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Encephalitogenic Peptides: Synthesis and ^{13}C N.M.R. of a Pentapeptide Fragment of Myelin Basic Protein of the (65–74) Region

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Abstract

The synthesis, by stepwise conventional method, of a fully protected pentapeptide, Boc-Ser(OBzl)-Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl, a fragment of the MBP 65–74 region, is described. ^{13}C n.m.r. of the intermediates are discussed, and it was observed that proline gave rise only to the *trans*-conformation. The benzyl ether protecting group of the serine side chain was found to cause the β-carbon to resonate at a lower field, whereas the α-carbon experienced an upfield shift.

Introduction

Experimental allergic encephalomyelitis (EAE) is an autoimmune, demyelinating disease affecting the central nervous system, similar in several respects to multiple sclerosis. EAE can be induced in animals by subcutaneous injection of myelin basic protein (MBP) in complete Freund's adjuvant. A complete sequence of MBP has been reported, consisting of 170 amino acid residues (bovine). It should be noted that there are different encephalitogenic determinants for different species.[1] The encephalitogenic determinant, active in rabbits, is a peptide with a sequence Thr-X-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys (MBP 65–74),* where X = Thr for bovine, and X = Ala for human.[2] Comparison of this peptide with the encephalitogenic determinant active in guinea pigs, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys (MBP 114–122), reveals that these two peptides have a feature in common, i.e. Gln-Lys, a dipeptide separated by four amino acid residues from an amino acid with an aromatic side chain. Deletion of the spacing tetrapeptides, Gly-Ser-Leu-Pro and Gly-Glu-Ala-Gly, from MBP 65–74 and MBP 114–122 respectively, resulted in loss of encephalitogenic activity; however, the delayed-type skin hypersensitivity (DTH) caused by these peptides was retained.[3] The structure–activity relationship of these peptides led Hashim *et al.*[4] to synthesize a non-encephalitogenic peptide, (Phe-Ser-Trp-Gln-Lys)$_4$-Gly, which suppresses and reverses EAE induced in guinea pigs.

The synthesis and ^{13}C n.m.r. of the pentapeptide, Boc-Ser(OBzl)-Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl, described in this paper, would provide a useful fragment in the preparation of the decapeptide, MBP 65–74.

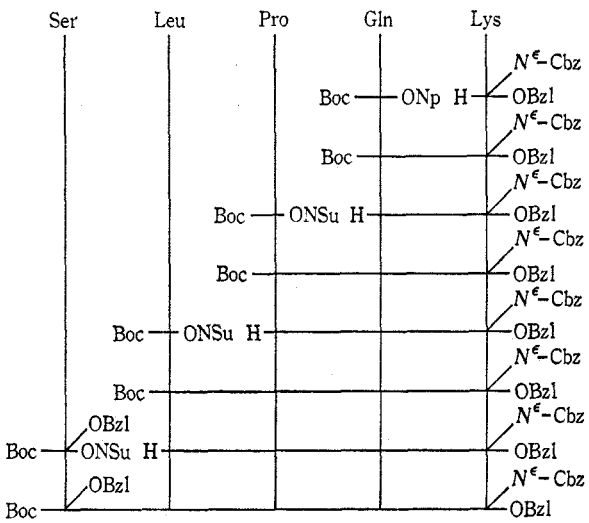
* Abbreviations follow IUPAC–IUB rules (1974): Bzl, benzyl; NSu, *N*-succinimido; Np, *p*-nitrophenyl. In addition, DCC stands for dicyclohexylcarbodiimide, and HOBt for benzotriazol-1-ol.

[1] Hashim, G. A., *Immunol. Rev.*, 1978, **39**, 60.
[2] Shapira, R., Chou, F. C.-H., McKneally, S., Urban, E., and Kibler, R. F., *Science*, 1971, **173**, 736.
[3] Hashim, G. A., and Sharpe, R. D., *Immunochemistry*, 1974, **11**, 633.
[4] Hashim, G. A., Sharpe, R. D., Carvalho, E. F., and Stevens, E. L., *J. Immunol.*, 1976, **116**, 126.

Results and Discussion

Synthesis

Although the synthesis of the MBP 65–74 fragment by the solid-phase method was reported, no data concerning the physical properties of this peptide were given.[3,5] Preference was given to the more time-consuming conventional solution method, because the solid-phase method often produces a side reaction which is difficult to remove from the desired product. Advantage was taken of the presence of glycine in the peptide MBP 65–74, to prepare two peptide fragments as intermediates, i.e. Boc-Ser(OBzl)-Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl, and a pentapeptide having Gly as a carboxyl terminal.



