Organic Process Research & Development **2000**, *4*, 194–198

Large-Scale Synthesis of Oligonucleotide Phosphorothioates Using 3-Amino-1,2,4-dithiazole-5-thione as an Efficient Sulfur-Transfer Reagent

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Abstract:

A commercially available and inexpensive compound, 3-amino-1,2,4-dithiazole-5-thione (ADTT), is discovered to be a new sulfur-transfer reagent for solid-phase synthesis of oligonucleotide phosphorothioates via the phosphoramidite method. The efficiency of ADTT was investigated by solid-phase syntheses of dinucleotide and oligonucleotide phosphorothioates. The results show that ADTT is a highly efficient sulfur-transfer reagent and fully compatible with automated solid-phase synthesis. ADTT has been applied in manufacture of oligonucleotide phosphorothioates to reduce the cost significantly.

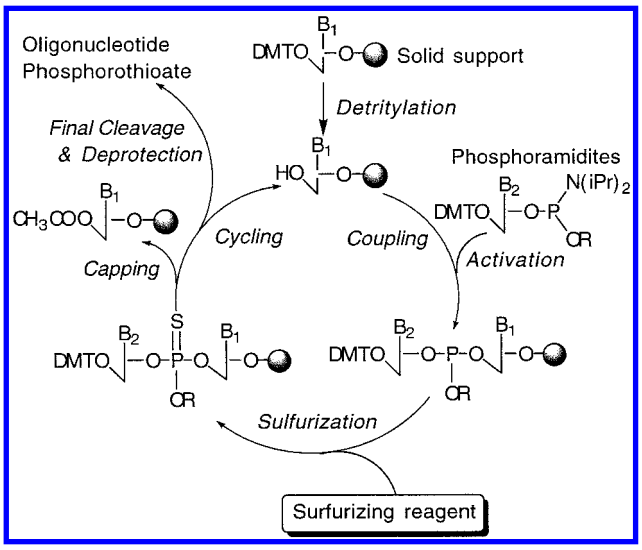
Introduction

As the first generation of modified oligonucleotides, phosphorothioate analogues are of considerable interest in nucleic acid research and are among the most promising analogues tested as oligonucleotide therapeutics.[3−6] In the second generation of antisense oligonucleotides under clinical trials or under development, full-length phosphorothioate backbone or portions of the phosphorothioate linkages have also been preserved.[7,8] Therefore, the major task of the current commercial-scale production of oligonucleotides is still the synthesis of phosphorothioate analogues.

The automated solid-phase approach dominates the oligonucleotide synthesis because of its flexibility with regard to different sequences. The automated solid-phase synthesis of phosphorothioates can be achieved using the H-phosphonate or phosphoramidite method. On the basis of 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. The solid phase synthesis of oligonucleotide phosphorothioates can now be routinely carried out at scales of 10−100 mmol.

The phosphoramidite method requires stepwise sulfurization to be carried out after each coupling (Scheme 1). It is imperative that an efficient sulfur-transfer reagent is used

Scheme 1



for the synthesis of oligonucleotide phosphorothioates via this approach. A number of reagents have been reported in recent years, which include phenylacetyl disulfide (PADS),[9] 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent),[10] tetraethylthiuram disulfide (TETD),[11] dibenzoyl tetrasulfide,[12] bis-(*O*,*O*-diisopropoxyphosphinothioyl) disulfide (S-Tetra),[13] benzyltriethylammonium tetrathiomolybdate (BTTM),[14] bis-(*p*-toluenesulfonyl) disulfide,[15] 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH),[16] 1,2,4-dithiazolidine-3,5-dione (DTSNH),[16] bis(ethoxythiocarbonyl)tetrasulfide,[17] and 3-methyl-1,2,4-dithiazolin-5-one (MEDITH).[18]

Of these compounds, Beaucage reagent has been widely used, but its synthesis and stability are not optimal. In addition, the by-product formed from Beaucage reagent during sulfurization, 3*H*-2,1-benzoxathiolan-3-one-1-oxide, is a potential oxidizing reagent that can lead to undesired

