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Synthesis of a Stimulus-responsive Processing Device and Its Application to a Nucleocytoplasmic Shuttle Peptide

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Temporally and spatially controlling the function of peptides/proteins by a stimulus has received increased attention due to its potential in various fields such as chemical biology and drug delivery. Recently, photo-induced processing (peptide bond cleavage) or conformational change has been successfully applied to convert inactive (or active) peptides and proteins into their corresponding active (or inactive) forms at a desired time and location.[1a-i] Increasing the diversity of the trigger involved in processing reaction may facilitate stimulus-responsive processing to become a general method for controlling peptide functions. Therefore, we attempted to design a molecular basis of an amino acid derivative, which induces a processing reaction as a response to a wide variety of stimuli. Inspired by the trimethyl lock system,[2,3] we designed stimulus-responsive peptide **1**, which can release a functional peptide after a stimulus-induced removal of a phenolic protective group (PG) and subsequent processing reaction (Scheme 1). Designed peptide **1** features the nucleophilic involvement of a regenerated phenolic hydroxyl group to an adjacent peptide bond to release functional peptides. In this paper, we chose a photo- removable *o*-nitrobenzyl (*o*-NB) and a phosphatase-removable phosphate group for phenolic protection to afford stimulus-responsive model peptides.

Scheme 2 shows the synthesis for photo-responsive model peptide **8**. Aldehyde **2**[2] was α-aminated with di-*tert*-butyl azodicarboxylate in the presence of pyrrolidine. After reduction of the aldehyde group with sodium borohydride, the resulting alcohol was protected with a TBS group to give silyl ether **3**. Trifluoroacetylation of the terminal nitrogen in **3** with trifluoroacetic anhydride (TFAA) and subsequent N-N bond cleavage by SmI_2 in the presence of HMPA and *tert*-BuOH gave amino alcohol **4**. The benzyl group on **4** was removed by hydrogenolysis and the generated phenolic hydroxyl group was protected with an *o*-nitrobenzyl group to afford *o*-NB ether **5**. After removing the TBS group of **5** under acidic conditions, a two-step oxidation was performed to give carboxylic acid **6**. The Boc group on **6** was removed with hydrogen chloride in ethyl acetate to yield an amine, which was reprotected with an Fmoc group to give Fmoc protected photo-responsive processing device **7**. The total yield of Fmoc derivative 7 amounted to 11% over 12 steps beginning from aldehyde **2**. Finally, incorporating amino acid **7** into the peptide by standard Fmoc solid phase peptide synthesis (SPPS) afforded photo-responsive model peptide **8** as a diastereomeric mixture.

To examine the photo-reactivity of model peptide **8**, we performed the photo-processing reaction outlined in Scheme 3. Peptide **8** in 20% MeCN/phosphate buffer (pH 7.6) was irradiated by UV light (>365 nm) for 3 min and then incubated at 37 °C. The reaction progress was monitored by HPLC and the peptides were characterized by ESI-MS (see Supporting Information, Figure S1). The *o*-nitrobenzyl group of peptide **8** was completely removed after 3 min of UV irradiation to generate deprotected intermediate **9**. Afterwards, intermediate **9** was converted to corresponding processing products **10** and **11** via incubation for 2 h. Monitoring the processing reaction clarified that the half-life of intermediate **9** was approximately 34 min.[4]

To demonstrate the applicability of this processing system to other stimuli, we designed phosphatase-responsive model peptide **12** in which the phenolic hydroxyl group was protected with phosphate (Scheme 4). Peptide **12** was synthesized by Boc SPPS (see Supporting Information). A phosphatase-responsive processing reaction of peptide **12** was examined in the presence of alkaline phosphatase derived from calf intestine. Model peptide **12** was incubated with alkaline phosphatase in Tris-HCl buffer (pH 7.9) at 37 °C. After 24 h, HPLC and ESI-MS analyses clarified that substrate **12** completely disappeared and processing products **10** and **13** were obtained in good purity (see Supporting Information, Figure S2). In the absence of an alkaline phosphatase, hydrolysis of the phosphate moiety was not detected under these conditions (data not shown).

