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Synthesis of 4-([^{18}F]Fluoromethyl)phenyl Isothiocyanate and its Use in Labelling Oligonucleotides

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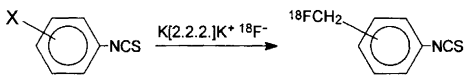
4-([^{18}F]Fluoromethyl)phenyl isothiocyanate **8** was obtained from [^{18}F]fluoride and 4-(4-toluenesulfonyloxymethyl)phenyl isothiocyanate **5** in 13–15% isolated, decay-corrected radiochemical yield within 40 min from the end of cyclotron bombardment. The precursor **8** was used to label the oligonucleotide 5′-GCT,AAG,CGA,TGC,CTC, CGT-3′, modified in the 5′-position with a hexylamine linker, in the 5′-position in up to 8% radiochemical yield.

Antisense oligodeoxynucleotides have been used extensively as specific probes in molecular biology. The possibility of using their hybridisation to the complementary mRNA strand in order selectively to block protein synthesis in cells has become an area of intense research activity which may result in a new generation of pharmaceutical agents. Consequently, it was of interest to label oligonucleotides with radionuclides suitable for positron emission tomography (PET). The PET method allows the non-invasive visualisation and quantification of biological functions *in vivo* by the use of appropriate compounds labelled with positron-emitting radionuclides. Commonly used radionuclides for PET are ^{15}O, ^{13}N, ^{11}C and ^{18}F with half-lives of 2.07, 9.97, 20.3 and 110 min, respectively.[1–4] Oligodeoxynucleotides (also commonly referred to as oligonucleotides) labelled with positron-emitting radionuclides, combined with the PET method might provide the possibility of studing *in vivo* hybridisation of antisense oligonucleotides and would also be interesting as a research tool for *in vitro* applications. One special feature in this context is the synthesis of labelled oligonucleotides with high specific radioactivity, which will increase the sensitivity of the detected hybridisation.

Oligonucleotides functionalised with an alkylamine in the 5′-position have been reacted with a wide range of electrophile containing conjugate groups.[5–9] The amine-reactive isothiocyanate functionality has been exploited in the radiolabelling of proteins,[10] the synthesis of derivatives of 5′-alkylamine functionalised oligonucleotides,[9] and in the ^{125}I-labelling of such oligonucleotides.[11] In this study, we focused on the possibility of incorporating the ^{18}F-label into oligonucleotides. To achieve this, a one-step synthesis of a labelled compound containing the isothiocyanate functionality, 4-([^{18}F]fluoromethyl)phenyl isothiocyanate **8**, was developed (Scheme 1). The reaction between **8** and oligonucleotides modified in the 5′-position with a hexylamine linker (Fig. 1) was then investigated (Scheme 2).

Experimental

General. ^{1}H NMR- (300 MHz) and ^{13}C NMR-spectra (75.4 MHz) were recorded on a Varian XL-300 spectrometer with chloroform-d_1 as the solvent. Either tetra-



1 X=2-$BrCH_2$
2 3-$BrCH_2$
3 4-$BrCH_2$
4 4-ICH_2
5 4-$TsOCH_2$

6 2-$^{18}FCH_2$
7 3-$^{18}FCH_2$
8 4-$^{18}FCH_2$

Scheme 1.

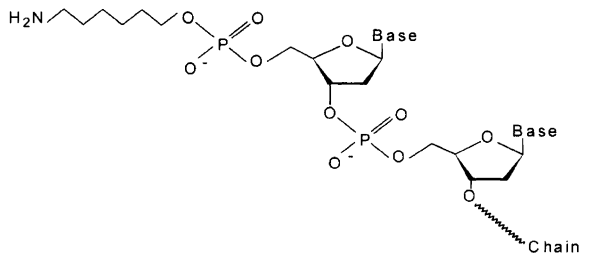


Fig. 1. Oligonucleotide modified in the 5′-position with hexylamine.

