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## **Pentadecaibins I–V: 15-Residue Peptaibols Produced by a Marine-Derived Trichoderma sp. of the Harzianum Clade**

Anne-Isaline van Bohemen, Nicolas Ruiz, Aurore Zalouk-Vergnoux, Aurore Michaud, Thibaut Robiou Du Pont, Irina Druzhinina, Lea Atanasova, Soizic Prado, Bernard Bodo, Laurence Meslet-Cladiere, et al.

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**Pentadecaibins I–V: 15-Residue Peptaibols Produced by a Marine-Derived *Trichoderma* sp. of the *Harzianum* Clade**

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9 **1 Pentadecaibins I–V: 15-Residue Peptaibols Produced by a**  
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12 **2 Marine-Derived *Trichoderma* sp. of the *Harzianum* Clade**  
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3 1 ABSTRACT: In the course of investigations on peptaibol chemodiversity from marine-derived  
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5 2 *Trichoderma* spp., five new 15-residue peptaibols named pentadecaibins I–V (1–5) were isolated  
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7 3 from the solid culture of the strain *Trichoderma* sp. MMS1255 belonging to the *T. harzianum*  
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9 4 species complex. Phylogenetic analyses allowed precise positioning of the strain close to  
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11 5 *T. lentiforme* lineage inside the *Harzianum* clade. Peptaibol sequences were elucidated on the basis  
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13 6 of their MS/MS fragmentation and extensive 2D-NMR experiments. Amino acid configurations  
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15 7 were determined by Marfey's analyses. The pentadecaibins are based on the sequences Ac-Aib1-  
16  
17 8 Gly2-Ala3-Leu4-Aib/Iva5-Gln6-Aib/Iva7-Val/Leu8-Aib9-Ala10-Aib11-Aib12-Aib13-Gln14-  
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19 9 Pheo15. Characteristic of the pentadecaibin sequences is the lack of the Aib-Pro motif commonly  
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21 10 present in peptaibols produced by *Trichoderma* spp. Genome sequencing of *Trichoderma* sp.  
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23 11 MMS1255 allowed the detection of a 15-module NRPS-encoding gene closely associated with  
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25 12 pentadecaibin biosynthesis. Pentadecaibins were assessed for their potential antiproliferative and  
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27 13 antimicrobial activities.  
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3 1 Numerous fungi belonging to the genus *Trichoderma* are known to produce bioactive linear  
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5 2 nonribosomal peptides (NRP) named peptaibols. Peptaibols are characterized by molecular masses  
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7 3 ranging from 500 to 2 000 Da containing 5-20 amino-acid (AA) residues, an *N*-acetyl terminus and  
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9 4 a *C*-terminal AA reduced into the corresponding amino-alcohol.<sup>1-4</sup> Peptaibol biosynthesis by non  
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11 5 ribosomal peptide synthetases (NRPS)<sup>5-7</sup> allows incorporation of non-proteinogenic AA into the  
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13 6 chain such as  $\alpha$ -aminoisobutyric acid (Aib) or isovaline (Iva).<sup>2</sup> In *Trichoderma* spp., peptaibols are  
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15 7 produced as complex microheterogeneous mixtures and can be classified into five main families  
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17 8 (11-, 14-, 18-, 19- and 20-residue peptaibols) according to their AA chain length. Peptaibols  
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19 9 represent the most important subgroup of the peptaibiotics group and numerous sequences have  
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21 10 already been published and listed in the *Comprehensive Peptaibiotics Database*.<sup>8,9</sup>  
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27 11 Peptaibols have been studied for their potential biological activities particularly as alternative  
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29 12 sources for antibiotic research or as new therapeutic agents.<sup>10,11</sup> Indeed, they have been shown to  
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31 13 exhibit a wide range of biological activities including antibacterial activity against Gram-positive  
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33 14 bacteria<sup>12-15</sup> and dormant mycobacteria,<sup>16,17</sup> antifungal activity,<sup>14,18,19</sup> antiviral activity -  
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35 15 particularly against infection caused by the tobacco mosaic virus,<sup>20-22</sup> and antiparasitic activity  
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37 16 against amoebae (*Dictyostelium* sp.)<sup>23</sup> and protozoa (*Plasmodium falciparum*).<sup>24</sup> They have been  
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39 17 also studied for their activities against fungal plant pathogens.<sup>25-28</sup> Peptaibols are known to act by  
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41 18 forming pores or voltage-dependent ion channels in cell membranes increasing membrane  
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43 19 permeability.<sup>23,29</sup>  
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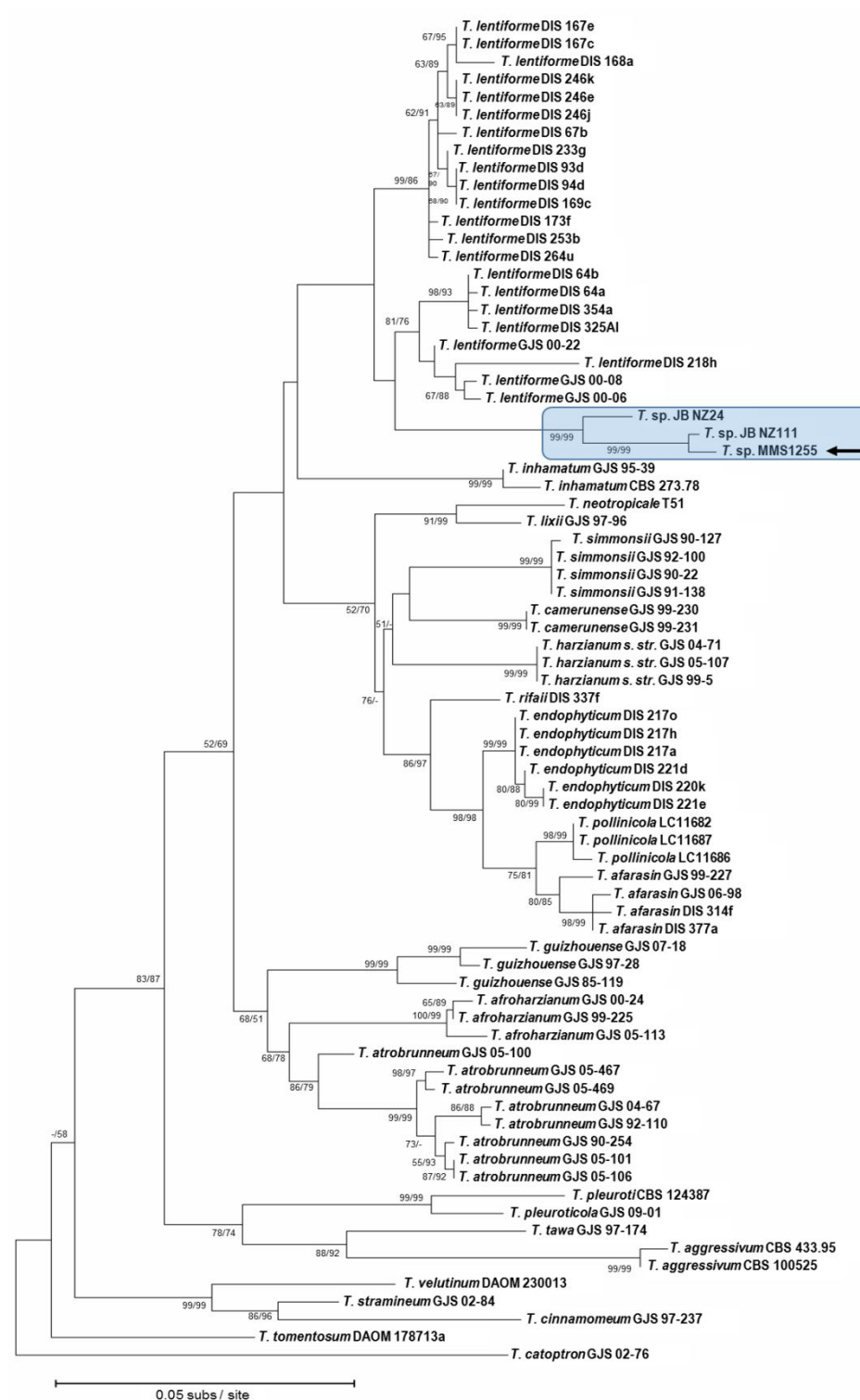
49 20 In a continuation of our investigations into peptaibol chemodiversity of French marine-derived  
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51 21 *Trichoderma*,<sup>30-33</sup> we focused in this study on the peptaibol production of the strain *Trichoderma*  
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53 22 sp. MMS1255. Phylogenetic analysis was performed to determine the precise taxonomic position  
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1 of the peptaibol-producing strain within the *Trichoderma* genus and more specifically in the  
2 *T. harzianum* species complex. Peptaibols isolated contained 15 AA and were characterized by the  
3 lack of the widespread Aib-Pro motif. Their sequences were determined by MS analysis and their  
4 complete structures were established by NMR spectroscopy. Marfey analysis and circular  
5 dichroism were used for the determination of the AA configurations. The genome sequencing of  
6 the peptaibol-producing strain revealed the presence of a new gene encoding a 15 module-NRPS.  
7 The organization of modules and domains in this gene has never been described before and may  
8 be involved in the biosynthesis of the isolated peptaibols. Cytotoxicity and antimicrobial activities  
9 were shown for these new compounds.

