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

Kumari, Geeta; Serra, Aida; Shin, Joon; Nguyen, Phuong Q. T.; Sze, Siu Kwan; Yoon, Ho Sup; Tam, James P.

2015

Kumari, G., Serra, A., Shin, J., Nguyen, P. Q. T., Sze, S. K., Yoon, H. S., & Tam, J. P. (2015). Cysteine-Rich Peptide Family with Unusual Disulfide Connectivity from *Jasminum sambac*. *Journal of Natural Products*, 78(11), 2791-2799.

<https://hdl.handle.net/10356/81859>

<https://doi.org/10.1021/acs.jnatprod.5b00762>

© 2015 The American Chemical Society and American Society of Pharmacognosy. This is the author created version of a work that has been peer reviewed and accepted for publication by *Journal of Natural Products*, 2015 The American Chemical Society and American Society of Pharmacognosy. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [<http://dx.doi.org/10.1021/acs.jnatprod.5b00762>].

Downloaded on 26 Apr 2023 19:17:54 SGT

Cysteine-rich Peptide Family With Unusual Disulfide Connectivity from *Jasminum sambac*

Geeta Kumari,[†] Aida Serra,[†] Joon Shin,[†] Phuong Q. T. Nguyen,^{†,‡} Siu Kwan Sze,[†] Ho Sup Yoon,[†] and James P. Tam^{†,*}

[†]School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551.

[‡]Current address: Department of Chemistry, University of Rochester, New York, 14627-0216

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, is 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 which 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 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.

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.¹ Traditionally, its leaves are reported to contain antipyretic properties, whereas their juice is useful for the treatment of ulcers. Leaf extracts exhibit analgesic and antiinflammatory activity in rats. Its roots have been used as purgative, analgesic, and anthelmintic against ringworm and tapeworm.²⁻⁴ Furthermore, small molecules such as flavonoids, steroids, and glycosides have been reported in jasmine plants.^{3, 5, 6}

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 gastrointestinal tracts after ingestion.

Plant cysteine-rich peptides (CRPs) ranging between 3-6 kDa, considered miniproteins, are most commonly found in medicinal plants.⁷ They have well-defined structures stabilized by three or more disulfide bonds which render them resistant to heat denaturation and enzymatic degradation.⁸ As such, plants 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.⁸⁻¹⁶ 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-4.¹⁷

Here, the discovery and characterization of a new family of CRPs, jasmintides (*Jasminum sambac*; peptide) of the Oleaceae family, is described. A combination of proteomic and

genomic methods were used to identify two jasmintides (jS1 and jS2) from the leaves of *J. sambac*. Jasmintides with an *N*-terminal pyroglutamic acid residue were shown to be resistant not only to heat and endopeptidase degradation, but also to exopeptidases. The precursor of jasmintides contained three domains similar to many CRPs, such as α -amylase inhibitors.¹⁰ Importantly, jasmintides displayed novel disulfide connectivity between C-1–C-5, C-2–C-4, and C-3–C-6 which is different from other known plant CRPs. Collectively, these findings provide new insights into the sequence, structure, and biosynthesis of jasmintides, a new plant CRP family with potentially useful bioactive peptides.

RESULTS AND DISCUSSION

Screening of the Oleacea 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.¹⁸ 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*.

Figure 1. MS fingerprint of jasminptides in *J. sambac*. Jasminptide jS1 is labeled at the top of the corresponding peak.

Isolation and Sequence Determination of Jasminptides from *J. sambac*. A scale-up extraction of the fresh leaves of *J. sambac* was performed and two putative CRPs, jasminptides, were identified. The major CRP with a MW of 3105 Da was named jasminptide **1** (jS1), and the minor CRP of 3181 Da jasminptide **2** (jS2). The most abundant jasminptide (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 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 (ZLCLQCRSNSDCNIIWRICRDGCCNVI) with an N-terminal pyroglutamic acid (Z; Figure 2). Isobaric amino acids Leu/Ile and Lys/Gln were assigned by gene cloning.

A **B** **C** **D**

Figure 2. MS/MS sequencing of jS1. (A) Enzymatic digestion of reduced jasmintide jS1 by trypsin yielded three fragments with m/z values 846, 1207, and 1095 Da; Z= pyroglutamic acid. (B-D) MS/MS spectra of digested fragments. Ile/Leu and Lys/Gln assignments were determined by jasmintide gene.

