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A novel N-terminal degradation reaction of peptides via N-amidination

Yoshio Hamada *

Medicinal Chemistry Laboratory, Kobe Pharmaceutical University, Motoyamakita, Higashinada, Kobe 658-8558, Japan

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ABSTRACT

The cleavage of amide bonds requires considerable energy. It is difficult to cleave the amide bonds in peptides at room temperature, whereas ester bonds are cleaved easily. If peptide bonds can be selectively cleaved at room temperature, it will become a powerful tool for life science research, peptide prodrug, and tissue-targeting drug delivery systems. To cleave a specific amide bond at room temperature, the decomposition reaction of arginine methyl ester was investigated. Arginine methyl ester forms a dimer; the dimer releases a heterocyclic compound and ornithine methyl ester at room temperature. We designed and synthesized *N*-amidinopeptides based on the decomposition reaction of arginine methyl ester. Alanyl-alanine anilide was used as the model peptide and could be converted into *N*-degraded peptide, alanine anilide, via an N-amidination reaction at close to room temperature. Although the cleavage rate in pH 7.4 phosphate buffered saline (PBS) at 37 °C was slow ($t_{1/2}$ = 35.7 h), a rapid cleavage rate was observed in 2% NaOH aq ($t_{1/2}$ = 1.5 min). To evaluate the versatility of this reaction, a series of peptides with Lys, Glu, Ser, Cys, Tyr, Val, and Pro residue at the N-terminal were synthesized; they showed rapid cleavage rates of $t_{1/2}$ values from 1 min to 10 min.

The selective cleavage of a specific peptide bond at room temperature will be a powerful tool for life science research, peptide prodrugs, and tissue-targeting drug delivery systems. However, the cleavage of amide bonds requires considerable energy,[1] whereas ester bonds can be easily cleaved. Many ester-type prodrugs that release the corresponding parent drugs with a hydroxy group under physiological conditions have been reported.[2,3] Previously, we reported a series of novel ester-type prodrugs that could be converted to the parent drugs under physiological conditions without using enzymes.[4–8] Although an amide bond is difficult to cleave at room temperature, selective amide bond cleavage reactions are known in nature. For example, a protein, intein, undergoes protein splicing that involves a particular peptide bond cleavage.[9,10] To develop a reaction for specific amide bond cleavage, we investigated the decomposition reaction of arginine methyl ester reported by Photaki et al.,[11] as shown in Figure 1A. In this reaction, the guanidino group of an arginine methyl ester attacks the ester carbonyl carbon of another arginine methyl ester, forming an arginine dimer. Next, the N-terminal amino group of the dimer attacks the guanidine carbon within the molecule, forming a heterocyclic compound and ornithine methyl ester. Surprisingly, this decomposition reaction proceeds at room temperature under neutral conditions. This *N*-aminoacyl guanidine structure of the dimer releases an N-terminal amino acid moiety at room temperature as shown in Figure 1B. However, the guanidine moiety does not contain an amide bond; this compound is also not a common peptide.

We assumed that the driving force of this decomposition reaction is the release of the heterocyclic compound that is stabilized because of a conjugate structure. Because the *N*-amidino aminoacyl structure in which the order of the acyl and guanidino groups is reversed is thought to release the same heterocyclic compound to the *N*-aminoacyl guanidine compound (Fig. 1B), we envisioned that the release of the heterocyclic compound would be the driving force for the cleavage reaction of an amide bond. The N-amidination of a peptide can be applied to the N-terminal degradation reaction of the peptide at room temperature. Hence, we designed a novel N-terminal cleavage reaction of peptides using this reaction and synthesized a series of *N*-amidinopeptides **1**–**8** and **18**.

Peptides **1**–**8** were synthesized using the common solution-phase peptide synthesis method as shown in Schemes 1 and 2. The peptide bond formation was performed using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) in the presence of 1-hydroxybenzotriazole (HOBt) as the coupling reagent. The amidination reactions for preparing **16a**–**g** were performed using *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine in acetonitrile or ethyl acetate. The Boc-deprotections of **9** and **11** were performed using anisole and 4 N HCl/dioxane. The

