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

I. Taxonomy, Fermentation, Isolation and Biological Activities†

KLAUS GEBHARDT[a], RÜDIGER PUKALL[b] and HANS-PETER FIEDLER[a,*]

[a] Mikrobiologisches Institut, Universitat Tübingen,
Auf der Morgenstelle 28, D-72076 Tübingen, Germany
[b] DSMZ German Collection of Microorganisms and Cell Cultures,
Mascheroder Weg 1b, D-38124 Braunschweig, Germany

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Four novel cyclic homodecapetide antibiotics, streptocidins A~D were detected in the mycelium extract of *Streptomyces* sp. Tü 6071 by HPLC-diode-array and HPLC-electrospray-mass-spectrometry screening. The peptides which were closely related in structure to the tyrocidines and gramicidins of *Bacillus brevis* show antibiotic activities against Gram-positive bacteria.

In the course of our search for novel secondary metabolites from freshly isolated actinomycetes using HPLC-DAD-MS screening, we investigated strain Tü 6071 which was isolated on glycerol - arginine agar[2] from a soil sample that was collected at Cape Coast, Ghana. A methanol - acetone extract of the mycelium was analysed by gradient reversed-phase HPLC coupled with a diode-array monitoring system (HPLC-DAD). The UV-visible spectra of the resulting peaks were compared with those of more than 600 reference compounds, mostly antibiotics, stored in our HPLC-UV-Vis-Database.[3] Four metabolites with retention times of 9.2, 9.5. 9.9 and 10.5 minutes – the latter two were the main compounds – were detected in the mycelium extract of strain Tü 6071 with UV maxima at 230 and 280 nm that showed a high conformity to the UV spectra of tyrocidines, cyclic decapeptide antibiotics produced by *Bacillus brevis*.[4] The analysis by HPLC-ESI-MS resulted in different molecular masses of these four metabolites compared to tyrocidines and other peptide antibiotics. They were named as streptocidins A, B, C and D.

This report deals with the taxonomy of the producing strain, fermentation, isolation and biological activities of streptocidins. Investigations on their chemical structure are reported in the following paper.[5] As indicated by these results, streptocidins are cyclic homodecapetide antibiotics, with a close relation to tyrocidines and gramicidins produced by bacilli.[6] Their structures are shown in Fig. 1.

Materials and Methods

Microorganisms

Strain Tü 6071 was isolated from a soil sample collected at Cape Coast, Ghana, using glycerol-arginine agar[2] with addition of cycloheximide (50 mg/liter) and nalidixic acid (20 ml/liter). The strain is deposited in the culture collection of our laboratory.

The standard strains for testing the biological activity spectrum and the minimal inhibition concentrations were obtained from DSMZ, ATCC and the stock collection of our laboratory in Tübingen.

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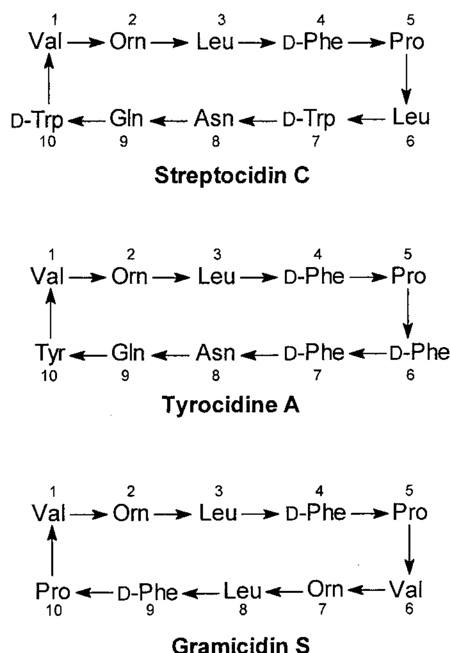
* Corresponding author: hans-peter.fiedler@uni-tuebingen.de

Fig. 1. Structures of streptocidin C, tyrocidine A and gramicidin S.



Taxonomy

Strain Tü 6071 was characterised by morphological and chemotaxonomic methods[7] as a member of the genus *Streptomyces*.