Scheme 1

The route of the synthesis of Boc-Ser(OBzl)-Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl is shown in Scheme 1. In this method the formation of the peptide bonds was mediated by nucleophilic substitution of the free amino group of the smaller peptides on the active ester of Boc-amino acid *N*-succinimido ester, as described by Anderson *et al.*[6] As in the case of the synthesis of nonapeptide, MBP 114–122, by conventional solution method,[7] the dipeptide, Boc-Gln-Lys(N^{ε}-Cbz)-OBzl, had the Boc protecting group removed by means of 98% formic acid,[8] and the free base was then obtained by neutralization to pH 9 by means of 1 M NH_4OH, followed by extraction with ethyl acetate. In this way partial deblocking of the N^{ε}-Cbz protecting group of lysine, when trifluoroacetic acid is used as a deblocking agent, is eliminated. Partial deblocking of the N^{ε}-Cbz protecting group of lysine can be overcome if the ε-amino side chain of lysine is protected by a ketimine protecting group[9] or an isonicotinyloxy-

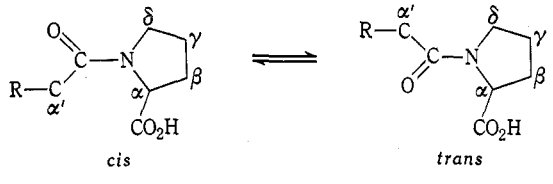
[5] Shapira, R., Chou, F. C.-H., and Kibler, R. F., in 'Peptides: Chemistry, Structure, Biology' (Ed. J. Meienhofer) p. 225 (Ann Arbor Science Publ.: Michigan 1972).
[6] Anderson, G. W., Zimmerman, J. E., and Callahan, F. M., *J. Am. Chem. Soc.*, 1964, **86**, 1839.
[7] Pasaribu, S. J., *Aust. J. Chem.*, 1977, **30**, 2533.
[8] Halpern, B., and Nitecki, D. E., *Tetrahedron Lett.*, 1967, 3031.
[9] Abdipranoto, A., Hope, A. P., and Halpern, B., *Aust. J. Chem.*, 1977, **30**, 2711.

carbonyl group,[10] both of which are resistant to trifluoroacetic acid. However, as these specific protecting groups were not readily available to us, they were not used. A dramatic increase in the reaction rate of the free base of Gln-Lys(N^{ε}-Cbz)-OBzl and Boc-Pro-ONSu occurred when benzotriazol-1-ol (HOBt) was added into the reaction mixture.[11] Therefore, HOBt was added to the reaction mixture in each coupling reaction stage of the synthesis. The completion of the coupling reactions was monitored by means of thin-layer chromatography, and it was noted that the free base generally moved slower (lower R_F) in solvent system (A) than did Boc-amino acid *N*-succinimido ester. The coupling of Boc-Ser(OBzl)-OH into the free base of the tetrapeptide, Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl, by means of *N*-succinimido ester or DCC/HOBt method, gave satisfactory results. At each stage the purity of the isolated peptide was determined by means of ^{13}C n.m.r. as well as thin-layer chromatography and element analysis.

^{13}C N.M.R. of Some Peptide Intermediates

To date, ^{13}C n.m.r. spectroscopy has provided one of the most sophisticated methods of analysis of amino acid residues in peptides. Extensive use of this facility has therefore been made in this work, and the ^{13}C n.m.r. chemical shifts of the amino acid residues in the peptide intermediates are shown in Table 1. The assignment was based on a comparison with the chemical shifts of amino acid residues incorporated into peptides, compiled by Wüthrich and Grathwohl.[12]

In many instances, interest in conformational studies has focused on the presence of the proline amino acid residue in a peptide, because of its rigid side chain and *cis/trans* isomers. The definition of *cis/trans* isomers of X-Pro reported in the literature[13,14] is based on (i) the position of the Cα carbon of Pro and the Cα′ carbon of the amino acid, X, around the peptide bond, or (ii) the arrangement of the Cδ carbon of Pro and the carbonyl group of the peptide bond, as shown in Scheme 2.



