^{13}C Shielding Effects at γ-Carbon Atoms in the Side-chains of α-Amino-acids

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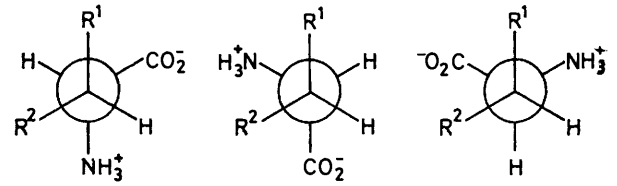
The ^{13}C shielding contributions at the γ-carbons from the α-carbon substituents in the side-chains of α-amino-acids have been estimated using data from 1-aminocyclohexanecarboxylic acid derivatives and amino-acids where the rotamer populations are known from studies of three-bond spin coupling constants. It is possible to use these values to estimate rotamer populations in amino-acids and in some cases to provide information not available from measuring $J_{\alpha\text{-}CH,\beta\text{-}CH}$ spin coupling constants. However, the estimated errors using the chemical shift method are somewhat larger than those encountered in the methods using spin coupling constants. For peptides and proteins additional errors will arise if there are shielding contributions at the γ-carbons from sources other than the immediate α-carbon substituents (such as ring current effects and electric field effects from neighbouring residues). In these cases the method will only become useful if corrections can be made for these other sources of shielding.

OVER the last few years considerable progress has been made in the application of high resolution n.m.r. techniques to the measurement of peptide conformations in solution. Most of the information has been deduced by relating measured three-bond spin–spin coupling conconstants to their appropriate dihedral angles. For example, $J_{\alpha\text{-}CH,NH}$ coupling constants provide conformational information on the backbone (φ dihedral angles) and similarly the $J_{\alpha\text{-}CH,\beta\text{-}CH}$ coupling constants lead to the side-chain conformations (χ dihedral angles).[1-7] Recently measurements of three-bond coupling constants involving ^{13}C–^{1}H[8] and ^{15}N–^{1}H[9] interactions have also been interpreted in a similar manner and have further extended the usefulness of this approach.

For large peptides where there is extensive overlap of the proton multiplets, these methods become progressively more difficult to apply. To some extent the problem can be alleviated by examining partially deuteriated peptides. However, in the case of longer peptides one must face up to the additional problem of line broadening caused by the shorter relaxation times. Clearly for such molecules information from multiplet structures is severely limited and it would be much more useful if conformational information could be obtained from chemical shift measurements.

It has been known for some time that the ^{13}C chemical shift of a carbon can be influenced by the spatial arrangements of its neighbours. Thus, for example, the shielding of a γ-carbon in a substituted alkane is markedly influenced by the orientation of the substituents on the α-carbon.[10,11] Potentially, these effects could form the basis of a method for estimating side-chain conformations about the C_α–C_β bond in amino-acids. A first step in such an analysis is the isolation of the γ-carbon shielding contributions related to the different orientations of α-carbon substituents. In this paper we have obtained these shielding contributions from studies of model compounds related to amino-acids and we have used them to explore the problems of peptide conformational analysis by this method.

The side chains of amino-acids are considered here as a rapidly interconverting mixture of the three minimum energy staggered rotamers (I)—(III) with fractional populations $p_{(I)}$—$p_{(III)}$.



(I) (II) (III)

