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^{1}H AND ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTRA OF SOME PEPTIDES WITH FIBRINOGEN-LIKE REACTIVITY

I.D. RAE* and H.A. SCHERAGA

Department of Chemistry, Cornell University, Ithaca, New York, U.S.A.

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The ^{1}H and ^{13}C spectra of four peptides, L-Phe-Val-Arg-pNA, D-Phe-Val-Arg-pNA, L-Phe-Pip-Arg-pNA and D-Phe-Pip-Arg-pNA (pNA = p-nitroaniline, Pip = pipecolic acid residue), have been examined, and deductions made about their conformation in solution. The D-Phe peptides, which are cleaved especially rapidly by thrombin in water, have structures (in deuterated DMSO) in which the aromatic ring of the D-Phe residue is folded back over the Val or Pip residue. This arrangement is not found in the L-Phe peptides. It is proposed that this feature (in which Phe could be situated near Val and near the Arg-Gly bond of the Aα chain in the three-dimensional structure of fibrinogen) may be specially advantageous for binding to the enzyme.

Key words: blood clotting; fibrinogen-like peptides; peptide conformation; phenylalanine residue.

In blood clotting, the enzyme thrombin cleaves specific Arg-Gly bonds in fibrinogen to produce fibrin monomer, which polymerizes to form the fibrin clot (Blombäck *et al.*, 1976). We have been interested in the rates at which thrombin cleaves the Arg-Gly bond in fragments of the fibrinogen molecule and in synthetic peptides in which the sequence resembles that near the Arg-Gly bonds of the native protein (Hageman & Scheraga, 1977; Scheraga, 1977; Van Nispen *et al.*, 1977). In general, the smaller molecules react more slowly than fibrinogen with thrombin, but the rates become comparable as the size of the peptide increases.

Reactivity comparable to that of fibrinogen has been reported recently (Claeson *et al.*, 1977) for several peptides of the type Phe-X-Arg-*p*NA, in which X is either Val or Pip (pipecolic acid), and the bond being cleaved is Arg-*p*NA, where *p*NA is *p*-nitroaniline. This end group was chosen (Claeson *et al.*, 1977) so as to permit spectrophotometric monitoring of the hydrolysis reaction. The reactivity is highest when the *N*-terminal residue is an unblocked D-Phe or an *N*-benzoyl-L-Phe. In fibrinogen itself, X is Val, and Phe is quite removed (toward the *N*-terminus of the molecule) from the Val-Arg-Gly moiety, and the reactivity of the peptides having the "natural" sequence approaches that of fibrinogen only when the peptide is long enough to include the Phe residue (Van Nispen *et al.*, 1977).

We have examined the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of two very active peptides, D-Phe-Val-Arg-*p*NA and D-Phe-Pip-Arg-*p*NA, and their less active L-Phe analogs, to try to identify any conformational features that might be related to their binding to, and reaction with, thrombin.

* Present Address: Department of Chemistry, Monash University, Clayton, Victoria, Australia 3168.

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EXPERIMENTAL PROCEDURES

Materials

The peptides L-Phe-Val-Arg-*p*NA, D-Phe-Val-Arg-*p*NA, L-Phe-Pip-Arg-*p*NA, and D-Phe-Pip-Arg-*p*NA were kindly supplied as the hydrochloride salts by Drs. G. Claeson and R. Simonsson of AB Kabi, Mölndal, Sweden. The Val, Pip and Arg residues were in the L configuration.

N-Acetyl-D, L-pipecolic acid was prepared as follows. DL-Pipecolic acid (Aldrich, 0.65 g, 5 mmol) was dissolved in a mixture of triethylamine (0.51 g, 5 mmol), water (25 ml) and dimethoxyethane (25 ml). *N*-Acetoxysuccinimide (0.79 g, 5 mmol) was added over a period of 2 h while the solution was stirred at room temperature. After a further 2 h, the solution was evaporated and the resulting syrup was taken up in 6 M HCl (25 ml) and extracted with ethyl acetate (2 x 20 ml). The ethyl acetate solution was dried (Na_2SO_4) and evaporated to give the solid product (0.80 g, 4.7 mmol, 94% yield). Recrystallization from ethyl acetate gave white crystals, m.p. 139.5–140.5°. The melting point (219° decomp.) quoted by Heilbron (1965), taken from Hess & Liebrandt (1917), is incorrect because it refers to the acetate salt rather than the free acid.

Anal. Calc. for $C_8H_{13}NO_3$: C, 56.13; H, 7.65; N, 8.18%. Found: C, 56.26; H, 7.77; N, 8.13%.

