Synthesis of Protegrin-Related Peptides and Their Antibacterial and Anti-human Immunodeficiency Virus Activity[1)]

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All disulfide analogs (types I, II and III) of protegrin (PG)-1, an 18-residue antimicrobial peptide having two intramolecular disulfide bonds, were synthesized using regioselective disulfide bond formation. Random air-oxidation of the fully reduced PG-1 formed the type III PG-1. In addition, a type III analog containing an amidated carboxy-terminal residue was also prepared. Each analog showed significant and different antibacterial and anti-human immunodeficiency virus (HIV) activity. Deletion of two disulfide bridges caused a significant decrease in activity.

Key words protegrin; antimicrobial activity; anti-human immunodeficiency virus activity; regioselective disulfide bond formation; defensin; tachyplesin

Protegrin (PG)-1, which was isolated from porcine leukocytes, is an 18-residue, Arg-rich peptide containing two intramolecular disulfide bonds. PG-1 has a broad spectrum of antimicrobial activity against gram-positive and gram-negative bacteria and fungi.[2)] Its primary structure combines features of defensin[3)] and tachyplesin[4)]; it especially shows substantial homology to the corticostatic rabbit defensin, NP-3a (Fig. 1). Defensins and tachyplesins are cationic, Arg-rich peptides, having structures stabilized by intramolecular disulfide bridges. These peptides exhibit not only antimicrobial activity against many gram-positive and gram-negative bacteria and fungi, but also antiviral activity against vesicular stomatitis virus and herpes simplex virus.[5,6)] Recently, these peptides were found to have anti-HIV activity.[7,8)] These findings prompted us to examine whether protegrin has anti-HIV activity. However, the positions of two disulfide bonds of PG-1 have not yet been determined. Additionally, the possibility that the carboxy-terminal residue is amidated can not be excluded.

In the present study, in order to examine the effects of disulfide pairing and the carboxy-terminal form on the biological activity, three types of disulfide isomers [PG-1(OH) types I, II and III] (Fig. 2), a linear analog [4Cys(Acm)–PG-1(OH)] and a type III analog containing an amidated carboxy-terminal residue [PG-1(NH_2) type III] were synthesized and subjected to biological assay.

Results and Discussion

Syntheses of Protegrin-Related Peptides The peptidyl resins of PG-1(OH) (types I, II and III) were synthesized using the Fmoc-based solid-phase method.[9)] For the synthesis of the type I analog, Cys(Trt)[10)] was incorporated at positions 13 and 15, and Cys(Acm)[11)] at positions 6 and 8. For the type II analog, Cys(Trt) was incorporated at positions 8 and 15, and Cys(Acm) at positions 6 and 13. For the type III analog, Cys(Trt) was incorporated at positions 8 and 13, and Cys(Acm) at positions 6 and 15. Treatment of the constructed peptide resins with 1 M TMSBr–thioanisole/TFA[12)] resulted in the cleavage of the peptides from the resins with the simultaneous removal of all protecting groups except for the S-Acm groups. Each resulting dihydropeptide, [2Cys(SH), 2Cys(Acm)]–PG-1(OH), was diluted to 6×10^{-5} M and air-oxidized to form the first disulfide bridge. The dihydropeptide of type I was diluted further to 7.5×10^{-6} M for air-oxidation, since two kinds of dimer products are generated along with the monomer at the usual concentration (6×10^{-5} M) (data not shown). Each crude monocyclic product, identified by ion spray mass spectrometry analysis, was subjected to iodine treatment[13)] in MeOH to construct the second disulfide bridge. Analytical HPLC of each reaction mixture showed a main peak having a different retention time (Fig. 3a—c). The integrity of each isomer was determined by amino acid analysis after 6 M HCl hydrolysis and ion spray mass spectrometry analysis. However, during the synthesis of the type I analog, iodine treatment of the crude monocyclic Cys(Acm)-peptide on a large scale (23 mg) exclusively gave the type III analog, which resulted from disulfide-scrambling, whereas iodine treatment on a small scale (1.2 mg) gave a mixture of types I and III in a ratio of 7 : 4. Since the selective preparation of PG-1(OH) type I was difficult using this procedure, we searched for conditions

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                1       5       10      15
HNP-1               A C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
PG-1        R G G R L C Y C R R R F C V C V G R
NP-3a               G I C A C R R R F C P N S E R F S G Y C R V N G A R Y V R C C S R R
tachyplesin I  K W C F R V C Y R G I C Y R R C R-NH2
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Fig. 1. Amino Acid Sequences of Human Defensin HNP-1, PG-1, Rabbit Defensin NP-3a and Tachyplesin I, with Alignment Based on Their Homology

