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Photochemical Control of DNA Structure through Radical Disproportionation

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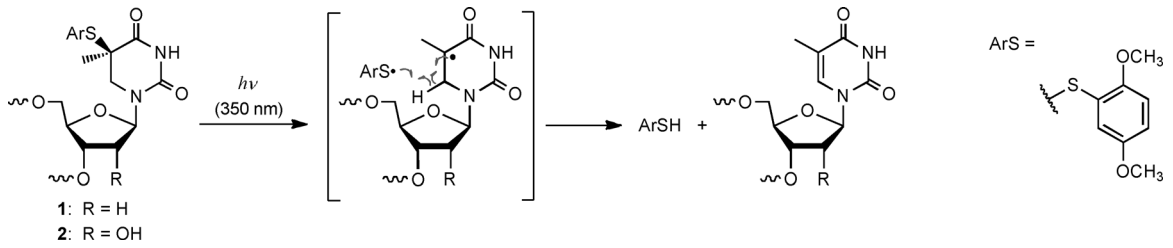
Photolysis of an aryl sulfide-containing 5,6-dihydropyrimidine (**1**) at 350 nm produces high yields of thymidine and products resulting from trapping of a 5,6-dihydrothymidin-5-yl radical by O_2 or thiols. Thymidine is believed to result from disproportionation of the radical pair originally generated from C–S bond homolysis of **1** on the microsecond timescale, which is significantly shorter than other photochemical transformations of modified nucleotides into their native forms. Duplex DNA containing **1** is destabilized, presumably due to disruption of π-stacking. Incorporation of **1** within the binding site of the restriction endonuclease EcoRV provides a photochemical switch for turning on the enzyme's activity. In contrast, **1** is a substrate for endonuclease VIII and serves as a photochemical off switch for this base excision repair enzyme. Modification **1** also modulates the activity of the 10–23 DNAzyme, despite its incorporation into a nonduplex region. Overall, dihydropyrimidine **1** shows promise as a tool to provide spatiotemporal control over DNA structure on the miscrosecond timescale.

Introduction

Transient modification of nucleotides is increasingly valuable in biotechnology and biophysics.[1–8] Modified nucleotides can alter RNA folding, hinder hybridization to complementary sequences in general, and inhibit enzyme activity.[9] In most applications, the modified nucleotide is introduced through chemical or enzymatic synthesis, but reversible modification can also be effected postsynthetically at a specific nucleotide within the biopolymer.[10] Although restoration of the native nucleotide is more common, control can also be effected by triggering the transformation of one nonnative nucleotide into another.[11]

Photochemical triggering provides spatiotemporal control over nucleic acid structure and function, whereas chemically labile modified nucleotides can be manipulated in a temporal manner.[12] Photochemical methods provide the additional opportunity of coupling to time-resolved detection methods.[7] The resolution afforded by such methods is limited by the timescale on which the unmasking reaction occurs. Herein, we report a method for disguising thymidine as a 5,6-dihydropyrimidine aryl sulfide (**1**) that disrupts base stacking. Conversion to thymidine occurs on the microsecond timescale through radical pair disproportionation (Scheme 1).

The *o*-nitrobenzyl photoredox reaction is most frequently used to reversibly modulate nucleic acid structure and function by altering the nucleotide's Watson–Crick hydrogen bonding face (Scheme 2). This is typically accomplished by protecting the exocyclic amine of cytosine or adenine or the O6 position of guanine.[7] The *o*-nitrobenzyl photoredox reaction is very robust and usually provides high yields of the desired product. However, the photochemical reaction yields the unmasked nucleotide via a metastable intermediate that hydrolyzes on the second to even minute timescale.[14] This limits the timescale of



Scheme 1. Photochemical unmasking of thymidine (5-methyluridine) from an aryl sulfide through radical pair disproportionation.

folding processes that one can follow. We recently described the utility of a dihydropyrimidine ribonucleotide (**2**) that produces 5-methyluridine in high yield upon photolysis at 350 nm (Scheme 1).[15] This method is amenable to following significantly faster folding events because the transformation proceeds through a radical disproportionation mechanism that occurs on the microsecond timescale. We have now applied

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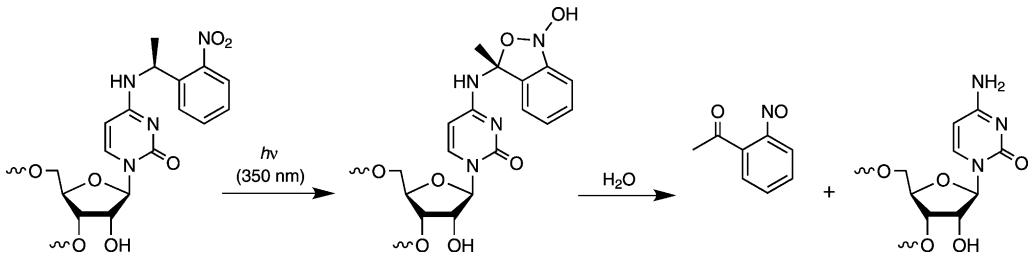


Scheme 2. Transient modification of cytidine through an *o*-nitrobenzyl photoredox reaction.[13]

this method to DNA by introducing a disguised form of thymidine into oligonucleotides.

