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ORIGINAL ARTICLE

# Identification of a new antimicrobial lysine-rich cyclolipopeptide family from *Xenorhabdus nematophila*

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**Entomopathogenic bacteria of the genus *Xenorhabdus* are known to be symbiotically associated with soil dwelling nematodes of the *Steinernematidae* family. These bacteria are transported by their nematode hosts into the hemocoel of the insect larvae, where they proliferate and produce insecticidal proteins, inhibitors of the insect immune system and antimicrobial molecules. In this study, we describe the discovery of a new family (PAX) of five antimicrobial compounds produced by fermentation of the *Xenorhabdus nematophila* F1 strain and purified by cation exchange chromatography and reversed phase chromatography. The chemical structure of PAX 3, a lysine-rich cyclolipopetide, was obtained from the analysis of homo and heteronuclear 2D NMR and confirmed by MS-MS experiments. The five members of the PAX family showed significant activity against plants and human fungal pathogens and moderate activity against few bacteria and yeast. No cytotoxicity was observed on CHO or insect cells.**
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**Keywords:** antimicrobial; cyclolipopetide; *Xenorhabdus*

## INTRODUCTION

Gram-negative bacterial strains of the genus *Xenorhabdus* are known to be symbiotically associated with soil dwelling nematodes of the *Steinernematidae* family.[1–3] After entering the insect larvae through natural openings, nematodes release bacteria from their intestine to the host's hemocoel.[4] Bacteria are involved in killing the insect host by producing insecticidal proteins[5] and inhibitors of the insect immune system.[6–8] The bacteria proliferate in the killed host and favor the reproduction of the nematode by degrading the insect biomass[9] and by producing antibiotics that inhibits the development of the other microorganisms present in the insects corpse (bacteria, fungi).[10] Boemare *et al.*[11] classified the antibiotic activities of *Xenorhabdus* into two categories: (i) antimicrobial molecules with broad spectrum and (ii) bacteriocins with very narrow spectrum and active only against bacteria closely related to *X. nematophila*. Only a few families of antimicrobial compounds have been described from *Xenorhabdus* in the literature: xenocoumacins,[12] xenorhabdins,[13] indole derivatives,[14,15] puromycin,[16] benzylidenacetone,[17] proteinaceous bacteriocins,[11,18] and xenortide and xenematide.[19] All *Xenorhabdus* strains spontaneously produce two distinct physiological states *in vitro*,[20] phase I and II variants.[21] Phase I variants produce several antibiotics and secrete a variety of proteins, whereas these properties are apparently absent or greatly reduced in phase II variants.

In our screening program, we found new cyclolipopeptidic antimicrobial compounds in the culture supernatant of the *X. nematophila* F1/1. These compounds possess significant activity against fungi and moderate activity against few Gram-negative and Gram-positive bacteria.

Nonribosomaly antimicrobial lipopetides are produced in bacteria and fungi during cultivation.[22–24] They are composed of a cationic or an anionic peptide covalently bound to a specifically modified aliphatic chain. Most of the peptidic moieties have complex cyclic structures. Some of these molecules are highly active against bacteria including multiresistant strains.[25–28] Others display solely antifungal activity[22,29] and a few both antifungal and antimicrobial activities.[22] Members of this family were approved for clinical use by the Food and Drug Administration: daptomycine, polymixine, echinochandine.[30,31]

This article describes the fermentation of the *X. nematophila* F1/1 strain, the isolation and biological activities of these active compounds named PAX (for peptide antimicrobial from *Xenorhabdus)*, and the chemical structure elucidation of PAX 3. This is the first example of lysine-rich cyclolipopetide characterized from the genus *Xenorhabdus*.

## RESULTS AND DISCUSSION

### Fermentation

*Xenorhabdus nematophila* F1/1 was cultivated for 48 h, at 28 °C with shaking in a 5 l Erlenmeyer flask containing 1 l of LB broth. The

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culture was inoculated with 0.1% (v/v) of a 24 h preculture in the same medium. The antibiotic production was controlled and quantified by diffusion test agar against *Micrococcus luteus*, and by analytical HPLC.

**Isolation**

Bacterial cells were removed by low-speed centrifugation (6000 *g*, 10 min at 4 °C) and supernatant was sterilized on 0.22 µm pore size filter. Supernatant was added (1:1; v/v) to a 0.1 M NaCl, 0.02 M Tris buffer (pH 9) and subjected to cation-exchange chromatography on a Sep Pack CarboxyMethyl cartridge (Acell Plus CM, Waters, Milford, MA, USA). Unbound material was removed by washes with a 0.1 M NaCl, 0.02 M Tris buffer (pH 9) and the antibiotic eluted with 0.5 M NaCl, 0.02 M Tris buffer (pH 9). This eluate was acidified with 0.1% (v/v) trifluoroacetic acid (TFA) and was then subjected to reversed-phase chromatography on a Sep Pack C18 cartridge (Sep-Pak Plus C18, Waters). Unbound material was removed by washing with $H_2O$-TFA 0.1%, and the antibiotic pool was eluted with acetonitrile. The eluate was concentrated by evaporation under reduced pressure and diluted in water (1:5; v/v). Pure compounds were isolated from the crude extract by reverse-phase HPLC using a semi-preparative C18 column (Waters; Symmetry Prep C18; 7 µm; 7.8×300 mm), a linear gradient of $H_2O$, 0.1% TFA-acetonitrile, 0.1% TFA starting from 20 to 80% in 30 min, a flow rate of 5 ml $min^{-1}$ and an UV detection at 214 nm, yielding pure PAX compounds with the following HPLC-retention times: PAX 1=19.9 min, PAX 2=20.94 min, PAX 3=21.1 min, PAX 4=21.3 min and PAX 5=22.3 min with roughly the 30/10/50/9/1 percentages. The collected fractions were freeze-dried.

