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Synthesis of fluorine-18 labeled rhodamine B: A potential PET myocardial perfusion imaging agent

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Abstract

There is considerable interest in developing an ^{18}F-labeled PET myocardial perfusion agent. Rhodamine dyes share several properties with ^{99m}Tc-MIBI, the most commonly used single-photon myocardial perfusion agent, suggesting that an ^{18}F-labeled rhodamine dye might prove useful for this application. In addition to being lipophilic cations, like ^{99m}Tc-MIBI, rhodamine dyes are known to accumulate in the myocardium and are substrates for Pgp, the protein implicated in MDR1 multidrug resistance. As the first step in determining whether ^{18}F-labeled rhodamines might be useful as myocardial perfusion agents for PET, our objective was to develop synthetic methods for preparing the ^{18}F-labeled compounds so that they could be evaluated *in vivo*. Rhodamine B was chosen as the prototype compound for development of the synthesis because the ethyl substituents on the amine moieties of rhodamine B protect them from side reactions, thus eliminating the need to include (and subsequently remove) protecting groups. The 2′-[^{18}F]fluoroethyl ester of rhodamine B was synthesized by heating rhodamine B lactone with [^{18}F]fluoroethyltosylate in acetonitrile at 165°C for 30 min.using [^{18}F]fluoroethyl tosylate, which was prepared by the reaction of ethyleneglycol ditosylate with Kryptofix 2.2.2, K_2CO_3, and [^{18}F]NaF in acetonitrile for 10 min. at 90°C. The product was purified by semi-preparative HPLC to produce the 2′-[^{18}F]-fluoroethylester in >97% radiochemical purity with a specific activity of 1.3 GBq/μmol, an isolated decay corrected yield of 35%, and a total synthesis time of 90 min.

Keywords

fluorine-18; positron emission tomography; rhodamine B; myocardial perfusion imaging

1. Introduction

There is considerable interest in developing an ^{18}F-labeled compound for PET myocardial perfusion imaging. This interest arises primarily because of the limitations of other PET radionuclides currently used for this application. These limitations include the high cost

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of ^{82}Rb (from the ^{82}Sr/^{82}Rb generator) and the limited availability of [^{13}N]NH_3 (because of the short half-life of ^{13}N). There is some interest in Cu radionuclides for this application (Packard *et al*., 2002, Wallhaus *et al*., 2001), primarily with ^{64}Cu because its half-life (12.7 h) is long enough to allow shipping from central production sites, but the positron yield of ^{18}F is greater than that of ^{64}Cu (97% vs. 18%), the shorter half-life of ^{18}F (110 min.) allows repeated studies within the same day, and the distribution networks that have been established for [^{18}F]FDG have demonstrated that production of ^{18}F-labeled radiopharmaceuticals at central sites is a reasonable alternative to on-site production. The ^{18}F compounds that have been reported to date as possible myocardial perfusion agents include quaternary ammonium salts (Studenov *et al.*, 2001), triphenylphosphonium compounds (Ravert *et al.*, 2004, Madar *et al.*, 2006, Madar *et al.*, 2007b, Shoup *et al.*, 2005, Madar *et al.*, 2007a), rotenone (Marshall *et al.*, 2004), and BMS-747158-02, a modified version of the pyridazinone insecticide pyridaben designed to target mitochondrial complex I (Huisman *et al.*, 2008).

There are several reasons that ^{18}F-labeled rhodamines (Fig. 1) are of interest as possible PET myocardial perfusion agents. First of all, as is the case for the single-photon myocardial perfusion agents ^{99m}Tc-MIBI and ^{99m}Tc-tetrofosmin and many of the ^{18}F-labeled compounds listed above, they are lipophilic cations. Other properties that non-radiolabeled rhodamines have in common with ^{99m}Tc-MIBI include accumulation in mitochondria in proportion to mitochondrial membrane potential (Lacerda *et al*., 2005,Reungpatthanaphong *et al.*, 2003,Hu *et al*., 2000,Lampidis *et al.*, 1985), accumulation in brain tumors (Powers *et al*., 1988,Packard *et al*., 1997), and being substrates for Pgp, a protein implicated in multidrug resistance (Rusiecka *et al*., 2008,De Moerloose *et al*., 1999). Perhaps most significantly, Vora and Dhalla showed that non-radiolabeled rhodamine 123 accumulated in the rat heart (Vora *et al.*, 1992). Additionally, Kassis and co-workers have investigated the potential use of radioiodinated rhodamine 123 in tumor diagnosis or therapy (Kinsey *et al.*, 1987,Kinsey *et al.*, 1989,Harapanhalli *et al*., 1998).

