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Red light-activated phosphorothioate oligodeoxyribonucleotides

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ABSTRACT

Hairpin-structured phosphorothioate oligodeoxyribonucleotides containing a singlet oxygen-sensitive linker in the loop were prepared. These compounds do not bind complementary nucleic acids in the dark. Upon irradiation with red light in the presence of chlorine e6 the linker within these compounds is cleaved and a single-stranded oligodeoxyribonucleotide is produced. The latter compound is an efficient binder of complementary nucleic acids. This is the first example of 'caged' phosphorothioate oligodeoxyribonucleotides, whose nucleic acid binding ability is triggered by red light.

'Caged' nucleic acids are compounds, which are not biologically active until uncaged by light. They may be used for spatially and temporally controlled photoregulation of biological processes. With few exceptions all reported 'caged' antisense oligodeoxyribonucleotides (ODNs), siRNAs, and plasmids, are activated by UV-light.[1] Light of this type is strongly absorbed by cellular components and is highly toxic to cells.[2] Haselton and co-workers have demonstrated that UV-light itself may inhibit gene expression.[3] This limits applications of 'caged' agents in cells and in vivo. We have recently reported 'caged' ODNs, which can be activated by light in any chosen spectral region, including, for example, red light.[4] Red light is significantly less toxic than UV-light and can deeply permeate into tissues.[5]

Herein, we expand our original concept to 'caged' phosphorothioate ODNs (PT-ODNs). In contrast to their natural counterparts, PT-ODNs are more suitable for cellular applications. These compounds are formally obtained by substitution of one oxygen atom in the phosphodiester group of the natural backbone for a sulfur atom. PT-ODNs are stable in the presence of cellular hydrolytic enzymes and permeate the cellular membrane better than ODNs.[6] These compounds are used as antisense agents and immunostimulants.[7] Correspondingly, 'caged' PT-ODNs may be potentially used for light-controlled regulation of gene expression and activity of immune system.

A concept of photo-activated PT-ODNs is presented in Figure 1. In the 'caged' PT-ODN sequence **A** is blocked by sequence **B**. The loop of this compound contains a $^1O_2$-sensitive linker. In the presence of a photosensitizer (**PS**) and upon illumination with light, $^1O_2$ is produced.[8] It induces cleavage of the linker that leads to formation of an unstable intermolecular duplex. The latter duplex dissociates forming the biologically active **A** strand.

We have used *p*-hydroquinone ether and 1,2-dithioethylene fragments as linkers. These moieties were expected to be sensitive to $^1O_2$.[4,9] They were introduced within PT-ODNs using phosphoramidites **L1** and **L2**. **L1** was synthesized in accordance with Scheme 1. First, 4,4′-dihydroxydiphenyl ether was alkylated by 3-bromopropan-1-ol, K_2CO_3 mixture in acetone to obtain 4,4′-(3-hydroxypropan-1-oxy)diphenyl ether. One of the hydroxyl groups of the latter compound was protected with 4,4′-dimethoxytrityl group and another one was phosphatylated to obtain phosphoramidite **L1**. Phosphoramidite **L2** was prepared as described elsewhere.[4] PT-ODNs **1** and **2** were prepared on an automated DNA synthesizer. Beaucage sulfurizing reagent was used in place of the oxidizer solution.

ODNs attached to controlled pore glass (CPG) were cleaved from the support and deprotected using aqueous ammonia (20%, 24 h,

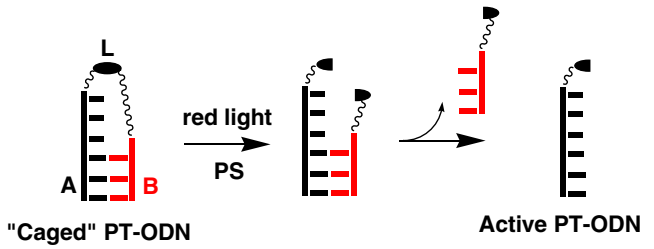

Figure 1. A concept of red light-activated PT-ODNs: **PS** is a red light-absorbing photosensitizer; **L** is a $^1O_2$-sensitive linker; **A** is a biologically active sequence; **B** is a blocker sequence.

