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Solid Phase Synthesis of Oligonucleotide Phosphorothioate Analogues Using Bis(ethoxythiocarbonyl)tetrasulfide as a New Sulfur-Transfer Reagent

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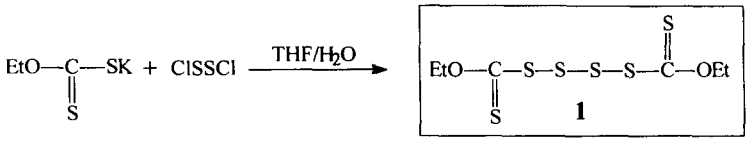
Abstract: A new sulfur-transfer reagent, bis(ethoxythiocarbonyl)tetrasulfide (**1**), has been prepared and used for the synthesis of oligonucleotide phosphorothioates via solid phase phosphoramidite approach.

Oligonucleotide phosphorothioates are of considerable interest in nucleic acid research and are investigated in several clinical trials as a new type of therapeutic.[2] The automated synthesis of phosphorothioates can be achieved using the *H*-phosphonate or phosphoramidite approach. The *H*-phosphonate approach allows a single sulfurization of all internucleotide linkages to be conducted after chain assembly using elemental sulfur (S_8) dissolved in a mixed solvent (CS_2/pyridine). The phosphoramidite approach requires stepwise sulfurization to be carried out after each coupling. Based on the superior coupling efficiency as well as the capability to control the state of each linkage in a site-specific manner, the phosphoramidite approach appears to be the method of choice. It is crucial that an efficient sulfur transfer reagent is employed for the stepwise sulfurization via the phosphoramidite approach. A number of sulfur-transfer reagents have also been reported.[3-10] Of these compounds, Beaucage reagent has been widely used and applied successfully in large scale synthesis[11], however, its synthetic accessibility and stability in solution[12] are not optimal. In order to support the increasing demand for preclinical and clinical trials as well as future commercialization, we are investigating an alternative sulfurizing reagent to further reduce costs. Herein we report our studies on a new sulfur-transfer reagent, *bis(ethoxythiocarbonyl) tetrasulfide* (**1**).

Compound **1** was prepared using potassium ethylxanthogenate and sulfur monochloride in 77% yield (Scheme 1).[13]



Scheme 1. The synthesis of bis(ethoxythiocarbonyl) tetrasulfide (**1**).

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The efficiency of sulfurizing reagent **1** was first investigated by solid-phase synthesis of dinucleotide phosphorothioates. Reagent **1** was also compared with five known sulfurizing reagents **2-6**[4-7, 9] (Figure 1).[14]

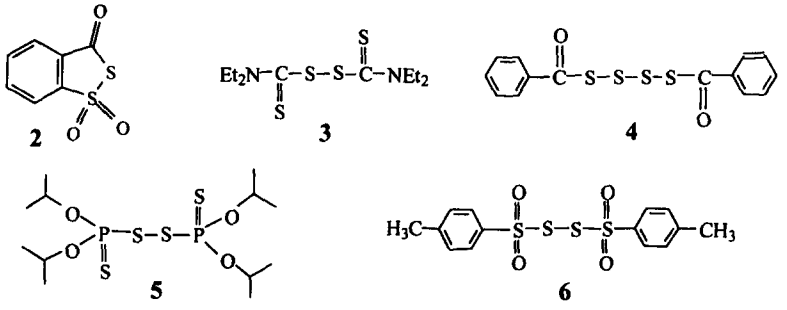

Figure 1. Sulfur-Transfer Reagents.

Synthesis of dinucleotide phosphorothioate d(TsT) was performed at the 1.0 μmol scale using an automated synthesizer, 8909 Expedite™ (Millipore, Bedford, MA). Solutions of the sulfurizing reagents **3-5** were dried over activated 4 **Å** molecular sieves overnight before use. The synthesis protocol "THIO 1 μmol" (Expedite™ software version 1.01) was used with the following modifications: 1. Capping was performed after the sulfurization step, since capping preceding the sulfurization may cause the formation of a small percentage of phosphodiester linkages[4]; 2. Delivery time of sulfurization reagents and acetonitrile wash step following sulfurization was extended in some cases as indicated. After cleavage and deprotection, the unpurified dimers were analyzed by reverse-phase HPLC. The reverse-phase HPLC profiles in Figure 2 show the correspoding dinucleotide phosphorothioate (PS) and dinucleotide phosphodiester (PO) present in the crude products from Experiments **a** and **h** (Table 1). The results obtained by using different sulfur-tranfer reagents are given in Table 1.

