Limnazine, the First Bacterial Azine Derivative from *Bacillus* sp. GW90a[⊥,1]

Ratnakar N. Asolkar,[†] Vijayendra P. Kamat,[‡] Irene Wagner-Döbler,[§] and Hartmut Laatsch*[,†]

Department of Organic Chemistry, University of Göttingen, Tammanstrasse 2, D-37077 Göttingen, Germany, Department of Chemistry, Goa University, Taleigao Plateau, Goa, 403 206, India, and Department of Microbiology at the National Research Institute for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany

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A novel dimeric azine derivative designated as limnazine (**1**) has been isolated from the culture broth of an aquatic *Bacillus* sp. strain GW90a. The structure has been elucidated, on the basis of spectral data, as *N*,*N*′-bis(2,2,6-trimethylchroman-4-ylidene)azine and was additionally confirmed by synthesis. Limnazine (**1**) is inactive against algae, fungi, and bacteria.

Microorganisms[1] are a prolific source of structurally unique and highly bioactive secondary metabolites that might represent leads in the development of new pharmaceutical agents. In the course of our investigations of biologically active substances from microorganisms, we have now isolated from the culture of an aquatic *Bacillus* sp. strain GW90a a novel compound having a symmetrical azine skeleton, for which we have given the name limnazine (**1**). Here we report the taxonomy of the producing strain, the isolation and structure determination of **1**, and its synthesis from 2-hydroxy-5-methylacetophenone.

The ethyl acetate extract obtained from the aquatic bacterium strain GW90a after usual workup of the culture broth was defatted with cyclohexane and chromatographed on silica gel to afford nine fractions.

Further separation yielded *o*-hydroxyacetophenone, *N*-(2′-phenylethyl)propionamide, *N*-(2′-phenylethyl)isobutyramide, *N*-(2′-phenylethyl)-3-methylbutyramide, *N*-(2′-phenylethyl)hexanamide,[2] uracil, and uridine, which were easily identified by their spectroscopic properties. While the latter are frequently occurring bacterial metabolites, *o*-hydroxyacetophenone was isolated from bacteria for the first time now.

Compound **1** was obtained from fraction 3 as a nonpolar light yellow colored solid. The ^{1}H NMR spectrum in deuteriochloroform showed two singlets at δ 1.40 (6 H) and 2.35 (3 H), indicating three methyl groups. The presence of an isolated methylene group was indicated by a singlet at δ 2.96. Three signals at δ 6.78 (d, J = 8 Hz, 1 H), 7.14 (dd, J = 8 Hz, 2 Hz, 1 H), and 7.95 (d, J = 2 Hz, 1 H) were indicative for a 1,2,4-trisubstituted benzene derivative. The ^{13}C NMR spectrum exhibited only 11 signals comprising seven sp^2 and four sp^3 carbon atoms, one of the latter with double intensity indicating two magnetically equivalent methyl groups. The EI mass spectrum showed a molecular ion peak at m/z 376.3, which was confirmed by CI-MS with peaks at m/z 377.3 $(M + 1)^+$ and m/z 753.6 $(2M + 1)^+$. The molecular formula of compound **1** was determined by HREIMS to be $C_{24}H_{28}O_2N_2$. On the basis of the above, the compound must be a symmetrical dimeric benzene derivative.

In the HMBC spectrum the methyl signal at δ 2.35 showed three-bond correlations with C-5 (δ 124.8) and C-7 (δ 133.1) and a two-bond correlation with C-6 (δ 119.3). Similarly the aromatic proton signal at δ 7.95 showed correlation with the carbon signals of C-11 (δ 20.1), C-7 (δ 133.1), and the two signals at δ 154.1 and 155.4, respectively. If one of the latter is due to an oxygen-attached ring atom, the second must be an imide group due to the empirical formula. Further correlations confirmed the substitution pattern in the benzene moiety as depicted in fragment **A**. Similarly, the methylene signal at δ 2.96 showed correlations with C-4a (129.8), C-9/10 (26.8), and C-4 (155.4), which led to the fragment **B**, as shown in the Figure 1.

