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Research Article

Synthesis and separation of tritiated inhibitors of aminopeptidase A and their prodrugs

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Summary

With the aim of studying the bioavailability of two related aminopeptidase A (APA) inhibitors, we have synthesized tritiated disulfide prodrugs. These molecules, 4,4′-dithiobis-(3,3′-amino)-1,1′-butanesulfonic acid (RB 150) and its 2,2-dimethylpropyl ester(RB 151) bearing one tritium atom per monomer in position 2 were obtained with high purity and a final specific activity of about 30 Ci/mmol. The active radiolabeled inhibitor EC 33, Ki = 270 nM for APA was obtained by reduction of the disulfide bond of RB 150. Copyright © 2004 John Wiley & Sons, Ltd.

Key Words: thiol compounds; ^{3}H; aminopeptidase A; inhibitors; cardiovascular

Introduction

Aminopeptidase A (APA, EC 3.4.11.7, glutamyl aminopeptidase) is a membrane bound zinc metalloprotease that selectively cleaves the N-terminal glutamyl or aspartyl residue of peptides. This enzyme is found in large amounts at the periphery in the kidney and the small intestine, but is also present in the brain.[1,2] Two physiological substrates of this enzyme have been identified in the central nervous system. The first is the sulfated CCK 8(Asp–Tyr(SO_3^-)–Met–Gly–Trp–Met–Asp–Phe–NH_2),[3] the second is angiotensin II (Asp–Arg–Val–Tyr–Ile–His–Pro–Phe)[4,5] which is matured by APA into angiotensin III. These two peptides exert different physiological effects. In the brain CCK 8 binds to the CCK_2 receptor[6] and is

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associated with, among other effects, the control of memory processes and anxiety.[7,8] Inhibition of APA by a thiol inhibitor EC 33[9] has enabled workers to demonstrate that in the central nervous system, angiotensin III is responsible for vasopressin release and increase in blood pressure.[10,11] Furthermore, overexpression of APA was recently observed in blood vessels of human tumors and APA inhibitors have been reported to inhibit angiogenesis.[12]

Thus APA inhibitors may find therapeutic applications in the regulation of behavior, in the treatment of cardiovascular diseases[13–15] or as antiangiogenic agents. For the two first applications, APA inhibition needs to be achieved at the nervous central system level and at the periphery for the regulation of angiogenesis. Cardiac failure remain one of the major health problems and the discovery of new targets to treat related pathologies is of major interest. We have previously demonstrated that intracerebroventricular injection of the APA inhibitor EC 33 induces a decrease of blood pressure in spontaneously hypertensive rats (SHR). In contrast, this molecule which is unable to enter the brain, did not affect blood pressure (BP) when given intravenously suggesting that the antihypertensive effect of EC 33 occurs at the brain level.[16]

In order to improve the bioavailability of EC 33, prodrugs were developed based on a previously demonstrated increase in brain penetration of thiol inhibitors by formation of their disulfide forms.[17] The designed prodrugs of EC 33 (RB 151 and RB 150) were found to be able to decrease BP in SHR after systemic or oral administration.[18,19]

As previously shown in the case of a mixed NEP/APN inhibitor[17] endowed with similar chemical characteristics by comparison with RB 150 and RB 151, the disulfide bridge of these latter prodrugs is reduced by a physiologically dependent process, leading to the active inhibitor (Figure 1). In our case we thought that a similar reaction could occur by esterase hydrolysis at the periphery or in the brain, of the neopentyl moiety protecting RB 152 and 151. Moreover, the disulfide bridge of RB 150 or 151 has been observed to be reduced in the brain.[17] Thus RB 151 is supposed to yield three compounds: RB 150, 152 and EC 33 (Figure 1). All these molecules are devoid of any chromophore thus precluding a quantitative estimation of their level using HPLC detection methods. Furthermore their derivatization using a fluorophore such as fluorescamin is ineffective for analytical purposes.[20] Generally, it is acknowledged that a tritium labeled version of the drug candidate provides the ideal standard for pharmacokinetics assays. Therefore, we have developed two tritium labeled prodrugs of EC 33 and a method to generate and separate the prodrugs and their metabolites, which will be useful for performing preclinical studies using RB 151 and RB 150.

