

Disulfide-Tethered Solid Supports for Synthesis of Photoluminescent Oligonucleotide Conjugates: Hydrolytic Stability and Labeling on the Support[†]

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Several new disulfide-tethered solid supports (**S1**–**S5**) were synthesized, and their resistance against ammonolysis was tested. Among these supports, only the one bearing an *N*-[15-[(4,4'-dimethoxytrityl)-oxy]-12,13-dithiapentadecanoyl] linker (**S4b**) tolerated ammonolysis and exhibited properties compatible with the oligonucleotide synthesis by phosphoramidite strategy. The applicability of this disulfide linker structure in postsynthetic oligonucleotide labeling on the support was demonstrated by introduction of two photoluminescent lanthanide chelates or two dansyl groups to the *N*³-(6-aminohexyl) amino-modified cytosine residues at the 5' end of the oligonucleotide sequence. Subsequent release of the resulting conjugates as their 3'-phosphates was achieved by reductive cleavage of the disulfide bond and precipitation of the conjugate from the solution with ethanol. The fluorescently tagged oligomer obtained showed hybridization properties similar to those of oligonucleotides labeled in solution.

INTRODUCTION

The introduction of polymerase chain reaction (PCR) (*1*) has been a launching pad for the development of an increasing number of hybridization assays for the detection of gene mutations. Methods utilizing fluorescent oligonucleotide conjugates have become the subject of particular interest (*2–7*). Besides organic fluorophores, photoluminescent lanthanide chelates have recently been exploited as markers in hybridization assays (*8–13*). These chelates offer some obvious advantages over conventional fluorescent dyes: (i) the luminescence lifetime is long (≈ 1 ms), allowing utilization of time-resolved measurement, (ii) the difference between the wavelength of excitation and main emission is large, (iii) the emission bands are narrow, and (iv) the concentration quenching is almost negligible (*14*). The most serious drawback is the rather laborious preparation of the oligonucleotide conjugates. Usually an oligonucleotide bearing a nucleophilic functionality, such as an aliphatic amino group, is reacted in solution with an isothiocyanatophenyl or 4,6-dichloro-1,3,5-triazin-2-ylaminophenyl group of the chelate (*15*). This necessarily leads to somewhat complicated chromatographic purifications. To simplify the purifications and to increase the efficiency of labeling, Kwiatkowski et al. (*16*) have prepared phosphoramidite building blocks that are compatible with the automated solid-phase synthesis of oligonucleotides and bear the desired ligand of the photoluminescent chelate in a fully protected form. After chain assembly and deprotection of the ligand and base moieties, the oligomer is released in solution, and the lanthanide ion is introduced by treat-

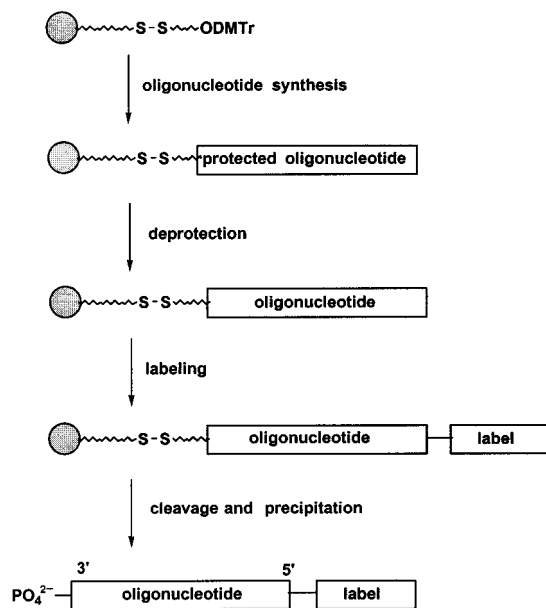
ment with lanthanide citrate. The final purification then includes desalting on NAP-10 Sephadex and RP FPLC.

While the labeling method described above undoubtedly is highly useful, it still depends on synthesis of a separate nucleosidic phosphoramidite building block for each luminescent chelate used as a marker. For some purposes, coupling of an activated chelate to a functional group of the oligomer that is still bound to the support would constitute a more versatile strategy. Obviously, the latter approach put high demands for the linker tethering the 3'-terminal nucleoside to the solid support. It should withstand, in addition to the conditions of the chain assembly, deprotection of the completed oligomer and reaction of the activated chelate with the appropriate conjugate group on the oligomer. Both of these steps are usually carried out under rather basic conditions. Furthermore, the linker should undergo cleavage under such mild conditions that the labeled oligomer is not modified upon the release in solution, and the final purification remains as simple as possible. Several linkers that allow synthesis of fully deprotected oligonucleotide on solid support and postsynthetic derivatization have been introduced, but in most cases their cleavage requires rather drastic conditions (*17, 18*). Disulfide-based tethers, however, may be expected to meet the requirements of solid-phase labeling with lanthanide chelates. They have been successfully used in automated oligonucleotide synthesis (*19–24*); they withstand basic conditions rather well, and they readily undergo reductive cleavage. Unfortunately, no data on postsynthetic transformation reactions of disulfide-immobilized oligonucleotides appear to exist. We now report on derivatization of polymeric solid supports (**S1**–**S5**) with a number of disulfide linkers. The stability of these linkers under basic conditions has been compared, and the best candidates (**S4b**) have been used in labeling of the *N*³-(6-aminohexyl)-cytosine residues of the support-bound oligonucleotide with a 4,6-dichloro-1,3,5-triazin-2-ylaminophenyl-acti-

