Xenofuranones A and B: Phenylpyruvate Dimers from *Xenorhabdus szentirmaii*

Alexander O. Brachmann,[†] Steven Forst,[‡] Ghazala M. Furgani,[§] Andras Fodor,[⊥] and Helge B. Bode*[,†]

Pharmazeutische Biotechnologie, Universität des Saarlandes, P.O. Box 151150, D-66041 Saarbrücken, Germany, Department of Biological Sciences, University of Wisconsin, Milwaukee, Wisconsin 53201, Department of Genetics, Eotvos University, Budapest, Hungary, and Department of Entomology, The Ohio State University, Wooster, Ohio 44691

Received August 18, 2006

Xenofuranones A (**1**) and B (**2**) have been isolated from cultures of the insect-pathogenic bacterium *Xenorhabdus szentirmaii*, and their structures were elucidated by NMR and mass spectroscopy. Both compounds show similarities to fungal furanones, and their biosynthesis was studied using a reversed approach by feeding putative ^{12}C precursors to an overall ^{13}C background in small-scale experiments followed by gas chromatographic analysis coupled to mass spectrometry.

Bacterial strains of the genera *Xenorhabdus* and *Photorhabdus* are model organisms to study nematode symbiosis and insect pathogenesis.[1−3] With one exception[4] these bacteria are always found in symbiosis with nematodes of the genera *Steinernema* or *Heterorhabditis*, respectively. After infection of insect larvae by these soil-dwelling nematodes the bacteria are involved in killing the insect host using insecticidal proteins[5,6] and other unknown factors. Additionally, the bacteria outcompete other bacteria living in the insect intestine by their high growth rate and antibiotic production and help to provide the nematodes with food supplies by degrading the biomass of the insect.[2] After several replication cycles of the nematode all food resources are used up and the nematode takes up some bacteria, develops into the free-living, nonfeeding form called infective juvenile, and exits the exploited carcass into the soil.[2] Although secondary metabolites play a crucial role in this symbiosis/pathogenesis life cycle of bacteria, nematode, and insect host (and in the protection of the insect cadaver from free-living soil bacteria and fungi), almost nothing is known about the nature and mode of action of the compounds involved. Moreover, neither their biosynthesis nor their regulation is known. Only six families of compounds have been described from *Xenorhabdus* and *Photorhabdus* in the literature.[7−13] Therefore we set up a screening program of this still unexplored resource, and we were able to isolate xenofuranones A (**1**) and B (**2**) as major metabolites from *Xenorhabdus szentirmaii*.

For the isolation of secondary metabolites, *Xenorhabdus szentirmaii* DSM 16338^T was cultivated in shaking flasks containing LB medium with 2% Amberlite XAD-16 adsorber resin. After cultivation for 3 days at 30 °C the resin was collected and extracted with MeOH repeatedly. The obtained brown, oily crude extract showed two lipophilic and highly UV-active compounds on TLC, which were isolated by preparative RP-HPLC to give pure xenofuranones A (**1**) and B (**2**).

The molecular formula $C_{18}H_{16}O_3$ of the main product **1** was derived from a HRESI mass spectrum (*m*/*z* ([M + H]$^+$) = 281.11735) indicating 11 double-bond equivalents. The structure of **1** was deduced from 1D (^1H, ^{13}C) and 2D ($^1H-^1H$ COSY, HSQC, HMBC) NMR data. In the ^{13}C NMR spectrum (Table 1) only 14 signals were identified, but four of the 10 signals between δ_C 127.1 and 141.0 showed very high intensities, indicating the presence of eight overlapping signals. Furthermore, a methoxy group at δ_C 58.5, a methylene group at δ_C 39.4, and a carboxyl group at δ_C 167.8 were observed. Characteristic signals in the ^1H NMR spectrum (Table 1) were the methoxy group at δ_H 3.92 and two aliphatic protons at δ_H 2.89 and 3.32, which could be correlated to the methylene group by HSQC correlation. From the coupling constants in the ^1H NMR and a $^1H-^1H$ COSY experiment three spin systems could be identified (Figure 1, bold lines), which could be connected with the remaining structural elements by HMBC correlation (Figure 1, arrows).

