Cysteine-Rich Peptide Family with Unusual Disulfide Connectivity from *Jasminum sambac*

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Supporting Information

**ABSTRACT:** Cysteine-rich peptides (CRPs) are natural products with privileged peptidyl structures that represent a potentially rich source of bioactive compounds. Here, the discovery and characterization of a novel plant CRP family, jasmintides from *Jasminum sambac* of the Oleaceae family, are described. Two 27-amino acid jasmintides (jS1 and jS2) were identified at the gene and protein levels. Disulfide bond mapping of jS1 by mass spectrometry and its confirmation by NMR spectroscopy revealed disulfide bond connectivity of C-1−C-5, C-2−C-4, and C-3−C-6, a cystine motif that has not been reported in plant CRPs. Structural determination showed that jS1 displays a well-defined structure framed by three short antiparallel β-sheets. Genomic analysis showed that jasmintides share a three-domain precursor arrangement with a C-terminal mature domain preceded by a long pro-domain of 46 residues and an intron cleavage site between the signal sequence and pro-domain. The compact cysteine-rich structure together with an N-terminal pyroglutamic acid residue confers jasmintides high resistance to heat and enzymatic degradation, including exopeptidase treatment. Collectively, these results reveal a new plant CRP structure with an unusual cystine connectivity, which could be useful as a scaffold for designing peptide drugs.

*Jasminum sambac* (L.) Ait. belongs to the Oleaceae family and is commonly known as Motia or Arabian jasmine. It is a small shrub well-known for its sweet fragrant flowers, which are used for the preparation of jasmine tea. Traditionally, its leaves are reported to contain antipteric properties, whereas their juice is useful for the treatment of ulcers. Leaf extracts exhibit analgesic and anti-inflammatory activity in rats. Its roots have been used as a purgative, analgesic, and anthelmintic against ringworm and tapeworm. Furthermore, small molecules such as flavonoids, steroids, and glycosides have been reported in jasmine plants.

Collectively, peptide- and protein-derived natural products in medicinal plants have not been seriously considered as sources of bioactive compounds in traditional medicine. Such products are generally perceived as unstable and not readily available as small-molecule metabolites. This bias could be attributed to the instability of peptides and proteins, which are denatured or degraded during the thermal process of decoction in traditional medicines and enzymatic degradation in the gastrointestinal tract after ingestion.

Plant cysteine-rich peptides (CRPs) ranging between 3 and 6 kDa, considered miniproteins, are most commonly found in medicinal plants. They have well-defined structures stabilized by three or more disulfide bonds, which render them resistant to heat denaturation and enzymatic degradation. As such, plant CRPs are an underexplored class of bioactive compounds in herbal medicine. Plant CRPs are classified according to their disulfide connectivity. Thus far, only four families of plant CRPs in the molecular weight (MW) range of 3–6 kDa are known, three of which contain a cystine-knot motif between C-1−C-4, C-2−C-5, and C-4−C-6. They include plant defensins, knottins, and heveins. The only exception is the family of thionins, which contain a symmetrical cystine-knot motif between C-1−C-6, C-2−C-5, and C-3−C-6.

Here, the discovery and characterization of a new family of CRPs, jasmintides (*Jasminum sambac*; peptide) of the Oleaceae family, are described. A combination of proteomic and genomic methods was used to identify two jasmintides (jS1 and jS2) from the leaves of *J. sambac*. Jasmintides with an N-terminal pyroglutamic acid residue confer jasmintides high resistance to heat and enzymatic degradation, including exopeptidase treatment. Collectively, these results reveal a new plant CRP structure with an unusual cystine connectivity, which could be useful as a scaffold for designing peptide drugs.

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RESULTS AND DISCUSSION

Screening of the Oleaceae Family. To determine the presence of CRPs in the Oleaceae family, seven plants (Osmanthus fragrans, J. multiflorum, J. sambac, J. biflorum, J. angustifolium, J. rex, and Nyctanthes arbor-tristis) were screened by mass spectrometry. The occurrence of CRPs was based on a mass shift before and after reduction of their disulfide bond with dithiothreitol (DTT) and S-alkylation with iodoacetamide (IAM), which results in an expected mass increment of 58 Da for each cysteine. Only J. sambac showed strong signals in the mass range of 2–4 kDa (Figure 1). The other six plant extracts showed a low abundance of CRPs under our screening conditions. After reduction and S-alkylation, a mass shift of 348 Da was observed, indicating the presence of three disulfide bonds in the putative CRPs of J. sambac.

