Thermolytic Release of Covalently Linked DNA Oligonucleotides and Their Conjugates from Controlled-Pore Glass at Near Neutral pH†

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The functionalization of long chain alkylamine controlled-pore glass (CPG) with a 3-hydroxypropyl-(2-cyanoethyl)thiophosphoryl linker and its conversion to the support **7** has led to the synthesis of DNA oligonucleotides and their 3′- or (3′,5′)-conjugates. Indeed, CPG support **7** has been successfully employed in the synthesis of both native and fully phosphorothioated DNA 20-mers. Unlike conventional succinylated CPG supports, this distinctively functionalized support allows oligonucleotide deprotection and removal of the deprotection side products to proceed without releasing the oligonucleotide into the aqueous milieu. When freed from deprotection side products, the DNA oligonucleotide is thermolytically released from the support within 2 h under nearly neutral conditions (pH 7.2, 90 °C). The quality of these oligonucleotides is comparable to that of identical oligonucleotides synthesized from succinylated CPG supports in terms of shorter than full length oligonucleotide contaminants and overall yields. The versatility of the thermolytic CPG support **7** is further demonstrated by the synthesis of a DNA oligonucleotide (20-mer) and its conjugation with an azido and alkynyl groups at both 5′-and 3′-termini, respectively. The functionality of the (3′,5′)-heteroconjugated oligonucleotide **18** is verified by its circularization to the DNA oligonucleotide **19** under "click" chemistry conditions.

INTRODUCTION

Solid supports have revolutionized the synthesis of natural products such as peptides (*1, 2*), oligonucleotides (*3–6*), and carbohydrates (*7, 8*), and that of organic compounds through the development of combinatorial chemistries (*9*). The functionality of solid-supported syntheses is however critically dependent on the design and implementation of linkers for the covalent attachment of organic building blocks to solid supports (*10*). When employed in the solid-phase synthesis of oligonucleotides and their analogues or conjugates, the solid support must be functionalized efficiently with a linker designed to allow synthesis to proceed while maintaining the availability of both 5′- and 3′-terminal hydroxyls for subsequent conjugation with various functional groups. The junction linker-to-oligonucleotide and linker-to-support should ideally be stable to the reagents and conditions used during oligonucleotide synthesis and conjugation in addition to those employed for oligonucleotide deprotection. Such an attribute would facilitate the removal of side products resulting from the cleavage of nucleobase and phosphate/thiophosphate protecting groups, including those generated from terminal 5′-/3′-hydroxyl and/or functional group deprotection, by simply washing the solid support. Release of the oligonucleotide, its conjugate or analogue, from the solid support would be effected under mild and neutral thermolytic conditions, free from side products. To the best of our knowledge, the thermolytic release of oligonucleotides with free 5′- and 3′-hydroxyls from solid supports under nearly neutral conditions has not, as yet, been described in the literature. Given that modified oligonucleotides and their conjugates are valuable tools for mechanistic investigations and for therapeutic or diagnostic applications (*11–13*), we have instigated the use of versatile supports designed to enable the preparation of oligonucleotides, conjugated or not with functional groups at either the 3′-terminus or both 3′- and 5′-termini. Specifically, nucleoside or dinucleotide precursors are covalently linked to long chain alkylamine controlled-pore glass (CPG) through either a 3′- or an internucleotidic phosphotriester function. After synthesis and deprotection, release of the oligonucleotides from the support is triggered by a thermolytic intramolecular cyclodeesterification reaction (*14*) in an aqueous buffer at pH 7.2. Details regarding the functionalization of CPG and the thermolytic release of dinucleotide models are outlined in Schemes 1 and 2, respectively. The solid-phase synthesis and thermolytic release of native and modified DNA oligonucleotides in addition to oligonucleotides conjugated with functional groups at the 3′-terminus or at both 3′-and 5′-termini are also described in this report.

EXPERIMENTAL PROCEDURES

Preparation of Functionalized CPG Support 3. CPG (500 Å, 500 mg) was suspended in Et_3N/MeCN (1:1 v/v, 2 mL) in a 4 mL glass vial and was manually agitated over a period of 2 min. The suspension was vacuum-filtered using a glass sintered funnel of coarse porosity. The CPG support was washed with MeCN (10 mL) and then dried under vacuum (3 min). The support was transferred to an 8 mL glass vial, which was stoppered with a rubber septum. The vial and its contents were flushed with a flow of dry argon prior to sequentially adding, by syringe, a solution of *O*-(2-cyanoethyl)-*O*-[3-(4,4′-dimethoxytrityl)oxy-1-propyl]-*N*,*N*-diisopropylphosphoramidite (**1**, 250

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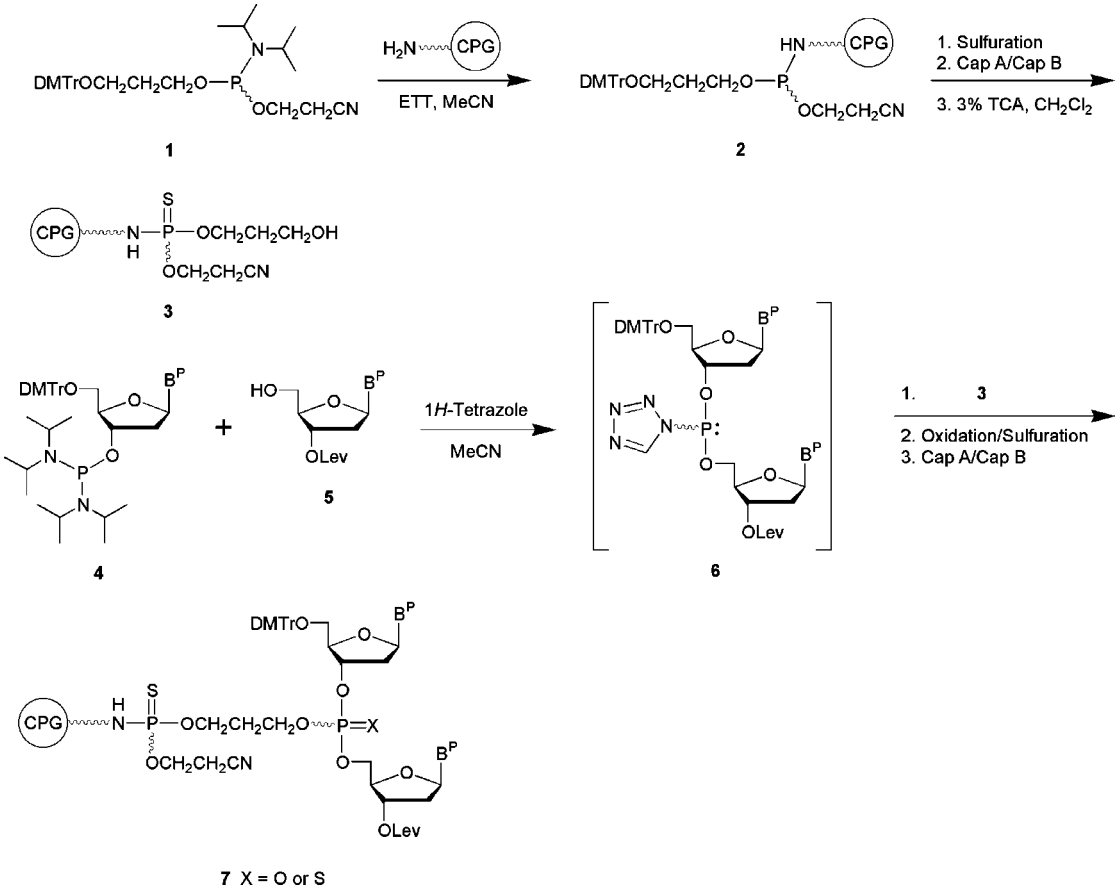
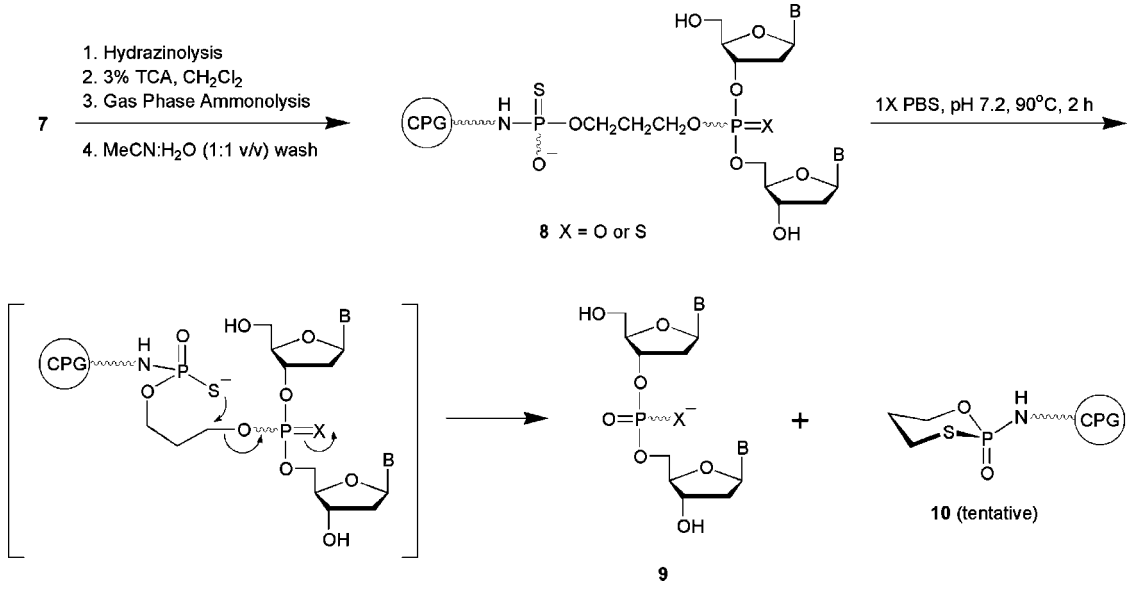
Scheme 1. Preparation of CPG Support 3 and Its Conversion to Support 7[a]



[a] Keys: DMTr, 4,4′-dimethoxytrityl; CPG, 500 Å long chain alkylamine controlled-pore glass; ETT, 5-ethylthio-1*H*-tetrazole; Cap A, acetic anhydride/pyridine/THF; Cap B, 10% 1-methylimidazole in THF; TCA, trichloroacetic acid; B^P, Thy (thymin-1-yl), Cyt^{Bz} (N^4-benzoylcytosin-1-yl), Ade^{Bz} (N^6-benzoyladenin-9-yl), or Gua^{iBu} (N^2-isobutyrylguanin-9-yl); Lev, levulinyl.

