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Xentrivalpeptides A–Q: Depsipeptide Diversification in *Xenorhabdus*

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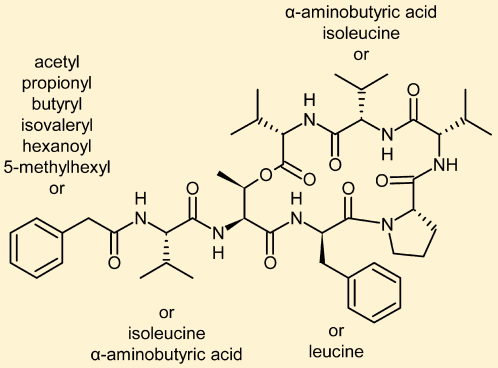
Supporting Information

ABSTRACT: Seventeen depsipeptides, xentrivalpeptides A–Q (**1**–**17**), have been identified from an entomopathogenic *Xenorhabdus* sp. Whereas the structure of xentrivalpeptide A (**1**) was determined after its isolation by NMR spectroscopy and the advanced Marfey's method, the structures of all other derivatives were determined using a combination of stable isotope labeling and detailed MS analysis.



Entomopathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus* live in symbiosis with nematodes of the genera *Steinernema* and *Heterorhabditis*, respectively, and together with them are able to infect and kill different insect larvae.[1−4] Although protein toxins are mainly responsible for larval mortality,[5,6] small molecules that are toxic to insect cells have also been identified.[7] It has been postulated that these bacteria produce antibiotics to protect the insect cadaver from competitors living in the soil, and compounds showing these activities have been identified recently.[7−9] Besides several small molecules such as isopropylstilbenes,[10,11] anthraquinones,[11,12] and xenofuranones,[13] recent work has shown that several peptides are produced by these bacteria. Among them are the highly polar PAX peptides,[14] the GameXPeptides,[15] and the depsipeptides xenematide[16,17] and szentiamide.[18] Of these, the PAX peptides,[14,19] GameXPeptides,[15] and xenematides[16,17] are produced as mixtures of several derivatives. Thus, *Xenorhabdus* and *Photorhabdus* have proven to be a rich source of bioactive natural products, and our goal is to identify additional bioactive natural products from them. Here we describe the identification and structure elucidation of the xentrivalpeptides, depsipeptides that show a much higher chemical diversity than the xentiamides[16,17] and szentiamide,[18] the two known depsipeptide classes from *Xenorhabdus*.

RESULTS AND DISCUSSION

During our search for new secondary metabolites from *Xenorhabdus* bacteria, we identified compound **1** with *m*/*z* 860.5 in extracts of *Xenorhabdus* sp. 85816 obtained from the Monsanto Company when grown in LB medium with 2% Amberlite XAD-16. From a 5 L culture grown under the same conditions, 22 mg of **1** was isolated from the XAD extract using preparative HPLC/MS. The molecular formula of **1** was determined from HRESIMS analysis (*m*/*z* 860.4880) as $C_{46}H_{65}N_7O_9$ (Table S1, Figure S1), and the structure of **1** was determined by detailed 1D (^{1}H,^{13}C) and 2D (COSY, HSQC, HMBC, TOCSY) NMR experiments (Table 1). Eight different spin systems were identified on the basis of coupling constants in the ^{1}H NMR spectrum and COSY data (Figure 1a). Several signals around 1 ppm were assigned to methyl groups from four valines and one threonine, and in addition, two phenyl groups were identified on the basis of the typical chemical shifts of δ_H 7.20−7.30 with total integration of 10 protons and typical δ_C shifts between 127 and 138 in the ^{13}C NMR spectrum. Each spin system could be connected with one of eight quaternary carbons in the range δ_C 169.5−174.9 by HMBC correlations (Figure 1b). In this way, the linear sequence derived from the connections of α-H with quaternary carbons and the connection between the β-H in Thr (3) and the quaternary carbon in Val (7) established the structure of the depsipeptide with a six-membered ring (Figure 1b).

