Synthesis and radioiodination of a stannyl oligodeoxyribonucleotide

Hayes Dougan*, John B. Hobbs1, Jeffrey I. Weitz2 and Donald M. Lyster3

TRIUMF, 4004 Wesbrook Mall, Vancouver, BC V6T 2A3, Canada, 1NAPS Unit, Biotechnology Laboratory, University of British Columbia, Vancouver, BC V6T 1Z3, Canada, 2McMaster University, Hamilton, Ontario L8V 1C3, Canada and 3Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC V6T 1W5, Canada

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ABSTRACT

Synthesis and radioiodination of a stannyl oligodeoxyribonucleotide were undertaken to evaluate a gamma ray emitting ODN ligand for thrombus imaging in vivo. Synthesis of the ODN was based on modified automated β-cyanoethyl phosphoramidite chemistry with an organotin nucleoside (dU*) coupled to a thrombin binding aptamer sequence to give d(U*GGTTGTGTTGG). The synthesis accommodated dU*, which is destannylation by iodine or acids. Fourteen standard synthesis cycles were followed by one ‘stannyl synthesis cycle’, distinguished by Fmoc protection, omission of capping, oxidation by an organic peroxide and cleavage by ammonium hydroxide. The organotin nucleoside phosphoramidite {5′-[fluorenymethoxycarbonyl]-5-(E)-[2-tri-n-butylstannylvinyl]-2′-deoxyuridine-3′-(2-cyanoethyl N,N-di-isopropyl phosphoramidite)} was prepared from 5-ido-2′-deoxyuridine. A customized mild rapid workup included deprotection with methylamine, and reverse phase HPLC with CH3CN/triethylammonium bicarbonate. Pure stannyl ODN was highly retained by reverse phase HPLC. Radioiodination of stannyl ODN (100 µg) provided 125I-labeling yields up to 97%. Five alternative oxidants were effective. High specific activity [125I]-ODN (15 000 Ci/mmol) was recovered, separated from unlabeled isomers. Excellent reverse phase HPLC resolution of ODN isomers (alternatively I, Cl, H or Br in vinyl deoxyuridine) was essential. The affinity of the iodovinyl aptamer analog (Kd = 36 nM) for human α-thrombin was similar to the native aptamer (Kd = 45 nM).

INTRODUCTION

We wish to label the aptamer sequence d(GGTTGTGTTGG) with a gamma emitting radioiodine, and evaluate it as a thrombus imaging radiotracer. The aptamer was selected by combinatorial chemistry for binding to human thrombin (Kd = 20 nmol) (1). The aptamer assumes a triple loop structure with two guanine quartets in solution; it functions in animal serum as an anticoagulant (2). The radioiodines permit labeling DNA with diverse gamma emitters (125I and 131I; SPECT imaging) and positron emitters (124I; PET imaging). Direct radioiodination of pyrimidine nucleosides at C5 is feasible, but it is unsatisfactory for polymeric DNA. In situ metellation/iodination with thallium (3) or mercury (4) can facilitate carrier-added radioiodination of DNA. A stable organotin precursor would improve the radioiodination of DNA. Vinyl and aromatic organotin compounds are highly reactive to no-carrier-added iododestannylation (5,6). Organotin compounds also possess notable hydrophobicity, so that a high specific activity iodide product is readily separated from the precursor. Organotin precursors are valuable for the preparation of radioiodine pharmaceuticals. We seek to incorporate an organotin analog of 2′-deoxyuridine (dU*) to obtain the sequence d(U*GGTTGTGTTGG), and then to radioiodinate the organotin sequence. The nucleoside of choice is IVDU (and its organotin analog). IVDU is an antiviral nucleoside (7) and radiotracer (8,9) dependent on herpesvirus thymidine kinase (10), that does not enter the DNA of uninfected cells (11). An applicable organotin nucleoside synthesis is available (12). Accessible automated β-cyanoethyl phosphoramidite DNA synthesis is the technology of choice (13,14). However the iodine and acidic reagents of the standard synthesis cycle will destannylate the organotin nucleoside residue prematurely. The available chemistries require modification for efficient synthesis of organotin DNA, consequently permitting organotin DNA to be evaluated as a precursor for radiohalogenated DNA pharmaceuticals.

