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Synthesis of Light-Responsive Bridged Nucleic Acid and Changes in Affinity with Complementary ssRNA

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The control of molecular properties through the use of external stimuli such as pH,[1] temperature,[2] or change in redox potential[3] is an attractive area of research for the regulation of various biological phenomena. Among such stimuli, light is an ideal trigger because of its potential for spatiotemporal control of cellular chemistry.[4] The compounds that can release an active molecule upon light irradiation are called "caged compounds".[5] In general, the properties of caged compounds, such as affinity for a specific molecule, can be changed only once (Figure 1 A).[6] If another trigger could cause additional alteration after photoirradiation, it would be possible to achieve control of molecular properties in two stages; this would allow for stricter regulation of biological phenomena (Figure 1 B). Recently, we reported the synthesis and properties of 2′-*O*,4′-*C*-methyleneoxymethylene-bridged nucleic acid (2′,4′-BNA^{COC}),[7] which is a 2′,4′-BNA/LNA[8] analogues (Scheme 1). It was observed that oligonucleotides containing 2′,4′-BNA^{COC} show high affinity for complementary single-stranded RNA (ssRNA) because the sugar conformation of 2′,4′-BNA^{COC} is prelocked in the N-type conformation, which is the major conformation in the A-form of RNA duplex structure.[9] Here, we focused on this property of 2′,4′-BNA^{COC} and designed light-responsive BNA **I** that contains a 2′-hydroxy group and a 4′-hydroxymethyl group that are both protected by a photolabile 6-nitroveratryl group (Scheme 1).[10] This compound is expected to retain its binding ability to complementary ssRNA, despite the presence of a large hydrophobic group at the minor groove,[11] because its sugar conformation is restricted to N-type. Irradiation with light results in cleavage of the bridged structure of **I**; this transforms the analogue to the 4′-*O*-benzoyl form **II**, which contains a bulky substituent at the 4′-*C* position[11] and loses its sugar conformation restriction. Hence, oligonucleotides containing **II** lose their binding ability to complementary ssRNA. The 4′-*O*-benzoyl group of **II** may be removed by treatment with various nucleophiles; this results in the formation of 4′-*C*-hydroxymethyl-RNA analogue **III**. 4′-*C*-hydroxymethyl-RNA analogue **III** restores the binding ability to complementary ssRNA, despite a flexible sugar conformation, because of a reduction in steric hindrance.[12] In other words, the affinity of oligonucleotides containing light-responsive BNA **I** for complementary ssRNA may be changed in two stages, unlike conventional caged compounds, triggered by light and treatment with nucleophiles.

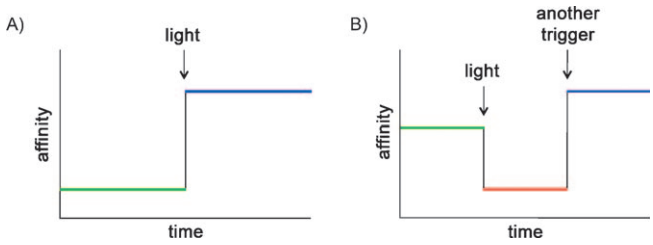
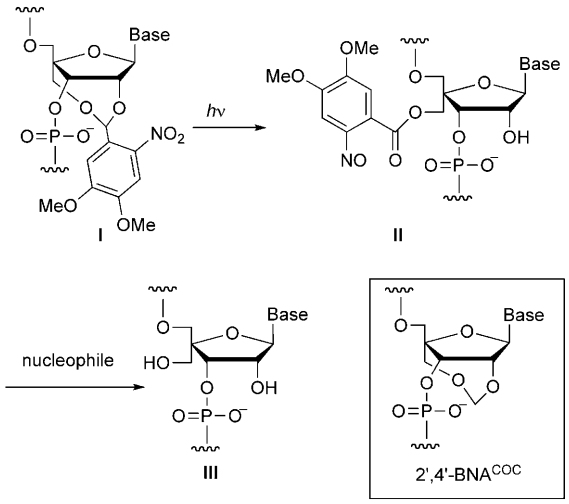


Figure 1. Property change caused by external stimulus. In this figure, changes in affinity for specific molecules are shown as an example. A) Caged compound with properties that can be changed by stimulation with light only once. B) Two-stage change triggered by light and another stimulus.