Results and Discussion

Design and synthesis of a photolabile dihydropyrimidine and its incorporation into chemically synthesized oligonucleotides

Molecular mechanics calculations predict that C5-aryl sulfide **1** will adopt the pseudo-axial conformation and disrupt π-stacking (Figure 1), just as **2** was predicted to do. As expected, the

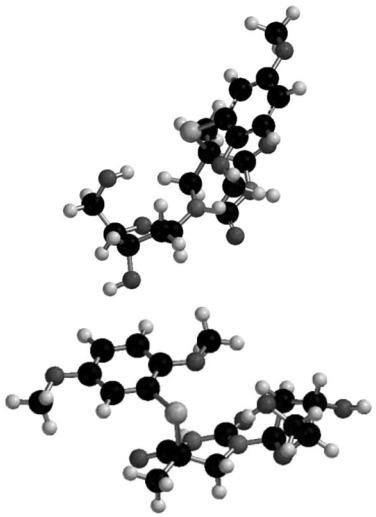
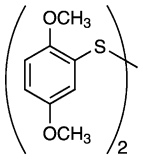


Figure 1. Energy minimized (Spartan) structure of **1**. The top perspective highlights the C2′-*endo* sugar pucker. The bottom perspective highlights the pseudo-axial orientation of the aryl sulfide.

sugar pucker in **1** is C2′-*endo*, which reduces the O4-C1′-N1-C6 dihedral angle to $<2^\circ$ from $\sim 26^\circ$ in **2**. There is ample precedent for photochemical radical generation from aryl sulfides.[15–19] Dimethoxy substitution of the aromatic rings helps to shift absorption to the red, reducing the direct excitation and potential damage of the DNA.

The aryl sulfide was synthesized from bis-silyl-protected 5,6-dihydrothymidine (**3**) by trapping the carbanion with symmetric disulfide **5**.[20] An inseparable 9:1 mixture of silylated diastereomers of **1** was obtained (Scheme 3). Assignment of the 5*R* configuration to the major isomer was based upon comparison to the respective compound obtained during the synthesis of



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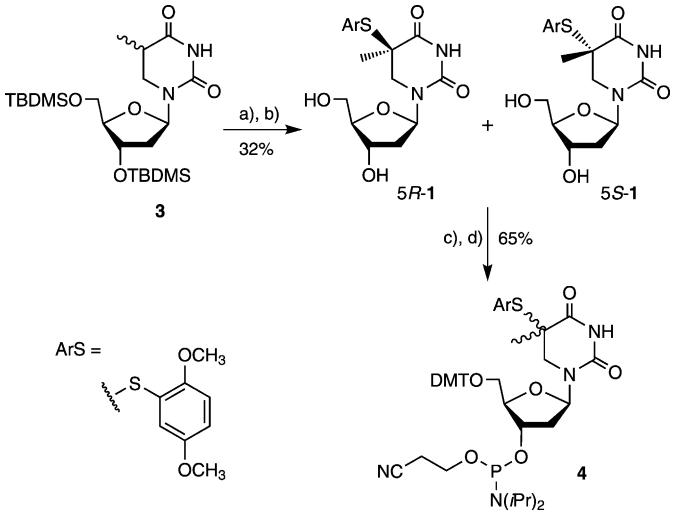
2 and the selectivity of other electrophilic additions to produce 5,6-dihydropyrimidines.[19,21]

Fluoride deprotection yielded **1**, which was used to establish the photochemistry at the monomer level. Nucleoside **1** was also carried on to the phosphoramidite (**4**) required for solid-phase oligonucleotide synthesis through standard methods. Five oligonucleotides (**6**–**10a**) containing **1** were synthesized by using

5′-d(GAG CTA GCT CAG GAT **1**TA CGA TCT GCA GCT)
6
5′-d(GAG CTA GCT CAG GAG **1**TA CGA TCT GCA GCT)
7
5′-d(GCA CCC AGG C**X**A GCT ACA ACG ACT CTC TCC)
8a, **b**
5′-d(GAG CTA GCT CAG GA**X** ATC CGA TCT GCA GCT)
9a, **b**
5′-d(CCA GGC **X**AG CTA)
10a,**b**
a X = **1**; **b X** = T



Scheme 3. Synthesis of photolabile aryl sulfide **1** and its respective phosphoramidite.[13] a) *sec*-BuLi then **5**, b) $Et_3N{\cdot}3HF$, c) DMTCl, d) phosphitylation.

