Linear and Cyclic Peptides from the Entomopathogenic Bacterium *Xenorhabdus nematophilus*

Gerhard Lang,[†] Tim Kalvelage,[†] Arne Peters,[‡] Jutta Wiese,[†] and Johannes F. Imhoff*[,†]

Kieler Wirkstoffzentrum am IFM-GEOMAR, Am Kiel-Kanal 44, 24106 Kiel, Germany, and e-nema, Gesellschaft für Biotechnologie and Biologischen Pflanzenschutz mbH, Klausdorfer Strasse 28-36, 24223 Raisdorf, Germany

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Three new peptides, xenortides A and B and xenematide, were isolated from a culture of the nematode-associated entomopathogenic bacterium *Xenorhabdus nematophilus*. Their structures were elucidated using NMR, MS, and chemical derivatization methods. Xenortides A and B are the *N*-phenethylamide and tryptamide derivatives, respectively, of the dipeptide (*N*Me-L-Leu−*N*Me-L-Phe). The cyclodepsipeptide xenematide has the sequence (Thr−Trp−Trp−Gly), with a 2-phenylacetamide substituent at the threonine residue and one D-tryptophan. The new peptides and the two known compounds xenocoumacin II and nematophin were tested for antibacterial, antifungal, insecticidal, and anti-*Artemia salina* activities. Xenematide and xenocoumacin II showed moderate antibacterial activities. Xenocoumacin II, nematophin, and the two xenortides were active in the *Artemia salina* assay, and xenematide acted weakly insecticidal.

The genus *Xenorhabdus* constitutes a group of bacteria associated with entomopathogenic nematodes. These bacteria live in a symbiotic relationship in the gut of the nematode host, which is able to infect insects and release bacteria into their hemolymph. The bacteria then proliferate and contribute to the death of the insect. There is good evidence that the insecticidal activity of *Xenorhabdus* bacteria is at least partially due to the production of insecticidal protein toxins.[1,2] Besides this, a number of low molecular weight metabolites with various biological activities have been isolated from *Xenorhabdus* cultures, e.g., xenorxides, xenorhabdins, xenocoumacins, and nematophin.[3]

In our search for novel metabolites from nematode-associated bacteria we performed a chemical screening of an extract of *X. nematophilus*. This screening led to the identification of three new peptides, xenortide A (**1**), xenortide B (**2**), and xenematide (**3**), as well as the known compounds xenocoumacin II[4] and nematophin.[5]

HPLC-MS analysis of an XAD extract of a liquid culture of *X. nematophilus* showed the presence of several compounds, with the most prominent one being readily attributed by its molecular mass and UV spectrum to nematophin, an antibacterial compound commonly found in cultures of *X. nematophilus*. Nematophin and four further components (**1**−**3** and xenocoumacin II) were purified using a combination of liquid−liquid and gel chromatography.

The ^{1}H and ^{13}C NMR spectra of xenortide A (**1**; Table 1) showed signals for two phenyl groups. By evaluation of the COSY and HMBC data these phenyl groups were attributed to one phenylalanine and one phenethylamine residue. A third ^{1}H spin system was characteristic of a leucine residue. The chemical shifts of the remaining signals from two methyl groups (δ_H 1.83 and 1.97; δ_C 33.6 and 32.2) indicated that they were attached to nitrogen atoms. Their positions at the amino groups of the leucine and the phenylalanine residue were evident from long-range H,C-correlations from the methyl protons to the α-carbon of the respective amino acids. HMBC correlations from the *N*-methyl group of phenylalanine to the carbonyl carbon of leucine and from one of the methylene groups of phenethylamine to the carbonyl carbon of phenylalanine showed the overall structure of **1** to be that of a doubly *N*-methylated leucyl-phenylalanine dipeptide with an *N*-phenethylamide C-terminus. The accurate mass of *m*/*z* 410.2794 measured for the $[M + H]^+$ ion was consistent with this structure. Both amino acids were found to have the L-configuration by acid hydrolysis, subsequent derivatization with D-FDVA [N^α-(2,4-dinitro-5-fluorophenyl)-D-valinamide],[6] and HPLC analysis. In the NMR spectra of **1** a signal set for a minor compound with only small differences in ^{1}H and ^{13}C chemical shifts (for a table with data see the Supporting Information) was discernible, although no additional peaks or masses were detectable in the HPLC-MS analysis. The possibility of the minor compound being a diastereomer of **1** was ruled out by the FDVA analysis. In the NOESY spectrum the signals of the major compound showed correlations with the respective signals of the minor compound. These cross-peaks had the same phase as the diagonal peaks, thus indicating chemical exchange. Consequently, the two signal sets presumably arise from slowly interconverting conformers of **1**.

