Nucleic Acids Research, 1998, Vol. 26, No. 21 **4983–4988**

Incorporation of terminal phosphorothioates into oligonucleotides

Stefan Alefelder, Bhisma K. Patel[+] and Fritz Eckstein*

Max-Planck-Institut für experimentelle Medizin, Hermann-Rein-Straße 3, D-37075 Göttingen, Germany

Received May 22, 1998; Revised and Accepted September 14, 1998

ABSTRACT

Considerable effort has been directed towards studying the structure and function of oligonucleotides and several approaches rely on the attachment of reporter groups to oligonucleotides. We report here the introduction of 3′- and 5′-terminal phosphorothioates into heptameric oligonucleotides and their post-synthetic modification with several reporter groups. The synthesis of terminal phosphorothioates is based on the coupling of a ribonucleoside phosphoramidite at the first or last nucleotide, respectively, which, after sulphurization, is removed by sequential oxidation of the vicinal hydroxyl groups and then β-elimination. Product formation is of the order of 95%. The ratio of phosphorothioate- versus phosphate-terminated oligodeoxynucleotides as analysed by electrophoresis on a Hg^{2+} gel is in general 85/15. Examples for the reactivity of the terminal phosphorothioates for conjugation with cholesterol, bimane and for sulphydryl exchange are described.

INTRODUCTION

The conjugation of reporter groups or groups promoting cellular uptake are useful in a number of applications (1–3). The attachment of such groups onto oligonucleotides can be achieved either by their incorporation during chemical synthesis (4) or by post-synthetic modifications (5,6). In the latter case it is desirable to have an oligonucleotide with amino or sulphydryl linkers which often are commercially available as phosphoramidites and can be incorporated during oligonucleotide synthesis (7–14) or oxidative phosphoramidation with substituted amines (15). Alternatively, reactive sulphydryls in the form of terminal phosphorothioates can be introduced. These have been introduced by chemical 5′-phosphorylation with different phosphoramidites and subsequent sulphurization (16–19) or by enzymatic transfer with ATPγS and T4 polynucleotide kinase (20,21). We found it desirable to develop an alternative method to introduce terminal phosphorothioates for further derivatization. We describe here such a method consisting of the introduction of a terminal ribonucleoside phosphorothioate which upon oxidation of the vicinal hydroxyl groups and subsequent β-elimination generates the desired phosphorothioate, either on the 5′- or the 3′-terminus of the oligonucleotide. The reactivity of the terminal phosphorothioates is demonstrated by coupling of cholesterol, bromobimane and dithiodipyridine derivatives to these oligonucleotides.

RESULTS AND DISCUSSION

The use of oligonucleotides often requires functionalization at the termini for the attachment of fluorescent probes like bromobimane (8,10,13) or hydrophobic groups like cholesterol (7,9,11,12,14,15). The application of reactive sulphydryl groups in the form of terminal phosphorothioates have proven suitable for this purpose as outlined in the Introduction. Often the incorporation of terminal phosphorothioates into oligonucleotides results in poor yields or during the chemical synthesis in side products (22). As an alternative, the possibility of introducing the phosphorothioate group initially as an internucleotidic linkage by standard methods and then conversion into a phosphorothioate monoester by removal of the terminal nucleoside was explored.

The introduction of terminal phosphates into oligonucleotides by protection with a ribnucleoside and its subsequent removal by oxidation and β-elimination was first described for a trinucleotide (23). The application of this procedure to the preparation of phosphorothioates such as ATPγS and ADPβS has been described, illustrating that oxidation conditions can be found where the phosphorothioate is stable (24).

Here an adaptation of these procedures is described to synthesise terminal phosphorothioate-containing oligonucleotides. For the synthesis of a 3′-terminal phosphorothioate-containing oligodeoxynucleotide, the synthesis is started with a uridine attached to CPG and sulphurization of the internucleotidic bond after the first coupling. Subsequent $NaIO_4$ oxidation cleaves the vicinal hydroxyl group to a dialdehyde which is subjected to a β-elimination reaction to generate the terminal phosphorothioate. An example for this sequence of reactions is given in Figure 1 for the synthesis of oligodeoxynucleotide **C**. An analogous sequence of reactions is applied for the introduction of a 5′-terminal phosphorothioate as outlined for the synthesis of oligodeoxynucleotide **F** in Figure 2.

The same reaction sequence for the introduction of a phosphorothioate at the 3′-end of oligoribonucleotides did not yield the expected product. Although the mechanism for the interfering

*To whom correspondence should be addressed. Tel: +49 551 3899 274; Fax: +49 551 3899 388; Email: eckstein@mail.mpiem.gwdg.de

[+]Present address: Department of Chemistry, Indian Institute of Technology, Guwahati-781 001, India

