Selenium modification of nucleic acids: preparation of phosphoroselenoate derivatives for crystallographic phasing of nucleic acid structures

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This protocol describes a simplified means of introducing an anomalously scattering atom into oligonucleotides by conventional solid-phase synthesis. Replacement of a nonbridging phosphate oxygen in the backbone with selenium is practically suitable for any nucleic acid. The resulting oligonucleotide P-diastereomers can be separated using anion exchange HPLC to yield diastereomerically pure phosphoroselenoates (PSes). The total time for the synthesis and ion-exchange HPLC separation of pure PSe is approximately 60 h.

INTRODUCTION

X-ray structure determination of nucleic acids has proven particularly challenging compared with protein crystallography owing to inherent difficulties in creating suitable heavy atom derivatives for crystallographic phasing. The conventional methods for heavy atom derivatization are soaking or co-crystallization with heavy metal ions[1–4] and covalent derivatization with halogens (Br^5U, Br^5C, I^5U)[5,6]. Oligonucleotides are generally devoid of binding sites for classic heavy atom derivatives and their crystals often lack the solvent channels that allow relatively large metal ion complexes to diffuse into the lattice. The major benefit of covalent modification compared with soaking or co-crystallization experiments is that once crystals are obtained, the presence of the heavy atom in the crystal is more or less guaranteed. However, brominated and iodinated oligonucleotides are light sensitive[7] and debromination of nucleic acids induced by X-rays has been reported[6]. Compared with derivatization with halogenated U or C residues, selenium modification of nucleic acids offers several advantages: (i) all 4 nucleotides (nt) can be chemically modified (i.e., 2′-SeMe-U, -C, -A or -G)[8–13]; (ii) any oxygen in a nucleotide can, in principle, be replaced with selenium (i.e., the nonbridging O1P and O2P phosphate oxygens, ribose O2′ and O4′ and uracil (thymine) O2 and O4; (iii) the 2′-SeMe modification is stable against oxidation, light and X-ray radiation; and (iv) in the case of phosphoroselenoate (PSe) derivative[14,15], there is no need for synthesizing specialized building blocks.

Selenium can be used for covalent derivatization of proteins in the form of selenomethionine (Se-Met)[16] that can be readily incorporated into proteins in place of Met[17]. This approach has greatly facilitated crystallographic structure determination of proteins in combination with multi-/single-wavelength anomalous dispersion (MAD/SAD) phasing[18]. The K absorption edge of selenium (12.6578 keV, 0.9795 Å) is readily accessible with synchrotron radiation. Selenium lies under group VI in the periodic table with a van der Waals radius of 2.00 Å (as compared with 1.85 Å for sulfur and 1.4 Å for oxygen). The structural isomorphism between numerous proteins containing Se-Met in place of Met and their natural counterparts has been demonstrated[19]. This article mainly discusses covalent modification of nucleic acids using selenium for crystallographic phasing, an approach that has proven very successful in the structural determination of proteins for more than a decade[16,17].

A few years ago, Huang, Egli and co-workers reported the incorporation of selenium at the C2′-position of 2′-deoxyuridine (2′-SeMe-U) using a chemical synthetic approach that is compatible with automated oligonucleotide synthesis[8]. (Throughout this article, the term oligonucleotides refers to oligodeoxynucleotides.) A detailed protocol for derivatization of oligonucleotides with 2′-SeMe-U can be found in this issue of *Nature Protocols*[20]. Initially, a DNA decamer sequence was synthesized and its structure was solved using the MAD technique[8,9]. However, modification at this site locks the sugar in a C3′-*endo* conformation, thus resulting in an A-form geometry of the duplex. Therefore, this approach is best suited for the modification of double helical DNA with an A-form geometry and RNA[9]. In addition to 2′-SeMe-U (ref. 8), syntheses for 2′-SeMe-C[10,12] -G[11] and -A[13] phosphoramidites have also been reported. Ligation of chemically synthesized oligonucleotides bearing 2′-SeMe-modified residues with longer RNAs obtained through *in vitro* transcription has been used to produce selenoated RNAs of up to 100 residues in length[12,13]. Recently, the first crystal structure determination of a new RNA motif based on 2′-SeMe-U derivatization has also been achieved[21].

