



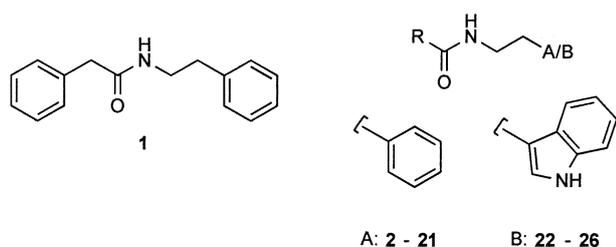
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**Cytotoxic fatty acid amides from Xenorhabdus  
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Cytotoxic Fatty Acid Amides from *Xenorhabdus*Anna Proschak,<sup>[a]</sup> Katharina Schultz,<sup>[b]</sup> Jennifer Herrmann,<sup>[b]</sup> Andrea J. Dowling,<sup>[c]</sup> Alexander O. Brachmann,<sup>[a]</sup> Richard ffrench-Constant,<sup>[c]</sup> Rolf Müller,<sup>[b, d]</sup> and Helge B. Bode<sup>\*[a]</sup>

During our search for novel natural products from entomopathogenic bacteria of the genus *Xenorhabdus*<sup>[1]</sup> we identified the known compound *N*-phenethyl-2-phenylacetamide (**1**) in

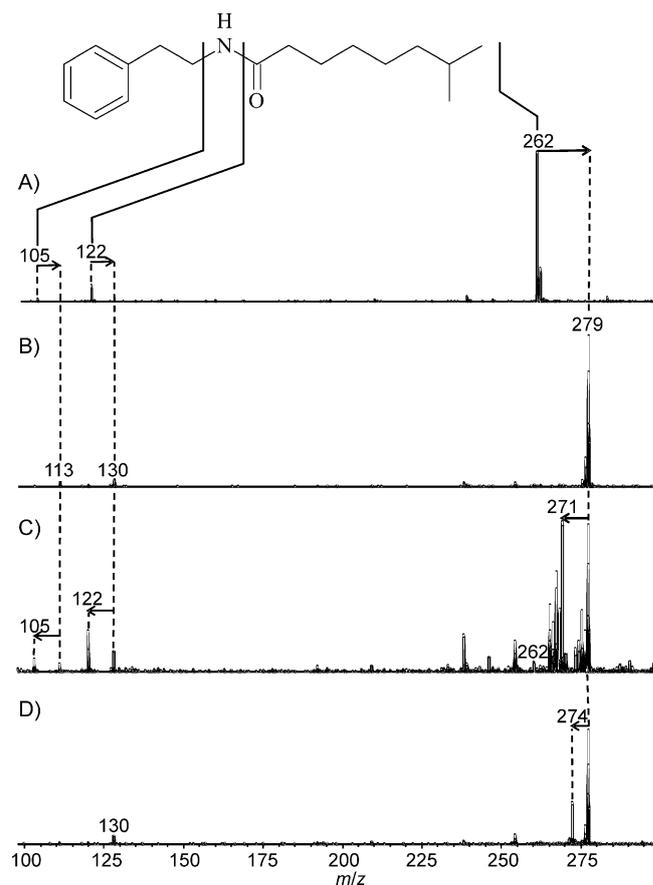


several different *Xenorhabdus* strains including *X. doucetiae* DSM17909. Acetamide **1** was originally isolated from *X. nematophilus* and showed weak cytotoxic activity, as it induced apoptosis.<sup>[2,3]</sup> A detailed search for other compounds produced by *X. doucetiae* revealed the presence of several similar amide derivatives. Unfortunately, most of these compounds are only produced in very small amounts but they could be easily synthesised. Thus, we tried to elucidate their structure without actually isolating them by following their chemical synthesis in order to obtain enough for bioactivity testing. From feeding experiments followed by HPLC/MS and GC/MS experiments we were able to elucidate the structure of 26 derivatives of this simple class of compounds. In order to determine structure–activity relationships, we synthesised two series of different acyl derivatives of the identified phenylethylamine and tryptamine derivatives. Determination of their cytotoxic activity revealed a clear correlation with their acyl chain length.

