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Nature-Inspired Chemistry of Complex Alkaloids: Combining Targeted Molecular Networking Approach and Semisynthetic Strategy to Access Rare Communesins in a Marine-Derived *Penicillium expansum*

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Abstract: Communesins are rare alkaloids isolated from fungi of the genus *Penicillium*. In this work, the extract of a marine-derived *Penicillium expansum* strain was studied using targeted molecular networking approach allowing to detect 65 communesins including 55 new ones. A fragmentation pattern for dimethylvinyl communesins was established and a script was implemented allowing to predict the structure and map all communesins in a global molecular network. A semisynthetic strategy was carried out to obtain some minor

congeners from the two isolated communesins A and B. Nine communesins were then synthesised: two of them were already described as produced by the studied strain; four are new natural products which occurrence in the extracts was confirmed; three are new semi-synthetic analogues never described so far. These communesins were evaluated for their cytotoxicity on two human cancer cell lines KB and MCF-7 leading to a preliminary study of their structure-activity relationships.

Introduction

Communesins are an emerging class of fungal metabolites produced by different species of the genus *Penicillium*. Communesins A (1) and B (2) were first identified from a strain of *Penicillium sp.* isolated from the marine alga *Enteromorpha intestinalis*.^[1] They have been reported later to be produced

with other communesin derivatives differing from each other by their substituents R¹, R² and R³ (Table 1).

Based on the presence or absence of one oxygen atom on the substituent R³, this class of compound is divided into two groups named "dimethylepoxy (DME)" and "dimethylvinyl (DMV)" communesins.

To date, twelve derivatives have been described from different species of *Penicillium* (*P. expansum*, *P. marinum*, *P. rivulum* and *P. buchwaldii*) from various origins (terrestrial or marine) (Table 1, Table 2). It is to note that only 3 DMV communesins have been described so far, which can be explained by the proposed biosynthesis suggesting they are non-oxidized precursors of the final DME communesins.^[2]

The original polycyclic system containing two vicinal quaternary centers coming from the coupling between a *N*-methylated aurantioclavine and an oxidized tryptamine derivative^[2] has inspired several synthetic chemists to establish strategies for the construction of this daunting skeleton.^[3] Furthermore, these compounds have presented different potential biological activities such as antiproliferative on human cancer cell lines, neurotoxicity on *Diptera* larvae, antimicrobial and antiviral, toxicity on *Artemia salina* and cardiovascular effects in a zebrafish model.^[1,3,4] However, the potential interest of this chemical class is currently limited by the low diversity of analogues described and available.

Access to a larger chemodiversity of natural products of this class would allow to better assess their structure-activity relationships.

Previous studies performed on a marine-derived *Penicillium expansum* Link MMS42 (isolated from a sediment sample

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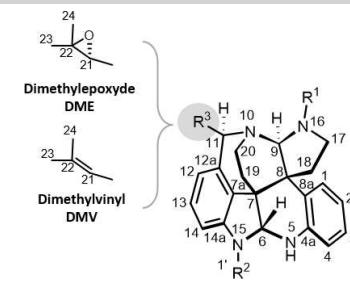
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Table 1. Structures of reported communesins (see references in Table 2).


Compounds	R ¹	Substituents R ²	R ³
Communesin A (1)	CO-CH ₃	CH ₃	DME
Communesin B (1)	CO-C ₃ H ₇	CH ₃	DME
Communesin C	CO-C ₃ H ₇	H	DME
Communesin D (9)	CO-C ₃ H ₇	CHO	DME
Communesin E	CO-CH ₃	H	DME
Communesin F (4)	CO-CH ₃	CH ₃	DMV
Communesin G	CO-C ₃ H ₅	CH ₃	DME
Communesin H	CO-C ₃ H ₇	CH ₃	DME
Communesin I-1 ^[a]	H	CH ₃	DME
Communesin I-2 ^[a]	CO-C ₃ H ₁₁ O	CH ₃	DME
Communesin J	CO-C ₃ H ₇	CH ₃	DMV
Communesin K	H	CH ₃	DMV

[a] Communesin I-1 and I-2 were described simultaneously by two different research teams, Lin et al. 2015 and Fan et al. 2015, respectively.

Table 2. Origin of reported communesins.

Species	Strains	Origin	Communesin													Reference	
			A	B	C	D	E	F	G	H	I-1	I-2	J	K			
<i>Penicillium</i> sp.		marine	x	x													Numata et al., 1993 ^[1]
<i>P. expansum</i>	^[a]	terrestrial		x													Andersen et al., 2004 ^[6b]
<i>P. expansum</i>	MK-57	terrestrial	x	x		x	x	x									Hayashi et al., 2004 ^[4c]
<i>Penicillium</i> sp.	ZMA.POR 15866	marine		x	x	x											Jadulco et al., 2004 ^[4b]
<i>P. rivulum</i>	IBT 24420	marine								x	x						Dalsgaard et al., 2005 ^[4d]
<i>P. marinum</i>	N934-53	terrestrial	x	x													Wigley et al., 2008 ^[3k]
<i>P. expansum</i>	MMS42	marine	x	x		x	x	x									Kerzaon et al., 2009 ^[4f]
<i>P. buchwaldii</i>	^[a]	terrestrial	x	x													Frisvad et al., 2013 ^[5]
<i>P. expansum</i>	NRRL 976	terrestrial	x	x	x			x	x			x			x	x	Lin et al., 2015 ^[2]
<i>P. expansum</i>	Y32	marine	x	x									x				Fan et al., 2015 ^[4g]