In search of a more general method for introducing selenium into DNA, RNA and nucleic acid analogs that is expected not to influence the backbone conformation, we turned our attention to PSe-DNA (**Fig. 1**). PSe-DNA was studied as a possible antisense modification, but had been found to be too unstable for *in vivo* applications[22]. However, we decided to test the suitability of the PSe modification as a means for crystallographic phasing despite the apparent instability due to oxidation and loss of selenium previously observed[22]. Nonstereoselective replacement of one of the nonbridging phosphate oxygen atoms with selenium produces a mixture of PSe P-diastereomers. Diastereomerically pure PSe containing a single internucleotide PSe linkage can be obtained using anion exchange HPLC of the diastereomeric mixture (**Fig. 1**). Enzymatic synthesis of longer stereo-defined PSe-oligonucleotides based on the use of both S_P and R_P diastereomers of α-seleno-NTPs (NTPαSe) has been achieved for both DNA[23]

and RNA[24]. Recently, a synthetic route for the synthesis of P-stereodefined PSe-oligonucleotides has also been described[25]. In a proof-of-principle study of the use of PSe-DNA for crystallographic phasing, we synthesized DNA hexamers of sequence 5′-CGCGCG-3′ with a single PSe moiety in their backbones, and the structure of the hexamer 5′-C_{PSe}GCGCG-3′ was determined using MAD and refined to atomic resolution[14]. This investigation demonstrated that PSe-modified oligonucleotides are stable on a crystallographic time scale. Also, we recently determined the crystal structure of homo-DNA using MAD with a single PSe derivative[15]. The structure of homo-DNA had resisted all attempts at phasing for many years, including those based on heavy atom soaking and bromination.

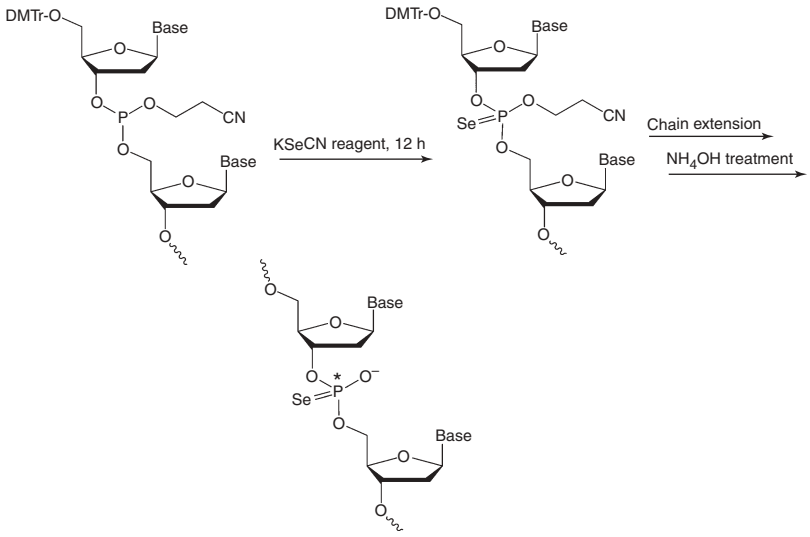

Figure 1 | Synthesis of oligonucleotides with incorporation of a phosphoroselenoate (PSe) linkage (the asterisk indicates a mixture of P-diastereomers).

This protocol describes the synthetic procedures for the derivatization of oligonucleotides using the PSe approach. For the derivatization of oligonucleotides with 2′-SeMe-U, see the protocol in this issue by Pallan and Egli[20].

In this approach, the PSe is introduced at the newly synthesized phosphite linkage during solid-phase oligonucleotide synthesis. For example, to synthesize the DNA sequence 5′-$^{6}C^{5}G_{PSe}{}^{4}C^{3}G^{2}C^{1}G$-3′ (where 5′-$^{5}G_{PSe}{}^{4}C$-3′ indicates the PSe linkage between 5′-^{5}G and ^{4}C-3′, and the numbers in superscript indicate the position of the nucleotide in the target oligonucleotide sequence), the researcher will edit the sequence 5′-$^{5}G^{4}C^{3}G^{2}C^{1}G$-3′ and start its synthesis with the trityl 'on' option, or, in other words, start the synthesis for the target oligonucleotide sequence 5′-dimethoxytrityl (DMTr)O-$^{5}G^{4}C^{3}G^{2}C^{1}G$-3′. It is worthwhile reminding at this juncture that on an automated DNA synthesizer, the synthesis takes place starting from the 3′-terminus and proceeds to the 5′-terminus. Soon after the final coupling step and the subsequent acetonitrile (CH_3CN) wash, the synthesis is paused. At this stage, the oligonucleotide is 5′-$^{5}G_{P(III)}{}^{4}C^{3}G^{2}C^{1}G$-3′, where the subscript P(III) refers to the fact that the newly generated linkage between 5′-^{5}G and ^{4}C-3′ is, at this point, a phosphite bridge (see **Fig. 1**). In the next step, to have selenium replace oxygen, while converting P(III) to P(V), a potassium selenocyanate (KSeCN)-based reagent is used in lieu of a standard oxidizing reagent (iodine/water/pyridine). Oxidation of PSes to phosphates and metallic selenium can occur, and as a precautionary measure, oligonucleotides were kept in the dark to minimize exposure to light.

