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Enzymatic bromination of marine fungal extracts for enhancement of chemical diversity

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ABSTRACT

This study reports for the first time the use of a vanadium chloroperoxidase (vCPO) enzyme to increase the chemical diversity of marine fungal extracts to generate new natural chemical entities. The vCPO used is a recombinant protein from the marine fungus *Hortaea werneckii* (*Hw*vCPO). It catalyzes the formation of hypohalous acid (HOX), a highly reactive compound that can react with electron-rich substrates. Here, four fungal extracts obtained from different marine fungal strains (*Penicillium expansum*, *Aspergillus pseudoglaucus*, *Trichoderma* sp. and *Hortaea werneckii*) were investigated for enhancement of their chemical diversity. The metabolomic study showed that the enzymatic treatment of extracts of *P.* expansum and *A. pseudoglaucus* significantly boosted the chemodiversity by increasing the number of halogenated molecules. Indeed, respectively 5.07 and 6.65 times more halogenated ions were detected in ESI-MS profile of the extracts compared to negative controls. The new chemistry generated allowed the identification of new brominated compounds, one of which was further purified and characterized as 12-bromocommunesin A (**2**). This new compound, in contrast to communesin A (**1**), exhibited moderate antimicrobial activity on the methillicin-resistant *Staphylococcus aureus* (IC_{50} of 62 μM). This study has clearly demonstrated the employment of the vCPO enzyme to be a promising and environmentally friendly strategy to enhance the chemical diversity of natural extracts.

1. Introduction

In February 2023, hundreds of thousands of molecules have been reported in the main natural product (NP) databases such as the naturaL prOducTs occUrrence databaSe (LOTUS, 276,518 NPs, https://lotus.naturalproducts.net/), the Dictionary of Natural Products (DNP 31.1, 324,967 NPs, https://dnp.chemnetbase.com/), the COlleCtion of Open Natural ProdUcTs (COCONUT, 407,270 NPs, https://coconut.naturalproducts.net/) or The Natural Products Atlas (33,372 NPs, https://www.npatlas.org/)(Rutz et al., 2021; Sorokina et al., 2021; van Santen et al., 2019). Entries in these databases are increasing over the time while new molecules are being discovered. Regarding Natural Products Atlas (NP Atlas), which describes molecules belonging to both fungal and bacterial reigns (with 20,304 (60.8 %) and 13,068 (39.2 %) molecules, respectively), 41.4 % of the described fungal molecules actually belong to the fungal genera *Aspergillus* (22.5 %) and *Penicillium* (18.9 %). Indeed, these two genera are the most studied worldwide because of their ubiquity, easy cultivability and adaptability to laboratory environment (Tsang et al., 2018). These two genera are of high importance

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in different fields, such as biotechnology or medicine (Fleming, 1929; Hussein, 2001; Maixner et al., 2021; Mapook et al., 2022). Therefore, major metabolites produced by these fungi are well-known and the description of new compounds therefrom has become challenging (Tsang et al., 2018). In order to prevent re-isolation of known NPs, modern analytical methods (such as High-Resolution Mass Spectrometry HRMS or Nuclear Magnetic Resonance NMR) are helpful for identification of known compounds in complex mixtures, the so-called dereplication process (El-Elimat et al., 2013). However, the unknown compounds often have low amounts in the extracts, hampering the efforts in their isolation for structure elucidation and biological testing. Chemical or biological modifications of crude natural extracts have been the subject of several studies to address this common issue in NP research (Ayelen Ramallo et al., 2019). Such strategies aim at increasing the chemodiversity of the processed natural extracts to allow the isolation of new bioactive hemisynthetic molecules based on naturally occurring scaffolds (Ayelen Ramallo et al., 2019). Regarding the larger chemical space occupied by natural products and their privileged interactions with biological targets, they are still considered as very interesting starting points for drug discovery (Newman and Cragg, 2020; Young et al., 2022). Thus, the value of diversifying the natural molecules in a natural extract becomes apparent.

Many strategies have been described in the literature for enhancement of chemodiversity of natural extracts from plants, bacteria or fungi. The "One Strain Many Compounds (OSMAC)" approach is used to increase chemodiversity of microorganisms by modifying the culture and/or environmental conditions (abiotic) to induce silent gene clusters and modulate metabolite expression (Romano et al., 2018). Another way to express silent gene clusters is the microbial co-cultivation which is a useful strategy to increase the production of communication and/or chemical defense metabolites (Arora et al., 2020). As mentioned before, chemicals can also be used to increase chemodiversity in extracts by using diverse reactions such as chemical bromination using Br_2 (García et al., 2016; Méndez et al., 2011) or using NaBr and H_2O_2 (Righi et al., 2019). Through this approach, Chemically Engineered Extracts (CEEs) are produced with potential novelty. In terms of biological strategies, biotransformation is also a tool which has been used to increase chemodiversity of plant extracts or sponge metabolites such as ilimaquinone. In this case, the extract (or the metabolite) is modified by an organism using its own enzymatic machinery (Boufridi et al., 2016; Dong et al., 2017; Jeong et al., 2020; Ju et al., 2020). In the same manner, the chemical composition of a natural extract can be modified by using purified enzymes (Bouhlel et al., 2012). Some studies in the literature combined several of the previously described methods to obtain new molecules of natural origin. Wu et al. described an efficient method combining microbial co-culture and biotransformation as an efficient pipeline for the discovery of novel fungal natural products (Wu et al., 2015).

In this study, we describe the efficient diversification of fungal extract metabolome by a recombinant vanadium chloroperoxidase obtained from the black yeast *Hortaea werneckii* (*Hwv*CPO), isolated from hydrothermal vents in the Atlantic Ridge (Burgaud et al., 2010; Cochereau et al., 2023). The protein has been overexpressed in *Escherichia coli* and belongs to the vanadium haloperoxidase family which catalyzes the production of hypohalous acid ($HO^{-}X^{+}$) in the presence of hydrogen peroxide. This hypohalous acid was described to diffuse to the proximal environment and then react with an electron-rich substrate to halogenate it in a non-specific manner, allowing multiple reactions at the same time (Agarwal et al., 2017). *Hwv*CPO possesses higher affinity for bromide (K_m = 26 μM) than chloride (K_m = 237 mM) (Cochereau et al., 2023), explaining why bromination of fungal extracts was here carried out.

Bromination as a NP enrichment method is consistent with usual strategies in medicinal chemistry. Indeed, halogenation is often used to modify drug candidates and thus, increase their absorption through biological membranes and potentially enhance their biological activities (Benedetto Tiz et al., 2022). In fact, halogens can have diverse impact on biological activity, e.g., increasing drug-target affinity by creating halogen-bonds (Xu et al., 2014). Halogen insertion can be also useful to optimize pharmacokinetics parameters of a molecule (Xu et al., 2014). Finally, halogen atoms can optimize or stabilize biomolecular conformations and mediation of protein–protein, protein–nucleic acid and receptor–ligand recognition by creating halogen-water-hydrogen bridges (Zhou et al., 2010). Furthermore, bromine atom insertion in metabolites has been described to readily modify and alter the physicochemical and antibacterial properties of tested molecules (Molchanova et al., 2020). Marine organisms are prolific producers of halogenated (especially brominated) compounds, due to high abundance of bromine in seawater (Cochereau et al., 2022; Gribble, 2010). Concerning fungi, approximatively 90% of the halogenated compounds described are bearing chlorine in their structure (Cochereau et al., 2022). This high rate is probably due to the many studies focusing on terrestrial derived strains instead of marine derived strains. Regarding the importance of natural products skeletons for drug discovery together with the importance of halogenation for medicinal chemistry, our strategy aims to diversify marine fungal extract metabolome by biohalogenation to enhance the discovery rate of new bioactive compounds.

The present study reports the bromination of complex extracts obtained from four marine fungal strains, namely *Penicillium expansum*, *Aspergillus Pseudoglaucus*, *Trichoderma* sp. and *Hortaea werneckii* using the recombinant *Hwv*CPO from *H. werneckii*. A metabolomics study highlighted the differential expression of halogenated metabolites in the different culture conditions and pinpointed the presence of a new brominated compound (**2**). The compound was isolated, then subjected to extensive structure elucidation and bioactivity assessment studies against multidrug-resistant human bacterial pathogens (ESKAPE pathogens) and pathogenic yeasts (*Candida* species).

