



An approach to the desulfurization of oligonucleotide phosphorothioates

Colin B. Reese* and Hongbin Yan

Department of Chemistry, King's College London, Strand, London WC2R 2LS, UK

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Abstract—Protected oligonucleotide phosphorothioates are converted by a two-step procedure into oligonucleotides with standard phosphodiester internucleotide linkages. The remaining protecting groups are then removed by ammonolysis. © 2003 Elsevier Science Ltd. All rights reserved.

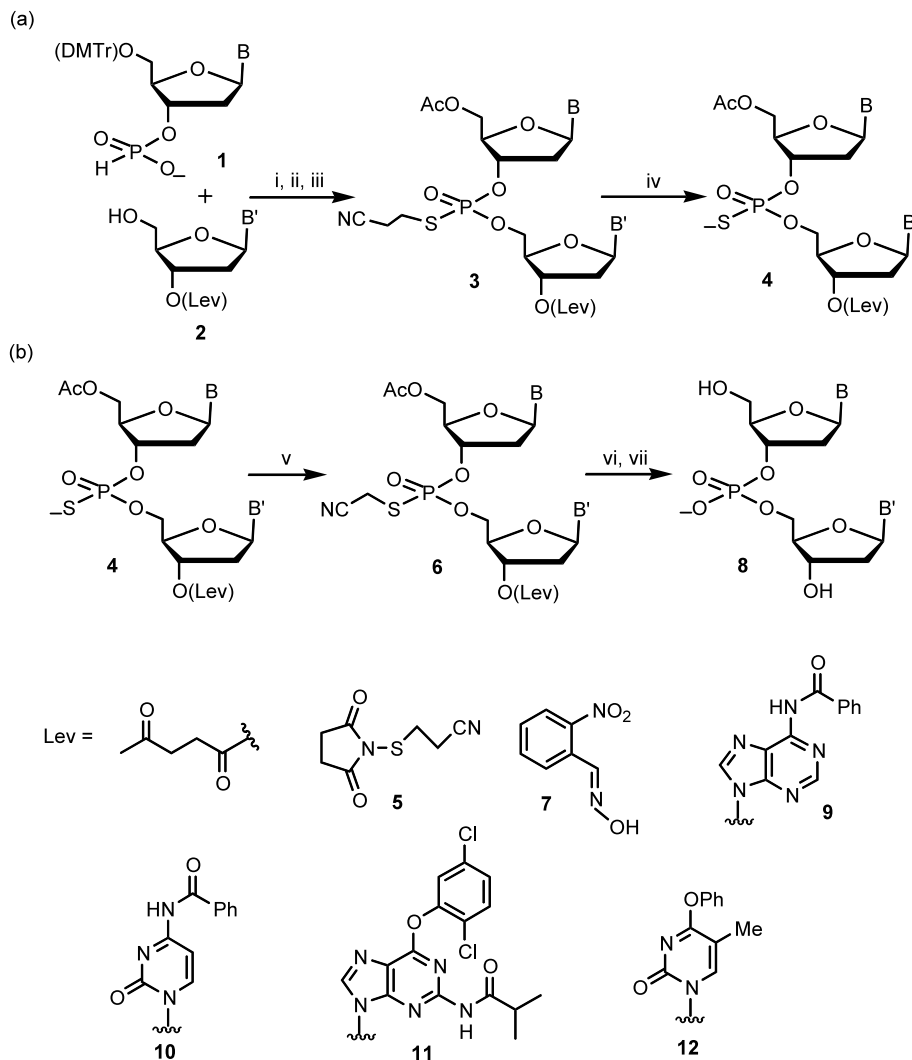
The synthesis of phosphorothioate analogues of oligonucleotides has, in recent years, become a matter of great importance in the context of the antisense approach^{1,2} to chemotherapy. Vitravene (ISIS 2922), a 21-mer oligodeoxyribonucleotide phosphorothioate, has already been licensed³ by the US Food and Drug Administration for the treatment of cytomegalovirus retinitis in AIDS patients and advanced clinical trials are being carried out on several other modified DNA sequences of this type. As potential drugs, oligonucleotide phosphorothioates have the advantage that they are not easily susceptible to digestion by phosphorolytic enzymes. However, this resistance to enzymatic digestion also has a disadvantage in that it prevents both the characterization of these oligonucleotide analogues by standard sequencing methods and the determination of their nucleoside ratios by total enzymatic digestion. With regard to both of these analytical procedures, it would be advantageous if oligonucleotide phosphorothioates could be readily and quantitatively desulfurized to give the corresponding unmodified oligonucleotides without any concomitant internucleotide cleavage. Several oxidative procedures involving, for example, iodine⁴ and potassium peroxydisulfate (oxone)⁵ have already been used to convert unprotected oligonucleotide phosphorothioates into the corresponding unmodified oligonucleotides. However, these desulfurization procedures appear to result in some accompanying internucleotide cleavage.

