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# Synthesis of oligonucleoside phosphorodithioates by the *H*-phosphonothioate method

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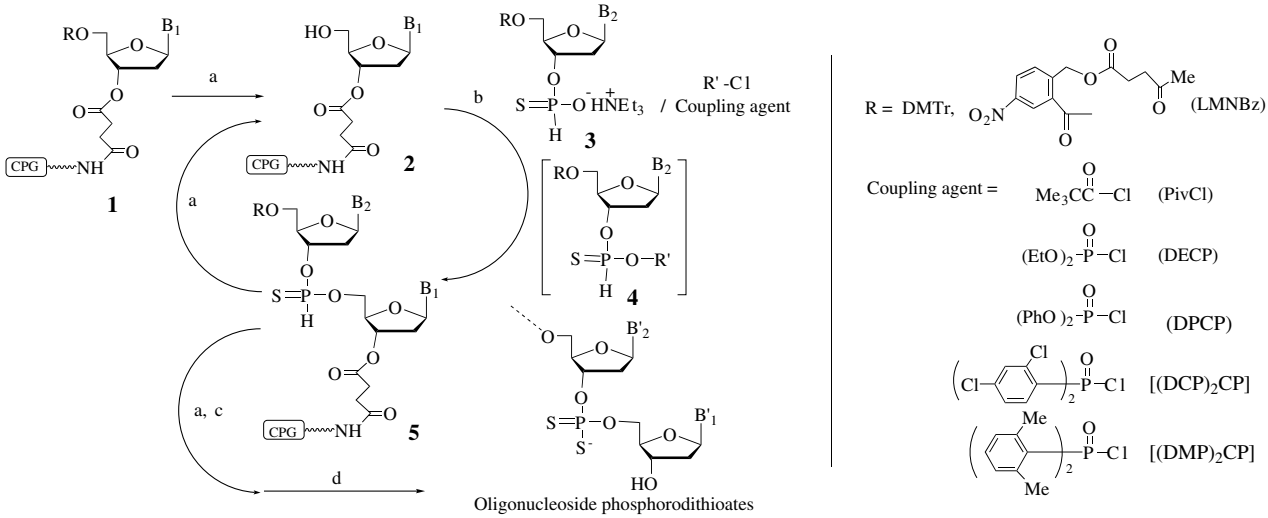
**Abstract**—The phosphorodithioate octamer [$(TpS_2)_7T$] was efficiently synthesized using bis(2,6-dimethylphenyl) phosphorochloridate as a coupling agent by application of the *H*-phosphonothioate method, where oxidation was facilitated using elemental sulfur following completion of oligonucleoside *H*-phosphonothioates assembly, as with the standard *H*-phosphonate method.


Oligonucleoside phosphorodithioates have proven useful as antisense probes.[1–4] Several approaches to the synthesis of these analogues involving the use of phosphorodithioate,[1a,b,d,5] thiophosphoroamidite[1d,2a,c,5b,6] *H*-phosphonothioate,[1d,5b,7] *H*-phosphonodithioate,[3,8] phosphorodithioate[2b,9] or dithiophospholane[10] monomers have been reported. The *H*-phosphonothioate method (Scheme 1) has advantages in that the *H*-phosphonothioate monomers are stable to hydrolysis and oxidation, the cycle time is short, and there is the possibility of




**Scheme 1.** Reaction cycle for assembly of oligonucleoside phosphorodithioates. Reagents and conditions: (a) 3% $CCl_3COOH/CH_2Cl_2$, 1 min (for deprotection of the DMTr group) or (i) 0.5 M $NH_2NH_2{\cdot}H_2O$, 1:4 $CH_3COOH$/pyridine, 15 min, (ii) 0.5 M imidazole, $CH_3CN$, 5 min (for deprotection of the LMNBz group); (b) 0.05 M **3**, 0.15–0.25 M coupling agent, 1:4 pyridine/$CH_3CN$, 10 min; (c) 0.3 M $S_8$, 1:9 2,6-lutidine/$CH_2Cl_2$, 1 h; (d) 1:1 concd $NH_4OH$/EtOH, rt, 3 h–55 °C, 5 h.

