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Photolytic Release of a Caged Inhibitor of an Endogenous Transcription Factor Enables Optochemical Control of CREB-Mediated Gene Expression

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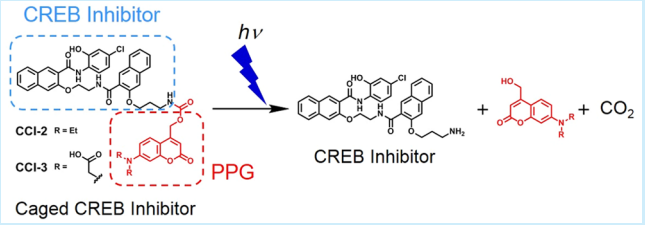
Supporting Information

ABSTRACT: A direct optochemical method for regulating gene function has been developed based on uncaging of an inactive caged precursor that fragments to produce a CREB (cAMP-response element binding protein) inhibitor that binds to an endogenous transcription factor responsible for regulating CREB-mediated gene expression levels.



Access to chemical biology tools that can be used to manipulate transcriptional activity is extremely useful for understanding gene function in cells. Optical tools for genetic manipulation have been used to generate spatiotemporal perturbations of targeted gene expression in cellular networks, which enables the role of the proteins they encode to be explored.[1] Recently, a variety of optogenetic systems have been developed that employ light-sensitive transcription factors and proteins to control gene function.[2,3] Alternative optochemical approaches that employ photoactivatable compounds have also been used as a method to achieve spatiotemporal modulation of transcription, without the need for genetically modified cells or animals.[4] Caged compounds are inactive precursors of biologically active molecules that are masked with a photoresponsive protecting group (PPG) that can be irreversibly activated by light.[5−8] A number of caged ligands/inhibitors targeting various transcription factors have been developed, whose ability to modulate transcriptional activity is based on photolytic generation of an activating or inhibitory ligand. For example, caged ligand systems based on photoaddressable doxycycline[9] and tamoxifen[10,11] precursors have previously been used for light-stimulated control of gene expression. However, these ligand systems are generally used in combination with exogenous gene expression systems (such as Tet-ON/OFF and CreER), which limits their general applicability for the direct regulation of endogenous gene expression. Therefore, the development of simple photoactivatable caged ligand systems that can be used to target endogenous gene expression, without the need for exogenous gene expression systems, is potentially of great interest.

This study reports the development of a photochemically addressable endogenous transcription system, based on photochemical release of an inhibitor of the cAMP-response element binding protein (CREB), which is a transcription factor that is endogenously expressed in various cell lines.[12] CREB is known to interact with a coactivator paralog CREB binding protein (CBP) to promote transcriptional initiation from cAMP-mediated response elements (CRE) downstream of cAMP/PKA and Ca^{2+} signaling pathways that lead to increased gene expression. CREB-mediated transcription activity is known to be important for promoting cell proliferation and cell differentiation, as well as the development of long-term memory networks in the nervous system.

Therefore, the availability of a simple optochemical tool that could be used for the spatiotemporal control of CREB activity would be very useful for probing the role of CREB in cellular development. CREB activity has previously been regulated by gene knockdown using small interfering RNA (siRNA) technology;[13] however, this approach only offers limited spatiotemporal control of CREB activity.

The aim of this study was to develop inactive caged CREB inhibitors (CCIs) that could be photolytically cleaved to release an active inhibitor of CREB-mediated transcriptional activity in cellular systems. Light activation of an inactive CCI within the cell would trigger intracellular release of the inhibitor cargo, thus enabling spatiotemporal suppression of CREB-dependent transcription to be achieved in real time

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(Figure 1). This would provide a simple chemical method to achieve controlled knockdown of CREB-mediated gene expression based on simple light stimulation of living cells.

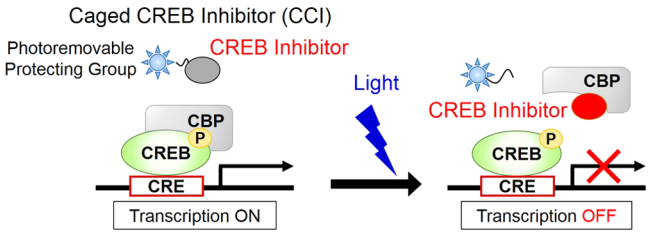


Figure 1. Caged inhibitor strategy for optochemical control of CREB-mediated gene expression.

