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SYNTHESIS OF N.C.A. CIS- AND TRANS-4-[^{18}F]FLUORO-L-PROLINE, RADIOTRACERS FOR PET-INVESTIGATION OF DISORDERED MATRIX PROTEIN SYNTHESIS

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SUMMARY

A radiosynthesis of n.c.a. (2S,4R)-4-[^{18}F]fluoroproline (trans-configuration) and its diastereomer (2S,4S)-4-[^{18}F]fluoroproline (cis-configuration) has been developed. It allows the routine production of up to 18 GBq of product for clinical purposes in a remote controlled system. The ^{18}F-labelled amino acids were prepared via cryptate mediated n.c.a. nucleophilic ^{18}F-fluorination starting from the corresponding N-Boc-4-(toluene-sulfonyloxy)proline methylester. N-deprotection and ester hydrolysis takes place under acidic conditions in presence of trifluoromethanesulfonic acid. HPLC-purification combined with on-line solid phase extraction yielded the diastereomerically pure 4-[^{18}F]fluoroprolines within 90 min with a radiochemical yield of 36 ± 7 % (n = 52). Configuration of both isomers was confirmed by comparison with standard compounds which were synthesized via fluorination of the related oxazolidinones with diethylamino sulfurtrifluoride, starting from (2S,4S)- and (2S,4R)-4-hydroxy proline, respectively.

Key words: ^{18}F-labelled amino acids, cis- and trans-[^{18}F]fluoroproline, radiosynthesis, PET-tracer, fluorine-18

INTRODUCTION

The aliphatic amino acid proline and its derivative 4-hydroxyproline represent the major constituents of the structural protein collagen with a content of 15 to 30%. In view of the potential suitability of labelled proline for investigating diseases with disordered metabolism of matrix protein synthesis, both diastereomeric 4-[^{18}F]fluoroprolines were synthesized in order to investigate their biochemical behavior (1).

It has recently been shown that both diastereomeric 4-[^{18}F]Fluoroprolines, (2S,4R)-4-[^{18}F]FPro (trans-FPro) and (2S,4S)-4-[^{18}F]FPro (cis-FPro) show high tumor uptake in osteosarcomas of mice reaching 12% iD/g for the cis-isomer compared to about 7% iD/g for

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trans-[^{18}F]FPro. Studies on tissue homogenates revealed protein co-precipitation only for the cis-isomer. However, due to the relatively slow protein incorporation tumor uptake of both compounds probably reflects mainly amino acid transport (2). Increased uptake of [^{18}F]fluoroproline has also been monitored in situ in a rabbit model of localized pulmonary fibroses by positron emission tomography showing the potential to provide a non-invasive and repeatable method for monitoring active matrix protein synthesis in interstitial lung disease (3,4).

Biodistribution and first results in patients with renal tumors showed [^{18}F]fluoroproline to be a useful complementary tracer in the decisive differentiation of renal masses, while FDG alone is not sufficient (5).

The synthesis of [^{18}F]fluoroproline as a mixture of both diastereomers was described firstly by van der Ley (6) starting with N-tosyl-4-(trifluoromethanesulfonyloxy)-L-proline methyl ester. However, the method described does not allow a reliable production of diastereomerically pure 4-[^{18}F]fluoroproline in amounts sufficient for clinical application. Recently, a semi-automatic synthesis of cis-[^{18}F]Fluoro-L-proline using a FDG-Microlab has been presented but leading only to moderate radiochemical yields of 8 to 13 % (7). Here, an optimized version of our initial method (1) is described for large-scale production of the title compounds.

EXPERIMENTAL

Materials

The diastereomeric compounds (2S,4R)- and (2S,4S) N-Boc-4-hydroxyproline methylester were purchased from Bachem Biochemica GmbH, Heidelberg, Germany, and recently the corresponding tosylate-precursors from Advanced Biochemical Compounds (ABX) GmbH, Dresden, Germany. Toluenesulfonyl chloride and diethylamino sulfurtrifluoride were delivered from Aldrich.

Kryptofix®2.2.2., potassium carbonate (Suprapur), acetonitrile (DNA-quality) and LiChrolut® SCX-cartridges were purchased from Merck , Darmstadt, Germany.

Melting points were measured on a Mettler FPG I melting point apparatus and are uncorrected. ^{1}H-n.m.r. spectra were recorded on a Bruker Avance DPX 200 spectrometer with samples dissolved in $CDCl_3$.