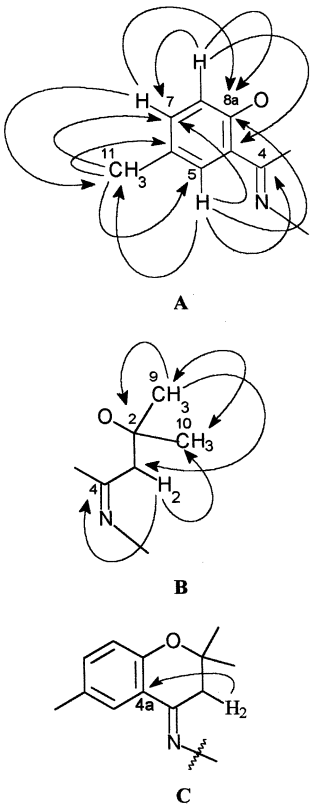

Figure 1. Fragments **A**−**C** of limnazine (**1**) deduced from HMBC correlations.

As fragment **C** is sufficient to explain all NMR shifts and correlations, yet contributes only half the molecular weight, two of such subunits must be existing and be linked together through the nitrogen atoms. Thus, the dimeric structure **1** was assigned to the yellow-colored compound

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* To whom correspondence should be addressed. Tel: +49-551-393211. Fax: +49-551-399660. E-mail: hlaatsc@gwdg.de.
[†] University of Göttingen.
[‡] Goa University.
[§] Microbiology Braunschweig.

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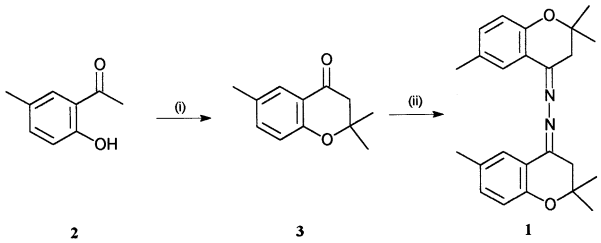

Figure 2. Reaction conditions: (i) acetone, piperidine, pyridine, reflux 72 h; (ii) hydrazine hydrate (99%), 2 h reflux.

designated as limnazine (**1**). The new azine was inactive against the bacteria *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*, the fungus *Mucor miehei*, and the microalgae *Chlorella vulgaris*, *Chlorella salina*, and *Chlorella sorokiniana*, using the agar diffusion test. Limnazine (**1**) was also inactive against *E. coli*, *B. subtilis*, and *C. vulgaris* in solution with concentrations up to 100 μg/mL.

It may be noted that hydrazine derivatives have been frequently encountered in nature; however, so far only one azine (agaricone[3]) has been reported from natural sources.[4] Although chromanone-based derivatives have already been reported as fungal metabolites,[5,6] the present report pertaining to the isolation of limnazine (**1**) constitutes the first example involving a dimeric chromane structure from bacteria. Limnazine (**1**) is closely related with lactarochromanoic acid from *Chrysothamnus viscidiflorus*[7] and lactarochromanal from the basidiomycete *Lactarius deliciosus*,[8] respectively.

To confirm the assigned structure, the synthesis of **1** was carried out by an unambiguous route as illustrated in the Figure 2.

Accordingly, *p*-cresyl acetate, prepared by acetylation of *p*-cresol with acetic anhydride in the presence of pyridine, was subjected to a Fries reaction as described in the literature[9] to afford 2-hydroxy-5-methylacetophenone (**2**). The latter was refluxed with excess acetone in the presence of piperidine and pyridine for 72 h. The reaction mixture, after usual workup and purification, provided the desired chromanone (**3**) in 77% yield. On refluxing with excess hydrazine hydrate,[10] **3** gave the corresponding azine derivative as a light yellow colored solid in a yield of 66.3%. The melting point and spectral data (IR, ^{1}H NMR, and MS) of the synthetic sample of **1** were identical with the data of the natural product, thereby confirming the assigned structure **1** of limnazine.

Experimental Section

General Experimental Procedures. The melting points were determined by capillary method and are uncorrected. IR spectra were recorded on a Shimadzu FTIR-8001 spectrophotometer. UV spectra were recorded on a HP8451 A diode array spectrophotometer; EIMS mass spectra, with a Varian MAT 731 (70 eV, high resolution with perfluoro kerosene for comparison). ^{1}H NMR spectra were recorded on a Varian VXR 300 (300 MHz) and a Varian 500 (499.8 MHz), and ^{13}C NMR spectra were measured on a Varian VXR 300 spectrometer at 75 MHz with TMS as internal standard. 2D spectra were run on a Varian VXR 300 instrument using a 5 mm inverse probe. Flash chromatography was carried out on silica gel (230−400 mesh), while Sephadex LH-20 (Pharmacia) was used for gel filtration.

