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Synthesis and Initial *In Vitro* Characterization of 6-[^{18}F]Fluoro-3-(2(*S*)-azetidinylmethoxy)pyridine, a High-Affinity Radioligand for Central Nicotinic Acetylcholine Receptors

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Summary

6-[^{18}F]Fluoro-3-(2(*S*)-azetidinylmethoxy)pyridine ([^{18}F]**1**), a novel analogue of the high-affinity nicotinic acetylcholine receptor ligand, A-85380, was prepared by a two-step procedure from an appropriate nitro precursor. In the first step, a Kryptofix 222-assisted ^{18}F nucleophilic heteroaromatic substitution in 6-nitro-3-((1-*tert*-butoxycarbonyl-2(*S*)-azetidinyl)methoxy)pyridine provided a radiofluorinated Boc-protected intermediate. Subsequent acidic deprotection of the intermediate gave [^{18}F]**1** with an overall radiochemical yield of 8 to 12% (non-decay-corrected). The typical synthesis time was *ca.* 110 min. Specific radioactivity of the final product ranged from 1000 to 4500 mCi/μmol, as calculated at the end-of-synthesis. *In vitro* studies demonstrated that the novel radioligand bound

Key Words: nicotinic acetylcholine receptors, A-85380, nucleophilic heteroaromatic substitution, fluorine-18, radioligand, receptor binding

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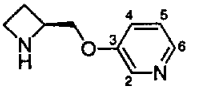
to a single population of sites in rat brain membranes, presumably, to the α4β2 subtype of nicotinic acetylcholine receptor. This binding was characterized by a K_d value of 28 pM, consistent with the K_i value of 25 pM, derived previously for unlabeled **1** in competition assays with (±)-[^{3}H]epibatidine.

Introduction

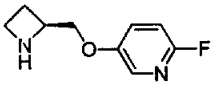
Over the past two decades, nicotinic acetylcholine receptors (nAChRs) have been the subject of much investigation and steadily growing interest. Accumulating evidence has implicated central nAChRs in a variety of brain functions, including cognitive processes (1), and showed them to be affected in a number of neurodegenerative disorders, including Alzheimer's and Parkinson's diseases (2). Accordingly, there has been significant interest in non-invasive imaging of central nAChRs using positron emission tomography (PET) and single photon emission computed tomography (SPECT). The ability to image these receptors *in vivo*, however, has long been hampered by the drawbacks of available radioligands, such as high non-specific binding, rapid clearance from the brain, and poor accumulation in nAChR-rich regions of the brain (3,4).

Epibatidine-based radioligands developed in the last few years (5–8) provided a remarkable advancement over earlier nAChR probes in terms of affinity, specificity of binding, and pharmacokinetics. Some radiolabeled epibatidine analogues were successfully used for imaging nAChRs *in vivo* in non-human primates (9–11). However, the narrow safety margin of epibatidine-based ligands (8,12), likely attributable to a poor nAChR-subtype selectivity of the prototype structure (13–15), has limited the widespread use of these compounds in PET/SPECT studies.

In 1996, a novel series of 3-pyridyl ethers with subnanomolar affinities for central nAChRs was described (16). One of these ethers, 3-(2(*S*)-azetidinylmethoxy)pyridine (A-85380, Figure 1), showed epibatidine-like affinity for the α4β2 subtype (15,16), the predominant variety of nAChRs in the mammalian brain (17). Furthermore, A-85380 was found to be highly selective in binding to α4β2 species but much less potent than epibatidine in activating this and other nAChR subtypes (15). These findings suggested that A-85380 could be a superior parent structure for developing nAChR probes (15,18).



A-85380



6-Fluoro-A-85380, **1**

Figure 1. Structures of A-85380 and 6-fluoro-A-85380.

We recently synthesized a series of halogenated analogues of A-85380, measured their affinities for nAChRs, and identified 2- and 6-fluoro-, 5-bromo-, and 5-iodo-derivatives as promising candidates for developing radioligands to study nAChRs using PET/SPECT (18). Subsequently, several research teams reported the radiosyntheses of 2-[^{18}F]fluoro- (19,20) and 5-[^{123}I]iodo-A-85380 (21,22), and their successful initial evaluation *in vivo* in rodents (22–24) and in non-human primates (25,26).

