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Synthesis and photochemical properties of photoactivated antitumor prodrugs releasing 5-fluorouracil†

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A new family of antitumor prodrugs (**1–3**) of 5-fluorouracil (5-FU) possessing photolabile 2-nitrobenzyl chromophores have been designed and synthesized to investigate the efficiency and mechanism of photoactivated 5-FU release upon UV-irradiation at $\lambda_{ex} = 365$ nm. The photoactivated prodrug **3** derived from conjugation of **2** with a tumor-homing cyclic peptide Cys-Asn-Gly-Arg-Cys (CNGRC) was so designed as to manifest a tumor-targeting function.

Introduction

Most antitumor drugs show poorly selective cytotoxicity which causes considerable limitations on their clinical applications. Several strategies for developing prodrugs have been explored to achieve better therapeutic effects and/or fewer side effects of antitumor drugs,[1–2] but there are still a number of problems which need to be overcome. Most prodrugs releasing antitumor drugs by hydrolysis usually exhibit poor stability and produce various side effects on normal cells.[1,3] For enzyme-activated prodrugs undergoing enzymatic hydrolysis, findings of tumor-specific enzymes and methods for selective delivery of enzymes or enzymatic genes to tumor tissues are still key subjects of investigation.[4–5]

Previously, we proposed a different class of antitumor prodrugs such as 1-(5′-fluoro-6′-hydroxy-5′,6′-dihydrouracil-5′-yl)-5-fluorouracil, 1-(2′-oxocycloalkyl)-5-fluorouracils, and 3-(2′-oxopropyl)-5-fluoro-2′-deoxyuridine that are activated to release 5-fluorouracil (5-FU) or 5-fluoro-2′-deoxyuridine (5-FdUrd) by hydrated electrons (e_{aq}^-) generated in water radiolysis under hypoxic conditions.[6] Unfortunately, due to competition with endogenous electron acceptors in the tumor tissues, these radiation-activated prodrugs produced less effective amounts of 5-FU or 5-FdUrd under practical conditions of limited radiation doses applicable to cancer radiotherapy.[6d,e,7] As an alternative molecular design, attention should be given to photoactivated prodrugs with a variety of photolabile protected groups, the triggering reactivity of which can be well controlled by selecting the wavelength of exciting light. Photolabile compounds have been widely applied to organic syntheses and biological investigations, since they can effectively release various active agents with outstanding spatial and temporal precision upon controlled photolysis.[8] Inheriting such advantages, photoactivated prodrugs are not only applicable to surface cancer treatment, but also fitted even to deep-seated cancer therapy employing endoscopes or optical fibers as used in photodynamic therapy.[9] Nevertheless, there are few studies on the development of photoactivated antitumor prodrugs. Moreover, all of the current photoactivated prodrugs releasing phosphoamide mustard, 5-FdUrd or leucyl-leucine methyl ester[10] were constructed based on labile linkages of phosphate, carbamate or ester that readily undergo hydrolysis.

In this study an attempt was made to design and synthesize a new family of photoactivated 5-FU prodrugs (**1–2**) that are not subject to hydrolysis, based on our experience from the development of radiation-activated prodrugs.[6] Focusing on further sophistication, we conjugated prodrug **2** with a tumor-homing cyclic peptide Cys-Asn-Gly-Arg-Cys (CNGRC)[11] to generate the first prototype compound **3** for a tumor-targeting photoactivated antitumor prodrug. In view of the functionality of the peptide CNGRC that recognizes a tumor marker of specific aminopeptidase N (APN/CD13) isoform over-expressing on the surface of tumor blood vessels,[11] the photoactivated prodrug **3** is expected to be accumulated in tumor tissues with high selectivity. In addition, a lysine structure was incorporated into the linker moiety in prodrug **3** to enhance the water solubility. Herein, we report the synthesis and photochemical reactivity of prodrugs **1–3** that effectively released 5-FU upon UV irradiation at $\lambda_{ex} = 365$ nm, and demonstrate the mechanism of photoactivated 5-FU release from **1** in both acetonitrile and aqueous solutions as investigated by means of nanosecond laser flash photolysis (LFP).

