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Modulated drug release from the stem-and-loop structured oligodeoxynucleotide upon UV-A irradiation in the presence of target DNA

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o-Nitrobenzyl photochemistry as induced by UV-A irradiation was applied to a photoactivated drug releasing system based on a molecular beacon strategy. A stem-and-loop structured oligodeoxynucleotide (ODN) possessing a photoreactive *o*-nitrobenzyl chromophore at the 3′-end and 1-aminonaphthalene quencher at the 5′-end underwent conformational change into a conventional double strand structure by hybridization with a specified target DNA. The intrinsic stem-and-loop structure suppressed photoactivated release of benzoic acid as a phantom drug from the *o*-nitrobenzyl chromophore because of intramolecular quenching by the 1-aminonaphthalene unit in close proximity to the chromophore. Formation of the double strand structure in the presence of perfectly matched target DNA minimized occurrence of intramolecular quenching and thereby enhanced the photoactivated drug release.

Introduction

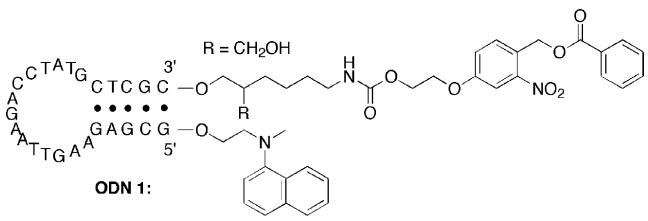
Smart drugs, which manifest a specific activity according to individual genetic information, are of particular significance for selective treatment of diseased cells.[1] This family of drugs can mediate effective medical treatment of diseased cells without inducing side-effects in normal cells. In this view, development of such drugs is recognized as the ultimate goal of chemotherapy.

A new strategy for creating these functional drugs involves the combination of a prescribed DNA hybridization sensor with a drug releasing system to set up a prodrug, the activation of which is modulated by sensing its target DNA sequence.[2] Recently, an attempt was made to develop a DNA sequence-specific drug-releasing system, using a photoreactive oligodeoxynucleotide (ODN) possessing phenacyl ester and naphthalene derivatives.[3] This photoreactive ODN consisted of a stem-and-loop structure to modulate the photoactivated drug release by a molecular beacon strategy.[4] Although the photoreaction of the phenacyl ester attachment for drug release could be effectively modulated whether or not there was target DNA present, the drawback to the previous photoreactive ODN was that UV-B irradiation (290–320 nm) is required for photoactivation and thus induces direct oxidative stress on normal cells to invoke a strong cytotoxic and mutagenic effect.[5] Furthermore, UV-B radiation has the demerit of less penetration through the epidermis and the upper dermis due to shorter wavelength. Therefore, an alternative photoactivation system with longer wavelengths of light to achieve deeper penetration through skin and avoid unfavorable direct radiation effect on normal tissues is essential.

We report here construction of a photoactivated drug releasing system based on a molecular beacon strategy and *o*-nitrobenzyl photochemistry,[6] using UV-A irradiation (320–400 nm) that does not cause direct excitation of DNA.[7,8] We synthesized a photoreactive ODN possessing an *o*-nitrobenzyl chromophore, which released benzoic acid as a phantom drug compound upon photoirradiation at 365 nm, and a 1-aminonaphthalene unit with an intramolecular quenching function.[9] Upon photoirradiation of the *o*-nitrobenzyl chromophore attached to the photoreactive ODN in the absence of complementary target DNA, the drug release was effectively suppressed because of quenching by the 1-aminonaphthalene unit that was located in close proximity to the *o*-nitrobenzene chromophore in the stem-and-loop structure. In contrast, the presence of complementary target DNA could switch over the stem-and-loop structure of the photoreactive ODN to an ordinary double-strand structure by hybridization with target, thereby resulting in the efficient photochemical drug release with decreased extent of intramolecular quenching due to separation of the *o*-nitrobenzene and 1-aminonaphthalene units. In addition, we obtained the first evidence that the enhanced drug release could not occur even in the presence of DNA with a single base mismatch.

