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## Pyrovobasine, hybrid alkylated pyrrolidine monoterpene indole alkaloid pseudodimer discovered using a combination of mass spectral and NMR-based machine learning annotations†

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A new vobasine–tryptamine-based monoterpene indole alkaloid pseudodimer was isolated from the stem bark of *Voacanga africana*. As a minor constituent occurring in a thoroughly investigated plant, this molecule was targeted based on a molecular networking strategy and a rational MS<sup>2</sup>-guided phytochemical investigation led to its isolation. Its structure was formally established based on HRMS, 1D/2D NMR data, and the application of the tool Small Molecule Accurate Recognition Technology (SMART 2.0). Its absolute configuration was assigned by the exciton chirality method and TD-DFT ECD calculations. Besides featuring an unprecedented intermonomeric linkage in the small group of vobasine/tryptamine hybrids, pyrovobasine also represents the first pyrrolidine-containing representative in the whole monoterpene indole alkaloids group. Biosynthetic hypotheses possibly underpinning these structural oddities are proposed here.

## Introduction

Fueled by the diverse ethnopharmacological claims related to *Voacanga africana* Stapf ex Scott-Elliot, decades of intensive phytochemical campaigns unveiled the array of specialized

products it shelters with a salient emphasis on its monoterpene indole alkaloid (MIA) content. Notwithstanding the long-held interest in *V. africana* chemistry, new appendages of MIAs are continuously being reported from this plant source.<sup>1–3</sup> The tremendous advances in MS<sup>2</sup>-based data processing strategies and in sensitivity of NMR techniques provide natural product chemists with ever-expanding capabilities for exploration of compounds occurring in minute amounts.<sup>4,5</sup> These refined analytical workflows pave the way for the re-investigation of deeply dug models using a state-of-the-art pipeline to gear the isolation efforts towards so-far overlooked chemical entities.<sup>6–12</sup> In the frame of our quest for new MIA-type specialized metabolites, and benefitting from our recent upload to the GNPS spectral libraries of the MIADB,<sup>13</sup> we embarked on a molecular-networking-based phytochemical investigation of *V. africana* stem bark. The observation of a molecular family clustered around a node tentatively tagged as ceridimine, a rare vobasine/tryptamine-type pseudodimer so far only known from *Pagiantha cerifera* (Pancher & Sebert) Markgr.,<sup>14</sup> sparked our interest and guided us to isolate the newly-reported pyrovobasine (**1**) following a MS<sup>2</sup>-streamlined isolation workflow. Besides representing the first instance of vobasine–tryptamine dimer featuring a C-3–C-2' interunit bonding,<sup>15,16</sup> pyrovobasine is also the first pyrrolidine-containing member in the huge phytochemical class of MIAs.

## Results and discussion

To reach a wide and untargeted insight into the indolomonoterpene alkaloid content of *V. africana*, both the alkaloid and crude ethanolic extracts of its stem bark were profiled by HPLC-HRMS<sup>2</sup> (positive polarity). These data were subsequently processed using the classical molecular-networking workflow<sup>17</sup> and dereplicated against the MIADB-implemented GNPS spectral libraries. A cautious examination of the obtained mole-

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† Electronic supplementary information (ESI) available: Cartesian coordinates, HRMS, and NMR data of **1**. See DOI: 10.1039/d1ob01791h

cular network (Fig. S1†) highlighted a molecular family consisting of four nodes (Fig. S2†) instigating a connection with a central node tentatively tagged as ceridimine. This constellation held a peculiar significance as only very few derivatives of ceridimine,<sup>14</sup> pertaining to the rare phytochemical class of vobasine–tryptamine pseudodimers, have been reported so far with none being consistent with the currently disclosed nominal masses. With this in mind, the LC-DAD-MS profile related to the alkaloidal extract of *V. africana* was monitored to check which of these compounds could be isolated in sufficient yield for full structure elucidation, leading us to guide our isolation efforts towards the ion detected at  $m/z$  605.303. Notwithstanding its relatively elevated molecular mass, the mass spectrum of this compound lacked the diagnostic isotopic pattern for doubly charged ion species encountered in most dimeric monoterpene indole alkaloids. This observation hinted that this molecule comprises a unique basic site, which strengthened our interest in isolating it. The molecular formula retrieved from this mass spectrometric signal,  $C_{37}H_{40}N_4O_4$ , returned no hit in the *Dictionary of Natural Products*,<sup>18</sup> deftly convincing us to undertake its MS<sup>2</sup>-streamlined isolation.