NMR spectra

The ^{1}H NMR spectra were recorded at 20° for solutions of 5 mg peptide in 0.5 ml of 100% DMSO-d_6 (Aldrich) on the HF-250 spectrometer at Carnegie-Mellon University, Pittsburgh. Tetramethylsilane (TMS) was used as an internal reference (approximately 1%) and lock substance, and the spectrometer was operated in the correlation mode.

The ^{13}C NMR spectra were recorded at 32° for solutions of approximately 40 mg peptide in 0.5 ml of DMSO-d_6 or D_2O. In DMSO-d_6, the central line of the solvent resonance was used as a reference (39.6 p.p.m. from the "internal TMS" position) and, in D_2O solutions, approximately 1% of dioxane was added as an internal standard (67.4 p.p.m., also from the "internal TMS" position). The spectra were recorded on a Varian CFT-20 spectrometer, using 8000 data points, 4000 Hz sweep width, pulse angles of 50–90° and delays of 1–3 s.

RESULTS AND DISCUSSION

*L-Phe-Val-Arg-*p*NA and D-Phe-Val-Arg-*p*NA*

The ^{1}H NMR spectral data for these two peptides in DMSO-d_6 are reported in Table 1. Because of overlaps of peaks, the Val $C^\beta H$, Arg $C^\beta H_2$, $C^\gamma H_2$, guanidine NH's, and the nitroaniline NH could not be assigned. The Val $C^\alpha H$-$C^\beta H$ coupling constants were measured in difference spectra obtained by decoupling the Val NH, i.e. by inspection of the Val $C^\alpha H$ resonances in the difference spectrum obtained by subtracting the normal spectrum from the decoupled spectrum, all these operations being performed with digitized spectra stored in the computer memory (Gibbons *et al.*, 1975 and references cited therein). For the L-Phe peptide, the resonances of the phenylalanine $C^\beta H_2$ (side-chain) hydrogens were observed in the difference spectrum (i.e. the original spectrum was subtracted from the spectrum obtained by irradiation of the Phe $C^\alpha H$) and were assigned as the AB part of an ABX spin system (Pachler, 1964), where hydrogens A, B and X are defined in Fig. 1. Making the usual assumption that the only important conformations about the C^α–C^β bond of the L-Phe residue are the three staggered forms shown in Fig. 1, the observed vicinal couplings (7.8 and 5.8 Hz) were used to calculate the proportions of each rotamer present in solution (Pachler, 1964; Ogura *et al.*, 1967). These populations are: (a) 0.47; (b) 0.29; (c) 0.24; using the parameters of Feeney (1976), the corresponding values are: (a) 0.52; (b) 0.36; (c) 0.12. In the D-Phe analog, the resonances of the two C^β hydrogens are coincident, and no such analysis is possible.

The methyl hydrogens of valine are shielded by 0.2 p.p.m. in the D-Phe peptide relative to their chemical shifts in the L-Phe peptide, probably because they are close to the face of the aromatic ring of the D-Phe residue. This phenomenon has been described for a dipeptide containing an L and a D residue, e.g. L-Phe-D-X (where X = Glu, Gln, Arg, N^ϵ-Ac-Lys) (Deber & Joshua, 1972). These L-D compounds were described as having "more compact structures" than L-L diastereomers (Deber & Joshua, 1972).

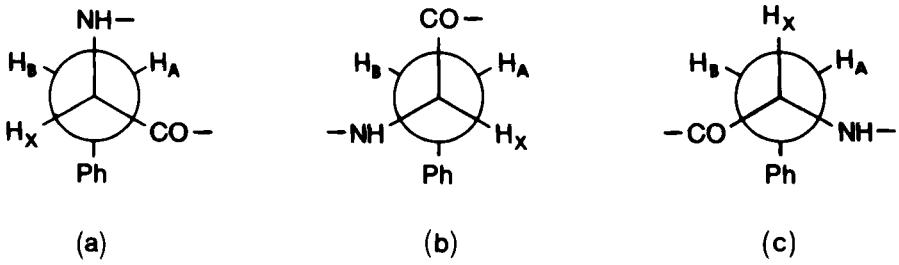

FIGURE 1

Side-chain conformations of the *N*-terminal L-Phe residue of these peptides. H_X is the C^{α} hydrogen, and H_A and H_B are the C^{β} hydrogens. According to standard nomenclature (IUPAC-IUB Commission on Biochemical Nomenclature (1970) *Biochemistry* 9, 3471), (a), (b) and (c) correspond to dihedral angles of $\chi^1 = 180°$, $-60°$, and $+60°$, and are designated t, g^-, and g^+, respectively.

This "compactness" refers to the way in which the aromatic ring of phenylalanine is folded back over the peptide chain, rather than to the shape of the backbone itself.

