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Certification of the Critical Importance of L-3-(2-Naphthyl)alanine at Position 3 of a Specific CXCR4 Inhibitor, T140, Leads to an Exploratory Performance of Its Downsizing Study

Hirokazu Tamamura,[a,*] Akane Omagari,[a] Kenichi Hiramatsu,[a] Shinya Oishi,[a] Hiromu Habashita,[a] Taisei Kanamoto,[b] Kazuyo Gotoh,[b] Naoki Yamamoto,[c] Hideki Nakashima,[d] Akira Otaka[a] and Nobutaka Fujii[a,*]

[a]*Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*
[b]*Department of Microbiology and Immunology, Kagoshima University Dental School, Sakuragaoka, Kagoshima 890-8544, Japan*
[c]*Tokyo Medical and Dental University, School of Medicine, Bunkyo-ku, Tokyo 113-8519, Japan*
[d]*St. Marianna University, School of Medicine, Miyamae-ku, Kawasaki 216-8511, Japan*

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Abstract—We have previously found that a 14-amino acid residue-peptide, T140, inhibits infection of target cells by T cell line-tropic HIV-1 (X4-HIV-1) through its specific binding to a chemokine receptor, CXCR4. Here, the importance of an L-3-(2-naphthyl)alanine (Nal) residue at position 3 in T140 for high anti-HIV activity and inhibitory activity against Ca^{2+} mobilization induced by stromal cell-derived factor (SDF)-1α-stimulation through CXCR4 has initially been shown by the synthesis and biological evaluation of several analogues, where Nal^3 is substituted by diverse aromatic amino acids. Next, the order of the N-terminal 3 residues (Arg^1-Arg^2-Nal^3) has been proved to be important from the structure–activity relationship (SAR) study shuffling these residues. Based on these results, we have found 10-residue peptides possessing modest anti-HIV activity by systematic antiviral evaluation of a series of synthetic, shortened analogues of T140.

Introduction

The life cycle of HIV-1, particularly at an early stage of HIV-infection such as viral entry, has been elucidated to develop many anti-HIV agents. Although the multiple drug-combination chemotherapy, 'highly active antiretroviral therapy (HAART)', which involves a combination of reverse transcriptase/protease inhibitors, has dramatically improved the clinical treatment of individuals with AIDS or HIV-infection, there still remained several serious problems including the emergence of viral strains with multi-drug resistance, significant adverse effects and high costs.[1] Discovery and development of different types of agents, such as inhibitors of HIV-entry or-fusion, are required for the combination therapy using multi-anti-HIV agents. The recent findings of chemokine receptors, CCR5 and CXCR4, as coreceptors for macrophage-tropic HIV-1 (R5-HIV-1)[2] and T cell line-tropic HIV-1 (X4-HIV-1),[3] respectively, provided an ideal therapeutic approach to discovery of different types of agents. We and others have reported several specific antagonists for the chemokine receptors.[4] We found that an 18-residue peptide amide, T22 ([$Tyr^{5,12}$ Lys^7]-polyphemusin II) (Fig. 1), which had previously been discovered as an anti-HIV peptide, is a specific CXCR4 antagonist that prevents X4-HIV-1 entry mediated by this coreceptor [anti-HIV activity: 50% effective concentration (EC_{50}) = 80 nM, antagonism of entry by X4-HIV-1: EC_{50} = 5.1 nM].[4a,b] T22 is derived from chemical conversions of horseshoe crab self-defense peptides, tachyplesins and polyphemusins. T22 takes an antiparallel β-sheet structure that is maintained by two intrachain disulfide bonds.[5] Based on the structure–activity relationship (SAR) study of T22, we synthesized several effective analogues, such as T134 (des-[$Cys^{8,13}$, $Tyr^{9,12}$]-[D-Lys^{10}, Pro^{11}, L-citrulline $(Cit)^{16}$]-T22 lacking the C-terminal amide) (Fig. 1), which has stronger anti-HIV activity and less cytotoxicity when compared to T22.[6] T134 is a 14-residue peptide with a single disulfide bridge and takes an

*Corresponding author. Tel.: +81-75-753-4551; fax: +81-75-753-4570; e-mail: tamamura@pharm.kyoto-u.ac.jp; nfujii@pharm.kyoto-u.ac.jp

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antiparallel β-sheet structure. Furthermore, the substitution for Trp[3] of T134 with L-3-(2-naphthyl)alanine (Nal) dramatically increased anti-HIV activity as well as inhibitory activity of X4-HIV-1 entry through CXCR4 to yield T140 ([Nal[3]]-T134) (Fig. 1).[7] T140 has the highest level of HIV-1 inhibition activity (anti-HIV activity: $EC_{50} = 3.5$ nM) based on antagonism of entry by X4-HIV-1 ($EC_{50} = 0.43$ nM). Herein, we initially investigated whether Nal[3] of T140 is critical for anti-HIV activity by a comparative study with several analogues, which have diverse aromatic amino acids at position 3. We also discuss the inevitability of the sequential order of the N-terminal 3 residues (Arg[1]-Arg[2]-aromatic residue[3]) using several synthetic analogues, in which the order of the Arg[1]-Arg[2]-aromatic residue[3] was shuffled. Furthermore, several shortened analogues were synthesized based on information concerning the requirement for anti-HIV activity[8] to find efficient small inhibitors.

Results and Discussion

Chemistry

The structures of synthetic T140 analogues are shown in Table 1, Figures 2–4 and Scheme 1. T134, T140 and TA14004 were previously synthesized.[6–8] Other T140 analogues were similarly synthesized by 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase syn-

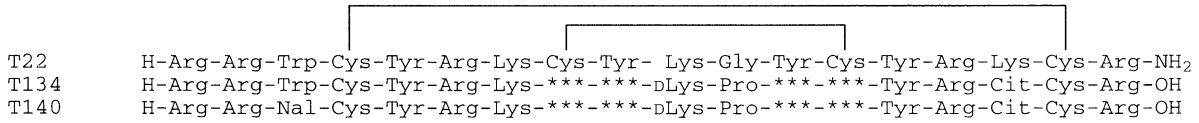
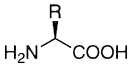
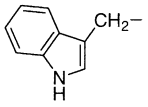
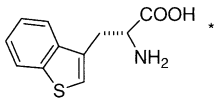
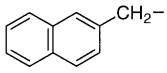
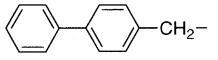
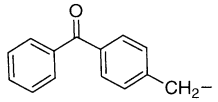
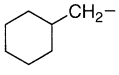
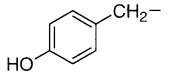
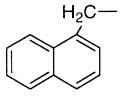
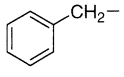
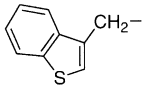


Figure 1. Amino acid sequences of T22, T134 and T140, with alignment based on their homology. The disulfide linkages are shown by solid lines. T22 have two disulfide linkages between Cys[4] and Cys[17] and between Cys[8] and Cys[13] whereas T134 and T140 have one disulfide linkages between Cys[4] and Cys[13]. (***) deletion of an amino acid residue.