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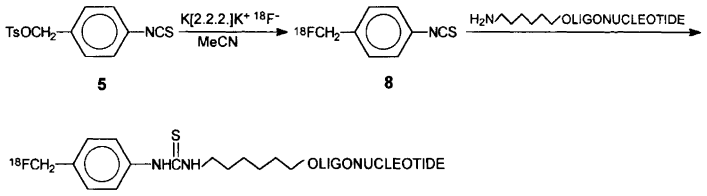

Scheme 2.

methylsilane or chloroform was used as internal standard. Infrared (IR) spectra were recorded on a Perkin–Elmer 1600 Series FTIR spectrophotometer. Melting points were determined using unsealed capillary tubes and are reported uncorrected. Thin-layer chromatography (TLC) was performed using Alugram Sil G/UV_{254} silica plates (Macherey-Nagel, Düren, Germany). The TLC plates were eluted with 100% dichloromethane. Ultraviolet (UV) absorbance was visualised with short- and long-wave UV light and radioactivity was imaged and quantified by storage-phosphorus autoradiography (Molecular Dynamics, Sunnyvale, CA, USA). Diol Isolute solid-phase extraction (SPE) columns, 500 mg, were obtained from Sorbent (Hengoed, UK). Acetonitrile used for labelling experiments was of Sure/Seal℠ grade supplied by Aldrich. Analytical HPLC was performed using two systems. System 1: two Waters 501 pumps, a Waters 440 UV detector with a β^+-flow detector in series, a Waters automated gradient controller and a Waters 745B Data Module. The column used was a Waters Hichrome ODS-1 C18 5 μm 250 × 4.6 mm ID column. Analytical conditions for system 1 were: 0.01 M KH_2PO_4 (A) and acetonitrile–water 50:7, v/v (B), isocratic 65% B, flow rate 2 ml min^{-1}. System 2: a Beckman 126 pump, a Beckman 166 UV detector in series with a β^+-flow detector and Beckman System Gold Chromatography Software Package. The column used was a TSK Gel Oligo DNA RP 300 Å 5 μm 200 × 4.6 mm ID column. Analytical conditions for system 2 were: 0.10 M NH_4OAc (pH unadj)–MeCN 95:5 v/v (a) and 0.10 M NH_4OAc (pH unadj)–MeCN 10:90 v/v (b), linear gradient 0–100% b 0–5 min 100% b 5–12 min, flow rate 0.7 ml min^{-1}.

Oligonucleotide synthesis. Oligonucleotides were synthesised on a 1 μmol scale using the phosphoramidite method,[12–14] on an Oligo 1000 DNA Synthesizer (Beckman, USA) with CED-phosphoramidites (Beckman, USA). The alkylamine was introduced into the 5′-position of the oligonucleotide as the last step in the synthesis cycle by standard procedures using Aminolink™ 2 obtained from ABI, Perkin Elmer, Sweden.[15] The crude oligonucleotides were purified by gel filtration (NAP-5 columns, Pharmacia Biotech, Uppsala, Sweden) followed by precipitation from 3 M pH 4.8 HOAc–NaAc buffer using cold ethanol.[16,17]

Synthesis of precursor and reference compounds.
(Bromomethyl)phenyl isothiocyanates **1–3.** Compound **1** was prepared following the procedure described by Gonda and Kristian[18] and compounds **2** and **3** by the procedures described by Litak and Kauffman.[19] Spectral data were in accordance with those reported in the literature.

4-(Iodomethyl)phenyl isothiocyanate **4.** 4-(Bromomethyl)phenyl isothiocyanate (228 mg, 1.00 mmol) in acetone (2 ml) was added to sodium iodide (165 mg, 1.10 mmol) in acetone (3.5 ml) at 0 °C. The mixture was stirred at 0 °C for 10 min and then kept at 8 °C for 10 h. The mixture was poured into water, $Na_2S_2O_5$ was added and the product was extracted with diethyl ether. The combined organic solutions were dried ($MgSO_4$) and the solvent was removed. The resulting light yellow solid was recrystallized from dichloromethane to yield (after drying under reduced pressure) 179 mg (65%) of **4** as white–yellow crystals: m.p. 122–123 °C. IR (CCl_4) 2045 cm^{-1} (NCS). ^{1}H NMR: δ 4.43 (2 H, s, CH_2I), 7.10–7.19 (2 H, m, H-2, H-6), 7.32–7.40 (2 H, m, H-3, H-5). ^{13}C NMR: δ 4.1 (CH_2I), 126.1 (C-2, C-6), 129.9 (C-3, C-5), 130.6 (C-1), 135.9 (NCS), 138.6 (C-4).

