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FRET-based Assay of the Processing Reaction Kinetics of Stimulus-Responsive Peptides: Influence of Amino Acid Sequence on Reaction Kinetics

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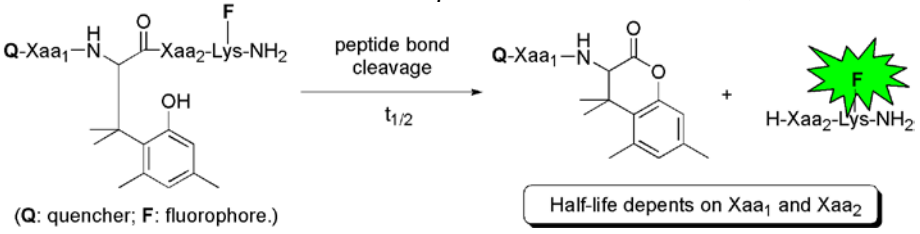

(**Q**: quencher; **F**: fluorophore.)

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FRET-based assay of the processing reaction kinetics of stimulus-responsive peptides: Influence of amino acid sequence on reaction kinetics

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Abstract—In the field of chemical biology, methods for controlling peptidyl function by a stimulus are attracting increasing attention. Recently, we reported a stimulus-responsive peptide which can be cleaved after exposure to a stimulus. In this study, we developed a FRET-based assay system to estimate the kinetics of the stimulus-induced processing (peptide bond cleavage) reaction. Based on the FRET system, it was clarified that introduction of a sterically less-hindered or polar residue at the position adjacent to the stimulus-responsive amino acid accelerates the processing reaction.

Keywords: FRET, stimulus responsive, peptide bond cleavage, UV irradiation.

1. Introduction

In the field of chemical biology, it is important to develop technology to control the function of intracellular peptides and proteins by an external stimulus. In this context, photo-responsive bond cleavage reaction[1] or conformational change[2] of a peptide/protein backbone has been successfully applied for spatiotemporal control of its function. Whereas these stimulus-responsive peptides and proteins can respond to specific stimuli, we developed a stimulus-responsive peptide which is applicable to arbitrary stimuli. Based on the trimethyl lock system,[3] we previously reported a stimulus-responsive processing (peptide bond cleavage) device with successful application for controlling peptidyl function that induces processing reaction after exposure to a stimulus such as UV-irradiation or enzymatic reaction.[4] When peptide **1** possessing the stimulus-responsive processing device was exposed to stimulus, removal of PG (protective group removable by a stimulus) followed by nucleophilic involvement of the generated phenolic hydroxyl group afforded processing products in good purity (Scheme 1). In a previous report, we briefly mentioned that the rate of the processing reaction was dependent on the amino acid adjacent to the stimulus-responsive residue. In the present paper, we report details of the influence of amino acid sequence on the kinetics of the processing reaction.

##Scheme 1##

2. Results and Discussions

2.1. Design of the FRET substrate

Fluorescence resonance energy transfer (FRET) is one of the most widely used techniques to evaluate kinetics of the cleavage reaction of a peptide.[5] It was reported that a fluorescein-5-thiocarbamoyl (FTC)-dabsyl (Dab) based FRET system is compatible with a photo-removable *o*-nitrobenzyl (*o*NB) group.[6] Therefore, we designed FTC-Dab based FRET substrate **2** possessing a photo-responsive amino acid protected by an *o*NB group (Scheme 2). If peptide **2** was irradiated by UV (>365 nm) for 3 min, it should be completely deprotected to generate intermediate **3** with weak fluorescence. Afterward, intermediate **3** would be converted to processing products **4** and **5** thereby emitting a strong fluorescence. Based on the time course of fluorescence intensity, kinetics of the processing reaction of intermediate **3** could be estimated.

