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PROTON MAGNETIC RESONANCE SPECTRA OF SOME PEPTIDES OF L-LEUCINE AND GLYCINE

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Abstract PMR spectra of all the di-, tri-, tetra- and pentapeptides of glycine containing a single L-leucyl residue have been recorded in D_2O at 60 Mc/s. Two of the corresponding hexapeptides and three peptides containing two L-leucyl residues were also studied. At least four types of glycyl methylene protons can be distinguished by their chemical shifts but only three types of α-methine leucyl protons. The protons of a glycyl residue next to the N-terminal residue have a characteristic chemical shift and are sometimes inequivalent. The results are discussed in terms of proton chemical-shift changes in an amino acid or peptide on acylation or amidation and also on variation of pH

INTRODUCTION

IN RECENT ORD studies,[1-3] some unexpected features were found in the curves of a number of di-, tri-, tetra- and pentapeptides of L-leucine and glycine which contained a single L-leucyl residue. The unusual aspects of the results may be summarized as follows. For the tetra- and pentapeptides, minima in the ORD curves were observed when the L-leucyl residue occupied the first internal position (i.e. adjacent to the N-terminal residue),[1,2] or the C-terminal position.[2,3] The four N-terminal leucyl compounds in neutral solution as zwitterions gave a set of closely related and featureless curves.[2] However at low pH, with a - COOH group on the C-terminal residue, the rotations decreased from their respective zwitterionic values by amounts which were linearly related to the peptide chain-length.[3]

These optical effects may result from conformational factors operating on the chromophores adjacent to the asymmetric C atoms in the peptides concerned, and it seemed that the PMR spectra of the compounds might also reveal the effect of such factors. In fact we can find no obvious correlation between the ORD and the PMR results. However, the PMR spectra themselves are of interest and they are presented here.

RESULTS AND DISCUSSION

Since the ORD data were obtained with aqueous solutions, D_2O was used as solvent for the PMR studies. In D_2O the peptides retain their dipolar ionic form and only protons attached to carbon are revealed in the spectra; those linked to amino and amido nitrogen are exchanging rapidly with D_2O and contribute to the HDO peak. The results are collected in Tables 1 and 2. The assignments in Table 1 for the glycyl methylene protons have been made by noting the systematic absences produced by incorporating L-leucyl or L-alanyl residues and by extension of Sheinblatt's assignments for glycylglycine and glycylglycylglycine.[4] His assignments were made on the basis of the changes in chemical shift as the pH was varied through the amino and carboxyl pK regions.

TABLE 1. CHEMICAL SHIFTS IN c/s AT 60 Mc/s OF GLYCYL METHYLENE PROTONS IN PEPTIDES OF GLYCINE AND OF GLYCINE WITH *L*-ALANINE, *L*-VALINE, OR *L*-LEUCINE

	Peptides	Glycyl residue					
		N-terminal	Internal				C-terminal
			1st	2nd	3rd	4th	
1	Glygly	231					228
2	Gly-L-ala	230					
3	Gly-L-val	232					
4	Gly-L-leu	229					
5	L-Alagly						227*
6	L-Valgly						230, 226.5*
7	L-Leugly						228.5, 225*
8	Glyglygly	233.5	241				226
9	Glygly-L-leu	233	240.5				
10	Gly-L-alagly	230.5					224
11	Gly-L-leugly	231.5					224
12	L-Alaglygly		240.5				225.5
13	L-Leuglygly		240, 239*				225
14	Glyglyglygly	234	242.5	238.5			225.5
15	Glyglygly-L-leu	233.5	241	236			
16	Glygly-L-leugly	232.5	241				224
17	Gly-L-leuglygly	231.5		237			225.5
18	L-Leuglyglygly		241.5	238			225
19	Glyglyglyglygly	233.5	243		239, 238		225.5
20	Glyglyglygly-L-leu	233	242.5		238, 236		
21	Glyglygly-L-leugly	233	241.5	238			224
22	Glygly-L-leuglygly	234	242		237		226
23	Gly-L-leuglyglygly	232			238(4H)		227
24	L-Leuglyglyglygly		243		240, 239		226
25	L-Leuglyglygly-L-leu		243		240, 237.5		
26	Glygly-L-leuglyglygly	233	242			238.5(4H)	225.5
27	Gly-L-leuglyglyglygly	231.5			238.5(6H)		225.5
28	Gly-L-leuglygly-L-leugly	231			237(4H)		223.5
29	L-Leuglyglygly-L-leuglyglygly		243 (1st internal)		238.5 (8H, other internals)		227

