# Structure of Antibacterial Peptide Microcin J25: A 21-Residue Lariat Protoknot

Marvin J. Bayro,[†] Jayanta Mukhopadhyay,[†] G. V. T. Swapna,[†] Janet Y. Huang,[†] Li-Chung Ma,[†] Elena Sineva,[†] Philip E. Dawson,[‡] Gaetano T. Montelione,[*,†] and Richard H. Ebright[*,†]

*Department of Molecular Biology and Biochemistry, Department of Chemistry, Center for Advanced Biotechnology and Medicine, Waksman Institute, and Howard Hughes Medical Institute, Rutgers University, Piscataway, New Jersey 08854, and Department of Cell Biology and Chemistry, The Scripps Research Institute, La Jolla, California 92037*

Received June 13, 2003; E-mail: guy@cabm.rutgers.edu; ebright@waksman.rutgers.edu

The antibacterial peptide microcin J25 (MccJ25)[1] inhibits bacterial transcription by binding within, and obstructing, the nucleotide-uptake channel of bacterial RNA polymerase.[2,3] MccJ25 is produced by *Escherichia coli* strains that harbor a plasmid-borne antibiotic-synthesis and antibiotic-export cassette, consisting of a gene for MccJ25 precursor (a 58-residue peptide containing a 37-residue N-terminal pro-sequence), two genes for factors that process MccJ25 precursor into MccJ25, and one gene for export of MccJ25.[4] Published covalent and three-dimensional structures indicate that MccJ25 is a 21-residue cycle.[5−7] Here, we show that the published covalent and three-dimensional structures[5−7] are incorrect, and that MccJ25 in fact is a 21-residue "lariat protoknot", consisting of an 8-residue cyclic segment followed by a 13-residue linear segment that loops back and threads through the cyclic segment.

The results in Figure 1 indicate that a 21-residue cycle synthesized according to the published structure ("21cyc")[8] is distinct from natural MccJ25,[5] being chromatographically distinct (different retention time in RP-HPLC), biochemically distinct (inactive in inhibiting transcription), and biologically distinct (inactive in inhibiting bacterial growth). We conclude that MccJ25 is not a 21-residue cycle.

The results in Figure 2 indicate that 21cyc and MccJ25 have identical molecular masses (Figure 2a) but different MS/MS fragmentation patterns (Figure 2b), indicating that 21cyc and MccJ25 are isomeric. For MccJ25, MS/MS yields no single-cleavage fragments for residues 1−8[10] (suggesting that residues 1−8 are part of a cyclic structure), but yields an unambiguous series of single-cleavage fragments for residues 9−21 (suggesting that residues 9−21 are not part of a cyclic structure) (Figure 2b, bottom). The MS/MS results are consistent with a "lariat" structure, consisting of an 8-residue cyclic segment--with a backbone−side-chain amide linkage between Gly1 and Glu8--followed by a 13-residue linear segment. Triple-resonance NMR experiments[11] directly confirm the presence of a backbone−side-chain amide linkage between Gly1 and Glu8 and indicate that the linkage has a trans conformation (through-bond coherence transfer between backbone nitrogen atom of Gly1 and side-chain $C^\beta$ and $C^\gamma$ atoms of Glu8; strong NOESY cross-peak between Gly1 $H^N$ and each Glu8 $H^\delta$ proton; Figure 2c). We conclude that MccJ25 is a 21-residue lariat.

Figure 3 shows the solution three-dimensional structure of MccJ25 determined in methanol by triple-resonance NMR.[11,12] In the three-dimensional structure, the linear segment of the lariat (residues 9−21) loops back, penetrates, and threads through the cycle of the lariat, as a thread through a needle eye, resulting in
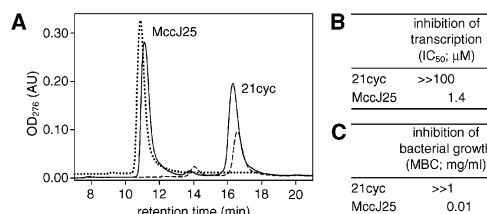