For molecular characterisation fresh bacteria cells were ground using a sterile micro pestle (Eppendorf) to get a uniform suspension. Preparation of genomic DNA from the pure culture was performed following the protocol given by ALTENBUCHER & CULLUM.[8] The amplification of the 16S rDNA was done as described by RAINEY *et al.*[9] The primer pair 27f (5′ GAGTTTGATCCTGGCTCAG 3′) and 1500r (5′ AGAAAGGAGGTGATCCAGCC 3′) was used for the amplification of the almost complete 16S rRNA gene.[10]

Sequencing was done by BioLux DNA-Analytik using the four primer 25f (5′ AGAGTTTGATCCTGGCTCAG 3′), 357f (5′ CTACGGGRSGCAGCAG 3′), 650f (5′ AAT-TCCTGGTGTAGCGGT 3′) and 926f (5′ AAACTCA-AAGGAATTGACGG 3′).

Fermentation

Streptomyces sp. Tü 6071 was cultivated in a 20-liter fermenter (type b20, Giovanola) equipped with an intensor system. The production medium consisted of: mannitol 2%, soybean meal 2% in tap water (pH 7.5, adjusted with 5 N NaOH). The fermenter was inoculated with 5 vol-% of shaking cultures, grown for 48 hours in 500 ml-Erlenmeyer flasks with one buffle on a rotary shaker at 120 rmp at 27°C in the same medium. For production of streptocidins the fermentation was carried out for 140 hours at 27°C with an aeration rate of 0.5 v/v/m and an agitation of 1000 rpm.

Isolation

Hyphlo Super-cel (2%) was added to the fermentation broth which was separated by multiple sheet filtration into culture filtrate and mycelium. The mycelium cake was extracted three times with MeOH - acetone (1 : 1). After concentration *in vacuo*, the aqueous residue was adjusted to pH 5 (1 N HCl) and extracted four times with the same volume of ethyl acetate. The organic extract was concentrated and the residue was applied to a diol-modified silica gel column (LiChroprep Diol, 40~63 μm, 400×25 mm; Merck) with CH_2Cl_2 - MeOH as eluent. Streptocidins were desorbed by linear gradient elution starting with CH_2Cl_2 to 100% MeOH within 4 hours at a flow rate of 4.5 ml/minute (medium pressure pump Mod. 381, gradient controller Mod. 388, Büchi). The fractions containing streptocidins were concentrated to dryness, dissolved in a small volume of MeOH and purified on a Sephadex LH-20 column (900×25 mm) using MeOH as eluent. Single streptocidin compounds were obtained after preparative reversed-phase HPLC using a stainless steel column (250×16 mm) filled with 10-μm LiChrospher RP-Select B (Merck), and linear gradient elution with 0.5% formic acid - MeOH starting from 70% MeOH to 100% MeOH within 20 minutes with a flow rate of 20 ml/minute. 20 mM triethylamine were added to MeOH. The preparative system consisted of two high-pressure pumps (Sepapress HPP-200/100; Kronwald), gradient unit (Sepacon GCU-311) and a Valco preparative injection valve (Mod. 6UW; VICI) with a 5-ml sample loop. The UV absorbance of the eluate was monitored at 280 nm by a Gilson spectrophotometer Mod. 116, equipped with a preparative cell. Streptocidins were obtained as white powders after lyophilisation.

HPLC-DAD-Analysis

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a built-in diode-array detector and a HP 79994B workstation (Hewlett-Packard). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360 and 435 nm; the spectrum range was from 200 to 600 nm with a 2-nm step and a sampling rate of 640 mseconds.

For analysing the mycelium, a 10 ml-sample of the fermentation broth was centrifuged (10 minutes at 13,000 *g*). The mycelium was extracted with MeOH - acetone (1 :

1), filtered, concentrated to dryness and re-suspended in 1 ml MeOH. Ten μl of the samples were injected onto a HPLC column (125×4.6 mm) fitted with a guard-column (20×4.6 mm) which was packed with 5-μm Nucleosil-100 C-18 (Grom). The samples were analysed by linear gradient elution using 0.1% phosphoric acid as solvent A and acetonitrile as solvent B at a flow rate of 2 ml/minute. The gradient was from 0% to 100% solvent B in 15 minutes with a 1-minute hold at 100% solvent B, followed by a 5-minute post-time at initial conditions.

HPLC-ESI-MS-Analysis

The system consisted of an ABI 140A HPLC gradient pump (Applied Biosystems) and an API III Taga 6000 E mass spectrometer equipped with a nebulizer-assisted electrospray source (Sciex). The HPLC column eluate was split by a T and introduced into the ion source at a constant flow rate of 40 μl/minute. Four μl of the samples were injected onto an HPLC column (100×2 mm) packed with 3-μm Nucleosil-100 C-18 (Grom). Samples were separated by linear gradient elution. Solvent A was 0.01% trifluoroacetic acid and solvent B was acetonitrile. The gradient was from 0% to 100% solvent B in 60 minutes at a flow rate of 200 μl/minute.

