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Total synthesis of [L40I, C90A, C109A]-human T-cell leukemia virus type 1 protease

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Abstract—[L40I, C90A, C109A]-Human T-cell leukemia virus type 1 protease, which contains 125 amino acid residues, was synthesized by means of the thioester method. The construction of the backbone of this protease, involved the synthesis of three building blocks, using the Boc solid-phase method. To suppress epimerization during the first segment coupling between Phe and Arg by the thioester method, DMF was employed as a solvent for the reaction. The amount of D-Phe-containing coupling product in the total product was less than 6% and the desired product was isolated by reversed-phase HPLC in 59% yield. The synthetic product showed enzymatic activities comparable to those reported for the [L40I, C90A, C109A]-human T-cell leukemia virus type 1 protease, prepared by an expression method.

Human T-cell leukemia virus type 1 (HTLV-1), a retrovirus associated with a number of human diseases, was the first human retrovirus isolated in the early 1980s from patients with adult T-cell leukemia/lymphoma by Gallo et al.[1] An HTLV-1 gene codes an asparatic protease (PR), which processes its own polyproteins which are transcripted owing to three reading frames. As the result of a series of *cis* processings, a set of proteins is produced, which are necessary for viral replication.[2] Thus, HTLV-1 PR plays a key role in the duplication of HTLV-1 in a manner analogous to the human immunodeficiency virus type 1 protease (HIV-1PR) in acquired immunodeficiency syndrome. In the design potent protease inhibitors for this virus, a knowledge of the characteristics of HTLV-1 PR itself and its substrate specificities is critical. We searched the synthetic conditions, especially conditions related to epimerization during segment condensation and estimated whether it is possible to set up an assay system using synthetic HTLV-1 PR.

HTLV-1 PR is consisted of 125 amino acids.[3] It has been reported that the mutant HTLV-1 PR, [L40I]-HTLV-1PR, is resistant to auto-digestion, and that [L40I, C90A, C109A]-HTLV-1 PR (C2A-HTLV-1 PR (**1**)) (Fig. 1), have equivalent enzymatic activity and substrate-specificity[4,5] to those of HTLV-1 PR. Thus, this auto-digestion resistant mutant was chosen as a synthetic target.

C2A-HTLV-1 PR was synthesized based on the route shown in Scheme 1. The three building blocks, Boc-[Lys(Boc)^{14,25}]-C2A-HTLV-1 PR (1-34)-SCH$_2$CH$_2$CO-Ala (**2**), Fmoc-[Lys(Boc)^{52,68}]-C2A-HTLV-1 PR (35-80)-SCH$_2$CH$_2$CO-Ala (**3**) and [Lys(Boc)^{95,119}]- C2A-HTLV-1 PR (81-125) (**4**), were prepared by the Boc solid-phase method.[6]

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Pro-Val-Ile-Pro-Leu-Asp-Pro-Ala-Arg-Arg-  10
Pro-Val-Ile-Lys-Ala-Gln-Val-Asp-Thr-Gln-  20
Thr-Ser-His-Pro-Lys-Thr-Ile-Glu-Ala-Leu-  30
Leu-Asp-Thr-Gly-Ala-Asp-Met-Thr-Val-Ile-  40
Pro-Ile-Ala-Leu-Phe-Ser-Ser-Asn-Thr-Pro-  50
Leu-Lys-Asn-Thr-Ser-Val-Leu-Gly-Ala-Gly-  60
Gly-Gln-Thr-Gln-Asp-His-Phe-Lys-Leu-Thr-  70
Ser-Leu-Pro-Val-Leu-Ile-Arg-Leu-Pro-Phe-  80
Arg-Thr-Thr-Pro-Ile-Val-Leu-Thr-Ser-Ala-  90
Leu-Val-Asp-Thr-Lys-Asn-Asn-Trp-Ala-Ile- 100
Ile-Gly-Arg-Asp-Ala-Leu-Gln-Gln-Ala-Gln- 110
Gly-Val-Leu-Tyr-Leu-Pro-Glu-Ala-Lys-Gly- 120
Pro-Pro-Val-Ile-Leu                      125
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Figure 1. The amino acid sequence of the C2A-HTLV-1 PR. The two arrows indicate the sites of segment couplings.

