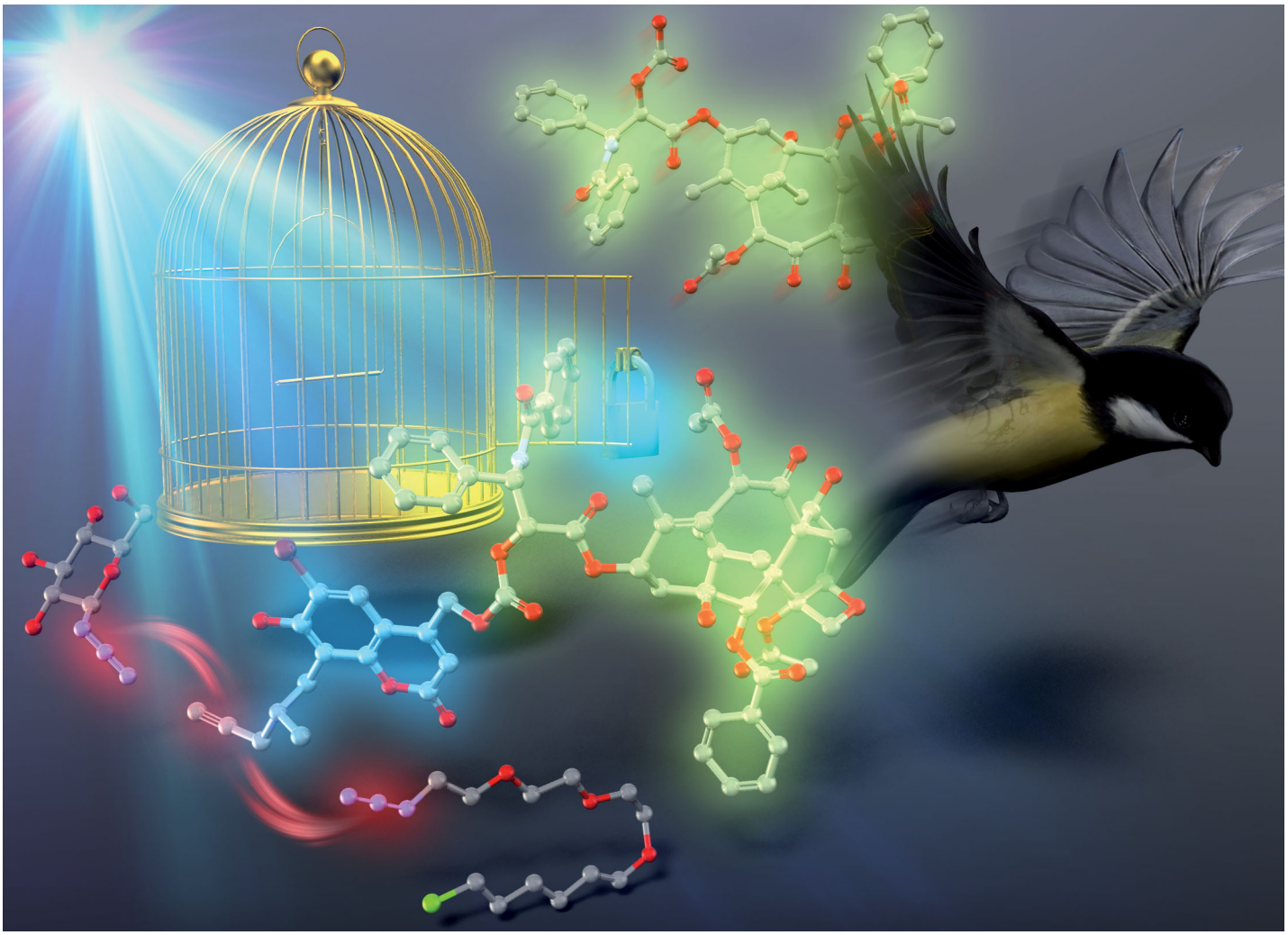

Showcasing research from Professor Furuta's laboratory, Department of Biomolecular Science, Toho University, Chiba, Japan.

A clickable caging group as a new platform for modular caged compounds with improved photochemical properties

Caged compounds allow photo-manipulation of cellular physiology with high spatiotemporal resolution. A click caging group capable of introducing diverse chemical properties was designed and applied to the synthesis of caged paclitaxels with improved photolysis efficiency.

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A clickable caging group as a new platform for modular caged compounds with improved photochemical properties†

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A 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) caged compound having a click-modifiable chemical handle was designed and synthesized. This molecule was applied to the synthesis of modular caged paclitaxels (PTXs) in which additional functional units could be easily installed. This system was used to prepare water-soluble caged PTXs with improved photolysis efficiencies.