Results and discussion

The synthesis of photoreactive ODN is outlined in Scheme 1. The *o*-nitrobenzyl ester **6** possessing a phantom drug compound of benzoic acid, and succinimidyl ester as connected to amino-modified DNA was synthesized from a 4-nitrophenol derivative **1**. A primary alcohol **8** prepared from *N*-methyl-1-aminonaphthalene **7**[10] was converted to phosphoramidite **9**, followed by introduction into DNA by automated DNA synthesis. Incorporation of the *o*-nitrobenzyl chromophore into DNA was achieved by coupling of the amino group in **10** and **6**. The formation of modified ODN was confirmed by MALDI–TOF mass spectrometry. The structures of the ODNs used in this study are shown in Fig. 1.



ODN 2: 5′-CATAGGTCTTAACTT-3′ (Complementary ODN)
ODN 3: 5′-CATAGGTGTTAACTT-3′ (Single base mismatched ODN)
ODN 4: 5′-CTGGTGCTCGACGCG-3′ (Non-complementary ODN)

Fig. 1 Structure and sequences of oligodeoxynucleotides (ODNs) used in this study.

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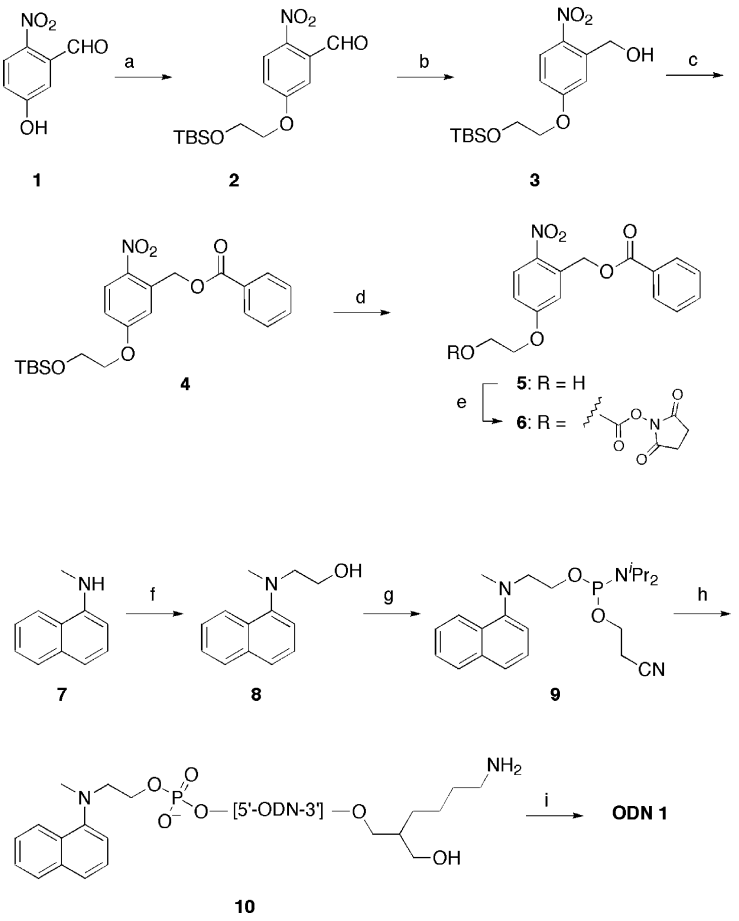

Scheme 1 *Reagents:* (a) 2-bromo-1-*tert*-butyldimethylsilyloxyethane, K_2CO_3, DMF, 76%; (b) $NaBH_4$, THF, 74%; (c) benzoyl chloride, Et_3N, CH_2Cl_2, 74%; (d) TBAF, acetic acid, THF, 94%; (e) *N*,*N*′-disuccinimidyl carbonate, Et_3N, CH_3CN, 66%; (f) 2-bromoethanol, Et_3N, toluene, 88%; (g) (iPr$_2$N)$_2$PO(CH$_2$)$_2$CN, 1*H*-tetrazole, acetonitrile, quant.; (h) DNA autosynthesizer, then 28% ammonia; (i) **6**, satd $NaHCO_3$ aq., acetonitrile–water (1 : 1).

