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Supplementary information

Structure elucidation and biosynthesis of PAX peptides in *Xenorhabdus nematophila* HGB081

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Materials and methods

Cultivation of strains: *X. nematophila* HGB081 (ATCC 19061) was maintained on LB-agar and grown at 30 °C for 48 h. For investigation of secondary metabolite production, 50 ml LB-medium (pH 7.5) in 500 ml flasks were inoculated with a single colony from solid medium and cultivated for 72 h under permanent shaking at 120 rpm. Liquid cultures were harvested by centrifugation at 4000 rpm for 20 min. The supernatant was frozen at -20°C.

Extraction/Enrichment of peptides: Culture supernatant was adjusted to 0.02 M TRIS/HCl pH 9.0, 0.1 M NH_4OH and loaded onto SEP-PAK-CM solid phase extraction (SPE)-cartridges (Waters, Eschborn, Germany). PAX peptides were eluted with 0.5 M HCl/50 % methanol, pH 1. After concentration of this fraction with a vacuum evaporator, the concentrate was diluted in 0.1 % TFA and loaded onto Strata C18E-SPE-cartridges (Phenomenex, Aschaffenburg, Germany) which were handled according to the manufacturer´s instructions. Loaded SPE-cartridges were washed with 40 % methanol/0.1 % trifluoroacetic acid (TFA). Peptides were eluted with 99.9 % methanol/0.1 % TFA. The eluate was completely dried with a vacuum centrifuge.

Mass spectrometry and sample preparation: Culture supernatants, extracted peptides, and derivatized peptides were analyzed with a MALDI LTQ Orbitrap XL (Thermo Fisher Scientific, Inc., Waltham, MA) equipped with a nitrogen laser at 337 nm and used in the Fourier transformation mode. The following instrument parameters were used: laser energy, 16 µJ; automatic gain control, on; auto spectrum filter, off; scan mode, full; resolution, 100000; plate motion, survey CSPS. Spectra were analyzed and monoisotopic mass lists were generated using Qual Browser (version 2.0.7; Thermo Fisher Scientific, Inc., Waltham, MA). This software was also used for the generation of elemental formulas from high accurate masses. The following adjustments were used for identification of PAX-like peptides: charge, 1; nitrogen rule, do not use; mass tolerance, 3 ppm; RDB equiv., 8-12; possible number of elements C, H, N, O, S, 52-56, 95-110, 13-15 (14 excluded), 5-10, respectively.
MS/MS analysis was performed with the MALDI LTQ Orbitrap XL using identical parameters as described above, except of the following: resolution, 15000; wide band activation, on;

precursor width range, optimized for every single precursor with regard to complete isolation. Optionally MS/MS experiments were performed using the ion trap of the MALDI LTQ Orbitrap XL as mass analyzer.
Furthermore a Voyager DE-STR mass spectrometer was used, which is equipped with a 337 nm nitrogen laser as well. MALDI-TOF parameters were polarity, positive; operation mode, reflector; accelerating voltage, 20 kV; grid voltage, 68.5%; delay time, 150 ns; mass range, 500-4,000 Da; low mass gate, 450 Da; bin size, 0.5 ns. Each mass spectrum resulted from accumulation of 500 single spectra. Data Explorer 4.9 (Applied Biosystems, Darmstadt, Germany) was used for analysis of the MS spectra using the following parameters: baseline correction; gaussian smooth, 5; minimum S/N ratio, 10; mass peak filter, monoisotopic peaks.
For measurement of peptides without prior processing, growth supernatants were diluted 1:2 in 30 % ACN/0.1 % TFA. Fractions from solid phase extraction and derivatized peptide samples were dried in a vacuum centrifuge and dissolved with 30 % ACN/0.1 % TFA. All samples were mixed 1:1 with 0.5 µl of a 20 mM 4-chloro-α-cyanocinnamic acid (ClCCA)[1,2] in 70 % ACN onto a polished stainless steel target and air-dried.

Derivatization of PAX-peptides: SPE-enriched PAX peptides were acetylated after dilution in 25 mM NH_4CO_3 (Roth, Karlsruhe) solution resembling the volume of the culture that was originally extracted by SPE. One part of the dissolved extract was mixed with three parts of acetic anhydride/methanol (1:3) and incubated for 12 h at 24 °C. For dimethylation of lysine sidechains, 6.3 mg SPE-enriched and dried PAX peptides were dissolved in 100 µl methanol and 5.6 mg para-formaldehyde, 4.5 mg sodium cyanoborohydride, and 10 µl acetic acid were added.[3] The mixture was stirred overnight at ambient temperature.

Feeding experiments: The incorporation of amino acids was tested by feeding L-lysine and L-arginine to HGB081 cultures cultivated in [U-^{13}C]medium (ISOGRO® C-powder growth medium 99 atom %C, Sigma Aldrich) background. The medium also contained 10 mM K_2HPO_4, 10 mM KH_2PO_4, 8 mM $MgSO_4 \cdot 7H_2O$, and 90 µM $CaCl_2 \cdot H_2O$. *X. nematophila* HGB081 was cultivated overnight at 30 °C in 5 ml LB-medium. After centrifugation for 5 min at 4000 rpm, the supernatant was removed and the pellet was washed once by resuspension in one culture volume of ^{13}C-medium. After centrifugation and removal of the supernatant, cells were again resuspended in one culture volume of ^{13}C-medium and used to inoculate 10 ml of ^{13}C-medium with a dilution of 1:100. After 4 h L-arginine and L-lysine (both biochemical grade, Roth, Karlsruhe) were added separately in portions of 1 mM. Feeding was repeated after 14, 24, 38, 48 and 56 h resulting in a final concentration of 5 mM. The nature of the fatty acid starter unit of the lipopeptides produced by *X. nematophila* HGB0181 was investigated by feeding of L-[5,5,5-$^2H_3$]leucine, DL-[2,3,4,4,4,5,5,5-$^2H_8$]valine, L-phenyl-$^2H_5$-alanine, and [U-$^2H_6$]propionic acid to LB-medium (deuterated compounds from Sigma-Aldrich, ≥ 98+ % deuterium). 1 ml of an overnight-culture was centrifuged for 1 ml and the pellet was washed with 1 ml of fresh LB-medium. The resuspended cells were used to inoculate 10 ml LB-medium with a dilution of 1:500. After 5 h of cultivation at 30 °C,

deuterated compounds were separately fed to a final concentration of 1 mM. Feeding to a concentration of 1 mM was repeated 24, 48, 72, and 96 h after inoculation of the culture resulting in a final concentration of 5 mM. Cultures were harvested 24 h after the last feeding by centrifugation at 12000 rpm for 5 min. The supernatant was frozen at -20 °C.

Determination of 3-OH-fatty acid stereochemistry: 3-OH-stereochemistry of the 14:0-3OH fatty acid was analyzed after derivatization of PAX-fatty acid-methylesters with (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride ((*R*)-(–)-MTPA-Cl).[4] For this purpose, SPE-enriched and dried PAX peptides were converted into their methylesters[5] and (*R*)-(–)-MTPA-Cl derivatization was performed as described before.[6] Consecutively, stereochemistry was analyzed by comparison of retention times with a (*R*)-(–)-MTPA-Cl derivatized BAME-standard (Sigma) containing racemic 14:0-3OH-fatty acids.

