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Bioorganic Synthesis of a Recombinant HIV-1 Fusion Inhibitor, SC35EK, with an N-Terminal Pyroglutamate Capping Group

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ABSTRACT

The bioorganic synthesis of an end-capped anti-HIV peptide from a recombinant protein was investigated. Cyanogen bromide-mediated cleavage of two Met-Gln sites across the target anti-HIV sequence generated an HIV-1 fusion inhibitor (SC35EK) analog bearing an N-terminal pyroglutamate (pGlu) residue and a C-terminal homoserine lactone (Hsl) residue. The end-capped peptide, pGlu-SC35EK-Hsl, had similar bioactivity and biophysical properties to the parent peptide, and an improved resistance to peptidase-mediated degradation was observed compared with the non-end-capped peptide obtained using standard recombinant technology.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is an enveloped virus that causes acquired immunodeficiency syndrome (AIDS) through the infection of immune cells. A number of anti-HIV drugs that target key enzymes in HIV-1 life cycle, including reverse transcriptase and viral protease, have been employed for highly active anti-retroviral therapy (HAART). Although combination therapy by HAART achieves prolonged viral suppression, resistant variants against these drugs often appear and compromise therapeutic efficiency.¹ In order to manage this disease, novel anti-HIV drugs that target existing classes of molecules as well as newly identified molecules in the viral replication cycle have been developed, such as entry inhibitors and HIV-1 integrase inhibitors.²

The fusion inhibitors are a new class of therapeutics for the treatment of HIV-1-infected patients. These drugs prevent viral entry into cells,³ which is mediated by the conformational transition of the viral envelope protein gp41⁴ that occurs after gp120 binds to its receptors on the host cell surface. The ectodomain of gp41, with two heptad repeat regions, HR1 and HR2, is folded into an anti-parallel coiled-coil structure of fusion-active conformation. Synthetic peptides derived from gp41 HR2, such as T-20 (enfuvirtide) and C34, exert potent anti-HIV activity by interfering with this viral gp41 folding and, therefore, the subsequent membrane fusion process.^{5,6} The mode of interaction between an inhibitory HR2 peptide and the viral HR1, including a representative peptide N36, has been elucidated and exploited to design the

second-generation of fusion inhibitors.⁷ Previously, we developed the potent anti-HIV peptides, T-20EK and SC35EK, which were designed by rearrangement of the bioactive α -helix structure of T-20 and C34, respectively.⁸ Substitutions of the non-interactive residues within T-20 and C34 with hydrophilic glutamic acids or lysines improved the anti-HIV activity of the original peptides as well as their biophysical properties.

T-20 is manufactured by chemical synthesis, in which a combination of solid-phase and solution-phase peptide synthesis methods is employed.⁹ Chemical synthesis of peptides allows optional modifications at the appropriate residues or positions by using non-proteinogenic amino acids and/or special amino acids with post-translational modifications which prolonged the effects of the peptide therapeutics in vivo. For example, N-terminal acyl- and/or C-terminal amide-modified peptides can be easily prepared, which can then contribute to the protection from enzymatic scissions that may occur in the circulatory system. However, step-wise elongation of a peptide-chain using protected amino acid components may be disadvantageous in terms of cost-effectiveness and environmental acceptability. The expression of recombinant proteins is an alternative approach used to prepare bioactive peptides and proteins,¹⁰ but the products are normally obtained without any functional modifications. Taking advantage of this approach, we synthesized an anti-HIV peptide, SC35EK, by a combination of the recombinant expression of fusion proteins in *Escherichia coli* and their subsequent treatment with chemical reagents to incorporate end-capping groups at both the N- and C-termini.

Among the several cleavage reactions available for peptides and proteins, cyanogen bromide (CNBr)-mediated cleavage at methionine (Met) residues is one of the most conventional, and is used for both sequence analysis and for the preparation of bioactive, short peptides from insoluble recombinant fusion proteins in *E. coli*. Such proteins include antibiotic peptides,¹¹ zinc finger peptides,¹² insulin-like peptides¹³ and pH-responsive self-assembling peptides.¹⁴ It is noteworthy that CNBr-mediated cleavage releases the first fragment containing a cyclic homoserine lactone (Hsl) at the C-terminus,¹⁵ and the second fragment without any N-terminal functional group. This Hsl residue was designed as a C-terminal protecting group for SC35EK. Pyroglutamic acid (pGlu) was chosen as the

N-terminal protecting group as this residue is important for the physiological stability of several mammalian peptide hormones and proteins.¹⁶ The cyclic structure of pGlu can be obtained by cyclization from a glutamine (Gln) residue mediated either by glutaminyl cyclase in vivo, or by treatment of Gln in non-enzymatic conditions.^{16,17}

In this study, we undertook the bioorganic synthesis of an SC35EK analog, which contains cyclic N-terminal pGlu and C-terminal Hsl end-capping structures.¹⁸ Using a model synthetic peptide, the conditions necessary for the cleavage and cyclization of a Gln residue to a pGlu residue were optimized. Recombinant His-tag fusion proteins containing either a single, or three consecutive anti-HIV sequences were expressed and purified from *E. coli*. The peptide, pGlu-SC35EK-Hsl, was cleaved from the resulting recombinant protein under optimized acidic conditions. We then assessed the biological and biophysical characteristics of pGlu-SC35EK-Hsl and its biostability in mouse serum.

