Renin Substrates. Part 2.[1] Rapid Solid Phase Synthesis of the Ratine Sequence Tetradecapeptide Using BOP Reagent

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A successful synthesis of Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[6]-His[9]-Leu[10]-Leu[11]-Tyr[12]-Tyr[13]-Ser[14] has been achieved by the classical stepwise solid-phase method using 1% crosslinked Merrifield resin and benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP reagent) respectively as solid support and coupling reagent. The coupling protocol included *in situ* neutralization of the amino partners after Boc-deprotection and the coupling times seldom exceeded 30 min. For His[6] and His[9], Boc-His(Boc). DCHA was neutralized *in situ* and allowed to couple. The peptide, purified by preparative h.p.l.c., was obtained in 29% overall yield based on the first residue attached to the solid support.

Since its discovery in our laboratories in 1975,[2] BOP reagent has been mainly utilized in the classical peptide syntheses in solution,[3] except for the single report of its use in a solid-phase coupling.[4] The usefulness of BOP reagent in liquid phase synthesis being well established, we decided to investigate its use in the now widely used solid-phase peptide synthesis first introduced by Merrifield.[5]

After a few successful preliminary results[6] in which BOP reagent exhibited very satisfactory behaviour in the presence of different kinds of resin supports, we report here the rapid stepwise solid-phase synthesis of a tetradecapeptide using BOP exclusively as the coupling reagent. The peptide, derived from ratine angiotensinogen† was synthesized manually on a 1% crosslinked polystyrene support using conventional N_α-Boc protection and benzyl-based side-chain protecting groups. Cleavage from the resin was achieved by treatment with hydrogen fluoride in the presence of *p*-cresol and purification was performed on a preparative h.p.l.c. column, according to the method recently described by Rivier *et al.*[7]

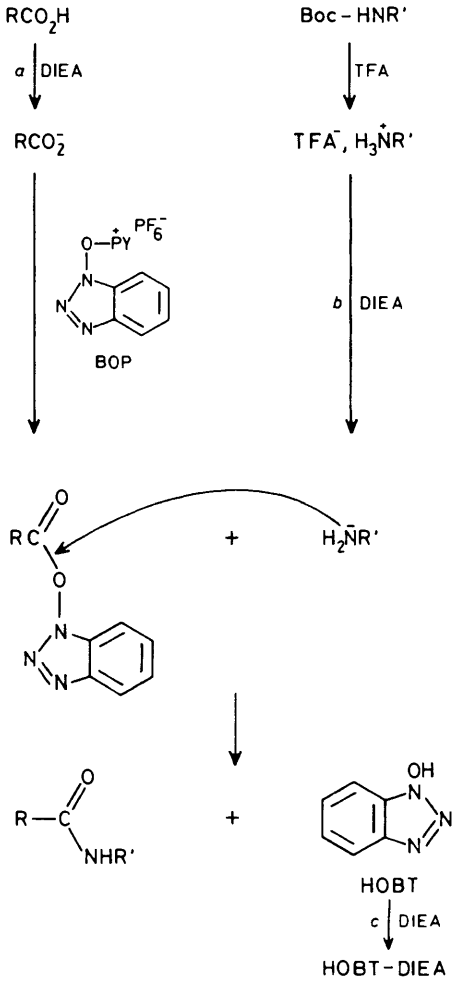
Discussion

All coupling cycles were satisfactorily achieved without recoupling during the solid-phase synthesis of the rat tetradecapeptide. All amino acids, introduced in a relatively low excess (2 equiv.) were completely coupled to the growing peptide chain within 30 min (Table 4), with the exception of Ile[5] (55 min) and Val[3] (75 min). These difficulties may be accounted for by the hindered nature of these residues and the length of the peptide chain to which they are coupled. Since the washing steps (DCM, MeOH, Pr^{i}OH) were achieved rapidly (0.5 min) with the use of a Teflon stirring rod, each of the coupling cycles shown in Table 1 lasted only little more than 1 h.

To avoid side-reactions caused by tertiary butyl carbocations, the TFA-deprotection steps were achieved in two stages including a pre-wash (1 min) and a 30 min reaction in the presence of EDT as scavenger. At the end of this step, the Pr^{i}OH wash led to considerable shrinking of the resin and almost complete elimination of TFA.

