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Effective lowly cytotoxic analogs of an HIV-cell fusion inhibitor, T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II)

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Abstract

A tachyplesin peptide analog, T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II), and its shortened congener, TW70 (des-[Cys^{8,13}, Tyr^{9,12}]-[D-Lys^{10}, Pro^{11}]-T22) have strong anti-human immunodeficiency virus (HIV) activity, comparable to that of 3′-azido-2′, 3′-dideoxythymidine (AZT). T22 and TW70 are extremely basic peptides, containing 5 Arg residues and 3 Lys residues. The number of positive charges might be related in part to high collateral cytotoxicities of T22 and TW70. Here we have synthesized several analogs, in which the number of positive charges has been reduced through amino acid substitutions using Glu or L-citrulline. As a result, several effective compounds have been found which possess higher selectivity indexes (SIs, 50% cytotoxic concentration/50% effective concentration) than those of T22 and TW70. Higher SIs were attributed mainly to a decrease in cytotoxicity.

Keywords: Anti-HIV peptide, T22, TW70, tachyplesin, low cytotoxicity.

1. Introduction

Acquired immunodeficiency syndrome (AIDS) [1] is the virally transmitted disease caused by HIV. To date, the life cycle of HIV-1 has been extensively elucidated using genetic engineering and cell biology, and thus, many effective agents for anti-HIV chemotherapy have been found. At present, the combined use of agents that have different antiviral mechanisms, such as reverse transcriptase inhibitors and HIV-1 protease inhibitors, is thought to be the optimum chemotherapeutic approach. Therefore further development of new agents, which affect different stages of HIV replication, is desired for use in combination therapies employing multiple drugs [2]. For this reason, we have sought effective anti-HIV compounds through chemical modifications of antimicrobial peptides, tachyplesin and polyphemusin.

Tachyplesin and polyphemusin, which are highly abundant in the hemocyte debris of Japanese horseshoe crabs (*Tachypleus tridentatus*) and American horseshoe crabs (*Limulus polyphemus*), respectively, inhibit the growth of Gram-positive and Gram-negative bacteria and some fungi [3]. These compounds also have antiviral activity against vesicular stomatitis virus, influenza A virus and HIV-1 [4]. Tachyplesins (I-III) and polyphemusins (I, II) are 17- and 18-residue peptide amides, respectively, possessing two disulfide bonds. In order to potentiate the anti-HIV activity of tachyplesins and polyphemusins, we have synthesized more than 100 peptide analogs [5]. Among these synthetic peptides, we found a novel compound, T22 ([Tyr^{5,12}, Lys^{7}]-poly-

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phemusin II), which showed strong anti-HIV activity in vitro. The effective concentration (EC_{50}) of T22 for 50% protection in an assay of HIV-induced cytopathogenicity is 2.6 nM, a value comparable to that of AZT (5.2 nM), and the cytotoxic concentration (CC_{50}) of T22 is 17 μM. T22, which is an 18-residue, Arg-rich peptide amide, takes an antiparallel β-sheet structure that is maintained by two disulfide bridges (Fig. 1) [6]. The previous experiments directed at elucidating its mechanism of action showed that T22 exerts its effect on a virus-cell fusion process [7], and that it binds specifically to both gp120 (an envelope protein of HIV) and CD4 (a T-cell surface protein) [8]. In a previous report [9], a 14-residue analog having one disulfide, TW70 (des-[$Cys^{8,13}$, $Tyr^{9,12}$]-[D-Lys^{10}, Pro^{11}]-T22), was found to exhibit highly potent activity (EC_{50} = 7.9 nM, CC_{50} = 26 μM) comparable to that of T22, and to take an antiparallel β-sheet structure similar to that of T22. This indicated that the molecular size of T22 could be reduced without loss of anti-HIV activity or significant change in its secondary structure, thereby making TW70 a potential novel lead compound. However, whereas we succeeded in shortening T22, the problem remained of relatively high cytotoxicities of T22 and TW70. Thus, our present research is focused on suppression of cytotoxicities of T22 analogs. Both T22 and TW70 are amphiphilic peptides containing both basic residues (5 Arg and 3 Lys residues) and hydrophobic residues (4 or 2 Tyr and 1 Trp residues), exhibiting total 9 positive charges throughout the entire in the molecule. Natural tachyplesin I is also an amphiphilic peptide, and its electrostatic and/or hydrophobic interaction with phospholipid bilayers is thought to cause its antimicrobial action [10]. T22's membrane permeability is related to its cytotoxicity [11]. Herein, in order to search for more effective analogs having low cytotoxicity, we have