2. Materials and methods

2.1. Fungal strains

Different fungal strains have been used for this experimentation. Three strains are coming from the MMS fungal collection: *Penicillium expansum* (MMS 42) isolated from marine sediments, Le Croisic, France; *Trichoderma* sp. (MMS 1255) isolated from marine sediments, Port-du-Bec, France and *Aspergillus pseudoglaucus* (MMS 1589) isolated from a culture of the microalgae *Prorocentrum lima* PL4V isolated in Galicia, Spain. One strain belongs to the UBO culture collection (UBOCC): *Hortaea werneckii* (UBOCC-A-208029,

Mo34, Hw) isolated from hydrothermal vents in the mid-Atlantic Ridge (Burgaud et al., 2010). Accession numbers for identification sequences for MMS 1255 available on GenBank are JQ653081.1, KU758964.1, MN450663.1, and MN428075.1 (van Bohemen et al., 2021). The ITS and β-tubulin sequences for MMS 1589 strain are available on GenBank under accession numbers MN134000.1 and MN164633.1, respectively (Berry et al., 2022). For MMS 42, accession numbers are JN794527 and JN794529 (Kerzaon, 2009). For Mo34, accession number is GU002279.1 (Burgaud et al., 2010).

2.2. Fungal cultures

The three stains from the MMS culture collection have been cultivated on a Potato Dextrose Agar medium (BD Difco™ Dehydrated Culture Media: Potato Dextrose Agar; 39 g/L) supplemented with 3% of artificial sea salt (Reef crystal™, Aquarium Systems) (abbreviated PDA3). The *Hortaea* werneckii strain which is a halotolerant strain has been cultivated on a PDA medium supplemented with 10% of artificial sea salts (abbreviated PDA10). All the strains have been cultivated for 14 days at 28 °C under natural light. Each strain has been cultivated in five replicates into Erlenmeyer flasks to obtain sufficient biomass.

2.3. Specialized metabolites extraction

Specialized metabolites of each strain were extracted two times using ethyl acetate (EtOAc). First, the culture media containing the strain was cut into thin pieces of culture media using a sterile scalpel. Then 100 mL of EtOAc were added to each individual culture Erlenmeyer. Then, the five Erlenmeyer flasks were pooled (for each strain). The total volume of EtOAc was 500 mL. Suspensions were treated 30 min in an ultrasonic bath to lyse the cells (S300 Elmasonic, Elma Schmidbauer GmbH, Germany). Then extractions were shaken for 1 h at 200 rpm. After 1 h, the solvent was filtrated on a classical filter paper. This first filtrate was again filtrated under vacuum using regenerated cellulose filters of 0.45 μM pore size (Sartorius Stedim Biotech GmbH, Germany). This first extraction was stored at 4 °C overnight. For the second extraction, 500 mL of EtOAc were added to the fungal biomass. Erlenmeyer flasks were shaken overnight at 200 rpm. Filtration steps were repeated following the same protocol as for the first extraction. First and second extractions were pooled and dried using a rotary evaporator. The dried extract was resuspended in a small amount of EtOAc, transferred to a tared flask, dried under nitrogen and stored at 4 °C before use. Medium control samples were prepared the same way from non-inoculated Erlenmeyer flasks for both PDA3 and PDA10 media.

For large scale purification of compounds produced by *P. expansum* (MMS 42), the fungal cultures were carried out in 39 Erlenmeyer flasks. Metabolites were extracted two times following the previous protocol. Organic phases were combined and dried using anhydrous sodium sulfate (Na_2SO_4) followed by the same filtration protocol to remove the solid matter. Finally, the organic solvents were evaporated to obtain the crude dried extract (1.067 g).

2.4. Halogenating enzyme

The *Hortaea werneckii* vanadium chloroperoxidase (*Hw*vCPO) is a recombinant enzyme produced by *E. Coli* transformed with the plasmid pQE81L + vHPO_Hw and pKJE7 (for chaperones expression) (GenBank ID: OP555106). The recombinant protein was purified using a Ni-NTA column followed by a gel filtration purification as previously described (Cochereau et al., 2023). The purified protein is composed of a mix of *Hw*vCPO and chaperone proteins and its concentration was estimated at 5 mg/mL. The enzyme was stored at - 20 °C before use. In addition to the biochemical characteristics and enzymatic constants already described (Cochereau et al., 2023), the enzyme was found stable for at least 79 h when stored at 4 °C or 20 °C (Fig. S1).

2.5. Bromination of the fungal extracts

For each fungal extract, the reaction with the enzyme was performed in triplicates in glass tubes with the following conditions. The reaction mixture was composed of acetate buffer 100 mM pH 7, 22 mM of KBr, 22 mM of H_2O_2, 100 μM of Na_3VO_4 and 20 μL of *Hw*vCPO (5 mg/mL). This mixture was added to the fungal extracts (12.33 mg for MMS 42, 8.76 mg for MMS 1255, 16.03 mg for MMS 1589, 12.23 mg for Mo34) and further completed with EtOH/H_2O (66:34 v/v) to reach a final volume of 2 mL and a final concentration of 50% ethanol. Tubes were incubated at 25 °C for 12 h at 175 rpm (overnight reaction to convert the maximum amount of extract). After 12 h, metabolites of each tube were extracted four times with 1 mL of CH_2Cl_2. Tubes were dried under nitrogen flow, weighted and stored at 4 °C before use. Medium control samples were treated the same way (on 5.75 mg of PDA3 and 1.34 mg of PDA10).

For control purposes, each fungal extract and medium control sample, was also treated in triplicates following the same conditions as before but without the enzyme. Finally, triplicates of each fungal extract and medium control samples were also resuspended in 2 mL of EtOH/H_2O (50:50 v/v) before being incubated, extracted and dried, for negative controls.

Following these experiments, 9 samples were then obtained for each fungal extract: 3 extracts treated with the enzyme (Enzyme-Treated Extracts or ETE), 3 extracts treated with the reaction medium but without the enzyme (Reaction Medium Treated Extracts or RMTE) and 3 untreated extracts as negative controls (or UE). For medium control samples, a total of 18 samples were obtained in the same way (9 samples for PDA3 and 9 samples for PDA10).

2.6. HPLC-HRMS analysis

Metabolic profiles were obtained using a 1200 infinity series® HPLC-system (Agilent Technologies, Santa Clara, CA, USA) coupled to an Exactive Orbitrap® MS (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray (H-ESI II) ion source (operating in polarity switch mode). The full instrument calibration was performed using a MSCAL6 ProteoMassT LTQ/FT-Hybrid ESI Pos/Neg® (Sigma–Aldrich, St. Louis, MO, USA). The software Xcalibur 2.2® (Thermo Fisher Scientific, San Jose, CA, USA) was used for data acquisition and analysis. Sample were prepared at 1 mg/mL in MeOH and 10 μL were injected on a Kinetex C18 column

(100 × 2.1 mm, 2.6 μm), heated at 40 °C. The gradient used for the HPLC was as follows: 0–2 min at 15 % of B, 2–25 min from 15 % to 100 % of B, 25–30 min at 100 % of B, 30–31 min from 100 % to 15 % of B and 31–35 min at 15 % of B (A: water + 0.1 % formic acid and B: acetonitrile + 0.1 % formic acid). All the solvents used were of ULC/MS grade (Biosolve BV, France). The MS source parameters were: spray voltage at 3.0 kV, capillary temperature at 350 °C, sheath gas flow rate at 55 (arbitrary units), auxiliary gas flow rate at 10 (arbitrary units), max spray current at 100.0 (μA) and heater temperature at 300 °C. A quality control sample was prepared combining 5 μL of each sample and injected every 10 samples to check instrument deviation along time and to assess the quality of the analyses during the sequence.

