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Bioorganic synthesis of end-capped anti-HIV peptides by simultaneous cyanocysteine-mediated cleavages of recombinant proteins

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ABSTRACT

Bioorganic synthesis of N- and C-terminal end-capped peptides by two simultaneous *S*-cyanocysteine-mediated cleavages of recombinant proteins is described. This approach is demonstrated in the preparation of anti-HIV fusion inhibitory peptides.

1. Introduction

The recent upsurge of successes in recombinant protein-based therapeutics, such as antibodies and cytokines, as well as advances in formulation technology, has rekindled an interest in the potential development of biomolecule-derived pharmaceuticals such as peptides and oligonucleotides.[1] In order to accommodate large-scale production for high daily dose requirements, facile access to prepare homogeneous polymeric compounds is needed.[2] Expression by recombinant technology is an alternative to chemical synthesis of bioactive peptides. This approach can overcome major drawbacks associated with chemical synthesis including concomitant production of chemical wastes derived from protecting groups, organic solvents and resin for solid-phase synthesis. Conversely, recombinant peptides from prokaryotes are usually produced without post-translational modifications. Such modifications often provide characteristic functions including bioactivity and biostability.[3,4]

Proteolytic cleavage by exopeptidases is one of the main pathways for degradation of bioactive peptides under physiological conditions. In order to maintain the prolonged effect of peptide therapeutics in vivo, the design of N-terminal acyl- and/or C-terminal amide-modified peptides has been attempted. Such modifications can prevent enzymatic scissions in the circulatory system. However, practical recombinant methodology to prepare bioactive peptides having two end-capping groups is not established. Site-specific cleavage at the *S*-cyanocysteine site within recombinant proteins **2** in the presence of ammonia has been reported (Scheme 1).[5] Such a reaction gives rise to peptide acids and amides **4**. This reaction concomitantly releases the tail peptide **5** with a 2-iminothiazolidine-4-carbonyl group at the N-terminus. On the basis of this chemistry, we envisaged that cleavages of **6** at two *S*-cyanocysteines across the target peptide sequence would generate a peptide amide modification **7** with an N-terminal 2-iminothiazolidine-4-carbonyl group. The current work represents the facile preparation of N- and C-terminally protected anti-HIV peptides by two simultaneous chemical cleavages of recombinant proteins.

2. Results and discussion

This type of cleavage presumably consists of nucleophilic attack of amines and the consecutive iminothiazolidine formation (Scheme 1). Since β-elimination of the thiocyanato group is a possible competing reaction of *S*-cyanocysteine, rapid progression of

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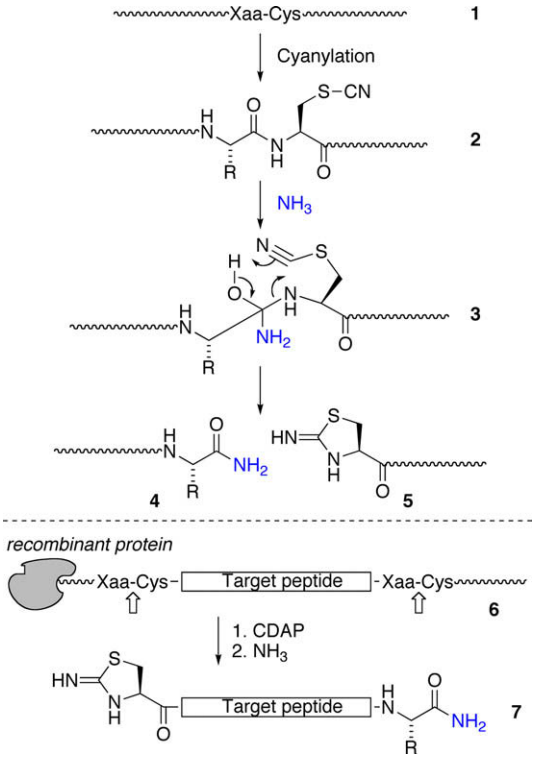
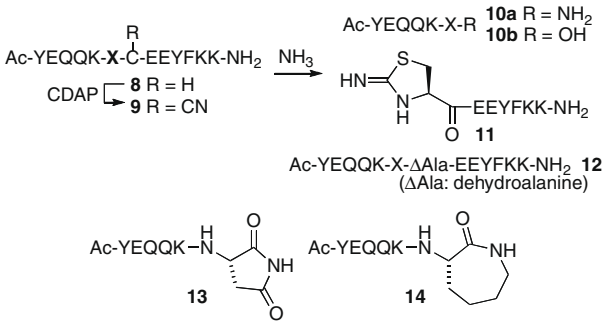


