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7-Azidomethoxy Coumarins as Profluorophores for Templated Nucleic Acid Detection

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Abstract

Templated nucleic acid detection is an emerging bioanalytical method that makes use of the target DNA or RNA strand to initiate a fluorogenic reaction. The Staudinger reduction holds particular promise for templated sensing of nucleic acids because the involved functional groups are highly chemoselective. Here, the azidomethoxy group, which can be removed under Staudinger conditions, is used to cage 7-hydroxycoumarin fluorophores. The reduction by phosphines and the subsequent loss of the azidomethoxy substituent induce a significant bathochromic shift of the major absorbance band in the near UV region. When excited at the appropriate wavelength, this change in the absorbance spectrum translates into a substantial fluorescence turn-on signal. The described profluorophores are readily conjugated to amino-modified DNAs and are rapidly uncaged by a triphenylphosphine-DNA probe under the control of a DNA template. In addition, turnover of the probes on the target strand is demonstrated, yielding substantial signal amplification.

Keywords

template synthesis; Staudinger reduction; α -azidoether; fluorescent probes; DNA recognition

Introduction

Combining nucleic acid templated reactions^[1] with fluorescence reporting provides a particularly attractive strategy to detect nucleic acid sequences.^[2] This sensing method relies on two functionalized oligonucleotide probes, which hybridize to a common target strand; the proximity of the probes accelerates a reaction between the attached functionalities, resulting in a fluorescence signal. Templated fluorescence activation can be selective to single mismatch polymorphisms^[3] and imposes minimal requirements for equipment and personnel. Furthermore, it enables the detection of nucleic acids in prokaryotic^[4] and mammalian^[5] cells. Detecting genetic sequences directly in host cells may reduce time and expenses associated with the characterization of pathogens and tissue samples. Previous studies of templated nucleic acid detection in live cells were based on a S_N2 quencher displacement strategy.^[6] In that approach, significant background fluorescence from the quenched probes restricted templated detection in cells to highly expressed RNAs.^[4,5]

Several fluorogenic reactions have been investigated to address the current limitations.^[7] However, most reaction schemes are not compatible with cellular RNA detection because of either low chemoselectivity or the dependence on external cofactors. The Staudinger

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reduction of organic azides by triphenylphosphine (TPP) holds particular promise for templated nucleic acid detection because of its exceptional degree of bioorthogonality.^[8] Besides having found numerous applications in chemical biology, this reaction is also compatible with DNA templated chemistry.^[9] The reaction kinetics are rapid, the preparation of the probes is straightforward, and the absence of probe ligation in principle enables amplification of the reporter signal. The primary challenge is to link the Staudinger reduction to a fluorescence turn-on event. Two fluorescence activation schemes have been applied toward this end. One strategy involves azide-modified peptide nucleic acid probes, which unmask a 2-(diphenylphosphino)benzoate derivative of fluorescein.^[10] Alternatively, a TPP-probe activates the fluorescence of a latent fluorophore, which contains an aromatic azide. The known examples include 7-azidocoumarin^[11] and azide-substituted rhodamines.^[12] However, certain drawbacks limit the scope of either caging strategy. The TPP-derivative of fluorescein is prone to phosphine oxidation and to hydrolysis of the phenolic ester, which hinders the application of this probe in cells. On the other hand, profluorophores with exocyclic azides are limited to few particular cases.

Here, an alternative strategy to couple the Staudinger reduction to a fluorescence turn-on event is presented. Derivatizing existing exocyclic functionalities of fluorophores with azide-based protecting groups allows the design of phosphine sensitive profluorophores. We have synthesized 7-hydroxycoumarins caged by azidomethyl-substituents (AzMe)^[13] and applied them to templated nucleic acid detection. TPP-modified DNA probes efficiently reduced 7-AzMeO-coumarin conjugates (Scheme 1), activating the fluorescence signal. This caging strategy for templated fluorescence reporting raises new possibilities for the detection of nucleic acids in vitro and in live cells.

Results

Design and synthesis of 7-azidomethoxy coumarins

Substituents at the C-7 position are well known to affect the fluorescence properties of coumarins. For example, alkylation quenches the emission of 7-hydroxycoumarins, which are highly fluorescent when deprotonated. Consequently, 7-alkoxycoumarins constitute profluorophores with excellent turn-on ratios upon dealkylation and have been applied as fluorogenic substrates for various enzymes.^[14] Pursuing this strategy, we hypothesized that the installment of an AzMe-protecting group^[13] at the phenolic position would convert 7-hydroxycoumarins into TPP-sensitive molecular probes. Carboxylic acid functionalities render these masked fluorophores water-soluble and make possible the straightforward conjugation to amino-modified DNAs. A chloro substituent at the C-6 position is expected to decrease the pK_a of the phenolic functionality and intensify the fluorescence brightness of the coumarin at physiological pH.^[15]

The synthesis of the designed 7-AzMeO-coumarins AzMeOHCoum (**7a**) and AzMeOCICoum (**7b**) was straightforwardly achieved in five steps (Scheme 2). The sulfuric acid catalyzed Pechmann condensation of dimethyl acetylsuccinate (**1**) with the corresponding resorcinol derivative (**2a** or **2b**) afforded the 7-hydroxycoumarins **3a** and **3b** in moderate yield. The AzMe caging group was installed by a three-step reaction sequence derived from a literature protocol.^[13a] Initially, reaction with bromomethyl 4-chlorophenyl sulfide converted the coumarins into the S,O-acetals **4a** and **4b**. Sulfuryl chloride replaced the (4-chlorophenyl)thio group of **4a** and **4b** by a chloro substituent. Nucleophilic substitution converted the chloromethylethers **5a** and **5b** to the corresponding azidomethylethers **6a** and **6b**. Hydrolysis of the methyl esters using lithium hydroxide provided the desired carboxylic acids AzMeOHCoum (**7a**) and AzMeOCICoum (**7b**), which were obtained in high purity by recrystallization from acetonitrile. Initial attempts to synthesize 6-chloro-7-azidomethoxycoumarin-4-acetic acid by hydrolysis of the

corresponding ethyl ester (Supporting Scheme S1) proved surprisingly difficult. The ester was unexpectedly inert, while the carboxylic acid was sensitive to decarboxylation when heated moderately.

