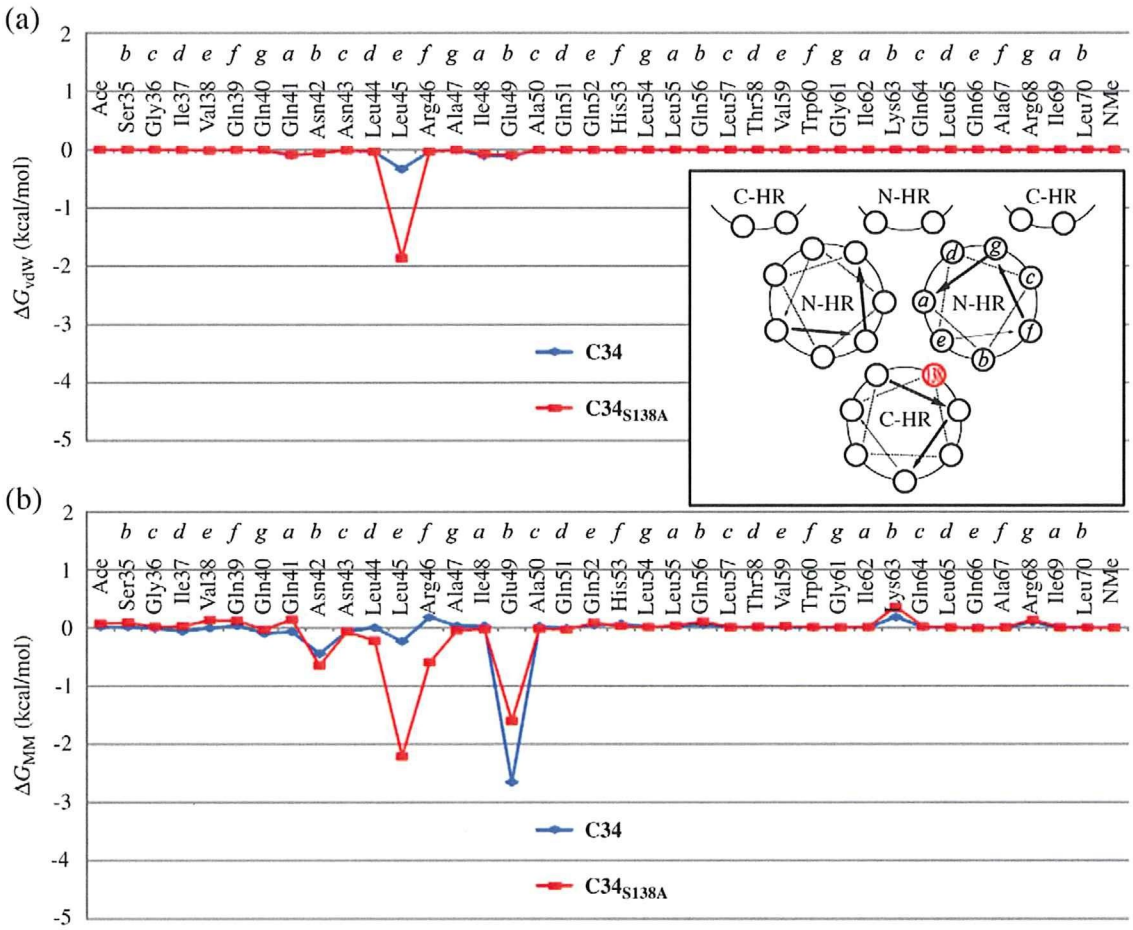


Fig. 5. Intermolecular interactions between residue 138 of the C-HR-derived peptides and one of the N-HR helices: (a) the van der Waals interaction term (ΔG_{vdW}); (b) the pair interaction term (ΔG_{MM}), which combines the van der Waals and electrostatic interactions ($\Delta G_{vdW} + \Delta G_{ele}$). Blue and red lines designate the interactions of C34 and $C34_{S138A}$, respectively. The assignments of the residues are presented above the amino acid sequence by the italicized letters.

replication may be required.[12,25] In this regard, we previously demonstrated that S138A acts as a secondary mutation and restores impaired replication kinetics by a primary mutation for T-20-resistant N43D.[19] Taken together, our observations are useful not only for the development of new peptidic and small molecular fusion inhibitors but also for the analysis of molecular mechanism of viral fusion.

Conclusion

We performed X-ray crystallographic analysis to evaluate the effects of the S138A substitution on the anti-HIV-1 activity of the C-HR-derived peptide C34. MM and PBSA calculations based on the X-ray coordinates were qualitatively supported by the difference between the experimental binding energies of the native and mutant C34 monomers. The N36/$C34_{S138A}$ complex was thermally more stable than the N36/C34 complex, and this result indicated that the S138A substitution accelerated the formation of the N-HR/C-HR six-helix bundle complex. This stability was found to be mainly caused by the difference between the desolvation energies of the C34 monomers with and without the S138A substitution. The desolvation energies depended on the hydrophobicity of residue 138. Therefore, the substitution of Ser138 to hydrophobic amino acids should enhance the anti-HIV-1 activities of the C-HR-derived peptides C34 and T-20.[19]

The crystal structure comparison between N36/C34 and N36/$C34_{S138A}$ complexes provided insights into the effect of S138A substitution on the binding affinities related with the anti-HIV-1 activities. We investigated whether the effects of the S138A substitution were predictable from the wild-type structure. The MM calculation of the wild-type X-ray structure after removing the Ser138 oxygen atom led to a different conclusion from that of the S138A X-ray structure. This result highlights the importance of atomic-level structure determination and indicates that the structural modeling of mutants starting from the wild-type X-ray structure should be carried out carefully. More reliable thermodynamic integration simulations are currently under investigation in order to predict the substitution effects *in silico*.

Materials and Methods

Peptide synthesis and purification

All peptides with an acetylated N-terminus and a C-terminal amide were synthesized by standard Fmoc-based peptide synthesis protocol using NovaSyn TGR resin. Fmoc amino acids had the following side-chain protections: *t*-butyl ester for Asp and Glu; trityl for His, Asn, and Gln; *t*-butyloxycarbonyl for Lys; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg; *t*-butyl ether for Ser, Thr, and Tyr. The Fmoc amino acids were coupled, employing *N*,*N*′-diisopropylcarbodiimide in the presence of 1-hydroxybenzotriazole hydrate. After final deprotection and cleavage from the resins using a trifluoroacetic acid/thioanisole/*m*-cresol/1,2-ethanedithiol/water (80:5:5:5:5) cocktail, the crude peptides were purified by reverse-phase HPLC to yield the desired peptides, which were characterized by mass spectrometry (Supplementary Data).

Determination of drug susceptibility of HIV-1

The peptide sensitivity of infectious clones was determined by the MAGI assay with HeLa-CD4-LTR-β-gal cells and NL4-3(GIV) strain. The activity of test compounds was determined as the concentration that blocked HIV-1 replication by 50% (EC_{50}).[19,24]

Measurement of T_m value

Each peptide (10 μM) was mixed with 5 mM Hepes buffer (pH 7.2). CD spectra were acquired on a Jasco spectropolarimeter (Model J-710, Jasco Inc., Tokyo, Japan). The thermal stability was assessed by monitoring the change in the CD signal at 222 nm. The middle point of the thermal unfolding transition [melting temperature (T_m)] of each complex was determined (Supplementary Data).

Crystallization and X-ray analysis

Crystallization attempts were made using Hampton Crystal Screens I and II. Trials were performed by the sitting-drop vapor-diffusion method at 293 K. The drop contained 1 μL solution of the N36/$C34_{S138A}$ complex (2 mg/mL in 100 mM AcOH/Na, pH 4.0) and an equal volume of reservoir solution. To grow crystals, we diluted (1:1) a 2-mg/mL stock of the complex in a sitting drop with 12% ethanol and 400 mM magnesium chloride. The obtained crystal was transferred into a cryoprotectant consisting of 12% ethanol, 400 mM magnesium chloride, and 30% polyethylene glycol 400. Several minutes later, it was scooped up in a cryoloop and frozen in liquid nitrogen. It was then mounted on a goniometer in a nitrogen stream at 113 K. X-ray diffraction was detected on an R-AXIS VII imaging-plate system attached to a Rigaku CuKα radiation rotating-anode generator (FR-E) with a crystal-to-detector distance of 120 mm. Data were collected to 2.2 Å resolution (0.5 frames) with an exposure time of 2 min. The diffraction data were processed and scaled with d*TREK.[26] The N36/$C34_{S138A}$ complex structure was built by a molecular replacement calculation with the program MOLREP,[27] using the deposited coordinates of the N36/C34 complex (PDB code: 1AIK). Subsequent manual rebuilding was carried out in the program Coot.[28] The structure was refined by using the CCP4 program Refmac5.[29,30]

Computational methods

The heavy-atom coordinates of six-helix bundles (hexamers) of the peptide complexes (N36/C34 and N36/$C34_{S138A}$) were constructed on the basis of the X-ray crystal structures. By using the MOE software package,[31] the C-termini of the peptides were capped as *N*-methylamide group, and hydrogen atoms of the whole structures were placed at ideal positions. Subsequently, the energy minimizations were performed with the FF99 force field.[32] During the minimizations, all heavy atoms were fixed to the X-ray coordinates or restrained using force constants of between 0 and 10,000 kcal/(mol·Å^2). Then, the peptides were assumed as under a standard ionization condition.

