Regular Article

Site-Specific Tritium Labeling at the Predefined Internal Position of the Chemically-Modified RNA

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In nucleic acid drug discovery, it is extremely important to develop a technology to understand the distribution in target organs and to trace the degradation process in the body in order to optimize the structure and improve the efficiency of the clinical trial process. Since nucleic acid drugs are essentially metabolically degraded into numerous fragments, labeling at the internal position is preferable to that at the terminus. Due to the high molar specific activity of tritium, various approaches for tritium-labeling have been studied for nucleic acid drugs. Nevertheless, a generally-applicable method for tritium labeling of the internal position of a nucleic acid has not been established. In this study, we have demonstrated a new and efficient method for site-specific tritium labeling of the cytosine base at a predefined internal position in nucleic acid drugs. This method was developed by the chemical modification of the cytosine 4-amino group with the pyridinyl vinyl keto group by the functionality-transfer reaction using the reactive oligodeoxynucleotide (ODN), followed by reduction with $NaBT_4$. Applicability to a variety of chemical structures, such as 5-methyl cytosine, 2′-*O*-methyl, 2′-fluoro ribose derivatives, Locked/Bridged nucleic acid (LNA/BNA) derivatives, as well as phosphorothioate bonds, has been evidenced using nine oligoribonucleic acid (ORN) substrates. It has been clearly demonstrated that this method is an excellent method for tritium-labeling of nucleic acid with an average conversion efficiency of 74%, an average isolated labeling yield of 60%, and an average specific activity of 61 GBq/mmol. This method is expected to contribute to the preclinical absorption, distribution, metabolism, excretion (ADME) studies of nucleic acid drug candidates.

Key words tritium labeling, nucleic acid, oligodeoxynucleotide, RNA modification, chemically-modified RNA

Introduction

It was demonstrated in 1978 that hybridization of mRNA with antisense oligonucleotides (ASOs) efficiently inhibited protein translation *in vitro*.[1] Since then, great deal of efforts has been expended for realization of nucleic acid drugs using synthetic oligonucleotides. Small interfering RNAs (siRNAs) and microRNAs (miRNAs) have become new mechanisms of nucleic acid drugs in the RNA-based regulation system. However, until recently, when more than thirteen ASO and siRNA drugs were approved and marketed since 2016, only very few nucleic acid drugs were clinically used.[2]

Small molecule drug discovery has made advances in the use of radiolabeled molecules for absorption, distribution, metabolism, excretion (ADME) research. In nucleic acid drug discovery, it is extremely important to develop a technology to understand the distribution in target organs and to trace the degradation process in the body in order to optimize the structure and improve the efficiency of the clinical trial process. Since nucleic acid drugs are essentially metabolically degraded into numerous fragments, labeling at the internal position is preferable to that at the terminus. Due to the high molar specific activity of tritium, various approaches for tritium-labeling have been studied for nucleic acid drugs. In the *N*-acetyl-D-galactosamine (GalNAc)-conjugated nucleic acid drug, tritium was introduced in the linker.[3] The C8 position of purines can be labeled by hydrogen-tritium exchange in tritium water, but C8 tritium readily reverts to hydrogen.[4] 5-Bromouridine derivatives can be inserted into the sequence and subsequently tritium-labeled by a palladium-catalyzed reaction in tritium gas, however, it is extremely difficult to apply to oligonucleotides with a phosphorothioate backbone.[5] A 5′-tritium-labeled nucleoside was synthesized by sequential reactions including the oxidation of the 5′-alcohol and the subsequent reduction of the formed aldehyde with $NaBT_4$, which was transformed into the 3′-phosphoramidite precursor for incorporation into the oligonucleotide by a solid-phase synthesizer.[6] The instability of the tritium-labeled phosphoramidite precursor, low synthesis yield, and low specific activity of the final product might be problems with this method. Ruthenium nanoparticles were used as a catalyst for the random hydrogen/tritium exchange in the purine rings at the C2 and C8 positions of oligodeoxynucleotide (ODN).[7] However, the randomly-radiolabeled ODN is not useful in metabolic studies because the label is uniformly distributed throughout the molecule.