The absolute configurations of the amino acids were determined using the advanced Marfey's method,[20] showing that only phenylalanine has the D-configuration (Table S2). We

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Table 1. NMR Spectroscopic Data (500 MHz (^{1}H), 125 MHz (^{13}C) in CD_3OD) of 1, δ in ppm[a]

subunit	position	δ_C	δ_H, mult (J in Hz)
PA	1	174.2	
	2	43.6	3.60, d (1.0)
	3	137.0	
	4	130.3[b]	7.29, m
	5	129.7	7.30, m
	6	128.2[c]	7.25, m
L-Val(1)	C=O	172.8	
	α	60.2	4.25, d (7.3)
	β	31.7	2.10, m
	γ	19.8	0.94, d (6.7)
	δ	18.6[d]	0.92, d (6.8)
L-Thr(2)	C=O	169.5	
	α	55.0	4.79, d (3.8)
	β	69.9	5.09, m
	γ	14.6	1.14, d (2.4)
D-Phe(3)	C=O	171.3	
	α	55.3	4.94, dd (5.2, 9.9)
	β1	40.5	2.91, dd (9.8, 12.6)
	β2	40.5	3.24, dd (5.0, 12.5)
	γ	137.0	
	δ	130.6[b]	7.27–7.29
	ε	129.7	7.27–7.29
	ζ	127.9[c]	7.20–7.24
L-Pro(4)	C=O	174.9	
	α	62.4	4.09, dd (4.9, 8.7)
	β1	30.7	1.79, m
	β2	30.7	1.89, m
	γ1	25.5	1.47, m
	γ2	25.5	1.79, m
	δ1	48.6	2.68, m
	δ2	48.6	3.44, m
L-Val(5)	C=O	173.6	
	α	65.5	3.64, d (11.3)
	β	30.8	2.31, m
	γ	20.3	0.89, d (6.4)
	δ	20.1	0.98, d (6.4)
L-Val(6)	C=O	173.5	
	α	58.0	4.42, d (5.9)
	β	33.6	2.04, m
	γ	18.7[d]	0.93, d (7.0)
	δ	19.7	0.99, d (7.0)
L-Val(7)	C=O	172.0	
	α	62.4	3.94, d (7.7)
	β	30.2	2.07, m
	γ	19.8	1.13, d (6.7)
	δ	18.9	1.04, d (6.8)

[a]PA: phenyl acetyl; d: doublet; m: multiplet. [b-d]Assignments are interchangeable.

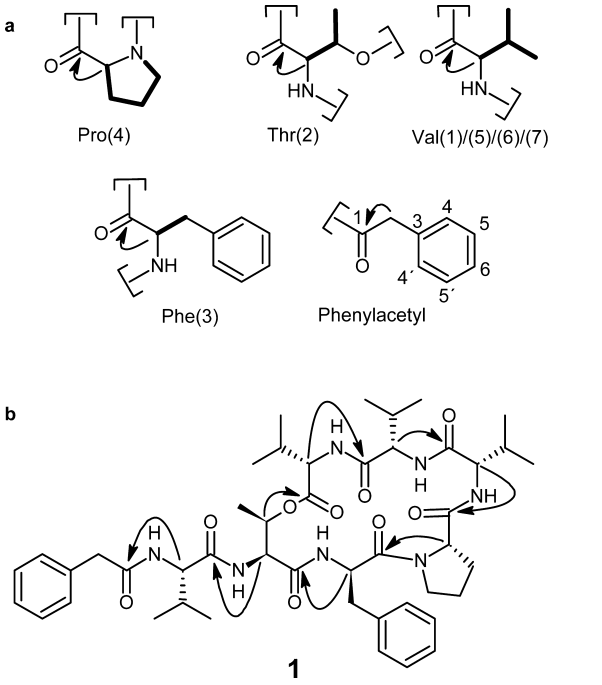

Figure 1. Subunits (a) with selected COSY (bold lines) and HMBC correlations (arrows) in xentrivalpeptide A (**1**) (b). Amino acid numbering from N- to C-terminus.

