SYNTHESIS OF THREE ^{11}C-LABELLED METHIONINE-CONTAINING ENKEPHALIN ANALOGUES

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SUMMARY

The synthesis of three S-[methyl-^{11}C]-labelled enkephalin analogues, Tyr-D-Ala-Gly-Phe-Met-NH_2, Tyr-D-Ala-D-Ala-Phe-Met-NH_2 and Tyr-D-Met-Gly-Phe-Pro-NH_2, from the corresponding S-benzyl-homocysteine-containing peptides is reported. The protected pentapeptide amides were prepared by (3+2) fragment condensations in solution. These peptides were subsequently deprotected with sodium in liquid ammonia and the sulphide anions formed alkylated with [^{11}C]-methyl iodide to give the S-[methyl-^{11}C]-labelled enkephalins. After purification by preparative LC, these labelled peptides were obtained in 55 to 75 % radiochemical yield, decay corrected, based on the [^{11}C]methyl iodide produced, within 30-40 min from start of the synthesis of this reagent. The radiochemical purities of the products were higher than 98 %, and the specific activity was in the order of 20-200 mCi/μmol.

Key words: S-[methyl-^{11}C]Tyr-D-Ala-Gly-Phe-Met-NH_2, S-[methyl-^{11}C]-Tyr-D-Ala-D-Ala-Phe-Met-NH_2, S-[methyl-^{11}C]Tyr-D-Met-Gly-Phe-Pro-HN_2, ^{11}C-labelled enkephalin analogues, ^{11}C-labelled peptides.

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0362-4803/88/020149–12$06.00

Received March 4, 1987
Revised May 28, 1987

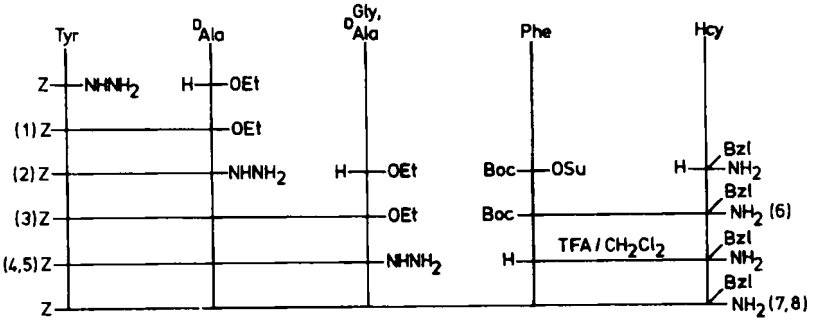
INTRODUCTION

The interest in small neuropeptides is steadily increasing as new ones continuously are isolated from living matter and analogues with increased biological activity and/or stability against degradation are prepared. The development of PET (positron emission tomography) now offers unique possibilities of studying the distribution and metabolism in vivo using such peptides labelled with short-lived, positron-emitting nuclides like ^{11}C and ^{13}N.

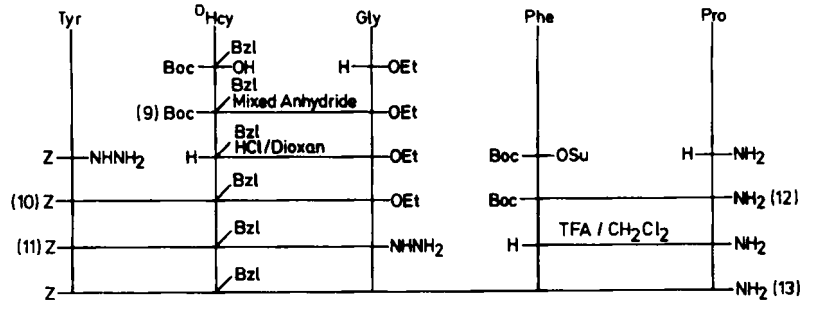
In this paper the synthesis of three ^{11}C-labelled methionine-containing enkephalin analogues, substituted with D-amino acids in the 2-position, is reported. The unlabelled peptides have previously been investigated and found to have increased activity and stability in biological tests.[1-3] Recently the ^{11}C-labelling of Met-enkephalin and two metabolic fragments[4] was reported utilizing the synthetic approach[5] originally developed in our laboratory for the synthesis of S-[methyl-^{11}C]methionine.[6] As Met-enkephalin is known to have a very short half-life in vivo,[7] the enkephalin analogues Tyr-D-Ala-Gly-Phe-Met-NH_2, Tyr-D-Ala-D-Ala-Phe-Met-NH_2 and Tyr-D-Met-Gly-Phe-Pro-NH_2, all labelled with ^{11}C in the methionine methyl group, have been prepared and studied, as has Met-enkephalin, in in vivo and in vitro studies in Rhesus monkeys and the results are reported elsewhere.[8]