Table 1. Structures of amino acid residues at the position 3 of T140 analogues



Compd	R	Compd	R
T134	 Trp	T154	 D-3-benzothienylalanine
T140	 L-3-(2-naphthyl)alanine (Nal)	T156	 L-3-(4-biphenyl)alanine
TA14004	H_3C– Ala	T157	 4-benzoyl-L-phenylalanine
T151	 L-3-cyclohexylalanine	T158	 Tyr
T152	 L-3-(1-naphthyl)alanine	T159	 Phe
T153	 L-3-benzothienylalanine		

The entire of D-3 benzothienylalanine is shown.

thesis. In the synthesis of a heterodimer TL14014, a protected pentapeptidyl resin **1** was constructed by Fmoc-based solid-phase synthesis, followed by treatment with TFA–*m*–cresol–thioanisole-H_2O (80:5:5:5, v/v) in the presence of 2,2′-dithiodipyridine to yield the *S*-(2-pyridylsulfenyl)-pentapeptide **2**. Z(OMe)-Cys(MeOBzl)-Arg(Mts)-NH_2 **3**, which was previously synthesized by solution-phase synthesis,[9] was deprotected by 1 M TMSBr-thioanisole/TFA in the presence of *m*-cresol. The resulting dipeptide **4** was treated with **2** in $AcONH_4$ buffer at pH 6.0 to yield the desired heterodimer TL14014. In the synthesis of a homodimer TL14015, the above protected resin **1** was treated with 1 M TMSBr-thioanisole/TFA in the presence of *m*-cresol and 1,2-ethanedithiol (EDT). The resulting SH-pentapeptide **5** was treated with the above *S*-(2-pyridylsulfenyl)-pentapeptide **2** in $AcONH_4$ buffer at pH 8.0 to yield the desired homodimer TL14015.

Anti-HIV activity and cytotoxicity

First, the anti-HIV activity and cytotoxicity of T140 analogues, where Nal[3] of T140 is substituted by diverse aromatic amino acids (Table 1), are summarized in

```
T140      H-Arg- Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH

TM14001   H-Phe- Arg-Arg-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TM14002   H-Trp- Arg-Arg-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TM14003   H-Nal- Arg-Arg-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TM14004  Benzoyl-Arg-Arg-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TM14005   H-Arg- Phe-Arg-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TM14006   H-Arg-DPhe-Arg-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TM14007   H-Arg- Trp-Arg-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TM14008   H-Arg-DTrp-Arg-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TM14009   H-Arg- Nal-Arg-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TM14010   H-Arg-DNal-Arg-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH

TD14001   H-Arg- Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-***-OH
TD14002   H-***- Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TD14003   H-***- ***-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TD14004   H-***- ***-***-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
```

Figure 2. Amino acid sequences of T140 analogues, TM14001–TM14010, where the order of Arg[1]-Arg[2]-aromatic residue[3] was shuffled each other, and TD14001–TD14004, where amino acid residue(s) in the N- or C-terminal region was deleted.

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T140      H-Arg- Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH

TD14011   H-Arg- Arg-Nal-Cys-***-***-Lys-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14012   H-Arg- Nal-Arg-Cys-***-***-Lys-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14013   H-Arg-DNal-Arg-Cys-***-***-Lys-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14014   H-Nal- Arg-Arg-Cys-***-***-Lys-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14021   H-Arg- Arg-Nal-Cys-Tyr-***-***-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14022   H-Arg- Nal-Arg-Cys-Tyr-***-***-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14023   H-Arg-DNal-Arg-Cys-Tyr-***-***-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14024   H-Nal- Arg-Arg-Cys-Tyr-***-***-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14031   H-Arg- Arg-Nal-Cys-***-***-Lys-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14032   H-Arg- Nal-Arg-Cys-***-***-Lys-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14033   H-Arg-DNal-Arg-Cys-***-***-Lys-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14034   H-Nal- Arg-Arg-Cys-***-***-Lys-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14041   H-Arg- Arg-Nal-Cys-Tyr-***-***-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14042   H-Arg- Nal-Arg-Cys-Tyr-***-***-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14043   H-Arg-DNal-Arg-Cys-Tyr-***-***-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14044   H-Nal- Arg-Arg-Cys-Tyr-***-***-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14051   H-Arg- Arg-Nal-Cys-***-Arg-***-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14052   H-Arg- Nal-Arg-Cys-***-Arg-***-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14053   H-Arg-DNal-Arg-Cys-***-Arg-***-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14054   H-Nal- Arg-Arg-Cys-***-Arg-***-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14061   H-Arg- Arg-Nal-Cys-***-Arg-***-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14062   H-Arg- Nal-Arg-Cys-***-Arg-***-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14063   H-Arg-DNal-Arg-Cys-***-Arg-***-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14064   H-Nal- Arg-Arg-Cys-***-Arg-***-DLys-Pro-***-***-Cit-Cys-Arg-OH
TD14071   H-Arg- Arg-Nal-Cys-***-Arg-***-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14072   H-Arg- Nal-Arg-Cys-***-Arg-***-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14073   H-Arg-DNal-Arg-Cys-***-Arg-***-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14074   H-Nal- Arg-Arg-Cys-***-Arg-***-DLys-Pro-Tyr-***-***-Cys-Arg-OH
TD14081   H-Arg- Arg-Nal-Cys-***-***-Lys-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14082   H-Arg- Nal-Arg-Cys-***-***-Lys-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14083   H-Arg-DNal-Arg-Cys-***-***-Lys-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14084   H-Nal- Arg-Arg-Cys-***-***-Lys-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14091   H-Arg- Arg-Nal-Cys-Tyr-***-***-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14092   H-Arg- Nal-Arg-Cys-Tyr-***-***-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14093   H-Arg-DNal-Arg-Cys-Tyr-***-***-DLys-Pro-***-Arg-***-Cys-Arg-OH
TD14094   H-Nal- Arg-Arg-Cys-Tyr-***-***-DLys-Pro-***-Arg-***-Cys-Arg-OH
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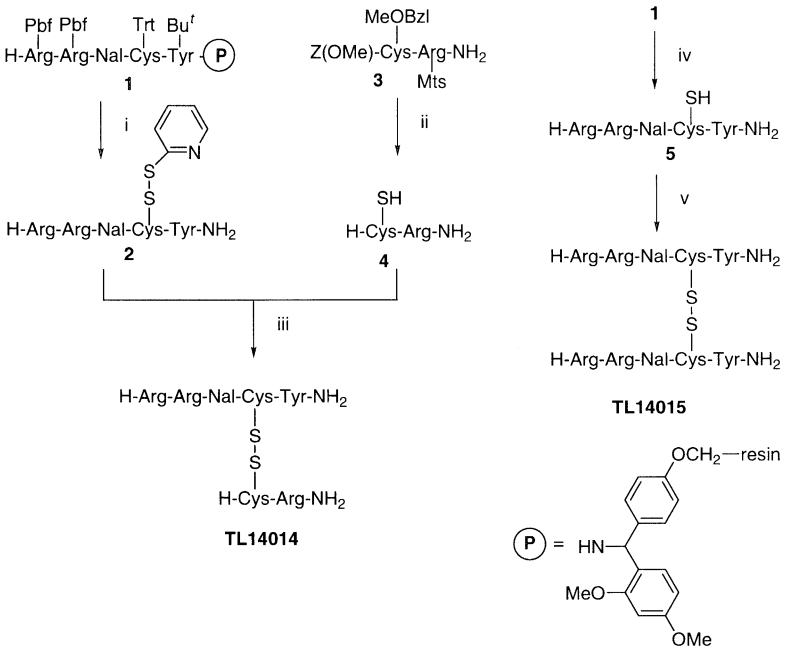
Figure 3. Amino acid sequences of shortened analogues of T140, TD14011–TD14094.



Scheme 1. Reagents: (i) TFA–*m*-cresol–thioanisole–H_2O (85:5:5:5, v/v),. 2,2′-dithiodipyridine; (ii) 1 M TMSBr-thioanisole/TFA, *m*-cresol; (iii) 0.4 M aq $AcONH_4$, pH 6.0; (iv) 1 M TMSBr-thioanisole/TFA, *m*-cresol, EDT; (v) **2**, 0.4 M aq $AcONH_4$, pH 8.0; Pbf = 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl.

Table 2. 50% inhibitory concentration (IC_{50}) values are based on the inhibition of Ca^{2+} mobilization induced by stromal cell-derived factor (SDF)-1α-stimulation through CXCR4.[10] EC_{50} values are based on the inhibition of HIV-1-induced cytopathogenicity in MT-4 cells. 50% cytotoxic concentration (CC_{50}) values are based on the reduction of the viability of mock-infected cells. The selectivity index (SI) is shown as CC_{50}/EC_{50}. As shown in Table 2, T140 exhibited the highest anti-HIV activity and inhibitory activity of Ca^{2+} mobilization. Both anti-HIV activity and inhibitory activity of Ca^{2+} mobilization are decreased in the order T140 > T134 (Trp^3-substitution) > T158 (Tyr^3-substitution) > T159 (Phe^3-substitution) > T154 (D-3-benzothienylalanine^3-substitution). T151 (L-3-cyclohexylalanine^3-substitution), T152 [L-3-(1-naphthyl)alanine^3-substitution], T153 (L-3-benzothienylalanine^3-substitution), T156 [L-3-(4-biphenyl)alanine^3-substitution] and T157 (4-benzoyl-L-phenylalanine^3-substitution) have neither high anti-HIV activity nor inhibitory activity of Ca^{2+} mobilization, although these analogues, containing an aromatic amino acid residue at position 3, have stronger anti-HIV activity and inhibitory activity of Ca^{2+} mobilization than TA14004 (Ala^3-substitution),[8] except for the inhibitory activity of Ca^{2+} mobilization of T157. T140 (Nal^3) showed much stronger anti-HIV activity and inhibitory activity of Ca^{2+} mobilization than T152 [L-3-(1-naphthyl)alanine^3-substitution], indicating that precise interaction is required between residue 3 and CXCR4. These results suggest that Nal is a critical residue at position 3, and that aromatic residues are at least preferred to Ala as residue 3. In addition, there is a clear correlation in these T140 analogues between anti-HIV activity and CXCR4-antagonism (inhibitory activity of Ca^{2+} mobilization), suggesting that these peptides inhibit the X4-HIV-1 infection mediated by CXCR4. These analogues do not possess significant cytotoxicity (CC_{50} > 80 μM, except for CC_{50} of T156 = 44 μM). Since the cytotoxicity of T134 and T140 was previously evaluated as CC_{50} > 40 μM, further estimation at high concentrations was omitted in this study.[6,7]

Second, in order to investigate the inevitability of the sequential order of the N-terminal 3 residues (Arg^1-Arg^2-aromatic residue^3), we synthesized several analogues, where the order of the Arg^1-Arg^2-aromatic residue^3 was shuffled. As aromatic residues, we selected Nal, Trp and Phe, which gave satisfactory results in the above experiment, and these D-isomers (Fig. 2; TM14001–TM14010). TM14004 possesses an α-benzoyl group instead of an N-terminal aromatic residue. As a

Table 2. Inhibitory activity on SDF-1-induced Ca^{2+} mobilization, anti-HIV activity and cytotoxicity of T140 analogues with substituted residues at the position 3