commercially available fast deprotecting phosphoramidites and **4**. A solution of pivaloyl anhydride and 2,6-lutidine in THF (1:1:8 by volume) was substituted for acetic anhydride and 2,6-

lutidine in order to prevent transamidation.[22] Finally, oligonucleotides containing **1** were deprotected and cleaved from the solid-phase support by using K_2CO_3 (0.1 M) in methanol for 7 h at 25 °C to guard against fragmentation of the 5,6-dihydropyrimidine ring. The prolonged time was necessary to remove all of the exocyclic amine protecting groups. All oligonucleotides containing **1** were analyzed by ESI-MS and nuclease digestion.[23] Although **4** consisted of a mixture (~9:1) of epimers at C5, HPLC analysis of enzyme-digested **6** and **7** indicated that (5*R*)-**1** accounted for >96% of the photolabile molecules (assuming that the extinction coefficients of the isomers are the same).[23]

Characterization of the photochemistry of monomeric 1

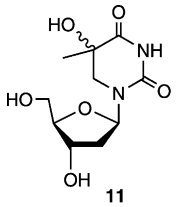
The photochemistry of **1** was initially analyzed by reverse phase HPLC (Table 1). Irradiation in a Rayonet photoreactor

Table 1. Product analysis by HPLC (UV detection) of photolyses of **1**.

O_2	[BME] [mM]	Thymidine [%]	Mass balance [%]
+	0	62.0 ± 0.8	62.1 ± 0.9
+	100	65.3 ± 1.4	65.9 ± 1.4
−	0	72.0 ± 4.4	72.4 ± 4.4
−	100	63.4 ± 3.6	64.7 ± 4.3

(λ_{max} = 350 nm) for 30 min led to >97% conversion. Although β-mercaptoethanol (BME, 100 mM) had no effect (within experimental error) on the yield of thymidine under aerobic conditions, it reduced the yield under anaerobic conditions. The yields of the expected thymidine product ranged from 62 to 72%, and mass balances were just slightly higher, thus indicating that a significant fraction of **1** was unaccounted for. These observations suggested that some of the intermediate radical (Scheme 1) was escaping from the cage and reacting with O_2 or thiol.

The anticipated radical trapping products (**11**–**13**) lack suitable chromophores for HPLC UV detection; this led us to ana-



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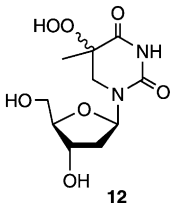

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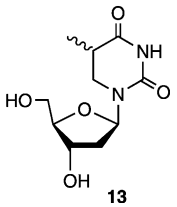

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lyze photolyses of **1** by ^{1}H NMR. Indeed, approximately equal amounts of the C5-epimers of **11** were observed in samples photolyzed under aerobic conditions.[21,23] The ratio of thymidine to **11** was 3:1, and the mass balance ranged from 73–78%. Mass spectral analysis of **11** formed from **1** photolyzed in $H_2{}^{18}O$ contained 100% ^{16}O, consistent with radical trapping by O_2. Hydroperoxide **12** was also detected by LC/MS, further supporting the radical source of **11**, which was presumably formed by thiol reduction of the hydroperoxide. Similarly, ^{1}H NMR analysis of anaerobic photolysates of **1** revealed the formation of **13**. Integration of the C5-methyl groups in the respective products indicated that the ratio of thymidine/**13** varied between 76:24 and 65:35 for 0.1 and 3.0 M BME, respectively, but did not follow any trend.[23] The lack of an effect of BME concentration on the ratio of products suggests that thymidine is formed in a radical cage process. Moreover, using the typical rate constant for thiol trapping of an alkyl radical ($>5\times10^5\ M^{-1}s^{-1}$) indicates that the lifetime of the radical pair (Scheme 1) is <1 μs.[15,19,24,25] Products **11**–**13** were attributed to cage escape. In the absence of added BME, the aromatic thiol produced from the disproportionation reaction is the presumptive reducing agent required for forming **11**–**13**.

The effect of 1 on duplex stability and the activities of naturally occurring enzymes that act on DNA

Aryl sulfide **1** was designed to destabilize duplex DNA by disrupting base stacking. Destabilization was verified by comparing the T_m of a dodecameric duplex containing **1** (**14a**, T_m = (43.9 ± 0.9) °C) with one in which thymidine was incorporated at the comparable position (**14b**, T_m = (51.3 ± 0.1) °C).[23] The melting temperature depression induced by **1** is smaller than that reported for thymidine glycol.[26] However, the latter was measured in a shorter oligonucleotide duplex in which the dihydropyrimidine is expected to have a proportionally larger effect.

5'-d(CCA GGC **X**AG CTA)
3'-d(GGT CCG ATC GAT)
14a: X = **1**
14b: X = Ta

Restriction enzyme activity was also affected by **1**. Less than 9% of duplex **15a**, containing **1** at the center of the recognition sequence for EcoRV (5′-GAT$^{\downarrow}$ATC), was incised, whereas almost 90% of the control substrate containing thymidine (**15b**) was cleaved (Figure 2). Following photolysis under aero-

5'-d(GAG CTA GCT CAG GA**X** ATC CGA TCT GCA GCT)
3'-d(CTC GAT CGA GTC CTA TAG GCT AGA CGT CGA)
15a: **X** = **1**, **b**: **X** = T

5'-d(GAG CTA GCT CAG GAT **1**TA CGA TCT GCA GCT)
3'-d(CTC GAT CGA GTC CTA AAT GCT AGA CGT CGA)
16

5'-d(GAG CTA GCT CAG GAG **1**TA CGA TCT GCA GCT)
3'-d(CTC GAT CGA GTC CTC AAT GCT AGA CGT CGA)
17

bic conditions, almost 68% of **15a** was incised by EcoRV. The incision yields are consistent with the yield of thymidine obtained from photolysis of monomeric **1**, and analysis by nondenaturing gel electrophoresis confirmed that the substrates were completely hybridized.

