Title: NEMAUCIN, AN ANTIBIOTIC PRODUCED BY ENTOMOPATHOGENIC XENORHABDUS CABANILLASII

Abstract: Antibiotic compounds purified from Xenorhabdus cabanillasii strain CNMC I-4418 and methods for producing said compounds and pharmaceutical compositions comprising said compounds and a pharmaceutically acceptable carrier for use in the treatment of fungal disease, Xenorhabdus cabanillasii strain CNMC I-4418.
The present invention relates to new antibiotic compounds, to a strain of *Xenorhabdus cabanillasii* producing said antibiotic compounds and to the use of these compounds in the treatment of microbial disease in humans and animals.

Antimicrobial resistance is a major public health problem with a significant impact on morbidity, mortality and healthcare-associated costs. The problem has been worsened by the restriction of antibiotic drug discovery and development programs. Nowadays, the most relevant multiresistant bacterial pathogens are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *enterococci* (VRE), extended spectrum β-lactamase formers (ESBL), multiresistant *Pseudomonas* and *Acinetobacter* species. For these bacteria, only a few of the existing antibiotics are efficient. There is an urgent need for new antibacterial compounds to ensure that bacterial infections can be effectively treated in the future. Living organisms have proven to be the most reliable source of bioactive chemicals with antimicrobial activity. Environmental microbes continue to be outstanding resources for the identification of new molecules. In this context, the discovery of new groups of antimicrobial peptides makes natural antibiotics the basic element of a novel generation of drugs for the treatment of bacterial and fungal infections. Antimicrobial peptides comprise a diverse class of molecules used in host defense by bacteria, plants, insects, and animals. Two examples of polypeptide antibiotics are polymyxin B and gramicidin S produced respectively by *Bacillus polymyxa* and *Bacillus brevis*.

The present application is based on research on an underexploited bacterial genus: *Xenorhabdus*. Gram-negative bacterial strains of the genus *Xenorhabdus* are known to be symbiotically associated with soil dwelling nematodes of the *Steinernematidae* family. After entering the insect larvae via natural openings, nematodes release bacteria from their intestine to the host’s hemocoel. Bacteria are involved in killing the insect host by producing insecticidal proteins and inhibitors of the insect immune system. The bacteria proliferate in the corpse of the host and favour the reproduction of the nematode by degrading the insect biomass and by producing antibiotics that inhibit the development of the other microorganisms present in the corpse of the insect (bacteria, fungi). Boemare et al. (Boemare N.E. et al, Appl. Environ. Microbiol. 58, 3032-3037 (1992)) classified the antibiotic activities of *Xenorhabdus* into two categories: antimicrobial molecules with broad spectrum of activity, and bacteriocins with very narrow spectrum of activity and active only against bacteria closely related to *X. nematophila*. Only a few families of compounds have been described from *Xenorhabdus* in the literature: xenocoumacins (Gregson R.P. et al, WO 86/01509; McInerney B.V. et al, J. Nat. Prod. 54, 774-84 (1991)), xenorhabdins (Rhodes S.H. et al, WO 84/01775; McInerney B.V. et al, J. Nat. Prod. 54,

In the present application, we describe new antimicrobial compounds, Nemaucins, produced by fermentation of a *Xenorhabdus campanilli* strain, and purified by cation exchange chromatography and reversed phase chromatography. The chemical structure of Nemaucins was obtained from the analysis of homo and heteronuclear 2D NMR and MS-MS experiments.

These molecules showed strong activity against Gram-positive and Gram-negative human bacterial pathogens, including some multi-resistant strains.

**Summary**

A first object of the present invention is a compound of formula (I):

```
Xaa₁—Xaa₂
\(\begin{array}{c}
\text{N} \\
\text{O} \\
\text{N} \\
\text{O}
\end{array}\)
Xaa₃
\(\begin{array}{c}
\text{N} \\
\text{H}
\end{array}\)
Xaa₄
```

wherein
Xaa₁ is aspartic acid or glutamic acid,
Xaa₂ is arginine, histidine or lysine,
Xaa₃ is serine, threonine, asparagine or glutamine,
Xaa₄ is selected in the group consisting of
R is H, OH or CH2-OH,
n is 1, 2, 3, 4 or 5.
Preferably, Xaa1 is aspartic acid.
Preferably, Xaa2 is histidine.
Preferably, Xaa3 is asparagine.

Preferably, Xaa4 is
Preferably, R is OH.
Preferably, n=4.

In preferred embodiments the compound of the present invention is the compound
of formula (II):

wherein n is 1, 2, 3, 4 or 5.
Preferably, n=4.

Preferably, the compounds of the present invention are for use as a medicament.
Preferably, the compounds of the present invention are for use as an antibiotic
agent.
Preferably, the compounds of the present invention are for use in treatment of
microbial disease.
Preferably, the compounds of the present invention are for use for treatment of a microbial disease caused by a microorganism selected from Staphylococcus, Enterococcus, Streptococcus, Bacillus, Clostridium, Chlamydia, Mycobacterium, Pasteurella, Escherichia, Enterobacter, Klebsiella, Citrobacter, Proteus, Serratia, Pseudomonas, Acinetobacter, Shigella, Salmonella, Stenotrophomonas, Yersinia, Burkholderia, Morganella, Moraxella, Propionibacterium, Listeria, Corynebacterium, Bifidobacterium, Lactobacillus, and Brucella.

More preferably, the compound of the present invention is for treatment of a microbial disease caused by a microorganism selected from Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella singapnaisis, Citrobacter koseri, Citrobacter freundii, Proteus penneri, Serratia marcescens and Pseudomonas aeruginosa.

Another object of the present invention is a composition comprising a compound of the present invention in association with another antibiotic compound.