T22	R	R	W	C	Y	R	K	C	Y	K	G	Y	C	Y	R	K	C	R	NH_2
TW70	R	R	W	C	Y	R	K			D-K	P			Y	R	K	C	R	NH_2
T121	R	R	W	C	Y	R	K			K	G			Y	R	K	C	R	OH
T122	R	R	W	C	Y	R	K			K	G			Y	R	K	C	E	OH
T123	R	R	W	C	Y	R	K			K	G			Y	R	E	C	R	OH
T124	R	R	W	C	Y	R	K			K	G			Y	E	K	C	R	OH
T125	R	R	W	C	Y	R	K			K	G			E	R	K	C	R	OH
T126	R	E	W	C	Y	R	K			K	G			Y	R	K	C	R	OH
T127	E	R	W	C	Y	R	K			K	G			Y	R	K	C	R	OH
T128	R	R	W	C	Y	E	K			K	G			Y	R	K	C	R	OH
T129	R	R	W	C	Y	E	K			K	G			Y	E	K	C	R	OH
T130	R	R	W	C	Y	R	K			K	G			E	R	K	C	E	OH
T131	R	R	Y	C	Y	R	K			D-K	P			Y	R	K	C	R	OH
T132	R	R	W	C	Y	R	K			D-K	P			Y	R	K	C	R	OH
T133	R	R	W	C	Y	R	K			D-K	P			Y	R	K	C	Ci	OH
T134	R	R	W	C	Y	R	K			D-K	P			Y	R	Ci	C	R	OH
T135	R	R	W	C	Y	R	K			D-K	P			Y	Ci	K	C	R	OH
T136	R	R	W	C	Y	Ci	K			D-K	P			Y	R	K	C	R	OH
T137	R	Ci	W	C	Y	R	K			D-K	P			Y	R	K	C	R	OH
T138	Ci	R	W	C	Y	R	K			D-K	P			Y	R	K	C	R	OH
T139	Ci	R	W	C	Y	Ci	K			D-K	P			Y	Ci	K	C	R	OH
Ac-T139	Ac-Ci	R	W	C	Y	Ci	K			D-K	P			Y	Ci	K	C	R	OH

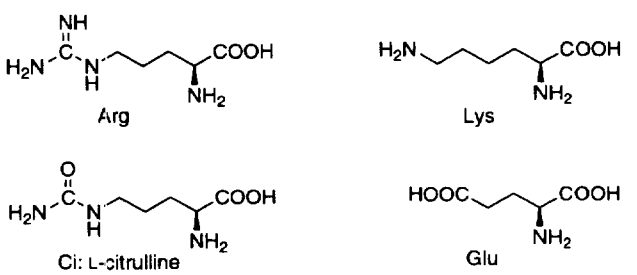

Fig. 1. Amino acid sequences of T22, TW70 and its analogs. Disulfide linkages in T22 are shown by solid lines. TW70 and its analogs have one disulfide bridge between Cys^{4} and Cys^{13}. The substituted amino acids are underlined. Ci: L-citrulline.

designed and synthesized several analogs, in which the number of total positive charges has been decreased by amino acid substitutions using Glu or L-citrulline. In this paper, we described the structure-activity relationship study on TW70.