Based on the resulting coordinates, the peptide–peptide pair interaction energies (ΔG_{MM}) were calculated by using the AMBER9 software package with the FF99 force field.[32,33] The solvation energies (ΔG_{solv}) were estimated by using the DELPHI program with PARSE radii,[20,34] AMBER partial charges, and dielectric constants of 1 (polypeptide interior) and 80 (water). The solvent-accessible surface areas were calculated using the MSMS program.[35] The desolvation energies (ΔG_{solv}) were defined as $\Delta G_{solv} = G_{solv}(\text{hexamer}) - G_{solv}(\text{pentamer}) - G_{solv}(\text{monomer})$. ΔG_{bind}, defined as a sum of the interaction energy (ΔG_{MM}) and the desolvation free energy (ΔG_{solv}), was obtained from $\Delta G_{bind} = G_{bind}(\text{hexamer}) - G_{bind}(\text{pentamer}) - G_{bind}(\text{monomer})$.

Accession code

Refined structure has been deposited in the PDB (PDB code: 2ZZO).

Acknowledgements

This work was supported by the Science and Technology Incubation Program in Advanced Regions from Japan Science and Technology Agency; a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and Health and Labour Sciences Research Grants (Research on HIV/AIDS).

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.07.027

References

1. Lu, M., Blacklow, S. C. & Kim, P. S. (1995). A trimeric structural domain of the HIV-1 transmembrane glycoprotein. *Nat. Struct. Biol.* **2**, 1075–1082.

2. Chan, D. C., Fass, D., Berger, J. M. & Kim, P. S. (1997). Core structure of gp41 from the HIV envelope glycoprotein. *Cell*, **89**, 263–273.
3. Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J. & Wiley, D. C. (1997). Atomic structure of the ectodomain from HIV-1 gp41. *Nature*, **387**, 426–430.
4. Melikyan, G. B., Markosyan, R. M., Hemmati, H., Delmedico, M. K., Lambert, D. M. & Cohen, F. S. (2000). Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. *J. Cell Biol.* **151**, 413–423.
5. Wild, C., Oas, T., McDanal, C., Bolognesi, D. & Matthews, T. (1992). A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. *Proc. Natl Acad. Sci. USA*, **89**, 10537–10541.
6. Wild, C. T., Shugars, D. C., Greenwell, T. K., McDanal, C. B. & Matthews, T. J. (1994). Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. *Proc. Natl Acad. Sci. USA*, **91**, 9770–9774.
7. Chen, C. H., Matthews, T. J., McDanal, C. B., Bolognesi, D. P. & Greenberg, M. L. (1995). A molecular clasp in the human immunodeficiency virus (HIV) type 1 TM protein determines the anti-HIV activity of gp41 derivatives: implication of viral fusion. *J. Virol.* **69**, 3771–3777.
8. Kilby, J. M., Hopkins, S., Venetta, T. M., DiMassimo, B., Cloud, G. A., Lee, J. Y. *et al.* (1998). Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nat. Med.* **4**, 1302–1307.
9. Matthews, T., Salgo, M., Greenberg, M., Chung, J., DeMasi, R. & Bolognesi, D. (2004). Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. *Nat. Rev., Drug Discov.* **3**, 215–225.
10. Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M. *et al.* (2002). Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother.* **46**, 1896–1905.
11. Marcelin, A. G., Reynes, J., Yerly, S., Ktorza, N., Segondy, M., Piot, J. C. *et al.* (2004). Characterization of genotypic determinants in HR-1 and HR-2 gp41 domains in individual with persistent HIV viraemia under T-20. *AIDS*, **18**, 1340–1342.
12. Xu, L., Pozniak, A., Wildfire, A., Stanfield-Oakley, S. A., Mosier, S. M., Ratcliffe, D. *et al.* (2005). Emergence and evolution of enfuvirtide resistance following long-term therapy involves heptad repeat 2 mutations within gp41. *Antimicrob. Agents Chemother.* **49**, 1113–1119.
13. Otaka, A., Nakamura, M., Nameki, D., Kodama, E., Uchiyama, S., Nakamura, S. *et al.* (2002). Remodeling gp41-C34 peptide leads to high effective inhibitors of the fusion of HIV-1 with target cells. *Angew. Chem., Int. Ed.* **41**, 2937–2940.
14. Oishi, S., Ito, S., Nishikawa, H., Watanabe, K., Tanaka, M., Ohno, H. *et al.* (2008). Design of a novel HIV-1 fusion inhibitor that displays a minimal interface for binding affinity. *J. Med. Chem.* **51**, 388–391.
15. Joyce, J. G., Hunri, W. M., Bogusky, M. J., Garsky, V. M., Liang, X., Citron, M. P. *et al.* (2002). Enhancement of α-helicity in the HIV-1 inhibitory peptide DP178 leads to an increased affinity for human monoclonal antibody 2F5 but does not elicit neutralizing responses in vitro. *J. Biol. Chem.* **277**, 45811–45820.
16. Dweyr, J. J., Wilson, K. L., Davison, D. K., Freel, S. A., Seedolff, J. E., Wring, S. A. *et al.* (2007). Design of helical, oligomeric HIV-1 fusion inhibitor peptides with potent activity against enfuvirtide-resistant virus. *Proc. Natl Acad. Sci. USA*, **104**, 12772–12777.
17. Chan, D. C., Chutkowski, C. T. & Kim, P. S. (1998). Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. *Proc. Natl Acad. Sci. USA*, **95**, 15613–15617.
18. Strockbine, B. & Rizzo, R. C. (2007). Binding of antifusion peptides with HIVgp41 from molecular dynamics simulations: quantitative correlation with experiment. *Proteins: Struct. Funct. Bioinform.* **67**, 630–642.
19. Izumi, K., Kodama, E., Shimura, K., Sakagami, Y., Watanabe, K., Ito, S. *et al.* (2009). Design of peptide-based inhibitors for human immunodeficiency virus type 1 strains resistant to T-20. *J. Biol. Chem.* **284**, 4914–4920.
20. Sitkoff, D., Sharp, K. A. & Honig, B. (1994). Accurate calculation of hydration free energies using macroscopic solvent models. *J. Phys. Chem.* **98**, 1978–1988.
21. Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A. & Martin, M. A. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59**, 284–291.
22. Judice, J. K., Tom, J. Y., Huang, W., Wrin, T., Vennari, J., Petropoulos, C. J. & McDowell, R. S. (1997). Inhibition of HIV type 1 infectivity by constrained α-helical peptides: implications for the viral fusion mechanism. *Proc. Natl Acad. Sci. USA*, **94**, 13426–13430.
23. Shu, W., Liu, J., Ji, H., Radigen, L., Jiang, S. & Lu, M. (2000). Helical interactions in the HIV-1 gp41 core reveal structural basis for the inhibitory activity of gp41 peptides. *Biochemistry*, **39**, 1634–1642.
24. Ueno, M., Kodama, E., Shimura, K., Sakurai, Y., Kajiwara, K., Sakagami, Y. *et al.* (2009). Synonymous mutations in stem–loop III of Rev responsive elements enhance HIV-1 replication impaired by primary mutations for resistance to enfuvirtide. *Antiviral Res.* **82**, 67–72.
25. Bai, X., Wilson, K. L., Seedorff, J. E., Ahrens, D., Green, J., Davison, D. K. *et al.* (2008). Impact of the enfuvirtide resistance mutation N43D and the associated baseline polymorphism E137K on peptide sensitivity and six-helix bundle structure. *Biochemistry*, **47**, 6662–6670.
26. Pflugrath, J. W. (1999). The finer things in X-ray diffraction data collection. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **55**, 1718–1725.
27. Vagin, A. & Teplyakov, A. (2000). An approach to multi-copy search in molecular replacement. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **56**, 1622–1624.
28. Emsley, P. & Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **60**, 2126–2132.
29. Collaborative Computational Project, Number 4. (1994). The CCP4 suite: programs for protein crystallography. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **50**, 760–763.
30. Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **53**, 240–255.
31. MOE software package from Chemical Computing Group Inc., http://www.chemcomp.com.
32. Wang, J. M., Cieplak, P. & Kollman, P. A. (2000). How well does a restrained electrostatic potential (RESP)

model perform in calculating conformational energies of organic and biological molecules. *J. Comput. Chem.* **21**, 1049–1074.
33. Pearlman, D. A., Case, D. A., Caldwell, J. W., Ross, W. R., Cheatham, T. E., DeBolt, S. *et al.* (1995). AMBER, a computer program for applying molecular mechanics, normal mode analysis, molecular dynamics and free energy calculations to elucidate the structures and energies of molecules. *Comp. Phys. Commun.* **91**, 1–41.
34. Honig, B. & Nicholls, A. (1995). Classical electrostatics in biology and chemistry. *Science*, **268**, 1144–1149.
35. Sanner, M. F., Olson, A. J. & Spehner, J. (1996). Reduced surface: an efficient way to compute molecular surfaces. *Biopolymers*, **38**, 305–320.