MATERIALS AND METHODS

[125I]NaI in 0.1 N NaOH was obtained from Nordion International. Gamma rays were detected with a well type NaI detector (Beckman), a dosimeter (Capintec) or (for HPLC effluent) a sodium iodide crystal (Bicron, 2 in) detector supported by Ortec amplifier (490B), ratemeter (541) and power supply (478) modules. The NMR Laboratory of the University of British Columbia (UBC) utilized a 200 MHz Bruker AC-200 (1H-NMR) or a 300 MHz Varian XL-300 (13P-NMR) for our specimens. Elemental analysis was performed by the UBC Microanalytical Chemistry Laboratory. The UBC Mass Spectroscopy Laboratory performed Desorption Chemical Ionization (DCI) mass spectrometry in the positive ion mode with methane gas in the Delsi Nermag R10-10 C. Inductively coupled plasma mass spectrometry (ICPMS) analysis of tin was performed by Elemental Research Inc. of North Vancouver, BC. ODNs were resolved by reverse phase HPLC (C18, 5 µm, 4 mm × 250 mm; Pharmacia P/N: 80-1266-38; or Phenomenex P/N: 000-G973-E0) with CH3CN 10–50% (42 min)

*To whom correspondence should be addressed. Tel: +1 604 222 1047; Fax: +1 604 222 1074; Email: dougan@triumf.ca
in triethylammonium acetate (TEAA) (0.13 M, pH 7.0) (1.0 ml min). [An alternative buffer was TEA bicarbonate (TEAB) (0.15 M, pH 7.0).] A variable wavelength UV detector was used.

Nucleosides were resolved by reverse phase HPLC (Waters Nova Pak C18 4 μm; 8 mm × 100 mm) in CH3CN 5–25% (42 min) (TEAA) (1.0 ml min). Some sources are listed: ‘Anhydrous grade’ acetonitrile, pyridine and toluene for nucleoside synthesis (Aldrich); ‘DNA synthesis grade’ acetonitrile and tetrzole (Applied Biosystems); 5-iodo-2′-deoxyuridine (Sigma); 2-cyoanoethyl N,N′,N′,N′-tetraisopropylphosphorodiamidite (Peninsula); 3-chloroanisole benzoic acid (10); iodogen and iodobeads (Pierce); silica TLC plates (IB2F) (Baker); and silica gel 7734 (Merck).

5′-fluorenylmethoxycarbonyl-5′-iodo-2′-deoxyuridine (2)

The standard Fmoc derivitization procedure was used (16) (5.3 g, 57% yield). 1H-NMR (DMSO-d6) (200 MHz): 2.15 (m, 2H, H-2′); 3.92 (d, 1H, H-4′); 4.18 (d, 1H, H-3′); 4.30 (m, 2H, H-5′); 4.33 (t, 1H, F-C′H); 4.55 (d, 2H, F-C′H2); 5.43 (d, 1H, OH′-3′; J = 4.09 Hz); 6.08 (t, 1H, H-1′; J = 6.59 Hz); 7.32 (t, 2H, Fmoc -′′-butylstannyl)ethene (17) (750 mg, 1.24 mmol), bis(triphenylphosphine) palladium (II) chloride (27 mg, 0.08 μmol), acetonitrile (10 ml) and toluene (5 ml). The mixture was divided between two 10 ml serum vials which were sealed, flushed with nitrogen, and heated at 80°C with magnetic stirring. A black precipitate appeared abruptly at 18 min; the mixture was stored at −20°C. The consumption of 2 was complete as judged by TLC (toluene/CH3CN; 1/1). The reaction mixture was purified by HPLC (TEAB). Nucleoside compositions were determined using snake venom phosphodiesterase.