Elucidation of amino acid configuration: Amino acid configuration was determined according to the advanced Marfey´s method.[7-9] Briefly, 1 mg of SPE-enriched PAX peptides was hydrolyzed in 6 M HCl at 110 °C overnight in an ACE-tube. After vacuum concentration of the hydrolyzed sample to dryness, the sample was diluted in 100 µl deionized water and split into two equal portions. 10 µl 1M $NaHCO_3$ and 100 µl 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) or D-FDLA were added and the reaction was allowed to proceed at ambient temperature in the dark for one hour. After stopping the reaction by addition of 10 µl 1 M HCl, samples were dried and individually diluted with 400 µl methanol. Stereochemistry of amino acids was determined by comparison of elution orders of the L-FDLA- and L-/D-FDLA-derivatized samples.

Construction of PAX-mutant: DNA isolation, plasmid preparation, restriction digest, PCR, gel electrophoresis and ligation reaction were performed according to standard methods[10]. PCR amplified fragments were isolated from agarose gels with the Fermentas GeneJET™ Gel Extraction Kit (Fermentas GmbH, St. Leon-Rot, Germany). PCR experiments were conducted with Phusion Polymerase (Finnzymes, Espoo, Finnland) and used according manufacturer´s instructions.
For construction of PAX negative strain, *xnc1_2781* was disrupted by plasmid integration. For that purpose an internal fragment of the desired gene (544bp) was amplified with PCR. The PCR was performed by using Phusion polymerase (Finnzymes) and primers Xn5620-fw (5'-ATCGGCATGCCCGTCACCAGGGTATCAGTTCA-3') and Xn5620-rv (5'-ATCGGAGCTCTGCCTCCACAATGCCTTTCA-3') containing *Sph*I and *Sac*I restriction sides (underlined). After purification of the PCR product and digest with *Sph*I and *Sac*I (Fermentas), the fragment was cloned into pDS132 plasmid[11] with chloramphenicol resistence gene. The resulting plasmid-construct was introduced into *E. coli S17-1 λ pir* by elctroporation and afterwards conjugated into rifampicin-resistent *X. nematophila* HGB081 strain as described previously.[12,14] Colonies were selected by growth on LB agar containing chloramphenicol and rifampicin. The genotype of the resulting HGB081- *xnc1_2781::cat* mutant was verified by PCR using the two plasmid-specific primers pDS132fw (5'-

GATCGATCCTCTAGAGTCGACCT-3') and pDS132rv (5'-ACATGTGGAATTGTGAGCGG-3') and two genome-specific pairs of primers vXn5620-fw (5'-TGGTGAAATACAAGCGATCCTC-3') and vXn5620-rv (5'-TTGGGGTTCACTGGTCTGGT-3').

The genome sequence of *X. nematophila* ATCC 19061 is available at the MaGe website (https://www.genoscope.cns.fr/agc/mage/wwwpkgdb/Login/log.php?pid=24).

Supplementary Figures and Tables

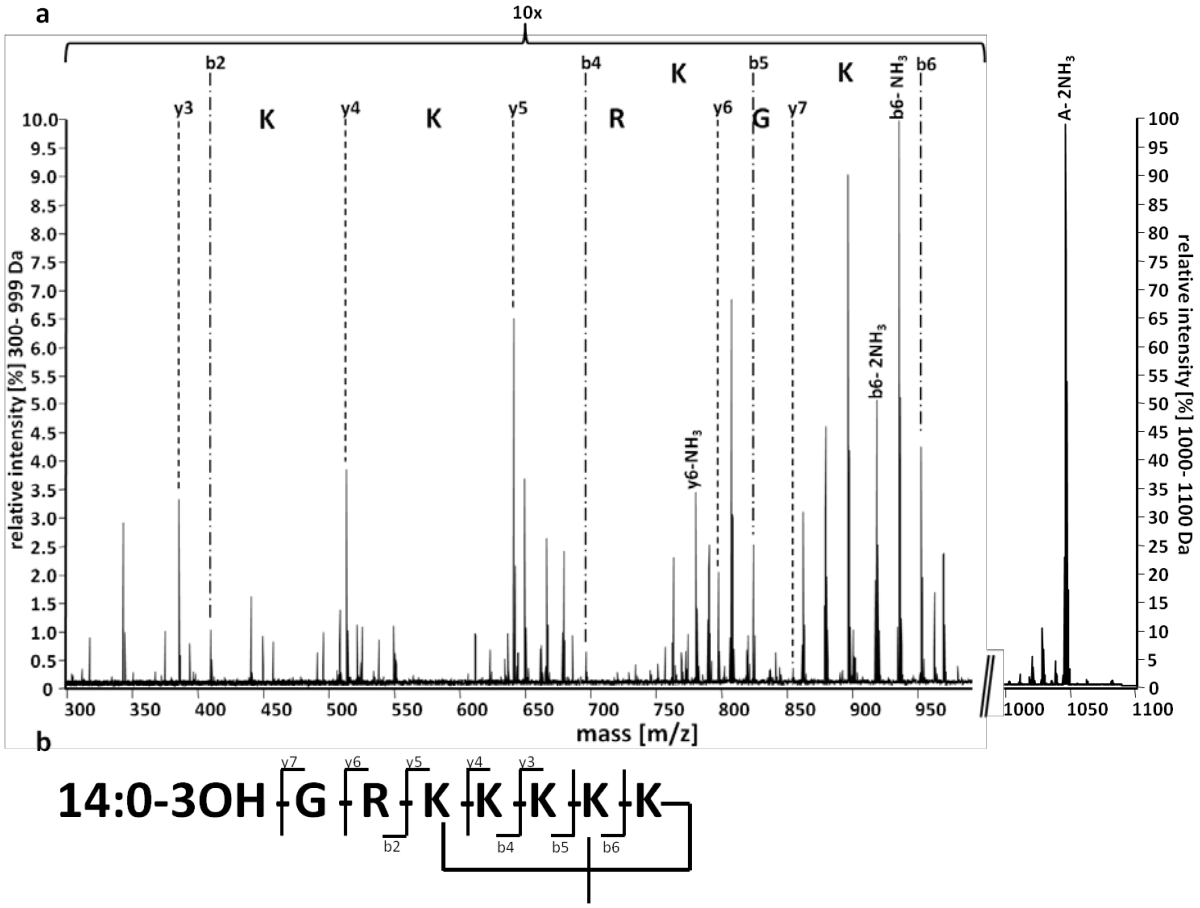


Figure S1. a) CID-ion-spectrum of PAX2´ [**5**] ($[A+H]^+$= 1080.7991 Da) recorded in FTMS-mode of a MALDI LTQ Orbitrap XL-mass spectrometer. b-ions are labeled with dashed/dotted lines and y-ions with dashed lines. For better visualization of fragment-ions below 1000 Da, this part of the spectrum is depicted in ten-fold magnification. b) Primary structure of PAX2´ with detected b- and y-ions. The fatty acid residue is depicted according to the calculation in table 1.

