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NMR STUDIES OF THE SOLUTION CONFORMATION OF AN ACTIVE TRI-PEPTIDE AND ANALOGUES

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Abstract—NMR spectra of threonyl-valyl-leucine (TVL; **1**) and a number of derivatives are presented and assigned for $CDCl_3$ and DMSO solutions, to determine the conformational mobility of the tri-peptide. At 400 MHz the ^1H spectrum of the leucyl side-chain can be completely analysed and the vicinal couplings show that the side-chain is predominantly in a single conformation. Analysis of the other NMR data, using vicinal (CH.CH and NH.CH) couplings, temperature dependence of the NH chemical shifts, and solvent titrations, indicates that there is considerable conformational mobility in DMSO solution, evidence of specific interactions in some compounds in $CDCl_3$ solution and that the parent peptide in D_2O solution shows some evidence of a preferred conformation.

The role of the protein α_2-globulin in schizophrenia has been repeatedly presented by Frohman *et al.*[1] Frohman's group has also reported properties of the α_2-globulin, namely its ability to increase the uptake of tryptophan, 5-hydroxytryptophan and pyrrolidone carboxylic acid by cells,[2] the dependence of these activities on the conformation of the α_2-globulin[3,4] and the isolation of another factor which controls both the conformation and the activity of the α_2-globulin[3,4] and its distribution in the human brain.[5]

This relationship between the conformation of the α_2-globulin and its activity showed (by ORD and CD measurements) that the random chain form or β-chain form, which is isolated from normal subjects, exhibits normal results when subjected to the above tests; however, α_2-globulin isolated from the plasma of *ca* 60% of unmedicated schizophrenics, within 7 yr of the onset of schizophrenia, possessed up to 60% α-helix structure, the amount of which correlated linearly with the elevation of tryptophan uptake and the lactate/pyruvate ratio.[3,4]

Recent studies have been directed at isolating the brain protein which appears to control the helical nature of the α_2-globulin and hence control the symptoms of schizophrenia.[6–8] A ready source of this "anti-S-protein" is beef hypothalamus, furthermore, digestion of this protein (MW *ca* 30,000) with proteolytic enzymes yields a segment which appears to have the ability to change the conformation of the active form of α_2-globulin to the normal form and hence affect the cellular uptake of tryptophan *in vitro* and the rate of intra-cranial self-stimulation in rats *in vivo*.[8] Isolation of the fragment showed it to be a tripeptide of the structure L-threonyl-L-valyl-L-leucine (TVL; **1**). Synthetic TVL and various derivatives also possess activity in the control of these indications of schizophrenia.[8]

These reports prompted us to commence an investigation into the synthesis and the characterisation of TVL and its derivatives and in particular to determine whether the tri-peptide exists in a preferred conformation in solution.

NMR spectroscopy is now the accepted technique for the study of peptide conformations in solution, and nuclear chemical shifts, coupling constants and relaxation times have all been used in such studies.[9–13]

Intensive investigations of the orientation dependence of the NH.CH coupling in peptides have led to the general acceptance of the Bystrov equation (1)[11]

$$^3J(NH.C_\alpha H) = 9.4\cos^2\theta - 1.1\cos\theta + 0.4 \qquad (1)$$

for peptides with *trans* amide bonds, which is the orientation normally encountered in acyclic peptides. The orientation dependence of the ^3J(CH.CH) couplings in the side-chain fragments of threonyl, valyl and leucyl can also be deduced with some degree of confidence.[9,11,22–24] Thus the determination of these chemical shifts and couplings in the tripeptide should provide some indication of the conformational rigidity of the molecule.

