Lett., 483–486 (1977).
(7) S. Masamune, G. S. Bates, and J. W. Corcoran, *Angew. Chem., Int. Ed. Engl.*, **16**, 585–607 (1977).
(8) (a) W. D. Celmer in "Biogenesis of Antibiotic Substances", Z. Vanek and Z. Hostalek, Ed., Publishing House of the Czechoslovak Academy of Sciences, Prague, and Academic Press, New York, N.Y., 1965, pp 99–130; (b) W. D. Celmer, *Pure Appl. Chem.*, **28**, 413–454 (1971).
(9) (a) Isolated from *P. brefeldianum* and first characterized by E. Harri, W. Loeffler, H. P. Sigg, H. Stähelin, and Ch. Tamm, *Helv. Chim. Acta*, **46**, 1235–1243 (1963); (b) H. P. Sigg, *ibid.*, **47**, 1401–1415 (1964); (c) as cyanein (V. Betina, J. Fuska, A. Kjaer, M. Kutkova, P. Nemec, and R. H. Shapiro, *J. Antibiot., Ser. A*, **19**, 115–117 (1966)); (d) as decumbin (V. L. Singleton, N. Bohonos, and A. J. Ullstrup, *Nature (London)*, **181**, 1072–1073 (1958)); (e) as ascotoxin (Y. Susuki, H. Tanaka, H. Aoki, and T. Tamura, *Agric. Biol. Chem.*, **34**, 395–413 (1970)).
(10) J. D. Bu'Lock and P. T. Clay, *Chem. Commun.*, 237–238 (1969).
(11) B. E. Cross and P. Hendley, *J. Chem. Soc., Chem. Commun.*, 124–125 (1975). [16-^{14}C]Palmitate was catabolized to [2-^{14}C]acetate before label entered **1**.
(12) Footnote in ref 11 and personal communication from Professor J. D. Bu'Lock, Jan 1977.
(13) (a) U. Handschin, H. P. Sigg, and Ch. Tamm, *Helv. Chim. Acta*, **51**, 1943–1965 (1968); (b) R. G. Coombe, P. S. Foss, and T. R. Watson, *Chem. Commun.*, 1229–1230 (1967); (c) R. G. Coombe, P. S. Foss, J. J. Jacobs, and T. R. Watson, *Aust. J. Chem.*, **22**, 1943–1950 (1969).
(14) H. P. Weber, D. Hauser, and H. P. Sigg, *Helv. Chim. Acta*, **54**, 2763–2767 (1971).
(15) The diol and diacetate analogues of **4** had molecular ion abundances too weak for satisfactory EIMS analysis.
(16) The structure of this pigment is not known; it often appeared on the third day of growth in 500-mL fermentations.
(17) Prepared according to C. R. Hutchinson and C. T. Mabuni, *J. Labelled Compd. Radiopharm.*, **13**, 571–574 (1977).
(18) Trial feeding experiments with sodium [2-^{3}H]acetate were done to determine that this method resulted in the minimal isotope dilution in labeled **1**.
(19) Of course, this oxygen also could have arisen from water.
(20) For example, the carboxyl oxygens of orsellinic acid were found to have one-half the ^{18}O enrichment present in the metabolite's phenolic groups from the incorporation of [$^{18}O_2$,1-^{14}C]acetate: S. Gatenbeck and K. Mosbach, *Acta Chem. Scand.*, **13**, 1561–1565 (1959); see also A. I. Scott and K. J. Wiesner, *J. Chem. Soc., Chem. Commun*, 1075–1077 (1972).
(21) A 4.12-fold dilution was stated in ref 1. This is an erroneous value resulting from a mistake in the original calculation method.
(22) (a) Shown by a separate feeding experiment with [1-^{14}C,2-^{2}H]acetate (^{3}H:^{14}C ratio = 9.18); **1** (^{3}H:^{14}C ratio = 2.58). (b) It was shown by Kuhn–Roth oxidation of this [^{3}H,^{14}C]-**1** that 30% of the molar radioactivity was contained in the acetate derived from C-15 and C-16. Therefore, each of the remaining C_2 units contain one-tenth of the total molar ^{3}H radioactivity.
(23) S. Samuels and B. L. Silver, *Adv. Phys. Org. Chem.*, **3**, 159–160 (1965); M. L. Bender, *J. Am. Chem. Soc.*, **73**, 1626 (1951).
(24) This could occur by reduction of $^{18}O_2$ to $H_2{}^{18}O_2$ followed by the disproportionation of $H_2{}^{18}O_2$ to $H_2{}^{18}O$. Cf. H. R. Mahler and E. H. Cordes, "Biological Chemistry", 2nd ed., Harper and Row, New York, N.Y., 1971, Chapter 15.
(25) R. W. Kelley and P. L. Taylor, *Anal. Chem.*, **48**, 465–467 (1976), and references cited therein.
(26) A relevant example of this type of experiment is the determination of the biological source of the cyclopentanol oxygens of PGE_1: B. Samuelsson, *J. Am. Chem. Soc.*, **87**, 3011–3013 (1965).
(27) Apparently a small leak was present in the system.
(28) O. Hayaishi, "Molecular Mechanisms of Oxygen Activation", Academic Press, New York, N.Y., 1974, pp 48–51.
(29) An analogous proton-initiated cyclization (i → 4-deoxy-iii) is a reasonable alternative hypothesis.
(30) P. D. G. Dean, P. R. Ortiz de Montellano, K. Bloch, and E. J. Corey, *J. Biol. Chem.*, **242**, 3014 (1967); J. D. Willett, K. B. Sharpless, K. E. Lord, E. E. van Tamelen, and R. B. Clayton, *ibid.*, **242**, 4182 (1967).
(31) Cf. ref 7 and (a) J. W. Corcoran, *Dev. Ind. Microbiol.*, **15**, 93–100 (1973); (b) A. Rosse and J. W. Corcoran, *Biochem. Biophys. Res. Commun.*, **50**, 597–601 (1973).
(32) E. J. Corey and J. W. Suggs, *Tetrahedron Lett.*, 2647–2651 (1975).
(33) K. Bieman, "Mass Spectrometry; Organic Chemical Applications", McGraw-Hill, New York, N.Y., 1962, pp 204–250.