Results and discussion

Synthesis

Photoactivated 5-FU prodrugs **1–2** were synthesized using a similar method as for the preparation of 1-(2′-oxocycloalkyl)-5-fluorouracils reported previously.[6b] As outlined in Scheme 1(a), 2,4-bis(trimethylsilyloxy)-5-fluoropyrimidine was refluxed for 4 h with 2-nitrobenzyl bromide or 4-bromomethyl-3-nitrobenzoic acid in acetonitrile under nitrogen in the dark. After cooling at room temperature, the reaction mixture was treated with methanol to give prodrugs **1** and **2** (see ESI†).

For the first prototype of a tumor-targeting photoactivated prodrug, compound **3** conjugated with a tumor-homing peptide CNGRC was synthesized as outlined in Scheme 1(b). The protected peptide KGCNGRC-resin was manually prepared by a standard Fmoc solid-phase synthesis method, using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIPEA) as the activators. The resulting protected peptide KGCNGRC-resin was condensed with compound **2**, followed by cleavage and disulfide cyclization with a trifluoroacetic acid (TFA)–trimethylsilyl chloride (TMSCl)–anisole–dimethylsulfoxide (DMSO) system in a one-pot procedure[12] to produce prodrug **3**.

† Electronic supplementary information (ESI) available: Experimental procedures for photoprodrugs **1** and **2**; Figures S1–S3: transient spectra and kinetic plots for laser flash photolysis of photoprodrug **1**. See http://www.rsc.org/suppdata/ob/b4/b417734g/

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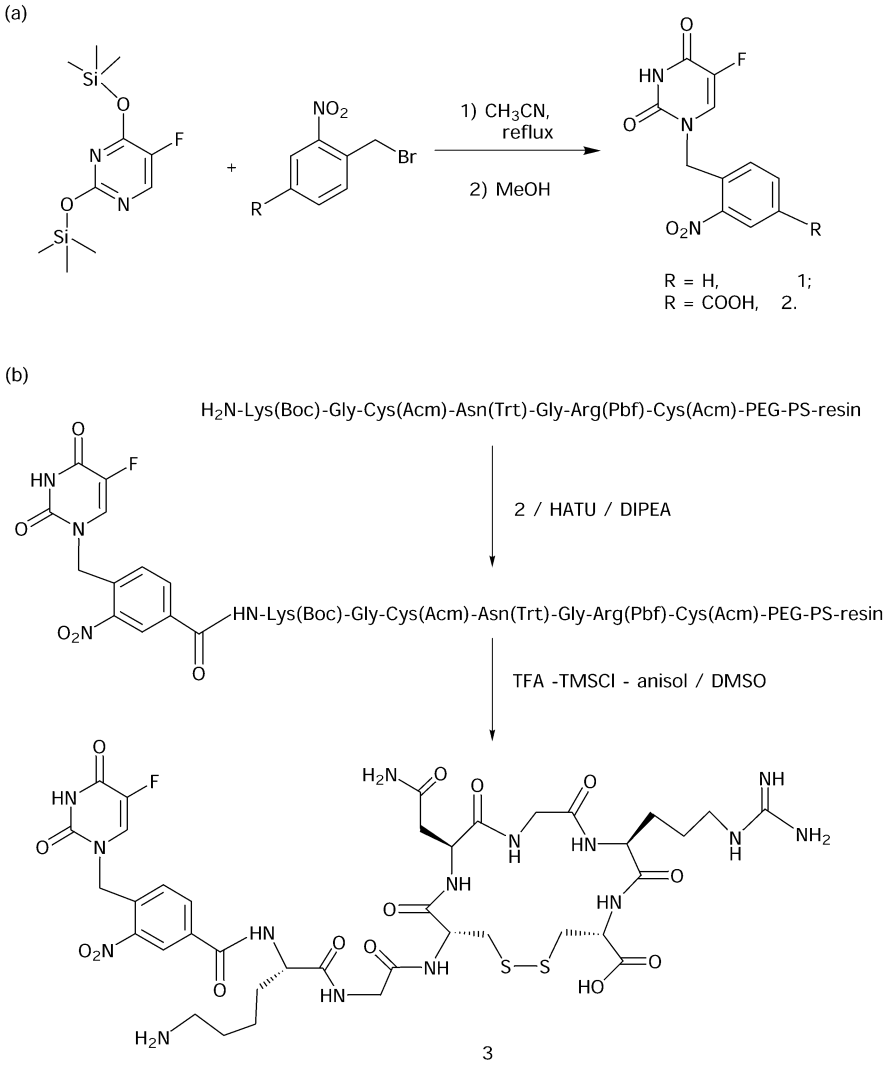


Scheme 1 (a) Syntheses of prodrugs **1** and **2**; (b) synthesis of prodrug **3**.