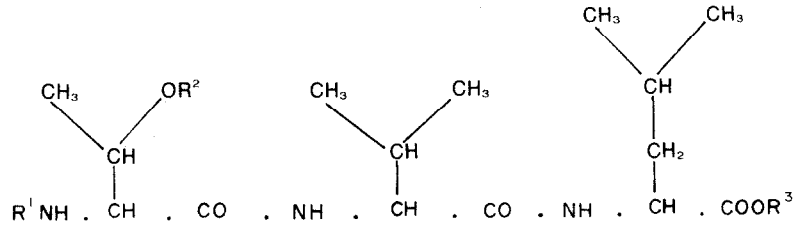
The solvent and temperature dependence of the amide protons chemical shift may be used to delineate exposed and shielded NH protons.[16–20] In DMSO solution shielded NH protons have temperature dependencies of 0–3 × 10^{-3} ppm/°C and exposed NH protons larger values. In chloroform solution[21] and in solvent titration studies[16] similar behaviour is observed with the exposed NH protons being most affected.

Here we concentrate on the amide proton chemical shifts and NH.CH couplings and their solvent and temperature dependence, although a complete assignment of the remaining proton signals and also of the ^{13}C spectra are given. A preliminary report on the complete analysis of the NMR spectrum of the leucyl side-chain in Ac-TVL has been presented,[14] and the results of molecular mechanics calculations on the tripeptide are given elsewhere.[15]

EXPERIMENTAL

The compounds synthesised are shown in Scheme 1. Full details of the synthesis and characterisation of the compounds are given elsewhere.[15]

The ^1H NMR spectra were obtained on a Perkin–Elmer R-34 spectrometer (220 MHz), probe temperature 27° in DMSO, $CDCl_3$ and D_2O, where possible. To reduce variations due to concen-



	R^1	R^2	R^3
I	H	H	H
II	Z	H	Bz
III	Boc	Bz	Bz
iv	H	bz	Bz
V	Ac	Bz	Bz
VI	Ac	H	H
VII	Boc	Bz	NH_2
VIII	H	Bz	NH_2
IX	Ac	Bz	NH_2
X	Ac	H	NH_2

Scheme 1. Analogues of TVL synthesised.

tration effects, the solutions were standardised at 50 mg/ml (*ca* 0.1M). Some spectra were obtained on a Bruker WH-400 spectrometer, typical operating conditions were (^{1}H spectra), PW 3.0 μs, SW 3200 Hz, AT 2.56 s, 16K data points, *ca* 50–100 accumulations. The ^{13}C spectra were obtained on a Varian XL-100 spectrometer at 25.2 MHz with PW 45 μs, SW 5000 Hz, AT 0.85 s, 8K data points, *ca* 50–100K accumulations, and on the Bruker WH-400 at 400 MHz, with PW8 μs, SW 20 KHz, AT 0.8 s, 32 K data points and *ca* 40–50 K accumulations.

RESULTS

The proton spectra consist of a number of well-separated groups of resonances, the NH protons at *ca* 6–8 δ, the αCH (and βCH of threonyl) at *ca* 4 δ, the valyl and leucyl side-chain protons at 1.5–2.0 δ and the methyl signals at *ca* 1.0 δ. Within these regions there are often many overlapping resonances and the subsequent assignment of the peaks was not trivial. Extensive double resonance experiments and in some cases titration experiments were necessary to assign the signals unequivocally. The C^β H_2-C^γ H fragment of the leucyl side-chain was a particularly complex closely coupled multiplet which defied analysis in all cases at 220 MHz (e.g. Fig. 1a).

The 400 MHz spectrum of one compound Ac–TVL (Fig. 1c) was fully analysed, using the iterative computer analysis programmes LAME[25] and NUMARIT.[26] The latter programme, by allowing simultaneous magnetic equivalence factoring and the X approximation, enabled the analysis of the entire side-chain spectrum, including the NH proton, to be performed as an $ABCRTX_3Y_3$ spin system. Proton–proton couplings over more than three bonds were taken as zero. The fitted couplings are given, with the computed standard deviations, in Fig. 2, and the observed and calculated (computer simulated) spectra in Fig. 1.