##Scheme 2##

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2.2. Synthesis of the FRET substrate

First, FRET substrates **2** were synthesized as shown in Scheme 3. The peptide was elongated on NovaSyn® TGR resin using Fmoc solid phase peptide synthesis (Fmoc SPPS), and the peptide was capped with dabsyl chloride to yield peptide resin **6**. After removal of the Alloc group on side chain of Lys, the regenerated amine was reacted with fluorescein-5-isothiocyanate (FITC) to introduce a fluorophore. Finally, peptide resin **7** was treated with a cleavage cocktail (TFA/H_2O/thioanisole = 95/2.5/2.5, v/v) to give FRET substrate **2** that was purified as a diastereomeric mixture before subsequent experiments.[7] Because the coupling reaction of Fmoc protected photo-responsive amino acid **8**[4] on sterically hindered residues such as Asn(Trt), Cys(Trt) and Ser(*t*-Bu) did not proceed well, Cys(MPM) (MPM: *p*-methoxybenzyl) and side chain unprotected Asn, Ser and Thr were used for the synthesis of peptide **2** (Xaa_1 = Gly; Xaa_2 = Asn, Cys, Ser and Thr), respectively. This result suggests that introduction of the photo-responsive amino acid on a sterically hindered residue should be avoided for facile synthesis.

##Scheme 3##

2.3. Kinetics measurement of the processing reaction

First, we monitored the processing reaction of peptide **2** (Xaa_1 = Gly; Xaa_2 = Tyr) using HPLC, and processing products were characterized by ESI-MS. The UV-induced processing reaction of the FRET substrate is outlined in Scheme 2. Peptide **2** in phosphate buffer (pH 7.6) with 30% MeCN was irradiated with UV light (>365 nm) for 3 min and then incubated at 37 ˚C. After 3 min of UV irradiation, substrate **2** (Xaa_1 = Gly; Xaa_2 = Tyr) was completely deprotected to yield intermediate **3** (Xaa_1 = Gly; Xaa_2 = Tyr) (data not shown). As shown in Figure 1, intermediate **3** (Xaa_1 = Gly; Xaa_2 = Tyr) was converted to processing products **4** (Xaa_1 = Gly) and **5** (Xaa_2 = Tyr) after 3 h of incubation.

##Figure 1##

Next, we monitored the time course of the fluorescence intensity of the reaction mixture at 37 ˚C using a plate reader (λ_{ex} = 495 nm; λ_{em} = 520 nm). Before UV irradiation, fluorescence intensity of the reaction mixture was recorded as time = 0 min. Then, the reaction mixture was irradiated by UV light (>365 nm) for 3 min, and the fluorescence intensities were recorded at 3 (just after UV irradiation), 5, 10, 15, 30, 60, 120 and 180 min. The percentage of remaining intermediate **3** was estimated based on relative fluorescence intensity. As shown in Figure 2, the processing reaction of intermediate **3** (Xaa_1 = Gly; Xaa_2 = Tyr) shows first order dependence on the concentration of the intermediate, and the half life of **3** (Xaa_1 = Gly; Xaa_2 = Tyr) was determined as 18.0 min.[8]

##Figure 2##

These results encouraged us to determine half lives of the stimulus-responsive peptides with various amino acids at a position adjacent to the stimulus-responsive residue. FRET substrates **2** possessing various amino acid sequences were synthesized as mentioned above. Their half-lives were estimated based on the FRET assay system, and results are summarized in Figure 3. When a sterically less-hindered amino acid had been introduced at Xaa_2 position, rapid processing reaction was observed. Interestingly, introduction of a polar residue at Xaa_2 position also accelerated the reaction regardless of its steric hindrance. The polar residue might assist the processing reaction by hydrogen bond-mediated activation of the carbonyl group of the peptide bond and/or deprotonation of the phenolic hydroxyl group. In the meantime, replacement of Xaa_1 with sterically less-hindered Gly or polar Glu or Lys also accelerated the reaction; however, the polarity effect of the side chain was smaller than that observed for Xaa_2.

##Figure 3##

3. Conclusion

In conclusion, the half-lives of the stimulus-responsive peptides were determined using a FRET-based assay system. Introduction of a sterically less-hindered or polar residue at position Xaa_2 significantly accelerated the reaction. Replacement of Xaa_1 with a sterically less-hindered Gly or polar Glu or Lys also accelerated the reaction whereas the polarity effect was smaller than that observed for Xaa_2. These results enable us to control the processing reaction rate for different purposes by choosing an appropriate residue at a position adjacent to the stimulus-responsive amino acid.

