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Research Article

Fluorine-18 labelling of oligonucleotides: Prosthetic labelling at the 5′-end using the *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide reagent

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Summary

Labelled oligonucleotides are new imaging tools to study gene expression at the nucleic acid and protein levels. We have previously developed a universal method to label oligonucleotides at their 3′-end with radiohalogens and particularly with fluorine-18, the most widely used positron-emitter, $t_{1/2}$: 109.8 min. Using the same strategy, we herein report the fluorine-18 labelling of oligonucleotides at their 5′-end. A 18-mer 2′O-methyl modified oligoribonucleotide, bearing a phosphorothioate group at its 5′-end, was conjugated to our fluorine-18-labelled reagent *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide. The whole synthetic procedure yielded up to 1 GBq of fluorine-18-labelled oligonucleotide with a specific radioactivity of 37–74 GBq/μmol in 160 min.

Key Words: fluorine-18; oligonucleotides; positron emission tomography

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Introduction

For many years, intense efforts have been devoted to the development of biological macromolecules such as oligonucleotides, peptides, proteins or antibody fragments as radiopharmaceuticals for positron emission tomography (PET). Labelling of these biological macromolecules is usually performed by conjugation of a prosthetic group, carrying the radioisotope, with a reactive function of the macromolecule. This strategy has the advantage of offering the possibility to use different chemical routes, including drastic chemical conditions for the preparation of the prosthetic group entity, followed by the conjugation of the latter with a macromolecule using mild conditions needed to preserve its integrity.

We have previously developed a universal method to label oligonucleotides with radioactive isotopes of halogens, such as fluorine-18 (the most widely used positron-emitter, $t_{1/2}$: 109.8 min), bromine-76 (another positron-emitter with a relatively long half life, $t_{1/2}$: 16.1 h) and iodine-125 (a low energy Auger-electron emitter used for high resolution autoradiography, $t_{1/2}$: 59.9 days). *N*-(4-halobenzyl)-2-bromoacetamide was designed as a general and radiochemically feasible reagent, the benzyl function offering the opportunity to act as the carrier of the radioactive halogens.[1,2] The method is based on the efficient and regioselective conjugation of this *N*-substituted-2-bromoacetamide with the oligonucleotide bearing a phosphorothioate monoester group at the 3′-end. This strategy has already been reliably and routinely applied to all the popular chemical modifications of oligonucleotides.[1–4] Besides natural phosphodiester DNA oligodeoxyribonucleotides, full length phosphorothioate diester internucleosidic-bond deoxyribonucleotides (the modification most favoured by industry for human antisense therapy[5]) were successfully labelled as well as hybrid methylphosphonate/phosphodiester internucleosidic-bond deoxyribonucleotides (a mixed-backbone oligonucleotide with interesting potential imaging properties[6,7]) or 2′O-methyl-modified oligoribonucleotides (confering resistance to nucleases and high efficiency of duplex formation with the complementary RNA[8,9]).

In the present study, we have investigated the labelling at the 5′-end of an *in vivo* stable 2′O-methyl-modified oligoribonucleotide (2′OMe-RNA) (bearing a phosphorothioate monoester group at its 5′-end) with our fluorine-18-labelled reagent *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide.

Results and discussion

Chemistry

2′O-methyl modified-oligoribonucleotides bearing a phosphorothioate group at their 5′-end (Figure 1) are commercially available.

N-(4-Fluorobenzyl)-2-bromoacetamide **(2)** was prepared in 60–65% non-optimized yields from commercially available 4-fluorobenzylamine and bromoacetyl bromide in the presence of *N*-methylmorpholine.[1] The conjugation of the *N*-(4-fluorobenzyl)-2-bromoacetamide **(2)** to the oligonucleotide **(1)**, bearing a phosphorothioate function at the 5′-end, was carried out in a mixture of methanol and phosphate buffer saline (PBS, 0.1 M aqueous, pH 8), at room temperature for 2 h (Scheme 1).

