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^{13}C NMR SPECTROSCOPIC STUDIES ON THE CONFORMATION DURING STEPWISE SYNTHESIS OF PEPTIDES BOUND TO SOLUBILIZING POLYMER SUPPORTS[a]

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Abstract—The C-13 {C-13–H-1} triple resonance technique allows constitutional and conformational studies on polypeptides bound to mono- and bi-functional solubilizing polyoxyethylene supports. High resolution shows distinct differences between random coil and α-helical conformations. This method is a valuable additional tool for control of the growing peptide during stepwise synthesis on soluble polymeric supports. Examples of partial sequences of the polypeptide antibiotic alamethicin and sequential analogs $(Aib\text{-}Ala)_n$ demonstrate the applicability for peptide polyoxyethylene esters with mean molecular masses between 2000 and 10,000.

One of the principal disadvantages of multistep syntheses on polymeric supports is the impossibility of direct spectroscopic characterisation of intermediate products. Thus the Merrifield synthesis of peptides[1] has been shown to yield mixtures of peptides of various lengths[2] and other side products which cannot be detected during synthesis. Clearcut analytical controls are possible only on the cleaved peptides. However this is a very impractical, time consuming procedure which leads to additional high losses in case of long sequences. Therefore, analytical methods are desirable allowing the characterisation of peptides without cleavage from its polymeric support or otherwise interrupting synthetic procedures. All of the numerous approaches for determining coupling yields or end group analysis are by no means satisfying or generally applicable to detect side reactions and non peptidic impurities so far. The application of high resolution NMR is not possible for the commonly used support of crosslinked insoluble polystyrene or the real solid supports such as modified glass beads.[3] A soluble and even peptide solubilizing support is used for the liquid-phase method according to Mutter and Bayer[4] which should allow recording of NMR spectra. For preparative reasons polyoxyethylene (POE) supports with mean molecular masses higher than 2000 must be used, since the resulting peptide polyoxyethylene ester should be recrystallizable in order to remove low molecular weight impurities. On the other hand, the polymer molecule should have a molecular mass lower than about 10,000 in order to reach a practicable loading; however, a low molecular mass of polyoxyethylene may result in decreasing solubilizing properties on increasing peptide chain length. Thus, in case of the synthesis of a fully protected tridecapeptidamide of the *N*-terminal secretin sequence bifunctional polyoxyethylene 6000 provided a suitable solubility in all solvents commonly used in peptide chemistry.[5] Polyoxyethylene 6000 is also suitable for the combined use of soluble supports and solid polymer reagents.[6,7] For the alternating liquid–solid phase peptide synthesis[8] polymer chains of a molecular mass lower than 4000 are required in connection with PBOC-amino acids attached to crosslinked polystyrene.

Nuclear magnetic resonance of polyoxyethylene

Polyoxyethylene has the advantage of showing only one signal in proton and carbon NMR spectra beside signals of terminal groups. This signal is too intense in the case of polyoxyethylenes with molecular masses higher than 1000, and the weak signals of the polymer bound peptide residue are not observable by techniques like block averaging. The POE signal will surmount the dynamic range of the currently used computers up to 20 bit word lengths. Thus one must prevent the polymer from giving rise to an induction signal in the receiver coil. This can be done by two techniques: firstly by selective non excitation of the POE signal, e.g. tailored excitation[9] or secondly by selective saturation of the POE resonance, which was the method we used to tackle our problem. As the peptide is covalently linked to the polymer, the relaxation times (T_1) of the peptide carbons are similar to those of the methylene carbons of polyoxyethylene. Thus saturation due to different T_1 is not possible.[10] However in a double resonance experiment the POE peak can be saturated selectively thus being eliminated from the spectrum. For most problems in peptide chemistry the carbon nucleus will be more advantageous since proton spectra up to 100 MHz are rather complicated even in cases of relatively short oligopeptides. Most of the singlets of the ^{13}C NMR spectrum will be unequivocally assignable for peptides up to about 10–20 residues. For control of stepwise peptide synthesis the signals of the last and second last residues are particularly interesting. In cases where weak additional signals are observed in the immediate neighbourhood of the second last residue one has to conclude

[a]This paper was partially referred to by D. L. at the 6th Int. Conf. on Magn. Res. on Biological Systems, St. Jovite, Canada (Sept. 76) and at the Chemiedozententagung in Marburg, Germany (March 1977).

