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Reactivity of 3*H*-1,2,4-dithiazole-3-thiones and 3*H*-1,2-dithiole-3-thiones as sulfurizing agents for oligonucleotide synthesis

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ABSTRACT

The reactivity of 5-amino-3*H*-1,2,4-dithiazole-3-thiones substituted at their amino group and 5-amino-3*H*-1,2-dithiole-3-thiones substituted at their amino group and C4 toward compounds containing P(III) atoms has been studied. *N*,*N*-Disubstituted-*N*′-(3-thioxo-3*H*-1,2,4-dithiazol-5-yl)methanimidamides were selected as novel efficient sulfur transfer reagents suitable for DNA and RNA synthesis.

1. Introduction

Oligonucleotides that contain unnatural internucleoside linkages where one of the non-bridging oxygen atoms of the phosphate group is replaced by a sulfur atom are referred to as oligonucleotide phosphorothioates. Due to their enhanced nucleolytic stability, improved biodistribution, and pharmacokinetic properties, oligonucleotide phosphorothioates are among the most commonly used analogs. Their widespread use has led to an increasing demand for more practical, inexpensive, and efficient methods and reagents for their preparation.

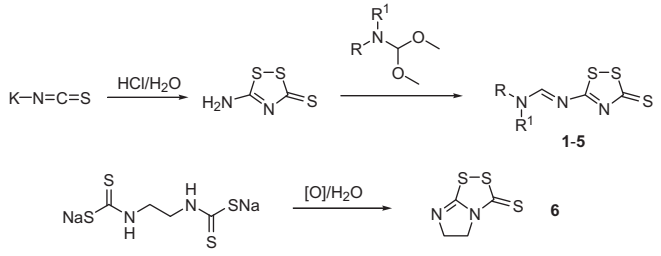
Examples of such agents include 3*H*-1,2-benzodithiol-3-one-l,l-dioxide, or the Beaucage reagent,[1] tetraethylthiuram disulfide,[2] phenylacetyl disulfide,[3] dibenzoyl tetrasulfide,[4] bis-(*O*,*O*-diisopropoxyphosphinothioyl) disulfide,[5] benzyltriethylammonium tetrathiomolybate,[6] bis-(*p*-toluenesulfonyl) disulfide,[7] 3-ethoxy-l,2,4-dithiazoline-5-one (EDITH) and 1,2,4-dithiazolidine-3,5-dione,[8] 3-amino-1,2,4-dithiazole-5-thione,[9] 3-methyl-1,2,4-dithiazolin-5-one,[8a,10] and 3-phenyl-1,2,4-dithiazoline-5-one.[11] However, when this study began, only the Beaucage reagent[1] and tetraethylthiuram disulfide[2] were commercially available. The widely used Beaucage reagent displays a rather low hydrolytic stability, and the reaction kinetics of tetraethylthiuram disulfide with solid support-bound phosphite triesters is somewhat slow, which makes it less useful in high-throughput applications. Consequently, more efficient sulfurizing agents are needed.

2. Results and discussion

2.1. Synthesis of 3*H*-1,2,4-dithiazole-3-thiones 1–5 and 3*H*-1,2-dithiole-3-thiones 6–15

Compounds **1**–**15** with the potential of becoming sulfurizing agents were synthesized starting from simple precursors as outlined in Schemes 1 and 2. As shown in Scheme 1, xanthane hydride was synthesized by acid-catalyzed trimerization of thiocyanic acid and was treated with the dimethylacetals of the corresponding commercial dialkylformamides to give compounds **1**–**5** in 80–90% yield.[12] Endodane **6** was prepared as reported previously[13](Scheme 1).

Compounds **7**–**9** were synthesized by the previously reported method[14] (Scheme 2) and were then converted to the *N*-(*N*′,*N*′-dimethylaminomethylene)-protected derivatives **10**–**12** by reaction with dimethylformamide dimethylacetal as described for compound **1**.[12] Additionally, compound **7** was converted to its



Scheme 1. Synthesis of 3*H*-1,2,4-dithiazole-3-thiones; **1**: R = R^1 = Me; **2**: R = R^1 = Bu; **3**: R + R^1 = $(CH)_4$; **4**: R + R^1 = $(CH)_5$; **5**: R + R^1 = $-(CH_2)_2O(CH_2)_2-$.

