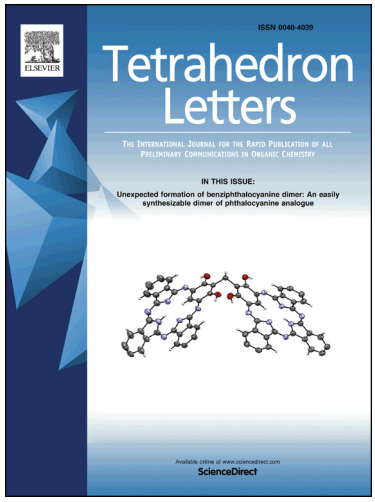

Reversible Diselenide Cross-links are Formed Between Oligonucleotides Containing 2'-Deoxy-6-selenoinosine

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Reversible Diselenide Cross-links are Formed Between Oligonucleotides Containing 2'-Deoxy-6-selenoinosine

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ABSTRACT

We have synthesized and characterized a phosphoramidite derivative of 2'-deoxy-6-selenoinosine ($d^{6Se}I$) and incorporated this modification into an oligonucleotide by solid-phase synthesis. During cleavage from the solid-support and deprotection, spontaneous dimerization of this oligonucleotide occurs via formation of a diselenide cross-link between the modified nucleobases. This cross-link can be readily reduced to restore the single-stranded oligonucleotide. UV thermal denaturation and circular dichroism spectroscopy of duplexes with $d^{6Se}I$ paired against all four native nucleobases revealed minor differences in stability and structure relative to 2'-deoxyinosine. This selenium containing nucleobase modification may be useful for applications in DNA nanomaterials and X-ray crystallography.

Introduction

Selenium (Se) derivatized nucleic acids, prepared by either enzymatic or solid-phase synthesis, are a powerful tool in solving the phase problem of X-ray crystallography due to the anomalous scattering properties of this element.[1] The substitution of oxygen (O) with Se for this purpose within the nucleic acid scaffold has been reported at the phosphate backbone[2], the 2', 4' and 5' positions of the sugar[3,4,5] and at the nucleobases.[6,7] Incorporation of Se at the nucleobase has advantages over other sites, including minimal structural perturbation, avoiding the introduction of an additional chiral center which would necessitate the separation of diastereomers and that incorporation is not limited at the termini of the oligonucleotide. Substitution of the O6-atom of guanine[7] and the O4-atom of thymine[6] with Se are examples of nucleoside modifications that have been prepared and incorporated into oligonucleotides. The crystal structures of Se-nucleobase modified oligonucleotides have been shown to be virtually identical to that of their native counterparts.[1] In addition, a nucleic acid-protein complex consisting of RNase H/Se-DNA/RNA, has been solved utilizing nucleobase Se incorporations.[8]

2'-Deoxyinosine (Figure 1a), containing the base hypoxanthine, is capable of pairing with the other natural nucleobases found in DNA.[9] Analogs of 2'-deoxyinosine have been used to introduce site-specific postsynthetic modifications within an oligonucleotide[10] and for applications as fluorescent probes.[11]

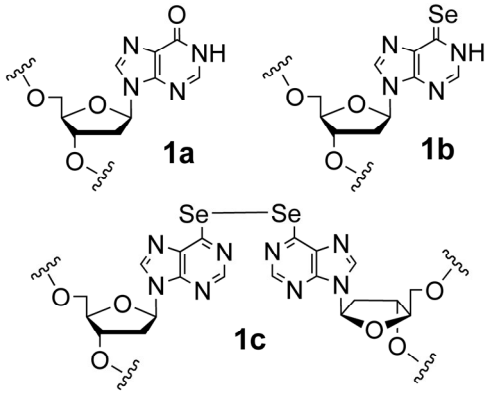

Figure 1 Structures of (1a) 2'-deoxyinosine, (1b) 2'-deoxy-6-selenoinosine, and (1c) diselenide bridged 2'-deoxy-6-selenoinosine.

