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A two-step sulfurization for efficient solution-phase synthesis of phosphorothioate oligonucleotides

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Abstract

A solution-phase approach for the synthesis of phosphorothioate oligonucleotides that circumvents the use of chromatographic purifications of protected phosphorothioate intermediates was developed. Implementation of a two-step sulfurization protocol in the phosphoramidite method allows efficient isolation of the intermediate phosphorothioates by extractions. The viability of this approach is demonstrated by the synthesis of a hexameric phosphorothioate oligonucleotide fragment.

Oligonucleotides are of interest as (potential) medicines and as tools in molecular biology and diagnostics.[1–7] This holds true especially for phosphorothioate (PS) oligonucleotides, in which one of the non-bridging oxygen atoms of the internucleotidic phosphate group is replaced by sulfur.[8,9] Various PS-oligonucleotides are currently in different stages of clinical trials as therapeutics for a range of diseases including cancer, cardiovascular diseases, autoimmune diseases, diabetes and infectious diseases.[10–14] The therapeutic and diagnostic potential of oligonucleotides has stimulated research on improved methods of oligonucleotide synthesis. The solid-phase methods by which oligonucleotides are currently synthesized produce oligonucleotides of high quality but in small quantities. Major limitations of solid-phase methodologies are the unpredictable results in terms of yield and purity after scaling up the synthesis of a specific sequence, the use of relatively large excesses of expensive reagents and high cost of the resins. A solution-phase approach may overcome these limitations, provided that time-consuming and expensive chromatographic purifications of intermediates can be avoided. Recently, we described an efficient solution-phase protocol for the synthesis of a hexameric oligonucleotide fragment, in which chromatographic purifications of protected oligonucleotide intermediates were replaced by extractions.[15] Other features of this method include the use of standard phosphoramidite chemistry with commercially available phosphoramidite monomers (except for thymidine), application of lipophilic protecting groups (i.e., an adamantane ester at the 3′-terminal nucleoside and the pivaloyloxymethyl (Pom) protective group at N^3 of thymidine) and HPLC-monitoring of the elongation procedure. Successful application of our solution-phase oligonucleotide synthesis protocol to the preparation of PS-oligonucleotides requires the availability of a suitable sulfurization procedure. The required sulfur transfer reagent should both sulfurize the phosphite triester intermediates obtained after each coupling step and convert the excess of monomeric building block derivatives, predominantly present as hydrogen phosphonates, into more polar diesters to facilitate their extractive removal.