[a] Studies on *P. expansum* by Andersen et al. (2004) and on *P. buchwaldii* by Frisvad et al. (2013) investigated 260 and 15 different isolates, respectively.^[6b,5]

collected on the west atlantic coast of France), have shown the huge potential of this strain in terms of production of new communesins.^[4f] Characteristic fragmentation pattern of DME communesins were then described and potentially new molecules of this series were detected by HPLC-MS/MS analyses. In the present work, we propose an automated strategy based on molecular networking (MN) approach to specifically detect communesins in samples. Application of this targeted molecular networking approach allowed to highlight additional DME communesins that were not detected in the first place^[4f] together with new DMV communesins, which structures could be proposed based on MS/MS fragmentation. Following this oriented molecular networking approach, a semisynthetic strategy was designed to access minor communesins and confirm their structure. Eleven communesins were then obtained by purification and semisynthesis. The biological activity of these compounds was evaluated on two human cancer cell lines.

Results and Discussion

As shown in Table 2, *P. expansum* is the main producer of communesins. A previous study identified 5 known and 7 new communesins in the extracts of the marine-derived *P. expansum* MMS42 but their structures were not elucidated because of limited quantities.^[4f] This latter study showed that the MMS42 strain was a potential source to access numerous new communesins. It was then chosen for a purification program to access these molecules. However, *P. expansum* is also known as a producer of different cytochalasan compounds, such as chaetoglobosins.^[6] In *P. expansum* MMS42, the extracted ion chromatograms at *m/z* 529.2702 and 531.2859 corresponding to chaetoglobosin A and E respectively showed that numerous chaetoglobosins were co-eluted with communesins (Figure S1) resulting in the difficult isolation of minor communesins. To overcome such difficulty and provide a better access to these specific compounds, a selective alkaloidic extraction step was

therefore developed allowing the elimination of chaetoglobosins (Figure S2). This protocol was applied on different fractions and the obtained fractions were subjected to LC-HRMS/MS analyses, on which a targeted molecular networking was considered.

MS/MS targeted molecular networking approach for the discovery of DME communesins chemodiversity

In order to selectively highlight communesins in samples (especially minor ones), an automated workflow was then developed, taking advantage of both molecular networking approach and characteristic fragmentation pattern of communesins. After a peak picking step applied to all LC-HRMS/MS data obtained on fractions enriched in communesins, a R script was devised for DME communesin detection. It calculated a score for each feature detected after the peak picking step, based on the presence of fragments within the MS/MS spectra. Fragments were corresponding to both characteristic neutral losses (-18.0106 , -58.0419 , -72.0575) and typical fragments (m/z $170.0844 + R^2$, $242.1419 + R^2$; R^2 being either $-H$, $-CH_3$ or $-CHO$) for DME communesins.^[4f] These scores were thus mapped into a molecular network obtained using MetGem software^[7] (Figure 1). Such an approach allowed to instantly highlight a cluster corresponding to DME communesins. Actually, the use of the Global Natural Product Social (GNPS) library^[8] assigned many nodes of this cluster as communesin A

analogues. The two known communesins A (1) and B (2) at m/z 457.2593 and 509.2904 were effectively detected in this cluster. This was further confirmed by comparison with previously isolated standards.^[4f] In addition, most communesins previously reported in trace amounts^[4f] were found in this cluster together with 40 putatively new ones as reported in Table S1. This validated the approach for targeted molecular networking. The two different visualizations of molecular networks (GNPS-like and t -SNE)^[7] were complementary as some singletons observed in the GNPS-like representation could be related to other nodes within the t -SNE representation (Figure 1).

Moreover, by taking into account the presence and the intensities of the different MS/MS fragments which m/z depends on R^2 , an additional score could be calculated for the 3 substituents corresponding to the most common groups on R^2 ($-H$, $-CH_3$ or $-CHO$). This allowed to determine a relative probability about the nature of R^2 , which could be visualized by applying a different color to each fragment score (for $-H$, $-CH_3$ or $-CHO$, respectively), displayed as pie charts for each node (Figure 2). Consequently, most nodes of our DME communesin cluster could have their R^2 substituent deduced, except for some nodes with low or weak fragmentation or with a R^2 substituent potentially different from $-H$, $-CH_3$ or $-CHO$ (blue nodes on Figure 2). The results obtained for the seven known DME communesins confirmed the accuracy of our approach. Thus, the DME communesin cluster was then composed mostly of analogues methylated at R^2 position whereas the two other

