Invention of stimulus-responsive peptide-bond-cleaving residue (Spr) and its application to chemical biology tools

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ABSTRACT

Elucidation of biological functions of peptides and proteins is essential for understanding peptide/protein-related biological events and developing drugs. Caged peptides and proteins that release a parent active peptide/protein by photo-irradiation have successfully been employed to elucidate the functions. Whereas the usual caged peptide/protein enables conversion of an inactive form to an active form (OFF-to-ON conversion) by photo-induced deprotection, photo-triggered main chain cleavage is reported to be applicable to ON-to-OFF conversion. These peptides and proteins are photo-responsive; however, if peptides and proteins could respond to other stimuli such as disease-related environment or enzymes, their range of application should be widened. To convert the photo-responsive peptide/protein into other stimulus-responsive peptide/protein, quite laborious *de novo* design and synthesis of the stimulus-responsive unit are required. In this context, we designed a stimulus-responsive peptide-bond-cleaving residue (Spr) in which the stimuli available for the main chain cleavage vary according to the choice of protecting groups on the residue. In this review, design and synthesis of Spr are introduced, and challenges to apply Spr to other fields to enable, for example, functional control, localization control, delivery of cargos, labeling of a protein of interest in living cells, and identification of target proteins of bioactive ligands are discussed.

KEYWORDS: artificial amino acid, amide bond cleavage, peptide bond cleavage, Spr, stimulus responsive, stimulus-responsive peptide-bond-cleaving residue, trimethyl lock

Abbreviations: Bn, benzyl; Boc, tert-butoxycarbonyl; CPP, cell penetrating peptide; CuAAC, copper-catalyzed azide-alkyne cycloaddition; Dab, 4-dimethylaminoazobenzene-4'-sulfonyl; Fmoc, 9-fluorenylmethoxycarbonyl; FmocOSu, Fmoc N-hydroxysuccinimide ester; Fmoc SPPS, Fmoc-based solid-phase peptide synthesis; FRET, fluorescence resonance energy transfer; FTC, fluorescein 5-aminothiocabonyl; HMPA, hexamethylphosphoric triamide; I_C*, C-intein;* I_N*, N-intein; MeCN, acetonitrile; NES, nuclear export signal; NLS, nuclear localization signal; oNB, o-nitrobenzyl; PCC, pyridinium chlorochromate; PG, protecting group that is removable by a stimulus; PNA, peptide nucleic acid; POI, protein of interest; SAv, streptavidin; SEAlide, N-sulfanylethylanilide; siRNA, small interfering RNA; Spr, stimulus-responsive peptide-bond-cleaving residue; Spr', Spr derivative; TBSOTf, tert-butyldimethylsilyl triflate;* T_m*, melting temperature; UV, ultraviolet.*

Introduction

Elucidation of biological functions of peptides and proteins is essential for understanding peptide/protein-related biological events and developing drugs. To clarify the function of peptides and proteins from a chemical point of view, photo-caging technology has been widely employed [1-3]. Generally, caged peptides/proteins bearing a photo-responsive protecting group on their side chain or a back bone amide remain inactive without photo-irradiation, being activated upon photo-induced removal of the protection [4-8]. Protecting groups employed for the caging prevent the caged molecules from interacting with their partner molecules; therefore, photo-induced removal of the protection restores the original function of parent peptides/proteins [9]. Whereas conventionally used protection strategies on a side chain or a backbone allow for the conversion of an inactive caged peptide/protein to its active form (OFF-to-ON conversion), photo-responsive main chain cleavage can be applied not only to OFF-to-ON, but also to ON-to-OFF conversion [10-12]. The most successful example of the photo-responsive main chain cleavage system is photo-responsive caspase-3, developed by Majima and co-workers, in which an inactive caspase-3 precursor possessing a photo-responsive *o*-nitrophenylglycine in its backbone can be converted to

an active matured caspase-3 via photo-induced main chain cleavage followed by assembly of the generated fragments [13].

Being inspired by the photo-responsive main-chain-cleavage system mentioned above, we envisioned that if the peptides/proteins can respond to a stimulus other than photo-irradiation, their range of application should be widened (e.g., therapeutics and delivery system activatable by disease related signals and microenvironment). To convert a photo-responsive peptide/protein into other stimulus-responsive peptide/protein, laborious *de novo* design and synthesis of the stimulus-responsive peptide-bond-cleaving unit are required. In this context, we designed a stimulus-responsive peptide-bond-cleaving residue (Spr), in which the stimuli available for the main chain cleavage vary according to the choice of protecting groups on the residue in a manner similar to environment-responsive fluorophores that can respond to various stimuli by changing their protection (Scheme 1) [14,15]. In this review, design, synthesis, and application of Spr are introduced.

Chemistry of the Spr

To use Spr in living cells, the main chain cleavage should occur under physiological conditions. In this context, a trimethyl lock system developed by Milstien

and Cohen was rediscovered as bases of the Spr (Scheme 2) [16]. Trimethyl lock **1** utilizes steric effect between three methyl groups to accelerate a lactonization reaction [17], and this results in the amide bond cleavage at a neutral pH and room temperature (X = NH). Because the trimethyl lock enables release of R unit **2** via stimulus-induced deprotection of the phenolic hydroxyl group under mild conditions, it has been utilized in the fields of chemical biology and drug delivery [18]. With these reports in mind, Spr based on the trimethyl lock was designed as shown in Scheme 3. A peptide/protein possessing Spr would be cleaved by stimulus-induced removal of the PG unit followed by lactonization of the trimethyl lock even under physiological conditions. In principle, this Spr-incorporated peptide/protein should respond to any stimulus by replacing the PG unit.

As a proof-of-concept experiment, we first designed racemic ultraviolet (UV)-responsive Spr **3** that possesses a photo-removable *o*-nitrobenzyl (*o*NB) group at the PG position [19] (Scheme 4). In this study, an α-amino group was protected by a 9-fluorenylmethoxycarbonyl (Fmoc) group for application to Fmoc-based solid-phase peptide synthesis (Fmoc SPPS). Starting from known aldehyde **4** that was prepared from commercially available 3,5-dimethylphenol via 4-step conversion [20], α-hydrazination was achieved in the presence of pyrrolidine to generate **5**. Resulting

aldehyde **5** was then converted to protected amino alcohol **6**, which is a key synthetic intermediate of Spr via a 5-step reaction. After protection of a phenolic hydroxyl group of **6** with a photo-removable *o*NB group, the obtained product was successfully employed for further transformation to generate Fmoc-protected UV-responsive Spr **3**. Whereas racemic **3** was prepared in the first preparation of UV-responsive Spr, enantioselective synthesis of (*S*)-**3** was achieved later by the use of a proline-based tetrazole catalyst (Scheme 4) [21-24]. We have yet to examine whether chirality of Spr affects activity and/or conformation of peptides/proteins possessing Spr; therefore, it should be addressed in due course. Synthesis of UV-responsive peptide **7** possessing the racemic Spr unit was accomplished by a usual Fmoc SPPS protocol (a structure of Spr-containing peptide **7** is shown in Scheme 5).

Next, UV-irradiation experiment of **7** was examined. After 3 min of UV-irradiation of **7** in a mixture of acetonitrile (MeCN) and phosphate buffer (pH 7.6) at 37 °C, the *o*NB group of **7** was completely removed and gradual conversion of the generated phenolic intermediate to the products via peptide bond cleavage was achieved. This result demonstrated that Spr can induce peptide bond cleavage after UV-triggered removal of the protecting group even under mild aqueous conditions.

Success of UV-responsive Spr encouraged us to develop new Spr derivatives that can respond to a stimulus other than UV. Simply replacing the *o*NB group to other stimulus-removable protecting groups, we have successfully developed seven Spr derivatives as summarized in Figure 1. These Spr derivatives can respond to photo-irradiation (UV [19,21]; two-photon near-infrared excitation [25]), enzymatic reaction (alkaline phosphatase: this Spr was introduced in a peptide using *tert*-butoxycarbonyl (Boc)-based solid-phase peptide synthesis [19]), intracellular environment (hypoxia [26]; thiol [27,28]: concentration of glutathione in cells is much higher than that of outer cells [29,30]; hydrogen peroxide [31]: a reactive oxygen species) and a chemical reagent (fluoride anion [32]).

