Design and synthesis of a hydrogen peroxide-responsive amino acid that induces peptide bond cleavage after exposure to hydrogen peroxide

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ARTICLE INFO

Article history:

Received

Received in revised form

Accepted

Available online

Keywords:

Amide bond cleavage

Hydrogen peroxide

Oxidative stress

Stimulus-responsive

Trimethyl lock

ABSTRACT

Oxidative stress-responsive compounds are attracting significant attention in the field of medicinal chemistry and chemical biology. Here, we disclose the development of a hydrogen peroxide (H₂O₂)-responsive amino acid that induces peptide bond cleavage in the presence of H₂O₂ that closely relates to the oxidative stress. The H₂O₂-responsive amino acid possessing a boronate or boronic acid moiety was incorporated into a peptide using Fmoc-based solid-phase peptide synthesis or that with minor modification, respectively, and the peptide bond cleavage of the obtained peptide was successfully triggered by the addition of H₂O₂.

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1. Introduction

Reactive oxygen species (ROS) play a wide variety of roles in health and disease.¹ Hydrogen peroxide (H₂O₂) is one of the ROS and its controlled generation is essential for living organisms, e.g., cellular signalling² and removal of invading pathogens.³ Loss of ROS regulation, however, can cause oxidative damage to cellular components, and thereby H₂O₂ is thought to be involved in numerous diseases and aging.⁴ Cancer is an oxidative stress related disease and production of ROS in most cancer cells is known to be elevated.⁵ For example, the intracellular concentration of H₂O₂ in cancer cells was estimated to be much higher than that in normal cells (in cancer cells: 10 to 100 μM; in normal cells: up to 0.7 μM).⁶ Therefore, it is of interest to develop H₂O₂-responsive fluorophores,⁷ prodrugs,⁸ and drug carriers⁹ to detect and treat cancer. With these in mind, we decided to prepare an H₂O₂-responsive amino acid that induces peptide bond cleavage in the presence of H₂O₂, because it would potentially be applicable to the development of oxidative stress-responsive peptidyl prodrugs and drug carriers.

Figure 1. Stimulus-responsive peptide bond cleavage system (PG: a protective group that can be removed by an appropriate stimulus; TBDPS: *t*-butyldiphenylsilyl).

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Previously, we had reported a stimulus-responsive amino acid and its application to control peptidyl function in living cells (Scheme 1).¹⁰ When peptide **1**, which possesses the stimulus-responsive amino acid, is exposed to an appropriate stimulus, removal of a **PG** (a protective group that can be removed by the stimulus) followed by lactonization of the trimethyl lock unit¹¹ will cause peptide bond cleavage. In principle, peptide **1** can respond to any stimuli simply by changing the **PG**. In this paper, the development of the H₂O₂-responsive amino acid and its application to the H₂O₂-responsive peptide are reported.

2. Results and discussion

As the H₂O₂-responsive protective group, a **4-methylphenylboronic acid** was employed because it is widely used as a triggering device that responds to intracellular H₂O₂ (Scheme 2).^{6a,7c-e,8a,b,d,9a} Hydrogen peroxide triggers oxidative conversion of the phenylboronic acid to a phenol, and following removal of a quinone methide from the intermediate releases a parent deprotected compound even under physiological conditions. In this study, 9-fluorenylmethoxy carbonyl (Fmoc) protected H₂O₂-responsive amino acid **2** possessing the **4-methylphenylboronic acid** unit was designed for Fmoc-based solid-phase peptide synthesis (Fmoc SPPS) (Figure 1).¹² Because we did not know initially whether a non-protected boronic acid is compatible with Fmoc SPPS, a methylimidodiacetic acid (MIDA)¹³ protected variant **3** was also prepared.

Scheme 2. Hydrogen peroxide-responsive oxidative removal of the **4-methylphenylboronic acid** unit.

Figure 1. Fmoc protected hydrogen peroxide-responsive amino acid building blocks.

Fmoc-protected H₂O₂-responsive amino acid derivatives **2** and **3** were prepared as shown in Scheme 3A. Since an oxidation reaction after the introduction of the boronate unit was expected, oxidation-tolerant MIDA protection was employed. Alkylation of phenol **4**¹⁴ with boronate derivative **6** prepared from **5**^{13b} was conducted, and a *t*-butyldimethylsilyl (TBS) group of the product **7** was removed under acidic conditions. Alcohol **8** was then oxidized to carboxylic acid **9** in a two-step manner. Next, boronic acid derivative **2** was synthesized as follow. An MIDA unit of **9** was removed in the presence of sodium bicarbonate in aqueous methanol, and the *t*-butoxycarbonyl (Boc) group was replaced with an Fmoc group by the usual manner to generate building block **2**. Conversely, preparation of MIDA derivative **3** was also

initiated from Boc derivative **9**. Removal of the Boc group by protic acids was first examined. However, partial deprotection of the boronate was observed (data not shown). In this study, therefore, aprotic conditions using *t*-butyldimethylsilyl triflate (TBSOTf) was employed,¹⁵ and the obtained amine was protected with an Fmoc group to generate MIDA protected building block **3**. Purification of **3** by open column chromatography was more difficult than that of **2**, because of its high polarity. Therefore, purification of **3** using preparative TLC was carefully performed, and the product was obtained in moderate yield.

