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Pharmacophore Identification of a Chemokine Receptor (CXCR4) Antagonist, T22 ([Tyr^{5,12}, Lys^{7}]-Polyphemusin II), which Specifically Blocks T Cell-line-tropic HIV-1 Infection

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Abstract—We have previously found that T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II) has strong anti-human immunodeficiency virus (HIV) activity, and that T22 inhibits T cell-line-tropic HIV-1 infection mediated by CXCR4/fusin. T22 is an 18-residue peptide amide, which takes an antiparallel β-sheet structure that is maintained by two disulfide bridges. Structure–activity relationship (SAR) studies on T22 have disclosed the contributions of each region of T22 to activity or cytotoxicity, and have provided the following useful information to develop new CXCR4 antagonists: The number of Arg residues in the N-terminal and C-terminal regions of T22 is closely related to anti-HIV activity. Addition of a variety of functional groups at the N-terminal end results in increases in activity. Disulfide rings, especially the major disulfide loop, are indispensable for anti-HIV activity and maintenance of the β-sheet structure. Trp^{3} can be replaced by other aromatic residues (Tyr, Phe and L-2-naphthylalanine). Between two repeats of Tyr-Arg-Lys, which are a characteristic structure in T22, Tyr-Arg-Lys in the N-terminal portion is more closely associated with anti-HIV activity and maintenance of the β-sheet structure. A positive charge in the side chain at the (i + 1) position of the β-turn region is necessary for strong activity. Through these studies, we have found several compounds having higher selectivity indexes (50% cytotoxic concentration/50% effective concentration) than that of T22.

Introduction

Currently, although many anti-HIV[1] agents are available, further development of new drugs is desired for multiple combined therapies that affect different stages of HIV-replication. Such combination therapy employing multi-types of drugs is thought to improve the clinical treatment of AIDS patients.[2] Thus, we have previously found an anti-HIV compound, T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II),[3] derived from chemical modifications of tachyplesin and polyphemusin, which are self-defense peptides of horseshoe crabs.[4] The effective concentration (EC_{50}) of T22 for 50% protection in an assay of HIV-induced cytopathogenicity was 2.6 nM, a value comparable to that of 3′-azido-2′,3′-dideoxythymidine (AZT) (5.2 nM). The cytotoxic concentration (CC_{50}) of T22 was 17 μM. Our previous studies showed that T22 exerts its effect on an HIV-cell fusion process,[5] and that T22 binds specifically to both an envelope protein of HIV, gp120, and a T cell surface

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protein, CD4.[6] We have disclosed very recently that T22 inhibits T cell-line-tropic (T-tropic) HIV-1 infection through its specific binding to a coreceptor (a chemokine receptor CXCR4/fusin) for the T-tropic HIV-entry.[7] At this moment, it cannot be clearly explained why T22 interacts with these three proteins which play an important role in HIV-cell fusion. T22 is an 18-residue peptide amide, which takes an antiparallel β-sheet structure with a type II β-turn, that is maintained by two intramolecular disulfide bridges.[8] In addition, T22 has 2 and 1 Arg residues in the N- and C-terminal regions, respectively, as well as two repeated motifs of Tyr-Arg-Lys, which face each other on the antiparallel β-strands. We wish to define pharmacophores of T22 which may aid in the design of more effective compounds and development of new HIV-1 coreceptor antagonists (CXCR4/fusin inhibitors). Recently, development of agents targeting chemokine receptors is desired. In a recent preliminary report,[9] several analogues of T22 were synthesized in order to investigate the biological effects. Herein, additional SAR studies have disclosed the contributions of each region of T22 to activity, cytotoxicity and conformational maintenance. In this paper, we report further investigations and experimental details with conformational studies using CD analysis.

Results and Discussion

Several T22 analogues were synthesized in order to examine the effects of the number of Arg residues and positive charges in the N-terminal and C-terminal regions, the peptide chain length, the disulfide ring structure, the aromatic ring of Trp^3, the two repeated Tyr-Arg-Lys motifs and a positive charge in the β-turn region (Figure 1).