4-(4-Toluenesulfonyloxymethyl)phenyl isothiocyanate **5.** Compound **5** was synthesised by converting the corresponding halide into the 4-toluenesulfonate (tosyl), using a modification of a reported procedure.[20] 4-(Bromomethyl)phenyl isothiocyanate (250 mg, 1.10 mmol) was added in portions to a solution of silver tosylate (370 mg, 1.33 mmol) in acetonitrile (7 ml, Sure/Seal™ grade) at 0 °C and the reaction vessel was protected from light. The mixture was stirred first at 8 °C for 40 min and then at room temperature for 11 h, after which it was cooled to 0 °C. A further 0.3 equiv. of silver tosylate was added, and the mixture was stirred at 0 °C for 10 min and then at room temperature for 2 h. The reaction mixture was poured into ice–water–diethyl ether and filtered through a layer of Celite. The filtrate was extracted with diethyl ether and the combined organic solutions were dried ($MgSO_4$). The solvent was removed and the resulting yellow solid was recrystallized from diethyl ether to yield (after drying under reduced pressure) 166 mg (47%) of **5** as yellow crystals, m.p. 95–97 °C. IR (CCl_4) 2050 cm^{-1} (NCS). ^{1}H NMR: δ 2.45 (3 H, s, CH_3–4′), 5.03 (2 H, s, CH_2–4), 7.16 (2 H, app d, J = 8.3 Hz, H-2, H-6), 7.24 (2 H, app d, J = 8.3 Hz, H-3, H-5), 7.34 (2 H, app d, J = 8.1 Hz, H-3′,H-5′), 7.78

(2 H, app d, J=8.1 Hz, H-2′, H-6′). ^{13}C NMR: δ 21.7 (CH_3), 70.7 ($TsOCH_2$), 125.9, 127.9, 129.7, 129.9, 131.9, 132.5, 133.0, 136.3 (NCS), 145.0 (C-4).

4-(Fluoromethyl)phenyl isothiocyanate. Potassium fluoride (18.5 mg, 0.318 mmol) and 18-crown-6 (88.2 mg, 0.334 mmol) were dried by azeotropic evaporation with acetonitrile (3 × 5 ml). 4-(4-Toluenesulfonyloxymethyl)phenyl isothiocyanate **5** (50 mg, 0.157 mmol) dissolved in acetonitrile (6 ml, Sure/Seal℠ grade) was added to the residue. The mixture was stirred at room temperature. After four days, another 0.5 equiv. of potassium fluoride and 18-crown-6 were dried as described above and added to the reaction mixture. The reaction was stirred at room temperature for 7 h. The solvent was removed, water was added to the residue and the product was extracted with dichloromethane. The combined organic solutions were dried ($MgSO_4$) and the solvent was removed. The resulting oil was purified by preparative TLC (eluent: pentane–diethyl ether 2:1, R_f=0.85) to give 7.6 mg (29%) of 4-(fluoromethyl)phenyl isothiocyanate as a colourless oil. IR (CCl_4) 2050 cm^{-1} (NCS). ^{1}H NMR: δ 5.37 (2 H, d, $J_{H,F}$=47.6 Hz, CH_2F), 7.25 (2 H, app br d, J=8.6 Hz, H-2, H-6), 7.36 (2 H, app dd, J=8.6 Hz, $^3J_{H,F}$=1.6 Hz, H-3, H-5). ^{13}C NMR: δ 83.6 (d, $^1J_{C,F}$=168 Hz, CH_2F), 125.9 (C-2, C-6), 128.5 (d, $^3J_{C,F}$=6 Hz, C-3, C-5), 131.6 (C-1), 135.3 (d, $^2J_{C,F}$=18 Hz, C-4).

Radiolabelling.

