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Fluorine-18 Labeling of Oligonucleotides Bearing Chemically - Modified Ribose - Phosphate Backbones

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Summary

We have recently described the labeling of a natural deoxyribose phosphodiester oligonucleotide with fluorine-18 ($t_{1/2}$: 109.8 min) and demonstrated its potential for *in vivo* imaging in a primate PET study. We here report that the methodology employed can be reliably and routinely applied to the most popular chemical modifications : (a) full length internucleosidic phosphorothioate diester bonds deoxyribose oligonucleotides (the modification most favoured by industry for human antisense therapy), (b) hybrid methylphosphonate/phosphodiester internucleosidic bonds deoxyribose oligonucleotides and (c) 2'O-Methyl modified ribose oligonucleotides. The whole fluorine-18 labeling procedure allows us to obtain 15 to 21 mCi (0.55 to 0.74 GBq) of pure labeled oligonucleotides (regardless the modification of the sugar phosphate backbone) in 180 minutes with a specific radioactivity of 0.8 to 2 Ci/μmol (30 to 70 GBq/μmol) at the end of synthesis.

Key Words : Fluorine-18, labeled oligonucleotide, positron emission tomography

Introduction

In vivo applications of oligonucleotides such as antisense therapy (1-3), anti-gene therapy (4,5) and aptamer diagnosis (6), require the use of chemically modified RNA or DNA because of the poor bioavailability of natural oligonucleotides (7,8). Of the hundreds of chemical modifications developed (9,10) few have been evaluated at the level of a whole intact living organism. Positron Emission Tomography (PET), a high-resolution, sensitive and non invasive imaging technique that can be used in humans, is the most advanced technology currently available for studying *in vivo* molecular interactions and represents a method of choice to assess the pharmacokinetics of new therapeutic agents such as modified oligonucleotides. Among the several methods to label oligonucleotides with γ or β^+ emitters for *in vivo* imaging (11-16), we described recently the labeling of a phosphodiester oligonucleotide with fluorine-18 ($t_{1/2}$: 109.8 min) and demonstrated its potential for *in vivo* imaging in a primate PET study (15,17).

We report here that this method can be reliably and routinely applied to the most popular chemical modifications : besides phosphodiester DNA, the natural deoxyribose oligonucleotide, a) a full length internucleosidic phosphorothioate diester bonds deoxyribose oligonucleotide, the modification most favoured by industry for human antisense therapy (2), b) a hybrid methylphosphonate/phosphodiester internucleosidic bonds deoxyribose oligonucleotide, a mixed backbone oligonucleotide with interesting imaging properties (18,19) and c) a 2'O-Methyl modified ribose oligonucleotide, confering resistance to nucleases and high efficiency of duplex formation with the complementary RNA (20,21).

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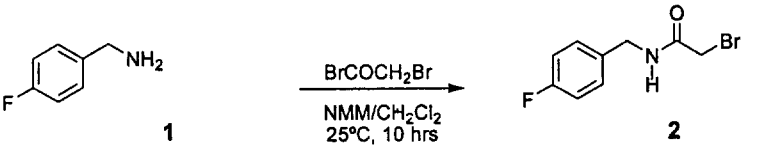
Results and discussion

Chemistry

The synthetic procedure of conjugation of oligonucleotides uses preparative scale commercially available 3'-end phosphorothioate oligonucleotides. It is based on the efficient coupling reaction of oligonucleotides containing a single phosphorothioate monoester with an electrophilic moiety such as 2-bromo- or 2-iodo *N*-substituted acetamides (22,23). *N*-benzyl-acetamides are, from a synthetic point of view, suitable to act as the carrier of the positron emitter fluorine-18.

Preparation of N-(4-fluorobenzyl)-2-bromoacetamide (2)

4-fluorobenzylamine (**1**) was reacted with 2-bromoacetylbromide in presence of *N*-methyl-morpholine to give *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) in yields of 60% to 65% (scheme 1).



