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

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Supplementary data

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

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13. Only partial S-cyanylations of two Cys residues in thioredoxin were observed. This was also verified by the recovery of C-terminal capped thioredoxin **24a**,**b** after basic treatment in the next step.
14. Peptides **20a**,**b** from **22a**,**b** were identical to the authentic samples, which were obtained by the cleavage reaction of the synthetic peptides.
15. Cytotoxicity of peptides **20a**,**b** was not observed even at 10 μM in MAGI assay.
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Bioorganic synthesis of a recombinant HIV-1 fusion inhibitor, SC35EK, with an N-terminal pyroglutamate capping group

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ABSTRACT

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

1. Introduction

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

The fusion inhibitors are a new class of therapeutics for the treatment of HIV-1-infected patients. These drugs prevent viral entry into cells,[3] which is mediated by the conformational transition of the viral envelope protein 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

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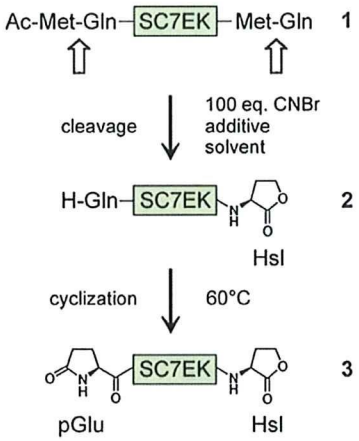


Scheme 1.

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.

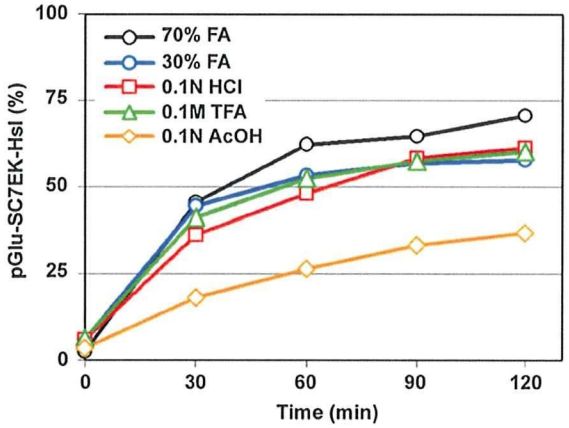


Figure 1. Time course of the cyclization process from Gln-SC7EK-Hsl to pGlu-SC7EK-Hsl. Cyclization of Gln to pGlu by heating the reaction at 60 °C under acidic conditions was monitored every 30 min for 2 h. The yields were calculated based on the combined peak areas at 220 nm of HPLC.

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%,

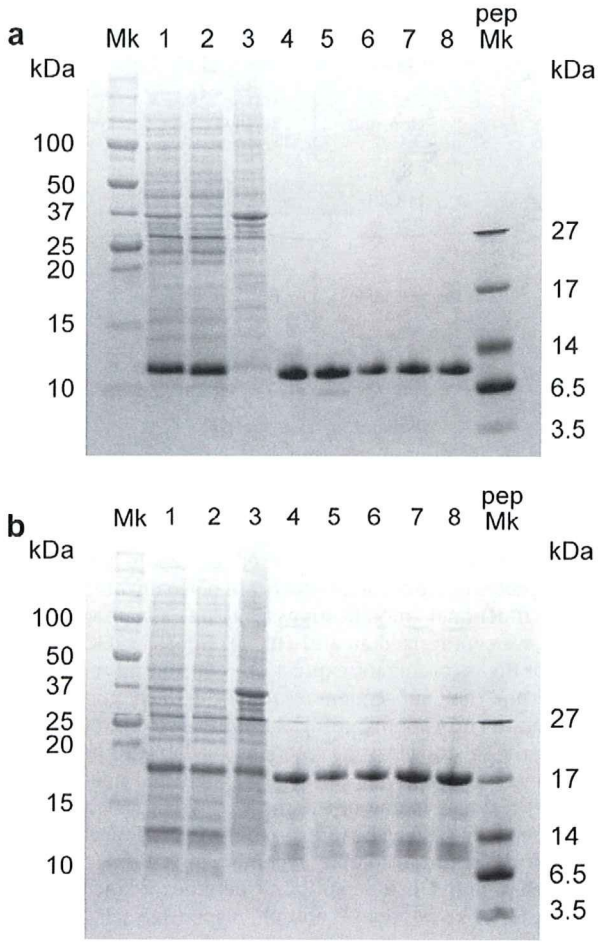


Figure 2. SDS–PAGE of recombinant proteins: (a) $(His)_6$-MQ-SC35EK-MQ **4** (7.0 kDa) and (b) $(His)_6$-M-(Q-SC35EK-M)$_3$-Q **5** (16.5 kDa). Lane Mk: molecular weight markers; lane 1: whole cell lysate; lane 2: supernatant of cell lysate; lane 3: precipitation of cell lysate; lane 4: pre-eluted resin;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

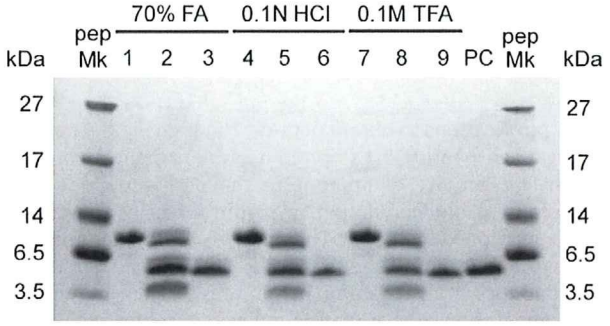


Figure 3. SDS-PAGE analysis of cleavage products and purified proteins. Lane Mk: polypeptide molecular weight marker; lanes 1, 4 and 7: $(His)_6$-MQ-SC35EK-MQ **4** (7.0 kDa); lanes 2, 5 and 8: after CNBr-mediated cleavage; lanes 3, 6 and 9: after HPLC purification; lane PC: chemically synthesized pGlu-SC35EK-Hsl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