RESULTS AND DISCUSSION

The synthesis of the protected pentapeptides 7, 8 and 13 is illustrated in Schemes 1 and 2. All synthetic steps leading to these pentapeptides were rather straightforward and proceeded in high yields. Different methods have been studied for some of the coupling and deprotection steps and attention has been paid to the ease of the work-up and crystallization procedures.



Scheme 1

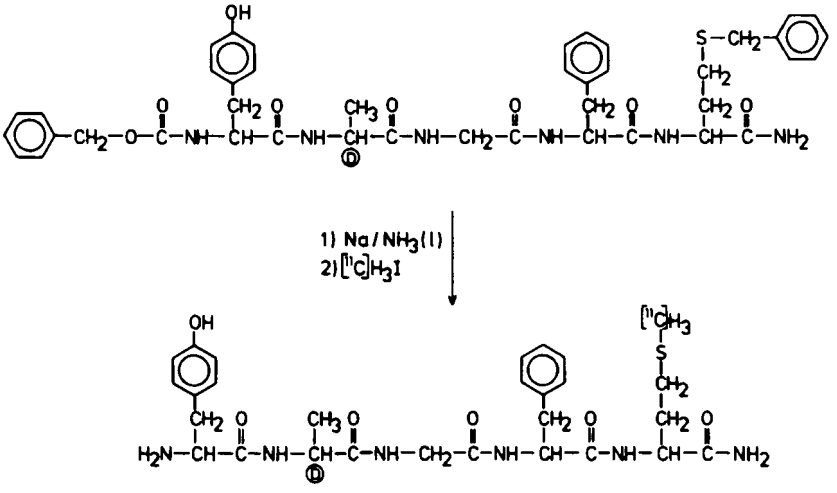


Scheme 2

In the coupling steps leading to the two peptides 6 and 12 the use of Boc-Phe-OSu gave products which could be purified by a single crystallization step, and was thus superior both to direct DCC-coupling with or without additives and to the mixed anhydride coupling method. The products obtained on removal of the Boc-group from 6 and 12 were checked for diketopiperazine formation on liberation of the amines from their salts, as Phe-Pro-NH_2 is reported to give this byproduct.[9] No significant byproducts were detected and the amines were therefore liberated before coupling, as the washing step removed traces of impurities.

The ^{11}C-labelled peptides, S-[methyl-^{11}C]Tyr-D-Ala-Gly-Phe-Met-NH_2 (14), S-[methyl-^{11}C]Tyr-D-Ala-D-Ala-Phe-Met-NH_2 (15) and S-[methyl-

[^{11}C]Tyr-D-Met-Gly-Phe-Pro-NH_2 (16) were prepared by analogy with the synthesis of S-[methyl-^{11}C]Met-enkephalin as exemplified in Scheme 3 for the synthesis of 14. They were all obtained in high radiochemical yield and purity as presented in Table 1. When precautions were taken with respect to the amount of sodium used in the deprotection step and to the exclusion of moisture from the reaction vessels, the syntheses were very reproducible. The sodium/ammonia reagent is known to cleave amide bonds in which proline amino groups are parts,[10] and such side reactions have been encountered in the synthesis of S-[methyl-^{11}C]-Substance P,[11] but this has not been observed in the synthesis of 16.



Scheme 3

Labelled Peptide	Yield (%) Crude[1]	Prep[2]	Time[3] (min)
S-[methyl-^{11}C]Tyr-D-Ala-Gly-Phe-Met-NH_2	70-75	55-60	30-40
S-[methyl-^{11}C]Tyr-D-Ala-D-Ala-Phe-Met-NH_2	83-94	70-75	30-40
S-[methyl-^{11}C]Tyr-D-Met-Gly-Phe-Pro-NH_2	70-87	55-65	30-40

1. The percentage of [^{11}C]methyl iodide that has reacted to the correct product.
2. As above, but also including preparative LC.
3. Total time of preparation, from start of synthesis of [^{11}C]methyl iodide to sterile filtered product.

