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Synthesis of Phosphorodithioate RNA by the H-Phosphonothioate Method

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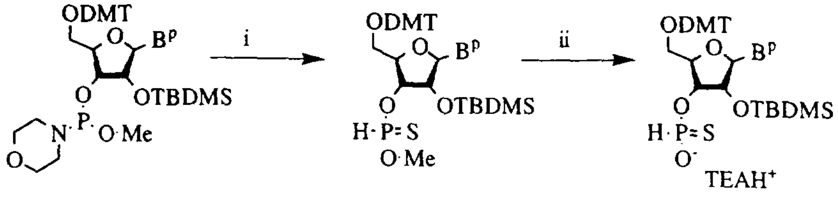
Abstract: Ribonucleoside 3'-H-phosphonothioates were used to synthesize phosphorodithioate RNA.

Phosphorodithioate DNA (S2DNA) displays biochemically interesting properties such as nuclease resistance,[1-3] the ability to stimulate RNase H[4] and inhibition of several enzymes.[5] The solution synthesis of a diribonucleoside phosphorodithioate also indicated stability toward alkaline hydrolysis.[6] These potentially useful properties prompted us to investigate the synthesis of phosphorodithioate RNA (S2RNA).



1 a-d **2 a-d** **3a-d**

Figure 1. Synthesis of Nucleoside H-phosphonothioate Monoesters. i = H_2S/tetrazole; ii = 2-carbamoyl-2-cyanoethylene-1,1-dithiolate/DMF; B^P = protected bases, a = U, b = C^{Bz}, c = A^{Bz}, d = G^{ibu}.

Recently deoxynucleoside 3'-H-phosphonothioate monoesters were developed as synthons in the preparation of phosphorothioate and phosphorodithioate DNA on a solid support.[7,8] These methods were adapted to the synthesis of ribonucleoside 3'-H-phosphonothioates (**3a-d**). Phosphoramidites (**1a-d**) (Fig. 1) were treated with H_2S to generate the H-phosphonothioate diesters (**2a-d**) which were then deprotected with 2-carbamoyl-2-cyanoethylene-1,1-dithiolate[9] to give the H-phosphonothioate monoesters **3a-d** in 35-70% yield. Among the derivatives examined, the morpholino compounds proved most useful because they were more stable to hydrolysis than conventional phosphoramidites. As a consequence, they could be prepared and stored with minimal hydrolysis or oxidation.

For the solid phase synthesis of S2RNA, a synthetic cycle was developed[10] that optimized the phosphorodithioate content of the final product (contamination with phosphorothioate was the major challenge). Synthesis begins with detritylation of the support with trichloroacetic acid (TCA) followed by activation of **3a-d** with diphenylchlorophosphate (1:1 molar ratio) to form **4** (Fig. 2). These steps were followed by sulfurization with 2,4-dichlorobenzylthiosuccinimide[11] to yield **5**, capping with acetic anhydride, and detritylation. Each cycle was complete in seven min with average coupling yield of 95-98% as measured by trityl cation release. Because this method is compatible with phosphoramidite chemistry, oligonucleotides having any combination of phosphorodithioate and phosphate diester internucleotide linkages can be synthesized.

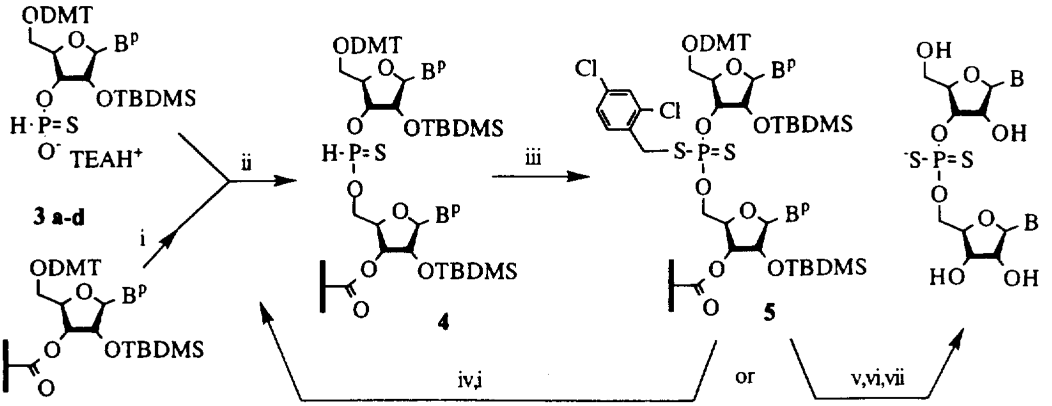


Figure 2. Automated Synthesis of S2RNA. i, TCA/DCM; ii, diphenylchlorophosphate/pyridine/MeCN; iii, 2,4-dichlorobenzylthiosuccinimide/triethylamine/DCM; iv, Ac$_2$O/ lutidine/NMI/THF; v, thiophenol/triethylamine/dioxane; vi, NH$_4$OH/ethanol; vii, triethylamine trihydrofluoride.