Chemistry

Structures of synthetic T22 analogues are shown in Figure 2. These peptides, except compounds **3a**–**c**, have two disulfide bridges each. During the synthesis of bis-disulfide bond-containing peptides, it is desirable that each disulfide bond be formed regioselectively in order to suppress the formation of disulfide-isomers. Particularly in amino acid-substituted analogues, disulfide bridges are not always formed by air-oxidation at the same position as those in the natural peptides. We recently developed a useful methodology for regioselective bis-disulfide bond formation using a combination of air-oxidation and an AgOTf-DMSO/HCl aq system.[10] Under these conditions, no significant side reactions were observed with oxidation-sensitive amino acids such as Met, Tyr and Trp. Therefore, this regioselective method was utilized for the synthesis of amino acid-substituted analogues of T22 examined in this study. For the synthesis of these analogues (**1a**, **1c**, **4a**–**d**, **5a**–**f** and **6a**–**d**), two different cysteinyl thiol-protecting groups, MBzl[11] and Acm,[12] were employed (Figure 3). The first disulfide bond was formed by air-oxidation between the Cys(SH)-residues resulting from deprotection of the MBzl groups with TMSBr treatment.[13] Then the second disulfide bond was regioselectively constructed between two Cys(Acm) residues using a stepwise-procedure consisting of AgOTf deprotection[14] and subsequent DMSO oxidation in aq HCl without affecting the first disulfide bond. The successful synthesis of these analogues demonstrated the usefulness of this combination protocol for rapid and convenient syntheses of many analogues containing Trp and two-disulfide bridges. On the other hand, for the synthesis of N- or C-terminal modified or deleted peptides (**1b** and **2a**–**f**), the air-oxidation method alone was used, since it had previously been confirmed that two disulfide bonds in T22 can be correctly formed by air-oxidation. The disulfide bond array of these peptides was subsequently confirmed.[15]

SAR study on T22

The anti-HIV activity and cytotoxicity of T22 analogues are summarized in Table 1. We initially examined SAR in N- and C-terminal regions of T22. T22 has 2 and 1

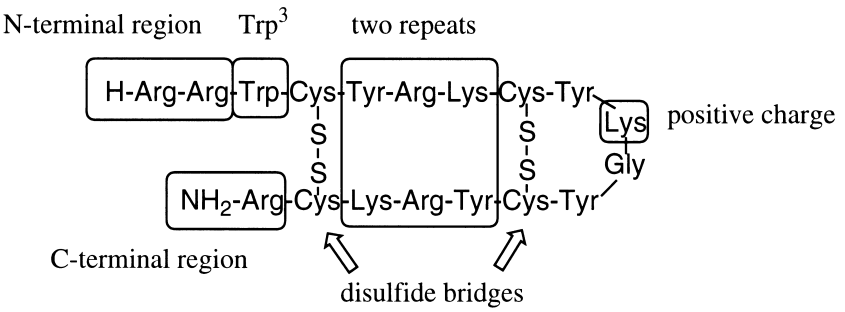

Figure 1. Structures of T22.

Figure 2. Amino acid sequences of T22 and its analogues, with alignment based on their homology. The disulfide linkages are shown by solid lines. However, **3a** and **3b** have one disulfide linkage between Cys^4 and Cys^{17}, and between Cys^8 and Cys^{13}, respectively. **3c** has no disulfide linkages. The substituted or modified sites are underlined. Nph: L-2-naphthylalanine, Orn: L-ornithine, Dap: L-2, 3-diaminopropionic acid.

Arg residues at the N- and C-termini, respectively, and thus, we investigated the biological effects of the number of Arg residues and positive charges in the N- and C-termini and the length of the peptide chain. Compound **1a** (C-terminus, no Arg) shows a slightly lower activity than T22. Compound **1b** (C-terminus, 2 Arg) shows 280 times less activity than T22. These results suggest that the C-terminal Arg residue of T22 is not necessary; however, the addition of an extra Arg residue to the C-terminus significantly decreases activity. Compound **1c** (C-terminal free carboxyl group) shows less cytotoxicity than T22, with half of the anti-HIV activity, and thus, its SI becomes higher than that of T22. The activity of T22 (N-terminus, 2 Arg) is 210 and 40 times higher than those of **2a** (N-terminus, 1 Arg) and **2b** (N-terminus, 3 Arg), respectively. The number of Arg residues at the N-terminus of T22 is related more closely to anti-HIV activity than to cytotoxicity. A delicate recognition of the peptide chain length or the number of positive charges may be required for the expression of anti-HIV activity. Compounds **2c**, **2d**, **2e** and **2f**, which have acetyl, octanoyl, palmitoyl and biotinyl groups at the N-terminus of T22, respectively, show somewhat higher, or the same, activity as compared with T22. This suggests that a positive charge in the N-terminal α-amino group is not needed for anti-HIV activity, and that addition of relatively large groups has no unfavorable effect on activity.