We have already developed a reaction that allows chemical modification of the desired position in RNA by recognizing sequences through the formation of a hybridized complex using artificial nucleic acids and by identifying bases with reactive base derivatives.[8–15] Following this reaction scheme, we developed an efficient method for the sequence- and base-selective chemical modification of guanine, cytosine,

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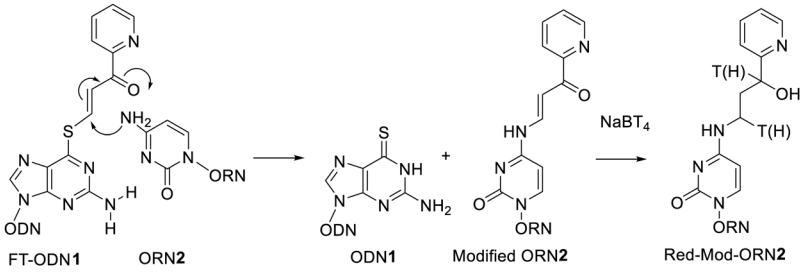

Chart 1. The Functionality Transfer Reaction of the Pyridine-Keto Group and Following Reduction with $NaBH_4$ to Produce the Corresponding Tritium-Labeled Oligonucleotide

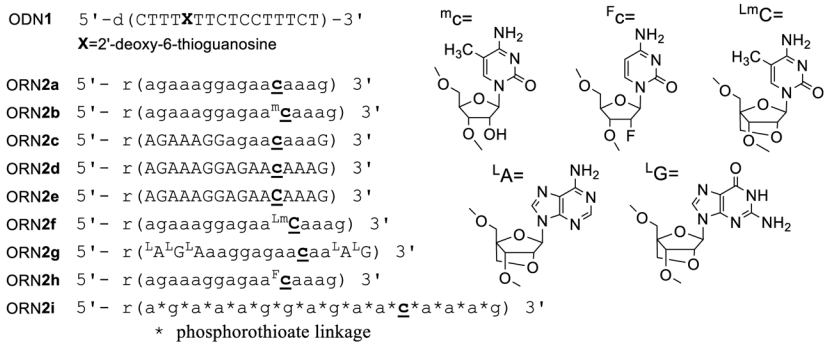

Fig. 1. Oligonucleotide Substrate Sequence and the Modified Nucleosides Used in This Study

The lowercase and uppercase letters represent ribonucleotides and 2′-*O*-methylribonucleotides, respectively. Bases with a superscript letter are indicated by the individual modified structure. The asterisk indicates the phoshporothioate linkage.

and adenine. Furthermore, this modified structure was efficiently converted into a tritium-labeled form by reduction with $NaBT_4$.[16] This method is a novel and highly progressive technology that allows tritium labeling of the desired internal positions of the already-synthesized RNA, such as nucleic acid drug candidates under development. The labeling procedure is simple and can be performed at typical radioisotope (RI) experimental facilities without special equipment or facilities, which is superior to existing technologies. The modified structure is sterically small and chemically stable, which is a better feature compared to the conventional labeling structure of radiometallic complexes with a sterically large size. In this paper, we report in detail the application of various modified oligonucleotides to tritium labeling. In particular, the labeling of phosphorothioate-based oligonucleotides has not been reported to the best of our knowledge.

Results and Discussion

Modification and $NaBH_4$-Reduction of the Chemically-Modified Oligoribonucleic Acid (ORN) We have already reported that the pyridinyl vinyl ketone unit on the 6-thioguanine of the FT-ODN**1** is transferred to the amino group of its facing cytidine of ORN**2** with its complementary sequence through the Michael reaction followed by a β-elimination reaction in the hybridized complex. In this study, the modified ORN**2** was reduced with $NaBT_4$ to produce the corresponding tritium-labeled ORN**2** (Chart 1).

