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Phototriggering of Caged Fluorescent Oligodeoxynucleotides

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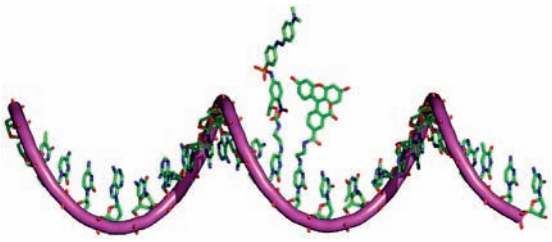
ABSTRACT



We describe a synthetic route for incorporating a photocleavable (PC) DABSYL moiety and fluorescein at adjacent cytidines in the middle of a 25-mer oligodeoxynucleotide. In hybridization studies, both fluorescein and the photocleavable DABSYL reduced the T_m about 6 °C separately. UV irradiation (355 nm, 2 min) removed the PC-DABSYL moiety, which increased the fluorescence intensity 51-fold and restored the melting temperature. Caged fluorescent oligodeoxynucleotides (CFOs) will allow many DNA processes to be controlled with light.

Controlling cellular chemistry with photoactivatable "caged" compounds has been an active area of research for almost three decades.[1] Caged DNA and RNA would have particular utility for biological investigations, but these compounds have not yet been fully developed, due, in part, to the challenges of accommodating a delicate photocleavable moiety during oligonucleotide solid-phase synthesis. In an effort to create DNA oligonucleotides whose function can be modulated with light, we have developed a new synthetic route for the site-specific incorporation of a photolabile leaving group. The additional attachment of a nearby fluorophore permits quantitative measurements of the uncaging yield, which facilitates both *in vitro* and *in vivo* experiments.

The utility of caged RNA for enzymatic studies was first demonstrated by modulating the reactivity of a single 2′-hydroxyl in the hammerhead ribozyme system.[2] However, photoillumination of the *O*-(2′-nitrobenzyl) caging group required laser wavelengths (308 nm) that are incompatible with most biological samples. To achieve photomediated gene activation *in vivo*, researchers circumvented this problem by incorporating multiple near-UV caging agents into large coding regions of DNA or RNA.[3] However, such caging strategies required prolonged photoillumination to produce activated nucleotides from heterogeneous mixtures. To cage DNA in a site-specific manner, the 6-nitroveratryloxycarbonyl (NVOC) group was attached to the N^3-position of thymidine and incorporated directly into oligonucleotide solid-phase synthesis.[4] Unfortunately, the NVOC group proved to be *too* stable, requiring irradiation for 5 h to reveal the unmodified oligonucleotide. Recently, the photocleavable 1-[5-(aminomethyl)-2-nitrophenyl]ethanol linker (PC) was attached to the 5-position of 2′-deoxyribouridine triphosphate[5] and was also incorporated near the 3′-end of a DNA oligonucleotide.[6] The heterobifunctional PC linker has high

