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SYNTHESIS OF ACHIRAL LINKER REAGENTS FOR DIRECT LABELLING OF OLIGONUCLEOTIDES ON SOLID SUPPORTS

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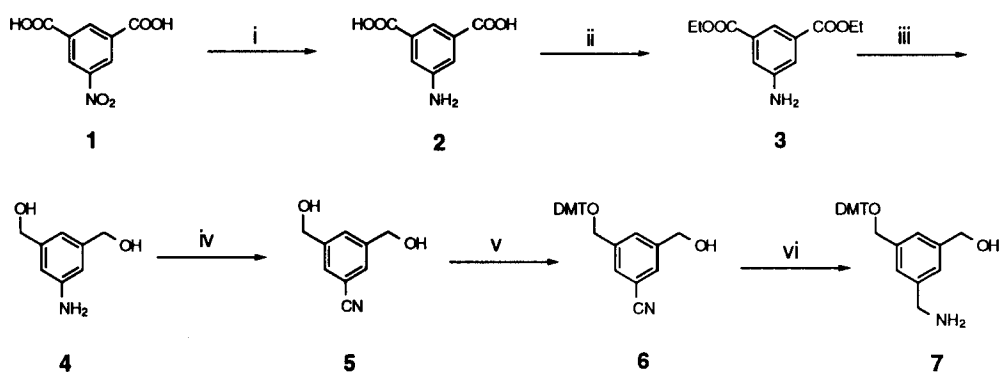
ABSTRACT: Full experimental procedures for the synthesis of a series of new functional linker reagents (14-16) and solid supports (11-13) are reported. The achiral linker reagents and supports can be used for high yield incorporation of free amino groups, fluorescein or biotin into DNA oligomers.

Modern DNA diagnostics rely extensively on chemical methods for attaching reporter groups to DNA fragments. For this purpose, labelling methods that can replace the hazardous radioisotopic ^{32}P - labelling are in great demand. Examples of nonradioactive reporter groups used in DNA diagnostics today include photo detectable dyes like fluorescein or rhodamine ¹ in addition to antibody / streptavidin detectable probes like digoxigenin ² and biotin. The first non-radioactive DNA labelling techniques reported were based on two step procedures where the DNA fragment initially was furnished with chemically reactive groups like thiols or primary aliphatic amines followed by subsequent chemical conjugation of desired bio-recognizable substances like biotin in aqueous solution ^{1,3,4}. In recent years numerous reagents allowing for direct attachment of nonradioactive reporter groups to DNA during solid phase synthesis have emerged ⁵. However, many of these reagents are only available as racemates leading to diastereomeric conjugates. We recently reported on an achiral amino modifying DNA linker reagent which could be introduced into oligonucleotides during solid phase synthesis without adding steric heterogeneity to the resulting conjugate ⁶. The linker reagent (8) could be either converted

into the phosphoramidite agent (**14**) allowing for multiple incorporation in various positions in the nucleotide sequence, or directly attached to the CPG support (**11**) rendering a 3'-modified oligonucleotide ⁶. We now report full experimental procedures for the synthesis and solid phase incorporation of the linker reagent (**8**), as well as experimental procedures for two analogues derivatized with fluorescein (**9**) and biotin (**10**). The hybridization properties of the modified oligodeoxyribonucleotides against DNA complements are also reported.