Bioorganic & Medicinal Chemistry 17 (2009) 7487–7492

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

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

Michinori Tanaka [a], Kazumi Kajiwara [a,b], Rei Tokiwa [a,b], Kentaro Watanabe [a], Hiroaki Ohno [a], Hiroko Tsutsumi [c], Yoji Hata [c], Kazuki Izumi [d], Eiichi Kodama [d], Masao Matsuoka [d], Shinya Oishi [a,*], Nobutaka Fujii [a,*]

[a] Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
[b] JST Innovation Plaza Kyoto, Japan Science and Technology Agency, Nishigyo-ku, Kyoto 615-8245, Japan
[c] Gekkeikan Research Institute, Gekkeikan Sake Company, Ltd, Fushimi-ku, Kyoto 612-8391, Japan
[d] Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

ARTICLE INFO

Article history:
Received 12 August 2009
Revised 5 September 2009
Accepted 10 September 2009
Available online 15 September 2009

Keywords:
Bioorganic synthesis
End-capped peptide
Fusion inhibitor
HIV-1

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]

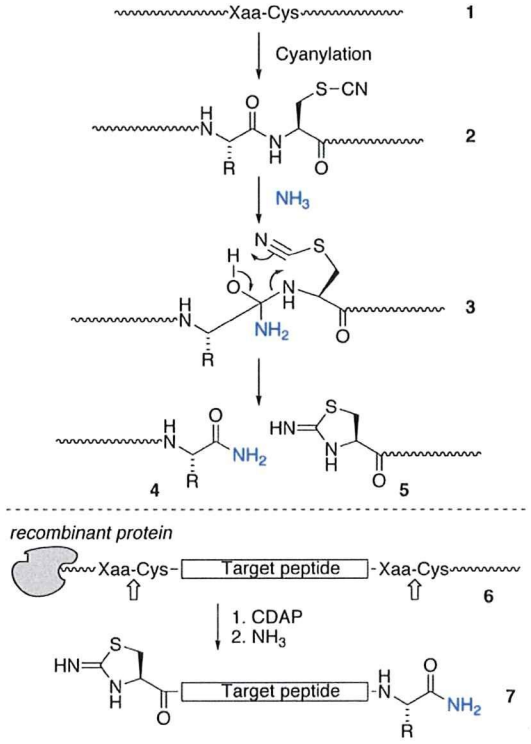
Proteolytic cleavage by exopeptidases is one of the main pathways for degradation of bioactive peptides under physiological conditions. In order to maintain the prolonged effect of peptide therapeutics in vivo, the design of N-terminal acyl- and/or C-terminal amide-modified peptides has been attempted. 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

* Corresponding authors. Tel.: +81 75 753 4551; fax: +81 75 753 4570 (S.O.).
E-mail addresses: soishi@pharm.kyoto-u.ac.jp (S. Oishi), nfujii@pharm.kyoto-u.ac.jp (N. Fujii).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.09.015

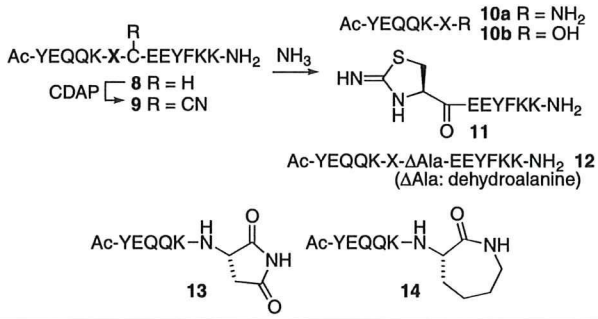


Scheme 1. Site-specific cleavage of recombinant proteins at *S*-cyanocysteine and the preparative illustration of N- and C-terminally capped peptide **7**.

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]

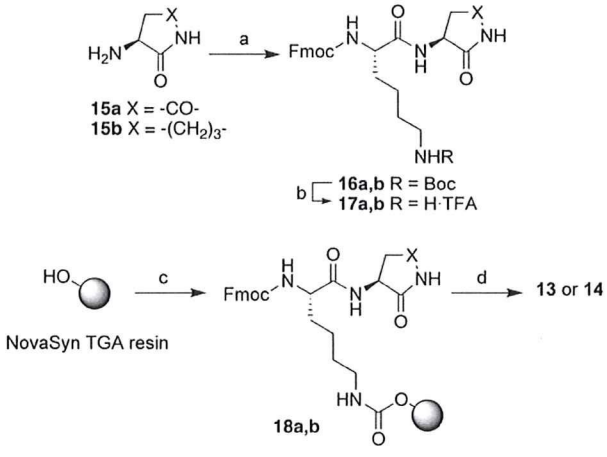
C-terminal cyclic structures in aspartimide **13** and lactam **14** were verified by ESI LC/MS/MS and by the comparative analysis using the peptides that were prepared by the alternative procedures (Scheme 2). Briefly, (*S*)-3-aminosuccinimide **15a** or (*S*)-3-amino-ε-caprolactam **15b** was coupled 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, $(i\text{-Pr})_2$EtN, DCM; (ii) **17a** or **17b**, $(i\text{-Pr})_2$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

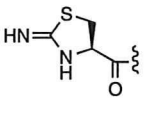
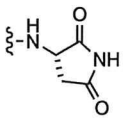
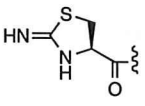
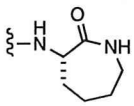
14. Comparative RP-HPLC analysis demonstrated that the peptides were coincident with the ones obtained by cyanocysteine-mediated cleavage (see Supplementary data).

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.

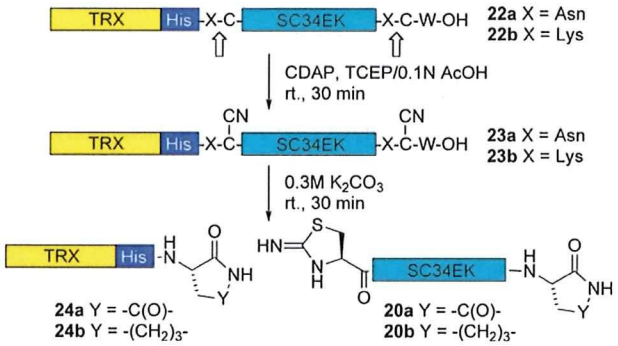
The established protocol was applied to bioorganic synthesis of anti-HIV peptides from recombinant proteins. As a model peptide, HIV fusion inhibitor SC34EK **19** was utilized, which was designed based on the bioactive α-helix conformation of the C-terminal heptad repeat in the envelop glycoprotein 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

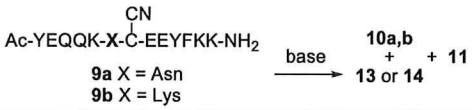
Peptide	R^1-WMEWDRKIEEYTKKIEELIKKSQEQQEKNEKELK-R^2 **19–21** R^1	R^2	EC_{50}[a] (nM)	T_m (°C)
SC34EK **19**	Ac	NH_2	0.60 ± 0.10	71.2
20a			0.48 ± 0.13	71.0
20b			0.58 ± 0.24	71.0
21	H	OH	0.68 ± 0.11	–

[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.

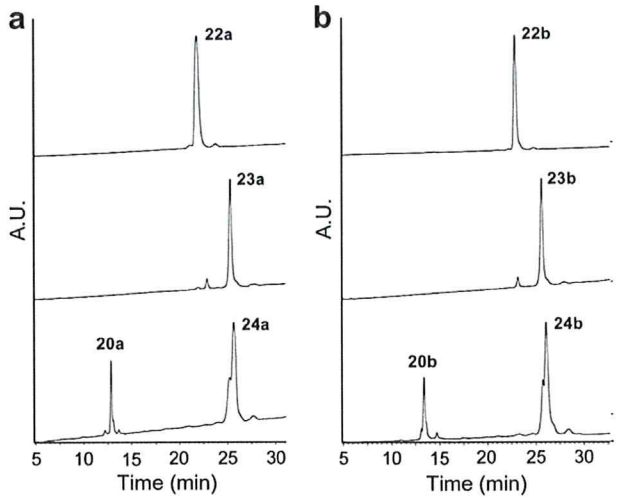


Figure 1. HPLC profiles of *S*-cyanocysteine-mediated cleavage of thioredoxin-fused proteins. (a) Asn-Cys(CN)-mediated cleavage; (b) Lys-Cys(CN)-mediated cleavage. Top: recombinant proteins **22a,b** purified by affinity chromatography; middle: S-cyanylated products **23a,b**; bottom: products from basic 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.

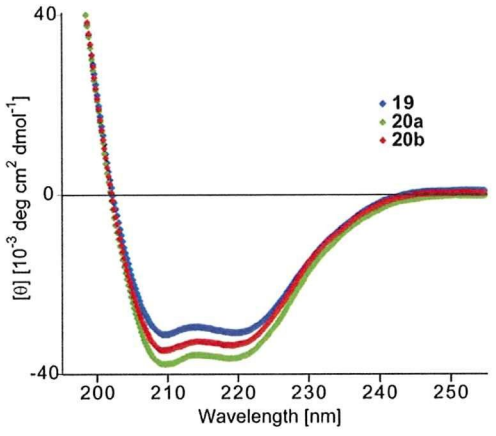


Figure 2. Circular dichroism spectra of N36-SC34EK analogue complexes.

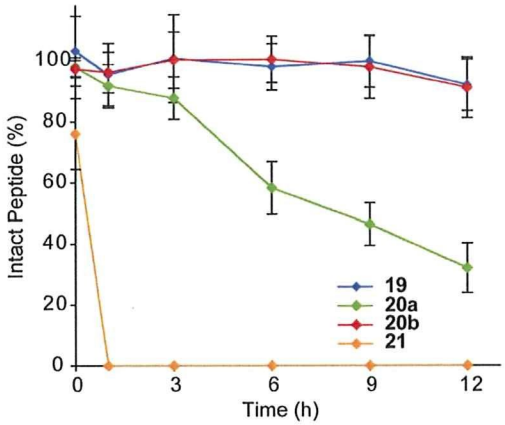


Figure 3. Degradation of SC34EK **19** and the analogues **20a,b**, **21** in mouse serum ($n = 5$).

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.

Acknowledgments

This work was supported by Science and Technology Incubation Program in Advanced Regions from Japan Science and Technology Agency, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Health and Labour Sciences Research Grants (Research on HIV/AIDS).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.09.015.