10

## 1 RESULTS AND DISCUSSION

2 **Morphological and Phylogenetic Analyses of *Trichoderma* sp. MMS1255.** The Dextrose  
3 Casein Agar (DCA) culture of strain MMS1255 was observed as successive concentric greenish to  
4 whitish zones on the agar surface plate. Microscopically, typically highly branched conidiophores  
5 were observed producing clustered phialides arising near 90° with respect to the others members  
6 (Figure S1). The taxonomic identification was performed by DNA Barcoding (Table S1, Figure  
7 S2). Preliminary molecular identification based on the internal transcribed spacer regions of the  
8 nuclear ribosomal RNA gene cluster (ITS1 and ITS2) led to the *Trichoderma harzianum* species  
9 complex. However, ITS is not sufficiently polymorphic to distinguish species in the *T. harzianum*  
10 complex,<sup>34</sup> thus we used the translation elongation factor 1- $\alpha$  (*tefl*) and RNA polymerase II subunit  
11 2 (*rpb2*) gene markers. This analysis localized the strain *Trichoderma* sp. MMS1255 among the  
12 *Trichoderma Harzianum* Clade (Figure S6).<sup>35</sup> For a deeper identification inside this infrageneric  
13 group, we performed a combined analysis of the marker genes *tefl* and calmodulin (*cal*). This  
14 resulted in the identification of the strain MMS1255 close to the *T. lentiforme* lineage and to two  
15 isolates previously described as *T. harzianum* JBNZ24 and JBNZ111 (Figure 1).<sup>36</sup> Considering the  
16 Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept,<sup>37</sup> further  
17 phenotyping characterizations are needed to clarify *Trichoderma* sp. MMS1255, *T. harzianum*  
18 JBNZ24 and JBNZ111 phylogenetic localization within or outside the borders of *T. lentiforme*.



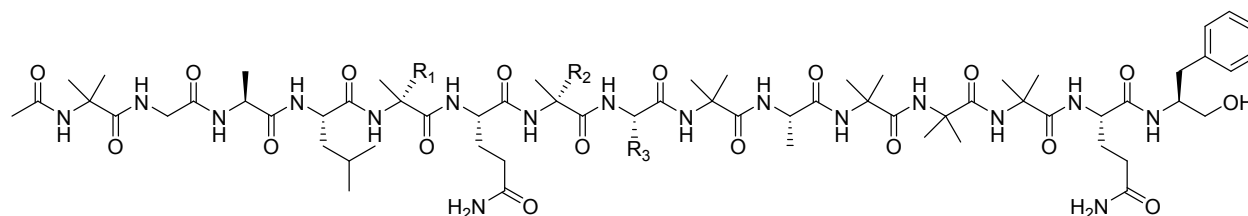
**Figure 1.** Phylogram of the best maximum likelihood tree (log likelihood: -4619.24) of *tefl* and *cal*, which includes only species in the *T. harzianum* complex. Values at nodes represent ML bootstrap (MLBP)/MP bootstrap (MPBP). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 76 nucleotide sequences. There were a total of 734 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.<sup>38</sup>



**1 Isolation and Structure Elucidation of Pentadecaibins (1–5).** A peptaibol-enriched fraction  
 2 extracted from a solid culture (DCA) of *Trichoderma* sp. MMS1255 was analyzed by MS and  
 3 revealed the presence of an unusual MS profile with  $[M + Na]^+$  ions ranging from  $m/z$  1466 to 1521  
 4 (Figure S3). Whereas ions ranging from  $m/z$  1466 to  $m/z$  1493 were supposed to correspond to 14-  
 5 residue peptaibols produced by *Trichoderma* strains,<sup>5,26,39,40</sup> ions  $m/z$  1493 and  $m/z$  1521 did not  
 6 match any known peptaibols. Herein, we report the discovery, structure elucidation and biological  
 7 activities of five 15-residue peptaibols (1-5) isolated from the marine-derived strain *Trichoderma*  
 8 sp. MMS1255.

### 9 Chart 1

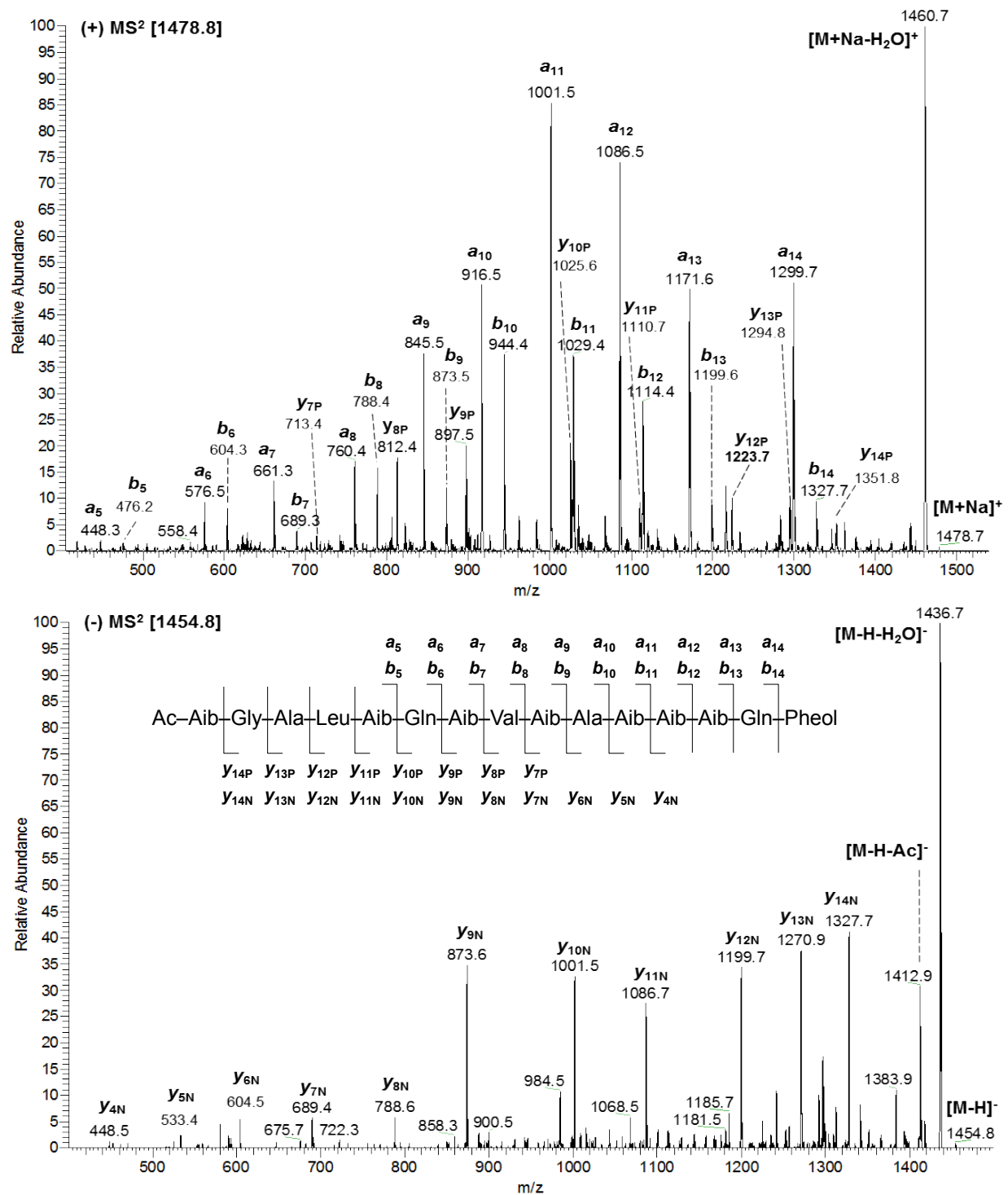
Ac - Aib<sup>1</sup> - Gly<sup>2</sup> - Ala<sup>3</sup> - Leu<sup>4</sup> - **Aib/D-Iva<sup>5</sup>** - Gln<sup>6</sup> - **Aib/D-Iva<sup>7</sup>** - **Val/Leu<sup>8</sup>** - Aib<sup>9</sup> - Ala<sup>10</sup> - Aib<sup>11</sup> - Aib<sup>12</sup> - Aib<sup>13</sup> - Gln<sup>14</sup> - Pheol<sup>15</sup>



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
<b>1</b>	Me (Aib <sup>5</sup> )	Me (Aib <sup>7</sup> )	<i>iso</i> -propyl (Val <sup>8</sup> )
<b>2</b>	Me (Aib <sup>5</sup> )	Me (Aib <sup>7</sup> )	<i>iso</i> -butyl (Leu <sup>8</sup> )
<b>3</b>	Me (Aib <sup>5</sup> )	Et (D-Iva <sup>7</sup> )	<i>iso</i> -propyl (Val <sup>8</sup> )
<b>4</b>	Me (Aib <sup>5</sup> )	Et (D-Iva <sup>7</sup> )	<i>iso</i> -butyl (Leu <sup>8</sup> )
<b>5</b>	Et (D-Iva <sup>5</sup> )	Et (D-Iva <sup>7</sup> )	<i>iso</i> -propyl (Val <sup>8</sup> )

11 Compound **1** was obtained as a white powder. The molecular formula was determined to be  
 12 C<sub>68</sub>H<sub>113</sub>N<sub>17</sub>O<sub>18</sub> on the basis of HRESIMS ( $m/z$  750.9132  $[M + 2Na]^{2+}$ ). The amino acid sequence  
 13 of **1** was determined on the basis of ESIMS<sup>2</sup> analyses by identifying fragment ions of the series a<sub>n</sub>,  
 14 b<sub>n</sub>, y<sub>nP</sub> generated in positive ionization (P refers to positive-ion mode) and y<sub>nN</sub> generated in negative