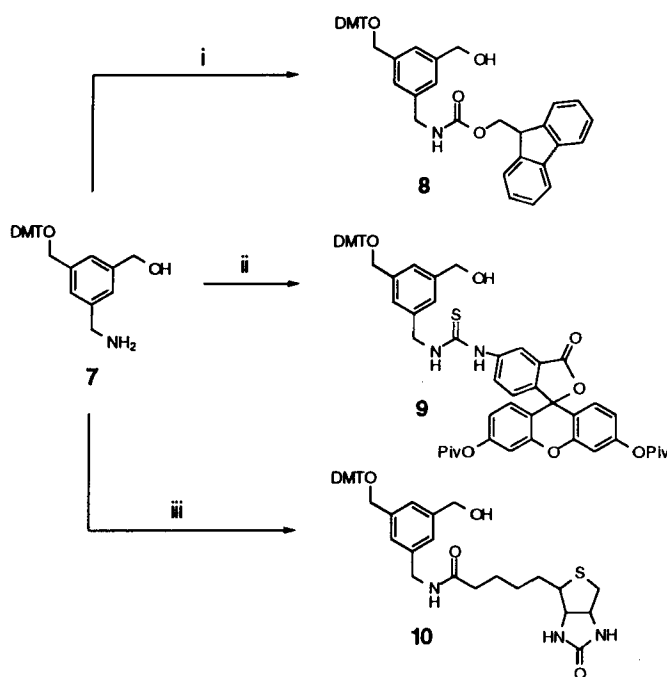
RESULTS AND DISCUSSION

The achirality of the linker reagent is based on the C_{2v} -symmetry of 5-(aminomethyl)-1,3-benzenedimethanol. Synthesis of the key intermediate **7** is depicted in **SCHEME 1**. Inexpensive 5-nitroisophthalic acid (**1**) initially was reduced to the amino diacid **2** using catalytic hydrogenation at 1 atm. over palladium on charcoal. Attempts to directly reduce **2** to the dialcohol **4**, using various metal hydrides or borane-dimethyl sulfide complexes proved unsuccessful. However, simple esterification of **2** with ethanol, followed by reduction of **3** with lithium aluminium hydride, gave the desired dialcohol **4** in more than 67 % overall yield after recrystallisation. It proved important to add **3** slowly to an excess of lithium aluminium hydride in order to obtain satisfying yields. The amino alcohol **4** then was converted to the diazonium salt and subsequently treated with sodium dicyanocuprate. The product was easily isolated by continuous extraction with dichloromethane, furnishing the cyano alcohol **5** in up to 74 % yield after recrystallisation from ethyl acetate. Using solvents more polar than dichloromethane resulted in extraction



SCHEME 1. *Reagents and conditions:* i) 1 atm. H_2 / Pd-C / MeOH, rt., 1 h (95 %). ii) EtOH, conc. H_2SO_4 , reflux, 16 h (82 %). iii) $LiAlH_4$ in THF, reflux, 3 h (80-82%). iv) $NaNO_2$, dil. HCl, $-5^\circ C$, 30 min. then $NaCu(CN)_2$, $0^\circ C$ 2h (66-74 %). v) DMT-Cl, anh. pyridine, rt., 16 h. (52 %). vi) $LiAlH_4$ in Et_2O , rt., 12 h, (79 %).

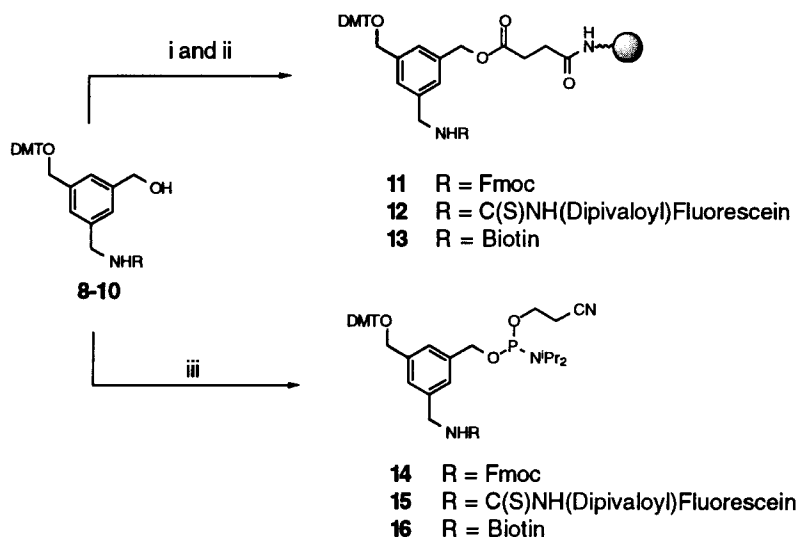
of side products which made the following crystallisation difficult. Attempts at reduction of the cyano group in **5** to the benzylic amine met with the same difficulties as the unsuccessful attempts with **2**. Although a range of reducing reagents and conditions were tried, in most cases the starting material was recovered unchanged. The problem seemed to be the low solubility of the initially formed metal alkoxides. This problem was solved by introducing the 4,4'-dimethoxytrityl group on one of the alcohol groups, thereby rendering the compound more soluble. Thus, **5** was treated with one equivalent of 4,4'-dimethoxytrityl chloride in pyridine to afford the mono protected dialcohol **6**. The cyano group in **6** easily underwent reduction with lithium aluminium hydride, and the benzylic amine **7** was obtained in fair yield. Chemoselective derivatization of **7** with either Fmoc-Cl, dipivaloyl-protected fluorescein isothiocyanate or biotin-NHS ester gave the functionalized derivatives **8**, **9** and **10** respectively (SCHEME 2). The derivatives **8-10** served as



SCHEME 2. *Reagents and conditions:* i) Fmoc-Cl / DIEA / DMF, rt., 40 min. (68 %). ii) Dipivaloylfluorescein isothiocyanate / THF, 0°C, 3h (78 %). iii) Biotin-NHS / DIEA / DMF, rt., 48 h (92 %).

precursors for synthesising the CPG-support bounded products **11-13** and the cyanoethyl phosphoramidites **14-16** as indicated in SCHEME 3. Derivatization of CPG followed a standard procedure: **8**, **9** or **10** were treated with succinic anhydride and the resulting acids converted into the p-nitrophenyl esters. The esters were reacted with LCA-CPG, and after

capping with acetic anhydride, the modified CPG supports **11-13** were obtained. Treatment of **8**, **9** or **10** with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite and tetrazole, followed by standard aqueous extraction and silica gel column chromatography or simple precipitation from hexane afforded the stable phosphoramidites **14**, **15** or **16** respectively.

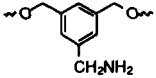


SCHEME 3. *Reagents and conditions:* i) succinic anhydride, DMAP, then p-nitrophenol / DCC / pyridine. ii) LCA-CPG, 2 h then Ac₂O / pyridine / DMAP (loading 22 - 33 μmol/g). iii) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite and tetrazole, acetonitrile, rt., 1h (66-85 %).

