Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 22 January 2010, Revised 3 May 2010, Accepted 8 June 2010 Published online 5 August 2010 in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jlcr.1811

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

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No-carrier added (n.c.a.) 2-[^{18}F]fluoromethyl-l-phenylalanine was found to be very sensitive to hydrolysis in aqueous solutions. This problem was solved partially by the addition of calcium ions (0.04 M), increasing the shelf-life to at least 6 h. In this paper the defluorination reaction was studied in detail to elucidate its mechanism. Therefore, L and D enantiomers of 2-[^{18}F]FMP and 4-[^{18}F]FMP were synthesized, as well as 2-[^{18}F]fluoromethyl-phenethylamine and 4-[^{18}F]fluoromethyl-phenethylamine, both decarboxylated 'mimetic' molecules of the amino acid analogues. Radiosynthesis, using a customized Scintomics automatic synthesis hot*box*^{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 L enantiomer of n.c.a 2-[^{18}F]FMP defluorinated seven times faster than the D enantiomer and 2-[^{18}F]fluoromethyl-phenethylamine. Both enantiomers of 4-[^{18}F]FMP and 4-[^{18}F]fluoromethyl-phenethylamine were stable. From these data, the reaction mechanism, involving two distinct intramolecular interactions, was derived. First, the interaction between the amine and the benzylic fluorine weakens the carbon–fluorine bond. Secondly, the formation of a second hydrogen bridge between the carboxyl group and one of the benzylic hydrogen atoms renders the fluorine atom even more susceptible to hydrolysis. The latter interaction induces an additional chiral center. The probability of its formation differs considerably between L and D enantiomers of n.c.a. 2-[^{18}F]FMP, which explains the difference in hydrolysis rate.

Keywords: L and D 2-[^{18}F]fluoromethyl-phenylalanine; non-enzymatic; hydrolysis; different; rates

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. We report for the first time the difference in non-enzymatic hydrolysis rate between L and D enantiomers, in casu: 2-[^{18}F]fluoromethyl-phenylalanine.

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.

Geometry optimizations and subsequent calculations of atomic charges, using the natural population analysis,[4–6] were performed at the B3LYP[7,8]/6-31G(d)[9] level of theory using the Gaussian 03 software package.[10]

Synthetic procedures

Fluorinated amino acids

The synthesis of the precursor molecules for the radiosynthesis of R-2-amino-3-(2-[^{18}F]fluoromethyl-phenyl)-propionic acid and

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S-2-amino-3-(2-[^{18}F]fluoromethyl-phenyl)-propionic acid as well as their 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.*[11] After silica gel column chromatography (50 g Si-gel (Merck), id 2 × 25 cm, 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):δ7.16–7.12 (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):δ7.20–7.10 (m, 4 H), 3.33 (m, 2 H), 2.73 (t, J = 7 Hz, 2 H), 2.35 (s, 3 H) and 1.43 (s, 9 H). 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):δ7.21–7.15 (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):δ7.14–7.01 (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):δ7.18–7.16 (m, 4 H), 5.52 (dd, J_1 = 10.0 Hz, J_2 = 15.0 Hz, $J_{[19F-1H]}$ = 50.0 Hz, 1 H), 5.42 (1H, dd, J_1 = 10.0 Hz, J_2 = 15.0 Hz, $J_{[19F-1H]}$ = 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):δ7.23–7.21 (m, 4 H), 5.52 (dd, J_1 = 10.0 Hz, J_2 = 15.0 Hz, $J_{[19F-1H]}$ = 50.0 Hz, 1 H), 5.42 (1H, dd, J_1 = 10.0 Hz, J_2 = 15.0 Hz, $J_{[19F-1H]}$ = 50 Hz), 3.38 (m, 2H), 2.86 (t, J = 7.0 Hz, 2 H) and 1.40 (s, 9 H). 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):δ7.10–6.99 (m, 4 H), 5.49 (s, $J_{[19F-1H]}$ = 50.0 Hz, ^{1}H), 5.39 (s, $J_{[19F-1H]}$ = 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%). ^{1}H NMR ($CDCl_3$, 250 MHz):δ7.20–7.01 (m, 4 H), 5.50 (s, $J_{[19F-1H]}$ = 47.0 Hz, ^{1}H), 5.37 (s, $J_{[19F-1H]}$ = 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 hot*box*^{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), 2-[^{18}F]fluoromethyl-D-phenylalanine (2-[^{18}F]FMDP), 4-[^{18}F]fluoromethyl-L-phenylalanine (4-[^{18}F]FMLP) and 4-[^{18}F]fluoromethyl-D-phenylalanine (4-[^{18}F]FMDP) 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 ($\sim 5.8 \times 10^{-10}$ M), the initial defluorination rate $V_{t=0}$ 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

