^{11}C-Labelling of Substance P. Preparation of a Homocysteine-containing Precursor and its Subsequent Application in the Synthesis of the Labelled Neuropeptide

Henry M. Franzén * and Ulf Ragnarsson
Institute of Biochemistry, Biomedical Center, University of Uppsala, Box 576, S-751 23 Uppsala, Sweden
Kjell Någren and Bengt Långström
Department of Organic Chemistry, Institute of Chemistry, University of Uppsala, Box 531, S-751 21 Uppsala, Sweden

The synthesis of a useful [Hcy^{11}]Substance P derivative (**16**), containing only two protecting groups both labile to sodium in liquid ammonia, by a multistep [3 + (4 + 4)] strategy in solution, is reported. This approach furnished solid intermediates in high yields. After removal of the protecting groups, the product was selectively methylated on sulphur *in situ* with the aid of [^{11}C]methyl iodide as previously described for Met-enkephalin to give [S-*methyl*-^{11}C]Substance P (**18**) in a radiochemical yield of 35%. The total time of preparation was 45—60 min from the start of the synthesis of [^{11}C]methyl iodide. After purification by h.p.l.c., the peptide exhibited a radiochemical purity higher than 98%. An intermediary Substance P_{4-11} fragment was also labelled.

Substance P (SP) is widely distributed in the peripheral and central nervous systems (CNS) of vertebrates. It has been proposed as a neurotransmitter or neuromodulator in the CNS, primarily in the sensory neurons.[1] From a structural point of view, SP is an undecapeptide amide with the sequence shown in Scheme 1.[2] It has been synthesized together with many fragments and analogues.[3]

Short-lived positron-emitting radionuclides such as ^{11}C and ^{18}F have proved useful for labelling of various compounds to be studied *in vivo* using positron emission tomography (PET).[4] Owing to the short half-life of ^{11}C (20.4 min) and the positron emission a very low radiation dose will be obtained which, combined with the possibility of external detection, permits even applications to humans.[5] Compounds of high specific radioactivity can be produced which allow tracer studies with endogenous or exogenous compounds—even toxic ones could be applied—with no, or small, pharmacological or physiological effects.

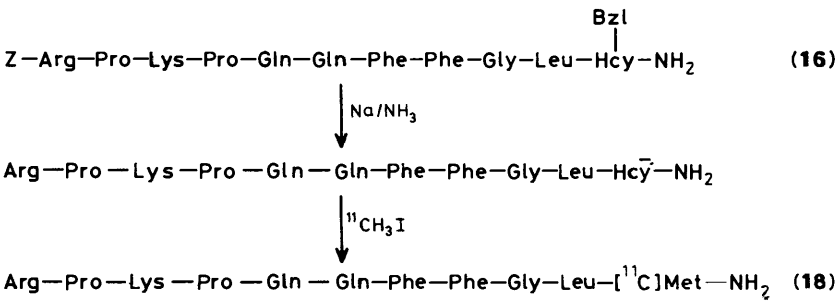
In order to reach a better understanding of the functional role of SP *in vivo*, a radioactive specimen was required. A labelled derivative should have a high specific activity and at the same time retain the biological activity. Since C-terminal SP-fragments have been shown to retain the biological activity of SP and, as a consequence of their hydrophobic character, might pass the blood-brain barrier more easily, a labelled fragment would also be of interest.

In the present study we will describe the syntheses of native ^{11}C-labelled SP as well as of the correspondingly labelled 4-11 fragment. The procedures used simultaneously exemplify a method of labelling biologically active methionine-containing peptides, first described by us for Met-enkephalin.[6] In this method *S*-benzylhomocysteine is incorporated instead of methionine in the protected precursor. The corresponding homocysteine peptide anion is then generated by removal of the *S*-benzyl group with sodium in liquid ammonia and directly alkylated with [^{11}C]methyl iodide (Scheme 1).

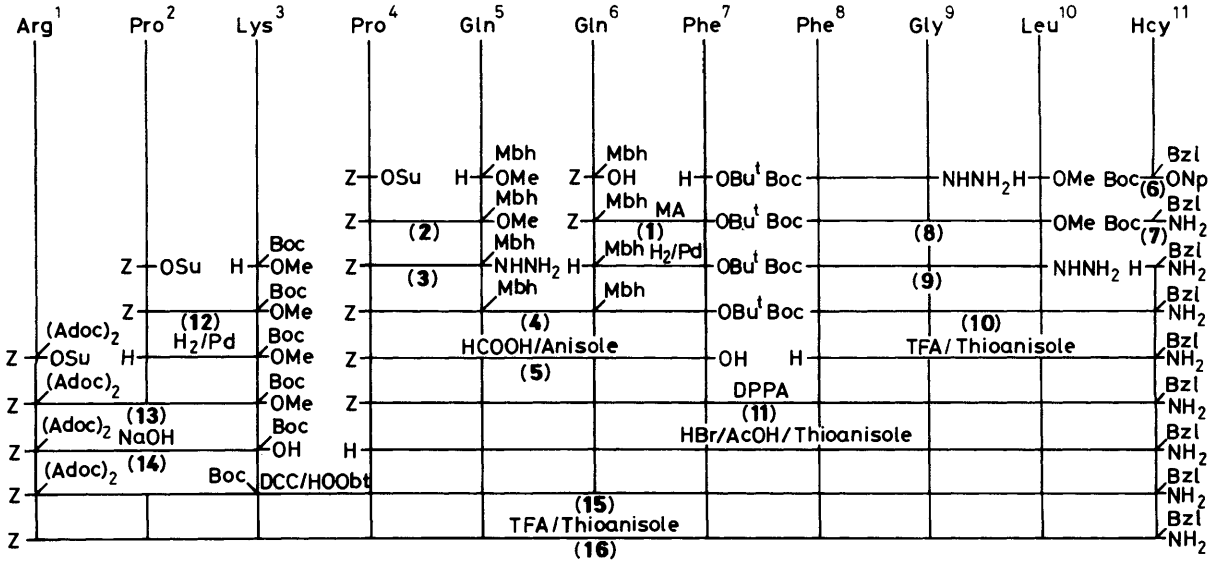
For the final ^{11}C-labelling experiments, the two precursor peptides (**11**) and (**16**) were used. The C-terminal octapeptide (4—11) was prepared from two tetrapeptides by the diphenylphosphoryl azide (DPPA) coupling method[7] and the undecapeptide by addition of the N-terminal tripeptide, using the dicyclohexylcarbodi-imide–3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one (DCC–HOObt) method (Scheme 2).[8] The octapeptide (**11**) was purified by repeated precipitations from DMF and the undecapeptide (**16**) by preparative reversed-phase h.p.l.c. Below, we will further comment on the preparation of our three major fragments as well as on their combination and subsequent labelling.