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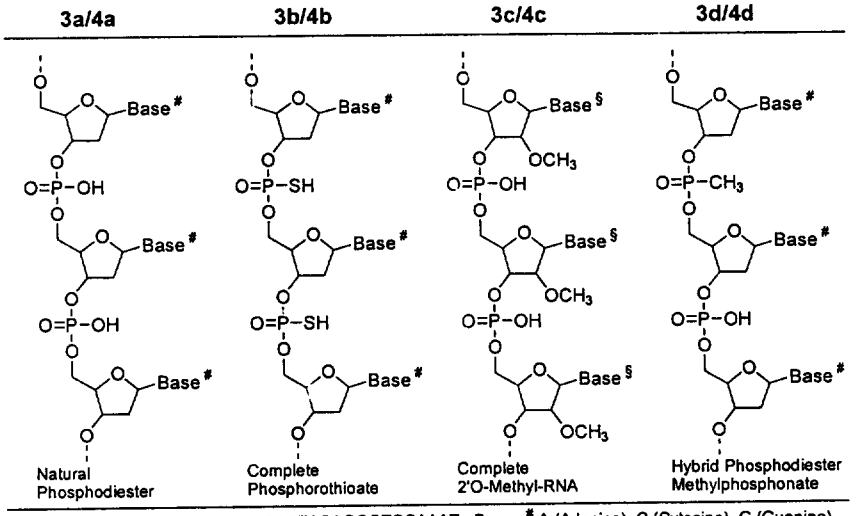
Scheme 1 : Synthesis of *N*-(4-fluorobenzyl)-2-bromoacetamide

Conjugation of oligonucleotides (3a-d) with N-(4-fluorobenzyl)-2-bromoacetamide (2)

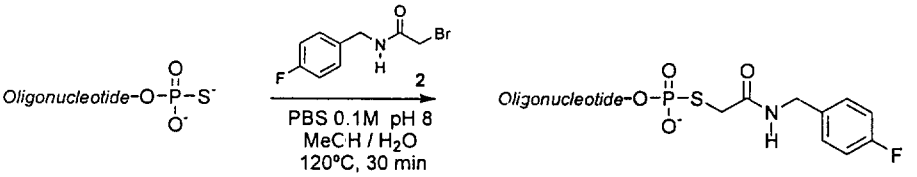
Various modified 18mer ribose- or deoxyribose-phosphate oligonucleotide backbones bearing one unique base sequence (scheme 2) were conjugated with *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**).



Length : 18 mer ; Sequence : AGAATACAGGGTCCAAAT ; Base : # A (Adenine), C (Cytosine), G (Guanine), T (Thymine), § T (Thymine) is replaced by U (Uracil).

Scheme 2 : Natural phosphodiester DNA and modified oligonucleotides

The 3'-substituted unlabeled oligonucleotides were prepared as described in scheme 3. The conjugation reaction between oligonucleotides **3a-d** and *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) was performed in a mixture of MeOH and phosphate buffer saline (PBS) in 30 minutes at 120°C to yield the 3'-end modified oligonucleotides **4a-d**. The modifications of the sugar phosphate backbone did not influence the yield of reaction (analytical yields : 70% to 75%, determined by HPLC). After RP-HPLC purification, the 3'-substituted oligonucleotides were produced as predominant compounds with higher retention times than the starting oligonucleotides due to the increased lipophilicity after addition of the halogenobenzyl substituent.



	Sequence and backbone chemistry		*Yields*
3a	AGAATACAGGGTCCAAAT	**4a**	75%
b	AsGsAsAsTsAsCsAsGsGsGsTsCsCsAsAsAsT	**b**	72%
c	[2'OMe]AGAAUACAGGGUCCAAAU	**c**	73%
d	AmpGmpAmpATACAGGGTCCAmpAmpAmpT	**d**	70%

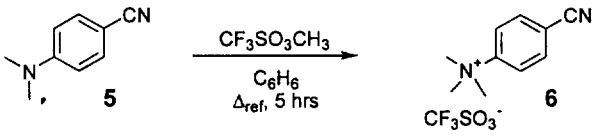
Scheme 3 : Conjugation of oligonucleotides with *N*-(4-fluorobenzyl)-2-bromoacetamide