Isolation and Sequence Determination of Jasminides from J. sambac. A scale-up extraction of the fresh leaves of J. sambac was performed, and two putative CRPs, jasmintides, were identified. The major CRP with a MW of 3105 Da was named jasmintide 1 (jS1), and the minor CRP of 3181 Da, jasmintide 2 (jS2). The most abundant jasmintide (jS1) was purified sequentially by strong cation exchange—high-pressure liquid chromatography (SCX-HPLC) and then by reversed-phase (RP)-HPLC. Characterization of jS1 by MALDI-TOF MS was performed after reduction and enzymatic digestion with chymotrypsin or trypsin. De novo sequencing of the digested fragments by MALDI-TOF gave the full sequence of jS1 (ZLCLQCRSNSDCNIWIRICRDGCNCV) with an N-terminal pyroglutamic acid (Z; Figure 2). Isobaric amino acids Leu/Ile and Lys/Gln were assigned by gene cloning.

Disulfide Connectivity Analysis of jS1 Shows a New Motif. Although disulfide bond mapping of CRPs has been traditionally assessed by regional partial reduction followed by differential alkylation using two different alkylation reagents, N-ethylmaleimide (NEM) and IAM, to generate fully reduced and S-alkylated fragments by enzymatic treatment suitable for sequencing, this strategy often meets with difficulty when there is a CC motif with an adjacent cysteine due to the lack of an enzyme capable of cleaving the peptide bond between the CC bond. Our early attempts to map the CC motif-containing jS1 failed, as all disulfide bonds of the CC motif were reduced simultaneously. Thus, a modified method involving careful partial reduction and alkylation followed by enzymatic digestion to generate multiple disulfide-bond-containing tryptic peptides was designed to determine the disulfide bond connectivity in jS1.

In our strategy, purified jS1 was first partially reduced with a limited amount of tris(2-carboxyethyl)phosphine to generate a series of fragments with one, two, or three reduced disulfide bonds, which were immediately alkylated with NEM under an acidic pH to avoid scrambling of disulfide linkages and then purified by RP-HPLC. The number of NEM-tagged cysteine bonds in the putative CRPs of J. sambac, which results in an expected mass increment of 58 Da with DTT and S-alkylation by mass spectrometry. The occurrence of CRPs was based on a mass shift before and after reduction of their disulfide bond with dithiothreitol (DTT) and S-alkylation with iodoacetamide (IAM), which results in an expected mass increment of 58 Da for each cysteine. Only J. sambac showed strong signals in the mass range of 2–4 kDa (Figure 1). The other six plant extracts showed a low abundance of CRPs under our screening conditions. After reduction and S-alkylation, a mass shift of 348 Da was observed, indicating the presence of three disulfide bonds in the putative CRPs of J. sambac.

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Disulfide mapping of jasmintide jS1. (A) RP-HPLC separation of partially S-reduced and S-NEM-labeled jasmintide jS1 derivatives. (B) Interpreted sequence of 3 (2SS) shows the disulfide connectivity C-1−C-5 established after the second alkylation with iodoacetamide (IAM denoted as CAM). (C) Schematic deconvolution of three possible scenarios by tryptic digestion patterns to arrive at C-2−C-4 and C-3−C-6 connectivity. MS showing two fragments of scenario 1 with m/z 1359.2 and 2053.3 generated after tryptic digestion of 3 performed under 2 M urea. Four small fragments generated after reduction and second alkylation using iodoacetamide (IAM) as an alkylating agent.

