Roseltide rT7 is a disulfide-rich, anionic, and cell-penetrating peptide that inhibits proteasomal degradation

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Running title: Disulfide-rich proteasome inhibitor

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\textbf{ABSTRACT}  
Disulfide-rich plant peptides with molecular weights of 2 to 6 kDa represent an expanding class of peptidyl-type natural products with diverse functions. They are structurally compact, hyperstable, and underexplored as cell-penetrating agents that inhibit intracellular functions. Here, we report the discovery of an anionic, 34-residue-peptide, the disulfide-rich roseltide rT7 from Hibiscus sabdariffa (of the Malvaceae family), that penetrates cells and inhibits their proteasomal activities. Combined proteomics and NMR spectroscopy revealed that roseltide rT7 is a cystine-knotted, 6C-hevein-like cysteine-rich peptide. A pair-wise comparison indicated that roseltide rT7 is > 100-fold more stable against protease degradation than its S-alkylated analog. Confocal microscopy studies and cell-based assays disclosed that after roseltide rT7 penetrates cells, it causes accumulation of ubiquitinated proteins, inhibits human 20S proteasomes, reduces tumor necrosis factor-\textalpha; degradation, and decreases expression levels of intercellular adhesion molecule-1. Structure--activity studies revealed that roseltide rT7 uses a canonical substrate-binding mechanism for proteasomal inhibition enabled by an IIML motif embedded in its proline-rich and exceptionally long intercysteine loop 4. Taken together, our results provide mechanistic insights into a novel disulfide-rich, anionic, and cell-penetrating peptide, representing a potential lead for further development as a proteasomal inhibitor in anti-cancer or anti-inflammatory therapies.

\textit{Hibiscus sabdariffa}, a medicinal plant that is native to Central and West Africa, is highly popular in Southeast Asia (Figure 1A). It belongs to the Malvaceae family and is known by the common name, roselle. The plant is used in the treatment of degenerative and inflammatory diseases, such as hypertension and cancer (1).

Previously, we reported the discovery of a family of 6-cysteine hevein-like peptides (6C-HLPs) from \textit{H. sabdariffa}, designated roseltides (rT1–rT8) (2). Of the eight members identified in this family, roseltide rT1 and rT7 are the two most abundant roseltides found in the aqueous extract of the calyces. Roseltide rT1 was first identified as a human neutrophil elastase inhibitor (2) and recently, as a cell-penetrating mitochondria-targeting peptide that modulates cellular bioenergetics via interaction with ATP synthase (3). However, roseltide rT7 has not yet been characterized.

Sequence comparison of roseltides rT1 and rT7 showed striking differences in the amino acid sequences and lengths of their intercysteine loop 4 (Figure 1B). Loop 4 of roseltide rT7 contains a 12-residue sequence, IIMLPTWPRYPV, which is proline-rich, hydrophobic, cationic, and eight amino acid residues longer than the corresponding loop 4 of rT1. A sequence search of roseltide rT7 loop 4 revealed that the hydrophobic IIML motif shares high sequence identity to epoxomicin and carmaphycin, two naturally-occurring proteasome...
inhibitors of the α',β'-epoxyketone peptide family (Figure 1C) (4-6). In addition, the presence of three proline residues and one bulky hydrophobic residue in loop 4 suggests that roseltide rT7 could penetrate cells and inhibit proteasomal activities.

The ubiquitin-proteasome system plays a major role in the recycling and degradation of unwanted proteins (7-10). It is also involved in important intracellular processing pathways, including apoptosis, antigen-presentation, differentiation, and NF-κB activation (11-17). The 26S proteasome is the largest intracellular protease complex of 2.5 MDa. Structurally, it consists of a cylindrical 20S proteolytic core, and one or two 19S regulatory particles which recognizes ubiquitinated substrates for proteolysis (11,18,19). The core, which comprises two pairs of 14 different subunits arranged into four stacked rings, is the site at which proteolysis occurs (11,18-20). Six of the 14 subunits are threonine proteases with characteristic proteolytic sites. They are chymotrypsin-, trypsin-, and caspase-like proteases (β5, β2, and β1, respectively), which cleave peptide bonds at the C-terminal of hydrophobic, basic, and acidic residues, respectively (11,18-20). Importantly, three proteasomal inhibitors have been clinically approved for anti-cancer treatment, especially against multiple myeloma, by promoting pro-apoptotic and inhibiting pro-inflammatory events.

In this study, we report the identification of roseltide rT7 as a hyper-stable cell-penetrating proteasome inhibitor. Plants produce diversified protease inhibitors that impact nearly all biological processes for their defense and development (21,22). However, no plant-derived cell-penetrating cysteine-rich peptide (CRP) inhibitor has been reported to target chymotrypsin-like proteasomes. In this regard, the cysteine- and proline-rich roseltide rT7 represents a “first-in-class” cell-penetrating proteasomal inhibitor with high preference for chymotrypsin-like activities.