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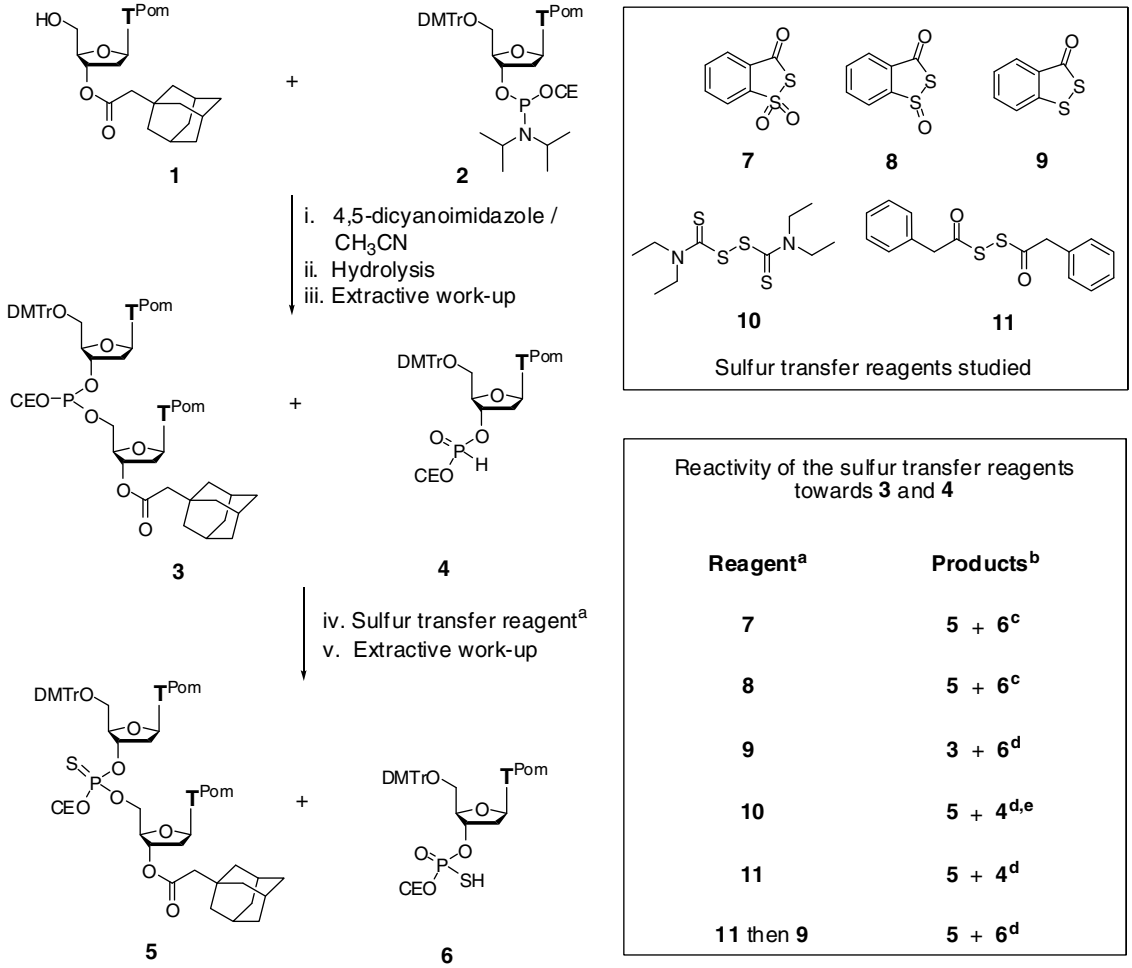
Moreover, any excess of the sulfur transfer reagent and the corresponding desulfurized reaction products should be removable by a simple extraction procedure.

Of the numerous sulfurizing reagents described in the literature[16–25] we selected 3*H*-1,2-benzodithiol-3-one 1,1-dioxide[16] (Beaucage reagent, **7**), 3*H*-1,2-benzodithiol-3-one 1-oxide[17] (**8**), 3*H*-1,2-benzodithiol-3-one[17] (**9**), tetraethylthuram disulfide[18,19] (TETD, **10**) and phenylacetyl disulfide[20,21] (PADS, **11**), and studied their efficiency in sulfurizing both phosphites and H-phosphonates that are present in solution after a condensation cycle (Scheme 1). These reagents were selected because they are known to facilitate the transformation of either phosphites or H-phosphonates into their sulfur derivatives under mild conditions. Initially, our attention was directed to the Beaucage reagent (**7**), the most widely used reagent for the solid-phase synthesis of PS-oligonucleotides.[16] To evaluate the properties of this reagent in our protocol (Scheme 1), a mixture of 3′-adamantylacetyl-N^3-pivaloyloxymethylthymidine **1** (1.0 equiv) and N^3-pivaloyloxymethylated thymidine phosphoramidite **2** (1.5 equiv) was treated with 4,5-dicyanoimidazole (DCI) (4.5 equiv). After complete consumption of **1**, as judged by HPLC, the excess of **2** was hydrolyzed by the addition of water to give H-phosphonate **4**. The mixture of phosphite **3** and H-phosphonate **4**, obtained after evaporation of the solvent, was treated with Beaucage reagent (**7**, 2.25 equiv) in 2% water/pyridine.[16] Analysis of the reaction mixture by ^{31}P NMR showed that phosphite **3** as well as H-phosphonate **4** was converted to a large extent into the corresponding phosphorothioate derivatives **5** (67 ppm) and **6** (57 ppm), respectively. However, the presence of signals at −2 ppm indicated the formation of significant amounts (up to 11%) of oxidation products.[26] These results are in agreement with the finding that 3*H*-2,1-benzoxathiolan-3-one-1-oxide, which is generated during the sulfurization, is an oxidizing agent that can lead to the formation of undesired internucleoside phosphate linkages.[17] The use of the mono-oxidized reagent **8** gave similar results as the Beaucage reagent (**7**). Stawinski and co-workers reported that compound **9** is a suitable reagent for the conversion of H-phosphonates into H-phosphonothioates.[17] Pilot experiments towards the sulfurization of trimethyl phosphite suggested that this reagent may also be suitable for the sulfurization of