In theory, the selenium moiety can be introduced in this fashion at any phosphate linkage, namely 3′-terminal, 5′-terminal or any of the positions in between. However, introduction of selenium at the 3′-terminus is challenging as the relatively sensitive PSe moiety has to withstand all subsequent oxidations required in the stepwise solid phase synthesis of an oligonucleotide. For this reason, the yields for PSe moieties placed at the 5′-terminal phosphate (e.g., see **Fig. 2a**) or near the 5′-terminal end are much higher compared with placement near the 3′-terminal end. Comparison of the relative intensities of the peaks in the HPLC chromatograms in **Figure 2a** and **b** illustrates the significant reduction in yield due to the implementation of a single additional oxidation step.

MATERIALS

REAGENTS

- KSeCN (Sigma-Aldrich, cat. no. 216186) **! CAUTION** Wear gloves and safety glasses while handling KSeCN. **! CAUTION** Is toxic and has to be handled with great care (search for material safety data sheet for cat. no. 216186 at http://www.sigma aldrich.com/catalog/search/AdvancedSearchPage). Handle all selenium reagents with gloves and in a fume hood. These precautions need to be observed during Steps 1–7.
- Ammonium hydroxide, 30% (NH_4OH; J.T. Baker cat. no. 9733)
- Drierite absorbents (Fisher Scientific, cat. no. 07-578-3A)
- Phosphorous pentoxide (P_2O_5; Acros; Fisher Scientific, cat. no. AC20089) **! CAUTION** Wear gloves and safety glasses while handling P_2O_5.
- Calcium hydride (CaH_2; Acros; Fisher Scientific, cat. no. AC19996) **! CAUTION** Wear gloves and safety glasses while handling CaH_2.
- CH_3CN, OmniSolv (EMD Chemicals Inc., cat. no. AX0142-1) **! CAUTION** Wear gloves and safety glasses while handling CH_3CN.
- De-blocking or detritylation reagent: 3% dichloroacetic acid/dichloromethane (Chem Genes Corp., MA 01887, cat. no. RN-1468) **! CAUTION** Wear gloves and safety glasses while handling this reagent; exercise caution when handling all DNA synthesis reagents.
- Activation reagent: 0.25 M 5-ethylthiotetrazole/CH_3CN (Chem Genes Corp., cat. no. RN-1466)
- Capping solution, CAP A: acetic anhydride/pyridine/tetrahydrofuran (THF) (Chem Genes Corp., cat. no. RN-1458)
- Capping solution, CAP B: 10% *N*-methylimidazole/THF (Chem Genes Corp., cat. no. RN-1481)
- Oxidation solution: 0.02 M iodine/pyridine/H_2O/THF (Chem Genes Corp., cat. no. RN-1455)
- Deoxycytidine-*N*-benzoyl 2-cyanoethyl-N,N-diisopropyl (CED) phosphoramidite (Chem Genes Corp., cat. no. ANP-5552)
- Deoxyguanosine-*N*-isobutyrl CED phosphoramidite (Chem Genes Corp., cat. no. ANP-5553)
- Deoxyguanosine-*N*-isobutyrl long-chain alkyl amino (lcaa) control pore glass (CPG) 500 Å (Chem Genes Corp., cat. no. N-5103-05) **! CAUTION** Wear gloves when handling the CPG column.
- Hydrochloric acid (HCl) solution, 1 N (EMD Chemicals Inc., cat. no. HX0603D-6)
- Triethylammonium acetate buffer, pH 7.0 (TEAAc; Fluka, cat. no. 90357)
- Methyl alcohol (Mallinckrodt Chemicals, cat. no. 3041)

