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Synthesis of Oligodeoxyribonucleoside Phosphorothioates Using Lawesson's Reagent for the Sulfur Transfer Step

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Abstract—2,4-Bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulfide (Lawesson's reagent) efficiently converts the triphosphite intermediate used in the solid-phase synthesis of oligonucleotides into an oligodeoxyribonucleoside phosphorothioate.

Oligodeoxyribonucleoside phosphorothioates are isoelectronic analogues of natural phosphodiesters in which one of the oxygen atoms not involved in the internucleotidic linkage is replaced by a sulfur atom. As this derivatization at phosphorus is sequence nonspecific, the high degree of specificity encoded by the four nucleotide bases can be preserved. However, unlike natural oligomers, phosphorothioate oligodeoxyribonucleotides are resistant to degradation by nucleases.[1] Oligodeoxyribonucleotide analogues complementary to mRNA have been used as antisense molecules to inhibit gene expression.[2] Thus, phosphorothioate oligodeoxyribonucleotide derivatives represent a promising class of potential therapeutic agents. For example, sequence specific phosphorothioate oligomers can decrease the cytopathic effect of HIV-1 in chronically infected H9 cells,[3] can potently inhibit HIV RT[4] and were effective against systemic CNS disease in a human lymphoma xenograft model.[5]

Initially, oligodeoxyribonucleotide thio-analogues were synthesized with a solid-phase automated DNA synthesizer using elemental sulfur (S_8) as the sulfurizing reagent.[6] However, the solubility of S_8 in organic solvents is low and its sulfurizing reaction rate is slow, making the synthesis of longer phosphorothioate oligodeoxyribonucleotides, or of shorter ones in large amounts, inefficient. Hence, the development of a sulfurizing reagent with high efficiency has been an urgent problem in bioorganic synthesis. In the past few years, a variety of sulfur transfer reagents have been designed and tested for the efficient synthesis of phosphorothioate oligodeoxyribonucleotides via the phosphoramidite approach: 3*H*-1,2-benzodithiole-3-one 1,1-dioxide (Beaucage reagent),[7] tetraethylthiuram disulfide (TETD),[8] phenylacetyl disulfide,[9] dibenzoyl tetrasulfide,[10] bis(*O*,*O*-diisopropoxyphosphinothioyl)disulfide (S-Tetra),[11] benzyltriethylammonium tetrathiomolybdate (BTTM),[12] and bis(4-methoxybenzenesulfonyl)disulfide and related derivatives.[13] Among these compounds, the Beaucage reagent is widely used because of its rapid sulfur transfer reaction rate and commercial availability. However, this reagent suffers from instability and other problems.[11,13] The sulfur transfer reagents 1,2,4-dithiazolidine-3,5-dione and 3-ethoxy-1,2,4-dithiazoline-5-one were recently reported to successfully produce 6–20 mer phosphorothioate oligodeoxyribonucleotides.[14]

2,4-Bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane 2,4-disulfide [Lawesson's reagent (LR)], an efficacious thionation reagent for a variety of oxo compounds,[15,16] can also transfer a sulfur atom to phosphite triesters, forming thiophosphates.[17] In this communication, we report model chemistry demonstrating the potential of LR as a replacement for S_8 in phosphorothioate oligodeoxyribonucleotide synthesis.

For our initial evaluation of LR, a protected phosphite (**1**) was synthesized and converted to the thiophosphonate **2** by treatment with a large molar excess of elemental sulfur in pyridine (Scheme 1).

