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Conjugatable and Bioreduction Cleavable Linker for the 5′-Functionalization of Oligonucleotides

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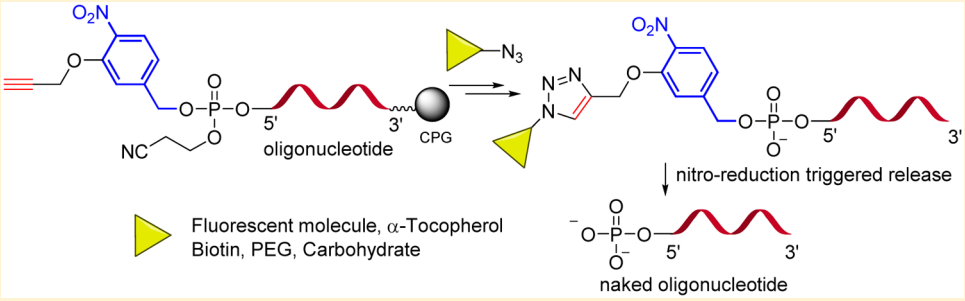
Supporting Information



ABSTRACT: An efficient conjugatable and bioreduction cleavable linker was designed and synthesized for the 5′-terminal ends of oligonucleotides. A phosphoramidite reagent bearing this linker was successfully applied to solid phase synthesis and incorporated at the 5′-terminal ends of oligonucleotides. The controlled pore glass (CPG)-supported oligonucleotides were subsequently conjugated to a diverse range of functional molecules using a CuAAC reaction. The synthesized oligonucleotide conjugates were then cleaved using a nitroreductase/NADH bioreduction system to release the naked oligonucleotides.

The conjugation of oligonucleotides with functional molecules is an important strategy for the construction of modified oligonucleotides with various applications in medicinal chemistry, diagnostics, and biological science.[1−6] Functional molecules can be attached to other compounds using a postsynthetic modification strategy, which requires the presence of a reactive group in the parent compound as a handle for the attachment of the functional molecule. This strategy has been widely used to attach a broad range of functional molecules (i.e., from small molecules to macromolecules) to various compounds and represents a simple and low-cost method for introducing functionality.[5−7] Several postsynthetic modification reactions have been reported in the literature,[5,6] including, most notably, the Cu(I)-catalyzed alkyne azide cycloaddition (CuAAC) reaction,[8−10] which has been used extensively in the field of nucleic acid chemistry[11−19] because of its high reaction efficacy and orthogonal reactivity profile.

Stimulus-responsive linkers are highly desired for the development of drug delivery systems for nucleic acid-based drugs.[20,21] To date, various cleavable linkers have been reported with numerous applications in the life sciences.[22,23] One of the most commonly used types of cleavable linkers are disulfide linkers, which can be cleaved by intracellular glutathione,[24] and linkers belonging to this class have been applied to several nucleic acid-based drugs.[20,25,26] As part of our research toward the development of new reduction-responsive cleavable linkers for oligonucleotides, we became interested in 4-nitrobenzyl-type structures[27] because we envisaged that they would be cleaved by a reductive-elimination reaction in cancer cells or bacterial cells.[28−30]

Herein, we report the design and synthesis of a phosphoramidite reagent bearing a 4-nitrobenzyl-type linker with an alkyne moiety. This reagent was used in a final capping reaction at the 5′-terminal end of an oligonucleotide prepared using a DNA/RNA synthesizer. The subsequent CuAAC reaction of this moiety with various functional molecules[6] could then be used to achieve the efficient preparation of oligonucleotide conjugates (Figure 1). These procedures could therefore be used to synthesize a diverse range of 5′-modified oligonucleotides from one parent oligonucleotide precursor. Furthermore, this linker can be readily cleaved under bioreductive conditions to release the naked oligonucleotide.

The synthesis of our phosphoramidite bearing a clickable/biocleavable linker started from the commercially available aldehyde **1**, which was reduced with $NaBH_4$ to give the known alcohol **2**.[31] The phenolic group of **2** was selectively alkylated with propargyl bromide in the presence of K_2CO_3 to give compound **3**, which was converted to phosphoramidite **4** for solid phase DNA synthesis using standard procedures (Scheme 1).

Phosphoramidite **4** was incorporated at the 5′-end of a series of model oligonucleotides (oligothymidylate and mixed sequence) using standard phosphoramidite chemistry (Scheme 2). Next, prior to the release and deprotection, we conducted