2. Results

2.1 Chemistry

The amino acid sequences of TW70 analogs are listed in Fig. 1. T22 and TW70 have been previously synthesized [5,9]. TW70 analogs were similarly synthesized by 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid phase synthesis involving diisopropylcarbodiimide (DIPCDI)-*N*-hydroxybenzotriazole (HOBt) activation [12]. *p*-Benzyloxybenzyl alcohol linker (Alko linker) [13] was used in combination with the following Fmoc-protected amino acids: Lys(Boc), D-Lys(Boc), Tyr(Bu^t), Arg(Mtr), Cys(MBzl) and Glu(OBu^t). In the synthesis of Ac-T139, the N-terminal α-amino group of the protected T139-resin was acetylated. The protected peptide resins were treated with a trimethylsilyl bromide (TMSBr) system to cleave the peptide from the resins and remove protecting groups [14]. The resulting crude Cys(SH)^{4,13}-peptides were air-oxidized and purified by preparative HPLC and gel-filtration. The peptides thus obtained were characterized and their purity was assessed by amino acid analysis, mass spectrometry and HPLC.

2.2 Anti-HIV activity and cytotoxicity

The anti-HIV activity and cytotoxicity of T22, TW70 and its analogs are summarized in Table 1. EC_{50} values are based on the inhibition 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 [15]. Based on the previous structure-activity relationship study [12], amino acid residues contained in TW70 were substituted by Glu or L-citrulline, except 2 residues involved in the β-turn region, 2 Cys residues and 3 residues which are indispensable for activity (Trp^3, Lys^5 and Tyr^7). Tyr^{10} was substituted by Glu, but not by L-citrulline, because L-citrulline-substitution for a Tyr residue does not result in a net change of charges. T121-T130 have the same β-turn sequence [(i+1) and (i+2); Lys^8-Gly^9] as that of T22,

Table 1
Anti-HIV activity and cytotoxicity of T22, TW70 and its analogs

Compound	Charges	SI	$CC_{50}(\mu M)$	EC_{50}(nM)	IC_{50}(nM)
T22	9	650	16	25	39
TW70	9	1300	16	12	N.T.
T121	8	2700	89	32	43
T122	6	>3600	>210	57	78
T123	6	940	160	170	520
T124	6	>140	>40	300	380
T125	7	3400	130	38	81
T126	6	>2.5	>210	91000	29000
T127	6	>1500	>210	130	84
T128	6	>3400	>210	61	89
T129	4	>32	>210	6700	9180
T130	5	950	110	110	140
T131	8	4200	84	20	56
T132	8	14000	75	5.4	81
T133	7	780	89	110	130
T134	7	33000	120	3.7	14
T135	7	4900	110	22	46
T136	7	14000	88	6.5	14
T137	7	120	99	880	1600
T138	7	15000	100	7.1	41
T139	5	>330	>210	630	760
AC-T139	4	>16	>210	13000	N.T.
AZT		1800	7.6	4.1	N.T.
ddC		8800	87	9.8	N.T.

Charges: the number of total positive charges of each peptide. EC_{50} values are based on the inhibition 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. N.T.: not tested.

