Preparation and ^{11}C-Labelling of a Substance P Analogue Containing D-Tryptophan in Positions 7 and 9

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An efficient synthesis of ^{11}C-labelled [D-Trp^{7,9}]-Substance P, a putative Substance P antagonist, is presented. The preparation of the precursor peptide, Z-[D-Trp,^{7,9} Hcy(Bzl)^{11}]-Substance P was achieved in solution according to a [(3 + 4) + 4] strategy. The yields in the various steps were generally high. A novel feature of the synthesis was the use of tryptophan intermediates protected on the indole nitrogen with Boc together with phenylisopropyloxycarbonyl (Ppoc) as a general N^{α}-protecting group to suppress acid-mediated side reactions during deprotection. After conventional removal of the acid-labile side-chain protective groups, the remaining benzyloxycarbonyl and *S*-benzyl groups were smoothly cleaved off with sodium in liquid ammonia and the generated homocysteine sulphide anion methylated with [^{11}C]methyl iodide. The resulting labelled undecapeptide [*S-methyl*-^{11}C][D-Trp^{7,9}]-Substance P was obtained in 25—50% yield after h.p.l.c. The radiochemical purity of the purified peptide exceeded 98%.

Among the various short-lived positron-emitting radionuclides applicable for labelling of organic compounds to be studied *in vivo* using positron emission tomography (PET) ^{11}C is of particular prominence. Although its half-life is only 20.4 min, we recently demonstrated that it can be specifically incorporated into synthetic peptides of moderate complexity. To accomplish this, the target molecule must contain a methionine residue which can be substituted by *S*-benzylhomocysteine in the synthesis of the protected precursor. After purification, at the very end of the synthesis, the *S*-benzyl group is removed by sodium in liquid ammonia to generate a sulphide anion which is then directly methylated with [^{11}C]methyl iodide to give the [*S-methyl*-^{11}C]methionine peptide. Among ^{11}C-labelled peptides prepared in this way are methionine-enkephalin [1] and Substance P (SP).[2]

Several authors have reported that antagonists of biologically active peptides could be obtained by substituting one or more amino acids with D-amino acids and this applies to SP as well. Many SP-analogues containing D-tryptophan in positions 7 and 9 have been claimed to be such antagonists.[3] Therefore, we decided to extend our previous work to include also the labelling of a putative SP-antagonist.

In this paper is presented the synthesis of [*S-methyl*-^{11}C][D-Trp^{7,9}]-SP by the general approach outlined above. Most procedures used in the synthesis of the corresponding protected precursor were taken over from our previous work dealing with SP itself, some features are, however, new. Thus, we chose to protect the indole nitrogen of both D-tryptophan residues by t-butoxycarbonyl (Boc), a procedure previously only applied to the synthesis of a small model peptide.[4] Although indole-Boc groups are much stabler to acid than normal N^{α}-Boc ones and, therefore, the latter can be cleaved off in the presence of the former, we preferred in this synthesis to use N^{α}-phenylisopropyloxycarbonyl (Ppoc)[5] in conjunction with indole-Boc, thereby additionally increasing the selectivity towards acids. The high acid-lability of Ppoc did not prevent its application as a protecting group under acidic conditions such as those used in the Honzl-Rudinger modified azide coupling method.[6]

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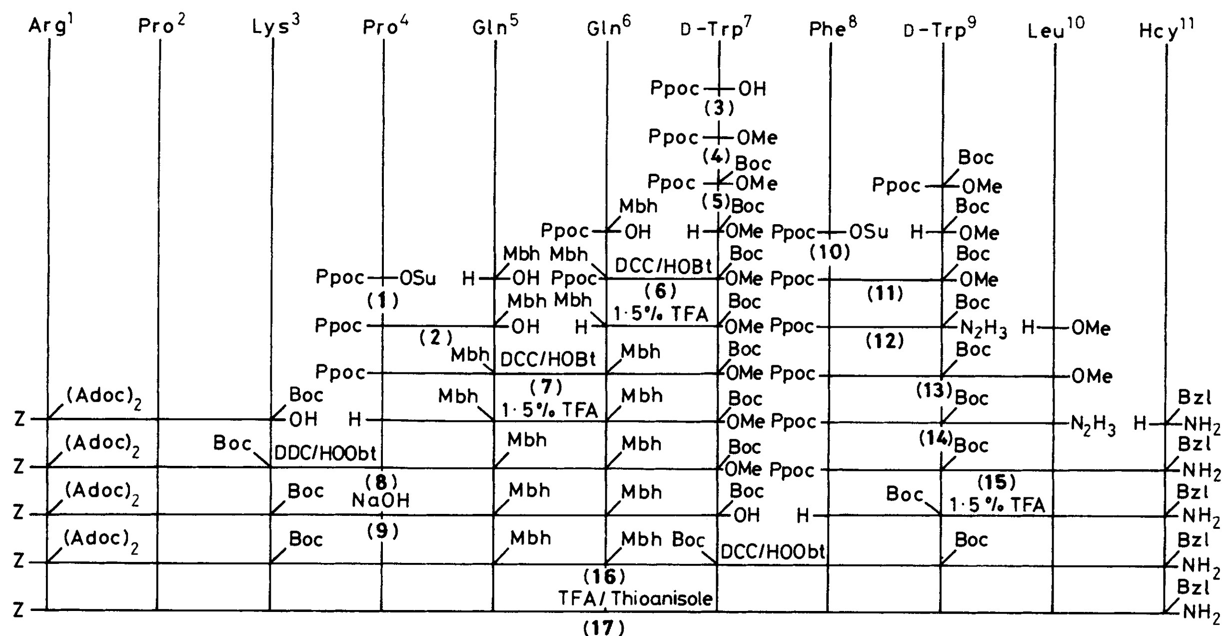
Results and Discussion