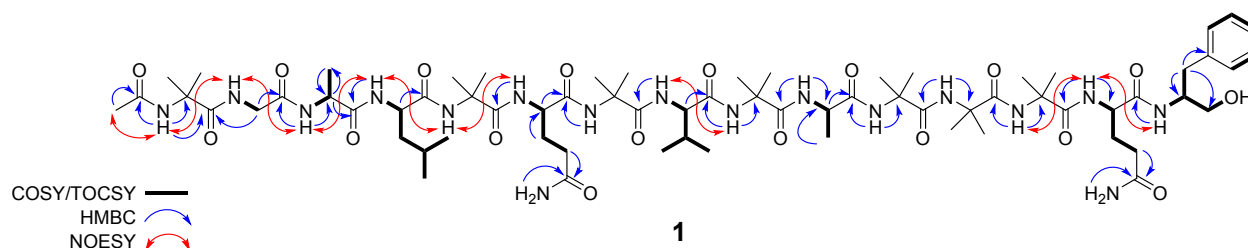
1 ionization (N for negative-ion mode) (Figure 2, Table S2). Fragmentation of the sodium adduct  
2  $[M + Na]^+$  produced a series of fragment ions  $a_5/b_5$  to  $a_{14}/b_{14}$  providing successive losses of Gln<sup>6</sup>,  
3 Aib<sup>7</sup>, Vxx<sup>8</sup>, Aib<sup>9</sup>, Ala<sup>10</sup>, Aib<sup>11</sup>, Aib<sup>12</sup>, Aib<sup>13</sup>, Gln<sup>14</sup> and the C-terminal Pheol<sup>15</sup> (phenylalaninol; Vxx  
4 = Val or Iva). In order to complete the sequence, ESI-MS<sup>2</sup> fragmentation of the deprotonated  
5 molecule  $[M - H]^-$  yielded diagnostic fragment ions  $y_{4N}$  to  $y_{14N}$ . Hence, the N-terminal part of the  
6 sequence was assigned as Ac-Aib<sup>1</sup>-Gly<sup>2</sup>-Ala<sup>3</sup>-Lxx<sup>4</sup>-Aib<sup>5</sup>-Gln<sup>6</sup>-Aib<sup>7</sup>-Vxx<sup>8</sup>-Aib<sup>9</sup>-Ala<sup>10</sup>-Aib<sup>11</sup> (Lxx =  
7 Leu or Ile). Fragment ions  $y_{7P}$  to  $y_{14P}$  assigned in positive mode supported the N-terminal sequence  
8 deduced from the  $y_{nN}$  ions series. Based on the above mass spectrometry analysis, the sequence of  
9 **1** was proposed to be Ac-Aib<sup>1</sup>-Gly<sup>2</sup>-Ala<sup>3</sup>-Lxx<sup>4</sup>-Aib<sup>5</sup>-Gln<sup>6</sup>-Aib<sup>7</sup>-Vxx<sup>8</sup>-Aib<sup>9</sup>-Ala<sup>10</sup>-Aib<sup>11</sup>-Aib<sup>12</sup>-  
10 Aib<sup>13</sup>-Gln<sup>14</sup>-Pheol<sup>15</sup>.



1 **Figure 2.** Positive and negative ESI-MS<sup>2</sup> fragmentation of pentadecaibin I (1).

2

The sequence determined from mass spectral fragmentations was thus verified by 1D and 2D-NMR correlations allowing in addition the assignment of the isomeric residues Val/Iva and Leu/Ile (Figure 3, Table 1).



**Figure 3.** NMR key correlations of pentadecaibin I (**1**) (COSY/TOCSY, HMBC and NOESY).

The  $^1\text{H}$  NMR spectrum of compound **1** exhibited in the range 6.7-9 ppm, 17 exchangeable signals characteristic of amide protons among which 11 singlets corresponding to seven Aib residues, two side-chain  $\text{NH}_2$  protons of the two Gln, one broad singlet characterising one Gly, five doublets (for Ala, Leu, two Gln and Val) and two multiplets (for Ala and Pheol). In addition, the aromatic protons of the mono-substituted phenyl ring of the Pheol were characterized by multiplets at  $\delta_{\text{H}}$  7.13, 7.18 and 7.27. Between  $\delta_{\text{H}}$  1.48 and 1.37 ppm were depicted 14 methyl singlets assigned to seven Aib residues.

$^1\text{H}$ - $^1\text{H}$  correlations from COSY and TOCSY spectra allowed to define seven spin systems corresponding to Gly, two Ala, Leu, Val and two Gln as represented in bold in Figure 3. An additional moiety was pointed out by  $^1\text{H}$ - $^1\text{H}$  correlations between a hydroxy group at  $\delta_{\text{H}}$  4.53, the methylene group at  $\delta_{\text{H}}$  3.37 ( $\delta_{\text{C}}$  63.2) and the methine at  $\delta_{\text{H}}$  3.89 ( $\delta_{\text{C}}$  52.4), which itself was linked to the NH at  $\delta_{\text{H}}$  7.13 and the methylene at  $\delta_{\text{H}}$  2.89/2.57 ( $\delta_{\text{C}}$  36.7).  $^1\text{H}$ - $^{13}\text{C}$  HMBC correlations allowed this methylene carbon to be linked to the protons of the phenyl ring, resulting in the characterization of a Pheol residue (Table 1). The  $\epsilon$ -amide protons of the two Gln were assigned from their HMBC correlations with the corresponding  $\gamma$ -carbon and their *syn* or *anti*-disposition

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3 1 from their NOE correlations with the respective  $\gamma$ -protons of the lateral chain (Figure S15). The  
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5 2 carbonyl at  $\delta_C$  171.0 was assigned to the *N*-terminal acetyl because its HMBC correlation with the  
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7 3 methyl at  $\delta_H$  1.93 and the NH of an Aib at  $\delta_H$  8.74, thus assigned to Aib<sup>1</sup>. The carbonyl of this Aib<sup>1</sup>  
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9 4 at  $\delta_C$  176.1 was correlated not only to the NH of Aib<sup>1</sup>, but also to the  $\alpha,\alpha'$ -protons of Gly<sup>2</sup>. The  
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11 5 CO of Gly<sup>2</sup> at  $\delta_C$  171.8 gave cross-peaks with these  $\alpha,\alpha'$ -protons and the NH at  $\delta_H$  8.00 (d,  $J =$   
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13 6 6.3) assigned to Ala<sup>3</sup>. Continuing this series of NH<sub>*i*+1</sub> to CO<sub>*i*</sub> correlations thus observed from Ac-  
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15 7 Aib<sup>1</sup> to Pheol<sup>15</sup>, the following completed sequence of **1** was finally confirmed as Ac-Aib<sup>1</sup>-Gly<sup>2</sup>-  
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17 8 Ala<sup>3</sup>-Leu<sup>4</sup>-Aib<sup>5</sup>-Gln<sup>6</sup>-Aib<sup>7</sup>-Val<sup>8</sup>-Aib<sup>9</sup>-Ala<sup>10</sup>-Aib<sup>11</sup>-Aib<sup>12</sup>-Aib<sup>13</sup>-Gln<sup>14</sup>-Pheol<sup>15</sup> and was named  
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19 9 pentadecaibin I (**1**). The sequences of the other isolated peptaibols were identified and  
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21 10 characterized in the same way.  
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1 **Table 1. <sup>1</sup>H and <sup>13</sup>C NMR of Pentadecaibin I (1) (<sup>1</sup>H 600 MHz, <sup>13</sup>C 150 MHz DMSO-*d*<sub>6</sub>)**

Pos.	δ <sub>C</sub> , type	δ <sub>H</sub> , mult. ( <i>J</i> in Hz)	Pos.	δ <sub>C</sub> , type	δ <sub>H</sub> , mult. ( <i>J</i> in Hz)	Pos.	δ <sub>C</sub> , type	δ <sub>H</sub> , mult. ( <i>J</i> in Hz)
Ac-Aib <sup>1</sup>			Gln <sup>6</sup>			Aib <sup>11</sup>		
C=O	176.1, C	-	C=O	173.1, C	-	C=O	175.2, C	-
N-H	-	8.74, s	N-H	-	7.83, d (2.6)	N-H	-	7.87, s
α C	55.6, C	-	α CH	56.7, CH	3.74, m	α C	55.5, C	-
β CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s	β CH <sub>2</sub>	26, CH <sub>2</sub>	2.02, m	β CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s
β' CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s		-	1.89, m	β' CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s
COCH <sub>3</sub>	171.0, C	-	γ CH <sub>2</sub>	31.1, CH <sub>2</sub>	2.22, m			
COCH <sub>3</sub>	23.1, CH <sub>3</sub>	1.93, s		-	2.09, m			
			δ C=O	173.1, C	-			
			εNH <sub>2</sub> anti	-	7.18, s			
			εNH <sub>2</sub> syn	-	6.73, s			
Gly <sup>2</sup>			Aib <sup>7</sup>			Aib <sup>12</sup>		
C=O	171.8, C	-	C=O	176.2, C	-	C=O	175.5, C	-
N-H	-	9.06, brs	N-H	-	8.54, s	N-H	-	7.50, s
α CH <sub>2</sub>	43.8, CH <sub>2</sub>	3.83, m (pro S)	α C	57.7, C	-	α C	56.0, C	-
	-	3.59, dd (16.1, 4.6) (pro R)	β CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s	β CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s
			β' CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s	β' CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s
Ala <sup>3</sup>			Val <sup>8</sup>			Aib <sup>13</sup>		
C=O	175.4, C	-	C=O	172.7, C	-	C=O	174.5, C	-
N-H	-	8.00, d (6.3)	N-H	-	7.80, d (5.0)	N-H	-	7.97, s
α CH	51.1, CH	4.04, dq (6.3, 7.5)	α CH	63.7, CH	3.37, m	α C	55.8, C	-
β CH <sub>3</sub>	16.1, CH <sub>3</sub>	1.39, d (7.5)	β CH	28.8, CH	2.08, m	β CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s
			γ CH <sub>3</sub>	18.8, CH <sub>3</sub>	0.98, d (6.4)	β' CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s
			γ' CH <sub>3</sub>	21.2, CH <sub>3</sub>	0.86, d (6.5)			
Leu <sup>4</sup>			Aib <sup>9</sup>			Gln <sup>14</sup>		
C=O	173.7, C	-	C=O	175.5, C	-	C=O	171.2, C	-
N-H	-	7.88, d (8.3)	N-H	-	8.00, s	N-H	-	7.48, d (7.2)
α CH	54.3, CH	4.01, m	α C	55.3, C	-	α CH	54.3, CH	3.84, m
β CH <sub>2</sub>	38.5, CH <sub>2</sub>	1.79, m	β CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s	β CH <sub>2</sub>	26.8, CH <sub>2</sub>	1.88, m
	-	1.55, m	β' CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s		-	1.80, m
γ CH	24.3, CH	1.53, m				γ CH <sub>2</sub>	31.8, CH <sub>2</sub>	2.16, m
δ CH <sub>3</sub>	22.4, CH <sub>3</sub>	0.83, d (6.5)					-	1.94, m
δ' CH <sub>3</sub>	21.6, CH <sub>3</sub>	0.87, d (6.5)				δ C=O	173.5, C	-
						εNH <sub>2</sub> anti	-	6.99, s
						εNH <sub>2</sub> syn	-	6.62, s
Aib <sup>5</sup>			Ala <sup>10</sup>			Pheol <sup>15</sup>		
C=O	176.2, C	-	C=O	174.4, C	-	NH	-	7.13, m
N-H	-	8.40, s	N-H	-	7.98, m	α CH	52.4, CH	3.89, m
α C	55.5, C	-	α CH	52.2, C	3.85, m	β CH <sub>2</sub>	36.7, CH <sub>2</sub>	2.89, dd (13.4, 4.3)
β CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s	β CH <sub>3</sub>	16.3, CH <sub>3</sub>	1.38, d (7.5)		-	2.57, dd (13.4, 9.2)
β' CH <sub>3</sub>	(*), CH <sub>3</sub>	(°) s				β CH <sub>2</sub> OH	63.2, CH <sub>2</sub>	3.37, m
							-	3.37, m
						OH	-	4.53, dd (6.1/6.1)
						C-1	139.3, C	-
						C-2,6	129.3, CH	7.27, m (8.2)
						C-3,5	127.8, CH	7.18, m (8.2, 8.2)
						C-4	125.7, CH	7.12, m (8.2, 8.2)

