*Journal of Labelled Compounds and Radiopharmaceuticals*
*J. Labelled Cpd. Radiopharm.* **44**, 89-98 (2001)

# Synthesis of [3(R)-amino-2(S)-sulfhydryl-5-sulfonate]-pentanoyl-(S)-3-[$^{125}$I]-iodotyrosyl-(S)-aspartic acid : a radiolabelled inhibitor of aminopeptidase A

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## SUMMARY

In order to investigate the localization of aminopeptidase A in the central nervous system and peripheral organs, we have synthesized the first radiolabelled inhibitor of this enzyme. This molecule, [3(R)-amino-2(S)-sulfhydryl-5-sulfonate]-pentanoyl-(S)-3-[$^{125}$I]-iodotyrosyl-(S)-aspartic acid, exhibited a Ki value of 4.8 nM for aminopeptidase A. A high purity was obtained with a specific activity of about 2,000 Ci/mmol at the end of the synthesis.

**Keywords.** Thiol compounds / $^{125}$I / Aminopeptidase A / Inhibitors.

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Received 15 June 2000
Revised 2 November 2000
Accepted 7 November 2000

## INTRODUCTION

Aminopeptidase A (APA, EC 3.4.11.7, glutamyl aminopeptidase), a membrane-bound zinc exopeptidase, is a widely distributed enzyme previously located in kidney and small intestine. In kidney, the highest levels of APA, detected by immunohistochemistry (1-6) are in the glomerulus and the proximal tubule of the brush border microvilli. In the intestine, APA has also been found in the apical pole of cells present in the brush border (2,7-11). In addition, APA appears to be associated with capillaries in some organs such as the lung (2) and the adrenal gland (12). Moreover, this enzyme was also shown to occur in the brain, especially on cerebral microvessels and the circumventricular organs lying outside the blood-brain barrier (2,13-17). Recently substantial APA activity was found in various microdissected brain nuclei, using a synthetic substrate and a selective inhibitor (17).

APA is involved in the enzymatic cascade of renin-angiotensin system (RAS) by cleaving angiotensin II into angiotensin III (18,19). The latter peptide was shown to be the most active peptide of the central RAS cascade, being responsible for the increase of blood pressure (20) and vasopressin release (21). Moreover, APA was demonstrated to participate in the in vivo metabolism of the sulfated octapeptide fragment of cholecystokinin (22) which plays an important role in memory (23), anxiety (24,25), feeding (26) and pain perception (27,28). In order to study the pharmacological effects of these biological systems in normal and physiopathological conditions, potent and selective inhibitors of APA have been synthesized (29). This led to investigate the possibility of designing radiolabelled inhibitors which have the advantage to be small and diffusible molecules able to interact quantitatively with the enzyme active site in all tissues and species. Moreover, due to its high sensitivity, the use of $^{125}I$ labelling requires only short exposure times to obtain autoradiograms, thus offering the possibility of a rapid and precise description of the enzyme localization.

With the aim to investigate the distribution of APA in the central nervous system and peripheral organs, we have developped the first highly potent and selective [$^{125}I$] radiolabelled APA inhibitor, [3(R)-amino-2(S)-sulfhydryl-5-sulfonate]-pentanoyl-(S)-3-[$^{125}I$]-iodotyrosyl-(S)-aspartic acid (Figure 1).

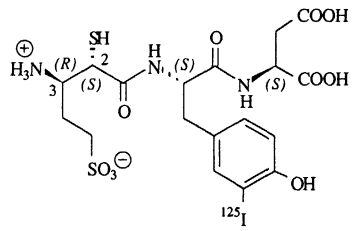

Figure 1. **Structure of [3(R)-amino-2(S)-sulfhydryl-5-sulfonate]-pentanoyl-(S)-3-[$^{125}$I]-iodotyrosyl-(S)-aspartic acid.**

## RESULTS AND DISCUSSION

In the course of this synthesis, the major difficulty consisted of the presence of the thiol moiety. Indeed, compounds in which a sulfur atom is present at a low oxidation state (thiols or thioethers) are sensitive to iodine or chloramine-T. This problem has been overcome for methionine-containing peptides (31) in which the thioether was first protected by oxidation to a sulfoxide, previous to the iodination step. This implicates the subsequent reduction of the sulfoxide. In our case, the free thiol was first protected as the corresponding disulfide which could not be further oxidized by chloramine-T. The disulfide can be subsequently reduced to give the free thiol. The whole synthesis can be seen in Figure 2.