We have recently introduced^{6–8} a method for the solution phase synthesis of oligonucleotide phosphoro-

thioates. We now report that, following the unblocking of the internucleotide linkages, it is possible to convert the resulting partially-protected intermediates into fully-unprotected unmodified oligonucleotides as well as into oligonucleotide phosphorothioates. No detectable internucleotide cleavage occurs in the course of the desulfurization process.

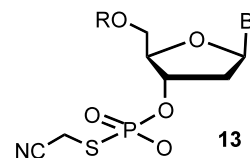
Our present approach⁸ to the synthesis of dinucleoside phosphorothioates in solution is illustrated in Scheme 1a. A 5'-protected nucleoside 3'-H-phosphonate **1** is coupled with a 3'-protected nucleoside derivative **2** in the presence of diphenyl phosphorochloridate and *N*-(2-cyanoethyl)sulfanyl)succinimide **5** in pyridine solution. Adenine, cytosine, guanine and thymine residues are protected as in **9**, **10**, **11** and **12**, respectively. At the beginning of the unblocking process, the 5'-*O*-DMTr protecting group is removed from the product and the released hydroxy function is acetylated (Scheme 1a, steps ii and iii) to give the fully-protected dinucleoside phosphorothioate **3**. The *S*-(2-cyanoethyl) protecting group is then removed by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dichloromethane solution to give a partially-protected dinucleoside phosphorothioate **4**. Following the removal of the protecting groups from the base residues and the terminal 3'- and 5'-hydroxy functions in the usual way, the fully-unblocked dinucleoside phosphorothioate is obtained. We now report that, if the desulfurized dinucleoside phosphate **8** is required, the excess DBU should first be neutralized with trifluoroacetic acid and the phosphorothioate internucleotide linkage of the intermediate **4** then alkylated with bromoacetonitrile in the presence of *N,N*-dimethylaniline (Scheme 1b, step v) to give the corresponding dinucleoside phosphoro-

* Corresponding author. Tel.: +44-20-7848-2260; fax: +44-20-7848-1771; e-mail: colin.reese@kcl.ac.uk



Scheme 1. Reagents and conditions: (i) $(\text{PhO})_2\text{POCl}$, **5**, $\text{C}_5\text{H}_5\text{N}$, rt; (ii) $\text{Cl}_2\text{CHCO}_2\text{H}$, pyrrole, CH_2Cl_2 , rt; (iii) Ac_2O , $\text{C}_5\text{H}_5\text{N}$, rt; (iv) DBU, CH_2Cl_2 , rt, 30 min; (v) (a) $\text{CF}_3\text{CO}_2\text{H}$, CH_2Cl_2 , rt, 5 min, (b) BrCH_2CN , PhNMe_2 , CH_2Cl_2 , rt, 15 h; (vi) **7**, $(\text{Me}_2\text{N})_2\text{C}=\text{NH}$ (TMG), MeCN, rt, 15 h; (vii) (a) conc. aq. NH_3 (d 0.88), 55°C , 15 h, (b) Amberlite IR 120 (Na^+) cation-exchange resin.

thioate **6** with an *S*-cyanomethyl-protected internucleotide linkage. If the appropriate stoichiometry is observed,⁹ quantitative and regiospecific *S*-alkylation of the phosphorothioate diester function occurs and no reaction takes place between bromoacetonitrile and any of the base residues.¹⁰ Treatment of the fully-protected intermediate **6** with *E*-2-nitrobenzaldoxime **7** and N^1,N^1,N^3,N^3 -tetramethylguanidine (TMG) in acetonitrile solution,¹² followed by the ammonolytic removal of the remaining protecting groups (steps vi and vii) leads solely to the unprotected dinucleoside phosphate **8**. It is clear that oximate treatment of *S*-cyanomethyl phosphorothioate triesters does not lead to internucleotide cleavage as *S*-cyanomethyl phosphorothioate diester functions **13** (or the corresponding 5'-diesters) cannot be detected in the ^{31}P NMR spectra¹³ of the desulfurized products obtained in this study. When an *S*-(2-cyanoethyl)-protected phosphorothioate (e.g. **3**) is treated directly with *E*-2-nitrobenzaldoxime **7** and TMG in acetonitrile solution, the corresponding phosphorothioate diester (e.g. **4**) is obtained as the only product.