RESULTS

Characterization and synthesis of roseltide rT7 — Roseltide rT7, the second most abundant CRP in H. sabdariffa aqueous calyces extract (Figure 1B), has an m/z value of 3696 (2,3). Figure S1 shows purified roseltide rT7 from the calyces extract using our previously established purification protocol for roseltide rT1. The primary amino acid sequence of roseltide rT7, CVSSGIVDACSECEPDKCIIMLPTWPRYYCV SV, was confirmed using de novo peptide sequencing (Figures S2-S4).

Figures 2A and 2B shows the nuclear magnetic resonance (NMR) structure of roseltide rT7 using the distance restraints obtained from 1H-1H two-dimensional nuclear Overhauser effect spectroscopy (2D 1H-1H NOESY). All 1H spin-spin systems of rT7 were identified, except proline 16, 24, 27, and 28 (Table S1). We observed roseltide rT7 has at least three conformations in solution. This is due to several Proline residues present in the peptide sequence. The major solution structure of rT7 was determined based on a total of 220 NMR-derived distance restraints. Figure 2A shows the NMR ensemble of the 17 lowest-energy rT7 structures. The root-mean-square deviation (RMSD) value of the 17 best structures for residues Cys1-Glu15, Cys19-Leu23, and Arg29-Ser33 was 0.81 ± 0.24 Å and that for all heavy atoms was 1.33 ± 0.33 Å (Table 1). The structure of rT7 is well-defined by a number of medium and long-range NOEs, which consists of two short extended anti-parallel β-strands (β1: Ser4-Val7 and β2: Try30-Ser33). The loop region (Pro24-Pro28) cannot be well-defined because there are three proline residues located within this loop. The solution structures of roseltide rT7 showed that the three disulfide bonds (Cys I-IV, II-V, and III-VI) adopted a cystine-knot fold similar to our previously published knottin family peptides of the same cysteine spacing pattern such as roseltide rT1 (2), bleogen pB1 (23), and alstotide as1 (24). Compared to roseltide rT1, roseltide rT7 has an unusually long intercysteine loop 4 in which the three prolines promote turn formation (Figures 2A-B).

In order to characterize the biological activity of roseltide rT7, we prepared its synthetic version via a stepwise solid-phase method using Fmoc chemistry (Figure 3). After the removal of protecting groups and cleavage from the solid support by trifluoroacetic acid, the linear precursor of roseltide rT7 was oxidatively folded using a combination of redox reagents consisting of cysteamine and cystamine in 0.1 M ammonium bicarbonate (pH 8) at 4 °C for 1 h before being subjected to RP-HPLC purification. The purified synthetic and natural roseltide rT7 were indistinguishable as demonstrated by a co-eluted
single peak using a reverse-phase high-performance liquid chromatography (RP-HPLC) and 2D NOESY spectra overlay (Figures S5-S7). Using similar methods, we also prepared the N-terminally tetramethylrhodamine (TAMRA)-labeled roseltide rT7 (TAMRA-rT7) as a chemical probe for biochemical and bioimaging studies. Synthetic roseltide rT7 was used throughout this study.

**Disulfide-rich roseltide rT7 displays high proteolytic and structural stability** — Cysteine-rich peptide crosslinked by multiple disulfides are known for their high stability against proteolytic degradation (2,3,23,25,26). Figures 4A–D shows that roseltide rT7 is stable against trypsin, pronase, neutrophil elastase, and pepsin. It is >100-fold more stable than the S-alkylated form of roseltide rT7, an analog with all disulfide bridges reduced and S-alkylated. Using far-UV circular dichroism (CD) spectroscopy, we showed that the CD spectrum of roseltide rT7 was not altered at temperature up to 90 °C, suggesting that roseltide rT7 is highly resistant to heat denaturation (Figure S8).

**Roseltide rT7 is cell penetrating** — A few cysteine-rich peptides, including roseltide rT1, alstotide as1, kalata B1, and MCoT-1, are known to be cell membrane permeable (3,24,27-30). However, these peptides are generally cationic as compared to the anionic roseltide rT7. In order to determine cellular roseltide rT7 uptake, flow cytometry and live-cell confocal microscopy were used. Figure 5A shows the live-cell images of A549 cells after a 2 h incubation with 1 µM TAMRA-rT7. The confocal images revealed that TAMRA-rT7 is internalized, distributed throughout the cell, and enters the nucleus. In order to provide a quantitative measurement for TAMRA-rT7 cellular uptake in A549 cells, we used flow cytometry, which showed an increase in fluorescence intensity of the cell population after incubation with 1 µM TAMRA-rT7 and reached a plateau by 2 h (Figure 5B).

To determine whether the cellular uptake is mediated by endocytosis, TAMRA-rT7 was incubated with A549 cells at 4 °C for 1 h. Figure 5C shows that the cellular uptake of TAMRA-rT7 at 4 °C was substantially reduced as compared to those at 37 °C. Additionally, cellular uptake of TAMRA-rT7 was reduced by endocytosis inhibitors, such as dynasore and ethyl-isopropyl amiloride (EIPA) (Figure S9). Roseltide rT7 did not cause significant membranolytic effects in A549 up to 100 µM as measured by lactate dehydrogenase leakage assay (Figure S10). These results suggested that the cellular uptake of TAMRA-rT7, at least in part, involves energy-dependent endocytosis.

