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Synthesis of Selenium-Derivatized Cytidine and Oligonucleotides for X-ray Crystallography Using MAD

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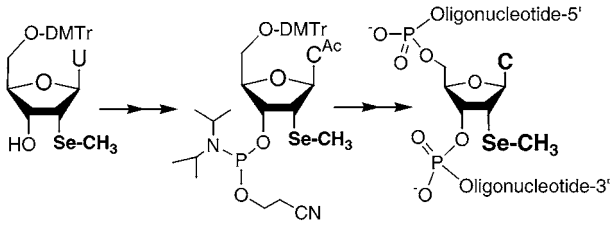
ABSTRACT



Synthesis of the novel 2′-Se-cytidine phosphoramidite was achieved via transformation of the uridine analogue to the cytidine derivative in high yield. This 2′-Se-cytidine phosphoramidite was used to synthesize selenium-derivatized DNA and RNA oligonucleotides for X-ray crystallography using MAD. The nucleotide coupling yield using this novel phosphoramidite was over 99% when 5-benzylmercaptotetrazole (5-BMT) was used as the coupling reagent.

Derivatization of DNA and RNA for phase determination in X-ray crystallography is a long-standing problem even though several conventional approaches are commonly used, such as heavy atom soaking, cocrystallization, and halogen derivatization.[1] Derivatization efforts via soaking and cocrystallization often fail, probably due to nucleotide cleavage by the heavy metal ions or nonspecific binding to polyanionic nucleic acids. Halogen derivatization is often limited to oligonucleotides.[2] Therefore, selenium derivatization of nucleic acids is of great importance because of the potential application in determination of 3-D structures of DNAs, ribozymes, other functional RNAs and nucleic acid−protein complexes by X-ray crystallography.[3]

Recently, Huang, Egli, and co-workers have established the principle of nucleic acid X-ray crystallography by the replacement of oxygen in nucleotide with selenium using multiwavelengh anomalous dispersion (MAD).[4−8] Previous X-ray crystallographic studies on the oligonucleotides containing the 2′-methylselenouridine have indicated that the 2′-methylseleno-substituted furanoses display C3′-endo puckers, consistent with the A-form geometry of RNA and A-form DNA. Furthermore, the duplexes of the modified oligonucleotides were as stable as the native ones. These results demonstrated that the 2′-selenium functionality is suitable for RNA and A-form DNA derivatization for X-ray

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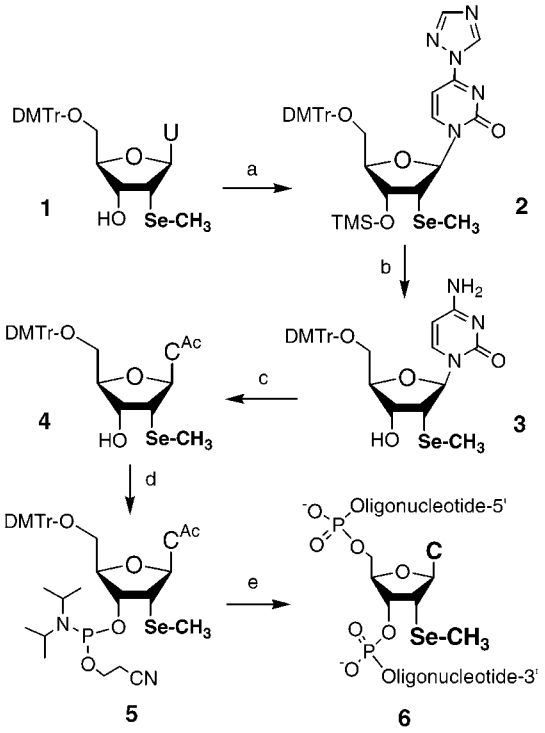
crystallography.[5,6] To further apply this strategy in nucleic acid derivatization, we designed and synthesized a novel building block, 2′-Se-cytidine phosphoramidite **5**, for derivatization of DNAs and RNAs using the solid-phase synthesis. We report here syntheses of the cytidine derivative with oxygen replacement with selenium at the 2′-position, the corresponding phosphoramidite, and the Se-DNAs and Se-RNAs for X-ray crystallography using MAD.