β/β' CH<sub>3</sub> Aib<sup>1</sup>, Aib<sup>5</sup>, Aib<sup>7</sup>, Aib<sup>9</sup>, Aib<sup>11</sup>, Aib<sup>12</sup>, Aib<sup>13</sup>.<sup>13</sup>C (\*) 26.9, 26.5, 26.1, 26.0, 25.9, 25.8, 25.8, 23.1, 22.8, 22.7, 22.6, 22.5, 22.4, 21.6, may be interchanged.<sup>1</sup>H (°) 1.48, 1.46, 1.46, 1.45, 1.45, 1.43, 1.43, 1.42, 1.39, 1.39, 1.38, 1.38, 1.37, 1.37, may be interchanged.

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2  
3 1 Compound **2** was obtained as a white powder. The molecular formula was determined to be  
4  
5 2  $C_{69}H_{115}N_{17}O_{18}$  on the basis of HRESIMS ( $m/z$  757.9172  $[M + 2Na]^{2+}$ ) indicating the presence of  
6  
7 3 an additional methylene group between **1** and **2**. This was confirmed by positive and negative mass  
8  
9 4 fragmentations (Figures S4-S5, Table S2), suggesting the presence of Ile/Leu in position 8 instead  
10  
11 5 of Val<sup>8</sup> for **1**. Detailed analyses of 1D and 2D NMR spectroscopic data were consistent with mass  
12  
13 6 fragmentation analyses and both residues were identified as leucine from COSY and TOCSY data  
14  
15 7 (Table S3). HMBC correlations confirmed the second Leu residue in position 8. Indeed,  $^1H$ - $^{13}C$   
16  
17 8 correlations were observed between the CO group of the Aib<sup>7</sup> at  $\delta_C$  176.3 was correlated to the NH  
18  
19 9 at  $\delta_H$  7.89 of the Leu<sup>8</sup> and the CO group of this Leu at  $\delta_C$  174.0 was correlated to the methine H $\alpha$   
20  
21 10 at  $\delta_H$  3.90 ( $\delta_C$  54.8) and to the NH at  $\delta_H$  8.03 of the Aib<sup>9</sup> (Figure S27). Therefore, the structure of  
22  
23 11 compound **2** was established as Ac-Aib<sup>1</sup>-Gly<sup>2</sup>-Ala<sup>3</sup>-Leu<sup>4</sup>-Aib<sup>5</sup>-Gln<sup>6</sup>-Aib<sup>7</sup>-Leu<sup>8</sup>-Aib<sup>9</sup>-Ala<sup>10</sup>-Aib<sup>11</sup>-  
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25 12 Aib<sup>12</sup>-Aib<sup>13</sup>-Gln<sup>14</sup>-Pheol<sup>15</sup> and named pentadecaibin II.

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31 13 Compound **3** was obtained as a white powder and exhibited the same molecular formula  
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33 14 ( $C_{69}H_{115}N_{17}O_{18}$ ) as compound **2**. On the basis of mass fragmentation analyses, compound **3** only  
34  
35 15 differed from **1** by the presence of a Val/Iva instead of an Aib in position 7 (Figures S6-S7, Table  
36  
37 16 S2). Mass spectrometry sequencing was confirmed by NMR investigations (Table S4) and the  
38  
39 17 TOCSY spectrum showed characteristic signals of one Iva and one Val isomeric residues. The CO  
40  
41 18 group of Gln<sup>6</sup> at  $\delta_C$  173.1 was correlated to the NH of Iva<sup>7</sup> at  $\delta_H$  7.76 and the CO of Iva<sup>7</sup> at  $\delta_C$  176.6  
42  
43 19 to the NH of Val<sup>8</sup> at  $\delta_H$  7.74 (Figure S36). Thus Aib<sup>7</sup> in compound **1** was changed into Iva<sup>7</sup> in **3**.  
44  
45 20 Compound **3** was identified as Ac-Aib<sup>1</sup>-Gly<sup>2</sup>-Ala<sup>3</sup>-Leu<sup>4</sup>-Aib<sup>5</sup>-Gln<sup>6</sup>-Iva<sup>7</sup>-Val<sup>8</sup>-Aib<sup>9</sup>-Ala<sup>10</sup>-Aib<sup>11</sup>-  
46  
47 21 Aib<sup>12</sup>-Aib<sup>13</sup>-Gln<sup>14</sup>-Pheol<sup>15</sup> and consequently named pentadecaibin III.

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53 22 Compound **4** was obtained as a white powder. On the basis of HRESIMS studies, the molecular  
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55 23 formula  $C_{70}H_{117}N_{17}O_{18}$  was deduced from the  $Na^+$ -adduct ion  $[M + 2Na]^{2+}$  at  $m/z$  764.9263. Mass  
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1 fragmentation analyses (Figures S8-S9) demonstrated that peptide **4** only differs from **3** by the  
2 replacement of the Val residue by a Ile/Leu in position 8 (Table S2). Detailed NMR analyses were  
3 consistent with mass fragmentation sequencing and revealed that compound **4** only differed from  
4 **3** by the presence of two Leu residues instead of one, the presence of an Iva and the absence of Val  
5 (Table S5). The CO of Gln<sup>6</sup> at  $\delta_C$  173.2 was correlated to the NH of the Iva<sup>7</sup> at  $\delta_H$  7.78, the CO of  
6 which at  $\delta_C$  176.8 was correlated to the NH at  $\delta_H$  7.85 of the second Leu, which was thus located  
7 at position 8 (Figure S45). Therefore, compound **4** was determined as Ac-Aib<sup>1</sup>-Gly<sup>2</sup>-Ala<sup>3</sup>-Leu<sup>4</sup>-  
8 Aib<sup>5</sup>-Gln<sup>6</sup>-Iva<sup>7</sup>-Leu<sup>8</sup>-Aib<sup>9</sup>-Ala<sup>10</sup>-Aib<sup>11</sup>-Aib<sup>12</sup>-Aib<sup>13</sup>-Gln<sup>14</sup>-Pheol<sup>15</sup> and named pentadecaibin IV.

9 Compound **5** was obtained as a white powder. HRESIMS analyses of the  $[M + 2Na]^{2+}$  at  
10  $m/z$  764.9180 confirmed that compound **5** exhibits the same molecular formula (C<sub>70</sub>H<sub>117</sub>N<sub>17</sub>O<sub>18</sub>) as  
11 compound **4**. Based on mass fragmentation sequencing, three Val or Iva residues were assigned in  
12 position 5, 7 and 8 (Figures S10-S11, Table S2). NMR investigations confirmed the mass  
13 fragmentation and revealed that **5** was characterised by the presence of two Iva and one Val (Table  
14 S6, Figure S54). The CO of Leu<sup>4</sup> at  $\delta_C$  173.5 was correlated to the NH at  $\delta_H$  8.10 of one of the Iva  
15 (Iva<sup>5</sup>). The CO of this Iva at  $\delta_C$  176.6 gave a cross-peak in the HMBC spectrum with the NH of  
16 Gln<sup>6</sup> at  $\delta_H$  7.80. As the CO of Gln<sup>6</sup> at  $\delta_C$  173.1 was correlated to the NH at  $\delta_H$  7.75 of the second  
17 Iva (Iva<sup>7</sup>) and the CO of which at  $\delta_C$  176.6 was correlated to the NH at  $\delta_H$  7.80 of a Val (Val<sup>8</sup>),  
18 compound **5** only differed from compound **4** by the replacement of an Aib by an Iva in position 5.  
19 Therefore, compound **5** was determined as Ac-Aib<sup>1</sup>-Gly<sup>2</sup>-Ala<sup>3</sup>-Leu<sup>4</sup>-Iva<sup>5</sup>-Gln<sup>6</sup>-Iva<sup>7</sup>-Val<sup>8</sup>-Aib<sup>9</sup>-  
20 Ala<sup>10</sup>-Aib<sup>11</sup>-Aib<sup>12</sup>-Aib<sup>13</sup>-Gln<sup>14</sup>-Pheol<sup>15</sup> and named pentadecaibin V.