To validate the modification efficiency and tritium labeling, a variety of modification nucleosides was incorporated to ORN**2(a**–**i)**. In Fig. 1, the lowercase and uppercase letters represent ribonucleotides and 2′-*O*-methylribonucleotides, respectively. Bases with a superscript letter are indicated by the individual modified structure. The asterisk indicates the phoshporothioate linkage. The transfer oligodeoxynucleotide probe (FT-ODN**1**) was prepared by the reaction of ODN**1** (50 μM) containing 6-thioguanine with the transfer group, (*E*)-3-iodo-1-(pyridin-2-yl)prop-2-en-1-one (0.5 mM), in carbonate buffer (100 mM, pH 10) at 0 °C for 30 min as previously described. The solution of FT-ODN**1** thus obtained was mixed with a solution of ORN**2a** in an *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer containing NaCl and $NiCl_2$ at 0 °C to make a solution with final concentrations ORN (5 μM), FT-ODN (7.5 μM), $NiCl_2$ (75 μM), HEPES buffer (50 mM), NaCl (100 mM). This mixture was incubated at 37 °C for 1 h, and the progress of the functionality-transfer reaction was followed by HPLC (Fig. 2). From the quantification of the UV charts, the modification yields were calculated to be 78% for ORN**2b**, 82% for ORN**2f** and 73% for ORN**2i**. All the ORN**2** substrates were successfully modified in good yields, and their HPLC charts are summarized in Supplementary Fig. S1, and the conversion yields are summarized in Table 1. The function transfer reaction was shown to be efficient for a variety of modified nucleotides, with an average modification efficiency of 77%.

The modified ORN**2** was isolated and subjected to reduction by $NaBH_4$. A solution of $NaBH_4$ in 2-propanol (2 mM) was added to a solution of the modified ORN**2a** in ultrapure water (MilliQ) to make a solution with the final concentrations of 200 μM $NaBH_4$ and 5 μM of the modified ORN**2**, which was reacted for 30 min at 25 °C. The reaction mixture was analyzed by HPLC and ultra performance liquid chromatography (UPLC)/QTOF-MS. Comparing the HPLC charts of the isolated and reduced products, it is clear that the major peak of the modified ORN**2** moved to a shorter retention time by reduction, indicating that the reduction quantitatively proceeded because the starting material, the modified ORN**2**,

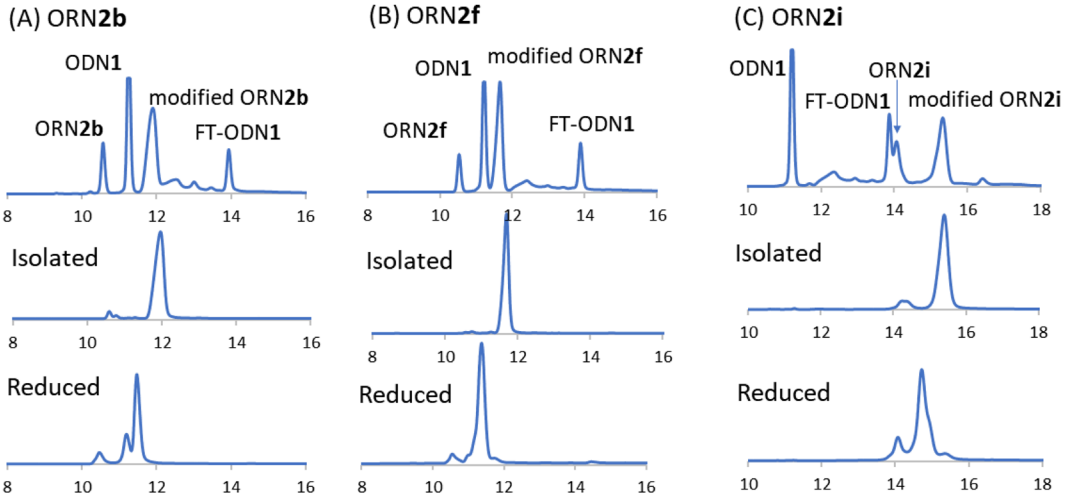

Fig. 2. HPLC Charts of the Modification, Isolation and $NaBH_4$-Reduction Using the ORN**2** Substrates

The horizontal axis indicates the time (min) after injection. The modification substrates are (A) ORN**2b**, (B) ORN**2f**, (C) ORN**2i**. HPLC Conditions: column, OSAKA SODA C18 MG, 4.6×250 mm; solvent, A: 0.1 M TEAA containing 5% CH_3CN, buffer, B: CH_3CN, B: 0 to 30%/20 min, 30 to 100%/25 min, linear gradient; column oven at 40 °C; flow rate, 1.0 mL/min; and monitored by UV at 254 nm.

