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Synthesis of *N*-Succinimidyl 4-[^{76}Br]Bromobenzoate and its Use in Conjugation Labelling of Macromolecules

Ulrika Yngve,[a,c] Elisabeth Hedberg,[a,c] Anna Lövqvist,[d] Vladimir Tolmachev[d] and Bengt Långström[a,b,c,*]

[a]Department of Organic Chemistry, Uppsala University, Box 531, SE-751 21 Uppsala, Sweden, [b]Uppsala University PET Centre, UAS, SE-751 83 Uppsala, Sweden, [c]Subfemtomole Biorecognition Project, Japan Science and Technology Corporation and Uppsala University PET Centre, and [d]Department of Radiation Science, Uppsala University

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The ^{76}Br radiobromination of proteins and modified oligonucleotides by the use of *N*-succinimidyl 4-[^{76}Br]bromobenzoate are described. The labelled *N*-succinimidyl bromobenzoate was synthesised from the corresponding trimethyltin compound, *N*-succinimidyl 4-trimethylstannylbenzoate, in 45–60% isolated radiochemical yield with a specific radioactivity of 20–200 GBq μmol^{-1}. The use of *N*-succinimidyl 4-[^{76}Br]bromobenzoate in conjugation with the proteins human serum albumin and chromogranin and with 5′-hexylamino-modified oligodeoxynucleotides (ODN) and its phosphorothioate analogues (S-ODN), is described. The oligonucleotide sequence chosen for this work is an antisense sequence for the m-RNA encoding for chromogranin in rat.

Oligonucleotides and proteins are important molecules in life sciences and might therefore be interesting as probes for various applications using positron emission tomography (PET). Such macromolecules may be labelled directly with halogens by oxidative methods[1–4] or indirectly by conjugation of a suitably labelled prosthetic group,[5,6] a bifunctional compound designed for facile radiolabelling and conjugation.

Conjugation is a relatively mild labelling technique and the same prosthetic group may be used for a large number of different macromolecules. Commonly used prosthetic groups for radiohalogenated compounds are, for example, activated esters of *N*-hydroxysuccinimide.[5] Trialkyltin compounds are often used for radiohalogenation through a metal–halogen exchange reaction on aromatic or vinylic substrates.[7] *N*-Succinimidyl 3-trimethylstannylbenzoate and *N*-succinimidyl tributylstannylbenzoate have previously been labelled with ^{211}At,[8] with ^{77}Br,[9] and with different isotopes of iodine, and coupled to proteins.[10,11] *N*-Succinimidyl 4-tributylstannylbenzoate has also been used in coupling reactions with oligonucleotides, and then labelled with ^{125}I.[12]

The activated ester may be coupled to different compounds containing a primary amine as a functional group, for example lysine residues and the *N*-terminal residue in proteins, or to oligodeoxynucleotides modified with an amino linker in the 5′-position. This technique allows different backbone modifications of the oligonucleotide and different base sequences.

^{76}Br ($t_{1/2} = 16$ h) decays 55% by positron emission and 45% by electron capture and has been considered for PET applications, since the half-life may allow a appropriate time frame for studying processes involving macromolecules. In this paper, the synthesis of *N*-succinimidyl 4-[^{76}Br]bromobenzoate (^{76}BrNHS) and its use in conjugation to the proteins human serum albumin and chromogranin[13] and to 5′-hexylamino-modified oligodeoxynucleotides (ODN) and their phosphorothioate analogues (S-ODN), are described. The oligonucleotide sequence chosen for this work is an antisense sequence for the m-RNA encoding for chromogranin in rat.[14]

Results and discussion

Synthesis of precursors. Different methods have been published for the preparation of *N*-succinimidyl 3-trimethylstannylbenzoate (*m*-Me$_3$SnNHS),[10] *N*-succinimidyl 3-tributylstannylbenzoate (*m*-Bu$_3$SnNHS)[10,15,16] and *N*-succinimidyl 4-tributylstannylbenzoate (*p*-Bu$_3$SnNHS).[10,11,17] *N*-Succinimidyl 4-trimethylstannylbenzoate (*p*-Me$_3$SnNHS) was synthesised by modifying the procedures described in the literature. Recently, a different method for preparing *p*-Me$_3$SnNHS has been described.[18] Ethyl 4-iodobenzoate was stannylated using

