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Automated Solid-Phase Synthesis of RNA Oligonucleotides Containing a Nonbridging Phosphorodithioate Linkage via Phosphorothioamidites

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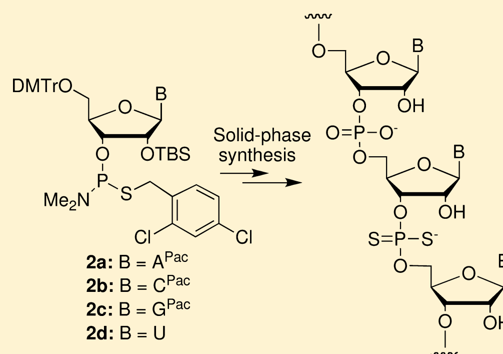
Supporting Information

ABSTRACT: This work describes a general method for the synthesis of oligoribonucleotides containing a site-specific nonbridging phosphorodithioate linkage via automated solid-phase synthesis using 5′-*O*-DMTr-2′-*O*-TBS-ribonucleoside 3′-*N*,*N*-dimethyl-*S*-(2,4-dichlorobenzyl) phosphorothioamidites (**2a–2d**). The 3′-phosphorothioamidites (**2a–2d**) can be conveniently prepared in good yields (86–99%) via a one-pot reaction from the corresponding 5′-*O*-DMTr-2′-*O*-TBS-ribonucleosides (**1a–1d**).



Oligonucleotides containing phosphoromonothioate (PS) linkages are among the most thoroughly characterized and widely used oligonucleotide analogues.[1] Both DNA and RNA PS oligonucleotides are incisive mechanistic probes that have helped to elucidate the catalytic mechanisms of both protein and nucleic acid enzymes.[2−7] PS linkages also confer nuclease resistance to oligodeoxynucleotides (ODNs) and oligoribonucleotides, increasing their half-life as antisense agents.[8−10] More recently, several investigations have studied the effects of PS linkages on the uptake and activity of small interfering RNA (siRNA) oligonucleotides.[11−15]

Less well investigated are phosphorodithioate (PS2) oligonucleotides, in which sulfur replaces both nonbridging phosphate oxygens. Unlike PS oligonucleotides, dithioate analogues are achiral at phosphorus and therefore sterochemically mimic their all-oxygen counterparts more closely than monothioates. PS2 oligonucleotides also do not require stereochemical resolution following synthesis, as do monothioates when species with defined stereochemistry are desired.[16]

Deoxynucleoside phosphorodithioate dimers have been prepared in several ways. One approach involves the reaction of a 5′-*O*-protected 2′-deoxynucleoside with a dithiophosphorylating agent, followed by coupling of the product to a 3′-*O*,*N*-protected 2′-deoxynucleoside.[17−19] A second approach, used to prepare dithymidine phosphorodithioate, involves the coupling reaction of the triethylammonium salt of a 3′-thymidine phosphonodithioate[20,21] or phosphorodithioate[22,23] to a 3′-*O*-protected thymidine. A third approach involves reaction of the corresponding dideoxynucleoside phosphoramidites (B^1-O-P(NPr-i_2)-O-B^2) with 4-chlorobenzylmercaptan (or hydrogen sulfide) followed by sulfurization.[24−26] For construction of oligodeoxynucleotides by solid-phase synthesis, 3′-phosphoramidites of dinucleoside phosphorodithioates[17,25−27] enabled access to sequences containing a single PS2 linkage, while 2′-deoxynucleoside 3′-phosphorothioamidites[28−30] enabled access to sequences containing exclusively PS2 linkages.