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Scheme 1. General Scheme for Labeling of Oligonucleotides on the Support


vated europium(III) chelate. The general protocol of this idea is presented in Scheme 1.

EXPERIMENTAL PROCEDURES

General. The reagents of oligonucleotide synthesis were products of Cruachem and Glen Research. The amino-modified polystyrene and Tentagel supports were from Applied Biosystems Inc. *N*⁴-(*N*-Trifluoroacetyl-6-aminoethyl)-2'-deoxycytidine 3'-(*N,N*-diisopropyl-*O*-cyanoethyl)phosphoramidite (**13**) and the photoluminescent europium chelate {2,2',2'',2'''-[[4'-{4''''-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]phenyl}-2,2':6'',2''-terpyridine-6,6''-diyl]-bis(methylenenitrilo)}tetrakis(acetato)}europium(III) were received from the laboratory of Wallac Oy. Adsorption column chromatography was performed on Silica Gel 60 (Merck). All the other reagents were purchased from Aldrich. NMR spectra were recorded on a JEOL GX-400 spectrometer operating at 399.8 and 100.5 MHz for ¹H and ¹³C, respectively. The oligonucleotides were analyzed by ion-exchange and RP HPLC (columns, Synchropak AX-300, 4.0 mm × 250 mm, Synchrom Inc., for the ion exchange, and Hypersil ODS 5 μm, 4.0 mm × 250 mm and Nucleosil 300-5C18, 4.0 mm × 250 mm, Macherey-Nagel, for the RP HPLC). Elemental analyses were performed on a Perkin-Elmer 2400 Series II instrument.

2-[(4,4'-Dimethoxytrityl)oxy]ethyl 2-Pyridyl Disulfide (5). Bis(2-pyridyl) disulfide (1.1 mmol, 250 mg) was dissolved in a mixture of pyridine and MeOH (10 mL, 1:99, v/v). 2-Mercaptoethanol (1.0 mmol, 72 μL) was added dropwise to the stirred solution, and the mixture was left to stand at ambient temperature until no 2-mercaptoethanol could be detected by TLC. The reaction mixture was evaporated to dryness and coevaporated three times with dry pyridine. The residue (**4**) was dissolved in dry pyridine (10 mL); 4,4'-dimethoxytrityl chloride (1.1 mmol, 375 mg) was added, and the reaction mixture was left to stand overnight at ambient temperature. The reaction was stopped by adding MeOH (0.5 mL) and aqueous NaHCO₃ (5 mL of a 5% solution). The mixture was evaporated to an oil in vacuo, dissolved in CH₂Cl₂ (40 mL), washed with water (2 × 20 mL), and dried over Na₂SO₄. The solvent was removed in vacuo, and **5** was separated on a silica gel column (30 mm ×

100 mm), eluting with a stepwise gradient system of CH₂-Cl₂ in hexane containing 0.1% pyridine (from 75 to 100% CH₂Cl₂). The overall yield was 81%. ¹H NMR (CDCl₃; δ, ppm): 8.41 (1H, m, Pyr H6), 7.68 (1H, m, Pyr H4), 7.54 (1H, m, Pyr H5), 7.43 (2H, m, DMTr), 7.32–7.15 (6H, m, DMTr), 7.04 (1H, m, Pyr H3), 6.83–6.78 (5H, m, DMTr), 3.77 (6H, s, OMe), 3.38 (2H, t, *J*^β = 6.4 Hz, DMTrOCH₂), 2.94 (2H, t, *J*^β = 6.4 Hz, PyrSSCH₂). TLC: *R*_f = 0.88 (19:1 CH₂Cl₂/MeOH), 0.62 (49:1 CH₂Cl₂/MeOH). MS: *m/z* at 489 (M⁺).

