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Synthetic peptides derived from the sequence of a lasso peptide microcin J25 show antibacterial activity

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ABSTRACT

Microcin J25 (MccJ25) is a plasmid-encoded, ribosomally synthesized antibacterial peptide with a unique lasso structure. The lasso structure, produced with the aid of two processing enzymes, provides exceptional stability to MccJ25. We report the synthesis of six peptides (**1**–**6**), derived from the MccJ25 sequence, that are designed to form folded conformation by disulfide bond formation and electrostatic or hydrophobic interactions. Two peptides (**1** and **6**) display good activity against *Salmonella newport*, and are the first synthetic derivatives of MccJ25 that are bactericidal. Peptide **1** displays potent activity against several *Salmonella* strains including two MccJ25 resistant strains. The solution conformation and the stability studies of the active peptides suggest that they do not fold into a lasso conformation and peptide **1** displays antimicrobial activity by inhibition of target cell respiration. Like MccJ25, the synthetic MccJ25 derivatives display minimal toxicity to mammalian cells suggesting that these peptides act specifically on bacterial cells.

1. Introduction

Microcin J25 (MccJ25) is a 21-residue ribosomally synthesized antimicrobial peptide that adopts a remarkable threaded-lasso structure.[1–3] MccJ25 displays bactericidal activity against a range of food-borne disease-causing Gram-negative pathogens including diarrheagenic *Escherichia coli* strains.[4] The lasso-structure of the peptide consists of a lactam ring (known as lariat ring) formed between the N-terminus Gly1 and the side chain of Glu8 (Fig. 1).[5–7] The C-terminal tail (Tyr9-Gly21) passes through the ring forming a β-hairpin structure made of a type I β-turn (Val11-Gly14) and two short double stranded antiparallel β-sheets, residues 6–7/19–20 and 10–11/15–16.

The active mature MccJ25 is produced and exported by four plasmid-encoded genes *mcjABCD*.[8] McjA encodes a linear 58-residue precursor peptide which is converted into mature peptide by two enzymes, McjB and McjC.[9,10] McjB, a putative peptidase, cleaves the 37-residue leader peptide from the precursor peptide, whereas, McjC is believed to catalyze the lactam bond formation. McjD helps in the transport of the active antibiotic peptide out of the producing cell.[11] The lasso-structure confers several properties to MccJ25, such as, resistance to proteolytic degradation,[12,13] stability towards chaotropes, organic solvents, and extreme temperatures.[14] Such properties make MccJ25 a potential candidate for a number of applications including food preservation and treatment of food-borne diseases.

Different variants of MccJ25, prepared by site directed mutagenesis, have been reported for studying structure–activity relationship (SAR).[15,16] Pavlova et al. performed a complete mutational scanning analysis of MccJ25 by making more than 380 MccJ25 variants with a single amino acid substitution.[16] Each single amino acid variant was tested for production and export from *E. coli*. In addition, the variants that were produced were analyzed for the ability to inhibit RNA polymerase (RNAP) and bacterial growth. Several residues in the lactam ring and the tail region were found to be important for the production of MccJ25 and inhibition of bacterial growth. Another study reported MccJ25 variants containing two or three amino acid substitutions where the peptides retain the lasso structure and the antimicrobial function.[15] These studies demonstrate that peptide lasso scaffold of MccJ25 is quite tolerant to amino acid substitutions. Additional SAR studies of MccJ25 have suggested that the lactam ring region and the tail β-hairpin loop play distinct roles in the peptide's antimicrobial activity.[17,18] In particular, using a thermolysin-digested MccJ25 cleaved between residues 10 and 11 it was demonstrated that the β-hairpin (residues 10–16) is crucial in the import of MccJ25 through FhuA, but not for RNAP and respiration inhibition.[18] The roles played by specific residues in the peptide have also been reported.[19,20] The C-terminal glycine was found to be important for RNAP inhibition,[19] while site specific mutation of His-5 demonstrated that this residue was important for recognition by the inner membrane receptor SbmA.[20]

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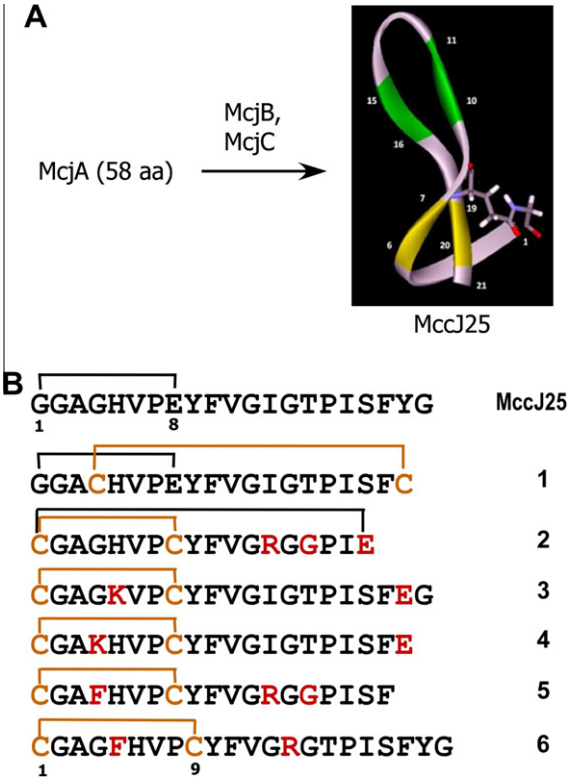


Figure 1. (A) A 58 amino acid McjA precursor peptide is converted into mature antimicrobial form, MccJ25 by McjB and McjC enzymes. The ribbon representation of the lasso structure of MccJ25 (PDB ID code 1Q71)[6] is shown. Antiparallel β-sheets are shown in green and yellow, and the amide bond between residues G1-E8 is shown as stick model. (B) Amino acid sequences of MccJ25 and derived peptides **1**–**6**. Substitutions of amino acids in the peptide derivatives are shown in yellow for cysteine and red for others.