The NMR data obtained for the remaining compounds examined is given in Tables 1–3. The parent peptide Compounds **II**, **III** and **V** were readily soluble in $CDCl_3$ and DMSO and complete assignments of the spectra in both solvents were eventually obtained. The free acid and amide (compounds **VI** and **IX**) were only soluble in DMSO but again complete assignments of the spectra could be obtained. In addition several solvent titrations were performed, originally to aid in the assignment of the spectra. Figure 3 shows the results obtained in a typical case with $CDCl_3$/DMSO and DMSO/TFE titrations. Finally the temperature coefficients of the NH protons were measured in all cases where this could be performed. These results are given in Table 3. The complete assignment of the ^{13}C (^{1}H) spectra of the compounds was performed with the aid of the extensive data on peptide ^{13}C chemical shifts.[13] This tabulation is given elsewhere[15] and is available on request.

DISCUSSION

It is convenient to consider first the conformations of the side-chains in the tripeptide. The rotamer distribution in the side-chains may be obtained from the observed couplings if the couplings in the discrete rotamers can be estimated. The couplings about the C^α–C^β bonds have been the subject of a number of investigations, all of which tend to give similar values of the couplings. We use the general method of Feeney *et al.*[23] in which the anti HH coupling is given by recourse to cyclohexanes with similar substituent groups, and the gauche HH couplings may be simply estimated by the procedure of Ref. 24. The rotamer distributions and couplings so obtained are shown in Fig. 4. For the leucyl side-chain, the observed couplings are then given by the equations

$$2.7n_A + 3.8n_B + 12.3n_C = J_{BR}$$

$$3.3n_A + 12.3n_B + 3.0n_C = J_{CR}$$

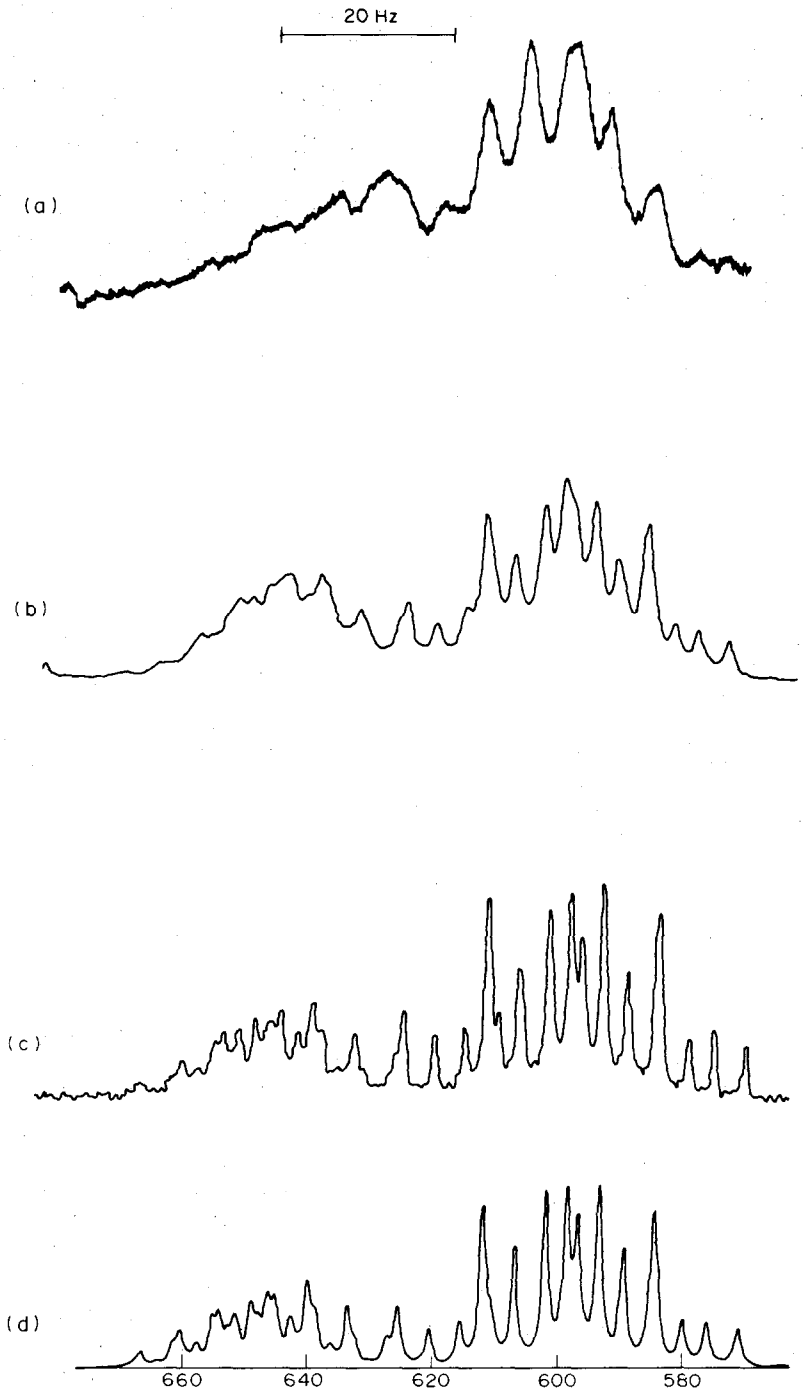
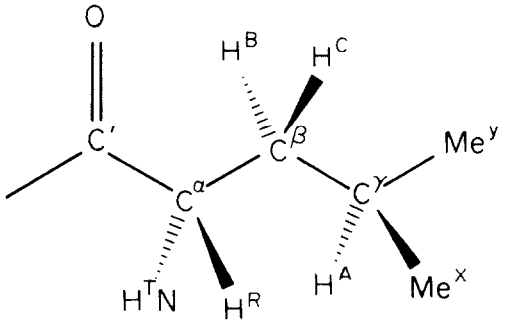