Scheme 3. Synthesis of hydrogen peroxide-responsive amino acids (A) and their incorporation into a model peptide (B). Reagents and conditions: a) **6**, K₂CO₃, KI, DMF, 92%; b) AcOH, THF, H₂O, 98%; c) PDC, DMF; d) NaClO₂, NaH₂PO₄, 2-methyl-2-butene, *t*-BuOH, acetone, H₂O, 71% (two steps); e) NaHCO₃, MeOH, H₂O; f) 4 M HCl in EtOAc; g) FmocOSu, Na₂CO₃, MeCN, H₂O, 77% (three steps) for **2**, 44% (two steps) for **3**; h) TBSOTf, 2,6-lutidine, CH₂Cl₂; i) SOCl₂, DMF, CH₂Cl₂, 67%; j) Fmoc SPPS; k) Fmoc SPPS. For removal of an Fmoc group, sat. Na₂B₄O₇ in 20% (v/v) piperidine/DMF was used; l) Fmoc SPPS. Before global deprotection, the resin was treated with sat. NaHCO₃ aq./H₂O/MeOH = 1/1/2 (v/v). Global deprotection conditions: TFA/triethylsilane/H₂O = 95/2.5/2.5 (v/v). (A: alanine; D: aspartic acid; G: glycine; Q: glutamine; R: arginine; S: serine; Y: tyrosine)

Next, the incorporation of H₂O₂-responsive units **2** and **3** into a model peptide was attempted (Scheme 3B). Peptide elongation was performed on NovaSyn® TGR resin and the H₂O₂-responsive unit was incorporated by usual Fmoc SPPS to afford protected peptide resin **10** or **11**. Elongation of a peptide on **11** was not problematic; however, the resin became sticky when **10** and its peptide-elongated derivatives were subjected to Fmoc removal with 20% (v/v) piperidine in DMF. It is known that trimerization of boronic acids are facilitated by addition of ligands such as amines.¹⁶ We thus speculate that the non-protected boronic acid on the peptide may cause the aggregation via formation of the trimer. Therefore, disodium tetraborate (Na₂B₄O₇), which would competitively prevent the boronic acid-induced aggregation, was added to the Fmoc removal cocktail

and suppression of the aggregation of the resin was successfully achieved to yield model peptide **12**. Conversion of peptide resin **11** to peptide **12** was also accomplished by the usual Fmoc SPPS followed by on-resin removal of the MIDA group and subsequent global deprotection. Regardless of which building block was used the chemical yield of peptide **12** was quite similar. Therefore, building block **2**, which can be easily purified and obtained in higher yield, was employed in the following experiments.

With H₂O₂-responsive peptide **12** in hand, we examined the H₂O₂-responsive cleavage of the peptide (Figure 2A). In this experiment, guanidine hydrochloride was added to dissolve the peptide in an aqueous sodium phosphate buffer (pH 7.4).^{17,18} Glycine and benzamide were also added to scavenge for an electrophilic quinone methide via 1,6-addition of an amino group¹⁹ and as an internal standard, respectively. H₂O₂ was added to the solution of peptide **12** and the obtained mixture was incubated at 37 °C (concentration of the components just after the addition of H₂O₂ is as follow: 10 μM peptide **12**, 6 M guanidine hydrochloride, 100 mM glycine, 25 ppm benzamide, 20 mM phosphate, 100 μM H₂O₂). The reaction progress was monitored by HPLC, and the peptides were characterized by ESI-TOF MS (Figure 2B). A compound eluted at 9.8 min was identified as quinone methide-glycine conjugate **17** by coinjection with an authentic sample prepared according to literature.²⁰ Within 1 h of the incubation, substrate **12** had disappeared completely (half-life = 11.4 min) (Figure 2C). Intermediate **13**, but not **14**, was observed; therefore, removal of the 4-hydroxyphenylmethyl group was estimated as a rate-limiting step. After the complete consumption of substrate **12**, disappearance of intermediate **13** showed first-order dependence on the relative amount of **13** and the half-life was determined as 231 min. The cleavage products **15**, **16** and **17** were generated in high purity after 24 h of the reaction. Then, the sensitivity of peptide **12** to H₂O₂ was examined. Concentration of H₂O₂ in cancer cells has been reported as 10 to 100 μM.⁶ Thus, the peptide was treated with 10 μM H₂O₂ (1 equivalent to peptide **12**). As shown in Figure 2C, the peptide bond cleavage proceeded reasonably, whereas almost no reaction was observed in the absence of H₂O₂. These results therefore suggest that the H₂O₂-responsive amino acid would have the potential as an H₂O₂-based cancer sensing unit.