Table 1. NMR Spectroscopic Data (500 MHz, $CDCl_3$) of Xenofuranones A (**1**) and B (**2**)

	xenofuranone A (**1**)		xenofuranone B (**2**)	
position	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)
2	167.8, qC		169.6, qC	
3	141.0, qC		137.1, qC	
4	130.0, qC		128.8, qC	
5	78.0, CH	5.56, dd (6.0, 3.5)	79.5, CH	5.61, dd (6.5, 3.5)
6	136.9, qC		130.0, qC	
7a/7b	127.6, CH	7.58, d (7.0)	127.1, CH	7.63, d (7.0)
8a/8b	128.9, CH	7.47, t (7.0)	129.0, CH	7.49, t (7.0)
9	129.4, CH	7.43, t (7.0)	129.2, CH	7.41, t (7.0)
10	39.4, CH_2	3.32, dd (14.5, 3.5) 2.89, dd (14.5, 6.0)	39.6, CH_2	3.39, dd (14.5, 3.5) 2.94, dd (14.5, 6.5)
11	134.7, qC		134.7, qC	
12a/12b	129.7, CH	7.02, m	129.7, CH	7.02, m
13a/13b	128.2, CH	7.21, m	128.3, CH	7.22, m
14	127.1, CH	7.21, m	127.6, CH	7.22, m
3-O*CH*$_3$	58.5, CH_3	3.92, s		
3-O*H*				3.80, s

Xenofuranone B (**2**) differed from **1** by a reduced mass of 14 amu in the molecular ion and the lack of the characteristic methoxy group at δ_H 3.92 and δ_C 58.5 and appearance of an additional singlet at δ_H 3.80, whereas all other signals (Table 1) were very similar to **1**, indicating that **2** is the 3-hydroxy derivative of **1**, as was additionally confirmed by $^1H-^1H$ COSY and HMBC correlations (Figure 1). Although both compounds have been described in a Japanese patent as minor compounds of *Aspergillus terreus*[14] and **2** had already been synthesized more than 40 years ago from phenylpyruvic acid, which allowed comparison of the physicochemical data,[15] both compounds have never been isolated from bacteria.

The only natural product with similarities to **1** and **2** that was investigated with respect to its biosynthesis is compound **3**, which was also isolated from an *A. terreus* species that additionally produces the antitumor compounds asteriquinone and aspulvinone D (**4**).[16] Feeding experiments with ^{14}C-labeled precursors showed that all these compounds in *A. terreus* are produced via condensation of the α-ketocarboxylic acids of tryptophan (asterriquinone) or tyrosine/phenylalanine (**3** and **4**). Because **1** and **2** lack the carboxylic acid moiety that is still present in **3**, the biosynthesis

* Corresponding author. Tel: ++49 681 3025494. Fax: ++49 681 3025473. E-mail: h.bode@mx.uni-saarland.de.
[†] Universität des Saarlandes.
[‡] University of Wisconsin.
[§] Eotvos University.
[⊥] The Ohio State University.

10.1021/np060409n CCC: $33.50 © 2006 American Chemical Society and American Society of Pharmacognosy
Published on Web 11/15/2006

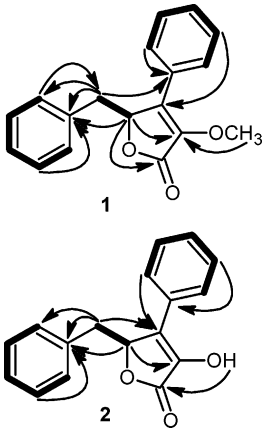
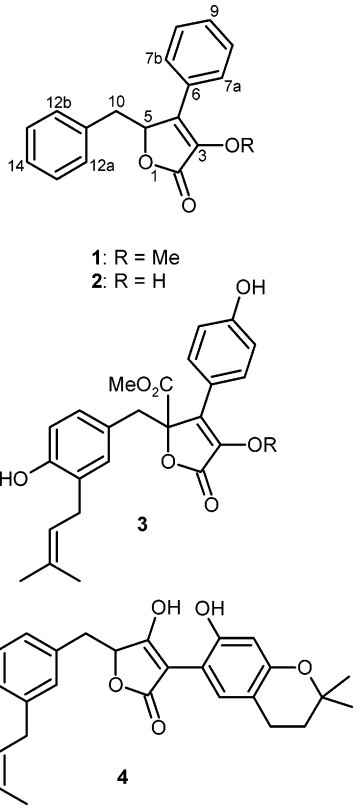


Figure 1. Selected $^1H-^1H$ COSY (bold lines) and HMBC (arrows) correlations of xenofuranones A (**1**) and B (**2**).