Scheme 2. Thermolytic Release of Dinucleoside Phosphate/Thiophosphate Diesters from CPG Support 8[a]



[a] Keys: B, thymin-1-yl, cytosin-1-yl, adenin-9-yl or guanin-9-yl; PBS, phosphate buffered saline.

mg, 0.43 mmol) in dry MeCN (1.2 mL) and 0.25 M 5-ethylthio-1*H*-tetrazole in dry MeCN (3.2 mL, 0.80 mmol). The suspension was agitated, manually, for 15 min under a positive pressure of argon and filtered under vacuum using a glass sintered funnel of medium porosity. The CPG support **2** was washed with MeCN (10 mL) and exposed, while in the funnel, to 0.1 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (5 mL) for 15 min. Excess sulfuration reagent was removed from the support by vacuum filtration. The functionalized CPG support was washed with MeCN (10 mL) and was then suspended in a solution of Cap A (THF/pyridine/Ac_2O, 2.5 mL) and Cap B (1-methylimidazole/THF, 2.5 mL) for a period of 10 min. Excess capping

reagents was removed by filtration; the support was washed with MeCN (10 mL) and dried under vacuum. Dedimethoxytritylation of the support by treatment with 3% TCA (trichloroacetic acid) in CH_2Cl_2 (3 mL, 3 min) afforded the functionalized CPG support **3**. Spectrophotometric determination of the 4,4′-dimethoxytrityl cation at 498 nm revealed a hydroxyl concentration of 69 μmol/g of **3**.

General Procedure for the Preparation of Deoxyribonucleoside Phosphorodiamidites (4) (*15*). A 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyribonucleoside (T, C^{Bz}, A^{Bz}, or G^{iBu}, 2.0 mmol) was dried by coevaporation with anhydrous pyridine (3 × 10 mL) under reduced pressure and was finally dissolved in a solution of anhydrous CH_2Cl_2 (15 mL) and *i*-Pr_2NEt (3.48 mL, 20.0 mmol). To the stirred solution was added bis((*N*,*N*-diisopropylamino)chlorophosphine (587 mg, 2.20 mmol). Progress of the reaction was monitored by TLC, which showed complete phosphinylation within 2 h at ∼25 °C. The reaction was quenched upon addition of water (15 mL); the mixture was vigorously stirred for ∼5 min prior to being transferred to a separatory funnel. The organic layer was collected and rotoevaporated to an oil under reduced pressure. The material was dissolved in a minimal amount (∼4 mL) of benzene/Et_3N (9:1, v/v) and loaded onto the top of a column packed with silica gel (∼30 g) pre-equilibrated in benzene/Et_3N (9:1, v/v). Elution of the reaction product was achieved using benzene/Et_3N (9:1, v/v) as the eluent. Fractions containing the product were identified by TLC and were pooled together. The solvent was removed by rotoevaporation under reduced pressure to afford the phosphoramidite **4** as a white foam. The material was dissolved in dry benzene (10 mL) and the resulting solution was frozen in a dry ice−acetone bath. The frozen solution was then lyophilized under high vacuum for ∼16 h to give triethylamine-free **4** as a white powder in yields of 85−95%.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-bis(*N*,*N*-diisopropylamino)phosphinyl-2′-deoxythymidine. ^{1}H NMR (700 MHz, C_6D_6): δ 7.58 (q, 1H, ^4J = 1.4 Hz, H-6), 7.57 (dd, 2H, J = 8.3, 1.4 Hz, DMTr), 7.41 (d, 2H, J = 9.1 Hz, DMTr), 7.39 (d, 2H, J = 9.1 Hz, DMTr), 7.15 (m, 2H, DMTr), 7.04 (tt, 1H, J = 7.5, 1.1 Hz, DMTr), 6.76 (m, 1H, H-1′), 6.74 (d, 2H, J = 9.1 Hz, DMTr), 6.73 (d, 2H, J = 9.1 Hz, DMTr), 4.61 (m, 1H, H-3′), 4.35 (m, 1H, H-4′), 3.55 (dd, 1H, J = 10.2, 2.8 Hz, H-5′/H-5″), 3.46−3.41 (m, 2H, NC**H**$(CH_3)_2$), 3.40 (dd, 1H, J = 10.2, 3.0 Hz, H-5′/H-5″), 3.40−3.35 (m, 2H, NC**H**$(CH_3)_2$), 3.29 (s, 3H, OC**H**$_3$), 3.28 (s, 3H, OC**H**$_3$), 2.51 (m, 1H, H-2′/H-2″), 2.23 (ddd, 1H, J = 13.3, 8.8, 5.8 Hz, H-2′/H-2″), 1.56 (d, 3H, ^4J = 1.4 Hz, 5-CH_3), 1.21 (d, 6H, J = 6.6 Hz, NCH(C**H**$_3$)$_2$), 1.14 (d, 6H, J = 6.6 Hz, NCH(C**H**$_3$)$_2$), 1.12 (d, 6H, J = 6.6 Hz, NCH(C**H**$_3$)$_2$), 1.09 (d, 6H, J = 6.6 Hz, NCH(C**H**$_3$)$_2$). ^{13}C NMR (175 MHz, C_6D_6): δ 164.2 (C-4), 159.32 (DMTr), 159.31 (DMTr), 151.0 (C-2), 145.2 (DMTr), 136.0 (DMTr), 135.9 (DMTr), 135.4 (C-6), 130.60 (DMTr), 130.58 (DMTr), 128.7 (DMTr), 128.2 (DMTr), 127.3 (DMTr), 113.64 (DMTr), 113.63 (DMTr), 111.1 (C-5), 87.3 (DMTr), 86.3 (d, J_{PC} = 5.4 Hz, C-4′), 85.5 (C-1′), 75.1 (d, $^2J_{CP}$ = 19.3 Hz, C-3′), 64.5 (C-5′), 54.8 (OCH_3), 45.0 (d, $^2J_{CP}$ = 12.9 Hz, N**C**H$(CH_3)_2$), 40.4 (d, J_{CP} = 5.4 Hz, C-2′), 24.5 (d, J_{CP} = 8.6 Hz, NCH(**C**H$_3$)$_2$), 24.3 (d, J_{CP} = 6.4 Hz, NCH(**C**H$_3$)$_2$), 24.2 (d, J_{CP} = 6.4 Hz, NCH(**C**H$_3$)$_2$), 12.2 (5-CH_3). ^{31}P NMR (121 MHz, C_6D_6): δ 116.2. +APESI-TOF MS: calcd for $C_{43}H_{59}N_4O_7P$ $(M+H)^+$ 775.4200, found 775.4195.

