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Characterization of a Phytotoxic Cyclotetrapeptide, a Novel Chlamydocin Analogue, from *Verticillium coccosporum*

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Abstract: A phytotoxic cyclotetrapeptide containing an unusual amino acid, 2-amino-8-oxo-9-hydroxydecanoic acid, was isolated from the fungus *Verticillium coccosporum*. Structurally, the peptide is closely related to the known peptide chlamydocin.

Chlamydocin (1), the first cyclotetrapeptide containing the unusual amino acid 2-amino-8-oxo-9,10-epoxydecanoic acid (aoe), was initially isolated as a cytostatic compound from the soil fungus *Diheterospora chlamydosporia*.[1] Since then, four other groups of cyclotetrapeptides containing aoe have been isolated from different sources with biological activity including host-specific phytotoxicity such as HC-toxin.[2] Based on structural analogy, it was inferred and experimentally demonstrated that 1 shows phytotoxic activity albeit not as host-specific as HC-toxin.[3] Based on structure-activity data on 1 and its congeners, it appears that the presence of the keto-epoxy unit in the molecule is a prerequisite for the biological activity which is a result of alkylation of nucleophilic substrates by these compounds. In this communication we report the isolation of a novel phytotoxic chlamydocin analogue 2 from the fungus *Verticillium coccosporum*,[4] which has 2-amino-8-oxo-9-hydroxydecanoic acid (aoh) as one of the residues. 2 is cyclo(aib-S-phe-R-pro-S-aoh).

The methylene chloride extract from the culture filtrate of *V. coccosporum* showed phytotoxic activity in *Lemna minor* assay.[5] Bioassay-guided separation resulted in the isolation of an active component[6] that was identified as 2 based on the following data. The high resolution FAB mass spectrum of 2 showed a protonated molecular ion at m/z 529.3028 that was in agreement with the elemental composition $C_{28}H_{41}N_4O_6$. IR spectrum showed absorptions at 3301 (NH), 1710 (CO), 1676, and 1664 (peptide CO) cm^{-1}. Acid hydrolysis (6N HCl, 110°C, 20 h) followed by chiral TLC (Machery Nagel, Chiralplate) and HPLC (Sumichiral OA5000) analysis showed the presence of S-phe and R-pro, respectively. The presence of aib was also confirmed by TLC analysis of the hydrolyzate.

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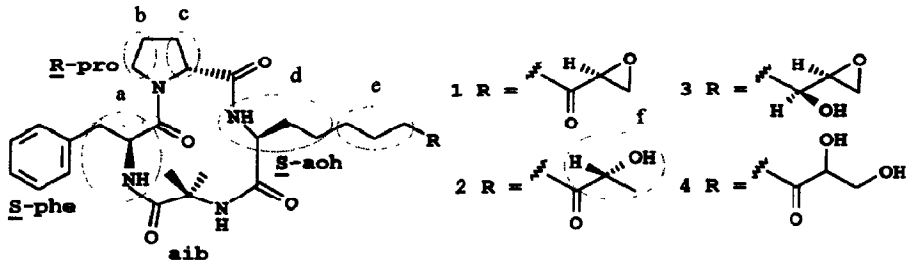

The structure elucidation was based mostly on NMR data.[7] The salient features of the ^1H NMR spectrum were the presence of one methyl doublet at 1.04 ppm (aoh), two methyl singlets at 1.22 and 1.73 ppm (aib), the α-protons at 4.29 (aoh), 4.45 (pro), and 5.36 (phe) ppm, and amide protons at 6.17 (aib), 7.46 (phe), and 7.83 (aoh) ppm. Multiplicities were confirmed by a HOM2DJ experiment. COSY analysis confirmed the presence of the spin systems a-f as shown for **2**. A LRCOSY experiment revealed the long range connectivities as shown in Figure 1, thus confirming the presence of aoh, phe, and pro units in the molecule.

