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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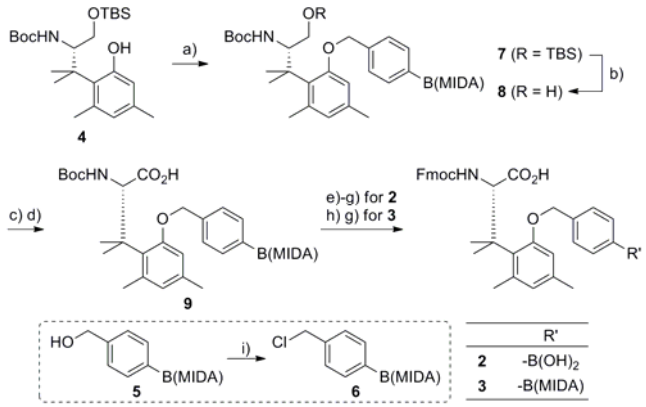
General Methods

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 $60F_{254}$). 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 ^{13}C (signals of ^{13}C connected to boron were not detected). For HPLC separations, a Cosmosil $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a Cosmosil $5C_{18}$-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min) or a Cosmosil $5C_{18}$-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 H_2O (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**, K_2CO_3, KI, DMF, 92%; b) AcOH, THF, H_2O, 98%; c) PDC, DMF; d) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene, *t*-BuOH, acetone, H_2O, 71% (two steps); e) $NaHCO_3$, MeOH, H_2O; f) 4 M HCl in EtOAc; g) FmocOSu, Na_2CO_3, MeCN, H_2O, 77% (three steps) for **2**, 44% (two steps) for **3**; h) TBSOTf, 2,6-lutidine, CH_2Cl_2; i) $SOCl_2$, DMF, CH_2Cl_2, 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 CH_2Cl_2 (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, H_2O and saturated aqueous solution of $NaHCO_3$ were added to the mixture. After extraction with EtOAc, the combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The product was purified by column chromatography (Et_2O/MeCN = 2/1 (v/v)) and 719 mg of chloride **6** (2.55 mmol, 67%) was obtained as a white solid: ^1H NMR (CD_3CN, 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) δ = 47.0, 48.4, 62.7, 129.1, 133.8, 139.8, 169.4; HRMS (ESI-TOF) m/z calcd for $C_{12}H_{14}BClNO_4$ ([M + 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: $[\alpha]^{20}_D$ -29.9 (*c* 1.22, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = -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) δ = -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 $C_{36}H_{55}BN_2NaO_8S$ ([M + 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 (×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 Et_2O/MeCN = 4/1 (v/v)) and 352 mg of alcohol **8** (98.9 μmol, 98%) was obtained as a white amorphousness: $[\alpha]^{20}_D$ -18.5 (*c* 1.04, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 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) δ = 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-dioxaza-borocan-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: $[\alpha]^{20}_D$ 7.53 (*c* 1.16, $CHCl_3$); ^{1}H 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-([{(9*H*-Fluoren-9-yl)methoxy}carbonyl]amino)-3-[2-{(4-boronobenzyl)oxy}-4,6-dimethyl-phenyl]-3-methylbutanoic acid (2)

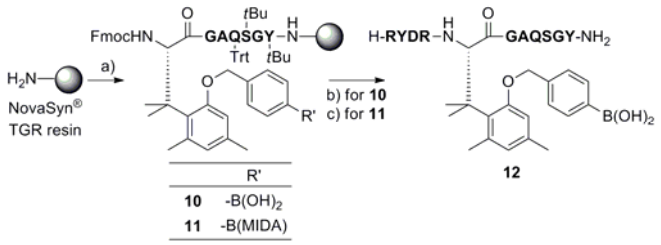
To an MIDA boronate **9** (210 mg, 0.361 mmol) in MeOH (5.6 mL) was added saturated aqueous solution of $NaHCO_3$ (2.78 mL), and the suspension was stirred for 3 h. After addition of saturated aqueous solution of NH_4Cl, the reaction mixture was extracted with EtOAc. The combined organic layer was washed 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 ($CHCl_3$/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 ($CHCl_3$/MeOH = 99/1 then 20/1 (v/v)) was employed: $[\alpha]^{20}_D$ -10.4 (*c* 1.03, $CHCl_3$); ^{1}H NMR (DMSO-d_6, 300 MHz, 80 °C) δ = 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) δ = 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 $C_{35}H_{37}BNO_7$ ($[M + H]^+$) 594.2663, found 594.2684.

(*S*)-2-([{(9*H*-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 (Et_2O/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: $[\alpha]^{20}_D$ -15.6 (*c* 1.00, $CHCl_3$); ^{1}H NMR (DMSO-d_6, 300 MHz, 100 °C) δ = 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) δ = 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 $C_{40}H_{42}BN_2O_9$ ($[M + H]^+$) 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. $Na_2B_4O_7$ in 20% (v/v) piperidine/DMF was used; c) Fmoc SPPS. Before global deprotection, the resin was treated with sat. $NaHCO_3$ aq./H_2O/MeOH = 1/1/2 (v/v). Global deprotection conditions: TFA/triethylsilane/H_2O = 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·H_2O, 3 eq.) in DMF for 2 h. Coupling of H_2O_2-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) piperadine 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·H_2O (3 eq.) in DMF for 2 h. Removal of an Fmoc group was achieved by using saturated solution of $Na_2B_4O_7$ in 20% (v/v) piperadine/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 Et_2O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with Et_2O 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 $Na_2B_4O_7$. Before global deprotection, the resin was treated with a mixture of saturated aqueous solution of $NaHCO_3$/H_2O/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 μL) were added 20 mM sodium phosphate buffer with 100 mM glycine and 6 M guanidine hydrochloride (pH 7.4, 1072.5 μL), and 0.025% (w/v) benzamide in 20 mM phosphate buffer with 100 mM glycine and 6 M guanidine hydrochloride (11 μL) (Final concentration: 10 μM peptide **12**, 6 M guanidine hydrochloride, 100 mM glycine, 25 ppm benzamide, 20 mM phosphate). For HPLC analysis (reaction time = 0 h), 99 μL of the reaction mixture was used. Aqueous solution of H_2O_2 (0, 1, or 10 mM, 10 μL) was then added to the remaining reaction mixture (990 μL), and the reaction was performed at 37 °C (final concentration of H_2O_2: 0, 10, or 100 μM 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.

References

[S1] E. P. Gillis, M. D. Burke, *J. Am. Chem. Soc.* **2008**, *130*, 14084-14085.

[S2] A. Shigenaga, J. Yamamoto, N. Nishioka, A. Otaka, *Tetrahedron* **2010**, *66*, 7367-7372.

[S3] M. Sakaitani, Y. Ohfune, *J. Org. Chem.* **1990**, *55*, 870-876.