References and notes

1. For reviews, see: (a) Frokjaer, S.; Otzen, D. E. *Nat. Rev. Drug Disc.* **2005**, *4*, 298; (b) Leader, B.; Baca, Q. J.; Golan, D. E. *Nat. Rev. Drug Disc.* **2008**, *7*, 21.
2. (a) Andersson, L.; Blomberg, L.; Flegel, M.; Lepsa, L.; Nilsson, B.; Verlander, M. *Biopolymers* **2000**, *5*, 227; (b) Bray, B. L. *Nat. Rev. Drug Disc.* **2003**, *2*, 587.
3. Dingermann, T. *Biotechnol. J.* **2008**, *3*, 90.
4. For recent reviews on the preparation of peptides and proteins having nonproteinogenic amino acid(s) or post-translational modification, see: (a) Muralidharan, V.; Muir, T. W. *Nat. Methods* **2006**, *3*, 429; (b) Bennett, C. S.; Wong, C. H. *Chem. Soc. Rev.* **2007**, *36*, 1227; (c) Ohta, A.; Yamagishi, Y.; Suga, H. *Curr. Opin. Chem. Biol.* **2008**, *12*, 159.
5. (a) Stark, G. R. *Methods Enzymol.* **1977**, *47*, 129; (b) Nakagawa, S.; Tamakashi, Y.; Hamana, T.; Kawase, M.; Taketomi, S.; Ishibashi, Y.; Nishimura, O.; Fukuda, T. *J. Am. Chem. Soc.* **1994**, *116*, 5513; (c) Nishimura, O.; Moriya, T.; Suenaga, M.; Tanaka, Y.; Itoh, T.; Koyama, N.; Fujii, R.; Hinuma, S.; Kitada, C.; Fujino, M. *Chem. Pharm. Bull.* **1998**, *46*, 1490; (d) Yang, Y.; Wu, J.; Watson, J. T. *J. Am. Chem. Soc.* **1998**, *120*, 5834; (e) Suenaga, M.; Itoh, T.; Miwa, M.; Koyama, N.; Hinuma, S.; Kitada, C.; Nishimura, O.; Fujino, M. *J. Chem. Soc., Perkin Trans. 1* **2000**, 1183; (f) Itoh, T.; Miwa, M.; Suenaga, M.; Ohtaki, T.; Kitada, C.; Nishimura, O.; Fujino, M. *J. Chem. Soc., Perkin Trans. 1* **2000**, 1333; (g) Nishimura, O.; Suenaga, M.; Yamada, T.; Itoh, T.; Koyama, N.; Wakimasu, M.; Ohtaki, T.; Kitada, C.; Fujino, M. *J. Chem. Soc., Perkin Trans. 1* **2001**, 1748; (h) Qi, J.; Wu, J.; Somkuti, G. A.; Watson, J. T. *Biochemistry* **2001**, *40*, 4531.
6. (a) Wu, J.; Watson, J. T. *Anal. Biochem.* **1998**, *258*, 268; (b) Tang, H. Y.; Speicher, D. W. *Anal. Biochem.* **2004**, *334*, 48.
7. Hayashi, Y.; Takayama, K.; Suehisa, Y.; Fujita, T.; Nguyen, J. T.; Futaki, S.; Yamamoto, A.; Kiso, Y. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5129.
8. Other side reactions including dimerization of the peptides by disulfide bond formation were also observed.
9. The cyclic imide formation with concomitant peptide bond cleavage in neutral and basic conditions was previously reported: (a) Geiger, T.; Clarke, S. *J. Biol. Chem.* **1987**, *262*, 785; (b) Patel, K.; Borchardt, R. T. *Pharm. Res.* **1990**, *7*, 787.
10. The ε-lactam formation accompanying amide bond cleavage was previously reported: (a) Takenawa, T.; Oda, Y.; Ishihama, Y.; Iwakura, M. *J. Biochem.* **1998**, *123*, 1137; (b) Ishihama, Y.; Ito, O.; Oda, Y.; Takenawa, T.; Iwakura, M. *Tetrahedron Lett.* **1999**, *40*, 3415.
11. A small amount of a β-elimination product was observed under the various cleavage conditions examined.
12. Otaka, A.; Nakamura, M.; Nameki, D.; Kodama, E.; Uchiyama, S.; Nakamura, S.; Nakano, H.; Tamamura, H.; Kobayashi, Y.; Matsuoka, M.; Fujii, N. *Angew. Chem., Int. Ed.* **2002**, *41*, 2937.
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.
16. The presence of a hydrolyzed ring-opening product indicates that the degradation presumably began with hydrolysis of the C-terminal aspartimide, Ref. 9.
17. (a) Kodama, E. I.; Kohgo, S.; Kitano, K.; Machida, H.; Gatanaga, H.; Shigeta, S.; Matsuoka, M.; Ohrui, H.; Mitsuya, H. *Antimicrob. Agents Chemother.* **2001**, *45*, 1539; (b) Maeda, Y.; Venzon, D. J.; Mitsuya, H. *J. Infect. Dis.* **1998**, *177*, 1207.

Bioorganic & Medicinal Chemistry 17 (2009) 7964–7970

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Bioorganic synthesis of a recombinant HIV-1 fusion inhibitor, SC35EK, with an N-terminal pyroglutamate capping group

Kazumi Kajiwara [a,b], Kentaro Watanabe [a], Rei Tokiwa [a,b], Tomoko Kurose [a,b], Hiroaki Ohno [a], Hiroko Tsutsumi [c], Yoji Hata [c], Kazuki Izumi [d], Eiichi Kodama [d], Masao Matsuoka [d], Shinya Oishi [a,*], Nobutaka Fujii [a,*]

[a] Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
[b] JST Innovation Plaza Kyoto, Japan Science and Technology Agency, Nishigyo-ku, Kyoto 615-8245, Japan
[c] Gekkeikan Research Institute, Gekkeikan Sake Company, Ltd, Fushimi-ku, Kyoto 612-8391, Japan
[d] Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

ARTICLE INFO

Article history:
Received 10 September 2009
Revised 7 October 2009
Accepted 8 October 2009
Available online 13 October 2009

Keywords:
Anti-HIV peptide
Fusion inhibitor
HIV
Pyroglutamate

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

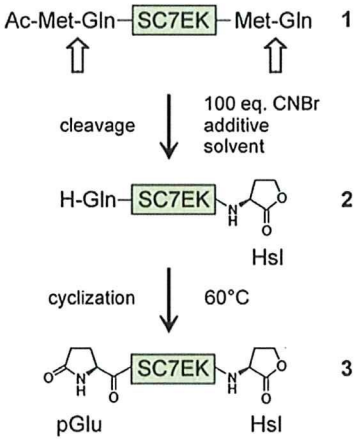
Human immunodeficiency virus type 1 (HIV-1) is an enveloped virus that causes acquired immunodeficiency syndrome (AIDS) through the infection of immune cells. A number of anti-HIV drugs that target key enzymes in HIV-1 life cycle, including reverse transcriptase and viral protease, have been employed 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 gp41[4] 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

* Corresponding authors. Tel.: +81 75 753 4551; fax: +81 75 753 4570.
E-mail addresses: soishi@pharm.kyoto-u.ac.jp (S. Oishi), nfujii@pharm.kyoto-u.ac.jp (N. Fujii).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.10.017



Scheme 1.

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 peptides[13] 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 Ni^{2+}-nitrilotriacetate (Ni-NTA)-agarose resin, and the expected proteins were eluted with either a standard imidazole buffer or an acidic solution con-

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	61.2
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.

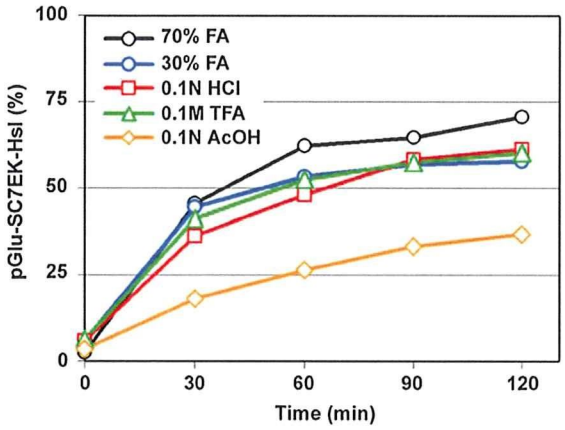


Figure 1. Time course of the cyclization process from Gln-SC7EK-Hsl to pGlu-SC7EK-Hsl. Cyclization of Gln to pGlu by heating the reaction at 60 °C under acidic conditions was monitored every 30 min for 2 h. The yields were calculated based on the combined peak areas at 220 nm of HPLC.

