New Diketopiperazine Alkaloids from *Penicillium fellutanum*

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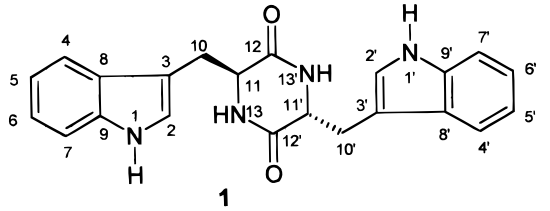
Four new diketopiperazine alkaloids (**1-4**) were isolated from cultures of *Penicillium fellutanum*, and their structures were determined by MS and NMR measurements.

Penicillium species produce numerous alkaloids, such as the roquefortines, rugulosuvines, and glandicolines.[1−3] Among them are diketopiperazine-type metabolites biosynthesized by condensation of two amino acids, such as tryptophane, proline, histidine, and phenylalanine. Interest in diketopiperazines is due to their activity in various pharmacological assay systems.[4] In this paper we report the structure of four new diketopiperazines named fellutanines A−D (**1**−**4**). Structure elucidation utilized physicochemical methods, including optical spectroscopy, MS, and NMR spectroscopy.

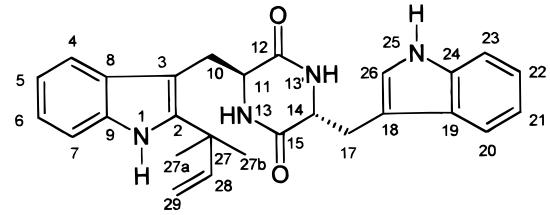
Penicillium fellutanum VKM-3020 was cultivated under submerged conditions in Erlenmeyer shake flasks for 10−12 days, as described earlier.[3] For the isolation of metabolites **1**−**4**, the culture liquid was extracted at pH 8.0 with $CHCl_3$, and the compounds were obtained by several subsequent chromatographic steps. The UV spectra of **1-4** displayed absorbances typical of indole and diketopiperazine chromophores,[2,5] while the spectrum of **4** showed bands typical of a dihydroindole.

The molecular formula of **1** ($C_{22}H_{20}N_4O_2$) was determined from the HREIMS ([M^+] *m/z* 372.1570; calcd 372.1585). A fragment ion with *m/z* 130 was attributed to α-cleavage of a 3-methylene indole.[4] Similarly, the formulas of **2** ($C_{27}H_{28}N_4O_2$; [M^+] *m/z* 440.2219; calcd 440.2202), **3** ($C_{32}H_{36}N_4O_2$; [M^+] *m/z* 508.2823; calcd 508.2808), and **4** ($C_{32}H_{36}N_4O_4$; [M^+] *m/z* 540.2728; calcd 540.2718) were determined by HREIMS. The fragment ion at *m/z* 198 in the EIMS of **2** and **3** showed that these compounds differed from **1** by having one additional dimethylallyl group or more linked with the indole moiety. A diagnostic feature in the MS of **4** was the loss of C_5H_9 from the M^+ ion to yield *m/z* 471 as the base peak. Diagnostic fragment ions such as *m/z* 270 and *m/z* 201 were ascribed to further fragmentation of *m/z* 471. Supporting evidence for the molecular weight of **1**−**4** was furnished by ESIMS, which showed the $[M + H]^+$ and $[M + Na]^+$ pseudomolecular ions.

The structures of **1**−**4** as shown were determined conclusively by 1D and 2D ^1H and ^{13}C NMR experiments (COSY, NOESY, TOCSY, DEPT, HSQC, HMBC). The ^{13}C NMR spectra of **1** and **3** displayed only 11 and 16 signals, respectively, as they contain two identical subunits. An olefinic methylene group was visible in the DEPT spectra of **2**, **3**, and **4**. Characteristic features of the $^1H-^1H$ COSY spectra of **1**−**4** were couplings of methylene protons