2.7. Data treatment

First, the files (*.raw) obtained from the HPLC-HRMS Exactive Orbitrap MS were converted in *.mzXML fromat in centroid mode by msconvert software to be further treated (Chambers et al., 2012). The output format was *.mzXML with a binary encoding precision of 64-bits. Peak Picking window was used to convert profile mode to centroid mode with the CWT algorithm (snr = 0.1 peakSpace = 0.1 msLevel = 1-). This operation was carried out separately for the two scan polarities: positive and negative. Then, the software Mzmine v2.53 was used to obtain the peak lists of each sample with specific parameters (Tables S1–S2, supplementary information). For each peak list, adducts, complexes and peaks coming from the culture media were deleted using a custom database created with the peaks from medium control samples triplicates. The number of features (*m/z*-RT) obtained for MMS 42 was 1,043 and 2,894 in ESI^+ and in ESI^-, respectively. For MMS 1589 2,610 and 1,235 features were obtained in ESI^+ and in ESI^-, respectively. For MMS 1255, 3,316 and 2,018 features were obtained in ESI^+ and in ESI^-, respectively. Finally, for Mo34, 4,487 and 4,490 features were obtained in ESI^+ and in ESI^-, respectively.

2.8. Statistical data treatments

Peak lists obtained with Mzmine 2.53 were exported in MetaboAnalyst readable files using Mzmine 2.53. Then, the online website MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) was used to carry out statistical analyses (Pang et al., 2022). Statistical Analysis (one factor) was chosen and the data were uploaded with data type set as "peak intensities" and the format as "samples in columns unpaired". No data filtering was performed (number of features under 5,000). Samples were normalized by the sum; and no further data transformation was applied. Data scaling was performed by Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable). Then, different univariate and multivariate analyses were conducted. Heat-maps were obtained by restraining to the most significant features with the "Use top ANOVA" option and with an adjusted p-value cutoff at 0.01 by using one-way ANOVA and post-hoc analysis: Fisher's LSD. Then, preset values were used to create the heat-maps except for the distance measure which was set to "Pearson" and the clustering method to "Complete". PCA score plots and loading plots were obtained by using preset values.

2.9. Halogenated features detection

To specifically detect potential halogenated features in the samples, the R script MeHaloCoA was used (Roullier et al., 2016). For the first step, the "matched filter" algorithm was used with the following parameters: peak fwmh set at 12, S/N ratio at 3, m/z diff at 0.005, maxnbofEIC at 50, Step at 0.1, Stepsat 2, profmethod at binlinbase, perfwhmat 1 and scan range being NULL. The second step, which actually detects specific halogenated (chlorinated and brominated) isotopic patterns, was carried out with the following parameters: m1 set at 0.003, m2 at 1.997, Tresh at 20, plotps at FALSE, mdiff at 0.005, ppmer at 16.5, m1need at FALSE, val at maxo, mdiff2 at 0.001 and deprof at FALSE. Potentially detected halogenated ions were then available in a *.csv file to be analyzed. Ions detected as brominated with high value of intensity were selected to search for potential non-halogenated precursors in the negative controls (UE). Monoisotopic *m/z* values for features of potential non-halogenated precursors were calculated with the following equation: M – 78.9183 (with M being the monoisotopic *m/z* value of the detected halogenated feature). The retention times (T_R) of the features matching the *m/z* values calculated were then checked because brominated features are supposed to be more lipophilic, and should then present a higher value of retention time. Only consistent features of potential non-halogenated precursors (with lower T_R) were conserved for interpretation. One-way ANOVA has been used to highlight significant differences in the number of halogenated features detected between the tested conditions. Student test has been used to highlight significant differences between two groups.

2.10. Annotation of compounds

Monoisotopic masses of compounds were calculated for all features of interest (halogenated and non-halogenated precursors) and compared with the "Natural Products Atlas" database to annotate already known features (van Santen et al., 2019). To identify putative non-halogenated precursors, the bromine atom (monoisotopic mass: 78.91834) was replaced by a hydrogen atom (monoisotopic mass: 1.00783) in the mass calculations.

2.11. Purification of compounds

Communesin A was purified from the large-scale extract of *P. expansum* MMS 42. A first step of alkaloidic extraction was performed. The extract was dissolved in 100 mL of EtOAc and extracted 3 times with 100 mL of H_2SO_4 solution (pH 1). The combined aqueous phases were alkalized up to pH 10 using 5 M NaOH solution. Then the aqueous fraction was extracted three times with 200 mL of CH_2Cl_2. The combined organic phases were dried using anhydrous Na_2SO_4, and concentrated using a rotary evaporator. The alkaloidic fraction weight was 249.3 mg.

Then, communesin A was purified by HPLC on a Luna $C_{18}(2)$ column (5 μm, 100 Å, 250 x 4.6 mm from Phenomenex, Torrance, California, USA). An isocratic elution was performed with CH_3CN/H_2O (60:40 v/v) for 60 min at a flow rate of 1 mL/min. Retention

time for communesin A was 27.922 min. An amount of 3.5 mg of communesin A was obtained and were added to the communesin A already purified in our in-house chemical library (Hoang et al., 2023; Kerzaon, 2009). The structure was confirmed by similar retention times and MS data, as well as consistent NMR data in comparison to the literature (Hoang et al., 2023; Lin et al., 2015; Numata et al., 1993) (Figs. S5 and S7). The total amount of communesin A available was 7.8 mg.

2.12. Bromination of communesin A using HwvCPO

The biocatalytic reaction was performed in a glass tube on 4.1 mg of communesin A in a 1 mL solution containing 100 mM acetate buffer pH 7, 2.2 mM of communesin A, 22 mM H_2O_2, 22 mM KBr, 100 μM Na_3VO_4 and 12.22 μL of *Hwv*CPO. All the components were diluted in CH_3CN/H_2O 50:50 (v/v). The final concentration of CH_3CN in the tube was 36.22%. The reaction was carried out for 4 h at 25 °C and 150 rpm planar rotary shacking. Then, the reactional media was extracted four times with 1 mL of ethyl acetate. The extract was dried under nitrogen and stored at 4 °C before analysis and purification. Bromo-communesin A was purified by HPLC on a Luna C_{18}(2) column (5 μm, 100 Å, 250 x 4.6 mm from Phenomenex, Torrance, California, USA). An isocratic elution was performed with CH_3CN/H_2O (43:57 v/v) for 60 min at a flow rate of 1 mL/min. Retention time for bromo-communesin A was 38.5 min, allowing to obtain of 1.4 mg of pure bromo-communesin A.

2.13. NMR analysis

NMR spectra were acquired at 293 K on a 700 MHz spectrometer equipped with a QCI cryo-probe ($^1H/^{13}C/^{15}N/^{19}F$) (Avance HD 700 MHz Bruker, Billerica, Massachusetts, États-Unis). The samples were prepared as follows: 0.65 mL of CD_2Cl_2 was added to the dried sample and then transferred to an NMR tube (a single-use tube specially designed for the SampleJet autosampler). The following 1D and 2D NMR analyses were performed: ^1H, ^{13}C, COSY and HSQC. ^1H analyses were systematically performed at the end of the ^{13}C analyses to check that the structure of the molecule remained intact throughout the experiments. The MestReNova v14.2 software was used to analyse the spectra (Willcott, 2009).

2.14. Biological assays

Both fungal extracts and pure compounds were evaluated for their antibacterial activities against the drug resistant ESKAPE panel consisting of six bacterial human pathogens (*Enterococcus faecium*, DSM 20477; methicillin-resistant *Staphylococcus aureus*, DSM 18827; *Klebsiella pneumoniae*, DSM 30104, *Acinetobacter baumannii*, DSM 30007; *Pseudomonas aeruginosa*, DSM 1128 and *Escherichia coli*, DSM 1576) and against two human pathogenic yeasts, *i.e., Candida glabrata* DSM 6425 and *Candida albicans* DSM 1386. The assays were performed by the broth dilution approach in 96-well microtiter plates as previously described (Magot et al., 2023; Oppong-Danquah et al., 2023). Bioactivity testing was carried out at effective test concentration of 100 μg/mL from crude extracts prepared at a concentration of 20 mg/mL in DMSO (Carl Roth), while the highest test concentration for pure compound **2** was 50 μg/mL. The positive controls used were ampicillin (*E. faecium)*, chloramphenicol (*S. aureus, K. pneumonia* and *E. coli*), doxycycline (*A. baumannii*), polymyxin B (*P. aeruginosa)* and nystatin (*C. glabrata* and *C. albicans*). Cultivation media and 0.5% DMSO were tested as negative controls. IC_{50} values were further calculated by Excel, for extracts that showed percentage inhibition of > 50% at the initial test concentration.