Attempts have also been made to chemically synthesize MccJ25 variants, such as, macrocyclic head-to-tail 21-residue MccJ25, a 21-residue variant with side chain-to-backbone lactam ring between α-amino of Gly1 and γ-carboxyl of Glu8, and a recently reported MccJ25 model peptide (cMccJ25) where Gly1 and Glu8 are replaced with nearly isosteric azidoacetic acid and propargyl glycine, respectively.[5,6,21] In the later variant (cMccJ25), the copper-catalyzed click reaction between the azide and the terminal alkyne permits cyclization of cMccJ25 without the influence of the maturation enzymes.[21] None of these synthetic peptides showed any antibacterial activity.

We hypothesized that peptides with antibacterial activity can be designed based on the MccJ25 sequence where a folded conformation is acquired by a combination of intra-peptide disulfide bond formation and electrostatic or hydrophobic interactions, instead of post-translational modification by McjB and McjC proteins. To test our hypothesis, six peptides (**1**–**6**) derived from the sequence of MccJ25 ranging in length 18–22 residues were designed and synthesized (Fig. 1). We report herein the results of antimicrobial activity, solution conformation, stability to proteases and cytotoxicity of this series of synthetic MccJ25 derivatives.

2. Results and discussion

Six peptides were designed on the basis of the MccJ25 sequence following the strategy that intra-peptide interactions will lead to active peptide derivatives. Peptides were designed to form folded conformation by intra-peptide (i) disulfide and amide bond formation (analogues **1** and **2**), (ii) disulfide and electrostatic interactions (**3** and **4**), or (iii) disulfide and hydrophobic interactions (**5** and **6**). In peptide **1**, the lactam ring of MccJ25 between G1 and E8 was maintained, and a disulfide bond between C4 and C20 (C-terminal) was introduced to hold the C-terminal of the peptide in place. In all other peptides (**2–6**), the isopeptide bond between G1 and E8 was replaced with a disulfide bond. Peptide **2,** an 18-residue peptide, utilized an amide bond to tie the N-terminal C1 with the C-terminal E18. Peptides **3** and **4** contain substitution of a ring residue and one residue from the tail region with positively and negatively charged amino acids, respectively, for electrostatic interactions (H5K, Y20E in **3** and G4K, Y20E in **4**). Similarly, peptides **5** and **6** contain substitution or insertion with hydrophobic amino acids, such as G4F in **5**. Peptide **6** consists of a larger 9-residue N-terminal ring, as expansion of the ring was thought to facilitate the insertion of the C-terminal into the N-terminal lariat ring by interaction between F5 and F19 and/or Y20. An aromatic hydrophobic Phe (F) residue was inserted between G4 and H5 for enhanced van der Waals and stacking interactions with the C-terminal F19 and Y20. In addition, I13R and/or T15G substitutions (replacement of hydrophobic Ile with basic guanidine containing Arg and Thr with a methyl side chain with Gly with no side chain) were introduced in peptides **2**, **5**, and **6** to increase solubility. These mutations have been reported to yield higher inhibitory activity compared to the wild-type MccJ25.[16]

Peptides **1**–**6** were synthesized as linear peptides using Fmoc solid-phase peptide synthesis. For peptides **1** and **2**, the lactam bond formation was achieved on the solid-phase, followed by cleavage from the resin. The second ring (disulfide bond) was then formed in solution. Peptides **3**–**6** were cleaved from the resin as linear peptides followed by oxidation to form disulfide bond. The electrostatic or hydrophobic interactions in the peptides were initiated when the peptides were cleaved from the solid phase and the side chain protections were removed. Native MccJ25 was expressed using pTUC202 plasmid in *E. coli* MC4100 in M9 minimal media.[12] Crude peptides were purified using semi-preparative reversed-phase HPLC prior to characterization by electrospray and/or MALDI-TOF mass spectrometry (Table S1, Supplementary data). All peptides were purified up to >95% purity as shown by analytical RP-HPLC and mass spectrometry (Figs. S1 and S2), and were obtained with an overall yield of 7–32%.

The antibacterial activity of MccJ25 and peptides **1**–**6** was evaluated against a number of Gram-negative pathogens. Twelve *Salmonella* strains and two *E. coli* strains were used to obtain the activity profile of the peptides using liquid growth inhibition assay (Table 1).[22] Peptides **1** and **6** displayed good activity (low micromolar range) against *Salmonella newport* with MIC values of 25 and 30 μM, respectively (Fig. 2). Compared to the native MccJ25 (MIC 0.5 μM), these peptides were 50–60-fold less potent. Interestingly, **1** also displayed activity against several (five) other *Salmonella* strains including two MccJ25 resistant strains. For all of these strains, MIC of **1** was in the range of 75–90 μM. The MIC of MccJ25 was also higher (compared to *S. newport*) for the three MccJ25 sensitive strains. Peptides **1–6** were also tested for inhibition of Gram-positive bacteria, such as *Staphylococcus aureus* ATCC 6538 and *Enterococcus faecalis* ATCC 19433. These peptides showed no activity against Gram-positive strains.

