Tyroscherin and tyroscherin analogs from Pseudallescheria boydii SNB-CN85 isolated from termite Termes cf. hispaniolae

Jonathan Sorres, Charlotte Nirma, Morgane Barthélemy, Véronique Eparvier, Didier Stien

Tyroscherin and tyroscherin analogs from *Pseudallescheria boydii* SNB-CN85 isolated from termite *Termes* cf. *hispaniolae*

Jonathan Sorres [a], Charlotte Nirma [a], Morgane Barthélemy [a],Véronique Eparvier [a,*], Didier Stien [b,*]

[a] *CNRS, Institut de Chimie des Substances Naturelles, UPR 2301, 1 avenue de la Terrasse, 91198 Gif-sur-Yvette, France*

[b] *Sorbonne Universités, UPMC Univ. Paris 06, CNRS, Laboratoire de Biodiversité et Biotechnologies Microbiennes (LBBM), Observatoire Océanologique, 66650 Banyuls/Mer, France*

Corresponding Authors

[*] Tel: +33 1 69 82 36 79; Fax: +33 1 69 82 37 84; E-mail: veronique.eparvier@cnrs.fr (V. Eparvier). Tel: +33 4 30 19 24 76; E-mail: didier.stien@cnrs.fr (D. Stien).

ABSTRACT

Two new tyroscherin derivatives: *N*-methyl-tyroscherone (**2**) and tyroscherin *N*,*O*-acetonide (**3**), as well as two known secondary metabolites tyroscherin (**1**) and pseurotin A (**4**), were isolated from a solid culture of the termite-borne fungus *Pseudallescheria boydii* SNB-CN85. The structures were elucidated by spectroscopic analysis and chemical modification. Compound **3** was synthesized from tyroscherin (**1**) in the free amine form by heating in deuterated acetone, followed by deuterium-proton exchange in MeOH. All compounds were tested for their antimicrobial activities on *C. albicans* and *S. aureus*, and **2** and **3** exhibited minimal inhibitory concentrations between 128 and 8 $\mu g.mL^{-1}$.

Keywords:

Pseudallescheria boydii; termite-borne fungus; tyroscherin; antimicrobial.

The exploration of secondary metabolites involved in microbial-host interactions has emerged as a successful strategy to identify novel chemical entities and to shed light on the ecology and evolution of defensive association (Beemelmanns et al., 2016; Cantley and Clardy, 2015). These associations are well described in social insects which include ants, bees, termites and wasps, although termites are somewhat less studied than others (Carr et al., 2012; Matsui et al., 2012; Nirma et al., 2015a, 2015b, 2013; Nowak et al., 2010; Sorres et al., 2017; Yan et al., 2011). Indeed, termites microorganisms interactions have been explored mainly to study trophobioses (Carr et al., 2012; Nowak et al., 2010; Yan et al., 2011). Our previous work on *Pseudallescheria boydii* SNB-CN73 strain had demonstrated that this fungus produced two antimicrobial compounds tyroscherin and *N*-methyl-tyroscherin along with ovalicin and several analogs of these metabolites (Nirma et al., 2013). Interestingly, the random isolation work of entomogenous microbes that was conducted in 2011 yielded six *P. boydii* strains from three different termite nests located in somewhat distant places in French Guiana. After our work on SNB-CN73, it was relevant to investigate other strains see whether or not these strains also produce tyroscherin and analogs.