Table 2. Data for Xentrivalpeptides A–Q (1–17)

no.	compound	t_R/min	amount[a]	fragment B[b] (m/z)
1	A	10.4	100.0	625.3
2	B	6.4	1.1	
3	C	7.6	<1.0	
4	D	8.8	3.0	
5	E	9.8	2.1	
6	F	12.7	<1.0	
7	G	11.5	<0.1	
8	H	9.8	<1.0	
9	I	11.3	3.5	
10	J	8.2	<1.0	591.3
11	K	9.2	<1.0	
12	L	9.8	2.1	
13	M	12.2	<0.1	
14	N	12.3	3.7	639.3
15	O	9.8	<1.0	611.3
16	P	7.6	<0.1	
17	Q	8.5	<0.1	526.3[c]

[a]Amount relative to **1** calculated from peak areas in HPLC/MS. [b]For details on fragment B see Figure 2. [c]**17** has a smaller ring than the other derivatives (see structure below and Figure S7). Retention time (t_R).

analyzed strain 85816 for other derivatives of this class of depsipeptides and could indeed identify several, which we named xentrivalpeptides because they were isolated from a *Xenorhabdus* strain and all contained at least three (tri) valines. All xentrivalpeptides showed similarity to **1** based on the MS fragmentation patterns (Table 2, Table S1). We performed extensive MS fragmentation experiments with **1** (Table S1, Figure S1), which revealed a general fragmentation pattern for this class of peptides (Figure 2) used for the structure elucidation of the other xentrivalpeptides. Additionally, we applied a combination of labeling and detailed MS analysis to strain 85816, as previously described, to differentiate isobaric building blocks such as leucine and isoleucine.[15] From the results of labeling experiments using deuterated or fluorinated building blocks in LB medium, and an inverse labeling experiment adding nonlabeled ^{12}C building blocks to a culture grown in fully ^{13}C-labeled medium, the building blocks of **1** could be confirmed (Figure 3). For example, labeling with *p*-fluorophenylalanine or *p*-fluorophenylacetic acid in LB medium showed the expected +18 Da shift to m/z 878.5 (Figure 3b and c), and labeling with phenylalanine in a ^{13}C-labeled culture

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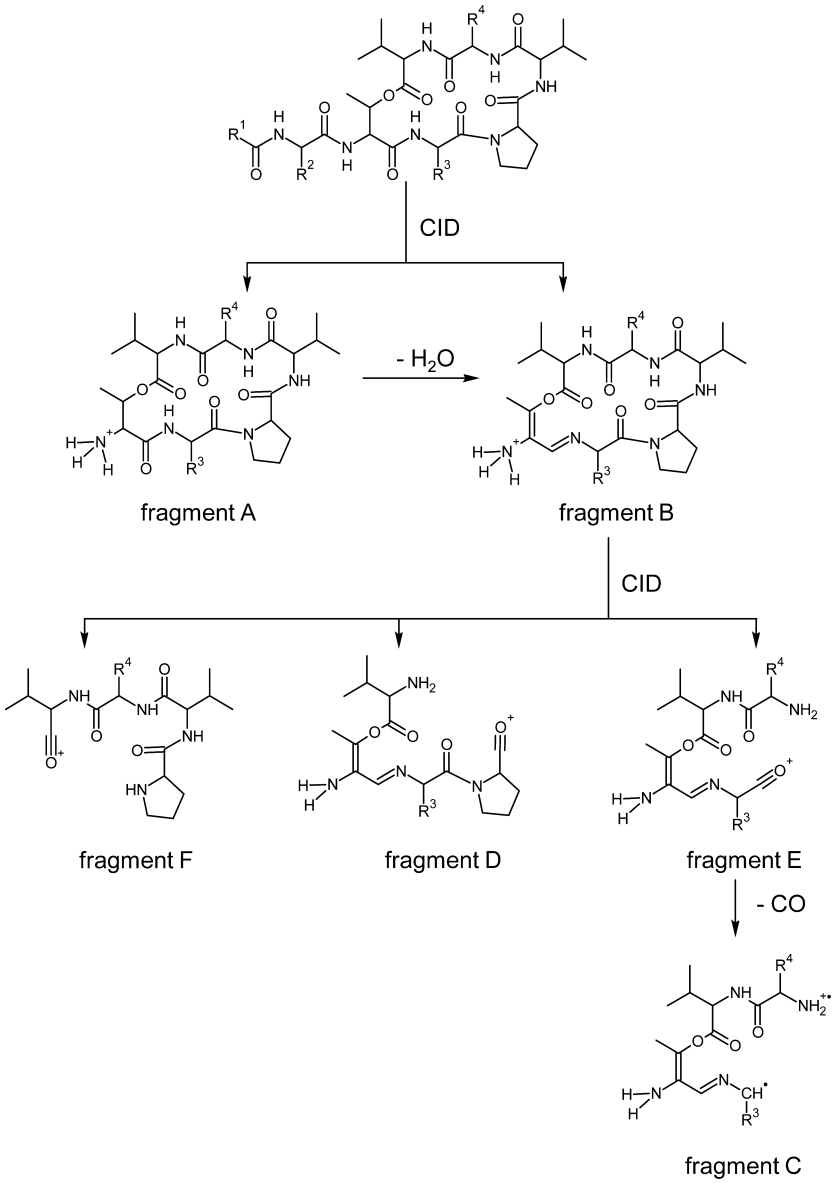