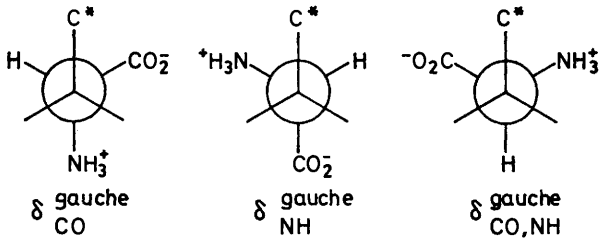
[1] W. A. Gibbons, G. Nemethy, A. Stern, and L. C. Craig, *Proc. Nat. Acad. Sci. U.S.A.*, 1970, **67**, 239.
[2] V. F. Bystrov, S. L. Portnova, V. I. Tsetlin, V. T. Ivanov, and Yu Ovchinnikov, *Tetrahedron*, 1969, **25**, 493.
[3] D. W. Urry, M. Ohnishi, and R. Walter, *Proc. Nat. Acad. Sci. U.S.A.*, 1970, **66**, 111.
[4] V. F. Bystrov, 'Spin-spin Coupling and Conformational States in Peptides,' in 'Progress in N.M.R. Spectroscopy,' eds. J. W. Emsley, J. Feeney, and L. H. Sutcliffe, Pergamon, London, 1976, vol. 10.
[5] K. G. R. Pachler, *Spectrochim. Acta*, 1964, **20**, 581.
[6] K. D. Kopple, M. Ohnishi, and A. Go, *Biochemistry*, 1969, **8**, 4087.
[7] J. Feeney, G. C. K. Roberts, and A. S. V. Burgen, 'Molecular and Quantum Pharmacology,' eds. E. Bergman and B. Pullman, Reidel, 1974, pp. 301—302.
[8] P. E. Hansen, J. Feeney, and G. C. K. Roberts, *J. Magnetic Resonance*, 1975, **17**, 249.
[9] J. A. Song, W. A. Gibbons, and E. W. Randall, *Biochemistry*, 1973, **12**, 2100.
[10] D. K. Dalling and D. M. Grant, *J. Amer. Chem. Soc.*, 1972, **94**, 5318.
[11] J. B. Stothers, 'Carbon-13 N.M.R. Spectroscopy,' Academic Press, New York, 1972.

The different orientations of the α-substituents give shielding contributions to the γ-carbon of δ_{NH}^{gauche}, δ_{CO}^{gauche}, and $\delta_{CO,NH}^{gauche}$ such that the observed shifts for the γ-carbons at R^1 and R^2 are given by equations (1) and (2) where the shielding parameters are defined as in

$$\delta R^1 = p_{(I)}\,\delta_{CO}^{gauche} + p_{(II)}\,\delta_{NH}^{gauche} + p_{(III)}\,\delta_{CO,NH}^{gauche} \quad (1)$$

$$\delta R^2 = p_{(I)}\,\delta_{NH}^{gauche} + p_{(II)}\,\delta_{CO,NH}^{gauche} + p_{(III)}\,\delta_{CO}^{gauche} \quad (2)$$

the Scheme. The shielding contributions at R^1 and R^2 are assumed to be equal [$\delta^g_{CO}(R^1) = \delta^g_{CO}(R^2)$] which is a

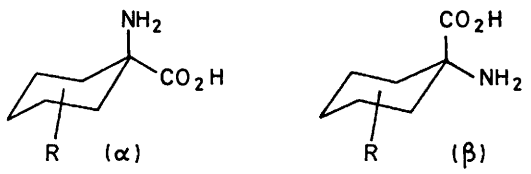


reasonable assumption when R^1 and R^2 are similar groups (CH_3 and CH_2 groups). In principle, if the component shielding contributions were available then the rotamer populations could be estimated from the observed γ-carbon shifts in amino-acids with two γ-carbon atoms.

EXPERIMENTAL

N.m.r. Measurements.—Carbon-13 proton noise-decoupled spectra were obtained at 34 °C on a Varian XL 100 spectrometer operating at 25.2 MHz in the Fourier transform mode using 8K data sets. A 2 000 Hz spectral width was used providing chemical shifts correct to ±0.02 p.p.m. The amino-acids were examined at concentrations of 5—10% by weight in D_2O solution and dioxan (1—5%) was used as an internal reference. The ^{13}C assignments were made on the basis of off-resonance ^1H decoupling experiments and the effects of ionisation on the ^{13}C chemical shifts.[12]

Materials.—All compounds other than 1-aminocyclohexanecarboxylic acids were obtained commercially and used without further purification. The methyl-substituted 1-aminocyclohexanecarboxylic acids were synthesized from the corresponding cyclohexanones by the Bucherer and Strecker reactions as described by Munday.[13] Edward and Jitrangsri[14] have shown that 1-amino-4-t-butylcyclohexanecarboxylic acids prepared in this manner have structures (α) and (β) respectively. We have found similar



structures for the methyl-substituted compounds and their ^{13}C spectra have been assigned by off-resonance decoupling experiments and the effects of ionisation on the ^{13}C chemical shifts. We have assumed that the dominant conformation is the one in which the methyl group is equatorial: the complexity of the ^1H n.m.r. spectra prevented us from substantiating this assumption using the ^1H–^1H coupling constants.

