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Schizotrin A; a Novel Antimicrobial Cyclic Peptide from a Cyanobacterium

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Abstract. A novel cyclic undecapeptide, Schizotrin A, possessing moderately strong antimicrobial activity is the major active metabolite in the cultured cyanobacterium *Schizotrix* sp. (TAU strain IL-89-2). The gross structure of Schizotrin A (1) was determined mainly by homonuclear- and inverse-heteronuclear-2D-NMR techniques and HRFABMS.

Cyanobacteria have been recognized in the last decade as a rich source for biologically active cyclic peptides. Many of these cyclic peptides contain long chain β-amino-acid residues. The microcystins[1] are by far the most common cyclic peptides associated with strains of freshwater blooms of cyanobacteria. Majusculamide C[2], scytonemin A[3], puwainaphycins[4], calophycin[5] and laxaphycins[6] are other examples of such peptides that have been found in this group of prokaryotic microorganisms. We report here the isolation and structure elucidation of a novel cyclic peptide, Schizotrin A (1), from a terrestrial *Schizotrix* sp.[7] (TAU strain IL-89-2) which possesses moderately strong antibacterial and antifungal activities.

The *Schizotrix* sp. (TAU strain IL-89-2) was isolated from a sample collected at a greenhouse in Kfar Azar, Israel. A pure clonal strain was grown in mass culture in the laboratory[8]. The freeze-dried cells were extracted with 70% ethanol in water and the resulting extract was subjected to repeated reverse-phase (C-18) chromatography, guided by antimicrobial activity-assay. In a typical isolation the freeze-dried cells (30 g from 100 L of culture) were extracted with a 7:3 EtOH/water solution. The crude extract was fractionated on a C_{18} column. Aliquots of each fraction were assayed for antimicrobial activity (*B. subtilis*). The active fractions were combined and applied to a preparative C_{18} HPLC column using 7:3 MeOH/water as the eluant. The final purification by HPLC on YMC RP-18 column with 3:1 MeOH/water afforded 29 mg of 1. Schizotrin A, 1, was isolated as an amorphous powder (0.1% of dry cells weight), $[\alpha]_D^{20}$ -64.7 ° (c 0.034, MeOH), λ_{max} (ε) 224 (19,750) nm and HRFABMS (thioglycerol/glycerol matrix): *m/z* 1490.779297 [(M+H)$^+$, calcd for $C_{72}H_{108}N_{13}O_{21}$ *m/z* 1490.778274, Δ -1.0 mDa]. The ^{1}H and ^{13}C NMR spectra of 1 in conventional NMR solvents[9] gave broad signals resulting from the equilibria of different conformers of the peptidic ring. The ^{1}H NMR signals of the valine methyls, for instance, appeared as two broad signals ($\nu_{1/2h}$ ~ 40 Hz) in these solvents. Only when 1 was dissolved in deuteromethanol/ water solutions and heated above 310°K could the later signals be recognized as two methyl doublets. Unfortunately, the combination of high temperature and presaturation of the water signal resulted in the disappearance of all of the amide proton signals, which are essential for determining the amino-acid sequence by NMR techniques. When the water in these mixtures was substituted with

a water/trifluoroacetic acid (TFA) mixture at pH 3 a sharp proton and carbon spectra was achieved at room temperature. For the structure elucidation of **1** 1:1- and 2:1-methanol-d3/water (with TFA) solutions were used[10].

Assignments of the signals for the thirteen acid residues that compose Schizotrin A[11] are based on the analysis of ^{1}H, ^{13}C, DEPT, COSY, TOCSY, HMQC and HMBC spectra. The thirteen residues that were assigned are: N-methyl alanyl (NMeAla), butyryl, didehydroaminobutyryl (Dhb), glutaminyl (Gln), glycyl (Gly), homotyrosinyl methylether (Htm), phenylalaninyl (Phe), two prolyl (Pro) residues, two serinyl (Ser) residues and a novel long chain β-amino acid residue; 3-amino-2,7,8-trihydroxy-10-methyl-5-oxyundecanoyl (Aound). Unusual chemical shifts of some proton signals were noticed. The H-5 signal for Aound (5.03 ppm) appeared at a much lower field than any of the other signals for protons on hydroxyl-bearing carbons of this residue and thus are assumed to be esterified. The signal for *proR* H-5 of Pro(2) is 0.3 ppm upfield shifted relative to the signals for the other H-5 protons of the two proline residues and the signals of the valine methyls are roughly 0.2 ppm higher field than normal (0.76 and 0.66 ppm). The upfield shift of the latter three signals appeared to be attributed to an anisotropic effect such as diamagnetic shielding by the ring current of a neighboring aromatic moiety. This was confirmed by NOE experiments.