taining 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%,

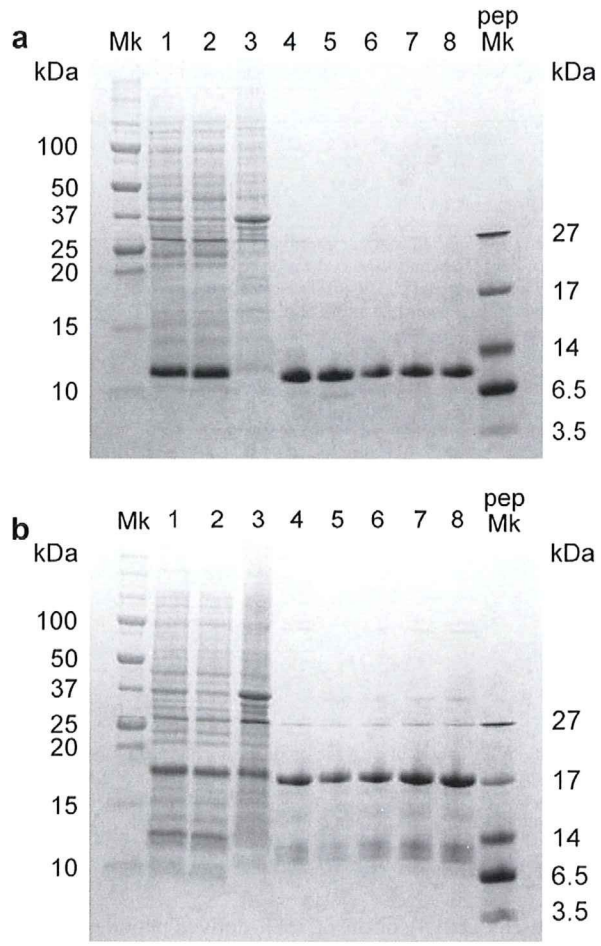


Figure 2. SDS–PAGE of recombinant proteins: (a) $(His)_6$-MQ-SC35EK-MQ **4** (7.0 kDa) and (b) $(His)_6$-M-(Q-SC35EK-M)$_3$-Q **5** (16.5 kDa). Lane Mk: molecular weight markers; lane 1: whole cell lysate; lane 2: supernatant of cell lysate; lane 3: precipitation of cell lysate; lane 4: pre-eluted resin; 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.

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(**7**) = 73.6 °C; T_m(**8**) = 75.8 °C; T_m(**9**) = 62.5 °C] (Fig. 5c and Table 3).

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[a] (mg)		Cyclization yield of **7** from **4** (%)[b,c]	Overall yield from 1 L culture of **4**[d] (mg)
		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.

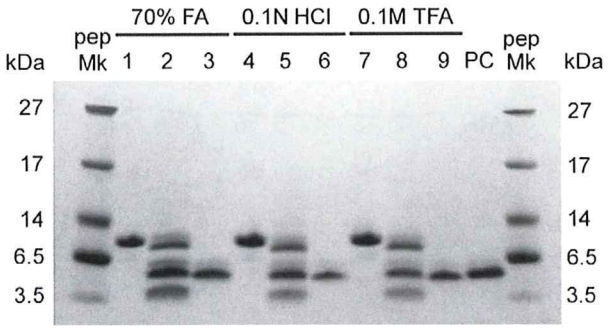


Figure 3. SDS-PAGE analysis of cleavage products and purified proteins. Lane Mk: polypeptide molecular weight marker; lanes 1, 4 and 7: $(His)_6$-MQ-SC35EK-MQ **4** (7.0 kDa); lanes 2, 5 and 8: after CNBr-mediated cleavage; lanes 3, 6 and 9: after HPLC purification; lane PC: chemically synthesized pGlu-SC35EK-Hsl **7** (control).

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}(**7**) = 0.57 nM; EC_{50}(**8**) = 0.50 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

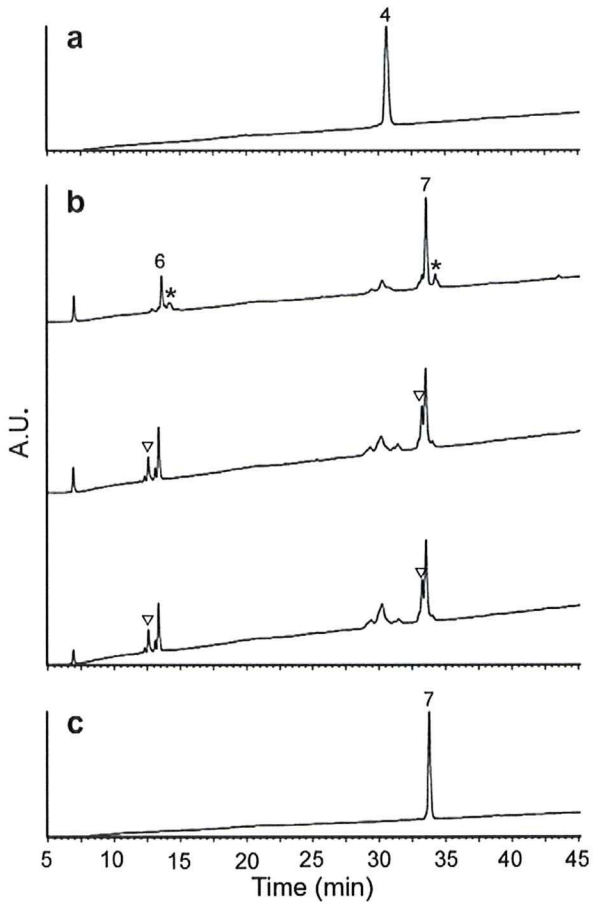
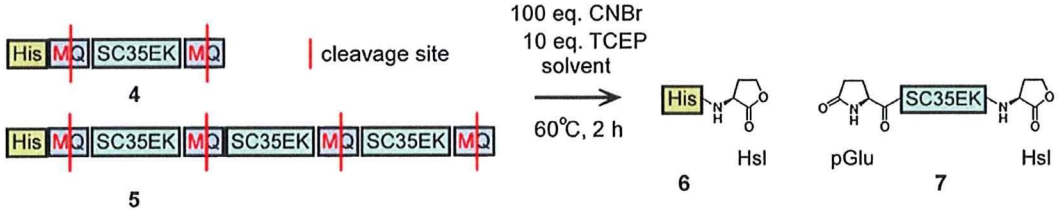


Figure 4. HPLC profiles of (a) $(His)_6$-MQ-SC35EK-MQ protein **4**; (b) the products of CNBr-mediated cleavage in (top) 70% FA, (middle) 0.1 N HCl, (bottom) 0.1 M TFA; (c) purified peptide **7**. Asterisk indicates the mono-formylated products of **6** and **7**. Inverted triangle indicates the ring-opened products at the Hsl of **6** and **7**, *HPLC conditions*: linear gradient 10–60% solvent B in solvent A over 50 min.



Scheme 2.

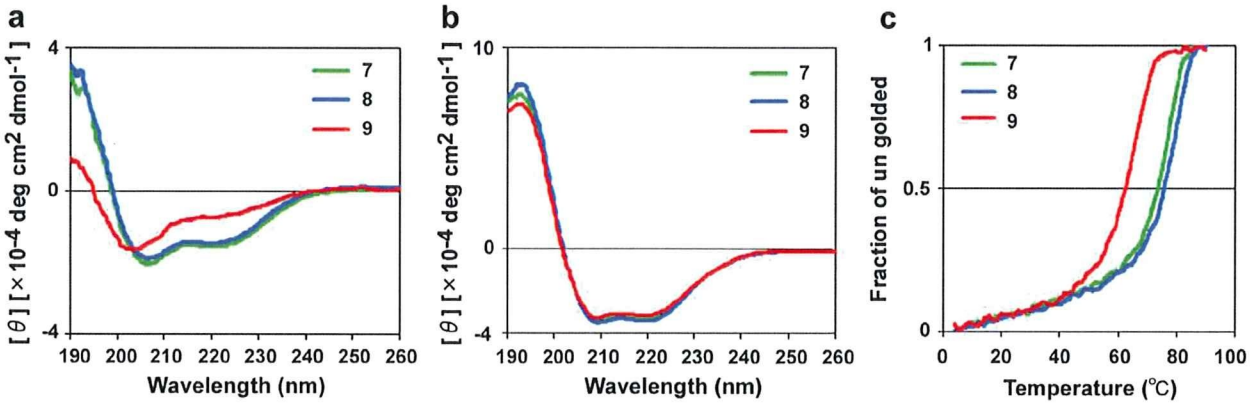
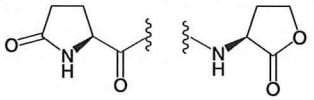


Figure 5. Secondary structure analysis using CD spectroscopy: CD spectra of (a) SC35EK-derived peptide; (b) SC35EK analog-N36 complex; and (c) thermostability of the SC35EK analog-N36 complex.

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

Peptide	R^1	R^2	EC_{50}[a] (nM)	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] EC_{50} was determined as the concentration that blocked HIV-1 infection by 50% in the MAGI assay.

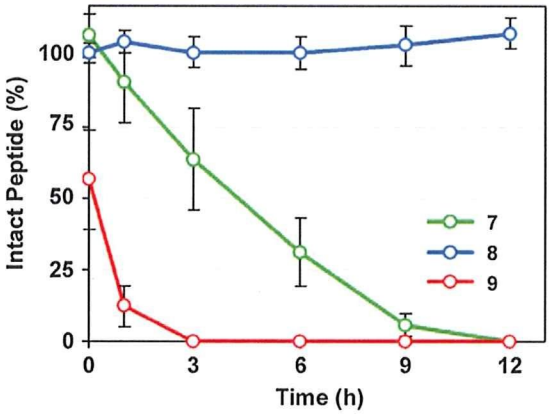


Figure 6. Degradation profile of peptides **7–9** by mouse serum. Each bar shows the mean ± SD (n = 5).