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that the coupling step was incomplete. When satellites of the peaks of the last *N*-protected residue appear one can be sure that the polymeric product still contains an excess of this amino acid. Solvent peaks arising from incomplete drying as well as protecting groups which were not removed completely are easily identified. Since the inner chain carbons of polyoxyethylene absorb at 71.5 ppm, this peak will not interfere with other signals from molecules relevant in peptide chemistry. In this region only the threonine C_β-signal and the methylene carbon of the benzyloxycarbonyl group are found, both absorbing at about 67 ppm. In this context the broadness of the applied frequency is of particular interest. In the case of the homo-decoupling FT technique with a single coil system very weak rf powers are needed for decoupling. Therefore a small band of only 20–25 Hz (about 1 ppm) will be wiped out. Commercial instruments are usually not designed for C-13{C-13} double resonance. A suitable spectrometer is described in the experimental part.

The ratio of the number of POE-carbons to the signal carbons of one amino acid residue is about 140:1 in the case of a polyoxyethylene 6000. Thus the molecular mass of a peptide polyoxyethylene ester with a relatively short sequence will be governed by the polymer support, while the relative amount of a single amino acid with respect to the whole molecule is very small. Therefore the concentration should be 0.05 mol/l or higher with respect to the peptide bound to the polymer in order to obtain reasonable signal to noise ratios. This requires a concentration of peptide polyoxyethylene ester of 1 g/ml and higher. The excellent solubilizing properties of polyoxyethylene for both polar and apolar sequences provide these conditions for NMR solvents such as water, methanol tri- and dichloromethane.

Application of the method

In the context of conformational and activity studies of the membrane modifying peptide antibiotics alamethicin,[11–13] suzukacillin[14,15] and trichotoxin A-40[16] we synthesized some partial sequences and model peptides on the polyoxyethylene support in order to study the conformational behaviour of these peptides. Due to their high content (40–60%) of the unusual sterically hindered α-branched amino acid 2-methylalanine (α-aminoisobutyric acid or Aib) these peptides exhibit a characteristic *N*-terminal α-helical region, which is a prerequisite for their insertion into the lipophilic region of lipid membranes. Circular dichroism measurements revealed about a doubling of the α-helix content in going from hydrophilic to lipophilic media.[11,14,16] However, the calculation of the percentage of α-helix content from CD data of these unusual non-protein peptides[11,12,14,16] alone is rather uncertain. It requires additional support by methods such as ^{13}C NMR. Therefore we measured the ^{13}C NMR of the decapeptide BOC-(Aib-L-Ala)$_5$-OPOE$_{6000}$ built as a model for the α-helices of membrane modifying peptides.[12,17] Figure 1 demonstrates the same resolution of peaks as in the case of low molecular weight compounds with the same digital resolution. Besides the decapeptide resonance there are four additional signals arising from the terminal methylene carbons of polyoxyethylene. These are free $-OCH_2-CH_2-OH$ groups and peptide ester groups $-OCH_2-CH_2-O-CO-CHR-NH-$. The relative intensities of these groups allow a rough estimation of the loading of the polymer end groups with peptide residues. In agreement with the amino acid analysis one may roughly estimate about 60%. Although the use of monofunctional polyoxyethylene may have some advantage we prefer bifunctional polymers for ^{13}C NMR studies since monoterminal methyl ether groupings give rise to additional strong signals and decrease the relative amount of the peptide. The spectrum of the *N*-protected polymer bound peptide (Fig. 1) clearly shows the three characteristic signals of the *t*-butyloxycarbonyl group and the carbons of alanine and 2-methylalanine appearing in the commonly observed shift regions.[11,14] There are different chemical shifts within the Ala-C_α region and within the Aib-C_β region (Table 1). This is an important sign for the onset of a partial α-helical conformation supporting our findings on alamethicin.[11] Thus Ala-C_α carbons in a helical environment are shifted 2 ppm to lower field compared to those in a random-coil environment.[11] On the other hand Aib-C_α or Ala-C_β carbons are not influenced to such an extent. In case of the decapeptide ester BOC-(Aib-L-Ala)$_5$-OPOE (Fig. 1) we observe the characteristic and relatively large shift difference between Aib-C_β carbons of about 3.2 ppm which is