Figure 2. General fragmentation pathway of xentrivalpeptides A–P (**1**–**16**) and the proposed fragment structures. For fragment data see Table S1.

showed the expected shift to a lower mass due to the incorporation of nine and eight carbons from incorporated phenylalanine and phenylacetic acid, respectively (Figure 3j). Moreover, from cultivation in ^{13}C and ^{15}N medium the number of carbon and nitrogen atoms of **1** could be determined easily, thus affording the correct molecular formula as shown previously[15] (Figure 3f and g).

The analysis of these labeling experiments followed by MS fragmentation experiments (Figures S2–S5) allowed the structure elucidation of xentrivalpeptides B–P (**2**–**16**). The absolute configurations of the amino acids were not determined experimentally due to their low production titer (Table 2), but are assumed to follow that of **1**.

The differences between **1**–**9** were only in the side chain, as shown by an identical ring fragment B of *m*/*z* 625.3 (Figure 2, Table S1) and confirmed by labeling experiments showing the presence of the same ring amino acids as in **1** (Figure S2). Xentrivalpeptides A–G (**1**–**7**) differed from each other at R^1 (acyl moiety) only. The nature of R^1 in these derivatives was unambiguously determined from labeling experiments: D_6-propionic acid was incorporated into **3**, as detected from a mass shift of +5 Da from *m*/*z* 798.5 to 803.5, ^{12}C-butyric acid was incorporated in a ^{13}C-labeled culture into **4**, as shown by a shift of −4 Da from *m*/*z* 854.5 (U-^{13}C-labeled **4**) to *m*/*z* 850.5, and five carbons from the ^{12}C-leucine-derived isovaleryl unit were incorporated into **5** and **6** in ^{13}C medium due to the presence of isovaleryl (in **5**) or 5-methylhexyl acyl groups (in **6**), resulting in a −5 Da shift in both compounds. Additionally, a sufficient amount of **4** was isolated allowing full characterization via NMR spectroscopy (Table S3, Figure S6). The presence of an acetyl moiety in **2** was deduced from the HRESIMS data, and the valine labeling results showed that one valine is present in the side chain (amino acid two), thus securing the acetyl group as the acyl moiety. The presence of a hexanoyl moiety in **7**, which was produced only in very minor amounts (Table 2), was proposed, as neither four carbons from valine (as isobutyryl) nor leucine were incorporated into the acyl chain and assuming that the acyl moiety is not branched (data not shown). For **8** and **9** the incorporations of α-aminobutyric acid and isoleucine in ^{13}C medium were detected at amino acid 1