Fig. 1. The ^{1}H NMR spectrum of the β and γ protons of the leucyl side chain of Ac-Thr-Val-LeuOH ($CDCl_3$ solution). (a) At 220 MHz; (b) at 400 MHz; (c) as (b) with resolution enhancement; (d) the computer simulated spectrum.

and this has been unequivocally proved for free leucine in both acid and base by ^{13}C and ^{15}N labelling.[28] Assuming the same assignment for the BR and CR couplings of compound VI (Fig. 2) and the free amino acid gives rotamer populations of 0.03, 0.18 and 0.78 for n_A, n_B and n_C.

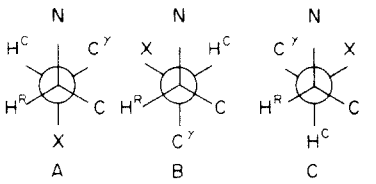
A similar treatment of the closely coupled system of the β and γ protons in **VI** is also possible for the first time in the leucyl side-chain.

The calculated couplings for the C^α anti Me^γ conformer of Fig. 2 are J_{AB} 3.8, J_{AC} 12.0; for the C^α anti H_A conformer J_{AB} 3.0, J_{AC} 3.0 and for the C^α anti Me^γ conformer J_{AB} 12.0 J_{AC} 3.8 Hz. These give with the observed couplings of Fig. 2 fractional populations of 0.66, 0.16 and 0.18 respectively. Furthermore, the large $\alpha\beta$ coupling is associated with the diastereotopic β proton which shows the small β, γ coupling and vice versa, thus the fractional rotamer populations of the entire



$^3J_{RT}$	7.707 (0.0082)
$^3J_{BR}$	10.405 (0.0116)
$^3J_{CR}$	4.720 (0.0136)
$^3J_{BC}$	-13.649 (0.0199)
$^3J_{AB}$	5.103 (0.0206)
$^3J_{AC}$	9.094 (0.0227)
$^3J_{AX}$	6.302 (0.0118)
$^3J_{AY}$	6.527 (0.0118)

Fig. 2. The favoured conformation of the leucyl side-chain with the nomenclature used and couplings obtained in the analysis (Hz). Values in parenthesis are computed standard deviations.