* Tel.: +81 78 441 7555.
E-mail address: pynden@gmail.com

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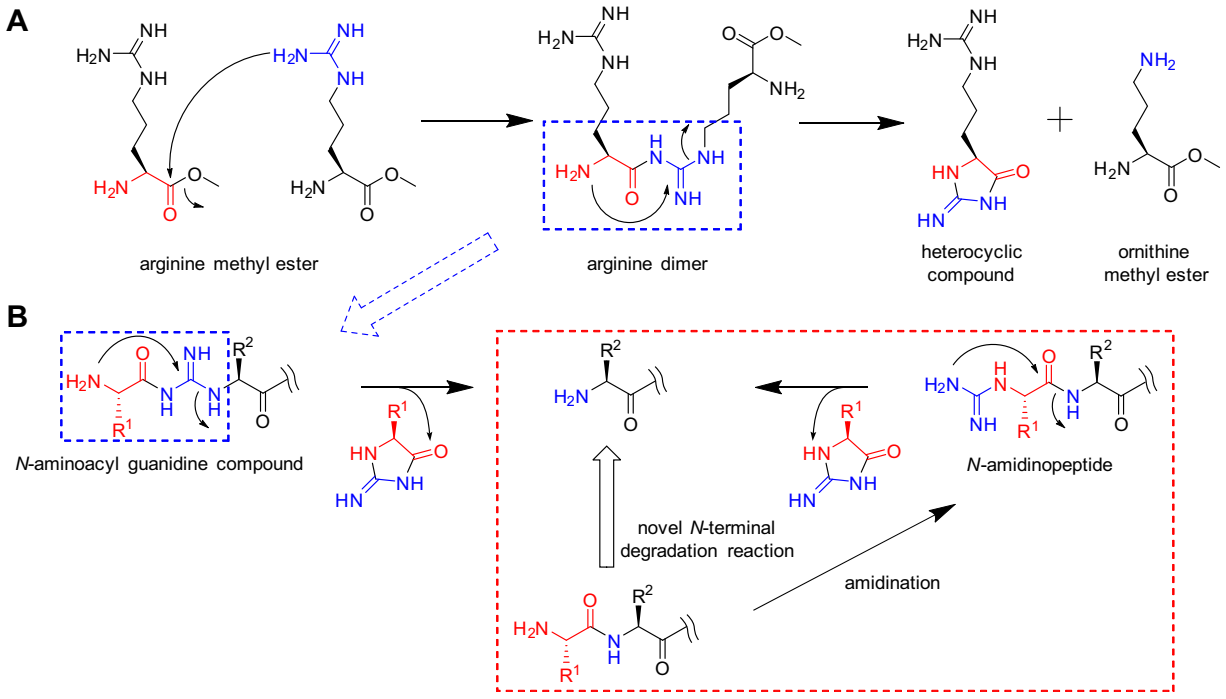
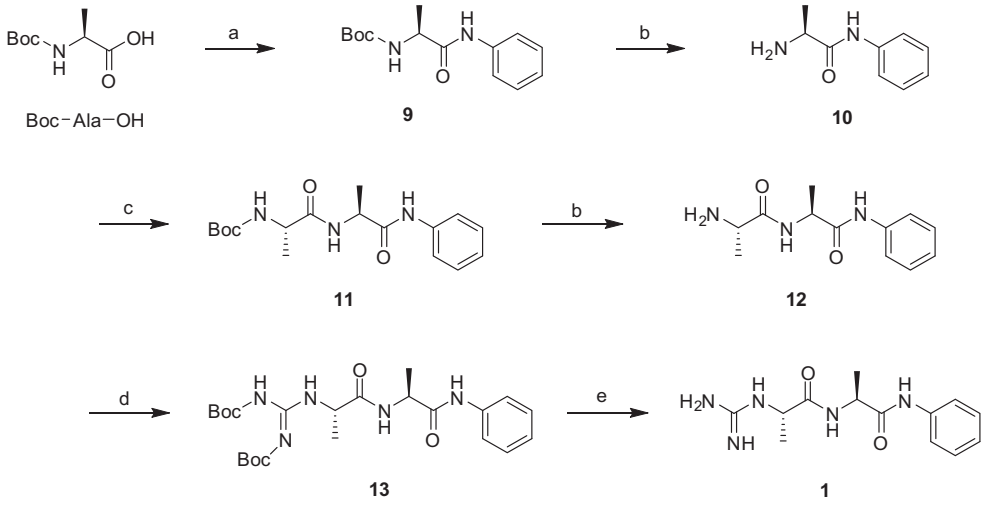