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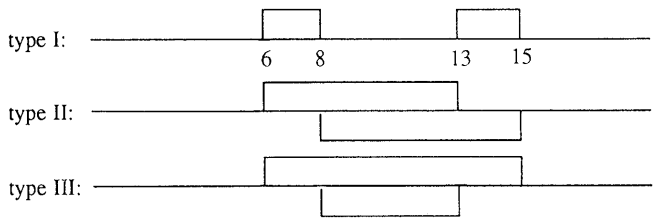

Fig. 2. Three Possible Disulfide Structures of PG-1

that would suppress the exchange of disulfide bonds during the second disulfide bond formation of type I. Several disulfide bond-forming reactions were preliminarily examined: iodine (1, 2, 4, 6 or 8 eq), DMSO–TMSCl,[14] AgOTf[15]–DMSO/HCl[16] and $Tl(TFA)_3$[17] oxidations. After each treatment, the ratio of individual disulfide isomers was estimated by measuring peak areas on analytical HPLC. The results are summarized in Table 1. Treatment with iodine (1 eq) did not completely convert the starting Cys(Acm)-peptide within 30 min. Treatment with iodine (2—8 eq) yielded a mixture of type I and III analogs. DMSO–TMSCl oxidation caused the exclusive formation of type III. In the AgOTf–DMSO/HCl system, formation of types I and III (5 : 6) was observed. $Tl(TFA)_3$ oxidation gave the best result; almost no disulfide exchange occurred during the oxidation (Fig. 3d). The variety of these results might be related to the character of the oxidant and the solvent. While both DMSO–TMSCl oxidation and $Tl(TFA)_3$ oxidation were performed in the same solvent (TFA), the former caused more disulfide scrambling than the latter. The exclusive formation of the correct disulfide bridge by $Tl(TFA)_3$ was not due to the character of the solvent, but rather to that of the oxidant. In the second disulfide bond formation of type I, DMSO was not a suitable oxidant; DMSO–TMSCl and AgOTf–DMSO/HCl systems gave undesirable results. Based on the above results, $Tl(TFA)_3$ oxidation was preferable for the larger-scale synthesis of the type I analog.

Next, a linear analog, 4Cys(Acm)–PG-1(OH), which contained Cys(Acm) instead of Cys at four modification sites, was synthesized using the Fmoc-based solid-phase method followed by deprotection with 1 M TMSBr–thioanisole/TFA. In order to prepare the fully reduced PG-1(OH), the Cys(Acm)-peptide was treated with AgOTf followed by reduction with DTT. Air-oxidation of the fully reduced peptide gave the type III analog without any other accompanying isomers (Fig. 3e). This result suggested that the type III disulfide bonds were preferentially formed under physiological conditions, and that type III might be the natural conformer.

Additionally, the carboxy-terminally amidated type III analog, PG-1(NH_2) type III, was synthesized in a manner similar to that used for the synthesis of PG-1(OH) type III (Fig. 3f).

We conducted a conformational study of these peptides in aqueous solution by CD spectroscopy. None of the spectra showed characteristic patterns due to a secondary structure (Fig. 4). Each disulfide isomer of PG-1(OH) exhibited a different spectrum. This result suggested that the regioselective disulfide bond formations of the three isomers of PG-1(OH) were successful. PG-1(OH) type III

