Neuropeptides labelled with ^{35}S

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Abstract

Methionine and cysteine containing peptides can be labelled with ^{35}S by coupling of ^{35}S-cysteine or ^{35}S-methionine with a large excess of suitably protected peptide precursors. This is illustrated for [pGlu^4, Cyt^6]AVP-(4-9), β-endorphin-(5-17) and the ACTH-fragment-analogue Org 2766. Using HPLC as final purification the peptides are obtained in radiochemical yields of about 5% and radiochemical purities between 90-97%. However the specific activities obtained are much lower than the specific activities of the starting ^{35}S-amino acids.

Keywords: peptide synthesis, radiolysis, memory effect.

Introduction

For the development of neuropeptides as potential drugs the availability of radioactive material is essential. While for metabolic and/or pharmacokinetic studies compounds labelled with tritium are often sufficient, materials of higher specific activity are needed for receptor binding studies[1]. For cysteine or methionine containing peptides, ^{35}S ($t_{\frac{1}{2}}$= 87,5 days)

Note: Nomenclature and symbolism for amino acids and protecting groups is according to the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature, *Biochem.J.* **219**, 345-373 (1984). Other abbreviations are: Cyt for cystine, and Scm for carbomethoxysulphenyl.

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in view of its high specific activity of 1500 Ci/mmole, is an attractive isotope since it can be incorporated into peptides using commercially available ^{35}S-amino acids obtained from bacteria grown on ^{35}S-sulphate. In addition to random labelling[2] only a few reports about specifically ^{35}S-labelled peptides have appeared: Neugebauer et al[3] described the synthesis of ^{35}S-enkephalin and ^{35}S-Met-ANF-(1-8) while we published[4] the preparation of ^{35}S-cysteine-[pGlu^4, Cyt^6]AVP(4-8). In this paper we describe the synthesis and characterisation of three ^{35}S-labelled neuropeptides, ^{35}S-[pGlu^4, Cyt^6]AVP-(4-9) (1, Org 30273) and the methionine containing peptides β-endorphin-(5-17) (2, Org 5970) and the ACTH-fragment-analogue Org 2766 (3). (See Figure 1).

*
H-Cys-OH
pGlu-Asn-Cys-Pro-Arg-Gly-NH_2
[4-pyroglutamic acid, 6-cystine] AVP(4-9)
Org 30273
1

H-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-OH
β-endorphin-(5-17)
Org 5970
2

H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH
ACTH-(4-9)-analogue
Org 2766
3

Figure 1

Results

Synthetic approaches

As described earlier[4] [pGlu^4, Cyt^6]AVP-(4-8) could be labelled with ^{35}S by direct coupling of S-activated [pGlu^4]AVP-(4-8) with ^{35}S-cysteine as obtained from the commercial supplier, i.e. in the presence of a "large" excess of dithiothreitol. Radiochemical yields were about 15% and the radiochemical purity (obtained after HPLC-purification) was 95%. Using a similar procedure (Figure 2) we also synthesized ^{35}S-[pGlu^4, Cyt^6]AVP-(4-9):

the symmetrical dimer of [pGlu^4]AVP(4-9) (4) was reacted with carbomethoxysulphenyl chloride to give the Scm-activated precursor 5. This material was reacted directly with 5 mCi ($\approx 10^{-3}$ equivalent) carrier free ^{35}S-cysteine in trifluoroethanol/methanol. Again the cysteine could be used as obtained from the supplier as a solution in aqueous sodium acetate containing mercaptoethanol. After 24 hours the crude product was purified by reversed phase HPLC to give ^{35}S-Org 30273 in a radiochemical yield of 10%. The final product had a radiochemical purity of about 90%. (see Figure 3). No attempts were made to increase the purity of the product since rechromatography with this kind of peptides does not improve the quality.