The regioselectivity of coupling was verified by ^{31}P NMR : as expected, the chemical shift of the 3'-end phosphorous atom was shifted from +42.00 ppm in the phosphorothioate monoester group to +15.20 ppm in the 3'-end phosphorothioate diester group. Other internucleosidic phosphodiester groups were between –3.50 ppm and –4.00 ppm, internucleosidic phosphorothioate diester groups around +50.00 ppm and the internucleosidic methylphosphonate diester groups between +28.00 ppm and +33.00 ppm. No reaction occured on the internucleosidic phosphorothioate diester groups in these conditions since no characteristic peak of a phosphorothioate triester group (+65.00 ppm to +72.00 ppm) appeared in the spectra. These values are in agreement with published ^{31}P NMR literature (24-26). Mass spectrometry analysis confirmed the conjugation of only one *N*-(4-fluorobenzyl)-2-bromoacetamide on the oligonucleotides.

Synthesis of the precursor 4-cyano-N,N,N-trimethylanilinium trifluoromethanesulfonate (6) for fluorine-18 labeling

4-Cyano-*N,N,N*-trimethylanilinium trifluoromethanesulfonate (**6**) was prepared from commercial 4-dimethylaminobenzonitrile (**5**) in a yield of 82% (scheme 4). The first radiochemical step in the fluorine-18 radiosynthesis (scheme 5) corresponded to the introduction of the fluorine into the benzonitrile (**6**), using a nucleophilic aromatic substitution on the trimethylammonium group which is activated by the para cyano function.

Trimethylammonium was chosen as leaving group for this substitution, not only for its high potential as leaving group in comparison with a corresponding halo- or better nitro substituent, but also for the expected superior precursor separation from the reaction product (4-[^{18}F]fluorobenzonitrile (7)) in HPLC due to the differences in the physico-chemical properties.

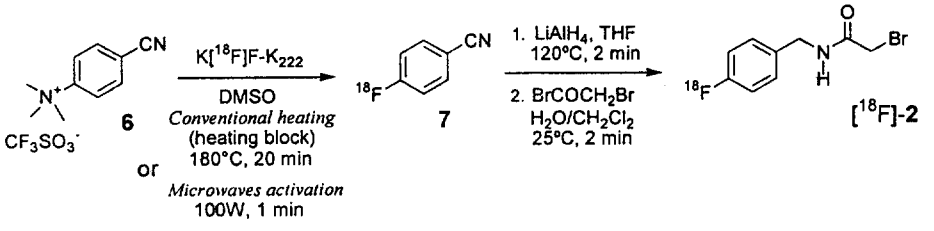


Scheme 4 : Synthesis of 4-cyano-*N,N,N*-trimethylanilinium trifluoromethanesulfonate

Radiochemistry

Fluorination and radiosynthesis of N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide [^{18}F]-(2)

The first radiochemical step was the introduction of fluorine-18 into the benzonitrile ring. This reaction was performed in hot DMSO, using the [^{18}F]KF-K_{222} complex, either by conventional heating at 180°C for 20 minutes or by microwaves activation at 100 Watt for 1 minute. The desired [^{18}F]fluorobenzonitrile (7) was provided in yields of 80-85% with conventional heating and up to 95% with microwaves activation. The use of microwaves allowed to reduce the synthesis time, a critical point with short-lived radioisotopes and to improve the yield of incorporation of fluorine-18.



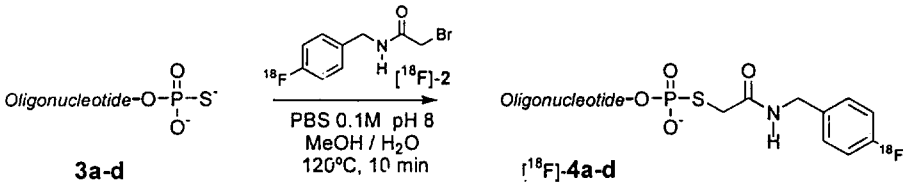
Scheme 5 : Synthesis of *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide

The second step was the reduction of the cyano function. Best results were obtained when the reduction was performed with $LiAlH_4$ in refluxing THF (120-130°C) for 2 minutes. The final step was the condensation with bromoacetyl bromide. The reaction occurred cleanly in 2 minutes 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). (Co-elution with authentic synthesized unlabeled reference compound). [^{18}F]-(2) was produced in 20-30% decay-corrected yield based on the starting [^{18}F]fluoride (scheme 5).