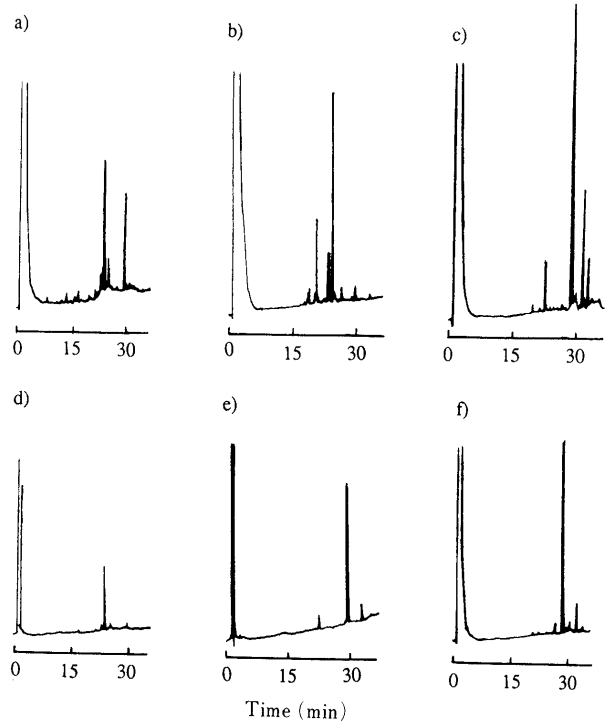

Fig. 3. Analytical HPLC (Gradient I) Profiles of Crude PG-1(OH) (Type I, a; Type II, b; Type III, c) and Crude PG-1(NH_2) Type III (f) Synthesized by Air and Iodine Oxidation, and Crude PG-1(OH) Type I Synthesized by Air and $Tl(TFA)_3$ Oxidation (d) and by Random Air-Oxidation of Tetrahydro-PG-1(OH) (e)

Table 1. Disulfide Bond Formation of PG-1(OH) Type I from [Cys(Acm)^{6,8}]–PG-1(OH) by Various Oxidation Systems

Oxidation system	Relative peak areas of the three disulfide isomers (%)			
	Type I	Type II	Type III	[Cys(Acm)^{6,8}] –PG-1(OH)
I_2 1 eq (30 min)	23	0	30	47
2 eq (30 min)	49	0	51	
4 eq (5 min)	73	0	27	
6 eq (5 min)	72	0	28	
8 eq (5 min)	70	0	30	
0.2 M DMSO–1 M TMSCl/TFA	0	0	100	
AgOTf/TFA–50% DMSO/ 1 M HCl	44	0	56	
$Tl(TFA)_3$ (1.2 eq)/TFA	100	0	0	

and PG-1(NH_2) type III showed almost the same pattern.

Antibacterial Activity The minimum inhibitory concentrations (*MIC*) of the above peptides against bacterial strains and fungi were determined using a previously reported method[18] (Table 2). The strains used in this study were *Escherichia coli* JC-2 (gram-negative bacteria), *Salmonella aureus* 209P (gram-positive bacteria) and *Candida albicans* TMD10 (fungi). In the antimicrobial assay against a strain of gram-negative bacteria, PG-1(OH) type III and PG-1(NH_2) type III possessed the most potent activity. PG-1(OH) type II exhibited lower activity, and PG-1(OH) type I and 4Cys(Acm)–PG-1(OH) had very weak or no activity. Against a strain of gram-positive bacteria, PG-1(NH_2) type III possessed the strongest activity. Bacteriostatic effects of PG-1(OH) types I and II were weak and those of 4Cys(Acm)–PG-1(OH)

and PG-1(OH) type III were very weak. PG-1(NH_2) type III has strong antimicrobial activity against both gram-negative and gram-positive bacteria, comparable to those of antibiotics kanamycin and ampicillin. Deletion of two disulfide bridges caused a significant decrease in antimicrobial activity against both gram-negative and gram-positive bacteria. These findings were similar to those reported for in tachyplesin I.[19] These results suggest that a structure maintained by disulfide bridges plays an important role in the activity of these antimicrobial peptides. However, none of these peptides exhibited activity against fungi at concentrations up to 50 μg/ml. On the other hand, Kokryakov *et al.* reported that PG-1 showed antimicrobial activity of about 40 units at the concentration of 500 μg/ml (activity of 10 units corresponds to a 1 mm diameter clear zone around the sample well).[2] This difference may be due to that in the methods of antimicrobial testing.

Anti-HIV Activity Next, the anti-HIV activity of these peptides was evaluated by the 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method[20] and the indirect immunofluorescence (IF) method[21] (Table 3). All of the protegrin-related peptides, except for 4Cys(Acm)–PG-1(OH), have almost the same potency in terms of anti-HIV activity; their antiviral activity was seen at concentrations 6 to 23 times lower than their cytotoxic concentration. The values of selectivity index (ratio of CC_{50} to EC_{50} (IC_{50})) were comparable to those of tachyplesins and polyphemusins.[22] Anti-HIV activity of protegrin-related peptides is a little stronger than those of defensins; the EC_{50}s of guinea-pig NP, rabbit NP-1 and rat NP-1 were 30.2, 38.1 and 28.4 μg/ml, respectively.[7] However, cytotoxicity of protegrin-related peptides is relatively strong, while no cytotoxicity of defensins was observed at concentrations up to 500 μg/ml. These results suggest that the amino-terminal 15 residues of defensins, covering the region homologous to protegrin, might be related to anti-HIV activity. Recently, a structure–activity relationship study on tachyplesin and polyphemusin analogs led us to find a novel synthetic compound, T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II), which showed strong anti-HIV activity and relatively low cytotoxicity *in vitro*.[22,23] The selectivity index of T22 exceeded 6000. The anti-HIV activity of protegrin-related peptides, demonstrated in this study, was weaker than that of T22. However, the chemical structure of protegrins resembles that of tachyplesin, polyphemusin and T22. The strategy employed in developing T22 may be applicable to the design of more active analogs based on protegrins.