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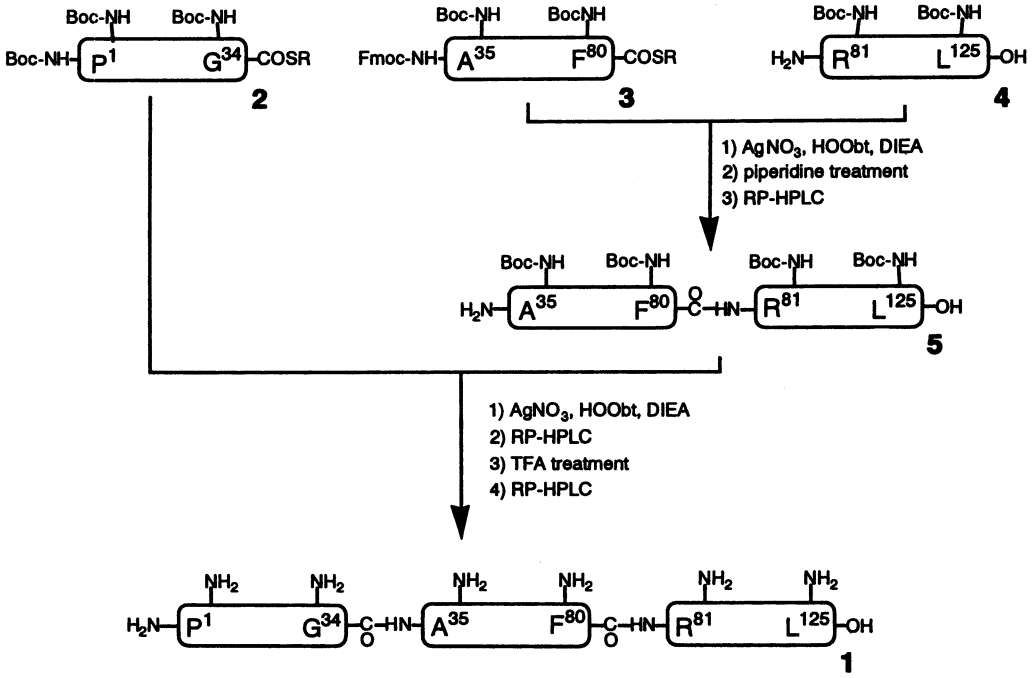

Scheme 1. Synthetic scheme for the preparation of the C2A-HTLV-1 PR.

When peptide thioester **3** and peptide **4** were condensed using silver nitrate in the presence of 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOObt) and DIEA in DMSO at room temperature for 48 h,[8] two coupling products, which had the expected mass number of the desired product, [Lys(Boc)^{52,68,95,119}]-C2A-HTLV-1 PR (35-125) (**5**), were detected by reversed-phase (RP) HPLC, after piperidine treatment, in 40.3% (**5a**) and 11.5% (**5b**) yields based on peptide **4**, respectively, as shown in Fig. 2. Gas-chromatographic analyses[9] of the pentafluoropropionyl amino acid *n*-butyl esters, derived from hydrolysates of each product, using a chiral capillary column, CHROMPACK™ (GL Science Inc., Netherlands), indicated that the minor product **5b** contained D-Phe. Taking it into account the fact that building blocks **3** and **4** did not contain D-Phe, epimerization likely occurred during segment condensation at the C-terminal Phe of peptide thioester **5**.

Phenylalanine is classified as a high-risk amino acid group with respect to epimerization during segment condensation. Thus, the factors that affect the epimerization of Phe during condensation between Phe and Arg via the thioester method were considered. Using the model peptide thioester, Boc-C2A-HLTV-1 PR (69-80)-SCH_2CH_2CO-Ala, and the peptide amide, C2A-HLTV-1 PR (81-87)-NH_2, some of the factors that might affect epimerization such as the structure of the thioester and its activation reagent, the solvent for condensation, and the ratio of base and active ester component were examined. Among these, a strong relationship was found only with the dielectric constant of the coupling solvent. The amounts of the D-Phe-containing product in the total coupling product were 23% in DMSO, 7% in *N*,*N*-dimethylacetamide and 4% in DMF. As a result, peptide **5** was re-synthesized using DMF as a solvent, keeping the other conditions the same. The yield of the D-Phe-free desired peptide **5** increased to 59% and the product that contained D-Phe decreased to 3.6% based on peptide **4**.