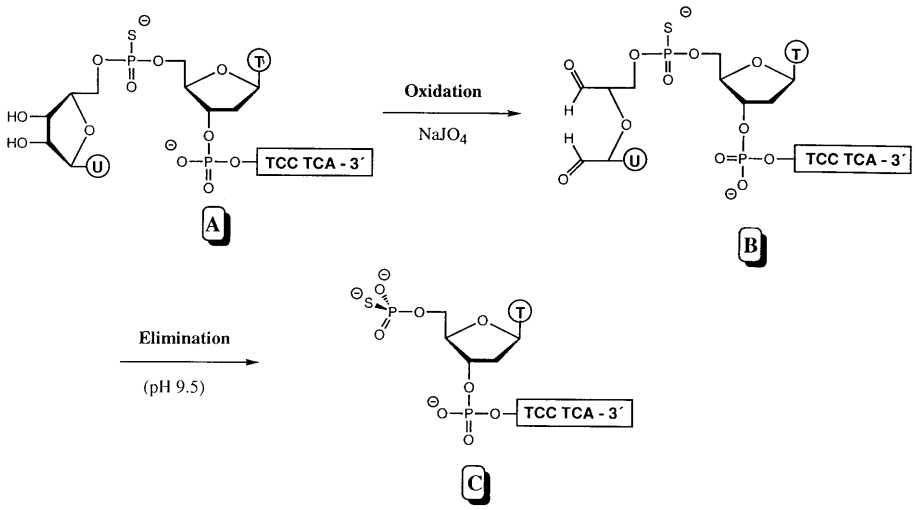

Figure 1. Synthesis of oligodeoxynucleotide 5′-phosphorothioate **C**.

reaction has not been elucidated, it is conceivable that the sulphur of the phosphorothioate adds to the β-elimination product, resulting in an oligonucleotide terminating with a 3′-phosphorothioate-S-ester (Fig. 3, **III**). This is analogous to the addition of thiophosphoric acid to acrylonitrile (25). Compound **III** would be subject to a phosphoryl transfer by nucleophilic attack of the 2′-OH group resulting in a terminal nucleoside 2′,3′-cyclic phosphate (Fig. 3, **IV** and **V**). There is precedence for the cyclisation reaction as it has been shown that nucleoside 3′-phosphorothioate-S-esters readily lead to nucleoside 2′,3′-cyclic phosphates as the S-esters are excellent leaving groups (23).

To prevent this undesired reaction in the synthesis of oligoribonucleotide 3′-phosphorothioate, a deoxynucleoside phosphoramidite was coupled to the penultimate position of the oligoribonucleotide as shown for the synthesis of **I** (Fig. 4). Thus the oligoribonucleotide has a deoxynucleotide at the 3′-terminal position which is generally not expected to interfere with application of such an oligonucleotide.

These reaction sequences proceed with good yields. The compounds give the calculated values in mass spectrometry and the ^{31}P NMR spectra show the expected distribution between phosphate and phosphorothioate signals (Fig. 5 gives an example). The ratio of phosphorothioate- and phosphate-terminated oligonucleotides was quantified by gel electrophoresis on a Hg^{2+} gel after ^{32}P-labelling (20). In this analysis the phosphorothioate-terminated oligonucleotides, in contrast to the phosphate-terminated ones, do not migrate in the gel because of their interaction with the Hg^{2+}. This separation allows for easy quantification. The ratio of phosphorothioate versus phosphate for the oligonucleotides prepared here was in general 85/15. The small content of phosphate presumably derives from some loss of sulphur during the oxidation step. The reactivity of an internucleotidic phosphorothioate group towards $NaIO_4$ oxidation under the conditions employed here was investigated with Tp(S)T. Judging by HPLC analysis ~15% reaction to TpT was observed. Thus, if the introduction of a terminal phosphorothioate group is carried out with an oligonucleotide containing phosphorothioate internucleotidic linkages a small loss of sulphur will have to be expected.

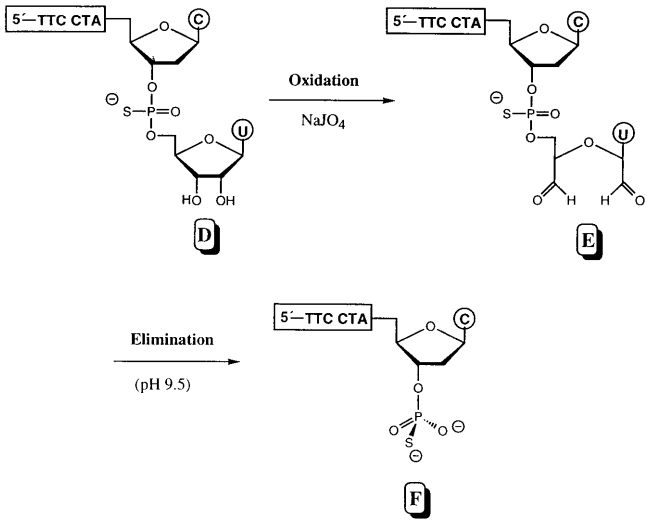

Figure 2. Synthesis of oligodeoxynucleotide 3′-phosphorothioate **F**.

The terminal phosphorothioates are phosphorothioate monoesters and as such react readily with thiophilic moieties (8,10,13, 18,21,26–29). This was confirmed for the compounds prepared by the oxidation–β-elimination procedure by derivatization of oligodeoxynucleotide **C** with bromobimane, dithiodipyridine, α-bromoacetamide 3-cholestesterol and 2-(5′-nitropyridyl)-3-cholesterol disulphide to compounds **J**–**M** which were also characterised by mass spectrometry (Fig. 6).