**Roseltide rT7 as a human 20S proteasome inhibitor** — In order to show that roseltide rT7 is a human proteasome inhibitor, we compared roseltide rT7 with a commercially available covalent proteasome inhibitor, MG132, and confirmed that they both promoted the accumulation of ubiquitinated proteins using western blotting in A549 cells (Figure 6A). Furthermore, using a commercially available purified human 20S proteasome, we showed that roseltide rT7 inhibited chymotrypsin-like human 20S proteasome activities with an IC$_{50}$ of 3.3 µM, whereas the commercially available covalent proteasome inhibitor, MG132, has an IC$_{50}$ of 0.13 µM (Figure 6B). In contrast, the negative control, roseltide rT1, did not inhibit chymotrypsin-like human 20S proteasome activities at concentrations up to 10 µM (Figure S11). Roseltide rT7 also weakly inhibited caspase-like 20S proteasome activity with a 20% inhibition at 10 µM (Figure S12) but not trypsin-like human 20S proteasome activity at concentrations up to 10 µM (Figure S13). Using proteasome activity probe, Me4BodipyFL-Ahx3Leu3VS, we further showed that roseltide rT7 inhibited human 20S proteasome activity at concentrations up to 10 µM (Figure 6C). Using MALDI-TOF MS, we showed that roseltide rT7 is stable against human 20S proteasome-mediated cleavage (Figure 6D). We also demonstrated that roseltide rT7 inhibited chymotrypsin-like human 26S proteasome activities (Figure 6E).

In order to provide mechanistic insights into the proteasome inhibitory effects of roseltide rT7, several substrate-like pentapeptide analogs with the Ile-Ile-Met-Leu-Xaa sequence, in which Leu-Xaa is the scissile bond, were chemically synthesized. Our results are summarized in Figure 6F. IIMLA, IIMLK, and IIMLP caused an inhibition of chymotrypsin-like activities with an IC$_{50}$ of 101 µM, 129 µM, and 140 µM, respectively. As Pro is not tolerated at the P1 position of the scissile bond, IIMPA and IIMPK were synthesized as the negative controls. Using HPLC and LC-MS analysis of pentapeptide analogs, we further showed that Ile-Ile-Met-Leu-Xaa is a proteasome substrate recognition sequence (Figures 6F, S14-S17).
Ixβα is a proteasome substrate and an inhibitor for nuclear factor (NF)-κB activation (31,32). Previous reports have shown that tumor necrosis factor (TNF) induced NF-κB activity and inflammatory markers by promoting the proteasomal degradation of IκBα (33-35). One of the well-studied NF-κB target gene is intercellular adhesion molecule (ICAM)-1 (36-38). **Figure 7A** shows that pre-treatment with roseltide rT7 or MG132 reduced TNF-mediated downregulation of IκBα expression in A549 cells. Furthermore, in order to determine the effects of roseltide rT7 on TNF-mediated inflammation, we examined its effect on TNF-induced ICAM-1 expression in A549 cells. **Figures 7B and 7C** shows that TNF caused downregulation of mRNA and protein expression of ICAM-1. Pre-treatment with roseltide rT7 or MG132 attenuated TNF-induced ICAM-1 mRNA and protein expressions.

**DISCUSSION**

The present study identified the 34-residue roseltide rT7 as a cell-penetrating 20S-proteasome inhibitor. To the best of our knowledge, this is the first report of a cysteine-rich peptide inhibitor against the chymotrypsin-like human 20S proteasome. A previous report described another plant-derived cysteine-rich peptide, *Momordica charantia* trypsin inhibitor (MCTI-1), which appeared to cross-react against the trypsin-like human 20S proteasome (39). However, roseltide rT7 does not share sequence homology with MCTI-1.

Roseltide rT7 belongs to the family of the non-chitin-binding, six-cysteine hevein-like peptides (6C-HLP) (2). 6C-HLPs have an evolutionarily conserved cysteine motif in which a tandemly-connecting CC at the Cys III and IV positions forms a cystine-knot disulfide connectivity (Cys I-IV, II-V, and III-VI) (2,3,23,26). Structurally stabilized by its cystine-knot scaffold, roseltide rT7 displays exceptional stability against heat denaturation and proteolytic degradation, a desirable feature that is highly sought after by pharmaceutical industries for biologics. In contrast, removing all three disulfide constraints, represented by the S-alkylated rT7, reduces its proteolytic stability by >100 fold.

Contrary to the general perception that cell-penetrating peptides need to be cationic (40-42), this study by using live-cell confocal microscopy and flow cytometry showed that the anionic roseltide rT7 internalizes into living cells partly through energy-dependent endocytosis mechanism. The cell-penetrating effect of roseltide rT7 is likely contributed by two major factors. First, the cystine scaffold is responsible for an “inside-out” structural feature of a CRP (25). This phenomenon is caused by the dense cystine core, which forces the hydrophobic side chains to point outwards. As such, within the same molecule, CRPs can display both hydrophilic and hydrophobic surface patches. The NMR structure of roseltide rT7 revealed the presence of this “inside-out” feature with all the side-chains pointing outwards. Because this, roseltide rT7 possesses both hydrophilic and hydrophobic surface properties (**Figure S18**).

