# An Organometallic Route to Oligonucleotides Containing Phosphoroselenoate

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*Unlike the widespread use of phosphorothioates in nucleic acid chemistry, complementary research on phosphoroselenoates has been severely limited. Previous routes to DNA and RNA that contain phosphoroselenoates employ elemental Se and KSeCN as Se transfer agents, although these reagents suffer from low or unselective reactivity. The metastability of the P–Se bond demands soluble, selective Se transfer reagents. The organometallic reagent ($iPrC_5H_4)_2TiSe_5$ satisfies these criteria, as we demonstrate by the synthesis of phosphoroselenoate derivatives of mono- and oligonucleotides of DNA and a dinucleotide of RNA. The new general method is compatible with high-throughput phosphoramidate oligonucleotide synthesis, which allows for the preparation of site-specifically labeled oligonucleotides. A $^{31}P$ NMR spectroscopy study shows that the phosphoroselenoate of (5′)-d(GGAATGTC$_{Se}$TGTCG)-(3′) selectively binds to soft $Cd^{2+}$ ions but not $Mg^{2+}$ ions.*

**KEYWORDS:**

DNA · RNA · selenium · synthetic methods

## Introduction

The role of selenium in biological systems is of increasing interest.[1–3] Not only is Se an essential element, it is emerging as a useful biochemical and biophysical probe of protein and nucleic acid structure. Selenium is softer than sulfur and thus provides an additional degree of discrimination between hard and soft metal ions for mechanistic studies on metalloenzymes. Oligonucleotides labeled with selenium are amenable to X-ray crystallography through use of multiwavelength anomalous dispersion.[4] Furthermore, $^{77}Se$ ($I = 1/2$) is NMR active and can be quite useful in structural characterizations.[5] Se-containing biomolecules are also more amenable to X-ray absorption spectroscopy study than their sulfur analogues because of the low energy of the Se X-ray absorption edge. Despite the numerous advantages of Se in biochemical studies, efficient protocols for the synthesis of Se-containing biomolecules or their analogues are lacking.

Significant opportunities for the use of selenium include studies of DNA and RNA that contain phosphoroselenoates. The chemistry of phosphorothioates is well developed,[6–11] whereas the corresponding phosphoroselenoates have received very limited attention.[5, 12–18] The increased softness of Se relative to S makes phosphoroselenoate-modified oligonucleotides particularly attractive species for studies of (deoxy)ribozyme activity. Additionally, Kool et al. showed that terminal phosphoroselenoates can be alkylated by iodo-modified riboses. This property has been used in ligation experiments to study the hybridization of DNA and detect mismatches in DNA sequences.[17]

The main problem in the synthesis of Se-labeled nucleotides is the nonavailability of soluble sources of $Se^0$. Red Se has only meager solubility in a limited range of solvents ($CS_2$ and benzene).[16] The more soluble $SeCN^-$ has emerged as the Se transfer agent of choice,[5, 14] however it is relatively inert. The use of $SePPh_3$ has recently been described[18] for the selenization of a dinucleotide phosphite in solution but it has not been employed for solid-phase synthesis, which is critical for studies on oligonucleotides. Perhaps the most significant selenization reagent is Stawinski's benzothioselenol-3-one, a species that is soluble in organic solvents and selenizes nucleoside phosphonates and triphosphites. In this report, we demonstrate that ($iPrC_5H_4)_2TiSe_5$ (**1**) is a particularly effective Se-atom transfer reagent since it serves as a highly soluble form of elemental selenium. We demonstrate that this reagent allows the synthesis of Se-labeled DNA and RNA oligonucleotides, including specifically labeled DNA oligonucleotides.[19] The use of phosphoroselenoate-modified DNA in spectroscopic studies of metal-ion interactions with DNA is also demonstrated.