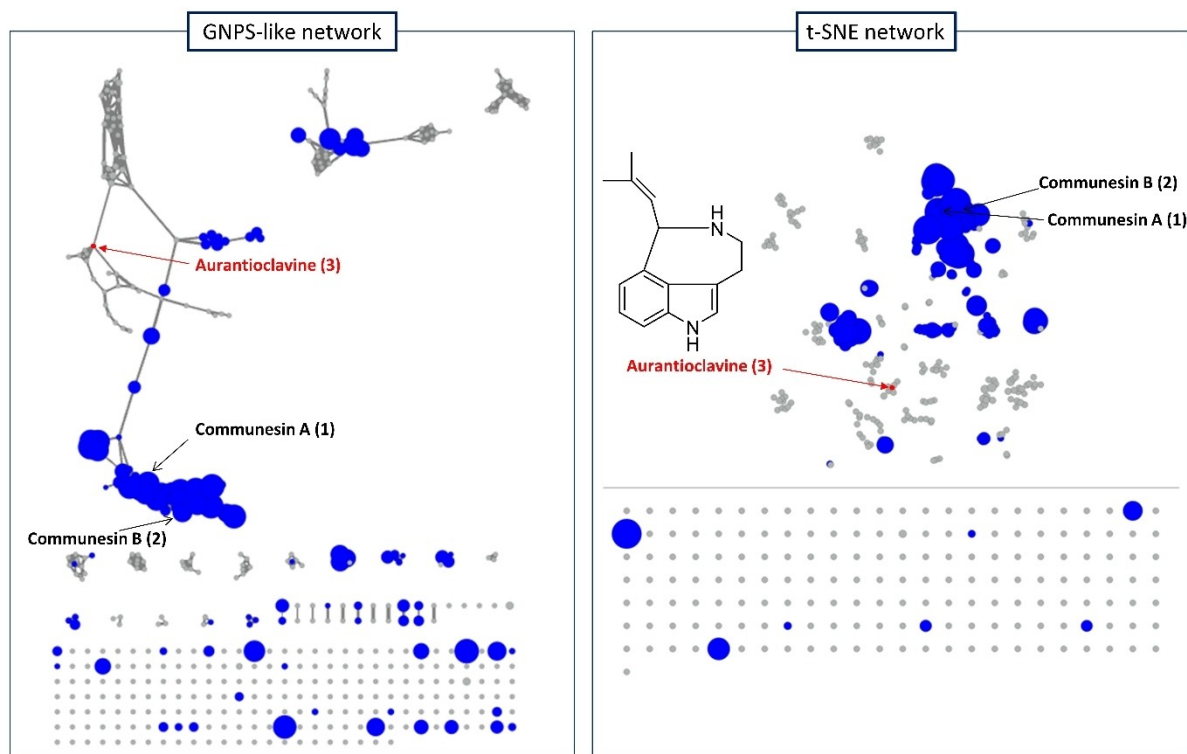


Figure 1. Molecular networks obtained using MetGem software^[6] with the DME communesin score calculated as node size. Nodes of putative DME-communesins are highlighted in blue.

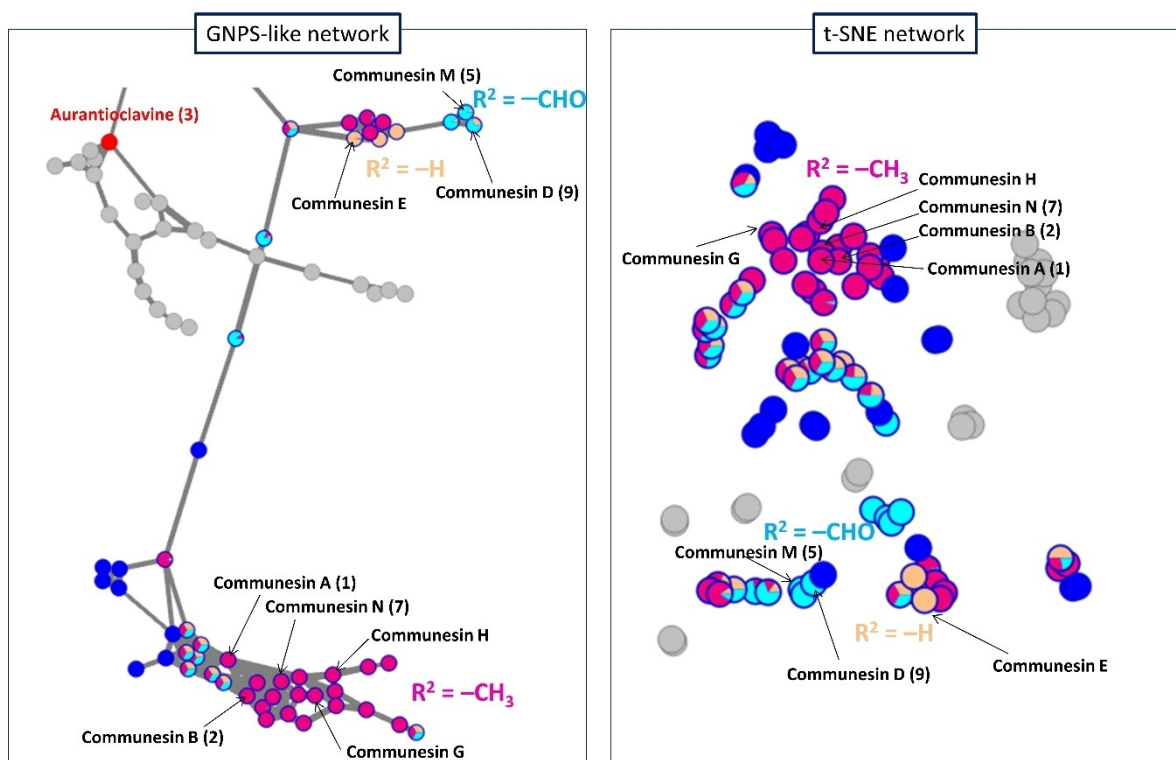


Figure 2. Determination of the most probable R^2 substituent based on the score calculated for each of the three possibilities: -H (orange), - CH_3 (pink) and -CHO (bright blue) and based on relative intensities of previously reported typical fragments observed for DME communesins.^[4f] Blue nodes correspond to DME communesins with unidentified R^2 substituent. Known and new communesins detected in this study are highlighted.

substituents were less detected, which is in accordance with the actual list of known communesins.