A number of different MIC values of MccJ25, ranging from 5 nM to 0.22 μM, against *S. newport* have been reported previously.[3,12,23,24] In this study, we found the MIC value of MccJ25 to be 0.5 μM against a clinical isolate of *S. newport*. This variation in MIC values could be due to the difference in the experimental conditions used, such as spot-on-lawn assay, liquid growth assay, media with or without supplements, or the difference in the strains used.

Furthermore, peptides **1** and **6** were found to be bactericidal similar to MccJ25.[4] The minimum bactericidal concentrations

Table 1
Antibacterial activity of MccJ25 and synthetic peptide derivatives (**1** and **6**) against Gram-negative bacterial strains

Strains[a]	MIC[b] (μM) of peptides		
	MccJ25	1	6
S. newport	0.5 ± 0.05	25 ± 3.0	30 ± 4.0
S. enteritidis PT8	1.5 ± 0.8	80 ± 3.0	—
S. enteritidis ATCC13076	1.8 ± 0.7	—	—
S. enteritidis AFLB 11	2.0 ± 0.6	—	—
S. enteritidis AFLB 41	1.6 ± 1.0	—	—
S. enteritidis AFLB 81	1.7 ± 1.2	—	—
S. typhimurium AFLB 25	2.8 ± 0.8	85 ± 5.2	—
S. typhimurium ATCC 13311	—	75 ± 6.5	—
S. heidelberg ATCC 8326	—	83 ± 6.1	—
S. montevideo 20	—	—	—
S. paratyphi	1.5 ± 1.0	—	—
S. choleresius ATCC 10708	1.5 ± 0.6	90 ± 5.7	—
E. coli DH5α	5.0 ± 1.0	—	—
E. coli MC4100	5.0 ± 2.0	—	—

[a] Details for the bacterial strains are provided in Table S2 (Supplementary data).
[b] MIC value is the last concentration of the peptide (μM) giving 100% inhibition of the strain. —, no activity detected up to 250 μM. Data are represented as means ± standard errors of the means. The values are results of at least three independent measurements.

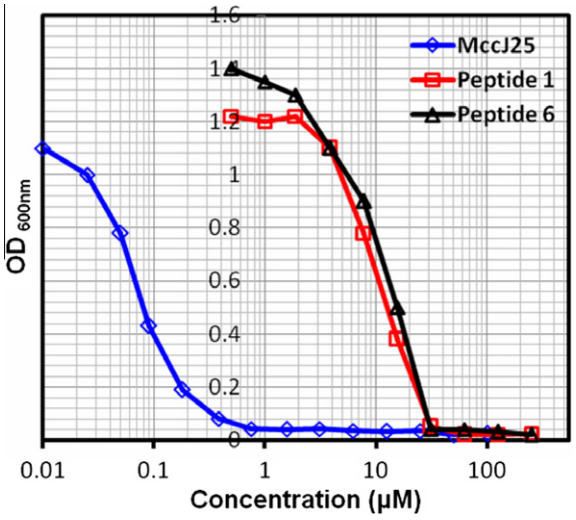


Figure 2. Growth curves of microcin J25, peptide **1**, and peptide **6** using broth assay against *Salmonella newport*. MICs were defined as the lowest peptide concentration that caused 100% growth inhibition (0.05 AU).

(MBCs) of peptides **1** and **6** against *S. newport* were 31.25 and 62.5 μM, respectively. The MBCs of peptide **1** against *Salmonella enteritidis* ATCC P76, *Salmonella typhimurium* ATCC 13311, and *Salmonella choleresius* ATCC 10708 were 125 μM each. A number of synthetic analogues of MccJ25 have been reported, however, none of them display activity against Gram-negative pathogens.[6,7,13,21] To our knowledge, peptides **1** and **6** are the first synthetic derivatives of MccJ25 that display antibacterial activity. Peptides **2**–**5** lacked antimicrobial activity against all of the tested strains up to 250 μM concentration. One striking feature of peptides **2**–**5** was the replacement of lactam bond with a disulfide linkage. Although the same is true for peptide **6**, but the replacement was better tolerated due to the expanded 9-residue lariat ring. A variant of peptide **6** with 8-residue lariat ring (CGAFHVPCYFVGRGTPISFYG) was synthesized (not shown here) and was found to be inactive suggesting larger lariat ring facilitates interaction between the C-terminal tail and the N-terminal ring. The reduced activity of **1** and **6** compared to the native