The deprotected terminal amino group of the growing peptide chain, obtained as its TFA salt, was ready for the following coupling step, without being liberated by a neutralization procedure; hence, it was less exposed to peptide loss due to ketopiperazine formation, especially during the introduction of residue 3 (Tyr[12]) of the peptide.[8]

The fact that bis(Boc-His) could be introduced in its DCHA salt form represented a further advantage of BOP. Indeed, the time-consuming operation to liberate the carboxylic function



Scheme 1. BOP-coupling pathway: role of DIEA

† Quinn and Burton[12] published the first 13 residues of rat angiotensinogen as Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]-His[9]-Leu[10]-Leu[11]-Tyr[12]-Lys[13]; further work by Bouhnik *et al.*[13] described the rat tetradecapeptide as Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]-His[9]-Leu[10]-Leu[11]-Tyr[12]-Tyr[13]-Ser[14].

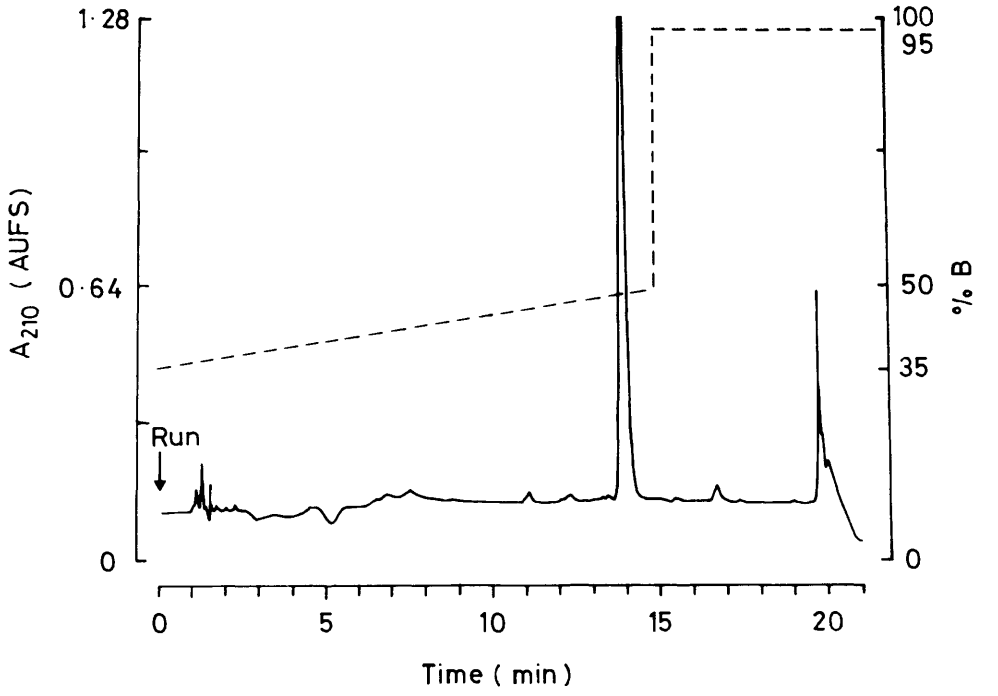


Figure 1. Load: lyophilized Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser from HF cleavage (20 μl *ca.* 200 μg). Column Lichrosorb 5 μm RP 18. 25 × 0.4 cm I.D. Buffers: TEAP 2.25 = A. 60% acetonitrile in A = B. Gradient: 35%–20′–55% B. Flow-rate: 2.0 ml min^{-1}. Detector: 1.28 (210 nm)

Table 1. Schedule of events for assembling the peptide–CM resin

1	DCM wash (2 × 0.5 min)
2	50% TFA–DCM + 5% EDT (1 min)
3	50% TFA–DCM + 5% EDT (30 min)
4	3% EDT–Pr^{i}OH wash (0.5 min)
5	DCM wash (2 × 0.5 min) Boc amino acid (2 equiv.) + BOP (2 equiv.) + DCM[a] + DIEA (6 equiv.) 10 min
6	pH check Ninhydrin test
7	MeOH wash (2 × 0.5 min)
8	DCM wash (2 × 0.5 min)

[a] Boc-Arg (Tos) was coupled in 20% DMF in DCM.