The entire synthesis of the required protected peptide precursor was carried out in solution and the corresponding intermediates were all obtained as solids, in yields generally in the range 75—100%. Throughout our work a global protection strategy, based upon three major fragments according to Scheme 1, was utilized. In contrast to the previously reported synthesis of a protected precursor used in the synthesis of ^{11}C-labelled SP,[2] where the fragments were coupled in the order 3 + (4 + 4) due to the need of the C-terminal octapeptide, we this time combined the fragments in the order (3 + 4) + 4. The reason for this altered strategy was that the homocysteine-containing C-terminal tetrapeptide could then be incorporated in the last coupling step, thereby reducing the risk of possible side-reactions due to the sulphide function. For the synthesis of the heptapeptide (**8**), the N-terminal tripeptide from our earlier work and the tetrapeptide (**7a**) were used. This order of assembly renders all intermediates readily soluble in the solvents normally used in synthesis and for chromatography. Furthermore, it takes full advantage of the properties of the N^{in}-Boc protected tryptophan analogue described earlier.[4] The utility of this tryptophan protection was further enhanced by substituting the Ppoc for the N^{α}-Boc protecting group, thus increasing the yield in the N^{α}-deprotection step from 60 to nearly 100%. N^{in}-Boc was completely stable under these conditions (1.5% TFA) unlike the situation when 100% TFA was used. Evidently, on the indole nitrogen the reaction takes place in two steps, affording the intermediate N^{in}-CO_2H derivative.[7] Conceivably this carboxy function serves as the effective protective group during the acidic cleavage step by blocking the nitrogen and deactivating the indole nucleus. The highly acid-labile Ppoc group was used as temporary N^{α}-protection throughout the synthesis of the protected precursor (**16**) in order to minimize possible side-reactions in the deprotection steps, *e.g.* alkylation of the indole moiety of tryptophan. The presence of the labile Ppoc in (**3**) required a mild method of esterification. In this synthesis, the corresponding caesium salt could be readily converted into (**4**) by methyl iodide in DMF in essentially quantitative yield without noticeable formation of undesired by-products.

Preparation of Ppoc-Pro-Gln(Mbh)-Gln(Mbh)-D-Trp(Boc)-OMe (**7**).—This tetrapeptide, corresponding to residues 4—7 of the SP analogue, was synthesized from Ppoc-Pro-Gln(Mbh)



Scheme 1.

and Gln(Mbh)-D-Trp(Boc)-OMe in 70% yield using the dicyclohexylcarbodi-imide–hydroxybenzotriazole (DCC–HOBt) procedure. Since the removal of contaminating dicyclohexylurea (DCU) from the reaction product was laborious on a larger scale, the crude material contained a minor amount of this impurity. The presence of DCU, however, presented no serious problems in the subsequent reaction steps, and the remaining traces of this compound could be more conveniently removed from (9) or (16) later in the synthesis. In comparison with the corresponding SP-fragment which was difficult to dissolve in organic solvents, this D-tryptophan analogue was very easily soluble in most solvents and could be purified by chromatography on silica. In addition to their use as protecting groups, this also demonstrates the favourable influence of N^{in}-Boc as well as of N^{α}-Ppoc on solubility.

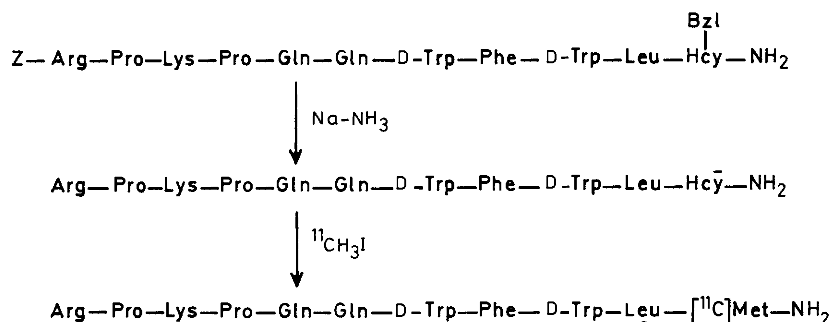
*Preparation of Z-Arg(Adoc)$_2$-Pro-Lys(Boc)-Pro-Gln(Mbh)-Gln(Mbh)-*D-*Trp(Boc)* (**9**).—The synthesis of peptide (**8**), corresponding to residues 1—7 of the target molecule, was accomplished from Z-Arg(Adoc)$_2$-Pro-Lys(Boc),[2] used in our previous synthesis of SP, and from (**7a**), using the DCC-3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one (DCC-HOObt) procedure.[8] If HOBt was exchanged for HOObt as additive in the coupling step, the yield was reduced and a by-product, probably the D-Lys derivative by analogy with our previous experience,[2] appeared at a slightly lower R_F value as judged from t.l.c. in system E. The yield after chromatography on silica, to remove excess of tetrapeptide amine and DCU, was 62%. The methyl ester was then easily hydrolysed with NaOH in 90% dioxane to give (**9**) in 76% yield, without cleavage of the N^{in}-Boc- and adamantyloxycarbonyl (Adoc)-protecting groups. This confirms our earlier finding that the N^{in}-Boc is stable under the conditions normally used for saponification of methyl esters in peptide synthesis.[4]