The precursor 3-benzyloxycarbonylamino-2-[4-methoxybenzylsulfanyl]-5-[2, 2-dimethylpropanoxysulfonyl]-pentanoic acid **1** was synthesized as previously described (29) and then coupled with the dipeptides H-(S)-Tyr(tBu)-(S)-Asp(OtBu)-OtBu or H-(S)-Tyr(3-I)-(S)-Asp(OtBu)-OtBu in the presence of EDCI/HOBt. These steps were followed by acidic cleavages of the protecting groups leading respectively to the precursor **5** and the non radioactive inhibitor **4**. In both cases, mixtures of two diastereomers were obtained.

These diastereomers were easily separated by semi-preparative HPLC as reported in the experimental section. The stereochemical assignment of the 2-sulfanyl-3-aminoacyl moiety of **4** and **5** (2S, 3R) was determined as previously reported (29,30).

 

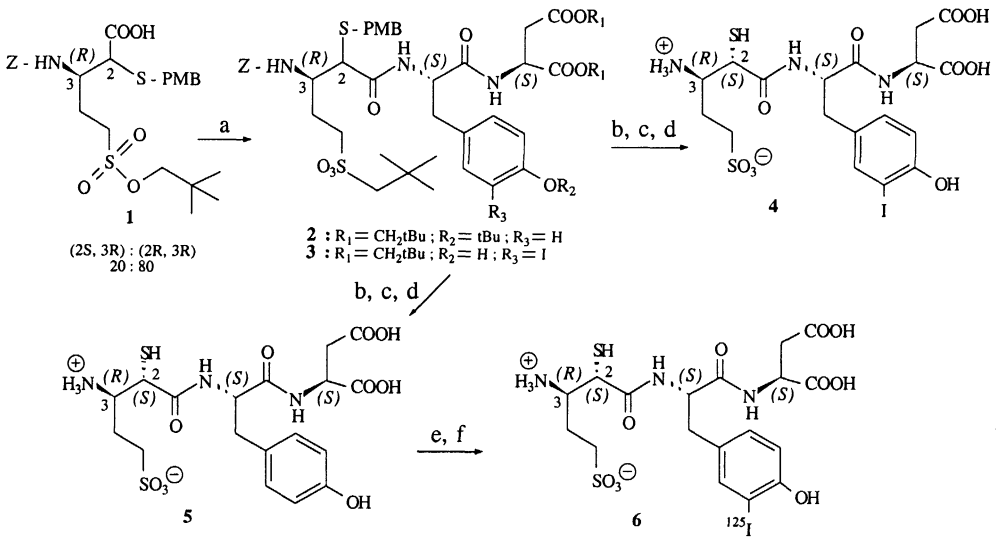


Figure 2. **Synthesis of the "cold" inhibitor 4 and the $^{125}I$ labelled inhibitor 6.** a : Coupling reagent, dipeptide, base ; b : TFA, $CH_2Cl_2$, anisole ; c : Liquid HF, m-cresol ; d : Separation of the two diastereomers by semi-preparative HPLC ; e : $I_2$, ethanol ; f : $Na^{125}I$ / chloramine-T, followed by DTT ; PMB : p-methoxybenzyl.

