METABOLIC PRODUCTS OF MICROORGANISMS. 243†

PYRIDAZOMYCIN, A NEW ANTIFUNGAL ANTIBIOTIC PRODUCED BY *STREPTOMYCES VIOLACEONIGER*

RALPH GROTE, YONGLE CHEN and AXEL ZEECK*

Institut für Organische Chemie, Universität Göttingen,
Tammannstr. 2, D-3400 Göttingen, FRG

ZENGXIANG CHEN and HANS ZÄHNER

Institut für Biologie II, Universität Tübingen,
Auf der Morgenstelle 28, D-7400 Tübingen, FRG

PETRA MISCHNICK-LÜBBECKE and WILFRIED A. KÖNIG

Institut für Organische Chemie und Biochemie, Universität Hamburg,
Martin-Luther-King-Platz 6, D-2000 Hamburg 13, FRG

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Pyridazomycin (**1**), a new antifungal antibiotic produced by *Streptomyces violaceoniger* sp. *griseofuscus* (strain Tü 2557), was detected in a selective screening against *Mucor hiemalis* (Tü 179/180). The amino acid side chain of **1** can be seen as L-ornithine, whose γ-nitrogen atom is part of a pyridazine ring building a quaternary ammonium system. The structure of **1** was established by spectroscopic analysis of the parent compound and degradation products. The occurrence of a pyridazine ring in microbial secondary metabolites is unique.

In the course of our screening for antifungal antibiotics especially active against *Mucor hiemalis* (Tü 179/180) we isolated pyridazomycin as a strongly basic compound from the culture filtrate of *Streptomyces violaceoniger* sp. *griseofuscus* (strain Tü 2557). In this report we describe the fermentation, isolation, physico-chemical characterization, structural elucidation and some biological properties of pyridazomycin[2].

Fermentation and Isolation

The producing organism was a new soil isolate collected near Tula, Mexico, and identified as *Streptomyces violaceoniger* sp. *griseofuscus* (strain Tü 2557). Production of pyridazomycin was conducted in 100-ml Erlenmeyer flasks. The cultures were harvested at 72 hours, when pyridazomycin production was maximal. The culture filtrate was adjusted to pH 3.0~3.5 and applied to Dowex 50W-X4. The active fractions were eluted with 4% aqueous ammonia solution and adsorbed on charcoal. After elution with methanol the crude extract was further purified by chromatography on silica gel (butanol - acetic acid - water, 2 : 1 : 1) and Sephadex G-10 (water) followed by cation exchange on an Amberlite IRC-50 column with 0.5 M sodium chloride solution as eluent. Repeated chromatography on Sephadex G-10 yielded pure pyridazomycin.

Characterization and Structure Elucidation

Pyridazomycin is a white, hygroscopic amorphous powder (dec above 119°C), insoluble in

† See ref 1.

Fig. 1. IR spectrum of pyridazomycin (1) in KBr.

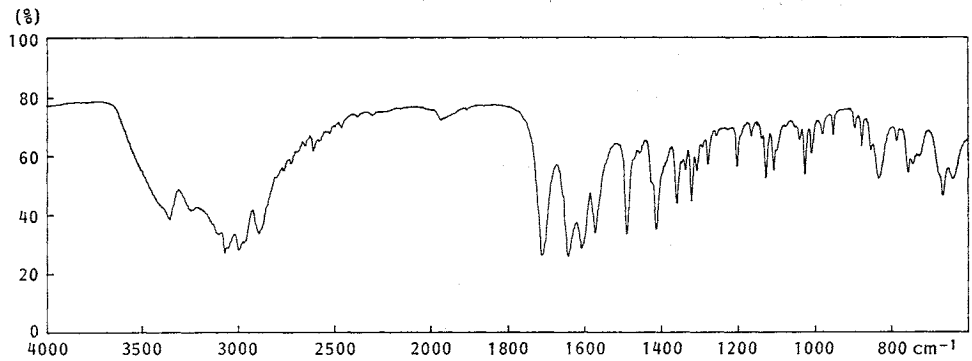


Table 1. ^{1}H and ^{13}C NMR data of pyridazomycin (1) (200 MHz, D_2O; coupling constants see Experimental section).