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 × 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. 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·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-ethandithiol (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. 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′-ctc**CATATG**CAGTGGGAAGAATGGGATAAAAAAATTGAAGA ATATACCAAAAAAATTGAAGAACTGATTAAAAAATCGGAAGAACAGCA AAAAAAAAATGAAGAAGAACTGAAAAAAATGCAGTAA**CTCGAG**cgtt-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′-ctc**GGATCCCATATGCAG**TGGGAGGAATGGGA TAAAAAAATCGAAGAATATACTAAGAAAATTGAAGAACTCATCAAGAA ATCCGAAGAACAACAGAAGAAAAACGAAGAGGAACTGAAAAAAATGC AATGGGAAGAGTGGGACAAAAAGATCGAAGAGTATACCAAAAAAATC GAAGAGTTGATTAAAAAGAGCGAAGAGCAGCAGAAAAAGAATGAAGA AGAGTTAAAAAAGATGCAGTGGGAAGAATGGGACAAGAAAATTGAGG AATACACTAAAAAGATCGAGGAACTGATTAAAAAATCTGAGGAACAGC AGAAAAAAAATGAGGAAGAATTGAAGAAAATGCAATAA**CTCGAG**cgtt-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 OD_{600} 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-dried. 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 [θ] 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; 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. 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 [EC_{50}]).

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 CH_3CN. 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.

Acknowledgements

This work was supported by Science and Technology Incubation Program in Advanced Regions from Japan Science and Technology Agency, and Health and Labour Sciences Research Grants (Research on HIV/AIDS).

Supplementary data

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

References and notes

1. Richman, D. D.; Morton, S. C.; Wrin, T.; Hellmann, N.; Berry, S.; Shapiro, M. F.; Bozzette, S. A. *AIDS* **2004**, *18*, 1393.
2. For a review, see: Flexner, C. *Nat. Rev. Drug Disc.* **2007**, *6*, 959.
3. Chan, D. C.; Kim, P. S. *Cell* **1998**, *93*, 981.
4. Eckert, D. M.; Kim, P. S. *Annu. Rev. Biochem.* **2001**, *70*, 777.
5. (a) Wild, C.; Oas, T.; McDanal, C.; Bolognesi, D.; Matthews, T. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10537; (b) Wild, C.; Greenwell, T.; Matthews, T. *AIDS Res.*

Hum. Retroviruses **1993**, *9*, 1051; (c) Wild, C. T.; Shugars, D. C.; Greenwell, T. K.; McDanal, C. B.; Matthews, T. J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9770.
6. For a review, see: Matthews, T.; Salgo, M.; Greenberg, M.; Chung, J.; DeMasi, R.; Bolognesi, D. *Nat. Rev. Drug Disc.* **2004**, *3*, 215.
7. (a) Izumi, K.; Kodama, E.; Shimura, K.; Sakagami, Y.; Watanabe, K.; Ito, S.; Watabe, T.; Terakawa, Y.; Nishikawa, H.; Sarafianos, S. G.; Kitaura, K.; Oishi, S.; Fujii, N.; Matsuoka, M. *J. Biol. Chem.* **2009**, *284*, 4914; (b) Watabe, T.; Terakawa, Y.; Watanabe, K.; Ohno, H.; Nakano, H.; Nakatsu, T.; Kato, H.; Izumi, K.; Kodama, E.; Matsuoka, M.; Kitaura, K.; Oishi, S.; Fujii, N. *J. Mol. Biol.* **2009**, *392*, 657.
8. (a) Otaka, A.; Nakamura, M.; Nameki, D.; Kodama, E.; Uchiyama, S.; Nakamura, S.; Nakano, H.; Tamamura, H.; Kobayashi, Y.; Matsuoka, M.; Fujii, N. *Angew. Chem., Int. Ed.* **2002**, *41*, 2937; (b) Oishi, S.; Ito, S.; Nishikawa, H.; Watanabe, K.; Tanaka, M.; Ohno, H.; Izumi, K.; Sakagami, Y.; Kodama, E.; Matsuoka, M.; Fujii, N. *J. Med. Chem.* **2008**, *51*, 388; (c) Nishikawa, H.; Oishi, S.; Fujita, M.; Watanabe, K.; Tokiwa, R.; Ohno, H.; Kodama, E.; Izumi, K.; Kajiwara, K.; Naitoh, T.; Matsuoka, M.; Otaka, A.; Fujii, N. *Bioorg. Med. Chem.* **2008**, *16*, 9184; (d) Nishikawa, H.; Nakamura, S.; Kodama, E.; Ito, S.; Kajiwara, K.; Izumi, K.; Sakagami, Y.; Oishi, S.; Ohkubo, T.; Kobayashi, Y.; Otaka, A.; Fujii, N.; Matsuoka, M. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 891; (e) Naito, T.; Izumi, K.; Kodama, E.; Sakagami, Y.; Kajiwara, K.; Nishikawa, H.; Watanabe, K.; Sarafianos, S. G.; Oishi, S.; Fujii, N.; Matsuoka, M. *Antimicrob. Agents Chemother.* **2009**, *53*, 1013.
9. Bray, B. L. *Nat. Rev. Drug Disc.* **2003**, *2*, 587.
10. Dingermann, T. *Biotechnol. J.* **2008**, *3*, 90.
11. (a) Rao, X. C.; Li, S.; Hu, J. C.; Jin, X. L.; Hu, X. M.; Huang, J. J.; Chen, Z. J.; Zhu, J. M.; Hu, F. Q. *Protein Expr. Purif.* **2004**, *36*, 11; (b) Park, T. J.; Kim, J. S.; Choi, S. S.; Kim, Y. *Protein Expr. Purif.* **2009**, *65*, 23; (c) Zorko, M.; Japelj, B.; Hafner-Bratkovic, I.; Jerala, R. *Biochim. Biophys. Acta* **2009**, *1788*, 314.
12. Zhao, D. X.; Ding, Z. C.; Liu, Y. Q.; Huang, Z. X. *Protein Expr. Purif.* **2007**, *53*, 232.
13. Chang, S. G.; Kim, D. Y.; Choi, K. D.; Shin, J. M.; Shin, H. C. *Biochem. J.* **1998**, *329*, 631.
14. Riley, J. M.; Aggeli, A.; Koopmans, R. J.; McPherson, M. J. *Biotechnol. Bioeng.* **2009**, *103*, 241.
15. (a) Gross, E.; Witkop, B. *J. Biol. Chem.* **1962**, *237*, 1856; (b) Kaiser, R.; Metzka, L. *Anal. Biochem.* **1999**, *266*, 1.
16. Abraham, G. N.; Podell, D. N. *Mol. Cell. Biochem.* **1981**, *38*, 181.
17. (a) Schilling, S.; Hoffmann, T.; Rosche, F.; Manhart, S.; Wasternack, C.; Demuth, H. U. *Biochemistry* **2002**, *41*, 10849; (b) Fernández, G. A.; Butz, P.; Trierweiler, B.; Zöller, H.; Stärke, J.; Pfaff, E.; Tauscher, B. *J. Agric. Food Chem.* **2003**, *51*, 8093; (c) Chelius, D.; Jing, K.; Lueras, A.; Rehder, D. S.; Dillon, T. M.; Vizel, A.; Rajan, R. S.; Li, T.; Treuheit, M. J.; Bondarenko, P. V. *Anal. Chem.* **2006**, *78*, 2370.
18. Recently, we have reported the preparation of HIV fusion inhibitor SC34EK by an alternative cleavage reaction using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) for the Cys residues: Tanaka, M.; Kajiwara, K.; Tokiwa, R.; Watanabe, K.; Ohno, H.; Tsutsumi, H.; Hata, Y.; Izumi, K.; Kodama, E.; Matsuoka, M.; Oishi, S.; Fujii, N. *Bioorg. Med. Chem.* **2009**, *17*, 7487.
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.
21. (a) Maeda, Y.; Venzon, D. J.; Mitsuya, H. *J. Infect. Dis.* **1998**, *177*, 1207; (b) Kodama, E. I.; Kohgo, S.; Kitano, K.; Machida, H.; Gatanaga, H.; Shigeta, S.; Matsuoka, M.; Ohrui, H.; Mitsuya, H. *Antimicrob. Agents Chemother.* **2001**, *45*, 1539.

Synthesis and biological evaluation of selective CXCR4 antagonists containing alkene dipeptide isosteres†

Tetsuo Narumi,[a,b] Ryoko Hayashi,[a] Kenji Tomita,[a] Kazuya Kobayashi,[a] Noriko Tanahara,[a] Hiroaki Ohno,[a] Takeshi Naito,[a] Eiichi Kodama,[c] Masao Matsuoka,[c] Shinya Oishi*[a] and Nobutaka Fujii*[a]

Received 21st August 2009, Accepted 31st October 2009
First published as an Advance Article on the web 4th December 2009
DOI: 10.1039/b917236j

A set of cyclic peptide analogues of a selective CXCR4 antagonist FC131 [*cyclo*(-D-Tyr-Arg-Arg-Nal-Gly-)] were synthesized and bioevaluated. Using (*E*)-alkene and (*Z*)-fluoroalkene dipeptide isosteres for Arg-Arg and Arg-Nal substructures, indispensable or the partial contribution of the two peptide bonds to the CXCR4 antagonism and anti-HIV activity was demonstrated. FC131 and the analogues were shown to selectively inhibit SDF-1 binding to CXCR4, whereas no inhibition of binding of SDF-1 to CXCR7 was observed.