MS/MS targeted molecular networking approach for the discovery of DMV communesin chemodiversity

Further development of the approach towards the scarcer DMV communesins was then considered. As aurantioclavine (**3**) (Figure 1) was shown to play a key role in the biosynthetic pathway of communesins and shared half the molecule core structure,^[2] its MS/MS fragmentation pattern was thus studied to predict the potential MS/MS fragmentation of DMV communesins. This was possible because the western part of aurantioclavine is composed of the DMV tricyclic skeleton of DMV communesins. The HRMS/MS spectrum of purified aurantioclavine (**3**) (Figure S3) showed a major product ion at m/z 210.1293 corresponding to the cleavage of the azepine ring with a loss of NH_3 (-17 u). The second product ion (neutral loss of -56 u, C_4H_8) is characteristic of the loss of the dimethylvinyl moiety and is a common feature of prenylated compounds.^[9] These two product ions at m/z 210.1293 and 171.0920 have been previously described for **3**.^[10] Two other main product ions were observed at m/z 198.1298 (- CH_3N) and 130.0660 (- $C_6H_{11}N$). A proposed fragmentation interpretation is shown in Figure S3.

In the molecular network, aurantioclavine (**3**) could be annotated and located with close MS/MS spectral similarity with

a cluster distinct from the previous DME communesin clusters (Figures 2 and 4). Most of the nodes included in this cluster presented the same typical neutral losses (17, 56 and 97) as aurantioclavine. One ion at m/z 441.2661 (compound **4**) was found in the latter cluster in the MN (Figure 4). It corresponded to the protonated ion of communesin F (theoretical mass: 441.2648 Da, mass error: 2.95 ppm), which is one of the most frequently reported DMV communesins in *P. expansum*.^[2,4c,f] HRMS/MS analysis showed the two product ions at m/z 424.2400 and 385.2041 corresponding to the losses of 17 and 56 u, respectively, as observed for **3** (Figure 3). The ion at m/z 385.2041 was the same as the $[M-((CH_3)_2CHO)]$ ion previously described for **1**,^[4f] indicating similar substituents on R^1 and R^2 . Another product ion observed at m/z 144.0827 ($C_{10}H_{10}N$) corresponded to the methylated analogue of the m/z 130.0660 product ion of aurantioclavine, indicating an indole moiety and the methylation of the nitrogen atom. All these observations led to the non-ambiguous identification of **4** as communesin F.^[4c] Interpretation of the fragmentations obtained by HRMS/MS for **4** was then proposed as the general fragmentation pattern for the DMV communesins (Figure 3). This fragmentation pattern allows to easily deduce the elemental composition of R^1 and R^2 substituents as for DME communesins.

The previous R script updated, taking into consideration this new fragmentation pattern allowed to calculate a new score for DMV communesin detection. Mapping this score in the MN as explained for DME communesins allowed to highlight several

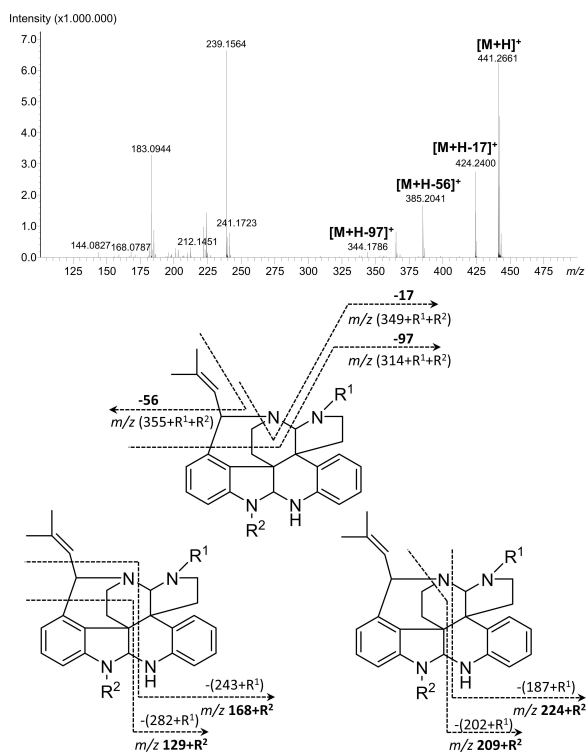


Figure 3. HRMS/MS spectrum of **4** and proposed fragmentation patterns for DMV communesins.

nodes putatively corresponding to DMV communesins in the extract of *P. expansum* MMS42 (Figure 4). In the same way, R^2 substituents were proposed for each of them. The three known DMV communesins F, J and K were thus annotated in the network and R^2 substituents were proposed for the 15 other highlighted nodes.

In total, our automated search of DME and DMV communesins among the samples analyzed through targeted MN approach, allowed to highlight 55 putatively new DME and DMV communesins in addition to the 7 known DME and 3 known DMV compounds (Table S1). For most new communesins, R^2 substituent could be deduced. However, as for DME, some substitutions of the DMV communesins (8 out of 56) could not be deduced from the MS/MS spectrum.

