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Development of novel water-soluble photocleavable protective group and its application for design of photoresponsive paclitaxel prodrugs

Mayo Noguchi,[a] Mariusz Skwarczynski,[a] Halan Prakash,[b] Shun Hirota,[b,c] Tooru Kimura,[a] Yoshio Hayashi[a,d] and Yoshiaki Kiso[a,*]

[a]*Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science and 21st Century COE Program, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan*
[b]*Graduate School of Materials Science, Nara Institute of Science and Technology, Nara 630-0192, Japan*
[c]*PRESTO, JST, Kawaguchi, Saitama 332-0012, Japan*
[d]*School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo 192-0392, Japan*

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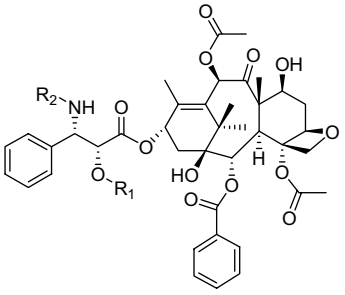
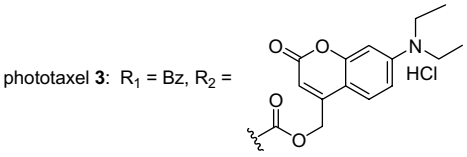
Abstract—A novel coumarin-based highly water-soluble photocleavable protective group was designed and synthesized, and then this photosensitive protecting group was used to design paclitaxel prodrugs. These novel paclitaxel conjugates demonstrated excellent water solubility, over 100 mg mL^{-1}. Thus, the use of a detergent in the formulation can be omitted completely, even at high doses. Phototaxel **11** released the parent drug, paclitaxel, quickly and efficiently by minimal tissue-damaging 365 nm UV light irradiation at low power, while laser activation at 355 nm led to extensive decomposition of the prodrug. The carbamate-type prodrug, phototaxel **11**, was stable in the dark prior to activation, whereas carbonate-type phototaxel **9** demonstrated poor stability under aqueous conditions. For such prodrugs, tumor-tissue targeting after administration could be achieved by selective light delivery, similar to that used in photodynamic therapy. In addition, newly designed coumarin derivative **8** can be applied in organic chemistry as a photosensitive protective group and for the design of caged compounds.

1. Introduction

The introduction of paclitaxel (**1**,[1] Fig. 1) in clinical cancer chemotherapy has markedly improved patient survival time.[2–4] Similar to most anticancer agents, dose-limiting toxicity corresponding to paclitaxel's side effects and poor water solubility are significant drawbacks. Its sparing water solubility requires coinjection of a detergent, Cremophor EL, which causes hypersensitivity reactions, and patients receiving this drug require premedication.[5] To overcome these problems, the prodrug strategy is promising.[6,7] Tumor-targeting prodrugs are designed to achieve a high local concentration of antitumor drugs to decrease undesired side effects caused by non-tissue selectivity of the drugs.[8] Water-sol-



1 (paclitaxel): R_1 = H, R_2 = Bz **2** (isotaxel): R_1 = Bz, R_2 = H



phototaxel **3**: R_1 = Bz, R_2 =

Figure 1. Structures of paclitaxel (**1**), isotaxel (**2**), and phototaxel **3**.

Keywords: Water-soluble photocleavable protective group; Prodrug of paclitaxel; Taxol; O–N intramolecular acyl migration.
* Corresponding author. Tel.: +81 75 595 4635; fax: +81 75 591 9900; e-mail: kiso@mb.kyoto-phu.ac.jp

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uble prodrugs are considered to improve water solubility of the parent drug and to avoid the use of toxic detergents in the drug formulation.[9] Additionally, according to the general nature of prodrugs, the pharmacokinetic profile of paclitaxel was also improved while reducing the side effects. However, the improved effectiveness of prodrugs over paclitaxel, often demonstrated in preclinical study, has not been confirmed in clinical trials.[6] This issue suggests that the development of novel prodrug strategies for paclitaxel is still needed.

Photodynamic therapy (PDT) has been used for cancer treatment for a long time. This technique is based on the administration of a sensitizer devoid of mutagenic properties, followed by exposure of the pathological area to a specific wavelength of light.[10] Two types of photoreaction mechanisms are invoked to explain photosensitizer action: free radical generation by electron or proton transfer, or singlet oxygen generation by energy transfer, from light-activated photosensitizers. The FDA has approved photosensitizing agents, porphyrin derivatives, such as porfimer sodium or verteporfin, for use in PDT.[11]

Photolabile 'caged' compounds are biologically inert precursors of active molecules. Their biological activity is masked by a covalently attached photocleavable group which can be selectively removed upon light irradiation to release parent bioactive molecules. Caged compounds have found wide application in life sciences to monitor biological processes.[12–19] For example, we applied this caged strategy for the controlled generation of intact amyloid β peptide 1–42 (Aβ 1–42) to study Alzheimer's disease. The photo-triggered Aβ 1–42 isopeptide ('click peptide') possessing a photocleavable 6-nitroveratryloxycarbonyl (Nvoc) group released parent Aβ 1–42 through photoirradiation by 355 nm light and subsequent spontaneous O–N intramolecular acyl migration reaction.[20–22]