*Preparation of Ppoc-Phe-*D-*Trp(Boc)-Leu-Hcy(Bzl)*-NH_2 (**15**).—The C-terminal tetrapeptide was synthesized in a stepwise manner from the N-terminus using the Honzl-Rudinger modified azide method,[6] except in the first step when the hydroxysuccinimide ester method [9] was used. This approach reduced the exposure of the homocysteine residue to a minimum. In addition it was most economical with respect to this amino acid. The yields were excellent with no loss of either N^{in}-Boc or N^{α}-Ppoc, again demonstrating the stability of the N^{in}-Boc group on hydrazinolysis and in azide couplings.

*Preparation of Z-Arg-Pro-Lys-Pro-Gln-Gln-*D-*Trp-Phe-*D-*Trp-Leu-Hcy(Bzl)*-NH_2 (**17**).—The synthesis of the fully protected peptide (**16**) was effected by coupling the heptapeptide (**9**) with the tetrapeptide amine (**15a**), again using the DCC–HOObt method. When the Honzl-Rudinger modified azide method was attempted the reaction was not complete despite prolonged reaction times as judged from t.l.c. The side-chain protecting groups were then removed by trifluoroacetic acid–thioanisole (99:1). The product was purified by semipreparative h.p.l.c. affording the pure product in 50% yield (Figure 1).

Preparation of [S-methyl-^{11}C]*Arg-Pro-Lys-Pro-Gln-Gln-*D-*Trp-Phe-*D-*Trp-Leu-Met*-NH_2 (**18**).—[^{11}C]Methyl iodide was recently successfully applied to the synthesis of Met-enkephalin,[1] a few enkephalin analogues,[10] and Substance P[2], all labelled with ^{11}C in the methionine methyl group. Only one problem has been encountered and this is related to the amino acid sequence of SP. The sodium–liquid ammonia reagent is known to cause some splitting of peptide bonds, especially the Lys-Pro sequence, *e.g.* when strictly anhydrous conditions are not used.[11] This instability explained the reduction of the radiochemical yield in the synthesis of [*S-methyl*-^{11}C] Substance P to 25—35% of the pure product compared with 50—80% in the enkephalin labellings.

The crude radiochemical yield of compound (**18**) varied in the range 40—65% decreasing to 25—50%, decay corrected, after purification by h.p.l.c. (Scheme 2). As a result of the above mentioned Lys-Pro cleavage, 25—50% of the C-terminal octapeptide was formed. The total time of preparation was 45—60 min, and the product was obtained in a radiochemical purity >98% in a sterile pyrogen-free solution ready for use in *in vitro* or *in vivo* experiments. The specific radioactivity of the labelled peptide is strongly correlated to the specific radioactivity of the [^{11}C]methyl iodide used. In our laboratory, this radioactivity has been in the order of 10—200 Ci/mmol at the end of the peptide labelling.



Scheme 2.

Experimental

The experimental conditions were essentially as described previously.[2] Analysis by t.l.c. was performed on pre-coated silica plates (Merck, F_{254}) in the following systems: A, CH_2Cl_2–acetone–AcOH (40:10:1); B, CH_2Cl_2–acetone–AcOH (5:5:1); C, $CHCl_3$–EtOH–H_2O (100:50:4); D, EtOAc–acetone–H_2O–AcOH (5:3:1:1); E, CH_2Cl_2–MeOH (9:1); F, CH_2Cl_2–acetone (7:1). The spots were all visualized with the chlorine–dicarboxidine[12] and/or the ninhydrin spray reagents. Preparative silica gel chromatography was performed using 500-ml (60 × 170 mm) columns with Merck Kieselgel 60. The analytical h.p.l.c. equipment consisted of two LDC Constametric pumps, an LDC gradient master, a Rheodyne 7125 injector, an LDC Spectromonitor III variable wavelength u.v.-detector, an Altex 400 mixer and a Shimadzu CR3A integrator. The ^{11}C was produced at the tandem Van de Graaff accelerator at the University of Uppsala by the $^{14}N(p, \alpha)^{11}C$ reaction using a nitrogen gas target. The [^{11}C]carbon dioxide was trapped in 4 Å molecular sieves and transported to the radiochemical laboratory in a lead shield. [^{11}C]Methyl iodide was prepared according to the routine laboratory procedure.[2]

Ppoc-Pro-OSu (**1**).—This compound was prepared according to a standard procedure[9] on a 15 mmolar scale using dioxane as solvent. The crude product was triturated twice with ether and crystallized from acetone–isopropyl alcohol (1:2, 80 ml) to give (**1**) (4.9 g, 87%), homogeneous by t.l.c. (A, E); m.p. 166—166.5 °C; $[\alpha]_D^{25}$ −40.5° (*c* 1.0 in DMF) (Found: C, 61.0; H, 5.9; N, 7.4. $C_{19}H_{22}N_2O_6$ requires C, 60.95; H, 5.92; N, 7.48%).