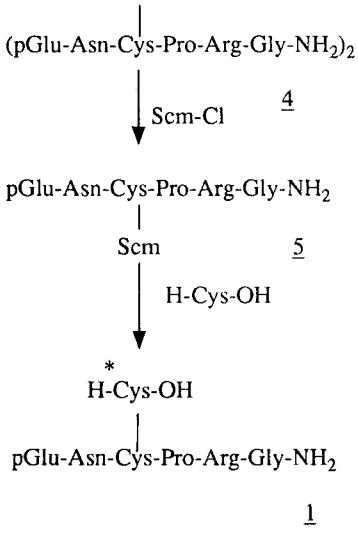


Figure 2: Reaction scheme for ^{35}S-[pGlu^4, Cyt^6]AVP-(4-9) (1)

Figure 4 gives the reaction scheme for Org 5970 (2): ^{35}S-methionine was converted in a radiochemical yield of 70-80% into Boc-methionine by reaction with excess $(Boc)_2O$. Reaction of this protected amino acid with an excess of peptide 7 (using hydroxybenzotriazole and dicyclohexylcarbodiimide for activation) gave the protected Org 5970- precursor 8 in radiochemical yields of 0,2 to 6,2 %. After deprotection with trifluoroacetic acid (in the presence of anisole and di-t-butyldisulphide) ^{35}S-Org 5970 (2) was obtained. The product was purified by HPLC resulting in a product with a radiochemical purity of $\geq$ 95% (see Figure 5).

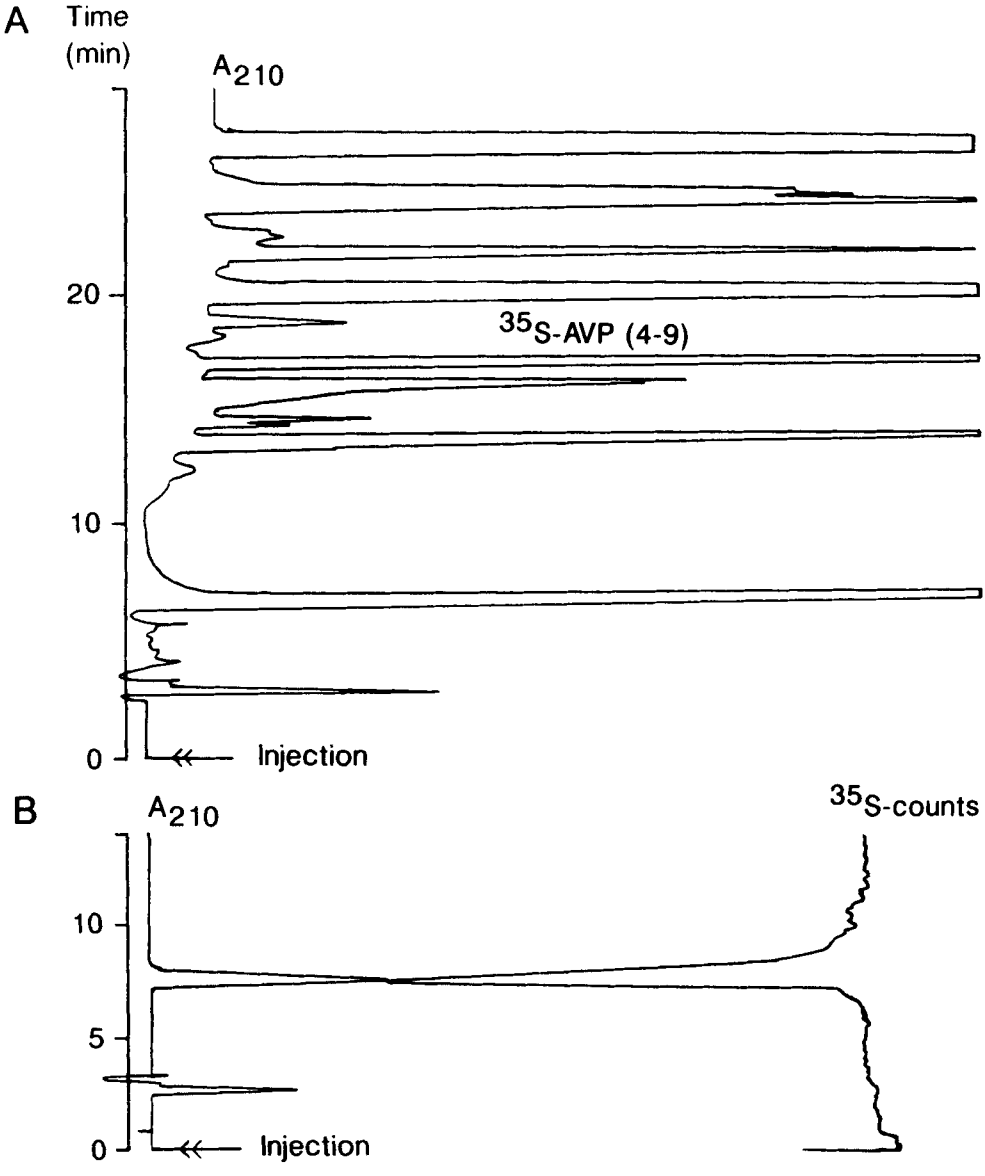
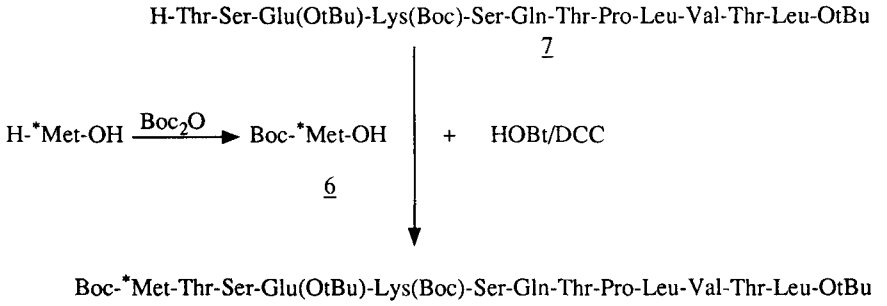
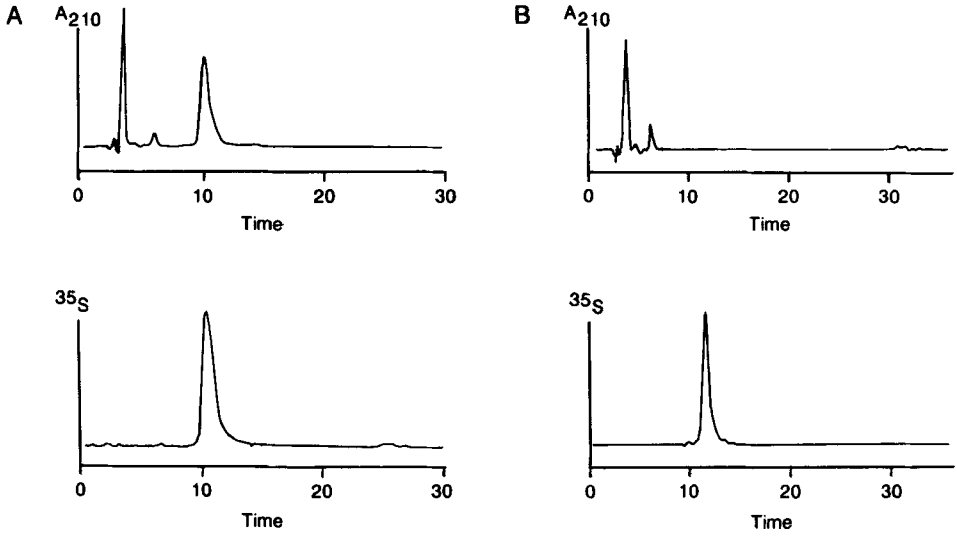