might also take place via condensation of phenylpyruvic acid with phenylacetaldehyde and subsequent cyclization. In order to elucidate the biosynthesis of **1** and **2** in bacteria, feeding of non-^{13}C-enriched precursors (^{12}C) to a ^{13}C-enriched culture of *X. szentirmaii* was performed on a small scale, followed by GC-MS analysis of the supernatant similar to a described method.[17] Whereas no incorporation of phenylacetaldehyde or phenlyacetic acid could be observed indicated by no mass shift to lower masses in the molecular ion, phenylalanine and phenylpyruvate were clearly incorporated once and twice into **1**, resulting in masses of *m*/*z* 290 (−8 amu), 289 (−9 amu), and 281 (−17 amu), compared to *m*/*z* 298 in the control experiment (Table 2). Moreover, the methyl group is derived from methionine as was shown by feeding [*methyl*-$^2H_3$]methionine to standard LB cultures, resulting in the expected +3 amu mass shift (Table 2). Additionally, feeding of **2** to *X. szentirmaii* growing in ^{13}C-labeled medium gave the expected mass of *m*/*z* 281 resulting from methylation of the added precursor. In summary it was shown that compounds **1** and **2** are derived from two phenylpyruvate moieties, and we propose a biosynthesis starting with enolate formation of one phenylpyruvate molecule followed by aldol-like condensation and cyclization to give the carboxylated furanone moiety, which can also be found in **3**. In *A. terreus*, the free carboxyl group is methylated and prenylation takes place before or after dimerization, resulting in the formation of **3**. Decarboxylation in *X. szentirmaii* results in the formation of **2**, which is methylated in a *S*-adenosylmethionine-dependent mechanism to give **1** as final product (Figure 2).

Compounds **1** and **2** were tested against different bacteria, yeast, and eukaryotic cell lines. However, only **1** showed a weak cytotoxic acitivity (IC_{50} 18 μg/mL) against eukaryotic cells (L929), whereas no additional biological activity was observed (data not shown). Nevertheless, these compounds might be involved directly in the nematode symbiosis or insect pathogenesis, and both possibilities are currently being tested by heterologous expression of the genes required for the biosynthesis of **1** and **2** and analysis of the biological activity of the heterologous producer.

Experimental Section

General Experimental Procedures. *Xenorhabdus szentirmaii* DSM 16338^T was described previously.[18] Melting points were determined on a Laboratory Devices MEL-TEMP II apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a UV−vis Specord 50 Analytik Jena photometer. IR spectra were obtained on a Bruker Tensor 27 spectrometer. NMR spectra were recorded on a Bruker DRX 500 spectrometer using $CDCl_3$ as solvent and internal standard. HRESIMS analysis was carried out on a Bruker APEX IV FTICR-MS. Preparative HPLC was performed on a Jasco HPLC (PU-2087, UV-2075) using a VP 250/21 Nucleosil 100-7 C18 column (Macherey & Nagel, Düren, Germany). GC-MS was carried out on an Agilent 6890N gas chromatograph with a 5973 electron impact mass selective detector (Agilent, Waldbronn, Germany) using a dimethyl-(5% phenyl)-polysiloxane capillary column (Agilent HP-5ms, 0.25 mm × 30 m × 0.25 μm) and helium as carrier gas at a flow rate of 1 mL/min. One microliter of the sample was injected in split mode (split ratio 10:1). The column temperature was kept at 70 °C for 5 min, then increased to 300 °C at a rate of 5 °C/min and held at 300 °C for 5 min. Other temperatures were as follows: inlet 250 °C, GC-MS transfer line 280 °C, ion source 230 °C, quadrupole 150 °C. The mass selective detector was operated in scan mode, scanning the mass range from *m*/*z* 40 to 700.

Fermentation. *Xenorhabdus szentirmaii* was cultivated at 30 °C and 280 rpm on a rotary shaker in two 5 L Erlenmeyer flasks each containing 1 L of Luria−Bertani (LB) broth (pH 7.0) and 2% (v/v) of XAD-16 (Sigma-Aldrich, Deisenhofen, Germany). These cultures were inoculated with 0.1% (v/v) of a 24 h preculture in the same medium without XAD-16. Cultures were harvested after 3 days, and XAD beads were separated from the supernatant by sieving.