The structure elucidation of phenylethylamine and tryptamine derivatives began with an HPLC/MS screen of a crude ex-

tract of *X. doucetiae* culture in LB medium, as this strain produces the largest number of these derivatives. However, we also identified these compounds in *X. kozodoii* DSM17907, *X. romanii* DSM17910 and *X. griffiniae*, DSM17911. Derivatives were identified by the characteristic fragmentation pattern of their dissociated protonated molecular ions  $[M+H]^+$  in positive-ion mode. Fatty acid amide derivatives of phenylethylamine showed the expected fragments of  $m/z$  105 and 122, which correspond to ethylbenzene and 2-phenylethylamine ions (Figure 1). A fragment ion of  $m/z$  144 characteristic of 3-ethylindole was observed in all tryptamine derivatives.

In order to elucidate the structure of the novel derivatives, we grew strain DSM17909 in  $[U-^{13}C]$  medium. The observed mass shift in comparison to a culture grown in LB medium was indicative of the number of carbons in the respective sum formula. Subsequently, we were able to determine the number of carbons for all phenylethyl-derived compounds. However,



**Figure 1.** Mass spectra of **6** showing the reverse feeding experiment in  $[U-^{13}C]$  medium with  $^{12}C$  precursors. Mass spectra of A) unlabelled compound in  $^{12}C$  medium. B)  $^{13}C$ -fermentation culture fed with C) phenylethylamine and D) L-leucine.

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due to their low production in the [U-<sup>13</sup>C] medium, not all tryptamine derivatives observed in LB medium could be analysed by using this approach. In order to elucidate the molecular structure of the identified compounds we used an inverse feeding approach, as described previously.<sup>[4,5]</sup> Precursors of natural abundance were fed to a culture of bacteria grown in [U-<sup>13</sup>C] medium.

The incorporation of precursors can be seen as a shift to lower masses, dependent upon the number of carbon atoms introduced by the precursor. With this approach, we could confirm 2-phenylethylamine or tryptamine to be the building blocks of the identified derivatives (Table 1). The nature of the acyl moiety could be determined from similar experiments by feeding <sup>13</sup>C-leucine (for *iso*-odd fatty acids) or <sup>13</sup>C-valine (for *iso*-even fatty acids) to [U-<sup>13</sup>C]-grown cells or [D<sub>6</sub>]propionic acid

to cells grown in LB medium (for uneven straight-chain fatty acids; Figure 1, Figure S1 in the Supporting Information, Table 1) as was previously done for the PAX peptides.<sup>[5]</sup> We observed a mass shift of five units in phenylethyl compounds **2**, **7**, **19** and **22**; this indicated the incorporation of <sup>12</sup>C-L-leucine. Therefore, we assumed that these compounds carry *iso*-odd fatty acid moieties. Incorporation of <sup>12</sup>C-L-valine was not observed. Supplementation with [D<sub>6</sub>]propionic acid resulted in labelled compounds **4**, **6**, **9**, **10**, **13**, **14**, **17** and **18** according to a shift to higher masses of five units. Due to the very low concentration of tryptamine derivatives, we had difficulty in determining the incorporation of <sup>12</sup>C-L-leucine and -valine into these compounds and, thus, assumed these derivatives to carry the same acyl chains as determined for the phenylethyl derivatives. A fatty acid analysis of strain DSM17909 (Table S1) revealed the

**Table 1.** Natural compounds identified from *Xenorhabdus doucetiae* and their relative amount to maximum production of **1** (100%) and the labelling status in <sup>12</sup>C-LB or [U-<sup>13</sup>C] medium.