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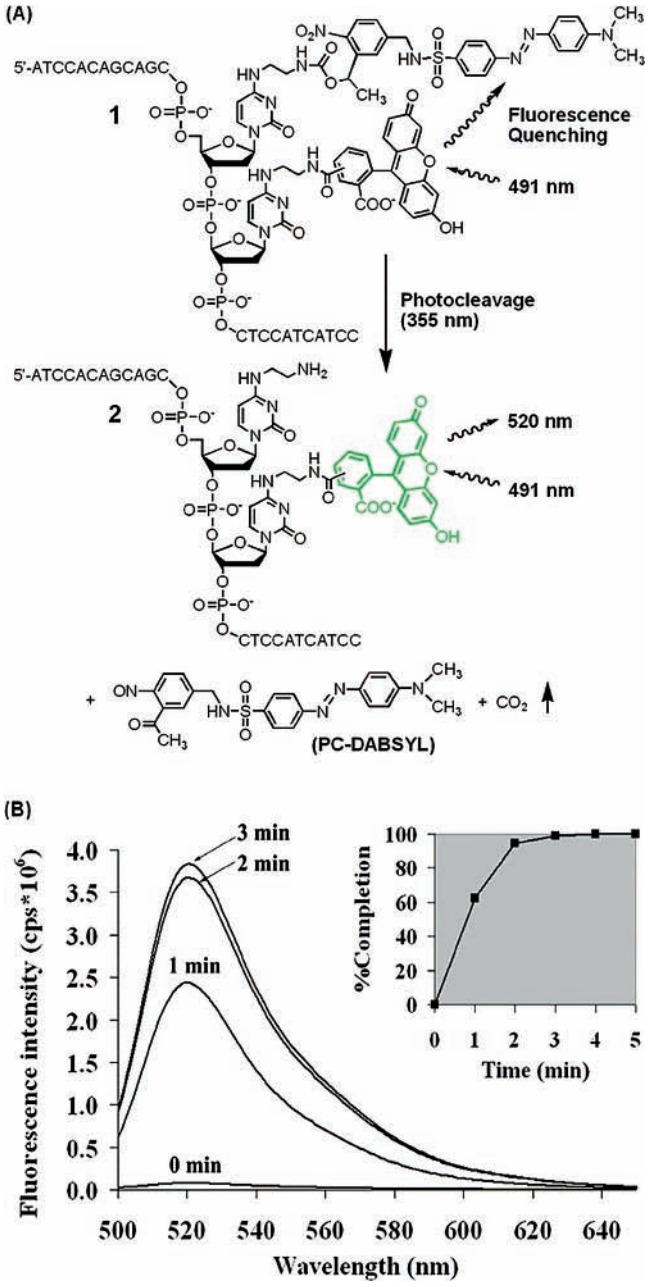

Figure 1. (A) Design of CFO with simultaneous triggering of uncaging and fluorescence. (B) Fluorescence spectra of CFO **1** (0.65 μM in SSC buffer, pH 7) at increasing irradiation times (λ_{irr} 355 nm, 0−3 min; λ_{ex} 491 nm). Inset: Photolysis was >99% complete by 3 min.

photocleavage efficiency at UV wavelengths (340−365 nm)[7] and allows the attachment of a wide variety of fluorophores,[5,6] quenchers,[8] or biomolecules[9] to the oligonucleotide.

Here, we report a general approach for the convergent synthesis of caged fluorescent oligodeoxynucleotides (CFOs) containing both the 2-nitrophenyl PC linker and a reporter fluorophore. The photocleavable linker modified with a dimethylamino-azobenzenesulfonyl (DABSYL) quencher can be attached after solid-phase synthesis to any DNA base with a pendant amine. To demonstrate this chemistry and the efficiency of photocleavage, we made CFO **1** with a DABSYL group and adjoining fluorescein, where the fluorophore reports on the caged state of the DNA (Figure 1). One long-term goal of these studies is to generate caged antisense molecules that bind their complementary target and fluoresce when triggered with light. As a proof-of-principle, oligodeoxynucleotides were synthesized to target the gene for chordin, a signaling protein important in establishing the dorsoventral axis in zebrafish and many other vertebrate organisms.[10]

To our knowledge, CFO **1** is the first example of a caged oligonucleotide whose photoactivation can be monitored in real time by fluorescence spectroscopy. Photochemical bond cleavage was achieved with modest exposure to UV light (355 nm, 36 mW/cm^2), releasing PC-DABSYL and CO_2. Removal of the PC-DABSYL unit was 95% complete after 2 min, as determined by a fluorometer (Figure 1B). HPLC analysis confirmed this result, where photocleavage of the hydrophobic PC-DABSYL group from **1** shortened its retention time on the C18 column from 11.3 to 6.1 min (Figure 2). Photogeneration of **2** to reveal the aminoethyl-

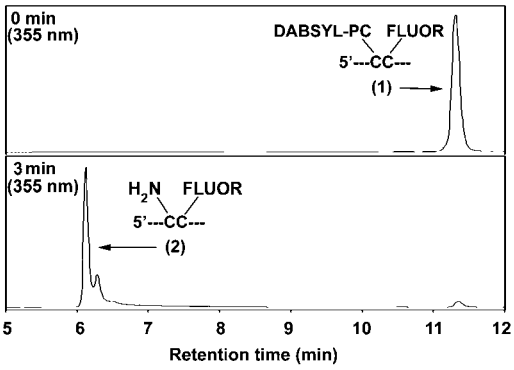

Figure 2. HPLC analysis of CFO **1**, before (above) and after 3 min of photolysis (λ_{irr} 355 nm). Detection channel: 260 nm.