A related compound, xenortide B (**2**), coeluted with **1** in reversed-phase HPLC, but was readily separated from it by employing gel chromatography on Sephadex LH-20. The ESIMS indicated a mass of 448.3 for **2**. The NMR spectra suggested a structural relationship to **1**, with significant differences apparent only in the aromatic region of the spectra (Table 1). Instead of signals of a second phenyl residue, as in the spectra of **1**, an ABCD system and a singlet were discernible, indicative of an indole residue. Analysis of the NMR data revealed **2** to be an analogue of **1** with a tryptamide group in place of the *N*-phenethylamide at the C-terminus. The amino acid residues in **2** both have the L-configuration, as determined by an FDVA analysis.

Xenematide (**3**), which was isolated by fast centrifugal partitioning chromatography (FCPC), had a molecular mass of 662.3. The ^{1}H NMR spectrum of **3** in DMSO-d_6 displayed signals for two indole moieties and one phenyl group. The resonances of four amide protons between 7.4 and 8.8 ppm coupling with signals between 2.5 and 4.7 ppm indicated a peptidic structure. By analyzing the COSY and HMBC spectra, β-alanine, two tryptophan residues, threonine, and a phenylacetyl (PAA) group were identified as the building blocks of **3**. The sequence of the amino acids was determined in a straightforward manner as PAA-Thr-Trp-Trp-β-Ala by using the $^2J_{HC}$ correlations of the amide protons with the carbonyl carbons of the respective adjoining amino acids. The HMBC correlation from the β-proton of threonine to the carbonyl carbon of β-alanine indicated a ring closure with an ester bond between these residues. After mild acid hydrolysis, with phenol and dithioerythritol added to prevent oxidation of tryptophan, the configurations of the amino acid residues were determined by the FDVA method. The threonine residue was found to have the normal (2*S*,3*R*)-configuration. However, the HPLC analysis of the FDVA derivatives revealed that both L- and D-tryptophan were present. An assignment of the correct configurations to the two tryptophan

* To whom correspondence should be addressed. Tel: +49-431-6004450. Fax: +49-431-6004452. E-mail: jimhoff@ifm-geomar.de.
[†] Kieler Wirkstoffzentrum.
[‡] e-nema.

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Table 1. ^{1}H and ^{13}C NMR Data (600 MHz, CH_3OD) for Xenortides A (**1**) and B (**2**)

		1		**2**	
position		δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
Leu	1	175.3, qC		175.3, qC	
	2	59.0, CH	3.57, dd (8.0, 5.9)	59.0, CH	3.57, dd (8.0, 5.9)
	3	42.7, CH_2	1.30−1.40, m	42.7, CH_2	1.30−1.40, m
	4	25.6, CH	1.71, m	25.6, CH	1.71, m
	5	23.8, CH_3	0.93, d (6.7)	23.8, CH_3	0.93, d (6.7)
	6	22.3, CH_3	0.91, d (6.7)	22.3, CH_3	0.91, d (6.7)
	NMe	33.6, CH_3	1.83, s	33.6, CH_3	1.83, s
Phe	1	171.9, qC		171.9, qC	
	2	59.5, CH	5.41, dd (11.0, 5.6)	59.5, CH	5.41, dd (11.0, 5.6)
	3	35.6, CH_2	3.19, dd (14.5, 5.6)	35.6, CH_2	3.19, dd (14.5, 5.6)
			3.00, dd (14.4, 11.0)		3.00, dd (14.4, 11.0)
	4	138.5, qC		138.5, qC	
	5/9	129.8[b], CH	7.25, m	129.8[a], CH	7.25, m
	6/8	130.2[b], CH	7.15−7.30, m	130.2[a], CH	7.15−7.30, m
	7	127.4[c], CH	7.15−7.30, m	127.4[b], CH	7.15−7.30, m
	NMe	32.2, CH_3	2.97, s	32.2, CH_3	2.97, s
PEA[a]	1	41.9, CH_2	3.43, t (7.5)		
	2	36.4, CH_2	2.78, t (7.3)		
	3	140.4, qC			
	4/8	129.5[b], CH	7.19, m		
	5/7	129.6[b], CH	7.15−7.30, m		
	6	127.8[c], CH	7.15−7.30, m		
tryptamine	1			41.3, CH_2	3.47 - 3.77, m
	2			26.2, CH_2	2.95, m
	3			113.1, qC	
	4			128.8, qC	
	5			119.3, CH	7.56, d (8.0)
	6			119.7, CH	7.02, t (7.5)
	7			122.4, CH	7.08, t (7.5)
	8			112.3, CH	7.33, d (8.0)
	9			138.2, qC	
	11			123.4, CH	7.04, s

[a] PEA = phenethylamine. [b] Chemical shifts with same superscripts may be interchanged. [c] Chemical shifts with same superscripts may be interchanged.