Results and Discussion

All peptides described below were synthesized in solution and obtained as solids in yields generally ranging from high to excellent. The routes adopted were dictated by our need for the



Scheme 1.



Scheme 2.

C-terminal (4—11) fragment. The synthetic strategy finally selected is based upon three major fragments according to Scheme 2.

Preparation of Z-Pro-Gln-Gln-Phe (**5**).—The middle tetrapeptide, corresponding to residues 4—7 of SP, was synthesized from Z-Pro-Gln(Mbh)-$NHNH_2$ and Gln(Mbh)-Phe-OBu^t in excellent yield using the Honzl-Rudinger modified azide method.[9] A first attempt to make (**5**) *via* its methyl ester without side-chain protection of the glutamine residues presented difficulties due to pyroglutamic acid-peptide formation and the purification problems ensuing. The original strategy to synthesize the octapeptide using the azide method also had to be abandoned in favour of the DPPA method, because of the very low solubility of the hydrazide with or without side-chain protection of the glutamines, even in DMF. On the other hand, the fully protected product (**4**) had a solubility that allowed purification by chromatography in CH_2Cl_2–MeOH on silica. The dimethoxybenzhydryl (Mbh) and the t-butyl ester groups could then be removed in one step using formic acid–anisole, that in this case proved to give a purer product than trifluoroacetic acid (TFA). The Mbh groups, no longer necessary to improve the solubility, were removed at this stage so as to avoid using the rather strong cleavage conditions for the octapeptide later, when the homocysteine-containing tetrapeptide had been incorporated.

Preparation of Boc-Phe-Gly-Leu-Hcy(Bzl)-NH_2 (**10**).—The C-terminal tetrapeptide (**10**) was synthesized in a stepwise manner beginning from the N-terminus. With respect to the tripeptide (**8**), the yield was increased in our hands by 31% compared with an earlier procedure.[10] The most troublesome part involved the preparation of Hcy(Bzl)-NH_2 (**7a**). Although the Boc-protected amide (**7**) was afforded *via* the *p*-nitrophenyl ester in high yield and purity, after removal of the Boc-protecting group, it was essential to improve the quality of the product (**7a**) by a careful washing procedure in order to avoid purification problems of the tetrapeptide (**10**). Chromatography on silica and crystallization then afforded the product (**10**) in 75% yield.

Preparation of Z-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Hcy(Bzl)-NH_2 (**11**).—As mentioned earlier our first attempts to make this octapeptide failed because of the solubility problems encountered with the tetrapeptide hydrazide. We therefore turned our attention to the DCC–hydroxybenzotriazole (DCC–HOBt) procedure.[11] This coupling reaction proceeded without problems using DMF as a solvent but it turned out to be very difficult to separate the product from the concomitant dicyclohexylurea (DCU). That was a misfortune arising from the very low solubility of (**11**) in anything but DMF and similar solvents. In order to overcome this problem DPPA was successfully tried as coupling reagent, affording the essentially pure product (**11**) in high yield after a simple washing procedure. The purity was determined by analytical h.p.l.c. and elemental analysis and the structure confirmed by n.m.r. and amino acid analysis.

Preparation of Z-Arg(Adoc)$_2$*-Pro-Lys(Boc)* (**14**).—This peptide, corresponding to SP_{1-3}, was obtained *via* its methyl ester by stepwise additions of the protected hydroxysuccinimide (OSu) ester of Pro and Arg to Lys(Boc)-OMe. The methyl ester was used since the coupling of Z-Arg(Adoc)$_2$-OSu[12] with Pro-Lys(Boc) only afforded the product (**14**) in low yield. The difficulties encountered were consequences of the very different polarities of the two components and, as a result of the slow reaction, hydrolysis of Z-Arg(Adoc)$_2$-OSu. Using the methyl ester the problems disappeared, again demonstrating the favourable influence of the adamantyloxycarbonyl (Adoc) groups on the solubility of arginine and arginine-containing peptides.[12]

Preparation of Z-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Hcy(Bzl)-NH_2 (**16**).—The synthesis was effected as shown in Scheme 2 by coupling the two fragments (**14**) and (**11a**) using the DCC–HOObt procedure. A first attempt to make (**15**) with HOBt instead as an additive or when using DPPA afforded 13—16% of the D-Lys derivative. Without additive it increased to 20—25%, but when the DCC–HOObt method was applied, the amount of the corresponding D-Lys derivative formed decreased to 3—4%. This is in agreement with König and Geiger's observation that the basic character of proline induces a high racemization in the C-terminal residue of the carboxyl component.[13] The product, therefore, had to be purified using preparative h.p.l.c. on a reversed-phase column with ethanol–triethylammonium formate as a mobile phase and could then be

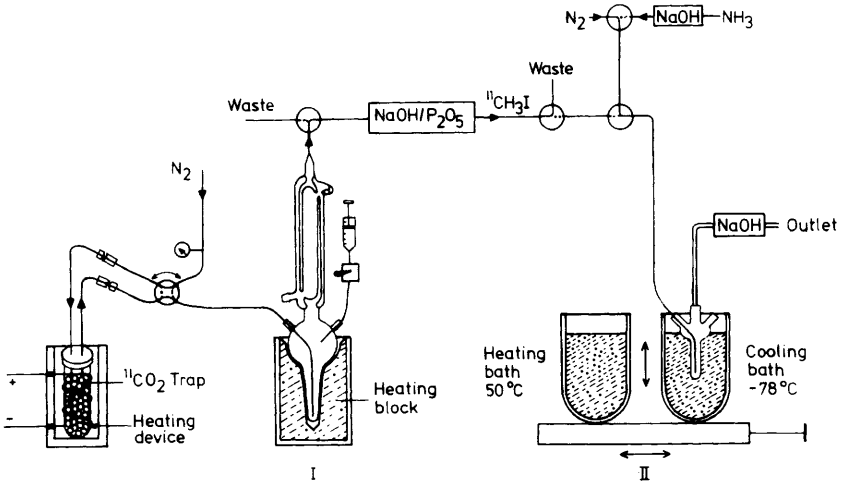


Figure 1. Experimental set-up for preparation of [^{11}C]methyl iodide and subsequent alkylation of sulphide anions. [^{11}C]Carbon dioxide is released from the molecular sieve by heating and carried by nitrogen gas to reaction vessel I, which contains a solution of lithium aluminium hydride (LAH) in THF. On addition of hydroiodic acid and heating the [^{11}C]methanol–LAH complex is hydrolysed and the liberated [^{11}C]methanol converted into [^{11}C]methyl iodide, which is transferred by nitrogen gas to reaction vessel II, which contains a solution of the sulphide anion to be alkylated

