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Tritium labeling of full-length small interfering RNAs

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A simple procedure is described for full-length single internal [^{3}H]-radiolabeling of oligonucleotides. Previous labeling strategies have been applied to large molecular weight compounds such as proteins and oligonucleotides, for example, iodination and ^{111}In labeling via covalently bounded chelators. However, a procedure has not yet been reported for single internal radiolabeling of oligonucleotides that preserves the molecular structure (^{3}H replacing a ^{1}H). In following our strategy, the radiolabel can be strategically placed within a stable and predetermined internal position of the siRNA. This placement was accomplished by placing a 5-bromouridine or 5-bromo-2′-O-methyluridine phosphoramidite building block into the middle of the antisense strand using standard phosphoramidite chemistry. The deprotected full-length antisense strand was tritium labeled by bromine/tritium exchange, catalyzed by palladium on charcoal in the predetermined 5-position of either uridine or 2′-O-methyluridine. Internal placement of the building block within the oligonucleotide sequence and label placement at 5-position decreases the likelihood of the label to be readily cleaved from the oligonucleotide *in vivo*, and loss of the label by spontaneous tritium/hydrogen exchange. The tritiated single-stranded and double-stranded RNAs were also shown to be radio and chemically stable for at least 6 months at −80 °C. This allows more than sufficient time to conduct pharmaceutical formulation and pharmacokinetic studies.

Keywords: oligonucleotides; siRNA; tritiation; halogen/tritium exchange; final step radiolabeling

Introduction

As current pharmaceutical drug research still largely focuses on interactions of small molecules with enzymes and proteins, nucleic acid-derived molecules involved with RNA interference (RNAi) offers a unique opportunity as novel drug candidates. These siRNA drugs would be designed to trigger the inhibition or breakdown of messenger RNA (mRNA).

The discovery of RNAi by Fire and Mello[1] has been considered a revolution in biotechnology, and the inventors were awarded the 2006 Nobel Prize. The RNAi pathway is initiated by double-stranded RNA (dsRNA) that, when introduced into cells from exogenous sources, are termed small interfering RNAs (siRNAs). In principle, RNAi can silence or slow down the production of any protein. In recent years, this role in cell regulation, which uses a more specific and targeted approach, has led to a significant interest in siRNA potential by the pharmaceutical industry. This area of research offers a new way to treat human diseases that may not be targetable with traditional approaches.

For the design and development of siRNAs throughout nonclinical and clinical pharmaceutical development, radiolabeled molecules offer unique and beneficial advantages. Although several oligonucleotide radiolabeling methods have been previously reported, limitations exist with respect to the choice of radioisotope, position of the label, and chemical modifications needed for coupling of the label.

Radiolabeled oligonucleotides labeled at chemically unstable positions or at the end of the oligonucleotide might pose the risk of the label being cleaved from the oligonucleotide *in vivo*. The limitations of these labeling methods could make it challenging to interpret pharmacokinetic and metabolic studies.

End-labeling has typically been accomplished by addition of a [^{32}P]-phosphate group to the 5′-end of the oligonucleotide,[2–7] or by using ^{14}C-labeled uridines in the 3′-end region of the molecule.[8] This is a valid approach, but the position of the label still may have some drawbacks for *in vivo* pharmacokinetics and absorption, distribution, metabolism and elimination studies. Uniform radiolabeling of phosphorothioate oligonucleotides[9,10] with ^{35}S is another option. However, this labeling technique is restricted to oligonucleotides with a phosphorothioate backbone. Facile removal of the radiolabel by exonucleases, short half-lives and radiation protection measures against strong beta radiations (^{32}P) are some drawbacks using these isotopes.[11]

Tritium radiolabeling procedures of oligonucleotides have also been reported in the literature, for example, internal labeling of deprotected phosphorothioate oligodeoxynucleotides randomly labeled at the unstable C8-position of the purines by exchange

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with tritiated water as described by Graham *et al.*[9] This procedure has been adapted to siRNAs[12,13] but has limitations in biological assays because of radiolytic degradation and formation of tritiated water. Sands *et al.*[14] described an enzymatic procedure for single internal final step labeling of deprotected oligodeoxynucleotides. [^{3}H]Methylation, which occurs at the C5-position of the first deoxycytidine in the GCGC recognition site of the enzyme (*Hha*I methylase). The procedure requires synthesis of a complementary sense strand to prevent tritiation of the sense strand. The sense strand is preferably made with 5-methyldeoxycytidine. Single internal labeling of the stable C5′-position of the sugar of oligodeoxynucleotide has been described by Tan *et al.*[15] This labeling occurred during oligonucleotide synthesis by an oxidation/reduction strategy. Le Doan *et al.*[11] have reported a procedure for final step labeling of deprotected oligodeoxynucleotides containing a terminal amino group linker reacting with [2,3-^{3}H]succinimidyl propionate, and an example with the amino linker attached to the C5-position of a terminal thymidine was also described.

Radioactive isotopes such as ^{111}In have also been used for radiolabeling. However, a major drawback of ^{111}In is the short half-life of the isotope (2.8 days), and the need of a cDTPA chelator covalently attached to the molecule of interest. The chelator as well as the bulky ^{111}In group could significantly affect the conformation or change the molecular properties of an oligonucleotide drug. As a consequence, this may alter or confound its pharmacodynamic and pharmacokinetic behavior. The resulting data may not be well understood, straight forward, representative or even accurate. Other labeling techniques such as fluorophores or direct coupling of the radio isotope ^{125}I have been used for labeling siRNAs. However, these labeling approaches may influence the pharmacokinetics and pharmacodynamics, as highlighted already for modified proteins (Swart *et al.*[16]).

Linear oligonucleotides are prepared by solid phase synthesis in a stepwise fashion, which suggests that the site-specific introduction of a radiolabel should be possible using correspondingly labeled monomers. To this end, we (J. H., F. N., H. A., unpublished results) originally explored the synthesis of a 5′-tritium labeled uridine phosphoramidite building block (5′-O-[(4,4′-dimethoxyphenyl) methyl]-2′-O-*tert*-butyldimethylsilyl-uridine-3′-O-[2-cyanoethyl N,N-(diisopropylamino)phosphor amidite). Although this compound could be used to incorporate a tritium label at low specific activity, any attempt to increase the tritium content in the building block led to failure of the oligonucleotide coupling reaction. The very same synthesis scheme, on the other hand, could be used for the successful incorporation of deuterium labels. We therefore presume a rapid, radioactivity-mediated decay of the phosphorous (III) building block at high tritium concentration.