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3 1 The solution conformation of pentadecaibins in DMSO- $d_6$  was tentatively examined by NMR,  
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5 2 based on the observed  $^3J_{\text{NH},\alpha\text{H}}$  coupling constants and the inter-residue NOE connectivities. The  
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7 3  $^3J_{\text{NH},\alpha\text{H}}$  coupling constants values mainly lower than 7 Hz agreed with a helical structure. The inter-  
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9 4 residue NOE patterns observed for the five pentadecaibins (**1–5**) on their NOESY spectra were  
10  
11 5 similar, and strong NOE correlations  $d_{\text{NN}(i, i+1)}$  and  $d_{\alpha\text{N}(i, i+1)}$  were observed, in agreement with the  
12  
13 6 proposed sequences (Figure 3, Figures S15, S23, S27, S32, S36, S41, S45, S50 and S54). A helical  
14  
15 7 structure was also obvious from the series of NOESY correlations between contiguous residues  
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17 8 such as strong  $d_{\text{NN}(i, i+1)}$ ,  $d_{\text{NN}(i, i+2)}$  and  $d_{\text{NN}(i, i+3)}$ . The prevalence of a helix stabilised by 4→1 intra-  
18  
19 9 molecular hydrogen bonds (3<sub>10</sub>-helix type) over an  $\alpha$ -helix which is stabilised by 5→1 intra-  
20  
21 10 molecular hydrogen bonds arose from the absence of  $d_{\text{NN}(i, i+4)}$  and the stronger peaks of  $d_{\text{NN}(i, i+2)}$   
22  
23 11 as compared to  $d_{\text{NN}(i, i+3)}$ . As similar conformational results were observed for all the five peptaibols,  
24  
25 12 it was concluded that the substitutions Aib → Iva (positions 5 and 7) and Val → Leu (position 8)  
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27 13 which were responsible for the microheterogeneity of the pentadecaibins were without significant  
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29 14 influence on their secondary structure.  
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36 15 In addition, on the basis of ECD spectra analyses (Figures S19, S28, S37, S46 and S55), the  
37  
38 16 occurrence of two negative maxima at 210 and 225 nm and a positive maximum near 200 nm  
39  
40 17 confirmed the right-handed helical conformation of the pentadecaibins.<sup>41</sup> In this case, for Iva  
41  
42 18 residues of pentadecaibins III (**3**), IV (**4**) and V (**5**), the large  $\Delta\delta_{\text{H}}$  values of  $^1\text{H}$  chemical shift  
43  
44 19 differences between the diastereotopic  $\beta$ -methylene protons ( $> 0.28$  ppm) and the chemical shifts  
45  
46 20 of  $\gamma$ -methyl protons and  $\beta$ -methylene carbons confirmed the presence of D-configured Iva (Table  
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48 21 2).<sup>42,43</sup>  
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**Table 2:  $^1\text{H}$  and  $^{13}\text{C}$  NMR Parameters of Iva Residues in Pentadecaibins III (3), IV (4) and V (5)**

NMR parameter	III (3)	IV (4)	V (5)	
	Iva <sup>7</sup>	Iva <sup>7</sup>	Iva <sup>5</sup>	Iva <sup>7</sup>
$\Delta\delta_{\beta\text{Hb}-\beta\text{Ha}}$	0.66	0.66	0.66	0.52
$\delta_{\text{H}} \gamma\text{-CH}_3$	0.76	0.76	0.76	0.75
$\delta_{\text{C}} \beta\text{-CH}_2$	25.0	24.9	25.0	25.0

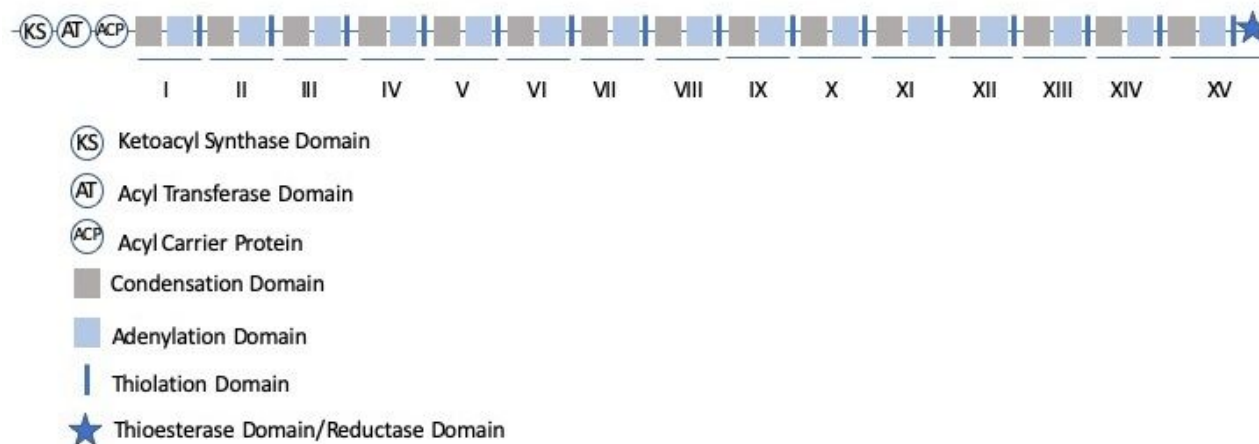
Moreover, the absolute configurations of the constituent chiral AA of pentadecaibins I–V (1–5) were determined after acid hydrolysis and subsequent derivatization of the amino acids with Marfey's reagent (L-FDAA)<sup>44</sup> (Figures S56-S60). Comparison of the L-DAA derivatives of 1–5 with appropriate L- and D- standard AA L-DAA derivatives by reversed-phase LC indicated the presence of L-configured AA for Ala, Val, Leu, Glu (resulting from Gln hydrolysis during Marfey's analysis) and Pheol. Marfey's analyses confirmed the presence of D-configured Iva for compounds 3–5. Consequently, all chiral AA present in pentadecaibins I–V possess the L-configuration except for the Iva residues (D-configuration).

This is the first report of the description of 15-residue peptaibols within the genus *Trichoderma*. These peptaibols are characterized by the lack of the highly conserved Aib-Pro motif which is generally characteristic for peptaibols produced by *Trichoderma* spp. Ampullosporins, chalciporins and tylopeptins, 15-residue peptaibols produced by *Sepedonium* species, are also characterized by the absence of the Aib-Pro motif.<sup>45-49</sup> However, these peptaibols exhibit sequence patterns which remain different from pentadecaibins.

Interestingly, no 18-residue peptaibols could be detected in *Trichoderma* sp. MMS1255 strain extracts, whereas *Trichoderma* species belonging to the *T. harzianum* complex are known to produce these peptaibol series.<sup>5,7</sup> Comparison of new 15- and already known 18-residue patterns produced by various *T. harzianum* strains showed a complete sequence similarity with an absence

1 of a 3 AA sequence (Aib<sup>12</sup>-Pro<sup>13</sup>-Leu<sup>14</sup>) (Figure S61). *Trichoderma* sp. MMS1255 genome  
 2 sequencing was performed in order to identify the putative NRPS gene encoding the  
 3 pentadecaibins.

4 **Genome Sequencing and NRPS Gene Analysis.** The genome was sequenced with the PacBio  
 5 technology. We obtained 228 contigs and the genome assembly size was estimated at 38.7 Mbp  
 6 which is coherent with the size of the other fungi within the *Trichoderma harzianum* complex.<sup>50</sup>  
 7 All sequences were submitted to the AntiSMASH software pipeline.<sup>51</sup> Interestingly, only one  
 8 NRPS gene encoding 15-module protein was identified in the genome, estimated to 53.5 kbp  
 9 (Figure 4).



10  
 11 **Figure 4.** Module organization of the putative NRPS gene encoding the pentadecaibins I–V (1–5). Modules are  
 12 indicated by numbers I–XV.

13 The presence of ketoacyl synthase and acyltransferase domain genes before the first module gene  
 14 was consistent with the typical *N*-terminal acetylation of peptaibols. The latest domain in the gene  
 15 corresponds to a thioesterase domain or reductase domain. This domain catalyzes the reduction of  
 16 the acyl thioester into its primary alcohol *via* the aldehyde intermediate,<sup>52</sup> which is in agreement  
 17 with the typical *C*-terminal amino-alcohol of peptaibols. This NRPS-encoding gene analysis was  
 18 completed by the prediction of incorporated AA based on the 10 AA signature sequences in the

1 adenylation domains of NRPS modules based on both NRPS/PKS Substrate predictor and by  
 2 NRSPredictor2 SVM (Table 7).<sup>53,54</sup>

3 **Table 3. Prediction of Incorporated AA in Pentadecaibins Based on the Signature Sequences**  
 4 **in 15-module NRPS Adenylation Domains**

Amino acid position in pentadecaibins	Signature sequence in NRPS module <sup>a</sup>	Amino acids predicted by <b>NRPS/PKS substrate predictor/NRSPredictor2 SVM</b> ( <i>Small clusters prediction</i> ) <sup>b</sup>	Amino acids detected in pentadecaibins
0	PKS-AT	Methylmalonyl-CoA	Ac
1	DLGYLAGVFK	<b>Iva</b> /Val,Leu,Ile,Abu,Iva	Aib
2	DLGiiCaVmK	Gly,Ala,Val,Leu,Ile,Abu,Iva/ <i>NP</i>	Gly
3	DVGfVAGVLK	<b>Ala</b> /Gly,Ala	Ala
4	DMGFLGGVaK	<b>Val</b> /Val,Leu,Ile,Abu,Iva	Leu
5	DLGivCGVyK	<b>NP</b> /Val,Leu,Ile,Abu,Iva	Aib/Iva
6	DGGMVGGNYK	<b>Gln</b> /Glu,Gln	Gln
7	DAawIVGVvK	<b>Val,Leu,Ile,Abu,Iva</b> / Val,Leu,Ile,Abu,Iva	Aib/Iva
8	DAFILGgViK	<b>Ala</b> /Val,Leu,Ile,Abu,Iva	Val/Leu
9	DLGYLAGCFK	<b>Iva</b> /Val,Leu,Ile,Abu,Iva	Aib
10	DVGyVAAVYK	Ser/ <i>Ser</i>	Ala
11	DLMYfagVAK	Gly,Ala,Val,Leu,Ile,Abu,Iva/ <i>NP</i>	Aib
12	DLGFLAGVFK	<b>Iva</b> /Val,Leu,Ile,Abu,Iva	Aib
13	DLGILAGLFK	<b>Iva</b> /Val,Leu,Ile,Abu,Iva	Aib
14	DGGMVGGNYK	<b>Gln</b> /Glu,Gln	Gln
15	DgFVIAGicK	<b>NP</b> /Pheol,Trpol	Pheol

<sup>a</sup> Shown in the single letter amino acid code. <sup>b</sup> Shown in the three letter amino acid code. NP: No prediction.

5 All signatures in the adenylation domains were consistent with the AA sequences of  
 6 pentadecaibins I-V. Based on these results, it can be assumed that pentadecaibins are synthesized  
 7 by a 15-module NRPS. This is the first detection of a 15-module peptaibol synthetase gene within  
 8 the *Trichoderma* spp. Contrary to other investigated *Trichoderma* strains within the *T. harzianum*  
 9 complex, MMS1255 is the only one producing 15-residue peptaibols associated with a 15-module  
 10 peptaibol synthetase.