Scheme 2

Dorman and Bovey[15] studied proline which has been incorporated into a peptide, X-Pro, and established that the Cγ carbon of proline resonated at δ 25·4 ± 0·5, downfield from $SiMe_4$, in the *trans* isomer, whereas in the *cis* isomer it resonated at δ 23·7 ± 0·3. As shown in Table 1, the ^{13}C chemical shift of the Cγ carbon of proline in Boc-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl resonated at δ 23·8 (*trans*) and at 23·0 (*cis*), values which are in excellent agreement with the criteria reported by Dorman

[10] Veber, D. E., Paleveda, W. J., Lee, Y. C., and Hirschmann, R., *J. Org. Chem.*, 1977, **42**, 3286.
[11] Konig, W., and Geiger, R., in 'Peptides: Chemistry, Structure, Biology' (Ed. J. Meienhofer) p. 343 (Ann Arbor Science Publ.: Michigan 1972).
[12] Grathwohl, Ch., and Wüthrich, K., *J. Magn. Reson.*, 1974, **13**, 217.
[13] Deber, C. M., Bovey, F. A., Carver, J. P., and Blout, E. R., *J. Am. Chem. Soc.*, 1970, **92**, 6191.
[14] Thomas, W. A., and Williams, M. K., *J. Chem. Soc., Chem. Commun.*, 1972, 994.
[15] Dorman, D. E., and Bovey, F. A., *J. Org. Chem.*, 1973, **38**, 2379.

Table 1. Carbon-13 chemical shifts (ppm) of some peptides downfield from $SiMe_4$ in $(CD_3)_2SO$
Compounds: Boc-Gln-Lys(N^{ε}-Cbz)-OBzl (1);[A] Boc-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl (2); Boc-Leu-Pro-OMe (3); Boc-Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl (4); Cbz-Ser-Leu-Pro-OMe (5); Boc-Ser-Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl (6); Boc-Ser(OBzl)-Leu-Pro-OMe (7); Boc-Ser(OBzl)-Leu-Pro-Gln-Lys-(N^{ε}-Cbz)-OBzl (8)

Residue	Carbon	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Lys	Cα	51·8	51·9	—	51·9	—	52·0	—	52·1
	Cβ	30·5	30·4	—	30·3	—	30·4	—	30·6
	Cγ	22·4	22·4	—	22·4	—	22·5	—	22·7
	Cδ	28·9	28·9	—	28·9	—	28·9	—	29·0
	Cε	40·0	40·0	—	39·8	—	40·0	—	40·4
	CO	171·8	171·4	—	171·2	—	170·6	—	170·6
Gln	Cα	53·7	51·9	—	51·9	—	52·0	—	52·1
	Cβ	27·8	28·0	—	28·1	—	28·0	—	28·1
	Cγ	31·5	31·4	—	31·3	—	31·4	—	31·5
	Cδ	173·8	173·8	—	173·8	—	173·9	—	174·4
	CO	172·1	172·3	—	171·7	—	171·7	—	171·8
Pro	Cα	—	59·4	58·5	59·2	58·6	59·4	58·6	59·7
	Cβ	—	30·8,[B] 29·4[C]	28·5	28·9	28·6	28·9	28·7	29·0
	Cγ	—	23·0,[B] 23·8[C]	24·7	24·4	24·7	24·4	24·7	24·6
	Cδ	—	46·5	46·3	46·6	46·6	46·6	46·6	46·8
	CO	—	171·6	171·1	171·4	170·6	171·5	170·2	171·6
Leu	Cα	—	—	50·3	50·3	48·8	48·7	48·6	48·7
	Cβ	—	—	39·8	39·4	40·5	40·1	40·5	40·3
	Cγ	—	—	24·1	24·1	24·1	23·9	24·0	24·0
	$C\delta^1$	—	—	23·1	23·1	23·2	23·2	23·3	23·3
	$C\delta^2$	—	—	21·4	21·1	21·6	21·4	21·7	21·5
	CO	—	—	172·3	171·3	172·1	171·3	172·3	171·5
Ser	Cα	—	—	—	—	57·2	56·7	54·5	54·5
	Cβ	—	—	—	—	62·2	61·9	70·2	70·0
	CO	—	—	—	—	170·1	170·1	169·6	169·8
OMe	Me	—	—	51·7	—	51·7	—	51·9	—
Boc	C_{tert}	78·0	78·5	77·9	77·8	—	78·2	78·5	78·5
	Me	28·1	28·0	28·1	28·1	—	28·1	28·3	28·1
	CO	155·2	153·8	155·4	155·4	—	155·1	155·2	155·3
Cbz	CH_2	65·0	65·0	—	65·0	65·9	65·1	—	65·3
	C1	137·2	137·2	—	137·2	137·0	137·2	—	137·4
	C2	128·3	128·3	—	128·3	128·3	128·3	—	128·3
	C3	127·7	127·7	—	127·7	127·7	127·7	—	127·8
	C4	127·7	127·7	—	127·7	127·7	127·7	—	127·8
	CO	156·0	156·0	—	156·0	155·9	156·0	—	156·2
Bzl ester	CH_2	65·9	65·9	—	65·8	—	65·9	—	66·0
	C1	135·9	135·9	—	135·8	—	135·9	—	136·0
	C2	128·4	128·4	—	128·3	—	128·4	—	128·3
	C3	128·0	128·0	—	127·9	—	127·9	—	127·9
	C4	127·7	127·7	—	127·6	—	127·6	—	127·5
Bzl ether	CH_2	—	—	—	—	—	—	72·1	72·2
	C1	—	—	—	—	—	—	138·3	138·3
	C2	—	—	—	—	—	—	128·2	128·3
	C3	—	—	—	—	—	—	127·5	127·5
	C4	—	—	—	—	—	—	127·5	127·5

