ORGANIC LETTERS

2005
Vol. 7, No. 25
5645−5648

Synthesis of a 2′-Se-uridine Phosphoramidite and Its Incorporation into Oligonucleotides for Structural Study

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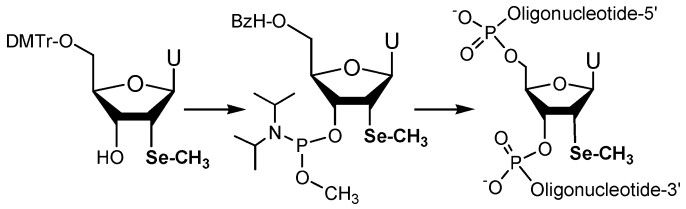
Received September 19, 2005

ABSTRACT



We report here the synthesis of the 5′-[benzhydryloxybis(trimethylsilyloxy)]silyl-2′-methylseleno-2′-deoxyuridine phosphoramidite and its incorporation into oligonucleotides by solid-phase synthesis. The coupling yield of this phosphoramidite into oligonucleotides is higher than 99%. We also demonstrate that this 2′-methylselenophosphoramidite is compatible with the 5′-silyl-2′-ACE chemistry, for longer Se-RNA solid-phase synthesis. Our preliminary NMR study on the synthesized 2′-Se-DNA has revealed a U_{Se}–A base pair and a duplex structure formation when its complementary strand was present.

Nucleic acids are important biomacromolecules in living systems and are involved in genetic information storage, expression, and regulation. Chemical modifications and structural studies have helped to reveal many insights on their biological functions and mechanisms.[1−5] To get insights of the structure and function of nucleic acids, we have recently developed selenium derivatization of DNA and RNA for phase determination,[6−10] a long-standing problem,[11] in X-ray crystallography. We have incorporated selenium atoms into nucleic acids chemically and enzymatically by replacing the nucleotide oxygen, such as the 5′, 2′, and nonbridging phosphate oxygen atoms[6,9,12] for X-ray crystal structure determination. Recently, several crystal structures of DNA and RNA molecules derivatized with the Se-labeling strategy have been reported by several laboratories using multiwavelength anomalous dispersion (MAD) phasing.[8,13,14]

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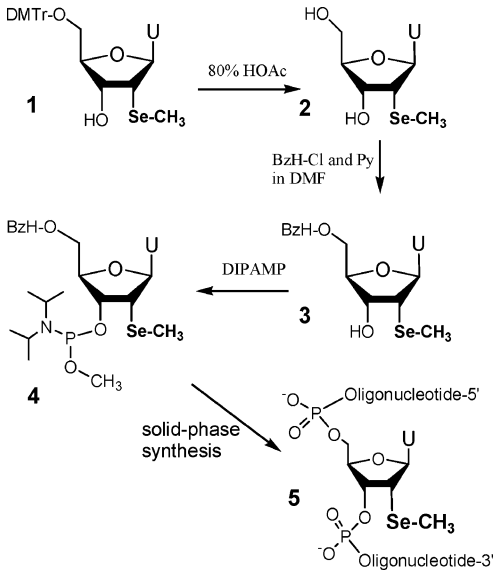
10.1021/ol052270y CCC: $30.25
Published on Web 11/16/2005

Our early experimental results on the structural determination of the Se-oligonucleotides by X-ray crystallography indicated that the 2′-Se modification favors the sugar pucker with the 2′-exo conformation, which is identical to that of A-form DNA or RNA.[8] Reliable and efficient synthesis of longer RNAs with this 2′-Se modification is desirable for X-ray crystal structural studies of catalytic RNAs. Preparation of longer RNAs with this Se derivatization is currently achieved via chemical synthesis of short RNA fragements and enzymatic ligation of these synthesized fragments.[15] This approach requires several purification steps and ligation steps, which is labor-intensive and also suffers low yield. Obviously, the current strategy is not practical for routine synthesis of large quantity (several miligrames) of longer RNAs, which limits the wide applications of the Se-derivatization of longer functional RNAs.

