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Solid Phase Synthesis of Oligonucleotide Phosphorothioate Analogues Using 3-Methyl-1,2,4-dithiazolin-5-one (MEDITH) as a New Sulfur-Transfer Reagent

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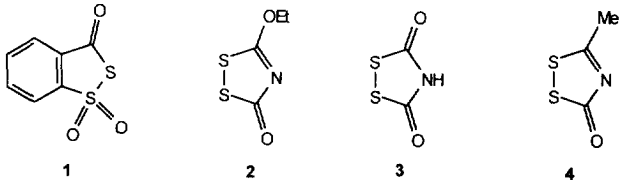
Abstract: A new sulfur-transfer reagent, 3-methyl-1,2,4-dithiazolin-5-one (MEDITH), has been used for the synthesis of oligonucleotide phosphorothioates via solid phase phosphoramidite approach.

Oligonucleotide phosphorothioates are of considerable interest in nucleic acid research and are investigated in several clinical trials as a new type of therapeutics.[2] In second generation antisense oligonucleotides under clinical trials or under development, the full length phosphorothioate backbone has been maintained or only a portion of the phosphorothioate linkages can be replaced.[2e] 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 synthetic method of choice. Since the phosphoramidite method requires stepwise sulfurization to be carried out after each coupling, it is crucial that an efficient sulfur transfer reagent is employed for the sulfurization step. A number of sulfur-transfer reagents have also been reported.[3-11] Of these compounds, Beaucage reagent (**1**) has been widely used,[4,11] however, its synthetic accessibility and stability in solution are not optimal. Two new sulfurizing reagents that are disulfide-containing five-membered heterocycles, 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH, **2**) and 1,2,4-dithiazoline-3,5-dione (DtsNH, **3**), are recently reported as advantageous alternatives to Beaucage reagent.[10] To develop new sulfurizing reagents and understand structure and activity relationships, we have investigated a series of disulfide-containing five- or six-membered heterocycles. Herein we report our studies on a new sulfur-transfer reagent, *3-methyl-1,2,4-dithiazolin-5-one* (MEDITH, **4**).



1 2 3 4

Figure 1. Sulfur-transfer reagents.

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MEDITH is a known compound that was first prepared by German scientists several decades ago and recently used to study mechanisms of dithiazolinone and thiadiazole formation.[13,14] However, to the best of our knowledge, no one has used this compound as a sulfur-transfer reagent in oligonucleotide synthesis. MEDITH was prepared by the reaction of thioacetamide with (chlorocarbonyl)sulfenyl chloride.[15] Unlike *O*-ethyl-thiocarbamate[16] required for the syntheses of EDITH and DtsNH, thioacetamide is a commercially available and inexpensive starting material. MEDITH is a crystalline compound, which can be stored for months at room temperature without any noticeable decomposition in solid state. MEDITH can be dissolved in acetonitrile as high as 4.0 M of concentration, which is an ideal property for the automated oligonucleotide synthesis.

The sulfurizing efficiency of MEDITH was first checked by solid-phase synthesis of dinucleotide phosphorothioates. Synthesis of dinucleotide phosphorothioate d(TsT) was performed at the 1.0 μmol scale using an automated synthesizer, 8909 Expedite™ (PerSeptive Biosystem, Franingham, MA). The sulfurization reaction was carried out using a 0.03 M solution of MEDITH in acetonitrile. The rate of the sulfurization was investigated, and the amounts of desired phosphorothioate (PS) and undesired phosphodiester (PO) were determined by reverse-phase HPLC. A comparison with some known sulfurizing reagents[17] is shown in Table 1.

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

Sulfurizing Reagents	Concentration (M)	Molar equivalent	Solvent	Reaction Time (min)	**P=O*** (%)	**P=S*** (%)
Beaucage	0.03	2	CH_3CN	1	13.65	86.35
		4		1	3.22	97.78
	0.06	11		1	0.68	99.32
				5	0.92	99.08
EDITH	0.03	2	CH_3CN	1	0.94	99.06
		4		1	0.41	99.59
MEDITH	0.03	2	CH_3CN	1	0.37	99.63
		4		1	0.32	99.68

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

As Table 1 shows, among these reagents MEDITH is a most efficient sulfurizing reagent under the conditions tested. A greater than 99.5% sulfurization efficiency can be achieved using 2 equivalent of MEDITH within one minute.