Compound **1** (Fig. 1) was isolated from the alkaloidic extract of *V. africana* stem bark by repetitive chromatographic separations following a MS<sup>2</sup>-guided phytochemical pipeline. Obtained as a brown amorphous solid, its molecular formula was confirmed to be  $C_{37}H_{40}N_4O_4$  based on the protonated molecular ion at  $m/z$  605.3122 (calcd for  $C_{37}H_{41}N_4O_4$ , 605.3122), indicating 20 degrees of hydrogen deficiency. The cursory examination of the NMR data of **1** revealed NMR landmarks evocative of a 3-vobasiny unit.<sup>19</sup> Accordingly, the <sup>1</sup>H and <sup>13</sup>C NMR data of **1** showcased the presence of four aromatic resonances, an upfield-shifted methyl ester group ( $\delta_H$  2.49/ $\delta_C$  169.2; 51.0), an *N*<sub>4</sub>-Me group ( $\delta_H$  2.89/ $\delta_C$  40.5) and an ethylidene side chain ( $\delta_H$  5.60/ $\delta_C$  126.6;  $\delta_H$  1.73/ $\delta_C$  13.0). Alongside the crosspeaks related to the contiguous aromatic hydrogens of the indole core, the COSY spectrum confirmed the occurrence of an isolated aminomethylene (C-21), of an ethylidene fragment, and of an extended CH<sub>2</sub>CHCHCH<sub>2</sub>CH spin system, displaying chemical shifts diagnostic of the C<sub>6</sub>–C<sub>5</sub>–C<sub>16</sub>–C<sub>15</sub>–C<sub>14</sub>–C<sub>3</sub> appendage of a vobasine-type MIA, further validated by the key HMBC crosspeaks outlined in Fig. 2B. The shielded chemical shift of the ester methyl linked to C-16 placed it within the shielding zone of the indole nucleus.<sup>6</sup>

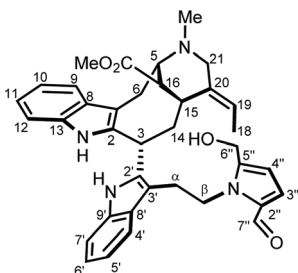


Fig. 1 Chemical structure of pyrrovobasine (**1**).

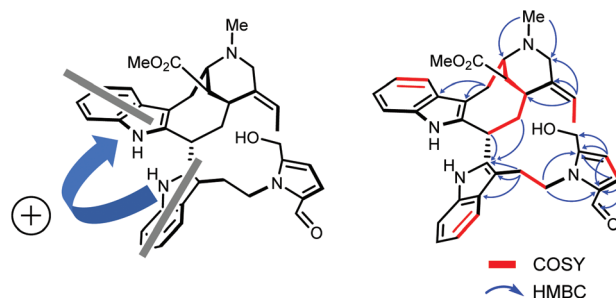


Fig. 2 (A) Two electronic dipoles denoting a positive chirality. (B) Selected 2D NMR correlations for **1**.

Consistent with the molecular network which determined **1** as a close derivative of ceridimine, the remaining signals hinted the occurrence of an additional free side chain-bearing tryptamine unit. Four additional resonances related to aromatic/olefinic protons substantiated this assumption, as well as the two vicinal methylenic protons that determined a CH<sub>2</sub>CH<sub>2</sub>N fragment. The structural elements elucidated so far determined **1** as a vobasine–tryptamine pseudodimer. This small phytochemical class is so far represented by three molecules, all obtained from an apocynaceous source: ceridimine (from *P. cerifera*),<sup>14</sup> demethylceridimine (from the by-then *Peschiera buchtienii* (H.J.P.Winkl.) Markgr.,<sup>20</sup> now known as *Tabernaemontana cymosa* Jacq.), and hunteriatryptamine (from *Hunteria zeylanica* (Retz.) Gardner ex Thwaites).<sup>21</sup> All these closely related molecules featured a linkage between the C-3 position of the vobasiny residue to the C-6' site of the tryptamine component. Regarding **1**, the HMBC correlations from the protons resonating at  $\delta_H$  2.02 (H-14), at  $\delta_H$  4.87 (H-3), and at  $\delta_H$  3.42 (H<sub>2</sub>- $\alpha$ ) to C-2' ( $\delta_C$  137.9) were diagnostic of a so-far unprecedented C-3–C-2' connection between the vobasine and tryptamine units. The  $\beta$ -orientation of H-3 was established based on the magnitude of the coupling constant values (dd,  $J$  = 13.4, 2.7 Hz), in excellent agreement with former literature reports.<sup>22</sup>