while T131-T139 and Ac-T139 possess the same structure (D-Lys^8-Pro^9) as that of TW70. T121 (substitution for the C-terminal amide by a carboxylic acid, total positive charges: +8) exhibited remarkably less cytotoxicity (1/5) than either T22 or TW70 (total: +9), yet maintained high anti-HIV activity. T122-T128 [total: +6 or +7(T125)] are T121 analogs substituted by 1 Glu residue. These analogs, except for T124, are all less cytotoxic than T121. T122, T125 and 128 have higher SIs than T121, suggesting that Arg^{6,14} and Tyr^{10} can be replaced by Glu. In sharp contrast, Arg^2 is indispensable for activity (see T126). The activity of T129 (total: +4), which is a T121 analog having 2 Glu residues substituted for Arg^{6,11}, is much lower than those of the corresponding 1 Glu-substituted analogs (T124 and T128, total : +6), whereas its cytotoxicity is weak. T130 (total: +5), a T121 analog having 2 Glu residues substituted for Tyr^{10} and Arg^{14}, also exhibits lower activity and selectivity indexes than the corresponding 1 Glu-substituted analogs (T122 and T125, total: +6 and +7, respectively). T132 (total: +8), which is a TW70 analog having a carboxylic acid substituted for the C-terminal amide, exhibited remarkably less cytotoxicity (1/5) than either T22 or TW70 (total: +9), yet maintained anti-HIV activity. T131 is a T132 analog having Tyr substituted for Trp^3, which maintains activity, indicating that Trp^3 can be replaced by Tyr. T133–T138 (total: +7) are T132 analogs containing 1 L-citrulline residue. These analogs all showed slightly less cytotoxicity than T132. T134, T135, T136 and T138 exhibited relatively high activities and SIs, indicated that Arg^{1,6,11} and Lys^{12} can be replaced by L-citrulline. On the other hand, T137, an analog having 1 L-citrulline substituted for Arg^2, has very weak activity. T139 (total: +5), an analog having 3 L-citrullines substituted for Arg^{1,6,11}, showed both very low activity and low cytotoxicity. A reduction of one additional charge resulted in a remarkable decrease in both activity and SI [see Ac-T139 (N^{α}-acetylated T139), total: +4].

2.3 CD spectroscopy of TW70 analogs

Conformational changes of analogs were analyzed by CD. All L-citrulline-substituted analogs (T131-T139 and Ac-T139) were found to form β-sheet structures, whereas CD spectra of Glu-substituted analogs (T121-T130) showed no characteristic patterns due to a preferred secondary structure. CD spectra of T125 and T134 in aqueous solution are shown as being representative (Fig. 2). T134 exhibited a strong negative band near 210 nm and a strong positive band near 197 nm. These bands, which are similar to those seen with TW70, are due to β-sheet structures [16]. Glu-substitution caused a change in solution structure, probably due to electrostatic interactions between Glu and Arg/Lys residues.

3. Discussion

T22 and TW70 are amphiphilic peptides containing 9 basic residues, resulting in overall strongly basic character, which might be related to observed cytotoxicities (as described in the Introduction section). In the present study, in an attempt to find analogs having reduced cytotoxicity, several compounds were designed and synthesized, in which the number of total positive charges was decreased by Glu- or L-citrulline-substitutions. Glu-substitution for a Lys or Arg residue brings about a decrease of two positive charges, whereas an L-citrulline-substitution results in a decrease of one positive charge. Since L-citrulline is an analog having an Arg-like isosteric structure with no charges, it is useful for substitutions of Arg-rich peptides such as T22 analogs. Initial findings indicated that substitution of the C-terminal amide by a carboxylic acid (total: +9 to +8) caused a remarkable decrease in cytotoxicity (see T121 and T132). It was subsequently found that reductions of total positive charges by Glu- or L-citrulline-substitutions resulted in even less cytotoxic analogs. Table 1 shows that there is an apparent correlation between the total positive charges and cytotoxicity (CC_{50}) which can be summarized as follows; total +9 charges: CC_{50} = ca. 15 μM, +8 charges: CC_{50} = ca. 80 μM, +7 charges: CC_{50} = ca. 100 μM, and < +7 charges: CC_{50} = ca. > 210 μM. It should be noted that there is no significant