The phosphorothioate internucleotidic linkage of an oligonucleotide can be alkylated at elevated temperature over several hours with thiophiles similar to those employed here (30). It was therefore of interest to compare the reactivity between a phosphorothioate diester and a monoester such as a terminal phosphorothioate described here. This was done by reacting Tp(S)T with bromobimane as an example as this is the most

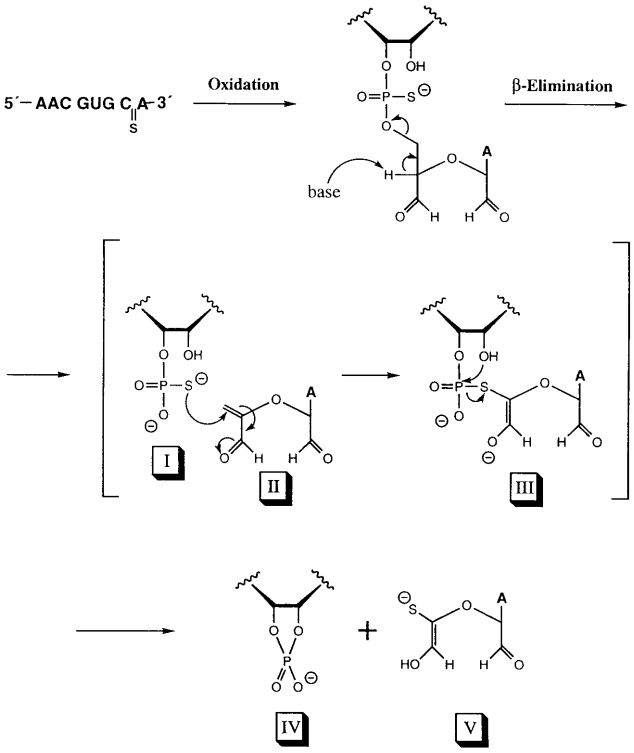

Figure 3. Cleavage of an internucleotidic phosphorothioate in an oligoribonucleotide.

reactive compound described for reaction with oligonucleotides containing one internucleotidic phosphorothioate. Interestingly, the terminal phosphorothioate of **F** reacted only marginally faster than that of Tp(S)T. Thus, caution has to be exercised when reacting terminal phosphorothioates of oligonucleotides which additionally also contain a phosphorothioate internucleotidic linkage.

The high yields of this synthetic method for the introduction of terminal phosphorothioates at either the 3′- or the 5′-end of an oligonucleotide establishes an attractive alternative to the use of linkers or enzymatic phosphorothioation.

MATERIALS AND METHODS

General

Synthesis of oligonucleotides was carried out by the phosphoramidite method on an Applied Biosystems DNA Synthesizer 380 B on a 1 µmol scale with nucleoside phosphoramidites from PerSeptive (Hamburg, Germany). 2′,3′-Diacetyluridine-5′-*O*-(2-cyanoethoxydiisopropylamino)phosphoramidite was either prepared by standard procedures (31) or obtained commercially from ChemGenes (Waltham, MA). Bromobimane was purchased from Sigma (Deisenhofen, Germany), dithiodipyridine from Fluka (Neu-Ulm, Germany), 1,8-bis-(dimethylamino)naphthalene, bromoacetyl bromide, bis-(5-nitro-2-pyridyl)disulphide and thiocholesterol from Aldrich (Steinheim, Germany) and SiO_2 (particle size 0.063–0.200 mm) from Merck (Darmstadt, Germany). All reactions of the phosphorothioate oligonucleotides were conducted in an argon atmosphere.

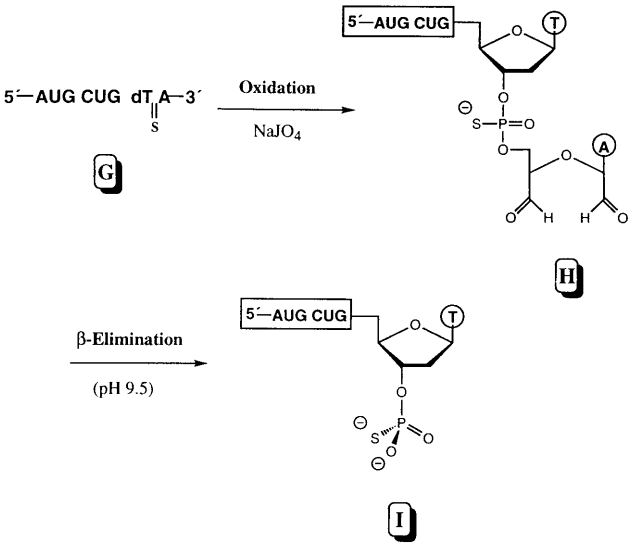

Figure 4. Synthesis of the oligoribonucleotide 3′-phosphorothioate **I**.

^1H and ^{13}C NMR were recorded in $CDCl_3$ on a Bruker AM 360L spectrometer at 360.13 and 90.55 MHz, respectively. Chemical shifts are reported in p.p.m., relative to tetramethylsilane at δ 0.0 p.p.m. ^{31}P NMR spectra were recorded at 145.79 MHz. Chemical shifts are reported in p.p.m., relative to 80% phosphoric acid as external standard at δ 0.0 p.p.m.