3. Results

3.1. Enzymatic bromination to modify extract compositions

Following the treatment of fungal extracts with *Hwv*CPO, initial principal component analysis revealed consistency in the data as replicates clustered together on score plots and no outlier could be identified allowing further interpretation (only one sample was deleted for MMS 1255 because it was too different from the others, being very similar to a blank) (Fig. S2). PCA score plots along principal components 1 and 2 (which explained more than 70% variability in most graphs) then showed systematic separation between negative controls (UE) and enzymatically treated extracts, revealing different compositions (Fig. 1, Fig. S2). In fact, whatever the extract or the ionization mode investigated, different metabolic profiles were observed. However, some extracts submitted to the reaction medium without the enzyme (such as for *Trichoderma* sp. MMS 1255 and *H. werneckii* Mo34), occurred to be very similar to enzymatically treated counterparts, meaning the differences were mainly due to the reactants used, probably driven by the high reactivity of hydrogen peroxide (Fig. S2).

The most consistent results were obtained for MMS 42 *P. expansum* and MMS 1589 *A. pseudoglaucus* extracts. Indeed, for these two strains, the ETE group (green dots on Fig. 1) is well separated on the PCA from the two control groups (RMTE and UE) (blue and red dots on Fig. 1). This observation is valid for both positive and negative ionization modes. The enzymatic treatment seemed to induce a change in the composition of both fungal extracts.

Then, looking more specifically to MS-detectable halogenated compounds (chlorinated and brominated), using the previously described automated tool "MeHaloCoA", a different number of putative halogenated molecules from one condition to another could be highlighted in the different strains (Roullier et al., 2016).

3.2. Bioinformatic detection of halogenated ions

In positive ionization mode, results for MMS 42 *P. expansum* extracts showed that halogenation with *Hwv*CPO was successfully conducted with 1.78 times more putative halogenated features detected. This result is significantly different from the control samples (Fig. 2). Similarly, a higher number of detected halogenated features was also obtained after enzymatic reaction of MMS 1589 *A. pseudoglaucus* extracts with 1.90 times more halogenated features detected, which is significantly different from the control samples

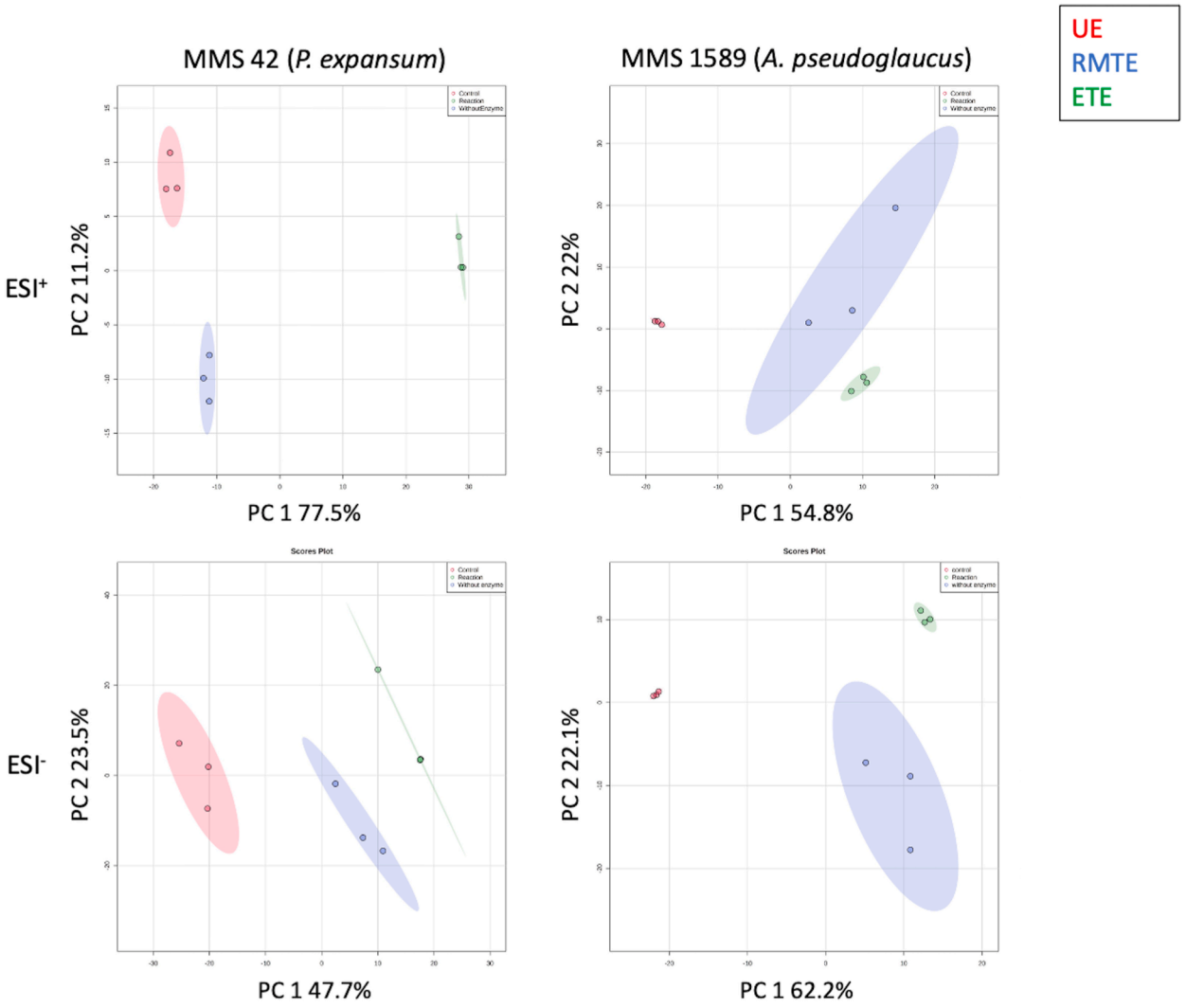

Fig. 1. Principal Component Analysis (PCA) score plots obtained with MetaboAnalyst for MMS 42 *P. expansum* extracts and MMS 1589 *A. pseudoglaucus* extracts, using LC-MS data matrices in positive ionization (ESI^+) and negative ionization (ESI^-). Plots were constructed along Principal Components (PC) 1 and 2 for each strain. Red dots represent UE samples (Untreated Extracts), blue dots represent RMTE (Reaction Medium Treated Extracts) samples and green dots represent ETE samples (Enzyme-Treated Extracts). Colored ellipses display 95 % confidence regions.

(Fig. 2). On the other hand, again, for MMS 1255 *Trichoderma* sp. and Mo34 *H. werneckii*, no significant change in the number of halogenated compounds was observed in positive ionization. Indeed, the number of halogenated features detected were in the same range (with 0.91 times less with a *p*-value: 0.103 and 0.92 times less with a *p*-value: 0.281, respectively). These results are consistent with the results of the PCA obtained in positive ionization (Fig. S2).