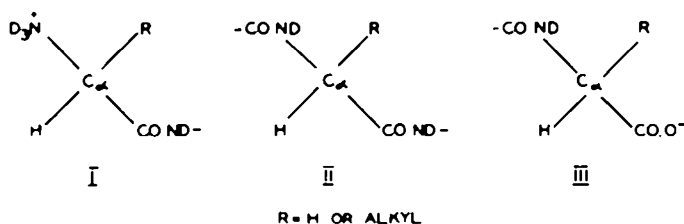
* These glycyl protons are inequivalent. The values listed are the frequencies of the two central members of the AB quartet in each case. See text for discussion of inequivalence. With L-alagly, splitting of 0.6 c/s was observed upon expansion.

TABLE 2. CHEMICAL SHIFTS, IN c/s, AT 60 mc/s OF α-METHINE and SIDE-CHAIN PROTONS IN PEPTIDE ZWITTERIONS

Peptide	Chemical shift			
	αCH	βCH multiplet*	CH_2· CH multiplet*	Side-chain —CH_3
Gly-L-ala	251·5 (J = 7)			80 (J = 7)
Gly-L-val	243·5 (J = 5·5)	123		54·5, 52·5 (J = 7)
Gly-L-leu	250·5 (J = 7·0)		98, 93	54 (J = 4)
L-Alagly	246·5 (J = 7·0)			92·2 (J = 7·5)
L-Valgly	228·5 (J = 6·0)	130		62·0 (J = 6·5)
L-Leugly	240 (J = 7·0)		105, 101	56·5 (J = 5·0)
Glygly-L-leu	253 (J = 7·0)		98, 93·5	55·5, 52
Gly-L-alagly	265 (J = 7·0)			84 (J = 7·0)
Gly-L-leugly	264 (J = 7·0)		101, 96	56, 52·5
L-Alaglygly	249·5 (J = 7·0)			93·0 (J = 7·0)
L-Leuglygly	θ		106, 102	57·0 (J = 5·0)
Glyglygly-L-leu	252 (J = 7)		97, 93	54·5, 51
Glygly-L-leugly	264 (J = 7·5)		101, 96·5	58, 55·5, 52·5
Gly-L-leuglygly	261·5 (J = 7·5)		101, 96·5	58·5, 56·5, 53
L-Leuglyglygly	θ		106·5, 102	57 (J = 6·5)
Glyglyglygly-L-leu	251·5 (J = 7)		97·5, 93·5	54·5, 51
Glyglygly-L-leugly	264·5 (J = 7·3)		101, 96·5	58, 55, 52·5
Glygly-L-leuglygly	262 (J = 7·5)		101, 97·0	58, 56, 53·5
Gly-L-leuglyglygly	264 (J = 7)		101, 97	56·5, 53·0
L-Leuglyglyglygly	θ		107, 102·5	57 (J = 5·5)
Gly-L-leuglyglyglygly	263 (J = 7)		101·5, 96·5	57, 52

* Complex multiplet; values are approximate centre positions.
θ Triplet masked by internal CH_2 resonances.

If the chemical shift of a proton attached to C_α in a peptide amino acid residue is determined mainly by the electronegativities of the three other attached groups and the magnetic anisotropies of these groups, then one might expect three distinct types of C_α protons, namely those of N-terminal (I), internal (II), and C-terminal residues (III).