RESULTS AND DISCUSSION

Estimation of δ_{NH}^{gauche}, δ_{CO}^{gauche}, *and* $\delta_{CO,NH}^{gauche}$.—The ideal method of estimating the contributions to the shielding of the γ-carbons in the three rotamers (I)—(III) would be to freeze out the rotamers at low temperatures. However, the energy barriers to interconversion are sufficiently low that for solutions of amino-acids in methanol–isopropyl alcohol mixtures we were unable to detect any evidence of freezing out of rotamers at −120 °C. In the absence of this direct information we have considered data from amino-acids and conformationally pure cyclic molecules.

For this purpose we have measured the ^{13}C chemical shifts of the γ-carbons of valine, 2-amino-3,3-dimethylbutyric acid (t-leucine), 2-aminobutyric acid, L- and allo-L-threonine, L- and allo-L-isoleucine, and several 1-aminocyclohexanecarboxylic acid derivatives (Tables 1 and 2). The contributions to the γ-carbon shifts

TABLE 1

^{13}C chemical shifts of γ-carbons in some amino-acids

Compound	pD	Chemical shifts † R^1	Chemical shifts † R^2	Substituent shift contribution (p.p.m.) R^1	Substituent shift contribution (p.p.m.) R
L-Valine	−0.65	49.00	49.40	6.1	6.5
	5.7	48.50	49.85	5.6	7.0
	11.9	47.40	49.80	4.5	7.9
2-Aminobutyric acid	0.6	58.00		6.4	
	5.7	57.9		6.3	
	11.7	57.1		5.5	
t-Leucine	0.2	40.91		5.3	
	7.5	40.63		5.0	
	12.1	40.47		4.9	
L-Threonine	0.1	47.48		4.9	
	5.7	47.02		4.4	
	11.1	47.33		4.7	
Allo-L-threonine	0.6		49.22		6.6
	5.8		50.21		7.5
	11.2		50.03		7.4
L-Isoleucine	0.1	52.33	41.74	7.0	6.3
	7.3	51.81	42.08	6.5	6.5
	12.0	50.96	42.45	5.7	6.85
Allo-L-isoleucine	0.1	41.54	53.03	5.9	7.7
	7.3	41.03	53.19	5.4	7.9
	12.0	40.29	53.15	4.65	7.9

† Chemical shifts in p.p.m. upfield from dioxan.

from the NH_2 and CO_2H substituents were estimated by comparing the γ-carbon chemical shifts with those of corresponding carbons in reference compounds in which the NH_2 and CO_2H substituents are replaced by hydrogen atoms. If the methyl-substituted 1-aminocyclohexanecarboxylic acids shown in Table 2 are regarded as rigid structures with the methyl substituents in equatorial positions, then the ^{13}C substituent shifts of the C-3, C-5, and CH_3 carbons would be expected to be similar to the corresponding γ-C substituent shifts in the amino-acid rotamers (I)—(III). These substituent shift parameters are given in Table 3 for the three

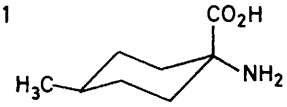
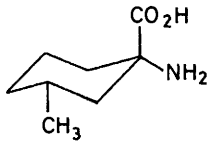
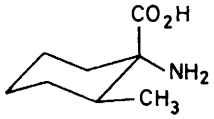
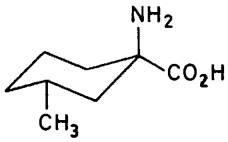
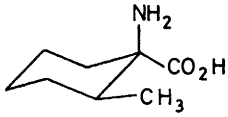
[12] J. G. Batchelor, J. Feeney, and G. C. K. Roberts, *J. Magnetic Resonance*, 1975, **20**, 19.

[13] L. Munday, *J. Chem. Soc.*, 1961, 4372.

[14] J. T. Edward and C. Jitrangsri, *Canad. J. Chem.*, 1975, **53**, 3339.

TABLE 2

^{13}C Chemical shifts of γ-carbons in some 1-aminocyclohexane carboxylic acid derivatives