C-atom	δ (C)	δ (H)	C-atom	δ (C)	δ (H)
C-1	174.0	—	C-3′	153.1	9.77
C-2	54.1	3.66	C-4′	139.6	—
C-3	27.2	1.94	C-5′	134.3	8.84
C-4	25.5	2.20	C-6′	150.7	9.93
C-5	65.1	4.96	C-7′	164.4	—

lipophilic organic solvents, slightly soluble in methanol and very well soluble in water or diluted acids. It is unstable at pH >7.5. On TLC-plates pyridazomycin gives brown spots with ninhydrin and green spots in the Barrolier test. The chirality of pyridazomycin is demonstrated by the specific rotation and a CD spectrum.

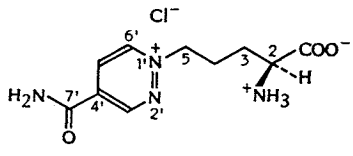
Since the electron impact mass spectrum (EI-MS) and the fast atom bombardment mass spectrum (FAB-MS) did not show a definite molecular ion, the molecular formula $C_{10}H_{17}N_4O_4Cl$ of the chloride as a monohydrate was estimated from the elemental analysis in accordance with the ten carbon atoms observed in the ^{13}C NMR spectrum (multiplicity assigned by attached proton test (APT)).

The IR spectrum (Fig. 1) illustrated the presence of NH/OH groups and displayed various C=C, C=N and C=O stretching vibrations. The wavenumbers and shapes of the observed absorptions were found to be related to the concentration of **1** and the duration of the pressing under reduced pressure. Broadened absorption bands were observed at high concentrations and long pressing durations. The UV spectra under neutral and acidic conditions showed an absorption band at 224 nm with a shoulder at 254 nm.

The ^{1}H NMR spectrum (Table 1) displayed resonances for ten protons. The signals at δ 1.94 (β-CH_2), 2.20 (γ-CH_2), 3.66 (α-CH) and 4.96 (δ-CH_2) resemble those of the basic amino acids arginine or ornithine as zwitterions. The strong downfield shifts and the coupling pattern as well as the coupling constants of three protons (δ 8.84, 9.77 and 9.93) suggested a 6-membered aromatic heterocycle with two protons *ortho* to each other and one in the *meta* position.

A $^{1}J_{C,H}$-heteronuclear shift correlation (HETCOR) NMR experiment allowed to assign seven of the ten resonances observed in the ^{13}C NMR spectrum to the corresponding protons (Table 1). The chemical shifts of the carbons C-1 to C-4 were in agreement with the published data for arginine and ornithine[3]. The three quaternary carbon atoms represent an amide carbonyl (δ 164.4), a carboxylate carbonyl (δ 174.0) and an aromatic carbon atom (δ 139.6).

Scheme 1.



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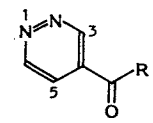
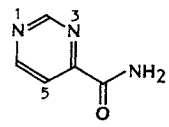


2a R = NH_2
2b R = OCH_3
2c R = OEt
2d R = $NHC(=NC_6H_5)NHC_6H_5$



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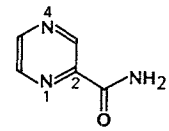

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Table 2. ^{1}H NMR data of 4-pyridazinecarboxamide (**2a**) (200 MHz, DMSO-d_6) and similar 2- or 4-substituted pyridazine, pyrimidine and pyrazine derivatives from literature (δ in ppm relative to internal TMS, J in Hz).