**Biological properties**

The PAX compounds show antifungal and antibacterial activities. They were tested for antimicrobial activity against a wide range of bacteria and fungi involved in nosocomial infection and phytopathologies (Tables 1 and 2). Regarding human pathogens (Table 1), PAX 1 and 2 did not show antibacterial activity except against *Staphylococcus epidermidis and M. luteus.* PAX 3 and 4 have weak activity against only few Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhimurium*), moderate activity against *S. epidermidis* and *B. cereus* and high activity against *M. luteus.* PAX 5 has moderate activity against few Gram-negative (*E. coli, P. aeruginosa and S. typhimurium*) including multiresistant strains and against few Gram-positive bacteria. However, high activity against the fungus *Fusarium oxysporum* was observed for PAX 1–5, whereas weak activity was observed against *Candida albicans*. The fungi belonging to the genus *Fusarium* are well-known plant pathogens and food contaminants that also cause superficial and subcutaneous infections in humans, such as onychomycosis and keratomycosis.[32] They have recently emerged as major opportunistic agents in immunocompromised hosts, especially in patients with hemopathy.[33,34] They are now considered as the third most common fungal genus (after *Candida* and *Aspergillus*) isolated from systemic infections in bone marrow transplantation patients.[35] *F. oxysporum* is responsible for about 30% of the human infection caused by this genus.[36] Regarding phytopathogenic fungi (Table 2), only PAX 1–3 were tested. At 10, 20 and 40 µg $ml^{-1}$, PAX 1 and 3 have strong activity against majority of fungi except *Botrytis cinerea myc* and *Piricularia oryzae sp.* PAX 2 has similar activity as PAX 1 and 3 against *Cladosporium sp.*, *F. culmorum sp.* and *Phytophthora*. Nevertheless, at 10 and 20 µg $ml^{-1}$, this molecule was less active against other fungi.

No cytotoxic activity with doses up to 1 mg $ml^{-1}$ against CHO cells was measured. Injections of the five PAX into the hemocoels of different species of insects did not result in increased mortality. Moreover, these molecules did not exhibit cytolytic activities against sheep erythrocytes or insect hemocytes. These results show that the PAX had no entomotoxic effects (data not shown).

**Chemical structure elucidation of PAXs**

Five compounds referred as PAX 1–5 were isolated, purified to homogeneity as a white powder and characterized by mass spectrometry. ESI-MS experiments revealed the molecular weights of different PAXs (PAX 1: 1051 Da, PAX 2: 1079 Da, PAX 3: 1065 Da, PAX 4: 1093 Da). PAXs are soluble in water and alcohols and show an UV λmax of 214 nm (methanol).

**Structure elucidation of PAX 3**

The acid hydrolysate of PAX 3 yielded seven amino acids, one glycine (Gly) and six lysine (Lys). Its NMR analysis carried out by homo and hetero nuclear experiments is described below.

The NMR data of PAX 3 (1065 Da) were recorded in DMSO-*d6* (Table 3). Its 1D spectrum showed eight amide signals spanning the 8.5–7.3 p.p.m. chemical shift area. Among them, one is a triplet (8.18 p.p.m., 5.7 and 5.7 Hz) thus suggesting the presence of a glycine and another displays an unusual doublet of doublet (7.36 p.p.m., 6.5 and 4.0 Hz) indicating that this amide group is bound with a methylene group. The six other amide signals are doublets with a $^3J_{HN\text{-}CH}$ coupling constant ranging from 5.2 to 7.1 Hz. The natural abundance $^1H$-$^{15}N$ HSQC confirmed the presence of these eight amide protons and the absence of a C-terminal amide group (data not shown). In agreement with the acid hydrolysis, the TOCSY experiment allowed us to unambiguously identify a glycine and six lysine residues (Figure 1a). These later being also characterized from the intense signal at 2.74 p.p.m. typical of the C$\varepsilon H_2$ protons. Surprisingly, the two-amide signals at 8.38 (doublet) and at 7.36 p.p.m. (doublet of doublet) were found to share the same spin system indicating that the ζ amino group of this lysine was involved in an amide bond to be identified. From the analysis of the DQF-COSY, TOCSY and NOESY data the assignment of the peptidic part was established on the basis of the classical sequential NOEs and is reported in Figure 1. The peptidic sequence was shown to consist of seven residues as following: $G^1K^2K^3K^4K^5K^6K^7$. Unexpectedly, this lysine-rich peptide includes an unusual 5-lysine macrocycle closed by an amide bond between the $K^7$ carboxyl group and the $\zeta K^3$ amino group. Such a cyclization which gives rise to a 5-residue macrolactame ring was mainly supported by the strong intensity dNN NOE between the $K^7$ and the $\zeta K^3$ amide protons and by the successive dNN NOEs observed all around the cycle. Owing to the overlap of the $K^6$ and $K^7$ amide signals, the $dNN_{6-7}$ NOE could not be observed in pure DMSO-*d6*. The addition of 17% water was enough to separate these two resonances and observe this essential NOE to confirm the cyclic structure giving rise to a 5-residue macrolactame ring. As a result, this cyclization constrains the $K^3$ side chain. This is in agreement with the strong inequivalency observed for the C$\varepsilon H_2$ resonances of $K^3$ at 3.45 and 2.77 p.p.m. (Figure 1a).