Reactivity of the sulfur transfer reagents towards **3** and **4**

Reagent[a]	Products[b]
7	**5** + **6**[c]
8	**5** + **6**[c]
9	**3** + **6**[d]
10	**5** + **4**[d,e]
11	**5** + **4**[d]
11 then **9**	**5** + **6**[d]

Scheme 1. Sulfurization strategy for phosphorothioates synthesis compatible with extractive purification. (a) Evaluated sulfur transfer reagents; (b) judged by ^{31}P NMR analysis; (c) 2.25 equiv sulfur transfer reagent, 2% H_2O/pyridine, 30 min, approximately 11% of oxidized by-product[26] was detected; (d) 1.5 equiv sulfur transfer reagent, dry CH_3CN, 30 min; (e) approximately 2% of oxidized by-product[26] was detected.

phosphites. However, when **9** was used in our protocol only H-phosphonate **4** was completely sulfurized to give **6** as shown by ^{31}P NMR analysis, whereas the phosphite triester **3** remained intact. Next, the mixture of phosphite **3** and H-phosphonate **4** in dry acetonitrile (ACN) was treated with TETD (**10**) to reveal, after 30 min, that **3** was completely converted into the corresponding phosphorothioate **5**. In contrast, H-phosphonate **4** remained stable towards **10** under the applied reaction conditions (1.5 equiv **10**, dry CH_3CN, 30 min).[27] The use of **10** also led to low, but persistent amounts of oxidation products. Analogous evaluation of PADS (**11**) showed effective sulfurization of the phosphite triester **3** into phosphorothioate **5** whereas the H-phosphonate **4** remained untouched. Contrary to TEDT (**10**) the use of PADS (**11**) did not result in the formation of oxidized by-products.

The outcome of these experiments guided us to a sulfurization scheme in which both PADS (**11**) and **9** were employed to sulfurize phosphite **3** and H-phosphonate **4**, respectively.[28] To this end, a mixture of phosphite triester **3** and H-phosphonate **4** (Fig. 1A) was treated with an excess (1.5 equiv) of **11** in dry ACN. ^{31}P NMR analysis after 30 min showed complete conversion of **3** into phosphorothioate **5** and the presence of unreacted H-phosphonate **4** (Fig. 1B). Addition of reagent **9** (1.5 equiv) afforded, after 30 min, a mixture of the desired phosphorothioate triester **5** and the phosphorothioate diester **6** (Fig. 1C).[29]

The excess of sulfur transfer reagents was depleted by the addition of tris-(2-carboxyethyl)phosphine (TCEP) in pyridine and water (Scheme 2).[30] The initially formed thio-