The C^{α}H-NH coupling constants for the two peptides fall in the range of 5.9–8.8 Hz, and this suggests that (the N–C^{α} portions of) the peptide chains are extended, or at least only a very small fraction of them are present in any other conformation (Bystrov, 1976).

These data indicate that the aromatic ring of the D-Phe residue is folded back over the Val side chain. In the other peptide, the L-Phe ring is not observed to be near the Val side chain.

In the amino acid sequence of the Aα chain of human fibrinogen, the L-Phe residue occurs nine residues before the thrombin-susceptible Arg-Gly bond (Blombäck & Blombäck, 1972; Blombäck *et al.*, 1972). The synthetic peptides with the same sequence, but with fewer than nine preceding residues, are cleaved relatively slowly by thrombin, but the reactivity undergoes a sharp increase when the Phe residue is added in the correct position (Van Nispen *et al.*, 1977). Thus, the Phe residue plays an important role, and this gives rise to speculation that thrombin might possess a large active site (Scheraga, 1977). The reactivity of D-Phe-Val-Arg-*p*NA is very high, however, whereas that of the L-Phe analog is essentially zero (Claeson *et al.*, 1977). The small active peptide has not only an "artificial" sequence, but also an "unnatural" D-Phe residue, and it might be significant that the aromatic ring of the D-Phe residue in this compound is in close proximity to the Val residue that immediately precedes the arginyl bond that is cleaved by thrombin. Thus, if there is a requirement for binding the aromatic ring, perhaps together with the Val side chain, near the active site, it can be met by the conformation of the D-Phe peptide that was deduced above, but not by its L-Phe analog. It should be noted that the amino acid sequence of the Aα chain of fibrinogen contains no other aromatic residues in the vicinity of the susceptible Arg-Gly bond, but the chain could fold so as to bring the aromatic ring close to this site. In this regard, it is interesting to note that the antithrombin activity of synthetic peptides containing phenylalanine falls off as the phenylalanine is moved even further from the cleavage site than it is in the Phe-Val-Arg sequence, and then rises as the "natural" position, some nine residues in the direction of the *N*-terminus, is approached (see Fig. 2 of Blombäck *et al.*, 1969).

The *N*-benzoyl derivatives of these two Phe-Val-Arg-*p*NA peptides show thrombin activity in the reverse sense to that of the peptides with free α-amino groups, i.e. Bz-L-Phe-Val-Arg-*p*NA is very reactive and the D-Phe analog is inactive (Claeson *et al.*, 1977). Without a careful study of their NMR spectra (the compounds not being available to us), it is difficult to say more. It would be interesting to know whether the benzoyl group is unique in producing this reversal, or whether, for example, an *N*-acetyl group would produce the same effect.

N-Acetyl-D, L-pipecolic acid (Ac-Pip-OH)

The ^{1}H and ^{13}C NMR spectra of *N*-acetyl-D, L-pipecolic acid in DMSO-d_6, in which solvent

TABLE 1

¹H spectra of peptides and model compounds in DMSO-d_6 at 20°[a, b]

	Hydrogen atom	Compound					
		L-Phe-Pip-Arg-*p*NA	D-Phe-Pip-Arg-*p*NA	Ac-Pip-OH *trans*	Ac-Pip-OH *cis*	L-Phe-Val-Arg-*p*NA	D-Phe-Val-Arg-*p*NA
Phe[c]	$C^{\alpha}H$	4.734	4.706 > 4.608[d]			4.183	4.265
	$C^{\beta}H_A$	2.938	2.96			2.956	3.032
	$C^{\beta}H_B$	3.142	2.96			3.130	3.032
	^{J}AB	13.0	–			13.7	–
	^{J}AX[e]	8.9	8.6 (Av.)			7.8	6.4 (Av.)
	^{J}BX[e]	6.2				5.8	
	aromatic[f]	7.312 ($\omega_{1/2}$ 33 Hz)	7.305 ($\omega_{1/2}$ 11.5)			7.300	7.321
Pip[g]	$C^{\alpha}H$ equat.	4.992	5.145 > 4.513[d]	5.057	4.651		
	$C^{\epsilon}H$ equat.	3.575	3.832 > 4.302[d]	3.731	4.312		
	$C^{\epsilon}H$ axial	2.938	~ 2.804 > 2.552[d]	3.146	2.474		
Val[g]	$C^{\gamma}H_3$ (1)					0.913	0.712
	$C^{\gamma}H_3$ (2)					0.938	0.733
	$C^{\alpha}H$					4.300	4.204
	$^{J}C^{\alpha}H-C^{\beta}H$					7.0	6.5
	NH					8.635	8.594
	$^{J}C^{\alpha}H-NH$					8.8	8.2

TABLE 1 (cont.)