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 alkylating reagents, *N*-ethylmaleimide (NEM) and IAM, to generate fully-reduced and *S*-alkylated fragments by enzymatic treatment suitable for sequencing,¹⁵ 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 (*S*-NEM) was determined by MALDI-TOF MS. *S*-NEM fragments caused a mass increase of 126 Da. Based on the mass shift, the four purified HPLC fractions were identified as follows: fraction one consisted of native jS1 with no reduced and alkylated cysteines; fraction two corresponded to peptide **3** with two *S*-NEM and two intact disulfide bonds

(2SS); fraction three contained peptide **4** with four *S*-NEM and one intact disulfide bond (1SS); and fraction four corresponded to peptide **5** with six *S*-NEM and no intact disulfide bonds (0SS) (Figure 3A). Peptide **3** was then fully reduced and *S*-alkylated with IAM and subjected to LC-MS/MS analysis to reveal its *S*-NEM connectivity at C-1–C-5 (Figure 3B).

To map the connectivity of the other two remaining disulfide bonds, peptide **3** was enzymatically digested with trypsin in a 2 M urea solution at pH 7 for 2 h. The use of urea during enzymatic hydrolysis was necessary as trypsin was not able to cleave the partially alkylated two-NEM-tagged specie in the urea-free solution. From that digestion, three possible combinations based on the remaining two disulfide bonds were calculated (Figure 3C). After digestion, two fragments with m/z of 1359 and 2053 Da, indicating the possibility of C-2–C-4 and C-3–C-6 pairs (Figure 3C, Scenario 1), were detected. These tryptic digested samples were fully reduced with DTT and alkylated with IAM, giving rise to four different short peptides which were further identified by LC-MS/MS (Figure S1, Supporting Information contains annotated MS/MS spectra). Based on 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 jasminptides 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

Figure 3. 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 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.

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 toxins,²⁰ (Table 1) all of which are substantially larger in size than jasmintides.

Carboxypeptidase inhibitor ACI inhibits carboxypeptidase A (CPA). Based on 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 μ M.

Table 1. Sequence alignment of peptides.

Peptide	Sequence	Ref
Plant		
jS1	QLCLQ----CRSNSDCN---IIWRI C---RDGC-CNVI-	This work
jS2	QLCLQ----CRSDDDCN---IIWRI C---RYGC-CNVI-	This work
H. Defensins		
HBD1	DHYN CVSSGGQCLYS-ACPIFTKIQGT CYRGKAKC-CK---	21, 22
HBD2	VFGGIGDPVTCCLKSGAICHPV-FCPRRYKQIGTCGLPGTKC-CKKP-	23
HBD3	GIINTLQKYYCRVRGGRCAVL-SCLPKEEQIGKCSTRGRKC-CRRKK	24
S. toxins		
k-BUTX-Tt2	GCMP--EY CAG--QCRGKVSQ-DY C-LKN--CRCIR--	20
Ts16	GC MK--EY CAG--QCRGKVSQ-DY C-LKH--CKCI PR-	20

The jasmintides jS1 and jS2 have been aligned against three human β -defensins (HBD1, HBD2 and HBD3) and two scorpion toxins (κ -BUTX-Tt2, and Ts16) based on similar disulfide connectivity pattern.

Solution Structure of the Jasmintide 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). Based on the strong NOE connectivities, sequential $d_{\alpha N(i,i+1)}$ NOEs together with 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 d_{NN} , $d_{\alpha N}$, and $d_{\alpha\alpha}$ 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 (β 1: L4-Q5; β 2: I18-R20; β 3: C23-N25) and two loops (L1: C6-R17 between β 1 and β 2; L2: R20-C24 between β 2 and β 3), 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 $d_{\beta\beta}(i,j)$ and $d_{\alpha\beta}(i,j)$ NOEs between cysteine pairs. An NOE signal between $H\beta$ - $H\beta$ 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 $H\beta$ - $H\beta$ from Cys6 and Cys19 (Figure 4B). Additionally, several long-range NOE correlations were observed between $HN/H\alpha$ atoms of Cys3 and $H\alpha/H\beta$ 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 peptide 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.