(Figure S1, Supporting Information, contains annotated MS/MS spectra). On the basis of the two detected tryptic digested peptides and four short peptides released after reduction and alkylation, disulfide linkages between C-2−C-4 and C-3−C-6...
were identified (Figure 3C). Thus, the disulfide connectivity of jS1 was found to be C-1−C-5, C-2−C-4, and C-3−C-6 (Figure 4A), providing unambiguous evidence that jasmintides do not share the C-1−C-4, C-2−C-5, and C-3−C-6 cystine-knot arrangement commonly found in many CRP families, such as plant defensins, knottins, and heveins.8−16

National Center for Biotechnology Information, European Bioinformatics Institute, and DNA Data Bank of Japan homology searches showed jS1 shared 43.5% homology with a carboxypeptidase inhibitor from Ascaris suum (ACI). Importantly, these searches failed to uncover the cystine motif of jasmintides in plant CRPs. However, similar disulfide connectivity was found in human β-defensins19 and in two scorpion toxins20 (Table 1), all of which are substantially larger in size than jasmintides.

Carboxypeptidase inhibitor ACI inhibits carboxypeptidase A (CPA). On the basis of the homology detected between jS1 and ACI the carboxypeptidase inhibitor activity of jS1 was tested against bovine carboxypeptidase A (bCPA). Nonetheless, jS1 does not inhibit bCPA1 up to 100 μM.

Solution Structure of the Jasmin tide 1 (jS1). In order to confirm that finding, the disulfide connectivity pattern of jS1 was further validated by NMR spectroscopy. The NMR spectrum of jS1 was characterized by well-dispersed resonances in the amide region signifying stable folded populations. All spin−spin systems of jS1 were identified, and most proton resonances were unambiguously assigned as ~98% completeness (Figures 2S and 3S, Table 1S, Supporting Information). The solution structure of jS1 was determined based on a total of 656 NMR-derived distance restraints and 22 dihedral angle restraints. The ensemble of 20 low-energy structures is shown in Figure 4C. Root-mean-square deviation values relative to the mean coordinate of 20 conformers were 0.18 Å for backbone atoms and 0.90 Å for all heavy atoms (Table 1). On the basis of the strong NOE connectivities, sequential δ δ(i,i+1) NOEs, and hydrogen bond patterns determined by amide−hydrogen exchange experiments, jS1 was determined to be mainly shaped by a combination of three short extended β-sheets and several loop elements. In addition, several long-range δ δ(i,j) NOEs were also observed, indicating folded β-sheet conformations for these regions. The structure of jS1 was found to consist of three short antiparallel β-sheets (β1: L4−Q5; β2: I18−R20; β3: C23−N25) and two loops (L1: C6−R17 between β1 and β2; L2: R20−C24 between β2 and β3), and its molecular shape is well-defined by a number of medium- and long-range NOEs (Figure 4D, Table 2). PROCHECK analysis indicated that all residues were distributed in the allowed region of the Ramachandran map (Table 2). The position of the disulfide bridges was further confirmed by characteristic δ δ(i,j) and δ δ(j) NOEs between cysteine pairs. An NOE signal between Hδ−Hδ from Cys3 and Cys23 (Figure 4B), which corresponds to the C-1−C-5 cysteine pair, was also identified, while the C-2−C-4 pair was identified based on an NOE between Hδ−Hβ from Cys6 and Cys19 (Figure 4B). Additionally, several long-range NOE correlations were observed between HN/Hα atoms of Cys3 and Hα/Hβ of Cys23. Similar long-range NOE correlations were identified for Cys6−Cys19 (C-2−C-4); all of these observations are in agreement with the formation of C-1−C-5 and C-2−C-4 disulfide bridges. These results confirm a new cystine motif relative to conventional cystine-knot peptides with disulfide connectivity between C-1−C-4, C-2−C-5, and C-3−C-6. The three disulfide bonds might play an important role in generating and stabilizing the overall structure of jS1.

Analysis of the solvent-exposed surface residues shows that the six cysteine residues were buried into the peptide core like other known cysteine-rich peptides such as cyclotides. However, hydrophobic residues (Ile14, Ile15, Trp 16) in the loop between β-sheet 1 and β-sheet 2 form a continuous hydrophobic surface combined with N- and C-terminal hydrophobic residues (Leu2, Leu4, Val26, and Ile27). The surface representation of the jS1 structure reveals the forming of these hydrophobic patches (Figure 4S). Additionally, most of the charged and polar residues are mainly exposed on the molecular surface.