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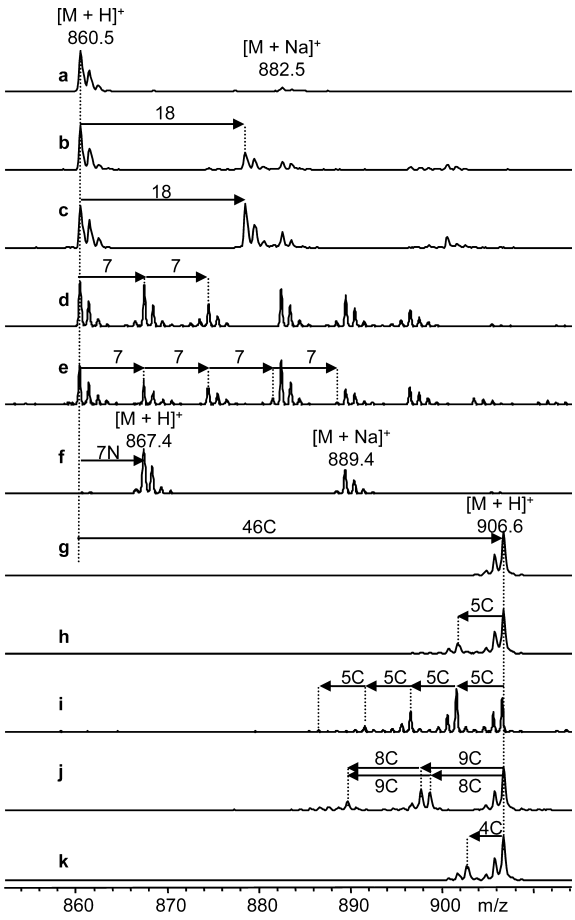
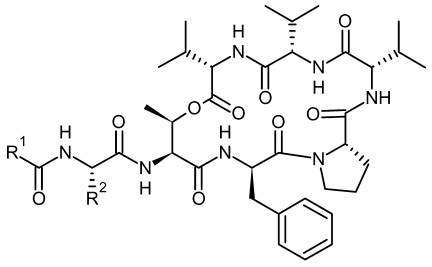
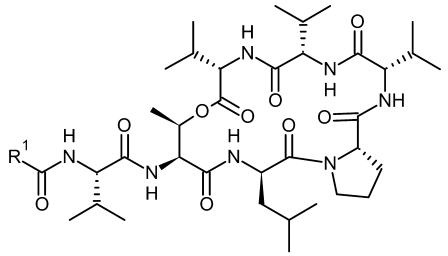


Figure 3. MS spectra from labeling experiments of xentrivalpeptide A (**1**). LB medium (^{12}C, a), LB medium with *p*-fluoro-DL-phenylalanine (^{12}C + *p*-F-Phe, b), with *p*-fluorophenylacetic acid (^{12}C + *p*-F-PAA, c), with additional L-[2,3,3,5,5′,6,6′,7-^{2}H]phenylalanine (^{12}C + $^{2}H_8$-Phe, d), with DL-[2,3,4,4,4,5,5,5-$^{2}H_8$]valine (^{12}C + $^{2}H_8$-Val, e), ^{15}N medium (^{15}N, f), ^{13}C medium (^{13}C, g), ^{13}C medium with L-proline (^{13}C + Pro, h), with L-valine (^{13}C + Val, i), with L-phenylalanine (^{13}C + Phe, j), and with L-threonine (^{13}C + Thr, k), respectively.



	R^1	R^2
xentrivalpeptide A (**1**)	Bn	*i*Pr
xentrivalpeptide B (**2**)	Me	*i*Pr
xentrivalpeptide C (**3**)	Et	*i*Pr
xentrivalpeptide D (**4**)	Pr	*i*Pr
xentrivalpeptide E (**5**)	*i*Bu	*i*Pr
xentrivalpeptide F (**6**)	*i*hexyl	*i*Pr
xentrivalpeptide G (**7**)	*n*pentyl	*i*Pr
xentrivalpeptide H (**8**)	Bn	Et
xentrivalpeptide I (**9**)	Bn	*sec*Bu

instead of the usual valine due to the mass shifts of 4 and 6 Da, respectively, and an unchanged fragment B for both compounds (Figure S2). As isoleucine is incorporated into **9**, the configuration in the isoleucine side chain is proposed to be *S*, as for the natural amino acid. The R^1 group was determined to be phenylacetyl in **8** and **9** from the 8 Da mass shifts from ^{12}C-phenylalanine in the ^{13}C medium and incorporation of *p*-fluorophenylacetic acid (Figure S2).