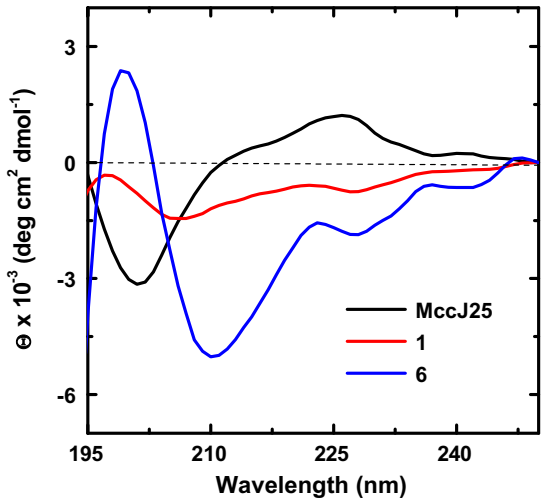
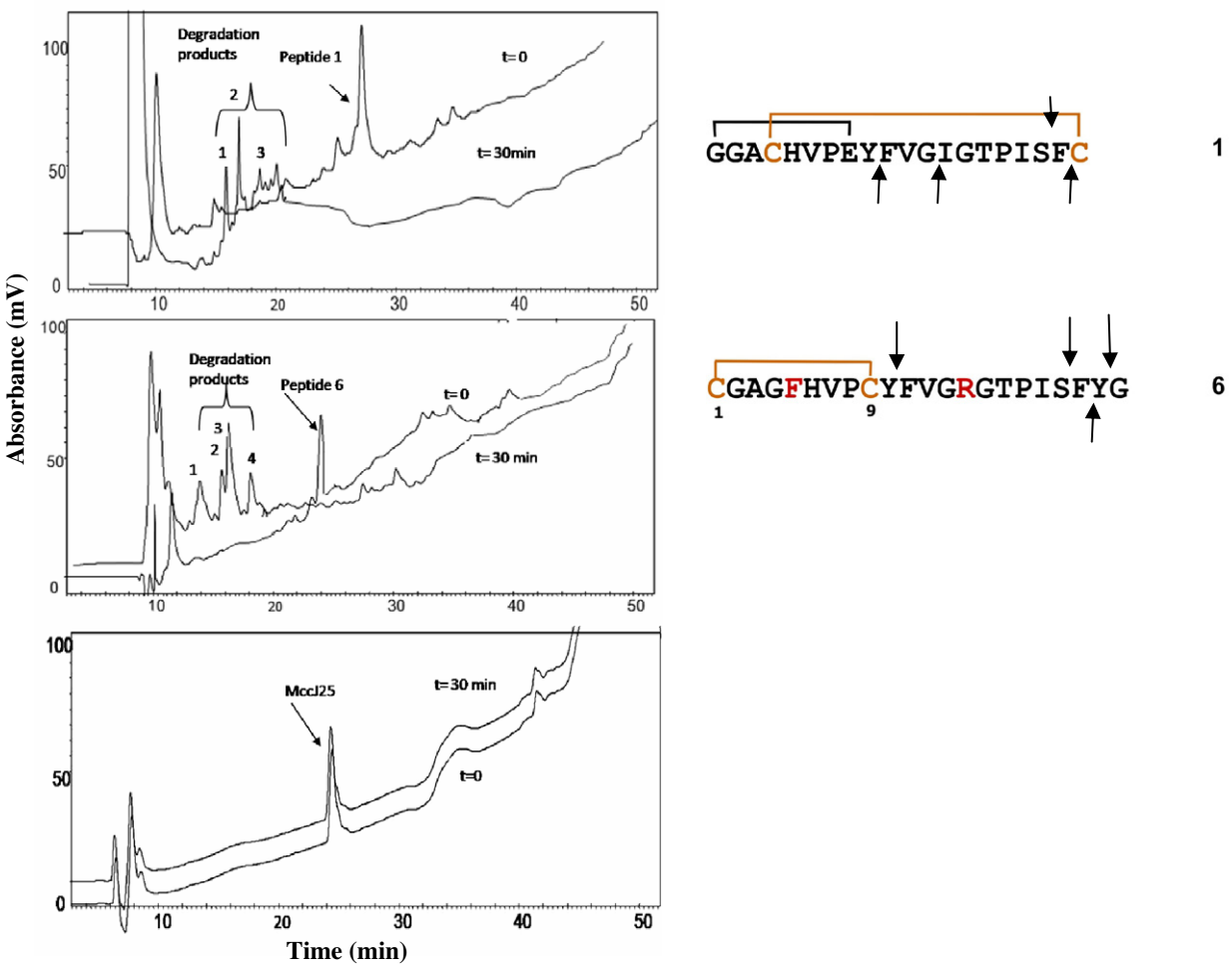


Figure 3. Circular dichroism spectra for MccJ25, **1** and **6** in methanol at 25 °C.

MccJ25 could be due to the loss of characteristic lasso conformation.

Circular dichroism (CD) spectroscopy was used to study the folding behavior of peptides **1** and **6** in solution. Methanol was used as a solvent for CD studies due to the limited solubility of the peptides in water. Moreover, methanol has been used previously to elucidate the solution structure (NMR structure) of MccJ25.[6] The CD spectra of peptides **1** and **6** in methanol were very different from MccJ25 suggesting different folded structures in solution (Fig. 3). The CD spectrum of MccJ25 in methanol displayed a minimum at 200 nm ($\Theta = -3.2 \times 10^3$) and a maximum at 225 nm ($\Theta = 1.1 \times 10^3$) with a positive shoulder at 215 nm (Fig. 3) consistent with the previously reported spectrum.[25] A negative peak at 200 nm is characteristic of unstructured peptides; however, this peak has also been attributed to small β-sheets or β-turn secondary structures such as in β-defensins.[26] The positive band at ca. 225 and 210 nm have been attributed to the Phe L_a and Tyr L_a transitions.[27] CD of peptide **1** showed a broad minimum at 208 nm with low intensity ($\Theta = -1.5 \times 10^3$), whereas, peptide **6** displayed a minimum at 210 nm ($\Theta = -5.0 \times 10^3$) with a shoulder at 226 nm ($\Theta = -1.8 \times 10^3$). Quantitative analysis of the secondary structure of peptide **6** with CDPro[28] suggested that there is little β-sheet conformation (~15% residues form β-sheet) compared to MccJ25 (39%). Peptide **6** showed the presence of increased helical folding. These results suggest that both the active peptides (**1** and **6)** may adopt a constrained and compact folded structure, however, do not adopt the true lasso structure as observed in MccJ25.

