

# SOME ASPECTS OF NMR TECHNIQUES FOR THE CONFORMATIONAL ANALYSIS OF PEPTIDES

V. F. BYSTROV\*, S. L. PORTNOVA, T. A. BALASHOVA, S. A. KOZ'MIN, YU. D. GAVRILOV and V. A. AFANAS'EV

*Shemyakin Institute for Chemistry of Natural Products, USSR Academy of Sciences, Moscow, USSR*

## ABSTRACT

An outline is given of some new NMR spectroscopic approaches to the spatial structure of peptides in solution which have been recently suggested and tested in the Shemyakin Institute for Chemistry of Natural Products. The previously derived angular dependence of the peptide vicinal  ${}^3J_{\text{NHCH}}$  coupling constant has been refined on the basis of the latest experimental data. It has been found that ion-dipole interaction of the type  $\text{C}=\text{O}\cdots\text{M}^+$  (where  $\text{M}^+$  is an alkali metal ion) leads to a low field shift of the  ${}^{13}\text{C}$  signal of the carbonyl group. This effect permits determination of the number and location of the ligand groups which form the internal cavity of peptide and depsipeptide complexones. The effect of 'shift reagents' on the NMR spectra sheds certain light on the spatial structure of peptides in solution, and, in particular, gives considerable information on the configuration of the amide bond and on the rotational states of the  $\text{C}^\alpha-\text{C}^\beta$  bond. The INDOR and signals on combination frequency have been used for detection of 'hidden' signals (their multiplicity, chemical shift and splitting) and on the assignment of the NH signals in the proton NMR spectra of peptides.

---

High resolution NMR spectroscopy is becoming the most powerful means for the study of the spatial structure of peptides in solution. The progress in this field has required the development of new spectroscopic approaches. The subject matter of this paper is to outline some of those which have been suggested and tested recently in the Shemyakin Institute for Chemistry of Natural Products.

## I. THE ANGULAR DEPENDENCE OF THE PEPTIDE VICINAL NH-C<sup>α</sup>H COUPLING CONSTANT

With the accumulation of more experimental data it has now become possible to refine the earlier proposed<sup>1,2</sup> dependence of the  ${}^3J_{\text{NHCH}}$  constant on the dihedral angle  $\theta$  between the  $\text{H}-\text{N}-\text{C}^\alpha$  and  $\text{N}-\text{C}^\alpha-\text{H}$  planes.

We assume as before<sup>1</sup> that our dependence is expected in the form of a Karplus-like equation<sup>3</sup>

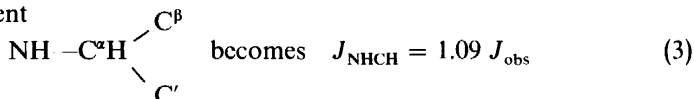
$${}^3J_{\text{NHCH}} = A \cos^2\theta - B \cos\theta + C \sin^2\theta \quad (1)$$

where  $A$ ,  $B$  and  $C$  are positive coefficients.

The corrections for the  $C^\alpha$  substituent electronegativity are made according to

$${}^3J_{\text{NHCH}} = J_{\text{obs}} (1 - \alpha \sum_i \Delta E_i)^{-1} \quad (2)$$

where  $J_{\text{obs}}$  is the experimental value for the  $\text{NH}-C^\alpha\text{H}$  coupling, and  $\Delta E_i$  is the electronegativity difference between the  $C^\alpha$  substituents and hydrogen. Assuming the value  $\alpha = -0.1$  in conformity with the experimental data for ethanes<sup>4</sup> and the Pauling scale of electronegativity<sup>5</sup>, equation 2 for the peptide fragment



In what follows only constants corrected according to equation 3 are used.

For any torsional potential of internal rotation as well as for free rotation about the  $\text{N}-\text{CH}_3$  bond in the  $\begin{array}{c} \text{O} \\ \parallel \\ \text{C} - \text{N} \\ \quad \quad \quad \diagup \quad \diagdown \\ \quad \quad \quad \quad \quad \text{CH}_3 \\ \quad \quad \quad \quad \quad \text{H} \end{array}$  fragment the averaged coupling constant is

$${}^3J_{\text{NHCH}_3} = (A + C)/2$$

The experimental data are 4.9 Hz for *N*-methylformamide<sup>6,7</sup> and 4.8 Hz for *N*-methylacetamide<sup>8</sup>.

In addition new maximum  ${}^3J_{\text{NHCH}}$  values for the regions  $0^\circ \leq \theta \leq 90^\circ$  and  $90^\circ \leq \theta \leq 180^\circ$  were selected from the data on peptides with *trans*-amide bonds. For the first region the value of 8.0 Hz was selected, being the constant of the  $\text{NH}-C^\alpha\text{H}$  fragment of the *D*-Val residue of valinomycin in the 'bracelet' conformation in non-polar media<sup>9</sup>. For the second region the maximum constant of 10.2 Hz was selected as that exhibited by the corresponding fragments of the *L*-Val and *L*-Leu residues of gramicidin S<sup>10</sup>.

We thus have the following boundary conditions for the coefficients of equation 1:

$$A + C = 9.8 \text{ Hz}$$

$$A - B \geq 8.0 \text{ Hz}$$

$$A + B \geq 10.2 \text{ Hz}$$

With these constraints computer calculation gave the angular dependence of the  ${}^3J_{\text{NHCH}}$  coupling constant presented in *Figure 1*. The refined dependence lies within the region of the one proposed earlier<sup>1,2</sup>, but confines permissible angles for a given experimental coupling constant to narrower limits and more definitely discriminates between the *cis*- and *trans*-orientation of the  $\text{NH}-C^\alpha\text{H}$  protons.

The mean permissible values shown in *Figure 1* as a hatched area are approximated by equation