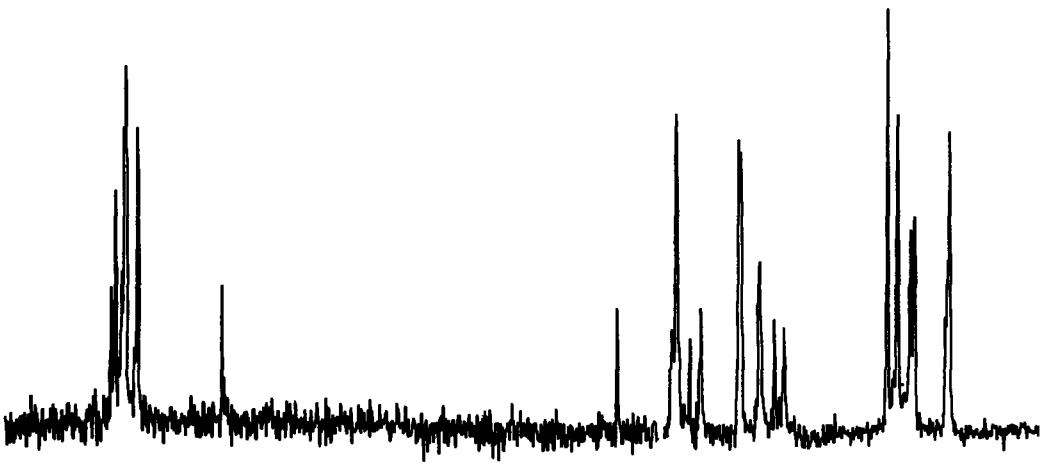

Fig. 1. ^{13}C NMR spectrum of BOC-(Aib-L-Ala)$_5$-OPOE$_{6000}$ in methanol (T = 303 K, C = 1 g/ml, 150 K interferograms).

Table 1. ^{13}C NMR Chemical shifts[a] of (Aib-Ala)$_n$ polyoxyethylene esters

	Ala		Aib					
	C_α	C_β	C_α	C_β	$\Delta\delta C_\beta$	CO	CO	
Boc-Ala-(Aib-Ala)$_2$-	52,7	17,7	57,5 (2x)	26,9		176,5	176,6	155,2 C=O
OPOE$_{6000}$	51,3	17,5	57,6	25,6 (2x)	1,3	175,8	175,9 (2x)	80,5 Cquart
	49,7	16,5		25,1				28,1 CH_3
BOC-Ala-(Aib-Ala)$_4$-	53,5 (3x)	18,6	57,3 (2x)	27,5 (2x)		176,5	176,1	156,2
OPOE$_{6000}$	50,3	17,8	57,3 (2x)	27,1 (2x)	3,3	177,0	176, (3x)	81,3
	45,2	17,2 (2x)		24,7		175,5	175,9 (2x)	28,0
				25,3 (3x)				
BOC-(Aib-Ala)$_5$-	53,3 (3x)	17,7	57,5 (3x)	27,0 (3x)		176,0	176,4	158,0
OPOE$_{6000}$	51,0	17,5	57,0 (2x)	24,6 (2x)	3,2	176,5 (2x)	174,6 (3x)	81,2
	49,3	16,9 (3x)		25,8 (2x)				28,0
$^+H_2$-(Aib-Ala)$_3$-	51,2	17,1	57,9	26,4		176,4	175,8 (3x)	
OPOE$_{6000}$	49,5	16,7 (2x)	57,5 (2x)	26,0	2,6	173,1		
	48,8			24,2 (3x)				
				23,8				
$^+H_2$-Ala-(Aib-Ala)$_3$	52,7	17,7 (2x)	58,0	25,3	2,0	177,0 (2x)	176,3	
OPOE$_{6000}$	51,3	17,1	57,6 (2x)	25,5	2,1	175,1 (2x)	174,0	
	50,5	16,8		25,3 (2x)		171,0		
	49,9			24,7				
$^+H_2$-(Aib-Ala)$_4$	52,8	17,5	57,9	26,5	26,3 2,5	177,5	176,8	
OPOE$_{6000}$	51,6	16,8 (3x)	57,5 2x)	25,5	24,4	176,3	175,5	
	50,8		57,3		(2x)	174,3	173,8 (2x)	
	49,2			24,0 (3x)		173,2		
$^+H_2$-Ala-(Aib-Ala)$_4$	53,3 (2x)	17,7 (3x)	57,8 (4x)	26,8 (3x)		178,2	177,2 (2x)	
OPOE$_{6000}$	51,0	17,5 (2x)		25,9	24,7 2,7	176,6	176,0	
	50,5	16,9		24,5	24,1	175,4	175,5 (2x)	
					(2x)	171,2		
	49,4							