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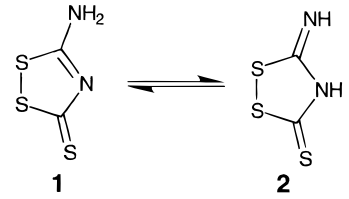
 10.1021/op990092g CCC: $19.00
Published on Web 02/10/2000

Scheme 2



phosphodiester linkages under certain conditions. Although these problems can be circumvented by using the freshly prepared solution and a shorter reagent contact time, the cost of Beaucage reagent has been a major issue in scale up of oligonucleotide phosphorothioates. On the other hand, EDITH has been considered as an advantageous alternative to Beaucage reagent and has been used in large-scale synthesis. However, its use has not offered much benefit in cost reduction. It is known that more than 80% of the bulk cost of oligonucleotide phosphorothioate production comes from raw materials, in which Beaucage reagent, solid support (CPG or Pharmacia Biotech polymer support), and nucleoside phosphoramidites have been the three major costs in raw materials. To support the increasing demand for preclinical and clinical trials as well as for future commercialization of oligonucleotide phosphorothioates, considerable efforts have been focused on the development of new reagents to further reduce costs. Herein we report our studies on a new sulfur-transfer reagent, 3-amino-1,2,4-dithiazole-5-thione (ADTT).[19]

Results and Discussion

The compound, 3-amino-1,2,4-dithiazole-5-thione (ADTT) or xanthane hydride, was first prepared by Wohler as long ago as 1821.[20] There exist two main tautomeric structures as shown in Scheme 2, in which structure **1** has been reported as the predominant form based on X-ray analysis[21] as well as NMR (^{15}N and ^{13}C) and IR spectroscopies.[22]

ADTT is commercially available from several chemical companies in bulk quantity, which has been used in the rubber industry as vulcanization reagents. However, to the best of our knowledge, it has not been applied in the synthesis of oligonucleotide phosphorothioates. In the process to find new sulfur-transfer reagents for large-scale synthesis of oligonucleotide phosphorothioates, we were especially interested in ADTT for three reasons. First, ADTT features a bisulfide-containing five-membered heterocycle with a thiocarbonyl group. On the basis of favorable kinetics and effectiveness demonstrated by the family of other bisulfide-containing five-membered heterocycles such as EDITH[16] and MEDITH,[18] it is evident that ADTT should be studied as a sulfur-transfer reagent. Second, it is a commercial product that is inexpensive and obtained in high purity (>98%). Third, it contains an amino group that provides a site for further modification as well as attachment on solid supports.[23]

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(23) The solid-supported sulfur-transfer reagent prepared from ADTT is the subject of another paper.