	Hydrogen atom	Compound					
		L-Phe-Pip-Arg-*p*NA	D-Phe-Pip-Arg-*p*NA	Ac-Pip-OH *trans*	Ac-Pip-OH *cis*	L-Phe-Val-Arg-*p*NA	D-Phe-Val-Arg-*p*NA
Arg[g]	$C^{\alpha}H$	4.445	4.458			4.456	4.408
	$C^{\delta}H_2$	3.152	3.160			3.151	3.154
	$N^{\alpha}H$	8.279	8.339 8.480			8.510	8.474
	$^{J}C^{\alpha}H-NH$	7.9	6.6 > 7.3[d]			5.9	7.0
CH_3CO	CH_3			2.024	1.960		
$-NHC_6H_4-4-NO_2$	$H_{2,6}$	7.920	7.938			7.919	7.945
	$H_{3,5}$	8.222	8.228			8.226	8.230

[a] Chemical shifts (δ) are in p.p.m. downfield for internal tetramethylsilane.
[b] Coupling constants (J) in Hz.
[c] NH_3^+ resonance was too broad for positive identification.
[d] a > b means that a and b are the chemical shifts or coupling constants for the major and minor isomers, respectively.
[e] These are coupling constants between the $C^{\alpha}H$ (designated as nucleus X) and H_A and H_B, respectively.
[f] This refers to the hydrogens of the aromatic ring of Phe, which yield one signal with half-width $\omega_{1/2}$.
[g] The signals for $C^{\beta}H_2$, $C^{\gamma}H_2$ and $C^{\delta}H_2$ of Pip, $C^{\beta}H$ of Val, $C^{\beta}H_2$, $C^{\gamma}H_2$, and guanidine NH's of Arg, and nitroaniline NH were too complex and overlapped for analysis.

TABLE 2

^{13}C NMR spectra of peptides and model compounds in DMSO-d_6 and D_2O at 32°[a]

	Carbon atom	Compound							
		L-Phe-Pip-Arg-*p*NA in D_2O	L-Phe-Pip-Arg-*p*NA in DMSO-d_6	D-Phe-Pip-Arg-*p*NA in D_2O	D-Phe-Pip-Arg-*p*NA in DMSO-d_6 *trans*	D-Phe-Pip-Arg-*p*NA in DMSO-d_6 *cis*	Ac-Pip-OH in DMSO-d_6 *trans*	Ac-Pip-OH in DMSO-d_6 *cis*	Ac-NHC_6H_4–4–NO_2 in DMSO-d_6
Phe	C^{α}	54.1	54.4	54.7	54.5	53.7			
	C^{β}	36.4		37.8					
	arom. C_1	134.1	134.6	134.2	134.2				
	arom. $C_{2,6}$	130.5	128.8	130.4	130.1				
	arom. $C_{3,5}$	130.0	127.4	130.1	128.6				
	arom. C_4	128.9	127.4	129.0	127.5				
	C′	169.7	168.9	170.5	168.0	168.2			
Pip	C^{α}	52.8	52.7	52.0	49.7	55.4	51.0	56.0	
	C^{β}	27.7		27.2			26.3	26.7	
	C^{γ}	20.0		19.8			21.4	21.4	
	C^{δ}	25.5		25.4			24.8	24.2	
	C^{ϵ}	44.7	45.5	45.1			43.4	–	
	C′	173.1	170.0	173.2	171.5	171.7	172.5	172.4	
Arg	C^{α}	55.2	49.9	55.4	51.9	51.0			
	C^{β}	29.0		28.2					
	C^{γ}	24.9		24.0					
	C^{δ}	41.4	40.1	41.3					
	C^{ϵ}	157.6	157.1	157.5	157.2				
	C′	173.4	171.4	173.8	169.8	171.0			

TABLE 2 (cont.)

	Carbon atom	Compound							
		L-Phe-Pip-Arg-*p*NA in D_2O	L-Phe-Pip-Arg-*p*NA in DMSO-d_6	D-Phe-Pip-Arg-*p*NA in D_2O	D-Phe-Pip-Arg-*p*NA in DMSO-d_6 *trans*	*cis*	Ac-Pip-OH in DMSO-d_6 *trans*	Ac-Pip-OH in DMSO-d_6 *cis*	Ac-NHC_6H_4-4-NO_2 in DMSO-d_6
CH_3CO	CH_3						20.5	21.2	24.2
	CO						169.8	169.6	169.3
$-NHC_6H_4-4-NO_2$	C_1[b]	144.2	145.3	144.6	145.4				145.8
	$C_{2,6}$	120.9	119.0	121.2	119.0				119.6
	$C_{3,5}$	125.9	125.0	125.3	125.0				123.6
	C_4	144.2	142.3	144.1	142.3				142.7

[a] Chemical shifts (δ) are in p.p.m. downfield from internal tetramethylsilane (in DMSO-d_6) or from dioxane taken as 67.4 p.p.m. (in D_2O solutions).
[b] C_1 is the carbon attached to NH.