These results highlight the huge chemodiversity of communesins produced by the *P. expansum* MMS42 strain. Regarding this important variety, the final steps of the biosynthesis of these compounds can be questioned. While the conserved heptacyclic core of the molecule is enzymatically controlled as previously described,^[2] two hypotheses can be proposed for the R^1 lateral substitution, with either spontaneous reactions between the free amine of the pyrrolidine ring and a large number of acylated compounds of various origins, or a very high tolerance of the promiscuous acyltransferase CnsK toward various substrates. Moreover, the range of DMV communesins observed here leads to assume that DME communesins occur through epoxidation of the corresponding DMV communesins rather than through acylation of a single DME intermediate

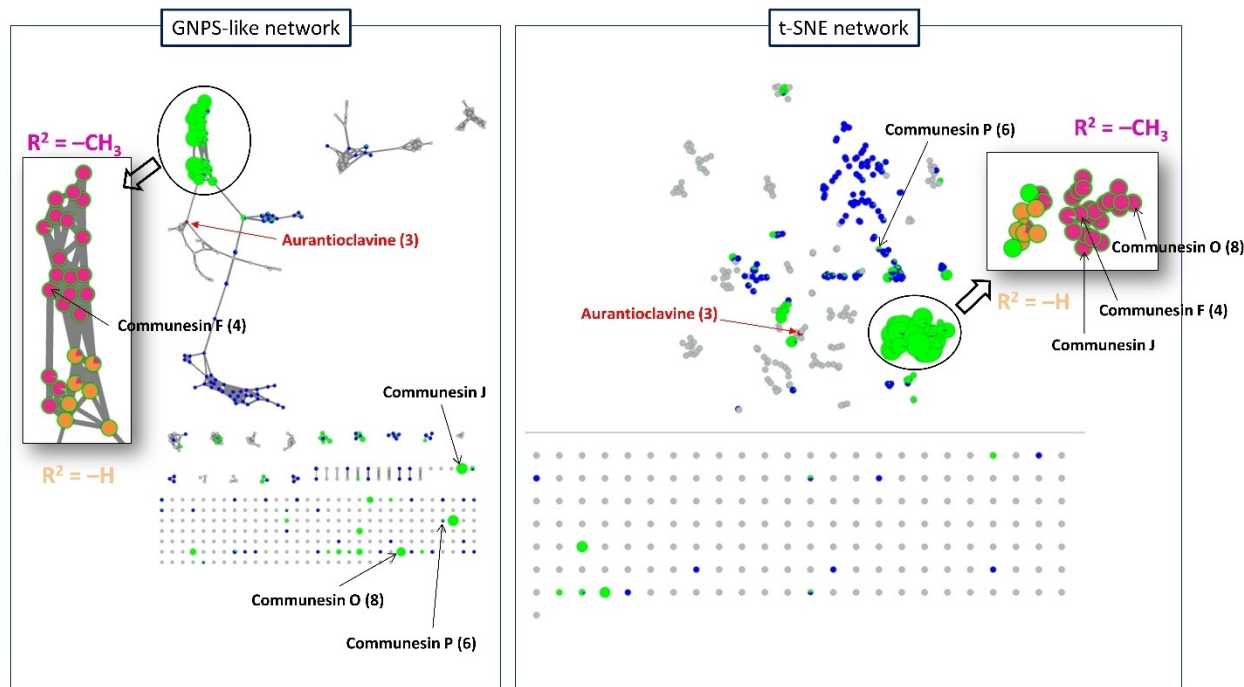


Figure 4. Molecular networks obtained using MetGem software^[6] with the DMV communesin score calculated as node size. Clusters of putative DMV communesins are highlighted in green. Aurantioclavine (**3**) is highlighted in red. In frame: Determination of the most probable R^2 substituent based on the score calculated for each of the three possibilities: $-H$ (orange), $-CH_3$ (pink) and $-CHO$ (bright blue) and based on relative intensities of typical fragments observed for DMV communesins. Green nodes correspond to DMV communesins with unidentified R^2 substituent. Annotation of known and new communesins detected in this study are highlighted.

(being communesin I-1) as reported.^[2] A new biosynthetic pathway for all described communesins is then proposed in Figure S67.

Limited isolation of natural communesins

In view of the wealth of new molecules potentially available in *P. expansum* MMS42, this strain was further investigated as it constituted an excellent model to generate a library of communesins. Despite the alkaloid extraction protocol at pH 1 allowing to remove chaetoglobosins, only one new communesin could be isolated, in very low amounts. Compound **5** was obtained as a light-yellow amorphous powder. HPLC-HRMS analysis of **5** displayed a $[M+H]^+$ ion at m/z 471.2435 eluting at $t_R = 9.4$ min, for which the molecular formula $C_{28}H_{30}N_4O_3$ was deduced requiring 15 degrees of unsaturation. Its HRMS/MS spectrum presented the characteristic fragmentations of DME communesins with the losses of 18, 58 and 72.^[4f] 1H NMR (400 MHz, $CDCl_3$) spectrum of **5** was very close to that of **1** except for the deshielding chemical shift of proton at 1' from 2.84 ppm (s, 3H) in **1** to 8.92 ppm (s, 1H) in **5** corresponding to

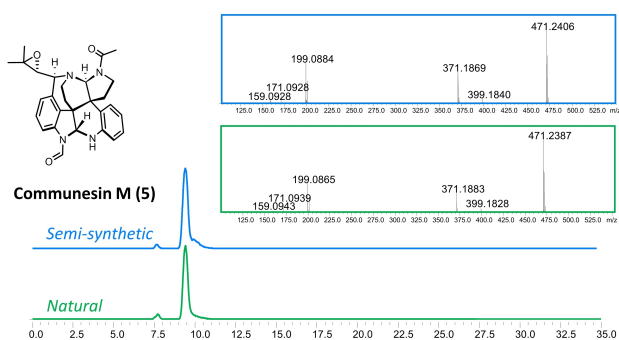


Figure 5. Confirmation of predicted structure of the natural communesin M (**5**) (detected at m/z 471.2387) based on the comparison of its extracted ion chromatogram from a fraction of MMS42 *P. expansum* (green trace) and its MS/MS spectrum (green trace) with its corresponding semi-synthetic compound (blue trace and blue box).