Scheme 1. Site-specific cleavage of recombinant proteins at *S*-cyanocysteine and the preparative illustration of N- and C-terminally capped peptide **7**.

these two steps is preferred.[6] We expected that the presence of an appropriate side-chain functional group in close proximity should assist the cleavage.[7] Using model synthetic peptides Ac-YEQQK-X-C-EEYFKK-NH_2 **8**, we evaluated the effect of the N-terminal-side residue (X) of the cysteine on peptide amide formation. After the standard Fmoc-based solid-phase peptide synthesis, the peptides **8** were treated with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) in a 0.1 N AcOH solution to provide S-cyanylated peptides **9**, which were purified by RP-HPLC. Cleavage reactions of **9** with aqueous 3 M NH_3 were monitored by RP-HPLC analysis (Table 1), in which the production of the first segment peptides **10a**,**b** and the second segment **11** with the N-terminal 2-iminothiazolidine-4-carbonyl group were expected. Among the 19 natural amino acids utilized for the X position (except Cys), bulky aliphatic amino acids such as Val, Ile, and Pro were unfavorable for the cleavage reaction. Peptides with an acidic amino acid such as Asp and Glu mainly provided a β-eliminated product **12**.[8] In contrast, Ser and Thr were appropriate residues for the cleavage reaction. Interestingly, the reaction of Asn- and Lys-containing peptides accompanied production of characteristic C-terminally protected peptides **13** and **14** in higher combined yields: from the Asn peptide, formation of C-terminal aspartimide **13** was observed along with the C-terminal peptide amide (**10a**:**13** = 53:47).[9] Cleavage of the Lys peptide produced a peptide **14** with a C-terminal seven-membered lactam preferentially over the C-terminal amide form (**10a**:**14** = 22:78).[10]

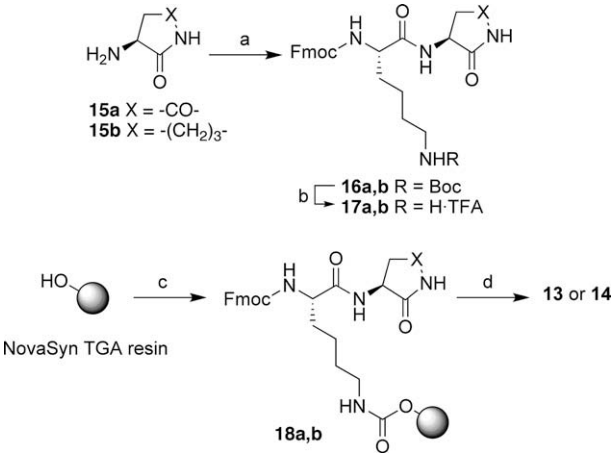
C-terminal cyclic structures in aspartimide **13** and lactam **14** were verified by ESI LC/MS/MS and by the comparative analysis using the peptides that were prepared by the alternative procedures (Scheme 2). Briefly, (*S*)-3-aminosuccinimide **15a** or (*S*)-3-amino-ε-caprolactam **15b** was coupled with Fmoc-Lys(Boc)-OH to give the protected C-terminal components **16a**,**b**. After Boc-deprotection of **16a**,**b**, the resulting amine **17a**,**b** were anchored onto *p*-nitrophenyl carbonate resin, which was prepared by treatment of NovaSyn TGA resin with 4-nitrophenyl chroloformate.