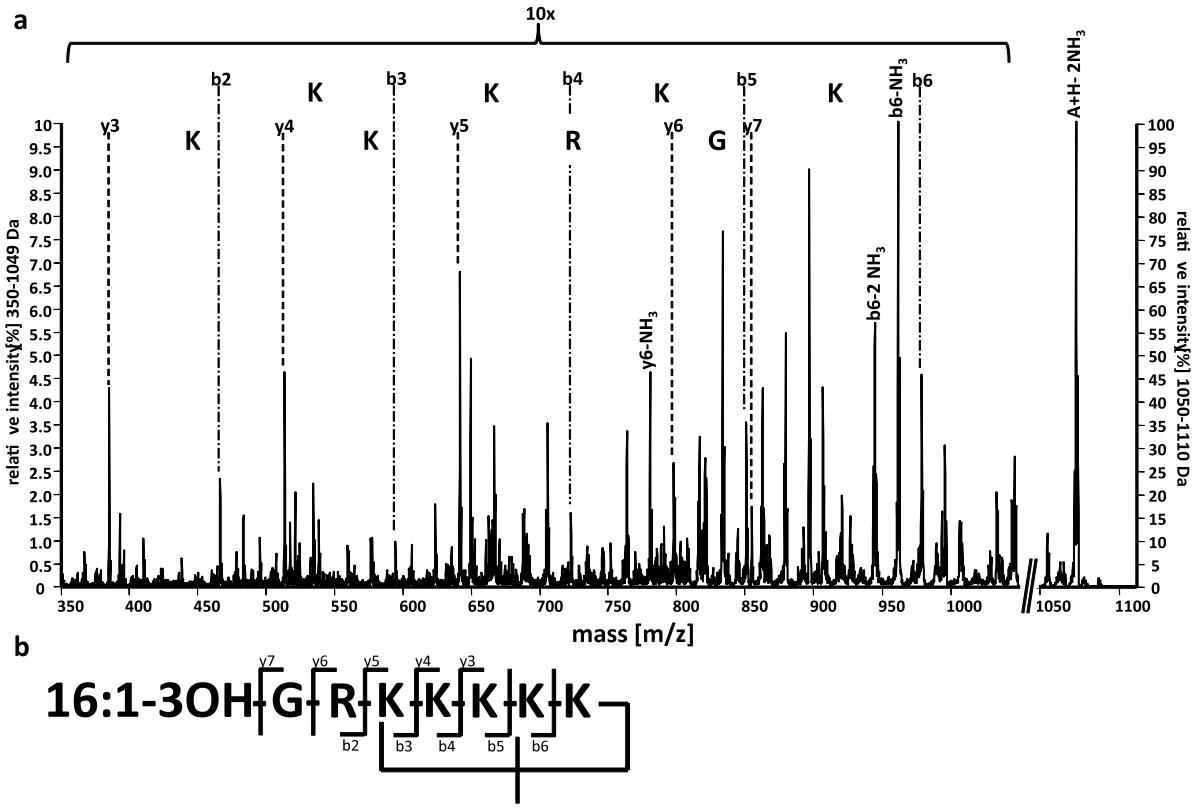


Figure S2. a) CID-ion-spectrum of PAX9 [**9**] ($[A+H]^+$= 1106.8145 Da) detected with the linear ion trap of a MALDI LTQ Orbitrap XL-mass spectrometer. b-ions are labeled with dashed/dotted lines and y-ions with dashed lines. For better visualization of fragment-ions below 1050 Da this part of the spectrum is depicted in ten-fold magnification. b) Primary structure of PAX9 with detected b- and y-ions. The fatty acid residue is depicted according to the calculation in table 1.

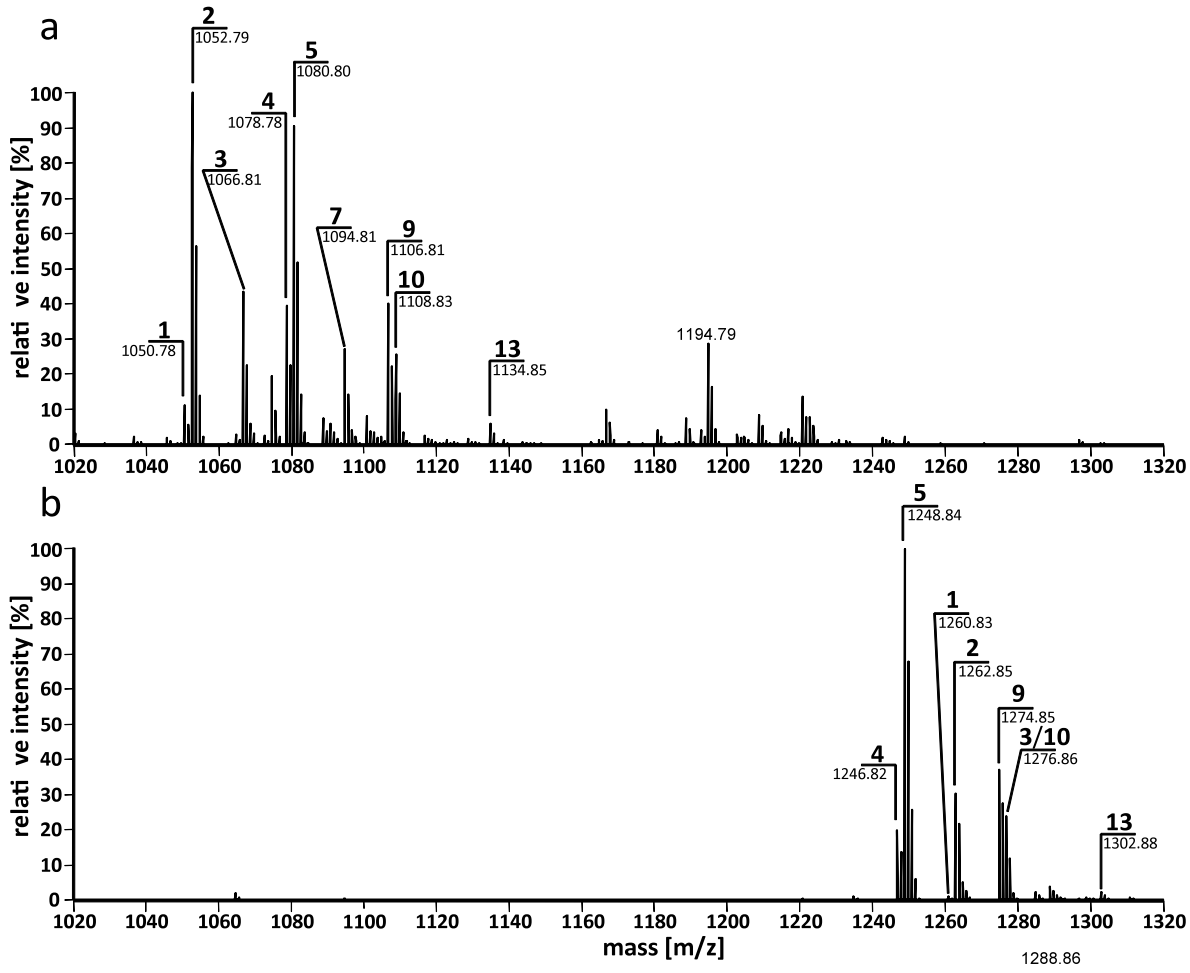


Figure S3. Positive ion mode MALDI-TOF mass spectrum of PAX-peptides extracted from *X. nematophila* HGB081 (a) and of the same sample after acetylation with acetic anhydride (b).