## Results and Discussion

### Synthesis of phosphoroselenoamidate mononucleotides

Initial experiments focused on the transfer of Se to $P^{III}$ intermediates relevant to automated nucleic acid synthesis. We found that the cyanoethoxy-protected dimethoxytritylated thymidyl phosphoramidite **A** reacted with **1** over the course of

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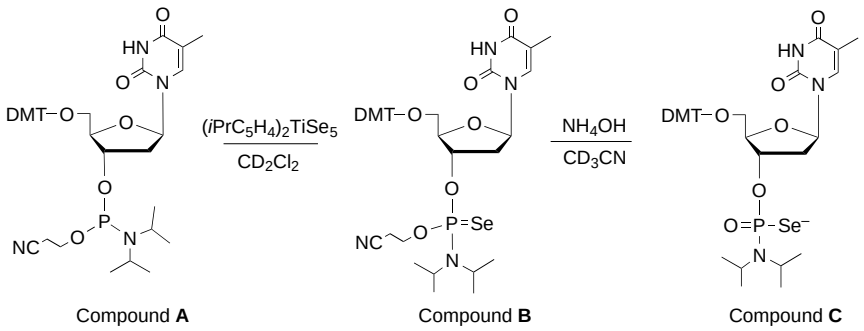
1 h to afford the protected phosphoroselenoamidate **B** quantitatively (Scheme 1). This reaction is much faster than the reactions of **A** with $Se^0$ and KSeCN[14] and is comparable to the reaction that uses $SePPh_3$ as the Se transfer agent.[18] The $^{31}P$ NMR spectrum of **B** consists of a pair of signals as a result of the presence of diastereomers (caused by the chirality at both the P and the ribose). Each signal shows $^{77}Se$ satellites with $J_{P\text{-}Se} = 909$ Hz. Base hydrolysis of **B** gave the deprotected phosphoroselenoamidate **C** (Scheme 1), again identified by the $^{31}P$ NMR spectrum, which showed $^{77}Se$ satellites with $J_{P\text{-}Se} = 687$ Hz (see the inset in Figure 1). The $^{31}P$ NMR signal of the phosphoroselenoate is approximately 50 ppm downfield of the signal for the phosphate groups in nucleic acids, which allows convenient product analysis. The chemical shift region of the phosphoroselenoate is approximately the same as the region for phosphorothioates ($\delta = 55-60$ ppm).[11]




***Scheme 1.*** *Selenization and deprotection of a mononucleotide (DMT = dimethoxytrityl).*

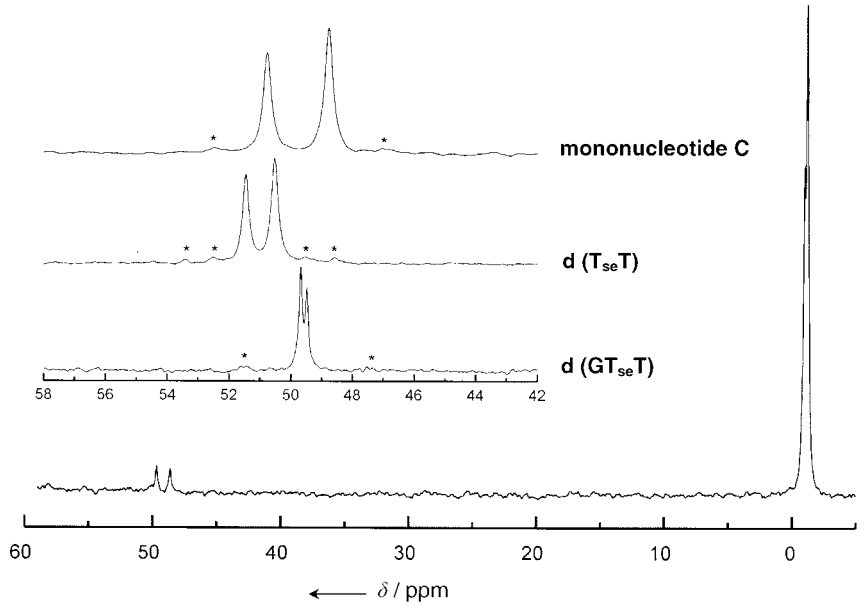



***Figure 1.*** *$^{31}P$ NMR spectrum (202 MHz) of the 13mer DNA modified with a unique phosphoroselenoate (2 mM DNA in 5 mM triethylamine (TEA; pH 7.8), 100 mM NaCl in $D_2O$). The inset shows the phosphoroselenoate signals for the three smaller oligonucleotides synthesized (* indicates $^{77}Se$ satellites).*