Table 2. ^{1}H and ^{13}C NMR Data (600 MHz, DMSO-d_6) for Xenematide (**3**)

position		δ_C, mult.	δ_H (J in Hz)	position		δ_C, mult.	δ_H (J in Hz)
β-Ala	1	169.2, qC		Trp2	4	109.3, qC	
	2	33.9, CH_2	2.52, m		5	127.0[b], qC	
			2.40, m		6	118.2, CH	7.55, d (7.8)
	3	34.6, CH_2	3.39, m		7	118.2, CH	7.00, td (7.8, 1.0)
			3.32, m		8	121.0, CH	7.08, m
	NH		7.39, t (6.1)		9	111.34, CH	7.36, bd (8.0)
Trp1	1	171.0, qC			10	136.0[c], qC	
	2	54.5, CH	4.19, ddd (10.0, 7.9,3.7)		11		10.7, s
	3	25.7, CH_2	3.18, m		12	123.4, CH	7.09, m
			2.87, m		NH		8.81, d (6.7)
	4	110.6, qC		Thr	1	170.2, qC	
	5	126.9[b], qC			2	54.02, CH	4.65, dd (9.2, 2.2)
	6	117.9, CH	7.49, d (7.8)		3	72.0, CH	5.11, qd (6.3, 2.3)
	7	118.2, CH	6.96, m		4	16.2, CH_3	1.05, d (6.2)
	8	120.8, CH	7.05, td (7.8, 1.0)		NH		8.09, d (9.5)
	9	111.28, CH	7.33, bd (8.0)	PAA[a]	1	170.6, qC	
	10	135.9[c], qC			2	41.7, CH_2	3.65, d (14.1)
	11		10.6, s				3.55, d (14.1)
	12	123.1, CH	6.96, m		3	136.3, qC	
	NH		8.72, d (6.7)		4/8	129.0, CH	7.26, m
Trp2	1	172.0, qC			5/7	128.1, CH	7.29, m
	2	54.0, CH	4.53, q (7.3)		6	126.1, CH	7.19, m
	3	25.7, CH_2	2.82 - 2.92, m				

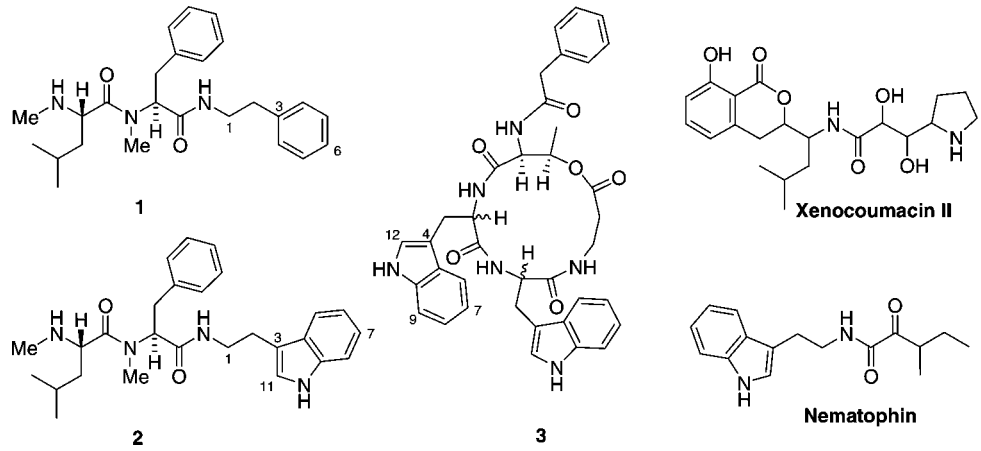
[a] PAA = 2-phenylacetic acid. [b] Chemical shifts with same superscripts may be interchanged. [c] Chemical shifts with same superscripts may be interchanged.

units by using NOE correlations was impossible because of the flexibility of the molecule.

Besides the three new peptides **1**−**3** the two known *Xenorhabdus* metabolites xenocoumacin II and nematophin were also isolated.

