VOL. 54 NO. 5, MAY 2001 THE JOURNAL OF ANTIBIOTICS pp. 434 — 440

# Streptocidins A~D, Novel Cyclic Decapeptide Antibiotics Produced by *Streptomyces* sp. Tü 6071

## II. Structure Elucidation†

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(Received for publication February 9, 2001)

The structures of the new antibiotics streptocidins A~D were elucidated as cyclic decapeptides cyclo[L-Val[1]-L-Orn[2]-L-Leu[3]-D-Phe[4]-L-Pro[5]-L-Leu[6]-X[7]-L-Asn[8]-L-Gln[9]-X[10]] with X[7]=D-Trp (A, B, C) or D-Phe (D) and X[10]=L-Tyr (A), L-Trp (B, D), or D-Trp (C). The amino acid composition (including the configuration) of the substances was determined by chiral-phase GC-MS of the hydrolysates. The sequences were established by EDMAN degradation following linearisation of the cyclic peptides upon treatment with $LiAlH_4$. NMR spectroscopic studies of streptocidins C and D confirmed the proposed sequences and provided conformational data which indicate a molecular topology of streptocidins C and D similar to those of tyrocidine A and gramicidin S.

The streptocidins are a family of closely related cyclic decapeptide antibiotics that were isolated from the culture broth of *Streptomyces* sp. Tü 6071. The fermentation, isolation, and biological activities of the streptocidins A~D are described in the preceding paper[1]. Here, we report the elucidation of the primary structure of the streptocidins (Fig. 1), together with NMR analysis of the primary structure and molecular topology of streptocidins C and D.

### Materials and Methods

Amino Acid Analysis by Chiral-Phase GC-MS

Samples (100~200 nmole peptide) were hydrolysed *in vacuo* for 24 hours at 110°C in 200 µl of 6 N HCl with the addition of 5 µl thioglycolic acid. Aliquots of the hydrolysate were derivatised to the *N*-trifluoroacetyl-*O*-ethyl ester derivatives and these were analysed by chiral-phase GC-MS on a 30% 2,6-dipentyl-3-butyryl-γ-cyclodextrin in PS255 (dimethyl-silicone gum) capillary and a Chirasil-L-Val capillary (both 25 m×250 µm fused silica).

Analysis was carried out on a Model 6890 gas chromatograph coupled to a Model 5973 mass spectrometer (Hewlett-Packard) using the manufacturers software and protocols. Amino acids were identified by their mass spectra and quantified by the technique of enantiomer labeling[2]. In order to compensate for oxidative losses of

† Art.No. 22 on 'Biosynthetic Capacities of Actinomycetes'. Art. No. 21: See ref. 1.

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tryptophan during hydrolysis, the appropriate Trp-antipode was added to the sample before hydrolysis. For quantitative analysis, selected specific ions for each amino acid were monitored (SIM-mode).

### Electrospray (ES)-MS

ES-mass spectra were collected using a VG Quattro II triple quadrupole mass spectrometer fitted with an atmospheric pressure ES source and analysed using MassLynx V2.2 Software (MicroMass). Peptides (10 $\mu$g/ml) were introduced in a continuous stream of 50% (v/v) aq. acetonitrile containing 0.1% (v/v) formic acid at flow rates of 5~50 $\mu$l/minute, and cone voltages in the range 20~50 eV were applied. The drying gas was air and the probe temperature was maintained at 70°C throughout the measurement.

### Reductive Ring Opening

For EDMAN sequencing the cyclic peptides were linearised by reductive cleavage of the peptidyl-prolyl amide bond by $LiAlH_4$, a reaction resulting in the generation of the corresponding peptide-aldehydes[3].

Linearised streptocidins were subsequently purified by HPLC using a stainless steel column (250×8 mm) filled with 5-$\mu$m Nucleosil C18 (Grom) and elution with water-0.1% TFA (v/v) (A) and acetonitrile-0.1% TFA (v/v) (B) applying a gradient of 10~90% B in 45 minutes at a flow rate of 3.5 ml/minute. The UV absorbance of the eluate was monitored at 214 nm. Peak fractions were collected manually, identified and characterised by ES-MS as above, and were lyophilised and stored at −20°C prior to further analysis.