The ^{13}C NMR analysis including DEPT showed the presence of 3 methyls, 9 methylenes, 9 methines, and 7 quaternary carbons (including 5 carbonyls) in the molecule. All the ^1J proton-carbon connectivities were established by a HETCOR experiment and a LRHETCOR experiment showed long range connectivities (Figure 1), confirming the assigned structure. The solution conformation of the molecule was studied by a NOESY experiment in C_6D_6 and the observed connectivities are shown in Figure 2. The absence of spatial proximity between the adjacent α-protons (accompanied by connectivities between the NH protons or substituent protons and the α-proton of the previous amino acid) indicated the presence of *trans* peptide bonds in the molecule. The observed connectivities also confirmed the relative positions of the amino acid residues. This is in accordance with the reported structure of **1**.

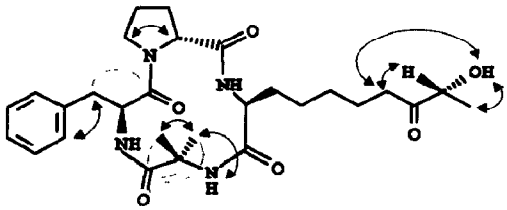

Fig. 1 LRCOSY (—) and LRHETCOR (- -) connectivities for **2**

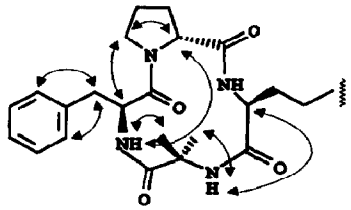

Fig. 2 NOESY connectivities for **2**

The absolute configuration at the chiral carbon (C-9) of the aoh unit bearing the secondary hydroxyl functionality was established by chiral derivatization with R- and S-O-methylmandelic acids.[8] The relevant ^1H NMR data from the two esters are shown in Figure 3. In particular, the upfield shift for the C-9 methyl (0.09 ppm) in the S-ester and the shift for the C-5 methylene (0.09 ppm) in the R-ester established the chirality as R at C-9 of aoh. The spectral assignments for both

the esters were confirmed by COSY analysis. The absolute configuration at the α-carbon of the aoh unit was not determined directly but was derived to be S based on the presence of all *trans* peptide bonds in the molecule and structural analogy with 1. That 2 is a natural product and not an artifact of the isolation procedure was confirmed by the absence of related compounds in the extract (as determined by the LC-MS analysis of the partially purified fractions) and by the use of ethyl acetate for extraction.

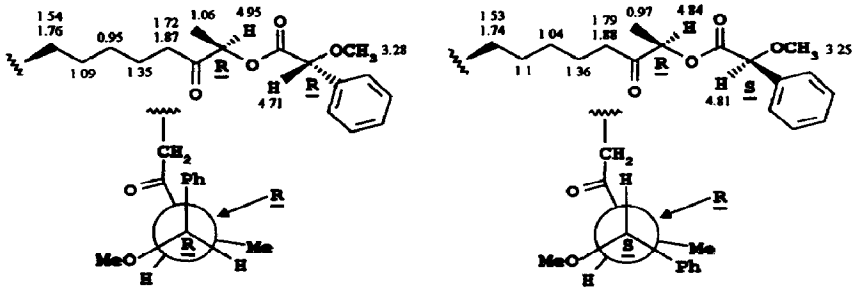

Fig. 3 ^{1}H NMR data on the R- and S-O-methylmandelates of 2 in C_6D_6 and extended Newman projections

2 exhibited activity in *L. minor* assay at 0.3 μM and at 1.7 μM in the *Brassica juncea* assay. In a preliminary greenhouse experiment, 2 showed activity against velvet leaf (*Abutilon theophrastii*) (70% control) and *B. juncea* (90% control) at 2 kg/hectare when applied postemergence. 1 was reported to show phytotoxic activity against maize. Interestingly, related compounds such as dihydrochlamydocin (3) and diol 4 were reported to be inactive when tested for cytostatic properties.[9] The proposed mechanism for the activity for 1 and related compounds is that they efficiently alkylate nucleophilic substrates (proteins) as has also been shown in the case of some highly active synthetic analogues containing halogens or alkylating substituents α to the C-8 ketone (lysine analogues) in the aoe unit.[10] 2 shows high phytotoxicity in spite of the presence of a hydroxyl α to the ketone functionality.

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(4) The fungal isolate was obtained from USDA-ARS collection of entomopathogenic fungi at Ithaca, New York. The fungus was originally isolated from an egg mass of *Lymantria dispar* from Oregon in 1985.