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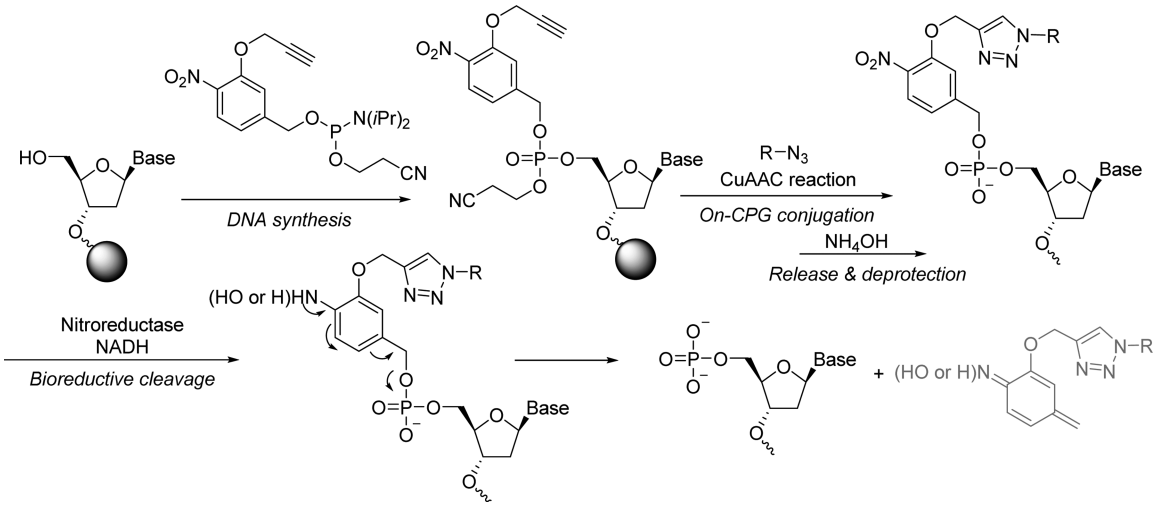
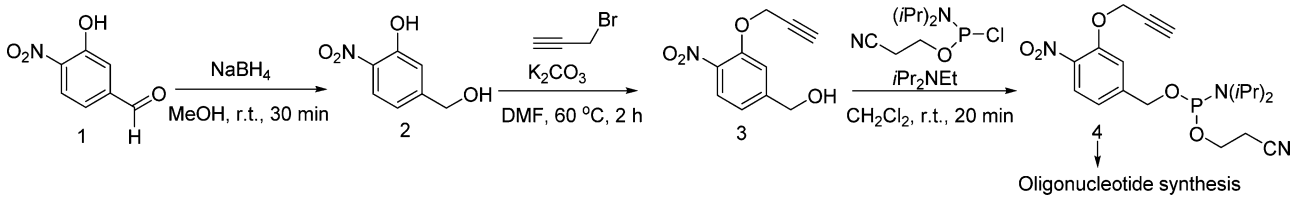
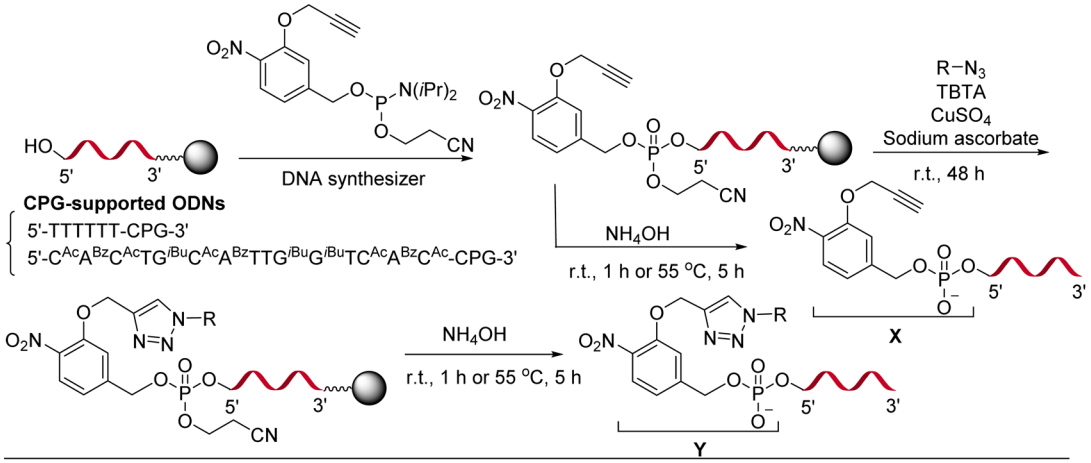


Figure 1. Conjugation/bioreductive cleavage strategy for nucleic acid.

Scheme 1. Synthesis of Phosphoramidite and Its Incorporation in Oligonucleotides



Scheme 2. Conjugation with Various Functional Molecules Using a CuAAC Reaction on the CPG Support



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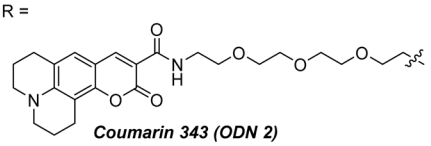
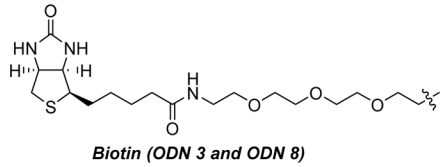
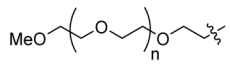


Coumarin 343 (ODN 2)

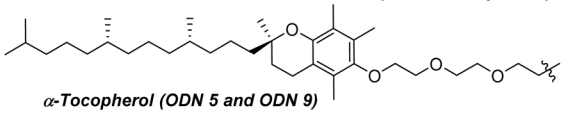


Biotin (ODN 3 and ODN 8)

