Imidazole Tethered Oligodeoxyribonucleotides: Synthesis and RNA Cleaving Activity

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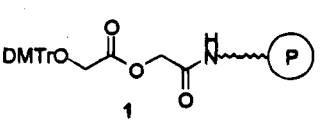
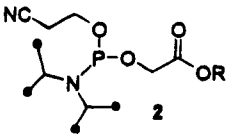
A solid support (**3**) and a non-nucleosidic phosphoramidite building block (**4**), both containing a thioester bond in their structure, were synthesized. They were used in the preparation of oligonucleotides tethered to an imidazole group at their 3′- or 5′-terminus, as well as at the 1′-position of nonterminal 3′-deoxypsicothymidine units. The desired functional groups were introduced during the deprotection of the oligonucleotide by using the primary amino group of histamine or 1-(3-aminopropyl)imidazole as a nucleophile. The ability of the tethered oligonucleotides to hydrolyze complementary RNA strands was tested. Of the oligonucleotides prepared, **12b**, bearing a histamine group at the 3′-end, was shown to promote a sequence specific strand scission of RNA in the presence of Zn^{2+} ions.

Introduction

Oligonucleotides tethered to catalytically active groups constitute a class of highly potent cleaving-agents, with which nucleic acids may be tailored in a predesigned manner. The oligonucleotide moiety recognizes a complementary base-sequence in the target nucleic acid, and the catalytic group cleaves the chain over a narrow range of nucleotide units, preferably at a single phosphodiester bond. The feasibility of this approach has been demonstrated by several metal-ion-based artificial nucleases that cleave DNA oxidatively in a sequence-specific manner.[1] By contrast, attempts to prepare oligonucleotide conjugates that could hydrolyze DNA- or RNA-sequences have been less successful. Only a few such sequence-specific cleaving-agents have been reported,[2-6] in spite of the fact that the hydrolysis of RNA,[7] and possibly also that of DNA,[8,9] may be promoted by various metal ions and their chelates.

Attachment of catalytically active groups, such as metal chelates or uncomplexed ligands, to oligonucleotides is not straightforward, and the lack of simple procedures with which to perform this has undoubtedly retarded the development of artificial nucleases. We have recently presented a rather versatile strategy for tethering of oligonucleotides:[10,11] an ester function is attached to the oligonucleotide during chain assembly, and upon completion of the synthesis, the desired functional group is introduced by treating the oligomer, when still anchored to the solid support, with an appropriate primary amine. For the 3′-tethering, the ester function is provided by a modified solid support[10] (**1**), and for the 5′-tethering (and 1′-tethers of 3′-deoxypsiconucleoside units) by a non-nucleosidic building block[11] (**2**).





R = CH_2CH_2Cl or
R = CH_2CF_3

This methodology actually suffers from only one limitation. The ester bond is hydrolyzed concurrent with its aminolysis, when amines of modest nucleophilicity are used to introduce the desired conjugate group. The most applicable support prepared, **1** allows the introduction of 3′-aminoalkyl tethers to synthetic oligonucleotide by treating the polymer bound material with 1 M aqueous diamines for 10 h at room temperature. However, tethering with less reactive nucleophiles needs longer reaction times or higher amine concentrations, which results in side reactions, such as transamination of N^4-benzoylated cytosine residues and hydrolysis of the ester bond. For example, attachment of a histamine spacer to the 3′-terminus of an oligonucleotide prepared on **1** could be accomplished by a 50% yield only. We now report that this problem may be overcome by replacing the leaving alkoxy function of the ester grouping with an alkylthio group. It is well known that thioesters, R^1COSR^2, are much more succeptible to nitrogen nucleophiles than their oxygen analogues, whereas the hydrolysis rate of these compounds are comparable.[12] The novel solid support used to functionalize the 3′-terminus thus

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(3) Magda, D.; Miller, R. A.; Sessler, J. L.; Iverson, B. L. *J. Am. Chem. Soc.* **1994**, *116*, 7439.