Threonyl X = OH	J_{CR}	4.2	10.5°	1.2
Valyl X = CH_3	J_{CR}	3.5	11.1°	2.4
Leucyl X = H_B	J_B	2.7	3.8	12.3°
	J_{CR}	3.3	12.3°	3.0°

Fig. 4. Rotamers and corresponding HH couplings about the C^α–C^β bond in the TVL side-chains.
(a) From J(ax – ax) in cyclohexane (13.2 Hz);[27] *trans* 4-acetylamido-t-butyl cyclohexane (12.3 Hz),[11] eq-cyclohexanol (11.4 Hz)[24] and *cis* 1,3 dimethylcyclohexane (12.0 Hz),[27] assuming additive substituent effects.

leucyl side-chain can now be determined. The preferred conformation of the leucyl side-chain is as shown in Fig. 2, and thus the leucyl residue may be considered essentially as the single conformation shown in spatial modelling or molecular mechanics calculations.

The conformations of the threonyl and valyl side-chains are not so clear-cut. There is only one coupling about the C^α–C^β bond, thus this does not permit any differentiation between rotamers A and C (Fig. 4), but only between B and (A + C). Also the observed couplings (Table 2) are not extreme values, indicating as would be expected, more rotational mobility in these side-chains than in the bulkier leucyl residue. In considering the

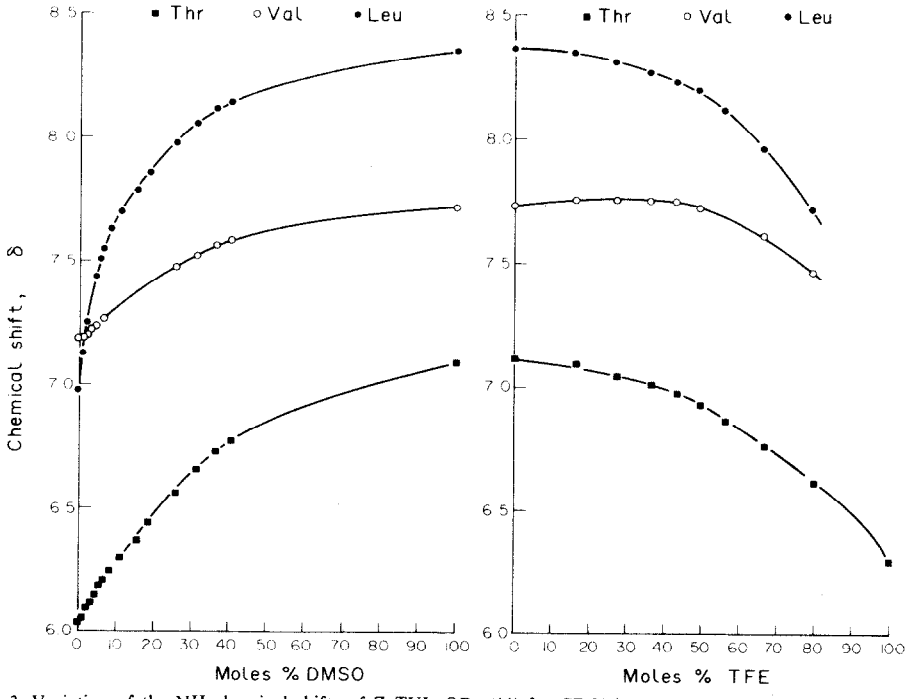


Fig. 3. Variation of the NH chemical shifts of Z-TVL-OBz (11) for $CDCl_3$/DMSO (left) and DMSO/TFE (right) solvent titrations (concentration of the solute 50 mg/ml).

Table 1. Proton chemical shifts (δ) of threonyl–valyl–leucyl (TVL) derivatives[a]