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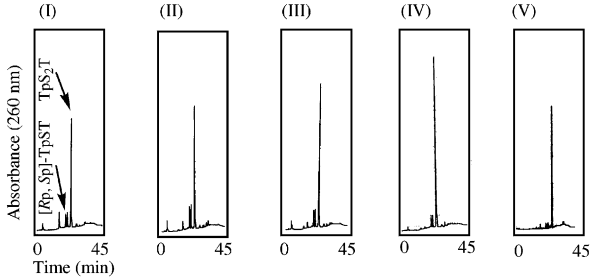
oxidation without using elemental sulfur in every cycle but only at the end of the synthesis, just as in the standard *H*-phosphonate method. The central issue for oligonucleoside phosphorodithioates synthesis by the *H*-phosphonothioate method involves the selective *O*-activation (**4**) of the *H*-phosphonothioate **3** by a coupling agent. Stawinski and co-workers reported studies on the synthesis of deoxynucleoside *H*-phosphonothioates **3** (R = DMTr) and their use in the synthesis of dinucleoside *H*-phosphonothioates.[7a,b,11] In a study pertaining to the synthesis of dinucleoside *H*-phosphonothioates they reported $^{31}$P NMR studies on the coupling reactions using nucleoside *H*-phosphonothioates with a variety of activators. When diphenyl phosphorochloridate (DPCP) or diethyl phosphorochloridate (DECP) were used as coupling agents, the *H*-phosphonothioate monomers could be selectively activated at the oxygen and coupled. In reactions when more than an equimolar amount of a hydroxylic component was used, some 5′-phosphorylation of the nucleoside by DPCP was observed. On the other hand, side products which may have been generated from the subsequent reaction of oligonucleoside *H*-phosphonothioate with coupling agents (phosphite triester or hypophosphates) were not observed. Caruthers and co-workers developed a new method for the synthesis of oligonucleoside phosphonothioates using *H*-phosphonothioates **3** (R = DMTr).[7e,f] They reported that oxidation with elemental sulfur at the end of the synthesis, just as in the standard *H*-phosphonate method, gave rise to the formation of significant amounts of desulfurized products. The synthesis of oligodeoxynucleoside phosphorodithioates was accomplished by using DPCP as a coupling agent and incorporating an oxidation step using 2,4-dichlorobenzyl thiosuccinimide in each coupling cycle.

On the other hand, we reported on the synthesis of oligonucleoside phosphorodithioates using nucleoside 3′-*H*-phosphonothioates **3** (R = LMNBz) by the standard *H*-phosphonate method, where oxidation was achieved using elemental sulfur following the completion of oligonucleoside *H*-phosphonothioates assembly.[12,13] The trimer ($TpS_2TpS_2T$) was efficiently synthesized using DECP as a coupling agent.[13] The successful synthesis of $TpS_2TpS_2T$ prompted us to further investigate the utility of this method. We report here further investigations on the synthesis of oligonucleoside phosphorodithioates by the *H*-phosphonothioate method through oxidation using elemental sulfur at the end of the synthesis.