Figure 2 can be found in page 4#

3. Conclusion

An H₂O₂-responsive amino acid was developed. It was successfully incorporated into a peptide by standard (for the MIDA boronate **3**) or slightly modified (for the boronic acid **2**) Fmoc SPPS that would be useful for preparation of peptides with a boronic acid. Finally, H₂O₂-triggered peptide bond cleavage of the obtained peptide was demonstrated even in the presence of 10 μM H₂O₂ (concentration of H₂O₂ in cancer cells: 10 to 100 μM).⁶ This result suggests that our H₂O₂-responsive system would become bases for peptidyl cancer-responsive fluorophores, prodrugs and drug carriers. Development of these cancer-responsive peptidyl tools, that are usable in mammalian cells, for medicinal chemistry and chemical biology is in progress.

Acknowledgments

This research was supported in part by PRESTO, Japan Science and Technology Agency (JST), a Grant-in-Aid for Scientific Research (C) and Innovative Areas "Fusion Material"

(No. 2206) from the Japanese Society for the Promotion of Science (JSPS) and the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT). Takeda Pharmaceutical Company is also acknowledged. JY and CK are grateful for a JSPS fellowship and scholarship from the Faculty of Pharmaceutical Science, Tokushima University, respectively.

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12. As the hydrogen peroxide-responsive amino acid, compound **18** was also designed and we first attempted to prepare it. However, mass of the product was identical to that of **19** but not **18** (ESI-Ion Trap MS, m/z calcd for $[M + Li, Na, \text{ or } K]^+$ 360.2, 376.2, or 392.2, found 359.7, 375.8, or 392.1, respectively). In a previous study, it was also observed that introduction of a sterically demanding substituent on the phenol of the trimethyl lock unit was problematic than that of benzyl derivatives.^{10b} In this study, therefore, we attempted to synthesize **2** and **3** that possesses the benzyl type protection on the phenol.

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17. To perform the reaction without guanidine, concentration of peptide **12** was reduced. However, the peptide was not dissolved in an aqueous solvent even if the concentration of **12** was 1 μM . Therefore, the use of guanidine is essential for the reaction of

peptide **12**. For application of the H_2O_2 -responsive peptide in cells, in our opinion, incorporation of a hydrophilic sequence such as cell penetrating peptides represented by an octaarginine would help to dissolve the peptide in an aqueous solvent without guanidine. About the octaarginine, see: Nakase, I.; Takeuchi, T.; Tanaka, G.; Futaki, S. *Adv. Drug Delivery Rev.* **2008**, *60*, 598–607 and references cited therein.

18. Whereas extracellular pH is acidified, intracellular pH is normally maintained above pH 7 in a certain tumors such as solid tumors. See: Vaupel, P.; Kallinowski, F.; Okunieff, P. *Cancer Res.* **1989**, *49*, 6449–6465, and references therein.
19. When concentration of glycine was reduced to 10 mM, addition of peptides to a quinone methide was observed (data not shown). Therefore, 100 mM of glycine was employed to completely suppress the reaction of peptides and a quinone methide for clarity of HPLC charts.
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Supplemental materials

Details for preparation of the hydrogen peroxide-responsive peptides and the oxidation induced peptide bond cleavage can be found in the Supporting Information.

Figure 2. Hydrogen peroxide-responsive peptide bond cleavage. A) Reagents and conditions: a) 100, 10, or 0 μM H_2O_2 , 10 μM **12**, 20 mM sodium phosphate buffer with 100 mM glycine and 6 M guanidine hydrochloride (pH 7.4), 25 ppm benzamide as an internal standard, 37 $^\circ\text{C}$. B) HPLC profiles of the reaction using 100 μM H_2O_2 . *Non-peptidyl peaks. **Benzamide as an internal standard. C) Time course of the disappearance of the substrate **12**, intermediate **13** and products **15** + **16**. The relative amount was calculated using the following equation in which PA_x means peak area of **x** in the HPLC chromatogram. Relative amount of **13** or **15** + **16** = $\{\text{PA}_{13} \text{ or } (\text{PA}_{15} + \text{PA}_{16})\} \times \text{PA}_{\text{IS}}(t=0) \div (\text{PA}_{\text{IS}} \times \text{PA}_{12}(t=0))$. The average and standard deviation of three measurements is shown at each time point.