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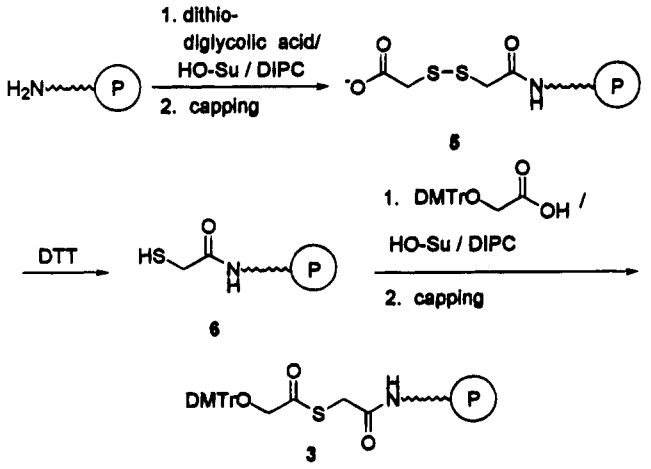
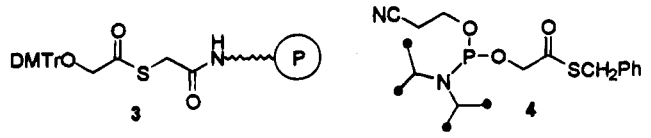
Scheme 1



P ≡ controlled pore glass; HO-Su = *N*-hydroxysuccinimide; DIPC = *N,N'*-diisopropylcarbodiimide; DTT = dithio-D,L-threitol

has the structure **3**, and the building block used for 5′- (or 1′-) functionalization has the structure **4**.



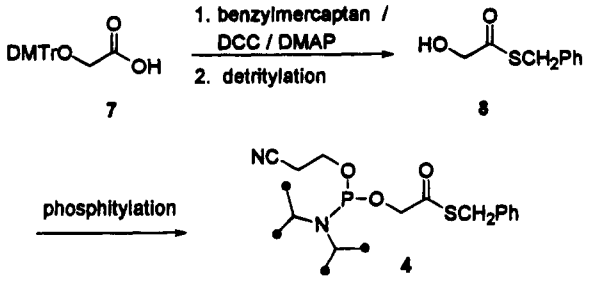
The applicability of **3** and **4** as tools for oligonucleotide tethering is demonstrated by using histamine and 1-(3-aminopropyl)imidazole as nucleophiles that displace the alkylthio group upon completion of the oligonucleotide synthesis. Furthermore, the potentiality of the oligonucleotide conjugates obtained as sequence-specific artificial RNA nucleases has been studied. An oligonucleotide bearing a 3′-histamino tether, **12b**, has been shown to cleave oligoribonucleotides in the presence of Zn^{2+} predominantly at a single site.

Results and Discussion

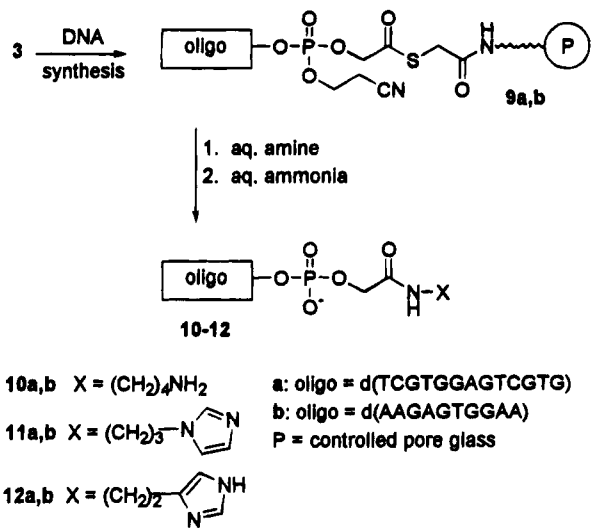
Synthesis of the Solid Support Bearing a Thioester Linkage. The solid support containing a thioester bond in its structure, **3**, was prepared as depicted in Scheme 1. Accordingly, dithiodiglycolic acid was initially coupled to the long chain alkylamine controlled pore glass (CPG) with the aid of *N,N'*-diisopropylcarbodiimide and *N*-hydroxysuccinimide, yielding **5**. Capping of the unreacted amino functions and reduction of the disulfide bond with 1,4-dithio-D,L-threitol gave the mercapto-derivatized support, **6**. Acylation with *O*-(4,4′-dimethoxytrityl)glycolic acid (**7**), using carbodiimide activation, then gave, after capping of the unreacted mercapto functions, support **3** as the final product. The loading, according to the dimethoxytrityl cation assay,[13] was 30 μmol of DMTr-groups per gram.