Structural perturbation by **1** was also examined by using DNA glycosylases involved in base excision repair. Endonuclease III (Nth) and endonuclease VIII (Nei) are bifunctional base excision repair enzymes that possess glycosylase and lyase activities. These catalysts hydrolyze the glycosidic bond of damaged pyrimidines, such as thymidine glycol, and induce β-elim-

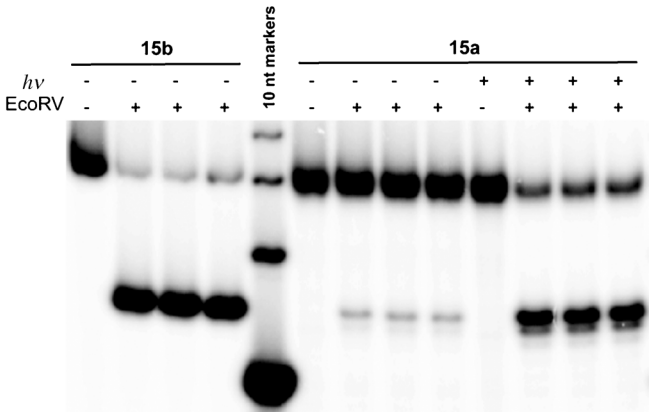


Figure 2. The effect of aryl sulfide **1** on EcoRV activity (the reaction is shown in triplicate).

ination of the formal abasic site that is produced, resulting in cleaved DNA.[27–29] Qualitatively, **1** is a poor substrate for Nth. No incision by Nth is detected in **17**, in which **1** is flanked by a 5′-dG (data not shown). Cleavage is observed when **1** is flanked by a 5′-T in **16** (Figure 3). However, the reaction is slow,

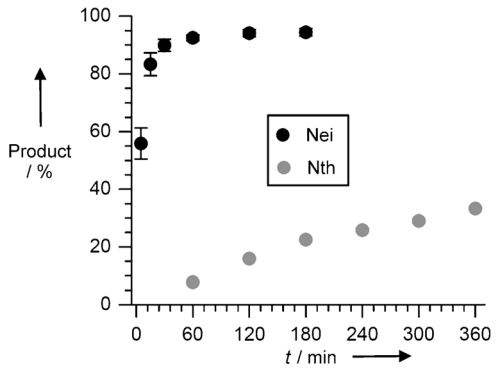
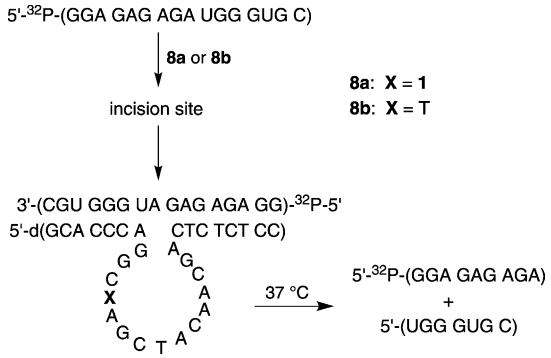


Figure 3. Excision of **1** from **16** by excess (16-fold) Nth and Nei.

and excess Nth is required. In contrast, Nei cleaves DNA containing **1** much more rapidly and is indifferent with respect to either flanking sequence employed (Figure 3 and the Supporting Information). Modest strand scission of **16** and **17** by Nei is even observed when the DNA is in excess.[23] Moreover, Nth incises less than 20% of **16** following photolysis, even after 6 h of incubation.[22] The residual activity is attributed to the formation of **11** upon photolysis of **1**.

Modulation of 10–23 DNA enzyme activity by 1

The 10–23 DNA enzyme (DNAzyme, **8b**), which hydrolyzes RNA, was the first such DNA molecule to be evolved and selected.[30] The DNAzyme uses two DNA arms that flank a 15-nucleotide catalytic domain to bind its RNA substrate through Watson–Crick base pairing (Scheme 4). Successful photochemical modulation of 10–23 activity was achieved by modifying the Watson–Crick face of a thymine within the catalytic domain using an *o*-nitrobenzyl photoredox reaction.[3]



Scheme 4. DNAzyme activity on an RNA substrate.