$${}^3J_{\text{NHCH}} = 9.8 \cos^2\theta - 1.1 \cos\theta + 0.4 \sin^2\theta$$

## NMR CONFORMATIONAL ANALYSIS

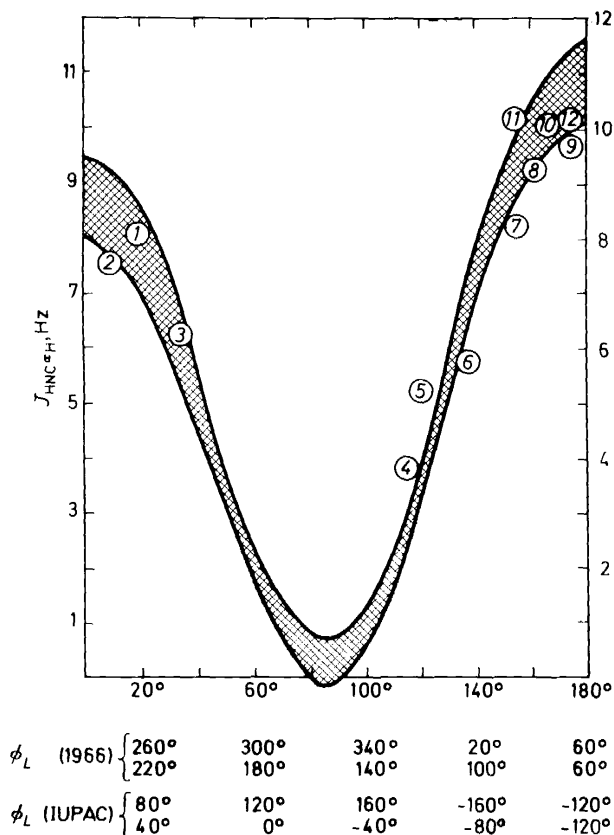


Figure 1. Refined angular dependence of the peptide NH—C<sup>α</sup>H coupling constant. Experimental data: 1 and 3, D- and L-Val residues of valinomycin in the 'bracelet' conformation<sup>9</sup>; 2 and 10, L- and D-Val of valinomycin in the 'propeller' conformation<sup>9, 11</sup>; 4, 9, 11 and 12, D-Phe, L-Orn, L-Leu and L-Val of gramicidine S<sup>10</sup>; 5, L- and D-Val of K<sup>+</sup>-valinomycin complex<sup>9, 11</sup>; 6–8, L-Orn<sub>2, 3, 1</sub> of alumichrome A<sup>12</sup> (x-ray diffraction data for ferrichrome A, see ref. 13).

which on rearrangement gives

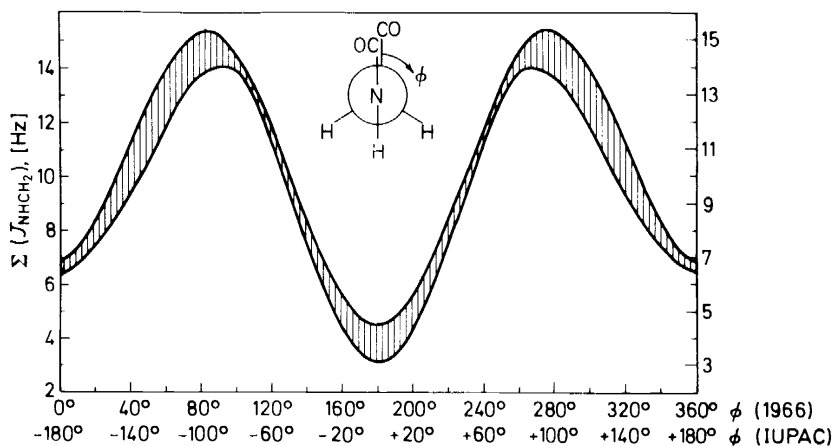
$${}^3J_{\text{NHCH}} = 9.4 \cos^2\theta - 1.1 \cos\theta + 0.4$$

Figure 1 also presents the most reliable experimental data obtained by the composite physicochemical method of conformational analysis in solution<sup>9–11</sup> and by x-ray analysis for peptides with *trans*-amide bonds<sup>12, 13</sup>. The extreme values for  ${}^3J_{\text{NHCH}}$  known up to now (2.7 Hz for evolidine<sup>14</sup>; less than 2.6 Hz for the Na<sup>+</sup>-antamanide complex<sup>15</sup> and the maximum value of 11.7 Hz for one of the forms of 'symmetric' Val<sup>(6)</sup>, Ala<sup>(9)</sup>-antamanide<sup>16</sup>) are in good accord with the general range of values for this newly derived dependence (Figure 1).

Vicinal NH—C<sup>α</sup>H<sub>2</sub> coupling in glycylic residues should be considered separately as the spectrum of these protons is either of the ABX or AA'X type. In this case the line separation of the NH signal (X-proton)—quartet or triplet—as a rule does not directly give the <sup>3</sup>J<sub>NHCH</sub> coupling constants<sup>17</sup>. Only the separation between the outer components is strictly equal to the sum of J<sub>AX</sub> and J<sub>BX</sub>(Σ<sup>3</sup>J<sub>NHCH<sub>2</sub></sub>). Assuming the projection angle between the NC<sup>α</sup>H glycylic planes to be the standard value 120°, one may then use the above <sup>3</sup>J<sub>NHCH</sub> dependence to calculate an analogous relationship for the overall constant of the glycylic residue protons from

$$\Sigma^3 J_{\text{NHCH}_2} = {}^3 J_{\text{NHCH}}(\theta) + {}^3 J_{\text{NHCH}}(120^\circ \pm \theta)$$

The result obtained is shown in *Figure 2* as a function of the conventional



*Figure 2.* Overall vicinal coupling constant in the glycylic NH—C<sup>α</sup>H<sub>2</sub> fragment plotted against the conformational angle  $\phi$ <sup>18,19</sup>.

conformational angles  $\phi$ <sup>18,19</sup>. The mean permissible values are approximated by the following function of the IUPAC—IUB angle  $\phi$ <sup>18</sup>

$$\Sigma^3 J_{\text{NHCH}_2} = -9.8 \cos^2 \phi - 1.3 \cos \phi + 15.0$$

The experimental glycylic coupling constants ranging from 13.5 Hz\* for glycylylanyl cyclopeptides<sup>20</sup> to 7.7 Hz for alumichrome<sup>12</sup> fall within the extremes of the  $\Sigma^3 J_{\text{NHCH}_2}$  curve (*Figure 2*).

Recently several other sets of coefficients for equation 1 have been proposed<sup>21-24</sup> (*Table 1*). The coefficients suggested by Schwyzer<sup>23</sup> practically coincide with those for a curve passing through the average values of our band dependence for the free rotation model<sup>2</sup>. The theoretically calculated dependence of Karplus and Barfield<sup>21</sup> does not reflect the actual difference between the *cis*- and *trans*-oriented NH—C<sup>α</sup>H protons. The empirical

\*Electronegativity corrections given according to expression  $\Sigma^3 J_{\text{NHCH}_2} = 1.04 \Sigma J_{\text{obs}}$ .

dependences of Thong *et al.*<sup>22</sup> and of Ramachandran *et al.*<sup>24</sup> give  $^3J_{\text{NHCH}}$  values too low for the *cis*-oriented NH and C $\alpha$ H bonds ( $\theta = 0^\circ$ ). This must be due to the fact that these authors used six-membered heterocycles with *cis*-amide bonds for defining the  $0^\circ \leq \theta \leq 90^\circ$  region of the curve. Quite possibly the strain occurring in these compounds, particularly in isoquinclidone (as shown in ref. 24), leads to somewhat smaller  $^3J_{\text{NHCH}}$  constants than would have been found for the *trans*-amide bonds. The preliminary theoretical INDO calculation shows that indeed the NH—C $\alpha$ H coupling constant is smaller for compounds with a *cis*-amide bond than with a *trans*-amide bond<sup>25</sup>.

Table 1. Coefficients proposed for equation 1.