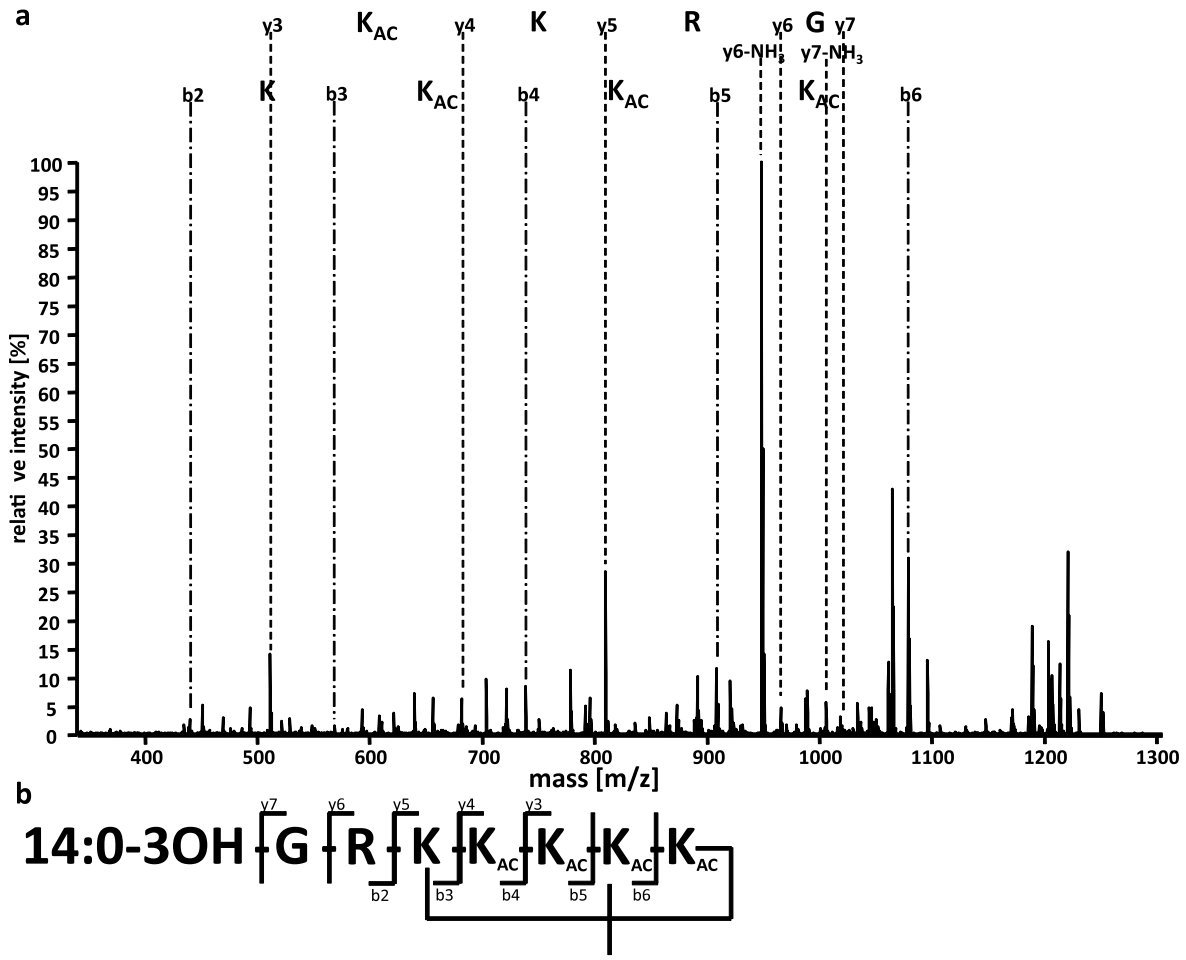


Figure S4. a) CID-ion-spectrum of acetylated **5** ($[A+H]^+$= 1248.841Da) recorded in FTMS-mode of a MALDI LTQ Orbitrap XL-mass spectrometer. b-ions are labeled with dashed/dotted lines and y-ions with dashed lines. b) Primary structure of acetylated PAX2´ with detected b- and y-ions. The fatty acid residue is depicted according to the calculation in table 2.