Table 2. Structural Statistics of NMR Structures of jS1

Number of NOE constraints	
All	656
Intra residues $ i-j =0$	165
Sequential, $ i-j =1$	195
Medium-range, $1< i-j <5$	86
Long-range, $ i-j \geq 5$	210
Number of Hydrogen Bond Constraints	10
Number of Dihedral Angle Constraints	22
Number of Constraint Violations ($>0.5\text{\AA}$)	0
Number of Angle Violations ($>5^\circ$)	0
Energies (kcal/mol)	
E_{NOE}	5.31 ± 1.10
E_{cdih}	0.14 ± 0.05
$E_{\text{bond}} + E_{\text{angle}} + E_{\text{improper}}$	23.57 ± 0.98
E_{VDW}	16.70 ± 1.39
RMS Deviation of the structural segment(Cys ³ -Ile ²⁷) for final 20 structures to REM structure	
Backbone (N,C α ,C')	$0.18 \pm 0.08\text{\AA}$
Heavy Atoms	$0.90 \pm 0.15\text{\AA}$

A **B** **C** **D**

Figure 4. Novel sequences of jasmintides and three dimensional structure of the jS1. (A) Alignment of jS1 and jS2. Disulfide connectivity is shown by bold solid lines; Z= pyroglutamic acid. Presence of β -sheets is shown by thick arrows. (B) 2D NOESY spectra showing the expanded aliphatic regions of jS1 obtained with 300-ms mixing in D₂O at 25 °C. Correlations between cysteine-forming disulfide bridges of jS1 (Cys³-H ^{β} /Cys23-H ^{β} , Cys⁶-H ^{β} /Cys¹⁹-H ^{β}) are represented by rectangular boxes. (C) Superposition of the backbone traces from the final 20 ensembles of solution structures and restrained energy minimized (REM) structures of jS1 by NMR spectroscopy. The REM structure of jS1 is highlighted in red. (D) Ribbon representation of jS1 REM structure. Three disulfide bridges C-1–C-5, C-2–C-4, and C-3–C-6 are shown by ball-and-stick representation.

Stability of jS1. CRPs are well-known for their stability against thermal and enzymatic degradation,^{8, 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}

A. Heat

B. Trypsin

C. Pepsin

D. Carboxypeptidase A

Figure 5. Stabilities of jasmintide jS1. (A) Thermal stability of at 100 °C. (B) Trypsin stability. (C) Pepsin stability. (C) Carboxypeptidase A stability. Error bars represent standard deviation.

Biological Activity of jS1

Next, we examined the cytotoxic, hemolytic, and antibacterial activity of jS1. To evaluate the cytotoxicity effect of jS1 on vero and Huvec cells, cell viability was measured using Presto blue reagent as an indicator. Jasmintide was nontoxic to the two tested cell lines at the concentration of 10 μ M. 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 upto 10 μ M. 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 Jasmintide 1 (jS1) did not exhibit antimicrobial activity against *E. coli*, *S. aureus*, and *C. albicans* at concentrations up to 100 μ M.

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 LCRDGCC 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'-RACE PCR. After assembling the 3'- and 5'-RACE clones, two full-length cDNA gene sequences were obtained that encoded jS1 and jS2 and named *jsac1* and *jsac2*, respectively. To better understand the DNA sequence of jasmintides, gene-specific primers targeting 3'- and 5'-UTRs of *jsac1* and *jsac2* were designed and used in PCR amplification of genomic DNA extracts. Comparison between mRNA and DNA revealed that these jasmintide genes contained a single intron located in the endoplasmic reticulum (ER) signal peptide region. Gene 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. Jasminptides 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 moities.

Genetic characterization revealed that a novel jasminptide gene encodes three major domains. The ER signal sequence plays a role in directing the protein to the secretory pathway.²⁷ 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 α -amylase inhibitors is cleaved between a Leu/Ile/Val-Xaa, where Xaa is a small amino acid. In comparison, the *N*-terminal cleavage site of our jasminptide precursor occurs between asparagine and glutamine residues (Figure 6) which could be mediated by an asparaginyl endopeptidase enzyme (AEP).²⁸ These characteristics show that jasminptides are ribosomally-synthesized peptides.¹³

Figure 6. Precursor and putative biosynthesis of jasminptides. (A) Alignments of deduced amino acid sequences of jasminptides 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 jasminptides.

The three-domain precursor is commonly found in many plants families producing cystine-knot peptides in the Rubiaceae, Violaceae, Solanaceae, Poaceae, and Apocynaceae families.^{10, 13-16} Jasminptides are found to contain a pyroglutamic acid at their *N*-termini. It remains to be determined whether the conversion of glutamine to pyroglutamic acid occurs spontaneously or is catalyzed by a specific glutamyl cyclase. A similar modification can be found in the squash trypsin inhibitor from *Momordica cochinchinensis*,²⁹ Phyb M isolated from *Petunia hybrid*,³⁰ and Ib-Amp from *Impatiens balsamin*.³¹ The incorporation of an *N*-terminal pyroglutamic acid unit could be an alternate strategy for backbone cyclization to protect jasminptides against exopeptidase degradation.