Thus, a novel full-length labeling procedure was developed for a single internal tritium labeling to avoid post labeling chemical and conformational changes of the siRNA. This procedure depends upon the existence of a uridine or a 2′-O-methyluridine residue in the sequence. Furthermore, the exact position of the radio label is known. This oligonucleotide radiolabeling procedure presents some advantages compared with previously reported methods. For instance, there is no chemical or molecular modification (^{3}H replacing a ^{1}H), that is, minimal or no change in the pharmacological activity, kinetics and metabolic behavior of the molecule. In addition, an important prerequisite was successfully demonstrated for the radiochemical stability of the tritiated product.

Experimental section

Materials and methods

All commercial reagents were used without further purification unless otherwise noted. DNA/RNA oligonucleotides were synthesized in-house (Novartis; Basel Switzerland). Deuterations and tritiations were performed using a tritium manifold system from RC Tritec (external partner company). All air and moisture-sensitive reactions were carried out in argon-flushed, two-neck flasks sealed with rubber septa, and the reagents were introduced by syringe and dried over molecular sieves 3 Å prior to use. Progress of reactions was monitored by thin layer chromatography on precoated Merck (Whitehouse Station, NJ, USA) silica gel plates (60 F-254). Visualization was accomplished by UV light (254 nm). Flash chromatography separations were performed on Merck silica gel 60 (0.040–0.063 mm). Melting points were recorded using Büchi 535 digital melting point apparatus (New Castle, DE, USA). Centrifugations were carried out using Thermo Scientific Heraeus Multifuge 3SR + centrifuge (Waltham, MA, USA) or Mistral 1000 centrifuge. HPLC analysis and purifications were performed on Agilent 1100 or 1200 Series systems (Santa Clara, CA, USA). Online radioactivity detection was performed in series to UV detection by adding 4 × LC flow of Flo-Scint A scintillation cocktail to the solvent stream. This mixture was subsequently investigated online with a Berthold LB 513 system using a 200 or 100-µl Z-cell. Offline radioactivity detection was performed by liquid scintillation counting (LSC) using Perkin Elmer Wallac 1414 Liquid Scintillation Counter or Packard 2200CA TRI-CARB Liquid Scintillation Analyzer (Waltham, MA, USA). NMR spectra were recorded using 400, 500, 600-MHz Bruker instruments. Coupling constants (*J*) are reported in Hz and chemical shifts (δ) in parts per million (ppm) relative to the residual proton signal of the deuterated solvent used ($CDCl_3$, DMSO-d6 or D_2O, 7.26, 2.50 (^{13}C 39.52) and 4.79, respectively). ^{3}H-NMR spectra were calibrated indirectly via the deuterated solvent in the ^{1}H-NMR spectra. The following abbreviations are used to denote multiplicities: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. MS (mass spectrometry) and high-resolution MS (HRMS) of monomers were performed by NIBR Analytical Sciences (Novartis) or recorded using Waters Micromass ZQ2000 or Thermo Finnigan LCQ Advantage MAX spectrometers. MS and HRMS of oligonucleotides were recorded using Waters LCT Premier XE or Thermo LTQ-Orbitrap XL mass spectrometers.

Synthesis of 5-bromo-2′O-methyluridine phosphoramidite

*Protected Nucleosides (**1–3**)*

5-bromo-3′,5′-O-benzoyl-2′-O-methyluridine (**1**): To a suspension of 1,3,5-tri-O-benzoyl-2-O-methyl-α/β-D-ribose (10.00 g, 21.0 mmol, 1.0 equiv) and 5-bromouracil (4.01 g, 21.0 mmol, 1.0 equiv) in abs. acetonitrile (80 ml) was added bis(trimethylsilyl)acetamide (10.41 ml, 8.54 g, 42.0 mmol, 2.0 equiv) under argon. The reaction mixture was heated to reflux upon which a clear solution was formed. After 30 min, trifluoromethanesulfonic acid trimethylsilylester (5.71 ml, 7.00 g, 31.5 mmol, 1.5 equiv) was added and heating was continued for 2 h. The reaction mixture was allowed to cool to room temperature (RT) and then diluted with chloroform (300 ml). The solution was washed first with water (150 ml) and then with saturated sodium bicarbonate (2 × 150 ml). The aqueous solutions were re-extracted with chloroform (150 ml). The combined organic layers were dried over sodium sulfate, filtered and concentrated *in vacuo*. The α/β diastereomeric ratio in the crude mixture was 1:1.8, the desired β-isomer was successfully isolated by washing the impurities (including the α-diastereomer) away with the following procedure: addition of heptane/EtOAc 1:1 (25 ml), vortex, centrifugation (2000 rpm, 3 min, 4 °C); removal of the supernatant, dissolving the precipitate in EtOAc; and removal of the solvent *in vacuo* yielded 6.07 g of **1** (53%) as a white solid. MF: $C_{24}H_{21}BrN_2O_8$; MW: 545.3361; M.p. 180–181 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 8.36 (br s, 1 H), 8.07 (dd, *J* = 12.6/8.3 Hz, 4 H), 7.80 (s, 1 H), 7.65–7.57 (m, 2 H), 7.48 (t, *J* = 7.6 Hz, 4 H), 6.04 (d, *J* = 3.8 Hz, 1 H), 5.43 (t, *J* = 5.7 Hz, 1 H), 4.81–4.64 (m, 3 H), 4.19 (t, *J* = 4.4 Hz, 1 H), 3.48 (s, 3 H); Confirmation of the β-isomer by ROESY

(500 MHz, $CDCl_3$): $H_{1'} \leftrightarrow H_{4'}$, $H_{1'} \leftrightarrow H_{2'\text{-OMe}}$, $H_{2'} \leftrightarrow H_6$, $H_{3'} \leftrightarrow H_6$; ^{13}C-NMR (151 MHz, DMSO-d6) δ (ppm): 165.52–164.87, 159.04, 149.69, 140.68, 133.70–133.50, 129.30–128.78, 96.77, 89.04, 80.02, 79.00, 71.03, 63.72, 58.38; HRMS (positive ESI) calculated for $C_{24}H_{22}BrN_2O_8$ $[M+H]^+$: *m/z* 545.0559; observed 545.0557 (0.37 ppm).