Solid Phase Synthesis

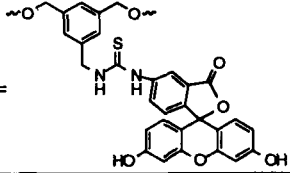
The modified CPG supports **11-13** and the cyanoethyl phosphoramidite linker reagents **14-16** were tested on a DNA synthesiser using standard protected deoxyribonucleoside phosphoramidites, Long Chain Alkylamine (LCA) CPG support and *tert*-butyl hydroperoxide as the oxidation reagent. The modified phosphoramidites **14-16** were manually coupled for 5 min. Initial experiments with iodine/water oxidation gave low yield of oligonucleotide products. This was in accord with literature reports on simple dibenzyl phosphoramidites, which with alcohols give low yields of phosphates when the intermediate dibenzyl phosphites are oxidized with iodine/water, but good yields when *tert*-butyl hydroperoxide or peracids are used as oxidants^{7,8}. Results from incorporation studies are seen in **TABLE 1-3**. With all reagents (**14-16**) and modified supports (**11-13**) the coupling efficiencies were good, and isolated yields of modified oligonucleotides were in the range of 60-75 % after ethanol precipitation, as calculated by optical density measurements.

TABLE 1

| Sequence | X =  | Reagent | Coupling efficiency | |
|---|---|---------|---------------------|----------------------|
| | | | X ^a | average ^b |
| 5' - X - GTA GAT CAC T - 3' | | 14 | 95 % | 97.5 % |
| 5' - GTA GAT CAC T - X - 3' | | 11 | ----- | 99.5 % |
| 5' - TGT ACG TXA CAA CTA - 3' | | 14 | 99 % | 98.2 % |
| 5' - TGT ACX TCA XAA CTA - 3' | | 14 | 98-99 % | 98.5 % |
| 5' - X TGT ACG TCA CAA CTA X - 3' | | 11 | ----- | 99.2 % |
| | | 14 | 98 % | |
| 5' - T ₁₀ XX T ₁₀ - 3' | | 14 | 97-98 % | 98.5 % |
| 5' - X - CAT GAT CTG ACA GAG GGA ACC CAG T - 3' | | 14 | 90 % | 99.2 % |
| 5' - CAT GAT CTG ACA GAG GGA ACC CAG T - X - 3' | | 11 | ----- | 99.3 % |
| 5' - XXX - GTA GAT CAC T - 3' | | 14 | 95-99 % | 98.8 % |
| 5' - GTA GAT CAC TXX A GT GAT CTA C - 3' | | 14 | 97-99 % | 99.2 % |

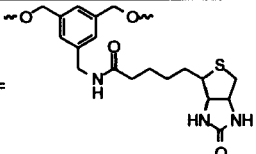
- a) Coupling efficiency for reagent 14 (determined from the DMT-cation release).
 b) Average coupling efficiency for the sequence indicated, including X.

TABLE 2

| Sequence | F =  | Reagent | Coupling efficiency | |
|---------------------------------------|---|---------|---------------------|----------------------|
| | | | F ^a | average ^b |
| 5' - FGT AGA TCA CT - 3' | | 15 | 99 % | 98.8 % |
| 5' - GTA GAT CAC TF - 3' | | 12 | ----- | 98.9 % |
| 5' - FFF GTA GAT CAC T - 3' | | 15 | 95-99 % | 98.8 % |
| 5' - FGC AGT TGA GTG CTT GCT AGG - 3' | | 15 | n.d. | 98.7 % |
| 5' - GGT AGC AGC CAG AGC GGT GGF-3' | | 12 | ----- | 98.6 % |

- a) Coupling efficiency for reagent 15 (determined from the DMT-cation release).
 b) Average coupling efficiency for the sequence indicated, including F.

TABLE 3

| Sequence | B =  | Coupling efficiency | |
|---------------------------------------|--|---------------------|--|
| | | Reagent | B ^a average ^b |
| 5' - BGT AGA TCA CT - 3' | 16 | 100 % | 98.8 % |
| 5' - GTA GAT CAC TB - 3' | 13 | ----- | 99.2 % |
| 5' - BBB GTA GAT CAC T - 3' | 16 | 100 % | 100 % |
| 5' - BGC AGT TGA GTG CTT GCT AGG - 3' | 16 | 100 % | 99.1 % |
| 5'- GGT AGC AGC CAG AGC GGT GGB-3' | 13 | ----- | 99.2 % |

a) Coupling efficiency for reagent **16** (determined from the DMT-cation release).

b) Average coupling efficiency for the sequence indicated, including **B**.

No base experiment

The linker, by virtue of its rigid 5 carbon atom spacer, is longer than the natural 3-carbon internucleotide phosphodiester bond distance. The effect on the stability of a double stranded DNA from incorporation of the linker element instead of a normal nucleoside unit (a no base effect) was evaluated by using **14** as the central monomer in a pentadecamer (**TABLE 4**). As seen from the melting temperatures (T_m , medium salt) the linker seriously disrupts the hybridisation. Compared to an unmodified oligomer (entry 5), the incorporation of one linker reagent lowers the thermal stability by some 20 °C. This reflects a general structural disturbance of the deoxyribose phosphate backbone as the effect is independent of the base in the complementary strand (entry 1-4).