3-[(4,4'-Dimethoxytrityl)oxy]propyl 2-Pyridyl Disulfide (8). Bis(3-hydroxypropyl) disulfide (**25**) (5.76 mmol, 1.05 g) was coevaporated with pyridine (3 × 20 mL) and dissolved in dry pyridine (50 mL). 4,4'-Dimethoxytrityl chloride (10.2 mmol, 3.45 g) in pyridine (50 mL) was added to the stirred solution and the mixture left to stand overnight at ambient temperature. The resulting **6** was worked up as described for **5** and used without further purification. **6** (0.6 g, 0.8 mmol) was dissolved in CH₂Cl₂ (10 mL), and methanolic DTT (0.8 mmol in 3 mL) was added. The reductive cleavage of **6** to **7** was followed by TLC. The reaction mixture was evaporated, dissolved in CH₂Cl₂, washed with water, dried, and concentrated to an oil. The oil was immediately added to a solution of bis(2-pyridyl) disulfide (1.6 mmol, 0.35 g) in MeOH (10 mL). After being stirred for 4 days at ambient temperature, the reaction mixture was evaporated to an oil and dissolved in a mixture of hexane and CH₂Cl₂ (25:75) containing 0.1% pyridine. **8** was isolated on a silica gel column (30 mm × 100 mm), eluting with a gradient of CH₂Cl₂ (75 to 100%) in hexane. The overall yield was 40%. ¹H NMR (CDCl₃; δ, ppm): 8.44 (1H, m, Pyr H6), 7.68 (1H, m, Pyr H4), 7.59 (1H, m, Pyr H5), 7.41 (2H, m, DMTr), 7.30–7.16 (6H, m, DMTr), 7.06 (1H, m, Pyr H3), 6.84–6.80 (5H, m, DMTr), 3.78 (6H, s, MeO), 3.14 (2H, t, *J*^β = 7.3 Hz, DMTrOCH₂), 2.93 (2H, t, *J*^β = 6.0 Hz, PyrSSCH₂), 1.98 (2H, q, *J*^β = 7.3 Hz CH₂CH₂CH₂). TLC: *R*_f = 0.88 (19:1 CH₂Cl₂/MeOH), 0.60 (49:1 CH₂Cl₂/MeOH). MS: *m/z* at 503 (M⁺).

15-[(4,4'-Dimethoxytrityl)oxy]-12,13-dithiapentadecanoic Acid (11). To a solution of thiolacetic acid (13 mmol, 1.0 g) in dioxane (1 mL) was added a few crystals (1–2 mg) of dibenzoylperoxide. The solution was mixed with a solution of 10-undecenoic acid (6.6 mmol, 1.21 g) in dioxane (20 mL), and the mixture was stirred overnight at ambient temperature. The completeness of the reaction was checked by TLC; water (20 mL) was added, and the mixture was acidified with aqueous hydrogen chloride. Extraction with ethyl acetate (2 × 30 mL) gave 11-acetylthioundecanoic acid (**9**) as a white solid (1.63 g, 95%). Anal. (C₁₃H₂₄O₃S): C, H, S. ¹H NMR (CDCl₃; δ, ppm): 2.86 (2H, t, *J*^β = 7.4 Hz, SCH₂CH₂), 2.35 (2H, t, *J*^β = 7.4 Hz, CH₂COOH), 2.32 (3H, s, SCOCH₃), 1.63 (2H, q, *J*^β = 7.4 Hz, CH₂CH₂COOH), 1.56 (2H, q, *J*^β = 7.0 Hz, SCH₂CH₂), 1.31 (12H, br m, (CH₂)₆CH₂COOH). TLC: *R*_f = 0.60 (1:2 EtOAc/hexane).

9 (0.50 g, 1.9 mmol) was dissolved in 20 mL of MeOH and deacetylated with an excess of 40% aqueous methyamine (5 mL), evaporated to dryness, and dissolved in water (30 mL). The pH was adjusted to 2 with aqueous hydrogen chloride, and the deprotected 11-mercaptopentadecanoic acid (**10**) was extracted in diethyl ether (3 × 30 mL) and dried with Na₂SO₄. From the crude **10**, a sample (0.070 g, 0.32 mmol) was then dissolved in a mixture of pyridine and MeOH (1:9, 0.9 mL) and added to a solution of **5** (0.2 g, 0.4 mmol) dissolved in a mixture of MeOH and MeCN (1:1, 5 mL), and the solution was stirred at room temperature overnight. The evaporation residue was dissolved in CH₂Cl₂ (5 mL) containing 5%

Et₃N and fractionated on a silica gel column, using a stepwise gradient of MeOH (0 to 5%) in Et₃N/CH₂Cl₂ (5:95) as an eluent. The yield of **11** (starting from **9**) as a triethylammonium salt was 0.15 g (72%). ¹H NMR (CDCl₃; δ, ppm): 7.46–6.78 (13H, m, DMTr), 3.79 (6H, s, OCH₃), 3.35 (2H, t, *J* = 7.5 Hz, OCH₂CH₂), 2.85 (2H, t, *J* = 6.4 Hz, CH₂CH₂S), 2.56 (2H, t, *J*^β = 7.7 Hz, CH₂-COO), 2.29 (2H, t, *J*^β = 7.3 Hz, SSCH₂), 1.60 (2H, br m, SSCH₂CH₂), 1.25–1.40 (14 H, br m, 7 × CH₂). The spectrum also exhibited signals of the triethylammonium group. TLC: *R*_f = 0.74 (5% Et₃N in 9:1 CH₂Cl₂/MeOH), *R*_f = 0.33 (5% Et₃N in 19:1 CH₂Cl₂/MeOH).