5-bromo-2′-O-methyluridine (**2**): **1** (5.06 g, 9.279 mmol, 1.0 equiv) was dissolved in methanol (70 ml) and stirred under argon. 7 N NH_3 in methanol (22.5 ml) was added and the mixture was stirred at RT under argon for 4 days. Removal of the solvent *in vacuo* was performed in a cautious manner. Flash chromatography (ethyl acetate/methanol) of the crude product yielded 2.79 g of **2** (89%) as a white solid. MF: $C_{10}H_{13}BrN_2O_6$; MW: 337.1240; M.p. 232–234 °C, literature[17] m.p. 235–237 °C; ^{1}H-NMR (400 MHz, DMSO-d6) δ (ppm): 11.83 (br s, 1 H), 8.53 (s, 1 H), 5.80 (d, *J* = 3.8 Hz, 1 H), 5.32 (t, *J* = 4.6 Hz, 1 H), 5.13 (d, *J* = 6.6 Hz, 1 H), 4.12 (q, *J* = 5.9 Hz, 1 H), 3.89–3.83 (m, 1 H), 3.81 (t, *J* = 4.3 Hz, 1 H), 3.75–3.54 (m, 2 H), 3.39 (s, 3 H), literature[17] ^{1}H-NMR (DMSO-d6) δ (ppm): 11.78 (s, 1 H), 8.50 (s, 1 H), 5.81 (d, *J* = 4 Hz, 1 H), 5.00–4.84 (m, 2 H), 3.41 (s, 3 H); ^{13}C-NMR (151 MHz, DMSO-d6) δ (ppm): 159.19, 149.78, 140.13, 95.78, 86.76, 84.82, 83.08, 67.79, 59.67, 57.65; HRMS (negative ESI) calculated for $C_{10}H_{12}BrN_2O_6$ $[M-H]^-$: *m/z* 334.9884; observed 334.9887 (0.90 ppm).

5-bromo-5′-[(4,4′-dimethoxyphenyl)methyl]-2′-O-methyluridine (**3**): **2** (2.79 g, 8.276 mmol, 1.0 equiv) was dissolved in pyridine (40 ml) under argon. 4,4′-dimethoxytrityl chloride (2.42 g, 7.143 mmol, 1.2 equiv) dissolved in pyridine (30 ml) was added portion wise during 20 min. The mixture was stirred at RT under argon for 24 h. Removal of the solvent *in vacuo* yielded a yellow oil, which was dissolved in chloroform (200 ml). The solution was first washed with saturated sodium bicarbonate (2 × 200 ml) and then washed with brine (200 ml). The aqueous solutions were re-extracted with chloroform (200 ml). The combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo* by azeotropic removal of pyridine with toluene (3 × 20 ml). Flash chromatography (heptane/ethyl acetate + 0.1% triethylamine) of the crude product yielded 3.16 g of **3** (60%) as a yellow solid. MF: $C_{31}H_{31}BrN_2O_8$; MW: 639.4904; M.p. 106–107 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 8.61 (br s, 1 H), 8.12 (s, 1 H), 7.43 (d, *J* = 7.1 Hz, 2 H), 7.37–7.27 (m, 6 H), 7.25–7.20 (m, 1 H), 6.85 (d, *J* = 8.1 Hz, 4 H), 5.94 (d, *J* = 2.8 Hz, 1 H), 4.50–4.44 (m, 1 H), 4.09–4.05 (m, 1 H), 3.95–3.91 (m, 1 H), 3.79 (s, 6 H), 3.64 (s, 3 H), 3.48 (d, *J* = 2.5 Hz, 2 H), 2.63 (d, *J* = 7.8 Hz, 1 H); ^{13}C-NMR (151 MHz, DMSO-d6) δ (ppm): 159.09, 158.14, 149.68, 144.69, 139.51, 135.45–135.25, 129.72, 127.93–127.65, 126.72, 113.29, 96.28, 87.57, 82.94, 82.29, 68.53, 62.91, 57.84, 55.04; HRMS (negative ESI) calculated for $C_{31}H_{30}BrN_2O_8$ $[M-H]^-$: *m/z* 637.1191; observed 637.1196 (0.78 ppm).

*Phosphoramidite (**4**)*

5-bromo-5′-[(4,4′-dimethoxyphenyl)methyl]-2′-O-methyluridine-3′-O-[2-cyanoethyl N,N-(diisopropylamino)phosphoramidite] (**4**): **3** (2.91 g, 4.551 mmol, 1.0 equiv) was dissolved in abs. dichloromethane (80 ml) under argon, and diisopropylammonium-tetrazolide (1.17 g, 6.826 mmol, 1.5 equiv) was added. Cyanoethyltetraisopropylphosphordiamidite (2.29 ml, 1.92 g, 6.371 mmol, 1.4 equiv) was added and the mixture was stirred at RT under argon for 7 h. Chloroform (100 ml) was added to the mixture and the solution was first washed with saturated sodium bicarbonate (2 × 50 ml), and then with brine (50 ml). The aqueous solutions were re-extracted with chloroform (50 ml). The combined organic layers are dried over sodium sulfate, filtered, and concentrated *in vacuo*. Flash chromatography (heptane/ethyl acetate + 0.1% triethylamine) of the crude product yielded 3.51 g of **4** (92%) as a yellow solid (phosphordiastereomers). MF: $C_{40}H_{48}BrN_4O_9P$; MW: 839.7083; M.p. 85–86 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 8.15 (br s, 1 H), 8.08 (s, 1 H), 7.43 (dd, *J* = 11.2/8.0 Hz, 2 H, H_c), 7.38–7.27 (m, 6 H), 7.26–7.20 (m, 1 H), 6.88–6.81 (m, 4 H), 6.01 and 5.94 (d, *J* = 4.8 & 3.3 Hz, 1 H), 4.62–4.46 (m, 1 H), 4.30–4.19 (m, 1 H), 4.08–4.03 (m, 1 H), 3.96–3.58 (m, 4 H), 3.80 (s, 6 H), 3–57/3.55 (3 H), 3.51–3.34 (m, 2 H), 2.65 (t, *J* = 6.2 Hz, 1 H), 2.41 (t, *J* = 5.7 Hz, 1 H), 1.23–1.02 (m, 12 H); ^{31}P-NMR (400 MHz, DMSO-d6) δ (ppm): 149.6 and 149.3, no internal reference; HRMS (positive ESI) calculated for $C_{40}H_{48}BrN_4NaO_9P$ $[M+Na]^+$: *m/z* 861.2240; observed 861.2239 (0.12 ppm).