Figure 3: HPLC-traces of ^{35}S-[pGlu^4, Cyt^6]AVP-(4-9) (1); for HPLC conditions see experimental part.
A: reaction mixture
B: after purification





Fig

Figure 5: HPLC-patterns of purified ^{35}S-Org 5970 (2)
A: with carrier added B: no carrier added
For HPLC conditions see experimental part.

The reaction scheme for the synthesis of ^{35}S-Org 2766 is given in Figure 6: [^{35}S] Boc-methionine was reacted with an excess (300-500 equivalents) of pentapeptide 10, freshly prepared from the fully protected pentapeptide 9 by removal of the benzyloxycarbonyl group by catalytic hydrogenation. To minimize radiolysis some mercaptoethanol was added as scavenger. The resulting peptide was purified by chromatography over silica gel yielding 11 in radiochemical yield of about 25% (based on Boc-methionine). The protecting groups in 11 were removed by treatment with H_3PO_4. Due to radiolysis the resulting peptide was partly oxidized; complete oxidation into the sulphone 12 was realized by reaction with $H_2O_2/(NH_4)_6Mo_7O_{24}$. After removal of the excess reagents by filtration over Seppak the product was purified by HPLC. The radiochemical yields for the total synthesis varied between 2,5 and 11%; these moderate yields are partly caused by the substantial losses of material during the HPLC-purification (50% loss). As shown in Figure 7 the resulting product had a radiochemical purity of ≥ 97%.

