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Cu(II)-mediated oxidative intermolecular *ortho* C–H functionalisation using tetrahydropyrimidine as the directing group†

Tsukasa Mizuhara, Shinsuke Inuki, Shinya Oishi, Nobutaka Fujii* and Hiroaki Ohno*

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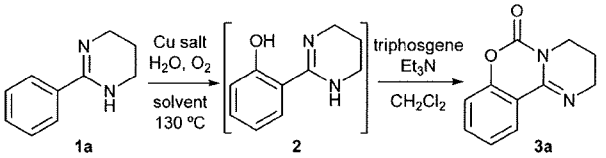
Tetrahydropyrimidine works efficiently as a directing group in Cu(II)-mediated oxidative aromatic C–H functionalisation for the selective introduction of oxygen or nitrogen to the *ortho*-position.

Transition metal-catalysed C–H functionalisation has received considerable attention in recent years. Notable progress has been made especially with Pd, Ru, Rh, and Pt catalysts, which allow atom-economical transformations using simple substrates. Directing group assisted intra/intermolecular C–H functionalisation[1] is considered to be one of the most promising approaches as a new carbon–carbon[1] or carbon–heteroatom bond[2,3] is selectively formed at a non-functionalised position proximal to the directing group. Recent research in this area has revealed that nitrogen-containing functional groups such as pyridines,[4] imines,[5] oximes (and ethers),[6] oxazolines[7] and amidines[8] as well as oxygen-containing functional groups such as amides,[9] esters,[10] ketones,[11] carboxylic acids[12] and phenols[13] effectively act as directing groups for regioselective C–H functionalisation. These functional groups and their equivalents are involved in biologically active natural and synthetic compounds while directing group-assisted C–H functionalisation serves as a powerful tool for the synthesis and modification of these molecules.

As part our ongoing program directed toward the development of C–H functionalisation reactions for the efficient construction of heterocyclic frameworks,[14] we designed an experiment for the oxidative introduction of a heteroatom by aromatic C–H functionalisation with the assistance of an *ortho*-tetrahydropyrimidinyl group. This group can be considered as a promising drug-like structure as well as a synthetic equivalent of a carboxylic group. Reinaud and co-workers previously reported a copper-catalysed *ortho*-hydroxylation reaction of benzoic acid using carboxyl as a directing group.[15] Yu *et al.*[16a] and Chatani *et al.*[16b] independently reported copper-mediated oxidative intermolecular C–H functionalisation using a pyridine moiety as the directing group. The latter reactions provide efficient access to functionalised pyridines with a biaryl structure by simply treating the biaryl substrates with a copper salt for several hours. More recently, copper-catalysed syntheses of benzimidazoles[17a] and benzoxazoles[17b] by oxidative intramolecular C–H functionalisation using amidine or amide as the directing/nucleophilic group were reported. Herein, we describe copper-mediated oxidative intermolecular C–H functionalisation using tetrahydropyrimidine as the directing group. In most cases, the reaction is complete within 1 h to provide 2-(tetrahydropyrimidinyl)phenol and aniline derivatives by the *ortho*-selective introduction of an oxygen or nitrogen atom.[18]

We initially investigated the reaction conditions for this C–H hydroxylation (Table 1). In the presence of H_2O (1.0 equiv.), treatment of 2-phenyl-1,4,5,6-tetrahydropyrimidine (**1a**) with CuO, $Cu(OH)_2$, $Cu(OTf)_2$ or $Cu(tfa)_2$ (1.0 equiv.) in DMF at 130 °C under an oxygen atmosphere led to the recovery of unchanged starting material and the desired C–H oxidation did not occur (entries 1–4). Using $Cu(OAc)_2$,[16a] however, led to the formation of the desired *ortho*-hydroxylated compound **2** (*ca.* 69% yield) although the isolation of **2** in its pure form was extremely difficult because of its basicity. We then attempted to isolate **3a** as the protected form: after the disappearance of **1a** (monitored by TLC), the reaction mixture was evaporated *in vacuo* and treated with triphosgene

Table 1 Optimisation of reaction conditions for C–H hydroxylation[a]



Entry	Cu salt (equiv.)	Solvent	Time/min	Yield (%)[b]
1	CuO (1.0)	DMF	20	N.r.[e]
2	$Cu(OH)_2$ (1.0)	DMF	20	N.r.[e]
3	$Cu(OTf)_2$ (1.0)	DMF	20	N.r.[e]
4	$Cu(tfa)_2$ (1.0)	DMF	20	N.r.[e]
5	$Cu(OAc)_2$ (1.0)	DMF	20	61
6	$Cu(OAc)_2$ (1.0)	Acetonitrile	60	11
7	$Cu(OAc)_2$ (1.0)	Dioxane	60	11
8	$Cu(OAc)_2$ (0.2)	DMF	60	30
9	$Cu(OAc)_2$ (2.0)	DMF	15	27
10[c]	$Cu(OAc)_2$ (1.0)	DMF	20	70
11[cd]	$Cu(OAc)_2$ (1.0)	DMF	20	56

[a] After completion of C–H hydroxylation (monitored by TLC), the reaction mixture was evaporated and treated with triphosgene (1.05 equiv.) and Et_3N (4.0 equiv.) in CH_2Cl_2 at 0 °C to rt for 1 h. [b] Isolated yields. [c] After completion of C–H hydroxylation (monitored by TLC), the reaction mixture was treated with TMEDA (4.0 equiv.) at 130 °C for 1 min. In this case, TMEDA (additional 4.0 equiv.) was used for the next step instead of Et_3N. [d] Reaction was carried out under air. [e] No reaction. Abbreviation: TMEDA = *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8501, Japan.
E-mail: hohno@pharm.kyoto-u.ac.jp; Fax: +81 75 753 4570; Tel: +81 75 753 4571

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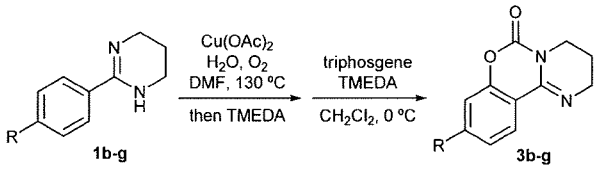
(1.05 equiv.) and triethylamine (4.0 equiv.) in CH_2Cl_2 to afford pure **3a** in a yield of 61% (entry 5). When acetonitrile or dioxane was used as the solvent instead of DMF, yields of **3a** decreased considerably (11%, entries 6 and 7). Lowering the loading of $Cu(OAc)_2$ to 0.2 equiv. also resulted in a decreased yield for **3a** (30%, entry 8), which indicates low catalyst efficiency. When using 2.0 equiv. of $Cu(OAc)_2$, the yield also decreased and this was contrary to our expectation (27%, entry 9). Considering that the *ortho*-hydroxylated product **2** may form a complex with the copper salt, we further optimised the reaction conditions including the carbonylation procedure. Initially, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) was added as a bidentate ligand to the oxidative C–H functionalisation reaction mixture and this resulted in the complete inhibition of the desired transformation. Similarly, use of TMEDA instead of triethylamine as the base for carbonylation did not improve the yield of **3a**. On the other hand, treatment with TMEDA (4.0 equiv.) at 130 °C for 1 min after the C–H hydroxylation followed by the carbonylation using additional TMEDA (4.0 equiv.) increased the yield to 70% (entry 10). The reaction under air resulted in a decreased yield (56%, entry 11), which indicates that molecular oxygen participates in the re-oxidation of the copper catalyst.

Using the optimised conditions (Table 1, entry 10), we examined the reaction of several substituted substrates (Table 2). Substitution with electron-donating groups such as methoxy (**1b**, entry 1) or methyl groups (**1c**, entry 2) was tolerated to afford the desired products **3b** and **3c** in 64% and 61% yields, respectively. The chemoselectivity of this reaction was evaluated by a reaction where aryl bromide **1d** was used and the desired product **3d** was obtained in a 45% yield (entry 3). Methoxycarbonyl (entry 4) and trifluoromethyl groups (entry 5) had a relatively small effect on the reactivity of these substrates and the use of the highly electron-deficient arene **1g** bearing a nitro group decreased the yield considerably (19%, entry 6). These results indicate that this reaction is sensitive to the presence of electron-withdrawing groups on the aromatic ring. In all cases, reactions without TMEDA gave less favourable results.

Table 2 Cu-catalysed C–H hydroxylation of 4-substituted 2-phenyl-1,4,5,6-tetrahydropyrimidines[a]



Entry	Substrate (R)	Product	Yield (%)[b]
1	**1b** (OMe)	**3b**	64 (53)
2	**1c** (Me)	**3c**	61 (54)
3	**1d** (Br)	**3d**	45 (37)
4	**1e** (CO_2Me)	**3e**	46 (43)
5	**1f** (CF_3)	**3f**	43 (38)
6	**1g** (NO_2)	**3g**	19 (16)

[a] These reactions were carried out using the optimised procedure (Table 1, entry 10). [b] Isolated yields. Yields in parentheses indicate those of the reactions containing Et_3N (as shown in Table 1, entry 5).

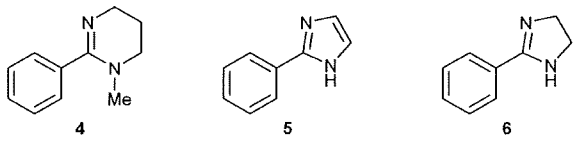
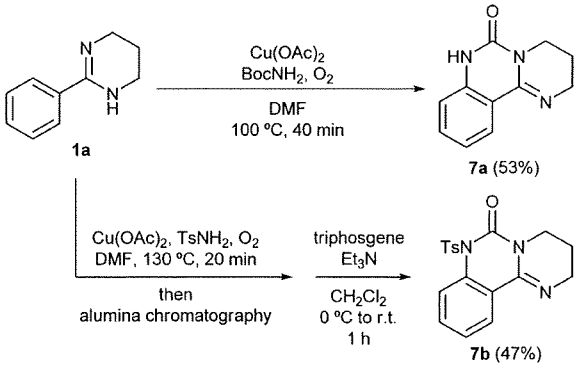


Fig. 1 Various amidine analogues.