Analogous to the 2′-Se-uridine synthesis,[5] the synthesis of the 2′-Se-cytidine derivatives was first attempted. Due to the low yield in the introduction of the methylseleno group, an alternative route (Scheme 1) was explored by converting

Scheme 1. Synthesis of the 2′-Se-Cytidine Phosphoramidite and Its Incorporation into DNA and RNA Oligonucleotides[a]



[a] Reagents and conditions: (a) TMS-Im, then $POCl_3$-triazole-TEA in CH_3CN; (b) NH_4OH; (c) TMS-Im, then Ac_2O, TEA, and DMAP in THF; (d) 2-cyanoethyl *N*,*N*-diisopropyl-chlorophosphoramidite and *N*,*N*-diisopropylethylamine in CH_2Cl_2; (e) synthesis of oligonucleotides on solid phase.

uridine to cytidine.[9] Transformation of uridine to cytidine consists of two major steps: activation of the position 4 of uridine and ammonia treatment. Initially, we encountered loss of the selenium functionality in the activation step. The deselenization probably was caused by the highly reactive species in the reagent-generating reaction, where phosphorus oxytriazolide was made in situ using phosphorus oxychloride ($POCl_3$) and triazole. This difficulty was later overcome by extending the reagent-making time and adding a large excess of dry triethylamine (TEA). After the in situ protection of

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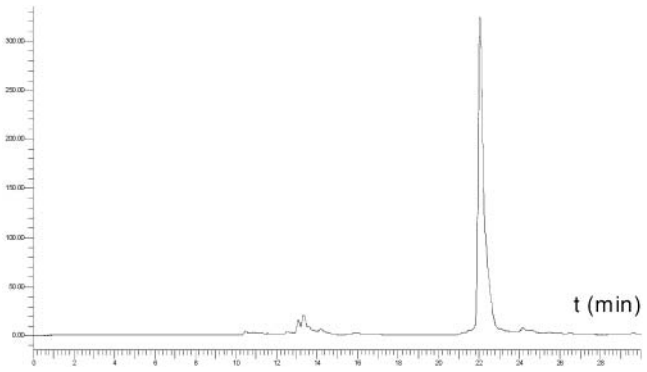

Figure 1. Reversed-phase HPLC analysis of crude DMTr-on 2′-Se-C RNA8mer (5′-DMTr-GGC$_{Se}$GUGCC-3′) after deprotection of the bases, backbone, and 2′-TOM groups. Its retention time is 22.1 min.

the 3′-hydroxyl group using 1-(trimethylsilyl)imidazole (TMS-Im) in dry acetonitrile, the position 4 of the uridine derivative was activated via formation of the triazolide (**2**) in dry acetonitrile. Intermediate **2** was directly converted to the cytidine derivative (**3**) without purification by aqueous ammonia treatment. The transient 3′-TMS protection was concomitantly removed during this treatment. The yield over these reactions in one-pot was 86%. After silylation of the 3′-OH group of **3** with TMS-Im in dry tetrahydrofuran (THF), the amino group of the cytidine derivative was acetylated using acetic anhydride in the presence of dry TEA and a catalytic amount of *N*,*N*-dimethylaminopyridine (DMAP). Under the same acetylation conditions, the acylation using benzoyl chloride was attempted without success. The transient silyl protection on the 3′-OH was removed to give **4** by tetrabutylammonium fluoride treatment in THF. Cytidine derivative **4** was converted to 2′-Se-cytidine phosphoramidite **5** in 92% yield by reacting it with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite in the presence of *N*,*N*-diisopropylethylamine, in dry CH_2Cl_2.[5]

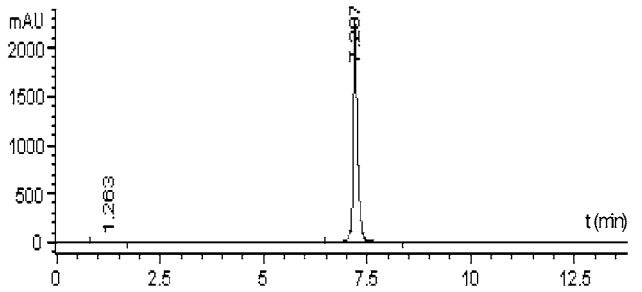

Figure 2. HPLC analysis of purified DMTr-off 2′-Se-C DNA12mer (5′-TATCGTTAATC$_{Se}$T-3′). The sample was analyzed on a 300SB-C8 column (4.5 × 150 mm), eluted (1 mL/min) with buffer A (5 mM ammonium acetate, pH 6.5) for 2 min, and then eluted with a linear gradient from buffer A to 100% buffer B (60% acetonitrile and 40% of buffer A) in 13 min. Its retention time is 7.2 min.