Compound	Solvent	Threonyl				Valyl				Leucyl				
		NH	$C^{\alpha}H$	$C^{\beta}H$	CH_3	NH	$C^{\alpha}H$	$C^{\beta}H$	CH_3	NH	$C^{\alpha}H$	$C^{\beta}H_2$	$C^{\gamma}H$	CH_3
(I) TVL	D_2O[b]	-	3.85	4.08	1.26	-	4.17	2.08	0.96, 0.95	-	4.20	1.7 - 1.6		0.90, 0.85
	D_2O/HCl	-	4.09	3.92	1.27	-	4.20	2.08	0.98, 0.98	-	4.41	1.7 - 1.6		0.94, 0.88
(II) Z-TVL-OBz	$CDCl_3$[c]	5.75	4.19	4.32	1.14	6.85	4.19	2.16	0.89, 0.84	6.42	4.65	1.7 - 1.5		0.89, 0.88
	DMSO	7.09	4.07	3.91	1.05	7.72	4.28	1.99	d	8.35	4.35	1.7 - 1.5		d
(III) Boc-T(Bz)VL-OBz	$CDCl_3$	5.48	ca. 4.25		1.19	6.98	ca 4.25	2.15	d	6.50	4.58	ca 1.6		d
	DMSO	6.61	4.15	3.90	1.10	7.80	ca 4.35	1.94	d	8.41	ca 4.35	"		d
(IV) T(Bz)VL-OBz	$CDCl_3$[e]	f	3.22	4.21	1.22	8.14	4.35	2.10	0.89, 0.85	6.89	4.61	"		0.86
(V) Ac-T(Bz)VL-OBz	$CDCl_3$	6.76	4.71	4.10	1.13	7.23	4.40	2.10	0.87, 0.79[e]	6.83	4.65	"		0.85, 0.89[e]
	DMSO	7.96	4.56	3.94	1.08	7.79	4.30	1.92	d	8.40	4.35	"		d
(VI) Ac-TVL	DMSO[g]	7.83	4.28	3.90	1.00	7.57	4.21	1.99	0.86, 0.81	8.11	4.17	1.52 1.47	1.62	0.88, 0.81
(IX) Ac-T(Bz)VL-NH_2	DMSO	7.97	4.50	3.92	1.08	7.78	4.28	2.00	d	7.91	4.28	ca 1.42	1.55	d

a 220 MHz, conc. 0.1M unless stated otherwise.

b saturated solution 400 MHz.

c 400 MHz, conc. ca. 3×10^{-2} M, δ (OH) 3.35 ($CDCl_3$); 4.82 (DMSO)

d 0.7 - 0.96

e 400 MHz

f exchanged with OH proton

g δ (OH) 4.75

Table 2. H–H couplings (Hz) in threonyl–valyl–leucyl (TVL) derivatives[a]

Compound	Solvent	Threonyl[b]		Valyl[c]		Leucyl[d]
		$^3J_{N\alpha}$	$^3J_{\alpha\beta}$	$^3J_{N\alpha}$	$^3J_{\alpha\beta}$	$^3J_{N\alpha}$
TVL	D_2O[e]	f	6.61	f	8.22	f
	D_2O/HCl	f	7.05	f	7.35	f
Z-TVL-OBz[e]	$CDCl_3$	7.80	2.93[g]	9.06	6.43[g]	8.23
	DMSO	8.56	4.69	8.89	6.57	7.72
Boc-T(Bz)VL-OBz	$CDCl_3$	6.00	f	8.95	6.15	7.99
	DMSO	8.93	4.05	8.91	f	7.30
T(Bz)VL-OBz	$CDCl_3$	f	2.62	9.21	6.81	f
Ac-T(Bz)VL-OBz	$CDCl_3$	6.85	3.75	9.09	6.58	8.16
	DMSO	8.79	4.13	8.61	6.68	7.59
Ac-TVL	DMSO	8.46	4.62	8.97	6.23	7.69
Ac-T(Bz)VL-NH_2	DMSO	8.74	4.32	8.76	6.73	8.53

a Conditions as in table 2.