Table 1. Modification Yield, Labeling Yield and Specific Activity for All the ORN**2** Substrates

ORN**2**	Modification yield (%)[a]	Labeling yield (%)[b]	Specific activity (GBq/mmol)[c]
ORN**2a**	80	62	61.5
ORN**2b**	78	58	61.1
ORN**2c**	66	58	66.5
ORN**2d**	77[d]	66	61.2
ORN**2e**	74	54	70.5
ORN**2f**	82	60	48.9
ORN**2g**	92[e]	74	50.4
ORN**2h**	72	60	59.9
ORN**2i**	73	48	67.3
Average	77	60	60.7

a) Modification yield was calculated based on the peak ratio of the non-reacted ORN**2** and the modified ORN**2** in the HPLC chart monitored at 254 nm as shown in Fig. 2. *b*) Labeling yield was obtained from the amount of the used substrate and the obtained radioactive materials, which were quantified by HPLC monitored at 254 nm as shown in Fig. 4. *c*) The specific activity was determined from the amount of material calculated from the UV chart of the HPLC and the amount of radioactivity of the whole fractions. *d*) Mixture with a portion of the remaining FT-ODN**1**. *e*) Mixture with a portion of the remaining ODN**1**.

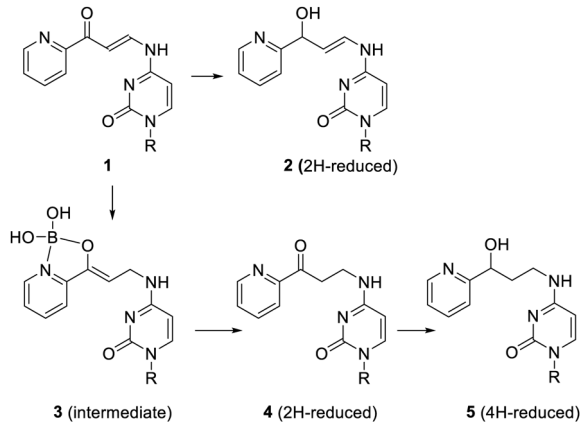

Chart 2. The Pathway for the Formation of the 2H- and 4H-Reduced Products

was not present. Details were obtained about the structure of the reduced products by the UPLC/QTOF-MS analysis. The 1,2-reduction of the modified structure **1** with $NaBH_4$ yields the 2H-reduced form **2**. The 1,4-reduction produces a boron chelate intermediate **3**, which gives the keto form **4**. Further reduction of **4** produces the 4H-reduced form **5**. The forms **2** and **5** are a mixture of the stereoisomers due to the hydroxyl group. Thus, the reduced ORN**2** products are obtained in a mixture of these various structures (Chart 2).

Figure 3 illustrates examples of the UPLC/MS charts and the high-resolution mass spectrum obtained in the negative mode. In Fig. 3A for the $NaBH_4$-reduction of the modified ORN**2b**, peaks 1 and 2 indicate the MS corresponding to the 4H-reduced and the 2H-reduced products, respectively. Although the 4H-reduced products were suggested by the molecular weight calculated by deconvolution for peaks 1 and 2 in Fig. 3B, these peaks may contain both 2H- and 4H-reduced products, because the isomers were not separated by UPLC. As all the phosphodiester bonds are replaced by the phosphorothioate in the ORN**2i** substrate, the corresponding reduced products are a mixture of the huge number of isomers, thus the peak in Fig. 3C is very broad. Nevertheless, the high-resolution mass spectra of peak 1 clearly indicates that the reduction takes place to produce the 2H- and 4H-reduced products. Similar results were obtained as shown in Supplementary Fig. S2, where the UPLC/MS charts and the high-resolution mass spectrum obtained by the negative mode for the reduction of all the modified ORN**2** as substrates are summarized.

Tritium Labeling by the Reduction with $NaBT_4$ We further investigated tritium labeling of the modified ORN**2** by reducing with $NaBT_4$. The modified ORN**2** was isolated as shown in Fig. 2, which was subjected to reduction by $NaBT_4$. An ampule containing $NaBT_4$ was opened in the hood at the RI facility, its entire contents were dissolved in 2-propanol, and the solution was stored at 4 °C. A solution of the modified ORN**2** in pure water (5 μM, final concentration) was made in a microtube, followed by the addition of the 2-propanol solution of $NaBT_4$ (200 μM, final concentration). The solution was allowed to stand at 25 °C for 1 h. The following procedure was performed to remove any radioactive materials with low mo-