crystallized from methanol and ether to afford a pure product in 45% overall yield, calculated from the octapeptide (**11a**). The yield is low since it was sacrificed in favour of purity in the chromatographic step. The purity was affirmed by t.l.c., analytical h.p.l.c., and elemental analysis and the structure was confirmed by n.m.r., mass spectrometry, and amino acid analysis. The presence of Hcy in (**15**) as well as in (**10**) and (**11**) could be established by amino acid analysis although its accurate quantitation presented problems. The deblocking of the arginine and lysine side-chains of (**15**) was easily accomplished in one step by TFA–thioanisole for 2 h at room temperature (r.t.), whereupon the product was precipitated with ether and washed before purification by preparative h.p.l.c., affording the pure product in 69% yield.

Preparation of [S-methyl-^{11}C]*Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met*-NH_2 (**17**) *and* [S-methyl-^{11}C]*Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met*-NH_2 (**18**).—The short half-life of ^{11}C is of course a great disadvantage in synthesis. This radionuclide therefore has to be produced in immediate connection with its use. ^{11}C Was produced by the ^{14}N(p,α)^{11}C nuclear reaction and was obtained as [^{11}C]carbon dioxide. The labelled carbon dioxide was then converted in a three-step on-line synthesis into [^{11}C]methyl iodide[14] (Figure 1), which was obtained in 60—90% radiochemical yield counted on the [^{11}C]carbon dioxide within 4—7 min. [^{11}C]Methyl iodide has previously been used in various alkylation reactions.[4] Using sulphide ions, generated *in situ* from the protected precursors, [^{11}C]methionine and ^{11}C-labelled methionine peptides have been synthesized[6,14] and the same strategy was adopted in the syntheses of (**17**) and (**18**). The ^{11}C-labelled octapeptide (**17**) was obtained in 55—60% radiochemical yield in 40—50 min from the start of the [^{11}C]methyl iodide preparation. Both the deprotection and alkylation steps proceeded successfully. In the corresponding labelling of (**16**) to give (**18**), however, problems arose in the deprotection step due to partial cleavage of peptide bonds involving proline nitrogens. On alkylation with [^{11}C]methyl iodide the main cleavage product was converted into (**17**). The structure of this by-product was established by amino acid analysis and mass spectrometry. The crude radiochemical yield of (**18**) varied between 40—55% decreasing to 25—35%, decay-corrected, after h.p.l.c.-purification. The total time of preparation in this case was 45—60 min.

Both peptides were obtained in at least 98% radiochemical purity in sterile pyrogen-free solutions ready for use in *in vitro* or *in vivo* experiments. The specific radioactivity of these labelled peptides is strongly correlated to the specific radioactivity of the [^{11}C]methyl iodide used which, in our laboratory, has been in the range 10—200 mCi μmol^{-1} at the end of the peptide labelling.

Experimental

The m.p.s are uncorrected. Optical rotations were measured using a 1-dm cell in a Perkin-Elmer 141 polarimeter. Microanalyses were performed by Centrala Analyslaboratoriet or Micro Kemi AB, both Uppsala and amino acid analyses, after acid hydrolysis, by the Central Amino Acid Analysis Laboratory, Department of Biochemistry, Uppsala. 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–AcOH–H_2O (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[15] and/or the ninhydrin spray reagents. Preparative silica gel chromatography was performed using 500 ml (55 × 170 mm) columns with Merck Kieselgel 60, no. 7734. 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. Mass spectrometry was performed using a JEOL DX 303 FAB mass spectrometer with a DA 5000 data system. Routine n.m.r.-spectra were recorded on a JEOL FX 90Q spectrometer.

All ^{11}C used in this study was produced at the tandem Van de Graaff accelerator at the University of Uppsala by means of the ^{14}N(p,α)^{11}C nuclear reaction on a nitrogen gas target. The [^{11}C]carbon dioxide produced was trapped in 4 Å molecular sieves and transported to the radiochemical laboratory. [^{11}C]Methyl iodide was prepared according to the standard procedure of our laboratory.[14] All radiochemical yields given are decay-corrected.

Z-Gln(Mbh)-Phe-OBu^t (**1**).—A solution of Z-Gln(Mbh)[16] (3.54 g, 7 mmol) and *N*-methylmorpholine (NMM) (770 μl, 7 mmol) in dry THF (20 ml) was cooled to −15 °C. Isobutyl-

oxycarbonyl chloride (909 μl, 6.95 mmol) was added followed 1 min later by a solution of Phe-OBu^t·HCl (1.86 g, 7.23 mmol) and NMM (795 μl, 7.23 mmol) in dry THF (40 ml). The mixture was stirred for 30 min at −15 °C and was then allowed to reach r.t. overnight. After evaporation, the white solid was dissolved in $CHCl_3$ (700 ml), the solution washed with portions (3 × 200 ml) of 1M aqueous $KHSO_4$, 1M aqueous $NaHCO_3$, and brine (saturated) and dried ($MgSO_4$). Evaporation gave a white crystalline solid that was triturated with ether. The residue was recrystallized from MeOH (80 ml) to give the product (**1**) (4.0 g, 81%), pure by t.l.c. (A, B, and C), m.p. 175.5—177 °C; $[\alpha]_D^{25}$ −5.9° (*c* 1.0 in DMF); amino acid analysis: $Glu_{1.02}$ and $Phe_{0.98}$ (Found: C, 69.5; H, 6.7; N, 5.9. $C_{41}H_{47}N_3O_8$ requires C, 69.38; H, 6.67; N, 5.92%).