### EDMAN Sequencing

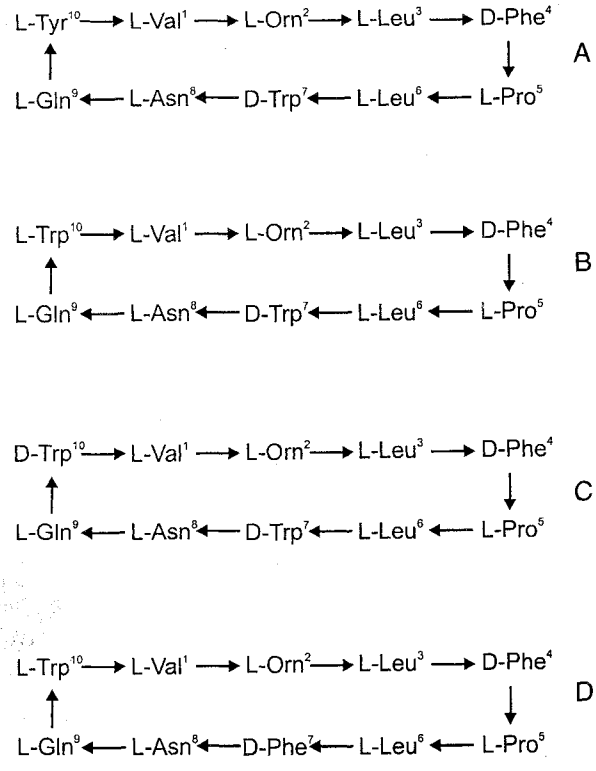
Aliquots of linearised streptocidins (typically 15 $\mu$l containing 200~300 pmol peptide), dissolved in 30% (v/v) aq. acetonitrile-0.1% (v/v) TFA, were applied to a TFA-treated glass fibre filter previously coated with 15 $\mu$l Biobrene Plus (Perkin-Elmer Applied Biosystems). The amino acid sequence was determined in a model 477A pulsed-liquid gas-phase protein sequencer coupled online to a model 120A narrow-bore phenylthiohydantoin (PTH)-amino acid analyser (Perkin-Elmer Applied Biosystems). For sequencing and data analysis the manufacturers chemicals, software and standard protocols for protein microsequencing were used. The relative retention time for ornithine (not contained in the PTH-amino acid standards) was estimated from the corresponding peak identified during sequencing of a synthetic peptide containing ornithine at defined positions. The *C*-terminal amino acid aldehyde could not be identified by this procedure, but could be inferred from the measured mass and the amino acid analysis data.

### NMR Spectroscopy

1D and 2D NMR spectra were recorded on a Bruker AMX2-600 spectrometer operating at a proton frequency of 600.13 MHz using a 5-mm inverse triple resonance probehead equipped with z-gradients. 7.4 mg of streptocidin C and 3.3 mg of streptocidin D, respectively, were each dissolved in 0.5 ml DMSO-$d_6$.

The data set acquired for each sample consisted of TOCSY (clean-MLEV spin lock of *ca.* 62 mseconds), P.E.COSY, NOESY (mixing time of 300 mseconds), and HSQC (with gradient selection) experiments acquired at 305 K as well as five 1D $^1$H NMR spectra acquired at 305 K, 310 K, 315 K, 320 K, and 325 K. The 1D NMR spectra were recorded with a time domain of 64 k complex points and a relaxation delay of 2 seconds. For 2D NMR spectra 2 k (TOCSY, NOESY, HSQC) or 4 k (P.E.COSY) complex data points in $t_2$ with 256 (NOESY, HSQC) or 512 (TOCSY, P.E.COSY) $t_1$ increments were acquired. The 2D NMR experiments were carried out with phase-sensitive detection in the indirect dimension by use of the TPPI (homonuclear experiments) and the States (HSCQ) method,

Fig. 1. Primary structures of streptocidins A~D.



respectively. The raw data were subjected to apodization by suitable window functions and zero filling prior to Fourier transformation, phase correction, and automatic baseline correction. The spectra were referenced to the signal of the solvent at $\delta$ ($^{1}$H)=2.50 ppm, and $\delta$ ($^{13}$C)=39.5 ppm.