The present invention is also directed to a pharmaceutical composition comprising:

- An effective amount of a compound of formula (I)

\[
\text{Xaa}_1-\text{Xaa}_2 \quad \text{O} \quad \text{Xaa}_3 \\
\text{O} \quad \text{Xaa}_4 \quad \text{NH} \quad \text{HN} \quad \text{NH} \quad \text{R} \quad \text{NH} \\
\text{NH}_2
\]

(1)

wherein

Xaa₁ is aspartic acid or glutamic acid,
Xaa₂ is arginine, histidine or lysine,
Xaa₃ is serine, threonine, asparagine or glutamine,
Xaa₄ is selected in the group consisting of

\[
\begin{align*}
\text{\textbullet} & \quad \text{\textbullet} \\
\text{\textbullet} & \quad \text{\textbullet} \\
\text{\textbullet} & \quad \text{\textbullet} \\
\end{align*}
\]

\[
\begin{align*}
\text{\textbullet} & \quad \text{\textbullet} \\
\text{\textbullet} & \quad \text{\textbullet} \\
\text{\textbullet} & \quad \text{\textbullet} \\
\end{align*}
\]

\[
\begin{align*}
\text{\textbullet} & \quad \text{\textbullet} \\
\text{\textbullet} & \quad \text{\textbullet} \\
\text{\textbullet} & \quad \text{\textbullet} \\
\end{align*}
\]

\[
\begin{align*}
\text{\textbullet} & \quad \text{\textbullet} \\
\text{\textbullet} & \quad \text{\textbullet} \\
\text{\textbullet} & \quad \text{\textbullet} \\
\end{align*}
\]
R is H, OH or -CH2-OH,
   n is 1, 2, 3, 4 or 5,
   - A pharmaceutically acceptable carrier.
Preferably, Xaa1 is aspartic acid.

Preferably, Xaa2 is histidine.
Preferably, Xaa3 is asparagine.

\[
\text{Preferably, Xaa}_4 \quad \text{is}
\]

Preferably, R is OH.

Preferably, n=4.

In preferred embodiments the compound of the present invention is the compound of formula (II):

\[
\text{wherein n is 1, 2, 3, 4 or 5. Preferably, n=4.}
\]

A further object of the present invention is a method for producing a compound according to formula (II) comprising the following steps:

- Growing *Xenorhabdus cabaillasii* strain CNCM I-4418 in a liquid culture medium,
- Purifying a compound according to formula (II)

\[
\text{wherein n=4.}
\]
In preferred embodiments, the compound of formula (II) wherein n=4, is purified from the culture supernatant after removal of the *Xenorhabdus cabanillasii* cells.

The present invention is also directed to the *Xenorhabdus cabanillasii* strain deposited at CNCM on 15-12-2010 having the accession number CNCM I-4418.

Another object of the present invention is a culture supernatant from the *Xenorhabdus cabanillasii* strain of the present invention having antibiotic activity.

A further object of the present invention is an extract from the *Xenorhabdus cabanillasii* strain of the present invention having antibiotic activity.

**Description of the invention**

In a first aspect, the present invention is related to *Xenorhabdus cabanillasii* strain JM26 deposited at CNCM (Collection Nationale de Cultures de Microorganismes) in the name of INRA (Institut National de la Recherche Agronomique) on 15-12-2010 having the accession number CNCM I-4418.

It has been found that *Xenorhabdus cabanillasii* strain CNCM I-4418 produces a compound exhibiting antibiotic or antimicrobial activity.

When *Xenorhabdus cabanillasii* strain CNCM I-4418 is grown in a liquid culture medium, the antibiotic compound of the present invention is secreted into the culture supernatant. In another aspect, the present invention is related to a culture supernatant from *Xenorhabdus cabanillasii* strain CNCM I-4418 exhibiting antibiotic activity. For the preparation of a culture supernatant having antibiotic activity *Xenorhabdus cabanillasii* strain CNCM I-4418 is grown in a liquid culture medium under standard conditions, the bacterial cells are removed and the supernatant is recovered. The bacterial cells may for example be removed by centrifugation or filtration.

In another aspect, the invention is related to extracts from *Xenorhabdus cabanillasii* strain CNCM I-4418 showing antibiotic activity. Cell extracts from *Xenorhabdus cabanillasii* may be prepared according to any appropriate method known to the skilled person.

The present invention also encompasses culture supernatant and extracts from *Xenorhabdus cabanillasii* strain CNCM I-4418 for use as medicament.

The present invention also encompasses culture supernatant and extracts from *Xenorhabdus cabanillasii* strain CNCM I-4418 for use as an antibiotic agent.

The terms “antibiotic”, “antibiotic activity” and “antimicrobial activity” as used herein refer generally to an effect in which a reduction, inhibition or a halt in the growth of a microorganism is achieved. Antibiotic activity may be tested according to any known method such as a microdilution method. The substances and compounds of the present invention inhibit or kill microorganism and more particularly kill or inhibit bacteria.
Interestingly, the substances and compounds of the present invention inhibit or kill multi-drug resistant bacteria which exhibit resistance to known antibiotics.

*Xenorhabdus cabanillasii* strain CNCM I-4418, culture supernatant from this strain and cell extracts derived from this strain exhibit antibiotic activity against different microorganism including human bacterial pathogens.

More particularly, *Xenorhabdus cabanillasii* strain CNCM I-4418, culture supernatant from this strain and cell extracts derived from this strain exhibit antibiotic activity against *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Klebsiella oxytica, Klebsiella singapona*, *Citrobacter koseri, Citrobacter freundii, Proteus penneri, Serratia marcescens* and *Pseudomonas aeruginosa*.

Compounds having antibiotic activity as described above have been purified from the culture supernatant of *Xenorhabdus cabanillasii* strain CNCM I-4418.

In another aspect, the present invention is related to a compound of formula (I):

\[
\begin{align*}
\text{Xaa}_1 & \quad \text{Xaa}_2 \quad \text{Xaa}_3 \quad \text{Xaa}_4 \quad \text{R} \quad \text{NH} & \quad \text{NH}_2 \\
\text{HN} & \quad \text{NH} & \quad \text{NH} & \quad \text{HN} & \quad \text{NH} & \quad \text{NH}_2 \\
\end{align*}
\]

wherein

- \( \text{Xaa}_1 \) is aspartic acid or glutamic acid,
- \( \text{Xaa}_2 \) is arginine, histidine or lysine,
- \( \text{Xaa}_3 \) is serine, threonine, asparagine or glutamine,
- \( \text{Xaa}_4 \) is selected in the group consisting of

\[
\begin{align*}
\text{NH}_2 \quad \text{OH} \quad \text{OH} \quad \text{HO} & \quad \text{HO} \\
\end{align*}
\]

- \( \text{R} \) is H, OH or CH2-OH,
- \( n \) is 1, 2, 3, 4 or 5,
a salt, a hydrate or a solvate thereof. These compounds have been named “Nemaucins”. The term “Nemaucins” refers to the compounds of formula I, a salt, a hydrate or a solvate thereof.

Preferably, Xaa₁ is aspartic acid.

Preferably, Xaa₂ is histidine.

Preferably, Xaa₃ is asparagine.

Preferably, Xaa₄ is

Preferably, R is OH.

Preferably, n=4.