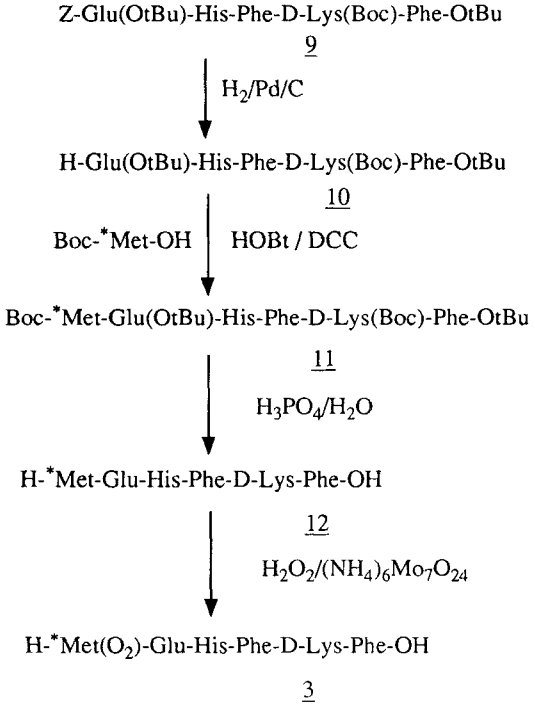


Figure 6: Reaction scheme for ^{35}S-Org 2766

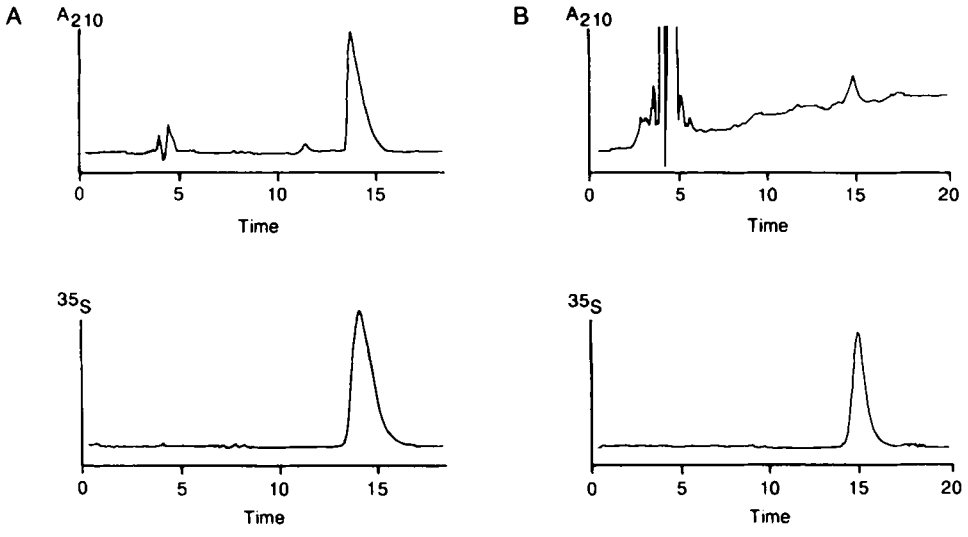


Figure 7: HPLC-traces of ^{35}S-Org 2766 (3)
A: with carrier; B: without carrier added
For HPLC-conditions see experimental part.

Table I: Radiochemical yield of ^{35}S-Org 5970 (2)

synthesis number	% yield
1	0,2
2	0,8
3	1,2
4	2,9
5	6,2
6	1,2

Quality control of the labelled peptides

Radioactive compounds are characterized by their radiochemical and chemical purity, position of the label and specific activity. As shown in the HPLC-pattern (Figures 3, 5 and 7) the ^{35}S-peptides had reasonable to good radiochemical purities while unlabelled, related peptides such as the precursors are absent. Information about specific activity, usually

obtained by mass spectrometry[5] or HPLC combined with liquid scintillation counting, could not be obtained due to the limited amount of material available (5 mCi ^{35}S peptide is about 5 μg).