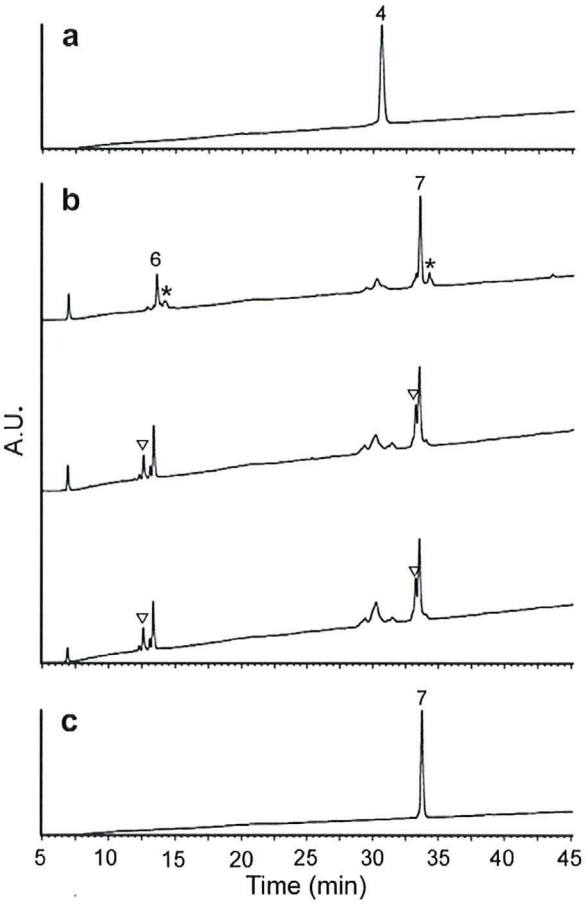
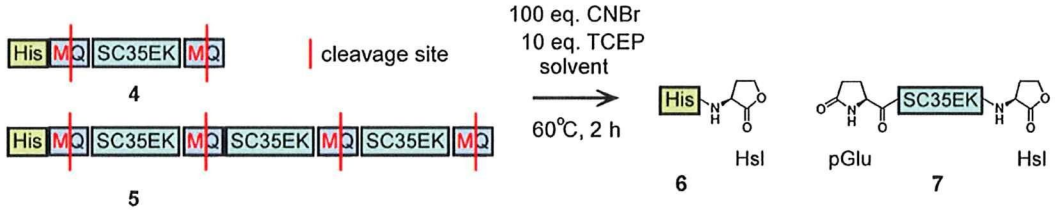


Figure 4. HPLC profiles of (a) $(His)_6$-MQ-SC35EK-MQ protein **4**; (b) the products of CNBr-mediated cleavage in (top) 70% FA, (middle) 0.1 N HCl, (bottom) 0.1 M TFA; (c) purified peptide **7**. Asterisk indicates the mono-formylated products of **6** and **7**. Inverted triangle indicates the ring-opened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



Scheme 2.

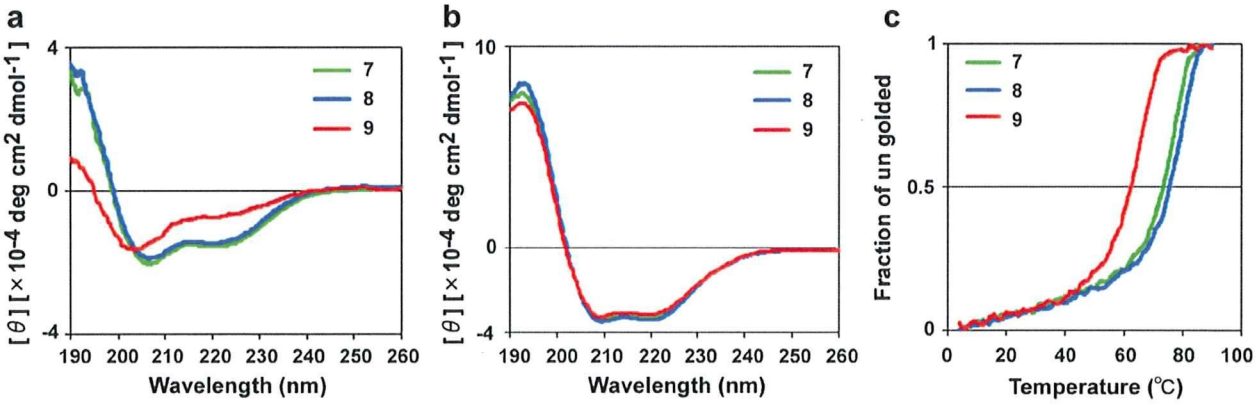
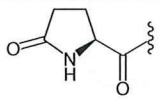
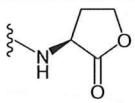


Figure 5. Secondary structure analysis using CD spectroscopy: CD spectra of (a) SC35EK-derived peptide; (b) SC35EK analog-N36 complex; and (c) thermostability of the SC35EK analog-N36 complex.

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.

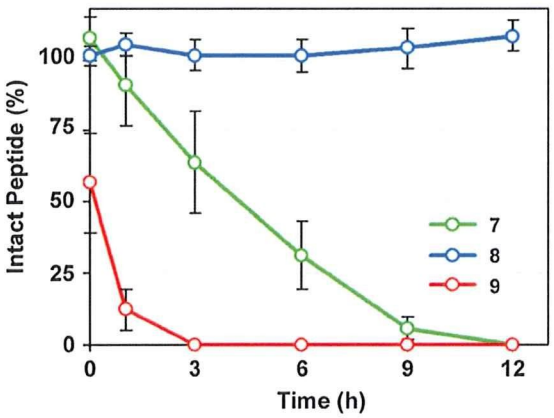


Figure 6. Degradation profile of peptides **7–9** by mouse serum. Each bar shows the mean ± SD (n = 5).

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**GGATCCCATATG**CAGTGGGAGGAATGGGA 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

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Supplementary data

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

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19. Non-end capped peptide **9** can be obtained by standard recombinant expression in prokaryotes. Peptide **9** for this experiment was obtained by the chemical synthesis.
20. The ring-opening C-terminal Hsl was verified by the observed +18 mass of the product, supporting the presence of Hsl in peptide **7**.[15] The ring-opened product may be degraded from the C-terminus by endopeptidases in serum.
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Synthesis and biological evaluation of selective CXCR4 antagonists containing alkene dipeptide isosteres†

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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.

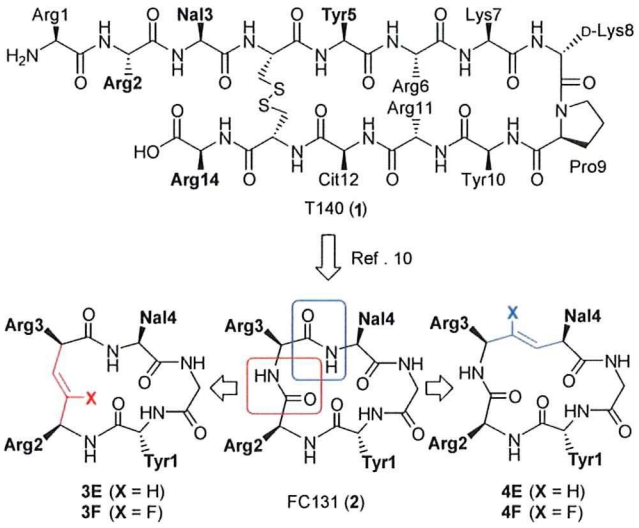

Fig. 1 Structures of T140 (**1**), FC131 (**2**), and the (*E*)-alkene and (*Z*)-fluoroalkene FC131 analogues. Bold residues of **1** are indispensable for the potent CXCR4-antagonistic activity. Nal = L-3-(2-naphthyl)alanine.