Scheme 1. Change in structure of light-responsive BNA **I**.

Phosphoramidite **7** was synthesized from nucleoside derivative **1** as shown in Scheme 2.[8a,13] The two benzyl groups of **1** were removed by hydrogenolysis by using palladium hydroxide on carbon, and the resulting hydroxyl groups were protected by a 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl (TIPDS) group to give **2**, which was treated with aqueous methylamine to give diol **3**. A 2′,4′-bridged structure was constructed by reacting diol **3** with 6-nitroveratraldehyde and zinc chloride in 1,1,1,3,3,3-hexafluoroisopropanol, and compound **4** was obtained as a single diastereomer. Configuration at the acetal carbon atom was assigned by HMBC and NOESY spectra (Supporting Information, S12).[14] Desilylation of **4** was carried out by using tetrabutylammonium fluoride (TBAF) to afford diol **5**, and tritylation of the primary hydroxyl group of **5** with 4,4′-dimethoxytrityl chloride gave **6**. Finally, phosphitylation of the secondary hydroxyl group of **6** with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tet-

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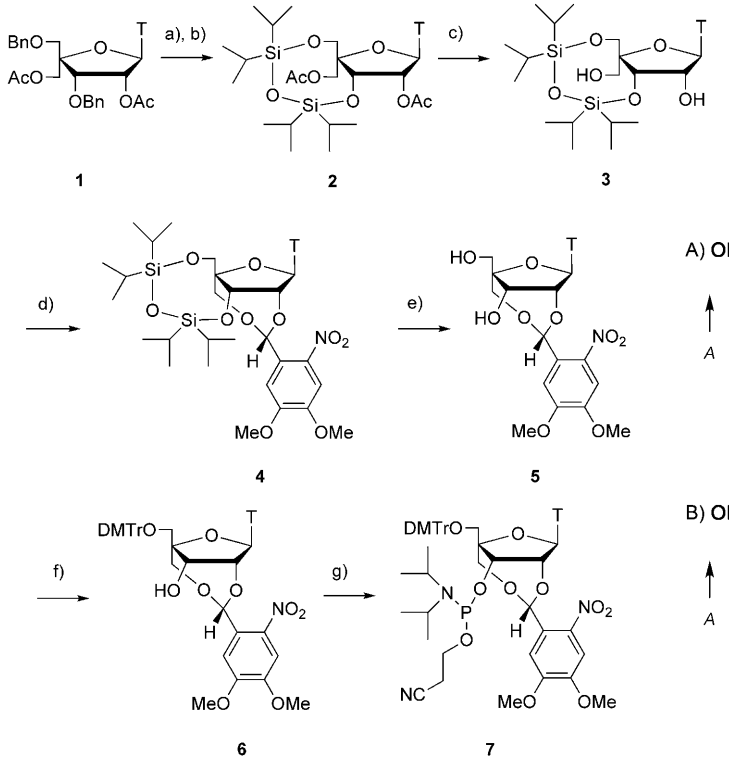

Scheme 2. Synthesis of light-responsive BNA monomer **5** and phosphoramidite **7**. Reagents and conditions: a) H_2, 20% $Pd(OH)_2$-C, AcOEt, RT, 9 h; b) $TIPDSCl_2$, imidazole, DMF, RT, 3.5 h (72% from **1**); c) 40% $MeNH_2$ aq., THF, 0 °C, 3 h (85%); d) 6-nitroveratraldehyde, $ZnCl_2$, HFIP, RT, 21 h (54%); e) TBAF, THF, RT, 0.5 h (89%); f) DMTrCl, pyridine, RT, 13 h (89%); g) $(iPr_2N)_2PO(CH_2)_2CN$, 4,5-dicyanoimidazole, MeCN, RT, 18 h (55%). Ac = acetyl, Bn = benzyl, DMTr = 4,4′-dimethoxytrityl, HFIP = 1,1,1,3,3,3-hexafluoroisopropanol, T = thymin-1-yl, TBAF = tetra-*n*-butylammonium fluoride, TIPDS = 1,1,3,3-tetraisopropyldisiloxane-l,3-diyl.

raisopropylphosphordiamidite yielded the desired thymine phosphoramidite **7**.