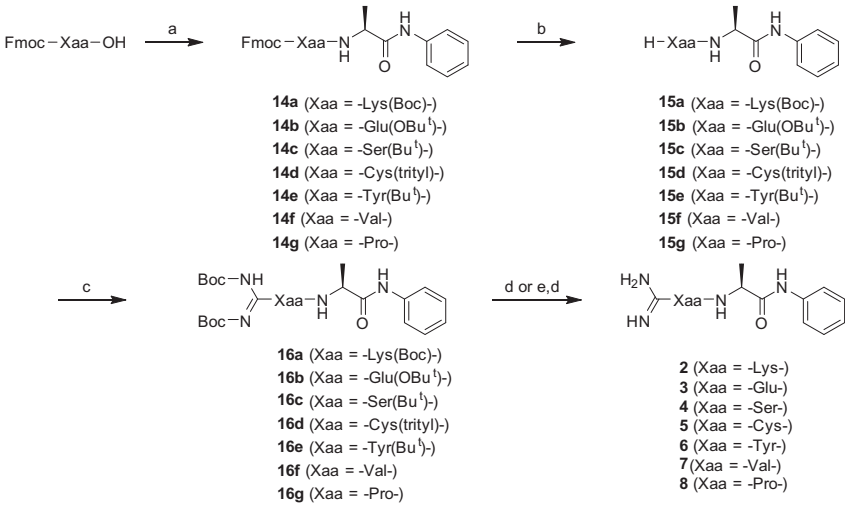
Figure 1. (A) Decomposition reaction of arginine methyl ester. (B) Design of an N-terminal degradation reaction of peptides via *N*-amidinopeptides.



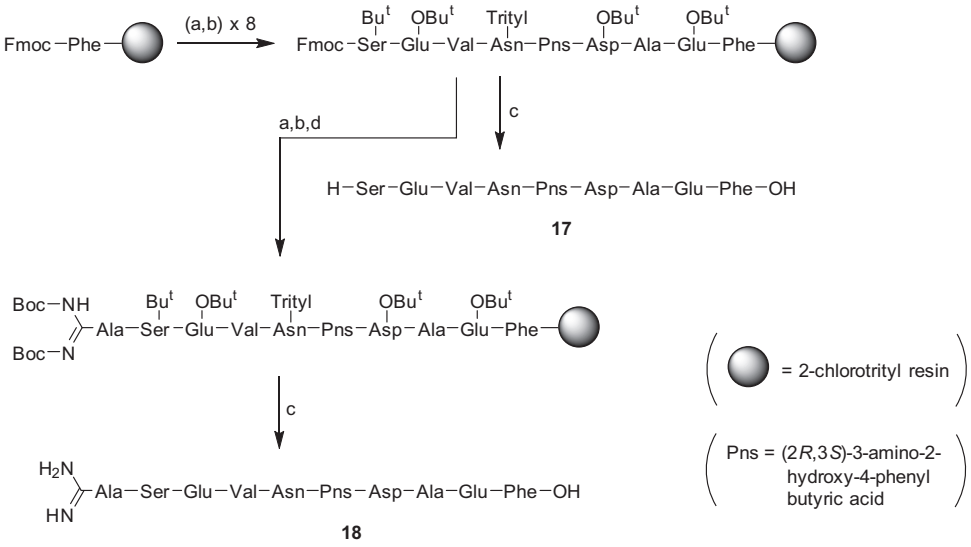
Scheme 1. Reagents and conditions: (a) aniline, EDC·HCl, HOBt, acetonitrile, 4 h; (b) anisole 4 N HCl/dioxane, 2 h; (c) Boc-Ala-OH, EDC·HCl, HOBt, acetonitrile, 4 h; (d) *N*,*N*′-bis (*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine, ethyl acetate, 1 day; (e) TFA, 2 h.

Fmoc-deprotections of **14a–g** were performed using 20% diethylamine in dioxane. The final deprotections for preparing of **1–8** were performed using trifluoroacetic acid (TFA) with/without cation scavengers, *m*-cresol, and ethyl mercaptan. Because the deprotection reaction for preparing **5** afforded some by-products with an *S*-trityl group or an *S*-*tert*-butyl group, the trityl group of **16d** was deprotected using iodide in ethyl acetate before the TFA treatment. Peptides **1–8** were obtained as the TFA salts and stored in a refrigerator. Peptides **17** and **18** were synthesized via 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis using a 2-chlorotritylchloride resin as shown in Scheme 3, and the details are given in the Supporting information. The Fmoc group was removed with 20% piperidine in dimethylformamide (DMF). Peptide bonds were formed using diisopropylcarbodiimide (DIPCDI) in the presence of HOBt as the coupling reagent. N-amidination of peptides was achieved using *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine in DMF. After the peptide chains were elongated to their desired length, they were cleaved from the resin using TFA in the presence of *m*-cresol, thioanisole, and water. The peptides were all purified by preparative reverse phase high-performance liquid chromatography (RP-HPLC).