Table 2. Amino acid analysis of h.p.l.c. purified product

	Asp	Arg	Val	Tyr	Ile	His	Pro	Leu	Ser	Phe
Theory	1	1	1	3	1	2	1	2	1	1
Found	1.00	0.97	0.89	2.95	0.76	1.89	1.09	2.03	0.99	1.02

became superfluous, the DCHA salt displaying satisfactory behaviour, similar to that observed in a previous synthesis in solution with BOP.[1] For the coupling step, as shown in Table 2. DIEA was used in sufficient amounts [6 equiv., with respect to the starting Ser (Bzl)-CM resin; 3 equiv., with respect to BOP and Boc amino acids] in order to satisfy the three following conditions (Scheme 1): (*a*) the carboxylic partner must be neutralized to allow the formation of its active ester; (*b*) the amino partner must be liberated from its salt to allow peptide bond formation; (*c*) the coupling reactions release hydroxybenzotriazole, inducing an acid-base reaction with DIEA which results in a decrease of the pH while the optimum pH in BOP coupling is between 8 and 9.

Furthermore, the trace-amount of TFA remaining after PrOH and DCM rinses can also consume DIEA; this fact also contributes to the utilization in large excess of this tertiary base. The presence of TFA is not an obstacle to the coupling reaction since TFA, unlike AcOH, is not activated by BOP[1]; no trifluoroacetylation can therefore occur.

Conclusion

We demonstrate in this paper that BOP reagent can be used in solid phase peptide synthesis with very satisfactory results. This method is both economical and non time-consuming since only a relatively small excess of acylating partners (2 equiv.) is used to complete the coupling reactions in *ca.* 30 min. Criticisms may be raised about the exposure of the coupling partners to a base during the coupling process, however, it is well known that Nα-urethane-blocked amino acids are well protected from racemization, and that DIEA does not promote this process at least *via* the oxazolone route.[9] Use of BOP reagent (which is commercially available, or can easily be prepared[10] in large scale) combined with preparative h.p.l.c. purification gives synthetic peptides rapidly.

Experimental

BOP reagent was obtained from Sempa Chimie*; all N_α-Boc amino acids were of the L form and obtained from Bachem or Novabiochem (Switzerland) with the exception of Boc-His-(Boc), DCHA which was prepared as reported earlier.[1] Reactive side chains were protected as follows: Asp, Tyr, and Ser with benzyl (Bzl); Arg with tosyl (Tos); Tyr with 2, 6-dichlorobenzyl (Dcb). Di-isopropylethylamine (DIEA), trifluoroacetic acid (TFA), and ethanedithiol (EDT) were purchased from Merck

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(France). Technical dichloromethane (DCM) was redistilled over phosphorus pentaoxide. Methanol and isopropyl alcohol (99%, Aldrich), hydrogen fluoride (Matheson) and acetonitrile (h.p.l.c. grade, Rathburn) were used without purification; 1% crosslinked chloromethylated polystyrene divinylbenzene (Merrifield resin) containing 1–2 mequiv. Cl^-/g was obtained from Pierce (Rockford, USA).

The reaction vessel used in this synthesis was similar to the Type A reaction vessel described by Stewart and Young[11] except for the top which was protected by a screw-cap. It was mounted on a shaker allowing a 360° rotation.

Preparative and analytical h.p.l.c. were performed on a low-pressure Hitachi h.p.l.c. apparatus (pump 655 A 11, gradient controller L 5000, spectrophotometer 655 A 22 and recorder-integrator D 2000). Analytical runs were on a reverse-phase Lichrosorb C-18 column (5 μm, 4 × 250 mm, Merck), using a triethylammonium phosphate (TEAP) buffer system: solution A contained TEAP (0.25M, pH 2.25) and solution B contained 60% MeCN in TEAP. Preparative h.p.l.c. of the peptide was carried out on a C-18 reverse-phase Partisil ODS3 Magnum 20 column (10 μm, 22 × 500 mm, Whatman). Solution A was 0.1% TFA in water and solution B contained 60% MeCN in water and 0.1% TFA, the linear gradient was from 45 to 60% solution B into solution A in 45 min; the flow rate was 10 ml min^{-1}. Purity monitoring of the collected fractions was achieved on a Waters apparatus consisting of two 6000A pumps, a 441 u.v. detector, a System Controller 720 programmer, a Wisp 710B automated sample injector, and an Houston Instrument Omniscribe chart recorder.