Description of the Organism. The strain GW90a was enriched from a sediment sample from the Georgswerder waste storage site close to Hamburg, Germany. A sample of ca. 50 mL was obtained manually from a seepage channel and contained 15 mg PCB kg^{-1} (dry weight). Slurry microcosms were set up as described previously[11] by mixing 2 g of the sample with 18 mL of M9 minimal medium and adding biphenyl crystals to yield a final concentration of ca. 650 μg L^{-1} in the liquid phase. Slurries were shaken gently for 6 months on a rotatory shaker. Evaporated water and consumed biphenyl crystals were periodically replaced. An aliquot of 100 μL was serially diluted in 0.85% (wt/vol) NaCl, and appropriate dilutions were spread on agar plates containing 0.1 strength Luria-Bertani (LB) medium (1 g of tryptone, 0.5 g of yeast extract, 1 g of NaCl in 1 L of tap water). Colonies that showed activity for the 2,3-dihydroxybiphenyl dioxygenase enzyme (spray test)[11] were picked and subcultivated on 0.1 strength LB plates.

The strain was investigated by FAME (fatty acid methyl ester) analysis and shown to belong to a tight cluster with a Euclidean distance <10. On the basis of the FAME profiles, the strain was assigned as a *Nocardiopsis* group of organisms but could not be identified further. Sequencing of the 16S rDNA showed GW90a to be an unknown *Bacillus* strain (96% sequence identity). Thus, this strain probably represents a new species or subspecies within the genus *Bacillus*. GW90a is deposited in the culture collection of the Department of Microbiology at the National Research Institute for Biotechnology in Braunschweig, Germany.

Fermentation. Two liters of culture medium composed of tryptone (20 g), yeast (10 g), and NaCl (20 g) were dissolved in 2 L of tap water, and the medium was adjusted to pH 7.2 with 2 N NaOH and distributed into 10 × 1 L Erlenmeyer flasks. After sterilization, the flasks were inoculated with a slant culture of strain GW90a and grown for 3 days at 29 °C with 95 rpm. This shaker culture served for the inoculation of a 20 L jar fermentor containing 18 L of the medium as above. Incubation was carried out for 3 days at 29 °C and 120 rpm with automatic addition of 2 N NaOH and 2 N HCl to maintain the pH at 6.5 ± 1.25. Niax PPG 2025 (Union Carbide Belgium N. V. Zwijndrecht) was used as antifoaming agent and sterile air (5 L/min) supplied. The culture broth of the fermentor was mixed with Celite (ca. 1 kg) and passed through a pressure filter. The culture filtrate and biomass were extracted three times with ca. 10 L of ethyl acetate, and the combined organic layer was evaporated to dryness to yield 4.12 g of crude extract.

The crude extract was dissolved in methanol (ca. 100 mL) and defatted with cyclohexane (ca. 100 mL). The methanol layer was concentrated, and the residue was dried in vacuo and subjected to silica gel column chromatography (70 × 3 cm) using a $CHCl_3$/CH_3OH gradient. The fractions were monitored by TLC using UV absorption and anisaldehyde/sulfuric acid reagent. Nine fractions were collected, which were further purified by using Sephadex LH-20 ($CHCl_3$/CH_3OH, 3:2) and PTLC.

Purification of the second fraction led to the isolation of *o*-hydroxyacetophenone (17 mg). The third fraction on purification with Sephadex LH 20 gave compound **1**. The fractions 4 and 5, on purification by HPLC and Sephadex LH 20, gave the known compounds *N*-(2′-phenylethyl)propionamide (19 mg), *N*-(2′-phenylethyl)isobutyramide (25 mg), *N*-(2′-phenylethyl)-3-methylbutyramide (24 mg), and *N*-(2′-phenylethyl)-hexanamide (35 mg), while fractions 8 and 9 on purification with Sephadex LH 20 gave uracil and uridine, respectively.