To evaluate our strategy for developing the N-terminal degradation reaction, we synthesized peptide **1**, in which the N-terminal amino group of the alanyl-alanine anilide **12** was used as the model peptide and converted to the guanidino group. First, peptide **1** was incubated with pH 7.4 phosphate buffered saline (PBS) buffer under physiological conditions at 37 °C and evaluated by high-performance liquid chromatography (HPLC) using a reverse-phase C18 column and a linear gradient system of acetonitrile and 0.1% aqueous TFA. Unfortunately, the cleavage rate of the N-terminal



Scheme 2. Reagents and conditions: (a) **10**, EDC·HCl, HOBt, DMF, 4 h; (b) 20% diethylamine, dioxane, 1 h; (c) *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine, acetonitrile, 1 day; (d) *m*-cresol, ethyl mercaptan, TFA, 2 h; (e) I_2, ethyl acetate.



Scheme 3. Reagents and conditions: (a) Fmoc-protected amino acid, HOBt, DIPCDI, DMF, 2 h; (b) 20% piperidine, DMF, 20 min; (c) thioanisole, *m*-cresol, H_2O, TFA; (d) *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine, DMF, 1 day.

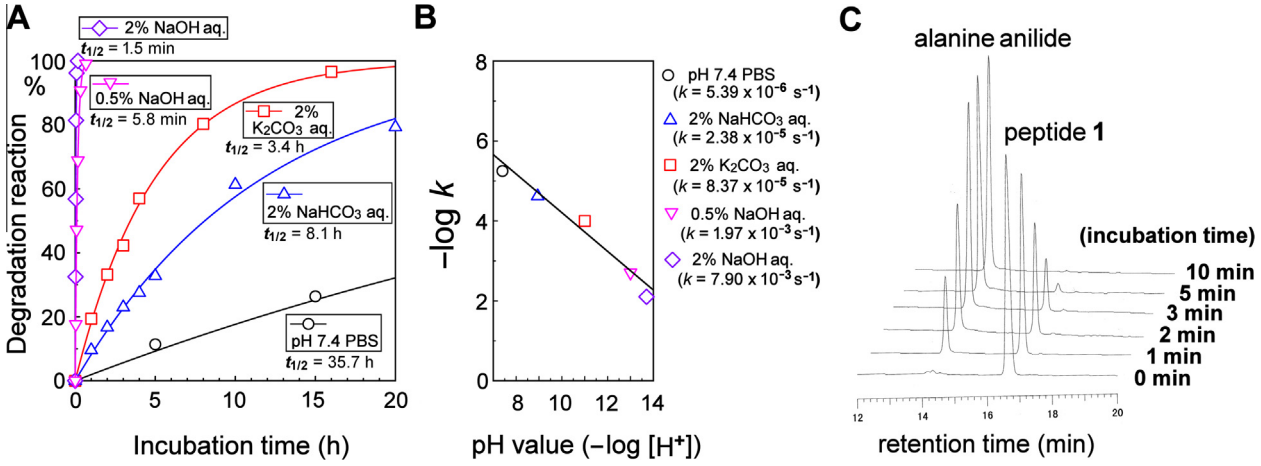


Figure 2. (A) Time course of peptide **1** in incubation mediums at 37 °C. (B) The correlation between the N-degradation reaction of peptide **1** and the respective pH values of the incubation media. The *k* values are the rate constants of the N-terminal degradation reaction. (C) HPLC profiles of peptide **1** in 2% NaOH aq at 37 °C.