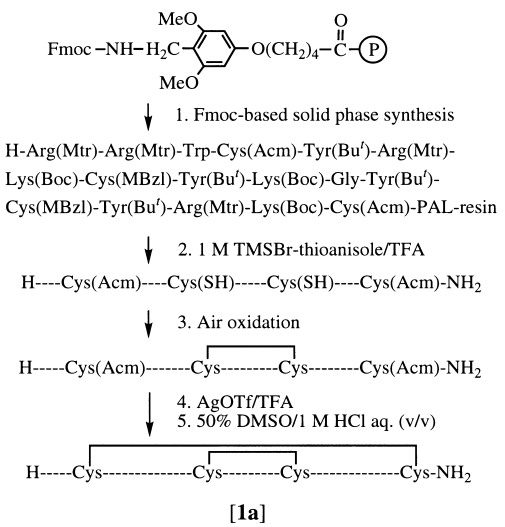

Figure 3. Synthetic scheme for compound **1a**.

Next, we examined biological and conformational effects of the two disulfide bridges contained in T22. Compounds **3a** [2 Ala-substituted T22 for the minor disulfide bridge ($Cys^{8,13}$)], **3b** [2 Ala-substituted T22 for

Table 1. Anti-HIV activity and cytotoxicity of T22 analogues

Compd	SI ratio	CC_{50} ratio	EC_{50} ratio	IC_{50} ratio
T22	1	1	1	1
1a	0.31	1.5	4.8	1.3
1b	0.012	3.2	280	N.T.
1c	2.9	5.7	2.0	2.5
2a	0.013	3.1	210	N.T.
2b	0.023	0.88	40	N.T.
2c	3.9	0.80	0.20	N.T.
2d	0.55	0.54	0.99	N.T.
2e	1.5	0.80	0.53	N.T.
2f	5.8	0.92	0.16	0.71
3a	0.17	1.0	6.0	2.1
3b	0.058	1.2	20	11
3c	0.21	5.0	24	50
4a	0.96	1.0	1.1	0.93
4b	1.0	0.72	0.69	0.99
4c	8.9	0.59	0.066	0.25
4d	0.10	0.95	9.1	6.5
5a	0.66	1.1	1.7	1.1
5b	0.022	0.99	45.6	8.9
5c	0.0063	0.99	160	42
5d	0.44	0.98	2.2	2.2
5e	0.017	1.1	63	37
5f	0.24	0.93	3.8	5.6
6a	1.6	0.63	0.40	0.64
6b	2.0	0.40	0.20	0.40
6c	1.0	0.80	0.78	0.57
6d	0.74	4.7	6.3	5.4

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}. IC_{50} values are based on the inhibition of viral antigen expression examined by the indirect IF method. CC_{50} ratio is CC_{50} of each analog/CC_{50} of T22 (M/M). EC_{50} ratio, IC_{50} ratio and SI ratio were also calculated similarly. All data are mean values for at least three experiments. N.T.: not tested.

the major disulfide bridge ($Cys^{4,17}$)] and **3c** [4 Ala-substituted T22 for all Cys residues] show 6, 20 and 24-fold less activity (EC_{50}) than T22, respectively. These results are compatible with those obtained in the inhibition assay of the viral antigen expression (IC_{50} ratios of **3a**, **3b** and **3c**: 2.1, 11 and 50, respectively). **3a** and **b** exhibit almost the same cytotoxicity as T22; however, **3c** shows fivefold less cytotoxicity than T22. These results suggest that the disulfide bridges, especially the major disulfide loop, are indispensable for activity, and that the presence of either disulfide bridge retains the cytotoxicity of T22. The CD spectra of **3a–c** in aqueous solution are shown compared with that of T22 in Figure 4A. T22 exhibits a strong negative band near 212 nm and a strong positive band near 202 nm. These bands are due to β-sheet structures,[16] and are compatible with the NMR data.[8] The CD spectra of **3b** and **c** are very different from that of T22. These analogues exhibit a strong negative band near 198 nm, which is due to random coil conformations.[16] The β-sheet structure compositions are reduced in the order T22 (73%) > **3a** (64%) > **3b** (58%) > **3c** (47%). The CD data suggest that the maintenance of the β-sheet structure of T22 requires disulfide bridges, especially the major loop, and that there is a correlation between anti-HIV activity and β-sheet compositions.