***N*^{4}-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-bis(*N*,*N*-diisopropylamino)phosphinyl-2′-deoxycytidine.** ^{1}H NMR (700 MHz, C_6D_6): δ 8.18 (bs, 1H, H-6), 7.92 (m, 2H, Bz), 7.62 (dd, 2H, J = 8.6, 1.4 Hz, DMTr), 7.46 (d, 2H, J = 8.9 Hz, DMTr), 7.45 (d, 2H, J = 8.9 Hz, DMTr), 7.24 (d, 1H J = 7.5 Hz, Ar/H-5), 7.22 (d, 1H J = 7.5 Hz, Ar/H-5), 7.10 (m, 2H, Ar), 7.02 (m, 2H, Ar), 6.83 (d, 2H, J = 9.1 Hz, DMTr), 6.82 (d, 2H, J = 9.1 Hz, DMTr), 6.51 (t, 1H, J = 6.2 Hz, H-1′), 4.58 (ddd, 1H, J = 6.4, 5.8, 3.3 Hz, H-3′), 4.41 (m, 1H, H-4′), 3.56 (dd, 1H, J = 10.5, 3.3 Hz, H-5′/H-5″), 3.50 (dd, 1H, J = 10.5, 4.1 Hz, H-5′/H-5″), 3.47−3.40 (m, 4H, NC**H**$(CH_3)_2$), 3.39 (s, 3H, OC**H**$_3$), 3.38 (s, 3H, OC**H**$_3$), 2.84 (m, 1H, H-2′/H-2″), 2.23 (ddd, 1H, J = 13.6, 6.4, 6.1 Hz, H-2′/H-2″), 1.18 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$), 1.15 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$), 1.14 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$), 1.12 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$). ^{13}C NMR (175 MHz, C_6D_6): δ 162.8 (C-4), 159.4 (DMTr), 145.1 (C-2), 143.8 (C-6), 136.0 (Ar), 135.84 (Ar), 135.83 (Ar), 134.6 (Ar), 132.3 (Ar), 130.7 (DMTr), 130.6 (DMTr), 128.7 (DMTr), 128.6 (DMTr), 128.26 (Ar), 128.25 (Ar), 127.34 (Ar), 127.32 (Ar), 113.69 (DMTr), 113.68 (DMTr), 101.7 (C-5), 87.8 (DMTr), 87.4 (C-1′), 86.7 (d, J_{CP} = 4.3 Hz, C-4′), 73.8 (d, $^2J_{CP}$ = 18.3 Hz, C-3′), 63.9 (C-5′), 54.9 (OCH_3), 45.1 (d, $^2J_{CP}$ = 12.6 Hz, N**C**H$(CH_3)_2$), 44.8 (d, $^2J_{CP}$ = 12.4 Hz, N**C**H$(CH_3)_2$), 41.5 (C-2′), 24.6 (d, J_{CP} = 8.6 Hz, NCH(**C**H$_3$)$_2$), 24.5 (d, J_{CP} = 8.6 Hz, NCH(**C**H$_3$)$_2$), 24.3 (d, J_{CP} = 6.4 Hz, NCH(**C**H$_3$)$_2$) 24.2 (d, J_{CP} = 6.4 Hz, NCH(**C**H$_3$)$_2$). ^{31}P NMR (121 MHz, C_6D_6): δ 117.1. +APESI-TOF MS: calcd for $C_{49}H_{62}N_5O_7P$ $(M+H)^+$ 864.4465, found 864.4477.

***N*^{6}-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-bis(*N*,*N*-diisopropylamino)phosphinyl-2′-deoxyadenosine.** ^{1}H NMR (700 MHz, C_6D_6): δ 8.74 (bs, 1H, H-2), 7.95 (s, 1H, H-8), 7.82 (m, 2H, Bz), 7.61 (dd, 2H, J = 8.5, 1.3 Hz, DMTr), 7.44 (d, 2H, J = 9.0 Hz, DMTr), 7.43 (d, 2H, J = 9.0 Hz, DMTr), 7.15 (m, 2H, Ar), 7.06−6.96 (m, 4H, Ar), 6.73 (d, 2H, J = 9.0 Hz, DMTr), 6.72 (d, 2H, J = 9.0 Hz, DMTr), 6.34 (t, 1H, J = 6.4 Hz, H-1′), 4.75 (ddd, 1H, J = 8.7, 5.9, 3.1 Hz, H-3′), 4.59 (m, 1H, H-4′), 3.59 (dd, 1H, J = 10.2, 4.1 Hz, H-5′/H-5″), 3.55 (dd, 1H, J = 10.2, 5.1 Hz, H-5′/H-5″), 3.51−3.42 (m, 4H, NC**H**$(CH_3)_2$), 3.33 (s, 3H, OC**H**$_3$), 3.32 (s, 3H, OC**H**$_3$), 2.87 (m, 1H, H-2′/H-2″), 2.56 (ddd, 1H, J = 13.6, 6.4, 3.1 Hz, H-2′/H-2″), 1.21 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$), 1.18 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$), 1.17 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$), 1.16 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$). ^{13}C NMR (175 MHz, C_6D_6): δ 166.2 (C═O), 159.18 (DMTr), 159.16 (DMTr,) 152.6 (C-2/C-6), 151.9 (C-6/C-2), 145.5 (C-4), 142.1 (C-8), 136.24 (DMTr), 136.21 (DMTr), 132.0 (Ar), 130.6 (DMTr), 130.5 (DMTr), 128.7(DMTr) 128.6 (DMTr), 128.3 (Ar), 128.1 (Ar), 127.1 (C-5), 113.5 (DMTr), 87.0 (DMTr), 86.9 (d, J_{CP} = 5.4 Hz, C-4′), 85.8 (C-1′), 74.9 (d, $^2J_{CP}$ = 18.3 Hz, C-3′), 64.8 (C-5′), 54.8 (OCH_3), 45.0 (d, $^2J_{CP}$ = 12.9 Hz, N**C**H$(CH_3)_2$), 44.9 (d, $^2J_{CP}$ = 12.9 Hz, N**C**H$(CH_3)_2$), 39.4 (C-2′), 24.6 (d, J_{CP} = 8.6 Hz, NCH(**C**H$_3$)$_2$), 24.5 (d, J_{CP} = 8.6 Hz, NCH(**C**H$_3$)$_2$), 24.3 (d, J_{CP} = 6.4 Hz, NCH(**C**H$_3$)$_2$), 24.2 (d, J_{CP} = 6.4 Hz, NCH(**C**H$_3$)$_2$). ^{31}P NMR (121 MHz, C_6D_6): δ 116.7. +APESI-TOF MS: calcd for $C_{50}H_{62}N_7O_6P$ $(M+H)^+$ 888.4577, found 888.4574.

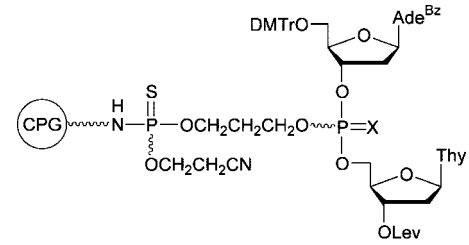
***N*^{2}-Isobutyryl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-bis(*N*,*N*-diisopropylamino)phosphinyl-2′-deoxyguanosine.** ^{1}H NMR (700 MHz, C_6D_6): δ 7.89 (s, 1H, H-8), 7.63 (dd, 2H, J = 8.6, 1.3 Hz, DMTr), 7.48 (d, 2H, J = 9.0 Hz, DMTr), 7.47 (d, 2H, J = 9.0 Hz, DMTr), 7.16 (m, 2H, DMTr), 7.04 (tt, 1H, J = 7.4, 1.3 Hz, DMTr), 6.77 (d, 2H, J = 9.0 Hz, DMTr), 6.76 (d, 2H, J = 9.0 Hz, DMTr), 6.45 (dd, 1H, J = 6.1, 5.9 Hz, H-1′), 4.66 (m, 1H, H-3′), 4.48 (m, 1H, H-4′), 3.56 (dd, 1H, J = 10.2, 3.3 Hz, H-5′/H-5″), 3.49−3.40 (m, 6H, H-5′/H-5″, COC**H**$(CH_3)_2$, NC**H**$(CH_3)_2$), 3.38 (s, 3H, OC**H**$_3$), 3.37 (s, 3H, OC**H**$_3$), 2.83 (m, 1H, H-2′/H-2″), 2.62 (ddd, 1H, J = 13.6, 5.9, 2.8 Hz, H-2′/H-2″), 1.19 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$), 1.15 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$), 1.14 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$), 1.13 (d, 6H, J = 6.7 Hz, NCH(C**H**$_3$)$_2$), 1.06 (d, 3H, J = 6.7 Hz, COCH(C**H**$_3$)$_2$), 1.05 (d, 3H, J = 6.7 Hz, COCH(C**H**$_3$)$_2$). ^{13}C NMR (175 MHz, C_6D_6): δ 159.24 (DMTr), 159.23 (DMTr), 159.1 (C-6), 149.4 (C-2), 145.5 (C-4), 137.2 (C-8), 136.3 (DMTr), 136.2 (DMTr), 130.7(DMTr), 130.6 (DMTr), 128.7

(DMTr), 128.3 (DMTr), 127.1 (DMTr), 122.3 (DMTr), 113.60 (DMTr), 113.58 (DMTr), 113.5 (C-5), 86.9 (DMTr), 86.7 (d, J_{CP} = 4.3 Hz, C-4′), 85.0 (C-1′), 74.5 (d, $^2J_{CP}$ = 17.2 Hz, C-3′), 64.8 (C-5′), 54.9 (OCH_3), 45.0 (d, $^2J_{PC}$ = 12.9 Hz, N**C**H$(CH_3)_2$), 44.9 (d, $^2J_{CP}$ = 12.9 Hz, N**C**H$(CH_3)_2$), 40.0 (d, J_{CP} = 4.3 Hz, C-2′), 35.9 (CO**C**H$(CH_3)_2$), 24.6 (d, J_{CP} = 8.6 Hz, NCH(**C**$H_3)_2$), 24.3 (d, J_{CP} = 6.4 Hz, NCH(**C**$H_3)_2$), 24.2 (d, J_{CP} = 6.4 Hz, NCH(**C**$H_3)_2$), 19.19 (COCH(**C**$H_3)_2$), 19.16 (COCH(**C**$H_3)_2$). ^{31}P NMR (121 MHz, C_6D_6): δ 116.9. +APESI-TOF MS: calcd for $C_{47}H_{64}N_7O_7P$ $(M+H)^+$ 870.4683, found 870.4687.

