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

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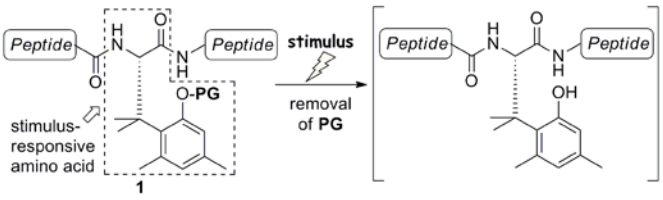
ABSTRACT

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 (H_2O_2)-responsive amino acid that induces peptide bond cleavage in the presence of H_2O_2 that closely relates to the oxidative stress. The H_2O_2-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 H_2O_2.

1. Introduction

Reactive oxygen species (ROS) play a wide variety of roles in health and disease.[1] Hydrogen peroxide (H_2O_2) is one of the ROS and its controlled generation is essential for living organisms, e.g., cellular signalling[2] and removal of invading pathogens.[3] Loss of ROS regulation, however, can cause oxidative damage to cellular components, and thereby H_2O_2 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 H_2O_2 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 H_2O_2-responsive fluorophores,[7] prodrugs,[6a,8] and drug carriers[9] to detect and treat cancer. With these in mind, we decided to prepare an H_2O_2-responsive amino acid that induces peptide bond cleavage in the presence of H_2O_2, because it would potentially be applicable to the development of oxidative stress-responsive peptidyl prodrugs and drug carriers.



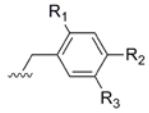
stimulus	PG	R_1	R_2	R_3
ultraviolet		NO_2	H	H
near-infrared two-photon excitation		NO_2	OMe	OMe
fluoride		H	OTBDPS	H
hypoxia		H	NO_2	H
thiol	4-nitrobenzenesulfonyl	-	-	-
phosphatase	phosphate	-	-	-

Figure 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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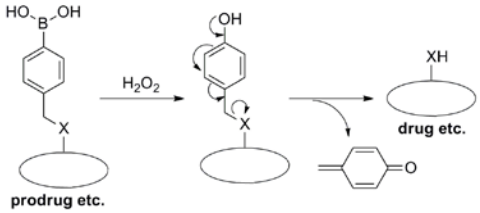
Previously, we had reported a stimulus-responsive amino acid and its application to control peptidyl function in living cells (Scheme 1).[10] When peptide **1**, which possesses the stimulus-responsive amino acid, is exposed to an appropriate stimulus, removal of a **PG** (a protective group that can be removed by the stimulus) followed by lactonization of the trimethyl lock unit[11] 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 H_2O_2-responsive amino acid and its application to the H_2O_2-reponsive peptide are reported.

2. Results and discussion

As the H_2O_2-responsive protective group, a 4-methylphenylboronic acid was employed because it is widely used as a triggering device that responds to intracellular H_2O_2 (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 H_2O_2-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.

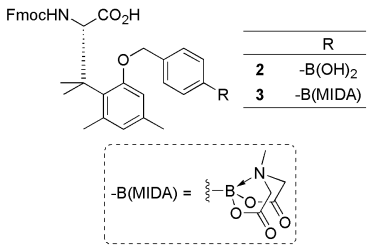
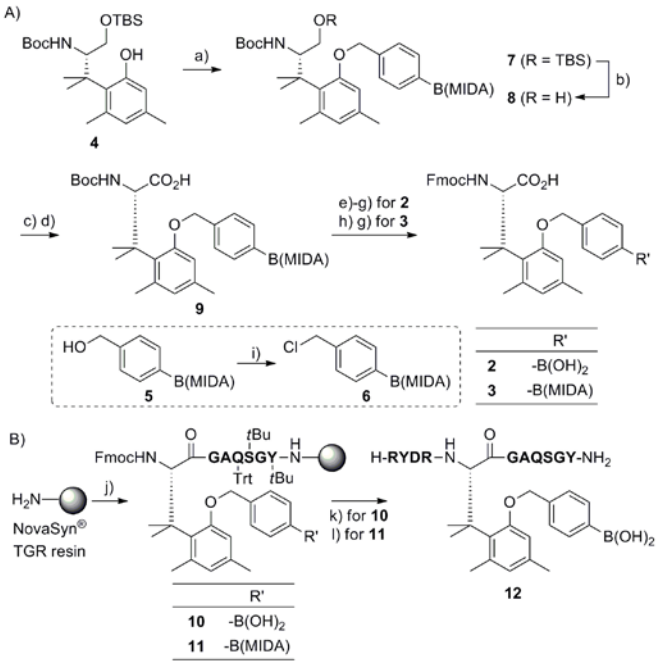