1



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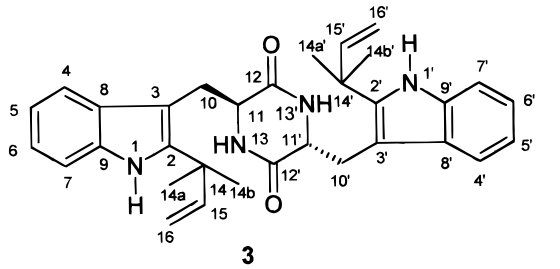

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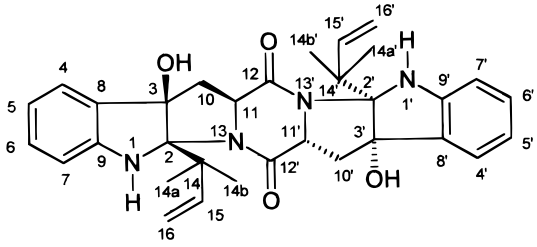

4

(H-10, H-10′, and H-17, respectively) with the protons of the diketopiperazine ring (H-11, H-11′). The quaternary carbon signal at δ 90.9 (C-2) in the ^{13}C NMR spectrum of **4** attested to the presence of an unusual linearly annelated structure in this symmetrical molecule. In addition, a signal at δ 89.0 was assigned to C-3 of **4** on the basis of C−H long-range measurements (HMBC).

The sequence of the carbon and hydrogen atoms was readily assigned by 2D NMR experiments. The C−H long-range couplings (HMBC) of the methyl and olefinic protons with the quaternary carbon atom C-27 in **2** and C-14 in **3** and **4**, with the heteroaromatic C-2 (C-2′) in **2** and **3** and

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the quaternary carbon C-2 (C-2′) in **4** indicated the point of attachment of the isopentenyl side chains to the heteroaromatic nucleus. Observable couplings of the indole N−H and aromatic protons supplied further supporting evidence for the structures of **1**−**4** as shown.

NOESY correlations were observed between H-4 (H-4′) and the hydrogen atoms H-11 (H-11′), H_A-10 (H_A-10′), and H_B-10 (H_B-10′), as a characteristic of **1** attributable to a special molecule conformation. The hydroxyl and isopentenyl groups in **4** are located on the same side of the molecule, as there were strong observable NOE correlations between H_B-10 (H_B-10′) and H-14b (H-14b′) and, respectively, H-14a (H-14a′) and H-14b (H-14b′) with 3-OH (δ 2.20). The position of H_B-10 relative to H-11 was determined by $^3J_{HB-10,H-11} = 13.0$ Hz.

Optical rotations of **1**−**4** all displayed negative values, suggesting that the stereochemistry of chiral carbon atoms of the diketopiperazine structures (C-11; C-11′) is the same as in L-tryptophane.[7] In addition, **1**−**4** displayed a strong negative Cotton effect in the 220−230 nm region due to the amide chromophore of the diketopiperazine ring. As in the related roquefortin, the comparably higher optical rotation of compound **4** ($[\alpha]_D = -383°$) is attributable to the presence of additional stereocenters (C-2, C-3, C-2′, C-3′).[7] Compound **4** was cytotoxic [IC_{50} (μg/mL): 11.6 (L-929 cells), 9.5 (K-562 cells), and 19.7 (HeLa cells)]. Compounds **1-3** were relatively inactive.

Experimental Section

General Experimental Procedures. EIMS were taken with a double-focusing mass spectrometer AMD 402 (AMD Intectra, Harpstedt, Germany) and ESIMS with a Quattro triple quadrupole instrument (VG Biotech, Altrincham, England). IR spectra were recorded on a Shimadzu IR-470 spectrophotometer and UV−vis spectra on a Beckman DU 60 scanning spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX 500 spectrometer using TMS as an internal standard. CD spectra were recorded in MeOH with a JASCO 460 instrument. Optical rotations were measured with a Propolis instrument (Dr. Kernchen Optical Works, Seelze, Germany).

Organism and Culture Conditions. *P. fellutanum* VKM F-3020 was obtained from the All-Russian Culture Collection (VKM) Pushchino, near Moscow, Russia. Spores from the 4-day malt-agar slopes were used for inoculation. They were cultivated under submerged conditions (24 ± 1 °C, a rotary shaker, 220−240 rpm) in 750-mL Erlenmeyer flasks containing 150 mL of medium of the following composition (g/L): mannitol (50), succinic acid (5.4), $MgSO_4 \cdot 7H_2O$ (0.3), and KH_2PO_4 (1), in distilled water adjusted to pH 5.4 using concentrated NH_4OH.