In contrast to the extensive investigations into the synthesis of PS2-bearing ODNs, only three reports describe the synthesis of the corresponding RNA oligonucleotides.[31−33] In 1990, Petersen et al. reported a solution synthesis of ribonucleoside phosphorodithioate dimers using 5′-*O*-DMTr-2′-*O*-TBS-ribonucleoside 3′-phosphorothioamidites (Figure 1, **I**) with tetrazole as activator.[32] In 1996, Greef et al. synthesized RNA containing exclusively PS2 linkages via a solid-phase approach that employed ribonucleoside 3′-*H*-phosphonothioates (Figure 1, **II**) with diphenylchlorophosphate as an activator and 2,4-dichlorobenzylthiosuccinimide as a sulfurizing reagent.[31] This method provides an effective approach for synthesizing oligoribonucleotides containing exclusively PS2 linkages, but synthesis of RNA containing individual PS2 linkages would require multiple activators and likely render the approach difficult to automate. Use of this method to prepare a 12-nt RNA containing a single phosphorodithioate linkage required a manual coupling step.[34] Following initial submission of this manuscript, Yang et al. reported a similar solid-phase synthesis of RNAs containing a single or multiple PS2 linkages using ribonucleoside 3′-pyrrolidino-*S*-[*β*-benzoylmercapto)ethyl]-3′-phosphorothioamidites (Figure 1, **III**).[33] Here we report the

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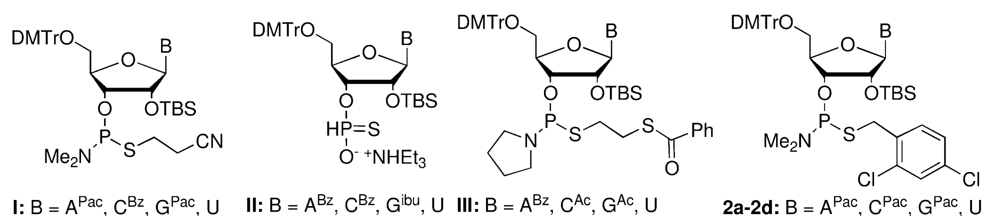
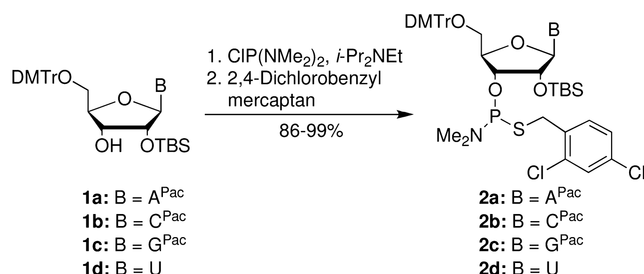


Figure 1. Phosphorus reagents used to synthesize RNAs containing PS2 linkages.

preparation and use of the 3′-phosphorothioamidites (Figure 1 and Scheme 1, **2a–2d**) for automated solid-phase synthesis of RNA oligonucleotides containing single PS2 linkages. The 3′-phosphorothioamidites (**2a–2d**) are prepared with greater convenience and efficiency (86–99% yield) compared to the preparation of 3′-*H*-phosphonothioates (**II**) (two steps, 35–77% yield).[31] They are also prepared more conveniently than the 3′-phosphorothioamidites described by Petersen et al.[32] and Yang et al. (Figure 1, **I** and **III**),[33] since all of the required starting materials are commercially available.

Scheme 1



The ribonucleoside 3′-phosphorothioamidites **2a–2d** were prepared from the corresponding 5′-*O*-DMTr-2′-*O*-TBS-protected ribonucleoside derivatives **1a–1d** in 86–99% yields according to the literature procedure described for the synthesis of **2d**[32] (Scheme 1).