Herein, the preparation of a 2'-deoxy-6-selenoinosine ($d^{6Se}I$, Figure 1b) phosphoramidite is described. This was achieved by using a convertible 2'-deoxyinosine nucleoside[12] and the Huang reagent (di(2-cyanoethyl) diselenide).[13] The Se containing phosphoramidite was incorporated into an oligonucleotide by solid-phase synthesis, deprotected and purified by strong anion exchange (SAX) HPLC to prepare the sequence 5'-dGGCT^{6Se}IGATCACCAG-3' (**dI-Se-ODN**). Interestingly, during the deprotection of the oligonucleotide from the controlled pore glass (CPG) solid-support, the serendipitous discovery of a cross-linked oligonucleotide homodimer (**dI-Se-ODN$_2$**) connected by a

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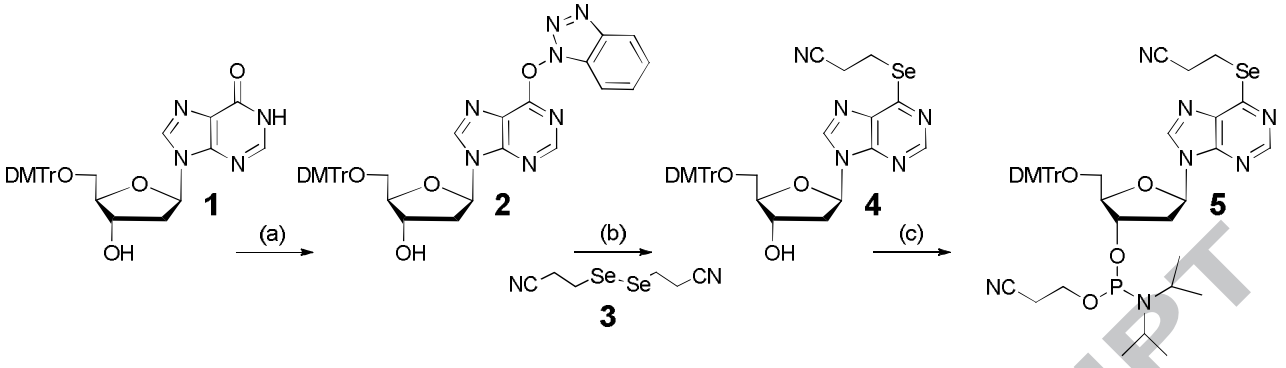


Scheme 1 Synthesis of the $d^{6Se}I$ phosphoramidite (**5**). Reagents and conditions: (a) PyBOP, DBU, DMF, 4 h, rt; (b) $NaBH_4$, EtOH, 2h, 0°C to rt; (c) Cl-POCENiPr_2, DIPEA, THF, 30 mins, rt.

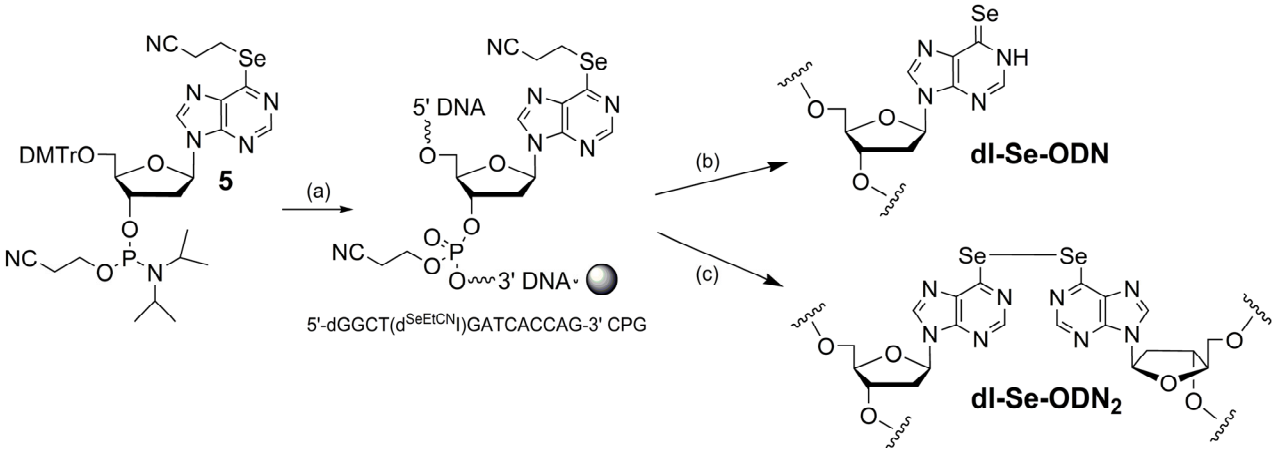
diselenide bridge (Figure 1c) between the modified nucleobases was made. This is the first instance of a Se derivatized nucleic acid where two oligonucleotides are cross-linked between the nucleobases with a diselenide linkage. **dI-Se-ODN$_2$** was studied by UV thermal denaturation, denaturing polyacrylamide gel electrophoresis (PAGE) and circular dichroism (CD). Time trials of oligonucleotide dimer formation after deprotection were monitored by SAX HPLC.