Z-Pro-Gln(Mbh)-OMe (**2**).—A solution of Z-Pro-OSu[17] (2.08 g, 6 mmol) in DMF (6 ml) was cooled to −10 °C and added to a solution of Gln(Mbh)-OMe* (2.55 g, 6.6 mmol) in DMF (6 ml) at −10 °C. The mixture was stirred for 2 h at this temperature and then allowed to reach r.t. overnight. The solvent was evaporated at 30 °C and the white solid dissolved in a mixture of $CHCl_3$ (40 ml) and EtOAc (200 ml). The organic phase was washed as for compound (**1**) and evaporated. The white solid was crystallized from EtOH (100 ml) to give compound (**2**) (3.34 g, 90%) pure by t.l.c. (A and B); m.p. 186.5—187 °C; $[\alpha]_D^{25}$ −23.4° (*c* 1.0 in DMF); amino acid analysis: $Pro_{0.99}$ and $Glu_{1.01}$ (Found: C, 65.6; H, 6.4; N, 6.8. $C_{34}H_{39}N_3O_8$ requires C, 66.11; H, 6.36; N, 6.80%).

Z-Pro-Gln(Mbh)-N_2H_3 (**3**).—Hydrazine hydrate (3.6 ml, 75 mmol) in MeOH (10 ml) was added to a solution of compound (**2**) (3.15 g, 5.1 mmol) in MeOH (70 ml) at 40 °C and stirred for 4 h at this temperature and then at r.t. overnight. The white precipitate was filtered off and washed with portions (2 × 20 ml) of MeOH and dry ether to give compound (**3**) (2.95 g, 94%), pure by t.l.c. (A and B); m.p. 240 °C; $[\alpha]_D^{25}$ −29.2° (*c* 1.0 in DMF); amino acid analysis: $Pro_{1.03}$ and $Glu_{0.97}$ (Found: C, 64.1; H, 6.4; N, 11.1. $C_{33}H_{39}N_5O_7$ requires C, 64.17; H, 6.36; N, 11.34%).

Z-Pro-Gln(Mbh)-Gln(Mbh)-Phe-OBu^t (**4**).—(a) *Deprotection step.* Compound (**1**) (3.8 g, 4.9 mmol) was dissolved with heating in MeOH (300 ml) and hydrogenated at r.t. over 5% Pd/C (0.3 g) for 2 h. The catalyst was then filtered off and the filtrate evaporated to give the amine (2.76 g, 98%) as a crystalline, white solid, pure by t.l.c. (B and D).

(b) *Coupling step.* A slurry of compound (**3**) (2.85 g, 4.61 mmol) in DMF (40 ml) was cooled to −30 °C, and HCl in dioxane (3.98M; 3.48 ml) and then isopentyl nitrite (IAN) (657 μl, 4.94 mmol) were added. The mixture was stirred for 30 min at this temperature. TEA (1.91 ml, 13.85 mmol) was then added, followed by a solution of the amine (2.76 g, 4.8 mmol) in DMF (40 ml) at −30 °C. The solution was stirred for 2 h at −20 °C, while the pH was kept at 7.5—8 with small portions of TEA (640 μl, 4.6 mmol in all), and then allowed to reach r.t. overnight. After the solution had been poured into water the precipitate was collected, rinsed with portions (4 × 30 ml) of 1M aqueous $KHSO_4$, 1M aqueous Na_2CO_3, and water and dried *in vacuo* at 40 °C to give the product (4.42 g, 85%) with traces of impurities [t.l.c. (B)]. Portions (1 g) were chromatographed on silica gel with CH_2Cl_2–MeOH (14:1) as the mobile phase and the combined products crystallized from MeOH (300 ml) to give (**4**) (3.2 g, 60%) pure by t.l.c. (B and E); m.p. >215 °C (decomp.); $[\alpha]_D^{25}$ −21.2° (*c* 1.0 in DMF); amino acid analysis: $Pro_{1.01}$, $Glu_{1.99}$, and $Phe_{1.00}$ (Found: C, 68.2; H, 6.6; N, 7.2. $C_{66}H_{76}N_6O_{13}$ requires C, 68.26; H, 6.60; N, 7.24%).

Z-Pro-Gln-Gln-Phe (**5**).—Compound (**4**) (4.0 g, 3.4 mmol) was dissolved in a mixture of HCO_2H and anisole (10:1, 50 ml) and heated to 80 °C for 1 h. The solution was then poured into water (100 ml) to give a fine-grained precipitate that was centrifuged and repeatedly digested with water (4 × 40 ml), MeOH (40 ml), and ether (4 × 40 ml) and centrifuged. It was then dried *in vacuo* to give (**5**) (2 g, 90%), pure by t.l.c. (B and D); m.p. >235 °C (decomp.); $[\alpha]_D^{25}$ −31.2° (*c* 1.0 in DMF) (Found: C, 58.4; H, 6.0; N, 12.6. $C_{32}H_{40}N_6O_9$ requires C, 58.89; H, 6.18; N, 12.88%).

Boc-Hcy(Bzl)-ONp (**6**).—A solution of Boc-Hcy(Bzl)[18] (16.3 g, 50 mmol) and *p*-nitrophenol (7.66 g, 55 mmol) in THF (80 ml) was cooled to −5 °C. After addition of a pre-cooled solution of DCC (10.3 g, 50 mmol) in THF (20 ml), the reaction mixture was stirred for 1 h at −5 °C and for 1 h at r.t. The urea by-product was then filtered off and the solvent evaporated. Trituration of the residue with light petroleum gave a solid crude product which was crystallized from ethanol–light petroleum to give the product (16.2 g, 72%) pure by t.l.c. (B); m.p. 98—99 °C; $[\alpha]_D^{25}$ −39.3° (*c* 1.0 in MeOH) (Found: C, 59.25; H, 5.8; N, 6.2; S, 7.2. $C_{22}H_{26}N_2O_6S$ requires C, 59.18; H, 5.87; N, 6.27; S, 7.18%).

Boc-Hcy(Bzl)-NH_2 (**7**).—Ammonia was bubbled through a stirred solution of compound (**6**) (15.2 g, 34 mmol) in THF (350 ml) at −10 °C for 15 min and then stirred for 1 h at r.t.; it was then evaporated. The residue was dissolved in EtOAc and the solution washed with 1M aqueous Na_2CO_3 until it was no longer yellow; it was then dried (Na_2SO_4) and evaporated. The residue was crystallized from ethanol–light petroleum to give the product (8.65 g, 84%) pure by t.l.c. (B); m.p. 134—135 °C; $[\alpha]_D^{25}$ −1.8° (*c* 2.0 in EtOH) (Found: C, 59.4; H, 7.5; N, 8.55; S, 9.8. $C_{16}H_{24}N_2O_3S$ requires C, 59.23; H, 7.46; N, 8.63; S, 9.88%).