Biological Assays

An agar plate diffusion assay was used to determine the antimicrobial spectrum of a mixture of streptocidins (B, C and D) and compared to the closely related peptide antibiotics tyrocidines (mixture of A, B and C; Serva) and gramicidin S (Sigma). Ten μl of the samples were applied to a filter disks (6 mm diameter). The test plates were incubated for 24~48 hours at a temperature that permitted an optimal growth of the microorganisms.

The broth dilution method was used to determine the minimal inhibition concentrations of the streptocidins C and D. The MIC concentrations were compared to those of a tyrocidine mixture and gramicidin D and S. The antibiotics were dissolved in DMSO with final DMSO concentrations in the cultures not to exceed 5%. The bacteria were grown in a medium which consisted of nutrient broth 0.8% and NaCl 0.5% prepared with tap water. Streptomycetes were grown in malt extract 1%, glucose 0.4% and yeast extract 0.4% in tap water (pH 7.3). Bacterial cells and spores at 10^6 cells/ml were used as inocula, and growth inhibition was evaluated after an incubation for 24 and 96 hours at 27°C and 37°C, respectively, on a rotary shaker.

Results

Taxonomy

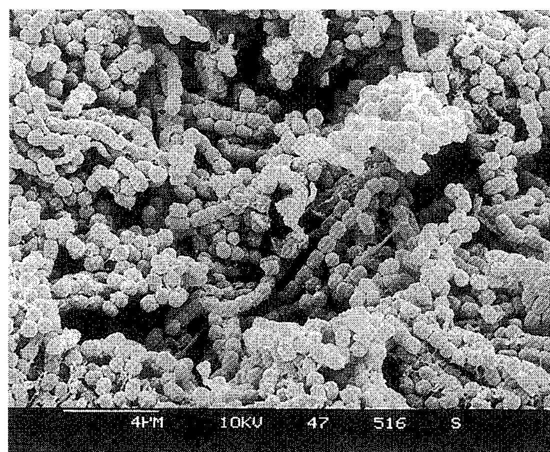
The chemotaxonomic analyses of diagnostic cell compounds revealed strain Tü 6071 to be a member of the genus *Streptomyces*, as characterised by the presence of LL-diaminopimelic acid in the peptidoglycan and the typical pattern of saturated *iso*- and *anteiso*-branched fatty acids. This genus affiliation was supported by morphological features. The spore mass colour of the aerial mycelium was white. The spore chains were straight with more than ten spores per chain (Fig. 2).

The almost complete 16S rDNA sequence of strain Tü 6071 was compared to sequences of other strains belonging to the family *Streptomycetaceae*, due to the presence of the family characteristic 16S rDNA signature nucleotides as described by STACKEBRANDT *et al.*[11] Nearest phylogenetic neighbour of strain Tü 6071 was determined as *Streptomyces thermocarboxydus* DSM 44293, showing a sequence similarity value of 98.1%.

Fermentation and Isolation

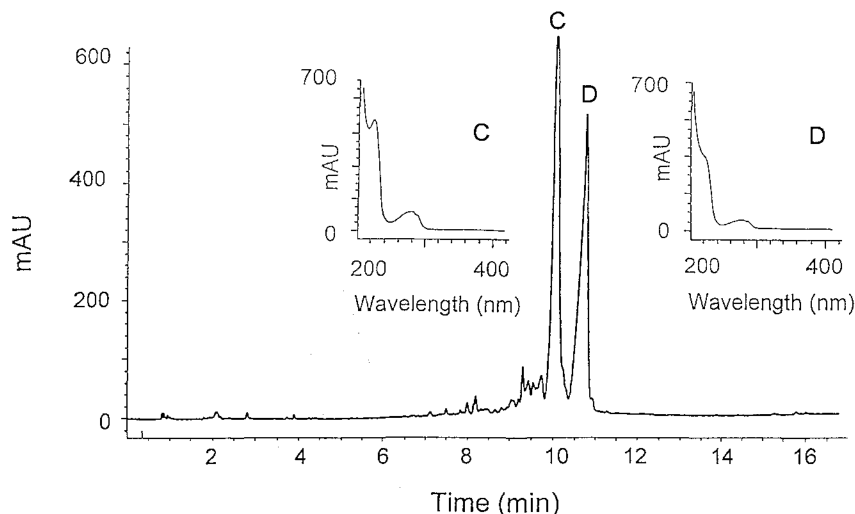
Batch fermentations of *Streptomyces* sp. Tü 6071 were carried out in a 20-liter fermenter equipped with a intensor system, using a complex medium that consisted of mannitol 2% and soybean meal 2% (pH 7.5). Maximal production of the compounds was observed after an incubation time of 120 hours. Streptocidins were isolated from the mycelium

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. Tü 6071.