	R^1
xentrivalpeptide J (**10**)	Pr
xentrivalpeptide K (**11**)	*i*Bu
xentrivalpeptide L (**12**)	Bn
xentrivalpeptide M (**13**)	*i*hexyl

Fragmentation of xentrivalpeptides J–M (**10**–**13**) revealed a fragment B with *m*/*z* 591.3 (Table S1). The difference of −34 Da compared to fragment B of **1** points to a leucine/isoleucine instead of a phenylalanine, which was confirmed by the incorporation of D_{10}-leucine into **10**–**13** (Figure S3) and no incorporation of phenylalanine into fragment B in these compounds. Moreover, MS fragmentation proved that **10**–**12** showed an identical fragmentation of fragment B with fragments D and E showing the mass shift of 34 Da. As valine incorporation could be observed in the side chain for **10**–**13**, these compounds again differed only in the acyl moiety, whose nature was assigned from the incorporation of butyric acid in **10**, leucine-derived isovalerate incorporation in **11** and **13**, and the incorporation of 4-fluorophenylacetic acid or phenylalanine in **12** (Figure S3), as described above for **4**, **5**, and **1**, respectively.

Xentrivalpeptide N (**14**, $C_{47}H_{67}N_7O_9$) showed a fragment B (Table S1) with *m*/*z* 639.3, indicating the presence of leucine/isoleucine instead of valine in the ring, as was confirmed by labeling with isoleucine in fully ^{13}C-labeled medium and by the expected mass shift of 6 Da in fragment B (Figure S4). The position of the isoleucine was readily identified from MS fragmentation experiments that confirmed the 14 Da mass shift only in fragments C, E, and F (Table S1, Figure 2), which is consistent with isoleucine (2*S*,3*S* configuration as in **9**) as amino acid 6. All other feeding experiments confirmed that **14** is otherwise identical to **1**.

Fragment B of xentrivalpeptides O (**15**) and P (**16**) showed *m*/*z* 611.3 (Table S1), indicating the loss of a methyl group compared to **1**. Thus the presence of α-aminobutyric acid instead of valine was proposed. This was confirmed in the respective feeding experiment (Figure S5). Fragments C, E, and F showed the −14 Da mass shift compared to **1**, thus again confirming amino acid 6 as the variable position (Table S1). Whereas the structure of **15** could be confirmed from labeling experiments and MS fragmentation experiments (Figure S5), labeling of **16** was very weak. However, as no labeling of leucine or isoleucine was observed and butyrate is an abundant starting

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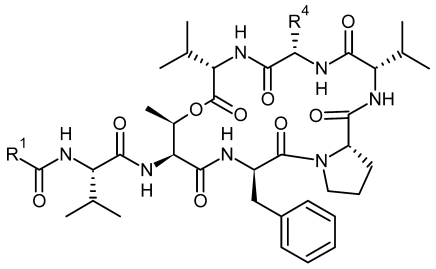
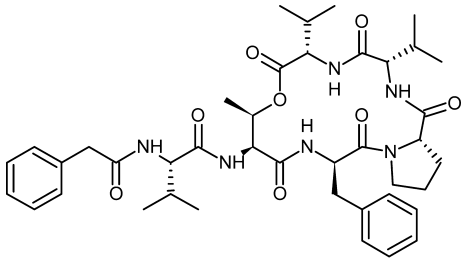


	R^1	R^4
xentrivalpeptide N (**14**)	Bn	*sec*Bu
xentrivalpeptide O (**15**)	Bn	Et
xentrivalpeptide P (**16**)	Pr	Et

unit for xentrivalpeptides (Table 2), we postulate that **16** has a butyryl unit as found in **4**.

The molecular formula of $C_{41}H_{56}N_6O_8$ for xentrivalpeptide Q (**17**) as determined by HRESIMS indicated the loss of one valine moiety compared to **1**. Fragmentation for **17** differed significantly from that of **1**−**16**. Fragments A′ and B′ with *m*/*z* 544.3 and 526.3, which correspond to fragments A and B in **1**−**16**, showed the expected loss of one valine moiety due to the loss of 99 Da. Additional fragments confirmed that only one valine in the ring is missing (Figure S7). Thus, **17** was confirmed to be a ring-contracted derivative of **1**.



xentrivalpeptide Q (**17**)