Oligonucleotides were characterised by MALDI-TOF mass detection on a PerSeptive Biosystems Voyager-DE Biospectrometry Workstation with 3-hydroxypicolinic acid in ammonium citrate as matrix.

HPLC analysis was carried out on a Waters Associates System with Model 6000A pumps, a Module 680 Automated System Controller, a Model 730 Data Module and a Model 481 LC Spectrophotometer. Reverse phase HPLC was performed on Hypersil ODS 5 µm columns (MZ Analysetechnik, Mainz, Germany) with a stepwise gradient of 0–16% acetonitrile in 50 mM triethyl ammonium acetate (pH 7.0) for 15 min, followed by an increase of acetonitrile from 16 to 70% from 15 to 20 min and keeping this buffer for up to 30 min for analytical runs. The same gradient was used for preparative runs but with 100 mM triethyl ammonium bicarbonate (pH 7.0).

Syntheses

α-*Bromoacetamide-3-cholesterol*. 3-α-Aminocholesterol (32) (20 mg, 0.052 mmol), 1,8-bis-(dimethylamino)naphthalene (16.72 mg, 0.078 mmol) (Aldrich) and bromoacetylbromide (12.56 mg, 5.42 µl, 0.062 mmol) (Aldrich) were dissolved in CH_2Cl_2 (5 ml). After 30 min at 25°C CH_2Cl_2 (50 ml) was added and the reaction mixture was extracted with 1 N NaOH (2 × 15 ml) and water (20 ml). The organic phase was dried over Na_2SO_4 and evaporated to dryness. The title compound was isolated by flash chromatography on SiO_2 (Merck) with a linear gradient of MeOH in CH_2Cl_2 from 0 to 2%. Yield, orange solid, 17.67 mg (70%). ^1H NMR: ($CDCl_3$) 0.68 (s, 3H, CH_3), 0.85 (d, 3H, J = 1.5 Hz, CH_3); 0.87 (d, 3H, J = 1.5 Hz, CH_3); 0.91 (d, 3H, J = 6.5 Hz, CH_3), 1.03 (s, 3H, CH_3); 1.07–2.04 (m, 25H); 2.69 (m, 1H); 3.85 (d, 2H,