In negative ionization mode (ESI^-), the results again highlight more putative halogenated features detected after enzymatic reaction for MMS 42 *P. expansum* and MMS 1589 *A. pseudoglaucus* extracts compared to negative controls (with 5.07 and 6.65 times more halogenated features, respectively) (Fig. 3). Interestingly, 5.25 times more putative halogenated features were also detected for Mo34 *H. werneckii* extracts after enzymatic treatment compared to untreated extracts, which is significant. As it can be observed on Fig. S2 for Mo34, it seems that the PCA does not show any difference between ETE and RMTE groups. Here, it seems that a significant difference between these two groups could not be observed on the first two components of the PCA (Fig. 3). This could be due to the fact that the halogenated compounds detected through MeHaloCoA algorithm might be present in very small amounts and the scaling method (namely Pareto) used might have reduced their importance in the PCA model. Moreover, as only PC1 and PC2 were taken into account, this difference may be visible on further principal components. In fact, a slight separation of the groups was observed according to PC 2 and 5. Some brominated features such as *m/z* 377.1704 at 20.00 min and *m/z* 375.1549 at 18.70 min seemed to account in the loadings of this PCs (Fig. S3). On the opposite, concerning MMS 1255, after enzymatic reaction, it seems that the number of putatively halogenated molecules is significantly reduced compared to the control (p-value: 0.037).

After this enzymatic treatment, the antibacterial activity of the different samples was evaluated against the antibiotic resistant bacterial pathogens (ESKAPE) and against the pathogenic yeasts *C. albicans* and *C. glabrata*. No clear tendencies in the antimicrobial

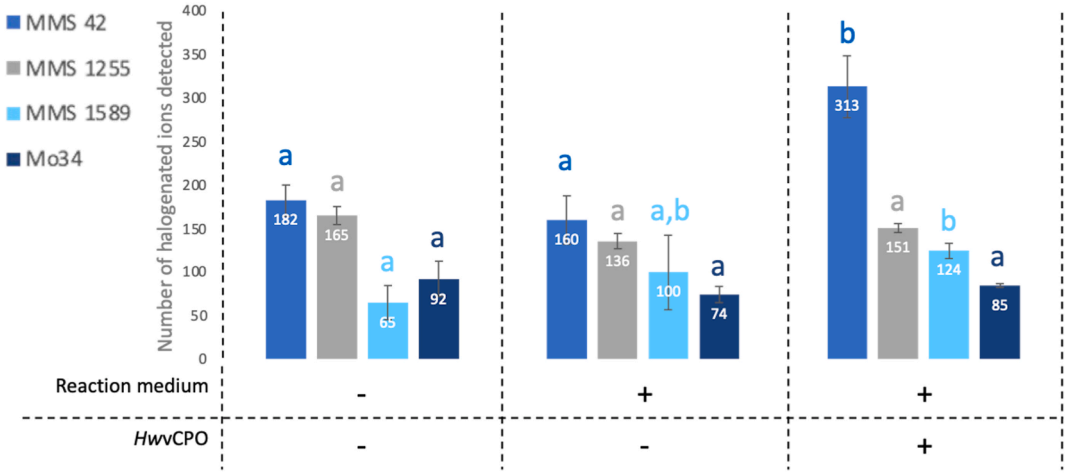

Fig. 2. Number of putative halogenated features detected using the R script "MeHaloCoA" in positive ionization (ESI^+) for each group of extracts from MMS 42 *Penicillium expansum* (blue), MMS 1255 *Trichoderma* sp. (grey), MMS 1589 *Aspergillus pseudoglaucus* (light blue) and Mo34 *Hortaea werneckii* (dark blue). For each strain, the means (average numbers of halogenated features) for each condition (untreated, reaction medium-treated or enzyme-treated) were compared two by two using a *t*-test and significantly different results (p-value < 0.05) were marked with different letters of the corresponding color. For each strain, if two conditions share the same letter (a, b, c), the results are not significantly different.

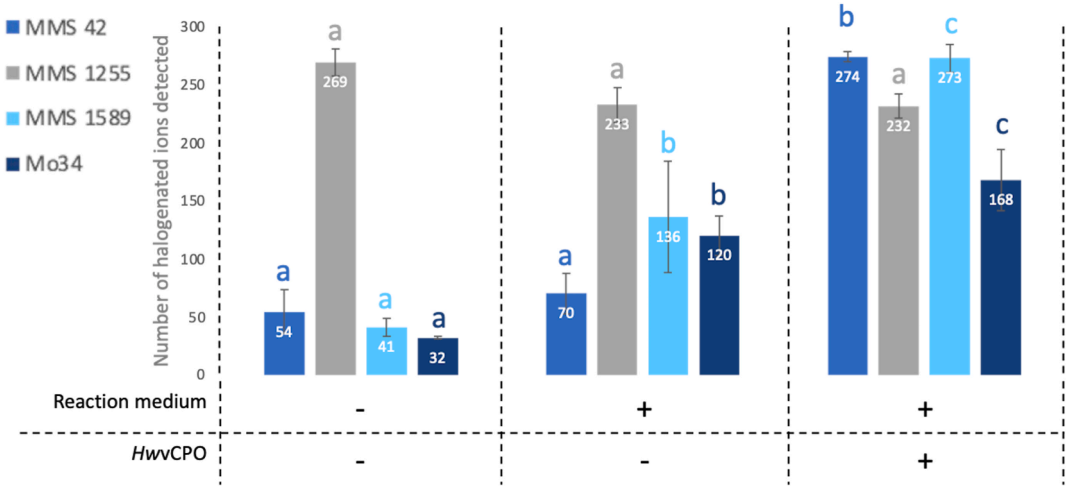

Fig. 3. Number of putative halogenated features detected using the R script "MeHaloCoA" in negative ionization (ESI^-) for each group of extracts from MMS 42 *Penicillium expansum* (blue), MMS 1255 *Trichoderma* sp. (grey), MMS 1589 *Aspergillus pseudoglaucus* (light blue) and Mo34 *Hortaea werneckii* (dark blue). For each strain, the means (average numbers of halogenated features) for each condition (untreated, reaction medium-treated or enzyme-treated) were compared two by two using a *t*-test and significantly different results (p-value < 0.05) were marked with different letters of the corresponding color. For each strain, if two conditions share the same letter (a, b, c), the results are not significantly different.

activity of halogenated and non-halogenated extracts was observed (Table S4). However, further chemical investigation was pursued as pure compounds are more interesting to study to obtain antimicrobial activities and describe new compounds.

3.3. Chemodiversity in *P. expansum* MMS 42 extract after enzymatic treatment

Regarding results obtained with MeHaloCoA on *P. expansum* MMS 42, features highlighted on the PCA loadings plot corresponding to brominated features were further investigated. Putative precursors of these features (with one or several bromine atoms less) were searched among untreated extracts and putatively annotated (Table 1). We chose to explore the results on MMS 42 in more detail as the results were the most significant (followed by those for MMS 1589).

Some of the ions presented in the table actually corresponded to the most intense peaks observed on chromatographic profiles of enzymatically treated extracts. For example, the annotated precursors communesin A (1), communesin B and the mycotoxin roquefortine C, which are naturally produced by our marine-derived stain of *P. expansum* as major metabolites seemed to be highly decreased in ETE while new peaks corresponding to putative brominated analogues appeared with significant intensity (Lin et al., 2015; Numata et al., 1993) (Fig. 4).