2. Results and discussion

2.1. Cleavage and cyclization of the model synthetic peptide

In order to obtain the end-capped SC35EK protein, we incorporated two Met-Gln dipeptide cleavage sites across the anti-HIV SC35EK sequence. A CNBr-mediated cleavage should provide a C-terminal Hsl residue and an N-terminal Gln residue, which could then be converted into pGlu under mildly acidic conditions. Using a model synthetic peptide Ac-MQ-WEEWDKK-MQ-OH (MQ-SC7EK-MQ) **1** derived from the N-terminal sequence of SC35EK, the acidic conditions for CNBr-mediated cleavage and cyclization were optimized (Scheme 1). The reaction products were analyzed using LC-MS and the yields of Gln-SC7EK-Hsl **2** and pGlu-SC7EK-Hsl **3** were calculated based upon the peak areas at 220 nm (Table 1). The pGlu formation was verified by the comparative analysis with the authentic sample obtained by chemical synthesis using pyroglutamic acid. CNBr-mediated cleavage of peptide **1** in the standard 70% formic acid (FA) solution yielded Gln-SC7EK-Hsl **2** without the oxidation of Met residues (entry 1). Significant Met oxidation, which disrupted the cleavage reaction, was observed under the other acidic conditions, including 30% FA, 0.1 N HCl, 0.1 M trifluoroacetic acid (TFA) and 0.1 N AcOH. This by-product formation was

prevented by the addition of tris(2-carboxyethyl)phosphine (TCEP) (entries 2–5). Partial production of the expected pGlu derivative **3** was observed in all cases in which this cyanylation step was carried out. The second cyclization, from N-terminal Gln to pGlu, was completed within 2 h. However, when 0.1 N AcOH solution was used, the reaction was incomplete (Fig. 1). Small amounts of formylated by-product were obtained along with peptide **3** in 70% FA solution, but peptide **3** was produced in higher yield (entry 1).

2.2. Preparation of recombinant His-tagged fusion protein

We used the pET28a(+) vector to express a hexa-histidine tagged [His-tag, (His)₆]-fusion protein in *E. coli*. The MQ-SC35EK-MQ sequence, or the tandem M-(Q-SC35EK-M)₃-Q sequence was spliced into the *NdeI-XhoI* restriction site downstream of the His-tag. This tandem sequence contains three consecutive anti-HIV peptides with five conjunctive Met-Gln cleavage sites designed to efficiently provide multiple SC35EK peptides from a single protein. Constructs were transformed into the *E. coli* strain BL21 (DE3)-RIL and protein expression was induced by IPTG. The resulting proteins were purified by affinity chromatography using Ni²⁺-nitrilotriacetate (Ni-NTA)-agarose resin, and the expected proteins were eluted with either a standard imidazole buffer or an acidic solution containing 70% FA, 0.1 N HCl or 0.1 M TFA. After elution using imidazole, the remaining imidazole was removed by gel-filtration. The sizes of the (His)₆-MQ-SC35EK-MQ **4** or (His)₆-M-(Q-SC35EK-M)₃-Q **5** fusion proteins on SDS-PAGE gels were 7.0 and 16.5 kDa, respectively (Fig. 2).

The (His)₆-MQ-SC35EK-MQ protein **4** was highly expressed in the soluble fraction and was obtained by elution with either imidazole or above acidic solutions from the affinity chromatography resin (Fig. 2a). Using the standard imidazole protocol, protein **4** was eluted in a moderate yield, however, approximately 100 mg of **4** was recovered from 1 L of bacterial culture under acidic solutions (Table 2). The lower yield obtained after elution using imidazole may be attributable either to incomplete protein elution from the column and/or protein loss during the desalting process. The purity of the (His)₆-MQ-SC35EK-MQ **4** was confirmed as >95% by HPLC (Fig. 4a). (His)₆-M-(Q-SC35EK-M)₃-Q **5** was expressed in both

the soluble and insoluble fractions (Fig. 2b) and this resulted in a decreased yield, regardless of the high expression level seen in the total fraction. Consequently, only 19 or 26 mg/L of protein **5** was obtained by elution with imidazole or acidic solutions, respectively, (including 70% FA, 0.1 N HCl, or 0.1 M TFA), with <80% purity confirmed by HPLC. Thus, (His)₆-MQ-SC35EK-MQ **4** was used for the further experiments.