Extraction and Isolation. After 12 days of growth, the culture broth was filtered. The filtrate (5.5 L) was extracted using the same volume of $CHCl_3$ at ca. pH 8.0 (basified with ammonia). The $CHCl_3$ extract was dried over Na_2SO_4 and evaporated in vacuo. The residue (160 mg) was fractionated by column chromatography (2.8 × 40 cm) on Si gel L5/40 (Chemapol, Czech Republic) with $CHCl_3$/MeOH/concentrated NH_4OH (90:10:1) as eluent (system I). Four fractions of UV absorbing metabolites (254 nm) were collected that stained blue with Ehrlich's reagent. Two metabolites [fellutanines A (**1**) and B (**2**)] were isolated and purified by TLC (system I) and recrystallization: **1** (R_f 0.18, system I), 30 mg and **2** (R_f 0.36, system I), 11 mg. The other two metabolites were separated on a Si gel plate run in C_6H_6/Me_2CO (3:1) (system II): fellutanine C (**3**; R_f 0.21), 4.5 mg, and fellutanine D (**4**; R_f 0.46), 6 mg.

Fellutanine A: colorless microcrystalline solid (CH_3CN), mp 270−272 °C; $[\alpha]^{20}_D$ −139° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 217 (4.61), 273.6 (3.96), 280.4 (3.99), 289.4 (3.92) nm; Cotton effect (0.11 mg/mL MeOH) $\Delta\epsilon$ (217 nm) −0.98; R_f (TLC, Si gel) 0.18, eluent: $CHCl_3$/MeOH/concentrated NH_4OH (90:10:1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.2 (1H, br, 1-NH), 7.45 (1H, d, J = 7.0 Hz, H-7), 7.28 (1H, d, J = 7.0 Hz, H-4), 7.12 (1H, dd, J = 7.0 Hz, 7.2 Hz, H-5), 7.05 (1H, dd, J = 7.0 Hz, 7.2 Hz, H-6), 6.42 (1H, d, J = 2.5 Hz, H-2), 4.05 (1H, dd, J = 3.6 Hz, 7.8 Hz, H-11), 3.05 (1H, dd, J = 3.6 Hz, 14.4 Hz, H_A-10), 2.25 (1H, dd, J = 7.8 Hz, 14.4 Hz, H_B-10);^{13}C NMR ($CDCl_3$, 125 MHz) δ 167.6 (s, C-12), 135.0 (s, C-9), 127.0 (s, C-8), 124.6 (d, C-2), 121.8 (d, C-5), 119.0 (d, C-6), 118.1 (d, C-7), 111.0 (d, C-4), 108.2 (s, C-3), 53.0 (d, C-11), 30.2 (t, C-10); chemical shift and coupling data of the right half of the molecule are identical; EIMS *m*/*z* 372.2 [M^+] (45), 130.1 (100); HREIMS *m*/*z* 372.1570 (calcd for $C_{22}H_{20}N_4O_2$, 372.1585).