Labeling synthesis

*[^{3}H]-Radiolabeling of monomer **2** by bromine/tritium exchange*

[5-^{3}H]2′-O-methyluridine (**5**): 5-bromo-2′-O-methyluridine **2** (3.43 mg, 0.010 mmol, 1.0 equiv) was dissolved dimethylformamide (DMF) (0.5 ml), the catalyst (10% Pd/C, 2.67 mg, 0.25 equiv) was added, then N,N-diisopropylethylamine (2 µl, 1.1 equiv). The reaction glass flask was connected to a tritium gas line. The reaction flask was then frozen in liquid nitrogen, evacuated, and filled with gaseous tritium (99 atom% $[^3H]$-H_2, up to a pressure of 450 mbar at thawed state). After defrosting, the Br/^{3}H-exchange reaction was performed with vigorous stirring of the reaction mixture at RT. The reaction was quenched after 2 h. The reaction solvents were lyophilized, and labile tritium was removed by lyophilizing twice with methanol (2 × ~1 ml). The dry crude product was redissolved in 0.22 µm filtered Millipore H_2O (~2 ml), filtered (0.2 µm, 10 mm Whatman Anotop), and diluted with ethanol (25 ml, 8.64 GBq) and stored at −80 °C until further use. Rotary evaporation, then flash chromatography (ethyl acetate/ethanol) of the crude product yielded **5**, redissolved in ethanol (25 ml), and stored at −80 °C until further use (2.34 mg, 7.25 GBq, 88% chemical yield, 77% radiochemical yield). Yield was calculated via obtained radioactivity and ^{3}H-incorporation. MF: $C_{10}H_{14}N_2O_6$; MW: 258.0852; ^{1}H-NMR (400 MHz, DMSO-d6) δ (ppm): 11.29 (br s, 1H), 7.91 (d, *J* = 8.5 Hz, 1H), 5.85 (d, *J* = 5.1 Hz, 1H), 5.63 (d, *J* = 8.0 Hz, 0.13% ^{1}H → 87% ^{3}H-incorporated (negative proof)), 5.17–5.05 (m, 2H), 4.11 (q, *J* = 5.1 Hz, 1H), 3.87–3.82 (m, 1H), 3.78 (t, *J* = 5.0 Hz, 1H), 3.67–3.52 (m, 2H), 3.35 (s, 3H); ^{3}H-NMR (400 MHz, DMSO-d6) δ (ppm): 5.65 (d, *J* = 8.3 Hz). HRMS (positive ESI) calculated for $C_{10}H_{15}N_2O_6$ $[M+H]^+$: *m/z* 259.0925; observed 259.0929 (1.54 ppm). Specific radioactivity was determined by both MS and ^{1}H-NMR: 86.4% and 87% ^{3}H-incorporation, respectively → 919 MBq/µmol, 3.53 GBq/mg.

Labeling of oligonucleotides by bromine/deuterium exchange

Prior to the tritiation experiments, the reaction conditions were optimized by means of deuterium labeling. Procedures as described for optimized tritium labeling have the following exceptions: connected to a deuterium gas line, reactions were quenched after maximum 8 h, the reaction solvents were lyophilized, the dry crude product was redissolved in 0.22-µm filtered Millipore H_2O, and filtered (0.2 µm, 25 mm Whatman Anotop).

*Characterization of [^{2}H]ssRNA-**1***

MF: $C_{197}H_{251}N_{69}O_{145}P_{20}$ (unlabeled acid); EM: 6521.9140; MS (ESI-TOFMS negative) calculated for $C_{197}H_{248}N_{69}O_{145}P_{20}$ $[M-H]^{3-}$: *m/z* 2173.0; observed 2173.0. Brominated starting material was not observed in the MS spectrum of [^{2}H]ssRNA-**1**.

*Characterization of [^{2}H]ssRNA-**2***

MF: $C_{202}H_{252}N_{68}O_{151}P_{20}$ (unlabeled acid); EM: 6664.8883; MS (ESI-TOFMS negative) calculated for $C_{202}H_{249}N_{68}O_{151}P_{20}$ $[M-H]^{3-}$: *m/z* 2220.6; observed 2220.7. Brominated starting material was not observed in the MS spectrum of [^{2}H]ssRNA-**2**.

*Characterization of [^{2}H]ssRNA-**3***

MF: $C_{206}H_{259}N_{75}O_{146}P_{20}$ (unlabeled acid); EM: 6737.9900; MS (ESI-TOFMS negative) calculated for $C_{206}H_{256}N_{75}O_{146}P_{20}$ $[M-H]^{3-}$: *m/z* 2245.0; observed 2245.1. Brominated starting material was not observed in the MS spectrum of [^{2}H]ssRNA-**3**.

Labeling of oligonucleotides by bromine/tritium exchange

*Optimized labeling procedure for [^{3}H]ssRNA-**1***

The brominated starting material of ssRNA-**1** was dissolved in 0.22-μm filtered Millipore H_2O (0.8 μmol, 5.5 mg), the catalyst (10% Pd/C, 75 equiv, 66.5 mg) was added, then N,N-diisopropylethylamine (147 equiv, 21 μl) and DMF was added to a final volume of 0.5 ml (DMF/H_2O ratio 80:20). The reaction glass flask was connected to a tritium gas line. The reaction flask was then frozen in liquid nitrogen, evacuated and filled with gaseous tritium (99 atom% [^{3}H]-H_2, up to a pressure of 170–270 mbar at frozen state → 280–520 mbar at thawed state, 6-ml dead volume). After defrosting, the Br/^{3}H-exchange reaction was performed with vigorous stirring of the reaction mixture at RT. The reaction was quenched after 6 h. The reaction solvents were lyophilized, and labile tritium was removed by lyophilizing twice with methanol (2 × ~1 ml). The dry crude product was redissolved in 0.22-μm filtered Millipore H_2O (~2 ml), filtered (0.2 μm, 25 mm Whatman Anotop) into a centrifugal filter device (Amicon Ultra – Millipore, 3 kDa cut-off) and centrifuged (4000 g, 60 min at RT) to the supernatant volume was <250 μl. The supernatant was pipette off and diluted to 5 ml (~85 MBq), and stored at −80 °C until further use.