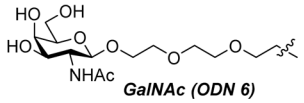


Methoxypolyethylene glycol 2000 (ODN 4)

ODN 1 : 5'-XTTTTTT-3' (Non CuAAC)
ODN 2 : 5'-YTTTTTT-3' (R = Coumarin 343)
ODN 3 : 5'-YTTTTTT-3' (R = Biotin)
ODN 4 : 5'-YTTTTTT-3' (R = Methoxypolyethylene glycol 2000)
ODN 5 : 5'-YTTTTTT-3' (R = α-Tocopherol)
ODN 6 : 5'-YTTTTTT-3' (R = GalNAc)
ODN 7 : 5'-XCACTGCATTGGTCAC-3' (Non CuAAC)
ODN 8 : 5'-YCACTGCATTGGTCAC-3' (R = Biotin)
ODN 9 : 5'-YCACTGCATTGGTCAC-3' (R = α-Tocopherol)



α-Tocopherol (ODN 5 and ODN 9)



GalNAc (ODN 6)

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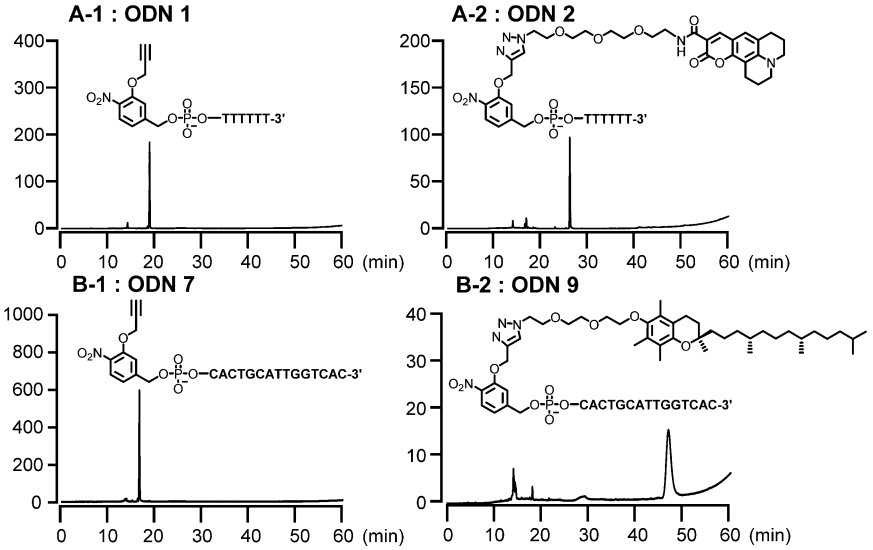


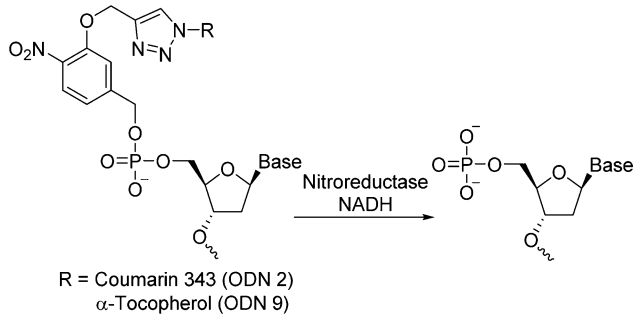
Figure 2. RP HPLC profiles of the crude conjugated ODNs before HPLC purification, which were prepared using an on-solid support CuAAC reaction. Panel A-1: **ODN 1**; Panel A-2: **ODN 2**; Panel B-1: **ODN 7**; Panel B-2: **ODN 9**. HPLC conditions: A buffer (0.1 M TEAA containing 5% CH_3CN), B buffer (CH_3CN), gradient (B) 0 to 100% (60 min). Flow rate: 1 mL/min.

an on-solid support CuAAC reaction[11,15,18] to allow for the attachment of several functional molecules, including biotin,[32] α-tocopherol,[33] *N*-acetylgalactosamine (GalNAc),[34] and polyethylene glycol (PEG)[35] to the oligonucleotide. These molecules were selected because they have been reported to enhance cellular uptake and prolong blood circulation. With the exception of the commercially available materials, the procedure used to prepare the azide described in this study is shown in Scheme S1. Upon completion of the reaction (48 h, r.t.), the remaining reagents were washed out of the reaction mixture, and the conjugated oligonucleotides were released from the CPG support and deprotected with aqueous NH_4OH. The resulting oligonucleotides (ODNs) were analyzed by reversed-phase HPLC (RP-HPLC), and the typical results are shown in Figure 2.

Panels A-1 and B-1 show the HPLC chromatograms of the nonconjugated oligonucleotides **ODN 1** and **ODN 7** before HPLC purification, which were used as controls. These chromatograms revealed that the linker was stable to the DNA synthesis and deprotection conditions. Panels A-2 and B-2 show the HPLC profiles of the conjugated oligonucleotides **ODN 2** and **ODN 9** before HPLC purification, respectively. The coupling yields for these reactions were generally good, and several other conjugates were also prepared in good yield, as described in Figure S1. The crude compound mixtures were purified by preparative HPLC, and the structures of the pure compounds were confirmed by MALDI-TOF mass spectroscopy (Figure S2).