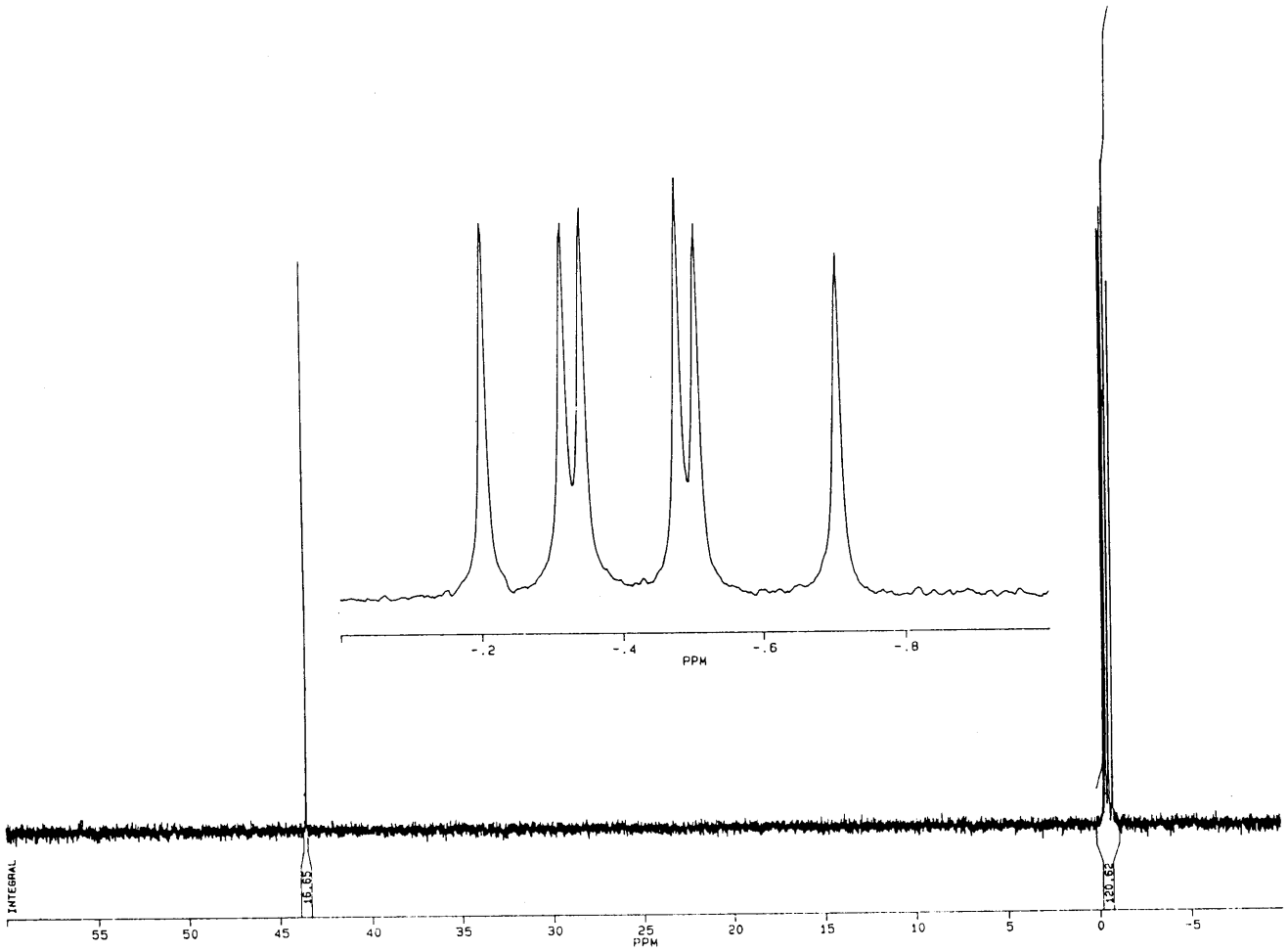

Figure 5. ^{31}P NMR spectrum of oligodeoxynucleotide 5′-phosphorothioate **C**.

J = 0.76 Hz, Br-CH_2), 4.11 (m, 1H), 5.45 (d, 1H, J = 5.2 Hz), 6.66 (d, 1H, NH, J = 7.3 Hz). ^{13}C NMR: ($CDCl_3$) 11.9, 18.8, 19.0, 20.9, 22.5, 22.6, 22.9, 23.9, 24.4, 26.1, 28.1, 28.3, 29.8, 31.9, 32.1, 34.5, 35.9, 36.3, 37.1, 37.5, 39.6, 39.8, 42.4, 46.5, 50.9, 56.3, 56.8, 124.4, 138.4, 164.3

2-(5′-Nitropyridyl)-3-cholesterol disulphide. Bis-(5-nitro-2-pyridyl)-disulphide (92.46 mg, 0.3 mmol) (Aldrich) and thiocholesterol (80 mg, 0.2 mmol) (Aldrich) were dissolved in pyridine (4 ml) and stirred at 25°C for 2 h. The solvent was removed *in vacuo*, the residue dissolved in CH_2Cl_2 (50 ml) and extracted with saturated $NaHCO_3$ (20 ml), 1 N NaOH (2 × 15 ml) and water (20 ml). The organic phase was dried over Na_2SO_4 and evaporated to dryness. The title compound was isolated by flash chromatography on SiO_2 (Merck) with a linear gradient of CH_2Cl_2 in *n*-hexane from 0 to 50% (v/v). Yield 88.87 mg (80%). ^{1}H NMR: ($CDCl_3$) 0.66 (s, 3H, CH_3), 0.84 (d, 3H, J = 1.6 Hz, CH_3), 0.86 (d, 3H, J = 1.6 Hz, CH_3), 0.89 (d, 3H, J = 6.5, CH_3), 0.98 (s, 3H, CH_3), 1.01–2.02 (m, 26H), 2.35 (m, 2H), 2.83 (m, 1H), 5.34 (d, 1H, J = 4.8 Hz), 7.96 (dd, 1H, J = 0.5 Hz, ArH), 8.40 (dd, 1H, J = 2.6 Hz, ArH), 9.25 (dd, 1H, J = 0.5 Hz, ArH). ^{13}C NMR: ($CDCl_3$) 11.9, 18.8, 19.4, 21.0, 22.6, 22.9, 23.9, 24.3, 28.1, 28.3, 29.0, 31.9, 31.9, 35.9, 36.3, 36.8, 38.9, 39.4, 39.6, 39.8, 42.4, 50.3, 51.0, 56.2, 56.8, 119.2, 122.3, 131.6, 140.8, 142.0, 145.0, 170.1.

Oligodeoxynucleotide 5′-phosphorothioate ***C***. The oligodeoxynucleotide dTTC CTC A was synthesized by standard methods with the deoxynucleoside phosphoramidites. After the last coupling of dT, 2′,3′-diacetyluridine-5′-*O*-(2-cyanoethoxydiisopropylamino)phosphoramidite was added, followed by sulphurization with Beaucage reagent. For purification the oligonucleotide was base deprotected and purified by preparative reverse phase HPLC to result in oligonucleotide **A** (analytical HPLC, retention time 12.16 min). $[M+H]^+$ calculated 2334.60; found 2332.5.