*Ion pair RP-HPLC purification of [^{3}H]ssRNA-**1***

The crude ssRNA-**1** was prepurified by semi-preparative IP-RP-HPLC-UV (Waters XBridge Prep, RP C-18, 5 μm, 10 × 250 mm; eluent A: 50 mM triethylammonium acetate (pH 7.0), eluent B: 30% A in acetonitrile; gradient: 5% B for 4′ → 0.94% B/min → 20% B after 16′ → 75% B/min → 95% B after 1′ → 95% B for 4′ → -900% B/min → 5% B after 0.1′ → 5% B for 4.9′; flow: 3.2 ml/min; λ = 260 nm; T = 30 °C; injection volume: 500 μl). In following the purification, the purity and integrity of the isolated material were tested against the pure nonradiolabeled standard using IP-RP-HPLC-UV-RA (Waters XBridge, RP C-18, 3.5 μm, 3.0 × 150 mm; eluent A: 16.4-mM triethylamine, 95-mM hexafluoroisopropanol (pH 8.4), eluent B: 16.4-mM triethylamine, 95-mM hexafluoroisopropanol in 40% methanol; gradient: 5% B for 0′ → 3.75% B/min → 80% B after 20′ → 150% B/min → 95% B after 0.1′ → 95% B for 2′ → -900% B/min → 5% B after 0.1′ → 5% B for 2.8′; LC flow: 0.5 ml/min; λ = 260 nm; T = 30 °C; injection volume: 5 μl, RA-flow: 2.0 ml/min). As deemed necessary, an additional purification was performed using semi-preparative IP-RP-HPLC-UV (same conditions as for analytical IP-RP-HPLC-UC-RA except; Waters XBridge Prep, RP C-18, 5 μm, 10 × 250 mm; flow: 2.0 ml/min; injection volume: 500 μl), followed by an analytical verification of the purity by IP-RP-HPLC-UV-RA (as described previously), and the corresponding fractions were combined, rotary evaporated, lyophilized (white solid) and redissolved in 0.22-μm filtered Millipore H_2O (1 ml) to yield [^{3}H]ssRNA-**1** (1.0 mg, 11.96 MBq, 18% chemical yield, 1% radiochemical yield). Quantity was measured by UV-absorption spectroscopy (34.42 OD, Epsilon 186460 [OD/mmol]) and stored at −80 °C until further use. MF: $C_{197}H_{251}N_{69}O_{145}P_{20}$ (unlabeled acid); EM: 6521.9140; HRMS (negative ESI) calculated for $C_{197}H_{247}N_{69}O_{145}P_{20}$ $[M-H]^{4-}$: *m/z* 1629.4707; observed 1629.4630 (4.39 ppm). Brominated starting material was not observed in the MS spectrum of [^{3}H]ssRNA-**1**. Purity of [^{3}H]ssRNA-**1** analyzed by analytical IP-RP-HPLC-UV-RA (same conditions as described above; radiochemical purity = 90.6%; chemical purity = 93.1% (UV, λ = 260 nm)). Specific radioactivity determined by LSC/HPLC linear regression method (calibration curve constituted from seven dilutions of ssRNA-**1** reference substance → R^2 = 0.9998) → 70 MBq/μmol, 10.8 MBq/mg.

*Annealing procedure of [^{3}H]siRNA-**1***

[^{3}H]ssRNA-**1** was lyophilized and redissolved in 0.9% NaCl solution (0.5 ml), resulting in a 325 nmol/ml solution. [^{3}H]ssRNA-**1** and the complementary sense strand were mixed equimolarly, and the mixture was heated and shaken for 3 min at 90 °C and then for 1 h at 37 °C in an Eppendorf thermomixer comfort (650 rpm). [^{3}H]siRNA-**1** was then stored at −80 °C until further use. Purity of [^{3}H]siRNA-**1** was analyzed by analytical IP-RP-HPLC-UV-RA (Waters XBridge, RP C-18, 3.5 μm, 3.0 × 150 mm; eluent A: 0.1 M NaCl, 16.4 mM triethylamine, 95 mM hexafluoroisopropanol (pH 8.4), eluent B: 0.1 M NaCl, 16.4 mM triethylamine, 95 mM hexafluoroisopropanol in 40% methanol; gradient: 5% B for 0′ → 2.33% B/min → 40% B after 20′ → 400% B/min → 80% B after 0.1′ → 80% B for 2′ → 750% B/min → 5% B after 0.1′ → 5% B for 2.8′; LC flow: 0.5 ml/min; λ = 254 nm; T = 30 °C; injection volume: 5 μl; RA-flow: 2.0 ml/min; radiochemical purity = 73.3%; chemical purity = 83.6%, and 4.0% of [^{3}H]ssRNA-**1** in, [^{3}H]siRNA-**1**). Specific radioactivity (determined previously for [^{3}H]MRP4 AS) → 70 MBq/μmol, 5.25 MBq/mg.

*[^{3}H]-radiolabeling, purification and annealing of [^{3}H]siRNA-**2***

Procedures as described for [^{3}H]siRNA-**1** have the following exceptions: labeling reaction time of 4 h, only prepurification performed, and annealing performed in phosphate buffered saline (PBS). [^{3}H]siRNA-**2** was then stored at −80 °C until further use.

[^{3}H]ssRNA-**2**: 2.0 mg, 9.41 MBq, 38% chemical yield, 1% radiochemical yield. Quantity was measured by UV-absorption spectroscopy (60.31 OD, Epsilon 203980 [OD/mmol]). MF: $C_{202}H_{252}N_{68}O_{151}P_{20}$ (unlabeled acid); EM: 6664.8883; ^{3}H-NMR (400 MHz, D_2O) δ (ppm): 5.73 (s) (decoupled spectrum to enhance the signals); HRMS (negative ESI) calculated for $C_{202}H_{242}N_{68}O_{151}P_{20}$ $[M-H]^{10-}$: *m/z* 665.4810; observed 665.4816 (0.89 ppm). Brominated starting material was not observed in the MS spectrum of [^{3}H]ssRNA-**2**. Purity of [^{3}H]ssRNA-**2** was analyzed by analytical IP-RP-HPLC-UV-RA (as described for ssRNA **1**, except used the solvents and gradient described for prepurification; LC flow: 0.9 ml/min; RA-flow: 3.6 ml/min); radiochemical purity = 60.9%; chemical purity = 86.5% (UV, λ = 260 nm). Specific radioactivity was determined by LSC/HPLC linear regression method (calibration curve constituted from eight dilutions of ssRNA-**2** reference substance → R^2 = 0.9605) → 26 MBq/μmol, 3.9 MBq/mg. [^{3}H]siRNA-**2**: radiochemical purity = 75.1%; chemical purity = 92.0%, and 4.8% of the sense strand in, [^{3}H]siRNA-**2** (UV, λ = 254 nm). Specific radioactivity (determined previously for [^{3}H]ssRNA-**2**) → 26 MBq/μmol, 1.9 MBq/mg.