Clearly, several remaining resonances in the $^1H$ spectrum do not belong to the peptidic part. In particular, the doublet at 0.84 p.p.m. and the multiplet at 1.49 p.p.m. belong to an isopropyl group. By using both homonuclear and heteronuclear data, starting from the sole methyl signal of the spectrum, the $(CH_3)_2$-CH-$(CH_2)_3$- spin system was unambiguously characterized (Figure 2). This is also the case for the proton resonance at 3.81 p.p.m. that belongs to a CH group whose $^{13}C$ signal is at 67.58 p.p.m.. These chemical shifts are typical for an alcohol or ether function indicating the presence of an

**Table 1 Chemical structure characteristics of PAX and minimal inhibitory concentration (MIC)[a] against bacteria and fungi including human pathogens**

| | *PAX 1* | *PAX 2* | *PAX 3* | *PAX 4* | *PAX 5* | *Vancomycin* | *Polymixin B* |
|---|---|---|---|---|---|---|---|
| Molecular weight (Da) | 1051 | 1079 | 1065 | 1093 | 1079 | | |
| *Chemical structure characteristics* | | | | | | | |
| Peptidic part: position 2 residue | K | R | K | R | ND | | |
| 3-hydroxy fatty acid part: isomerization and length | iso-14:0 | iso-14:0 | iso-15:0 | iso-15:0 | | | |
| *Activity against Gram-negative bacteria* | | | | | | | |
| *P. aeruginosa* CIP 76.*110* | >100[a] | >100 | 100 | 50 | 25 | >100 | 1.56 |
| *P. aeruginosa* H41308 (L, Q) | >100 | >100 | 100 | 100 | 50 | >100 | 0.78 |
| *E. coli* CIP 76.24 | 100 | >100 | 100 | 50 | 12.5 | >100 | 0.78 |
| *E. coli* H35393 (L,C,Q)[b] | >100 | 100 | 50 | 100 | 25 | >100 | 1.56 |
| *S. typhimurium* H23212 | 100 | 100 | 100 | 100 | 50 | >100 | 1.56 |
| *S. maltophilia* H38058 | >100 | >100 | >100 | >100 | >100 | 50 | 1.56 |
| *Klebsiella pneumoniae* H35150 | >100 | >100 | >100 | >100 | >100 | >100 | 1.56 |
| *E. aerogenes* H35956 | >100 | >100 | >100 | >100 | >100 | >100 | 1.56 |
| *M. morganii* H45543 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| *P. vulgaris* CIP 58.60 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| *Activity against Gram-positive bacteria* | | | | | | | |
| *S. aureus* CIP 76.25 | >100 | 100 | 50 | 50 | 50 | 1.56 | >100 |
| *S. epidermidis* CIP 68.21 | 100 | 50 | 25 | 12.5 | 12.5 | 1.56 | 100 |
| *E. faecalis* H37812 | >100 | >100 | >100 | >100 | >100 | 1.56 | >100 |
| *B. cereus* ATCC 14579 | | | 25 | 25 | | 1.56 | >100 |
| *S. pneumoniae* CIP 103.566 | >100 | >100 | >100 | >100 | >100 | 1.56 | >100 |
| *M. luteus* CIP 53.45 | 3.125 | 3.125 | 3.125 | 3.125 | 1.56 | 0.78 | 6.25 |
| *Activity against fungi and yeast* | | | | | | | |
| *F. oxysporum* H3012 | 1.56 | 1.56 | 1.56 | 3.12 | 0.78 | | |
| *C. albicans* CIP 48.72 | 50 | 50 | 50 | 50 | 50 | | |

Abbreviation: ND, not determined.
[a]In $\mu g\,ml^{-1}$.
[b]Resistance to L: β lactam, Q: quinolone, C: Cycline.

**Table 2 Antifungal activity of PAX (percent inhibition of fungal growth)**

| | *PAX 1* | | | *PAX 2* | | | *PAX 3* | | |
|---|---|---|---|---|---|---|---|---|---|
| | *10 $\mu g\,ml^{-1}$* | *20 $\mu g\,ml^{-1}$* | *40 $\mu g\,ml^{-1}$* | *10 $\mu g\,ml^{-1}$* | *20 $\mu g\,ml^{-1}$* | *40 $\mu g\,ml^{-1}$* | *10 $\mu g\,ml^{-1}$* | *20 $\mu g\,ml^{-1}$* | *40 $\mu g\,ml^{-1}$* |
| *Alternaria brassicae* | 82 | 84 | 83 | 25 | 47 | 83 | 82 | 82 | 83 |
| *Botrytis cinerea* | 38 | 95 | 95 | 12 | 53 | 83 | 52 | 95 | 94 |
| *Botrytis cinerea myc* | 21 | 34 | 60 | 0 | 6 | 34 | 40 | 70 | 93 |
| *Cladosporium sp* | 94 | 94 | 94 | 74 | 95 | 95 | 94 | 94 | 94 |
| *Cladosporium myc* | 89 | 90 | 89 | 0 | 40 | 91 | 70 | 92 | 91 |
| *F. culmorum* | 96 | 96 | 96 | 66 | 97 | 97 | 96 | 96 | 96 |
| *Helminthosporium teres* | 70 | 91 | 95 | 33 | 47 | 80 | 76 | 94 | 92 |
| *Rhizoctonia solani myc* | 9.7 | 85 | 93 | 6 | 7 | 64 | 56 | 91 | 93 |
| *Phytophthora myc* | 79 | 78 | 77 | 71 | 81 | 78 | 76 | 78 | 77 |
| *P. oryzae* | 18 | 22 | 45 | 12 | 15 | 21 | 30 | 35 | 45 |
| *S. tritici* | 88 | 88 | 85 | 0 | 86 | 89 | 87 | 88 | 86 |