a) in ^{2}H-methanol relativ to internal tetramethyl silane (δ = 0ppm); relative intensities are given in brackets.

comparable to that in alamethicin with 3.5 ppm.[11] Also the absolute chemical shifts of 23.8 and 27.0 ppm agree well with those of alamethicin (23.1 and 26.6 ppm). The same agreement is found between Ala-C_α carbons located in the middle of the decapeptide appearing at 53.8 ppm and the corresponding alamethicin signals at 53.4 ppm. On the other hand Ala-C_α carbons in a random coil environment resonate at about 51.5 ppm in methanolic solutions. If the peptide chain length is too short for formation of an α-helix, e.g. in the case of the pentapeptide ester BOC-L-Ala-(Aib-Ala)$_2$-POE (Fig. 2) the difference in chemical shift of the Aib-C_β carbons is only 1.8 ppm and it is mainly due to intrinsic diastereotopicity induced by the neighbouring L-alanine residues. In this context one should notice that it is only the low-field signal which shifts back to that region normally occupied by Aib-C_β resonances (Table 1). The C_α resonance of the middle alanine in the pentapeptide occurs at 51.8 ppm as in other short alanine oligopeptides.[11,18]

The validity of these rules has also been examined and confirmed for other model compounds. The hexapeptide ester BOC-(Aib-L-Ala)$_3$-OPOE exhibits predominantly random coil conformation. The nonapeptide ester BOC-L-Ala-(Aib-Ala)$_4$-POE forms appreciable amounts of α-helix. The *N*-terminal protonated nonapeptide ester $^+H_2$-Ala-(Aib-Ala)$_4$-OPOE also has some tendency for partially α-helical conformation. However, the interpretation of the spectra of protonated oligopeptides is more difficult because the positive charge produces charge shifts in the last and second last amino acid of the *N*-terminus. For example the spectrum of $^+H_2$-L-Ala-(Aib-Ala)$_3$-OPOE alone is not sufficient for assigning the conformation. Yet, comparing the higher and lower homologs one can conclude that the heptapeptide assumes no α-helix.