No	Amount [%]	M <sub>w</sub>	L-Leu	Phenyl-ethylamine	Try	[U-D <sub>6</sub> ]propionate	R	A/B
1	100	C <sub>16</sub> H <sub>17</sub> NO	239	-	✓	-		A
2	5	C <sub>13</sub> H <sub>19</sub> NO	205	✓	✓	-		A
3	4	C <sub>14</sub> H <sub>21</sub> NO	219	-	✓	-		A
4	10	C <sub>15</sub> H <sub>23</sub> NO	233	-	✓	✓		A
5	30	C <sub>16</sub> H <sub>25</sub> NO	247	-	✓	-		A
6	13	C <sub>17</sub> H <sub>27</sub> NO	261	-	✓	✓		A
7	13	C <sub>17</sub> H <sub>27</sub> NO	261	✓	✓	-		A
8	85	C <sub>18</sub> H <sub>29</sub> NO	275	-	✓	-		A
9	8	C <sub>19</sub> H <sub>29</sub> NO	287	-	✓	✓		A
10	51	C <sub>19</sub> H <sub>31</sub> NO	289	-	✓	✓		A
11	57	C <sub>20</sub> H <sub>31</sub> NO	301	-	✓	-		A
12	66	C <sub>20</sub> H <sub>33</sub> NO	303	-	✓	-		A
13	13	C <sub>21</sub> H <sub>33</sub> NO	315	-	✓	✓		A
14	7	C <sub>21</sub> H <sub>35</sub> NO	317	-	✓	✓		A
15	27	C <sub>22</sub> H <sub>35</sub> NO	329	-	✓	-		A
16	12	C <sub>22</sub> H <sub>37</sub> NO	331	-	✓	-		A
17	3	C <sub>23</sub> H <sub>37</sub> NO	343	-	✓	✓		A
18	1	C <sub>23</sub> H <sub>39</sub> NO	345	-	✓	✓		A
19	1	C <sub>23</sub> H <sub>39</sub> NO	345	✓	✓	-		A
20	3	C <sub>24</sub> H <sub>39</sub> NO	357	-	✓	-		A
21	1	C <sub>24</sub> H <sub>41</sub> NO	359	-	✓	-		A
22	22	C <sub>17</sub> H <sub>24</sub> N <sub>2</sub> O	272	✓	-	✓		B
23	3	C <sub>19</sub> H <sub>28</sub> N <sub>2</sub> O	300	n.d.	-	✓		B
24	4	C <sub>21</sub> H <sub>32</sub> N <sub>2</sub> O	328	n.d.	-	✓		B
25	4	C <sub>22</sub> H <sub>32</sub> N <sub>2</sub> O	340	n.d.	-	✓		B
26	2	C <sub>23</sub> H <sub>34</sub> N <sub>2</sub> O	354	n.d.	-	✓		B