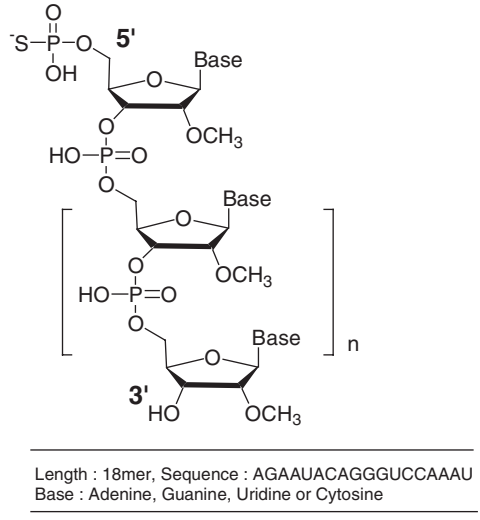
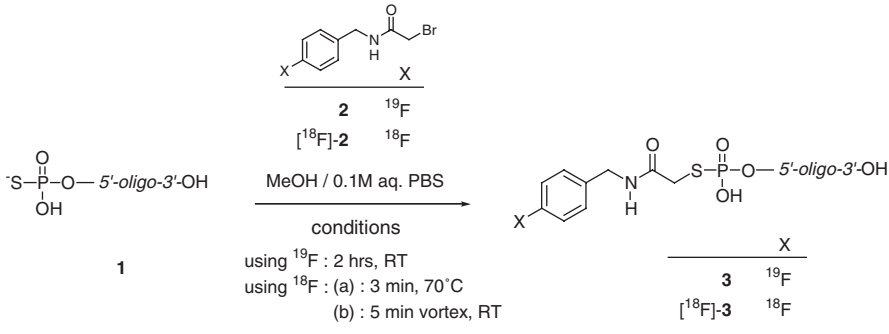
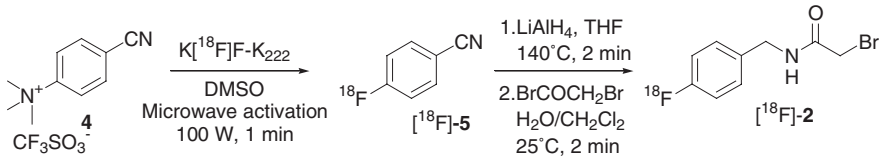


Figure 1. Chemical structure of a 5′-end phosphorothioate monoester 2′O-methyl modified-oligoribonucleotide



Scheme 1. Synthesis of conjugated oligonucleotides 3 and [^{18}F]-3



Scheme 2. Synthesis of *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-2)

These conditions gave the desired conjugated oligonucleotide (**3**) in about 90% yield (determined by HPLC). The conjugated oligonucleotide (**3**) was purified by semi-preparative HPLC and desalted, using a Sephadex® cartridge for characterization. The regioselectivity of the coupling was verified by ^{31}P-NMR: we observed that the chemical shift of the 5′-end phosphorus atom was moved from +41.20 ppm in the 5′-phosphorothioate monoester group to +15.57 ppm in the 5′-end phosphorothioate diester group after alkylation. These results are in agreement with published ^{31}P-NMR tables[10,11] and previously reported data.[1–3] Mass spectrometry analysis also confirmed the mono-conjugation of the *N*-(4-fluorobenzyl)-2-bromoacetamide on the oligonucleotide: calculated mass: 6299.0, measured mass: 6300.3.