Table 1
Cleavage reaction of *S*-cyanocysteine-containing peptides **9** by aqueous NH_3[a]



X	Conversion[b] (%)
Gly	50
Ala	62
Val	36
Leu	53
Ile	46
Pro	42
Met	66
Phe	63
Tyr	55
Trp	57
Ser	68
Thr	71
Asp	45
Glu	46
Asn	79[c]
Gln	65
Lys	82[d]
Arg	71
His	64

[a] All cleavage reactions were carried out in 3 M NH_3 for 20 min at 20 °C.
[b] The conversion yields were calculated based on the combined peak areas of peptides **10a** and **11** by RP-HPLC analysis.
[c] Including C-terminal aspartimide product **13** (**10a**:**13** = 53:47).
[d] Including C-terminal ε-lactam product **14** (**10a**:**14** = 22:78).



Scheme 2. Alternative synthesis of peptides **13** and **14**. Reagents and conditions: (a) Fmoc-Lys(Boc)-OH, HOBt·H_2O, WSC·HCl, (*S*)-3-aminosuccinimide for **16a** (94%) or (*S*)-3-amino-ε-caprolactam for **16b** (99%); (b) 95% aqueous TFA (quant.); (c) (i) 4-nitrophenyl chloroformate, (*i*-Pr)$_2$EtN, DCM; (ii) **17a** or **17b**, (*i*-Pr)$_2$EtN, DMF (**18a**: 51% loading, **18b**: 45% loading); (d) (i) Fmoc-based SPPS; (ii) TFA/H_2O/*m*-cresol/thioanisole/1,2-ethanedithiol (80:5:5:5:5).

Fmoc-based solid-phase synthesis of the peptide sequence followed by final deprotection gave the expected peptides **13** and

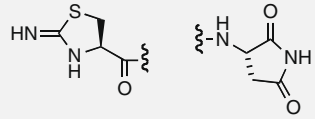
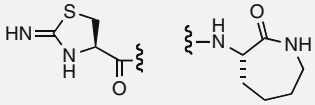
14. Comparative RP-HPLC analysis demonstrated that the peptides were coincident with the ones obtained by cyanocysteine-mediated cleavage (see Supplementary data).

Both the ring structures of **13** and **14** were formed by intramolecular cyclization of characteristic side-chains of Asn and Lys under basic conditions. Since intramolecular imide or amide formation was expected to assist the rapid cleavage, we searched for appropriate conditions for two simultaneous ring-closing reactions including C-terminal aspartimide **13** or lysine ε-lactam **14** as well as N-terminal iminothiazolidine formations (Table 2). Tertiary amines such as Et_3N and $(i\text{-Pr})_2EtN$ generated the expected two peptides **13** and **14** with cyclic structures. Treatment of **9** with alkaline metal-based weak bases such as $NaHCO_3$ and AcONa recovered the starting material, while the expected **13** or **14** was predominantly obtained by aqueous carbonates.[11] Aqueous 0.3 M K_2CO_3, which provided **13** or **14** most efficiently, was employed for further experiments.

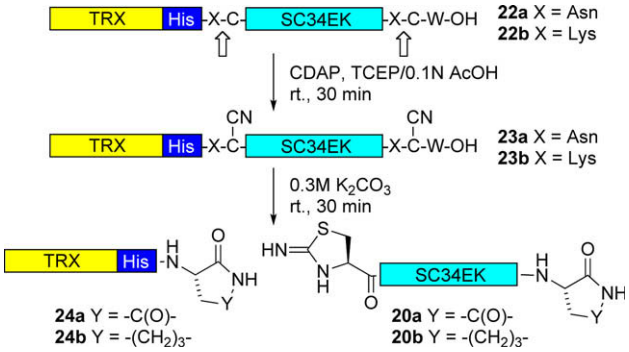
The established protocol was applied to bioorganic synthesis of anti-HIV peptides from recombinant proteins. As a model peptide, HIV fusion inhibitor SC34EK **19** was utilized, which was designed based on the bioactive α-helix conformation of the C-terminal heptad repeat in the envelop glycoprotein gp41 (Table 3, Scheme 3).[12] Peptide **19** exerts anti-HIV activity by preventing formation of the fusogenic six-helical bundle of gp41 and is potent against wild-type and enfuvirtide-resistant HIV-1 viruses. The recombinant thioredoxin-fused proteins **22a**,**b** containing anti-HIV sequence were expressed using the *Escherichia coli* BL21 strain and were purified by affinity chromatography using Ni-NTA resin. After the removal of imidazole by gel filtration, the protein concentration was quantified by the standard Bradford assay. S-Cyanylation of **22a**,**b** was carried out with 10 mM CDAP in 0.1 N AcOH containing 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), which was added to maintain reducing condition for keeping Cys residues.[6b,13] The resulting cyanylated proteins **23a**,**b** were treated in a K_2CO_3 solution (0.3 M) to provide the expected end-capped peptides **20a** and **20b** in 24% and 21% isolated yields, respectively (Fig. 1) and were accompanied with the thioredoxin parts **24a**,**b**.[14]