Bar represents 4 μm

Fig. 3. HPLC analysis of a diol-silica column fraction of streptocidin C (9.9 minutes) and D (10.5 minutes), monitored at 210 nm.



Insert: UV-visible spectra of streptocidins C and D.

Table 1. Antimicrobial spectrum of streptocidins (mixture of B, C and D) compared to tyrocidine (mixture of A, B and C), gramicidin S and gramicidin D, determined by the agar plate diffusion assay at a concentration of 1 mg/ml (inhibition zones in mm).

Organisms	Streptocidin B,C,D	Tyrocidine A,B,C	Gramicidin S	Gramicidin D
Arthrobacter aurescens DSM 20166	10	n.d.	n.d.	n.d.
Bacillus subtilis DSM 10	9	8	12	-
Staphylococcus aureus DSM 20231	6	9	10	-
Streptomyces viridochromogenes Tü 57	8.5	7	10	7

by extraction with MeOH - acetone (1 : 1). The extract was concentrated and re-extracted with ethyl acetate (pH 5) and subjected to a diol-modified silica gel column. The HPLC analysis of a streptocidin C and D containing fraction, which were the main compounds produced by *Streptomyces* sp. Tü 6071, is shown in Fig. 3. Further purification was done by Sephadex LH-20 chromatography. Finally, pure streptocidins were obtained after preparative reversed-phase HPLC using LiChrospher RP-Select B material and 0.5% formic acid - MeOH gradient elution, resulting in four fractions containing single streptocidins as white powders after lyophilisation.

Biological Properties

The antimicrobial spectrum of a streptocidin mixture (B, C and D) was determined by an agar plate diffusion assay and compared with the activities of the structurally related cyclic decapeptide antibiotics tyrocidines and gramicidins (Table 1). The minimal inhibition concentration of the main compounds, streptocidins C and D was determined by a broth dilution method (Table 2). Streptocidins showed antibiotic activities against Gram-positive bacteria in the same order as tyrocidine and gramicidin S. Gram-negative bacteria, such as *Escherichia coli* K12, *Proteus mirabilis*

Table 2. Minimal inhibition concentrations (MIC) of streptocidins C and D in comparison to tyrocidine and gramicidin S as determined by the broth dilution method.

Organism	MIC (μg/ml)			
	Streptocidin C	Streptocidin D	Tyrocidine A,B,C	Gramicidin S
Bacillus subtilis DSM 10	3	3	1	1
Staphylococcus aureus DSM 20231	3	10	3	1
Mycobacterium phlei DSM 750	>100	>100	>100	30
Streptomyces viridochromogenes Tü 57	1	3	1	3
Streptomyces sp. Tü 6071	3	10	30	3

ATCC 35501 and *Pseudomonas fluorescens* DSM 50090 were not inhibited. Eucaryotic test organisms, such as *Saccharomyces cerevisiae* ATCC 9080, *Botrytis cinerea* Tü 157 and the green algae *Chlorella fusca* were not sensitive to streptocidins, tyrocidines and gramicidins.

Discussion

The search for cyclic decapeptide antibiotics in commercial databases result only in a few hits, *e.g.* gramicidins, tyrocidines and loloatins.[12] These peptides were usually produced by bacilli and are often used as model compounds for various investigations, *e.g.* gramicidin S is the most intensive studied example for a non-ribosomally synthesised peptide antibiotic.[13] All these cyclic decapetide antibiotics show an antibiotic activity against Gram-positive bacteria. Gramicidin S is used in the topical treatment of infections in the case of pharyngitis.[14] In this report we describe the first time the production of four close ancestors of bacilli-formed decapetide antibiotics by a streptomycete strain which were named streptocidins A~D. The compounds showed an antibiotic activity against Gram-positive bacteria too. In comparison to tyrocidine and gramicidin S their inhibitory activity was a little bit weaker. This may result from the slightly different amino acid composition of the molecules. Those differences in the decapeptide structure have a significant impact on the antimicrobial activity.[15,16] Several data indicate tyrocidine as a regulatory element in the sporulation of bacilli.[17,18] It will be of interest if streptocidins play also a similar role in the differentiation process of streptomycetes.