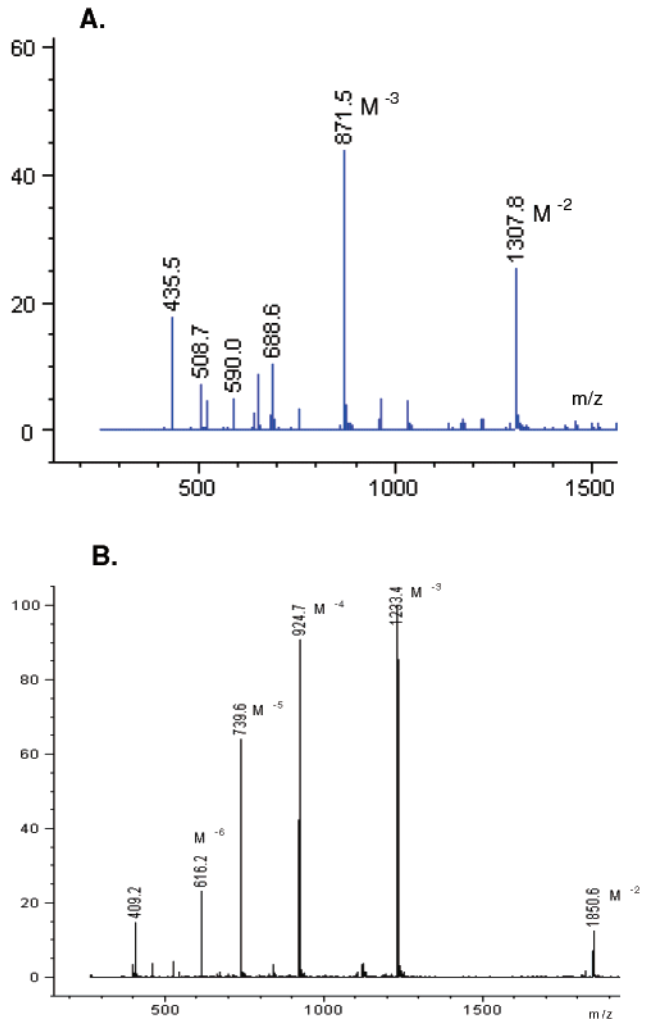

Figure 3. Electrospray MS analysis of 2′-Se-C oligonucleotides: (A) DMTr-off 2′-Se-C RNA8mer ($C_{77}H_{98}N_{31}O_{54}P_7Se$), isotopic mass 2617.3, M^{-2} 1307.8 (1307.7), M^{-3} 871.5 (871.4); (B) DMTr-off 2′-Se-C DNA12mer ($C_{119}H_{153}N_{38}O_{73}P_{11}Se$), isotopic mass 3702.6, M^{-2} 1850.6 (1850.3), M^{-3} 1233.4 (1233.2), M^{-4} 924.7 (924.6), M^{-5} 739.6 (739.5), M^{-6} 616.2 (616.1).

To demonstrate the compatibility of **5** with the solid-phase synthesis, several DNA and RNA oligonucleotides were designed and synthesized. Selenium-derivatized RNAs were synthesized using 2′-O-triisopropylsilyloxymethyl (TOM)-protected nucleoside phosphoramidites,[10] while Se-derivatized DNAs were synthesized by following standard solid-phase synthesis and using 5-benzylmercaptotetrazole (5-BMT) as the coupling reagent.[11] The coupling yield using 5-BMT activator was higher than that using tetrazole (data not shown). Like the previous report on the synthesis of 2′-Se-U DNAs,[5] the protected selenide functionality in 2′-Se-C oligonucleotides was also stable under mild I_2 treatment (20 mM, 20 s) for the phosphite oxidation. This selenium functionality was stable even when it was close to the 3′-end, where it experienced multiple cycles of the I_2 oxidation (data not shown). In addition, like the 2′-Se-U phosphoramidite, the solid-phase coupling yields of RNAs and DNAs using this novel 2′-Se-C phosphoramidite were higher than 99%. The reversed-phase HPLC analysis of the crude RNA8mer synthesized using 2′-Se-C phosphoramidite **5** is shown in Figure 1.

Chemically synthesized RNA and DNA oligonucleotides containing 2′-Se-C derivatization were purified twice by HPLC (DMTr-on and DMTr-off). The reversed-phase HPLC purifications were performed by a Zorbax C8 column (21.2 × 250 mm). Samples were eluted (10 mL/min, Figure 1) with a linear gradient from buffer A [50 mM triethylammonium acetate (TEAAc), pH 7.1] to 90% buffer B (50% aqueous acetonitrile, 50 mM TEAAc, pH 7.1) in 25 min. A typical HPLC profile of purified Se-RNAs and Se-DNAs is shown in Figure 2. Purified 2′-Se-C-derivatized oligonucleotides were confirmed by electrospray mass spectrometry. MS spectral examples of 2′-Se-C-RNAs and 2′-Se-C-DNAs are shown in Figure 3, and the molecular peaks with several different charges are observed. The MS analytical data are shown in Table 1.