## Results

The primary structures of streptocidins A~D (Fig. 1) were elucidated by a combination of chiral-phase GC-MS of the hydrolysates, ES-MS, and EDMAN sequencing after

Table 1. Amino acid composition of streptocidins A~D.

| | Streptocidin | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | |
| | Found | Integer | Found | Integer | Found | Integer | Found | Integer |
| L-Asx | 0.99 | 1 | 0.92 | 1 | 1.00 | 1 | 0.97 | 1 |
| L-Glx | 0.95 | 1 | 1.12 | 1 | 0.91 | 1 | 1.16 | 1 |
| D-Phe | 0.86 | 1 | 0.87 | 1 | 1.25 | 1 | 1.79 | 2 |
| L-Leu | 1.71 | 2 | 1.80 | 2 | 2.20 | 2 | 2.14 | 2 |
| L-Orn | 1.19 | 1 | 1.06 | 1 | 1.44 | 1 | 1.23 | 1 |
| L-Pro | 1.00 | 1 | 1.00 | 1 | 1.00 | 1 | 1.00 | 1 |
| L-Val | 0.86 | 1 | 0.82 | 1 | 1.09 | 1 | 1.15 | 1 |
| L-Trp | - | - | 1.05 | 1 | - | - | 1.11 | 1 |
| D-Trp | 0.99 | 1 | 1.04 | 1 | 2.09 | 2 | - | - |
| L-Tyr | 0.89 | 1 | - | - | - | - | - | - |
| Total | - | 10 | - | 10 | - | 10 | - | 10 |

Table 2. ES-MS and EDMAN sequencing data obtained for streptocidins A~D.

| | Mass | | | | |
|---|---|---|---|---|---|
| | Measured | | Calculated | | |
| Streptocidin | Linear | Cyclic | Linear | Cyclic | N-Terminal Sequence |
| A | 1277.6 +/- 0.1 | 1275.1 +/- 0.4 | 1277.5 | 1275.5 | PLWNQYVOL(F)[a] |
| B | 1300.8 +/- 0.1 | 1298.2 +/- 0.4 | 1300.6 | 1298.6 | PLWNQWVOL(F)[a] |
| C | 1300.4 +/- 0.1 | 1298.1 +/- 0.3 | 1300.6 | 1298.6 | PLWNQWVOL(F)[a] |
| D | 1261.7 +/- 0.1 | 1259.1 +/- 0.4 | 1261.5 | 1259.5 | PLFNQWVOL(F)[a] |

[a] *C*-terminal phenylalanine-aldehyde (generated by reductive ring opening) could not be identified by amino acid sequencing, but was inferred from both the measured mass and the amino acid composition data.

linearisation. The data obtained are summarised in Tables 1 and 2 and allowed us to unambiguously deduce the primary structure of streptocidins A, C, and D. The relative positions of L-Trp and D-Trp in streptocidin B could not be inferred from the presented data. Based on biosynthetic considerations, the primary structure of streptocidin B was therefore proposed in analogy to streptocidins A, C, and D with the D-amino acid in position 7.

NMR spectroscopic studies were carried out for the model systems streptocidins C and D in order to both confirm the proposed sequences and to provide additional, conformational data. The amino acid residues of streptocidins C and D were identified by their unique spin systems (Val, Orn, Leu, Pro) from TOCSY spectra alone or with additional information from intraresidue NOEs (Phe, Trp, Asn, Gln). The assignment within the spin systems of the residues was completed by means of P.E.COSY and HSQC spectra. For both compounds, a complete sequential walk ($H_i^{\alpha} \leftrightarrow H_{i+1}^{N}$, and $H^{\alpha}$-Phe$^4 \leftrightarrow H^{\delta 2}$-Pro$^5$ for the Phe$^4$-Pro$^5$ bond, respectively) was observed in the respective NOESY spectrum. Tables 3 and 4 comprise the assignment of the $^1$H and of the proton-bound $^{13}$C atoms for streptocidins C and D.

$^3J_{N\alpha}$ coupling constants were extracted from P.E.COSY

Table 3. $^1$H and $^1$H-$^{13}$C chemical shifts (ppm) of streptocidin C (DMSO-$d_6$, 305 K).