Table 1

EXPERIMENTAL

General: Melting points are uncorrected. Optical rotations were measured on a Perkin Elmer 141 polarimeter. Elemental analyses were performed at Mikro Kemi AB, Uppsala (one decimal) or Novo Microanalytical Laboratory, Bagsvaerd, Denmark (two decimals). Amino acid analyses were carried out after acid hydrolysis at the Institute of Biochemistry, Biomedical Center. When not otherwise stated, ethyl acetate extracts of evaporated reaction mixtures were washed twice with aqueous solutions of sodium hydrogen carbonate (1 M), potassium hydrogen sulphate (1 M) and sodium chloride (sat.) and dried over magnesium or sodium sulphate.

The purity of all compounds was checked by TLC using the following systems: A: methylene chloride ($MeCl_2$)/acetone/acetic acid, 5/5/1; B: chloroform/ethanol/water, 100/50/4; C: $MeCl_2$/methanol, 9/1 and D: $MeCl_2$/acetone/acetic acid, 40/10/1. The protected pentapeptides (7, 8 and 13) were also analysed by LC on an LDC system with a 228 x 4.6 mm Spherisorb ODS 10 μm column and u.v. detection at 210 nm using gradients of E: aqueous NaH_2PO_4 (0.1 M, pH 2.98) and F: acetonitrile/aqueous NaH_2PO_4 (0.1 M, pH 2.98), 70/30 (v/v).

The ^{11}C was produced at the tandem Van de Graaff accelerator of the University of Uppsala by means of the $^{14}N(p,\alpha)^{11}C$ reaction on a nitrogen gas target. The [^{11}C]carbon dioxide produced was trapped in 4Å molecular sieves and transported to the radiochemistry laboratory. [^{11}C]Methyl iodide was prepared according to the standard procedure of our laboratory.[6] Analytical LC for the ^{11}C-syntheses was carried out on an HP 1084B with a variable wavelength detector, or an HP 1090 with a diode array detector, at 274 nm, equipped with a 250 x 4.6 mm Spherisorb C-18 10 μm column at 40 °C and a β-flow detector. Preparative LC was performed, using a 250 x 10 mm Spherisorb 30 μm column, on a Waters system equipped with a u.v. detector M-441 (254 nm) and a GM detector. Aqueous ammonium formate, 0.1 M, pH 3.5 (G) and methanol were used as solvents. Sterile aqueous solutions of hydrochloric acid (0.1 M), sodium hydroxide (0.1 M) and phosphate buffer (pH 7.4, physiological buffer) and sterile

septum-equipped flasks were obtained from the Hospital Pharmacy, University Hospital of Uppsala. Millipore Millex-GS 0.22 μm filters were used for sterile filtration.

Precursor synthesis: N-Z-Tyr-D-Ala-OEt (1): N-Z-Tyr-$NHNH_2$ (3.29 g, 10 mmol) was dissolved in 30 mL of dimethyl formamide (DMF) and the solution cooled to -20 °C before the addition of 12.5 mL of a 4 M solution of HCl in dioxan and 1.93 mL of isoamyl nitrite (IAN). The solution was stirred for 90 min at this temperature and was then cooled to -30 °C before the addition of 1.62 g (10.5 mmol) of D-Ala-OEtxHCl and 8.5 mL of triethylamine (TEA) in 15 mL of DMF. The temperature was allowed to rise to 4 °C during one hour, at which temperature the reaction mixture was finally stirred for 43 hours. A total of 1.0 mL of TEA was added in portions to maintain the pH at 7.5-8.0. The residue obtained after standard work-up was crystallized from methanol/ether/petroleum ether (PE) to give 3.42 g (83 %) of the pure (A) product, m.p. 135.5-6.5 °C, $[\alpha]_D^{25}$ + 9.0° (c 1.0, EtOH).

N-Z-Tyr-D-Ala-$NHNH_2$ (2): Compound 1 (3.31 g, 8 mmol), was dissolved in 20 mL of ethanol and 4.8 mL of hydrazine hydrate (HH) added. After stirring for 18 h at room temperature a solid mass was obtained and 50 mL of ether was added. The product was filtered off and washed with small portions of ether. Drying yielded 3.01 g (94 %) of the pure (A) product, m.p. 205-6 °C, $[\alpha]_D^{25}$ -20.3° (c 1.0, DMF).