The spectrum of gly_3 is in accord with this expectation. However, the spectra of gly_4 and gly_5 show that at 60 Mc/s up to five individual methylene resonances can be distinguished and that the internal category must be subdivided. In fact with all the tetra- and higher peptides the glycyl methylene protons of the first internal position are easily distinguished from those of the other internal glycyl residues. Sometimes the second and third internal glycyl protons are well-enough separated to be resolved (gly_4-L-leu, 2 c/s; gly_5, 1 c/s; L-leugly_4, 1 c/s) and sometimes not (gly-L-leugly_3).

In Table 2 the chemical shifts for the α-methine proton and side-chain protons of the residues other than glycine are listed. As with the glycyl residues, the N-terminal, C-terminal and internal residues each have characteristic α-methine proton chemical-shift values. But in contrast to the subdivision of the glycyl internal residues, there is no obvious distinction between the first internal and other internal leucyl residues, despite the fact that an ORD minimum is associated with L-leucine in the first-internal position in the tetra- and pentapeptides. Neither is the ORD distinction between gly_2-L-leu and gly_3-L-leu reflected by any gross difference in the chemical shifts of the α-methine proton of the C-terminal residue in these two compounds.

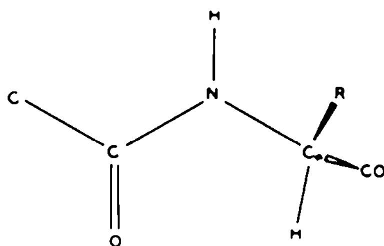
The distinctions within the internal glycyl category probably stem from the N-terminal amino acid residues, either directly through long-range shieldings or indirectly from preferred conformations involving the two flanking peptide groups and the N-terminal residue. For the gly_4 zwitterion, the internal glycyl protons are separated by 4 c/s, but this difference drops to 2·5 c/s in the anion where the terminal amino group is uncharged (Table 3). Again, if the N-terminal residue is replaced by an acetyl group to make acetyl-gly_3 then the corresponding internal glycyl residues are not distinguishable.

The assignments given in Tables 1 and 2 are self-consistent and it seems that at least four distinct glycyl environments do persist as the position in the chain of the leucyl residue is changed.

Chemical shifts on peptide formation

From a given amino acid, two types of dipeptides can be made, one by acylation of the $\cdot\dot{N}H_3$ group and the other by conversion of COO^- into a peptide linkage. Both of these conversions result in downfield shifts of the protons on C_α and these shifts (called N-acylation and C-amidation) are summarized in Table 4.

For N-acylation of an amino acid zwitterion, the shifts of the two glycyl protons (~0·2 ppm) are sharply distinguished from the shifts of the methine proton in an α-substituted residue (~0·4 ppm). The difference between the two shifts is similar to, but smaller than, the differential observed upon acylation (esterification) of primary and secondary alcohols, where the shifts are about 0·5 and 1·0 ppm respectively. The explanation advanced for the esters was that in the secondary ester the C_α proton is in or near the plane of the —OCO·C— group and subject to considerable magnetic-anisotropy deshielding.[5] These N-acylation shifts suggest a small preference for a similar conformation (IV) involving the C-terminal residue and adjacent amide group in these C_α-substituted peptides.