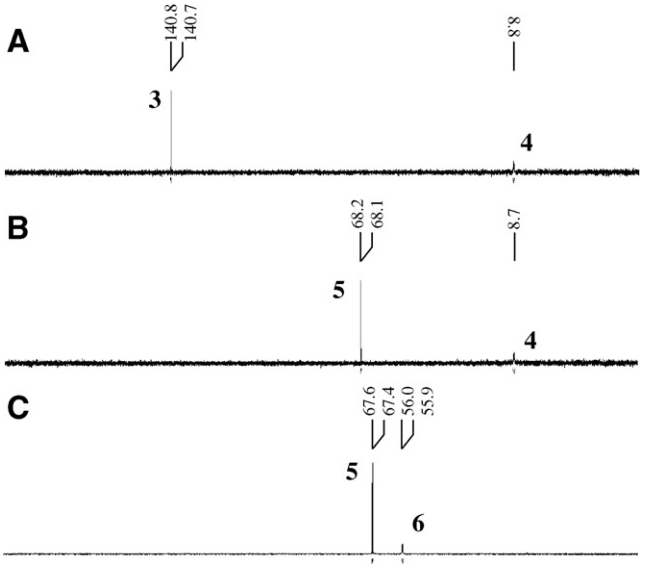
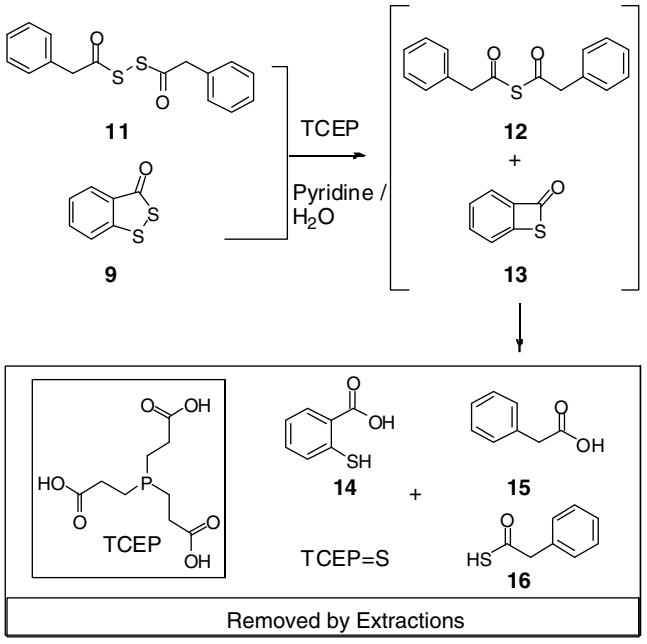


Fig. 1. ^{31}P NMR spectra of the two-step sulfurization. ^{31}P NMR (80.7 MHz, CH_3CN and H_3PO_4 as internal standard) spectra of (A) the reaction mixture after dicyanoimidazole mediated coupling and subsequent hydrolysis of the excess phosphoramidite monomer **2**, (B) the reaction mixture after the addition of PADS (**11**), (C) the reaction mixture after the sequential addition of PADS (**11**) and reagent **9**.



Scheme 2. Quenching of excess sulfur transfer reagents (putative products).[17,20]

anhydride **12** and thiolactone **13** were hydrolyzed in situ giving the water-soluble acids **14**–**16**.[17,20] Compounds **5** and **6** were isolated after extractive work-up involving dilution of the reaction mixture with EtOAc/THF (5/2 v/v) and extraction with water, 10% $KHSO_4$ (to remove pyridine) and 10% $NaHCO_3$ [to remove DCI, reagent side products originating from the sulfurizing agents, TCEP and tris-(2-carboxyethyl)thiophosphine (TCEP=S)].

The mixture of **5** and **6** was detritylated with 0.1 M HCl in ACN/MeOH followed by extractive work-up and further processing as described in detail in the earlier paper[16] for the preparation of regular phosphodiester oligonucleotides. The crude detritylated dinucleotide was isolated in >95% yield. Analysis by mass spectrometry, HPLC and ^{31}P NMR (Fig. 1S, Supplementary data) indicated the presence of trace amounts of H-phosphonate **4** (<5%).

The applicability of the two-step sulfurization protocol is illustrated by the assembly of the hexameric oligothioate **17** ($^{HO}G_{ps}A_{ps}C_{ps}$ $G_{ps}T_{ps}T_{OH}$), representing the minimal sequence able to activate Toll-like receptor 9 in mice.[31]

The synthesis started from monomer **1** at 0.5 mmol scale and after five elongation cycles, crude PS-hexamer $^{HO}G^{iBu}A^{Bz}C^{Bz}G^{iBu}T^{Pom}T^{Pom}_{OH}$ was obtained in 67% overall yield (based on **1**). After ammonolysis of this hexamer, HPLC showed one major product (**17**, Fig. 2A). The mixture was subjected to preparative HPLC purification using triethylammonium acetate buffer at 60 °C.[32] The purified PS-hexanucleotide **17** was obtained in 31% overall yield (based on **1**, Fig. 2B).[33]