No.	Compound	pD	Chemical shifts *			Substituent shift contribution (p.p.m.)		
			C-3	C-5	CH_3	C-3	C-5	CH_3
1		0.2	35.20	35.20	46.01	4.2	4.2	1.9
		6.4	35.55	35.55	46.67	4.5	4.5	2.6
		11.0	35.00	35.00	45.41	4.0	4.0	1.3
2		0.35	38.34	44.87	45.08	4.6	4.6	1.0
		7.0	37.83	44.32	44.91	4.1	4.0	0.8
		11.0	36.81	43.16	44.47	3.1	2.9	0.4
3		0.25	34.30	45.34	51.34	3.3	4.9	7.2
		6.2	34.74	44.85	51.00	3.7	4.4	6.9
		11.2	35.01	45.06	50.75	4.0	4.7	6.6
4		0.1	38.9	38.9	45.63	7.9	7.9	1.5
		7.0	37.98	37.98	45.19	7.0	7.0	1.1
		11.1	37.01	37.01	44.73	6.0	6.0	0.6
5		0.4	40.35	46.81	45.11	6.6	6.5	1.0
		6.0	39.74	46.19	44.96	6.3	5.9	0.9
		11.0	39.47	45.50	44.29	5.8	5.2	0.2
6		0.7	38.65	47.23	51.10	7.6	6.8	7.0
		6.4	38.10	46.55	51.07	7.1	6.2	7.0
		11.2	37.38	45.88	50.50	6.4	6.5	6.4

* Chemical shifts in p.p.m. upfield from dioxan.

possible combinations of the substituent ionisation states (anion, zwitterion, and cation). Because the amino-acids and reference compounds are examined in different solvents there will be some errors in determining the substituent shifts (see later). We have used these substituent shift values in conjunction with rotamer populations deduced previously from coupling constant data [8,15] to estimate the γ-^{13}C shifts for the amino-acids listed in Table 4. The calculated and observed values are only in moderate agreement indicating that the substituent shift parameters require some correction to allow for the fact that they were derived from cyclic systems. In particular, the $\delta^{gauche}_{CO,NH}$ values which are derived from consideration of CH_3 carbon shifts in cyclohexanes (3 and 6 in Table 2) are expected to be underestimated considerably.[10]

We have obtained an alternative set of parameters by combining the cyclohexane derived values with the results of regression analysis of the data for valine, threonine, and 2-aminobutyric acid using populations calculated from coupling constants (excluding the errors in the populations). From the regression analysis, five of the calculated parameters (δ^{gauche}_{NH} at all pH values and $\delta^{gauche}_{CO,NH}$ at neutral and base pH values) have small residual

TABLE 3

γ-Carbon ^{13}C shift contributions (p.p.m.) in 1-aminocyclohexanecarboxylic acids

	δ^{gauche}_{NH} [a]	δ^{gauche}_{CO} [b]	$\delta^{gauche}_{CO,NH}$ [c]
Acid	7.2 ± 0.7	4.3 ± 1.0	7.1 ± 0.1
Neutral	6.6 ± 0.7	4.2 ± 0.3	7.0 ± 0.1
Base	6.0 ± 0.8	3.8 ± 0.9	6.5 ± 0.1

[a] Estimated from C-3 and C-5 shifts in compounds 4—6 in Table 2. [b] Estimated from C-3 and C-5 shifts in compounds 1—3 in Table 2. [c] Estimated from CH_3 shifts in compounds 3 and 6 in Table 2.

[15] J. Feeney, *J. Magnetic Resonance*, 1976, **21**, 473.

errors (within ± 0.3) and their values are given in Table 5. The other parameters have larger residual errors (within ± 1.6) and it therefore seems better to use other methods to estimate these values. For the δ_{CO}^{gauche} parameters the cyclohexane derived values are of the charges. These effects are fairly small (see Table 3) and thus we have estimated the γ-carbon shift parameters for a peptide by adding to the values for the amino-acid zwitterion (see Table 5) the shifts caused by protonating the carboxy- group and deprotonating the