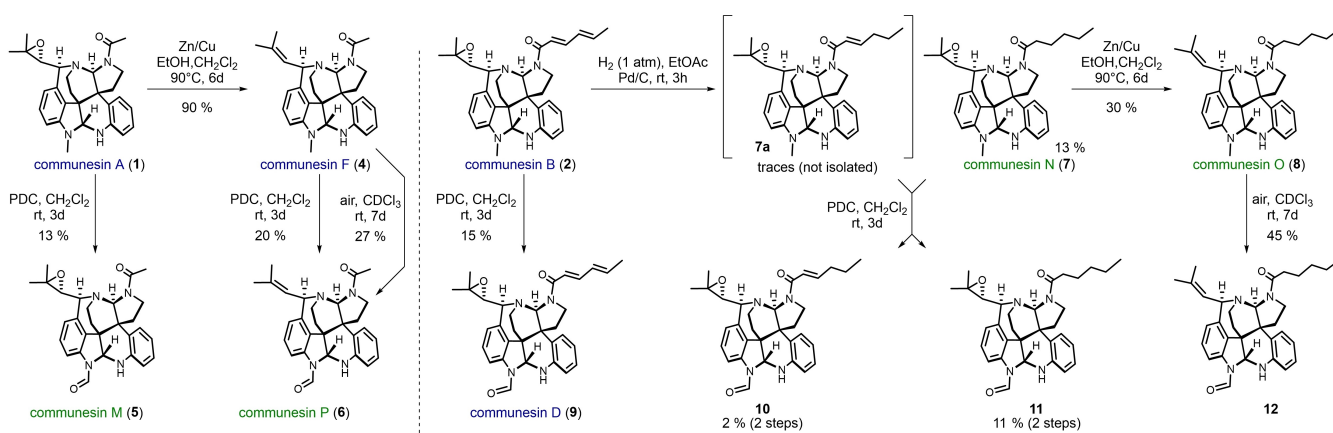
the substitution of *N*-methyl in **1** by an *N*-formyl group in **5**. The structure of this compound was then further confirmed by the comparison of its LC-HRMS/MS and NMR data with that of the semisynthetic product (Figure 5).

Semisynthetic strategy to confirm the structure of detected natural communesins

As the amounts of other putatively new communesins in the *P. expansum* MMS42 extract were very low, confirmation of their hypothesized structure by isolation did not seem appropriate. Therefore, a semisynthetic work from the two isolated major communesins **1** and **2** was then considered to access some of the new communesins detected here and in the previous study on the marine-derived *P. expansum* MMS42.^[4f] To this purpose, the isolation of **1** and **2** was first carried out on a large-scale culture leading to 114.8 and 92.2 mg of **1** and **2** respectively.

Then a semisynthetic work from **1** and **2** was conducted toward accessible communesins to further confirm their structures. Three reactions (Scheme 1) were developed: i) the oxidation of the *N*-methyl group into *N*-formyl derivatives by pyridinium dichromate (PDC); ii) the hydrogenation of the side chain of **2** into saturated analogues; iii) the epoxide removal into olefin mediated by zinc-copper alloy in a dichloromethane/ethanol mixture at 90 °C (sealed tube).

Starting from milligram scale of **1** and **2**, nine communesins were then synthesized (Scheme 1) including seven new analogues (**5–12**) and two known communesins (**4**, **9**). Among these, compound **7a** was obtained during the hydrogenation of **2** as a trace product and could not be separated from **7**. After the oxidation step, products **10** and **11** were obtained as pure forms. The structures of these semisynthetic compounds were established by 2D NMR analyses (Tables S2–S5 and Figures S9–S62). Surprisingly, spontaneous *N*-oxidation at R^2 of two DMV communesins (compounds **4** and **8**) produced two new other compounds **6** and **12** respectively (Figure S4). 1H NMR spectral analyses of compounds **4** and **8** after 7 days in $CDCl_3$ clearly showed the presence of a new proton at 8.88 ppm correspond-



Scheme 1. Semisynthetic reaction scheme carried out on the isolated communesins **1** and **2**. Reported natural communesins are highlighted in blue, while new natural communesins (**5–8**) are highlighted in green.

ing to the formyl group. After purification, the new compounds produced from compounds **4** and **8** were confirmed to be **6** and **12**. This phenomenon was not observed for the DME communesins when they were conserved using the same condition, nor for DMV when these compounds were conserved in MeOH at room temperature for 7 days. To the best of our knowledge, this type of reaction has never been described before. An unusual methylenedioxy ring formation was previously observed leading the authors to hypothesize spontaneous oxidation.^[11] Here, the data also suggest a spontaneous formation of the *N*-formyl function of natural communesins (see below).

All semisynthetic communesin derivatives were analyzed by HPLC-HRMS/MS to confirm the production of these compounds by the studied strain *P. expansum* MMS42. By comparison of retention times and MS/MS spectra of natural and semisynthetic products (Figures 5 and S63–S65), we could thus confirm that this species effectively produces the two known communesins F and D (**4**, **9**) and four new others (**5–8**). The four new natural communesins **5–8**, were then named communesin M, P, N and O respectively. Communesin M actually corresponds to the (+)-*N*⁸-formyl communesin E, which has been recently described in a patent and claimed to be obtained by synthesis.^[3m] All these communesins were annotated in the molecular network (Figures 2 and 4).