Preparation of [^{18}F]fluoride. Aqueous [^{18}F]fluoride was produced using the ^{18}O(p,n)^{18}F reaction by irradiation of [^{18}O]H_2O with 17 MeV protons in a low pressure silver target using a Scanditronix MC-17 Cyclotron at the Uppsala University PET Centre. The aqueous [^{18}F]fluoride solution was added to Kryptofix® 2.2.2. (K[2.2.2.]) (3.8 mg, 10 μmol) and K_2CO_3 (0.7 mg, 5 μmol) and the water was removed by repeated azeotropic evaporation with acetonitrile at 105 °C. The dried residue was finally dissolved in acetonitrile (250 μl) and used as described below.

([^{18}F]Fluoromethyl)phenyl isothiocyanates **6–8**, *general procedure.* The K[2.2.2.]/K^+ complex of [^{18}F]fluoride in acetonitrile was added to the isothiocyanates **1–5** (25 μmol). The reaction mixture was heated at 110–120 °C in a closed reaction vial, and samples from the reaction mixture were diluted with acetonitrile and analysed by radio-HPLC.

4-([^{18}F]Fluoromethyl)phenyl isothiocyanate **8** *used for oligonucleotide labelling.* Isothiocyanate **5** (8.0 mg, 25 μmol) was reacted with the K[2.2.2.]/K^+ complex of [^{18}F]fluoride in acetonitrile at 115–120 °C for 15–20 min in a closed reaction vial. The acetonitrile was evaporated off and the residue was dissolved in dichloromethane (0.2 ml), diluted with pentane (2.8 ml) and passed through a diol SPE column. The product was analysed by HPLC. Retention times for **8** were 4.0–4.3 and 9.5–9.7 min using HPLC system 1 and 2, respectively. The R_f value of **8** was 0.77–0.80.

Oligonucleotide labelling. The eluate from the diol SPE column containing **8** was concentrated, transferred to an Eppendorf vial and evaporated to a residue using nitrogen. DMF (30 μl) was added to the residue, followed by the oligonucleotide (10 OD_{260} units, ≈70 nmol) dissolved in buffer (60 μl) (Table 2). The reactions were carried out at specified temperatures (Table 2) for 30–100 min. Samples from the reaction mixture were diluted with water and analysed by reversed-phase HPLC using system 2. The retention time for [^{18}F]fluoride was 2.9 min and for the oligonucleotide (starting material) and the labelled product 6.3–6.4 min.

Results and discussion

Isothiocyanates **3–5** containing the leaving groups bromide, iodide and tosyl were synthesised and used as substrates in nucleophilic substitution reactions with [^{18}F]fluoride to produce compound **8** (Scheme 1). The main reason also for evaluating iodide and tosylate as leaving groups in the [^{18}F]fluoride-substitution reaction was that unsatisfactory radiochemical yields were obtained using bromide as leaving group. No improvements in radiochemical yields compared with using bromide as the leaving group were observed, however, for the other two leaving groups (Table 1). We have at present no explanation for the low radiochemical yield for this transformation.

Starting with the previously reported compound **3**,[19] substrate **4** was prepared by a Finkelstein reaction[21] and compound **5** by tosylation.[20] The reference compound, 4-(fluoromethyl)phenyl isothiocyanate, was synthesised from **5** by nucleophilic substitution using KF/18-crown-6.[22]

The labelled isothiocyanate **8** was prepared from **3–5** (Scheme 1) in 13–36% radiochemical yield in 15 min reaction time (Table 1). The best yields were obtained when acetonitrile was used as the solvent, while THF,

Table 1. Radiochemical yields of 4-([^{18}F]fluoromethyl)phenyl isothiocyanate **8**.

Entry	Substrate	T/°C[a]	Radiochemical yield (%)[b]	n
1	**3**	115	21–23	2
2	**4**	115	23–36	2
3	**5**	115	13–17	2
4	**5**	120	18–27	3

[a] Reaction temperature. [b] Determined by TLC analysis of the reaction mixture as a percentage of the total amount of radioactivity in the sample. Reaction conditions were: 25 μmol substrate, 10 μmol K[2.2.2.] and 5 μmol K_2CO_3 in 250 μl acetonitrile, heated for 15 min.