SNB-CN85 is one of these *P. boydii* strains. It was isolated from host *Termes* cf. *hispaniolae*, whereas SNB-CN73 was isolated from *Nasutitermes corniger*. In addition to being from different termite species, both nests were located 3 km apart from each other as the crow flies. Technically, SNB-CN85 was isolated from a surface-sterilized worker placed in a Petri dish containing a solid potato dextrose agar medium. It was identified by amplification and sequencing of the nuclear ribosomal internal transcribed spacer region ITS4 and comparison with NCBI database of sequences. The EtOAc extract of a solid culture of *P. boydii* SNB-CN85 exhibited significant activity against *C. albicans* (MIC = 8 $\mu g.mL^{-1}$). Chemical

investigation of this extract led to the isolation of tyroscherin (**1**) (Hayakawa Y, Yamashita T, Mori T, Nagai K, Shin-Ya K, 2004; Katsuta et al., 2008; Nirma et al., 2013) and pseurotin A (**4**) (Bloch and Tamm, 1981; Hayashi et al., 2003) along with two new tyroscherin analogs *N*-methyl-tyroscherone (**2**) and tyroscherin *N*,*O*-acetonide (**3**).

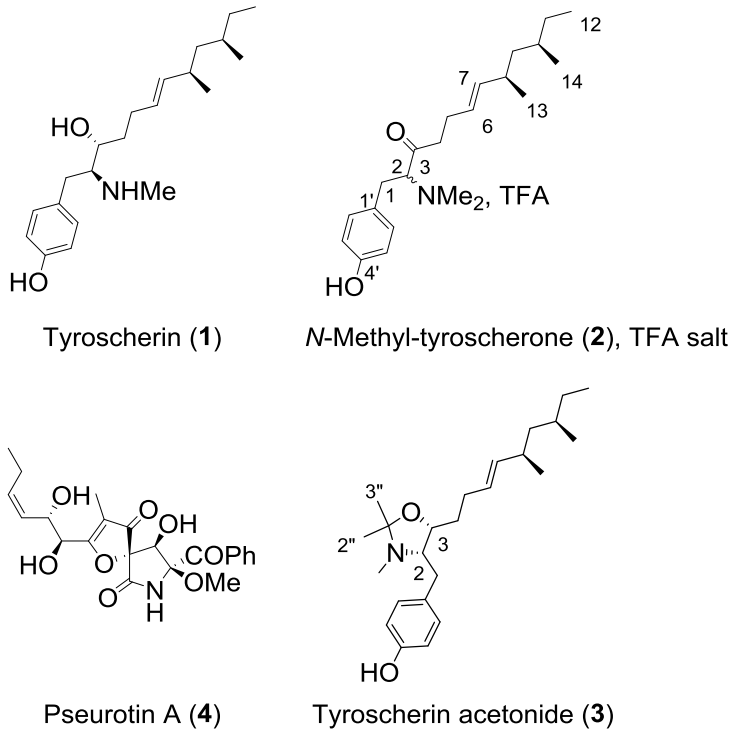


Fig. 1. Structures of compounds **1**-**4** with configurations presented as absolute

Compound **2** TFA salt was obtained as white amorphous powder. HRESIMS analysis indicated a molecular formula of $C_{22}H_{35}NO_2$ (*m/z* for 346.2749 for $[M+H]^+$), implying 6 degrees of unsaturation, i.e., one more than for *N*-methyl-tyroscherin with the same number of carbons. Preliminary inspection of the ^1H and ^{13}C NMR spectra, along with the analysis of the HSQC correlations confirmed that compound **2** was a tyroscherin analog (Table 1). The main difference between these compounds was for ^1H and ^{13}C chemical shifts in the C-2–C-4 spin system. In particular, the tyroscherin α-hydroxyl proton H-3 at δ_H 3.85 ppm was absent