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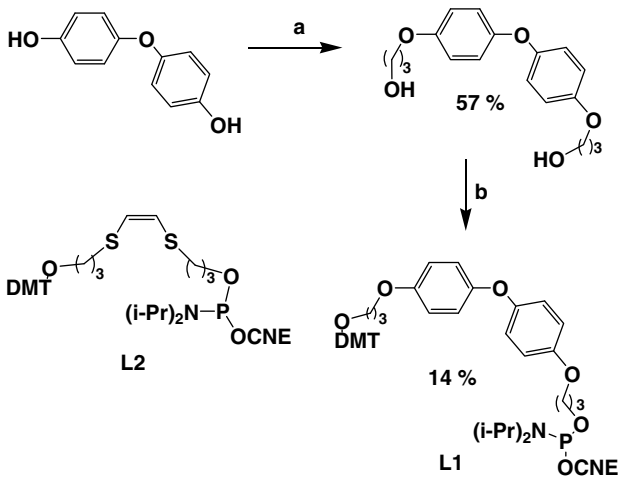


PT-ODN1: TCG AGC GTT CTC **L1** ACG CTC GA
PT-ODN2: TCG AGC GTT CTC **L2** ACG CTC GA
PT-ODN3: TCG AGC GTT CTC
PT-ODN4: ACG CTC GA

c_ODN: TAMRA~GAG AAC GCT CGA
c_RNA: TAMRA~UUU UGA GAA CGC UCG AUU UU

Scheme 1. Synthesis of phosphoramidite **L1** and sequences of PT-ODNs and complementary to them **c_ODN**; TAMRA – *N,N,N′,N′*-tetramethylrhodamine. Reagents and conditions: (a) $Br(CH_2)_3OH$, K_2CO_3, acetone; (b) 1—DMT-Cl, pyridine, 2—$ClP(OCH_2CH_2CN)N(i\text{-}Pr)_2$, DIEA; CNE: 2-cyanoethyl.

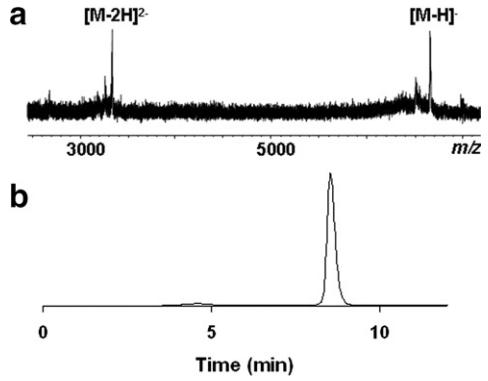


Figure 2. Purity of HPLC purified **PT-ODN2**; (A) MALDI-TOF mass spectrum; 2,4,6-trihydroxyacetophenone (0.3 M in CH_3CN), diammonium citrate (0.1 M in water), 2/1 (v/v) was used as a matrix, matrix/probe 2/1 (v/v) (B) HPLC profile; gradient and other conditions are given in Ref. 10.

25 °C) and then purified by HPLC. Fractions containing >90% pure PT-ODNs were combined and lyophilized (Fig. 2).[10]

Photodecomposition of the linkers within the PT-ODNs was studied by using MALDI-TOF mass spectrometry and HPLC. In the typical assay a mixture of PT-ODN (5 μM) and chlorine e6 (1 equiv) in ammonium acetate buffer (150 mM, pH 7), which contained **PT-ODN4** as an internal standard (IS), was exposed to red light (mercury lamp, red filter) for 1.5 h. Then the sample was treated with dithiotreitol (DTT) (10 mM) and analyzed by mass spectrometry (Fig. 3). Chlorine e6 was selected since this photosensitizer is efficiently excited by red light (λ_{max} ~410 and 650 nm), is soluble in water and has good membrane permeability. The latter property is important for cellular applications of 'caged' PT-ODNs. **PT-ODN1** containing the hydroquinone ether moiety is stable at these