Precursor synthesis

(2S,4R)-N-tert.-butoxycarbonyl-4-(p-toluenesulfonyloxy)proline methylester

2.6 g (10.5 mmol) of (2S,4R)-N-Boc-4-hydroxyproline methylester was dissolved in 5.2 ml of dry pyridine and cooled to 0°C. 2.2 g (11.5 mmol) of p-toluenesulfonyl chloride was added in the course of about 15 min and the reaction mixture kept at 0°C for 4 hours. The solution was then allowed to warm to room temperature and stirred overnight. The organic phase was mixed with approximately 30 ml cold water; the water phase was decanted and the oily residue washed with 5 ml of cold water. The crude product was dissolved in 10 ml of acetonitrile and the solution evaporated to dryness. Residual pyridine was extracted by washing the product three times with n-hexane. The colorless oil (3.25 g) was crystallized from 35 ml of n-hexane yielding 2.34 g (56%), mp. 73.8 °C, ν_{max}(KBr) 1746s, 1699s, 1398s, 1173s, 900s.

^{1}H-NMR ($CDCl_3$): 7.8O (2H, Tos), 7.36 (2H, Tos), 3.75 (s, 3H, OCH_3), 1.42 (s, 9H, $C(CH_3)$

Analytical calculations for $C_{18}H_{25}NO_7S$ (M = 399.55 g/mol): C, 54.11; H, 6.31; N, 3.51; S, 8.03. Found: C, 54.04 H, 6.28 N, 3.72

(2S,4S)-N-tert.-butoxycarbonyl-4-(p-toluenesulfonyloxy)proline methylester

0.52g (2 mmol) of (2S,4S)-N-tert.-Butoxycarbonyl-4-hydroxyproline methylester was dissolved in 1 ml of dry pyridine and stirred at 0°C in the presence of 0.21g (2.2 mmol) of p-toluenesulfonyl chloride. Product purification was performed as for the (2S,4R)-isomer.
Yield: 0.57 g (71%), mp. 90.4 °C, ν_{max} (KBr) 1731s, 1699s, 1400s, 1171s, 896s.
^{1}H-NMR ($CDCl_3$): 7.82 (2H, Tos), 7.36 (2H., Tos), 3.73 (s, 3H, OCH_3), 1.43 (s, 9H, $C(CH_3)_3$

Analytical calculation for $C_{18}H_{25}NO_7S$ (M = 399.55 g/mol): C, 54.11; H, 6.31; N, 3.51; Found: C, 53.84 H, 6.42 N, 3.24

Synthesis of standard compounds: (2S,4R) and (2S,4S)-4-fluoroproline

(6S,7aS)- and (6R,7aS)-6-hydroxy-3,3 bis(trifluoromethyl) tetrahydro-pyrrolo[1,2-c]oxazol-1-on **1**

At room temperature a gentle stream of gaseous hexafluoroacetone (HFA) was passed through a suspension of 0.65 g (5 mmol) of (2S,4R)- or (2S,4S)-4-hydroxyproline, respectively, in dry DMSO. To prevent the evaporation of volatile and poisonous hexafluoroacetone the round bottom flask was connected to a reflux condenser cooled with dry ice. A gradual dissolution of the hydroxyproline took place until saturation of the solvent with HFA. When reflux started, the inlet of HFA was interrupted. Dissolution of hydroxyproline could be accelerated by gentle heating to max. 50 °C, 30 min after mixing. The reaction was continued for 3 h under reflux, preventing the evaporation of HFA.
Surplus hexafluoroacetone was then depleted with a stream of He for 10 min and the toxic gas absorbed in water. The solution was diluted with water and extracted with dichloromethane (3x50 ml). The organic phase was washed with $NaHCO_3$-solution and water, dried over anhydrous Na_2SO_4 and evaporated to dryness yielding a colorless viscous oil which can be stored at -20 °C only for a couple of days. The products obtained were used for fluorination without further purification.

(6S,7aS)-6-fluoro-3,3 bis(trifluoromethyl) tetrahydro-pyrrolo[1,2-c]oxazol-1-on **2**
and (6R,7aS)-isomer.