### Synthesis of a DNA phosphoroselenoate dinucleotide

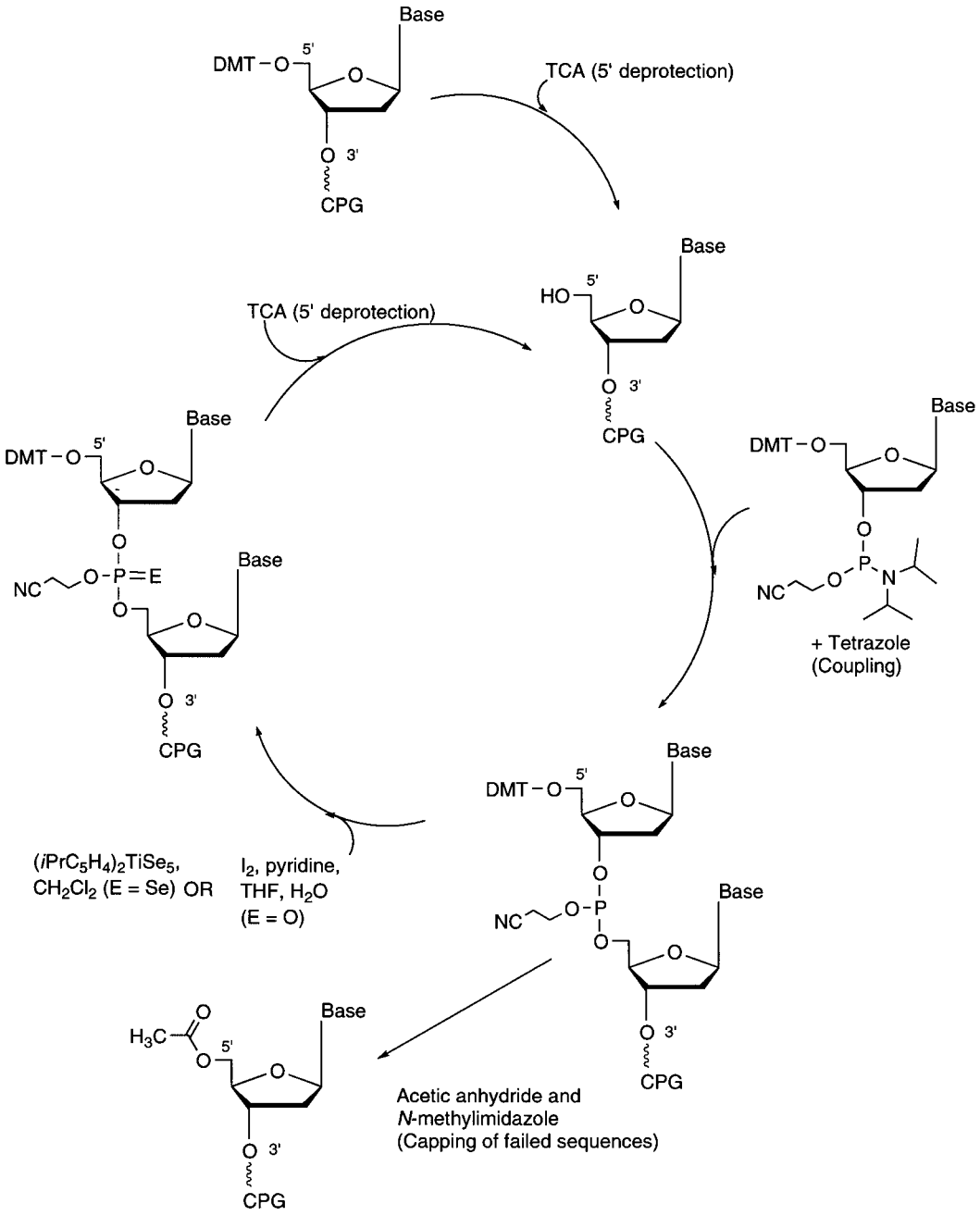
We also found that **1** efficiently selenizes phosphite-linked dinucleotides. In one experiment, we employed a dinucleotide d(TT) that was fully protected for phosphoramidate DNA synthesis and anchored on CPG functionalized beads (Scheme 2).[20] Se transfer was complete in 24 h. ESI MS (*m/z*: calculated: 910.7; observed: 911.4) and $^{31}P$ NMR spectroscopy ($J_{P\text{-}Se} = 808$ Hz; see inset of Figure 1) analysis confirmed that the $NH_4OH$ cleaved product was pure phosphoroselenoate.