Ppoc-Pro-Gln(Mbh) (**2**).—(a) *Deprotection step.* Z-Gln(Mbh)·DCHA[13] (4.82 g, 7.0 mmol) in AcOH–MeOH (1:1; 30 ml) was hydrogenated over 5% Pd/C for 2 h and then filtered. The filtrate was evaporated and the resulting clear oil solidified upon the addition of ether. The product was collected by filtration and dried *in vacuo* to afford Gln(Mbh) (2.45 g, 94%), homogeneous by t.l.c. (C, D). No trace of DCHA could be detected by n.m.r.

(b) *Coupling step.* Solid compound (**1**) (1.95 g, 5.2 mmol) was added to a stirred solution of Gln(Mbh) (2.29 g, 6.1 mmol) and *N*-methylmorpholine (NMM) (671 µl, 6.1 mmol) in DMF (50 ml) at −10 °C. After being stirred at this temperature for 1 h and overnight at 4 °C, the solution was evaporated and the residue partitioned between EtOAc (800 ml) and 0.2M citric acid (150 ml); the organic phase was separated, washed with portions (3 × 150 ml) of 0.2M citric acid and saturated brine, dried ($MgSO_4$), and evaporated to give a solid. This was triturated with ether and then crystallized from EtOAc–light petroleum (10:7; 170 ml) to give the title compound (**2**) (2.89 g, 88%), homogeneous by t.l.c. (B), m.p. >108 °C (decomp.); $[\alpha]_D^{25}$ −19.4° (*c* 1.0 in DMF); amino acid analysis: $Pro_{0.99}$ and $Glu_{1.01}$ (Found: C, 66.2; H, 6.7; N, 6.4. $C_{35}H_{41}N_3O_8$ requires C, 66.55; H, 6.54; N, 6.65%).

Ppoc-D-Trp (**3**).—A 13.1% (w/w) solution of Ppoc-F in ether (22.4 g, 16.1 mmol) at −80 °C was added to a stirred solution of D-Trp (3.00 g, 14.7 mmol) in THF (25 ml), 4M NaOH (4.5 ml), and 10% $NaHCO_3$ (18 ml) at 0 °C during 1 h. After a further hour at 0 °C the product was worked up essentially as described earlier.[5] Crystallization from MeOH–ether–light petroleum (1:3:6, 150 ml) afforded the title compound (**3**) (4.65 g, 86%), homogeneous by t.l.c. (A, E); m.p. >130 °C (decomp.); $[\alpha]_D^{25}$ + 6.4° (*c* 1.0 in DMF) (Found: C, 68.9; H, 6.1; N, 7.6. $C_{21}H_{22}N_2O_4$ requires C, 68.84; H, 6.05; N, 7.65%).

*Ppoc-D-Trp-*OMe (**4**).—Cs_2CO_3 (1.63 g, 5.0 mmol) in water (6 ml) was added to a solution of compound (**3**) (3.66 g, 10.0 mmol) in 90% EtOH (55 ml) at room temperature. After being stirred for 10 min, the mixture was evaporated and twice re-evaporated from absolute EtOH, finally using a vacuum pump. The salt was dissolved in DMF (45 ml) and MeI (1.56 g, 11 mmol) in DMF (5 ml) added; the solution was then stirred for 1.5 h at room temperature. Evaporation gave a foam which was partitioned between ether (750 ml) and 0.2M citric acid (150 ml). The organic phase was washed with portions (3 × 150 ml) of 0.2M citric acid, $NaHCO_3$, and saturated brine, dried ($MgSO_4$), and evaporated to afford the title compound (**4**) as a white foam (3.61 g, 95%) homogeneous by t.l.c. (E, ether); $[\alpha]_D^{25}$ +10.5° (*c* 1.0 in DMF) (Found: C, 69.5; H, 6.5; N, 7.3. $C_{22}H_{24}N_2O_4$ requires C, 69.46; H, 6.36; N, 7.36%).

*Ppoc-D-Trp(Boc)-*OMe (**5**).—$(Boc)_2O$ (2.05 g, 9.4 mmol) was added to a solution of (**4**) (3.42 g, 9.0 mmol) and 4-dimethylaminopyridine (DMAP) (110 mg, 0.90 mmol) in MeCN (36 ml). After the mixture had been stirred for 2 h, the volume was reduced to one half and the clear, oily residue taken up in ether and washed as described for compound (**4**). A white foam (4.33 g, 99%) resulted, which was homogeneous by t.l.c. (ether); $[\alpha]_D^{25}$ +12.7° (*c* 1.0 in DMF) (Found: C, 67.6; H, 7.0; N, 5.6. $C_{27}H_{32}N_2O_6$ requires C, 67.48; H, 6.71; N, 5.83%).