^1H N.m.r. data were recorded on a Bruker 360 MHz instrument. The spectra were recorded in dimethyl sulphoxide and assigned by the usual spin decoupling method and 2D COSY and relayed COSY (RELSY). Amino acid analysis was carried out by Service d'Analyses d'Amino Acides, Hôpital Saint-Antoine, Paris, France.

General Methods.—The first residue Ser was anchored to the resin by the caesium salt method[14] to yield a substitution of 0.45 mmol Ser g^{-1}. The succeeding amino acid derivatives were coupled to the esterified resin (3 g, 1.35 mmol Ser) at pH 8—9 (monitored with moist pH paper). After 10 min, the pH was checked and readjusted if necessary with DIEA. Boc-deprotection was achieved by treatment with 50% TFA in DCM

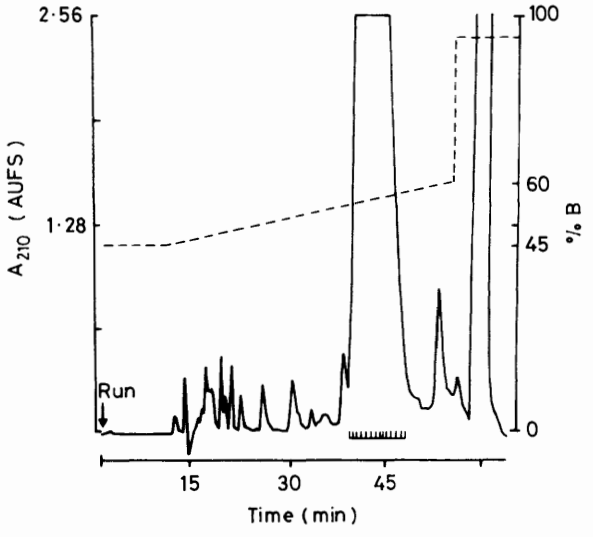

Figure 2. Load: lyophilized Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser (25 ml, 50 mg). Column: Magnum 20. Partisil ODS 3 10 μm C_{18}. 50 × 2.2 cm I.D. Buffers: 0.1% TFA in water = A: 60% acetonitrile in A = B. Gradient: 45% (load 2.5)–10′–45%—45′–60%. Flow-rate 9.9 ml min^{-1}. Detector 2.58 (210 nm)

Table 3. ^1H N.m.r. spectral results for rat tetradecapeptide renin substrate in $(CD_3)_2SO$ at 302 K

Residue	NH	C_α-H	C_β-H	C_γ-H	C_δ-H	Others
Asp 1		4.12	2.80—2.64			
Arg 2	8.58	4.34	1.63—1.48	1.48	3.08	ε 7.69
Val 3	7.81	4.16	1.93	0.76		
Tyr 4	8.02	4.49	2.80—2.66			
Ile 5	7.85	4.14	1.63	1.35—1.04	0.76	β′ 0.75
His 6	8.32	4.69	2.93			
Pro 7		4.28	1.97—1.73	1.73	3.58—3.32	
Phe 8	8.39	4.47	2.98—2.86			
His 9	8.17	4.59	3.07—2.97			
Leu 10	7.99	4.28	1.43	1.48	0.83	
Leu 11	8.12	4.24	1.36	1.51	0.79	
Tyr 12	7.69	4.36	2.84—2.64			
Tyr 13	7.91	4.52	2.94—2.69			
Ser 14	8.15	4.28	3.73—3.63			

Table 4. Summary of the different coupling stages during the synthesis[a] of Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]-His[9]-Leu[10]-Leu[11]-Tyr[12]-Tyr[13]-Ser[14]