Entry	Compd	IC_{50} (nM)	EC_{50} (nM)	CC_{50} (μM)	SI
1	T134	30	8.3	> 10	> 1200
2	T140	6.9	3.4	> 10	> 2900
3	TA14004	2000	7400	> 80	> 11
4	T151	420	360	> 80	> 220
5	T152	290	290	> 80	> 280
6	T153	1200	1100	> 80	> 72
7	T154	110	91	> 80	> 870
8	T156	650	990	44	44
9	T157	2900	620	> 80	> 130
10	T158	35	16	> 80	> 5100
11	T159	42	73	> 80	> 1100
12	AZT	NT	79	240	3000
13	ddC	NT	7500	120	16

IC_{50} values are the concentrations for 50% inhibition of Ca^{2+} mobilization induced by SDF-1-stimulation through CXCR4. EC_{50} values are the concentrations for 50% protection of HIV-induced cytopathogenicity in MT-4 cells. CC_{50} values are based on the reduction of the viability of mock-infected cells. SI is shown as CC_{50}/EC_{50}. All data are mean values of at least two experiments for IC_{50} or three experiments for EC_{50} and CC_{50}.

result, analogues with high anti-HIV activity (EC_{50} <0.79 μM) could not be found, suggesting that the sequential order of the Arg^1-Arg^2-aromatic residue^3 is critical for high anti-HIV activity (Table 3). Since anti-HIV activity of all these analogues is very weak, we did not estimate the inhibitory activity of Ca^{2+} mobilization of these analogues.

Third, we examined whether the amino acid residues in the N- and C-terminal regions of T140 can be deleted with maintenance of the anti-HIV activity. A C-terminally deleted analogue, TD14001 (Arg^{14}-deletion), and N-terminally deleted analogues, TD14002 (Arg^1-deletion), TD14003 (Arg^1-Arg^2-deletion) and TD14004 (Arg^1-Arg^2-Nal^3-deletion), were synthesized to evaluate anti-HIV activity. As a result, these analogues did not possess high anti-HIV activity, showing that even Arg^1 or Arg^{14} can not be deleted to exhibit high activity. According to our previous SAR study, Arg^2, Nal^3, Tyr^5 and Arg^{14} are found to be the indispensable residues for the strong anti-HIV activity of T140.[8] Our previous results are compatible with the present results showing that the anti-HIV activities of TD14001, TD14003 and TD14004 are relatively weak.

Fourth, since it has been proven that the N-terminal 3 residues and the C-terminal residue of T140 can not be deleted, we investigated whether amino acid residues between two Cys residues of T140 can be deleted with maintenance of the anti-HIV activity. According to our previous conformational analysis,[11] T140 takes an antiparallel and pleated β-sheet structure extending from residue 3 to 14 with a type II′ β-turn formed by Lys^7 (*i*), D-Lys^8 ($i+1$), Pro^9 ($i+2$) and Tyr^{10} ($i+3$). In the synthesis of shortened analogues, two residues from each β-strand of T140 (total four residues) were deleted to design 10-residue peptides. In combination, we also investigated the inevitability of the sequential order of the N-terminal 3 residues (Arg^1-Arg^2-Nal^3) by shuffling these residues to obtain double assurance. In this case, D-Nal was also utilized as an alternative to Nal. Thus, 36 analogues (Fig. 3; TD14011–TD14094) were synthesized to evaluate anti-HIV activity (Table 4). As shown in Table 4, all these analogues possess modest anti-HIV activity (EC_{50} <60 μM), except for TD14084. TD14041 (N-terminus: Arg^1-Arg^2-Nal^3, deletion of Arg^6-Lys^7 and Arg^{11}-Cit^{12}) is the top analogue having relatively high activity ($EC_{50}=590$ nM), whereas TD14041 is more than two orders of magnitude less potent than T140 ($EC_{50}=3.4$ nM), suggesting the importance of residues between two Cys residues.

Fifth, the biological effect of deletion of amino acid residues contained in T140 was investigated using several linear peptides (Fig. 4; TL14001–TL14013). TL14001 ([$Ala^{4,13}$]-T140), where two Cys residues of T140 were substituted by Ala, showed more than 3000 times lower anti-HIV activity ($EC_{50}=11$ μM) (Table 5), when compared to T140. TL14002, where two Cys residues of the above TD14041 were substituted by Ala, showed two orders of magnitude lower anti-HIV activity ($EC_{50}=74$ μM) than TD14041. Comparison between EC_{50}s of T140 and TL14001 and between those of TD14041 and TL14002 suggests that globally conformational restriction by the disulfide bridge is necessary for high anti-HIV activity. TL14002–TL14013, which are linear-shortened analogues containing at least the N-terminal 3 residues of T140, did not exhibit high anti-HIV activity. Since the disulfide bridge is thought

Table 3. Anti-HIV activity and cytotoxicity of T140 analogues (TM14001–TM14010 and TD14001–TD14004)

Entry	Compd	EC_{50} (μM)	CC_{50} (μM)	SI
1	TM14001	0.79	>160	>210
2	TM14002	7.4	>160	>22
3	TM14003	2.5	>160	>65
4	TM14004	12	>160	>15
5	TM14005	7.4	>160	>22
6	TM14006	1.6	>160	>10
7	TM14007	24	>160	>7
8	TM14008	3.7	>160	>44
9	TM14009	4.5	>160	>36
10	TM14010	7.9	>160	>20
11	TD14001	2.5	>160	71
12	TD14002	2.8	>160	65
13	TD14003	17	>160	12
14	TD14004	>160	>160	ND

EC_{50} and CC_{50} values are calculated in the same way as in Table 2. ND, not determined.