The 10–23 DNAzyme presents an interesting challenge for **1** to modulate function. The modified thymidine is not part of a duplex, potentially reducing the structural disruption imparted by the aryl sulfide (**1**). Indeed, incorporation of **1** at T11 in **8a** greatly reduced RNA hydrolysis but did not eliminate the reaction (Figure 4). As expected, DNAzyme activity is rapidly restored upon photolysis. The activity is lower than independently synthesized 10–23 DNAzyme (**8b**), but this is expected, given the less than 100% yield of thymidine from photolysis of **1**. Consistent with a previous report, photolysis (30 min) prior to hybridization with the target RNA results in even greater restoration of activity.[3,23] This might be due to preferential binding of the native DNAzyme, which is in excess, to the target RNA. However, other considerations, including populations of one or more unproductive conformations in the complex that is hybridized prior to photolysis are possible.[3]

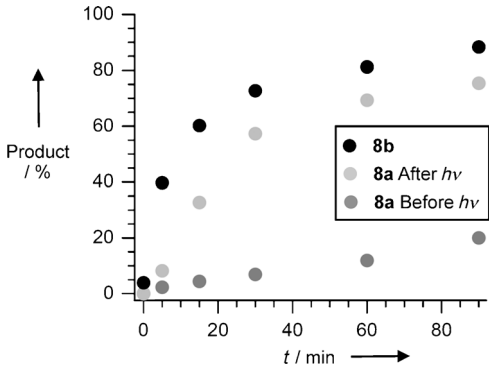


Figure 4. Photochemical modulation of 10–23 DNAzyme activity by **1**.

Conclusions

In summary, we have shown that incorporation of **1** into oligonucleotides turns off enzyme activity that requires native thymidine. The dihydropyrimidine is efficiently recognized and excised by one of two bifunctional base excision repair enzymes (Nei) examined. These data and a UV melting experiment indicate that **1** destabilizes and perturbs duplex DNA structure. Importantly, the activities of the enzymes are reversed when DNA containing **1** is photolyzed. The extent of the photochemical

switch is consistent with the yield of thymidine (~70%) generated from photolysis of monomeric **1**. Unlike other photochemical modulators of nucleic acid function that alter the Watson–Crick hydrogen bonding pattern, **1** perturbs DNA by disrupting base stacking. Its ability to reduce DNAzyme activity suggests that the catalytic core of this molecule contains some stacking at the position at which **1** is substituted. Finally, **1** offers potential advantages over other photolabile modified nucleotides in time-resolved experiments, due to its significantly faster (microsecond timescale) transformation into thymidine following irradiation.

Experimental Section

General methods: All solvents were distilled before reactions. THF was dried over benzophenone. Dichloromethane, TEA, and pyridine were dried over CaH_2. All chemicals were purchased from either Sigma–Aldrich or Fisher and were used without further purification. Oligonucleotides were synthesized on an Applied Biosystems Incorporated 394 oligonucleotide synthesizer. Oligonucleotide synthesis reagents were purchased from Glen Research (Sterling, VA). The RNA substrate for DNAzyme studies was purchased from Integrated DNA Technologies. T4 polynucleotide kinase was obtained from New England Biolabs, and γ-^{32}P-ATP was purchased from PerkinElmer. C18-Sep-Pak cartridges were obtained from Waters, and Poly-Prep columns were obtained from Bio-Rad. Quantification of radiolabeled oligonucleotides was carried out by using a Molecular Dynamics Phosphorimager 840 equipped with ImageQuant version 5.1 software. ESI-MS was carried out on a Thermoquest LCQDeca. LC/MS analyses were carried out with a Waters Xevo G2 Tof mass spectrometer with UPLC/MS^E and QuanTof technology. HPLC analysis was carried out by using a Waters 2487 dual absorbance detector and 515 HPLC pumps. UV spectra and T_m measurements were obtained by using a Beckman DU 640 spectrophotometer equipped with a temperature control unit. All photolyses were carried out in Pyrex tubes by using a Rayonet photoreactor fitted with 16 lamps, with a maximum output at 350 nm. All anaerobic photolyses were carried out in sealed Pyrex tubes, which were degassed and sealed by using a standard freeze-pump-thaw degassing technique (three cycles).