Stability of jS1. CRPs are well-known for their stability against thermal and enzymatic degradation,10,25 This high resistance is an important feature for CRP biologics in traditional medicine preparations. In this work, 96% of jS1 remained intact after incubation in boiling water for 1 h,
whereas the KL and RV control peptides were completely degraded after 1 h of incubation with trypsin or 4 h treatment with pepsin or carboxypeptidase A at 37°C. Native jS1 also showed high resistance to proteolytic degradation, maintaining more than 90% of the initial peptide intact under pepsin and carboxypeptidase A treatment and maintaining 73% of the initial concentration intact with trypsin (Figure 5). Interestingly, jS1 was resistant to both thermal denaturation and proteolytic degradation, including exopeptidase treatment. These stability features make jasmintides comparable to cystine-knot peptides, such as α-amylase inhibitors.8,10

Biological Activity of jS1. Next, we examined the cytotoxic, hemolytic, and antimicrobial activity of jS1. To evaluate the cytotoxicity effect of jS1 on Vero and Huvec cells, cell viability was measured using PrestoBlue reagent as an indicator. Jasminidine was nontoxic to the two tested cell lines at the concentration of 10 μM. The hemolytic effects were investigated on human type O+ erythrocytes. Triton X-100 (0.1%) was used as positive control. Compound 1 did not show any hemolytic effect up to 10 μM. Finally, we examined antimicrobial activity against Escherichia coli, Staphylococcus aureus, and Candida albicans using the radial diffusion assay. The peptide dendrimer D4R, with broad antimicrobial activity, was used as a positive control.26 Jasminidine 1 (jS1) did not exhibit antimicrobial activity against E. coli, S. aureus, or C. albicans at concentrations up to 100 μM.

Biosynthesis of Jasmintides. The full-length gene of jS1 using 3′- and 5′-rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR) was obtained to gain insight into its biosynthesis. A degenerate primer targeting the LCRD GCC sequence was designed for 3′-RACE PCR, which resulted in the 3′-end partial gene sequence. Based on this partial gene sequence, a specific primer from the 3′-untranslated region (UTR) was designed and used for 5′-

Table 1. Sequence Alignment of Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Ref</th>
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<tr>
<td>jS1</td>
<td>QLGQ---CRSNSD--IINR---RCG---CNVI-</td>
<td>This work</td>
</tr>
<tr>
<td>jS2</td>
<td>QLGQ---CRSDDN--IINR---RCG---CNVI-</td>
<td>This work</td>
</tr>
<tr>
<td>H. Defensins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBD1</td>
<td>DHYLVSSGGCLYS-ACPIPTKIQGTYRGGAKK-CK---</td>
<td>21, 22</td>
</tr>
<tr>
<td>HBD2</td>
<td>VFGIGPVLKSKGACHPV-FGPRKYQITGCLPGTKK-EKPF-</td>
<td>23</td>
</tr>
<tr>
<td>HBD3</td>
<td>GIINTLQKYVRVGRPLAVL-SQLPKEEQIKGEQGRKG-BKDRK-</td>
<td>24</td>
</tr>
<tr>
<td>S. toxins</td>
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<td></td>
</tr>
<tr>
<td>κ-BUTX-T2</td>
<td>CEP---IELAG---QEGKVSQ-DYEC---LKNH---EPR1---</td>
<td>20</td>
</tr>
<tr>
<td>Ts16</td>
<td>CEMK---IELAG---QEGKVSQ-DYEC---LKNH---EPR1PR---</td>
<td>20</td>
</tr>
</tbody>
</table>

“*The jasmintides jS1 and jS2 have been aligned against three human β-defensins (HBD1, HBD2, and HBD3) and two scorpion toxins (κ-BUTX-T2 and Ts16) based on a similar disulfide connectivity pattern.