General Procedure for Preparation of Dinucleoside Phosphorotetrazolide Intermediates (6) in Situ and for Their Covalent Attachment to CPG Support 3. Phosphorodiamidite **4** (B^P = Thy, Cyt^{Bz}, Ade^{Bz}, or Gua^{iBu}, 50 μmol), 3′-*O*-levulinyl-2′-deoxyribonucleoside **5** (B^P = Thy, Cyt^{Bz}, Ade^{Bz}, or Gua^{iBu}, 50 μmol), and sublimed 1*H*-tetrazole (11 mg, 150 μmol) were placed in a flame-dried 4 mL glass vial, which was stoppered with a rubber septum and purged with a flow of dry Argon. Anhydrous MeCN (300 μL) was syringed into the vial through the rubber septum. The coupling reaction was allowed to proceed over a period of 10 min at ~25 °C to generate the putative dinucleoside phosphorotetrazolide intermediate **6** (*16*). The reaction mixture was withdrawn from the vial and through a synthesis column filled with CPG support **3** (14.5 mg, 1 μmol) using a luer-tipped syringe connected to one end of the synthesis column and a 16-G hypodermic needle connected to the other end of the synthesis column. The coupling reaction was performed for a period of 10 min. Excess reagent was expelled from the column, which was washed with a solution of 20% 1-methylimidazole in H_2O/MeCN (1:1 v/v) for 2 min. An oxidation reaction was carried out using either 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN or 0.02 M I_2 in THF/pyridine/H_2O for 2 min. Unreacted hydroxyl groups of support **3** were acylated when exposed to Cap A and Cap B solutions (1 mL each) for 2 min. Excess capping reagents was removed from CPG support **7** by washing with MeCN (10 mL). Spectrophotometric determination (498 nm) of the 4,4′-dimethoxytrityl cation released from **7** under acidic conditions revealed a dinucleoside phosphate triester concentration of 40 μmol/g of support.

General Procedure for the Automated Solid-Phase Synthesis of DNA Oligonucleotides Using CPG Support 7. Solid-phase synthesis of native and phosphorothioated DNA oligonucleotides was performed on a 0.2 μmol scale using a DNA/RNA synthesizer and 2-cyanoethyl deoxyribonucleoside phosphoramidites as 0.1 M solutions in dry MeCN. The reagents and conditions employed for solid-phase synthesis were those recommended by the instrument's manufacturer. However, the preparation of fully thioated oligodeoxyribonucleotides required replacement of the iodine oxidation step in the synthesis cycle with a sulfuration step employing a 0.05 M solution of 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN, as recommended in the literature (*17, 18*). The capping step was performed before the oxidation step when synthesizing native DNA oligonucleotides and after the sulfuration step when synthesizing phosphorothioated DNA oligonucleotides.

General Procedure for Deprotection, Thermolytic Release, and RP-HPLC Analysis of DNA Oligonucleotides Synthesized from CPG Support 7. 5′-*O*-Dedimethoxytritylated DNA oligonucleotides that were synthesized using CPG support **7** on a 0.2 to 1 μmol scale were subjected to pressurized ammonia gas (~10 bar) for 16 h at 25 °C (*19*) to remove the nucleobase, phosphate/thiophosphate, and 3′-*O*-levulinyl protecting groups. The solid support was washed with 50% aqueous MeCN (1 mL) to eliminate the deprotection side product and was air-dried. The CPG support was transferred to a 4 mL



7 X = O or S

screw-capped glass vial to which was added 1 × PBS (pH 7.2, 0.5 mL); the suspension was heated for 2 h at 90 ± 2 °C. The purity of each unpurified native or phosphorothioated DNA oligonucleotide released in the aqueous buffer was assessed by RP-HPLC and compared with that of identical oligonucleotides prepared from unmodified CPG supports (data shown in Figures 1 and 2). Typically, an aliquot (20 μL) of the PBS solution of each native DNA oligonucleotide was analyzed using a 5 μm Supelcosil LC-18S column (25 cm × 4.6 mm) according to the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 0.7% MeCN/min was pumped at a flow rate of 1 mL/min for 30 min and was followed by a linear gradient of 4% MeCN/min for 5 min, which was held isocratically for 5 min.

An aliquot (60 μL) of the PBS solution of each unpurified phosphorothioated DNA oligonucleotide was analyzed under conditions similar to those used for the native DNA oligonucleotide with the following modification: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min was pumped at a flow rate of 1 mL/min for 40 min. Peak heights were all normalized to the highest peak, which was set to 1 arbitrary unit.

5′-d(CGACTGTGAATCGATGCCAT) [MALDI-TOF MS]: Calcd for $C_{195}H_{227}N_{75}O_{118}P_{19}$ $(M-H)^-$ 6097, found 6102.

5′-d($C_{PS}G_{PS}A_{PS}C_{PS}T_{PS}G_{PS}T_{PS}G_{PS}A_{PS}A_{PS}T_{PS}C_{PS}G_{PS}A_{PS}T_{PS}G_{PS}C_{PS}A_{PS}T$) [MALDI-TOF MS]: Calcd for $C_{195}H_{227}N_{75}O_{99}P_{19}S_{19}$ $(M-H)^-$ 6402, found 6406.

Polyacrylamide Gel Electrophoresis Analysis of DNA Oligonucleotides Synthesized from CPG Support 7. To each unpurified native or phosphorothioated DNA oligonucleotide (0.25 OD_{260}) in a 1.5 mL microfuge tube was added 10 μL of Loading Buffer (10 × Tris Borate EDTA, pH 8.3, in formamide (1:4 v/v) containing 2 mg/mL bromophenol blue). The dark blue solution was vortexed vigorously and centrifuged at 16 000*g* for 5 s. Each DNA solution was syringed into a 2 cm wide well of a 20% polyacrylamide−7 M urea gel (20 cm × 40 cm × 0.75 mm). Electrophoresis was performed at 350 V, using 1 × Tris Borate EDTA (pH 8.3) as the electrolyte, until the bromphenol blue dye traveled 75% of the gel's length. The gel was immersed in a staining solution (250 mL) composed of formamide/isopropyl alcohol/ddH_2O (1:5:20 v/v/v) to which was added a solution (10 mL) of Stains-all (1 mg/mL) in formamide. The gel was stained for ~4 h in the dark. The staining solution was discarded and the gel was rinsed three times with 250 mL distilled water. The gel was exposed to natural light until the purple background disappeared. The native DNA oligonucleotides were visualized as sharp blue bands, whereas fully phosphorothioated DNA oligonucleotides appeared as dark purple bands. Photographs of the gels are shown in Figure 3.

Hydrolysis of Native DNA Oligonucleotides Catalyzed by Snake Venom Phosphodiesterase and Bacterial Alkaline Phosphatase. To 1.0 OD_{260} of lyophilized oligonucleotide was added 1 M Tris·HCl (pH 9.0, 6 μL), 1 M $MgCl_2$ (8 μL), ddH_2O to a total volume of 89 μL, snake venom phosphodiesterase (*Crotallus adamanteus*, 5 μL, 2 U/mL), and bacterial alkaline

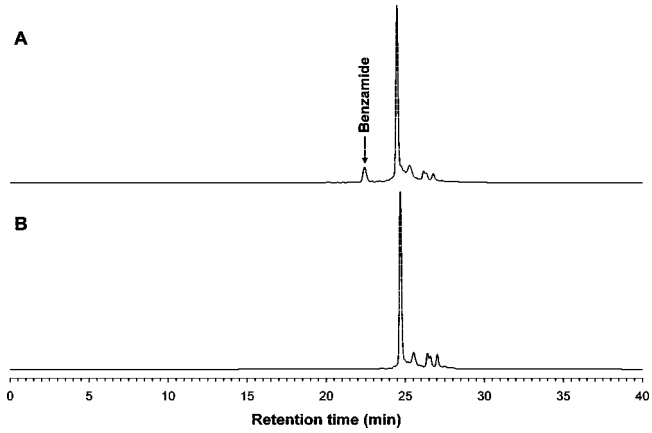


Figure 1. RP-HPLC analysis of unpurified 5′-d(CGACTGTGAATC-GATGCCAT). (A) Chromatogram of the native 20-mer that was synthesized employing unmodified CPG under standard conditions. Nucleobase and phosphate deprotection, and release of the oligomer from the support were effected by treatment with pressurized NH_3 gas (∼10 bar) for 16 h at 25 °C, as described in the Experimental Procedures section. (B) Chromatogram of the 20-mer that was synthesized using CPG support **7** under standard conditions. After nucleobase and phosphate deprotection under the conditions described in (A), the deprotection side products were washed away from the modified CPG support with MeCN:H_2O (1:1 v/v). The 20-mer was thermolytically cleaved from the support upon heating at 90 °C in 1 × PBS (pH 7.2) for 2 h. Chromatographic conditions are described in the Experimental Procedures section.