To characterize the functionality of 1-aminonaphthalene as an intramolecular quencher influencing on the photoactivated release of benzoic acid, we performed photoirradiation at 365 nm of **ODN 1** in 10 mM sodium cacodylate (pH 7.0) for 30 min at various temperatures in the range 0–60 °C. As shown in Fig. 2, photoactivated drug release was suppressed at temperatures lower than 20 °C, where **ODN 1** would favor the stabilized stem-and-loop structure. In contrast, the elevated temperatures enhanced the drug releasing efficiency to a substantial amount up to a five-fold increase at 60 °C. These results strongly suggest that the 1-aminonaphthalene attachment to the 5′-end of **ODN 1** can display an effective intramolecular quenching ability for the photochemical reaction of the *o*-nitrobenzyl chromophore in the stem-and-loop structure stabilized at lower temperatures, in which the *o*-nitrobenzyl chromophore remains in close proximity to the 1-aminonaphthalene attachment. At higher temperatures, however, such a quenching by 1-aminonaphthalene becomes minor to induce efficient release of benzoic acid, because the stem strand dissociates to separate the average distance between chromophore and quencher.[11]

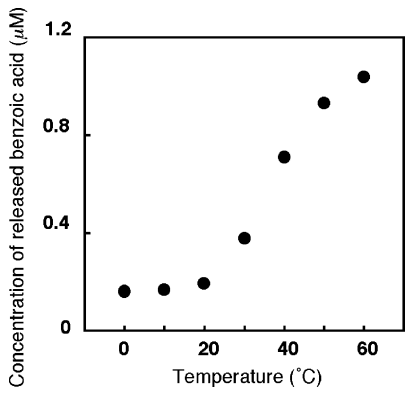

Fig. 2 Influence of the reaction temperature on the photoactivated release of benzoic acid from **ODN 1**. Irradiation of 10 μM **ODN 1** with 365 nm light obtained from transilluminator was carried out in 10 mM sodium cacodylate (pH 7.0) for 30 min at various temperatures. The photoreaction was monitored by reversed-phase HPLC.

To identify the functionality of the loop strand to hybridize with its target DNA possessing a specified base sequence, we further compared photoactivated releases of benzoic acid from **ODN 1** at 0 °C in the presence and absence of complementary DNA. The time courses of the photoreactions are shown in Fig. 3. **ODN 1** released benzoic acid about twice as efficiently in the presence of complementary **ODN 2** as in its absence. This result indicates that hybridization of **ODN 1** with **ODN 2** into a duplex structure gives rise to separation of the *o*-nitrobenzyl chromophore and 1-aminonaphthalene quencher. A smaller efficiency of the apparent photoactivated release of benzoic acid from **ODN 1** in the presence of **ODN 2** at 0 °C (Fig. 3), relative to the heat denatured **ODN 1** at 60 °C (Fig. 2), suggests that the stem-and-loop formation of **ODN 1** may still occur to a considerable extent at lower temperatures in competition with the duplex formation between **ODN 1** and **ODN 2**. In the separate experiments, we also confirmed that the presence of a single-base mismatched **ODN 3** or a non-complementary **ODN 4**, instead of the complementary **ODN 2**, was ineffective for the enhanced release of benzoic acid, the efficiency of which was practically the same as in the photoirradiation of **ODN 1** alone. Thus, the characteristic photoreactivity of **ODN 1** with a stem-and-loop structure could discriminate a single-base mismatched DNA.