- Molecular sieves 3 Å, 3–5 mm beads (Alfa Aesar, cat. no. L05359) (for drying of molecular sieves see REAGENT SETUP)
- Tris(hydroxymethyl)aminomethane (TRIS base, J.T. Baker cat. no. 4109)
- Molecular sieves 4 Å, 3–5 mm beads (Alfa Aesar, cat. no. L05466)
- Tris–HCl buffer (see REAGENT SETUP)

EQUIPMENT

- SpeedVac (Thermo Electron Corp., model no. SC110)
- DNA synthesizer (see EQUIPMENT SETUP)
- Plastic syringes and plastic hypodermic syringes (all polypropylene)
- Screw cap vials (2 ml capacity)
- ColorpHast pH 5–10 (pH paper) (EMD Chemicals Inc., cat. no. M95883)
- Teflon tape
- Drying tube (Fisher Scientific, cat. no.09-215A)
- Muffle furnace (Barnstead International, model no. FB1415M, 100–1,100 °C, 1450 W)
- HPLC system with a UV detector (see EQUIPMENT SETUP)
- HPLC column strong anion exchange (SAX)-column (e.g., Dionex DNAPac PA-100 analytical column, 4 mm × 250 mm quaternary-ammonium functionalized, 100-nm-sized microbeads bound to a 13-µm diameter nonporous substrate; Dionex Corp., cat. no. 043010)
- Corning sterile filter system
- Sep-Pak C-18 cartridges (Waters Corp., cat. no. WAT051910)

REAGENT SETUP

Drying of molecular sieves Molecular sieves 3 and 4 Å, approximately same quantities (200 g each) are mixed in a beaker and heated at 350 °C in a furnace for 3 h. Switch off the heating, and when the temperature drops to approximately 200 °C, transfer the beakers into a vacuum desiccator (desiccator containing drying agent, drierite, placed below the desiccator plate), and allow it to cool for several hours (typically 3 h or more). The molecular sieves activated in this fashion are active for approximately 2 weeks. ! CAUTION Care should be taken while handling molecular sieves. Search for material safety data sheet for cat. nos L05359 and L05466 at http://www.alfa.com/webapps/ec120w.pgm. Use thermal protection (insulated mittens) and safety glasses while handling the beakers at 200 °C. Use insulated tongs to avoid slipping of the beaker. The vacuum desiccator is released with argon gas to take out the dried molecular sieves.

Dry CH_3CN should be used throughout the oligonucleotide synthesis as well as for Steps 1–4 and Step 6. Dry CH_3CN (purity 98%) by distillation over P_2O_5, followed by reflux and distillation over CaH_2 pellets[26]. For the most part, 0.5–1% (wt/vol) P_2O_5 is added to the distilling flask containing CH_3CN to remove the remaining water. Excess P_2O_5 should be avoided as it leads to the formation of an orange polymer. To achieve this, add approximately 30–40 g P_2O_5 to 3 l CH_3CN, reflux for 3–4 h and distill. To the distillate, add approximately 15 g CaH_2 pellets, and reflux the solvent for 3–4 h, followed by distillation. Transfer the dried CH_3CN to 1 l bottles and seal with Teflon tape. Prior to use, add dried molecular sieves (mixture of molecular sieves 3 and 4 Å), and use the solvent after 2–4 h. On the other hand, CH_3CN with low water content (10 p.p.m. or less) is considered suitable for oligonucleotide synthesis, and the traces of moisture present can be removed using activated molecular sieves. ! CAUTION Always wear gloves and safety glasses while handling CH_3CN, P_2O_5 and CaH_2. (For P_2O_5 and CaH_2, see material safety data sheets at https://fscimage.fishersci.com/msds/96386.htm and https://fscimage.fishersci.com/msds/95942.htm, respectively.) Take precaution to exclude atmospheric moisture during the distillation procedure using fresh $CaCl_2$ drying tubes.

Preparation of Tris–HCl buffer, pH 7.8 Dissolve 60.57 g Tris base in 300 ml deionized (DI) water, adjust the pH to 7.8 by adding HCl (1 N) dropwise with stirring, and make up the volume (using DI water) to 500 ml in a volumetric flask. Filter the buffer, which has a concentration of 1 M, through a Corning sterile filter system with nylon membrane, and store at 4 °C.

EQUIPMENT SETUP

DNA synthesizer For oligonucleotide synthesis, it is advisable to charge the automated DNA synthesizer with freshly prepared phosphoramidite solutions and reagents. The phosphoramidite solutions can be further protected from moisture by adding activated 3 and 4 Å molecular sieves.