In preferred embodiments the compound of the present invention is the compound of formula (II):

![Chemical Structure](image)

wherein n is 1, 2, 3, 4 or 5,

a salt, a hydrate or a solvate thereof.

Preferably, n=4. This compound has been named “Nemaucin”.

The term “Nemaucin” refers to the compound of formula II wherein n=4, a salt, a hydrate or a solvate thereof.

In preferred embodiments, the antibiotic compounds according to the present invention are for use as a medicament, for use as an antibiotic agent, for use as an antimicrobial agent, or for use in the treatment of microbial disease in particular of bacterial infection caused by pathogenic bacteria.

The compounds of the present invention are preferably for use in the treatment of bacterial infection and in particular in the treatment of hospital-acquired infections or nosocomial bacterial infections.

The invention is also related to the use of compounds of formula (I) for the manufacture of a medicament for treatment of microbial infection or microbial disease.
The invention is also related to the use of compounds of formula (I) for the manufacture of an antibiotic.

The present invention also provides methods for treating microbial/bacterial infection and/or microbial/bacterial disease including administering an effective amount of a compound of formula (I) to a human or to a patient in need thereof. In a preferred embodiment, the invention relates to methods for treatment of bacterial disease and/or bacterial infection.

In one embodiment, the patient is a mammal, i.e., a living mammal. In one embodiment, the patient is a human, i.e., a living human, including a living human child and a living human adult.

The term “treatment,” as used herein in the context of treating a condition, refers generally to treatment and therapy, whether of a human or an animal (e.g., in veterinary applications), in which some desired therapeutic effect is achieved, for example, the inhibition of the progress of the condition, and includes a reduction in the rate of progress, a halt in the rate of progress, amelioration of the condition, and cure of the condition. Treatment as a prophylactic measure (i.e., prophylaxis) is also included. For example, use with subjects who have not yet developed the condition, but who are at risk of developing the condition, is encompassed by the term “treatment.”

The term “therapeutically-effective amount,” as used herein, refers to the amounts of the active agents (compounds of formula (I) or a salt, hydrate, or solvate thereof) that is effective for producing some desired therapeutic effect, commensurate with a reasonable benefit/risk ratio, when administered in accordance with a desired treatment regimen.

The term “treatment” includes combination treatments and therapies, in which two or more treatments or therapies are combined, for example, sequentially or simultaneously.

For example, the active agents of the present invention may also be used in further combination therapies, e.g., in conjunction with other agents, for example, other antimicrobial or antibiotic agents, etc.

Another aspect of the present invention is a pharmaceutical composition comprising:

- an effective amount of a compound of formula (I)
wherein
Xaa\textsubscript{1} is aspartic acid or glutamic acid,
Xaa\textsubscript{2} is arginine, histidine or lysine,
Xaa\textsubscript{3} is serine, threonine, asparagine or glutamine,
Xaa\textsubscript{4} is selected in the group consisting of
\begin{itemize}
  \item \begin{tikzpicture}
    \draw [thick] (0,0) -- (0.5,0.5) -- (0,1) -- (-0.5,0.5) -- (0,0);
    \node at (0,0) {\text{Xaa}_2};
  \end{tikzpicture}
  \item \begin{tikzpicture}
    \draw [thick] (0,0) -- (0.5,0.5) -- (0,1) -- (-0.5,0.5) -- (0,0);
    \node at (0,0) {\text{Xaa}_3};
  \end{tikzpicture}
  \item \begin{tikzpicture}
    \draw [thick] (0,0) -- (0.5,0.5) -- (0,1) -- (-0.5,0.5) -- (0,0);
    \node at (0,0) {\text{Xaa}_4};
  \end{tikzpicture}
\end{itemize}
and
R is H, OH or CH\textsubscript{2}-OH,
n is 1, 2, 3, 4 or 5,
and
- a pharmaceutically acceptable carrier.

Preferably, Xaa\textsubscript{1} is aspartic acid.
Preferably, Xaa\textsubscript{2} is histidine.
Preferably, Xaa\textsubscript{3} is asparagine.

Preferably, Xaa\textsubscript{4} is
Preferably, R is OH.
Preferably, n=4.
In preferred embodiments the compound is the compound of formula (II):

![Chemical Structure](image)

wherein n is 1, 2, 3, 4 or 5,
a salt, a hydrate or a solvate thereof. Preferably, n=4.

As used herein, "pharmaceutically acceptable carriers" includes any and all solvents, dispersion media, coatings, and the like that are physiologically compatible.

The compositions of the invention may be in a variety of forms. These include for example liquid, semi-solid, and solid dosage forms, but the preferred form depends on the intended mode of administration and therapeutic application.

These pharmaceutical compositions are preferably for oral, topical, percutaneous or parenteral administration.

The compositions as described herein may be orally administered.

As solid compositions for oral administration, tablets, pills, powders (gelatine capsules, sachets) or granules may be used. In these compositions, the active ingredient according to the invention is mixed with one or more inert diluents, such as starch, cellulose, sucrose, lactose or silica, under an argon stream. These compositions may also comprise substances other than diluents, for example one or more lubricants such as magnesium stearate or talc, a coloring, a coating (sugar-coated tablet) or a glaze. Preferred oral compositions include coated and uncoated tablets, hard and soft gelatin capsules, sugar-coated pills, lozenges, pellets, and powders.

As liquid compositions for oral administration, there may be used pharmaceutically acceptable solutions, suspensions, emulsions, syrups and elixirs containing inert diluents such as water, ethanol, glycerol, vegetable oils or paraffin oil. These compositions may comprise substances other than diluents, for example wetting, sweetening, thickening, flavouring or stabilizing products.

In preferred embodiments, the pharmaceutical compositions for oral use contain Nemaucin together with the usual excipients as diluting agents like mannitol, lactose and sorbitol; binding agents like starches, gelatins, sugars, cellulose derivatives, natural gums and polyvinylpyrroldone; lubricating agents like talc, stearates, hydrogenated vegetable
oils, polyethylene glycol and colloidal silicon dioxide; disintegrating agents like starcks, celluloses, alginites, gums and reticulated polymers; coloring, flavoring and sweetening agents.

In other embodiments, the compositions comprise Nemaucin according to the present invention with carriers or excipients suitable for topical administration. Preferred compositions for topical administration of Nemaucin according to the invention include ointments, pomades, creams, gels, and lotions.