Photoactivation efficiency of 5-FU release

When solutions of **1** and **2** in acetonitrile–H_2O (3 : 1 v/v) and an aqueous solution of **3** were allowed to stand overnight at 37 °C in the dark, neither decomposition of the prodrugs nor release of 5-FU could be detected by analytical HPLC. In contrast, UV irradiation at λ_{ex} = 365 nm of these solutions resulted in 5-FU release. Although the 2-nitrobenzyl chromophores showed UV absorption maxima λ_{max} at wavelengths around 265 nm, the longer wavelength of λ_{ex} = 365 nm in the UV-A (320–400 nm) was employed in this study because UV-B (290–320 nm) and UV-C (100–290 nm) with wavelengths below 320 nm induce direct effects such as sub-lethal and lethal damage of DNA/RNA bases in both normal and tumor cells.[13] As shown in Fig. 1, the photochemical conversion to 5-FU was efficient and highly selective for all of the prodrugs. Apparently, prodrug **1** showed about 1.4 times higher photoactivation efficiency than the carboxylated analog **2**. This is mainly due to the UV spectral characteristic that the absorption maximum in acetonitrile–H_2O (3 : 1 v/v) (λ_{abs}^{max} = 264 nm) of **2** bearing a carboxylic group was slightly blue-shifted relative to that of **1** (λ_{abs}^{max} = 267 nm). On the other hand, the KGCNGRC-conjugated prodrug **3** showed about 6 times smaller efficiency of photoactivated 5-FU release in aqueous solution (λ_{max} = 262 nm) than prodrug **1** in acetonitrile–H_2O (3 : 1 v/v). Prodrug **3** produced some unknown byproducts in the photolysis, while still showing high efficiency and selectivity of 5-FU release: 365 nm UV irradiation of **3** for 6 hours resulted in 80% decomposition and 60% yield (75% selectivity) of 5-FU. This result greatly encourages further investigation of tumor-targeting photoactivated prodrugs conjugated with various tumor-homing peptides by photolabile linkers. The novel class of tumor-targeting photoactivated anticancer prodrugs reported herein show resistance to hydrolysis could be easily accumulated within tumor tissue and then controlled to effectively release active antitumor agents by applied light. So these prodrugs could selectively work within antitumor tissues and minimize side effects on normal tissues. These prodrugs also show higher efficiency and selectivity to release active agents in comparison to radiation-activated prodrugs.[6]

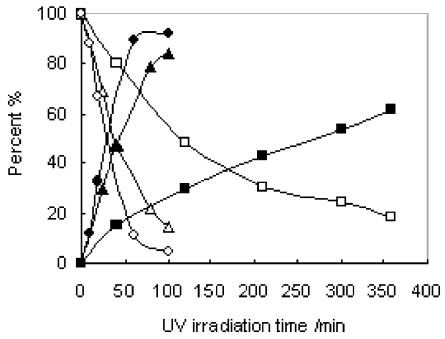


Fig. 1 Photolysis of prodrugs **1** and **2** in acetonitrile–H_2O (2 : 1, v/v) and **3** in H_2O at λ_{ex} = 365 nm: decrease of prodrugs ((○) **1**; (△) **2**; (□) **3**) and 5-FU release from prodrugs ((●) **1**; (▲) **2**; (■) **3**).