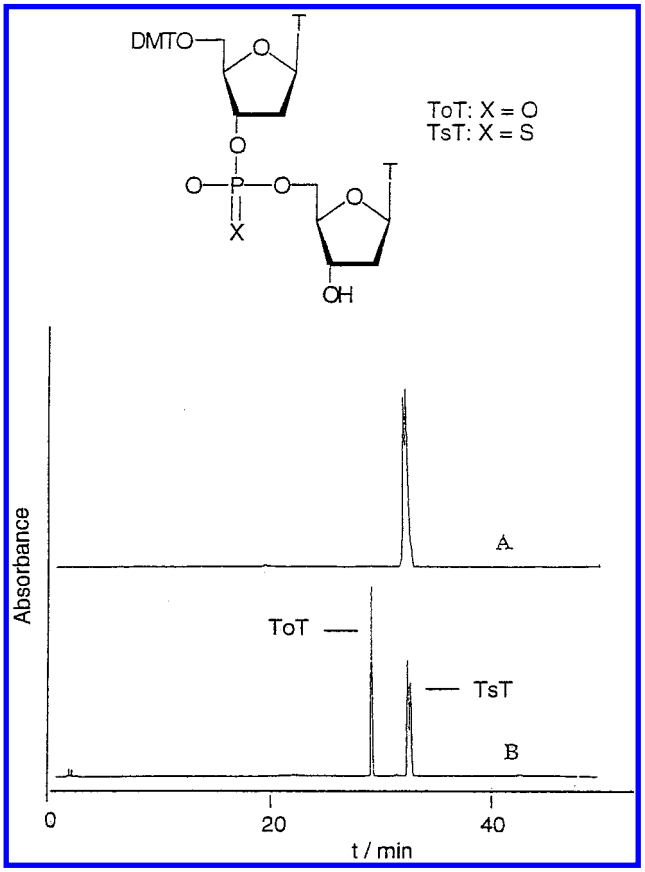

Figure 1. **The reverse-phase HPLC analyses of dinucleotide phosphorothioate (TsT) and phosphodiester (T_OT). (A) Sulfurization was carried out using 4 equiv of ADTT for 1 min; (B) co-injection of the dinucleotide phosphodiester (T_OT) and phosphorothioate (TsT).**

ADTT can be dissolved in acetonitrile to form a 0.01 M solution. The solubility increases with the addition of pyridine. ADTT is stable in acetonitrile and acetonitrile−pyridine solutions. No precipitation and color change occurred during two weeks. We have found that a 0.02 M solution in acetonitrile−pyridine (9:1) is appropriate for the automated solid-phase synthesis, in which no clogging and other problems were observed during the syntheses relative to this solution.

The sulfurizing efficiency of ADTT was first checked by solid-phase synthesis of dinucleoside phosphorothioates. Synthesis of dinucleoside phosphorothioate TsT was performed on a 1.0 μmol scale. The sulfurization reaction was carried out using a 0.02 M solution of ADTT in acetonitrile−pyridine (9:1). After cleavage and deprotection, the unpurified dimers were analyzed by reverse-phase HPLC (Figure 1). A dinucleoside phosphodiester T_OT was also synthesized as an authentic sample for the HPLC analysis (Figure 1A). The efficiency of sulfurization was investigated, and the amounts of desired phosphorothioate (TsT) and undesired phosphodiester (T_OT) were determined by reverse-phase HPLC. A comparison with two other sulfurizing reagents, Beaucage reagent and EDITH, is shown in Table 1.

As Table 1 shows, ADTT is a highly efficient sulfurizing reagent under the conditions tested. A greater than 99.5% sulfurization efficiency can be achieved using only 4 equiv of ADTT within 1 min.

Scheme 3

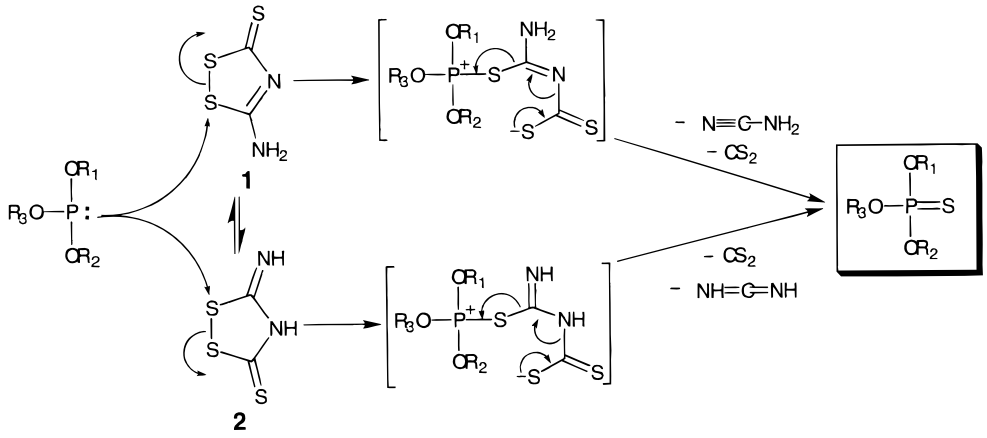

Table 1. **Sulfur-transfer efficiency of various reagents for synthesis of the dimer 5′-d(TsT)-3′**

sulfurizing reagents	concen (M)	molar equiv	solvent	reaction time (min)	T_OT[a] (%)	TsT[b] (%)
Beaucage	0.03	4	CH_3CN	1	3.22	97.78
	0.06	11		1	0.68	99.32
				5	0.92	99.08
EDITH	0.03	4	CH_3CN	1	0.41	99.59
ADTT	0.02	4	CH_3CN/ pyridine (9:1)	1	0.32	99.68
				5	0.28	99.72

[a] T_OT indicates the dimer phosphodiester. [b] TsT indicates the dimer phosphorothioate.