Initially we assumed that the specific activity would be equal to the specific activity of the starting materials, however this assumption is not correct. During preparative and analytical HPLC-runs (e.g. see Figure 7) we observed larger mass peaks for the ^{35}S-peptides than could be expected based on a specific activity of about 1000 Ci/mmol. Because of the availability of a radioimmunoassay for Org 2766 we could measure the binding of the ^{35}S-peptide to antibodies in comparison with ^{3}H-Org 2766 of known specific activity. This indicated for one typical batch a specific activity between 150 and 200 Ci/mmol, a value in agreement with the intensity of the mass peaks observed during HPLC. This means that during the synthetic procedure dilution with cold peptide had occurred. For the other peptides we could not measure the specific activity with this method because no antibodies for these peptides were available, but judged from the HPLC-traces dilution with these materials was less serious; e.g. in the HPLC-patterns of the purified material (Figures 3B and 5B) no mass peaks were visible. The dilution with unlabelled peptides was probably caused by memory effects in the final HPLC-purification; the columns used were calibrated and more important, deactivated by the injection of unlabelled peptide. Losses of proteins and peptides in HPLC-systems is a known problem[6] and although in this study special peptide-columns were used, deactivation of HPLC-equipment was still essential for the obtained yields as illustrated in Table I; the yields of Org 5970 increased (mainly because of enhanced recovery in the HPLC-systems) with the number of the syntheses performed, but a dramatical decrease in yield was obtained when a new pre-column was introduced in the HPLC-system (synthesis number 6).

Concluding remarks

As illustrated in this article, peptide synthesis on a μg scale can be used to synthesize ^{35}S-methionine and cysteine containing peptides in acceptable radiochemical yields. However, improved methods (capillary zone electrophoresis) should be found to purify

these peptides without negative effects on the specific activities of the final products.
In contrast to the synthesis of ^{3}H or ^{14}C-labelled compounds radioactive contamination is a problem in working with ^{35}S. Recently Klein et al.[7] reported that ^{35}S-labelled amino acids release volatile ^{35}S-compound(s) resulting in serious contamination of equipment. Once incorporated in peptides this uncontrolled release of radioactivity is no longer a problem and the usual safety measures when working with β-emitters are sufficient.

Experimental details

^{35}S-cysteine (spec. activity 1110 Ci/mmol; 41,1 TBq/mmol) was obtained from Amersham Int. plc as an aqueous solution in 20 mM potassium acetate and 5 mM dithiothreitol. ^{35}S-methionine (specific activity 1100-1400 Ci/mmol; ≈ 40 TBq/mmol) was obtained from NEN as an aqueous solution containing 10 µM 2-mercaptoethanol.
Peptide syntheses were carried out in polypropylene Eppendorf-microtubes. HPLC-runs were performed on a Waters apparatus equipped with a Berthold LB 282/283 or Ramona D radioactivity detector and a Pye-Unicam UV detector.
Peptide precursors 4, 7 and 9 were prepared at the Medicinal Chemistry Labs of Organon Int. B.V., Oss, The Netherlands.
Dodecapeptide 4: MS(FAB; glycerol; pos. mode): m/z= 1309, $[M+H]^+$.
Dodecapeptide 7: MS(FAB; glycerol; pos. mode): m/z= 1516, $[M+H]^+$.
Pentapeptide 9: MS (FAB glycerol$^+$; pos. mode): m/z= 1053, $[M+H]^+$; mz= 998; $[M-C_4H_8+H]^+$; MS(FAB; glycerol; neg. mode): m/z= 1051; $[M-H]^-$; m/z= 995 , $[M-C_4H_8-H]^-$.

Org 30273 (1):
The symmetrical dimer of [p-Glu^4]AVP-(4-9) (4) (2 mg) was dissolved in trifluoroethanol (200 µl) and carbomethoxysulphenyl chloride (6 µl) was added. After 45 minutes at room temperature the reaction mixture was filtered over hyflo and diethyl ether was added to the filtrate. The precipitate was collected by centrifugation, washed with diethyl ether and dried in vacuo yielding product 5.
To a solution of 5 mCi ^{35}S-cysteine in 0,5 µl water (containing 20 mM KOAc and 5 mM dithiotreitol) 3 mg of the peptide precursor 5 in 200 µl trifluoroethanol/methanol (2:1, v/v) were added and the reaction mixture was left overnight at room temperature. The volume of the reaction mixture was reduced to 500 µl by evaporation under a gentle stream of nitrogen and the residue was purified in four injections on HPLC (Supelco LC-18-DB with a 0-24% gradient of acetonitrile in water with 0,1% trifluoroacetic acid). The fractions containing the pure peptide were freeze-dried and the residue was dissolved in Milli Q-water in a concentration of about 100 µCi/0,5 ml. The peptide was stored at 4°C.