N-Z-Tyr-D-Ala-Gly-OEt (3): A solution of 2 (2.00 g, 5.0 mmol) in 20 mL of DMF was cooled to -20 °C and 5.5 mL of 4.54 M HCl in dioxan and 0.83 mL of IAN was added. After 1 h at -20 °C the temperature was lowered to -30 °C and a pre-cooled solution of 733 mg of Gly-OEtxHCl and 4.23 mL of TEA in 5 mL of DMF was added. The temperature was then increased to 4 °C during 1 h and, while being stirred for 45 h, a total of 675 μL of TEA was added in portions to keep the pH at 7.5-8.0. After removing the precipitated amine salt by filtration and the solvent by evaporation, the crude product was worked up in the standard manner. Crystal-

lization from ethanol/ether/PE gave 1.93 g (82 %) of pure (D) 3, m.p. 176-7 °C, $[\alpha]_D^{25}$ + 31.2° (c 1.0, EtOH).

N-Z-Tyr-D-Ala-Gly-$NHNH_2$ (4): Compound 3 (1.88 g, 4.0 mmol) was dissolved in 35 mL of methanol and 2.4 mL of HH added. After stirring for 9 h at room temperature, the slurry was placed in a freezer overnight. Ether (50 mL) was then added to the thick mass and the product collected by filtration and washed with small portions of ether. Drying yielded 1.37 g (75 %) of the pure (C) product, m.p. 184-6 °C, $[\alpha]_D^{25}$ -20.9° (c 1.0, DMF).

N-Z-Tyr-D-Ala-D-Ala-OEt: Compound 2 (1.20 g, 3.0 mmol) and D-Ala-OEtxHCl (484 mg, 3.15 mmol) were coupled in 15 mL of DMF as described for 3. Several attempts to crystallize the oily residue obtained after evaporation of the ethyl acetate failed, so the crude product (homogeneous on TLC (D)) was used directly in the synthesis of 5.

N-Z-Tyr-D-Ala-D-Ala-$NHNH_2$ (5): The crude N-Z-Tyr-D-Ala-D-Ala-OEt was dissolved in 10 mL of methanol and 1.8 mL of HH added. After 11 hours at room temperature the reaction mixture was placed in a freezer and the product isolated as described for 4, giving 1.06 g (75 %) of pure (C) 5, m.p. 217-9 °C, $[\alpha]_D^{25}$ -14.0° (c 1.0, DMF).

Boc-Phe-Hcy(Bzl)-NH_2 (6): Solutions of Boc-Phe-OSu (4.26 g, 12.3 mmol) in 15 mL of DMF and Hcy(Bzl)-NH_2[11] (2.90 g, 12.9 mmol) in 10 mL of DMF were prepared and cooled to -20 °C. The latter solution was then added during 3 min to the former one, and the mixture stirred for 2 h at -20 °C and 6 h at 4 °C. Evaporation gave a solid residue which was worked up in the standard manner and finally crystallized from ethanol/PE to give 4.94 g (85 %) of the pure (C) product, m.p. 162-4 °C, $[\alpha]_D^{25}$ -25.1° (c 1.0, DMF). Anal. $C_{25}H_{33}N_3O_4S$. Found/calc: C: 63.7/63.67; H: 7.1/7.05; N: 9.0/8.91 and S: 6.8/6.80.

Phe-Hcy(Bzl)-NH_2: Compound 6 (1.41 g, 3.0 mmol) was dissolved in trifluoroacetic acid (TFA, 30 % in $MeCl_2$ (v/v), 30 mL) and the mixture stirred for 15 min at room temperature. After evaporation, the residue

was distributed between ethyl acetate (300 mL) and K_2CO_3 (30 % aq (w/w), 30 mL). The organic phase was then washed with the same amount of K_2CO_3, and dried over $MgSO_4$. Following evaporation, the product (100 %) was used directly in the synthesis of 7.