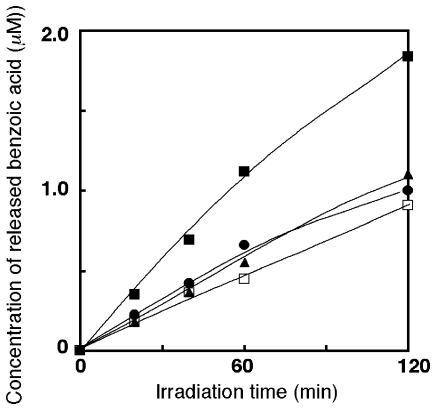

Fig. 3 Release of benzoic acid upon photoirradiation of **ODN 1**. Photoirradaition at 365 nm of 10 μM **ODN 1** for 0, 20, 40, 60 and 120 min at 0 °C in 10 mM sodium cacodylate (pH 7.0) was carried out in the presence of 50 μM complementary **ODN 2** (■), 50 μM single-base mismatched **ODN 3** (▲), or 50 μM noncomplementary **ODN 4** (●). Release of benzoic acid upon photoirradiation of **ODN 1** alone is also indicated by (□). The reaction was monitored by HPLC.

Compared with the previous photo-induced drug release system with functionalized ODN possessing phenacyl ester as a chromophore,[3] the difference in efficiency between the drug releases in the presence and absence of complementary DNA is smaller in the present *o*-nitrobenzyl photoreaction system. This may be attributed to the evidence that photoreaction of the *o*-nitrobenzyl chromophore occurring both in the singlet and triplet excited states[12] was partly quenched by aminonaphthalene quencher *via* long-range dipole–dipole interaction.[13] Thus, such a long-range quenching could occur even in the double strand structure of **ODN 1** with complementary **ODN 2** to reduce efficiency of drug release in the present system.

Conclusion

In summary, we improved a photoactivated drug releasing system modulated by a molecular beacon strategy. The presented molecular system consisted of an *o*-nitrobenzyl chromophore, a 1-aminonaphthalene quencher, and an ODN with a stem-and-loop structure, the base sequence of which was so arranged that it can hybridize with a given target DNA. The ODN underwent conformational change from a stem-and-loop structure into double strand structure in the presence of complementary DNA that had a perfect base match, and thereby resulted in efficient drug release. Although the present system showed a smaller extent of enhancement of photo-induced drug release in the presence of target complementary DNA relative to the previous system, a major advantage is that we could selectively excite the *o*-nitrobenzyl chromophore of functionalized ODN by a less harmful light of UV-A. In addition, nitrobenzyl photochemistry employed herein has been demonstrated to be applicable to the development of photoactivated prodrugs releasing actual drugs such as 5-fluorouracil, phosphoramide mustard and L-leucyl-L-leucine methyl ester.[6,14] In these views the present system would be a promising candidate for effective medical treatment without serious side-effects.

Experimental

General methods

Melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H NMR spectra were measured with JEOL JNM-AL 300 (300 MHz), or JEOL JMN-EX-400 (400 MHz) spectrometers. The chemical shifts are expressed in ppm downfield from tetramethylsilane, using residual chloroform ($\delta = 7.24$ in ^{1}H NMR) and residual dimethyl sulfoxide ($\delta = 2.49$ in ^{1}H NMR) as internal standards. ^{13}C NMR spectra were measured with JEOL JNM-AL 300 (300 MHz), JEOL JMN-EX-400 (400 MHz) or JEOL JNM-A500 (500 MHz) spectrometers. Mass spectra were recorded on a JEOL JMS-SX102A spectrometer. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry of oligonucleotides were obtained on JEOL JMS-ELITE MALDI-TOF MASS spectrometer with 2′,3′,4′-trihydroxyacetophenone as matrix. Calf intestine alkaline phosphotase (AP), snake venom phosphodiesterase (svPDE) and nuclease P1 (P1) were purchased from PROMEGA, YAMASA and ICN, respectively. The oligonucleotide was purchased from INVITROGEN. The reagents for the DNA synthesizer such as A, G, T, C and 3′-Amino-Modifier C7 CPG support were purchased from Glen Research. The purity and concentration of all oligodeoxynucleotides were determined by complete digestion with svPDE, AP and P1 to 2′-deoxymononucleotides. Reversed phase HPLC was performed on an Inertsil ODS-3 column (4.6 × 150 mm) with Shimadzu 10A or HITACHI D-7000 HPLC system using a UV detector at 230 or 260 nm. Photoirradiation at 365 nm was carried out using TFX-40.M. transilluminator.