Anti-HIV activity of the peptides **20a**,**b** derived from recombinant proteins was evaluated along with the parent SC34EK **19** and the end-capping free **21**, which is usually expressed in prokaryotes (Table 3). Peptides **20a**,**b** reproduced the bioactivity of the original peptide **19** [EC_{50}(**20a**) = 0.48 nM; EC_{50}(**20b**) = 0.58 nM],[15] indicating that the original anti-HIV activity was not

Table 3
Sequences and anti-HIV activity of N- and C-terminally capped SC34EK analogs

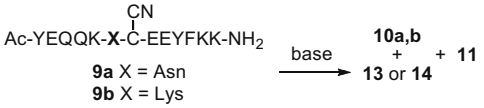
Peptide	R^1-WMEWDRKIEEYTKKIEELIKKSQEQQEKNEKELK-R^2 **19–21** R^1	R^2	EC_{50}[a] (nM)	T_m (°C)
SC34EK **19**	Ac	NH_2	0.60 ± 0.10	71.2
20a			0.48 ± 0.13	71.0
20b			0.58 ± 0.24	71.0
21	H	OH	0.68 ± 0.11	—

[a] EC_{50} was determined as the concentration that blocked HIV-1 replication by 50% in a MAGI assay.



Scheme 3. Bioorganic synthesis of anti-HIV peptide SC34EK analogs **20a**,**b** including N- and C-terminal capping moieties.

Table 2
Cleavage reaction of Asn-Cys(CN) or Lys-Cys(CN)-containing peptides by basic treatment[a]



Entry	Base	From peptide **9a**		From peptide **9b**	
		Conversion[b] (%)	Ratio (**13**/**10**)[c]	Conversion[b] (%)	Ratio (**14**/**10**)[c]
1	3 M NH_3	79	0.9	82	3.6
2	0.5 M NH_3	59	1.7	72	12.4
3	1 M Et_3N	70	3.6	64	>30
4	1 M $(i\text{-Pr})_2EtN$	63	3.1	65	>30
5	1 M AcONa	—[d]	—	—[d]	—
6	1 M $NaHCO_3$	—[d]	—	—[d]	—
7	1 M Na_2CO_3	85	2.5	64	5.6
8	0.3 M Na_2CO_3	83	3.3	74	13.5
9	1 M K_2CO_3	85	1.8	68	9.7
10	0.3 M K_2CO_3	83	3.5	73	15.6

[a] All cleavage reactions were carried out for 20 min at 20 °C.
[b] The conversion yields were calculated based on the combined peak areas of peptides **10a** (entries 1 and 2)/**10b** (entries 3–10), **11**, and **13** or **14** by RP-HPLC analysis.
[c] The ratios of the peak areas of aspartimide **13** or ε-lactam **14** to peptide **10**.
[d] The starting material was recovered.