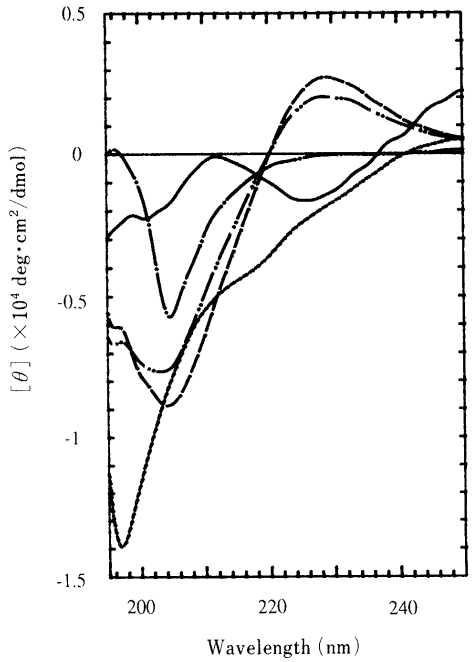

Fig. 4. Circular Dichroism Spectra of Protegrin-Related Peptides

Solid line, PG-1(OH) type I; dotted line, PG-1(OH) type II; dashed line, PG-1(OH) type III; center-dotted line, 4Cys(Acm)–PG-1(OH); center-double dotted line, PG-1(NH_2) type III.

Conclusion

In this study, three types of disulfide isomers of PG-1(OH) were synthesized using the regioselective disulfide bond formation technique. Disulfide bonds of types II and III were formed by the combination of air and iodine oxidation, while regioselective formation of type I disulfide bonds could not be achieved by this method. The combination of air and Tl$(TFA)_3$ oxidation was the best method for the correct disulfide bond formation of the type I analog, among the several oxidation methods examined in this study. Random air-oxidation of the fully reduced PG-1(OH) gave the type III analog. This result suggests that type III might be the natural conformer. The three disulfide isomers have significant and different antimicrobial activity, while a linear analog, which contains no disulfide bridges, is almost inactive. These results indicated the biological importance of the structure maintained by two disulfide bridges. The type III analog with an amidated carboxy-terminal residue (PG-1(NH_2) type III) exhibited the strongest bactericidal activity among the peptides examined. Furthermore, these peptides also showed anti-HIV activity comparable to those of tachyplesin and polyphemusin. The information obtained in this study may assist in rational design of compounds possessing higher activity. Direct comparison

Table 2. *MIC* of Protegrin-Related Peptides against *E. coli*, *S. aureus*, and *C. albicans*

Strain	PG-1(OH)			4Cys(Acm)–PG-1(OH)	PG-1(NH_2) Type III	Kanamycin	Ampicillin
	Type I	Type II	Type III				
E. coli JC-2	>50	25	12.5	50	12.5	12.5	12.5
S. aureus JC-1	25	25	>50	>50	6.25	6.25	3.12
C. albicans	>50	>50	>50	>50	>50		

MIC: μg/ml.

Table 3. Anti-HIV Activity of Protegrin-Related Peptides

Compound	IC_{50} (μg/ml)[a]	EC_{50} (μg/ml)[b]	CC_{50} (μg/ml)[b]
PG-1(OH) type I	2.3	2.1	16.8
PG-1(OH) type II	2.4	1.3	19.1
PG-1(OH) type III	3.1	1.6	14.6
4Cys(Acm)-PG-1(OH)	3.4	17.7	398.7
PG-1(NH_2) type III	1.0	1.6	9.9
T22	0.016	0.0076	52.1
AZT	0.0027	0.0018	14.8
Dextran sulfate	0.31	0.59	>1000

a) Indirect IF assay. *b*) MTT assay.

between PG-1 (NH_2 or OH) type III and natural PG-1 will disclose the position of disulfide bonds and the carboxy-terminal form of natural PG-1.