Figure 1. Fmoc protected hydrogen peroxide-responsive amino acid building blocks.

Fmoc-protected H_2O_2-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**, 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%; j) Fmoc SPPS; k) Fmoc SPPS. For removal of an Fmoc group, sat. $Na_2B_4O_7$ in 20% (v/v) piperidine/DMF was used; l) 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). (**A**: alanine; **D**: aspartic acid; **G**: glycine; **Q**: glutamine; **R**: arginine; **S**: serine; **Y**: tyrosine)

Next, the incorporation of H_2O_2-responsive units **2** and **3** into a model peptide was attempted (Scheme 3B). Peptide elongation was performed on NovaSyn® TGR resin and the H_2O_2-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 ($Na_2B_4O_7$), 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 H_2O_2-responsive peptide **12** in hand, we examined the H_2O_2-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 group[19] and as an internal standard, respectively. H_2O_2 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 H_2O_2 is as follow: 10 μM peptide **12**, 6 M guanidine hydrochloride, 100 mM glycine, 25 ppm benzamide, 20 mM phosphate, 100 μM H_2O_2). 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 H_2O_2 was examined. Concentration of H_2O_2 in cancer cells has been reported as 10 to 100 μM.[6] Thus, the peptide was treated with 10 μM H_2O_2 (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 H_2O_2. These results therefore suggest that the H_2O_2-responsive amino acid would have the potential as an H_2O_2-based cancer sensing unit.

Figure 2 can be found in page 4#

3. Conclusion

An H_2O_2-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, H_2O_2-triggered peptide bond cleavage of the obtained peptide was demonstrated even in the presence of 10 μM H_2O_2 (concentration of H_2O_2 in cancer cells: 10 to 100 μM).[6] This result suggests that our H_2O_2-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

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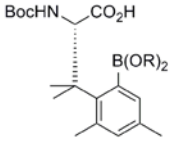
12. As the hydrogen peroxide-responsive amino acid, compound **18** was also designed and we first attempted to prepare it. However, mass of the product was identical to that of **19** but not **18** (ESI-Ion Trap MS, *m*/*z* calcd for [M + Li, Na, or K]$^+$ 360.2, 376.2, or 392.2, found 359.7, 375.8, 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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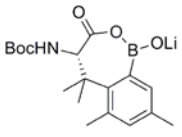

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17. To perform the reaction without guanidine, concentration of peptide **12** was reduced. However, the peptide was not dissolved in an aqueous solvent even if the concentration of **12** was 1 μM. Therefore, the use of guanidine is essential for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