2.3. Production of the anti-HIV peptide by cleavage and cyclization of the recombinant protein

The optimized cleavage protocol established above was applied to (His)₆-MQ-SC35EK-MQ **4**. Purified protein **4** was cleaved and cyclized simultaneously by CNBr treatment under acidic conditions at 60 °C for 2 h (Scheme 2, and Fig. 3). All the LC-MS profiles indicated the formation of two major products corresponding to the tag fragment **6** and pGlu-SC35EK-Hsl **7** (Fig. 4b, top). The formylated by-products of **6** and **7** were only obtained by reaction in 70% FA. This result agrees with that obtained using the model peptide, and also with previous reports.^{15b} Significant amounts of ring-opened products at the C-terminal Hsl of **6** and **7** were observed when the cleavage reaction was carried out in either 0.1 N HCl or 0.1 M TFA (Fig. 4b, middle and bottom). pGlu-SC35EK-Hsl **7** obtained under all conditions was purified by HPLC with >99% purity (Fig. 4c). Peptide **7** was characterized by ESI-MS measurement and by the comparative analysis with the one obtained by chemical synthesis using pGlu (see Supplementary data). The cyclization yields of pGlu-SC35EK-Hsl **7** obtained from the reaction in 70% FA, 0.1 N HCl, or 0.1 M TFA solutions were 16%, 15%, and 14%, respectively, and the overall yields from 1 L of *E. coli* culture were 10.4 mg, 10.2 mg, and 8.7 mg, respectively (Table 2).

2.4. Analysis of the SC35EK analog with end-capping groups by circular dichroism

The peptide conformation of pGlu-SC35EK-Hsl **7** was evaluated by measurement of the CD spectrum, along with SC35EK **8** and the non-end-capped peptide **9** (Fig. 5a, Table 3).¹⁹ SC35EK **8** exhibits an α -helical conformation and interacts directly with an NHR-derived peptide, N36.^{8a} The similar spectra with two characteristic spectrum minima at 208 and 222

nm were observed for peptides **7** and **8**. Peptide **9** showed significantly less α -helix formation compared with the other peptides, suggesting that the improved α -helical conformation of SC35EK is affected by the presence of the capping groups, but not by their structure. Potential six-helical bundle structure formation consisting of SC35EK derivatives **7–9** and N36, and the stability of the peptides, were also evaluated using CD analysis. The similar, stabilized α -helix conformations were verified within three complexes of six-helical bundle structures by the CD spectra (Fig. 5b). However, the thermal stability of the peptide **9**-N36 was less than those of the other two complexes [$T_m(\mathbf{7}) = 73.6\text{ }^\circ\text{C}$; $T_m(\mathbf{8}) = 75.8\text{ }^\circ\text{C}$; $T_m(\mathbf{9}) = 62.5\text{ }^\circ\text{C}$] (Fig. 5c and Table 3).

2.5. Anti-HIV activity

The anti-HIV activity of the SC35EK-derived peptides was evaluated using the MAGI assay (Table 3). pGlu-SC35EK-Hsl **7** reproduced the anti-HIV activity of SC35EK **8** [$EC_{50}(\mathbf{7}) = 0.57\text{ nM}$; $EC_{50}(\mathbf{8}) = 0.50\text{ nM}$], indicating that the original anti-HIV activity is not disrupted by the presence of the N- and C-terminal end-capping functional groups derived from the Met-Gln cleavage sites. The fivefold reduction in anti-HIV activity exhibited by peptide **9** compared with two other peptides was consistent with the less stable α -helix structures, both in the peptide itself and in the six-helical bundle complex.

2.6. Stability of the end-capped peptide in mouse serum

The ability of the N- and C-terminal capping moieties to protect the SC35EK analog **7** from biodegradation was assessed by incubating the peptides in mouse serum (Fig. 6). Rapid degradation of the non-end-capped peptide **9** was observed. Although pGlu-SC35EK-Hsl **7** was more stable than peptide **9**, ring-opening of the C-terminal Hsl in this peptide, followed by degradation at the C-terminus was observed.²⁰ This suggests that the pGlu end-capping group is able to provide protection equivalent to that of an N-terminal acyl group. The γ -lactone structure of the C-terminal Hsl may be unfavorable for in vivo biostability compared with the C-terminal amide of peptide **8**, although the structure did not affect the in vitro anti-HIV activity.

3. Conclusions

The bioorganic synthesis of an end-capped anti-HIV peptide was achieved. The CNBr-mediated cleavages at the Met-Gln dipeptide sites of recombinant protein **4** afforded the end-capped SC35EK analog **7** bearing an N-terminal pGlu residue and a C-terminal Hsl residue. The acidic solution used for elution from the affinity chromatography resin to obtain the purified recombinant protein was also used for the cleavage-cyclization reactions. This facilitated the synthetic process and removed the need for repeated purifications to obtain peptide **7** in high yield. The resulting end-capped peptide **7** exhibited a stable α -helical conformation, anti-HIV activity equipotent to the parent peptide **8** and was resistant to biodegradation in serum when compared with the non-end-capped peptide **9**. The methods outlined in this paper are directly applicable to the preparation of end-capped anti-HIV fusion inhibitors from recombinant proteins, which may provide the next generation of therapeutic molecules active against multi-drug resistant strains of HIV-1.