b ^3J (β .CH_3) = 6.37 ($\pm$ .05) $CDCl_3$; 6.02 ($\pm$ .05) DMSO, 6.46 (D_2O)

c ^3J (β .CH_3) = 6.80 ($\pm$ .05) $CDCl_3$, 6.75 (D_2O)

d ^3J (γ .CH_3) = 6.10 ($\pm$ .05) $CDCl_3$; 6.30, 6.53, DMSO; 6.31, 6.45 (D_2O)

e ^3J (β .OH) ca. 3.0 ($CDCl_3$); 5.6 (DMSO)

f unobserved due to exchange or complex multiplets

g at 400 MHz

Table 3. Temperature coefficients of the amide protons of TVL derivatives

Compound		Solvent	Temp. Coeff.[a] (p.p.m. x 10^3 $^0C^{-1}$)		
			Threonyl	Valyl	Leucyl
II	Z-TVL-OBz	$CDCl_3$	8.3	7.2	12.4
		DMSO	7.4	4.0	5.4
III	Boc-T(Bz)VL-OBz	$CDCl_3$	1.2	1.5	7.4
		DMSO	6.8	4.4	5.2
V	Ac-T(Bz)VL-OBz	$CDCl_3$	10.4	9.2	16.1
		DMSO	4.8	5.0	5.2
VI	Ac-TVL	DMSO	5.0	3.3	5.2
IX	Ac-T(Bz)VL-NH_2[b]	DMSO	3.8	3.7	3.6

a All the NH protons measured move to high field with increasing temperature, thus all the above coefficients should formally be negative.

b The leucinamide protons temp. coeff. are ca. 3.9 and 4.0.

values of the C^{α}–C^{β} couplings it is convenient to compare the observed values with both the calculated rotamer couplings of Fig. 4 and the average coupling for "free rotation" of the side-chain (i.e. $n_A = n_B = n_C = \frac{1}{3}$). The average couplings for the threonyl and valyl fragments are 5.3 and 5.7 Hz respectively.

The observed threonyl $\alpha\beta$ couplings are all less than J_{AV} in $CDCl_3$ and DMSO solution. In DMSO solution the range of the observed couplings is small (4.69–4.05 Hz) indicating considerable rotational mobility with a slight preference for rotamers (A + C). The values in $CDCl_3$ solution are much smaller (2.6–3.7 Hz), suggesting certainly for the lower value, a strongly preferred conformation. However, most interestingly, the values of the coupling in the parent tripeptide in D_2O and D_2O/HCl are quite different from all the others and indicate a strong preference for rotamer B.

The $\alpha\beta$ coupling in the valyl side-chain is always larger than J_{AV}, indicating a small preference for rotamer B, and again there is a considerable enhancement of the coupling in TVL in D_2O. These couplings suggest therefore that there is general rotational mobility of the side-chains in DMSO solution, considerably less for the threonyl residue in $CDCl_3$ solution, possibly due to the formation of an intramolecular hydrogen bond involving the threonyl OH, but TVL in D_2O appears to be much more biased towards a particular conformation than any of the analogues in $CDCl_3$ or DMSO solution.

Making the usual assumption that only *trans* amide bonds are present in this acyclic peptide, the ^3J ($NH.C^{\alpha}H$) couplings are the major source of information on the main-chain conformation. In DMSO these couplings are very similar in all the compounds investigated, and the averages of the observed couplings (Table 2) are for the threonyl, valy and leucyl residues 8.70 (+0.15); 8.83 (+0.12) and 7.60 (+0.13) with the single exception of the leucyl coupling in **IX** of 8.5 Hz. These values may be used with eqn (1) to obtain the dihedral angles about the N.C bond. However, for most values of the coupling there will be two values of θ satisfying this equation, and thus four values of the $CO.N.C^{\alpha}.C^{\beta}$ dihedral angle (ϕ), as positive and negative values of θ result in different values of ϕ. The couplings in the threonyl and valyl fragments indicate values of θ of *ca* 0° or 150° and corresponding values of ϕ of 60, −150 and −90°. The lower value of the coupling in the leucyl fragment gives values of θ of 20° or 140° and corresponding values of ϕ of 40, 80, −160 and −80°. Consideration of these alternatives will be deferred until they can be considered together with the molecular mechanics calculations.