IV

TABLE 3 PROTON CHEMICAL SHIFTS, IN C/S, AT 60 MC/S OF AMINO ACID AND PEPTIDE CATIONS, ZWITTERIONS AND ANIONS

*Cation, *Zwitterion, or Anion	αCH	βCH multiplet†	Side-chain CH_2–CH multiplet†	Side-chain CH_3	CH_3CO	CH_2(N)[a]	CH_2(I)[b]	CH_2(C)[c]
*L-Leucine	245 (J = 6·7)		111·5, 107	61·5, 56				
*L-Leucine	223 (J = 6·7)		105·0, 100·5	60·0, 54·5				
L-Leucine	194 (J = 7·0)		90, 85	56·0, 50·5				
Acetylgly⁻					122			223·5
Acetyl-L-ala	245 (J = 7·3)			77 (J = 7·4)	117			
Acetyl-L-val	241 (J = 5·5)	121·5		54, 52 (J = 7·0)	121·5			
Acetyl-L-leu⁻	248·5 (J = 7·0)		97·5, 92·0	55·5, 52·0	120			
*Gly-L-val	260 (J = 5·5)	130		58·5 (J = 6·5)		234·5		
*Gly-L-val	244 (J = 5·5)	123		54·5, 52·5 (J = 7·0)		232		
Gly-L-val	244 (J = 5·5)	123		55·0, 53·5 (J = 7·0)		201		
*L-Valgly	233·5 (J = 6·0)	132		63·0 (J = 6·5)				245
*L-Valgly	228·5 (J = 6·0)	130		62·0 (J = 6·5)				230, 226·5•
L-Valgly⁻	189·5 (J = 6·0)	115		65·5, 64·0 (J = 6·5)				226
*L-Leugly	(242) (J = 6·5)		106·5, 102	57 (J = 5·5)				242·5
*L-Leugly⁻	240 (J = 7·0)		105, 101	56·5 (J = 5·0)				228·5, 225•
L-Leugly	204·6 (J = 7·0)		92·5, 86	54 (J = 4·5)				224·5
*L-Leuglygly	θ		107, 103	57·5 (J = 5·0)			241	241
*L-Leuglygly	θ		106·5, 102	57 (J = 5·0)			240, 239•	225
L-Leuglygly	207 (J = 7·1)		94, 88	55 (J = 4·5)			237·5	225·5

	CH_3CO	CH_2(N)[a]	1st CH_2(I)[b]	2nd CH_2(I)[b]	CH_2(C)[c]
*Glyglyglygly		234	242	240	240
*Glyglyglygly		234	242·5	238·5	225·5
Glyglyglygly		202·5	239	237·5	225·5
Acetylglyglygly⁻	123			237·5 (4H)	225·5

[a] N = N-terminal glycyl. [b] I = internal glycyl [c] C = C-terminal glycyl.

• These glycyl protons are inequivalent – see discussion in text

† Complex multiplet; values are approximate centre positions.

θ Triplet masked by internal glycyl resonances

TABLE 4. DOWNFIELD SHIFTS (PPM) OF α-METHYLENE AND α-METHINE PROTON RESONANCES OF N-ACYLATED MOIETIES AND C-AMIDATED RESIDUES ON AMIDE FORMATION

N-acylation $(D_3\overset{+}{N}C\underline{H}R\cdot CO- \rightarrow -COND C\underline{H}R\cdot CO-)$

Acylating residue							Acylated moiety
Glycyl	Acetyl	Alanyl	Valyl	Leucyl	Glycyl-glycyl	Diglycyl-glycyl	
0·23	0·16	0·18	0·23	0·21	0·21	0·20	glycine
0·16		0·16		0·15	0·16	0·15	glycyl-glycine
0·41	0·3	0·38*			0·41*		alanine
0·47	0·43						valine
0·46	0·42				0·50	0·48	leucine
0·31							alanyl-glycine
0·40					0·40	0·40	leucyl-glycine

C-amidation $(D_3\overset{+}{N}C\underline{H}R\cdot COO^- \rightarrow D_3\overset{+}{N}C\underline{H}R\cdot COND-)$

Amidated residue	Amidating moiety				
	Glycine	Alanine	Valine	Leucine	Glycyl-glycine
Glycyl	0·28	0·27	0·30	0·25	0·32
Alanyl	0·32	0·32*		0·37	
Valyl	0·22				
Leucyl	0·28			0·28	

* Values from Ref 8.

The N-acylation shifts of the dipeptides, when acylated by either an amino acid or glycylglycine, show much the same differential as the amino acids themselves. Thus a confirmation similar to (IV), in which the α-methine proton of a first or second internal C_α-substituted residue is nearly coplanar with the peptide group, may also be preferred in the higher peptides.

There is no consistent distinction between the C-amidation shifts of glycine and the other amino acids.

Effect of charge on proton chemical shifts of peptides

In D_2O the amino acids and their peptides exist in the zwitterionic form. Acidification converts $-COO^-$ to —COOD and this change results in a downfield shift of the protons attached to C_α. Removal of the positive charge on $-ND_3^+$ by addition of alkali causes an upfield shift of the C_α protons. These ionization shifts are summarized in Table 5, for amino acids, di- and tripeptides.