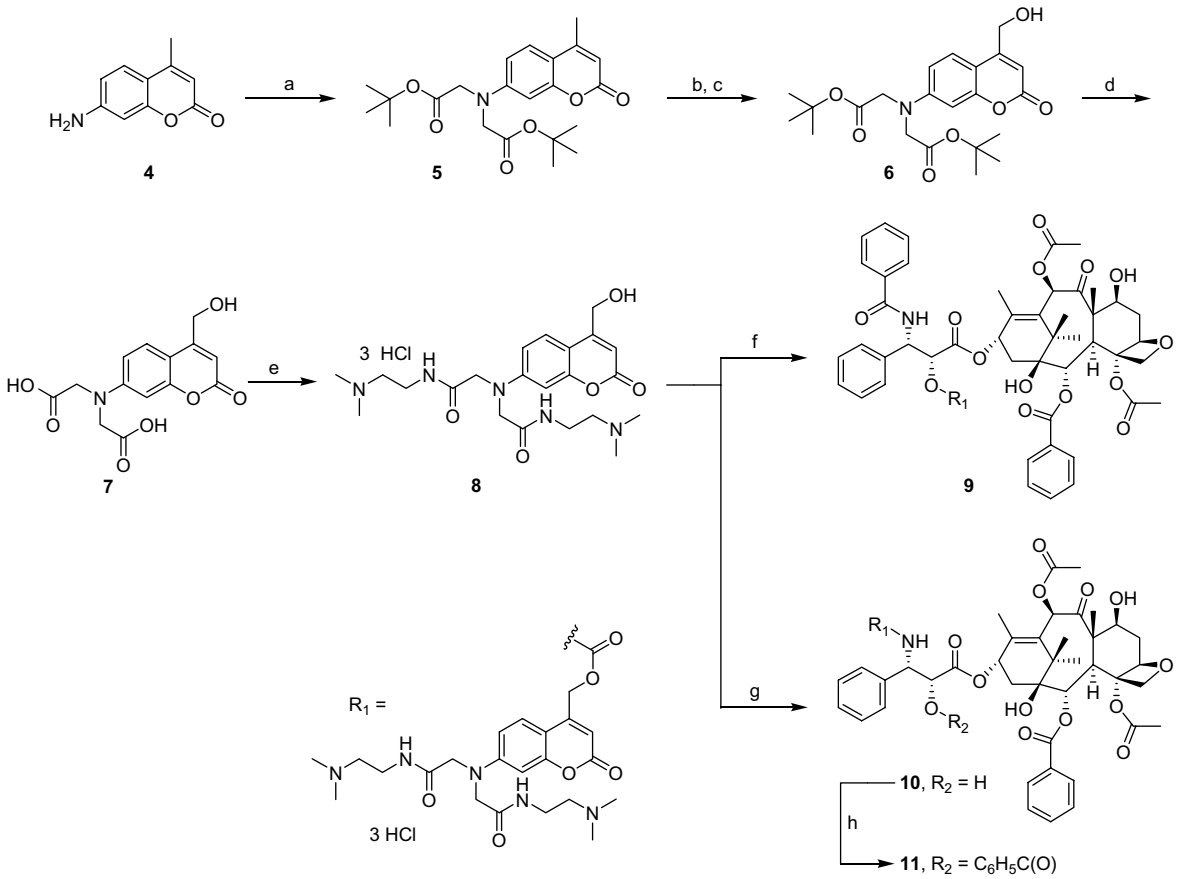
Previously, we developed a unique synthetic water-soluble paclitaxel prodrug, isotaxel (**2**), which produces paclitaxel through O–N intramolecular acyl migration.[23–25] This prodrug, a 2′-*O*-benzoyl isoform of paclitaxel, showed promising results in higher water solubility and appropriate kinetics for parent drug release, which proceeded without additional functional auxiliaries released during conversion to the parent drug. However, the lack of tumor-targeting properties of isotaxel can still be considered a serious shortcoming for practical application of such a prodrug. Thus, taking into consideration three strategies: prodrug, photodynamic therapy, and caged compound chemistry, we recently reported the first photo-triggered paclitaxel prodrug, phototaxel **3**,[26] which has a coumarin derivative conjugated via a 3′-carbamate to an amino moiety of isotaxel (**2**) (Fig. 1). Phototaxel **3**: (a) was selectively activated by visible light irradiation (430 nm) leading to cleavage of coumarin, then (b) released paclitaxel via subsequent spontaneous O–N intramolecular acyl migration reaction.[22] Tumor-tissue targeting for such a prodrug after its administration could be achieved by selective light delivery, for example, by utilizing an endoscope or optic fibers. 7-*N*,*N*-Diethylamino-4-hydroxymethyl coumarin (DECM) was chosen as the photolabile group since DECM-caged compounds have been reported to be water-soluble, non-toxic, thermally stable, and rapidly photolyzed by visible light.[27–29] Nonetheless, phototaxel **3** was completely insoluble in water,[26] which would cause serious problems in the practical application of such an agent.

Thus, we decided to develop a new photocleavable protective group for the design of water-soluble photoresponsive prodrugs. This group can be also applied for caged compound design and in organic chemistry, especially in green chemistry and native chemical ligation where water-soluble protective groups are highly desired. Herein, we report a new coumarin-based highly water-soluble photocleavable protecting group (**8**, Scheme 1) that was applied for the design of two new phototaxels (**9** and **11**, Scheme 1), one of which demonstrated high water solubility, high stability in the dark and quick release of the parent drug upon UV light irradiation.

2. Design and synthesis

Initially, we synthesized the reported DECM derivative 7-*N*,*N*-[bis(*tert*-butoxycarbonylmethyl)-amino]-4-hydroxymethyl coumarin (**6**), which has been used for the preparation of water-soluble caged phosphates and which allowed the photorelease of caged compound upon one- and two-photon excitation.[30,31] As depicted in Scheme 1, compound **6** was prepared from commercially available 7-amino-4-methyl coumarin (**4**), according to the slightly modified procedure reported by Hagen and coworkers.[31] Thus, compound **4** was treated with an excess of bromoacetic acid *tert*-butyl ester in the presence of diisopropylethylamine to yield **5** with monoalkylated coumarin as a major by-product. Oxidation of **5** with SeO_2 and subsequent reduction with $NaBH_4$, according to the previously described method, gave alcohol **6**.[26,29,31] Then, we tried to synthesize phototaxels with derivative **7** as a photosensitive group (data not shown). Alcohol **6** was coupled at 4-position via a carbonate or carbamate bond to the 2′-hydroxy group of paclitaxel or 3′-amino group of 3′-*N*-debenzoylpaclitaxel, respectively. The attempt to obtain free carboxyl groups of coumarin moiety in conjugates was unsuccessful. Decomposition of conjugates was observed even under very mild deprotection conditions.