4. Experimental

4.1. General

Exact mass spectra were recorded on Bruker Esquire200T or Waters MICROMASS® LCT PREMIER™. For HPLC separations, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1 mL/min) or a 5C_{18}-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA in water (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution. Infinite M200 flexible microplate reader (TECAN Austria GmbH) was used for fluorescence measurements. Photolysis was performed using Moritex MUV-202U with the filtered output (>365 nm) of a 3000 mW/cm^2 Hg-Xe lamp.

4.2. General procedure for a preparation of FRET substrate 2

Elongation of peptides on NovaSyn® TGR resin (0.25 mmol/g, 8.7 mg) was performed as described in previous

report.[4] For the synthesis of peptide **2** (Xaa_1 = Gly; Xaa_2 = Asn, Cys, Ser or Thr), Fmoc-Asn-OH, Fmoc-Cys(MPM)-OH, Fmoc-Ser-OH or Fmoc-Thr-OH was used as a building block respectively. Obtained resin was reacted with dabsyl chloride (2.1 mg) and triethylamine (1.8 μL) in DMF (70 μL) for 3 h. After washing with DMF followed by dichloromethane, Alloc group was removed by a treatment with $(Ph_3P)_4Pd$ (4.0 mg) and 1,3-dimethyl-barbituric acid (3.4 mg) in 1,2-dichloroethane (172 μL) overnight. Then the resin was washed with dichloromethane followed by DMF. To obtained resin were added fluorescein-5-isothiocyanate (FITC, 2.0 mg) and DIEA (10 μL) in DMF (50 μL), and the reaction was performed overnight. After filtration, the resin was treated with FITC (2.0 mg) and DIEA (0.88 μL) in DMF (50 μL) for additional 24 h. Finally, global deprotection of the peptide with TFA/thioanisole/H_2O (95/2.5/2.5, v/v) afforded a crude product. After trituration with diethyl ether, the precipitate was purified by HPLC to give FRET substrate **2** as a diastereomeric mixture in 10 to 20% yield. Analytical HPLC coditions: linear gradient of solvent B in solvent A, 30 to 90% over 30 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 40 to 70 over 30 min.

##Table 1##

4.3. Reaction monitoring of the processing reaction by using HPLC

The experiment was performed as described in previous report.[4] Analytical HPLC conditions: linear gradient of solvent B in solvent A, 30 to 90% over 30 min. ESI-MS: **4** (Xaa_1 = Gly) calcd for $[M + H]^+$ 564.2, obs 563.9. **5** (Xaa_2 = Tyr) cald for $[M + H]^+$ 698.2, obs 697.9. Retention time = 23.5 or 5.4 min, respectively for **4** (Xaa_1 = Gly) or **5** (Xaa_2 = Tyr).

4.4. Kinetics measurement based on FRET system

Fluorescence intensity of FRET substrate **2** (final concentration = 8 μM) in phosphate buffer (pH 7.6, 20 mM) with 30% MeCN was measured before photolysis and it was defined as time = 0 min (λ_{ex} = 495 nm; λ_{em} = 520 nm). Then, the reaction mixture was irradiated by UV light (>365 nm) for 3 min and the fluorescence of the reaction mixture at 37 ˚C was recorded at 3, 5, 10, 15, 30, 60, 120 and 180 min. Percentage of remaining intermediate **3** was estimated based on relative fluorescence intensity. Remaining intermediate **3** (%) = 100 × (1 – relative fluorescence intensity)

Acknowledgments

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7. Racemic **8** was used for a synthesis of FRET substrates **2**.
8. Because pH dependence of the processnig reaction kinetics was observed, kinetics of this reaction should be described by pseudo-first-order (half life of **3** (Xaa_1 = Gly, Xaa_2 = Tyr) in 30% MeCN/phosphate buffer (pH 5.5): 26.9 min).