Apart from glycine itself, the neutralization of $-ND_3^+$ generally has a larger effect on chemical shifts than the neutralization of $—COO^-$. This differential effect is also felt by (i) side-chain protons further away from the charged site, e.g. β, γ, δ protons in a leucyl residue and (ii) protons of residues adjacent to the one whose charge is

TABLE 5 IONIZATION SHIFTS OF αCH PROTONS OF AMINO ACIDS AND PEPTIDES

	ppm downfield for $-COO^- \rightarrow COOD$	ppm upfield for $-\overset{+}{N}D_3 \rightarrow -ND_2$
Glycine	0·39	0·39
Alanine	0·39	0·46
Valine	0·38	0·53
Leucine	0·37	0·48
Glygly	0·28	0·48
Leugly	0·26	0·64
Leugly$_2$	0·27	0·65
Valgly	0·28	0·65
Glyval	0·27	0·52
Gly$_3$	0·27	0·52
Gly$_4$	0·23	0·52
Alagly	0·26*	0·62*
Glyala	0·27*	0·53*
Gly$_2$ala	0·23*	0·52*
Alagly$_2$	0·27*	0·62*

* Values from Ref. 8.

being changed, e.g. the two internal CH_2 resonances in gly$_4$ move slightly more on anion formation than on cation formation.

The difference between the carboxylate-neutralization shift and the C-amidation shift is small (about 0·1 ppm) and reflects the similarity in magnetic shielding effects of the —COOH and —CONH— groups. It also accounts for the greater number of distinct glycyl-residue positions recognized in this work in D_2O at 60 Mc/s compared with that of Bovey and Tiers.[6] Since their spectra were obtained at 40 Mc/s in trifluoracetic acid, the distinction between the internal residues (—$CH_2CO \cdot NH$—) and C-terminal residues, present as —$CH_2CO \cdot OH$, is very small. Gly$_5$ was run here in trifluoroacetic acid and showed perhaps three methylene resonances at 258, 255 and 253·5 c/s in contrast to its five α-proton resonances in D_2O.

Proton inequivalences

Mandel has reported that the glycyl CH_2 protons adjacent to the L-leucyl residue in L-leugly and L-leugly$_2$ are inequivalent in D_2O,[7] and recently van Gorkom has observed a similar inequivalence of the methylene protons in L-alanylglycine.[8] We have also observed these inequivalences for the zwitterions and, in addition, find that the glycyl protons of L-valylglycine are inequivalent (Table 1). The line-widths in the L-leugly$_3$ zwitterion indicate that the splitting of the methylene protons of the first-internal glycyl residue is less than 1 c/s.

The AB quartets from the glycyl CH_2 protons were identified in the spectra of the zwitterions of L-valgly and L-leugly and the AB chemical-shift differences were calculated as 0·17 and 0·19 ppm respectively, both with J_{AB} = 17·5 c/s. The weaker outermost lines of the quartet for L-leugly$_2$ were not clear, but those of the central

pair were separated by about 2 c/s which, with J = 17·5 c/s, indicates an AB chemical-shift difference of about 0·1 ppm. For L-leugly$_3$ the difference is less than 0·03 ppm.

Thus in the N-terminal L-leucyl peptides the chemical-shift difference between the methylene protons of the adjacent glycyl residue is dependent on the chain-length of the peptide. In trying to determine the origin of this inequivalence we have found that in the anions and cations of valgly, leugly and leugly$_2$ the inequivalence has disappeared (see Table 3), but for the valgly zwitterion the inequivalence remains up to 90°.

The chemical-shift difference between the protons of the two isopropyl Me groups in the L-valine dipeptides gives an indication of the magnetic asymmetry in another part of these molecules. Chemical-shift differences for these Me protons, and for some related valine compounds in various ionic forms, are given in Table 6. L-Valylglycine

TABLE 6. CHEMICAL-SHIFT DIFFERENCE BETWEEN METHYL GROUPS IN L-VALINE COMPOUNDS

Compound	Chemical-shift difference in c/s at 60 Mc/s		
	Anion	Zwitterion	Cation
L-Valine	4·0	3·0	1·8
Acetyl-L-valine	2·0		
L-Valgly	1·5	0	0
Gly-L-val	1·5	2·0	<0·5
L-Valine methyl ester hydrochloride	·		0

is unique among these in that the Me groups are equivalent in the zwitterionic form.