Synthesis of the Phosphoramidite. The preparation of the building block, **4**, aimed at introducing tether groups to the 5′-terminus of oligonucleotides, as well as to the nonterminal hydroxy groups of 3′-deoxypsiconucleoside units, is depicted in Scheme 2. Accordingly, benzylmercaptan was coupled to **7** with the aid of DCC, followed by detritylation. The product, benzyl thioglycolate (**8**), was then converted to the corresponding phosphoramidite, **4**, with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite in the presence of 1*H*-tetrazole.

Scheme 2



Scheme 3



Synthesis of 3′-Tethered Oligonucleotides. To study the applicability of **3** for the preparation of 3′-modified oligonucleotides and to compare the results with those obtained previously with ester bond derivatized solid supports,[10] two sequences d(GCCGTGGAGTCTGTT) and d(AAGAGTGGAA) were prepared. No difference between the dimethoxytrityl responses of **3** and commercial nucleoside derivatized columns were found, demonstrating the stability of the thioester bond of **3** toward oligonucleotide chain assembly steps. Upon completion of the oligonucleotide synthesis, the desired functional groups were introduced as shown in Scheme 3.

The high succeptibility of the thioester bond of **3** toward nucleophiles is clearly seen from the reaction times and amine concentrations needed to introduce the aminoalkyl tether group. Accordingly, treatment of **9a,b** with 1 M aqueous diamine for 30 min at ambient temperature was sufficient to introduce the desired tether. When histamine or 1-(3-aminopropyl)imidazole were used as cleaving agents, lower concentrations (0.2 M solutions in water) and longer reaction times (6 h at rt) were used. In all cases, conventional ammonialysis removed the remaining protecting groups, giving the 3′-tethered oligonucleotides, **10-12**, in a high yield.

Synthesis of 5′-Tethered Oligonucleotides. To evaluate how well the building block **4** fits to normal machine-assisted oligonucleotide synthesis, two sequences d(TCCGTGGAGTCGTG) and d(AAGAGTGGAA) were assembled (Scheme 4). **4** was used in the last

(13) Atkinson, T.; Smith, M. In *Oligonucleotide Synthesis: A Practical Approach*; Gait, M. J., Ed.; IRL Press: Oxford, 1984; p 47.

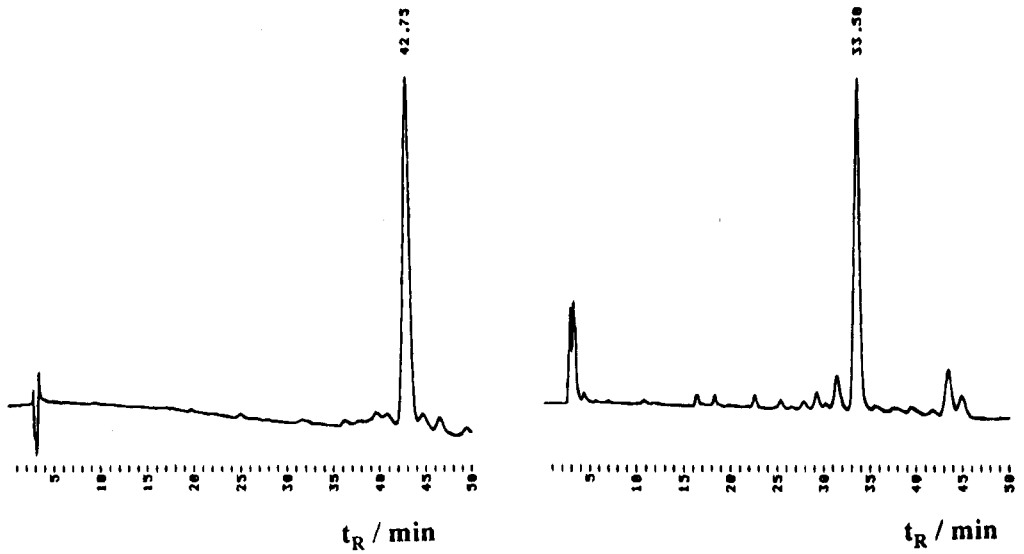
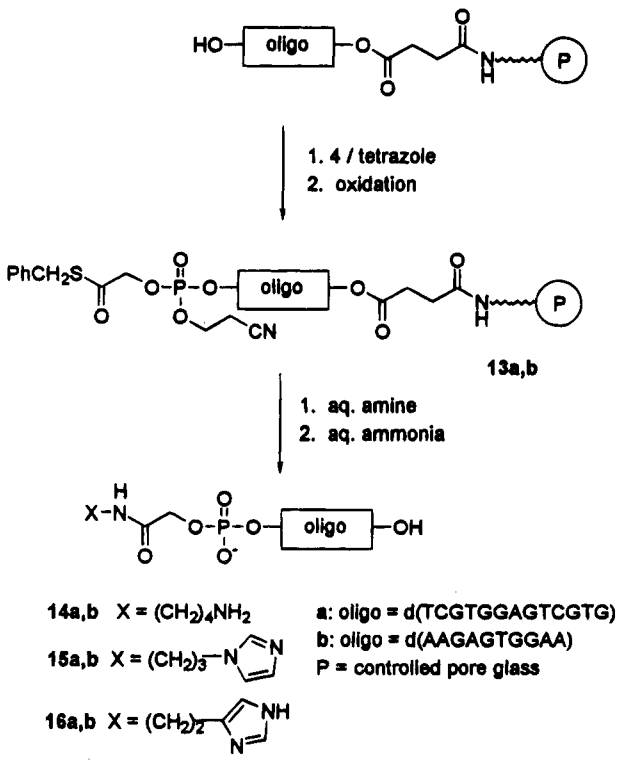