Photophysical properties of 7-azidomethoxy coumarins

The absorbance and fluorescence emission spectra of the synthesized 7-AzMeO-coumarins **7a** and **7b** were analyzed (0.1 M sodium phosphate buffer, pH 7.55) and compared to those of the corresponding 7-hydroxycoumarins **8a** and **8b** (Figure 1 and Table 1). Both masked 7-AzMeO-coumarins displayed absorbance bands in the near UV range with maxima at 319 nm ($\epsilon_{\text{max}} = 15200 \text{ cm}^{-1} \text{ M}^{-1}$) for AzMeOHCoum and 325 nm ($\epsilon_{\text{max}} = 14000 \text{ cm}^{-1} \text{ M}^{-1}$) for AzMeOClCoum. The absorbance band of HOClCoum experienced a significant bathochromic shift relative to the band of AzMeOClCoum with the maximal absorbance at 364 nm ($\epsilon_{\text{max}} = 16200 \text{ cm}^{-1} \text{ M}^{-1}$). This red-shifted absorbance band corresponds to the deprotonated coumarin. Conversely, at pH 7.55 the major absorbance band of HOHCoum was centered at 324 nm ($\epsilon_{\text{max}} = 13600 \text{ cm}^{-1} \text{ M}^{-1}$) with a shoulder at longer wavelengths. The differences between the absorbance spectra of HOHCoum and HOClCoum likely result from the different pKa values of the phenolic functionality, which is significantly lower for 6-chloro substituted 7-hydroxycoumarins.^[15] At higher pH the absorbance spectrum of HOHCoum indeed displayed a bathochromically shifted absorbance band (Supporting Figure S1).

Both 7-hydroxycoumarins (**8a**, **8b**) emitted light at 450 nm when excited at 375 nm. In contrast, the caged coumarins **7a** and **7b** were nearly non-fluorescent. The fluorescence intensity of HOClCoum was approximately 4 times that of HOHCoum, correlating with the difference in extinction at 375 nm, while the quantum yields were the same within experimental error. Unexpectedly, at pH 7.55 AzMeOHCoum displayed a distinctively higher fluorescence enhancement upon removal of the AzMe-group (62-fold) than did AzMeOClCoum (32-fold), despite the fact that only a fraction of HOHCoum is deprotonated at this pH. Apparently, a chloro substituent at the C-6 position of the coumarin heterocycle decreases the quenching efficiency imposed by alkylation of the 7-hydroxy group. AzMeOClCoum has a faint absorbance at 375 nm and the quantum yield is insignificantly lower than that of HOClCoum. Of course, the photophysical properties of 7-hydroxycoumarins depend strongly on the pH; increasing the basicity increases the fluorescence of HOHCoum (Supporting Figure S1) and the fluorescence turn-on ratio. DNA templated fluorophore uncaging experiments were performed with AzMeOHCoum in order to ensure minimal background fluorescence.

DNA templated profluorophore activation

A pair of modified DNA-probes was prepared to investigate the template dependent uncaging of AzMeOHCoum (Scheme 3). One probe contained the AzMeOHCoum profluorophore at its 3'-terminus (AzMeOCoum-DNA) while a TPP molecule was appended to the 5'-terminus of the second probe (TPP-DNA). The modified probes were complementary to adjacent sequences at a single nucleotide polymorphism site of the H-*ras* oncogene.^[17] Conjugation of the reactants to the DNAs entailed standard amide bond formation. The in situ generated *N*-hydroxysuccinimide ester of AzMeOHCoum was reacted post-synthetically with 3'-amino modified DNA and. TPP coupling to the 5'-amino modified DNA was performed on solid support followed by cleavage/deprotection.^[9] Reactive DNA probes were purified by reverse phase HPLC and confirmed by MALDI-TOF mass-spectrometry (Supporting Information).

The templated reaction between AzMeOCoum-DNA and TPP-DNA and the associated increase in fluorescence was assessed (Figure 2). AzMeOCoum-DNA (200 nM) was

incubated with the template strand *mut A* (200 nM) at 37 °C (10 mM MgCl₂ + 70 mM tris-borate buffer, pH 7.55) and the fluorescence intensity ($\lambda_{ex} = 375$ nm and $\lambda_{em} = 450$ nm) was measured as a function of time after the addition of TPP-DNA (400 nM). The TPP-probe rapidly induced a major enhancement of the fluorescence emission before it reached a constant level within few minutes. After 30 min, the emission level exceeded the fluorescence signal of the caged AzMeOCoum-DNA by a factor of 29.0 ± 1.5 . Post-reactive addition of excess TPP-DNA did not further increase the fluorescence, which indicates that the AzMeCoum-DNA probes had reacted near-quantitatively. This increase was inferior to that of the uncaging of AzMeOHCoum (Table 1), indicating that the fluorescence turn-on was limited by incidental coumarin uncaging during conjugation and purification of the probe. An uncaging experiment performed at pH 8.5 provided a comparable turn-on ratio but higher fluorescence intensity, which supports this hypothesis (Supporting Figure S2). To exclude that a non-templated reaction between TPP-DNA and AzMeOHCoumDNA caused the observed unmasking, the experiment was repeated in absence of the *mut A* template. The fluorescence intensity remained unchanged within the 30 min experimental interval, demonstrating that the reaction was template-dependent and that background uncaging was negligible. To assess the mismatch sensitivity, the reaction was repeated in the presence of *wt C*, a template strand that contains a C-T mismatch at the center of the AzMeOHCoum-DNA binding site. In this experiment, the rate of reaction was considerably reduced relative to the rate in the presence of the fully matched template *mut A*. The kinetic mismatch discrimination was estimated at 115 ± 8 -fold based on the initial rates of reaction. This mismatch sensitivity rivals values reported for the best current templated detection schemes.