Nanosecond laser flash photolysis (LFP) of prodrug 1

A mechanistic study on the photoactivated release of 5-FU was performed by nanosecond 266 nm LFP of prodrug **1** (0.1 mM) in both Ar-saturated acetonitrile and aqueous acetonitrile solutions. An intense fluorescence emission was observed immediately after 266 nm laser flash excitation of prodrug **1** in acetonitrile. Along with the fluorescence decay, buildup of a transient with $\lambda_{max} = 410$ nm was observed (see Fig. S1(a) in ESI†). By reference to a previous study,[14] this transient is attributable to an intermediate of *aci*-nitro tautomer (**A**) formed by photoinduced intramolecular hydrogen transfer, as shown in Scheme 2. The decay of intermediate **A** is well represented in terms of first-order kinetics with a rate constant of $k_{a/b} = (1.4 \pm 0.1) \times 10^4$ s^{-1} (see Fig. S1(b) in ESI†). The absorption of transient **A** decayed to the baseline level (see the inset of Fig. S1(b)) on a sub-ms timescale without leaving any residual absorption at wavelengths above 300 nm. Subsequently, slow buildup of a new transient was observed with absorption at around 320 nm to attain its maximum value 2 s after LFP (see Fig. S1(a) and (c)). This transient may be assigned to an intermediate **C** generated from the preceding intermediate B that shows no absorption at wavelengths above 300 nm (see Scheme 2).[14c,d] Formation of **C** is also supported by the concomitant growth of a very weak absorption at 740 nm, which is characteristic of nitroso compounds.[15] The intermediate C (see Fig. S1(c)) showed first-order kinetic growth and decay with the respective rate constants of $k_{b/c} = 1.2 \pm 0.1$ s^{-1} and $k_{c/4} = 0.13 \pm 0.02$ s^{-1} to generate the final products 5-FU and 2-nitrosobenzaldehyde (**4**) (Scheme 2).[14c,d] These products were confirmed by HR-FABMS in a separate experiment with the 365 nm UV irradiation of **1** (1 mM) in Ar-saturated acetonitrile solution.

The transient absorption spectrum of *aci*-nitro tautomer **A** with $\lambda_{max} = 410$ nm was also observed upon LFP of **1** in acetonitrile–H_2O (2 : 1 v/v) solution, as in bulk acetonitrile solution, while λ_{max} was quickly shifted to a longer wavelength of 460 nm (Fig. S2(a) and S1(b)). This red-shift is attributed to ionization of **A** to $\mathbf{A}^-$ with the first-order kinetic rate constant of $k_{a/a-} = (1.0 \pm 0.1) \times 10^6$ s^{-1} (see Scheme 3). Similar ionization processes were also observed in the LFP of several 2-nitobenzyl compounds in aqueous solution.[14] This conclusion is also in accord with observations in the LFP of **1** in acetonitrile–H_2O (2 : 1 v/v) solution at various pH values. While the absorption with $\lambda_{max} = 410$ nm was observed in acidic solutions (pH $\leq$ 2.5) until it decayed to baseline and subsequent buildup at around 320 nm appeared (see Fig. S2(c)), the absorption with $\lambda_{max} = 460$ nm appeared quickly after the LFP in neutral and basic solutions (see Fig. S2(d)). The deprotonated intermediate $\mathbf{A}^-$ formed in acetonitrile–H_2O (2 : 1 v/v) solution was more stable than the protonated intermediate **A** formed in acetonitrile. Following the first-order kinetics with $k_{a/b} = (1.4 \pm 0.1) \times 10^3$ s^{-1}, $\mathbf{A}^-$ underwent ring-closure to $\mathbf{B}^-$ that is transparent at wavelengths above 300 nm. Subsequently, slow ring-opening of $\mathbf{B}^-$ into **C** ($\lambda_{max} = 320$ nm) occurred with $k_{b/c} = 1.1 \pm 0.1$ s^{-1}, followed by decomposition of **C** to release 5-FU and 2-nitrosobenzaldehyde with $k_{c/4} = 0.13 \pm 0.02$ s^{-1} (see Fig. S2(a) and Scheme 3).