Compounds **1**−**3**, xenocoumacin II, and nematophin were tested for antimicrobial activity against various Gram-positive and Gram-negative bacteria as well as the yeast *Candida glabrata*. The only compounds exhibiting a noticeable antibacterial effect were xenematide (**3**) and xenocoumacin II, the latter showing an MIC of 40 μg/mL against the phytopathogens *Xanthomonas campestris*, *Ralstonia solanacearum*, and *Pseudomonas syringae*, and an MIC of 20 μg/mL against *Bacillus subtilis*. At a concentration of 20 μg/mL xenocoumacin II inhibited the growth of *S. lentus* by 70%. Xenematide (**3**) had an MIC value of 10 μg/mL against *Bacillus subtilis*, *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Erwinia amylovora*, and *Ralstonia solanacearum*. At a maximum concentra-

Chart 1



tion of 40 μg/mL the other three compounds did not inhibit microbial growth. It is noteworthy that we were not able to reproduce the previously described strong antistaphylococcal effect of nematophin.[5,7]

Xenocoumacin II also was notably active in the *Artemia salina* assay (IC_{50} = 9 μg/mL). In the same assay nematophin showed an IC_{50} value of 50 μg/mL, while **1** and **2** were weakly active, with an IC_{50} of about 200 μg/mL. Among the isolated compounds, only xenematide (**3**) exhibited insecticidal activity against *Galleria mellonella*, with two out of five larvae dead after a six-day period.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. NMR spectra were recorded on a 600 MHz spectrometer (Bruker AV600). For calibration of ^{13}C and ^{1}H NMR chemical shifts the carbon signals and the residual proton signals of the solvent were used (CD_3OD: δ_H 3.31 and δ_C 49.0; DMSO-d_6: δ_H 2.50 and δ_C 39.52). HPLC analyses were performed using a C18 column (Phenomenex Luna C18(2), 4.6 × 250 mm, 5 μm) applying a H_2O/MeCN gradient with 0.1% (v/v) HCOOH added to both solvents. For MS detection the HPLC was coupled to an ESI-ion trap system (Esquire 4000, Bruker). HRMS were acquired on a FT-ICR spectrometer (7 T Apex-Qe, Bruker) using positive electrospray ionization.

Cultivation and Extraction. The strain *Xenorhabdus nematophilus* was isolated from the nematode *Steinernema carpocapsae* according to Akhurst et al. (1980).[10] The identity of this isolate was confirmed by the 16S rDNA sequence analysis. The strain was cultivated in a previously described medium (20 L).[8] After 30 h at 30 °C XAD-16N resin (200 g) was added to the culture, which was left shaking for another 20 h, before washing the resin with H_2O (800 mL) and eluting with MeOH (400 mL).

Isolation of Metabolites. The extract was dried, redissolved in H_2O (400 mL), and consecutively extracted with EtOAc (2 × 400 mL) and *n*-BuOH (2 × 400 mL). After drying, the BuOH extract was subjected to gel chromatography on Sephadex LH-20 (2.5 × 110 cm; MeOH; 2.5 mL/min). Xenortide A (**1**; 35 mg), xenortide B (**2**; 7.8 mg), and xenocoumacin II (7.6 mg) were eluted from 102 to 132 min, from 138 to 150 min, and from 88 to 95 min, respectively. The EtOAc fraction was separated by liquid−liquid chromatography on a fast centrifugal partitioning chromatography system (FCPC; Kromaton) using the solvent system H_2O/MeOH/EtOAc/*n*-heptane (4.5:5.5:4.5:5.5) with the upper layer as the stationary phase (7 mL min^{-1}; 1400 rpm). Xenematide (**3**; 6.7 mg) and nematophin (39 mg) eluted after 18 and 129 min, respectively.

Xenortide A (1): amorphous, white solid; $[\alpha]^{20}_D$ −54 (*c* 0.2, MeOH); for ^{1}H and ^{13}C NMR data, see Table 1; COSY and HMBC data are available as Supporting Information; HRESIMS *m*/*z* 410.2794 $[M + H]^+$ (calc for $C_{25}H_{36}N_3O_2$ 410.2807).

Xenortide B (2): amorphous, yellowish solid; $[\alpha]^{20}_D$ −58 (*c* 0.3, MeOH); for ^{1}H and ^{13}C NMR data, see Table 1; COSY and HMBC data are available as Supporting Information; HRESIMS *m*/*z* 449.2908 $[M + H]^+$ (calc for $C_{27}H_{37}N_4O_2$ 449.2916).