Fellutanine B: colorless microcrystalline solid (MeOH); mp 281−283 °C; $[\alpha]^{20}_D$ −91° (*c* 0.14, DMSO); UV (MeOH) λ_{max} (log ϵ) 227 (4.69), 273.6 (shoulder; 4.01), 282.2 (4.05), 289.6 (3.99) nm; Cotton effect (0.13 mg/mL MeOH) $\Delta\epsilon$ (227 nm) −9.3; R_f (TLC, Si gel) 0.36, eluent $CHCl_3$/MeOH/concentrated NH_4OH (90:10:1); ^{1}H NMR ($CDCl_3$; 500 MHz) δ 8.21 (1H, br, 16-NH), 7.95 (1H, d, J = 7.1 Hz, H-7), 7.41 (1H, m, H-6), 7.40 (1H, m, H-5), 7.61 (1H, d, J = 7.0 Hz, H-4), 7.55 (1H, d, J = 7.0 Hz, H-23), 7.28 (1H, dd, J = 7.0 Hz, 7.0 Hz, H-22), 7.15 (1H, dd, J = 7.0 Hz, 7.0 Hz, H-21), 6.95 (1H, d, J = 7.0 Hz, H-20), 6.93 (1H, s, H-26), 6.25 (dd, J = 7.0 Hz, 18.0 Hz, H-28), 5.25 (2H, ddd, J = 0.5 Hz, 7.0 Hz, 18 Hz), 4.21 (1H, dd, J = 10.0 Hz, 2.0 Hz, H-11), 4.03 (1H, dd, J = 7.0 Hz, 2.1 Hz, H-14), 3.05 (1H, dd, J = 14.0 Hz, 10.1 Hz, H_B-10), 3.07 (1H, dd, J = 15 Hz, 2.1 Hz, H_B-17), 2.11 (1H, dd, J = 14.0 Hz, 2.0 Hz, H_A-10), 2.05 (1H, dd, J = 7.0 Hz, 15 Hz, H_A-17), 1.41 (3H, s, H-27a), 1.42 (3H, s, H-27b); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.8 (s, C-12), 166.8 (s, C-15), 145.4 (s, C-2), 139.0 (s, C-8), 134.1 (s, C-24), 129.9 (s, C-17a), 127.7 (d, C-26), 127.0 (s, C-9), 120.6 (d, C-5), 118.1 (d, C-6), 117.1 (d, C-7), 110.4 (d, C-4), 107.2 (s, C-3), 107.3 (s, C-18), 126.0, d (C-21), 119.8 (d, C-22), 117.8 (d, C-23), 109.4 (d, C-20), 109.8 (t, C-29), 105.0 (d, C-28), 55.7 (d, C-11), 55.1 (d, C-14), 38.0 (s, C-27), 29.2 (t, C-10), 26.4 (q, C-27a), 26.4 (q, C-27b); EIMS *m*/*z* 440.2 [M^+] (90), 198.2 (100), 183.2 (40), 168.1. (15), 130.1 (35); HREIMS *m*/*z* 440.2219 (calcd for $C_{27}H_{28}N_4O_2$, 440.2202).

Fellutanine C: colorless microcrystalline solid ($CHCl_3$/hexane); mp 178−180 °C, $[\alpha]^{20}_D$ −79 °C (*c* 0.1, MeOH), UV (MeOH) λ_{max} nm (log ϵ) 224 (4.69), 275.0 (shoulder; 4.09), 283.0 (4.08), 290.6 (4.04); Cotton effect (0.105 mg/mL MeOH) $\Delta\epsilon$ (224 nm) −18.2; R_f (TLC, Si gel) 0.21, eluent benzene/acetone (3:1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.05 (1H, s, 1-NH), 7.50 (1H, d, J = 7.3 Hz, H-7), 7.38 (1H, d, J = 7.75 Hz, H-4), 7.15 (1H, dd, J = 8.0 Hz, 7.5 Hz, H-5), 7.1 (1H, d, J = 8.0 Hz, 7.3 Hz, H-6), 6.18 (1H, dd, J = 17.0 Hz, 11.1 Hz, H-15), 5.72 (1H, s, 13-NH), 5.21 (1H, dd, J = 11 Hz, 0.5 Hz, H-16b), 5.20 (1H, dd, J = 17.0, 0.5 Hz, H-16a), 3.80 (1H, dd, J = 14.5 Hz, 3.5 Hz, H_B-10), 3.25 (1H, dd, J = 12.0 Hz, 14.5 Hz, H_A-10), 1.62 (3H, s, H-14a), 1.60 (3H, s, H-14b); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 167.2 (s, C-12), 145.7 (d, C-15), 141.7 (s, C-2), 135.5 (s, C-8), 128.8 (s, C-9), 121.2 (d, C-6), 118.1 (d, C-7), 112.6 (t, C-16), 112.2 (d, C-5), 110.8 (d, C-4), 104.5 (s, C-3), 54.8 (d, C-11), 39.1 (s, C-14), 28.1 (q, C-14a), 29.9 (q, C-14b); chemical shift and coupling data of the left half of the molecule are identical with the above values; EIMS *m*/*z* 508.5 [M^+] (25), 198.2 (100), 183.1 (30). 168.1 (15), 130.1 (10); HREIMS *m*/*z* 508.2823 (calcd $C_{32}H_{36}N_4O_2$ for 508.2808).