We next examined influence of an amino acid neighboring to the Spr on kinetics of the Spr-induced peptide-bond-cleavage reaction [33]. In this study, a fluorescence resonance energy transfer (FRET)-based monitoring system was constructed as shown in Scheme 6. UV-irradiation to non-fluorescent **8** possessing a fluorophore (FTC) and a quencher (Dab) generates non-fluorescent intermediate **9**, which was spontaneously converted to a fluorescent cleavage product. It was observed that the peptide bond cleavage was much slower than that of photo-removal of the *o*NB group; therefore, kinetics of the peptide bond cleavage could be estimated based on

time-course of fluorescence intensity. Details of the results are shown in the literature [33], but introduction of sterically less-hindered or polar amino acids at $\mathbf{X_1}$ and $\mathbf{X_2}$ positions accelerated the cleavage reaction (half-lives of **9**: fastest, 3.1 min ($\mathbf{X_1}$ = Gly, $\mathbf{X_2}$ = His); slowest, 74.5 min ($\mathbf{X_1}$ = Ile, $\mathbf{X_2}$ = Tyr)).

As mentioned above, we successfully developed Spr in which the stimuli available for the main chain cleavage vary according to the choice of protecting groups on the phenol. Its application to chemical biology tools is discussed in the following section.

Application of Spr to functional control of peptides in living cells

We first applied Spr to functional control of a peptide in living cells. As represented by caged peptides [1-3], functional control of a peptide developed so far enables ON-to-OFF or OFF-to-ON conversion of its activity. In contrast, we envisioned the construction of an ON-to-ON conversion system in which a functional peptide would be transformed to another functional peptide by an external stimulus. Molecular design of the ON-to-ON system is shown in Scheme 7. Peptide **10** possesses UV-responsive Spr that connects a functional peptide A and a functionally suppressed *O*-acyl isopeptide (an inactive functional peptide B). Therefore, peptide **10** exhibits

peptide A-derived function. UV-irradiation of **10** would induce peptide bond cleavage at C-terminal position of Spr, and the generated *O*-acyl isopeptide **11** undergoes O–N acyl transfer reaction similar to a click peptide reported by Kiso's group [34] to generate an active linear peptide B: that is, conversion of functional peptide A to other functional peptide B by UV-irradiation.

As a proof-of-concept experiment, a nuclear cytoplasmic peptide that localizes in nucleus before UV-irradiation but goes back to cytoplasm after UV-irradiation was designed [19]. In this study, an octaarginine [35] as a cell-penetrating peptide (CPP) sequence and a nuclear localization signal sequence (NLS) were incorporated as the peptide A (see Table in Scheme 7, upper line); therefore, peptide **10** was concentrated in nucleus when cells were incubated with **10**. UV-irradiation then triggered the main chain cleavage followed by O–N acyl transfer afforded nuclear export signal (NES) sequence as the peptide B to go back to cytoplasm. These results suggested that our ON-to-ON strategy successfully works in living cells.

We next collaborated with Kwon's group to apply this system to UV-responsive visualization of a protein of interest (POI) in living cells [36]. As shown in the lower line of the table in Scheme 7, UV-responsive Spr was incorporated between CPP and a functionally suppressed C-intein (I_C) sequence that possesses a quencher at

$B_{1/2}$ position and a fluorophore at $B_{2/2}$ position. This non-fluorescent peptide **10** first internalized in a cell that expresses POI fused with N-intein (I_N). UV-irradiation then triggered generation of a non-fluorescent matured I_C. Finally, protein trans-splicing [37] between the matured I_C and the I_N-fused POI successfully yielded a fluorophore-labeled fluorescent POI in living cells.

Another application of Spr in living cells is visualization of an intracellular environment. We developed hypoxia-responsive peptide **11** that is composed of Dab as a quencher, hypoxia-responsive Spr, FTC as a fluorophore, and octaarginine as CPP (Scheme 8). After addition of peptide **11** to cells, it entered inside the cells, but strong fluorescence was observed only in hypoxic cells. This result suggests that replacement of the protecting group of Spr enables, in principle, visualization of intracellular environment other than hypoxia.

As mentioned in this section, it is demonstrated that Spr can be used to control a peptidyl function in living cells. Application of Spr to the functional control outside cells is introduced in the next section.

Application of Spr to functional control outside cells

The O–N acyl transfer strategy mentioned in the previous section is also applicable to functional control of small molecules. One example is Spr-based caged ceramide **12**, which generates ceramide by UV-irradiation (Scheme 9) [38]. UV-irradiation of caged ceramide **12** induced amide bond cleavage at C-terminal position of UV-responsive Spr, and the generated amine **13** underwent O–N acyl transfer reaction to generate a bioactive parent ceramide. Caged ceramide **12** would enable introduction of functional unit such as a localization signal or a solubilizing unit at an R position. It should be mentioned that application of our caged ceramide to living cells has yet to be completed; however, successful synthesis and biological study of caged ceramide that possesses a straightforward photo-removable protecting group on ceramide was reported by Bittman et al. in the same year [39].

Another application of UV-responsive Spr is a photo-responsive growth system of peptide nanofibers that can be addressed by DNA hybridization (Figure 2) [40]. In collaboration with our group, Matsuura et al. designed precursor peptide **12**, which possesses a DNA (dA_{20}), UV-responsive Spr, and a β-sheet forming peptide. Peptide **12** was addressed by hybridization with complementary DNA (dT_{20}) immobilized on glass, but it does not form microfibers because of intermolecular electrostatic repulsion of the DNA unit. Upon UV-irradiation, peptide bond cleavage afforded a free β-sheet-forming

peptide, and dT20-addressed microfiber formation was observed. This system could be applicable to a highly sensitive DNA detecting device.

Spr is also applicable to stimulus-responsive DNA-releasing system [27]. It is known that intracellular concentration of thiol (glutathione) is much higher than the extracellular one [29,30]. Therefore, we envisioned that a non-viral thiol-responsive DNA-releasing device might be valuable for gene therapy and genetic recombination. In this study, peptide nucleic acid (PNA) bearing a peptide-based scaffold as a replacement of sugars and phosphates of DNA was employed (Figure 3) [41,42]. Thiol-responsive PNA **13** was prepared, and melting temperature (T_m) of a **13**-complementary DNA (dA_9) hybrid was measured. The result suggested that addition of a thiol had triggered release of the complementary DNA as we designed.

Recently, Mei et al. utilized two-photon near-infrared excitation-responsive Spr to polymer nanoparticle-based targeted cancer therapy [43]. For selective introduction of cargo molecules to a target acidic tumor by near-infrared irradiation, they applied two-photon near-infrared excitation-responsive Spr to modify polymer nanoparticles (Figure 4). The nanoparticle was modified with conjugate **14** composed of phospholipid, positively charged CPP, Spr, and negatively charged CPP-binder that suppresses CPP activity. After addition of the nanoparticle followed by near-infrared irradiation to a

target tumor, cleavage at Spr followed by protonation-induced removal of CPP-binder under acidic conditions activated CPP to carry a cargo molecule to a target acidic tumor. In this system, conjugate **14** acts as an AND logic gate (near-infrared irradiation AND low pH) to achieve strict targeting. The authors utilized this system to successfully introduce a small interfering RNA (siRNA) to target tumor *in vivo*.

The following example is not an application of Spr, but trimethyl lock strategy might enable control of thiol protease activity in an ON-to-OFF-to-ON manner. A concept of the ON-to-OFF-to-ON strategy is shown in Scheme 10A [44]. By the addition of a UV-removable alkylating reagent with a substrate-mimicking moiety, an active cysteine protease would be converted to an inactivated form via alkylation of the active center thiol (ON-to-OFF conversion). UV-triggered removal of the alkylating reagent would then induce recovery of the activity of the protease (OFF-to-ON conversion). As bases for the ON-to-OFF-to-ON strategy, we designed reagent **15**, which can catch and photo-release a thiol (Scheme 10B). In this study, a peptide with a cysteinyl thiol was employed as a model of a thiol protease. Addition of **15** to the model peptide afforded *S*-alkylated catch product **16** via Michael addition. UV-irradiation of **16** induced removal of the *o*NB group followed by lactonization of a trimethyl lock-like moiety to generate intermediate **17**, which spontaneously released the parent model

peptide via β-elimination and aromatization. Application of the ON-to-OFF-to-ON system to thiol proteases is in progress.

Application of Spr to identification of target proteins of bioactive ligands

We thus far reviewed challenges to apply Spr to control biological functions. In this section, development of a traceable linker that would facilitate identification of target proteins of bioactive ligands is reported. Identification of the target proteins is essential for drug development and chemical biology study. The identification usually involves the following processes (Scheme 11): (1) introduction of an alkyne-incorporated ligand derivative to the target protein by photo-affinity labeling [45] or activity based probe technology [46]; (2) copper-catalyzed azide-alkyne cycloaddition (CuAAC) [47] of the alkyne-modified-target protein and a linker with azide and biotin; (3) enrichment of the biotinylated target protein using streptavidin (SAv)-coated beads [48]; and (4) sequence analysis of the target. In this procedure, elution of the biotinylated target from SAv-coated beads requires harsh conditions because of their high affinity [49]. The harsh conditions cause co-elution of the target with non-specifically adsorbed non-targets that would hamper subsequent sequencing. Therefore, cleavable linkers that can be cleaved under mild conditions have been

developed to surmount this problem [50]. Whereas successful application of the cleavable linkers to the target identification has been achieved, contamination of non-targets was sometimes observed even if the cleavable linkers were employed.