Feeding Experiments. Feeding experiments were carried out in 2 mL Eppendorf tubes containing 1 mL of ISOGRO-^{13}C (Sigma-Aldrich) medium also containing 10 mM K_2HPO_4, 10 mM KH_2PO_4, 8 mM $MgSO_4 \cdot 7H_2O$, and 90 μM $CaCl_2 \cdot H_2O$. Feeding cultures were inoculated with 0.1% of a 24 h LB preculture that was first washed with ISOGRO-^{13}C to minimize the ^{12}C background. Feeding of [methyl-$^2H_3$]methionine (Sigma-Aldrich) was performed in nonlabeled LB medium. Precursors were added after 6 h of incubation to a final concentration of 2 mM each. After 3 days of incubation at 30 °C and 1050 rpm on an Eppendorf Thermomixer the compounds were extracted with 1 mL of ethylacetate. For further GC-MS analysis 100 μL of the ethylacetate extract was evaporated to dryness, redissolved in 175 μL of chloroform, and derivatized with 25 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA).

Isolation. XAD beads were extracted with MeOH (3 × 25 mL), and the MeOH extract was concentrated to dryness on a rotary evaporator, yielding a dark brown oily residue and an amorphous precipitate. The residue was redissolved in 5 mL of MeOH and filtered through a piece of cotton wool to exclude the amorphous precipitate. Pure compounds were isolated from the crude extract by preparative RP-HPLC using a 25 min gradient from 50 to 95% MeOH and UV detection at 254 nm, yielding pure xenofuranone A (**1**; t_R (HPLC) = 19.8 min; 28 mg/L) and xenofuranone B (**2**; t_R (HPLC) = 17.3 min; 10 mg/L). The collected fractions were dried and checked for purity

Table 2. Mass Spectroscopic Data of Xenofuranone A (**1**) Obtained from Different Feeding Experiments[a]

		m/z									
label	background	298	297	296	290	289	284	283	282	281	280
	^{13}C-glucose	90	8								2
^{12}C-phenylalanine	^{13}C-glucose	31	6		22	23				15	5
^{12}C-phenylpyruvate	^{13}C-glucose	30	7		22	21				14	6
2	^{13}C-glucose	56	11						4	29	
	LB[b]								3	16	81
[methyl-$^{2}H_3$]methionine	LB[b]						6	33	2	9	48

[a] Isotopomers with *m/z* between 285−288 and 291−295 were only present as trace compounds (<1%). [b] The feeding experiment was performed in nonlabeled LB medium.

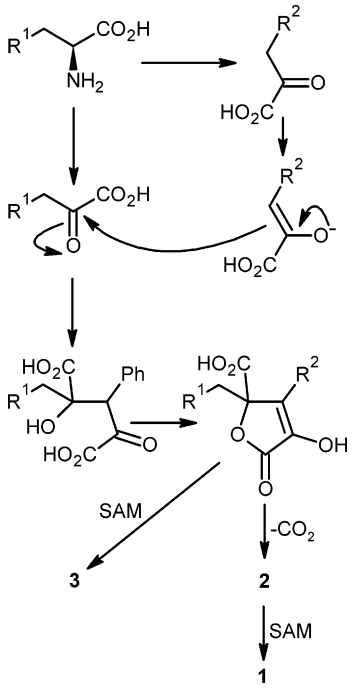


Figure 2. Proposed biosynthesis of xenofuranones A (**1**) and B (**2**). SAM = *S*-adenosylmethionine. For R^1 and R^2 see structures of **1**, **2**, and **3**.

by TLC (silica gel/TLC-cards, Sigma-Aldrich) and HPLCMS before NMR analysis.

Xenofuranone A (5-benzyl-3-methoxy-4-phenylfuran-2(3*H*)-one; 1): colorless solid; mp 77 °C; $[\alpha]^{20}_D$ +96 (*c* 0.03 in $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 204 (3.98), 282 (3.86), UV (MeOH/NaOH) 209 (4.48), 282 (3.84), UV (MeOH/HCl) 207 (3.85), 282 (3.82) nm; IR ν_{max} 2945, 1736, 1181, 766, 724, 694 cm^{-1}; EIMS (70 eV) *m/z* 280 $[M]^+$ (55), 252 (21), 189 (86), 161 (20), 146 (13), 133 (6), 118 (20), 105 (3), 91 (100), 77 (8); HRESIMS *m/z* 281.11735 (calcd for $C_{18}H_{16}O_3$ + H^+, 281.11722); t_R (GC) 37.6 min, R_f ($CHCl_3$/MeOH, 99:1) 0.87.