Caged compounds are synthetic molecules whose biological activity is temporally masked by covalently attached photoremovable protecting groups.[1–3] Photoremovable protecting groups that can be used to cage various functional groups have already been reported, for example 2-nitrobenzyls,[4,5] 2-(2-nitrophenyl)ethyls,[6] and phenacyls.[7] However, coumarin-4-ylmethyl-type caging groups are widely regarded as having the most practically useful chemical and physical properties.[8,9]

Our group has previously reported the 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) group as a photocaging group with improved photolysis efficiency under one- and two-photon excitation conditions.[10–12] Bhc and its derivatives have been applied to the synthesis of caged compounds with various functional groups, including amines, carboxylates, alcohols, and phosphates. Since the photophysical and chemical properties of caged compounds depend primarily on the nature of the caging groups used to prepare them, photoremovable protecting groups with diverse physical and chemical properties are required. For example, some caged compounds need to be more hydrophobic to achieve better membrane permeability without sacrificing their favourable photochemical properties, while others need to be more water soluble for use in a biological context. Thus, the limited structural diversity of currently available photocaging groups with high photolysis efficiency and difficulties in their synthetic modification are obstacles to expanding the repertoire and usefulness of caged compounds.

Chemical modification of coumarin-type caging groups is a promising approach to overcome these problems. Studies on this approach have led to the development of 7-bis(carboxymethyl)amino coumarin,[13] 8-bis(carboxymethyl) aminomethyl Bhc,[14] and 8-azacoumarins,[15] all of which exhibit improved water solubility.

Our group has previously addressed the structural diversity problem by introducing a modular Bhc group, which can be modified with various functional units without the need for laborious synthetic procedures.[16] As a precursor of modular caged nucleotides, NHS-Bhc-hydrazone comprises three components: a photolabile Bhc core, NHS for the introduction of additional functional units, and a hydrazone as a latent diazomethyl moiety for chemoselective caging of phosphates. This precursor was applied to the facile preparation of caged siRNAs and dsDNAs in which additional functional modules such as an affinity tag or a ligand for cellular targeting can be easily installed.[16,17] Nevertheless, the NHS-Bhc-hydrazone system has drawbacks owing to its photophysical properties, especially its relatively short absorption maximum (330 nm) compared to those of other coumarin-type photocages.

To expand the repertoire of Bhc-type caged compounds, we report here a new platform of modular caged compounds that can be used for the preparation of caged carboxylates, amines and alcohols. The new precursor molecule (paBhc, Fig. 1b) has a terminal alkyne moiety as a chemical handle for further modification. Thus, after being introduced into a biologically active molecule, the paBhc group makes the caged compound "clickable." To demonstrate this concept, we have applied the clickable caging group to the preparation of new caged paclitaxels with diverse physical and chemical properties.

We synthesized the new precursor molecule paBhcCH_2OH (**1**) as a clickable platform for modular caged compounds (Scheme 1). An alkyne substituent was introduced at the C8

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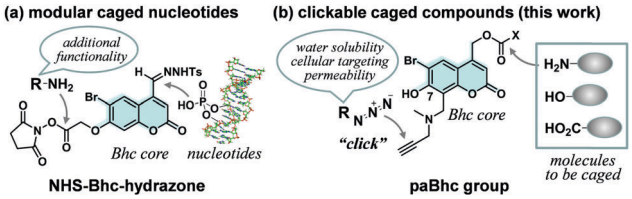


Fig. 1 Clickable caging groups for modular caged compounds. Chemical structure of (a) the NHS-Bhc-hydrazone as a precursor for modular caged nucleotides and (b) paBhc group for clickable caged compounds.

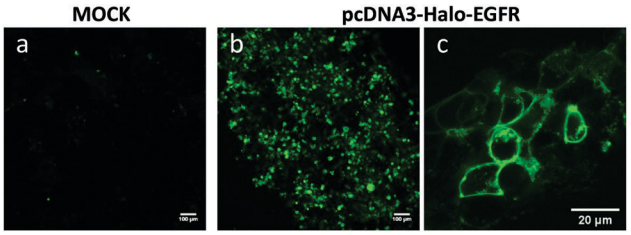
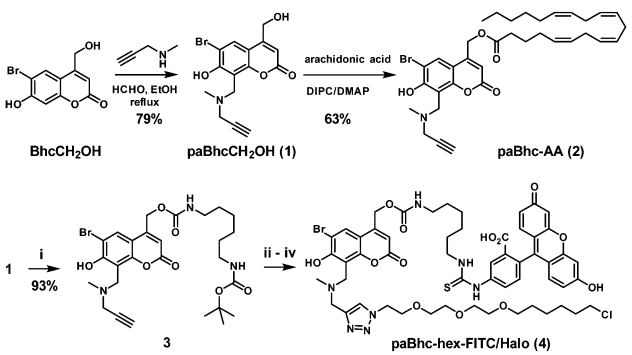