Figure(s)

	R
2	$-\text{B}(\text{OH})_2$
3	$-\text{B}(\text{MIDA})$

Figure(s)

Figure(s)

stimulus	PG	R ₁	R ₂	R ₃
ultraviolet		NO ₂	H	H
near-infrared two-photon excitation		NO ₂	OMe	OMe
fluoride		H	OTBDPS	H
hypoxia		H	NO ₂	H
thiol	4-nitrobenzenesulfonyl	-	-	-
phosphatase	phosphate	-	-	-

Figure(s)

Figure(s)

Supporting Information for

Design and synthesis of a hydrogen peroxide-responsive amino acid that induces peptide bond cleavage after exposure to hydrogen peroxide

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General Methods

All reactions were carried out under a positive pressure of argon at room temperature unless otherwise noted. For column chromatography, Silica Gel 60 N (spherical, neutral, Kanto Chemical Co., Inc.) was employed. Preparative TLC was performed on precoated plates (1 mm silica gel, Merck KGaA 60F₂₅₄). Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™ (ESI-TOF). NMR spectra were recorded using a Bruker AV400N at 400 MHz frequency for ¹H, and JEOL JNM-AL300 at 300 MHz frequency for ¹H and 75 MHz frequency for ¹³C (signals of ¹³C connected to boron were not detected). For HPLC separations, a Cosmosil 5C₁₈-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a Cosmosil 5C₁₈-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min) or a Cosmosil 5C₁₈-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10.0 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% (v/v) TFA in H₂O (solvent A) and 0.1% TFA (v/v) in MeCN (solvent B) was used for HPLC elution. Optical rotations were measured using a JASCO P-2200 polarimeter (concentration in g/100 mL).

Synthesis of hydrogen peroxide-responsive amino acid derivatives **2** and **3**

Scheme S1. Reagents and conditions: a) **6**, K₂CO₃, KI, DMF, 92%; b) AcOH, THF, H₂O, 98%; c) PDC, DMF; d) NaClO₂, NaH₂PO₄, 2-methyl-2-butene, *t*-BuOH, acetone, H₂O, 71% (two steps); e) NaHCO₃, MeOH, H₂O; f) 4 M HCl in EtOAc; g) FmocOSu, Na₂CO₃, MeCN, H₂O, 77% (three steps) for **2**, 44% (two steps) for **3**; h) TBSOTf, 2,6-lutidine, CH₂Cl₂; i) SOCl₂, DMF, CH₂Cl₂, 67%.

2-{4-(Chloromethyl)phenyl}-6-methyl-1,3,6,2-dioxazaborocane-4,8-dione (**6**)

To a stirred solution of benzyl alcohol **5**^[S1] (1.00 g, 3.80 mmol) in DMF (8.0 μL) and CH₂Cl₂ (7.6 mL) was added thionyl chloride (331 μL, 4.45 mmol) at 0 °C. The reaction mixture was stirred for 17 h, and then EtOAc, MeCN, H₂O and saturated aqueous solution of NaHCO₃ were added to the mixture. After extraction with EtOAc, the combined organic layer was washed with brine, dried over Na₂SO₄, and concentrated in vacuo. The product was purified by column chromatography (Et₂O/MeCN = 2/1 (v/v)) and 719 mg of chloride **6** (2.55 mmol, 67%) was obtained as a white solid: ¹H NMR (CD₃CN, 400 MHz) δ = 2.50 (3H, s), 3.90 (2H, d, *J* = 17.0 Hz), 4.07 (2H, d, *J* = 17.0 Hz),

4.68 (2H, s), 7.43 (2H, d, $J = 8.0$ Hz), 7.52 (2H, d, $J = 8.0$ Hz); ^{13}C NMR (CD_3CN , 75 MHz) $\delta = 47.0, 48.4, 62.7, 129.1, 133.8, 139.8, 169.4$; HRMS (ESI-TOF) m/z calcd for $\text{C}_{12}\text{H}_{14}\text{BClINO}_4$ ($[\text{M} + \text{H}]^+$) 282.0704, found 282.0690.