TABLE 4

Observed and calculated γ-carbon ^{13}C shifts in some amino-acids

	Rotamer populations						Chemical shift contributions (p.p.m.)					
Compound	pD	$p_{(I)}$	$p_{(II)}$	$p_{(III)}$	R^1	R^2	R^1(obs)	R^2(obs)	R^1(calc) *	R^2(calc) *	R^1(calc) †	R^2(calc) †
L-Valine	0.5	0.17	0.50	0.33	CH_3	CH_3	6.1	6.5	5.94	6.49	6.67	6.19
	5.7	0.17	0.59	0.24	CH_3	CH_3	5.6	7.0	5.43	6.85	6.29	6.26
	11.9	0.23	0.61	0.16	CH_3	CH_3	4.5	6.9	4.78	6.92	5.57	5.95
2-Aminobutyric acid	0.6	0.33	0.33	0.34	CH_3	H	6.4		5.89		6.21	
	5.7	0.18	0.41	0.41	CH_3	H	6.3		6.13		6.33	
	11.7	0.29	0.37	0.34	CH_3	H	5.5		5.61		5.53	
L-Threonine	0.1	0.12	0.80	0.08	CH_3	OH	4.9		5.04		6.84	
	5.1	0.21	0.72	0.07	CH_3	OH	4.4		4.72		6.12	
	11.1	0.22	0.68	0.10	CH_3	OH	4.7		4.50		5.57	
Allo-L-threonine	0.6	0.09	0.53	0.38	OH	CH_3		6.6		6.57		6.05
	5.8	0.15	0.77	0.08	OH	CH_3		7.5		7.63		6.72
	11.2	0.18	0.74	0.08	OH	CH_3		7.4		7.55		6.19
L-Isoleucine	0.1	0.12			CH_3	CH_2	7.5	6.2				
	7.3	0.12			CH_3	CH_2	6.5	6.5				
	12.0	0.24	0.56	0.20	CH_3	CH_2	5.7	6.85	4.97	6.67	5.57	5.84
Allo-L-isoleucine	0.1	0.11			CH_2	CH_3	5.9	7.7				
	7.3	0.08			CH_2	CH_3	5.4	7.9				
	12.0	0.13	0.75	0.12	CH_2	CH_3	4.65	7.9	4.63	7.59	5.77	6.11

* Using corrected substituent shifts given in Table 5. † Using substituent shifts from cyclic amino-acids in Table 3.

selected (Table 5). The $\delta_{CO,NH}^{gauche}$ (acid) parameter was estimated from the average of values obtained from using the data of t-leucine and 2-aminobutyric acid (Table 1).

TABLE 5

Corrected set of γ-carbon ^{13}C shift contributions in amino-acid side-chain rotamers

	δ_{NH}^{gauche}	δ_{CO}^{gauche}	$\delta_{CO,NH}^{gauche}$
Acid	4.8 [a]	4.3 [b]	8.5 [c]
Neutral	4.5 [a]	4.2 [b]	8.6 [a]
Base	4.1 [a]	3.8 [b]	8.8 [a]

[a] Regression analysis of Val, Thr, and 2-aminobutyric acid data. [b] Cyclohexane derived values. [c] Derived from t-leucine and 2-aminobutyric acid data.

When the substituent parameters given in Table 5 are used to calculate the γ-carbon ^{13}C shifts for the amino-acids using the rotamer populations given in Table 4, much better agreement is obtained between observed and calculated ^{13}C chemical shifts at all pH values. Discrepancies still exist and it is clear that the substituent effects at the γ-carbons also depend to some extent on the presence of other substituents on the β-carbon and also on the δ-substituents. However, while each amino-acid will have a unique set of substituent parameters, the set of values in Table 5 can be regarded as reasonable estimates for the amino-acids considered here.

From consideration of the shifts caused by acetylation of model amines and amidation of carboxylic acids * it is seen that the difference between the γ-carbon shifts of amino-acids and peptides will be due mainly to removal amino-group. This leads to the values δ_{NH}^{gauche} 4.4, δ_{CO}^{gauche} 4.2, $\delta_{CO,NH}^{gauche}$ 8.3.

We must now consider to what extent these substituent shift values can be used to provide conformational information about amino-acid side-chains.

Estimation of Side-chain Conformations.—For amino-acids with two assigned γ-carbons [16] such as valine one can deduce the rotamer populations from the γ-^{13}C chemical shifts using equations (1) and (2). This is not possible for amino-acids containing only a single γ-carbon. However, because the parameters for δ_{NH}^{gauche} and δ_{CO}^{gauche} are very similar at each pH value, an estimate of one of the rotamer populations ($p_{(II)}$ or $p_{(III)}$) can be obtained from the γ-^{13}C shift in these compounds. Since $p_{(I)}$ can be estimated from the $J_{\alpha\text{-}CH,\beta\text{-}CH}$ values then, by combining this result with that from the γ-carbon shift data, all three rotamer populations can be obtained. Table 6 gives the rotamer populations for several amino-acids calculated from the γ-^{13}C shifts and also from the coupling constant data. The overall agreement is quite good although differences of ± 0.1 are noted for some of the calculated populations.

Included in Table 6 are the valine side-chain rotamer populations for two small peptides, Lys-Pro-Val-Gly NH_2 [17] and HCO Val-Pro-Val-Gly-OMe [18] calculated from the valine γ-^{13}C shifts assuming that the γ-carbon assignments are the same as in the amino-acid; the conformations are quite similar to those in *N*-acetyl-Val NH_2.