Considering the problems with the final deprotection of conjugates, we decided to apply prodrug design in which final deprotection can be omitted. A few years ago, we reported a prodrug of HIV protease inhibitors that possessed simple water-soluble tertiary amine moieties.[32,33] Thus, by combining the structure of coumarin derivative **7** and *N*-[2-(dimethylamino)ethyl]succinamic acid,[33] we designed a new photosensitive group **8**. Compound **8** was prepared from alcohol **6** via the cleavage of *t*-Bu protective groups with TFA (trifluoroacetic acid) to afford **7**, and subsequent coupling of crude **7** with *N*,*N*-dimethylethylenediamine by mixed-anhydride method using isobutyl chloroformate. Coumarin derivative with only one carboxylic group substituted was a major by-product of coupling. Photosensitive protective group **8**



Scheme 1. Synthesis of phototaxels **9** and **11**. Reagents and conditions: (a) $BrCH_2COO$*t*-Bu, *i*-Pr_2EtN, NaI, CH_3CN, reflux, 4 days, 68%; (b) SeO_2, *p*-xylene, reflux, 24 h; (c) $NaBH_4$, EtOH, rt, 30 min, 61% over two steps; (d) TFA/$CHCl_3$/H_2O 74:25:1, rt, 20 min; (e) isobutyl chloroformate, *N*-methylmorpholine, DMF, −30 °C, 2 h, then $(CH_3)_2NCH_2CH_2NH_2$, *N*-methylmorpholine, −15 °C, 1 h, then rt overnight, 65% over two steps; (f) **8**, 4-nitrophenyl chloroformate, DMAP, CH_3CN/DMSO, rt, 5 h, then **1**, DMAP, 22 h, then HCl, 63%; (g) **8**, 4-nitrophenyl chloroformate, DMAP, DMF, rt, 3 h, then 3′-*N*-debenzoylpaclitaxel, DMAP, 4 h, then HCl, 59%; (h) benzoic acid, EDC·HCl, DMAP, CH_3CN, rt, 2 h, then HCl, 82%.

was activated by coupling to 4-nitrophenyl chloroformate in the presence of DMAP (4-(dimethylamino)pyridine),[34] and then allowed to react with paclitaxel (**1**) to yield **9**. The final product was purified by HPLC with a binary solvent system, a linear gradient of CH_3CN in 1% aqueous acetic acid, due to prodrug partial decomposition when 12 mM HCl aqueous solution was used. Then, the product was converted to HCl salt via lyophilization using only a slight excess of 1 N HCl. Prodrug **11** was synthesized in a similar manner. Compound **8** was activated by coupling to 4-nitrophenyl chloroformate in the presence of DMAP, then allowed to react with 3′-*N*-debenzoylpaclitaxel, which was synthesized by a previously described method.[23,35] Finally, benzoylation of the resultant carbamate **10** with benzoic acid by the EDC–DMAP method (EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), followed by HPLC purification with ion exchange by elution with 1% AcOH and then salt exchange with 1 N HCl gave phototaxel **11** as an HCl salt (Scheme 1).

3. Results and discussion

The water solubility of **9** and **11** was determined as a value higher than 100 mg mL^{-1}, which was at least 400,000-fold higher than that of paclitaxel (0.00025 mg mL^{-1}),[25] and at least 200-fold higher than that of isotaxel (0.45 mg mL^{-1}). The water solubility of prodrugs with coumarin derivative **8** drastically increased in comparison to phototaxel **3** and was even greater than that of water-soluble prodrug **2**. Thus, the use of a detergent for the formulation of **9** or **11** can be omitted even with a high dose of prodrugs.

Because we expected that carbonate prodrug **9** can be less stable than carbamate **11**, before studying the parent drug release, the stability of prodrugs was examined. Prodrug **9** showed poor stability in the dark, 20% of paclitaxel was released in phosphate-buffered saline (PBS, pH 7.4, 37 °C) after 8 h of incubation, and only 18% of the prodrug remained when **9** was stored at 4 °C for 13 days, while in the solid form, the prodrug was stable at −20 °C for at least 4 months. Thus, compound **9** can be water-soluble prodrug of **1** but is not an ideal candidate for selectively activated prodrug since it decomposed under physiological conditions. In contrast, prodrug **11** was stable under physiological conditions (PBS, pH 7.4, 37 °C, 8 h) as well as under storage conditions (PBS, pH 7.4, 4 °C, for at least 1 month) and in a solid state at −20 °C for at least 4 months. Thus, for further detailed study, only phototaxel **11** was chosen (Fig. 2).

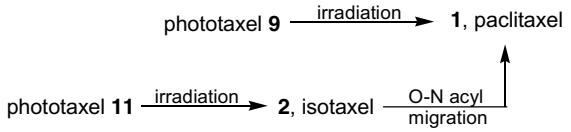


Figure 2. Releasing mechanism of paclitaxel (**1**) from its photoresponsive prodrugs **9** and **11**.

The high water solubility of new phototaxel **11** allowed us to study the kinetics of photoconversion under physiological conditions in phosphate-buffered saline (PBS, pH 7.4), in contrast to phototaxel **3**, for which an organic solvent was necessary for prodrug solubilization.[26] Prodrug **11** was irradiated with UV pulses (355 nm, 10 Hz, 5 mJ), at 23 °C. This irradiation wavelength was chosen as coumarin derivative **8** had intensive maxima at 353 nm and prodrug **11** at 361 nm. Thus, modification of the coumarin moiety in prodrug **3** (DECM has intensive maxima at 385 nm)[26] caused a shift in the absorption maximum to a shorter wavelength in compound **7** (376 nm)[31] and even a further shift in **8** (353 nm). The reason for such a shift may be related to the electron-withdrawing properties of *N*-substituted groups in coumarin. Irradiation at wavelengths above 300 nm is less likely to be absorbed by the biological environment.[36] Thus, photoirradiation with long-wavelength UV light (UV-A) has been mainly used for prodrug activation.[37–43] UV-A is already used clinically in the treatment of atopic dermatitis[44,45] and in photochemotherapy (PUVA therapy).[46] In comparison to visible or infrared light irradiation (above 600 nm) used in standard photodynamic therapy, UV-A light has significantly lower tissue penetration.[47–49] On the other hand, therapy with red light is associated with side effects such as burning pain and heating due to thermal irradiation. Buchczyk and coworkers demonstrated that the application of UV-A to photodynamic therapy can be highly promising.[50] In addition, many promising new prodrug strategies target only a subset of cells in tumors, and their therapeutic utility is associated with 'bystander effects, by which cell killing extends from targeted cells to untargeted tumor cells in the vicinity.[51–54] Thus, a prodrug does not need to be activated by light at the whole tumor site as a drug can usually diffuse within tumor tissue.