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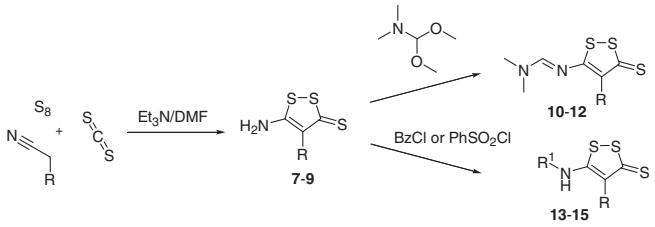
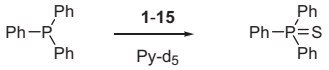


Scheme 2. Synthesis of 3*H*-1,2-dithiole-3-thiones; **7**, **10**, **13**: R = CO_2Et; **8**, **11**, **14**: R = CN; **9**, **12**, **15**: R = SO_2Ph; **13**: R^1 = SO_2Ph; **14**: R^1 = Bz; **15**: R^1 = Bz.



Scheme 3. Sulfurization of PPh_3 with compounds **1**–**15** in solution.

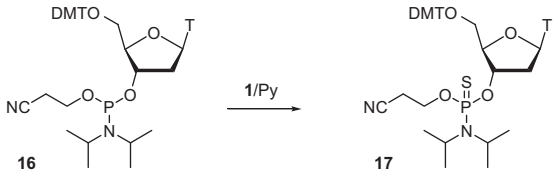
phenylsulfonyl derivative **13** (Scheme 2), and compounds **8** and **9** were *N*-benzoylated to give compounds **14** and **15**, respectively.

2.2. Testing of compounds 1–15 as sulfurizing agents in solution

The efficiency of compounds **1**–**15** as sulfur transfer reagents was first evaluated for their ability to convert PPh_3 into $Ph_3P{=}S$ in solution. Equimolecular amounts of **1**–**15** were mixed with triphenylphosphine in Py-d_5 and the progress of the reaction was monitored by ^{31}P NMR (Scheme 3). Compounds **1**–**5** and **7**–**12** reacted quantitatively with the substrate in less than 5 min to give triphenylphosphine sulfide in more than 99.9% yield along with a minor amount of triphenylphosphine oxide (<0.1%). In contrast, compounds **6** and **13**–**15** failed to produce good yields of $Ph_3P{=}S$ over a period of 5 min. The results obtained thus warranted continued testing of compounds **1**–**5** and **7**–**12** as sulfur transfer reagents in solid-phase oligonucleotide synthesis while compounds **6** and **13**–**15** were excluded from further study.

In a more detailed investigation, the stoichiometry of the sulfur transfer was ascertained by reacting aliquoted amounts of *N*,*N*-dimethyl-*N*′-(3-thioxo-3*H*-1,2,4-dithiazol-5-yl)methanimidamide (DDTT, **1**) with triphenylphosphine (1–5 equiv) and determining the ratio of $Ph_3P/Ph_3P{=}S$ by ^{31}P NMR. It was found that **1** transferred sulfur to PPh_3 in pyridine with a stoichiometric ratio of **1** to PPh_3 of 1:2.

In a similar manner, compound **1** was reacted with an equimolecular amount of 5′-*O*-(4,4′-dimethoxytrityl)thymidine 2-cyanoethyl 3′-*O*-(*N*,*N*-diisopropyl)phosphoramidite **16** (Scheme 4) to



Scheme 4. Sulfurization of **16** using compound **1**.

give thionophosphoramidate **17** as a mixture of diastereomers in more than 99.9% yield as judged by ^{31}P NMR.