Limnazine (1): light yellow solid, 8 mg, mp 204 °C, R_f 0.90 (CH_2Cl_2/5% CH_3OH); UV ($CHCl_3$) λ_{max} (log ϵ) 350 (4.08), 307 (3.93), 280 (3.97), 271 (3.96) nm; IR (KBr) ν_{max} 2995, 2920, 1610, 1540, 1490, 1450, 1325, 1290, 1260, 1210, 1160, 1120, 920, 615 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.95 (2 H, d, J = 2 Hz, H-5,5′), 7.14 (2 H, dd, J = 8, J' = 2 Hz, 2 Hz, H-7,7′), 6.78 (2 H, d, J = 8 Hz, H-8,8′), 2.96 (4 H, s, CH_2-3,3′), 2.35 (6 H, s, CH_3-11,11′), 1.40 (12 H, s, $(CH_3)_2$-2,2′); ^{1}H NMR (acetone-d_6, 200 MHz) δ 7.92 (2 H, d, J = 2 Hz, H-5,5′), 7.15 (2 H, dd, J = 8 Hz, 2 Hz, H-7,7′), 6.77 (2 H, d, J = 8 Hz, H-8,8′), 3.00 (4 H, s, CH_2-3,3′), 2.30 (6 H, s, CH_3-11,11′), 1.37 (12 H, s, $(CH_3)_2$-2,2′); ^{13}C NMR ($CDCl_3$, 75.5 MHz; assignments from HMQC) δ 155.4 (C_q-4,4′), 154.1 (C_q-8a,8a′), 133.1 (CH-7,7′), 129.8 (C_q-4a,4a′), 124.8 (CH-5,5′), 119.3 (C_q-6,6′), 117.9 (CH-8,8′), 75.9 (C_q-2,2′), 37.1 (CH_2-3,3′), 26.8 (CH_3-9,9′, CH_3-10,10′), 20.1 (CH_3-11,11′); HMBC ($CDCl_3$, IN4LPLRND, F1 75.5 MHz, F2 300.1

MHz) (H → C), see Figure 1; EIMS (70 eV) *m*/*z* (%) 376.3 ([M^+], 56), 361.2 ([$M^+ - CH_3$], 100), 190.1 (10), 187.1 (20), 173.1 (10); DCI-MS (NH_3) *m*/*z* (%) 377.3 ([M + H]$^+$, 100), 753.5 ([2M + H]$^+$, 10); HREIMS *m*/*z* 376.2156 (calcd 376.2156 for $C_{24}H_{28}O_2N_2$).

Synthesis of 2,2,6-Trimethyl-4-chromanone (3). A mixture of 2-hydroxy-5-methylacetophenone **2** (1 g, 6.66 mmol), acetone (2.5 mL), piperidine (0.56 mL, 6.66 mmol), and pyridine (2.5 mL) was refluxed for 72 h. The mixture was then cooled, concentrated under reduced pressure, and poured into a mixture of 2 N NaOH (25 mL) and ice. The resulting solution was extracted with ethyl acetate, and the organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (petroleum ether/diethyl ether, 9:1) to afford **3** (0.98 g, 77%) as a light yellow oil: IR (neat) ν_{max} 2990, 1687, 1610, 1565, 1475, 1450, 1375, 1320, 1270, 1250, 1210, 1190, 1170, 1110, 930, 840 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.65 (1 H, d, J = 2 Hz, H-5), 7.28 (1 H, dd, J = 8.5, J' = 2 Hz, H-7), 6.82 (1 H, d, J = 8.5 Hz, H-8), 2.72 (2 H, s, CH_2−H), 2.30 (3 H, s, CH_3-11), 1.44 (6 H, s, 2 × CH_3-2); EIMS (70 eV) *m*/*z* (%) 190 ([M^+], 70), 175 ([$M^+ - CH_3$], 100), 134 (50), 133 (28), 107 (16), 106 (44), 105 (28).

Synthesis of Limnazine (1). A mixture of chromanone **3** (0.1 g, 0.526 mmol) and hydrazine hydrate (10 mL) was heated under reflux for 2 h. The excess hydrazine hydrate remaining in the reaction mixture was distilled off, and the residue after cooling was poured on crushed ice and extracted with diethyl ether (3 × 25 mL). The combined ether extract was washed with water and saturated aqueous sodium chloride and dried (Na_2SO_4). The solvent was removed and the crude product was purified by silica gel column chromatography using petroleum ether/diethyl ether (1:19) as the eluent to give a light yellow crystalline solid **1** (0.065 g, 66.3%), mp = 204 °C.

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