N-[7-[(4,4'-Dimethoxytrityl)oxy]-4,5-dithiaheptanoyl] Supports (S1a–c). Long-chain aminoalkyl controlled pore glass (LCAA-CPG) was treated overnight with 3% dichloroacetic acid in CH₂Cl₂, subjected to successive washings with CH₂Cl₂, 20% Et₃N in MeOH, MeOH, and Et₂O, and finally dried in vacuo. Polystyrene and Tentagel supports were used as received. The supports were suspended in dry pyridine and degassed under reduced pressure (water pump). *N*-Hydroxysuccinimide (2–3 mmol) and 3,3'-dithiodipropionic acid (1–2 mmol, 20–100-fold excess with respect to the support-bound amino groups) were dissolved in pyridine, dried by coevaporation with pyridine, and dissolved again in a minimum amount of pyridine. The resulting solution was added to the suspension of the support in pyridine, and *N,N*-diisopropylcarbodiimide (2–3 mmol) and a catalytic amount of 4-(dimethylamino)pyridine were added. The suspension was shaken overnight, and the support was washed on a filter with pyridine, CH₂Cl₂, MeOH, and Et₂O. The support dried in the air was suspended in methanol and degassed. DTT (0.30 g, 1.9 mmol) and 30 μL of a 0.3 mol L⁻¹ solution of Et₃N in methanol were added, and the solution was shaken for several hours. After the mixture was washed with methanol, **5** (0.45 g) was immediately added to the suspension of the support in a mixture of MeOH and MeCN (1:1, 5 mL), and the suspension was shaken overnight. The support was washed on the filter with MeOH. The unreacted amino groups of the support (**S1a–c**) were finally capped by treating the support for 3 × 10 min with a 1:1 mixture of solutions A and B (A is 1:1:8 acetic anhydride/2,6-lutidine/THF and B is 16:84 *N*-methylimidazole/THF). The loading of the washed and dried support was determined by the dimethoxytrityl cation assay (26). The loadings ranged from 8 to 33 μmol g⁻¹.

N-[8-[(4,4'-Dimethoxytrityl)oxy]-4,5-dithiooctanoyl] Supports (S2a–c). These supports were prepared as described for **S1a–c** by using **8** instead of **5**.

N-[16-[(4,4'-Dimethoxytrityl)oxy]-12,13-dithiahexadecanoyl] Supports (S5a,b). 10-Undecenoic acid was attached to amino-modified polystyrene or Tentagel support by the *N*-hydroxysuccinimide and *N,N*-diisopropylcarbodiimide assisted acylation as described above for supports **S1a–c**. The *N*-acylated support was suspended in dioxane, and thioacetic acid diluted with dioxane (1:1, 800 μL) and a few crystals (1–2 mg) of dibenzoylperoxide were added. The suspension was shaken overnight, washed with dioxane, and dried on the filter. The *S*-acetyl group was removed with 1 mol L⁻¹ butylamine in MeOH, and the released mercapto function was coupled with **8** and capped to give supports **S5a,b**, as described for supports **S1a–c**. The loading determined by the dimethoxytrityl cation assay ranged from 7 to 16 μmol g⁻¹.

N-[15-[(4,4'-Dimethoxytrityl)oxy]-12,13-dithiapentadecanoyl] Supports (S4a,b). These supports were

prepared as described for supports **S5a,b** by using **5** instead of **8**. The support **S4b** was also obtained in two alternative manners. First, the Tentagel support was acylated with the triethylammonium salt of **11**, according to the procedure described for supports **S1a–c** and capped. Second, the Tentagel support was acylated with 11-acetylthioundecanoic acid (**9**), deacetylated with 1 mol L⁻¹ aqueous butylamine, reacted with **5**, and capped.

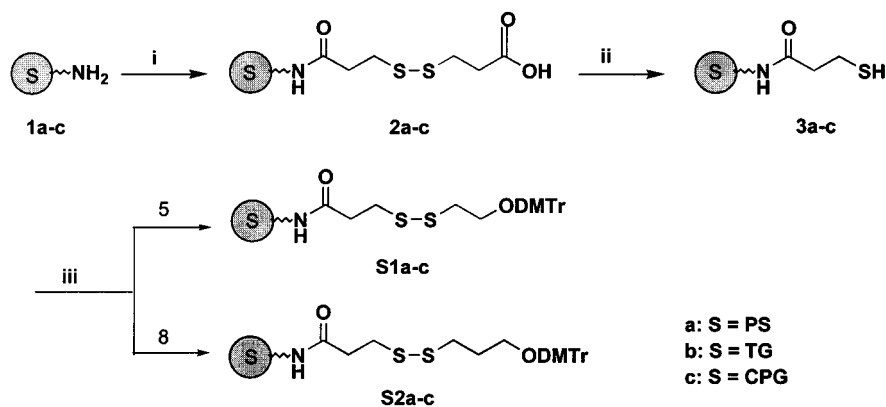
N-[10-[(4,4'-Dimethoxytrityl)oxy]-7,8-dithiadecanoyl] Support (S3). The support was prepared as described for supports **S5a,b** by using 5-hexenoic acid instead of 10-undecenoic acid and **3** instead of **7**.