Figure 1. Ion-exchange HPLC profiles of crude **16a** (left) and **11b** (right). Column: Synchropak AX-300 4.6 × 250 mm, 6 μm; buffer A = 0.05 M KH_2PO_4 in 50% (v/v) aqueous formamide, pH 5.6; buffer B = A + 0.6 M $(NH_4)_2SO_4$, pH = 5.6; from A to 60% B in 50 min, flow rate 1.0 mL min^{-1}, λ = 260 nm. The large peaks ($t_R \sim 3$ min) on the profile of **11b** are results of non-nucleosidic material.

Scheme 4

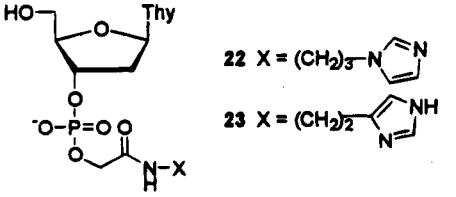


coupling step in a standard manner (0.1 M **4** in acetonitrile, coupling time 30 s). After oxidation with iodine, the desired tethers were introduced as described above for the preparation of 3′-tethered oligonucleotides. Accordingly, treatment of the polymer-bound material **13a,b** with 0.1 M diamine, 0.2 M aqueous histamine, or 1-(3-aminopropyl)imidazole for 6 h at ambient temperature introduced the desired tether groups. Ammonialysis completed the deprotection in all cases.

Synthesis of 1′-Tethered Oligonucleotides. The methodology described above was also applied to the 1′-derivatization of oligonucleotides containing a 3′-psicothymidine unit (Π). Accordingly, two sequences d(TCCGTGGAGΠCGTG) and d(AAGAGΠGGAA) were prepared as described previously[11] (Scheme 5). After chain assembly, the 1′-*O*-levulinyl group used to protect the 1′-hydroxy function was selectively removed with hydrazinium acetate, and phosphoramidite **4** was coupled to the 1′-*O*-deprotected material in a standard manner. Iodine oxidation, followed by detritylation of the 5′-terminus, yielded oligonucleotides **18a,b**. The 1′-tether groups were introduced to **18a,b** as described above.

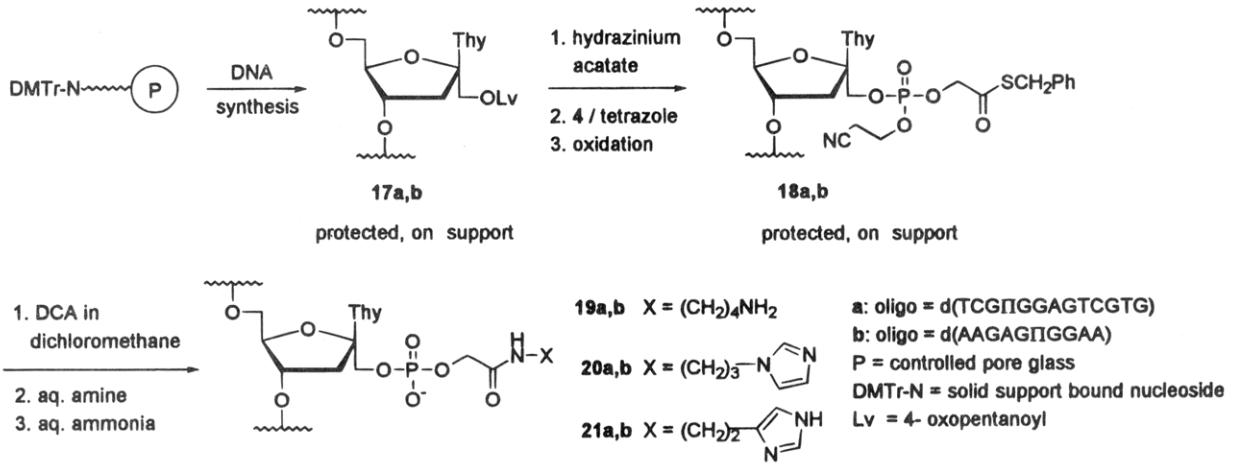
Characterization of the Oligonucleotides Prepared. The tethered oligonucleotides prepared (**10-12**, **14-16**, and **19-21**) were isolated by anion-exchange HPLC, purified on an RP-column, and desalted by gel filtration.[10,11] The ion-exchange HPLC profiles of **11b** and **16a** (crude reaction mixtures) are shown in Figure 1 as illustrative examples.