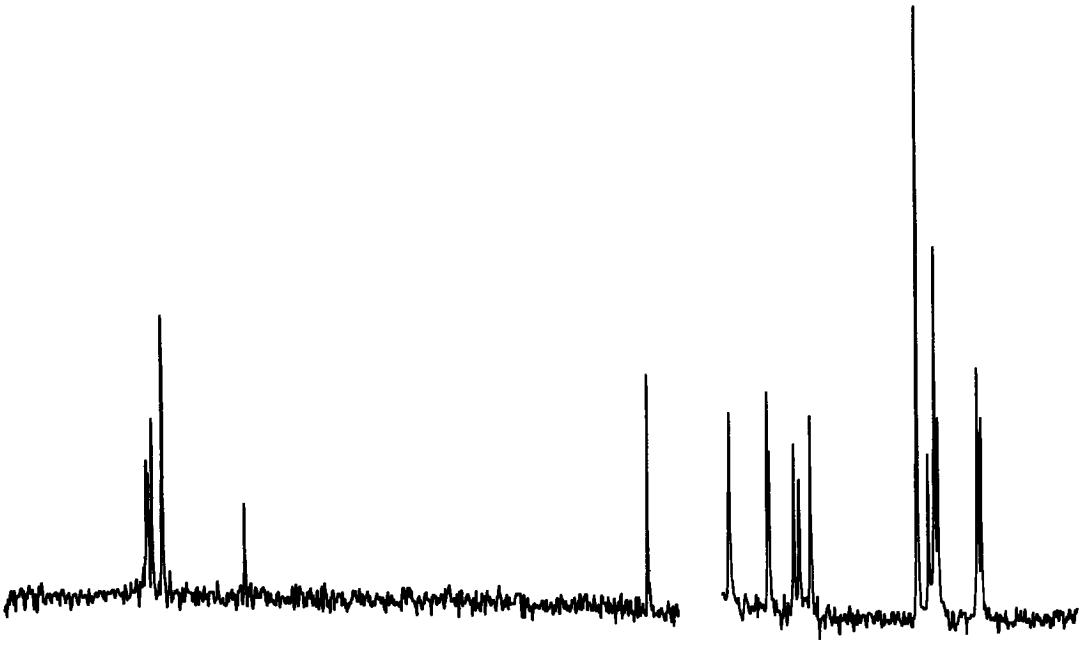

Fig. 2. ^{13}C NMR Spectrum of BOC-L-Ala-(Aib-Ala)$_2$-OPOE$_{6000}$ in methanol (T = 303 K, C = 1 g/ml, 100 K interferograms).

For the longer peptides there could be a change between random coil and α-helical conformations. Since no signal splittings can be observed these conformational transitions would have to be fast on the NMR time scale. In addition excellent agreement between the chemical shifts of alamethicin and the long-chain model peptides indicates that such an equilibrium indeed exists. Circular dichroism and optical rotatory dispersion data on natural and synthetic sequences of alamethicin[17] give proof of a helical structure in alamethicin and its conformational mobility. Thus these studies may also serve as a basis for attempts to quantitatively correlate CD and ^{13}C NMR data for conformational problems of Aib peptides.

Finally some examples for the application of NMR in the control of liquid phase peptide synthesis are discussed. The purity of the *t*-butyloxycarbonyl-L-glutamine-polyoxyethylene ester (Fig. 3) had to be controlled before the first coupling step. The terminal methylene POE-carbons appear between 65 and 70 ppm, and one clearly observes various signals arising from impurities besides the regular signals of BOC-Gln-OPOE (Table 2). Two of these originate from ethanol, which was the solvent used for recrystallization. In the immediate neighbourhood of glutamine peaks there are satellite signals. Comparison with known values and the fact that a carboxyl signal appears at low field these satellites must be attributed to non coupled BOC-L-Gln-OH not removed by one recrystallization from ethanol. In this case relatively short chain polyoxyethylene (mean molecular mass 2000) was tested for synthesis. This support has a melting point too low for efficient purification by recrystallization. If the molecular mass is below 4000 relatively high losses in the crystallization step are observed, furthermore low molecular weight components, e.g. excess of BOC-amino acids, are difficult to remove. Since the distance between signals of the desired product and those of the satellites is different one should be able to recognize any such impurities. Figure 4 shows that the dipeptide polyoxyethylene ester BOC-L-Leu-Gly-OPOE$_{10,000}$ was synthesized with sufficient purity, since the spectrum reveals only the expected signals (Table 2). In case of doubtfull peaks in the noise a repeated recording gives final proof. According to our experiences a polymer with mean molecular mass 6000 was found to be best for practical peptide synthesis and for spectroscopic control of synthesis. The concentration limits for recognizing impurities depend of course on the relative ratio of peptide polymer and impurity. Impurities in the range of 5% are no problem, and for the detection of 1% impurities sample cells with diameters of 15 mm and more are required. Since the substance can be used for further synthesis after measurement the use of large amounts of samples is acceptable. Of course this method should be applied as an additional tool to the well established methods in peptide chemistry. However it is obvious that the method may be applied to a variety of similar problems involving NMR spectroscopic measurements of substances bound to soluble polymers.