We then proceeded to investigate the cleavability of the linker to determine whether the functional molecules could be readily separated from the conjugated oligonucleotides by bioreduction (Scheme 3). The conjugates were treated with nitroreductase (from *Escherichia coli*) and NADH, which were selected as model reductive conditions, and the reaction was monitored by HPLC. Time-course profiles for the deprotection reaction are shown in Figure 3. Panels A (bottom) and B (bottom) show the peaks for the DNA conjugates **ODN 2** and **ODN 9** as well as the internal standard (asterisk) and NADH (5−12 min). The addition of the enzyme led to the cleavage of the linker to give the corresponding 5′-phosphorylated ODNs

Scheme 3. Release of the Naked Oligonucleotide via a Nitro-Reduction Reaction



(Panel A, upper; Panel B, upper) in a time-dependent manner. The conversion yields were estimated from the HPLC data, and the results were determined to be as follows: 15 min, 35%; 30 min, 53%; 60 min, 69%; 120 min, 85% for panel A and 15 min, 22%; 30 min, 32%; 60 min, 38%; 120 min, 46% for panel B. The newly formed peak (naked ODN, as indicated by the black allow) was isolated by preparative HPLC, and its structure was confirmed by MALDI-TOF mass spectroscopy. The linker was stable in a buffer containing NADH or enzyme (Figure S3). These results indicated that the reduction-elimination reaction performed effectively for the elimination of the functionalized molecules attached to the oligonucleotides. Several other conjugate molecules (**ODN 3**−**6** and **8**) were also cleaved under the same conditions, as shown in Figure S4. To assess the applicability of these functionalized oligonucleotides to in vivo systems, we need to investigate the cleavage reaction under the hypoxic conditions typically observed in tumor cells. These studies go beyond the scope of the current study but are currently in progress in our laboratory and will be reported elsewhere in due course.

To summarize, a phosphoramidite monomer bearing a conjugatable and bioreduction cleavable linker was prepared to functionalize the 5′-terminal ends of oligonucleotides. This system was successfully applied to solid phase synthesis and incorporated at the 5′-terminal ends of oligonucleotides. The

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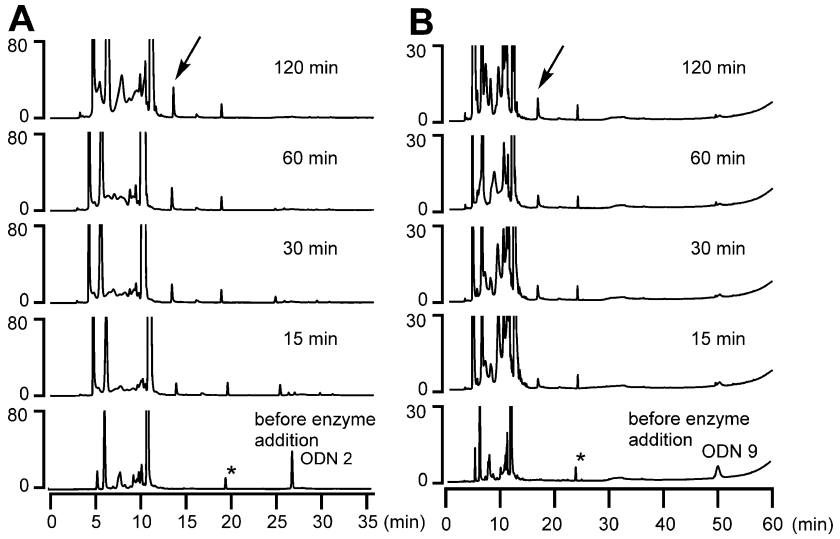


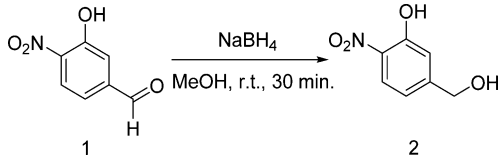
Figure 3. Time-dependent RP-HPLC profiles for the nitroreductase-triggered cleavage of the linkers in the ODNs: Panel A: **ODN 2**; Panel B: **ODN 9**; *internal standard (N6-Bz-2′-dA). HPLC conditions: Panel A: A buffer (0.1 M TEAA containing 5% CH_3CN), B buffer (CH_3CN), gradient (B) 0 to 60% (36 min). Flow rate: 1 mL/min. Panel B: A buffer (0.1 M TEAA containing 5% CH_3CN), B buffer (CH_3CN), gradient (B) 0 to 20% (20 min)and then 20 to 100% (40 min). Flow rate: 1 mL/min.

subsequent conjugation of these CPG-supported oligonucleotides with a diverse range of functional molecules was achieved using an on-CPG CuAAC reaction. The resulting oligonucleotide-functionalized conjugates were successfully cleaved using a nitroreductase/NADH system to release the naked oligonucleotides. This new method could be used for the functionalization of oligonucleotides and bioactive nucleotides at their 5′-termini, and the biocleavable properties could be useful for medicinal chemistry and biotechnology applications. For example, the linker reported in this study could be used like a prodrug for bioreductive cleavage leading to the release of a 5′-phosphorylated guide strand RNA, which is a component of the siRNA used for selective RNA interference in cancer cells.

