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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.

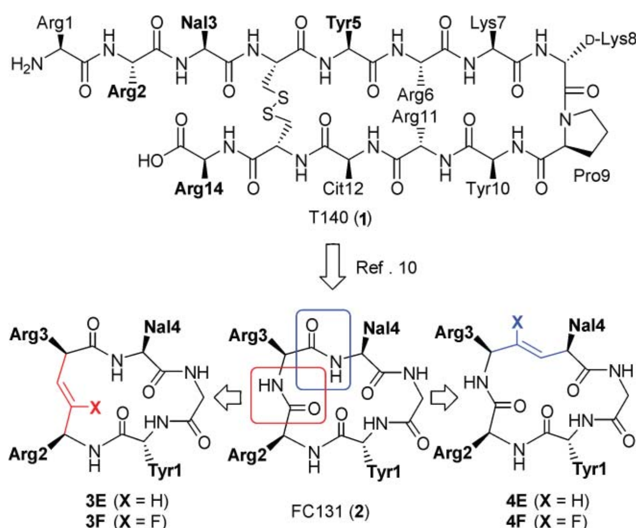

Fig. 1 Structures of T140 (**1**), FC131 (**2**), and the (*E*)-alkene and (*Z*)-fluoroalkene FC131 analogues. Bold residues of **1** are indispensable for the potent CXCR4-antagonistic activity. Nal = L-3-(2-naphthyl)alanine.

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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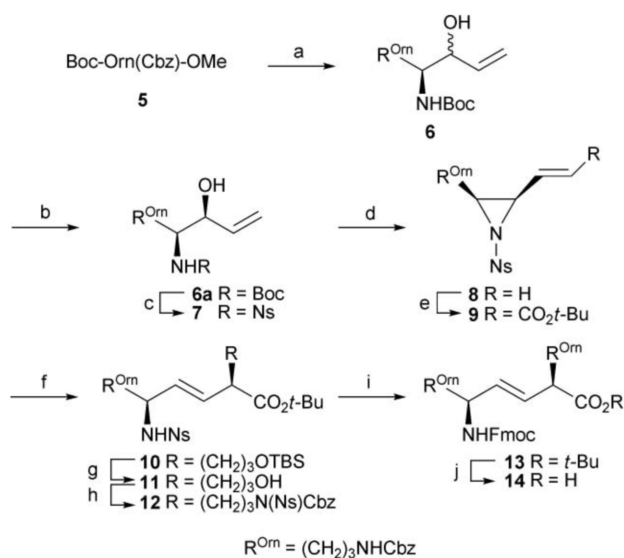
Results and discussion

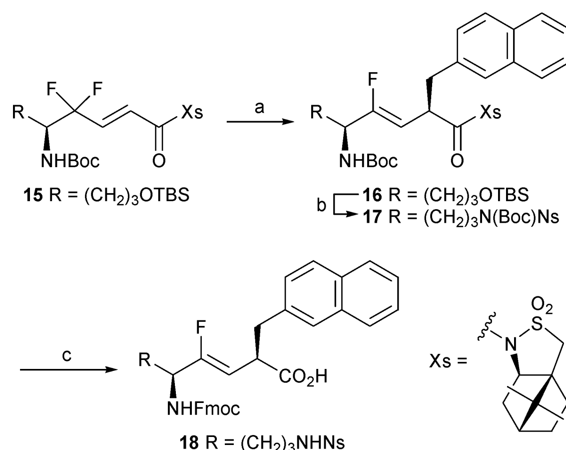
Synthesis of alkene dipeptide isosteres and the application to FC131 analogues

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 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.



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, $(i\text{-Pr})_2NEt$, 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%).

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).

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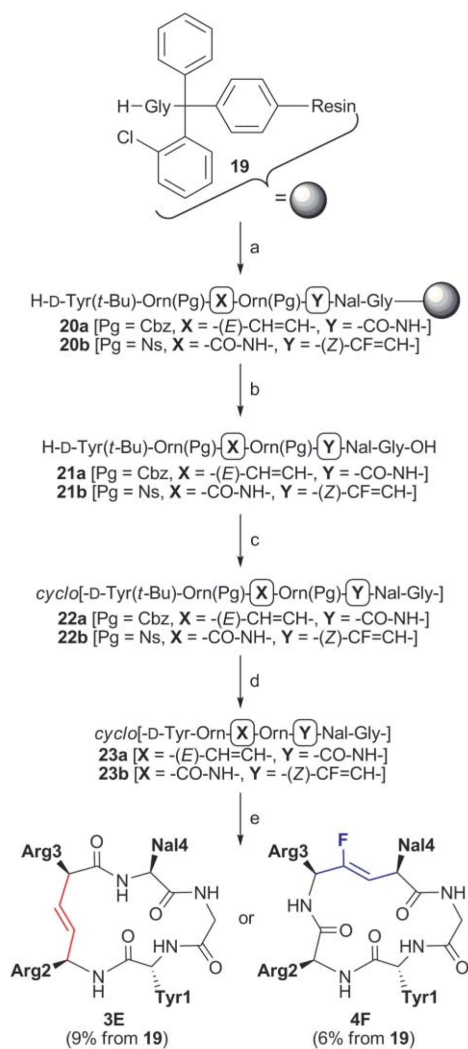

Scheme 3 Synthesis of the alkene analogues of FC131. Reagents and conditions: (a) Fmoc-based SPPS; (b) 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), CH_2Cl_2; (c) diphenylphosphoryl azide (DPPA), $NaHCO_3$, DMF, –40 °C to rt; (d) **23a**: 1 M TMSBr/thioanisole in TFA, *m*-cresol, 1,2-ethandithiol, 6 h; **23b**: (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

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.

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.

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%.

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[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

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(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,

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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}]).

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