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[*] To whom correspondence should be addressed.

hexamethylditin and tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$] as a catalyst to give **2** (93%).[11,17] The stannylbenzoate **2** was hydrolysed and then esterified with *N,N'*-disuccinimidylcarbonate to give the desired *p*-Me_3SnNHS (16%) (Scheme 1).[16] The low yield might be due to impurities in compound **3** since compound **2** was difficult to obtain pure using flash chromatography and **3** was used without further purification.

When the tin compounds were purified by flash chromatography on silica gel, triethylamine (Et_3N) was added to the mobile phase in order to prevent the tin from interacting too strongly with the residual silanol groups on the silica gel. In the purification of the corresponding tributylstannyl compound, *p*-Bu_3SnNHS, the product and the intermediate ethyl ester were bound irreversibly to the silica column if Et_3N was not added to the mobile phase.

N-Succinimidyl 4-trimethylstannylbenzoate was chosen over *N*-succinimidyl 4-tributylstannylbenzoate as substrate for radiobromination since recrystallisation was possible, giving higher purity and higher labelling yields. It has also been reported that the trimethylstannyl compound is more reactive than the tributylstannyl compound.[10] The *p*-Me_3SnNHS was stable for at least 6 months when stored at −20 °C.

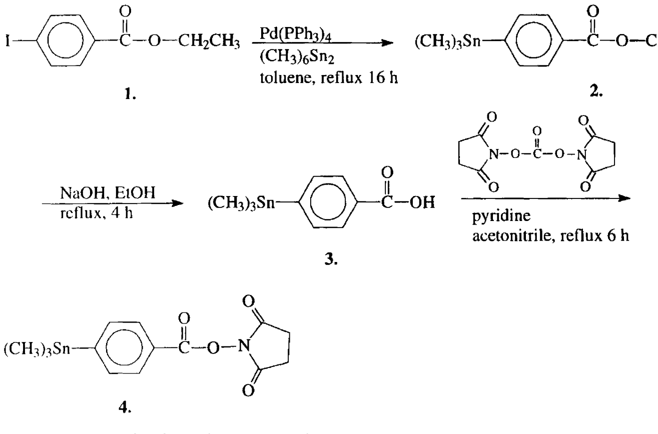
[^{76}Br]Bromide production. [^{76}Br]Bromide was produced by proton irradiation of a [^{76}Se]selenium-enriched Cu_2Se-target.[19] For practical and radiation safety reasons, the target was irradiated in the afternoon and removed manually from the cyclotron vault the following morning. The radioactive peak from the [^{76}Br]bromide solution coeluted with Br^- from NaBr using ion exchange chromatography (HPLC system 5). Tolmachev *et al.* have reported that the radioactive peak coelutes with Br^- using aluminium oxide TLC and anion exchange HPLC.[19]

Radiolabelling. *p*-Me_3SnNHS was radiobrominated in 45–60% isolated radiochemical yield (not corrected for decay) by an electrophilic substitution reaction using chloramine-T (*N*-chloro-4-toluenesulfonamide sodium salt) as oxidation agent (Scheme 2). *tert*-Butyl hydroperoxide, *N*-chlorosuccinimide and IODO-BEADS® were tested as the oxidation agents but all gave lower yields. Chlorination of the tin compound, yielding *N*-succinimidyl 4-chlorobenzoate (ClNHS), was a drawback with the use of chloramine-T.