Table 2. Structural Statistics of NMR Structures of jS1

<table>
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<tr>
<th>Statistics</th>
<th>Value</th>
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<tr>
<td>Number of NOE constraints</td>
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<tr>
<td>Intraresidues li – &amp; j = 0</td>
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</tr>
<tr>
<td>Sequential, li – &amp; j = 1</td>
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</tr>
<tr>
<td>Medium range, 1 &lt; li – &amp; j &lt; 5</td>
<td>86</td>
</tr>
<tr>
<td>Long range, li – &amp; j ≥ 5</td>
<td>210</td>
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<tr>
<td>Number of hydrogen bond constraints</td>
<td>10</td>
</tr>
<tr>
<td>Number of dihedral angle constraints</td>
<td>22</td>
</tr>
<tr>
<td>Number of constraint violations (&gt;0.5 Å)</td>
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</tr>
<tr>
<td>Number of angle violations (&gt;5°)</td>
<td>0</td>
</tr>
<tr>
<td>Energies (kcal/mol)</td>
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</tr>
<tr>
<td>E_{NOE}</td>
<td>5.31 ± 1.10</td>
</tr>
<tr>
<td>E_{nhh}</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>E_{total} + E_{angle} + E_{improper}</td>
<td>23.57 ± 0.98</td>
</tr>
<tr>
<td>E_{VDW}</td>
<td>16.70 ± 1.39</td>
</tr>
<tr>
<td>RMS deviation of the structural segment(Cys3-Ile27) for final 20 structures to REM structure</td>
<td></td>
</tr>
<tr>
<td>Backbone (N,CαC′)</td>
<td>0.18 ± 0.08 Å</td>
</tr>
<tr>
<td>Heavy atoms</td>
<td>0.90 ± 0.15 Å</td>
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</table>

Figure 5. Stabilities of jasmintide jS1. (A) Thermal stability at 100°C. (B) Trypsin stability. (C) Pepsin stability. (C) Carboxypeptidase A stability. Error bars represent standard deviation.
plays a role in directing the protein to the secretory pathway. The ER signal sequence encodes three major domains. The ER signal sequence asparagine moieties. The cysteine, four isoleucine, three arginine, two leucine, and two other by only three residues (Figure 6); they contained six shared high sequence homology (88%), di after the mature peptide domain. Jasmintides jS and jS2 contain a single residue di terminus (Figure 6). The signal peptide sequence and pro-domain and a 27-residue jasmintide domain at the C-terminus (Figure 6). The signal peptide from the precursor to release the pro-peptide. Generally, signal peptidase I (Spase I) is involved in cleaving the signal peptide from the precursor to release the pro-peptide. (A) Alignments of deduced amino acid sequences of jasmintide precursors. The endoplasmic reticulum signal peptide was predicted using SignalP 4.1. The intron is depicted by a triangle within the endoplasmic reticulum (ER) signal peptide region. Gen sequences showed that jasmintide precursors contained a 27-residue ER signal peptide region followed by a 46-residue pro-domain and a 27-residue jasmintide domain at the C-terminus (Figure 6). The signal peptide sequence and pro-domain of jS1 and jS2 precursors were highly similar, with only a single residue difference, and had a stop codon immediately after the mature peptide domain. Jasmintides jS1 and jS2 shared high sequence homology (88%), differing from each other by only three residues (Figure 6); they contained six cysteine, four isoleucine, three arginine, two leucine, and two asparagine moieties.

Genetic characterization revealed that a novel jasmintide gene encodes three major domains. The ER signal sequence plays a role in directing the protein to the secretory pathway. Generally, signal peptidase I (Spase I) is involved in cleaving the signal peptide from the precursor to release the pro-peptide. The knottin family of CRPs with an unusual cysteine motif from the Oleaceae family in the present study serves to expand our knowledge of the occurrence of cysteine motifs in plant CRPs. Understanding the structure and stability of jasmintides could lead to their use as a scaffold for designing new peptide drugs.

### EXPERIMENTAL SECTION

**General Experimental Procedures.** NMR experiments were performed on 600 or 700 MHz Bruker Avance spectrometers equipped with 5 mm triple-resonance cryogenic probe heads and a z-axis gradient coil at 25 °C. Mass spectrometry analysis of plant CRPs with a MW of 3 kDa and a cysteine connectivity motif of C-1–C-5, C-2–C-5, and C-3–C-6 reported. Currently, the C-1–C-4, C-2–C-5, and C-3–C-6 cystine-knot arrangement in the knottin family is most common. The proline-rich, carbon-bond-containing α-amylase inhibitors with 30–32 amino acids in the knottin family represent the closest relatives to jasmintides. Other six-cysteine-containing knottin CRPs include both linear and cyclic cyclotides that contain 29–37 amino acids. The isolation and characterization of a new family of CRPs with an unusual cysteine motif from *J. sambac* of the Oleaceae family in the present study serves to expand our knowledge of the occurrence of cysteine motifs in plant CRPs. Understanding the structure and stability of jasmintides could lead to their use as a scaffold for designing new peptide drugs.