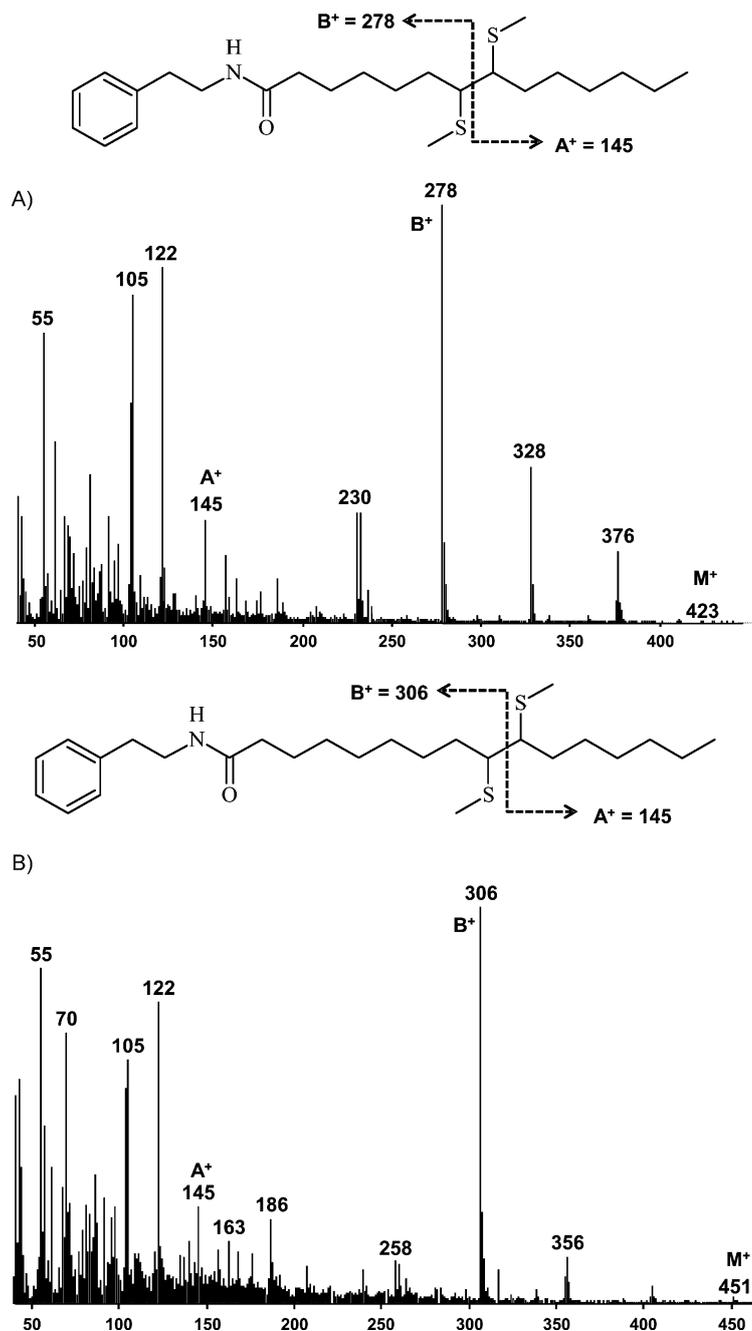
n.d.: not determined.

presence of 16:0 9-10-CH<sub>2</sub> cyclopropyl fatty acid, and therefore we also fed ([D<sub>3</sub>]methyl)methionine to LB-grown cells. However, no incorporation of label was observed.

As the sum formulas of compounds **9**, **11**, **13**, **15**, **17**, **20**, **25** and **26** are indicative of unsaturated acyl side chains, we tried to determine the position of the double bond. Thus, we derivatised the crude extract with dimethyldisulfide (DMDS)<sup>[6]</sup> and searched for the expected DMDS derivatives. Due to the low amount of unsaturated derivatives (Table 1) only compounds **15** and **20** were identified as DMDS adducts. The investigated compounds gave distinct EI mass spectra, which display specific fragments that allow the localisation of the double bonds (Figure 2). Mass peaks M<sup>+</sup>, A<sup>+</sup>, B<sup>+</sup> are characteristic of the DMDS adduct. For both compounds, mass peak A<sup>+</sup> was identified as a fragment ion of *m/z* 145. The characteristic ion B<sup>+</sup> was determined as *m/z* 278 for compound **15** or *m/z* 306 for **20**. Molecular ion M<sup>+</sup>, which occurred at very low intensity, was observed as a fragment ion of *m/z* 423 (**15**) or *m/z* 451 (**20**). Thus, both compounds were revealed to be ω<sub>7</sub>-desaturated fatty acid derivatives; this is consistent with the detection of ω<sub>7</sub>-desaturated fatty acids in the whole-cell fatty profile (Table S1).

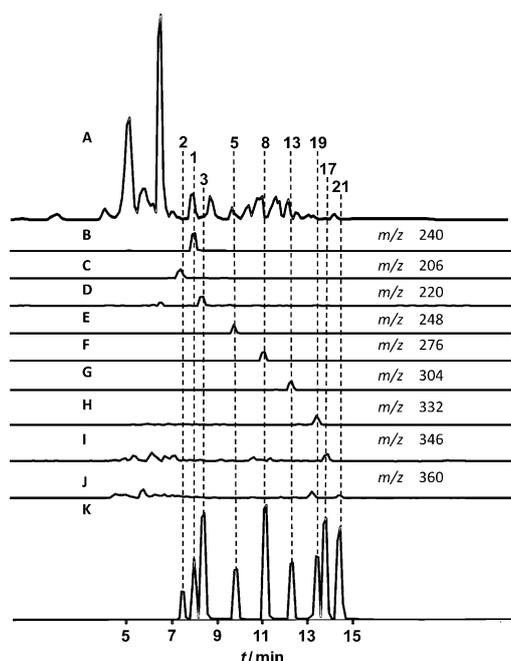
In order to investigate the bioactivity of the identified alkylamide derivatives in biological assays, we synthesised several derivatives from *Xenorhabdus* (see the Supporting Information). This allowed the unambiguous identification of compounds **1–3**, **5**, **8**, **13**, **17**, **19** and **21**, as they showed the same retention times and fragmentation patterns as the corresponding natural product (Figure 3).