N-Z-Tyr-D-Ala-Gly-Phe-Hcy(Bzl)-NH_2 (7): The two fragments 4 (1.14 g, 2.5 mmol) and Phe-Hcy(Bzl)-NH_2 (1.12 g, 3.0 mmol) were coupled in 22 mL of DMF using the same procedure as in the synthesis of 3. As the product was sparingly soluble in ethyl acetate and other organic solvents suitable for phase separations with aqueous solutions, a different work-up procedure was used. The reaction solution was poured slowly into $KHSO_4$ (1.0 M, 200 mL) and the solution placed in a refrigerator overnight. After collection by filtration of the precipitated material, it was reprecipitated from DMF, twice with $KHSO_4$, once with distilled water and once with ether. Drying gave the pure (C) product 7 (1.34 g, 67 %), m.p. 189-92 °C, $[\alpha]_D^{25}$ -25.3° (c 1.0, DMF). Anal. $C_{42}H_{48}N_6O_8S$. Found/calc: C: 62.7/63.30; H: 6.2/6.07; N: 10.6/10.55 and S: 3.97/4.02. Amino acid analysis: $Tyr_{0.96}Ala_{1.01}Gly_{0.99}Phe_{1.04}$.

N-Z-Tyr-D-Ala-D-Ala-Phe-Hcy(Bzl)-NH_2 (8): Coupling of the two fragments 5 (943 mg, 2.0 mmol) and Phe-Hcy(Bzl)-NH_2 (893 mg, 2.4 mmol) and the subsequent work-up of the product were performed as described for 7. The yield of the pure (C) dry product was 787 mg (49 %), m.p. 216-20 °C, $[\alpha]_D^{25}$ -25.7° (c 1.0, DMF). Anal. $C_{43}H_{50}N_6O_8S$. Found/calc: C: 63.6/63.69; H: 6.3/6.21; N: 10.3/10.36 and S: 3.96/3.95. Amino acid analysis: $Tyr_{0.97}Ala_{2.02}Phe_{1.01}$.

Boc-D-Hcy(Bzl)-Gly-OEt (9): A solution of 1.71 g (5.25 mmol) of Boc-D-Hcy(Bzl), prepared as described for the L-isomer,[13] and 0.55 mL of N-methyl morpholine (NMM) in 20 mL of THF was cooled to -15 °C, and 0.69 mL of isobutyl chloroformate added. After an activation time of 1 min, a pre-cooled solution of 0.69 g (5.0 mmol) of Gly-OEtxHCl and 0.55 mL of NMM in 10 mL of DMF was added, and the reaction mixture stirred for 30 min at -15 °C and for 4 h at room temperature. After

filtration and evaporation the residue was worked up in the standard manner. Evaporation gave an oily residue which was triturated with PE to give a solid product. Crystallization from ether/PE, finally, gave 1.43 g (70 %) of the pure (A) product, m.p. 65.5-6.5 °C, $[\alpha]_D^{25}$ + 12.2° (c 1.0, EtOH). Anal. $C_{20}H_{30}N_2O_5S$. Found/calc: C: 58.43/58.51; H: 7.41/7.37; N: 6.77/6.82 and S: 7.90/7.81.

D-Hcy(Bzl)-Gly-OEtxHCl: To a solution of 1.36 g (3.3 mmol) of 9 in 10 mL of dioxan, 16 mL of a 4.1 M solution of HCl in dioxan was added and the reaction mixture stirred for 2.5 h at room temperature. Evaporation of the solvent gave an oily residue, pure by TLC (A), which failed to crystallize when triturated with ether. It was used directly in the synthesis of 10.

N-Z-Tyr-D-Hcy(Bzl)-Gly-OEt (10): N-Z-Tyr-$NHNH_2$ (1.04 g, 3.15 mmol) and crude Hcy(Bzl)-Gly-OEtxHCl (3.3 mmol) were reacted in 20 mL of DMF as described for 1 (coupling completed at 4 °C for 25 h). Evaporation of the ethyl acetate resulted in a semicrystalline product which was crystallized from EtOH/PE giving 1.36 g (71 %) of the pure (A) product, m.p. 131-2 °C, $[\alpha]_D^{25}$ + 50.9° (c 1.0, EtOH). Anal. $C_{32}H_{37}N_3O_7S$. Found/calc: C: 63.28/63.24; H: 6.20/6.14; N: 6.90/6.91 and S: 5.28/5.28.