The xentrivalpeptides are the third and by far the most diverse class of depsipeptides isolated from *Xenorhabdus*[8,16−18] and thus a nice example of natural combinatorial biochemistry, which in addition to the N-acyl variability is in part due to relaxed amino acid specificities in the corresponding adenylation (A) and condensation (C) domains of the nonribosomal peptide synthetase (NRPS) responsible for biosynthesis.[21−23] Whereas this was sometimes interpreted as a "mistake" of the respective enzymes in the past, it might in fact be beneficial for the producing organism to generate a large chemical diversity with a minimal set of building blocks and enzymes.[24] As these derivatives might also have different biological activities, the resulting compounds might allow *Xenorhabdus* to kill several different insect larvae as well as to protect them against different food competitors. However, so far no biological activity has been detected for **1** in standard bioactivity tests (antibiotic Gram-negative or Gram-positive, antifungal, cytotoxic against eukaryotic cells). Whereas biological activity for other *Xenorhabdus*-derived compounds has been detected at low concentrations,[7] only a high concentration (100 μg/mL) of **1** led to actin ruffling, pointing to the cytoskeleton as the target for the xentrivalpeptides (Figure S8), as will be investigated in the future in more detail.

Because the xentrivalpeptides lack any unusual amino acids, their synthesis should be facile using solid-phase methods.[8] Therefore, current efforts in our group are directed toward synthesizing the xentrivalpeptides to verify the proposed absolute configuration and to broaden the scope of biological testing.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined with a MPM-H2 melting point meter by Schropp Gerätetechnik and are uncorrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded with a GE NanoVue Plus photometer. IR spectra were obtained with a Perkin-Elmer Spectrum Two spectrometer. NMR spectra were recorded with a Bruker AV 400 spectrometer using deuterated methanol as solvent. Collision-induced dissociation (CID) was performed on the ion trap in the amaZon X in positive ion mode, and HRESIMS analysis was carried out using an LTQ Orbitrap (Thermo Fisher). Preparative HPLC was performed using an Waters autopurification system.

Strain Cultivation and Extract Preparation. *Xenorhabdus* sp. 85816 was identified as *X. stockiae* based on its *recA* sequence (Genbank accession number JX485977) and was cultivated in LB medium at 30 °C. For extract analysis, 20 mL of LB medium was inoculated with 1% overnight culture, and 2% Amberlite XAD-16 was added. After cultivation for 72 h, the XAD beads were collected, washed once with 5 mL of H_2O, and extracted with 20 mL of MeOH. To isolate the peptides, XAD beads from a 5 L culture were extracted with 200 mL of MeOH three times. The extract was fractionated on silica gel with $CHCl_3$ and MeOH with a linear gradient of 5% to 40% MeOH. The fractions were analyzed by HPLC-MS, and fractions eluted with ∼20% MeOH contained the xentrivalpeptides. The enriched fraction containing xentrivalpeptides was used for HPLC purification.

Feeding Experiments in Nonlabeled Medium. The cell pellet from a 1 mL overnight *Xenorhabdus* sp. 85816 culture was washed once and resuspended with 1 mL of LB medium. The 5 mL feeding culture was inoculated with a 1% (50 μL) solution of washed cells. After incubation for 6 h at 30 °C, 200 rpm, 50 μL of a stock solution (100 mM) of substrate (*p*-fluoro-DL-phenylalanine, *p*-fluorophenylacetic acid, L-[2,3,3,5,5′,6,6′,7-$^2H_8$]phenylalanine, DL-[2,3,4,4,4,5,5,5-$^2H_8$]valine, L-[2,3,3,4,5,5,5,5′,5′,5′-$^2H_{10}$]leucine, or [U-$^2H_6$]propionic acid) was added. Two further feedings of substrate were carried out after 24 and 48 h to a final concentration of 3 mM. Cultures were harvested after 72 h of incubation by extraction with 5 mL of EtOAc. The extracts were evaporated to dryness and dissolved in 500 μL of MeOH. Diluted solutions were analyzed by HPLC-MS, and control cultivation was carried out without feeding.