deoxycytidine occurred rapidly and was concomitant with a 51-fold increase in fluorescence at 520 nm. This corresponds to >98% quenching efficiency of fluorescein by DABSYL, in agreement with other oligonucleotide-quenched probes that similarly employ this donor−acceptor pair.[11] Spectral overlap between the donor fluorescein emission and acceptor DABSYL absorption leads to efficient intramolecular resonance

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energy transfer (RET) at distances approximating the Förster distance, $R_0 = 39$ Å.[12] However, in CFO **1**, the short distance between donor and acceptor, as well as the high quenching efficiency, strongly suggests a contact-mediated quenching mechanism.[13]

Caged oligodeoxynucleotides **1** and **3** (Table 1) were synthesized by first incorporating 2-aminoethyl deoxycytidine into the oligonucleotide, and, after solid-phase synthesis, subsequently attaching the photocleavable DABSYL unit. The aminolinked dC provides a convergent strategy for attaching a variety of electrophiles under mild conditions,[14] as the final step in CFO synthesis. The photolabile DABSYL unit was covalently attached to the amine under mild conditions in 50−54% yield. Yields were further improved by increasing the duration of the reaction, and unreacted oligonucleotide was readily recovered by HPLC. The CFO synthesis serves as a mild alternative to the convertible nucleoside approach for modifying bases in the middle of a synthetic oligodeoxynucleotide.[15] We believe that this currently offers the best route for incorporating photolabile leaving groups into the middle of a synthetic oligodeoxynucleotide.

Table 1. Oligodeoxynucleotides with Melting Temperatures

	sequence (5′ → 3′)[a]	T_m	T_m post[b]
1	ATCCACAGCAGC**ZY**CTCCATCATCC	66.0 (0.2)	66.8 (0.3)
2	ATCCACAGCAGC**XY**CTCCATCATCC	67.3 (0.2)	
3	ATCCACAGCAGC**Z**CCTCCATCATCC	69.4 (0.5)	74.2 (0.2)
4	ATCCACAGCAGC**Y**CCTCCATCATCC	69.1 (0.2)	
5	ATCCACAGCAGC**X**CCTCCATCATCC	74.6 (0.3)	
6	ATCCACAGCAGCCCCTCCATCATCC	75.1 (0.4)	
Tg	GGATGATGGAGGGGCTGCTGTGGAT		

[a] Modified nucleosides in bold; **X**, aminolinked dC; **Y**, fluoresceinated aminolinked dC; **Z**, PC-DABSYL aminolinked dC. [b] T_m measured after complete photocleavage of PC-DABSYL. Uncertainties in parentheses.

In B-DNA, the non-base-paired substituent on the N^4-position of dC extends into the center of the major groove. Thus, the modifications to dC were expected to show only a modest steric effect in duplex formation to the target 25-mer chordin sequence, **Tg**. Oligodeoxynucleotides with PC-DABSYL-modified dC, **3**, and fluorescein-modified dC, **4**, exhibited melting temperatures 6 °C lower than unmodified **6** in complexes with **Tg**. This destabilization energy is comparable to most single-site mismatches and single-base modifications observed in DNA duplexes.[4,15] Photolysis of **3** for 5 min at 355 nm to generate **5** produced the expected increase in melting termperature: $T_m = 74.2$ °C for photolyzed **3** vs 74.6 °C for **5**. Irradiation of **1** also yielded a melting temperature virtually identical to its cognate **2**. Incorporation of both PC-DABSYL- and fluorescein-modified deoxycytidines into the middle of **1** produced the lowest T_m, 66 °C. This represents a 9 °C destabilization relative to the native duplex, **6**-**Tg**.

Association between fluorescein and DABSYL appears to stabilize the **1**-**Tg** duplex, causing the T_m to increase by only 1 °C upon photocleavage of the DABSYL moiety: $T_m = 66.0$ °C for **1**-**Tg** vs 66.8 °C for photolyzed **1**-**Tg** and 67.3 °C for **2**-**Tg**. Such stabilization may occur through hydrogen bonding or hydrophobic interactions, as has been seen previously for other fluorophore-quencher pairs.[13] Thus, future efforts to prevent DNA hybridization with blocking groups must consider the position and number of photocleavable moieties on the oligonucleotide, in addition to their hydrophobicity and charge.