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.

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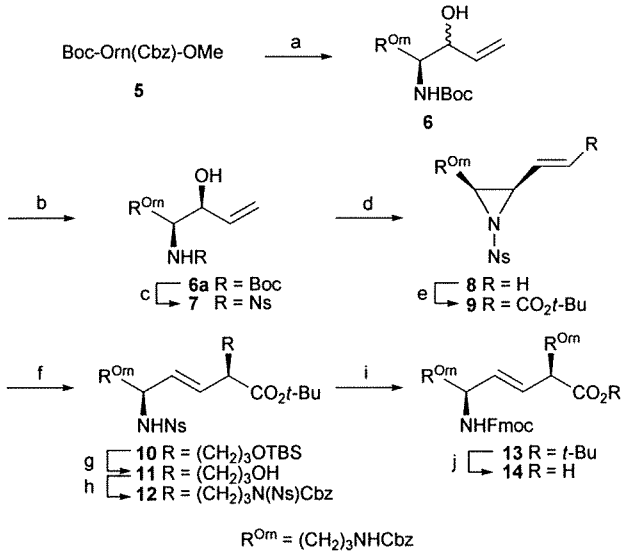
† Electronic supplementary information (ESI) available: Additional experimental procedures, NMR spectra and HPLC charts. See DOI: 10.1039/b917236j

Results and discussion

Synthesis of alkene dipeptide isosteres and the application to FC131 analogues

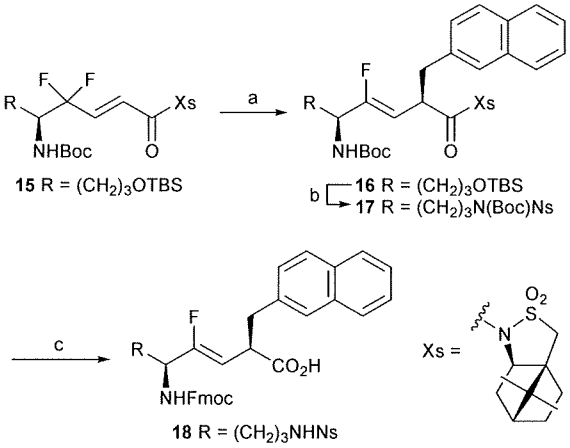
In our previous synthesis of the Arg-Nal type (*E*)-alkene dipeptide isostere (EADI),[15] a protected arginine was employed as the starting material. However, the derivatives were not experimentally tractable in the same synthetic process due to the presence of the protected guanidino group. Consequently, 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%).

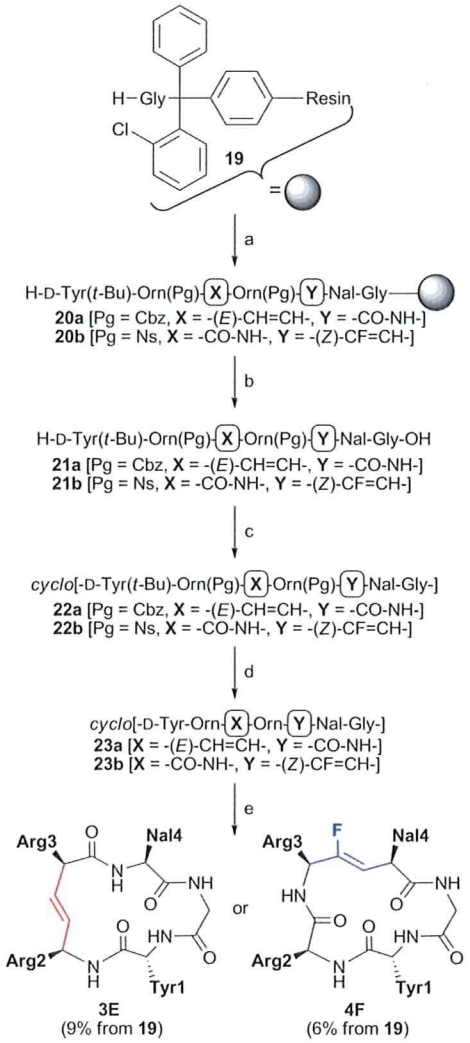
of **10** and the subsequent Mitsunobu reaction using CbzNHNs to give a bis(sulfonamide) **12**. The expected Fmoc-Orn(Cbz)-ψ[(*E*)-CH=CH]-Orn(Cbz)-OH **14** was obtained by sequential manipulation of the protecting groups including cleavage of two Ns groups in **12** and *N*-Fmoc protection and deprotection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

[IC_{50}(**3E**) = 0.234 μM (NL4-3) and 0.295 μM (IIIB); IC_{50}(**3F**) = 0.332 μM (NL4-3) and 0.403 μM (IIIB)]. The potency was significantly less compared with the original FC131 **2** [IC_{50}(**2**) = 0.014 μM (NL4-3) and 0.019 μM (IIIB)]. Substitutions of Arg3-Nal4 dipeptides with EADI and FADI resulted in the loss of the anti-HIV activity [IC_{50}(**4E**/**4F**) > 10 μM (NL4-3 and IIIB)], which also correlates with the observation of no CXCR4 antagonistic activity of these peptides. For the Ba-L strain, that utilizes CCR5 for entry, all peptides showed no inhibitory activity at 10 μM.

Conclusions

In conclusion, Orn-Orn type EADI **14** and Orn-Nal type FADI **18** were synthesized and incorporated into FC131 analogues. Comparative bioevaluation of a set of peptides containing EADI or FADI at Arg2-Arg3 and Arg3-Nal4 positions revealed the significant contribution of these peptide bonds to FC131 bioactivity. Although substitutions with alkene isosteres resulted in a decrease in bioactivity, the structural and functional requirements of the corresponding amide bonds to biological activity was shown. The results will be useful for the development of cyclic pentapeptide-based CXCR4 antagonists. Additionally, it was demonstrated that FC131 and the analogous were selective CXCR4 antagonists, which did not inhibit SDF-1 binding to CXCR7. Further studies on the synthesis and biological evaluation of CXCR4 antagonists with peptide bond mimetics are the subject of an ongoing investigation.