Scheme 1 C–H amidation with $BocNH_2$ and $TsNH_2$.

Next, we investigated the ability of other amidine analogues to function as directing groups (Fig. 1). The reaction of the N-methylated analogue **4** and 2-phenylimidazole **5** did not produce the desired *ortho*-hydroxylated products under standard reaction conditions and the starting materials were recovered. Interestingly, the five-membered ring amidine in **6** was not effective as a directing group either. These results suggest that subtle differences in the intermediate formed by a copper salt and a directing group strongly affect the reactivity of the substrates.

Finally, we investigated C–H amidation (Scheme 1). We found that the reaction of amidine **1a** with $Cu(OAc)_2$ (1.0 equiv.) and *tert*-butyl carbamate (3.0 equiv.) in DMF at 100 °C for 40 min directly afforded the tricyclic aniline derivative (**7a**) in 53% yield. This reaction occurred by cyclisation involving the elimination of *tert*-butoxide. *p*-Toluenesulfonamide also reacted with **1a** under identical conditions to afford **7b** in 47% yield after alumina column chromatography[19] followed by treatment with triphosgene–Et_3N.

Although the exact mechanism of the *ortho* C–H oxidation is unclear, a single electron transfer (SET) pathway *via* a radical-cation intermediate[16a] is supported by the fact that the presence of an electron-withdrawing group on the benzene ring considerably decreased the product yields. The observed *ortho*-selectivity can be attributed to an intramolecular transfer of the coordinating group on the copper atom.

In conclusion, we have developed a copper-mediated oxidative *ortho* C–H functionalisation using tetrahydropyrimidine as a directing group. This reaction applies to 2-phenyl-1,4,5,6-tetrahydropyrimidines having an electron-donating or a weak electron-withdrawing group and affords the corresponding phenol derivatives within 1 h. Use of *tert*-butyl carbamate or tosylamide instead of H_2O promotes the introduction of a nitrogen functionality to give aniline derivatives. As far as we are aware, this is the first example of an oxidative

intermolecular C–H functionalisation using an amidine moiety as the directing group. Further studies that include an investigation of the exact reaction mechanism and the application to synthesis of biologically-active compounds are now in progress.

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Peptide bond mimicry by (*E*)-alkene and (*Z*)-fluoroalkene peptide isosteres: synthesis and bioevaluation of α-helical anti-HIV peptide analogues†

Shinya Oishi,*[a] Hirotaka Kamitani,[a] Yasuyo Kodera,[a] Kentaro Watanabe,[a] Kazuya Kobayashi,[a] Tetsuo Narumi,[a] Kenji Tomita,[a] Hiroaki Ohno,[a] Takeshi Naito,[b] Eiichi Kodama,[b] Masao Matsuoka[b] and Nobutaka Fujii*[a]

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The α-helix structures of the anti-HIV fusion inhibitory peptides are stabilized by the amino acid sequence and by intrachain hydrogen bonds. The study of peptide analogues using (*E*)-alkene and (*Z*)-fluoroalkene dipeptide isosteres demonstrated the substantial, yet position-dependent, contribution of hydrogen bonds to the α-helix stability and anti-HIV bioactivity.

Introduction

The α-helix represents one of the largest classes of secondary structure elements found in protein and peptide structures.[1] The cylindrical structures are stabilized by intrachain hydrogen bond (H-bond) networks which are formed between the C=O of residue *i* and the amide N–H of the *i*+4 residue to generate 13-membered pseudocyclic structures. The functional and/or interactive surface(s) of the α-helix are revealed by the arrangement of the distribution residues in the linear sequence upon folding.

In order to stabilize the α-helix structure of bioactive peptides, there are two possible approaches: (1) bridging side-chains by covalent or non-covalent bond(s) or (2) mimicking intrachain H-bond(s).[2] Recently, we reported a novel design concept of fusion inhibitory peptides active against HIV-1 by utilizing an X-EE-XX-KK motif (X: original residue; E: glutamic acid; K: lysine).[3,4] This motif contributes to the stabilization of the bioactive α-helix conformation by forming two potential salt bridges between Glu and Lys side-chains without altering the location of residues that form the interactive surface with the viral protein gp41.[4c] The peptides, named SC35EK and T-20EK, exhibit highly potent anti-HIV activity by inhibition of the rearrangement of HIV-1 gp41 that facilitates fusion between the host cellular and viral membranes. In addition, a structure–activity relationship study identified a novel amphiphilic peptide, SC29EK, with a minimal sequence for anti-HIV activity.[5] In light of its high potency of SC29EK, it was of interest to estimate the effect of intrachain H-bond(s) on α-helix stabilization in the presence of the X-EE-XX-KK motifs. Accordingly, efforts herein have been undertaken to comparatively evaluate the anti-HIV activity and biophysical properties of SC29EK analogues containing peptide bond mimetics.

(*E*)-Alkene dipeptide isostere (EADI) **1** and (*Z*)-fluoroalkene dipeptide isostere (FADI) **2** of Lys-Lys were chosen as planar peptide bond surrogates for positional scanning of each Lys-Lys dipeptide in four repeat motifs (Fig. 1).[6] Two potential H-bonds may be missing when replacing the peptide bond in Lys-Lys with the olefin congeners: (1) between the C=O of the first Lys (*i*) and the N–H of the downward Glu (*i*+4), (2) between the N–H of the second Lys (*i*+1) and the C=O of the upward Glu (*i*−3) (Fig. 1). In the case of FADI substitution, the presence of the first H-bond was expected, because of the potential ability of a fluorine atom to act as a H-bond acceptor.[7]

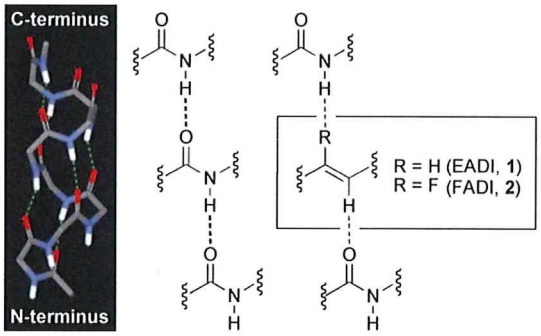


Fig. 1 Structures of (*E*)-alkene and (*Z*)-fluoroalkene dipeptide isosteres and the potential mimicry of H-bonds stabilizing the α-helix structure.

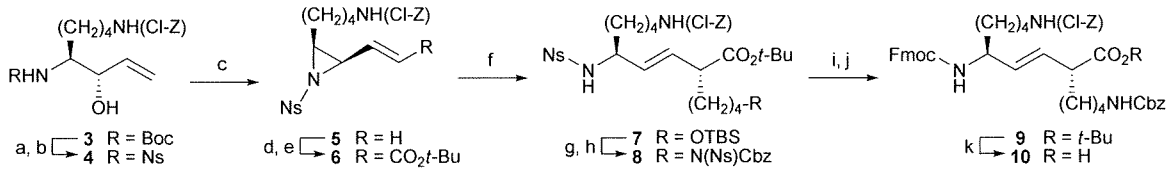
Results and discussion

Lys-Lys EADI[8] and FADI[9] were prepared by the established procedures shown in Schemes 1 and 2, respectively. Briefly, allyl alcohol **3**[10] derived from a protected amino acid was converted into Ns-amide **4**. Aziridination of **4** by the Mitsunobu reaction followed by C-1 elongation afforded the β-aziridinyl-α,β-unsaturated ester **6**. Organocopper-mediated alkylation of **6** provided an α-alkyl adduct **7** regio- and stereoselectively. Subsequent functional group manipulations generated the expected Fmoc-protected EADI **10**.

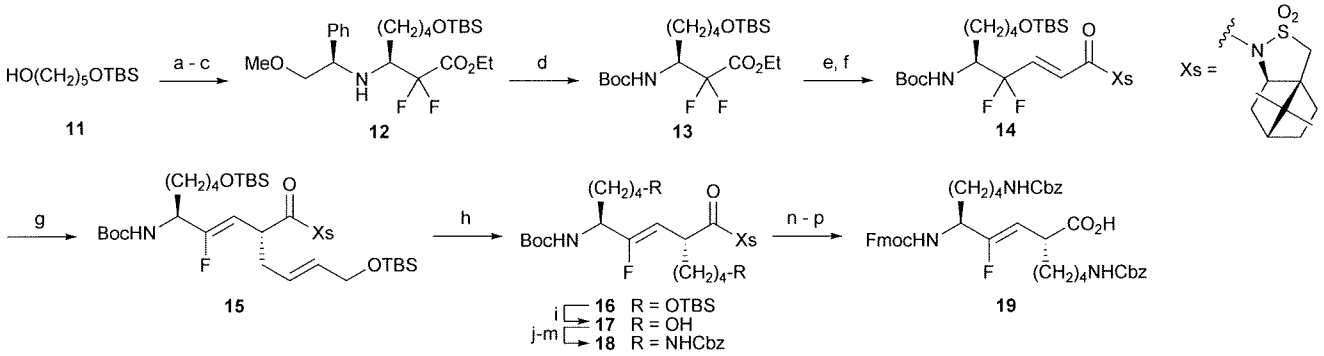
FADI synthesis began with mono-TBS-protected 1,5-pentanediol **11**. Rh-catalyzed Reformatsky–Honda reaction[11] of the corresponding aldehyde gave α,α-difluoro-β-amino ester **12**. The simultaneous hydrogenolysis and Boc protection followed by C-2 elongation using the Horner–Wadsworth–Emmons reaction produced a key γ,γ-difluoro-α,β-enoyl sultam **14**. One-pot reduction/asymmetric alkylation *via* transmetalation with allyl bromide formed the FADI scaffold **15**. Selective

[a]*Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8501, Japan. E-mail: soishi@pharm.kyoto-u.ac.jp, nfujii@pharm.kyoto-u.ac.jp; Fax: +81-75-753-4570; Tel: +81-75-753-4551*
[b]*Laboratory of Virus Control, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto, 606-8507, Japan*

† Electronic supplementary information (ESI) available: Additional experimental procedures, NMR spectra and HPLC charts. See DOI: 10.1039/b907983a



Scheme 1 Synthesis of the Lys-Lys-type alkene dipeptide isostere. *Reagents and conditions*: (a) 4 N HCl/dioxane; (b) NsCl, 2,4,6-collidine, $CHCl_3$, 65% (2 steps); (c) DIAD, PPh_3, THF/toluene, 0 °C, 84%; (d) O_3, AcOEt, –78 °C, then Me_2S; (e) $(EtO)_2P(O)CH_2CO_2t$-Bu, LiCl, DIEA, CH_3CN, 0 °C, 46% (2 steps); (f) $TBSO(CH_2)_4I$, *t*-BuLi, CuCN, LiCl, *n*-pentane/Et_2O/THF, –78 °C, 60%; (g) H_2SiF_6 aq., CH_3CN/CH_3OH, 0 °C; (h) CbzNHNs, PPh_3, DIAD, THF/toluene, 81% (2 steps); (i) PhSH, K_2CO_3, DMF; (j) Fmoc-OSu, Et_3N, DMF, 84% (2 steps); (k) 4 N HCl/dioxane, 96%.