Proton/coupling	**2a**	**2b**[7),a]	**2c**[8),b]	**2d**[9),a]	**3**[5),a]	**4**[6),a]
3-H/2-H	9.54	9.67	9.77	9.54	9.37	9.25
5-H	7.99	7.99	8.10	7.97	8.07	8.89
6-H	9.42	9.44[c]	9.53	9.38	9.1	8.75
$J_{3,5}/J_{2,5}$	2.3			2.0		0.01
$J_{3,6}/J_{2,6}$	1.3			1.3	1.5	1.51
$J_{5,6}$	5.3			5.3	5.5	2.49

[a] In DMSO-d_6; [b] in $CDCl_3$.
[c] It is obvious from the context that the false value given in the original publication (δ 7.44) is due to a printing error.

Hydrolysis of pyridazomycin in methanolic sodium hydroxide at pH 9 resulted in a variety of products. Out of these one extinguished UV light (254 nm) on TLC-plates and was isolated. On the basis of high resolution mass spectroscopy the molecular formula $C_5H_5N_3O$ was assigned. The IR spectrum indicated the presence of an amide (1695 and 1650 cm^{-1}). In the downfield region of the ^{1}H NMR spectrum (200 MHz, DMSO-d_6) three well resolved double doublets were found at δ 7.99, 9.42 and 9.54 besides two broad NH resonances at δ 7.96 and 8.48. Only 6-membered heterocycles containing two nitrogen atoms are consistent with these data. The discrimination between the three possible isomeric diazabenzenes 4-pyridazinecarboxamide[4] (**2a**), 4-pyrimidinecarboxamide[5] (**3**) and 2-pyrazinecarboxamide[6] (**4**) (Scheme 1) was done by comparison of the ^{1}H NMR chemical shifts and coupling constants (Table 2). Although there were no NMR data reported for **2a** it is obvious that those of the pyridazine ring (shown by the shifts and coupling constants of **2b**[7], **2c**[8] and **2d**[9]) fit much better than those of the pyrimidine or pyrazine systems.

The exact linkage position of the partial structures was proved by ^{1}H NMR double resonance

Scheme 2.

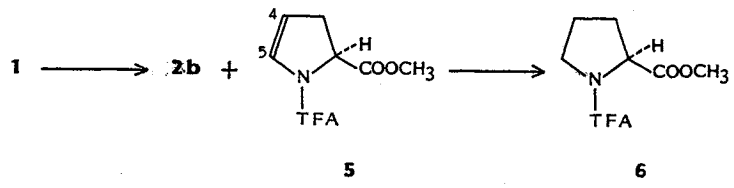

Table 3. Antifungal and antibacterial assays of pyridazomycin (disk diffusion assay).

Test microorganism	Inhibition diameter (mm)				MIC (μg/ml)[a]
	Concentration (mg/ml)				
	1.0	0.3	0.1	0.03	
Mucor hiemalis (Tü 179/180)	40	35	31	26	0.01
Yarrowia lipolytica	36	30	23	16	0.6
Paecilomyces variotii	50	41	32	22	1
Bacillus subtilis	17	11	—	—	13

[a] Estimated.

experiments. Irradiation at 5-H_2 (δ 4.96) showed effects on 4-H_2, 3′-H and 6′-H, thus suggesting a connection of the carbon at position 5 of the amino acid residue to one of the heterocyclic N-atoms. The discrimination between N-1′ and N-2′ was made possible by one-dimensional heteronuclear decoupling (5-H_2/C-6′) and confirmed by a $^{n}J_{C,H}$-heteronuclear shift correlation (COLOC) NMR experiment[10]. All observed long range couplings are in accordance with structure **1**. In the case of the linkage of the amino acid side chain at N-1′ a $^{3}J_{C,H}$-coupling between 5-H_2 and C-6′ would occur, as it was detected. The alternative linkage at N-2′ would give a $^{4}J_{C,H}$-coupling, which would be much smaller and virtually undetectable. A correlation between 6-H′ and C-5 was not observed.