Results and discussion

The [^{3}H]-radiolabeling method of oligonucleotides is based on catalytic halogen/tritium exchange, which is an established means to introduce tritium within a molecule. Either 5-brominated uridine or 2′-O-methyluridine (**2**) was used for the halogenated precursors. The chemical modification 2′-O-methyl of RNA is an existing modification.[18] This modification was shown to be well tolerated throughout the duplex.[19] With the use of two precursors, that is, uridine as unmodified and chemically modified 2′-O-methyl, additional flexibility has been provided for siRNA labeling. The likelihood that the siRNA contains at least one uridine or 2′-O-methyluridine is significant. The brominated precursors were built into the oligonucleotides by conversion to their respective phosphoramidites via standard phosphoramidite chemistry. The bromine/tritium exchange catalyzed by palladium on charcoal under tritium gas occurs on the fully deprotected full-length oligonucleotide strand at the predetermined 5-position of either the uridine or 2′-O-methyluridine (Figure 1).

Synthesis of the brominated 2′-O-methyluridine phosphoramidite

5-bromouridine phosphoramidite were obtained from Glen Research. 5-Bromo-2′-O-methyluridine **2** has been described in literature.[17] The 5-bromo-2′-O-methyluridine phosphoramidite **4** was synthesized via a four-step synthesis route (Figure 2). The benzoyl-protected sugar and the 5-bromouracil base in the presence of bis(trimethylsilyl)acetamide and trimethylsilyl triflate resulted in the protected nucleoside **1** and its α-diastereoisomer (ratio 1.8:1). Separation of the two diastereoisomers was not successful by flash chromatography. However, the diastereoisomers were successfully separated via solvent extraction with a heptane / ethyl acetate mixture (1:1), which resulted in a 53% yield of the

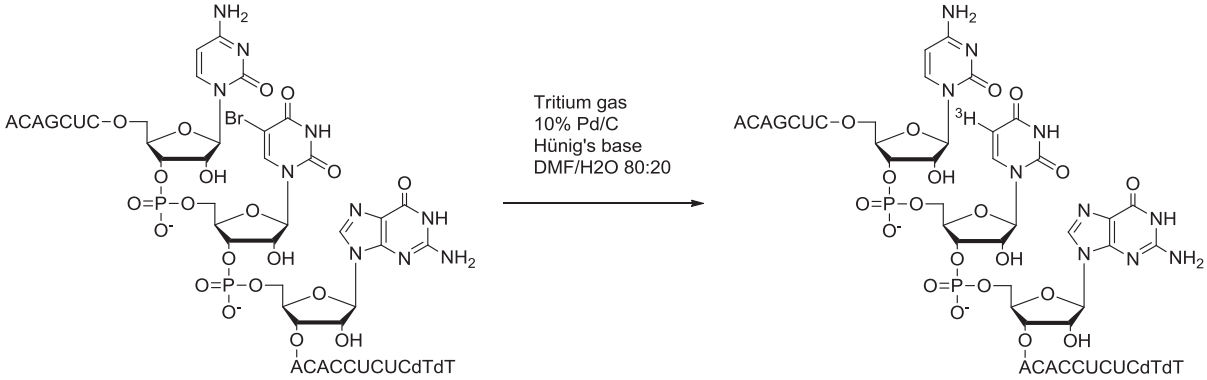

Figure 1. [^{3}H]-Radiolabeling of oligonucleotides (ssRNA-**1**) by bromine/tritium exchange.

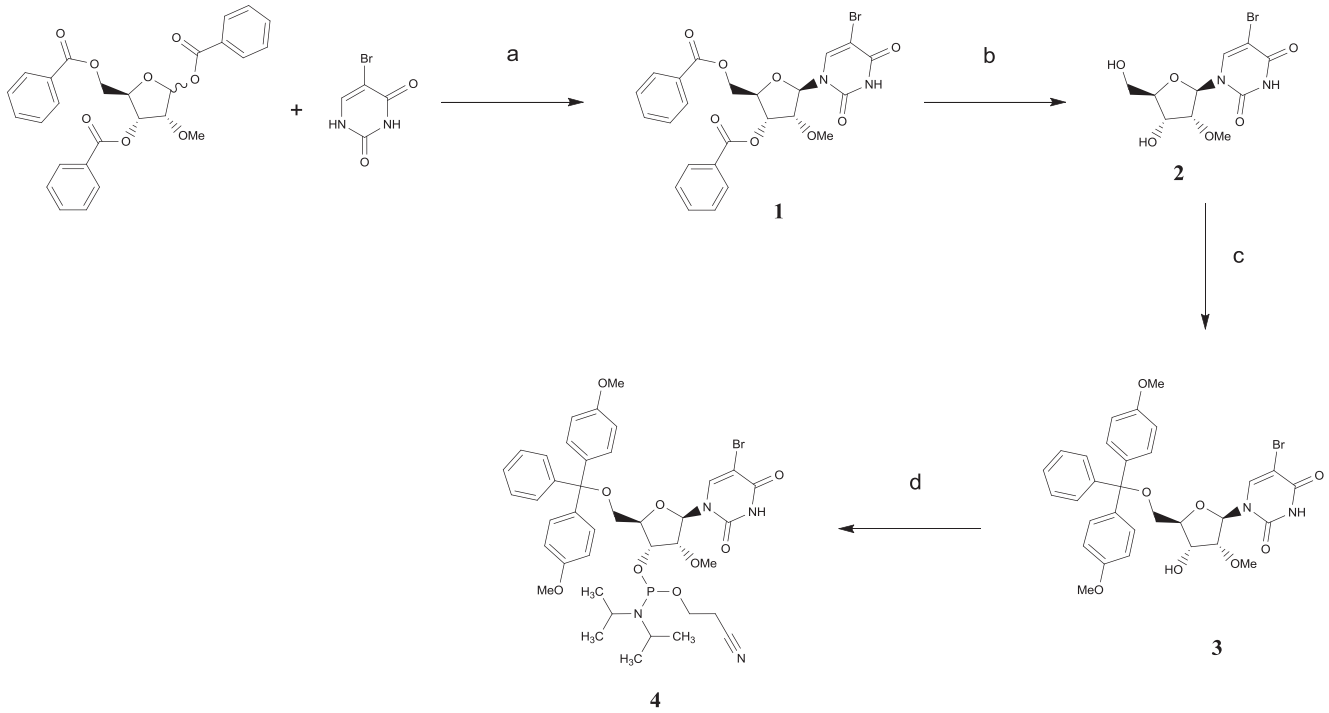

Figure 2. Synthesis route of 5-brominated-2′-O-methyluridine phosphoramidite (**4**). (a) bis(trimethylsilyl)acetamide (2.0 equiv), Trifluoromethanesulfonic acid methylsilylester (1.5 equiv), CH_3CN, argon, reflux, 2 h, 53%; (b) 7N NH_3 in MeOH, MeOH, argon, RT, 4 days, 89%; (c) DMT-Cl (1.2 equiv), pyridine, argon, RT, 24 h, 60%; d: Diisopropylammonium-tetrazolide (1.5 equiv), cyanoethyltetraisopropylphosphoramidite (1.4 equiv), CH_2Cl_2, argon, RT, 7 h, 92%.