For the synthesis of the terminal phosphorothioate the oligonucleotide (20 A_{260} units, 250 nmol) was evaporated to dryness in a Speed-Vac and the residue dissolved in borax/boric acid buffer (17.5 µl, 0.06 M, pH 8.6). Sodium periodate was added (2.5 µl, 200 mM in water) and the reaction was incubated in the dark at room temperature for 10 min. After this time glycerol (2 µl) was added, followed again by an incubation for 10 min at room temperature. For the β-elimination the reaction mixture was concentrated in a Speed-Vac and then dissolved in NaOH/borax/boric acid buffer (50 µl, 0.055 M, pH 9.5) and kept at 45°C for 90 min. Analytical HPLC indicated 95% conversion to **C** (retention time 11.32 min). The crude product was purified by

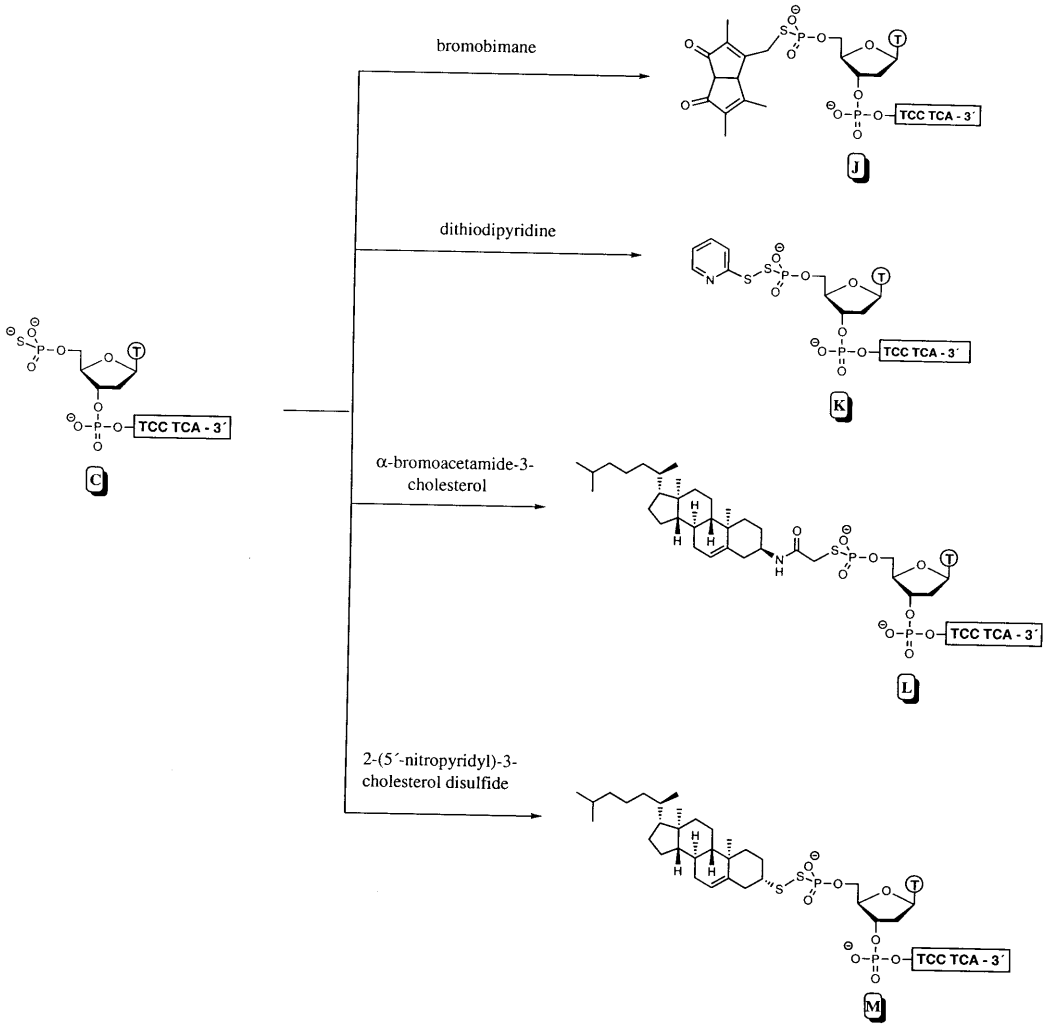

Figure 6. Derivatizations of oligodeoxynucleotide 5′-phosphorothioate **C**.

reverse phase chromatography (Sep-Pak 18 cartridge) as follows. The Sep-Pak 18 cartridge was prewashed with acetonitrile (10 ml) followed by triethylammonium bicarbonate (TEAB, 100 mM, pH 7.5, 10 ml). After loading the reaction mixture on the cartridge, it was first washed with TEAB/4% acetonitrile (100 mM, pH 7.5, 20 ml) followed by acetonitrile/methanol/water (35:35:30 v/v, 2 ml). The first 0.4 ml were discarded and the product collected in the following 1.6 ml. The eluant was evaporated to dryness and the residue dissolved in water (200 µl). Dithiothreitol (DTT, 5 µl, 250 mM) was added to this solution to reduce any disulphide. DTT was removed by extraction with ethyl acetate (2 × 200 µl). HPLC retention time of **C**, 11.32 min (of disulphide, 12.25 min). Yield 83%. ^{31}P NMR (H_2O) 45 p.p.m. (PS) and 0 p.p.m. (PO) (Fig. 5); $[M+H]^+$ calculated 2124.41; found 2129.7.