in **2**. Also, ^{13}C NMR spectrum of **2** displayed a carbonyl signal at δ_C 207.7 ppm, and we also observed the lack of the carbinol at δ_C 68.7 ppm in tyroscherin. In HMBC, methylene protons in position 1 at δ_H 3.37 and 2.96 ppm, methylene protons in position 4 at δ_H 2.37 and 2.00 ppm, and the methine proton at δ_H 4.45 ppm all correlated with this carbonyl. Consequently, it was clear that compound **2** had a carbonyl in position 3. The complete proton assignment of compound **2** was deduced from the careful examination of COSY, HSQC and HMBC correlations. However, although the overall integration of protons was correct, it was noticed that many signals in the ^1H and ^{13}C NMR experiments were split. Attempts to separate two different compounds by HPLC failed. Since all NMR data were almost identical, we assumed that the position 2 in compound **2** may in fact epimerize due to the presence of the carbonyl group at C-3, yielding a mixture of epimers. Treatment of **2** with NaOD in CD_3OD at room temperature provided *N*-methyl-tyroscherone-2-*d* (**2-*d***) with 69% conversion (as measured by ^1H NMR by integration of the residual H-2 proton signal), confirming that carbon C-2 is most prone to epimerization. We concluded then that compound **2** was isolated in the form of a mixture of 2*R* and 2*S* epimers. Biosynthetic considerations would provide the configurations of other asymmetric centres as being identical to those observed for tyroscherin (8*R* and 10*R*). Compound **2** was named *N*-methyl-tyroscherone from a putative oxo analog of tyroscherin.

The molecular formula of **3** was determined to be $C_{24}H_{39}NO_2$ by HRESIMS (*m/z* 374.3046 for $[M+H]^+$), indicating the presence of three additional carbons compared to the molecular formula of **1**. Compound **3** was originally isolated as TFA salt, and was converted to the free base for 1D and 2D NMR analyses. The ^1H NMR spectra of compounds **1** and **3** presented some similarities, indicating that **3** was also a tyroscherin analog (Table 1). For example,

compound **3** had *para*-substituted aromatic systems characteristic signals at δ_H 7.06 (d, J = 8.6 Hz, 2H) and δ_H 6.71 (d, J = 8.6 Hz, 2H). It also had the tyroscherin series methyl signals δ_H 0.90 (d, J = 6.7 Hz, 3H), 0.86 (t, J = 7.3 Hz, 3H), and 0.81 (d, J = 6.5 Hz, 3H), ethylenic protons at δ_H 5.31 and δ_H 5.10, a carbinol at δ_H 3.92, and a proton α to a nitrogen at δ_H 3.23. On the other hand, the remarkable difference between compounds **1** and **3** in ^{1}H NMR was the presence of 2 additional singlet signals integrating for 3H at δ_H 1.33 and 1.14 in compound **3**.

Table 1

NMR spectroscopic data for *N*-methyl-tyroscherone (**2**) TFA salt (two diastereoisomers 1:1) and tyroscherin acetonide (**3**) at 500 MHz in CD_3OD.