Determination of the Hydrolytic Stability of S1–S5. The loading of each batch of **S1–S5** was determined by the dimethoxytrityl cation assay. The supports were then weighed into a synthesizer column and subjected to ammonolysis according to the protocol of manual deprotection and cleavage by a double-syringe method (27). The columns were kept at room temperature for 24 or 48 h. At the end of this period, the ammonia was withdrawn and the support was washed with water, aqueous MeCN, and MeCN, and dried. The dimethoxytrityl cation assay was repeated.

Oligodeoxynucleotide Synthesis. Supports **S4b** were used as supports in the automated oligonucleotide synthesis. The protected oligonucleotides were assembled on an Applied Biosystems 392 DNA synthesizer in 0.2 and 1.0 μmol scales, using the standard phosphoramidite protocol and unaltered coupling times even when the modified building block (**13**) was introduced (Scheme 6). The support-bound oligonucleotides were deprotected under standard deprotection conditions: treatment with concentrated ammonia (33%) at 55 °C for 8 h or at room temperature for 48 h. The support, still bearing the deprotected oligonucleotide, was washed with water and acetonitrile and dried.

Labeling of the Oligonucleotides on the Solid Support. An appropriate amount of the dried support, containing ~2 OD of oligomer on 0.5 mg of the support, was weighed in a microcentrifuge tube, and the labeling reagent, either 5-(dimethylamino)-1-naphthalenesulfonyl chloride (DNSCl) (7.7 mg, 0.28 μmol) or **14** (3.0 mg, 3.2 μmol), from Scheme 6, in 0.05:1:2.95 DBU/MeCN/H₂O (50 μL, v/v/v) was added (28). The tubes were shaken from 16 to 48 h in the dark at ambient temperature. The solid phase was filtered and washed with water and acetonitrile. The labeled oligonucleotides were detached from the supports with slightly basic 0.5 mol L⁻¹ aqueous DTT (100 μL, a 3 μmol L⁻¹ Et₃N/methanol solution in 1 mL of aqueous DTT or DTT in 0.1 mol L⁻¹ Tris buffer at pH 8.4). The reaction was performed either in a synthesizer column by a double-syringe method or in a 1.5 mL microcentrifuge tube. The support was removed by filtration and rinsed with water, aqueous MeCN, and MeCN. The filtrate and washings were combined and evaporated to dryness on a SpeedVac or rotary evaporator. The labeled oligonucleotide conjugate was precipitated with EtOH, and the quality and identity of the product were verified by HPLC. In the case of labeling with **14**, the extent of labeling was determined by releasing Eu(III) ion in solution with a fluorescence enhancement solution and measuring the fluorescence intensity on a time-resolved fluorometer against a europium(III) chloride standard solution (Delfia protocol) (29).

Hybridization Experiments. The hybridization experiments were carried out as described previously (30).

Scheme 2^a

^a (i) 3,3'-Dithiodipropionic acid/*N*-hydroxysuccinimide/*N,N*-diisopropylcarbodiimide/Pyr/DMAP; (ii) DTT/MeOH/Et₃N; (iii) for **S1a-c**, **5**/MeOH/MeCN; for **S2a-c**, **8**/MeOH/MeCN. DTT, 1,4-dithio-D,L-threitol; DMAP, *N,N*-(dimethylamino)pyridine.

Table 1. Results of the DMTr Cation Assay of the Supports before and after Hydrolysis

Symbol	Support material	Linker structure	loading before NH ₃ -treatment μmol/g	loading after NH ₃ -treatment μmol/g (% of original)	treatment time/h
S1a	Polystyrene		7.9	5.4 (68)	24
S1b	Tentagel	"	33	15 (45)	24
S1c	CPG	"	13	3 (23)	24
S2a	Polystyrene		3.2	2.4 (75)	24
S2b	Tentagel	"	50	23 (46)	24
S2c	CPG	"	32	12 (38)	24
S3	Tentagel		43	33 (77)	48
S4a	Polystyrene		3.3	3.8 (100)	48
S4b ^a	Tentagel	"	14	13 (93)	48
S4b ^b	Tentagel	"	19	16 (84)	48
S4b ^c	Tentagel	"	41	41 (100)	48
S5a	Polystyrene		6.8	6.3 (93)	48
S5b	Tentagel	"	16	15 (94)	48

^a From Scheme 4. ^b From Scheme 5, route A. ^c Scheme 5, route B.