Radiochemistry

N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide [^{18}F]-(**2**) (Scheme 2), was synthesized in three steps from 4-cyano-*N*,*N*,*N*-trimethylanilinium trifluoromethanesulfonate (**4**, prepared from commercial 4-dimethylaminobenzonitrile).[1] The first radiochemical step, the introduction of fluorine-18 into the benzonitrile ring, was performed in hot DMSO, using the K[^{18}F]F-K_{222} complex by microwave activation at 100 W for 1 min, giving the desired 4-[^{18}F]fluorobenzonitrile ([^{18}F]-**5**). The second step, the reduction of the cyano function, was performed with $LiAlH_4$ in refluxing THF (140°C) for 2 min, giving the desired labelled 4-[^{18}F]fluorobenzylamine. The final step, the condensation with bromoacetyl bromide, occurred cleanly in 2 min at room temperature in a 10/1 (v/v) mixture of CH_2Cl_2/H_2O. Semi-preparative HPLC gave pure *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide [^{18}F]-(**2**).

Typically, starting from a batch of 22–24 GBq of [^{18}F]fluoride, we routinely produced 2.2–2.4 GBq of HPLC-purified *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**) in about 90 min.

The conjugation of the *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**) with the oligonucleotide **1** was performed in a mixture of

MeOH and PBS (0.1 M aqueous, pH 8) (Scheme 1). The reaction mixture was first gently heated at 70°C for 3 min and then vortexed for another 5 min at room temperature. The fluorine-18-labelled conjugated oligonucleotide [^{18}F]-**3** was purified by semi-preparative RP-HPLC and desalted using a Sephadex® cartridge. The decay-corrected radiochemical yield of the conjugation was about 60%.

As demonstrated by HPLC analysis, radiosynthesized [^{18}F]-**3** co-eluted with the authentic synthesized reference compound **3**. The fluorine-18-labelled conjugated oligonucleotide [^{18}F]-**3** was found to be >95% chemically and radiochemically pure. The preparation was shown to be free of non-radioactive precursor and radiochemically stable for at least 120 min.

Conclusion

Using the general methodology that we have developed for the labelling of oligonucleotides, we have achieved the fluorine-18-labelling of a 18-mer 2′O-methyl-modified oligoribonucleotide at its 5′-end. The whole synthetic procedure, including the preparation of the fluorine-18-labelled reagent *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide, the coupling to the macromolecule and the HPLC-purification, allows us to produce up to 1 GBq of fluorine-18-labelled oligonucleotide with a specific radioactivity of 37–74 GBq/μmol in 160 min.

Experimental

General

Chemicals. Chemicals were purchased from Aldrich, Sigma and Fluka and were used without further purification. The 18mer-oligonucleotide of sequence $^{5'}p_s$-AGAAUACAGGGUCCAAAU (2′OMe-RNA) was purchased from Eurogentech (Belgium).

Analytical methods. Thin Layer Chromatography (TLC) was run on precoated plates of silica gel $60F_{254}$ (Merk). The compounds were localized at 254 nm using a UV lamp. Flash chromatography was conducted on silica gel 63–200 μm (Merck) at 0.3 bar (compressed air). HPLC systems: HPLC **A**: semi-preparative RP-HPLC: column

C18 μBondapak® Waters (300 × 7.8 mm, porosity 10 μm), 600 Controller Gradient system Waters, UV detector multiwavelength 490E Waters, Geiger-Müller detector; solvents: triethylammonium acetate, 100 mM, pH 7 (TEAA) and acetonitrile. HPLC **B**: semi-preparative normal phase HPLC; column Prep Nova-Pak® HR Silica Waters (7.8 × 300 mm, 60 Å, 6 μm), UV detector 440 Waters, Geiger-Müller detector; solvents: CH_2Cl_2/EtOAc (95/5). HPLC **C**: analytical RP-HPLC, column C18 Symmetry® Waters (50 × 4.6 mm, 5 μm), a 600 Pump and 600 Controller Waters, a UV detector Series 1100 (254 nm) Hewlett Packard and a Flow One Scintillation Analyzer Packard equipped with a positron-dedicated cell for radioactivity monitoring; solvents: triethylammonium acetate, 100 mM, pH 7 (TEAA) and acetonitrile. NMR spectra were recorded on a Bruker AMX (300 MHz) spectrometer using the hydrogenated residue of the deuterated solvents (DMSO-d_6, $\delta = 2.50$ ppm; CD_2Cl_2, $\delta = 5.32$ ppm) and/or TMS as internal standards for ^{1}H-NMR as well as the deuterated solvents (DMSO-d_6, $\delta = 39.5$ ppm; CD_2Cl_2, $\delta = 53.8$ ppm) and/or TMS as internal standards for ^{13}C-NMR and TMP as internal standard for ^{31}P-NMR. The chemical shifts are reported in ppm, downfield from TMS (^{1}H and ^{13}C) or TMP (^{31}P) (s, d, t, q, dd, m, b for singlet, doublet, triplet, quadruplet, doublet of doublet, multiplet (or multi sharp-peak system) and broad respectively). The mass spectra were measured on a Nermag R10-10 apparatus and a Quattro VG (Fison, Manchester, UK) for electrospray ionization (negative mode).