Experimental

Synthesis

***tert*-Butyl (2*R*,5*S*,3*E*)-8-[*N*-(benzyloxycarbonyl)amino]-2-[3-(*tert*-butyldimethylsiloxy)prop-1-yl]-5-[*N*-(*o*-nitrobenzenesulfonyl)amino]oct-3-enoate (10).** 1.57 M *t*-BuLi in *n*-pentane solution (28.7 cm^3, 45 mmol) was added dropwise to a stirred solution of $I(CH_2)_3OTBS$ (6.78 g, 22.5 mmol) in dry Et_2O (10.6 cm^3) under argon at −78 °C. Following stirring at −78 °C for 30 min, the mixture was stirred at room temperature for 10 min. To a stirred solution of CuCN (1.26 g, 14.1 mmol) and LiCl (1.19 g, 28.1 mmol) in dry THF (20 cm^3) under argon at −78 °C, the above 0.5 M $TBSO(CH_2)_3Li$ in THF–Et_2O–*n*-pentane solution (28.2 cm^3) was added dropwise, and the mixture was further stirred at 0 °C for 10 min. To the above mixture, a solution of the enoate **9** (1.92 g, 3.51 mmol) in dry THF (20 cm^3) was added dropwise at −78 °C, and the mixture was further stirred for 2 h at −78 °C. The reaction was quenched by the addition of a saturated NH_4Cl/28% NH_4OH solution (1/1, 30 cm^3), with additional stirring at room temperature for 1 h. After the mixture was concentrated under reduced pressure, the residue was extracted with Et_2O. The extract was washed with water and brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–*n*-hexane (1/5) gave the title compound **10** (1.68 g, 66%) as a colorless oil: $[\alpha]_D^{24}$ −89.8 (*c* 1.00, $CHCl_3$); δ_H (500 MHz, $CDCl_3$, Me_4Si) 0.00 (6 H, s), 0.85 (9 H, s), 1.22–1.26 (2 H, m), 1.34 (9 H, s), 1.46–1.51 (6 H, m), 2.59–2.64 (1 H, m), 3.12–3.14 (2 H, m), 3.45–3.48 (2 H, m), 3.89–3.93 (1 H, m), 4.79–4.87 (1 H, m), 5.04 (2 H, s), 5.22 (1 H, dd, *J* 15.5 and 7.4), 5.34 (1H, dd, *J* 15.5 and 8.6), 5.42 (1 H, d, *J* 8.0), 7.23–7.31 (5 H, m), 7.61–7.65 (2 H, m), 7.74–7.80 (1 H, m) and 7.99–8.06 (1 H, m); δ_C (125 MHz, $CDCl_3$, Me_4Si) −5.4 (2 C), 18.2, 25.9 (3 C), 26.0, 27.9 (3 C), 28.7, 29.9, 33.0, 40.3, 49.0, 56.5, 62.5, 66.5, 80.6, 125.2, 128.0 (3 C), 128.4 (2 C), 130.9, 131.2, 132.8, 133.2, 133.3, 134.8, 136.5, 147.7, 156.4 and 172.6; HRMS (FAB), *m*/*z* calcd for $C_{35}H_{52}N_3O_9SSi$ ($[M - H]^-$) 718.3199, found 718.3190.

(2*R*,5*S*,3*E*)-8-[*N*-(Benzyloxycarbonyl)amino]-2-{3-[*N*-(benzyloxycarbonyl) amino]prop-1-yl}-5-[*N*-(fluorenylmethoxycarbonyl)amino]oct-3-enoic acid (14). Compound **13** (610 mg, 0.790 mmol) was dissolved in 4 N HCl–dioxane (8 cm^3) and the mixture was stirred at room temperature for 8 h. After the mixture was concentrated under reduced pressure, the residue was extracted with EtOAc. The extract was washed with 1 N HCl and brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–*n*-hexane–AcOH (1/1/0.02) gave the title compound **14** (367 mg, 65%) as a white solid: mp 162–163 °C; $[\alpha]_D^{24}$ −16.6 (*c* 1.02, DMSO); δ_H (500 MHz, DMSO, Me_4Si) 1.38–1.40 (7 H, m), 1.55–1.66 (1 H, m), 2.87 (1 H, m), 2.97 (4 H, m), 3.93 (1 H, m), 4.17–4.24 (1 H, m), 4.24–4.31 (1 H, m), 4.96–5.03 (5 H, m), 5.47 (2 H, m), 7.28–7.41 (17 H, m), 7.65–7.69 (2 H, m), 7.86–7.88 (2 H, m) and 12.20 (1 H, s); δ_C (125 MHz, DMSO, Me_4Si) 26.1, 27.0, 29.2, 30.9, 40.0 (2 C), 46.7, 47.8, 51.9, 65.1, 65.2 (2 C), 120.0 (2 C), 125.2 (2 C), 127.0 (2 C), 127.5 (2 C), 127.6 (3 C), 127.7 (3 C), 128.3 (4 C), 133.2, 137.2 (2 C), 140.7, 143.8 (2 C), 143.9 (2 C), 156.1 (3 C) and 174.8; HRMS (FAB), *m*/*z* calcd for $C_{42}H_{44}N_3O_8$ ($[M - H]^-$) 718.3134, found 718.3125.

(2*R*,5*S*,3*Z*)-5-[(*tert*-Butoxycarbonyl)amino]-8-(*tert*-butyldimethylsiloxy)-4-fluoro-2-(naphthalen-2-ylmethyl)oct-3-enoyl (*S*)-sultam (16). To a suspension of CuI (2.22 g, 11.6 mmol) in THF (250 cm^3) at −78 °C under argon was added dropwise a solution of MeLi·LiBr complex in Et_2O (1.5 M, 15.5 cm^3, 23.2 mmol), and the mixture was stirred for 10 min at 0 °C. To the solution of the above organocopper reagent at −78 °C was added dropwise a solution of the *N*-enoyl sultam **15** (1.80 g, 2.90 mmol) in THF (70 cm^3). The mixture was stirred for 30 min at −78 °C and HMPA (8.31 cm^3, 46.4 mmol) was added dropwise to the mixture. After stirring for 30 min at −78 °C, a solution of triphenyltin chloride (2.24 g, 5.80 mmol) in THF (20 cm^3) was added dropwise, and the mixture was subsequently stirred for 10 min at −40 °C. 2-(Bromomethyl)naphthalene (5.13 g, 23.2 mmol) in THF (30 cm^3) was added dropwise and the mixture was stirred for 20 h at −40 °C. The reaction was quenched at −40 °C by the addition of a saturated NH_4Cl/28% NH_4OH solution (1/1, 50 cm^3) and the mixture was stirred at room temperature for an additional 30 min. The mixture was extracted with Et_2O and the extract was washed with brine and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–*n*-hexane (1/3) gave the title compound **16** (1.71 g, 79%) as a colorless oil: $[\alpha]_D^{24}$ −74.3 (*c* 1.00, $CHCl_3$); δ_H (500 MHz, $CDCl_3$, Me_4Si) 0.02 (6 H, s), 0.30 (3 H, s), 0.76 (3 H, s), 0.88 (9 H, s), 1.18–1.30 (2 H, m), 1.38–1.48 (11 H, m), 1.52–1.66 (4 H, m), 1.70–1.82 (2 H, m), 1.91 (1 H, dd, *J* 13.7 and 8.0), 2.97 (1 H, dd, *J* 13.7 and 6.9), 3.24–3.36 (3 H, m), 3.46–3.56 (2 H, m), 3.64–3.79 (1 H, m), 4.08–4.21 (1 H, m), 4.48–4.60 (1 H, m), 4.67 (1 H, d, *J* 8.6), 5.06 (1 H, dd, *J* 36.1 and 9.2), 7.38–7.44 (3 H, m), 7.64 (1 H, s) and 7.71–7.78 (3 H, m); δ_C (125 MHz, $CDCl_3$, Me_4Si) −5.3