Next, we applied photo-responsive processing device **7** to a nucleocytoplasmic shuttle peptide as shown in Scheme 5. Shuttle peptide **14** possesses a cell penetrating peptide (CPP) sequence[5] and a nuclear localization signal (NLS)[6] at the *N*-terminal position and *O*-acyl isopeptide part as a functionally suppressed nuclear export signal (NES)[7] precursor. Once added to a cell, shuttle peptide **14** should penetrate a cell membrane and localize into the nucleus by the function of CPP and NLS. Upon UV irradiation and subsequent removal of CPP and NLS, generated *O*-acyl isopeptide intermediate **16** can easily isomerize

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to afford a matured NES sequence via *O*-*N* acyl shift reaction[1c,e] and return to the cytoplasm.

Shuttle peptide **14** was synthesized by Fmoc SPPS (see Supporting Information). For a localization study, a FTC (5-fluorescein-5-thiocarbamoyl) group was introduced at the *N*-terminal of the isopeptide through a 4-aminobutyric acid (Abu) linker. Next, we examined a photo-processing reaction of shuttle peptide **14** in a manner similar to that of photo-responsive peptide **8**. Upon UV irradiation and subsequent incubation at 37 °C, the shuttle peptide was completely consumed to generate processing products **15** and **17** as the major products (see Supporting Information, Figure S3). In this case, intermediate **16** was not detected, presumably due to its high lability under basic conditions.[1c,e,8] We obtained a small amount of NES derivatives, which may be caused by a reaction with a singlet oxygen.[9]

The above results encouraged us to evaluate the subcellular localization of the shuttle peptide in living cells. Chinese hamster ovary (CHO)-K1 cells were incubated in a serum medium containing FTC-labelled shuttle peptide **14** (10 μM) for 1 h at 37 °C. After incubation, the cells were treated with UV irradiation. As shown in Figure 1, most of the cells, which were transduced by shuttle peptide **14** in the control experiment, exhibited a remarkable co-localization with Hoechst 33258-stained nuclei [UV (-)]. After UV irradiation (>365 nm, 4 min) and an additional 1 h of incubation, the cells exhibited cytoplasmic fluorescence but not a nuclear pattern due to the export of shuttle peptide [UV (+)]. In contrast, treating with leptomycin B (LMB, 200 nM)[10] as an NES inhibitor after UV irradiation [UV (+) + LMB] inhibited the release of the fluorescent peptide form nuclei. These results suggest that photo-induced *O*-*N* acyl shift product **17**, which possesses an NES sequence, was generated in living cells.

In conclusion, we developed a photo- and phosphatase-responsive self-processing peptide. The processing reaction was triggered by the deprotection of the phenolic hydroxyl moiety and the processing products were obtained in good purity. Reaction monitoring revealed that the reaction rate depends on the amino acid next to the stimulus-responsive residue. Then, photo-responsive processing device **7** was successfully combined with the isopeptide rearrangement system to afford the nucleocytoplasmic shuttle peptide. A localization assay clarified that the shuttle peptide **14** localized inside the nucleus before UV irradiation, but it returned to a cytoplasm after UV irradiation. This unprecedented peptide manipulation consisting of processing and isopeptide rearrangement system would have an extraordinary impact on peptide-based chemical biology fields.

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Keywords: hydrolysis · peptide · photoreaction · stimulus responsive · trimethyl lock

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[4] Replacement of Tyr [7] adjacent to the photo-responsive residue with Gly enhanced the reaction rate, presumably due to the smaller steric hindrance of Gly compared to that of Tyr (Half-life: 5 min).

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[8] The retention time of processing product **17** was identical to that of authentic sample which was synthesized separately.

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Legends for Schemes and Figure

Scheme 1. Design of a stimulus-responsive peptide.

Scheme 2. Reagents and conditions. (i) di-*tert*-butyl azodicarboxylate, pyrrolidine, CH_2Cl_2, 85%; (ii) $NaBH_4$, MeOH, 100%; (iii) TBSOTf, Et_3N, CH_2Cl_2, 89%; (iv) TFAA, pyridine, CH_2Cl_2; (v) SmI_2, HMPA, *tert*-BuOH, THF, 74% (2 steps); (vi) H_2, Pd/C, MeOH, 88%; (vii) *o*-nitrobenzyl bromide, K_2CO_3, DMF, 98%; (viii) AcOH, THF, H_2O, 94%; (ix) PCC, CH_2Cl_2, 60%; (x) $NaClO_2$, NaH_2PO_4, *tert*-BuOH, H_2O, acetone, 2-methyl-2-butene; (xi) HCl, AcOEt; (xii) FmocOSu, Na_2CO_3, 1,4-dioxane, H_2O, 40% (3 steps); (xiii) Fmoc SPPS.