Miscellaneous: Radiosyntheses using fluorine-18 were performed in a 7.5-cm-lead shielded cell using a computer-assisted Zymate robot system (Zymark corporation, USA). Microwave activation was performed with a MicroWell 10 oven (2.45 GHz), Labwell AB, Sweden. Specific radioactivity was determined as follows: the area of the absorbance peak corresponding to the radiolabelled product was measured on the HPLC chromatogram and compared to a standard curve relating mass to absorbance.

Radioisotope availability: No-carrier-added aqueous [^{18}F]fluoride ion was produced on a CGR-MeV 520 cyclotron by irradiation of a 2 ml water target using a 17 MeV proton beam on 95% enriched [^{18}O]water by the [^{18}O(p,n)^{18}F] nuclear reaction and was transferred to the appropriate hot cell. Typical production: 550–650 mCi (20.3–24.0 GBq) of [^{18}F]F^- at the end of bombardment for a 20 μA, 30 min (36,000 μC) irradiation. A complete description of the target hardware and operation can be found in Dollé *et al.*[12]

Chemistry

*N-(4-fluorobenzyl)-2-bromoacetamide (**2**).* Synthesized from commercially available 4-fluorobenzylamine according to references 1 and 3. R_f (heptane/EtOAc: 50/50): 0.35. ^{1}H-NMR (DMSO-d_6, 300.0 K): δ: 8.80 (bt, 1 H); 7.32 (dd, *J*: 8.1 Hz and 5.70 Hz, 2 H); 7.15 (t, *J*: 8.1 Hz, 2 H); 4.31 (d, *J*: 6 Hz, 2 H); 3.92 (s, 2H). ^{13}C-NMR (DMSO-d_6, 300.0 K): δ: 166.1 [C]; 161.3 [C, d, *J*: 249 Hz]; 135.0 [C, d, *J*: 2 Hz]; 129.2 [CH, d, *J*: 8 Hz]; 115.1 [CH, d, *J*: 23 Hz]; 41.9 [CH_2]; 29.4 [CH_2]. MS: 265 [$M + NH_4^+$]; 263 [$M + NH_4^+$]; 248 [$M + H^+$]; 246 [$M + H^+$].

*4-Cyano-N, N, N-trimethylanilinium trifluoromethanesulfonate (**4**).* Synthesized from commercially available 4-dimethylaminobenzonitrile according to Dollé *et al.*[1] R_f (MeOH/AcOH: 50/50): 0.4. ^{1}H-NMR (DMSO-d_6, 338.0 K): δ : 8.30–8.00 (2b, 4H); 3.70 (s, 9H). ^{13}C-NMR (DMSO-d_6, 338.0 K): δ : 150.0 [C]; 133.9 [CH]; 121.8 [CH]; 120.6 [CF_3, q, *J*: 325 Hz]; 116.9 [C]; 113.2 [C]; 56.4 [CH_3].