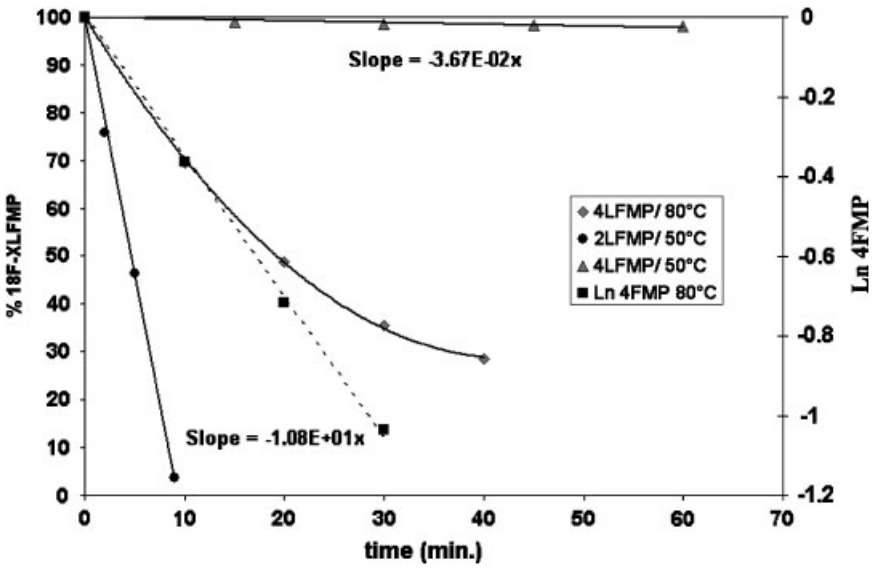

Figure 1. Defluorination of 2-[^{18}F]FMLP and 4-[^{18}F]FMLP. Y-axis left: percentage of original compound a.f.o. time at 50 and 80°C. *Y*-axis right: ln (% 4-[^{18}F]FMLP) a.f.o. time and represented by the dotted line.

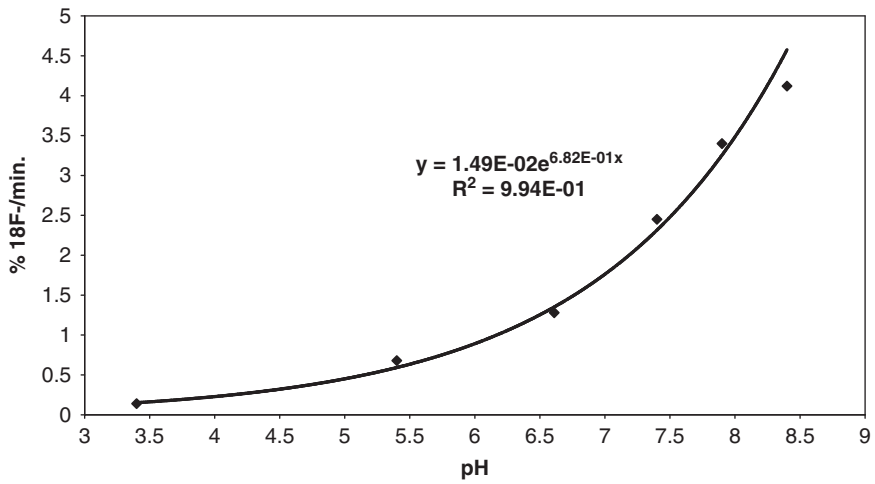

Figure 2. Defluorination of N.C.A. 2-[^{18}F]FMLP at room temperature at different pH values.