Figure 6. Precursor and putative biosynthesis of jasmintides. (A) Alignments of deduced amino acid sequences of jasmintide precursors. The endoplasmic reticulum signal peptide was predicted using SignalP 4.1. The intron is depicted by a triangle within the endoplasmic reticulum signal peptide. (B) Biosynthetic pathway of jasmintides.
ultraperformance liquid chromatography (UPLC) were performed on Shimadzu systems. Grace Vydac C8 columns (particle size, 5 μm; pore size, 300 Å; Hesperia, CA, USA) with dimensions of 250 × 22 mm, 250 × 10 mm, and 250 × 4.6 mm were used for preparative, semipreparative, and analytical RP-HPLC, respectively. PolyLC polysulphonyl A columns (250 × 9.4 mm and 250 × 4.6 mm) were used for SCX-HPLC. LC-MS/MS was performed in an Orbitrap Elite mass spectrometer (Thermo Scientific Inc., Bremen, Germany) coupled with a Dionex UltiMate 3000 UHPLC system (Thermo Scientific Inc.) into an Acclaim PepMap RSL column (75 μm × 15 cm; 2 μm particles; Thermo Scientific Inc., Bremen, Germany). Chemical reagents used in this study were of analytical grade and purchased from Sigma-Aldrich (MO, USA).

**Plant Material.** *J. sambac* was collected from May 2014 to April 2015 from the Nanyang Technological University herb garden (Singapore). The plant was identified by Paul Leong at the Singapore Botanic Garden. A voucher specimen (SING 2015-202) was deposited at Singapore Botanic Garden Herbarium.

**Screening of Oleaceae Family Plants. Plant Extraction.** Leaves of seven different plants from the Oleaceae family (*Osmanthus fragrans*, *J. multiflorum*, *J. sambac*, *J. balfouria*, *J. angustifolium*, *J. rex* and *Nyctanthes arbor-tristis*; 300 mg of each) were homogenized in 900 μL of 50% EtOH and centrifuged at 1000 g for 10 min at 4 °C. The extracts were diluted twice in order to reduce the percentage of EtOH.

**Reduction and Alkylation.** The number of disulfide bonds in each peptide was determined from the mass difference after reduction–alkylation treatment of crude extracts. Reduction and alkylation were performed as previously described. Briefly, aliquots of leaf extracts were incubated at 37 °C with 20 mM DTT in 20 mM Tris-HCl buffer (pH 7.2) for 1 h. Subsequently, peptides were alkylated by incubating the reduced extract with 40 mM IAM for 45 min in the dark.

**Isolation and Purification of the Most Abundant Jasminitide from *J. sambac*. Scale-up Extraction.** Fresh *J. sambac* leaves (4 kg) were homogenized and extracted twice in 50% (v/v) EtOH. After centrifugation (8500 g, 10 min, 4 °C), the supernatant was filtered through 1 μm and 0.45 μm pore-size filters, diluted to 20% in EtOH, and purified in a C8 flash column (Grace Davison, Columbia, MD, USA) by washing with 20% EtOH and eluting with 70% EtOH.

**Purification by SCX.** For purification of the most abundant jasminitide (jS1) several dimensions of SCX- and RP-HPLC were performed. Removal of small molecules by SCX-HPLC was conducted with a linear gradient from 0% to 100% with buffer A (0.1% TFA in 20 mM phosphate buffer) and buffer B (20% MeCN, 10 mM phosphate buffer, and 1 M NaCl; pH 3). SCX-containing fractions were subsequently purified by RP-HPLC using a linear gradient from 25% to 45% with buffer A (0.1% TFA) and buffer B (0.1% TFA in MeCN). The yield for jS1 was ~100 mg.

**Sequence Characterization.** Purified jS1 (40 μg) dissolved in 100 μL of 50 mM NH4HCO3 buffer (pH 7.8) was reduced in 20 mM DTT for 1 h at 37 °C. Jasminitide jS1 was digested with trypsin or chymotrypsin at 1:10 (protein:enzyme). Enzymatic digestions were performed at 37 °C for 30 min and alkylated with 40 mM IAM for 2 h at room temperature.