Table 4. Anti-HIV activity and cytotoxicity of T140 analogues (TD14011–TD14094)

Entry	Compd	EC_{50} (μM)	CC_{50} (μM)	SI
1	TD14011	34	>110	>3
2	TD14012	24	>110	>5
3	TD14013	41	>110	>3
4	TD14014	42	>110	>3
5	TD14021	55	>110	>2
6	TD14022	14	>110	>8
7	TD14023	55	>110	>2
8	TD14024	50	>110	>2
9	TD14031	43	>110	>3
10	TD14032	30	>110	>4
11	TD14033	48	>110	>2
12	TD14034	52	>110	>2
13	TD14041	0.59	>110	>200
14	TD14042	19	>110	>6
15	TD14043	50	>110	>2
16	TD14044	39	>110	>3
17	TD14051	28	>110	>4
18	TD14052	21	>110	>5
19	TD14053	54	>110	>2
20	TD14054	46	>110	>2
21	TD14061	6.6	>110	>17
22	TD14062	11	>110	>10
23	TD14063	18	>110	>6
24	TD14064	14	>110	>8
25	TD14071	13	>110	>9
26	TD14072	27	>110	>4
27	TD14073	7.3	>110	>14
28	TD14074	9.8	>110	>11
29	TD14081	13	>110	>8
30	TD14082	4.8	>110	>23
31	TD14083	35	>110	>3
32	TD14084	>110	>110	ND
33	TD14091	11	>110	>10
34	TD14092	20	>110	>6
35	TD14093	17	>110	>7
36	TD14094	37	>110	>3

EC_{50} and CC_{50} values are calculated in the same way as in Table 2.

to be necessary for small-sized peptides to express high anti-HIV activity, a heterodimer TL14014 and a homodimer TL14015 were synthesized (Scheme 1). TL14014 possesses the minimum indispensable structure unit containing the N-terminal 3 residues, the C-terminal Arg^{14} and the disulfide bond of T140. TL14015 has a similar structure unit containing two Arg residues instead of the C-terminal Arg^{14} of T140. TL14014 exhibited weak anti-HIV activity ($EC_{50} = 55$ μM), whereas its corresponding monomer TL14013 was essentially inactive even at 100 μM. TL14015 showed much higher anti-HIV activity ($EC_{50} = 1.2$ μM) than TL14013. These results indicate that the moiety containing Arg residue(s), which is linked to H-Arg-Arg-Nal-Cys(S-)-Tyr-NH_2 by the disulfide bridge, is indispensable for high anti-HIV activity.

In conclusion, we have shown that Nal^3 of T140 is critically important for both high anti-HIV activity and antagonism of SDF-1-induced Ca^{2+} mobilization through CXCR4, that the sequential order of the N-terminal 3 residues (Arg^1-Arg^2-Nal^3) is also critical for

Table 5. Anti-HIV activity and cytotoxicity of T140 analogues (TL14001–TL14015)

Entry	Compd	EC_{50} (μM)	CC_{50} (μM)	SI
1	TL14001	11	>100	>9
2	TL14002	74	>100	>1
3	TL14003	>100	>100	ND
4	TL14004	>100	>100	ND
5	TL14005	>100	>100	ND
6	TL14006	>100	>100	ND
7	TL14007	82	>100	>1
8	TL14008	>100	>100	ND
9	TL14009	>100	>100	ND
10	TL14010	65	>100	>1
11	TL14011	>100	>100	ND
12	TL14012	>100	>100	ND
13	TL14013	>100	>100	ND
14	TL14014	55	>100	>2
15	TL14015	1.2	59	48

EC_{50} and CC_{50} values are calculated in the same way as in Table 2.

Table 6. Characterization data of the synthetic T140 analogues (No. 1, T151–T159, TM14001–TM14010 and TD14001–TD14024)

Entry	Compd	Yield (%)	$[\alpha]_D$	*c*	Temperature	Formula	IS-MS	
							Found	Calcd
1	T151	26	−0.016	1.05	28	$C_{86}H_{145}N_{33}O_{18}S_2$	1993.0	1993.4
2	T152	22	−0.016	1.15	28	$C_{90}H_{141}N_{33}O_{18}S_2$	2036.5	2037.4
3	T153	51	−21.3	1.60	24	$C_{88}H_{142}N_{34}O_{17}S_3$	2044.1	2044.5
4	T154	29	−0.017	0.875	28	$C_{88}H_{142}N_{34}O_{17}S_3$	2042.0	2044.5
5	T156	13	−7.05	0.850	23	$C_{92}H_{143}N_{33}O_{18}S_2$	2062.7	2063.5
6	T157	4	0.012	0.051	26	$C_{93}H_{143}N_{33}O_{19}S_2$	2090.2	2091.5
7	T158	50	−20.0	1.55	25	$C_{86}H_{139}N_{33}O_{19}S_2$	2003.6	2003.4
8	T159	34	−22.9	1.05	25	$C_{86}H_{139}N_{33}O_{18}S_2$	1987.5	1987.4
9	TM14001	23	−28.0	0.501	27	$C_{86}H_{139}N_{33}O_{18}S_2$	1987.2	1987.4
10	TM14002	18	−33.3	0.616	30	$C_{88}H_{140}N_{34}O_{18}S_2$	2026.7	2026.4
11	TM14003	3	−93.3	0.150	30	$C_{90}H_{141}N_{33}O_{18}S_2$	2037.0	2037.4
12	TM14004	6	−20.0	0.324	29	$C_{84}H_{134}N_{32}O_{18}S_2$	1944.0	1944.3
13	TM14005	21	−8.92	0.560	29	$C_{86}H_{139}N_{33}O_{18}S_2$	1987.2	1987.4
14	TM14006	36	−27.8	0.395	25	$C_{86}H_{139}N_{33}O_{18}S_2$	1987.7	1987.4
15	TM14007	21	−12.2	0.410	26	$C_{88}H_{140}N_{34}O_{18}S_2$	2026.2	2026.4
16	TM14008	35	−8.52	0.469	25	$C_{88}H_{140}N_{34}O_{18}S_2$	2026.2	2026.4
17	TM14009	8	−14.0	0.570	27	$C_{90}H_{141}N_{33}O_{18}S_2$	2037.0	2037.4
18	TM14010	16	−15.6	0.514	24	$C_{90}H_{141}N_{33}O_{18}S_2$	2037.0	2037.4
19	TD14001	19	−11.3	0.354	23	$C_{84}H_{129}N_{29}O_{17}S_2$	1881.5	1881.2
20	TD14002	17	18.6	0.590	23	$C_{84}H_{129}N_{29}O_{17}S_2$	1881.0	1881.2
21	TD14003	36	2.92	0.342	23	$C_{78}H_{117}N_{25}O_{16}S_2$	1725.0	1725.1
22	TD14004	23	−21.9	0.593	22	$C_{65}H_{106}N_{24}O_{15}S_2$	1527.0	1527.8
23	TD14011	23	−48.9	0.613	28	$C_{63}H_{97}N_{21}O_{12}S_2$	1405.0	1404.7
24	TD14012	16	−45.6	0.417	30	$C_{63}H_{97}N_{21}O_{12}S_2$	1405.0	1404.7
25	TD14013	8	−73.1	0.219	30	$C_{63}H_{97}N_{21}O_{12}S_2$	1405.0	1404.7
26	TD14014	18	−19.6	0.459	30	$C_{63}H_{97}N_{21}O_{12}S_2$	1405.0	1404.7
27	TD14021	5	−15.6	0.128	26	$C_{63}H_{96}N_{22}O_{13}S_2$	1434.5	1433.7
28	TD14022	11	−7.19	0.278	25	$C_{63}H_{96}N_{22}O_{13}S_2$	1434.0	1433.7
29	TD14023	9	−21.8	0.229	25	$C_{63}H_{96}N_{22}O_{13}S_2$	1434.0	1433.7
30	TD14024	18	−4.38	0.456	25	$C_{63}H_{96}N_{22}O_{13}S_2$	1435.2	1433.7