Compound **5** was oxidized as its disulfide, and the radioiodination was performed by using 5 mCi of $Na^{125}I$ in aqueous sodium hydroxide solution (pH 7-11) with chloramine-T in 50 mM phosphate buffer at pH 7.2. The reaction mixture was stirred for 5 minutes at 0°C and the reaction was then quenched by addition of 10 eq. of $Na_2S_2O_5$ and the disulfide reduced by 100 eq. of DTT.

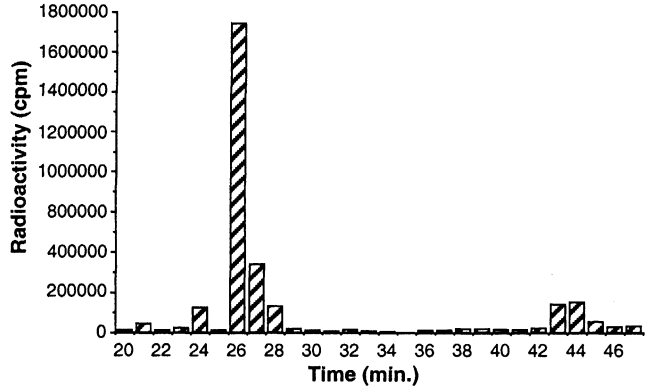


Figure 3. **HPLC separation of compound 6 (peak centered at 26-28) from the bis-iodinated derivative of 4 (peak centered at 43-45).**

The radiolabelled compound **6** was purified by HPLC. The mono-iodinated inhibitor was obtained in 17.6% yield and the bis-iodinated inhibitor in 1.5% yield (Figure 3). It was stored in the presence of 10 eq. of DTT.

In conclusion, [3(R)-amino-2(S)-sulfhydryl-5-sulfonate]-pentanoyl-(S)-3-[$^{125}$I]-iodotyrosyl-(S)-aspartic acid **4**, a new iodinated compound in the series of thiol inhibitors, was synthesized and evaluated *in vitro* for inhibition of recombinant APA (32) using α-(S)-glutamyl-β-naphtylamide (βGluNA) as substrate. This compound was found to have a high affinity towards APA (Ki = 4.8 nM) and good selectivity versus other exopeptidases. A selectivity factor greater than 1000 was obtained versus APN (Aminopeptidase N, EC 3.4.11.2) and up to 160 versus ACE (Angiotensin converting enzyme, EC 3.4.15.11), two metallopeptidases involved in the enzymatic renin-angiotensin cascade. Moreover, a good selectivity was found versus aspartyl-aminopeptidase (S. Wilk, unpublished results). Conversely, the selectivity factor of this inhibitor was only 10 versus NEP (Neutral endopeptidase, EC 3.4.24.11). The corresponding $^{125}$I labelled inhibitor **6** was prepared in good yields by using the oxidative chloramine-T method. This new radiolabelled compound should provide a useful tool for distribution studies of APA in brain and periphery and for *in vivo* experiments (33). Such studies are currently in progress in our laboratory.

## EXPERIMENTAL SECTION

The natural amino acid derivatives were purchased from Bachem (Budendorf, Switzerland). Reagents were from Aldrich (Strasbourg, France). Na$^{125}$I was from Amersham. Solvents were from SDS (Peypin, France). TLC were stained with UV or iodine vapor. Final compounds were purified by HPLC on a reverse phase column (4.6 x 250 mm) with 0.05% of trifluoroacetic acid in $H_2O$ and $CH_3CN$ as mobile phase, and all retention times were determined on a LC-10AT Shimadzu apparatus (Touzard & Matignon, France). The eluted compounds were monitored at 210 nm.

The structures of all compounds were confirmed by $^1$H NMR spectroscopy (Brüker AC 270 MHz) using HMDS as internal reference. Melting points of the compounds were measured on a Büchi B-540 and are reported uncorrected.

 

The following abbreviations are used : cHex, cyclohexane ; EtOAc, ethyl acetate ; THF, tetrahydrofuran ; BOP, benzotriazole-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate ; HOBt, N-hydroxybenzotriazole ; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide methiodide ; Z, benzyloxycarbonyl ; DTT, dithiothreitol.