The importance of Trp^3 was investigated. Compounds **4a** (Tyr-substituted T22) and **4b** (Phe-substituted T22) exhibit almost the same activity (EC_{50} and IC_{50}) as that of T22, whereas compound **4d** (Ala-substituted T22) shows lower activity than T22. The activity of **4c** (L-2-naphthylalanine-substituted T22) is remarkably higher than that of T22. The CD spectra of **4a–d** are shown in Figure 4B. **4a–c** show spectra similar to that of T22, whereas **4d** exhibits a little different pattern. A negative band is shifted to shorter wave lengths (212 nm to 208 nm), and a positive band near 202 nm is weak. The β-sheet compositions of **4a**, **b**, **c** and **d** are 72%, 76%, 78% and 50%, respectively. **4a–d** have almost the same cytotoxicity as that of T22. These results indicate that an aromatic residue is required at the 3 position in T22.

Next, two repeats of the Tyr-Arg-Lys sequence were selected as targets for investigation. The previous study showed that the tachyplesin analogues, which contained these repeats, exhibited relatively strong activity,[3] suggesting that these unique structures seem to be related to high activity. Compound **5a** (Phe-substituted T22 for Tyr^{12}) exhibits almost the same potency (EC_{50} and IC_{50}) as T22. However, compound **5b** (2 Phe-substituted T22 for $Tyr^{5,12}$) shows much lower activity (EC_{50} and IC_{50}) as compared with T22. The CD spectra of **5a** and **b** are similar to that of T22 (data not shown), indicating that this difference in activity is not related to the secondary structures of these peptides, and that the phenol group itself in the side-chain of Tyr^5 is indispensable for anti-HIV activity. Compound **5c** [(Lys^5-Arg^6-Tyr^7)-substituted T22 for (Tyr^5-Arg^6-Lys^7)] is much less active (EC_{50} and IC_{50}) than T22; however, a similar replacement for (Tyr^{14}-Arg^{15}-Lys^{16}) (**5d**) does not induce such a decrease in activity. The CD spectra of **5c** and **d** in aqueous solution are shown in Figure 4C. **5c** and **d** exhibit a strong negative band near 212 nm and a positive band near 202 nm. However, **5c** spectra are markedly different from that of T22, because a positive band near 202 nm is weaker than that of T22 and **5c** has a negative band near 198 nm. The β-sheet compositions of **5c** and **d** are 45% and 55%, respectively. A Lys-substitution for Tyr^5 or Tyr^{14} in the two Tyr-Arg-Lys sequences (**5e** or **5f**, respectively) reduces the anti-HIV activity (EC_{50} and IC_{50}) in a different manner; that is, **5e** is less active than **5f**. CD spectra of **5e** and **f** do not have