There are no previous reports of the synthesis of an ^{18}F-labeled rhodamine, so the first step in this investigation was the development of a method by which rhodamines could be labeled with ^{18}F. Rhodamine B was chosen as the prototype compound in this series primarily because the ethyl substituents on the amines of the xanthene ring system preclude the need to protect them from possible side reactions. An additional consideration is that rhodamine B is itself a substrate for Pgp (Rusiecka *et al*., 2008) and a marker of mitochondrial membrane potential (Reungpatthanaphong *et al.*, 2003).

The general synthetic approach used was to prepare the 2′-[^{18}F]fluoroethyl ester of the benzoic acid moiety of the parent compound, a method analogous to the route used by Yoon, et al. for the preparation of ^{18}F-labeled flumazenil (Yoon *et al.*, 2003). In the present work, we describe the one-pot synthesis of 2′-[^{18}F]fluoroethylrhodamine B (**1**) from rhodamine B lactone (**2**) using [^{18}F]fluoroethyl tosylate ([^{18}F]FETos) as the fluorination reagent as well as the synthesis and characterization of the non-radiolabeled analog. A preliminary account of this work was presented in abstract form (Heinrich *et al.*, 2007, Heinrich *et al.*, 2008).

2. Experimental

2.1. General

Rhodamine B lactone (>97%) was purchased from MP Biomedicals (Solon, OH). Ethylene glycol ditosylate (>97%) was purchased from Sigma-Aldrich (St. Louis, MO). Extra dry reagent grade acetonitrile and Kryptofix® (K2.2.2) (98%) were purchased from Fluka (St. Louis, MO). Potasssium carbonate (99.997%) was purchased from Alfa Aesar (Ward Hill, MA). Other solvents and reagents were of the highest grade commercially available and were used as received unless otherwise noted. Thin-layer chromatography (TLC) was performed

using Silicagel IB-F coated plastic sheets from J.T. Baker (Phillipsburg, NJ). ^{1}H-Nuclear magnetic resonance (NMR) spectra were obtained using a Varian 400 spectrometer (Palo Alto, CA). Chemical shifts are reported as δ values. Coupling constants are reported in hertz. Multiplicity is defined by s (singlet), d (doublet), t (triplet), q (quadruplet) and m (multiplet). Mass spectra were obtained through the courtesy of Prof. Elena V. Rybak-Akimova (Tufts University) using a Thermo- Finnigan LTQ Mass Spectrometer is ESI-MS mode. Fluorine-18 (in water) was purchased from Cardinal Healthcare (Woburn, MA) or obtained as a gift from Massachusetts General Hospital.

2.2 Purification and quality control

Analytical high-performance liquid chromatography (HPLC) was carried out using an HITACHI 7000 system including an L-7455 diode array detector, an L-7100 pump, and a D-7000 interface. The radiometric HPLC detector was comprised of Canberra nuclear instrumentation modules and optimized for 511 keV photons. A Hitachi LaChrom PuroSphere Star C18e column (4 × 30 mm; 3 μm) was used for analytical measurements. The solvent system was 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) at a flow rate of 1 mL/min at room temperature. The solvent gradient was 0-15 min (30%-70% B), 15-25 min. (70% B).

For semi-preparative HPLC, an ISCO system comprised of an ISCO V^{4} variable wavelength uv-visible detector (operated at λ=550 nm), an ISCO 2300 HPLC pump, a Canberra gamma detector, and a Grace Apollo C18 column (10 × 250 mm; 5μm) was used. Preparative HPLC method (isocratic): 40% 0.1% trifluoroacetic acid (TFA) in water (solvent A); 60% 0.1% TFA in acetonitrile; flow rate — 5 mL/min.; room temperature.

Radiofluorination yields were determined by thin-layer chromatography using silica gel plates and chloroform:methanol (8:1 v/v) as the solvent. After they were developed, the TLC strips were cut into 1 cm pieces and counted with a Packard Cobra gamma counter.