Conformational Analysis by Nuclear Magnetic Resonance Spectroscopy: ^{15}N NMR of a Cyclic Pentapeptide

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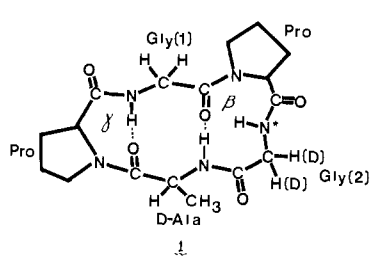
Abstract: The cyclic pentapeptide *cyclo*-(Gly(1)-Pro-Gly(2)-D-Ala-Pro), which has been shown by ^{1}H and ^{13}C NMR to incorporate both β and γ turns, has been used as a model system to explore the use of ^{15}N NMR to analyze the conformations of peptides. Assignments of ^{15}N resonances to specific amino acids have been made by analogy with similar peptides and confirmed by ^{15}N labeling. Nitrogen chemical shifts of the peptide, which is soluble in a wide variety of solvents, are sensitive to solvent changes. In water, two conformations corresponding to different cis–trans configurations of the peptide bonds are present. By means of ^{15}N labeling and an analysis of ^{15}N chemical shifts the involvement of Gly(1) in this cis–trans isomerism has been established. These results indicate substantial utility for ^{15}N NMR in the conformational analysis of peptides.

The cyclic pentapeptide *cyclo*-(glycyl-L-prolyl-glycyl-D-alanyl-L-prolyl), **1** [*cyclo*(Gly(1)-Pro-Gly(2)-D-Ala-Pro)],



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has been proposed on the basis of ^{1}H and ^{13}C NMR data[2] to adopt a rigid conformation containing two distinct types of intramolecular hydrogen bonds, one forming a seven-membered ring γ turn[3] and one forming a ten-membered ring β turn.[4]

The primary purpose of the present work was to explore the use of ^{15}N NMR in determining conformations of polypeptides, and the cyclic pentapeptide **1** provides a model system with favorable solubility characteristics and well-defined conformational populations. The procedure was to obtain spectra in a variety of solvents and to assign the ^{15}N resonances to specific amino acid residues. Solvent shifts were interpreted in terms of the relative strengths of hydrogen bonds and the results correlated with the structure of the peptide as indicated by ^{1}H and ^{13}C NMR, circular dichroism,[2] and X-ray crystallography.[5]