Preparation of [^{18}F]-labeled oligonucleotides [^{18}F]-4a-d

The reaction of conjugation of oligonucleotides (**3a-d**) with *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide [^{18}F]-(**2**) is described in scheme 6. The reaction occured in 10 minutes in mild conditions to prevent the decomposition of the oligonucleotides.

The labeled oligonucleotides [^{18}F]-**4a-d** were provided in a yield of 40% regardless the modification of the sugar phosphate backbone.



sequence and backbone chemistry : (a) : AGAATACAGGGTCCAAAT ; (b) : AsGsAsAsTsAsCsAsGsGsGsTsCsCsAsAsAsT ; (c) : [2'OMe]AGAAUACAGGGUCCAAAU ; (d) : AmpGmpAmpATACAGGGTCCAmpAmpAmpT

Scheme 6 : Fluorine-18 labeling of oligonucleotides

The whole fluorine-18 labeling procedure allows us to obtain 15 to 21 mCi (0.55 to 0.74 GBq) of pure labeled oligonucleotides in 180 minutes with a specific radioactivity of 0.8 to 2 Ci/μmol (30 to 70 GBq/μmol) at the end of synthesis.

The procedure for labeling oligonucleotides with fluorine-18 is largely automated and the production of [^{18}F]-labeled oligonucleotides ([^{18}F]-**4a-d**) is nearly performed in routine. This method allows to prepare a radioactive probe, in good yields with a sufficient specific radioactivity, that can be used in PET experiments. As a control, an aliquot of each radioactive probe was co-injected with unlabeled reference onto analytical RP-HPLC. Radioactivity- and UV detection demonstrated that [^{18}F]-labeled oligonucleotides ([^{18}F]-**4a-d**) eluted at the same retention time as authentic synthetized unlabeled reference compounds (**4a-c**). This control was also performed several hours after end of synthesis showing that the radioactive probe was not degraded in saline and that no dehalogenation of the aromatic ring occured in contrast to the labeling method with halogeno-alkyl compounds where the solvolysis of the C-F bond was noticed (16).

Experimental

General

Chemicals *:* Chemicals were purchased from Aldrich, Sigma and ICN Biochemicals and were used without further purification. A 18mer phosphodiester oligonucleotide (AGAATACAGGGTC-CAAAT$^{3'}$-p$_s$) was purchased from Eurogentec (Seraing, Belgium) and the chemically modified ribose-phosphate backbone oligonucleotides too : phosphorothioate, 2'O-Methyl and hybrid-phosphodiester-methylphosphonate oligonucleotides. This sequence is complementary to nucleotides 1209-1227 of the *env* sequence of the Lilly and Steeves strain of murine SFFV Freind erythroleukemia virus.