4. Experimental

4.1. General.

For HPLC separations of synthetic peptides, a Cosmosil 5C18-ARII analytical column (4.6 x 250 mm, flow rate 1 mL/min, Nacalai Tesque, Kyoto, Japan) or a Cosmosil 5C18-ARII preparative column (20 x 250 mm, flow rate 10 mL/min) was employed. The eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) were used for HPLC elution.

4.2 Peptide synthesis.

Protected peptide-resins were manually constructed by standard Fmoc-based SPPS on Rink amide resin (Novabiochem, 83 mg, 0.05 mmol). *t*-Bu for Tyr, Ser and Thr; *t*-Bu ester for Asp and Glu; Boc for Lys; and Trt for Asn and Gln were employed for side-chain protection, respectively. Fmoc-amino acids were coupled using five equivalents of reagents [Fmoc-amino acid, *N,N'*-diisopropylcarbodiimide and HOBt·H₂O] to free amino group in DMF for 1.5 h.

Fmoc deprotection was performed by 20% piperidine in DMF (2 x 1 min, 1 x 20 min). The resulting protected resin was treated with TFA/H₂O/*m*-cresol/thioanisole/1,2-ethanedithiol (80:5:5:5:5) at room temperature for 2 h. After removal of the resin by filtration, ice-cold dry Et₂O (30 mL) was added to the residue. The resulting powder was collected by centrifugation and then washed with ice-cold dry Et₂O (3 x 15 mL). Purification of the crude product by preparative HPLC afforded a colorless powder of the desired peptide. All peptides were characterized by an ESI-MS (micromassZQ2000, Waters), and the purity was calculated as >95% by HPLC.

4.3. Cleavage and cyclization of the model peptide

The model synthetic peptide MQ-SC7EK-MQ **1** was treated with CNBr (100 equiv) in the presence of TCEP (10 equiv) under acidic conditions at room temperature for 2 h. After cleavage at the Met residue, the reaction mixture was heated at 60 °C for 2 h. The reaction products were analyzed every 30 min using LC-MS (Fig. 1). The Gln-SC7EK-Hsl **2** or the pGlu-SC7EK-Hsl **3** peptides were quantified based on the combined peak areas at 220 nm of peptides after HPLC.

4.4. Preparation of recombinant (His)₆-fused proteins.

The cDNA sequences encoding the MQ-SC35EK-MQ or M-(Q-SC35EK-M)₃-Q proteins were amplified by PCR using the following chemically synthesized 139-mer or 361-mer oligonucleotides, respectively:

5'-ctcCATATGCAGTGGGAAGAATGGGATAAAAAAATTGAAGAATATACCAA
AAAATTGAAGAACTGATTAATAAAATCGGAAGAACAGCAAAAAAAAAAATGAAGAA
GAACTGAAAAAAAAATGCAGTAACTCGAGcgtt-3' (both end of sequences in small letters
indicate a flanking sequence for efficient restriction enzyme digestion of *Nde*I (CATATG) and
*Xho*I (CTCGAG)) or
5'-ctcGGATCCCATATGCAGTGGGAGGAATGGGATAAAAAAATCGAAGAATATACTA
AGAAAATTGAAGAACTCATCAAGAAATCCGAAGAACAACAGAAGAAAAACGAAG
AGGAACTGAAAAAAAAATGCAATGGGAAGAGTGGGACAAAAAGATCGAAGAGTATA

CCAAAAAATCGAAGAGTTGATTA AAAAGAGCGAAGAGCAGCAGAAAAAGAATG
AAGAAGAGTTAAAAAGATGCAGTGGGAAGAATGGGACAAGAAAATTGAGGAAT
ACACTAAAAAGATCGAGGAACTGATTA AAAAATCTGAGGAACAGCAGAAAAAAA
ATGAGGAAGAATTGAAGAAAATGCAATAACTCGAGcggtt-3' (both end of sequences in
small letters indicate a flanking sequence for efficient restriction enzyme digestion of *Bam*HI
(GGATCC), *Nde*I (CATATG) and *Xho*I (CTCGAG)).