Results and Discussion

Electrophilic O^6-(benzotriazol-1-yl)-2'-deoxyinosine[12] (**2**), first described by Lakshman, was synthesized from commercially available 5'-*O*-dimethoxytrityl-2'-deoxyinosine (**1**) with a yield of 74% (Scheme 1). Reduction of diselenide (**3**)[13], generating nucleophilic selenolate, allowed for the displacement of the benzotriazoyl group to produce (**4**), with a yield of 66%. Phosphitylation of (**4**) afforded phosphoramidite (**5**), with a yield of 88%. The phosphoramidite was introduced into the CPG-bound DNA sequence 5'-dGGCT^{6Se}IGATCACCAG-3' by solid-phase synthesis (Scheme 2). All trityl values after phosphoramidite coupling were above 90% indicating successful incorporation of the modified phosphoramidite.

Conventional deprotection with NH_4OH/EtOH (3:1, v/v) was found to be too harsh to accomplish removal of the protective groups while retaining Se in the oligonucleotide, necessitating the use of a milder *t*-butylamine/H_2O (1:3, v/v) treatment followed by the addition of an equimolar amount of glacial acetic acid to neutralize the amine base (Scheme 2). Unexpectedly, rather than the desired oligonucleotide **dI-Se-ODN,** the dimerized **dI-Se-ODN$_2$** was the major product after deprotection and liberation from the solid-support. The formation of this dimer was first suspected during the SAX HPLC purification of the crude oligonucleotide. Elution times for sequences 14 nucleotides in length are expected to be in the range of 18-22 minutes under the buffer conditions employed by SAX HPLC (specified in Figure 2); however, the major species generated had an elution time closer to 26 minutes (Figure 2a). Furthermore, 4-selenothymidine and 2'-deoxy-6-selenoguanosine containing oligonucleotides have characteristic UV absorptions at 369 nm and 360 nm, respectively.[7,14] Absorption at 352 nm was observed for the $d^{6Se}I$ containing oligonucleotides (Supplementary Figure 10); however, the major species generated had minimal absorbance at this wavelength (Figure 2a), especially when compared to the minor species. In order to account for such a drastic decrease in absorbance intensity at 352 nm, it was hypothesized that a modification occurred at or near the Se position of the oligonucleotide. The major species was collected, desalted, and analysed by ESI-MS, which confirmed a higher than expected molecular mass. The mass observed corresponded to a diselenide bridged oligonucleotide homodimer (Supplementary Figure 12). Unfortunately, attempts to digest **dI-Se-ODN$_2$** by methods previously used in our group were not successful.[15] This may be due to the nuclease not being able to digest the oligonucleotide at the cross-linked site or that buffer used for digestion compromises the Se-Se cross-link.

In order to isolate **dI-Se-ODN** and confirm the presence of a



Scheme 2 Synthesis of the **dI-Se-ODN** and formation of **dI-Se-ODN$_2$**. Reagents and conditions: (a) Solid-phase synthesis (see supplementary data, page S-4); (b) (i) *t*-butylamine/H_2O (1:3, v/v) in 50mM DTT, 6 h, 60°C; (ii) glacial acetic acid; (c) (i) *t*-butylamine/H_2O (1:3, v/v), 6 h, 60°C (ii) glacial acetic acid.