For example, the feature with m/z 457.2580 at 11.44 min, which corresponds to the alkaloid communesin A, is consumed in the ETE and a putative brominated derivative is appearing with a 535.1729 m/z at 14.10 min (+ 77.9149 m/z corresponding to a H sub-

Table 1
Proposed annotations for putative precursors calculated from the most intense brominated ions identified in MMS 42 extracts after enzymatic treatment (*Hwv*CPO) and highlighted with the R script "MeHaloCoA". Annotations were added using the database Natural Products Atlas.

m/z (Brominated ion)	T_R	ionization	Monoisotopic mass of the putative precursor	NP Atlas (Putative identification)
305.0038	1.1	ESI^-	228.0997	Cephalosporolide J, Decarestrictine E
314.9877	10.7	ESI^-	238.0836	46-dimethylcurvulinic acid, (+)-formylanserinone B, Sclerotinin B
317.0022	8.7	ESI^-	240.0981	Hydroxymethylanserinone B, Penicyclone A, Pyrenocine E
412.9869	16.6	ESI^+	334.0685	Brocaenol A, Lapidosin
430.1156	13.8	ESI^+	351.1972	Penicimutamide C, Deoxybrevianamide E
446.1433	16.0	ESI^+	367.2249	Brevicompanine B
446.1434	15.7	ESI^+	367.2250	
466.0901	14.6	ESI^-	389.1860	Epolactaene, Roquefortine C
466.0903	11.3	ESI^-	389.1862	
466.0903	14.1	ESI^-	389.1862	
468.1051	11.0	ESI^+	389.1867	
468.1053	11.3	ESI^+	389.1869	
468.1053	14.1	ESI^+	389.1869	
470.1204	9.7	ESI^+	391.2020	Roquefortine D
482.082	10.5	ESI^-	405.1779	(16R)-hydroxyroquefortine C, (16S)-hydroxyroquefortine C, 6-[5-(1-carboxy-4-N-
484.1020	10.5	ESI^+	405.1836	carboxylate)-3-methylpent-2-enyl]-7-hydroxy-5-methoxy-4-methylphthalan-1-one
484.1020	10.1	ESI^+	405.1836	
484.1021	10.9	ESI^+	405.1837	
521.1550	12.0	ESI^+	442.2366	Austalide X
535.1747	**14.1**	**ESI^+**	**456.2563**	**Communesin A**
549.1537	12.6	ESI^+	470.2353	Arigsugacin I, Arisugacin M, Commnesin 470, Pentacecilide C
549.1885	15.0	ESI^+	470.2701	Communesin G, Prugosene B1
587.2021	17.2	ESI^+	508.2837	Communesin B, Fellutanine C
591.2311	18.3	ESI^+	512.3127	Kadhenrischinin H
617.1732	15.5	ESI^-	540.2691	Fellutanine D

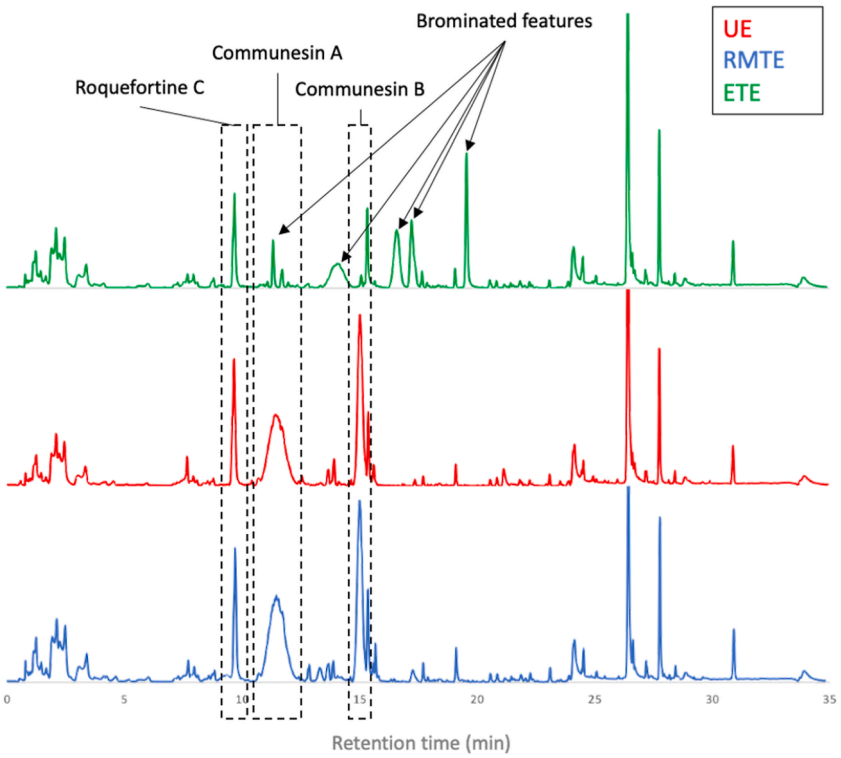

Fig. 4. Base peak chromatograms (HPLC-HRMS) of extracts originating from *P. expansum* MMS 42 strain in positive ionization (ESI^+). The results are shown for one replicate (1) of ETE (green), UE (red) and RMTE (blue).

stituted by a Br), possessing a typical mono-brominated isotopic profile (Fig. 5). Going further, a new peak appearing at 16.63 min with a 613.0824 *m/z* (+ 77.9095 *m/z* corresponding to another H substituted by a Br) could also correspond to the dibrominated version of communesin A. Consistently, this new peak possesses a typical isotopic profile for a dibrominated ion. The conversion of major peaks is also observed for communesin B and the mycotoxin roquefortine C with the production of putative brominated derivates.

To validate these hypothetic structures and determine the position of the bromine substitution, purification of the compounds was needed. Concerning the putative new bromo-communesin A (**2**), as purification couldn't be performed directly on treated extracts because of insufficient amounts (21.9 mg of extract), direct halogenation by *Hwv*CPO of the purified communesin A was then considered. After reaction, 1.2 mg of the new metabolite bromo-communesin A (**2**) was obtained. Comparative analyses of NMR data of communesin A (1) (Lin et al., 2015) and its bromo-derivative **2** allowed to place the bromine atom at C-12 (Fig. 6). Conversion rate for bromination of 1 mg of communesin A was 96 % according to the area under the curve obtained with a diode array detector (Fig. S4).

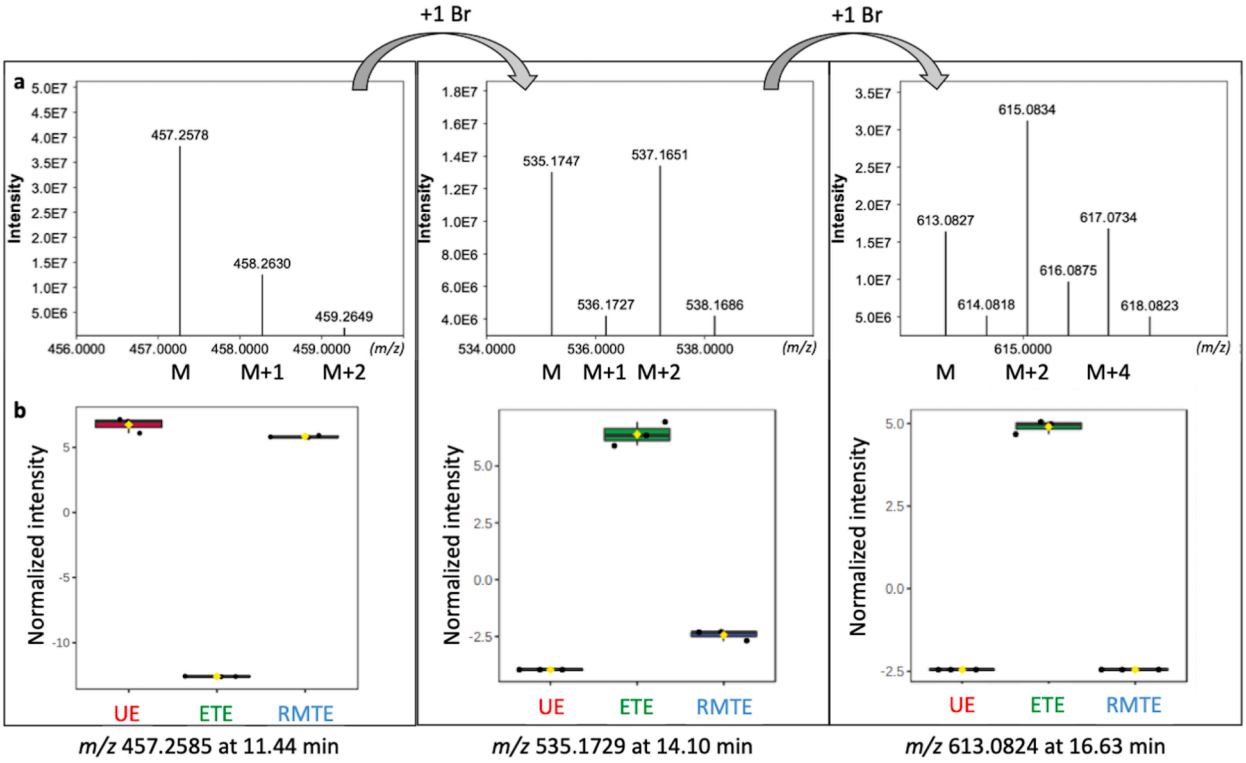

Fig. 5. Isotopic profiles of the three features *m/z* 457.2585 at 11.44 min, *m/z* 535.1729 at 14.10 min and *m/z* 613.0824 at 16.63 min obtained with Mzmine 2.53 in ESI^+. Box plots represent their original concentrations and normalized concentrations into each group UE, RMTE and ETE.