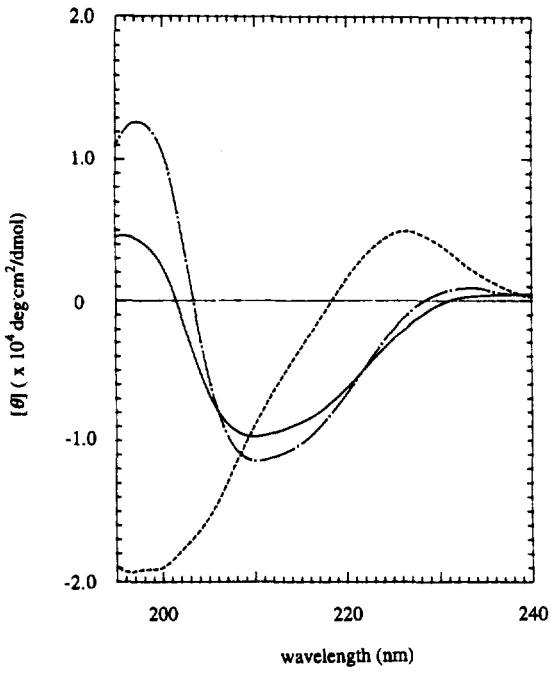

Fig. 2. CD spectra of TW70, T125 and T134. Solid line: TW70, dotted line: T125, center-dotted line: T134.

correlation between position of replaced residues and cytotoxicity.

In addition to cytotoxicity, however, effect of each substitution upon anti-HIV activity must be considered. The indispensability of the Arg^2 residue was shown by a remarkable decrease in the activities of Glu- or L-citrulline-substituted analogs (T126 or T137).

$Arg^{1,6,14}$ can be replaced by both Glu and L-citrulline (see T122, T127, T128, T133, T136 and T138). However, analogs having L-citrulline substituted for Arg^1 or Arg^6 (T138 or T136) showed higher activity than the corresponding Glu-substituted analogs (T127 or T128), suggesting that L-citrulline is a more suitable replacement for Arg residues than Glu. This may be due to the Arg-like structure of L-citrulline which has no charges. Arg^{11} and Lys^{12} can be replaced by L-citrulline (see T135 and T134), but not by Glu (see T124 and T123), indicating that an Arg-like structure or a neutral charge might be important. Taken together, Glu-substituted analogs (total: +6, except T125) have lower cytotoxicity than L-citrulline-substituted analogs (total: +7). However, analogs possessing higher activity (EC_{50} and IC_{50} < 15 nM) can be found among L-citrulline-substituted analogs (T134 and T136), but not among Glu-substituted analogs. In addition, CD analyses of TW70 analogs suggest that a β-sheet structure is not indispensable for a certain degree of activity (EC_{50} and IC_{50} = 40–90 nM, T122, T125 and T128), although it might be required for higher activity (EC_{50} and IC_{50} < 15 nM, T134 and T136).

In further studies, we investigated the possibility of multiple substitutions using Glu or L-citrulline. T129 is a T121 analog having 2 Glu residues substituted for $Arg^{6,11}$ These face each other on antiparallel β-strands. The side chains of these Arg residues and 3 additional residues which are indispensable for activity (Trp^3, Lys^5 and Tyr^7) are directed toward opposite sides of the plane of the β-sheet, indicating that the side of the plane containing $Arg^{6,11}$ might not be related to activity. The activity of T129 (total: +4) is much weaker than those of the corresponding 1 Glu-substituted analogs (T124 and T128, total : +6), whereas its cytotoxicity is weak. Similarly T130 (2 Glu for Tyr^{10} and Arg^{14}, total: +5) showed lower activity than T122 (1 Glu for Arg^{14}, total: +6) or T125 (1 Glu for Tyr^{10}, total: +7). An analog having 3 L-citrullines, T139 (3 L-citrullines for $Arg^{1,6,11}$, total: +5) also exhibited much lower activity than the corresponding 1 L-citrulline-substituted analogs, T135 (1 L-citrulline for Arg^{11}, total: +7), T136 (1 L-citrulline for Arg^6, total: +7) and T138 (1 L-citrulline for Arg^1, total: +7). Furthermore, an N^{α}-acetylated analog, Ac-T139 (total: +4) showed much lower activity than T139. Since N^{α}-acetylated TW70 exhibited almost the same activity as that of TW70 in our recent study [9], a decrease in the activity of Ac-T139 may not be due to the functionality of the acetyl group itself, but rather due to a reduction of positive charges (+5 to +4). It has been found that analogs possessing total +5 charges (T130 and T139) have low activity (EC_{50} and IC_{50} = 100–800 nM), with analogs having +4 charges (T129 and Ac-T139) showing even lower activity (EC_{50} and IC_{50} = ca. 10 μM). These results suggest that a minimum of 6 positive charges are indispensable for the expression of high anti-HIV activity, and that analogs with total +6 or +7 charges exhibit a suitable balance between activity and cytotoxicity.