β-diastereoisomer. The protected nucleoside **1** was deprotected with ammonia to give the deprotected 5-bromo-2′-O-methyluridine **2** in an 89% yield after purification. The 5-bromo-2′-O-methyluridine **2** was converted into its phosphoramidite **4** in two steps. First, the 5′-hydroxy group was protected with a DMT-group to afford 5-bromo-5′-[(4,4′-dimethoxyphenyl)methyl]-2′-O-methyluridine **3** in 60% yield after purification. Subsequent phosphorylation of **3** with cyanoethyltetraisopropylphosphordiamidite in the presence of diisopropylammonium-tetrazolide gave the desired 5-brominated-2′-O-methyluridine phosphoramidite **4** in a 92% yield.

Tritiation of 5-Br-2′-O-methyluridine

Tritiation of 5-Br-2′-O-methyluridine **2** with 10% palladium on charcoal in DMF in the presence of Hünig base was completed within 2 h. The obtained [5-^{3}H]2′-O-methyluridine **5** had a high specific radioactivity of 919 GBq/mmol (3.53 GBq/mg). Hünig base was added to sequester tritium bromide (^{3}HBr). The catalyst did not hydrogenate the double bond, and the reaction went to completion; thus, there was no need to evaluate alternative catalysts for the siRNA models. For DNA models, one approach using the catalyst without the carrier (Pd black) and another with $Pd/BaSO_4$ were successful. However, these approaches were not as efficient as the 10% Pd/C. When turning from the monomer to the DNA oligonucleotide models (data not shown), we decided to proceed with a DMF/H_2O solvent system in the ratios 80:20. Pure DMF would have been preferred, but we anticipated a 21-mer RNA oligonucleotide would not be soluble under those terms.

Stability of [5-^{3}H]2′-O-methyluridine

The chemical stability of the carbon–tritium bond in the C5-position of the tritiated monomer [5-^{3}H]2′-O-methyluridine **5** was followed for 17 days in phosphate buffer (PBS, pH 7.4) at 37 °C, that is,

mimicking physiological conditions. Two different analytical methods (LSC and ^{1}H- & ^{3}H-NMR) gave consistent results of less than 5% tritiated water observed after 17 days. In addition, the compound was stable after 1 year in EtOH at −80 °C and gave results of less than a 5% loss of the tritium label.

Synthesis and tritiation of ssRNA

Three siRNAs (Table 1) with different sequences and two nucleotide 3′-end overhangs were synthesized using standard phosphoramidite chemistry. siRNA-**1** is a 21-mer siRNA, which has the rat MRP4 as target and is a chemically unmodified 21-mer oligonucleotide (van de Water *et al.*[20]). The 5-Br-uridine was incorporated at position 9 of the antisense strand. siRNA-**2** sequence is also known as SSB, and directs against the ubiquitous gene Sjögren Syndrome antigen B. The antisense contained 5-Br-2′-OMeU in position 7. siRNA-**3** is a dummy sequence with a relative high 'u' density. This sequence was selected to study the effect of the oligonucleotide sequences on success of the labeling.

In following their synthesis and purification, the brominated ssRNAs were analyzed by liquid chromatography–mass spectrometry. For all three siRNAs, we could confirm the incorporation of the brominated monomers. The deuteration and tritiation experiments for the full-length ssRNA were performed in the low mg scale of approximately 5–6 mg, and were performed using three ssRNAs.

During optimization of the ssRNA-**1** tritiation, the quantity of the catalyst and the base to obtain full conversion of the starting material was found to be of major importance. A large amount of catalyst and base equivalents (75 and 150, respectively) were needed for a full conversion of the ssRNA within 4 h. Other parameters such as temperature, and gas pressure during the tritiation had no or limited impact on the conversion of the starting material.

However, a deviation of more than ±5% from the 80:20 ratio of the DMF/H_2O solvent system had a negative effect on the conversion. The explorative studies on the tritiation conditions showed a full conversion on the bromide into tritium/hydrogen on the final conditions within a 6-h reaction time. The final tritiated ssRNA-**1** had a specific radioactivity of 70 MBq/μmol (10.8 MBq/mg). For further stability and kinetic studies, the ssRNA was annealed equimolarly with the complementary sense strand to afford [^{3}H]siRNA-**1**. Similar conditions were applied for the tritiation for ssRNA-**2**, containing the 5-bromo-2′-O-methyluridine precursor in position 7, instead of the 5-bromouridine, resulting

Table 1. 21-mer RNA oligonucleotides studied

Name		Sequence (Antisense and Sense)	Precursor
siRNA-**1**	AS	5′-ACAGCUCCU$_9$GACACCUCUCdTdT-3′	5-Br-U
	S	5′-GAGAGGUGUCAGGAGCUGUdTdT-3′	
siRNA-**2**	AS	5′-UuAcAUu$_7$AAAGUCUGUUGUuu-3′	5-Br-2′-OMeU
	S	5′-AcAAcAGAcuuuAAuGuAAuu-3′	
siRNA-**3**	AS	5′-UGAACcuAGUu$_{11}$AAcuUAAGuu-3′	5-Br-2′-OMeU
	S	5′- cuuAAGuuAAcuAGGuucAuu-3′	

dX = DNA, X = RNA, and x = 2′-OMe RNA.