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** : sem. preparative RP-HPLC : column C18 μBondapak® Waters (300 x 7.8 mm, porosity 10 μm), 600 Controller Gradient system Waters, UV detector (254 nm) multiwavelength 490E Waters and a Geiger Müller Detector; solvents : triethylammonium acetate (TEAA) 100 mM, pH 7, and acetonitrile ; HPLC **B** : semi preparative normal phase HPLC : column Prep Nova-Pak® HR Silica Waters (7.8 x 300 mm, 60 Å, 6 μm), UV detector Waters, a Geiger Müller detector; solvents : CH_2Cl_2/EtOAc (95/5) ; HPLC **C** : analytical RP-HPLC : column C18 μBondapak® Waters (300 x 3.9 mm, porosity 10 μm), a 600 Pump and 600 Controller Waters, a UV detector Series 1100 (254 nm) Hewlett Packard and a Flow One Scintillation Analyzer Packard equiped with a Positron-dedicated cell for radioactivity monitoring; solvents : triethylammonium acetate (TEAA) 100 mM, pH 7, and acetonitrile. NMR spectra were recorded on a Bruker AMX (300 MHz) apparatus using the hydrogenated residue of the deuterated solvents (DMSO-d_6, δ = 2.50 ppm ; CD_2Cl_2, δ = 5.32 ppm) and/or TMS as internal standards for ^1H NMR as well as the deuterated solvents (DMSO-d_6, δ = 39.5 ppm ; CD_2Cl_2, δ = 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 were reported in ppm, downfield from TMS (^1H 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 5 cm-lead shielded cell using a computer assisted Zymate robot system (Zymark corporation, USA). Microwaves activation were performed with a MicroWell 10 oven (2.45 GHz), Labwell AB, Sweden.

Isotope 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 [$^{18}O(p,n)^{18}F$] and was transferred to the appropriate hot cell. Typical production : 700-800 mCi (25.9-29.6 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 reference 27.

Chemistry

***N*-(4-fluorobenzyl)-2-bromoacetamide (2)**. To a solution containing 13.0 mL of 4-fluorobenzylamine (**1**) (114 mmol) in 100 mL CH_2Cl_2 and 10.2 mL of *N*-methylmorpholine (1 eq), were dropwise added 10.0 mL of bromoacetyl bromide (1 eq) while maintaining the temperature under 30°C. After addition, the reaction mixture was stirred at room temperature overnight. The mixture was then filtered, concentrated to dryness and the residue was chromatographed on silica

gel. Elution with heptane/EtOAc (90/10 to 60/40) provided 16.8 g of pure *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) as a white solid (60% yield). Rf (heptane/EtOAc : 50/50) : 0.35. ^{1}H NMR (DMSO-d_6, 300.0K) : δ : 8.80 (bt, 1H) ; 7.32 (dd, J : 8.1 Hz and 5.70 Hz, 2H) ; 7.15 (t, J : 8.1 Hz, 2H) ; 4.31 (d, J : 6 Hz, 2H) ; 3.92 (s, 2H). ^{13}C NMR (DMSO-d_6, 300.0K) : 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^+].

General procedure for conjugating N-(4-fluorobenzyl)-2-bromoacetamide (2) to 3'phosphorothioate monoester modified oligonucleotides (3a-d).

100 OD (1 OD is the UV absorbance of 33 µg of an oligonucleotide regardless of the sequence of bases) of 18mer phosphodiester-, phosphorothioatediester-, 2'O-Methyl- or hybrid methylphosphonatediester/phosphodiester 3'phosphorothioate monoester oligonucleotides (**3a-d**) in water were reacted with an excess (3 eq) 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 30 min at 120°C. The solvents were evaporated and the conjugated oligonucleotides (**4a-d**) were purified by RP-HPLC.

***N*-(4-fluorobenzyl)-2-(AGAATACAGGGTCCAAAT$^{3'}$-p$_s$)-acetamide (4a).** The general procedure described above was used with AGAATACAGGGTCCAAAT$^{3'}$-p$_s$ (**3a**) and *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) to provide *N*-(4-fluorobenzyl)-2-(AGAATACAGGGTCCAAAT$^{3'}$-p$_s$)-acetamide (**4a**) in a yield of 75% (determined by HPLC) after RP-HPLC purification. 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 85/15, then wash-out for 10 min at 50/50; flow rate : 6 mL/min; retention time 14.0-14.5 min. ^{31}P NMR (D_2O, 298.0K) : δ : + 15.22 (-OP(O)(OH)(S-acetamide)) ; - 4.00 (-OP(O)(OH)O-). MS (electrospray) : 5801.0 (theor.), 5799.4 (exp.).