2.3. Animal studies

Animal studies were carried out under a protocol approved by the Children's Hospital Boston Institutional Animal Care and Use Committee. Imaging studies were carried out using a Siemens Focus 120 microPET system. The HPLC-purified compound was evaporated to dryness (to remove acetonitrile and trifluoractic acid) and redissolved in a solution of 10% ethanol in saline for injection. Animals were injected with 3.7-7.4 MBq (100-200 μCi) of 2′-[^{18}F]fluorethylrhodamine B in 100 μL of 10% ethanol in saline. Immediately after injection, the animals were anesthetized with isofluorane (2-4% in air) and transferred to the microPET system. Image acquisition was initiated approximately 5 min. after injection. The total imaging time was 1 h.

2.4. Synthesis of non-radioactive 2′-fluoroethylrhodamine B

Synthesis of 2-fluoroethyl tosylate (FETos)—FETos was prepared from 2-fluoroethanol and p-toluene sulfonyl chloride in dry pyridine under nitrogen at 0°C for 4.5 h as described by Parenty, et al. (Parenty *et al.*, 2005). The product was obtained as an oil, which solidified after several days in the refrigerator.

Synthesis of 2′-fluoroethyl rhodamine B (FERhB)—The nonradioactive 2′-fluoroethyl ester of rhodamine B (**2**) was prepared by transesterification of rhodamine B lactone (**1**) with FETos (Scheme 1). 250 mg of **1** (0.56 mmol) were dissolved in 10 mL acetonitrile to which 146 mg FETos (0.67 mmol, previously dissolved in 2 mL acetonitrile) and 0.5 mL (2.94 mmol) diisopropylethylamine (DIPEA) were added. The solution was refluxed and the reaction was monitored by HPLC. After 24 h, 97% of the rhodamine B lactone starting material had been

converted to the 2′-fluoroethyl ester, at which time the heating was discontinued, the reaction mixture was allowed to cool to room temperature, and then evaporated to dryness to give approx. 400 mg of crude product as a dark red oil. 10 mg of the crude product was purified by semi-preparative HPLC to provide 9.5 mg (95%) of purified FERhB as a purple oil.

Compound 1: TLC: R_f = 0.5 (chloroform/methanol, 8:1). ^{1}H-NMR ($CDCl_3$, 600 MHz): δ=1.33 (t, ^3J=7.1 Hz, 12H, $4CH_2C\mathbf{H}_3$), 3.62 (q, ^3J=7.1 Hz, 8H, $4C\mathbf{H_2}CH_3$), 4.21–4.24 (m, 1H, $C\mathbf{H}_2CH_2$), 4.25–4.27 (m, 1H, $C\mathbf{H}_2CH_2$), 4.34–4.36 (m, 1H, $C\mathbf{H}_2CH_2$), 4.44–4.46 (m, 1H, $C\mathbf{H}_2CH_2$), 6.82-6.86(m, $4H_{Ar}$), 7.05–7.09 (m, $2H_{Ar}$), 7.34 (d, ^3J=7.2 Hz, $1H_{Ar}$), 7.75 (dd, ^3J=7.1Hz, $1H_{Ar}$), 7.82 (dd, ^3J=7.1Hz, $1H_{Ar}$), 8.34 (d, ^3J=7.2 Hz, $1H_{Ar}$). Mass spec: calculated 489.25 Found 489.38

2.5. Radiolabeling

Drying procedure for [^{18}F]fluoride—The aqueous [^{18}F]fluoride solution (100 μL to 1 mL, 75 MBq to 750 MBq ^{18}F) was added to a solution of Kryptofix®2.2.2. (5 mg, 13.3 μmol) and potassium carbonate (0.9 mg, 6.7 μmol) in 0.5 mL acetonitrile-water (9:1 v/v) and dried azeotropically by addition of 10 mL CH_3CN (3 × 3.3 mL) under a stream of nitrogen at 90°C. A final azeotropic drying was performed with 3 × 0.5 mL of extra dry CH_3CN.

Synthesis of [^{18}F]FETos—Ethyleneglycol-1,2-distosylate (2.5 mg in 0.5 mL extra dry acetonitrile) was added to the dried Kryptofix®2.2.2./K_2CO_3[^{18}F]fluoride mixture and heated at 100°C for 10 min. in a sealed Pierce “V-vial” to produce the [^{18}F]FETos in 80-90% yield. The product may be purified by diluting the reaction mixture with 4 mL diethyl ether and passing through a Sep-Pak® silica gel cartridge. Kryptofix®, K_2CO_3, and excess [^{18}F]fluoride are retained on the cartridge, and the product is eluted with 5 mL diethyl ether, which is then evaporated at 50°C under a steam of nitrogen. Excess ethyleneglycol-1,2- distosylate is not separated from [^{18}F]FETos using this procedure. The procedure requires approximately 25 min. and provides [^{18}F]FETos in >95% radiochemical purity with an overall radiochemical yield of 30 to 50%.