Incorporation of phosphorothioamidites **2a–2d** into oligonucleotides at specific sites was performed on an Expedite Nucleic Acid Synthesis System (8900) via a modified 1 μM RNA protocol using phosphoramidite chemistry (Scheme 2). The protocol was modified for phosphorothioamidites **2a–2d** as follows: (1) The coupling step was repeated once (double coupling); (2) sulfurization with 3-(*N*,*N*-dimethylaminomethylidene)amino-3-*H*-1,2,4-dithiazole-5-thione (DDTT) was executed prior to the standard capping procedure. As expected, coupling of the ribonucleoside phosphorothioamidites **2a–2d** was less efficient than for the corresponding 2′-deoxyribonucleoside phosphorothioamidites.[29] However, when double coupling was applied, the trityl data showed that the coupling yields with **2a–2d** were comparable to those of commercially available RNA phosphoramidites. We also found that sulfurization was more efficient with DDTT than with the Beaucage reagent.[35,36] For the incorporation of standard nucleotides, all commercially available phosphoramidites could be used except the N^2-isobutyrylguanosine phosphoramidite. To minimize the PS2 RNA degradation under basic conditions, we chose PAC-protected phosphoramidites to allow deprotection of the oligonucleotides by treatment with ammonium hydroxide at 55 °C for only two hours. After solid-phase synthesis, the solid supports were deprotected via a three-step protocol as shown in Scheme 2.[31] The crude

Scheme 2

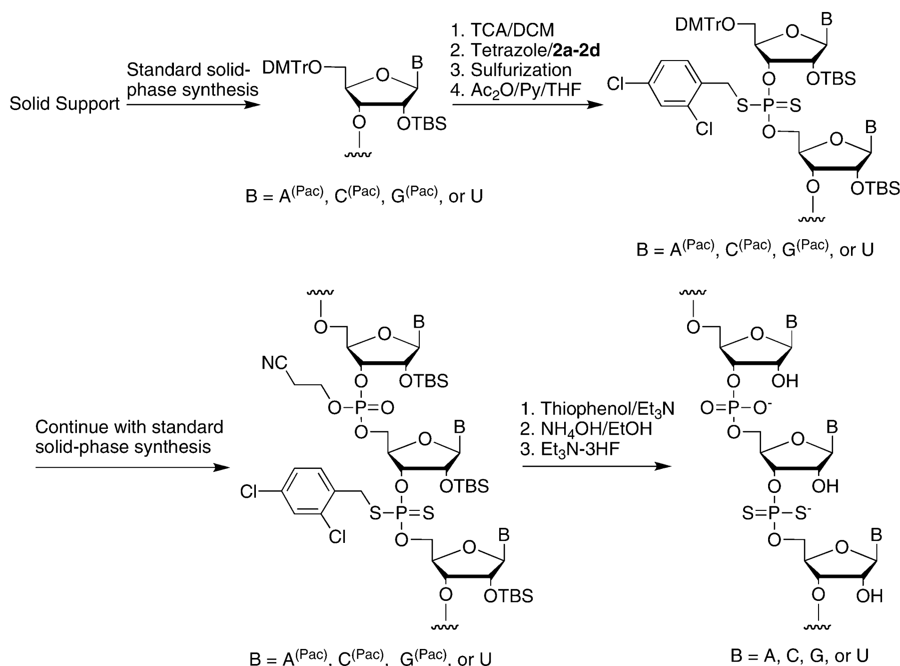


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Table 1. Yields and MALDI-TOF MS Data of the Purified Oligonucleotides Containing Single PS2 Linkages (3a−3f)

oligos	yield (%)	MALDI-TOF MS calculated	MALDI-TOF MS found
3a	5.2	2846.4 (M^+), 2847.4 (MH^+), 2890.4 ($M - 2H + 2Na^+$)	2847.5, 2890.4
3b	5.8		2846.8, 2888.7
3c	5.5		2847.9, 2888.8
3d	5.3		2847.9, 2889.8
3e	3.3	6788.9 (M^+), 6789.9 (MH^+), 6811.9 (MNa^+), 6832.9 ($M - 2H + 2Na^+$), 6854.9 ($M - 3H + 3Na$)	6789.2, 6790.6, 6811.4, 6812.6, 6829.7, 6853.8
3f	3.6		6787.4, 6809.6, 6831.2, 6853.2, 6854.7