**Biological Activities of Pentadecaibins.** Biological activities of pentadecaibins I-V (**1–5**) were assessed against cancer cells (KB), Gram-positive (*Staphylococcus aureus*) and negative (*Escherichia coli*) bacteria and human-pathogenic yeast (*Candida albicans*) (Table 4).

**Table 4. Biological Activities of Pentadecaibins I-V (1–5)**

Compound	Cytotoxicity – IC <sub>50</sub> ( $\mu$ M)	Antimicrobial activity – MIC ( $\mu$ g/mL)		
	KB cells	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
<b>1</b>	2.4 $\pm$ 0.1	25 $\pm$ 0	> 100	> 100
<b>2</b>	4.3 $\pm$ 1.1	> 100	> 100	> 100
<b>3</b>	0.8 $\pm$ 0.5	25 $\pm$ 0	> 100	> 100
<b>4</b>	0.8 $\pm$ 0.2	> 100	> 100	> 100
<b>5</b>	0.7 $\pm$ 0.2	25 $\pm$ 0	> 100	> 100
Alm F50/5 <sup>a</sup>	9 $\pm$ 1	n.t. (12.5) <sup>b</sup>	n.t.	n.t.

n.t. not tested.  
<sup>a</sup> Synthetic alamethicin F50/5<sup>55</sup>; <sup>b</sup> value from <sup>56</sup>

The Cytotoxic activities of the pentadecaibins were consistent with previous reports of cell growth inhibitory activity of 11- and 20-residue peptaibols from a marine-derived *T. longibrachiatum* strain.<sup>30,32</sup> Pentadecaibins III-V (**3 – 5**) incorporating a D-Iva<sup>7</sup> residue instead of Aib<sup>7</sup> exhibited higher growth inhibitory activity against KB cells than pentadecaibins I–II (**1–2**). Interestingly, similar observations were obtained with trichodermites A–E assessed on SW620 human colorectal carcinoma cells.<sup>57</sup> Pentadecaibins did not exhibit noticeable growth inhibition against Gram-negative *E. coli* or yeast *C. albicans*. However, moderate antibacterial activity against *S. aureus* was observed for pentadecaibins I (**1**), III (**3**) and V (**5**) with MIC values of 25  $\mu$ g/mL, being in agreement with previous observations in the literature for antimicrobial activity.<sup>30,56</sup>

In conclusion, in this study we have identified a new series of 15-residue peptaibols named pentadecaibins I–IV (**1–5**). They are produced by a marine-derived strain belonging to the *Harzianum* Clade. The phylogenetic position of *Trichoderma* sp. MMS1255 within the

1 *Trichoderma* genus was studied using sequence alignment of combined genes of representatives  
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6 2 *Trichoderma* strains. No precise identification can be proposed, but the phylogenetic analysis  
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8 3 clearly demonstrates the relation of *Trichoderma* sp. MMS1255 to the *T. lentiforme* lineage. The  
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10 4 pentadecaibins discovered differ between them by AA exchange in positions 5 (Aib/Iva), 7  
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12 5 (Aib/Iva) and 8 (Val/Leu). They are all characterized by the lack of the Aib-Pro motif widespread  
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14 6 in peptaibols produced by *Trichoderma* spp. Genome sequencing allowed the identification for the  
15  
16 7 first time within *Trichoderma* spp. of a 15-module peptaibol synthetase encoding gene closely  
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18 8 related to pentadecaibin biosynthesis, according to the prediction of the incorporated AA. The  
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20 9 pentadecaibins exhibit moderate cytotoxicity on KB cells and antibacterial activity against  
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22 10 *S. aureus*.  
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## 1 EXPERIMENTAL SECTION

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6 **General Experimental Procedures.** The specific rotation was measured with a Perkin Elmer  
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8 model 341 polarimeter at 589 nm. UV spectra were obtained from a Shimadzu 1605 UV/visible  
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10 spectrophotometer, at room temperature over a wavelength range of 200–400 (peptaibols 0.1 mM,  
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12 MeOH). ECD experiments were carried out using a Jasco J-810 CD spectropolarimeter. Spectra  
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14 were recorded at room temperature over a wavelength range of 195–260 nm using a 0.2 cm path  
15  
16 length cuvette (peptaibols 0.1 mM, MeOH). Measurements were performed using a step scan  
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18 scanning mode with data acquisition interval of 0.1 nm, bandwidth of 2 nm and accumulation of 5.  
19  
20 ECD sample spectra are shown after subtracting the baseline, smoothing and data normalization.  
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22 IR spectra were recorded on a Shimadzu IR Affinity FTIR spectrometer fitted with an ATR  
23  
24 MiRacle 10. One- and two-dimensional NMR spectra were performed on a Bruker Avance III HD  
25  
26 600 MHz spectrometer operating at 600.193 MHz and using a triple resonance TCI cryoprobe,  
27  
28 equipped with shielded gradients z. NMR spectra were recorded in DMSO-*d*<sub>6</sub> solution and were  
29  
30 processed using the Bruker TOPSPIN 3.2 software. Chemical shifts are expressed in  $\delta$  (ppm) and  
31  
32 are referenced to the residual non-deuterated solvent signals (for DMSO-*d*<sub>6</sub>  $\delta_{\text{H}}$  2.49 and  $\delta_{\text{C}}$  39.5).  
33  
34 For the HMBC experiments the delay ( $1/2J$ ) was 70 ms and for the NOESY experiments the mixing  
35  
36 time was 500 ms. Mass analyses were carried out using a LCQ™ electrospray ionization ion-trap  
37  
38 mass spectrometer instrument (ESI-IT/MS, Thermo Fischer Scientific) in positive and negative  
39  
40 mode with capillary temperature of 160°C, capillary voltage of 3.44 V (positive mode) or – 9.81 V  
41  
42 (negative mode), spray voltage of 4.51 kV, and sheath gas (N<sub>2</sub>) flow rate of 19.50 AU. Compounds  
43  
44 were infused as methanolic solutions (0.5  $\mu\text{g/mL}$ ) directly into the ESI probe with a 500- $\mu\text{L}$   
45  
46 micrometrically automated syringe (Hamilton) at a flow rate of 3  $\mu\text{L}/\text{min}$ . All spectra were acquired  
47  
48 and analyzed by LCQ Xcalibur software (Thermo Fischer Scientific). Total current ion mass  
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1 spectra (Fullscan mode) were acquired in the range  $m/z$  150 to 2000. Charge state and isotopic  
2 distribution were analyzed by a narrow-scan range mode (Zoomscan mode). MS fragmentation  
3 was performed by positive and negative ionizations under the same experimental conditions  
4 described above for regular MS analysis *via* infusion of methanolic solutions (0.5  $\mu\text{g/mL}$ ). The  
5 sodium adduct ions  $[\text{M} + \text{Na}]^+$  and the deprotonated molecules  $[\text{M} - \text{H}]^-$  were selected as precursor  
6 ions for  $\text{MS}^2$ . Peptaibols were sequenced by assignation of the diagnostic fragment ions of the  
7 series  $a_n / b_n$  and  $y_{nP}$  produced in positive-ion mode and  $y_{nN}$  produced in negative-ion mode.  
8 HRESIMS analyses were conducted using an electrospray ionization ion-trap time-of-flight  
9 multistage mass spectrometer (IT-TOFMS, Shimadzu). Organic solvents used for extraction and  
10 purification of compounds were purchased from Carlo Erba SDS and distilled prior to use. Water  
11 was purified to HPLC-grade quality with a Millipore-QRG ultrapure water system (Millipore). MS  
12 analyses were performed using LCMS-grade MeOH (Biosolve). Semi-preparative HPLC  
13 purifications and AA Marfey's analysis were carried out on Agilent 1200 HPLC instrument  
14 connected to corresponding quaternary pump, fraction collector, DAD detector and Chemstation  
15 software. All *D*- and *L*- amino acids, Gly and Aib were purchased from Sigma-Aldrich, except *D*-  
16 and *L*-Iva, purchased from Thermo Fisher Scientific. Acetonitrile ( $\text{CH}_3\text{CN}$ ) and trifluoroacetic  
17 acid (TFA) used for AA Marfey's analysis were purchased from Carlo Erba and Sigma-Adrich,  
18 respectively.

19 **Isolation, Fungal Strain Identification and Phylogenetic Analysis.** The fungal strain was  
20 isolated from marine sediment collected in a shellfish-farming area from the estuary of the river  
21 Loire at Port-du-Bec, France. The isolated strain is deposited in the laboratory fungal collection  
22 (MMS-Marine Fungal Collection, University of Nantes) under the reference number MMS1255 as  
23 well as in the TU Wien Collection of Industrially Important Microorganisms under the reference



1 TUCIM 5509. Strain MMS1255 was identified as belonging to the genus *Trichoderma* on the basis  
2 of macroscopic and microscopic morphological features (Figure S1). The identification was  
3 completed at the molecular level by amplification and sequencing of internal transcribed spacers  
4 (ITS) and DNA partial sequences of the fourth large intron of translation elongation factor 1- $\alpha$   
5 (*tefl*); RNA polymerase II subunit 2 (*rpb2*) and calmodulin (*cal*). Sequences were deposited in  
6 GenBank under accession numbers JQ653081.1, KU758964.1, MN450663.1 and MN428075.1,  
7 respectively. Briefly, mycelium for DNA extraction was grown on potato dextrose agar (PDA) and  
8 harvested after seven days. Genomic DNA was extracted according to the microwave mini-prep  
9 procedure described by Goodwin and Lee (1993)<sup>58</sup> using 100  $\mu$ L lysis buffer (50 mM Tris-HCl pH  
10 7.5, 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol). The final DNA pellet was supplemented  
11 into 100  $\mu$ L TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and stored at  $-20^{\circ}\text{C}$  until used.  
12 Gene sequences were obtained by polymerase chain reaction (PCR) amplification (GoTaq® G2  
13 Hot Start Polymerase, Promega Corporation) and sequenced using Eurofins Genomics facility.  
14 DNA partial sequences of *cal*, ITS, *rpb2* and *tefl* were amplified using the following primers:  
15 CAL-228F/CAL-737R,<sup>59</sup> ITS1/ITS4,<sup>60</sup> fRPB2-5f/fRPB2-7cr<sup>61</sup> and EF1-728f<sup>59</sup>/TEF1LLERev,<sup>62</sup>  
16 respectively. ITS sequence (JQ653081) allowed to assign MMS1255 strain to *Trichoderma* genus.  
17 A first phylogenetic analysis aiming to affiliate MMS1255 strain to the *Trichoderma* subclades  
18 defined by Jaklitsch and Voglmayr<sup>35</sup> was performed using 112 *Trichoderma* spp. combined  
19 sequences of *tefl* and *rpb2* (Table S1). For the *Trichoderma* subclade affiliation tree,  
20 *Protocrea farinosa* sequences were used as outgroup taxa. A second analysis was performed to  
21 determine MMS1255 strain lineages inside the *Trichoderma Harzianum* clade or to the  
22 *T. harzianum* complex defined by Jaklitsch and Voglmayr<sup>35</sup> and Chaverri *et al.*,<sup>34</sup> respectively.  
23 According to Chaverri *et al.*,<sup>34</sup> *T. catoptron* is one of the more distant species from the