A number of fully-protected dinucleoside phosphorothioates, trinucleoside diphosphorothioates and oligonucleoside phosphorothioates were unblocked (Table 1) by this desulfurization procedure. These transformations were carried out on a relatively small (0.05–0.088 g) scale and the percentage yields of fully-unblocked desulfurized products, which were isolated as their sodium salts, were on the whole satisfactory. However, none of these transformations has yet been optimized. The resulting products were chromatographically (HPLC) relatively homogeneous even though they were not further purified after unblocking; when they were digested¹⁴ first with *Crotalus adamanteus* snake venom

Table 1. Desulfurization of oligonucleotide phosphorothioates

Entry	Substrate ^a	Quantity desulfurized mg (μ mol)	Yield (%) ^b	t_R ^c (min)	dA:dC:dG:T ratios ^d	
					Found	Calculated
1	Ac-Cp(s)G-Lev	50 (46)	75	5.44 (A)	0:1.00:1.00:0	0:1.00:1.00:0
2	Ac-Gp(s)A-Lev	60 (49)	68	7.40 (A)	1.05:0:1.00:0	1.00:0:1.00:0
3	Ac-Ap(s)Tp(s)T-Lev	70 (49)	68	10.60 (A)	1.04:0:0:2.00	1.00:0:0:2.00
4	Ac-Cp(s)Tp(s)T-Lev	45 (33)	55	8.96 (A)	0:1.00:0:1.94	0:1.00:0:2.00
5	Ac-(6-mer)-Lev ⁸	50 (16.3)	45	9.52 (A)	0:2.00:2.00:1.97	0:2.00:2.00:2.00
6	Ac-(12-mer)-Lev ⁸	88 (15.2)	66	10.46 (A)	0:4.00:2.06:5.89	0:4.00:2.00:6.00
7	Ac-(21-mer)-Lev ⁸	87 (8.4)	56	6.72 (B)	0:6.00:5.27:10.13	0:6.00:5.00:10.00

^a The abbreviations for the substrates in entries 1–4 are explained below.¹¹ Using the same abbreviations, the substrates in entries 5–7 are Ac-Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-Lev, Ac-[Cp(s)Tp(s)Tp(s)]₃Gp(s)Cp(s)G-Lev and Ac-Gp(s)Cp(s)Gp(s)Tp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)[Cp(s)Tp(s)-Tp(s)]₃Gp(s)Cp(s)G-Lev, respectively.

^b The desulfurized oligonucleotides were isolated as their sodium salts.

^c Reversed-phase HPLC was carried out on a 250×4.6 mm Hypersil ODS 5 μ column. The column was eluted with 0.1 M triethylammonium acetate buffer (pH 7.0)–acetonitrile mixtures. The HPLC programmes used are indicated in parentheses [(A): linear programme of buffer–acetonitrile (95:5 v/v to 85:15 v/v) over 10 min and then isocratic elution; (B): linear programme of buffer–acetonitrile (95:5 v/v to 60:40 v/v) over 10 min and then isocratic elution].

^d The nucleoside ratios were determined by reversed-phase HPLC [programme (A)], following digestion of the desulfurized oligonucleotides according to the procedure indicated below.¹⁴

phosphodiesterase and then with *E. coli* alkaline phosphatase, they underwent quantitative digestion and the proportions of the constituent nucleosides obtained were in close correspondence to the calculated proportions (Table 1, final column; entries 1–7). Furthermore, satisfactory ¹H and ³¹P NMR spectra were obtained for all of the products. It is particularly noteworthy that signals in the region of 56 ppm, which may be assigned to the resonances of phosphorothioate phosphorus atoms, were either absent or very weak indeed in the ³¹P NMR spectra of all the products. The HPLC profiles of the fully-unblocked hexamer [d(CTTGCG)] and 21-mer [d(GCGTTTGCTCTTCTTCTTGCG)] (Table 1, entries 5 and 7, respectively) and their digestion products are illustrated in Figure 1; the ³¹P NMR spectra of these two oligonucleotides are illustrated in Figure 2. The detailed experimental procedure for the unblocking and desulfurization of the 21-mer (entry 7) is provided below.¹⁵