Reference	Coefficients in Hz			$^3J_{\text{NHCH}}$ in Hz	
	A	B	C	<i>Cis</i> ( $\theta = 0^\circ$ )	<i>Trans</i> ( $\theta = 180^\circ$ )
This paper <sup>a</sup>	9.8	-1.1	0.4	8.0-9.4	10.2-11.6
Bystrov <i>et al.</i> <sup>1, a</sup>	9.3	-0.5	0.9	8.0-9.8	8.0-11.6
Bystrov <i>et al.</i> <sup>2, a</sup>	9.6	-0.2	0.4	8.9-9.8	8.9-10.7
Barfield and Karplus <sup>21</sup>	12.0	0.0	0.2	12.0	12.0
Thong <i>et al.</i> <sup>22, b</sup>	9.3	-3.5	0.3	5.8	12.8
Schwyzler <sup>23</sup>	9.68	-0.42	0.12	9.26	10.10
Ramachandran <i>et al.</i> <sup>24, b</sup>	8.6	-1.7	1.5	6.9	10.3

<sup>a</sup>The coefficients correspond to a curve in the middle of the allowed region. <sup>b</sup>Electronegativity corrections made according to equation 3.

The good agreement between the experimental data and the derived curves gives ground to believe that the proposed angular dependence will provide a more precise conformational coordinate and will find ever increasing use in studies of peptides by proton NMR. Dihedral angles  $\theta$  can be converted into the conventional conformational angles  $\phi$ <sup>18, 19</sup> as shown in Table 2.

## II. DETECTION OF ION-DIPOLE INTERACTIONS IN PEPTIDE COMPLEXES WITH ALKALI METAL BY $^{13}\text{C}$ NMR

It is to be expected that the intensively developing field of  $^{13}\text{C}$  NMR will open up new possibilities in studies of the spatial structure of peptides. As a first step in this direction we reported<sup>26</sup> on the effect on the  $^{13}\text{C}$  spectra of the formation by cyclic depsipeptides and peptides of stable alkali ion complexes (for the independent communication on this effect see ref. 27).

It is known that the distinctive characteristic of the peptidic complexones (see, for example, refs. 9, 11, 15, 16) is the location of the ion within the central cavity of the cyclic molecule so that the former is held in place by ion-dipole interaction with those carbonyls that are oriented towards the centre of the cavity. Such compounds are being widely used as tools for the study of processes associated with ion transport through membranes. Among the most popular of the alkali ion complexones are the depsipeptide antibiotics valinomycin and the enniatins, and the cyclic decapeptide antamanide.

Table 2. Relation between the dihedral angles  $\theta$  and the conventional

	$\theta$	$0^\circ$	$20^\circ$	$40^\circ$
According to the nomenclature (1966) <sup>18</sup>	L-	$240^\circ$	$260^\circ$	$280^\circ$
	$\phi$		$220^\circ$	$200^\circ$
	D-	$120^\circ$	$140^\circ$	$160^\circ$
			$100^\circ$	$80^\circ$
According to the IUPAC nomenclature <sup>19</sup>	L-	$60^\circ$	$80^\circ$	$100^\circ$
	$\phi$		$40^\circ$	$20^\circ$
	D-	$-60^\circ$	$-40^\circ$	$-20^\circ$
			$-80^\circ$	$-100^\circ$

From general considerations it is to be expected that with ion-dipole interaction of the type  $C=O \cdots M^+$ , where  $M^+$  stands for the monovalent cation ( $Na^+$  or  $K^+$ ), an additional shift of electron density on the carbonyl bond toward the oxygen will take place. This should decrease the  $^{13}C$  screening only of those carbonyls of the peptidic complexone that are sufficiently near to the cation.

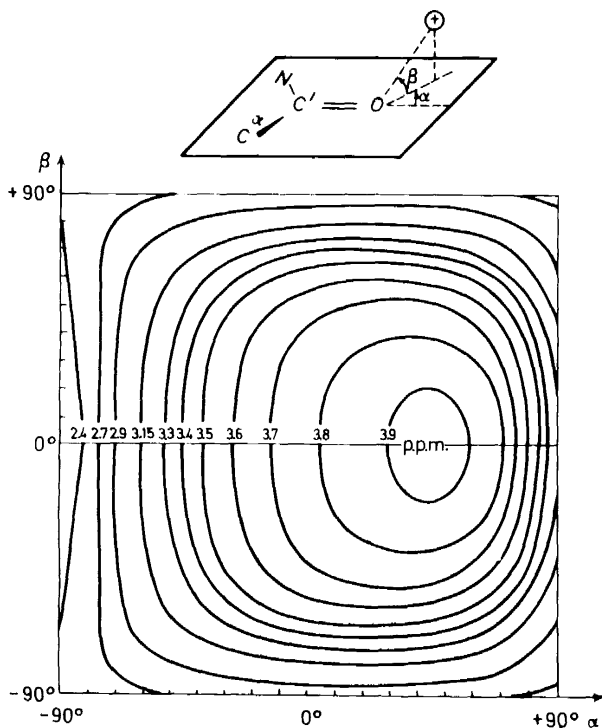


Figure 3. Influence of the positive charge on the  $^{13}C$  screening of carbonyl as a function of the angles  $\alpha$  and  $\beta$  for the  $O \cdots M^+$  distance 2.8 Å.

## NMR CONFORMATIONAL ANALYSIS

angles  $\phi$  for L- and D-amino acid residues in peptides.

60°	80°	100°	120°	140°	160°	180°
300°	320°	340°	360°, 0°	20°	40°	60°
180°	160°	140°	120°	100°	80°	
180°	200°	220°	240°	260°	280°	300°
60°	40°	20°	0° 360°	340°	320°	
120°	140°	160°	± 180°	- 160°	- 140°	- 120°
0°	- 20°	- 40°	- 60°	- 80°	- 100°	
0°	20°	40°	60°	80°	100°	120°
- 120°	- 140°	- 160°	± 180°	160°	140°	

The rough estimation of the expected  $^{13}\text{C}$  shift of the carbonyl has been carried out by the theory of magnetic screening associated with chemical bond polarization under the influence of a point electrostatic charge<sup>28, 29</sup>. Taking into account only the first order effects one may express the change

of  $^{13}\text{C}$  screening for the amide bond  $\begin{matrix} \text{N} \\ \diagup \\ \text{C}'=\text{O} \cdots \text{M}^+ \\ \diagdown \\ \text{C}^\alpha \end{matrix}$

$$\Sigma\Delta\sigma = -A_{\text{C}'-\text{O}}E_{\text{C}'=\text{O}} - A_{\text{C}'-\text{N}}E_{\text{C}'-\text{N}} - A_{\text{C}'-\text{C}^\alpha}E_{\text{C}'-\text{C}^\alpha}$$

where  $A$  denotes the coefficients depending on the bond polarizability; and  $E$ , the components of the electrostatic field of the point charge  $\text{M}^+$  along with the direction of the corresponding chemical bond:

$$E_{\text{C}'-\text{X}} = |e|r^{-2} \cos \chi_{\text{X}-\text{C}'}$$

Here  $r$  is the distance from the positive charge to the carbonyl carbon atom, and  $\chi_{\text{X}-\text{C}'}$  is the angle between the direction  $\text{M}^+ \cdots \text{C}'$  and the  $\text{X}-\text{C}'$

bond). The screening for the ester fragment  $\begin{matrix} \text{O} \\ \diagup \\ \text{C}'=\text{O} \cdots \text{M}^+ \\ \diagdown \\ \text{C}^\alpha \end{matrix}$  is expressed in a similar manner.