The side-chain Me protons of the leucyl peptides also show some indication of inequivalence. For two equivalent Me groups coupled with one CH proton, a Me doublet is expected and this is most nearly realized in the peptides with N-terminal L-leucine. In C-terminal leucyl peptides and in peptides containing internal leucyl residues the methyl resonances are more complex. (Table 2).

Line-width changes during ionization of ammonium group

It has been noted before that during dissociation of the NH_3^+ group of amino acids the line-width of the protons on the adjacent α-C atom increases.[9] The spectra of gly$_2$ and gly$_3$ in the pH range 5 to 11 show a considerable broadening of the N-terminal CH_2 resonance. This becomes a maximum around the pK value (Fig. 1). It seems that the line-shapes can be accounted for by exchange between the two sites, —$CH_2ND_3^+$ and —CH_2ND_2, with their respective populations changing in accordance with the pH and pK values.

Summary

In the peptides studied here, the chemical shifts of the glycyl and leucyl protons are largely understandable in terms of the immediate protonic environment. However the spectra also show a number of features which reflect the long-range environment of the protons and something of the conformational balance in solution. Whereas the ORD results pick out two special leucyl positions in the tetra- and higher peptides, the PMR results show the first internal position as special when occupied by a glycyl residue in the tetra- and higher peptides. The two techniques appear to be complementary, rather than supplementary

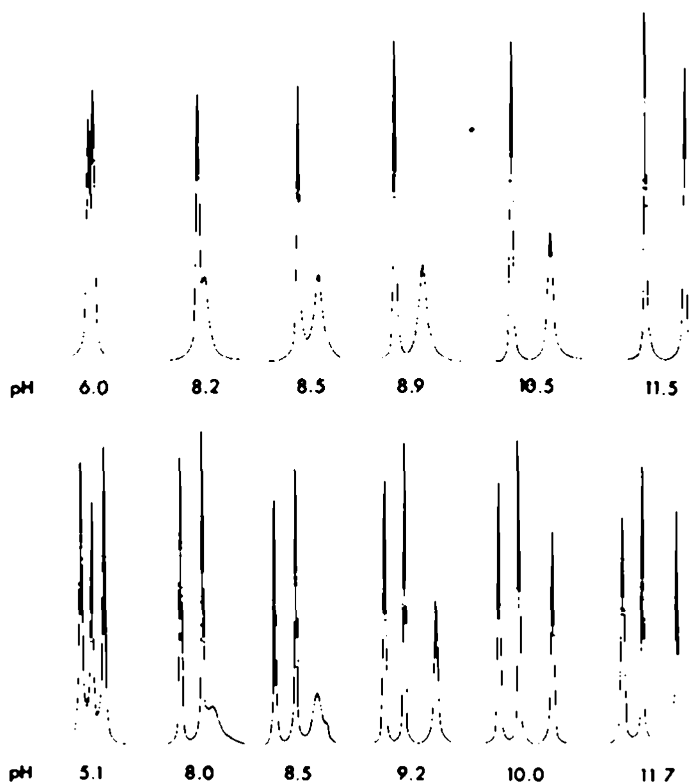


FIG. 1 60 Mc/s PMR spectra of gly_2 (upper) and gly_3 (lower) showing the variation of line-width of the N-terminal glycyl protons as the pH is changed through the ammonium pK value. Magnetic field increases to the right (see Tables 1 and 5 for chemical-shift values). The spectra were recorded in D_2O and the pH values, determined with a glass electrode, have not been corrected for this

EXPERIMENTAL

Syntheses for the new compounds discussed in the text have already been reported, together with their physical constants and rotational characteristics.[10] The PMR spectra were recorded on a Varian A-60 spectrometer in D_2O soln with sodium 3-trimethylsilylpropane-1-sulphonate as internal reference

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