*Ppoc-Gln(Mbh)-D-Trp(Boc)-*OMe (**6**).—(a) *Deprotection step.* Compound (**5**) (6.73 g, 14 mmol) was dissolved in a solution of TFA in CH_2Cl_2 (1.5%; 210 ml) and stirred for 1 h. The mixture was then partitioned between ether (800 ml) and 30% K_2CO_3–saturated brine (1:1; 150 ml) and the organic phase separated, washed twice with saturated brine, dried (Na_2SO_4), and evaporated to give the amine (**5a**) (4.46 g, quantitative) essentially homogeneous by t.l.c. (B, C) and pure by n.m.r.

(b) *Coupling step.* Ppoc-Gln(Mbh)[14] (3.74 g, 7.0 mmol), D-Trp(Boc)-OMe (2.32 g, 7.3 mmol) and HOBt·H_2O (1.3 g, 8.4 mmol) were dissolved in CH_2Cl_2 (8 ml) and the solution cooled to 0 °C before a pre-cooled solution of DCC (1.45 g, 7.0 mmol) in CH_2Cl_2 (5 ml) was added in one portion. The solution was stirred at 0 °C overnight and then allowed to assume room temperature. Subsequently it was cooled to −20 °C, the precipitated dicyclohexylurea (DCU) filtered off, and the filtrate

partitioned between EtOAc and 0.2M citric acid. Work-up as described for compound (**4**) provided a light-yellow foam which was dissolved in ether and the solution filtered. Light petroleum was then added until precipitation started whereupon the solution was left at 0 °C overnight. Filtration and repeated addition of light petroleum afforded a total of 4.09 g (70%) of the title compound (**6**), slightly contaminated with DCU. An analytical sample was obtained by recrystallization from ether–light petroleum, homogeneous by t.l.c. (A, E); m.p. >95 °C (decomp.); $[\alpha]_D^{25}$ +6.9° (*c* 1.0 in DMF); amino acid analysis: $Glu_{1.00}$ and $Trp_{0.90}$ (Found: C, 67.7; H, 6.6; N, 6.7. $C_{47}H_{54}N_4O_{10}$ requires C, 67.61; 6.52; N, 6.71%).

*Ppoc-Pro-Gln(Mbh)-Gln(Mbh)-*D*-Trp(Boc)*-OMe (**7**).—(a) *Deprotection step.* Compound (**6**) (3.84 g, 4.6 mmol) was dissolved in a solution of TFA in CH_2Cl_2 (1.5%, 90 ml) and then treated as described for compound (**5a**), except that the reaction time was 2 h. Evaporation gave the amine (**6a**) as a colourless oil, homogeneous by t.l.c. (A, C), and used as such.

(b) *Coupling step.* Compound (**2**) (2.73 g, 4.32 mmol), the amine (**6a**), and HOBt·H_2O (793 mg, 5.18 mmol) were dissolved in CH_2Cl_2 (60 ml) and the solution cooled to 0 °C. A pre-cooled solution of DCC (892 mg, 4.32 mmol) was then added and the solution stirred for 3 h at the same temperature and then at room temperature overnight. The resulting mixture was partitioned between $CHCl_3$ (800 ml) and 0.2M citric acid (100 ml) and washed as described for compound (**4**), except that 30% K_2CO_3–brine (1:1) was used instead of $NaHCO_3$. The product was chromatographed on a 500-ml silica column using first 1 500 ml of CH_2Cl_2–acetone (9:1) to elute DCU and then CH_2Cl_2–MeOH (9:1) to give 4.79 g (86%) of the title compound (**7**), slightly contaminated with DCU. An analytical sample was obtained by rechromatography as above, homogeneous by t.l.c. (A, E); m.p. 198.5—199 °C (decomp.); $[\alpha]_D^{25}$ −14.1° (*c* 1.0 in DMF); amino acid analysis: $Pro_{1.00}$, $Glu_{2.00}$, and D-$Trp_{0.89}$ (Found: C, 67.2; H, 6.5; N, 7.7. $C_{72}H_{83}N_7O_{15}$ requires C, 67.22; H, 6.50; N, 7.63%).

*Z-Arg(Adoc)$_2$-Pro-Lys(Boc)-Pro-Gln(Mbh)-Gln(Mbh)-*D*-Trp(Boc)*-OMe (**8**).—(a) *Deprotection step.* Compound (**7**) (710 mg, 550 μmol) was deblocked as described for compound (**6**). Evaporation left solid (**7a**), homogeneous by t.l.c. (B, C), apart from trace amounts of DCU, and was used as such.