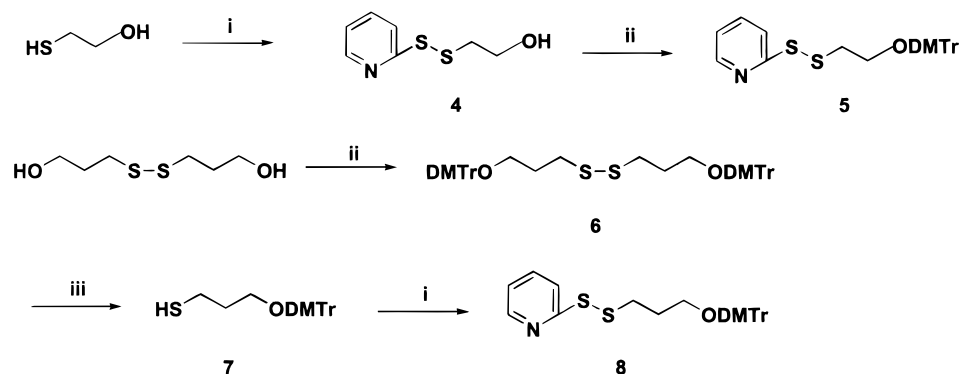
RESULTS AND DISCUSSION

Synthesis of the Supports. Solid supports **S1** and **S2** were prepared as depicted in Scheme 2. Accordingly, the amino functions of polystyrene, Tentagel, and LCAA CPG supports were first acylated with 3,3'-dithiodipropionic acid; the disulfide bond was reductively cleaved with DTT, and the released mercapto function was reacted with either 2-[(4,4'-dimethoxytrityl)oxy]ethyl 2-pyridyl disulfide (**5**; supports **S1a-c**) or 3-[(4,4'-dimethoxytrityl)oxy]propyl 2-pyridyl disulfide (**8**; supports **S2a-c**). The loadings determined by the dimethoxytrityl cation method are listed in Table 1. The 2-pyridyl disulfide-activated ω-[(4,4'-dimethoxytrityl)oxy]thiols, **5** and **8**, were prepared from 2-mercaptoethanol and bis-(3-hydroxypropyl) disulfide, as described in Scheme 3.

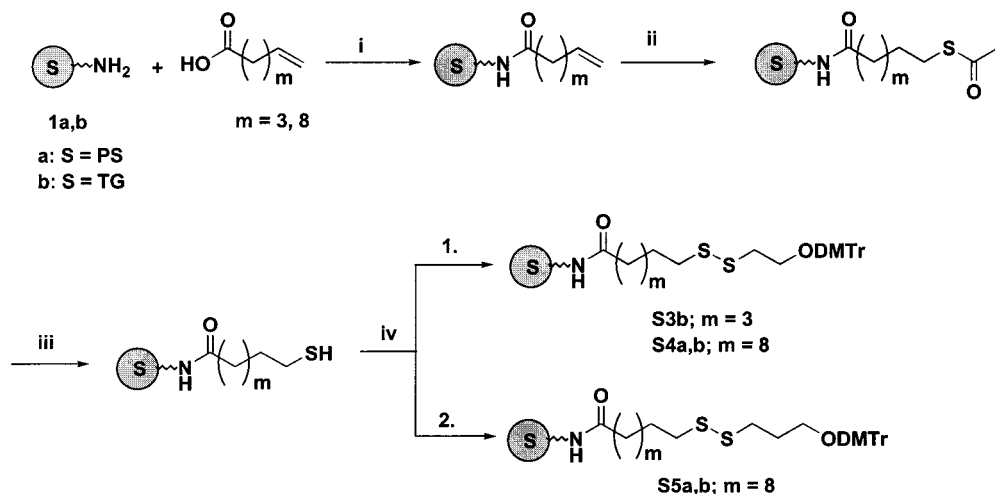
The rest of the supports employed (**S3-S5**) were obtained via initial acylation with ω-alkenoic acid, followed by radical addition of thiolacetic acid to the terminal double bond (*3l*) (Scheme 4). The *S*-acetyl

group was then removed by aminolysis with butylamine, and the released mercapto group was reacted with **5**. The Tentagel-based support **S4b** was also prepared in two alternative manners shown in Scheme 5. First, the disulfide linker (**11**) was synthesized in solution and attached to the amino-modified Tentagel by an *N*-acylation assisted by *N,N*-diisopropylcarbodiimide, *N*-hydroxysuccinimide, and 4-(dimethylamino)pyridine. Second, 11-(acetylthio)undecanoic acid (**9**) was prepared by radical addition in solution, and only the aminolysis and coupling with **5** were performed on the support (for the loadings, see Table 1).