Legends

Figure 1. HPLC profiles (a) before UV irradiation (b) after 3 min of UV irradiation followed by 3 h of incubation at 37 ˚C (Xaa_1 = Gly; Xaa_2 = Tyr). Diastereomeric mixture of **2** was used. Peptides were

detected by UV absorbance at 220 nm. Asterisked peak is an *o*-nitrobenzyl derived small molecule.

Figure 2. Kinetic measurement of the processing reaction of **3** (Xaa_1 = Gly; Xaa_2 = Tyr). (a) Change in fluorescence intensity in phosphate buffer (pH 7.6, 20 mM) with 30% MeCN at 37 ˚C after UV irradiation (λ_{ex} = 495 nm; λ_{em} = 520 nm). (b) First-order kinetic treatment of the data. Percentage of intermediate **3** was calculated as follow. **3** (%) = 100 × (1 – relative fluorescence intensity).

Figure 3. Half lives of the intermediates **3** in the processing reaction. Ser(Ac) is an *O*-acetyl serine.

Scheme 1. Stimulus-responsive processing system (PG: protective group removable by a stimulus).

Scheme 2. FRET-based assay system for the kinetics of the processing reaction. (*o*NB: *o*-nitrobenzyl)

Scheme 3. Reagents and conditions. a) Fmoc SPPS. b) Dabsyl chloride, Et_3N, DMF. c) $(Ph_3P)_4Pd$, 1,3-dimethylbarbituric acid, 1,2-dichloroethane. d) FITC, DIEA, DMF. e) TFA/thioanisole/H_2O = 95/2.5/2.5 (v/v). (*o*NB: *o*-nitrobenzyl)

Table 1. [a]Ser(Ac) = *O*-acetyl serine.