**[3(R)-Benzyloxycarbonylamino-2(S)-(4-methoxybenzylsulfanyl)-5-(2,2-dimethyl-propanoxysulfonyl)-pentanoyl]-(S)-Tyr(tBu)-(S)-Asp(OtBu)-OtBu : 2**

To a solution of 3(R)-benzyloxycarbonylamino-2-(S)-(4-methoxybenzylsulfanyl)-5-(2,2-dimethylpropanoxysulfonyl)-pentanoic acid **1** (1.0 g, 1.8 mmol) in $CH_2Cl_2$ (24 ml) were added the dipeptide H-(S)Tyr(tBu)-(S)Asp(OtBu)-OtBu (1.03 g, 2.2 mmol), BOP (1.12 g, 2.5 mmol) and diisopropylethylamine (0.95 ml, 5.4 mmol) and the mixture was stirred for 4 hours at room temperature. After evaporation to dryness, the oily residue was taken up in EtOAc, washed with citric acid, 10% $NaHCO_3$, brine, dried over $Na_2SO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography on silica gel (Eluent : cHex, EtOAc 4/1, R*f* (7/3) = 0.3 and 0.21) giving 1.5 g of **2** (84%) as a white powder. Mp = 127-129 °C. $^1$H NMR ($CDCl_3$) δ 0.9 (s, 9H, $CH_2$-tBu), 1.3 (s, 9H, $C_6H_4$-OtBu), 1.4 (2s, 18H, 2 x COOtBu), 1.75 to 2.15 (m, 2H, CH-$CH_2$-$CH_2$), 2.5 to 2.9 (m, 2H, $CH_2$β Asp), 2.8 to 3.1 (m, 4H, $CH_2$β Tyr, CH-$CH_2$-$CH_2$), 3.1 and 3.2 (2d, 1H, CH-S), 3.2 to 3.6 (m, 2H, S-$CH_2$), 3.7 (s, 3H, $OCH_3$), 3.75 (s, 2H, $CH_2$-tBu), 4.05 (m, 1H, CH-$CH_2$-$CH_2$), 4.55 (m, 2H, CHα Tyr, CHα Asp), 5.05 (m, 2H, $C_6H_5$-$CH_2$), 6.2 and 6.2 (2d, 1H, Z-NH), 6.75 (2d, 3H, CONH Asp, CH arom. ortho $OCH_3$), 6.9 (d, 2H, CH arom. ortho OtBu), 7.0 (d, 2H, CH arom. meta $OCH_3$), 7.1 (d, 2H, CH arom. meta OtBu), 7.3 (m, 6H, $C_6H_5$-$CH_2$, CONH Tyr). HPLC $C_{18}$ Kromasil (5μ, 100A°) $CH_3CN/H_2O$ (TFA) 80/20, $t_R$=11.9 (2S, 3R) and 12.5 min. (2R, 3R).

**[3(R)-Benzyloxycarbonylamino-2(S)-4-methoxybenzylsulfanyl)-5-(2,2-dimethyl-propanoxysulfonyl)-pentanoyl]-(S)-(3-I)Tyr-(S)-Asp(OtBu)-OtBu : 3**

To a solution of **1** (0.246 g, 0.44 mmol) in THF (2 ml) was added successively the dipeptide H-(3-I)-(S)Tyr-(S)Asp(OtBu)-OtBu (0.242 g, 0.44 mmol) in $CHCl_3$ (2 ml), HOBt (60 mg, 0.44 mmol) in THF (0.3 ml), EDCI (198 mg, 0.67 mmol) in $CHCl_3$ (2 ml) and triethylamine (60 μl, 0.44 mmol). The mixture was stirred for 3 hours at