Scheme 2 Synthesis of the Lys-Lys-type fluoroalkene dipeptide isostere. *Reagents and conditions*: (a) DMSO, $(COCl)_2$, Et_3N, CH_2Cl_2, –78 °C; (b) (*R*)-2-methoxy-1-phenylethylamine, 3 Å MS, THF, 0 °C; (c) $BrCF_2CO_2Et$, $RhCl(PPh_3)_3$, Et_2Zn, 0 °C, 43% (3 steps); (d) $Pd(OH)_2$, H_2, Boc_2O, EtOH, 87%; (e) DIBAL-H, CH_2Cl_2/toluene –78 °C; (f) $(EtO)_2P(O)CH_2COXs$, LiCl, DIEA, CH_3CN, 0 °C, 87% (2 steps); (g) $Me_2CuLi{\cdot}LiI$, THF/Et_2O, –78 °C, then HMPA, then Ph_3SnCl, –78 °C to –40 °C, then $BrCH_2$–(*E*)-CH=CH–CH_2OTBS, –40 °C, 78%; (h) 4.5% Pd/C(en), EtOH, H_2; (i) aq. H_2SiF_6, CH_3CN/CH_3OH, 78% (2 steps); (j) TsCl, Et_3N, CH_2Cl_2; (k) NaN_3, DMF; (l) PPh_3, THF/H_2O; (m) Cbz-OSu, Et_3N, DMF, 65% (4 steps); (n) 1 N LiOH, 50% H_2O_2, THF/H_2O; (o) TFA, CH_2Cl_2; (p) Fmoc-OSu, Et_3N, MeCN/DMF/H_2O, 64% (3 steps).

hydrogenation in the presence of Pd/C(en)[12] and step-wise modifications afforded the Fmoc-protected FADI **19**. The resulting isosteres **10** and **19** were incorporated into the KK dipeptide of SC29EK sequence by standard Fmoc-based solid-phase peptide synthesis.

Anti-HIV activities of the isostere-containing peptides **20E–23E** and **20F–23F** were examined using the MAGI assay (Table 1). Substitutions of the first and second N-terminal Lys-Lys dipeptides with EADI (**20E** and **21E**) resulted in the loss of the anti-HIV activity (EC_{50} >10 μM). In contrast, the FADI congeners exhibited weak or moderate anti-HIV activities (**20F**: EC_{50} = 5.2 μM; **21F**: EC_{50} = 599 nM). Both EADI and FADI analogues with substitution at the third Lys-Lys showed slightly lower anti-HIV potency than wild-type C29[5] without the α-helix inducible XEEXXKK motifs (**22E**: EC_{50} = 865 nM; **22F**: EC_{50} = 663 nM). The best peptide analogues were obtained by replacement of the C-terminal Lys-Lys with the isosteres (**23E**: EC_{50} = 43 nM; **23F**: EC_{50} = 37 nM); however, the potency was lower than the original SC29EK peptide (EC_{50} = 2.2 nM). Similar bioactivities of peptide **20E–23E** and **20F–23F** were also observed against the other HIV-1 strains (Table 2). These observations suggest that all the peptide bonds within the Lys-Lys and the related H-bonding are essential for the potent anti-HIV activity of SC29EK.

The α-helix properties of these peptides were determined by circular dichroism (CD) analysis (Fig. 2a,b). The stable α-helix

Table 1 Sequences and anti-HIV activities of C29 and its derivatives and T_m values of the mixture with N36

	EADI analogues **E**		FADI analogues **F**	
Sequence[a]	EC_{50} (nM)[b]	T_m (°C)[c]	EC_{50} (nM)[b]	T_m (°C)[c]
WMEWDREINNYTSLIHSLIEESQNQQEKN C29	308 ± 144	51.7	—	—
WEEWDKKIEEYTKKIEELIKKSEEQQKKN SC29EK	2.2 ± 0.2	67.4	—	—
WEEWDKKIEEYTKKIEELIKKSEEQQKKN **20E/20F**	>10000	43.9	5220 ± 202	44.1
WEEWDKKIEEYTKKIEELIKKSEEQQKKN **21E/21F**	>10000	40.1	599 ± 96	49.5
WEEWDKKIEEYTKKIEELIKKSEEQQKKN **22E/22F**	865 ± 317	62.2	663 ± 242	60.9
WEEWDKKIEEYTKKIEELIKKSEEQQKKN **23E/23F**	43 ± 7	64.1	37 ± 6	64.8

[a] The underlined KK dipeptide indicates the position of the dipeptide isostere. [b] EC_{50} was determined as the concentration that blocked HIV-1 (NL4-3 strain) replication by 50%. [c] T_m values were defined by the midpoint of the thermal unfolding transition state as determined from $[\theta]_{222}$ readings.

Table 2 Anti-HIV activities of C29 and its derivatives against three HIV-1 strains

Peptides	EC_{50} (nM)[a]		
	NL4–3	IIIB	Ba-L
C29	308 ± 144	396 ± 83	42 ± 8
SC29EK	2.2 ± 0.2	6.5 ± 0.9	1.9 ± 0.2
20E	>10000	>10000	>10000
20F	5220 ± 202	>10000	5580 ± 1920
21E	>10000	>10000	>10000
21F	599 ± 96	3010 ± 554	600 ± 302
22E	865 ± 317	5110 ± 2,750	2630 ± 386
22F	663 ± 242	2200 ± 712	527 ± 95
23E	37 ± 6	153 ± 27	33 ± 2
23F	43 ± 7	237 ± 16	51 ± 7

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

structure of SC29EK was disrupted by a single substitution of the second or third Lys-Lys peptide bond with the isosteres in **21E/21F** and **22E/22F**. This suggests that the contribution of the H-bonds to the stability of the α-helix is likely to be superior to the multiple introductions of the X-EE-XX-KK motifs at these positions. Conversely, the effects of N- and C-terminal substitution were less significant as observed in **20E/20F** and **23E/23F**. This may be rationalized by the fact that these peptide bonds of SC29EK are positioned at the edge of the helix and that C-terminal Lys-Lys is involved in only upward H-bonding through the donor N–H moiety. CD spectra of SC29EK analogues in the presence of an interactive counterpart N36 indicated the formation of stable six-helix coiled-coil structures (Fig. 2c,d).[13] This observation supports the concept that SC29EK analogues exert their anti-HIV activity by inhibiting the folding process of the HIV-1 envelop protein gp41.

Binding affinity of SC29EK analogues to a viral protein was determined by the thermal stability of the six-helix complexes formed between SC29EK and N36 peptides. The melting temperature (T_m), representing 50% disruption of the six-helix bundle, was comparatively evaluated by monitoring the change in the circular dichroism signal at 222 nm as a function of increasing temperature (Table 1). The complexes involving peptides **20E/20F** and **21E/21F** showed significantly lower thermal stability, which correlates with the observed absent or low anti-HIV activities of these peptides. In contrast, potent analogues **23E/23F** form stable complexes with N36 with T_m values comparable to the value measured for SC29EK (**23E**: T_m = 64.1 °C; **23F**: T_m = 64.8 °C). The N-terminal tryptophan-rich domain (WRD) of inhibitory peptides such as C34 is essential for binding to the cavity formed by the N36 coiled-coil.[11] H-Bonds linked by the first and second Lys-Lys peptide bonds in SC29EK would reinforce the arrangement of these tryptophans. Interestingly, less potent anti-HIV activity of peptide **22E/22F** was observed compared with C29, whereas the complexes with N36 showed higher thermal stability. This result suggests that the loss of crucial H-bonds could reduce the anti-HIV activity, even though the X-EE-XX-KK motifs apparently aid the conformational stability of the six-helix bundle.

In terms of the mimicking ability of the two-peptide-bond isosteres, FADI peptides **20F–23F** exhibited slightly more potent anti-HIV activity and formed more stable complexes with N36 (except for **22F**). Although a fluoroalkene with a large dipole moment imperfectly reproduces the H-bonds needed for α-helix stabilization, FADI is an appropriate peptide bond surrogate to investigate structural requirements in bioactive peptides.

