Anthranicine, an Unusual Cyclic Hexapeptide from *Acremonium* sp. A29-2004

Johannes C. Liermann[a], Eckhard Thines[b], Heidrun Anke[b], and Till Opatz[a]

[a] Universität Hamburg, Institut für Organische Chemie, Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany
[b] Institut für Biotechnologie und Wirkstoff-Forschung (IBWF), Erwin-Schrödinger-Str. 56, D-67663 Kaiserslautern, Germany

Reprint requests to Prof. Dr. Till Opatz. Fax: +49-40-42838-3834.
E-mail: opatz@chemie.uni-hamburg.de

Z. Naturforsch. **2009**, *64b,* 727 – 730; received March 12, 2009

Dedicated to Professor Gerhard Maas on the occasion of his 60th birthday

Anthranicine, a cyclic hexapeptide containing anthranilic acid and pipecolic acid, was isolated from a mycophilic *Acremonium* species. The structure and stereochemistry of the compound featuring two D-configurated amino acids are discussed.

Key words: Cyclopeptide, Structure Elucidation, NMR, Anthranilic Acid, D-Amino Acids

Introduction

During our ongoing search for novel secondary metabolites from fungi, the mycophilic strain *Acremonium* sp. A29-2004 was identified as a prolific producer of antifungal compounds. Among the metabolites identified were ilicicolins [1, 2] C [3], D [4], F [5], H [6], and the structurally related 4′,5′-dihydro-4′-hydroxyascochlorin [7]. During the isolation of these metabolites, HPLC fractions were obtained which contained yet another compound which differed in structure from the mentioned terpenoids, as judged by LC-MS analysis. Therefore, compound **1** was purified and its structure elucidated by a combination of spectroscopic techniques.

Results and Discussion

Compound **1** was isolated from the culture broth of a 20 L fermentor as described in the Experimental Section. HRMS experiments indicated a molecular formula of $C_{35}H_{54}N_6O_6$ while the UV spectrum suggested the presence of an aromatic system. Strong bands in the IR spectrum at 3436, 1686, and 1644 cm^{-1} gave evidence for the presence of amide bonds, and it was concluded that the compound might be of peptidic nature. The rather lipophilic compound **1** was well soluble in $CDCl_3$, and no conformational

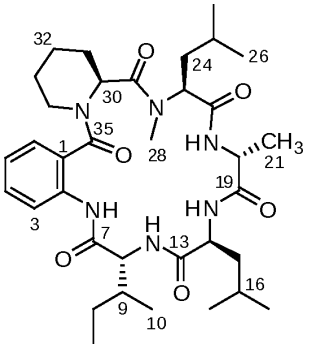

Fig. 1. Structure of Anthranicine (**1**).

transitions were observed in the NMR spectra. Six amide carbonyls were found in the ^{13}C NMR spectra, along with five C_α carbons. ^1H NMR spectra showed the signals of a 1,2-substituted benzene system along with four sharp amide N–H resonances with varying multiplicities. One *N*-methyl group was also found, confirming the presence of a pseudo-hexapeptide with one cyclic and one unusual amino acid.

2D NMR spectroscopy allowed to identify an anthranilic acid moiety as part of the cyclic backbone the sequence of which could be assigned by HMBC correlations and by the scalar coupling of H_α with the respective amide protons. The side chains were assigned by TOCSY and COSY experiments to find two

0932–0776 / 09 / 0600–0727 $ 06.00

leucines, one isoleucine, one alanine, and pipecolic acid, with one of the leucines being *N*-methylated (Fig. 1).

The absolute configuration of all five α-amino acids was established by hydrolysis, reaction with Marfey's reagent, and HPLC comparison with authentic standards. Remarkably, isoleucine and alanine were found to have D-configuration.

Cyclopeptides containing anthranilic acid are infrequently found in nature. The isolation and structures of some cyclic dipeptides and didepsipeptides with modified tryptophan from *Aspergillus* sp. have been reported [8 – 13], along with few examples for cyclic pentapeptides and hexapeptides [14 – 17]. A compound of identical constitution to anthranicine (**1**) was mentionend as a hypolipidemic metabolite of a *Hamigera* species in a patent filed by Meiji Sekai Kaisha Ltd., although no information on the stereochemistry has been given [15]. No antibacterial, antifungal, cytotoxic, phytotoxic or nematicidal activities were detected for **1** up to concentrations of 50 μg mL^{-1}.