As the 5′-silyl-2′-ACE chemistry is currently the most effective strategy for synthesizing longer RNAs,[16,17] we have recently explored this ACE chemistry using the previously developed 5′-O-DMTr-2′-Se-Me uridine and cytidine phosphoramidites.[7,10] Due to differences in the 5′-deprotection and phosphite oxidation steps, however, these two Se-phosphoramidites could not be conveniently used in the 5′-silyl-2′-ACE chemistry. To overcome these shortcomings and to meet the needs of longer Se-RNA synthesis, we have synthesized 5′-benzhydryl-2′-methylselenophosphoramidite (**4**, Scheme 1). This development allows milligram-scale synthesis of longer RNAs derivatized with selenium, without ligation. Similarly, this synthesis can be extended to syntheses of the other three Se-derivatized phosphoramidites (A, C, and G) for the ACE chemistry. To conveniently demonstrate that 5′-BzH-2′-Se-uridine phosphoramidite **4** is compatible with 5′-silyl-2′-ACE chemistry, syntheses of 2′-Se-RNAs and 2′-Se-DNAs were designed as a model system to examine the compatibility of **4** with the reagents and conditions used in the 5′-silyl-2′-ACE chemistry. The synthesized Se-DNAs were also studied by NMR to obtain the insight of duplex formation.

Scheme 1. Synthesis of 5′-BzH-2′-Se-Me-uridine Phosphoramidite (4) and Oligonucleotides Containing the Se Derivatization (**5**)



We report here the synthesis of the 5′-O−BzH-2′-Se-Me-uridine phosphoramidite (**4**), its incorporation into oligonucleotides via solid-phase synthesis, and Se−DNA duplex study by NMR. Uridine derivative **1**, synthesized previously,[10] was treated with 80% acetic acid to remove the 5′-DMTr group (96% yield), followed by protection of the 5′-hydroxy group with benzhydryloxybis(trimethylsilyloxy)silyl (BzH) group using BzH-Cl in dry DMF (86% yield).[17] Partially protected uridine derivative **3** was converted to the corresponding phosphoramidite **4** in dry CH_2Cl_2 solvent using bis(*N*,*N*-diisopropylamino)methoxyphosphine (DIPAMP) in the presence of 5-(benzylthio)-1*H*-tetrazole (88% yield).[10, 17]

The incorporation cycle of phosphoramidite **4** in oligonucleotide solid-phase synthesis[10] was modified in order to successfully synthesize the 2′-Se-derivatized DNAs and RNAs, which also served as testing models for the compatibility with 5′-silyl-2′-ACE chemistry. These following modifications represent the reagents and treatments in the 5′-silyl-2′-ACE chemistry. First, 5-benzylmercapto-1*H*-tetrazole was used as the reagent for the coupling reaction of phosphoramidite **4**,[18] instead of 1*H*-tetrazole. Second, after the coupling of Se-modified phosphoramidite **4**, we examined the phosphite oxidation with *tert*-butyl hydroperoxide (BHPO) in toluene for 45 s.[17] This BHPO treatment did not cause oxidation of the 2′-selenide functionality, which is consistent with the iodine oxidation.[10] MS and HPLC analyses indicated that the selenide oxidation was not detectable (Figures 1 and 2). Third, the 5′-BzH deprotection was performed with triethylammonium fluoride in DMF for 60 s, instead of the 5′-DMTr deprotection with 3% trichloroacetic acid. Fourth, after the oligonucleotide synthesis was completed, the immobilized oligonucleotides were treated with disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate[17] in DMF for 15 min to remove the methoxy group on the 3′-phosphate group of the Se-modified nucleotide, followed by water and acetonitrile washing. Finally, the oligonucleotide RNAs and DNAs containing the 2′-Se derivatization were cleaved off the support and fully deprotected with concentrated ammonia at 55 °C overnight for DNAs and with methylamine for RNAs.[10] The stability of the Se-oligonucleotides under these conditions and treatments used in the 5′-silyl-2′-ACE chemistry indicates the compatibility of this Se-uridine phosphoramidite (**4**) with the ACE chemistry for RNA synthesis.

The crude 2′-Se-derivatized DNA and RNA oligonucleotides with DMTr-on were analyzed by HPLC. Representative reversed-phase HPLC elution profiles are shown in