Experimental Section

The cyclic peptide[2b] is soluble in acetonitrile, water, chloroform,

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Table I. ^{15}N Chemical Shifts of *cyclo*-[Gly(1)-Pro-Gly(2)-D-Ala-Pro] (Relative Peak Areas in Parentheses)

solvent	chemical shifts, ppm[a] prolines	D-alanine	glycine(2)	glycine(1)	repetition rate, s
CH_3OH	237.8 (0.67)	252.7 (1.00)	266.7 (0.64)[c]	268.4 (1.81)	5
	237.8 (0.32)	252.7 (1.00)	266.7 (0.90)[c]	268.4 (1.20)	1
$CHCl_3$[b]	237.8 (0.33)	254.9 (1.00)	269.6 (2.08)[c]		5
	240.5 (0.37)				
7.9% acetone 92.1% $CHCl_3$[b]	238.3 (0.91)	254.9 (1.00)	269.2 (1.19)	270.0 (1.42)	4
50% acetone 50% $CHCl_3$[b]	238.6 (0.16)	255.1 (1.00)	269.5 (0.99)	270.7 (1.07)	4
75% acetone		254.8 (~1)	269.4 (~1)	270.7 (~1)	1
25% $CHCl_3$[b]		254.8 (1.00)	269.2 (0.73)	270.3 (1.21)	4
H_2O[d]		249.3	265.1	264.6	3
			265.6	267.0	

[a] Chemical shifts in parts per million upfield from 1.0 M $H^{15}NO_3$ in D_2O. [b] Gly(2) dideuterated at α carbon. [c] Broad. [d] Gly(2) 3% enriched with ^{15}N.

methanol, dimethyl sulfoxide, and, to some extent, acetone. All spectra were run at the same concentration (0.1 M) by dissolving 900 mg of the peptide (mol wt 381) in 22 mL of solvent. Two different samples were used, one dideuterated at the α (methylene) carbon of Gly(2) and the other enriched (3%) with ^{15}N at the Gly(2) amide nitrogen (see **1**). The ^{15}N spectra were obtained with a Bruker WH-180 NMR spectrometer at 18.25 MHz in 25-mm o.d. spinning sample tubes containing a 5-mm diameter concentric tube filled with a 1.0 M solution of enriched $H^{15}NO_3$ in D_2O which provided both the reference and the lock signals. Chemical shifts are reported in parts per million upfield from $H^{15}NO_3$ and are considered accurate to ±0.3 ppm. Samples were proton noise decoupled at 3.5 W, the pulse angle was 30°, and the pulse repetition rate was varied from 0.8 and 5 s. Adequate signal to noise ratios could be obtained in from 5 to 12 h.

Results

The ^{15}N resonances of the three different amino acids of this peptide fall in a 33-ppm range with no overlap. In model *N*-acetyl tripeptides, the chemical shifts of the amide nitrogens of Gly, Ala, and Pro (1 M Me_2SO solutions) are 271, 255, and 249 ppm, respectively,[6] of Gly(2) in *N*-acetyltriglycine 264.6 ppm (aqueous solution),[7] and of C-terminal Gly and C-terminal Ala 258 and 245 ppm, respectively (0.2 M aqueous solutions).[8] In *cyclo*-(Pro-Gly)$_3$, the nitrogens of the glycines come at 266.4 ppm and the proline nitrogens at 243.1 ppm (0.14 M chloroform solution).[9] Finally, in gramicidin S, the Pro nitrogen resonance is at 238 ppm in methanol and 240 ppm in 4:1 dimethyl sulfoxide/methanol.[6] From these data, we have assigned the nitrogen resonances of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) in the 237–239-ppm region to proline, those in the 249–256-ppm region to alanine, and those in the 264–271-ppm region to glycine. The glycine assignment has been further confirmed by ^{15}N labeling (see below).

The spectrum of the peptide in chloroform was taken using a 5-s repetition rate. Because the proline nitrogens bear no hydrogen atoms they are expected to have longer relaxation times than the other amino acid nitrogens. At this repetition rate, the two proline peaks have the smallest areas (0.33 and 0.37).[10] The two glycine peaks overlap to give the peak at 269.6 ppm, the largest peak in the spectrum (relative area 2.08) (see Table I and Figure 1a).