In conclusion, a reduction of the number of total positive charges by Glu- or L-citrulline-substitutions was proven to be a useful strategy for developing several effective analogs possessing low cytotoxicity. Compounds, T121, T122, T125, T127, T128, T131, T132, T134, T135, T136 and T138, were found to have higher SIs than those of T22 and TW70, with their high SIs being attributable mainly to an increase in CC_{50}. Furthermore, there is a remarkable correlation between the number of the total positive charges and associated CC_{50} values, with total +6 or +7 charges representing a good balance between activity and cytotoxicity. These findings will aid in the rational design of effective compounds possessing much higher potency and lower cytotoxicity.

4. Experimental

Amino acid analyses were conducted using a Hitachi 835 instrument (Tokyo, Japan). HPLC solvents were H_2O and CH_3CN, both containing 0.1% (v/v) TFA. For analytical HPLC, a Waters μBondasphere 5μC18–100Å column (3.9×150 mm, Nihon Millipore Ltd., Tokyo, Japan) was eluted with a linear gradient of CH_3CN (10–20%, 30 min) at a flow rate of 1 ml/min on a Waters LC Module I equipped with a Waters 741 Data Module. Preparative HPLC was performed on a Waters Delta Prep 4000 equipped with a Cosmosil 5C18-AR column (20×250 mm, Nacalai Tesque Inc., Kyoto, Japan) 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 mass spectra were obtained with a Sciex API*III*E triple quadrupole mass spectrometer (Toronto, Canada). Optical rotations of peptides as 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). AZT and ddC were obtained from Sigma Chemical Co. (St. Louis, MO). All the other chemicals were purchased from either Nacalai Tesque Inc. or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

4.1 Synthesis of TW70 analogs

4.1.1 Representative T125.

Fmoc-Arg(Mtr)-OH (1.16 g, 1.75 mmol), 4-dimethylaminopyridine (42.8 mg, 0.35 mmol) and DIPCDI (270 μl, 1.75 mmol) were added to Alko resin (500 mg, 0.35 mmol) in DMF (5 ml). The mixture was shaken at r.t. for 3 h to give 690 mg of Fmoc-Arg(Mtr)-Alko-resin (0.41 mmol/g) following filtration. Subsequently, the protected T125-resin was manually constructed using Fmoc-based solid-phase methodology on Fmoc-Arg(Mtr)-Alko-resin (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 [17]. The following side-chain protected Fmoc amino acids were used: Cys(MBzl), Lys(Boc), Arg(Mtr), Glu(OBu^t) and Tyr(Bu^t). The resulting protected T125-resin (148 mg, 27.6 μmol) was treated with 1 M TMSBr-thioanisole/TFA (5 ml) in the presence of *m*-cresol (250 μl, 100 equiv.) and 1,2-ethanedithiol (100 μl, 60 equiv.) at 4°C for 2 h. After removal of the resin by