[A] Ref. 7; see also Table 3. [B] *cis*, [C] *trans*.

and Bovey.[15] However, the ^{13}C chemical shift of the Cβ carbon of proline is less consistent than that of the Cγ carbon of proline. A characteristic correlation between the ^{13}C chemical shifts of the Cβ carbon and the Cγ carbon was cited by Deslauriers *et al.*[16] as 1·3–6·0 ppm for a *trans* X-Pro bond, and 8·3–10·0 ppm for a *cis* X-Pro bond. The difference between the ^{13}C chemical shift of the Cβ carbon and that of the Cγ carbon of proline in the tripeptide, Boc-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl, was found to be 7·8 ppm for the *cis* conformer and 5·6 ppm for the *trans* conformer. It can be seen that the *trans* conformer is within, and the *cis* conformer close to, the range of characteristic correlation between the Cβ and Cγ carbons of proline mentioned earlier.

Table 2. Carbon-13 chemical shifts of proline incorporated into peptides, expressed in ppm downfield from $SiMe_4$

Solvent was $(CD_3)_2SO$ unless stated otherwise. The conversion of chemical shifts from CS_2 into $SiMe_4$ was made by the equation

$$\delta(SiMe_4) = 192{\cdot}5 - \delta(CS_2)$$

Compound	Cα	Cβ	Cγ	Cδ	CO
Boc-Pro-OH *cis*	58·7	30·4	23·2	46·2	174·4
trans	58·8	29·5	23·9	46·2	174·0
Boc-Pro-OH[A] *cis*	58·8	30·7	23·5	46·2	178·3
trans	58·8	29·3	24·1	46·7	176·6
Pro-OMe(HCl)	58·2	27·6	23·0	44·9	169·0
Boc-Leu-Pro-OMe	58·5	28·5	24·7	46·4	171·1
Boc-Pro-Met-OMe *cis*	59·3	30·9	23·0	46·5	172·6
trans	59·3	30·3	23·8	46·5	172·6
Boc-Gly-Pro-OH[B] *cis*	58·3	31·4	22·1	46·8	173·7
trans	58·9	29·0	24·7	45·7	173·7
Boc-Ala-Pro-OH[B]	58·7	28·8	24·8	46·5 47·7	173·6
Boc-Pro-Pro-OH[C] (*i*)	57·5	28·7	23·3	46·6	170·4
(*i*+1)	58·8	29·6	24·8	46·6	171·0
Boc-Gly-Pro-OH[D] *cis*	59·1	31·0	21·9	46·5	174·4
trans	59·1	28·7	24·3	45·9	174·4

[A] Voelter, W., Fuchs, St., Seuffer, R. H., and Zech, K., *Monatsh. Chem.*, 1974, **105**, 1110. In $CDCl_3$.

[B] Voelter, W., and Oster, O., *Org. Magn. Reson.*, 1973, **5**, 547.

[C] Voelter, W., Oster, O., and Breitmeier, E., *Z. Naturforsch. B*, 1973, **28**, 370. The symbols (*i*) and (*i*+1) are used to distinguish between the proline attached to the Boc group (*i*) and the proline adjacent to Boc-Pro (*i*+1). In general terms this can be described as (*i*+*n*), where *n* stands for the position of another proline from proline *i*.

[D] Dorman, D. E., and Bovey, F. A., *J. Org. Chem.*, 1973, **38**, 2379. In D_2O.

The incorporation of Boc-Leu into a tripeptide, to form Boc-Leu-Pro-Gln-Lys-(N^{ε}-Cbz)-OBzl, resulted in a singlet peak of each carbon of the proline amino acid residue, as can be seen in Table 1. Voelter and Oster[17] studied the effect of the side chain of an amino acid, X, on the *cis*/*trans* isomerism in the peptide, Boc-X-Pro,

[16] Deslauriers, R., Smith, I. C. P., Levy, G. C., Orlowski, R., and Walter, R., *J. Am. Chem. Soc.*, 1978, **100**, 3912.