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. The subsequent nucleophilic hydroxylation is related to the solvation of [^{18}F]fluoride ions and not to the formation of HF (pK_a 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) and a proton of the positively charged ammonium group (the pK_a of the amino group in phenylalanine is 9.5), in which a hydrogen bridge is formed resulting in an intermediate H_2N^+–H---F $^{\delta-}$–C_{BzI} (Bzl = benzyl). This model can explain the zero-order kinetics whereby the role of the intramolecular hydrogen bridge 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 tropane –N 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.[12,13]

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 $^{\delta-}$–C_{BzL} (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. As depicted in Figure 4, the defluorination rate of 2-[^{18}F]FMPAM at room temperature was comparable to that of 2-[^{18}F]fluoromethyl-D-phenylalanine (2-[^{18}F]FMDP). Remarkably, there was a large difference in hydrolysis rate between the L and D enatiomers of [^{18}F]-2-Fluoromethyl-phenylalanine: 2-[^{18}F]FMDP was hydrolysed approximately seven times slower than 2-[^{18}F]FMLP. To our knowledge it is the first time that a considerable difference is reported between the rates of non-enzymatic hydrolysis of the L and D enantiomers of a fluorobenzyl analogue of phenylalanine.

This stereospecificity requires either the interaction with a chiral reaction partner or the presence of two chiral centers[14] in the fluoromethyl-phenylalanine analogue, the chiral alfa carbon being one of them. Because the OH^- ion, the second partner in the hydrolysis reaction, is not chiral, the origin of the apparent stereospecificity must be linked to an additional intramolecular interaction. If one assumes that besides the NH_3^+F $^{\delta-}{}_{BzL}$ internal bridge, a second hydrogen bridge type interaction can occur between the O^- of the carboxylic group and a proton of the benzylic carbon atom, a second apparent asymmetric carbon atom appears. According to the Gutman rules[15] 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), increasing its leaving group capacity or nucleofugality.[16,17] This results in a faster hydrolysis by the OH^- ions. Similar induction of an asymmetric environment due to H-bonding

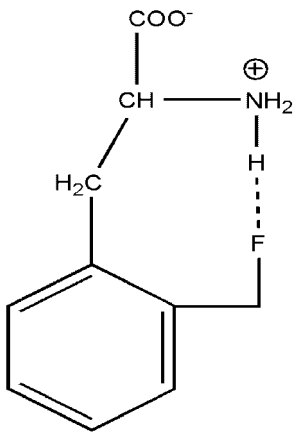

Figure 3. Interaction of the amino group with the benzylic fluorine in 2-[^{18}F]FMLP.

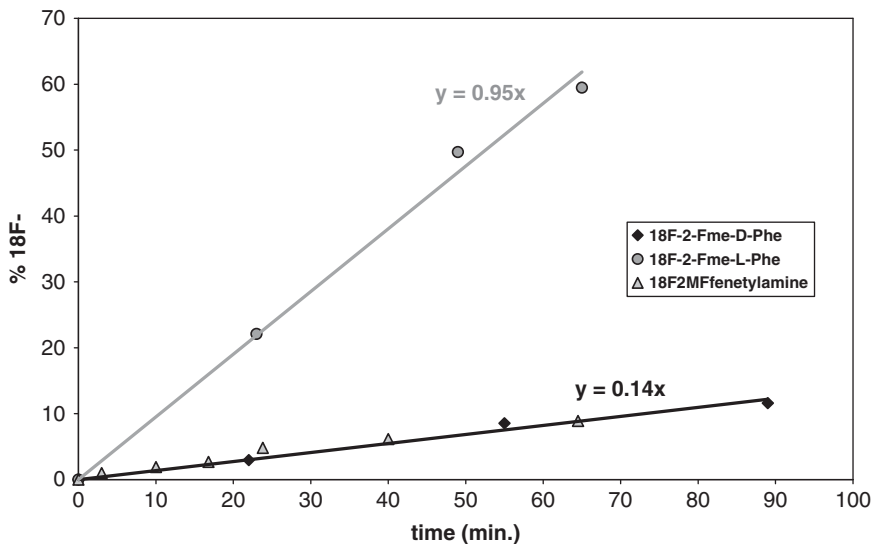

Figure 4. Defluorination of 2-[^{18}F]FMLP and 2-[^{18}F]FMDP in water (pH 6.5) at room temperature.