Following a conventional phosphoramidite coupling protocol, oligonucleotides were synthesized using phosphoramidite **7** and natural DNA amidite building blocks in an automated DNA synthesizer (Applied Biosystems Expedite 8909). The concentration of the phosphoramidite **7** was 0.067 M and 5-ethylthio-1*H*-tetrazole was used as an activator. The coupling time of phosphoramidite **7** was prolonged to 40 min from 90 seconds. Coupling yields were determined by trityl monitoring and were found to be 95–100%. The oligonucleotide sequences synthesized for this study are given in Figure 2. The oligonucleotides were purified by reversed-phase HPLC (RP-HPLC)

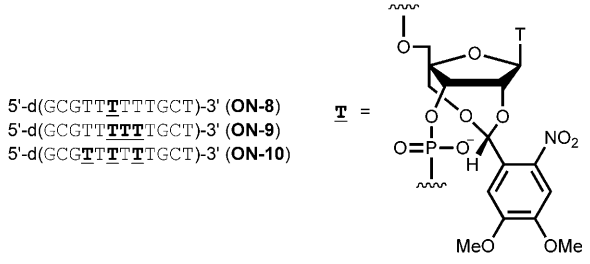

Figure 2. Sequences of oligonucleotides used in this study.

and characterized by MALDI-TOF mass spectroscopy (mass spectral data and yields of oligonucleotides are provided in Supporting Information).

We analyzed **ON-8** by using RP-HPLC after photoirradiation (Figure 3).[15] When irradiation was carried out at 365 nm for 5 seconds, one peak (24.0 min),

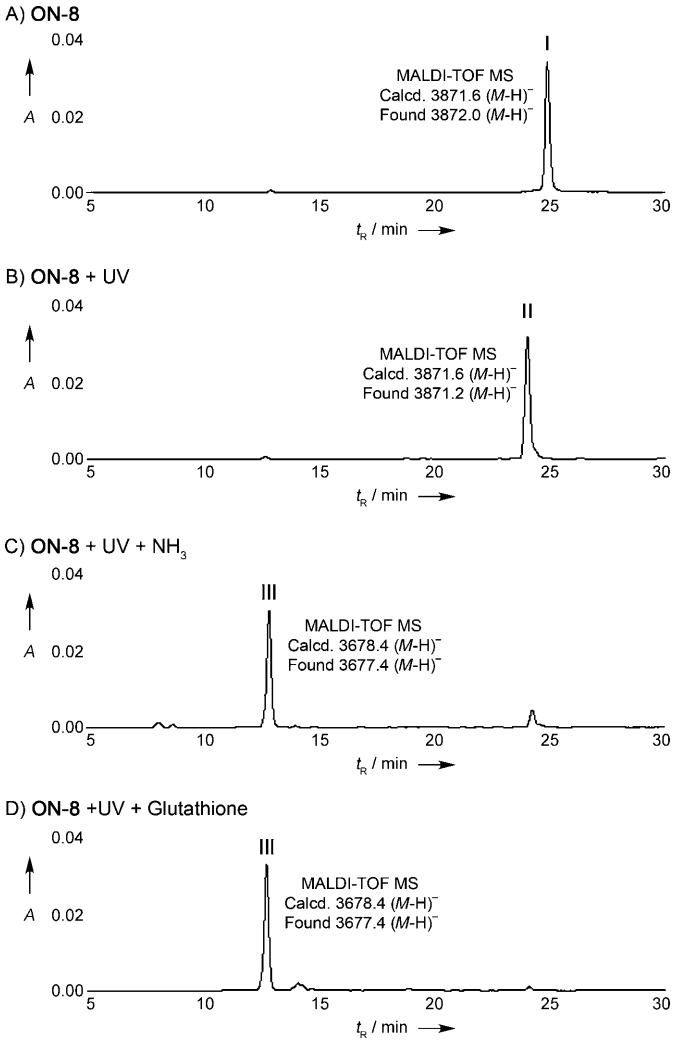

Figure 3. RP-HPLC analysis of **ON-8**. A) **ON-8** (10 μM) before photoirradiation. B) **ON-8** (10 μM) after irradiation at 365 nm for 5 s. C) **ON-8** (10 μM) after irradiation at 365 nm for 5 s and subsequent reaction with excess ammonia for 60 min. D) **ON-8** (10 μM) after irradiation at 365 nm for 5 s and subsequent reaction with glutathione (10 equiv) for 60 min. The reaction mixture was analyzed by RP-HPLC using an XTerra MS C_{18} column (4.6×50 mm) with a linear gradient of MeCN (from 6% to 12% over 30 min) in 0.1 M triethylammonium acetate (pH 7.0).

