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Solution Structure of Polytheonamide B, a Highly Cytotoxic Nonribosomal Polypeptide from Marine Sponge

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Abstract: Polytheonamide B (pTB), a highly cytotoxic polypeptide, is one of the most unusual nonribosomal peptides of sponge origin. pTB is a linear 48-residue peptide with alternating D- and L-amino acids and contains a total of eight types of nonproteinogenic amino acids. To investigate the mechanisms underlying its cytotoxic activity, we determined the three-dimensional structure of pTB by NMR spectroscopy, structure calculation, and energy minimization. pTB adopts a single right-handed $\beta^{6.3}$-helical structure in a 1:1 mixture of methanol/chloroform with a length of approximately 45 Å and a hydrophilic pore of ca. 4 Å inner diameter. These features indicate that pTB molecules form transmembrane channels that permeate monovalent cations as gramicidin A channels do. The strong cytotoxicity of pTB can be ascribed to its ability to form single molecule channels through biological membranes.

Introduction

Marine sponges (phylum Porifera), often called the most primitive multicellular animals, are the first metazoans that evolved about 600 million years ago. They have proved to be one of the most prolific sources of secondary metabolites with novel chemical and biological properties.[1] In particular, their nonribosomal peptides, which are probably produced by endophytic microbial symbionts,[2] not only show interesting biological activities but also contain D-amino acids and nonproteinogenic amino acids.[3] Recently, we have isolated polytheonamide B (pTB), a 48-residue nonribosomal polypeptide from the Japanese marine sponge *Theonella swinhoei*.[4] pTB exhibits cytotoxicity against P388 murine leukemia cells at extremely low concentrations (IC_{50} value of 70−80 pg/mL[4c] in the MTT assay; doxorubicin exhibited an IC_{50} value of 40−90 ng/mL in the parallel experiments). pTB was also highly cytotoxic against L1210 murine lymphocytic leukemia cells (IC_{50} < 4 ng/mL)[4b] and Neuro-2a mouse neuroblastoma cells (IC_{50} < 1 ng/mL). We therefore believe that pTB is a nonspecific cytotoxin. This potency is extraordinary for linear peptides. Furthermore, pTB has an unprecedented primary structure: The *N*-terminus is blocked with a 5,5-dimethyl-2-oxo-hexanoyl group and 23 out of the 48 residues are constituted by unusual amino acids; one *allo*-threonine, eight *tert*-leucines, one β-methylisoleucine, three β-hydroxyvalines, six γ-*N*-methylasparagines, two γ-*N*-methyl-*threo*-β-hydroxyasparagines, one β-methylglutamine, and one β,β-dimethylmethionine sulfoxide (Figure 1A). The residues show alternating D/L stereochemistry throughout the chain.[4a]

The predominantly hydrophobic nature of its constituent amino acid residues and the blocked *N*-terminus make the peptide virtually insoluble in water. As it is typical for membrane proteins, pTB is soluble only in the presence of detergents or lipid vesicles. pTB forms a monovalent cation-selective ion channel[5] with the selectivity of permeability $H^+ > Cs^+ > Rb^+ > K^+ > Na^+$. This and the presence of alternating D/L amino acid residues suggest a structural and functional similarity