Boc-Phe-Gly-Leu-OMe (**8**).—A solution of Boc-Phe-Gly-$NHNH_2$[19] (10.8 g, 32 mmol) in DMF (120 ml) was cooled to −20 °C, and HCl in dioxane (4.51M; 35.5 ml) and then IAN (4.86 ml, 35.2 mmol) were added. After being stirred for 45 min at −20 °C the solution was cooled to −35 °C and neutralized with TEA (22.4 ml) before the addition of a pre-cooled solution of Leu-OMe·HCl (6.22 g, 34.2 mmol) and TEA (4.5 ml) in DMF (25 ml). The reaction mixture was allowed to reach 4 °C during 1 h at which temperature it was finally stirred for 20 h. Some additional TEA was added at times to maintain the pH slightly basic. After filtration and evaporation the residue was dissolved in EtOAc, and the solution washed and dried as for compound (**1**). Evaporation gave an oil which was crystallized from toluene–light petroleum to give the product (11.9 g, 83%) pure by t.l.c. (E); m.p. 126—127 °C; $[\alpha]_D^{25}$ −30.7° (*c* 1.0 in MeOH) (lit.,[10] 52%, m.p. 119—121 °C).

Boc-Phe-Gly-Leu-$NHNH_2$ (**9**).—Hydrazine hydrate (12.3 ml, 0.25 mol) was added to a solution of compound (**8**) (11.2 g, 25 mmol) in MeOH (60 ml) and the reaction mixture stirred for 14 h at r.t. and placed in a refrigerator overnight. Filtration of the precipitated product, washing with small portions of pre-cooled MeOH and ether, and drying, yielded the product (9.0 g, 80%) pure by t.l.c. (E); m.p. 123—125 °C; $[\alpha]_D^{25}$ −25.7° (*c* 1.0 in MeOH).

Boc-Phe-Gly-Leu-Hcy(Bzl)-NH_2 (**10**).—(a) *Deprotection step.* A solution of compound (**7**) (1.30 g, 4 mmol) in 30% TFA in methylene dichloride (40 ml) was stirred for 15 min at r.t.

* Obtained from Z-Gln(Mbh)-OMe[16] by hydrogenation over Pd/C in MeOH. The chromatographically pure, crude product was used as such after evaporation.

After evaporation the residue was distributed between chloroform (20 ml) and 1M $NaHCO_3$ (100 ml). The organic layer was extracted once with a further portion (100 ml) of 1M aqueous $NaHCO_3$ and could then to our surprise be discarded. In this way we got rid of several impurities otherwise interfering in the coupling step. The combined aqueous phases, saturated with NaCl, were extracted with chloroform (6 × 100 ml). The extract was dried ($MgSO_4$) and evaporated to give compound (**7a**) (738 mg, 82%) pure by t.l.c. (C), which was used directly in the synthesis of compound (**10**).

(b) *Coupling step.* A solution of compound (**9**) (1.34 g, 3.0 mmol) in DMF (20 ml) was cooled to −20 °C. After addition of HCl in dioxane (4.51M; 3.31 ml) and IAN (516 μl) the mixture was treated as described for compound (**8**). The crude product was purified by chromatography on silica using methylene dichloride–methanol (20:1). The eluate from 600—1 100 ml contained the pure product which, after evaporation, was crystallized from MeOH–ether–light petroleum to give compound (**10**) (1.44 g, 75%) pure by t.l.c. (E); m.p. 189.5—190.5 °C; $[\alpha]_D^{25}$ −29.0° (*c* 1.0 in MeOH); amino acid analysis: $Phe_{1.00}$, $Gly_{0.99}$, and $Leu_{1.01}$ (Found: C, 61.7; H, 7.5; N, 10.9; S, 4.8. $C_{33}H_{47}N_5O_6S$ requires C, 61.76; H, 7.38; N, 10.91; S, 5.00%).

Z-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Hcy(Bzl)-NH_2 (**11**).—(a) *Deprotection step.* A solution of compound (**10**) (1.35 g, 2.1 mmol) in TFA–CH_2Cl_2 (3:7, 24 ml) was stirred for 15 min at r.t. Evaporation of the solvent left an oil which was partitioned between EtOAc (150 ml) and K_2CO_3 (10 ml, 30%). The organic phase was washed once with aqueous K_2CO_3 (30%) and once with brine (saturated) (10 ml) and was then dried (Na_2SO_4). Evaporation gave the amine (**10a**) (1.14 g, 100%), pure by t.l.c. (E), as a solid which was used as such.

(b) *Coupling step.* A solution of compound (**5**) (1.21 g, 1.85 mmol) and the amine (1.14 g, 2.1 mmol) in dry DMF (40 ml) was cooled to 0 °C before addition of DPPA (611 mg, 2.22 mmol) and TEA (311 μl, 2.22 mmol). After the mixture had been stirred for 48 h at 4 °C and 24 h at r.t. the solvent volume was reduced to 10 ml and the product was precipitated by addition of cold MeOH (50 ml). The precipitate was washed by repeated centrifugations with MeOH (3 × 40 ml) and ether (3 × 40 ml) to afford a fairly pure product (1.80 g, 83%) as judged by analytical h.p.l.c. To improve the purity the product was again dissolved in DMF (10 ml) and was then precipitated and washed as above. This re-precipitation afforded essentially pure product (1.57 g, 72%) (Figure 2); m.p. 243—245 °C (decomp.); $[\alpha]_D^{25}$ −44.3° (*c* 1 in DMF); amino acid analysis: $Pro_{1.05}$, $Glu_{1.99}$, $Phe_{1.97}$, $Gly_{1.00}$, and $Leu_{1.01}$ (Found: C, 61.0; H, 6.7; N, 13.1; S, 2.7. $C_{60}H_{77}N_{11}O_{12}S$ requires C, 61.26; H, 6.60; N, 13.10; S, 2.73%).