[17] Voelter, W., and Oster, O., *Org. Magn. Reson.*, 1973, **5**, 547.

and found that the bulky side chain of the amino acid, X, e.g. in Boc-Ala-Pro, has a significant influence on the *cis/trans* isomers formation of the proline residue, as cited in Table 2. From the results shown in Table 2, it is apparent that a bulky side chain of the leucine amino acid residue in the peptide, Boc-Leu-Pro-OMe, exerts a high degree of steric effect on the equilibrium of the *cis/trans* isomers of proline, with only one resonance for each of the carbons in the proline moiety, in contrast to Boc-Gly-Pro-OH, which has both *cis* and *trans* isomers. It would be

Table 3. ^{13}C chemical shifts (ppm) of the α-carbons of some Cbz- and Boc-amino acids incorporated into peptides [for (1)–(4)], downfield from $SiMe_4$

In CD_3SOCD_3 unless stated otherwise. The values in *italics* are the ^{13}C chemical shifts of the amino acids (X) in peptides CF_3-CO-Gly-Gly-X-Ala-OMe reported in ref. 12

Fully protected peptides Boc-X-X-X-X-R	Cα chemical shifts (1)	(2)	(3)	(4)
Boc-Gln-Lys(N^{ε}-Cbz)-OBzl[A]	*51·7*, 53·7[B]	*51·8*, 51·8[B]		
Boc-Asn-Lys(N^{ε}-Cbz)-OBzl	*49·3*, 51·0	*51·8*, 51·9		
Boc-Gln-Arg(NO_2)-OBzl	*51·7*, 53·6	*51·7*, 51·5		
Boc-Asn-Arg(NO_2)-OBzl	*49·3*, 51·0	*51·7*, 51·6		
Boc-Gln-Gly-OBzl	*51·7*, 53·8	*42·1*, 40·7		
Boc-Gly-Lys(N^{ε}-Cbz)-OBzl	*42·1*, 42·9	*51·8*, 51·9		
Boc-Trp-Gly-OBzl[A]	*53·1*, 54·8	*42·1*, 40·7		
Boc-Leu-Pro-OMe	*50·5*, 50·3	*59·1*,[C] *58·7*,[D] 58·5		
Boc-Leu-Gly-OEt	*50·5*, 52·5	*42·1*, 40·9		
Cbz-Ser-Lys(N^{ε}-Boc)-OMe[E]	*54·8*, 57·2	*51·8*, 51·9		
Cbz-Pro-Leu-Gly-OEt[F]	*59·1*,[C] *58·7*,[D] 61·5,[C] 60·7[D]	*50·5*, 52·3	*42·1*, 42·5	
Boc-Gly-Asn-Lys(N^{ε}-Cbz)-OBzl	*42·1*, 43·3	*49·3*, 49·2	*51·8*, 52·0	
Boc-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl	*59·1*,[C] *58·7*,[D] 59·4	*51·7*, 51·9	*51·8*, 51·9	
Boc-Ser-Trp-Gly-OBzl[A]	*54·8*, 56·7	*53·1*, 53·0	*42·1*, 40·7	
Boc-Gly-Gln-Lys(N^{ε}-Cbz)-OBzl[A]	*42·1*, 43·1[B]	*51·7*, 51·5	*51·8*, 52·0	
Cbz-Cys(Bzl)-Pro-Leu-Gly-NH_2[F]	*51·6*, 54·1	*59·1*,[C] *58·7*,[D] 61·5	*50·5*, 53·0	*42·1*, 43·6
Boc-Glu(OBzl)-Gly-Gln-Lys(N^{ε}-Cbz)-OBzl[A]	*51·4*, 53·4[B]	*42·1*, 40·6	*51·7*, 51·6[B]	*51·8*, 52·0[B]
Boc-Phe-Ser-Trp-Gly-OEt[A]	*53·6*, 55·5[B]	*54·8*, 54·7[B]	*53·1*, 53·3	*42·1*, 40·7
Boc-Phe-Ser-Trp-Gly-OH[G]	*53·6*, 55·5	*54·8*, 54·7	*53·1*, 53·3	*42·1*, 40·6
Boc-Phe-Ser(OBzl)-Trp-Gly-OBzl[G]	*53·6*, 55·4	*54·8*, 53·1	*53·1*, 52·5	*42·1*, 40·6
Boc-Phe-Ser(OBzl)-Trp-Gly-OH[G]	*53·6*, 55·4	*54·8*, 53·2	*53·1*, 52·6	*42·1*, 40·6