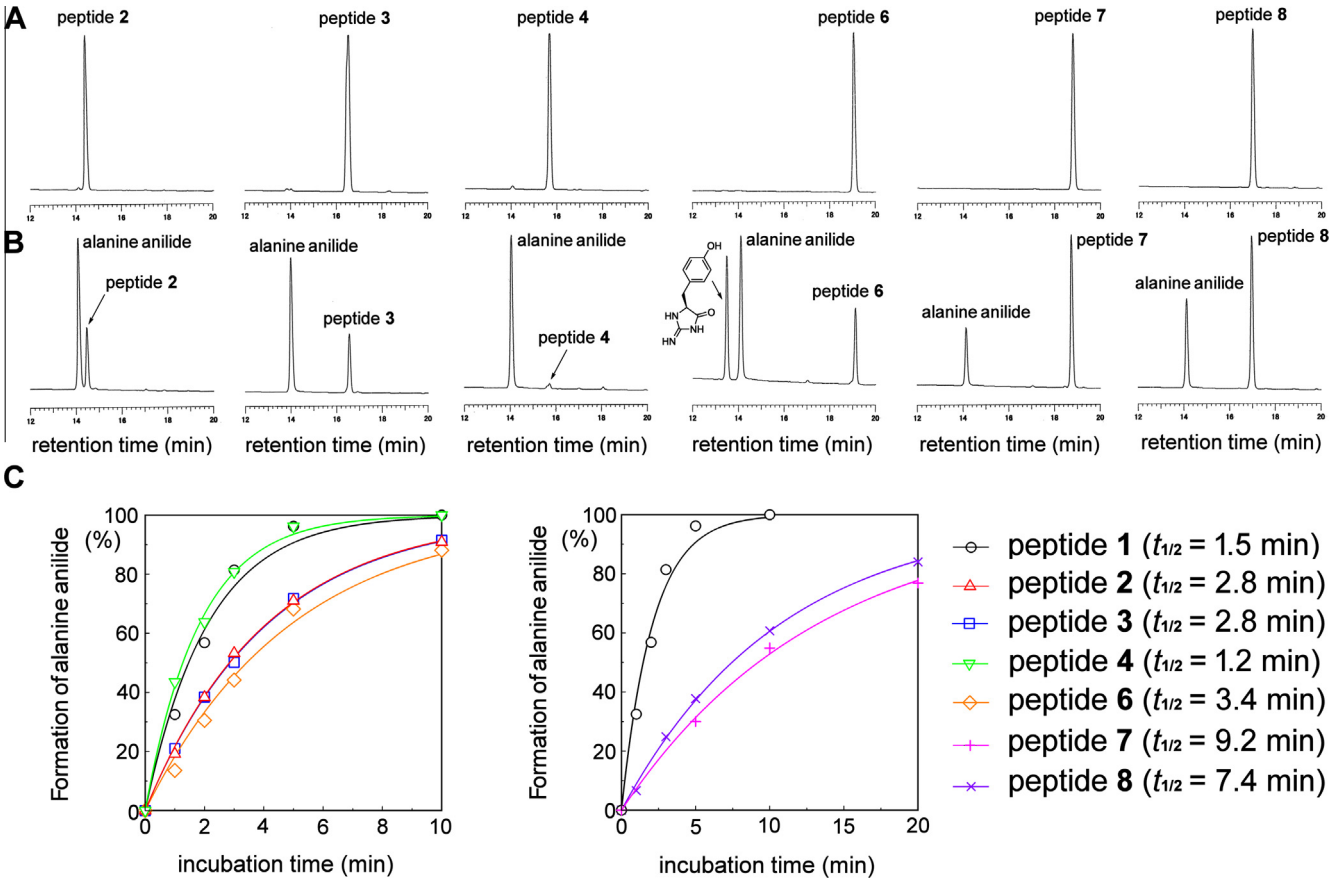


Figure 3. (A) HPLC profiles of peptides **2**–**4** and **6**–**8**. (B) HPLC profiles of peptides **2**–**4** and **6**–**8** after the incubation in 2% NaOH aq for 5 min at 37 °C. (C) Time course of peptides **1**–**4** and **6**–**8** in 2% NaOH aq at 37 °C.

alanine residue was very slow in pH 7.4 PBS at 37 °C, as shown in Figure 2A ($t_{1/2}$ value: 35.7 h). This is the first N-terminal degradation reaction of peptides at room temperature. The N-terminal cleavage reaction, Edman degradation,[12,13] requires acidic and heating conditions after the treatment of a peptide with phenylisothiocyanate. Next, the cleavage rates of peptide **1** in alkaline media, with pH values ranging from 8.0 to 13.7 were measured. The cleavage rates (given as $t_{1/2}$ values) in 2% aq $NaHCO_3$ (pH = 8.9), 2% aq K_2CO_3 (pH = 11), 0.5% aq NaOH (pH = 13) and 2% aq NaOH (pH = 13.7) were 8.1 h, 3.4 h, 5.8 min, and 1.5 min, respectively. Higher pH values appeared to accelerate the *N*-degradation reaction, as shown in Figure 2B. To investigate the pH dependency of the N-terminal degradation reaction, the rate constants of peptide **1** in the respective incubation media were calculated using the fitting Eq. 1

$$[A]_t = A_{MAX} \times (1 - \mathrm{Exp}(-k \times t)) \quad (1)$$

where, t is the incubation time; k is the rate constant of the N-terminal degradation reaction; A_{MAX} is the maximum concentration of alanine anilide; and $[A]$ is the concentration of alanine anilide.