*N-(4-fluorobenzyl)-2-($^{5'}p_s$-AGAAUACAGGGUCCAAAU)-acetamide (**3**).* 100 OD (1 OD is the UV absorbance at 260 nm of 38 µg of an oligonucleotide regardless of the sequence of bases) of 2′OMe-RNA **1** were reacted with an excess (3 equivalent) of *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) in 1.0 ml of a 1/1 mixture of methanol and PBS (0.1 M pH 8) (v/v) for 2 h at room temperature. The solvents were evaporated under reduced pressure and the conjugated 2′OMe-RNA (**3**) was purified by RP-HPLC. HPLC A: gradient elution: linear in 5 min from 95/5 to 90/10 (TEAA/acetonitrile, see *analytical methods*), then linear in 10 min from 90/10 to 75/25, then wash-out for 10 min at 50/50; flow rate: 6 ml/min; retention time: 13.5–14.0 min (starting 2′OMe-RNA (**1**): 12.0–12.5 min). The HPLC-fraction containing **3** was concentrated under reduced pressure and transferred in a volume of 1 ml onto a NAP-10® G25 Sephadex Column (Amersham Pharmacia Biotech). Pure labelled and desalted **3** was eluted with 1.5 ml of water according to the manufacturer's instructions. ^{31}P-NMR (D_2O, 298.0 K): δ : +15.57 (-OP(O)(OH)(S-acetamide)); −4.62 (-OP(O)(OH)O-). MS (electrospray): 6300.3 (theor.), 6299.0 (exp.).

Radiochemistry

Preparation of the K[^{18}F]F-K_{222}-complex. In order to recover and recycle the [^{18}O]water target, the 2 ml of aqueous [^{18}F]fluoride from the

target holder were passed through an anion exchange resin (Sep-Pak® Light Waters Accell™ Plus QMA Cartridge in the chloride form, washed with 5 ml 1 M aqueous $NaHCO_3$ and then rinsed with 50 ml of water) by Helium pressure (1.5–2.0 bar). Helium is blown through the column to maximally extract the [^{18}O]water. See Dollé *et al.*[12,13] for more practical details. The [^{18}F]fluoride ion were then eluted from the resin, using 1.0 ml of a 4.5 mg/ml aqueous K_2CO_3 solution, into a Vacutainer® tube containing 12.0 to 15.0 mg of Kryptofix®222 (K_{222}: 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane). The resulting solution was then gently concentrated to dryness at 145–150°C under a nitrogen stream for 10 min to give no-carrier-added K[^{18}F]F-K_{222} complex as a white semi-solid residue.[14]

*N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**).* Freshly distilled DMSO (600 µl) containing 8 mg of the triflate salt of 4-trimethylammoniumbenzonitrile (**4,** labelling precursor), was directly added into the Vacutainer® tube containing the dried K[^{18}F]F-K_{222} complex. The tube (not sealed) was placed in a microwave oven. Microwaves, 100 W, were applied to the system for 1 min. The resulting yellow-brown reaction mixture was diluted with 1 ml of water and transferred onto a C18 Sep-Pak cartridge (PrepSep™ R-C18, Fisher Scientific). The tube was rinsed twice with 1 ml of water which was also transferred and added to the diluted reaction mixture on the cartridge. The whole was then passed through the cartridge. The cartridge was washed with 1 ml of water and partially dried for 3 min by applying a nitrogen stream. 4-[^{18}F]Fluorobenzonitrile ([^{18}F]-**5**) was eluted with 3 ml of THF onto a column containing 1.0 g of oven-dried ground 4 Å molecular sieve and the cartridge was rinsed twice with 1 ml of THF. The combined THF eluate and rinsings solution (5 ml) was left on the molecular sieve column for 3 min and then eluted into a reaction vial containing 20 mg of dry powdered $LiAlH_4$. Another 3 times 1 ml of THF was used to rinse the column and to completely transfer the 4-[^{18}F]fluorobenzonitrile ([^{18}F]-**5**). The vessel was then tightly closed and heated for 2 min at 140°C (strong reflux). The resulting grey suspension was cooled for 2 min using an ice/water bath and concentrated to dryness under a nitrogen stream. The amine–aluminium complex as well as the excess of $LiAlH_4$ were destroyed by adding 300 µl of H_2O. The white aqueous mixture was then diluted with 3 ml of CH_2Cl_2. After addition of 1 ml of a solution of bromoacetyl bromide in CH_2Cl_2 (10 µl/ml or 114 µmoles of $BrCOCH_2Br$), the white milky suspension was allowed to react for