The pendant aminoethyl group on deoxycytidine has remarkably little effect on the hybridization energy: compare **5**-**Tg** and **6**-**Tg** ($\Delta T_m = -0.5$ °C). Collectively, the data from Table 1 suggest that the PC-DABSYL group and resulting amino-linked photochemical product have little effect on DNA duplex structure. Because only subtle structural changes occur at the DNA backbone and the large DABSYL moiety occupies the major groove, this linker and leaving group provide an attractive route for modulating the binding of many proteins. Experiments directed along these lines are currently underway.

The ability to photoactivate CFOs on the seconds-to-minute time scale is well suited for many *in vivo* applications, since it minimizes damage to the biological sample and maximizes spatial and temporal resolution. On the basis of UV−vis spectra, roughly 60% of the absorbed light between 340 and 360 nm in CFO **1** is due to absorption by the 2-nitrophenyl PC linker, with DABSYL and fluorescein making much smaller contributions. Our results show that this is sufficient for quantitative photocleavage of the PC linker, and activation can be monitored by fluorescence both *in vitro* and *in vivo*.

CFO **1** is stable in ambient light over several minutes, which facilitates its synthesis and handling. This compound was microinjected into living zebrafish embryos while illuminated with a standard light microscope; no apparent increase in fluorescence was observed even 24 h later, indicating that the DABSYL moiety remained intact until irradiated with UV light. Complete uncaging of **1** was achieved in living zebrafish embryos within milliseconds using a UV ion laser (Coherent II, 351 nm, 80 mW) and inverted confocal microscope. However, the *in vivo* fluorescence intensity proved to be difficult to quantify, due to concomitant photobleaching at high laser power. Additionally, the attenuated fluorescence of CFO **1** made it impossible to localize the oligonucleotide within the embryo prior to photolysis.

However, photoactivation of CFO **1** with a low-power UV lamp identified regions within the embryo that had incorporated the compound 24 h postfertilization (Figure 3). Confocal laser scanning microscopy using a 32 anode photomultiplier tube for fluorescence detection (Zeiss 510

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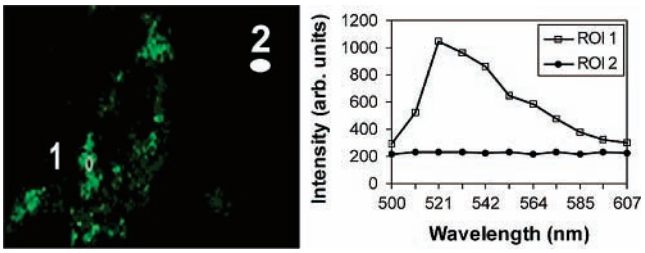

Figure 3. Green fluorescence in region 1 generated by photoactivating CFO **1** inside a living zebrafish embryo (λ_{max} = 521 nm, corresponding to fluorescein). No fluorescence was observed before photoirradiation. The spectrum of CFO **1** in region of interest (ROI) 1 is referenced to background autofluorescence, ROI 2. Embryos were irradiated with a UV lamp (365 nm, 500 μW/cm^2, 2 h) before confocal imaging.

Meta system) revealed emission profiles within these embryos clearly corresponding to fluorescein (Figure 3, ROI 1). Neither the compounds themselves nor photoirradiation (with the generation of free radicals) appeared to harm the development of several dozen zebrafish embryos. The *in vivo* stability and low phototoxicity of the 2-nitrophenyl PC linker should allow many biological studies.

In conclusion, a synthetic route for photoactivatable oligodeoxynucleotides has been developed that will facilitate the site-specific incorporation of many different fluorophores, quenchers, and biologically active molecules. The utility of the 1-[5-(aminomethyl)-2-nitrophenyl]ethanol PC linker has been validated for cellular studies on the basis of its general stability, efficient photocleavage, and low phototoxicity. With a careful choice of leaving groups, it will be possible to generate CFOs for which binding to nucleic acids or proteins can be modulated with light.

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Supporting Information Available: Experimental details, synthesis, and characterization of all oligodeoxynucleotides, melting temperatures, and *in vivo* studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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