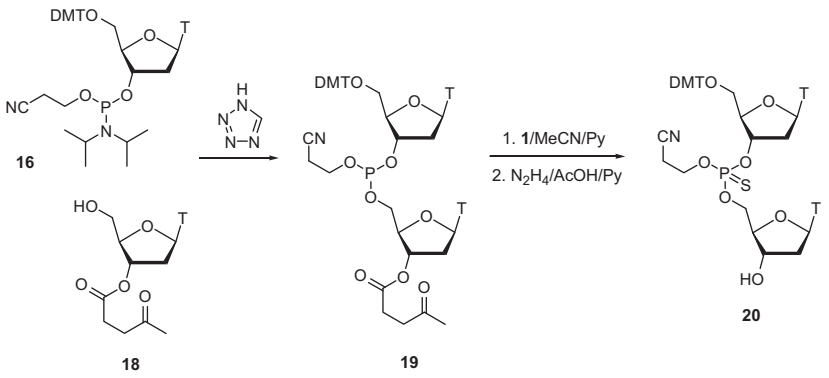
To further evaluate the usefulness of **1** as a sulfur transfer reagent, the protected dithymidylyl monophosphorothioate **20** was synthesized by reacting the phosphoramidite **16** with 3′-*O*-levulinylthymidine **18**[15] in the presence of 1*H*-tetrazole (Scheme 5).[16] Without isolation, the protected phosphite triester **19** was treated with a 1.2 molar excess of **1**, and the 3′-*O*-levulinyl protecting group was removed by treatment with hydrazinium acetate in a mixture of pyridine and AcOH to give compound **20** as a mixture of R_p and S_p diastereomers. Upon aqueous work-up, the crude **20** was analyzed by ^{31}P NMR and reverse-phase HPLC to show a 99.3% efficiency of the sulfur transfer. In order to characterize the compound, a portion of **20** was separated using reverse-phase HPLC to give the individual diastereomers **20a** and **20b**.[16] The latter compounds were decyanoethylated with a mixture of aqueous ammonium hydroxide and pyridine (1:5) followed by removal of the DMT protection with 80% aqueous AcOH resulting in the individual R_p and S_p diastereomers of thiothymidylyl-(3′ → 5′)-thymidine (**21a**[17] and **21b**,[18] ^{31}P NMR δ 56.65 and 56.28, respectively). Based on the reported data,[19] the R_p configuration was assigned to **21a** having a larger chemical shift for its P-atom.

Titration of compounds **16** and **19** with DDTT (**1**) showed that the stoichiometric ratio of **1** to a phosphite in these reactions was 2:3 (^{31}P NMR).

2.3. Testing of compounds 1–15 as sulfurizing agents in oligonucleotide synthesis on solid-phase

To test compounds **1**–**5** and **7**–**12** as sulfurizing agents in oligonucleotide synthesis, the oligonucleotide DMT-T_{10} phosphorothioate (**22**) was assembled on a 1 μmol scale using the standard protocol of the chain assembly, 0.05 M solutions of **1**–**5** and **7**–**12** in pyridine, and 4 min sulfurization time in each synthetic cycle.

After the solid-phase-bound material was released and deprotected with concentrated aqueous ammonium hydroxide, the crude oligonucleotide phosphorothioates were analyzed by re-



Scheme 5. Solution-phase synthesis of 2-cyanoethyl 5′-*O*-(4,4′-dimethoxytrityl)thiothymidylyl-(3′ → 5′)-thymidine **20**.

verse-phase HPLC and ESMS. The results showed that, with compounds **1**–**5**, more than 99.5% sulfur transfer efficiency was achieved in each step. Compounds **7** and **10**–**12** displayed an unacceptably slow kinetics of sulfur transfer while the use of compound **8** resulted in the extensive formation of side products.