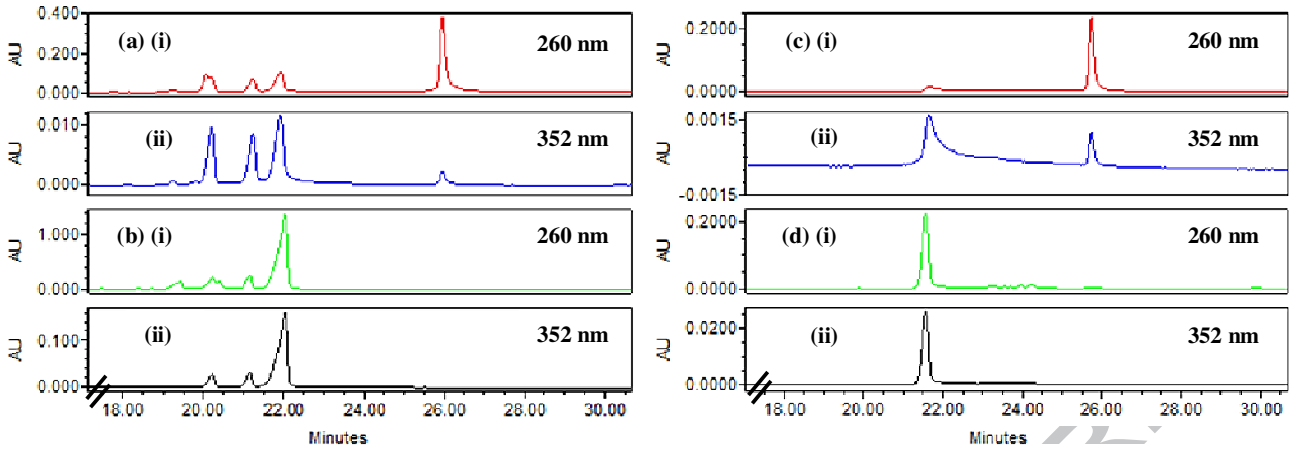


Figure 2 SAX HPLC traces of (a) crude **dI-Se-ODN$_2$** monitored at (i) 260 nm and (ii) 352 nm (b) crude **dI-Se-ODN** monitored at (i) 260 nm and (ii) 352 nm (c) pure **dI-Se-ODN$_2$** monitored at (i) 260 nm and (ii) 352 nm (d) pure **dI-Se-ODN** monitored at (i) 260 nm and (ii) 352 nm. The column was eluted at room temperature using a linear gradient of 0-52% buffer B over 30 minutes (buffer A: 100 mM Tris HCl, pH 7.5, 10% acetonitrile; buffer B: 100 mM Tris HCl, pH 7.5, 10% acetonitrile, 1 M NaCl).

diselenide bond, deprotection of the CPG-bound oligonucleotide was repeated, but with the addition of the reducing agent dithiothreitol (DTT). The major species in the SAX HPLC profile now had a reduced elution time of approximately 22 minutes and an intense absorbance at 352 nm (Figure 2b), which were characteristic of the d^{6Se}I containing 14-mer. No peak at 26 minutes was observed. After collecting and desalting the major species, SAX HPLC indicated the dimer had reformed (Figure 2c). Re-addition of DTT to the solution allowed for the isolation of pure **dI-Se-ODN** (Figure 2d), which was confirmed by ESI-MS (Supplementary Figure 14). Storage of **dI-Se-ODN** at 4^{o}C in the absence of DTT was shown to prevent dimerization, even after 1 week (data not shown).

To test if the deprotection condition used contributed to dimerization, deprotection was repeated with another mild treatment: 0.05 M K_2CO_3/MeOH for 4 h at room temperature, with and without the addition of an equimolar amount of glacial acetic acid. In both instances, the major species was once again **dI-Se-ODN$_2$** (data not shown). Furthermore, removal of DTT during desalting of crude **dI-Se-ODN** initiated re-dimerization. These results suggest spontaneous (without the addition of an oxidizing agent) diselenide bridge formation is occurring. Interestingly, 2'-deoxy-6-selenoguanosine containing oligonucleotides have not been reported to readily self-dimerize.[7] Potentially, the C2 exocyclic amino group of guanine sterically prevents diselenide bond formation between the 6-seleno atoms. Thio-nucleobase-modified oligonucleotides have been shown to form disulfide bridges in complementary sequences where the bridging atoms are in proximity to one another after hybridization.[16,17]

In order to observe the timescale of dimerization after cleavage from the solid-support, time trials were performed by analysing the crude **dI-Se-ODN** oligonucleotide by SAX HPLC after deprotection in the absence of DTT. Dimerization was however, too fast under these experimental conditions. After only 2 hours of deprotection, **dI-Se-ODN$_2$** was already the predominant species, with no evidence of **dI-Se-ODN** accumulating over time (Supplementary Figure 17). This suggests that at elevated temperatures (60^{o}C), diselenide bridge formation is rapid and occurs soon after cleavage from the solid-support and removal of the cyanoethyl group protecting the 6-seleno atom. After 18 hours of deprotection, incubation of the crude oligonucleotide with 50 mM DTT for 2 hours quantitatively reverted the dimer (**dI-Se-ODN$_2$**) to the single-stranded species (**dI-Se-ODN**) (Supplementary Figure 17).