Table 1. MS Analytical Data of RNA and DNA Oligonucleotides

entry	Se-oligonucleotides	measured (calcd) *m/z*
a	2′-Se-C RNA8mer	M^{2-}: 1307.8 (1307.7)
	(5′-GGC$_{Se}$GUGCC-3′)	M^{3-}: 871.5 (871.4)
	$C_{77}H_{98}N_{31}O_{54}P_7Se$: FW 2617.3	
b	2′-Se-C RNA14mer	M^{3-}: 1500.6 (1500.5)
	(GC$_{Se}$UGACGAUACACC)	M^{4-}: 1125.3 (1125.2)
	$C_{134}H_{169}N_{54}O_{93}P_{13}Se$: FW 4504.6	
c	2′-Se-C DNA8mer	M^{2-}: 1254.7 (1254.7)
	(5′-AC$_{Se}$TGACAG-3′)	M^{3-}: 836.1 (836.1)
	$C_{79}H_{100}N_{33}O_{44}P_7Se$: FW 2511.4	
d	DMTr-on 2′-Se-C DNA12mer	M^{2-}: 2001.8 (2001.4)
	(DMTr-TATCGTTAATC$_{Se}$T)	M^{3-}: 1334.2 (1333.9)
	formula: $C_{140}H_{171}N_{38}O_{75}P_{11}Se$	M^{3-}: 1334.2 (1333.9)
	formula isotopic weight: 4004.7	M^{4-}: 1000.3 (1000.2)
		M^{5-}: 800.1 (799.9)
		M^{6-}: 666.6 (666.5)
e	DMTr-off 2′-Se-C DNA12mer	M^{2-}: 1850.6 (1850.3)
	(5′-TATCGTTAATC$_{Se}$T-3′)	M^{3-}: 1233.4 (1233.2)
	formula: $C_{119}H_{153}N_{38}O_{73}P_{11}Se$;	M^{4-}: 924.7 (924.6)
	formula isotopic weight: 3702.6	M^{5-}: 739.6 (739.5)
		M^{6-}: 616.2 (616.1)

To investigate the duplex stability of RNA containing the 2′-Se derivatization, the UV melting study was performed using the RNA8mer and its analogues (Table 2). Solutions of the duplex RNAs (1.5 μM) were prepared by dissolving the RNAs in a buffer containing NaCl (200 mM), sodium phosphate (5 mM, pH 7.4), and EDTA (1 mM). The solutions were then heated to 95 °C for 1 min, cooled slowly to room temperature, and stored at 5 °C overnight before measurement.[12] Denaturation curves were acquired at 260 nm at a heating rate of 0.5 °C/min (from 10 to 70 °C) using a 8453 UV−vis Spectrometer from Agilent Technologies, which was

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Table 2. UV Melting Temperatures of the RNA8mers

entry	RNA8mer	melting temperature (°C)
a	native RNA8mer (5′-GGCGUGCC-3′)	36.6
b	2′-MeO−RNA8mer (5′-GGC$_O$GUGCC-3′)	32.9
c	2′-MeSe−RNA8mer (5′-GGC$_{Se}$GUGCC-3′)	31.7
d	2′-MeSe−RNA8mer (5′-GGCGU$_{Se}$GCC-3′)	32.4

equipped with a Peltier temperature controller. The result shows that the melting temperatures of the 2′-Se-C and 2′-Se-U RNA8mers were almost the same as that of the 2′-MeO RNA8mer (Table 2), which was slightly lower than that of the native. This melting study indicates that the 2′-Se modification had just minor effect on RNA duplex stability. This is consistent with the stability study of the A-form DNA duplex derivatized with the 2′-Se functionality investigated previously, which indicated that the selenium derivatization had no significant effect on stability of the A-form DNA duplex.[6]

In conclusion, we have developed a route to synthesize the novel 2′-Se-cytidine phosphoramidite via conversion of the uridine to the 2′-Se-cytidine derivative. This 2′-Se-C phosphoramidite has been successfully used to derivatize RNAs and DNAs by the solid-phase synthesis. As the large-scale synthesis of Se-derivatized oligonucleotides is possible, the chemical synthesis of Se-DNAs and Se-RNAs is of great importance in nucleic acid X-ray crystallography using MAD. As one selenium atom has anomalous phasing power over approximately 30 nucleotides, long DNAs and RNAs (e.g., over 100 nt) containing multiple selenium labels can be prepared through a ligation of one synthetic fragment containing multiple selenium atoms with a polymerized DNA or transcribed RNA fragment.[13,14] In addition, the derivatization strategy of nucleic acids with selenium can be used to derivatize nucleic acid−protein complexes via derivatization of nucleic acids instead of the protein counterparts for X-ray crystallography.

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Supporting Information Available: Experimental procedures and ^{1}H, ^{13}C, and ^{31}P NMR, and HRMS analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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