2.4. The use of DDTT (1) as the sulfurizing agent in DNA and 2′-OMe RNA synthesis

While compounds **1**–**5** all displayed acceptable kinetics of sulfur transfer in DNA synthesis, only DDTT (**1**) was considered to be of practical and commercial value due to the ease and the low cost of its preparation. Accordingly, the protocols employing compound **1** as the sulfur transfer reagent in oligonucleotide synthesis on solid-phase were optimized with respect to the concentration and excess of **1**, the contact time, and the nature of the solvent. The following oligonucleotides were selected as model compounds:

DMT-T_{10} all PS (**22**);
DMT-d(5′-TGTGAGTACCACTGATTC-3′) all PS (**23**);
DMT-d(5′-TAGTGAAGTACACTATGATGT-3′) all PS (**24**);
DMT-(5′-$U^{OMe}G^{OMe}U^{OMe}G^{OMe}A^{OMe}$dGTdAdCdCdAdCTG^{OMe} $A^{OMe}U^{OMe}U^{OMe}C^{OMe}$-3′) all PS (**25**).[20]

The oligonucleotides were synthesized by the phosphoramidite method on a 0.2 and a 1.0 μmol scale using the standard protocol of the chain assembly, 0.02–0.1 M solutions of **1** in the appropriate mixtures of MeCN and pyridine (see below), and 0.5, 1, 2, or 2.5 min sulfurization time in each synthetic cycle. To minimize the negative impact of basic deprotection on the PO/PS ratio, the solid-phase-bound material was released and the protecting groups were removed with concentrated aqueous ammonium hydroxide under the conditions described previously.[22] Upon evaporation, the crude oligonucleotide phosphorothioates were analyzed by reverse-phase HPLC and ESMS.

The results showed that, in the solid-phase synthesis of the oligonucleotide phosphorothioates by the phosphoramidite method on a small scale, DDTT (**1**) can be used efficiently in a 5–6 molar excess as a 0.1 M, 0.05 M, 0.03 M, and 0.02 M solution in an appropriate aprotic solvent (see below) using a contact time of 30 s, 1 min, 2 min, and 2.5 min, respectively. Under these optimized conditions, the efficiency of the sulfur transfer reaction for the oligodeoxynucleotides **22**–**24** and the 2′-*O*-Me analog **25** was greater than 99.8% per step (total PO/PS ratio greater than 2:98) while the ESMS and the RP HPLC profiles of the products were indistinguishable from those obtained using EDITH[8] as a sulfur transfer reagent.

Several measures aimed at the reduction of the extent of PO formation are worth noting:

1. Regardless of the sulfurization agent used, the presence of water in the reaction mixtures increased the degree of oxidation thus leading to higher PO/PS ratios. The use of anhydrous solvents for the preparation of the solutions of DDTT (**1**) was most preferable.
2. It is known from the literature that the sulfurization step is best performed prior to the capping step.[21] Indeed, addition of 10% (v/v) of the capping reagent or 0.01 M Ac_2O to 0.05 M solution of compound **1** in pyridine-THF (20:80) resulted in the formation of oligonucleotides containing up to 20% of PO linkages. Similarly, when the capping mixture was not removed from synthesis columns by an excessive wash with MeCN prior to the delivery of the sulfurizing agent, an increased PO/PS ratio was observed.
3. The use of bases stronger than pyridine (2-picoline, 2,6-lutidine, 2,4,6-collidine, and 1-methylimidazole) as co-solvents for **1** resulted in increased PO/PS ratios and thus is not recommended.

2.5. The use of DDTT (1) as the sulfurizing agents in RNA synthesis

The usefulness of **1** in the synthesis of full-length RNA phosphorothioates was evaluated using the RNA sequences **26** and **27**.

UGU GAG UAC CAC UGA UUC phosphorothioate (**26**);
GAG UAG CAG GAG AAG GAU phosphorothioate (**27**).

The oligonucleotides **26** and **27** were assembled using 2′-*O*-*t*BDMS-protected RNA phosphoramidites and a 0.05 M or a 0.1 M solution of **1** in pyridine, with a contact time of 1, 2, and 4 min. Anion-exchange HPLC analysis of the crude deprotected products showed that, under less than optimal conditions, the incomplete sulfurization of the internucleosidic linkages resulting in the formation of shorter oligonucleotides after the final deprotection was the complication more often observed than that of the formation of PO side products. Therefore, the success of sulfurization is best described in terms of the yield of the full-length product rather than of the PO/PS ratio. Under the optimized conditions (0.05 M solution of **1**, 2 min contact time), a yield of the full-PS RNA **26** greater than 90% (>99.4% per step) was obtained. The RNA sequence **27** containing a high content of purine nucleotide residues was more difficult to sulfurize irrespective of whether EDITH[8] or compound **1** was used. To obtain a high degree of sulfurization in this instance, a 0.1 M solution of **1** and a 4 min contact time was required.