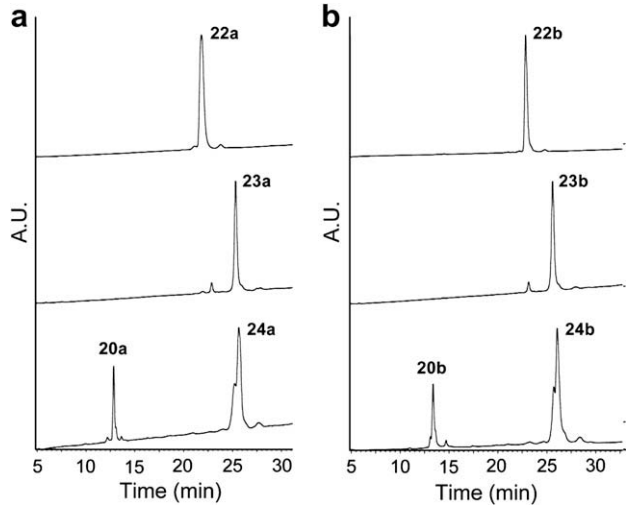


Figure 1. HPLC profiles of *S*-cyanocysteine-mediated cleavage of thioredoxin-fused proteins. (a) Asn-Cys(CN)-mediated cleavage; (b) Lys-Cys(CN)-mediated cleavage. Top: recombinant proteins **22a**,**b** purified by affinity chromatography; middle: S-cyanylated products **23a**,**b**; bottom: products from basic treatment of **23a**,**b**. *HPLC conditions*: linear gradient of 30–60% solvent B in solvent A over 30 min.

disturbed by the N- and C-terminal functional end-capping groups. Biophysical characterization of these peptides was investigated by circular dichroism (CD) analysis (Fig. 2 and Supplementary data). The improved α-helix property of **19** is retained in **20a** and **20b**, and similar thermal stability of the six-helical bundle with N36 was observed (Table 3).

The protecting ability of iminothiazolidine for the N-terminus as well as lysine ε-lactam and aspartimide for the C-terminus from the biodegradation by potential exopeptidases was assessed using peptides **19–21**. The quantity of the intact peptide during incubation in mouse serum was monitored by RP-HPLC (Fig. 3). Rapid degradation was observed for peptide without capping groups at both ends. The isolated digested product had two C-terminal residues deleted. Similarly, C-terminal aspartimide peptide **20a** was also gradually degraded at the C-terminus.[16] In contrast, peptide **20b** with a C-terminal ε-lactam was stable during the 24-h incubation. As such, a combination of iminothiazolidine at the N-terminus and lysine ε-lactam at the C-terminus is beneficial for stabilizing the anti-HIV peptide against potential exopeptidase-mediated degradation without alteration of the biological and biophysical characters.

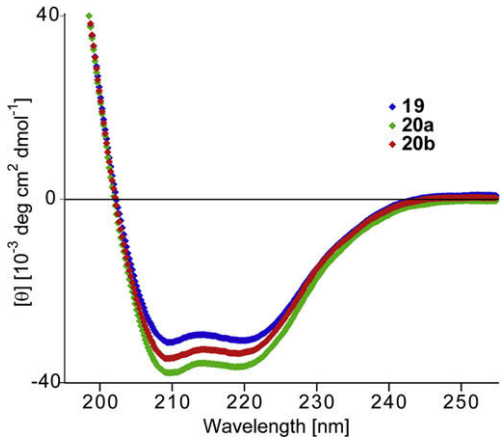


Figure 2. Circular dichroism spectra of N36-SC34EK analogue complexes.

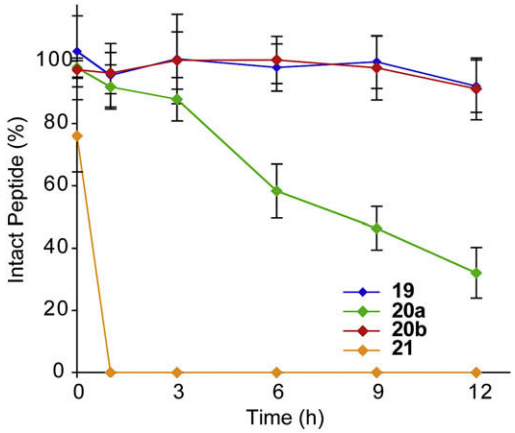


Figure 3. Degradation of SC34EK **19** and the analogues **20a**,**b**, **21** in mouse serum ($n = 5$).

3. Conclusions

Reported herein is the bioorganic synthesis of anti-HIV peptides with two end-capping groups from recombinant proteins. *S*-Cyanocysteine-mediated cleavage at the Asn-Cys(CN) and Lys-Cys(CN) sites provided the characteristic C-terminal ring structures, aspartimide and lysine ε-lactam, respectively. These ring structures are not found in recombinant proteins and peptides produced from prokaryotes. In the current anti-HIV peptide study, capping functional groups did not disturb the original potent bioactivity or modify the biophysical character. This approach is applicable to the preparation of plural end-capped peptides from a single protein molecule having tandem target sequences in conjunction with Lys-Cys(CN) sites.