The synthesised compounds were then tested in a standardised cell-viability assay by using a tetrazolium salt (MTT).<sup>[7]</sup> The compounds were tested against mouse fibroblasts (L929 cells; subcutaneous connective tissue) and human promyelocytic leukaemia cells (HL-60); details of their activity are given in Table S2. We observed a strong dependency of the cytotoxic activity on the acyl chain length. Furthermore, the tryptamine derivatives were more potent than the corresponding *N*-phenylethylamine ones. The results for the HL-60 cells are illustrated in Figure 4; similar results have also been observed for L929 cells (Figure S2). In general, the cytotoxic activity of several derivatives was greater than the activity of the previously known compound **1**. We have also tested some of these derivatives against insect haemocytes, which represent the first line of defence pathogens like *Xenorhabdus* must overcome in order to establish insect infection.<sup>[8,9]</sup> Here we tested **1** as well as short- (**30** and **36**), medium- (**12** and **41**) and long-chain derivatives (**21** and **44**) of phenylethylamine and tryptamine derivatives, respectively. The short- and medium-chain tryptamine derivatives **36** and **41** showed an LD<sub>50</sub> values of 2.8 and 4.3 μg mL<sup>-1</sup>, respectively, against insect haemocytes from *Galleria mellonell-*



**Figure 2.** Mass spectra of DMDS adducts of A) (*Z*)-*N*-phenylethyltetradec-7-enamide (**15**) and B) (*Z*)-*N*-phenylethylhexadec-9-enamide (**20**).

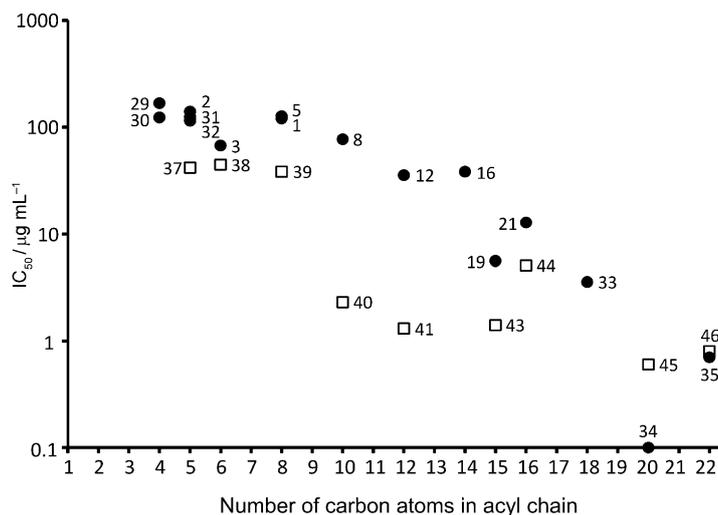
*la*, and haemocyte viability was strongly affected by these two compounds (Figure 5). Losses in cell number and mitochondrial viability were accompanied by a massive alteration in cytoskeletal phenotype, with massive contraction in the case of **41** (Figure 5B) or the formation of stress fibres caused by **36** (Figure 5C). No activity was observed for the other compounds even at the highest concentration tested (100 μg mL<sup>-1</sup>). Thus, we might conclude that, as in the cytotoxicity assay against L929 or HL-60 cells, the tryptamine derivatives are more active. However, against insect haemocytes, the derivatives with the short and medium acyl chains are significantly more active;