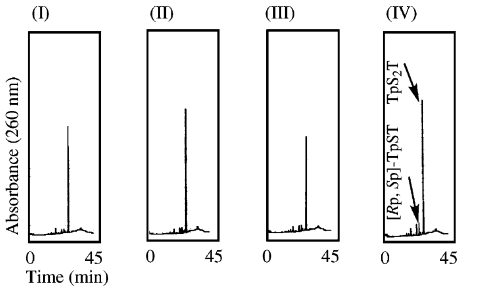
Based on the aforementioned reports,[7a,b,e,f,11,13] the reaction of *H*-phosphonothioate **3** with hydroxylic component **2** using the bulky phosphorochloridate derivative as a coupling agent could be expected to be selectively activated at oxygen and coupled without side reactions, that is the generation of phosphorothioate oligomers and the 5′-phosphorylation of nucleotide by a coupling agent. Additionally, using bulky diphenylphosphorochloridate derivatives having substituents on the phenyl groups as a coupling agent, it was expected that the selectivity relating to *O*-activation (**4**) of the phosphonothioate **3** by a coupling agent could be improved by the electronic effect of substituents on the phenyl groups. The commercially available bis(2,4-dichlorophenyl) phosphorochloridate [$(DCP)_2CP$] and bis(2,6-dimethylphenyl) phosphorochloridate [$(DMP)_2$CP] were considered as candidates for the effective coupling agent.

At first, a comparative study was performed of the various coupling agents, pivaloyl chloride (PivCl), DPCP, DECP, $(DCP)_2CP$, and $(DMP)_2CP$. These were employed in the synthesis of oligonucleoside phosphorodithioates as exemplified by the synthesis of dimer $TpS_2T$ using 5′-*O*-DMTr-thymidine 3′-*H*-phosphonothioate **3**[7a,b] on CPG support **1** (R = DMTr) through oxidation using elemental sulfur as shown in Scheme 1 by manual synthesis.[14] Following deprotection of the DMTr group of 5′-*O*-DMTr-thymidine linked to CPG support **1**, *H*-phosphonothioate **3** (R = DMTr) was coupled to thymidine (**2**) in the presence of either PivCl, DPCP, DECP, $(DCP)_2CP$, or $(DMP)_2CP$. Following *H*-phosphonothioate dimer **5** assembly and subsequent removal of the DMTr group, oxidation was performed using a 0.3 M solution of elemental sulfur in 1:9 2,6-lutidine/dichloromethane for 1 h to yield the desired phosphonodithioate dimer, cleavage of $TpS_2T$ from the support, and analysis by reversed-phase HPLC. The effectiveness of $(DMP)_2CP$ can clearly be seen from the HPLC profiles shown in Figure 1. Given the low reactivity of the *S*-nucleophile toward the phosphorus center,[7b] the decrease in reactivity of the diphenylphosphorochloridate derivative, due to the effect of the




| Coupling agent | PivCl | $(DCP)_2CP$ | DPCP | DECP | $(DMP)_2CP$ |
|---|---|---|---|---|---|
| [*Rp*, *Sp*]-TpST/$TpS_2T$ | 8.4+9.6 / 82.0 | 12.2+12.7 / 75.1 | 8.8+8.8 / 82.4 | 5.9+5.5 / 88.6 | 3.3+4.1 / 92.6 |

**Figure 1.** Reversed-phase HPLC profiles of crude products of $TpS_2T$ preparations using the 5′-*O*-DMTr-thymidine *H*-phosphonothioate **3** on CPG **1** (R = DMTr) in the presence of Piv-Cl (I), $(DCP)_2CP$ (II), DPCP (III), DECP (IV), and $(DMP)_2CP$ (V) as a coupling agent, respectively.




| Time (h) | 15 min | 1 | 4 | 8 |
|---|---|---|---|---|
| [*Rp*, *Sp*]-TpST/TpS$_2$T | 3.3+3.0 / 93.7 | 3.3+4.1 / 92.6 | 6.0+5.5 / 88.5 | 7.4+7.1 / 85.5 |

**Figure 2.** Reversed-phase HPLC profiles of crude products of TpS$_2$T preparations using the 5′-*O*-DMTr-thymidine *H*-phosphonothioate **3** on CPG **1** (R = DMTr) in the presence of (DMP)$_2$CP as a coupling agent through treatment with 0.3 M solution of $S_8$ in 1:9 2,6-lutidine/pyridine for 15 min (I), 1 h (II), 4 h (III), and 8 h (IV), respectively.

electron-donating substituents on the phenyl groups, is likely to have enhanced the selectivity of *O*-activation (**4**) of *H*-phosphonothioate **3**. DECP, which possesses the electron-donating substituents in the ester moiety, also showed highly selective *O*-activation (**4**).