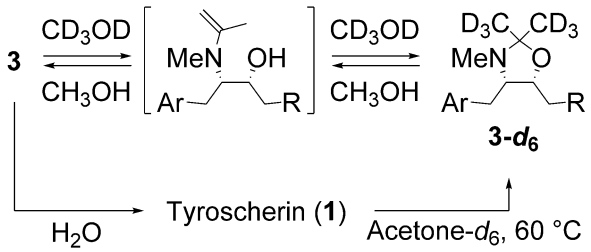
	N-Methyl-tyroscherone (**2**)				Tyroscherin *N*,*O*-acetonide (**3**)			
position	δ_C, type	δ_H (*J* in Hz)	COSY	HMBC[a]	δ_C	δ_H (*J* in Hz)	COSY	HMBC[a]
1	34.33/34.28, CH_2	a: 3.361/3.365, dd (13.7, 5.8)	1b, 2	2, 3, 1', 2'	34.4, CH_2	a: 2.83, dd (14.3, 5.6)	1b, 2	2, 3, 1', 2'
		b: 2.956/2.964, dd (13.7, 9.7)	1a, 2	2, 3, 1', 2'		b: 2.57, dd (14.3, 9.1)	1a, 2	2, 3, 1', 2'
2	74.2, CH	4.445/4.452, dd (9.7, 5.8)	1a, 1b	1, 3, 1'	66.8, CH	3.23, ddd (9.1, 7.0, 5.6)	1a, 1b, 3	1
3	207.75/207.68, C				77.6, CH	3.92, ddd (10.3, 7.0, 3.1)	2, 4	
4	44.9/44.8, CH_2	a: 2.37, m	4b, 5a, 5b	3, 5, 6	32.0, CH_2	1.55, m	3, 5a	3, 6
		b: 2.00, m	4a, 5a, 5b	3, 5, 6				
5	26.7, CH_2	a: 2.12, m	4a, 4b, 5b, 6	3, 4, 6, 7	29.5, CH_2	a: 2.12, m	4, 5b	4, 6, 7
		b: 1.99, m	4a, 4b, 5a, 6	3, 4, 6, 7		b: 1.93, m	4, 5a, 6	4, 6, 7
6	127.3, CH	5.17, m	5a, 5b, 7	4, 5, 7, 8	137.7, CH	5.31, m	5a, 5b, 7	5, 8
7	139.21/139.18, CH	5.14, m	6, 8	5, 6, 8, 9, 13	129.0, CH	5.10, ddt (15.3, 8.4, 1.4)	6, 8	5
8	35.82/35.79, CH	2.11, m	7, 9a, 9b, 13	6, 7	35.7, CH	2.10, m		
9	45.59/45.58, CH_2	a: 1.21, m	8, 9b, 10	7, 8, 10, 11, 13, 14	45.5, CH_2	a: 1.21, m	9b	
		b: 0.97, ddd (13.5 8.6 5.0)	8, 9a, 10	7, 8, 10, 11, 13, 14		b: 0.97, m	9a, 10	7, 8, 10, 11
10	33.3, CH	1.26, m			32.9, CH	1.30, m	14	
11	31.3, CH_2	a: 1.29, m	11b, 12		31.2, CH_2	a: 1.27, m	12	
		b: 1.13, m	12, 11a	9, 10, 12, 14		b: 1.14, m	12	
12	11.81/11.79, CH_3	0.85/0.86, t (7.3)	11a, 11b	10, 11	11.6, CH_3	0.86, t (7.3)	11a, 11b	10, 11
13	22.32/22.29, CH_3	0.898/0.899, d (6.7)	8	7, 8, 9	22.4, CH_3	0.90, d (6.7)	8	7, 8, 9
14	19.44/19.41, CH_3	0.804/0.806, d (6.5)	10	9, 10, 11	19.3, CH_3	0.81, d (6.5)	10	9, 10, 11
1'	125.43/125.40, C				130.6, C			
2'/6'	131.7, CH	7.10, bd (8.7)	3'/5'	1, 4', 2'/6'	130.7, CH	7.06, d (8.6)	3'/5'	1, 4', 2'/6'
3'/5'	117.3, CH	6.80, bd	2'/6'	1', 4', 3'/5'	116.0, CH	6.71, d (8.6)	2'/6'	1', 4', 3'/5'
4'	158.7, C				156.5, C			
NMe	42.6 (broad) , CH_3	2.90, bs		2	34.5, CH_3	2.20, s		2, 1''
1''					95.6, C			
2''					26.8, CH_3	1.33, s		1''
3''					20.0, CH_3	1.14, s		1'', 2''

[a] HMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon.

These two additional methyls were in geminal position, both linked to C1" as witnessed by HMBC. The correlation between the NMe and C1" as well as the C1" chemical shift at δ_C 95.6 confirmed the existence of an oxazolidine ring including carbons C2, C3 and C1". Compound **3** was named tyroscherin *N*,*O*-acetonide. Its presence was confirmed in the HPLC profile of the crude extract, and we therefore assumed that this compound did not originate from an unwanted addition of tyroscherin onto acetone after extraction (no acetone was used in the process).