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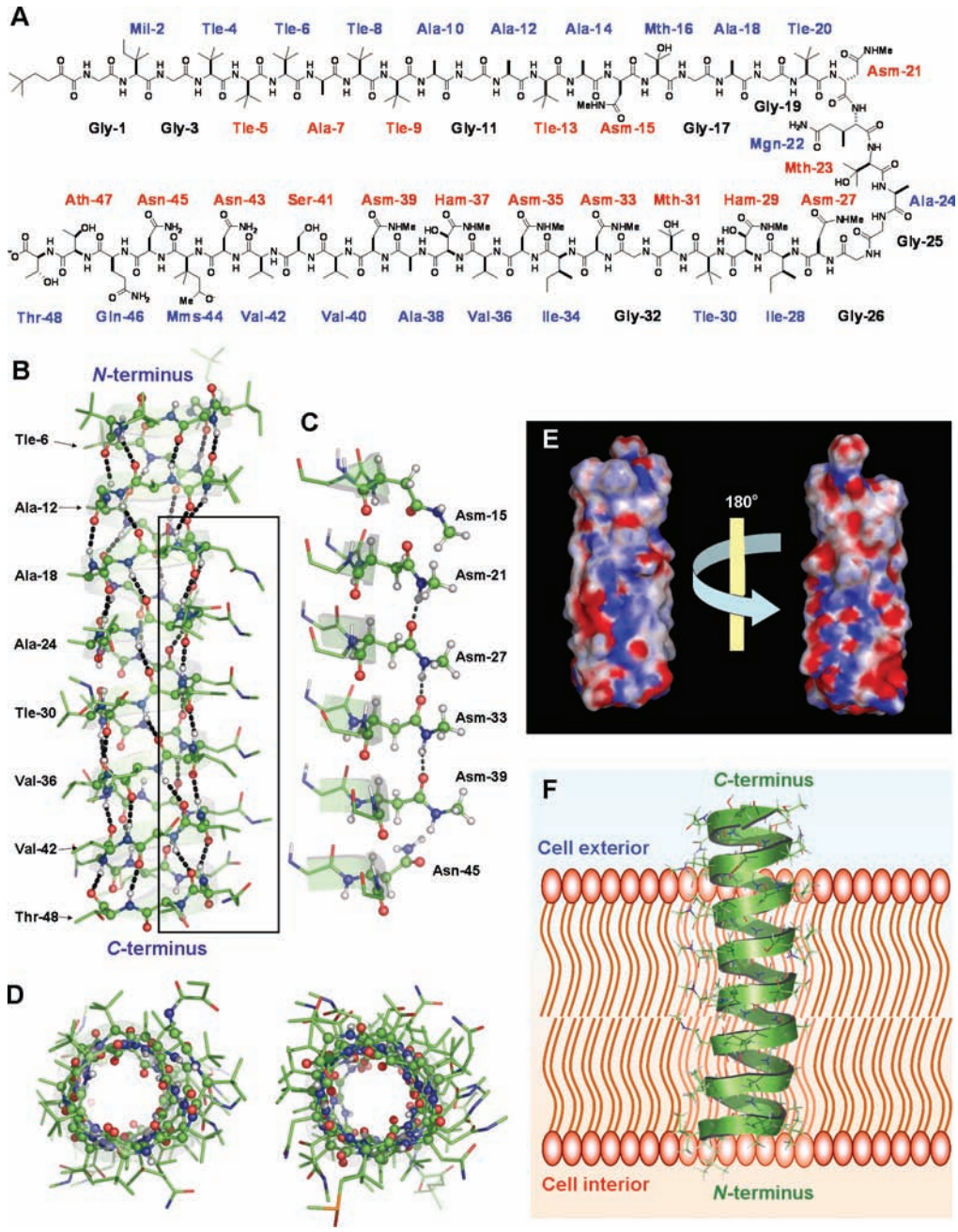

Figure 1. Structure of polytheonamide B (pTB). (A) Primary structure. Unusual amino acids are represented with the following three letter codes: Mil, β-methylisoleucine; Tle, *tert*-leucine; Asm, γ-*N*-methylasparagine; Mth, β-hydroxyvaline; Mgn, β-methylglutamine; Ham, γ-*N*-methyl-*threo*-β-hydroxyasparagine; Mms, β,β-dimethylmethionine sulfoxide; Ath, *allo*-threonine. D- and L-amino acid residues are shown with red and blue labels, respectively. (B) Schematic representation of the solution structure of pTB in a 1:1 chloroform/methanol mixture. The right-handed β-helical backbone is shown as a green ribbon. Dashed lines indicate hydrogen bonds. (C) Region of the boxed part in B. The ball-and-stick models represent the side chains of residues Asm-15, Asm-21, Asm-27, Asm-33, Asm-39, and Asn-45. Dashed lines indicate side chain−side chain hydrogen bonds. (D) Top views of the pTB solution structure from the *N*-terminal side (left) and from the *C*-terminal side (right), showing the hydrophilic core. (E) Electrostatic surface potential of pTB; positive and negative values are represented in blue and red, respectively. Color saturation corresponds to an electrostatic energy of $\pm 50 k_B T$. (F) Schematic representation of pTB inserted in the membrane. In this model, the *N*-terminal part of pTB penetrates into the cell membrane from the exterior of the cell. The hydrophobic *N*-terminus anchors pTB in the lipophilic interior of the membrane. Although in this model the *C*-terminus sticks out of the membrane, it might be located within the membrane if the membrane is thicker than assumed here.