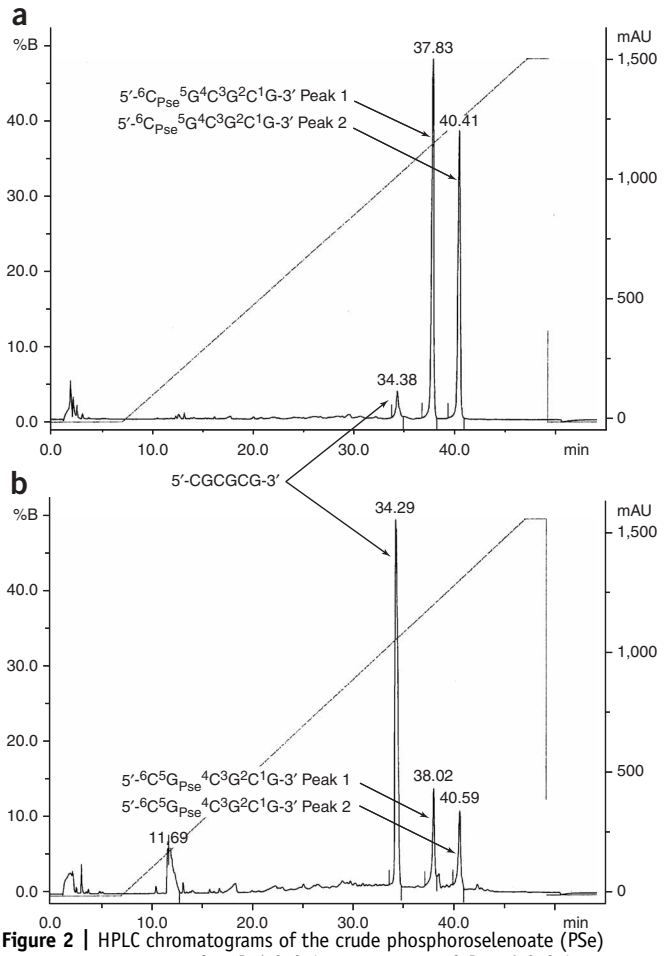

Figure 2 | HPLC chromatograms of the crude phosphoroselenoate (PSe) oligonucleotides (**a**) 5′-$^{6}C_{Pse}{}^{5}G^{4}C^{3}G^{2}C^{1}$G-3′ and (**b**) 5′-$^{6}C^{5}G_{Pse}{}^{4}C^{3}G^{2}C^{1}$G-3′.

In our laboratory, we use an ABI 381A DNA synthesizer, and a concentration of 0.1 M for the phosphoramidite solutions. Other reagents used are listed in the MATERIALS section. Syntheses are normally performed on a 1-µmol scale, on CPG supports with 500 Å pore size with the nucleoside immobilized through an lcaa linker. Standard protocols for the synthesis of oligonucleotides are followed for all steps namely (i) de-blocking, (ii) base condensation or coupling, (iii) capping and (iv) oxidation, following the manufacturer's recommendations. In accordance with the literature[27], we also found that use of 0.25 M 5-ethylthiotetrazole/CH_3CN gives better coupling efficiencies and overall yields and this reagent is therefore used as the activator.

HPLC setup for oligonucleotide purification The PSe oligonucleotide synthesized can be purified, and the P-diastereomers can be separated using anion exchange HPLC. Buffer A: 25 mM Tris–HCl (pH 7.8), buffer B: 25 mM Tris–HCl, 1.0 M NaCl (pH 7.8). Turn the HPLC on and equilibrate the column (Dionex, DNAPac PA-100 analytical column used for anion exchange HPLC, quaternary-ammonium functionalized, 100-nm-sized microbeads bound to a 13-µm diameter nonporous substrate) with 30 ml (approximately 10-column volumes) of buffer A with a flow rate of 1 ml min^{-1}. (Filter the buffers through a Corning sterile filter system with nylon membrane prior to use.) The UV lamp is also switched on, allowed to stabilize for 10 min and auto-zeroed before sample injection.

PROCEDURE

Oligonucleotide synthesis with incorporation of a PSe linkage

1| To 100 mg KSeCN in a 2-ml screw cap vial, add 1 ml CH_3CN; close the cap tightly and seal with Teflon tape.

■ **PAUSE POINT** Place the vial on a heating block maintained at 60 °C for 12 h in the dark (e.g., wrapped in aluminum foil) as the compound appears to be either air- or light sensitive (appearance of pink residue).

