Pharacine, a Natural *p*-Cyclophane and Other Indole Derivatives from *Cytophaga* sp. Strain AM13.1[1]

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From the ethyl acetate extract of the bacterial strain *Cytophaga* sp. AM13.1, among many known compounds, the new natural products 2,5-bis(3-indolylmethyl)pyrazine (**2**) and a highly symmetrical *p*-cyclophane named pharacine (**5**) were identified. In addition, tryptamine isovalerate (**1**) and *p*-hydroxyphenylacetamide (**4**), known as plant metabolites, were isolated and characterized from a microorganism for the first time. The new natural products showed no activity against three microalgae, the fungus *Mucor miehei*, the yeast *Candida albicans*, and the bacteria *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Streptomyces viridochromogenes*.

Indole derivatives are common secondary metabolites from microorganisms, plants, and other sources. More than 1000 derivatives have been reported from microorganisms,[2] most of them being substituted at C-3.[2] In the course of a screening of microorganisms from the North Sea, a new indole derivative, 2,5-bis(3-indolylmethyl)pyrazine (**2**), and a natural *p*-cyclophane named pharacine (**5**) were isolated from the extracts of a *Cytophaga* strain AM13.1, and their structures were assigned spectroscopically. Additionally we report tryptamine isovalerate (madugin, **1**)[3] and *p*-hydroxyphenylacetamide (**4**)[4] as secondary metabolites from a microorganism for the first time. The known compounds tryptamine acetate,[5] 2-phenylethylacetamide,[6] 3-indolylcarboxylic acid,[7,8] *cyclo*(tyrosylprolyl),[9] *cyclo*(phenylalanylprolyl),[10] *cyclo*(valylprolyl),[11] *cyclo*(isoleucylvalyl),[12] 2-(3-indolyl)ethanol,[13] 2-phenylethylisovaleramide,[1] 3-indolylacetic acid,[8,14] 2,2-dimethylbenzopyrimid-4-one,[15] *o*-aminobenzamide,[16] *o*-acetylaminobenzamide (**3**),[17] phenylacetamide,[18] 2-(*p*-hydroxyphenyl)ethanol,[19] phenylacetic acid,[20] 3-phenyllactic acid,[21] uracil, and thymidine were also found in the extracts. The dark yellow color of the colonies was due to tryptanthrin (**6**), a pigment known previously only from yeast.[22]

Results and Discussion

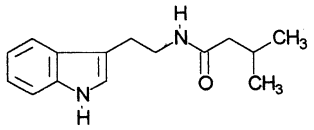
The ^{1}H NMR spectrum of compound **1** from fraction 2 showed five aromatic proton signals between δ 7.0 and 7.6, four of which belonged to a 1,2-disubstituted benzene ring, and the last one, with a chemical shift of δ 7.04, was indicative for H-2 of an indole system. A triplet and a quartet at δ 2.98 and 3.62, respectively (the last one giving a triplet after D_2O exchange), were typical for two methylene groups in vicinal position to each other. In addition, an isobutyl system was present due to signals at δ 2.10 (m, 1 H), 1.98 (d, 2 H), and 0.90 (d, 6 H).

The CI and EI mass spectra of compound **1** led to a molecular weight of 244. A search in AntiBase[2] with the molecular weight and the substructures determined from the ^{1}H NMR data resulted in the identification of compound **1** as tryptamine isovalerate (**1**), a compound already reported from the plant *Clausena indica*, which was confirmed by direct comparison of the NMR data.[3]

The ^{1}H NMR spectrum of a second compound from fraction 2 showed five aromatic proton signals with a coupling pattern identical to that of **1**, tryptamine acetate, and 2-(3-indolyl)ethanol: two doublets and two triplets of an *ortho*-disubstituted benzene ring and a doublet with a relatively small coupling constant, as typical of the pyrrole part of the indole ring. The indole skeleton was further supported by the pink color formation with Ehrlich's reagent and the brown coloration with anisaldehyde/sulfuric acid.

Compound **2** showed an additional signal at deep field (δ 8.4) in the proton NMR spectrum. This suggested an electron-deficient heteroaromatic ring. In addition to the pattern of an indole skeleton, the ^{13}C NMR spectrum contained signals of a methylene group at δ 30.8, and at δ 153.7 and 142.9 two aromatic carbons of which the first must be connected to nitrogen, as the formula contained no oxygen. The NMR data suggested the partial structure 1a (Figure 1).

