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Cyclipostins and Cyclophostin analogs are multi-target inhibitors that impair growth of *Mycobacterium abscessus*

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Twelve new Cyclophostin and Cyclipostins analogs (CyC19-30) were synthesized, thus extending our series to 38 CyCs. Their antibacterial activities were evaluated against four pathogenic mycobacteria (Mycobacterium abscessus, Mycobacterium marinum, Mycobacterium bovis BCG and Mycobacterium tuberculosis) and two Gram negative bacteria. The CyCs displayed very low toxicity towards host cells and were only active against mycobacteria. Importantly, several CyCs were active against extracellular M. abscessus (CyC17/CyC18p/CyC25/CyC26) or intramacrophage residing mycobacteria (CyC7(α,β)/CyC8(α,β)) with minimal inhibitory concentrations (MIC50) values comparable to or better than those of amikacin or imipenem, respectively. An activity-based protein profiling combined with mass spectrometry allowed identification of the potential target enzymes of CyC17/CyC26, mostly being involved in lipid metabolism and/or in cell wall biosynthesis. Overall, these results strengthen the selective activity of the CyCs against mycobacteria, including the most drug-resistant M. abscessus, through the cumulative inhibition of a large number of Ser- and Cys-enzymes participating in key physiological processes.

**Keywords**: Total synthesis; drug susceptibility; activity based-protein profiling; proteomics analysis.
Produced by *Streptomyces* species, natural Cyclophostin (CyC1 – Figure 1) is characterized by a unique seven-membered cyclic phosphate triester fused to a butyrolactone ring. This bicyclic core also contains a unique enol phosphate and chiral centers at both phosphorus and C-3a carbon atom. The structurally related natural Cyclipostins (e.g., Cyclipostin P CyC18β - Figure 1), which varied in the nature of the lipophilic chain attached to the phosphate, were described as bacterial growth inhibitors of various mycobacteria. Together, compelling evidence suggests the great potential for cyclic enolphosphate and phosphonate analogs of Cyclophostin (and the Cyclipostins) as anti-mycobacterial agents.

To explore this hypothesis, we have reported the synthesis of Cyclipostins and Cyclophostin analogs (namely the CyCs – Figure 1). We first demonstrated that this first series of 26 CyCs (Figure 1) was able to efficiently inhibit the growth of *Mycobacterium tuberculosis* H37Rv, *in vitro* as well as in infected macrophages, with very low toxicity towards the host cells. These studies also strongly support the assumption that CyC compounds are multi-target inhibitors impairing various mycobacterial Ser- or Cys-containing enzymes involved in important physiological processes via the formation of a covalent bond between the enol-phosphorous atom and the catalytic residue. The efficiency/selectivity of these CyCs was investigated further towards three slow-growing species (i.e., *M. marinum*, *M. bovis* BCG, and *M. tuberculosis*) and various mycobacterial clinical isolates and bacteria responsible for nosocomial infections, including Gram-negative and Gram-positive bacteria as well as rapidly-growing mycobacteria (RGM) belonging to the *Mycobacterium chelonae-abscessus* clade. Remarkably, the inhibitory activity of these CyCs was exclusively restricted to mycobacteria. Notably, the most potent inhibitor (CyC17) exhibited minimal inhibitory concentrations (MIC) values comparable to those of most classical antibiotics used to treat *M. tuberculosis* and *M. abscessus* infections. Collectively, these results emphasized the attractiveness of this new family of compounds as valid drug candidates to be exploited in future therapeutic developments against mycobacterial-associated infections, especially against *M. abscessus* the most drug-resistant mycobacterial species.
Figure 1. Chemical structure of natural Cyclipostins and Cyclophostin, and related CyC analogs. Natural Cyclophostin (CyC₁), Cyclipostin P (CyC₁₈₈) and its trans diastereoisomer (CyC₁₈₈α). Cyclophostin phosphonate analog (CyC₂), monocyclic enolphosphorus analogs to either Cyclophostin (CyC₃₋₁₀; 1₅₋₁₆) or Cyclipostins (CyC₁₁₋₁₄; 1₇). CyC₆₋₁₀ and CyC₁₃ were obtained as two diastereoisomers, being either in a trans (α-isomer) or cis (β-isomer) conformation between the OMe on the phosphorus and the H-substituent on the C-5 carbon atom. Adapted from ⁷.