Codons were replaced by more frequently used ones based on *E. coli* codon usage. The synthetic cDNA fragments contained *Nde*I and *Xho*I restriction sites at the 5' and 3' ends, respectively, and an extra ATGCAG or ATGCAA sequence (encoding Met-Gln, *underlined*) at their 5' and 3' termini across the SC35EK sequence to facilitate cleavage and cyclization. Each segment was digested with *Nde*I and *Xho*I and inserted into the pET28a (+) vector (Novagen). The plasmids [pET28a-MQ-SC35EK-MQ or pET28a-M(Q-SC35EK-M)₃-Q] were then transformed into the *E. coli* strain BL21(DE3)-RIL (Stratagene) for expression. Isolated colonies were picked and cultured overnight in 10 mL of LB culture containing 0.100 mg/mL kanamycin at 30 °C, with shaking. This culture was then transferred into 1 L of LB culture in the presence of 0.100 mg/mL kanamycin. When the OD₆₀₀ reached 0.6–0.8, protein expression was initiated by the addition of 1 mM IPTG. After an additional 6-h incubation at 25 °C, the cells were harvested by centrifugation at 4000 rpm for 30 min. Cells were resuspended in B-PER solution (PIERCE) and disrupted by sonication. After centrifugation at 12,000 rpm for 30 min, the supernatant was transferred to a Ni-NTA agarose column (QIAGEN). The column was washed with wash buffer (20 mM phosphate, pH 6.0, containing 0.5 M NaCl) and the protein eluted with imidazole buffer (50–200 mM imidazole in phosphate buffer (pH 6.0)), 70% FA, 0.1 N HCl or 0.1 M TFA. The expression and purification of the proteins was analyzed by SDS–PAGE (15–20% gradient gel). The protein eluted with imidazole buffer was desalted by gel-filtration and freeze-dry. The freeze-dried protein was reconstituted in water to a concentration of 1 mM. The yield of the eluted proteins was calculated using a Protein Assay Kit (BIO-RAD Laboratories, Hercules, CA).

4.5. Preparation of the end-capped anti-HIV peptide from the recombinant protein.

The protein eluted with an acidic solution was reconstituted to a concentration of 1 mM. The protein was treated with CNBr (100 equiv) in the presence of TCEP (10 equiv) under acidic conditions (as shown in Table 2) at 60 °C for 2 h and the products were analyzed by LC–MS. Preparative HPLC of the product provided the expected end-capped peptide. The yield of purified peptide was calculated by measuring the UV absorbance at 280 nm.

4.6. Measurement of CD spectra.

Peptides **7–9** were dissolved in 5 mM HEPES buffer (pH 7.2) to a final concentration of 10 μM. For CD measurement of a mixture of the NHR peptide (N36) and SC35EK analogs, the peptides were incubated at 37 °C for 30 min beforehand. The wavelength-dependent molar ellipticity $[\theta]$ was monitored at 25 °C as the average of 8 scans in a Jasco spectropolarimeter (Model J-710, Jasco Inc., Tokyo, Japan). Thermal unfolding of the potential six-helical bundle in the presence of N36 was monitored by the $[\theta]_{222}$ values at intervals of 0.5 °C after a 15-s equilibration at the desired temperature and an integration time of 1.0 s. The midpoint of the thermal unfolding transition of each complex was defined as the melting temperature (T_m).

4.7. Determination of drug susceptibility of HIV-1.

The peptide sensitivity of infectious clones was determined by the MAGI assay with some modifications.²¹ Briefly, the target cells (HeLa-CD4/CCR5-LTR-β-gal; 10⁴ cells/well) were plated in 96-well flat microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 clone (NL4-3, 60 MAGI U/well, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of drugs in fresh medium. After (48 h) viral exposure, all the blue cells stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were counted in each well. The activity of test compounds was determined as the concentration that blocked HIV-1 replication by 50% (50% effective concentration [EC₅₀]).

4.8. Stability of SC35EK peptide or analogs in mouse serum.

Peptides **7–9** (0.5 mM in PBS) were incubated at 37 °C in 50% mouse serum in the presence of 0.1% *m*-cresol (internal standard). 0.010 mL samples were collected at 0, 0.5, 1, 3, 6, 9 and 12 h and the reaction was terminated by the addition of 1 µL 0.1 N HCl and 0.040 mL of CH₃CN. Samples were deproteinized by centrifugation at 12,000 rpm for 10 min and 0.010 mL of the supernatant was injected into LC–MS. The percentage of intact peptides was calculated by peak area and corrected against the internal standard.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.10.017.

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19. Non-end capped peptide **9** can be obtained by standard recombinant expression in prokaryotes. Peptide **9** for this experiment was obtained by the chemical synthesis.
20. The ring-opening C-terminal Hsl was verified by the observed +18 mass of the product, supporting the presence of Hsl in peptide **7**¹⁵. The ring-opened product may be degraded from the C-terminus by endopeptidases in serum.
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Table 1. Cleavage and cyclization reactions of a model synthetic peptide, MQ-SC7EK-MQ **1**, under acidic conditions.

entry	solvent	additive	yield by CNBr treatment (%) ^{a,c}		yield of pGlu formation (%) ^{b,c}	
			2	3	2	3
1	70% FA	-	78.3	2.4	-	70.7
2	30% FA	TCEP	60.5	5.7	-	57.7
3	0.1 N HCl	TCEP	63.8	5.8	4.5	60.0
4	0.1 M TFA	TCEP	62.7	6.5	3.5	60.1
5	0.1 N AcOH	TCEP	53.9	3.6	16.9	37.0

^a CNBr treatment (100 equiv.) was carried out for 2 h at room temperature. ^b All cyclizations were carried out for 2 h at 60 °C. ^c The yields were calculated based on the combined peak areas of the peptides at 220 nm after HPLC.