(b) *Coupling step.* Z-Arg(Adoc)$_2$-Pro-Lys(Boc)[2] (495 mg, 500 μmol), the amine (**7a**), and HOObt (82 mg, 500 μmol) were dissolved in DMF (2 ml) and cooled to 0 °C before DCC(s) (104 mg, 500 μmol) was added. The solution was stirred at 0 °C for 40 h and then at room temperature for 18 h; it was then worked up as for compound (**7**). The crude product was chromatographed on a 500-ml silica column, using first CH_2Cl_2–acetone (7:1; 2 500 ml) to elute DCU and then CH_2Cl_2–MeOH (20:1) to afford the product (**8**) (650 mg, 62%). An analytical sample was rechromatographed on a 250-ml (45 × 170 mm) silica column, eluted with CH_2Cl_2–acetone (3:1), to give (**8**) as an oil, homogeneous by t.l.c. (A, E) which crystallized upon addition of ether; it had m.p. >164 °C (decomp.); $[\alpha]_D^{25}$ −18.8° (*c* 0.5 in DMF); amino acid analysis: $Arg_{1.02}$, $Pro_{2.03}$, $Lys_{0.97}$, $Glu_{1.98}$ and $Trp_{0.70}$ (Found: C, 65.3; H, 6.8; N, 9.3. $C_{114}H_{146}N_{14}O_{24}$ requires C, 65.31; H, 7.02; N, 9.35%).

*Z-Arg(Adoc)$_2$-Pro-Lys(Boc)-Pro-Gln(Mbh)-Gln(Mbh)-*D*-Trp(Boc)* (**9**).—Aqueous NaOH (1.045M; 166 μl, 173 μmol) was added to a solution of compound (**8**) (355 mg, 167 μmol) in 90% dioxane (3 ml). The solution was stirred for 2 h at room temperature and then partitioned between EtOAc (100 ml) and 1M $KHSO_4$ (25 ml). The organic extract was washed with 1M $KHSO_4$ (3 × 25 ml) and saturated brine (2 × 25 ml), dried ($MgSO_4$), and evaporated to leave an oil which contained no trace of (**8**); this was chromatographed on a 100-ml (25 × 200 mm) silica column using CH_2Cl_2–MeOH (15:1) as eluant. Fractions 200—600 ml were evaporated and then precipitated from EtOAc with cold, light petroleum to give the title compound (**9**) (265 mg, 76%), homogeneous by t.l.c. (B, E); m.p. >160 °C (decomp.); $[\alpha]_D^{25}$ −13.7° (*c* 0.5 in DMF) (Found: C, 64.9; H, 6.9; N, 9.3. $C_{113}H_{144}N_{14}O_{24}$ requires C, 65.18; H, 6.97; N, 9.42%).

Ppoc-Phe-OSu (**10**).—Compound (**10**) was prepared on a 7 mmolar scale as described for compound (**1**) except that THF was used as solvent and gave the product (2.93 g, 99%), as a foam contaminated with a few percent of HOSu as judged by n.m.r. An analytical sample was obtained by chromatography on silica with CH_2Cl_2–acetone (4:1) as eluant; $[\alpha]_D^{25}$ −44.6° (*c* 1.0 in DMF) (Found: C, 64.4; H, 5.7; N, 6.6. $C_{23}H_{24}N_2O_6$ requires C, 65.08; H, 5.70; N, 6.60%).

*Ppoc-Phe-*D*-Trp(Boc)*-OMe (**11**).—Compound (**10**) (2.00 g, 4.7 mmol) was added to a solution of the amine (**5a**) (1.5 g, 4.76 mmol) in CH_2Cl_2 (12 ml) at −10 °C. The solution was stirred and allowed to reach room temperature overnight before being partitioned between ether (800 ml) and 0.2M citric acid (150 ml) and washed as for compound (**4**). Crystallization from ether–light petroleum (1:3; 150 ml) afforded the product (**11**) (2.74 g, 93%), homogeneous by t.l.c. (E, ether); m.p. 131—132 °C; $[\alpha]_D^{25}$ +24.6° (*c* 1.0 in DMF) (Found: C, 69.3; H, 6.6; N, 6.6. $C_{36}H_{41}N_3O_7$ requires C, 68.88; H, 6.58; N, 6.69%).

*Ppoc-Phe-*D*-Trp(Boc)*-NH-NH_2 (**12**).—Compound (**11**) (1.94 g, 3.0 mmol) was added to a solution of hydrazine hydrate (1.41 ml, 30 mmol) in MeOH (10 ml) at room temperature and stirred for 2 h when t.l.c. (E) indicated complete reaction. The solution was then evaporated and re-evaporated twice from MeOH to give a white foam which was partitioned between ether (100 ml) and water (50 ml). The ether extract was then washed with portions (2 × 50 ml) of water and saturated brine and dried ($MgSO_4$). Evaporation left a white foam (1.94 g, 100%), essentially homogeneous by t.l.c. (E, F); $[\alpha]_D^{25}$ +19.3° (*c* 1.0 in DMF) (Found: C, 66.8; H, 6.6; N, 11.2. $C_{35}H_{41}N_5O_6$ requires C, 66.97; H, 6.58; N, 11.16%).