Synthesis of bis-TBDMS-5,6-dihydro-5-(2,5-dimethoxythiophenyl)thymidine: Bis-TBDMS-5,6-dihydrothymidine (**3**, 178.2 mg, 0.38 mmol) was azeotropically dried with pyridine (2 mL).[20] LDA (0.83 mmol) was formed in situ from a solution of dry diisopropylamine (86 mg, 0.83 mmol) and *n*BuLi (53 mg, 0.83 mmol) in THF (0.5 mL) at −78 °C. The reaction was stirred for 10 min, warmed to room temperature for 40 min, then cooled again to −78 °C. Bis-TBDMS-5,6-dihydrothymidine in THF (2 mL) was added, and the solution was stirred for 1 h at −78 °C. After 1 h, a solution of di-(2,5-dimethoxy)phenyl disulfide (**5**, 85 mg, 0.41 mmol) in THF (1 mL) was added.[15] The reaction was monitored by TLC (EtOAc/hexanes 3:7) and quenched by the addition of saturated NH_4Cl (50 mL). The product was extracted with EtOAc (3×50 mL), and the combined organic layers were washed with brine and dried with anhydrous $MgSO_4$. The product was purified by flash column chromatography (EtOAc/hexanes, 1:6–1:1) to give a mixture of diastereomers (9:1) of the coupling product as a white solid (81.4 mg, 34%): ^{1}H NMR ($CDCl_3$): δ = 7.98 (br s, 1H), 7.00 (d, J = 3.2 Hz, 1H), 6.91 (dd, J = 3.2, 9.2 Hz, 1H), 6.79–6.81 (m, 1H), 6.37 (dd, J = 6, 8 Hz, 1H), 4.36–4.39 (m, 1H), 3.76–3.78 (m, 1H), 3.72–3.75 (m, 8H), 3.69–3.70 (m, 2H), 3.44–3.52 (m, 2H), 2.18–2.25 (m, 1H), 1.99–2.04 (m, 1H), 1.37 (s, 3H), 0.92 (s, 9H), 0.89 (s, 9H), 0.09 (s, 3H), 0.08 (s, 6H), 0.07 ppm (s, 3H); ^{13}C NMR ($CDCl_3$): δ = 169.8, 155.9, 153.3, 153.22, 152.2, 125.1, 125.0, 117.7, 117.6, 117.4, 112.4, 112.3, 87.0, 86.5, 84.6, 83.8, 72.7, 72.1, 63.2, 56.5, 56.4, 56.0, 56.0, 49.5, 49.44, 47.9, 47.4, 38.0, 26.2, 26.2, 26.0, 20.9, 20.7, 18.6, 18.59, 18.21, 18.20, −4.4, −4.4, −4.58, −4.6, −5.1 ppm; IR (neat): ν = 3207, 3084, 2953, 2928, 2898, 2856, 1691, 1491, 1471, 1436, 1361, 1257, 1217, 1094, 1025, 831, 775, 734 cm^{-1}; HRMS: *m/z* calcd.: 640.3028 $[M]^+$, found: 640.3024.

Synthesis of 5,6-dihydro-5-(2,5-dimethoxythiophenyl)thymidine (1): Triethylamine·trihydrofluoride (99 mg, 0.6 mmol) was added to a solution of Bis-TBDMS-5,6-dihydro-5-(2,5-dimethoxythiophenyl)-thymidine (81 mg, 0.12 mmol) in THF (0.8 mL). The reaction was stirred overnight at room temperature. The solution was then concentrated in vacuo and purified by flash column chromatography ($MeOH/CH_2Cl_2$ 1:19–1:6) to give a mixture of diastereomers (9:1) of **1** as a white foam (47 mg, 94% yield): ^{1}H NMR ($CDCl_3$): δ = 8.61 (br s, 1H), 6.99 (d, J = 2.8 Hz, 1H), 6.90 (dd, J = 2.8, 8.8 Hz, 1H), 6.79 (d, J = 8.8 Hz, 1H), 6.31 (t, J = 6.8 Hz, 1H), 4.36–4.38 (m, 1H), 3.86–3.87 (m, 2H), 3.72–3.74 (m, 8H), 3.47–3.55 (m, 4H), 2.34–2.37 (m, 1H), 2.16–2.17 (m, 1H), 1.34 ppm (s, 3H); ^{13}C NMR ($CDCl_3$): δ = 170.3, 155.8, 153.2, 153.0, 125.0, 117.7, 117.2, 112.4, 85.7, 84.2, 71.6, 62.8, 56.5, 56.1, 50.9, 49.6, 48.3, 37.1, 20.5 ppm; IR (neat): ν = 3388 (broad), 3086, 2970, 2939, 1691, 1488, 1456, 1379, 1217, 1039, 734 cm^{-1}; HRMS: *m/z* calcd.: 412.1299 $[M]^+$, found: 412.1316.

Synthesis of 5′-DMT-3′-hydroxy-5,6-dihydro-5-(2,5-dimethoxythiophenyl)thymidine: 5,6-Dihydro-5-(2,5-dimethoxythiophenyl)thymidine (**1**, 200 mg, 0.49 mmol) was azeotropically dried with pyridine (2×2 mL). A solution of 4,4′-dimethoxytritylchloride (214 mg, 0.63 mmol) and 4-(dimethylamino)pyridine (11.8 mg, 0.1 mmol) in pyridine (4 mL) was added to the dried nucleoside. The reaction was stirred overnight at room temperature. The solution was then concentrated in vacuo and purified by flash column chromatography ($MeOH/CH_2Cl_2$ 1:20, containing 0.1% triethylamine) to give the major diastereomer as a white foam (251 mg, 72%): ^{1}H NMR ($CDCl_3$): δ = 7.42–7.44 (d, J = 7.2 Hz, 2H), 7.29–7.33 (m, 7H), 7.23–7.25 (m, 2H), 7.01 (d, J = 3.2 Hz, 1H), 6.93 (dd, J = 3.2, 8.8 Hz, 1H), 6.83–6.87 (m, 5H), 6.43 (t, J = 7.2 Hz, 1H) 4.46–4.47 (m, 1H), 3.87–3.88 (m, 1H), 3.87 (s, 6H), 3.76 (s, 3H), 3.75 (s, 3H), 3.32–3.34 (m, 2H), 2.44–2.46 (m, 1H), 2.15–2.25 (m, 1H), 2.01–2.02 (m, 1H), 1.22 ppm (s, 3H); ^{13}C NMR ($CDCl_3$): δ = 169.3, 158.6, 155.7, 153.1, 151.9, 135.7, 135.7, 130.10, 130.06, 128.1, 128.0, 127.0, 124.9, 117.5, 113.3, 112.1, 86.6, 84.2, 83.4, 72.6, 63.7, 56.2, 55.9, 55.2, 49.2, 47.9, 37.5, 20.3 ppm; IR (neat): ν = 3460 (broad), 3214, 3069, 2999, 2958, 2931, 2836, 1689, 1607, 1508, 1490, 1438, 1250, 1219, 1178, 1093, 1035, 956, 911, 828, 733 cm^{-1}; HRMS: *m/z* calcd.: 737.2503 $[M+Na]^+$, found: 737.2507.