***N*-(4-fluorobenzyl)-2-(AsGsAsAsTsAsCsAsGsGsGsTsCsCsAsAsAsT$^{3'}$-p$_s$)-acetamide (4b).** The general procedure described above was used with AsGsAsASTsAsCsAsGsGsGsTsCsCsAsAs-AsT$^{3'}$-p$_s$ (**3b**) and *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) to provide *N*-(4-fluorobenzyl)-2-(AsGsAsAsTsAsCsAsGsGsGsTsCsCsAsAsAsT$^{3'}$-p$_s$)-acetamide (**4b**) in a yield of 72% (determined by HPLC) after RP-HPLC purification. 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. ^{31}P NMR (D_2O, 298.0K) : δ : + 15.22 (-OP(O)(OH)(S-acetamide)) ; + 53.40 (-OP(S)(OH)O-). MS (electrospray) : 6073.0 (theor.), 6074.0 (exp.).

***N*-(-4-fluorobenzyl)-2-([2'OMe]AGAAUACAGGGUCCAAAU$^{3'}$-p$_s$)-acetamide (4c).** The general procedure described above was used with [2'OMe]AGAAUACAGGGUCCAAAU$^{3'}$-p$_s$ (**3c**) and *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) to provide *N*-(4-fluorobenzyl)-2-([2'OMe]AGAAUACAGGGUCCAAAU$^{3'}$-p$_s$)-acetamide (**4c**) in a yield of 73% (determined by

HPLC) after RP-HPLC purification. 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 14.0-14.5 min. ^{31}P NMR (D_2O, 298.0K) : δ : + 15.22 (-OP(O)(OH)(S-acetamide)) ; - 4.00 (-OP(O)(OH)O-). MS (electrospray) : 6300.3 (theor.), 6299.0 (exp.).

***N*-(4-fluorobenzyl)-2-($A_{MP}G_{MP}A_{MP}$ATACAGGGTCC$A_{MP}A_{MP}A_{MP}T^{3'}$-p_s)-acetamide (4d).** The general procedure described above was used with $A_{MP}G_{MP}A_{MP}$ATACAGGGTCC-$A_{MP}A_{MP}A_{MP}T^{3'}$-p_s (**3d**) and *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) to provide *N*-(4-fluorobenzyl)-2-($A_{MP}G_{MP}A_{MP}$ATACAGGGTCC$A_{MP}A_{MP}A_{MP}T^{3'}$-p_s)-acetamide (**4d**) in a yield of 70% (determined by HPLC) after RP-HPLC purification. 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 15.0-15.5 min. ^{31}P NMR (D_2O, 298.0K) : δ :+ 15.22 (-OP(O)(OH)(S-acetamide)) ; +32.40 (-OP(CH_3)(OH)O-) ; - 4.00 (-OP(O)(OH)O-). MS (electrospray) : 5789.0 (theor.), 5787.2 (exp.).

4-Cyano-*N,N,N*-trimethylanilinium trifluoromethanesulfonate (6). A solution of 21.1 g of 4-dimethylaminobenzonitrile (**5**) (144 mmol) and 22.6 mL of methyl trifluoromethanesulfonate (1.4 eq, 200 mmol) in 300 mL of dry benzene was refluxed for 5 h under an argon atmosphere. The solid residue was filtered off and dissolved in 300 mL of water. This aqueous solution was washed once with 200 mL of CH_2Cl_2, once with 200 mL of $CHCl_3$ and concentrated to dryness. The residue was recrystallized twice from CH_2Cl_2 containing a few drops of MeOH to give 36.6 g of pure 4-cyano-*N,N,N*-trimethylanilinium trifluoromethanesulfonate (**6**) as pale yellow needles (82% yield). Rf (MeOH/AcOH : 50/50) : 0.4. ^1H NMR (DMSO-d_6, 338.0K) : δ : 8.30-8.00 (2b, 4H) ; 3.70 (s, 9H). ^{13}C NMR (DMSO-d_6, 338.0K) : δ : 150.0 [C] ; 133.9 [CH] ; 121.8 [CH] ; 120.6 [q, J : 325 Hz, CF_3] ; 116.9 [C] ; 113.2 [C] ; 56.4 [CH_3].