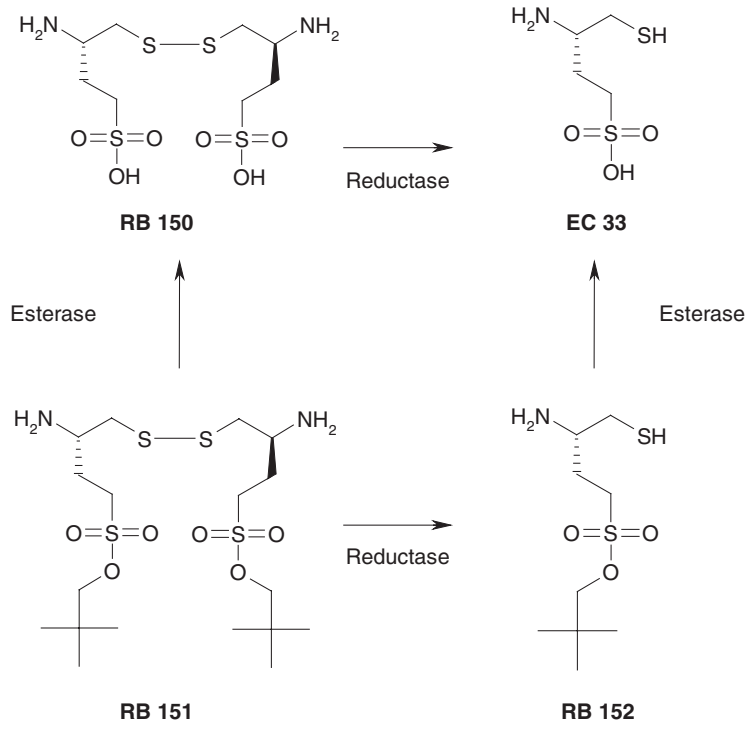

Figure 1. Structure of RB 151 and its metabolites

Results and discussion

The previously described synthetic pathway to obtain EC 33 was not a good starting point for the synthesis of tritium labeled RB 151, because of the number of reaction steps and some stringent reaction conditions.[9] We have thus developed a shorter and new synthetic approach in order to obtain this compound (Figure 2). Esterification of commercially available (*S*)-*N*-tertiobutoxycarbonylamino-*S*-trityl-cysteine with trimethylsilyldiazomethane yields quantitatively compound **1**[21] which was then used for the introduction of the sulfonate side chain by a previously described one pot reaction[22] involving a DIBAl-H induced reduction of the methyl ester moiety leading to an aluminoxyacetal intermediate. The latter was not isolated and was submitted to a Wittig–Horner reaction[23] leading to compound **2** after semi-preparative HPLC purification. The reduction of the α–β-unsaturated sulfonate **2** (23 mg) was carried out using 10 Ci of sodium boro[^{3}H]hydride in ethanol affording, after HPLC purification, 400 mCi of the radiolabelled intermediate **3**. This last compound was deprotected using TFA and triisopropylsilane and air oxidized to yield, after HPLC purification, 50 mCi of tritiated RB 151 with a specific activity of 29 Ci/mmol and a radiochemical purity $>97\%$. Deprotection of 5 mCi of the sulfonate **3** in 1 ml ethanol with 1 ml 12N HCl at 80°C for three