The structure of the streptocidins showed the same amino acid backbone as gramicidins and tyrocidines (Fig. 2). This fact raises a lot of interesting questions about the enzymes being involved in the biosynthesis, *e.g.* is the multienzyme complex similar to tyrocidine and gramicidin S synthetase found in *Bacillus brevis*.[13] Last but not least it can be speculated about the possibility of a horizontal gene transfer between the two bacterial genera and the presence of identical gene clusters in bacilli and streptomycetes.

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References

1) FIEDLER, H.-P.; P. KRASTEL, J. MÜLLER, K. GEBHARDT & A. ZEECK: Enterobactin: the characteristic catecholate siderophore of Enterobacteriaceae is produced by *Streptomyces* species. FEMS Microbiol. Lett. 196: 147~151, 2001
2) EL-NAKEEB, M. A. & H. A. LECHEVALIER: Selective isolation of aerobic actinomycetes. Appl. Microbiol. 11: 75~77, 1963
3) FIEDLER, H.-P.: Biosynthetic capacities of actinomycetes. 1. Screening for secondary metabolites by HPLC and

UV-visible absorbance spectral libraries. Nat. Prod. Lett. 2: 119~128, 1993

4) HOTCHKISS, R. D. & R. J. DUBOS: The isolation of bactericidal substances from cultures of *Bacillus brevis*. J. Biol. Chem. 141: 155~162, 1941
5) HÖLTZEL, A.; R. W. JACK, G. J. NICHOLSON, G. JUNG, K. GEBHARDT, H.-P. FIEDLER & R. D. SÜSSMUTH: Streptocidins A~D, novel cyclic decapeptide antibiotics produced by *Streptomyces* sp. Tü 6071. II. Structure elucidation. J. Antibiotics 54: 434~440, 2001
6) KLEINKAUF, H. & H. VON DÖHREN: Biochemistry of Peptide Antibiotics. De Gruyter, Berlin, 1990
7) GOODFELLOW, M. & A. G. O'DONNEL: Chemical Methods in Prokaryotic Systematics. John Wiley & Sons, Chichester, 1994
8) ALTENBUCHNER, J. & J. CULLUM: DNA amplification and an unstable arginine gene in *Streptomyces lividans* 66. Mol. Gen. Genet. 195: 134~138, 1984
9) RAINEY, F. A.; N. WARD-RAINEY, R. M. KROPPENSTEDT & E. STACKEBRANDT: The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. Int. J. Syst. Bacteriol. 46: 1088~1092, 1996
10) LANE, D. J.: 16S/23S rRNA sequencing. *In* Nucleic Acid Techniques in Bacterial Systematics. *Eds*., E. STACKEBRANDT & M. GOODFELLOW, pp. 125~175, Wiley, Chichester, 1991
11) STACKEBRANDT, E.; F. A. RAINEY & N. L. WARD-RAINEY: Proposal for a new hierarchic classification system, actinobacteria classis nov. Int. J. Syst. Bacteriol. 47: 479~491, 1997
12) Dictionary of Natural Products on CD-ROM, Version 8.2. Chapman & Hall, Hampden Data Services, London, 2000
13) VATER, J.: Gramicidin S synthetase. *In* Biochemistry of Peptide Antibiotics. *Eds*., H. KLEINKAUF & H. VON DÖHREN, pp. 33~55, De Gruyter, Berlin, 1990
14) JACK, R. W. & G. JUNG: Natural peptides with antimicrobal activity. Chimia 52: 48~55, 1998
15) GERARD, J. M.; P. HADEN, M. T. KELLY & R. J. ANDERSEN: Loloatins A~D, cyclic decapeptide antibiotics produced in culture by a tropical marine bacterium. J. Nat. Prod. 62: 80~85, 1999
16) WAKI, M. & N. IZUMIYZ: Chemical synthesis and bioactivity of gramicidin S and related peptides. *In* Biochemistry of Peptide Antibiotics. *Eds*., H. KLEINKAUF & H. VON DÖHREN, pp. 205~244, De Gruyter, Berlin, 1990
17) BOHG, A. & H. RISTOW: Tyrocidine-induced modulation of DNA conformation in *Bacillus brevis*. Eur. J. Biochem. 170: 253~258, 1987
18) DANDERS, W.; M. A. MARAHIEL, M. KRAUSE, N. KOSUI, T. KATO, N. IZUMIYA & H. KLEINKAUF: Antibacterial action of gramicidin S and tyrocidines in relation to active transport, *in vitro* transcription, and spore outgrowth. Antimicrob. Agents Chemother. 22: 785~790, 1982