Biological activity evaluation

All obtained compounds were submitted to an antiproliferative evaluation against the two KB and MCF-7 human cancer cell lines. These results showed moderate cytotoxicity of all compounds in comparison to the positive control docetaxel, with compounds **10** and **11** being the most active, with IC₅₀ values of 4.8 μM and 9 μM on KB and MCF-7 respectively for **10** and 6.3 μM on MCF-7 for **11** (Figure S66). While these results are still moderate in comparison to the positive control docetaxel (with IC₅₀ values of 38 nM and 10.3 nM for KB and MCF-7 cell lines respectively), some observations could be raised such as the importance of the length of the side chain R¹ for the activity as well as the epoxidation at R³ and the *N*-formylation of the indoline which seemed to confer a higher activity to the communesins.

Conclusion

The present work highlighted the great potential in the production of a series of complex indole alkaloids, by a marine-derived strain *P. expansum* MMS42, which could then constitute an alternative platform for the obtention of nature-inspired semisynthetic communesins when compared to complex total synthetic strategies.^[3c,12] The development of a targeted alkaloid extraction protocol allowed to isolate one new communesin **5** and facilitated the detection of many minor communesins. This work confirmed the performance of molecular networking approach in detecting new communesins in the extracts as well

as in predicting their structures. Following structural hypotheses raised for these compounds, and using the two major communesins **1** and **2** as starting materials for semisynthesis, nine communesin analogues could be obtained including seven new ones. This allowed to confirm the structure of four natural minor communesins detected by HPLC-HRMS/MS. The antiproliferative activity of these compounds was carried out on two human cancer cell lines KB and MCF-7 and allowed to establish preliminary SAR in the communesin series. Further work to obtain other communesin analogues together with the investigation of their biological target and mechanism of action is ongoing.

Experimental Section

General experimental procedures: Optical rotations were performed with an MCP 300 Anton Paar polarimeter at 589 nm at 18 °C. 1D and 2D NMR spectra were recorded in CDCl₃ on Bruker Avance 400 MHz spectrometers. Chemical shifts were referenced using residual solvents CDCl₃ (δ_H 7.26 and δ_C 77.16). HPLC was performed on an Agilent 1200 series apparatus using C₁₈-bonded silica columns (Interchim, 250×10 mm; Luna Phenomenex, 5 μm, 250×10 mm). Vacuum liquid chromatography (VLC) was performed using commercial silica gel (pore size 60 Å, 35–70 mesh particle size, Carlo Erba). The evolution of reactions was followed by thin-layer chromatography on silica gel plates with fluorescence indicator at UV₂₅₄ (pore size 60 Å, thickness 0.2 mm, Macherey-Nagel).

Chemicals: All components used for fungal culture media were purchased from Becton-Dickinson (Sparks, USA) and Laboratorios CONDA (Madrid, Spain). The artificial seawater (Reef crystals) was purchased from Aquarium Systems. Solvents used for extraction and purification were redistilled technical grade solvents from Carlo Erba (Val de Reuil, France), and ultrapure H₂O was obtained by filtering on the arium comfort system (Sartorius, Germany). All materials used for the tests were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Reagents including zinc-copper alloy (Zn/Cu) and pyridinium dichromate (PDC) were purchased from ACROS Organics (Fisher Scientific, France) and Sigma-Aldrich respectively. Solvents used for synthesis were purchased from Aldrich (France) and required no further purification. Solvents used for HPLC-HRMS were of ULC-MS grade from Biosolve Chimie (Dieuze, France).

Fungal strain: The studied fungal strain was isolated from a sediment sample collected in a shellfish farming area of the Loire estuary (Le Croisic, France). It was identified as *Penicillium expansum* Link by sequencing the internal transcribed spacers (ITS 1 and 2) and the beta-tubulin regions (GenBank accession number JN794527 and JN794529, respectively). The strain is conserved in the laboratory fungal collection under the reference number MMS42 (UR2160 ISOMer, Nantes Université, France).

Culture, fermentation and extraction: Cultures were performed in Erlenmeyer flasks (250 mL) containing 50 mL of Dextrose Casein Agar medium (DCA). The composition of medium included 40 g/L glucose, 10 g/L casein enzymatic digest, 15 g/L agar and 36 g/L artificial seawater. The fungal cultures were incubated for 12 days at 27 °C under natural light before extraction. Extraction processing using CH₂Cl₂/EtOAc 1:1 (v/v) was carried out following the protocol previously described.^[13]

Alkaloid extraction protocol: 100 mL of H₂SO₄ solution at pH 1 were added to the fungal culture extract and well stirred. Then

100 mL of ethyl acetate (EtOAc) were added. This mixture was decanted giving EtOAc and aqueous phases. The EtOAc phase was extracted for the second time using 100 mL of H₂SO₄ pH 1. The aqueous phases were collected and alkalized until pH 10 by a NaOH concentrated solution. The obtained mixture was extracted three times by CH₂Cl₂ (200 mL each time). The CH₂Cl₂ phases were collected and dried giving the alkaloid fraction.