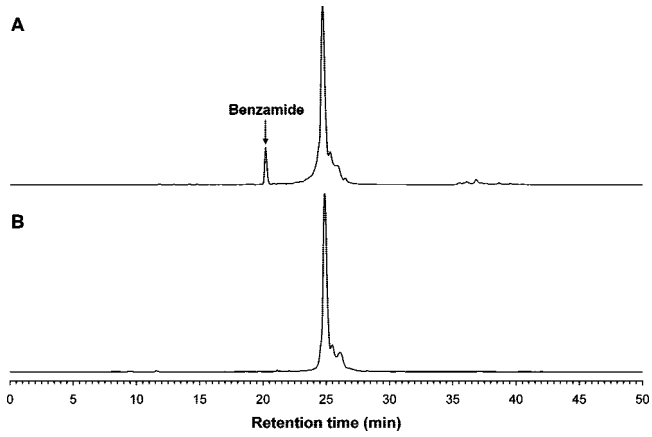


Figure 2. RP-HPLC analysis of unpurified 5′-d($C_{PS}G_{PS}A_{PS}C_{PS}T_{PS}G_{PS}$-$T_{PS}G_{PS}A_{PS}A_{PS}T_{PS}C_{PS}G_{PS}A_{PS}T_{PS}G_{PS}C_{PS}C_{PS}A_{PS}T$). (A) Chromatogram of the fully phosphorothioated 20-mer that was synthesized employing unmodified CPG under standard conditions. Nucleobase and thiophosphate deprotection was effected by treatment with pressurized NH_3 gas (∼10 bar) over a period of 16 h at 25 °C, as described in the Experimental Procedures section. (B) Chromatogram of the 20-mer that was synthesized using CPG support **7** under standard conditions. After nucleobase and thiophosphate deprotection under the conditions described in (A), the deprotection side products were washed away from the modified CPG support with MeCN:H_2O (1:1 v/v). The 20-mer was thermolytically cleaved from the support upon heating at 90 °C in 1 × PBS (pH 7.2) for 2 h. Chromatographic conditions are described in the Experimental Procedures section.

phosphatase (6 μL, 0.7 U). The digestion reaction was heated for 16 h in a water bath kept at 37 °C. The digest was finally heated at 100 °C for 1 min to denature the enzymes and was centrifuged at 16 000*g* for 2 min prior to immediate analysis by RP-HPLC. The analysis was performed using a 5 μm Supelcosil LC-18S column (25 cm × 4.6 mm) according to the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min is pumped

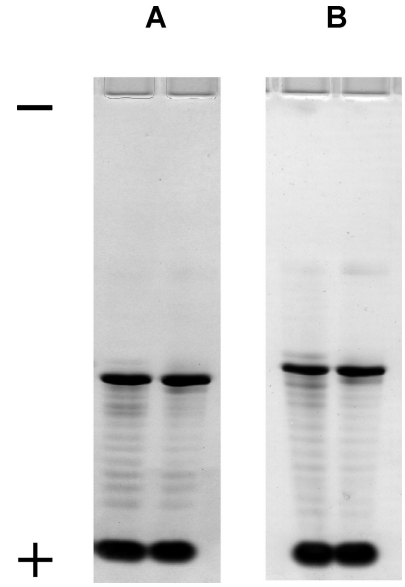


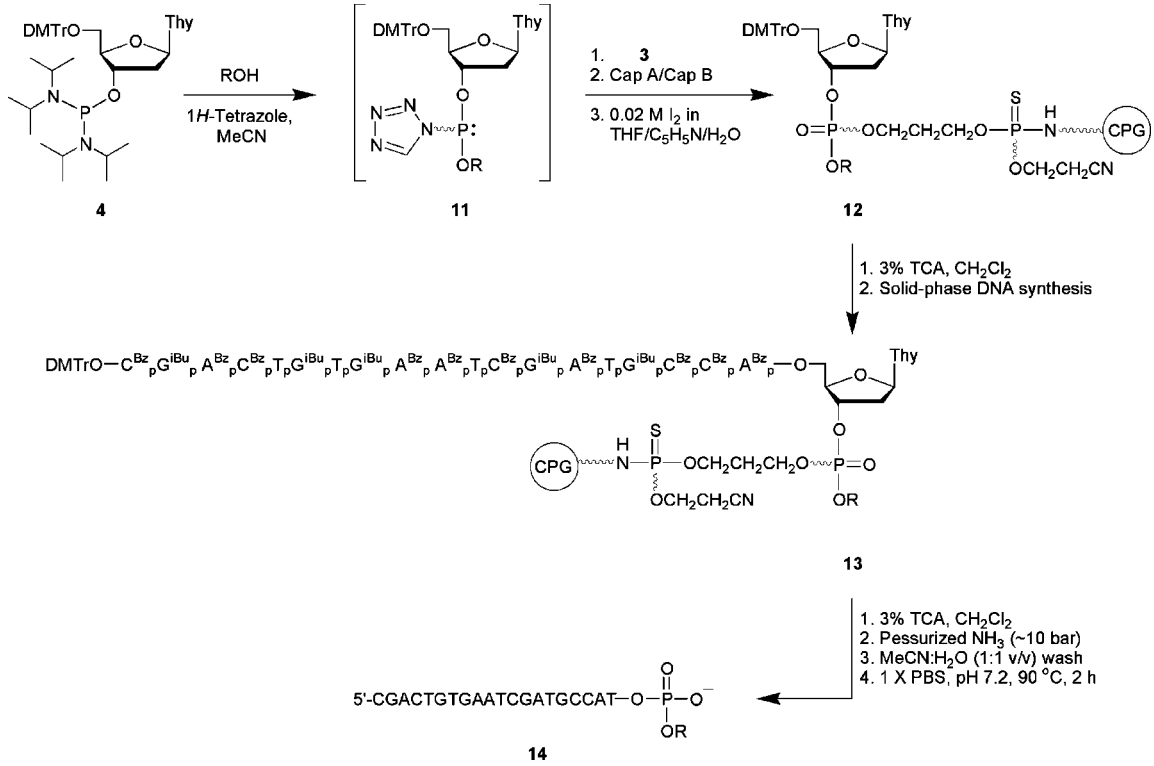
Figure 3. Polyacrylamide gel electrophoresis analysis of unpurified 5′-d(CGACTGTGAATCGATGCCAT) and 5′-d($C_{PS}G_{PS}A_{PS}C_{PS}T_{PS}G_{PS}T_{PS}$-$G_{PS}A_{PS}A_{PS}T_{PS}C_{PS}G_{PS}A_{PS}T_{PS}G_{PS}C_{PS}C_{PS}A_{PS}T$) under denaturing conditions. (A) Left lane; electrophoretic profile of the 20-mer that was synthesized employing unmodified CPG and deprotected under standard conditions, as described in the legend of Figure 1. Right lane: electrophoretic profile of the 20-mer that was synthesized using the CPG support **7** under standard conditions and deprotected as described in the legend of Figure 1. (B) Left lane: electrophoretic profile of the fully phosphorothioated 20-mer that was synthesized employing unmodified CPG and deprotected under standard conditions, as described in the legend of Figure 2. Right lane: electrophoretic profile of the fully phosphorothioated 20-mer that was synthesized using the CPG support **7** under standard conditions and deprotected as recommended in the legend of Figure 2. Electrophoretic conditions are described in the Experimental Procedures section.

at a flow rate of 1 mL/min for 40 min. Peak heights were normalized to the highest peak, which was set to 1 arbitrary unit.

Preparation of CPG-Linked Nucleotide 12. Phosphorodiamidite **4** (B^P = T, 39 mg, 50 μmol), 1-pyrenebutanol (14 mg, 50 μmol), and sublimed 1*H*-tetrazole (11 mg, 150 μmol) were placed in a flame-dried 4-mL glass vial, which was stoppered with a rubber septum and purged with a flow of dry argon. Anhydrous MeCN (300 μL) was syringed into the vial through the rubber septum. The coupling reaction was allowed to proceed for 10 min at ∼25 °C to generate the deoxyribonucleoside phosphorotetrazolide intermediate **11** (Scheme 3.) The condensation of **11** with the CPG support **3** (29 mg, 2 μmol) and all subsequent processing steps were performed under conditions identical to those described above for the attachment of dinucleoside phosphorotetrazolide intermediates **6** to CPG support **3**. A fraction of CPG support **12** (2.6 mg, 0.1 μmol) was 5′-dedimethoxytritylated by treatment with 3% TCA in CH_2Cl_2 (3 mL, 3 min), washed with MeCN (10 mL), and suspended in 1 × PBS (pH 7.2, 0.5 mL). The suspension was heated for 2 h at 90 ± 2 °C. The RP-HPLC profile of thymidine 3′-*O*-(1-pyrenylbutyl)phosphate is presented in the Supporting Information.

Preparation of CPG-Linked Oligonucleotide 13. 5′-Dedimethoxytritylation of the support-linked deoxyribonucleoside phosphate triester **12** (26 mg, 1 μmol) was effected by slowly pushing 3% TCA in CH_2Cl_2 (3 mL) through the synthesis column over a period of 3 min. Automated solid-phase oligonucleotide synthesis leading to the CPG-linked oligonucleotide **13** (Scheme 3) was carried out according to the general procedure described above when employing CPG support **7**.

Scheme 3. Preparation of CPG Support 13 for the Solid-Phase Synthesis of DNA Oligonucleotide 3′-Conjugates[a]



[a] Keys: R, 1-pyrenebutyl; p, 2-cyanoethyl phosphoryl.

