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Mechanistic approach of the difference in hydrolysis rate between the 2- and 4-isomers of no-carrier-added [^{18}F]fluoromethyl-L-phenylalanine

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No-carrier-added (n.c.a.) 2-[^{18}F]fluoromethyl-l-phenylalanine (2-[^{18}F]FMP) was found to be very sensitive to hydrolysis in aqueous solutions. In this paper, the defluorination reaction was studied in detail to elucidate its mechanism. Therefore, besides 2-[^{18}F]FMP and 4-[^{18}F]FMP, 2-[^{18}F]fluoromethyl-phenethylamine (2-[^{18}F]FMPAM) and 4-[^{18}F]FMPAM were synthesized, both 'mimetic' molecules of the decarboxylated amino acid analogues. Radiosynthesis, using a customized Scintomics automatic synthesis hotbox^{three} module, resulted in a high overall yield and a radiochemical purity of >99%. The defluorination rates of all compounds were studied by HPLC. The defluorination rate of 2-[^{18}F]FMLP at 50°C was approximately 300 times faster than that of n.c.a. 4-[^{18}F]FMLP. The defluorination rate of 2-[^{18}F]FMPAM is somewhat lower than of 2-[^{18}F]FMP but still very high in comparison with 4-[^{18}F]FMPAM, which is virtually stable. It allowed to elucidate the reaction mechanism ruled by two distinct intramolecular interactions. First, the hydrogen bond interaction between the amine and the benzylic fluorine weakening the carbon–fluorine bond. Secondly, the formation of a second hydrogen bond between the carboxyl oxygen atom and one of the benzylic hydrogen atoms rendering the benzyl fluoride group even more susceptible to hydrolysis.

Keywords: 2 and 4-[^{18}F]fluoromethyl-l-phenylalanine; hydrolysis; different; rates; mechanism; hydrogen bonds

Introduction

Our group has previously shown that 2-amino-(*S*)-3-(2-[^{18}F]fluoromethyl-phenyl)-propionic acid (2-[^{18}F]fluoromethyl-L-phenylalanine; 2-[^{18}F]FMLP) showed a high uptake in cancer cells both *in vitro* and *in vivo*. However, in its no-carrier-added (n.c.a.) form it suffered from considerable non-radiolytic radiodefluorination in water at room temperature.[1,2] No-carrier added 2-amino-(*S*)-3-(4-[^{18}F]fluoromethyl-phenyl)-propionic acid (4-[^{18}F]fluoromethyl-L-phenylalanine; 4-[^{18}F]FMLP) was stable in the same conditions. This suggested that the fast hydrolysis was due to the ortho-position of the fluoromethyl group on the aromatic ring and more specifically to an intra-molecular interaction between the negatively charged benzyl fluorine atom and the positively charged NH_3^+ of the amino acid group.[2,3] The problem of radiodefluorination in the radiopharmaceutical formulation was solved for the larger part by the addition of calcium ions (0.04 M) ensuring a shelf-life of at least 6 h. This stabilizing effect was assumed to be related to the formation of a complex between the Ca^{2+} ions and the negatively charged fluorine and oxygen atom of the dissociated carboxylic acid.[2] In this paper, we study the mechanism of the fast defluorination of n.c.a. 2-[^{18}F]FMLP.

Materials and methods

All products were at least of analytical grade. All solvents were of HPLC quality or better. NMR data were obtained using an AVANCE DRX 250 instrument and MS data were acquired on a Fisons VG II Quattro Mass Spectrometer.

Synthetic procedures

Fluorinated amino acids

The synthesis of the precursor molecules for the radiosynthesis as well as of non-radioactive fluorinated analogues was described in detail in earlier publications.[2,3]

Synthesis of 2/4-fluoromethyl-phenethylamine

The amine group of commercially available 2/4-methylphenethylamine (Sigma-Aldrich) was protected with N-Boc by reaction with Boc_2O in the presence of triethylamine in tetrahydrofurane, as described by Leftheris *et al.*[4] After silica gel column chromatography (50 g Si-gel (Merck), id 2 × 25 cm,