At this stage of the structure elucidation process, a few signal patterns were still pending assignment: two contiguous aromatic/olefinic protons disclosing an unusual coupling constant value of 3.9 Hz [ $\delta_H$  6.97 (1H, d,  $J$  = 3.9 Hz)/ $\delta_C$  124.3;  $\delta_H$  6.30 (1H, d,  $J$  = 3.9 Hz)/ $\delta_C$  110.7], a shielded aldehydic proton [ $\delta_H$  9.56 (1H, s)/ $\delta_C$  179.9], a set of oxygenated methylenic protons [ $\delta_H$  4.63 (1H, d,  $J$  = 13.5 Hz) and 4.77 (1H, d,  $J$  = 13.5 Hz)/ $\delta_C$  56.7], and finally two low-field shifted quaternary carbons ( $\delta_C$  141.9 and 132.9). To expedite the elucidation of this specific fragment, the HSQC data retrieved from it were collated in a .csv file prior to being uploaded into the SMART 2.0 (Small Molecule Accurate Recognition Technology) platform. This artificial intelligence-based tool is used to generate structure hypotheses from HSQC data through a deep convolutional neural network architecture that includes the <sup>1</sup>H–<sup>13</sup>C HSQC spectra related to more than 53 000 natural products (encompassing approximately 15% of the known natural products as of 2020).<sup>23,24</sup> SMART analysis revealed 5-(hydroxy-

methyl)pyrrole-2-carbaldehyde, or pyrraline, to be the preferred hit (cosine value  $\approx 0.97$ ), guiding us to consider it to account for the so far unassigned structural elements. Accordingly, SMART assistance tended to elucidate the structure of **1** as indicated in Fig. 1, in good agreement with molecular formula requirements. The key HMBC correlations outlined in Fig. 2B confirmed this tentative structure as the nitrogen atom of the pyrrole nucleus was shown to correspond to that of the side chain of the tryptamine unit, as evidenced based on the HMBC correlations from the protons resonating at  $\delta_{\text{H}}$  4.84 ( $\text{H}_2\text{-}\beta$ ) to both C-2'' ( $\delta_{\text{C}}$  132.9) and C-5'' ( $\delta_{\text{C}}$  141.9). A rapid literature survey indicated that pyrraline-containing natural products display highly similar chemical shifts values, also for aromatic quaternaries left unconsidered throughout SMART analyses.<sup>25</sup> The absolute configuration of **1** was determined using the exciton chirality CD method.<sup>26</sup> The positive sign of the first Cotton effect [ $\lambda_{\text{max}}$  237 nm ( $\Delta\epsilon + 31$ )] and the negative sign of the second one [ $\lambda_{\text{max}}$  222 nm ( $\Delta\epsilon - 21$ )] (Fig. 3) indicated a right-handed screw between the two indole chromophores (Fig. 2A).<sup>27</sup> The good fit between the calculated ECD spectrum of the lowest-energy conformer of **1** and the experimental ECD spectrum confirmed the proposed absolute configuration (Fig. 3). These structural features allowed to elucidate the structure of **1**, namely pyrrovobasine, as shown in Fig. 1.