Over the last decade, non-tuberculous mycobacterial (NTM) infections have increased worldwide, leading to an emerging public health problem, particularly in industrialized countries, sometimes surpassing tuberculosis.¹¹ Among them, M. abscessus represents the most important rapidly growing mycobacteria (RGM).¹² M. abscessus is indeed responsible for a wide spectrum of clinical syndromes ranging from skin infections to severe pulmonary infections in humans with compromised natural defenses, such as patients with cystic fibrosis (CF).¹³⁻¹⁴ The M. abscessus complex, along with Mycobacterium avium complex, represents over 90% of all reported NTM-pulmonary infection cases in CF patients.¹⁵⁻¹⁶ Reports also show that M. abscessus isolated from CF patients ranged from 16% and up to 68% of all isolated NTM.¹⁷⁻¹⁹ In addition, the presence of M.
infection prior to lung transplantation represents a risk factor for developing lung infection or a disseminated disease after transplantation.\textsuperscript{14} \textit{M. abscessus} displays rough (R) and smooth (S) colony morphotypes.\textsuperscript{20-21} The S morphotype is related to the abundance of the surface-associated glycopeptidolipids (GPL), which is associated with sliding motility and biofilm formation.\textsuperscript{20} In contrast, a remarkable reduction in GPL amount in the cell wall of the R variant was correlated with cord formation.\textsuperscript{22} In addition, S and R variants can be considered as two representatives of the same isolate, which may coexist and/or evolve differently in response to host immunity, resulting in different fates for the mycobacteria in its host.\textsuperscript{20} Several studies have confirmed the correlation between colony morphology and virulence. The S variants are less virulent than R variants,\textsuperscript{23} which are frequently associated with severe pulmonary infections\textsuperscript{24-25} and are able to persist for years especially in infected CF patients.\textsuperscript{26} In the light of these findings, \textit{M. abscessus} is known to transition from a smooth (S) morphotype with cell surface-associated GPL to a rough (R) morphotype lacking GPL. Accordingly, \textit{M. abscessus} is notorious for being one of the most drug-resistant mycobacterial species, refractory to standard antimicrobials used for the treatment of Gram-positive and Gram-negative bacteria.\textsuperscript{27} In addition, most antitubercular agents (\textit{i.e.}, rifampin, isoniazid, and ethambutol) are also ineffective against \textit{M. abscessus}, referred to as an “antibiotic nightmare”.\textsuperscript{28} This intrinsic polyresistance to drugs results from multiple factors,\textsuperscript{29} including the presence of a highly impermeable cell envelope preventing the penetration of drug, multiple-drug efflux pumps,\textsuperscript{30-31} inability to convert prodrugs into their active metabolites,\textsuperscript{32} expression of numerous enzymes which can modify the drug-target or inactivate the drug itself,\textsuperscript{27} and the acquisition of mutations in the antibiotic targets. Hence, the development of new therapeutic approaches and/or discovery of new chemical entities to fight this pathogen are needed.

Herein, we have synthesized 12 new analogs, \textit{i.e.} \textbf{CyC19} to \textbf{CyC30}, by varying the lipophilicity of the R\textsuperscript{3} chain located on the 7-membered enolphosphorus ring in our phosphate and phosphonate analogs. This structural modification is also supported by the isolation of the anti-malarial Salinipostins,\textsuperscript{33-34} which are essentially Cyclipostins with variations in the enol alkyl substituent.
Their respective antibacterial activity was further assessed against a panel of pathogenic mycobacteria, including the *M. abscessus* R and S variants. Importantly, the MIC of all (26+12) CyCs determined against either extracellular or intracellular *M. abscessus* growth, revealed the efficacy of eight new candidates. The potential targets of CyC17 and CyC26, the two most potent inhibitors against extracellularly-growing bacteria, were identified via an activity-based protein profiling (ABPP) approach.
RESULTS AND DISCUSSION

Synthesis of new Cyclophostin phosphate and phosphonate analogs. We incremented the already available library comprising 26 CyC compounds (i.e., CyC1-18 including the cis–(α) and trans–(β) isomers),7,10 by synthesizing 12 additional analogs (CyC19-30) from various 3-keto esters following previously described synthetic routes (Scheme 1).3-6

The general approach previously used for the synthesis of phosphonate analogs CyC4-10 was applied (Scheme 1A) to the synthesis of cyclic phosphonates with variation in the enol alkyl substituent (CyC19-22). The palladium-catalyzed substitution reaction of methyl or ethyl acetoacetate derivatives (2)35 with the allylic carbonate (1) gave the vinyl phosphonates (3) as an E/Z mixture in good yields (35-63%). The E and Z isomers could be separated by silica gel chromatography, but were generally taken into the next reaction as mixture. Quantitative hydrogenation of (3a-d) led to the saturated phosphonates (4a-d), which after selective demethylation and cyclization gave the racemic forms of monocyclic enolphosphonates (5a-d) corresponding to compounds CyC19 to CyC22 (17-62%, 2 steps).