The target caged CREB inhibitors **CCI-1**, **CCI-2**, and **CCI-3** were designed based on a naphthol AS-E derivative (**1**) (Figure 2), which is known to possess high CREB inhibitory activity

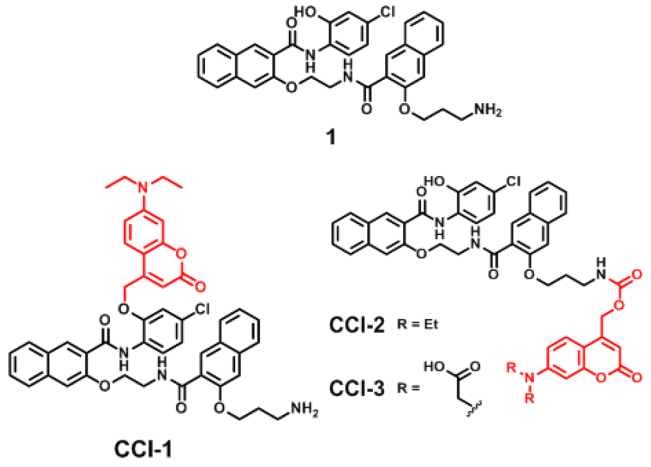
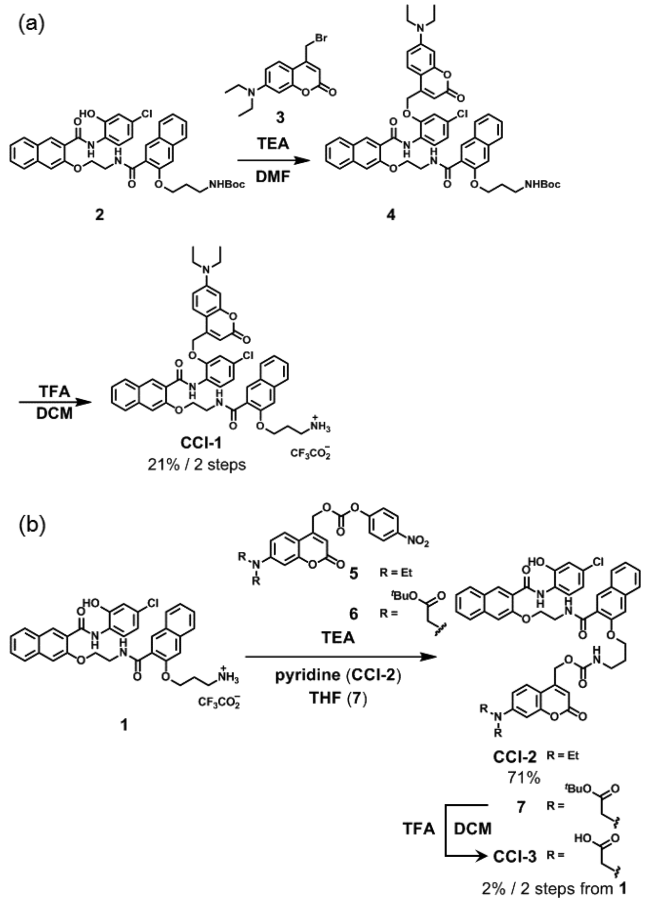


Figure 2. Structures of CREB inhibitor **1** and caged CREB inhibitors (CCIs) whose PPG fragments are highlighted in red.

(IC_{50} = 81 nM).[14] The 7-(diethylamino)coumarin-4-yl methyl (DEACM) group was selected as PPG owing to its high decomposition efficiency at long wavelengths above 400 nm.[15] The phenolic hydroxyl group of the CREB inhibitor **2** was then functionalized with bromo-DEACM **3**, followed by TFA-mediated deprotection of the *N*-Boc group to afford **CCI-1** in 21% yield over two steps (Scheme 1a). The carbamate linkers of **CCI-2** (containing a diethylamino group) and **CCI-3** (containing a bis(carboxymethyl)amino group) were synthesized by direct conjugation of the free amino group of **1** with the corresponding *p*-nitrophenyl carbonate esters of DEACM in 71% and 2% yields over two steps, respectively (Scheme 1b).

The photochemical properties of **CCI-1**, **CCI-2**, and **CCI-3** were then explored through uncaging of their DEACM groups at 405 nm in HEPES buffer, which resulted in products with absorption maxima below 380 nm (Figure S1). HPLC analysis of the photolytic cleavage products of **CCI-1** revealed that inhibitor **1** had been formed in the presence of a number of other byproducts (Figure S2). Mass spectrometric analysis revealed that the major byproduct gave an identical molecular ion to **CCI-1** ($[M + H]^+$ = 813.29), indicating that a competing intramolecular photo-Claisen rearrangement of **CCI-1** had occurred to afford a rearranged phenolic isomer

Scheme 1. Synthesis of CREB Inhibitors CCI-1 (a), CCI-2, and CCI-3 (b)



(Figure S2).[16] However, photolytic cleavage reactions of amino-protected **CCI-2** and **CCI-3** at 405 nm proceeded cleanly, affording inhibitor **1** in high yield, with no evidence of any photo-Claisen rearrangement products having been formed (see HPLC traces shown in Figure 3). Analysis of the time course of the photolytic reactions of **CCI-2** and **CCI-3** enabled their cleavage rates to be determined (Figure 4) with their relatively rapid photocleavage rate constants (Table 1), indicating that they would be suitable as photolytically labile caged CREB inhibitors for cellular studies.