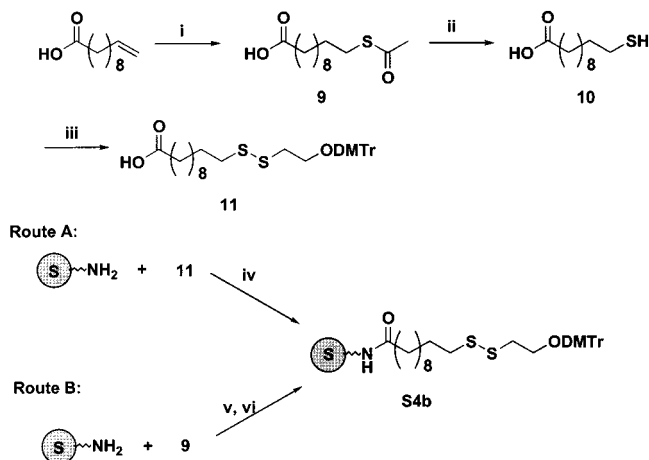
Hydrolytic Stability of the Disulfide-Tethered Supports (S1-S5). To evaluate the applicability of the supports in machine-assisted oligonucleotide synthesis, they were subjected to ammonolysis. According to the standard protocol, the base moiety protections are removed by treating the support-bound oligomer with concentrated aqueous ammonia for 48 h at room tem-

Scheme 3^a

^a (i) Bis(2-pyridyl) disulfide/MeOH/Pyr; (ii) DMTr-Cl/Pyr; (iii) DTT/MeOH/Et₃N.

Scheme 4^a

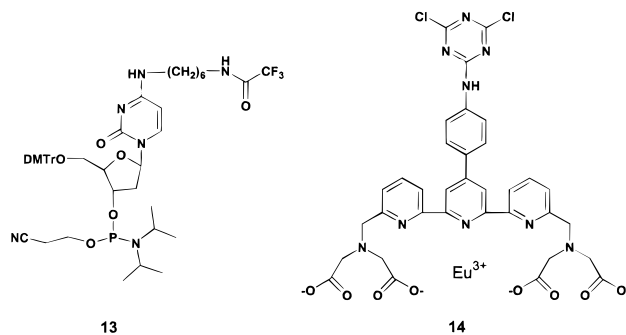
^a (i) *N*-Hydroxysuccinimide/*N,N*-diisopropylcarbodiimide/Pyr/DMAP; (ii) CH₃COSH/dioxane/dibenzoylperoxide; (iii) 1 M *n*-BuNH₂/MeOH; (iv) for **S3b** and **S4a,b**, 5/MeOH/MeCN; for **S5a,b**, 8/MeOH/MeCN.

Scheme 5^a

^a (i) CH₃COSH/dioxane/dibenzoylperoxide; (ii) aqueous MeNH₂/MeOH; (iii) 5/MeOH/Pyr; (iv) *N*-hydroxysuccinimide/*N,N*-diisopropylcarbodiimide/Pyr/DMAP; (v) 1 M *n*-BuNH₂/MeOH; (vi) 5/MeOH/Pyr.

perature or for 8 h at 55 °C. We applied the double-syringe method for 24–48 h at room temperature. Table 1 records the dimethoxytrityl loadings before and after this ammonolysis. These results indicate that the material of the support is of considerable importance. The loading of the CPG supports, **S1c** and **S2c**, decreased dramatically during the ammonolysis, which in all likelihood may be attributed to partial dissolution of glass.

Scheme 6. Structures of the Modified Phosphoramidite 13 and the Photoluminescent Lanthanide Chelate 14



Another important factor appears to be the length of the linker molecule; the resistance to ammonolysis increases with the length of the hydrocarbon chain connecting the disulfide group to the carbonyl carbon of the linker. Supports containing a dimethylene bridge are considerably more labile than those containing a nonamethylene bridge. This difference in stability is most clearly seen with the Tentagel-based supports **S2b** and **S5b**. Evidently, the carbonyl function and the disulfide group must be separated by at least three carbon atoms to prevent β -elimination. The distance between the disulfide linkage and the dimethoxytrityloxy group seems to be of lesser importance.

Synthesis of Oligonucleotides on Supports. A model sequence of T₆ was synthesized on those supports

that showed considerable resistance toward ammonolysis. The support-bound oligos were then subjected to ammonia treatment, and after 48 h the ammonia was washed from the supports and collected. These washings were then investigated by ion-exchange HPLC. The oligonucleotide that remained bound to the support was finally released with DTT under mildly basic conditions. The oligomer content of the washings was again determined by HPLC. The general conclusion of these studies was that assembly of an oligonucleotide on the deprotected hydroxy function of the supports still decreased the stability of the disulfide bond toward ammonolysis. Support **S4b**, in particular the batches obtained by route B in Scheme 5, exhibited the desired properties and was selected for further studies. More than 85% of the assembled oligomer remained bonded to the support after ammonolysis and could be released in 84% yield by subsequent reductive cleavage. Furthermore, the released oligomer was more homogeneous than with the other supports tried.