(S)-tert-Butyl [1-((tert-butyldimethylsilyl)oxy)-3-(2,4-dimethyl-6-[[4-(6-methyl-4,8-dioxo-1,3,6,2-dioxazaborocan-2-yl)benzyl]oxy]phenyl)-3-methylbutan-2-yl]carbamate (7)

To a stirred solution of phenol **4**^[S2] (400 mg, 0.914 mmol) in DMF (8.0 mL) were added K_2CO_3 (303 mg, 2.19 mmol), benzyl chloride derivative **6** (514 mg, 1.83 mmol), and potassium iodide (364 mg, 2.19 mmol), and the resulting suspension was stirred for 2 d. After addition of EtOAc and saturated aqueous solution of NH_4Cl , the reaction mixture was stirred for additional 30 min. The mixture was then extracted with EtOAc, and the combined organic layer was washed with water, saturated aqueous solution of NH_4Cl and brine. The extract was dried over Na_2SO_4 and concentrated in vacuo. The product was purified by column chromatography (hexane/EtOAc = 2/1 then 1/6 (v/v)) and 574 mg of MIDA boronate **7** (0.841 mmol, 92%) was obtained as a pale yellow amorphousness: $[\alpha]_{\text{D}}^{20} -29.9$ (c 1.22, CHCl_3); ^1H NMR (CDCl_3 , 400 MHz) $\delta = -0.06$ (3H, s), -0.05 (3H, s), 0.84 (9H, s), 1.39 (9H, s), 1.49 (3H, s), 1.53 (3H, s), 2.20 (3H, s), 2.53 (3H, s), 2.55 (3H, s), 3.48 (1H, dd, $J = 10.4$ and 4.0 Hz), 3.55 (1H, dd, $J = 10.4$ and 4.0 Hz), 3.80 (2H, d, $J = 16.8$ Hz), 4.07 (2H, d, $J = 16.8$ Hz), 4.60 (1H, br m), 4.84 (1H, d, $J = 10.0$ Hz), 5.06 (1H, d, $J = 12.4$ Hz), 5.13 (1H, d, $J = 12.4$ Hz), 6.56 (1H, s), 6.59 (1H, s), 7.49 - 7.52 (4H, m); ^{13}C NMR (CDCl_3 , 75 MHz) $\delta = -5.6, -5.5, 18.1, 20.7, 25.8, 25.9, 27.5, 28.4, 29.2, 44.8, 47.6, 56.3, 61.7, 63.8, 70.8, 78.3, 112.7, 127.3, 127.7, 131.0, 132.4, 136.2, 138.5, 139.3, 156.0, 158.6, 167.9$; HRMS (ESI-TOF) m/z calcd for $\text{C}_{36}\text{H}_{55}\text{BN}_2\text{NaO}_8\text{S}$ ($[\text{M} + \text{Na}]^+$) 705.3718, found 705.3693.

(S)-tert-Butyl {3-(2,4-dimethyl-6-[[4-(6-methyl-4,8-dioxo-1,3,6,2-dioxazaborocan-2-yl)benzyl]-oxy]phenyl)-1-hydroxy-3-methylbutan-2-yl}carbamate (8)

Glacial acetic acid (3.41 mL) and water (3.41 mL) were added to a solution of silyl ether **7** (430 mg, 0.630 mmol) in THF (10 mL). The reaction mixture was stirred for 14 h and was diluted with water. After extraction with EtOAc, the organic phase was washed with water ($\times 2$) followed by brine, dried over Na_2SO_4 , and concentrated in vacuo. The crude product was purified by column chromatography (hexane/EtOAc = 1/10 then $\text{Et}_2\text{O}/\text{MeCN} = 4/1$ (v/v)) and 352 mg of alcohol **8** (98.9 μmol , 98%) was obtained as a white amorphousness: $[\alpha]_{\text{D}}^{20} -18.5$ (c 1.04, CHCl_3); ^1H NMR (CDCl_3 , 400 MHz) $\delta = 1.36$ (9H, s), 1.47 (3H, s), 1.49 (3H, s), 2.21 (3H, s), 2.50 (3H, s), 2.55 (3H, s), 3.45 (1H, dd, $J = 10.4$ and 8.4 Hz), 3.58 (1H, d, $J = 9.6$ Hz), 3.81 (2H, d, $J = 16.6$ Hz), 4.04 (2H, d, $J = 16.6$ Hz), 4.49 - 4.53 (1H, m), 4.91 (1H, d, $J = 8.4$ Hz), 5.06 (1H, d, $J = 12.0$ Hz), 5.10 (1H, d, $J = 8.4$ Hz), 6.57 (1H, s), 6.64 (1H, s), 7.49 (2H, d, $J = 7.8$ Hz), 7.54 (2H, d, $J = 7.8$ Hz); ^{13}C NMR (CDCl_3 , 75 MHz) $\delta = 20.7, 25.9, 27.9, 28.3, 28.6, 43.8, 47.6, 59.0, 61.8, 64.3, 71.0, 79.2, 112.8, 127.6, 127.9, 130.3, 132.6,$

136.6, 138.2, 138.8, 157.2, 158.5, 167.9; HRMS (ESI-TOF) m/z calcd for $C_{30}H_{42}BN_2O_8$ ($[M + H]^+$) 569.3034, found 569.3051.