^{35}S-Org 5970 (2):

The pH of the solution of ^{35}S-methionine (10 mCi) in 10 µM aqueous dithiothreitol was adjusted to 8 by the addition of 5% aq. $NaHCO_3$-solution (≈ 30 µl). A solution of 4 mg t-butyloxycarbonyl anhydride in dioxane (200 µl) was added and the mixture was left for 16 hours at room temperature.

The pH of the reaction mixture was adjusted to 2,5 with an aqueous solution of $KHSO_4$ and the solution was extracted with 3x500 µl ethyl acetate (containing 0,1% β-mercaptoethanol). The organic layer was washed with aq. NaCl-solution and dried on sodium sulphate. DMF (50 µl) containing 0,1% 2-mercaptoethanol was added and the ethyl acetate was removed by a gentle stream of nitrogen. To this solution of Boc-methionine in DMF 5 mg of the dodecapeptide 7 were added together with hydroxybenzotriazole (5 mg) and dicyclohexylcarbodiimide (5,5 mg) dissolved in DMF (100 µl) containing 0,2% triethylamine. After 16 hours at room temperature the reaction mixture was freeze-dried and the residue was chromatographed over Seppak C18 (washing of the column with acetonitrile/water (7:3, v/v), containing 0,1% β-mercaptoethanol; elution of the peptide with acetonitrile/water (1:1, v/v) with 0,1% β-mercaptoethanol). The crude peptide was purified by HPLC (Supelcosil LC-18-DB with a pelliguard precolumn using a mixture of acetonitrile/water (57:43, v/v) containing 0,1% trifluoroacetic acid and 0,02% β-mercaptoethanol). The fractions containing pure 8 were freeze-dried and the residues dissolved in a mixture of trifluoroacetic acid/water (9:1, v/v; 100 µl); anisole (10 µl of a solution of 100 µl anisol in 1 ml trifluoroacetic acid) and di-t-butyldisulphide (10 µl of a solution of 330 µl in 1 ml trifluoroacetic acid) were added. After 2 hours at room temperature the mixture was evaporated to dryness under a gentle stream of nitrogen and the residue was purified by HPLC (Supelcosil LC-18-DB with a mixture of acetonitrile and water (33:67, v/v) containing 0,1% trifluoroacetic acid and 0,02% β-mercaptoethanol). The fractions containing pure 2 were freeze-dried and the residue was dissolved in water containing 0,2% β-mercaptoethanol (200-300µCi/ml) and stored at 4°C.

^{35}S-Org 2766 (3):

To a solution of ^{35}S-Boc-methionine a solution of pentapeptide 10 (4 mg; prepared from pentapeptide 9 by reaction with H_2 using Pd/C as the catalyst) in DMF (200 µl) was added followed by triethylamine (0,7 µl), hydroxybenzotriazole (4 mg) and dicyclohexylcarbodiimide (4 mg). The reaction mixture was left overnight under nitrogen. After freeze-drying the residue was chromatographed over silica gel with a gradient of methanol in dichloromethane (5-10%, v/v). The fractions containing the ^{35}S-peptide were evaporated to dryness (with a gentle stream of nitrogen) and the residue was dissolved in a mixture of 85% H_3PO_4/H_2O (1:1, v/v; 200 µl). After 24 hours at 4°C under nitrogen H_2O_2 (100 µl) and an aqueous solution of $(NH_4)_6\ Mo_7O_{24}$ (100 µl; 26 mg/ml) were added and the mixture was heated at 55°C for 45 minutes.

The reaction mixture was chromatographed over Seppak C18 (washing of the column with water, elution of the peptide with water/acetonitrile). After removal of the acetonitrile by

freeze-drying the crude peptide was purified by HPLC (Supelcosil LC-18-DB with a guard column using a 80:20 (v/v) mixture of H_2O/CH_3CN containing 0,1% (v/v) trifluoroacetic acid and 0,01% (v/v) 2-mercaptoethanol). The fractions containing pure peptide were freeze-dried and the residue was dissolved in water containing 15% ethanol (100 μCi/ml).

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