**Figure 1.** MccJ25 is not a 21-residue cycle. RP-HPLC[9] analysis (dashed line, 21cyc; dotted line, MccJ25; solid line, both 21cyc and MccJ25). (b) Inhibition of transcription (IC$_{50}$ from in vitro transcription assays with *E. coli* RNA polymerase; methods as in ref 3). (c) Inhibition of bacterial growth (MBC from minimum-bacteriocidal-concentration assays with *E. coli* strain DH5α; methods as in ref 3).
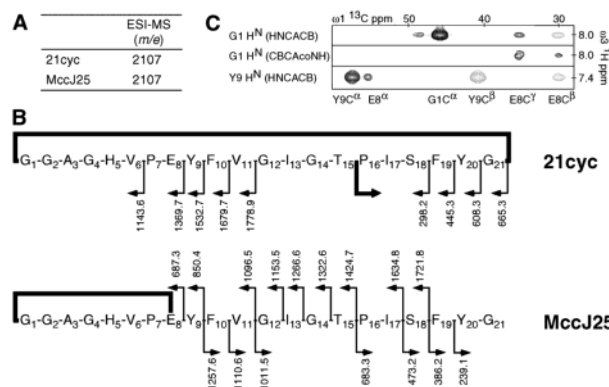


**Figure 2.** MccJ25 is a 21-residue lariat. (a) ESI-MS analysis. (b) ESI-MS/MS analysis. All fragments for 21cyc result from double cleavage, with one cleavage at residue 15 (bold arrow). (c) Strip plots from three-dimensional HNCACB and three-dimensional CBCAcoNH spectra[11] of MccJ25 showing Gly1-H$^N$/Glu8-C$^\gamma$ and Gly1-H$^N$/Glu8-C$^\beta$ cross-peaks due to coherence transfer across the Gly1-backbone/Glu8-side-chain amide linkage.

formation of a "protoknot"[13] (also known as an "entanglement"[14,15]) (Figure 3a−c). The protoknot has a linking number of Lk = −1 (conventions for chain topology as in ref 16). The four C-terminal residues (residues 18−21) are in contact with, and encircled by, the cycle, and a short antiparallel β-sheet is formed comprising C-terminal residues (residues 19−20; β2) and residues of the cycle (residues 6−7; β1)] (Figure 3a−c). The connector between the four C-terminal residues and the cycle contains a chain reversal (residues 11−14), the backbone conformation of which is less well defined and possibly dynamically disordered (Figure 3a,b). We conclude that MccJ25 is a 21-residue lariat protoknot.

Phe19 and Tyr20 bracket the cycle, with the aromatic side-chain of Phe19 being located on one face of the cycle, and the aromatic side-chain of Tyr20 being located on the other face of the cycle

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† Rutgers University.
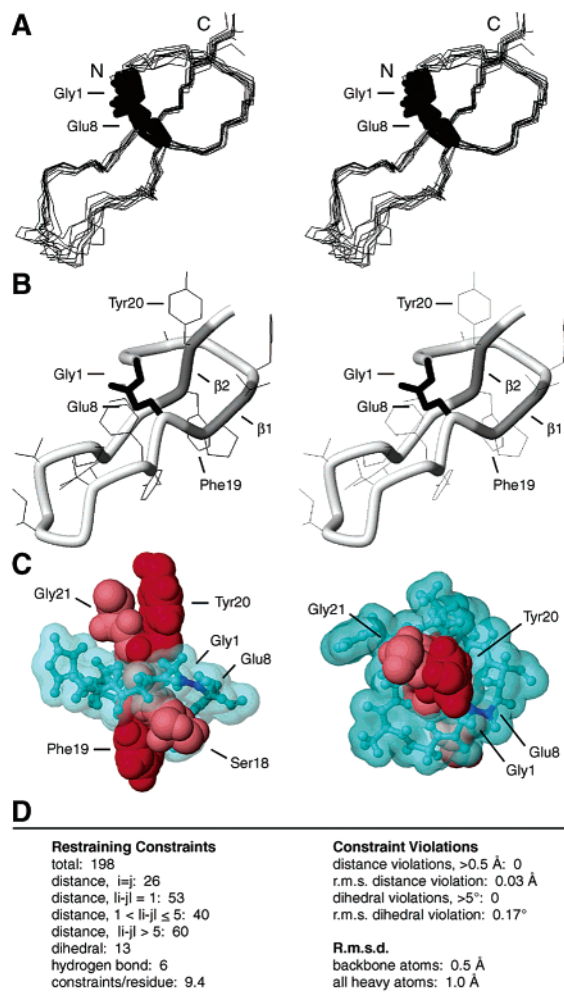‡ Scripps Research Institute.

**Figure 3.** MccJ25 is a 21-residue lariat protoknot. (a) Stereodiagram of seven superimposed structures of MccJ25 determined by NMR.[11,12] (b) Representative structure of MccJ25. (c) Two orthogonal views of threading of the C-terminal segment (residues 18−21 in pink; side-chains of Phe19 and Tyr20 in red) through the cycle (residues 1−8 in cyan; backbone−side-chain bond in blue). (d) NMR structure−quality statistics.