2| Transfer the vial to the fume hood, allow it to cool to room temperature (25–27 °C, throughout the procedure) and store in the dark. Throughout this protocol, the reagent thus prepared is referred to as 'KSeCN reagent'. For one PSe modification on a 1-µmol scale, two vials of reagents are required.

3| To introduce the PSe linkage using solid-phase synthesis, pause the synthesis soon after the phosphoramidite coupling at the position where selenium is to be placed. The synthesis is paused after the phosphoramidite coupling, at the end of CH_3CN wash, and just before the oxidation step starts. Remove the column (the one containing the CPG) from the synthesizer, attach a 10-ml syringe filled with CH_3CN and wash the column with CH_3CN.

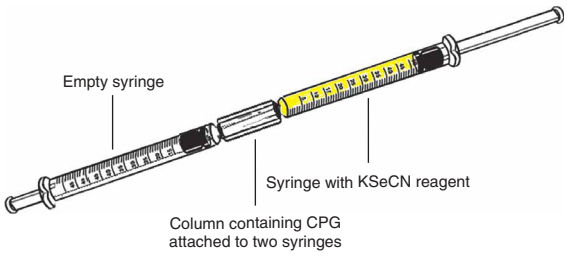

Figure 3 | Schematic representation of two tuberculin syringes attached to the column containing control pore glass (CPG) for treatment with potassium selenocyanate (KSeCN) reagent.

4| Draw 0.9 ml KSeCN solution to a 1-ml tuberculin syringe and attach it to one end of the column, with an empty 1-ml tuberculin syringe at the other end of the column (see schematic representation of the setup in **Fig. 3**). Flush KSeCN solution through the column by moving the syringe plunger to and fro (two to three times). This ensures contact between the selenium reagent and the densely packed CPG.

▲ **CRITICAL STEP** Using Teflon tape around the syringe nozzles helps to prevent moisture intervention in the reaction. While attaching the syringe nozzle to either ends of the CPG column, care should be taken to avoid damaging the frits or filter membranes of the CPG column. For the same reason, while moving the syringe plunger to and fro, when the first plunger is pushed in, the second plunger at the other end of the column is drawn out, and vice versa.

■ **PAUSE POINT** Leave the system overnight (12–16 h) in the dark (e.g., wrapped in aluminum foil).

5| Drain out KSeCN reagent from the column, and repeat Step 4 to ensure complete conversion of 5′-$^{5}G_{P(III)}{}^{4}C$-3′ to 5′-$^{5}G_{PSe}{}^{4}C$-3′. Leave the system for at least 2 h (and for up to 6 h) in the dark (e.g., wrapped in aluminum foil).

6| Attach a 10-ml syringe filled with CH_3CN and wash the column with 10 ml CH_3CN.

7| Attach the column back to the synthesizer and continue the oligonucleotide synthesis after the capping step. To achieve this, edit the sequence 5′-$^{6}C^{5}G$-3′ with trityl 'off' (i.e., start the synthesis for the target sequence 5′-HO-$^{6}C^{5}G_{PSe}$, where $^{5}G_{PSe}$ is bound to the CPG), perform the capping step manually and start the synthesis.

▲ **CRITICAL STEP** On our synthesizer (ABI 381 A DNA synthesizer), the capping step is carried out manually by the function numbers *16* (capping reagent preparation, 3 s) and *22* (delivery of capping reagent to CPG column, 15 s). We prefer to use the trityl 'off' option as the DMTr function would interfere with the subsequent separation of diastereoisomers in the purification step via SAX HPLC.

8| After completing the synthesis, take out the column and transfer the CPG to a screw cap vial and add 1 ml NH_4OH. Cap the vial tightly, vortex the solution and keep it on a heating block at 60 °C for 8 h in the dark.

■ **PAUSE POINT** The vial can be left on the heating block at 55 °C for a total of 12 h, if desired.

9| Cool the vial in an ice bath for 30 min.

■ **PAUSE POINT** Unscrew the cap, and leave the vial in the fume hood overnight at room temperature (12–16 h) so that the oligonucleotide solution becomes free of ammonia.