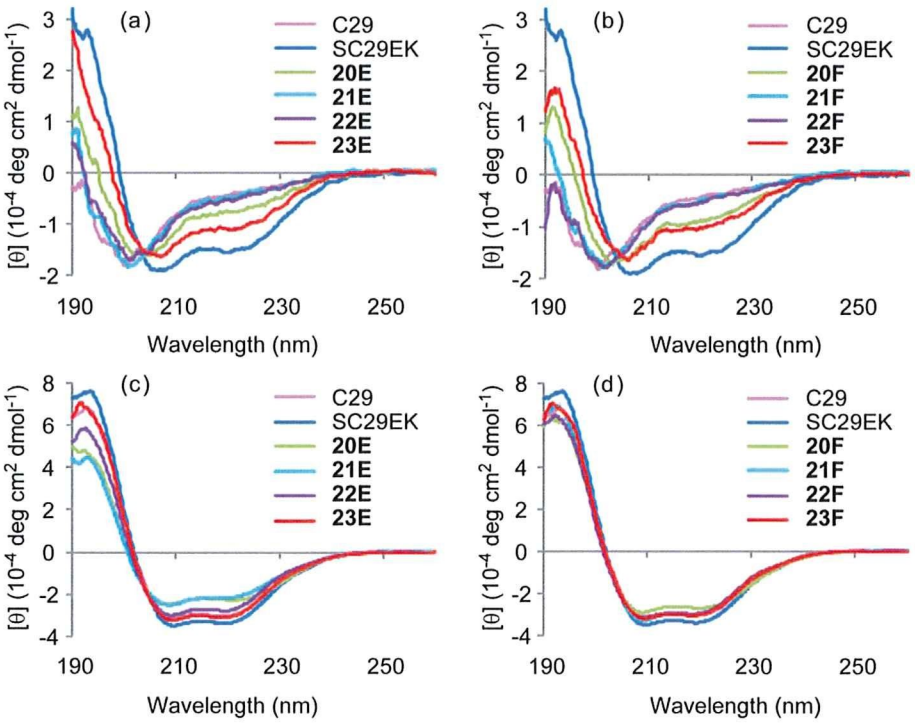


Fig. 2 CD spectra of EADI- and FADI-containing SC29EK analogues in the absence (a,b) and presence (c,d) of N36.

Conclusions

The effects of H-bonds on the stability of the α-helix of an HIV-1 fusion inhibitor were investigated by positional-scanning of the Lys-Lys dipeptides using EADI and FADI. As demonstrated by CD analysis of the SC29EK analogues, H-bonds in the middle of the sequence contribute significantly to the stabilization of the α-helix. In contrast, the effect of H-bonds on the anti-HIV activity of the peptides depends on the distance from the crucial interactive domain. As such, we have shown that EADI and FADI can be used for conformational evaluation of bioactive and/or functional α-helical peptides.

Experimental section

Synthesis

***tert*-Butyl (2*R*,5*S*,3*E*)-2-[4-(*tert*-butyldimethylsiloxy)butyl]-9-[*N*-(2-chlorobenzyloxycarbonyl)amino]-5-[*N*-(2-nitrophenylsulfonyl)amino]non-3-enoate (7).** To a stirred solution of TBSO-$(CH_2)_4$I (236 mg, 0.75 mmol) in dry Et_2O (0.5 cm^3), was added dropwise 1.59 M *t*-BuLi in Et_2O solution (1.0 cm^3, 1.58 mmol) under Ar at –78 °C. After being stirred at this temperature for 30 min, the mixture was stirred at 0 °C for 30 min. To a stirred solution of CuCN (61 mg, 0.61 mmol) and LiCl (52 mg, 1.23 mmol) in dry THF (0.8 cm^3), was added dropwise the above 0.5 M TBSO$(CH_2)_4$Li in THF solution (1.2 cm^3) under Ar at –78 °C, and the mixture was stirred at 0 °C for 10 min. A solution of aziridinyl enoate **6** (91 mg, 0.15 mmol) in dry THF (1.0 cm^3) was added dropwise to the above mixture at –78 °C with stirring, and the stirring was continued for 1.5 h followed by quenching with saturated NH_4Cl/28% NH_4OH solution (1/1, 2.0 cm^3). The mixture was washed with H_2O and brine and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with *n*-hexane-EtOAc (3:1) gave the title compound **7** (72 mg, 60%) as a colorless oil; $[\alpha]^{23}_D$ –73.2 (*c* 0.87 in $CHCl_3$); ν_{max}/cm^{-1} 3349 (NHCO), 1725 (CO); δ_H (500 MHz; $CDCl_3$) 0.04 (6H, s), 0.89 (9H, s), 1.08–1.64 (21H, m), 2.64 (1H, dt, *J* 8.0 and 6.3), 3.07–3.21 (2H, m), 3.55 (2H, t, *J* 6.3), 3.86–3.96 (1H, m), 4.92 (1H, br s), 5.21 (2H, s), 5.26 (1H, dd, *J* 15.5 and 7.5), 5.39 (1H, d, *J* 8.0), 5.40 (1H, dd, *J* 15.5 and 8.0), 7.22–7.30 (2H, m), 7.37 (1H, dd, *J* 5.7 and 2.3), 7.42 (1H, dd, *J* 5.7 and 2.3), 7.65–7.74 (2H, m), 7.83 (1H, dd, *J* 6.9 and 2.3), 8.09 (1H, dd, *J* 6.9 and 2.3); δ_C (100 MHz; $CDCl_3$) –5.3 (2C), 18.3, 22.5, 23.3, 25.9, 28.0 (3C), 29.3 (3C), 32.3, 32.6, 35.4, 40.6, 49.3, 56.7, 62.8, 63.9, 80.6, 125.3, 126.9, 129.3, 129.5, 129.8, 130.9 (2C), 131.2, 132.8, 133.3, 133.5, 134.3, 135.1, 147.8, 156.2, 172.8; *m/z* (FAB) 782.3246 ([M + H]$^+$, $C_{37}H_{57}ClN_3O_9SSi$ requires 782.3273).

(2*R*,5*S*,3*E*)-2-{4-[*N*-(*tert*-Butoxycarbonyl)amino]butyl}-9-[*N*-(2-chlorobenzyloxycarbonyl)amino]-5-[*N*-(9-fluorenylmethoxycarbonyl)amino]non-3-enoic acid (10). To the Fmoc-protected amine **9** (435 mg, 0.52 mmol) was added 4 N HCl/dioxane (5.0 cm^3) at 0 °C, and the mixture was stirred for 20 h at room temperature. Concentration under reduced pressure followed by flash chromatography over silica gel with *n*-hexane-EtOAc (1:1) gave the title compound **10** (391 mg, 96%) as a semisolid; $[\alpha]^{22}_D$ –18.0 (*c* 0.87 in $CHCl_3$); ν_{max}/cm^{-1} 3324 (NHCO), 1703 (CO); δ_H (500 MHz; $CDCl_3$) 1.09–1.81 (12H, m), 2.85–3.01 (1H, m), 3.03–3.21 (4H, m), 4.01–4.21 (2H, m), 4.30–4.54 (2H, m), 4.81–5.26 (6H, m), 5.30–5.81 (3H, m), 7.19–7.41 (13H, m), 7.52–7.59 (2H, m), 7.74 (2H, d, *J* 7.5); δ_C (125 MHz; $CDCl_3$) 22.5, 24.0, 29.2, 29.3, 31.5, 34.3, 40.6 (2C), 47.1, 48.4, 52.3, 63.7, 66.4 (2C), 119.8 (2C), 124.9 (2C), 126.7 (2C), 126.9 (2C), 127.5 (2C), 127.7, 127.9, 128.3, 129.2 (2C), 129.3 (2C), 129.5, 133.3, 134.2, 136.5, 141.1, 143.7 (2C), 143.8 (2C), 155.8, 156.3, 156.4, 178.0; *m/z* (FAB) 782.3201 ([M + H]$^+$, $C_{44}H_{49}ClN_3O_8$ requires 782.3208).

(2*R*,5*S*,3*Z*)-5-[*N*-(*tert*-Butoxycarbonyl)amino]-2-[(*E*)-4-(*tert*-butyldimethylsiloxy)but-2-enyl]-9-(*tert*-butyldimethylsiloxy)-4-fluoronon-3-enoyl (*S*)-sultam (15). To a suspension of CuI (180 mg, 0.94 mmol) in THF (4.8 cm^3) at –78 °C under argon was added dropwise a solution of MeLi·LiBr complex in Et_2O (1.5 M, 1.3 cm^3, 1.89 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 **14** (150 mg, 0.24 mmol) in THF (4.8 cm^3). The mixture was stirred for 30 min at –78 °C and HMPA (0.66 cm^3, 3.78 mmol) was added dropwise to the mixture. After stirring for 30 min at –78 °C, a solution of triphenyltin chloride (182 mg, 0.47 mmol) in THF (3.0 cm^3) was added dropwise, and the mixture was then stirred for 30 min at –40 °C. (*E*)-(4-Bromobut-2-enyloxy)(*tert*-butyl)dimethylsilane (501 mg, 1.89 mmol) in THF (3.0 cm^3) was added dropwise and the mixture was stirred for 20 h at –40 °C. The reaction was quenched at –40 °C by addition of a saturated NH_4Cl/28% NH_4OH solution (1/1, 6.0 cm^3) and the mixture was stirred at room temperature for 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 *n*-hexane-EtOAc (5:1) gave the title compound **15** (148 mg, 78% yield) as a colorless oil; $[\alpha]^{25}_D$ –47.1 (*c* 1.00 in $CHCl_3$); ν_{max}/cm^{-1} 3317 (NHCO), 1693 (CO); δ_H (500 MHz; $CDCl_3$) 0.03 (6H, s), 0.04 (6H, s), 0.88 (9H, s), 0.89 (9H, s), 0.96 (3H, s), 1.15 (3H, s), 1.24–1.64 (17H, m), 1.83–1.91 (3H, m), 2.02–2.05 (2H, m), 2.33–2.37 (1H, m), 2.51–2.55 (1H, m), 3.41 (1H, d, *J* 13.7), 3.49 (1H, d, *J* 13.7), 3.58 (2H, t, *J* 6.3), 3.86 (1H, t, *J* 6.3), 4.06 (2H, d, *J* 3.4), 4.12–4.21 (2H, m), 4.60–4.72 (1H, m), 4.97 (1H, dd, *J* 36.7 and 8.6), 5.58 (2H, m); δ_C (125 MHz; $CDCl_3$) –5.3 (4C), 18.2, 18.3, 19.8, 20.7, 21.9, 25.9 (6C), 26.4, 28.3 (3C), 32.2, 32.3, 32.8, 36.9, 38.3, 41.0, 44.6, 47.6, 48.2, 51.6 (d, *J* 27.6), 53.0, 62.8, 63.5, 65.1, 79.4, 103.3 (d, *J* 12.0), 125.9, 132.7, 154.8, 158.6 (d, *J* 261.5) 172.2; δ_F (470 MHz; $CDCl_3$) –119.1—119.8 (m); *m/z* (FAB) 801.4732 ([M + H]$^+$, $C_{40}H_{74}FN_2O_7SSi_2$ requires 801.4739).