Long-wavelength emission FRET probes

For cellular applications, the signal for fluorescence RNA detection using a single probe color depends on parameters other than analyte concentration such as cellular probe concentration, making experimental results difficult to interpret. Conversely, two-color reporter systems are independent of probe concentration and allow straightforward sequence calls for single nucleotide variations. For example, a pair of probes that emit in different spectral regions when excited at a common wavelength has been used recently to discriminate bacterial strains based on single nucleotide differences.^[4c]

With this objective in mind, we tested whether uncaged 7-AzMeO-coumarins could be used to yield a secondary signal by Förster resonance energy transfer (FRET). We prepared a AzMeOHCoum-DNA probe (AzMeOCoum-FRET-DNA, Scheme 3), which contains a fluorescein, a preferred FRET-partner for coumarins,^[18] at an internal thymidine site (Scheme 4). TPP-DNA induced activation of FRET-acceptor emission in the presence of the target strand *mut A* was investigated (Figure 3). AzMeOCoum-FRET-DNA and *mut A* (200 nM) were combined in a hybridization buffer (10 mM MgCl₂ + 70 mM tris-borate, pH 8.5) and the fluorescence was measured before and after 30 min incubation with TPP-DNA (400 nM) at 37 °C. AzMeCoum-FRET-DNA exhibited a strong acceptor emission band with a maximum at 521 nm after the templated reaction with TPP-DNA. The intensity of fluorescence at 521 nm increased 3.2-fold relative to the level of the probe prior to reduction by TPP-DNA. Coumarin emission at 450 nm, on the other hand, was strongly quenched compared to uncaged AzMeOCoum-DNA. These results demonstrate efficient FRET between the uncaged coumarin and fluorescein. The sequence-specificity of the Staudinger fluorescence activation was unaffected by the modification with fluorescein (Supporting Figure S3). Thus, the results show that the AzMe-caged coumarins can be employed in a two-color, single-nucleotide sequence detection system.

Template turnover

The Staudinger reaction of the present azidoethers (and recent other examples⁹⁻¹²) likely involves an iminiophosphorane intermediate¹⁹ which decomposes rapidly, leaving two unjoined product probes as the major products. Accordingly, at physiological temperature, the DNA probes can be expected to dissociate after the reaction takes place, allowing the target strand to act as a template for further Staudinger reductions. Such turnover of multiple probes on the template yields useful levels of signal amplification.^[6b,11,20] To investigate turnover of the template for the described coumarin unmasking reaction, the intensity of fluorescence as a function of time was measured in the presence of substoichiometric concentrations of the template oligonucleotide *mut A* (Figure 4) under the same conditions as described for stoichiometric coumarin uncaging. After 30 min, the measured signal clearly exceeded the emission level expected for a stoichiometric template dependence. For example, a solution containing 40 nM of the *mut A* template, corresponding to 20 % of the AzMeOCoum-DNA concentration, exhibited nearly the same fluorescence intensity after 30 min as aliquots containing one equivalent of *mut A* (200 nM). This result implies that on average each template catalyzed the uncaging of approximately five coumarins under these conditions, assuming that stoichiometric coumarin activation is quantitative. Further decreasing the concentration of *mut A* additionally enhanced the turnover of the probes. After 30 min, a sample containing 2.5 nM of *mut A* exhibited approximately 25 % of the fluorescence intensity of a solution containing one equivalent of *mut A* (200 nM). This outcome corresponds to approximately 20 turnovers of probes on the *mut A* template in 30 minutes. Importantly, the fluorescence signal in the presence of 2.5 nM of *mut A* exceeded that measured for a stoichiometric amount of the mismatched strand *wt C* (Figure 2). This outcome demonstrates that nanomolar concentrations of the target oligonucleotides can be reliably sensed in the presence of mismatched sequences.

Discussion

Our data show that 7-AzMeO-coumarins hold substantial potential as phosphine-sensitive profluorophores. Removal of the AzMe-protecting group from the 7-hydroxycoumarin's phenol functionality is coupled to a considerable increase of the fluorescence intensity when excited at 375 nm. The prepared 7-AzMeO-coumarins **7a** and **7b** are suitable for bioconjugation through standard amide bond formation as demonstrated by the post-synthetic labelling of an amino-modified DNA probe with AzMeOHCoum. Applications of these reporters in biochemistry and biology can be envisioned beyond the illustrated DNA-templated reaction scheme.

The TPP-induced, fluorogenic deprotection of 7-AzMeO-coumarins was successfully implemented in a DNA-templated reaction scheme. This outcome adds support to recent reports that promote the Staudinger reduction as a prospective reaction for templated nucleic acid detection,^[10-12] complementary to the S_N2 quencher displacement strategy developed by our laboratory.^[6] In particular, the rapid reaction kinetics of the reductive deprotection represents a considerable rate-advantage over templated S_N2 displacement. The outlined templated reaction also displays a favorable fluorescence turn-on ratio, which significantly exceeds the value reported in another study of TPP-induced coumarin uncaging.^[11] The measured templated fluorescence enhancement of AzMeOCoum-DNA was less than that for AzMeOHCoum **7a**. It is possible that, despite the evident stability of the α -azidoether functionality, a minor fraction of the coumarin-probes was deprotected (but undetected) during conjugation and purification; a turn-on ratio of 29 could correspond to 3-4 % of the fluorophore-conjugates being unmasked.

Post-reactive dissociation of the probes releases the target strand for additional rounds of reaction templation. Indeed, the present results indicate efficient turnover of the template, at

30 min significantly exceeding values reported for S_N2 displacement using a destabilizing linker.^[6b] Template recycling translates into an amplified fluorescence signal and will significantly enhance the detection sensitivity of templated nucleic acid sensing at low target concentrations. The proposed α -azidoethers are particularly suitable for amplified Staudinger reductions. In earlier Staudinger probes, the inertness of the phosphorous-ylide intermediate^[19] interfered with dissociation of the probes and amplification of the signal. Basic reaction conditions^[9] or alternative reductants^[12] were required to overcome this limitation. In contrast, the lability of the current phosphorous-ylide intermediate of α -azidoether reduction^[21] reduces product inhibition and enhances signal amplification. We observed 22 turnovers in 30 min; it seems likely that longer reaction times and greater probe excesses could yield higher levels of amplification. For example, Grossman and Seitz reported a turnover number of 402 within 24 h using a 10^4 -fold excess of probes for a quencher transfer reaction.^[7f]

The AzMeO-functional group may be applied to cage other fluorophores containing phenolic groups such as fluorescein, resorufin, or Tokyo green derivatives.^[22] Furthermore, alternative azide-based protecting groups that can be removed by mild reducing agents^[23] have been described and could be applied to mask fluorophores. Consequently, the outlined strategy to cage fluorophores by modifying existing exocyclic substituents with phosphine-sensitive protecting groups is highly versatile in contrast to the reduction of aromatic azides, which is limited to specific cases only.^[11,12] The outlined FRET strategy, which enables long wavelength detection, further adds to the versatility of the current approach. We have shown that FRET-probes can be applied to simple, color-based visual calling of single nucleotide sequence variations.^[4c]

The described phosphine-induced unmasking of AzMe-caged profluorophores has promise for the templated detection of native RNAs and DNAs. The high degree of bioorthogonality of the Staudinger reaction may enable to use of this system with cellular RNA targets.^[12] The fast reaction kinetics of the present fluorogenic Staudinger reaction combined with the efficient signal amplification through template turnover will improve the sensitivity of these caged probes. Future work will be directed to employing them in identifying pathogens and characterizing human sequence variations.