Support **S4b** prepared by route B in Scheme 5 was used to assemble a 15-mer oligonucleotide, 5'-d(GAA-CATCATGGTCGT)-3', and ammonolyze it in the dimethoxytrityl-on mode. According to RP HPLC, 10% of the full-length tritylated sequences were released during ammonolysis and 90% by the DTT treatment. The latter treatment also resulted in cleavage of the *O*-(2-mercaptoethyl) from the 3'-terminal phosphate group of the released oligomer. This reaction has been suggested to proceed by an intramolecular nucleophilic displacement of the phosphate group by the mercapto function (32). That this really happened under the conditions employed was verified by comparing a dT₆ sequence prepared on **S4b** to an authentic sample of 3'-phosphorylated dT₆ synthesized on a commercially available universal solid support producing oligonucleotide 3'-phosphates. These two materials exhibited identical retention times on ion-exchange HPLC, and when co-injected, gave only a single peak.

Labeling of Oligodeoxyribonucleotides on the Support. The labeling experiments were carried out with an 18-mer sequence, 5'-d(X₂ATATCATCTTTGGTGT)-3', assembled on **S4b** [X = N⁶-(6-aminoethyl)-2'-deoxycytidine] (33). The synthesis gave an average stepwise yield of 99%, calculated by the dimethoxytrityl cation release after the ammonia treatment. The free aliphatic amino groups of the 5'-terminal N⁶-aminoalkylated cytosine bases were labeled either with a prompt fluorophore, 5-(dimethylamino)-1-naphthalenesulfonyl chloride, or a dichlorotriazine derivative of a photoluminescent europium(III) chelate. The appropriate labeling reagent was dissolved in a minimum amount (<50 μL) of a mixture of DBU [1,8-diazabicyclo(5,4,0)undec-7-ene], water, and MeCN (0.05:1:2.95 v/v/v) and added to 0.5 mg of support-bound oligo (~2 OD) in a microcentrifuge tube. After incubation for 16 h in a shaker, the excess of the reagents was washed, and the support-bound, labeled oligonucleotides were released into solution with dithiothreitol. An ethanol precipitation of the oligonucleotides gave a DTT-free fraction of the labeled oligonucleotide. The extent of labeling of this oligonucleotide conjugate was determined according to the Delfia protocol (29). Accordingly, the europium(III) ion was released in solution and quantified against europium chloride standards. The amount of oligonucleotide was determined spectrophotometrically at 260 nm. The extent of labeling was 1.6, *i.e.*, about 80% of the theoretical value, 2. The presence of the prompt fluorophore (dansyl) was, in turn, verified by measuring the UV spectrum of the ethanol-

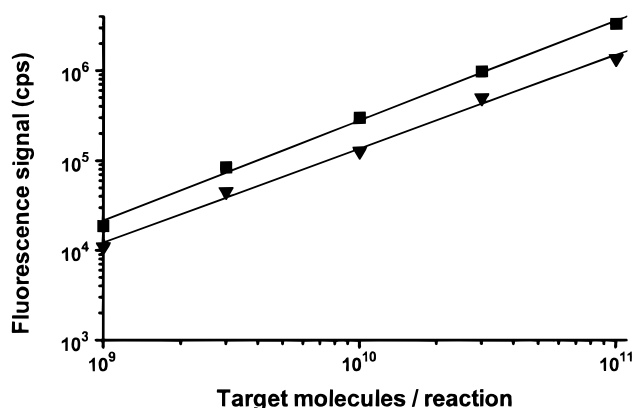


Figure 1. Sandwich hybridization on microparticles bearing a sequence complementary to labeled oligonucleotide. The fluorescence signal is measured from one particle as a function of target molecules added to the hybridization reaction mixture. A comparison of in-solution labeled oligo containing five chelates per molecule (line with squares) and solid-phase labeled oligo (maximum of two chelates per oligo) (line with triangles).

precipitated labeled oligo against water. The observed ratio of the absorbance at 360 nm (DNS moiety) and at 260 nm (base moieties) agreed with the almost theoretical extent of labeling. Additionally, the presence of a DNS oligoconjugate was verified with RP HPLC.

Hybridization Properties of the Multilabeled Oligonucleotides. The identity of the oligomer conjugate derived from the photoluminescent europium chelate was further verified by performing two parallel hybridization assays by the previously reported technique based on time-resolved fluorescence detection on microparticles (30). In one of the experiments, an oligonucleotide conjugate prepared in the present work was used. The signal intensity was then compared to that observed when an oligomer labeled previously in solution was used in the same assay (30). As seen from Figure 1, the ratio of the two sets of signals is consistent with the determined extent of labeling.

In summary, the methodology described in the present paper allows preparation of satisfactorily pure oligonucleotide 3'-monophosphates labeled with a photoluminescent europium chelate to the amino groups of N⁶-(aminoalkyl)cytosine residues. The chain assembly and labeling take place on a single solid support, and the resulting conjugate may be purified simply by washing, release in solution, and precipitation with ethanol.

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