| | N | α | β | Others |
|---|---|---|---|---|
| L-Val[1] | 7.54 | 4.60 | 2.11 | γ: 0.99 |
| | | 56.7 | 31.0 | γ: 18.3 |
| L-Orn[2] | 8.71 | 5.25 | 1.80 | γ: 1.67; δ: 2.88/2.75; ε: 8.30 |
| | | 50.3 | 30.7 | γ: 22.4 ; δ: 38.5 |
| L-Leu[3] | 7.71 | 4.38 | 1.02 | γ: 1.27; δ: 0.86/0.82 |
| | | 49.9 | 40.2 | γ: 24.0; δ: 21.6/23.1 |
| D-Phe[4] | 9.23 | 4.29 | 2.92/2.80 | 2,6: 7.21; 3,5: 7.25; 4: 7.21 |
| | | 53.5 | 35.2 | 2,6: 128.8; 3,5: 127.7; 4: 126.6 |
| L-Pro[5] | - | 4.13 | 1.83/1.42 | γ: 1.40; δ: 3.45/2.37 |
| | | 59.5 | 28.6 | γ: 22.9; δ: 45.7 |
| L-Leu[6] | 7.12 | 4.17 | 0.85/0.07 | γ: 1.15; δ: 0.58/0.57 |
| | | 50.0 | 38.4 | γ: 23.3; δ: 20.1/22.7 |
| D-Trp[7] | 8.54 | 5.42 | 3.05/2.95 | 1: 10.68; 2: 7.00; 6: 7.18; 7: 6.91; 8: 6.71; 9: 7.51 |
| | | 51.4 | 29.7 | 2: 124.2; 6: 110.5; 7: 120.3; 8: 117.8; 9: 118.2 |
| L-Asn[8] | 9.11 | 4.59 | 3.29/2.98 | δ: 8.00/7.39 |
| | | 48.8 | 35.5 | |
| L-Gln[9] | 8.58 | 3.82 | 1.67/1.58 | γ: 1.89; ε: 7.16/6.81 |
| | | 54.6 | 25.3 | γ: 30.7 |
| D-Trp[10] | 8.54 | 4.50 | 3.23/3.13 | 1: 10.80; 2: 7.02; 6: 7.31; 7: 7.04; 8: 6.97; 9: 7.49 |
| | | 55.1 | 27.8 | 2: 122.9; 6: 111.1; 7: 120.4; 8: 117.8; 9: 117.9 |

Table 4. $^{1}H$ and $^{1}H$-$^{13}C$ chemical shifts (ppm) of streptocidin D (DMSO-$d_6$, 305 K).

| | N | α | β | Others |
|---|---|---|---|---|
| L-Val[1] | 7.47 | 4.56 | 2.03 | γ: 0.93/0.92 |
| | | 56.5 | 31.0 | γ: 18.2 |
| L-Orn[2] | 8.80 | 5.25 | 1.81/1.72 | γ: 1.69; δ: 2.90/2.77; ε: 8.29 |
| | | 49.9 | 30.4 | γ: 22.3; δ: 38.2 |
| L-Leu[3] | 7.79 | 4.48 | 1.31/1.17 | γ: 1.39; δ: 0.90/0.88 |
| | | 49.7 | 40.9 | γ: 24.1; δ: 21.6/22.6 |
| D-Phe[4] | 9.27 | 4.32 | 2.95/2.83 | 2,6: 7.23; 3,5: 7.31; 4: 7.24 |
| | | 56.3 | 35.2 | 2,6: 128.9; 3,5: 127.7; 4: 126.3 |
| L-Pro[5] | - | 4.17 | 1.86/1.44 | γ: 1.43; δ: 3.49/2.38 |
| | | 59.4 | 28.4 | γ: 22.5; δ: 45.5 |
| L-Leu[6] | 7.19 | 4.26 | 1.10/0.55 | γ: 1.32; δ: 0.67/0.66 |
| | | 49.8 | 39.1 | γ: 23.7 ; δ: 22.8/19.8 |
| D-Phe[7] | 8.80 | 5.48 | 2.98/2.68 | 2,6: 7.13; 3,5: 7.07; 4: 7.07 |
| | | 52.4 | 39.6 | 2,6: 128.8; 3,5: 127.2; 4: 125.5 |
| L-Asn[8] | 9.00 | 4.50 | 3.33/3.00 | δ: 8.00/7.39 |
| | | 48.6 | 34.9 | |
| L-Gln[9] | 8.64 | 3.81 | 1.62/1.57 | γ: 1.88; ε: 7.17/6.80 |
| | | 54.6 | 25.3 | γ: 30.7 |
| L-Trp[10] | 8.47 | 4.49 | 3.19/3.12 | 1: 10.80; 2: 7.01; 6: 7.31; 7: 7.04; 8: 6.97; 9: 7.48 |
| | | 54.9 | 27.8 | 2: 122.7; 6: 110.9; 7: 120.3; 8: 117.9; 9: 117.4 |