Radiochemistry

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

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 were passed through an anion exchange resin (AG1X8, Bio-Rad, 100-200 mesh). See reference 28 for more practical details. The [^{18}F]fluoride ion was then eluted from the resin using 1.0 mL of a 4.5 mg/mL aqueous K_2CO_3 solution. After addition of 11.0 to 15.0 mg of Kryptofix® 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.

Procedure A : conventional heating. The residue was then dissolved in 200 μL of freshly distilled DMSO and transferred to a 2 mL reaction vial containing 8 mg of the triflate salt of 4-trimethylammoniumbenzonitrile (**6**). The evaporation tube was rinsed twice with 200 μL of DMSO which was then added to the former reaction mixture. Resolubilization yields were about 60-80% of the original [^{18}F]fluoride ion radioactivity. The reaction vial was then tightly sealed with a Teflon cap and heated in a heating block without stirring at 180°C for 20 min. The resulting yellow-brown reaction mixture was cooled using an ice/water bath, diluted with 3 mL of water and passed through a C18 Sep-pak cartridge (Waters). The cartridge was washed with 0.5 mL of water and partially dried for 5 min by applying a nitrogen stream. 4-[^{18}F]Fluorobenzonitrile was eluted with 4 mL of THF (less than 5% of the total radioactivity amount was left on the cartridge) onto a column containing 1.0 g of oven-dried 4Å ground molecular sieve. The yield of substitution varied from 60% to 80% with respect to [^{18}F]fluoride ion (yields were determined after the Sep-pak elution by the THF over DMSO/H_2O radioactivity counting ratio followed by radiochromatography (SiO_2-TLC, eluent : heptane/EtOAc : 95/5, Rf : 4-[^{18}F]fluorobenzonitrile (**7**) : 0.35 and Rf : [^{18}F]fluoride ion : 0.0 or eluent : heptane/EtOAc : 50/50, Rf : 4-[^{18}F]fluorobenzonitrile (**7**) : 0.80 and Rf : [^{18}F]fluoride ion : 0.0).

The mentioned THF solution was left on the molecular sieve column for 5 min and then eluted into a 8 mL reaction vial containing 20 mg of dry powdered $LiAlH_4$. Another 3 mL of THF was used to wash the column and to completely transfer the 4-[^{18}F]fluorobenzonitrile. The vessel was then tightly closed and heated for 2 min at 120-130°C (strong reflux). The yield of reduction was usually quantitative as determined by radiochromatography (SiO_2-TLC, eluent : heptane/EtOAc : 50/50, Rf : 4-[^{18}F]fluorobenzonitrile (**7**) : 0.80 and Rf : 4-[^{18}F]fluorobenzylamine-aluminium complex : 0.0). The resulting gray-white suspension was cooled using an ice/water bath and concentrated to dryness. The amine-aluminium complex as well as the excess of $LiAlH_4$ were destroyed by adding 300 μL of H_2O. The white aqueous residue was then diluted with 3 mL of CH_2Cl_2 (SiO_2-TLC, eluent : CH_2Cl_2/MeOH/TEA : 90/10/3, Rf : 4-[^{18}F]fluorobenzylamine : 0.25). After addition of 1 mL of a solution of bromoacetyl bromide (15.5 μg/mL, 80 nmol) in CH_2Cl_2, the white milky suspension was allowed to react 2-3 min at room temperature with a smooth air-bubbling agitation. The yield of condensation with $BrCOCH_2Br$ varied from 65% to 90% as determined by radiochromatography (SiO_2-TLC, eluent : heptane/EtOAc : 50/50, Rf *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**) : 0.35 and Rf : 4-[^{18}F]fluorobenzylamine : 0.0). The reaction mixture was then filtered on cotton and the precipitate washed twice with 1.0 mL of CH_2Cl_2. The filtrate was concentrated to dryness, the residue dissolved in 1-2 mL of CH_2Cl_2 (or 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, Rt : 5.0-5.5 min.