Compounds **10a**, **14a**, and **19a** coeluted both on an RP and ion-exchange column with the authentic samples synthesized and characterized previously.[10,11] The identity of oligonucleotides **11a** and **12a** was verified by digestion with phosphodiesterase I and alkaline phosphatase, followed by analysis of the product distribution by RP HPLC. The enzymatic digestions gave in both cases, in addition to natural nucleosides, the tethered thymidine 3′-phosphate (**22**, **23**), the identity of which was verified by spiking with an authentic sample (see Experimental Section for details). The product distributions were in agreement with the expected values.



RNA Hydrolysis. The ability of oligonucleotide conjugates (**11b**, **12b**, **15b**, **16b**, **20b**, **21b**) to promote the sequence specific cleavage of a complementary synthetic RNA fragment, 5′-^{32}p(U)$_5$CCACUC(U)$_5$ (**24**), in the presence or absence of Zn^{2+} ions was investigated. None of the oligodeoxyribonucleotides prepared were able to promote hydrolysis of **24** in the absence of Zn^{2+} within

Scheme 5



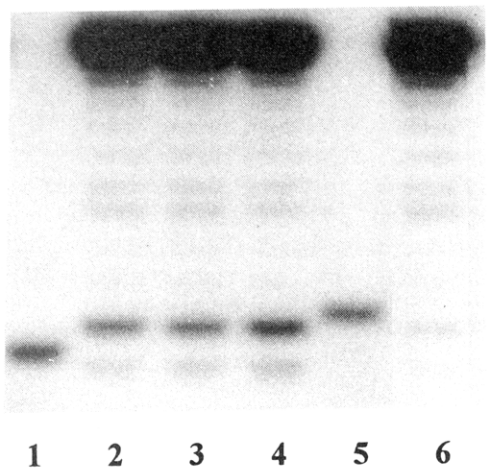


Figure 2. Autoradiogram of a 20% denaturing polyacrylamide gel showing hydrolysis pattern of oligoribonucleotide **24**. Lane 1: 5′-^{32}pUUUUU; lane 2: hydrolysis pattern of **24** in the presence of **12b** (5-fold excess) and Zn^{2+} (50 mM) after 1 h at rt; lane 3: after 3 h at rt; lane 4: after 19 h at rt; lane 5: 5′-^{32}pUUUUUC; lane 6: **24** in the presence of Zn^{2+} (50 mM) after 24 h at rt.

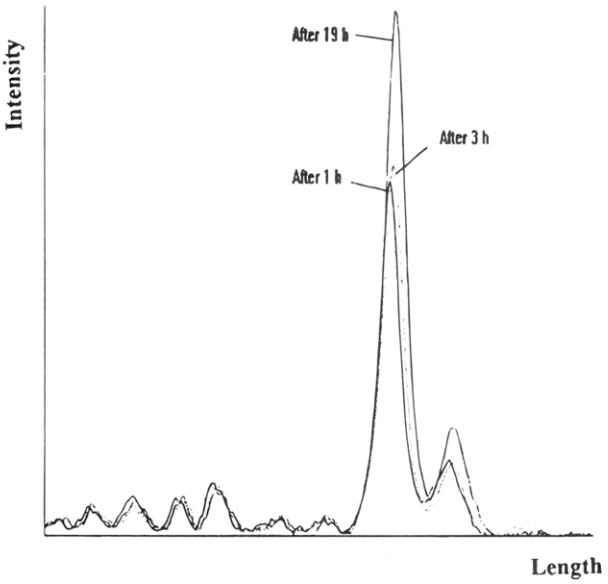


Figure 3. Densitometric traces of autoradiograms of PAGE of the main hydrolysis products of **24** as a function of time (from lanes 2–4 of Figure 2): The larger peaks correspond to 5′-^{32}pUUUUUC>p and the smaller ones to 5′-^{32}pUUUUU>p.