Synthesis of phosphoramidite 4: Diisopropylethylamine (DIPEA) (172 mg, 1.33 mmol) was added to a solution of 5′-DMT-5,6-dihydro-5-(2,5-dimethoxythiophenyl)thymidine (237 mg, 0.33 mmol) in CH_2Cl_2 (4 mL). β-Cyanoethyl diisopropylchlorophosphoramidite (94 mg, 0.40 mmol) was then added to the solution. After 1 h, TLC (EtOAc) showed disappearance of the starting material. The solution was quenched with saturated $NaHCO_3$ (30 mL) and extracted with EtOAc (3×30 mL). The combined organic layers were washed with brine and dried with anhydrous Na_2SO_4. The product was purified by flash column chromatography (hexanes/EtOAc 1:3, containing 0.1% triethylamine) to give a white foam (258 mg, 85%). ^{1}H NMR ($CDCl_3$): δ = 7.43–7.44 (m, 2H), 7.30–7.34 (m, 6H), 7.20 (m, 1H), 7.01–7.03 (dd, J = 2.4, 3.2 Hz, 1H), 6.92–6.93 (m, 1H), 6.81–6.86 (m, 5H), 6.40–6.44 (m, 1H), 4.52–4.62 (m, 1H), 4.0–4.1 (m, 1H), 3.80 (s, 3H), 3.79 (s, 3H), 3.76 (s, 3H), 3.74 (s, 3H), 3.59–3.63 (m, 4H), 3.41–3.50 (m, 1H), 3.32–3.42 (m, 1H), 3.18–3.25(m, 1H), 2.41–2.50

(m, 2H), 1.16–1.20 (m, 12H), 1.05–1.07 ppm (d, $J=6.8$ Hz, 3H); ^{31}P NMR ($CDCl_3$): $\delta=148.64$, 148.25 ppm; IR (neat): $\nu=3205$, 3063, 2965, 2932, 2872, 2836, 1694, 1607, 1508, 1490, 1437, 1249, 1219, 1179, 1036, 978, 829, 734 cm^{-1}; HRMS: *m/z* calcd.: 937.3582 $[M+Na]^+$, found: 937.3565.

HPLC analysis of the photochemistry of monomeric 1: Solutions containing monomeric **1** (50 μM) and internal standard 2′-deoxyuridine (30 μM) were photolyzed in a Rayonet photoreactor ($\lambda_{max}=$ 350 nm) for 30 min. For anaerobic photolyses, samples were subjected to three freeze-pump-thaw degas cycles before sealing with a flame torch under vacuum. Samples containing BME were concentrated under reduced pressure and redissolved in water (50 μL) prior to injection. All other samples were directly analyzed by HPLC. HPLC samples were analyzed by using an Agilent Microsorb MV 100-5 C18 column (250×4.6 mm). Samples were detected at 260 and 310 nm following separation with water (solvent A) and CH_3CN (solvent B; flow rate = 1 mL min^{-1}) by using the following linear gradient: (time, % B) 0, 3; 1, 3; 25, 20; 30, 40; 35, 40.

NMR analysis of the photochemistry of monomeric 1: A solution of **1** (5 mM) in water/CH_3CN (1:1), together with a known amount of internal standard (cytidine) was photolyzed for 5–6 h at 350 nm in an open Pyrex tube. After photolysis, the solution was concentrated in vacuo and dissolved in CD_3OD for ^{1}H NMR analysis. When examining the effect of BME on product distribution, the thiol (0.1–3 M) was added to a solution of **1**, prepared as described above. The solution was degassed by using three freeze-pump-thaw degas cycles, sealed under vacuum, and photolyzed (350 nm) for 3–6 h. The solution was concentrated in vacuo overnight, redissolved in D_2O, and analyzed by ^{1}H NMR.