After isolating peptide **5** by RP-HPLC, followed by freeze-drying, peptide thioester **2** was condensed with peptide **5** using DMSO as the solvent in the presence of silver nitrate, HOObt and DIEA with stirring for 48 h at room temperature.[10] This reaction mixture was subjected to RP HPLC column to give the product, Boc-

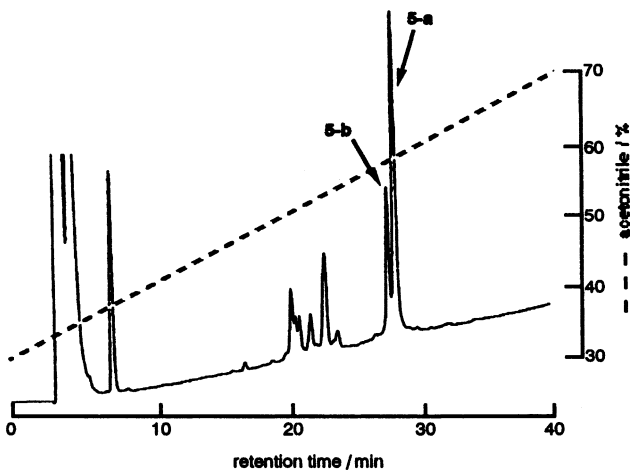

Figure 2. RP-HPLC elution profile of the coupling product, [Lys(Boc)^{52,68,95,119}]-C2A-HTLV-1 PR (35-125) (**5a** and **5b**). Column: Cosmosil 5C_4AR-300 (4.6×150 mm) (Nacalai tesque, Kyoto, Japan); eluent: 0.1% TFA in aqueous acetonitrile using linear gradient indicated on the chromatogram (1.0 mL min $^{-1}$).

[Lys(Boc)^{14,25,52,68,95,119}]-C2A-HTLV-1 PR, which was treated with TFA to remove all Boc groups on the peptide. The free peptide, C2A-HTLV-1 PR, was once purified by the same RP HPLC to obtain a highly pure C2A-HTLV-1 PR (**1**) in 40% yield based on the peptide **5** as shown in Fig. 3; MS observed (MH^+) = 13395.9 amu, calculated (MH^+) = 13396.53 amu.

The purified powder of peptide **1** was dissolved with aqueous Gdn-HCl and stored. An aliquot of this enzyme solution was diluted with 40-fold volume of formate buffer. Then the solution was diluted 500-fold excess PIPES buffer. This solution was used for enzyme assay. The enzymatic activities and substrate-selectivities of the synthetic HTLV-1 PR were then examined using the synthetic peptide substrates. The enzymatic assay was initiated by mixing an enzyme solution, incubation buffer and a substrate. An aliquot of the reaction mixtures was subjected to RP-HPLC. The concentrations of substrate and the enzyme were determined by amino acid analysis. The substrates containing the cleavage sites in HTLV-1, PR/p3 (KGPPVIL-PIQAP) and MA/CA (APQVL-PVMHP), were digested by treatment with synthetic protease to produce KGPPVIL and PIQAP from PR/p3, and APQVL and PVMHP from MA/CA, respectively. No hydrolysis, however, observed for the substrate containing the cleavage sites in HIV-1, PR/RT (CTLNF-PISP) or p1/p6 (RPGNF-LQSRP). The kinetic parameters for the PR/p3 substrate were determined by measuring the concentration of the produced cleavage products. (Table 1).

Table 1 shows that this synthetic protease has comparative enzymatic activities which are comparable with those reported for the C2A-HTLV-1 PR and the intact one, HTLV-1 PR, prepared by an expression method.