10| Spin the vial in a centrifuge at 4,000 r.p.m. (1,306*g*, 3 min) at room temperature, pipette out the supernatant to a 2-ml Eppendorf tube and lyophilize the sample to dryness. Resuspend the oligonucleotide in 200 µl DI water, remove a 5 µl aliquot from the suspension and dilute it with 995 µl DI water. Measure the UV absorbance at 260 nm, and estimate the concentration of the oligonucleotide solution. Please note that the concentration of the oligonucleotide solution can be expressed in terms of UV OD at a wavelength of 260 nm. The total OD is calculated using the following formula:

$$\mathrm{OD} = A_{260} \times d \times V,$$

where A_{260} is the UV absorbance at 260 nm, d is the dilution factor (in this case 1,000/5 = 200), V is the volume in milliliters in which the oligonucleotide was resuspended (in this case, 0.20 ml).

11| Switch on the HPLC, attach the SAX column (4 mm × 250 mm, Dionex, Dionex, DNAPac PA-100) and equilibrate the column with buffer A (see EQUIPMENT SETUP).

▲ **CRITICAL STEP** For best results, the HPLC purification must be carried out on an SAX column (ion-exchange HPLC purification).

12| Based on absorbance data from Step 10, inject a measured volume containing 10 OD of the oligonucleotide into the column and subject it to a gradient run using the following conditions: 0–60% buffer B (see EQUIPMENT SETUP) in 60 min with a 1 ml min^{-1} flow rate (approximately 20 column volumes). Monitor the UV absorbance continuously at 260 and 280 nm. The fully oxidized oligonucleotide (PO, instead of PSe) elutes first, followed by two peaks (similar to peaks 1 and 2 shown in **Fig. 2**) that correspond to the diastereomeric selenoates. If the HPLC peaks of the oligonucleotides containing the PSe moiety (labeled as peaks 1 and 2 in **Fig. 2**) are not well resolved, make the gradient less steep and standardize the HPLC run.
? TROUBLESHOOTING

13| Once the HPLC run is standardized for reasonably resolved peaks of PSe, repeat the HPLC purifications and collect peaks 1 and 2 separately.

14| Pool the fractions containing the oligonucleotide peaks 1 and 2 separately.

15| Oligonucleotides purified by HPLC can be desalted on Sep-Pak C-18 cartridges (Waters Inc.). Attach the cartridge to a 10-ml syringe with a three-way Leur-lock in between. Flush the cartridge with 10 ml CH_3CN, and then with 10 ml DI water. Apply the oligonucleotide solution to the cartridge, and wash with 10 ml DI water to remove the salts. Elute the desalted oligonucleotide (which is now bound to the C-18 cartridge) with 5 ml 60% (vol/vol) methanol in water and collect in 2-ml Eppendorf tubes.

16| Lyophilize the fractions and resuspend in measured amount of DI water to estimate concentration (as described in Step 10), characterize by mass spectrometry, and perform crystallization experiments.
▲ **CRITICAL STEP** The oligonucleotides bearing selenium are stored in the dark (e.g., wrapped in aluminum foil). Store the sample at –20 °C after drying in a SpeedVac. For crystallization experiments, after the drops have been setup, protect them from light (e.g., wrapped in aluminum foil).
? TROUBLESHOOTING

● **TIMING**
Step 1, 12 h; Step 2, 1 h; Step 3, approximately 15 min nt^{-1}; Step 4, 12–16 h; Step 5, 2–6 h; Step 6, 5 min; Step 7, approximately 15 min nt^{-1}; Step 8, 8–12 h; Step 9, 30 min; Step 10, 10 min; Step 11, 1 h; Step 12, 2 h; Step 13, 5 h; Step 14, 5 min; Step 15, 10 min; Step 16, 4 h

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
12	Lots of peaks and uncertainty in deciding the identity of individual oligonucleotide peaks	The reagents are of poor quality and/or the phosphoramidite solutions are not dry	Repeat the synthesis with freshly prepared phosphoramidite solutions and add dried molecular sieves 3 and 4 Å. Also test the synthesizer for proper flow of reagents (flow test) and do the 'self-test' if available for the machine
12	Difficulty in separating the phosphoroselenoate (PSe) diastereomers	Gradient too steep	Use a more shallow gradient and/or increase the run time. If problem persists, perform an HPLC column regeneration by following the manufacturer's instructions
16	Following storage of the PSe oligonucleotides, their solutions appear pink	Oxidation of PSes to phosphates and metallic selenium	Always store the oligonucleotide solutions in the dark (by wrapping the vial in aluminum foil), at –20 °C, and whenever possible keep the oligonucleotides as dried samples at –20 °C

ANTICIPATED RESULTS

Yields

The yields for PSe linkage introduction using solid-phase syntheses in the case of DNA hexamer sequences 5′-$^{6}C_{PSe}{}^{5}G^{4}C^{3}G^{2}C^{1}G$-3′ and 5′-$^{6}C^{5}G_{PSe}{}^{4}C^{3}G^{2}C^{1}G$-3′ are approximately 32 and 6%, respectively (peaks 1 and 2 are obtained approximately in equal quantities).