Xenematide (3): amorphous, yellowish solid; $[\alpha]^{20}_D$ +45 (*c* 0.2, MeOH for ^{1}H and ^{13}C NMR data, see Table 2; COSY and HMBC data are available as Supporting Information; HRESIMS *m*/*z* 663.2923 $[M + H]^+$ (calc for $C_{37}H_{39}N_6O_6$ 663.2931).

Preparation and Analysis of D- and L-FDVA Derivatives. Compounds **1** and **2** (3 mg each) were hydrolyzed by heating in HCl (6 M; 1 mL) for 20 h at 110 °C. The solutions were then evaporated to dryness and redissolved in H_2O (250 μL). A 1% (w/v) solution (100 μL) of D-FDVA[6] in acetone was added to an aliquot (50 μL) of the acid hydrolysate solution (or to 50 μL of a 50 mM solution of the respective amino acid). After addition of a $NaHCO_3$ solution (1 M; 40 μL) and DMSO (70 μL) the mixture was incubated at 60 °C (2 h). The reactions were quenched by addition of HCl (2 M; 30 μL). The reaction mixtures were then diluted 100-fold with MeOH−H_2O (1:1) and analyzed by HPLC (Phenomenex Onyx monolithic C18, 100 × 3.0; solvents: A water + 0.1% HCOOH, B MeCN + 0.1% HCOOH; linear gradient: 0 min 5% B, 4 min 60% B; 6 min 100% B; 40 °C; 2 mL min^{-1}). The amino acid standards were derivatized with both D- and L-FDVA. Retention times (min) of the amino acid derivatives were as follows: D-FDVA-L-*N*Me-Leu (3.85), L-FDVA-L-*N*Me-Leu (3.62), D-FDVA-L-*N*Me-Phe (3.61), and L-FDVA-L-*N*Me-Phe (3.52). HPLC analyses of the peptide hydrolysates derivatized with D-FDVA both showed peaks for L-*N*Me-Leu and L-*N*Me-Phe at 3.85 and 3.61 min, respectively. Xenematide (**3**; 200 μg) was hydrolyzed with HCl (400 μL; 110 °C; 1 h) containing phenol (3% w/v) and dithioerythritol (1% w/v). The hydrolysate was derivatized with L-FDVA as described above. For the HPLC analysis of the tryptophan derivatives the HPLC conditions were the same as above. The threonine derivatives were analyzed using a different gradient (0 min 10% B, 5.5 min 30% B). The retention times of the derivatized amino acid standards were as follows: L-FDVA-L-Thr (2.87), L-FDVA-L-allo-Thr (3.10), D-FDVA-L-Thr (4.38), D-FDVA-L-allo-Thr (3.77), L-FDVA-L-Trp (3.24), and D-FDVA-L-Trp (3.51). The derivatized hydrolysate showed peaks corresponding to L-Thr, L-Trp, and D-Trp.

Antimicrobial Assays. The antimicrobial assays were performed as described before[9] with the following strains: *Bacillus subtilis* (DSM 347), *Escherichia coli* K12 (DSM 498), *Staphylococcus lentus* (DSM 6672), *Pseudomonas syringae* var. *aptata* (DSM 50252), *P. fluorescens* (NCIMB 10586), *Xanthomonas campestris* (DSM 2405), *Ralstonia solanacearum* (DSM 9544), and *Candida glabrata* (DSM 6425).

Insecticidal Assay. The isolated compounds, dissolved at 1 mg/mL in a 50% aqueous EtOH solution, were tested for oral toxicity to larvae of the greater wax moth (*Galleria mellonella*). Five larvae were placed in a plastic box (2 cm diameter) and fed with 1 g of a diet composed of 0.8 mL of the substance, 0.2 mL of glycerol (86%), and 1 g of wheat bran. An untreated control group were fed with 1 g of a mixture of 0.4 mL of distilled H_2O, 0.4 mL of EtOH, 0.2 mL of glycerol, and 1 g of wheat bran. The larvae were incubated at 35 °C for 6 days, and the mortality and the ability to spin silk were recorded. The experiment was done in four replicates except for nematophin (two replicates).

***Artemia salina* Assay.** A series of 2-fold dilutions of the test compounds with amounts from 50 to 0.781 μg/well were prepared in 96-well microplates, which were then dried. To each well were added between 10 and 20 freshly hatched *A. salina* nauplii in artificial seawater (300 μL). After 24 h the number of immotile nauplii per well was counted.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra and tables of data of xenortides A (**1**) and B (**2**) as well as xenematide (**3**) are available free of charge via the Internet at http://pubs.acs.org.

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