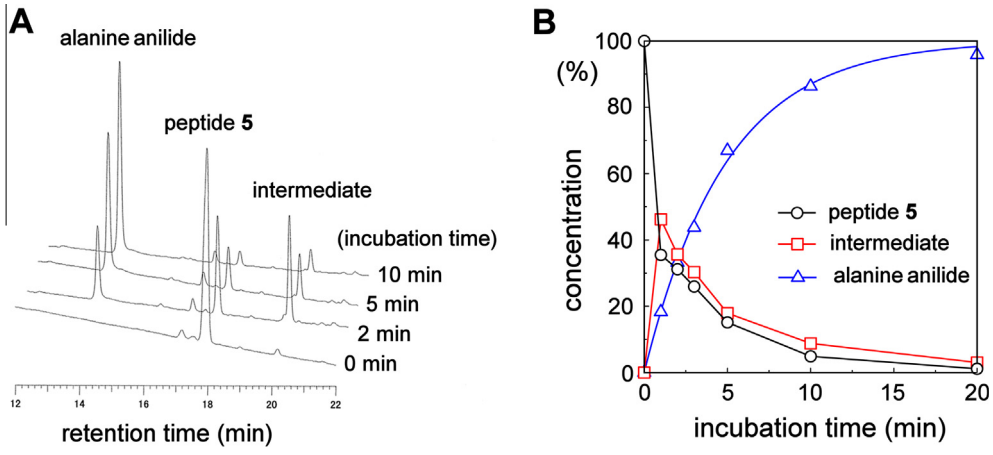


Figure 4. (A) HPLC profile of peptide **5** in 2% NaOH aq at 37 °C. (B) Time course of peptide **5** in 2% NaOH aq at 37 °C.

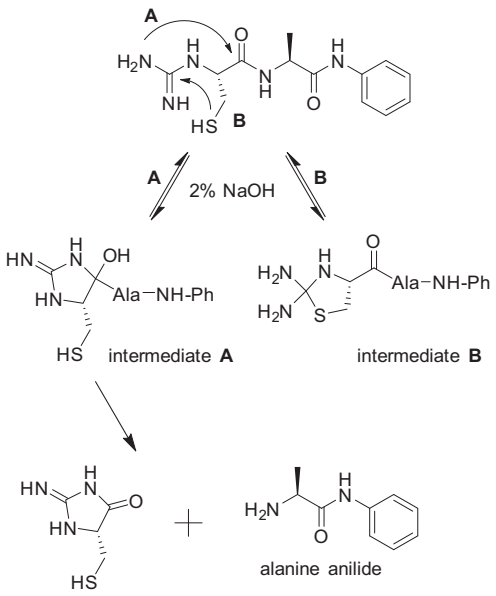


Figure 5. N-terminal degradation reaction and intermediates of peptide **5** with a Cys residue.

The pH values ($-\log[H^+]$) of the respective incubation media showed a negative correlation (coefficient of correlation, r = –0.9814) with the –log *k* values (*k*, rate constant) of the N-terminal degradation reaction, as shown in Fig. 2B—which means that the rate constants of the N-terminal degradation reaction appeared to be positively correlated to the concentration of the hydroxide ions [OH^-] in the incubation media. Thus, the incubation in 2% NaOH aq showed the most rapid cleavage rate, as shown in Figure 2A. This peptide is stable as a TFA salt and released the N-terminal-degraded peptide, alanine anilide **10**, time-dependently without the formation of any by-product as shown in Figure 2C.