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petroleum ether/diethyl ether: 2/8 (v/v)) (2-o-Tolyl-ethyl)-carbamic acid tert-butyl ester was obtained as a colourless oil (88% yield). ^{1}H NMR ($CDCl_3$, 500 MHz):δ 7.16–7.12 (m, 4 H), 2.91 (t, J = 8 Hz, 2 H), 2.51 (t, J = 7.5 Hz, 2 H), 2.34 (s, 3 H) and 1.46 (s, 9 H). MS (EI) m/z 236 (MH^+). (4-*o*-Tolyl-ethyl)-carbamic acid tert-butyl ester was obtained as a colourless oil (88% yield). ^{1}H NMR ($CDCl_3$, 500 MHz):δ 7.20–7.10 (m, 4 H), 3.33 (m, 2H), 2.73 (t, J = 7 Hz, 2H), 2.35 (s, 3H) and 1.43 (s, 9H). MS (EI) m/z 236 (MH^+).

For the radical bromination of the methyl side chain, (2 or 4-*o*-tolyl-ethyl)-carbamic acid tert-butyl ester (270 mg, 1.49 mmol) was reacted with *N*-bromosuccinimide (245 mg, 1.38 mmol), using azobisisobutyronitrile (28 mg, 0.17 mmol) in dichloromethane (50 mL) at 50°C for 2 h. The crude product was purified via silica gel column chromatography (50 g Si-gel (Merck), id 2 × 25 cm, petroleum ether/diethyl ether: 1/9 (v/v)) to yield [2-(2-bromomethyl-phenyl)-ethyl]-carbamic acid tert-butyl ester as a yellow oil in 48% yield. ^{1}H NMR ($CDCl_3$, 500 MHz):δ 7.21–7.15 (m, 4 H), 4.58 (s, 2 H), 2.96 (t, J = 7.5, 2 H), 2.54 (t, J = 7.0 Hz, 2 H) and 1.42 (s, 9 H). MS (EI) m/z 314 (M^+). [2-(4-bromomethyl-phenyl)-ethyl]-carbamic acid tert-butyl ester was obtained as a yellow oil in (yield 46%). ^{1}H NMR ($CDCl_3$, 250 MHz):δ 7.14–7.01 (m, 4 H), 4.58 (s, 2 H), 3.27 (m, 2 H), 2.76 (t, J = 6.5 Hz, 2 H) and 1.40(s, 9 H). MS (EI) m/z 314 (M^+).

In the next step, [2-(2 or 4-bromomethyl-phenyl)-ethyl]-carbamic acid tert-butyl ester (106 mg, 0.339 mmol) was treated with an excess of AgF (180 mg, 1.42 mmol) in dry acetonitrile (5 mL) for 3 h at 65°C. The reaction product was purified via silica gel column chromatography (50 g Si-gel (Merck), id 2 × 25 cm) using a gradient of ethyl acetate 1–10% (v/v) in petroleum ether (40–60°C). [2-(2-fluoromethyl-phenyl)-ethyl]-carbamic acid tert-butyl ester was obtained as a white amorphous solid (yield 54%). ^{1}H NMR ($CDCl_3$, 250 MHz):δ 7.18–7.16 (m, 4 H), 5.52 (dd, J_1 = 10.0 Hz, J_2 = 15.0 Hz, $J[^{19}F–^1H]$ = 50.0 Hz, 1 H), 5.42 (1 H, dd, J_1 = 10.0 Hz, J_2 = 15.0 Hz, $J[^{19}F–^1H]$ = 50 Hz), 3.37 (m, 2H), 2.88 (t, J = 7.5 Hz, 2 H) and 1.41 (s, 9 H). MS (EI) m/z 254 (MH^+). [2-(4-fluoromethyl-phenyl)-ethyl]-carbamic acid tert-butyl ester was obtained as a white amorphous solid (yield 58%). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.23–7.21 (m, 4 H), 5.52 (dd, J_1 = 10.0 Hz, J_2 = 15.0 Hz, $J[^{19}F–^1H]$ = 50.0 Hz, 1 H), 5.42 (1 H, dd, J_1 = 10.0 Hz, J_2 = 15.0 Hz, $J[^{19}F–^1H]$ = 50 Hz), 3.38 (m, 2 H), 2.86 (t, J = 7.0 Hz, 2H) and 1.40 (s, 9H). MS (EI) m/z 254 (MH^+).