The EIHRMS data of **2** led to the molecular formula $C_{22}H_{18}N_4$, which contained double the number of carbon, nitrogen, and hydrogen atoms shown in the partial structure 1a. This suggested that the structure is a symmetrical dimer of 1a with the final structure **2**.



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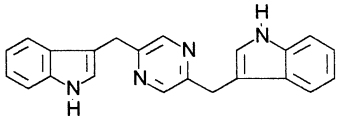

2

Simple pyrazines are known as urinary signaling pheromones in the tree shrew (*Tupaia belangeri*) and the pine vole (*Microtus pinetorum*)[23] or as pheromones of various ants.[24] 2,5-Dimethylpyrazine, 2-hydroxymethyl-5-methylpyrazine,[15] and 2,5-dihydroxymethylpyrazine[25] have been

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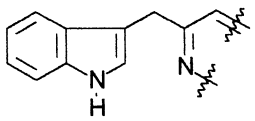


Figure 1. Partial structure 1a of compound **2** derived from NMR data and comparison with known metabolites.

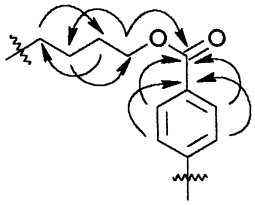


Figure 2. Partial structure of compound **5** derived from 2D NMR measurements (→: HMBC couplings).

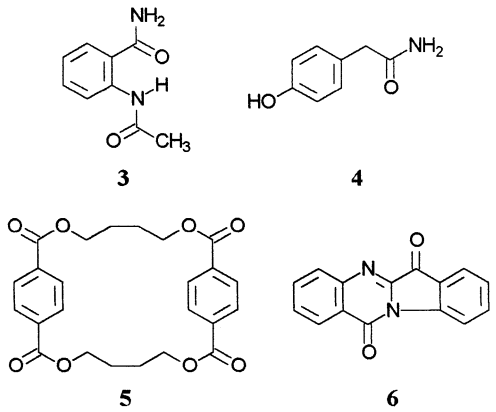
previously isolated from marine Streptomycetes in our laboratory. GC/MS investigations of bacterial flavor components[26] show that these and others are very widespread. More complex pyrazines, however, are rare, and a hydroxy substitution of the aromatic system as in septorin[27] and flavacol[28] seems to indicate an origin from piperazinediones, i.e., simple cyclodimers of amino acids. The latter are rather abundant in marine bacteria and were often isolated in our group from marine Streptomycetes and other marine bacteria, three alone from the *Cytophaga* strain AM13 investigated here.

The structure of the metabolite from fraction 3 was determined to be *o*-acetylaminobenzamide (**3**) by comparison of the spectral data with those of anthranilic acid, *o*-pyruvoylaminobenzamide,[29] and NI15501A.[30] Comparison with data of **3** isolated previously from a terrestrial Streptomycete[17] accompanied by the molecular formula (EIHRMS) confirmed the structure.

The high-resolution mass spectrum of compound 3 showed a molecular ion at *m*/*z* 151 and diagnostic signals at *m*/*z* 107 ($[M - CONH_2]^+$) and 44 ($[CONH_2]^+$). The mass and the fragmentation pattern were used to identify this compound as *p*-hydroxyphenyl acetamide (**4**). It has previously been reported from plants,[31] and the structure was confirmed by direct comparison of the NMR data with reference values.[31]

The ^{1}H NMR spectrum of compound **5** showed only three signals each of relative intensity 1. A singlet at δ 7.86 can be interpreted as the β-proton of a double bond conjugated with a carbonyl group or as an aromatic proton in conjugation with an electron-withdrawing substituent. Two broad singlets at δ 4.41 and 2.02 were observed, of which the first results from a group connected to oxygen. The ^{13}C NMR spectrum displayed a CO signal of a carboxylic acid, ester, or amide at δ 165.5, two carbon signals at δ 133.9 (C_q) and 129.4 (CH), and two signals for methylene carbons at δ 64.6 (OCH_2) and 25.8 (CCH_2C), corresponding to the proton signals mentioned above. The resonance of the sp^2-carbons indicated that the molecule contained more likely a symmetrically substituted aromatic ring than a conjugated olefinic double bond. These few signals first pointed to a simple structure; however, the EI and CI mass spectra led to the molecular mass of 440 Da. The high resolution of the molecular ion delivered the formula $C_{24}H_{24}O_8$, which suggested the structure to be highly symmetrical. The interpretation of 1D and 2D NMR spectra (COSY, HSQC, HMBC) led to the partial structure as in Figure 2. Here, the more plausible three-bond correlations have been drawn; however, two-bond correlations cannot be excluded completely due to identical shifts.