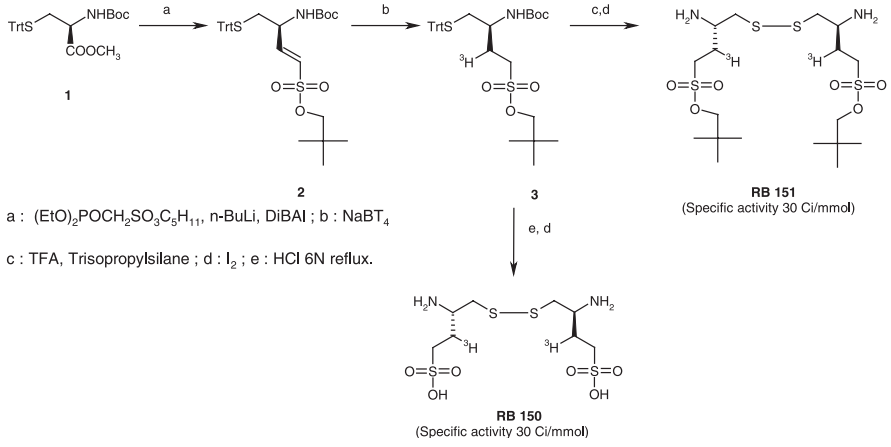

Figure 2. Synthetic scheme for the inactive standard and the ^{3}H prodrugs RB 150 and 151

days, followed by iodine oxidation, yielded, after HPLC purification, 2.1 mCi of RB 150 with a specific activity of 29 Ci/mmol.

Furthermore the possible metabolites of RB 150 and RB 151, respectively EC 33 and RB 152, could be generated using a 10 fold excess of dithiothreitol as reducing agent.[24] The formation of EC 33 and RB 152 was monitored by TLC separation in isopropanol/water/acetic acid 8-2-1 as eluent followed by four days exposure of the plate on a Bas TR2025 plate and measurement with a Bas 5000 phosphoimager. Moreover, this separation method of RB 151 and their by-products can be helpful when the metabolic studies of RB 151 will be undertaken. The sensitivity of this autoradiographic method was tested using RB 151. After 10 days exposure, 0.1 nCi of RB 151 could be easily detected (Figure 3 right).

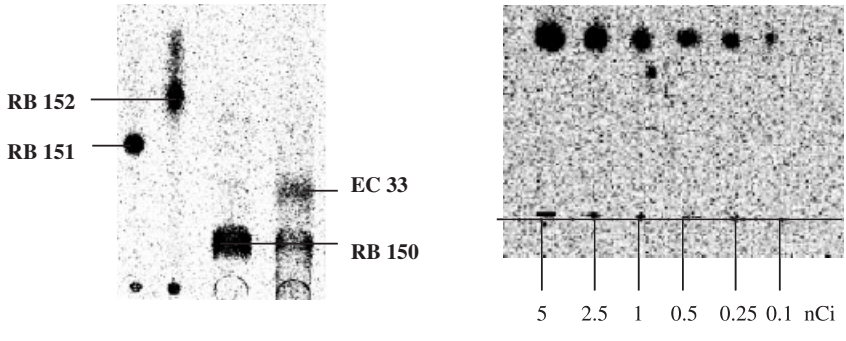

Figure 3. Autoradiographic plate of RB 151 metabolites (left TLC) and sensitivity study with RB 151 (right TLC)

In conclusion, three tritiated prodrugs and their parent APA inhibitor have been synthesized. Furthermore, a TLC separation and a quantification method of the prodrug and of the different metabolites was carried out. These compounds and the separation method should provide useful tools for pharmacokinetic studies of APA inhibitors.

Experimental part

General

All reagents and solvents are commercially available and used directly without purification. The natural amino acid derivatives were purchased from Bachem (Budendorf, Switzerland). Reagents were from Aldrich (Strasbourg, France). Solvents were from SDS (Peypin, France). The tritiation was undertaken by Amersham Biosciences (Cardiff, Walles). TLC was performed on Merck 60 F254 precoated silica plates. Analytical HPLC was performed on a LC 10 ADvp Shimadzu instrument (Touzart & Matignon, France) equipped with a Kromasil C18 or a spherisorb S5CN column (4.6 × 150 mm) and an UV detector (210 and 254 nm, Shimadzu SPD10Avp detector). Semi-preparative HPLC was performed on a Waters 600 instrument (Milford, MA, USA) equipped with a Kromasil C18 column (21 × 150 mm) and peak detection was achieved by UV at 210 and 254 nm. Radioactivity measurements were performed on a Wallac 1409 liquid scintillation counter (Turku, Finland) using Beckman scintillant. A Bruker 400 MHz instrument (Wissenbourg, France) was used for proton NMR characterization using TMS as internal reference. TLC plates were exposed on a Bas-TR2025 Fuji imaging plate (Raytest, Paris) and the levels of radioactive material evaluated using a Fuji Bas 5000 phosphoimager (Tokyo, Japan). Electropray mass spectra were recorded on a Esquier–Bruker spectrometer (Wissenbourg, France).