Oligonucleotide deprotection and thermolytic release from the CPG support were performed following the general procedure described above for DNA oligonucleotides synthesized from CPG support **7**. The purity of the 3′-pyrenylated DNA oligonucleotide conjugate **14** was greater than 90%, as determined by RP-HPLC (data shown in the Supporting Information). The identity of unpurified and desalted **14** was confirmed by MALDI-TOF MS: Calcd for $C_{215}H_{243}N_{75}O_{121}P_{20}$ $(M-H)^-$ 6432, found 6437.

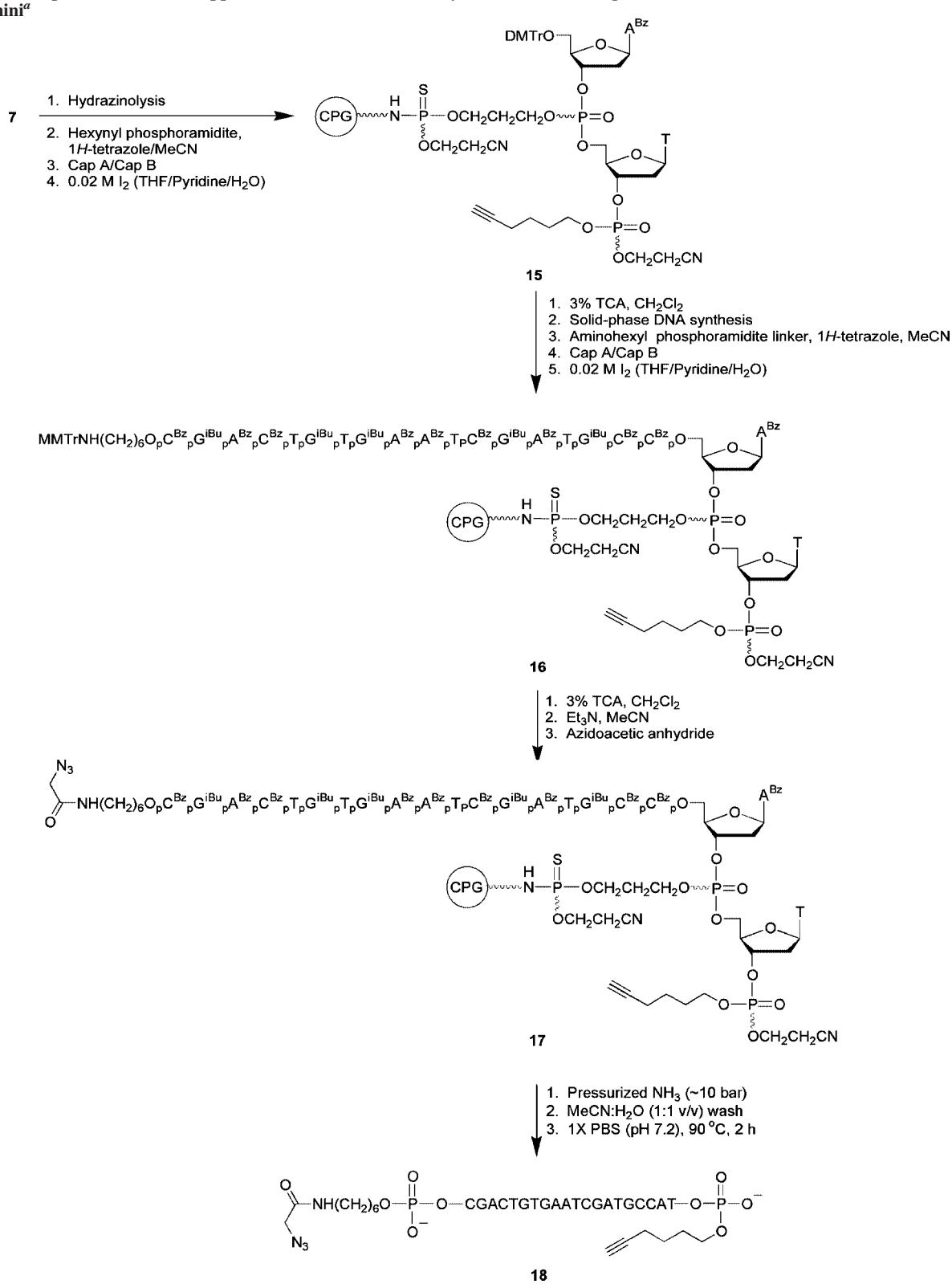
Preparation of CPG-Linked Dinucleotide 15. A solution of 0.5 M hydrazine hydrate in pyridine/acetic acid (3:2 v/v, 2 mL) was pushed through a synthesis column, packed with the support-linked dinucleoside phosphate triester **7** (0.2 μmol), for a period of 30 min to completely remove the 3′-*O*-levulinyl group. Dry MeCN (20 mL) was flushed through the support by syringe to carefully eliminate residual hydrazinolysis reagents and side-product. A premixed solution of 0.1 M *O*-(2-cyanoethyl)-*O*-(5-hexyn-1-yl)-*N*,*N*-diisopropylphosphoramidite (10 μmol) and 0.45 M 1*H*-tetrazole (67 μmol) in dry MeCN was syringed into the synthesis column. The suspension was manually agitated for 3 min. Excess reagents were discarded with MeCN (10 mL) and a premixed solution of Cap A and Cap B reagents (1 mL each) were syringed through the synthesis column. After a total exposure time of 1 min, the capping reagents were replaced with a solution of 0.02 M iodine in THF/pyridine/H_2O (1 mL). An oxidation time of 1 min was sufficient to complete the preparation of the CPG-linked dinucleotide **15** (Scheme 4).

Preparation of CPG-Linked DNA Oligonucleotide 16. 5′-Dedimethoxtritylation of the CPG-linked dinucleotide **15** was carried out by treatment with 3% TCA in CH_2Cl_2 (3 mL) for 3 min. A 4 mL glass vial containing 0.1 M *O*-(2-cyanoethyl)-*O*-[6-(4-methoxytrityl)amino-1-hexyl]-*N*,*N*-diisopropylphosphoramidite in dry MeCN was connected to the synthesizer at position #5 and was incorporated as such into the DNA synthesis program. The automated solid-phase oligonucleotide synthesis was achieved according to the general procedure delineated above when using CPG support **7**.

Preparation of CPG-Linked DNA Oligonucleotide 17. Removal of the terminal 4-methoxytrityl group from **16** was achieved by passing 3% TCA in CH_2Cl_2 (10 mL) through the synthesis column for a period of 10 min. Excess acid was eliminated from the column with an acetonitrile wash (20 mL) followed by a brief (30 s) Et_3N-MeCN (1:1 v/v) wash (3 mL). The resulting free terminal amino group was acylated with a solution (0.5 mL) of azidoacetic anhydride/pyridine/THF (1:1:8 v/v/v) and Cap B (0.5 mL) to produce the CPG-linked DNA oligonucleotide **17** (Scheme 4). Azidoacetic anhydride was prepared in situ from azidoacetic acid (202 mg, 2.00 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (217 mg, 1.05 mmol) in dry THF (3 mL) under conditions similar to those reported for the preparation of levulinic anhydride (*20*). Oligonucleotide deprotection and thermolytic release from the CPG support were performed following the general procedure described above for DNA oligonucleotides synthesized from the CPG support **7**. The (3′,5′)-heteroconjugated DNA oligonucleotide **18** was RP-HPLC purified using the chromatographic conditions outlined above for the analysis of phosphorothioated DNA oligonucleotides; the RP-HPLC profile of **18** is shown in the Supporting Information. Identity of the desalted oligonucleotide **18** was corroborated by MALDI-TOF MS: Calcd for $C_{209}H_{249}N_{79}O_{125}P_{21}$ $(M-H)^-$ 6517, found 6522.

Conversion of Oligonucleotide 18 to Circular DNA Oligonucleotide 19. The circularization of the DNA oligonucleotide **18** was performed essentially as reported by Kumar et al. (*21*). To a solution of *tris*(benzyltriazolylmethyl)amine (1.4 μmol), sodium ascorbate (2.0 μmol), and $CuSO_4 \cdot 5H_2O$ (0.2 μmol) in 0.2 M NaCl (2.4 mL) was added **18** (5 nmol) in 0.2 M NaCl (100 μL). The reaction mixture was left standing at 25 °C for 2 h and was desalted through

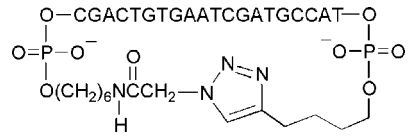
Scheme 4. Preparation of CPG Support 15 for the Solid-Phase Synthesis of DNA Oligonucleotide Functionalized at Both 5′- and 3′-Termini[a]



[a] Keys: MMTr, 4-methoxytrityl.

a PD-10 (Sephadex G-25M) column. The crude circular DNA oligonucleotide **19** was RP-HPLC purified under chromatographic conditions similar to those employed for the analysis of phosphorothioated DNA oligonucleotides. The RP-HPLC profile of the purified circular DNA oligonucleotide **19** is shown in the Supporting Information. The RP-HPLC retention time of **19** (t_R = 21.2 min) is shorter than that of **18** (t_R = 23.8 min) but, as expected, the molecular weight of **19**

(MALDI-TOF MS: Calcd for $C_{209}H_{249}N_{79}O_{125}P_{21}$ $(M-H)^-$ 6517, found 6521) is identical to that of **18**.