To date, there are no six-cysteine-containing linear plant CRPs with a MW of 3 kDa and a cysteine connectivity motif of C-1–C-5, C-2–C-4, 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-carbon-bond-containing α -amylase inhibitors with 30-32 amino acids in the knottin family represent the closest relatives to jasminptides.⁸ Other six-cysteine-containing knottin CRPs include both linear and cyclic cyclotides which contain 29-37 amino acids.^{15, 16, 32} 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 jasminptides 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 extracts, reduction and alkylation, enzymatic digestion, and stability were monitored by a MALDI-TOF/TOF ABI 4800 (Applied Biosystems, Framingham, MA, USA). Data acquisition was performed in reflectron mode using a mass range of 1000–6000 Da and a focusing mass of 3000 Da. A saturated solution of α -cyano-4-hydroxycinnamic acid prepared in 75% MeCN and 0.1% TFA was used as the MALDI matrix. Each spot contained 0.5 μ L of α -cyano-4-hydroxycinnamic acid solution and 0.5 μ L of sample. Desalting of samples, if needed was carried out using C₁₈ zip-tips. HPLC and ultra-performance liquid chromatography (UPLC) were performed on Shimadzu systems. Grace Vydac C₁₈ columns (particle size, 5 μ m; pore size, 300 Å; Hesperia, CA, USA) with dimensions of 250 x 22 mm, 250 x 10 mm, and 250 x 4.6 mm were used for preparative, semi-preparative, and analytical RP-HPLC, respectively. PolyLC polysulfoethyl A columns (250 x 9.4 mm and 250 x 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 μ m $\times$ 15 cm; 2 μ m 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 Oleacea Family Plants.

Plant Extraction. Leaves of seven different plants from the Oleacea family (*Osmanthus fragrans*, *J. multiflorum*, *J. sambac*, *J. biflorum*, *J. angustifolium*, *J. rex*, and *Nyctanthes*

arbor-tristis; 300 mg of each) were homogenized in 900 μ L 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.³² 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 Jasmintide 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- μ m and 0.45- μ m pore-size filters, diluted to 20% in EtOH, and purified in a C₁₈ 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 jasmintide (jS1) several dimensions of SCX- and RP-HPLC were performed. Removal of small molecules by SCX-HPLC was conducted with a linear gradient from 0-100% with buffer A (20% MeCN and 10 mM phosphate buffer; pH 3), and buffer B (20% MeCN, 10 mM phosphate buffer, and 1M NaCl; pH 3). jS1-containing fractions were subsequently purified by RP-HPLC using a linear gradient from 25-45% 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 μ g) dissolved in 100 μ L of 50 mM NH₄HCO₃ buffer (pH 7.8) was reduced in 20 mM DTT for 1 h at 37 °C. Jasmintide jS1 was digested

with trypsin or chymotrypsin at 1:10 (protein:enzyme). Enzymatic digestions were performed at 37 °C for 10 min, and then sequenced by MALDI MS/MS as previously described.³²

Ile/Leu and Lys/Gln were assigned based on gene sequence.

Gene Cloning. Total RNA extracted using Trizol reagent (Life technologies, Carlsbad, CA, USA) from fresh leaves was converted to 3' and 5'-RACE cDNA libraries using 3'-RACE system (Invitrogen, Carlsbad, CA, USA) and SMARTerTM RACE Amplification kits (Clontech, Takara biotechnology, Dalian, China), respectively. The degenerate primer targeting LCRDGCC (5'-CTCTGTAGRGAYGGNTGYTG-3') and RDGCCNV (5'-CGCGATGGN TGYTGYYAAYGT-3') sequences were used for RACE PCR. The PCR product obtained was gel-purified, cloned into pGEM-T Easy Vectors (Promega, Madison, WI, USA), and sequenced. Based on the newly obtained partial sequence, a specific reverse primer targeting the 3'-UTR was designed to amplify the remaining encoding gene in 5'-RACE PCR.