Experimental Section

Methods and materials

Anhydrous solvents were purchased from Fisher Scientific and used without further purification. Bromomethyl 4-chlorophenyl sulfide was synthesized as reported^[24] and used within few days after preparation; all other chemicals were purchased from either Sigma-Aldrich, Alfa-Aesar, or Acros and used without further purification. Chemicals used for the solid-phase synthesis of oligonucleotides such as phosphoramidites, solid-supports, amino-modifiers, and synthesizer reagent-solutions were acquired from Glen Research. All ^1H - and ^{13}C -NMR spectra were recorded on either a Varian Innova 500 MHz or a Varian Mercury 400 MHz NMR spectrometer. ^1H - and ^{13}C -NMR Spectra were internally referenced to the residual solvent signal. High-resolution mass spectrometry analysis was performed by the UC Riverside Mass Spectrometry Facility. Semi-preparative high performance liquid chromatography was performed on a LC-CAD Shimadzu liquid chromatograph, equipped with a SPD-M10A VD diode array detector and a SCL 10A VP system controller. Fluorescence measurements were performed on a Fluorolog Jobin Yvon fluorophotospectrometer equipped with an external temperature controller. UV-absorbance spectra were collected on a Cary 100 Bio UV-visible spectrophotometer. Oligonucleotide masses were determined by the Stanford University Protein and Nucleic Acid Facility on a

Perspective Voyager-DE RP Biospectrometry MALDI-TOF mass-spectrometry instrument using a 3-Hydroxypicolinic acid/di-ammonium hydrogen citrate matrix.

Methyl 7-hydroxy-4-methylcoumarin-3-acetate (3a)

Concentrated sulfuric acid (15 mL) was added to a suspension of triturated resorcinol (10.4 g, 92 mmol) in dimethyl acetylsuccinate (17.8 g, 92 mmol). The viscous liquid was stirred at room temperature for 18 h. The reaction mixture was blended with MeOH, which induced the precipitation of the product within few minutes. After filtration, the precipitate was washed with MeOH/H₂O and dried under vacuum to provide the product in a yield of 10.2 g (44 %). ¹H-NMR (500 MHz, [D₆]DMSO): δ = 2.33 ppm (s, 3H; CH₃), 3.60 ppm (s, 3H; CH₃), 3.63 ppm (s, 2H; CH₂), 6.70 ppm (d, ⁴J(H,H) = 2.5 Hz, 1H; Ar-H), 6.81 ppm (dd, ³J(H,H) = 9.0 Hz, ⁴J(H,H) = 2.5 Hz, 1H; Ar-H), 7.64 ppm (d, ³J(H,H) = 9.0 Hz, 1H; Ar-H), 10.53 ppm (bs, 1H; ArOH); ¹³C-NMR (500 MHz, [D₆]DMSO): δ = 15.06, 32.36, 51.86, 102.01, 112.19, 113.11, 115.07, 126.99, 149.73, 153.48, 160.75, 161.00, 170.77 ppm. HRMS [-Scan]; calculated mass for C₁₃H₁₁O₅ 247.0606; observed mass: 247.0603.

Methyl 6-chloro-7-hydroxy-4-methylcoumarin-3-acetate (3b)

Concentrated sulfuric acid (7.5 mL) was added to a suspension of triturated 4-chlororesorcinol (6.8 g, 47 mmol) in dimethyl acetylsuccinate (8.9 g, 47 mmol). The viscous liquid was stirred at room temperature for 24 h. The reaction mixture was blended with MeOH and the precipitate was filtrated. Recrystallization from MeOH/H₂O afforded the product in a yield of 3.0 g (23 %). ¹H-NMR (500 MHz, [D₆]DMSO): δ = 2.35 ppm (s, 3H; CH₃), 3.60 ppm (s, 3H; CH₃), 3.64 ppm (s, 2H; CH₂), 6.89 ppm (s, 1H; Ar-H), 7.81 ppm (s, 1H; Ar-H), 11.38 ppm (bs, 1H; ArOH); ¹³C-NMR (500 MHz, [D₆]DMSO): δ = 15.14, 32.42, 51.89, 103.14, 113.01, 116.24, 117.11, 126.47, 149.01, 151.63, 155.87, 160.57, 170.57 ppm. HRMS [+Scan]; calculated mass for C₁₃H₁₂ClO₅ 283.0373; observed mass: 283.0372.

Methyl 7-(4-chlorophenylthio)methoxy-4-methylcoumarin-3-acetate (4a)

Calcinated potassium carbonate (1.1 g, 8.2 mmol) was added to a solution of **3a** (1.0 g, 6 mmol) in anhydrous acetone (15 mL) followed by the addition of a solution of bromomethyl 4-chlorophenyl sulfide (1.2 g, 5.1 mmol) in acetone (2.5 mL). The reaction mixture was heated to reflux for 2 h. Precipitates were removed by filtration and the solvent evaporated. The residue was dissolved in a minimal amount of DCM and the product precipitated by the addition of hexanes. After filtration, the precipitate was dried under vacuum to provide the product as a white solid in a yield of 1.55 g (95%). ¹H-NMR (500 MHz, CDCl₃): δ = 2.40 ppm (s, 3H; CH₃), 3.72 ppm (s, 3H; CH₃), 3.73 ppm (s, 2H; CH₂), 5.47 ppm (s, 2H; CH₂), 6.90 ppm (d, ⁴J(H,H) = 2.5 Hz, 1H; Ar-H), 6.92 ppm (dd, ³J(H,H) = 9.0 Hz, ⁴J(H,H) = 2.5 Hz, 1H; Ar-H), 7.30 ppm (d, ³J(H,H) = 8.5 Hz, 2H; Ar-H), 7.41 ppm (d, ³J(H,H) = 9.0 Hz, 2H; Ar-H), 7.58 ppm (d, ³J(H,H) = 9.0 Hz, 1H; Ar-H); ¹³C-NMR (400 MHz, CDCl₃): δ = 15.46, 32.74, 52.33, 73.25, 103.14, 113.43, 114.93, 117.11, 126.07, 129.35, 132.44, 132.64, 133.96, 149.01, 153.73, 158.96, 161.60, 170.78 ppm. HRMS [+Scan]; calculated mass for C₂₀H₁₈O₅SCl 405.0563; observed mass: 405.0566.