between pTB and gramicidin A (gA) channels.[6] To elucidate the mechanism of the cytotoxicity and the ion channel activity of pTB, we carried out analysis of the three-dimensional NMR structure of pTB in organic solvents that mimic the membrane environment.

Results

pTB is soluble in dimethyl sulfoxide (DMSO), 2,2,2-trifluoroethanol (TFE), and methanol/chloroform mixtures between 1:9 and 7:3 (v/v). We chose a 1:1 methanol/chloroform

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mixture (v/v) for the nuclear magnetic resonance (NMR) structural analysis of pTB. This solvent system has been proposed as a low dielectric organic solvent that mimics the environment in the interior of a membrane.[7] In fact, the ^{1}H NMR spectrum of pTB in this solvent was more dispersed than that in DMSO-d_6 (Figure S1 of Supporting Information), which indicates an ordering of the macromolecular structure in a 1:1 mixture of methanol/chloroform.

By use of homonuclear double quantum-filtered correlation spectroscopy (DQF-COSY, Figure S2 of Supporting Information), total correlation spectroscopy (TOCSY, Figure S3 of Supporting Information), and nuclear Overhauser effect spectroscopy (NOESY, Figure 2 and Figure S4 of Supporting Information) of 2 mM pTB in $CDCl_3/CD_3OH$ (1:1, v/v) measured at 300 K, all resonances of the aliphatic protons and the backbone amide protons could be assigned (Table T1). Several NMR spectroscopic features disclosed the secondary structure of pTB in $CDCl_3/CD_3OH$ (1:1, v/v) (Figure S5 of Supporting Information): (1) all scalar coupling constants between NH and αH except for those in the *N*-terminus were larger than 8 Hz, which is a range observed exclusively for extended β structures; (2) NOE cross peaks corresponding to $d_{\alpha N}(i, i + 1)$ were observed throughout the molecule, while $d_{NN}(i, i + 1)$ cross peaks were hardly seen (Figure S4 of Supporting Information); (3) NOE connectivities spanning six residues were observed throughout the sequence for $d_{\alpha N}(i, i + 6)$ with even residue numbers i and $d_{\alpha N}(i, i - 6)$ with odd residue numbers i (Figure 2). Therefore, we concluded that pTB has extended β conformation throughout the chain and forms a helix with six residues per turn. In deuterium exchange experiments monitored by homonuclear TOCSY spectra most of the backbone amide protons (filled squares in Figure S5b of Supporting Information) exchanged slowly, indicating their involvement in hydrogen bonds. Only the five NH-αH TOCSY signals of Gly-1, Gly-25, Tle-30, Gly-32, and Ala-38 disappeared within 10 h after dissolving pTB in $CDCl_3/CD_3OD$ (1:1, v/v) at 300 K because their main chains are solvent-accessible (Figure S6 of Supporting Information).

A total of 501 NOE distance restraints including 247 long-range restraints were used in the final structure calculations with torsion angle dynamics[8] and in the subsequent restrained energy refinement with the program OPALp.[9] The structures were well-defined and showed excellent agreement with the experimental data (Table 1). No violations of NOE distance restraints larger than 0.1 Å were observed in the final NMR structure. The precision of the structure was characterized by rmsd values to the mean coordinates of 0.21 Å for the backbone atoms and 0.71 Å for all heavy atoms in the structured region of residues 3−44 (Figure S7 of Supporting Information).

pTB forms a right-handed $\beta^{6.3}$ helix in $CDCl_3/CD_3OH$ solution (Figure 1B). Most of the backbone hydrogen bonds run nearly parallel to the axis of the helix: NH(i) − CO($i + 5$) for odd i residues and NH(j) − CO($j - 7$) for even j residues. Because of the alternating D- and L-residues and the formation of the β-helix, all side chains point toward the exterior of the helix, thereby creating a hollow tubular structure (Figure 1D). The pore diameter is about 4 Å (based on van der Waals surfaces), and the interior provides a hydrophilic environment capable of permeating small ions, as in the case of gA channels.[6]