EXPERIMENTAL

^{13}C NMR measurements

The spectra were recorded on a Bruker HFX-90 NMR spectrometer (15" magnet, 22.63 MHz) with broad band decoupling. For the C-13{C-13} double resonance experiments a second C-13 frequency was generated with a Schomandl decade ND-100 M (synchronized to the master quarz) with a 1V output. It is fed into the multipulse generator B-SV 3 PM via an additional power attenuator. The proton frequency goes directly into the broad band amplifier. Similar arrangements are described by Mann.[19] With respect to the desired resolution 4 K or 8 K interferograms have been accumulated. Depending on the concentration 50–150 K interferograms were accumulated. For 1 g samples we used the normal 10 mm cells, larger amounts were measured in 15 mm cells. The samples were measured in ^2H_2O, $^{12}C^2H_3O^2H$ or $^{12}C^2HCl_3$, and the cell temperature was 303 K.

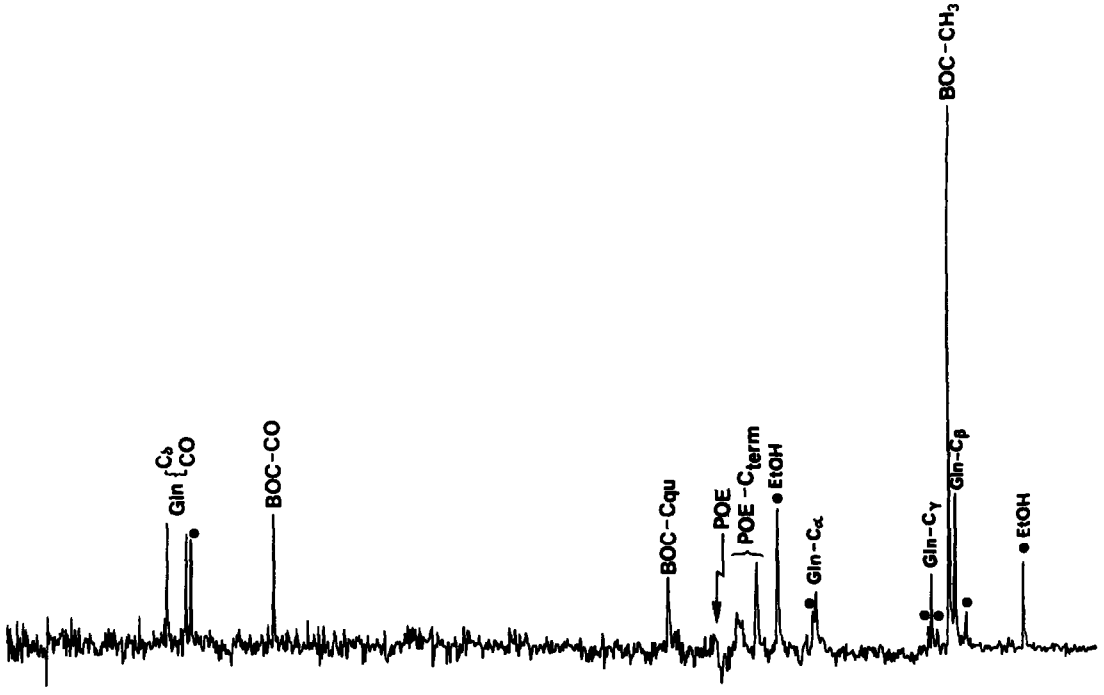

Fig. 3. ^{13}C NMR spectrum of impure *t*-butyloxycarbonyl-L-glutamine polyoxyethylene ester (mean molecular mass 2000) in deuterated water (T = 303 K, C = 1 g/ml, 50 K interferograms).