Adopting the  $A$  values as in refs. 29, 30 one could calculate the total shift  $\Sigma\Delta\sigma$  as a function of the angular coordinates  $\alpha$  and  $\beta$  of the  $\text{M}^+$  ion with regard to the carbonyl bond (Figure 3). As the result we have found that (1) the  $^{13}\text{C}$  signal must undergo the low field shift; (2) the total shift has the maximum value  $-3.9$  p.p.m. (for  $r = 2.8 \text{ \AA}$ ) and depends on the  $\alpha$  and  $\beta$  angles; (3) the  $\Sigma\Delta\sigma$  values and the general appearance of their dependence on the angles are similar for the amide and ester groups.

These conclusions are confirmed by the following experimental data.

With valinomycin†  $[(\text{D-Val-L-Lac-L-Val-D-HyIV})_3]$  we have the following: there are four  $^{13}\text{C}=\text{O}$  signals (see Table 3) of which, owing to the symmetry of the chemical and spatial structure of the molecule<sup>9, 11</sup>, each should correspond to the carbonyls of three identical amino or hydroxy acid

†The following abbreviations for residues are used: HyIlo,  $\alpha$ -hydroxyisovaleric acid; Lac, lactic acid; MePhe,  $N$ -methylphenylalanine; MeLeu,  $N$ -methylleucine; Melle,  $N$ -methylisoleucine.

Table 3. Chemical shifts ( $\pm 0.2$  p.p.m.) of  $^{13}\text{C}=\text{O}$  signals of depsipeptidic and peptidic complexones (with reference to carbon disulphide as internal standard)<sup>a</sup>.

Valinomycin		Beauvericin			"Symmetric" Val <sup>(6)</sup> , Ala <sup>(9)</sup> -antamanide	
Non-complexed	K <sup>+</sup> complex	Non-complexed	K <sup>+</sup> complex	Na <sup>+</sup> complex	Non-complexed	Na <sup>+</sup> complex
20.4 (ester)	16.8	21.6	20.2	19.9	19.0	16.7
20.9 (amide)	19.8	22.9	20.9	20.6	20.8	19.3
21.7 (ester)	17.3				21.5	21.0
22.3 (amide)	21.2				21.5	21.7
					21.8	22.1

<sup>a</sup>The  $^{13}\text{C}$  spectra were obtained at 22.63 MHz on a Bruker HX 90/18-18" spectrometer in the FT mode (2000 to 8000 scans at 0.8 s/scan). Solutions of 100 to 300 mg of substance in 1.4 ml of a 1:1 (v.v) mixture of  $\text{CDCl}_3$  with  $\text{CD}_3\text{OD}$  were used. The complexes were formed by adding KNCS or NaNCS in 1:2.5 to 1:5 mole ratios to the compound.

residues. Now in the spectrum of the  $\text{K}^+$  complex only one  $^{13}\text{C}=\text{O}$  signal falls within the  $\text{C}=\text{O}$  region of the non-complexed valinomycin spectrum. According to the assignment given by Ohnishi *et al.*<sup>27</sup> the two ester  $^{13}\text{C}=\text{O}$  signals undergo a larger downfield shift ( $-3.6$  and  $-4.4$  p.p.m.) than the two amide  $^{13}\text{C}=\text{O}$  signals (both  $-1.1$  p.p.m.). This is in complete agreement with the spatial structure of the  $\text{K}^+$ -valinomycin complex [Figure 4(a)] of

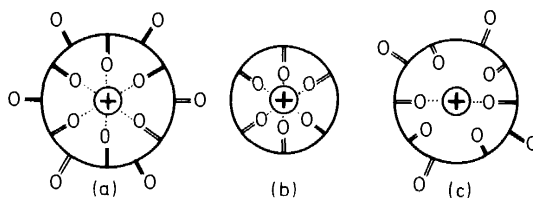


Figure 4. Schematic representation of the carbonyl conformations in alkali ion complexes of valinomycin (a), enniatin B and beauvericin (b), and antamanide (c). For detailed conformation of the compounds see refs. 9, 31, 15, respectively.

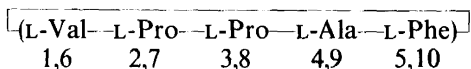
which only ester carbonyls are engaged in strong ion-dipole interaction with the  $\text{K}^+$  ion located in the molecular cavity (the  $\text{O}\cdots\text{K}^+$  distance is 2.7 to 2.9 Å). The direction and the values of these carbonyl shifts are consistent with the above rough calculations. The noticeable paramagnetic shift of the amide  $^{13}\text{C}=\text{O}$  signals seems to mean that, besides taking part in the intramolecular hydrogen bonding, these carbonyls may also be participating in a weak ion-dipole interaction with the  $\text{K}^+$  ion (here the  $\text{O}\cdots\text{K}^+$  distance is around 4.0 to 4.5 Å).



In conformity with the theory of magnetic screening associated with electrostatic bond polarization it follows that the induced chemical shift should decrease with decrease in the  $C=O \cdots M^+$  angle from  $180^\circ$  to  $90^\circ$ . In cyclohexadepsipeptidic enniatin complexes where all six carbonyls are symmetrically located around the central cation<sup>31</sup> [Figure 4(b)], these angles are much less than  $180^\circ$  (in contrast with valinomycin where the corresponding angles are approximately  $180^\circ$ <sup>9, 11</sup>). Hence, despite the practically identical  $O \cdots K^+$  distance in both types of complexes, the change in  $^{13}C=O$  resonance should be less for enniatins. In fact, in the  $K^+$  complex of beauvericin† [(L-MePhe-D-HyIv)<sub>3</sub>]<sup>-</sup> the  $^{13}C=O$  signals undergo less change in position than in the valinomycin complex (Table 3).

When the  $K^+$  cation is replaced by the smaller  $Na^+$  cation, there is a characteristic change in the enniatin conformation, similar to the closing of a flower<sup>31</sup>. This is connected with a decrease in the  $O \cdots M^+$  distance and a straightening out of the  $C=O \cdots M^+$  angle. Both of these spatial structure changes should cause an increase in the downfield shift of the corresponding  $^{13}C=O$  signals. Indeed, the  $^{13}C=O$  resonances for the  $Na^+$  complex of beauvericin are in lower field than those for the  $K^+$  complex (Table 3).

In the  $^{13}C$  spectrum of the  $Na^+$  complex of a symmetrical analogue of antamanide -Val<sup>(6)</sup>,Ala<sup>(9)</sup>-antamanide:



a significant shift to lower field (not less than  $-2.3$  p.p.m.) is observed for only one  $^{13}C=O$  line corresponding to two symmetrically located identical amino acid residues (Table 3). The shifts of the other four  $^{13}C=O$  signals are within the limits of only  $+0.5$  to  $-1.5$  p.p.m. Hence the carbonyls of only two identical residues (apparently Val<sup>(1)</sup> and Val<sup>(6)</sup>) of the cyclodecapeptide approach the cation located within the internal cavity. This conclusion is in complete agreement with the earlier proposed conformation of the  $Na^+$ -antamanide complex<sup>15</sup> of which only two carbonyls of the symmetrically situated Val<sup>(1)</sup> and Phe<sup>(6)</sup> residues are in close contact (*ca.* 2.6 Å) with the central ion [Figure 4(c)].