With these issues in mind, some groups [51-53], including ours [28,32], designed a traceable linker that enables not only target elution by linker cleavage as similar to the cleavable linker, but also selective labeling of the target for discrimination of the target from contaminated non-targets. Design of our Spr-based traceable linker **18** is depicted in Scheme 12. After CuAAC of the linker and an alkynylated target protein followed by adsorption on SAv-coated beads, exposure to a corresponding stimulus induces cleavage at a C-terminal position of Spr to elute the target protein with an aminooxy group. The resulting aminooxy group reacts with an aldehyde bioorthogonally [54]; therefore, target-selective labeling would be achieved by the addition of a reporter (e.g., fluorescein) with an aldehyde moiety. So far we reported thiol-responsive [28] and fluoride-responsive [32] Spr-based traceable linkers. Recently, an *N*-sulfanylethylanilide (SEAlide) developed by us as a crypto-thioester for chemical protein synthesis [55,56] was also applied to the traceable linker [57]. Details are not mentioned in this review because it does not utilize Spr, but we believe that Spr-based and SEAlide-based traceable linkers both should contribute to the target identification.

Application of the traceable linkers to the target identification will be reported in due course.

Summary

Development and application of Spr were overviewed. An advantage of Spr over other stimulus-responsive units is that the stimuli available for the main chain cleavage vary according to the choice of the phenolic protection. We have already developed seven Spr, and new Spr should emerge as need arises. Applications of Spr in the chemical biology field have been examined to enable, for example, functional control, localization control, delivery of cargos, labeling of POI in living cells, and identification of target proteins of bioactive ligands. We hope that the Spr-based technologies described in this review will become practical technologies in many research fields. We are also expecting that invention of other valuable Spr-based tools will emerge in the near future.

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References

1 H.-M. Lee, D. R. Larson, D. S. Lawrence, Illuminating the chemistry of life: design, synthesis, and application of “caged” and related photoresponsive compounds. *ACS Chem. Biol*. 2009; **4**: 409-427.

2 H. Yu, J. Li, D. Wu, Z. Qiu, Y. Zhang, Chemistry and biological application of photo-labile organic molecules. *Chem. Soc. Rev.* 2010; **39**: 464-473.

3 C. Brieke, F. Rohrbach, A. Gottschalk, G. Mayer, A. Heckel, Light-controlled tools. *Angew. Chem. Int. Ed.* 2012; **51**: 8446-8476.

4 Technology for photo-control of peptide/proten activity other than the photo-caging, see references 5-8.

5 A. A. Beharry, G. A. Woolley, Azobenzene photoswitches for biomolecules. *Chem. Soc. Rev.* 2011; **40**: 4422-4437.

6 T. Fehrentz, M. Schönberger, D. Trauner, Optochemical genetics. *Angew. Chem. Int. Ed.* 2011; **50**: 12156-12182.

7 W. Szymański, J. M. Beierle, H. A. V. Kistemaker, W. A. Velema, B. L. Feringa, Reversible photocontrol of biological systems by the incorporation of molecular photoswitches. *Chem. Rev.* 2013; **113**: 6114-6178.

8 A. Gautier, C. Gauron, M. Volovitch, D. Bensimon, L. Jullien, S. Vriz, How to control proteins with light in living systems. *Nat. Chem. Biol.* 2014; **10**: 533-541.

9 K. Ebisuno, M. Denda, K. Ogura, T. Inokuma, A. Shigenaga, A. Otaka, Development of caged non-hydrolyzable phosphoamino acids and application to photo-control of binding affinity of phosphopeptide mimetic to phosphopeptide-recognizing protein. *Bioorg. Med. Chem.* 2014; **22**: 2984-2991.

10 L. L. Parker, J. W. Kurutz, S. B. H. Kent, S. J. Kron, Control of the yeast cell cycle with a photocleavable α-factor analogue. *Angew. Chem. Int. Ed.* 2006; **45**: 6322-6325.

11 M. Toebes, M. Coccoris, A. Bins, B. Rodenko, R. Gomez, N. J. Nieuwkoop, W. van de Kasteele, G. F. Rimmelzwaan, J. B. A. G. Haanen, H. Ovaa, T. N. M. Schumacher, Design and use of conditional MHC class ligands. *Nat. Med.* 2006; *12*: 246-251.

12 H. Li, J.-M. Hah, D. S. Lawrence, Light-mediated liberation of enzymatic activity: "small molecule" caged protein equivalents. *J. Am. Chem. Soc.* 2008; **130**: 10474-10475.

13 M. Endo, K. Nakayama, Y. Kaida, T. Majima, Design and synthesis of photochemically controllable caspase-3. *Angew. Chem. Int. Ed.* 2004; **43**: 5643-5645.

14 H. Kobayashi, M. Ogawa, R. Alford, P. L. Choyke, Y. Urano, New strategies for fluorescent probe design in medical diagnostic imaging. *Chem. Rev.* 2010; **110**: 2620-2640.

15 Y. Tang, D. Lee, J. Wang, G. Li, J. Yu, W. Lin, J. Yoon, Development of fluorescent probes based on protection-deprotection of the key functional groups for biological imaging. *Chem. Soc. Rev.* 2015; **44**: 5003-5015.

16 S. Milstien, L. A. Cohen, Rate acceleration by stereopopulation control: models for enzyme action. *Proc. Natl. Acad. Sci. U.S.A.* 1970; **67**: 1143-1147.

17 M. E. Jung, G. Piizzi, gem-Disubstituent effect: theoretical basis and synthetic applications, *Chem. Rev.* 2005; **105**: 1735-1766.

18 M. N. Levine, R. T. Raines, Trimethyl lock: a trigger for molecular release in chemistry, biology, and pharmacology, *Chem. Sci.* 2012; **3**: 2412-2420.

19 A. Shigenaga, D. Tsuji, N. Nishioka, S. Tsuda, K. Itoh, A. Otaka, Synthesis of a stimulus-responsive processing device and its application to a nucleocytoplasmic shuttle peptide. *ChemBioChem* 2007; **8**: 1929-1931.

20 K. L. Amsberry, R. T. Borchardt, The lactonization of 2'-hydroxyhydrocinnamic acid amides: a potential prodrug for amines. *J. Org. Chem.* 1990; **55**: 5867-5877.

21 A. Shigenaga, J. Yamamoto, N. Nishioka, A. Otaka, Enantioselective synthesis of stimulus-responsive amino acid via asymmetric α-amination of aldehyde. *Tetrahedron* 2010; **66**: 7367-7372.

22 H. Kotsuki, N. Sasakura, Proline-related secondary amine catalysts and application. In *Comprehensive Enantioselective Organocatalysi*; P. I. Dalko Ed.; Wiley-VCH, 2013; Vol. 1, pp 3-31.

23 H. Torii, M. Nakadai, K. Ishihara, S. Saito, H. Yamamoto, Asymmetric direct aldol reaction assisted by water and a proline-derived tetrazole catalyst. *Angew. Chem. Int. Ed.* 2004; **43**: 1983-1986.

24 A. Hartikka, P. I. Arvidsson, Rational design of asymmetric organocatalysts - increased reactivity and solvent scope with a tetrazolic acid. *Tetrahedron Asymmetry* 2004; **15**: 1831-1834.

25 A .Shigenaga, J. Yamamoto, Y. Sumikawa, T. Furuta, A. Otaka, Development of photo-responsive peptide-bond-cleavage reaction of two-photon near-infrared excitation-responsive peptide. *Tetrahedron Lett.* 2010; **51**: 2868-2871.

26 A. Shigenaga, K. Ogura, H. Hirakawa, J. Yamamoto, K. Ebisuno, L. Miyamoto, K. Ishizawa, K. Tsuchiya, A. Otaka, Development of a reduction-responsive amino acid that induces peptide bond cleavage in hypoxic cells. *ChemBioChem* 2012; **13**: 968-971.

27 A. Shigenaga, J. Yamamoto, H. Hirakawa, K. Ogura, N. Maeda, K. Morishita, A. Otaka, Development of thiol-responsive amide bond cleavage device and its application for peptide nucleic acid-based DNA releasing system. *Tetrahedron Lett.* 2010; **51**: 2525-2528.