Table 2. Reaction conditions and radiochemical yields for labelled oligonucleotides synthesised from **8**.

Entry	Solvent[a]	pH of buffer	t/°C	Reaction time/min	Radiochemical yield (%)[b]	Amount of [^{18}F]fluoride (%)[b]
1	A	9.0	37	30	3	55
2	A	9.0	37	60	0	75
3	B	7.5	45	70	0	10
4	B	8.0	40	35	6.8	44
5	B	8.0	40	60	6.8	54
6	B	8.0	40	100	7.7	66
7	B	8.5	45	70	0	100
8	B	9.0	45	70	0	100
9	C	7.0	35	50	0	20
10	C	8.0	35	80	8.0	62

[a] A, 1:2 DMF–0.1 M $NaHCO_3/Na_2CO_3$ buffer; B, 1:2 DMF–0.05 M $H_3BO_3/Na_2B_4O_7$ buffer; C, 1:2 DMF–0.1 M NaH_2PO_4/Na_2HPO_4 buffer. [b]Determined by HPLC analysis (system 2) of the reaction mixture as a percentage of the total amount of radioactivity in the sample.

DMF, DMSO or mixtures of these with acetonitrile all gave considerably lower yields. The influence of reaction temperature, substrate concentration and concentration of Kryptofix® 2.2.2./K^+ on the radiochemical yield of **8** was also studied. Reaction conditions used in Table 1 gave the highest radiochemical yields of **8**. Substitution with [^{18}F]fluoride on isothiocyanates **1** and **2** gave labelled products in 9 and 22% radiochemical yields, respectively (reaction conditions as in Table 1).

Tosylate **5** was chosen as the substrate for the preparation of **8**, for oligonucleotide labelling experiments. This substrate was preferred over the iodine analogue **4**, since **5** could easily be separated from **8** using a diol SPE column. The reactions of unlabelled isothiocyanate compounds with the amino-linked oligonucleotides might render the separation of the labelled oligonucleotides from non-labelled oligonucleotides more difficult. Compound **8** was prepared in 13–15% isolated radiochemical yield (decay-corrected, counted from EOB) and >99% radiochemical purity within 40 min synthesis time, using substrate **5** and the reaction conditions specified in Table 1.

An unactivated aliphatic position of the ^{18}F-label was also investigated using 3-bromopropylbenzene and 4-(3-bromopropyl)phenyl isothiocyanate[19] as substrates with reaction conditions as specified in Table 1. [3-^{18}F]-3-Fluoropropylbenzene was obtained from 3-bromopropylbenzene in >96% radiochemical yield from [^{18}F]fluoride. The other compound, 4-(3-bromopropyl)phenyl isothiocyanate, could not be labelled using the reaction conditions specified in Table 1.

Oligonucleotide 5′-GCT,AAG,CGA,TGC,CTC,CGT-3′ was labelled in up to 8% radiochemical yield from **8**. The remaining radioactivity after 30–100 min reaction time consisted of [^{18}F]fluoride (10–100%) and **8** (Table 2). When gel filtration using NAP-5 Sephadex columns was performed on the crude products, all of the radioactivity was found to come from [^{18}F]fluoride in high- and low-molecular weight fractions (HPLC system 2). It is evident that the labelled oligonucleotide was dehalogenated, presumably by solvolysis. This conclusion is further supported by the result obtained from solvolysis of [3-^{18}F]-3-fluoropropyl benzene with carbonate buffer in DMF. In this reaction, 34% of the radioactivity was found as [^{18}F]fluoride after 1 h at 40 °C (reaction conditions as in Table 2, entries 1 and 2).

Solvolysis of alkylfluorides in aqueous media at neutral to basic pH is a slow process.[23] In our study this side reaction causes major losses of [^{18}F]fluoride from labelled products, presumably due to the low concentrations of the labelled compounds.

In summary, 5′-alkylamine functionalised oligonucleotides can be labelled using **8**. Competing solvolysis of the benzyl fluoride function was, however, the main reaction within the pH interval used.

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