Here, we describe the radiosynthesis and *in vitro* characterization of 6-[^{18}F]fluoro-A-85380 (6-[^{18}F]fluoro-3-(2(*S*)-azetidinylmethoxy)pyridine, [^{18}F]**1**), a ligand whose affinity for central nAChRs (K_i = 25 pM (18)) is almost twice as high as that of its previously radiolabeled isomer, 2-fluoro-A-85380 (K_i = 46 pM (18)). The radiosynthesis, reported in the present work, uses a nitro compound as the labeling precursor. An alternative synthesis of [^{18}F]**1** *via* an iodo precursor will be described elsewhere.

Experimental

Chemistry. Methods and Materials. All reagents and solvents used were ACS or HPLC grade and were purchased from Aldrich Chemical Co. (Milwaukee, WI). ^{1}H NMR spectra were recorded on a Bruker AM 300 instrument at 300 MHz. Chemical shifts are reported in ppm downfield from internal tetramethylsilane at 0.00 ppm. Flash chromatography was carried out on Merck silica gel (grade 9385, 230–400 mesh, 60Å) obtained from Aldrich Chemical Co.

HPLC analysis and purification were performed on equipment consisting of Rheodyne 7125 injectors, Waters 610 pumps, Waters 486 and 2487 UV detectors (254 nm), Bioscan B-FC-series scintillation detectors, and Rainin control interface modules connected to Macintosh computers with Rainin Dynamax data acquisition and reprocessing software. HPLC columns and mobile phases used were: (**A**) Hamilton PRP-1 columns (10 µm, 7 × 305 mm) eluted with MeCN–MeOH–water–TFA (6:4:90:0.2) at a flow rate of 7 mL/min, and (**B**) Waters Nova-Pak Silica radial compression cartridges (6 µm, 25 × 100 mm) eluted with *i*-PrOH–hexane (8:92) at a flow rate of 7 mL/min.

[^{18}F]Fluoride ion was produced by irradiation of 98%-enriched [^{18}O]water with an 11 MeV negative-ion beam in a CTI RDS-111 cyclotron. Dry K[^{18}F]F–Kryptofix 222 complex was prepared by a standard procedure described in detail elsewhere (5,19). A Capintec CRC-35R dose calibrator was used for radioactivity measurements.

1-*tert*-Butoxycarbonyl-2(*S*)-azetidinemethanol, 6-fluoro-3-(2(*S*)-azetidinylmethoxy)pyridine (**1**), and 6-nitro-3-pyridinol were synthesized according to procedures reported in the literature (16, 18,27).

The following abbreviations are used in the experimental section: DMSO for dimethyl sulfoxide, EtOAc for ethyl acetate, TFA for trifluoroacetic acid, t_R for retention time.

6-Nitro-3-((1-*tert*-butoxycarbonyl-2(S)-azetidinyl)methoxy)pyridine (4). To a stirred at 0 °C solution of 6-nitro-3-pyridinol (420 mg, 3 mmol) and 1-*tert*-butoxycarbonyl-2(*S*)-azetidinemethanol (840 mg, 4.5 mmol) in anhydrous tetrahydrofuran (12 mL) was added in one portion Ph_3P (1.18 mg, 4.5 mmol) followed by the dropwise addition of neat diethyl azodicarboxylate (783 mg, 4.5 mmol). The resulting solution was allowed to warm gradually and was stirred at room temperature for 3 days. The solvent was removed under reduced pressure. The residue was extracted with four 20-mL portions of an 80:20 mixture of hexane and EtOAc. The combined extract was washed with saturated $NaHCO_3$ (15 mL) and water (15 mL) and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo*. The crude material was purified by flash chromatography with a 60:40 mixture of hexane and EtOAc to yield 743 mg (80%) of the desired product as a colorless syrup. ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 9H), 2.26–2.45 (m, 2H), 3.90 (m, 2H), 4.26 (m, 1H), 4.45–4.61 (m, 2H), 7.49 (dd, J = 2.9, 9.0 Hz, 1H), 8.27 (d, J = 9.0 Hz, 1H), 8.29 (d, J = 2.9 Hz, 1H).