-CH(OH)- or -CH(OR)- group. In addition, from this alcohol or ether group the CO-$CH_2$-CH(O)-$(CH_2)_3$- spin system was clearly characterized (Figure 2). Interestingly, these two well-identified spin systems share an identical intense cross-peak at ($^1$H) 1.24/($^{13}$C) 28.86 p.p.m. corresponding to several -$CH_2$- groups, suggesting that the two spin systems could be linked together by an aliphatic chain. Thus, the acyl fragment would be a 3-hydroxy fatty acid. Owing to the overlap of several methylene groups the acyl chain length was deduced

**Table 3 NMR data of PAX 3 (dimethyl sulfoxide, 32 °C) and of PAX 4 (dimethyl sulfoxide, 27 °C)**

| | *Pax 3* | | *Pax 4* | |
|---|---|---|---|---|
| *Residue* | *$^{13}$C (p.p.m.)* | *$^{1}$H (p.p.m.)* | *Residue* | *$^{1}$H (p.p.m.)* |
| *iso-15:0 (3-hydroxy) fatty acid* | | | *iso-15:0 (3-hydroxy) fatty acid* | |
| $(CH_3)2$ | 22.29 | 0.845 | $(CH_3)2$ | 0.841 |
| $C_{13}H$ | 27.15 | 1.489 | $C_{13}H$ | 1.500 |
| $C_{12}H2$ | 38.21 | 1.140 | $C_{12}H2$ | 1.135 |
| $C_{11}H2$ | 26.55 | 1.124 | $C_{11}H2$ | 1.240 |
| $C_{6-10}H2$ | 28.86 | 1.249 | $C_{6-10}H2$ | 1.250 |
| $C_5H2$ | 24.78 | 1.243 | $C_5H2$ | 1.238 |
| $C_4H2$ | 36.78 | 1.356 | $C_4H2$ | 1.354 |
| $C_3H(OH)$ | 67.58 | 3.807 | $C_3H(OH)$ | 3.803 |
| $C_2H2$ | 43.25 | 2.233 | $C_2H2$ | 2.228 |
| $C_1O$ | 171.45 | | | |
| *Gly$^1$* | | | *Gly$^1$* | |
| HN | | 8.178 | HN | 8.190 |
| $H_\alpha$ | 41.92 | 3.738 | $H_\alpha$ | 3.755 |
| $H_{\alpha'}$ | | 3.700 | $H_{\alpha'}$ | 3.688 |
| *Lys$^2$* | | | *Arg$^2$* | |
| HN | | 8.015 | HN | 8.063 |
| $H_\alpha$ | 52.68 | 4.180 | $H_\alpha$ | 4.199 |
| $C_\beta H2$ | 30.41 | 1.615/1.557 | $C_\beta H2$ | 1.662/1.563 |
| $C_\gamma H2$ | 21.96 | 1.320 | $C_\gamma H2$ | 1.499/1.420 |
| $C_\delta H2$ | 26.35 | 1.518 | $C_\delta H2$ | 3.097 |
| $C_\varepsilon H2$ | 38.38 | 2.740 | $HN_\varepsilon$ | 7.768 |
| *Lys$^3$* | | | *Lys$^3$* | |
| HN | | 8.377 | HN | 8.423 |
| $H_\alpha$ | 53.53 | 4.093 | $H_\alpha$ | 4.104 |
| $C_\beta H2$ | 30.77 | 1.653 | $C_\beta H2$ | 1.646 |
| $C_\gamma H2$ | 22.18 | 1.430 | $C_\gamma H2$ | 1.325/1.233 |
| $C_\delta H2$ | 28.68 | 1.455/1.324 | $C_\delta H2$ | 1.420 |
| $C_\varepsilon H2$ | 37.95 | 3.448/2.774 | $C_\varepsilon H2$ | 3.458/2.756 |
| $HN_\varepsilon$ | | 7.358 | $H_\varepsilon N$ | 7.353 |
| *Lys$^4$* | | | *Lys$^4$* | |
| HN | | 7.589 | HN | 7.600 |
| $H_\alpha$ | 52.76 | 4.097 | $H_\alpha$ | 4.087 |
| $C_\beta H2$ | 29.68 | 1.656/1.628 | $C_\beta H2$ | 1.645 |
| $C_\gamma H2$ | 21.67 | 1.213 | $C_\gamma H2$ | 1.293/1.212 |
| $C_\delta H2$ | 26.25 | 1.518 | $C_\delta H2$ | 1.517 |
| $C_\varepsilon H2$ | 38.38 | 2.740 | $C_\varepsilon H2$ | 2.750 |
| *Lys$^5$* | | | *Lys$^5$* | |
| HN | | 8.165 | HN | 8.174 |
| $H_\alpha$ | 52.86 | 4.073 | $H_\alpha$ | 4.064 |
| $C_\beta H2$ | 30.52 | 1.750 | $C_\beta H2$ | 1.745/1.590 |
| $C_\gamma H2$ | 21.69 | 1.320 | $C_\gamma H2$ | 1.321 |
| $C_\delta H2$ | 26.25 | | $C_\delta H2$ | 1.543 |
| $C_\varepsilon H2$ | 38.38 | 2.740 | $C_\varepsilon H2$ | 2.740 |
| *Lys$^6$* | | | *Lys$^6$* | |
| HN | | 7.922 | HN | 7.926 |
| $H_\alpha$ | 52.15 | 4.077 | $H_\alpha$ | 4.064 |
| $C_\beta H2$ | 29.35 | 1.765/1.512 | $C_\beta H2$ | 1.700 |
| $C_\gamma H2$ | 22.02 | 1.318/1.264 | $C_\gamma H2$ | 1.324 |
| $C_\delta H2$ | 26.23 | 1.518 | $C_\delta H2$ | 1.525 |
| $C_\varepsilon H2$ | 38.38 | 2.740 | $C_\varepsilon H2$ | 2.760 |
| *Lys$^7$* | | | *Lys$^7$* | |
| HN | | 7.904 | HN | 7.926 |
| $H_\alpha$ | 53.23 | 4.077 | $H_\alpha$ | 4.064 |
| $C_\beta H2$ | 30.04 | 1.717/1.640 | $C_\beta H2$ | 1.700 |
| $C_\gamma H2$ | 22.12 | 1.378 | $C_\gamma H2$ | 1.324 |
| $C_\delta H2$ | 26.23 | 1.518 | $C_\delta H2$ | 1.525 |
| $C_\varepsilon H2$ | 38.38 | 2.740 | $C_\varepsilon H2$ | 2.762 |