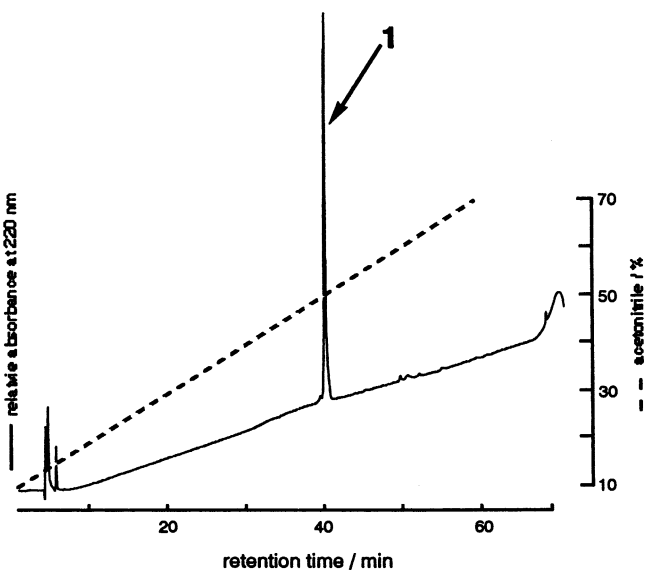

Figure 3. RP-HPLC elution profile of C2A-HTLV-1 PR, 1. Column: Cosmosil 5C_4AR-300 (4.6×150 mm) (Nacalai tesque, Kyoto, Japan); eluent: 0.1% TFA in aqueous acetonitrile using linear gradient indicated on the chromatogram (1.0 mL min^{-1}).

Table 1. Kinetic parameters for PR/p3

	This work	Reported[4]	
		C2A-HTLV-1 PR	HTLV-1 PR
k_{cat} (s^{-1})	8.2±0.9	11.82±0.89	6.91±2.4
K_m (μM)	47±5	41±1	30±6

Table 2. K_i values for three inhibitors

	Pepstatin	SI	cSI
K_i (nM)	11200±900	242±18	250±84

The substrate-mimetic protease inhibitor,[4] SI, and its cyclized product cSI were prepared in which the P1′ and P1 amino acid were substituted to statin, based on the amino acid sequence of MA/CA. The K_i value of these three inhibitors were determined by using least-squares method in the Dixon plot,[11] as summarized in Table 2.

SI and cSI show a 50-fold increase in potency over pepstatine. No significant difference, however, was observed between the linear inhibitor SI and the cSI, the cyclized form of SI.

In conclusion, C2A-HTLV-1 PR successfully prepared by the thioester method with minimal epimerization and the resulting synthetic C2A-HTLV-1 PR could be converted to an active form that had enzymatic activity and substrate-specificity. The synthetic enzyme will be used for the construction of an assay system in an attempt to develop more effective and specific viral inhibitors.

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6. Typical procedure of the preparation of building blocks [Lys(Boc)^{14,25}]-Boc-C2A-HTLV-1 PR(1-34)-SCH_2CH_2-CO-Ala (**2**). A Boc-Gly-SCH_2CH_2CO-Ala-OCH_2-Pam resin (1.09 g) was prepared form Boc-Ala-OCH_2-Pam resin (1.00 g, Ala; 0.77 mmol), according to a previously