28 J. Yamamoto, M. Denda, N. Maeda, M. Kita, C. Komiya, T. Tanaka, W. Nomura, H. Tamamura, Y. Sato, A. Yamauchi, A. Shigenaga, A. Otaka, Development of a traceable linker containing a thiol-responsive amino acid for the enrichment and selective labelling of target proteins. *Org. Biomol. Chem.* 2014; **12**: 3821-3826.

29 M. E. Anderson, Glutathione: an overview of biosynthesis and modulation. *Chem. Biol. Interact.* 1998; **112**: 1-14.

30 D. P. Jones, J. L. Carlson, P. S. Samiec, P. Stemberg Jr., V. C. Mody Jr., R. L. Reed, L. A. Brown, Glutathione measurement in human plasma: evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC. *Clin. Chim. Acta* 1998; **275**: 175-184.

31 M. Kita, J. Yamamoto, T. Morisaki, C. Komiya, T. Inokuma, L. Miyamoto, K. Tsuchiya, A. Shigenaga, A. Otaka, Design and synthesis of a hydrogen peroxide-responsive amino acid that induces peptide bond cleavage after exposure to hydrogen peroxide. *Tetrahedron Lett.* 2015; **56**: 4228-4231.

32 J. Yamamoto, N. Maeda, C. Komiya, T. Tanaka, M. Denda, K. Ebisuno, W. Nomura, H. Tamamura, Y. Sato, A. Yamauchi, A. Shigenaga, A. Otaka, Development of a fluoride-responsive amide bond cleavage device that is potentially applicable to a traceable linker. *Tetrahedron* 2014; **70**: 5122-5127.

33 A. Shigenaga, J. Yamamoto, H. Hirakawa, K. Yamaguchi, A. Otaka, FRET-based assay of the processing reaction kinetics of stimulus-responsive peptides: influence of amino acid sequence on reaction kinetics. *Tetrahedron* 2009; **65**: 2212-2216.

34 Y. Sohma, T. Yoshiya, A. Taniguchi, T. Kimura, Y. Hayashi, Y. Kiso, Development of *O*-acyl isopeptide method. *Biopolymers (Pept. Sci.)* 2007; **88**: 253-262.

35 S. Futaki, Membrane-permeable arginine-rich peptides and the translocation mechanism. *Adv. Drug Deliv. Rev.* 2005; **57**: 547-558.

36 D. Jung, K. Sato, K. Min, A. Shigenaga, J. Jung, A. Otaka, Y. Kwon, Photo-triggered fluorescent labelling of recombinant proteins in live cells. *Chem. Commun.* 2015; **51**: 9670-9673.

37 Y. Li, Split-inteins and their bioapplication. *Biotechnol. Lett.* 2015; **37**: 2121-2137.

38 A. Shigenaga, H. Hirakawa, J. Yamamoto, K. Ogura, M. Denda, K. Yamaguchi, D. Tsuji, K. Itoh, A. Otaka, Design and synthesis of caged ceramide: UV-responsive ceramide releasing system based on UV-induced amide bond cleavage follwed by O–N acyl transfer. *Tetrahedron* 2011; **67**: 3984-3990.

39 Y. A. Kim, D. M. C. Ramirez, W. J. Costain, L. J. Johnston, R. Bittman, *Chem. Commun.* 2011; **47**: 9236-9238.

40 M. Furutani, A. Uemura, A. Shigenaga, C. Komiya, A. Otaka, K. Matsuura, A photoinduced growth system of petide nanofibres addressed by DNA hybridization. *Chem. Commun.* 2015; **51**: 8020-8022.

41 P. E. Nielsen, Peptide nucleic acids and the origin of life. *Chem. Biodivers.* 2007; **4**: 1996-2002.

42 P. E. Nielsen, Peptide nucleic acids (PNA) in chemical biology and drug discovery. *Chem. Biodivers.* 2010; **7**: 786-804.

43 Y. Yang, X. Xie, Y. Yang, Z. Li, F. Yu, W. Gong, Y. Li, H. Zhang, Z. Wang, X. Mei, Polymer nanoparticles modified with photo- and pH-dual-responsive polypeptides for enhanced and targeted cancer therapy. *Mol. Pharmaceutics* 2016; **13**: 1508-1519.

44 A. Shigenaga, K. Morishita, K. Yamaguchi, H. Ding, K. Ebisuno, K. Sato, J. Yamamoto, K. Akaji, A. Otaka, Development of UV-resonsive catch-and-release system of a cysteine protease model peptide. *Tetrahedron* 2011; **67**: 8879-8886.

45 D. J. Lapinsky, Tandem photoaffinity labeling-bioorthogonal conjugation in medicinal chemistry. *Bioorg. Med. Chem.* 2012; **20**: 6237-6247.

46 N. Li, H. S. Overkleeft, B. I. Florea, Activity-based protein profiling; an enabling technology in chemical biology research. *Curr. Opin. Chem. Biol.* 2012; **16**: 227-233

47 J. E. Hein, V. V. Fokin, Copper-catalyzed azide-alkyne cycloaddition (CuAAC) and beyond: new reactivity of copper(I) acetylides. *Chem. Soc. Rev.* 2010; **39**: 1302-1315.

48 K. Hoffmann, Y. Kiso, An approach to the targeted attachment of peptides and proteins to solid supports. *Proc. Natl. Acad. Sci. U.S.A.* 1976; **73**: 3516-3518.

49 N. M. Green, Avidin. *Adv. Protein Chem.* 1975; **29**: 85-133.

50 R. Bielski, Z. Witczak, Strategy for coupling molecular units if subsequent decoupling is requred. *Chem. Rev.* 2013; **113**: 2205-2243.

51 K. D. Park, R. Liu, H. Kohn, Useful tools for biomolecule isolation, detection, and identification: acylhydrazone-based cleavable linkers. *Chem. Biol.* 2009; **16**: 763-772.

52 A. Dirksen, S. Yegneswaran, P. E. Dawson, Bisaryl hydrazones as exchengeable biocompatible linkers. *Angew. Chem. Int. Ed.* 2010; **49**: 2023-2027.

53 S. Lee, W. Wang, Y. Lee, N. S. Sampson, Cyclic acetals as cleavable linkers for affinity capture. *Org. Biomol. Chem.* **2015**; *13*: 8445-8452.

54 S. M. Agten, P. E. Dawson, T. M. Hackeng, Oxime conjugation in protein chemistry: from carbonyl incorporation to nucleophilic catalysis. *J. Pept. Sci.* 2016; **22**: 271-279.

55 A. Otaka, K. Sato, H. Ding, A. Shigenaga, One-pot/sequenctial native chemical ligation using *N*-sulfanylethylanilide peptide. *Chem. Record* 2012; **12**: 479-490.

56 A. Otaka, K. Sato, A. Shigenaga, Chemical synthesis of proteins using *N*-sulfanylethylanilide peptides, based on N-S acyl transfer chemistry. *Top. Curr. Chem.* 2015; **363**: 33-56.

57 T. Morisaki, M. Denda, J. Yamamoto, D. Tsuji, T. Inokuma, K. Itoh, A. Shigenaga, A. Otaka, An *N*-sulfanylethylanilide-based traceable linker for enrichment and selective labelling of target proteins. *Chem. Commun.* 2016; **52**: 6911-6913.

Figure Legends

Scheme 1. A concept of Spr-based functional control of a peptide/protein that can respond to any stimulus by replacing the PG unit (PG: a protecting group that is removable by a stimulus; Spr': a Spr derivative).

Scheme 2. Lactonization of a trimethyl lock under mild conditions. Three methyl groups that are essential to acceleration of lactonization are depicted in red.

Scheme 3. Stimulus-responsive peptide-bond-cleavage induced by Spr.

Scheme 4. Synthesis of UV-responsive Spr (FmocOSu: Fmoc *N*-hydroxysuccinimide ester; HMPA: hexamethylphosphoric triamide; PCC: pyridinium chlorochromate; TBSOTf: *tert*-butyldimethylsilyl triflate).

Scheme 5. UV-responsive peptide bond cleavage induced by UV-responsive Spr.

Scheme 6. FRET-based kinetic study of Spr-induced peptide-bond-cleavage reaction

(Dab: 4-dimethylaminoazobenzene-4'-sulfonyl; FTC: fluorescein 5-aminothiocabonyl).

Scheme 7. ON-to-ON control of peptidyl function based on combination of Spr and a click peptide (CPP: cell penetrating peptide; I_C: C-intein; I_N: N-intein; NES: nuclear export signal; NLS: nuclear localization signal; POI: protein of interest).