2.4 g (15 mmol) of diethylamino sulfurtrifluoride was added dropwise to a solution of 1.4 g (5 mmol) of compound **1** in 40 ml of dry dichloromethane at 0 °C. After 5 h at 0 °C, stirring was continued at room temperature for 15 h. The solution was added to ice-water, extracted two times with $NaHCO_3$-solution and water and dried over anhydrous Na_2SO_4. After removing the solvent, the crude product was solubilized in 10 to 15 ml of hexane / ethylacetate (7:3) and filtered through a silica gel cartridge. The solvent was evaporated and the residual brown-coloured liquid (1.3 g) was vacuum distilled (< 0.1 mbar) with a bath temperature of about 100 °C, yielding 0.7 g (50%) of slightly yellow oil.

Fluoroxazolon with (6S,7aS)-configuration: 1.26 g (85 %)
Fluoroxazolon with (6R,7aS)-configuration: 0.57 g (38 %)

analytical HPLC: column: Nucleosil 100-5 C18 (250x4 mm)
eluent: methanol/water/acetic acid (88/12/0.4) (v/v/v), flow: 1 ml/min
UV-detection: 220 nm; k'= 1.42 (both isomers)

(2S,4S)-4-fluoroproline **3**

1 g (3.4 mmol) of (6S,7aS)-6-Fluoro-3,3 bis(trifluoromethyl) tetrahydro-pyrrolo[1,2-c] oxazol-1-on was solubilized in 5 ml of propanol, 2.5 ml of water was added and the solution stirred at room temperature for 2 days. After evaporation to dryness ethanol (2 ml / 0.1 g residue) was added and the solution heated under reflux. The dissolution was completed by adding a few drops of water. Slow cooling led to the formation of white crystals of the diastereomerically pure product 0.18 g (40 %), mp. 265 °C (mp. 271 °C, (8)).

(2S,4R)-4-fluoroproline

(2S,4R)-4-fluoroproline was produced in an identical manner to the cis-isomer starting with 0.6 g (2 mmol) of (6R,7aS)-6-fluoro-3,3 bis(trifluoromethyl)tetrahydro-pyrrolo[1,2-c]oxazol-1-on. 60 mg (22 %) of crystalline amino acid was obtained; mp. 246 °C
(mp. 243...246 °C (8)).

^{1}H-NMR-spectral parameters of cis- and trans-4-fluoro-L-proline are in agreement with the literature data (9).

Radiochemistry

The radio-fluorinated amino acids were synthesized by remote control in a modified FDG-synthesis device (10) which was connected on-line to a preparative HPLC-system and solid phase extraction unit.

Nucleophilic radio-fluorination

The aqueous solution of [^{18}F]fluoride was passed through a column (1.5x25 mm) containing about 0.1 g of wet anion exchange resin (AG1-X8, carbonate form). Elution of the activity was performed by washing the column with 0.3 ml of potassium carbonate solution (67 mmol/l). The ^{18}F-containing solution was mixed with 15 mg (40 µmol) of Kryptofix®222 in 1 ml of acetonitrile and evaporated to dryness as reported earlier (10).

A solution of 16 mg (4 µmol) of the precursor in 1 ml of acetonitrile (DNA-quality) was added to the dry cryptate at a temperature of 85 °C. After a reaction time of 10 min the solution was evaporated to dryness.

Deprotection and crude product separation

The deprotection of the intermediate product takes place under isometric conditions in the presence of 0.5 ml of 2 M trifuoromethanesulfonic acid at 125-130°C for 10 min. After cooling to room temperature the solution was neutralized by passing through a column (8.5 mm×20 mm) containing 0.8 g of AG1-X8 resin in the hydrogen carbonate form. The reaction vessel was washed with 0.5 ml of water which was also passed on to the resin. The [^{18}F]fluoroproline still remaining on the solid phase was desorbed by washing with 2.5 ml of 0.02 M sodium glycinate solution. The combined solutions (approx. 3 ml) were used for HPLC.

HPLC-purification and product formulation

Chromatographic purification of [^{18}F]fluoroprolines solubilized in 3 to 3.5 ml was performed using Nucleosil 100-10 NH_2 (250x20 mm, precolumn 20x20mm) (Macherey-Nagel, Düren, Germany). Eluent: acetonitrile/0.01 M phosphate buffer, pH 7 (60/40);

flow rate: 20 ml/min.

k'(trans-4-[^{18}F]fluoroproline) = 2.46

(cis-4-[^{18}F]fluoroproline) = 3.36

The eluent fraction containing the pure radiotracer (about 30 ml) was passed through a SCX-cartridge in the H^+-form (LiChrolut® SCX, 1g, Merck, Darmstadt) over the course of 8 min. After washing the cartridge with 12 ml of sterile water for elimination of residual acetonitrile, the n.c.a. [^{18}F]fluoroproline was eluted with 6 ml of 0.1 M trisodium phosphate

yielding a product solution with a pH in the range of 6.5 to 8. The solution was finally passed through a Millipore filter (0.22 μm).