Fig. 2 Fluorescence images of HEK293T cells incubated with a paBhc compound **4**. Cells treated with MOCK (a) or cells transiently expressed Halo-EGFR (b and c) were incubated with **4** (50 μM for (a) and (b), 2 μM for (c)) at 37 °C for 30 min. The images were taken after repeated washing with PBS. The scale bar is 100 μm for (a) and (b), and 20 μm for (c).

position of the coumarin ring so that the phenolic hydroxy moiety at the C7 position was free from modification. This was achieved employing a regioselective Mannich alkylation using *N*-methylpropargyl amine and formaldehyde.[14,18,19] Introduction of the tertiary amine moiety on the C8 position of the 7-hydroxycoumarin ring lowers the pK_a of the phenolic hydroxy group.[19] Practically, the pK_a of the C7 hydroxy in **1** is 3.8, which is more than two units lower than that of the parent Bhc group (pK_a 6.2).[10] Consequently, almost all the paBhc is ionized at neutral pH, and its absorption profile is independent of pH above 5 (Fig. S1, ESI†). In addition, steric bulkiness of the C8 substituent would hinder the C7 hydroxy from modification. As expected, a caged arachidonic acid, paBhc-AA (**2**), and a caged diamine **3** were prepared in a single step without using any protecting groups for C7 OH. The results also indicate the paBhc group can be used as a precursor of caged carboxylates and amines.

The paBhc-caged diamine **3** was further modified by a fluorescent tag FITC to test whether an additional functionality is installed. Introduction of a functional module to **3** was performed by copper-catalysed Huisgen cyclization of the terminal alkyne with an azide having a HaloTag ligand for cellular targeting. Thus, a caged amine paBhcmoc-hex-FITC/Halo (**4**) was synthesized (Scheme 1). The compound was applied to HEK293T cells which had been transiently transfected with a HaloTag/EGFR (pcDNA3-Halo-EGFR). Since EGFR is a membrane-bound single chain peptide whose N-terminus is located outside the cells, the HaloTag protein fused to the N-terminus of EGFR should be exposed to the extracellular surfaces. No significant fluorescence was observed from the non-transfected cells (Fig. 2a). When **4** was applied to the transfected cells, green fluorescence was detected only from the plasma membranes even after the extensive washing (Fig. 2b, c and Fig. S3, ESI†). The results indicated that an additional functionality, such as cell targeting ability, was installed to a paBhc-caged compound.



Scheme 1 Synthesis of paBhcCH_2OH and chemical structure of a paBhc-caged carboxylate and an amine. i. CDI then Boc$NH(CH_2)_6NH_2$, ii. TFA, iii. FITC, iv. $N_3(CH_2CH_2O)_3$–$(CH_2)_6$–Cl/$CuSO_4$/sodium ascorbate.

Next, we applied the paBhc group to the synthesis of caged paclitaxels with additional properties. Paclitaxel (PTX) is one of the most potent anticancer agents in clinical use. It binds to β-tubulin to stabilize polymerized microtubules and prevents cells from proliferation. Since the dynamic instability of microtubules is essential for cell mitosis, PTX is also used to a chemical tool to elucidate the mechanisms underlying cellular chemistry mediated by the dynamics of microtubules.

It was reported that the free 2′-hydroxyl group was required for the microtubule-stabilizing activity of PTX.[20] Kiso and Hayashi reported the first examples of photoactivatable and water-soluble prodrugs of paclitaxel having 7-dialkylamino-coumarin caging groups on the C3′ position of the side chain.[21,22] Furthermore, del Campo reported that caging at both the C7 and C2′ hydroxyls with the 4,5-dimethoxy-2-nitroveratoryloxy-carbonyl (NVOC) group was necessary to mask the biological activity of paclitaxel.[23]

We synthesized three new Bhc-caged paclitaxels: 2′-Bhcmoc-PTX (**5**), 2′-Bmcmoc-PTX (**6**) and 2′-paBhcmoc-PTX (**7**). All the products were obtained as a single regioisomers. The position of modification was confirmed by ^{1}H NMR analysis. The chemical shifts of the H2′ and H3′ protons of **5** are shifted to lower field by 0.91 ppm for H2′ and by 0.19 ppm for H3′ compared with those of the parent PTX (4.59 ppm for H2′ and 5.41 ppm for H3′), inferring that the Bhcmoc group was introduced at the 2′-position of PTX. Since similar lower-field shifts were observed for **6** and **7** (ESI†), we concluded that all the caged products in this study were modified at the C2′ position (Fig. 3).