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RESULTS AND DISCUSSION

Functionalization of CPG through 3-Hydroxypropyl Thiophosphoramidation. The amino groups of long chain alkylamine controlled-pore glass (CPG) were first activated by treatment with a solution of Et_3N in MeCN prior to reaction with a 36 mM solution of *O*-(2-cyanoethyl)-*O*-[3-(4,4′-dimethoxytrityl)oxy-1-propyl]-*N*,*N*-diisopropylphosphoramidite (**1**) and 0.25 M 5-ethylthio-1*H*-tetrazole in dry MeCN. The functionalized support **2** (Scheme 1) was washed with MeCN to remove excess reagents and was immersed into a 0.1 M solution of 1,2-benzodithiol-3-one 1,1-dioxide to oxidize the phosphoramidite entity to a thiophosphoramidate function. The unreacted amino groups of CPG were deactivated upon *N*-acetylation with an equal volume of each of the Cap A (THF/pyridine/Ac_2O) and Cap B (1-methylimidazole/THF) solutions. Subsequent dedimethoxytritylation of the support **2** under acidic conditions revealed an essentially quantitative functionalization of CPG based on an hydroxyl concentration of 69 μmol/g of **3**, which was determined by the standard trityl assay.

Attachment of Leader Dinucleoside Phosphate/Thiophosphate to CPG Support 3. The conversion of CPG support **3** to CPG support **7** began with the preparation of the deoxyribonucleoside phosphorodiamidites **4** (Scheme 1), which was reported in the literature more than 20 years ago (*15*). Typically, a 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxribonucleoside (B^P = Thy, Cyt^{Bz}, Ade^{Bz}, or Gua^{iBu}) was condensed with bis(*N*,*N*-diisopropylamino)chlorophosphine and *N*,*N*-diisopropylethylamine in anhydrous CH_2Cl_2. Complete phosphinylation was accomplished within 2 h, whereupon the crude reaction product was subjected to aqueous extractive workup and subsequent purification by chromatography on silica gel. It was necessary to equilibrate silica gel in benzene/Et_3N (9:1 v/v) to neutralize its inherent acidity and prevent acid-mediated decomposition of the reaction product. The phororodiamidites **4** were isolated as white foams, which were freed from residual moisture and Et_3N by lyophilization from dry benzene. However, depending on the variable amounts of residual moisture and Et_3N contaminating the purified phosphorodiamidites **4**, it is often preferable to precipitate **4** in cold (−78 °C) hexanes followed by lyophilization of the white precipitates from dry benzene to ensure complete removal of these contaminants. Triethylamine-free **4** were obtained as white powders, the yields of which were in the range 85−95%. The phosphorodiamidites **4** were characterized extensively using one-dimensional ^1H, ^1H decoupled ^{13}C, ^1H and ^{31}P decoupled ^{13}C, and ^{31}P NMR techniques, which were complemented with two-dimensional $^1H-^1H$ total correlation (TOCSY) and $^1H-^{13}C$ correlation (HSQC) data, and accurate mass determination by atmospheric pressure electrospray ionization time-of-flight mass spectrometry (APESI-TOF MS). The selection of an appropriate solvent for NMR characterization of the diamidites **4** is critical; the stability of **4** in a nonpolar solvent, such as benzene, is considerably higher than that observed in the relatively polar DMSO. Thus, NMR analysis of the deoxyribonucleoside phosphorodiamidites **4** was carried out in deuterated benzene (C_6D_6). All characterization data are presented in the Experimental Procedures and Supporting Information sections of this report. When pure, each deoxyribonucleoside phororodiamidite **4** is stable for months at −20 °C.

When phosphorodiamidite **4** (B^P = Thy, Cyt^{Bz}, Ade^{Bz}, or Gua^{iBu}), 3′-*O*-levulinyl-2′-deoxyribonucleoside **5** (B^P = Thy, Cyt^{Bz}, Ade^{Bz}, or Gua^{iBu}), and sublimed 1*H*-tetrazole were mixed together in a respective molar ratio of (1:1:3) in anhydrous MeCN, the putative dinucleoside phosphorotetrazolide **6** (Scheme 1) was generated within 10 min. Without isolation, **6** was mixed with support **3** and was allowed to react for 10 min at ∼25 °C. The support was washed with 20% 1-methylimidazole in H_2O/MeCN (1:1 v/v) to cleave any nucleobase adducts that might have been formed during the coupling reaction. Exposure of the support to an oxidant, namely, 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN or 0.02 M I_2 in THF/pyridine/H_2O gave CPG support **7** (Scheme 1). The unreacted hydroxyl groups of CPG support **3** were deactivated when reacted with equal volumes of Cap A and Cap B solutions. Under these conditions, the concentration of a dinucleoside phosphate/thiophosphate on CPG support **7** averaged 40 μmol/g of support, as determined by the standard trityl assay, and is comparable to the concentration of deoxyribonucleosides linked to unmodified CPG supports.

Deprotection and release of the dinucleoside phosphate/thiophosphate triester covalently attached to CPG support **7** was initiated to evaluate the purity of each of the sixteen combinatorial dinucleoside phosphorothioate diesters and that of two exemplary dinucleoside phosphate diesters (data shown in the Supporting Information). The deprotection steps included: (i) acid-mediated cleavage of the 5′-*O*-DMTr group and (ii) exposure to pressurized ammonia gas to deprotect the nucleobase, 3′-hydroxyl, and eliminate the 2-cyanoethyl group from the phosphorothioamidate linker. The deprotection side products were conveniently eliminated from the support by washing **8** (Scheme 2) with MeCN/H_2O (1:1 v/v). The support-bound dinucleoside phosphate/thiophosphate triester **8** also served as a model for assessing the thermolytic cleavage of oligonucleotides from the CPG support. The support **8** was suspended in 1 × phosphate buffered saline (PBS, pH 7.2) and the resulting suspension was heated in a closed vial at 90 °C for 2 h. This process induced a thermolytic intramolecular cyclodeesterification reaction (*14, 22–28*) resulting in the release of the dinucleoside phosphate/thiophosphate diester **9** in the aqueous buffer, whereas the putative cyclodeesterification side product remained covalently linked to CPG support **10** (Scheme 2).

Reversed-phase high performance liquid chromatography (RP-HPLC) analysis of dinucleoside phosphate/thiophosphate diesters **9** revealed clean reaction products (data shown in the Supporting Information). The purity of the dinucleoside phosphorothioate and dinucleoside phosphate diesters exceeded 95% and 98%, respectively. These encouraging results prompted the solid-phase synthesis of the native DNA oligonucleotide 5′-d(CGACTGTGAATCGATGCCAT) and that of its fully phosphorothioated analogue 5′-d($C_{PS}G_{PS}A_{PS}C_{PS}T_{PS}G_{PS}T_{PS}G_{PS}A_{PS}A_{PS}T_{PS}C_{PS}G_{PS}A_{PS}T_{PS}G_{PS}C_{PS}C_{PS}A_{PS}T$) employing the thermolytic support **7**.

Solid-Phase Oligonucleotide Synthesis on CPG Support 7 and Thermolytic Release of DNA Oligonucleotides from the Modified CPG Support. Solid-phase synthesis of DNA oligonucleotides employing the thermolytic CPG support **7** and the conventional CPG support was carried out under standard conditions (see Experimental Procedures section). Oligonucleotides sharing identical DNA sequences were prepared to demonstrate comparability of the oligonucleotides produced from the two supports in terms of identity, purity, and overall yields. The DNA oligonucleotides synthesized through CPG support **7** and the unmodified CPG support were deprotected on exposure to pressurized ammonia gas at 25 °C (*19*). After

washing away the deprotection side products from the modified support, DNA oligonucleotides were thermolytically released from the support under the conditions outlined in Scheme 2. RP-HPLC analysis of the unpurified oligonucleotides released from the modified support confirmed the absence of deprotection side products (Figures 1B and 2B) when compared to oligonucleotides prepared from the unmodified CPG support showing contamination with benzamide, one of the deprotection side products (Figures 1A and 2A). The RP-HPLC retention times of either the two native oligonucleoside phosphate diesters or the two oligonucleoside phosphorothioate diesters were identical within experimental variability (Figures 1 and 2). The polyacrylamide gel electrophoresis (PAGE) profiles of the two 20-mers produced from the use of support **7** are comparable to those produced when employing the unmodified CPG support on the basis of the amounts of less than full-length oligonucleotides contaminating the DNA oligonucleotide products (Figure 3). Each unpurified oligonucleotide was also characterized by MALDI-TOF mass spectrometry; mass determination results were consistent with the DNA sequence of each oligonucleotide (see Experimental Procedures section).

The unpurified native oligonucleoside phosphate diesters synthesized from either support **7** or the unmodified CPG support were further characterized by treatment with snake venom phosphodiesterase and bacterial alkaline phosphatase. RP-HPLC analysis of the digests revealed the expected deoxyribonucleosides without detectable nucleobase modification. These results collectively demonstrate that the idendity, purity, and overall yield of DNA oligonucleotides synthesized through the thermolytic CPG support **7** are comparable to those of identical oligonucleotides prepared from an unmodified CPG support.