Upon double incorporation of the linker element into central positions of a pentadecamer, the modified oligonucleotide lost its ability to hybridize completely (**FIG. 1a**). However, when added to the end positions of a pentadecamer, the linker element gave no significant disturbance of the hybridization properties (**FIG. 1b**).

In conclusion a new set of achiral linker reagents (**14-16**) and a set of modified CPG supports (**11-13**) designed for incorporating either free amino groups, fluorescein or biotin into oligonucleotides without adding steric heterogeneity to the resulting product have been synthesised and evaluated. The linker reagents (**14-16**) can be incorporated into

5' TAG TTG TZA CGT ACA 3'
3' ATC AACAYT GCA TGT 5'

TABLE 4: T_m values for a series of pentadecamers hybridized to a modified oligonucleotide carrying the linker element ($Y = X$) at the central position. The measurements were made in a medium salt buffer (see experimental section). $X = 5$ -(aminomethyl)-1,3-benzenedimethanol.

| | Z | Y | T_m |
|---|---|---|-------|
| 1 | G | X | 32.5 |
| 2 | A | X | 33.0 |
| 3 | C | X | 31.5 |
| 4 | T | X | 31.0 |
| 5 | G | C | 54.0 |

| | | |
|----|-------------------------------|------------------------------------|
| a) | 5' TAG TTG TGA CGT ACA 3' | $T_m < 10\text{ }^\circ\text{C}$ |
| | 3' ATC AAX ACT XCA TGT 5' | |
| b) | 5' TAG TTG TGA CGT ACA 3' | $T_m = 53.0\text{ }^\circ\text{C}$ |
| | 3' X ATC AAC ACT GCA TGT X 5' | |

FIGURE 1: T_m values for two sets of oligonucleotide duplexes carrying the modified linker. $X = 5$ -(aminomethyl)-1,3-benzenedimethanol

the oligonucleotide at any position using standard phosphoramidite chemistry; however when incorporated into the central part of the oligonucleotide, a marked reduction in the oligonucleotide binding property is observed. Apart from the necessity of using *tert*-butyl hydroperoxide in the oxidation step, the linker reagents and solid supports are fully compatible with the standard automated DNA synthesis protocol, and have been used with success in repetitive coupling cycles.

EXPERIMENTAL

General remarks

N-Hydroxysuccinimidobiotin [35013-72-0] was obtained from Sigma, and fluorescein isothio-cyanate [3326-32-7] and 5-nitroisophthalic acid [618-88-2] from Aldrich. 2-Cyanoethyl *N,N,N',N'*-tetraisopropyl-phosphorodiamidite [102691-36-1] and DMT-Cl [40615-36-9] were obtained from K.J. Ross-Petersen AS DK-2970 Hørsholm, Denmark. Protected deoxyribonucleoside phosphoramidites and supports were from Cruachem. All other reagents (Aldrich or Fluka) and solvents (LabScan) were of standard quality and used without further purification unless indicated. NMR spectra were recorded on either a Bruker 250 MHz or a Varian 400 MHz spectrometer with tetramethylsilane (^1H and ^{13}C) as internal standard and 85% H_3PO_4 (^{31}P) as external standard. IR spectra was recorded on a Perkin Elmer FTIR 1760 instrument. Melting points were recorded on a Büchi melting point apparatus and are uncorrected. Oligonucleotide synthesis was performed on a Biosearch 8750 DNA synthesiser at 0.2 - 1.0 μmol scale. Standard synthesis cycles were used but with *tert*-butylhydroperoxide (1.1 M in acetone / dichloromethane (1:1), 3min) as the oxidation reagent. The phosphoramidites **14-16** were coupled manually (10-20 eq. for 5 min). After standard ammonia treatment the products were ethanol precipitated and analyzed by capillary electrophoresis.

5-Aminoisophthalic acid(2):

5-Nitroisophthalic acid (9.98g, 47.7 mmol) was dissolved in methanol (100 ml), and the solution was flushed with nitrogen. Palladium, 10% on carbon (0.50 g), was added, and the mixture was hydrogenated at 1 atm., 20°C, for 1 h. The mixture was filtered, and the filter cake washed with hot methanol (2 x 50 ml). The combined extracts were taken to dryness, and the residue used without further purification. Yield: 8.21 g (95%). Mp > 260 °C. ^1H NMR ($\text{DMSO}-d_6$): δ 5.60 (bs, 2H); 7.36 (s, 2H); 7.64 (s, 1H); 12.60 (bs, 2H). ^{13}C NMR ($\text{DMSO}-d_6$): δ 117.53; 188.27; 131.75; 149.31; 167.30. MS (FAB+) m/z 182.04 ($\text{M} + \text{H}^+$) ($\text{C}_8\text{H}_7\text{NO}_4$ requires 182.045). Anal. Calc. for $\text{C}_8\text{H}_7\text{NO}_4$: C, 53.04; H, 3.80; N, 7.73. Found: C, 53.26; H, 3.80; N, 7.68.