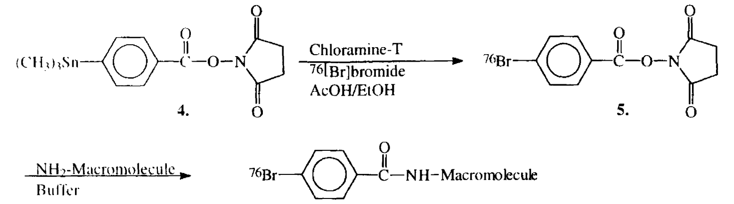
Purification of the [^{76}Br]bromide, after recovery from the target, by solid phase extraction was crucial in order to achieve good and reproducible yields in the synthesis of ^{76}BrNHS. The recovery of radioactive material in the purification step was 90–95%. Without purification of the bromide solution prior to use the radiochemical yields of ^{76}BrNHS were 10–35% due to large amounts of non-polar by-products. If only a small portion of the non-purified bromide batch (approximately 2%) was used together with 1 mg *p*-Me_3SnNHS for labelling experiments, the relative amount of non-polar by-products was much lower, indicating that the [^{76}Br]bromide-solution contained contaminating substances. The formation of labelled by-products might be caused by oxidised selenium species released from the target pellet.

Removal of all the solvent from the crude reaction mixture in the synthesis of ^{76}BrNHS prior to injection onto the straight phase HPLC column was necessary in order to achieve baseline separation of *p*-Me_3SnNHS and ^{76}BrNHS. ^{76}BrNHS was stable for at least 24 h in the HPLC-eluting solvent (system 2) which allowed conjugation experiments to be performed for two days using ^{76}BrNHS from the same synthesis.

Conjugation to macromolecules. ^{76}BrNHS was evaporated to dryness and the macromolecule was added in buffer or DMF–buffer pH 8.5–9 to give labelled macromolecules in varying yields (Table 1). The remaining radioactivity consisted of [^{76}Br]bromobenzoic acid and unidentified labelled by-products. All macromolecules were identified as high molecular weight products using gel chromatography, system 4. The labelled ODN and S-ODN were also identified by electrospray ionisation



Scheme 1. Synthesis of *N*-succinimidyl 4-trimethylstannylbenzoate.



Scheme 2. Synthesis of *N*-succinimidyl 4-[^{76}Br]bromobenzoate and conjugation to macromolecule.

Table 1 Radiochemical yields for the conjugation of ^{76}BrNHS to proteins and oligonucleotides.

Macromolecule	Isolated radiochemical yield (%)	*n*
HSA	39–61	4
Chromogranin	23–28	3
ODN 20-mer	12	1
S-ODN 20-mer	13–25	5

mass spectrometry after syntheses where carrier BrNHS was added.[20]

Labelled ODN or S-ODN was separated from its unlabelled counterpart using reversed phase perfusion chromatography with TEAA as buffer. Separation from unlabelled ODN was crucial in order to obtain high specific radioactivity and high chemical purity since a large excess of the ODN was used in the conjugation step to achieve acceptable incorporation of radioactivity.

Determination of specific radioactivity. The mass of the sample for determination of the specific radioactivity was calculated from BrNHS and the pseudo carrier ClNHS since both can be expected to react with the macromolecules. The specific radioactivity was determined to be 20–200 GBq µmol^{-1} for ^{76}BrNHS by HPLC, method 1b. As an example, the specific radioactivity for [^{76}Br]bromide was determined to be 180 GBq µmol^{-1} before purification and 76 GBq µmol^{-1} after purification (using HPLC-method 5) giving a specific radioactivity of 70 GBq µmol^{-1} for ^{76}BrNHS. Because of the good separation between labelled and unlabelled oligonucleotide in the purification step, the specific radioactivity of the oligonuclotide should be of the same magnitude as the specific radioactivity for BrNHS and ClNHS. The specific radioactivity of the proteins was determined by the amount of protein added to the reaction mixture since the labelled product was not separated from the unlabelled.