The pharmaceutical compositions for topical use contain Nemaucin together with the usual excipients like white petrolatum, white wax, lanolin and derivatives thereof; stearic alcohol, propylene glycol, sodium lauryl sulfate, ethers of the fatty polyoxyethylene alcohols, sorbitan monostearate, glycerol monostearate, propylene glycol monostearate, polyethylene glycols, methylcellulose, hydroxymethylpropylcellulose, sodium carboxymethylcellulose, colloidal aluminium and magnesium silicate, sodium alginate. Any topical preparation may be used in the present invention, for instance ointments, pomades, creams, gels and lotions.

The doses of Nemaucin depend on the desired effect, the duration of the treatment and the route of administration used.

In preferred embodiments, the pharmaceutical compositions according to the present invention are for use as an antimicrobial agent, for use as antibiotic or for use in the treatment of microbial disease in particular of microbial disease caused by bacteria.

The pharmaceutical compositions of the present invention are preferably for use in the treatment of bacterial infection and in particular for use in the treatment of hospital-acquired infections or nosocomial bacterial infections.

Preferably, the compound of the present invention is for use for treatment of a microbial disease caused by a microorganism selected from *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Bacillus*, *Clostridium*, *Chlamydia*, *Mycobacterium*, *Pasteurella*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Proteus*, *Serratia*, *Pseudomonas*, *Acinetobacter*, *Shigella*, *Salmonella*, *Stenotrophomonas*, *Yersinia*, *Burkholderia*, *Morganella*, *Moraxella*, *Propionibacterium*, *Listeria*, *Corynebacterium*, *Bifidobacterium*, *Lactobacillus*, and *Brucella*.

More preferably, the compound of the present invention is for treatment of a microbial disease caused by a microorganism selected from *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella singapounasis*, *Citrobacter koseri*, *Citrobacter freundii*, *Proteus penneri*, *Serratia marcescens* and *Pseudomonas aeruginosa*. 
The compounds of the present invention may be combined with other active compounds exhibiting an antimicrobial/antibiotic activity. The pharmaceutical compositions encompassed by the present invention may also contain a further therapeutic agent for the treatment of bacterial disease or bacterial infection.

Another aspect of the present invention is a method for producing a compound according to formula (II) comprising the following steps:
- Growing *Xenorhabdus cabanillasii* strain CNCM I-4418 in a liquid culture medium,
- Purifying a compound according to formula (II):

![Chemical Structure](image)

wherein n=4.

The active compounds according to the present invention may be purified from the *Xenorhabdus cabanillasii* cells of the present invention. Advantageously, the active compounds of the present invention may be purified from the culture supernatant after removal of the *Xenorhabdus cabanillasii* cells. For the preparation of a culture supernatant having antifungal activity *Xenorhabdus cabanillasii* strain CNCM I-4418 is grown in a liquid culture medium under standard conditions, the bacterial cells are removed and the supernatant is recovered. The bacterial cells may for example be removed by centrifugation or filtration.

Further purification of the active compounds according to the present invention may be carried out by any known method including cation-exchange chromatography, reversed-phase chromatography and/or reverse phase HPLC.

In preferred embodiments, the active compound is purified from the culture supernatant of *Xenorhabdus cabanillasii* strain CNCM I-4418 by successive cation-exchange chromatography, reversed-phase chromatography and reverse phase HPLC.

**Figures**
- Figure 1: HPLC chromatogram of the *Xenorhabdus cabanillasii* growth medium
- Figure 2: Bactericidal effects of Nemaucin on growing *S.aureus* CIP 76.24
- Figure 3: Viability of hTERT HME-1 at 8 concentrations of Nemaucin
- Figure 4: Viability of PC-3 at 8 concentrations of Nemaucin
Figure 5: ESI-MS experiments
Figure 6: UV absorption spectra of Nemaucin
Figure 7: Peptidic part of Nemaucin
Figure 8: MSMS fragmentation of Nemaucin
Figure 9: Chemical structure of Nemaucin

EXAMPLES

A. Materials and methods

Producing organism

*Xenorhabdus cabanillasi* CMCM I-4418 was grown on Luria-Bertani medium (LB, composed of bactotryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) 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) 31 g/L, bromothymol blue 25 mg/L and 2, 3, 5-triphenyl tetrazolium chloride 1% 40 mg/L) and measuring antibacterial activity against *Micrococcus luteus*. *Xenorhabdus* exhibit 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, while 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.

Antibacterial susceptibility testing methods

The following reference strains were used for evaluation of antimicrobial activity: *Pseudomonas aeruginosa* CIP 76.110, *Escherichia coli* CIP 76.24, *Staphylococcus aureus* CIP 76.25, and clinical isolates obtained from patients with infection at the University Hospital of Nîmes. Vancomycin and polymyxin (Sigma-Aldrich) were provided as standard powders by the manufacturers. MIC and MBC determination procedures were determined as recommended by the CLSI (National Committee for Clinical Laboratory Standards. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Sixth Edition: Approved Standard M07-A6. NCCLS, Villanova, PA, USA. (2003)).

Antibiotics were tested at final concentrations (prepared from serial twofold dilutions) ranging from 0.01 to 100 µg/mL. The minimal inhibitory concentration (MIC) was defined as the lowest antibiotic concentration which yielded no visible growth. The test medium was Mueller-Hinton broth (MHB), the inoculum was 5x10⁵ CFU/mL. The inoculated microplates were incubated at 37°C for 18 h before reading.
Minimal bactericidal concentrations (MBCs) were established by extending the MIC procedure to the evaluation of bactericidal activity. After 24 hours, 10 μL were drawn from the wells, serially diluted and then spotted onto suitable agar plates. The plates were incubated at 37°C overnight. The MBC was read 18 h later as the lowest concentration of antibiotic which resulted in 0.1 % survival in the subculture. All the experiments were done in triplicates.

Protein binding.

The impact of serum protein binding was assessed by examining the impact of human serum albumin (HSA) on the activity of Nemaucin in vitro. The MICs for S. aureus CIP 76.25 and E. coli CIP 76.24 were determined in MHB + 4% HSA. Concentrations of HSA used for in vitro studies were selected on the basis of normal physiological concentration. A reduced potency (higher MIC) in MHB + HSA was presumed due to drug binding to serum protein.