Experimental Section

General methods

Melting points were determined with a Dr. Tottoli apparatus and are uncorrected. Optical rotations were measured with a Krüss P8000 polarimeter at 589 nm. UV and IR spectra were measured with a Perkin-Elmer Lambda-16 spectrophotometer and a Bruker IFS48 FTIR spectrometer, respectively. NMR spectra were recorded on a Bruker DRX-500 instrument. The spectra were measured in $CDCl_3$, and the chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.26 ppm, δ_C = 77.16 ppm) [18]. APCIMS spectra were measured with a Hewlett Packard MSD1100. ESI-HRMS spectra were recorded on a Finnigan MAT95 spectrometer using PEG as internal reference.

Microorganisms

The mycophilic strain A29-2004 was isolated from fruiting bodies of *Pulvinula constellatio* collected near Annot, France [19]. It was grown and kept on YMG-medium consisting of glucose (1 %), malt extract (1 %), and yeast extract (0.4 %) in tap water at 22 °C. For solid media, 1.5 % agar was added. The morphological features of the strain were in accordance with those described for members of the genus *Acremonium* with simple philalides and ovoid conidia in chains or slimy heads [20]. Several species of this genus are known to inhabit fruiting bodies of asco- or basidiomycetes [21].

Fermentation

Fermentations in 500 mL Erlenmeyer flasks containing 200 mL YMG medium were inoculated with four pieces (1 cm × 1 cm) cut from agar slants. The flasks were incubated on a rotary shaker at 120 rpm and 22 °C. Fermentations on a larger scale were carried out in a Biostat (Braun, Melsungen, Germany) containing 20 L of malt medium (2 % malt extract) with stirring (120 rpm) and aeration (3 L air per minute) at 21 °C. To prevent foaming, silicone antifoam (Merck, Darmstadt) was added. The fermentor was inoculated with 200 mL of a well grown culture in YMG medium. Samples were withdrawn daily and assayed for pH, glucose and maltose content. When the carbon sources were used up, the fermentation was terminated. The culture broth was separated by filtration. Mycelia contained neither active compounds nor compound **1** and were discarded.

*Isolation of compound **1***

The broth was passed through a column of HP21 resin in water (Mitsubishi Chemical Industry LTD, Düsseldorf). The eluate was discarded. After washing with water (2 L), compound **1** was eluted with MeOH (2 L), followed by acetone (2 L). After concentration *in vacuo* and passage over Chromabond (Macherey and Nagel, C18ec, 15 mL/2000 mg) in acetonitrile-water, 830 mg of crude extract was obtained. Final purification was achieved by preparative HPLC with a Jasco modular HPLC system (Groß-Umstadt, Germany) consisting of two binary pumps (PU-1586) and the multiwavelength detector UV-1570M. The HPLC was fitted with a LiChroSpher 100, RP 18 column (Merck, 250 × 25 mm^2, particle size 5 μm). Elution with an H_2O-acetonitrile gradient (10 min equilibration 60 % MeCN, linear gradient to 95 % MeCN within 12 min, followed by 13 min 95 % MeCN) resulted in 2.2 mg of compound **1** (r. t. 14.1 min) from 70 mg of crude product. The total yield from 15 L fermentation broth was 25 mg of compound **1**.

Amino acid analysis

About 100 μg of **1** was hydrolyzed with 50 μL 6M HCl in a sealed glass capillary for 2 h at 120 °C. After evaporation to dryness, the sample was reacted with Marfey's reagent as described elsewhere [22]. The sample was injected onto a RP column (Zorbax Eclipse XDB-Phenyl, 3.5 μm, 3 × 150 mm^2, Agilent) with a mixture of 65 % water-containing 0.1 vol-% formic acid and 35 % acetonitrile at 0.6 mL min^{-1} as mobile phase, using detection by UV at 340 nm and LCMS. Amino acids were identified by their molecular weight and by comparison with standards (standards: L-alanine R_t = 2.90 min, D-alanine R_t = 3.41 min, L-pipecolic acid R_t = 5.65 min, D-pipecolic acid R_t = 4.97 min, L-leucine R_t = 7.08 min, D-leucine R_t = 10.17 min, L-isoleucine R_t = 6.86 min, D-isoleucine R_t =

9.91 min, NMe-L-leucine R_t = 8.55 min, NMe-D-leucine R_t = 9.91 min, anthranilic acid R_t = 5.08 min; found: D-alanine R_t = 3.47 min, anthranilic acid R_t = 5.04 min, L-pipecolic acid R_t = 5.60 min, L-leucine R_t = 7.02 min, NMe-L-leucine R_t = 8.50 min, D-isoleucine R_t = 9.76 min). Derivatives of secondary amines gave UV spectra different from those of primary amines and could be distinguished in case of identical retention times and molecular weights.