Besides the ion-dipole interaction induced shifts of the  $^{13}C=O$  signal discussed above, marked changes are observed also in the positions of a number of other signals<sup>26</sup>. For instance, both  $C^\alpha$  signals from D- and L-Val residues of valinomycin are shifted by 2.1 to 3.4 p.p.m. to the low field on complex formation, whereas the  $C^\alpha$  signals from L-Lac and D-HyIv residues are shifted by only  $-1.2$  and  $-0.8$  p.p.m. Obviously such selective shifts are due to a conformational rearrangement of the molecule on complex formation<sup>9, 11</sup>.

Undoubtedly  $^{13}C$  NMR will become a powerful new method among the physicochemical techniques used for investigating the secondary and tertiary structures of peptide systems. A particular stress is to be placed on the considerably higher 'resolving power' of  $^{13}C$  spectroscopy compared with proton NMR. For instance, in the  $^{13}C$  spectra of the most complicated

† Beauvericin stands very close in conformational parameters and complexing properties to the thoroughly investigated<sup>31</sup> enniatin B [(L-MeVal-D-HyIv)<sub>3</sub>]<sup>-</sup>.

compound in this study, 'symmetric' antamanide and its  $\text{Na}^+$  complex, individual signals are clearly visible from all of the 25 structurally non-equivalent carbon atoms. Evidently, the main difficulty that can be seen so far is the assignment of the  $^{13}\text{C}$  signals, in particular those of the carbonyl groups. This can, of course, be overcome by synthesis of compounds selectively labelled with the  $^{13}\text{C}$  or  $^{15}\text{N}$  isotope.

### III. THE APPLICATION OF 'SHIFT REAGENTS' IN THE NMR SPECTROSCOPY OF PEPTIDES†

Another intensively developing field of NMR spectroscopy is the use of the so-called 'shift reagents', a rapidly expanding class of substances, mostly lanthanide complexes such as tris(dipivaloylmetanato)europium(III) [ $\text{Eu}(\text{DPM})_3$ ] as aids in spectral and structural interpretations<sup>32</sup>. Their name stems from the ability of these reagents to associate with polar functional groups and thereby cause substantial specific shifts of the NMR signals of the closely lying nuclei, the magnitude of the shift being largely determined by the distance of the nucleus from the lanthanide ion.

In peptides, such polar groups would be the carbonyls and one could have expected that the 'shift reagents' could shed some light on the spatial arrangement of magnetic nuclei, mainly protons and  $^{13}\text{C}$  surrounding these groups. In particular, we have made use of the shift reagents for determining the configuration of the amide bond and of the rotational states of the  $\text{C}^\alpha\text{-C}^\beta$  fragments.

The assignment of NMR signals to isomers with *cis*- and *trans*-amide bonds is a matter of some difficulty. Apparently it is only the 'shift reagents' which permit a general solution of this problem. It has been shown<sup>33</sup> that with the simple *N*-alkylamides the 'shift reagent' moves the signal of the  $\text{N-CH}_3$  group *cis* to the carbonyl oxygen much farther downfield ( $-9.3$  p.p.m.) than the *trans*- $\text{N-CH}_3$  signal ( $-4.0$  p.p.m.)‡. Obviously this is due to the *cis*-methyl group being located considerably nearer to the complexing position than the *trans*-methyl group. We have obtained similar results for some model and biologically active peptides and depsiptides with *N*-methylated amide bonds.

One of the simplest linear peptides, dipeptide *N*-benzyloxycarbonyl-D-alanyl-L-*N*-methylalanine methyl ester (I) exists as a 3:7 mixture of *cis*- and *trans*-isomers with respect to the *N*-methylamide bond<sup>1, 34</sup>:



†This part has been done in collaboration with Dr V. P. Zvolinskii.

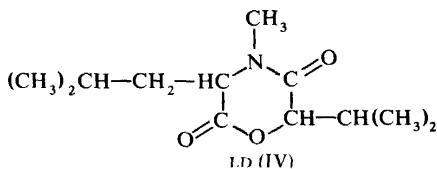
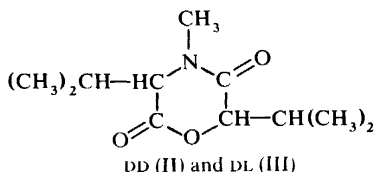
‡The shifts of the signals are extrapolated to 1:1 mole 'reagent'—compound ratio.

## NMR CONFORMATIONAL ANALYSIS

As expected, the addition of  $\text{Eu}(\text{DPM})_3$  shifts the  $\text{N}-\text{CH}_3$  signal of the *cis*-isomer much more ( $-9.1$  p.p.m.) than that of the *trans*-isomer ( $-2.2$  p.p.m.)<sup>†</sup>. This then is a direct and convenient method for assigning signals to configurational isomers of *N*-alkylated peptides.

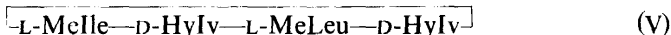
It is noteworthy that the ester  $\text{CH}_3$  signal undergoes a rather small shift:  $-0.4$  and  $-1.6$  p.p.m. for *trans*- and *cis*-isomers of the dipeptide (I), respectively. Apparently the ester group forms a less stable complex with  $\text{Eu}(\text{DPM})_3$  than does amide carbonyl<sup>35</sup>. This conclusion is important for the NMR study of depsipeptides.

The diketomorpholines [for example (II) to (IV)], the simplest cyclic depsipeptides, represent convenient model compounds with *cis*-amide bonds. The  $\text{Eu}(\text{DPM})_3$  reagent



caused a downfield shift of the  $\text{N}-\text{CH}_3$  signals in accord with the values cited above for *cis*- $\text{NCH}_3$  groups: (II)  $-9.3$  p.p.m., (III)  $-9.2$  p.p.m. and (IV)  $-9.5$  p.p.m.

The cyclotetradepsipeptide (V) contains two amide bonds



and the observed shifts when extrapolated to the ratio of one mole  $\text{Eu}(\text{DPM})_3$  per one amide bond were found to be  $-10.2$  and  $-9.9$  p.p.m. indicative of *cis*-configuration, both amide bonds showing them to be of the same configuration in a solution of the depsipeptide (V) as determined by x-ray analysis<sup>36</sup> for the solid state.

The *trans*-configuration of the amide bonds in biologically active cyclohexadepsipeptide enniatin B<sup>31</sup> (VI) was confirmed by the relatively low  $\text{Eu}(\text{DPM})_3$  induced shift of



$\text{N}-\text{CH}_3$  signal:  $-2.0$  p.p.m. per one amide bond. This value closely agrees with the above shift for the *trans*-isomer of (I).

The good correlation of the 'shift reagent' induced shifts for the cyclic depsipeptides (II) to (VI) on the one hand and for dipeptide (I) and the *N*-alkylamides<sup>33</sup> on the other hand confirms the above observation that the ester groups associate more weakly with  $\text{Eu}(\text{DPM})_3$  than do the amide groups.