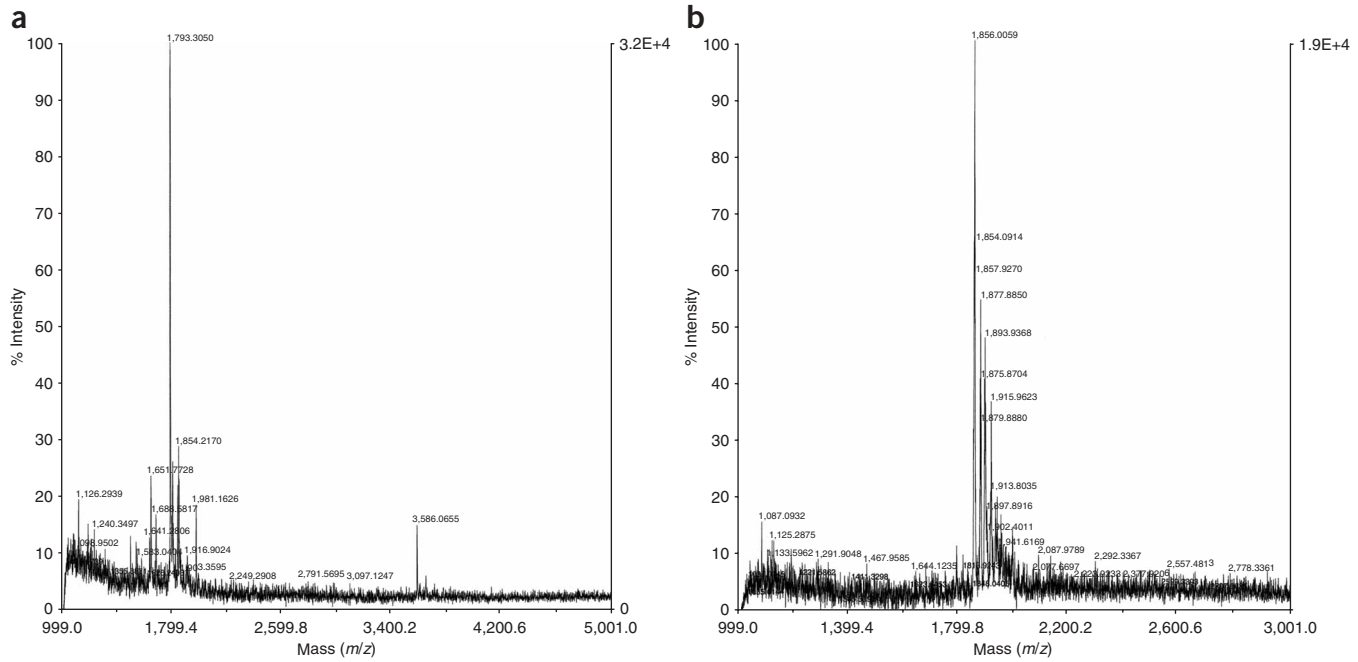

Figure 4 | Matrix-assisted laser desorption/ionization—time of flight (MALDI-TOF) mass spectra of (**a**) 5′-$^{6}C^{5}G^{4}C^{3}G^{2}C^{1}$G-3′ (wild type) and (**b**) 5′-$^{6}C_{PSe}{}^{5}G^{4}C^{3}G^{2}C^{1}$G-3′ (slower eluting diastereomer). Reproduced with permission from the American Chemical Society (ref. 14, supporting information, pages 5–6).

Mass spectrometry

The PSe oligonucleotides synthesized are characterized by matrix-assisted laser desorption/ionization—time of flight (MALDI-TOF) mass spectrometry (**Fig. 4**). The molecular weight of PSe derivatives is higher by 62.9 atomic mass units (amus) than the wild type. The expected difference of 62.9 amu can be seen between molecular masses of the wild type and the PSe oligonucleotides.

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COMPETING INTERESTS STATEMENT The authors declare no competing financial interests.

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