[A] Ref. 7. [B] They were interchanged in ref. 7. [C] *trans*. [D] *cis*. [E] De Marco, A., and Gatti, G., *Int. J. Pept. Protein Res.*, 1975, **7**, 437. [F] Deslauriers, R., Walter, R., and Smith, I. C. P., *Biochem. Biophys. Res. Commun.*, 1972, **48**, 854. [G] The synthesis of the compound was reported in ref. 7.

worthwhile to note that the α-carbon of leucine in the dipeptide, Boc-Leu-Pro-OMe, and the tetrapeptide, Boc-Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl, was found to resonate at a higher field (50·3 ppm; see Tables 1 and 3). The cause of this upfield shift is probably due to the γ-effect of CH_2 of proline, in the δ-position, on the α-carbon of leucine, i.e. *trans* configuration. As expected, the replacement of the Boc group by Boc-Ser(OBzl) or Boc-Ser-OH to form a pentapeptide or tripeptide, as shown in Table 1, resulted in an upfield shift of the α-carbon of leucine, with a magnitude of 1·7 ppm, due to the Boc neighbouring effect.

The α-carbons of Gln and Lys listed in Table 1 were found to be magnetically equivalent in all peptides, i.e. 1·8 ppm upfield from one of the α-carbons of the dipeptide, Boc-Gln-Lys(N^{ε}-Cbz)-OBzl (53·7 ppm). From the data collected in Table 3, it is clear that the α-carbons of some Boc-amino acids incorporated into a peptide generally resonate at *c.* 1–2 ppm downfield from the ^{13}C chemical shift of the corresponding α-carbon incorporated in the peptide, CF_3CO-Gly-Gly-X-Ala-OMe, reported by Wüthrich and Grathwohl.[12] As reported by Deslauriers *et al.*,[18] it was observed that Pro was not sensitive to this change. However, when the Boc group of a Boc-dipeptide, Boc-Trp-Gly-OBzl (Cα, Trp, 54·8 ppm), was removed following the coupling reaction with Boc-amino acid, such as Boc-Ser-OH, to form a tripeptide, Boc-Ser-Trp-Gly-OBzl, the α-carbon of the penultimate residue, Trp (Cα, 53·0 ppm), was observed as being 1·8 ppm upfield, i.e. it moved to the ^{13}C chemical shift of the amino acid residue incorporated into the peptide, CF_3CO-Gly-Gly-X-Ala-OMe. A similar Cbz neighbouring effect on the α-carbon of the penultimate residue in a fully protected peptide was reported by Deslauriers *et al.*[18] in the case of the coupling of Cbz-Gln with Cbz-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2, where the α-carbon of Asn moved upfield by 2·0 ppm in Cbz-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2. The magnetical equivalence of the α-carbons of glutamine and lysine in all peptides listed in Table 1 appears to be due partly to the steric effect of proline and partly to the disappearance of the Boc neighbouring effect in Boc-Gln-Lys(N^{ε}-Cbz)-OBzl during the subsequent elongation stages in the synthesis of the fully protected pentapeptide. This reasoning is supported by the fact that there is a difference of 0·5 ppm in the chemical shifts of the α-carbons of glutamine and lysine in the tripeptide, Boc-Gly-Gln-Lys(N^{ε}-Cbz)-OBzl (see Table 3).

The coupling of Boc-Ser(OBzl)-OH with tetrapeptide produced interesting results, affecting the chemical shifts of the α-carbon and β-carbon, as shown in Table 1, i.e. the α-carbon of serine in the pentapeptide, Boc-Ser(OBzl)-Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl, resonated at a higher field (54·5 ppm) compared to that of the α-carbon of serine (56·7 ppm) in the unprotected side chain, and the β-carbon resonated at a lower field (70·0 ppm) compared to that of the β-carbon in the unprotected peptide (61·9 ppm). These parameters proved to be useful during the removal by hydrogenolysis (Pd/C catalyst) of the benzyl protecting group from Boc-Phe-Ser(OBzl)-Trp-Gly-OBzl, where the benzyl ester protecting group was removed selectively.[7] Thus, ^{13}C n.m.r. provides a useful means in monitoring the removal of the benzyl protecting group by hydrogenolysis in peptides containing Ser(OBzl), particularly in cases where incomplete removal occurs.