**Confirmation by LC-MS/MS.** The sequence of reduced and alkylated digested peptides was confirmed by LC-MS/MS. LC separation was performed with 0.1% formic acid (eluent A) and 90% MeCN in 0.1% formic acid (eluent B), respectively, in a 60 min gradient starting at 3% eluent B for 1 min, 3–35% eluent B over 47 min, 35–50% eluent B over 4 min, 50–80% eluent B over 6 s, and 80% eluent B for 78 s and then reverted to initial conditions in 6 s and maintained isocratically for 6.5 min. Spray was generated with a Michrom Thermo Captive Spray nanoelectrospray ion source (Bruker-Michrom Inc., Auburn, CA, USA) with a source voltage of 1.5 kV and a capillary temperature of 250 °C. Data were collected in positive ion mode using LTQ Tune Plus software (Thermo Scientific Inc.) alternating between full-scan MS (350–1600 m/z; 60,000 resolution at 400 m/z, 1 microscan per spectrum) and full-scan MS/MS (150–2000 m/z; 15,000 resolution at 400 m/z, 1 microscan averaged per spectrum). High-energy collisional dissociation fragmentation was performed for the 10 most intense ions with a 500-count threshold using 32% normalized collision energy per spectrum and a 2 Da isolation window. Automatic gain control for full-scan MS and MS/MS was set to 1 × 104 ions.

**Data Analysis.** Data from LC-MS/MS were analyzed using PEAKS Studio (version 7.0, Bioinformatics Solutions, Waterloo, Canada). A precursor mass tolerance of 10 ppm and a fragment mass error tolerance of 0.05 Da were applied.

**NMR Spectroscopy.** NMR samples were prepared by dissolving 1 mg of lyophilized peptide in 500 μL of 90% H2O/10% D2O or 99.99% D2O at pH 7.0 in a 20 mM Na2PO4 buffer containing 50 mM NaCl and 0.01% NaN3. The concentration of the NMR sample was approximately 0.7–1 mM. Homonuclear 2D NOESY experiments were performed with mixing times of 200 and 300 ms in collecting NOE spectra for jS1. Data were also recorded in both H2O and D2O solutions with a mixing time of 78 ms using MLEV17 spin-lock pulses. Vicinal coupling constants were determined using DQF COSY and 1H NMR experiments. All 2D NMR data were recorded in the phase-sensitive mode using the time-proportional phase increment method with 2048 data points in the t2 domain and 512 points in the t1 domain. Slowly exchanging amide protons were identified by lyophilization of a fully protonated sample in H2O solution to dryness, resuspension in 99.99% D2O solution, and immediate acquisition of a series of 1D spectra. The strong solvent resonance was suppressed by water-gated pulse sequences or excitation sculpting combined with pulsed-field gradients. All NMR data were processed using Bruker TOPSPIN 2.1 (Bruker Instruments) or NMRPipe programs on a Linux workstation and analyzed by using Sparky 3.12 software. The DQF-COSY spectra were processed to 8192 × 1024 data matrices to obtain a maximum digital resolution for coupling constant measurements. The proton chemical
shifts were referenced to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

**Structure Calculations.** Solution structures of jS1 were calculated by hybrid distance geometry and simulated annealing protocols in torsion angle space with CNS 1.2.6 The three disulphide bonds were restrained in accordance with the disulphide bonding patterns based on the observed 1H−1H NOEs from NOESY spectra by generation of covalent disulphide linkages during the initial molecular topology file generation stage using a CNS script. A total of 656 distance and 22 torsion angle constraints were used for structure calculations. NOE distance restraints were classified as strong (1.8−3.0 Å), medium (1.8−3.5 Å), weak (1.8−5.0 Å), or very weak (1.8−6.0 Å) based on the intensities derived from NOEYS spectra recorded in 90% H2O/10% D2O or 99.99% D2O at 25 °C. Corrections for pseudoatom representations were used for noncovalent specifically assigned methylene, methyl group, and aromatic ring protons.61 Backbone dihedral angle restraints were derived from 1Hα±1Hα coupling constants in DQF-COSY or 1H NMR spectra in H2O solution.62,63 Backbone dihedral restraints were used as −55° ± 45° (1Hα±1Hα < 6 Hz) and −120° ± 50° (1Hα±1Hα > 8 Hz). Hydrogen bond donors were identified from proton−deuterium exchange experiments, and hydrogen bond acceptors were determined at the preliminary structure calculation stage. The 200 starting structures were generated and refined using a hybrid distance geometry-simulated annealing protocol by the CNS 1.2 program.64−66 Finally, 20 final structures were selected by their total energy values for display and structural analysis. MOLMOL51 and PyMol52 programs were used for structure visualization, and PROCHECK-NMR53 was used for structure validation. The jS1 solution structure solved for 20 ensembles is available in the Protein Data Bank under accession number 2NSQ.