The identity and homogeneity of purified hexamer **17** were confirmed by LC–MS,[34,35] analytical HPLC analysis, ^{31}P NMR (Fig. 3) and MALDI TOF mass spectrometry (found: 1886.67, expected: 1886.23). Moreover, **17** was

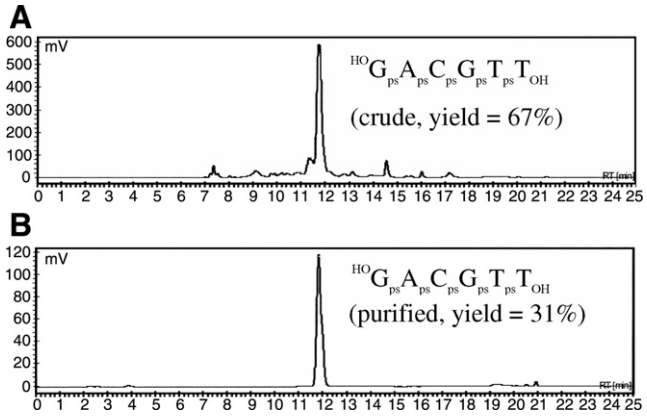


Fig. 2. HPLC trace (254 nm) of: (A) crude PS-hexamer **17**, (B) purified **17**.

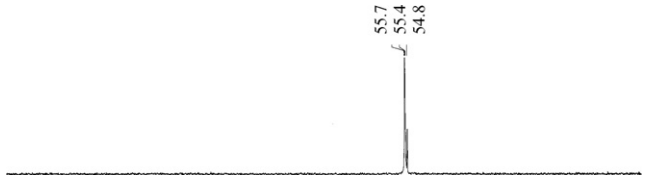


Fig. 3. ^{31}P NMR (80.7 MHz, CH_3CN and H_3PO_4 as internal standard) spectrum of purified PS-hexamer **17** ($^{HO}G_{ps}A_{ps}C_{ps}G_{ps}T_{ps}T_{OH}$).

identical (HPLC, MS) with the same hexamer prepared via standard solid-phase synthesis.

In conclusion, the sequential use of phenylacetyl disulfide (PADS, **11**) and 3*H*-1,2-benzodithiol-3-one (**9**) is an efficient procedure for the one-pot sulfurization of phosphite and H-phosphonate intermediates. Implementation of this sulfurization procedure in a solution-phase approach for the synthesis of phosphorothioate oligonucleotides allows the replacement of chromatographic purifications of protected phosphorothioate intermediates by simple extractions. This solution-phase approach was demonstrated by the synthesis of hexameric oligothioate **17** which was obtained in both good yield and purity on a 0.5 mmol scale, with no apparent formation of side products caused by oxidation.

Acknowledgements

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Supplementary data

Experimental procedures, spectroscopic and chromatographic data for all new compounds. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.03.025.

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26. The detected oxidation by-products represent the corresponding phosphates of either **3** or **4**.
27. It is reported that TEDT (**10**) is able to convert H-phosphonates into H-phosphonothioates under basic conditions (0.5 M TEDT (**10**) in carbon disulfide/triethylamine 9:1 (v/v) for 10 min).[22]
28. Attempts to oxidize H-phosphonate **4** into the extractable phosphate diester by the addition of iodine was accompanied by oxidation by-products of **5** due to desulfurization of the corresponding phosphorothioate diester linkage, which is generated from the phosphorothioate triester due to some premature loss of the 2-cyanoethyl phosphate protective group.
29. The overall reaction rate of the two-step sulfurization protocol was negatively affected in terms of yield and purity by the simultaneous addition of PADS (**11**) and **9** or the reversal of the order of addition, that is, first **9** and then **11**.
30. TCEP was sulfurized by both PADS (**11**) and reagent **9**.
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32. The HPLC-purification was performed at 60 °C with triethylammonium acetate to suppress peak broadening due to P-diastereomers.
33. The yield was estimated by A_{260} absorption units using $\varepsilon = 58\,200$ L mol^{-1} cm^{-1}.
34. Application of a hexafluoroisopropanol/triethylamine buffer in MeOH/H_2O suppressed peak broadening due to P-diastereomers.
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