4. Experimental

4.1. General

For HPLC separations of synthetic peptides, a Cosmosil 5C18-ARII analytical column (4.6 × 250 mm, flow rate 1 mL/min, Nacalai Tesque, Kyoto Japan) or a Cosmosil 5C18-ARII preparative column (20 × 250 mm, flow rate 10 mL/min) was employed. For HPLC analysis of recombinant proteins **22a**,**b**, **23a**,**b** and the cleaved products **20a**,**b** (Fig. 1), a Cosmosil Protein-R analytical column (4.6 × 150 mm, flow rate 1 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. All peptides were characterized by a MALDI-TOF-MS (AXIMA-CFR plus, Shimadzu, Kyoto, Japan) or by a QqTof (QSTAR pulsar i, Applied Biosystems). NMR spectra were recorded on Bruker AVANCE500.

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; Trt for Cys, His, Asn and Gln; and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg were employed for side-chain protection, respectively. Fmoc-amino acids were coupled using 5 equiv of reagents [Fmoc-amino acid, *N*,*N*′-diisopropylcarbodiimide and HOBt·H_2O] to free amino group in DMF for 1.5 h. Fmoc deprotection was performed by 20% piperidine in DMF (2 × 1 min, 1 × 20 min). The resulting protected resin was treated with TFA/H_2O/*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 Et_2O (30 mL) was added to the residue. The resulting powder was collected by centrifugation and then washed with ice-cold dry Et_2O (3 × 15 mL). Purification of the crude product by preparative HPLC afforded a colorless powder of the desired peptide.

4.3. General procedure for the preparation of *S*-cyanocysteine-containing peptides 9

To a solution of peptide **8** (X = Lys, 5.8 mg) in 0.1 N AcOH (0.58 mL) was added the solution of CDAP in 0.1 N AcOH (10 mg/mL, 182 μL). After being stirred at room temperature for 30 min, the solution was purified by preparative HPLC to give freeze-dried powder of peptide **9** (X = Lys, 5.7 mg, 97%).

4.4. General procedure for cleavage reaction of *S*-cyanocysteine-containing peptides 9 by aqueous NH_3

Peptide **9** (ca. 1 mg) was dissolved in 100 μL of 3 M NH_3 solution. After standing at 20 °C for 20 min, the reaction was monitored by RP-HPLC. The results are summarized in Table 1.

4.5. Synthesis of compound 16a

To a solution of Fmoc-Lys(Boc)-OH (2.34 g, 5.0 mmol) and HOBt·H_2O (0.77 g, 5.0 mmol) in DMF (20 mL) was added WSC·HCl (0.96 g, 5.0 mmol) at 0 °C. After being stirred for 5 min. at room temperature, a solution of (*S*)-3-aminosuccinimide **15a** [prepared by catalytic hydrogenation of (*S*)-3-*N*-carbobenzoxyaminosuccinimide (1.61 g, 6.5 mmol)] in DMF (5 mL) was added. The reaction mixture was stirred for 1 h and was poured into ice-cold water. The resulting precipitate was extracted with AcOEt and the organic layer was washed with citric acid solution and brine. After drying over $MgSO_4$, the solvent was evaporated under reduced pressure. The residue was purified by silica-gel column chromatography to provide the compound **16a** (2.65 g, 94% yield) as a colorless powder: $[\alpha]_D^{20}$ −31.4 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.22 (s, 1H), 8.51 (d, *J* = 8.0 Hz, 1H), 7.92–7.80 (m, 2H), 7.75–7.66 (m, 2H), 7.52 (d, *J* = 8.0 Hz, 1H), 7.44–7.36 (m, 2H), 7.36–7.27 (m, 2H), 6.78 (t, *J* = 5.5 Hz, 1H), 4.59–4.46 (m, 1H), 4.34–4.14 (m, 3H), 3.98–3.83 (m, 1H), 2.94–2.78 (m, 3H), 2.42 (dd, *J* = 17.5, 5.5 Hz, 1H), 1.69–1.43 (m, 2H), 1.42–1.11 (m, 13H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 177.4, 176.3, 172.1, 155.9, 155.5, 143.8, 143.6, 140.6, 127.5, 127.0, 125.3, 120.0, 77.2, 65.6, 54.3, 49.2, 46.6, 38.9, 36.0, 31.4, 29.0, 28.2, 22.6. Anal. Calcd for $C_{30}H_{36}N_4O_7\cdot H_2O$: C, 61.84; H, 6.57; N, 9.62. Found: C, 61.92; H, 6.23; N, 9.72.