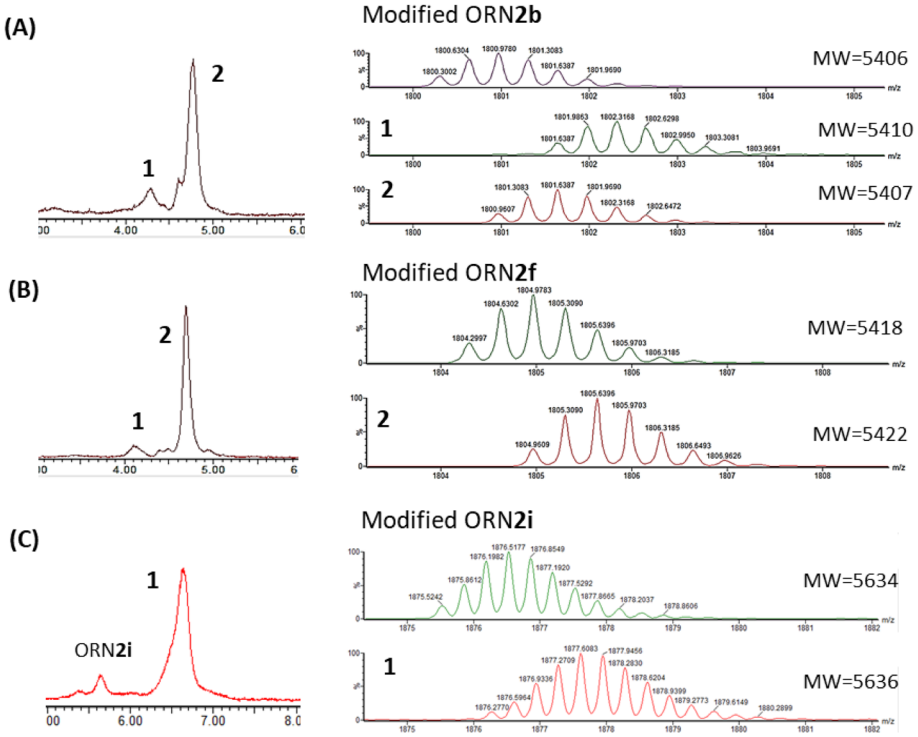

Fig. 3. UPLC Charts and the High-Resolution Mass Spectrum of the Reduced Products with $NaBH_4$

The molecular weight noted on the right side of the MS was obtained by deconvolution based on MS derived from the trivalent negative ions observed by UPLC/QTOF-MS.

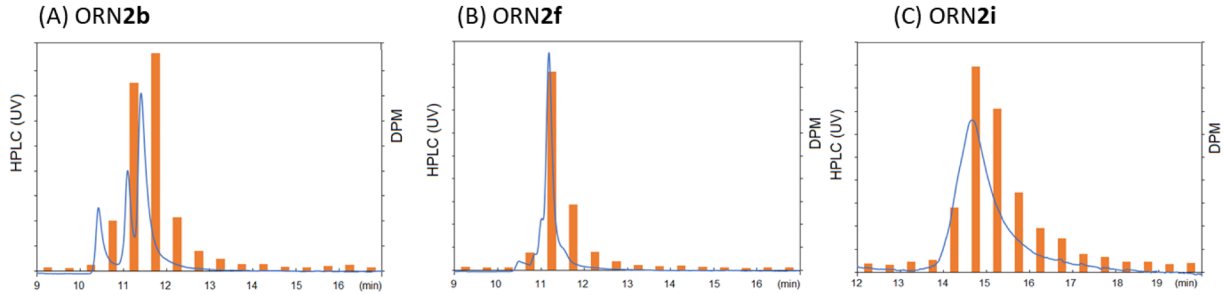

Fig. 4. Results of the Reduction of the Modified ORN**2** with $NaBT_4$

The blue line shows the HPLC charts detected by UV at 254 nm and the red bar represents the radioactivity of the fraction collected every 30 s measured by the liquid scintillation counter.