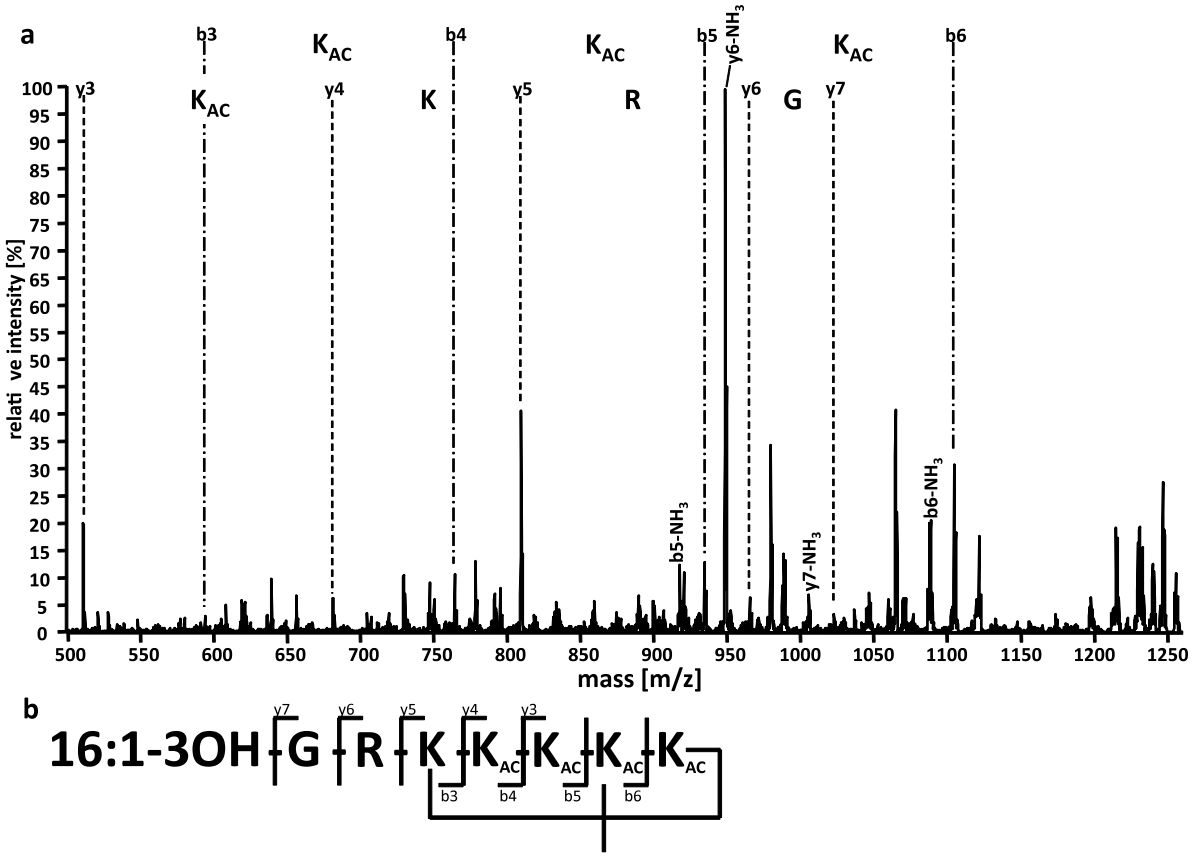


Figure S5. a) CID-ion-spectrum of acetylated **9** ($[A+H]^+$= 1274.8531Da) recorded in the linear ion trap of a MALDI LTQ Orbitrap XL-mass spectrometer. b-ions are labeled with dashed/dotted lines and y-ions with dashed lines. b) Primary structure of acetylated PAX9 with detected b- and y-ions. The fatty acid residue is depicted according to the calculation in table 2.

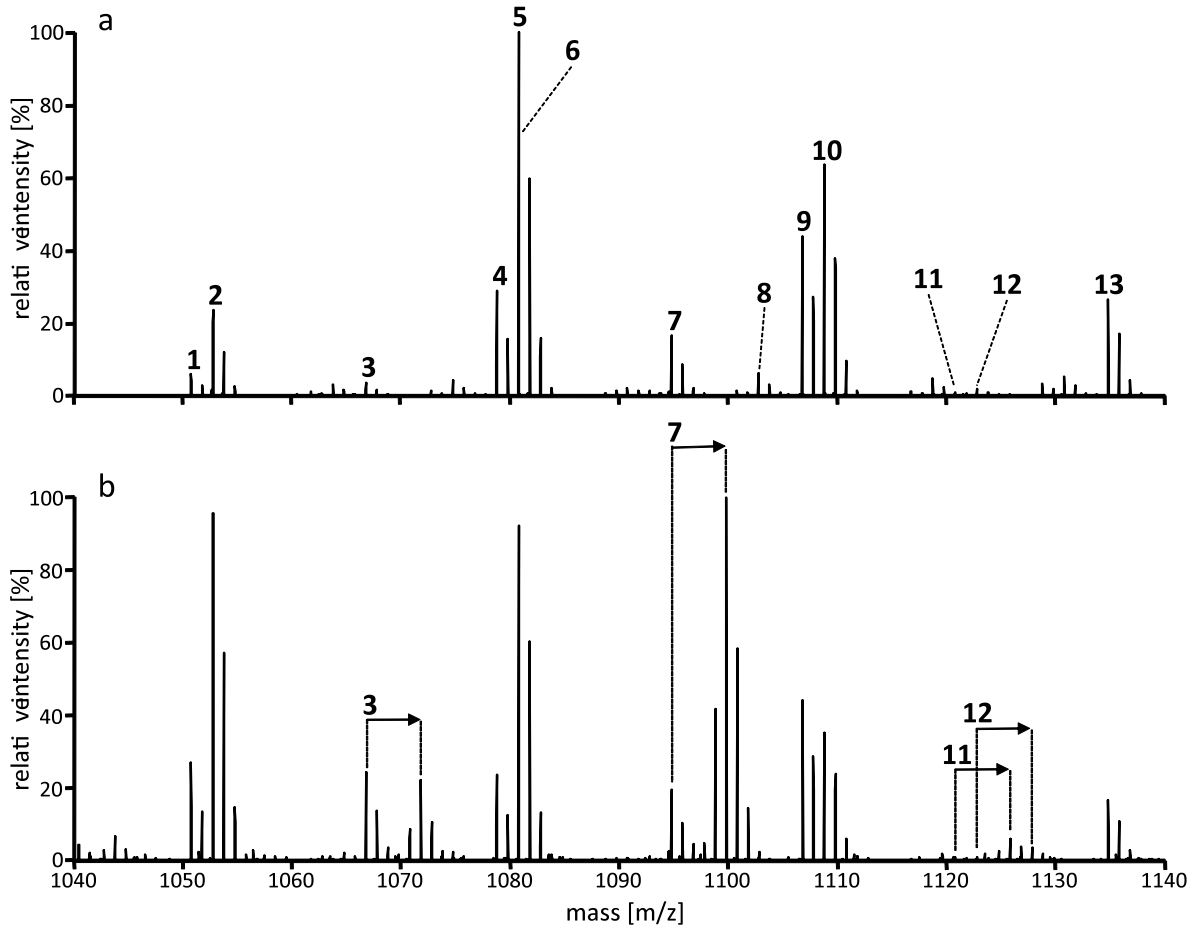


Figure S6. MALDI-Orbitrap-mass spectra of culture supernatants from *X. nematophila* HGB081 cultivated in LB-medium (a) and after supplementation with [U-$^{2}H_{6}$]propionate (b). Incorporation of feeding substrates is indicated by arrows (Table S1).

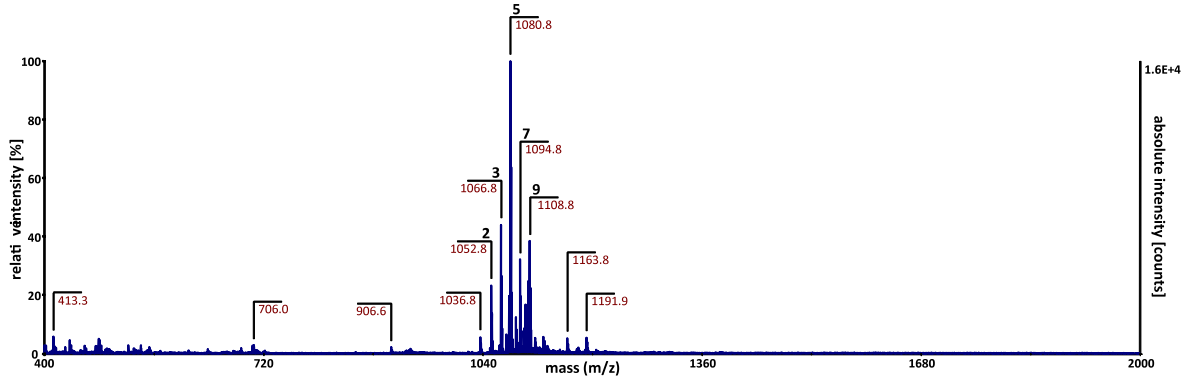


Figure S7. Positive ion mode MALDI-TOF mass spectrum of enriched PAX peptides from *X. nematophila* HGB081. Enrichment was performed by means of weak cation exchange solid phase extraction (SPE) followed by reversed phase SPE.