In methanol, two spectra were run, one with a repetition rate of 5 s and the other with a repetition rate of 1 s. As seen in Table I and Figures 1b and 1c, the area of the proline peak decreased considerably at the faster repetition rate as expected for the longer relaxation time of tertiary nitrogen atoms. In addition, the change of solvent from chloroform to methanol caused the two proline peaks to merge and caused the glycine peak to split. Enrichment of the Gly(2) nitrogen (3.0 atom % excess ^{15}N) allows unequivocal assignment of the downfield peak of the pair to Gly(2). In addition, it is of note that in the deuterated material (Gly(2)-d_2) the downfield peak is always

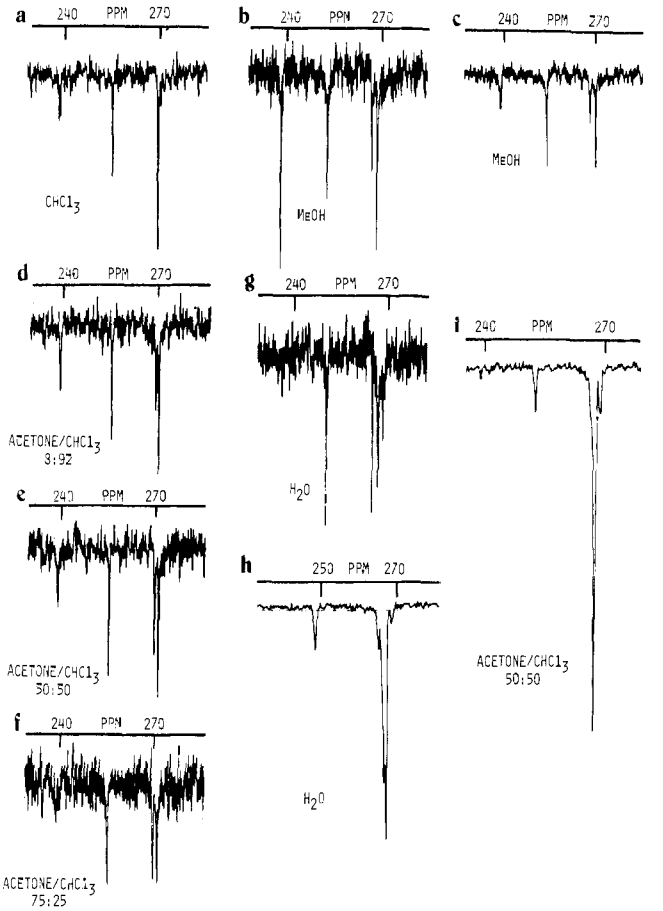

Figure 1. ^{15}N NMR spectra of 0.1 M *cyclo*-(Gly(1)-Pro-Gly(2)-D-Ala-Pro) in various solvents. Gly(2) labeled with d_2 in a–g and with ^{15}N in h and i. (a) Chloroform, 5-s repetition time (rep); (b) methanol, 5-s rep; (c) methanol, 1-s rep; (d) 7.9% acetone/92.1% chloroform, 4-s rep; (e) 50% acetone/50% chloroform, 4-s rep; (f) 75% acetone/25% chloroform, 4-s rep; (g) water, 3-s rep; (h) water, 5-s rep, ^{15}N Gly(2); (i) 50% acetone/50% chloroform, 5-s rep, ^{15}N Gly(2).

the broader of the pair because of small unresolved ^{15}N–deuterium two-bond couplings.

In aqueous solution, when a repetition time of 2 s is employed, four peaks are observed: three in the glycine region, one in the alanine region, and none in the proline region (Figure 1g). An expansion of the glycine region reveals four peaks, the central two being barely resolved. In the Gly(2) ^{15}N-enriched sample, the two central peaks in the glycine region can easily be assigned to Gly(2) (Figure 1h).

To determine the degree to which ^{15}N resonances in peptides are sensitive to the participation of the amide hydrogen-bonding interactions, a series of ^{15}N spectra of the cyclic peptide was run in chloroform containing various amounts of added acetone. The acetone is an effective hydrogen-bond acceptor and thus might be expected to perturb the resonances of the amide hydrogens which are exposed to solvent. By comparison, chloroform is a very weak hydrogen-bond acceptor solvent.

As acetone was added to the chloroform solution, the glycine resonances were observed to split apart. Over the range of acetone concentrations (0–75% v/v in chloroform), one glycine signal shifts upfield about 1 ppm and the other shifts downfield about 0.3 ppm (see Table I and Figures 1d–f). The spectrum of the ^{15}N-enriched peptide allows an assignment of the signal which moves downfield to the Gly(2) nitrogen (Figure 1i). The Gly(2) also provides the broader peak in the Gly(2)-d_2 sample. Note that the proline peaks decrease in intensity as the acetone concentration rises until, in 75% acetone/25% chloroform, the peaks are not significantly above the noise.

Discussion

The ^{15}N resonances of the three amino acids in this cyclic peptide cover a range of 33 ppm, and even though an individual resonance can vary as much as 5 ppm in different solvents, there is no overlap of the chemical-shift range for each amino acid. Assignments could reliably have been made by reference to simple model compounds, but were confirmed in the case of glycine by ^{15}N labeling.