EXPERIMENTAL SECTION

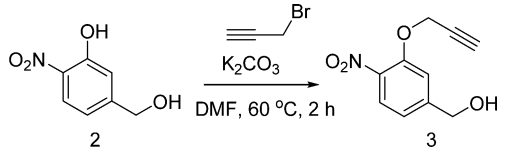
General Experimental Procedure. Chemicals were purchased from a commercial supplier and used without further purification. NMR spectra were recorded at 500 or 600 MHz for ^{1}H NMR, 126 or 151 MHz for ^{13}C NMR, and 243 MHz for ^{31}P NMR. Chemical shifts were measured from tetramethylsilane for ^{1}H NMR spectra and ^{13}C NMR spectra and 85% phosphoric acid (0.0 ppm) for ^{31}P NMR spectra. The coupling constant (J) was reported in hertz. Abbreviations for multiplicity were: s, singlet; d, doublet; t, triplet; sext, sextet; m, multiplet; br, broad. Column chromatography was carried out with a silica gel C-60 or NH silica gel. Thin-layer chromatography (TLC) analyses were carried out on preparative TLC.

5-(Hydroxymethyl)-2-nitrophenol[31] **2.** 3-Hydroxy-4-nitrobenzaldehyde (1.02 g, 6.11 mmol) was dissolved in MeOH (20 mL). To



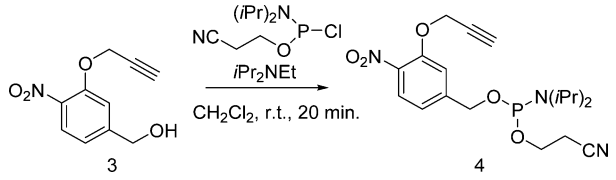
the solution was carefully added $NaBH_4$ (462 mg, 12.2 mmol), and the solution was stirred at room temperature for 30 min. The solution was quenched with 1 M HCl aq, evaporated in vacuo, dissolved in EtOAc, and washed with H_2O. The organic solution was dried (Na_2SO_4), filtered, and evaporated in vacuo. The residue was purified by column chromatography on a silica gel eluted with $CHCl_3$/MeOH (95:5, v/v) to give (967 mg, 94%) as a yellow solid: mp 96.5−97.0 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.66 (1H, s), 8.10 (1H, d, J = 8.3 Hz), 7.18 (1H, s), 6.98 (1H, dd, J = 8.2, 1.4 Hz), 4.77 (2H, d, J = 5.5 Hz), 1.89 (1H, t, J = 5.5 Hz).

(4-Nitro-3-(prop-2-yn-1-yloxy)phenyl)methanol 3. Compound **2** (580 mg, 3.43 mmol) and K_2CO_3 (1.42 g, 10.3 mmol)



were dissolved in DMF (10 mL), and a solution was stirred at 60 °C for 1 h. To the solution was added propargyl bromide (368 μL, 4.12 mmol), and the solution was stirred at 60 °C for 1 h. The solution was diluted with EtOAc and washed with H_2O (twice). The organic solution was dried ($MgSO_4$), filtered, and evaporated in vacuo. The residue was purified by column chromatography on a silica gel eluted with hexane:EtOAc (80:20 → 70:30, v/v) to give **3** (670 mg, 94%) as a pale yellow solid: mp 83.5−84.5 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.86 (1H, d, J = 8.6 Hz), 7.28 (1H, s), 7.05 (1H, d, J = 8.3 Hz), 4.86 (2H, d, J = 2.4 Hz), 4.79 (2H, d, J = 5.5 Hz), 2.60 (1H, t, J = 2.4 Hz), 2.23 (1H, t, J = 5.8 Hz); ^{13}C NMR (151 MHz, $CDCl_3$) δ 151.2, 148.2, 139.0, 126.1, 118.9, 113.0, 77.2, 77.1, 64.0, 57.2.; HRMS (ESI-TOF) m/z: [M + Na^+] calcd for $C_{10}H_9N\,NaO_4^+$: 230.0424, found: 230.0426.

2-Cyanoethyl (4-Nitro-3-(prop-2-yn-1-yloxy)benzyl) Diisopropylphosphoramidite 4. Compound **3** (52 mg, 250 μmol) was



coevaporated with pyridine (5 times) and toluene (twice) and dissolved in CH_2Cl_2 (5 mL). To the solution were added iPr$_2$NEt (87 μL, 500 μmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (83 μL, 375 μmol) under argon. The mixture was stirred at room temperature for 20 min and diluted with CH_2Cl_2, dried (Na_2SO_4), filtered, and evaporated in vacuo. The residue was purified by column chromatography on a NH-silica gel eluted with hexane:EtOAc (50:50, v/v) to give **4** (52 mg, 53%) as a clear oil; ^{1}H NMR (500 MHz,