*(S)-N-tertiobutoxycarbonylamino-S-trityl-cysteine methyl ester (**1**).* (Trimethylsilyl)diazomethane (7 ml) was added dropwise at room temperature to a stirred solution of (*S*)-*N*-Boc-*S*-trityl-cysteine 5 g (10.7 mmol) in a mixture of ether 80 ml and methanol 20 ml. After 6 h reaction time the solvent was removed under reduced pressure to afford a white foam which was used in the next step without further purification. 5.15 g (100%). TLC (Eluent cHex, EtOAc 6/4 Rf = 0.57. ^{1}H NMR ($CDCl_3$) δ 1.35 (s, 9H, C($C\underline{H}_3$)$_3$), 2.5 (d, J = 5.9 Hz, 2 H, S–$C\underline{H}_2$), 3.6 (s, 3H, O–$C\underline{H}_3$), 4.2 (td, 1H, C$\underline{H}$NH), 4.95 (d, 1H, CHN$\underline{H}$), 7.15 (td, 3H, CH arom.), 7.2 (td, 6H, CH arom.), 7.35 (d, 6H, CH arom.). HPLC C_{18} Kromasil (5 µ 100 Å) CH_3CN/H_2O (TFA) 85/15, t_R = 8.3 min.

(3S)-3-tertiobutoxycarbonylamino-4-tritylsulfanyl-but-1-ene-1-sulfonic acid 2,2-dimethyl-propyl ester (**2**). To a solution of neopentyl diethoxyphosphoryl-methanesulfonate 3.22 g (1.2 equiv.) in 20 ml dry THF was added dropwise, at

−78°C, 8 ml (1.2 equiv.) of a solution of *n*-butyllithium (1.6 M in hexane). After the mixture was stirred for 30 min at this temperature, a solution of *S*-*N*-Boc-*S*-trityl-cysteine methyl ester (5.1 g in 10 ml dry THF) was added, immediately followed by a dropwise addition of 8 ml diisobutyl aluminum hydride (1.5M in toluene). The resulting mixture was stirred overnight. The solvents were removed under reduced pressure. 180 ml of diethylether and 200 ml 1M hydrochloric acid were added to the residue and the mixture stirred for 1 h. The organic layer was separated, washed with brine, dried (Na_2SO_4), filtered and concentrated *in vacuo*. The product was purified by semi preparative HPLC C_{18} Kromasil (5 µ 100 Å, 20 × 250 mm) CH_3CN/H_2O (TFA) 85/15 to afford 2.4 g (37%) of a white solid. TLC (Eluent cHex, EtOAc 9/1 Rf = 0.14. ^{1}H NMR ($CDCl_3$) δ 0.9 (s, 9H, $CH_2C(C\underline{H}_3)_3$), 1.35 (s, 9H, $C(C\underline{H}_3)_3$), 2.4 (dd, 2H, S–$C\underline{H}_2$), 3.6 (s, 2H, SO_2O–$C\underline{H}_2$), 4.2 (td, 1H, $C\underline{H}NH$), 4.55 (d, 1H, $CHN\underline{H}$), 6.1 (dd, 1H, $C\underline{H}$ = CH–SO_3), 6.55 (d, 1H, CH–SO_3), 7.15−7.3 (m, 15H, CH arom.). HPLC C_{18} Kromasil (5 µ 100 Å) CH_3CN/H_2O (TFA) 80/20, t_R = 22.8 min. $[M + Na]^+$ = 618.3. $[\alpha]_D$ = −12.13, (*c* = 0.52 in EtOH) λ = 589 nm.