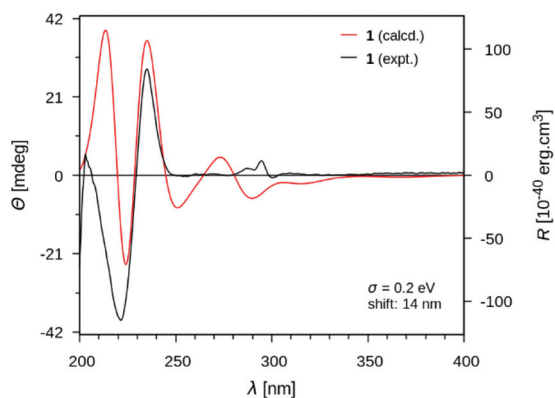


Fig. 3 ECD spectra of **1** (experimental and calculated at the B3LYP/6-31G\* level).

Pyrrovobasine features several structural oddities which are worth being stressed out. As already pinpointed, the nature of the connection between the tryptamine and the vobasine sub-units meets no precedents in this small group of pseudodimers. The electrophilic nature of vobasine C-3 (Fig. 4) is illustrious and, as such, dimeric MIA structures disclosing a 3-vobasiny residue following its attack by an electron-rich center are of considerable generality.<sup>15</sup> As per the tryptamine component, a literature survey indicates that an array of different substituents can be encountered at a tryptaminic C-2 position such as prenyl groups,<sup>28</sup> bromine atoms,<sup>29</sup> methyl groups,<sup>30</sup> a beta-carboline,<sup>31</sup> or may even lead to disulfide-

Table 1  $^1\text{H}$  and  $^{13}\text{C}$  NMR Data for **1** ( $\text{CDCl}_3$ , 600/150 MHz)

No.	$\delta_{\text{H}}$ , mult ( $J$ , Hz)	$\delta_{\text{C}}$	No.	$\delta_{\text{H}}$ , mult ( $J$ , Hz)	$\delta_{\text{C}}$
1 (NH)	8.24, br s		1' (NH)	8.30, br s	
2		135.8			
3	4.87, dd (13.4, 2.7)	35.8	2'		137.9
5	4.42, t (8.5)	60.7	3'		107.6
6	3.63, m	21.5	4'	7.72, m	118.9
	3.74, m				
			5'	7.08, ov.	120.0
7		106.8			
8		129.1	6'	7.08, ov.	122.9
9	7.55, d (7.7)	117.7			
10	7.11, ov.	120.0	7'	7.16, ov.	110.8
11	7.15, ov.	122.9	8'		128.4
12	7.16, ov.	110.8	9'		135.8
13		136.4	$\alpha$	3.42, m	26.7
14	2.02, ddd (15.0, 6.7, 3.1)	35.2	$\beta$	4.67, ov.	46.9
	2.75, q (13.4)			4.84, dd (13.7, 6.7)	
15	3.89, m	32.5	2''		132.9
16	2.99, br s	43.8	3''	6.97, d (3.9)	124.3
			4''	6.30, d (3.9)	110.7
18	1.73, d (6.9)	13.0	5''		141.9
			6''	4.63, d (13.5)	56.7
19	5.60, q (6.9)	126.6		4.67, d (13.5)	
			7''	9.56, s	179.9
20		132.9			
21	4.23, d (13.7)	51.4			
	3.45, ov.				
		169.2			
	COOMe	169.2			
	COOMe	51.0			
	N-Me	40.5			

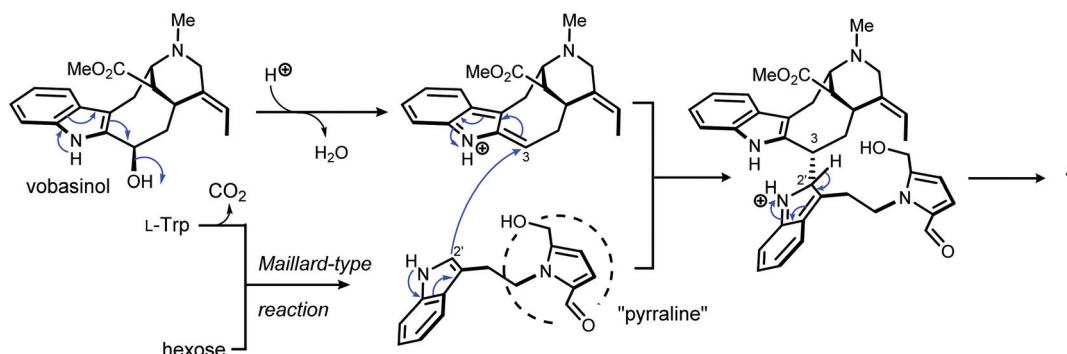


Fig. 4 Possible biosynthetic logic of pyrrovobasine (**1**).