Preparation of [^{18}F]FERhB (1)—5 mg rhodamine B lactone dissolved in 0.8 mL anhydrous acetonitrile and 20 μL of DIPEA were added sequentially to the unpurified [^{18}F]FETos (above) in the original reaction vial, the vial was sealed, the septum in the cap was fitted with a ventilation needle (30 g), and reaction mixture was heated for 30 min. at 165°C. The final product, [^{18}F]FERhB, was obtained in an overall radiochemical yield of 35% after purification by solid-phase extraction (silica gel Sep-Pak® cartridge (Waters), $CHCl_3$:methanol, 4:1) or semi-preparative HPLC (t_R=13.2 min). The total reaction time using this method, including the drying of the [^{18}F]fluoride and preparative HPLC, is approximately 90 min. The identity of the product was confirmed by analytical HPLC and TLC using non-radioactive compound 2′-fluoroethyl rhodamine B as a reference. On analytical HPLC, the retention time of [^{18}F] FERhB is 9.6 min., and the R_f (TLC) is 0.5 (chloroform/methanol 8:1).

3. Results and Discussion

3.1. Synthesis of the non-radioactive compound

An authentic sample of 2′-fluoroethyl ester of rhodamine B (FERhB, **2**) was prepared and chemically characterized for use as a reference compound for the HPLC characterization of the ^{18}F-labeled compound. The synthetic route is outlined in Scheme 1. The nonradioactive fluoroethyl tosylate (FETos) was synthesized according to literature (Parenty *et al.*, 2005), and the non-radioactive 2′-fluorethyl ester of rhodamine B was prepared by fluoroethylation of rhodamine B lactone (**1**) with FETos in the presence of DIPEA. The conversion from the lactone to the 2′-fluoroethylester was monitored by HPLC and was determined to be 97% after

24 h under reflux. The product was purified by semi-preparative HPLC, isolated as a purple oil in 95% yield, and characterized by NMR and mass spectroscopy.

3.2. Synthesis of ^{18}F-labeled rhodamine B

The radiosynthesis of [^{18}F]FERhB was accomplished using a one-pot synthesis in which the aqueous [^{18}F]fluoride solution was evaporated in a Pierce "V" vial after addition of Kryptofix® and K_2CO_3, ethylene glycol ditosylate dissolved in acetonitrile was added to the dried residue, and the solution was heated for 10 min. to give [^{18}F]fluoroethyltosylate. This reaction mixture was cooled to room temperature, and a solution of rhodamine B lactone in acetonitrile and DIPEA were added to the unpurified precursor. This solution was then heated for 30 min. to give the ^{18}F-labeled rhodamine. The one-pot synthesis was developed after it was observed that a large amount (>50%) of the [^{18}F]FETos precursor was lost during evaporation of the diethyl ether solvent when solid-phase extraction was used to purify this material. While optimizing the reaction conditions, we observed that the yield of the reaction was a much higher if the solvent was allowed to slowly evaporate during the reaction, so a small gauge needle was inserted in the reaction vial in all subsequent studies. Possible reasons for this include the increased concentration of the precursors as the solvent evaporates and opening of the lactone ring on heating. These optimized conditions produced the final product in a decay corrected yield of 35%, 97% radiochemical purity, specific activity - 1.3 GBq/μmol, and a total synthesis time of approximately 90 min.

3.3. Small-animal PET imaging

The biodistribution of 2′-[^{18}F]fluorethylrhodamine B was examined in mice using microPET. An example of one of these studies is shown in Figure 2. The microPET study reveals that there is no appreciable uptake in the myocardium. Small, but noticeable, uptake of ^{18}F was observed in the ribs and long bones. This is presumably due to the presence of a small amount of free ^{18}F fluoride in the [^{18}F]FERhB preparation (<3% by TLC). There is significant uptake in the gall bladder, and subsequently, the intestine. This observation is consistent with the results obtained by Harapanhalli, et al., who also observed excretion into the small intestine with minimal accumulation in the heart (Harapanhalli *et al.*, 1998). We suspect that this may be due to hydrolysis of the ester *in vivo*. To evaluate this possibility, a sample of bile was collected from a mouse sacrificed 1 h post-injection and assayed using the TLC system described above the assay the HPLC purified product. This analysis revealed that less than 10% of the ^{18}F activity in the bile was present as [^{18}F]FERhB and less than 20% was free ^{18}F-fluoride. The remainder of the activity (>70%) was present as a new peak (R_f=0.3-0.4), presumably 2-[^{18}F]fluoroethanol and/or one or more of its metabolites. Additional studies are currently underway to more completely characterize this material. More importantly, we are evaluating alternative labeling strategies that may be less susceptible to *in vivo* degradation.