Table 2. **Synthesis of GEM 91 using ADTT as a sulfurizing reagent**

				IEX HPLC		CE purity (%)	
sulfurizing reagents	scale (μmol)	molar equiv	reaction time (min)	DMT-ON[a] (%)	PO[b] (%)	n[c]	$n-1$[d]
ADTT	341	4	5	81	0.28	77	2
	336	4	6.8	82	0.26	80	2
	336	2	6.8	81	0.27	77	2

[a] DMT-ON is the 25mer oligonucleotide phosphorothioate. [b] PO indicates the 25mer which has a single phosphodiester internucleotide linkage at a random position along with 23 of phosphodiester linkages. [c] n is the full-length 25mer product. [d] $n-1$ is one nucleotide deletion sequences.

To further evaluate the usefulness of ADTT as a sulfur-transfer reagent, a 25mer oligodeoxynucleotide phosphorothioate, 5′-CTCTCGCACCCATCTCTCTCCTTCT-3′ (GEM-91), was also synthesized on a 1.0 μmol scale. The synthesis was carried out under the same conditions as previously described for syntheses of the dinucleotide. A similar sulfurizing efficiency was achieved. Subsequently, the scale was increased to 336−341 μmol. After ammonolytic cleavage from CPG and deprotection, the crude oligodeoxynucleotide phosphorothioate was analyzed by ^{31}P NMR, ion-exchange HPLC and capillary gel electrophoresis. The results obtained from ion-exchange HPLC and capillary gel electrophoresis are summarized in Table 2.

^{31}P NMR analysis shows that a greater than 99.5% sulfur-transfer efficiency was achieved in the synthesis at each step under these conditions.

Table 3. **Diastereomer ratio of dimer phosphorothioates**

	Beaucage reagent		ADTT	
dimer	Rp (%)	Sp (%)	Rp (%)	Sp (%)
TT	55.10	44.90	54.97	45.03
CT	54.87	45.13	55.03	44.97
GT	49.15	50.85	49.04	50.96
AT	57.02	42.98	57.21	42.79

The proposed sulfur-transfer mechanisms for ADTT are shown in Scheme 3.

To study the stability of ADTT in solution at room temperature, we have compared the sulfurization efficiency of freshly prepared ADTT with a two-week old solution in the synthesis of the 25mer oligonucleotide phosphorothioate at the 1.0 μmol scale. The results showed that in both cases the same sulfur-transfer efficiency (>99.5%) was achieved.

Oligonucleotide phosphorothioates contain internucleotide linkages in which one of the nonbridging oxygen atoms of the phosphate group is replaced by a sulfur atom to form a chiral center on the phosphorus atom. The oligonucleotide phosphorothioates currently used in clinical trials are a mixture of P-chiral diastereomers. The biological effects of different diastereomers are still not fully understood. Since ADTT works through a different sulfur-transfer mechanism from Beaucage reagent, it is important to know whether it would change the chirality population of oligonucleotide phosphorothioates. To investigate the stereochemical ratio of Rp and Sp diastereomers, four dinucleoside phosphorothioates were synthesized as model compounds by using ADTT and Beaucage reagent. The results (Table 3) indicate that there are no significant differences in isomer ratio among four dimers synthesized from using ADTT to Beaucage reagent.

Based on these favorable results, ADTT was further utilized in the manufacture of a 18mer oligonucleotide phosphorothioate, GCGUGCCTCCTCACUGGC[24] (GEM-231), at 100 mmol scale. During sulfurization, 3.5 equiv of ADTT solution as a 0.02 M solution in acetonitrile−pyridine (9:1, v/v) was used. After synthesis, the crude oligonucleotide phosphorothioate was analyzed by ^{31}P NMR and capillary

(24) Underlining indicates the 2′-*O*-methyl nucleotides.