Secondly, roseltide rT7 contains four prolines within its 34 residues, three of which are embedded in loop 4 and account for 25% of the residues in this loop. The hydrophobic surface region of roseltide rT7 located at the Pro-rich region together with the Arg residue at loop 4 creates an exposed cationic hydrophobic surface that could be important for driving its cell-penetration. This finding is similar to the observations from our and others previous studies on bactenecin-7 and pyrrhocoricin into which cationic Pro-rich peptides can penetrate cells (43,44).

The crosslinked disulfide bonds of 6C-HLPs resulted in a topological resemblance to a four-leaf clover consisting of four intercysteine loops (2,3,23). In 6C-HLP, these loops are evolvable and generally differ from each other in sequence and size. Importantly, they are often functional sites contributing to their multiple biological activities (25,45,46). This feature has also been extensively exploited for the design of hyperstable and multi-targeting peptide biologics for extracellular and intracellular targets (47-54). Roseltide rT7 has a long intercysteine loop 4 with an amino acid sequence of IIMLPWPRYYV that is absent in roseltide rT1. The IIML motif in roseltide rT7 showed sequence similarity to the tetrapeptide guiding sequence of the α', β'-epoxyketone-type proteasome inhibitors epoxomicin and carmaphycin. We showed that roseltide rT7 inhibits chymotrypsin-like human 20S proteasome activities, but roseltide rT1, missing the IIML motif, does not display any inhibition. Our results suggest that loop 4 of roseltide rT7 is the reactive-site loop for its effects against human 20S proteasome.
Epoxomicin and carmaphycin are the α',β'εpoxo- ketone-type covalent 20S proteasome inhibitors derived from microorganisms (5,6,55). They have two important features of a tetrapeptide guiding sequence and a C-terminal epoxyketone group (4,20,56-58). The tetrapeptide guiding sequence determines its binding specificity through the formation of an antiparallel β sheet. Previous studies have shown that the P1 and P3 positions in the β5 proteasome subunit are important for its binding specificity (20,56,57,59-61). The P1 position favors Leu, while the P3 position prefers a hydrophobic amino acid residue (61). In contrast, the P2 position does not participate in the interaction of the binding pocket, and the P4 position accepts a wide range of incoming groups with preference for hydrophobic moieties (20,62). The C-terminal epoxyketone group serves as a “warhead” that links covalently to the catalytic threonine residue of the proteasome subunit (20). As the IIML motif of roseltide rT7 is homologous to the tetrapeptide guiding sequence, we envisioned that it could bind to the β5 proteasome subunit. Structure–activity studies using synthetic pentapeptides with the Ile-Ile-Met-Leu-Xaa sequence motif provided mechanistic insights into its inhibitory activities. Most importantly, roseltide rT7 represents a conceptual lead for the development of other proteasome inhibitors through epitope-grafting of substrate sequence into structurally stable scaffolds.

**EXPERIMENTAL PROCEDURES**

**Materials** — All chemicals and solvents were purchased from Sigma-Aldrich, USA, and ThermoFisher Scientific, USA, unless specified otherwise.

**Extraction and purification of roseltide rT7** — Dried calyces (1 kg) of *H. sabdariffa* were extracted with water and centrifuged at 9000 rpm for 10 min at 4 °C (Beckman Coulter, USA). The supernatant was filtered, and the filtrate loaded onto a C18 flash column (GE Healthcare, USA) and eluted with 60% ethanol/0.01% trifluoroacetic acid (TFA). The eluted fractions were loaded onto an SP Sepharose resin column (GE Healthcare, USA) and eluted with 1 M NaCl (pH 3.0) followed by ultrafiltration (ViVaflow 200, 2000 molecular weight cut-off hydrostat). Further purification was done by RP-HPLC (Shimadzu, Japan). A linear gradient of mobile phase A (0.05% TFA in H2O) and mobile phase B (0.05% TFA in acetonitrile [ACN]) was used with the C18 column (250 x 22 mm, 5 μm, 300Å; Grace Davison, USA). Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) was used to identify the presence of roseltide rT7 in the eluted fractions.

**S-reduction and S-alkylation** — Purified translocation of NF-κB subunits and its activation for protein synthesis (Figure 7D). Activation of NF-κB has been reported to upregulate ICAM-1, a cell-surface adhesion molecule that promotes the recruitment of immune cells to trigger inflammatory response (36-38). Using ICAM-1, a TNF-inducible cell adhesion molecule as a marker, we showed that roseltide rT7 accumulated IκBα and suppressed the upregulated expressions of both ICAM-1 mRNA and protein in TNF-treated A549 cells, similar to MG132 and other proteasome inhibitors (67).