To optimize the potent observed antibacterial activity of compound CyC17,7,10 the synthesis of various monocyclic phosphate analogs has also been completed. Following a literature procedure36-37 β-ketoesters (8a-d) were prepared by alkylation of t-butyl acetoacetate derivatives (7a-d) with iodide (6)38 (Scheme 1B). The tert-butyl ester was chosen so as to minimize the risk of lactonization upon deprotection of the alcohol function. Reaction of compounds (8a-d) with dimethyl chlorophosphite followed by oxidation of crude material with I2 and methanol gave the enolphosphates (9a-d) in modest yield along with recovered starting material (21-49%, 49-81% BRSM). The 4-methoxybenzyl ether (PMB) protecting group was then removed with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) under standard conditions to afford alcohols (10a-d). Demethylation and subsequent cyclization using 1-mesitylene-sulfonyl-3-nitrotriazole (MSNT) gave the monocyclic phosphate tert-butyl esters CyC27 to CyC30 (11a-d). Cleavage of the tert-butyl moiety with TFA in anhydrous conditions followed by TMSCHN2 treatment resulted in the formation of the
corresponding racemic cyclic enolphosphate methyl esters CyC\textsubscript{23} to CyC\textsubscript{26} (12a-d) in good yields (79-100%).

**Antibacterial activity of the new CyC<sub>19</sub>-CyC<sub>30</sub> compounds.** The antimicrobial activity of the 12 newly synthesized derivatives was evaluated as previously reported.<sup>10</sup> Briefly, a preliminary screen on solid medium (30 µM final concentration) was performed on a selected panel of four pathogenic mycobacterial species (*i.e.*, *M. marinum*, *M. bovis* BCG, *M. tuberculosis* mc²6230, and both S/R variants of *M. abscessus*) and 2 Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). Following CFU count, 5 out of the 12 CyCs inhibited growth in the range of 50-100% relative to the positive growth control (*i.e.*, bacteria without antibiotics). Moreover, and as previously observed with CyC<sub>1</sub> to CyC<sub>18</sub>,<sup>10</sup> this inhibitory activity was restricted to the four mycobacteria tested, while growth of *E. coli* and *P. aeruginosa* was not impacted (see Supplementary Material Table S1). The MIC of these selected CyC<sub>21</sub>, CyC<sub>22</sub>, CyC<sub>25</sub>, CyC<sub>26</sub> and CyC<sub>30</sub> towards each mycobacterial strain were next determined using the resazurin microtiter assay (REMA)<sup>7,10</sup> (**Table 1**). Among the abovementioned CyCs, only CyC<sub>26</sub> inhibited growth of *M. marinum*, *M. bovis* BCG and *M. tuberculosis*, with MIC<sub>50</sub> values ranging from 1.8 to 26.4 µM (**Table 1**). Interestingly, CyC<sub>21</sub> and CyC<sub>22</sub> exhibited the best activity (MIC<sub>50</sub> 5.6-8.7 µM) against *M. tuberculosis* mc²6230. These latter compounds represent two new potential candidates against this bacterium, therefore extending the first series of CyC growth inhibitors previously identified.<sup>7,10</sup>

Despite the fact that only CyC<sub>25</sub> and CyC<sub>26</sub> were active against *M. abscessus* on solid medium (**Table 1**), we also determined the respective MIC<sub>50</sub> values of all CyCs including the first series of 26 analogs against each S/R variant of *M. abscessus* to investigate deeper their efficiency/selectivity towards this pathogen (see Supplementary Material Table S2). Among the 38 tested compounds including the 12 new synthesized analogs, the best growth inhibitors against *M. abscessus* S variant still remained CyC<sub>17</sub> and CyC<sub>18β</sub>, which displayed similar MIC<sub>50</sub> values (mean MIC<sub>50</sub> = 11.7 ±0.94 µM)<sup>10</sup> comparable to that of amikacin (AMK) (**Table 1**). In all other cases, MIC<sub>50</sub> were indicative either of a poor (MIC<sub>50</sub> ≥117 µM for CyC<sub>1-4;6-9;11;16;19;21;27-28</sub>), or a moderate (MIC<sub>50</sub> = 45-97 µM for CyC<sub>15;18α;20;22;25-26;29-30</sub>) antibacterial activity (see Supplementary Material Table S2). By contrast, nearly all CyCs were found more active against *M. abscessus* R variant as compared to the S variant.
Although most of the CyCs exhibited quite weak MIC\textsubscript{50} in the range 100 to >200 µM; eight analogs displayed moderate activities (mean MIC\textsubscript{50} = 51.7 ±7.4 µM for CyC\textsubscript{6a;8a;9a:18a:21-22;29-30}) (see Supplementary Material Table S2). CyC\textsubscript{25} (MIC\textsubscript{50} = 13.9 µM) and CyC\textsubscript{26} (MIC\textsubscript{50} = 6.9 µM) were confirmed as very good antimycobacterial molecules, in addition to CyC\textsubscript{17} (MIC\textsubscript{50} = 0.37 µM) and CyC\textsubscript{18β} (MIC\textsubscript{50} = 8.9 µM).\textsuperscript{10} Of interest, these latter four best inhibitors of \textit{M. abscessus} growth were all phosphate esters bearing a long lipophilic C10/C16 alkyl chain either at the R\textsuperscript{1} or R\textsuperscript{3} position (Figure 1 and Scheme 1).