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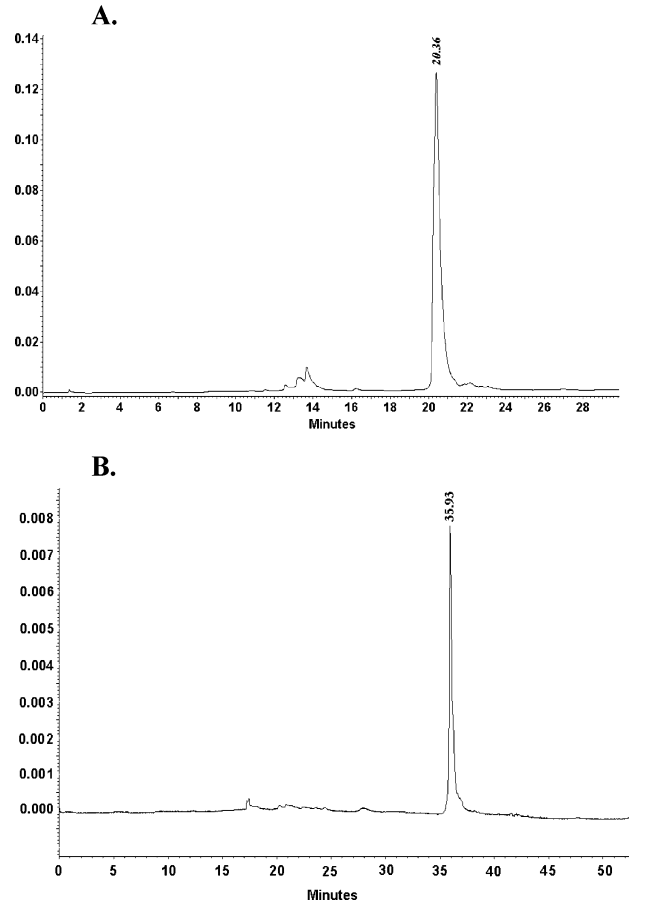

Figure 1. Reversed-phase HPLC analysis of Se-oligonucleotides. (A) Crude DMTr-on 2′-Se-DNA (5′-DMTr-GAGCU$_{Se}$CCAT-3′) after cleavage of the oligonucleotide from the solid support and the deprotection of the bases and backbone. The sample was analyzed on a Zorbax SB-C18 column (4.6 × 250 mm), eluted (1 mL/min) with a linear gradient from buffer A (20 mM triethylammonium acetate, pH 7.1) to 100% buffer B (50% acetonitrile, 20 mM triethylammonium acetate, pH 7.1) in 30 min. Its retention time is 20.4 min. (B) crude DMTr-on 2′-Se-RNA (5′-DMTr-AUGGU$_{Se}$GCUC-3′) after cleavage of the oligonucleotide from the solid support and the deprotection of the bases, 2′-TOM groups, and backbone. The sample was analyzed on the same column with the same buffers A and B. The sample was eluted (1 mL/min) with a linear gradient from buffer A to 100% buffer B in 45 min. Its retention time is 35.9 min.

Figure 1. Less than 7% short oligonucleotides were formed on average, which suggested a high coupling yield per synthetic cycle (higher than 99% on average). As expected, the coupling yields of this Se-phosphoramidite in both Se-DNA and Se-RNA syntheses are the same due to the almost identical coupling of the Se-phosphoramidite incorporation into both Se-DNA and Se-RNA. The synthesized oligonucleotides were purified twice by HPLC (DMTr-on and DMTr-off). The reversed-phase HPLC purification was performed by a Zorbax C18 column (21 × 250 mm), eluted (10 mL/min) with a linear gradient from buffer A (20 mM triethylammonium acetate, pH 7.1) to 100% buffer B (50% acetonitrile, 20 mM triethylammonium acetate, pH 7.1) in 30 min. These oligonucleotides were also confirmed by MS analysis.