**Heat Stability.** Jasminide jS1 was heated in boiling H2O for 1 h and then subjected to UPLC. A linear peptide (RV) of 14 residues (RLYYRGRGLYRRNHV) was subject to the same treatment of 20:1 (w/w) at 37 °C for 8 h. A nine-residue (KL) peptide (RRPPGFSL) treated in the same manner was used as a positive control. Treated and control peptides were subjected to UPLC at intervals, and collected peaks were monitored by MALDI-TOF.

In the carboxypeptidase A stability assay, the peptide was incubated with or without trypsin in 100 mM NH4HCO3 buffer (pH 7.8) and pepsin in 100 mM sodium citrate buffer (pH 2.5) at a final peptide−enzyme ratio of 20:1 (w/w) at 37 °C for 8 h. A nine-residue (KL) peptide (KRPFGFSPL) treated in the same manner was used as a positive control. Treated and control peptides were subjected to UPLC at intervals, and collected peaks were monitored by MALDI-TOF.

**Cytotoxicity Assay.** The cytotoxicity of the peptide on African green monkey kidney (Vero) and human umbilical vein endothelial cells (HUVEC) was measured using standard PrestoBlue reagent (Invitrogen). Briefly, cells were seeded in a 96-well plate at a concentration of 8×103 cells per well in DMEM in 10% FBS and 1% penicillin and streptomycin and incubated at 37 °C with 5% CO2 in air. After 24 h, varying concentrations of jS1 in the range 0.01−10 μM were added in triplicate and then incubated for 24 h. PrestoBlue (10 μL) was added into each well and maintained for 1 h at 37 °C. The absorbance was read using the Tecan plate reader at an absorbance of 570 nm. Triton X-100 (1%) and PBS served as positive and negative control, respectively.

**Hemolysis Assay.** Fresh blood type O+ was donated by a healthy volunteer. To obtain erythrocytes, blood was centrifuged (1000 rpm, 15 min). The pellet was isolated and washed with phosphate buffer saline (PBS) three times. A stock suspension of erythrocytes was prepared via 100x dilution in fresh PBS buffer. Various concentrations of jS1 were added, with an equal volume of treated blood sample, into a 96-well plate. After incubation at 37 °C for 4 h, the plate was centrifuged (1000 rpm, 5 min), and supernatants were transferred into a new plate. After transferring supernatants into a new plate, absorbance was measured at 415 nm using a Tecan Infinite M200 plate reader. A 0.1% Triton X-100 solution and PBS solution served as the negative and positive controls, respectively.

**Antimicrobial Activity Using Radical Diffusion Assay.** Two bacterial strains and one fungal strain from ATCC were used, namely, Staphylococcus aureus ATCC 12600, Escherichia coli ATCC 25922, and Candida albicans 11006. All strains were cultured in trypticase soy broth. The antimicrobial activity of jS1 was examined using a radial diffusion assay as described previously under low salt conditions (10 mM Na3PO4).54 D4R was used as positive control.

**Carboxypeptidase Inhibition Assay.** The assay was performed as reported.34 Briefly, various concentrations of jS1 (0.1 to 100 μM) were added to the 96-well plate with 0.1 mM N-(4-methoxyphenylazoformyl)phenylalanine substrate. The reaction was started by adding 25 nM bovine carboxypeptidase A. The substrate hydrolysis was monitored at 350 nm in 50 mM Tris HCL and 1 M NaCl buffer using end point measurement, quenched with 1 M Na2CO3 after 5 min.

**REFERENCES**