Table 1. Antibacterial activities of the selected active CyC compounds against four pathogenic mycobacterial strains\textsuperscript{a}

<table>
<thead>
<tr>
<th>Cpds</th>
<th>M. marinum ATCC BAA-535/M</th>
<th>M. bovis BCG</th>
<th>M. tuberculosis mc\textsuperscript{2}6230</th>
<th>M. abscessus CIP 104536\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S variant</td>
<td>R variant</td>
<td>S variant</td>
<td>R variant</td>
</tr>
<tr>
<td>AMK</td>
<td>1.1 ±0.05</td>
<td>0.54 ±0.03</td>
<td>0.42 ±0.02</td>
<td>3.9 ±0.19</td>
</tr>
<tr>
<td>INH</td>
<td>20.5 ±0.71</td>
<td>0.71 ±0.03</td>
<td>1.5 ±0.07</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CyC\textsubscript{17} \textsuperscript{b}</td>
<td>1.5 ±0.04</td>
<td>0.54 ±0.01</td>
<td>0.55 ±0.02</td>
<td>12.7 ±0.26</td>
</tr>
<tr>
<td>CyC\textsubscript{18β} \textsuperscript{b}</td>
<td>23.1 ±1.07</td>
<td>6.0 ±0.29</td>
<td>1.6 ±0.07</td>
<td>10.8 ±0.43</td>
</tr>
<tr>
<td>CyC\textsubscript{21}</td>
<td>&gt;100</td>
<td>13.8 ±0.69</td>
<td>8.7 ±0.28</td>
<td>139.0 ±4.80</td>
</tr>
<tr>
<td>CyC\textsubscript{22}</td>
<td>&gt;100</td>
<td>24.9 ±1.16</td>
<td>5.6 ±0.25</td>
<td>65.0 ±2.19</td>
</tr>
<tr>
<td>CyC\textsubscript{25}</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>85.0 ±0.86</td>
</tr>
<tr>
<td>CyC\textsubscript{26}</td>
<td>1.8 ±0.08</td>
<td>12.4 ±0.44</td>
<td>26.4 ±0.60</td>
<td>60.0 ±2.50</td>
</tr>
<tr>
<td>CyC\textsubscript{30}</td>
<td>&gt;100</td>
<td>14.2 ±0.60</td>
<td>&gt;100</td>
<td>62.6 ±2.39</td>
</tr>
</tbody>
</table>

\textsuperscript{a} MIC\textsubscript{50} corresponding to the concentration leading to 50% growth inhibition as determined by the REMA assay, are expressed as mean values of two independent assays performed in triplicate (CV% <5%). \textsuperscript{b} Data for CyC\textsubscript{17} and CyC\textsubscript{18β} are from \textsuperscript{10}. AMK, amikacin. INH, isoniazid.

\textit{M. abscessus} surface-exposed GPL drives the antibacterial potency of CyC compounds. From these results, \textit{M. abscessus} R appeared to be 6.1- to 34-times more sensitive than \textit{M. abscessus} S to CyC\textsubscript{25}, CyC\textsubscript{26} and CyC\textsubscript{17} (Table 1). As previously mentioned, the major difference between these two variants resides in the loss of surface-associated glycopeptidolipids (GPL) in the R form.\textsuperscript{20,23,39} It may be inferred that the GPL covering the surface of \textit{M. abscessus} S acts as a protective shield.
towards the CyCs, although the natural Cyclipostin P CyC_{18β} was not strongly affected by the GPL profile.

To confirm the possible relationship between CyC potency and GPL production, drug susceptibility was assessed using the GPL-deficient ΔmmpL4b mutant generated in an S background of the type strain CIP104536^T, and its complemented counterpart.\textsuperscript{40-42} The mmpL4b gene encodes for the MmpL4b membrane protein, which allows translocation of GPL across the plasma membrane.\textsuperscript{40, 43} Mutations in this gene result in the loss of GPLs and acquisition of a R morphotype.\textsuperscript{23, 40-41} Both the wild-type \textit{M. abscessus} S and the ΔmmpL4b complemented strain responded similarly to CyC_{17}, CyC_{25} and CyC_{26}, with a more pronounced resistance level to these compounds as compared to the GPL-deficient ΔmmpL4b mutant, which behaved like the wild-type R variant (Figure 2 and Table 2). Accordingly and as observed with \textit{M. abscessus} S vs. R variant, the MIC\textsubscript{50} of CyC_{25}, CyC_{26} and CyC_{17} reached on the ΔmmpL4b complemented strain were 4.8- to 32-times higher than those determined on the mmpL4b deletion mutant (Table 2). In contrast, there was a low influence of the GPL content in the activity of CyC_{18β}, as judged by the obtained MIC\textsubscript{50} values against different strains (Table 2).