In conclusion, this study describes the discovery of roseltide rT7 as a novel cell-penetrating hyperstable proteasome inhibitor. Collectively, our results suggested that the 12-residue hydrophobic and proline-rich loop 4 of roseltide rT7 is responsible for its functional properties. Also, our studies of the Ile-Ile-Met-Leu-Xaa sequence motif provided mechanistic insights into its inhibitory activities. Most importantly, roseltide rT7 represents a conceptual lead for the development of other protease inhibitors through epitope-grafting of substrate sequence into structurally stable scaffolds.
roseltide rT7 (1 mg/mL) was S-reduced by 10 mM dithiothreitol (DTT) in ammonium bicarbonate buffer (25 mM) pH 8 at 37 °C for 30 min followed by S-alkylation with 60 mM of iodoacetamide (IAM) at 37 °C for 45 min. MALDI-TOF MS was used to confirm the mass shift after S-reduction and S-alkylation.

De novo peptide sequencing — S-alkylated roseltide rT7 (1 mg/mL) was digested with Lys-C, Arg-C or Glu-C in 5:1 (w/w) ratio in ammonium bicarbonate buffer (25 mM), pH 8 at 37 °C for 10 min. The digested peptide fragments were then analyzed by MALDI-TOF MS followed by MS/MS (AB SCIEX 4800 MALDI-TOF/TOF). De novo peptide sequencing was performed using the b- and y-ions.

Solid-phase peptide synthesis and oxidative folding of roseltide rT7 — Roseltide rT7 was synthesized by Fmoc-based solid-phase peptide synthesis on chlorotriyl (Cl-MPA) Proteide resin (LL) using an automatic microwave-assisted peptide synthesizer. The linear precursor peptide was cleaved using a cocktail consisting of 92.5 % TFA, 2.5 % H₂O, 2.5 % 1,2-ethanedithiol, and 2.5 % triisopropylsilane at room temperature for 2 h followed by precipitation with diethyl ether. The crude cleavage product was folded in 10% dimethyl sulfoxide (DMSO), 90% 0.1 M NH₄HCO₃ (pH 8), cystamine (10 equivalents), and cysteamine (100 equivalents) for 1 h at 4 °C. Folded roseltide rT7 was purified by preparative HPLC (250 x 21 mm, 5 µm; Phenomenex, USA). A linear gradient of mobile phase A (0.1 % TFA in H₂O) and mobile phase B (0.1 % TFA in ACN) was used. The folded TAMRA-rT7 was identified using MALDI-TOF MS. The folding yield was approximately 70%. RP-HPLC and 2-dimensional-nuclear magnetic resonance (2D NMR) were performed to compare the physical properties of synthetic roseltide rT7 to its native form.

N-terminal 5-TAMRA labeling of rT7 was performed on a peptide resin with a mixture of 5-TAMRA (4.0 eq.), N, N-diisopropylethylamine (DIPEA; 6.0 eq.), PyAOP; (4.0 eq.) in 50% dimethylformamide (DMF), and 50% 1-methyl-2-pyrrolidinone (NMP) for 2 h at room temperature. After 2 h reaction, the TAMRA-labeled-rT7 was cleaved and oxidatively-folded as described above. The folded TAMRA-rT7 was purified by preparative HPLC and identified using MALDI-TOF MS.

Disulfide-rich proteasome inhibitor

Chemical synthesis of linear pentapeptide analogs — The linear pentapeptide with the amino acid sequences IIMLP, IIMLA, IIMLK, IIMPA, and IIMPK were synthesized by Fmoc-based solid-phase peptide synthesis on rink-amide Protide resin (LL) using an automatic microwave-assisted peptide synthesizer. The linear precursor peptide was cleaved in a cocktail consisting of 92.5% TFA, 2.5% H₂O, 2.5% 1,2-ethanedithiol, and 2.5% triisopropylsilane at room temperature for 2 h and precipitated using diethyl ether. The pentapeptide was purified by preparative HPLC and identified using an Orbitrap Elite mass spectrometer (Thermo Scientific Inc., Bremen, Germany) coupled with a Dionex UltiMate 3000 UHPLC system (Thermo Scientific Inc., Bremen, Germany) and RP-HPLC (Table S2, Figures S19-S28).

NMR spectroscopy and structural determination — All NMR experiments were conducted at 25 °C on a BRUKER Avance 800 NMR spectrometer with a cryogenic probe. The concentrations of natural and synthetic roseltide rT7 were approximately 1 mM and contained 5% D₂O and 95% H₂O. For ¹H, ¹H-2D TOCSY and NOESY, the mixing times were 80 and 200 msec, respectively. The spectral width was 12 ppm for both dimensions. The NMR spectra were processed using NMRPipe software (68). All data analyses were performed using Sparky software based on the 2D NOESY and TOCSY experiment (69). The proton chemical shift assignments for each amino acid residue were achieved by 2D ¹H-¹H TOCSY and ¹H-¹H NOESY while the proton-proton distances restraints were obtained from 2D ¹H-¹H NOESY based on the intensities of the NOE cross-peaks.