5-[2-(*tert*-Butyldimethylsilyloxy)ethyloxy]-2-nitrobenzaldehyde (2)

To a solution of 5-hydroxy-2-nitrobenzaldehyde **1** (2.01 g, 12.03 mmol) in dry DMF (30 ml) was added 2-bromoethoxy-*tert*-butyldimethylsilane **3** (3.0 g, 12.59 mmol) and K_2CO_3 (1.89 g, 13.67 mmol), and the mixture was stirred at 80 °C for 1.5 h. The resulting mixture was diluted with sat. NH_4Cl and extracted with ethyl acetate. The extract was washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 20% ethyl acetate–hexane) to give **2** (2.97 g, 76%) as a yellow oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.45 (d, 1H, J = 4.0 Hz), 7.31 (dd, 1H, J = 2.4, 4.0 Hz), 7.15 (dd, 1H, J = 8.8, 2.4 Hz), 4.17 (t, 2H, J = 4.6 Hz), 3.98 (t, 2H, J = 4.6 Hz), 0.86 (s, 9H), 0.06 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 188.3, 163.4, 142.1, 134.2, 127.1, 119.0, 113.9, 70.6, 61.6, 25.9, 18.4, −5.2; FABMS (NBA) m/z 326 [(M + H)$^+$]; HRMS calcd. for $C_{15}H_{24}NO_5Si$ [(M + H)$^+$] 326.1424, found 326.1422.

5-[2-(*tert*-Butyldimethylsilyloxy)ethyloxy]-2-nitrobenzyl alcohol (3)

To a solution of **2** (1.63 g, 5.02 mmol) in THF (25 ml) was added $NaBH_4$ (188 mg, 4.84 mmol), and the mixture was stirred at 0 °C for 2 h. The resulting mixture was diluted with water and extracted with ethyl acetate. The extract was washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 20% ethyl acetate–hexane) to give **3** (1.22 g, 74%) as a yellow solid: mp 51–52 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.11 (d, 1H, J = 9.2 Hz), 7.21 (d, 1H, J = 2.8 Hz), 6.86 (dd, 1H, J = 9.2, 2.8 Hz), 4.95 (s, 2H), 4.12 (t, 2H, J = 5.0 Hz), 3.97 (t, 2H, J = 5.0 Hz), 0.87 (s, 9H), 0.06 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.5, 140.3, 140.1, 127.8, 114.4, 113.5, 70.0, 62.8, 61.7, 25.9, −5.2; FABMS (NBA) m/z 328 [(M + H)$^+$]; HRMS calcd. for $C_{15}H_{26}NO_5Si$ [(M + H)$^+$] 328.1580, found 328.1588.

5-[2-(*tert*-Butyldimethylsilyloxy)ethyloxy]-2-nitrobenzyl benzoate (4)

To a solution of **3** (1.01 g, 3.09 mmol) in CH_2Cl_2 (20 ml) was added benzoyl chloride (520 mg, 3.70 mmol) and triethylamine (374 mg, 3.69 mmol), and the mixture was stirred at 0 °C for 2 h. The resulting mixture was diluted with sat. $NaHCO_3$ and extracted with ethyl acetate. The extract was washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 13% ethyl acetate–hexane) to give **4** (983 mg, 74%) as a yellow solid: mp 78–79 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.21 (d, 1H, J = 9.2 Hz), 8.10 (dd, 2H, J = 8.0, 1.2 Hz), 7.59 (m, 1H), 7.47 (dd, 2H, J = 8.0, 8.0 Hz), 7.13 (d, 1H, J = 2.8 Hz),