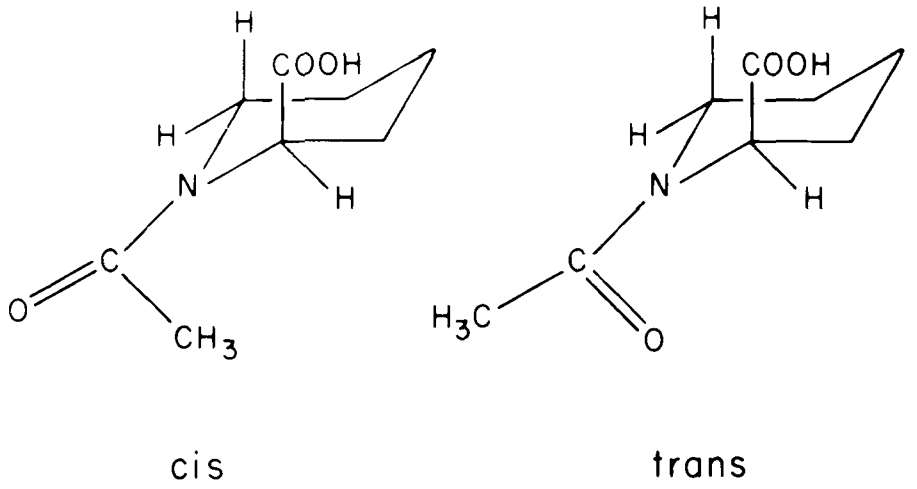

FIGURE 2
Stereochemistry of *cis* and *trans* *N*-acetylpipecolic acid.

the compound should exist as the un-ionized acid, were examined as aids in the interpretation of the spectra of the L- and D-Phe-Pip-Arg-*p*NA peptides (Tables 1 and 2). The ^{1}H spectrum of *N*-acetyl-D,L-pipecolic acid was mentioned briefly without a full analysis (Thomas & Williams, 1972; Thomas, 1976). As in the case of *N*-acetylproline compounds, both *cis* and *trans* isomers (about the Ac-N bond) (Fig. 2) exist in solution. *N*-Acetyl-D, L-pipecolic acid is thought (Thomas, 1976) to adopt a chair conformation, with the carboxyl group in an axial position, and our analysis below is in broad agreement with these conclusions. We find a ratio of *trans* to *cis* of 68 : 32, calculated from the areas of the acetylmethyl proton resonances.

In the spectrum of the major (*trans*) isomer, the $C^{\alpha}H$ signal is a broad doublet with J = 4.8 Hz. This small coupling constant definitely identified the $C^{\alpha}H$ as an equatorial (rather than an axial) hydrogen (Williams & Fleming, 1973), but we would have expected the signal to be a triplet because of coupling of the $C^{\alpha}H$ to the *two* C^{β} hydrogens. Some flattening of the piperidine ring, associated with a near-planar conformation about the nitrogen, could give a structure in which the dihedral angle between $C^{\alpha}H$ and one of the C^{β} hydrogens is close to 90°. The resulting coupling of $C^{\alpha}H$ to this particular $C^{\beta}H$ would be small, and quite possibly masked by line broadening, leaving only a doublet splitting visible at the $C^{\alpha}H$ signal. One $C^{\beta}H$ could be assigned to a broad triplet, J = 12 Hz, at 2.124 p.p.m. (not listed in Table 1) but the signal of the other $C^{\beta}H$ was obscured. The major splittings of this signal arise from geminal and axial-axial coupling (both expected to be large) but the absence of a 4.8 Hz splitting means that it is not the $C^{\beta}H$ whose coupling to $C^{\alpha}H$ is discussed above. Thus, this signal belongs to a pseudo-axial $C^{\beta}H$, and its coupling with $C^{\alpha}H$ is close to zero. The $C^{\alpha}H$ hydrogen of the γ-methyl proline residue in monamycin H_1 also shows coupling to only one $C^{\beta}H$ (Hassall *et al.*, 1977).

