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# Development of thiol-responsive amide bond cleavage device and its application for peptide nucleic acid-based DNA releasing system

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ABSTRACT

To develop a thiol-responsive DNA-releasing system, a thiol-responsive amino acid capable of inducing an amide bond cleavage in the presence of a thiol was developed. It was successfully combined with peptide nucleic acid (PNA), and thiol-induced release of DNA from the thiol-responsive PNA/DNA complex was observed.



In the field of gene therapy and genetic recombination, development of a non-viral methodology for controlled delivery of DNA or RNA to prevent potential infection resulting from the use of viral vectors has been attracting increasing attention.[1] In this context, release of an entrapped or encapsulated nucleic acid by a non-viral vector only after entering target cells is indispensable. An intracellular reductive environment, mainly due to the presence of thiols like glutathione, is one of the most attractive triggering substances for intracellular DNA/RNA releasing systems[2] because the concentration of thiols is significantly different between the intracellular and extracellular environments (concentration of glutathione: 0.2–10 mM in cytoplasm; 2 μM in plasma).[3] Previously, we reported a stimulus-responsive peptide which can induce a processing (peptide bond cleavage) reaction after stimulus-induced removal of a phenolic protective group followed by lactonization of the trimethyl lock moiety.[4,5] In this Letter, we report the development of a thiol-responsive amino acid and its application for a thiol-responsive DNA releasing system.

It is known that a nitrobenzenesulfonyl amide and ester can be easily cleaved in the presence of thiol,[6] and thiol-induced cleavage of an aryl nitrobenzenesulfonate has been successfully applied as a quantitative thiol probe.[7] With this in mind, we designed thiol-responsive peptide nucleic acid (PNA[8]) **1** possessing a *p*-nitrobenzenesulfonyl (*p*Ns) group on the phenolic hydroxyl group of the stimulus-responsive amino acid (Scheme 1). Addition of PNA **1** to a solution of complementary DNA should induce hybridization to form a stable PNA/DNA double strand. In the presence of thiol, removal of the *p*Ns group followed by a lactonization of the trimethyl lock moiety should cause fragmentation of PNA **1** at the C-terminal position of the thiol-responsive amino acid releasing the complementary DNA.

First, we attempted to synthesize model peptide **2**, possessing a thiol-responsive amino acid, to examine its reactivity and selectivity against thiol. Scheme 2 shows the synthesis of thiol-responsive model peptide **2**. The phenolic hydroxyl group on substrate **3**[4b] was sulfonylated with a *p*-nitrobenzenesulfonyl chloride in the presence of $K_2CO_3$ to afford *p*Ns derivative **4**. The TBS group on **4** was removed under acidic conditions to afford alcohol **5**, and it was oxidized with PCC to give aldehyde **6**. After subsequent oxidation of aldehyde **6** with $NaClO_2$, followed by Boc removal by the action of TFA, the generated amine was re-protected with the Fmoc group to afford Fmoc-protected thiol-responsive amino acid **7**. The total yield of Fmoc derivative **7** amounted to 64% over six steps beginning from phenol **3**. Finally, incorporation of amino acid derivative **7** into a peptide by Fmoc solid phase peptide synthesis (Fmoc SPPS) afforded thiol-responsive model peptide **2**.[9]

To examine the reactivity and selectivity of model peptide **2** against thiol, we tested the processing reaction outlined in Scheme 3. Diastereomerically purified peptide **2** (0.02% w/v) in 30% v/v acetonitrile/phosphate buffer (20 mM, pH 7.6 or 9.0) was incubated with or without 0.1% w/v nucleophiles including thiol and amine at 37 °C for 24 h. The reaction progress was monitored by HPLC, and the resulting peptides were characterized by electrospray ionization mass spectrometry (ESI-MS). Yields of processing products were calculated based on peak areas in the HPLC chart, and the results are summarized in Table 1. In the presence of sodium 2-mercaptoethanesulfonate **11**,[10] processing products **8**, **8′** (an epimer of **8** at a carbon asterisked in Scheme 3),[11] and **9** were obtained at both pH 7.6 and 9.0 (entries 1 and 2, respectively). In these reactions, *p*Ns-deprotected intermediate **10** was not observed because lactonization was possibly faster than removal of the *p*Ns group.[4a] The yield of processing products at pH 9.0 was higher than that at pH 7.6, presumably due to an efficient generation of a nucleophilic thiolate anion under basic conditions. To accelerate the processing reaction under physiological conditions, introduction of the electron-withdrawing group on *p*Ns group or treatment of peptide