Methyl 6-chloro-7-(4-chlorophenylthio)methoxy-4-methyl-coumarin-3-acetate (4b)

A solution of bromomethyl 4-chlorophenyl sulfide (2.8 g, 11.8 mmol) in anhydrous DMF (2 mL) was added to a solution of **3b** (2.8 g, 10 mmol) and diisopropylethylamine (1.52 g, 11.8 mmol) in DMF (20 mL). The solution was stirred at 50°C until the starting coumarin had disappeared (5 h). The solution was concentrated on the rotary evaporator and added to MeOH (50 mL). The precipitate was filtrated, washed with MeOH, and dried under vacuum. The product was obtained as a white solid in a yield of 3.8 g (92 %). ¹H-NMR (500 MHz,

CDCl₃): δ = 2.37 ppm (s, 3H; CH₃), 3.7(18) ppm (s, 3H; CH₃), 3.7(23) ppm (s, 2H; CH₂), 5.52 ppm (s, 2H; CH₂), 6.92 ppm (s, 1H; Ar-H), 7.31 ppm (d, $^3J(\text{H,H})$ = 8.5 Hz, 2H; Ar-H), 7.46 ppm (d, $^3J(\text{H,H})$ = 8.5 Hz, 2H; Ar-H), 7.65 ppm (s, 1H; Ar-H); ¹³C-NMR (500 MHz, CDCl₃): δ = 15.58, 32.84, 52.46, 74.25, 103.15, 115.33, 118.25, 120.31, 126.18, 129.43, 132.27, 132.83, 134.29, 148.09, 151.88, 154.06, 161.13, 170.55 ppm. HRMS [-Scan]; calculated mass for C₂₀H₁₄O₅SCl₂ 437.0017; observed mass: 437.0013.

Methyl 7-chloromethoxy-4-methylcoumarin-3-acetate (5a)

A solution of sulfuryl chloride (0.56 g, 4.15 mmol) in anhydrous DCM (2 mL) was added dropwise to a solution of **4a** (1.4 g, 3.5 mL) in DCM (15 mL) cooled 0°C, causing the appearance of a yellow coloration. The solution was incubated at 0°C for 45 min before cyclohexene (0.34 g, 4.2 mmol) diluted with DCM (2 mL) was added dropwise accompanied by complete decoloration of the solution. The mixture was stirred for additional 15 min, concentrated by rotary evaporation, and added dropwise to hot hexanes (50 mL). The forming precipitate was filtrated off affording the product in 0.98 g yield (95 %). ¹H-NMR (500 MHz, CDCl₃): δ = 2.41 ppm (s, 3H; CH₃), 3.72 ppm (s, 3H; CH₃), 3.73 ppm (s, 2H; CH₂), 5.91 ppm (s, 2H; CH₂), 7.04 ppm (dd, $^3J(\text{H,H})$ = 9 Hz, $^4J(\text{H,H})$ = 2.5 Hz, 1H; Ar-H), 7.07 ppm (d, $^4J(\text{H,H})$ = 2.5 Hz, 1H; Ar-H), 7.62 ppm (d, $^3J(\text{H,H})$ = 9.0 Hz, 1H; Ar-H); ¹³C-NMR (400 MHz, CDCl₃): δ = 15.55, 32.82, 52.41, 76.08, 103.62, 113.15, 115.94, 117.85, 126.22, 148.85, 153.69, 157.79, 161.47, 170.73 ppm. HRMS [+Scan]; calculated mass for C₁₄H₁₄O₅Cl 297.0530; observed mass: 297.0523.

Methyl 6-chloro-7-chloromethoxy-4-methylcoumarin-3-acetate (5b)

A solution of sulfuryl chloride (0.55 g, 4.1 mmol) in anhydrous DCM (1 mL) was added dropwise to a solution of **4b** (1.5 g, 3.4 mmol) in DCM (20 mL) cooled to 0°C. The solution was incubated at 0°C for 45 min before cyclohexene (0.34 g, 4.1 mmol) dissolved in DCM (1 mL) was added dropwise, causing complete decoloration of the solution. The mixture was incubated for additional 15 min at 0°C, concentrated by rotary evaporation, and added dropwise to hot hexanes (50 mL). The forming precipitate was filtrated off affording the product in 1.12 g yield (99 %). ¹H-NMR (500 MHz, CDCl₃): δ = 2.39 ppm (s, 3H; CH₃), 3.73 ppm (s, 3H; CH₃), 3.72 ppm (s, 2H; CH₂), 5.95 ppm (s, 2H; CH₂), 7.22 (s, 1H; Ar-H), 7.67 ppm (s, 1H; Ar-H); ¹³C-NMR (500 MHz, CDCl₃): δ = 15.06, 32.88, 52.49, 76.23, 103.67, 116.39, 118.96, 120.10, 126.34, 147.87, 151.87, 153.11, 160.96, 170.45 ppm. HRMS [-Scan]; calculated mass for C₁₄H₁₁O₅Cl₂ 328.9984; observed mass: 328.9981.

Methyl 7-azidomethoxy-4-methylcoumarin-3-acetate (6a)

Tetrabutylammonium azide (0.96 g, 3.4 mmol) was added to a solution of **5a** (0.8 g, 2.7 mmol) in anhydrous THF (10 mL). The solution was incubated at room temperature for 4 h. The solvent was removed by rotary evaporation and the residue purified by column chromatography (DCM + 2 % acetone). Product containing fractions were combined and evaporated to provide the product in a yield of 0.69 g (84 %). ¹H-NMR (500 MHz, CDCl₃): δ = 2.36 ppm (s, 3H; CH₃), 3.68 ppm (s, 5H; CH₃ and CH₂), 5.19 ppm (s, 2H; CH₂), 6.88 ppm (s, 1H; Ar-H), 6.92 ppm (d, $^3J(\text{H,H})$ = 9.0 Hz, 1H; Ar-H), 7.55 ppm (d, $^3J(\text{H,H})$ = 8.5 Hz, 1H; Ar-H); ¹³C-NMR (500 MHz, CDCl₃): δ = 15.38, 32.66, 52.25, 79.37, 103.07, 112.86, 115.20, 117.26, 126.11, 148.88, 153.60, 158.76, 161.43, 170.67 ppm. HRMS [+Scan]; calculated mass for C₁₄H₁₄N₃O₅ 304.0933; observed mass: 304.0932.