For further quantitative analysis the oligonucleotide was subjected to gel electrophoresis on Hg^{2+} PAGE ([(*N*-acryloamino)-phenyl]mercuric chloride (APM) affinity gel) (20). The oligonucleotide was 3′-end-labelled with [^{32}P]pCp (Amersham; 300 Ci/mmol) using T4 RNA ligase (33) and the sample loaded on the gel. In this gel the phosphorothioate oligonucleotide barely moves out of the well. The gel was analysed using a Fuji BAS-2000 Bio-Imaging PhosphorImager system. The content of the 5′-phosphorothioate oligonucleotide was 85%. The remaining 15% was 5′-phosphate oligodeoxynucleotide with faster mobility.

*Oligodeoxynucleotide 3′-phosphorothioate **F***. The synthesis was started with a uridine–CPG column. After the first coupling of the deoxycytidine phosphoramidite the phosphite triester was reacted with the Beaucage reagent. The following phosphoramidite couplings and oxidations to obtain **D** (retention time 12.09 min) were carried out as usual. The oligonucleotide was purified by the normal RNA purification procedure described previously (34). $[M+H]^+$ for **D** calculated 2334.60; found 2338.6. Subsequent oxidation and β-elimination steps to **E** (retention time 11.60 min) and **F** (retention time 11.00 min), respectively, and Sep-Pak purification were carried out as described for **C**. Yield of **F** 85%; $[M+H]^+$ calculated 2124.41; found 2129.4.

For phosphorothioate quantification on the APM affinity gel, the oligonucleotide was 5′-labelled with [γ-^{32}P]ATP (35). Content of the 3′-terminal phosphorothioate oligonucleotide was 83%.

*Oligoribonucleotide 3′-phosphorothioate **I***. The synthesis started with an adenosine–CPG column. After the first coupling of the

deoxythymidine phosphoramidite the phosphite triester was reacted with the Beaucage reagent. The following phosphoramidite couplings and further purification to obtain **I** (retention time 11.94 min) were carried out as described above. $[M+H]^+$ for **I** calculated 2522.640; found 2525.1.

Subsequent oxidation and β-elimination to **H** (retention time 11.45 min) and **I** (retention time 10.69 min), respectively, and Sep-Pak purification were carried out as described for **C**. Yield of **I** 68%; ^{31}P NMR (H_2O) 43 p.p.m. (PS) and 0 p.p.m. (PO); $[M+H]^+$ calculated 2256.4; found 2261.5.

For phosphorothioate quantification on the APM affinity gel, the oligonucleotide was 5′-labelled with [γ-^{32}P]ATP as described for **F**. Content of the 3′-terminal phosphorothioate oligonucleotide was 85%.

Derivatisation of oligodeoxynucleotide 5′-phosphorothioate

*Oligodeoxynucleotide 5′-bimane derivative **J***. A solution of 1.5 A_{260} units (21.4 nmol) of **C** in HEPES buffer (10 µl, 500 mM, pH 7.5) was treated with a bromobimane solution (10 µl, 100 mM in acetonitrile). The reaction was incubated in the dark at room temperature for 2 h, resulting in a 85% conversion to product. Yield of **L** after purification by HPLC 64% (retention time 14.03 min); λ_{max} 214, 253 and 388 nm; $[M+H]^+$ calculated 2315.62; found 2320.5.

*Oligodeoxynucleotide 5′-thiopyridine derivative **K***. A solution of 1.5 A_{260} units of **C** (21.4 nmol) in boric acid/borax buffer (10 µl, 0.06 M, pH 8.6) was treated with dithiodipyridine (10 µl, 100 mM in acetonitrile). After 2 h at room temperature, the reaction was 86% complete to give **K**. Yield after HPLC purification 60%; retention time 14.91 min; $[M+H]^+$ calculated 2234.57; found 2240.4.

*Oligodeoxynucleotide 5′-cholesteryl derivative **L***. A solution of 1.5 A_{260} units of **C** (21.4 nmol) in boric acid/borax buffer (3 µl, 0.06 M, pH 8.6) and DMF (7 µl) was treated with a solution of α-bromoacetamide-3-cholesterol (10 µl, 100 mM in DMF). The reaction was terminated after 4 h at 37°C when the reaction showed 72% conversion to **L**. Yield after HPLC purification 31%; retention time 20.99 min; $[M+H]^+$ calculated 2551.12; found 2557.1.

*Oligodeoxynucleotide 5′-disulphide cholesteryl derivative **M***. A solution of 1.5 A_{260} units of **C** (21.4 nmol) in boric acid/borax buffer (3 µl, 0.06 M, pH 8.6) and DMF (7 µl) was treated with 2-(5′-nitropyridyl)-3-cholesterol disulphide (10 µl, 25 mM in DMF). After 24 h at 37°C, HPLC analysis indicated ~52% conversion to **M**. Yield after HPLC purification 22% (retention time 21.31 min); $[M+H]^+$ calculated 2526.13; found 2528.8.

The corresponding oligodeoxynucleotide 3′-derivatives were synthesized under the same conditions starting with oligodeoxynucleotide 3′-phosphorothioate **F**. For the oligoribonucleotide 3′-phosphorothioate **I** only the cholesteryl derivatives were synthesized. In all the syntheses comparable yields were obtained. All derivatives were characterised by mass spectrometry.