from the molecular weight as being an iso-15:0 3-hydroxy fatty acid. Moreover, the 'sequential' NOE between the $C_2H_2$ (2.23 p.p.m.) of the 3-hydroxy fatty acid and the amide proton of $G^1$ unambiguously characterized the amide bond link between the fatty acid and the peptidic fragments. Finally, the PAX 3 structure was fully corroborated by MS-MS fragmentation (Figure 3).

Altogether, these data suggest the presence of the iso-15:0 3-hydroxy fatty acid linked to the cyclopeptidic part at the $G^1$ residue to yield a lysine-rich cyclolipopeptide with a 5-residue macrolactame ring (Figure 4). The configurations of the α-carbons of the amino acids as well as that of the $C_3$ of the iso-15:0 3 hydroxy acid were not determined.

The PAX 1, PAX 2 and PAX 4, $^1$H NMR spectra in DMSO (dimethyl sulfoxide) were very similar to that of PAX 3. The two main changes were easily observed and characterized by the combination of $^1$H NMR and ESI/MS data. The first one with regard to the peptidic part with the $K^2R$ mutation, whereas the second one with regard to the length of the fatty acid. The PAX 2 (1079 Da) and PAX 4 (1093 Da) chemical structures consist of the $K^2R$ mutation (Tables 1 and 3) with the iso-14:0 and the iso-15:0 3-hydroxy acid, respectively. In contrast, PAX 1 (1051 Da) displays a K2 residue and the iso-14:0 3-hydroxy acid.

Although the $^1$H NMR spectrum of PAX 5 could not be obtained, its molecular weight identical to PAX 2 (1079 Da) suggested that it could be its normal isomer. However, when compared with PAX 3, the +14 Da delta mass could be explained either by one methylene group extension of the 3-hydroxy fatty acid to yield a 16:0 homolog or by a simultaneous presence of a K2R mutation (+28 Da) and a 14:0 3-hydroxy fatty acid (−14 Da). To choose between these two hypothesis, more material should be isolated to record the PAX 5 NMR spectrum.

Notice that in cyclolipopeptides there are two types of cyclizations involving the carboxylic group, one involving an amide bond and another an ester bond leading to a macrolactame or a macrolactone ring, respectively.[37]

## Conclusion

This report describes the production, the purification and the characterization of a new antimicrobial family from *X. nematophila*. Their significant antifungal activity and their lack of cytotoxicity and entomotoxicity increase the potential interest of these molecules for vegetal or human health application.

## METHODS

### Producing organism

*Xenorhabdus nematophila* F1 (Ecologie Microbienne des Insectes et Interactions Hôtes-Pathogène collection) was grown on Luria-Bertani medium (LB,