two weeks at room temperature. Autoradiograms of PAGE showed the ^{32}P-labeled oligoribonucleotide **24** to remain practically intact in all the cases (data not shown). By contrast, **12b**, bearing a histamine tether at the 3′-terminus of oligodeoxyribonucleotide chain, promoted hydrolysis of the complementary oligoribonucleotide **24** in the presence of Zn^{2+} in a rather specific manner. Figures 2 and 3 show the autoradiogram of PAGE exhibiting a pattern of oligoribonucleotide **24** (0.5 μM) scission in the presence of a 5-fold molar excess of **12b** at $[Zn^{2+}] = 50$ μM.

It is clearly seen that the cleavage predominantly takes place at a single site. Comparison of the electrophoretic mobility of the 5′-^{32}P labeled product to that of pentauridylic acid and 5′-^{32}p-U_5C suggests that a hexameric fragment is cleaved from the 5′-terminus of **24**. Most likely this major product is a 2′,3′-cyclic phosphate of 5′-^{32}pUUUUUC>p, the minor product being the corresponding pentamer, 5′-^{32}pUUUUU>p. Accordingly, the target oligoribonucleotide is not cleaved at the O5′-P bond of the last complementary nucleotide, but the cleaved fragment seems to be three units longer. Finally, it should be noted that only the 3′-histamine tethered oligomer, **12b**, exhibited this cleaving activity, not the corresponding 5′- (**16b**) or 1′-tethered oligonucleotides (**21b**), nor any of the 1-(3-aminopropyl)imidazole tethered oligonucleotides (**11b**, **15b**, **20b**).

The cleaving efficiency of **12b** in the presence of Zn^{2+} is comparable to that of the other artificial nucleases recently reported: for the Cu^{2+} terpyridine conjugate 11% cleavage in 72 h at 37 °C,[4] for the Eu^{3+}/porphyrin conjugate 30% cleavage in 24 h at 27 °C,[3] and Lu^{3+}/iminoacetic acid conjugate 17% in 8 h at 37 °C.[5] Under the experimental conditions indicated above, **12b** cleaved 2–5% of **24** in 19 h at room temperature.

Experimental Section

General. Long chain alkylamino CPG, histamine, and 1-(3-aminopropyl)imidazole were products of Sigma. Reagents for the machine-assisted oligonucleotide synthesis were purchased from Cruachem. Oligonucleotides were assembled on an Applied Biosystems 392 DNA Synthesizer (ABI) using phosphoramidite chemistry and recommended protocols (DMTr-Off synthesis). Oligonucleotides were purified by HPLC techniques as described previously in detail.[10]

Synthesis of the Solid Support 3. Long chain alkylamine-controlled pore glass (1.0 g) was treated with 80% aqueous ethanol (v/v) containing 10% Et_3N (v/v), washed with acetonitrile, and dried. Dithiodiglycolic acid (100 mg; 0.5

mmol), *N,N'*-diisopropylcarbodiimide (1.0 mmol, 157 μL), and *N*-hydroxysuccinimide (0.5 mmol; 58 mg) were added to the suspension of solid support in dry pyridine (5 mL), and the mixture was shaken overnight at ambient temperature. The suspension was filtered, washed with pyridine, kept in a mixture of Ac_2O:pyridine:*N*-methylimidazole (1:5:1, v/v) for 10 min, and washed with pyridine and diethyl ether. The material obtained was suspended in methanol (5 mL) containing 1,4-dithio-D,L-threitol (10 mg), shaken 2 h at room temperature, filtered, and dried. The material was then suspended in dry pyridine (5 mL), and compound **7** was added (as pyridinium salt; 0.5 g, 1.0 mmol). Coupling by the carbodiimide method was performed as described above. After capping of the unreacted mercapto groups, the solid support was washed with pyridine and diethyl ether and then dried.