To study intron location, genomic DNA was extracted from *J. sambac* using cetyltrimethyl ammonium bromide DNA extraction protocols³³. Two specific primers targeting UTRs of jS1 were designed, JS3105-R1 (5'- CCATTGTGCAGCCATTATGA-3') and JS3105-5UTR-Fn (5'- TTAAACATGGAGAAGGCTTCA-3'). The ER signal peptides were predicted with SIGNALP 4.1 server.³⁴ The nucleotide sequences reported for jS1 and jS2 in this paper have been deposited in GeneBank database under accession numbers [KT438682](#) and [KT438683](#), respectively.

Disulfide Mapping.

Partial Reduction and Alkylation. Jasminide jS1 (150 µg) was partially reduced in 100 mM citrate buffer (pH 3.0) with 15 mM tris(2-carboxyethyl)phosphine at 37 °C for 30 min. Subsequently, 30 mM NEM was added and incubated at room temperature for 2 h. The

reaction was quenched by immediate injection of sample into analytical RP-HPLC.

HPLC Fractionation. Reduced and alkylated species were separated with a linear gradient of 20-60% buffer B (0.1% TFA in MeCN). Fractions were collected and analyzed by MALDI-TOF MS to verify NEM-alkylated intermediate species. The two-NEM-alkylated intermediate specie **3** (2SS) was lyophilized and redissolved in 2 M urea. For determining the other two disulfide connectivities, **3** (2SS) was enzymatically digested with trypsin for 2 h at 37 °C. Disulfide bond-containing digested fragments were fully reduced with 20 mM DTT at 37 °C for 30 min and alkylated with 40 mM IAM for 1 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, 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 was 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 1×10^6 ions.

Data Analysis. Data from LC-MS/MS were analyzed using PEAKS Studio³⁵ (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 μ L 90% H_2O /10 % D_2O or 99.99% D_2O at pH 7.0 in a 20 mM Na_3PO_4 buffer containing 50 mM NaCl and 0.01% NaN_3 . 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.^{36, 37} COSY³⁸ data were also recorded in both H_2O and D_2O solutions with a mixing time of 78 ms using MLEV17 spin-lock pulses.³⁹ Vicinal coupling constants were determined using DQF COSY⁴⁰ and ^1H NMR experiments. All 2D-NMR data were recorded in the phase-sensitive model using the time-proportional phase increment method⁴¹ with 2048 data points in the t_2 domain and 512 points in the t_1 domain. Slowly exchanging amide protons were identified by lyophilization of a fully protonated sample in H_2O solution to dryness, resuspension in a 99.99% D_2O solution, and immediate acquisition of a series of 1D-spectra. The strong solvent resonance was suppressed by water-gated pulse sequences⁴² or excitation sculpting⁴³ combined with pulsed-field gradients. All NMR data were processed using a Bruker TOPSPIN 2.1 (Bruker Instruments) or NMRPipe⁴⁴ programs on a Linux workstation and analyzed by using Sparky 3.12 software. The DQF-COSY spectra were processed to 8192 x 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 simulated annealing protocols in torsion angle space with CNS 1.2.⁴⁵ The three disulfide bonds were restrained in accordance with the disulfide bonding patterns based on the observed $\text{H}\beta$ - $\text{H}\beta$ NOEs from NOESY spectra by generation of

covalent disulfide 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 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 NOESY spectra recorded in 90% H₂O/10% D₂O or 99.99% D₂O at 25 °C. Corrections for pseudo atom representations were used for nonstereospecifically-assigned methylene, methyl group, and aromatic ring protons.⁴¹ Backbone dihedral angle restraints were derived from ³J_{HN-Hα} coupling constants in DQF-COSY or ¹H NMR spectra in H₂O solution.^{46, 47} Backbone dihedral restraints were used as -55° ± 45° (³J_{HN-Hα} < 6 Hz) and -120° ± 50° (³J_{HN-Hα} > 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.⁴⁸⁻⁵⁰ Finally, 20 final structures were selected by their total energy values for display and structural analysis. MOLMOL⁵¹ and PyMOL programs were used for structure visualization, and PROCHECK-NMR⁵² were used for structure validation. The jS1 solution structure solved for 20 ensembles is available in the Protein Data Bank under accession number 2N5Q.

Heat Stability. Jasminide jS1 was heated in boiling H₂O for 1 h and then subjected to UPLC. A linear peptide (RV) of 14 residues (RLYRRGRLYRRNHV) was used as the control. Peaks were collected from UPLC and monitored by MALDI-TOF MS. Peak areas in UPLC were compared to evaluate stability.