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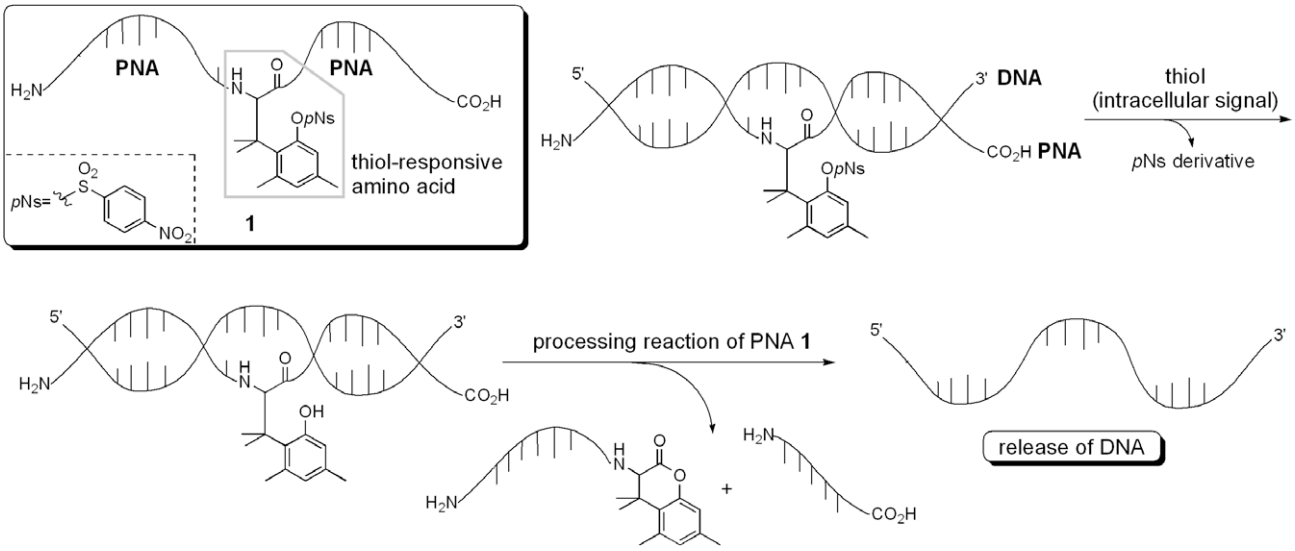
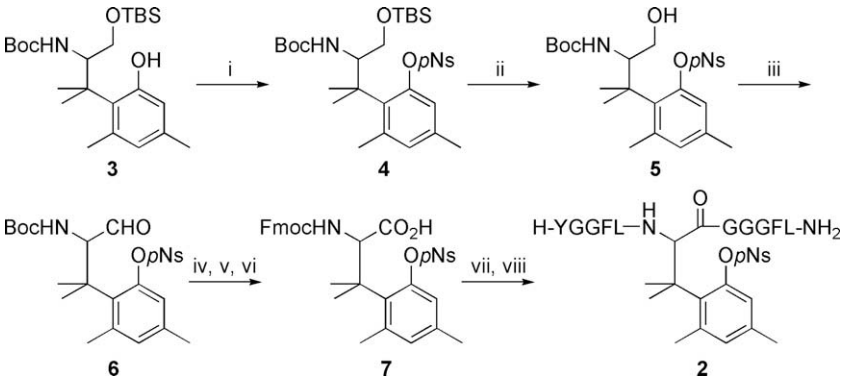
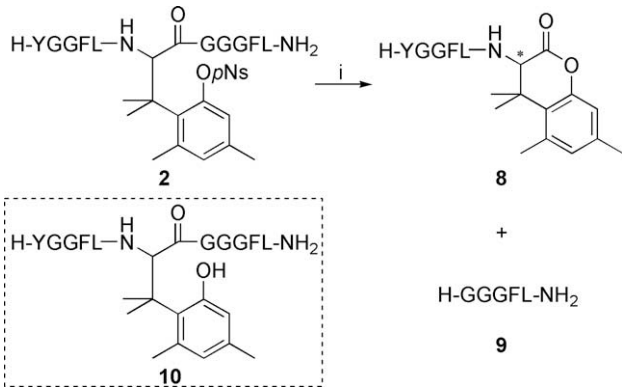