Experimental

General. All chemicals used were commercially available and used without further purification unless otherwise indicated. Deionised water (18.2 MΩ) and 99.5% ethanol were used for labelling reactions. Glassware for reactions performed under nitrogen was dried for at least 6 h at 120 °C. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer (300 MHz) with chloroform-d_1 as both solvent and internal standard. Thin-layer chromatography (TLC) was performed using DC-Alufolien Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany). Overpressured layer chromatography (OPLC) was performed using OE-340 Chrompres 25 (Hungary) and precoated TLC plates, 20 x 20 cm, Kieselgel 60 F_{254} (Merck). Ultraviolet (UV) absorbance was visualised using short- and long-wave ultraviolet light. Radioactivity on TLC-plates was measured by means of storage-phosphor autoradiography plates using a Molecular Dynamics Phosphor Imager® (Sunnyvale, CA, USA). Electrospray ionisation mass spectroscopy, ESI-MS, was performed using a Fisons Platform (Micromass, Manchester, UK). The reported radiochemical yields are uncorrected with respect to decay because of the relatively long half-life compared with synthesis time. *N*-Succinimidyl 4-bromobenzoate (BrNHS) and *N*-succinimidyl 4-chlorobenzoate (ClNHS) were prepared according to the literature.[21]

Liquid chromatography. Analytical liquid chromatography separations were performed using a Beckman (Fullerton, CA, USA) System (a 126 pump and a 166 UV detector) with a β^+-flow detector in series. Data collection was performed using the Beckman System Gold Chromatography Software Package. Semi-preparative HPLC was performed using equipment from Waters (Milford, MA, USA): a modified M 6000A pump, an automated gradient controller, a 440 UV detector with a β^+-flow detector in series and two 745B Data Modules. A Dionex ion chromatography instrument (Sunnyvale, CA, USA) equipped with a conductivity detector and a β^+-flow detector in series was used for determining the specific radioactivity of [^{76}Br]bromide.

The following columns and mobile phases were used: System 1: Ultrasphere ODS C-18 HPLC column (Beckman) 250 x 4.6 mm ID, 5 µm, flow 1.5 ml min^{-1}, A = 10 mM formic acid, B = MeCN. (1a) 50% B 0–7 min, linear gradient 50–95% B 7–10 min, linear gradient 95–50% B 20–25 min. (1b) Isocratic 50% B.

System 2: Ultrasphere Silica HPLC column (Beckman) 250 x 10 mm ID, 5 µm, flow 4 ml min^{-1}, hexane–ethyl acetate–acetic acid 70:30:0.1.

System 3: Fast desalting HR 10/10 fast protein liquid chromatography (FPLC) gel filtration column (Pharmacia Biotech, Uppsala, Sweden), flow 2.0 ml min^{-1}, 50 mM NaH_2PO_4–Na_2HPO_4, 0.16 M NaCl, pH 7.3.

System 4: Poros R2/H perfusion chromatography reversed-phase column (PerSeptive Biosystems, Cambridge, MA, USA) 100 × 2.1 mm ID, 10 μm, flow 1.5 ml $\min^{-1}$, A = 50 mM triethylammonium acetate (TEAA), B = MeCN. (4a) 0% B 0–2 min, linear gradient 0–15% B 2–22 min, 15% B 22–28 min, linear gradient 15–60% B 28–31 min, linear gradient 60–0% B 31–34 min. (4b) 0% B 0–2 min, linear gradient 0–10% B 2–22 min, 10% B 22–28 min, linear gradient 10–60% B 28–31 min, linear gradient 60–0% B 31–34 min.

System 5: Ionpack® AS4A-SC column (Dionex), 4 mm, flow 1 ml $\min^{-1}$, isocratic 50% 50 mM NaOH, 50% H_2O.