The $C^{\epsilon}H$ signals of the *trans* isomer were identified as a broad doublet, J = 12.6 Hz, arising from the equatorial hydrogen, and a triplet of doublets (J = 12.9 and 2.8 Hz, respectively) arising from the axial $C^{\epsilon}H$. The relationship between this geminal pair of C^{ϵ} protons was confirmed by mutual spin decoupling experiments.

A similar analysis was made for the minor (*cis*) isomer. In this isomer, the $C^{\alpha}H$ signal was a broadened doublet, J = 3.7 Hz, and the $C^{\epsilon}H$ signals were a broad doublet (J = 12.8 Hz) for the equatorial $C^{\epsilon}H$ and a triplet of doublets (J = 11.2 and 2.5 Hz, respectively) for the axial $C^{\epsilon}H$.

The chemical shifts of the corresponding

hydrogens in the *trans* and *cis* isomers confirm our assignments. Thus, the $C^{\alpha}H$ is 0.406 p.p.m. upfield in the *cis* relative to the *trans* isomer, and the equatorial $C^{\epsilon}H$ is 0.581 p.p.m. upfield in the *trans* relative to the *cis* isomer, as a result of deshielding by the amide carbonyl (Jackman & Sternhell, 1969), i.e. $C^{\alpha}H$ is deshielded in the *trans* isomer and $C^{\epsilon}H$ (equatorial) is deshielded in the *cis* isomer. The axial $C^{\epsilon}H$ is shielded by 0.672 p.p.m. in the *cis* isomer relative to the *trans*, and also seems shielded when compared with the chemical shifts of $C^{\delta}H$ in *N*-acetylproline compounds (Pogliani *et al.*, 1975). This shielding probably arises from the carboxyl to which the axial $C^{\epsilon}H$ stands in a 1,3-diaxial relationship (Fig. 2), and the conformation of the carboxyl must be quite different in the *cis* and *trans* isomers.

This first-order analysis of the ^1H spectra of the two isomers of *N*-acetyl-D, L-pipecolic acid is similar to that of *N*-nitrosopipecolic acid (Lijinsky *et al.*, 1970). The chemical shift differences for hydrogens in the two isomers are similar in sign and magnitude for both the *N*-acetyl and *N*-nitroso compounds. In the only other published work on the ^1H spectra of pipecolic acid residues in cyclic dipeptides (Vicar *et al.*, 1973), the assignments differed from ours in having the $C^{\alpha}H$ resonances at 3.6–3.9 p.p.m. and the equatorial $C^{\epsilon}H$ resonances at 4.6–4.7 p.p.m. Since our assignments are in the reverse sense, i.e. $C^{\alpha}H$ less shielded than equatorial $C^{\epsilon}H$, and are confirmed by spin decoupling, we would suggest the reassignment of the resonances reported by Vicar *et al.* (1973).

The effects of *cis-trans* isomerism about the Ac-N bond in Ac-Pip-OH are also seen in the ^{13}C NMR spectrum (Table 2). In DMSO-d_6 solution, the *trans* to *cis* isomer ratio is approximately 2:1 (average of intensity ratios for several resonances), and we have assigned the individual carbon resonances in the same way as Thomas (1976), although the chemical shifts differ consistently because of different referencing systems. We were unable to locate the C^{ϵ} resonance of the *cis* isomer among the resonances of the solvent and, in our spectrum, the acetyl CO resonance of the *cis* isomer was 0.2 p.p.m. to high field of the *trans* isomer, rather than to low field as shown in the published spectrum (Thomas, 1976). It is interesting to compare the resonances of respective carbons in the *cis* and *trans* isomers, as has been done for *N*-acetylproline isomers (Dorman & Bovey, 1973; Deslauriers & Smith, 1976). We quote here the chemical shift difference, $\delta_{cis} - \delta_{trans}$, in p.p.m. for each carbon of *N*-acetyl-D, L-pipecolic acid, followed by the corresponding figures for *N*-acetyl-L-proline (Dorman & Bovey, 1973): C^{α}, 5.0, 1.9; C^{β}, 0.4, 1.4; C^{γ}, 0.0, −1.7; C^{δ}, −0.6, no Pro equivalent; C^{ϵ}, −4.9, −1.8 [the value of −4.9 was obtained from data of Thomas (1976)]. The differences for the carbons adjacent to nitrogen in *N*-acetyl-D, L-pipecolic acid are more like those seen in acyclic amides (Fritz *et al.*, 1977), i.e. larger than in proline, and this should make the detection of X-Pip isomers more straightforward, although we do not yet know the range of variation of the C^{α} and C^{ϵ} resonances of pipecolic acid derivatives.

The study of this model compound furnished us with "reference values" for coupling constants and chemical shifts in both *cis* and *trans* *N*-acyl-Pip residues, and confirmed the existence in these solutions of *cis* and *trans* isomers about the acyl-Pip bond.