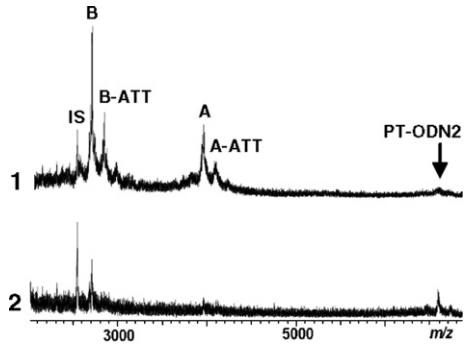


Figure 3. Photocleavage of **PT-ODN2** in the presence of chlorine e6 monitored by MALDI-TOF mass spectrometry; spectrum 1, irradiated probe; spectrum 2, probe kept in the dark; a mixture of 6-aza-2-thiothymine (ATT, saturated solution in CH_3CN) with diammonium citrate (0.1 M in water), 2/1 (v/v) was used as a matrix, matrix/probe 2/1 (v/v); IS is an internal standard, **PT-ODN4**. Other experimental details are given in the text. Nature of fragments **A** and **B** is clear from Fig. 1.

conditions. Therefore, it was not further studied. In contrast, **PT-ODN2** containing 1,2-dithioethylene moiety is cleanly cleaved forming expected fragments **A** and **B** (Figs. 1 and 3). Additional peaks in the mass spectrum of the irradiated mixture correspond to gas phase adducts of the PT-ODNs with ATT matrix (**A**-ATT, **B**-ATT, Fig. 3). When **PT-ODN2** is kept in the dark it is stable for at least 48 h.

Length of the blocking sequence **B** was optimized to achieve that (a) **PT-ODN2** exists in solution in the hairpin form and (b) products of **PT-ODN2** photocleavage (compounds **A** and **B**) do not bind to each other at 22 °C. **PT-ODN2** with an 8-mer blocking sequence exhibited the optimal properties. In particular, melting point (T_m) of **PT-ODN2** in phosphate buffer (10 mM) containing NaCl (150 mM) is well above 22 °C: 65.0 ± 0.4 °C. Moreover, it is not dependent on the concentration of the PT-ODN. The latter fact excludes the possibility of formation of intermolecular associates in the **PT-ODN2** solution, while the former one indicates that ~100% of **PT-ODN2** exists in the hairpin form at 22 °C. Furtheron, products of **PT-ODN2** photocleavage do not bind to each other at room temperature, since the duplex formed between **PT-ODN3** (sequence **A**) and **PT-ODN4** (sequence **B**) melts below 20 °C. Thus,

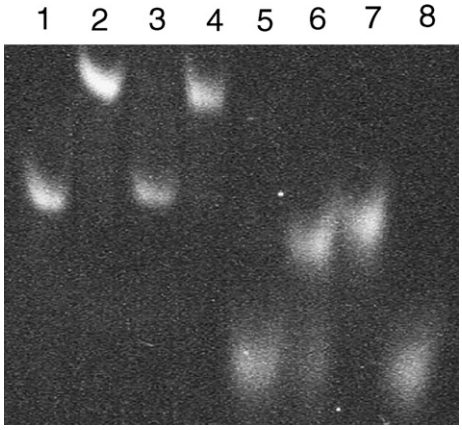
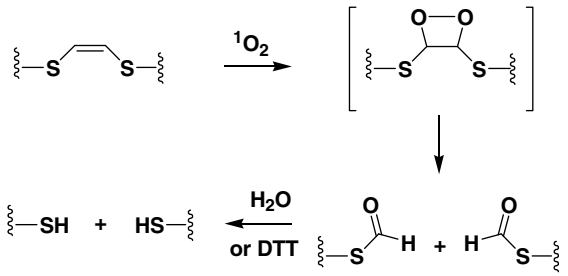