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Preparation of 5′-[fluorenylmethoxycarbonyl]-5′-[E]-[2-tri-n-butylstannyllvinyl]-2′-deoxyuridine-3′-2-cyanoethyl N,N-diisopropylphosphorodiamidite) (5) in solution

The protected nucleoside 3 (21.4 mg, 28 μmol) was dried by two evaporations from dry acetonitrile/toluene (300/25, v/v; 325 μl), and then dissolved under argon in dry acetonitrile/toluene (115/15, v/v; 130 μl). Tetrazole (0.45 M in acetonitrile, 84 μl, 37.8 μmol) and 2-cyanoethyl N,N′,N′,N′-tetraisopropylphosphorodiamidite [4] (18) (12 μl, 37.8 μmol) were added and stirred for 6 h at ambient temperature. The reaction was terminated by filtration of the precipitate (disisopropylammonium tetrazolate), giving a solution ~0.1 M in 5. TLC (CH3Cl2/EtAc/Py: 80/20/2) revealed (UV) two nearly equal spots (5) near the front (Rf = 0.66 and 0.78), and a very faint spot of substrate (3) was noted (Rf = 0.13). A narrow band of 3′,3′-dinucleoside monophosphate was identified (Rf = 0.26). 31P-NMR (H3PO4 standard) was performed with a 6 h product diluted in CH3CN/CD3CN (19,20).
phosphodiesterase (SVDE) and alkaline phosphatase, following a literature procedure (24).

**Determination of aptamer affinity for thrombin**

Human α-thrombin (Enzyme Research) was derivatized with fluorescein-labeled D-Phe-Pro-Ala-CH$_2$Cl (PPACK) (Haematologic Technologies) blocking the active site (29). Thrombin solution (20 mM Tris, 150 mM NaCl, pH 7.4) was titrated with aptamer. The fluorescence intensity was monitored using a Luminescence Spectrophotometer (LS50b; Perkin Elmer) (Fig. 1), and fit by non-linear regression analysis to equation 10 of ref. (30) to estimate $K_d$.

**Radioiodination**

Radioiodinations were carried out in a fumehood, in miniaturized stoppered glass tubes. Literature procedures were performed with Chloramine T (100 µg) (12), Iodogen (10 µg) (25), laccoperoxidase/Seaphores (Worthington) (27) or peracetic acid (28). (Table 2). Reactions utilized ODN 7 (100 µg) and [123$I$]NaI (4.82 mCi; 8 µl; 0.1 M NaOH) with an Iodobead. Following 5 min, the reaction solution was quenched in water (160 µl) with Na$_2$S$_2$O$_3$ (10 µg). The product (3.87 mCi) was purified by HPLC (TEAB), and the ODN fraction (3.20 mCi; 90% labeling yield) was recovered. A portion of the product (2.86 mCi) was divided among four plastic tubes and evaporated in a vacuum centrifuge. The recovered yield at 110 min was 2.53 mCi (52% uncorrected). The product was 98% ODN 9 with 2% inorganic iodide. One ODN portion was re-run on HPLC. The total ODN absorbance was estimated 4 x 6.48 x 10$^{-3}$ OD$_{254}$ or 166 pmol, and the specific activity for 2.53 mCi was 15 000 Ci/mmol. Physiological solutions were formulated in potassium phosphate buffer (0.05 M, pH 7.0).

**RESULTS**

An alternative ‘stannyl synthesis cycle’ was developed in order to accommodate the stannyl reactivity. An organotin nucleoside was prepared with base-cleavable fluorenylmethyl (Fmoc) protection. Scheme 1 illustrates how 5-iodo-2$'$-deoxyuridine (1) was treated with 9-fluorenylmethyl chloroformate/pyridine to give 5$'$-[fluorenylmethoxy carbonyl]-5-ido-2$'$-deoxyuridine (2) (57%). Treatment of 2 with E-1,2,-bis(tri-(n-butyl)stannyl)ethene and a catalytic quantity of (Ph$_3$P)$_2$PdCl$_2$ in acetonitrile/toluene provided 5$'$-[fluorenylmethoxy carbonyl]-5-(E)-[2-tri-n-butylstannylvinyl]-2$'$-deoxyuridine (3) in 25% yield. Analytical, mass spectroscopy and NMR results were satisfactory.

**In situ phosphorylation** was used to give the desired organotin nucleoside phosphoramidite, 5$'$-[fluorenylmethoxy carbonyl]-5-(E)-[2-tri-n-butylstannylvinyl]-2$'$-deoxyuridine-3$'$-(2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodi amidite) (5). The desired level of nucleoside phosphorylation (85–90%) was obtained when the ratio of 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodi amidite (4) to
nucleoside was 1.35:1. Two Fmoc phosphoramidite diastereomer spots were observed near the solvent front following TLC (31, 32). The reaction required 5 h to reach completion. $^{31}$P-NMR analysis identified the nucleoside diastereomers (148.6 and 149.0 p.p.m.) and phosphoramidous acid (14 p.p.m.). The $^{31}$P peak of reagent or of 9 was not observed in the product solution (18–20). The combined TLC/NMR observations led to the important inference that the tributylstannyl moiety of the nucleoside remained intact until it was incorporated into DNA.