Figure 1

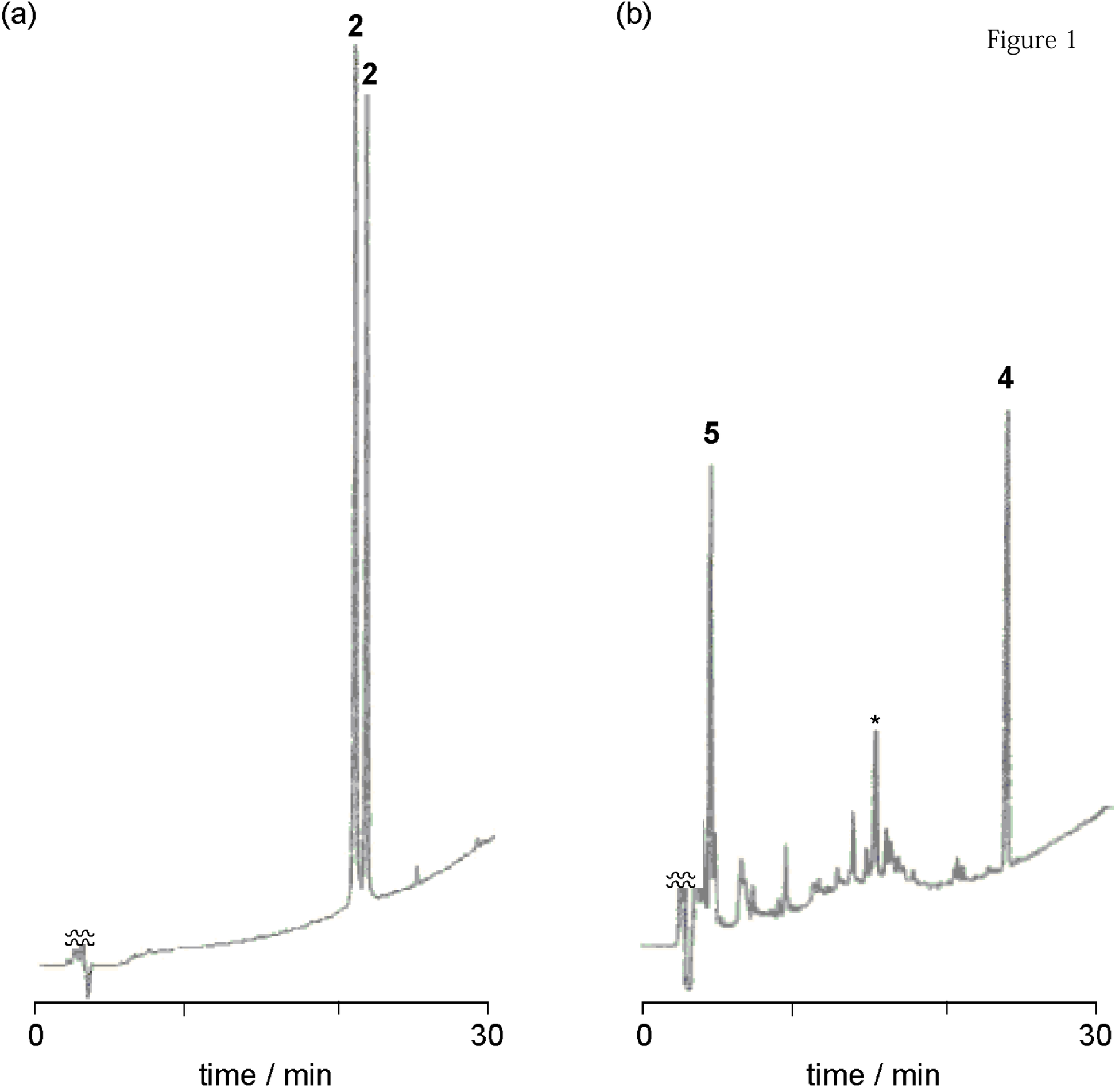