In addition to the method discussed above for determining the amide bond configuration, one might expect that the 'shift reagents' could be used

<sup>†</sup>The  $^1\text{H}$  NMR spectra were obtained for 0.1 to 0.2 mol/l. solutions in  $\text{CDCl}_3$  with increasing amounts of  $\text{Eu}(\text{DPM})_3$  up to 0.4 mole ratio of 'shift reagent' to compound.

as a supplementary tool for determining the rotational states of the  $C^\alpha-C^\beta$  bond in the amino and hydroxy acid residues. From the NMR spectra it is as yet impossible to define which of the rotamers b or c (Figure 5) is predominant. However, the 'shift reagent' on associating with the carbonyl should be

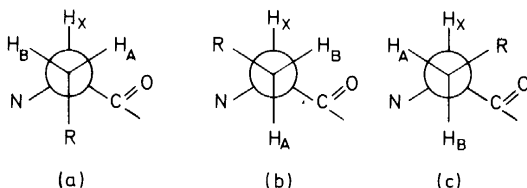


Figure 5. Rotational states of the  $C^\alpha H_X-C^\beta H_A H_B$  fragment in the amino or hydroxy acid sidechain.

expected to cause an equal and considerable shift of both magnetically non-equivalent  $H_A$  and  $H_B$  protons in rotamer b but considerably differing shifts for these protons in rotamer c.

This is confirmed by the following example. The sidechain of the HyIv residue of the diketomorpholines (II) to (IV) could assume the rotational states shown in Figure 6. The values of the vicinal  $^3J(C^\alpha H_X C^\beta H_A)$  coupling constant for compounds (III) and (IV) (2.0 and 2.3 Hz, respectively) indicate

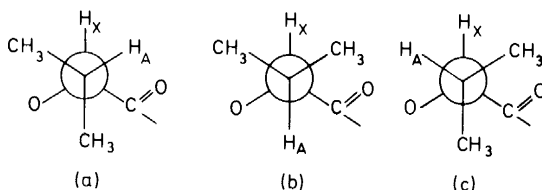


Figure 6. Rotational states of the  $C^\alpha-C^\beta$  bond in the sidechain of the HyIv residue of the diketomorpholines (II)-(IV).

that one of the gauche rotamers a or c is predominant, whereas for compound (II) ( $^3J_{a\beta} = 6.2$  Hz) apparently all three or two (a and b or b and c) rotational states are approximately equally populated. The 'shift reagent'  $Eu(DPM)_3$  induces a substantial shift of the  $C^\beta H_A$  signals in compounds (III) and (IV):  $-11.5$  and  $-11.4$  p.p.m., respectively, practically coinciding with the  $C^\alpha H_X$  shifts:  $-11.6$  and  $-12.0$  p.p.m., respectively. Evidently the large  $C^\beta H_A$  shifts indicate predominance of the rotamers a of which the  $H_A$  proton is situated more closely to the amide carbonyl than in the case of the c rotamer. The differences in the induced shifts of the  $CH_3$  signals [ $-5.7$  and  $-2.3$  p.p.m. for (III) and  $-6.0$  and  $-2.6$  p.p.m. for (IV)] also favour the preference for the a rotamer. If the c rotamer were predominant, these signals would have moved downfield for about the same distance.

With diketomorpholine (II), as could have been expected for a levelling of the distribution of the  $C^\alpha-C^\beta$  rotamers, there is a substantially lower  $C^\beta H_A$  shift ( $-8.4$  p.p.m.) and an equalizing of the  $CH_3$  shifts ( $-4.6$  and  $-3.5$  p.p.m.).

It is to be stressed that an addition of  $\text{Eu}(\text{DPM})_3$  up to a molar ratio of 2:5 does not affect the relative weights of the spatial forms of the molecule. This is evident from the constant integral intensities of the *cis*- and *trans*-isomer signals of dipeptide (I) and from the constancy of the  $^3J_{\alpha\beta}$  coupling constants for diketomorpholines (II) to (IV).

#### IV. ASSIGNMENT OF NH SIGNALS AND DISCLOSING OF 'HIDDEN' LINES IN THE NMR SPECTRA OF PEPTIDES

Undoubtedly an urgent problem in the NMR spectroscopy of peptides is a reliable assignment of the signals. For the proton NMR difficulties arise mainly in the overlap of the  $\text{C}^\alpha\text{H}$  and  $\text{C}^\beta\text{H}$  signals and in the assignment of the NH doublets to the proper amino acid residue. The most direct way of assigning the NH signals is determination of the multiplicity of the signal from the adjacent  $\text{C}^\alpha\text{H}$  group. In this way one may distinguish between amino acid residues with  $\text{NH}-\text{C}^\alpha\text{H}-\text{C}^\beta\text{H}$ ,  $\text{NH}-\text{C}^\alpha\text{H}-\text{C}^\beta\text{H}_2$  and  $\text{NH}-\text{C}^\alpha\text{H}-\text{CH}_3$  fragments. Following this there remains to be determined the chemical shift and multiplicity of the  $\text{C}^\beta\text{H}$  signal.

The most direct and convenient approach to this problem is the use of the INDOR<sup>37</sup> and detection of signals on combination frequencies<sup>38</sup>. In both techniques one of the radiofrequency fields is swept over the region of the hidden signal. To illustrate their use we shall discuss the interpretation of the proton spectrum of 'symmetric' Val<sup>(6)</sup>,Ala<sup>(9)</sup>-antamanide.

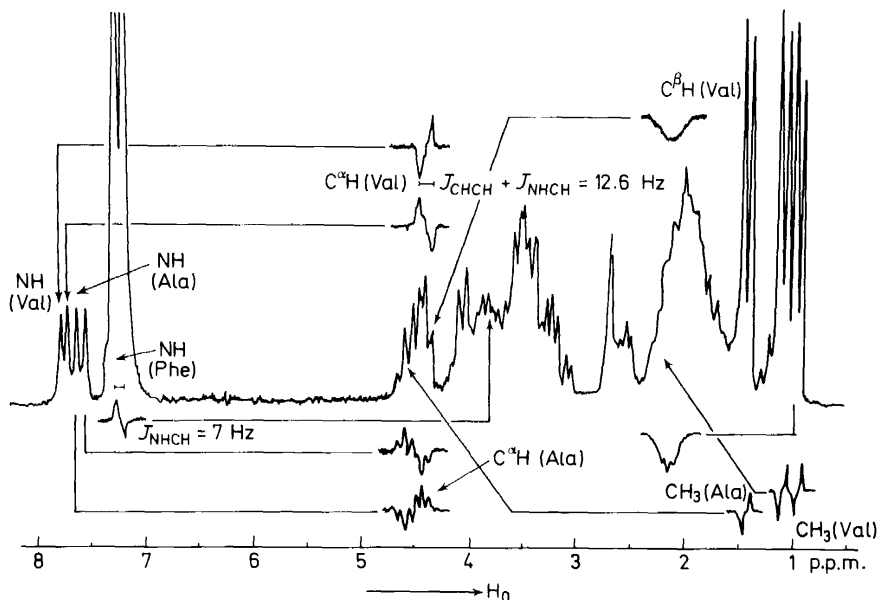


Figure 7. Application of INDOR for signal assignments in the  $^1\text{H}$  NMR spectrum of 'symmetric' Val<sup>(6)</sup>,Ala<sup>(9)</sup>-antamanide (100 MHz,  $\text{CDCl}_3$ ).