Table 2. ^{13}C NMR shifts of *N*-protected amino acid derivatives bound to polyoxyethylene

BOC-Gln-OPOE$_{2000}$ [b]	C_α 54,5	C_β 32,5	C_γ 27,7	C_δ 179,6	CO 174,6	BOC 158,9; 83,2; 29,0	
BOC-Gln-OPOE$_{3000}$ [a]	C_α 54,5	C_β 32,2	C_γ 28,4	C_δ 176,5	CO 173,5	BOC 157,1; 80,1; 28,8	
BOC-Val-OPOE$_{2000}$ [a]	C_α 60,4	C_β 31,8	C_γ 19,8/18,9		CO 173,2	BOC 157,4; 80,1; 29,1	
BOC-Leu-Gly-OPOE$_{10000}$ [a]	Leu: C_α 54,1	C_β 41,9	C_γ 25,3	C_δ 22,2/23,7	CO 175,5	BOC:157,1; 80,2; 28,9	
	Gly: C_α 42,4				CO 170,9		

a) in ^{2}H-methanol relativ to internal tetramethylsilane (δ = 0ppm)

b) in ^{2}H-water

Substances

The *N*-protected peptide polymer esters BOC-L-Ala-(Aib-L-Ala)$_2$-OPOE, BOC-(Aib-L-Ala)$_3$-OPOE, BOC-(Aib-L-Ala)$_5$-OPOE and the N-protonated peptide derivatives $^+H_2$-L-Ala-(Aib-L-Ala)$_3$-OPOE, $^+H_2$-(Aib-L-Ala)$_4$-OPOE and $^+H_2$-L-Ala-(Aib-L-Ala)$_4$-OPOE were synthesized as described elsewhere[17] using polyoxyethylene 6000 as solubilizing support. These substances were carefully recrystallized and checked by various analytical controls, e.g. amino acid analysis and TLC and spectroscopic methods such as CD, ORD and IR. Typical examples for synthetic procedures are given for the esterification of BOC-L-Gln-OH with polyoxyethylene-2000 and for the synthesis of the dipeptide ester BOC-L-Leu-Gly-OPOE$_{10,000}$.

t-Butyloxycarbonyl-L-glutamine polyoxyethylene ester. 2.0 g Dicyclohexylcarbodiimide are added to a stirred solution of 4.9 g BOC-L-Gln-OH in 50 ml tetrahydrofuran at 0°C. After 20 min the dicyclohexylurea was removed filtrating the mixture directly into a stirred solution of 4 g polyoxyethylene (mean molecular mass 2000). After 2 days at room temperature BOC-L-Gln-OPOE was precipitated at 0°C with 250 ml diethylether and recrystallized twice from ethanol ether. Amino acid analysis indicated an esterification of 72%. This impure preparation was measured by ^{13}C NMR (Fig. 3). After removal of excess low molecular weight components by several precipitations from acetic acid/ether amino acid analysis revealed only 11% esterification. However, it should be pointed out, that other esterification procedures using monofunctional POE$_{3000}$ yielded pure BOC-L-Gln-OPOE with reasonable loading of 80%. This polymer ester differed more from BOC-Gln-OH in its solubility than the badly crystallizable polyoxyethylene 2000. By similar procedures BOC-L-Val-OPOE$_{2000}$ with bifunctional polymer and BOC-L-Gln-OPOE$_{3000}$ with monofunctional support were synthesized. The latter clearly shows O–CH_3 resonance at 59.1 ppm between Gln-C_α and the terminal methylene POE-carbons (Table 2).