### Synthesis of a DNA phosphoroselenoate trinucleotide

Once we had demonstrated that **1** was sufficiently reactive to form phosphoroselenoates from both phosphoramidites and phosphites, we investigated the compatibility of this methodology with the other steps in the automated synthesis of oligonucleotides. Selenization of a CPG-anchored d(TT) dinucleotide was carried out as described above (24 h, $CH_2Cl_2$ solution), followed by detritylation, attachment of G, and oxidation of the second phosphite to phosphate (Scheme 2). The critical question here was the stability of the preformed phosphoroselenoate to the $I_2/py/H_2O$ reagent employed in the oxidation of the $P^{III}$ atom to a $P^V$ species. Analysis by ESI MS (*m/z*: calculated: 1241.2; observed: 1240.4) and $^{31}P$ NMR spectroscopy of the resulting trinucleotide showed that the product was about 88% Se,O molecules, that is, (5′)-d($GT_{se}$T)-(3′), and approximately 12% O,O species, that is, (5′)-d(GTT)-(3′). Independent experiments showed that in solution the phosphoroselenoate of the trinucleotide is fully converted to phosphates within 20 min of exposure to $I_2/py/H_2O$ at room temperature. Overall, however, the successful solid-phase synthesis of the mixed phosphate – phosphoroselenoate trinucleotide encouraged us to explore the modification of higher oligonucleotides, paying particular attention to the possible loss of Se during the $I_2/py/H_2O$ steps of the automated synthesis.

### Synthesis of DNA phosphoroselenoate tridecanucleotides

We selected a 13mer DNA analogue of the RNA substrate of a hammerhead ribozyme as our synthetic target, with Se at the site of cleavage of the substrate.[21] We started from CPG-anchored (5′)-d(CTGTCG)-(3′) with a phosphite group between the last two bases, introduced Se (by using **1**) at the phosphite




***Scheme 2.*** *Phosphoramidate automated DNA synthesis cycle. CPG = controlled pore glass; TCA = trichloroacetic acid; THF = tetrahydrofuran.*

position, and then returned the resin to the synthesizer to complete the synthesis of (5′)-d(GGAATGTC$_{Se}$TGTCG)-(3′). Optimization experiments revealed that it is critical to introduce Se into the growing oligomer before the detritylation step in order to avoid oxidization of the $P^{III}$ species prior to addition of **1**. It also appears that **1** may cause partial depurination of the bases that neighbor the phosphoroselenoate modification. For example, in one set of experiments, (5′)-d(GGAATGTC$_{Se}$GGTCG)-(3′) showed loss of the G base on the 3′ side, just prior to the phosphoroselenoate in the sequence.

When Se was introduced prior to detritylation, the desired Se-modified 13mer was the predominant product, as verified by MALDI MS (*m/z*: calculated: 4066; observed: 4066.6). $^{31}P$ NMR analysis was consistent with the MS results and showed a pair of signals from the diastereomers of the 13mer in the phosphoroselenoate region and a broader signal in the phosphate region (Figure 1). Integration of these two sets of resonances indicates that 84% of the product is the phosphoroselenoate-modified oligonucleotide, whilst the remainder is unmodified oligonucleotide. The phosphoroselenoate withstands seven treatments with the $I_2$/py/$H_2O$ oxidant, which indicates that it is more resistant to oxidative deselenization than suggested by experiments with the modified trinucleotide.