**Scheme 1.** Design of thiol-responsive peptide nucleic acid (PNA).




**Scheme 2.** Reagents and conditions: (i) *p*-nitrobenzenesulfonyl chloride (*p*NsCl), $K_2CO_3$, acetone, reflux, quant; (ii) AcOH, $H_2O$, THF, quant; (iii) PCC, $CH_2Cl_2$, 91%; (iv) $NaClO_2$, $NaH_2PO_4$, 2-methyl-2-butene, *tert*-BuOH, $H_2O$, acetone; (v) HCl, AcOEt; (vi) FmocOSu, $Na_2CO_3$, $H_2O$, MeCN, 70% (three steps); (vii) Fmoc SPPS; (viii) TFA/triethylsilane/$H_2O$ = 95:2.5:2.5 v/v/v.




**Scheme 3.** Reagents and conditions: (i) 0.1% w/v nucleophile, phosphate buffer (20 mM), 30% v/v MeCN, 37 °C, 24 h under Ar. Peptide **8′** is an epimer of **8** at the asterisked carbon.

**2** with glutathione S-transferase[7b] is in progress. When peptide **2** was incubated without nucleophile or with a taurine as an amine nucleophile, the processing products were not detected (entries 3 and 4). These results suggest that the processing reaction of model peptide **2** occurs thiol-selectively.

**Table 1**
Effects of pH and nucleophile on the processing reaction of peptide **2** as shown in Scheme 3

| Entry | Nucleophile | pH | Yield (%) |
|---|---|---|---|
| 1 | $HS(CH_2)_2SO_3Na$ (**11**) | 7.6 | 37 |
| 2 | **11** | 9.0 | 68 |
| 3 | $H_2N(CH_2)_2SO_3H$ | 9.0 | 0 |
| 4 | None | 9.0 | 0 |

Next, we monitored the time course of the thiol-responsive processing reaction of peptide **2** in the presence of 0.1% w/v thiol **11** at pH 9.0 (Fig. S1). After 24 h of incubation at 37 °C, the yield of processing products almost reached a plateau. It might be caused by oxidative decomposition of thiol **11** under basic conditions to generate a disulfide by-product. In order to complete the processing reaction, we tried stepwise addition of thiol in the two-portion manner described below. Peptide **2** (0.02% w/v) in 30% v/v acetonitrile/phosphate buffer (pH 9.0, 20 mM) was incubated with 0.1% w/v thiol **11** at 37 °C for 24 h, and additional 0.1% w/v thiol **11** was added to the reaction mixture. It was incubated at the same temperature for an additional 24 h, and the reaction progress was monitored by HPLC (Fig. 1). Whereas the substrate remained after incubation with 0.1% w/v thiol **11** at 37 °C for 24 h (Fig. 1b),