bonded dimeric structures.<sup>32</sup> The depiction of the resonance contributors related to the indolic system reveals that all non-bridgehead carbons can serve as functional carbanion equivalents.<sup>33,34</sup>

At last, 5-hydroxymethylpyrrole-2-carbaldehyde, sometimes referred to as pyrraline, is known from a wealth of natural sources, but also from cooked foods.<sup>35</sup> No enzymatic biosynthetic pathway to this nucleus has been proposed so far and it is rather believed that this ring system arises by condensation of amines and sugars, following the so-called Maillard reaction (Fig. 4).<sup>36</sup> Consistent with this viewpoint, 2-formylpyrrole-containing natural substances are ubiquitous in nature and these molecules – recently reviewed by Brimble *et al.* are classified based on the nature of their amine component, falling into three major types: amino acids, biogenic amines or amino sugars.<sup>35</sup> Tryptamine-based pyrrole alkaloids have already been reported from macromycetes in particular.<sup>37,38</sup> Yet, to the best of our knowledge, pyrrovobasine represents the first example of pyrraline-containing monoterpene indole alkaloid.

The *in vitro* antibacterial activities of compound **1** were evaluated against *Mycobacterium smegmatis*, *M. abscessus*, *M. bovis* BCG, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The results showed that pyrrovobasine (**1**) exerts no antibacterial activity (MIC > 50  $\mu\text{g mL}^{-1}$ ).

## Conclusions

In summary, we have described the first member of the pyrrovobasine family of natural products from the stem bark of *V. africana*, representing the first example of a pyrraline-containing monoterpene indole alkaloid. The isolation process was enabled by exploiting a molecular networking approach and the structure elucidation was assisted by an NMR-based machine learning tool.

## Experimental section

### General experimental procedures

Optical rotations were measured at 25 °C on a Polarar 32 polarimeter. UV spectra were recorded on a Lightwave II + WPA 7126 V. 1.6.1 spectrophotometer. ECD spectra were measured at 25 °C on a JASCO J-810 spectropolarimeter. IR spectra were recorded with a PerkinElmer type 257 spectrometer. The NMR spectra were recorded on a Bruker AM-600 (600 MHz) NMR spectrometer equipped with a TCI 5 mm cryoprobe using  $\text{CDCl}_3$  as a solvent. The solvent signal was used as a reference. Sunfire preparative  $\text{C}_{18}$  columns (150 × 19 mm, i.d. 5  $\mu\text{m}$  and 150 × 30 mm, i.d. 5  $\mu\text{m}$ ; Waters) were used for preparative HPLC separations using a Waters Delta Prep equipped with a binary pump (Waters 2525) and a UV-visible diode array detector (190–600 nm, Waters 2996). Silica 330 g, 120 g, and 24 g Grace cartridges were used for flash chromatography using an Armen Instrument spot liquid chromatography flash appar-

atus. Chemicals and solvents were purchased from Sigma-Aldrich.

### Plant material

The fresh bark of *V. africana* (Yaounde National Herbarium voucher specimen No. HNY/1949; Victor Nana (collector)) was collected from the campus of the University of Yaounde I, Cameroon in December 2019.