(2*R*,5*S*,3*Z*)-2-{4-[*N*-(Benzyloxycarbonyl)amino]butyl}-9-[*N*-(benzyloxycarbonyl)amino]-5-[*N*-(9-fluorenylmethoxycarbonyl)-amino]-4-fluoronon-3-enoic acid (19). To a solution of the sultam **18** (376 mg, 0.34 mmol) and aqueous 50% H_2O_2 (0.12 cm^3, 1.75 mmol) in THF/H_2O (5/1, 6.0 cm^3) at 0 °C was added aqueous 1 N LiOH (0.67 cm^3, 0.67 mmol), and the mixture was stirred at room temperature for 2 h. After being diluted with EtOAc (20 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 reaction without purification. To a solution of the above acid in CH_2Cl_2 (15 cm^3) at 0 °C was added TFA (4.0 cm^3), and the mixture was stirred at room temperature for 0.5 h. Concentration under reduced pressure gave an oily residue, which was dissolved in MeCN/DMF/H_2O

(10/9/1, 20 cm^3). Fmoc-OSu (159 mg, 0.472 mmol) and Et_3N (0.094 cm^3, 0.675 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 (70 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 *n*-hexane-EtOAc (1:1) gave the title compound **19** (267.3 mg, 65% yield) as a semisolid; $[\alpha]^{24}_D$ −19.6 (*c* 1.13 in DMSO); ν_{max}/cm^{-1} 3333 (OH), 1693 (CO); δ_H (500 MHz; DMSO-d_6) 1.04–1.70 (12H, m), 2.89–3.02 (4H, m), 3.21 (1H, dt, *J* 9.7 and 7.5), 3.98–4.10 (1H, m), 4.22 (1H, t, *J* 6.9), 4.30 (2H, d, *J* 6.9), 4.85 (1H, dd, *J* 37.2 and 9.7), 4.99 (4H, s), 7.19–7.44 (16H, m), 7.65–7.74 (3H, m), 7.89 (2H, d, *J* 7.5), 12.35 (1H, br s); δ_C (125 MHz; DMSO-d_6) 22.6, 23.7, 28.9, 29.0, 30.9, 31.9, 40.0, 40.1, 40.3, 46.6, 51.3 (d, *J* 31.2), 65.0, 65.1, 65.4, 104.0 (d, *J* 12.0), 120.0 (2C), 125.1 (2C), 127.0 (2C), 127.6 (2C), 127.6 (4C), 127.7 (2C), 128.3 (4C), 137.2 (2C), 140.7 (2C), 143.7, 143.8, 155.6, 156.0 (2C), 159.4 (d, *J* 257.9), 174.4; δ_F (470 MHz; DMSO-d_6) −117.9– −118.5 (m); *m/z* (FAB) 766.3512 ([M + H]$^+$, $C_{44}H_{49}FN_3O_8$ requires 766.3504).

General procedure for preparation of peptide by Fmoc-SPPS

The protected peptide chains were constructed on the Novasyn® TGR resin (0.26 mmol g^{-1}, 96 mg, 0.025 mmol). *t*-Bu ester for Asp and Glu; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg; *t*-Bu for Thr, Tyr and Ser; Boc for Lys; and Trt for Gln, Asn and His were employed for side-chain protection. Fmoc-amino acids (0.075 mmol) were coupled by using *N*,*N*′-diisopropylcarbodiimide (DIC; 0.012 cm^3, 0.075 mmol) and *N*-hydroxybenzotriazole monohydrate (HOBt·H_2O, 11.5 mg, 0.075 mmol) in DMF for 2 h. Coupling of dipeptide isosteres (EADI **10**: 49 mg, 0.063 mmol; FADI **19**, 48 mg, 0.063 mmol) was carried out with DIC and HOBt·H_2O for 12 h. The peptide resins were treated with 1 M TMSBr-thioanisole/TFA in the presence of *m*-cresol and 1,2-ethanedithiol as scavengers. The reaction mixture was precipitated with diethyl ether. The resulting powder was collected by centrifugation and then washed three times with diethyl ether. The crude product was purified by preparative HPLC to afford the expected peptides as a colorless powder. The purity of each compound was assessed analytical RP-HPLC prior to the CD analysis and biological testing (>98%).

Anti-HIV-1 activity

Anti-HIV-1 activity was determined by the multinuclear activation of a galactosidase indicator (MAGI) assay as described previously.[14] Briefly, the MAGI cells (10^4 cells $well^{-1}$) were seeded in flat-bottom 96-well microtitre plates. The following day, the cells were inoculated with HIV-1 (60 MAGI units/well, yielding 60 blue cells after 48 h incubation) and cultured in the presence of various concentrations of peptide inhibitors in fresh medium. After 48 h incubation, all the blue cells stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in each well were counted. The activity of inhibitors was determined as the concentration that blocked HIV-1 replication by 50% (50% effective concentration [EC_{50}]).

Measurement of CD spectra

Peptides 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 at intervals of 0.5 °C was performed after a 0.25-min equilibration at the desired temperature and an integration time of 1.0 s. The mid point of the thermal unfolding transition (melting temperature, T_m) of each complex was determined from the maximum of the first derivative, with respect to the reciprocal of the temperature, of the $[\theta]_{222}$ values.

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Structure-activity relationship study on artificial CXCR4 ligands possessing the cyclic pentapeptide scaffold: the exploration of amino acid residues of pentapeptides by substitutions of several aromatic amino acids†

Tomohiro Tanaka,[a] Wataru Nomura,*[a] Tetsuo Narumi,[a,b] Ai Esaka,[b] Shinya Oishi,[b] Nami Ohashi,[a] Kyoko Itotani,[a] Barry J. Evans,[c] Zi-xuan Wang,[c,d] Stephen C. Peiper,[c] Nobutaka Fujii[b] and Hirokazu Tamamura*[a]

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Previously, downsizing of a 14-residue peptidic CXCR4 antagonist **1** has led to the development of a highly potent CXCR4 antagonist **2** [*cyclo*(-D-Tyr[1]-Arg[2]-Arg[3]-Nal[4]-Gly[5]-)]. In the present study, cyclic pentapeptide libraries that were designed by substitutions of several amino acids for D-Tyr[1] and Arg[2] in peptide **2** were prepared and screened to evaluate binding activity for CXCR4. The above structure-activity relationship study led to the finding of several potent CXCR4 ligands.

Introduction

The chemokine receptor CXCR4, which has an endogenous ligand, stromal-cell derived factor-1α (SDF-1α)/CXCL12,[1,2] belongs to the G-protein coupled receptor (GPCR) family.[3,4] The CXCR4-CXCL12 axis plays an important role in various physiological functions: chemotaxis,[5] angiogenesis[6,7] and neurogenesis[8,9] in embryonic stage. However, CXCR4 is also relevant to multiple intractable diseases: AIDS,[10,11] cancer metastasis,[12] progress of leukemia,[13] and rheumatoid arthritis[14] in adulthood. Thus, CXCR4 is thought to be an attractive drug target against these diseases, and CXCR4 antagonists would be useful for the development of potent therapeutic agents.[15–17] Various CXCR4 antagonists such as AMD3100[18,19] and KRH-1636[20] have been reported to date. A 14-residue cyclic peptide CXCR4 antagonist **1** was previously found by structure optimization of an 18-residue bicyclic peptide polyphemusin analogue (Fig. 1).[21,22] Furthermore, the downsizing of **1** using its pharmacophore residues [Arg × 2, L-3-(2-naphthyl)alanine (Nal), Tyr] brought the development of cyclic pentapeptide **2** as a CXCR4 antagonist.[23]

In addition, a biologically stable analogue **3** was derived from **1** with the addition of a 4-fluorobenzoyl group as a new pharmacophore moiety at the N-terminus.[24] We have studied structure-activity-relationship (SAR) of **2** through various modifications such as changes of the ring size and amino acid substitutions.[25–27]

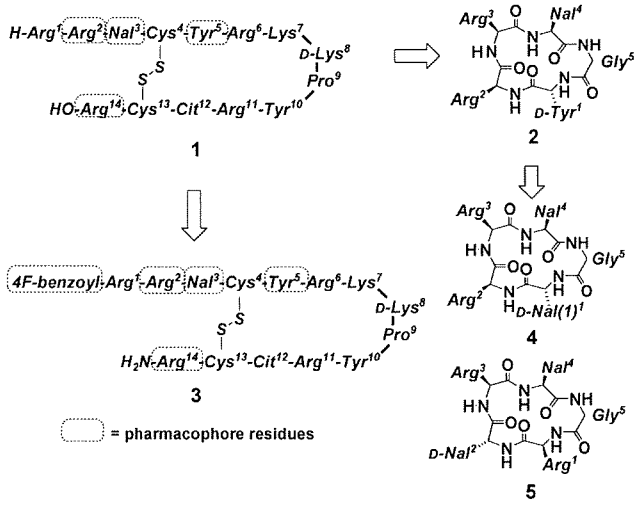


Fig. 1 Development of a cyclic pentapeptide **2** based on the pharmacophore of a CXCR4 antagonistic peptide **1**. Further conversion from **1** into a biostable derivative **3** and from **2** into new cyclic pentapeptide leads **4** and **5**. Cit = L-citrulline, Nal = L-3-(2-naphthyl)alanine, Nal(1) = L-3-(1-naphthyl)alanine.