HPLC-HRESIMS analysis: HPLC-HRESIMS/MS analyses were carried out on a UFLC-MS (IT-TOF) Shimadzu instrument (combining Ion trap and Time of Flight analyzers, Shimadzu, Japan). A C₁₈ column (2.6 μm, 100×2.1 mm, Phenomenex, France) was used and analysis conditions were previously described.^[13–14]

Molecular networking: HPLC-HRMS/MS profiles were converted from *.raw data to *.mzXML format using MSconvert software. These raw data were processed by using MZmine2 software as detailed in supplementary information. Export from MZmine2 generated a *.mgf file with 459 peaks with associated HRMS/MS data and a *.csv file with the integration values for each peak in each sample. Automated detection of communesin patterns was performed on the *.mgf file by using an in-house developed R script, using the CluMSID package (version 1.6.0)^[15] searching for characteristic fragments of both DME and DMV communesins (see Supporting Information). The script allowed to calculate specific scores for each peak, based on their probability to correspond to DME or DMV communesins, as well as for the probability for R² to be either -H, -CH₃ or -CHO. These scores were implemented directly in the previously generated *.csv file. Molecular network was then built using MetGem (see Supporting Information for details), which allowed to visualize spectral similarities between the peaks by using both the cosine score (GNPS-like) and the t-SNE algorithm.^[7] Search for known compounds was carried out by using libraries of available experimental or in silico MS/MS spectra.^[8,16] The scores previously calculated were used to highlight the information on the networks.

Isolation of 1, 2, 3 and 5: The DCA large scale cultivation was performed on 484 Erlenmeyer flasks yielding 11.2 g of extract. A part of extract (6.6 g) was fractionated by silica gel vacuum liquid chromatography (VLC) eluting by mixtures of hexane/acetone (from 95:5 to 0:100 (v/v)) and then MeOH to yield 8 fractions. A final step of pH 1 MeOH with acetic acid (glacial) allowed to obtain pure aurantioclavine (**3**). Fraction VLC5 (1.5 g) was purified by reversed-phase HPLC (column C₁₈ Interchim 5 μm, 250×10 mm), with a mobile phase of CH₃CN/H₂O 55:45 (v/v) at a flow rate of 2.5 mL/min to afford **2** (51.9 mg) and a subfraction (VLC5-2). Fraction VLC5-2 (980.1 mg) was subjected to an alkaloid extraction at pH 1. The alkaloid fraction VLC5-2-2 (142 mg) was purified by reversed-phase HPLC (column C₁₈ Interchim 5 μm, 250×10 mm, mobile phase CH₃CN/H₂O (0.1% acid formic) 55:45 (v/v), 2.5 mL/min) to afford **1** (38.2 mg). Fraction VLC6 (515.1 mg) was subjected to an alkaloid extraction at pH 1 and the alkaloid fraction VLC6-2 (132.6 mg) was then purified by reversed-phase HPLC in the same conditions to yield **1** (24.4 mg) and **2** (5.5 mg). Another part of fungal extract (4.6 g) was subjected to an alkaloid extraction at pH 1 to yield 0.87 g of an alkaloid fraction (EA1B2). One part of EA1B2 (0.48 g) was purified by reversed-phase HPLC (column C₁₈ Interchim 5 μm, 250×10 mm, mobile phase CH₃CN/H₂O 55:45 (v/v), flow rate of 2.5 mL/min) to afford **1** (20.7 mg) and **2** (10.9 mg). The rest of EA1B2 (0.39 g) was subjected to an alkaloid extraction at pH 1 to yield 2 subfractions (EA1B2-1 and EA1B2-2). Fraction EA1B2-1 (123.4 mg) was purified by reversed-phase HPLC (column C₁₈ Interchim 5 μm, 250×10 mm, mobile phase CH₃CN/H₂O 60:40 (v/v), flow rate of 2.5 mL/min) to afford **2** (2.8 mg). Fraction EA1B2-2 (156.5 mg) was also purified by reversed-phase HPLC (column C₁₈ Interchim 5 μm, 250×10 mm, mobile phase CH₃CN/H₂O 65:35 (v/v),

flow rate of 2.5 mL/min) to afford **1** (31.5 mg), **2** (11.1 mg) and **5** (2.5 mg).

Communesin M (5): light yellow amorphous powder; [α]_D¹⁸ +31 (c 0.12, CHCl₃); ¹H and ¹³C NMR data, see Table S2; UV (MeOH) λ_{max} 228 nm; HRESIMS *m/z* 471.2412 [M+H]⁺ (Δppm=3.4, calcd for C₂₈H₃₁N₄O₃⁺).

Preparation of 4–12: All details are provided in Supporting Information, together with corresponding spectroscopic data. Note that all DMV communesins **4**, **6**, **8**, **12** presented two rotamer forms (*cis* and *trans* R1 side chain, with the *cis* form favored in all cases) on NMR spectra acquired in CDCl₃ as already reported for compound **4**.^[3c]

Biological activities: The KB and MCF-7 cell lines were purchased from the European Collection of Animal Cell Cultures (ECACC). Cytotoxicity assays were performed using the MTT assay, as previously described.^[17] Stock solutions were prepared in EtOH at a concentration of 2.5 mg/mL. Each compound was tested in different concentrations in the range 1.4–56.7 μM. The absorbance at 570 and 630 nm was measured on a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices). Negative and positive controls were culture medium in 1% EtOH and docetaxel, respectively. Docetaxel showed IC₅₀ values of 38 nM and 10.3 nM against KB and MCF-7 cell lines respectively.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: communesins · marine-derived fungi · mass spectrometry · natural products · *Penicillium expansum* · semisynthesis

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