The ^{15}N chemical shifts are obviously sensitive to both environment and conformation. Witness the glycine resonances which occur over a range of 264.3–270.7 ppm as a function of the nature of the solvent. Addition of acetone, a hydrogen-bond acceptor, to a chloroform solution of the peptide should perturb the amide hydrogens exposed to the acetone while those involved in intramolecular hydrogen bonds should be unaffected. Inspection of the formula of the cyclic peptide **1** reveals that the Gly(1) amide nitrogen is hydrogen bonded to the Ala carbonyl as part of the γ turn. As expected, proton spectra of **1** in chloroform on titration with acetone show little change in the Gly(1) NH resonance but a marked downfield shift of the Gly(2) NH resonance as the acetone concentration increases.[2] In contrast, the ^{15}N shift of Gly(1) moves upfield more than 1 ppm, while the Gly(2) resonance shifts downfield a bit less than 0.3 ppm, and the alanine amide nitrogen (which is involved in intramolecular hydrogen bonding) does not shift significantly as acetone is added to the chloroform solution. We have no consistent rationale for these observations.[11] Nonetheless, ^1H NMR spectroscopy indicates that no change in conformation is occurring during this titration.[2]

The gradual decrease in intensity of the proline ^{15}N peak in the acetone titration is undoubtedly due to the changes in aggregation of the cyclic peptide which result in decreasing the effective molecular weight along with a concomitant increase in the spin–lattice relaxation times of the proline nitrogens.

In aqueous solution, four peaks are observed in the glycine region of the ^{15}N-enriched Gly(2) spectrum: a small peak at 264.6 ppm, a large shoulder at 265.1 ppm, a large peak at 265.6 ppm, and a small peak at 267.0 ppm (see Figure 1h). Clearly, the two large peaks, separated by 0.5 ppm, come from the ^{15}N-enriched Gly(2) and the two small peaks, separated by 2.4 ppm, come from the Gly(1). The ^{13}C spectrum indicates that the cyclic pentapeptide, which exists as an all-trans conformer in most solvents, exists in water in equilibrium with a conformation which contains one cis peptide bond (the trans/cis ratio is about 4:1). Molecular models suggest that, for steric reasons, the Gly(1)–Pro bond is more likely to isomerize than the D-Ala–Pro bond.[2] The present study shows the Gly nitrogen shifts to vary markedly between the two conformers with a larger chemical-shift difference between the Gly(1) cis and trans peaks (264.6 and 267.0 ppm) than between the Gly(2) cis and trans peaks (265.1 and 265.6 ppm). This supports strongly the involvement of Gly(1) in the peptide-bond isomerization.

The wide chemical-shift range for different amino acids and the sensitivity of amide nitrogen shifts to solvent and to changes in molecular conformation in this model peptide indicate that ^{15}N NMR is a useful addition to the tools used to investigate the solution structure of peptides.

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References and Notes

(1) (a) Mount Holyoke College; Fellow of the John Simon Guggenheim Foundation, 1975–1976. (b) Amherst College. (c) California Institute of Technology.
(2) (a) L. G. Pease and C. Watson, Proceedings of the 5th American Peptide Symposium, June 1977, M. Goodman and J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., in press; (b) L. G. Pease and C. Watson, *J. Am. Chem. Soc.*, **100**, 1279 (1978).
(3) G. Nemethy and M. P. Printz, *Macromolecules*, **5**, 755 (1972).
(4) C. M. Venkatachalam, *Biopolymers*, **6**, 1425 (1968).
(5) I. L. Karle, *J. Am. Chem. Soc.*, **100**, 1286 (1978).
(6) G. E. Hawkes, E. W. Randall, and C. H. Bradley, *Nature (London)*, **257**, 767 (1975).
(7) A. Lapidot and C. S. Irving, *J. Am. Chem. Soc.*, **99**, 5488 (1977).
(8) T. B. Posner, V. F. Markowski, P. Loftus, and J. D. Roberts, *J. Chem. Soc., Chem. Commun.*, 769 (1975).
(9) D. Giannini, personal communication.
(10) To facilitate comparisons, peak areas were arbitrarily normalized so that the ^{15}N resonance of alanine always has area 1.00 in a given spectrum.
(11) The peptide is known to be involved in intermolecular association at the concentration used in chloroform. It is possible that addition of acetone disrupts peptide–peptide associations, and that the electron density of the peptide bond shifts concomitantly. The changes in chemical shift of the nitrogen resonances may be in part reflecting this indirect solvent effect.