L-Phe-Pip-Arg-pNA and D-Phe-Pip-Arg-pNA

The resonances of the Phe residues in the ^1H NMR spectra of both of these peptides in DMSO-d_6 are different from those in the Val peptides discussed above, in that the aromatic hydrogens give a broad multiplet which must arise because of greater differences in the individual chemical shifts of the ortho, meta and para hydrogens. These differences probably arise from differences in solvation or other intermolecular effects, but it is difficult to draw any firm conclusions.

For L-Phe-Pip-Arg-*p*NA, the side chain ^1H–^1H couplings were analyzed as described above (Pachler, 1964; Ogura *et al.*, 1967), and the rotamer proportions found to be: (a) 0.57; (b) 0.33; (c) 0.10. Using the parameters of Feeney (1976), the corresponding values are: (a) 0.69; (b) 0.31; (c) 0.00. These values are similar to those found in L-Phe-Val-Arg-*p*NA. For the D-Phe isomer, the two $C^{\beta}H$ resonances are again coincident and analysis is impossible.

The most striking feature of these ^1H spectra,

however, is the doubling of all of the analyzable CH resonances of Pip in D-Phe-Pip-Arg-*p*NA, which we ascribe to the existence of *trans* and *cis* isomers about the D-Phe-Pip- peptide bond, in the ratio 2:1. The chemical shifts of signals assigned to C^{α} and C^{ϵ} hydrogens correspond well to those in the *N*-acetyl-D, L-pipecolic acid isomers, with the exception that the axial $C^{\epsilon}H$ in the *trans* isomer of the peptide seems to be shielded by 0.34 p.p.m. with respect to that in the model compound. This is probably the result of the proximity of this $C^{\epsilon}H$ to the D-Phe aromatic ring in this *trans* isomer of D-Phe-Pip-Arg-*p*NA, as is the case for the Val methyl groups in D-Phe-Val-Arg-*p*NA. In both the Val and Pip series, this shielding of Val or Pip hydrogens means that they must be close to the face of the aromatic ring of the D-Phe residue, and we deduce that a similarly folded conformation exists for both (Deber & Joshua, 1972). In the L-Phe peptides, the Phe ring is too far away to shield the Val or Pip hydrogens.

The ^{13}C spectra of these two peptides have been recorded for D_2O and DMSO-d_6 solutions (Table 2), and assigned by reference to the values for Ac-D, L-Pip-OH (Table 2) and literature compilations (Rosenthal & Fendler, 1976). In D_2O, there was a single isomer of each peptide, and the assignment of most resonances was straightforward with the usual exceptions of the carbonyl carbons, and these latter assignments are tentative. For the DMSO-d_6 solutions, which were necessarily rather dilute, we were unable to assign the high-field resonances because of technical difficulties with the spectrometer. It was confirmed from doubling of the C^{α} resonances, however, that the D-Phe-Pip-Arg-*p*NA existed as a *trans-cis* mixture in DMSO-d_6, while the L-Phe-Pip-Arg-*p*NA is wholly *trans*. In the spectrum of the D-Phe peptide, the C^{α} and C′ (carbonyl) carbon resonances are doubled, and it was possible to assign the C^{α} resonances with certainty and to estimate the isomer ratio as *trans*:*cis* of approximately 2:1. This difference in the isomer ratio for D-Phe-Pip and L-Phe-Pip moieties is the reverse of that observed in the corresponding Phe-Pro dipeptides (Grathwohl & Wüthrich, 1976), where the L-Phe-L-Pro bond is more likely to be *cis* than that in D-Phe-L-Pro. However, both D- and L-Phe-Pip-Arg-*p*NA exist in D_2O with the Phe-Pip bond *trans*; hence, the isomerism is probably without effect in reactions with thrombin in aqueous systems. It has also been observed that Gly-Pro-Ala-NH_2 exists as a 3:1 mixture of *trans* and *cis* isomers in DMSO-d_6, but is all *trans* in D_2O (Von Dreele *et al.*, 1978).

Our investigations have shown pronounced differences between the conformations of the D-Phe and L-Phe series in DMSO-d_6. In the D-Phe, but not in the L-Phe peptides, the aromatic ring appears to be folded back over the Val or Pip residue. These differences in conformation might be related to the differences in hydrolysis rates if binding of an aromatic residue in close proximity to valine, near the cleavage site, is an essential step in enzymic hydrolysis.