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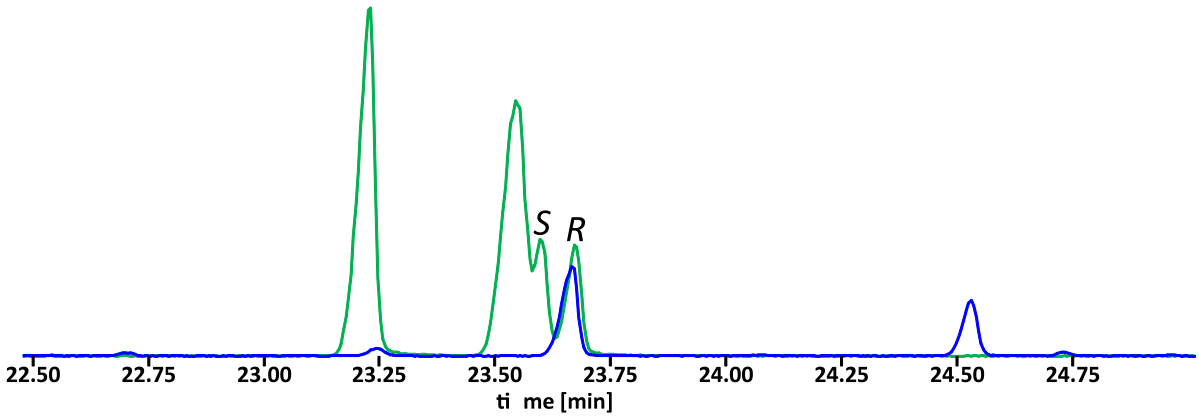


Figure S8. Chromatographic determination of β-carbon stereochemistry of PAX-14:0-3OH fatty acids after derivatization with (*R*)-(–)-MTPA-Cl. Depicted are EICs (189.1 Da) of a derivatized fatty acid standard (green) containing *S*-14:0-3OH-fatty acid (*S*) and *R*-14:0-3OH-fatty acid (*R*) as well as of a derivatized sample of PAX peptide fatty acids (blue). Comparison of retention times in both samples revealed, that PAX peptides exclusively contain the *R*-enantiomer of the 14:0-3OH fatty acid.

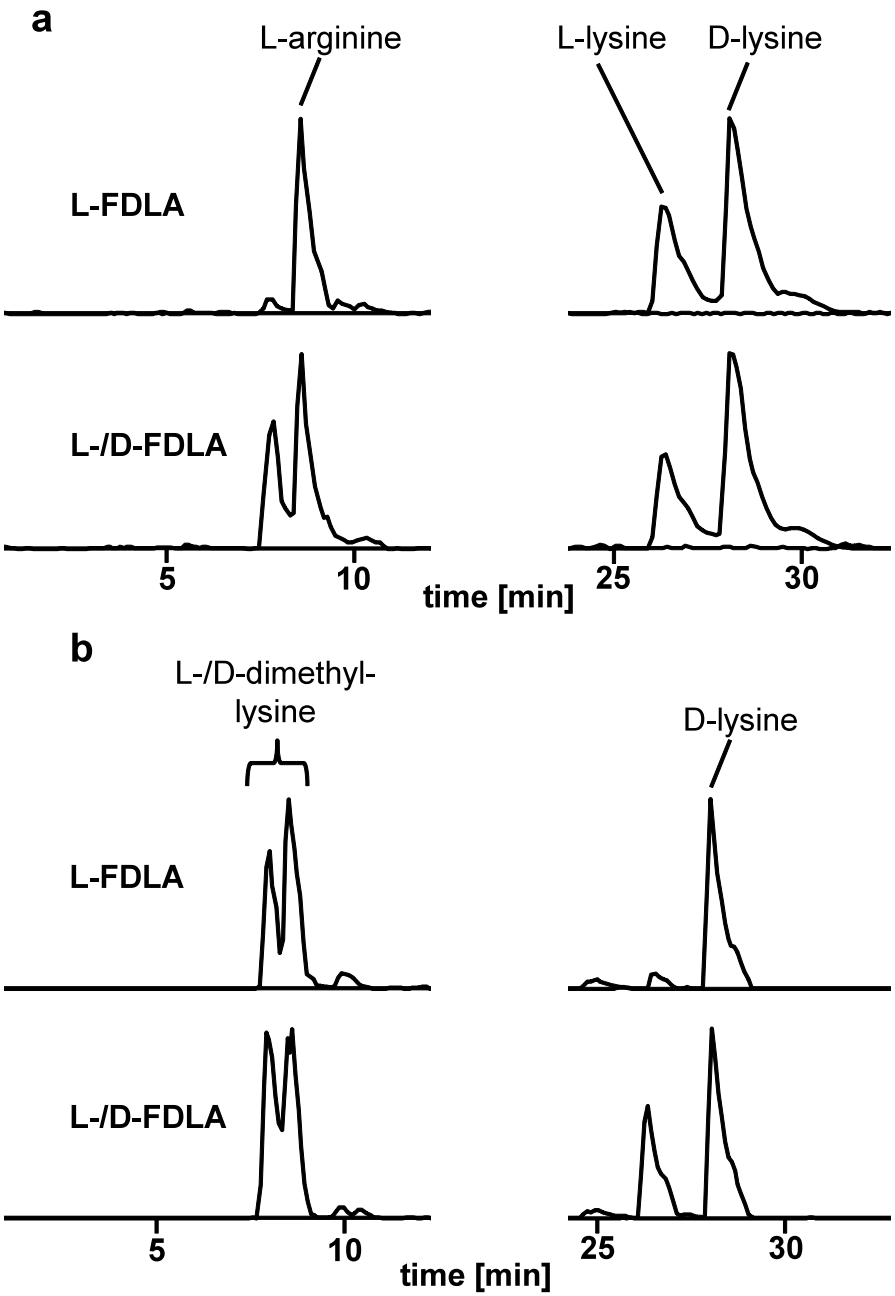


Figure S9. RP-HPLC-analysis of hydrolyzed PAX-peptides before (a) and after methylation (b) and subsequent derivatization with L-FDLA (upper) and D-FDLA (lower). Depicted are EIC of derivatized amino acids. According to Fujii *et al*.[8,13] the elution order of the FDLA derivatized amino acids reveals their stereochemistry. L-FDLA derivatized D-arginine elutes prior to the L-enantiomer whereas L-lysine elutes prior to its D-enantiomer. D-FDLA derivatized amino acids behave inversely.