t-Butyloxycarbonyl-L-leucyl-glycine-polyoxyethylene ester. The esterification of BOC-Gly-OH with POE$_{10,000}$ was carried out by a similar procedure as described above and yielded 97% esterified OH-groups. For removal of the BOC-group 10 g BOC-Gly-OPOE are stirred in 100 ml 1.2 N HCl/acetic acid. After 20 min the solution was evaporated and $^+H_2$-Gly-OPOE × HCl was precipitated with dry ether. The product was recrystallized twice from ethanol/ether and dried over KOH *in vacuo*. Separate solutions of 2.3 g dry BOC-L-Leu-OH and 1 g dicyclohexylcarbodiimide in some dichloromethane are cooled to 0°C combined and stirred for 20 min. Dicyclohexylurea is removed by filtrating the mixture directly into a solution of 5 g $^+H_2$-Gly-OPOE. The reaction mixture was neutralized with *N*-methyl-morpholine. After 2 h again a filtered solution of 1.1 g BOC-L-Leu-OH and

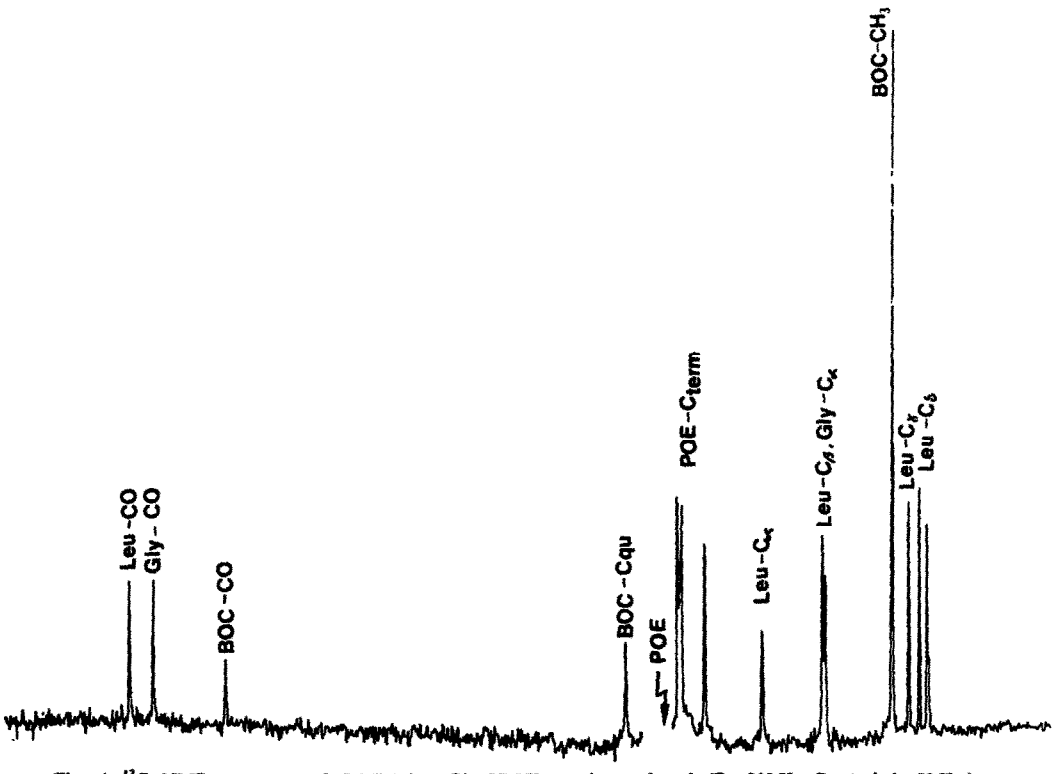

Fig. 4. ^{13}C NMR spectrum of BOC-L-Leu-Gly-OPOE$_{10,000}$ in methanol (T = 303 K, C = 1 g/ml, 50 K interferograms).

0.5 g dicyclohexylcarbodiimide was added. After 2 h the dipeptide ester was precipitated with ether in order to remove low molecular weight components. Amino acid analysis showed a loading of 91% and a Gly:Leu ratio 1.00/0.97. Titration revealed less than 1% non reacted free amino groups.

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