(S)-2-((tert-Butoxycarbonyl)amino)-3-(2,4-dimethyl-6-[[4-(6-methyl-4,8-dioxo-1,3,6,2-dioxaborocan-2-yl)benzyl]oxy]phenyl)-3-methylbutanoic acid (9)

Pyridinium dichromate (937 mg, 2.49 mmol) was added to a solution of alcohol **8** (283 mg, 0.497 mmol) in DMF (2.4 mL). The reaction mixture was stirred for 13 h. After addition of Celite 535, the obtained mixture was filtered through Celite 535. The filtrate was washed with 5% (v/v) aqueous solution of $KHSO_4$ followed by brine, dried over Na_2SO_4 , and concentrated in vacuo. The resulting crude aldehyde was subjected to subsequent reaction without further purification. To a solution of the crude aldehyde in acetone (9.34 mL)/*t*-BuOH (6.41 mL)/ H_2O (1.54 mL) were added 2-methyl-2-butene (0.355 mL, 3.35 mmol), NaH_2PO_4 (89.5 mg, 0.746 mmol) and $NaClO_2$ (236 mg, 2.61 mmol), and the mixture was stirred for 3 h. After addition of saturated aqueous solution of NH_4Cl , the reaction mixture was extracted with EtOAc. The organic phase was dried over Na_2SO_4 and was concentrated in vacuo. The obtained crude product was purified by column chromatography ($Et_2O/MeCN = 5/1$ then $1/1$ (v/v)) and 207 mg of Boc amino acid **9** (0.354 mmol, 71% (two steps)) was obtained as a pale yellow amorphousness: $[\alpha]_D^{20}$ 7.53 (c 1.16, $CHCl_3$); ^1H NMR (DMSO- d_6 , 400 MHz, 60 °C) δ = 1.25 (9H, s), 1.44 (3H, s), 1.52 (3H, s), 2.13 (3H, s), 2.42 (3H, s), 2.49 (3H, s), 4.06 (2H, d, J = 17.1 Hz), 4.28 (2H, d, J = 17.1 Hz), 5.06 (1H, d, J = 12.6 Hz), 5.13 (1H, d, J = 12.6 Hz), 5.21 (1H, d, J = 9.6 Hz), 6.18 (1H, br s), 6.46 (1H, s), 6.70 (1H, s), 7.43 (2H, d, J = 8.0 Hz), 7.51 (2H, d, J = 8.0 Hz); ^{13}C NMR (DMSO- d_6 , 75 MHz, 60 °C) δ = 20.4, 25.3, 27.8, 27.9, 28.1, 43.3, 47.6, 58.9, 61.7, 70.2, 77.7, 112.7, 126.4, 127.1, 129.7, 132.4, 135.4, 137.3, 138.2, 155.4, 158.2, 169.3, 173.5; HRMS (ESI-TOF) m/z calcd for $C_{30}H_{39}BN_2NaO_9$ ($[M + Na]^+$) 605.2646, found 605.2670.

(S)-2-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-[2-((4-boronobenzyl)oxy)-4,6-dimethylphenyl]-3-methylbutanoic acid (2)

To an MIDA boronate **9** (210 mg, 0.361 mmol) in MeOH (5.6 mL) was added saturated aqueous solution of $NaHCO_3$ (2.78 mL), and the suspension was stirred for 3 h. After addition of saturated aqueous solution of NH_4Cl , the reaction mixture was extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4 , and concentrated in vacuo. The crude material was dissolved in hydrogen chloride in EtOAc (4 M, 2.78 mL) and the mixture was stirred for 1 h. After concentration in vacuo, MeCN (5.92 mL) and 10% (v/v) aqueous solution of Na_2CO_3 (1.93 mL) were added to the resulting residue. After addition of FmocOSu (134 mg, 0.397 mmol), the mixture was stirred for 14 h. Then the reaction mixture was acidified with 5% (v/v) aqueous solution of $KHSO_4$. The resulting mixture was extracted with EtOAc. The organic layer was washed with brine,