lecular weights. 1 × phosphate buffered saline (PBS)(−) Buffer was added to the reaction mixture, and the whole solution was ultrafiltrated using Amicon®Ultra (10000 × ***g***, 25 °C, 20 min). This procedure was repeated 5 times to obtain a sample of the 1 × PBS(−) Buffer solution. The solution was injected into an HPLC column and fractions were collected every 30 s. A portion of the fraction was mixed with a scintillation cocktail (Ultima Gold), and the radioactivity was measured by a liquid scintillation counter. Figure 4 summarizes the labeling results using the same substrates summarized in Fig. 2. The blue line represents the UV chart detected at 254 nm, showing that $NaBT_4$ produces almost the same reduction products as $NaBH_4$. The fact that the radioactivity peaks overlap with the UV peaks strongly supports that tritium labeling takes place by the reduction with $NaBT_4$. Figure 4A represents the HPLC analysis for the $NaBT_4$-reduction of RNA**2b**. The area under the UV curve was quantified to determine the total amount of the labeled ORN**2b** to be 28.9 pmol, showing the labeling yield to be 58% based on the modified ORN**2b** (50 pmol) as a starting material. The sum of the radioactivity of fractions collected between 10.5–13.5 min was 1767 Bq. From these values (1767 Bq/28.9 pmol), the specific radioactivity of ORN**2b** was calculated to be 61.1 Bq/pmol (61.1 GBq/mmol). For all the ORN**2** substrates, the HPLC charts observed at 254 nm and the radioactivity bar graph are shown in Supplementary Fig. S3, the labeling yields and the specific activities are summarized in Table 1. The average labeling yield of 60% and the average specific activity of 60.7 GBq/mmol for nucleic acids with various modified structures clearly indicate that this labeling method is an excellent method for synthesizing labeled nucleic acids with high specific activity.

Conclusion

In this study we have demonstrated a new and efficient method for site-specific tritium labeling of the cytosine base at a predefined internal position in nucleic acid drugs. This method is developed by the chemical modification of the cytosine 4-amino group with the pyridinyl vinyl keto group by

the functionality-transfer reaction using the reactive ODN, followed by reduction with $NaBT_4$. Applicability to a variety of chemical structures, such as 5-methyl cytosine, 2′-*O*-methyl, 2′-fluoro ribose derivatives, locked/bridged nucleic acid (LNA/BNA) derivatives, as well as phosphorothioate bonds has been evidenced using nine ORN**2** substrates. It has been clearly demonstrated that this method is an excellent method for tritium-labeling of nucleic acids, with an average conversion efficiency of 74%, an average isolated labeling yield of 60%, and an average specific activity of 61 GBq/mmol. *In vivo* stability and biochemical effects of the labeled structure are now under investigation and will be reported elsewhere. This method is expected to contribute to the preclinical ADME studies of nucleic acid drug candidates.

Experimental

General The UPLC/MS measurements were performed using a Waters ACQUITY UPLC equipped with a Xevo G2-XS Qtof and Oligonucleotide BEH C18 Column, 130 Å, 1.7 μm, 2.1 × 50 mm. HPLC was performed by a JASCO LC-2000 PLUS series equipped with an OSAKA SODA CAPCELL PAK C18 MG (4.6 × 250 nm). The ORNs and ODN were purchased from Gene Design, GeneNet or Nihon Bioservice. The concentrations of the ODN and ORN were determined using NonoDrop One by measuring the absorbance at 260 nm and 25 °C based on the sum of the molar absorbance coefficients of the nucleobases multiplied by 0.9: adenine, 15300; guanine, 11800; thymine, 9300; cytosine, 7400; uracil, 10000; and 6-thioguanine, 11800.

Preparation of the Functionality-Transfer ODN, FT-ODN(1) (Chart 1) The sequence of ODN is 5′ dCTTT-X-TTCTCCTTTCT, in which X represents 2′-deoxy-6-thioguanosine. At 0 °C, an aqueous stock solution of ODN**1** (150 μM, 30 μL, 4.50 nmol) was added to a carbonate buffer (500 mM, 18 μL, pH 10.0) and diluted with ultrapure water (19.5 μL). To this solution, a solution of ((*E*)-3-iodo-1-(pyridin-2-yl)-prop-2-en-1-one) (2 mM, 22.5 μL, 4 nmol) in CH_3CN was added. After 30 min at 0 °C, the reaction progress was followed by HPLC. Final concentrations: ODN**1** (50 μM), (*E*)-3-iodo-1-(pyridin-2-yl)prop-2-en-1-one (500 μM), and carbonate buffer (100 mM). The reaction solution was used for the functionality-transfer reaction without purification. The HPLC analysis conditions were as follows: column: OSAKA SODA C18, 4.6 × 250 mm and eluent solvents: A: 0.1 M TEAA buffer and 5% CH_3CN, B: CH_3CN, B: 0 to 30%/20 min, 30 to 100%/25 min, linear gradient; column oven: 40 °C; flow rate: 1.0 mL/min; and UV: 254 nm.