Cytotoxicity test

100 μL of cell suspension of PC-3 (Human prostatic carcinoma) or hTERT HME-1 (Human normal mammary epithelium) prepared in RPMI + 10% SVF + 1% glutamine were inoculated into 96-well plates. The inoculating cell number was 3500 cells per well for PC-3 and 7500 cells per well for hTERT HME-1. The microplates were incubated at 37°C for 24h with 5% of CO₂. After 24h of culture, the medium was removed by aspiration and 100 μL of RPMI + 10% SVF + 1% glutamine with 8 concentrations of Nemaucin (from 0.78 μg/ml to 100 μg/mL) were added to each well. Microplates were incubated at 37°C for 48h with 5% of CO₂. After 48h of culture, the medium was removed by aspiration and 100 μL of RPMI + MTT (0.5 mg/mL) were added to each wells. Microplates were inoculated at 37°C during 150 min. Next, all the medium was removed by inverting and tapping the plate and 100 μL of DMSO were added in each plate. The spectrophotometric absorbance was then measured at 550 nm.

The Lethal Dose 50 corresponds to DL50 = ((50-(Y2-((Y1-Y2)/(X1-X2)))*X2))/((Y1-Y2)/(X1-X2))). X1 represents the concentration for which viability was superior to 50%, X2 represents the concentration for which viability was inferior to 50%, Y1 represents the percentage of viability at the concentration X1 and Y2 the percentage of viability at the concentration X2.

Experiments were done in triplicate.

Bactericidal effects of Nemaucin on growing S. aureus

Bacterial killing curves were carried out by inoculating S. aureus CIP 76.24 with Nemaucin concentrations equal to the MIC, two-fold the MIC and four-fold the MIC.
Vancomycin was used at four-fold its MIC. The *S. aureus* inoculums was prepared from colonies grown overnight in MHB. Antibiotics concentrations in the flask were adjusted in MHB according to the desired concentration. Culture tubes containing 10 mL were inoculated with *S. aureus* at an approximate inoculum of 10^5 cfu/mL. Samples were drawn and bacteria were counted at 0, 1, 2, 3, 4, 6 and 24h of incubation at 37°C. Thus, after vortexing the culture tubes, two 50 µL samples were removed and serially diluted with MHB. After each dilution step, 20 µL was plated onto LB agar plates, which were incubated for 24 h at 37°C. Afterwards the colonies were counted and back-extrapolated to the original volume to determine the initial concentration (cfu/mL).

**NMR and MS analysis**

The purified compound was analyzed by Mass Spectroscopy and NMR to determine its chemical structure.

The NMR study was carried out on Bruker Avance 500 and 700 MHz spectrometers equipped with cryoprobes. The sample (5-7 mM) was solubilized in water (95/5 H2O/D2O v/v) at pH 4.3. Protons chemical shifts are expressed with respect to sodium 4,4 dimethyl-silapentane-1-sulfonate, according to IUPAC recommendations. LC-MS was first performed in order to obtain the m/z value of the protonated molecules of Nemaucin. MS-MS fragmentation was then carried out on the Nemaucin. 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 Symmetry C18 5 µm 4.6X150 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. The mobile phase composition was 100% A at 0 min, ramped to 30% B at 30 min. Samples were dissolved in solvent A (100 µL). Sample injection volume was 10 µL. UV–Visible detection was by absorbance at 200–400 nm. Solvent flow to the MS was diverted to waste for the first 5 min to minimise salt build-up. MS-MS fragmentation data were obtained on a Waters Micromass Q-Tof micro mass spectrometer.

**B. Results**

**Fermentation**

*Xenorhabdus cabanillasii* CNCM I-4418 was cultivated for 48 h, at 28°C with shaking in a 2 L Erlenmeyer flask containing 500 mL of medium broth composed of bactopeptone 10 g/L, K2HPO4 5 g/L, MgSO4, 7H2O 1 g/L, (NH4)2SO4 2g/L, CaCO3 2 g/L, and NaCl 10 g/L. The culture was inoculated with 0.1% (v/v) of a 24 h preculture in the same medium. The antibiotic production was controlled by analytical HPLC. (Figure 1)
**Isolation**

Bacterial cells were removed by low-speed centrifugation (6000 × g, 10 min at 4°C) and supernatant was sterilized onto 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 7), and subjected to cation-exchange chromatography on a Sep Pack CarboxyMethyl cartridge (Acell™ Plus CM, Waters). Unbound material was removed by washes with a 0.1 M NaCl-0.02 M Tris buffer (pH 7) and the antibiotic activity eluted with 0.5 M NaCl-0.02 M Tris buffer (pH 7). This eluate was acidified with 0.1% (v/v) trifluoroacetic acid (TFA) and was then subjected to reverse-phase chromatography on a Sep Pack C18 cartridge (Sep-Pak Plus C18, Waters). Unbound material was removed by washing with H₂O-TFA 0.1% and the antibiotic pool was eluted with acetonitrile. The eluate was freeze-dried then resuspended in water (1:5; v/v). Pure compounds were isolated from the crude extract by reverse phase HPLC using a C18 column (Waters; Symmetry Symmetry C18, 5μm; 4.6X150 mm), a linear gradient of H₂O, 0.1% TFA-acetonitrile starting from 0% to 30% in 30 min, a flow rate of 1 mL/min and an UV detection from 200 to 400 nm, yielding pure Nemaucin with the following HPLC-retention time: 27.16 min.

**Biological properties**

Nemaucin compounds demonstrate strong antibacterial activities. The Nemaucins were tested for antimicrobial activity against a wide range of bacteria involved in nosocomial and animal infection. Regarding reference strains (Table 1), Nemaucin has strong activity against *S. aureus* CIP 76.24 and *E. coli* CIP 76.25 (inferior to 1μg/mL) but has moderate activity against *P. aeruginosa* CIP 76.110. Nemaucin was also tested against multi-resistant bacteria including Gram-positive and Gram-negative bacteria. They also have a very strong activity against Gram-positive multi-resistant bacteria such as MRSA, VRE and Gram-negative multi-resistant bacteria such as ESBL *Enterobacter cloacae*, *E. coli*, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Klebsiella singapponasis* or carbapenem resistant *K. pneumoniae*.