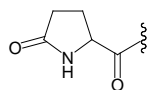
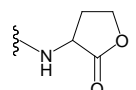
Table 2. Purification of proteins **4** and **5** by affinity chromatography and the subsequent CNBr-mediated cleavage and cyclization reactions of **4**.

entry	solvent	protein yield from 1 L culture (mg) ^a		cyclization yield of 7 from 4 (%) ^{b,c}	overall yield from 1 L culture of 4 (mg) ^d
		4	5		
1	imidazole	35	19	- ^e	- ^e
2	70% FA	92	26	16	10.4
3	0.1 N HCl	100	26	15	10.2
4	0.1 M TFA	94	26	14	8.7

^a The yield was quantified using Bradford protein assay. ^b CNBr treatment (100 equiv.) and cyclization were carried out for 2 h at 60 °C. ^c The yield was quantified by UV absorbance at 280 nm. ^d Peptide yields (mg) from 1 L culture of **4**. ^e Not tested.

Table 3. Structures and anti-HIV activity of peptides **7-9**.

R¹-WEEWDKKIEEYTKKIEELIKKSEEQQKKNEEELKK-R²

Peptide	R ¹	R ²	EC ₅₀ (nM) ^a	T _m (°C)
7			0.57 ± 0.24	73.6
SC35EK 8	Ac	NH ₂	0.50 ± 0.16	75.8
9	H	OH	2.43 ± 0.22	62.5

^a EC₅₀ was determined as the concentration that blocked HIV-1 infection by 50% in the MAGI assay.

Figure 1. Time course of the cyclization process from Gln-SC7EK-Hsl to pGlu-SC7EK-Hsl. Cyclization of Gln to pGlu by heating the reaction at 60 °C under acidic conditions was monitored every 30 min for 2 h. The yields were calculated based on the combined peak areas at 220 nm of HPLC.

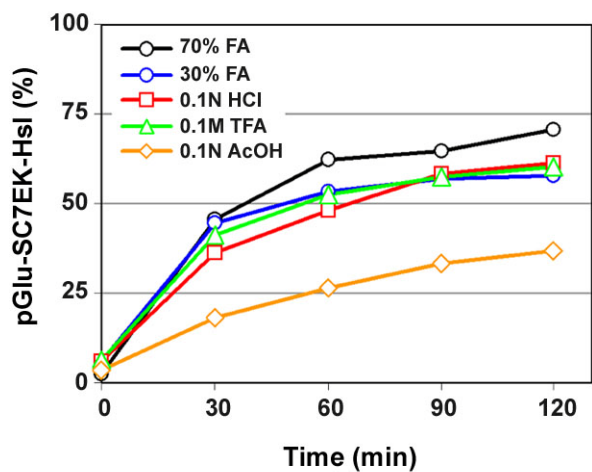


Figure 2. SDS-PAGE of recombinant proteins. (a) (His)₆-MQ-SC35EK-MQ **4** (7.0 kDa) and (b) (His)₆-M-(Q-SC35EK-M)₃-Q **5** (16.5 kDa). Lane Mk: molecular weight markers; lane 1: whole cell lysate; lane 2: supernatant of cell lysate; lane 3: precipitation of cell lysate; lane 4: pre-eluted resin; lanes 5-8: purified fractions from imidazole solution, 70% FA, 0.1 N HCl or 0.1 M TFA, respectively; lane pep Mk: polypeptide molecular weight markers.