4. Conclusions

Using rhodamine B as the prototype compound, we have developed a synthetic method for radiolabeling rhodamine dyes with ^{18}F as a first step in evaluating these compounds as possible PET tracers for myocardial perfusion imaging. Fluorination was accomplished by synthesis of the 2′-fluoroethyl ester using rhodamine B lactone as the starting material and 2-tosylethylfluoride as the prosthetic group. Both the non-radioactive and ^{18}F-labeled compounds were prepared using the same general method. The ^{18}F-labeled compound was obtained in reasonably high radiochemical yield (35%) and high radiochemical purity (>97%). The specific activity is 1.3 GBq/μmol, and the total synthesis time is approximately 90 min. Preliminary microPET studies of the biodistribution of the ^{18}F-labeled compound in mice, however, showed that rather than accumulating in the heart, the compound was rapidly excreted through the gall bladder and into the intestines. TLC analysis of the bile revealed that activity

in the bile was not primarily either intact [^{18}F]FERhB or ^{18}F-fluoride suggesting *in vivo* hydrolysis of the ester to produce 2′-[^{18}F]fluoroethanol and its metabolites. Additional studies are currently underway to more completely characterize the route of decomposition and to determine if this may be the reason for this unexpected result thereby providing a basis for deciding if these compounds merit further examination as potential myocardial perfusion agents.

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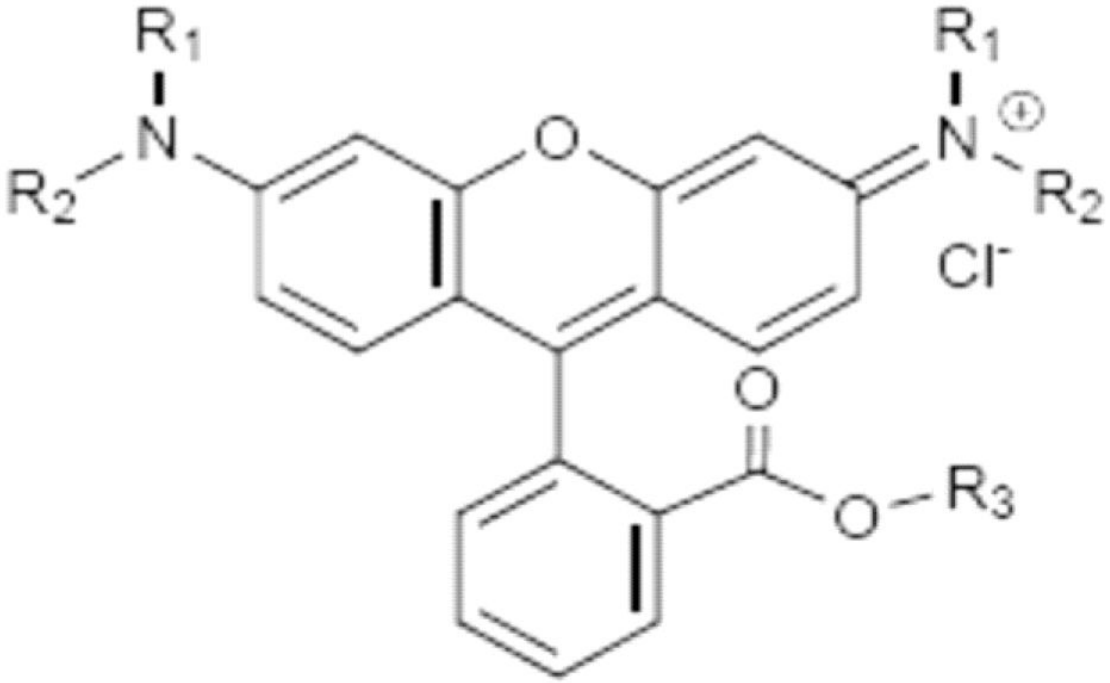