Introduction

Chemokine receptor CXCR4 belongs to the G-protein coupled receptor family[1] and plays important roles in physiological functions including angiogenesis,[2] chemotaxis,[3] and neurogenesis.[4] CXCR4 is associated with various pathological conditions including cancer metastasis,[5] HIV-1 infection[6] and rheumatoid arthritis.[7] The broad spectrum of biological activities has led to extensive research towards the development of specific inhibitors directed against CXCR4.[8,9]

We have previously identified a highly potent CXCR4 antagonist, T140 **1**, which is a β-sheet-like 14-mer peptide with a single disulfide bridge (Fig. 1).[10] The indispensable residues for bioactivity are four amino acids positioned across the disulfide bridge: Arg2, L-3-(2-naphthyl)alanine3 (Nal3), Tyr5 and Arg14. These residues were used for further molecular-size reductions. Using these critical residues for a characteristic combination of cyclic pentapeptide libraries, a potent CXCR4 antagonist FC131 **2** was identified, which exerts comparable anti-HIV activity to T140.[11]

Structure–activity relationship (SAR) studies of FC131 by various modifications such as amino acid substitution,[12] tuning of the ring structure,[13] and backbone modifications,[14,15] demonstrated that the potent bioactivity of FC131 is attributed to the ideal spatial dispositions of the side-chain functional groups. For example, *N*-methylation of the peptide bonds of FC131 and the epimeric congeners significantly altered the bioactivity.[14] The appropriate combination of sequence, chirality and auxiliary groups on the cyclic pentapeptide backbone can accommodate the bioactive conformations.

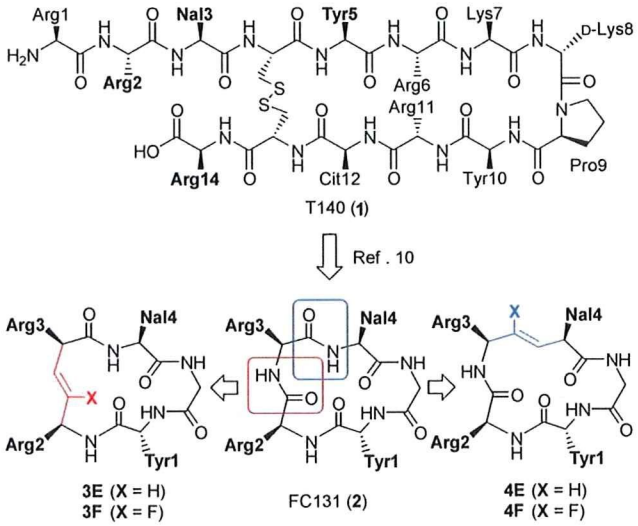


Fig. 1 Structures of T140 (**1**), FC131 (**2**), and the (*E*)-alkene and (*Z*)-fluoroalkene FC131 analogues. Bold residues of **1** are indispensable for the potent CXCR4-antagonistic activity. Nal = L-3-(2-naphthyl)alanine.

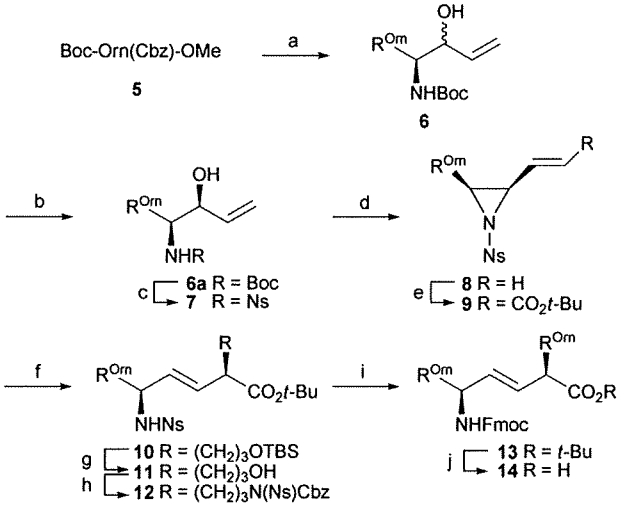
Replacement of the planar amide bond with a surrogate alkene substructure, including unsubstituted,[15,16] fluorinated,[17] multi-substituted,[18] and trifluoromethylated[19] alkenes, represents a promising approach to probe structural and electrostatic requirements in bioactive peptides. In particular, fluorinated or substituted alkene isosteres are considered to be more appropriate peptide bond mimetics when compared with unsubstituted alkene isosteres because of the favorable electrostatic and steric properties.[20] In this study, the contributions of the Arg2-Arg3 and Arg3-Nal4 peptide bonds to the bioactivity of FC131 were investigated through the synthesis and bioevaluation of alkene analogues of FC131, *cyclo*[(-D-Tyr-Arg-ψ[*trans*-CX=CH]-Arg-Nal-Gly-)] **3E/3F** and *cyclo*[(-D-Tyr-Arg-Arg-ψ[(*trans*-CX=CH]-Nal-Gly-)] **4E/4F** (X = H or F). The comparative study using unsubstituted and fluorinated isosteres aimed to reveal the electrostatic contributions of the amide carbonyl groups of these peptide bonds to the bioactivity of FC131.

[a]*Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. E-mail: soishi@pharm.kyoto-u.ac.jp, nfujii@pharm.kyoto-u.ac.jp; Fax: +81-75-753-4570; Tel: +81-75-753-4551*
[b]*Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan*
[c]*Laboratory of Virus Control, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan*
† Electronic supplementary information (ESI) available: Additional experimental procedures, NMR spectra and HPLC charts. See DOI: 10.1039/b917236j

Results and discussion

Synthesis of alkene dipeptide isosteres and the application to FC131 analogues

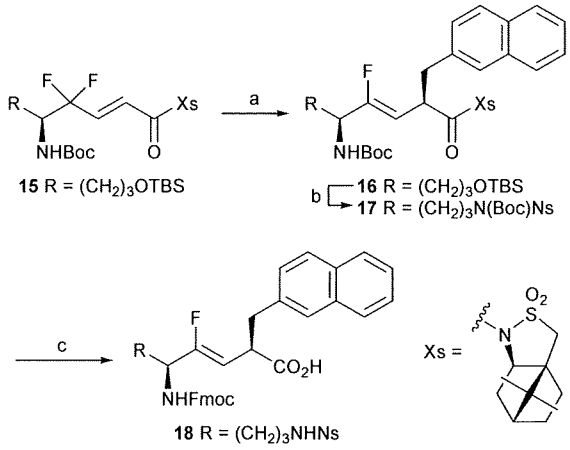
In our previous synthesis of the Arg-Nal type (*E*)-alkene dipeptide isostere (EADI),[15] a protected arginine was employed as the starting material. However, the derivatives were not experimentally tractable in the same synthetic process due to the presence of the protected guanidino group. Consequently, the synthesis of FC131 analogue **3E** bearing Arg-Arg type EADI began with Boc-Orn(Cbz)-OMe **5** (Orn = L-ornithine, Scheme 1). Ornithine includes a 3-aminoprop-1-yl group that can be used as a precursor of the arginine side-chain. Successive treatment of the ester **5** with diisobutylaluminium hydride (DIBAL-H) and vinylzinc chloride, gave a *syn* and *anti*-mixture of allylic alcohols **6** (*syn*:*anti* = 87:13). The *syn*-isomer **6a** was obtained by recrystallization. Boc cleavage of **6a** with TFA followed by *N*-2-nitrobenzenesulfonyl (Ns) protection produced a Ns-amide **7**. The intramolecular Mitsunobu reaction of **7** proceeded to provide 2,3-*cis*-aziridine **8** in high yield. Ozonolysis of **8** and the subsequent Horner–Wadsworth–Emmons reaction predominantly afforded the (*E*)-isomer of β-aziridinyl-α,β-enoate **9** in 57% yield. Organocopper-mediated *anti*-S_N2' type alkylation of **9** gave the α-alkylated product **10** with a TBS-protected 3-hydroxyprop-1-yl group, that can be modified to provide another Arg side-chain. Transformation to the Orn side-chain was performed by TBAF-mediated deprotection



Scheme 1 Synthesis of the Orn-Orn-type (*E*)-alkene dipeptide isostere. Reagents and conditions: (a) (i) Diisobutylaluminium hydride (DIBAL-H), CH_2Cl_2–toluene, –78 °C, 1 h; (ii) H_2C=CHMgCl, $ZnCl_2$, LiCl, –78 °C, 3 h (42%, *syn*:*anti* = 87:13); (b) recrystallization; (c) (i) TFA, CH_2Cl_2, 0 °C, 1 h; (ii) 2-nitrobenzenesulfonyl chloride (NsCl), Et_3N, CH_2Cl_2, rt, 1 h (74%); (d) diethyl azodicarboxylate (DEAD), PPh_3, THF, rt, 9 h (93%); (e) (i) O_3, EtOAc, –78 °C, then Me_2S; (ii) $(EtO)_2P(O)CH_2CO_2t$-Bu, LiCl, (*i*-Pr)$_2$NEt, MeCN, 0 °C, 4 h (57%); (f) $TBSO(CH_2)_3Li$, CuCN, LiCl, THF–Et_2O–*n*-pentane, –78 °C, 2 h (66%); (g) tetrabutylammonium fluoride (TBAF), THF, 0 °C, 14 h (85%); (h) CbzNHNs, DEAD, PPh_3, THF, 0 °C, 24 h (93%); (i) (i) PhSH, K_2CO_3, MeCN–DMSO, 50 °C, 2 h; (ii) *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu), Et_3N, THF–H_2O, 0 °C, 4 h (quant); (j) 4 N HCl–dioxane, rt, 8 h (65%).