(*3S*)-*3-tertiobutoxycarbonylamino-4-tritylsulfanyl-butane-1-sulfonic acid 2,2-dimethyl-propyl ester (**3**)*. To a stirred solution of compound **2**, 1 g (1.68 mmol) in 5.7 ml EtOH (95%) was added 28.5 mg (0.84 mmol) of sodium borohydride in 6 ml ethanol. The resulting mixture was stirred overnight at room temperature. The reaction was quenched by addition of 2 ml water. After concentration of the solvent under reduced pressure the residue was purified by semipreparative HPLC C_{18} Kromasil (5 µ 100 Å, 20 × 250 mm) CH_3CN/H_2O (TFA) 85/15 to afford 0.85 g (85%) of a white solid. TLC (Eluent cHex, EtOAc 9/1 Rf = 0.14. ^{1}H NMR ($CDCl_3$) δ 0.9 (s, 9H, $CH_2C(C\underline{H}_3)_3$), 1.35 (s, 9H, $C(C\underline{H}_3)_3$), 1.9 (m, 2H, $C\underline{H}_2$–CH_2–SO_3), 2.8 (d, *J* = 7 Hz, 2H, S–CH_2), 2.9 (t, *J* = 7 Hz, 2H, CH_2–$C\underline{H}_2$–SO_3), 3.55 (m, 1H, $C\underline{H}NH$), 3.7 (s, 2H, SO_2O–$C\underline{H}_2$), 4.55 (d, 1H, $CHN\underline{H}$), 7.15–7.3 (m, 15H, CH arom.). HPLC C_{18} Kromasil (5 µ 100 Å) CH_3CN/H_2O (TFA) 80/20, t_R = 21.1 min. $[M + Na]^+$ = 620.3. $[\alpha]_D$ = −2.5 (*c* = 0.64 in EtOH) λ = 589 nm.

2-^{3}H-(3S)-3-tertiobutoxycarbonylamino-4-tritylsulfanyl-butane-1-sulfonic acid 2,2-dimethyl-propyl ester (*3*). After HPLC purification 400 mCi of tritiated compound **3** was obtained as described above using 23 mg of compound **2** dissolved in 100 µl EtOH and 10 Ci of sodium boro[^{3}H]hydride.

(*3S*)-*3-amino-4-sulfanyl-butane-1-sulfonic acid 2,2-dimethyl-propyl ester trifluoroacetate salt (RB 152)*. To a solution of 0.23 g (0.38 mmol) of compound **2** in 1.6 ml of dry dichloromethane were added 180 µl (0.84 mmol) of triisopropylsilane and 1.6 ml (20 mmol) trifluoroacetic acid. The mixture was stirred under argon for 2 h. The solvents were removed under reduced

pressure. The residue was dissolved in water (10 ml) and extracted three times with 5 ml ether. The aqueous phase was lyophilyzed to afford a white powder 0.1 g (71%). TLC (Eluent *i*PrOH, H_2O, Acetic Acid 8/2/1 Rf = 0.6. ^1H NMR (DMSO) δ 0.85 (s, 9 H, $CH_2C(C\underline{H}_3)_3$), 2.0 (m, 2H, $C\underline{H}_2$–CH_2–SO_3), 2.7 (m, 2H, S–CH_2), 3.3 (m, 1H, $C\underline{H}NH_3$), 3.4 (t, J = 7 Hz, 2H, CH_2–$C\underline{H}_2$–SO_3), 3.8 (s, 2H, SO_2O–$C\underline{H}_2$), 7.9 (s, 3H, $C\underline{H}NH_3$). HPLC C_{18} Kromasil (5 μ 100 Å) CH_3CN/H_2O (TFA) 30/70, t_R = 7.6 min. $[\alpha]_D$ = 6.4 (c = 0.77 in H_2O) λ = 589 nm. [RB 152 + H]$^+$ = 509.3.