Furthermore, the absence of lasso conformation in peptides **1** and **6** was confirmed by enzyme stability studies. MccJ25 is known to be highly stable to degradation in the presence of proteolytic enzymes, such as chymotrypsin, trypsin, carboxypeptidase and pepsin.[12] In contrast, both **1** and **6** were not stable in the presence of chymotrypsin and pepsin. During the antimicrobial assay, the circular growth inhibition zones of **1** and **6** were distorted to a crescent-shape in the presence of a drop of chymotrypsin. Likewise, reversed-phase HPLC analysis of peptides **1** and **6** incubated with pepsin for 30 min showed complete degradation of the peptides (Fig. 4). For peptide **1**, the presence of fragments FVGIGTPIS and FVGIGTPISF showed that the cleavage occurred before and after the C-terminal Phe residue (Phe19). This suggests that the C-terminal cysteine is present outside the ring (N-terminal ring between Gly1 and Glu8) allowing access to the C-terminal residues for cleavage by enzymes such as pepsin. Peptide **6** also gave similar



Fragment #	Sequence	Mass Found
*Peptide **1***		
1	FVGIGTPIS and FVGIGTPISF	889.1 and 1036.5
2	GGACHVPEY-C and GGACHVPEY-FC	1034.6 and 1181.2
3	GGACHVPEY-IGTPISFC	1748.8
*Peptide **6***		
1	FVGRGTPIS	932.2
2	CGAGFHVPCY	1052.1
3	FVGRGTPISF and FVGRGTPISFY	1079.2 and 1242.6
4	CGAGFHVPCYFVGRGTPIS, CGAGFHVPCYFVGRGTPISF, and CGAGFHVPCYFVGRGTPISFY	1966.9, 2112.9 and 2276.0

Figure 4. RP-HPLC chromatograms of peptides **1** (top), **6** (middle) and MccJ25 (bottom) after incubation with pepsin at time 0 and 30 min. On the right side are shown the cleavage sites for the peptides. A linear gradient was run on a C18 Vydac column (4.6 × 250 mm) over 35 min from 15% to 50% isopropyl alcohol/water 0.05% (v/v) TFA with a flow rate of 2 mL/min.

degradation products after incubation with pepsin. However, fragments from cleavage near Phe5 residue, which is present inside the N-terminal ring (between Cys1 and Cys9) of **6**, were not detected. Interestingly, the evaluation of thermostability of **1** and **6** showed that only peptide **1** was active after incubation in boiling water for 3 h. This may be due to its rigid bicyclic ring structure allowing high heat resistance similar to wild-type MccJ25. The disulfide bond was found to be intact for peptide **1** after boiling treatment. Peptides **1** and **6** with reduced cysteine residues (absence of disulfide bond) were found to display no antimicrobial activity.

MccJ25 displays antibacterial activity by inhibiting the RNA polymerase (RNAP) and/or the respiratory chain in the target organism, depending on the bacterial species.[23,19,29,30] For *Salmonella* species MccJ25 targets both the RNAP and the cell respiration, however, for *E. coli* the target is mainly RNAP. Peptides **1** and **6** most likely do not inhibit RNAP as they are inactive against *E. coli* as well as the lasso structure essential for RNAP inhibition is absent in these peptides. Different structure–activity relationship studies of MccJ25 showed that lasso conformation (tail locked in ring) is important in RNAP interaction and inhibition.[19,17] Thermolysin-cleaved MccJ25 and MccJ25 lacking amino acids 13–17 inhibit RNAP transcription suggesting that the unusual lasso ring-tail part of the MccJ25 molecule interacts with RNAP, and blocks the secondary channel of the enzyme.[17] Whereas, amidation

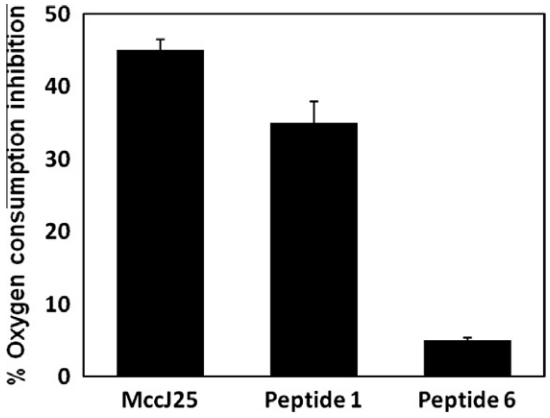


Figure 5. Effects of treatment with MccJ25, peptide **1** and peptide **6** on the oxygen consumption. Oxygen consumption of bacteria growing in the presence of peptides was expressed as a percentage of the oxygen concentration of the control culture (without peptides). Data correspond to mean values of three independent experiments. Error bars correspond to the standard deviations.