Methyl 7-azidomethoxy-6-chloro-4-methylcoumarin-3-acetate (6b)

A solution of sodium azide (0.26 g, 4.05 mmol) in H₂O (15 mL) was added to a solution of **5b** (1.075 g, 3.2 mmol) in MeCN (50 mL). The solution was stirred at 60°C for 4 h. After rotary evaporation of the MeCN, the aqueous suspension was extracted with EtOAc, dried

over Na₂SO₄, filtrated, and evaporated to provide the product in a yield of 0.99 g (90 %). ¹H-NMR (400 MHz, CDCl₃): δ = 2.38 ppm (s, 3H; CH₃), 3.71(8) ppm (s, 3H; CH₃), 3.72(3) (s, 2H; CH₂) 5.28 ppm (s, 2H; CH₂), 7.02 ppm (s, 1H; Ar-H), 7.65 ppm (s, 1H; Ar-H); ¹³C-NMR (400 MHz, CDCl₃): δ = 15.57, 32.85, 52.45, 80.41, 103.52, 115.90, 118.61, 119.95, 126.20, 147.95, 152.00, 154.16, 161.03, 170.51 ppm. HRMS [+Scan]; calculated mass for C₁₄H₁₃N₃O₅Cl 338.0544; observed mass: 338.0540.

7-azidomethoxy-4-methylcoumarin-3-acetic acid (7a)

An aqueous solution of lithium hydroxide monohydrate (0.27 g, 6.4 mmol in 5 mL) was added to a solution of **6a** (0.27 g, 0.89 mmol) in THF (5 mL) cooled to 0°C. The biphasic system was stirred at 0°C for 90 min until all starting product had disappeared. The mixture was acidified with aqueous HCl and extracted with EtOAc. The organic layer was dried over Na₂SO₄, filtrated, and evaporated to provide the product as a white solid in a yield of 195 mg (76 %). Minor contamination of 7-hydroxy-4-methylcoumarin-3-acetic acid (< 2 %) was removed by recrystallization from MeCN. ¹H-NMR (500 MHz, [D₆]DMSO): δ = 2.37 ppm (s, 3H; CH₃), 3.58 ppm (s, 2H; CH₂), 5.52 ppm (s, 2H; CH₂), 7.07 ppm (dd, ³J(H,H) = 9.0 Hz, ⁴J(H,H) = 2.5 Hz, 1H; Ar-H), 7.11 ppm (d, ⁴J(H,H) = 2.5 Hz, 1H; Ar-H), 7.80 ppm (d, ³J(H,H) = 9.0 Hz, 1H; Ar-H), 12.48 ppm (bs, 1H; COOH); ¹³C-NMR (500 MHz, [D₆]DMSO): δ = 15.12, 32.79, 78.77, 102.61, 112.98, 114.73, 117.64, 127.03, 148.84, 153.00, 158.37, 160.77, 171.50 ppm.

7-azidomethoxy-6-chloro-4-methylcoumarin-2-acetic acid (7b)

A solution of lithium hydroxide monohydrate (0.65 g, 15.5 mmol) in H₂O (5 mL) was added to a solution of **6b** (0.65 g, 1.92 mmol) in THF (13 mL) cooled at 0°C. The biphasic mixture was vigorously stirred at 0°C and allowed to warm to room temperature for 90 min until all starting product had disappeared. The mixture was acidified with aqueous HCl and extracted with EtOAc. The organic layers were dried over Na₂SO₄, filtrated, and evaporated to provide the product as a white solid in a yield of 0.60 g (97 %). Minor contamination of 6-chloro-7-hydroxy-4-methylcoumarin-2-acetic acid (< 2 %) was removed by recrystallization from MeCN. ¹H-NMR (500 MHz, [D₆]DMSO): δ = 2.37 ppm (s, 3H; CH₃), 3.58 ppm (s, 2H; CH₂), 5.60 ppm (s, 2H; CH₂), 7.31 ppm (s, 1H; Ar-H), 7.93 ppm (s, 1H; Ar-H), 12.49 ppm (bs, 1H; COOH); ¹³C-NMR (500 MHz, [D₆]DMSO): δ = 15.57, 32.85, 52.45, 80.41, 103.52, 115.90, 118.61, 119.95, 126.20, 147.95, 152.00, 154.16, 161.03, 170.51 ppm. HRMS [+Scan]; calculated mass for C₁₃H₁₁N₃O₅Cl 324.0387; observed mass: 324.0381.

Synthesis of oligonucleotides

All oligonucleotides were synthesized on a 1 μmol scale on an ABI model 392 synthesizer using standard β-cyanoethylphosphoramidite coupling chemistry. Removal of the protecting groups and cleavage from the CPG-support were carried out by incubation in concentrated aqueous NH₄OH solution at 55 °C for 14 h. The oligonucleotides were purified using Poly-Pak II cartridges. Oligonucleotide concentrations were determined by UV-absorbance using extinction coefficients derived by the nearest neighbour approximation. The identity of the strands was confirmed by MALDI-TOF mass spectrometry.

Preparation of DNA-conjugates of AzMeOHCoum

Oligonucleotides were synthesized on a 3'-PT-amino-modifier C3 CPG solid support, cleaved/deprotected, and purified as described for standard oligonucleotides. A DMSO solution containing **8a** (0.1 M), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.1 M), and *N*-hydroxysuccinimide (0.1 M) was incubated for 2.5 h at room temperature in the dark. A mixture of 3'-amino DNA solution (200 μL, 1-2 mM), borate buffer (600 μL, 0.1 M, pH 8.5), and the preincubated *N*-hydroxysuccinyl AzMeOHCoum

ester solution (140 μL) was shaken gently over night protected from light. The solution was centrifuged, decanted, and extracted with CHCl_3 . The oligonucleotides were precipitated with EtOH and purified by reverse-phase HPLC. Formation of the intact conjugates was confirmed by MALDI-TOF mass spectrometry (AzMeOCoum-DNA: calc. mass = 2504.5 m/z; exp. mass = 2507.8 m/z). The oligonucleotide concentrations were determined photospectrometrically at 260 nm assuming an extinction coefficient for AzMeOHCoum of $\epsilon_{260} = 3800 \text{ M}^{-1} \text{ cm}^{-1}$. AzMeOCoum-FRET-DNA was prepared and purified analogously using the fluorescein-dT phosphoramidite (MALDI-TOF analysis of AzMeOCoum-FRET-DNA: calc. mass = 3016.6 m/z; exp. mass = 3015.3 m/z).