*Ppoc-Phe-*D*-Trp(Boc)-Leu*-OMe (**13**).—A solution of compound (**12**) (1.88 g, 3.0 mmol) in DMF (24 ml) was cooled to −30 °C, and HCl in dioxane (2.29M; 5.9 ml), pre-cooled in DMF (6 ml), and then isopentyl nitrite (IAN) (471 μl, 3.6 mmol) were added. The mixture was stirred for 30 min at this temperature after which NMM (1.48 ml, 13.5 mmol) was added, followed by Leu-OMe·HCl (655 mg, 3.6 mmol) and NMM (395 μl, 3.6 mmol) in DMF (3 ml) at −30 °C. The mixture was stirred for 2 h at −15 °C, while the pH was kept around 7.5 with small portions of NMM (330 μl, 3.0 mmol in all), and then at room temperature overnight. The solution was evaporated and partitioned between ether (400 ml) and 0.2M citric acid (100 ml) and washed as for compound (**4**). Crystallization from ether–light petroleum (2:3; 150 ml) afforded the title compound (**13**) (2.08 g, 94%), homogeneous by t.l.c. (F, ether); m.p. 122—122.5 °C, $[\alpha]_D^{25}$ +1.2° (*c* 3.0 in DMF); amino acid analysis: $Phe_{0.97}$, $Leu_{1.03}$ and $Trp_{0.78}$ (Found: C, 68.2; H, 7.2; N, 7.6. $C_{42}H_{52}N_4O_8$ requires C, 68.09; H, 7.08; N, 7.56%).

*Ppoc-Phe-*D*-Trp(Boc)-Leu*-NH-NH_2 (**14**).—This compound was prepared as described for compound (**12**) on a 1.5 mmol scale and yielded after precipitation from ether into light petroleum an amorphous powder (950 mg, 86%), homogeneous by t.l.c. (E, F); $[\alpha]_D^{25}$ −7.9° (*c* 0.8 in MeOH).

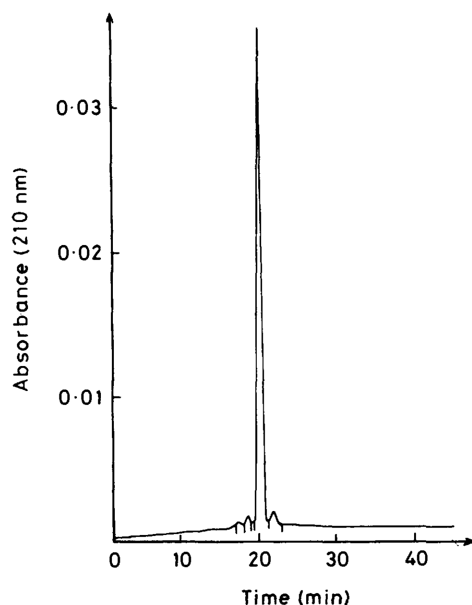


Figure 1. H.p.l.c. of purified undecapeptide (**17**). Mobile phase: A gradient of acetonitrile (35—90%) over 15 min in 0.1M phosphate buffer (pH 3.0), flow-rate 2.0 ml/min. Support: Spherisorb ODS-2 (250 × 4.6 mm, 10 μm); detection: 210 nm

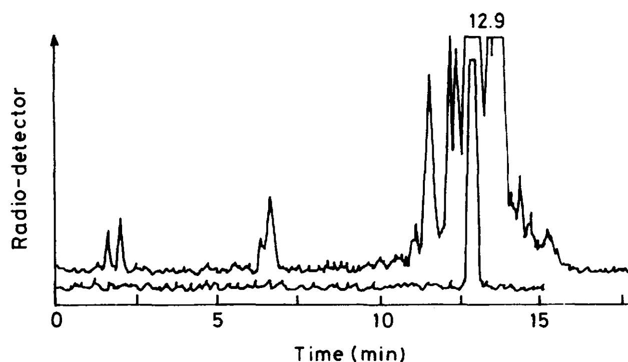


Figure 2. H.p.l.c. of crude and purified ^{11}C-labelled undecapeptide (**18**). Mobile phase: A gradient of acetonitrile (B) in 0.1M phosphate buffer (pH 3.0). Time 0—10 min, linear gradient 17—35% B; 10—12 min, linear gradient to 56% B; flow-rate 2.0 ml/min. Support: Hewlett Packard RP-18 (250 × 4.6 mm, 5 μm), β-flow detector

*Ppoc-Phe-*D-*Trp(Boc)-Leu-Hcy(Bzl)*-NH_2 (**15**).—This compound was prepared as described for compound (**13**) on a 1 mmol scale from compound (**14**) and Hcy(Bzl)NH_2.[2] The reaction mixture was partitioned between EtOAc and 0.2M citric acid and washed as described for compound (**4**). Crystallization from EtOAc afforded the title compound (**15**) (650 mg, 72%), homogeneous by t.l.c. (A, E); m.p. 189—190 °C (decomp.); $[\alpha]_D^{25}$ −29.7° (*c* 1.0 in DMF); amino acid analysis: $Phe_{0.98}$, D-$Trp_{0.74}$, $Leu_{1.02}$, and $Hcy(Bzl)_{0.95}$ (Found: C, 66.9; H, 6.9; N, 9.1; S, 3.3. $C_{52}H_{64}N_6O_8S$ requires C, 66.93; H, 6.91; N, 9.01; S, 3.44%).