Diethyl 5-aminoisophthalate(3):

5-Aminoisophthalic acid (**2**, 25.0 g, 0.14 mol) was dissolved in abs. ethanol (400 ml), and conc. sulfuric acid (20 ml) was added. After refluxing for 16 h the mixture was poured on ice-water containing sodium hydrogen carbonate (50 g). The aqueous solution was extracted with diethyl ether (3 x 100 ml). The organic phase was washed with aq. saturated sodium chloride solution, and dried over anhydrous magnesium sulphate. Evaporation *in vacuo*, followed by recrystallisation of the residue from ethanol/water (1:1) yielded 26.8 g (82 %) of diethyl 5-aminoisophthalate as white needles. Mp: 88-90 °C. ¹H NMR (DMSO-*d*₆): δ 1.30 (t, 6H); 4.29 (q, 4H); 5.72 (s, 2H); 7.40 (s, 2H); 7.64 (s, 1H). ¹³C NMR (DMSO-*d*₆): δ 14.24; 60.83; 116.67; 118.09; 130.96; 149.54; 165.57. MS (FAB⁺) *m/z* 238.12 (M + H)⁺ (C₁₂H₁₆NO₄ requires 238.108). Anal. Calc. for C₁₂H₁₅NO₄: C, 60.76; H, 6.33; N, 5.91. Found: C, 60.83; H, 6.31; N, 5.84.

5-Amino-1,3-benzenedimethanol(4):

Lithium aluminium hydride (6.0 g, 158 mmol) was added to a reaction flask charged with dry tetrahydrofuran (220 ml) under nitrogen, and the resulting suspension heated to reflux. A solution of diethyl 5-aminoisophthalate (**3**, 10.0 g, 42.2 mmol) in dry tetrahydrofuran (100 ml) was added to the refluxing mixture over a period of 1 h. After refluxing for an additional 2 h, the mixture was cooled to room temperature, and methanol (8 ml), then water (12 ml), was added. The mixture was stirred for 2 h and filtered. The filtrate was evaporated to dryness *in vacuo*, and the residue recrystallized from ethyl acetate. Yield 5.19 - 5.32 g (80 - 82 %). Mp: 110-112 °C. ¹H NMR (D₂O): δ 4.47 (s, 4H); 6.68 (s, 2H); 6.80 (s, 1H). ¹³C NMR (D₂O): δ 66.44; 114.07; 116.78; 141.68; 146.46. MS (FAB⁺) *m/z* 154.06 (M + H)⁺ (C₈H₁₂NO₂ requires 154.087). Anal. Calc. for C₈H₁₁NO₂: C, 62.74; H, 7.19; N, 9.15. Found: C, 62.77; H, 7.11; N, 9.14.

3,5-Bis(hydroxymethyl)benzonitrile(5):

5-Amino-1,3-benzenedimethanol (**4**, 5.00 g, 32.7 mmol) was dissolved in hydrochloric acid (2N, 100 ml), cooled to -5 °C, and stirred for 30 min. A cold sodium nitrite solution (2.48 g, 36 mmol in 50 ml water) was slowly added, while the temperature was kept at -5 °C. The solution was stirred for 30 min., and subsequently neutralized with solid sodium carbonate (6.80 g, 64 mmol). A solution of cuprous cyanide (3.20 g, 35.7 mmol) and sodium cyanide (3.52 g, 71.8 mmol) in water (50 ml) was prepared, and after cooling to 0°C, added in one portion to the stirred diazonium salt solution. The temperature was kept at 0°C for 2 h, while the solution changed colour from yellow to black. After stirring overnight, followed by filtration, the dark solution was extracted continuously with methylene chloride (230 ml) for 36 h. The organic phase was dried over anhydrous sodium

sulphate and evaporated to dryness *in vacuo*. The residue was recrystallized from ethyl acetate. Yield 3.52 - 3.94 g (66-74 %). Mp: 138-140 °C. ^1H NMR (D_2O): δ 4.64 (s, 4H); 7.61 (s, 1H); 7.64 (s, 2H). ^{13}C NMR (D_2O): δ 62.51; 111.18; 119.23; 129.71; 130.42; 141.82. IR(KBr): ν = 2230 cm^{-1} . MS (FAB $^+$) m/z 164.04 ($\text{M} + \text{H}^+$) ($\text{C}_9\text{H}_{10}\text{NO}_2$ requires 164.071). Anal. Calc. for $\text{C}_9\text{H}_9\text{NO}_2$: C, 66.26; H, 5.52; N, 8.59. Found: C, 66.24; H, 5.65; N, 8.59.