Table 2
Characterization of the synthetic TW70 analogs

	Yield (%)	r.t. (min)	$[\alpha]_D$(°) (H_2O)	Formula	Ion spray mass (reconstructed)	
					Found	Calculated
T121	24.3	19.6	−8.11 (c = 0.2, 30°C)	$C_{85}H_{137}N_{33}O_{17}S_2$	1956.5	1956.0
T122	50.3	22.0	−12.3 (c = 0.4, 30°C)	$C_{84}H_{132}N_{30}O_{19}S_2$	1929.5	1929.0
T123	33.2	21.5	−10.4 (c = 0.4, 30°C)	$C_{84}H_{132}N_{32}O_{19}S_2$	1957.7	1957.1
T124	28.9	21.4	−6.93 (c = 0.1, 30°C)	$C_{84}H_{132}N_{30}O_{19}S_2$	1929.5	1929.0
T125	53.6	17.3	−25.9 (c = 0.4, 30°C)	$C_{81}H_{135}N_{33}O_{18}S_2$	1922.2	1922.1
T126	51.2	20.4	−14.3 (c = 0.4, 30°C)	$C_{84}H_{132}N_{30}O_{19}S_2$	1929.5	1929.0
T127	40.6	20.3	0 (c = 0.3, 30°C)	$C_{84}H_{132}N_{30}O_{19}S_2$	1929.5	1929.0
T128	10.1	22.6	16.6 (c = 0.1, 16°C)	$C_{84}H_{132}N_{30}O_{19}S_2$	1929.0	1929.0
T129	13.9	27.1	0 (c = 0.1, 16°C)	$C_{83}H_{127}N_{27}O_{21}S_2$	1903.5	1901.9
T130	19.7	25.8	0 (c = 0.2, 16°C)	$C_{80}H_{130}N_{30}O_{20}S_2$	1895.7	1895.1
T131	34.2	20.6	−24.5 (c = 0.2, 26°C)	$C_{86}H_{140}N_{32}O_{18}S_2$	1974.0	1973.2
T132	20.4	24.2	−21.6 (c = 0.2, 29°C)	$C_{88}H_{141}N_{33}O_{17}S_2$	1996.2	1996.1
T133	25.3	26.2	−21.1 (c = 0.1, 26°C)	$C_{88}H_{140}N_{32}O_{18}S_2$	1998.3	1997.0
T134	27.9	26.1	0 (c = 0.1, 16°C)	$C_{88}H_{140}N_{34}O_{18}S_2$	2025.0	2025.1
T135	14.0	24.7	−8.82 (c = 0.1, 26°C)	$C_{88}H_{140}N_{32}O_{18}S_2$	1998.0	1997.0
T136	30.9	24.8	−24.9 (c = 0.2, 26°C)	$C_{88}H_{140}N_{32}O_{18}S_2$	1998.0	1997.0
T137	52.0	24.7	−39.5 (c = 0.1, 26°C)	$C_{88}H_{140}N_{32}O_{18}S_2$	1998.0	1997.0
T138	49.3	23.8	−17.9 (c = 0.1, 26°C)	$C_{88}H_{140}N_{32}O_{18}S_2$	1998.0	1997.0
T139	47.5	25.2	−4.15 (c = 0.2, 16°C)	$C_{88}H_{138}N_{30}O_{20}S_2$	1999.3	1999.0
Ac-T139	51.6	28.0	−22.9 (c = 0.2, 16°C)	$C_{90}H_{140}N_{30}O_{21}S_2$	2041.8	2041.0

Yield was calculated from the corresponding protected peptide resin. r.t. was a retention time on analytical HPLC eluted with a linear gradient of CH_3CN (10–20%, 30 min).