Upon UV light irradiation (pulse laser, 355 nm, 10 Hz, 5 mJ), prodrug **11** quickly released isotaxel (**2**) and subsequent spontaneous O–N intramolecular acyl migration ($t_{1/2}$ = 15.1 min)[23–25] formed paclitaxel (**1**) (Fig. 3). In comparison, prodrug **9** can release paclitaxel without any delay (Fig. 2). However, as **9** released the parent drug in a non-specific manner prior to photoactivation, it could not be employed as a selectively activated prodrug of paclitaxel. The delay of parent drug release from **11** after irradiation (related to migration of the benzoyl group) is supposed to be short enough to avoid intermediate (**2**) diffusion from the tumor tissue before the parent drug is released. Moreover, we recently demonstrated faster O–N intramolecular acyl (or alkoxycarbonyl[55,56]) migration in other highly potent taxoids.[22,23,35] Phototaxoids derived from these types

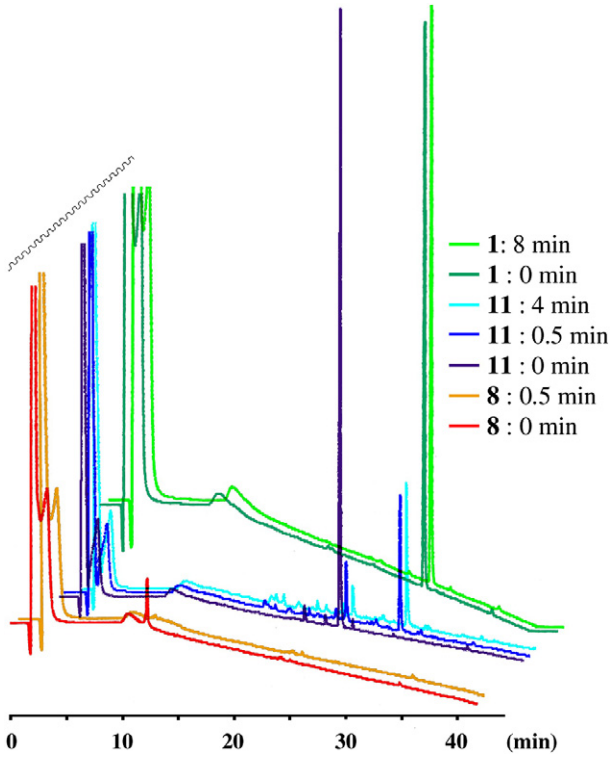


Figure 3. HPLC charts for compound **8**, prodrug **11**, and paclitaxel (**1**) subjected to UV light irradiation (355 nm, 10 Hz, 5 mJ) detected at 230 nm.

of taxoids would be more effective without risking diffusion from the irradiation site. In addition, prodrug **11** is not expected to be active prior to photoconversion, based on previous SAR studies on paclitaxel derivatives.[57,58]

The recovery yield of paclitaxel (**1**) after 4 min of irradiation was 24%, significantly smaller than that of previously reported phototaxel **3** (69%).[26] This low recovery was probably related to the observed partial decomposition of prodrug **11** upon UV light irradiation (pulse laser, 355 nm, 10 Hz, 5 mJ), because the coumarin moiety of the prodrug was highly unstable under irradiation conditions. After 0.5 min of irradiation of coumarin derivative **8**, its complete decomposition was observed (Fig. 3), while **1** was almost stable under these photoirradiation conditions, and only a small amount of paclitaxel decomposition (about 2%) was observed after 8 min of irradiation (Fig. 3). In contrast, when prodrug **11** was irradiated with a light of a UV lamp (365 nm, 6 W, UV Ray Lamp, Model UV-LS, Ishii Laboratory Work Co., Ltd), instead of an intense light of a pulse laser, the recovery yield was much higher (69%), retaining a fast rate of prodrug conversion to the parent drug. Thus, 50% of the reaction was completed after 4.0 ± 0.6 min under the above continuous irradiation conditions (Figs. 4 and 5). HPLC analysis indicated one noteworthy minor by-product formation under these irradiation conditions; however, we were not able to characterize it (signal at 14 min, Fig. 4). This by-product might be formed from coumarin **8**, as release of the photosensitive group **8** from **11** was very poor

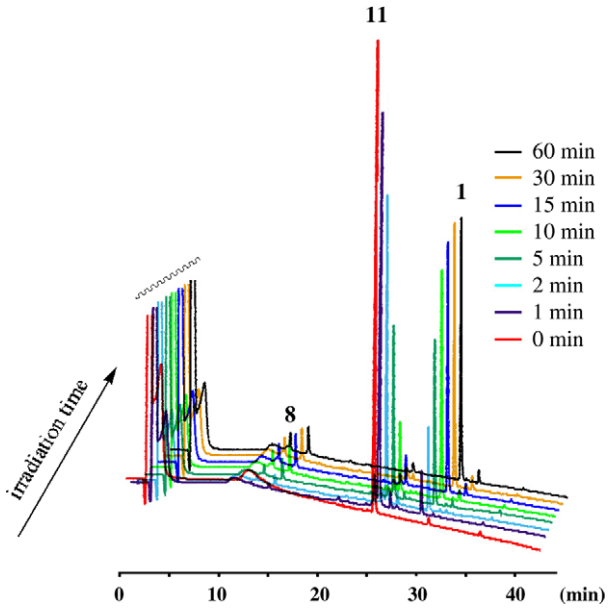


Figure 4. HPLC charts for prodrug **11** subjected to UV light irradiation (365 nm, 6 W) detected at 230 nm.