(2 C), 18.3, 19.6, 19.8, 26.0 (3 C), 26.3, 28.3 (3 C), 28.6, 28.7, 32.8, 38.2, 40.6 (d, *J* 2.4), 43.0, 44.5, 47.3, 48.0, 51.7, 52.9, 62.5, 64.9, 79.6, 103.7 (d, *J* 13.1), 125.3, 125.7, 127.5, 127.6, 127.8, 127.9, 127.9, 132.4, 133.4, 135.1, 154.9, 158.8 (d, *J* 261.1) and 172.2; δ_F (125 MHz, $CDCl_3$, $CFCl_3$) −119.5; HRMS (FAB), *m/z* calcd for $C_{40}H_{58}FN_2O_6SSi$ ([M − H]$^-$) 741.3774, found: 741.3768.

(2*R*,5*S*,3*Z*)-5-[*N*-(Fluorenylmethoxycarbonyl)amino]-4-fluoro-2-(naphthalen-2-ylmethyl)-8-[*N*-(*o*-nitrobenzenesulfonyl)amino]oct-3-enoic acid (18). To a solution of the sultam **17** (986 mg, 1.08 mmol) and aqueous 50% H_2O_2 (0.383 cm^3, 5.62 mmol) in THF–H_2O (5/1, 15 cm^3) at 0 °C was added aqueous 1 N LiOH (2.16 cm^3, 2.16 mmol). The mixture was stirred at room temperature for 2 h. Following dilution with EtOAc (50 cm^3), the mixture was washed with 0.1 N HCl and dried over $MgSO_4$. Concentration under reduced pressure gave the corresponding acid, which was used in the next step without purification. TFA (5 cm^3) was added to a solution of the acid in CH_2Cl_2 (5 cm^3) at 0 °C, and the mixture was stirred at room temperature for 30 min. Concentration under reduced pressure gave an oily residue, which was dissolved in MeCN–DMF–H_2O (10/9/1, 40 cm^3). Fmoc-OSu (584 mg, 1.73 mmol) and Et_3N (0.332 cm^3, 2.38 mmol) were added to the mixture at 0 °C and the mixture was stirred at room temperature for 12 h. After being diluted with EtOAc (280 cm^3), the reaction mixture was washed with 1 N HCl and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–*n*-hexane–AcOH (1/1/0.02) gave the title compound **18** (673 mg, 85%) as a colorless semisolid: $[\alpha]_D^{25}$ −27.4 (*c* 1.00, $CHCl_3$); δ_H (500 MHz, $CDCl_3$, Me_4Si) 1.31–1.40 (2 H, m), 1.41–1.55 (2 H, m), 2.93–2.99 (3 H, m), 3.28 (1 H, dd, *J* 13.7 and 6.3), 3.78–3.87 (1 H, m), 4.08–4.16 (2 H, m), 4.28 (1 H, dd, *J* 10.3 and 6.9), 4.40 (1 H, dd, *J* 10.3 and 6.9 Hz), 4.81 (1 H, d, *J* 9.2), 4.93 (1 H, dd, *J* 36.1 and 9.7), 5.38 (1 H, t, *J* 5.7), 7.26–7.79 (18 H, m) and 8.02–8.07 (1 H, m); δ_C (125 MHz, $CDCl_3$, Me_4Si) 25.6, 28.9, 38.4, 42.9, 47.1, 51.6 (d, *J* 27.6), 62.3, 66.7, 104.7 (d, *J* 14.4), 120.0 (2 C), 124.4, 124.9, 125.0 (2 C), 125.2 (2 C), 125.5 (2 C), 125.9, 127.1, 127.5 (2 C), 127.7, 127.8, 130.9, 132.2, 132.7, 133.3, 133.5, 133.5, 135.6, 141.3 (2 C), 143.7, 143.8, 147.9, 158.0 (d, *J* 262.0), 163.0 and 177.0; δ_F (125 MHz, $CDCl_3$, $CFCl_3$) −120.8; HRMS (FAB), *m/z* calcd for $C_{40}H_{35}FN_3O_8S$ ([M − H]$^-$) 736.2134, found: 736.2137.

Peptide synthesis

The protected linear peptides **20a,b** were constructed on H–Gly–(2-Cl)Trt resin (0.8 mmol g^{-1}, 38 mg, 0.03 mmol). *t*-Bu was employed for Tyr side-chain protection. Fmoc-protected amino acids (0.3 mmol) were coupled by using DIC (0.046 cm^3, 0.3 mmol) and HOBt·H_2O (46 mg, 0.3 mmol) in DMF. Coupling of EADI **14** (33mg, 0.045 mmol) was carried out with HOAt (6.3 mg, 0.045 mmol), HATU (17 mg, 0.045 mmol) and (*i*-Pr)$_2$NEt (0.009 cm^3, 0.045 mmol). Completion of each coupling reaction was ascertained using the Kaiser ninhydrin test. The Fmoc-protecting group was removed by treating the resin with a DMF/piperidine solution (80/20, v/v).