Recently INDOR spectroscopy has been used to obtain the  $^{13}\text{C}$  spectra of amino acids<sup>29</sup> and to illustrate the coupling of the sidechain CH—CH protons of amino acids and their derivatives<sup>39</sup>, and to reduce the sidechain,  $\text{C}^\alpha$  and amino-aromatic proton regions in the peptidic antibiotics spectra<sup>40</sup>.

Here we apply the INDOR technique to the assignment of the NH signal in the peptide  $^1\text{H}$  NMR spectrum (Figure 7). On monitoring the components of the low field NH doublet (7.76 p.p.m.), the INDOR response in the  $\text{C}^\alpha\text{H}$  region has the appearance of a doublet which is specific for a  $\text{C}^\alpha\text{H}$ — $\text{C}^\beta\text{H}$  fragment with one  $\beta$  proton. For the given compound this fragment corresponds to the L-Val residues. The separation between the doublet components is the sum of the  $^3J_{\text{NHCH}}$  and  $^3J_{\alpha\beta}$  coupling constants. On determining the former from the corresponding NH signal one can obtain the latter which cannot be determined from the ordinary spectrum. Further consecutive use of INDOR permits determination of the  $\text{C}^\beta\text{H}$  signal and after this of the  $\text{CH}_3$  signals of the L-Val residues.

For the other NH doublet at 7.61 p.p.m. the  $\text{C}^\alpha\text{H}$  INDOR response reveals a sextet, corresponding to the  $\text{C}^\alpha\text{H}$ — $\text{CH}_3$  fragment, i.e. to the alanyl residue.

The third NH signal stemming from the remaining phenylalanyl residues of the 'symmetric' antamanide is obscured by signals of the aromatic and solvent protons. In order to obtain its INDOR response one must test several peaks in the 3.1 to 4.2 p.p.m. region. Only with two of them at *ca.*

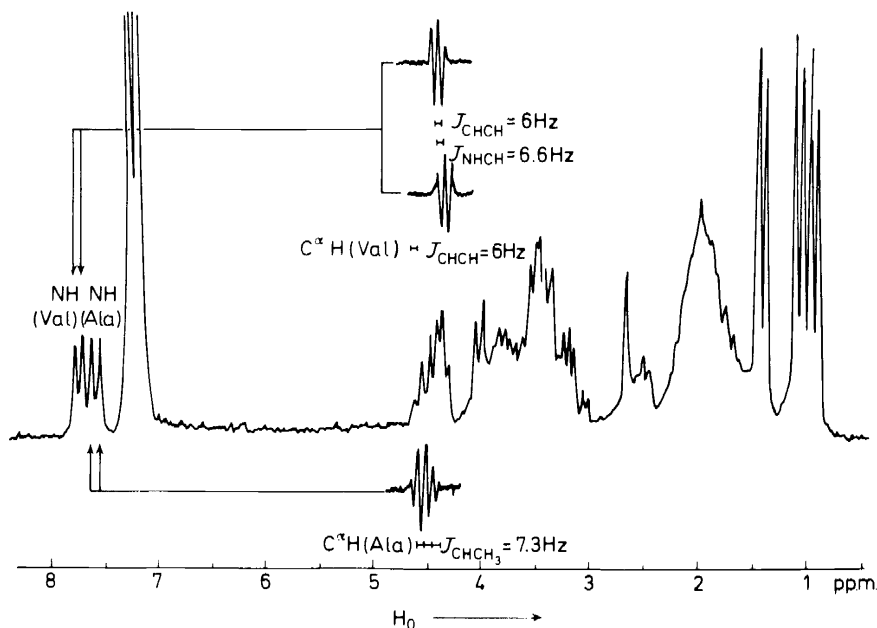


Figure 8. Application of the combination frequency technique for NH signal assignments in the  $^1\text{H}$  NMR spectrum of Val<sup>(6)</sup>,Ala<sup>(9)</sup>-antamanide (100 MHz,  $\text{CDCl}_3$ ).

3.8 p.p.m. was it possible to obtain the NH INDOR response. From the splitting of this doublet response one could determine the phenylalanyl  $^3J_{\text{NHCH}}$  coupling constant.

From the viewpoint of the instruments used, the combination frequency signal technique is more complicated than the INDOR technique but it gives a clearer response with higher resolution and sensitivity. *Figure 8* shows the application of this technique for the assignment of the NH signal. When both components of the low field NH doublet are irradiated, the combination frequency C $^{\alpha}$ H response appears as a doublet<sup>38†</sup> which corresponds to the valyl C $^{\alpha}$ H—C $^{\beta}$ H $\leftarrow$  fragment. The separation between the response components is directly equal to the  $^3J_{\alpha\beta}$  coupling constant of the protons in this fragment. The distances between the responses which are detected on the two alternative combination frequencies ( $\nu_{\text{sweep}} - \nu_1 + \nu_2$  and  $\nu_{\text{sweep}} + \nu_1 - \nu_2$ , where  $\nu_{\text{sweep}}$  is the sweep frequency, and  $\nu_1$  and  $\nu_2$  are the irradiation frequencies) is equal to the  $^3J_{\text{NHCH}}$  coupling constant. The combination frequency response for the other NH signal is a quartet, which is specific for the alanyl C $^{\alpha}$ H—CH $_3$  fragment.

It goes without saying that the INDOR and combination frequency signals techniques do not exclude the simpler spin decoupling. But without doubt they have their advantages in the accuracy and elegance of the experiment.

## V. CONCLUSION

The approaches presented above have far from exhausted the capacity of NMR in studies of peptide conformations. The problems next in turn are a detailed analysis of the  $^{13}\text{C}$  chemical shifts with respect to conformation, use of relaxation parameters, application of the spin labels and paramagnetic probes, elucidation of the angular dependence of the vicinal  $^{15}\text{N}$ —H coupling constant for evaluation of the  $\psi$  angle and other techniques which are only looming in the background.

## REFERENCES

- <sup>1</sup> V. F. Bystrov, S. L. Portnova, V. I. Tsetlin, V. T. Ivanov and Yu. A. Ovchinnikov, *Tetrahedron*, **25**, 493 (1969).
- <sup>2</sup> V. F. Bystrov, S. L. Portnova, T. A. Balashova, V. I. Tsetlin, V. T. Ivanov, P. V. Kostetzky and Yu. A. Ovchinnikov, *Tetrahedron Letters*, 5225 (1969); *Zh. Obshch. Khim.* **41**, 407 (1971) (in Russian).
- <sup>3</sup> M. Karplus, *J. Chem. Phys.* **30**, 11 (1959); *J. Amer. Chem. Soc.* **85**, 2870 (1963).
- <sup>4</sup> R. J. Abraham and K. G. R. Pachler, *Molec. Phys.* **7**, 165 (1963-1964).
- <sup>5</sup> L. Pauling, *The Nature of the Chemical Bond*, 3rd ed. Cornell University Press: Princeton (1960).
- <sup>6</sup> J. G. Powles and J. H. Strange, *Disc. Faraday Soc.* **34**, 30 (1962).
- <sup>7</sup> A. J. R. Bourn and E. W. Randall, *Molec. Phys.* **8**, 567 (1964).
- <sup>8</sup> L. A. La Planche, *Ph. D. Thesis*, University of Michigan (1963).
- <sup>9</sup> V. T. Ivanov, I. A. Laine, N. D. Abdullaev, V. Z. Pletnev, G. M. Lipkind, S. F. Arkhipova, L. B. Senyavina, E. N. Meshcheryakova, E. M. Popov, V. F. Bystrov and Yu. A. Ovchinnikov, *Khim. Prir. Soed.* 221 (1971) (in Russian).