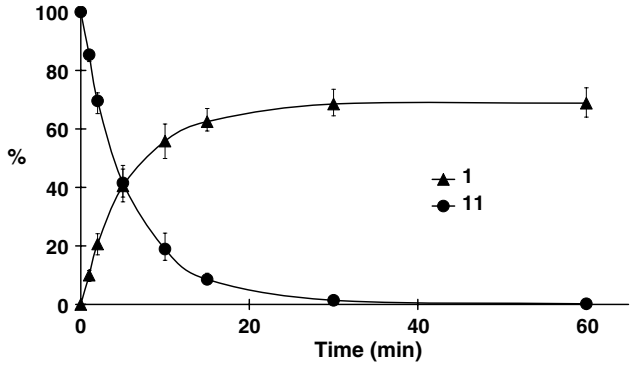


Figure 5. Time course of photolysis of prodrug **11** and release of **1** in 10 mM PBS at 365 nm. The percentage was determined by HPLC. Data are the average (±standard error) of three assays.

(with 11% yield after 1 h of irradiation). Paclitaxel was stable under these conditions (less than 2% decomposition after 1 h) and, in contrast to pulse laser application, coumarin derivative **8** alone was completely stable for at least 15 min under UV irradiation. Thus, the reason for the non-quantitative yield of **1** release could be decomposition of the prodrug and/or non-specific absorption of compounds on the surface of experimental tubes, as was suggested in the case of prodrug **3**.[26] The higher yield and purity of paclitaxel release via irradiation with a UV lamp can be related to weaker UV lamp energy compared to the pulse laser as well as its longer wavelength of irradiation, 365 nm compared to 355 nm by the laser. Thus, the 355-nm pulse laser is the third harmonic of the YAG laser and exhibits a very intense pulse light of a single wavelength. The wavelength cannot be adjusted to 365 nm. The UV lamp has a relatively broad wavelength and its center wavelength is 365 nm. The intensity of the UV lamp is much weaker compared with that of the laser. Thus, the choice of an appropriate UV source is highly important for the practical application of water-soluble protective group **8**. Nonetheless, even longer wavelengths could be used for prodrug activation, because **11** absorbs light up to the visible light wavelength (see Supplementary data Figure 1S). In addition, coumarin derivative **8** may also be cleaved with IR light two-photon photoirradiation in the same manner as derivative **7**.[31]

4. Conclusion

We designed and synthesized a coumarin-based novel highly water-soluble protective group. This masking group can also be applied to organic chemistry to protect hydroxy or amine moiety, in caged compound chemistry,and finally in photoresponsive prodrug strategies. Herein, this photosensitive coumarin derivative was used in the design of paclitaxel prodrugs. The carbamate-type prodrug, phototaxel **11**, released the parent drug, paclitaxel, quickly with minimal tissue-damaging 365 nm UV-A light irradiation at low power and was stable prior to activation, whereas carbonate-type prodrug **9** demonstrated poor stability under aqueous conditions. Both prodrugs demonstrated excellent water solubility and thus can be easily formulated for biological study. We also demonstrated that the choice of appropriate irradiation conditions is the key issue in light-triggered prodrug strategies.

5. Experimental

5.1. Materials

Reagents and solvents were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Kanto Chemical Co. Inc. (Tokyo, Japan), and Aldrich Chemical Co. Inc. (Milwaukee, WI), and were used without further purification. Column chromatography was performed on Merck 107734 silica gel 60 (70–230 mesh). TLC was performed using Merck Silica gel 60 F_{254} precoated plates. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1}, detected at 230 nm. Preparative HPLC was carried out on a C18 reverse phase column (20 × 250 mm; YMC Pack ODS SH343-5) with a binary solvent system: a linear gradient of CH_3CN in 12 mm aqueous HCl or 1% aqueous acetic acid at a flow rate of 5.0 mL min^{-1}, detected at 230 nm. Solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade or better. ^1H NMR spectra were obtained using a 400 MHz Varian UNITY INOVA 400NB spectrometer with TMS as an internal standard at 25 °C. FAB-MS was performed on a JEOL JMS-SX102A spectrometer equipped with the JMA-DA7000 data system. EI-MS was performed on a JEOL JMS-GCmate spectrometer.

5.2. Chemistry

5.2.1. 7-[Bis(*tert*-butoxycarbonylmethyl)amino]-4-methylcoumarin (5). [31] 7-Amino-4-methylcoumarin (4.4 g, 25 mmol), NaI (3.8 g, 25 mmol), diisopropylethylamine (21.9 mL, 126 mmol), and bromoacetic acid *tert*-butyl ester (37.1 mL, 0.25 mol) in 120 mL dry CH_3CN were refluxed for 4 days under an argon atmosphere. The mixture was cooled to room temperature, filtered, and the solvent was removed under reduced pressure. The residue was dissolved in AcOEt, washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo. The resulting oil was purified by silica-gel column chromatography (AcOEt/hexane 1:3) to yield **5** (6.90 g, 17.1 mmol, 68%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.42 (d, ^3J(H,H) = 8.8 Hz, 1H, CH), 6.52 (dd, ^4J(H,H) = 2.6 Hz, ^3J(H,H) = 8.8 Hz, 1H, CH), 6.45 (d, ^4J(H,H) = 2.6 Hz, 1H, CH), 6.02 (d, ^4J(H,H) = 1.3 Hz, 1H, CH), 4.06 (s, 4H, CH_2), 2.35 (d, ^4J(H,H) = 1.3 Hz, 3H, CH_3), 1.48 (s, 18H, CH_3). EI MS: calcd for $C_{22}H_{29}NO_6$ [M^+]: 403.1995, found: 403.2001. Anal. Calcd for $C_{22}H_{29}NO_6$, C: 65.49; H: 7.24; N: 3.47, found: C: 65.45, H: 7.26, N: 3.55. Purity was 95% (HPLC analysis at 230 nm).