6.91 (dd, 1H, J = 9.2, 2.8 Hz), 5.79 (s, 2H), 4.09 (t, 2H, J = 4.8 Hz), 3.95 (t, 2H, J = 4.8 Hz), 0.85 (s, 9H), 0.04 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.7, 163.3, 140.1, 135.6, 133.3, 129.7, 129.6, 128.5, 128.0, 114.4, 113.0, 70.0, 63.6, 61.6, 25.9, 18.4, −5.2; FABMS (NBA) m/z 432 [(M + H)$^+$]; HRMS calcd. for $C_{22}H_{30}NO_6Si$ [(M + H)$^+$] 432.1842, found 432.1845.

5-[2-Hydroxyethyloxy]-2-nitrobenzyl benzoate (5)

To a solution of **4** (621 mg, 1.44 mmol) in THF (25 ml) was added acetic acid (432 mg, 7.20 mmol) and TBAF (2.88 mL, 1.0 M in THF, 2.88 mmol), and the mixture was stirred at ambient temperature for 10 h. The resulting mixture was diluted with sat. $NaHCO_3$ and extracted with ethyl acetate. The extract was washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 3% methanol–$CHCl_3$) to give **5** (429 mg, 94%) as a white solid: mp 75–76 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.20 (d, 1H, J = 9.2 Hz), 8.10 (dd, 2H, J = 8.4, 0.8 Hz), 7.59 (m, 1H), 7.47 (dd, 2H, J = 8.4, 8.4 Hz), 7.13 (d, 1H, J = 2.4 Hz), 6.91 (dd, 1H, J = 9.2, 2.4 Hz), 5.78 (s, 2H), 4.13 (t, 2H, J = 4.6 Hz), 3.96 (t, 2H, J = 4.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.7, 162.8, 140.4, 135.7, 133.4, 129.7, 129.5, 128.5, 128.0, 114.4, 112.8, 69.9, 63.6, 61.0; FABMS (NBA) m/z 318 [(M + H)$^+$]; HRMS calcd. for $C_{16}H_{16}NO_6$ [(M + H)$^+$] 318.0977, found 318.0985.

5-[2-(2,5-Dioxopyrrolidinyloxycarbonyloxy)ethyloxy]-2-nitrobenzyl benzoate (6)

To a solution of **5** (295 mg, 0.93 mmol) in CH_3CN (3 ml) was added *N*,*N*′-disuccinimidyl carbonate (1.19 g, 4.65 mmol) and triethylamine (471 mg, 4.65 mmol), and the mixture was stirred at ambient temperature for 24 h. The resulting mixture was diluted with sat. $NaHCO_3$ and extracted with ethyl acetate. The extract was washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 5% acetone–$CHCl_3$) to give **6** (279 mg, 66%) as a white solid: mp 47–48 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.23 (d, 1H, J = 9.2 Hz), 8.11 (dd, 2H, J = 8.4, 1.6 Hz), 7.59 (m, 1H), 7.48 (dd, 2H, J = 8.4, 8.4 Hz), 7.17 (d, 1H, J = 2.4 Hz), 6.93 (dd, 1H, J = 9.2, 2.4 Hz), 5.79 (s, 2H), 4.66 (t, 2H, J = 4.4 Hz), 4.31 (t, 2H, J = 4.4 Hz), 2.81 (s, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.2, 165.8, 162.1, 151.5, 140.9, 135.8, 133.3, 129.7, 129.5, 128.6, 128.0, 114.5, 113.1, 68.4, 65.6, 63.5, 25.5; FABMS (NBA) m/z 459 [(M + H)$^+$]; HRMS calcd. for $C_{21}H_{19}N_2O_{10}$ [(M + H)$^+$] 459.1039, found 459.1044.