Fellutanine D: colorless microcrystalline solid ($CHCl_3$/hexane); mp 198−201 °C, $[\alpha]^{20}_D$ −383° (*c* 0.1, MeOH); UV (MeOH) λ_{max} nm (log ϵ) 214 (4.10), 244 (3.2), 297.0 (3.53); Cotton effect (0.16 mg/mL MeOH) $\Delta\epsilon$ (214 nm) −9.7; $\Delta\epsilon$ (244 nm) −10.2; R_f (TLC, Si gel) 0.46, eluent benzene/acetone (3:1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.20 (1H, d, J = 7.0 Hz, H-7), 7.12 (1H, dd, J = 7.0 Hz, 7.0 Hz, H-5), 6.75 (1H, dd, J = 7.0 Hz, 7.0 Hz, H-6), 6.65 (1H, d, J = 7.0 Hz, H-4), 6.40 (1H, dd, J = 17.0 Hz, 10.0 Hz, H-15), 6.1 (1H, s, 1-NH), 5.10 (1H, dd, J = 17.0 Hz, 0.5 Hz, H_A-16), 5.15 (1H, dd, J = 10.0 Hz, 0.5 Hz, H_B-16), 3.51 (1H, dd, J = 13.0 Hz, 7.1 Hz, H-11), 2.75 (1H, dd, J = 13.0 Hz, 13.5 Hz, H_B-10), 2.62 (1H, dd, J = 13.5 Hz, 7.1 Hz, H_A-10), 1.40 (3H, s, C-14a), 1.35 (3H, s, C-14b); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.4 (s, C-12), 148.8 (s, C-8), 144.2 (s, C-15), 130.9 (d, C-5), 123.6 (d, C-7), 129.8 (s, C-9), 120.1 (d, C-6), 113.3 (t, C-16), 111.4 (d, C-4), 91.0 (s, C-2), 89.1 (s, C-3), 61.4 (d, C-11), 45,1 (s, C-14), 35.6 (t, C-10), 27.1 (q, C-14a), 23.1 (q, C-14b); chemical shift and coupling data of the left half of the molecule are identical with the above values; EIMS *m*/*z* 540.4 [M^+] (40), 471.3 (100), 340.3 (15), 270.1 (15), 201.1 (15); HREIMS *m*/*z* 540.2728 [(calcd for $C_{32}H_{36}N_4O_4$, 540.2718).

Cell Cultures and Measurements of Cytotoxicity. Cytotoxicity was determined by use of the adherent mouse fibroblast cell line L-929, the nonadherent human leukemia cell line K-562, and the adherent human cell line HeLa after 72 h of cultivation. The cultivation conditions were the same as was described previously.[6]

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References and Notes

(1) Kozlovsky, A. G. *Proc. Jpn. Mycotoxicol.* **1990**, *32*, 31−34.
(2) Kozlovsky, A. G.; Marfenina, O. G.; Vinokurova, N. G.; Zhelifonova, V. P.; Adanin, V. M. *Mikrobiologiya* (in Russian) **1997**, *66*, 112−116.
(3) Kozlovski, A. G.; Soloveva, T. F. *Prikl. Biokhim Mikrobiol.* (in Russian) **1985**, *21*, 579−586.
(4) Kozlovsky, A. G.; Marfenina, O. G.; Vinokurova, N. G.; Zhelifonova, V. P.; Adanin, V. M. *Mycotoxins* **1998**, *48*, 37−43.
(5) Sangster, A. W.; Stuart, K. L. *Chem. Rev.* **1965**, *65*, 69−130.
(6) Ritzau, M.; Heinze, S.; Fleck, W. F.; Dahse, H. W.; Gräfe, U. *J. Nat. Prod.* **1998**, *61*, 1337−1339.
(7) Laatsch, H. *Antibase*, Database of Microbial Products; Chemical Concepts: Weinheim, Germany, 1998.

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