Yields were calculated from the corresponding Alko-resins. IS-MS (Found) is reconstructed mass.

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T140       H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH

TL14001    H-Arg-Arg-Nal-Ala-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Ala-Arg-OH
TL14002    H-Arg-Arg-Nal-Ala-Tyr-***-***-DLys-Pro-Tyr-***-***-Ala-Arg-OH
TL14003    H-Arg-Arg-Nal-Ala-***-***-***-DLys-Pro-Tyr-***-***-Ala-Arg-OH
TL14004    H-Arg-Arg-Nal-Ala-Tyr-***-***-DLys-Pro-***-***-***-Ala-Arg-OH
TL14005    H-Arg-Arg-Nal-Ala-***-***-***-DLys-Pro-***-***-***-Ala-Arg-OH
TL14006    H-Arg-Arg-Nal-***-***-***-***-DLys-Pro-***-***-***-***-Arg-OH
TL14007    H-Arg-Arg-Nal-Ala-Tyr-***-***-DLys-Pro-***-***-***-***-Arg-OH
TL14008    H-Arg-Arg-Nal-***-***-***-***-DLys-Pro-Tyr-***-***-Ala-Arg-OH
TL14009    H-Arg-Arg-Nal-***-***-***-***-DLys-Pro-Tyr-***-***-***-Arg-OH
TL14010    H-Arg-Arg-Nal-***-DTyr-***-***-***-Pro-***-***-***-***-Arg-OH
TL14011    H-Arg-Arg-Nal-***-DTyr-***-***-***-***-***-***-***-***-Arg-OH
TL14012    H-Arg-Arg-Nal-Ala-Tyr-***-***- ***-***-***-***-***-***-***-Arg-OH
TL14013    H-Arg-Arg-Nal-Ala-Tyr-***-***- ***-***-***-***-***-***-***-OH
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Figure 4. Amino acid sequences of linear analogues of T140, TL14001–TL14013.

Table 7. Characterization data of the synthetic T140 analogues (No. 2, TD14031–TD14094)

Entry	Compd	Yield (%)	$[\alpha]_D$	*c*	Temperature	Formula	IS-MS	
							Found	Calcd
31	TD14031	21	−7.61	1.182	20	$C_{60}H_{99}N_{23}O_{12}S_2$	1399.0	1398.7
32	TD14032	15	−13.85	0.794	22	$C_{60}H_{99}N_{23}O_{12}S_2$	1399.0	1398.7
33	TD14033	27	−8.58	1.397	22	$C_{60}H_{99}N_{23}O_{12}S_2$	1399.0	1398.7
34	TD14034	18	−27.13	0.479	23	$C_{60}H_{99}N_{23}O_{12}S_2$	1399.0	1398.7
35	TD14041	10	91.60	0.262	23	$C_{60}H_{99}N_{23}O_{12}S_2$	1398.0	1398.7
36	TD14042	9	18.34	0.218	24	$C_{60}H_{99}N_{23}O_{12}S_2$	1399.0	1398.7
37	TD14043	11	−25.83	0.271	24	$C_{60}H_{99}N_{23}O_{12}S_2$	1399.0	1398.7
38	TD14044	16	−35.44	0.395	24	$C_{60}H_{99}N_{23}O_{12}S_2$	1398.0	1398.7
39	TD14051	39	−22.81	1.096	21	$C_{60}H_{100}N_{26}O_{11}S_2$	1426.1	1425.7
40	TD14052	23	−33.43	0.628	24	$C_{60}H_{100}N_{26}O_{11}S_2$	1426.1	1425.7
41	TD14053	13	−28.08	0.356	24	$C_{60}H_{100}N_{26}O_{11}S_2$	1425.3	1425.7
42	TD14054	11	−32.36	0.309	25	$C_{60}H_{100}N_{26}O_{11}S_2$	1426.0	1425.7
43	TD14061	27	−21.24	0.706	25	$C_{60}H_{99}N_{25}O_{12}S_2$	1426.7	1426.7
44	TD14062	15	−7.59	0.395	26	$C_{60}H_{99}N_{25}O_{12}S_2$	1426.7	1426.7
45	TD14063	19	−27.23	0.514	26	$C_{60}H_{99}N_{25}O_{12}S_2$	1426.5	1426.7
46	TD14064	14	−20.99	0.381	26	$C_{60}H_{99}N_{25}O_{12}S_2$	1426.7	1426.7
47	TD14071	28	−25.40	0.748	26	$C_{63}H_{97}N_{23}O_{12}S_2$	1433.0	1432.7
48	TD14072	12	9.61	0.312	26	$C_{63}H_{97}N_{23}O_{12}S_2$	1433.0	1432.7
49	TD14073	3	−46.05	0.152	24	$C_{63}H_{97}N_{23}O_{12}S_2$	1433.0	1432.7
50	TD14074	3	−18.40	0.163	27	$C_{63}H_{97}N_{23}O_{12}S_2$	1434.0	1432.7
51	TD14081	34	−27.71	0.938	25	$C_{60}H_{100}N_{24}O_{11}S_2$	1398.0	1397.7
52	TD14082	28	−33.37	0.779	28	$C_{60}H_{100}N_{24}O_{11}S_2$	1398.0	1397.7
53	TD14083	21	−49.31	0.588	28	$C_{60}H_{100}N_{24}O_{11}S_2$	1397.6	1397.7
54	TD14084	23	−31.84	0.628	28	$C_{60}H_{100}N_{24}O_{11}S_2$	1397.6	1397.7
55	TD14091	37	−19.62	0.968	26	$C_{63}H_{97}N_{23}O_{12}S_2$	1432.1	1432.7
56	TD14092	16	−4.68	0.427	27	$C_{63}H_{97}N_{23}O_{12}S_2$	1432.8	1432.7
57	TD14093	24	−20.63	0.630	27	$C_{63}H_{97}N_{23}O_{12}S_2$	1432.8	1432.7
58	TD14094	23	−16.10	0.621	27	$C_{63}H_{97}N_{23}O_{12}S_2$	1432.1	1432.7