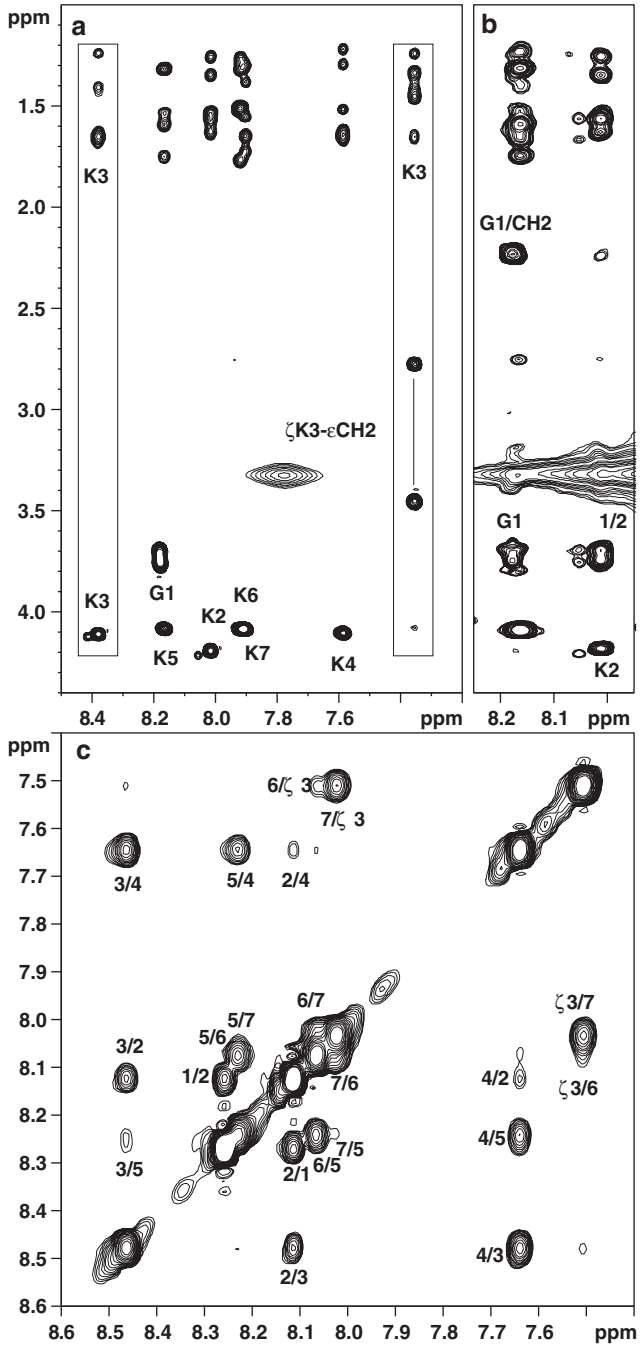



**Figure 1** $^{1}$H NMR data illustrating the assignment of the peptidic moiety. (**a**) Part of the TOCSY showing the different spin systems (DMSO, 305 K). ζK$^{3}$ is for the zeta amide proton of K$^{3}$. The side chain spin system of K$^{3}$ observed both from the amide and the ζK$^{3}$ amide proton is boxed. Due to the length of the side chain the εCH$_2$ cross-peaks are of weak intensity from the amide chemical shift and not observed in this plot level. (**b**) Part of the NOESY showing the 'sequential' NOE between the αCH$_2$ of the fatty acid and the amide proton of G$^{1}$ labeled G$^{1}$/CH$_2$ (DMSO-*d6*, 305 K, 250 ms of mixing time). (**c**) Part of the NOESY showing the dNN NOEs (DMSO-*d6* with 17% of water, 285 K, 250 ms of mixing time). The strong intensity NOE between HN K$^{7}$ and ζHN K$^{3}$ amide protons (7/ζ3) characterizes the cyclization. Notice that the dNN6–7 was not observed in pure DMSO due to the overlap of the K$^{6}$ and K$^{7}$ amide signals (part **a**).

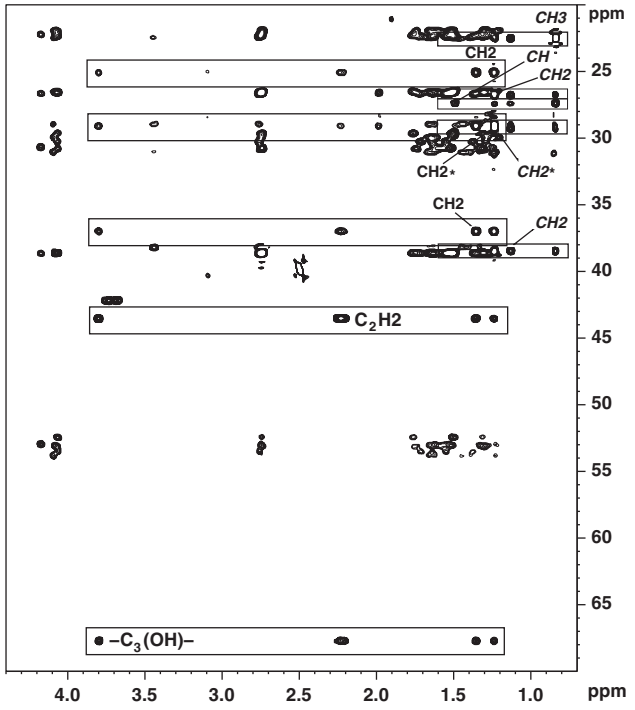



**Figure 2** Part of the $^{1}$H-$^{13}$C HSQC-TOCSY showing the spin system of the iso-15:0 3-hydroxy fatty acid. The two partial spin systems identified from the carboxyl (large rectangles) and from the methyl (small rectangles with labels in italics) groups are boxed. They share the same CH$_2$ cross-peak (twofold labeled CH$_2$*) corresponding to the overlap of several equivalent methylene groups at 1.24/28.86 p.p.m. located between the two extremities of the fatty acid. The length of the acyl chain was deduced from the molecular weight obtained by mass spectrometry.

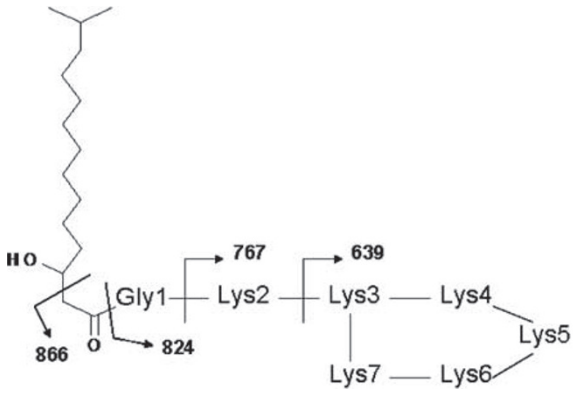
composed of bactotryptone 10 g l$^{-1}$, yeast extract 5 g l$^{-1}$ and NaCl 10 g l$^{-1}$) for liquid culture and on LB-agar for solid cultures. The phase status (I or II) of this strain was determined by culturing on NBTA (Nutrient agar (Difco, Detroit, MI, USA) 31 g l$^{-1}$, bromothymol blue 25 mg l$^{-1}$ and 2, 3, 5-triphenyl tetrazolium chloride 1% 40 mg l$^{-1}$) and measuring antibacterial activity against *M. luteus*. *Xenorhabdus* exhibits two colony forms or variants when cultured *in vitro*. Modifications of the outer membrane induce differential adsorption of dyes by variants. Phase I variants absorb dyes and are blue on NBTA plates, whereas phase II colonies are red. Phases I and II of strains are indicated as suffixes (/1 and /2, respectively) attached to strain designations. This strain was maintained at 15 °C on NBTA medium.