Z-Pro-Lys(Boc)-OMe (**12**).—A solution of Lys(Boc)-OMe [20] (2 g, 7.7 mmol) in CH_2Cl_2 (10 ml) was cooled in ice, Z-Pro-OSu [17] (2.5 g, 7.2 mmol) added and the resulting clear solution stirred with ice cooling for 4 h and overnight at r.t. The solution was then partitioned between EtOAc (400 ml) and 1M aqueous $KHSO_4$ (100 ml) and washed as for compound (**1**). Evaporation gave a crisp foam which was crystallized from EtOAc–light petroleum (1:3, v/v; 50 ml g^{-1}) to afford the pure product (**12**) (3.3 g, 93%); t.l.c. (A, E), m.p. 92—93 °C; $[\alpha]_D^{25}$ −24.4° (*c* 1 in DMF); amino acid analysis: $Pro_{0.98}$ and $Lys_{1.02}$ (Found: C, 60.7; H, 7.6; N, 8.5. $C_{25}H_{37}N_3O_7$ requires C, 61.08; H, 7.59; N, 8.55%).

Z-Arg(Adoc)$_2$-Pro-Lys(Boc)-OMe (**13**).—(a) *Deprotection step.* To a solution of compound (**12**) (2.53 g, 5.15 mmol) in dry DMF (10 ml/mmol) was added Pd/C (5%, 0.25 g) and the mixture hydrogenated at r.t. and normal pressure for 75 min. After removal of the catalyst by filtration through Celite, the filtrate was cooled to 0 °C and used immediately for coupling to minimize the formation of contaminating dioxopiperazine. The product was pure by t.l.c. (B and C).

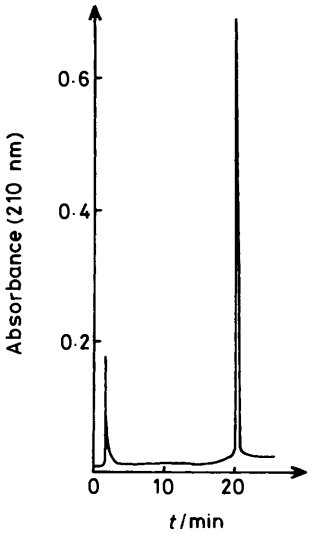


Figure 2. H.p.l.c. of the purified octapeptide (**11**). Mobile phase: A gradient of acetonitrile (18—67%) over 25 min in 0.1M phosphate buffer (pH 3.0), flow-rate 2.0 ml min^{-1}. Support: Spherisorb ODS (250 × 4.6 mm, 10 μm); detection: 210 nm

(b) *Coupling step.* Z-Arg(Adoc)$_2$-OSu [12] (3.81 g, 5 mmol) was added to a solution of the amine (5.15 mmol) in DMF (70 ml) at 0 °C. After being stirred for 2 h at this temperature the mixture was allowed to reach r.t. overnight. It was then evaporated and the resulting clear oil partitioned between ether (400 ml) and 1M $KHSO_4$ (100 ml). The organic extract was washed as for compound (**1**) and evaporated to give a white, crispy foam that was chromatographed in two portions on silica (500 ml) using CH_2Cl_2–acetone (7:1) as eluant. Evaporation gave a crisp foam (4.2 g, 84%), pure by t.l.c. (A and F); m.p. 94—96 °C; $[\alpha]_D^{25}$ −14.9° (*c* 1 in DMF); amino acid analysis: $Arg_{1.01}$, $Pro_{1.01}$, and $Lys_{0.99}$ (Found: C, 62.6; H, 7.9; N, 9.4. $C_{53}H_{77}N_7O_{12}\cdot H_2O$ requires C, 62.27; H, 7.79; N, 9.59%).

Z-Arg(Adoc)$_2$-Pro-Lys(Boc) (**14**).—To a solution of compound (**13**) (4 g, 4 mmol) in 90% dioxane (50 ml), was added 1M aqueous NaOH (4.2 ml, 4.2 mmol). The resulting mixture was stirred for 3 h at r.t. and then concentrated on an evaporator. After partitioning between EtOAc (400 ml) and 1M aqueous $KHSO_4$ (100 ml), the organic extract was washed with 1M $KHSO_4$ (3 × 50 ml) and brine (saturated) (2 × 50 ml) and dried ($MgSO_4$). Evaporation gave a clear oil which was dissolved in CH_2Cl_2 (10 ml) and added dropwise to cold (−20 °C) light petroleum (800 ml), to yield compound (**14**) (4 g, 100%), pure by t.l.c. (A, E); m.p. 130—132 °C (decomp.); $[\alpha]_D^{25}$ −9.3° (*c* 1 in DMF) (Found: C, 62.0; H, 7.6; N, 9.8. $C_{52}H_{75}N_7O_{12}\cdot H_2O$ requires C, 61.95; H, 7.70; N, 9.73%).

Z-Arg(Adoc)$_2$-Pro-Lys(Boc)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Hcy(Bzl)-NH_2 (**15**).—(a) *Deprotection step.* To a slurry of compound (**11**) (1.47 g, 1.25 mmol) in AcOH–thioanisole (6:1; 11 ml) was added HBr–AcOH (45%; 5.4 ml) which gave a clear solution. After being stirred for 2 h at r.t. the solution was added dropwise to ether (40 ml) to give a fine-grained precipitate which was collected by centrifugation and washed with ether (4 × 40 ml). The product (**11a**) (1.47 g, 105%) was used as such after being dried *in vacuo* over solid NaOH for 24 h.

(b) *Coupling step.* The amine salt (1.42 g, 1.2 mmol) was dissolved in dry DMF (9 ml) and neutralized with NMM (470 μl, 4.3 mmol) before addition of compound (**14**) (1.31 g, 1.32 mmol) and HOObt (215 mg, 1.32 mmol). The solution was cooled to −5 °C and DCC (273 mg, 1.32 mmol) added. An additional amount of NMM (100 μl, 0.91 mmol) was required after 3 h to keep the pH at 7. After being stirred for 72 h at 0 °C the reaction mixture was poured into ether (50 ml) and the fine-grained precipitate collected by centrifugation. After being washed with ether (6 × 50 ml) the product was applied, in portions (130 mg) in DMF (400 μl), to a Waters Delta Prep 3000 h.p.l.c. system, equipped with a column (250 × 20 mm, Spherisorb ODS 2, 10 μm) and a guard column, using 69% EtOH in 0.1M triethylammonium formate, pH 3.0 as an eluant. The flow-rate was 20 ml min^{-1} and the eluate was monitored at 230 nm. The main peak was eluted at 1 440—1 600 ml and contained altogether 920 mg (45% recovery) (Figure 3); $[\alpha]_D^{25}$ −43.7° (*c* 1 in MeOH); amino acid analysis: $Arg_{1.02}$, $Pro_{1.96}$, $Lys_{1.01}$, $Glu_{2.05}$, $Phe_{1.96}$, $Gly_{0.97}$, and $Leu_{1.03}$ (Found: C, 61.2; H, 7.2; N, 12.4; S, 1.5. $C_{104}H_{144}N_{18}O_{21}S{\cdot}H_2O$ requires C, 61.46; H, 7.24; N, 12.40; S, 1.58%); *m/z* 2 015.3 ($M{\cdot}H^+$).