A General Procedure for the Functionality Transfer Reaction to Modify ORN (Chart 1, Fig. 2) The sequences of ORN**2** are described in Fig. 1, in which the common sequence is 5′-agaaagaagaa-**c**-aaag, and the underlined c indicates the target cytosine for the modification. A general procedure for the functional group transfer reaction to ORN is described for the reaction between FT-ODN**1** and ORN**2a**. A stock solution of ORN**2a** (100 μM, 21.5 μL, 2.15 nmol) was added to a HEPES buffer solution (250 mM HEPES, 120 μL, pH 7.2), followed by the addition of an aqueous NaCl solution (1 M, 60 μL, 60 μmol), and ultrapure water (218.5 μL). Aqueous solutions of FT-ODN**1** (50 μM, 90 μL, 4.5 nmol) and aqueous $NiCl_2$ solution (500 μM, 90 μL, 45 nmol) were added to this solution at 0 °C, and the mixture was incubated at 37 °C for 1 h. Final concentrations: ORN (5 μM), FT-ODN (7.5 μM), $NiCl_2$ (75 μM), HEPES buffer (50 mM), NaCl (100 mM). The reaction progress was followed by HPLC using the following conditions: column: OSAKA SODA C18, 4.6 × 250 mm and eluent solvents: A: 0.1 M TEAA buffer and 5% CH_3CN, B: CH_3CN, B: 0 to 30%/20 min, 30 to 100%/25 min, linear gradient; column oven: 40 °C; flow rate: 1.0 mL/min; and UV: 254 nm. Some examples of the HPLC charts are summarized in Fig. 2. The peak corresponding to the modified ORN**2** was isolated, the solution was ultrafiltrated using Amicon®Ultra-0.5 mL 3K device (14000 × ***g***, 25 °C, 20 min) to exchange the buffer and concentrate the modified ORN**2**. This solution was for the reduction with $NaBH_4$ and $NaBT_4$. The modification yield was calculated from the peak area ratio of the unreacted ORN**2** to the modified ORN**2**, which are summarized in Table 1.

A General Procedure for the Reduction of the Modified ORN2 with $NaBH_4$ (Chart 1, Figs. 2, 3) A general procedure for the reduction of the modified ORN**2** with $NaBH_4$ is described for the reaction of the modified ORN**2a**. A stock solution of the modified ORN**2a** (27.5 μM, 36.3 μL, 100 pmol) was added to ultrapure water (MilliQ, 14.37 μL), followed by the addition of a $NaBH_4$ solution in 2-propanol (2 mM, 2 μL, 4 nmol) at 25 °C, and the mixture was allowed to stand for 30 min at 25 °C. The reaction progress was followed by HPLC using the following conditions: column: OSAKA SODA C18, MG, 5 μm, 4.6 × 250 mm and eluent solvents: A: 0.1 M TEAA buffer and 5% CH_3CN, B: CH_3CN, B: 0 to 30%/20 min, 30 to 100%/25 min, linear gradient; column oven: 40 °C; flow rate: 1.0 mL/min; and UV: 254 nm. Some examples of the HPLC charts are summarized in Fig. 2. The exact MS of the reduced products were measured by UPLC/QTof-MS measurement using a Waters ACQUITY UPLC equipped with a Xevo G2-XS Qtof. UPLC conditions: Column: Oligonucleotide BEH C18 Column, 130 Å, 1.7 μm, 2.1 × 50 mm, column temperature: 40 °C; eluents: A: 15 mM TEA, 400 mM HFIP pH 7.9, B: A/MeOH = 1/1, B: 35 to 70%/10 min, 0.2 mL/min. MS Conditions: mass range: 450–3000 Da; internal standard: LE, mode: ESI negative; resolution: continuum; cone voltage: 40 V; capillary voltage: 4 kV; desolvation: 600 °C; and desolvation gas flow: 700 L/h. Some examples are shown in Fig. 3, and all the data are summarized in Table 2.