1 *T. harzianum* complex, thus the *T. catoptron* strain GJS 02-76 sequences were used to root  
2 phylogenetic trees. For the *Trichoderma* sp. MMS1255 lineage affiliation to the *T. harzianum*  
3 complex, the combined sequences *tefl* and *cal* gene of 76 representative strains were used (Table  
4 S1). As described by Jaklitsch and Voglmayr,<sup>35</sup> all alignments were produced with the server  
5 version of MAFFT ([www.ebi.ac.uk/Tools/mafft](http://www.ebi.ac.uk/Tools/mafft)), with a gap open penalty of 1.0 and a gap  
6 extension penalty in the range of 0.05 to 0.1, with a tree building number = 100 and maxiterate =  
7 100. The resulting alignments were checked and refined using BioEdit version v. 7.0.5.3.<sup>63</sup>  
8 Maximum likelihood (ML) analysis was performed with MEGA 7.0.26. The general time-  
9 reversible model (GTR)<sup>64</sup> with gamma-distributed substitution rates, additionally assuming a  
10 proportion of invariant sites (GTR+I+G) was selected according to the Akaike information  
11 criterion (AIC). Initial tree(s) for the heuristic search were obtained automatically by applying  
12 Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the  
13 Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior  
14 log likelihood value. Maximum likelihood bootstrap proportions (MLBP) were computed with  
15 1000 replicates. Maximum parsimony (MP) trees were obtained by MEGA 7.0.26 using 1000  
16 replicates of heuristic search with random addition of sequences and tree bisection–reconnection  
17 (TBR) as the branch-swapping algorithm. All characters were weighted equally. Maximum  
18 parsimony bootstrap proportions (MPBP) were calculated from 1000 replicates, each with 10  
19 replicates of random addition of taxa. Trees were visualized with MEGA 7.0.26 with MLBP and  
20 MPBP above 50% as shown at the nodes.

21 **Analytical Cultivation and Peptaibol Profiling by ESI-IT-MS.** For peptaibol chemical  
22 profiling, *Trichoderma* sp. MMS1255 was inoculated onto a Petri dish (10-cm diameter) containing  
23 20 mL of marine DCA medium (dextrose 40 g/L, enzymatic digest of casein 10 g/L, agar 15 g/L,

1 Difco, VWR, synthetic sea salt 36 g/L, Reef Crystals, Aquarium Systems). Culture was incubated  
2 for 7 days at 27 °C. After incubation, culture was harvested and mycelia and conidia were scraped  
3 from the agar surface. The harvested biomass was extracted by CH<sub>2</sub>Cl<sub>2</sub>/MeOH 1:2 then 2:1 (v/v,  
4 30 mL each) for 30 min at room temperature. The combined extracts were filtered, washed with  
5 distilled H<sub>2</sub>O (10 mL) and evaporated to dryness to provide an organic extract. The extract was  
6 fractionated by vacuum liquid chromatography (VLC) on Chromabond adsorbant (OH) 2 Diol (10  
7 × 50 mm, 60 Å, 45 µm, Macherey-Nagel) with CH<sub>2</sub>Cl<sub>2</sub>/EtOH mixtures (98:2 to 85:15, v/v). The  
8 peptaibol-enriched fraction eluting with CH<sub>2</sub>Cl<sub>2</sub>/EtOH 90:10 (v/v) was subjected to ESI-MS  
9 analysis in infusion mode.

10 **Preparative Cultivation, Extraction and Purification of Compounds 1-5.** Strain MMS1255  
11 was inoculated onto 35 Petri dishes (20-cm diameter) containing 125 mL of marine DCA medium.  
12 Cultures were incubated in natural light for 11 days at 27 °C prior to harvesting for biomass  
13 extraction, as previously mentioned (see above). After incubation, the harvested biomass was  
14 extracted by CH<sub>2</sub>Cl<sub>2</sub>/MeOH 1:2 then 2:1 (v/v, 1200 mL each) for 2 h at room temperature. The  
15 combined extracts were filtered, washed with distilled H<sub>2</sub>O (600 mL) and evaporated to dryness to  
16 provide an extract (2.8 g). The extract was partitioned in aliquots of 276 mg and fractionated by  
17 repetitive VLC, with CH<sub>2</sub>Cl<sub>2</sub>/EtOH mixtures (98:2, 95:5, 92:8, 90:10 and 85:15, v/v). Peptide-  
18 containing fractions (CH<sub>2</sub>Cl<sub>2</sub>/EtOH 95:5 to 85:15, v/v) were combined to obtain the fraction 2 (940  
19 mg) and subjected to a liquid chromatography on an open silica gel column (Chromagel, 200 × 20  
20 mm, 60 Å, 35-70 µm, SDS), with CH<sub>2</sub>Cl<sub>2</sub>/MeOH mixtures (100:0 to 80:20, v/v). Fifteen-residue  
21 peptaibols were mainly eluted in the 2-9 and 2-10 CH<sub>2</sub>Cl<sub>2</sub>/MeOH mixtures (85:15, v/v).  
22 Chromatographic separation of the fraction 2-10 (433 mg) by repetitive reversed-phase preparative  
23 HPLC (Luna RP18 column, 250 × 10 mm, 100 Å, 5 µm, Phenomenex) yielded five compounds

(1–5) eluting respectively in sub-fractions 2-10-4 to 2-10-8. Mobile phase (MeOH/H<sub>2</sub>O 85:15, v/v) was delivered at a constant flow rate of 5 mL/min. Volumes of 900 μL of 10 mg/mL peptaibol solution resuspended in mobile phase were injected. Detection was performed at 230 nm. All data were acquired by HP ChemStation for LC.

*Pentadecaibin I (1)*: white powder,  $[\alpha]^{20}_D - 21$  (*c* 0.2, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 204 nm (3.46) nm; IR  $\nu_{\max}$  3304, 2929, 1645, 1531, 1454, 1384, 1363, 1294, 1220, 1176, 1043 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR Data, Table 1; ESIMS *m/z* 750.9 [M + 2Na]<sup>2+</sup>, *m/z* 1478.8 [M + Na]<sup>+</sup>. HRESIMS *m/z* 750.9132 [M + 2Na]<sup>2+</sup> (calcd for ½ C<sub>68</sub>H<sub>113</sub>N<sub>17</sub>O<sub>18</sub>Na<sub>2</sub>, 750.9117, Δ 2.0 ppm).

*Pentadecaibin II (2)*: white powder,  $[\alpha]^{20}_D - 17.3$  (*c* 0.34, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 204 nm (3.45) nm; IR  $\nu_{\max}$  3304, 2930, 1649, 1533, 1452, 1384, 1360, 1298, 1225, 1188, 1043 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C-NMR Data, Table S3; ESIMS *m/z* 757.9 [M + 2Na]<sup>2+</sup>, *m/z* 1492.8 [M + Na]<sup>+</sup>. HRESIMS *m/z* 757.9172 [M + 2Na]<sup>2+</sup> (calcd for ½ C<sub>69</sub>H<sub>115</sub>N<sub>17</sub>O<sub>18</sub>Na<sub>2</sub>, 757.9195, Δ 3.03 ppm).

*Pentadecaibin III (3)*: white powder,  $[\alpha]^{20}_D - 15.1$  (*c* 1.0, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 204 nm (3.42) nm; IR  $\nu_{\max}$  3304, 2935, 1649, 1531, 1456, 1384, 1363, 1290, 1219, 1174, 1043 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR Data, Table S4; ESIMS *m/z* 757.9 [M + 2Na]<sup>2+</sup>, *m/z* 1492.8 [M + Na]<sup>+</sup>. HRESIMS *m/z* 757.9174 [M + 2Na]<sup>2+</sup> (calcd for ½ C<sub>69</sub>H<sub>115</sub>N<sub>17</sub>O<sub>18</sub>Na<sub>2</sub>, 757.9195, Δ 2.77 ppm).

*Pentadecaibin IV (4)*: white powder,  $[\alpha]^{20}_D - 17.9$  (*c* 1.0, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 204 nm (3.42) nm; IR  $\nu_{\max}$  3304, 2937, 1649, 1531, 1456, 1384, 1361, 1290, 1220, 1176, 1045 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR Data, Table S5; ESIMS *m/z* 764.9 [M + 2Na]<sup>2+</sup>, *m/z* 1506.8 [M + Na]<sup>+</sup>. HRESIMS *m/z* 764.9263 [M + 2Na]<sup>2+</sup> (calcd for ½ C<sub>70</sub>H<sub>117</sub>N<sub>17</sub>O<sub>18</sub>Na<sub>2</sub>, 764.9273, Δ 1.31 ppm).