Figure 4. Gel-electrophoresis (native conditions, 20% acrylamide); in all lanes: acetate buffer 100 mM, pH 7, DTT 10 mM, NaCl 1 M. Lane 1, **c_RNA** (1 μM) – negative control; lane 2, **c_RNA** (1 μM), **PT-ODN3** (10 μM) – positive control; lane 3: **c_RNA** (1 μM), **PT-ODN2** (10 μM); chlorine e6 (50 μM), kept in the dark for 1.5 h; lane 4: **c_RNA** (1 μM), **PT-ODN2** (10 μM); chlorine e6 (50 μM), irradiated with red light for 1.5 h; lane 5, **c_ODN** (1 μM), **PT-ODN2** (10 μM); chlorine e6 (50 μM), kept in the dark for 1.5 h; lane 6, **c_ODN** (1 μM), **PT-ODN2** (10 μM); chlorine e6 (50 μM), irradiated with red light for 1.5 h; lane 7, **c_ODN** (1 μM), **PT-ODN3** (10 μM) – positive control; lane 8: **c_ODN** (1 μM) – negative control.



Scheme 2. A mechanism of singlet oxygen induced decomposition of —SCH=CHS— group in **PT-ODN2**.

compound **A** is expected to be in the single stranded, biologically active form after photoactivation of **PT-ODN2**.

UV-melting data were corroborated by gel-electrophoresis experiments (Fig. 4). One observes that sequence **A** in **PT-ODN2** is blocked and can bind neither complementary RNA (**c_RNA**) nor DNA (**c_ODN**), whereas the product of red light-induced **PT-ODN2** photocleavage forms a stable duplex with both RNA and DNA targets.

The mechanism of **L2** cleavage is illustrated in Scheme 2. $^1O_2$ is first produced in the result of red light-induced excitation of chlorine e6 to the triplet state followed by its relaxation to the ground state via energy transfer to $^3O_2$. Singlet oxygen forms 2 + 2 addition product with the —SCH=CHS— fragment of **PT-ODN2**. This product is unstable. It decomposes to formic acid thioesters, which are transformed in the presence of DTT or water into thiols.[4] It has been reported that singlet oxygen can be quenched by sulfur-containing compounds.[11] Therefore, photoactivation of 'caged' PT-ODNs could be slowed down due to interactions of the phosphorothioate groups with singlet oxygen. Fortunately, this effect seems to be less important. For example, we observed that $^1O_2$-induced activation of 'caged' phosphorothioate ODNs is only 1.5 times slower than that of 'caged' natural ODNs.

In summary, we have prepared 'caged' phosphorothioate oligodeoxyribonucleotides. These compounds are inert in the dark, whereas they are activated by red light in the presence of chlorine e6. The 'uncaged' form of PT-ODNs is an efficient binder of single-stranded nucleic acids. This is the first example of phosphorothioate oligodeoxyribonucleotides, whose nucleic acid binding ability is triggered by red light.

These compounds can potentially exhibit antisense activity. We are currently testing them for red light-controlled gene expression in HeLa cells.