Experimental

The protected amino acids used were of L-configuration and were purchased from Bachem Inc., Marina del Rey, California. Melting points were determined on a Reichert hot-stage microscope, and are uncorrected. Element analyses were performed by the Australian Microanalytical Service, Melbourne. Thin-layer chromatography on Kieselgel $HF_{254+366}$ (Merck) supported on a glass plate (20 by 10 cm) was used, with solvent systems (A) chloroform/methanol/acetic acid, 14 : 2 : 1; (B) butan-1-ol/pyridine/acetic acid/water, 30 : 20 : 6 : 24. The spots were visualized by ninhydrin spray, after exposure to HCl vapour. The ^{13}C n.m.r. spectra were measured on a Bruker HX-90 spectrometer, operating at 22·625 MHz; noise-decoupled experiments were obtained with a spectral

[18] Smith, I. C. P., Deslauriers, R., and Walter, R., in 'Peptides: Chemistry, Structure, Biology' (Ed. J. Meienhofer) p. 29 (Ann Arbor Science Publ.: Michigan 1972).

width of 5000 Hz and 8192 data points, requiring approximately 60000 transients with concentrations 80 mg/1·5 ml. Chemical shifts are reported in ppm, downfield from tetramethylsilane ($SiMe_4$). Optical rotations were measured on a Perkin–Elmer 241 polarimeter, with a microcell.

Butyloxycarbonyl–Dipeptide Esters (Method A)

The amino acid benzyl or other ester (1 mmol) was liberated from the salt by means of *N*-ethylmorpholine (1 mmol), and by using $HCONMe_2$ (5 ml) as a solvent. To that solution was added t-butyloxycarbonyl amino acid succinimido active ester (1 mmol) (or *p*-nitrophenyl active ester), followed by benzotriazol-1-ol (1 mmol); then the reaction mixture was allowed to stand at room temperature until the reaction was completed. Thin-layer chromatography was then carried out. The reaction mixture was poured into water, the product was extracted with ethyl acetate, and the organic layer washed with 1 M NH_4OH, water and dried ($MgSO_4$). After removal of the solvent the purity of the product was checked by thin-layer chromatography and ^{13}C n.m.r.

t-Butyloxycarbonyl-L-asparaginyl-N$^{\varepsilon}$-benzyloxycarbonyl-L-lysine Benzyl Ester

This was prepared, as described in method A, with Boc-Asn-ONp (0·5 mmol) and Lys(*N*$^{\varepsilon}$-Cbz)-OBzl HCl salt (0·5 mmol) as starting materials. The *product* was then isolated by filtration from the aqueous chilled mixture to give a white substance (214 mg, 73%), m.p. 203–205° (Found: C, 61·3; H, 7·0; N, 9·4. $C_{30}H_{40}N_4O_8$ requires C, 61·6; H, 6·8; N, 9·6%).

t-Butyloxycarbonyl-L-glutaminyl-N$^{\varepsilon}$-benzyloxycarbonyl-L-lysine Benzyl Ester

This dipeptide was prepared in a similar manner to that described by Lefrancier and Bricas,[19] giving a yield of 70–75%. It crystallized from ethyl acetate/n-hexane, giving a white product, m.p. 136–138° (lit.[19] 108–109°), homogeneous on thin-layer chromatography, with R_F(A) 0·8.

t-Butyloxycarbonyl-L-prolyl-L-glutaminyl-N$^{\varepsilon}$-benzyloxycarbonyl-L-lysine Benzyl Ester

Boc-Gln-Lys(*N*$^{\varepsilon}$-Cbz)-OBzl (598 mg, 1 mmol) was deprotected by means of 98% formic acid, and worked up as previously reported to obtain the corresponding free base.[7] The isolated free base was then dissolved in $HCONMe_2$ (5 ml), and to that solution was added benzotriazol-1-ol (135 mg, 1 mmol), followed by Boc-Pro-ONSu (312 mg, 1 mmol). The reaction mixture was allowed to stand at room temperature until the free base had reacted quantitatively, then it was checked by thin-layer chromatography and worked up as described in the general procedure. The isolated *product* was crystallized from ethyl acetate/light petroleum (40–60°), giving a creamy white powder (360 mg, 51%), m.p. 82–84°, $[\alpha]_{20}^{D}$ −39·9° (*c*, 1·0 in $HCONMe_2$) (Found: C, 62·0; H, 7·2; N, 10·0. $C_{36}H_{49}N_5O_9$ requires C, 62·2; H, 7·0; N, 10·0%). ^{13}C n.m.r., see Table 1. Homogeneous on thin-layer chromatography before and after deprotection of the Boc group (solvent A).

t-Butyloxycarbonyl-L-leucyl-L-prolyl-L-glutaminyl-N$^{\varepsilon}$-benzyloxycarbonyl-L-lysine Benzyl Ester