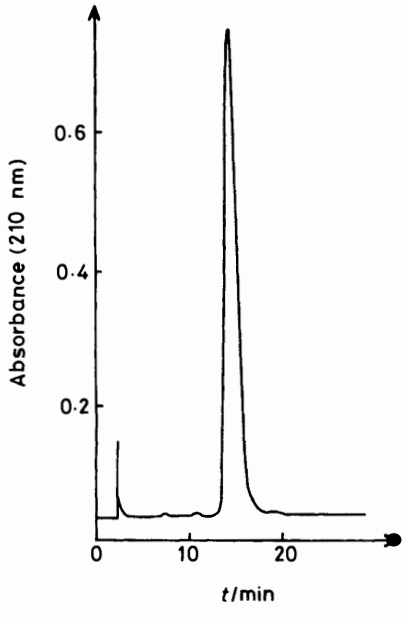


Figure 3. H.p.l.c. of the purified protected undecapeptide (**15**). Mobile phase: Isocratic 70% acetonitrile in 0.05M phosphate buffer (pH 3.0). Otherwise as in Figure 2

Z-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Hcy(Bzl)-NH_2 Acetate (**16**).—Compound (**15**) (350 mg, 0.17 mmol) was dissolved in TFA–thioanisole (99:1; 3.5 ml) and stirred for 2 h at r.t. The reaction was terminated by addition of dry ether (75 ml) giving a fine-grained precipitate which was collected by centrifugation and, after decanting, repeatedly digested with ether (5 × 20 ml) and centrifuged. An aliquot was then applied, in portions (6 mg) to the LDC h.p.l.c. system equipped with a guard column and a 250 × 10 mm Spherisorb ODS 2, 10 μm column. The mobile phase used was a gradient of MeCN (31—39%, 15 min) in 0.1M triethylammonium formate, pH 3.0 with a flow-rate of 8 ml min^{-1}. The product was eluted at 96—120 ml and, after evaporation of the acetonitrile, was twice freeze-dried from dilute AcOH. The product (**16**), 69% overall yield, was essentially pure as judged from analytical h.p.l.c. (Figure 4) on Spherisorb ODS, 10 μm (linear gradient of 30—60% MeCN in 0.1M Na phosphate, pH 3.0, for 15 min, flow-rate 2 ml min^{-1}). Amino acid analysis: $Arg_{1.02}$, $Pro_{1.95}$, $Lys_{1.03}$, $Glu_{1.99}$, $Phe_{1.98}$, $Gly_{1.00}$, and $Leu_{1.03}$. Peptide content: 92% (theory requires 92.8% for the diacetate).

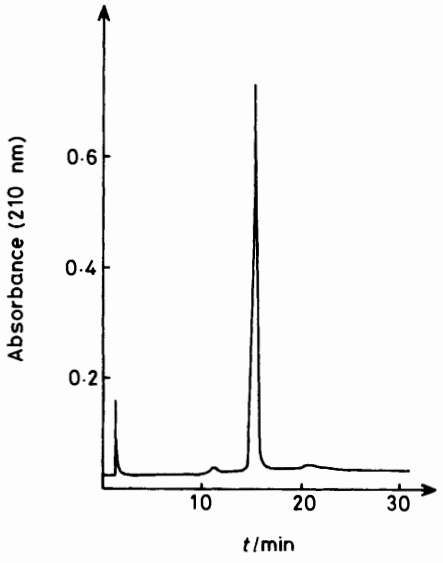


Figure 4. H.p.l.c. of the purified undecapeptide (**16**). Conditions as in Figure 2 except that a gradient of 30—60% acetonitrile over 15 min was used

[S-methyl-^{11}C]*Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-*NH_2 (**17**) *and* [S-methyl-^{11}C]*Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-*NH_2 (**18**).—The removal of the Z and S-Bzl groups from the protected precursors (**11**) and (**16**), made shortly before the [^{11}C]carbon dioxide arrived at the radiochemical laboratory, was performed in a three-neck septum-equipped reaction vial (originally developed for the synthesis of [S-*methyl*-^{11}C]methionine).[14] Compound (**11**) or (**16**) (5—10 mg) and sodium (2—3 mg) were added to the reaction vial and a drying tower with sodium hydroxide was connected to one of the necks as a gas outlet. Ammonia, passed through another drying tower with sodium hydroxide, was condensed at −78 °C up to a volume of 1.5—2 ml. If a persistent blue colour was obtained, during or after ending the condensation, a small amount of ammonium chloride was added in order to destroy excess of sodium. When the synthesis of [^{11}C]methyl iodide was finished it was transferred with a nitrogen gas stream and trapped in the reaction vial (Figure 1). After 1—2 min a constant level of radioactivity was obtained in the vial. Ammonia was then removed using nitrogen gas and gentle heating. The solid residue obtained was dissolved in 10% aqueous acetic acid (2 ml) 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. detector and a GM detector. The mobile phase used was a mixture of ammonium formate (0.1M, pH 3) and methanol [50/50, v/v for (**17**) and 45/55 for (**18**)] with a flow-rate of 8 ml min^{-1}. After evaporation of the appropriate h.p.l.c.-fractions, the residue was dissolved in ethanol–propylene glycol solution, pH-adjusted, and sterile filtered using a Millipore Millex-GS 0.22 μm filter. Analyses of the crude and the purified products were performed on a Hewlett Packard 1090, 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 (Figures 5 and 6).[21] Gradients of sodium phosphate (0.1M, pH 3) and acetonitrile or the above mentioned system were used. Mass spectra gave for (**17**): *m/z* 966.3 ($M{\cdot}H^+$) and for (**18**): *m/z* (1 347.5 ($M{\cdot}H^+$).