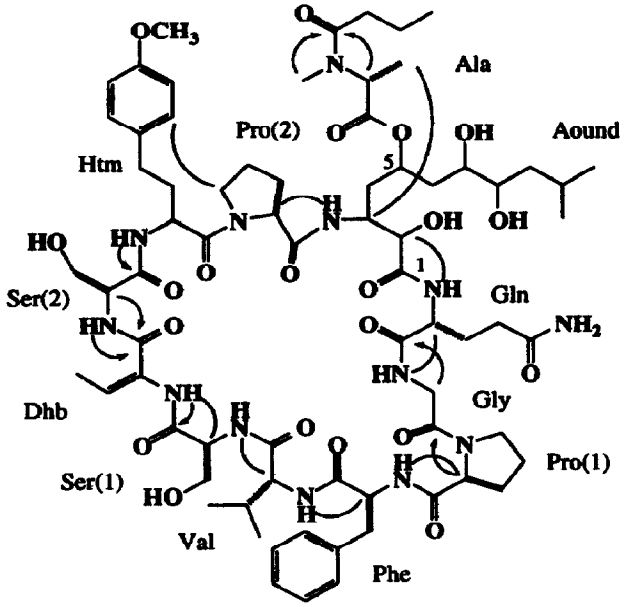

Figure 1. Selected HMBC (→) and NOESY (——) correlations of schizotrin A (1).

HMBC correlations (see Figure 1) allowed the construction of three residues: (i) N-butyryl-N-methyl Ala, (ii) Pro(1)-Gly and (iii) Hmt-Ser(2)-Dhb-Ser(1). The signals for the Gln and Phe carbonyls overlapped, thus the correlations between the Gly α protons and Gln-CO in the HMBC map could not be assigned unequivocally. The cross peaks in the NOESY spectrum, however, were of great help in the assignment of the amino acid sequence (See Figure 1). The Htm-Ser(2)-Dhb-Ser(1) fragment could be extended through the NOESY correlations of: (i) Htm H-6/6' with Pro(2) *pro-R* H-5 and *pro-S* H-5; (ii) Pro(2) H-2 and Aound NH; (iii) Aound H-2 and Gln NH, and (iv) Gln H-2 and Gly NH, to the Gly-Gln-Aound-Pro(2)-Htm-Ser(2)-Dhb-Ser(1) fragment. The attachment of the Gly to Pro(1) was confirmed by the NOE connectivities between Pro(1) *pro-R* H-5 and *pro-S* H-5 and the Gly α protons. Correlations between Pro(1) H-2 and Phe NH, Phe H-2 and Val NH, and Val H-2 and Ser(1) NH, close the peptidic ring. The N-butyryl-N-methyl-Ala fragment was assigned as

the esteric group attached to C-5 of the Aound chain on the basis of NOESY correlations between Aound H-3, and the Ala methyl and H-2.

The absolute configurations of the common amino acids were analyzed by Marfey's HPLC method[12]. The amino acids were determined as: L-Proline (2), L-Serine (2), L-Phenylalanine, D-Valine, N-methyl-D-Alanine and D-Glutamine, by comparison with authentic D and L standards of each amino acid. Studies are in progress to assign the stereochemistry of the chiral centers in the Htm and Aound residues.