 

room temperature. The same work-up as for compound **2** gave the crude product, which was purified by flash chromatography on silica gel (Eluent : cHex, EtOAc 6/4, R*f* (4/6) = 0.58) giving **3** as a white foam, 0.25g (52%). $^1$H NMR ($CDCl_3$) δ 0.9 (s, 9H, $CH_2$-tBu), 1.3 (s, 27H, $C_6H_4$-OtBu, 2 x COOtBu), 1.75 to 2.15 (m, 2H, CH-$CH_2$-$CH_2$), 2.5 to 2.9 (m, 2H, $CH_2$β Asp), 2.8 to 3.1 (m, 4H, $CH_2$β Tyr, CH-$CH_2$-$CH_2$), 3.1 and 3.2 (2d, 1H, CH-S), 3.5 to 3.7 (m, 7H, S-$CH_2$, $OCH_3$, $CH_2$-tBu), 4.0 (m, 1H, CH-$CH_2$-$CH_2$), 4.4 to 4.65 (m, 2H, CHα Tyr, CHα Asp), 5.05 (m, 2H, $C_6H_5$-$CH_2$), 5.6 and 6.1 (2d, 1H, Z-NH), 6.4 to 7.2 (m, 14H, CONH Asp, CH arom. ortho $OCH_3$, CH arom. ortho OtBu, CH arom. meta $OCH_3$, CH arom. meta OtBu, $C_6H_5$-$CH_2$, CONH Tyr). HPLC $C_{18}$ Kromasil (5μ, 100A°) $CH_3CN/H_2O$ (TFA) 80/20, $t_R$=11.2 (2S, 3R) and 12.6 min. (2R, 3R).

**[3(R)-Benzyloxycarbonylamino-2(S)-(4-methoxybenzylsulfanyl)-5-(2,2-dimethyl -propanoxysulfonyl)-pentanoyl]-(S)-Tyr-(S)-Asp-OH**

To a solution of 1.05 g of compound **2** (1.05 mmol) in $CH_2Cl_2$ (8.8 ml) and anisole (125 μl) was added at 0 °C, trifluoroacetic acid (2.5 ml, 31.6 mmol). The mixture was stirred for 3.5 hours at room temperature. After evaporation to dryness, the residue was solidified using ether and filtered off to give the pure product as a white powder, 836 mg (95 %) Mp = 163-165 °C. $^1$H NMR ($d_6$-DMSO + TFA) δ 1.9 (s, 9H, $CH_2$-tBu), 1.6 to 2.0 (m, 2H, CH-$CH_2$-$CH_2$), 2.5 to 2.7 (m, 2H, $CH_2$β Asp), 2.6 to 2.9 (m, 2H, $CH_2$β Tyr), 3.0 to 3.2 (m, 2H, CH-$CH_2$-$CH_2$), 3.4 to 3.7 (m, 6H, CH-S, $OCH_3$, S-$CH_2$), 3.7 to 3.9 (m, 3H, $CH_2$-tBu, CH-$CH_2$-$CH_2$), 4.5 (m, 2H, CHα Tyr, CHα Asp), 5.0 (m, 2H, $C_6H_5$-$CH_2$), 6.6 (d, 2H, CH arom. ortho OH), 6.75 (d, 2H, CH arom. ortho $OCH_3$), 6.95 (d, 2H, CH arom. meta $OCH_3$), 7.05 and 7.4 (2d, 1H, Z-NH), 7.1 (d, 2H, CH arom. meta OH), 7.3 (m, 5H, $C_6H_5$-$CH_2$), 8.2 and 8.35 (2d, 1H, CONH Tyr), 8.4 (d, 1H, CONH Asp). HPLC $C_{18}$ Kromasil (5μ, 100A°) $CH_3CN/H_2O$ (TFA) 50/50, $t_R$=16.3 (2S, 3R) and 18.6 min. (2R, 3R).