Name	R_1	R_2	R_3
Rhodamine B	Et	Et	H
Rhodamine 123	H	H	Me
Rhodamine 110	H	H	H

Fig. 1.
Examples of rhodamine dyes

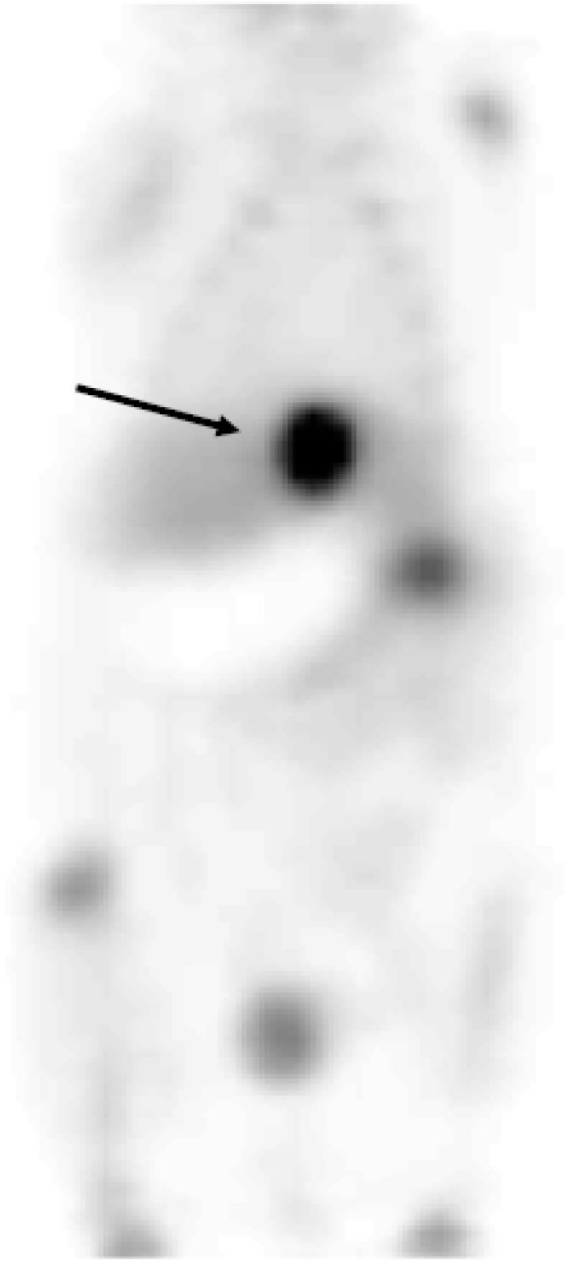
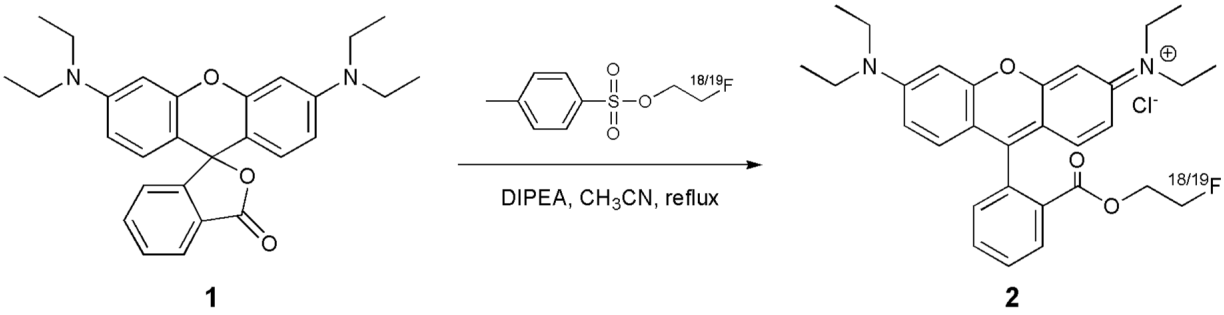


Fig. 2.
MicroPET image (1 h) of a mouse injected with [^{18}F]2′-fluoroethylrhodamine B. Note high concentration of radioactivity in the gall bladder (arrow).



1 2

Scheme 1.
Synthesis of 2′-fluoroethyl rhodamine B.