### Extraction and isolation

The air-dried and powdered bark of *V. africana* (2.3 kg) was extracted three times with MeOH at room temperature. The resulting extract was concentrated under reduced pressure to obtain a crude extract (231 g). The obtained extract was dissolved in the mixture MeOH–H<sub>2</sub>O 10% and alkalized with NH<sub>3</sub> (pH = 12) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 1.5 L, 1 h each, 20 °C, atmospheric pressure). The mixture was concentrated under vacuum at 38 °C to yield 50.5 g of an alkaloid extract. A part of this residue (VADCM, 25 g) was subjected to flash chromatography using a silica 330 g Grace cartridge with a gradient of CH<sub>2</sub>Cl<sub>2</sub>–MeOH (100:0 to 0:100) at 80 mL min<sup>-1</sup> to afford fifteen fractions, VA1–VA15, according to their TLC profiles. Fractions VA4–VA6 were gathered (14.7 g) and were subjected to a second flash chromatography using a silica 120 g Grace cartridge with a gradient of CH<sub>2</sub>Cl<sub>2</sub>–MeOH (100:0 to 0:100) at 80 mL min<sup>-1</sup> to afford thirteen fractions VAB1–VAB13. Among them, VAB6 (1.7 g) was selected for further fractionation and submitted to Sephadex LH20 and eluted with a mixture of MeOH:CH<sub>2</sub>Cl<sub>2</sub> (8:2) to afford fifteen fractions VAB61–VAB615. VAB67 (761 mg) was submitted again to Sephadex LH-20 (4 cm × 90 cm) and eluted with a mixture of MeOH:CH<sub>2</sub>Cl<sub>2</sub> (8:2) to afford thirteen fractions VAB671–VAB6713. VAB6713 underwent a preparative HPLC separation using a gradient of MeOH–H<sub>2</sub>O with 0.1% formic acid (grad 10–80% in 25 min) to give compound **1** (3.2 mg, yield 0.0005%).

### Physical and spectroscopic data of **1**

**Pyrrovobasine (1).** Brown amorphous solid;  $[\alpha]_{\text{D}}^{25.0} + 160$  (*c* 0.05, MeOH); IR  $\nu_{\text{max}}$  3400, 1720  $\text{cm}^{-1}$ ; UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 232 (2.42), 296.1 (3.3) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; HR-ESI-MS *m/z* 605.3122 [*M* + H]<sup>+</sup> (calcd for C<sub>37</sub>H<sub>41</sub>N<sub>4</sub>O<sub>4</sub>, 605.3122); MS/MS spectrum was deposited in the GNPS spectral library under the identifier: CCMSLIB00006710432.

### Data dependent LC-HRMS<sup>2</sup> analyses

LC-ESI-HRMS<sup>2</sup> analyses were achieved by coupling the LC system to a hybrid quadrupole time of-flight mass spectrometer Agilent 6530 (Agilent Technologies, Massy, France) equipped with an ESI<sup>+</sup> source, operating in positive-ion mode. Source parameters were set as follows: capillary temperature at 320 °C, source voltage at 3500 V, sheath gas flow rate at 10 L min<sup>-1</sup>. The divert valve was set to waste for the first 3 min. MS scans were operated in full-scan mode from *m/z* 100 to 1700 (0.1 s scan time) with a mass resolution of 11 000 at *m/z* 922. MS<sup>1</sup> scan was followed by MS<sup>2</sup> scans of the three most intense

ions above an absolute threshold of 5000 counts. Selected parent ions were fragmented with on collision energie fixed at 50 eV and an isolation window of 1.3 amu. Calibration solution, containing two internal reference masses (purine, C<sub>5</sub>H<sub>4</sub>N<sub>4</sub>, *m/z* 121.050873, and HP-921 [hexakis-(1*H*,1*H*,3*H*-tetrafluoropentoxy)phosphazene], C<sub>18</sub>H<sub>18</sub>O<sub>6</sub>N<sub>3</sub>P<sub>3</sub>F<sub>24</sub>, *m/z* 922.0098). A permanent MS/MS exclusion list criterion was set to prevent oversampling of the internal calibrants. LC-UV and MS data acquisition and processing were performed using MassHunter Workstation software (Agilent Technologies, Massy, France).