Investigations were then performed in an effort to ascertain the stability of phosphorodithioate under oxidation conditions. In the synthesis of oligonucleoside phosphonodithioates using *H*-phosphonothioates **3** (R = DMTr), significant amounts of desulfurized products were formed during oxidation using elemental sulfur following completion of *H*-phosphonothioate

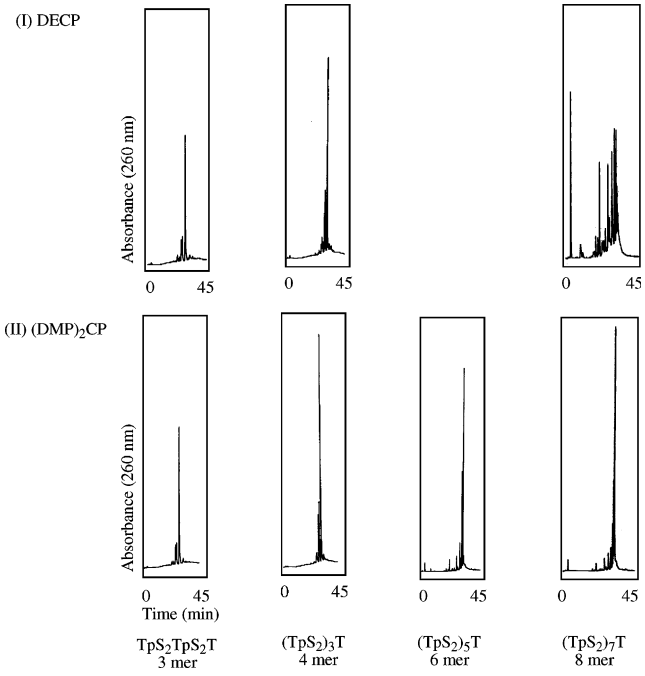



**Figure 3.** Reversed-phase HPLC profiles of crude products of phosphorodithioate oligomer [(TpS$_2$)$_n$T] preparations using the 5′-*O*-DMTr-thymidine 3′-phosphonothioate **3** on CPG **1** (R = DMTr) in the presence of DECP (I) and (DMP)$_2$CP (II), respectively, through oxidation with $S_8$ for 1 h at the end of the synthesis. Conditions of reversed-phase HPLCs: column μ BONDASPHERE 5 μ C18 (3.9 mm ID × 150 mm L); elution buffer 7.25–50% $CH_3CN$/0.1 M TEAA (pH 7); flow rate 1 mL/min; detection UV at 260 nm.

oligomer assembly as reported by Caruthers and co-workers, although detailed data was not shown.[7f] We investigated the stability of TpS$_2$T under oxidation conditions. Following *H*-phosphonothioate dimer **5** (B$_1$ = B$_2$ = T) assembly using (DMP)$_2$CP as a coupling agent and subsequent removal of the DMTr group, oxidation was performed using elemental sulfur for either 15 min, 1, 4 or 8 h. TpS$_2$T was cleaved from the support and subjected to analysis by reversed-phase HPLC. It was determined that sulfurization was complete within 15 min and that the amount of desulfurized products ([*Rp*,*Sp*]-TpST: [*Rp*]- and [*Sp*]-phosphorothioate dimers) increased slightly with increased time (Fig. 2).

Based on the studies described above, we extended our studies to the synthesis of long-chain oligonucleoside phosphonodithioates. Using the same procedure employed with DECP and (DMP)$_2$CP as coupling agents through oxidation using elemental sulfur for 1 h at the end of the synthesis, oligomers [(TpS$_2$)$_n$T:trimer, tetramer, hexamer, and octamer] were assembled and HPLC tracings of the each of the resulting mixtures are shown in Figure 3.[15] With (DMP)$_2$CP as a coupling agent, the oligonucleoside phosphonodithioates were efficiently synthesized.