reported protocol.[7] Starting from an aliquot of this resin (650 mg, Gly; 0.45 mmol, Ala; 0.48 mmol), a protected peptide resin (2.58 g) corresponding to the sequence C2A-HTLV-1 PR (1-34), Pro-Val-Ile-Pro-Leu-Asp-(O*c*Hex)-Pro-Ala-Arg(Tos)-Arg(Tos)-Pro-Val-Ile-Lys(Cl-Z)-Ala-Gln-Val-Asp(O*c*Hex)-Thr(Bzl)-Gln-Thr(Bzl)-Ser-(Bzl)-His-Pro-Lys(Cl-Z)-Thr(Bzl)-Ile-Glu(O*c*Hex)-Ala-Leu-Leu-Asp(O*c*Hex)-Thr(Bzl)-Gly-SCH_2CH_2CO-Ala-OCH_2-Pam resin, was prepared on a peptide synthesizer (430A; Applied Biosystems Inc., Foster City, CA) using the 0.5 mmol-scale double-coupling protocol of the benzotriazole active ester method of the system software version 1.40 NMP/HOBt *t*-Boc. The ends were capped by reaction with acetic anhydride after the introduction of each amino acid. An aliquot of the resin (527 mg) was treated with a reagent containing anhydrous HF (8.5 mL), anisole (0.75 mL) and 1,4-butanedithiol (0.75 mL) with stirring at 0°C for 90 min. After evaporation of the HF, ether (10 mL) was added to the reaction cylinder to precipitate the peptide, which was further washed with ether (10 mL×2) and dissolved in aqueous acetonitrile. The peptide solution was passed through a disposable ODS cartridge, TOYO-PACK ODS M (Tosoh, Tokyo). The resulting solution was lyophilized to give a powder (473.7 mg), which was suspended in deionized water and then solved by mixing with an equal volume of TFA/acetonitrile (1:1) solution just prior to applying it to RP-HPLC. The resulting solution was injected onto a RP-HPLC column (Cosmosil $5C_{18}$-AR-300, 20×250 mm (Nacalai Tesque, Kyoto)). The fractions were freeze-dried to give a powder, C2A-HTLV-1 PR (1-34)-SCH_2CH_2CO-Ala (107.3 mg). To a solution of this peptide thioester in DMSO (1.5 mL) were added Boc-OSu (54 mg, 252 μmol) and DIEA (30 μL, 170 μmol). After stirring for 90 min at room temperature, the solvent was evaporated and ether was added to wash the residual solid. The slurry was washed with ether two times, to give [Lys(Boc)^{14,25}]-Boc-C2A-HTLV-1 PR (1-34)-SCH_2CH_2CO-Ala; Boc-Pro-Val-Ile-Pro-Leu-Asp-Pro-Ala-Arg-Arg-Pro-Val-Ile-Lys(Boc)-Ala-Gln-Val-Asp-Thr-Gln-Thr-Ser-His-Pro-Lys(Boc)-Thr-Ile-Glu-Ala-Leu-Leu-Asp-Thr-Gly-SCH_2CH_2CO-Ala (126.2 mg) (**2**). Observed (MH^+) = 4137.97 amu, calcd (MH^+) = 4138.78 amu. The yield was 24% based on the Ala in the starting resin.

[Lys(Boc)^{52,68}]-Fmoc-C2A-HTLV-1 PR (35-80)-SCH_2CH_2CO-Ala (**3**). Starting from Boc-Ala-OCH_2-Pam resin, Fmoc-Ala-Asp-Met-Thr-Val-Ile-Pro-Ile-Ala-Leu-Phe-Ser-Ser-Asn-Thr-Pro-Leu-Lys(Boc)-Asn-Thr-Ser-Val-Leu-Gly-Ala-Gly-Gly-Gln-Thr-Gln-Asp-His-Phe-Lys-(Boc)-Leu-Thr-Ser-Leu-Pro-Val-Leu-Ile-Arg-Leu-Pro-Phe-SCH_2CH_2CO-Ala (**3**) was synthesized in the same manner as was used for the preparation of the peptide thioester **2** except that the N-terminal Ala was loaded with Fmoc-Ala. An MS analysis was performed after the TFA treatment. Observed (MH^+) = 5290.73 amu, calcd (MH^+) = 5293.17 amu. The yield was 5.1% based on the Ala in the starting resin.

[Lys(Boc)^{95,119}]-C2A-HTLV-1 PR (81-125) (**4**). Starting from Boc-Leu-OCH_2-Pam resin, Arg-Thr-Thr-Pro-Ile-Val-Leu-Thr-Ser-Ala-Leu-Val-Asp-Thr-Lys(Boc)-Asn-Asn-Trp-Ala-Ile-Ile-Gly-Arg-Asp-Ala-Leu-Gln-Gln-Ala-Gln-Gly-Val-Leu-Tyr-Leu-Pro-Glu-Ala-Lys(Boc)-Gly-Pro-Pro-Val-Ile-Leu (**4**) was synthesized employing the same synthetic protocol as was used for the preparation of peptide **2**. The mass measurement of peptide **4** was carried out after the removal of the N-terminal Fmoc group by treatment with piperidine. Observed (MH^+) = 5043.56 amu calcd (MH^+) = 5043.86 amu. The yield was 6.0% based on the Ala in the starting resin.