and concentrated in vacuo. The crude product was purified by preparative TLC ($\text{CHCl}_3/\text{MeOH} = 10/1$ (v/v)) and 165 mg of boronic acid **2** (0.728 mmol, 77% (three steps)) was obtained as a pale yellow amorphousness. For large scale purification, column chromatography ($\text{CHCl}_3/\text{MeOH} = 99/1$ then $20/1$ (v/v)) was employed: $[\alpha]_{\text{D}}^{20} -10.4$ (c 1.03, CHCl_3); ^1H NMR (DMSO-d_6 , 300 MHz, 80 °C) $\delta = 1.79$ (3H, s), 1.86 (3H, s), 2.36 (3H, s), 2.75 (3H, s), 4.30-4.36 (2H, m), 4.47 (1H, dd, $J = 13.0$ and 10.4 Hz), 5.33 (1H, d, $J = 12.5$ Hz), 5.39 (1H, d, $J = 12.5$ Hz), 5.66 (1H, d, $J = 9.7$ Hz), 6.73 (1H, s), 6.96 (1H, s), 7.09 (1H, br s), 7.53 (2H, t, $J = 7.5$ Hz), 7.64 (2H, t, $J = 7.2$ Hz), 7.74 (2H, d, $J = 7.9$ Hz), 7.84 (1H, d, $J = 9.3$ Hz), 7.87 (1H, d, $J = 9.3$ Hz), 7.93 (2H, s), 8.03 (2H, d, $J = 7.9$ Hz), 8.09 (2H, d, $J = 7.5$ Hz), 12.3 (1H, br s); ^{13}C NMR (DMSO-d_6 , 75 MHz, 80 °C) $\delta = 20.3, 25.3, 27.8, 27.9, 43.6, 46.6, 59.4, 65.7, 70.2, 112.5, 119.9, 120.0, 125.3, 125.6, 126.2, 126.9, 127.0, 127.2, 127.5, 127.6, 129.5, 134.1, 135.4, 137.3, 139.3, 140.5, 140.6, 143.7, 143.9, 156.1, 158.1, 173.3$; HRMS (ESI-TOF) m/z calcd for $\text{C}_{35}\text{H}_{37}\text{BNO}_7$ ($[\text{M} + \text{H}]^+$) 594.2663, found 594.2684.

(S)-2-([(9H-Fluoren-9-yl)methoxy]carbonyl)amino)-3-(2,4-dimethyl-6-[[4-(6-methyl-4,8-dioxo-1,3,6,2-dioxazaborocan-2-yl)benzyl]oxy]phenyl)-3-methylbutanoic acid (3**)**

To a solution of Boc derivative **9** (100 mg, 0.172 mmol) in CH_2Cl_2 (3.8 mL) were added 2,6-lutidine (0.120 mL, 1.03 mmol) and TBSOTf (0.158 mL, 0.668 mmol).^[S3] The reaction mixture was stirred for 3 h. After concentration in vacuo, Fmoc protection of the obtained crude material was carried out as similar to that described for preparation of boronic acid **2**. The product was purified by preparative TLC ($\text{Et}_2\text{O}/\text{MeCN} = 3/1$ (v/v)) and 53.1 mg of MIDA ester **3** (57.4 μmol , 44% (two steps)) was obtained as a pale yellow amorphousness: $[\alpha]_{\text{D}}^{20} -15.6$ (c 1.00, CHCl_3); ^1H NMR (DMSO-d_6 , 300 MHz, 100 °C) $\delta = 1.52$ (3H, s), 1.59 (3H, s), 2.09 (3H, s), 2.47 (3H, s), 2.49 (3H, s), 4.02-4.18 (3H, m), 4.04 (2H, d, $J = 16.7$ Hz), 4.28 (2H, d, $J = 16.7$ Hz), 5.07 (1H, d, $J = 12.6$ Hz), 5.13 (1H, d, $J = 12.6$ Hz), 5.45 (1H, d, $J = 9.6$ Hz), 6.46 (1H, s), 6.70 (1H, s), 7.08 (1H, br s), 7.27 (2H, t, $J = 7.2$ Hz), 7.36-7.63 (8H, m), 7.84 (2H, d, $J = 7.5$ Hz); ^{13}C NMR (DMSO-d_6 , 75 MHz, 100 °C) $\delta = 20.3, 25.3, 27.8, 27.9, 43.6, 46.6, 47.5, 59.2, 61.7, 65.7, 70.2, 112.6, 119.9, 120.0, 125.3, 125.6, 126.5, 126.9, 127.0, 127.2, 127.5, 127.6, 129.5, 132.3, 135.4, 137.3, 138.1, 140.5, 140.6, 143.6, 143.9, 156.0, 158.1, 169.3, 173.3$; HRMS (ESI-TOF) m/z calcd for $\text{C}_{40}\text{H}_{42}\text{BN}_2\text{O}_9$ ($[\text{M} + \text{H}]^+$) 705.2983, found 705.2964.