Proteolytic Stability Test. Jasminide jS1 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

(KRPPGFSPL) 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 enzyme [50:1 (w/w)] in Tris buffer (50 mM Tris and 100 mM NaCl; pH 7.5) at 37 °C for up to 6 h. A 14-residue RV peptide (RLYRRGRLYRRNHV) was subject to the same treatment and served as the control. Collected peaks were monitored by MALDI-TOF. Peak areas in UPLC were compared to evaluate their stability.

Cytotoxicity Assay

The cytotoxicity of peptide on African green monkey kidney (Vero) and human umbilical vein endothelial cells (HUVEC) was measured using standard PrestoBlue TM reagent (Invitrogen). Briefly, cells were seeded in 96-well plate at a concentration of 8×10^3 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 ranging 0.01-10 µM were added in triplicate and then incubated for 24 h. Presto blue (10 µL) was added into each wells and maintained for 1 h at 37 °C. The absorbance was read using the Tecan plate reader at absorbance of 570 nm. 1% Triton X-100 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) thrice. A stock dispersion 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 Tecan Infinite® M200 plate reader. 0.1% Triton X-100 solution and PBS solution served as the negative and positive controls, respectively.

Antimicrobial Activity Using Radial Diffusion Assay

Two bacterial strains and one fungal strain from ATCC were used including *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 radial diffusion assay as described previously under low salt condition (10 mM Na₃PO₄).⁵³ D4R was used as positive control .²⁶

Carboxypeptidase Inhibition Assay

The assay was performed as reported.⁵⁴ Briefly, various concentration of jS1 (0.1 to 100 µM) were added to the 96 well plate with 0.1 mM of N-(4-methoxyphenylazoformyl) phenylalanine substrate. The reaction was started by adding 25 nM of bovine carboxypeptidase A (bCPA). The substrate hydrolysis was monitored at 350 nm in 50 mM Tris HCL, 1M NaCl buffer using end point measurement, quenched with 1 M of Na₂CO₃ after 5 min.

ASSOCIATED CONTENT

Supporting Information

MS/MS spectra for fragment identification of disulfide connectivity in jS1. 2D-NOESY spectrum and sequential NOE connectivities for jS1.

AUTHOR INFORMATION

Corresponding author:

* Tel: (+65) 6316-7042. Fax: (+65) 6515-1632. Email: jptam@ntu.edu.sg

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by the Competitive Research Grant by National Research Foundations in Singapore (NRF-CRP8-2011-05). We thank Leong, P., from the Singapore Botanic Garden for identifying the plant.

REFERENCES

- (1) Ito, Y.; Sugimoto, A.; Kakuda, T.; Kubota, K., *J. Agric. Food Chem.* **2002**, 50, (17), 4878-4884.
- (2) Bhagat, A.; Khainar, A.; Tenpe, C.; Upaganlawar, A.; Yeole, P., *Indian J. Nat. Prod.*

2007, 23, (3), 25-28.

(3) Lim, T. K., *Jasminum sambac*. In *Edible Medicinal and Non Medicinal Plants*, Springer Netherlands: 2014; Vol. 8, pp 529-540.

(4) Bhangale, J.; Patel, R.; Acharya, S.; Chaudhari, K., *Am. J. PharmTech. Res.* **2012**, 2, (4), 804-812.

(5) Zhang, Y.-J.; Liu, Y.-Q.; Pu, X.-Y.; Yang, C.-R., *Phytochemistry* **1995**, 38, (4), 899-903.

(6) Tanahashi, T.; Nagakura, N.; Inoue, K.; Inouye, H., *Tetrahedron Lett.* **1988**, 29, (15), 1793-1796.

(7) Laverigne, V.; J Taft, R.; F Alewood, P., *Curr. Top. Med. Chem.* **2012**, 12, (14), 1514-1533.

(8) Nguyen, P. Q.; Luu, T. T.; Bai, Y.; Nguyen, G. K.; Pervushin, K.; Tam, J. P., *J. Nat. Prod.* **2015**, 78, (4), 695-704.

(9) Tam, J. P.; Lu, Y.-A.; Yang, J.-L.; Chiu, K.-W., *Proc. Natl. Acad. Sci.* **1999**, 96, (16), 8913-8918.

(10) Nguyen, P. Q.; Wang, S.; Kumar, A.; Yap, L. J.; Luu, T. T.; Lescar, J.; Tam, J. P., *FEBS J.* **2014**, 281, (19), 4351-4366.