***cyclo*(−D−Tyr−Arg−Ψ[(*E*)-CH=CH]−Arg−Nal−Gly−)·2TFA (3E).** The obtained resin **20a** was treated with HFIP/CH_2Cl_2 (2/8, 15 cm^3) at room temperature for 2 h. After removal of the resin by filtration, the filtrate solution was concentrated under reduced pressure to give a crude protected peptide **21a**. To a mixture of **21a** and $NaHCO_3$ (21 mg, 0.25 mmol) in DMF (20 cm^3) was added DPPA (0.0270 cm^3, 0.13 mmol) at −40 °C. The mixture was stirred for 66 h with warming to room temperature and then filtered. The filtrate was concentrated under reduced pressure to give the protected cyclic peptide **22a**. The peptide **22a** was treated with 1 M TMSBr/thioanisole in TFA (10 cm^3) in the presence of *m*-cresol and 1,2-ethandithiol (0.117 cm^3) for 6 h at 0 °C. The mixture was poured into ice-cold dry Et_2O. The resulting powder was collected and washed three times with ice-cold dry Et_2O. To a stirred solution of the precipitant **23a** in DMF (1 cm^3) were added (*i*-Pr)$_2$NEt (0.014 cm^3, 0.08 mmol) and 1*H*-pyrazole-1-carboxamidine·HCl (12 mg, 0.04 mmol), and the mixture was stirred at room temperature for 60 h. After concentration under reduced pressure, purification by preparative HPLC gave the bis-trifluoroacetate salt of the title peptide **3E** (1.9 mg, 9% yield based on H–Gly–(2-Cl)Trt resin, >98% purity by HPLC analysis) as a colorless freeze-dried powder: HRMS (FAB), *m/z* calcd for $C_{37}H_{49}N_{10}O_5$ ([M+H]$^+$) 713.3882, found 713.3886.

***cyclo*(−D−Tyr−Arg−Arg−Ψ[(*Z*)-CF=CH]−Nal−Gly−)·2TFA (4F).** Cyclic peptide **4F** was synthesized by a procedure identical with that described for the synthesis of **3E**. The protected peptide **22b** (32.0 mg, 0.0270 mmol) was treated with aqueous TFA/H_2O (95/5, 10 cm^3) for 3 h. Concentration under reduced pressure gave an oily residue. To a solution of the residue in DMF (8 cm^3) were added 2-mercaptoethanol (0.0191 cm^3, 0.270 mmol) and DBU (0.0809 cm^3, 0.540 mmol), and the mixture was stirred at 50 °C for 2.5 h. After concentration under reduced pressure, the residue **23b** was treated with Et_3N (0.112 cm^3, 0.810 mmol) and 1*H*-pyrazole-1-carboxamidine·HCl (39.6 mg, 0.270 mmol) in DMF (2 cm^3). After concentration under reduced pressure, purification by preparative HPLC gave the bis-trifluoroacetate salt of the title peptide **4F** (3.6 mg, 6% yield based on H–Gly–(2-Cl)Trt resin, 89% purity by HPLC analysis): HRMS (FAB), *m/z* calcd for $C_{37}H_{48}FN_{10}O_5$ ([M+H]$^+$) 731.3788, found 731.3796.

[^{125}I]-SDF-1 binding and displacement

Membrane extracts were prepared from CHO-K1 cell lines expressing either CXCR4 or CXCR7. For ligand binding, 0.050 cm^3 of the inhibitor, 0.025 cm^3 of [^{125}I]-SDF-1α (0.3 nM, Perkin-Elmer Life Sciences) and 0.025 cm^3 of the membrane/beads mixture [CXCR4: 7.5 μg well^{-1} of membrane, 0.5 mg well^{-1} of PVT WGA beads (Amersham); CXCR7: 3 μg well^{-1} of membrane, 0.25 mg well^{-1} of PVT-PEI type A beads (Amersham)] in assay buffer (25 mM HEPES pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 140 mM NaCl, 250 mM sucrose, 0.5% BSA) were incubated in the wells of an Optiplate places (Perkin-Elmer Life Sciences) at room temperature for 1 h. The bound radioactivity was counted for 1 min well^{-1} in a TopCount (Packard). Inhibitory activity of the test compounds was determined based on the inhibition of [^{125}I]-SDF-1 binding to the receptors (IC_{50}).

Determination of anti-HIV activity

The peptide sensitivity of three HIV-1 strains was determined by the MAGI assay with some modifications.[22] Briefly, the target cells (HeLa-CD4/CCR5-LTR-β-gal; 10^4 cells well^{-1}) were plated in 96-well flat microtiter culture plates. On the following day,

the cells were inoculated with the HIV-1 (60 MAGI U/well, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of the 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}]).

Acknowledgements

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Efficient Synthesis of Pyrimido[1,2-*c*][1,3]benzothiazin-6-imines and Related Tricyclic Heterocycles by S_NAr-Type C−S, C−N, or C−O Bond Formation with Heterocumulenes

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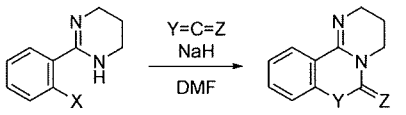

A simple and practical synthetic method of pyrimido[1,2-*c*]-[1,3]benzothiazin-6-imines and related tricyclic heterocycles has been developed. Treatment of 2-(2-haloaryl)-tetrahydropyrimidines with NaH and a heterocumulene such as carbon disulfide, isothiocyanates, and isocyanates in DMF provides the desired cyclization products through a regioselective S_NAr-type reaction. This method provides direct access to PD 404182 and related compounds.

The pyrimidobenzothiazine derivative PD 404182 (**1**) was recently discovered to be an antibiotic agent (Figure 1).[1,2]

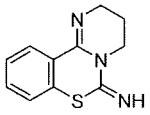

FIGURE 1. Structure of PD 404182.

This compound inhibits 3-deoxy-D-*manno*-octulosonic acid 8-phosphate (KDO 8-P) synthase, which catalyzes the condensation of phosphoenolpyruvate and arabinose 5-phosphate in the first committed step in the synthesis of KDO (an integral part of the lipopolysaccharide layer in Gram-negative bacteria). PD 404182 is considered to be an important lead in the development of structurally novel antibiotics effective against multidrug-resistant bacteria.[2b] Extensive study of the structure−activity relationship (SAR) of PD 404182 has not been carried out, presumably due to the lack of an efficient synthetic method suitable for lead optimization, as well as the cost of commercially available PD 404182.[3]

To develop a reliable, short-step synthetic method of tricyclic heterocycles related to PD 404182, we planned a novel strategy based on carbon (sp^2)−heteroatom bond formation using 2-(2-haloaryl)tetrahydropyrimidine derivatives. The carbon−heteroatom bond formation reaction is becoming a powerful methodology for construction of various heterocycles, providing several biologically active compounds.[4] The nucleophilic aromatic substitution (S_NAr) reaction is a well-established transition metal-free[5,6] carbon−heteroatom bond formation reaction.[7,8] In general, the S_NAr reaction requires harsh conditions (> 100 °C) and/or sufficiently activated aromatic rings by powerful electron-withdrawing group(s) (e.g., nitro). We describe a direct synthesis of tricyclic heterocycles related to PD 404182 by a regioselective S_NAr-type reaction of tetrahydropyrimidine-substituted haloarenes with heterocumulene in the absence of additional electron-withdrawing groups.[9] The efficient short-step synthesis of PD 404182 is also presented.