6-Nitro-3-(2(S)-azetidinylmethoxy)pyridine (5), salt with TFA. To a solution of 4 (93 mg, 0.3 mmol) in CH_2Cl_2 (1.5 mL), was added TFA (1.5 mL). The reaction mixture was stirred at ambient temperature for 3 h and concentrated *in vacuo* at 40 °C using a rotary evaporator. The residue was purified using HPLC system A. The title compound eluted with a retention time of 5.8 min. The product-containing fraction was concentrated *in vacuo* at 40 °C using a rotary evaporator. During the evaporation, HPLC-grade acetonitrile was repeatedly added to the product for azeotropical removal of water. After drying in a vacuum-desiccator over P_2O_5 at 0.1 mm Hg, the product was obtained as a viscous colorless syrup. The yield was 101 mg (77%). Composition by CHN-analysis: $C_9H_{11}N_3O_3 \cdot 2C_2HF_3O_2$. ^{1}H NMR (acetone-$d_6$) δ 2.68 (m, 1H), 3.02 (m, 1H), 4.81–5.13 (m, 4H), 5.79 (m, 1H), 7.84 (dd, J = 2.9, 9.0, Hz, 1H), 8.35 (d, J = 9.0, Hz, 1H), 8.39 (d, J = 2.9, Hz, 1H).

6-[^{18}F]Fluoro-3-((1-*tert*-butoxycarbonyl-2(S)-azetidinyl)methoxy)pyridine ([^{18}F]6). A solution of 4 (4 mg) in anhydrous DMSO (0.4 mL) was added to the dry K[^{18}F]F–Kryptofix 222 complex, and the reaction mixture was heated in an air-open vial at 150 °C for 20 min. After being cooled, the mixture was diluted with water (3 mL) and passed through a Waters C18 Sep-Pak cartridge. The cartridge was washed with water (5 mL), blown through with argon gas for 1 min and eluted with CH_2Cl_2 (5 mL). The organic solution was evaporated to dryness on a rotary evaporator. The residue was dissolved in an appropriate mobile phase (2 × 0.4 mL) and injected onto HPLC system B to give [^{18}F]6 (t_R 8.5–9.3 min), which was free of the labeling precursor (t_R 17–20 min).

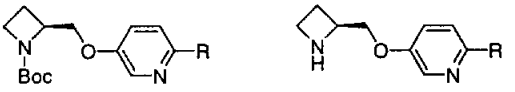
6-[^{18}F]Fluoro-3-(2(S)-azetidinylmethoxy)pyridine ([^{18}F]**1**). The HPLC-fraction, containing intermediate [^{18}F]**6**, was collected into a flask with TFA (1 mL), and the solvent was removed *in vacuo* at 55 °C using a rotary evaporator. The residue was dissolved in a 20:80 mixture of TFA and CH_2Cl_2 (5 mL). The solution was stirred at ambient temperature for 10 min and concentrated to dryness on a rotary evaporator *in vacuo* at 55 °C. The residue was redissolved in an appropriate mobile phase (2 × 0.4 mL) and injected onto HPLC system **A**. The radioactive fraction with a retention time of 5.5–6.8 min corresponding to the unlabeled **1** was collected, and the solvent was removed *in vacuo* at 55 °C using a rotary evaporator. The product was redissolved in 0.9% sterile saline (2 × 3 mL) and filtered through a Millex GS sterile filter into a sterile vial. For the determination of specific radioactivity and radiochemical purity, an aliquot of the final solution of known volume and radioactivity was injected onto analytical HPLC system **A**. The area of the UV peak at 5.5 min, corresponding to the radioactive product, was measured and correlated with a standard calibration curve relating concentration of **1** to its UV absorption.