spectra. Amide hydrogen temperature coefficients were calculated from the chemical shifts observed in five 1D $^{1}H$ NMR spectra acquired at temperatures from 305 K to 325 K. $^{3}J_{N\alpha}$ coupling constants and amide hydrogen temperature coefficients of streptocidins C and D are summarised in Table 5. Qualitatively, NOE and coupling constant data of streptocidins C and D agree with an approximately antiparallel $\beta$-pleated sheet moiety formed by residues Val[1]-Orn[2]-Leu[3] and Leu[6]-Trp[7]/Phe[7]-Asn[8] whose strands are interconnected by two $\beta$ turns. This structural model has been proposed for the closely related tyrocidine A[4,5], which is cyclo[L-Val[1]-L-Orn[2]-L-Leu[3]-D-Phe[4]-L-Pro[5]-L-Phe[6]-D-Phe[7]-L-Asn[8]-L-Gln[9]-L-Tyr[10]], and for gramicidin S, whose sequence consists of the repeated L-Val[1]-L-Orn[2]-L-Leu[3]-D-Phe[4]-L-Pro[5] motif[6]. The observation of nearly identical intense NOEs between $H^{\alpha}$-Phe[4] and both $H^{\delta 2}$- and $H^{\delta 1}$-Pro5, which has been reported for gramicidin S and tyrocidine A, reflects a similar type II′ geometry around D-Phe[4]-L-Pro[5] in streptocidins C and D. Streptocidin D follows tyrocidine A in featuring a type I $\beta$ turn around

Table 5. $^3J_{N\alpha}$ coupling constants[a] (Hz) and amide hydrogen temperature coefficients[b] (ppb/K) of streptocidins C and D.

| | $^3J_{N\alpha}$ | | $\Delta\delta(H^N)/\Delta T$ | |
|---|---|---|---|---|
| | C | D | C | D |
| Val[1] | 9.3 | 10.7 | +0.50 ± 0.09 | +0.30 ± 0.03 |
| Orn[2] | 9.1 | 8.5 | -7.10 ± 0.15 | -4.80 ± 0.06 |
| Leu[3] | 9.1 | 10.6 | +2.12 ± 0.08 | -0.66 ± 0.02 |
| Phe[4] | 4.5 | 4.3 | -7.84 ± 0.06 | -7.86 ± 0.05 |
| Pro[5] | - | - | - | - |
| Leu[6] | 9.2 | 8.6 | -1.65 ± 0.40 | -1.44 ± 0.04 |
| Trp[7]/Phe[7] | 9.1 | 10.6 | -3.66 ± 0.11 | -7.30 ± 0.15 |
| Asn[8] | 7.0 | 6.5 | -3.88 ± 0.04 | -2.80 ± 0.00 |
| Gln[9] | 4.6 | 4.3 | -2.24 ± 0.02 | -2.54 ± 0.05 |
| Trp[10] | 9.2 | 8.6 | -5.08 ± 0.06 | +3.10 ± 0.02 |

[a] Coupling constants are given with an accuracy of about 60.5 Hz.
[b] Amide hydrogen temperature coefficients were calculated by linear regression of the temperature dependent amide hydrogen shifts and are given with their standard errors.