To evaluate the versatility of the N-terminal degradation reaction, peptides **2**–**8** possessing Lys, Glu, Ser, Cys, Tyr, Val, and Pro residues at the N-terminal position were synthesized. Because these peptides contain a basic group, an acidic group, an OH group, an SH group, an aromatic ring, a sterically hindered branched alkyl group, and a cyclic structure, respectively, on the side chains, they cover all types of amino acid residues for evaluating the N-terminal degradation reaction. Peptides **2**–**8** were incubated in 2% NaOH aq at 37 °C and measured by HPLC. Peptides **2**–**4** and **6**–**8** rapidly released the N-terminal-degraded peptide, alanine anilide, without any by-product as shown in Figure 3A and B. Although the heterocyclic compounds that were released from most of the *N*-amidinopeptides had a high hydrophilicity and low UV absorption coefficient and could not be identified by the HPLC analysis using an ODS column and a UV detector. Although the heterocyclic compound that was released from peptide **6** with a Tyr residue could be identified by HPLC, as shown in Figure 3B, all products released from *N*-amidinopeptides **1**–**8** were identified using ESI-Mass analysis (see Supporting information). In the case of peptide **5** with a Cys residue, an unidentified peak appeared on the HPLC trace at ~20.2 min after the incubation in 2% NaOH aq at 37 °C as shown in Figure 4A. The peak at ~20.2 min immediately appeared on the HPLC trace after the incubation and then disappeared slowly. Because the peaks of the unidentified compound and peptide **5** showed an almost equal rate (55:45, Fig. 4B), they may be in equilibrium. There are two possibilities for the unidentified compound—intermediate **A** and **B** as shown in Figure 5. A peak that showed the same molecular weight to the intermediate

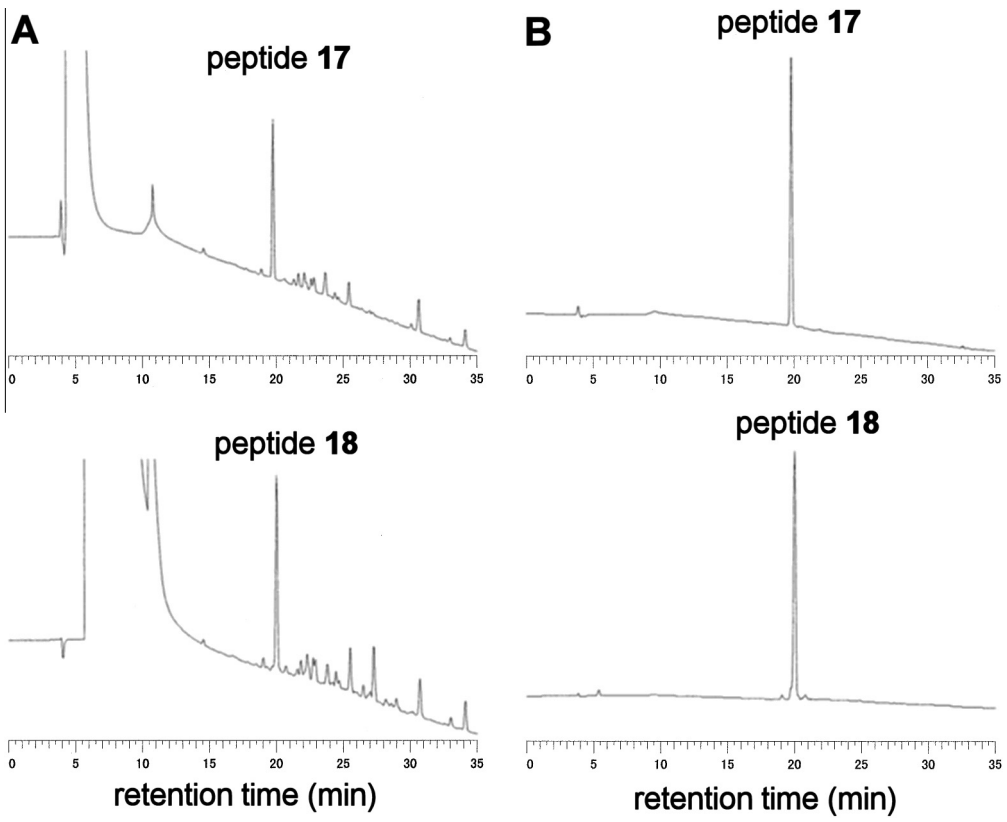


Figure 6. (A) HPLC profiles of the crude peptides, **17** and **18**, after their cleavage from the resin. (B) HPLC profiles of the preparative RP-HPLC purified peptides, **17** and **18**.