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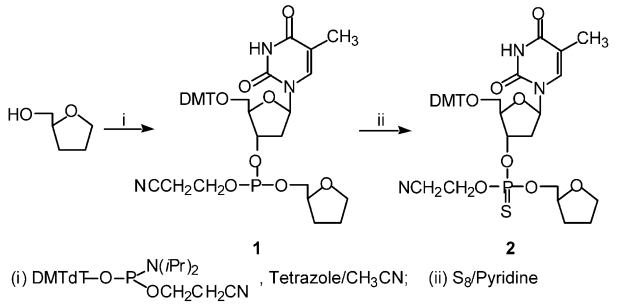
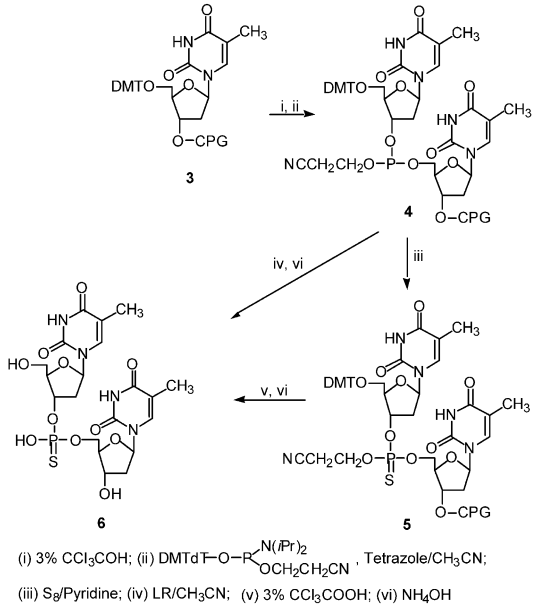
Using **2** as a standard, the reaction of **1** with commercial-grade LR (1.05 equiv) in $CDCl_3$ was then investigated by ^{31}P NMR. LR dissolved immediately and the solution became pale red. After 3 min, TLC analysis indicated complete consumption of the starting material. Analysis by ^{31}P NMR showed that the phosphite triester signals were replaced by several new peaks at δ 61.7–84.7 ppm, indicative of a quantitative sulfurization of the phosphite ester. However, the expected phosphorothioate ester **2** could not be readily isolated by the usual procedure, indicating that protecting group cleavage might have occurred.

The protecting groups on **1**, 4,4′-dimethoxytrityl (DMT) and $NCCH_2CH_2$–, are removed by acid and base, respectively. The red color seen in the reaction mixture suggested release of the DMT cation (see below).

To investigate further the potential of LR for thio-oligonucleotide synthesis, the dimer S-d(T_PT) **6** was first prepared from solid-phase controlled pore glass (CPG)-linked 5′-*O*-dimethoxytritylated 2′-deoxythymidine **3** using a large molar excess of S_8 to convert the intermediate phosphite dimer **4** into the corresponding phosphorothioate **5**, which was then deprotected and released from the support by successive treatment with



Scheme 1.



Scheme 2.

CCl_3CO_2H and NH_4OH (Scheme 2). Use of LR (acetonitrile) in place of S_8 followed by NH_4OH treatment converted **4** directly to **6**, identified by ^{31}P NMR. In solid-phase DNA synthesis, acid cleavage of the DMT group protecting the 5′-OH of the nucleoside releases trityl cation, $\lambda_{max} = 503$ nm. Absorption spectral analysis of acid wash (Cl_3CO_2H in CH_2Cl_2) solutions from the solid phase product with LR confirmed the absence of DMT, consistent with the results obtained for model compound **1**.[18] The sulfurization reaction of **4** with LR was complete within 5 min. This reaction time was significantly shorter than that for S_8 (30 min), although a much larger excess of the latter reagent was used.

In summary, the potential utility of LR as a replacement for S_8 in the synthesis of oligodeoxynucleoside phosphorothioates has been investigated. LR was found to rapidly and cleanly sulfurize a model phosphite triester intermediate, and could replace S_8 as the sulfur-transfer reagent in the synthesis of a dinucleoside phosphorothioate, S-d(T_PT). The reactions with LR (using as little as 5% excess) were completed within 5 min.[19] The trityl group protecting the 5′-OH moiety of the substrate was removed during LR sulfurization, obviating the standard separate detritylation step with CCl_3CO_2H.

NMR spectra were measured at 360.14 MHz (^1H) and 145.78 MHz (^{31}P). Chemical shifts were reported in ppm versus $CHCl_3$ (^1H: 7.24 ppm), and 85% H_3PO_4 (^{31}P: 0.00 ppm). UV–vis spectra were recorded on a Shimadzu UV-260 spectrometer. Reagents were purchased from Pharmacia, CPG (**3**) or Aldrich (LR). Sulfurizations were conducted at room temperature.