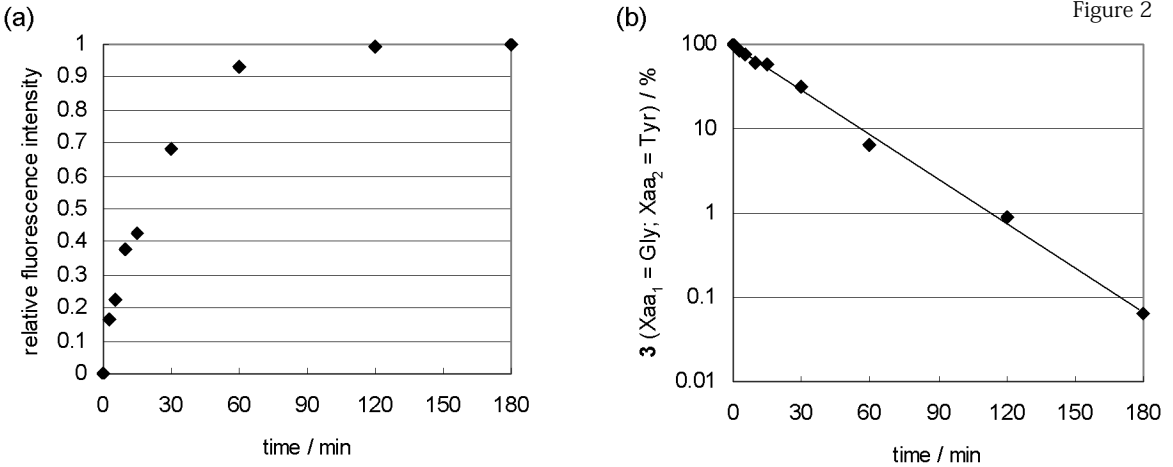
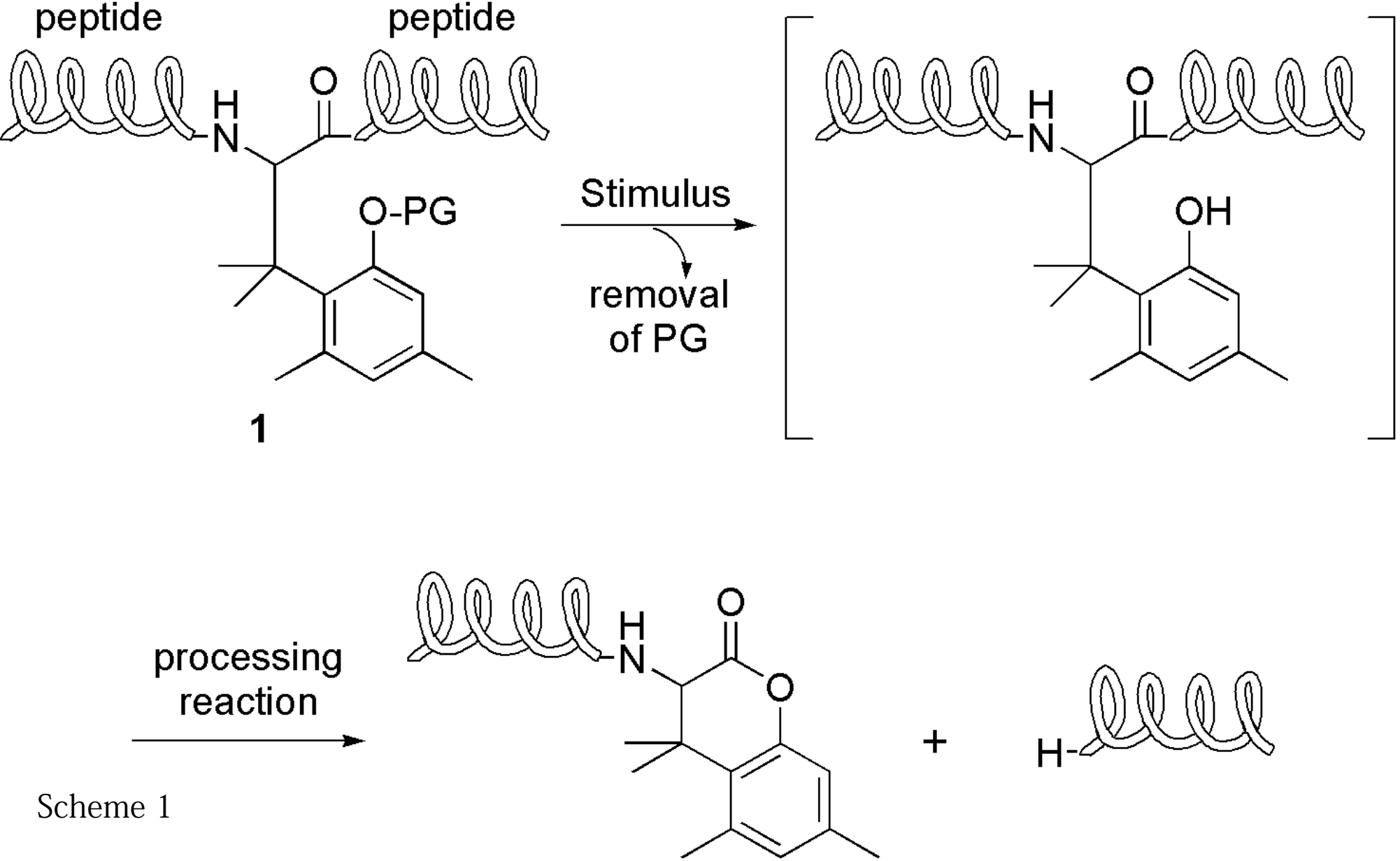