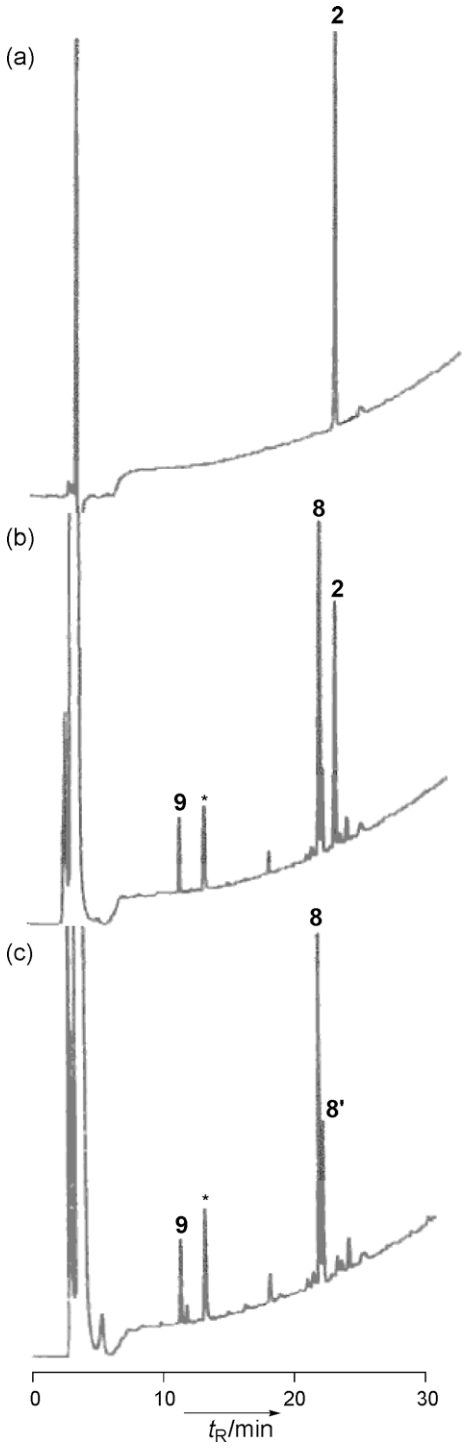



**Figure 1.** HPLC profiles (a) before treatment with thiol **11**, (b) after 24 h incubation with 0.1% w/v thiol **11** at 37 °C under Ar, and (c) after 24 h incubation with 0.1% w/v thiol **11** at 37 °C under Ar, followed by subsequent incubation with additional 0.1% w/v thiol **11** at 37 °C for 24 h under Ar. Peptides were detected by UV absorbance at 220 nm. Asterisked peak is not a peptidic compound.

substrate **2** completely disappeared after stepwise addition of thiol **11** to afford processing products **8**, **8′** and **9** (Fig. 1c). When 0.2% w/v thiol **11** was added in one portion, the reaction did not finish within 48 h of incubation, and the yield of the processing products was 76%. Therefore we decided to use the two-portion protocol for a thiol treatment in subsequent experiments.

These results encouraged us to develop a thiol-responsive PNA. PNA **12** depicted in Scheme 4 was designed to possess a Gly-thiol-responsive amino acyl residue with length similar to that of thymine derived **t** residue (Fig. 2). Additionally, a lysine residue was incorporated, which was expected to provide high solubility in water and to interact with negatively charged DNA. Fmoc SPPS using PyBOP (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate), HCTU (*O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate), or HATU (*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate) allowed PNA **12**[12] to be synthesized.[13] Then we examined a thiol-responsive processing reaction of PNA **12** in phosphate buffer (pH 9.0) (Scheme 4). After treatment with thiol **11** in the two-portion manner, PNA **12** completely disappeared and processing products **13** and **14** were obtained in good purity (Fig. S2). Next we examined the ability of PNA **12** to hybridize with complementary DNA and to release the DNA from the complex by treatment with a thiol. Melting temperature ($T_m$) of PNA or DNA/DNA complex was measured in phosphate buffer (10 mM phosphate, 100 mM NaCl, pH 9.0);[14] the results are summarized in Table 2. As a thiol-treated sample (depicted as 'thiol +' in Table 2), hybridized nucleic acids were incubated with 0.1% w/v of sodium 2-mercaptoethanesulfonate **11** at 37 °C for 24 h, and were subsequently incubated with additional thiol **11** (0.1% w/v) at the same temperature for 24 h. Melting curves were recorded using a fluorescence spectrometer ($\lambda_{ex}$ = 525 nm, $\lambda_{em}$ = 600 nm) in the presence of ethidium bromide.[15] In the absence of thiol, the melting curve of PNA **12**/DNA($A_9$) complex was observed under these conditions, and $T_m$ was estimated as 25.0 °C. (DNA($N_9$) refers to 5′-d(NNNNNNNNN)-3′). As expected, $T_m$ of PNA **12**/DNA($A_9$) complex was lowered in the presence of thiol (entries 1 and 2).[16] In contrast, DNA($T_9$)/DNA($A_9$) hybridization was not affected by thiol treatment (entries 3 and 4). Furthermore, $T_m$ of PNA **12**/DNA($A_9$) complex was