Number of residue	Boc amino acid	Amount of BOP and Boc amino acid (equiv.)	Solvent	Total coupling time (min)	Amount of DIEA (equiv.)
13	Tyr (Dcb)	2	DCM	20	6
12	Tyr (Dcb)	2	DCM	20	6
11	Leu (1 H_2O)	2	DCM	15	6
10	Leu (1 H_2O)	2	DCM	15	6
9	His(Boc)·DCHA	2	DCM	20	6
8	Phe	2	DCM	20	6
7	Pro	2	DCM	20	6
6	His(Boc)·DCHA	2	DCM	30	6
5	Ile (0.5 H_2O)	2	DCM	55	6
4	Tyr (Dcb)	2	DCM	30	6
3	Val	2	DCM	15	6
2	Arg (Tos)[b]	2	DMF–DCM (20:80)	40	6
1	Asp (OBzl)	2	DCM	30	6

[a] Starting from Boc-Ser (Bzl)-CM Resin (3 g, 0.45 mmol (Ser/g). [b] Obtained from Bachem as Boc-Arg (Tos). 0.5 H_2O. 0.75 AcOEt.

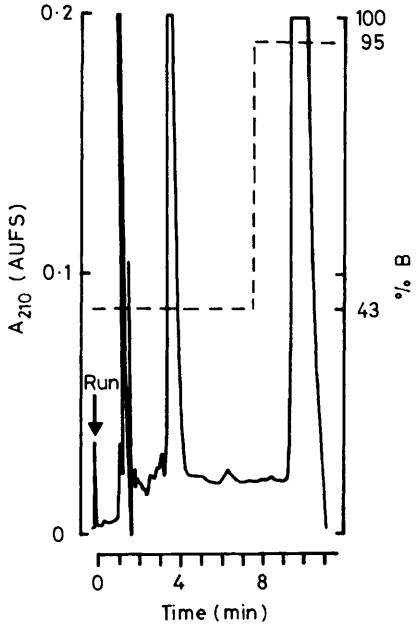

Figure 3a. Load: lyophilized Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser (10 μg). Column Lichrosorb 5 μm RP 18. 25 × 0.4 cm I.D. Buffers: TEAP 2.25 = A. 60% acetonitrile in A = B. Gradient: 43% B isocratic. Flow-rate 2 ml min^{-1}. Detector: 0.2 (214 nm)

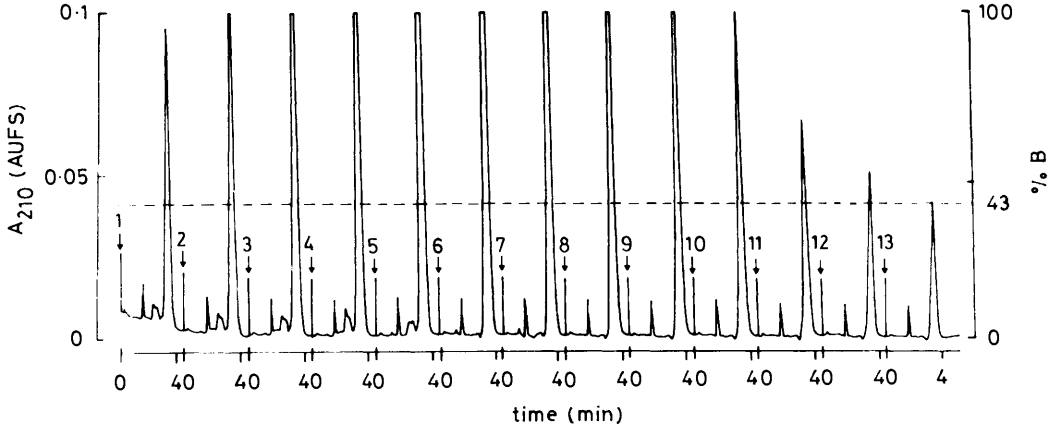

Figure 3b. Load: fractions 1—13 (20 μl) from preparative purification shown in Figure 2. Column Lichrosorb 5 μm RP18. 25 × 0.4 cm I.D. Buffers: TEAP 2.25 = A: 60% acetonitrile in A = B. Gradient 43% B isocratic Flow-rate 2 ml min^{-1}. Detector 0.1 (214 nm)

containing 5% EDT. Coupling reactions were carried out with 2 equiv. of BOP and Boc amino acids; monitoring with ninhydrin tests[15] showed that most couplings were complete within 30 min, no recoupling was necessary. The details of the synthetic cycle are given in Table 1.