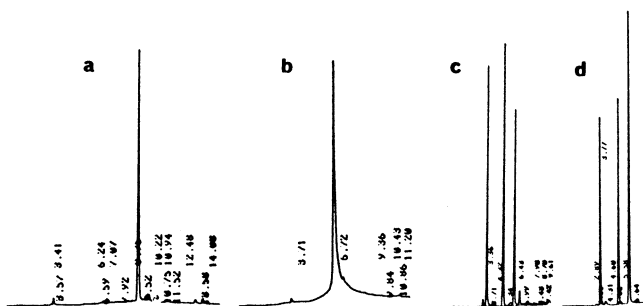


Figure 1. Reversed-phase HPLC (see Table 1) profiles of (a) d(CTTGCG), (b) d(GCGTTTGCTCTTCTTCTTGCG), (c) enzymatic digest¹⁴ of d(CTTGCG) and (d) enzymatic digest¹⁴ of d(GCGTTTGCTCTTCTTCTTGCG). In (c) and (d), the HPLC detector was set at 260 nm and the order of elution was dC, dG, dT.

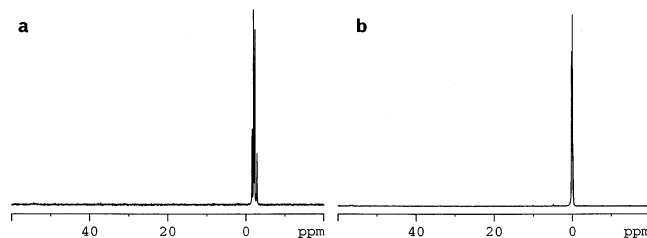


Figure 2. ³¹P NMR spectra (145.78 MHz, D₂O) of (a) d(CTTGCG) and (b) d(GCGTTTGCTCTTCTTCTTGCG).

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9. For each (2-cyanoethyl)-protected internucleotide linkage, ca. 10 mol equiv. of DBU is used (e.g. 10 mol equiv. for an *S*-(2-cyanoethyl) dinucleoside phosphorothioate and 20 mol equiv. for a bis-(2-cyanoethyl) trinucleoside diphosphorothioate). Neutralization is effected with ca. 9 mol equiv. of trifluoroacetic acid per internucleotide linkage. Alkylation is effected with ca. 3 mol equiv. each of bromoacetonitrile and *N,N*-dimethylaniline per internu-