The molecular formula suggested the molecule to consists of four equivalent parts resulting in structure **5**, which we named pharacine. No other isomer is consistent with the chemical shifts and the long-range couplings. The structure was further confirmed by hydrolysis in methanol/2 N HCl followed by methylation with diazomethane, which delivered terephthalic acid dimethyl ester with a molecular ion at *m*/*z* 194. The broadened singlets (narrow multiplets after expansion) for the butanediol bridges may not correspond to the expectation; however, the data were identical with published values[32] and very similar to simulated ^{13}C and ^{1}H NMR spectra,[33] while other isomers such as the cyclic phenylenediacetic acid glycol ester were clearly different.



3 **4**

5 **6**

Terephthalic acid and several of its esters have been isolated from plants.[2] Pharacine (**5**) is the first cyclic terephthalic acid ester from a natural source; however, it has already been described as a cyclization product from terephthaloyl chloride and butanediol[34] or as trace components of poly(1,4-butylene terephthalate).[35] To the best of our knowledge, NMR data have not been published; however, our mass spectra were nearly identical with those in the literature.[36] As terephthalic esters are often used as additives in plastics, pharacine (**5**) could be present in the materials used in the laboratory and extracted as an artifact during the workup. We were able to reproduce the isolation from *Cytophaga* sp. AM13.1 strictly avoiding all contacts with plastic materials, yet we could not isolate it from other bacterial strains. To definitely rule out an artificial origin of **5**, biosynthetic studies should be done, which are, however, presently not possible due to the very low yield.

The known metabolites tryptamine acetate,[5] 2-phenylethylacetamide,[6] 3-indolylcarboxylic acid,[7] *cyclo*(tyrosylprolyl),[9] *cyclo*(phenylalanylprolyl),[10] *cyclo*(valylprolyl),[11] *cyclo*(isoleucylvalyl),[12] tryptanthrin (**6**),[22] 2-(3-indolyl)ethanol,[13] 2-phenylethylisovaleramide,[1] 3-indolylacetic acid,[8,14] 2,2-dimethylbenzopyrimid-4-one,[15] *o*-aminobenzamide,[16] phenylacetamide,[18] 2-(*p*-hydroxyphenyl)ethanol,[19] phenylacetic acid,[20] 3-phenyllactic acid,[21] uracil, and thymidine were also identified as constituents of *Cytophaga* sp. AM13.1 by NMR and MS data using AntiBase.[2] While **6** contributes most to the antibacterial and antifungal activity of the crude extracts, the phenylethyl amides are responsible for the weak phycotoxicity against the microalgae *Chlorella vulgaris*, *Chlorella salina*, and *Scenedesmus subspicatus*.[1] Although also various biological activities were reported for the piperazinediones and most of the simple indole derivatives,[2] we could not find any antibiotic or phytotoxic activity for **2** or **5**.

Experimental Section

General Experimental Procedures. NMR spectra were measured on a Varian Unity 300 (300.145 MHz) and a Varian Inova 500 (499.876 MHz) spectrometer in $CDCl_3$ with TMS as internal standard. CIMS was recorded on a Finnigan MAT 95 A instrument using NH_3 as reactant gas. EIMS was recorded on a Varian MAT 731 (70 eV), a Varian 311A (70 eV), and an AMD-402 (70 eV), and perfluorokerosene was used as reference substance in HREIMS. IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR in KBr pellets. Preparative HPLC was performed using an RP18 column (Eurochrom Eurospher RP 100-C18, 5 μm) using a Jasco diode array multiple wavelength detector (MD-910) in a scanning range of 195−650 nm. Retention times were measured on a 4 × 250 mm Eurochrome RP18 column (60 Å, 5 μm) with a linear acetonitrile-water azeotrope/water gradient (t = 0: 10% azeotrope, t = 25 min: 100% azeotrope; isocratic for a further 50 min). Flash chromatography was carried out on silica gel (30−60 μm, J. T. Baker). R_f values were measured on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.) with the solvent used for column chromatography when not stated otherwise. Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia).

Luria-Bertani Medium. Tryptone (10 g), yeast extract (5 g), and NaCl (10 g) were dissolved in 1 L of tap water, and the medium was adjusted to pH 7.2 with 2 N NaOH and sterilized for 33 min at 121 °C. After sterilization an end pH of 6.5 of the medium is attained.