Solid-Phase Synthesis and Thermolytic Release of DNA Oligonucleotides Functionalized with Functional Groups at the 3′-Terminus or at Both 5′- And 3′-Termini. The functionalization of the 3′-terminus or both 5′- and 3′-termini of DNA oligonucleotides with various functional groups is useful in the preparation of bioconjugates or in applications where both termini are required to achieve a specific function. The functionalization of a DNA oligonucleotide (20-mer) synthesized through the use of CPG support **7** was attempted after hydrazinolysis of the 3′-terminal levulinyl group. Phosphinylation of the free 3′-hydroxyl group with a commercial fluorescein-labeled phosphoramidite was unsuccessful presumably due to intermolecular crowding near the surface of the support that is commensurate with the chain length of neighboring oligonucleotides. A convenient method for the functionalization of oligonucleotides at the 3′-terminus consisted of generating a deoxyribonucleoside phosphorotetrazolide functionalized with a ligand, in situ, and covalently attaching the modified phosphorotetrazolide to CPG support **3** (Scheme 3). The ligand must however be compatible with the conditions used during oligonucleotide synthesis, oligonucleotide deprotection, and thermolytic release of the oligonucleotide from the support. Typically, condensation of the deoxyribonucleoside phosphorodiamidite **4** (B^P = Thy) with and equimolar amount of 1-pyrenebutanol and 3 molar equiv of sublimed 1*H*-tetrazole in anhydrous MeCN resulted in the formation of the pyrenylated deoxyribonucleoside phosphorotetrazolide **11** (Scheme 3) within 10 min. The hydroxylated support **3** was mixed with **11** and was allowed to react for 10 min at ~25 °C. The functionalized CPG support **12** was obtained after treatment with 20% 1-methylimidazole in H_2O/MeCN (1:1 v/v) to cleave any nucleobase adducts that might have been formed during the coupling reaction, and after oxidation effected by 0.02 M I_2 in THF/pyridine/H_2O. The unreacted CPG support **3** was suspended in Cap A/Cap B (1:1 v/v) solutions to acetylate any free hydroxyls. 5′-Dedimethoxytritylation of **12** under acidic conditions and spectrophotometric determination of the 4,4′-dimethoxytrityl cation at 498 nm indicated that the concentration of the pyrenylated deoxyribonucleoside phosphate, covalently attached to the CPG support, was 38 μmol/g of **12**. A small fraction of the CPG support **12** was heated in 1 × PBS (pH 7.2) at 90 °C for 2 h to release thymidine 3′-*O*-(1-pyrenebutyl) phosphate, the identity and purity of which was demonstrated by RP-HPLC analysis at 260 and 340 nm (data shown in the Supporting Information). The characteristic UV absorptivity of 1-pyrenebutanol at 340 nm and the absence of absorptivity at 260 nm allows for the unambiguous identification of pyrenylated nucleic acid conjugates.

Solid-phase oligonucleotide synthesis employing the CPG support **12** was carried out on a 0.2 μmol scale using commercial deoxyribonucleoside 3′-phosphoramidites under standard conditions. After the final 5′-*O*-dedimethoxytritylation, oligonucleotide deprotection was initiated by exposing the solid support to pressurized ammonia gas to cleave the nucleobase and the 2-cyanoethyl phosphate protecting groups. Removal of the deprotection side products was effected by washing the CPG support with MeCN/H_2O (1:1 v/v). The support-linked oligonucleotide was heated in 1 × PBS (pH 7.2) at 90 °C for 2 h to release the DNA oligonucleotide conjugate **14**, the identity of which was confirmed by MALDI-TOF mass spectrometry. RP-HPLC profiles of **14** at 260 nm and at 340 nm confirmed the presence of the pyrenyl group in the conjugate and captured the purity of the pyrenylated DNA oligonucleotide, which was 78% based on total peak area at 260 nm (data shown in the Supporting Information).

The versatility of this approach to the preparation of DNA oligonucleotide 3′-conjugates enables one to employ a large variety of commercial hydroxylated ligands or functional groups in the synthesis of these biomolecules, thereby avoiding the restrictions created by the use of commercial supports functionalized with a limited number of ligands or functional groups.

Solid-Phase Synthesis and Thermolytic Release of DNA Oligonucleotides Conjugated with Functional Groups at the 3′-Terminus or at Both 5′- And 3′-Termini. CPG support **7** can also be employed in the solid-phase synthesis of DNA oligonucleotides conjugated with functional groups at both 5′- and 3′-termini (Scheme 4). Hydrazinolysis of **7** followed by condensation of the free 3′-hydroxyl group with *O*-(2-cyanoethyl)-*O*-(5-hexyn-1-yl)-*N*,*N*-diisopropylphosphoramidite and 1*H*-tetrazole in MeCN produced the corresponding 3′-phosphite. Unreacted 3′-hydroxyls were acylated when treated with the Cap A/Cap B solutions and oxidation of the 3′-phosphite to the desired CPG-support **15** was effected by a solution of 0.02 M I_2 in THF/pyridine/water. 5′-Dedimethoxytritylation of **15** was performed under acidic conditions and the solid-phase synthesis of an oligonucleotide sharing a DNA sequence identical to that of conjugate **13** was initiated. Phosphitylation of the terminal 5′-hydroxyl with *O*-(2-cyanoethyl)-*O*-[6-(4-methoxytrityl)amino-1-hexyl]-*N*,*N*-diisopropylphosphoramidite in the presence of 1*H*-tetrazole gave the solid support-bound oligonucleotide **16** after standard capping and oxidation steps. Removal of the 5′-monomethoxytrityl group under acidic conditions followed by acylation of the aminoalkylated oligonucleotide by treatment with azidoacetic anhydride, generated in situ from azidoacetic acid and *N*,*N*′-dicyclohexylcarbodiimide in THF, afforded the CPG-linked oligonucleotide **17** carrying azido and alkynyl groups at the 5′- and 3′-terminus, respectively. Oligonucleotide deprotection was carried out by exposing the solid support to pressurized ammonia gas to remove the nucleobase and phosphate protecting groups, which were discarded by washing the solid support with MeCN/H_2O (1:1 v/v). The deprotected, support-linked oligonucleotide was heated in 1 × PBS at 90

°C for 2 h to release the DNA oligonucleotide **18**. The identity of RP-HPLC-purified **18** was unequivocally confirmed by MALDI-TOF mass spectrometry and the functional characterization of **18** was demonstrated by its circularization through the copper-catalyzed azide-alkyne cycloaddition reaction (*29, 30*). The circularization of **18** was carried out at a concentration of 2 μM in the presence of the Cu(I) catalyst, which was prepared in situ from Cu(II) sulfate pentahydrate, sodium ascorbate, and *tris*(benzyltriazolylmethyl)amine (*30*) in 0.2 M NaCl (*21*). The formation of the cyclic DNA oligonucleotide **19** was assessed by RP-HPLC and MALDI-TOF mass spectrometry (data shown in the Supporting Information).

In summary, the functionalization of long chain alkylamine CPG with an hydroxyalkylated thiophosphoramidate linker produced the CPG support **3**. This support allowed the covalent attachment of combinatorial dinucleoside phosphate/thiophosphate that were generated in situ from activated deoxyribonucleoside phosphorodiamidites (**4**) and 3′-*O*-levulinated deoxyribonucleosides (**5**) to produce the functionalized CPG support **7**, which in turn enabled the solid-phase synthesis of native or modified DNA oligonucleotides. These oligonucleotides can be freed from nucleobase, hydroxyl, and phosphate/thiophosphate protecting groups while being still attached to the support. The very unique feature of CPG support **7** is that fully deprotected DNA oligonucleotides can be released from the support according to a heat-driven cyclodeesterification pathway under nearly neutral conditions. In addition to the orthogonality of oligonucleotide release conditions relative to those employed in conventional solid-phase oligonucleotide synthesis, the versatility of CPG support **7** has been demonstrated in the preparation and full deprotection of a DNA oligonucleotide conjugated with two different functional groups at the 5′- and 3′-termini while still being covalently bound to the support. This approach to the preparation of DNA oligonucleotide conjugates allows the selection of a wide variety of ligands or functional groups in the synthesis of these biomolecules assuming stability of the ligands or functional groups to the conditions employed during synthesis, deprotection, and thermolytic release of the DNA conjugates. The application of the CPG support **3** to the solid-phase synthesis of oligoribonucleotides and their analogues in the context of RNA interference studies is currently being evaluated in our laboratory.

Supporting Information Available: Materials and methods; ^{1}H, ^{13}C, and ^{31}P NMR spectra of deoxyribonucleoside phosphorodiamidites **4**; ^{1}H−^{1}H TOCSY spectra and ^{1}H−^{13}C HSQC spectra of phosphorodiamidites **4**; high resolution mass spectra of phosphorodiamidites **4**; RP-HPLC chromatograms of dinucleoside phosphate diesters and dinucleoside phosphorothioate diesters that were thermolytically released from the CPG support **7**; enlarged Figures 1 and 2; RP-HPLC chromatogram of 5′-d(CGACTGTGAATCGATGCCAT) after treatment with snake venom phosphodiesterase and bacterial alkaline phosphatase; RP-HPLC chromatograms of unpurified thymidine 3′-*O*-(1-pyrenebutyl) phosphate that was thermolytically released from the CPG support **12**; RP-HPLC chromatograms of unpurified 3′-pyrenylated DNA oligonucleotide **14**; RP-HPLC chromatograms of the (3′,5′)-heteroconjugated DNA oligonucleotide **18** and of the circular DNA oligonucleotide **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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