Experimental

Amino acid analysis was conducted using a Hitachi 835 instrument. HPLC was performed on a Waters model 600E or a Waters LC Module I equipped with a Waters 741 Data Module. The solvents for HPLC were H_2O and CH_3CN, both containing 0.1% (v/v) TFA. For analytical HPLC, μBondasphere 5μC18-100 Å (3.9×150 mm) was eluted with a linear gradient of CH_3CN (gradient I, 15—30%, 30 min; gradient II, 15—45%, 30 min) at a flow rate of 1 ml/min. Preparative HPLC was performed on a YMC Packed Column (ODS, 20×250 mm) at a flow rate of 7 ml/min. The eluate was monitored by UV absorption measurements at 220 nm. Ion spray mass spectra were obtained with a Sciex API *III*E Biomolecular Mass Analyzer. Optical rotations of the peptides in water were measured with a JASCO DIP-360 digital polarimeter. Fmoc-Arg(Mtr)-*p*-benzyloxybenzyl alcohol (Alko) resin and 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid resin (PAL™-resin) were purchased from Watanabe Chemical Industries, Ltd. and Millipore, respectively.

Three Types of Disulfide Isomers of PG-1(OH) (Types I, II and III) The protected peptide resins of three disulfide isomers of PG-1(OH) (types I, II and III) were constructed using the Fast Moc™ procedure with an Applied Biosystems model 431A automated peptide synthesizer. The Fmoc-amino acid derivative (5 eq) in DMF–NMP (1:1, 4.2 g) was successively condensed using HBTU[24]-HOBt (5 eq, each) in the presence of DIPEA (5 eq) on Fmoc–Arg(Mtr)–Alko resin (0.40 mmol/g, 0.2 mmol scale) for 50 min in combination with 20% piperidine/NMP (v/v, 5 ml) treatment (6 min) to remove the Fmoc group. The following side-chain-protected Fmoc amino acids were used: Arg(Mtr) and Tyr(Bu^t). For the S-protection of the four Cys residues, two kinds of protecting groups were employed; in type I, Trt groups for Cys^{13} and Cys^{15}, and Acm groups for Cys^6 and Cys^8, in type II, Trt groups for Cys^8 and Cys^{15}, and Acm groups for Cys^6 and Cys^{13}, and in type III, Trt groups for Cys^8 and Cys^{13}, and Acm groups for Cys^6 and Cys^{15}. Each protected resin (200 mg, 30 μmol) was treated with 1 M TMSBr–thioanisole/TFA (20 ml) in the presence of *m*-cresol (1 ml, 400 eq) and EDT (400 μl, 240 eq) at 4 °C for 2 h. After removal of the resin by filtration, the filtrate was concentrated *in vacuo*. Dry ether was added to precipitate the product. The product was dissolved in 50% (v/v) AcOH (5 ml). The total volume of solution was brought to 500 ml or 4 l (type I) with H_2O and its pH was adjusted to 7.5 with concentrated NH_4OH. After 2 d, the pH was adjusted to 5 with AcOH, followed by the addition of Diaion HP-20 resin (*ca.* 10 g). The mixture was stirred for 1 h, and the resin was collected by filtration. The peptide was eluted from the resin with 80% (v/v) CH_3CN in 1 M AcOH (100 ml). The solvent was removed by evaporation and lyophilization to give a crude powder; [Cys(Acm)^{6,8}–PG-1(OH) (type I)] yield: 69.2 mg (30.0 μmol) 100% (based on the protected peptide resin), retention time on analytical HPLC (gradient I): 23.3 min, ion spray mass (reconstructed) *m/z*: 2299.8 (calcd 2299.3 for $C_{94}H_{158}N_{38}O_{22}S_4$), [Cys(Acm)^{6,13}–PG-1(OH) (type II)] yield: 69.5 mg (30.0 μmol) 100%, retention time (gradient I): 22.3 min, ion spray mass (reconstructed) *m/z*: 2299.7 (Calcd 2299.3 for $C_{94}H_{158}N_{38}O_{22}S_4$), [Cys(Acm)^{6,15}–PG-1(OH) (type III)] yield: 60.4 mg (26.1 μmol) 87.0%, retention time (gradient I): 27.0 min, ion spray mass (reconstructed) *m/z*: 2299.7 (Calcd 2299.3 for $C_{94}H_{158}N_{38}O_{22}S_4$).