Scheme 3 illustrates a proposed mechanism by which prodrug **1** is photoactivated to release 5-FU *via* a series of reaction steps in acetonitrile–H_2O (2 : 1 v/v) solution at various pH values. The observed rate constant $k_{a/b}$ for the ring-closure of **A** (or $\mathbf{A}^-$) is strongly pH-dependent and is more accelerated with increasing acidity in the pH range <10 (see Fig. S3(a) in ESI†) Two sigmoidal pH–rate profiles are clearly seen in Fig. S3(a), which correspond to the acid–base equilibrium constants for $\mathbf{A}/\mathbf{A}^-$ ($pK_{a/a-} \approx 3.8$) and $\mathbf{A}^-/\mathbf{A}^{2-}$ ($pK_{a-/a2-} \approx 10$), respectively (see Scheme 3). The rate of ring-closure from $\mathbf{A}^{2-}$ to $\mathbf{B}^{2-}$ occurring at pH > 10 seems to be extremely small ($k_{a/b} \approx 20$ s^{-1}). In the wide pH range of 1 to 12, $k_{a/b}$ is about 50–10000 times greater than $k_{b/c}$ and $k_{b/c}$ is about 2.5–10 times greater than $k_{c/4}$. Obviously, formation of **C** involves acid catalysis at pH < 7, while decomposition of **C** is weakly catalyzed by acid at pH < 3 and by base at pH > 7 (see Fig. S3(b)).

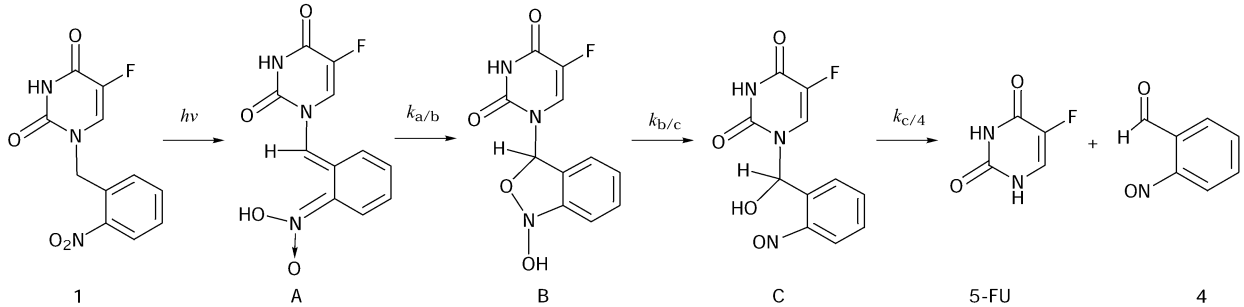
Conclusion

A new family of photoactivated 5-FU prodrugs **1–3** with photolabile 2-nitrobenzyl chromophores could release quantitative amounts of 5-FU in solution upon UV irradiation at $\lambda_{ex} =$ 365 nm, while being quite stable in the dark. A photoactivation mechanism by which the representative prodrug **1** releases 5-FU was clarified by means of nanosecond LFP. The prodrug **3** derived from conjugation of **2** with a typical tumor-homing cyclic peptide CNGRC is a prototype for the first tumor-targeting photoactivated antitumor prodrug. In view of the evidence that the prodrug **3** was effectively photoactivated to release 5-FU, further investigations on the synthesis of related compounds and evaluation of their anti-tumor activity are in progress.