(11) Hernandez, J.-F.; Gagnon, J.; Chiche, L.; Nguyen, T. M.; Andrieu, J.-P.; Heitz, A.; Trinh Hong, T.; Pham, T. T. C.; Le Nguyen, D., *Biochemistry* **2000**, 39, (19), 5722-5730.

(12) Broekaert, W. F.; Marien, W.; Terras, F. R.; De Bolle, M. F.; Proost, P.; Van Damme, J.; Dillen, L.; Claeys, M.; Rees, S. B., *Biochemistry* **1992**, 31, (17), 4308-4314.

(13) Jennings, C.; West, J.; Waine, C.; Craik, D.; Anderson, M., *Proc. Natl. Acad. Sci. U.S.A.* **2001**, 98, (19), 10614-9.

(14) Dutton, J. L.; Renda, R. F.; Waine, C.; Clark, R. J.; Daly, N. L.; Jennings, C. V.; Anderson, M. A.; Craik, D. J., *J. Biol. Chem.* **2004**, 279, (45), 46858-46867.

(15) Nguyen, G. K. T.; Zhang, S.; Wang, W.; Wong, C. T. T.; Nguyen, N. T. K.; Tam, J. P.,

J. Biol. Chem. **2011**, 286, (52), 44833-44844.

(16) Nguyen, G. K. T.; Lian, Y.; Pang, E. W. H.; Nguyen, P. Q. T.; Tran, T. D.; Tam, J. P., *J. Biol. Chem.* **2013**, 288, (5), 3370-3380.

(17) Stec, B., *Cell. Mol. Life Sci.* **2006**, 63, (12), 1370-1385.

(18) Gruber, C. W.; Elliott, A. G.; Ireland, D. C.; Delprete, P. G.; Dessein, S.; Göransson, U.; Trabi, M.; Wang, C. K.; Kinghorn, A. B.; Robbrecht, E., *Plant Cell* **2008**, 20, (9), 2471-2483.

(19) Selsted, M. E.; Ouellette, A. J., *Nat. Immunol.* **2005**, 6, (6), 551-557.

(20) Saucedo, A. L.; Flores-Solis, D.; Rodriguez de la Vega, R. C.; Ramirez-Cordero, B.; Hernandez-Lopez, R.; Cano-Sanchez, P.; Noriega Navarro, R.; Garcia-Valdes, J.; Coronas-Valderrama, F.; de Roodt, A.; Brieba, L. G.; Domingos Possani, L.; del Rio-Portilla, F., *J. Biol. Chem.* **2012**, 287, (15), 12321-30.

(21) Bensch, K. W.; Raida, M.; Magert, H. J.; Schulz-Knappe, P.; Forssmann, W. G., *FEBS Lett.* **1995**, 368, (2), 331-5.

(22) Liu, L.; Zhao, C.; Heng, H. H. Q.; Ganz, T., *Genomics* **1997**, 43, (3), 316-320.

(23) Harder, J.; Bartels, J.; Christophers, E.; Schroder, J. M., *Nature* **1997**, 387, (6636), 861.

(24) Jia, H. P.; Schutte, B. C.; Schudy, A.; Linzmeier, R.; Guthmiller, J. M.; Johnson, G. K.; Tack, B. F.; Mitros, J. P.; Rosenthal, A.; Ganz, T.; McCray, P. B., Jr., *Gene* **2001**, 263, (1-2), 211-8.

(25) Colgrave, M. L.; Craik, D. J., *Biochemistry* **2004**, 43, (20), 5965-5975.

(26) Tam, J. P.; Lu, Y.-A.; Yang, J.-L., *Eur. J. Biochem.* **2002**, 269, (3), 923-932.

(27) Mergaert, P.; Nikovics, K.; Kelemen, Z.; Maunoury, N.; Vaubert, D.; Kondorosi, A.; Kondorosi, E., *Plant Physiol.* **2003**, 132, (1), 161-173.

(28) Nguyen, G. K.; Wang, S.; Qiu, Y.; Hemu, X.; Lian, Y.; Tam, J. P., *Nat. Chem. Biol.* **2014**, 10, (9), 732-738.