Nemaucin has a wide spectrum antibacterial activity including multi-resistant bacteria.
Table 1: MIC (µg/mL) of Nemaucin, Polymyxin, and Vancomycin against bacterial strains, including multiresistant clinical isolates (Nimes University Hospital)

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
<th>Resistance</th>
<th>Nemaucin</th>
<th>Polymyxin</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>CIP 76.25</td>
<td>-</td>
<td>0.08</td>
<td>-</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>21840</td>
<td>MRSA</td>
<td>0.05</td>
<td>-</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>20681</td>
<td>MRSA</td>
<td>0.05</td>
<td>-</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>20364</td>
<td>MRSA</td>
<td>0.05</td>
<td>-</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>23305</td>
<td>MRSA</td>
<td>0.05</td>
<td>-</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>16666</td>
<td>MRSA</td>
<td>0.05</td>
<td>-</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>N5</td>
<td>VRE</td>
<td>0.05</td>
<td>-</td>
<td>&gt;50</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>CIP 76.24</td>
<td>-</td>
<td>0.78</td>
<td>0.31</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>PEC21</td>
<td>ESBL/SHV</td>
<td>0.78</td>
<td>0.31</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>NEC20</td>
<td>ESBL/TEM</td>
<td>0.39</td>
<td>0.02</td>
<td>-</td>
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<tr>
<td><em>Escherichia coli</em></td>
<td>MEC12</td>
<td>ESBL/TEM</td>
<td>0.78</td>
<td>0.07</td>
<td>-</td>
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<tr>
<td><em>Escherichia coli</em></td>
<td>MEC63</td>
<td>ESBL/CTX M15</td>
<td>0.78</td>
<td>0.31</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>EC Abwa</td>
<td>ESBL/CTX M15</td>
<td>0.78</td>
<td>0.15</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>AEC7</td>
<td>ESBL/CTX M14</td>
<td>0.78</td>
<td>0.31</td>
<td>-</td>
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<tr>
<td><em>Enterobacter cloacae</em></td>
<td>11370</td>
<td></td>
<td>0.78</td>
<td>0.62</td>
<td>-</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>BEB 1</td>
<td></td>
<td>1.56</td>
<td>1.25</td>
<td>-</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>BEB 4</td>
<td></td>
<td>0.78</td>
<td>0.08</td>
<td>-</td>
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<tr>
<td><em>Enterobacter cloacae</em></td>
<td>41206</td>
<td></td>
<td>1.56</td>
<td>0.08</td>
<td>-</td>
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<tr>
<td><em>Enterobacter cloacae</em></td>
<td>36265</td>
<td></td>
<td>0.78</td>
<td>0.08</td>
<td>-</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>PEB1</td>
<td></td>
<td>0.39</td>
<td>0.62</td>
<td>-</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>MEB 11</td>
<td></td>
<td>0.78</td>
<td>0.62</td>
<td>-</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>NEB 2</td>
<td></td>
<td>0.78</td>
<td>0.39</td>
<td>-</td>
</tr>
<tr>
<td>Organism</td>
<td>MIC (µg/mL)</td>
<td>MBC (µg/mL)</td>
<td>MIC (µg/mL)</td>
<td>MBC (µg/mL)</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>NEB 4</td>
<td>0.78</td>
<td>5.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>KPC2 475</td>
<td>1.56</td>
<td>1.25</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella oxytica</em></td>
<td>PEB 4</td>
<td>0.78</td>
<td>0.63</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella singaponesis</em></td>
<td>NEB 9</td>
<td>0.78</td>
<td>0.63</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Citrobacter koseri</em></td>
<td>BEB 6</td>
<td>0.39</td>
<td>0.07</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>37553</td>
<td>1.56</td>
<td>0.15</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Proteus penneri</em></td>
<td>12042</td>
<td>0.78</td>
<td>&gt;12.5</td>
<td>-</td>
<td></td>
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<tr>
<td><em>Serratia marescens</em></td>
<td>31674</td>
<td>&gt;12.5</td>
<td>0.03</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Serratia marescens</em></td>
<td>31626</td>
<td>&gt;12.5</td>
<td>0.01</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>CIP 76.110</td>
<td>-</td>
<td>&gt;12.5</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>5618</td>
<td>MDR</td>
<td>&gt;12.5</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>401681</td>
<td>MDR</td>
<td>&gt;12.5</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>35170</td>
<td>MDR</td>
<td>&gt;12.5</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>42162</td>
<td>MDR</td>
<td>&gt;12.5</td>
<td>0.62</td>
<td></td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>512232</td>
<td>MDR</td>
<td>&gt;12.5</td>
<td>0.62</td>
<td></td>
</tr>
</tbody>
</table>

As evidenced by its value of MIC and MBC, Nemaucin is bactericidal (Table 2). The Bactericidal effects of Nemaucin on growing *S. aureus* CIP 76.24 was determined. At two-fold and four-fold the MIC, Nemaucin kills 100% of bacteria within 6 h, making this molecule a fast and powerful bactericidal agent. (Figure 2) Their activities are reduced by a factor of 2 in presence of HSA, suggesting weak interaction between HSA and Nemaucin. (Table 2)

**Table 2:** MIC (in µg/mL), MBC (in µg/mL) and MIC in presence of 4% of HSA (in µg/mL) against reference bacteria (*MIC/MBC*)

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nemaucin</td>
<td>0.04 µg/ml/0.08 µg/ml</td>
<td>0.78/0.78*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nemaucin +4% (w/v) HSA</td>
<td>0.08</td>
<td>1.56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The LD50 of Nemaucin A was 36.8 ± 3.54 μg/mL against Human prostatic carcinoma cells (PC-3) and 28.42 ± 3.61 μg/mL against Human normal mammary epithelium cells (hTERT HME-1) (Table 3). 100 % of cell viability was observed up to 12.5μg/mL. (Figures 3 and 4)

Table 3: LD 50 of Nemaucin A against PC-3 and hTERT HME-1

<table>
<thead>
<tr>
<th>Cells</th>
<th>LD50 in μg/mL</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>36.79</td>
<td>3.54</td>
</tr>
<tr>
<td>hTERT HME-1</td>
<td>28.42</td>
<td>3.61</td>
</tr>
</tbody>
</table>

Physico-chemical properties of nemaucins

One compound referred to as Nemaucin was isolated, purified to homogeneity as a white powder and characterized by mass spectrometry. ESI-MS experiments revealed the molecular weight of Nemaucin. (Figures 5 and 6)

Nemaucin A : White powder, UV λMeOHmax nm 214, ESI-MS (m/z) 1303 [M+H]⁺;

Chemical structure elucidation

The ¹H spectrum revealed the features of a peptidic moiety with amide signals in the 9.1-6.9 ppm area, alpha proton signals in the 4.8-3.5 ppm, beta proton signals in the 3.7-2.0 ppm and methyl signals at 1.2-1.1 ppm. To identify the spin systems of the different residues an homonuclear data set (COSY, TOCSY, NOESY and ROESY) was recorded, as well as ¹H-¹³C data set (HSQC, HSQC-TOCSY, HMBC) and a ¹H-¹⁵N HSQC in natural abundance. The combined analysis of all these data allowed us to identify spin systems of the peptidic sequence which contains at least 6 residues, including 2 unusual, derived from Threonine (Thr) and Proline (Pro) alterations.