of the C-terminal glycine threaded through the MccJ25 lariat ring specifically blocks the RNAP inhibition. The amidated MccJ25 peptide inhibits cell respiration.[19]

To assess the effect of peptides **1** and **6** on the inhibition of cell respiration, the oxygen consumption of *S. newport* in the presence of MccJ25, **1** or **6** was assayed. Peptide **1** inhibited oxygen consumption, whereas, peptide **6** had no effect on oxygen consumption (Fig. 5). The findings from this experiment suggest that peptide **1** inhibits cell respiration in a manner analogous to MccJ25, and peptide **6** that did not inhibit cell respiration has an alternative mechanism of action. Peptide **1**, which lacks the lasso structure, seems to act primarily by inhibition of cell respiration which may also account for its decreased antimicrobial activity compared to MccJ25.

Finally, the cellular toxicity of MccJ25 and peptides **1**–**6** was investigated as a lead antibiotic must display low toxicity to mammalian cells. The cytotoxicity of the peptides was evaluated using cell viability MTT assay against two cancer cell lines, MDA-MB-435 and MCF-7 cells (Fig. S3). MccJ25 and the peptide derivatives displayed very low cytotoxicity (>80% cell viability) up to the highest concentration tested (100 μM). Under similar conditions, a known antitumor agent doxorubicin showed only 20% cell viability at low concentration (3 μM). Peptides **3**–**4** with charged residues or involving electrostatic interactions displayed slightly higher cytotoxic effect (75% cell viability) compared to the other peptides (>90% cell viability). In general, the toxicity values observed were not significant when compared to the known antitumor agents. In this context, it is of particular interest to note that Lopez et al. observed low hemolytic activity for MccJ25 against red blood cells.[24] Although MccJ25 displays antiapoptotic properties with isolated mitochondria,[31] the low cytotoxicity could be due to its inability to permeate human cells.

To summarize, two active peptides (**1** and **6**) derived from the sequence of MccJ25 are reported. The design strategy used for obtaining active synthetic sequences from the MccJ25 sequence suggests that (i) the folding of the C-terminal tail (in order to constraint the C-terminal tail near the lactam ring) can be achieved with a covalent disulfide bond formation (peptide **1**) and (ii) the lactam (or lariat) ring can be substituted with a ring formed by a disulfide bond, however, it may require ring expansion (peptide **6**). The CD and resistance to proteases experiments suggest that the active peptides (**1** and **6**) do not fold into a lasso conformation, and peptide **1** displays antimicrobial activity by inhibition of target cell respiration. Finally, the synthetic peptide derivatives of MccJ25 display minimal toxicity to mammalian cells and act specifically on bacterial cells.

3. Experimental section

3.1. Reagents and equipment

Wang resin (0.58 mmol/g), N-α-Fmoc-amino acids, benzotriazol-L-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), and (1-hydroxybenzotriazole) HOBt were purchased from NovaBiochem (San Diego, CA). The side chains of amino acids were protected as follows: *tert*-butyl (tBu) for serine, threonine, tyrosine, *tert*-butyl ester (OtBu) or allyl ester (OAll) for glutamic acid, *tert*-butoxycarbonyl (Boc) for lysine, trityl (trt) for cysteine and histidine. 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU), 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent), *N*-methyl morpholine (NMM), triisopropylsilane (TIS), diisopropylethylamine (DIPEA) and trifluoroacetic acid (TFA) were purchased from Aldrich, while piperidine was purchased from Caledon (Canada). All other reagents were purchased from Sigma–Aldrich. All commercial reagents and solvents were analytical grade and were used as received.

RP-HPLC purification and analysis were carried out on a Varian Prostar (210 USA) HPLC system using Vydac semi-preparative C18 (1 × 25 cm, 5 μm), analytical C8 (0.46 × 25 cm, 5 μm) and Agilent preparative C18 (21.2 × 25 cm, 7 μm) columns. Compounds were detected by UV absorption at 220 nm. Mass spectra were recorded on a MALDI Voyager time-of-flight (TOF) spectrometer (Voyager™ Elite) or on a Waters micromass ZQ. Absorbance of the purple formazan product observed during MTT assay was measured using VERSA max microplate reader (Molecular Devices, Sunnyvale, CA, USA). All the procedures regarding the cell culture maintenance and treatment of cells were carried out in a level II biosafety cabinet.

3.2. Peptide synthesis and purification

3.2.1. General procedure

Stepwise synthesis of peptides **1–6** was done manually on a 0.2-mmol scale of Wang resin (1.0% DVB cross-linked), following the standard Fmoc solid-phase peptide chemistry as described previously.[32] Attachment of the first amino acid (5 equiv) was carried out using 2,6-dichlorobenzoylchloride (DCB) in DMF/pyridine mixture. Successive couplings were performed by dissolving an excess (2 equiv) of Fmoc-protected amino acid and Castro reagent (BOP) in presence of (HOBt) to prevent racemization in DMF. Coupling efficiency was monitored using the ninhydrin (Kaiser) test.[33] The coupling step was repeated (double coupling) if Kaiser test was found positive. In addition, a test cleavage was performed after each five residues were coupled, and the desired product was confirmed by MALDI-TOF mass spectrometry. Each peptide was cleaved from the resin using a mixture of 95% TFA, 5% triisopropylsilane, 5% water, for 120 min at room temperature with mechanical shaking. The filtrate from the cleavage reactions was collected, combined with TFA washes (3 × 2 min, 1 mL), and concentrated in vacuum. Cold diethyl ether (∼15 mL) was added to precipitate the crude cleaved peptide. After triturating for 2 min, the peptide was collected upon centrifugation and decantation of the ether. The peptides were then purified using RP-HPLC.