*Anthranicine (**1**)*

Brownish solid, m. p. = 186 °C. – UV (MeOH): λ_{max} ($\log\varepsilon$) = 248 (sh, 4.00), 290 (3.36) nm. – $[\alpha]_D^{25}$ = +27.9 (c = 0.31, $CDCl_3$). – IR (KBr): ν = 3436, 3347, 2960, 1686, 1644, 1520, 1293, 761 cm^{-1}. – ^{1}H NMR, COSY, TOCSY, NOESY (500 MHz, $CDCl_3$): δ = 9.44 (s, 1H, NH-7), 8.32 (d, J = 8.3 Hz, H-3), 8.00 (d, J = 7.9 Hz, 1H, NH-19), 7.63 (d, J = 9.7 Hz, 1H, NH-22), 7.46 (dd, J = 8.3, 7.9 Hz, 1H, H-4), 7.39 (d, J = 7.4 Hz, 1H, NH-13), 7.19 (d, J = 6.7 Hz, 1H, H-6), 7.12 (dd, J = 7.9, 6.7 Hz, 1H, H-5), 4.81 (m, 1H, H-20), 4.57 – 4.52 (m, 1H, H-14), 4.44 (dd, J = 7.4, 3.1 Hz, 1H, H-8), 4.13 (dd, J = 12.9, 2.8 Hz, 1H, H_a-34), 3.69 (dd, J = 11.0, 2.7 Hz, 1H, H-30), 3.48 (dd, J = 9.3, 4.3 Hz, 1H, H-23), 3.19 (s, 3H, H_3-28), 3.19 – 3.13 (m, 1H, H_b-34), 2.42 (ddd, J = 14.4, 7.2, 3.0 Hz, 1H, H-9), 2.20 (ddd, J = 14.1, 9.1, 4.7 Hz, 1H, H_a-24), 2.11 – 1.97 (m, 3H, H_a-15, H_2-31), 1.93 (ddd, J = 14.1, 8.9, 5.1 Hz, 1H, H_b-24), 1.82 – 1.73 (m, 2H, H_b-15, H-16), 1.69 – 1.60 (m, 1H, H-25), 1.59 – 1.53 (m, 1H, H_a-33), 1.39 (quin, J = 7.2 Hz, 2H, H_2-11), 1.31 – 1.24 (m, 3H, H_b-33, H_2-32), 1.28 (d, J = 7.3 Hz, 3H, H_3-21), 0.99 – 0.86 (m, 15H, H_3-10, H_3-12, H_3-18, H_3-26, H_3-27), 0.88 (d, J = 5.9 Hz, 3H, H_3-17) ppm. – ^{13}C NMR, HSQC, HMBC (126 MHz, $CDCl_3$): δ = 174.4 (C-19), 174.2 (C-13), 171.2 (C-7), 170.3 (C-35), 169.4 (C-22), 169.0 (C-29), 137.3 (C-2), 131.9 (C-4), 127.2 (C-6), 124.0 (C-3), 123.4 (C-5), 122.8 (C-1), 65.3 (C-23), 61.7 (C-30), 57.4 (C-8), 52.7 (C-34), 51.0 (C-14), 48.0 (C-20), 38.0 (C-28), 37.9 (C-24), 36.6 (C-9), 36.4 (C-15), 28.2 (C-31), 27.6 (C-32), 27.0 (C-11), 25.7 (C-25), 24.64 (C-33), 24.62 (C-16), 23.5 (C-27), 23.2 (C-18), 22.2 (C-26), 21.9 (C-17), 18.6 (C-21), 14.1 (C-10), 11.9 (C-12) ppm. – MS ((+)-APCI): *m/z* (%) = 655.3 (100) $[M+H]^+$. – HRMS ((+)-ESI): *m/z* = 677.4002 (calcd. 677.4003 for $C_{35}H_{54}N_6O_6Na$, $[M+Na]^+$).

Acknowledgements

We thank Dr. V. Sinnwell (University of Hamburg, Germany) for measuring the NMR data, Dr. S. Franke (University of Hamburg, Germany) for mass spectrometric analyses, as well as H. Kolshorn (University of Mainz, Germany) for helpful discussions. The expert technical assistance of N. Heil and A. Meffert is gratefully acknowledged. This work was financially supported by BASF SE and the Competence Center for Integrated Natural Products Research Rheinland-Pfalz.

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