***In Vitro* Binding Assay.** (±)-[^{3}H]Epibatidine (48 mCi/μmol) was purchased from New England Nuclear Corp. (Boston, MA). Male Fischer-344 rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). Preparation of rat brain membranes and competition binding assays with (±)-[^{3}H]epibatidine were performed as described previously (18). Saturation binding assays were carried out analogously to the reported procedure (21). Briefly, rat brain membranes (P2 fraction, 20–30 μg protein) were incubated in the presence of 1–1000 pM of [^{18}F]**1** in HEPES–salt solution (1 mL, pH 7.4) at 22 °C for 4h. Bound ligand was separated by filtration through Whatman GF/B filters pretreated with 1% polyethyleneimine. Nonspecific binding was determined in the presence of 300 μM (–)-nicotine. Radioactivity was measured using a Packard Cobra gamma-counter calibrated against the Capintec CRC-35R dose calibrator.

Results and Discussion

6-[^{18}F]Fluoro-3-(2(*S*)-azetidinylmethoxy)pyridine, a high-affinity radioligand for nAChRs, was prepared by a two-step procedure from an appropriate nitro precursor in up to 12% overall radiochemical yield (non-decay-corrected). The typical synthesis time was *ca.* 110 min. Specific radioactivity of the final product ranged from 1000 to 4500 mCi/μmol as calculated at the end-of-synthesis (EOS). *In vitro* binding studies in rat brain membranes showed that the novel radioligand bound with a single population of binding sites characterized by a K_d value of 28 ± 3 pM ($n = 4$).

Previously, we demonstrated that 2-[^{18}F]fluoro-A-85380, a lower-affinity isomer of the target radioligand, [^{18}F]**1**, could be prepared in a satisfactory radiochemical yield from 2-iodo-3-((1-*tert*-butoxycarbonyl-2(*S*)-azetidinyl)methoxy)pyridine via halogen exchange at 190 °C followed by removal of the Boc-group (19). The use of an analogous iodo compound (**2**) as the labeling precursor in the synthesis of [^{18}F]**1**, however, appeared less favorable. First, we found that the 6-iodo-3-pyridyloxy moiety was notably less reactive than the 2-iodo function in the nucleophilic iodo-to-fluoro substitution under the same reaction conditions (18). Hence, we reasoned that **2** might not provide sufficient radiochemical yield as the ^{18}F-labeling precursor. Another point to consider was potentially possible contamination of radioligand [^{18}F]**1** with its iodo analogue **3**, which would result from the deprotection of excess of precursor **2**. Compound **3** manifests subnanomolar affinity for nAChRs (K_i = 150 pM (18)) and could confound the interpretation of PET data. Therefore, our efforts were directed toward developing a precursor with a more reactive leaving group at position 6 of the pyridine ring and with a low-affinity deprotected form. Here, we turned our attention to the nitro function as it is widely used in nucleophilic ^{18}F-labeling and recently has been successfully employed in the radiosynthesis of another fluoro analogue of A-85380 (20).

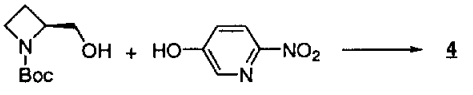


2, R = I
4, R = NO_2

3, R = I
5, R = NO_2

Figure 2. Structures of the considered precursors for the synthesis of 6-[^{18}F]fluoro-A-85380, and the corresponding secondary amines.

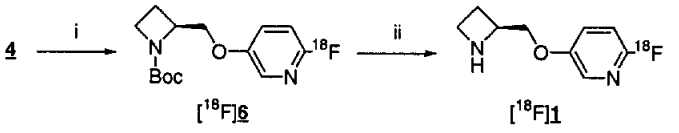
Nitro compound **4**, synthesized from the known 1-*tert*-butoxycarbonyl-2(*S*)-azetidinemethanol (16) and 6-nitro-3-pyridinol (27) (Scheme 1), was found to possess the desired features. Its deprotected derivative, the secondary amine **5**, demonstrated a far more modest affinity for nAChRs (K_i = 3.7 ± 0.2 nM vs. (±)-[^{3}H]epibatidine in rat brain membranes) as compared with iodo analogue **3**.