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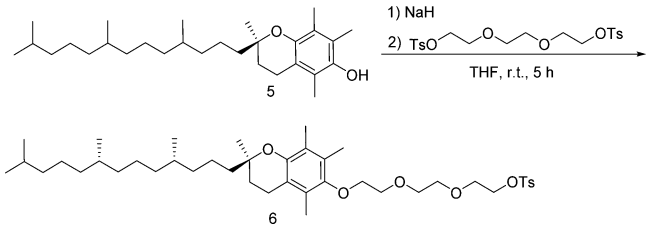
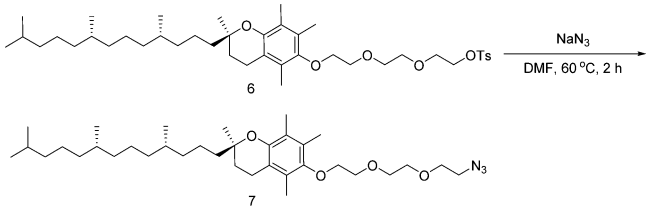
$CDCl_3$) δ 7.87 (1H, d, J = 8.6 Hz), 7.29 (1H, brs), 7.05−7.03 (1H, m), 4.86 (2H, d, J = 2.6 Hz), 4.83−4.73 (2H, m), 3.95−3.89 (1H, m), 3.86−3.80 (1H, m), 3.74−3.63 (2H, m), 2.67 (2H, t, J = 6.6 Hz), 2.59 (1H, t, J = 2.3 Hz), 1.23−1.21 (12H, m); ^{13}C NMR (126 MHz, $CDCl_3$) δ 151.1, 146.52, 146.47, 139.1, 125.9, 119.1, 117.6, 113.3, 77.26, 77.02, 64.5, 64.3, 58.5, 58.3, 57.2, 43.4, 43.3, 24.74, 24.68, 24.6, 20.52, 20.46; ^{31}P NMR (243 MHz, $CDCl_3$) δ 149.7 HRMS (ESI-TOF) m/z: [M + Na^+] calcd for $C_{19}H_{26}N_3NaO_5P^+$: 430.1502, found: 430.1506.

2-(2-(2-(((*R*)-2,5,7,8-Tetramethyl-2-((4*S*,8*S*)-4,8,12-trimethyltridecyl)chroman-6-yl)oxy)ethoxy)ethoxy)ethyl 4-

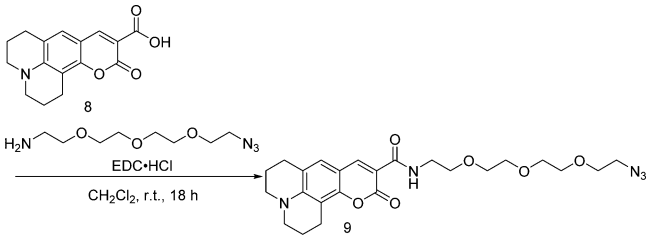


Methylbenzenesulfonate 6. α-Tocopherol (312 mg, 0.724 mmol) was dissolved in THF (10 mL) under argon. To the solution was added 55% NaH (65 mg, 1.46 mmol), and the solution was stirred at room temperature for 5 min. To the mixture was added triethylene glycol bis(p-toluenesulfonate) (995 mg, 2.17 mmol), and it was stirred at room temperature for 5 h. Crushed ice was added to the reaction mixture, which was extracted with EtOAc. The organic solution was washed with brine, dried (Na_2SO_4), filtered, and evaporated in vacuo. The residue was purified by column chromatography on a silica gel eluted with hexane:EtOAc (90:10 → 80:20, v/v) to give **6** (495 mg, 95%) as a yellow syrup. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.81 (2H, d, J = 8.3 Hz), 7.33 (2H, d, J = 8.3 Hz), 4.18 (2H, t, J = 4.8 Hz), 3.80−3.77 (4H, m), 3.74 (1H, t, J = 4.8 Hz), 3.69−3.67 (2H, m), 3.65−3.63 (2H, m), 2.57 (2H, t, J = 6.2 Hz), 2.43 (3H, s), 2.16 (3H, s), 2.12 (3H, s), 2.07 (3H, s), 1.83−1.72 (2H, m), 1.56−1.04 (24H, m), 0.87−0.84 (12H, m); ^{13}C NMR (151 MHz, $CDCl_3$) δ 148.0, 147.8, 144.8, 133.0, 129.8, 128.0, 127.8, 125.8, 122.8, 117.5, 74.8, 72.1, 70.9, 70.6, 69.3, 68.8, 40.1, 39.4, 37.49, 37.47, 37.44, 37.3, 32.8, 32.7, 31.3, 28.0, 24.8, 24.5, 23.9, 22.7, 22.6, 21.6, 21.1, 20.6, 19.8, 19.7, 12.7, 11.84, 11.79.; HRMS (ESI-TOF) m/z: [M + Na^+] calcd for $C_{42}H_{68}NaO_7S^+$: 739.4578, found: 739.4588.