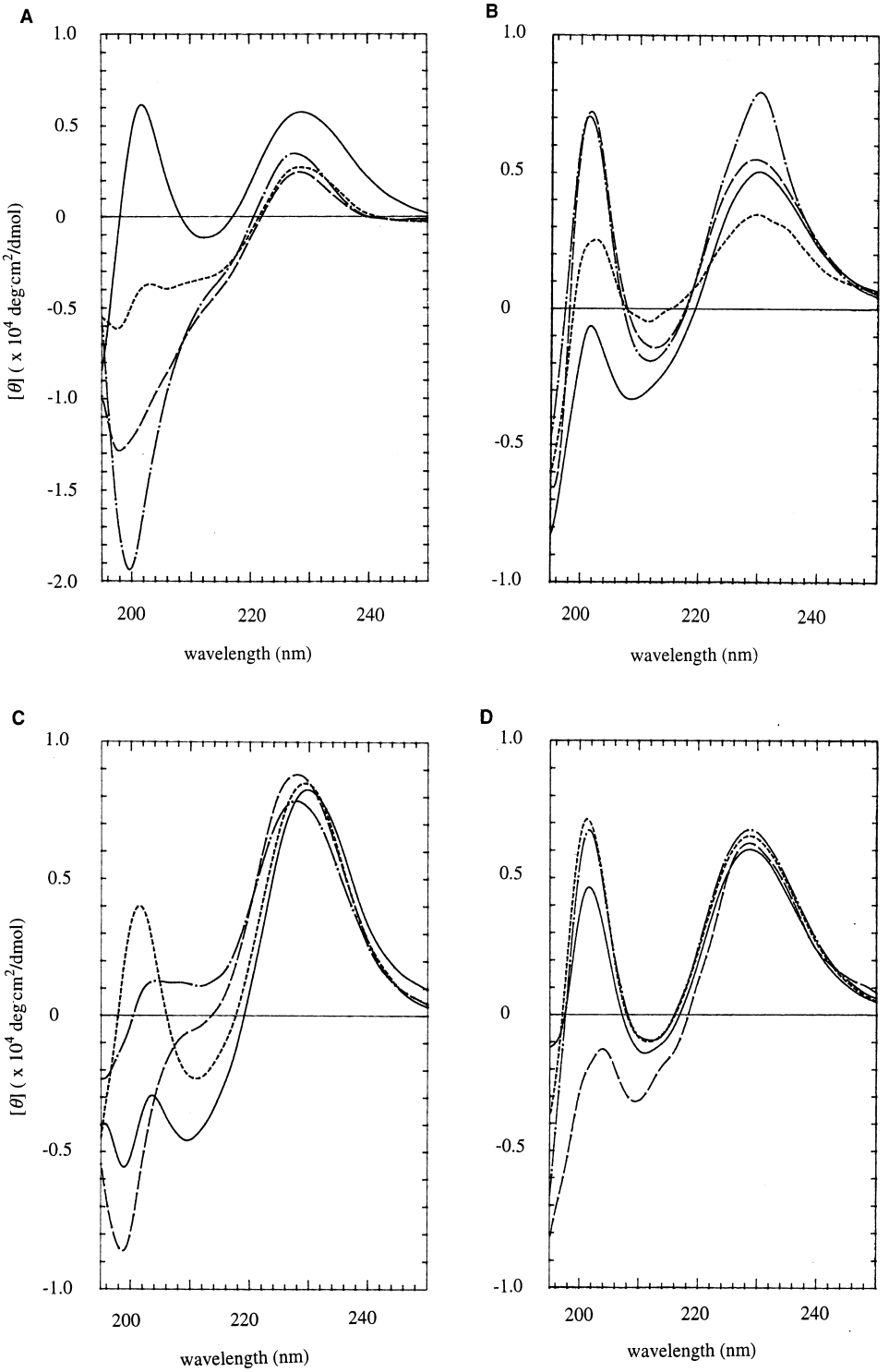

Figure 4. CD spectra of T22 analogues. (A) Solid line: T22, dotted line: **3a**, dashed line: **3b**, center-dotted line: **3c**, (B) solid line: **4d**, dotted line: **4a**, dashed line: **4b**, center-dotted line: **4c**, (C) solid line: **5c**, dotted line: **5d**, dashed line: **5e**, center-dotted line: **5f**, (D) solid line: **6a**, dotted line: **6b**, dashed line: **6d**, center-dotted line: **6c**.

clear positive bands near 202 nm, and **5e** shows a strong negative band near 198 nm (Figure 4C). The β-sheet compositions of **5e** and **f** could not be calculated because Yang's calculated curves did not fit the observed curves tightly. However, the β-sheet compositions of **5e** and **f** may not be so high (**5e** < **5f**), judging from these spectra. These results suggest that Tyr^5-Arg^6-Lys^7 is more closely related to anti-HIV activity and maintenance of the β-sheet structure than Tyr^{14}-Arg^{15}-Lys^{16}, and that the order of Tyr, Arg and Lys in T22 (positions 5–7) is important. The same kind of amino acids and the same number of charges as those of T22 are not sufficient for activity. In addition, substitutions for the two Tyr-Arg-Lys repeats cause no change in cytotoxicity (**5a**–**f**).

T22 has a type II β-turn formed by Tyr^9, Lys^{10}, Gly^{11} and Tyr^{12}. The side-chain of Lys^{10} is directed straight out of the β-sheet through the β-strand (Cys^4–Tyr^9). Finally, the effects of the side-chain amino group on activity and conformation were examined. Compounds **6a**–**c**, which are the T22 analogues substituted by L-ornithine, Arg and L-2, 3-diaminopropionic acid, respectively, have slightly higher activities (EC_{50} and IC_{50}) than T22, while an Ala-substitution for Lys^{10} causes a significant decrease in activity (**6d**). The CD spectra of **6a**–**d** are shown in Figure 4D. **6a**–**c** show spectra similar to that of T22, whereas **6d** exhibits a different pattern. The β-sheet compositions of **6a**–**d** are 75%, 73%, 72% and 45%, respectively. These results suggest that a side-chain positive charge at the (i + 1) position in the β-turn region is indispensable for activity and conformational stability, but the length of the side-chains is not as crucial. **6d** exhibits lower cytotoxicity than the other analogues. Comparing it with **3c**, the excessive collapse of β-sheet structures might also bring about a decrease in cytotoxicity.