Table 8. Characterization data of the synthetic T140 analogues (No. 3, TL14001–TL14015)

Entry	Compd	Yield (%)	$[\alpha]_D$	*c*	Temperature	Formula	IS-MS	
							Found	Calcd
59	TL14001	6	−53.97	0.352	23	$C_{90}H_{143}N_{33}O_{18}$	1975.7	1975.3
60	TL14002	29	−21.66	1.200	25	$C_{66}H_{96}N_{20}O_{13}$	1376.7	1377.6
61	TL14003	36	−25.64	1.365	25	$C_{57}H_{87}N_{19}O_{11}$	1214.0	1214.4
62	TL14004	39	−38.72	1.472	24	$C_{57}H_{87}N_{19}O_{11}$	1214.0	1214.4
63	TL14005	31	−41.54	1.059	24	$C_{48}H_{78}N_{18}O_9$	1051.2	1051.3
64	TL14006	66	−19.90	2.010	24	$C_{42}H_{68}N_{16}O_7$	909.0	909.1
65	TL14007	46	−17.56	1.651	24	$C_{54}H_{82}N_{18}O_{10}$	1143.0	1143.4
66	TL14008	40	−15.07	1.459	24	$C_{54}H_{82}N_{18}O_{10}$	1143.0	1143.4
67	TL14009	41	−15.68	1.403	24	$C_{51}H_{77}N_{17}O_9$	1072.1	1072.3
68	TL14010	35	−29.98	1.034	24	$C_{45}H_{65}N_{15}O_8$	943.8	944.1
69	TL14011	39	−8.55	1.052	23	$C_{40}H_{58}N_{14}O_7$	846.0	847.0
70	TL14012	14	−9.18	1.416	23	$C_{43}H_{63}N_{15}O_8$	918.0	918.1
71	TL14013	45	−0.95	1.051	23	$C_{37}H_{51}N_{11}O_7$	761.7	761.9
72	TL14014	16	−27.39	0.365	27	$C_{46}H_{70}N_{18}O_8S_2$	1067.7	1067.3
73	TL14015	92	−7.01	0.713	26	$C_{74}H_{102}N_{24}O_{12}S_2$	1584.0	1583.9

The yields of TL14014 and TL14015 were calculated from the protected dipeptide **3** and the protected pentapeptidyl resin **1**, respectively.

high anti-HIV activity, and that these three residues and Arg^{14} are indispensable. Furthermore, in the downsizing study we have found novel lead compounds: a 10-residue analogue, TD14041, and a homodimer analogue, TL14015, both possessing modest anti-HIV activity ($EC_{50} = 590$ nM and 1.2 μM, respectively). Although both analogues are less potent than T140, the present results will give insight into the rational design of a new type of small molecule anti-HIV drugs based on CXCR4 antagonists.

Experimental

HPLC solvents were H_2O and CH_3CN, both containing 0.1% (v/v) TFA. For analytical HPLC, a Cosmosil 5C18-AR column (4.6 × 250 mm, Nacalai Tesque Inc., Kyoto, Japan) was eluted with a linear gradient of CH_3CN at a flow rate of 1 mL/min on a Waters LC Module I equipped with a Waters 741 Data Module (Nihon Millipore, Ltd., Tokyo, Japan). Preparative HPLC was performed on a Waters Delta Prep 4000 equipped with a Cosmosil 5C18-AR column (20 × 250 mm, Nacalai Tesque Inc.) using a linear gradient of CH_3CN at a flow rate of 7 mL/min. For gel-filtration, the solution was applied to a column of Sephadex G-15 (2.1 × 30 cm), which was eluted with 1 M AcOH. Ion-spray (IS)-mass spectrum was obtained with a Sciex API*III*E triple quadrupole mass spectrometer (Toronto, Canada). Optical rotation of a peptide in aqueous solution was measured with a JASCO DIP-360 digital polarimeter (Tokyo, Japan) or a Horiba high-sensitive polarimeter SEPA-200 (Kyoto, Japan). Fmoc-protected amino acids, *p*-benzyloxybenzyl alcohol (Alko)-resins and 4-(2′,4′-dimethoxyphenylaminomethyl)phenoxy (SAL) resin[12] were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan) or Calbiochem-Novabiochem Japan, Ltd. (Tokyo, Japan). All the other chemicals were purchased from either Nacalai Tesque Inc. or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Synthesis of T140 analogues

Representative compound T151. The protected T151-resin was manually constructed using Fmoc-based solid-phase synthesis on an Fmoc-Arg(Pbf)-Alko-resin (0.64 meq/g, 0.1 mmol scale, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl). Fmoc-protected amino acid derivatives (2.5 equiv) were successively condensed using 1,3-diisopropylcarbodiimide (DIPCDI) (2.5 equiv) in the presence of *N*-hydroxybenzotriazole (HOBt) (2.5 equiv). The following side-chain protecting groups were used: Pbf for Arg, Trt for Cys, Bu^t for Tyr and Boc for Lys and D-Lys. The resulting protected T151-resin (50 μmol) was treated with 1 M TMSBr-thioanisole/TFA (5 mL) in the presence of *m*-cresol (250 μL, 55 equiv) and EDT (100 μL, 33 equiv) at 4 °C for 2 h. After removal of the resin by filtration, the filtrate was concentrated in vacuo. Ice-cold dry diethyl ether (30 mL) was added to the residue. The resulting powder was collected by centrifugation and then washed three times with ice-cold dry diethyl ether (20 mL × 3). The crude reduced peptide was dissolved in 50% AcOH (2 mL). Subsequently, the solution was diluted to total volume 400 mL with H_2O, and then pH was adjusted to 7.8 with concentrated NH_4OH. After air-oxidation for 1 day, the pH of the solution was adjusted to 5 with AcOH. The crude product in the solution was purified by preparative HPLC and gel-filtration to afford a fluffy white powder of T151; yield 32 mg [13 μmol, 26% based on the Fmoc-Arg(Pbf)-Alko-resin]. Characterized data of all the synthetic peptides are listed in Tables 6–8. In the synthesis of TM14004, benzoic acid, instead of an N-terminal Fmoc-protected amino acid derivative, was condensed with DIPCDI and HOBt. In the synthesis of TL14001–TL14013, after deprotection and cleavage from the resin, the crude product was purified by preparative HPLC and gel-filtration.