Table 3
Amino acid ratios of the synthetic TW70 analogs after 6 M HCl hydrolysis

	L-Citrulline	Glu	Gly	Tyr	Lys	Arg	Pro
T121			1.00 (1)	2.02 (2)	3.00 (3)	4.70 (5)	
T122		0.98 (1)	1.01 (1)	2.02 (2)	3.00 (3)	3.83 (4)	
T123		0.97 (1)	1.01 (1)	1.99 (2)	2.00 (2)	4.77 (5)	
T124		0.98 (1)	1.01 (1)	2.06 (2)	3.00 (3)	3.81 (4)	
T125		1.00 (1)	1.02 (1)	0.98 (1)	3.00 (3)	4.74 (5)	
T126		0.94 (1)	1.01 (1)	2.02 (2)	3.00 (3)	3.86 (4)	
T127		0.97 (1)	1.00 (1)	2.00 (2)	3.00 (3)	3.86 (4)	
T128		1.03 (1)	1.06 (1)	2.02 (2)	3.00 (3)	3.94 (4)	
T129		1.98 (2)	1.03 (1)	2.00 (2)	3.00 (3)	2.97 (3)	
T130		2.38 (2)	1.07 (1)	1.01 (1)	3.00 (3)	3.59 (4)	
T131				2.87 (3)	3.00 (3)	4.85 (5)	0.95 (1)
T132				1.99 (2)	3.00 (3)	5.49 (5)	1.06 (1)
T133	0.99 (1)			1.92 (2)	3.00 (3)	4.02 (4)	0.97 (1)
T134	1.01 (1)			1.96 (2)	2.00 (2)	4.83 (5)	1.21 (1)
T135	0.94 (1)			1.89 (2)	3.00 (3)	4.01 (4)	0.96 (1)
T136	0.97 (1)			1.93 (2)	3.00 (3)	4.03 (4)	0.98 (1)
T137	0.95 (1)			1.92 (2)	3.00 (3)	4.00 (4)	0.98 (1)
T138	0.94 (1)			2.05 (2)	3.00 (3)	4.09 (4)	0.94 (1)
T139	3.01 (3)			2.13 (2)	3.00 (3)	1.86 (2)	0.95 (1)
Ac-T139	3.08 (3)			2.08 (2)	3.00 (3)	1.90 (2)	0.90 (1)

Cystine and Trp were not determined. Lys contains D-Lys.

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 total solution volume adjusted to 200 ml with H_2O and then pH was adjusted to 7.8 with concentrated NH_4OH. After 1 day's air-oxidation, the pH of the solution was adjusted to 5 with AcOH. The crude product was purified by preparative HPLC and gel-filtration to afford a white powder following lyophilization; yield 36.5 mg (14.8 μmol, 53.6% based on the protected T125-resin).

Additional analogs were synthesized in similar fashion. For acetylation of the N-terminal α-amino group of the protected T139-resin during the synthesis of Ac-T139, the protected T139-resin (25 μmol) was treated with acetic anhydride (236 μl, 1000 equiv.) and pyridine (201 μl, 1000 equiv.) in DMF (5 ml) at r. t. for 2 h. Yields, physical constants and analytical data are listed in Tables 2 and 3.

4.2 Evaluation of anti-HIV activity and cytotoxicity

The HIV-1(III_B) strain of HIV-1 was used for anti-HIV evaluation. This virus was obtained from the culture supernatant of HIV-1 persistently infected MOLT-4/HIV-1(III_B) cells. Antiviral activity against HIV-1 was determined based on the protection against virus-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 (2.5×10^4/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 [18]. Cytotoxicity of compounds was determined based on the viability of mock-infected cells using the MTT method. Anti-HIV-1 activity was also determined as the inhibitory effect on the virus-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 IF [15] with polyclonal anti-HIV-1 antibody as a probe, and monitored by laser flow cytometry (Epics profile II; Coulter Electronics, Inc., Hialeah, FL).

4.3 CD spectroscopy of TW70 analogs

Peptides were dissolved in H_2O at concentrations of 10 μM. CD spectra were recorded on a JASCO J-720 spectropolarimeter (Tokyo, Japan) using 1 cm cells at 1 nm intervals, with 5 scans averaged for each.

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References and Notes

[1] Abbreviations used: T22, [Tyr^{5,12} Lys^{7}]-polyphemusin II; TW70, des-[Cys^{8,13}, Tyr^{9,12}]-[D-Lys^{10}, Pro^{11}]-T22); HIV, human immunodeficiency virus; AZT, 3′-azido-2′, 3′-dideoxythymidine; AIDS, acquired immunodeficiency syndrome; EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration; Fmoc, 9-fluorenylmethyloxycarbonyl; DIPCDI, 1,3-diisopropylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; Alko, *p*-benzyloxybenzyl alcohol; Bu^{t}, *tert*-butyl; Mtr, 4-methoxy-2,3,6-trimethylphenylsulfonyl; MBzl, 4-methoxybenzyl; TMSBr, trimethylsilyl bromide; SI, selectivity index; IC_{50}, 50% inhibitory concentration; IF, immunofluorescence; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; ddC, 2′,3′-dideoxycytidine.

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