Introduction of functional modules to 2′-paBhcmoc-PTX (**7**) was performed by "click" chemistry with one of two azides, one having a sugar for water solubility and the other a halo tag ligand. Thus, two new caged PTX with additional functional units, 2′-Glc-paBhcmoc-PTX (**8**) and 2′-halo-paBhcmoc-PTX (**9**) were synthesized. As proof of concept experiments, the water solubility, photochemistry, and biochemical activity of **8** were investigated.

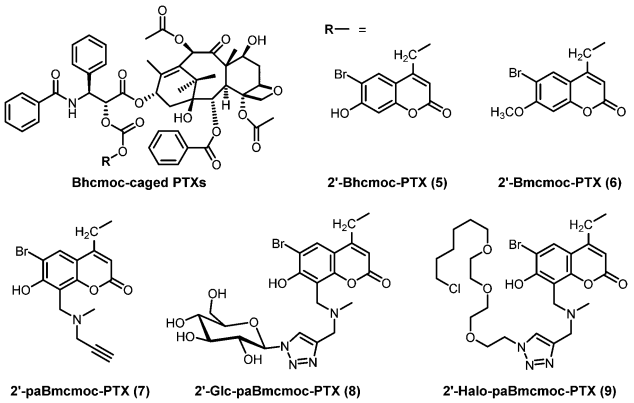


Fig. 3 Bhc-caged paclitaxels synthesized in this study.

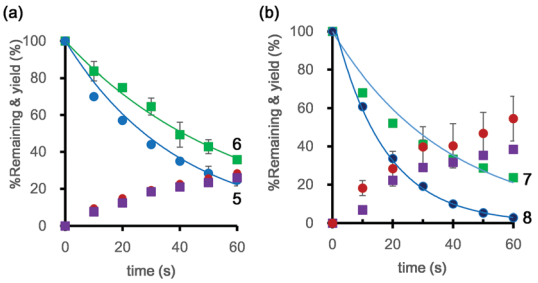


Fig. 4 Time course for the photolysis of Bhc-caged PTXs. Samples (10 μM) were irradiated at 350 nm (10 mJ cm^{-2}) under simulated physiological conditions. (a) **5** (blue, solid circle), PTX released from **5** (red, solid circle), **6** (green, solid square), PTX from **6** (purple, solid square), (b) **7** (green, solid square), PTX from **7** (purple, solid square), **8** (blue, solid circle), PTX from **8** (red, solid circle).

Table 1 Physical and chemical properties of the caged PTXs in this study

Compounds	λ_{max} [a]	ε_{max} [b]	Φ_{350} [c]	$\varepsilon\Phi_{350}$ [d]	Solubility [e]
5	340	10 500	4.0 ± 0.4	400	48
6	331	8100	3.5 ± 0.2	220	7.2
7	359	9300	5.9 ± 0.1	670	
8	373	12 100	14 ± 0.2	1280	450
2	366	10 300	8.3	750	
Bhc-AA [f]	341	10 800	3.8	390	

[a] Absorption maximum (nm). [b] Molar absorptivity (M^{-1} cm^{-1}). [c] Quantum yield of disappearance of starting materials upon 350 nm irradiation (% ±SE). [d] Product of molar absorptivity and quantum yield of disappearance. [e] Concentration of the saturated solution in KMOPS buffer (pH 7.2) (μM). [f] Ref. 24.

The photophysical and chemical properties of the four caged PTXs **5–8** are summarized in Table 1. The absorption maximum of 2′-Bhcmoc-PTX (**5**) is 340 nm, which is 30 nm shorter than those of other previously reported Bhc-caged compounds. This blue shift indicates that most of the phenolic 7-OH groups of **5** are protonated owing to the hydrophobic character of PTX. Similar blue shifts have been previously observed for Bhc-arachidonic acid (Bhc-AA)[24] (Table 1). In contrast, the absorption maximum of 2′-Glc-paBhcmoc-PTX (**8**) is observed at 373 nm, which is comparable to those of other Bhc-caged compounds, such as Bhc-cAMP (λ_{max} = 375 nm).[25]

All the caged PTXs were photolyzed upon 350 nm irradiation under a simulated physiological condition (10 mM K-MOPS buffer solution at pH 7.2) to produce the parent paclitaxel. The production of PTX is observed with the consumption of the starting material, which is approximated as single exponential decay (Fig. 4 and Fig. S2, ESI†). The yield of PTX from **8** after 60 s irradiation is approximately 60%, which is lower than expected. Some of this discrepancy may be due to the fact that PTX is photosensitive to UV irradiation and approximately 20% of the PTX decomposes upon 60 s irradiation (data not shown). This disadvantage can be avoided by using a light source having a wavelength of 405 nm or longer which is not absorbed by PTX.