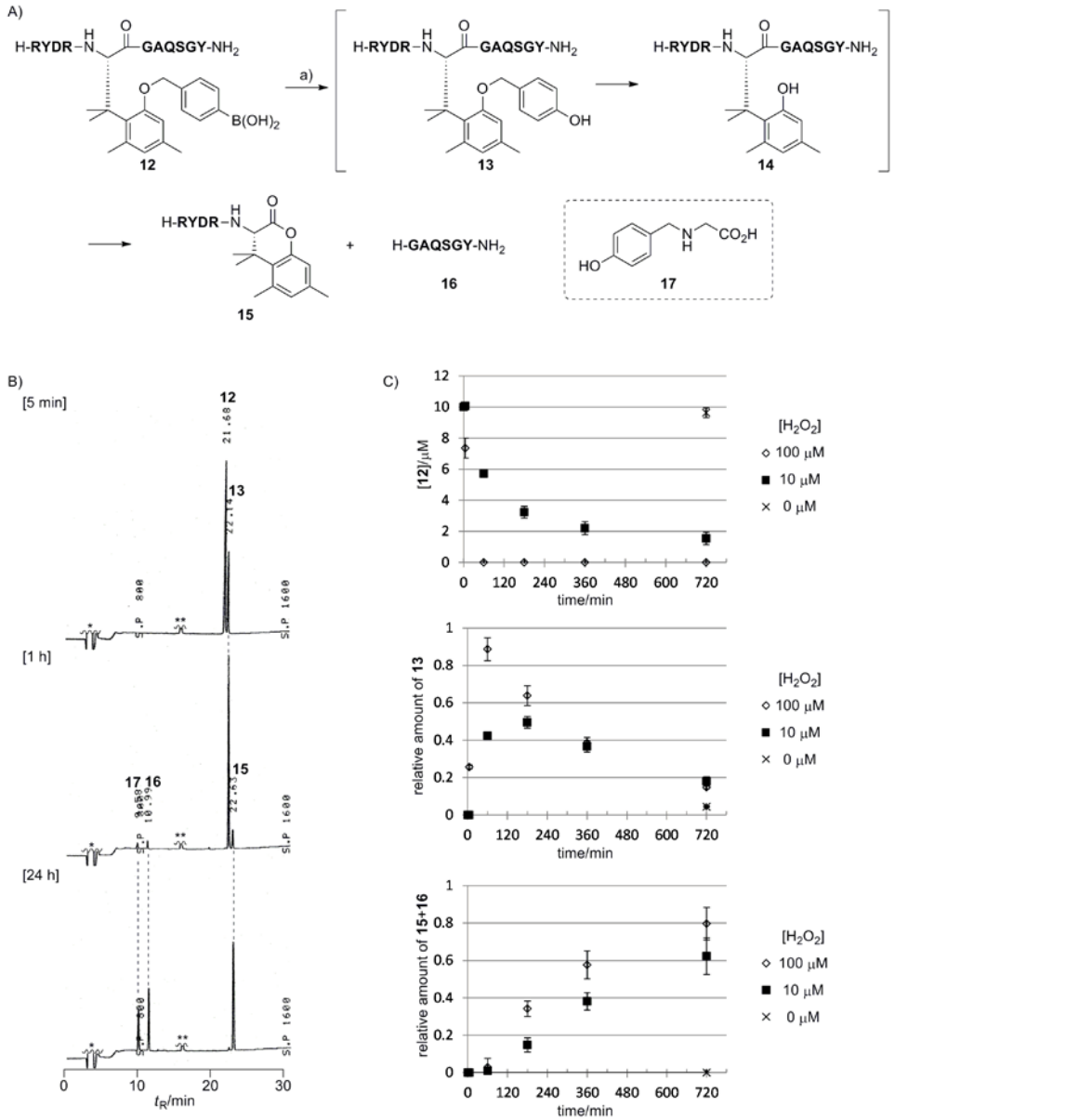

Figure 2. Hydrogen peroxide-responsive peptide bond cleavage. A) Reagents and conditions: a) 100, 10, or 0 μM H_2O_2, 10 μM **12**, 20 mM sodium phosphate buffer with 100 mM glycine and 6 M guanidine hydrochloride (pH 7.4), 25 ppm benzamide as an internal standard, 37 °C. B) HPLC profiles of the reaction 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 $PA_{\mathbf{x}}$ means peak area of **x** in the HPLC chromatogram. Relative amount of **13** or **15** + **16** = {$PA_{\mathbf{13}}$ or ($PA_{\mathbf{15}}$ + $PA_{\mathbf{16}}$)} × $PA_{IS\,(t\,=\,0)}$ ÷ (PA_{IS} × $PA_{\mathbf{12}\,(t\,=\,0)}$). The average and standard deviation of three measurements is shown at each time point.