The solution structures of roseltides were calculated using CNSsolve 1.3 software (70). The proton-proton distance restraints and three disulphide bond restraints were employed in a standard simulated annealing protocol. The distance restraints were divided into three classes based on the intensities of NOE cross-peaks: (1) strong: 1.8 < d < 2.9 Å; (2) medium: 1.8 < d < 3.5 Å; and (3) weak: 1.8 Å < d < 5 Å. A total of 100 structures were calculated, and the 17 lowest energy structures were chosen for data statistics and presentation. The structure was verified using the PROCHECK program and presented using Chimera version 1.6.2 (71). The PDB code for this deposition is 6KLM. The BMRB code for this deposition is 36273.

Cell cultures — Human alveolar basal epithelial adenocarcinoma A549 cells were
cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/mL of penicillin and streptomycin.

**Cellular uptake analyses by flow cytometry** — In order to study cellular TAMRA-rT7 uptake using flow cytometry, cells were incubated with 1 μM TAMRA-rT7 in serum-free medium at 37 °C. Following incubation, cells were harvested and collected by centrifugation at 500 g for 5 min. In order to quench extracellular fluorescence, cells were treated with 150 μg/mL of trypan blue, and the samples were analyzed by flow cytometry. A total of 10,000 cells was analyzed using a BD LSRFortessaTM X-20 flow cytometer. For temperature-dependent uptake studies, A549 cells were incubated at 4 °C for 30 min prior to incubation with TAMRA-rT7 for 1 h at 4 °C. For endocytosis inhibitor studies, A549 cells were pretreated with endocytosis inhibitors, including dynasore (50 μM), ethylisopropylamiloride (50 μM), and nystatin (50 μg/mL) for 30 min followed by incubation with TAMRA-rT7 for 1 h at 37 °C.

**Confocal microscopy analysis** — In order to examine the intracellular TARMA-rT7 distribution, cells were seeded on an 8-well chamber slide (Ibidi, Germany). Prior to incubation with TARMA-rT7, cells were stained with Hoechst 333241. TARMA-rT7 was incubated on cells in phenol red-free and serum-free medium at 37 °C. The slides were washed gently with phosphate-buffered saline (PBS) three times, and the media was replaced prior to imaging. Slides were observed using a Zeiss LSM 710 confocal microscope.

**Proteasome inhibition assay** — Proteasome-Glo™ chymotrypsin-, trypsin-, and caspase-like assay kit (Promega, USA) was used to determine proteasome inhibition. Briefly, purified human 20S proteasome (100 pM, BostonBiochem, USA) or human 26S proteasome (500 pM, BostonBiochem, USA) was incubated with MG132 or roseltide rT7 for 1 h at 37 °C. 500 nM Me4BodipyFL-Ahx3Leu3VS, was then added for 30 min at 37 °C. The resultant mixture was mixed with 6x loading dye with 2-mercaptoethanol and heated for 10 min at 85 °C. The samples were resolved using a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V constant for 120 min. In-gel fluorescence detection was performed using Bio-rad ChemiDoc MP system (Bio-rad, USA).

**Western blot analysis** — A549 cells treated with roseltide rT7 or MG132 were harvested and lysed in CelllyticTM M lysis buffer supplemented with both protease and phosphatase inhibitor cocktails on ice with frequent agitation for 30 min. The cell homogenates were centrifuged at 12,000 rpm for 30 min at 4 °C, and supernatants were collected. Protein concentrations were determined using bicinchoninic acid (BCA) reagent. Total protein (30 μg) mixed with 6x loading dye with 2-mercaptoethanol was heated for 10 min at 85 °C. The resultant mixture was centrifuged at 12,000 g for 1 min and resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V constant for 120 min. Blot transfer was performed onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Sweden) at 250 mA for 120 min on ice. The blot was blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline and Tween 20 (TBST) before being incubated overnight at 4 °C with a mouse anti-ubiquitin (P4D1) (1:200 in 5 % BSA-TBST; Santa Cruz Biotechnology, USA), rabbit anti-IκBα (1:500 in 5% TBST; GeneTex, USA), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (1:10000 in 5 % BSA-TBST; Avivas Systems Biology, USA), or rat mouse anti-B-actin antibodies (1:10000 in 5% TBST; Merck, USA). After overnight incubation, the membrane was washed with TBST at room temperature three times for 10 min each. The blot was then incubated with secondary mouse or rabbit anti-horseradish peroxidase (1:10000 in 5 % BSA-TBST; Cell Signaling Technology, USA) for 1 h at room temperature. The blot was washed five times for 10 min each with TBST at room temperature before addition of chemiluminescence substrate (Advansa, USA) and exposure on X-ray film (Fujifilm, Japan).
Gene expression analysis — Total RNA was extracted from A549 using PureLink™ RNA mini kit (Thermo Fisher Scientific, US). First-strand cDNA was synthesised from 600 ng of total RNA using SuperScript™ II Reverse Transcriptase and Oligo(dT)12–18 (Thermo Fisher Scientific, US) according to the manufacturer instructions. Quantitative PCR (qPCR) was performed with iTaq Universal SYBR Green Supermix (Bio-Rad, US) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, US) for 40 cycles. The PCR reaction (20 µL) consisted of 3 µL cDNA, 1 µL primer mix (10 µM), 6 µL diethyl polycarbonate (DEPC)-treated water, and 10 µL Mastermix. The pre-designed primer pairs (Origene, US) used in the qPCR reactions consisted of two sets of primers: (1) ICAM-1 (NM_000201): 5ʹ- AGC GGC TGA CGT GTG CAG TAA T -3 primer (forward) and 5ʹ- TCT GAG ACC TTC GTC A-3 (reverse) and (2) reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH; NM_001256799): 5ʹ- GTC TCC TCT GAC TTC AAC AGC G -3 (forward) and 5ʹ- ACC ACC CTG TTG CTG TAG CCA A-3 (reverse). GAPDH was used as a housekeeping gene for normalization. Fold changes of gene expressions with ICAM-1 were calculated using the 2−ΔΔCT method (72).