## Bacterial strains and antimicrobial agents

The following reference strains were used for the evaluation of antimicrobial activity: *P. aeruginosa* CIP 76.110, *E. coli* CIP 76.24, *Proteus vulgaris* CIP 58.60, *S. aureus* CIP 76.25, *S. epidermidis* CIP 68.21, *Bacillus cereus* ATCC 14579, *Streptococcus pneumoniae* CIP 103.566, *M. luteus* CIP 53.45 and *C. albicans* CIP 48.72 and clinical isolates (strains are indicated as suffixes H and a number) obtained from patients with infection at the University Hospital of Nîmes. Phytopathogenic fungi were obtained from Rhobio (Lyon, France). Vancomycin and polymyxin (Sigma-Aldrich, St Louis, MO, USA) were provided as standard powders by the manufacturers.

## Antibacterial susceptibility testing methods

The minimal inhibitory concentration (MIC) was defined as the lowest antibiotic concentration, which yielded no visible growth. MIC was determined as recommended by the Clinical and Laboratory Standards Institute.[38] Antibiotics were tested at final concentrations (prepared from serial twofold dilutions) ranging from 100 to 0.78 mg l$^{-1}$. The test medium was Mueller–Hinton broth, and the inoculum was 5×10$^{5}$ CFU ml$^{-1}$. The inoculated microplates were incubated at 37 °C under shaking for 18 h before reading.




| Fragment ion | |
|---|---|
| 129 | $[Lys^7 + H]^+$ |
| 185 | $[Gly^1Lys^2 + H]^+$ |
| 257 | $[Lys^7Lys^6 + H]^+$ |
| 385 | $[Lys^7Lys^6Lys^5 + H]^+$ |
| 513 | $[Lys^7Lys^6Lys^5Lys^4 + H]^+$ |
| 554 | $[M+H]^+ - Lys^7Lys^6Lys^5Lys^4$ |
| 639 | figure |
| 641 | $[Lys^7Lys^6Lys^5Lys^4Lys^3 + H]^+$ |
| 682 | $[M+H]^+ - Lys^7Lys^6Lys^5$ |
| 767 | figure |
| 769 | $[Lys^7Lys^6Lys^5Lys^4Lys^3Lys^2 + H]^+$ |
| 810 | $[M+H]^+ - Lys^7Lys^6$ |
| 824 | figure |
| 826 | $[Lys^7Lys^6Lys^5Lys^4Lys^3Lys^2Gly^1 + H]^+$ |
| 866 | figure |
| 938 | $[M+H]^+ - Lys^7$ |
| 1048 | $[M+H]^+ - H_2O$ |

**Figure 3** Key fragmentations of the 1066 $[M+H]^+$ ions of PAX 3.

### Antifungal susceptibility testing methods

The PAX activities were tested against the human pathogen *F. oxysporum* and against different phytopathogenic fungi by the M38-A microdilution method[39] with RPMI 1640 medium as recommended in the Clinical and Laboratory Standards Institute M23-A document.[40] The NCCLS M27-A2 broth microdilution method was used when *C. albicans* was tested.[41]

Fungal inoculi were prepared from 7-day cultures grown on potato dextrose agar and adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.17 and diluted (1:50) in RPMI 1640 broth. The density of the inoculum suspension of *C. albicans* isolate was adjusted to a density of a 0.5 McFarland standard and diluted 1:1000 in RPMI 1640 broth.[41] Microdilution trays (96 U-bottom shaped) containing 100 µl antifungal dilutions were added with 100 µl PAX solutions (final concentration ranging from 100 to 0.78 mg $l^{-1}$ for *F. oxysporum* and *C. albicans*; 10, 20 and 40 µg $ml^{-1}$ for other fungi). After inoculation of the trays, all microdilution trays were incubated at 30 °C in ambient air. As described in the M38-A method,[39] MICs for *F. oxysporum* were determined by visual examination at 48 h. MICs were defined as the lowest drug concentration that showed absence of growth or complete growth inhibition (100%). MICs for *C. albicans* were determined at 48 h and corresponded to 100% of growth inhibition.[41] Regarding phytopathogenic fungi, the culture absorbance was measured at 600 nm, 5 days after the beginning of the experiments. The activity results correspond to a percentage of growth inhibition $((1-Abs_{600\,nm}$ culture with PAX)/$Abs_{600\,nm}$ culture without PAX).

### Cytotoxicity test

Chinese Hamster Ovary (CHO) cells were grown in RPMI medium supplemented with 5% (v/v) fetal calf serum. The cells were incubated for 24 h at 37 °C in the absence of serum and in the presence of PAX (final concentration ranging from 1000 to 7.8 mg $l^{-1}$).

The cytotoxicity was measured using the cell cytotoxicity Kit I (Roche Applied Sciences, Meylan, France).