†The number of the response components is determined as the least of the 'up' and 'down' peaks<sup>38</sup>.

- <sup>10</sup> Yu. A. Ovchinnikov, V. T. Ivanov, V. F. Bystrov, A. I. Miroshnikov, E. N. Shepel, N. D. Abdullaev, E. S. Efremov and L. B. Senyavina, *Biochem. Biophys. Res. Commun.* **39**, 217 (1970).
- <sup>11</sup> V. T. Ivanov, I. A. Laine, N. D. Abdullaev, L. B. Senyavina, E. M. Popov, Yu. A. Ovchinnikov and M. M. Shemyakin, *Biochem. Biophys. Res. Commun.* **34**, 803 (1969).
- <sup>12</sup> M. Llines, M. P. Klein and J. B. Neilands, *J. Molec. Biol.* **52**, 399 (1970).
- <sup>13</sup> A. Zalkin, J. D. Forrester and D. H. Templeton, *J. Amer. Chem. Soc.* **89**, 1810 (1966).
- <sup>14</sup> K. D. Kopple, *Biopolymers*, **10**, 1139 (1971).
- <sup>15</sup> V. T. Ivanov, A. I. Miroshnikov, N. D. Abdullaev, L. B. Senyavina, S. F. Arkhipova, N. N. Uvarova, K. Kh. Khalilulina, V. F. Bystrov and Yu. A. Ovchinnikov, *Biochem. Biophys. Res. Commun.* **42**, 654 (1971).
- <sup>16</sup> V. T. Ivanov, A. I. Miroshnikov, S. A. Koz'min, E. N. Meshcheryakova, L. B. Senyavina, N. N. Uvarova, K. Kh. Khalilulina, V. A. Zabrodin, V. F. Bystrov and Yu. A. Ovchinnikov, *Khim. Prir. Soed.* in press (in Russian).
- <sup>17</sup> J. W. Emsley, J. Feeney and H. L. Sutcliffe, *High Resolution NMR Spectroscopy*, Vol. I, Chapters 8.13.2 and 8.15.1. Pergamon: Oxford (1965).
- <sup>18</sup> J. T. Edsall, P. J. Flory, J. C. Kendrew, A. M. Liquori, G. Nemethy, G. N. Ramachandran and H. A. Scheraga, *J. Molec. Biol.* **15**, 399 (1966) and **20**, 589 (1966); *J. Biol. Chem.* **241**, 1004 and 4167 (1966); *Biopolymers*, **4**, 121 and 1149 (1966).
- <sup>19</sup> IUPAC-IUB Commission on Biochemical Nomenclature, *J. Molec. Biol.* **52**, 1 (1970); *J. Biol. Chem.* **245**, 6489 (1970); *Biochemistry*, **9**, 3471 (1970); *Europ. J. Biochem.* **17**, 193 (1970).
- <sup>20</sup> S. L. Portnova, T. A. Balashova, V. F. Bystrov, V. V. Shilin, Ya. Bernat, V. T. Ivanov and Yu. A. Ovchinnikov, *Khim. Prir. Soed.* 323 (1971) (in Russian).
- <sup>21</sup> M. Barfield and M. Karplus, *J. Amer. Chem. Soc.* **91**, 1 (1969).
- <sup>22</sup> C. M. Thong, D. Canet, P. Grenger, M. Marraud and J. Neel, *C.R. Acad. Sci., Paris, Sér. C*, **269**, 580 (1969).
- <sup>23</sup> R. Schwyzer, private communication cited by R. J. Weinkam and E. C. Jorgensen, *J. Amer. Chem. Soc.* **93**, 7038 (1971).
- <sup>24</sup> G. N. Ramachandran, R. Chandrasekaran and K. D. Kopple, *Biopolymers*, **10**, 2113 (1971).
- <sup>25</sup> V. Solkan and V. F. Bystrov, *Tetrahedron Letters*, in press (1973).
- <sup>26</sup> V. F. Bystrov, V. T. Ivanov, S. A. Koz'min, I. I. Mikhaleva, K. Kh. Khalilulina and Yu. A. Ovchinnikov, *FEBS Letters*, **21**, 34 (1972).
- <sup>27</sup> M. Ohnishi, M.-C. Fedarko, J. D. Baldeschwieler and M. F. Johnson, *Biochem. Biophys. Res. Commun.* **46**, 312 (1972).
- <sup>28</sup> A. D. Buckingham, *Canad. J. Chem.* **38**, 300 (1960).
- <sup>29</sup> W. J. Horsley and H. Sternlicht, *J. Amer. Chem. Soc.* **90**, 3738 (1968).
- <sup>30</sup> K. G. Denbigh, *Trans. Faraday Soc.* **36**, 936 (1940).
- <sup>31</sup> Yu. A. Ovchinnikov, V. T. Ivanov, A. V. Evstratov, V. F. Bystrov, N. D. Abdullaev, E. M. Popov, G. M. Lipkind, S. F. Arkhipova, E. S. Efremov and M. M. Shemyakin, *Biochem. Biophys. Res. Commun.* **37**, 665 (1969).
- <sup>32</sup> C. C. Hinckley, *J. Amer. Chem. Soc.* **91**, 5160 (1969).
- <sup>33</sup> L. R. Isbrandt and M. T. Rogers, *Chem. Commun.* 1378 (1971).
- <sup>34</sup> S. L. Portnova, V. F. Bystrov, T. A. Balashova, V. T. Ivanov and Yu. A. Ovchinnikov, *Izvest. Akad. Nauk SSSR, Ser. Khim.* 825 (1970) (in Russian).
- <sup>35</sup> J. K. M. Sanders and D. H. Williams, *J. Amer. Chem. Soc.* **93**, 641 (1971).
- <sup>36</sup> J. Konnert and I. L. Karle, *J. Amer. Chem. Soc.* **91**, 4888 (1969).
- <sup>37</sup> E. B. Baker, *J. Chem. Phys.* **37**, 911 (1962).
- <sup>38</sup> V. F. Bystrov, *J. Molec. Spectrosc.* **28**, 81 (1968);  
V. A. Afanas'ev and V. F. Bystrov, *J. Magn. Res.* **3**, 357 (1970).
- <sup>39</sup> W. A. Gibbons, H. Alms, J. Song and H. R. Wyssbröd, *Proc. Nat. Acad. Sci., Wash.* **69**, 1261 (1972).
- <sup>40</sup> W. A. Gibbons, H. Alms, R. S. Bockman and H. R. Wyssbröd, *Biochemistry*, **11**, 1721 (1972).