Each crude 2Cys(Acm)–PG-1(OH) (1.22 mg, 0.5 μmol) was dissolved in MeOH:H_2O=1:3 (v/v, 500 μl) in the presence of 15 eq of HCl. MeOH solution (75 μl) containing I_2 (15 eq) was added at room temperature.[13] At intervals (5, 15, 30, 45, 60 and 120 min), part of the reaction mixture (50 μl each) was sampled and the reaction was stopped by adding saturated ascorbic acid solution (50 μl). The progress of the reaction was monitored by analytical HPLC. The HPLC (gradient I) elution pattern of each sample for a 5—15 min treatment (type I: 5 min, type II: 5 min, type III: 15 min) is shown in Fig. 3a—c; retention time of main peak: 23.9 min (type I), 24.7 min (type II), 29.0 min (type III). The product corresponding to each main peak was subjected to ion spray mass spectrometric analysis; (reconstructed) *m/z*: 2155.8 (type I), 2155.7 (type II), 2155.7 (type III) (Calcd 2155.2 for $C_{88}H_{146}N_{36}O_{20}S_4$). Iodine treatment of Cys(Acm)^{6,8}–PG-1(OH) (type I) gave PG-1(OH) types I and III in a ratio of 7:4 (HPLC peak area).

Each crude 2Cys(Acm)–PG-1(OH) (23 mg, 10 μmol) was dissolved in MeOH:H_2O=1:3 (v/v, 10 ml) in the presence of 15 eq of HCl. MeOH solution (1.5 ml) containing I_2 (15 eq) was added at room temperature. After a 5—15 min treatment (type I: 5 min, type II: 5 min, type III: 15 min), the reaction was stopped by adding saturated ascorbic acid solution (10 ml). The type II or III product was isolated by adsorption on Diaion HP-20 resin (*ca.* 5 g) as described above, and purified by HPLC (gradient of CH_3CN, type II: 20—23%, 30 min, type III: 24—27%, 30 min); [PG-1(OH) (type II)] yield: 5.5 mg (2.6 μmol) 25.5% (based on the protected peptide resin), amino acid ratios after 6 M HCl hydrolysis (values in parentheses are theoretical): Gly 3.00 (3), Cys N. D. (2), Val 1.93 (2), Leu 0.99 (1), Tyr 0.90 (1), Phe 0.96 (1), Arg 5.80 (6), $[\alpha]_D^{20}$ −114.43° (*c*=0.2, H_2O), [PG-1(OH) (type III)] yield: 4.3 mg (2.0 μmol) 17.4% (based on the protected peptide resin), amino acid ratios after 6 M HCl hydrolysis (values in parentheses are theoretical): Gly 3.00 (3), Cys N. D. (2), Val 1.92 (2), Leu 0.98 (1), Tyr 0.85 (1), Phe 0.97 (1), Arg 5.69 (6), $[\alpha]_D^{20}$ 0° (*c*=0.2, H_2O). Iodine treatment of the crude Cys(Acm)^{6,8}–PG-1(OH) (type I) mainly gave the type III peptide instead of the desired type I peptide.

Disulfide Bond Formation of PG-1(OH) Type I The crude Cys(Acm)^{6,8}-PG-1(OH) (type I) (1.22 mg, 0.5 μmol each) was treated with the following oxidation systems; i) I_2 (1, 2, 4, 6, or 8 eq) in MeOH:H_2O=1:3 (v/v, 500 μl) containing HCl (15 eq) for 5—30 min, ii) 0.2 M DMSO-1 M TMSCl in TFA (3 ml) for 1.5 h, iii) AgOTf (40 eq)/TFA (500 μl)–50% (v/v) DMSO/1 M HCl (1.2 ml) for 9 h, iv) $Tl(TFA)_3$ (1.2 eq) in TFA (2.5 ml) for 1 h. Each crude product was analyzed by HPLC, and the ratio of disulfide isomers was estimated from the corresponding peak areas on analytical HPLC. Results are shown in Table 1. The HPLC (gradient I) elution pattern of the crude sample oxidized by the $Tl(TFA)_3$ method, which gave the best result, is shown in Fig. 3d.

The crude Cys(Acm)^{6,8}–PG-1(OH) (type I) (26.6 mg, 11 μmol) was treated with $Tl(TFA)_3$ (10.6 mg, 1.2 eq) in TFA (32.4 ml) in the presence of anisole (270 μl, 200 eq) at 4 °C for 1 h. The product was precipitated by the addition of dry ether and purified by HPLC (gradient of CH_3CN: 21—24%, 30 min); yield: 4.1 mg (1.9 μmol) 17.4% (based on the protected peptide resin), amino acid ratios after 6 M HCl hydrolysis (values in parentheses are theoretical): Gly 3.00 (3), Cys N. D. (2), Val 1.86 (2), Leu 1.01 (1), Tyr 0.84 (1), Phe 1.01 (1), Arg 5.55 (6), $[\alpha]_D^{20}$ 18.39° (*c*=0.1, H_2O).