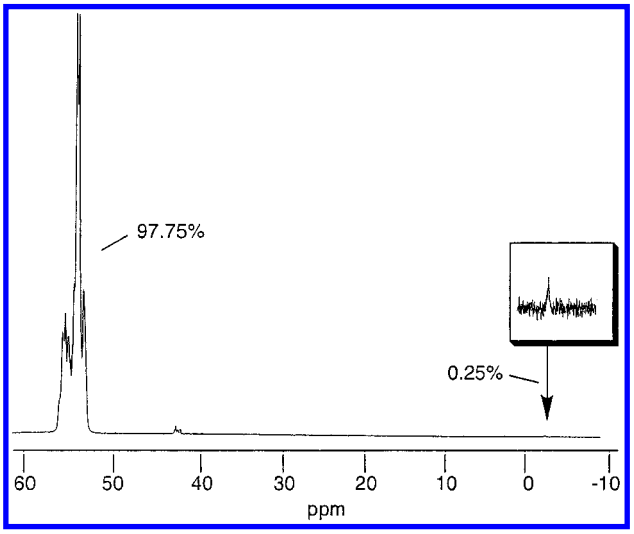

Figure 2. **Analysis of the crude 18mer oligonucleotide phosphorothioate (GEM-231) by ^{13}P NMR.**

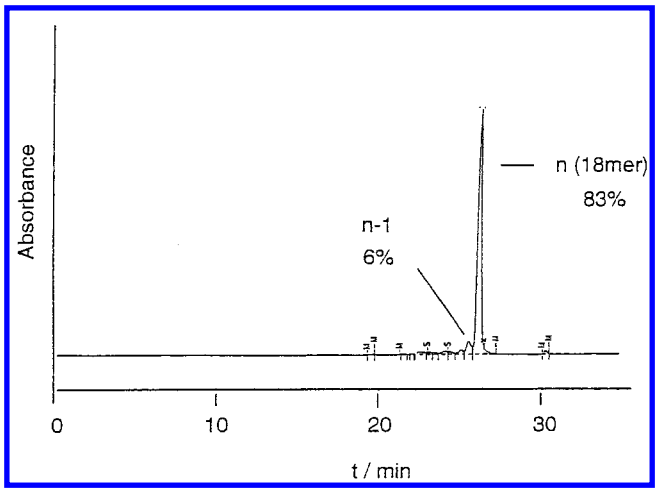

Figure 3. **Analysis of the crude 18mer oligonucleotide phosphorothioate (GEM-231) by capillary gel electrophoresis.**

gel electrophoresis (Figures 2 and 3). As Figure 2 shows, the ratio of the integrals of the signals around δ 53 ppm (phosphorothioate) and δ −2.0 ppm (phosphodiester) was 99.75/0.25. The NMR result indicates that a greater than 99.7% sulfur-transfer efficiency was achieved in the synthesis at each step under these conditions. The profile of capillary gel electrophoresis (Figure 3) shows that in the synthesis the full-length 18mer product (*n*) was obtained in 83% yield and the corresponding one nucleotide deletion sequences (*n* − 1)-mers were 6%. After purification[25] 290 g of the final product was obtained, in which 94% purity was determined by CE analysis. The ^{31}P NMR result indicated that the ratio of phosphorothioate and phosphodiester linkages was 99.97/0.03 in the final product.

Conclusions

In conclusion, we find that ADTT is a highly efficient sulfur-transfer reagent and fully compatible with standard automated oligonucleotide synthesis. ADTT has been successfully used in large-scale of manufacture of oligonucleotide phosphorothioates. Since ADTT is an inexpensive compound and commercially available from several chemical companies in bulk quantity, its use as a sulfur-transfer reagent in large-scale synthesis of oligonucleotide phosphorothioates to replace Beaucage reagent has significantly reduced the cost of raw materials.