with a different retention time from that of **ON-8** (24.9 min), appeared quantitatively. Furthermore, when **ON-8** was treated with ammonia or glutathione after photoirradiation, another peak appeared at 12.7 min, while the peak with a retention time of 24.0 disappeared. The compounds with retention times of 24.0 min and 12.7 min were characterized by MALDI-TOF mass spectroscopy, and it was found that—as expected—light-responsive BNA **I** in **ON-8** was transformed to the 4′-*O*-benzoyl

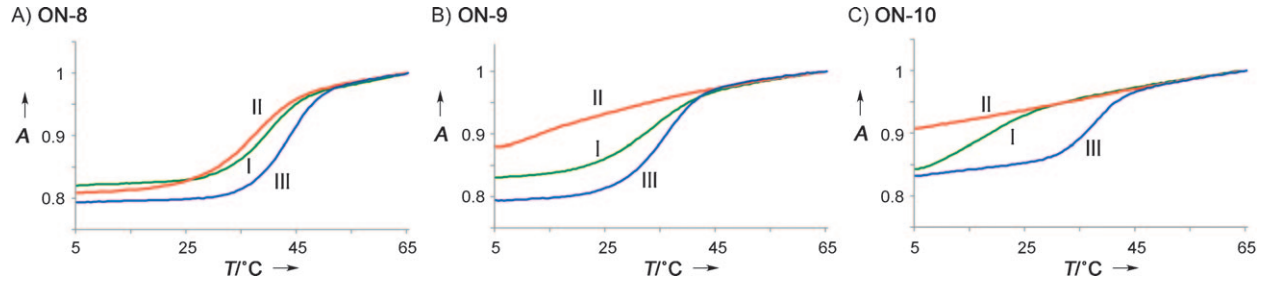

Figure 4. UV melting curves (260 nm) for A) **ON-8**, B) **ON-9**, C) **ON-10**. ——: before photoirradiation; ——: after irradiation at 365 nm for 5 s (**ON-8**) and 15 s (**ON-9** and **ON-10**); ——: after irradiation at 365 nm for 5–15 s, treatment with ammonia (excess) or glutathione (**ON-8**: 10 equiv, **ON-9** and **ON-10**: 20 equiv) and purification by RP-HPLC. Conditions: 10 mM sodium phosphate buffer solution (pH 7.2) containing 100 mM NaCl; each strand concentration = 4 μM; scan rate 0.5 °C min^{-1}.

form **II** (24.0 min) by using photoirradiation and then to 4′-*C*-hydroxymethyl-RNA analogue **III** (12.7 min) by subsequent treatment with ammonia or glutathione. **ON-8** (**I**) (24.9 min) was successfully converted to **ON-8** (**III**) (12.7 min), and no other reaction was found to have taken place.

The affinity of **ON-8** to complementary ssRNA was expected to deteriorate upon photoirradiation and be restored by subsequent treatment with ammonia or glutathione. We evaluated the change in affinity of **ON-8** with complementary ssRNA through UV melting experiments (Figure 4). After photoirradiation, **ON-8** (**II**) showed lower affinity with complementary ssRNA as compared with **ON-8** (**I**) (T_m: 40→38 °C) and after subsequent treatment with ammonia or glutathione, the affinity of **ON-8** (**III**) was higher (T_m: 38→44 °C; Table 1).

Table 1. T_m values (°C) of duplexes formed with complementary ssRNA.[a,b,c]

Oligonucleotides Form	−UV (**I**)	+UV (**II**)	+UV, +NH_3 or glutathione (**III**)[d,e]
ON-8	40	38	44
ON-9	33	–[f]	36
ON-10	–[f]	–[f]	38

[a] Irradiation at 365 nm for 5 s (**ON-8**) or 15 s (**ON-9** and **ON-10**). Treatment with ammonia (excess) and glutathione (**ON-8**, 10 equiv; **ON-9** and **ON-10**, 20 equiv) was carried out for 60 min. [b] Conditions: 10 mM sodium phosphate buffer solution (pH 7.2) containing 100 mM NaCl; each strand concentration = 4 μM; scan rate 0.5 °C min^{-1}. [c] The number is average of three independent measurements. [d] The form **III** oligonucleotides were purified before T_m measurements. [e] Synthesizing the control oligonucleotides by a previously reported method[12] would be difficult. [f] T_m < 15 °C or not detectable.