TL14014. The protected pentapeptidyl resin **1** was manually constructed using Fmoc-based solid-phase synthesis on SAL resin (0.59 meq/g, 0.2 mmol scale). The resulting protected resin **1** (60 μmol) was treated with TFA–*m*–cresol–thioanisole–H_2O (85:5:5:5, v/v, 5 mL) in the presence of 2,2′-dithiodipyridine (26 mg, 2 equiv) at room temp. for 2 h. After removal of the resin by filtration, the filtrate was concentrated in vacuo. Ice-cold dry diethyl ether (30 mL) was added to the residue. The resulting powder was collected by centrifugation and then washed three times with ice-cold dry diethyl ether (20 mL × 3) to yield the crude *S*-(2-pyridylsulfenyl)-pentapeptide **2** (65 mg, 52 μmol, 86% based on the SAL resin) [IS-MS (reconstructed) found: *m*/*s* 899.0 (calcd for $C_{42}H_{55}N_{13}O_6S_2$: 901.3)]. Z(OMe)-Cys(MeOBzl)-Arg(Mts)-NH_2 **3** (57 μmol)[9] was treated with 1 M TMSBr-thioanisole/TFA (2.5 mL) in the presence of *m*-cresol (80 μL, 15 equiv) at rt for 3 h. After deprotection, the mixture was concentrated in vacuo. Ice-cold dry diethyl ether (30 mL) was added to the residue. The resulting powder was collected by centrifugation and then washed three times with ice-cold dry diethyl ether (20 mL × 3) to yield the crude dipeptide **4** (24 mg, 47 μmol, 82%) [IS-MS (reconstructed) found: *m*/*s* 275.0 (calcd for $C_9H_{20}N_6O_2S$: 276.1)]. The SH-peptide **4** (47 μmol) in 0.4 M $AcONH_4$ aqueous buffer (pH 6.0, 5 mL) was added dropwise to a stirred solution of the pentapeptide **2** (52 μmol) in 0.4 M $AcONH_4$ aqueous buffer (pH 6.0, 5 mL) at 0 °C under Ar. The mixture was allowed to warm to rt and stirring was continued for 17 h. The mixture was purified by preparative HPLC and gel-filtration to afford a fluffy white powder of TL14014; yield 13 mg (9.5 μmol, 16% based on the protected dipeptide **3**).

TL14015. The protected pentapeptidyl resin **1** (28 μmol) was treated with 1 M TMSBr-thioanisole/TFA (3.5 mL) in the presence of *m*-cresol (180 μL, 63 equiv) and EDT (70 μL, 38 equiv) at 4 °C for 2 h. The resulting crude SH-pentapeptide **5** in 0.4 M $AcONH_4$ aqueous buffer (pH 8.0, 3.5 mL) was added to a stirred solution of the pentapeptide **2** (28 μmol) in 0.4 M $AcONH_4$ aqueous buffer (pH 8.0, 3.5 mL) at rt. Stirring was continued for 17 h. The mixture was purified by preparative HPLC and gel-filtration to afford a fluffy white powder of TL14015; yield 51 mg (26 μmol, 92% based on the protected pentapeptidyl resin **1**).

Cell culture

Human T-cell lines, MT-4 and MOLT-4 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin.

Virus

A strain of X4-HIV-1, HIV-1_{IIIB}, was used for the anti-HIV assay. This virus was obtained from the culture supernatant of HIV-1 persistently infected MOLT-4/HIV-1_{IIIB} cells, and stored at −80 °C until used.

Anti-HIV-1 assay

Anti-HIV-1 activity was determined based on the protection against HIV-1-induced cytopathogenicity in MT-4 cells. Various concentrations of test compounds were added to HIV-1-infected MT-4 cells at a multiplicity of infection (MOI) of 0.01, and placed in wells of a flat-bottomed microtiter tray (1.5×10^4 cells/well). After 5 days' incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (EC_{50}).[13] Cytotoxicity of compounds was determined based on the viability of mock-infected cells using the MTT method (CC_{50}). 3′-Azido-3′-dideoxythymidine (AZT)[14] and 2′, 3′-dideoxycytidine (ddC)[15] were tested as controls.

Calcium fluorimetry

The stable CXCR4-transfected Chinese hamster ovary (CHO) cell lines (3×10^4 cells/100 μL/well) were placed in wells of a flat-bottomed microtiter tray. After 1 day's incubation at 37 °C in a CO_2 incubator, the cells were loaded with 5 μM of Fura2-AM (Dojin, Kumamoto, Japan), 2.5 mM Probenecid (Sigma) and 20 mM Hepes (pH 7.4) in Ham's F-12 (80 μL/well) for 1 h at 37 °C, and then twice washed with Hank's balanced salt solution (100 μL × 2), and inserted into a spectrofluorometer (96-well Fluorescence Drug Screening System, Hamamatsu Photonix, Japan). 30 s after start of measurement, the cells were incubated with various concentrations of T140 analogues in Hank's balanced salt solution (10 μL/well), and after 3 min, recombinant SDF-1α (PreproTech, 30 nM/40 μL/well) was added. Real time recording of $[Ca^{2+}]_i$ changes in the stable CXCR4-transfected CHO cell lines loaded with Fura2-AM was performed by a modified procedure of the Fura-2 method.[10e] Inhibitory activity of T140 analogues was determined based on the inhibition of Ca^{2+} mobilization induced by SDF-1α-stimulation through CXCR4 (IC_{50}).

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