The final step was the deprotection of [2-(2-fluoromethyl-phenyl)-ethyl]-carbamic acid tert-butyl ester (137 mg, 0.541 mmol) in 20 mL dichloromethane/trifluoroactic acid: 1/1 (v/v) (30 min at room temperature). After the final reaction, solvents were removed by rotatory evaporation and 2-(2-fluoromethyl-phenyl)-ethylamine was obtained as a pale yellow oil (82%).^{1}H NMR ($CDCl_3$, 250 MHz):δ 7.10–6.99 (m, 4 H), 5.49 (s, $J[^{19}F–^1H]$ = 50.0 Hz, 1 H), 5.39 (s, $J[^{19}F–^1H]$ = 50 Hz, 1 H), 3.16 (t, J = 8 Hz, 2 H), 2.73 (t, J = 8.0 Hz, 2 H), 1.20 (s, 2 H). MS (EI) m/z 154 (MH^+). 2-(4-Fluoromethyl-phenyl)-ethylamine was obtained as a pale yellow oil (84%). 1H NMR ($CDCl_3$, 250 MHz):δ 7.20–7.01 (m, 4 H), 5.50 (s, $J[^{19}F–^1H]$ = 47.0 Hz, 1 H), 5.37 (s, $J[^{19}F–^1H]$ = 47 Hz, 1 H), 3.16 (t, J = 8 Hz, 2 H), 2.73 (t, J = 8.0 Hz, 2 H), 1.22 (s, 2 H). MS (EI) m/z 154 (MH^+).

Radiosynthesis

All radioactive compounds were synthesized using the synthetic pathways and the customized modular Scintomics hotbox^{three} system (Scintomics, Fürstenfeldbruck, Germany) as described earlier[3] using the appropriate time for recovery of the n.c.a. [^{18}F]-labeled fully protected compounds after HPLC separation (for the new compounds 2-[^{18}F]FMPAM and 4-[^{18}F]FMPAM this was 8.2 min).

Quality control

Quality control of the radiofluorinated amino acid analogues was performed as described earlier.[2,3]

Quality control of the n.c.a. 2-(2/4-[^{18}F]fluoromethyl-phenyl)-ethylamine was achieved by HPLC analysis, performed on a Polaris C8 125 × 4 mm, 5 μ column (Varian) using an EtOH/aqueous solution of 1 mM CH_3COONH_4 and 1 mM NaF: 5/95 (v/v) of pH 6.5 as mobile phase with a flow rate of 1 mL/min while monitoring both radioactivity (NaI(Tl), Harshaw Chemie) and UV absorption at 254 nm (Shimadzu). The k' values were 0.6 and 8.3 for [^{18}F]fluoride and 2-(2/4-[^{18}F]fluoromethyl-phenyl)-ethylamine, respectively.

Shelf-life study experiments

Quantities of 2.86 GBq of each radiofluorinated compound were synthesized as described earlier. Immediately after the radiosynthesis $CaCl_2$ was added to a final concentration of 40 mM in a volume of 5 mL. Solutions were stored at room temperature. Follow-up of the stock solution showed that the defluorination rate was limited to 0.5% per hour.

From the stock solution 10 μL aliquots were added to a 20 mL vial containing 9.990 mL of water, resulting in a solution containing 5.72 MBq of radiofluorinated compound and 0.04 mM $CaCl_2$. At this concentration of $CaCl_2$, the stabilizing effect is negligible, as was demonstrated by comparison with a sample without $CaCl_2$. Samples of 10 μL, containing about 5.72 kBq at time zero, were directly taken from this solution and injected on the HPLC system at regular time points. The HPLC conditions applied were the same as described in the Quality Control subsection, but using only the radioactivity detector. For each series a t = 0 analysis was performed and the results corrected for the free $^{18}F^-$ if present. The results were expressed as a fraction of the activity at t = 0, using the surface under the peaks of $^{18}F^-$ and the radiofluorinated compound. Collection of the radioactive peak of a calibrated $^{18}F^-$ solution showed that in presence of 1 mM NaF, the amount of $^{18}F^-$ absorbed in the HPLC system was negligible.