### Synthesis of an RNA phosphoroselenoate dinucleotide

We also demonstrated the utility of **1** in the preparation of Se-modified RNA dinucleotides. The dinucleotide r(UU) anchored on CPG beads was treated with **1** (24 h), followed by removal from the resin. HPLC purification gave the desired phosphoroselenoate, as confirmed by ESI MS (*m/z*: calculated: 613; observed: 613.2). $^{31}$P NMR analysis showed two signals in the phosphoroselenoate chemical shift region consistent with the formation of two diastereomers. The synthesis of r($U_{Se}$U) was achieved directly on the synthesizer,[22] which indicates that this method could be optimized for complete automation.

### Metal binding studies

We wished to demonstrate the utility of phosphoroselenoate-modified DNA in spectroscopic studies of nucleic acid interactions with metal ions. We carried out a $^{31}$P NMR spectroscopy study of $Cd^{2+}$ binding to the oligonucleotide (5′)-d(GGAAT-GTC$_{Se}$TGTCG)-(3′) discussed above. As shown in Figure 2, titration of the 13mer with up to 5 equivalents $Cd^{2+}$ ions resulted in a 4.0-ppm upfield shift of the $P_{Se}$ signals. The direction of the chemical shift is consistent with previous observations for interactions with phosphorothioates[11, 23] and the fact that this particular signal is cleanly separated from the remainder of the phosphate signal simplifies the interpretation of the effect. When the 13mer was treated with $Mg^{2+}$ ions, the $P_{Se}$ signal did not move significantly, which indicates that the phosphoroselenoate preferentially binds $Cd^{2+}$ rather than $Mg^{2+}$ ions.

## Summary

This work demonstrates a powerful approach to the modification of nucleic acids with selenium. In particular, reagent **1** is much more efficient than the common selenization reagents $Se^0$ and $SeCN^-$. Our methodology with reagent **1** clearly demonstrates that specific phosphoroselenoate modifications of DNA and RNA oligonucleotides can be achieved with reasonable yield by using high-throughput phosphoramidate synthesis techniques. The successful use of **1** within the automated synthesizer to synthesize r($U_{Se}$U) has prompted us to look into methods to synthesize other phosphoroselenoate oligonucleotides by complete automation with the use of reagent **1**.

Reagent **1** provides a versatile means to introduce Se in a manner that is compatible with both solution-phase and semiautomated oligonucleotide syntheses of both phosphoroselenoate DNA and phosphoroselenoate RNA. Furthermore, the phosphoroselenoate-modified 13mer DNA was shown by $^{31}$P NMR spectroscopy to bind metals selectively at the phosphoroselenoate. These results should allow future studies aimed at evaluation of the effect of the Se softness on (deoxy)ribozyme reactivity and should enable the use of $^{77}$Se NMR spectroscopy and Se extended X-ray absorption fine structure spectroscopy for structural characterization and metal binding studies of nucleic acids.