Recently, we reported that the Cys^8-Tyr^9 and Tyr^{12}-Cys^{13} sequences could be deleted from T22 without loss of activity or significant change in the secondary structure, leading to a 14-residue analogue, TW70 (des-[$Cys^{8,13}$, $Tyr^{9,12}$]-[D-Lys^{10}, Pro^{11}]-T22).[17] TW70 has a type II′ β-turn induced by a D-amino acid-Pro sequence at the (i + 1, i + 2) turn positions. For formation of a β-turn, either Gly^{11} (type II) or Pro^{11} (type II′) is required. Taken together, the present SAR study examines the contributions of each region of T22 to activity, cytotoxicity or conformational maintenance. In this study, several compounds possessing higher SIs than that of T22 have been found: C-terminal free T22 (**1c**), N-terminus-acylated T22 (**2c**, **2e** and **2f**) and [L-2-naphthylalanine^3]-T22 (**4c**). Their high SIs are attributed to a decrease in EC_{50} (**2c**, **2e**, **2f** and **4c**) or an increase in CC_{50} (**1c**). In addition, a β-sheet structure is thought to be indispensable for high activity of T22 analogues. These new findings will aid in elucidating the basis for the high anti-HIV activity of T22, and may lead to the rational designing of more effective compounds containing the essential pharmacophores of T22. Furthermore, since T22 is a selective CXCR4/fusin inhibitor and specifically inhibits T-tropic HIV-1 infection,[7] our present results will be useful for developing new anti-HIV agents.

Experimental

Preparative HPLC was performed on a Waters Delta Prep 4000 (Nihon Millipore Ltd., Tokyo, Japan) equipped with a Cosmosil 5C18-AR column (20×250 mm, Nacalai Tesque Inc., Kyoto, Japan) or a YMC packed column (Kyoto, Japan) at a flow rate of 7 mL/min. HPLC solvents were H_2O and CH_3CN, both containing 0.1% (v/v) TFA. 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 spectra were obtained with a Sciex API*III*E triple quadruple mass spectrometer (Toronto, Canada). Optical rotations of peptides in aqueous solution were measured with a JASCO DIP-360 digital polarimeter (Tokyo, Japan). Fmoc-protected amino acids and *p*-benzyloxybenzyl alcohol (Alko) resin were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)-valeric acid resin (PAL™-resin)[18] was purchased from Millipore. All the other chemicals were purchased from either Nacalai Tesque Inc. or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Synthesis of T22 analogues

Representative compound 1a. The protected **1a**-resin was manually constructed using Fmoc-based solid-phase synthesis on a PAL™-resin (0.30 meq/g, 0.1 mmol scale). Fmoc-protected amino acid derivatives (2.5 equiv.) were successively condensed using DIPCDI (2.5 equiv.) in the presence of HOBt (2.5 equiv.) according to the reported schedule.[19] The following side-chain protecting groups were used: Boc for Lys, Mtr for Arg, Bu^t for Tyr, MBzl for $Cys^{8,13}$ and Acm for $Cys^{4,17}$. The resulting protected **1a**-resin (209 mg, 29.1 μmol) was treated with 1 M TMSBr-thioanisole/TFA (20 mL) in the presence of *m*-cresol (1 mL, 380 equiv.) and 1,2-ethanedithiol (400 μL, 230 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 then added, and the resulting powder was collected by centrifugation. After washing three times with ice-cold dry diethyl ether (20 mL×3), the product 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 d, the pH of the solution was adjusted to 5 with AcOH. Diaion HP-20 resin (ca. 10 g) was added into the solution, followed by stirring for 2 h. After collection of the resin by filtration, the peptide was eluted from the resin with 80% CH_3CN in 1 M AcOH aq. (v/v, 100 mL). The solvent was removed by evaporation and lyophilization to give a white powder of crude monocyclic peptide [Cys(Acm)^{4,17}]-**1a**: yield, 48.2 mg (16.3 μmol). The crude peptide [Cys(Acm)^{4,17}]-**1a** (14.0 mg, 4.74 μmol) was treated with AgOTf (145 mg, 100 equiv.) in TFA (2 μL)-anisole (20 μL) at 4 °C for 2 h. After addition of ice-cold dry diethyl ether (30 mL), the resulting precipitate was washed three times with ice-cold dry diethyl ether (20 mL×3), and then treated with 50% DMSO/1 M HCl aq. (v/v, 14 mL) at room temperature for 7 h. After removal of the precipitate AgCl by filtration, the filtrate was diluted to 150 mL with H_2O. The crude product was purified by preparative HPLC (isocratic mode of CH_3CN: 19%) and gel-filtration to afford a fluffy white powder of **1a**; yield 2.46 mg (0.876 μmol, 10.4% based on the protected **1a**-resin). The disulfide bond array was confirmed by ion spray mass analysis of peptide fragments derived from the tryptic digest of the synthetic peptides.[15]