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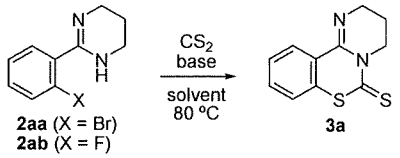
TABLE 1. Optimization of Reaction Conditions with CS_2[a]



entry	X	base (equiv)	solvent	time (h)	yield (%)[b]
1	Br	NaH (5)	MeCN	4	trace
2	Br	NaH (5)	THF	4	trace
3	Br	NaH (5)	DMF	6	75
4	Br	NaH (2)	DMF	12	88
5	Br	none	DMF	12	12
6	Br	Et_3N (2)	DMF	12	trace
7	Br	KH (2)	DMF	6	trace
8	Br	NaO*t*-Bu (2)	DMF	6	27
9	F	NaH (2)	DMF	12	86

[a]All reactions were carried out at 80 °C with 2 or 5 equiv of CS_2 (corresponding to the base loading). [b]Isolated yields.

Initial experiments were carried out with bromoarene **2aa**, which can be readily obtained by oxidative amidination[10] of 2-bromobenzaldehyde with propanediamine, and carbon disulfide as a heterocumulene (Table 1). Exposure of **2aa** with sodium hydride (5.0 equiv) and carbon disulfide (5.0 equiv) in acetonitrile or THF afforded only a trace amount of desired compound **3a** (entries 1 and 2). The desired reaction was efficiently promoted in DMF to give **3a** in 75% yield (entry 3). A decreasing amount of sodium hydride and carbon disulfide (2.0 equiv) slightly improved the yield of **3a** (88%) under the reaction for 12 h (entry 4).[11] The reaction in the absence of sodium hydride provided a yield of **3a** of only 12%. We next screened several bases such as triethylamine, potassium hydride[12] and sodium *tert*-butoxide (entries 6–8): sodium hydride was the most effective (entry 4). The fluoride **2ab** gave a comparable result with the bromide **2aa** to afford **3a** in 86% yield under optimized conditions (entry 9).

With knowledge of the optimized conditions, we examined the reaction of several substituted substrates (Table 2). Substrates **2b–d** having a methoxy, methyl, or fluoro group at the 4-position provided the corresponding cyclized products **3b–d** in good-to-excellent yields (76–95%, entries 1–3). Whereas the reaction of **2e** bearing the 4-nitro group at 80 °C resulted in formation of a complex mixture, the reaction at room temperature gave the cyclization product **3e** in 73% yield (entry 4). A methoxy group on the 5-position considerably diminished the reactivity, affording **3f** in only 17% yield (entry 5). This was presumably due to increased electron density at the carbon substituted by a bromine atom. In the case of **2g** bearing a 5-nitro group, the corresponding product **3g** was obtained by the reaction at room temperature (entry 6), similarly to **2e** (entry 4). Pyridine derivatives **4** and **6** showed different reactivity depending on the position of the nitrogen atom: the 2-bromopyridine derivative **6** gave a better result (71%, entry 8) than the 3-bromopyridine derivative **4** (18%, entry 7). The

TABLE 2. Reaction of Substituted 2-(2-Halophenyl)-1,4,5,6-tetrahydropyrimidines[a]

entry	substrate	product	yield (%)[b]
1	**2b** (R = OMe, X = F)	**3b** (R = OMe)	95
2	**2c** (R = Me, X = Br)	**3c** (R = Me)	88
3	**2d** (R = F, X = Br)	**3d** (R = F)	76
4	**2e** (R = NO_2, X = F)	**3e** (R = NO_2)	–[c] (73)[d]
5	**2f** (R = OMe)	**3f** (R = OMe)	17
6	**2g** (R = NO_2)	**3g** (R = NO_2)	–[c] (57)[d]
7	**4**	**5**	18
8	**6**	**7**	71
9	**8**	**9**	quant.

[a]Unless otherwise stated, reactions were carried out with CS_2 (2.0 equiv) and NaH (2.0 equiv) in DMF at 80 °C for 12 h. [b]Isolated yields. [c]A complex mixture formed. [d]Yields in parentheses indicate those of the reactions at rt.

naphthalene derivative **8** afforded the tetracyclic compound **9** in quantitative yield (entry 9).

To further expand our methodology for construction of other heterocyclic frameworks, we investigated the reaction using isothiocyanates or isocyanates[13,14] as heterocumulene (Table 3). When benzylisothiocyanate was employed, the reaction of **2aa** or **2ab** efficiently proceeded to give the corresponding *N*-arylated product **10** in 82% and 97% yields, respectively (entries 1 and 2). The reaction with *tert*-butylisothiocyanate exclusively furnished an *S*-arylated product **11** as a single isomer (entry 3). These results indicate

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(11) Larger amounts of unidentified byproduct were formed when using 5 equiv of NaH than in the reaction with 2 equiv of NaH (entry 4).
(12) A reason for the significant countercation effect (NaH vs. KH) on the reactivity is unclear.

(13) For related reactions of electron-deficient (haloaryl)isothiocyanates, see: (a) Muthusamy, S.; Paramasivam, R.; Ramakrishnan, V. T. *J. Heterocycl. Chem.* **1991**, *28*, 759–763. (b) Zambounis, J. S.; Christen, E.; Pfeiffer, J.; Ribs, G. *J. Am. Chem. Soc.* **1994**, *116*, 925–931. (c) Huang, S.; Connolly, P. J. *Tetrahedron Lett.* **2004**, *45*, 9373–9375.
(14) For related transition metal-catalyzed reactions, see: (a) Ferraccioli, R.; Carenzi, D. *Synthesis* **2003**, 1383–1386. (b) Benedí, C.; Bravo, F.; Uriz, P.; Fernández, E.; Claver, C.; Castillón, S. *Tetrahedron Lett.* **2003**, *44*, 6073–6077. (c) Yang, D.; Liu, H.; Yang, H.; Fu, H.; Hu, L.; Jiang, Y.; Zhao, Y. *Adv. Synth. Catal.* **2009**, *351*, 1999–2004. (d) Murru, S.; Mondal, P.; Yella, R.; Patel, B. K. *Eur. J. Org. Chem.* **2009**, 5406–5413. (e) Qiu, J.-W.; Zhang, X.-G.; Tang, R.-Y.; Zhong, P.; Li, J.-H. *Adv. Synth. Catal.* **2009**, *351*, 2319–2323. (f) Shen, G.; Lv, X.; Bao, W. *Eur. J. Org. Chem.* **2009**, 5897–5901.