Preparation of TPP-DNA conjugates

Oligonucleotides containing a 5'-amino modifier 5 were synthesized as described for standard oligonucleotides. The monomethoxy-trityl protecting group was removed on the synthesizer using alternating cycles of deprotection reagent (3 % trichloroacetic acid in DCM) and DCM washes. The solid support was added to a solution containing 4-(diphenylphosphino)benzoic acid (0.1 M), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.1 M), and diisopropylethylamine (0.1 M) in DMF. The mixture was vacuumized and filled with argon to remove air trapped in the solid support and incubated at 37°C for 2.5 hours. The DMF was decanted, the resin washed twice with MeCN, and dispersed in aqueous $\text{NH}_4\text{OH}/\text{MeNH}_2$ deprotection/cleavage solution (1 ml) containing the sacrificial oxygen scavenger tris-(2-carboxyethyl)phosphine (4 mg) and incubated for 2 h at 55°C. Beads were eliminated by filtration and TPP-DNA was purified by reverse phase HPLC and stored at -78°C. (MALDI-TOF analysis of TPP-DNA: calc. mass: 2919.6 m/z, calc. mass for TPP-DNA oxidized: 2935.6 m/z, exp. Mass: 2935.6 m/z). TPP-DNA was sensitive to oxidation by atmospheric oxygen. The TPP-DNA stock solution was kept under argon at -78°C and used within two weeks after preparation to ensure the reactivity of the probe.

Templated fluorescence uncaging studies

TPP-DNA (400 nM) was added to samples containing AzMeOCoum-DNA or AzMeOCoum-FRET-DNA (200 nM) and the corresponding template strand (200 nM unless indicated differently) in buffer (70 mM tris-borate + 10 mM MgCl_2 , pH 7.5 for AzMeOCoum or pH 8.5 for AzMeOCoum-FRET-DNA) at 37°C. The change in fluorescence was determined as a function of time with probe excitation at $\lambda_{ex} = 375 \text{ nm}$ and measurement of emission at $\lambda_{ex} = 450 \text{ nm}$ or 521 nm for AzMeOCoum-DNA or AzMeOCoum-FRET-DNA, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. a) Goodwin JT, Lynn DG. J Am Chem Soc. 1992; 114:9197–9198. b) Gryaznov SM, Letsinger RL. J Am Chem Soc. 1993; 115:3808–3809. c) Xu Y, Kool ET. Tetrahedron Lett. 1997; 38:5595–5598. [PubMed: 19924262] d) Li X, Liu DR. Angew Chem. 2004; 116:4956–4979. Angew Chem Int Ed. 2004; 43:4848–4870.

2. a) Silverman AP, Kool ET. *Chem Rev.* 2006; 106:3775–3789. [PubMed: 16967920] b) Ihara T, Mukae M. *Anal Sci.* 2007; 23:625–629. [PubMed: 17575342]
3. Xu Y, Karalkar NB, Kool ET. *Nat Biotechnol.* 2001; 19:148–152. [PubMed: 11175729]
4. a) Sando S, Kool ET. *J Am Chem Soc.* 2002; 124:9686–9687. [PubMed: 12175209] b) Silverman AP, Kool ET. *Nucleic Acids Res.* 2005; 33:4978–4986. [PubMed: 16284198] c) Silverman AP, Barton EJ, Kool ET. *ChemBioChem.* 2006; 7:1890–1894. [PubMed: 17031884] d) Miller GP, Silverman AP, Kool ET. *Bioorg Med Chem.* 2008; 16:56–64. [PubMed: 17502150]
5. Abe H, Kool ET. *Proc Natl Acad Sci.* 2006; 103:263–268. [PubMed: 16384914]
6. a) Sando S, Kool ET. *J Am Chem Soc.* 2002; 124:2096–2097. [PubMed: 11878946] b) Abe H, Kool ET. *J Am Chem Soc.* 2004; 126:13980–13986. [PubMed: 15506759]
7. a) Oser A, Valet G. *Angew Chem.* 1990; 102:1197–1200. *Angew Chem Int Ed.* 1990; 29:1167–1169. b) Kitamura Y, Ihara T, Tsujimura Y, Tazaki M, Jyo A. *Chem Lett.* 2005; 34:1606–1607. c) Cai J, Li X, Taylor JS. *Org Lett.* 2005; 7:751–754. [PubMed: 15727432] d) Yoshimura Y, Noguchi Y, Sato H, Fujimoto K. *ChemBioChem.* 2006; 7:598–601. [PubMed: 16502477] e) Tanabe K, Tachi Y, Okazaki A, Nishimoto S. *Chem Lett.* 2006; 35:938–939. f) Grossmann TN, Seitz O. *J Am Chem Soc.* 2006; 128:15596–15597. [PubMed: 17147362] g) Dose C, Seitz O. *Bioorg Med Chem.* 2008; 16:65–77. [PubMed: 17499998] h) Huang Y, Coull JM. *J Am Chem Soc.* 2008; 130:3238–3239. [PubMed: 18302371] i) Franzini RM, Kool ET. *Org Lett.* 2008; 10:2935–2938. [PubMed: 18549220]
8. Kohn M, Breinbauer R. *Angew Chem.* 2004; 116:3168–3178. *Angew Chem Int Ed.* 2004; 43:3106–3116.
9. Sakurai K, Snyder TM, Liu DR. *J Am Chem Soc.* 2005; 127:1660–1661. [PubMed: 15700999]
10. Cai J, Li X, Yue X, Taylor JS. *J Am Chem Soc.* 2004; 126:16324–16325. [PubMed: 15600325]
11. Pianowski ZL, Winssinger N. *Chem Commun.* 2007:3820–3822.
12. Abe H, Wang J, Furukawa K, Oki K, Uda M, Tsuneda S, Ito Y. *Bioconjugate Chem.* 2008; 19:1219–1226.
13. a) Loubinoux B, Tabbache S, Gerardin P, Miazimbakana J. *Tetrahedron.* 1988; 44:6055–6064. b) Young T, Kiessling LL. *Angew Chem.* 2002; 114:3599–3601. *Angew Chem Int Ed.* 2002; 41:3449–3451.
14. a) Gao W, Xing B, Tsien RY, Rao J. *J Am Chem Soc.* 2003; 125:11146–11147. [PubMed: 16220906] b) Gutiérrez MC, Slegers A, Simpson HD, Alphand V, Furstoss R. *Org Biomol Chem.* 2003; 1:3500–3506. [PubMed: 14599010] c) Leroy E, Bense N, Reymond J-L. *Bioorg Med Chem Lett.* 2003; 13:2105–2108. [PubMed: 12798314]
15. Matoo BN. *Z Phys Chem.* 1957; 12:232–240.
16. Sun W-C, Gee KR, Haugland RP. *Bioorg Med Chem Lett.* 1998; 8:3107–3110. [PubMed: 9873685]
17. Reddy EP, Reynolds RK, Santos E, Barbacid M. *Nature.* 1982; 300:149–152. [PubMed: 7133135]
18. a) Takakusa H, Kikuchi K, Urano Y, Sakamoto S, Yamaguchi K, Nagano T. *J Am Chem Soc.* 2002; 124:1653–1657. [PubMed: 11853439] b) Takakusa H, Kikuchi K, Urano Y, Kojima H, Nagano T. *Chem Eur J.* 2003; 9:1479–1485. [PubMed: 12658644]
19. Soellner MB, Nilsson BL, Raines RT. *J Am Chem Soc.* 2006; 128:8820–8828. [PubMed: 16819875]
20. Dose C, Ficht S, Seitz O. *Angew Chem.* 2006; 118:5495–5499. *Angew Chem Int Ed.* 2006; 45:5369–5373.
21. Guo J, Xu N, Li Z, Zhang S, Wu J, Kim DH, Marma MS, Meng Q, Cao H, Li X, Shi S, Yu L, Kalachikov S, Russo JJ, Turro NJ, Ju J. *Proc Natl Acad Sci.* 2008; 105:9145–9150. [PubMed: 18591653]
22. Urano Y, Kamiya M, Kanda K, Ueno T, Hirose K, Nagano T. *J Am Chem Soc.* 2005; 127:4888–4894. [PubMed: 15796553]
23. a) Loubinoux B, Gerardin P. *Tetrahedron Lett.* 1991; 32:351–354. b) Griffin RJ, Evers E, Davison R, Gibson AE, Layton D, Irwin WJ. *J Chem Soc Perkin Trans.* 1996; 11:1205–1211. c) Pothukanuri S, Winssinger N. *Org Lett.* 2007; 9:2223–2225. [PubMed: 17480087]