The configuration of the chiral center at C-2 was derived on two independent ways. The CD spectrum of pyridazomycin showed a small positive cotton effect at 210 nm in accordance with comparable values in the same region of L-configurated 2-amino acids[11], thus indicating the same configuration at C-2 of pyridazomycin. After hydrolysis of pyridazomycin in aqueous ammonia followed by esterification and trifluoroacetylation, two main products were detected by GC-MS (Scheme 2). Besides the expected methyl 4-pyridazinecarboxylate (**2b**) *N*-trifluoroacetyl-4,5-dehydro-L-proline methyl ester (**5**) was found, whose structure was confirmed by comparison with the isomeric 3,4-dehydro derivative. The mass spectrum of **5** revealed an abundant fragment at *m/z* 163 (53%) corresponding to the loss of $HCOOCH_3$ from the molecular ion, which is caused by the generation of a relatively stable *N*-trifluoroacetylpyrrol cation, besides the base peak at *m/z* 164 ($M-COOCH_3$, 100%). In contrast the 3,4-dehydro derivative (*m/z* 164, $M-COOCH_3$, 100%) did not give a peak at *m/z* 163, because the elimination is not favorable in this case. The occurrence of **5** can be rationalized by a Hofmann-like elimination process of the cationic heterocycle followed by intramolecular cyclization using the amino group and loss of a hydride ion. Catalytic hydrogenation of the mixture of **2b** and **5** gave *N*-trifluoroacetyl-L-proline methyl ester (**6**). Chiral phase capillary GC showed by comparison with *N*-trifluoroacetyl-DL-proline methyl ester and co-injection of the racemate together with **6** that only the peak corresponding to the L-enantiomer was enhanced.

Biological Activity

Pyridazomycin exhibits a significant biological activity against fungi in the disk diffusion assay (see Table 3). Besides a small inhibitory effect on *Bacillus subtilis* no other bacteria like *Streptomyces*, *Escherichia coli* or *Clostridium pasteurianum* seem to be sensitive. More details on the biological activity will be given in a forthcoming publication.

Discussion

Pyridazomycin (1) represents the first naturally occurring antibiotic with a pyridazine core. Although antibiotics with 3-substituted 2,3,4,5-tetrahydropyridazine or hexahydropyridazine acids are known in depsipeptides, for example antrimycin[12], azinothricin[13] and cirratiomycins[14], there are no antibiotics described which derive formally from 4-substituted pyridazines. Synthetic derivatives with the pyridazine or hydropyridazine core — mostly 3- or 6-oxo-substituted — are biologically active in a variety of applications, ranging from antihypertensive to herbicidic and cytostatic effects. Besides its antifungal activity pyridazomycin exhibits antagonistic behavior against some basic amino acids (arginine, ornithine, lysine and histidine).

Experimental

General

MP's were determined using a Reichert hot stage microscope. UV spectra were recorded using a Kontron Uvikon 860 spectrometer. IR spectra were obtained in pressed KBr disks using a Perkin-Elmer Model 298 spectrometer. The NMR spectra were determined with a Varian VXR-200 or Bruker WM 400, respectively. The mass spectra were obtained on a Varian MAT 731 or a Varian MAT 311 A, respectively, using direct probe insert, exact mass measurements with perfluorokerosine as a standard. The GC-MS coupling was performed using a Hewlett Packard HP 5185 instrument (25 m SE-54 fused silica capillary column, He, chemical ionization with isobutane, EI: 70 eV). For capillary GC a Carlo Erba 2101 (25 m Chirasil-val capillary column, H_2, injector/detector: 270°C, 100°C isothermal, flame ionization detector) was used. CD spectra were recorded using a Jasco J 500 A spectrometer in combination with a BMC if 800 personal computer. Optical rotations were taken with a Perkin-Elmer Model 241 polarimeter. TLC was performed on silica gel plates (Macherey-Nagel Sil G/UV 254+366, 0.25 mm silica gel on glass), low pressure column chromatography on Silica gel 60 (0.063~0.04 mm Macherey-Nagel) with N_2 as carrier gas, desalting on Sephadex G-10 (Pharmacia).