Flow cytometric analyses for ICAM-1 expressions — Surface expressions of ICAM-1 were determined by flow cytometry. Briefly, A549 cells were exposed to TNF (50 ng/mL) (Peprotech, USA) for 6 h with or without 2 h rosetide rT7 or MG132 pretreatment. Cells were harvested and collected by centrifugation at 500 g for 5 min. The cell pellet was stained with anti-ICAM-1-PE (ebioscience, USA) in serum containing medium for 30 min on ice. The pellet was then washed three times, and the samples were subjected to flow cytometric analyses. Ten thousand cells were analysed using the BD LSRFortessa™ X-20 flow cytometer. The results were analysed by FlowJo vX.0.7 (FlowJo, USA) and presented as mean fluorescence intensity.

Peptide stability assay — Purified rosetide rT7 (0.1 M) and S-alkylated rT7 (iodoacetamido-) were tested in the following stability studies with respective buffer conditions. Pepsin stability: Pepsin (Roche Applied Science, US) of 50:1 (w/v) ratio in 0.2 M HCl at 37 °C. Pronase stability: Pronase (0.2 mg/mL; Roche Applied Science, US) in PBS at 37 °C. Neutrophil elastase stability: Human neutrophil elastase (0.05 mg/mL; Molecular Innovations, US) in PBS at 37 °C. Trypsin stability: Trypsin (0.2 mg/mL; Sigma Aldrich, US) in PBS at 37 °C. Analysis for stability assays: All samples collected at various time points from the stability assays were analyzed by RP-HPLC with a linear gradient of mobile phase A (0.05% TFA/H2O) and mobile phase B (0.05% TFA/ACN) on aeris peptide XB-C18 column (Phenomenex, US). The resulting peaks were collected and identified by MALDI-TOF MS. The results were expressed as percentage of initial concentration using the peak area of the HPLC profile.

Circular Dichroism Spectroscopy — Far-UV CD spectra were recorded using a Chirascan™ CD spectrometer (Applied Photophysics, Leatherhead, UK). CD Spectra were acquired between 190 and 280 nm from 20 to 90 °C using a 10 mm path-length quartz cuvette, a 1 nm spectral bandwidth, and a 0.5 nm step size.

20S proteasome cleavage of pentapeptide — 20S proteasome cleavage of pentapeptide was performed in a 50 µL reaction mixture containing 10 mM HEPES (pH 7.6), human 20S proteasome (5 nM) and pentapeptide (50 µM). Each reaction was performed at 37°C. The samples were separated using RP-HPLC. The identity of the HPLC peak was analyzed using an Orbitrap Elite mass spectrometer (Thermo Scientific Inc., Bremen, Germany) coupled with a Dionex UltiMate 3000 UHPLC system (Thermo Scientific Inc., Bremen, Germany).