Scheme 3. Photo-processing reaction of model peptide **8**.

Scheme 4. Alkaline phosphatase-induced processing reaction.

Scheme 5. Design of a nucleocytoplasmic shuttle peptide. (Abu: 4-aminobutyryl; CPP: cell penetrating peptide; FTC: fluorescein-5-thiocarbamoyl; NES: nuclear export signal; NLS: nuclear localization signal; *o*-NB: *o*-nitrobenzyl)

Figure 1. Confocal microscopic images of CHO-K1 cells treated with the FTC-labelled shuttle peptide. UV (-): CHO-K1 cells were incubated with shuttle peptide **14** (10 μM) in Ham's F-10 FBS (-) at 37 °C for 1 h. UV (+): After incubation with the shuttle peptide, cells were irradiated by UV light (>365 nm) for 4 min, and then incubated at 37 °C for additional 1 h. UV (+) + LMB: Before an additional 1 h of incubation, cells were treated with leptomycin B.

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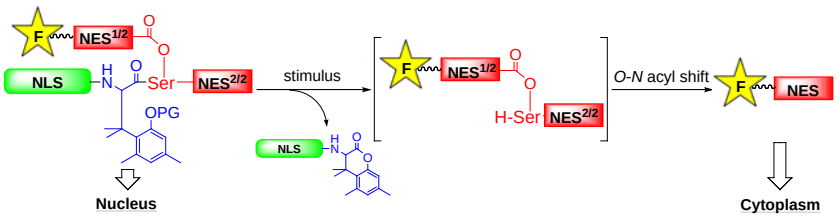
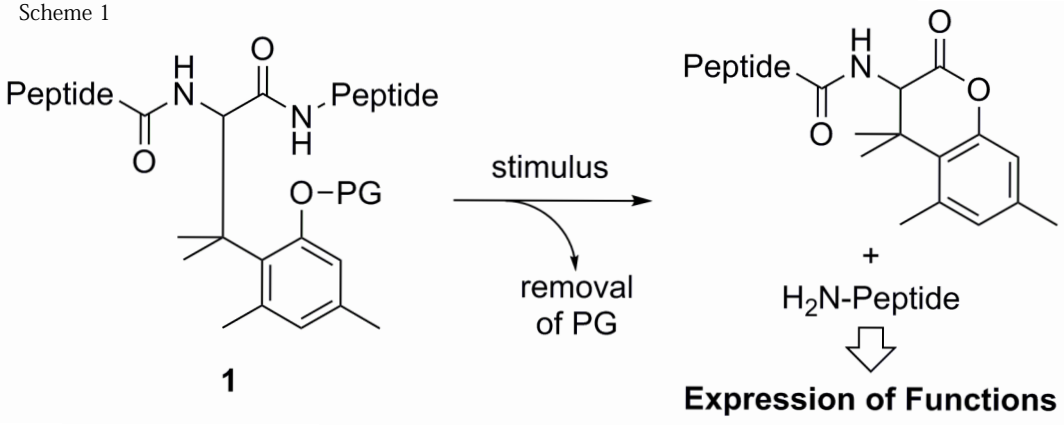
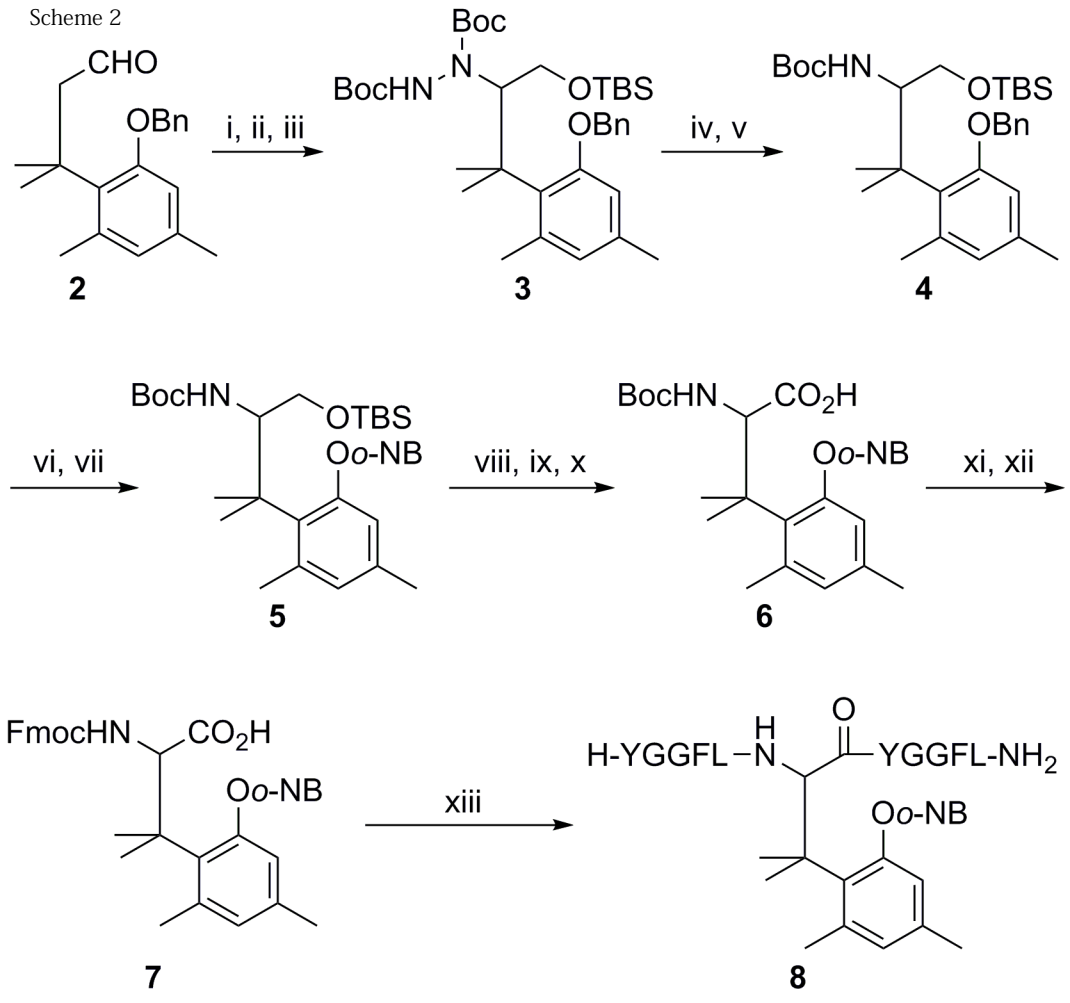


Stimulus-responsive processing (peptide bond cleavage) devices were developed. The processing reaction was triggered by stimulus-induced removal of a PG and the processing products were obtained in good purity. A photo-responsive processing device was successfully applied to develop a nucleocytoplasmic shuttle peptide. (F: fluorophore, NES: nuclear export signal. NLS: nuclear localization signal. PG: stimulus-responsive protective group)

Scheme 1


Peptide
H
N
O
O
N
H
Peptide
O–PG
1
stimulus
removal
of PG
Peptide
H
N
O
O
O
+
H2N-Peptide
Expression of Functions