Potent CXCR4 ligands contain aromatic and cationic groups,[28] suggesting that these groups are involved in binding to CXCR4 mediated by hydrophobic and electrostatic interactions. In a previous study, D-Tyr[1] and Arg[2] in peptide **2** were replaced by a bicyclic aromatic amino acid and a cationic amino acid to identify novel pharmacophores and to find new lead compounds. Compounds **4**, with replacement of D-Tyr[1] by D-3-(1-naphthyl)alanine (D-Nal(1)), and **5** with the sequence of Arg[1]-D-Nal[2] based on shuffling cationic and aromatic amino acids at positions 1 and 2 of compound **2** showed high CXCR4 binding activity.[29] Thus, in this study, the design of a cyclic pentapeptide library based on substitutions of several aromatic amino acids at positions 1 and 2 led to the development of novel analogues of **2** to explore new pharmacophore moieties.

[a]*Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo, 101-0062, Japan. E-mail: nomura.mr@tmd.ac.jp, tamamura.mr@tmd.ac.jp; Fax: +813 5280 8039; Tel: +813 5280 8036*
[b]*Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8501, Japan*
[c]*Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107, USA*
[d]*Department of Surgery, Thomas Jefferson University, Philadelphia, PA 19107, USA*
† Electronic supplementary information (ESI) available: Characterization data (MS and $[\alpha]_D$) and HPLC charts of novel synthetic compounds. See DOI: 10.1039/b908286g

Table 1 Inhibitory activity of the synthetic compounds **6–10** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-**Xaa^1**-**Xaa^2**-Arg^3-Nal^4-Gly^5-). **Xaa^1-Xaa^2**	IC_{50}/μM[a]
2	D-Tyr^1-Arg^2	0.0079
6	D-Phe(4-F)^1-Arg^2	0.22
7	D-Phe(4-F)^1-D-Arg^2	0.31
8	Phe(4-F)^1-Arg^2	0.22
9	Phe(4-F)^1-D-Arg^2	2.2
10	*cyclo*(-D-Tyr^1-Arg^2-Arg^3-Nal^4- Phe(4-F)^5-)	4.4

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

Biological results and discussion

SAR of analogues with L/D-Phe(4-F)^1-L/D-Arg^2

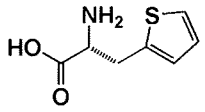
Since compound **6**, where D-Tyr^1 of **2** was replaced by 4-fluoro-D-phenylalanine [D-Phe(4-F)] relevant to the new pharmacophore 4-fluorobenzoyl group, showed relatively potent CXCR4-binding activity as reported previously,[26] initially, **6** and its three analogues **7–9**, [L/D-Phe(4-F)^1, L/D-Arg^2]-**2**, were synthesized to evaluate the configuration effects of amino acids at positions 1 and 2 (Table 1). These analogues except for **9** showed high CXCR4 binding activity (IC_{50} = 0.2–0.4 μM, Table 1), although the potencies were much lower than that of **2**. Compound **9** showed moderate potency (IC_{50} = 2.2 μM), suggesting that the combination of Phe(4-F)^1 and D-Arg^2 is not suitable. In addition, compound **10** was synthesized, where Gly^5 of **2** was replaced by Phe(4-F) with maintenance of D-Tyr^1, since both Phe(4-F) and D-Tyr are thought to be important pharmacophore residues. However, compound **10** did not show high potency (IC_{50} = 4.4 μM), probably due to a conformational change.

SAR of analogues with replacement of D-Phe(4-F)^1 of 6 by an aromatic D-amino acid

Based on the configuration of [D-Phe(4-F)^1, L-Arg^2] of **6**, a series of analogues with replacement of D-Phe(4-F)^1 by several aromatic amino acids were synthesized. The order of preference of halogen atoms as a substituent of position 4 on D-Phe^1 is fluorine, chlorine and bromine as shown in activity of **6**, **11** and **12** (IC_{50} = 0.22, 1.2 and 2.3 μM, respectively, Tables 1 & 2). It suggests that a small or electron-withdrawing group is favorable for a substituent of position 4 on D-Phe^1. Next, preference of positions of fluorine on the phenyl ring of D-Phe^1 was investigated. As a result, the order of preference is *ortho*, *meta* and *para*-positions, as shown in activity of **13**, **14** and **6** (IC_{50} = 0.059, 0.088 and 0.22 μM, respectively). In the previous paper, a D-Nal(1)^1-substituted analogue **4** (IC_{50} = 0.043 μM) showed much higher CXCR4 binding activity than a D-Nal^1-substituted analogue, [D-Nal^1]-**2** (IC_{50} > 2.0 μM).[29] Taken together, a *para*-substituent on the phenyl ring of the D-amino acid residue at position 1 is not appropriate for high potency, possibly due to the steric hindrance between the para-substituent on the phenyl ring and CXCR4. In addition, two other analogues were prepared. Compound **15**, [L/D-Phg^1]-**2** (racemic), did not show high CXCR4 binding activity. Compound **16**, [β-(2-thienyl)-D-alanine (D-Thi)^1]-**2**, showed very potent CXCR4 binding activity, suggesting a thienyl group is relatively suitable for the side-chain of the amino acid at position 1. New leads, **13** and **16**, having D-Phe(2-F)^1 and D-Thi^1, respectively, were found although the potencies were approximately one-eighth of that of **2**.

Table 2 Inhibitory activity of the synthetic compounds **11–16** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-**Xaa^1**-Arg^2-Arg^3-Nal^4-Gly^5-). **Xaa^1**	IC_{50}/μM[a]
2	D-Tyr^1	0.0079
11	D-Phe(4-Cl)^1	1.2
12	D-Phe(4-Br)^1	2.3
13	D-Phe(2-F)^1	0.059
14	D-Phe(3-F)^1	0.088
15	L/D-Phg^1	1.1
16	D-Thi^1	0.056



β-(2-thienyl)-D-alanine (D-Thi)

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

SAR of analogues with Arg^1-aromatic D-amino acid^2

Since **5**, an analogue with the sequence of Arg^1-D-Nal^2 based on shuffling cationic and aromatic amino acids at positions 1 and 2 of compound **2**, showed high CXCR4 binding activity,[29]a series of analogues with the sequence of Arg^1-aromatic D-amino acid^2 (substitution for D-Nal^2) were synthesized. Among halogen substituents at position 4 on the phenyl ring of D-Phe^2, fluorine is the most suitable, whereas chlorine or bromine is not preferable as shown in activity of **17**,[26] **18** and **19** (IC_{50} = 0.035, 0.79 and 0.57 μM, respectively, Table 3). In addition, a 4-nitro group is not suitable although this group is an electron-withdrawing group, possibly due to steric hindrance (**20**, IC_{50} = 0.94 μM). A 4-hydroxy group with electron-donating action and a 4-amino group with strong electron-donating action is not favorable (**21**, IC_{50} = 0.97 μM, **22**, IC_{50} = 15 μM). As a result, fluorine is the most suitable substituent at position 4 on D-Phe^2 among these atoms and groups. In the investigation of the preference of positions of fluorine on the phenyl ring of D-Phe^2, *para*-position is superior to

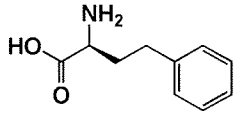
Table 3 Inhibitory activity of the synthetic compounds **17–24** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-**Xaa^1**- **Xaa^2**-Arg^3-Nal^4-Gly^5-). **Xaa^1-Xaa^2**	IC_{50}/μM[a]
2	D-Tyr^1-Arg^2	0.0079
17	Arg^1-D-Phe(4-F)^2	0.035
18	Arg^1-D-Phe(4-Cl)^2	0.79
19	Arg^1-D-Phe(4-Br)^2	0.57
20	Arg^1-D-Phe(4-NO_2)^2	0.94
21	Arg^1-D-Tyr^2	0.97
22	Arg^1-D-Phe(4-NH_2)^2	15
23	Arg^1-D-Phe(2-F)^2	7.1
24	Arg^1-D-Phe(3-F)^2	6.1

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

Table 4 Inhibitory activity of the synthetic compounds **25–31** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-D-Tyr ^{1}-**Xaa^2-Xaa^3**-Nal^4-Gly^5-). **Xaa^2-Xaa^3**	IC_{50}/μM[a]
2	Arg^2-Arg^3	0.0079
25	Hph^2-Arg^3	0.075
26	D-Phg^2-Arg^3	6.0
27	Phg^2-Arg^3	0.17
28	His^2-Arg^3	0.037
29	D-His^2-Arg^3	0.035
30	Arg^2-His^3	5.0
31	His^2-His^2	12

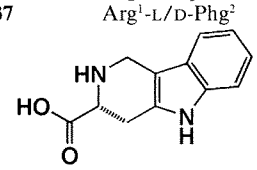


L-homophenylalanine (Hph)

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

Table 5 Inhibitory activity of the synthetic compounds **32–37** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-**Xaa^1**- **Xaa^2**-Arg^3-Nal^4-Gly^5-). **Xaa^1-Xaa^2**	IC_{50}/μM[a]
2	D-Tyr^1-Arg^2	0.0079
32	Arg^1-His^2	0.40
33	Arg^1-D-His^2	0.96
34	Arg^1-D-Thi^2	1.7
35	Arg^1-D-Tpi^2	8.1
36	Arg^1-D-Hph^2	5.0
37	Arg^1-L/D-Phg^2	5.9



(3*R*)-2,3,4,9-tetrahydro-1*H*-β-carboline -3-carboxylic acid (D-Tpi)

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

ortho or *meta* as shown in the activity of **17**, **23** and **24** (IC_{50} = 0.035, 7.1 and 6.1 μM, respectively). In the previous paper, a D-Nal^2-substituted analogue **5** (IC_{50} = 0.045 μM) showed much higher CXCR4 binding activity than a D-Nal(1)^2-substituted analogue, [Arg^1-D-Nal(1)^2]-**2** (IC_{50} > 2.0 μM).[29] Taken together, a *para*-substituent on the phenyl ring of the D-amino acid residue at position 2 is suitable for high potency, possibly due to hydrophobic or π interaction between the *para*-substituent on the phenyl ring and the receptor CXCR4.