Scheme 1. Reagents and conditions: Ph_3P, diethyl azodicarboxylate, THF, mixing at 0 °C, then room temperature, 72 h.

Incorporation of the ^{18}F label into the target structure of **1** was accomplished by a two-step procedure (Scheme 2). In the first step, the ^{18}F nucleophilic heteroaromatic substitution in **4** performed

at 150 °C in an air-open vial afforded intermediate [^{18}F]**6** in up to 25% radiochemical yield after HPLC purification (non-decay-corrected). A normal-phase HPLC, used in this case, ensured a complete separation of the labeled compound from the labeling nitro precursor (retention times 8.5–9.3 and 17–20 min, respectively). The average time for the synthesis and purification of the intermediate was 55 min. It should be stressed here that the possibility to carry out the radiofluorination in an air-open reaction vessel at moderately high temperature would facilitate future automation of the synthesis.



Scheme 2. Reagents and conditions: (i) [^{18}F]F$^-$, K_2CO_3, Kryptofix 222, DMSO, 150 °C, 20 min; (ii) TFA, CH_2Cl_2, room temperature, 10 min.

The second step, acidic deprotection of [^{18}F]**6**, followed by a reverse-phase HPLC purification gave the target radioligand, [^{18}F]**1**, with an overall radiochemical yield of 8–12% (EOS). The average time for the second step was 55 min. The obtained radioligand co-eluted with the authentic unlabeled **1**, displayed no foreign UV peaks detectable on the HPLC chromatogram (Figure 3), and was estimated to be 99% radiochemically pure. Specific radioactivity of [^{18}F]**1**, prepared using 100–150 mCi of [^{18}F]fluoride ion, ranged from 1000 to 4500 mCi/μmol (EOS). The reported radiochemical yields and synthesis times were not optimized in this study.

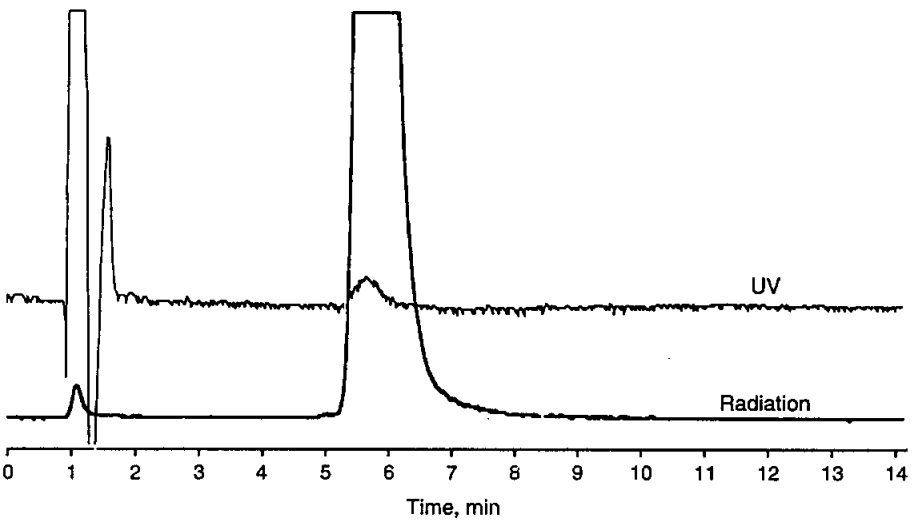


Figure 3. A typical analytical HPLC chromatogram used for the determination of radiochemical purity and specific radioactivity of 6-[^{18}F]fluoro-A-85380.