Boc-Pro-Gln-Lys(*N*$^{\varepsilon}$-Cbz)-OBzl (2·04 g, 2·9 mmol) was deprotected by means of 98% formic acid and worked up as described previously to obtain the free base.[7] The isolated free base tripeptide was dissolved in $HCONMe_2$ (10 ml), and to that solution was added benzotriazol-1-ol (39·6 mg, 2·9 mmol), followed by Boc-Leu-ONSu (965 mg, 2·9 mmol). The reaction mixture was left to stand at room temperature until the reaction was completed. It was then checked by means of thin-layer chromatography and worked up as described in the general procedure. The crude product was purified by means of a Sephadex LH-20 column and eluted with $HCONMe_2$/MeOH (2 : 1 volume ratio) to give a pure *product* which solidified on refrigeration from ethyl acetate/light petroleum (40–60°) (1·5 g, 62%), m.p. 64–67°, $[\alpha]_{20}^{D}$ −49·6° (*c*, 1·0 in $HCONMe_2$) (Found: C, 60·8; H, 7·5; N, 9·6. $C_{42}H_{60}N_6O_{10},H_2O$ requires C, 61·0; H, 7·5; N, 10·1%). For ^{13}C n.m.r., see Table 1; homogeneous on thin-layer chromatography before and after the removal of the Boc group.

[19] Lefrancier, P., and Bricas, E., *Bull. Soc. Chim. Biol.*, 1967, **49**, 1257.

*t-Butyloxycarbonyl-*O*-benzyl-*L*-seryl-*L*-leucyl-*L*-prolyl-*L*-glutaminyl-*N$^{\varepsilon}$*-benzyloxycarbonyl-*L*-lysine Benzyl Ester*

Boc-Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl (760 mg, 0·94 mmol) was treated with 98% formic acid and worked up as previously reported to isolate the corresponding free base.[7] The isolated free base was then dissolved in $HCONMe_2$ (5 ml), and to that solution was added benzotriazol-1-ol (135 mg, 1 mmol), followed by Boc-Ser(OBzl)-ONSu (384 mg, 0·9 mmol). Other experimental conditions were the same as those described for the preparation of Boc-Leu-Pro-Gln-Lys(N^{ε}-Cbz)-OBzl. A creamy white *product* was obtained (486 mg, 53%), m.p. 103–105°, $[\alpha]_{20}^{D}$ −22·4° (*c*, 1·0 in $HCONMe_2$) (Found: C, 62·8; H, 7·5; N, 9·7. $C_{52}H_{71}N_7O_{12}$ requires C, 63·3; H, 7·2; N, 9·9%). For ^{13}C n.m.r., see Table 1; homogeneous on thin-layer chromatography before and after the removal of the Boc group.

*t-Butyloxycarbonyl-*L*-leucyl-*L*-prolyl Methyl Ester*

To a solution of prolyl methyl ester hydrochloride salt (497 mg, 3 mmol) in $HCONMe_2$ (10 ml) was added *N*-methylmorpholine (0·38 ml, 3 mmol), followed by Boc-Leu-ONSu (656 mg, 2 mmol). The reaction mixture was stirred, and left to stand at room temperature. The completion of the reaction was followed by thin-layer chromatography, then it was worked up as described in method A, to give an oily product (620 mg, 93%); R_F(A), 0·8. For ^{13}C n.m.r., see Table 1.

*Benzyloxycarbonyl-*L*-seryl-*L*-leucyl-*L*-prolyl Methyl Ester*

Boc-Leu-Pro-OMe (1·0 g, 2·9 mmol) was deblocked by means of 98% formic acid, and the free base was isolated as described previously.[7] Then it was dissolved in $HCONMe_2$ (4 ml), cooled down to a temperature of −10 to −15° (ice/NaCl bath). In the meantime a mixed anhydride solution of benzyloxycarbonyl serine was prepared by dissolving Cbz-Ser(OH) (700 mg, 3 mmol) in $HCONMe_2$ (10 ml), followed by the dropwise addition of *N*-methylmorpholine (0·38 ml, 3 mmol); then the reaction mixture was cooled to −15° (dry ice/methanol bath). To this solution was added ethyl chloroformate (0·28 ml, 3 mmol). After 10 min at −15° the solution of the pre-cooled free base of Leu-Pro-OMe was added dropwise. The temperature was maintained for 4 h, then the reaction mixture was diluted with ethyl acetate (150 ml), washed with $NaHCO_3$ solution (5%), citric acid (5%), water and dried ($MgSO_4$). After the removal of the solvent, an oily product was obtained (1·2 g, 86%) which was homogeneous on thin-layer chromatography; for ^{13}C n.m.r., see Table 1.

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