Xenofuranone B (5-benzyl-3-hydroxy-4-phenylfuran-2(3*H*)-one; 2): colorless solid; mp 64 °C; $[\alpha]^{20}_D$ +19.5 (*c* 0.0017 in $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 203 (3.92), 286 (3.83), UV (MeOH/NaOH) 208 (4.44), 321 (3.81), UV (MeOH/HCl) 207 (3.81), 286 (3.83) nm; IR ν_{max} 3265, 1724, 1151, 760, 730, 692 cm^{-1}; NMR (see Table 1); EIMS (TMS derivative; 70 eV) *m/z* 338 $[M]^+$ (49), 323 (10), 293 (15), 279 (7), 261 (3), 247 (47), 221 (75), 203 (30), 193 (20), 178 (6), 163 (6), 145 (4), 135 (8), 115 (13), 102 (5), 91 (27), 73 (100), 65 (5), 45 (8); HREISMS *m/z* 267.10172 (calcd for $C_{17}H_{14}O_3$ + H^+, 267.10157); t_R (GC) 38.4 min, R_f ($CHCl_3$/MeOH, 99:1) 0.34.

Acknowledgment. This work was supported by a research grant of the Saarland University (to H.B.B.), and the authors are grateful to H. Frauendorf for HRESIMS measurements and A. Mathe Fodor for help with the initial isolation of the strain.

References and Notes

(1) ffrench-Constant, R.; Waterfield, N.; Daborn, P.; Joyce, S.; Bennett, H.; Au, C.; Dowling, A.; Boundy, S.; Reynolds, S.; Clarke, D. *FEMS Microbiol. Rev.* **2003**, *26*, 433−456.
(2) Forst, S.; Dowds, B.; Boemare, N. E.; Stackebrandt, E. *Annu. Rev. Microbiol.* **1997**, *51*, 47−72.
(3) Forst, S.; Nealson, K. *Microbiol. Rev.* **1996**, *60*, 21−43.
(4) Gerrard, J.; Waterfield, N.; Vohra, R.; ffrench-Constant, R. *Microbes Infect.* **2004**, *6*, 229−237.
(5) Bowen, D.; Rocheleau, T. A.; Blackburn, M.; Andreev, O.; Golubeva, E.; Bhartia, R.; ffrench-Constant, R. H. *Science* **1998**, *280*, 2129−2132.
(6) Brown, S. E.; Cao, A. T.; Dobson, P.; Hines, E. R.; Akhurst, R. J.; East, P. D. *Appl. Environ. Microbiol.* **2006**, *72*, 1653−1662.
(7) Ciche, T. A.; Blackburn, M.; Carney, J. R.; Ensign, J. C. *Appl. Environ. Microbiol.* **2003**, *69*, 4706−4713.
(8) McInerney, B. V.; Gregson, R. P.; Lacey, M. J.; Akhurst, R. J.; Lyons, G. R.; Rhodes, S. H.; Smith, D. R.; Engelhardt, L. M.; White, A. H. *J. Nat. Prod.* **1991**, *54*, 774−784.
(9) McInerney, B. V.; Taylor, W. C.; Lacey, M. J.; Akhurst, R. J.; Gregson, R. P. *J. Nat. Prod.* **1991**, *54*, 785−795.
(10) Li, J. X.; Chen, G. H.; Webster, J. M. *Can. J. Microbiol.* **1997**, *43*, 770−773.
(11) Li, J.; Chen, G.; Webster, J. M.; Czyzewska, E. *J. Nat. Prod.* **1995**, *58*, 1081−1086.
(12) Richardson, W. H.; Schmidt, T. M.; Nealson, K. H. *Appl. Environ. Microbiol.* **1988**, *54*, 1602−1605.
(13) Li, J.; Chen, G.; Wu, H.; Webster, J. M. *Appl. Environ. Microbiol.* **1995**, *61*, 4329−4333.
(14) Morishima, H.; Fujita, K.; Nakano, M.; Atsumi, S.; Ookubo, M.; Kitagawa, M.; Matsumoto, H.; Okunyama, A.; Okabe, T.; Suda, H.; Nishimura, S. Jpn. Kokai Tokkyo Koho 1994.
(15) Sakan, T.; Kato, M.; Miwa, T. *Bull. Chem. Soc. Jpn.* **1964**, *37*, 1171−1174.
(16) Kiriyama, N.; Nitta, K.; Sakaguchi, Y.; Taguchi, Y.; Yamamoto, Y. *Chem. Pharm. Bull.* **1977**, *25*, 2593−2601.
(17) Brady, S. F.; Clardy, J. *Angew. Chem., Int. Ed.* **2005**, *44*, 7045−7048.
(18) Lengyel, K.; Lang, E.; Fodor, A.; Szallas, E.; Schumann, P.; Stackebrandt, E. *Syst. Appl. Microbiol.* **2005**, *28*, 115−122.

NP060409N