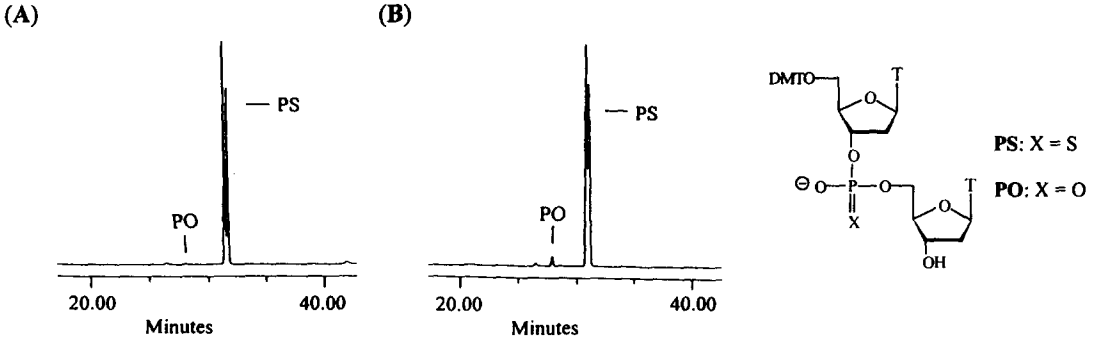

Figure 2. The reverse-phase HPLC analyses of dinucleotide phosphorothioate d(TsT) using the different sulfur transfer reagents: **(A) 1** (Experiment **a**, see Table 1); **(B) 4** (Experiment **h**).

TABLE 1. Sulfur-transfer efficiency for synthesis of the dimer 5'-d(TsT)-3'

Experiment	Sulfurizing Reagents	Concentration (M)	Molar equivalent	Solvent	Reaction Time(min)	P=O* (%)	P=S* (%)
a	**1**	0.08	15	Pyridine	1	0.63	99.37
b			10	Pyridine	5	0.31	99.69
c	**2**	0.06	11	CH_3CN	1	0.68	99.32
d					5	0.92	99.08
e	**3**	0.50	90	CH_3CN	1	4.55	95.45
f					5	3.00	97.00
g	**4**	0.40	72	THF	1	4.42	95.58
h					5	2.21	97.79
i	**5**	0.20	37	Pyridine	1	1.55	98.45
j					4	0.71	99.29
k	**6**	0.20	37	DCM:Py(9:1)	1	2.07	97.93

* P=O indicates the phosphodiester linkage; P=S indicates the phosphorothioate linkage.

Table 1 shows that (a) compound **1** is a highly efficient sulfurizing reagent under the conditions tested. (b) in our hands, the compounds **3-6** were much less effective even at high concentration (or molar equivalent); (c) the sulfurization yield was improved by increasing the reaction time for **1**, however, the prolonged reaction time may lower the sulfurization yield for Beacage reagent **2**.

To further evaluate the usefulness of **1** as an efficient sulfurizing reagent, a 25mer (5'-CTCTCGCACCCATCTCTCTCCTTCT-3') oligonucleotide phosphorothioate was synthesized on the 1.0 μmol using an automated synthesizer, 8909 Expedite™ (Millipore, Bedford, MA). The synthesis was carried out under experimental condition **a** in Table 1. After ammonolytic release from CPG and deprotection, the unpurified oligonucleotide phosphorothioate was analyzed by ion exchange-HPLC and gel-capillary electrophoresis (Figure 3).