Figure 2

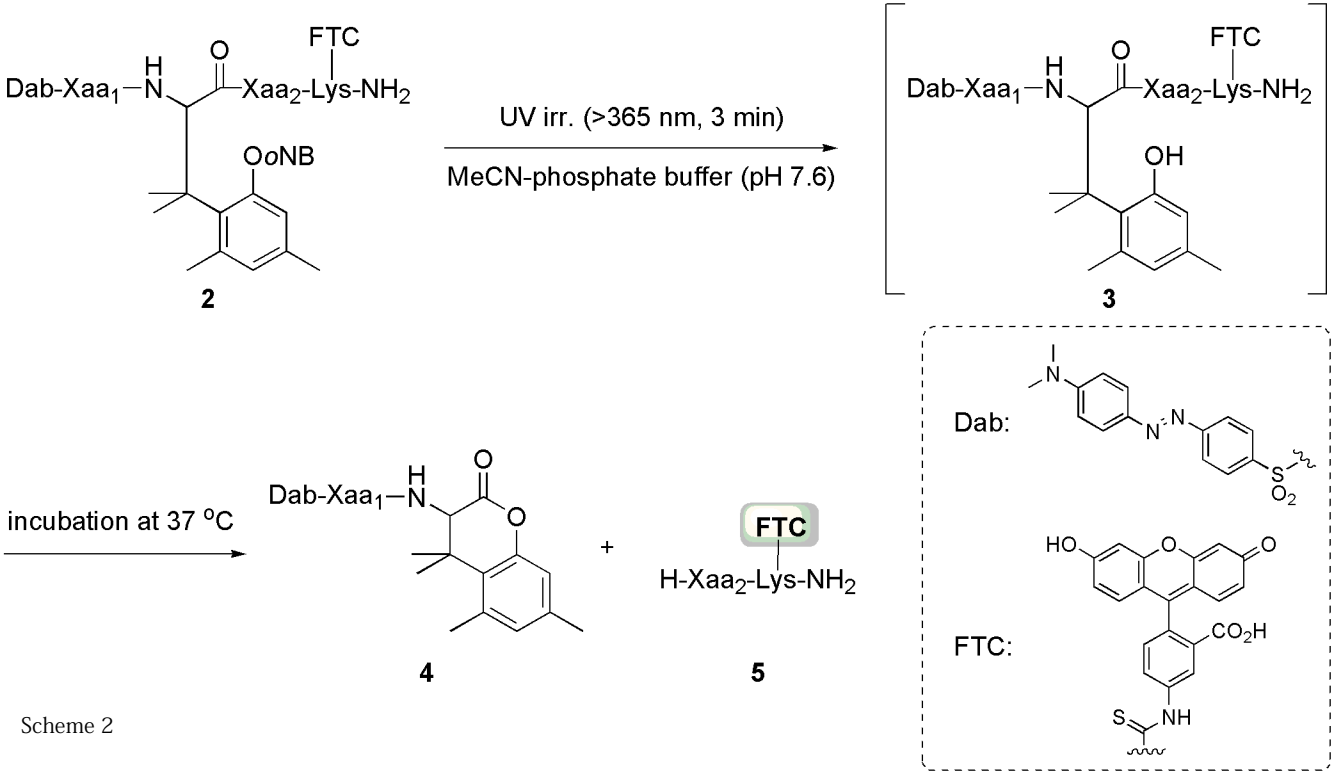

(a)
relative fluorescence intensity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
30
60
90
120
150
180
time / min
(b)
100
10
1
0.1
0.01
3 (Xaa1 = Gly; Xaa2 = Tyr) / %
0
30
60
90
120
150
180
time / min

80
70
60
50
40
30
20
10
0
t1/2 / min
Ala
Arg
Asn
Asp
Cys
Gln
Glu
Gly
His
Ile
Leu
Lys
Met
Phe
Pro
Ser
Ser(Ac)
Thr
Trp
Tyr
Val
Glu
Ile
Lys
Xaa1 = Gly; Xaa2 =
Xaa2 = Tyr; Xaa1 =