The organotin nucleoside phosphoramide (5) was coupled with DNA. First, d(GGTGTTGGTTG)G (1 µmol) was prepared by 14 standard phosphoramidite synthesis cycles; the oligonucleotide was left attached to the CPG support. The stannyl synthesis cycle reactions were then carried out using syringe technique. The resin was dehydrated with dry CH$_3$CN, and then treated with 5 (28 µmol) ion the presence of tetrazole catalyst. The reaction required 5 h to reach completion. 31 P-NMR analysis indicated (ICPMS) in ODN 7 (100 µg, $^{123}$I)NaI (1 mCi), and five different oxidants. Table 2 lists the radioiodination yields. The N-chloro oxidants led to various quantities of ODN 10. Destannylated ODN 8 was found in all the reactions. Most ODN 7 originated from frozen ODN 7 samples. ODN 8 was elevated with peracetic acid. Some $^{125}$I was found to be the lactoperoxidase matrix.

HPLC of the product revealed the unmodified ODN 6 and two additional peaks, ODNs 7 (~20%) and 8 (~70%). Elemental tin mass cluster was identified (ICPMS) in ODN 7 and not in ODNs 6 or 8. Upon acetic acid treatment, ODN 7 was converted to ODN 8, while ODNs 6 and 8 were inert. Upon treatment with iodine/CH$_3$CN, ODN 7 was converted to the new ODN 9, while ODNs 6 and 8 were inert. This led to the identification of ODN 7 as the organotin species and ODN 8 as the destannylated product as indicated in Table 1.

ODN 7 was apparently destannylated during workup and HPLC. Increased yield of ODN 7 followed revised, briefest deprotection with a mixture of methylamine and ammonium hydroxide (1:1; N-AMA) (20 min at 55°C). ODN 7 remained intact during standard deprotection (1 h at 20°C in 35% ammonium hydroxide). Destannylations of 15–30% were obtained with the revised workup. A ‘satellite’ isomer of ODN 7 (~20% of 7) appeared on HPLC following cleavage with methylamine or AMA. To suppress the new isomer, cleavage was always carried out in ammonium hydroxide. Deprotection was divided into two stages: a 10 min treatment with ammonium hydroxide was followed by a 20 min treatment with AMA. Standard evaporation in a vacuum centrifuge concluded the workup. Since ODN 7 was unstable in TEAA (with a half-life of ~5 h), the HPLC was improved by adopting TEAB, leading to pure intact ODN 7.

Iodinated ODN 9 was prepared by treating ODN 7 with N-iodosuccinimide. Brominated ODN 11 was likewise prepared using N-bromosuccinimide. ODN 8 was prepared by treating ODN 7 with acetic acid. The nucleoside composition of the derived DNA molecules showed only the standard nucleosides dG and T and the appropriate vinyl nucleoside (BrVDU, IVDU or VDU) in the correct molar ratio (9:6:1; dG: T: vinyl nucleoside).

The binding and $K_a$ of ODNs 6 and 9 were determined with human α-thrombin. A fluorometric binding assay was based on a fluorescein-PPACK active-site-blocked derivative of human α-thrombin (Fig. 1). A $K_a$ of 36 nM was found for ODN 9, while a $K_a$ of 45 nM was found for ODN 6. No binding to thrombin was observed with the scrambled aptamer sequence, d(GGTGTTGGTTG).

A preliminary radioiodination trial (with Chloramine T) demonstrated that purified ODN 7 substrate led to a pure ODN 9 product. Non-purified ODN 7 substrate led to numerous small $^{123}$I peaks (in addition to ODN 9) following radioiodination. Subsequent trials were performed to determine whether ODN 7 is very accessible to diverse radioiodination reactions. Radioiodinations were carried out with pure ODN 7 (100 µg, $^{123}$I)NaI (1 mCi), and five different oxidants. Table 2 lists the radioiodination yields. The N-chloro oxidants led to various quantities of ODN 10. Destannylated ODN 8 was found in all the reactions. Most ODN 7 originated from frozen ODN 7 samples. ODN 8 was elevated with peracetic acid. Some $^{125}$I was found to be the lactoperoxidase matrix.