Oligonucleotides were deprotected and removed from the support using a three step process. First, the dichlorobenzyl group was removed using thiophenol in triethylamine. Treatment with ethanolic ammonia then cleaved the S2RNA from the support and removed exocyclic amino protecting groups. The final step involved treatment with triethylamine trihydrofluoride[12,13] to cleave the 2'-t-butyldimethylsilyl group. The S2RNA was purified by either reverse-phase HPLC or polyacrylamide gel electrophoresis. For oligonucleotides containing less than 50% dithioate internucleotide linkages, ion-exchange HPLC can also be used. Generally, oligomers synthesized by this approach contained 90-96% phosphorodithioate linkages (115-118 ppm) with the remainder being phosphorothioate (56-58 ppm) (Fig. 3).

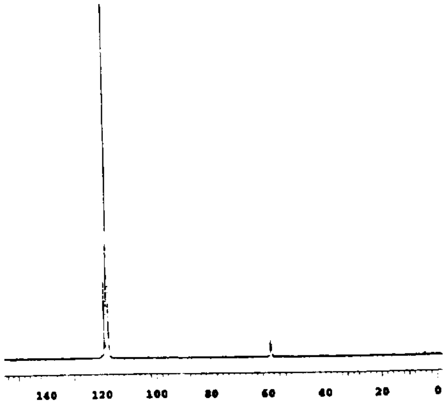


Figure 3. ^{31}P NMR of Deprotected S2U$_{15}$.

Several steps in the synthetic process exist where phosphorothioate impurities may be introduced. Recently gel-phase NMR was reported as a useful technique for developing resin-based chemistries.[14] We have used ^{31}P gel phase NMR to systematically examine the points of introduction of these impurities during the synthetic cycle.[15] Typical results are shown in Fig. 4 for the thiophenol deprotection step. Panel A illustrates results from a polystyrene support for a completely protected phosphorodithioate uridine pentamer (S2U$_5$).

Peaks at 98-100 ppm correspond to the phosphorodithioate triesters and those at 28-32 ppm the S-dichlorobenzyl phosphorothioate triester impurity. Deprotection of this resin with thiophenol and analysis by NMR gave the spectrum shown in panel B showing the phosphorodithioate diester (115-118 ppm) with approximately 5% phosphorothioate impurity (56-58 ppm). Integration of the peaks indicated greater than 99% removal of the dichlorobenzyl protecting groups. Similar analysis of the coupling reaction by this method indicated the presence of the H-phosphonothioate diester (74 ppm) and the corresponding H-phosphonate diester (10 ppm). Integration of these signals and the final phosphorothioate contamination indicates that the phosphorothioate impurity is primarily introduced during the coupling reaction — presumably due to partial activation of **3a-d** through sulfur by diphenylchlorophosphate. Further research using different procedures may lead to exclusive activation through oxygen.

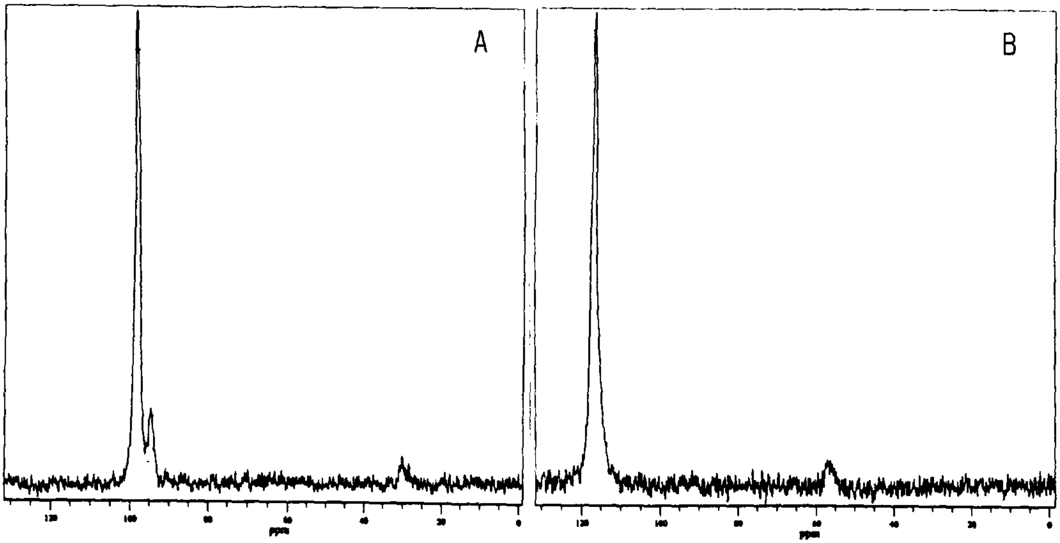


Figure 4. ^{31}P NMRs of Solid Support Linked Oligomers: A, S2U$_5$-Triester; B, S2U$_5$ Product of Thiophenol Deprotection.