4Cys(Acm)–PG-1(OH) The protected peptide resin was manually constructed using the Fmoc-based solid-phase method on Fmoc–Arg(Mtr)–Alko resin (0.40 mmol/g, 0.2 mmol scale). The Fmoc-amino acid derivative (2.5 eq) was successively condensed using DIPCDI (2.5 eq) in the presence of HOBt (2.5 eq) according to the reported schedule.[25] The following side-chain-protected Fmoc amino acids were used: Arg(Mtr), Cys(Acm) and Tyr(Bu^t). The protected resin (200 mg, 33 μmol) was treated with 1 M TMSBr–thioanisole/TFA (10 ml) in the presence of *m*-cresol (500 μl, 180 eq) and EDT (200 μl, 110 eq) at 4 °C for 2 h. After removal of the resin by filtration, the filtrate was concentrated *in vacuo*. Dry ether was added to precipitate the product. The crude product was purified by HPLC (gradient of CH_3CN: 19—22%, 30 min); yield: 49.6 mg (20.3 μmol) 61.6% (based on the protected peptide resin), retention time on analytical HPLC (gradient II): 16.1 min, amino acid ratios after 6 M HCl hydrolysis (values in parentheses are theoretical): Gly 3.00 (3), Cys(Acm) N. D. (4), Val 1.93 (2), Leu 0.98 (1), Tyr 0.90 (1), Phe 0.99 (1), Arg 5.82 (6), ion spray mass (reconstructed) *m/z*: 2444.0 (Calcd 2443.4 for $C_{100}H_{170}N_{40}O_{24}S_4$), $[\alpha]_D^{20}$ −57.06°

($c=0.3$, H_2O).

Random Air-Oxidation of Tetrahydro-PG-1(OH) The crude 4Cys-(Acm)–PG-1(OH) (20 mg, 8.2 μmol) obtained above was treated with AgOTf (168 mg, 80 eq) in TFA (2 ml) in the presence of anisole (35 μl, 40 eq) at 4 °C for 2 h. Dry ether was added to precipitate the product. The product was dissolved in 0.2 M Tris–HCl buffer containing 6 M guanidine hydrochloride (pH 7.5) (1 ml) and DTT (126 mg, 100 eq) was added. The mixture was stirred at room temperature overnight, followed by centrifugation. The supernatant was applied to a column of Sephadex G-15 (2.1 × 43 cm), which was eluted with 4 M AcOH. The fractions corresponding to the front main peak (monitored by UV absorption measurement at 280 nm) were collected; the total volume of solution was brought to 200 ml with H_2O and its pH was adjusted to 7.5 with concentrated NH_4OH. The HPLC (gradient I) elution pattern of the solution after 2 days' treatment is shown in Fig. 3e. This main product was co-eluted with the type III disulfide isomer on analytical HPLC. The product was isolated by adsorption on Diaion HP-20 (*ca.* 2 g) and purified by HPLC (gradient of CH_3CN: 24—27%, 30 min); yield: 8.2 mg (3.8 μmol) 34.8% (based on the protected resin), retention time on analytical HPLC (gradient I): 28.9 min, amino acid ratios after 6 M HCl hydrolysis (values in parentheses are theoretical): Gly 3.00 (3), Cys N. D. (2), Val 1.94 (2), Leu 0.98 (1), Tyr 0.91 (1), Phe 1.03 (1), Arg 5.81 (6), ion spray mass (reconstructed) *m/z*: 2156.0 (Calcd 2155.2 for $C_{88}H_{146}N_{36}O_{20}S_4$), $[\alpha]_D^{20}$ 0° ($c=0.1$, H_2O).

PG-1(NH_2) (Type III) The protected peptide resin was constructed on PAL™–resin (0.31 mmol/g, 0.2 mmol scale) in essentially the same way as in the synthesis of PG-1(OH) (type III). The protected peptide was cleaved from the resin (200 mg, 27 μmol) and similarly deprotected. The product was air-oxidized and isolated by adsorption on Diaion HP-20 (*ca.* 10 g); [crude Cys(Acm)^{6,15}–PG-1(NH_2) (type III)] yield: 25.2 mg (11 μmol) 40.6% (based on the protected resin), retention time on analytical HPLC (gradient I): 22.7 min, ion spray mass (reconstructed) *m/z*: 2298.0 (Calcd 2298.3 for $C_{94}H_{159}N_{39}O_{21}S_4$).