*Pentadecaibin V (5)*: white powder,  $[\alpha]^{20}_D - 10.2$  (*c* 0.28, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 204 nm (3.46) nm; IR  $\nu_{\max}$  3305, 2933, 1647, 1531, 1455, 1384, 1360, 1288, 1219, 1172, 1043 cm<sup>-1</sup>;

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3 1  $^1\text{H}$  and  $^{13}\text{C}$  NMR Data, Table S6; ESIMS  $m/z$  764.9  $[\text{M} + 2\text{Na}]^{2+}$ ,  $m/z$  1506.8  $[\text{M} + \text{Na}]^+$ . HRESIMS  
4  
5 2  $m/z$  764.9249  $[\text{M} + 2\text{Na}]^{2+}$  (calcd for  $\frac{1}{2} \text{C}_{70}\text{H}_{117}\text{N}_{17}\text{O}_{18}\text{Na}_2$ , 764.9273,  $\Delta$  3.14 ppm).  
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9 3 **Marfey's Derivatization.** Compounds **1–5** (0.5 mg) and 500  $\mu\text{L}$  of 6 M HCl were heated for  
10  
11 4 24 h at 110  $^\circ\text{C}$  in 4 mL sealed tubes (Supelco Analytical; Sigma-Aldrich). Hydrolyzed solutions of  
12  
13 5 peptaibols were evaporated to dryness under a stream of nitrogen and solubilized with 100  $\mu\text{L}$  of  
14  
15 6  $\text{H}_2\text{O}$  prior their derivatization with the FDAA Marfey's reagent (Thermo Fisher Scientific). Ten  
16  
17 7 microliters of each aqueous solution of hydrolyzed peptaibol were transferred in insert for 1 mL  
18  
19 8 HPLC vial and completed by 4  $\mu\text{L}$  of 1 M sodium bicarbonate and 20  $\mu\text{L}$  of Marfey's reagent (1%  
20  
21 9 in acetone). The resulting mixture was sealed and then heated at 40  $^\circ\text{C}$  during 2 h. After cooling,  
22  
23 10 samples were neutralized with 4  $\mu\text{L}$  of 1 M HCl and diluted 1:3 with MeOH. The same treatment  
24  
25 11 was performed for the Marfey's derivatization of standard AA (Gly, Aib, *D*- and *L*- standards of  
26  
27 12 Glu, Ala, Val, Iva, Leu and Pheol). Aliquots (10  $\mu\text{L}$ ) of hydrolyzed peptaibols and standard AA  
28  
29 13 were analyzed by HPLC-UV on a reversed-phase analytical column (Inertsil ODS-3 RP18, 250  $\times$   
30  
31 14 4.6 mm, 5  $\mu\text{m}$ , Interchim). The elution was insured at a constant flow rate of 0.5 mL/min using  
32  
33 15  $\text{CH}_3\text{CN}$  / 0.05% TFA in  $\text{H}_2\text{O}$  as mobile phase. The gradient started at 20%  $\text{CH}_3\text{CN}$  ramping up to  
34  
35 16 50% over 20 min and was then held for 15 min. Detection was performed at 430 nm. All data were  
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37 17 acquired by HP ChemStation for LC.  
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44 18 **Genome Sequencing and 15-Module NRPS-encoding Gene Analysis.** *Trichoderma* sp.  
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46 19 MMS1255 was cultivated in the dark at 25 $^\circ\text{C}$  on PDA medium (potato 4 g/L, dextrose 20 g/L, agar  
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48 20 15 g/L, Difco Laboratories). Spore suspensions were produced and concentrations were adjusted  
49  
50 21 to  $10^7$  to  $10^8$  spores/mL prior to storage at -80  $^\circ\text{C}$  until use. Genomic DNA was extracted from fresh  
51  
52 22 mycelium, following the CTAB method proposed by the Joint Genome Institute with an optional  
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54 23 step using Qiagen genome-tips.<sup>65</sup> Genome was sequenced with PacBio technology. Sequences  
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1 were quality checked with FastQC.<sup>66</sup> The genome was assembled using canu.<sup>67</sup> Secondary  
2 metabolism associated genes (polyketide synthase -PKS-, non ribosomal peptide synthetase -  
3 NRPS-, terpene synthase -TPS-, dimethylallyl tryptophan synthase -DMATS-) and genes  
4 potentially involved in adaptation to the environment were searched in each species. Gene cluster  
5 associated with secondary metabolites were searched with FungiSMASH.<sup>51</sup>

6 **Cytotoxicity Assays.** KB cells (human oral epidermoid carcinoma ATCC CCL 17, American  
7 Type Culture Collection,) were cultivated in RPMI (Roswell Park Memorial Institute medium)  
8 supplemented with 5% (v/v) fetal calf serum, 1% (v/v) glutamine 200 mM and 1% (v/v)  
9 streptomycin (10 mg/mL) / penicillin (1000 U) (all Sigma-Aldrich,). Cells were cultivated in plastic  
10 flasks (Falcon; Becton Dickinson Labware,) at 37 °C in a 5% CO<sub>2</sub> enriched atmosphere. After an  
11 incubation period of 48 h, trypsinized cells were suspended as a 2 × 10<sup>5</sup> cells/mL suspension and  
12 50 µL were put in each well of 96-well microplates (Nunclon Delta Surface; Thermo Fisher  
13 Scientific). After an incubation of 48h, 50 µL of peptaibol samples were added to the initial 50-µL  
14 cell suspension. Peptaibol samples were tested in well as a final 5% (v/v) methanolic solution in  
15 supplemented RPMI with concentrations ranging from 0.6 to 25 µg/mL. A final 5% (v/v)  
16 methanolic solution in supplemented RPMI was used as solvent control. Synthetic alamethicin<sup>55</sup>  
17 was used as positive control. After 76 h of incubation, the cell viability was evaluated by the  
18 colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-  
19 Aldrich) bioassay.<sup>68,69</sup>

20 **Antimicrobial Assays.** The *C. albicans* clinical isolate was obtained from the Angers University  
21 Hospital (Angers, France), *E. coli* (CIP54.8T) and *S. aureus* (CIP53.156) strains are part of the  
22 Pasteur Institute Collection. Precultures were inoculated with loop-fulls of cells from agar plates

1 and incubated 20 h at the appropriate temperature 30 °C (for *C. albicans*) or 37 °C (for bacterial  
2 strains) on a rotator in 5 mL YDP medium (yeast dextrose peptone) (for *C. albicans*) or LB medium  
3 (Lysogeny Broth) (for bacterial strains). Overnight precultures were then harvested by  
4 centrifugation, washed in sterile H<sub>2</sub>O and inoculated to an optical density (OD) 0.5 in fresh YPD  
5 or LB media. The microplate wells were filled with calibrated suspensions and peptaibols to be  
6 tested at the desired concentration (ranging from 1 to 100 μg/mL in 1% MeOH, 300 μL/well) and  
7 microbial growth was automatically recorded at 600 nm using a multiplate spectrophotometer  
8 (Spectrostar nano; BMG Labtech). The plates were subjected to permanent shaking at 200 rpm and  
9 OD measurements were taken every 10 min during a 24 h period. For each condition, the area  
10 under the growth curve representative of the lag phase and the maximal growth rate was calculated  
11 as previously described.<sup>70</sup> A percentage of growth inhibition was calculated for each independent  
12 experiment ( $100 - ((AUC_{treated}/AUC_c) \times 100$  where  $AUC_{treated}$  is the Area Under the growth  
13 Curve after exposure to the selected peptaibol or antibiotic, and  $AUC_c$  is the area under the growth  
14 curve of a untreated culture). All data presented herein was obtained from two independent  
15 biological repetitions and each repetition included three technical replicates. Amphotericin B  
16 (100% inhibition of *C. albicans* at 30 μg/mL in 1% MeOH) and gentamycin (100% inhibition of  
17 *E. coli* and *S. aureus* at 40 μg/mL in 1% MeOH) were used as positive controls.

## 18 ASSOCIATED CONTENT

19 **Supporting Information.** The Supporting Information is available free of charge on the ACS  
20 Publications website at DOI: Morphological, genomic and phylogenetic analyses of *Trichoderma*  
21 sp. MMS1255, MS and MS<sup>2</sup> spectra, 1D and 2D NMR spectra, ECD data and HPLC-UV data for  
22 Marfey's analysis of compounds **1–5**

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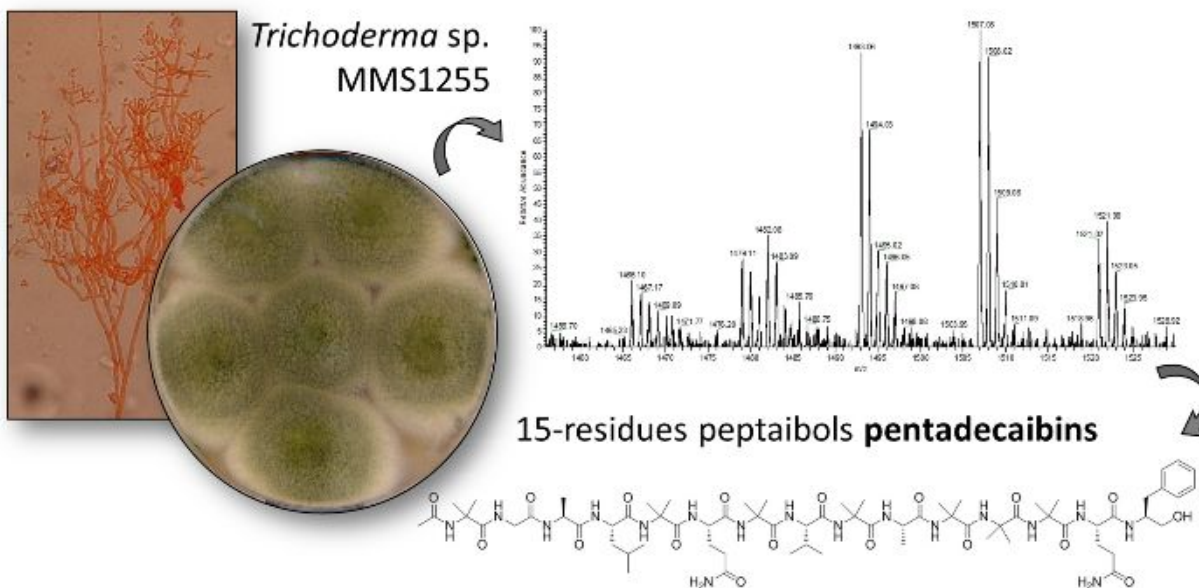
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