Other analogues (**1c**, **4a–d**, **5a–f** and **6a–d**) were synthesized in similar fashion. In the synthesis of **1b** and **2a–f**, MBzl groups were used for the protection of side-chains of four Cys-residues, and two disulfide bonds were formed by air-oxidation. In the synthesis of **3a** and **3b**, MBzl groups were used for the protection of side-chains of two Cys-residues, and one disulfide bond was formed by air-oxidation. In the synthesis of **3c**, the crude peptide, which was obtained by TMSBr-treatment, was purified by preparative HPLC. For acetylation of the N-terminal α-amino group of the protected T22-resin during the synthesis of **2c**, the protected T22-resin (25 μmol) was treated with acetic anhydride (1000 equiv.) and pyridine (1000 equiv.) in DMF (5 mL) at r.t. for 2 h. For acylation of the N-terminal α-amino group of the protected T22-resin during the synthesis of **2d–f**, the protected T22-resin (25 μmol) was treated with the corresponding carboxylic acids (5 equiv.), DIPCDI (5 equiv.) and HOBt (5 equiv.) in DMF (5 mL) at r.t. for 2 h. Characterized data are listed in Table 2.

Cell culture

Human T-cell lines, MT-4 and MOLT-4 cells were grown in RPMI 1640 medium containing 10% heat-inactivated

Table 2. Characterized data of the synthetic T22 analogues

Compd	$[\alpha]_D$(°)(in H_2O)	Formula	IS-MS (reconstructed)	
			Found	Calcd
1a	−4.87 (c = 0.1, 20 °C)	$C_{103}H_{152}N_{34}O_{21}S_4$	2330.5	2329.1
1b	9.60 (c = 0.1, 16 °C)	$C_{115}H_{176}N_{42}O_{23}S_4$	2641.5	2641.3
1c	9.52 (c = 0.1, 20 °C)	$C_{109}H_{163}N_{37}O_{23}S_4$	2488.2	2486.2
2a	28.5 (c = 0.2, 16 °C)	$C_{103}H_{152}N_{34}O_{21}S_4$	2329.7	2329.1
2b	6.52 (c = 0.1, 16 °C)	$C_{115}H_{176}N_{42}O_{23}S_4$	2641.8	2641.3
2c	24.3 (c = 0.1, 27 °C)	$C_{111}H_{166}N_{39}O_{23}S_4$	2529.3	2527.2
2d	7.85 (c = 0.1, 27 °C)	$C_{117}H_{178}N_{38}O_{23}S_4$	2613.4	2611.3
2e	1.73 (c = 0.1, 27 °C)	$C_{125}H_{194}N_{38}O_{23}S_4$	2724.8	2723.4
2f	9.33 (c = 0.3, 23 °C)	$C_{119}H_{118}N_{40}O_{24}S_5$	2712.0	2711.3
3a	−8.72 (c = 0.1, 27 °C)	$C_{109}H_{166}N_{38}O_{22}S_2$	2424.0	2423.2
3b	−33.3 (c = 0.1, 30 °C)	$C_{109}H_{166}N_{38}O_{22}S_2$	2424.0	2423.2
3c	−51.6 (c = 0.2, 30 °C)	$C_{109}H_{168}N_{38}O_{22}$	2362.2	2361.3
4a	3.50 (c = 0.1, 17 °C)	$C_{107}H_{163}N_{37}O_{23}S_4$	2463.0	2462.3
4b	1.16 (c = 0.1, 31 °C)	$C_{107}H_{163}N_{37}O_{22}S_4$	2446.7	2446.3
4c	15.8 (c = 0.1, 31 °C)	$C_{111}H_{167}N_{37}O_{22}S_4$	2497.2	2496.2
4d	−13.9 (c = 0.1, 31 °C)	$C_{111}H_{159}N_{37}O_{22}S_4$	2371.2	2370.3
5a	0 (c = 0.1, 20 °C)	$C_{109}H_{164}N_{38}O_{21}S_4$	2470.2	2469.2
5b	2.92 (c = 0.1, 20 °C)	$C_{109}H_{164}N_{38}O_{20}S_4$	2454.0	2453.2
5c	7.68 (c = 0.5, 20 °C)	$C_{109}H_{164}N_{38}O_{22}S_4$	2486.5	2485.2
5d	0 (c = 0.1, 20 °C)	$C_{109}H_{164}N_{38}O_{22}S_4$	2486.5	2485.2
5e	17.4 (c = 0.1, 24 °C)	$C_{106}H_{167}N_{39}O_{21}S_4$	2450.6	2450.2
5f	27.7 (c = 0.1, 24 °C)	$C_{106}H_{167}N_{39}O_{21}S_4$	2451.0	2450.2
6a	10.3 (c = 0.1, 31 °C)	$C_{108}H_{162}N_{38}O_{22}S_4$	2472.0	2471.2
6b	13.8 (c = 0.1, 31 °C)	$C_{109}H_{164}N_{40}O_{22}S_4$	2514.0	2513.3
6c	0 (c = 0.1, 31 °C)	$C_{106}H_{158}N_{38}O_{22}S_4$	2443.7	2443.1
6d	−18.7 (c = 0.2, 17 °C)	$C_{106}H_{157}N_{37}O_{22}S_4$	2428.7	2428.3