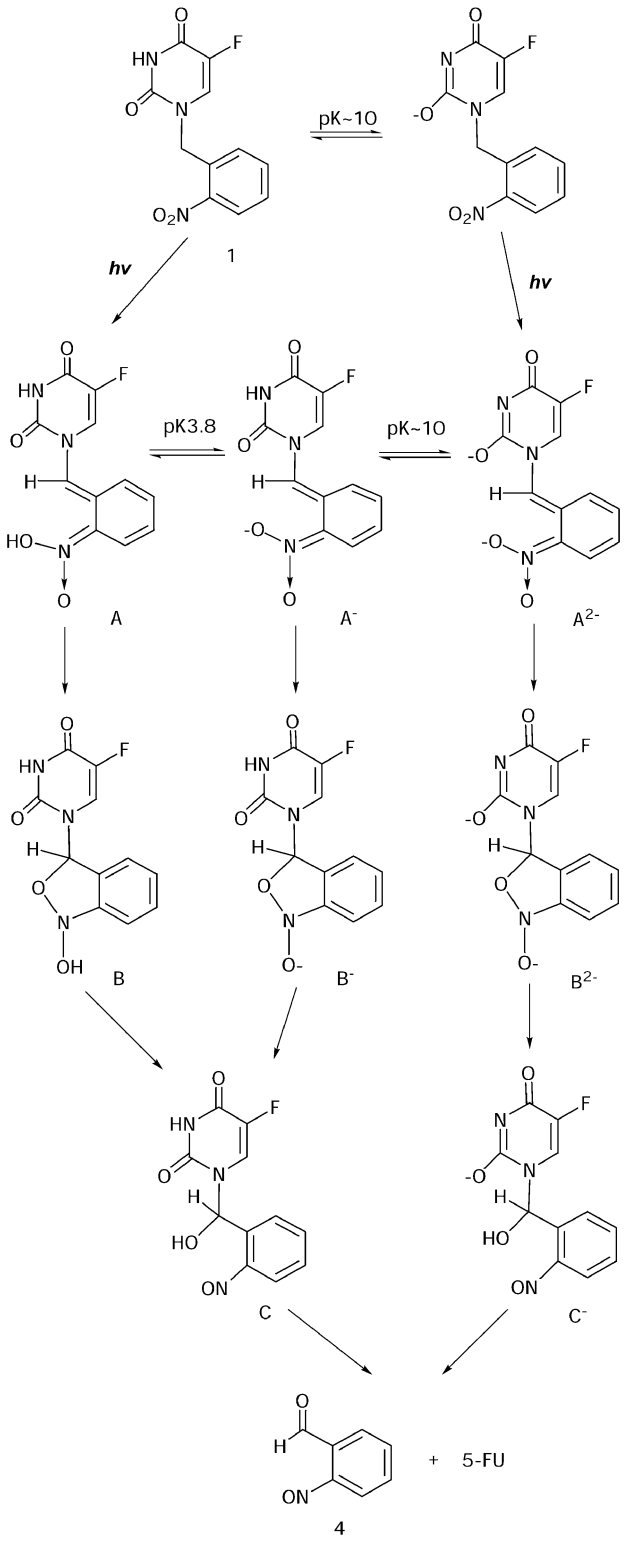
Experimental

General

Thin-layer chromatography was performed on a silica gel coated glass sheet (Silica Gel 60 F_{254} TLC plates, Merck & Co.). Wakogel C-300 silica gel (45–75 μm, Wako Pure Chemical Industries Ltd.) was used for column chromatography. HPLC was performed on a Hitachi D-7000 HPLC system. An Interstil ODS-3 column (ϕ 4.6 mm × 250 mm, GL Science Inc., Japan) was used for analytical HPLC. Prodrugs and photoproducts were eluted with acetonitrile–H_2O mixed solvents at a flow rate of 0.6 mL min^{-1} and detected by UV absorbance at 220 nm. A preparative Interstil ODS-3 column (ϕ 10 mm × 250 mm, GL Science Inc., Japan) was used for preparative HPLC. Products were eluted with a linear gradient of acetonitrile (10.8–50%, 30 min) containing 0.05% TFA at a flow rate of 3.0 mL min^{-1} and detected by UV absorbance at 260 nm. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-EX-400 (400 MHz) or a JNM-AL300 (300 MHz) spectrometer. Fast atom bombardment mass spectrometry (FAB MS) was performed on a JEOL JMS-SX102A mass spectrometer, using glycerol matrix or 3-nitrobenzyl alcohol (NBA) matrix.



Scheme 2 Proposed mechanism of photoactived 5-FU release from prodrug **1** in acetonitrile.



Scheme 3 Proposed mechanism of photoactived 5-FU release from prodrug **1** in acetonitrile–H_2O (2 : 1 v/v) solution.