Synthesis of ethyl 4-trimethylstannylbenzoate **2**. Ethyl 4-iodobenzoate **1** (0.705 g, 2.54 mmol) was dissolved in freshly distilled toluene (15 ml). Hexamethylditin (1.453 g, 4.44 mmol) and $Pd(PPh_3)_4$ (0.050 g, 0.043 mmol) were added and the mixture was refluxed for 14 h under nitrogen atmosphere. The solvent was removed *in vacuo* and the product was purified by flash chromatography (silica; pentane containing 2% Et_3N to wash out the unreacted tin followed by pentane–ethyl acetate 15:1 containing 2% Et_3N to elute the product) to yield 0.739 g (93%) of **2**. TLC 10:1 hexane–ethyl acetate, $R_f = 0.32$. ^{1}H NMR: δ 8.0 (d, 2 H), 7.6 (d, 2 H), 4.4 (q, 2 H), 1.4, (t, 3 H), 0.3 (s, 9 H). ^{13}C NMR: δ 167, 149, 136, 130, 128, 60.8, 14.3, −9.6.

Synthesis of 4-trimethylstannylbenzoic acid **3**. The ethyl 4-trimethylstannylbenzoate **2** (0.739 g, 2.36 mmol) was hydrolysed in refluxing ethanol (15 ml) and 1 M NaOH (4.7 ml) for 4.5 h. Most of the ethanol was removed *in vacuo*; the residue was acidified with 1 M HCl and extracted with ether (5 × 10 ml). The combined extracts were dried over Na_2SO_4 and filtered, and the solvent was removed *in vacuo* to give 0.642 g (95%) of a white solid of crude **3**. ^{1}H NMR: δ 8.0 (d, 2 H), 7.6 (d, 2 H), 0.3 (s, 9 H).

Synthesis of N-*succinimidyl 4-trimethylstannylbenzoate* **4**. Crude **3** (0.458 g, 1.61 mmol), *N,N′*-disuccinimidyl carbonate (0.494 g, 1.93 mmol) and pyridine (distilled from calcium hydride) (0.16 ml, 1.9 mmol) were dissolved in dry acetonitrile (8 ml) and refluxed under a nitrogen atmosphere for 6 h. The solvent was removed on a rotary evaporator and the product was purified by flash chromatography (silica; pentane–ether 2:8 containing 2% Et_3N), to give 98 mg (16%) of *N*-succinimidyl 4-trimethylstannylbenzoate **4**. The product was recrystallised from hexane–ethyl acetate to give white crystals. TLC ether–hexane 8:2, $R_f = 0.18$. ^{1}H NMR: δ 8.05 (d, 2 H), 7.65 (d, 2 H), 2.8 (s, 4 H), 0.35 (s, 9 H). ^{13}C NMR: δ 169, 161, 153, 136, 129, 124, 26.6, −9.5.

Production of ^{76}*Br*. ^{76}Br was produced by a ^{76}Se(p,n)^{76}Br reaction using the Scanditronics MC-17 cyclotron at Uppsala University PET Centre and a ^{76}Se enriched Cu_2Se pellet as target.[19] For production of a normal batch of [^{76}Br]bromide, the target was irradiated for 1 h with a 10 μA beam current. The target was removed manually from the cyclotron vault the following day and placed in a separation oven. The bromide was recovered from the target using a thermochromatographic method whereby the target was heated to 1090 °C under a flow of argon and the [^{76}Br]bromide released was deposited on the walls of a piece of PTFE tubing. The tubing was rinsed with water or ethanol (200 μl) providing 20–25 MBq [^{76}Br]bromide per μAh (40–50 MBq/μAh at EOB, end of bombardment).

The [^{76}Br]bromide solution was purified using a C-18 SPEC-column (Ansys Inc., Irvine, CA, USA) (15 mg). The column was conditioned with ethanol (200 μl) followed by water (200 μl) and the bromide was loaded onto the column in 200 μl of water and eluted with water (100 μl). The water was removed by azeotropic distillation with acetonitrile (0.1–0.2 ml) at 70 °C under a flow of helium, and the residue was redissolved in ethanol.