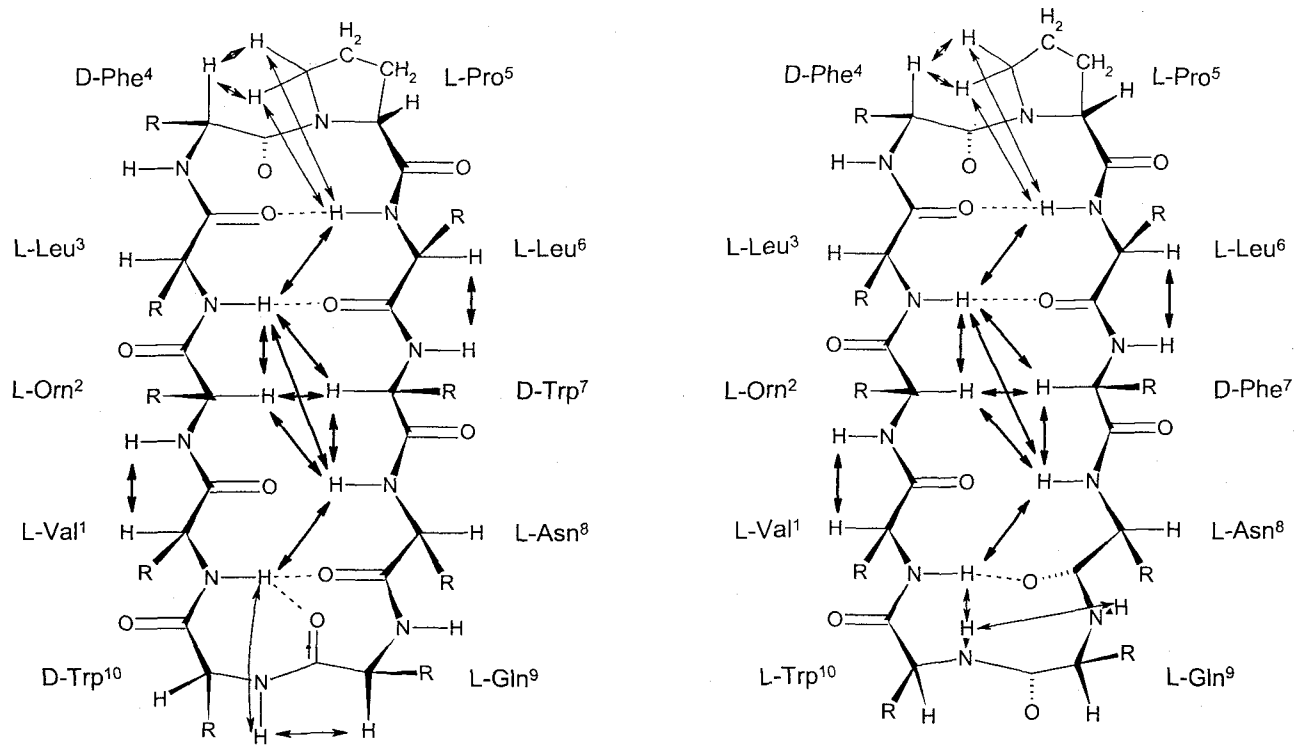
L-Gln[9]-L-Trp[10] indicated by the intense NOEs $H^N$-Trp[10] ↔ $H^N$-Val[1] and $H^N$-Gln[9] ↔ $H^N$-Trp[10]. The latter NOE is missing in the NOESY spectrum of streptocidin C suggesting that the presence of the D-amino acid in position 10 in streptocidin C leads to the preference of a type II turn around L-Gln[9]-D-Trp[10] [7]. Fig. 2 shows conformational models for streptocidins C and D indicating the observed NOEs characteristic of secondary structure[8] and the likely position of hydrogen bridges.

## Discussion

Streptocidins A~D can be structurally classified to a family of antibiotically active cyclic decapeptides composed of the tyrocidines (A~E) produced by *Bacillus brevis* and the loloatins (A~D), which have recently been isolated from an as yet unidentified tropical marine bacterium, possibly also of the genus *Bacillus*[9]. The common sequence of tyrocidines, streptocidins, and loloatins can be formulated as cyclo[L-Val[1]-L-Orn[2]-L-Leu[3]-D-(Phe/Tyr)[4]-L-Pro[5]-L-X[6]-D-(Phe/Trp)[7]-L-Asn[8]-L-(Gln/Asp)[9]-D/L-X[10]][†††]. Streptocidins share with the tyrocidines the occurrence of D-Phe[4] and L-Gln[9], whereas loloatins contain D-Tyr[4] and L-Asn[9]. Tyrocidines and streptocidins therefore comprise the complete L-Val[1]-L-Orn[2]-L-Leu[3]-D-Phe[4]-L-Pro[5] motif of gramicidin S and are positively charged (L-Orn[2]) molecules. Due to the presence of both, ornithine and aspartic acid residues, loloatins are zwitterionic. L-Tyr and L-Trp are the preferred amino acids for position 10 in tyrocidines, streptocidins and loloatins, with the exception of tyrocidine E (L-Phe[10]) and streptocidin C (D-Trp[10]). The main structural characteristic of streptocidins is the occurrence of L-Leu in position 6 as opposed to tyrocidines and loloatins, which have either L-Phe[6] or L-Trp[6].

[†††] Loloatin D contains L-hydroxyproline in position 5.

Fig. 2. Conformational models proposed for streptocidin C (left) and streptocidin D (right).




Arrows symbolize the observed backbone NOEs, which are indicative of secondary structure (thick: antiparallel $\beta$-pleated sheet, normal: $\beta$-turns)[8]. Broken lines symbolize hydrogen bonds whose presence was supported by observation of amide hydrogen temperature coefficients $\leq |2|$ ppb/K (Table 5).

Acknowledgements

This work was supported by the Deutsche Forschungs-gemeinschaft (SFB 323).

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