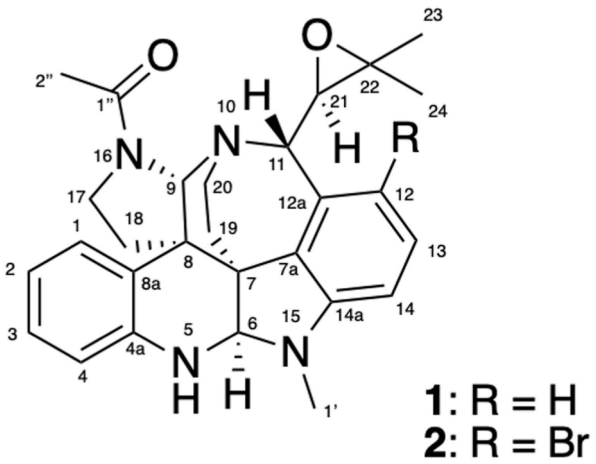

Fig. 6. Structures of communesin A (**1**) and 12-bromo-communesin A (**2**). Numbering of carbon is based on Lin et al. (Lin et al., 2015).

Indeed, the characteristic doublet of H-12 (δ_H 6.09) from communesin A (**1**) was absent on the new compound **2** and the related C-12 signal (δ_C 113.7 for 1 and δ_C 105.6 for 2) appeared more shielded and as a quaternary carbon, which is consistent with the substitution with a bromine in this position (Figs. S5–S8, Table S3). Analysis of 2D-NMR further confirmed the structure of compound **2** and allowed us to assign it as the new 12-bromo-communesin A (**2**). Retention times and MS spectra of both the compound highlighted after treatment of the crude extract of *P. expansum* MMS 42 and the newly formed compound **2** were identical allowing to confirm the nature of the compound produced initially from the extract (Fig. S9). Antimicrobial activity of 12-bromo-communesin A was assessed against *E. faecium*, methillicin resistant *S. aureus* and the two pathogenic yeasts *C. albicans* and *C. glabrata*. Compounds **1** and **2** were inactive against pathogenic yeasts. Interestingly, 12-bromo-communesin A (**2**) presented moderate activity against methicillin-resistant *Staphylococcus aureus* (MRSA) with an IC_{50} value of 62 μM (33 μg/mL), while communesin A was inactive, suggesting beneficial effect of the bromine. However, this activity is not very high, probably explaining why the extract containing this compound did not show any activity against MRSA (cf. MMS 42 *P. expansum* ETE, Table S4)

4. Discussion

The use of the newly described vHPO from *Hortaea werneckii* for biocatalysis is highlighted in this study. Indeed, vHPOs have already been used in biocatalysis because of their useful characteristics to perform unspecific halogenation of compounds (Höfler et al., 2019). vHPOs enzymes are described to be stable in organic solvents such as acetonitrile, which was used here for the reaction on communesin A or in ethanol for the bromination of fungal extracts (Höfler et al., 2019). Keeping an aqueous part in the reactional media is important because enzymes are biological entities. However, mixtures with organic solvents can be used and, in this context, *Hwv*CPO already showed a suitable preservation of its enzymatic activity (Cochereau et al., 2023). Because the enzyme retains some activity in organic solvents, it increases the polarity range for compounds which can be halogenated. One other important characteristic of vHPOs for biocatalysis is that the vanadate cofactor is not degraded by the strong oxidant hydrogen peroxide, which is a powerful advantage compared to heme-dependent haloperoxidases, for which hydrogen peroxide can inactivate the heme cofactor (Conesa et al., 2002; Höfler et al., 2019).

We report here for the first time the biocatalysed bromination of complex matrices (marine fungal extracts), which are composed of a multitude of complex specialized metabolites. Regarding our results, it appeared that the success of this type of experimentation, which was clearly demonstrated for *P. expansum* MMS 42 and *A. pseudoglaucus* MMS 1589, depends on the molecular composition of each fungal extract. Indeed, vHPOs do not catalyze specific halogenation. They catalyze oxidation of halides to produce and release the corresponding hypohalous acid (Cochereau et al., 2022), which reactivity is mostly directed towards electron-rich substrates in theory (Gérard et al., 2023; Höfler et al., 2019). We can therefore hypothesize that the order of halogenation of the compounds in the extract may depend on their reactivity to halogenation by hypohalous acid. In this case, if the molecules produced by one strain are not enough reactive toward hypohalous acids, no bromination will be observed. It seemed to be the case for extracts from *Trichoderma* sp. MMS 1255. This strain was actually described to produce high amounts of peptaibols (a specific class of non-proteogenic peptides) and the structure of these compounds does not provide sufficient reactivity to hypobromous acid (van Bohemen et al., 2021).

The enzyme effect on the detection of new halogenated features in *H. werneckii* extracts was not as straightforward as for *P. expansum* MMS 42 or *P. pseudoglaucus* MMS 1589, either. Here, it is more difficult to raise similar hypotheses as for *Trichoderma* extracts as very few specialized metabolites are described from this yeast, with only seven compounds described in NP Atlas database in the Teratosphaeriaceae family and only one (hortein, not detected in our samples) in this species (Fig. S10). Nonetheless, in the negative mode, even though the percentage increase in halogenated features is weaker (up to 1.4 times more between RMTE and ETE) compared to MMS 42 (3.91 times more) and MMS 1589 (2 times more), in these three cases, the number of detected brominated features remains significantly higher compared to RMTE group (in ESI^-, Mo34 *p*-value: 0.027, MMS 42 *p*-value: 1.91E-05, MMS 1589 *p*-value: 4.34E-03). For the results obtained for the strains MMS 1255 and Mo34, it is also possible that the enzyme is inhibited due to compounds produced by these fungi and present in the extracts. In this case, the effects of the presence of the enzyme could be weak or null. If this were the case, it would be interesting to couple the biocatalytic approach with an OSMAC approach (Romano et al., 2018). Thus, the expression of some metabolites would be modulated by the culture medium. By repeating the biocatalysis step on extracts of the same strain grown on a different medium, the composition of which would be different, it might be possible to identify the metabolites responsible for this enzymatic inhibition (if the enzyme shows activity on one extract).

One important thing to notify is that the results presented for negative mode ESI-HRMS analyses can be skewed by the formation of chlorinated and brominated adducts in the MS source, leading to improper annotation as chlorinated or brominated features (de Laeter et al., 2003). However, bromine adducts are rarer compared to chlorine adducts. Therefore, the comparison of the treatments with enzyme and without enzyme (ETE and RMTE) provides more reliable data. Indeed, these conditions contain the same concentration of starting potassium bromide (22 mM). Considering these statements, it may be concluded that the results are still significant (*p*-value < 0.05, Fig. 3) after enzymatic treatments for the strains *P. expansum*, *A. pseudoglaucus* and *H. werneckii* (respectively 3.9, 2 and 1.4 times more putative halogenated compounds detected).

The enzyme *Hwv*CPO has been successfully used to increase chemodiversity in fungal extracts from MMS 42 and MMS 1589. Indeed, principal component analysis obtained for these two fungal extracts highlights different groups discriminated after each treatment. Treatment with reaction medium without *Hwv*CPO is enough to differentiate this group from the negative control group (Fig. 1). The changes induced by the treatment with the reaction medium (RMTE) may be attributed to the presence of hydrogen peroxide which is a strong oxidant. In samples being only treated with the reaction medium, brominated features can also be observed compared to the control extracts (Figs. 2 and 3). It can be assumed that oxidation of bromide by hydrogen peroxide complexed to vanadate might occur spontaneously leading to the formation of hypobromous acid which can then react with metabolites from the ex-

tract. Indeed, it has been described that some complexes of vanadium can mimic the reaction catalyzed by vHPOs but less efficiently (Mendoza et al., 2016; Saikia et al., 2016). This hypothesis can then explain the detection of brominated features in these extracts.