Compound **1** was not active against *Staphyloccocus aureus*, *S. albus*, and *Escherichia coli* at 67.1 nM/6 mm disc but gave a 15 mm zone of inhibition against *Bacillus subtilis* at 6.7 nM/6 mm disc. Compound **1** gave 7, 7, 9 and 8 mm zones of inhibition against *Saccharomyces cerevisiae*, *Candida albicans*, *C. tropicalis* and *Rhodotorula rubra* at 16.7, 13.4, 13.4 and 13.4 nM /6 mm disc, respectively, but not against *C. paropsillosis* at 67.1 nM/6 mm disc. Compound **1** also gave 28%, 25%, 37% and 47% reduction in radial growth of the colonies of the fungi *Sclerotium rolfsii*, *Rhizoctonia solani*, *Fusarium oxysporum* and *Colletotrichum gloeosporioides* at 13.4, 13.4, 33.5 and 13.4 nM/6 mm disc, respectively.

Interestingly, the structure of schizotrin A (**1**) resembles three other cyanobacterial cyclopeptides: scytonemin A[3], puwainaphycin C[4], and calophycin[5]. The latter deca- and undecacyclic peptides, although having a different amino acid profile, share a common segment, i.e. a proline residue attached to the amino group of a 2-hydroxy-3-amino-long chain acid residue. This segment may be responsible for the biological activity of these compounds.

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9. The NMR solvents that were examined were: chloroform-d, methanol-d4, acetone-d_6, pyridine-d_5, acetonitrile-d_3, benzene-d_6 and DMSO-d_6.