5.2.2. 7-[Bis(*tert*-butoxycarbonylmethyl)amino]-4-(hydroxymethyl)coumarin (6). [31] Selenium dioxide (0.43 g, 3.8 mmol) and **5** (1.03 g, 2.55 mmol) in 25 mL *p*-xylene were refluxed with vigorous stirring under an argon atmosphere. After 24 h, the mixture was filtered and concentrated under reduced pressure. The dark brown residual oil was dissolved in ethanol (30 mL), then sodium borohydride (48.3 mg, 1.28 mmol) was added, and the solution was stirred for 1 h at room temperature. Thereafter, the suspension was carefully hydrolyzed with 1 M HCl (2 mL), diluted with H_2O, and partially concentrated under reduced pressure to remove EtOH. The resulting mixture was extracted with AcOEt. The organic phase was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The resulting oil was purified by silica-gel column chromatography (AcOEt/hexane 1:3) to yield **6** (0.65 g, 1.55 mmol, 61%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.28 (d, ^3J(H,H) = 9.0 Hz, 1H, CH), 6.48 (dd, ^4J(H,H) = 2.7 Hz, ^3J(H,H) = 8.9 Hz, 1H, CH), 6.44 (d, ^4J(H,H) = 2.6 Hz, 1H, CH), 6.37 (t, ^4J(H,H) = 1.4 Hz, 1H, CH), 4.78 (d, ^4J(H,H) = 1.3 Hz, 2H, CH_2), 4.06 (s, 4H, CH_2), 1.49 (s, 18H, CH_3). EI MS: calcd for $C_{22}H_{29}NO_7$ [M^+]: 419.1944, found: 419.1939. Anal. Calcd for $C_{22}H_{29}NO_7$, C: 62.99, H: 6.97, N: 3.34, found: C: 62.87, H: 6.84, N: 3.37. Purity was 97% (HPLC analysis at 230 nm).

5.2.3. 7-[Bis-[2-[[2-(dimethylamino)ethyl]amino]-2-oxoethyl]amino]-4-(hydroxymethyl) coumarin hydrochloride (8). Deprotection of compound **6** was achieved in an identical manner as reported by Hagen et al.,[31] and the desired product **7** was used for the next step without any purification. Compound **7** (148 mg, 0.482 mmol) in 15 mL dehydrated DMF was cooled down to −30 °C under an argon atmosphere. *N*-Methylmorpholine (159 μL, 1.45 mmol) and isobutyl chlorocarbonate (187 μL, 1.45 mmol) were added, and the solution was stirred for 2 h. Then *N*,*N*-dimethylethylenediamine (184 μL, 1.69 mmol) and *N*-methylmorpholine (159 μL, 1.45 mmol) were added, the solution was slowly warmed up to −15 °C, stirred 1 h, slowly warmed up to room temperature, and stirred overnight. The reaction mixture was directly applied to preparative HPLC, which was eluted with 12 mm aqueous HCl over 10 min, and then a linear gradient of 0–25% CH_3CN in 12 mm aqueous HCl over 25 min at a flow rate of 5 mL/min. The desired fraction was collected and lyophilized to give **8** as an HCl salt (176 mg, 0.316 mmol, 65%). ^{1}H NMR (CD_3OD, 400 MHz): δ = 7.52 (d, ^3J(H,H) = 9.0 Hz, 1H, CH), 6.59 (dd, ^4J(H,H) = 2.5 Hz, ^3J(H,H) = 8.9 Hz, 1H, CH), 6.44 (d, ^4J(H,H) = 2.5 Hz, 1H, CH), 6.30 (s, 1H, CH), 4.77 (d, ^4J(H,H) = 1.3 Hz, 2H, CH_2), 4.36 (s, 4H, CH_2), 3.64 (t, ^3J(H,H) = 5.6 Hz, 4H, CH_2), 3.32 (t, ^3J(H,H) = 5.6 Hz, 4H, CH_2, partially overlapping with signal from CD_3OD), 2.93 (s, 12H, CH_3). EI MS: calcd for $C_{22}H_{33}N_5O_5$ [M^+]: 447.2481, found: 447.2476. Anal. Calcd for $C_{22}H_{36}Cl_3N_5O_5 2H_2O$, C: 44.56, H: 6.80, N: 11.81, found: C: 44.77, H: 6.62, N: 11.71. Purity was 99% (HPLC analysis at 230 nm).

5.2.4. 7-[Bis-[2-[[2-(dimethylamino)ethyl]amino]-2-oxoethyl]amino]-4-[(2′- *O*-paclitaxel)carbonyloxymethyl] coumarin hydrochloride (9). The whole procedure was carried out in the dark. Compound **8** (16 mg, 0.031 mmol), 4-nitrophenyl chloroformate (7.8 mg, 0.038 mmol), and 4-(dimethylamino)pyridine (7.5 mg, 0.062 mmol) in 4 mL of dry acetonitrile were stirred at room temperature for 3 h under an argon atmosphere. For complete solubilization, 1 mL of DMSO was added and the mixture was stirred for an additional 2 h. Then, another portion of 4-(dimethylamino)pyridine (1.9 mg, 0.015 mmol) and paclitaxel (6.6 mg, 0.0077 mmol) were added. The reaction mixture was stirred at room temperature for 22 h, quenched with 1 mL of MeOH, and applied to preparative HPLC, which was eluted with 10% CH_3CN in 1% aqueous AcOH over 5 min, and then a linear gradient of 30–50% CH_3CN in 1% aqueous AcOH over 20 min at a flow rate of 5 mL/min. The desired fraction was collected and lyophilized to give **9** as an AcOH salt (7.0 mg, 0.0049 mmol). Then, the product was lyophilized from a mixture of CH_3CN (2 mL) and water (1 mL) with 1 N HCl (17.2 μL, 0.0172 mmol) to afford **9** as an HCl salt (7.0 mg, 0.00487 mmol, 63%). ^{1}H NMR (CD_3OD, 400 MHz): δ = 8.11–8.09 (m, 2H, CH), 7.82–7.79 (m, 2H, CH), 7.68–7.64 (m, 1H, CH), 7.59–7.41 (m, 10H, CH), 7.29 (br t, ^3J(H,H) = 7.3 Hz, 1H, CH), 6.59 (dd, ^4J(H,H) = 2.6 Hz, ^3J(H,H) = 9.0 Hz, 1H, CH), 6.44 (d, ^4J(H,H) = 2.4 Hz, 1H, CH), 6.37 (s, 1H, CH), 6.21 (s, 1H, CH), 6.04 (br t, ^3J(H,H) = 8.5 Hz, 1H, CH), 5.86 (d, ^3J(H,H) = 6.4 Hz, 1H, CH), 5.62 (d, ^3J(H,H) = 7.3 Hz, 1H, CH), 5.58 (d, ^3J(H,H) = 6.4 Hz, 1H, CH), 5.44, 5.38 (2d, ^2J(H,H) = 15.3 Hz, 2H, CH_2), 4.97 (br d, ^3J(H,H) = 7.7 Hz, 1H, CH), 4.38, 4.33 (2d, ^2J(H,H) = 19.4 Hz, 4H, CH_2), 4.25 (dd, ^3J(H,H) = 6.7, 11.1 Hz, 1H, CH), 4.18 (s, 2H, CH_2), 3.77 (d, ^3J(H,H) = 7.1 Hz, 1H, CH), 3.63 (t, ^3J(H,H) = 5.3 Hz, 4H, CH_2), 3.33–3.27 (t, 4H, CH_2, overlapping with signal from CD_3OD), 2.92 (s, 12H, CH_3), 2.57–2.50 (m, 1H, CH_2), 2.38 (s, 3H, CH_3), 2.20–2.12 (m, 1H, CH_2), 2.17 (s, 3H, CH_3), 1.91–1.80 (m, 2H, CH_2), 1.77 (d,