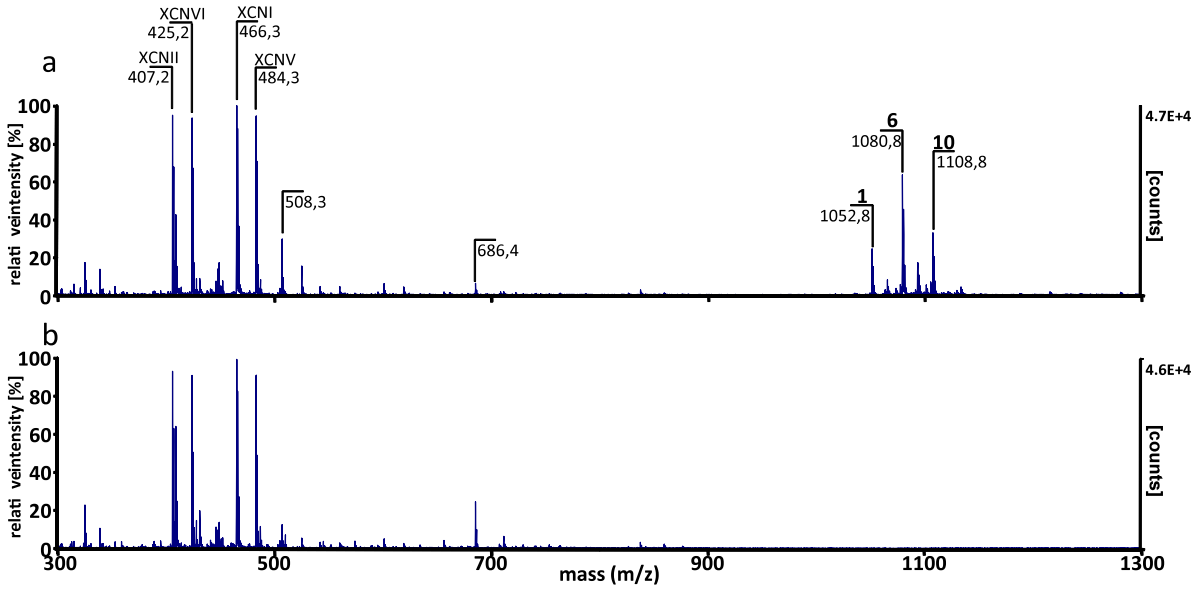


Figure S10. MALDI-TOF mass spectrum of an extracted culture of *X. nematophila* HGB081 (a) and of the same strain carrying a knockout of gene *xnc1_2782* (b). Monoisotopic *m/z* of PAX-peptides are labeled with their compound number. XCN I-IV indicate the different xenocoumacin-derivatives that were detected.[14]

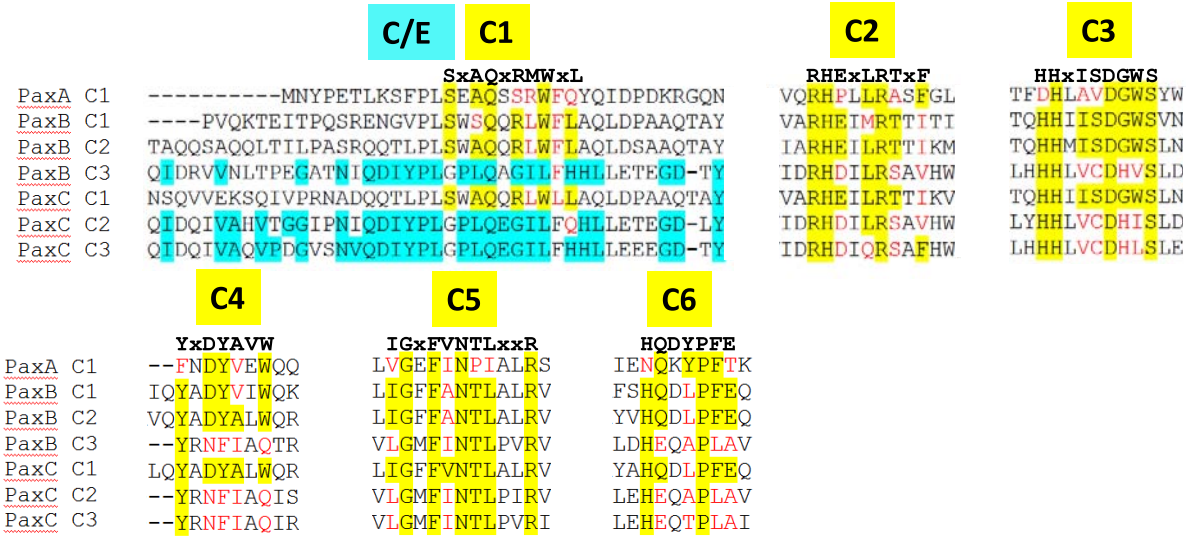


Figure S11. ClustalW-alignment of C-domains in the NRPS-proteins that have been shown to catalyze PAX-biosynthesis. Consensus sequences of C-domains[15] are highlighted with a yellow background, whereas the consensus motive of dual C/E-domains[16] are highlighted in blue.

Table S1. PAX peptides in a culture supernatant of *X. nematophila* HGB081 identified by means of a MALDI-FTMS-analysis. Deviations of the calibrated *m/z* to theoretical *m/z* of PAX-peptides with two possible variables (fatty acid chain and aa-residue 2) are calculated in ppm. Furthermore the detected *m/z* as well as the relative error [Δppm] of PAX-peptides are depicted, which were produced after feeding of [U-$^{2}H_{6}$]propionate as well as in ^{13}C-medium after feeding of L-[U-^{12}C]lysine or L-[U-^{12}C]arginine in [U^{13}C] medium.

no.	chem. formular	fatty acid	aa2	12C		12C acetylated		12C+D5-prpionate		13C		13C+C12-Lys		13C+C12-Arg	
				mass [m/z]	Δppm	mass [m/z]	Δppm	mass [m/z]	Δppm	mass [m/z]	Δppm	mass [m/z]	Δppm	mass [m/z]	Δppm
1	C52H100O9N13	14:1-3OH	K	1050.7772	1	1260.832	4.42	n.d.		n.d.				n.d.	
2	C52H102O9N13	14:O-3OH	K	1052.7926	-1.78	1262.85	4.07	n.d.		1104.9659	-2.74	1098.945 1092.925 1086.99047 1080.8847	-1.04 -0.96 -1.15 -1.06	n.d.	
3	C53H104O9N13	15:O-3OH	K	1066.8085	0.99	1276.862	3.27	1071.838	-0.5	n.d.				n.d.	
4	C52H100O9N15	14:1-3OH	R	1078.7839	1.49	1246.827	3.56	n.d.		1130.9533	-3.05			n.d.	
5	C52H102O9N15	14:O-3OH	R	1080.7991	1.07	1248.841	1.99	n.d.		1132.973	0.53	1126.9519 1120.9322 1114.9119 1108.8917 1102.871	-0.35 0 -0.18 -0.27 -0.81	1126.953	0.18
6	C54H106O9N13	16:O-3OH	K	1080.8232	0.09	n.d.		n.d.		1135.0036	-0.58	1128.9827 1122.9628 1116.9431 1110.9227 1104.9025	-1.29 -1.12 -0.77 -1.04 -1.14	n.d.	
7	C53H104O9N15	15:O-3OH	R	1094.8145	0.83	n.d.		1099.844	-0.89	1147.991	-0.44			n.d.	
8	C56H104O9N13	18:O-3OH	K	1102.8068	-0.59			n.d.		n.d.				n.d.	
9	C54H104O9N15	16:1-3OH	R	1106.8145	0.82	1274.853	-0.44	n.d.		1160.9942	-0.48	1154.9724 1148.9528 1142.9327 1136.9119	-1.95 -1.53 -1.54 -2.16	1154.974	-0.65
10	C54H106O9N15	16:O-3OH	R	1108.8306	1.22	1276.862	-5.96	n.d.		1163.0105	0.08	1156.9996 1144.9494 1138.929 1132.9087	-0.61 -0.62 -0.89 -0.11	1156.99	-0.09
11	C55H106O9N15	17:1-3OH	R	1120.8305	1.12	1288.857	-9.78	1125.859	-1.09	n.d				n.d.	
12	C55H108O9N15	17:0-3OH	R	1122.8447	-0.17	n.d.		1127.876	-0.43	n.d.				n.d.	
13	C56H108O9N15	18:1-3OH	R	1134.8458	0.8	1302.883	-1.74	n.d.		1191.0328	0.03	1185.0114 1178.991 1172.971 1166.9508	-1.07 -1.33 -1.25 -1.35	1185.012	-0.81

Table S2. *m/z* and mass errors of b- and y-ions generated by CID of **2**, **3**, **5**, **9**, **10**, and **13** as well as of acetylated derivatives from **5** and **9**.