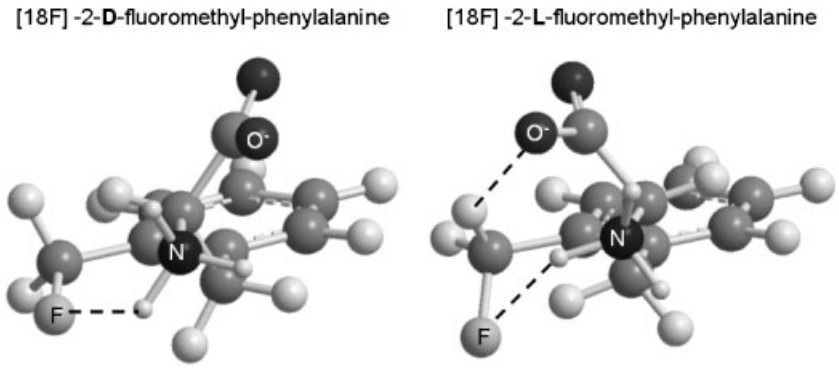

Figure 5. Molecular mechanics result of the optimized geometry of 2-[^{18}F]FMLP(right) and 2-[^{18}F]FMDP (left), showing the potential intramolecular interactions.

(conformational induction of asymmetry) has recently been studied by some of the authors in the case of the influence of conformation on the local dissimilarity of the N atom of the amino group in the enantiomers of alanine and serine.[18,19] Both types of interaction are dynamic processes, and their probability depends on the conformation of the different atoms and groups within the molecular structures. 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.

The lower defluorination rate of 2-[^{18}F]FMDP compared with 2-[^{18}F]FMLP can be explained by the lower probability of hydrogen bridge-like interaction of the D compared with the L enantiomer. This is represented as a 'static' image in Figure 5 by a molecular mechanics result of the optimized geometry of the D and L enantiomers, in which a second chiral center was introduced by replacing one of the hydrogen atoms of the benzylic carbon atom by a deuterium atom (CS Chem3D pro 7.0 representation[20]). At room temperature and neutral pH, the defluorination rate of 2-[^{18}F]FMDP is comparable to that of 2-[^{18}F]FMPAM, lacking the acid group, suggesting that the probability of hydrogen bridge interaction between O^- of the acid group and the benzylic proton in the D enantiomer is very low and that the defluorination in that case is due for the larger part to the interaction between the NH_3^+ and the negatively charged benzylic F-atom.

These findings can be of great practical importance for tumour diagnosis with PET. It was shown by our group that 2-D-[^{123}I]Iodo-phenylalanine compared with the L enantiomer showed an equal tumour uptake but longer tumour retention and a faster clearance from non-target tissues, increasing the tumour contrast.[21] The high *in vitro* affinity of 2-[^{18}F]FMDP for LAT1 transport (non-published results) and increased *in vivo* stability creates a potential for n.c.a. 2-[^{18}F]FMDP as a new tumour tracer for PET.

Conclusion

A considerable difference in reaction rate is reported for the non-enzymatic hydrolysis of the L and D enantiomers of no-carrier added 2-[^{18}F]FMP. The study of this unique difference in reaction kinetics leads to the elucidation of the reaction mechanism, which is governed by two distinct intramolecular interactions. First, the interaction between the amine and the benzylic fluorine weakens the carbon–fluorine bond. Secondly, the formation of an additional hydrogen bridge between the carboxyl group and one of the benzylic hydrogen atoms renders the fluorine atom even more susceptible to hydrolysis. The latter interaction induces an additional chiral center and the probability of its formation differs greatly between L and D enantiomers of no-carrier added 2-[^{18}F]FMP.

The gained insights are important, not only with regard to the design of future radiofluorinated compounds but also for the possible application of 2-[^{18}F]FMDP as a potential tumour tracer as it is expected to be more stable *in vivo* than its L enantiomer.

Acknowledgements

K. Kersemans wishes to thank the Research Unit High Resolution NMR and the department of Organic Chemistry of the Vrije Universiteit Brussel for performing the ^{1}H NMR and ESI-MS analysis. Finally we thank Prof. Dr Bengt Langström for the helpful discussion on the chiral aspects in our hypothesis.

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