Scheme 8. Visualization of hypoxic environment in living cells using hypoxia-responsive Spr.

Scheme 9. Caged ceramide that generates parent ceramide by UV-irradiation.

Scheme 10. Functional controlling system for a cysteine protease. A) A concept of ON-to-OFF-to-ON strategy. B) A key unit and reactions of ON-to-OFF-to-ON strategy.

Scheme 11. Schematic representation of a straightforward identification protocol of a target protein of a bioactive ligand.

Scheme 12. A Spr-based traceable linker that enables enrichment and selective labeling of a target protein.

Figure 1. Spr derivatives developed so far (*chiral (*S*)-Spr was synthesized).

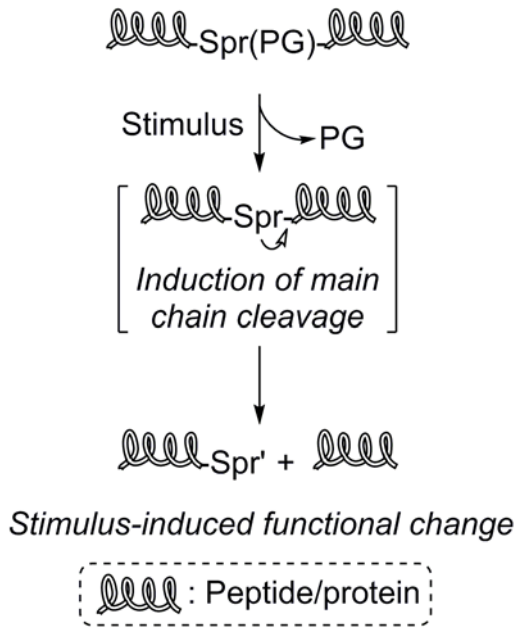
Figure 2. Photo-responsive growth system of peptide nanofibers that can be addressed by DNA hybridization.

Figure 3. Thiol-responsive DNA-releasing device. Melting temperature (T_m) of hybridized PNA **13** and complementary DNA (dA_9) are depicted in the table.

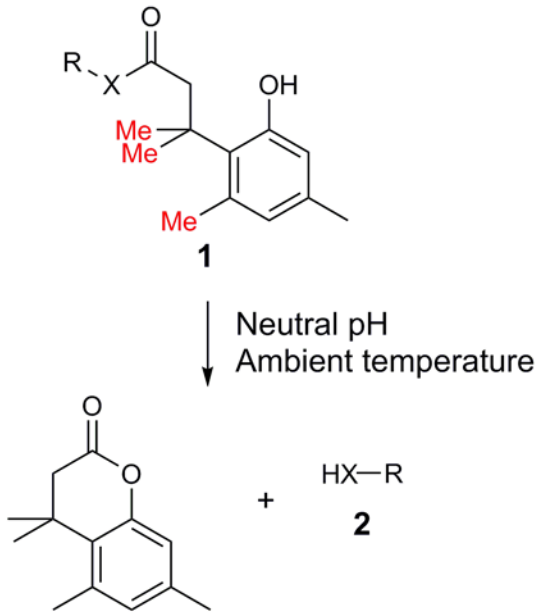
Figure 4. Near-infrared irradiation- and low pH-responsive delivery system for tumor-targeting therapy.

Schemes and Figures

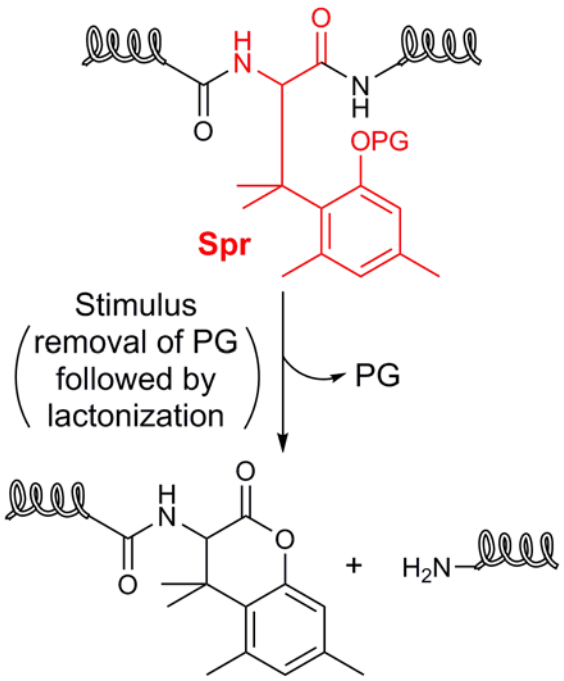
Scheme 1



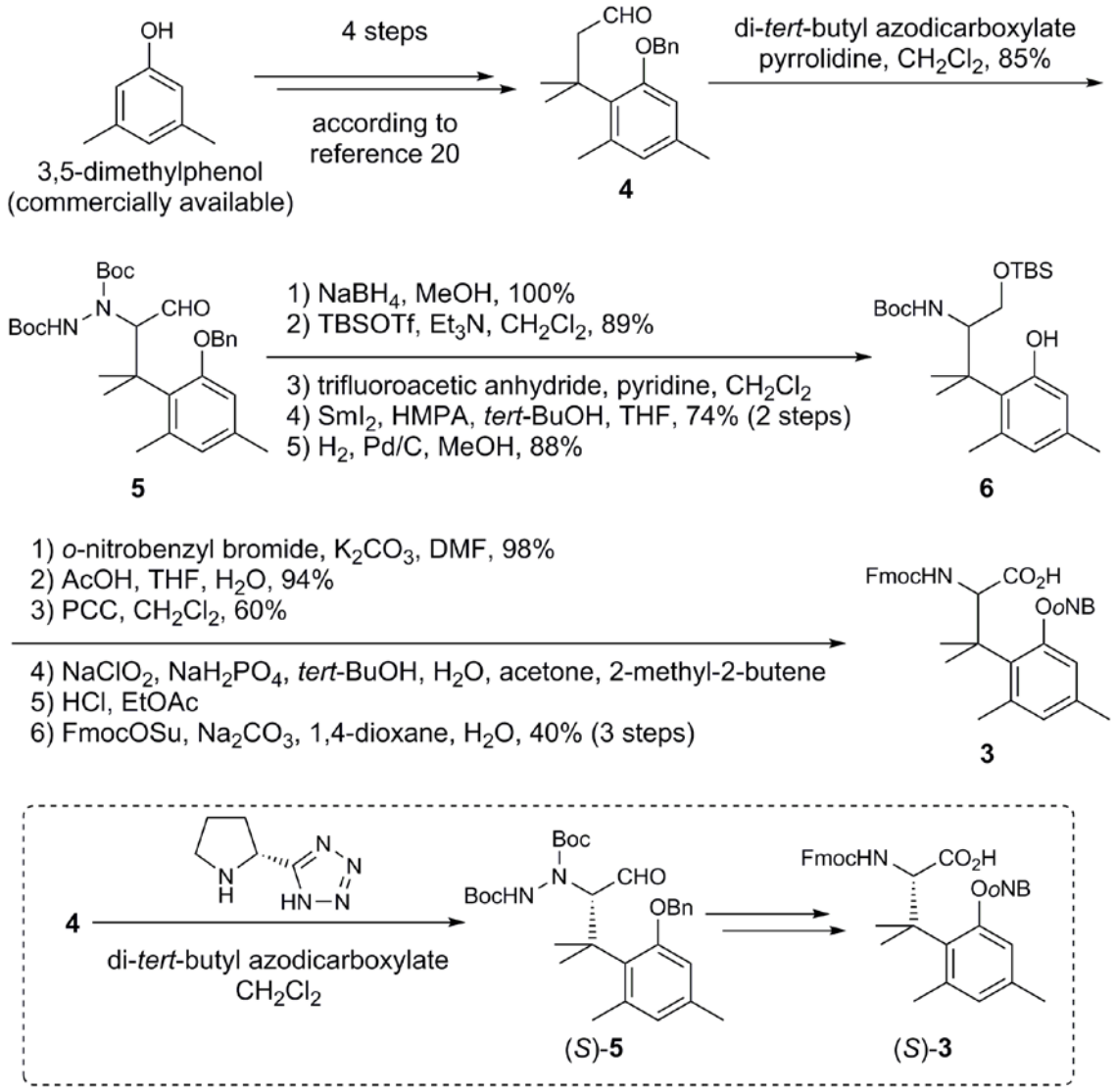
Scheme 2



Scheme 3


H
N
O
O
N
H
O
OPG
Spr
Stimulus
removal of PG
followed by
lactonization
PG
H
N
O
O
O
+
H2N