### Table 2. Variation of MIC\textsubscript{50} (µM) of CyC_{17}, CyC_{18β}, CyC_{25} and CyC_{26} against both R & S variants of \textit{M. abscessus} as well as mmpL4b mutant strains

<table>
<thead>
<tr>
<th></th>
<th>\textit{M. abscessus} S</th>
<th>\textit{M. abscessus} R</th>
<th>\textit{M. abscessus} S_ΔmmpL4b</th>
<th>\textit{M. abscessus} S_ΔmmpL4b::C</th>
<th>\textit{ΔmmpL4b} R → S</th>
<th>\textit{ΔmmpL4b} R → S_ΔmmpL4b::C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyC_{17}</td>
<td>12.7 ±0.26</td>
<td>0.37 ±0.01</td>
<td>0.60 ±0.03</td>
<td>19.2 ±0.62</td>
<td>×34.3</td>
<td>×32.0</td>
</tr>
<tr>
<td>CyC_{18β}</td>
<td>10.8 ±0.43</td>
<td>8.9 ±0.09</td>
<td>3.3 ±0.16</td>
<td>5.4 ±0.26</td>
<td>×1.2</td>
<td>×1.6</td>
</tr>
<tr>
<td>CyC_{25}</td>
<td>85.0 ±0.86</td>
<td>13.9 ±0.56</td>
<td>15.7 ±0.73</td>
<td>74.9 ±3.4</td>
<td>×6.1</td>
<td>×4.8</td>
</tr>
<tr>
<td>CyC_{26}</td>
<td>60.2 ±2.50</td>
<td>6.9 ±0.16</td>
<td>7.2 ±0.28</td>
<td>67.3 ±3.1</td>
<td>×8.7</td>
<td>×9.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} MIC\textsubscript{50} corresponding to the concentration leading to 50% growth inhibition as determined by the REMA assay, are expressed as mean values of two independent assays performed in triplicate (CV\% <5%).
Figure 2. Dose-response activity of CyC\textsubscript{17} and CyC\textsubscript{26} against \textit{M. abscessus} S, \Delta\textit{mmpL4b} mutant (\textit{M. abscessus} S\_\Delta\textit{mmpL4b}), \Delta\textit{mmpL4b} complemented (\textit{M. abscessus} S\_\Delta\textit{mmpL4b}:C), and \textit{M. abscessus} R strains replicating in broth medium, expressed as normalized relative fluorescence units (RFU\%). For each concentration, data are means ± SD of at least two independent assays performed in duplicate (CV% <5%).
A significant reduced susceptibility to CyC$_{17}$ has been reported previously in a *M. smegmatis*Δlipg mutant strain in which GPLs were overexpressed due to disruption of *lipG*, resulting in a change vs WT in MIC$_{50}$ value of ×15.7 fold.$^{44}$ Both results are also in agreement with a study$^{45}$ reporting that *M. avium* GPL-positive serovar was less sensitive to ethambutol than GPL-negative one, presumably because hydrophobic GPL may alter cell-wall fluidity/permeability and correlate with antibiotic tolerance.$^{45-46}$ Together, these findings underline the importance of the surface-exposed GPL in the CyC-dependent inhibition of *M. abscessus* growth. Remarkably, CyC$_{18}$, which differs from CyC$_{17}$ by the presence of a butyrolactone ring fused to the seven-membered cyclic phosphate triester (Figure 1), failed to show a high GPL-dependent activity profile. Accordingly, the presence of the lactone may be responsible for this behavior, different from the other monocyclic CyC analogs, towards *M. abscessus* variants.

Activity-based protein profiling (ABPP) approach for targets identification. In light of our previous study demonstrating the strong affinity of the CyCs for (Ser/Cys)-enzymes bearing a catalytic serine or cysteine residue,$^{4-9}$, $^{37}$, $^{47-48}$ we presumed that these inhibitors would also target/impair the activity of enzymes participating in a wide range of physiological processes of the *M. abscessus* life cycle, resulting in bacterial growth inhibition, as previously shown in *M. tuberculosis*.$^7$ Hence, to gain access to their putative targets, activity-based protein profiling (ABPP)$^7$ was employed to capture and identify candidate target enzyme(s) impacted by CyC$_{17}$ and CyC$_{26}$, the two best hits acting against extracellular *M. abscessus*.

In the first approach, a crude lysate of *M. abscessus* R was incubated with either a CyC inhibitor (or DMSO as a control) and subjected to a competitive probe labeling/enrichment assay with a Desthiobiotin-FP probe (see Supplementary Material Figure S1A).$^7$ The enriched mixtures were then digested with trypsin and the resulting peptides analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), followed by subsequent label free quantification analysis. The proteins that were also found in the control experiment (*i.e.*, DMSO alone for unspecific binding to streptavidin-magnetic beads) were discarded. At first, proteins identified with a permutation false
discovery rate (pFDR) up to 10% were selected, leading to a panel of 806 distinct protein candidates in the case of CyC17, which was narrowed down to 30 when applying a pFDR of 1%, as compared to only 4 with CyC26 (14 at a pFDR of 10%) (see Supplementary Material Tables S3-S8).