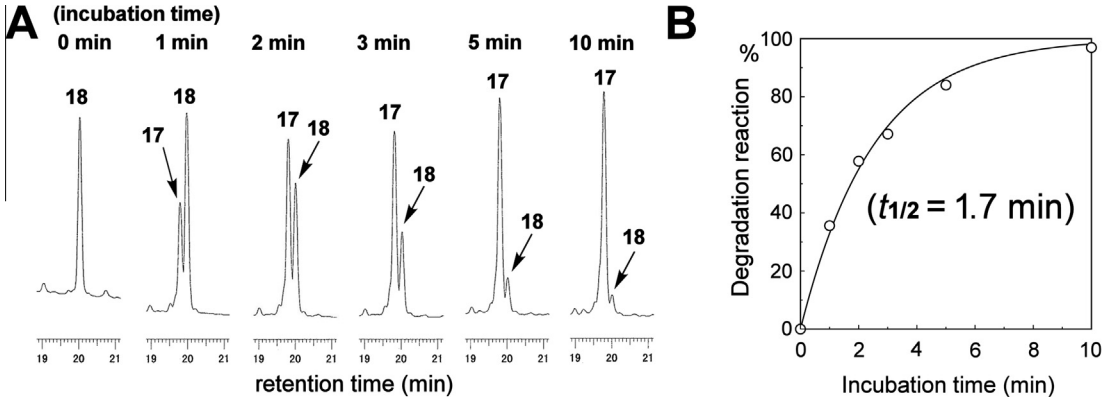


Figure 7. (A) HPLC profile of *N*-amidino-decapeptide **18** in 2% aq NaOH at 37 °C. (B) Degradation reaction progress of *N*-amidino-decapeptide **18** in 2% aq NaOH at 37 °C.

A and **B** was identified on the ESI-Mass trace obtained from the incubated solution of peptide **5**. Intermediate **A**, which was immediately produced before the N-terminal cleavage reaction, is unlikely, because such a peak did not appear on the HPLC traces of other *N*-amidinopeptides. As peptide **5** with a Cys residue can only form an intermediate with a five-membered ring, thiazolidine, the unidentified peak at ~20.2 min seem to be intermediate **B**. The $t_{1/2}$ value of peptide **5** in 2% NaOH aq at 37 °C was 3.4 min, given the existence of intermediate **B**. As the cleavage of peptide **7** ($t_{1/2}$ = 9.2 min) and **8** ($t_{1/2}$ = 7.4 min) showed slower cleavage rates than peptides **1**–**6** with a $t_{1/2}$ value from 1.2 min to 3.4 min as shown in Figure 3C. The sterically hindered group and ring structure of Val and Pro residues, respectively, may prevent the formation of a five-membered ring transition state.

We applied the N-terminal degradation reaction to a long-chain peptide. Peptide **17** contains an amino acid sequence from our previously reported β-secretase inhibitory peptide and an unusual amino acid, (2*R*,3*S*)-3-amino-2-hydroxy-4phenylbutyric acid (Pns), as a transition-state analog.[14–19] We designed and synthesized *N*-amidino-decapeptide **18** based on peptide **17**. The HPLC profiles of **18**, before and after purification via preparative RP-HPLC, are shown in Figure 6. *N*-Amidino-decapeptide **18** was incubated in 2% aq NaOH at 37 °C and analyzed by HPLC. As shown in Figure 7, peptide **18** released the N-terminal-degraded peptide **17** in a time-dependent manner, and it was identified using HPLC retention time and ESI-Mass experiment. Peptide **18** released **17** rapidly with a $t_{1/2}$ value of 1.7 min and this is close to that of peptide **1** ($t_{1/2}$ = 1.5 min), which has the same N-terminal amino acid residue, Ala. These results indicated that the length of a peptide chain does not affect the N-terminal degradation reaction.

In conclusion, we designed a novel N-terminal degradation reaction and synthesized a series of *N*-amidinopeptides, in which the order of the acyl and guanidino groups of *N*-aminoacyl guanidine structure in the dimer of arginine methyl ester was reversed. The driving force of this reaction may be the release of a heterocyclic compound that is stabilized because of a conjugate structure. A specific amide bond, an N-terminal amino acid residue could be successfully cleaved at almost room temperature with rapid rates of $t_{1/2}$ values from 1 min to 10 min. Moreover, we applied the N-terminal degradation reaction to a long-chain peptide. *N*-Amidino-decapeptide **18** showed a rapid N-terminal degradation rate, with a $t_{1/2}$ value of 1.7 min. Our degradation reaction provided the same product as the Edman degradation. This degradation reaction proceeds in neutral-to-weak alkaline media, whereas Edman degradation requires acidic and heating conditions. Because our degradation proceeds under different reaction conditions than Edman degradation, it has great potential to become an important tool for life science research.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.02.058.

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