Synthesis of N-*succinimidyl 4-[*^{76}*Br]bromobenzoate* **5**. **4** (0.8–1.0 mg, 2.1–2.6 μmol) was dissolved in 1% acetic acid in ethanol (70 μl) and added to [^{76}Br]bromide in ethanol (200 μl). Chloramine-T in ethanol (6 mg ml^{-1}, 15 μl, 0.4 μmol) was added and the mixture was heated at 70 °C for 10 min. A sample was removed from the reaction mixture and analysed by HPLC system 1a to check for unreacted [^{76}Br]bromide. The solvent was evaporated under a stream of nitrogen and the residue was redissolved in hexane–ethyl acetate 70:30 and purified by semi-preparative HPLC system 2 to give pure ^{76}BrNHS in ≈5 ml eluent. The identity of the product was confirmed by coelution with BrNHS, using TLC or OPLC, hexane–ethyl acetate 50:50 and HPLC-system 1a. Isolated radiochemical yields were 45–60%, radiochemical purity >99% and the specific radioactivity 20–200 GBq μmol^{-1} (using HPLC system 1b). Me_3SnNHS system 1: $t_R = 13.7$ min, $k' = 8.1$; system 2: $t_R = 10.3$ min; ^{76}BrNHS system 1: $t_R = 5.7$ min, $k' = 2.8$; system 2: $t_R = 13.0$ min.

Oligonucleotides. Oligonucleotides were synthesised on a 1 μmol scale using the phosphor amidite method,[22–24] on an Oligo 1000 DNA Synthesizer (Beckman) with CED-phosphor amidites (Beckman). The hexylamine 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 Applied Biosystems (Foster City, CA, USA).[25] The crude oligonucleotides were purified by gel filtration (NAP-5 columns, Pharmacia Biotech) followed by precipitation from a 3 M pH 4.8 AcOH–NaAc buffer using cold ethanol.[26,27]

Phosphorothioate oligonucleotides with a hexylamine linker in the 5′-position were purchased from Scandinavian Gene Synthesis AB (Köping, Sweden).

The oligonucleotide sequence chosen as a model for this work was 5′-CA CCT TAG TGT CCC CTT TTG, which is an antisense sequence for rat chromogranin.[14]

Macromolecule labelling. The solvents were removed from ^{76}BrNHS, prepared as described above, under a stream of nitrogen and the residue containing ^{76}BrNHS was used as follows.

(A) Oligonucleotides: the residue was dissolved in DMF (20 μl) followed by the ODN (10 A_{260} OD units, ≈70 nmol) in 50 mM borate buffer pH 8.5 (80 μl). The mixture was heated at 40 °C for 30 min, until all ^{76}BrNHS was consumed according to HPLC system 1b, then diluted with 50 mM TEAA (100 μl) before perfusion chromatography purification, system 4b. ODN: $t_R = 17.2$–19.2 min, $k' = 50.4$–51.6; ^{76}BrODN: $t_R = 23.3$–23.5 min, $k' = 68.7$–69.9.

(B) Phosphorothioate oligonucleotide: the residue was dissolved in DMF (30 μl) followed by the S-ODN (≈70 nmol) in water (35 μl) and 1 M carbonate buffer pH 9 (35 μl). The mixture was heated at 50 °C for 30 min and was diluted with TEAA (100 μl) before purification using perfusion chromatography, system 4a. S-ODN: $t_R = 22.2$–22.8 min, $k' = 62.4$–64.1; ^{76}BrS-ODN: $t_R = 29.8$–30.0 min, $k' = 84.1$–84.7.

(C) Proteins: a protein solution of human serum albumin (HSA) or chromogranin (100 μl, 1 mg ml^{-1}) in 50 mM borate buffer pH 8.5 was added and the mixture was heated at 35–40 °C for 40 min. The protein was purified using gel chromatography, system 3. The high molecular weight fraction was collected, $t_R = 1.4$ min.

Conclusions

N-Succinimidyl 4-[^{76}Br]bromobenzoate was synthesised from *N*-succinimidyl 4-trimethylstannylbenzoate by oxidative bromination and conjugated to macromolecules containing a primary amine functionality. The synthesis of ^{76}BrNHS presented was reliable, giving isolated radiochemical yields in the range 45–60% and specific radioactivity in the range 20–200 GBq μmol^{-1}.

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