(Figure 3c). We propose that steric constraints imposed by the side-chains of Phe19 and Tyr20 lock the register of the threaded segment relative to the cycle, irreversibly trapping the threaded segment within the cycle. Consistent with this proposal, the NMR spectra[11,12] provide no indication of multiple slowly interconverting conformers or conformational exchange in this region of the structure.

The proposed presence of an irreversible protoknot, with irreversible trapping of residues 18−21 within the cycle, has three implications. First, it accounts for the observations that led to the incorrect proposal that MccJ25 was a 21-residue cycle: i.e., the observation that MccJ25 is resistant to carboxypeptidase, and the observation that cleavage of MccJ25 between residues 10 and 11 yields one product, not two.[5,7,16] Second, it accounts for the observed exceptional stability of MccJ25 to denaturation (stable for ≫10 h in 8 M urea at 95 °C).[7] Third, it implies that, during biosynthesis of MccJ25, the MccJ25 precursor must prefold--at least transiently adopting a native or near-native conformation, presumably a $\beta$-hairpin containing the $\beta1/\beta2$ $\beta$-sheet--prior to formation of the backbone−side-chain amide linkage.[17]

MccJ25 is the first example of a lariat protoknot involving a backbone−side-chain amide linkage. Like Cys−Cys side-chain−side-chain linkages[18] and Cys−Phe and Cys−Thr side-chain−backbone linkages,[19] lariat protoknots involving a backbone−side-chain amide linkage provide an effective means of conferring defined, stable three-dimensional structure to short peptides.

**Supporting Information Available:** MS and MS/MS data and details of experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) C18, 5 $\mu$m, 300 Å column (Rainin, Inc.); solvent A = 0.1% TFA, solvent B = 90% acetonitrile and 0.1% TFA; gradient = 30−35% solvent B in solvent A over a period of 20 min, flow rate = 1 mL/min.
(10) Residues are numbered as $n_{gene} - 37$, where $n_{gene}$ is the codon number in the gene for MccJ25 precursor.[4]
(11) Samples contained 2 mM [$^{13}$C;$^{15}$N]MccJ25 (prepared essentially as described for MccJ25[5]) in $^{13}$CD$_3$OH. Triple-resonance NMR data were collected at 25 °C as described[20] using a Varian INOVA 500 NMR spectrometer. Resonance assignments were determined for all assignable atoms by manual analysis of triple-resonance NMR spectra and have been deposited in the BioMagResDataBase (accession 5859).
(12) NOESY data were collected at 25 °C using a Varian INOVA 600 NMR spectrometer. Structure determination was performed using AutoStructure[21] and DYANA.[22] Input included manually edited peak lists from three-dimensional $^{15}$N-edited NOESY and $^{13}$C-edited NOESY spectra (recorded with mixing times of 175 ms) and 13 $^3J$(H$^N$−H$^\alpha$) scalar coupling constants. Amino acid libraries of DYANA were modified to model the side-chain−backbone bond. The final NMR structure is represented by the 10 (of 60) structures having lowest values of the DYANA target function after 14 cycles of iterative analysis of NOESY peak assignments with AutoStructure (Figure 3a). All peptide bonds are in the trans conformation, and all backbone dihedral angles are in low-energy regions of the Ramachandran map. Atomic coordinates and constraint lists have been deposited in the PDB (accession 1PP5).
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(16) MS and MS/MS analyses indicate that the product of cleavage of MccJ25 between residues 10 and 11 (thermolysin-cleaved MccJ25[5,7]) has MW = 2125 and consists of two associated, presumably threaded, chains: a 10-residue lariat chain and an 11-residue linear chain (Supporting Information).
(17) PSI-BLAST analysis indicates that the MccJ25 processing factor McjC[4] exhibits sequence similiarity to amidotransferases of the Asn-synthase/Gln-hydrolase class, which catalyze transfer of ammonia or an amine from an amide donor to a carboxyl acceptor[23] (Ebright, R. H., unpublished results). Processing of MccJ25 involves two reactions: removal of the 37-residue N-terminal pro-sequence (residues −37 to −1) and cyclization of residues 1−8. We suggest that McjC carries out both reactions, acting on pre-folded MccJ25 precursor to catalyze transfer of the α-amino of residue 1 from the backbone amide of residue −1 to the side-chain carboxyl of residue 8.
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