The fact that oligonucleotides containing one light-responsive BNA **I** moiety showed a small but measurable change in affinity with complementary ssRNA prompted us to evaluate the binding properties of **ON-9** and **ON-10**, in which three light-responsive BNA **I** moieties were introduced either consecutively or alternately.

At first, it was confirmed that photoirradiation of **ON-9** and **ON-10** quantitatively gave **ON-9** (**II**) and **ON-10** (**II**), respectively, and subsequent treatment with glutathione successfully afforded **ON-9** (**III**) and **ON-10** (**III**) (Supporting Information, S16). Then, the change in affinity of **ON-9** and **ON-10** for complementary ssRNA was evaluated through UV melting experiments (Figure 4). As a result, very interestingly, different behaviors of oligonucleotides were observed depending on the position of three light-responsive BNA **I** moieties in the sequences. **ON-9** (**I**), which contains three consecutive BNA **I** units, bound to complementary ssRNA (T_m = 33 °C), whereas no binding was observed after photoirradiation (form **II**). The binding affinity was clearly recovered after treatment with glutathione (form **III**, T_m = 36 °C; Figure 5 A, [on]→[off]→[on]). This change in

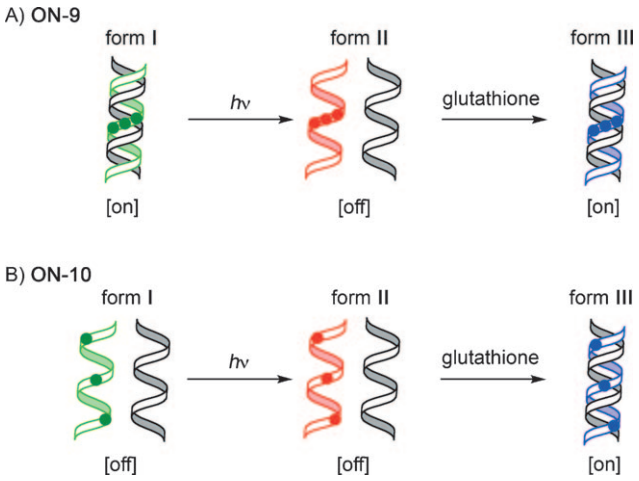

Figure 5. Change in affinity of A) **ON-9** and B) **ON-10** for complementary ssRNA. Red strand: after photoirradiation; blue strand: after treatment with ammonia or glutathione; gray strand: complementary ssRNA. Filled circles on the colored strands indicate modified residues.

thermal stability can be anticipated from that of **ON-8**. On the other hand, **ON-10** (**I**), which contains three alternating BNA **I** units, showed almost no binding to its RNA complement (T_m < 15 °C). As expected, no duplex formation was observed at all after photoirradiation (form **II**). However, **ON-10** (**III**), obtained by treatment with glutathione, acquired duplex-forming ability with ssRNA (T_m = 38 °C; Figure 5 B, [off]→[off]→[on]).

From these features, light-responsive BNA **I** would be applicable to a variety of biotechnologies. For example, the oligonucleotides with consecutive BNA **I** units (like **ON-9**) could trap functional ssRNA, for example, miRNA or ribozyme, and release it upon photoirradiation only in the cells with low glutathione levels. These oligonucleotides could work as a novel drug delivery agent, which releases functional ssRNA in a specific place at just the desired time. On the other hand, the oligonucleotides containing alternating BNA **I** units (like **ON-10**) could be a good antisense agent to treat cancer spatiotemporally with little side effects, because it is well known that the glutathione concentration is nearly threefold higher in some cancer cells compared to the level of 0.1–10 mmol L^{-1} in normal cells.[16]

In summary, we successfully synthesized light-responsive BNA **I**, which contains a bridged structure with a photolabile protecting group, and evaluated the change in affinity of BNA **I**-containing oligonucleotides with complementary ssRNA. By adjusting the number and position of BNA **I** moieties in the oligonucleotides, it is possible to regulate the binding affinity to complementary ssRNA in different ways. Light-responsive BNA **I** is expected to allow fine spatiotemporal gene regulation by sensing the environment.