24. Fors KS, Gage JR, Heier RF, Kelly RC, Perrault WR, Wicnienski N. J Org Chem. 1998; 63:7348–7356. [PubMed: 11672382]

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Figure 1. Absorbance (dotted lines) and fluorescence emission (solid lines) spectra of A) AzMeOHCoum and B) AzMeOCiCoum and the corresponding 7-hydroxycoumarins HOHCoum and HOCiCoum (light grey lines). Dotted vertical lines indicate excitation and maximal emission wavelengths. Measurements were performed in sodium phosphate buffer (0.1 M, pH 7.55) and probe concentrations for emission spectra were 0.5 μM .

Figure 2. Time courses of the fluorescence activation of AzMeOCoum-DNA by TPP-DNA in the presence of the fully matched template *mut A* (solid line), the mismatch containing template *wt C* (dashed line), and in absence of template (dotted line). Experiments were performed in a hybridization buffer (70mM tris-borate, 10 mM MgCl₂, pH 7.55) at 37°C; probe concentrations were 200 nM for AzMeOCoum-DNA and templates and 400 nM of TPP-DNA.

Figure 3.

Fluorescence emission spectra of AzMeOCoum-DNA (dark grey traces) and AzMeOCoum-FRET-DNA (light grey traces) before (dotted line) and after (solid line) uncaging by TPP-DNA in the presence of the template *mut A* at 37°C. Experiments were performed in hybridization buffer (10 mM MgCl₂ + 70 mM tris-borate, pH 8.5) with AzMeOCoum-DNA (200 nM), AzMeOCoum-FRET-DNA (200 nM), *mut A* (200 nM), and TPP-DNA (400 nM).

Figure 4. TPP-DNA induced activation of AzMeOCoum-DNA fluorescence in the presence of substoichiometric amounts of the template strand *mut A*. A) Representative time courses of fluorescence activation. B) Turnover numbers after 30 min incubation with variable concentrations of *mut A*. Error bars represent standard error from triplicate experiments.

Scheme 1.

Conceptual representation of DNA-templated fluorescence activation of 7-azidomethoxycoumarin, induced by Staudinger reduction.

Scheme 2.

Synthesis of 7-azidomethoxy-4-methylcoumarin-3-acetic acids. a) H_2SO_4 ; b) bromomethyl 4-chlorophenyl sulfide, K_2CO_3 (for **4a**) or DIEA (for **4b**); c) SO_2Cl_2 then cyclohexene; d) tetrabutylammonium azide, THF; e) NaN_3 , MeCN/ H_2O . f) LiOH, THF/ H_2O .

Scheme 3.

DNA-probes and target strands for template dependent activation of AzMeOHCoum fluorescence. Underlined bases indicate a single nucleotide polymorphism site. ^{5Fl}T specifies a thymine base with a fluorescein attached to the C-5 position.

Scheme 4.

Conceptual representation of the long-wavelength FRET probe AzMeOCoum-FRET-DNA. A) Fluorophore uncaging without FRET reporting. Upon reaction with triphenylphosphine the uncaged coumarin emits at 450 nm when excited at 375 nm. B) Excitation of uncaged coumarin AzMeOCoum-FRET-DNA leads to an efficient energy transfer and emission at the fluorescein emission wavelength at 521 nm. (TPP: triphenylphosphine; TPPO: triphenylphosphine oxide)

Table 1

Photophysical properties of coumarin dyes.

Fluorophore	$\lambda_{abs,max}$ [nm]	ϵ_{max} [M ⁻¹ cm ⁻¹]	ϕ^a	Fluorescence Increase ^{b]}
AzMeOHCoum	319	15200	- ^{c]}	62 X
HOHCoum	324	13600	0.69	
AzMeOCICoum	325	14000	0.60	32 X
HOICoum	364	16200	0.67	

^{a]}Quantum yields were determined using 7-hydroxy-4-methylcoumarin as fluorescence standard.^[16]^{b]}Excitation at λ_{ex} = 375 nm and emission at λ_{em} = 450 nm.^{c]}Emission spectrum was unsuitable for integration.