2.6. Stability and solubility of DDTT (1) in solution

The solubility of DDTT (**1**) in mixtures of anhydrous pyridine and MeCN or THF is relatively limited and increases with an increasing concentration of pyridine. Some useful compositions are listed in Table 1.

Under these conditions, **1** forms stable solutions that do not display any precipitation of the reagent for a period of over 6 months. To prepare solutions of the desired concentration, **1** was first dissolved in the calculated amount of pyridine, which may necessitate using mild heat followed by diluting the resulting solution with the required volume of MeCN or THF.

Stability studies were carried out by keeping the 0.02 M solution of **1** in anhydrous pyridine-MeCN (20:80) at 25 °C. Every two weeks, the solution was installed on the synthesizer and was used as the sulfurizing reagent in the preparation of the oligonucleotide **22**. Upon completion of the synthesis, the solid-phase-bound material was released with concentrated aqueous ammonium hydroxide for 2 h, the solution was evaporated, and the crude oligonucleotide obtained was detritylated and analyzed by ion-exchange HPLC. Comparison of the HPLC traces showed no change in the activity of **1** over a period of 6 months, at which point the experiment was terminated.

Table 1
Solubility of DDTT (**1**) in Organic Solvents

Concentration of **1** (M)	Solvents and their Ratio (v/v)	
	Py–MeCN	Py–THF
0.1	100:0	40:60
0.06	50:50	—
0.05	40:60	20:80
0.03	30:70	—
0.02	20:80	0:100

3. Conclusions

Compounds **1**–**5** demonstrated excellent properties as efficient sulfurizing agents for DNA synthesis. Among these, DDTT (**1**) proved to be particularly useful. The compound afforded excellent yields and kinetics of sulfurization in routine DNA and RNA synthesis, was stable in solution for at least 6 months, and was readily prepared on large scale using simple chemical methods.