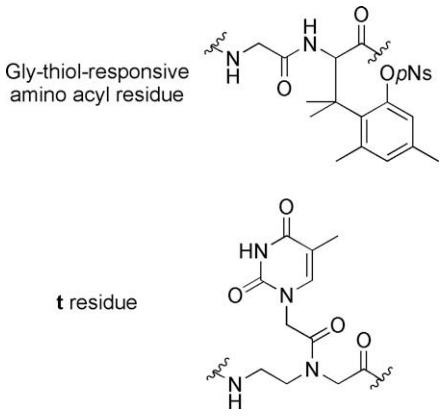
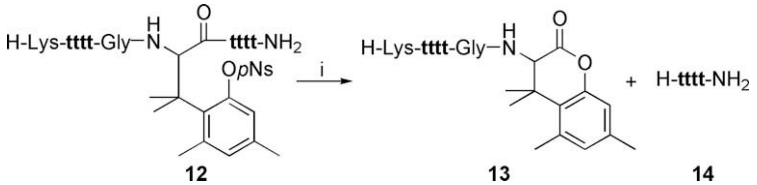



**Figure 2.** Structural comparison of Gly-thiol-responsive amino acyl residue and **t** residue.




**Scheme 4.** Reagents and conditions: (i) 0.1% w/v thiol **11**, phosphate buffer (pH 9.0, 20 mM), 30% v/v MeCN, 37 °C, 24 h under Ar. Subsequent addition of 0.1% w/v thiol **11**, 37 °C, 24 h under Ar, as per the two-portion manner.

**Table 2**
Results of melting temperature experiments[a]

| Entry | Nucleic acid | Thiol | $T_m$ (°C) |
|---|---|---|---|
| 1 | PNA **12**/DNA($A_9$) | – | 25.0 |
| 2 | PNA **12**/DNA($A_9$) | + | <10.0 |
| 3 | DNA($T_9$)/DNA($A_9$) | – | 18.5 |
| 4 | DNA($T_9$)/DNA($A_9$) | + | 18.5 |

[a] Conditions: 4.0 μM concentration for each strand, 10 mM phosphate buffer (pH 9.0), 100 mM NaCl. Thiol –: The sample was not treated with thiol **11**. Thiol +: The sample was treated with thiol **11** (0.1% w/v at 37 °C for 24 h under Ar, followed by subsequent treatment with thiol **11** (0.1% w/v) again at 37 °C for an additional 24 h under Ar. Melting curves were recorded in the presence of ethidium bromide ($\lambda_{ex}$ = 525 nm, $\lambda_{em}$ = 600 nm). $T_m$s are the average of two runs. DNA($N_9$) denotes 5′-d(NNNNNNNNN)-3′.

higher than that of corresponding DNA($T_9$)/DNA($A_9$) complex (entries 1 and 3). These results suggest that PNA **12** is potentially applicable to a thiol-responsive DNA releasing system.

In conclusion, we developed a thiol-responsive self-processing amino acid which induces an amide bond cleavage reaction after treatment with thiol. With its successful combination with a peptide nucleic acid, thiol-responsive PNA was generated. Melting temperature experiments clarified that thiol-responsive PNA **12** can bind to complementary DNA($A_9$), and the $T_m$ of the thiol-responsive PNA **12**/DNA($A_9$) complex was drastically decreased by treatment with thiol. These results suggest that thiol-responsive PNA is potentially applicable as a DNA-binding moiety of a thiol-responsive gene delivery system. Development of the thiol-responsive PNA with sequence specificity and high sensitivity to thiols, and its application for gene delivery system are in progress.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.03.006.

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9. *Thiol-responsive model peptide* **2**: Analytical HPLC condition (Cosmosil $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate: 1 mL/min)): linear gradient of solvent B in solvent A, 10–80% over 30 min. Retention time = 22.6 and 23.2 min, respectively for each diastereomer of peptide **2**. MS (ESI-IT) calcd for $C_{68}H_{88}N_{13}O_{17}S$ ($[M+H]^+$): 1390.6, found; 1390.7 and 1390.7.
10. Because glutathione and its disulfide derivative make HPLC chart complicated, thiol **11** was used as a model thiol.
11. Time dependent epimerization of processing product **8** to its epimer **8′** was observed under reaction conditions.
12. *Thiol-responsive PNA* **12**: Analytical HPLC condition (Cosmosil $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate: 1 mL/min)): linear gradient of solvent B in solvent A, 1–50% over 30 min. Retention time = 22.0 min. MS (ESI-IT) calcd for $C_{115}H_{152}N_{38}O_{40}S$ ($[M+2H]^{2+}$): 1369.1, found; 1369.1.
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