Given the fact that orthologs typically perform equivalent functions in the respective organisms,51 the corresponding orthologs in M. tuberculosis H37Rv genome of the various identified proteins were reported, adding reliable predictions of the gene functions in terms of essentiality, genomic location and activity. With each inhibitor, except for a few uncharacterized proteins, most of the identified hits were (Ser/Cys)-based enzymes participating in mycobacterial lipid metabolism (see Supplementary Material Tables S4-S5). This was substantiated with CyC26 for which 8 out of 14 identified proteins are (Ser/Cys)-based enzymes. These included the putative β-lactamase MAB_2833 (i.e., Rv1367c), possibly involved in cell wall biosynthesis; the D-amino acid aminohydrolase MAB_2605c (i.e., Rv2913c) and the probable peptidase MAB_4130 (i.e., Rv0457c); three members of the lipase family Lip, LipH (MAB_2039), LipN (MAB_3270c) and LipW (MAB_0826c); and two Cutinase-like proteins, Cut1 (MAB_3272c) and Cut4 (MAB_3809c). Interestingly the histidinol-phosphate aminotransferase (i.e., MAB_2669c or HisC1), possibly involved in the histidine pathway and annotated as an essential enzyme in M. tuberculosis,52-53 was also uncovered with CyC26. All target-proteins of CyC26 were also identified with CyC17, thus confirming this latter as a broader multi-target inhibitor. Given the putative mechanism of action of HisC1 (MAB_2669c) together with the recent resolution of the 3D structure of its M. tuberculosis ortholog Rv1600 in complex with its natural substrate, i.e. pyridoxal-5'-phosphate (PDB id: 4R8D),54 one can hypothesize that the two phosphate analogs CyC17 and CyC26 may bind to the active site, thus acting as substrate-like adducts.

In the second approach, the penetration/diffusion of CyC17 inhibitor through the mycobacterial cell wall was taken into account in order to reduce the list of potential targets. Thus, a similar experiment was performed on living bacterial cells.55 M. abscessus R cells were grown to log phase and incubated with CyC17 (or DMSO as a control). After cell lysis, part of the lysate was processed
as described above with Desthiobiotin-FP probe and streptavidin magnetic beads (see Supplementary Material Figure S1B). In parallel, the CyC17-treated lysate was also incubated with TAMRA-FP to reveal by in-gel fluorescence on an SDS-PAGE scanning, the (Ser/Cys)-enzyme candidates that presumably had reacted with this inhibitor7 (see Supplementary Material Figure S1C). At this stage, around 20 distinct bands labeled by TAMRA-FP were detected in the fluorescence readout and also visible by Coomassie staining after release of the enzymes captured by Desthiobiotin-FP (see Supplementary Material Figure S1C-D). In contrast, pre-treatment with CyC17 resulted in a strong decrease in fluorescence intensity of all visible bands; the CyC17-enzyme complex being unable to react with the TAMRA-FP probe. Tryptic digestion followed by tandem mass spectrometry analysis led to the identification of lower numbers of protein candidates as compared to a total lysate; i.e., 39 vs. 208 at a pFDR of 5%, respectively (Supplementary Material Table S6). It is noteworthy that among these 39 potential hits, 24 were previously detected in the CyC17-treated total lysate (see Supplementary Material Tables S3-4): 16 at a pFDR of 10%, 1 at a pFDR of 5%, and 7 at a pFDR of 1%; thus implying that 15 proteins had not been detected in the previous treated M. abscessus total lysate, or at least for a pFDR > 10%.

Consistent with previous work involving CyC17,7 a variety of hydrolases were detected, including one amidase AmiC (MAB_2181c), two Lip-family members LipI (MAB_2814) and LipT (MAB_3336c), two Cutinase-like proteins Cut1 (MAB_3272c) and Cut3 (MAB_3765), a possible hydrolase (MAB_3034), and MAB_176 (Ag85A) and MAB_177 (Ag85-A/B/C precursor) two members of the antigen 85 (Ag85) complex, which catalyzes the formation of trehalose dimycolate and adds mycolic acids to arabinogalactan9, 56-57 (see Supplementary Material Table S6). Interestingly, 9 out of the 39 identified proteins are annotated as essential enzymes in M. tuberculosis genome (Table 3).52-53 Among them, MAB_0172 (i.e., Rv3807c) & MAB_3612 (i.e., Rv3265c) are possibly involved in the arabinogalactan biosynthesis;58-59 and MAB_0944 (i.e., Rv0896) is a probable citrate synthase required for the tricarboxylic acid cycle.60
Table 3. CyC<sub>17</sub> target proteins identified in <i>M. abscessus</i> R culture by LC-ESI-MS/MS analysis and annotated as essential<sup>a</sup> for <i>M. tuberculosis</i> orthologs.