TABLE 3. Reaction with Isothiocyanates or Isocyanates[a]

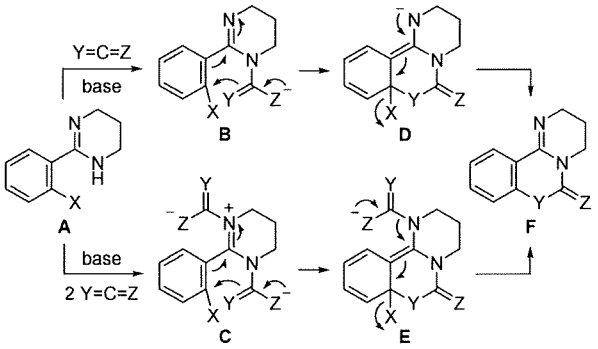
entry	substrate	R-NCX	product	yield (%)[b]
1	**2aa** (X = Br)	BnNCS	**10**	82
2	**2ab** (X = F)			97
3[c]	**2ab**	*t*-BuNCS	**11**	62[d,e]
4	**2ab**	BnNCO	**12**	quant.
5[c]	**2ab**	*t*-BuNCO	**13** (Y = N*t*-Bu, Z = O) **14** (Y = O, Z = N*t*-Bu)	54 18[e]
6	**2ab**	PhNCO	**15**	quant.
7	**16**	*t*-BuNCS	**17**	49[e]

[a]Unless otherwise stated, reactions were carried out with R-NCX (2.0 equiv) and NaH (2.0 equiv) in DMF at rt for 2–3 h. [b]Isolated yields. [c]These reactions were carried out at 80 °C. [d]A trace amount of regioisomeric *N*-arylation product was also formed. [e]Isolated as a single isomer.

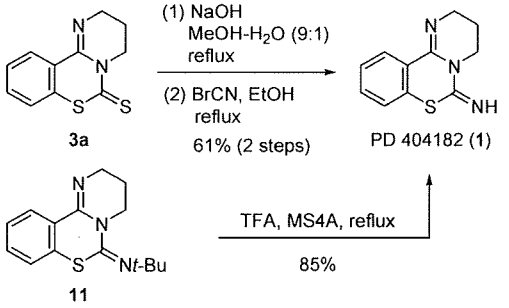
that the regioselectivity of the reaction can be perfectly switched by changing a substituent on the nitrogen atom. As expected, the reaction of **2ab** with benzylisocyanate provided an *N*-arylated product **12** in quantitative yield (entry 4) as in the case with isothiocyanate (entries 1 and 2). Interestingly, *tert*-butylisocyanate showed moderate selectivity to mainly afford an *N*-arylation product **13** (54%), formed by the arylation at the more bulky position, as well as an *O*-arylation product **14** (18%, entry 5). Phenylisocyanate also provided an *N*-arylated product **15** (entry 6). The 2-phenylimidazoline derivative **16** (a 5-membered-ring amidine congener) also provided the corresponding *S*-arylated product **17** in a slightly decreased yield (49%, entry 7).

This reaction would proceed via nucleophilic addition of the amidine moiety to heterocumulene followed by an intramolecular S_NAr reaction of the resulting adducts such as **B** (Scheme 1). Nonactivated aromatic rings efficiently reacted under relatively mild conditions, so two molecules of

SCHEME 1. Proposed Reaction Mechanisms



SCHEME 2. Synthesis of PD 404182



the heterocumulene may be involved in the reaction to form the intermediate **C** in which the amidine moiety can be a more powerful electron-withdrawing group suitable for the S_NAr-type reaction. The regioselectivity in the nucleophilic attack on the aromatic ring (Y vs. Z) is controlled by a subtle balance of inherent nucleophilicity and steric hindrance of these functionalities.

We finally focused on the synthesis of PD 404182 (**1**) (Scheme 2). Hydrolysis of the carbamodithioate derivative **3a** followed by treatment with cyanogen bromide[15] readily afforded the desired compound **1**. The same compound was also obtained in a single step by heating compound **11** in trifluoroacetic acid in the presence of molecular sieves.

In conclusion, we developed a simple and practical synthetic method for tricyclic heteroarenes related to PD 404182. This reaction provides divergent access to several related heterocycles under mild conditions without a powerful activating group. Further investigations including SAR study of these derivatives are currently underway.

Experimental Section

General Procedure for Synthesis of 3,4-Dihydro-2*H*-pyrimido-[1,2-*c*][1,3]benzothiazine-6-thione (3a) (Table 1, Entry 4). To a mixture of **2aa** (59.8 mg, 0.25 mmol) and NaH (20.0 mg, 0.50 mmol; 60% oil suspension) in DMF (0.83 mL) was added carbon disulfide (30.5 μL, 0.50 mmol) under an Ar atmosphere. After being stirred at 80 °C for 12 h, the mixture was concentrated in vacuo. The residue was purified by flash chromatography over silica gel with *n*-hexane–EtOAc (9:1) to give compound **3a** as a pale-yellow solid (51.4 mg, 88%): mp 139–141 °C (from $CHCl_3$–*n*-hexane); IR (neat) (cm^{-1}) 1624 (C=N); ^1H NMR (400 MHz, $CDCl_3$) δ 2.01–2.07 (m, 2H,

(15) Peter, S.; Gerhard, S. *Ger. Offen.* DE2811131, 1979.

CH_2), 3.76 (t, J = 5.6 Hz, 2H, CH_2), 4.45 (t, J = 6.2 Hz, 2H, CH_2), 7.03 (dd, J = 7.8, 1.5 Hz, 1H, Ar), 7.28−7.33 (m, 1H, Ar), 7.41 (ddd, J = 8.0, 7.6, 1.5 Hz, 1H, Ar), 8.20 (dd, J = 8.0, 1.2 Hz, 1H, Ar); ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.6, 45.5, 48.6, 121.6, 126.5, 127.5, 128.9, 131.1, 131.8, 144.2, 189.8. Anal. Calcd for $C_{11}H_{10}N_2S_2$: C, 56.38; H, 4.30; N, 11.95. Found: C, 56.23; H, 4.44; N, 11.85.

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Supporting Information Available: Experimental procedures, full characterization, and ^{1}H and ^{13}C NMR charts of substrates and cyclization products. This material is available free of charge via the Internet at http://pubs.acs.org.

研究成果の刊行に関する一覧表

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雑誌

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