**Figure 3.** A) HPLC/MS analysis of a crude extract of *Xenorhabdus doucetiae* (base peak chromatogram). B)–J) Extracted ion chromatograms of natural alkyl amides of phenylethyl derivatives. K) Base peak chromatogram of synthesised phenylethyl derivatives.

this is in accordance with their being more abundant in *Xenorhabdus* cultures (Table 1) where they can add to the overall insecticidal activity of these bacteria. Compound 1 was shown to induce apoptosis through cytochrome *c*-dependent caspase-3 activation in U937 cells.<sup>[2]</sup> Whether the other amide derivatives have the same mode of action and whether the same mode of action also applies to the insect haemocytes will be investigated in the future.

In this study we have demonstrated the structure elucidation of 26 biologically active fatty acid amide derivatives of

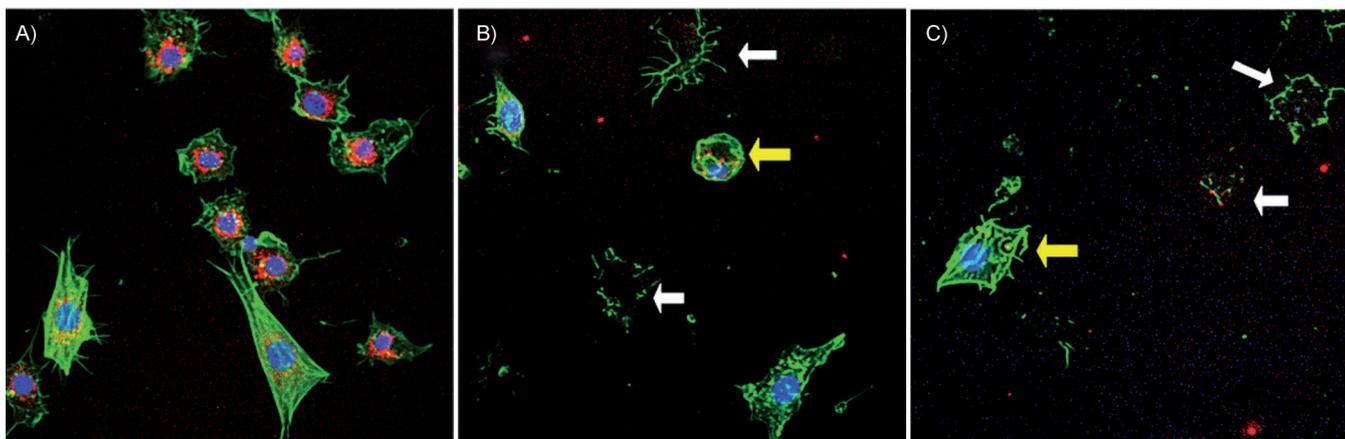


**Figure 4.** Structure–activity relationships of *N*-phenylethylamine (●) and tryptamine (□) derivatives in HL-60 cells.

phenylethylamine and tryptamine produced by *Xenorhabdus doucetiae*. We achieved this by using a combined analytical approach of HPLC/MS analysis, labelling experiment and fatty acid analysis of DMDS adducts by GC/MS. Since the bioactivity testing of natural products from natural systems is often hampered by the low yields obtained from traditional purification methods, our approach of structure elucidation followed by synthesis in order to obtain enough material for testing might be more efficient and timesaving than the actual isolation. However, the approach is clearly limited by the size and complexity of the compounds to be analysed.

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**Figure 5.** Haemocyte morphology following treatment with compounds 36 and 41. A) Control haemocytes treated with 2% DMSO. Haemocytes treated with 100  $\mu\text{g mL}^{-1}$  of B) 41 and C) 36. White arrows indicate cytoskeletal corpses with absent nucleus, and yellow arrows show haemocytes with dramatic cytoskeletal rearrangements. Blue = nucleus, green = filamentous actin cytoskeleton, red = viable mitochondria.

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**Keywords:** cytotoxicity · fatty acid alkylamides · natural products · structure elucidation · symbiotic bacteria

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