ACKNOWLEDGEMENTS

We thank R. Jacob and Innovir, Göttingen for recording the mass spectra, U. Kutzke for expert technical assistance and P. A. Heaton for reading the manuscript. This work was financially supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1 Verma,S. and Eckstein,F. (1998) *Annu. Rev. Biochem.*, **67**, 99–134.
2 Uhlmann,E. and Peyman,A. (1990) *Chem. Rev.*, **90**, 543–584.
3 Goodchild,J. (1990) *Bioconjugate Chem.*, **1**, 165–187.
4 MacKellar,C., Graham,D., Will,D.W., Burgess,S. and Brown,T. (1992) *Nucleic Acids Res.*, **20**, 3411–3417.
5 Manoharan,M. (1993) In Crooke,S.T. and Lebleu,B. (eds), *Antisense Research and Applications*. CRC Press, Boca Raton, FL, pp. 303–349.
6 Agrawal,S. (1995) In Agrawal,S. (ed.), *Methods in Molecular Biology*, 26. *Protocols for Oligonucleotide Conjugates*. Humana Press, Totowa, NJ.
7 Boutorine,A.S., Le Doan,T., Battioni,J.P., Mansuy,D., Dupré,D. and Hélène,C. (1990) *Bioconjugate Chem.*, **1**, 350–356.
8 Cosstick,R., McLaughlin,L.W. and Eckstein,F. (1984) *Nucleic Acids Res.*, **12**, 1791–1810.
9 Desjardins,J., Mata,J., Brown,T., Graham,D., Zon,G. and Iversen,P. (1995) *J. Drug Target.*, **2**, 477–485.
10 Gupta,K.C., Sharma,P., Kumar,P. and Sathyanarayana,S. (1991) *Nucleic Acids Res.*, **19**, 3019–3025.
11 Manoharan,M., Tivel,K.L. and Cook,P.D. (1995) *Tetrahedron Lett.*, **36**, 3651–3654.
12 Oberhauser,B. and Wagner,E. (1992) *Nucleic Acids Res.*, **20**, 533–538.
13 Zuckermann,R., Corey,R.M. and Schultz,P. (1987) *Nucleic Acids Res.*, **15**, 5305–5321.
14 Zelphati,O., Wagner,E. and Leserman,L. (1994) *Antiviral Res.*, **25**, 13–25.
15 Letsinger,R.L., Zhang,G., Sun,D.K., Ikeuchi,T. and Sarin,P.S. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 6553–6556.
16 Uhlmann,E. and Engels,J. (1986) *Tetrahedron Lett.*, **27**, 1023–1026.
17 Horn,T. and Urdea,M.S. (1986) *Tetrahedron Lett.*, **27**, 4705–4708.
18 Gryaznov,S.M. and Letsinger,R.L. (1993) *Nucleic Acids Res.*, **21**, 1403–1408.
19 Thuong,N.T. and Chassgnol,M. (1987) *Tetrahedron Lett.*, **28**, 4157–4160.
20 Igloi,G.L. (1988) *Biochemistry*, **27**, 3842–3849.
21 Kang,S.-H., Xu,X., Heidenreich,O., Gryaznov,S. and Nerenberg,M. (1995) *Nucleic Acids Res.*, **23**, 2344–2345.
22 Kuijpers,W.H.A. and van Boeckel,C.A.A. (1993) *Tetrahedron Lett.*, **49**, 10931–10944.
23 Kathawala,F. and Cramer,F. (1968) *Liebigs Ann. Chem.*, **712**, 195–200.
24 Richard,J.P. and Frey,P.A. (1982) *J. Am. Chem. Soc.*, **104**, 3476–3481.
25 Burgers,P.M.J. and Eckstein,F. (1979) *Biochemistry*, **18**, 592–596.
26 Herrlein,M.K., Nelson,J.S. and Letsinger,R.L. (1995) *J. Am. Chem. Soc.*, **117**, 10151–10152.
27 Herrlein,M.K. and Letsinger,R.L. (1994) *Nucleic Acids Res.*, **22**, 5076–5078.
28 Gryaznov,S.M., Schultz,R., Chaturvedi,S.K. and Letsinger,R.L. (1994) *Nucleic Acids Res.*, **22**, 2366–2369.
29 Gryaznov,S.M. and Letsinger,R.L. (1993) *Nucleic Acids Res.*, **21**, 1403–1408.
30 Fidanza,J.A., Ozaki,H. and McLaughlin,L.W. (1992) *J. Am. Chem. Soc.*, **114**, 5509–5517.
31 Sinha,N.D., Biernat,J., MacManus,J.P. and Köster,H. (1984) *Nucleic Acids Res.*, **12**, 4539–4557.
32 Bose,A.K., Kistner,J.F. and Farber,L. (1962) *J. Org. Chem.*, **27**, 2925–2927.
33 Uhlenbeck,O.C. and Gumport,R.I. (1982) In Boyer,P.D. (ed.), *The Enzymes*, 3rd Edn. Academic Press, New York, NY, Vol. 15, pp. 31–58.
34 Tuschl,T. and Eckstein,F. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 6991–6994.
35 Richardson,C.C. (1971) In Cantoni,G.L. and Davies,D.R. (eds), *Procedures in Nucleic Acid Research*. Harper & Row, New York, NY, Vol. 2, pp. 815–828