- (29) Mylne, J. S.; Chan, L. Y.; Chanson, A. H.; Daly, N. L.; Schaefer, H.; Bailey, T. L.; Nguyencong, P.; Cascales, L.; Craik, D. J., *Plant Cell* **2012**, 24, (7), 2765-2778.
- (30) Poth, A. G.; Mylne, J. S.; Grassl, J.; Lyons, R. E.; Millar, A. H.; Colgrave, M. L.; Craik, D. J., *J. Biol. Chem.* **2012**, 287, (32), 27033-27046.
- (31) Tailor, R. H.; Acland, D. P.; Attenborough, S.; Cammue, B. P.; Evans, I. J.; Osborn, R. W.; Ray, J. A.; Rees, S. B.; Broekaert, W. F., *J. Biol. Chem.* **1997**, 272, (39), 24480-24487.
- (32) Nguyen, G. K. T.; Zhang, S.; Nguyen, N. T. K.; Nguyen, P. Q. T.; Chiu, M. S.; Hardjojo, A.; Tam, J. P., *J. Biol. Chem.* **2011**, 286, (27), 24275-24287.
- (33) Doyle, J. J., *Phytochem. Bull.* **1987**, 19, 11-15.
- (34) Petersen, T. N.; Brunak, S.; von Heijne, G.; Nielsen, H., *Nat. Methods* **2011**, 8, (10), 785-786.
- (35) Han, X.; He, L.; Xin, L.; Shan, B.; Ma, B., *PeaksPTM: J. Proteome Res.* **2011**, 10, (7), 2930-2936.
- (36) Jeener, J.; Meier, B.; Bachmann, P.; Ernst, R., *J. Chem. Phys.* **1979**, 71, (11), 4546-4553.
- (37) Kumar, A.; Ernst, R.; Wüthrich, K., *Biochem. Biophys. Res. Commun.* **1980**, 95, (1), 1-6.
- (38) Davis, D. G.; Bax, A., *J. Am. Chem. Soc.* **1985**, 107, (9), 2820-2821.
- (39) Bax, A.; Davis, D. G., *J. Magn. Reson. (1969)* **1985**, 65, (2), 355-360.
- (40) Rance, M.; Sørensen, O.; Bodenhausen, G.; Wagner, G.; Ernst, R.; Wüthrich, K., *Biochem. Biophys. Res. Commun.* **1983**, 117, (2), 479-485.
- (41) Wüthrich, K.; Billeter, M.; Braun, W., *J. Mol. Biol.* **1983**, 169, (4), 949-961.
- (42) Liu, M.; Mao, X.-a.; Ye, C.; Huang, H.; Nicholson, J. K.; Lindon, J. C., *J. Magn. Reson.* **1998**, 132, (1), 125-129.
- (43) Hwang, T.-L.; Shaka, A., *J. Magn. Reson., Ser. A* **1995**, 112, (2), 275-279.

- (44) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A., *J. Biomol. NMR* **1995**, 6, (3), 277-293.
- (45) Brünger, A. T.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J.-S.; Kuszewski, J.; Nilges, M.; Pannu, N. S., *Acta Crystallogr. Sect. D: Biol. Crystallogr.* **1998**, 54, (5), 905-921.
- (46) Pardi, A.; Billeter, M.; Wüthrich, K., *J. Mol. Biol.* **1984**, 180, (3), 741-751.
- (47) Driscoll, P. C.; Gronenborn, A. M.; Beress, L.; Clore, G. M., *Biochemistry* **1989**, 28, (5), 2188-2198.
- (48) Nilges, M.; Gronenborn, A. M.; Brünger, A. T.; Clore, G. M., *Protein Eng.* **1988**, 2, (1), 27-38.
- (49) Nilges, M.; Clore, G.; Gronenborn, A., *FEBS Lett.* **1988**, 229, 317-324.
- (50) Nilges, M., *Proteins: Struct., Funct. and Bioinf.* **1993**, 17, (3), 297-309.
- (51) Koradi, R.; Billeter, M.; Wüthrich, K., *J. Mol. Graphics* **1996**, 14, (1), 51-55.
- (52) Laskowski, R. A.; MacArthur, M. W.; Moss, D. S.; Thornton, J. M., *J. Appl. Crystallogr.* **1993**, 26, (2), 283-291.
- (53) Lehrer, R. I.; Rosenman, M.; Harwig, S. S. S. L.; Jackson, R.; Eisenhauer, P., *U J. Immunol. Methods* **1991**, 137, (2), 167-173.
- (54) Ryan, C. A.; Hass, G. M.; Kuhn, R. W., *J. Biol. Chem.* **1974**, 249, (17), 5495-5499.

TOC/Abstract graphic