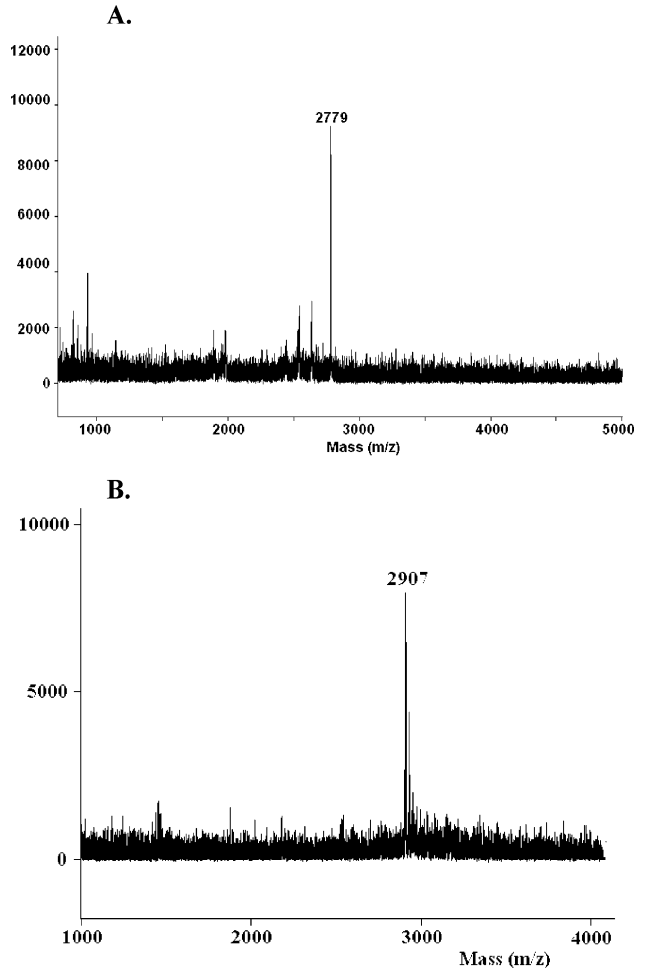

Figure 2. MALDI-TOF MS analysis of the Se-oligonucleotides: (A) DMTr-off 2′-Se-DNA9mer (5′-GAGCU$_{Se}$CCAT-3′; molecular formula: $C_{87}H_{111}N_{33}O_{52}P_8Se$), FW 2777.8; $[M + H]^+$: 2779 (calcd 2778.8). (B) DMTr-off 2′-Se-RNA9mer (5′-AUGGU$_{Se}$-GCUC-3′; molecular formula: $C_{86}H_{108}N_{32}O_{62}P_8Se$), FW 2908.7; $[M - H]^-$ 2907 (calcd 2907.7).

Typical MS spectra of DMTr-off DNAs and RNAs are shown in Figure 2, and all MS data are collected in Table 1.

To understand the effect of Se modification on oligonucleotide structure, we examined the consequence of a single derivatization. The host DNA duplex forms a B-type duplex with 9 base pairs (5′-GAGCTCCAT-3′ and 5′-ATGGAGCTC-3′, Figure 3A).[19] Figure 3 shows the imino proton NMR spectra of the unmodified host duplex, the Se-modified duplex and the constituent strands. The Se-modified single strand (5′-GAGCU$_{Se}$CCAT-3′) is largely unstructured in solution (Figure 3B), while the complement strand (Figure 3C) exhibits some base pair formation by itself, indicative of homoduplex formation. In the presence of the Se-modified counterpart, however, the heteroduplex is formed (Figure 3D). The minor peak observed at 14.05 ppm in Figure 3D indicates the presence of small amount of homoduplex.

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Table 1. MALDI-TOF MS Analytical Data of Se-oligonucleotides

entry	Se-oligonucleotides	measured (calcd) *m/z*
a	Se-DNA1 (5′-GAGCU$_{Se}$CCAT-3′) $C_{87}H_{111}N_{33}O_{52}P_8Se$: FW 2777.8	$[M + H]^+$: 2779 (2778.8)
b	Se-DNA2 nontreated with BHPO (5′-ATGGU$_{Se}$GCTC-3′) $C_{88}H_{112}N_{32}O_{54}P_8Se$: FW 2808.9	$[M + H]^+$: 2810 (2809.9)
c	Se-DNA2 treated with BHPO (5′-ATGGU$_{Se}$GCTC-3′) $C_{88}H_{112}N_{32}O_{54}P_8Se$: FW 2808.9	$[M + H]^+$: 2810 (2809.9)
d	Se-DNA3 nontreated with BHPO (5′-CCTU$_{Se}$GACAAAG-3′) $C_{107}H_{135}N_{43}O_{62}P_{10}Se$: FW 3404.2	$[M + H]^+$: 3405 (3400 avg) $[M + Na]^+$: 3427 (3422 avg)
e	Se-DNA3 treated with BHPO (5′-CCTU$_{Se}$GACAAAG-3′) $C_{107}H_{135}N_{43}O_{62}P_{10}Se$: FW 3404.2	$[M + H]^+$: 3405 (3400 avg) $[M + Na]^+$: 3427 (3422 avg)
f	Se-RNA9mer treated with BHPO (5′-AUGGU$_{Se}$GCUC-3′) $C_{86}H_{108}N_{32}O_{62}P_8Se$: FW 2908.7	$[M + H]^-$: 2907 (2907.7)
g	Se-RNA11mer treated with BHPO (5′-CCUU$_{Se}$GACAAAG-3′) $C_{106}H_{133}N_{43}O_{72}P_{10}Se$: FW 3550.1	$[M - H]^-$: 3549 (3549.1)