Bacterial Strains

The standard strains for the activity spectrum of Tü 2557 were obtained from the stock culture collection in our laboratories or from ATCC. The antibiotic producing organism (Tü 2557) was a new soil isolate from Tula, Mexico, classified according to HÜTTER[15] and BERGEY'S[16] as *S. violaceoniger* sp. *griseofuscus*.

Biological Assay

The disk diffusion assay was used for measuring the antibiotic content of the cultures and to determine the biological activity (see Table 3). Minimal medium for fungi; KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.02%, NaCl 0.01%, glutamate 0.15%, glucose 0.1%, biotin 0.0003%, agar-agar (Difco) 2%, pH 7.0. Minimal medium for bacteria; glucose 0.8%, ammonium tartrate 0.4%, NaCl 0.5%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCl_2$ 0.02%, $MnSO_4 \cdot 7H_2O$ 0.001%, ferrioxamine 0.002%, agar 2%.

Fermentation and Isolation

S. violaceoniger sp. *griseofuscus* was cultured for 72 hours at 27°C in a medium consisting of mannitol 2.0% and soybean meal 2% (NL 19) (200×100 ml in 500-ml Erlenmeyer flasks with one intrusion). The culture broth was adjusted to pH 3.0~3.5 with formic acid. After addition of 2% Celite the filtrate was stirred with Dowex 50W-X4 and the resin washed three times with deionized water.

The active fractions were eluted with 4% aqueous ammonia solution and stirred successively with 170 and 60 g charcoal. Elution proceeded with 7 liters of 90% aqueous MeOH in three steps. After concentration under reduced pressure and filtration the crude extract (~10 ml) was chromatographed under pressure (2 bar) on a column (50×2 cm) of silica gel with BuOH - AcOH - H_2O (2:1:1) as eluent, followed by column chromatography (20×1 cm) on Sephadex G-10 with water as solvent. The active fractions were applied on an Amberlite IRC-50 column (20×1 cm), washed with deionized water and the antibiotic eluted with 0.5 M NaCl solution. Repeated chromatography on a Sephadex G-10 column (20×1 cm) with water resulted in 15 mg pyridazomycin (**1**; (*S*)-5-(2-azonia-5-carbamoyl-2-pyridyl)-2-aminopentanoic acid chloride).

MP 119°C (dec); $[\alpha]_D^{20}$ +94.6° (*c* 0.93, MeOH - H_2O, 20:1); Rf 0.28 (BuOH - AcOH - H_2O, 4:3:3), 0.15 (BuOH - AcOH - H_2O, 2:1:1), 0.28 (BuOH - pyridine - AcOH - H_2O, 17:12:6:15); IR (KBr, Fig. 1) cm^{-1} 3400~2900 (br), 1710, 1640, 1610, 1570; UV λ_{max} (H_2O and H_2O - HCl) nm (ε) 254 (3,700), 224 (7,900); ^1H NMR (200 MHz, D_2O, Table 1, reference DOH δ 4.67) δ 1.94 (2H, m, 3-H_2), 2.20 (2H, m, 4-H_2), 3.66 (1H, t, *J*=6.0 Hz, 2-H), 4.96 (2H, t, *J*=7.0 Hz, 5-H_2), 8.84 (1H, dd, *J*=6.0 and 2.5 Hz, 5'-H), 9.77 (1H, d, *J*=2.5 Hz, 3'-H), 9.93 (1H, d, *J*=6.0 Hz, 6'-H); ^{13}C NMR (50.3 MHz, D_2O, Table 1, reference acetone-H_3 δ 30.6) δ 25.5 (t, *J*=132 Hz, C-4), 27.2 (t, *J*=122 Hz, C-3), 54.1 (d, *J*=146 Hz, C-2), 65.1 (t, *J*=148 Hz, C-5), 134.3 (d, *J*=182 Hz, C-5'), 139.6 (s, C-4'), 150.7 (d, *J*=198 Hz, C-6'), 153.1 (d, *J*=197 Hz, C-3'), 164.4 (s, C-7'), 174.0 (s, C-1); CD $\lambda_{extreme}$ (H_2O) nm ($[\theta]^{22}$) 210 (+680).