Benzyl Thioglycolate 8. Compound **7** (as triethylammonium salt; 10.0 mmol, 4.79 g), benzyl mercaptan (9.50 mmol, 1.11 mL), and a catalytic amount of DMAP were dissolved in dichloromethane (15 mL). DCC (10 mmol, 2.06 g; predissolved in 5 mL of CH_2Cl_2) was added, and the mixture was stirred for 2 h at ambient temperature. The dicyclohexylurea formed was filtered, and the filtrate was concentrated and dissolved in a mixture of dichloromethane:dichloroacetic acid:ethanol (85:5:10, v/v; 30 mL). After 30 min at rt, the mixture was poured with vigorous stirring into saturated aqueous $NaHCO_3$. The organic layer was separated and dried over Na_2SO_4. Purification on a silica gel column (gradient: from 0 to 3% ethanol in dichloromethane) yielded the title compound as an oil (1.51 g; 83%). ^{1}H NMR (400 MHz; $CDCl_3$): 7.27 (5 H, s); 4.29 (2 H, s); 4.16 (2 H, s), 2.99 (1 H, br s). IR (film, cm^{-1}): 3423, 1686. Anal. Found: C, 59.0; H, 5.58; S, 17.2%. Calcd for $C_9H_{10}O_2S_1$: C, 59.3; H, 5.53; S, 17.6%.

Synthesis of the Phosphoramidite 4. Predried **8** (0.59 g, 3.10 mmol) and 2-(cyanoethyl)-*N,N,N',N'*-tetraisopropylphosphorodiamidite[14] (1.39 g, 4.60 mmol) were dissolved in acetonitrile (1.5 mL). 1*H*-Tetrazole (3.10 mmol; 6.90 mL, 0.45 M in acetonitrile) was added, and the mixture was stirred for 30 min at ambient temperature. It was then poured into 5% aqueous $NaHCO_3$ (150 mL) and extracted with dichloromethane (2 × 75 mL). The organic extracts were dried over Na_2SO_4 and concentrated to an oil. Cold hexane (−80 °C) was added to the residue, and the mixture was shaken vigorously. After decantation the product, **4** was obtained as an oil. It was stored at −20 °C. ^{1}H NMR (400 MHz; $CDCl_3$): 7.30 (5 H, s); 4.27 (2 H, m); 4.13 (2 H, s); 3.93 (2 H, m); 3.65 (2 H, m); 2.65 (2 H, m); 1.19 (12 H, dd, *J* 6.4). ^{31}P NMR (161.9 MHz; $CDCl_3$): 149.6 ppm.

Introduction of the 3'-Tether groups in the Oligonucleotides. The oligonucleotides were assembled on solid support **3** using standard protocols in 0.2 μmol scale. Upon completion of the oligonucleotide chain assembly, the protected oligonucleotides **9a,b** were treated with appropriate nucleophiles in water [1 M butane-1,4-diamine for 30 min or 0.2 M histamine or 1-(3-aminopropyl)imidazole for 6 h] by using a two-syringe method.[10] The solution was then transferred into a seal-cap vial, diluted with concentrated aqueous ammonia, and kept 7 h at 55 °C. When the deprotection was completed, all volatile materials were evaporated under reduced pressure. The remaining material was dissolved in water, and pH of the solution was adjusted to 7 with acetic acid. Purification was performed by HPLC techniques as described previously.[10]

Introduction of the 5'-Tether Groups in the Oligonucleotides. The oligonucleotides were assembled on commercial nucleoside derivatized columns using standard protocols. Phosphoramidite **4** was used in the last coupling step (0.1 M **4** in acetonitrile; coupling time 30 s). After oxidation with iodine, the desired tether groups were introduced, and the products were purified as described above, except in the case of butane-1,4-diamine when lower concentration (0.1 M) and longer reaction time (6 h) was used.

Introduction of the 1'-Tether Groups in the Oligonucleotides. The oligonucleotides having a psicothymidine unit in their structure were prepared, and their 1'-*O*-protecting groups were cleaved as described previously.[11] Coupling of **4**, oxidation, introduction of the tether, and purification of the target oligonucleotide was performed as described above.