The interaction of radioligand [^{18}F]**1** with central nAChRs *in vitro* was studied using brain membranes from Fischer-344 rats. The assays revealed that the specific binding of [^{18}F]**1** in the rat brain was saturable (Figure 4). The linearity of Scatchard plots (Figure 4, insert) indicated that the interaction was represented by a single population of binding sites over a broad range of radioligand concentrations (1 to 900 pM). These binding sites were characterized by a K_d value of 28 ± 3 pM and a B_{max} value of 3.2 ± 0.3 pmol/g tissue (132 ± 12 fmol/mg protein; $n = 4$). The obtained K_d value closely matched a K_i value of 25 pM derived previously for unlabeled **1** in competition assays with (±)-[^{3}H]epibatidine (18). The measured maximal density of binding sites, B_{max}, agreed well with the known density of the α4β2-subtype nAChRs in the rat brain (28) determined using the subtype-selective nAChR radioligand, (–)-[^{3}H]cytisine. This agreement suggests that [^{18}F]**1** binds to the α4β2 subtype of nAChRs.

In the present work, the binding assays were also used for an alternative determination of specific radioactivity and radiochemical purity of [^{18}F]**1** in addition to those measured using analytical HPLC. For this purpose, an aliquot of known radioactivity, withdrawn from the final solution of [^{18}F]**1**, was mixed with a known number of moles of non-radioactive **1**. The amount of the unlabeled compound taken was far in excess of that of the radioligand so that the total quantity might be approximated with a reasonable accuracy by the amount of **1** alone (in practice, *ca.* 20-fold excess was used). The resulting radioactive composition was used in saturation binding assays to measure the quantity of binding sites (expressed in pmol) in a given amount of receptor membranes. Simultaneously and under the identical conditions, binding assays were performed with the undiluted [^{18}F]**1** to determine the maximal amount of radioactivity capable of specific binding with the same amount of receptor membranes. Hence, specific radioactivity of the radioligand was derived as the

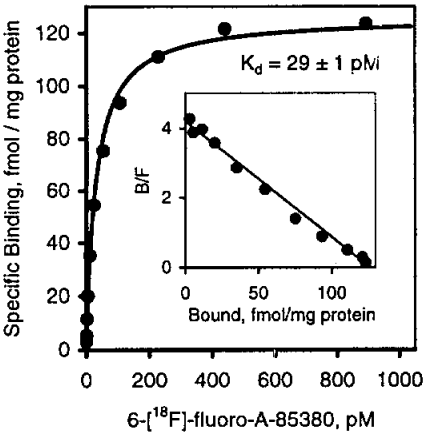


Figure 4. Specific binding of 6-[^{18}F]fluoro-A-85380 to rat brain membranes (P2 fraction). The graph represents data from a single experiment performed in quadruplicate (SEM < 2%). Analogous results were obtained in three additional independent experiments. Average values from the four experiments were: K_d, 28 ± 3 pM, B_{max}, 3.2 ± 0.3 pmol/g tissue (132 ± 12 fmol/mg protein).

ratio of the bound radioactivity to the quantity of binding sites. The obtained value agreed well with the specific radioactivity determined from the HPLC data.

The derived specific radioactivity was then used for the calculation of a K_d value from the data of saturation binding assays with the undiluted [^{18}F]**1**. The calculated constant was compared with the K_d yielded by the assays with the mixture of [^{18}F]**1** and non-radioactive **1**. The ratio of the two K_d values was typically 0.95 ± 0.05, thus indicating a high degree of radiochemical purity of the radioligand. As an example, the ratio of 0.8 would mean that 20% of total radioactivity belong to a compound that does not bind with the receptors.

Conclusion

6-[^{18}F]Fluoro-3-(2(*S*)-azetidinylmethoxy)pyridine, a novel analogue of the high-affinity nAChR ligand, A-85380, was prepared from an appropriate nitro precursor using Kryptofix 222-assisted ^{18}F nucleophilic heteroaromatic substitution, performed in an air-open reaction vessel. The labeled product was obtained in sufficient yield with high chemical and radiochemical purity and high specific radioactivity. *In vitro* binding studies demonstrated that the novel radiofluorinated ligand bound to central nAChRs with high affinity. Therefore, 6-[^{18}F]fluoro-A-85380 holds promise as a radioligand for studying these receptors *in vivo* with PET.

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