Anal Calcd for $C_{10}H_{15}N_4O_3 \cdot Cl \cdot H_2O$: C 41.03, H 5.85, N 19.14, Cl 12.11.
Found: C 40.92, H 5.80, N 18.47, Cl 13.98.

4-Pyridazinecarboxamide (**2a**)

A MeOH solution (2 ml) of **1** (15 mg) was adjusted to pH 9 with a few drops of 2 M NaOH. After stirring 4 hours at room temperature the mixture was neutralized with 2 M HCl and the solvent removed. Repeated extraction of the residue with CH_2Cl_2 - MeOH (9:1) and column chromatography on silica gel (10×0.5 cm) with CH_2Cl_2 - MeOH (9:1) as eluent yielded 3.5 mg **2a**.

MP 190°C (literature[4]) 191~192°C); Rf 0.35 ($CHCl_3$ - MeOH, 9:1), 0.80 (PrOH - H_2O, 6:4); IR (KBr) cm^{-1} 3360, 3140, 2920, 1695 (s), 1650, 1595; UV λ_{max} (MeOH and MeOH - NaOH) nm (ε) 325 (180), 250 (1,700); (MeOH - HCl) 250 (1,800); ^1H NMR (200 MHz, DMSO-d_6, reference internal TMS) δ 7.96 (1H, br, NH), 7.99 (1H, dd, *J*=5.3 and 2.3 Hz, 5-H), 8.48 (1H, br, NH), 9.42 (1H, dd, *J*=5.3 and 1.3 Hz, 6-H), 9.54 (1H, dd, *J*=2.3 and 1.3 Hz, 3-H); EI-MS (70 eV) *m/z* (abundance) 123 (50%, M^+, high resolution calcd for $C_5H_5N_3O$ and found: 123.0433), 96 (27%), 95 (43%), 44 (100%).

Hydrolysis by Aqueous Ammonia

Approx 1 mg **1** was treated with 500 μl conc ammonia solution (28%) for 10 minutes at 100°C in a sealed tube. After evaporation to dryness the residue was esterified with methanolic hydrogen chloride (1.5 M) for 10 minutes at 100°C. After removal of the solvent 200 μl of CH_2Cl_2 and 50 μl of trifluoroacetic acid anhydride were added and the mixture kept at room temperature for 1 hour. After evaporation to dryness two substances were detected by GC-MS (solvent CH_2Cl_2): Methyl 4-pyridazinecarboxylate (**2b**) and *N*-trifluoroacetyl-4,5-dehydro-L-proline methyl ester (**5**). **2b**: *m/z* (abundance) 138 (100%, M^+), 123 (15%, M−CH_3), 108 (11%), 107 (15%). **5**: *m/z* (abundance) 223 (27%, M^+), 164 (100%, M−$COOCH_3$), 163 (53%, M−$COOCH_3$−H), 126 (17%, M−CF_3CO), 116 (15%), 94 (36%). For comparison: *N*-trifluoroacetyl-3,4-dehydro-L-proline methyl ester: About 1 mg (L)-3,4-dehydroproline was treated under the same conditions as above. EI-MS *m/z* (abundance) 223 (18%, M^+), 165 (8%), 164 (100%, M−$COOCH_3$), 94 (13%).

Hydrogenation of **5**

The mixture obtained from the above reaction was dissolved in CH_2Cl_2 and approx 2 mg Pd/charcoal catalyst was added. For 10 minutes hydrogen was bubbled through the solution, an equivalent of which was then applied onto the chiral GC capillary column and compared with the reaction product obtained from DL-proline under the same conditions.

Acknowledgment

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