To further evaluate the usefulness of MEDITH as an efficient sulfur-transfer reagent, a 25mer oligodeoxynucleotide phosphorothioate, 5'-CTCTCGCACCCATCTCTCTCCTTCT-3', was also synthesized on the 1.0 μmol using an automated synthesizer, 8909 Expedite™. The synthesis was carried out under the same condition as previously described for synthesis of the dinucleotide (0.03 M solution, 2 equivalent of MEDITH and one minute of reaction time). After ammonolytic cleavage from CPG and deprotection, the crude oligodeoxynucleotide phosphorothioate was analyzed by ^{31}P NMR, ion exchange-HPLC and gel-capillary electrophoresis (Figure 2).

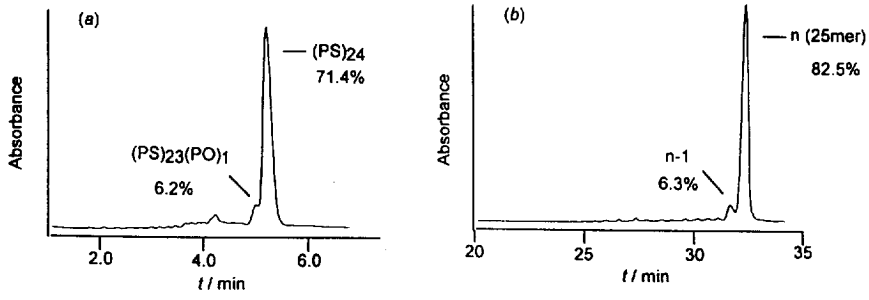
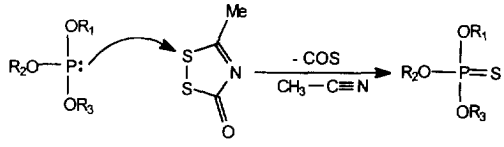


Figure 2. Analyses of the crude 25mer oligonucleotide phosphorothioate by (a) ion exchange-HPLC; (b) gel-capillary electrophoresis.

^{31}P NMR analysis showed that the ratio of the integrals of the signals around δ 55.2 ppm (phosphorothioate) and δ -1.5 ppm (phosphodiester) is 99.67/0.33. The results show that a greater than 99.5% sulfur transfer efficiency was achieved in the synthesis at each step using 2 equivalents of MEDITH and one minute of reaction time. On the other hand, almost no full length 25mer oligonucleotide phosphorothioate can be obtained under the similar condition using Beacauge reagent.

To study the stability of MEDITH in solution at room temperature, we also compared the sulfurization efficiency of freshly prepared MEDITH with a three-week old solution in the synthesis of the 25mer oligonucleotide phosphorothioate. The results show that in all cases the same sulfur transfer efficiency (>99.5%) was achieved.

MEDITH has the same core heterocyclic ring as DtsNH and EDITH, which is similarly a planar and aromatic molecule. The mechanism for the reaction of MEDITH with the trivalent phosphorus intermediates should be similar as proposed for EDITH[10]. Since both of the co-products, carbonyl sulfide (COS) and acetonitrile (CH_3CN), are stable molecules, sulfur-transfer from MEDITH should be a more favorable process.



Scheme 1.

In conclusion, by comparing several sulfur transfer reagents we find MEDITH is a highly efficient reagent and fully compatible with standard automated DNA synthesis. This compound is also relatively easy to prepare and stable upon prolonged room temperature storage in acetonitrile solution. Due to its high efficiency and stability, MEDITH can be considered an advantageous alternative to Beaucage reagent, especially in large-scale preparation of oligonucleotide phosphorothioates. Further optimization of synthetic conditions and scale up are actively underway.

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15. The compound was prepared following the procedure in Reference 14. The compound was further purified by recrystallization (ethyl acetate and hexane) to give a pale yellow solid. ^{1}H NMR ($CDCl_3$) δ 2.73 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 189.5, 186.5, 22.7.
16. *O*-Ethyl-thiocarbamate is not commercially available. See Chem Sources-USA, 1998 Edition.
17. Beaucage reagent (**1**) was purchased from R. I. Chemical (Orange, CA); EDITH (**2**) was purchased from PerSeptive Biosystems (FRAMINGHAM, MA).