### Molecular networking

The MS<sup>2</sup> data files related to the alkaloid extract of the barks of *V. africana* were converted from the .d (Agilent) standard data-format to .mzXML format using the MSConvert software, part of the ProteoWizard package.<sup>39</sup> A molecular network was created using the online Molecular Networking workflow (version release\_8) at GNPS<sup>17</sup> (<http://gnps.ucsd.edu>) with a parent mass tolerance of 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine score above 0.65 and more than 6 matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS spectral libraries. All matches kept between network spectra and library spectra were required to have a score above 0.6 and at least 6 matched peaks. The molecular networking data were analyzed and visualized using Cytoscape (ver. 3.6.0).<sup>40</sup>

### Computational methods

The lowest energy conformer of compound **1** was fully optimized *in vacuo* and without constraint using DFT<sup>41,42</sup> with the hybrid Becke-3-parameter-Lee-Yang-Parr<sup>43,44</sup> exchange–correlation functional and the 6-31G\* basis,<sup>45</sup> as implemented in the Gaussian 16 revB.01 software package.<sup>46</sup> Upon geometrical optimization convergence, a frequency calculation within the harmonic approximation was conducted at the same level of theory and conformer was characterized as a minimum by the absence of imaginary frequency. TDDFT at the same level of theory was then employed to predict energies as well as rotational strengths of the first 60 electronic transitions. The UCSF Chimera v1.11<sup>47</sup> software package was used for the depiction of the most stable conformer of compound **1**. ECD spectrum envelope was then calculated using SpecDis v1.71<sup>48</sup> using sigma/gamma value of 0.2 eV and a shift of +14 nm and rendered using Gnuplot v5.2.<sup>49</sup>

### Biological evaluation

**Antibacterial activity assay by microdilutions.** The antibacterial activity was assessed as previously described<sup>50</sup> against *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442), *Mycobacterium smegmatis* (CIP 7326) and *Mycobacterium abscessus* (*M. abscessus* subsp. *bolletii*, ITM-M001006, BCCM, Belgium). The assays were performed in 96-well plates using a broth microdilution method in order to identify the minimal inhibitory concentration (MIC). Two-fold

serial dilutions (100 μL in 200 μL final volume) of the molecules were first performed in triplicates in Mueller Hinton broth (or in the 7H9 medium when working with *M. abscessus*). Then, 100 μL bacteria (100 fold dilution of 0.5 Mc Farland) were added into each well. Negative (without bacteria) and positive (without additional compounds) controls were present in each plate. The MIC value were first interpreted after 24 h incubation at 37 °C (or 2–3 days incubation for *M. abscessus*), corresponding to the lowest concentration inhibiting bacteria growth after visual inspection. This visual interpretation was always verified by adding MTT reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, (0.5 mg mL<sup>-1</sup> final concentration) to allow formazan crystal formation in viable cells during 4 h additional incubation period. A final naked eye inspection was performed to record the results. This assay was performed in triplicate in three separate experiments. Positive control drugs were vancomycin hydrochloride for *Staphylococcus aureus*, ceftriaxime for *Pseudomonas aeruginosa*, and rifampicin for *Mycobacterium smegmatis*.

**Activity assay against *M. bovis* BCG.** To assess the antimycobacterial activity of our compounds on *M. bovis* BCG, the MIC was assessed by the microdilution method as previously described,<sup>51</sup> performed in 7H9 medium supplemented with 10% albumin-dextrose complex and 0.2% glycerol in a polycarbonate tube to reach a final volume of 1 ml. Briefly, 0.5 mL of 2-fold serial drug dilutions were inoculated with 0.5 mL (at 5 × 10<sup>5</sup> CFU per mL) early exponential culture. Vancomycin at a final concentration of 25 μg mL<sup>-1</sup> (far under its MIC > 500 mg mL<sup>-1</sup>) was eventually added to investigate the possible synergy between the two drugs. The MIC was interpreted as the lowest drug concentration that inhibited visible bacterial growth, defined by clump formation, when growth of the 1% inoculum drug-free control became visible (after 5–10 days growth).

The antimycobacterial activity was further assessed to investigate whether at the MIC, compounds could have an irreversible antimycobacterial activity (bactericidal activity) or a reversible effect (bacteriostatic activity) on *M. bovis* BCG. This was assessed by spreading 10 μL out of the 1 mL tubes from the broth microdilution method on 7H11 agar plate. Proliferation of bacteria colonies was interpreted as bacteria having encountered mycobacteriostatic compounds at their MIC, while absence of proliferation was interpreted as bacteria having encountered mycobactericidal compounds at their MBC (minimal bactericidal concentration).

## Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

## Conflicts of interest

There are no conflicts to declare.

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