R.D. and Kopple, K. (1975) Chem. Commun. 33—34
4 Wasylishen, R.E. (1977) Annu. Rep. NMR Spectrosc. 7, 245—291
5 Bertrand, R.D., Moniz, W.B., Garroway, A.N. and Chingas, G.C. (1979) J. Magn. Reson., in press
6 Bertrand, R.D., Moniz, W.B., Garroway, A.N. and Chingas, G.C. (1978) J. Am. Chem. Soc. 100, 5227—5229
7 Schnabel, E. (1967) Ann. Chem. 702, 1476—1482
8 Nitecki, D.E., Halpern, B. and Westley, J.W. (1968) J. Org. Chem. 33, 864—869
9 Deslauriers, R., Grzonka, Z., Schaumberg, K., Shiba, T. and Walter, R. (1975) J. Am. Chem. Soc. 97, 5093—5100
10 Karplus, S., and Karplus, M. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3204—3206
11 Tran-Dinh, S., Fermandjian, S., Sala, E., Mermet-Bouvier, R., Cohen, M., and Fromageot, P. (1974) J. Am. Chem. Soc. 96, 1484—1493
12 Markowski, V., Posner, T.B., Loftus, J. and Roberts, J.D. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1308—1309
13 Lapidot, A., Irving, C.S. and Malik, Z. (1976) J. Am. Chem. Soc. 98, 632—634
14 Niu, C., Matsuura, S., Shindo, H. and Cohen, J.S. (1978) J. Biol. Chem., in press