Discussion

pTB adopts a long β-helical structure with 6.3 residues per turn in a membrane-mimetic organic solvent. The backbone helical structure overlays closely with that of the gA channel structure (PDB accession code 1JNO),[12b] giving an rmsd variation of 0.3 Å for the backbone heavy atom coordinates (Figure S8 of Supporting Information). The length of the helix axis is ca. 45 Å, almost three times longer than that of monomeric gA observed in membrane. This allows a single molecule of pTB to form a pore spanning the membrane. The $\beta^{6.3}$-helix of pTB is rigid and stable in solution. Many DL-alternating peptides have been synthesized by expecting that they would form a pore similar to gA channels. However, none of these designed peptides formed a $\beta^{6.3}$-helix pore. Instead they formed either a more stable smaller helix with 4.4 residues per turn[10] or intertwined double helices either in solution or in membrane.[11] Even for gA, transmembrane *N*-terminal-to-*N*-terminal $\beta^{6.3}$-helix dimers have been identified only in micelles[12] and lipid membranes.[13] One of the reasons for the stability of the β-helical structure of pTB is the spatial arrangement of asparagine-related residues such as γ-*N*-methylasparagine (Asm) and asparagine (Asn). The β-helical structure of pTB exhibits a stack of five Asm and one Asn residues in the same row (residues 15, 21, 27, 33, 39, and 45) whose side chain amide groups are within hydrogen-bonding distance to form a stabilizing network (Figure 1C). Although in the deuterium exchange experiment the side-chain amide hydrogen atoms of the Asm residues exchanged more rapidly than those of the main chain, NOESY cross peaks such as between δNH-21 and ϵCH_3-27 and between δNH-33 and ϵCH_3-39, suggested a tendency to form hydrogen bonds between these side chains. This string of hydrogen bonds runs parallel to the helix axis of the molecule and covers two-thirds of the whole length of pTB, suggesting its role for stabilizing the long β-helical structure as in other coiled helical peptides and proteins.[14]

Voltage-dependent gating of pTB was reported by the Oiki group[5] who studied the channel activities of pTB using the planar lipid bilayer method. The ionic current was observed down to pM concentrations of pTB. The pTB channel showed monovalent cation selectivity, and the selectivity sequence was $H^+ > Cs^+ > Rb^+ > K^+ > Na^+$. These results parallel the selectivity observed for gA channels[6b] and are in agreement with the similar inner diameter and weak electrostatic field inside the β-helical pore. On the other hand, the orientation of the peptide in the membrane was fixed when the peptide was added to one side of the chamber. The asymmetric behavior should be correlated to the polarization of the pTB molecule (Figure 1E). The *N*-terminal half of pTB forms an apolar side, because six out of the nine *N*-terminal residues (one β-methylisoleucine