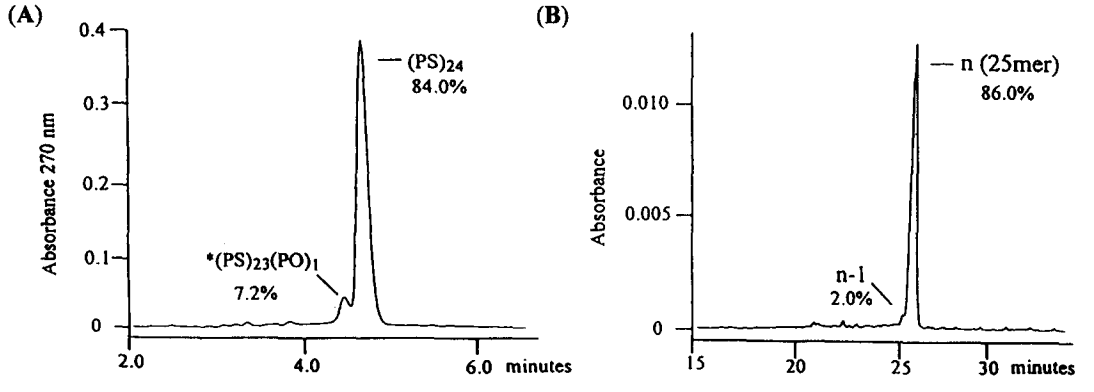

Figure 3. Analyses of the unpurified 25mer oligonucleotide phosphorothioate by (**A**) ion exchange-HPLC; (**B**) Gel-capillary electrophoresis. *$(PS)_{23}(PO)_1$ indicates the 25mer which has a single phosphodiester internucleotide linkage at a random position along with 23 of phosphorothioate linkages.

The ion exchange-HPLC analysis (Figure 3-A) indicates that in the synthesis of the 25mer more than 99.5% sulfur transfer efficiency was achieved at each step using **1**. To confirm this result we have also examined the the crude 25mer oligonucleotide by NMR. ^{31}P NMR analysis shows that the ratio of the integrals of the signals at δ 55.4 ppm (phosphorothioate linkage) and δ -2.2 ppm (phosphodiester linkage) is 99.62/0.38, which also indicates a more than 99.5% sulfur transfer efficiency. The profile of gel-capillary electrophoresis (Figure 3-A) shows that in the synthesis using **1**, the full-length 25mer product (n) was obtained in 89% yield and the corresponding one nucleotide deletion sequence (n-1)-mer was only 2%. The same results are obtained in olignucleotide phosphorthioate synthesis using the solution of **1** stored more than one week.

In conclusion, by comparing several sulfur transfer reagents we find **1** is a highly efficient reagent. This compound is also very easy and inexpensive to prepare. Due to its high efficiency and low cost, compound **1** can be considered an advantageous alternative to Beaucage reagent, especially in large-scale preparation of oligonucleotide phosphorothioates. Further optimization of reaction conditions and scale up are actively underway.

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12. Prolonged storage of Beaucage reagent in acetonitrile may cause the formation of precipitates and an inferior sulfurization efficiency.
13. To a solution of potassium ethylxanthogenate (40 g., 0.25 mol) in THF (100 mL) and H_2O (100 mL) was added dropwise sulfur monochloride (33.76 g., 20 mL, 0.25 mol) at 0 °C. The reaction mixture was stirred at room temperature overnight, and extracted with 300 mL of CH_2Cl_2. The organic phase was washed with 5% Na_2CO_3 solution followed by saturated NaCl solution, and dried over anhydrous potassium sulfate for 3 h. The solution was filtered, and the solvent was removed at reduced pressure to give the product as a pale yellow oil (29.6 g, 96.6 mmol, 77.3%): ^1H NMR ($CDCl_3$) δ 4.68 (m, 4H), 1.45 (m, 6H).
14. Beaucage reagent (**2**) was purchased from R. I. Chemical (Orange, CA); TETD (**3**) was from Applied Biosystems (Foster City, CA); dibenzoyl tetrasulfide (**4**)[6] and bis(O,O-diisopropoxyphosphinothioyl) disulfide (S-Tetra, **5**)[7] were prepared by literature's procedure; bis(*p*-toluenesulfonyl)disulfide (**6**) was provided by Aronex Pharmaceuticals (The Woodlands, TX).