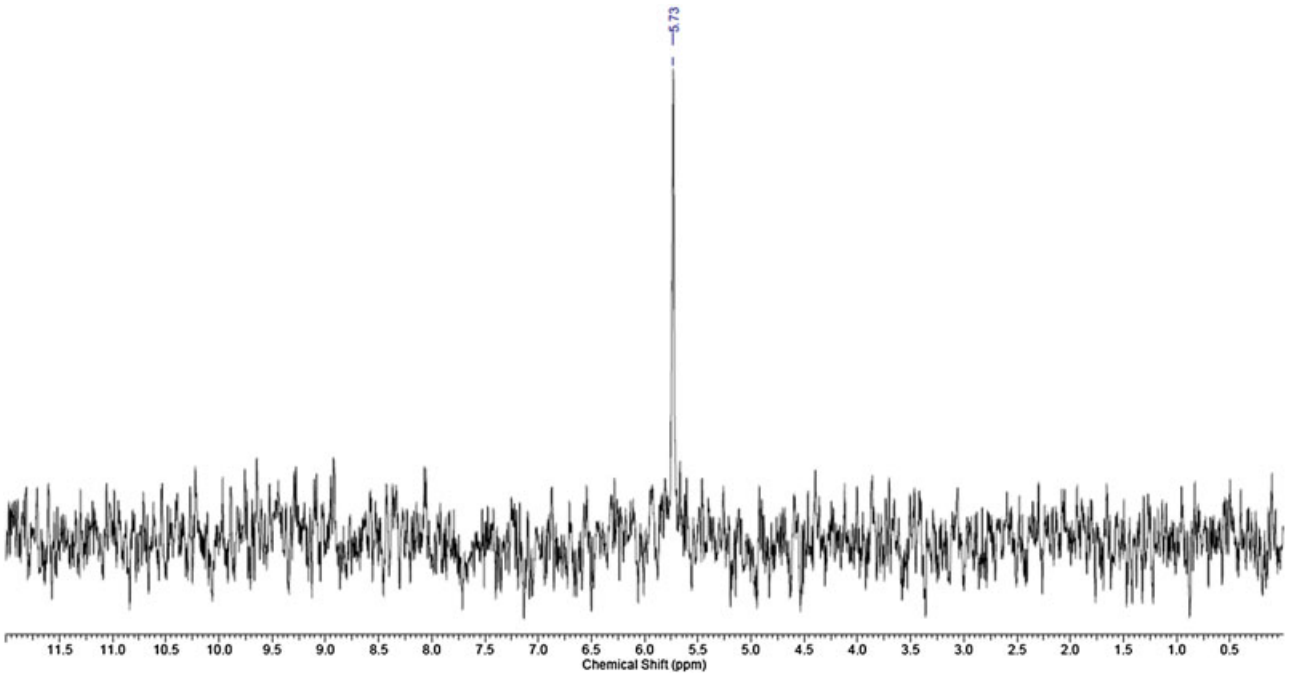

Figure 3. ^{3}H-NMR spectrum of ssRNA-**2**.

in full conversion of the starting material within 4 h, with a specific radioactivity of 26 MBq/μmol (3.9 MBq/mg). A decoupled ^{3}H-NMR spectrum of ssRNA-**2** has been obtained (Figure 3), and the tritium distribution observed is localized on one shift only (5.73 ppm), which correspond to the 5-position of 2′-O-methyluridine **5**. This also indicates that no random incorporation (scrambling) of tritium into the molecule has occurred. The chemical yields of the [^{3}H]-radiolabeling reactions of ssRNA-**1** and ssRNA-**2** were 18% and 38%, respectively, even though in both cases all starting material was converted. This was mostly because considerable amounts of material were lost during work-up and purification; the reaction scale (5–6 mg) itself might also have had an impact. Several batches of [^{3}H]ssRNA-**1** and [^{3}H]ssRNA-**2** have been conducted, with consistent results, and thereby the reproducibility of the labeling procedure has been confirmed.

By testing the developed labeling method with the aforementioned ssRNAs, the conversion of the starting material did not seem to be affected much by the sequence or the position of the brominated precursor or the choice of precursor. A lower incorporation of tritium into ssRNA-**2**, than ssRNA-**1** was observed (26 and 70 MBq/μmol, respectively); this might be due to the sequence, position of precursor, and/or choice of precursor. However, the observed differences do not appear significant. Attempts were made to characterize the impurities with HRMS but were unsuccessful.

Stability of tritiated ssRNAs and dsRNAs

The stability of the [^{3}H]-radiolabeled single strands (ssRNA-**1** and ssRNA-**2**) and duplexes (siRNA-**1** and siRNA-**2**) have been studied by ion pair RP-HPLC with radio detection at +4 and −80 °C. The single strands were stored in water or in 10% EtOH. After 8-month storage at +4 °C, less than 10% tritiated water was observed and less than 5% at −80 °C for both solutions. The duplexes were stored in PBS. After 30-month storage at +4 °C and −80 °C, less than 5% tritiated water was observed, allowing batch wise synthesis of tritiated siRNA for multiple pharmaceutical formulation and pharmacokinetic studies.

Conclusions

The described final step labeling procedure for single internal [^{3}H]-radiolabeling of oligonucleotides, which exclusively depends upon the existence of a uridine or a 2′-O-methyluridine residue in the sequence is a very promising approach. The starting point for development of the labeling method was the monomer. The method was then optimized for DNA models with minor adjustments and successfully transferred to the RNA oligonucleotides.

This radiolabeling procedure presents some definite advantages over previously reported methods of labeling oligonucleotides. This labeling did not alter the chemical or molecular characteristics (^{3}H replacing a ^{1}H), that is, the reactivity and metabolism of the molecule should remain unaffected compared with the unlabeled compound. The radiolabel is placed in a stable and predetermined internal position, which decreases the likelihood of the label to be readily cleaved from the oligonucleotide *in vivo*. Storage of either the radiolabeled single strands or the duplexes does not seem to be an issue. The relatively low specific radioactivity obtained for both ssRNAs was most likely due to the partially aqueous solvent system. To address this, a third building block with iodine in the C6-position of 2′-O-methyluridine is currently in progress. This building block is anticipated to increase the specific radioactivity, because of a more efficient exchange *versus* bromine. Furthermore, the chemical stability of the carbon–tritium bond in the C6-position of pyrimidines is known in the literature[21,22] to be more stable than the C5-position. As the method is dependent upon the existence of a uridine or a 2′-O-methyluridine residue in the sequence, this method should also be transferable to chemically modified as well as unmodified phosphodiester oligonucleotides. This work provides a good indication that the method might be universal for chemically modified as well as unmodified phosphodiester oligonucleotides.

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Conflict of Interest

The authors did not report any conflict of interest.

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