Synthesis of 3'-Derivatized Thymidine 3'-Phosphates 22 and 23. 3'-(Methoxycarbonylmethyl 2-chlorophenyl) ester of 5'-*O*-(4,4'-dimethoxytrityl)thymidine 3'-monophosphate[10] (100 mg, 0.12 mmol) was dissolved in pyridine (500 μL). An aqueous solution of histamine or 1-(3-aminopropyl)imidazole (5 M, 5 mL) was added, and the mixture was kept 2 days at room temperature. All volatile materials were removed *in vacuo*, and the residue was dissolved in dichloromethane and extracted with aqueous KH_2PO_4. After concentration, the organic phase was dissolved in 80% aqueous acetic acid and kept 30 min at room temperature. The mixture was concentrated, dissolved in water, and extracted with diethyl ether. The aqueous phase was concentrated and purified on a semipreparative HPLC [Hypersil C-18; 250 × 10 mm, 5 μm, isocratic elution of 0.05 M ammonium acetate containing 3% (v/v) acetonitrile, flow rate 3.0 mL min^{-1}]. The pure fractions were pooled, concentrated, and desalted by using the same chromatographic system but omitting the ammonium acetate from the eluent **22**. ^{1}H NMR (400 MHz; D_2O): 8.28 (1 H, s); 7.46 (1 H, d, *J* < 1); 7.05 (1 H, s); 6.09 (1 H, dd, *J* 7.8 and 6.4); 4.61 (1 H, m); 4.14 (2 H, d, *J* 7.8); 3.98 (1 H, m); 3.64 (1 H, dd, *J* 3.4 and 12.7); 3.58 (1 H, dd, *J* 4.9 and 12.7); 3.37 (2 H, t, *J* 6.4); 2.77 (2 H, t, *J* 6.4); 2.32 (1 H, ddd, *J* 2.9, 6.4 and 14.1); 2.20 (1 H, m, *J* 6.8 and 14.1); 1.69 (3 H, d, *J* < 1). ^{31}P NMR (161.9 MHz; D_2O) −1.84. **23**. ^{1}H NMR (400 MHz; D_2O): 8.41 (1 H, s); 7.47 (1 H, s); 7.30 (1 H, s); 7.22 (1 H, s); 6.11 (1 H, dd, *J* 6.8 and 6.8); 4.62 (1 H, m); 4.17 (2 H, d, *J* 7.3); 4.09 (2 H, t, *J* 6.8); 4.01 (1 H, m); 3.66 (1 H, dd, *J* 3.4 and 12.2); 3.60 (1 H, dd, *J* 4.9 and 12.2); 3.15 (2 H, t, *J* 6.8); 2.335 (1 H, ddd, *J* 3.4, 6.4 and 14.2); 2.23 (1 H, m, *J* 6.8, 7.3 and 14.2); 1.98 (2 H p, *J* 6.8); 1.70 (3 H, s). ^{31}P NMR (161.9 MHz; D_2O) −1.84.

Enzymatic Digestions. Compounds **11a** and **12a** (1.0 OD) were digested with the mixture of phosphodiesterase I and alkaline phosphatase as described previously.[10] The product distribution was analyzed by reversed phase HPLC (column: LiChospher C-18, WP 300, 5 μm; eluent 0.05 M ammonium acetate containing 4% (v/v) acetonitrile, flow rate 1.0 mL min^{-1}).

RNA Synthesis and Purification. RNA fragment $(U)_5$-CCACUC$(U)_5$ was assembled on an ABI Synthesizer using commercial 2'-*O*-[1-(2-fluoro-4-methylphenyl)-4-methoxypiperidin-4-yl] protected phosphoramidites and an uridine derivatized column. Deblocking was performed as recommended by Cruachem.[15] The deprotected oligoribonucleotide was purified by reversed phase HPLC (column: Nucleosil 300-5-C18, 5 μm; gradient: from 0 to 17.5% acetonitrile in 0.1 M TEAA in 60 min, flow rate 1.0 mL min^{-1}) and desalted.[10] ^{32}P labeling of oligoribonucleotide was performed as described previously.[16] The product **24** was purified by reversed phase HPLC and desalted as described above for unlabeled oligonucleotide.

RNA Hydrolysis. The substrate 16-mer RNA (**24**; 5 pmol μL^{-1} in water, 2 μL) was mixed with **12b** (33 pmol μL^{-1} in water, 1.5 μL), Tris·HCl buffer (300 mM, containing 1 M NaCl; pH 7.0; 2 μL), and water (12.5 μL). The mixture was heated to 65 °C for 2 min and then allowed to cool slowly to room temperature for annealing. Hydrolysis was initiated by addition of aqueous $Zn(OAc)_2$ (500 μM, 2 μL).

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Supplementary Material Available: Copies of ^{1}H and ^{31}P NMR spectra of **22** and **23** (9 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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