Z-Arg(Adoc)$_2$*-Pro-Lys(Boc)-Pro-Gln(Mbh)-Gln(Mbh)*-D-*Trp(Boc)-Phe*-D-*Trp(Boc)-Leu-Hcy(Bzl)*-NH_2 (**16**).—(a) *Deprotection step.* Compound (**15**) (140 mg, 150 μmol) was deprotected as described for compound (**6**), affording compound (**15a**) as a solid which was used as such.

(b) *Coupling step.* DCC (20.7 mg, 100 μmol) was added to a solution of compound (**9**) (211 mg, 100 μmol), (**15a**) (80 mg, 103 μmol), and HOObt (20 mg, 120 μmol) in DMF (400 μl) at 0 °C. The solution was stirred for 40 h at the same temperature and then partitioned between EtOAc (100 ml) and 1M $KHSO_4$ (25 ml) and washed as described for compound (**7**). After evaporation, the product was chromatographed on a 100-ml (25 × 200 mm) silica column using CH_2Cl_2–acetone (3:1) as eluant to afford the title compound (**16**) (200 mg, 71%), essentially homogeneous by t.l.c. (C, E); m.p. >136 °C (decomp.); $[\alpha]_D^{25}$ −28.9° (*c* 0.61 in DMF); amino acid analysis: $Arg_{1.01}$, $Pro_{2.08}$, Lys $_{1.01}$, $Glu_{2.18}$, $Trp_{1.73}$, $Phe_{0.84}$, $Leu_{0.86}$ and $Hcy(Bzl)_{1.03}$ (Found: C, 65.1; H, 6.8; N, 9.7; S, 0.91. $C_{155}H_{196}N_{20}O_{29}S$ requires C, 65.66; H, 6.97; N, 9.88; S, 1.13%).

Z-Arg-Pro-Lys-Pro-Gln-Gln-D-*Trp-Phe*-D-*Trp-Leu-Hcy-(Bzl)*-NH_2 (**17**).—Compound (**16**) (150 mg, 53 μmol) was added to a stirred mixture of TFA and anisole (99:1; 2.25 ml). The reaction was terminated after 1 h by pouring the mixture into ether (40 ml). The resulting precipitate was collected by centrifugation and then washed 3 times by first dissolving the precipitate in MeOH (1 ml) and then precipitating it by the addition of ether (15 ml) and centrifuging again. Drying afforded impure title compound (**17**) (100 mg) which was purified in portions of 10 mg by semipreparative h.p.l.c., using the analytical LDC equipment with a 250 × 10 mm Spherisorb ODS-2, 10-μm column and guard column. The mobile phase used was a gradient of EtOH (50—67%, 25 min) in 0.1M triethylammonium formate, pH 3.0 with a flow-rate of 5.5 ml/min. The product was eluted at 80 ml and, after evaporation of the ethanol, was twice freeze-dried from dilute AcOH. The product (**17**) (50 mg, 50%) was essentially pure as judged from analytical h.p.l.c. (Figure 1) on Spherisorb ODS, 10 μm (linear gradient, 35—90% MeCN in 0.1M sodium phosphate, pH 3.0, for 15 min, flow-rate 2 ml/min). Amino acid analysis: $Arg_{1.03}$, $Pro_{2.00}$, $Lys_{0.94}$, $Glu_{2.00}$, $Trp_{1.77}$, $Phe_{1.03}$, $Leu_{1.01}$ and $Hcy(Bzl)_{0.68}$. Mass-spectra gave *m/z*: 1 725.255 $(MH)^+$, $C_{88}H_{116}N_{15}O_{20}S\cdot H$ requires 1 725.873. Peptide content 77.4% (theory requires 94% for the diacetate).

[S-methyl-^{11}C]*Arg-Pro-Lys-Pro-Gln-Gln*-D-*Trp-Phe*-D-*Trp-Leu-Met*-NH_2 (**18**).—Compound (**17**) (5—7 mg) was deprotected with sodium (2—3 mg) in liquid ammonia (0.5—1.0 ml) and directly alkylated with [^{11}C]methyl iodide as previously described for the synthesis of [S-*methyl*-^{11}C] Substance P.[2] The crude product, obtained on removal of the ammonia, was dissolved in 20% aqueous acetic acid and purified by h.p.l.c. on a Waters system using a 250 × 10 mm Nucleosil C18, 30-μm column equipped with a u.v.- and a GM-detector. The mobile phase used was a mixture of ammonium formate (0.1M; pH 3.5) and MeCN (70/30, v/v) with a flow-rate of 8 ml/min. After evaporation of the appropriate h.p.l.c.-fractions, the residue was dissolved in aqueous ethanol–propylene glycol and the solution, pH-adjusted, and sterile-filtered, using a Millipore Millex-GS 0.22 μm filter was used in biological experiments.* Analyses of the crude and the purified products were performed on a Hewlett Packard 1090 instrument, equipped with a 250 × 4.6 mm Hewlett Packard RP-18, 5-μm column and a diode array detector in series with a β-flow detector (Figure 2). Gradients of sodium phosphate (0.1M; pH 3) and MeCN were used.

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