1 min at room temperature with a smooth air-bubbling agitation. The reaction mixture was then filtered on cotton and the precipitate rinsed twice with 1 ml of CH_2Cl_2. The combined filtrates were concentrated to dryness at 80°C under a nitrogen stream, the residue dissolved in 1 ml of the HPLC solvent used for purification and the crude was injected onto a SiO_2 semi-preparative HPLC. HPLC B: isocratic elution: flow rate: 5 ml/min, R_t: 10.0–10.5 min.

Typically, 2.2–3.3 GBq (60–90 mCi) of pure *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**) could be obtained in 85–95 min starting from a 20.3–24.0 GBq (550–650 mCi) aliquot of a [^{18}F]F^- cyclotron production batch.

*N-(4-[^{18}F]fluorobenzyl)-2-($^{5'}p_s$-AGAAUACAGGGUCCAAAU)-acetamide ([^{18}F]-**3**)*. The HPLC-collected fraction containing *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**) was concentrated to dryness at 80°C under a nitrogen stream and then taken up with 0.4 ml of MeOH. A 55 OD aliquot of the 18mer 2′OMe-RNA (**1**) in 0.3 ml of water and 0.2 ml of phosphate buffer saline (0.1 M aqueous, pH 8) were successively added. The reaction vial was then tightly sealed with a Teflon cap and heated in a heating block without stirring at 70°C for 3 min and then vortexed at room temperature for another 5 min. After cooling for 2 min using an ice/water bath, the reaction mixture was concentrated at 80°C under a nitrogen stream. The [^{18}F]-conjugated 2′OMe-RNA ([^{18}F]-**3**) was separated from unlabelled 2′OMe-RNA (**1**) and unreacted *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**) by RP-HPLC using the same conditions as for the purification of the cold reference conjugated 2′OMe-RNA (HPLC A, see analytical methods). The retention time of the fluorine-18-labelled conjugated 2′OMe-RNA ([^{18}F]-**3**) was 13.5–14.0 min.

The whole synthetic procedure allows one to produce up to 1 GBq of fluorine-18-labelled oligonucleotide in 160 min with a specific radioactivity of 37–74 GBq/μmol at the end of synthesis.

Formulation and quality control. The HPLC-fraction containing [^{18}F]-**3** was concentrated under reduced pressure and transferred in a volume of 1 ml onto a NAP-10® G25 Sephadex Column (Amersham Pharmacia Biotech). Pure labelled and desalted [^{18}F]-**3** was eluted with 1.5 ml of water or buffer according to the manufacturer's instructions.

As demonstrated by HPLC analysis (HPLC C, see Analytical methods), radiosynthesized [^{18}F]-**3** co-eluted with the authentic synthe-

sized reference compound **3**. The radiolabelled product was found to be >95% chemically and radiochemically pure. The preparation was shown to be free of non-radioactive precursor and radiochemically stable for at least 120 min. HPLC C: gradient elution: linear in 5 min from 95/5 to 90/10 (TEAA/acetonitrile, see *analytical methods*), then linear in 10 min from 90/10 to 75/25, then wash-out for 10 min at 50/50; flow rate: 1.5 ml/min; retention time 10.0–10.5 min.

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