^4J(H,H) = 1.1 Hz, 3H, CH_3), 1.65 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 1.11 (s, 3H, CH_3). HRMS (FAB^+): calcd For $C_{70}H_{83}N_6O_{20}$ [M^++H]: 1327.5662 found: 1327.5654. Anal. Calcd for $C_{70}H_{85}Cl_3N_6O_{20}\cdot 7H_2O$, C: 53.79, H: 6.38, N: 5.38, found: C: 53.72, H: 6.19, N: 5.11. Purity was 99% (HPLC analysis at 230 nm).

5.2.5. 7-[Bis-[2-[[2-(dimethylamino)ethyl]amino]-2-oxoethyl]amino]-4-[[3′-*N*-(3′-*N*-debenzoylpaclitaxel)]carbonyloxymethyl] coumarin Hydrochloride (10). The whole procedure was carried out in the dark. Compound **8** (15.2 mg, 0.0273 mmol), 4-nitrophenyl chloroformate (5.5 mg, 0.0273 mmol), and 4-(dimethylamino)pyridine (6.7 mg, 0.0546 mmol) in 2 mL of dry DMF were stirred at room temperature for 3 h under an argon atmosphere. Then, another portion of 4-(dimethylamino)-pyridine (3.4 mg, 0.027 mmol) and 3′-*N*-debenzoylpaclitaxel[23,35] (6.8 mg, 0.0091 mmol) were added. The reaction mixture was stirred at room temperature for 4 h, and applied directly to preparative HPLC, which was eluted with 10% CH_3CN in 1% aqueous AcOH over 5 min, and then a linear gradient of 25–45% CH_3CN in 1% aqueous AcOH over 20 min at a flow rate of 5 mL/min. The desired fraction was collected and lyophilized to give **10** as an AcOH salt (7.2 mg, 0.0053 mmol). Then, the product was lyophilized from a mixture of CH_3CN (2 mL) and water (1 mL) with 1 N HCl (17.6 μL, 0.0176 mmol) to afford **10** as an HCl salt (7.1 mg, 0.0053 mmol, 59%). ^{1}H NMR (CD_3OD, 400 MHz): δ = 8.08 (d, ^3J(H,H) = 6.8 Hz, 2H, CH), 8.01 (d, ^3J(H,H) = 9.7 Hz, 1H, CH), 7.68–7.64 (m, 1H, CH), 7.52–7.39 (m, 7H, CH), 7.31–7.25 (m, 2H, CH), 6.49 (dd, ^4J(H,H) = 2.2 Hz, ^3J(H,H) = 9.5 Hz, 1H, CH), 6.44 (s, 1H, CH), 6.37 (d, ^4J(H,H) = 2.0 Hz, 1H, CH), 6.24 (br t, ^3J(H,H) = 9.0 Hz, 1H, CH), 6.18 (s, 1H, CH), 5.61 (d, ^3J(H,H) = 7.3 Hz, 1H, CH), 5.32, 5.17 (2d, ^2J(H,H) = 16.4 Hz, 2H, CH_2), 5.28 (d, ^3J(H,H) = 3.7 Hz, 1H, CH), 4.98 (dd, ^3J(H,H) = 1.8, 7.9 Hz, 1H, CH), 4.64 (d, ^3J(H,H) = 4.2 Hz, 1H, CH), 4.34 (br s, 5H, CH and CH_2), 4.16, 4.14 (2d, ^2J(H,H) = 14.1 Hz, 2H, CH_2), 3.84 (d, ^3J(H,H) = 7.3 Hz, 1H, CH), 3.63 (t, ^3J(H,H) = 5.2 Hz, 4H, CH_2), 3.34–3.26 (t, 4H, CH_2, overlapping with signal from CD_3OD), 2.93 (s, 12H, CH_3), 2.48–2.41 (m, 1H, CH_2), 2.41 (s, 3H, CH_3), 2.33–2.27 (m, 1H, CH_2), 2.18 (s, 3H, CH_3), 2.00–1.94 (m, 1H, CH_2), 1.94 (s, 3H, CH_3), 1.83–1.76 (m, 1H, CH_2), 1.65 (s, 3H, CH_3), 1.17 (s, 3H, CH_3), 1.15 (s, 3H, CH_3). HRMS (FAB^+): calcd For $C_{63}H_{79}N_6O_{19}$ [M^++H]: 1223.5400 found: 1223.5409. Anal. Calcd for $C_{63}H_{81}Cl_3N_6O_{19}\cdot 8.5H_2O$, C: 50.93, H: 6.65, N: 5.66, found: C: 51.05, H: 6.54, N: 5.18. Purity was 99% (HPLC analysis at 230 nm).