- cleotide linkage. Both the removal of *S*-(2-cyanoethyl) and the introduction of *S*-cyanomethyl groups may be monitored by NMR spectroscopy. The phosphorus atoms in *S*-(2-cyanoethyl)-protected (e.g. **3**), unprotected (e.g. **4**) and *S*-cyanomethyl-protected (e.g. **6**) phosphorothioates resonate at ca. 28, 56 and 25 ppm, respectively.
10. Following the treatment of Ac-Cp(s)*G*-Lev¹¹ **4**; B=**10**, B'=**11** and Ac-Tp(s)*A*-Lev¹¹ **4**; B=**12**, B'=9 with bromoacetonitrile and *N,N*-dimethylaniline, it was clear from the ¹H NMR spectra of the products that no alkylation of the NH protons of the cytosine, guanine and adenine residues had occurred. It was also clear from the ³¹P NMR spectra of both products [Ac-Cp(s')*G*-Lev¹¹ **6**; B=**10**, B'=11 and Ac-Tp(s')*A*-Lev¹¹ **6**; B=**12**, B'=9] that quantitative *S*-cyanomethylation of the phosphorothioate diester internucleotide linkages had occurred.
 11. In a system of abbreviations for protected oligonucleotide phosphorothioates that we have introduced,^{6,7} nucleoside residues and internucleotide linkages are italicized if they are protected in a defined way. In the present context, *A*, *C*, *G* and *T* represent 2'-deoxyadenosine protected with a benzoyl group on *N*-6 of its adenine residue (as in **9**), 2'-deoxycytidine protected with a benzoyl group on *N*-4 of its cytosine residue (as in **10**), 2'-deoxyguanosine protected with an isobutyryl group on *N*-2 and a 2,5-dichlorophenyl group on *O*-6 of its guanine residue (as in **11**) and thymidine protected with a phenyl group on *O*-4 of its thymine residue (as in **12**), respectively; *p*(s), *p*(s') and *p*(s') represent an unprotected phosphorothioate internucleotide linkage, an *S*-(2-cyanoethyl)- and an *S*-cyanomethyl-protected phosphorothioate internucleotide linkage, respectively.
 12. For the unblocking of each *S*-cyanomethyl-protected phosphorothioate internucleotide linkage and each guanine and thymine base residue, ca. 4.5 mol equiv. of *E*-2-nitrobenzaloxime **7** and ca. 4 mol equiv. of TMG were used.
 13. 5'-*O*-DMTr-4-*O*-phenylthymidine 3'-(*S*-cyanomethyl) phosphorothioate **13**; R=DMTr, B=**12** has $\delta_p[(CD_3)_2SO]$ 10.5. The only significant signals in the ³¹P NMR spectra of the desulfurized products obtained in this study are in the region of 0 ppm.
 14. Stock enzyme solutions were prepared by dissolving *Crotalus adamanteus* snake venom phosphodiesterase (0.2–0.4 unit) and *E. coli* alkaline phosphatase (ca. 1.0 unit) separately in 0.1 M tris hydrochloride buffer (pH 8.3, 0.5 ml; 0.01 M with respect to magnesium chloride). Phosphodiesterase stock solution (14 μ l) was added to a solution of substrate (ca. 1.0 A₂₆₀ unit) in water (10 μ l), and the resulting solution was incubated at 37°C for 20 h. Alkaline phosphatase stock solution (15 μ l) was then added and incubation was continued for a further period of 20 h. The digestion products were analyzed by HPLC (see Table 1, footnote^d).
 15. DBU (0.25 ml, 1.67 mmol) was added to a stirred solution of Ac-Gp(s)*Cp*(s)*Gp*(s)*Tp*(s)*Tp*(s)*Tp*(s)*Gp*(s)*Cp*(s)-Tp(s)[*Cp*(s)*Tp*(s)*Tp*(s)]₃Gp(s)*Cp*(s)*G*-Lev (0.087 g, 8.4 μ mol) in dichloromethane (1.5 ml) at rt. After 30 min, a solution of trifluoroacetic acid (TFA) in dichloromethane (10% w/v, 1.73 ml, 1.5 mmol of TFA) was added. After 5 min, diethyl ether (40 ml) was added and the resulting precipitate was collected by centrifugation. This material was reprecipitated by diethyl ether (30 ml) from its dichloromethane (1.5 ml) solution two times, and was finally dried in vacuo; it was then dissolved in dichloromethane (1.5 ml), and *N,N*-dimethylaniline (0.106 ml, 0.84 mmol) followed by bromoacetonitrile (0.0585 ml, 0.84 mmol) were added to the stirred solution at rt. After 15 h, diethyl ether (30 ml) was added. The resulting precipitate was collected by centrifugation, redissolved in dichloromethane (1.0 ml) and reprecipitated with diethyl ether (30 ml). This material was then fractionated by short column chromatography on silica gel: the appropriate fractions, which were eluted with dichloromethane–methanol (92:8 v/v), were combined and evaporated under reduced pressure to give a colourless froth (0.063 g). *E*-2-Nitrobenzaloxime **7** (0.164 g, 0.99 mmol) and TMG (0.11 ml, 0.88 mmol) were added to a stirred solution of this material in acetonitrile (1.0 ml) and dichloromethane (1.0 ml) at rt. After 16 h, the products were concentrated under reduced pressure and the residue was heated with concentrated aq. NH₃ (*d* 0.88, 1.5 ml) at 55°C for 15 h. The cooled products were concentrated under reduced pressure and re-evaporated with absolute ethanol (2×2 ml). Ethyl acetate (40 ml) was added to a solution of the residue in methanol (3 ml). The resulting precipitate was collected by centrifugation and was reprecipitated from its methanol (3.0 ml) solution by ethyl acetate (40 ml) two further times. A solution of the resulting solid in water (4 ml) was applied to a column (3×1.5 cm diameter) of Amberlite IR 120 (Na⁺ form) cation-exchange resin. The column was eluted with water: the appropriate fractions were combined and concentrated under reduced pressure to give the sodium salt of d(GCGTTTGCTCTTCTTCTTGCG) (0.030 g, 56%) as a colourless solid.