Experimental Section

ADTT was purchased from Lancaster (Windham, NH), Crescent Chemicals (Haupauge, NY), and Maybridge (Cornwall, U.K.) and used as received. Beaucage reagent was purchased from R.I. Chemical (Orange, CA). EDITH was purchased from PerSeptive Biosystems (Framingham, MA). ^{31}P NMR spectra (121.65 MHz) were recorded on a Varian UNITY 300, and the chemical shift was correlated to 85% H_3PO_4. Reverse phase HPLC was performed on a Waters 600E pump with a Waters 440 absorbance detector and Waters 746 integrator. Ion-exchange HPLC analysis was performed on a Beckman System Gold 126 with a Beckman 166 absorbance detector on a NUCEOPAC PA-100 column (4 × 50 mm) using a linear gradient of buffer A (25 mM of Tris-HCl and 1 mM EDTA in CH_3CN, pH = 8) and buffer B (2 M NaCl and buffer A) from 100% A to 100% B over 5 min, and then maintained at 100% B for 3 min at flow rate 2 mL/min and detection at 254 nm. Capillary gel electrophoresis was performed on a Beckman P/ACE System 5010. Samples were injected for 5 s and analyzed for 40 min.

Syntheses of Dinucleoside Phosphorothioates. Dinucleoside phosphorothioates were synthesized on a 1.0 μmol scale using an automated DNA/RNA synthesizer, 8909 Expedite (PerSeptive Biosystem, Framingham, MA). The protocol "Thio 1 μmol " (Expedite software version 1.01) was used with the modifications on sulfurization steps depending on the sulfur-transfer reagent, the equivalents, and the time applied. Sulfurization was carried out under the conditions indicated in Table 1. The solid support was DMT-T-CPG (500 Å, 60 μmol/g). When the synthesis was completed, the cleavage and deprotection were carried out by treatment with concentrated ammonium hydroxide solution (1 mL/1 μmol) at 55 °C for 8 h. CPG was removed by filtration, and the ammonium hydroxide solution was dried by lyophilization. The crude product was analyzed by reverse-phase HPLC using a Nova-Pak C18 (3.9 × 150 mm) column and a linear gradient of buffer A (0.1 M ammonium acetate) and buffer B (80:20, v/v, acetonitrile:0.1 M ammonium acetate) from 90:10 to 40:60 over 40 min (flow rate 1.0 mL/min, detection at 260 nm).

The diastereomeric ratios of four dinucleoside phosphorothioates were determined by reverse-phase HPLC using 8 NV C18-4μm Radial Pak cartridge column and linear gradient of buffer A (0.1 M ammonium acetate) and buffer B (80:20, v/v, acetonitrile:0.1 M ammonium acetate) from 100:0 to 40:60 over 60 min (flow rate 1.0 mL/min, detection at 260 nm).

Syntheses of the 25mer Oligonucleotide Phosphorothioate GEM-91. When the synthesis was carried out at the 1.0 μmol scale, the same procedure was used as described in syntheses of dinucleotide phosphorothioates. The crude

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product was evaluated by ion-exchange HPLC and CE analyses.

When the synthesis was carried out at 336−341 μmol scale, an automated DNA/RNA synthesizer, OligoPilot II (Amersham Pharmacia Biotech), was used. The solid support was DMT-T-CPG (500 Å, 88 μmol/g). In each coupling step, 2 equiv of the amidite (0.2 M solution in acetonitrile) was used. After the chain assembly, a cleavage and deprotection, similar to that described in syntheses of dinucleotide phosphorothioates, proceeded. The crude products were evaluated by ion-exchange HPLC, CE, and ^{31}P NMR analyses.

Synthesis of the 18mer Oligonucleotide Phosphorothioate GEM-231. The synthesis was carried out at a 100 mmol scale using an automated synthesizer, OligoProcess (Amersham Pharmacia Biotech). A methylacrylate and ethylene glycol copolymer support[26] (90 μmol/g) was used. In each coupling step, 2 equiv of the amidite (0.2 M solution in acetonitrile) was used. After the synthesis and final cleavage as well as deprotection, a sample from the crude product was submitted to ^{31}P NMR and CE analyses. The crude product was purified by hydrophobic interaction chromatography with phenyl sepharose, followed by anion-exchange chromatography on a DEAE-5PW column. After purification, the final product was analyzed by ^{31}P NMR spectroscopy and CE.

Received for review October 18, 1999.

OP990092G

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