(*R*)-6-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)-2,5,7,8-tetramethyl-2-((4*S*,8*S*)-4,8,12-trimethyltridecyl)chromane 7. Com-

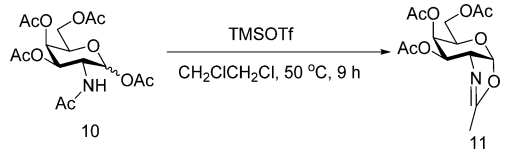


pound **6** (120 mg, 0.167 mmol) was dissolved in DMF (2 mL). To a solution was added NaN_3 (109 mg, 1.68 mmol), and the solution was stirred at 60 °C for 2 h. The reaction mixture was diluted with EtOAc and washed with H_2O and brine. The organic solution was dried (Na_2SO_4), filtered, and evaporated in vacuo. The residue was purified by column chromatography on a silica gel eluted with hexane:EtOAc (9:1, v/v) to give azide derivative **7** (79 mg, 81%) as a yellow syrup. ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.83 (4H, s), 3.77−3.76 (2H, m), 3.73−3.71 (4H, m), 3.41 (2H, t, J = 4.8 Hz), 2.58 (2H, t, J = 6.9 Hz), 2.18 (3H, s), 2.14 (3H, s), 2.07 (3H, s), 1.83−1.72 (2H, m), 1.56−1.04 (24H, m), 0.87−0.83 (12H, m); ^{13}C NMR (151 MHz, $CDCl_3$) δ 148.1, 147.8, 127.8, 125.8, 122.8, 117.5, 74.8, 72.1, 71.0, 70.8, 70.7, 70.2, 50.8, 40.1, 39.4, 37.5, 37.4, 37.3, 32.8, 32.7, 31.3, 28.0, 24.8, 24.5, 23.9, 22.7, 22.6, 21.0, 20.7, 19.8, 19.7, 12.7, 11.84, 11.79.; HRMS (ESI-TOF) m/z: [M + Na^+] calcd for $C_{35}H_{61}N_3NaO_4^+$: 610.4554, found: 610.4569.



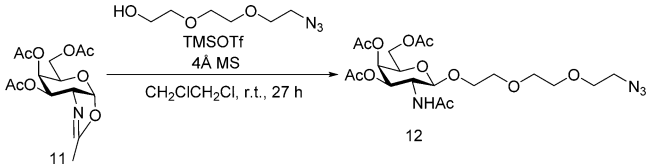
***N*-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl)-11-oxo-2,3,6,7-tetrahydro-1*H*,5*H*,11*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]-quinoline-9-carboxamide 9.** Coumarin 343 (50 mg, 0.175 mmol) and 1-amino-11-azido-3,6,9-trioxaundecane (50 mg, 0.229 mmol) were dissolved in CH_2Cl_2 (2 mL). To the solution was added EDC·HCl (38 mg, 0.198 mmol). The resulted mixture was stirred at room temperature for 18 h. The mixture was evaporated in vacuo. The residue was purified by column chromatography on a silica gel eluted with $CHCl_3$/MeOH (50:1, v/v) to give **9** (83 mg, 98%) as a yellow syrup. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.08−9.07 (1H, m), 8.58 (1H, s), 7.00 (1H, s), 3.70−3.63 (14H, m), 3.40 (2H, t, J = 4.8 Hz), 3.34 (4H, t, J = 6.9 Hz), 2.89 (2H, t, J = 6.2 Hz), 2.78 (2H, t, J = 6.2 Hz), 2.00 (4H, sext, J = 6.9 Hz); ^{13}C NMR (151 MHz, $CDCl_3$) δ 163.4, 162.9, 152.7, 148.1, 148.0, 127.0, 119.6, 109.0, 108.2, 105.6, 70.74, 70.68, 70.6, 70.0, 69.9, 50.7, 50.2, 49.8, 39.4, 27.5, 21.1, 20.2, 20.1; HRMS (ESI-TOF) m/z: [M + Na^+] calcd for $C_{24}H_{31}N_5NaO_6^+$: 508.2167, found: 508.2174.

Compound 11.[34] D-Galactosamine pentaacetate **10** (2.00 g, 5.14 mmol) was dissolved in 1,2-dichloroethane (20 mL) under argon.



TMSOTf (1 mL, 5.53 mmol) was added to the mixture, which was stirred at 50 °C for 9 h. Saturated $NaHCO_3$ aq was added to the mixture, which was washed with saturated $NaHCO_3$ aq (twice) and brine (twice). The organic solution was dried (Na_2SO_4), filtered, and evaporated in vacuo. The residue was purified by column chromatography on a silica gel eluted with $CHCl_3$/MeOH (50:1, v/v) to give oxazoline derivative **11** (925 mg, 54%) as a clear syrup. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.00 (1H, d, J = 6.9 Hz), 5.47 (1H, t, J = 3.1 Hz), 4.92 (1H, dd, J = 7.6, 3.4 Hz), 4.26 (1H, td, J = 6.9, 2.8 Hz), 4.21 (1H, dd, J = 11.7, 7.6 Hz), 4.13 (1H, dd, J = 11.7, 6.2 Hz), 4.01 (1H, td, J = 7.6, 1.4 Hz), 2.13 (3H, s), 2.07 (6H, s), 2.06 (3H, d, J = 1.4 Hz).