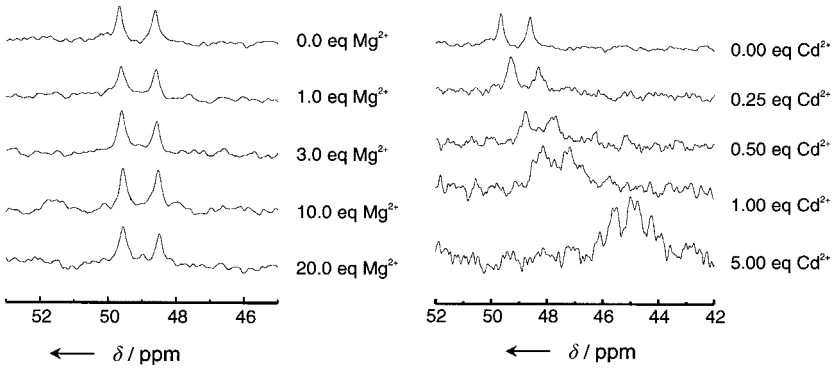



***Figure 2.*** *$^{31}$P NMR spectra of the 13mer DNA modified with a unique phosphoroselenoate (2 mM DNA in 5 mM TEA (pH 7.8), 100 mM NaCl in $D_2O$). The plot on the right shows a shift of the phosphoroselenoate signals upon addition of $Cd^{2+}$ ions (304 MHz), whereas the plot on the left shows no shift of the signals upon addition of $Mg^{2+}$ ions (202 MHz).*

## Experimental Section

**Materials**: dT phosphoramidite was purchased from Glen Research. Oligonucleotide syntheses (dimer, trimer, 13mer) were performed at the W. M. Keck Genomics Center at the University of Illinois. The reagent (*i*Pr$C_5H_4$)$_2$Ti$Se_5$ (**1**) was synthesized according to the literature.[19] $CH_2Cl_2$ was dried over $CaH_2$ and distilled under $N_2$. All other reagents were used as purchased. The $^{31}$P NMR spectra were externally referenced to 85% $H_3PO_4$ except for the metal binding experiments, which were referenced internally to $PPh_3$ in $CH_2Cl_2$ ($\delta = -5.786$ ppm). *The materials described in this work should be handled with care because phosphoroselenoated oligonucleotides have been reported to be highly toxic to cells.*[1]

**Handling of (*i*Pr$C_5H_4$)$_2$Ti$Se_5$**: Solid samples of **1** are indefinitely stable in air, but its solutions are less stable and were made with nitrogen-sparged solvents.

**Synthesis of phosphoroselenoamidate mononucleotides**: Under a nitrogen atmosphere, dT phosphoramidite (**A**; 50 mg, 0.067 mmol) was added to (*i*Pr$C_5H_4$)$_2$Ti$Se_5$ (50 mg, 0.076 mmol) and dissolved in deuterated methylene chloride (5 mL). The $^{31}$P NMR spectrum was obtained within one hour and showed that the reaction had completely formed the cyanoethoxy-protected phosphoroselenoamidate **B**. Removal of the cyanoethoxy-protecting group was accomplished by dissolution of **B** in deuterated acetonitrile (500 μL) followed by addition of concentrated ammonium hydroxide (500 μL). The $^{31}$P NMR spectra were obtained after 15 min, 24 h, and 4 days to show that the compound had been fully deprotected to give the phosphoroselenoamidate **C**. Elevated temperatures (55 °C for 8 h) may also be used to facilitate removal of the cyanoethoxy groups. $^{31}$P NMR spectroscopy: **B**, $\delta = 74.7$, 73.6 ppm ($J_{P\text{-}Se} = 909$ Hz); **C**, $\delta = 50.4$, 48.5 ppm ($J_{P\text{-}Se} = 687$ Hz).

**Synthesis of a DNA phosphoroselenoate dinucleotide**: Dinucleotide with a $P^{III}$-linked d(TT) unit (approximately 10 μmol) was synthesized on a 394 Synthesizer (ABI/Perkin Elmer) by using phosphoramidate synthesis methods. The oxidation and detritylation steps were omitted to preserve the internucleotide phosphite linkage. The column containing the dimer bound to the solid support (CPG) was taken off the synthesizer and its contents were transferred to a round-bottom Schlenk flask in a nitrogen dry box, then

($i$PrC$_5$H$_4$)$_2$TiSe$_5$ (6.2 mg, 9.4 μmol) was added to the flask. Dry, deoxygenated $CH_2Cl_2$ (12 mL) was added to the reaction mixture, which was then stirred for 24 h. The solution was filtered on a glass frit to isolate the beads from the titanium compounds. Concentrated ammonium hydroxide (10 mL) was added to the beads and the mixture was stirred overnight (15 h). The solution was centrifuged to separate the beads and the supernatant was evaporated in a SC110A Savant Speedvac Concentrator, with addition of triethylamine to prevent precipitation caused by acidification of the solution during evaporation of the ammonium hydroxide. ESI MS showed the highest mass signal at $m/z = 911.4$ (calcd DMT-bound dinucleotide mass: 910.7). $^{31}$P NMR spectroscopy showed two signals at $\delta = 51.5$ and 50.5 ppm ($J_{P\text{-}Se} = 808$ Hz).