Results and discussion

Radiosynthesis

No-carrier-added 2-[^{18}F]fluoromethyl-L-phenylalanine (2-[^{18}F]FMLP) and 4-[^{18}F]fluoromethyl-L-phenylalanine (4-[^{18}F]FMLP) were synthesized as described earlier[3] with a 30% yield and a radiopharmaceutical purity of at least 99%. The 'mimetic molecules' n.c.a. 2-[^{18}F]fluoromethyl-phenethylamine (2-[^{18}F]FMPAM) and n.c.a. 4-[^{18}F]fluoromethyl-phenethylamine (4-[^{18}F]FMPAM) were synthesized with a 40% yield and 99% radiopharmaceutical purity using the same automated modular system and similar synthesis strategies. All radiopharmaceuticals were stabilized by addition of $CaCl_2$. The samples used in the stability experiments were diluted to obtain a final $CaCl_2$ concentration of 40 μM. The defluorination in these solutions was proven to be the same as in pure water.

Hydrolysis study

Defluorination of n.c.a. 2-[^{18}F]FMLP and n.c.a. 4-[^{18}F]FMLP in water at neutral pH at 50°C or 80°C is shown in Figure 1. At equal activities and concentrations (~5.8 × 10^{-10} M), the

initial defluorination rate Vt = 0 of 2-[^{18}F]FMLP at 50°C was approximately 300 times faster than that of n.c.a. 4-[^{18}F]FMLP. At room temperature the defluorination of 4-[^{18}F]FMLP is too slow to allow kinetic measurements (< 0.5% after 3 h). These results show that the fluorobenzyl moiety was more activated for nucleophilic hydroxylation when the benzylic carbon atom was present in the ortho position compared with the para position of the aromatic ring. Remarkably, a difference in the order of reaction kinetics between the ortho- and para-substituted analogues was observed. The defluorination of 4-[^{18}F]FMLP followed a pseudo-first-order reaction (dotted line showing ln % 4-[^{18}F]FMLP a.f.o. time), indicating that the concentration of the second reactant remained constant during the reaction. This reactant could be water ([H_2O] = 55 M) or the hydroxyl ions, since the ratio [OH]/[4-[^{18}F]FMLP] at pH 7.0 amounts to at least 5×10^2 and [OH^-] can assumed to be constant.

Less evident was the fact that the hydrolysis of 2-[^{18}F]FMLP showed zero-order kinetics. Therefore, defluorination of 2-[^{18}F]FMLP was studied at room temperature as a function of pH, ranging from pH 3.5 to pH 8.0. The reaction rate constant K_{obs} (% $^{18}F^-$/min.) was calculated for each pH value and the results presented in Figure 2. Within the recorded pH range the hydrolysis rate constant of 2-[^{18}F]FMLP increases exponentially with the [OH^-] concentration, ruling out H_2O as the nucleophile. On the other hand, the fact that at pH 3.5 K_{obs} is approximately 25 times lower than at pH 8.0 eliminates acid hydrolysis by the bulk protons as the initial step in the overall hydrolysis reaction.

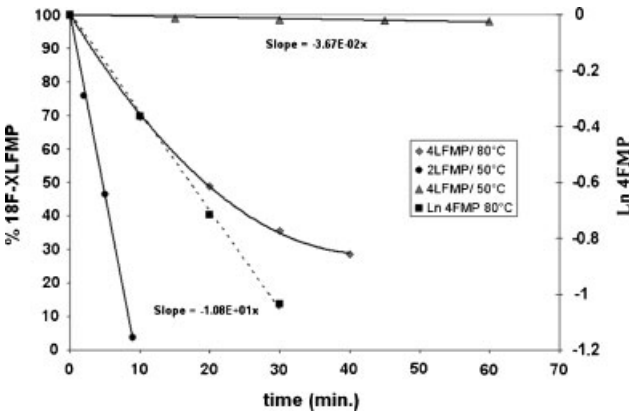

Figure 1. Defluorination of 2-[^{18}F]FMLP and 4-[^{18}F]FMLP. Y-axis left: percentage of original compound a.f.o. time at 50 and 80°C. Y-axis right: ln (% 4-[^{18}F]FMLP) a.f.o. time and represented by the dotted line.

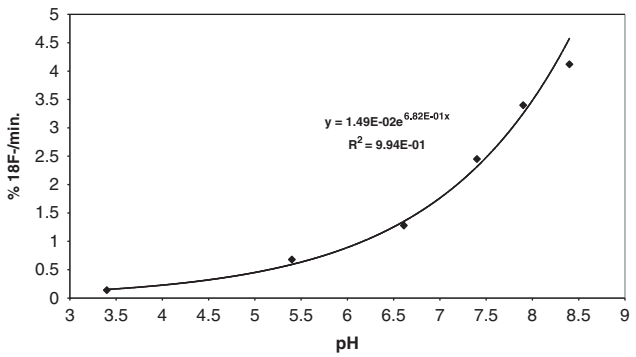

Figure 2. Defluorination of N.C.A. 2-[^{18}F]FMLP at room temperature at different pH values.