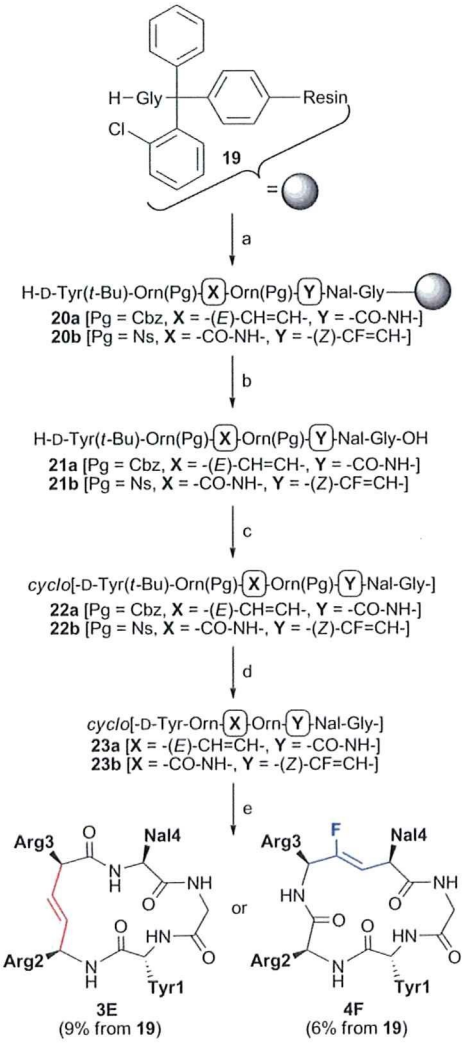
of **10** and the subsequent Mitsunobu reaction using CbzNHNs to give a bis(sulfonamide) **12**. The expected Fmoc-Orn(Cbz)-ψ[(*E*)-CH=CH]-Orn(Cbz)-OH **14** was obtained by sequential manipulation of the protecting groups including cleavage of two Ns groups in **12** and *N*-Fmoc protection and deprotection of the *t*-Bu ester.

Diastereoselective synthesis of (*Z*)-fluoroalkene dipeptide isosteres (FADI) has recently been accomplished.[17e] The key step in this synthesis is the one-pot reaction involving organocopper-mediated reduction/asymmetric alkylation *via* transmetalation to establish the α-alkylated isostere with appropriate configuration. According to the previous synthetic study of peptide **3F** bearing the Arg-Arg type FADI,[17i] the preparation of the Orn-Nal type FADI was carried out (Scheme 2). The one-pot reaction of γ,γ-difluoro-α,β-enoyl sultam **15**[17i] with 2-(bromomethyl)naphthalene yielded the corresponding α-alkylated sultam **16**. Cleavage of the TBS group with aqueous H_2SiF_6 followed by the Mitsunobu reaction with BocNHNs afforded the sulfonamide **17**. The sulfonamide **17** was converted to the Fmoc-protected FADI **18** by a standard deprotection/protection manipulation.



Scheme 2 Synthesis of the Orn-Nal-type (*Z*)-fluoroalkene dipeptide isostere. Reagents and conditions: (a) (i) Me_2CuLi·LiI·2LiBr, THF–Et_2O, –78 °C, 0.5 h; (ii) Hexamethylphosphoric triamide (HMPA), –78 °C, 0.5 h; (iii) Ph_3SnCl, THF, –40 °C, 10 min; (iv) 2-(bromomethyl)naphthalene, –40 °C, 20 h (79%); (b) (i) H_2SiF_6 aq. MeCN–MeOH, 0 °C, 1 h; (ii) BocNHNs, DEAD, PPh_3, THF, rt, 12 h (98%); (c) (i) 1 N LiOH, H_2O_2, THF–H_2O, rt, 2 h; (ii) TFA, CH_2Cl_2, rt, 0.5 h; (iii) Fmoc-OSu, Et_3N, DMF–H_2O–MeCN, rt, 12 h (85%).

The resulting isosteres **14** and **18** were incorporated into the peptide-chain by standard Fmoc-based solid-phase peptide synthesis (Scheme 3). Briefly, the protected peptides **21a,b** were cleaved off the resins **20a,b** with 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). After diphenylphosphoryl azide (DPPA)-mediated cyclization, the Cbz- or Ns-groups on the ornithine δ-amino group(s) of **22a,b** were deprotected by treatment with 1 M TMSBr/thioanisole in TFA or with 95% aqueous TFA followed by 2-mercaptoethanol/1,8-diazabicyclo[5,4,0]-7-undecene (DBU), respectively. Subsequently, the amino group(s) of **23a,b** were modified using 1*H*-pyrazole-1-carboxamidine to provide the expected peptidomimetics **3E** and **4F** with the Arg guanidino group(s).



Scheme 3 Synthesis of the alkene analogues of FC131. Reagents and conditions: (a) Fmoc-based SPPS; (b) 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), CH_2Cl_2; (c) diphenylphosphoryl azide (DPPA), $NaHCO_3$, DMF, –40 °C to rt; (d) **23a**: 1 M TMSBr/thioanisole in TFA, *m*-cresol, 1,2-ethandithiol, 6 h; **23b**: (i) TFA-H_2O, 3 h; (ii) 2-mercaptoethanol, 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), DMF, 50 °C, 2.5 h; (e) **3E**: 1*H*-pyrazole-1-carboxamidine·HCl, (*i*-Pr)$_2$NEt, DMF; **4F**: 1*H*-pyrazole-1-carboxamidine·HCl, Et_3N, DMF.

Biological evaluation of FC131 analogues with EADI and FADI

The biological activities of cyclic pseudopeptides **3E/3F**[17i] and **4E**[15]**/4F** were comparatively evaluated, in which the Arg2-Arg3 and Arg3-Nal4 dipeptide sites were substituted with EADI or FADI. The inhibitory potency against [^{125}I]-SDF-1-binding to CXCR4 or CXCR7 was measured (Table 1). Both EADI and FADI analogues (**3E** and **3F**) with substitution at the Arg2-Arg3 dipeptide moderately inhibited the SDF-1 binding to CXCR4 [IC_{50}(**3E**) = 1.46 μM; IC_{50}(**3F**) = 1.78 μM]. The potency was approximately 20-fold lower than the original FC131 **2** [IC_{50}(**2**) = 0.068 μM], indicating the partial contribution of the amide bond within the Arg2-Arg3 dipeptide to the bioactivity of FC131. This is consistent with the bioactivity of the FC131 analogue containing the Arg2-MeArg3 dipeptide substructure,[14] suggesting that the less potent activity may be attributed to the loss of the H-bonding amide hydrogen of Arg3 and/or the conformational change by the backbone modification. Comparison of the biological activities of the two analogues **3E** and **3F** showed that the unsubstituted alkene analogue **3E** was essentially equipotent in inhibiting the binding of SDF-1 to CXCR4 to the fluoroalkene analogue **3F**. This observation indicates that the presence of the fluorine atom did not aid the appropriate mimicry of the steric and electrostatic effects of the Arg2 carbonyl group.

Table 1 Inhibitory activity of FC131 and the derivatives against [^{125}I]-SDF-1 binding to CXCR4 and CXCR7

Peptide	IC_{50}/μM[c]	
	CXCR4	CXCR7
FC131 **2**	0.068	> 10
cyclo[(-D-Tyr-Arg-**Ψ**^{E}-Arg-Nal-Gly-)] **3E**[a]	1.46	> 10
cyclo[(-D-Tyr-Arg-**Ψ**^{F}-Arg-Nal-Gly-)] **3F**[b]	1.78	> 10
cyclo[(-D-Tyr-Arg-Arg-**Ψ**^{E}-Nal-Gly-)] **4E**[a]	> 10	> 10
cyclo[(-D-Tyr-Arg-Arg-**Ψ**^{F}-Nal-Gly-)] **4F**[b]	> 10	> 10

[a] The **Ψ**^{E} indicates the isosteric ψ[(*E*)-CH=CH] substructure. [b] The **Ψ**^{F} indicates the isosteric ψ[(*Z*)-CF=CH] substructure. [c] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 or CXCR7 transfectants of CHO-K1 cells.

Our previous studies on *N*-methylamino acid-scanning[14] and EADI replacement[15] (**4E**) revealed that the modification of Arg3-Nal4 peptide bond resulted in a significant loss of CXCR4-binding inhibition activity. This is possibly due to the absence of the amide hydrogen and/or the dissolution of the pseudo-1,3-allylic strain between the Arg3 carbonyl group and the Nal4 side chain. Although the possible mimicking ability of the fluorine atom was expected,[20] the introduction of the FADI into the Arg3-Nal4 dipeptide (**4F**) also led to the loss of CXCR4-binding activity again [IC_{50}(**4F**) > 10 μM]. This result indicates that the amide hydrogen within the Arg3-Nal4 dipeptide of FC131 may contribute to a critical interaction required for binding to CXCR4.

Furthermore, inhibitory activity of the peptides for CXCR7, which is also a target receptor of SDF-1, was also examined; however, no inhibition was observed even at 10 μM. This observation showed that FC131 and the related analogues are selective CXCR4 antagonists and show similar target specificity as the T140 derivatives.[21]

Anti-HIV activity based on the inhibition of HIV-1 entry into the target cells was examined by the MAGI assay using three strains including NL4-3, IIIB and Ba-L (Table 2). As in the case of CXCR4-binding inhibition, moderate anti-HIV activity against NL4-3 and IIIB strains was observed for peptides **3E/3F** containing EADI and FADI for the Arg2-Arg3 dipeptide

Table 2 Anti-HIV activities of FC131 and the derivatives

Peptide	EC_{50}/μM[a]		
	NL4-3	IIIB	Ba-L
2	0.014 ± 0.002	0.019 ± 0.003	> 10
3E	0.234 ± 0.004	0.295 ± 0.069	> 10
3F	0.332 ± 0.073	0.403 ± 0.051	> 10
4E	> 10	> 10	> 10
4F	> 10	> 10	> 10

[a] EC_{50} is the concentration that blocks HIV-1 infection by 50%.