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(6) The crude extract was subjected to flash silica gel chromatography (CH_2Cl_2-CH_3OH 98:2). The active fraction was subjected to HPLC (ODS, H_2O-CH_3OH gradient) and final purification was achieved by HPLC on silica gel (CH_2Cl_2-CH_3OH 98:2).

(7) **2**, δ_H (300 MHz, C_6D_6) 1.04 (3H, d, J = 7 Hz, C<u>H</u>$_3$ aoh), 1.37 (2H, m, C<u>H</u>$_2$CH$_2$CO aoh), 1.73 (3H, s, C<u>H</u>$_3$ aib), 0.97 and 2.1 (1H each, each m, C<u>H</u>$_2$CH pro), 1.12 and 1.94 (1H each, each m, CH$_2$C<u>H</u>$_2$CH$_2$ pro), 1.15 (2H, m, CHCH$_2$C<u>H</u>$_2$ aoh), 1.22 (3H, s, C<u>H</u>$_3$ aib), 1.03 (2H, m, CHCH$_2$CH$_2$C<u>H</u>$_2$ aoh), 1.56 and 1.77 (1H each, each m, CHC<u>H</u>$_2$ aoh), 2.91 and 3.37 (1H each, m, dd, J = 10.1, 13.1 Hz, CHC<u>H</u>$_2$ phe), 1.79 and 1.88 (1H each, each m, C<u>H</u>$_2$CO aoh), 2.84 and 4.02 (1H each, each m, NC<u>H</u>$_2$ pro), 5.36 (1H, m, C<u>H</u> phe), 4.29 (1H, m, C<u>H</u>NH aoh), 4.45 (1H, m, C<u>H</u> pro), 3.86 (1H, m, C<u>H</u>OH aoh), 6.98 (1H, m, ar C<u>H</u> p- phe), 7.08 (2H, m, ar 2 x C<u>H</u> m- phe), 7.19 (2H, m, ar 2 x C<u>H</u> o- phe), 3.7 (1H, m, O<u>H</u> aoh), 6.17 (1H, s, N<u>H</u>, aib), 7.46 (1H, d, J = 10.1Hz, N<u>H</u> phe), 7.83 (1H, d, J = 10.4 Hz, N<u>H</u> aoh). δ_C (75 MHz, C_6D_6) 19.84 (<u>C</u>H$_3$ aoh), 23.38 (<u>C</u>H$_2$CH$_2$CO aoh), 23.69 (<u>C</u>H$_3$ aib), 24.68 (<u>C</u>H$_2$CH pro), 25.06 (CH$_2$<u>C</u>H$_2$CH$_2$ pro), 25.63 (CHCH$_2$<u>C</u>H$_2$ aoh), 26.71 (<u>C</u>H$_3$ aib), 28.94 (CHCH$_2$CH$_2$<u>C</u>H$_2$ aoh), 29.25 (CH<u>C</u>H$_2$ aoh), 36.31 (CH<u>C</u>H$_2$ phe), 36.96 (<u>C</u>H$_2$CO aoh), 46.95 (N<u>C</u>H$_2$ pro), 53.95 (<u>C</u>H phe), 54.59 (<u>C</u>HNH aoh), 58.01 (<u>C</u>H pro), 58.91 (q <u>C</u> aib), 72.65 (<u>C</u>HOH aoh), 126.89 (ar <u>C</u>H p phe), 128.79 (ar 2 x <u>C</u>H m phe), 129.56 (ar 2 x <u>C</u>H o phe), 137.87 (ar q <u>C</u> phe), 172.16, 172.96, 174.64, and 175.7 (all peptide <u>C</u>O), 211.94 (<u>C</u>OCHOH aoh). Complete NMR assignments were also achieved in $CDCl_3$ that supported the assigned structure.

(8) The esters were prepared by the 1-hydroxybenzotriazole-DCC method and purified by HPLC (ODS, H_2O-CH_3OH gradient). The NMR spectra were recorded in C_6D_6. B. M. Trost, J. L. Belletire, S. Godleski, P. G. McDougal, J. M. Balkovec, J. J. Baldwin, M. E. Christy, G. S. Ponticello, S. L. Varga, J. P. Springer, *J. Org. Chem.* **1986**, *51*, 2370.

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