Isolation of *Cytophaga* sp. Strain AM13.1. Strain AM13.1 was isolated from a 30 L water sample taken ca. 2 km offshore the island of Helgoland, Germany (54°08′N and 7°52′E) at a depth of 10 m using a Ruttner sampler.[37] The sample was immediately filtered through a nylon mesh filter (pore size 5 μm). The filtered water was stored at 4 °C in the dark for 1 year. An aliquot of 20 mL was amended with M13 medium components[38] (0.25 g L^{-1} yeast extract, 0.25 g L^{-1} peptone, 0.25 g L^{-1} glucose). Cycloheximide and ampicillin (Sigma, Germany) were added to a final concentration of 0.02 g L^{-1} each to prevent growth of fungi and to favor Planctomycete growth, respectively. The enriched sample was incubated at 4 °C in ambient light on a shaker for 4 weeks. A 100 μL sample of the enrichment culture was serially diluted in autoclaved, filter-sterilized (cellulose nitrate filters, pore size 0.22 μm, Sartorius AG, Göttingen) North Sea water and spread on agar plates containing M13 medium with cycloheximide. Plates were incubated at room temperature in the light for several days. Individual colonies were picked and subcultured. Sequencing of the 16S rDNA showed strain AM13.1 to be most similar but not identical to *Cytophaga marinoflava* (97% sequence identity).

The strain is deposited in the culture collection of the Department of Microbiology at the National Research Institute for Biotechnology (GBF) in Braunschweig, Germany.

Fermentation. Ten 1 L Erlenmeyer flasks each containing 200 mL of Luria-Bertani medium (adjusted to pH 7.2 before sterilization) were inoculated with the producing strain *Cytophaga* sp. AM13.1 and grown for 72 h at 28 °C while shaking at 95 rpm (circular shaker ITE, Infors, Germany). This culture served for the inoculation of a 25 L jar fermentor containing 18 L of the same medium. Incubation was continued for 3 days at 29 °C with agitation at 120 rpm and automatic addition of 2 N NaOH and 2 N HCl to maintain a pH of 6.5 ± 1.25. Niax PPG 2025 (Union Carbide, Belgium) was used as antifoaming agent, and sterile air was supplied at 5 L/min. The culture broth was mixed with diatomaceous earth (ca. 1 kg) and filtered through a press filter. The culture filtrate and biomass were each extracted separately with ethyl acetate. The biomass was additionally extracted with acetone. The combined organic phases were evaporated to dryness to yield 13.2 g of extract.

Separation. The crude extract was subjected to column chromatography on silica gel with a stepwise $CHCl_3$/MeOH/ gradient (1 L of $CHCl_3$, 1 L of $CHCl_3$/1% MeOH, 1 L of $CHCl_3$/3% MeOH, 1 L of $CHCl_3$/5% MeOH, 1 L of $CHCl_3$/10% MeOH, 0.5 L of $CHCl_3$/20% MeOH, 0.5 L of $CHCl_3$/50% MeOH) to deliver five fractions (Figure 3).

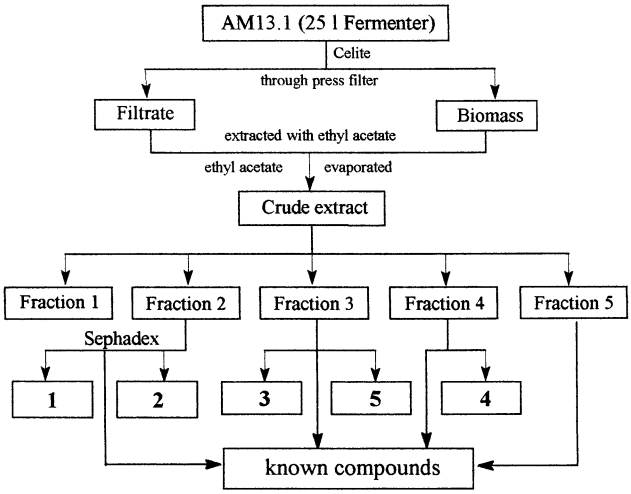


Figure 3. Workup scheme for the strain *Cytophaga* sp. AM13.1

Further purification of fraction 2 on Sephadex (3 × 60 cm, MeOH) and by HPLC RP18 (MeCN/H_2O) delivered 12 mg of 2,5-bis(3-indolylmethyl)pyrazine (**2**, t_R = 15.01 min), 2 mg of tryptamine isovalerate (**1**, t_R = 21.10 min), and the known compounds *cyclo*(tyrosylprolyl) (5 mg, t_R = 13.80 min), 3-indolylcarboxylic acid (20 mg, t_R = 14.10 min), *cyclo*(phenylalanylprolyl) (4 mg, t_R = 14.90 min), *cyclo*(prolylvalyl) (3 mg, t_R = 15.60 min), 2-(3-indolyl)ethanol (12 mg, t_R = 16.10 min), 2-phenylethylacetamide (3 mg, t_R = 16.80 min), tryptamine acetate (50 mg, t_R = 17.10 min), tryptanthrin (**6**, 10 mg, t_R = 19.00 min), and *cyclo*(isoleucylvalyl) (2 mg, t_R = 20.20 min).