The CREB inhibitory activities of uncaged **CCI-2** and **CCI-3** were then evaluated in living human embryo kidney (HEK293T) cells that had been cotransfected to express a dual luciferase reporter system. These cells contained a firefly luciferase reporter gene that was under the control of a CREB-responsive promoter containing a cAMP response element (CRE), and a renilla luciferase gene whose luminescence activity served as an internal control. Use of this HEK293T cell-line enabled CREB-dependent gene expression activity to be determined by normalizing the luminescent activity of the firefly luciferase reporter against the known luminescent activity of the renilla luciferase reporter, thus minimizing any experimental variability caused by differences in cell viability and/or transfection efficiency. Prior to photoactivation, **CCI-2** (5 μM) and **CCI-3** (10 μM) were shown to have no effect on CREB-mediated firefly luciferase expression levels in HEK293T cells (Figure S3). In contrast, treatment of HEK293T cells with CREB inhibitor **1** (IC_{50} = 614 nM) (Figure S4), or uncaged **CCI-2** (IC_{50} = 872 nM) or **CCI-3**

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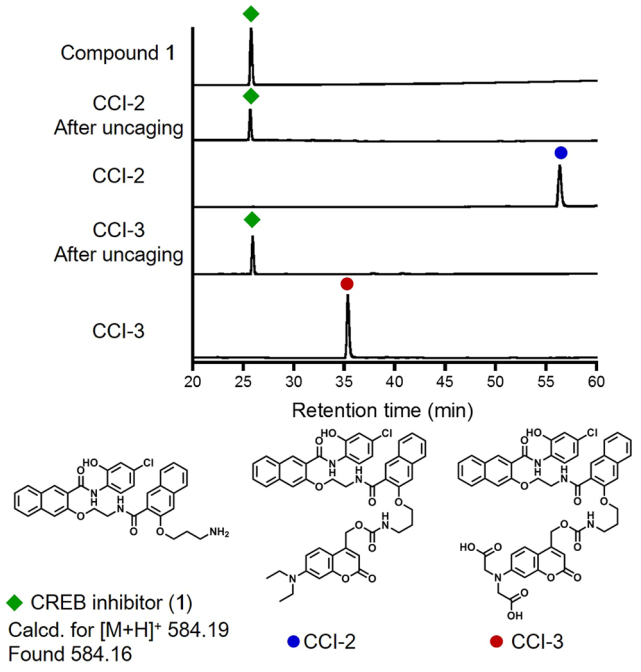

Figure 3. HPLC traces of the photoreaction products produced from uncaging of compounds **1**, **CCI-2**, and **CCI-3**. Reaction conditions: 50 μM compound **1** in 20 mM HEPES (10% DMSO), 50 μM **CCI-2** in 20 mM HEPES buffer/CH_3CN = 3/7 (10% DMSO), or 50 μM **CCI-3** in 20 mM HEPES buffer/CH_3CN = 7/3 (10% DMSO). Light intensity: 20 mW/cm^2 for 15 min, λ_{ex} = 405 nm.

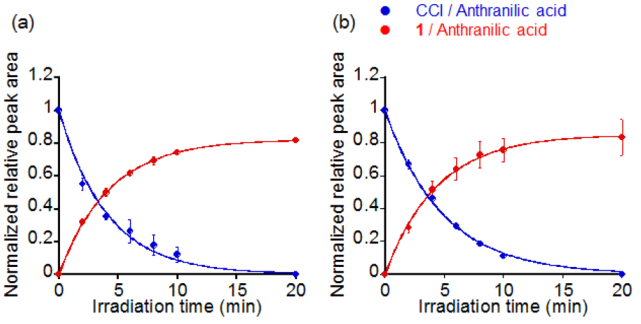

Figure 4. Photolysis rates of **CCI-2** and **CCI-3**. Reaction conditions: (a) 50 μM **CCI-2** in 20 mM HEPES buffer (pH = 7.2)/CH_3CN = 3/7 (10% DMSO) or (b) 50 μM **CCI-3** in 20 mM HEPES buffer (pH = 7.2)/CH_3CN = 7/3 (10% DMSO). Light intensity: 10 mW/cm^2, λ = 405 nm. Error bars denote ± SD.