The Thr derived residue located in position 5 of the sequence results from the change of the γ oxygen atom for a nitrogen atom, giving rise to an unusual β amino group.

Moreover, this amino group is involved in a peptidic bond. This is clearly supported first, by the TOCSY and COSY data and confirmed by the ¹H-¹⁵N HSQC which shows the two H-N cross peaks. Such an unusual residue was referred to later as Thn.

The Pro derived residue in position 6 was shown to contain an additional -CH(OH)-CH2- moiety between the alpha and carbonyl carbons. The molecular weight of such a residue is 141.1 Da (159.1 Da in its carboxylic and amino form). It was referred to later as P141. The ¹H-¹³C HSQC and HSQC-TOCSY data were particularly helpful to identify its spin system. The configuration of the asymmetric carbons of these two non-natural residues was not determined.
By using sequential NOEs, the peptidic sequence was found to contain 6 residues as following:

\[ \text{Asp}^1-\text{His}^2-\text{Asp}^3-\text{Asn}^4-\text{Thn}^5-\text{P141}^6-\text{CH}_2-\text{CH}_2-\text{R'} \]

Proton and \(^1\text{H}-^{13}\text{C} \) HSQC spectra were recorded at different pH ranging from 4.34 to 2.15. Of the two Asp residues only the Asp\(^1\) was sensitive to the ionisation meaning that its \( \gamma \) carboxyl group is free. The signals of the Asp\(^3\) spin system were insensitive to pH suggesting that its \( \gamma \) carboxyl group was involved in an amide bond. The NOE between the \( \beta \) amide of Thn\(^5\) and the \( \beta \) protons of Asp\(^3\) indicates that the side chains of these two residues were bound by a peptidic bond to form a cyclic peptide.

We observed that the P141\(^6\) residue is linked to a seventh one, the amide signal of which was a triplet. Its spin system contains a \(-\text{HN}-\text{CH}_2-\text{CH}_2-\) moiety (COSY, TOCSY, HSQC-TOCSY).

Chemical structure of the peptidic part of Nemaucin was confirmed by MSMS fragmentation. (Table 5 and Figure 8)

**Table 4: Chemical shifts of the identified peptidic part (water, 280 K)**

<table>
<thead>
<tr>
<th>Spin system</th>
<th>Group</th>
<th>(^1\text{H} ) (ppm)</th>
<th>(^{13}\text{C} ) (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp(^1)</td>
<td>CH</td>
<td>4.02</td>
<td>51.01</td>
</tr>
<tr>
<td></td>
<td>CH(_2)</td>
<td>2.88</td>
<td>35.15</td>
</tr>
<tr>
<td>His(^2)</td>
<td>HN</td>
<td>8.69</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C(\alpha)H</td>
<td>4.67</td>
<td>52.19</td>
</tr>
<tr>
<td></td>
<td>C(\beta)H(_2)</td>
<td>3.23/3.13</td>
<td>26.34</td>
</tr>
<tr>
<td>Asp(^3)</td>
<td>HN</td>
<td>8.65</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C(\alpha)H</td>
<td>4.49</td>
<td>52.35</td>
</tr>
<tr>
<td></td>
<td>C(\beta)H(_2)</td>
<td>2.69/2.50</td>
<td>39.01</td>
</tr>
<tr>
<td>Asn(^4)</td>
<td>HN</td>
<td>9.08</td>
<td>-</td>
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<tr>
<td></td>
<td>C(\alpha)H</td>
<td>4.18</td>
<td>51.77</td>
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<td></td>
<td>C(\beta)H(_2)</td>
<td>3.10/2.83</td>
<td>33.73</td>
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<tr>
<td>Thn(^5)</td>
<td>HN</td>
<td>7.71</td>
<td>-</td>
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<tr>
<td></td>
<td>C(\alpha)H</td>
<td>4.45</td>
<td>57.14</td>
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<tr>
<td></td>
<td>C(\beta)H</td>
<td>4.40</td>
<td>46.48</td>
</tr>
<tr>
<td></td>
<td>HN(_\beta)</td>
<td>8.31</td>
<td>-</td>
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<tr>
<td></td>
<td>CH(_3)</td>
<td>1.19</td>
<td>15.80</td>
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<td>P141(^6)</td>
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<td>61.75</td>
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<td>C(\beta)H(_2)</td>
<td>1.98/1.85</td>
<td>26.09</td>
</tr>
<tr>
<td></td>
<td>C(\gamma)H</td>
<td>1.99</td>
<td>23.58</td>
</tr>
<tr>
<td></td>
<td>C(\delta)H(_2)</td>
<td>3.99/3.68</td>
<td>48.24</td>
</tr>
<tr>
<td></td>
<td>C(\beta)'H</td>
<td>4.19</td>
<td>69.64</td>
</tr>
<tr>
<td></td>
<td>C(\gamma)H(_2)</td>
<td>2.41/2.33</td>
<td>40.27</td>
</tr>
</tbody>
</table>
Table 5: Key fragmentations of the 1303 [M+H]^+ ions of Nemaucin A.

<table>
<thead>
<tr>
<th></th>
<th>Fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>Immonium ions His^2</td>
</tr>
<tr>
<td>142</td>
<td>[P141^6]^+</td>
</tr>
<tr>
<td>253</td>
<td>[Asp^1-His^2]^+</td>
</tr>
<tr>
<td>312</td>
<td>[Asp^3-Asn^4-Thn^5]^+</td>
</tr>
<tr>
<td>345</td>
<td>[Asp^1-His^2-Asp^3-Asn^4-Thn^5-P141^6 - NH_3^+ + 2H]^2+</td>
</tr>
<tr>
<td>353.5</td>
<td>[Asp^1-His^2-Asp^3-Asn^4-Thn^5-P141^6 + 2H]^2+</td>
</tr>
<tr>
<td>361.9</td>
<td>[Asp^1-His^2-Asp^3-Asn^4-Thn^5-P141^6 + NH_3^+ + 2H]^2+</td>
</tr>
<tr>
<td>370.4</td>
<td>[Asp^1-His^2-Asp^3-Asn^4-Thn^5-P141^6 + NH_3^+ + NH_3^+ + 2H]^2+</td>
</tr>
<tr>
<td>449</td>
<td>[His^2-Asp^3-Asn^4-Thn^5]^+</td>
</tr>
<tr>
<td>564</td>
<td>[Asp^1-His^2-Asp^3-Asn^4-Thn^5]^+</td>
</tr>
<tr>
<td>594</td>
<td>[M+2H]^2+ - [Asp^1+2H]^2+</td>
</tr>
<tr>
<td>652</td>
<td>[M+2H]^2+</td>
</tr>
<tr>
<td>671</td>
<td>[Asp^1-His^2-Asp^3-Asn^4-Thn^5-P141^6 - NH_3^+ - NH_3^+]</td>
</tr>
<tr>
<td>688</td>
<td>[Asp^1-His^2-Asp^3-Asn^4-Thn^5-P141^6 - NH_3^+]</td>
</tr>
<tr>
<td>705</td>
<td>[Asp^1-His^2-Asp^3-Asn^4-Thn^5-P141^6]^+</td>
</tr>
<tr>
<td>722</td>
<td>[Asp^1-His^2-Asp^3-Asn^4-Thn^5-P141^6 + NH_3]^+</td>
</tr>
<tr>
<td>739</td>
<td>[Asp^1-His^2-Asp^3-Asn^4-Thn^5-P141^6 + NH_3^+ + NH_3^+]</td>
</tr>
<tr>
<td>1051</td>
<td>[M+H]^+ - Asp^1 - His^2</td>
</tr>
<tr>
<td>1188</td>
<td>[M+H]^+ - Asp^1</td>
</tr>
</tbody>
</table>