Experimental Section

Synthesis of oligonucleotides: Synthesis of the light-responsive BNA modified oligonucleotides (**ON-8**–**ON-10**, on the 0.2 μmol scale) was performed on Applied Biosystems Expedite 8909 Nucleic Acid Synthesis System (Foster City, USA) according to a phosphoramidite coupling protocol using 5-ethylthio-1*H*-tetrazole as the activator. The coupling time of phosphoramidite **7** was 40 min and the coupling reaction was efficient as well as natural amidite. The solid supported oligonucleotides were then treated with concentrated ammonium hydroxide solution at 55 °C for 12 h, and then concentrated. The crude oligonucleotides were primarily purified by GE Healthcare Nap 10 column and finally purified by using reversed-phase HPLC with a Waters XTerra MS C_{18} column 2.5 cm (10× 50 mm, Mitford, USA) by using MeCN in triethylammonium acetate buffer (0.1 M, pH 7.0). The purified oligonucleotides were analyzed by reversed-phase HPLC with Waters XTerra MS C_{18} column 2.5 cm (4.6×10 mm) for their purity and characterized by MALDI-TOF mass spectroscopy.

Photoirradiation experiments: Photoirradiation at 365 nm was performed on a ZUV-C30H UV-LED lamp as light source and ZUV-L8H as a lens unit (Omron, Kyoto, Japan). Photoreaction was performed at a distance of 4 cm from the lens unit. Equimolecular amounts of the oligonucleotides were dissolved in sodium phosphate buffer (25 mM, pH 7.2) to give a final strand concentration of 10.0 μM. **ON-8** was irradiated for 5 s and **ON-9** and **10** were for 15 s.

Nucleophile treatment experiments: After irradiation **ON-8** was dissolved in sodium phosphate buffer (25 mM, pH 7.2) and NH_3 (excess) or glutathione (10 equiv) was added to give a final strand concentration of 10.0 μM. The mixture was analyzed by RP-HPLC after a reaction time of 60 min at room temperature. **ON-9** and **10** were dissolved in sodium phosphate buffer (25 mM, pH 7.2) after irradiation and glutathione (10 equiv) was added to give a final strand concentration of 10.0 μM. After a reaction time of 30 min at room temperature, glutathione (10 equiv) was added and the reaction was allowed to proceed for 30 min at room temperature and then analyzed by RP-HPLC.

UV melting experiments: UV melting experiments were carried out on a Shimadzu UV-1650B and Shimadzu UV-1650PC spectrometer equipped with a T_m analysis accessory (Kyoto, Japan). Equimolecular amounts of the target ssRNA and **ON-8**–**10** were dissolved in sodium phosphate buffer (10 mM, pH 7.2) containing NaCl (100 mM) to give final strand concentration of 4.0 μM. The samples were annealed by heating at 90 °C followed by slow cooling to room temperature. The melting profile was recorded at 260 nm from 5–90 °C at a scan rate of 0.5 °C min^{-1}. T_m values for form **II** were measured without purification and form **III** were after purification with RP-HPLC.

Acknowledgements

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Keywords: antisense agents · BNA · nucleic acids · photoirradiation · RNA

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[14] NOESY spectrum of compound **4** showed no correlation between acetal-H and 1′-H and strong correlation between acetal-H, 1″-H and TIPDS-H; this data strongly supports the indicated configuration. It should be impossible to show such a NOESY spectrum for the other diastereomer.
[15] To evaluate the structure of photoproduct, compound **5** was irradiated at 365 nm (LED). There are two C–O bonds in the benzylidene moiety of **5**. However, only one regioisomer was obtained, although the reason is unknown. The structure of the photoproduct was characterized by ^{1}H–^{1}H COSY NMR experiments, which showed that the photoproduct has two secondary hydroxyl groups (2′-OH and 3′-OH) and a benzoyl substituent at the 4′-*C*-hydroxymethyl group. See Supporting Information, S14.
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