<table>
<thead>
<tr>
<th>Protein Ids</th>
<th>Mol. Weight [kDa]</th>
<th>Rv number</th>
<th>Essentiality</th>
<th>Location&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Activity / Function</th>
<th>Functional category&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAB_0172</td>
<td>19.3</td>
<td>Rv3807c</td>
<td>Macrophages</td>
<td>M; WCL</td>
<td>Putative decaprenylphosphoryl-5-phosphoribose phosphatase</td>
<td>CW/CP</td>
</tr>
<tr>
<td>MAB_4481</td>
<td>28.8</td>
<td>Rv0224c</td>
<td>essential gene</td>
<td>WCL</td>
<td>Possible methyltransferase</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_3612c</td>
<td>32.9</td>
<td>Rv3265c</td>
<td>essential gene</td>
<td>M; WCL</td>
<td>Putative dTDP-rhamnosyltransferase WbbL1</td>
<td>CW/CP</td>
</tr>
<tr>
<td>MAB_1588c</td>
<td>39.0</td>
<td>Rv3230c</td>
<td>in vitro growth</td>
<td>M; WCL</td>
<td>Putative dTDP-rhamnosyltransferase WbbL1</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_4876c</td>
<td>45.0</td>
<td>Rv2017</td>
<td>in vitro growth</td>
<td>M; WCL</td>
<td>Probable oxidoreductase</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_1524c</td>
<td>46.9</td>
<td>Rv1232c</td>
<td>essential gene</td>
<td>M; WCL</td>
<td>Uncharacterized protein</td>
<td>-</td>
</tr>
<tr>
<td>MAB_0944</td>
<td>47.6</td>
<td>Rv0896</td>
<td>in vitro growth</td>
<td>CW; M; WCL</td>
<td>Probable citrate synthase I GltA2</td>
<td>IM/R</td>
</tr>
<tr>
<td>MAB_3511c</td>
<td>74.8</td>
<td>Rv3198c</td>
<td>essential gene</td>
<td>WCL</td>
<td>Probable citrate synthase I GltA2</td>
<td>IP</td>
</tr>
<tr>
<td>MAB_2486c</td>
<td>190.8</td>
<td>Rv3859c</td>
<td>in vitro growth</td>
<td>CF; CW; WCL</td>
<td>Probable gltB, ferredoxin-dependent glutamate synthase</td>
<td>IM/R</td>
</tr>
</tbody>
</table>

<sup>a</sup> from<sup>52-53</sup>.  
<sup>b</sup> CF: Culture filtrate; CW: Cell wall; M: Membrane fraction; WCL: Whole cell lysate.  
<sup>c</sup> IM/R: Intermediary metabolism/respiration; CW/CP: cell wall/cell processes;  
LM: Lipid metabolism; V/D/A: Virulence, detoxification, adaptation.
Of particular interest, the Ag85A/B/C proteins from *M. tuberculosis* have been recently validated as real targets of CyC. Herein, we confirm that the recombinant *M. abscessus* Ag85C protein, which shares nearly 60% amino acid sequence identity with its *M. tuberculosis* ortholog and possesses the same conserved catalytic triad (i.e., Ser^{124}-Glu^{228}-His^{260}), is also inactivated by CyC. Ag85C<sub>Mabs</sub> (50 µM) was incubated in its native form with 1 mM (i.e., enzyme/inhibitor molar ratio of 1:20) CyC and then treated with 10 µM TAMRA-FP fluorescent probe for 1 h. Equal amounts of proteins (2 µg) were separated by 12% SDS-PAGE and visualized by Coomassie staining or in-gel fluorescence for TAMRA detection (see Supplementary Material Figure S2 for details). Quantification of labeling normalized to protein load revealed that pre-incubation with CyC resulted in a significant decrease in fluorescence intensity by around 70% as compared with the non-treated protein. This implies that reaction with the TAMRA probe is strongly impaired in the Ag85C<sub>Mabs</sub>-CyC adduct, resulting in a significant loss of fluorescence emission.

**Activity of CyC analogs against intracellular *M. abscessus***. Similar to *M. tuberculosis*, following lung infection *M. abscessus* can grow and survive intracellularly inside macrophages. However, MIC values determined in broth medium do not always correlate with the activity of the compounds against intracellular bacteria. Such discrepancy between extra- and intracellular activity has been reported in the case of *M. tuberculosis* with the CyCs as well as another family of inhibitors, the Oxadiazolone-core derivatives. From these findings, all 38 CyC analogs were further tested for their antibacterial activity against *M. abscessus* growth inside infected macrophages.

The cytotoxicity of the 12 new compounds was first assessed using murine Raw264.7 macrophages. Except CyC<sub>26</sub> (CC<sub>50</sub> = 50 µM), all other inhibitors CyC<sub>19-25,27-30</sub> showed very low toxicity towards the cells with CC<sub>50</sub> > 100 µM, similarly to AMK (CC<sub>50</sub> ≥ 150 µM). Taking into account our previous toxicity results obtained with CyC<sub>1-18</sub>, the 38 synthesized analogs exhibited low cytotoxic towards host mammalian cells.

Due to its high clumping capability, infection with *M. abscessus* R results in the lysis of nearly all macrophages at 24 h post-infection, making it very difficult to quantify in a reliable manner the
The intracellular effect of the CyCs. This is, however, not the case with *M. abscessus* S which does not aggregate like the R form, thus enabling the preparation of homogenous and dispersed suspensions.