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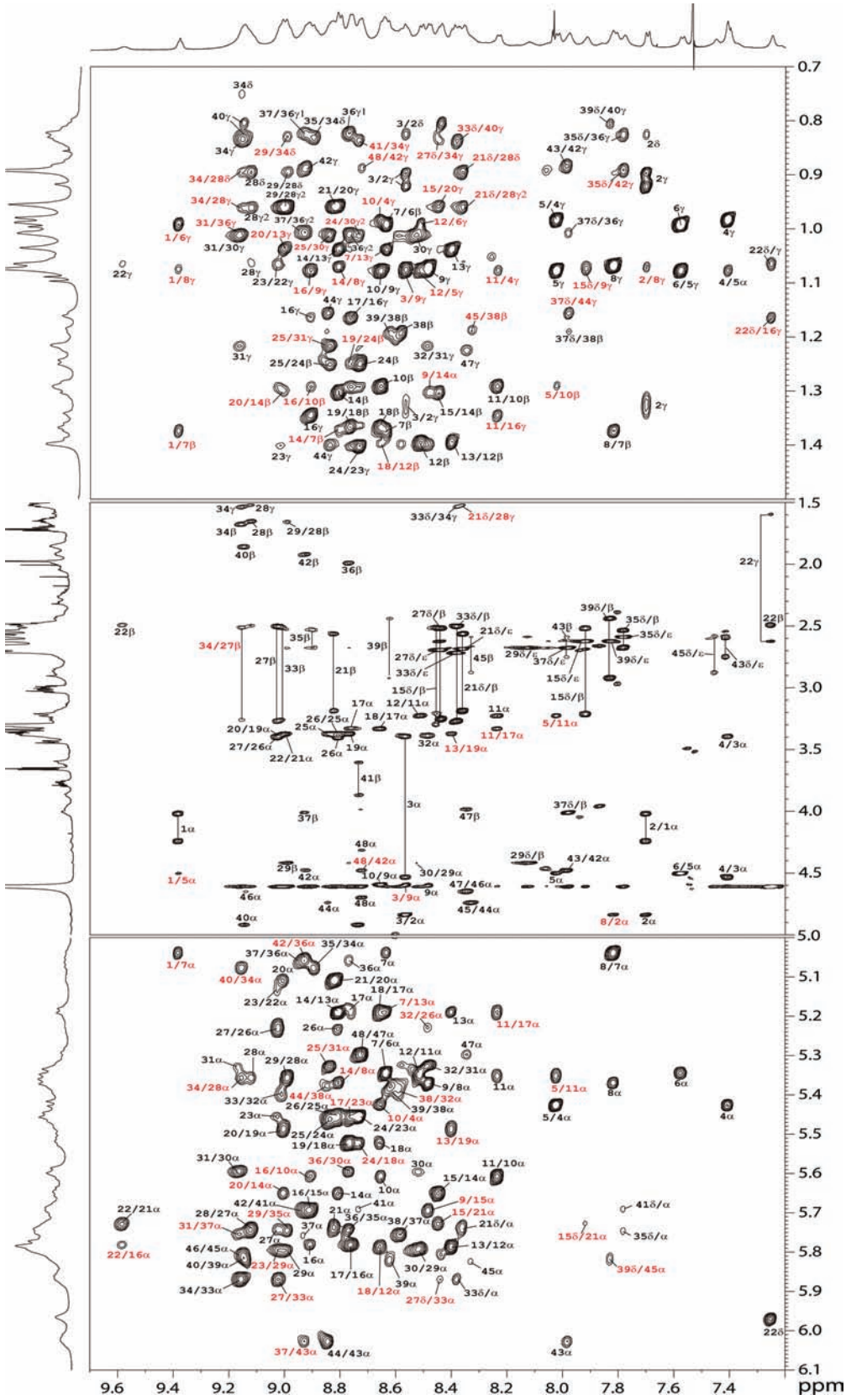

Figure 2. NOESY spectrum of pTB in a 1:1 mixture of $CDCl_3$ and CD_3OH. The region of cross peaks between amide protons and aliphatic protons is shown. Assigned cross peaks are labeled; long-range NOE cross peaks are labeled in red.

and five *tert*-leucines) have a bulky and hydrophobic side chain. In addition, the unique *N*-terminal 2-oxo-acyl group is hydrophobic. To the contrary, predominantly hydrophilic residues (cf. Asn-43 to Thr-48) in the *C*-terminal half of pTB form a polar side. These features suggest that pTB penetrates into the cell membrane with the hydrophobic *N*-terminal side (Figure 1F).

Table 1. Statistics for the NMR Solution Structure of pTB

distance restraints	
number	501
intraresidual, $\|i - j\| = 0$	159
sequential, $\|i - j\| = 1$	95
medium range, $1 < \|i - j\| < 5$	0
long range, $\|i - j\| \geq 5$	247
maximal violation	0.10 ± 0.01 Å
torsion angle restraints (ϕ/ψ)	
number	92
maximal violation	1.50 ± 0.38°
final CYANA target function value	0.14 ± 0.01 Å^2
AMBER energy	−1821 ± 19 kcal/mol
rms deviations from ideal geometry	
bond lengths	0.0442 ± 0.0001 Å
bond angles	5.09 ± 0.36°
rmsd to mean coordinates	
backbone atoms N, C$^\alpha$, C′ of residues 3−44	0.21 ± 0.06 Å
all heavy atoms of residues 3−44	0.71 ± 0.06 Å