The phosphorodithioate oligomers were easily purified by reversed-phase HPLC, and the electrophoretic profiles gave clear single bands (Fig. 4). The structure of the products were consistent with data derived from $^{31}$P NMR (Fig. 5) and ESI-TOF MS analyses.[16]

Synthesis of the phosphorodithioate octamer [(TpS$_2$)$_7$T] was accomplished by employing (DMP)$_2$CP as a coupling

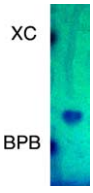



**Figure 4.** Electrophoresis of the octamer [(TpS$_2$)$_7$T] on a 20% polyacrylamide gel containing 7 M urea, visualized by UV-shadowing.

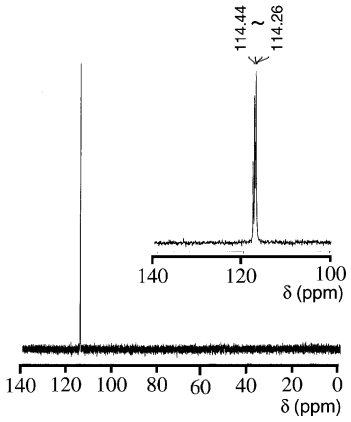



**Figure 5.** $^{31}$P NMR of the octamer [(TpS$_2$)$_7$T] in $D_2O$.

agent in the synthetic cycles using the *H*-phosphonothioate method, and where oxidation using elemental sulfur was achieved following completion of oligonucleoside *H*-phosphonothioates assembly. Application of the approach detailed in this investigation will be useful in the synthesis of phosphorodithioate-type short oligonucleotides.

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15. Yield of $TpS_2T$: 11.9 (DECP) and 12.2 [$(DMP)_2CP$] $A_{260}$ units from **1** (R = DMTr, 1 μmol). Yield of $(TpS_2)_2T$: 11.8 (DECP) and 13.7 [$(DMP)_2CP$] $A_{260}$ units from **1** (R = DMTr, 1 μmol). Yield of $(TpS_2)_3T$: 9.5 (DECP) and 13.4 [$(DMP)_2CP$] $A_{260}$ units from **1** (R = DMTr, 1 μmol); Yield of $(TpS_2)_5T$: 11.6 [$(DMP)_2CP$] $A_{260}$ units from **1** (R = DMTr, 1 μmol); Yield of $(TpS_2)_7T$: 17.5 [$(DMP)_2$ CP] $A_{260}$ units from **1** (R = DMTr, 1 μmol) (Fig. 3).
16. $TpS_2T$: $^{31}P$ NMR ($D_2O$) $\delta$ 114.55 (s, $PS_2$, 1P); ESI-TOF MS $m/z$ 601 $(M+Na)^+$. $(TpS_2)_2T$: $^{31}P$ NMR ($D_2O$) $\delta$ 114.39 and 114.77 (2s, $PS_2$, 2P); ESI-TOF MS $m/z$ 937 $(M+Na)^+$. $(TpS_2)_3T$: $^{31}P$ NMR ($D_2O$) $\delta$ 114.38 (s, $PS_2$, 1P) and 114.59 (s, $PS_2$, 2P); ESI-TOF MS $m/z$ 1273 $(M+Na)^+$. $(TpS_2)_5T$: $^{31}P$ NMR ($D_2O$) $\delta$ 114.29, 114.32, 114.38, 114.40, and 114.46 (5s, $PS_2$, 5P); ESI-TOF MS $m/z$ 1945 $(M+Na)^+$. $(TpS_2)_7T$: $^{31}P$ NMR ($D_2O$): $\delta$ 114.26–114.44 (m, $PS_2$, 7P) (Fig. 4); ESI-TOF MS $m/z$ 2617 $(M+Na)^+$.