Although the Se-modified duplex is thermodynamically less stable than the control duplex, we clearly observe the presence of nine base pairs. The imino protons of the 5 G−C base pairs resonate at 12.6−12.9 ppm, while the 3 A−T and 1 A−U$_{Se}$ base pairs are in the region of 13−14.3 ppm. As anticipated, the spectrum of the control duplex (5 G−C and 4 A−T, Figure 3A) is similar to the modified duplex. In particular, the chemical shifts of 1, 2, 8 A−T base pairs remain the same. While the chemical shift of the no. 5 A−T imino proton in native duplex is at 13.9 ppm, the modified A−U$_{Se}$ base pair is at 13.7 ppm. This chemical shift demonstrates that the central Se-modified U is capable of base pairing with A in the context of a B type host duplex. This comparison of the Se-modidified and control duplexes reveals that the Se-modification does not cause significant perturbation, which is consistent with the UV−melting temperature study.[12] Further NMR study will reveal the structural nature of the modified duplex.

In conclusion, we have synthesized the 2′-Se-uridine phosphoramidite (**4**). More importantly, we have successfully incorporated it into oligonucleotides with a high coupling yield (over 99%). As expected, the 2′-Se functionality is stable under conventional oligonucleotide synthesis, indicated by HPLC and MS analyses. We have also demonstrated that

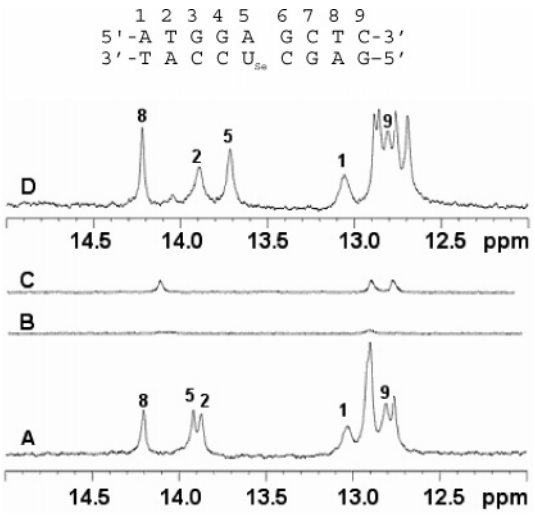

Figure 3. NMR study of Se-DNA, the complementary strand, and Se-derivatized DNA duplex (100 μM each sequence) in 50 mM NaCl, 0.1 mM EDTA, 10 mM NaH_2PO_4−Na_2HPO_4 (pH 6.5) at 283 K: (A) NMR spectrum of the unmodified heteroduplex as a control; (B) NMR spectrum of 5′-GAGCU$_{Se}$CCAT-3′ single strand; (C) NMR spectrum of 5′-ATGGAGCTC-3′ single strand; (D) NMR spectrum of the modified heteroduplex.

this phosphoramidite (**4**) and the synthesized 2′-Se-oligonucleotides are stable and compatible with the reagents used in the 5′-silyl-2′-ACE chemistry for RNA synthesis. As longer Se-RNA synthesis with satisfactory quantity and quality is desired in studying functional RNAs, this phosphoramidite (**4**) meets the need for site-specific RNA derivatization with selenium in X-ray crystal structure study of longer RNAs.[20]

Our NMR data demonstrate the formation of a DNA duplex structure in aqueous solution between the 2′-Se−U modified sequence and its unmodified complementary strand. The U$_{Se}$−A base pair formation is consistent with our previous crystal structure study.[8] Further NMR experiments will provide conformation parameters of 2′-Se derivatized nucleotides in solution and will also help in determining optimal positions of Se derivatization for nucleic acid X-ray crystal structure study. Moreover, our results have suggested that the 2′-Se labels can be useful in NMR spectrum assignment and structure determination, especially for longer RNAs (over 60 nt). The selenium derivatization of DNA and RNA will significantly facilitate structure and mechanism studies of nucleic acids.

Acknowledgment. This work was supported by the GSU Research Program and the National Institutes of Health (GM069703). We thank Dr. Abdalla Hassan and Julianne Caton-Williams for carefully reading this manuscript.

Supporting Information Available: Experimental procedures, ^1H and ^{13}C NMR, HPLC, and HRMS analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

OL052270Y

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