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8. [Lys(Boc)^{52,68,95,119}]-C2A-HTLV-1 PR (35-125) (**5**). HOObt (3 mg, 18 μmol), DIEA (2.1 μL, 12 μmol), $AgNO_3$ (0.34 mg, 2 μmol), [Lys(Boc)^{52,68}]-Fmoc-C2A-HTLV-1 PR (35-80)-SCH_2CH_2CO-Ala (**3**) (0.6 μmol) and [Lys(Boc)^{95,119}]-C2A-HTLV-1 PR(81-125) (**4**) (0.3 μmol) were dissolved in DMSO (750 μL) and the resulting solution was stirred for 48 h at room temperature. After piperidine treatment, the reaction mixture was subjected to RP-HPLC. Two peaks, **5a** and **5b**, which had the same mass number as the expected coupling product, were isolated. Portions of the peptides in these two peaks were treated with TFA and subjected to MS analysis. **5a** Observed (MH^+) = 9727.73 amu, calcd (MH^+) = 9736.33 amu. **5b** Observed (MH^+) = 9727.73 amu calcd (MH^+) = 9736.33 amu. The peptides contained in these peaks were hydrolyzed in constant boiling point HCl (Nacalai Tesque Co., Kyoto) at 110°C for 4 h and subjected to amino acid analysis.
9. 1-Butanol containing 10% HCl (500 μL, Nacalai Tesque, Kyoto) was added to a hydrolysate of a peptide and the mixture was incubated for 2 h at 110°C in a sealed vial. The solution was evaporated and pentafluoroacetic acid anhydride (50 μL, Nacalai Tesque, Kyoto) was then added to the vial, after which, the solution was incubated for 30 min at 110°C. Excess reagent was removed by a stream of Ar gas. The resulting slurry was dissolved in CH_2Cl_2 and the solution was filtrated through a cotton plug. This sample was analyzed using a G-3500 gas chromatography (Hitachi Ltd., Tokyo) equipped with a L-Val coated fused silica capillary column. Gradient conditions; initial 90°C for 2 min and liner increase of the oven temperature from 90 to 190°C at a rate of increase of 2.0°C min^{-1}.
10. C2A-HTLV-1 PR (1-125) (**1**); HOObt (2.4 mg, 18 μmol), DIEA (4.2 μL, 18 μmol), $AgNO_3$ (0.2 mg, 1.2 μmol), [Lys(Boc)^{14,25}]-Boc-C2A-HTLV-1 PR (1-34)-SCH_2CH_2CO-Ala (**2**) (600 nmol) and [Lys(Boc)^{52,68,95,119}]-C2A-HTLV-1 PR (35-125) (**5**) (200 nmol) were dissolved in DMSO (200 μL) The DMSO solution was stirred for 48 h at room temperature. This reaction mixture was directly subjected to RP-HPLC resulting in the isolation of the coupling product, [Lys(Boc)^{14,25,52,68,95,119}]-Boc-C2A-HTLV-1 PR (1-125). This material was treated with TFA, containing deionized water (5% v/v) for 30 min. After evaporation of the reagents, the residue was dissolved in 4 M aqueous Gdn-HCl and the solution was again subjected to RP-HPLC to give purified **1** in 40% yield based on **5**. Amino acid analysis: $Asp_{7.6}Thr_{8.3}Ser_{4.9}Glu_{6.3}Gly_6Ala_{8.0}Val_{6.7}Met_{0.5}Ile_{4.9}Leu_{12.5}$-$Tyr_{0.8}Phe_{3.9}Lys_{3.8}His_{1.2}Arg_{2.7}Pro_{8.2}$.

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