3-(4,4'-Dimethoxytrityloxymethyl)-5-(hydroxymethyl)benzonitrile (6):

3,5-Bis(hydroxymethyl)benzonitrile (**5**, 1.00 g; 6.13 mmol) was dissolved in anhydrous pyridine (25 ml), and 4,4'-dimethoxytrityl chloride (2.29 g; 6.76 mmol) was added. The mixture was stirred overnight at 20 °C under a nitrogen atmosphere. Methanol (5 ml) was added, and the mixture stirred for another 10 min. Pyridine was removed by evaporation *in vacuo*, and the residue suspended in diethyl ether (50 ml). After filtration of unreacted starting material, the filtrate was concentrated to 4 ml, and applied on a silica gel column (25 x 4 cm). The column was eluted with hexane / ethyl acetate (800 ml; 3 : 1) followed by hexane / ethyl acetate (400 ml; 1 : 1). Fractions showing a single spot on TLC at R_f 0.6 (ethyl acetate / hexane 1:1) were pooled, and the solvent removed by rotary evaporation to give 1.49 g (52.3 %) of an yellow oil. The oil solidified to an amorphous compound by evaporation to dryness from diethyl ether. ^1H NMR ($\text{DMSO}-d_6$): δ 3.72 (s, 6H); 4.15 (s, 2H); 4.53 (d, 2H); 5.48 (t, 1H); 6.89 (d, 4H); 7.2-7.7 (m, 12 H). ^{13}C NMR ($\text{DMSO}-d_6$): δ 55.12; 62.03; 64.33; 86.43; 111.19; 113.49; 119.12; 127.00; 127.78; 128.16; 128.47; 128.69; 129.50; 129.89; 135.60; 140.47; 144.51; 144.88; 158.37. IR(KBr): ν = 2232 cm^{-1} . Anal. Calc. for $\text{C}_{30}\text{H}_{27}\text{NO}_4$: C, 77.39; H, 5.84; N, 3.00. Found: C, 77.07; H, 5.95; N, 3.03.

3-(4,4'-Dimethoxytrityloxymethyl)-5-(hydroxymethyl)benzylamine (7):

3-(4,4'-Dimethoxytrityloxymethyl)-5-(hydroxymethyl)benzonitrile (**6**, 357 mg; 0.76 mmol) was dissolved in dry diethyl ether (50 ml), and lithium aluminium hydride (90 mg, 2.40 mmol) was added. The mixture was stirred overnight, and then quenched with methanol (3.0 ml). Water (0.5 ml) was added, and the mixture stirred for a further 30 min. The inorganic salts were filtered off and the solvent removed by evaporation *in vacuo*. The hygroscopic foam obtained was sufficiently pure to be used in later reactions. Yield: 280 mg (79 %). ^1H NMR ($\text{DMSO}-d_6$): δ 3.54 (broad s; 3H); 3.82 (s, 6H); 3.85 (s, 2H); 4.11 (s, 2H); 4.58 (s, 2H); 6.98-7.53 (m, 16H). ^{13}C NMR($\text{DMSO}-d_6$): δ 55.08; 62.94; 65.21; 67.05; 85.91; 113.30; 123.41; 124.40; 124.57; 126.74; 127.70; 127.93; 129.67; 135.71; 138.28; 141.87; 142.60; 144.94; 158.13.

N-Fmoc-3-(4,4'-dimethoxytrityloxymethyl)-5-(hydroxymethyl)benzylamine (8):

3-(4,4'-Dimethoxytrityloxymethyl)-5-(hydroxymethyl)benzylamine (**7**, 460 mg; 0.98 mmol) was dissolved in dry DMF (6 ml), and cooled on a ice bath. Diisopropylethylamine (153 mg; 1.19 mmol) and 9-fluorenylmethyl chloroformate (280 mg; 1.08 mmol) was added, and the mixture stirred for 40 min. during which time the temperature reached 20 °C. The mixture was poured into a cold solution of 1 N sodium hydrogen carbonate (100 ml), and the precipitated solid collected by filtration. The solid was applied on a silica gel column (20 g), which was eluted with n-hexane / ethyl acetate (400 ml; 1:1). Fractions showing one spot on TLC at R_f 0.23 (ethyl acetate / hexane 1:1) were collected and the solvent removed on a rotary evaporator. The resulting oil was solidified by addition of water (5 ml). Yield 462 mg (68 %). Mp: 68-72 °C. ^1H NMR(DMSO- d_6): δ 3.80 (s, 6H); 4.10 (s, 2H); 4.27 (t, 2H); 4.36 (d, 2H); 4.39 (t, 1H) 4.56 (d, 2H); 5.26 (t, 1H); 6.96-7.97 (m, 24 H). ^{13}C NMR(DMSO- d_6): δ 43.96; 46.79; 55.16; 63.00; 65.22; 65.62; 113.41; 120.24; 123.58; 124.27; 125.28; 126.87; 127.16; 127.73; 128.05; 129.80; 135.81; 158.25. (only strong signals are reported). Anal. Calc. for $\text{C}_{45}\text{H}_{41}\text{NO}_6$: C, 78.12; H, 5.97; N, 2.02. Found: C, 77.84; H, 5.96; N, 2.02.