10. In these solvent mixtures **1** appears to have one major species and three minor ones (at a ratio of 20:2:2:1) as is evidenced from the upfield region of the spectrum, and ca. a 3:1 rotamers mixture of the N-butyryl-N-methyl Ala residue. The NH signals of Ser(1), Dhb and Phe appear at one tenth of the intensity due to their exchage with the presaturated water. All of the NH signals are seen when **1** is dissolved in a 1:1 methanol-*d3*/water solution at pH 3. At pH 1.8, **1** decomposes slowly.
11. COSY, TOCSY, NOESY, DEPT, HMQC, and HMBC spectra were thaken on a Bruker ARX 500 instrument. ^{1}H NMR (500.13 MHz, 2:1 methanol-d3/(H_2O + TFA pH 3)) δ (multiplicity, J in Hz, H assignment): Ser(1) 4.39 (m, H-2), 3.94 (dd -11.4, 5.2, H-3), 3.88 (dd -11.4, 4.0, H-3'), 8.48 (d 7.8, NH); Dhb 5.89 (q 7.4, H-3), 1.89 (d 7.4, H_3-4), 9.43 (brs, NH); Ser(2) 4.52 (m, H-2), 3.98 (dd -11.8, 5.6, H-3), 3.88 (dd -11.8, 3.7, H-3'), 8.05 (d 7.6, NH); Htm 4.48 (m, H-2), 1.98 (m, H-3), 1.93 (m, H-3'), 2.64 (ddd 13.1, 7.2, 5.3, H-4), 2.51 (dt 13.1, 8.1, H-4'), 7.14 (d 8.7, H-6/6'), 6.85 (d 8.7, H-7/7'), 8.15 (d 7.6, NH), 3.74 (s, 8-OCH_3); Pro(2) 4.29 (dd 8.4, 3.7, H-2), 2.00 (m, H-3), 2.08 (m, H-3'), 1.88 (m, H-4), 1.92 (m, H-4'), 3.25 (dt 10.1, 7.4, proR-H-5), 3.57 (m, proS-H-5); Aound 4.14 (d 2.4, H-2), 4.21 (m, H-3), 1.86 (m, H-4), 1.96 (m, H-4'), 5.09 (ddt 10.2, 8.4, 3.1, H-5), 1.74 (m, H-6), 1.54 (m, H-6'), 3.43 (ddd 11.5, 3.1, 1.5, H-7), 3.52 (m, H-8), 1.25 (ddd 14.0,9.5, 3.0, H-9), 1.30 (ddd 14.0, 9.9, 4.7, H-9'), 1.68 (m, H-10), 0.90 (d 6.7, H_3-11), 0.86 (d 6.6, H_3-12), 7.84 (d 9.2, NH); N-MeAla 4.84(mj)* and 4.78(mi)* (q 7.3, H-2), 1.40(mj) and 1.47(mi) (d 7.3, H_3-3), 2.99(mj), and 2.76(mi) (s, N-CH_3); Butyl 2.35(mj) and 2.36(mi) (t 7.5, H_2-2), 1.59 (tq 7.5, 7.3, H_2-3), 0.93 (t 7.3, H_3-4); Gln 4.37 (m, H-2), 2.10 (m, H-3), 2.00 (m, H-3'), 2.31 (m, H_2-4), 8.20 (d 7.1, NH), 7.69 (d 2.5, NH_2), 6.82 (d 2.5, NH_2); Gly 4.07 (dd -17.1, 4.6, H-2), 4.00 (dd -17.1, 5.4, H-2'), 8.18 (dd 4.6, 5.4, NH), Pro(1) 4.34 (dd 8.6, 4.1, H-2), 2.10 (m, H-3), 2.09 (m, H-3'), 1.91 (m, proS-H-4), 1.70 (m, proR-H-4), 3.57 (m, proS-H-5), 3.53 (m, proR-H-5); Phe 4.60 (m, H-2), 3.14 (dd -13.6, 7.5, H-3), 3.05 (dd -13.6, 8.5, H-3'), 7.24 (d 7.2, H-5/5'), 7.29 (t 7.2, H-6/6'), 7.21 (t 7.2, H-7), 8.28 (d 8.1, NH); Val 4.14 (dd 8.2, 6.2, H-2), 2.12 (m, H-3), 0.74 (d 6.7, H_3-4), 0.63 (d 6.8, H_3-5), 7.87 (d 8.2, NH). The five expected hydroxyl protons were not observed. * mj = major and mi = minor conformers of the alanyl C(2)-N bond. ^{13}C NMR (125.77 MHz, 2:1 methanol-d3/(H_2O + TFA pH 3)) δ (multiplicity, C assignment): Ser(1) 71.78 (d, C-1), 57.88 (d, C-2), 62.53 (t, C-3); Dhb 167.19 (s, C-1), 128.67 (s, C-2), 129.74 (d, C-3), 13.68 (q, C-4); Ser(2) 172.21 (s, C-1), 57.17 (d, C-2), 62.66 (t, C-3); Htm 172.21 (s, C-1), 51.91 (d, C-2), 33.67 (t, C-3), 31.24 (t, C-4), 134.12 (s, C-5), 130.56 (d, C-6/6'), 114.79 (d, C-7/7'), 158.77 (s, C-8), 55.93 (q, C-9); Pro(2) 174.03 (s, C-1), 61.62 (d, C-2), 30.25 (t, C-3), 25.17 (t, C-4), 47.81 (t, C-5); Aound 174.03 (s, C-1), 73.53 (d, C-2), 49.83 (d, C-3), 37.11 (t, C-4), 70.73 (d, C-5), 38.05 (t, C-6), 72.36 (d, C-7), 73.70 (d, C-8), 42.08 (t, C-9), 25.55 (t, C-10), 23.98 (q, C-11), 21.86 (q, C-12); N-MeAla 173.30 (s, C-1), 54.93 (d, C-2, 14.38 (q, C-3), 33.53 (q, C-4); Butyl 176.33 (s, C-1), 35.97 (t, C-2), 19.29 (t, C-3), 13.95 (q, C-4); Gln 173.96 (s, C-1), 54.19 (d, C-2), 28.78 (t, C-3), 32.49 (t, C-4), 178.58 (s, C-5); Gly 170.29 (s, C-1), 42.96 (t, C-2); Pro(1) 174.65 (s, C-1), 61.71 (d, C-2), 30.31 (t, C-3), 25.29 (t, C-4), 48.30 (t, C-5); Phe 173.96 (s, C-1), 56.65 (d, C-2), 37.24 (t, C-3), 137.59 (s, C-4), 129.93 (d, C-5/5'), 129.57 (d, C-6/6'), 127.89 (d, C-7); Val 174.65 (s, C-1), 60.47 (d, C-2), 30.36 (d, C-3), 18.27 (q, C-4), 19.25 (q, C-5).
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