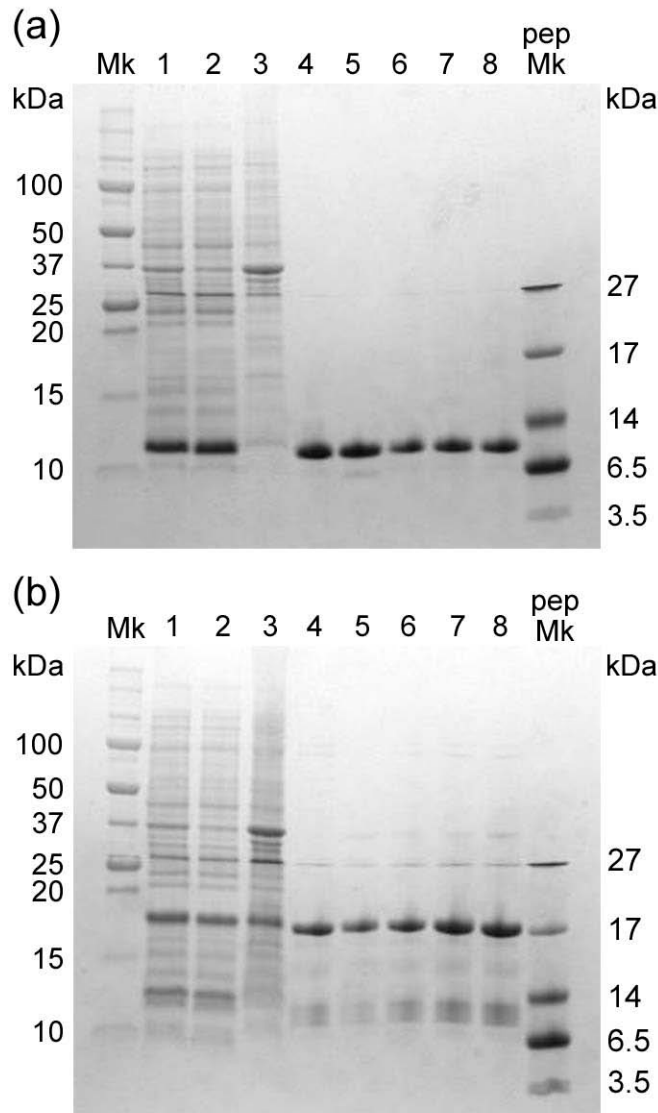


Figure 3. SDS-PAGE analysis of cleavage products and purified proteins. Lane Mk: polypeptide molecular weight marker; lanes 1, 4 and 7: (His)₆-MQ-SC35EK-MQ **4** (7.0 kDa); lanes 2, 5 and 8: after CNBr-mediated cleavage; lanes 3, 6 and 9: after HPLC purification; lane PC: chemically synthesized pGlu-SC35EK-Hsl **7** (control).

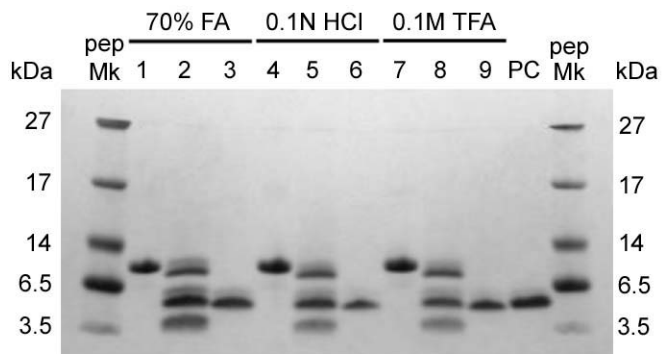


Figure 4. HPLC profiles of (a) (His)₆-MQ-SC35EK-MQ protein **4**; (b) the products of CNBr-mediated cleavage in (top) 70% FA, (middle) 0.1 N HCl, (bottom) 0.1 M TFA; (c) purified peptide **7**. Asterisk indicates the mono-formylated products of **6** and **7**. Inverted triangle indicates the ring-opened products at the Hsl of **6** and **7**, *HPLC conditions*: linear gradient 10-60% solvent B in solvent A over 50 min.

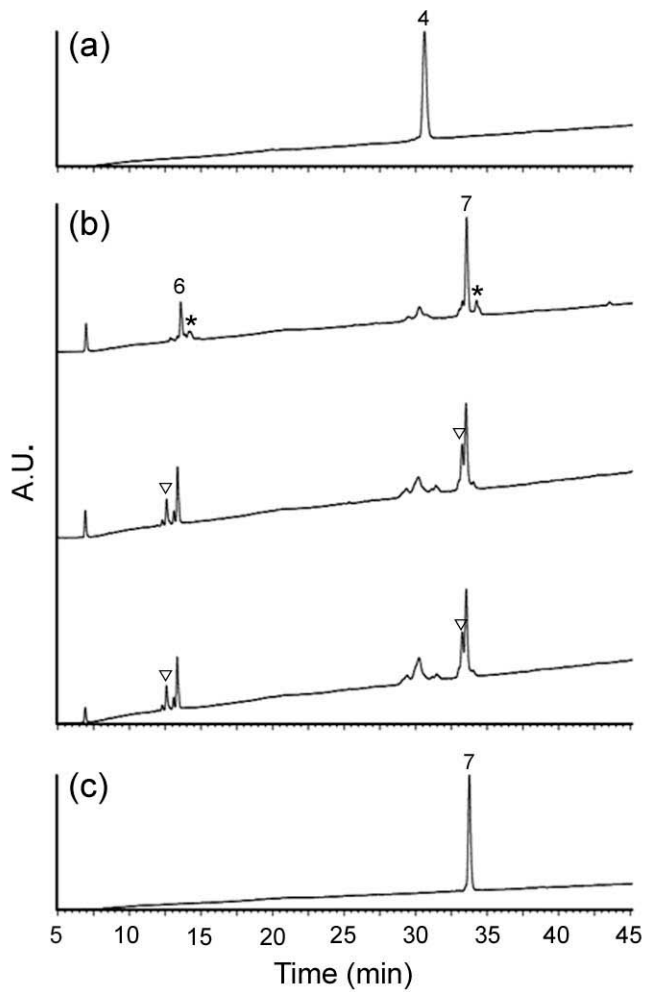


Figure 5. Secondary structure analysis using CD spectroscopy: CD spectra of (a) SC35EK-derived peptide; (b) SC35EK analog-N36 complex; and (c) thermostability of the SC35EK analog-N36 complex.