Quality control

Radiochemical and chemical purity were controlled using the following HPLC-system: Nucleosil 100-5 NH_2 (250x4.6 mm), eluent: acetonitrile/0.01 M phosphate buffer, pH 7 (60:40), flow rate: 1 ml/min; UV-detection at 220 nm;
k'-values: trans-isomer (1.77), cis isomer (2.61).
The acetonitrile content was measured via gas chromatography using the column Permabond OV-1701-DF-1.0 (50 m x 0.32 mm) with the precolumn: FS-Phe-Sil Desact (10 m x 0.32 mm) with a hydrogen flow of 80 ml/min and an injector temperature of 200 °C. The temperature programme started at 80 °C with an increase of 5 °C/min up to 200 °C. The retention time of acetonitrile was 6.86 min. The upper limit of acetonitrile concentration should not exceed 0.1 g/ml of phosphate buffer, equivalent to a total content of 0.6 mg of acetonitrile in the final (6 ml) injection solution.

RESULTS AND DISCUSSION

Synthesis of diastereomerically pure 4-fluoroprolines

α-Functionalized carboxylic acids, like amino acids, can be converted into bis(trifluormethyl) substituted five-membered lactones using the fluorinated ketone hexafluoroacetone. These lactones which are readily available and relatively stable against weak acids and increased temperature can be used under mild conditions in the presence of nucleophilic agents like alcohols and amines (11). As functional groups in the side chain, e.g. of amino acids remain unaffected, this method can be applied for regioselective functional group transformation. According to this strategy both diastereomeric 4-hydroxyprolines, the (2S,4R)- and (2S,4S)-isomer, have been converted into the corresponding 1.3-oxazolidinones like the (6R,7aS)-6-hydroxy-3,3-bis(trifluoromethyl) tetrahydro-pyrrolo[1,2-c]oxazol-1-on 1 starting with (2S,4R)-4-hydroxyproline (see Fig.1). Subsequent reaction with diethylamino sulfurtrifluoride leads to the formation of the

corresponding fluoro-compound with inversed configuration. Diastereomerically pure 4-fluoroprolines were obtained by hydrolysis of the fluorinated oxazolidinones in the presence of propanol-2/water in an overall yield of about 35 % (cis) and 10 % (trans). The identity and purity of both standard compounds was determined by ^{19}F-NMR spectroscopy (9) and HPLC.

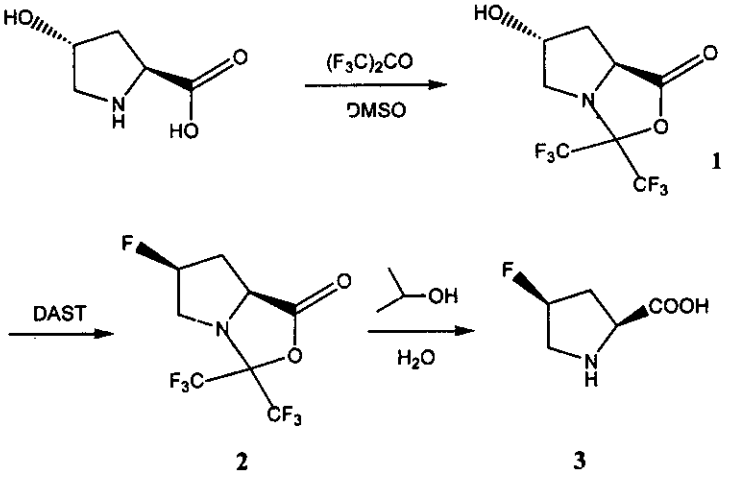

Fig. 1 Synthesis of cis-4-fluoroproline (2S,4S-configuration)

Radiochemical synthesis

In order to investigate the biochemical properties of 4-[^{18}F]fluoro-L-proline a reliable n.c.a.-synthesis of both diastereomers (2S,4R) and (2R,4S) has been developed allowing us to produce the n.c.a. fluorinated aliphatic amino acids for routine clinical purposes. A radiochemical yield of 36 ± 7 % and a radiochemical purity of >98 % (n = 52) was obtained within about 90 min.