3.2.2. On resin amide cyclization (peptides 1 and 2)

For peptides **1** and **2**, side chain of Glu was protected as allyl ester (OAll). After complete assembly of the protected peptide on the

resin, the side chain protecting group (allyl) of Glu8 (peptide **1**) or Glu18 (peptide **2**) was first removed. Deprotection of the allyl from carboxyl group was carried out with $Pd(PPh_3)_4$ (0.08 equiv) and PhSiH3 (8 equiv) in DCM/DMF (45 min × 3) under nitrogen. The N-terminus Fmoc group was removed using 20% piperidine/DMF. The desired lactam linkage was then formed using HATU/DIPEA (2 equiv) as the activating agents for 3 h in DMF, after which complete cyclization was achieved. It was confirmed using ninhydrin test and MALDI-TOF.

3.2.3. Oxidative folding and disulfide bond formation

The purified linear (analogues **3**–**6**) or lactam cyclized peptides (**1**–**2**) were dissolved in 50 mM Tris buffer (pH 8.3) to a final concentration of 0.1 mM. The peptide solution was then treated with 20% DMSO to assist in oxidation and enhance peptide solubility. The reaction mixture was left stirring overnight at room temperature in an open flask. The oxidation was monitored using Ellman test,[34] and MALDI-TOF mass analysis. The oxidation was complete in 48 h. A mass spectrum with M-2 indicated loss of two protons and formation of the oxidized peptide.

3.2.4. Peptide purification

Purification of the peptides was done in two stages. First, the crude linear or lactam cyclized peptides were reconstituted in 30–50% aqueous acetonitrile and purified on a semi-preparative Vydac C18 reversed-phase (RP) HPLC column (10 × 250 mm, 5 μm, flow rate = 2 mL/min, monitored at 220 nm) using different linear gradients of acetonitrile/water or isopropyl alcohol/water (0.05% TFA, v/v) mixtures. The peptides were oxidized to form the disulfide bond, and were subjected to another RP-HPLC purification using preparative C18 HPLC column (21.2 × 250 mm, 7 μm). This purification was essentially done to remove the salts from the reaction mixture. The collected fractions were evaporated on the rotary evaporator followed by lyophilization to obtain the pure peptide. The identity and purity of the peptides were assessed by analytical HPLC (Fig. S1) and MALDI-TOF mass spectrometry (Fig. S2). The details of the purification methods and the elution time of each peptide are listed in Table S1. In general, pure peptides were obtained with an overall yield of 7-32% and purity greater than 95%.

3.3. Microcin J25 expression and purification

The expression and purification of native MccJ25 were performed as described previously with some modifications.[12] Briefly, the high copy number plasmid pTUC202 (a gift from Rutgers University, USA) which carries the MccJ25 biosynthetic gene cluster was transformed to competent *E. coli* MC4100 cells. The cells were grown in M9 minimal media (2 L) for 24 h at 37 °C. The culture supernatant was obtained by centrifugation at 4000 g for 15 min, and then subjected to three successive purification steps. First, the supernatant was applied to flex column filled with XAD16 resin (Aldrich, CA). Followed by elution in two successive steps with 30:70 (v/v) and 80:20 (v/v) methanol/water mixtures. Antibacterial assay was used to follow the peptide. MccJ25 found in 80:20 (v/v) methanol/water fraction was next applied to C18 Megabond column (Varian, USA). Three successive elution steps were carried out with 40:60 (v/v), 60:40 (v/v) and 80:20 (v/v) methanol/water mixtures. The 80:20 (v/v) fraction was further purified using C18 RP-HPLC (2 mL/min flow rate and absorbance monitored at 220 nm) under linear gradient from 55% to 80% methanol/water (0.05% TFA) in 45 min. The concentration of the peptide was determined spectrophotometrically at 278 nm (molar absorptivity 3340 M/L),[18] and the yield was 2 mg.

3.4. Antimicrobial activity assay

The minimum inhibitory concentrations (MICs) of peptides **1**, **6** and MccJ25 were determined by liquid growth inhibition assays in sterile 96 well plate using LB as growth media.[22] Peptide stock solutions were prepared in 20% methanol/water (1 mM for MccJ55 and 2.5 mM for **1** and **6**), and were serially double diluted in sterile water to give concentrations ranging from 0.01 to 100 μM for MccJ25, and 0.5 to 250 μM for **1** and **6**. Similar concentration of methanol/water alone without the peptide did not change the growth of bacteria. Bacterial culture (180 μL) diluted in LB media were added to the microplate wells to obtain optical density (OD_{620}) of 0.02–0.05, followed by addition of peptide solutions (20 μL). Each peptide concentration was tested in triplicate. Control wells contained the peptide in LB media as well as untreated bacterial cells. After incubation of the microplates for 24 h at 37 °C, the turbidity (OD) was read at 620 nm on a plate reader. MICs were defined from a growth curve as the lowest peptide concentrations that caused 100% growth inhibition (0.05 AU). Three representative growth curves of microcin J25, peptide **1**, and peptide **6** obtained using broth assay against *S. newport* are shown in Figure S4. Standard deviations derived from MIC outcome of three graphs for three repeated experiments were calculated. Concentrations of peptides stocks solutions were determined by absorbance at 280 nm described previously.[35] The minimum bactericidal concentration (MBC) was determined from wells showing complete inhibition. An LB agar plate was seeded on the surface with 10 μL from each clear well and incubated (24 h, 37 °C). The MBC was defined as the lowest concentration giving no growth on an LB plate afterwards.