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REFERENCES

Blombäck, B. & Blombäck, M. (1972) *Ann. N.Y. Acad. Sci.* **202**, 77–97

Blombäck, B., Blombäck, M., Olsson, P., Svendsen, L. & Åberg, G. (1969) *Scand. J. Clin. Lab. Invest.*, Suppl. **107**, 59–64

Blombäck, B., Hessel, B., Iwanaga, S., Reuterby, J. & Blombäck, M. (1972) *J. Biol. Chem.* **247**, 1496–1512

Blombäck, B., Hogg, D.H., Gardland, B., Hessel, B. & Kudryk, B. (1976) *Thrombosis Res.*, Suppl. II, **8**, 329–346

Bystrov, V.F. (1976) *Progr. Nucl. Magnetic Resonance Spectr.* **10**, 41–81

Claeson, G., Aurell, L., Karlsson, G. & Friberger, P. (1977) in *New Methods for the Analysis of Coagulation Using Chromogenic Substrates*, Proc. Symp. Deut. Ges. Klin. Chem., Titisee, Breisgau, July 1976 (Witt, I. ed.), pp. 37–54, W. de Gruyter, Berlin–New York

Deber, C.M. & Joshua, H. (1972) *Biopolymers* **11**, 2493–2503

Deslauriers, R. & Smith, I.C.P. (1976) in *Topics in Carbon-13 NMR Spectroscopy* (Levy, G.C., ed.), vol. 2, pp. 1–80, Wiley-Interscience, New York

Dorman, D.E. & Bovey, F.A. (1973) *J. Org. Chem.* **38**, 2379–2383

Feeney, J. (1976) *J. Magnetic Resonance* **21**, 473–478

Fritz, H., Hug, P., Sauter, H., Winkler, T. & Logemann, E. (1977) *Org. Magnetic Resonance* **9**, 108–112

Gibbons, W.A., Crepaux, D., Delayre, J., Dunand, J.J., Hajdukovic, G. & Wyssbrod, H.R. (1975) in *Peptides: Chemistry, Structure and Biology* (Walter, R. & Meienhofer, J., eds.), pp. 127–137, Ann Arbor Science Publishers, Ann Arbor, Mich.

Grathwohl, C. & Wüthrich, K. (1976) *Biopolymers* **15**, 2025–2041

Hageman, T.C. & Scheraga, H.A. (1977) *Arch. Biochem. Biophys.* **179**, 506–517

Hassall, C.H., Thomas, W.A. & Moschidis, M.C. (1977) *J. Chem. Soc., Perkin* **I**, 2369–2376

Heilbron, I. (1965) in *Dictionary of Organic Compounds*, 4th ed., vol. 5, p. 2761, Oxford University Press, New York

Hess, K. & Liebrandt, F. (1917) *Ber. Deut. Chem. Ges.* **50**, 385–389

Jackman, L.M. & Sternhell, S. (1969) *Applications of Nuclear Magnetic Resonance in Organic Chemistry*, 2nd ed., p. 89, Pergamon Press, Oxford

Lijinsky, W., Keefer, L. & Loo, J. (1970) *Tetrahedron* **26**, 5137–5153

Ogura, H., Arata, Y. & Fujiwara, S. (1967) *J. Mol. Spectr.* **23**, 76–85

Pachler, K.G.R. (1964) *Spectrochim. Acta* **20**, 581–587

Pogliani, L., Ellenberger, M., Valat, J. & Bellocq, A.M. (1975) *Intern. J. Peptide Protein Res.* **7**, 345–360

Rosenthal, S.N. & Fendler, J.H. (1976) *Adv. Phys. Org. Chem.* **13**, 279–424

Scheraga, H.A. (1977) in *Chemistry and Biology of Thrombin*, (Lundblad, R.L., Fenton, J.W., II & Mann, K.G., eds.), pp. 145–158, Ann Arbor Science Publishers, Ann Arbor, Mich.

Thomas, W.A. (1976) *Ann. Rep. NMR Spectr.* **6B**, 1–41

Thomas, W.A. & Williams, M.K. (1972) *J. Chem. Soc. Chem. Commun.*, 788–789

Van Nispen, J.W., Hageman, T.C. & Scheraga, H.A. (1977) *Arch. Biochem. Biophys.* **182**, 227–243

Vicar, J., Budesinsky, M. & Blaha, K. (1973) *Coll. Czech. Chem. Commun.* **38**, 1940–1956

Von Dreele, P.H., Rae, I.D. & Scheraga, H.A. (1978) *Biochemistry* **17**, 956–962

Williams, D.H. & Fleming, I. (1973) *Spectroscopic Methods in Organic Chemistry*, 2nd ed., pp. 101–104, McGraw-Hill, London

Address:

H.A. Scheraga
Dept. of Chemistry
Cornell University
Ithaca, NY 14853
U.S.A.