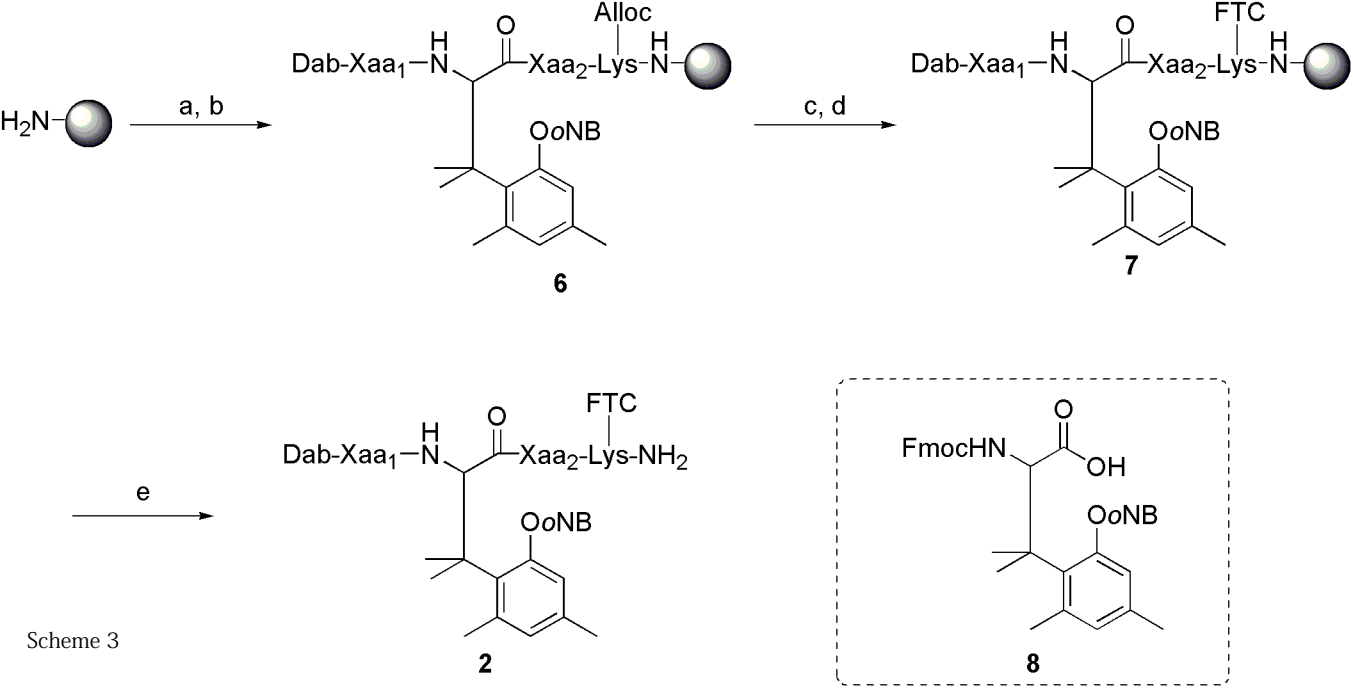
Figure 3


peptide
peptide
H
N
O
O-PG
1
Stimulus
removal
of PG
OH
processing
reaction
+
H-

Scheme 1


FTC
Dab-Xaa1–N
H
O
Xaa2-Lys-NH2
OoNB
2
UV irr. (>365 nm, 3 min)
MeCN-phosphate buffer (pH 7.6)
OH
3
incubation at 37 °C
4
+
FTC
H-Xaa2-Lys-NH2
5
Dab:
FTC:
HO
CO2H
NH

Scheme 2


H2N
a, b
Dab-Xaa1
N
H
O
Xaa2-Lys-N
H
Alloc
OoNBO
6
c, d
FTC
7
e
NH2
2
FmocHN
OH
8

Scheme 3

Table 1

2		ESI MS ($[M + 2H]^{2+}$)	
Xaa_1	Xaa_2	calc.	obs.
Gly	Ala	652.7	652.5
Gly	Arg	695.5	695.1
Gly	Asn	674.4	674.4
Gly	Asp	674.7	674.7
Gly	Cys	668.7	668.9
Gly	Gln	681.3	681.6
Gly	Glu	681.8	682.1
Gly	Gly	645.7	645.5
Gly	His	685.8	686.0
Gly	Ile	673.8	673.9
Gly	Leu	673.8	673.6
Gly	Lys	681.3	681.4
Gly	Met	682.8	682.9
Gly	Phe	690.8	690.9
Gly	Pro	665.8	666.0
Gly	Ser	660.7	660.9
Gly	Ser(Ac) [a]	681.8	681.9
Gly	Thr	667.8	667.9
Gly	Trp	710.3	710.4
Gly	Tyr	698.8	699.0
Gly	Val	666.8	666.7
Glu	Tyr	734.8	735.4
Ile	Tyr	726.5	727.0
Lys	Tyr	734.3	734.5