Scheme 4 図中にリファレンスあり



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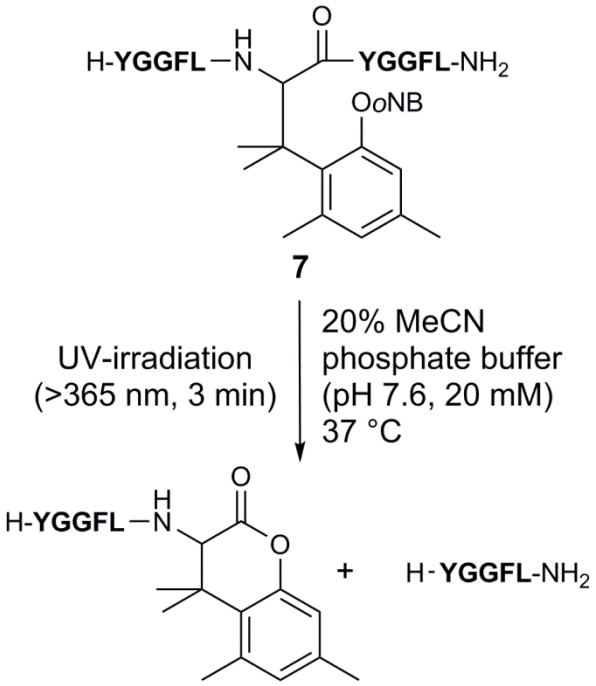
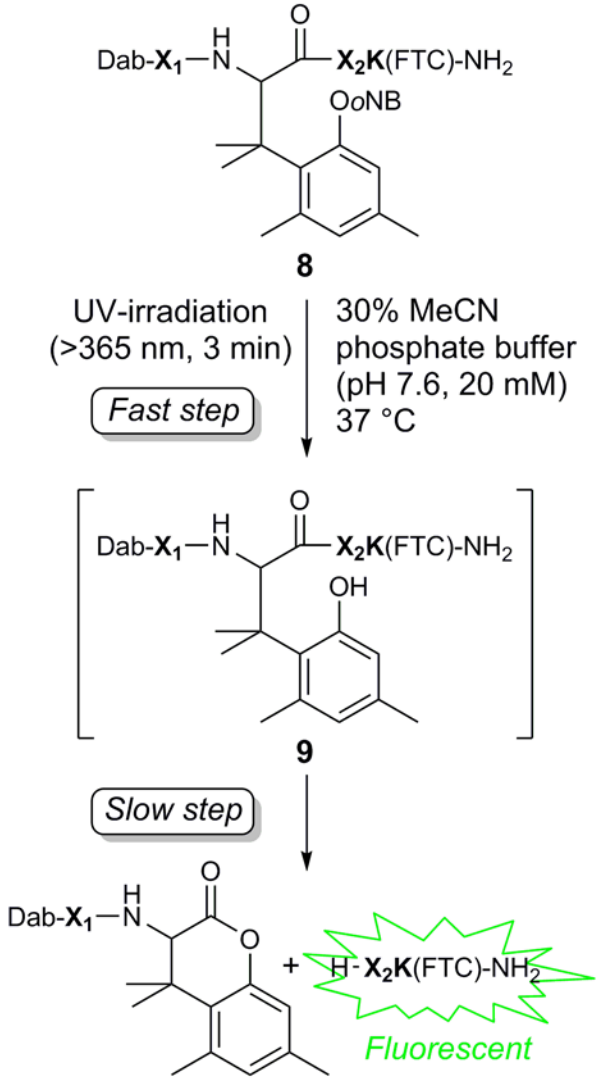
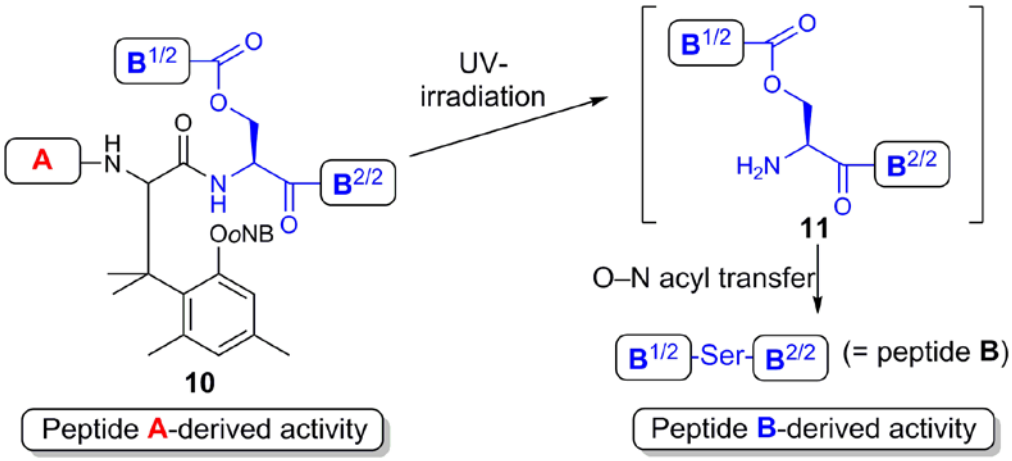

H-YGGFL
H
N
O
YGGFL-NH2
OoNB
7
UV-irradiation
(>365 nm, 3 min)
20% MeCN
phosphate buffer
(pH 7.6, 20 mM)
37 °C
H-YGGFL
H
N
O
O
+
H-YGGFL-NH2

Scheme 6

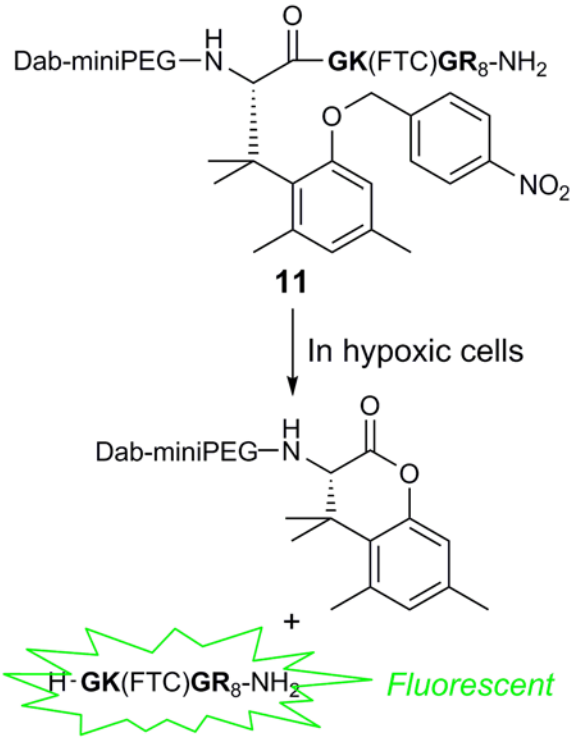

Dab-X1—N
H
O
X2K(FTC)-NH2
OoNB
8
UV-irradiation
(>365 nm, 3 min)
Fast step
30% MeCN
phosphate buffer
(pH 7.6, 20 mM)
37 °C
OH
9
Slow step
+
H-X2K(FTC)-NH2
Fluorescent

Scheme 7 図中にリファレンスあり

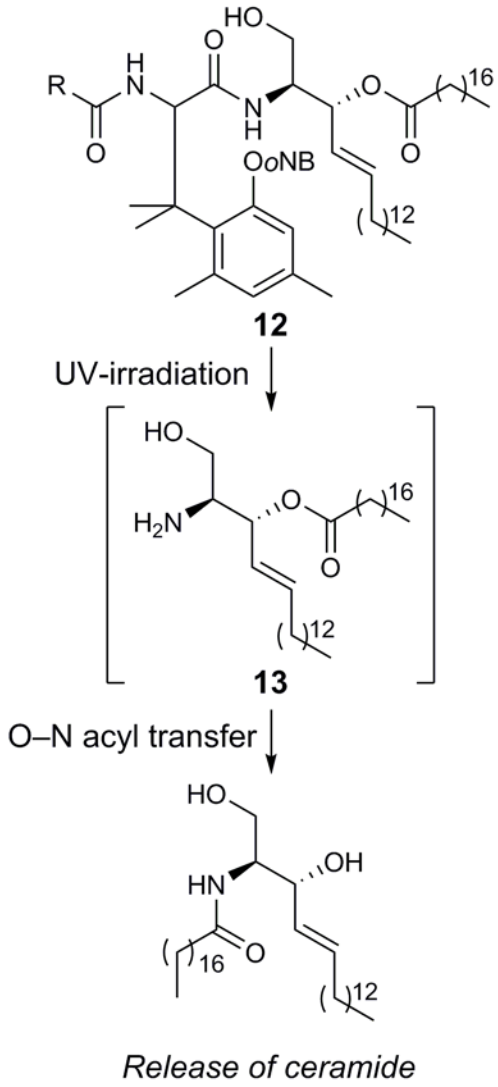


	A { Sequence (Function)	B { Sequence (Function)
Photo-control of localization of peptide in cell [19]	CPP and NLS (Localization in nucleus)	NES (Escape from nucleus to cytoplasm)
Photo-triggered fluorescent labeling of POI in cell [36]	CPP (Internalization in cell)	I_C with quencher at $B_{1/2}$ and fluorophore at $B_{2/2}$ (Labeling of I_N fused POI)