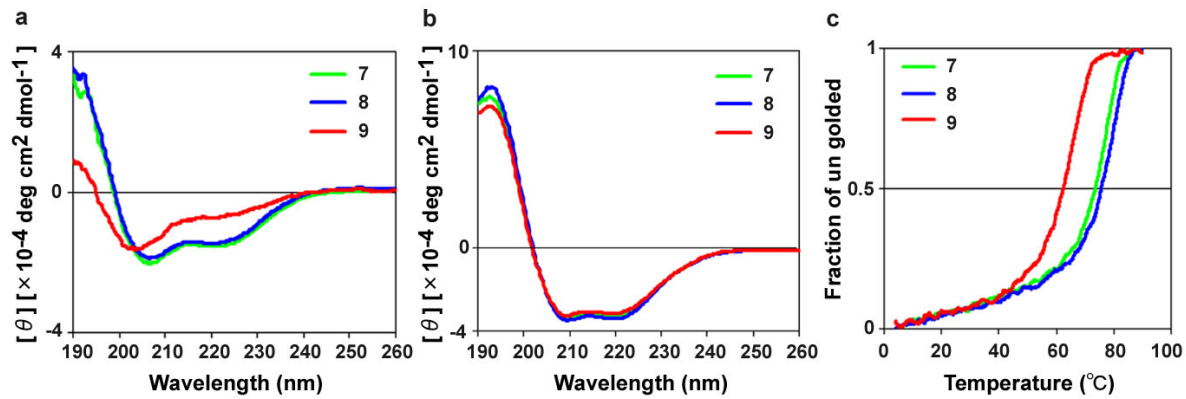
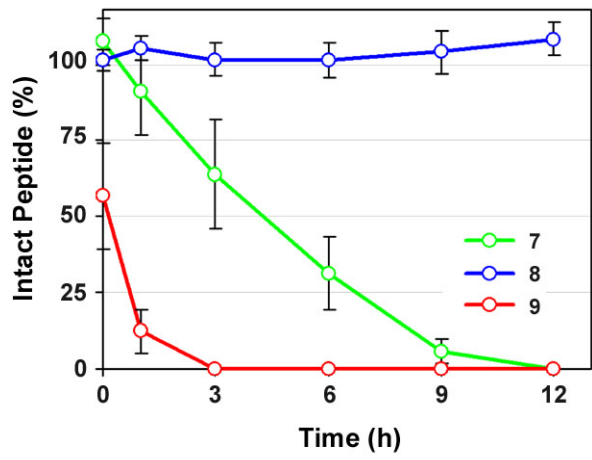
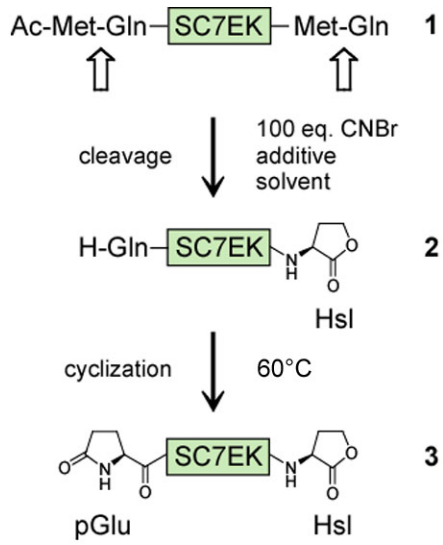


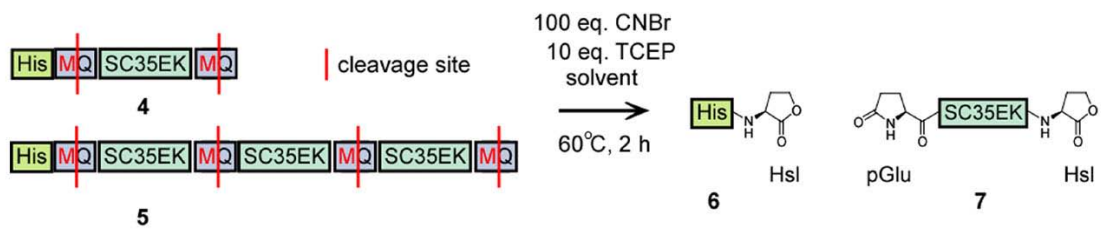
Figure 6. Degradation profile of peptides 7–9 by mouse serum. Each bar shows the mean \pm SD (n = 5).



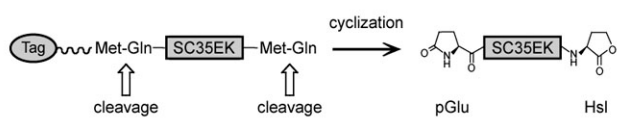
Scheme 1.



Scheme 2.



Graphical Abstract



An anti-HIV peptide with N- and C-terminal end-capping groups was synthesized by cyanogen bromide-mediated cleavage of a recombinant protein.