The quantum yield of the photolytic disappearance (Φ_{dis}) of **8** is 14%, and therefore the photolysis efficiency upon 350 nm irradiation ($\varepsilon\Phi_{350}$) is 1280, which is approximately 3-times better than that of **5** ($\varepsilon\Phi_{350}$ = 400). A similar improvement in photolysis quantum yields were observed for paBhc caged compounds **7** and **2** compared with their Bhc-caged counterparts **5** and Bhc-AA, respectively. The observed improvements in the photolysis efficiencies of the 2′-Glc-paBhc and paBhc groups could be accounted for by the increased water solubilities of their corresponding caged compounds. Ion-pair intermediates have been proposed for the photolytic cleavage of (coumarin-4-yl)methyl type cages.[26] Thus, the hydrophobic character of the attached PTX or AA would provide the Bhc group with a less-polar environment in which the formation of the ion pair intermediates is less favourable. Therefore, the increased water solubility of the Glc-paBhc group promotes the photolytic release of PTX. The aqueous solubilities of the caged PTXs in a simulated physiological solution (pH 7.2) are also summarized in Table 1. The saturated concentration of **8** is approximately ten-times higher than that of **5**.

Finally, we performed an *in vitro* tubulin polymerization assay[27] to test whether the modification of the 2′ hydroxyl group with modular Bhc photocages sufficiently suppresses the stabilizing effect of PTX and whether exposure to UV light restores this activity. The polymerization of tubulin was monitored by measuring turbidity at 340 nm. Under our assay conditions, the turbidities of the tubulin solutions reach a plateau at 6 μM PTX (Fig. S4, ESI†). None of the caged PTXs (8 μM) synthesized in this study cause absorption changes, indicating that they have no pre-activation effect on tubulin polymerization (Fig. 5a). However, for all the caged PTXs, the polymerized-tubulin-stabilizing effect is restored upon exposure to 350 nm light (10 mJ cm^{-2}, 60 s). The degree of restored activity is approximately proportional to the expected concentration of PTX photo-chemically released from each caged PTX. For example, the maximum activity observed for **8** is identical to that for the same concentration of PTX (Fig. 5b).

In summary, we have developed a novel modular brominated-coumarin-type caging group with a propargylamine moiety at the C8 position. Introduction of this group lowers the pK_a of the C7 phenolic hydroxy group by more than two units, allowing almost complete ionization of the paBhc groups at physiological pH. The absorption maximum and molar absorptivity of the paBhc group is not affected by solution pH when it is 5 or higher.

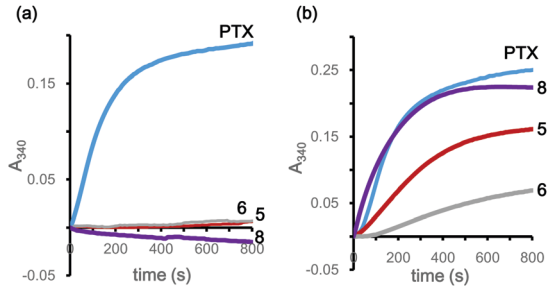


Fig. 5 *In vitro* turbidity assay for tubulin polymerization. Tubulin polymerization was performed using porcine brain tubulin in the presence of 8 μM of the indicated samples (a) without irradiation or (b) with 60 s irradiation using 350 nm light. Absorption at 340 nm was recorded every 10 s for 15 min. Data are presented as the mean of three independent experiments.

Additional functional units were successfully installed on the terminal alkyne handle using copper-catalysed Huisgen cyclization without sacrificing the favourable photophysical and chemical properties of the parent Bhc group. Thus, caging groups that can be easily modified using click chemistry represent a useful platform to expand the repertoire of caged compounds, allowing diverse chemical properties to be installed.

This method was applied to the preparation of novel water-soluble caged PTXs with improved photochemical properties. Furthermore, since the paBhc group can be used for the protection of other functional groups such as carboxylic acid and amine, previously reported Bhc caged compounds[10–12,24,25,28] can now be modified with a wide range of groups using the strategy presented here to make their caging group "clickable".[29]

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Conflicts of interest

There are no conflicts of interests to declare.

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