The synthesis of both diastereomeric 4-[^{18}F]fluoroprolines is based on the commonly used method of cryptate mediated nucleophilic ^{18}F-fluorination (12). Under large scale production conditions of up to 18 GBq, the synthesis was performed in a remote controlled device which is based on the FDG-synthesizer (10) and connected on-line to a preparative HPLC-system and solid phase extraction unit. Splitting of the acid labile tert. butyloxycarbonyl

function at room temperature and hydrolysis of the methyl ester group (>100°C) was performed in the presence of 2 M trifluoromethanesulfonic acid. This acid is stronger and less volatile than hydrochloric acid. The attempt to do the ester hydrolysis under alkaline conditions led to the formation of a couple of side products, decreasing the radiochemical yield. Furthermore, it is uncertain whether alkaline hydrolysis induces partial racemization of the n.c.a. ^{18}F-labelled amino acid. Neutralization of the acidic solution after ester hydrolysis was performed by using the anion exchange resin AG1X8 in the hydrogen carbonate form. The fluorinated proline bound to the anion exchange resin can be selectively desorbed by elution with sodium glycinate solution. The binding selectivity of glycine is similar to that of proline, thus leading to a preferred elution of [^{18}F]fluoroprolinate whereas stronger anions like [^{18}F]fluoride and trifluoromethanesulfonate remain on the resin.

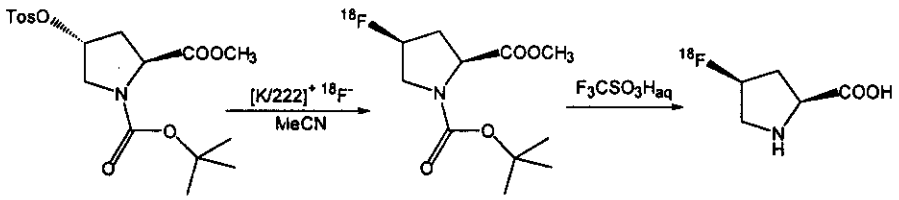

Fig. 2 ^{18}F-labelling of proline exemplified for the synthesis of (2S,4S)-4-[^{18}F]fluoroproline (cis-configuration)

Although the fluoroproline precursor is diastereomerically pure, both [^{18}F]fluoroproline isomers are formed in the course of nucleophilic ^{18}F-fluorination. Starting with the (2S,4R)-precursor 82.3 ± 4.9 % (n = 32) of the 4-[^{18}F]fluoroproline has the expected (2S,4S)-configuration (cis-form). When using the (2S,4S)-precursor 83.0 ± 6.7 % (n = 20) of the radiotracer formed has the desired (2S,4R)-configuration. The fluctuation of the relative content of the undesired isomer, which was in the range of 7.1 to 28.8 %, is equal for both diastereomers. It may be due to the fact that the nucleophilic substitution does occur according to an S_N2 as well as S_N1 mechanism. Gottlieb et al. (8) have reported the same extent of epimerisation, although the reason for this stereochemical unselectivity is not yet clear. It may be that the influence of the adjacent ester function and /or the small content of residual water determines the behavior of the nucleofugic tosylate group. HPLC purification

and separation of (2S,4S)- and (2S,4R)-[^{18}F]fluoroproline was performed on an aminopropyl phase with baseline resolved separation of both diastereomers within 10 min. Subsequent solid phase extraction using a cationic exchange resin in the H^+-form allows complete extraction of the radiotracer which can be desorbed with trisodium phosphate solution. The amount of sulfonated groups on the silica gel phase is in accordance with the amount of the strong basic trisodium solution used for the desorption of the radiotracer, thus leading to [^{18}F]fluoroproline solubilized in phosphate buffer with a pH of 6.5 to 8, ready for human application (5). A semi-automatic synthesis of cis-[^{18}F]fluoroproline in an adapted FDG Microlab has recently been reported, where the nucleophilic ^{18}F-fluorination via solid phase mediated substitution led to the formation of cis-[^{18}F]fluoroproline with a diastereomeric excess of about 99 % without the need of preparative HPLC (7). However, the radiochemical yield obtained was lower by a factor of 3 to 4 by comparison to our results described herein.

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