The fully protected peptide resin with its *N*-terminal Boc group removed to avoid side reactions during HF cleavage weighed 5.5 g. Roughly 25% of this product (1.5 g) was treated with anhydrous HF (15 ml) in the presence of *p*-cresol (150 mg) for 30 min at 0 °C. After removal of the HF, the peptide resin was triturated with diethyl ether and the crude deprotected tetradecapeptide was extracted with dilute acetic acid and lyophilized (yield 360 mg, *ca.* 54%). This low yield was due to the poor cleavage and isolation conditions; the synthesis was not at fault since ninhydrin tests after each coupling were satisfactory, and the quality of the crude material (h.p.l.c. monitoring) tended to confirm this assumption.

H.p.l.c. Purification and Homogeneity.—Analytical h.p.l.c. runs of the crude product showed the presence of a major peak corresponding to a 79% content of the expected tetradecapeptide (Figure 1). The crude product (250 mg) was dissolved in 15% MeCN in 0.1%TFA–water (125 ml) and injected in 5 runs through inlet C of the gradient controller into the Magnum 20 preparative column (22 × 500 mm) by means of 25 ml per injection. The gradient preparative run in 0.1% TFA–acetonitrile detected at 210 nm is illustrated in Figure 2. After isocratic analysis of the different cuts from the main peak (Figure 3), fractions 6—9 were pooled and lyophilized. Thus, five successive preparative runs yielded 110 mg of a *ca* 99% pure peptide (Figure 4) (percentage purity is defined as the ratio of the integrated area of a peak over that of total integrated areas, excluding loading artefacts, when detection is at 210 mm). Fractions 1—5 containing a hydrophilic side-peak were also pooled and lyophilized to yield 20 mg of 96% pure peptide.

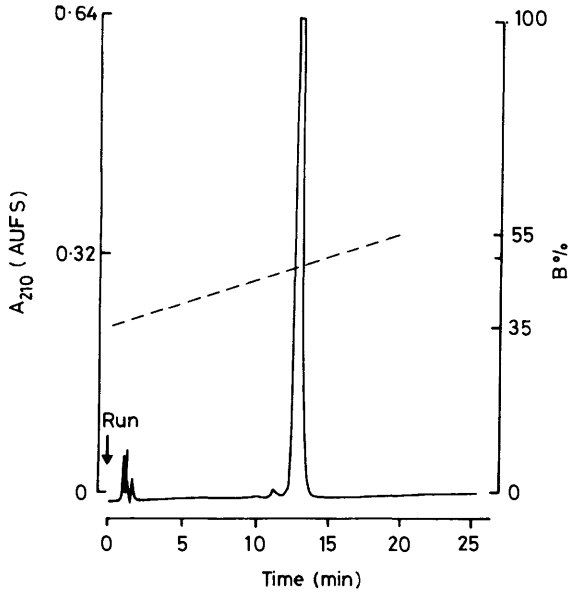

Figure 4. Load: lyophilized pool of fractions 6—13 (10 µl, *ca.* 50 µg) from 5 preparative purifications. Column Lichrosorb 5 µm RP 18 25 × 0.4 cm I.D. Buffers: TEAP 2.25 = A. 60% acetonitrile in A = B. Gradient: 35–20′–55% B. Flow-rate = 2 ml min^{-1}. Detector 0.64 (210 nm)

Total recovery of peptide was 130 mg corresponding to 53% yield based on the lyophilized crude peptide. The overall yield calculated from the starting Ser-resin was 29% (*M*, 1 790).

Amino acid analysis (Table 2) revealed that the purified product had the composition required by the expected sequence. ^{1}H N.m.r. measurements achieved with a solution containing 10 mg of the purified peptide in deuteriated dimethyl sulphoxide (0.5 ml) using the usual spin decoupling method, 2D COSY and relayed COSY (RELSY) confirmed the expected peptide composition, and the amino acid sequence was ascertained by n.O.e. measurments following the procedure described by Wuthrich.[16] Table 3 summarises the spectral data allowing the proton assignment of the tetradecapeptide.

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