**Synthesis of a DNA phosphoroselenoate trinucleotide and tridecanucleotides**: Syntheses of the trinucleotide and 13mer oligonucleotides were also performed as described above. The trinucleotide sequence was (5′)-d(GT$_{Se}$T)-(3′). The two 13mer DNA sequences used were: (5′)-d(GGAATGTC$_{Se}$TGTCG)-(3′) and (5′)-d(GGAATGTC$_{Se}$GGTCG)-(3′). The first bases of the sequence (from the 3′-end) were synthesized normally until the creation of the phosphite linkage to be selenized (base 2 for the trinucleotide, and base 6 for the 13mers). The steps following the last coupling reaction were omitted (capping and detritylation) to preserve the phosphite linkage. The column containing the reagent was removed from the synthesizer and placed under an inert atmosphere. Reagent **1** (2 mL of a 6 mM solution in $CH_2Cl_2$) was introduced into the column with syringes. The mixture was allowed to react under nitrogen for 24 h. The column was rinsed with $CH_2Cl_2$ and dried with air before being placed back on the synthesizer to complete the synthesis of the remainder of the oligonucleotide. Cleavage from the support was achieved in the synthesizer by introduction of ammonium hydroxide (4 – 8 mL) to the column. Deprotection and lyophilization were performed as described for the dinucleotide.

ESI MS of the trinucleotide showed the highest mass fragment at $m/z = 1240.4$ (calcd product mass: 1241.2). The $^{31}$P NMR spectrum of the trinucleotide showed resonances at $\delta = 49.7$ and 49.5 ppm (intensity: 25.3 and 18.9) for $P_{Se}$ ($J_{P\text{-}Se} = 800$ Hz) and $\delta = -0.8$ and $-0.9$ ppm (intensity: 36.2 and 19.7) for $P_O$. MALDI MS of the tridecanucleotide (5′)-d(GGAATGTC$_{Se}$TGTCG)-(3′) showed the highest mass fragment at $m/z = 4066$ (calcd product mass: 4066). The $^{31}$P NMR spectrum of the tridecanucleotide showed signals at $\delta =$ 50.1 and 49.1 ppm (total intensity: 7) for $P_{Se}$ and $\delta = -0.9$ ppm (total intensity: 93) for $P_O$.

**Synthesis of an RNA phosphoroselenoate dinucleotide**: The dinucleotide r(U$_{Se}$U) was synthesized by using the 2′-*O*-bis(2-acetoxyethoxy)methyl) method developed by Dharmacon Research, Inc. (Boulder, Colorado, USA). The phosphite linkage was selenized directly on the machine (8 injections of reagent **1** (6 mM), each followed by a 3-h interval). Reversed-phase HPLC separation on a C18 column allowed separation of the two diastereomers of the phosphoroselenoate dimer from the phosphate dimer by elution with $NH_4C_2H_3O_2$ (5 – 10 mM) and addition of a gradient of MeCN from 0 – 5 % in 15 minutes; the products eluted at 14 min for r(UU) and at 17 min and 20 min for the two r(U$_{Se}$U) diastereomers. ESI MS showed the highest mass fragment at $m/z = 613.2$ (calcd product mass: 613). The $^{31}$P NMR spectrum showed two resonances at $\delta = 50.9$ and 49.9 ppm; the small amount of phosphate signal at $\delta = -0.4$ ppm agreed with the HPLC calculation that the product is 93 % r(U$_{Se}$U).

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