UV thermal denaturation of duplexes between **dI-Se-ODN** with complementary strands where d^{6Se}I is base paired with dA, dC, dG and dT nucleotides were performed. The buffers for these experiments contained 2.5 mM DTT in order to avoid the self-dimerization of the d^{6Se}I containing oligonucleotide. The denaturation profiles revealed T_m values which were comparable to duplexes containing unmodified 2'-deoxyinosine (Supplementary Figure 18). This suggests there is no significant destabilization of the duplex upon Se modification at the 6-position of 2'-deoxyinosine. Furthermore, the CD spectra of the same **dI-Se-ODN** containing duplexes showed typical B-form DNA signatures, with a wavelength maximum at 280 nm, an intercept near 260 nm and a minimum around 250 nm, similar to the control 2'-deoxyinosine containing duplexes (Supplementary Figure 19). This suggests the Se modification has minimal influence on the global DNA structure.

UV thermal denaturation experiments of **dI-Se-ODN$_2$** in the presence of 2 equivalents of the complementary strands produced T_m curves which were biphasic in nature and the CD spectra also showed typical B-form DNA signatures (Supplementary Figures 20 and 21). The biphasic T_m curves may be due to the dissociation of the first, then second equivalent, of the complementary strand hybridized to **dI-Se-ODN$_2$**. Denaturing PAGE was used in order to evaluate the efficiency of other reducing agents on diselenide bridge removal of **dI-Se-ODN$_2$**, specifically $NaBH_4$ and glutathione (GSH) (Supplementary Figure 22). Both of these reagents caused the loss of the dimer band after only 2 hours of incubation at room temperature, with migrations similar to **dI-Se-ODN**. A significant smearing in the GSH lane was observed, perhaps due to incomplete removal of the GSH in loading of the oligonucleotide onto the gel. In the untreated **dI-Se-ODN$_2$** lane, two bands were observed. Electrolytic reduction of the diselenide bond is believed to occur, producing the lower band which migrates with the **dI-Se-ODN** control in lane 4; a significant amount of dimer remained. Electrolytic reduction of insulin disulfide bonds has previously been reported.[18]

Conclusion

In summary, the synthesis and characterization of a novel Se modified 2'-deoxyinosine phosphoramidite has been described. In the absence of reducing agents such as DTT, the crude

oligonucleotide forms a homodimer linked by a diselenide bridge. This dimer can readily be reduced to restore the single stranded oligonucleotide upon treatment with agents such as DTT, $NaBH_4$ and GSH. Duplexes containing $d^{6Se}I$ paired with any of the four natural nucleobases of DNA revealed minimal perturbation to duplex thermal stability and structure relative to 2'-deoxyinosine.

Future work will involve crystallizing and solving the X-ray crystal structure of duplexes containing this novel Se containing 2'-deoxyinosine derivative. Formation and crystallization of a diselenide cross-linked DNA duplex and structural determination by X-ray crystallography may be advantageous in solving the structures of larger nucleic acid assemblies given that the Se phasing power is proportional to the number of Se atoms incorporated (one Se atom per 30 nucleotides[19]).

Beyond X-ray crystallography, these diselenide cross-linked oligonucleotides may find applications as stimuli responsive materials given their reactivity relative to species containing disulfide bonds.[20,21,22] These materials may be attractive as a molecular switch able to respond to the intracellular reducing environment.[23]

Acknowledgments

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at (insert doi link)

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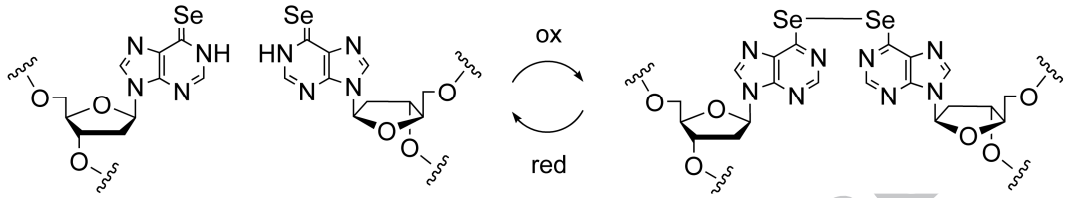

Highlights

- 2'-Deoxy-6-selenoinosine has been synthesized.
- This modification was incorporated into DNA by solid-phase synthesis.
- Site-specific diselenide cross-links between the DNA strands were formed.
- Mild reductive conditions readily remove these cross-links.