oligonucleotides containing single phosphorodithioate linkages were analyzed and purified by ion exchange HPLC. HPLC profiles of the crude oligonucleotides showed that the phosphorodithioate oligonucleotides were the major products (see Supporting Information). The following oligonucleotides containing single PS2 linkages were prepared and characterized: 5′-ACG UA-O-P(S)2-O-C GUU-3′ (**3a**), 5′-ACG UAC-O-P(S)2-O-GUU-3′ (**3b**), 5′-ACG-O-P(S)2-O-UAC GUU-3′ (**3c**), ACG U-O-P(S)2-O-AC GUU-3′ (**3d**), 5′-UGG UAA U-O-P(S)2-O-AA GCU GAC GGA CAU-3′ (**3e**) and 5′-UGG UA-O-P(S)2-O-A UAA GCU GAC GGA CAU-3′ (**3f**). The purified phosphorodithioate oligonucleotides were also analyzed and confirmed by MALDI-TOF mass spectrometry (Table 1).

In summary, in contrast to the well developed methods for preparing ODNs bearing PS2 linkages, approaches for synthesizing the corresponding RNAs until now have lacked automation or the capacity for site-specific incorporation. To enable convenient access to these modifications, we constructed the four ribonucleoside 3′-phosphorothioamidites (**2a**−**2d**) in a one-pot synthesis from commercially available starting materials and used them to prepare RNA containing site-specific PS2 linkages by automated solid-phase synthesis. Two recent applications of PS2 RNAs in RNAi[33] and RNA structure−function analysis[37] likely will heighten future interest in these molecules. The current work simplifies access to RNA bearing site-specific PS2 linkages by enabling automated, solid-phase synthesis using ribonucleoside 3′-phosphorothioamidites (**2a**−**2d**).[38] Following submission of this work, Yang et al. demonstrated the use of 3′-pyrrolidino-*S*-[β-benzoylmercapto)-ethyl]-3′-phosphorothioamidites for constructing PS2 RNA, but our approach offers the convenience of phosphorothioamidite access in one-pot from commercially available starting materials.

■ EXPERIMENTAL SECTION

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-N^6-phenoxyacetyladenosine 3′-*N*,*N*-dimethyl-*S*-(2,4-dichlorobenzyl) Phosphorothioamidite (2a). To a solution of 5′-*O*-(dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-N^6-phenoxyacetyladenosine (**1a**) (0.408 g, 0.50 mmol) in dry acetonitrile (2.5 mL) were simultaneously added *i*-Pr_2NEt (0.14 mL, 0.80 mmol) and $ClP(NCH_3)_2$ (93 mg, 0.60 mmol). After the reaction mixture was stirred at room temperature for 30 min, 2,4-dichlorobenzyl mercaptan (0.10 mL, 0.70 mmol) was added, and stirring continued at room temperature for an additional 30 min. The reaction was quenched with aqueous saturated $NaHCO_3$, and the product was extracted with ethyl acetate and dried over anhydrous magnesium sulfate. The solvent was removed, and the residue was precipitated in degassed hexane to give a white solid. The white solid was rinsed with hexane (2 × 5 mL) and dried over vacuum to give **2a** in 86% yield (468 mg, >90% purity as assessed by ^{31}P NMR). ^{31}P NMR (CD_3CN): δ 179.9, 173.5. HRMS calcd for $C_{54}H_{61}N_6O_8NaSiPSCl_2$ (MNa^+): 1105.3053, found 1105.3056.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-N^4-phenoxyacetylcytidine 3′-*N*,*N*-dimethyl-*S*-(2,4-dichlorobenzyl) Phosphorothioamidite (2b). Phosphoramidite **2b** was obtained from 5′-*O*-(dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-N^4-phenoxyacetylcytidine (**1b**) (397 mg, 0.50 mmol) in 94% yield (499 mg, >90% purity as assessed by ^{31}P NMR) according to the procedure described for the synthesis of **2a**. ^{31}P NMR (CD_3CN): δ 180.9, 176.5. HRMS: calcd for $C_{53}H_{61}N_4O_9NaSiPSCl_2$ (MNa^+) 1081.2935, found 1081.2966.