Synthesis of 1 and 2. dT Phosphoramidite (75 mg, 0.1 mmol) in 1.5 mL dry CH_3CN was added to tetrahydrofurfuryl alcohol (15 mg, 0.5 mmol) in 1 mL dry CH_3CN, followed by tetrazole (35 mg, 0.5 mmol) in 1.5 mL dry CH_3CN. After 3 min, **1** was isolated by preparative TLC (silica gel, ethyl acetate): ^{31}P NMR ($CDCl_3$): δ 139.6, 143.1 ppm; ^1H NMR ($CDCl^3$): δ 3.81 (s, 6H, CH_3O), 1.38 (s, 3H, CH_3), 2.55–6.36 (m, ribose ring), 6.70–7.60 (m, Ph), 8.81 ppm (s, br, 1H, NH). Elemental sulfur (32 mg, 1.0 mmol) in 0.5 mL pyridine/CS_2/Et_3N (1:1:1) was added to **1** (10 mg, 0.013 mmol) in 1 mL CH_3CN. After 15 min, the phosphite peaks (δ = 139, 142 ppm) were replaced by signals at δ 66.9, 67.1 and 56.2, 56.3 ppm. Phosphorothioate **2** (9 mg) was isolated by prep. TLC: ^{31}P NMR ($CDCl_3$): δ 66.9, 67.1 ppm (P = S, diastereomers); ^1H NMR ($CDCl_3$): 1.38 (s, 3H, CH_3), 2.55–6.36 (m, ribose ring), δ 3.72 (6H, CH_3O), 6.72–7.63 (m, Ph), 8.81 ppm (s, br, 1H, NH). Reaction of **1** (10 mg, 0.013 mmol) with LR (2.71 mg, 0.007 mmol in 2 mL $CDCl_3$) was done in a 10 mm NMR tube.

Preparation of 6. The CPG-linked protected deoxythymidine **3** (200 mg, 6.54 μmol) was detritylated with 3% CCl_3COOH in CH_2Cl_2. After washing with CH_3CN and drying in vacuo for 5 min, 10 equiv of 5′-*O*-DMT-2′-deoxythymidine-*N*,*N*′-diisopropyl cyanoethyl phosphoramidite (2 M, 1 mL CH_3CN solution) and tetrazole

(0.45 M, 1 mL CH_3CN solution) were added and the suspension was shaken for 30 min. After flushing (argon) and washing (CH_3CN), 5 mL 5% S_8 in pyridine/CS_2/Et_3N was added and the rection was allowed to proceed (30 min). After flushing (Ar) and washing (5×3 mL pyridine, 5×3 mL CH_2Cl_2), treatment with 3% CCl_3CO_2H in CH_2Cl_2 (4×2 mL) followed by NH_4OH (10 mL, overnight) gave, after evaporation, **6**: ^{31}P NMR (D_2O): δ 55.84, 56.21 ppm (P=S, diastereomers); ^1H NMR (D_2O): δ 1.71, 1.82 (s, 6H, CH_3), 2.18–2.87 (m, 6H, *H*-2′, 3′), 3.67–4.00 (m, 4H, *H*-5′), 4.42 (m, 2H, *H*-4′), 6.05–6.17 (m, 2H, *H*-1′), 7.50, 7.56 ppm (s, 2H, *H*-6); ^{13}C NMR (D_2O): δ 18.94 and 21.11 (s, *C*H_3), 36.17–38.55 (C-2′), 60.66 and 70.40 (C-3′), 64.72–65.19, 74.88–75.25 (C-5′), 84.73–84.88 (C-4′), 84.95–85.38 (C-1′), 111.27 and 111.37 (C-5), 137.08 and 137.12 (C-6), 151.57 (C-4), 166.24 ppm (C-2). With LR (11 mg in 5 mL CH_3CN) in place of S_8, reaction was complete in 5 min; after washing with warm (50 °C) 5×5 mL CH_3CN and NH_4OH deprotection, the product was **6**: ^{31}P NMR (D_2O): δ 55.84, 56.21 ppm (P=S, diastereomers).

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18. Commercial LR is 97% pure and gives a pH <2 when an acetonitrile solution is diluted with H_2O.
19. In solid phase multi-step syntheses, excess reagents are routinely employed to maximize the yield for each step. Owing to the relatively low solubility of LR in acetonitrile at room temperature, in the second model reaction the effective excess of LR was smaller than the nominal excess (8 equiv). The same solubilty consideration prompted the use of warm acetonitrile in the washing step, and would need to be accommodated if this facile sulfurization chemistry is adapted to automated DNA synthesis. This should be technically feasible by use of a thermal wrap or similar means to warm the solid phase reaction column.