Dipivaloylfluorescein isothiocyanate:

Fluorescein isothiocyanate (200 mg; 0.51 mmol) was dissolved in dry THF (25 ml). Diisopropylethylamine (270 μl ; 1.53 mmol) was added, and the solution was cooled to 0°C on an ice bath. Pivaloyl chloride (155 mg; 1.28 mmol) was added, and the mixture was stirred for 10 min. at 0°C, and then for 12 h at room temperature. Methanol (5 ml) was added, and stirring was continued for an additional 30 min. Solvents were removed *in vacuo*, and the residue purified by silica gel column chromatography with dichloromethane as the eluent. Yield: 217 mg (76 %) R_f 0.6 (dichloromethane). ^1H NMR (CDCl_3): δ 1.35 (s, 18 H); 6.79 (s, 4 H); 7.05 (s, 2 H); 7.14 (d, 1 H, $J = 8.0$ Hz); 7.47 (dd, 1H, $J_1 = 8.0$ Hz, $J_2 = 1.7$ Hz); 7.82 (d, 1H, $J_1 = 1.7$ Hz). ^{13}C NMR(CDCl_3): δ 26.96; 39.11; 81.00; 110.36; 115.33; 117.75; 121.67; 125.31; 127.64; 128.53; 132.38; 133.94; 139.41; 150.85; 151.39; 152.67; 167.42; 176.31.

N-(Dipivaloylfluoresceinyl)-N'-3-(4,4'-dimethoxytrityloxymethyl)-5-(hydroxymethyl)benzyl thiourea (9):

3-(4,4'-Dimethoxytrityloxymethyl)-5-(hydroxymethyl)benzylamine (**7**, 790 mg; 1.68 mmol) was dissolved in Al_2O_3 -dried tetrahydrofuran (20 ml), and 2 drops of triethylamine was added. The reaction flask was cooled on an ice bath, and a solution of dipivaloylfluorescein isothiocyanate (937 mg; 1.68 mmol) in dry THF (30 ml) was added

over 3 min. The mixture was stirred at 0°C for 3 h, then taken to dryness by evaporation *in vacuo*. The white foam obtained was purified by column chromatography on silica (eluent: 5% triethylamine / 5% methanol / 90 % dichloromethane), the purified fractions evaporated, and the residue dissolved in a small amount of dichloromethane and precipitated into hexane, to give pure **9** as a yellow powder. Yield: 1.32 g (78 %). ¹H NMR (DMSO-*d*₆): δ 1.33 (s, 18 H); 3.01 (bs, 1H); 3.75 (s, 6H); 4.15 (s, 2H); 4.64 (d, 2H); 4.85 (bs, 2H); 5.26 (t, 1H); 6.95-7.80 (m, 25H); 8.52 (s, 1H). ¹³C NMR (DMSO-*d*₆): δ 27.06; 55.21; 64.89; 65.36; 110.37; 113.16; 115.95; 117.75; 118.24; 124.60; 124.86; 125.72; 126.77; 127.85; 128.12; 128.88; 130.05; 136.08; 158.46; 176.47; 181.08 (only strong signals are reported). Anal. Calc. for C₆₁H₅₈N₂O₁₁S: C, 71.33; H, 5.69; N, 2.73. Found: C, 70.00; H, 6.46; N, 3.60.

***N*-Biotinyl-(4,4'-dimethoxytrityloxymethyl)-5-(hydroxymethyl)benzylamine (10):**

3-(4,4'-Dimethoxytrityloxymethyl)-5-(hydroxymethyl)benzylamine (**7**, 470 mg; 1.0 mmol), was dissolved in dry DMF (8.0 ml), and diisopropylethylamine (196 μl) was added followed by *N*-hydroxysuccinimidobiotin (375 mg, 1.1 mmol). The resulting clear solution was stirred at room temperature for 48 h. The reaction mixture was partitioned between water and dichloromethane, the phases separated, and the water phase extracted twice with dichloromethane. The combined organic phases were washed twice with saturated aqueous sodium bicarbonate solution and once with brine. After drying with anhydrous sodium sulfate, the solvents were removed to leave an oily residue. Further purification by column chromatography on silica using 5 % triethylamine and 5 % methanol in dichloromethane as the eluent gave 650 mg (92 %) of the title compound as a hygroscopic glass. ¹H NMR (DMSO-*d*₆): δ 1.2-1.6 (m, 6H); 2.15 (t, 2H); 2.56 (d, 1H); 2.76 (dd, 1H); 3.05 (m, 1H); 3.74 (s, 6H); 4.02 (s, 2H); 4.08 (m, 1H); 4.25 (s, 2H); 4.27 (s, 1H); 4.48 (d, 2H); 5.19 (t, 1H); 6.35 (s, 1H); 6.40 (s, 1H); 6.90-7.45 (m, 16H); 8.32 (t, 1H). ¹³C NMR (DMSO-*d*₆): δ 25.39; 28.08; 28.30; 35.27; 42.05; 45.63; 55.11; 55.44; 59.26; 61.07; 62.90; 65.10; 85.91; 113.31; 123.36; 124.06; 124.27; 126.78; 127.70; 127.95; 129.69; 135.71; 138.43; 139.70; 142.70; 144.96; 158.15; 162.73; 172.02. Anal. Calc. for C₄₀H₄₅N₃O₆S: C, 69.04; H, 6.51; N, 6.04; S, 4.60. Found: C, 66.00; H, 6.58; N, 5.98; S, 4.76.