Scheme 2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OBn
i, ii, iii
Boc
N
BocHN
OTBS
OBn
iv, v
BocHN
OTBS
OBn
2
3
4
vi, vii
BocHN
OTBS
Oo-NB
viii, ix, x
BocHN
CO2H
Oo-NB
xi, xii
5
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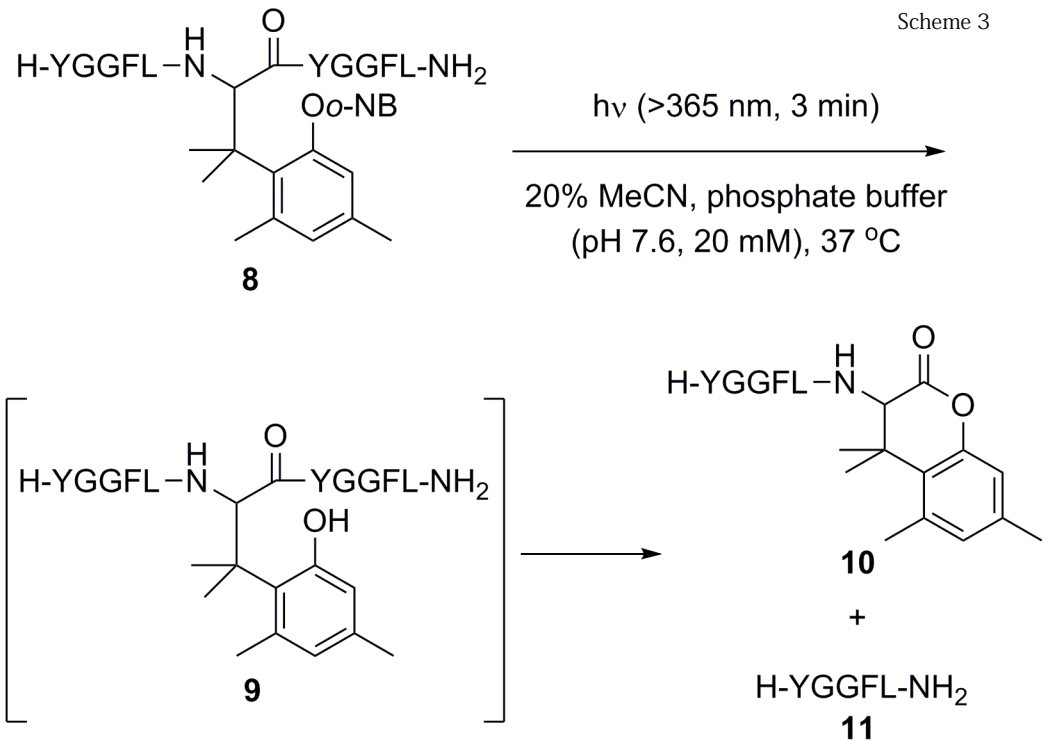
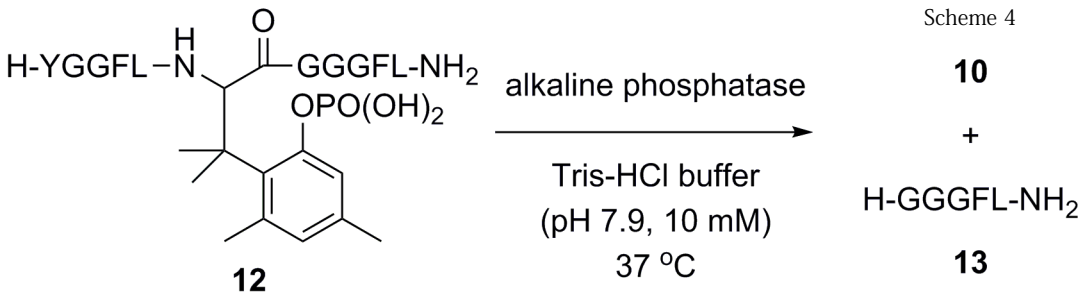
Oo-NB
7
8


H-YGGFL–N
H
O
YGGFL-NH2
Oo-NB
8
hν (>365 nm, 3 min)
20% MeCN, phosphate buffer
(pH 7.6, 20 mM), 37 °C
H-YGGFL–N
H
O
YGGFL-NH2
OH
9
H-YGGFL–N
H
O
O
10
+
H-YGGFL-NH2
11

Scheme 4


H-YGGFL
N
H
O
GGGFL-NH2
OPO(OH)2
alkaline phosphatase
Tris-HCl buffer
(pH 7.9, 10 mM)
37 °C
10
+
H-GGGFL-NH2
13