**Table 1. Structure of the stannyl ODN d(U*GGTTGGTTG)G and related analogs**

<table>
<thead>
<tr>
<th>ODN</th>
<th>dU* analog</th>
<th>R</th>
<th>$\tau_b$</th>
</tr>
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<tbody>
<tr>
<td>6</td>
<td>absent</td>
<td>–</td>
<td>14.5</td>
</tr>
<tr>
<td>7</td>
<td>Bu$_3$SnVDU</td>
<td>Bu$_3$Sn</td>
<td>41.0</td>
</tr>
<tr>
<td>8</td>
<td>VDU</td>
<td>H</td>
<td>15.5</td>
</tr>
<tr>
<td>9</td>
<td>IVDU</td>
<td>I</td>
<td>18.0</td>
</tr>
<tr>
<td>10</td>
<td>CV DU</td>
<td>Cl</td>
<td>16.5</td>
</tr>
<tr>
<td>11</td>
<td>BrVDU</td>
<td>Br</td>
<td>17.3</td>
</tr>
</tbody>
</table>

$^{a}$dU* is a 5-E-[2-R-vinyl]-2′-deoxyuridine analog; R is the functional group. $^{b}$The typical HPLC retention time is given in min.

**Table 2. Products following radioiodination of ODN 7 with [123I]NaI**

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>µg</th>
<th>Ref.</th>
<th>Min</th>
<th>ODN 9</th>
<th>ODN 9</th>
<th>ODN 8</th>
<th>ODN 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramine T</td>
<td>100 (12)</td>
<td>5</td>
<td>96</td>
<td>12</td>
<td>68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodogen</td>
<td>10 (31)</td>
<td>5</td>
<td>94</td>
<td>20</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodobead</td>
<td>(32)</td>
<td>5</td>
<td>97</td>
<td>9.2</td>
<td>9.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peracetic acid</td>
<td>(34)</td>
<td>1</td>
<td>64</td>
<td>93</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>(33)</td>
<td>10</td>
<td>80</td>
<td>12</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$Each reaction contained ODN 7 (100 µg) and [123I]NaI (1 mCi).
$^{b}$Labeling yield % is given for ODN 9.
$^{c}$ODNs 8 and 10 are given as % of total ODN.

The Iodobead mediated labeling yield was determined with different concentrations of ODN 7 (100 µg, 97%; 50 µg, 86%; 25 µg, 73%; 10 µg, 12%). Figure 2 illustrates HPLC resolution of the products at 1 and 5 min following the radioiodination of ODN 7 (100 µg). The increase in ODN 10 (2.5 and 9.4%) can be seen. Roughly 1% of the unlabeled material trailed into the ODN 9 fraction. An illustrative 5 min reaction with 4.8 mCi [123I]NaI gave 90% labeling. A portion was evaporated and provided 2.53 mCi product at 110 min (52% uncorrected yield). HPLC analysis indicated 98% ODN 9, 2% iodide, and specific activity 15 000 Ci/mmol.

**DISCUSSION**

Stannyl ODN 7 was obtained by the following strategy. Fourteen rounds of standard synthesis cycle were followed by a single
round of ‘stannyl synthesis cycle’ distinguished by Fmoc protection, omission of capping, oxidation by an organic peroxide and cleavage by ammonium hydroxide. A mild workup utilized protection, omission of capping, oxidation by an organic peroxide and the HPLC resolution cannot be attributed to one single factor (13, 33–35).

The net result of the DNA synthesis operation is that one organotin nucleoside is located at the 5’ terminal of ODN 7. One (and no more) organotin site is desired for radioiodination. Multiple organotins would lead to multiple pharmaceutical products and leave residual organotin in the labeled ODN. The aptamer consensus structure is effective in ODN 9, and that ODN 9 should be evaluated for thrombin imaging.

ACKNOWLEDGEMENTS

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