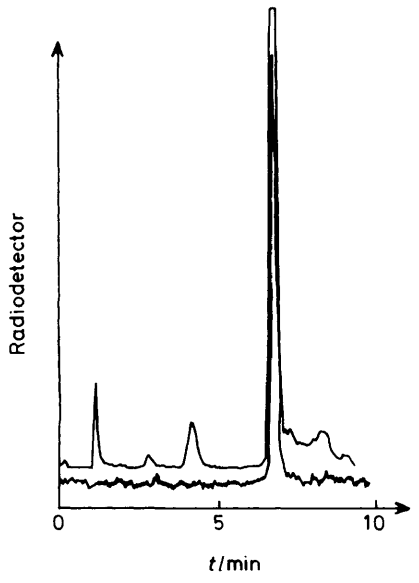


Figure 5. H.p.l.c. of crude and purified ^{11}C-labelled octapeptide (**17**). Mobile phase: A gradient of methanol (B) in 0.05M ammonium formate buffer (pH 3.5). Time 0—4 min, 40% B; 4—8 min, linear gradient to 85% B; 8—10 min, 85% B; flow-rate 2.0 mol min^{-1}. Support: Nucleosil C18 (250 × 4.6 mm, 10 μm), β-flow detector

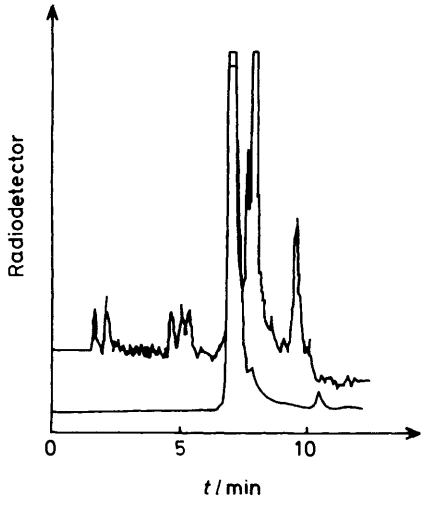


Figure 6. H.p.l.c. of the crude and purified ^{11}C-labelled undecapeptide (**18**). Mobile phase: A gradient of acetonitrile (B) in 0.1M phosphate buffer (pH 3.0). Time 0—10 min, linear gradient 17—35% B; 10—12 min linear gradient to 56% B; 12—14 min, 56% B; flow-rate 2.0 ml min^{-1}. Support: Hewlett Packard RP-18 (250 × 4.6 mm, 5 μm), β-flow detector

Acknowledgements

This work was supported by the National Swedish Board for Technical Development and the Swedish Natural Science Research Council. In addition we thank Dr. P. Malmborg for radionuclide production and Mr. A. Sandström for his skilful recording of mass spectra.

References

1 (*a*) M. R. Hanley and L. L. Iversen, in 'Neurotransmitter Receptors part I,' eds. S. J. Enna and H. I. Yamamura, Chapman and Hall, London, 1980, vol. 9, ser. B, p. 71; (*b*) B. E. B. Sandberg and L. L. Iversen, *J. Med. Chem.*, 1982, **25**, 1009; (*c*) T. M. Jessell, *Nature*, 1982, **295**, 551; (*d*) B. Pernow, *Pharmacol. Rev.*, 1983, **35**, 85; (*e*) R. von Gamse, *Arzneim. Forsch.*, 1984, **34**, 1074.
2 M. M. Chang, S. E. Leeman, and H. D. Niall, *Nature*, 1971, **232**, 86.
3 (*a*) G. W. Tregear, H. D. Niall, J. T. Potts, S. E. Leeman, and M. M. Chang, *Nature*, 1971, **232**, 87; (*b*) R. W. Bury and M. L. Mashford, *J. Med. Chem.*, 1976, **19**, 854; (*c*) R. Couture and D. Regoli, *Pharmacology*, 1982, **24**, 1.
4 J. S. Fowler and A. P. Wolf, in 'Positron Emission Tomography and Autoradiography: Principles and Applications for the Brain and Heart,' eds. M. Phelps, I. Mazziotta, and H. Schelbert, Raven Press, New York, 1986, p. 391.
5 (*a*) T. Greitz, D. H. Ingvar, and L. Widén, 'The Metabolism of the Human Brain Studied with Positron Emission Tomography,' Raven Press, New York, 1985; (*b*) M. Phelps, I. Mazziotta, and H. Schelbert, ref. 4; (*c*) O. Hayaishi and K. Torizuka, 'Biomedical Imaging,' Academic Press, New York, 1986.
6 (*a*) K. Någren, B. Långström, and U. Ragnarsson, *Acta Chem. Scand.*, 1985, **B39**, 157; (*b*) K. Någren, U. Ragnarsson, and B. Långström, *Int. J. Appl. Radiat. Isot.*, 1986, **37**, 537.
7 T. Shioiri, K. Ninomiya, and S. Yamada, *J. Am. Chem. Soc.*, 1972, **94**, 6203.
8 W. König and R. Geiger, *Chem. Ber.*, 1970, **103**, 2034.
9 J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, 1961, **26**, 2333.
10 H. Künzi, D. Gillessen, A. Trzeciak, R. O. Studer, B. Kerdelhué, M. Jutisz, and W. Lotz, *Helv. Chim. Acta*, 1974, **57**, 2131.
11 W. König and R. Geiger, *Chem. Ber.*, 1970, **103**, 788.
12 L. Grehn, B. Fransson, and U. Ragnarsson, *J. Chem. Soc., Perkin Trans. 1*, 1987, 529.
13 W. König and R. Geiger, *Chem. Ber.*, 1970, **103**, 2024.
14 B. Långström, G. Antoni, P. Gullberg, C. Halldin, K. Någren, A. Rimland, and H. Svärd, *J. Nucl. Med.*, in the press.
15 C. M. Svahn and J. Gyllander, *J. Chromatogr.*, 1979, **170**, 292.
16 W. König and R. Geiger, *Chem. Ber.*, 1970, **103**, 2041.
17 G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, 1964, **86**, 1839.
18 B. Långström, S. Sjöberg, and U. Ragnarsson, *J. Labelled Compd. Radiopharm.*, 1981, **18**, 479.
19 E. Schröder, *Ann. Chem.*, 1964, **673**, 186.
20 (*a*) J. W. Scott, D. Parker, and D. R. Parrish, *Synth. Commun.*, 1981, **11**, 303; (*b*) R. Schwyzer and W. Rittel, *Helv. Chim. Acta*, 1961, **44**, 159.
21 B. Långström and H. Lundqvist, *Radiochem. Radioanal. Lett.*, 1979, **41**, 375.

Received 29th September 1986; *Paper* 6/1916