4.6. Synthesis of compound 16b

To a solution of Fmoc-Lys(Boc)-OH (2.34 g, 5.0 mmol) and HOBt·H_2O (0.77 g, 5.0 mmol) in DMF (20 mL) was added WSC·HCl (0.96 g, 5.0 mmol) at 0 °C. After being stirred for 5 min at room temperature, a solution of (*S*)-3-amino-ε-caprolactam **15b** (0.77 g, 6.0 mmol) in DMF (5 mL) was added. The reaction mixture was stirred for 1 h, and was poured into ice-cold water. The resulting precipitate was extracted with AcOEt and the organic layer was washed with citric acid solution and brine. After drying over $MgSO_4$, the solvent was evaporated under reduced pressure. The residue was purified by silica-gel column chromatography to provide the compound **16b** (2.86 g, 99% yield) as a colorless powder: $[\alpha]_D^{20}$ −8.6 (c 1.0, $CDCl_3$); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.92–7.82 (m, 3H), 7.81 (d, *J* = 6.5 Hz, 1H), 7.76–7.69 (m, 2H), 7.67 (d, *J* = 8.0 Hz, 1H), 7.45–7.38 (m, 2H), 7.36–7.25 (m, 2H), 6.78 (t, *J* = 5.5 Hz, 1H), 4.40–4.30 (m, 1H), 4.30–4.13 (m, 3H), 4.00–3.89 (m, 1H), 3.23–3.14 (m, 1H), 3.10–3.00 (m, 1H), 2.96–2.81 (m, 2H), 1.90–1.80 (m, 1H), 1.80–1.69 (m, 2H), 1.69–1.56 (m, 2H), 1.56–1.46 (m, 1H), 1.42–1.12 (m, 15H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 174.0, 170.8, 155.9, 155.5, 143.8, 143.6, 140.6, 127.5, 125.3, 125.2, 120.0, 77.2, 65.6, 54.9, 51.2, 46.6, 40.5, 31.3, 30.9, 29.1, 28.7, 28.2, 27.5, 22.8. Anal. Calcd for $C_{32}H_{42}N_4O_6$: C, 66.41; H, 7.32; N, 9.68. Found: C, 66.17; H, 7.03; N, 9.71.

4.7. Synthesis of resin 18a

Compound **16a** (0.56 g, 1 mmol) was dissolved in 95% aqueous TFA (5 ml) and the solution was stirred at room temperature for 1 h. The solvent was removed under reduced pressure to give compound **17a** (0.58 g, quant.) as a colorless oil, which was utilized for the next step without further purification. A solution of 0.3 M 4-nitrophenyl chloroformate and 0.3 M (*i*-Pr)$_2$EtN in DCM (2 mL) was added to NovaSyn TGA resin (0.26 mmol/g, 192 mg, 0.05 mmol). The mixture was stirred at room temperature for 4 h, and the resin was washed with DCM (×3) and DMF (×3). A solution of 0.3 M compound **17a**, 0.3 M (*i*-Pr)$_2$EtN in DMF (2 mL) was added to the resin and the mixture was stirred at room temperature for 6 h. The resin was washed by DMF (×5), DCM (×3) and MeOH (×3) and dried to give the expected resin **18a** (51% loading).