NMR spectral data of **3** were recorded in CD_3OD for 12 hours. Interestingly, after this period, we observed that both methyl signals at δ_H 1.33 (H2") and 1.14 ppm (H3") had disappeared in ^1H NMR. Deuteration had occurred in these positions yielding **3-*d*$_6$**, most likely via a transient acyclic enamine resulting from the opening of the oxazolidine ring (Scheme 1). Besides, deuteriums were exchanged back to protons in anhydrous methanol. Also, after a long time in solution (~1 year), it was found that compound **3** had been converted into tyroscherin, therefore confirming the absolute configuration of **3** as 2*S*, 3*R*, 8*R*, 10*R*. Further confirmation was obtained by reacting tyroscherin with acetone-d_6 under reflux for 50 hours (Scheme 1). **3-*d*$_6$** was obtained and was converted in **3** in methanol. The overall yield was 88%. The NMR data for **3-*d*$_6$** and **3** obtained with this process were identical to those of natural **3** and of **3-*d*$_6$** obtained by deuteration of natural **3**. Note that acetonides are rather uncommon in Nature (Shao et al., 2011; Yu et al., 2016). Compound **3** was isolated without using acetone, neither for extraction, nor for purification. Also, condensation of tyroscherin with acetone-d_6 did not occur at room temperature. Fungal cultures can produce acetone (Scotter et al., 2005), which we suppose was trapped by tyroscherin in the culture medium. We feel that compound **3**

should therefore be considered as a genuine natural product although the condensation may not have been enzyme-catalyzed.



Scheme 1

The antimicrobial potential of compounds **1**-**4** was evaluated against human pathogens *Staphylococcus aureus* and *Candida albicans* (Table 2). In accordance with the literature, tyroscherin (**1**) was strongly active on both pathogens, and pseurotin A (**4**) was inactive (Lu et al., 2014; Nirma et al., 2013). Interestingly, both tyroscherin analogs were significantly active, with minimal inhibitory concentrations of 8 $\mu g.mL^{-1}$ for **2** on *S. aureus*, and 16 and 8 $\mu g.mL^{-1}$ for **3** on *C. albicans* and *S. aureus*, respectively. In addition, compounds **1**-**3** showed a moderate cytotoxicity towards MRC5 cells with IC_{50} in the 20-50 $\mu g.mL^{-1}$ range.

Table 2. Antimicrobial activity of compounds **1**-**4**

Molecule	MIC ($\mu g.mL^{-1}$) *C. albicans*[a]	*S. aureus*[b]
Tyroscherin (**1**)	16	8
N-Methyl-tyroscherone (**2**)	128	8
Tyroscherin *N*,*O*-acetonide (**3**)	16	8
Pseurotin A (**4**)	> 128	> 128
Oxacillin	-	4
Fluconazole	4	-

[a] *Candida albicans* ATCC 10213

[b] *Staphylococcus aureus* ATCC 29213

In conclusion, this article reports *N*-methyl-tyroscherone and tyroscherin *N*,*O*-acetonide

for the first time. These compounds were isolated along with pseurotin A and tyroscherin from the termite-borne fungus *Pseudallescheria boydii* SNB-CN85. Their relative and absolute configurations were determined by chemical modifications and comparison with tyroscherin. Along with our previous report,(Nirma et al., 2013) this article further demonstrates that tyroscherin-producing *Pseudallescheria* fungal strains can be found in several termite nests in French Guiana. The exact occurrence of *Pseudallescheria* sp. and the ability of this strain to deliver tyroscherin to its host termites are currently being investigated.

Acknowledgment

This work has benefited from an "Investissement d'Avenir" grant of the Agence Nationale de la Recherche (CEBA, ref. ANR-10-LABX-0025, France).

Appendix A. Supplementary data

Supplementary data associated with this article include experimental information, full spectroscopic data, and NMR spectra of compounds **1**-**4**. It can be found at http://

References

Beemelmanns, C., Guo, H., Rischer, M., Poulsen, M., 2016. Natural products from microbes associated with insects. Beilstein J. Org. Chem. 12, 314–327.

Bloch, P., Tamm, C., 1981. Isolation and structure of pseurotin A, a microbial metabolite of *Pseudeurotium ovalis* Stolk with an unusual heterospirocyclic system. Helv.

Chim. Acta 64, 304–315.