Preparation of hydrogen peroxide-responsive model peptide **12**

Scheme S2. Reagents and conditions: a) Fmoc SPPS; b) Fmoc SPPS. For removal of an Fmoc group, sat. Na₂B₄O₇ in 20% (v/v) piperidine/DMF was used; c) Fmoc SPPS. Before global deprotection, the resin was treated with sat. NaHCO₃ aq./H₂O/MeOH = 1/1/2 (v/v). Global deprotection conditions: TFA/triethylsilane/H₂O = 95/2.5/2.5 (v/v).

Preparation of peptide resin **10** or **11**

On NovaSyn® TGR resin (0.22 mmol amine/g) were coupled Fmoc protected naturally occurring amino acid derivatives (3 eq., a protective group of a side chain: *t*-Bu for serine and tyrosine) in the presence of *N,N'*-diisopropylcarbodiimide (DIC, 3.2 eq.) and 1-hydroxybenzotriazole hydrate (HOBt·H₂O, 3 eq.) in DMF for 2 h. Coupling of H₂O₂-responsive unit **2** or **3** (2 eq.) was performed by the use of *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (1.95 eq.) and *N,N*-diisopropylethyamine overnight. For Fmoc removal of the peptide resin, 20% (v/v) piperidine in DMF (10 min) was employed.

Preparation of model peptide **12** from boronic acid derivative **10**

Fmoc protected amino acid derivatives (3 eq., a protective group of a side chain: *t*-Bu for aspartic acid and tyrosine; Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) for arginine; Trt for Gln) were coupled on peptide resin **10** in the presence of DIC (3.2 eq.) and HOBt·H₂O (3 eq.) in DMF for 2 h. Removal of an Fmoc group was achieved by using saturated solution of Na₂B₄O₇ in 20% (v/v) piperidine/DMF for 10 min. The resulting completed resin was treated with TFA/triethylsilane/water (95/2.5/2.5 (v/v)) for 2 h. After filtration of the resin, cooled Et₂O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with Et₂O and was purified by preparative HPLC to give model peptide **12** as a white lyophilized powder. Analytical HPLC conditions: linear gradient of solvent B in solvent A, 1 to 50% over 30 min. Retention time = 21.7 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 22 to 25% over 30 min. LRMS (ESI-TOF) *m/z* calcd for [M + 2H]²⁺ 762.9, found 762.7.

Preparation of model peptide **12** from MIDA ester derivative **11**

On resin **11**, a peptide chain was elongated as similar to that on **10**. Removal of an Fmoc group was achieved without Na₂B₄O₇. Before global deprotection, the resin was treated with a mixture of saturated aqueous solution of NaHCO₃/H₂O/MeOH (1/1/2 (v/v)) overnight to remove MIDA unit.

Then global deprotection of the peptide resin was conducted as mentioned above.

Hydrogen peroxide-responsive peptide bond cleavage reaction

To a solution of 2.0 mM peptide **12** in 6 M guanidine (5.5 μ L) were added 20 mM sodium phosphate buffer with 100 mM glycine and 6 M guanidine hydrochloride (pH 7.4, 1072.5 μ L), and 0.025% (w/v) benzamide in 20 mM phosphate buffer with 100 mM glycine and 6 M guanidine hydrochloride (11 μ L) (Final concentration: 10 μ M peptide **12**, 6 M guanidine hydrochloride, 100 mM glycine, 25 ppm benzamide, 20 mM phosphate). For HPLC analysis (reaction time = 0 h), 99 μ L of the reaction mixture was used. Aqueous solution of H₂O₂ (0, 1, or 10 mM, 10 μ L) was then added to the remaining reaction mixture (990 μ L), and the reaction was performed at 37 °C (final concentration of H₂O₂: 0, 10, or 100 μ M respectively). Progress of the reaction was monitored by HPLC and the peptides were characterized by ESI-TOF MS. Percentage of remaining **12** was estimated based on HPLC peak area. Relative amount of **13** and **15** + **16** was calculated using the following equation in which PA_x means peak area of x in HPLC chromatogram.

$$\text{Relative amount of } \mathbf{13} \text{ or } \mathbf{15} + \mathbf{16} = \{PA_{\mathbf{13}} \text{ or } (PA_{\mathbf{15}} + PA_{\mathbf{16}})\} \times PA_{IS(t=0)} \div (PA_{IS} \times PA_{\mathbf{12}(t=0)})$$

12: Retention time = 21.7 min.

13: Retention time = 22.1 min. LRMS (ESI-TOF) m/z calcd for [M + 2H]⁺ 748.9, found 748.7.

15: Retention time = 22.6 min. LRMS (ESI-TOF) m/z calcd for [M + 2H]⁺ 405.7, found 405.8.

16: Retention time = 11.0 min. LRMS (ESI-TOF) m/z calcd for [M + H]⁺ 581.3, found 581.3.

Benzamide (internal standard): Retention time = 15.5 min.

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