SAR of analogues with replacement of Arg^2 of 2 by an aromatic amino acid

Since [D-Tyr^1-Phe(4-F)^2]-**2** showed high CXCR4 binding activity in the previous paper,[26] analogues having incorporation of an aromatic amino acid into position 2 were synthesized. An L-homophenylalanine (Hph)-substituted analogue **25** showed potent CXCR4 binding activity (IC_{50} = 0.075 μM, Table 4), whereas an L-phenylglycine (Phg)-substituted analogue **27** showed lower CXCR4 binding activity (IC_{50} = 0.17 μM), although a D-phenylglycine (D-Phg)-substituted analogue **26** showed even lower CXCR4 binding activity (IC_{50} = 6.0 μM). Since His has both basic and aromatic character, it would be a useful amino acid substitution at position 2. Practically, L- and D-His-substituted analogues **28** and **29** showed high potency (IC_{50} = 0.037 and 0.035 μM, respectively), indicating that the chirality of L/D-His at position 2 does not affect CXCR4 binding. The potencies are approximately one-fourth of that of **2**. Next, we extended the His-substitution to position 3 (Arg^3). Analogues with the sequences of Arg^2-His^3 and His^2-His^3 were synthesized (**30** and **31**, respectively). However, these analogues did not show potent CXCR4 binding activity (IC_{50} = 5.2 and 12 μM, respectively), suggesting that His-substitution for Arg^3 is not appropriate.

SAR of analogues with Arg^1-aromatic-amino acid^2

Among analogues with a combination of the sequences of Arg/His^2-Arg/His^3, **28** having the sequence of His^2-Arg^3 is the most potent compound, with almost the same CXCR4 binding activity as **29** (D-His^2-Arg^3). Thus, **32** and **33**, where D-Tyr^1 of **28** and **29** was replaced by Arg, respectively, were synthesized. However, **32** and **33** are more than 10 fold weaker than **28** and **29** (Table 5), indicating that Arg^1 is not suitable in these analogues. D-Thi, (3*R*)-2,3,4,9-tetrahydro-1*H*-β-carboline-3-carboxylic acid (D-Tpi), D-Hph and L/D-Phg (racemic)-substituted analogues did not show high potency as shown in **34**, **35**, **36** and **37**, respectively. It suggests that these series of analogues with the sequence of Arg^1-aromatic-amino acid^2 are not potent compounds, although **5** and **17** showed high potency.

SAR of analogues with D-Phe(4-F)^1-Arg/His^2-Arg/His^3 or Arg/His^1-D-Phe(4-F)^2-Arg/His^3

Since **6**, [D-Phe(4-F)^1]-**2**, showed moderate CXCR4 binding activity,[26] a series of analogues with the sequence of D-Phe(4-F)^1-Arg/His^2-Arg/His^3 were synthesized (**38**, **39** and **40**). However, significantly potent analogues could not be found (IC_{50} > 10 μM, Table 6). Thus, to interchange the order of positions 1 and 2, a series of analogues with the sequence of Arg/His^1-D-Phe(4-F)^2-Arg/His^3 were synthesized (**41**, **42** and **43**). CXCR4 binding

Table 6 Inhibitory activity of the synthetic compounds **38–43** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-**Xaa^1**- **Xaa^2-Xaa^3**-Nal^4-Gly^5-). **Xaa^1-Xaa^2-Xaa^3**	IC_{50}/μM[a]
2	D-Tyr^1-Arg^2-Arg^3	0.0079
38	D-Phe(4-F)^1-Arg^2-His^3	22
39	D-Phe(4-F)^1-His^2-Arg^3	10
40	D-Phe(4-F)^1-His^2-His^3	>100
41	Arg^1-D-Phe(4-F)^2-His^3	>100
42	His^1-D-Phe(4-F)^2-Arg^3	5.7
43	His^1-D-Phe(4-F)^2-His^3	>100

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1 binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

activities of these analogues are relatively weak (IC_{50} > 5 μM), indicating that incorporation of D-Phe(4-F) at position 1 and His-substitution for Arg^3 are not suitable.

Conclusion

In this paper, SAR of several cyclic pentapeptides having CXCR4 binding activity was studied to discover useful lead compounds. (1) Of the analogues with replacement of D-Tyr^1 of **2** by an aromatic D-amino acid, a D-$Phe(2\text{-}F)^1$-substituted analogue, **13**, and a D-Thi^1-substituted analogue, **16**, have potent CXCR4 binding activity. A para-substituent on the phenyl ring of the D-amino acid residue at position 1 is not favorable for high potency. (2) Among a series of analogues based on shuffling cationic and aromatic amino acids at positions 1 and 2, an [Arg^1-D-$Phe(4\text{-}F)^2$]-containing analogue, **17**, showed the most potent CXCR4 binding activity. A *para*-substituent on the phenyl ring of the D-amino acid residue at position 2 is suitable for high potency. (3) Analogues, where Arg^2 of **2** was replaced by L/D-His which have both basic and aromatic characters, have high CXCR4 binding activity. (4) Arg^1-substituted analogues or His^3-substituted analogues are not potent leads. Taken together, in the present study several new leads were found, and aromatic amino acid residues, Phe(2-F), Phe(4-F), Thi and His, were identified to be new pharmacophore residues in addition to Arg, Nal, Nal(1) and Tyr. The present data will be important for the development of CXCR4 antagonists. In future, the introduction of fluorophenyl, thienyl, imidazoyl groups, *etc.* involving the combinational use of the above groups into the cyclic pentapeptide templates and into low molecular weight linear type scaffolds will bring us the development of new-type leads of CXCR4 antagonists. The present data of preferences of the target CXCR4 such as inclination of aromatic and basic groups will be useful to disclose an unknown detail binding mode of CXCR4 and cyclic pentapeptide-type ligands on the cell membrane.

Experimental

Chemistry

Cyclic peptides were synthesized by Fmoc-based solid-phase synthesis followed by cleavage from the resin, cyclization with the diphenylphosphoryl azide and deprotection, as reported previously.[23]

General. The protected peptide resin (0.100 mmol), which was constructed on H-Gly-(2-chloro)trityl resin manually by Fmoc-based solid phase peptide synthesis (SPPS). *t*-Bu for L/D-Tyr and Pbf for L/D-Arg were used for side-chain protection. Fmoc deprotection was achieved by 20% (v/v) piperidine in DMF (10 mL, 2 × 1 min, 1 × 20 min). Fmoc amino acids were coupled by treatment with five equivalents of reagents [Fmoc-amino acid, *N*,*N*′-diisopropylcarbodiimide (DIPCDI) and HOBt·H_2O] to free amino group in DMF (5 mL) for 1.5 h. The constructed protected peptide resin was subjected to AcOH/TFE/CH_2Cl_2 (1 : 1 : 3 (v/v/v), 10 mL) treatment at room temperature for 2 h. After filtration of the residual resin, the filtrate was concentrated under reduced pressure to give a crude protected peptide. To a stirred mixture of the protected peptide and $NaHCO_3$ (57.1 mg, 0.680 mmol) in DMF (41 mL) was added diphenylphosphoryl azide (DPPA, 0.0879 mL, 0.408 mmol) at –40 °C. The mixture was stirred for 36 h with warming to room temperature and filtered. The filtrate was concentrated under reduced pressure to give an oily residue, which was subjected to solid phase extraction over basic alumina in $CHCl_3$-MeOH (9 : 1 (v/v)) to remove inorganic salts derived from DPPA. The resulting cyclic protected peptide was treated with 95% TFA solution for 1.5 h at room temperature. Concentration under reduced pressure and purification by preparative HPLC gave a cyclic peptide.

CXCR4 receptor binding assay[30]

Stable CHO cell transfectants expressing CXCR4 were prepared as described previously.[31] CHO transfectants were detached by treatment with trypsin-EDTA, allowed to recover in complete growth medium (MEM-R, 100 μg/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 10% FBS (v/v)), and then washed in cold binding buffer (PBS containing 2 mg/mL BSA). For ligand binding, the cells were resuspended in binding buffer at 1×10^7 cell/mL, and 100 μL aliquots were incubated with 0.1 nM of [^{125}I]-SDF-1 (Perkin-Elmer Life Sciences) for 1 h on ice under constant agitation. Free and bound radioligands were separated by centrifugation of the cells through an oil cushion, and bound radioactivity was measured with a gamma-counter (Cobra, Packard, Downers Grove, IL, USA). Inhibitory activity of test compounds was determined based on the inhibition of [^{125}I]-SDF-1 binding to CXCR4 transfectants (IC_{50}).