**[3(R)-Benzyloxycarbonylamino-2(S)-(4-methoxybenzylsulfanyl)-5-(2,2-dimethyl propanoxysulfonyl)-pentanoyl]-(S)-(3-I)Tyr-(S)-Asp-OH**

Using the reaction conditions described above, but starting from 0.24 g (0.22 mmol) of **3**, 169 mg of product (79%) were obtained as a white powder. $^1$H NMR ($d_6$-DMSO

 

+ TFA) δ 1.85 (s, 9H, $CH_2$-tBu), 1.6 to 1.9 (m, 2H, CH-$CH_2$-$CH_2$), 2.5 to 2.7 (m, 2H, $CH_2$β Asp), 2.6 to 2.9 (m, 2H, $CH_2$β Tyr), 3.0 to 3.2 (m, 2H, CH-$CH_2$-$CH_2$), 3.2 to 3.8 (m, 6H, CH-S, $OCH_3$, S-$CH_2$), 3.75 (s, 2H, $CH_2$-tBu), 3.95 (m, 1H, CH-$CH_2$-$CH_2$), 4.5 (m, 2H, CHα Tyr, CHα Asp), 4.85 to 5.0 (m, 2H, $C_6H_5$-$CH_2$), 6.5 to 7.3 (m, 7H, CH arom. ortho OH, CH arom. meta OH, CH arom. ortho $OCH_3$, CH arom. meta $OCH_3$, Z-NH), 7.2 (m, 5H, $C_6H_5$-$CH_2$), 7.5 and 7.6 (s, 1H, CH arom. ortho I), 8.1 and 8.35 to 8.5 (m, 2H, CONH Tyr, CONH Asp). HPLC $C_{18}$ Kromasil (5μ, 100A°) $CH_3CN/H_2O$ (TFA) 50/50, $t_R$=28.2 (2S, 3R) and 31.3 min. (2R, 3R).

**(3(R)-Amino-2(S)-sulfhydryl-5-sulfonate-pentanoyl)-(S)-Tyr-(S)-Asp-OH : 5**

729 mg of [3(R)-benzyloxycarbonylamino-2-(S)-(4-methoxybenzylsulfanyl)-5-(2,2-dimethylpropanoxysulfonyl)-pentanoyl]-(S)-Tyr-(S)-Asp-OH (0.88 mmol) in the presence of dry m-cresol (1 ml) were treated with anhydrous liquid hydrogen fluoride (10 ml) and stirred at 0 °C for one hour. After removal of HF *in vacuo*, the residue was taken up in trifluoroacetic acid and precipitated with a cold mixture of ether/n-hexane 50/50. After centrifugation, the precipitate was taken up in water and freeze-dried. The crude product consisted of two diastereomers which were separated by semi-preparative HPLC $C_8$ Kromasil (10 μ, 100 Å, 250x20 mm), using a linear gradient from 10 to 60%. Both diastereoisomers were obtained as white powders, 203 mg for (2R, 3R) and 94 mg for (2S, 3R) (67%). (2S, 3R) : $^1$H NMR (DMSO-$d_6$ + TFA) δ 1.9 (m, 2H, CH-$CH_2$-$CH_2$), 2.5 to 2.7 (m, 4H, $CH_2$β Asp, $CH_2$β Tyr), 2.8 (d, 1H, SH), 2.9 (m, 2H, CH-$CH_2$-$CH_2$), 3.5 (m, 1H, CH-$CH_2$-$CH_2$), 3.7 (m, 1H, CH-S), 4.5 (m, 2H, CHα Tyr, CHα Asp), 6.6 (d, 2H, CH arom. ortho OH), 7.0 (d, 2H, CH arom. meta OH), 8.0 (broad s, 3H, $NH_3^+$), 8.4 (d, 1H, CONH Asp), 8.5 (d, 1H, CONH Tyr). HPLC $C_8$ Kromasil (5 μ, 100 A°) $CH_3CN/H_2O$ (TFA) 20/80, $t_R$ = 1.79 min. $[M + H]^+ = 507.7$, $[M + Na]^+ = 529.6$.