2-(*N*-Methyl-*N*-1-naphthylamino)ethanol (8)

To a solution of **7**[10] (295 mg, 2.00 mmol) in toluene (3 ml) was added triethylamine (405 mg, 4.00 mmol) and 2-bromoethanol (501 mg, 4.01 mmol), and the mixture was refluxed for 2 h. The resulting mixture was diluted with sat. NH_4Cl and extracted with ethyl acetate. The extract was washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 20% ethyl acetate–hexane) to give **8** (353 mg, 88%) as a pale purple solid: mp 53–54 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.28 (dd, 1H, J = 7.0, 2.0 Hz), 7.82 (dd, 1H, J = 6.9, 2.7 Hz), 7.58 (d, 1H, J = 8.4 Hz), 7.50–7.36 (3H), 7.17 (dd, 1H, J = 7.4, 1.1 Hz), 3.80 (dt, 2H, J = 5.4, 5.4 Hz), 3.30 (t, 2H, J = 5.4 Hz), 2.87 (s, 3H), 2.41 (t, 1H, J = 5.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 149.4, 134.6, 129.3, 128.2, 125.6, 125.4, 125.4, 123.8, 123.3, 116.0, 59.2, 58.0, 42.9; FABMS (NBA) m/z 202 [(M + H)$^+$]; HRMS calcd. for $C_{13}H_{16}NO$ [(M + H)$^+$] 202.1232, found 202.1225.

N-Methyl-*N*-[2-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyloxy)ethyl]-1-aminonaphthalene (9)

To a solution of **8** (40 mg, 0.20 mmol) and tetrazole (18 mg, 0.26 mmol) in dry CH_3CN was added 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (60 mg, 0.20 mmol), and the mixture was stirred at ambient temperature for 30 min. After the reaction, crude product **9** was used for automated DNA synthesis without further purification.

Synthesis of modified oligodeoxynucleotides (10)

Modified oligodeoxynucleotides possessing amino group at 3′-terminal and aminonaphthalene quencher at 5′-terminal were prepared by the β-cyanoethylphosphoramidite method on 3′-Amino-Modifier C7 controlled pore glass support (1 μmol) by using an Applied Biosystems Model 392 DNA/RNA synthesizer. After the automated synthesis, oligomers were deprotected by heating the solutions at 55 °C for 12 h. The synthesized oligomers were purified by reversed phase HPLC, elution with a solvent mixture of 0.1 M triethylamine acetate (TEAA), pH 7.0, linear gradient over 60 min from 0% to 30% acetonitrile at a flow rate 3.0 mL min^{-1}. The synthesized oligodeoxynucleotides was identified by MALDI-TOF mass (**10**: calcd. 8162.39, found 8162.59).

Incorporation of nitrobenzyl chromophore into oligodeoxynucleotides (ODN 1)

To a solution (total volume 20 μL) of **10** was added the solution of **6** (550 μg, 1.20 μmol) and sat. $NaHCO_3$ (10 μL), and the mixture was incubated at 25 °C for 12 h. After the reaction, the crude product was purified by reversed phase HPLC to give **ODN 1**. The formation of **ODN 1** was confirmed by MALDI–TOF mass (**ODN 1**: calcd. 8505.68, found 8505.27).

Photoreactions of ODN 1

Before irradiation, we carried out formation of stem-and-loop structure or hybridization with target DNA (**ODN 2**, **ODN 3** or **ODN 4**) of **ODN 1**, which was achieved by heating the sample at 90 °C for 5 min and slowly cooing to room temperature. Photoreactions of 10 μM **ODN 1** in the presence or absence of 50 μM target DNA at given temperatures were carried out in a buffer containing 10 mM sodium cacodylate (pH 7.0), using 365 nm light from transilluminator. After the reaction, the released benzoic acid was determined by reversed phase HPLC (HITACHI D-7000) equipped with Intersil ODS-3 column (4.6 × 150 mm, GL Sciences) using a UV detector (L-7455) at 230 nm.

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