The values of the couplings in $CDCl_3$ solution differ considerably from those in DMSO and also show more diversity between the different compounds. The threonyl couplings change markedly from 7.8 Hz in **II** to 6.0 Hz in **III**. There was also some concentration dependence of the couplings in this solvent, indicating intermolecular complexation, very likely due to hydrogen bonding, which would of course be removed in DMSO solution. The values of the valyl and leucyl couplings are more consistent, averaging at 9.1 (±0.1) Hz and 8.1 (±0.1) Hz. The value for the valyl fragment is on the limit for ϕ values of 0°, when it is considered that the observed value in a mobile molecule such as an acyclic tripeptide will always be the result of some conformational averaging, and suggests that the correct values of θ to consider are ±160°, i.e. ϕ equals −100 or −140. The leucyl couplings are also slightly higher than in DMSO, and again this is consistent with the deduction made from the side-chain couplings that the peptide is more biased towards one conformation in $CDCl_3$ than in DMSO. It is unfortunate that the corresponding couplings in D_2O solution could not be obtained despite several attempts, as these would have been of particular interest.

The temperature dependences of the NH protons in $CDCl_3$ and DMSO solution are given in Table 3. The temperature coefficients of the NH protons in DMSO vary over only small ranges, threonyl 3.8–7.4, valyl 3.3–5.0 and leucyl 3.6–5.4. These are all in the range for NH protons largely exposed to the solvent and provide no evidence of strong intramolecular hydrogen bonding in DMSO solution. In contrast to the DMSO results, the range of observed temperature coefficients in $CDCl_3$ solution is much larger, threonyl 1.2–10.4, valyl 1.5–9.2 and leucyl 7.4–16.1. The low values of the threonyl and valyl NH protons in **III** strongly suggests that these NH's are shielded from the solvent, but they contrast sharply with the very large values obtained for the same protons in **V**, in which the only change from **III** is that Boc is replaced by acetyl. This suggests the onset of some conformational mobility in **V**, or some intermolecular association and this would be consistent with the observed changes in the couplings with concentration noted previously in $CDCl_3$ solution.

The contrasting results in $CDCl_3$ and DMSO solution may be illustrated by the results of a solvent titration in which the solvent ratio is continuously varied whilst maintaining a constant solute concentration (Fig. 3). There is the expected downfield shift of the NH protons, but the leucyl NH shows a large solvent induced shift and a very pronounced chemical shift change for the addition of small amounts of DMSO. Clearly the leucyl NH is the preferred site of attack of the DMSO and now this can be seen to be consistent with the large temperature coefficient of this NH proton in $CDCl_3$ solution. Large temperature coefficients in $CDCl_3$ solution are due to the break-down of intermolecular hydrogen bonds,[21] thus the leucyl NH forms intermolecular hydrogen bonds in $CDCl_3$ solution which are the first to be broken by competition from DMSO. The analogous DMSO/TFE titration (Fig. 3) again supports the "exposed" sites for the threonyl and leucyl NH's in DMSO. The valyl NH proton curve indicates a more shielded site to TFE, and the temperature coefficient of this proton is, in agreement with this, less than the others. However, there is still no evidence for a strong intramolecular hydrogen bond in DMSO solution.

CONCLUSION

The NMR data presented do begin to provide a consistent picture of the conformational mobility of the tripeptide, though there are still considerable gaps to fill.

In DMSO there is general conformational mobility and no evidence for an overwhelmingly preferred conformation. In particular there is no evidence for any intramolecular hydrogen bonding in this solvent, and this is not unexpected as competition with the solvent to form intermolecular hydrogen bonds would be greatest in this solvent. What conformational preference there is is the result of steric factors, which can be considerable in particular sections of the molecule. For example in the leucyl side-chain steric factors give rise to the predominant side-chain conformation described here.

The situation in the other solvents is less clear. In $CDCl_3$ there is eivdence for intramolecular hydrogen

bonding (or shielded NH sites, which is not necessarily synonymous) in some of the compounds studied. Also the conformations, as indicated by the coupling constants are both more varied than in DMSO and also at times more biased towards one conformation, again suggesting specific chemical interactions within the molecule.

In D_2O from the sparse data of the parent tripeptide spectrum again more conformational bias than in DMSO appears to exist, and most interestingly from the evidence of the side-chain couplings this appears to be quite a different conformation than in the non-polar solvent.

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