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

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

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

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ABSTRACT

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

1. Introduction

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

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

2. Results and discussion

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

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

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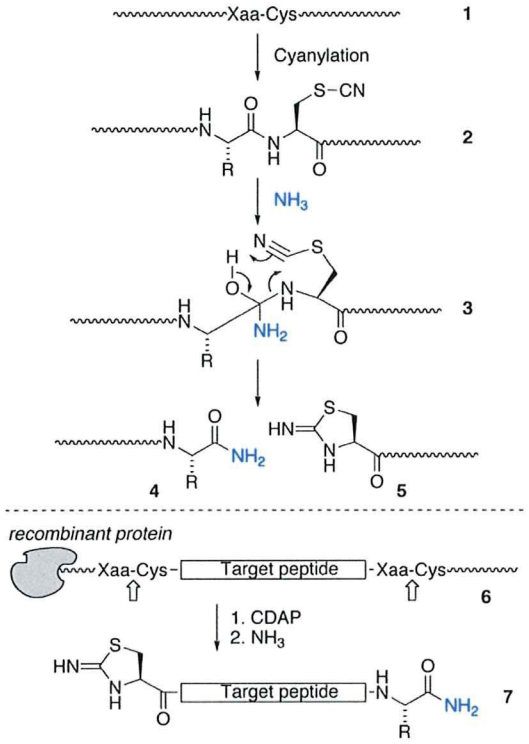
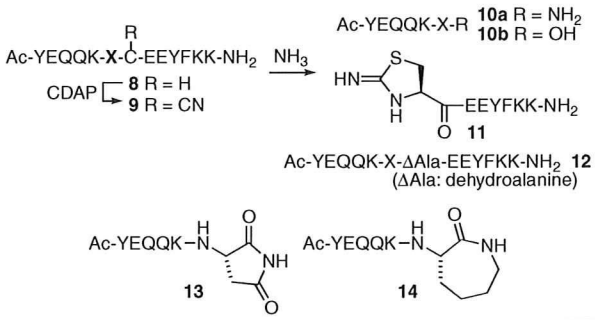


Scheme 1. Site-specific cleavage of recombinant proteins at *S*-cyanocysteine and the preparative illustration of N- and C-terminally capped peptide **7**.

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

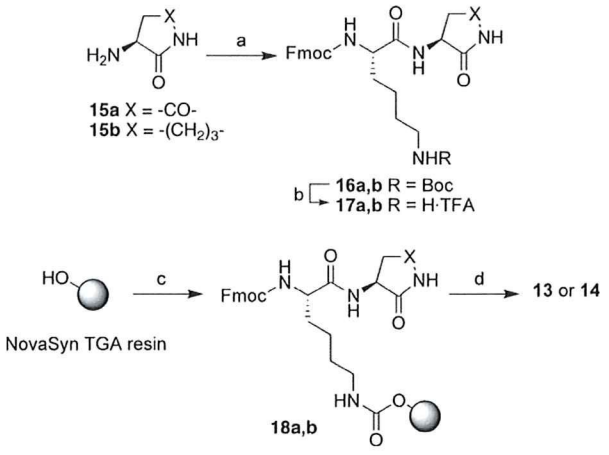
C-terminal cyclic structures in aspartimide **13** and lactam **14** were verified by ESI LC/MS/MS and by the comparative analysis using the peptides that were prepared by the alternative procedures (Scheme 2). Briefly, (*S*)-3-aminosuccinimide **15a** or (*S*)-3-amino-ε-caprolactam **15b** was coupled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

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



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

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



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

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

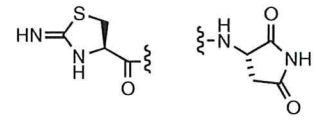
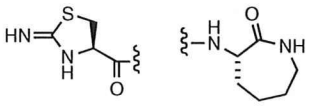
14. Comparative RP-HPLC analysis demonstrated that the peptides were coincident with the ones obtained by cyanocysteine-mediated cleavage (see Supplementary data).

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

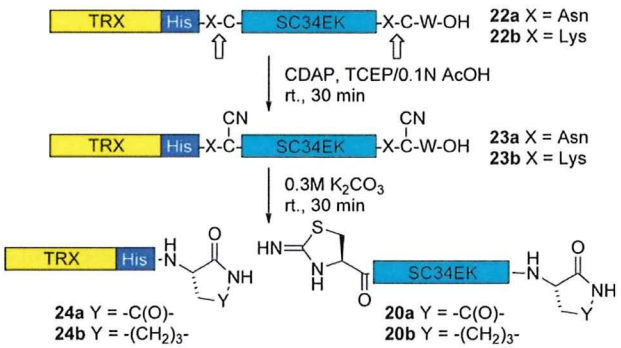
The established protocol was applied to bioorganic synthesis of anti-HIV peptides from recombinant proteins. As a model peptide, HIV fusion inhibitor SC34EK **19** was utilized, which was designed based on the bioactive α-helix conformation of the C-terminal heptad repeat in the envelop glycoprotein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

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

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

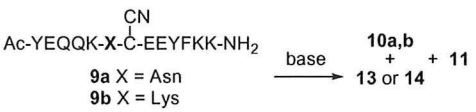
Peptide	R^1-WMEWDRKIEEYTKKIEELIKKSQEQQEKNEKELK-R^2 **19–21** R^1	R^2	EC_{50}[a] (nM)	T_m (°C)
SC34EK **19**	Ac	NH_2	0.60 ± 0.10	71.2
20a			0.48 ± 0.13	71.0
20b			0.58 ± 0.24	71.0
21	H	OH	0.68 ± 0.11	–

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



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

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



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

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

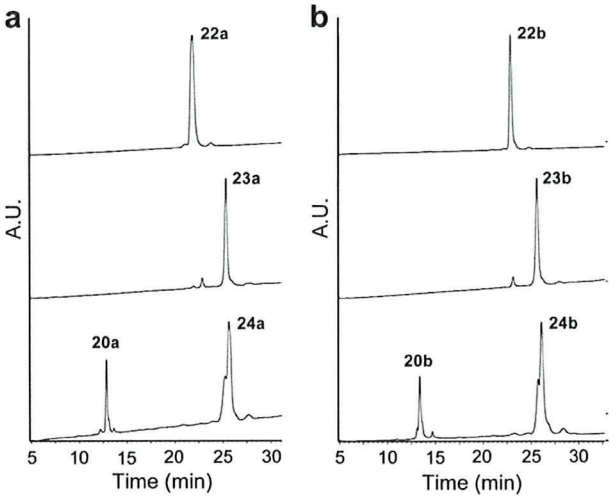


Figure 1. HPLC profiles of *S*-cyanocysteine-mediated cleavage of thioredoxin-fused proteins. (a) Asn-Cys(CN)-mediated cleavage; (b) Lys-Cys(CN)-mediated cleavage. Top: recombinant proteins **22a**,**b** purified by affinity chromatography; middle: S-cyanylated products **23a**,**b**; bottom: products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

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

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

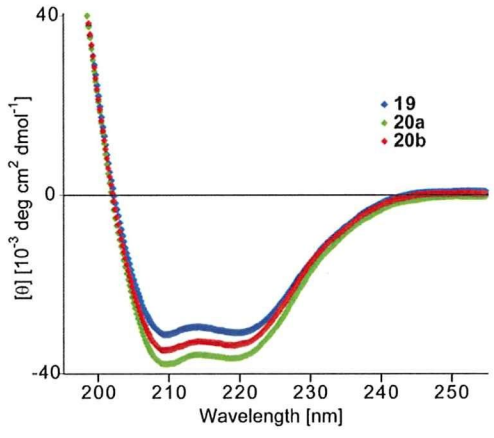


Figure 2. Circular dichroism spectra of N36-SC34EK analogue complexes.

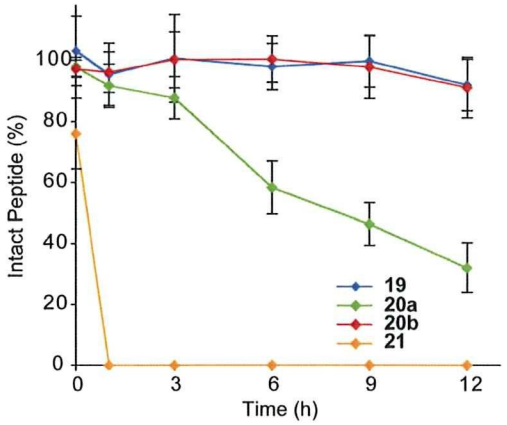


Figure 3. Degradation of SC34EK **19** and the analogues **20a**,**b**, **21** in mouse serum ($n = 5$).

3. Conclusions

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

4. Experimental

4.1. General

For HPLC separations of synthetic peptides, a Cosmosil 5C18-ARII analytical column (4.6 × 250 mm, flow rate 1 mL/min, Nacalai Tesque, Kyoto Japan) or a Cosmosil 5C18-ARII preparative column (20 × 250 mm, flow rate 10 mL/min) was employed. For HPLC analysis of recombinant proteins **22a**,**b**, **23a**,**b** and the cleaved products **20a**,**b** (Fig. 1), a Cosmosil Protein-R analytical column (4.6 × 150 mm, flow rate 1 mL/min) was employed. The eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) were used for HPLC elution. All peptides were characterized by a MALDI-TOF-MS (AXIMA-CFR plus, Shimadzu, Kyoto, Japan) or by a QqTof (QSTAR pulsar i, Applied Biosystems). NMR spectra were recorded on Bruker AVANCE500.

4.2. Peptide synthesis

Protected peptide-resins were manually constructed by standard Fmoc-based SPPS on Rink Amide resin (Novabiochem, 83 mg, 0.05 mmol). *t*-Bu for Tyr, Ser and Thr; *t*-Bu ester for Asp and Glu; Boc for Lys; Trt for Cys, His, Asn and Gln; and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg were employed for side-chain protection, respectively. Fmoc-amino acids were coupled using 5 equiv of reagents [Fmoc-amino acid, *N*,*N*′-diisopropylcarbodiimide and HOBt·H_2O] to free amino group in DMF for 1.5 h. Fmoc deprotection was performed by 20% piperidine in DMF (2 × 1 min, 1 × 20 min). The resulting protected resin was treated with TFA/H_2O/*m*-cresol/thioanisole/1,2-ethanedithiol

(80:5:5:5:5) at room temperature for 2 h. After removal of the resin by filtration, ice-cold dry Et_2O (30 mL) was added to the residue. The resulting powder was collected by centrifugation and then washed with ice-cold dry Et_2O (3 × 15 mL). Purification of the crude product by preparative HPLC afforded a colorless powder of the desired peptide.

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

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

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

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

4.5. Synthesis of compound 16a

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

4.6. Synthesis of compound 16b

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

4.7. Synthesis of resin 18a

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

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

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

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

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