A General Procedure for the Reduction of the Modified ORN2 with $NaBT_4$ (Chart 1, Fig. 4) A general procedure for the reduction of the modified ORN**2** with $NaBT_4$

Table 2. Molecular Weights of the Major Peak Obtained by the Reduction of the Modified ORN**2** with $NaBH_4$

	Modified, found*	4H-redn, calcld	2H-redn, calcld	Found*
ORN**2a**	5392	5396.5	5394.5	5395
ORN**2b**	5406	5410.6	5408.6	5407
ORN**2c**	5504	5508.8	5506.8	5506
ORN**2d**	5602	5607.0	5605.0	5604
ORN**2e**	5617	5621.0	5619.0	5619
ORN**2f**	5418	5422.6	5420.6	5422
ORN**2g**	5452	5456.5	5454.5	5453
ORN**2h**	5393	5398.5	5396.5	5394, 5395
ORN**2i**	5634	5637.5	5635.5	5636

*The molecular weights were obtained by deconvolution based on the high-resolution mass spectra derived from the trivalent negative ions observed by UPLC/QtofMS.

is described for the reaction of the modified ORN**2a**. An ampule containing $NaBT_4$ (3.7 GBq, ART012D, purchased from Japan Radioisotope Association; JRIA) was opened in the hood at the RI facility, its entire materials were dissolved in 2-propanol (300 μL), and the solution was stored at 4 °C. A portion (1.5 μL) of this solution is calculated to contain 33.3 mM $NaBT_4$ and 12.33 MBq/μL based on the catalog data, which was diluted with 2-propanol (23.5 μL) to make a stock solution of $NaBT_4$ (2 mM). A stock solution of the modified ORN**2a** (9.47 μM, 10.6 μL, 100 μmol) was diluted with ultrapure water (7.4 μL) in a microtube, followed by the addition of the 2-propanol solution of $NaBT_4$ (2 mM, 2 μL). A solution of 20 μL total volume contains 5 μM of the modified ORN**2a** and 200 μM $NaBT_4$ as the final concentrations. The solution was allowed to stand at 25 °C for 1 h. The following procedure was performed to remove any radioactive materials with low molecular weights. 1 × PBS(−) Buffer (350 μL) was added to the reaction mixture, and the whole solution was ultrafiltrated using Amicon®Ultra-0.5 mL 3K device (10000 × ***g***, 25 °C, 20 min). This procedure was repeated 5 times to obtain a sample of the 1 × PBS(−) Buffer solution, which was diluted with 1 × PBS(−) Buffer solution and ethanol to make a 50 μL solution containing 1% ethanol. A portion (1 μL) was taken from the 50 μL sample solution and mixed with a liquid scintillation cocktail (Ultima Gold, 5 mL), the radioactivity of which was measured by a liquid scintillation counter (Tri-Carb 2800TR, Perkin-Elmer) to give 76.8 Bq for a 1 μL sample solution. Thus, the radioactivity of the isolated labeled ORN**2a** was calculated to be 3.83 kBq. Half of this solution (25 μL) was diluted with 25 μL of ultrapure water, and whole solution was injected into a HPLC column and fractions were collected every 30 s. HPLC conditions: column: OSAKA SODA C18, MG, 5 μm, 4.6 × 250 mm and eluent solvents: A: 0.1 M TEAA buffer and 5% CH_3CN, B: CH_3CN, B: 0 to 30%/20 min, 30 to 100%/25 min, linear gradient; column oven: 40 °C; flow rate: 1.0 mL/min; and UV: 254 nm. The UV chart and the bar graph of the radioactivity of each fraction are shown in Fig. 4A. The area under the UV curve was quantified to determine the total amount of the labeled ORN**2a** to be 30.8 pmol. As this analysis was done using half of the product, the total yield of the reduction was 61.6 pmon (62% yield based on the modified ORN**2a** as the starting material). The sum of the radioactivity of the fractions collected between 10.5–12.5 min was 1893 Bq. Based on these values (1893 Bq/30.8 pmol), the specific radioactivity of ORN**2a** was calculated to be 61.5 Bq/pmol (61.5 GBq/mmol). Based on the radioactivity of the product (3.83 kBq) after the ultrafiltration through Amicon, 92% of the radioactivity was recovered after the HPLC purification. The yields of the labeling and specific activity thus obtained for all the ORN**2** substrates are summarized in Table 1.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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