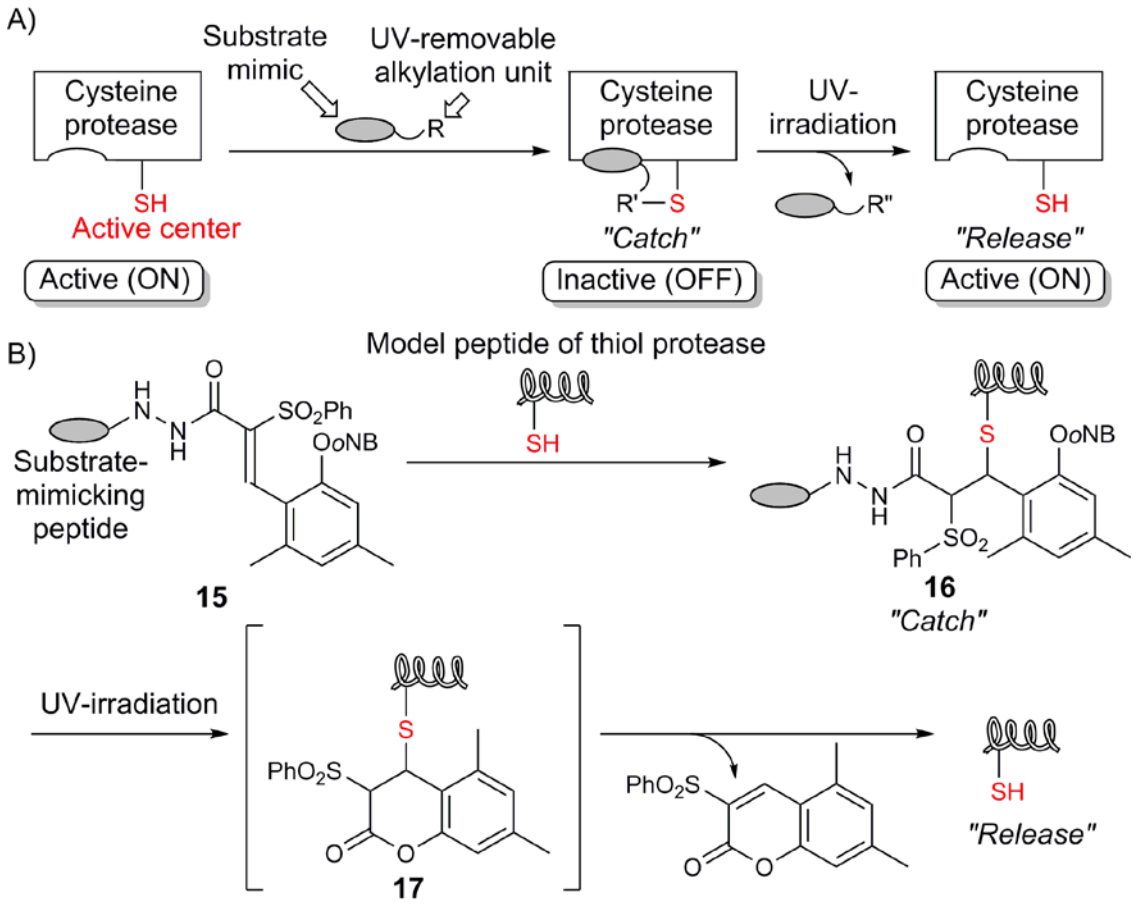
Scheme 8


Dab-miniPEG
H
N
O
GK(FTC)GR8-NH2
O
NO2
11
In hypoxic cells
Dab-miniPEG
H
N
O
O
+
H-GK(FTC)GR8-NH2
Fluorescent

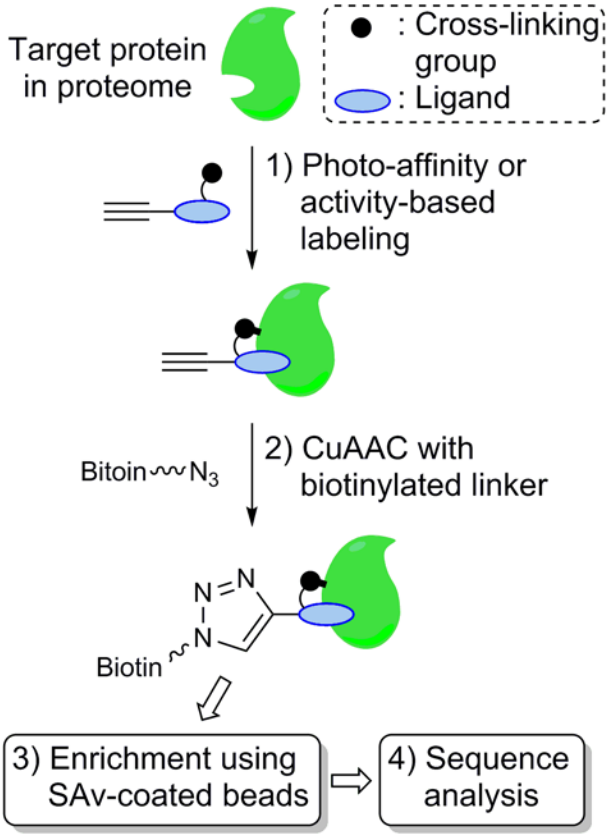
Scheme 9


HO
O
R
H
N
O
N
H
O
16
O
OoNB
12
12
UV-irradiation
HO
H2N
O
16
O
12
13
O–N acyl transfer
HO
OH
HN
16
O
12
Release of ceramide

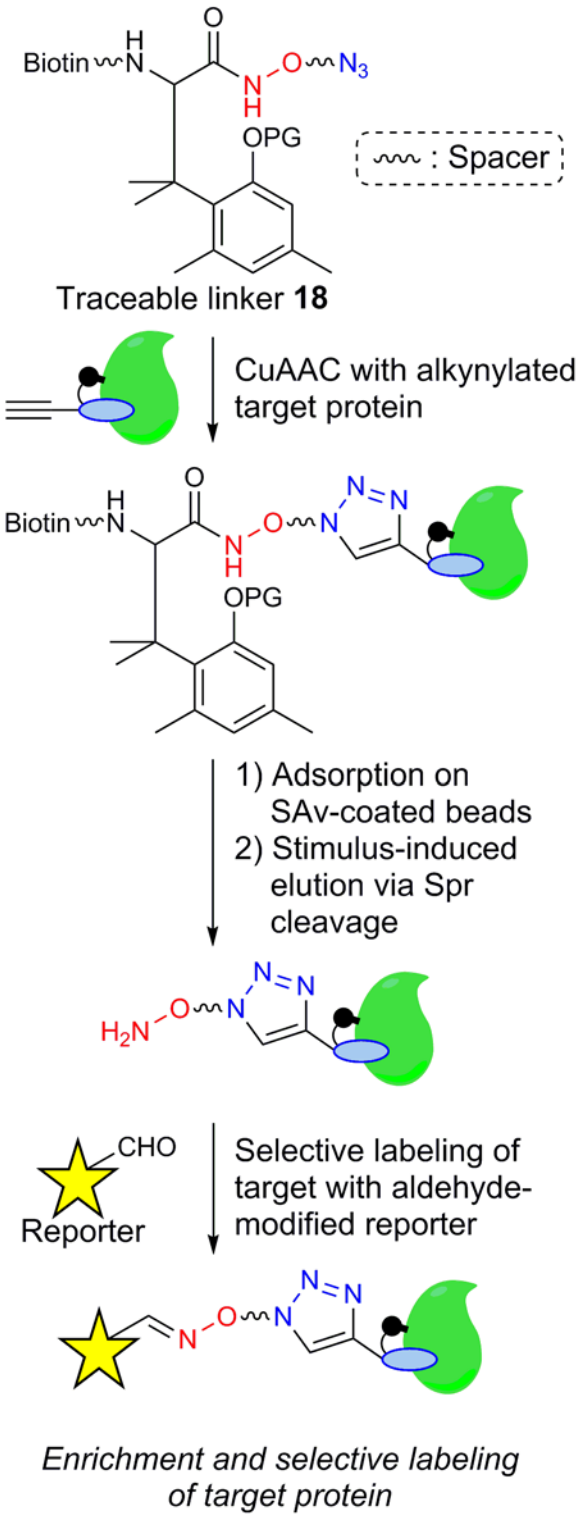
Scheme 10


A)
Substrate mimic
UV-removable alkylation unit
Cysteine protease
SH
Active center
Active (ON)
R
Cysteine protease
R'—S
"Catch"
Inactive (OFF)
UV-irradiation
R"
Cysteine protease
SH
"Release"
Active (ON)
B)
Model peptide of thiol protease
SH
Substrate-mimicking peptide
SO2Ph
OoNB
15
16
"Catch"
UV-irradiation
PhO2S
17
PhO2S
SH
"Release"