1-(2′-Nitro-4′-carboxybenzyl)-5-fluorouracil–KGCNGRC conjugate (3)

A protected peptide KGCNGRC-resin was manually prepared by an Fmoc solid phase synthesis method, using Fmoc-Cys(Acm)-PEG-PS resin (100 mg, 0.02 mmol) and 5 equivalent Fmoc-amino acids, Arg(Pbf), Asn(Trt), Cys(Acm), Gly and Lys(Boc), along with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU, 5 equivalent) and *N*,*N*-diisoproylethylamine (DIPEA, 10 equivalent) as activators. The protected peptide KGCNGRC-resin was condensed with compound **2** (30 mg, 0.1 mmol) under activation by HATU and DIPEA. After filtration and washing with *N*,*N*-dimethylformamide (5 × 4 mL) and dichloromethane (4 × 4 mL), the resulting protected 5-FU–KGCNGRC-resin was treated with a mixture of trimethylsilyl chloride (TMSCl, 0.22 mL)–anisole (0.3 mL)–trifluoroacetic acid (TFA, 26 mL) at room temperature. After 1 hour, dimethylsulfoxide (DMSO, 3.8 mL, 300 equivalent) was added to the reaction mixture which was then stirred for another 1 h at 4 °C.[12] After removal of the resin by filtration, ice-cooled dry diethyl ether (80 mL) was added to the filtrate. The precipitate was collected by centrifugation and washed with ice-cooled dry diethyl ether (4 × 20 mL). The crude product was purified by preparative HPLC (R_t = 19.8 min) and freeze-dried to give a fluffy pale yellow powder of 5-FU–KGCNGRC conjugate **3** (3.1 mg, 15% calculated based on the Fmoc-Cys(Acm)-PEG-PS-resin), as identified by FAB-HRMS (positive mode, glycerol matrix): *m*/*z* 1026.3320 [MH^+], $C_{38}H_{53}N_{15}O_{14}FS_2$ requires 1026.3322.

Nanosecond laser flash photolysis (LFP)

The nanosecond spectroscopic LFP experiment for prodrugs **1**–**3** was performed with a Unisoku TSP-601 flash spectrometer. A Continuum Surelite-I Nd–YAG (Q-switched) laser with fourth harmonic generation at 266 nm was used for the nanosecond flash excitation. Further details of the laser flash system have been described previously.[6c]

Solutions of **1** (0.1 mM) in acetonitrile or acetonitrile–H_2O (2 : 1 v/v) were deaerated by argon bubbling prior to the laser flash photolysis. In the pH–rate profile experiment, solutions of **1** (0.1 mM) in acetonitrile–H_2O (2 : 1 v/v) at various pH values were prepared using 10 mM phosphate buffer with acidification by 0.001–0.1 M perchloric acid or basification by 0.001–0.1 M sodium hydroxide. The first-order rate constants $k_{a/b}$ were evaluated from the decay of the absorption at 410 nm (pH ≤ 3.8) or 460 nm (pH ≥ 3.5), while $k_{b/c}$ and $k_{c/4}$ were determined from the growth or decay of the absorption at 320 nm, respectively.

UV irradiation of photoprodrugs and products analysis

A 25 W ultraviolet transilluminator (λ_{ex} = 365 nm, Model TFL-40, UVP Inc.) was used for steady-state UV irradiation. For a product study, Ar-saturated solution of **1** (1 mM) in acetonitrile was photoirradiated at λ_{ex} = 365 nm for 20 min and the final products of 5-FU and *o*-nitrosobenzaldehyde **4** were determined by FAB-HRMAS (positive mode, glycerol matrix): for 5-FU, *m*/*z* 131.0252 [MH^+], $C_4H_4O_2N_2F$ requires 131.0251; for **4**, *m*/*z* 136.0404 [MH^+], $C_7H_6O_2N$ requires 136.0399.

Solutions of **1** (1 mM) in acetonitrile–H_2O (3 : 1 v/v), **2** (1 mM) in acetonitrile–H_2O (3 : 1 v/v) and **3** (1 mM) in H_2O were irradiated at λ_{ex} = 365 nm in air at 37 °C, while the corresponding control solutions were kept overnight in the dark at 37 °C. At various time intervals, an aliquot (10 μL) was sampled and examined by analytical HPLC. A mixed solvent of acetonitrile–H_2O (1 : 1 v/v) with HCl (pH 3) was used as eluent in the HPLC analyses for **1** and **2**, while a linear gradient elution with acetonitrile–H_2O mixed solvents (5.9–50%, 30 min) containing 0.05% (v/v) trifluoroacetic acid (TFA) was applied for the HPLC analysis of **3**. The concentrations of **1–3** and released 5-FU were quantified from the HPLC peak areas, using the respective calibration curves prepared by reference to authentic samples.

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