Feeding Experiments in Labeled Medium. The cell pellet from a 1 mL overnight *Xenorhabdus* sp. 85816 culture was washed once and resuspended with 1 mL of ISOGRO-^{13}C medium. ISOGRO-^{13}C medium was prepared with 1 g of ISOGRO-^{13}C powder (Sigma-Aldrich), 1.8 g/L K_2HPO_4, 1.4 g/L KH_2PO_4, and 11.1 mg/L $CaCl_2·H_2O$ in 100 mL of H_2O. The feeding culture was started by inoculation of a 1% (50 μL) solution of washed cells in 5 mL of ISOGRO-^{13}C medium. After incubation for 6 h at 30 °C, 200 rpm, 50 μL of a stock solution (100 mM) of substrate (L-α-aminobutyric acid, butyric acid, L-α-valine, L-α-leucine, L-α-proline, L-α-threonine, L-α-phenylalanine, or L-α-isoleucine) was added. Two further feedings of every substrate were carried out after 24 and 48 h of incubation to a final concentration of 3 mM. Cultures were harvested after 72 h of incubation by extraction with 5 mL of EtOAc. The extracts were evaporated to dryness and dissolved in 500 μL of MeOH. Diluted solutions were analyzed by HPLC-MS. Control cultivation was carried out without feeding. *Xenorhabdus* sp. 85816 was also cultivated in ^{15}N-labeled medium without feeding of substrate. The ^{15}N-labeled medium was prepared in the same manner as the ^{13}C-labeled medium.

HPLC and Mass Spectrometry. Analysis of the extracts was carried out on an Ultimate 3000 LC system from Dionex, coupled to an amaZon X electrospray ionization mass spectrometer from Bruker Daltonics. Peptides were separated on a C_{18} column (Acquity UPLC

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BEH, 1.7 μm 2.1 × 50 mm, flow rate 0.6 mL/min, Waters). Acetonitrile−H_2O containing 0.1% HCOOH was used as the mobile phase under a linear gradient from 35% to 55% CH_3CN over 11 min. The relative amount of derivatives was obtained by comparison of the peak areas to the peak area of **1**. For preparative purification an Xbridge column (Waters, OBD, 5 μm, 19 × 150 mm) was used with CH_3CN−H_2O containing 0.1% HCOOH as the mobile phase with a linear gradient from 35% to 55% CH_3CN over 22 min for the separation.

Xentrivalpeptide A (1): colorless solid; mp 140 °C; $[\alpha]^{20}_D$ −33 (*c* 0.36; $CHCl_3$); UV (MeOH/H_2O) λ_{max} (log ε) 218 (4.34), 258 (3.82); IR ν_{max} 3280, 2967, 1756, 1633, 1531, 1454, 1157 cm^{-1}; for NMR data see Table 1; HRESIMS *m/z* 860.4880 $[M + H]^+$ (calcd for $C_{46}H_{66}N_7O_9$, 860.4922).

Determination of the Absolute Amino Acid Configurations (ref 20). Approximately 0.5 mg of **1** was hydrolyzed with 0.8 mL of 6 M HCl in an ACE high-pressure tube at 110 °C for 16 h. The hydrolysate was evaporated to dryness and resuspended in 100 μL of H_2O. To each half-portion (50 μL) were added 10 μL of 1 M $NaHCO_3$ and 100 μL of 1% FDLA N_α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide or D-leucinamide (L-FDLA or D-FDLA, solution in acetone), respectively. The reaction vials were closed and placed in a water bath at 40 °C for 1 h. After that, the reactions were cooled to room temperature, quenched with 10 μL of 1 M HCl, and evaporated to dryness. The residue was dissolved in 400 μL of MeOH. The analyses of L- and LD-FDLA-derivatized amino acids were carried out with LC-MS. Acetonitrile−H_2O containing 0.1% HCOOH was used as solvent with a linear gradient from 20% to 60% CH_3CN over 34 min.

Bioactivity Tests. Xentrivalpeptide A (**1**) was tested against Gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis*) and Gram-positive bacteria (*Staphylococcus aureus, S. epidermidis, Micrococcus luteus, Bacillus subtilis*), the yeast *Saccharomyces cerevisiae*, the eukaryotic cell lines L-929 (mouse connective tissue fibroblast; ACC 2) and HL-60 (human acute myeloid leukemia; ACC 3), and *Galleria mellonella* hemocytes as described previously.[7]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HRMS and fragmentation data for all compounds, NMR data for **1** and **4**, and activity of **1** against *G. mellonella* hemocytes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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