Cantley, A.M., Clardy, J., 2015. Animals in a bacterial world: opportunities for chemical ecology. Nat. Prod. Rep. 32, 888–892.

Carr, G., Poulsen, M., Klassen, J.L., Hou, Y., Wyche, T.P., Bugni, T.S., Currie, C.R., Clardy, J., 2012. Microtermolides A and B from termite-associated *streptomyces* sp. and structural revision of vinylamycin. Org. Lett. 14, 2822–2825.

Hayakawa Y, Yamashita T, Mori T, Nagai K, Shin-Ya K, W.H., 2004. Structure of tyroscherin, an antitumor antibiotic against IGF-1-dependent cells from *Pseudallescheria* sp. J. Antibiot. 57, 634–638.

Hayashi, Y., Shoji, M., Yamaguchi, S., Mukaiyama, T., Yamaguchi, J., Kakeya, H., Osada, H., 2003. Asymmetric total synthesis of pseurotin A. Org. Lett. 5, 2287–2290.

Katsuta, R., Shibata, C., Ishigami, K., Watanabe, H., Kitahara, T., 2008. Synthesis and structure revision of tyroscherin, a growth inhibitor of IGF-1-dependent tumor cells. Tetrahedron Lett. 49, 7042–7045.

Lu, Q.-Q., Tian, J.-M., Wei, J., Gao, J.-M., 2014. Bioactive metabolites from the mycelia of the basidiomycete *Hericium erinaceum*. Nat. Prod. Res. 28, 1288–1292.

Matsui, T., Tanaka, J., Namihira, T., Shinzato, N., 2012. Antibiotics production by an actinomycete isolated from the termite gut. J. Basic Microbiol. 52, 731–735.

Nirma, C., Eparvier, V., Stien, D., 2015a. Antibacterial ilicicolinic Acids C and D and ilicicolinal from *Neonectria discophora* SNB-CN63 isolated from a termite nest. J. Nat. Prod. 78, 159–162.

Nirma, C., Eparvier, V., Stien, D., 2015b. Reactivation of antibiosis in the entomogenous fungus *Chrysoporthe* sp. SNB-CN74. J. Antibiot. 68, 586–590.

Nirma, C., Eparvier, V., Stien, D., 2013. Antifungal agents from *Pseudallescheria boydii*

SNB-CN73 isolated from a *Nasutitermes* sp. termite. J. Nat. Prod. 76, 988–991.

Nowak, M.A., Tarnita, C.E., Wilson, E.O., 2010. The evolution of eusociality. Nature 466, 1057–1062.

Scotter, J.M., Langford, V.S., Wilson, P.F., McEwan, M.J., Chambers, S.T., 2005. Real-time detection of common microbial volatile organic compounds from medically important fungi by Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS). J. Microbiol. Methods 63, 127–134.

Shao, C.L., Wu, H.X., Wang, C.Y., Liu, Q.A., Xu, Y., Wei, M.Y., Qian, P.Y., Gu, Y.C., Zheng, C.J., She, Z.G., Lin, Y.C., 2011. Potent antifouling resorcylic acid lactones from the gorgonian-derived fungus *Cochliobolus lunatus*. J. Nat. Prod. 74, 629–633.

Sorres, J., Nirma, C., Eparvier, V., Stien, D., 2017. Pseudallicins A-D, Four Complex Ovalicin Derivatives from *Pseudallescheria boydii* SNB-CN85. Org. Lett. 19, 3978–3981.

Yan, S., Li, S., Wu, W., Zhao, F., Bao, L., Ding, R., Gao, H., Wen, H.A., Song, F., Liu, H.W., 2011. Terpenoid and phenolic metabolites from the fungus *Xylaria* sp. associated with termite nests. Chem. Biodivers. 8, 1689–1700.

Yu, J.S., Moon, E., Choi, S.U., Kim, K.H., 2016. Asarotonide, a new phenylpropanoid with a rare natural acetonide group from the rhizomes of *Acorus gramineus*. Tetrahedron Lett. 57, 1699–1701.