The ^1H NMR spectra also showed 18 methylenes including 9 CH_2 bonded to nitrogen. After a detailed examination of the 2D-NMR spectra (COSY, HSQC, HMBC, TOCSY), the following structure is suggested for the R'-part:

![Chemical structure](image)

The assignment of ^1H in D_2O is:

^1H NMR (500 MHz, D_2O) δ 3.38-3.21 (18H, m, H-2, 7, 10, 15, 18, 23, 26, 31, and 34), 1.75-1.58 (16H, m, H-8, 9, 16, 17, 24, 25, 32, and 33)

The complete chemical structure of Nemaucin is shown in Figure 9.
This report describes the production, the purification and the characterization of a novel peptide antibiotic produced by *Xenorhabdus cabanillasii*. Its significant antibacterial activity, including against multi-resistant bacteria, increases the potential interest of this molecule for human and animal health applications.

REFERENCES

J. Nat. Prod. 54, 774-84 (1991)

PATENT REFERENCES

WO 86/01509
WO 84/01775
US 5,569,668
0-1 Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)

| 0-1-1 | Prepared Using | PCT Online Filing Version 3.5.000.225 MT/FOP 20020701/0.20.5.20 |

0-2 International Application No. PCT/EP2011/073738

0-3 Applicant’s or agent’s file reference 359890D28953

1 The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:

| 1-1 | page | 6 |
| 1-2 | line | 4 |

1-3 Identification of deposit

| 1-3-1 | Name of depositary institution | CNCM Collection nationale de cultures de micro-organismes |
| 1-3-2 | Address of depositary institution | Institut Pasteur, 28, rue du Dr Roux, 75724 Paris Cedex 15, France |
| 1-3-3 | Date of deposit | 15 December 2010 (15.12.2010) |
| 1-3-4 | Accession Number | CNCM I-4418 |

1-4 Additional Indications Xenorhabdus cabanillasii JM 26

1-5 Designated States for Which Indications are Made All designations

FOR RECEIVING OFFICE USE ONLY

| 0-4 | This form was received with the international application: (yes or no) | Yes |
| 0-4-1 | Authorized officer | Krista Delimon |

FOR INTERNATIONAL BUREAU USE ONLY

| 0-5 | This form was received by the international Bureau on: |
| 0-5-1 | Authorized officer |
CLAIMS

1. Compound of formula (I):

\[ X_{aa_1} - X_{aa_2} - \text{Xaa}_3 - \text{Xaa}_4 - \text{NH}_2 \]

wherein

- Xaa\(_1\) is aspartic acid or glutamic acid,
- Xaa\(_2\) is arginine, histidine or lysine,
- Xaa\(_3\) is serine, threonine, asparagine or glutamine,
- Xaa\(_4\) is selected in the group consisting of

\[ \begin{align*}
&\text{N} & \text{C} & \text{O} \\
&\text{N} & \text{C} & \text{O} \\
&\text{N} & \text{C} & \text{O} \\
&\text{N} & \text{C} & \text{O} \\
&\text{N} & \text{C} & \text{O} \\
&\text{N} & \text{C} & \text{O} \\
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&\text{N} & \text{C} & \text{O} \\
&\text{N} & \text{C} & \text{O} \\
&\text{N} & \text{C} & \text{O} \\
\end{align*} \]

and

- R is H, OH or \(-\text{CH}_2\)-OH,
- n is 1, 2, 3, 4 or 5.

2. Compound according to claim 1 wherein Xaa\(_1\) is aspartic acid.

3. Compound according to anyone of claims 1-2 wherein Xaa\(_2\) is histidine.

4. Compound according to anyone of claims 1-3 wherein Xaa\(_3\) is asparagine.

5. Compound according to anyone of claims 1-4 wherein Xaa\(_4\) is
6. Compound according to anyone of claims 1-5 wherein R is OH.

7. Compound according to anyone of claims 1-6 wherein n=4.

8. Compound according to anyone of claims 1-7 of formula (II):

   ![Chemical Structure](image)

   wherein n is 1, 2, 3, 4 or 5.

9. Compound according to claim 8 wherein n=4.

10. Compound according to anyone of claims 1-9 for use as a medicament.

11. Compound according to anyone of claims 1-10 for use as an antibiotic agent.

12. Composition comprising a compound according to anyone of claims 1-11 and an antibiotic compound.

13. Method for producing a compound according to formula (II)

   comprising the following steps:

   a. Growing *Xenorhabdus* cabanillasii strain CNCM I-4418 in a liquid culture medium,

   b. Purifying a compound according to formula (II)

   ![Chemical Structure](image)

   wherein n=4.
14. *Xenorhabdus cabanillasii* strain deposited at CNCM having the accession number CNCM I-4418.

15. Culture supernatant from the *Xenorhabdus cabanillasii* strain of claim 14 having antibiotic activity.
FIG. 1
FIG. 4
FIG. 5

Retention time = 27.1 mn
FIG. 6
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

inv. C07K7/56 A61P31/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"A" document member of the same patent family

Date of the actual completion of the international search

15 February 2012

Date of mailing of the international search report

22/02/2012

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Authorized officer

Schleifenbaum, A

Form PCT/ISA/210 (second sheet) (April 2005)
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<thead>
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<th>Category</th>
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