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12. Dimethylformamide dimethylacetal (154.2 g, 1.29 mol) was added dropwise to a stirred solution of 5-amino-3*H*-1,2,4-dithiazole-5-thione (185.2 g, 1.23 mol) in DMF (700 mL) over 30 min at 20–25 °C. The reaction mixture was stirred at room temperature for 5 h and ether (600 mL) was slowly added. The precipitate was filtered off, washed with ether (3 × 200 mL), and dried in vacuo to give compound **1** (213.9 g, 84.5%) as a lemon-yellow crystalline material, mp 178–179 °C (dioxane). ^{1}H NMR (DMSO-d_6): δ 8.72 (1H, m), 3.31 (3H, d, J = 0.6 Hz), 3.16 (3H, d, J = 0.6 Hz). ^{13}C NMR (DMSO-d_6): δ 209.36, 191.89, 160.01, 41.84, 36.14. Anal. Found: C, 28.91; H, 3.41; N, 20.45; S, 47.20. Calcd for $C_5H_7N_3S_3$: C, 29.25; H, 3.44; N, 20.47; S, 46.85.
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16. A mixture of **16** (2.23 g, 3.0 mmol), **18** (1.021 g, 3.0 mmol), and 1*H*-tetrazole (0.4 M in MeCN, 15.0 mL) was stirred for 45 min. Saturated aqueous $NaHCO_3$ (30 mL) was added and the mixture was extracted with CH_2Cl_2 (3 × 75 mL). The extracts were dried over Na_2SO_4 and evaporated in vacuo and the residual oil was dried on an oil pump. A portion of the material obtained (2.36 g, 2.40 mmol) was dissolved in pyridine (15 mL) and treated with **1** (0.59 g, 2.88 mmol) at room temperature. The reaction was monitored by ^{31}P NMR and found to be complete in 10 min. Acetic acid (1.14 g, 19 mmol) and hydrazine hydrate (380 mg, 7.6 mmol) were added, and the reaction mixture was left overnight. Saturated aqueous $NaHCO_3$ (100 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 × 75 mL). The extracts were dried over Na_2SO_4 and evaporated in vacuo, and the residual oil was dried on an oil pump to give 1.74 g (99%) of the crude **20**. ^{31}P NMR, (CD_3CN–Py-d_5): δ 70.87 (**20a**, R_p, 45.2%); 70.80 (**20b**, S_p, 54.1%); 1.69 (P═O, 0.7%). An aliquot of this mixture was dissolved in 30% aqueous MeCN and analyzed by reverse-phase HPLC on a Waters Exterra C18 column, 3.5 μm (4.6 × 100 mm) using 0.05 M aqueous NH_4OAc as Buffer A, MeCN as Buffer B, and a linear gradient from 30% to 60% B over a period of 30 min at a flow rate 1.0 mL/min. The R_p and S_p diastereomers **20a** and **20b** were eluted at 28.6 and 29.12 min, respectively. The P═O dimer (0.7%) was eluted at a retention time of 26.5 min. Compound **20a**, ^{31}P NMR ($CDCl_3$): δ 65.49. Compound **20b**, ^{31}P NMR ($CDCl_3$): δ 65.04.
17. *R_p-Thiothymidylyl-(3′ → 5′)-thymidine* (**21a**),[19] ^{1}H NMR (D_2O): δ 7.70 (1H, d, J = 0.9 Hz), 7.63 (1H, d, J = 0.9 Hz), 6.29 (1H, t, J = 6.7 Hz), 6.18 (1H, t, J = 6.9 Hz), 4.94 (1H, m), 4.57 (1H, m), 4.15 (4H, m), 3.85 (1H, dd, J = 3.0 and 12.6 Hz), 3.79 (1H, dd, J = 3.0, and 12.6 Hz), 2.54 (1H, ddd, J = 3.6, 6.0, 14.1 Hz), 2.34 (3H, m), 1.92 (3H, d, J = 0.9 Hz), 1.86 (3H, d, J = 0.9 Hz).^{13}C NMR (D_2O): δ 168.82, 168.73, 154.12, 153.94, 139.86, 139.81, 114.15, 114.0, 88.19 (d, J_{CP} = 10.0 Hz), 87.77, 87.74, 87.62, 87.55, 78.13 (d, J_{CP} = 6.8 Hz), 73.31, 67.64 (d, J_{CP} = 8.0 Hz), 63.53, 41.49, 40.18 (d, J_{CP} = 4.2 Hz), 14.50, 14.36. ^{31}P NMR (D_2O): δ 56.65.
18. *S_p-Thiothymidylyl-(3′ → 5′)-thymidine* (**21b**),[19] ^{1}H NMR (D_2O): δ 7.72 (1H, s), 7.65 (1H, s), 6.31 (1H, t, J = 6.7 Hz), 6.21 (1H, t, J = 6.7 Hz), 4.95 (1H, m), 4.59 (1H, m), 4.17 (4H, m), 3.84 (1H, dd, J = 4.2, and 12.4 Hz), 3.79 (1H, dd, J = 4.2, and 12.4 Hz), 2.55 (1H, m), 2.39 (3H, m), 1.92 (3H, s), 1.86 (3H, s). ^{13}C NMR (D_2O): δ 168.87, 168.77, 154.16, 154.00, 139.88, 139.86, 114.13, 114.01, 88.1 (d, J_{CP} = 10.0 Hz), 87.77, 87.61, 87.56, 77.80 (d, J_{CP} = 8.4 Hz), 73.27, 68.0 (d, J_{CP} = 7.6 Hz), 63.56, 41.49, 40.43, 14.48, 14.36. ^{31}P NMR (D_2O): δ 56.28.
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