2-(*p*-Hydroxyphenyl)ethanol (5 mg, t_R = 12.79 min), *o*-acetylaminobenzamide (**3**, 4 mg, t_R = 13.04 min), 3-indolylacetic acid (500 mg, t_R = 14.70 min), 2,2-dimethyl benzopyrimid-4-one (2 mg, t_R = 15.00 min), *p*-phenylacetic acid (3 mg, t_R = 18.01 min), *o*-aminobenzamide (50 mg, t_R = 13.04 min), phenylacetamide (2 mg, t_R = 20.80 min), and pharacine (**5**, 2 mg, t_R = 24.50 min) were isolated from fraction 3 by column chromatography on Sephadex (3 × 60 cm, $CHCl_3$/40% MeOH). Fractions 4 and 5 gave *p*-hydroxyphenyl acetamide (**4**, 3 mg, t_R = 2.40 min), uracil (30 mg, t_R = 3.67 min), thymidine (15 mg, t_R = 5.02 min), and 3-phenyllactic acid (3 mg, t_R = 12.10 min).

2,5-Bis(3-indolylmethyl)pyrazine (2): R_f = 0.22 ($CHCl_3$/5% MeOH), R_f = 0.45 ($CHCl_3$/10% MeOH); IR (KBr) ν_{max} 3267, 3057, 2921, 2856, 1621, 1489, 1457, 1429, 1344, 1221, 1098, 1042, 922, 794, 735 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.43 (2 H, s, H-3,6), 8.05 (2 H, s br, 2 NH), 7.53 (2 H, dd, ^3J = 7.9 Hz, ^4J = 1.1 Hz, H-7′,7″), 7.35 (2 H, dd, ^3J = 8.3 Hz, ^4J = 1.1 Hz, H-4′,4″), 7.18 (2 H, dt, ^3J = 7.2 Hz, ^4J = 1.2 Hz, H-6′,6″), 7.07 (2 H, dt, ^3J = 7.2 Hz, ^4J = 1.1 Hz, H-5′,5″), 7.05 (2 H, d, ^3J = 0.8 Hz, H-2′,2″), 4.26 (4 H, s, 2 CH_2); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 153.7 (C_q-2,5), 142.9 (CH-3,6), 136.2 (C_q-7a′,7a″), 126.8 (C_q-3a′,3a″), 123.4 (CH-2′,2″), 121.0 (CH-4′,4″), 118.4 (CH-5′,5″,6′,6″), 111.6 (C_q-3′,3″), 111.4 (CH-7′,7″), 30.8 (2 CH_2); EIMS (70 eV) m/z 338 $[M]^+$ (100), 221 (9), 208 (4), 169 (8), 154 (4), 130 (48), 83 (8); CIMS (NH_3) m/z 356 $[M + NH_4]^+$ (3), 339 $[M + H]^+$ (100); HREIMS m/z 338.1520 (calcd for $C_{22}H_{18}N_4$, 338.15315).

Pharacine (5): R_f = 0.90 ($CHCl_3$/5% MeOH); IR (KBr) ν_{max} 2962, 2928, 1721, 1635, 1383, 1280, 1124, 1104, 1019, 946, 874, 729 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.86 (8 H, s, ar-H), 4.41 (8 H, s br, 4 OCH_2), 2.02 (8 H, s br, 4 CH_2); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ 165.5 (CO), 133.9 (C_q), 129.4 (CH), 64.6 (OCH_2), 25.8 (CH_2); EIMS (70 eV) m/z 440 $[M]^+$ (73), 369 (100), 324 (20), 176 (20), 149 (63), 132 (93), 104 (37), 54 (87); CIMS (NH_3) m/z 898 $[2M + NH_4]^+$ (11), 458 $[M + NH_4]^+$ (100); EIHRMS 440.1468 (calcd for $C_{24}H_{24}O_8$, 440.14712).

Biological Tests. All tests were performed using the agar diffusion method with bacteria, fungi, or microalgae as test organisms, as described previously.[1,39]

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