Optical rotations of **2c–e** were measured in 1 M AcOH solution.

fetal calf serum, 100 IU/mL penicillin and 100 mg/mL streptomycin.

Virus

A strain of HIV-1, HIV-1(III_B), was used for the anti-HIV assay. This virus was obtained from the culture supernatant of HIV-1 persistently infected MOLT-4/HIV-1(III_B) 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 MT-4 cells at the same time when the cells were infected at a multiplicity of infection (MOI) of 0.01, and placed in wells of a flat-bottomed microtiter tray (2.5×10^4/well). After a 5 d 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}).[20] Cytotoxicity of compounds was determined based on the viability of mock-infected cells using the MTT method (CC_{50}). Anti-HIV-1 activity was also determined as the inhibitory effect on the HIV-specific antigen expression. HIV-1-infected MT-4 cells (MOI = 0.01) were cultured with various concentrations of test compounds, and the viral antigen expression was then examined by indirect immunofluorescence (IF)[21] with polyclonal anti-HIV-1 antibody as a probe, and monitored by laser flow cytometry (Epics profile II; Coulter Electronics, Inc., Hialeah, FL, USA) (IC_{50}).

CD spectroscopy of T22 analogues

CD spectra were recorded on a JASCO J-720 spectropolarimeter (Tokyo, Japan). Peptides were dissolved in H_2O at concentrations of 10 μM. Measurements were conducted using 1-cm cells at 1-nm intervals, with five scans averaged for each.[16] The β-sheet compositions of peptides were calculated by Yang's method.[22]

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References and Notes

1. Abbreviations used: T22, [$Tyr^{5,12}$, Lys^7]-polyphemusin II; HIV, human immunodeficiency virus; SAR, structure–activity relationship; AIDS, acquired immunodeficiency syndrome; EC_{50}, 50% effective concentration; AZT, 3′-azido-2′, 3′-dideoxythymidine; CC_{50}, 50% cytotoxic concentration; T-tropic, T cell line-tropic; CD, circular dichroism; AgOTf, silver trifluoromethanesulfonate; DMSO, dimethylsulfoxide; MBzl, 4-methoxybenzyl; Acm, acetamidomethyl; TMSBr, trimethylsilylbromide; IC_{50}, 50% inhibitory concentration; TW70, (des-[$Cys^{8,13}$, $Tyr^{9,12}$]-[D-Lys^{10}, Pro^{11}]-T22); SI, selectivity index; IS, ion-spray; PAL, 5-(4-Fmoc-aminomethyl-3, 5-dimethoxyphenoxy)-valeric acid; Fmoc, 9-fluorenylmethyloxycarbonyl; DIPCDI, 1, 3-diisopropylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; Boc, *tert*-butyloxycarbonyl; Mtr, 4-methoxy-2, 3, 6-trimethylphenylsulfonyl; Bu^t, *tert*-butyl; TFA, trifluoroacetic acid; MOI, multiplicity of infection; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; IF, immunofluorescence.

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