Synthesis and deprotection of oligonucleotides containing 1: Commercially available fast deprotecting phosphoramidites were employed for native nucleotides. Pivaloyl anhydride/2,6-lutidine/THF (1:1:8, *v/v/v*) was substituted for the typical capping agent, and *t*BuOOH (5–6 M in decane) in toluene (final concentration of 1 M) was used as the oxidant instead of I_2. Capping and oxidation times were increased to 25 and 40 s respectively, whereas the coupling time with **4** was increased from 25 s to 10 min. Oligonucleotides were deprotected with K_2CO_3 (0.1 M) in dry MeOH for a total of 7 h (with mixing every 30 min) at room temperature. After deprotection, the solution was neutralized with glacial acetic acid, followed by concentration under reduced pressure. The dried products were resuspended in formamide loading buffer and purified by using 20% denaturing PAGE.

UV melting experiments: UV melting experiments were carried out in quartz cells with a path length of 1 cm. The oligonucleotide samples were prepared by mixing 1 μM of each of the complementary strands in buffer (200 μL; 100 mM NaCl, 10 mM sodium phosphate pH 7.2, 10 mM $MgCl_2$). The DNA solution was hybridized by heating at 70 °C for 5 min and allowing to cool to room temperature overnight. During the melting experiment, the temperature was held at 20 °C for 10 min, then increased linearly to 75 °C at a rate of 1.0 °C min^{-1} while monitoring the change in UV absorbance at 260 nm. The data were processed with Origin 6.1 by plotting the absorbance versus temperature and fitting the curve to the equation

$$y = \frac{a}{1 + e^{(b-cx)}} + d$$

here *a*, *b*, *c*, and *d* are constants, *x* is the temperature, and *y* is the absorbance. The melting temperature was calculated by determining the value of *x* at the average of the minimum and maximum absorbance.

DNA studies containing 1: The appropriate oligonucleotide was radiolabeled at its 5′ terminus with γ-^{32}P-ATP (45 min, 37 °C) by using T4 polynucleotide kinase in buffer (70 mM Tris·HCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, pH 7.6). Unincorporated radioactive ATP was removed by passing the solution through a Sephadex G-25 column. Labeled strands were hybridized to the complementary strand (1.5 equiv) in PBS (0.1 M NaCl, 10 mM sodium phosphate, pH 7.2) by heating at 70 °C for 3 min and slowly cooling to room temperature (~2.5 h). DNA was photolyzed (350 nm) for 30 min under aerobic conditions. Aliquots (photolyzed or unphotolyzed) were treated with Nei (427 nM, 10 mM Tris·HCl, 75 mM NaCl, 1 mM EDTA, pH 8, 5–180 min, 37 °C), Nth (208 nM, 20 mM Tris·Cl, 1 mM EDTA, 1 mM DTT, pH 8, 1–6 h, 37 °C), or EcoRV-HF (20 units, 100 mM NaCl, 50 mM Tris·Cl, 10 mM $MgCl_2$, 1 mM DTT, 100 μg mL^{-1} BSA, pH 7.9, 1 h, 37 °C). A control duplex containing thymidine in place of **1** was subjected to identical reaction conditions. All samples were precipitated (0.3 M NaOAc, pH 5.2, 0.1 mg mL^{-1} calf thymus DNA) with EtOH. Samples were analyzed by dissolving in formamide loading buffer prior to running in 20% denaturing PAGE.

The effect of 1 on DNAzyme activity: The RNA substrate (300 pmol) was 5′-labeled with T4 polynucleotide kinase and γ-^{32}P-ATP as described above. After labeling, unincorporated radioactive ATP was removed from the solution by using a Sephadex G-25 column, exchanging buffer with RNAse free water. The labeled RNA was further purified by 20% denaturing PAGE. The radioactive band corresponding to the full 16-mer was excised from the gel and eluted overnight in 1 mL of elution buffer (0.2 M NaCl, 1 mM EDTA, 25 mM Tris, pH 7.5) at room temperature. The eluted RNA was concentrated by using an Amicon Ultra 3K (Millipore) by spinning (14000 *g*, 25 min) in two portions of 500 μL to an approximate total final volume of 100 μL. The concentrated RNA was precipitated twice, first with the addition of cold EtOH (300 μL) to the concentrated RNA, and the second precipitation in the presence of NaOAc (0.3 M, pH 5.2). DNAzyme experiments were performed under single turnover conditions (DNAzyme **8a** or **8b** (640 nM) and RNA (64 nM)) in the reaction buffer (2 mM $MgCl_2$, 150 mM NaCl, 25 mM Tris·Cl, pH 7.5). DNAzyme and RNA were incubated separately in the reaction buffer at 37 °C for 15 min. After equilibration, DNAzyme **8a** or **8b** was added to the RNA substrate at 37 °C. For photolyzed samples, reactions were carried out inside the Rayonet photoreactor after mixing. Samples that were photolyzed prior to annealing with RNA were irradiated for 30 min. Aliquots of the reaction were removed at 0, 5, 15, 30, 60, and 90 min, and the reactions were quenched by mixing with formamide loading buffer (90% formamide, 10 mM EDTA). A control DNAzyme containing thymidine in place of **1** was subjected to identical reaction conditions. Samples were separated by 20% denaturing PAGE and analyzed by using a phosphorimager.

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