N-L-Tyr-D-Hcy(Bzl)-Gly-$NHNH_2$ (11): Compound 10, (1.22 g, 2.0 mmol) was dissolved in 50 mL of ethanol and 1.2 mL of HH added. After stirring for 48 h at room temperature, the solution was placed in a refrigerator overnight and the precipitated product then collected by filtration. Washing with small portions of cold ethanol and ether and drying yielded 985 mg (83 %) of the pure (A) product, m.p. 149.5-50.5 °C, $[\alpha]_D^{25}$ + 14.1° (c 1.0, DMF).

Boc-Phe-Pro-NH_2 (12): Boc-Phe-OSu (1.82 g, 5.25 mmol) was dissolved in 7 mL and Pro-NH_2 (630 mg, 5.5 mmol) in 8 mL of DMF, and the latter solution added dropwise during a couple of minutes to the former one at -20 °C. The temperature was increased during 90 min to a final value of

4°, at which temperature the solution was stirred for 22 h. After evaporation of the solvent the residue was worked up in the standard manner. Crystallization from ethanol/PE gave 1.71 g (90 %) of the pure (C) product, m.p. 76-7 °C, $[\alpha]_D^{25}$ -33.5° (c 1.0, EtOH).

Phe-Pro-NH_2: Compound 12 (651 mg, 1.8 mmol) was deprotected and the product worked up as described for Phe-Hcy(Bzl)-NH_2. The crude product (550 mg, 100 %), pure as judged by TLC (A), was used as such in the synthesis of 13.

N-Z-Tyr-D-Hcy(Bzl)-Gly-Phe-Pro-NH_2 (13): The coupling of the two fragments 11 (890 mg, 1.5 mmol) and crude Phe-Pro-NH_2 (1.8 mmol) and the subsequent work-up were performed as described for 7. The yield of the pure (A) dry product was 765 mg (62 %), m.p. 143-6 °C, $[\alpha]_D^{25}$ -4.8° (c 1.0, DMF). Anal. $C_{44}H_{50}N_6O_8S$. Found/calc: C: 63.86/64.22; H: 6.15/6.12; N: 9.84/10.21 and S: 3.81/3.90. Amino acid analysis: $Tyr_{0.96}Gly_{1.00}Phe_{1.04}Pro_{1.01}$.

Synthesis of labelled peptides: S-[methyl-^{11}C]Tyr-D-Ala-Gly-Phe-Met-NH_2 (14), S-[methyl-^{11}C]Tyr-D-Ala-D-Ala-Phe-Met-NH_2 (15) and S-[methyl-^{11}C]-Tyr-D-Met-Gly-Phe-Pro-NH_2 (16): Immediately before the production of [^{11}C]methyl iodide, 1-5 mg (approx. 5 μmol) of the proper protected peptide (7 for the synthesis of 14, 8 for 15 and 13 for 16) was placed in a three-neck septum-equipped reaction vial (originally developed for the synthesis of S-[methyl-^{11}C]methionine[6]). Sodium (1-2 mg, previously suspended and stored under pentane) was added and the vessel cooled to -78 °C. A drying tower containing sodium hydroxide was connected as an outlet to the vessel, and ammonia (passed through another drying tower with sodium hydroxide) was condensed, within 2 min, up to a volume of 1-2 mL. If a persistent blue colour was obtained during or after ending the condensation, some ammonium chloride was added in order to destroy the excess sodium. The [^{11}C]methyl iodide was then transferred to the reaction vessel in a nitrogen gas stream. After a transfer time of 1-2

min, a steady state of radioactivity was reached. The ammonia was then removed using nitrogen gas and gentle heating, giving a solid residue. This was dissolved in 1.5 mL of physiological buffer and purified by preparative LC using an isocratic system of solvents G/MeOH, 70/30 (v/v) for peptides 14 and 15, and 60/40 for peptide 16, with a flow rate of 8 mL/min. After evaporation of the appropriate LC-fractions, the residue was dissolved in physiological buffer, pH-adjusted and sterile filtered. Analysis for radiochemical and chemical purity were performed by analytical LC using the following LC-programme: Time 0-7 min, linear gradient 80-40 % G, and then a washing program time 7-9, linear gradient to 20 % G, time 9-11, 20 % G isocratic, with a flow rate of 3 mL/min (retention times for 14, 15 and 16 were 4.03, 4.34 and 5.87 min respectively).

Acknowledgements. We are indebted to Dr P. Malmborg for the radionuclide production. This work forms part of projects financially supported by the Swedish Natural Science Research Council by grants K-DT 1777-100, K-KU 3020 and K-KU 3464.

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