The subsequent nucleophilic hydroxylation is related to the solvation of [^{18}F]fluoride ions and not to the formation of HF (pKa of 3.2). These findings can only be explained by the earlier proposed intramolecular interaction followed by $^-$OH substitution. This intramolecular interaction must then occur between the negatively charged benzyl fluorine atom (natural charge 0.4[5]) and a proton of the positively charged ammonium group (the pKa of the amino group in phenylalanine is 9.5), in which a hydrogen bond is formed resulting in an intermediate H_2N^+–H–$F^{\delta-}$–C_{Bzl} (Bzl = benzyl). This model can explain the zero-order kinetics whereby the role of the intramolecular hydrogen bond formation in the reaction kinetics can be compared with the common known keto–enol tautomerism in the halogenation of ketones, the typical example of a zero-order reaction. Our hypothesis is supported by literature through analogy of the role of the positively charged tropaneN atom (R_3NH^+) in the non-enzymatic acid catalyzed hydrolysis of the methyl ester group of cocaine in water, proven experimentally and by quantum chemical first-principle electronic structure calculations.[6,7]

In order to further our hypothesis we also studied the hydrolytic properties of n.c.a 2-[^{18}F]FMPAM as a 'mimetic' compound, since it is in fact decarboxylated 2-fluoromethyl-phenylalanine. This means that both the position of the NH_3^+ group vis à vis the fluorobenzyl group and the probability of AA–NH_3^+–$F^{\delta-}$–CBzL (benzyl) interaction are comparable to these in 2-[^{18}F]FMP. In water at pH 6.5 n.c.a. 2-[^{18}F]FMPAM showed a defluorination rate of 8.5 and 24% per hour at room temperature and 37°C, respectively (Figure 3). N.c.a. 4-[^{18}F]FMPAM was stable under these conditions. This proves our hypothesis that a protonated amine in close proximity of the benzylic fluoromethyl group activates defluorination by an intramolecular interaction.

Moreover, at room temperature and neutral pH, the defluorination rate of 2-[^{18}F]FMPAM, lacking the acid group, is lower than 2-[^{18}F]FMLP (0.14%/min relative to 0.95%/min, respectively). This finding suggests an additional hydrogen bond involving the acid group. It let us to assume that besides the AA-NH_3^+–$F^{\delta-}$-C_{Bzl} internal bridge, a second hydrogen bond type interaction can occur between the O of the carboxylic group and a proton of the benzylic carbon atom. According to the Gutman rules[8] on electron donor–acceptor complexes, confirmed by *ab initio* quantum chemical calculations on H-bonding, this interaction transfers the negative charge to the F-atom (the spill-over effect),

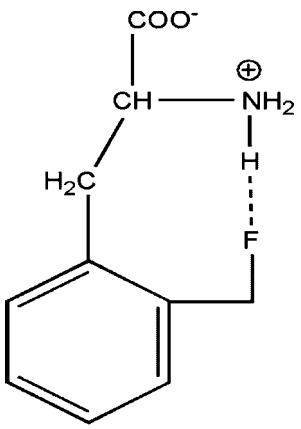

Figure 3. Interaction of the amino group with the benzylic fluorine in 2-[^{18}F]FMLP.

increasing its leaving group capacity or nucleofugality.[9,10] This mechanism also explains the stabilizing role of Ca^{2+} ions.[2] The O^- atom of the dissociated carboxylic acid and the negatively charged F-atom of the fluorobenzyl entity are involved as ligands in a bidentate Ca^{2+} complex, preventing the occurrence of aforementioned interaction.

Conclusion

The considerable difference in reaction rate for the hydrolysis of n.c.a. 2- and 4-[^{18}F]FMP was studied. The study of the reaction kinetics lead to the elucidation of the reaction mechanism, which is governed by two distinct intramolecular interactions. First, the hydrogen bond interaction between the amine and the benzylic fluorine weakens the carbon–fluorine bond. Secondly, the formation of an additional hydrogen bond between the carboxyl negatively charged oxygen atom and one of the benzylic hydrogen atoms renders the fluorine atom even more susceptible to hydrolysis.

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