12

Scheme 5

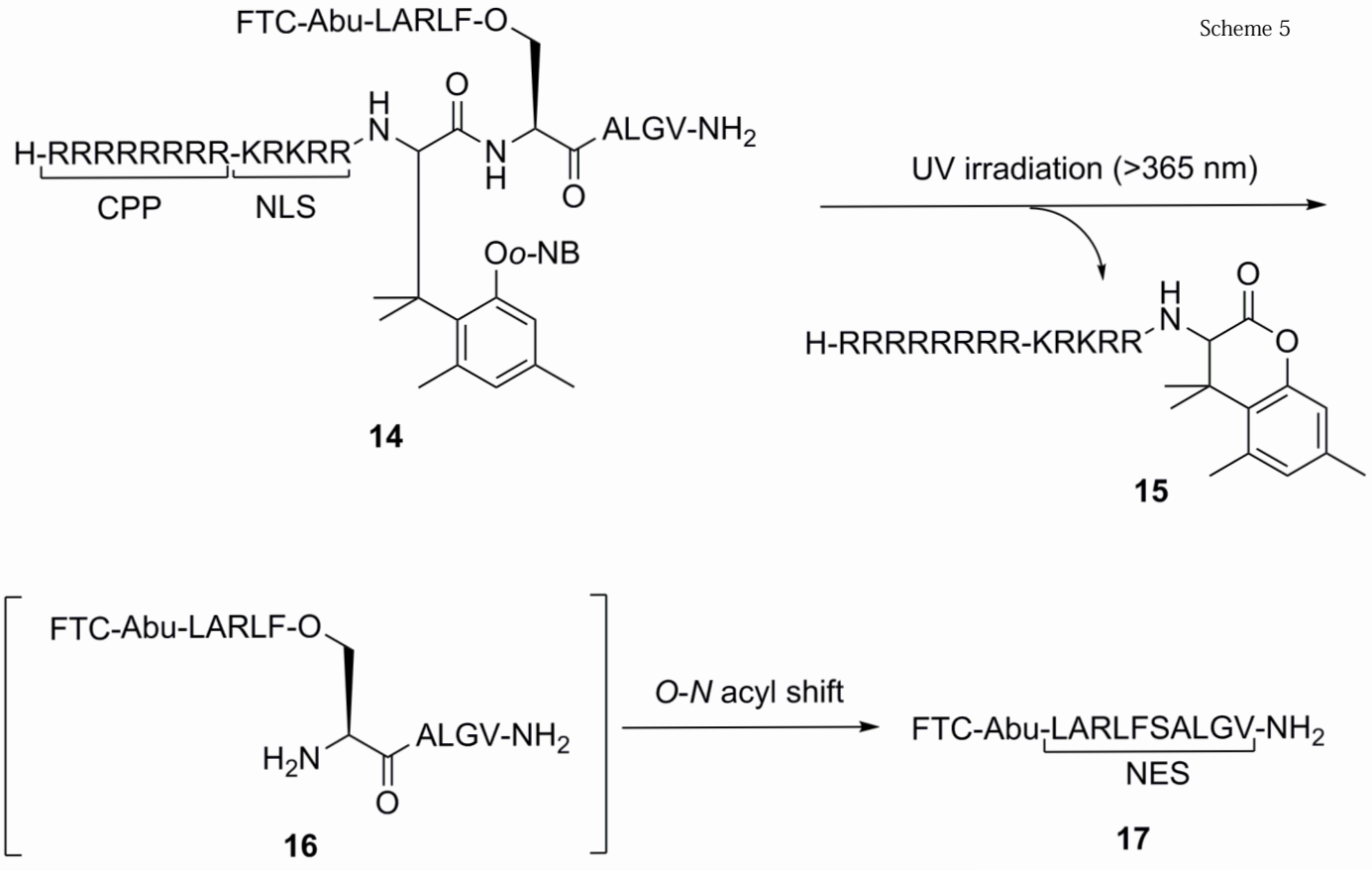

FTC-Abu-LARLF-O
H-RRRRRRRR-KRKRR
CPP
NLS
ALGV-NH2
Oo-NB
14
UV irradiation (>365 nm)
H-RRRRRRRR-KRKRR
15
FTC-Abu-LARLF-O
H2N
ALGV-NH2
16
O-N acyl shift
FTC-Abu-LARLFSALGV-NH2
NES
17

Figure 1