5.2.6. 7-[Bis-[2-[[2-(dimethylamino)ethyl]amino]-2-oxoethyl]amino]-4-[[3′-*N*-(2′-*O*-benzoyl-3′-*N*-debenzoylpaclitaxel)] carbonyloxymethyl] coumarin hydrochloride (11). The whole procedure was carried out in the dark. Compound **10** (6.8 mg, 0.0051 mmol), benzoic acid (1.9 mg, 0.0153 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.9 mg, 0.0153 mmol), and 4-(dimethylamino)pyridine (0.6 mg, 0.0051 mmol) in 2 mL of dry acetonitrile were stirred at room temperature for 2 h under an argon atmosphere. Then, the reaction mixture was applied directly to preparative HPLC, which was eluted with 10% CH_3CN in 1% aqueous AcOH over 20 min, and then a linear gradient of 10–50% CH_3CN in 1% aqueous AcOH over 40 min at a flow rate of 5 mL/min. The desired fraction was collected and lyophilized to give **11** as an AcOH salt (6.0 mg, 0.0042 mmol). Then, the product was lyophilized from a mixture of CH_3CN (2 mL) and water (1 mL) with 1 N HCl (13.4 μL, 0.0134 mmol) to afford **11** as an HCl salt (6.0 mg, 0.0042 mmol, 82%). ^{1}H NMR (CD_3OD, 400 MHz): δ = 8.68 (d, ^3J(H,H) = 9.7 Hz, 1H, CH), 8.10–8.07 (m, 3H, CH), 7.64 (br t, ^3J(H,H) = 7.4 Hz, 1H, CH), 7.56–7.42 (m, 9H, CH), 7.29 (br t, ^3J(H,H) = 7.3 Hz, 1H, CH), 7.20 (d, ^3J(H,H) = 9.0 Hz, 1H, CH), 6.46 (s, 1H, CH), 6.46–6.43 (dd, 1H, CH, partially overlapping with previous signal), 6.35 (d, ^4J(H,H) = 2.0 Hz, 1H, CH), 6.20 (br t, ^3J(H,H) = 9.1 Hz, 1H, CH), 6.17 (s, 1H, CH), 5.67–5.59 (m, 3H, CH), 5.39, 5.17 (2d, ^2J(H,H) = 16.8 Hz, 2H, CH_2), 5.00 (br d, ^3J(H,H) = 8.2 Hz, 1H, CH), 4.37 (dd, ^3J(H,H) = 6.6, 10.9 Hz, 1H, CH), 4.33 (br s, 4H, CH_2), 4.17, 4.15 (2d, ^2J(H,H) = 12.0 Hz, 2H, CH_2), 3.86 (d, ^3J(H,H) = 7.3 Hz, 1H, CH), 3.64 (t, ^3J(H,H) = 5.3 Hz, 4H, CH_2), 3.33–3.27 (t, 4H, CH_2, overlapping with signal from CD_3OD), 2.94 (s, 12H, CH_3), 2.49 (s, 3H, CH_3), 2.47–2.43 (m, 1H, CH_2), 2.34–2.26 (m, 1H, CH_2), 2.17 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 1.94–1.88 (m, 1H, CH_2), 1.84–1.77 (m, 1H, CH_2), 1.65 (s, 3H, CH_3), 1.13 (s, 6H, CH_3). HRMS (FAB^+): calcd For $C_{70}H_{83}N_6O_{20}$ [M^++H]: 1327.5662 found: 1327.5670. Anal. Calcd for $C_{70}H_{85}Cl_3N_6O_{20}\cdot 3.5H_2O$, C: 56.05, H: 6.18, N: 5.60, found: C: 56.04, H: 6.18, N: 5.54. Purity was higher than 98% (HPLC analysis at 230 nm).

5.3. Water solubility

The prodrugs (**9**, **11**) were saturated in distilled water and shaken vigorously. The saturated solutions were sonicated for 15 min at 25 °C and passed through the centrifugal filter (0.45 μm filter unit, Ultrafree®-MC, Millipore). The filtrate was analyzed, using RP-HPLC. The water solubility was determined using a calibration curve prepared from standard solutions containing known quantities of prodrugs.

5.4. Photolysis of 1, 8, and 11 with a laser

The conversion profiles of **11** were determined in phosphate-buffered saline (10 mM PBS, pH 7.4). To 990 μL of PBS (pH 7.4) was added 10 μL of solution, including **1**, **8** and **11** (2 mM in DMSO), and the mixture was irradiated with UV pulses (355 nm, 10 Hz, 5 mJ) obtained from the third harmonic of an Nd-YAG laser (continuum, Minilite II; pulse energy, 5 mJ; pulse width, 5 ns) at 23 °C through a quartz cell. At the desired time points, 1 mL of MeOH was added to the samples and in the case of **11**, the samples were kept in the dark at room temperature for a few hours to induce complete O–N intramolecular acyl migration to the parent paclitaxel (**1**). Finally, 200 μL of the mixture was directly analyzed by RP-HPLC. HPLC was performed using a C18 (4.6 × 150 mm; YMC Pack ODS AM302) reverse phase

column with binary solvent system: linear gradient of CH_3CN (0–100%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL/min, detected at UV 230 nm.

5.5. Photolysis of 1, 8, and 11 with a UV lamp

The conversion profiles of the **11** were determined in phosphate-buffered saline (10 mM PBS, pH 7.4). To 297 μL of PBS (pH 7.4) was added 3 μL of solution including, **1**, **8** and **11** (2 mM in DMSO), and the mixture was irradiated with a UV lamp (365 nm, 6 W, UV Ray Lamp, Model UV-LS, Ishii Laboratory Work Co., Ltd) at room temperature. At the desired time points 300 μL of MeOH was added to the samples, and in the case of **11**, the samples were kept in the dark at room temperature for a few hours to induce complete O–N intramolecular acyl migration to the parent paclitaxel (**1**). Finally, 200 μL of the mixture was directly analyzed by RP-HPLC. HPLC was performed using a C18 (4.6×150 mm; YMC Pack ODS AM302) reverse phase column with a binary solvent system: linear gradient of CH_3CN (0–100%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL/min, detected at UV 230 nm.

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Supplementary data

Absorption spectra of phototaxel **11**, and spectral and analytical data for compounds. This material is available online at www.sciencedirect.com. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.04.022.

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