	2		**3**		**5**		**9**		**10**		**13**		**5** acetylated		**9** acetylated	
CID-ion	det. m/z	Δ m/z	det. m/z	Δ m/z	det. m/z	Δ m/z	det. m/z	Δ m/z	det. m/z	Δ m/z	det. m/z	Δ m/z	det. m/z	Δ m/z	det. m/z	Δ m/z
y3	385.33	0.04	385.33	0.04	385.29	0.00	385.33	0.04	385.33	0.04	385.33	0.04	511.33	0.01	511.33	0.01
y4	513.42	0.03	513.42	0.03	513.38	-0.01	513.42	0.03	513.42	0.03	513.42	0.03	681.5	0.07	681.5	0.07
y5	641.5	0.02	641.5	0.02	641.48	0.00	641.5	0.02	641.5	0.1	641.5	0.1	809.58	0.06	809.58	0.06
y6	769.58	0.01	769.58	0.09	797.58	0.00	797.67	0.09	797.67	0.09	797.67	0.09	965.67	0.04	965.67	0.04
y7	826.58	-0.01	826.58	0.07	854.6	-0.01	854.67	0.06	854.58	-0.02	854.58	-0.02	1022.67	0.02	1022.67	0.02
b1	n.d.		n.d.		n.d.		n.d.		n.d.		338.33	0.06	n.d.		n.d.	
b2	412.33	0.02	426.33	0.00	440.32	0.00	466.42	0.07	468.42	0.07	494.42	0.05	440.32	0.00	466.33	-0.01
b3	540.42	0.00	554.42	-0.01	568.42	0.03	594.5	0.05	596.5	0.05	622.5	0.03	568.42	0.00	594.42	-0.01
b4	668.58	0.08	682.58	0.06	696.51	0.01	722.58	0.13	724.67	0.13	750.67	0.11	738.52	0.00	764.58	0.04
b5	796.67	0.06	n.d.		824.61	0.01	850.67	0.03	852.67	0.03	878.67	0.02	908.63	0.00	934.75	0.10
b6	924.75	0.05	938.75	0.04	952.7	0.01	978.75	0.1	980.83	0.1	1006.67	-0.08	1078.74	0.00	1104.75	0.00

Table S3. Fatty acid composition of cells from *X. nematophila* HGB081. All fatty acids were converted into their methylesters. Hydroxyl groups were trimethylsyliated.

Fatty acid	Percentage of total fatty acids [%]
12:0	1.29
iso-13:0	0.43
13:0	0.04
14:0	6.58
12:0 3-OH	0.12
iso-15:0	0.85
15:1	0.38
15:0	1.85
13:0 3-OH	0.04
16:1ω7c	4.76
16:0	40.75
14:0 3-OH	7.16
iso-15:0 3-OH	0.28
16:0 9,10cyclopropyl	20.07
17:0	0.72
18:1w7c	6.50
iso-16:1 3-OH	0.20
18:0	1.50
16:0 3-OH	0.43
18:0 11,12cyclopropyl	6.05

Table S4. Chromatographic determination of β-carbon stereochemistry of PAX-14:0-3OH fatty acids after derivatization with (*R*)-(–)-MTPA-Cl. Retention times (t_R) and configuration of hydroxylated fatty acids in the BAME standard are given as well as the retention time and deduced stereochemistry of the PAX-14:0-3OH fatty acids.

sample	fatty acid	t_R [min]	enantiomer
BAME	14:0-2OH	23.2	S
	14:0-2OH	23.5	R
	14:0-3OH	23.6	S
	14:0-3OH	23.7	R
PAX	14:0-3OH	23.7	R

Table S5. Determination of absolute amino acid configuration in PAX peptides by means of the advanced Marfey´s method.[8,13] Depicted are the retention times (t_R) of amino acids after derivatization with L-FDLA and L-FDLA/ D-FDLA. The elution orders of the amino acid stereoisomers are shown according to the literature.[13]

sample	amino acid	t_R L-FDLA [min]	t_R L-/D-FDLA [min]	elution order F-FDLA[13]
unmodified	lysine	26.5/28.2	26.5/28.2	L→D
	arginine	8.5	7.8/8.5	D→L
methylated	lysine	28.2	26.5/28.2	L→D
	dimethyl-lysine	8.1/8.6	8.1/8.7	?

Table S6. Identified genes in the PAX-biosynthesis gene-cluster in *X. nematophila* HGB081. Depicted are the names of the encoded proteins as well as the predicted NRPS-domains, their position in the protein, and presumed function. Furthermore the closest homologues of each gene with identities/ positivities as well as the origin-species and accession number are depicted.

gene	protein	presumed function	domain	position	specificity/motiv	closest homologue	identity/ positivity	origin	accession no.
xcn1_2784	PaxT	ABC-transporter				ABC transport protein (XpsD; Fragment; modular protein)	78/88	*X. bovienii* SS-2004	YP_003468053.1
xcn1_2783	PaxA	NRPS	C	10-446		peptide synthetase XpsA	62/75	*X. bovienii* SS-2004	AAL57599.1
			A	457-982	NosC-M2-Gly/D I L Q I G L I				
			T	994-1058					
xcn1_2782	PaxB	NRPS	C	46-486		peptide synthetase XpsB	68/81	X. bovienii SS-2004	YP_003468051.1
			A	497-1030	no hit/D T S D I G S V				
			T	1042-1106					
			C	1128-1566					
			A	1577-2110	no hit/D T S D I G S V				
			T	2122-2185					
			C/E	2224-2670					
			A	2681-3213	no hit/D T S D I G T V				
			T	3225-3289					
xcn1_2781	PaxC	NRPS	C	42-480		peptide synthetase XpsB	64/78	X. bovienii SS-2004	YP_003468050.1
			A	491-1024	no hit/D T S D I G S V				
			T	1036-1099					
			C/E	1138-1557					
			A	1590-2123	no hit/D T S D I G S V				
			T	2135-2198					
			C/E	2237-2681					
			A	2692-3225	no hit/D T S D I G S V				
			T	3237-3301					
			TE	3327-3586					

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