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10. *Synthesis of 4,4′-(3-hydroxypropan-1-oxy)diphenyl ether:* A suspension of 4,4′-dihydroxydiphenyl ether (1.0 g, 4.9 mmol) and K_2CO_3 (1.4 g, 9.8 mmol) in acetone (10 mL) was warmed up to 50 °C. Then solution of 3-brom-1-propanol (0.9 mL, 10.3 mmol) in acetone (5 mL) was slowly added. The reaction mixture was stirred overnight at reflux temperature. After addition of water (10 mL) the reaction mixture was heated until everything was dissolved. The product was crystallized from this solution upon cooling down to 22 °C. The white crystals were filtered, washed with cold water, and dried in vacuo. Yield 0.89 g (57%); ^{1}H NMR (MeOD, 200 MHz) δ 1.98 (m, 4H), 3.75 (t, 4H, J = 6.2), 4.06 (t, 4H, J = 6.2), 6.88 (d, 8H, J = 0.6); ^{13}C NMR (MeOD, 50 MHz) δ33.5, 59.6, 66.3, 116.6, 120.5, 153.1, 156.3; Calcd. for $C_{18}H_{23}O_5$ $[M+H]^+$ 319.2; found (ESI-MS$^+$): 319.2.
Synthesis of 3-[4-(4-{3-[Bis-(4-methoxyphenyl)-phenyl-methoxy]-propoxy}-phenoxy)-phenoxy]-propan-1-ol: A mixture of 4,4′-bis[(3-phenoxy-propan-1-ol)] ether (0.70 g, 2.2 mmol) and 4-dimethylaminopyridine (DMAP, 0.18 g, 1.5 mmol) was dissolved in pyridine (25 mL) and diisopropylethylamine (DIEA, 255 μL, 1.5 mmol) was added. Then 4,4′-dimethoxytrityl chloride (0.73 g, 2.2 mmol) in pyridine (15 mL) was added to the reaction mixture over 40 min. The solution was stirred overnight at 22 °C. Volatile components were removed and the residue was co-evaporated twice with toluene (15 mL, each portion). The crude product was purified by column chromatography (silica gel, ethyl acetate:hexane:triethylamine (TEA) = 69:30:1; R_f = 0.35). Yield 0.26 g (19%). ^{1}H NMR ($CDCl_3$, 200 MHz) δ 2.00 (m, 4H), 3.22 (t, 2H, J = 6.0), 3.73 (s, 6H), 3.82 (t, 2H, J = 6.0), 4.06 (t, 2H, J = 5.8), 4.09 (t, 2H, J = 7.2), 6.71–6.89 (m, 12H), 7.10–7.32 (m, 7H), 7.34–7.42 (m, 2H).
*Synthesis of **L1** (all operations were conducted at anaerobic conditions):* The above described alcohol (0.26 g, 0.4 mmol) was dissolved in CH_2Cl_2 (10 mL) and DIEA (0.4 mL, 2.3 mmol) was added. 2-Cyanoethyldiisopropyl-amidochloridophosphite (0.3 mL, 1.3 mmol) was added slowly. After 2 h the reaction mixture was poured into saturated aqueous solution of $NaHCO_3$ (15 mL) and extracted with CH_2Cl_2 (3× 15 mL). The combined organic layers were dried over magnesium sulfate. Volatile components were removed. The crude product was purified by column chromatography (silica gel, ethyl acetate:hexane:TEA = 68:30:2; R_f = 0.71). Yield 0.26 g (76%). ^{31}P NMR ($CDCl_3$) δ 147.7; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.16 (t, 12H, J = 6.4), 1.96–2.09 (m, 4H), 2.59 (t, 2H, J = 6.4), 3.25 (t, 2H, J = 6.0), 3.53–3.61 (m, 2H), 3.76 (s, 6H), 3.77–7.85 (m, 4H), 4.04 (t, 2H, J = 6.0), 4.11 (t, 2H, J = 6.4), 6.73–6.91 (m, 12H), 7.12–7.31 (m, 7H), 7.39–7.41 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.3 (d, J = 6.9), 24.5 (d, J = 7.6), 24.6 (d, J = 7.6), 30.0, 31.0 (d, J = 7.3), 43.0 (d, J = 12.3), 55.2, 58.3 (d, J = 19.3), 59.7, 60.2 (d, J = 17.4), 65.0, 65.4, 85.9, 113.0, 115.4, 117.6, 119.4, 119.5, 126.6, 127.7, 128.2, 130.0, 136.4, 145.2, 151.4, 151.6, 154.6, 154.7, 158.3.
***PT-ODN1**:* Yield 8.6%. HPLC (Column: Macherey-Nagel Nucleosil 300-5, C18, 250/4.6; solvent A: 0.1 M (NEt_3H)(OAc) in water, pH 7.0; solvent B: CH_3CN, gradient: 0% B for 5 min, in 30 min to 25% B, in 10 min to 90% B, 90% B for 9 min) R_t = 32–35 min. MALDI-TOF MS: Calcd for $C_{211}H_{266}N_{71}O_{107}P_{20}S_{20}$ $[M-H]^-$: 6765, found 6764.
***PT-ODN2**:* Yield 4.4%. HPLC (Column: Macherey-Nagel Nucleosil 100-5, C4, 125/2; gradient: 0% B for 0.5 min, in 9.5 min to 35% B, in 2 min to 100% B) R_t = 8.53 min. MALDI-TOF MS: Calcd for $C_{201}H_{260}N_{71}O_{104}P_{20}S_{22}$ $[M-H]^-$: 6655, found 6659.
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