### Insect toxicity tests

The common cutworm, *Spodoptera littoralis*, was reared on an artificial diet[42] at 24 °C, and the wax moth, *Galleria mellonella*, was reared on pollen and wax at 28 °C. A locust, *Locusta migratoria*, was reared on grass at 28 °C. Eggs of the tobacco hornworm, *Manduca sexta*, were obtained from Monika Stengl (University of Regensburg, Regensburg, Germany). *M. sexta* larvae were reared on an artificial diet[43] at 27 °C with light-dark cycles consisting of 16 h of light and 8 h of darkness. Fifth-instar larvae of each insect species were selected and surface sterilized with 70% (v/v) ethanol before intrahemocoelic injection. The larvae were divided into groups of 12 larva, and each larva was injected with 10 µl of one of the purified PAX, corresponding to a dose of 0.1 µg per insect, or with phosphate-buffered saline. The treated larvae were incubated individually for up to 96 h, and then the number of dead insects was recorded.

A liquid hemolysis assay with sheep erythrocytes[44] was used to determine hemolytic activity of purified PAX. Cytolytic assays were performed with insect hemocytes by collecting hemolymph samples from *S. littoralis* larvae in an anticoagulant buffer.[45] Hemocytes were centrifuged, rinsed in phosphate-buffered saline to remove plasmatic factors, and resuspended in the same buffer ($2\times10^4$ hemocytes $ml^{-1}$). The suspensions (10 µl) were each mixed with 10 µl of a purified PAX, corresponding to a 0.1-µg dose, deposited on a slide, and incubated for 20 min at 28 °C. Hemocytes with phosphate-buffered saline were used as a control. Cell lysis was observed with a light microscope and was recorded.

### NMR and MS analysis

The NMR samples were prepared from the lyophylized lipopeptide. They were disolved in DMSO-*d6* to yield 1.0–1.5 mM solution. Chemical shifts are expressed with respect to the DMSO-*d6* residual signal set at 2.50 and 39.5 p.p.m. for $^1H$ and $^{13}C$ spectra, respectively. All NMR experiments were carried out on a Bruker Avance 600 spectrometer (Bruker Analytik GmbH, Rheinstetten, Germany) equipped with a triple resonance cryoprobe, and spectra were recorded at temperatures ranging from 295 to 310 K. Double-quantum filtered-COSY (DQF-COSY),[46,47] z-filtered total-correlated spectroscopy (z-TOCSY)[48,49] and NOESY[50] spectra were acquired in the phase-sensitive mode, using the States-TPPI method.[51] We obtained z-TOCSY spectra with a mixing time of 90 ms and NOESY spectra with mixing times of 150 and 250 ms, respectively. The $^1H$-$^{15}N$ HSQC, $^1H$-$^{13}C$ HSQC, $^1H$-$^{13}C$ HMBC, $^1H$-$^{13}C$ HSQC-TOCSY experiments[52–54] were carried out with the same sample.

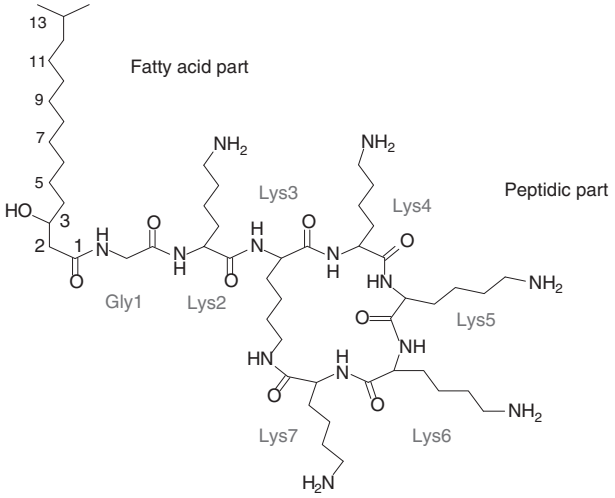



**Figure 4** Chemical structure of PAX 3.

All data were processed with XWINNMR software (Bruker Analytik GmbH). The assignment of the peptidic part was achieved using the general strategy described by Wüthrich.[55] Owing to the close proximity of the $K^6$ and $K^7$ amide signals in DMSO-*d6*, the $dNN_{6-7}$ NOE could not be observed. Thus, two other data sets were recorded with 17 and 33% of water at several temperatures ranging from 285 to 310 K. In these conditions, these two amide signals were enough separated to observe the $dNN_{6-7}$ NOE.

The assignment of the non-peptidic part was obtained from the analysis of the homo and heteronuclear data. Owing to the overlap of $^1H$ resonances as well as the $^{13}C$ resonances of the central methylene groups of the fatty acid, giving rise to an unique HSQC cross-peak, the length of the fatty acid chain was calculated to be in agreement with the molecular weight measured by mass spectrometry. For PAX 1, 2 and 4, only $^1H$-NMR data were recorded.

LC-MS was first performed to obtain the *m/z* value of the protonated PAX derivatives. ESI-LC-MS data were obtained in the positive mode on a Waters alliance LC-MS system (Waters ZQ mass detector, Waters photodiode array detector 2696, Waters alliance HPLC systems 2790). The HPLC column used was a C18 column (Waters; X-terra RP18; 5 µm; 4.6×250 mm) maintained at 35 °C. Solvents were (A) water +0.1% TFA (B) acetonitrile +0.1% TFA and the flow rate was 1 ml $min^{-1}$. The mobile phase composition was 80% A from 0 to 5 min, ramped to 80% B at 35 min. Samples were dissolved in solvent A (100 µl). Sample injection volume was 10 µl. UV-visible detection was by absorbance at 200–600 nm. Solvent flow to the MS was diverted to waste for the first 5 min to minimize salt build-up. PAX 3 MS-MS fragmentation data were obtained on a Waters Micromass Q-T of micro mass spectrometer.

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