Procedure B : Microwaves activation. Freshly distilled DMSO (600 μL) containing 8 mg of the labeling precursor (**6**) were directly added into the tube containing the dried K[^{18}F]F-K_{222} complex. The tube (not sealed) was placed in a microwave oven. Microwaves, 100 Watt, were applied to the system for 1 min. The remainder of the synthesis used the same procedure as described above.

Typically, 66-95 mCi (2.4-3.5 GBq) of pure [^{18}F]-**2** (and up to 114 mCi (4.2 GBq)) could be obtained in 85-95 min starting from a 600-650 mCi (22.2-24.0 GBq) aliquot of a cyclotron [^{18}F]F^- production batch.

General procedure for labeling oligonucleotides (3a-d).

30 OD (ca. 1 mg) of 18mer oligonucleotides (**3a-d**) in water were added into a vessel containing the dried purified *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**) and diluted in a 1/1 mixture of methanol/PBS (0,1 M, pH 8) (v/v). After 10 min of reaction at 120°C, the solvents were evaporated under a stream of nitrogen and labeled oligonucleotides [^{18}F]-**4a-d** were separated from unlabeled oligonucleotides (**3a-d**) 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 18mer oligonucleotides **4a-d** (HPLC A). Pure labeled 18mer oligonucleotides were collected and desalted on a G25 Sephadex Column (Amersham Pharmacia Biotech) after evaporation of the solvents.

N-(4-[^{18}F]fluorobenzyl)-2-(AGAATACAGGGTCCAAAT$^{3'}$-p$_y$)-acetamide ([^{18}F]-4a), N-(4-[^{18}F]fluorobenzyl)-2-(AsGsAsAsTsAsCsAsGsGsGsTsCsCsAsAsAsT$^{3'}$-p$_y$)-acetamide ([^{18}F]-4b), N-(-4-[^{18}F]fluorobenzyl)-2-([2'OMe]AGAAUACAGGGUCCAAAU$^{3'}$-p$_y$)-acetamide ([^{18}F]-4c), N-(4-[^{18}F]fluorobenzyl)-2-(A$_{MP}$G$_{MP}$A$_{MP}$ATACAGGGTCCA$_{MP}$A$_{MP}$A$_{MP}$T$^{3'}$-p$_y$)-acetamide ([^{18}F]-4d).

Labeled synthesized oligonucleotides co-elute with authentic synthesized unlabeled reference compound **4a** to **4d**, respectively. HPLC B : gradient elution : linear in 3 min from 95/5 to 90/10 (TEAA/acetonitrile, see *analytical methods*), then linear in 27 min from 90/10 to 70/30, then wash-out for 10 min at 50/50 ; flow rate : 1.5 mL/min ; retention times : [^{18}F]-**4a** : 14.2 min ; [^{18}F]-**4b** : 19.0 min ; [^{18}F]-**4c** : 22.3 min ; [^{18}F]-**4d** : 16.6 min.

Typically, the whole fluorine-18 labeling procedure allows us to obtain 15 to 21 mCi (0.55 to 0.74 GBq) of pure labeled oligonucleotides **4a-d** in 180 min with a specific radioactivity of 0.8 to 2 Ci/μmol (30 to 70 GBq/μmol) at the end of synthesis starting from a 600-650 mCi (22.2-24.0 GBq) aliquot of a cyclotron [^{18}F]F$^-$ production batch.

Conclusion

The increasing interest in oligonucleotides as new drugs (3) or as diagnostic agents in nuclear medicine (29,30) has led to the development of modified molecules with improved biological properties. A simple and reliable labeling method to evaluate *in vivo* these properties is required. We have demonstrated that it is possible to label oligonucleotides possessing chemical modifications of the sugar-phosphate backbone (modified phosphate groups or -ribose) with a positron emitter. This method allows production of radioactive tracers in a time compatible with the half life of fluorine-18, and in sufficient quantity and with high specific radioactivity for PET studies. The fact that this synthetic procedure has been routinely implemented in our laboratory suggests that it will be easily applied to other chemically modified oligonucleotides.

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