Table 1. Photochemical Properties of CCI-2 and CCI-3[a]

	CCI-2	CCI-3
ε (405 nm) [M^{-1} cm^{-1}]	1.0 × 10^4	1.0 × 10^4
k [s^{-1}]	0.24	0.20
k' [s^{-1}]	0.19	0.19

[a] k and k' are the rate constants for consumption of the CCIs and production of inhibitor **1**, respectively.

(IC_{50} = 702 nM) resulted in the gene expression levels being decreased in a concentration-dependent manner (Figure S3). We confirmed that CREB inhibitor **1** does not directly affect the activity of firefly luciferase (Figure S5).

Then, light-induced inhibition of CREB-dependent gene expression was examined in living cells by using a dual luciferase reporter system. Calibrated luminescent data shown in Figure 5 reveal that the background inhibitory activities of **CCI-2** and **CCI-3** at a concentration of 5 μM in HEK293T

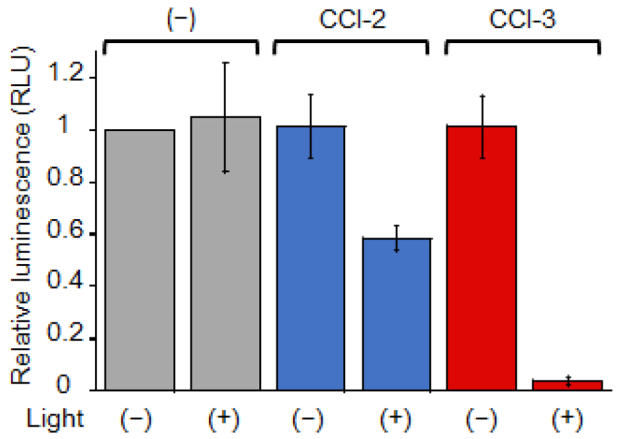

Figure 5. Inhibition of cellular CREB activity by uncaged **CCI-2** and **CCI-3**. Reaction conditions: 5 μM **CCI-2** or **CCI-3** in cells were illuminated by light. The dual-luciferase reporter assay was performed at ~6 h after light illumination with either blank or 5 μM **CCI-2** or **CCI-3** in DMEM (1% DMSO) (N = 3). Error bars denote ± SD.

cells were negligible. However, photoactivation of HEK293T cells treated with **CCI-2** and **CCI-3** at 405 nm resulted in a large decrease in CREB-dependent luciferase expression levels to 47% and 3% of their original values, respectively. The reason for the enhanced photoinhibitory activity of **CCI-3** is currently unclear; however, it may be due to its significantly better aqueous solubility resulting in a more efficient photolysis cleavage reaction. These results clearly indicated that illumination of the inactive **CCI-2** and **CCI-3** precursors had resulted in photolytic cleavage to afford an active inhibitor **1** that then transiently inhibits CREB-dependent gene expression in cells. However, since CREB and CBP are both expressed within cells, we considered the possibility that the low inhibitory activity of **CCI-2** and **CCI-3** might be caused by their low cellular permeabilities, with photolytic cleavage to afford the cell permeable inhibitor **1** actually occurring outside the cell. Consequently, we performed a fluorescence cell imaging experiment on HEK293T cells that had been incubated with **CCI-2** and **CCI-3** for 30 min. The resultant images revealed the presence of fluorescent signals derived from the DEACM chromophore in the cytosolic fraction of the cells (Figure S6), thus demonstrating that both **CCI-2** and **CCI-3** can effectively penetrate the cell membrane and that their photolytic cleavage occurs intracellularly. Although **CCI-3** has two anionic carboxyl groups that might cause electrostatic repulsion with cell membranes, the compound still maintains its cell permeability. This result might be attributed to the increased hydrophobicity of **CCI-3** upon conjugation with CREB inhibitor **1**.

In conclusion, we have developed new caged derivatives of a CREB inhibitor (CCIs) that can be used as direct optochemical tools for light-triggered knockdown of CREB transcriptional activity in living cells. Use of the more water-soluble **CCI-3** potentially allows for spatiotemporal knockdown of endogenous CREB-mediated transcription activity in different cell types. However, light-triggered activation of the gene expression is highly desired, which could be achieved with a caged CREB activator. Caged CREB modulators will provide a useful tool for investigating the role of CREB-controlled

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pathways in the differentiation and growth of cancer cells and in long-term memory.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b03568.

Full experimental procedures, characterization data, and NMR spectra (PDF)

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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