In conclusion we have shown that appropriately protected nucleoside 3'-H-phosphonothioates can be used to successfully prepare phosphorodithioate RNA via a solid phase synthesis cycle on polystyrene, controlled pore glass, or tentagel supports. To date, oligomers up to 15 mononucleotides have been prepared. The use of ^{31}P gel phase NMR to monitor nucleic acid chemistry has allowed stepwise analysis of intermediates in the synthesis cycle. We anticipate that this methodology will prove useful for monitoring solid phase synthesis strategies for other analogs.

Acknowledgements: This research was supported by the NIH (Grant 25680) and Amgen Inc. This is number 43 in a nucleotide chemistry series. Paper 42: Wiesler, W.; Caruthers, M.. *J. Org. Chem.*, submitted.

References and Notes:

1. Grandas, A.; Marshall, W. S.; Nielsen, J.; Caruthers, M. H. *Tetrahedron Lett.* **1989**, *30*, 543-546.
2. Porritt, G. M.; Reese, C. B. *Tetrahedron Lett.* **1989**, *30*, 4713-4716.
3. Okruszek, A.; Sierzchala, A.; Feason, K. L.; Stec, W. J. *J. Org. Chem.* **1995**, *60*, 6998-7005.
4. Caruthers, M. H.; Beaton, G.; Cummins, L.; Dellinger, D.; Graff, D.; Ma, Y.-X; Marshall, W. S.; Sasmor, H.; Shankland, P.; Wu, J. V.; Yau, E. K. *Nucleosides and Nucleotides* **1991**, *10*, 47-59.

5. Marshall, W. S.; Caruthers, M. H. *Science* **1993**, *259*, 1564-1590.
6. Petersen, K. H.; Nielsen, J. *Tetrahedron Lett.* **1990**, *31*, 911-914.
7. Seeberger, P. H.; Bankaitis-Davis, D. M.; Beaton, G.; Caruthers, M. H., manuscript in preparation.
8. Zain, R.; Stromberg, R.; Stawinski, J. *J. Org. Chem.* **1995**, *60*, 8241-8244.
9. Dahl, B. H.; Bjergarde, K.; Nielsen, J.; Dahl, O. *Tetrahedron Lett.* **1990**, *31*, 3489-3492.
10. Syntheses were performed on an Applied Biosysterms 394 instrument. The automated cycle contained the following steps:
    i. Detritylation: a. 4 x 5s delivery 2% TCA/DCM. b. 20s rinse dichloromethane (DCM).
    ii. Coupling: a. 2 x 5s simultaneous delivery of monomer **3a-d** at 0.1 M in 1:1 MeCN/pyridine and diphenylchlorophosphate at 0.1 M in 95:5 MeCN/pyridine with 10s wait following each delivery. b. 10s rinse with 1:1 MeCN/pyridine.
    iii. Oxidation: a. 2 x 30s simultaneous delivery of 2,4 dichlorobenzylthiosuccinimide at 0.1 M in DCM and 5% TEA/DCM with 45s wait following each delivery. b. 3 x 10s rinse with DCM
    iv. Capping: a. 1 x 20s delivery of capping reagents (followed by 20s wait). b. 2 x 10s rinse with DCM.
11. Dreef, C. E.; Dreef-Tromp, C. M.; Marel, G. A.v.d.; van Boom, J. J. *Synlett* **1990**, 481-483.
12. Markiewicz, W. T.; Biala, E.; Adamiak, R. W.; Grzeskowiak, K.; Kierzek, R.; Kraszewski, A.; Stawinski, J.; Wiewiorowski, M. *Nucleic Acids Res. Symposium Series No. 7* **(1980)** pp 115-127.
13. Gasparutto, D.; Livache, T; Bazin, H.; Duplaa, A.-M.; Guy, A.; Khorlin; A.; Molko, D.; Roget, A.; Téoule, R. *Nucleic Acids Res.* **1992**, 5159-5166.
14. Look, G. C.; Holmes, C. P.; Chinn, J. P.; Gallop, M. A. *J. Org. Chem.* **1994**, *59*, 7588-7590.
15. Polystyrene (30 mg) or tentagel (5 mg) was suspended in DMSO in a 5 mm NMR tube containing an insert which matched the magnetic susceptibility of the sample (purchased from Shigami, Inc.). ^{31}P NMR were recorded usually overnight on a GE Omega 300 MHz instrument. Spectra were referenced to 85% phosphoric acid.

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