3.5. Circular dichroism (CD) spectroscopy

The CD measurements for the peptides (MccJ25, **1** and **6**) were made on an Olis CD spectrometer (Georgia, USA) at 25 °C in a thermally controlled quartz cell over 190–260 nm. All samples were dissolved in 100% methanol with a final concentration of 200 μM. The length of the cuvette was 0.02 cm and number of scans was set to 10. Smoothing and correction of the background spectra was performed afterwards. The CD data was normalized and expressed in terms of mean residue ellipticity (deg cm^2 $dmol^{-1}$). CD spectra were analyzed using quantitative curve fitting using the CDPro software analysis program as described previously.[36]

3.6. Proteolytic stability

The enzymatic stability of peptides **1** and **6** compared to native MccJ25 was evaluated against two digestive enzymes (pepsin and chymotrypsin). The susceptibility of **1** and **6** for chymotrypsin cleavage was assessed using spot on lawn method as reported previously.[13] Peptide analogues **1** and **6** (10 μL of 200 μM) were spotted onto LB plates and chymotrypsin solution (10 μL of 0.5 mg/mL) was placed 1 cm away from the peptide spots. After the drops had dried, the plates were overlaid with 4 mL of soft agar inoculated with 10^7 cells of a clinical isolate of *Salmonella enterica* serovar Newport. After overnight incubation at 37 °C, the growth inhibition zones around the drops were recorded. MccJ25 was used as control at 5 μM concentration.

Pepsin was dissolved in 0.1 N HCl (4% w/w) and then peptide **1** or **6** was added (150 μM).[37] The solution was incubated at 37 °C and the degradation was stopped at specific time points 0, 30 min, 1 h, by taking 100 μL aliquots into 10 μL 0.1 N NaOH containing 70% methanol to precipitate the enzyme. Sample was centrifuged at 16000 g for 10 min, and the supernatant containing

the cleavage mixture was injected in RP-HPLC. Fractions were collected for mass detection using MALDI-TOF to determine the labile sites. Also fragments were analyzed for antibacterial activity using spot on Lawn as described earlier. For sake of comparison the same experiment was repeated for native MccJ25.

3.7. Oxygen consumption determination

Oxygen consumption was determined as previously described.[38] *S. newport* strain were grown to exponential phase (OD_{600} 0.4–0.5) in LB media. Samples were diluted in LB media to an OD_{600} of 0.2. Cells were then incubated at 37 °C with either MccJ25 (0.25 μM), **1** (25 μM), or **6** (30 μM) for 35 min. Thereafter, the average rate of respiration for subsequent 5 min was polographically measured with a 2 mL Gilson Clarke electrode oxygraph at 37 °C. A control experiment in the absence of peptide was carried out similarly.

3.8. Cell viability assay

3.8.1. Cell lines

Tumor cell lines used in this study included MDA-MB-435 and MCF7 breast cancer cell lines. MDA-MB-435 cells were cultured in RPMI 1640 medium (Sigma) supplemented with FBS (10%), penicillin (50 μg/mL), and streptomycin (0.05 g/mL) in a humidified atmosphere (5% CO_2) at 37 °C. MCF7 cells were grown in DMEM media supplemented with same additives as stated above.

3.8.2. MTT cytotoxicity assay

The cellular toxicity of MccJ25 and the peptide derivatives was determined by measuring the cell growth inhibition using MTT assay.[39] Two cancer cell lines were used, MDA-MB-435 and MCF7 cells. Cells were seeded in 96 well plates (Corning Inc., MA, USA) at a concentration of 1×10^4 cells/well (100 μL) in complete media and were incubated at 37 °C in 5% CO_2 atmosphere. After 24 h, the cells were treated with aseptic samples. Samples (1 mM, MccJ25 and peptides) were prepared in sterile 10% DMSO/water followed by filtration through 0.2 μM filter. The cell culture media were added to the 96 well plate (180 μL/well) followed by addition of peptide sample solution (20 μL/well), serially double diluted to give concentrations ranging from 0.3125 to 100 μM. Doxorubicin was used as a positive control and untreated cells were used as a negative control. The plates were incubated for another 24 h following which the culture media was aspirated and replaced with 100 μL MTT solution (5 mg/mL in media). The cells were incubated for 3.5 h to allow interaction with MTT solution. Following incubation, the media was removed by vacuum, and the purple formazan product precipitated in each well was solubilized in DMSO (100 μL), and the absorbance was measured at 570 nm using microtitre reader. The percentage cell viability was expressed as the absorbance ratio of cells treated with peptides to untreated cells dissolved in complete media. All experiments were done in triplicate, and the data is presented in the form of mean.

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Supplementary data

Supplementary data (characterization of new peptides using reversed phase HPLC and mass spectrometry, details of the bacterial strains, and results from the cell viability MTT assays (Tables S1–S2 and Figs. S1–S3)) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.061.

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