4.8. Preparation of recombinant thioredoxin-fused proteins 22a,b

The cDNA sequence encoding KKC-SC34EK-KCW and KNC-SC34EK-NCW was utilized as template for PCR amplification, KNC-SC34EK-NCW: 5′-CTC**GGATCC**AAAAATTGCTGGATGGAATGGG ATCGTAAAATTGAAGAATATACCAAAAAAATTGAAGAACTGATTAAAA AAAGCCAGGAACAGCAGGAAAAAAATGAAAAAGAACTGAAAAATTGC TGGTAA**CTCGAG**-3′; KKC-SC34EK-KCW: 5′-CTC**GGATCC**AAAAAATG CTGGATGGAATGGGATCGTAAAATTGAAGAATATACCAAAAAAATTGA AGAACTGATTAAAAAAAAGCCAGGAACAGCAGGAAAAAAATGAAAAA GAACTGAAAAAATGCTGGTAA**CTCGAG**GAG-3′. The two restriction sites for BamHI and XhoI are shown in bold. Codons were replaced by more frequently used ones based on *E. coli.* codon usage. Each segment was digested with BamHI and XhoI and inserted into pET32a vector. Then the plasmids (pET32a-KKC-SC34EK-KCW and pET32a-KNC-SC34EK-NCW) were transformed into *E. coli* BL21 (DE3)-RIL strain for expression. Isolated colonies were picked up and cultured overnight in 10 mL of LB culture with 50 μg/mL ampicillin at 30 °C with shaking. This culture was transferred into 1 L of LB culture in the presence of 50 μg/mL ampicillin. When the OD_{600} reached 0.6–0.8 at 30 °C, protein expression was initiated by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM). After an additional 6 h cultivation at 25 °C, cells were harvested by centrifugation at 4000 rpm for 20 min. Cells were resuspended in B-PER (PIERCE) solution, and disrupted by sonication. After centrifugation at 12,000 rpm for 30 min, the supernatant, supplemented 0.5 mM TCEP, was transferred to column with Ni-NTA agarose (QIAGEN). The column was washed with wash buffer (20 mM phosphate, pH 6.0, containing 0.5 M NaCl and 0.5 mM TCEP). Protein was eluted from the column by the 150 mM imidazole in phosphate buffer (pH 6.0) containing 0.5 mM TCEP. The expression and purification of the fusion protein was analyzed by SDS–PAGE (10–20% gradient gel). The yield of eluted protein was calculated using Protein Assay Kit (BIO-RAD).

4.9. General procedure for the preparation of the end-capped anti-HIV peptide from recombinant protein

The eluted protein **22a** (6.8 mg quantified by Bradford assay) from the NAP column (GE healthcare) was cyanylated by 10 mM CDAP in the 0.1 N AcOH containing 0.5 mM TCEP for 30 min. After

desalting by gel-filtration and freeze-drying, cyanylated protein was treated with 0.3 M K_2CO_3 for 30 min. The reaction products were analyzed using LC–MS. Purification of the product by preparative HPLC provided the expected end-capped peptide **20a** (0.40 mg, 24%) that was quantified by UV absorbance at 280 nm.

4.10. Determination of drug susceptibility of HIV-1

The peptide sensitivity of infectious clones was determined by the MAGI assay with some modifications.[17] Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^4 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. Forty-eight hours after 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 [EC_{50}]).

4.11. Measurement of CD spectra

Peptides **19** and **20a**,**b** were incubated at 37 °C for 30 min (the final concentrations of peptides were 10 μM in 5 mM HEPES buffer, pH 7.2). CD spectra were acquired on a Jasco spectropolarimeter (Model J-710, Jasco Inc., Tokyo, Japan) at 25 °C as the average of 8 scans. Thermal unfolding of potential six-helical bundle in the presence of N36 was monitored by the $[\theta]_{222}$ values at intervals of 0.5 °C after a 0.25-min 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).

4.12. Stability of SC34EK peptide or analogs in mouse serum

Peptides **19**-**21** (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, 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 CH_3CN. Samples were deproteinized by centrifugation at 12000 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.2009.09.015.

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13. Only partial S-cyanylations of two Cys residues in thioredoxin were observed. This was also verified by the recovery of C-terminal capped thioredoxin **24a**,**b** after basic treatment in the next step.
14. Peptides **20a**,**b** from **22a**,**b** were identical to the authentic samples, which were obtained by the cleavage reaction of the synthetic peptides.
15. Cytotoxicity of peptides **20a**,**b** was not observed even at 10 μM in MAGI assay.
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