In contrast, gA is believed to form a membrane-spanning channel by head-to-head dimerization with each monomer unit inserted into the membrane from different sides.[15]

Polytheonamides exhibit potent cytotoxicity against P388 murine leukemia cells with an IC_{50} value of 70−80 pg/mL, which are 1000 times more potent than gA (0.1 μg/mL), when doxorubicin was used for the positive control (IC_{50} 40−90 ng/mL). We assume that the strong cytotoxicity of pTB can be ascribed to its ability to penetrate biological membranes and to form single molecule channels. Thus pTB and its three-dimensional structure will deepen our understanding of ion conductance and the mechanism underlying the stabilization of pore structures in biological membranes.

Experimental Section

NMR Spectroscopy. The source and isolation of pTB were described previously.[4a] pTB was dissolved at a concentration of 2 mM (4 mg per 400 μL) in $CDCl_3/CD_3OH$ (1:1, v/v). All NMR spectra were acquired at 300 K on a Bruker Avance 800 spectrometer. Data processing was performed with XWINNMR software (Bruker). DQF-COSY was measured with 8192 (t_2) by 1024 (t_1) data points. The mixing times in NOESY experiments were 100, 200, and 400 ms, and the spin-lock time in the homonuclear TOCSY was 100 ms. For the deuterium exchange experiment, eight TOCSY spectra, with measurement times of 3 h each, were started 0.5, 3.5, 6.5, 9.5, 12.5, 15.5, 18.5, and 21.5 h after dissolving the protein in $CDCl_3/CD_3OD$ (1:1, v/v).

Structure Calculations. The structure calculations used 465 proton−proton distance restraints obtained from NOESY spectra, 36 distance restraints for 18 hydrogen bonds identified by an analysis of the amide proton exchange rates and NOE patterns, and 92 ϕ and Ψ torsion angle restraints from the three-bond scalar coupling constants $^3J_{HN\alpha}$ between HN and H$^\alpha$ protons and patterns of strong $d_{\alpha N}(i, i + 1)$ and weak or missing $d_{NN}(i, i + 1)$ sequential NOESY cross peaks.[16] The JML-S1000 MolSkop system (JEOL)[17] equipped with the DADAS90 program[18] was applied for creating over 2000 randomized initial structures and intermediate structure calculations. The program CYANA[19] was applied for the final structure calculations. New entries were added to the CYANA library for the nonstandard residues. The standard CYANA protocol of seven iterative cycles of NOE assignment[20] and structure calculation[21] followed by a final structure calculation was applied. NOE distance restraints involving ^{1}H atoms with degenerated chemical shifts, e.g., methyl groups, were treated as ambiguous distance restraints using $1/r^6$ summation over the distances to the individual ^{1}H atoms.[22] Nonstereospecifically assigned methyl and methylene protons were treated by automatic swapping of restraints between diastereotopic partners[23] during the seven cycles of automated NOE assignment and by pseudoatom correction and symmetrization[24] for the final structure calculation. In each cycle the structure calculations started from 100 randomized conformers, and 10000 torsion angle dynamics steps were performed per conformer. The 20 structures with the lowest final CYANA target function values were subjected to restrained energy refinement in explicit solvent against the AMBER force field[25] using the program OPALp.[9] A maximum of 3000 steps of restrained conjugate gradient minimization were applied, using the standard AMBER force field and a pseudopotential for NOE upper distance bounds that was proportional to the sixth power of the restraint violation. The force constant was chosen such that a distance restraint violation of 0.1 Å contributed 0.3 kcal/mol to the potential energy. Figures were generated with PyMOL (Delano Scientific; http://www.pymol.org) and MOLMOL.[26] The atomic coordinates have been deposited in Protein Data Bank, www.rcsb.org (PDB ID code 2RQO).

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Supporting Information Available: ^{1}H NMR chemical shift table, NMR spectra, secondary structure information, and structure representations. This material is available free of charge via the Internet at http://pubs.acs.org.

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