The crude Cys(Acm)^{6,15}–PG-1(NH_2) (type III) (23 mg, 10 μmol) was subjected to iodine-oxidation in the same way as in the synthesis of PG-1(OH) (type III) (Fig. 3f), followed by HPLC purification (gradient of CH_3CN: 23—26%, 30 min); [PG-1(NH_2) (type III)] yield: 3.4 mg (1.6 μmol) 6.5% (based on the protected resin), retention time on analytical HPLC (gradient I): 29.1 min, amino acid ratios after 6 M HCl hydrolysis (values in parentheses are theoretical): Gly 3.00 (3), Cys N. D. (2), Val 1.92 (2), Leu 0.98 (1), Tyr 0.89 (1), Phe 1.02 (1), Arg 5.71 (6), ion spray mass (reconstructed) *m/z*: 2154.0 (Calcd 2154.2 for $C_{88}H_{147}N_{37}O_{19}S_4$), $[\alpha]_D^{20}$ −14.49° ($c=0.1$, H_2O).

CD Spectroscopy The CD spectra were obtained using a JASCO spectropolarimeter, model J-720, according to the previously reported method[19] (Fig. 4). The concentration of peptides in water was 10^{-5} M.

Bacterial Strains and Culture Media *Escherichia coli* JC-2, *Staphylococcus aureus* 209P and *Candida albicans* TMD10 were used. Mueller Hinton broth (Difco Laboratories, Detroit, MI, U.S.A.) or Sabouraud liquid medium (Difco) was employed for the subculture or antibactericidal activity test. The microorganisms were subcultured at 37 °C overnight.

Antibacterial Assay *MIC*s were determined using the broth dilution method using a 0.5 ml medium of serial dilutions of antimicrobial agent and an inoculum of 5 μl of an early-log-phase culture (approximately 5×10^5/ml).[18] The medium with inoculated microorganisms was incubated for 24 h at 37 °C, and bacterial growth was determined visually or by measuring the absorbance at 550 nm. *MIC* was expressed as the lowest final concentration (μg/ml) at which no growth was observed (Table 2).

Anti-HIV Assay The antiviral activity against HIV-1 was determined on the basis of the protection against virus-induced cytopathogenicity in MT-4 cells.[23] Various concentrations of the test compounds were added to each HIV-1-infected MT-4 cell aliquot at a multiplicity of infection (MOI) of 0.01, in the wells of a flat-bottomed microtiter tray (2.5×10^4 cells/well). After 5 days' incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined by the MTT method.[20] The cytotoxicity of the compounds was determined on the basis of the viability of mock-infected cells by the MTT method. The anti-HIV activity was also determined as the inhibitory effect on virus-specific antigen expression.[21] HIV-1-infected MT-4 cells (MOI = 0.01) were cultured with various concentrations of test compounds, and the viral antigen expression was examined by IF with polyclonal anti-HIV-1 antibody as a probe, monitored by laser flow cytometry (Epics Profile II; Coulter Electronics, Inc., Hialeah, FL).

References and Notes

1) The amino acids used here are of the L-configuration. The following abbreviations are used: PG = protegrin, HIV = human immunodeficiency virus, Fmoc = 9-fluorenylmethyloxycarbonyl, Trt = triphenylmethyl, Acm = acetamidomethyl, TMSBr = trimethylsilyl bromide, TFA = trifluoroacetic acid, DMSO = dimethylsulfoxide, TMSCl = trimethylsilyl chloride, AgOTf = silver trifluoromethanesulfonate, DTT = dithiothreitol, CD = circular dichroism, *MIC* = minimum inhibitory concentration, MTT = 3′-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide, IF = indirect immunofluorescence, CC_{50} = 50% cytotoxic concentration, EC_{50} = 50% effective concentration, IC_{50} = 50% inhibitory concentration, Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl, Alko = *p*-benzyloxybenzyl alcohol, DMF = *N*,*N*-dimethylformamide, NMP = *N*-methylpyrrolidone, HBTU = 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt = *N*-hydroxybenzotriazole, DIPEA = *N*,*N*-diisopropylethylamine, Bu^t = *tert*-butyl, EDT = 1,2-ethanedithiol, DIPCDI = 1,3-diisopropylcarbodiimide.

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