5′-*O*-(4,4-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-N^2-phenoxyacetylguanosine 3′-*N*,*N*-dimethyl-*S*-(2,4-dichlorobenzyl) Phosphorothioamidite (2c). Phosphoramidite **2c** was obtained from 5′-*O*-(dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-N^2-phenoxyacetylguanosine (**1c**) (417 mg, 0.50 mmol) in 95% yield (523 mg, >90% purity as assessed by ^{31}P NMR) according to the procedure described for the synthesis of **2a**. ^{31}P NMR (CD_3CN): δ 180.3, 173.4. HRMS: calcd for $C_{54}H_{62}N_6O_9SiPSCl_2$ (MH^+) 1099.3177, found 1099.3166.

5′-*O*-(4,4-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-uridine 3′-*N*,*N*-dimethyl-*S*-(2,4-dichlorobenzyl) Phosphorothioamidite (2d).[32] Phosphoramidite **2d** was obtained from 5′-*O*-(dimethoxytrityl)-2′-*O*-(*tert*-butyldimthylsilyl)uridine (**1d**) (660 mg, 1.0 mmol) in 99% yield (917 mg, >90% purity as assessed by ^{31}P NMR) according to the procedure described for the synthesis of **2a**. ^{31}P NMR (CD_3CN): δ 180.5, 174.2.

Incorporation of Phosphorothioamidites 2a−2d into Oligoribonucleotides by Solid-Phase Synthesis. Oligonucleotides were synthesized on a 1 μmol scale with standard phosphoramidites (Pac-A-CE, Pac-C-CE, Pac-G-CE, U-CE) using an Expedite Nucleic Acid Synthesis System (8900) and a modified RNA protocol. Each phosphorothioamidite (**2a**−**2d**) (~0.1 M, 100 mg in 1 mL of CH_3CN) was double-coupled, followed by sulfurization with DDTT (0.05 M solution in 40% pyridine/CH_3CN) for 400 s to form the phosphorodithioate linkage. Capping, detritylation and various washes were the same as those described in the standard RNA protocol. The oligonucleotides were deprotected according to the following steps: (1) the solid support inside the column was treated with a mixture of thiophenol (0.4 mL), triethylamine (0.8 mL) and 1,4-dioxane (0.8 mL) at room temperature for 2 h; (2) the thiophenolate solution was removed, and the solid support was rinsed with methanol and subsequently with ether; (3) the support was treated with 2 mL of ammonium hydroxide/ethanol (3:1 v/v) at 55 °C for 2 h; (4) after cooling to room temperature, the supernatant was removed and the support was rinsed with 2 × 1 mL of an ethanol:acetonitrile:water mixture (3:1:1 v/v/v); (5) the rinses were combined with the supernatant, and the resulting solution was evaporated to dryness; (6) the oligo was desilylated with triethylamine trihydrofluoride/*N*-methylpyrrolidinone solution (~300 μL) [made from *N*-methylpyrrolidinone (NMP) (135 μL), triethylamine (70 μL) and TEA-3HF (95 μL)] at 65 °C for 1.5 h. The crude oligonucleotides were precipitated from *n*-butanol (1 mL), redissolved into water (500 μL) and purified via ion exchange HPLC (Dionex DNAPac PA-100, 9 × 250 mm; buffer A, 0.25 M tris, pH 8.93; B, water; C, 1.0 M NaCl; flow rate: 2.0 mL/min).

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra of phosphorothioamidites **2a–2d**; HRMS spectra of new phosphorothioamidites **2a–2c**; MALDI-TOF mass spectra of oligonucleotides **3a–3f**; HPLC profiles of the crude and purified oligonucleotides (**3a**, **3c**, **3e** and **3f**); trityl yields of **3e** and **3f** from the solid-phase synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(38) An attempt to extend the scope of our approach to prepare 5′-Ups2Ups2Ups2Ups2Ups2U-3′ containing exclusively phosphorodithioate linkages was not successful. The yields dropped significantly even though double coupling was used for each linkage. The reasons are still unclear. Therefore, our method may not be suitable for synthesizing oligonucleotides containing exclusively phosphorodithioate linkages.