A total of 658 characteristic features are listed in Table S5 and represent the ions highlighted by the metabolomic study as being more significantly expressed in the ETE group for MMS 42 and MMS 1589 strains (p-value < 0.05). Among these features, 107 could be annotated thanks to the NP Atlas database using the filter of molecules from the Aspergillaceae family. It only represents 16 % of the listed compounds, highlighting potential new molecules to isolate and describe in future studies. Communesin A and B are the main compounds produced by the *P. expansum* marine derived strain MMS 42 as well as the terrestrial derived strain (Andersen et al., 2004). Detection of these compounds is no more observable after the enzymatic reaction with *Hwv*CPO (Fig. 4). Indeed, ions corresponding to the brominated forms of these two communesins appear after enzymatic treatment and correspond to important variables in projection (VIP) in partial least square discriminant analysis (PLS-DA), further performed to discriminate the enzymatic reaction group from the two other groups (Fig. 7). This shows how important the conversion rate was for these compounds, which is consistent with what was observed for the reaction on the MMS 42 extract, and allows to consider future investigations for biocatalysis of complex molecules of this type with *Hwv*CPO.

To isolate and describe 12-bromo-communesin A, it was not possible to purify it directly from the samples treated with *Hwv*CPO, as the amount of extract was insufficient. Isolation of communesin A (**1**) from another extract with enough mass was then carried out to use the purified molecule in an *in vitro* reaction with *Hwv*CPO and subsequently produce the brominated version of communesin A. In fact, even though a large-scale culture from *P. expansum* MMS 42 was obtained, its treatment by *Hwv*CPO would not have been possible because of the quantity of enzyme needed. Nevertheless, the strategy developed in the present study allows to produce a high number of new molecules in one pot, which identification is facilitated by the characteristic isotopic profile of brominated compounds. Whenever one of this generated molecule appears of interest (because of its novelty and/or activity), their access becomes easier as the reaction conditions have already been proven successful. Considering more specifically the bromination mechanism of communesin A, we can assume the enzyme released hypobromous acid (Br^+OH^-), as usually described for this type of enzyme (Agarwal et al., 2017), which then reacted with communesin A by electrophilic aromatic substitution to form the brominated analogue. The position 12 is in fact the most reactive one (most electrophilic), as revealed by theoretical electron-density calculations (Table S6).

Initial antimicrobial assays on 12-bromo-communesin A (**2**) showed its moderate inhibitory activity against MRSA (IC_{50} value of 62 μM), while the non-brominated version was inactive. Antimicrobial activity is mainly acquired because of the presence of a bromine atom in the structure. Indeed, bromine presence is often used to increase antimicrobial properties of natural compounds (Molchanova et al., 2020; Raimondi et al., 2006). However, we need to be careful on this assumption as for the fungal extracts, antimicrobial activities were variable.

Indeed, our results showed that bromination of the extracts did not always lead to better activities after halogenation. Sometimes, bromination even reduced the antimicrobial activity on the ESKAPE strains panel. Most of the activity was observed towards MRSA strain for all the extracts. For MMS 1589, 100 % of inhibition was observed on *E. faecium* after treatment of the extract with the reaction medium without enzyme (IC_{50} value of 39.7 μg/mL) (Table S4). This result is interesting because the control extract and the brominated extract do not display any activity against *E. faecium*. Major changes observed in the fungal extract treated without en-

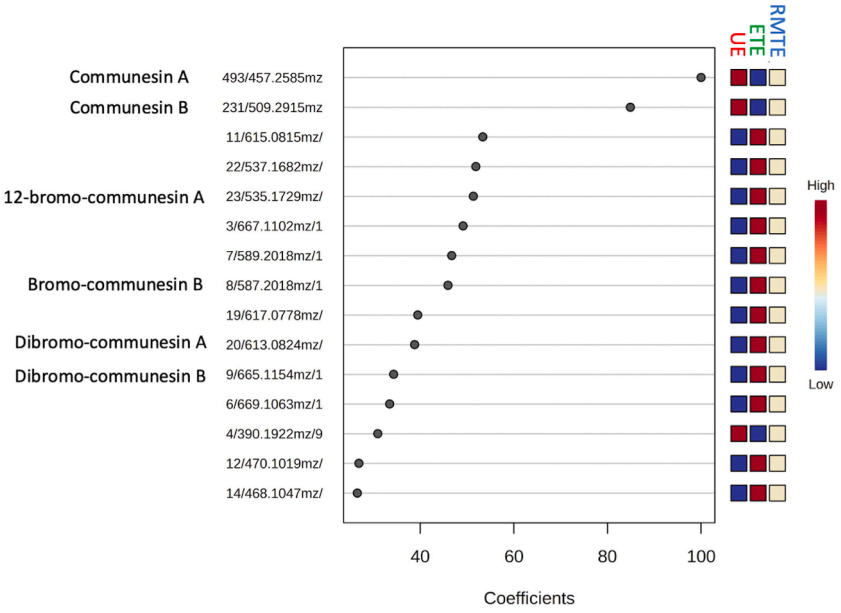

Fig. 7. Variable importance in projection (VIP) in PLS-DA obtained for MMS 42 in positive ionization ESI^+. Color intensity represents the relative concentrations of the corresponding metabolite in each group under study. Putative annotations have been added for brominated communesin A and B.

zyme is probably due to the presence of hydrogen peroxide which can cause oxidation of the compounds and modify their properties. Further analysis will be performed to identify compounds responsible of the antimicrobial observed with this "chemical" treatment without enzyme. The same extract is described to possess the best activity against MRSA with an IC_{50} of 7 μg/mL. Future investigation beyond the scope of this publication will focus on fractionation of this extract and target the active compound(s) for purification.

5. Conclusion

This article describes the successful use of the recombinant vanadium chloroperoxidase from *Hortaea werneckii* to increase chemodiversity in fungal extracts, a strategy never described before. The *Hw*vCPO emerged as a useful tool to modify and halogenate electron-rich specialized metabolites from fungal extracts. As highlighted by the statistical analyses, especially for *P. expansum* MMS 42 and *A. pseudoglaucus* MMS 1589 extracts, some features were specific to the ETE group and were not present in the control extracts (UE and RMTE). This strategy allowed to highlight and produce a new analogue of communesin A, namely 12-bromo-communesin A, with a good conversion rate at laboratory scale. With this study, we showed that bromination of extracts does not always modify their antimicrobial activity, which is consistent with the complex nature of extracts and of bacterial targets, which can undergo multiple mechanisms of action, with both synergistic and antagonist effects. However, this strategy allowed modifying the chemodiversity in most fungal extracts with the production of new compounds never described in databases so far. While this study more specifically focused on the formation of 12-bromo-communesin as a proof of concept, it paves the way to the production of many other potentially new halogenated natural compounds. In fact, further work is in progress to describe other new brominated compounds identified in this study. Interestingly, while the production of brominated metabolites is clearly dependent on the reactivity of the compounds present in fungal extracts, as weak bromination rate was observed when few electron-rich compounds were present in the extract, some spontaneous bromination were also observed with the reaction medium only (without the enzyme), which would need more study. Moreover, further investigations will also be considered to identify the features responsible for the variation of bioactivities with biochemometrics tools such as the R FiBiCo script and isolate the compounds of interest (Ory et al., 2019).

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Author contributions

Bastien Cochereau: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization; Thibaut Robiou du Pont: Resources; Yves-François Pouchus: Supervision, Funding acquisition; Deniz Tasdemir: Investigation, Writing - Review & Editing; Laurence Meslet-Cladière: Supervision, Investigation, Writing - Review & Editing; Catherine Roullier: Conceptualization, Methodology, Validation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2023.102786.

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