* The differences between the ^{13}C shifts of the CH_3 carbons in $(CH_3)_3CCH_2COOH$ and $(CH_3)_3CCH_2CONHCH_3$ is 0.24 p.p.m. and the difference between $(CH_3)_3CCH_2$–NH_2 and $(CH_3)_3CCH_2$-$NHCOCH_3$ is <0.4 p.p.m.

[16] J. G. Batchelor and J. Feeney, *J.C.S. Chem. Comm.*, 1975, 503.

[17] A. F. Bradbury, A. Boicelli, and J. Feeney, unpublished results.

[18] D. W. Urry, L. W. Mitchell, and T. Ohnishi, *Biochemistry*, 1974, **13**, 4083.

Estimation of Errors.—In assessing the usefulness of this approach to conformational analysis it is necessary to consider the possible errors in the measured chemical shifts and also those in the estimated substituent shift parameters. There are problems in determining the substituent shifts because these are differences between the shifts of amino-acid resonances measured in aqueous solutions and those of reference compounds (with NH_2 and CO_2H groups replaced by protons) examined in non-aqueous media. Such problems could introduce errors of ± 0.2 p.p.m. on the measured values. If we assume that the substituent shift parameters are also in error by ± 0.5 p.p.m. we can readily estimate that the maximum errors involved in calculating the fractional populations for the amino-acids in Table 6 are: $p_{(I)}$ ± 0.22, $p_{(II)}$ ± 0.10, $p_{(III)}$ ± 0.12.

TABLE 6

Calculated rotamer populations

Compound	pD	From coupling constants $p_{(I)}$	$p_{(II)}$	$p_{(III)}$	From ^{13}C chemical shifts $p_{(I)}$	$p_{(II)}$	$p_{(III)}$
L-Valine	0.5	0.17	0.50	0.33	0.07	0.53	0.40
	5.7	0.17	0.59	0.24	0.08	0.56	0.36
	11.9	0.23	0.61	0.16	0.30	0.61	0.09
2-Aminobutyric acid	0.6	0.33	0.33	0.34			0.50
	5.7	0.18	0.41	0.41			0.45
	11.7	0.29	0.37	0.34			0.31
L-Threonine	0.1	0.12	0.80	0.08			0.06
	5.1	0.21	0.72	0.07			0.00
	11.1	0.22	0.68	0.10			0.14
Allo-L-threonine	0.6	0.09	0.53	0.38		0.56	
	5.8	0.15	0.77	0.08		0.74	
	11.2	0.18	0.74	0.08		0.71	
L-Isoleucine	0.1	0.12			0	0.34	0.66
	7.3	0.12			0	0.50	0.50
	12.0	0.24	0.56	0.20	0.05	0.61	0.34
Allo-L-isoleucine	0.1	0.11			0	0.86	0.14
	7.3	0.08			0.11	0.88	0.01
	12.0	0.13	0.75	0.12	0.05	0.82	0.13
N-Acetyl-Val-NH_2 *	~7.0	0.37			0.16	0.55	0.29
Lys-Pro-Val-Gly-NH_2 †	5.85				0.31	0.40	0.29
HCO-Val-Pro-Val-Gly-OMe ‡					0.3 0.13	0.63 0.54	0.34 0.33

* γ-C shifts 5.44 and 6.55 p.p.m. † γ-C shifts 5.48 and 5.91 p.p.m.[17] ‡ γ-C shifts (5.7 and 6.8) and (5.7 and 6.4) p.p.m.[18]

For peptides, a further error arises if the observed γ-carbon chemical shifts have contributions from sources other than the local effects of the α-carbon substituents (for example, from ring currents [19] or from electric fields associated with charged groups in neighbouring residues.[15]) Unfortunately such effects could produce shielding contributions comparable in magnitude to the differences between the γ-carbon shift parameters given in Table 5. Clearly, this approach could only be used with confidence for such molecules if corrections could be made for these other sources of shielding.

Conclusions.—The different orientations of the α-carbon substituents in amino-acid side-chains have been shown to give rise to different γ-carbon shielding parameters. These have been estimated and should prove useful in rationalising γ-^{13}C chemical shifts of amino-acids in terms of side-chain conformations. However, for peptides and proteins the possibility of shielding contributions from sources other than the α-carbon substituents considerably limits the usefulness of this method of conformational analysis.

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