Statistical analyses — Statistical comparisons were performed using GraphPad Version 6.0d (USA). The data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test. Data are expressed as the mean ± standard error of the mean (SEM). P <0.05 was considered statistically significant.
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Figure 1. (A) The calyx of Hibiscus sabdariffa. (B) Sequence comparison between rosel tide rT1 and rT7 as described previously (2). (C) Sequence comparison between the IIML motif of rosel tide rT7, epoxomicin, and car maphycin.
Figure 2. Roseltide rT7 structure based on nuclear magnetic resonance (NMR). (A) Left: 17 lowest energy structures of roseltide rT7. Right: Cartoon view of roseltide rT7 with disulfide bonds in yellow. The side chains of the three proline residues located in loop 4 are shown. (B) Structure of roseltide rT1 (PDB entry 5GSF) and rT7 (PDB entry 6KLM). Loop 4 is colored in green. IIML motif is colored in red.
Figure 3. Synthesis and site-specific labeling of roseltide rT7. Synthetic scheme for roseltide rT7 by solid-phase peptide synthesis, as well as N-terminally fluorescent-labeled roseltide rT7.
Figure 4. Roseltide rT7 is stable against proteolytic degradation. Comparison of the peptide stability of roselude rT7 and S-alkylated rT7 (iodoacetamido-) under (A) trypsin, (B) pronase, (C) pepsin, and (D) human neutrophil elastase treatment as analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC); n=3. N.D. Not Detected.
Figure 5. Cellular uptake of tetramethylrhodamine (TAMRA)-rT7 is endocytosis-dependent. (A) A549 cells after incubation with TAMRA-rT7 (1 μM) using live-cell confocal microscopy at 37 °C. (B) Flow cytometry analysis of A549 cells after incubation with TAMRA-rT7 (1 μM) at 37 °C. (C) Flow cytometry analysis of A549 cells incubated at 4 °C for 30 min prior to incubation with TAMRA-rT7 (1 μM) for 1 h at 4 °C. N=3; P <0.05 compared to control.
Figure 6. Roseltide rT7 is a human 20S proteasome inhibitor. (A) Representative western blot analysis on the ubiquitinated protein expressions in A549 cells for 2 h with 10 µM roseltide rT7; 1 µM MG132 was used as positive control. (B) Dose-response effect of roseltide rT7 and MG132 on human 20S chymotrypsin-like proteasome activities using Proteasome-Glo chymotrypsin-using assay kit. The IC₅₀ for roseltide rT7 and MG132 against human 20S chymotrypsin-like proteasome activities are 3.3±0.04 µM and 0.13±0.03 µM, respectively. (C) In-gel fluorescence image of human 20S proteasome treated with roseltide rT7 or MG132 for 1 h followed by 30 min with Me4BodipyFL-Ahx3Leu3VS probe. (D) MALDI-TOF-MS profiles of roseltide rT7 (10 µM) without (upper panel) or with human 20S proteasome (5 nM) (lower panel) for 24 h at 37 °C. (E) Dose-response effect of roseltide rT7 and MG132 on human 26S chymotrypsin-like proteasome activities using Proteasome-Glo chymotrypsin-using assay kit. The IC₅₀ for roseltide rT7 and MG132 against human 26S chymotrypsin-like proteasome activities are 14.93±0.04 µM and 0.05±0.02 µM, respectively. (F) Effects of linear pentapeptides, IIMLP, IIMLA, IIMLK, IIMPA, and IIMPK, on human 20S chymotrypsin-like proteasome activities using Proteasome-Glo chymotrypsin-using assay kit. Proteasome cleavage of P1′-P1 site was determined using RP-HPLC and LC-MS analysis. P(X) refers to N-terminal to the substrate cleavage site. P(X)′ refers to C-terminal to the substrate cleavage site.
Figure 7. Roseltide rT7 inhibits TNF-induced IκBα degradation and intercellular adhesion molecule (ICAM)-1 expressions. (A) Representative western blot analysis on the IκBα expression in A549 cells following 30 min tumor necrosis factor (TNF; 50 ng/mL) incubation with or without 10 µM roseltide rT7 for 2 h; MG132 was used as positive control. (B) The gene expressions of ICAM-1 in A549 cells following 4 h TNF (50 ng/mL) incubation with or without 10 µM roseltide rT7 for 2 h as determined by real-time polymerase chain reaction (qPCR); 1 µM MG132 was used as positive control. (C) The surface protein expressions of ICAM-1 in A549 cells following 4 h TNF (50 ng/mL) incubation with or without 10 µM roseltide rT7 for 2 h as determined by flow cytometry using anti-ICAM-1-PE antibody; 1 µM MG132 was used as positive control. (D) Schematic diagram for the inhibitory effects of roseltide rT7 on TNF-mediated IκBα proteasomal degradation and ICAM-1 expressions. All results were expressed as mean ± SEM; n=3; *p <0.05 compared to control group.
Table 1. Structural statistics for the final 17 conformers of rT7\textsuperscript{a}

| Distance restraints | Count |
|---------------------|-------|
| Intra-residue ($i-j = 0$) | 107   |
| Sequential ($|i-j| = 1$) | 67    |
| Medium range ($2 \leq |i-j| \leq 4$) | 12    |
| Long range ($|i-j| \geq 5$) | 34    |
| Hydrogen bond | 0     |
| Total | 220   |

Average rmsd to the mean structure (Å)\textsuperscript{b}

|                      |       |
|---------------------|-------|
| Backbone atoms      | 0.81 ± 0.24 |
| Heavy atoms         | 1.33 ± 0.33 |

\(\phi/\psi\) space\textsuperscript{c}

| Region                              | Percentage |
|-------------------------------------|------------|
| Most favored region (%)             | 64.7       |
| Additionally allowed region (%)     | 30.9       |
| Generously allowed region (%)       | 2.2        |
| Disallowed region (%)               | 2.2        |

rmsd from covalent geometry

| Component     | Value             |
|---------------|-------------------|
| Bonds (Å)     | 0.0081 ± 0.0001   |
| Angles (deg.) | 0.706 ± 0.0247    |
| Impropers (deg.) | 0.434 ± 0.015 |

rmsd from experimental restraints

| Component   | Value             |
|-------------|-------------------|
| NOEs (Å)    | 0.0251 ± 0.0007   |

\textsuperscript{a} Selected from 100 calculated conformers according to overall energy.

\textsuperscript{b} Calculated with MOLMOL using range 1-15, 19-23, 29-33.

\textsuperscript{c} Calculated with PROCHECK-NMR.
Roseltide rT7 is a disulfide-rich, anionic, and cell-penetrating peptide that inhibits 
proteasomal degradation
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