Scheme 11

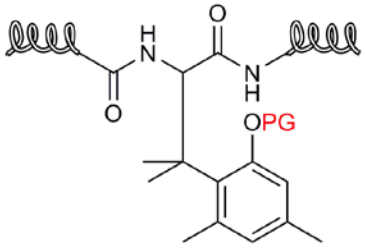

Target protein in proteome
: Cross-linking group
: Ligand
1) Photo-affinity or activity-based labeling
Bitoin
N3
2) CuAAC with biotinylated linker
N
N
N
Biotin
3) Enrichment using SAv-coated beads
4) Sequence analysis

Scheme 12



Enrichment and selective labeling of target protein

Figure 1



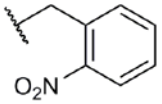
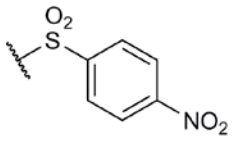
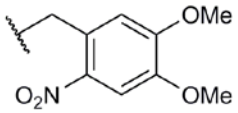
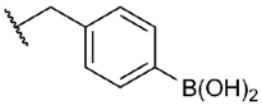
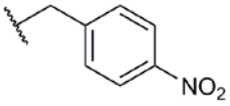
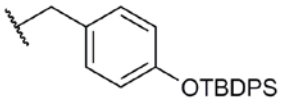
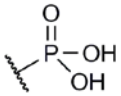
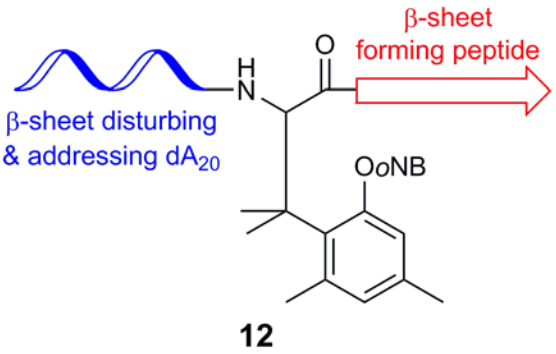
Stimulus	PG	Stimulus	PG
UV*		Thiol*	
Two-photon near-infrared excitation		Hydrogen peroxide*	
Hypoxia in cell*		Fluoride anion*	
Alkaline phosphatase			

Figure 2



1) Hybridization with dT_{20} that is immobilised on glass
2) UV-induced peptide bond cleavage followed by release of β-sheet forming peptide
3) Nanofiber formation

Figure 3

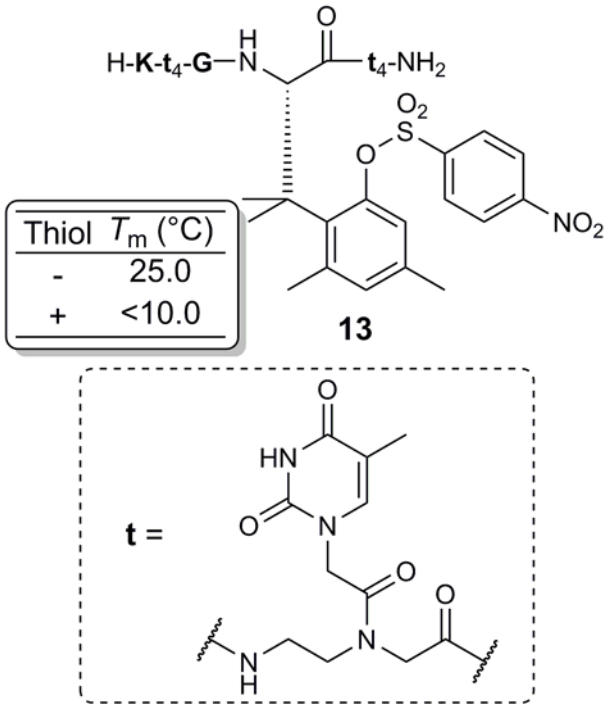
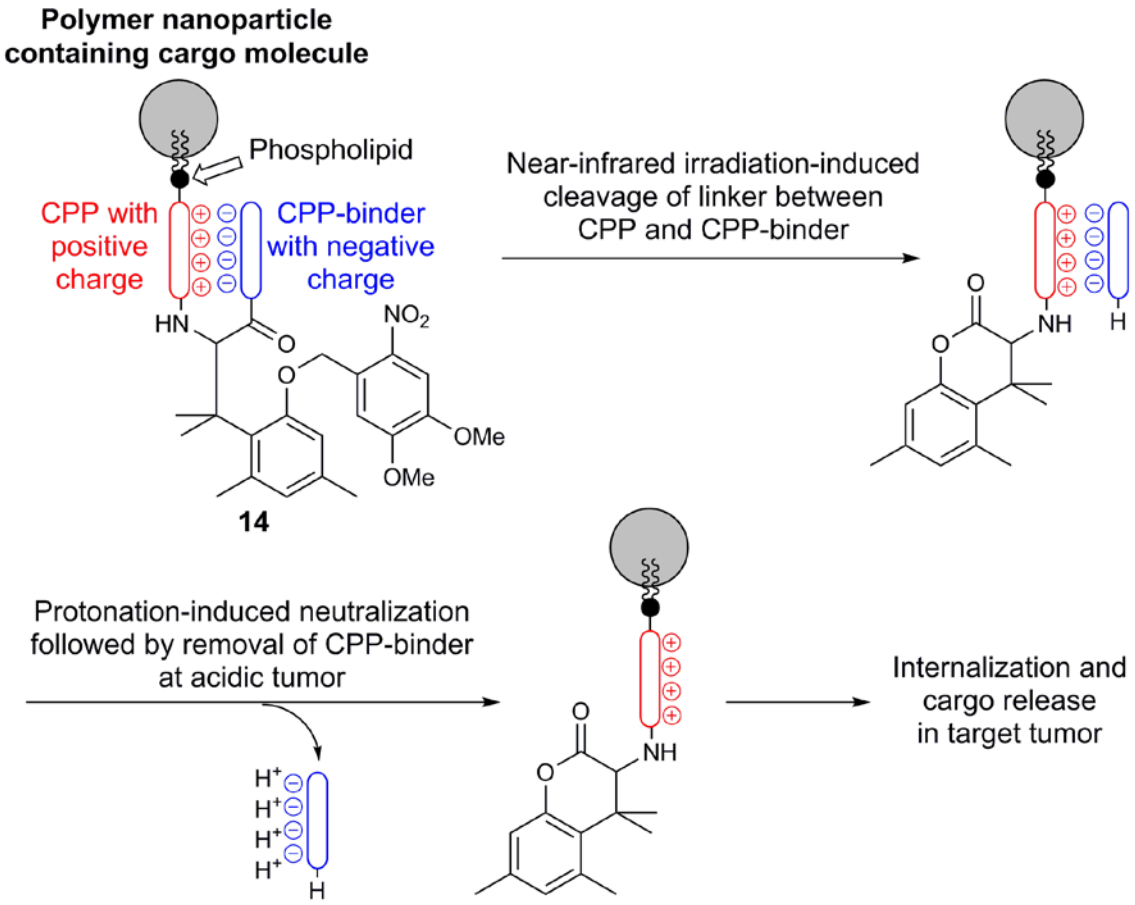


Figure 4


Polymer nanoparticle containing cargo molecule
Phospholipid
CPP with positive charge
CPP-binder with negative charge
HN
O
O
NO2
OMe
OMe
14
Near-infrared irradiation-induced cleavage of linker between CPP and CPP-binder
O
O
NH
H
Protonation-induced neutralization followed by removal of CPP-binder at acidic tumor
H+
H+
H+
H+
H
O
O
NH
Internalization and cargo release in target tumor