General procedure for the preparation of modified CPG - supports (11-13):

The functionalized derivative **8**, **9** or **10** (0.20 mmol) was dissolved in pyridine (2.0 ml). Succinyl anhydride (20 mg; 0.20 mmol) and 4-*N,N*-dimethylaminopyridine (10 mg; 0.08 mmol) was added, and the mixture was stirred at room temperature overnight. The solvent was removed, and the residue redissolved in ethyl acetate (10 ml). The organic phase was

washed with brine (2 x 10 ml), dried with anhydrous sodium sulfate, and taken to dryness by evaporation *in vacuo*. Traces of water were removed by co-evaporation from pyridine (2 x 5.0 ml), and the oily residue was dissolved in anhydrous DMF (2.0 ml). Pyridine (200 μ l), 4-nitrophenol (43 mg; 0.31 mmol) and dicyclohexylcarbodiimide (45 mg; 0.22 mmol) was added. The mixture was stirred for 4 h at room temperature. Long chain alkylamine CPG (300 mg, 89 μ mol/g) previously dried by evaporation from dry acetonitrile, was added. The mixture was placed in an orbital shaker overnight. The CPG was filtered off and washed with DMF (2x20ml), methanol (3x15ml), and diethyl ether (2x15ml). The modified CPG was capped by treatment with a solution of acetic anhydride / pyridine / dichloromethane (6 ml, 1/2/2) for 2 h. After filtration, the CPG was washed with pyridine (20 ml), DMF (3x20ml), water (3x20ml), methanol (3x20ml) and diethyl ether (5x20ml) and finally dried *in vacuo*. The loadings were determined spectrophotometrically from the absorbance of the DMT cation following standard procedures. The loadings were 25.0, 33.1 and 22.3 μ mol/g for **11**, **12** and **13** respectively.

General procedure for the preparation of cyanoethyl N,N-diisopropylphosphoramidites (14-16):

The functionalized derivative **8**, **9** or **10** (0.70 mmol) was dissolved in dry dichloromethane (4.0 ml). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (241 mg; 0.80 mmol), and a solution of tetrazole (56 mg; 0.80 mmol) in acetonitrile (2.0 ml) were added. The mixture was stirred for 1 h at room temperature, before diisopropylethylamine (0.7 ml) was added. The reaction mixture was diluted with dichloromethane (5.0 ml), and washed with a 5% solution of sodium bicarbonate in water (2 x 10 ml). The organic phase was dried with anhydrous magnesium sulfate, and evaporated to dryness *in vacuo*. The residue was either purified by column chromatography on silica using ethyl acetate / dichloromethane / triethylamine (50:45:5) as the eluent or dissolved in a minimum of dichloromethane and precipitated from cold hexane. Yield: 66-85 %.

Fmoc-protected cyanoethyl N,N-diisopropylphosphoramidite (14):

^{31}P NMR (CDCl_3): δ 148.5. ^1H NMR (CDCl_3) δ 1.19 (t, 12H); 2.57 (t, 2H); 3.63 (dt, 2H); 3.78 (s, 6H); 3.82 (m, 2H); 4.18 (s, 2H); 4.22 (t, 1H); 4.39 (d, 2H); 4.43 (d, 2H); 4.71 (ddd, 2H); 5.15 (t, 1H); 6.82-7.75 (m, 24H). Anal. Calc. for $\text{C}_{54}\text{H}_{58}\text{N}_3\text{O}_7\text{P}$: C, 72.71; H, 6.55; N, 4.71. Found: C, 72.65; H, 6.45; N, 4.70.

Fluoresceinyl modified cyanoethyl N,N-diisopropylphosphoramidite (15):

^{31}P NMR (CDCl_3): δ 148.4. ^1H NMR ($\text{DMSO}-d_6$): δ 1.15 (t, 12H), 1.35 (s, 18 H); 2.74

(t, 2H); 3.01 (bs, 1H); 3.53 (t, 2H); 3.72 (m, 2H); 3.75 (s, 6H); 4.15 (s, 2H); 4.64 (d, 2H); 4.85 (dd, 2H); 6.95-7.80 (m, 25H); 8.91 (bs, 1H).

Biotinyl modified cyanoethyl N,N-diisopropylphosphoramidite (16):

³¹P NMR (DMSO-*d*₆): δ 148.6. ¹H NMR (DMSO-*d*₆): δ 1.14 (t, 12H); 1.2-1.6 (m, 6H); 2.13 (t, 2H); 2.57 (s, 1H); 2.74 (t, 2H); 2.84 (m, 1H); 3.04 (m, 1H); 3.58 (m, 4H); 3.73 (s, 6H); 4.02 (s, 2H); 4.08 (m, 1H); 4.25 (d, 2H), 4.27 (s, 1H); 4.71 (dd, 2H); 6.34 (s, 1H); 6.38 (s, 1H); 6.89 (d, 4H); 7.11 (s, 1H); 7.23 (m, 4H); 7.30 (d, 4H); 7.40 (m, 3H); 8.31 (t, 1H).

Thermal transition studies:

The modified oligonucleotides (0.5 OD) and their complements (0.5 OD) were dissolved in buffer solution { 100 mM NaCl, 10 mM KH₂PO₄, 0.1 mM EDTA, pH = 7.0 (dil. NaOH adjustment)}, heated to 90 °C for 5 min. and slowly cooled to 4 °C. The thermal melting curves of the samples were recorded at a speed of 1 °C/min following the absorptivity change at λ = 260 nm. The melting temperature was determined as the maximum of the first order derivative of the melting curve.

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