2-(2-(2-Azideethoxy)ethoxy)ethyl-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranoside 12.[34] Compound **11** (847



mg, 2.57 mmol) and 0.5 M 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol in t-butyl methyl ether (7.7 mL, 3.35 mmol) was coevaporated with pyiridine (thrice) and toluene (thrice) and dissolved in 1,2-dichloroethane (10 mL) under argon. To the solution was added 4 Å molecular sieves (1 g), and the mixture was stirred at room temperature for 30 min. TMSOTf (232 μL, 1.29 mmol) was added, and the mixture was stirred at room temperature for 19 h, and TMSOTf (232 μL, 1.29 mmol) was further added. The mixture was stirred at same temperature for 8 h, and Et_3N was added. The mixture was evaporated in vacuo. The residue was purified by column chromatography on a silica gel eluted with hexane:EtOAc (1:9, v/v) to give **12** (796 mg, 61%) as a clear syrup. ^{1}H NMR (600 MHz, $CDCl_3$)

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δ 6.35 (1H, d, J = 8.9 Hz), 5.34 (1H, d, J = 3.4 Hz), 5.10 (1H, dd, J = 11.0, 3.4 Hz), 4.78 (1H, d, J = 8.3 Hz), 4.24−4.11 (3H, m), 3.93−3.91 (2H, m), 3.88−3.84 (1H, m), 3.75−3.64 (9H, m), 3.50−3.48 (2H, m), 2.17 (3H, s), 2.05 (3H, s), 2.00 (3H, s), 1.99 (3H, s).

Oligonucleotide Synthesis and On-CPG Conjugation by CuAAC Reaction. Oligonucleotide synthesis was carried out on the Applied Biosystems 394 DNA/RNA Synthesizer according to the manufacturer's recommendations. 2-Cyanoethyl phosphoramidite (dT, dC, dA, and dG) and phosphoramidite **4** were used as 0.1 M solutions in dry acetonitrile. Synthesized solid-supported ODNs were used for conjugation as described below.

Condition A (5′-XTTTTT-3′). CPG support (135 nmol on the 5 mg CPG), azide (1 μmol), copper(II) sulfate pentahydrate (10 nmol), Tris[1-benzyl-1*H*-1,2,3-triazol-4-yl]methyl]amine (10 nmol), and sodium ascorbate (200 nmol) in 100 μL of a mixed solution (MeOH:THF:H_2O:*t*BuOH (50:25:24.5:0.5)) were allowed to stand at room temperature for 48 h.

Condition B (5′-YCACTCGATTGGTCAC-3′). CPG support (135 nmol on the 5 mg CPG), azide (1 μmol), copper(II) sulfate pentahydrate (144 nmol), Tris[1-benzyl-1*H*-1,2,3-triazol-4-yl]methyl]-amine (430 nmol), and sodium ascorbate (430 nmol) in 100 μL of a mixed solution (MeOH:THF:H_2O:*t*BuOH (50:25:21:4)) were allowed to stand at room temperature for 48 h.

After the conjugation reaction was completed, excess reagents were washed out using CH_3CN (1 mL × 5) and CH_2Cl_2 (1 mL × 5). Then, CPG-supported ODN was dried in vacuo and released from the CPG support and deprotected by NH_4OH at room temperature for 1 h (**ODN 1**−**6**) or at 55 °C for 5 h (**ODN 7**−**9**). ODNs were purified by HPLC with a reversed-phase silica gel column.

The structure of each ODN was confirmed by MALDI-TOF mass spectrometry using refection negative mode. (Matrix for ionizing samples was used as a mixture (10:1:1; saturated 3-hydroxy-2-picolic acid, 2-picolic acid/H_2O (50 mg/mL), and ammonium citrate/H_2O (50 mg/mL)). MALDI-TOF mass data: **ODN 1**: calcd [M − H]: 2030.32; found: 2030.16; **ODN 2**: calcd [M − H]: 2515.55; found 2514.81; **ODN 3**: calcd [M − H]: 2474.54; found 2474.88; **ODN 4**: calcd [M − H]: 3830−4230; found 3761−4250; **ODN 5**: calcd [M − H]: 2617.79; found 2618.37; **ODN 6**: calcd [M − H]: 2408.50; found 2408.20; **ODN 7**: calcd [M − H]: 4793.80; found 4795.80; **ODN 8**: calcd [M − H]: 5238.01; found 5240.54; **ODN 9**: calcd [M − H]: 5381.25; found 5384.89

Bioreductive Cleavage of Linker for Releasing Naked Oligonucleotides. ODN (10 μM), NADH (10 mM), and nitro-reductase (20 or 80 μg for **ODN 4**) in 200 μL of 50 mM sodium phosphate (pH 7.0) were incubated at 37 °C. Aliquots of sample solution were analyzed by reverse-phase HPLC at appropriate times. The appeared peak was fractionized and identified by MALDI-TOF mass spectroscopy. 5′-PO_4-TTTTTT-3′ (calcd [M − H]: 1841.28; found: 1841.73). 5′-PO_4-CACTCGATTGGTCAC-3′ (calcd [M − H]: 4604.75; found: 4597.49).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b02527.

HPLC profiles, stability under various conditions, synthetic routes, and copies of ^{1}H, ^{13}C, and ^{31}P NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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