**(3(R)-Amino-2(S)-sulfhydryl-5-sulfonate-pentanoyl)-(S)-(3-iodo)Tyr-(S)-Asp-OH : 4**

Using the reaction conditions described above, from 130 mg (0.13 mmol) of [3(R)-benzyloxycarbonylamino-2(S)-(4-methoxybenzylsulfanyl)-5-(2,2-dimethylpropanoxy-sulfonyl)-pentanoyl]-(S)-(3-I)Tyr-(S)-Asp-OH, the two diastereomers of **4** were separated by semi-preparative HPLC $C_{18}$ Kromasil (5 μ, 100 A°, 20 x 250 mm)

 

giving two white powders, 20 mg (2R, 3R) and 15 mg (2S, 3R) (44%). (2S, 3R) : $^1$H NMR (DMSO-$d_6$ + TFA) δ 1.5 to 1.9 (m, 2H, CH-$\underline{CH_2}$-$CH_2$), 2.5 to 2.7 (m, 4H, $CH_2$β Asp, $CH_2$β Tyr), 2.7 (d, 1H, SH), 2.9 (dd, 2H, CH-$CH_2$-$\underline{CH_2}$), 3.5 (m, 1H, $\underline{CH}$-$CH_2$-$CH_2$), 3.8 (m, 1H, CH-S), 4.5 (m, 2H, CHα Tyr, CHα Asp), 6.7 (d, 2H, CH arom.ortho OH), 7.0 (d, 2H, CH arom. meta OH), 7.5 (d, CH arom. ortho I), 8.0 (broad s, 3H, $NH_3^+$), 8.5 (d, 1H, CONH Asp), 8.7 (d, 1H, CONH Tyr). HPLC $C_{18}$ Kromasil (5 μ, 100 A°) $CH_3CN/H_2O$ (TFA) 15 / 85, $t_R$ = 13.3 min. $[M + H]^+$ = 634.7, $[M + Na]^+$ = 672.7.

**(3(R)-Amino-2(S)-sulfhydryl-5-sulfonate-pentanoyl)-(S)-3-[$^{125}$I]-iodoTyr-(S)-Asp-OH : 6**

To a solution of **5** (6 mg, 0.012 mmol) in ethanol (1 ml) was added a solution of $I_2$ (3.9 ml of a 1.1 mM solution in ethanol). The solvent was removed *in vacuo* and the residue was taken up in water and freeze-dried, giving a pale yellow powder, corresponding to **5** as the disulfide, 5.9 mg (98%). HPLC $C_{18}$ Kromasil (5 μ, 100 A°) $CH_3CN/H_2O$ (TFA) 20/80, $t_R$ = 3.4 min. To a solution of sodium [$^{125}$I] iodide (5 mCi, 2.3 nmol) in aqueous sodium hydroxide solution were added at 0°C, [3(R)-amino-2(S)-sulfhydryl-5-sulfonate-pentanoyl]-(S)-tyrosine-(S)-aspartic acid **5** under disulfide form (2.3 nmol) in 11.7 μl of 50 mM phosphate buffer at pH 7.2 and 2.3 nmol of chloramine-T in 40 μl of the same buffer. After stirring for 5 min, the reaction was stopped by addition of 23 nmol of an aqueous solution of $Na_2S_2O_5$ and 230 nmol of a solution of DTT in 50 mM phosphate buffer. The desired radiolabelled inhibitor was purified by HPLC on a reverse phase Kromasil $C_8$ (5μ, 100Å) column eluted under linear gradient conditions from 10 to 55% $CH_3CN$ in 40 min and then in isocratic mode for 20 min at a flow rate of 1 ml/min to provide 885 μCi of product with a specific activity of 2,000 Ci/mmol.

## ACKNOWLEDGEMENTS

We thank Dr. C. Llorens-Cortès and N. De Mota for experiments with APA and S. DaNascimento for experiments with APN, NEP and ACE. We gratefully acknowledge Dr. S. Wilk (New York University) for determination of the inhibitory potency of **4** on aspartyl-aminopeptidase.

 

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