(3S, 3′S)-4,4′-dithiobis-3,3′-amino-1,1′-butanesulfonic acid 2,2-dimethylpropyl ester trifluoroacetate salt (RB 151). 20 μl of an ethanolic iodide solution (37 mg l^{-1}) was added to a solution of RB 152 (4 mg) in 250 μl EtOH. The colorless solution turned orange. The solvent was removed *in vacuo* to yield pure RB 151 (4 mg, yield 100%). TLC (Eluent *i*PrOH, H_2O, Acetic Acid 8/2/1) Rf = 0.5. ^1H NMR (DMSO) δ 0.85 (s, 18H, $CH_2C(C\underline{H}_3)_3$), 2.05 (td, 2H, $C\underline{H}_2$–CH_2–SO_3), 2.9 (dd, 2H, S–CH_2), 3.05 (dd, 2H, S–CH_2), 3.5 (m, 6H, $C\underline{H}NH_3$, CH_2–$C\underline{H}_2$–SO_3), 3.85 (s, 4H, SO_2O–$C\underline{H}_2$), 8.1 (s, 6H, $CHN\underline{H}_3$). Mass spectroscopy: [RB 151 + H]$^+$ = 509.1852. HPLC : HPLC C_{18} Kromasil (5 μ 100 Å) CH_3CN/H_2O (TFA) 30/70, t_R = 2.6 min. Spherisorb Cyano S5CN; 150 × 4.6 mm 5 μ Solvent A: 0.05% TFA in CH_3CN, solvent B 0.05% TFA in H_2O. Gradient 30% B to 100% B over 10 min, t_R = 6.9 min, $[\alpha]_D$ = +78.7 (c = 0.48 in H_2O), λ = 589 nm.

2,2′-^{3}H-4,4′-dithiobis-(3-amino)-1,1′-butanesulfonic acid 2,2-dimethylpropyl ester trifluoroacetate salt (RB 151). Deprotection of 400 mCi of intermediate **3** in a mixture of 100 μl CH_2Cl_2, 100 μl TFA and 10 μl trisisopropylsilane yields tritiated RB152. After removal of the solvents the crude product was subjected to air oxidation in 100 μl EtOH. After HPLC purification 50 mCi of ^3H RB151 were obtained with a specific activity (determined by mass spectrometry) of 1.07 TBq/mmol (29Ci/mmol) [(^3H)RB 151 + H]$^+$ = 513.2029.

2,2′ ^{3}H-(3s,3′s)-4,4′-dithiobis-(3,3′-amino)-1,1′-butanesulfonic acid RB 150. To 7 mg of compound **2** as a tracer were added 5 mCi of the tritiated form in 1 ml EtOH and 1 ml 12N HCl in a sealed vial. The reaction mixture was stirred at 80°C for five days. After cooling to room temperature and 3 h speedvac concentration, the residue was dissolved in 100 μl water. The desired radiolabeled compound was purified by HPLC on a reverse phase kromasil C18 (5 μ 100 Å) column (mobile phase: 0.05%TFA in water/CH_3CN) eluted in isocratic mode at 90% CH_3CN at a flow rate of 1 ml/min in 10 min. Fractions of 1 ml were collected each minute to provide 2.1 mCi of product with a specific activity of 29 Ci/mmol.

Inactive RB 150 was used as a reference and had the following spectroscopic features: TLC (Eluent *i*PrOH, H_2O, Acetic Acid 8/2/1) Rf = 0.26. ^1H NMR

(D_2O) δ 2.15 (td, 4H, C<u>H</u>$_2$–CH_2–SO_3), 2.85 (dd, 2H, S–CH_2), 2.95 (t, 4H, CH_2–C<u>H</u>$_2$–SO_3), 3.15 (dd, 2H, S–CH_2), 3.75 (m, 2H, C<u>H</u>NH_3). HPLC: Spherisorb Cyano S5CN; 150 × 4.6 mm 5 μ Solvent A : 0.05% TFA in CH_3CN, solvent B 0.05% TFA in H_2O. 20% B, t_R = 3.1 min. $[\alpha]_D$ = +194.5 (c = 1.33 in H_2O) λ = 589 nm.

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