The intracellular growth of *M. abscessus* S was next assessed following a 24 h exposure of infected Raw264.7 cells to the CyC compounds at a final concentration of 30 µM. To avoid growth of extracellular mycobacteria, cells were extensively washed and treated with amikacin (200 µg/mL = 340 µM; 87 × MIC<sub>50</sub>) prior to treatment with the CyC analogs. Imipenem (IMP; 80 µg/mL = 267 µM; 67 × MIC<sub>50</sub>) was used as positive control for this intracellular killing assay. In each case, the viability of infected macrophages was checked by addition of trypan blue before cell lysis and plating for CFU counting. Nearly all compounds, including the CyC<sub>19-30</sub> new series, failed to show an effect at the concentration investigated. In agreement with previous work with *M. tuberculosis*,<sup>7</sup> both (α) and (β) isomers of CyC<sub>7</sub> and CyC<sub>8</sub>, which are not active against extracellular bacilli, showed a 40-50% and 63-65% decrease in intramacrophagic CFUs 24 h post infection in the presence of CyC<sub>7(α,β)</sub> and CyC<sub>8(α,β)</sub>, respectively.

Interestingly, although the phosphate analogs CyC<sub>17</sub>, CyC<sub>18β</sub>, CyC<sub>25</sub> and CyC<sub>26</sub> exclusively impaired extracellular growth of *M. abscessus*, they however remained fully inactive towards infected macrophages. In contrast, the phosphonate analogs CyC<sub>7-8</sub> were found active only against intramacrophagic *M. abscessus*. Similar properties between the phosphonate (*i.e.*, CyC<sub>7-8</sub>) vs. phosphate (*i.e.*, CyC<sub>17</sub>) chemical groups have been reported for *M. tuberculosis*.<sup>7</sup> In addition, the fact that only CyC<sub>7(α,β)</sub> and CyC<sub>8(α,β)</sub> showed a clear preference against intracellularly-replicating mycobacteria may imply that the intracellular mode of action of these CyCs differs from that of CyCs acting exclusively on extracellularly-replicating bacilli. Another hypothesis is that accessibility and/or vulnerability of their corresponding target(s) may be more apparent and crucial during the intracellular lifestyle of *M. abscessus*. Moreover, a specific stringent response of the macrophage, such as possible host cell metabolism, stimulated by the action of these latter compounds and leading to bacterial clearance cannot, however, be excluded.
To confirm this screen, *M. abscessus* S infected macrophages were subjected to a dose-response assay using the latter selected CyC7(α,β) and CyC8(α,β) analogs or IMP (Figure 3), taken as reference intracellular drug, in order to determine their respective intracellular MIC50Raw values (Table 4). Both CyC7α and CyC7β displayed a moderate activity against intracellular *M. abscessus* S (Figure 3A) with a calculated MIC50Raw of 29.3 µM and 65.3 µM, respectively, similar to that of IMP (MIC50Raw = 28.3 µM) used as reference (Table 4). By contrast a 24 h-treatment with either CyC8α or CyC8β led to a 68.4 ±6.4% (30-60 µM) and 73.5 ±1.9% (60 µM) reduction in mycobacteria, respectively; which was comparable to that elicited by IMP, *i.e.*, 74.0 ±4.4% reduction following treatment with 60 µM (Figure 3C). Regarding CyC8β, a plateau value corresponding to 64.7 ±3.8% bacterial killing was reached when the infected cells were treated with the compound at a 5-30 µM concentration range. Remarkably, MIC50Raw values of CyC8α (7.9 µM) as well as CyC8β (2.0 µM) towards intramacrophagic bacilli were 3.6- and 14-fold lower than that of IMP (28.3 µM) (Table 4). These MIC50Raw are of the same order of magnitude than those previously obtained with CyC8(α,β) acting against intracellular *M. tuberculosis* (*i.e.*, MIC50Raw_Mtb around 4-12 µM).7 The selectivity index (SI = CC50/MIC50Raw) of the four best inhibitors on intracellular *M. abscessus* vs. Raw264.7 cells was thus found to be in a range from 1.1 and up to 35 for CyC8β. Overall, these results indicate that the (α) and (β) isomers of CyC7 and CyC8 could enter the macrophages and arrest bacterial replication without exhibiting significant toxicity for the host cell, with comparable effects to IMP.
Figure 3. Intracellular activity of (A) CyC_{7α} & CyC_{7β}, and (B) CyC_{8α} & CyC_{8β} as compared to (C) imipenem (IMP). The activity of selected CyCs on intracellular *M. abscessus* was tested in Raw264.7 murine macrophages. Cells were infected at a multiplicity of infection (MOI) of 10 with *M. abscessus* S variant and treated with various concentrations of each inhibitor or IMP for 24 h. Then, surviving bacteria were enumerated by plating serial dilutions of macrophage lysates. Untreated infected macrophages were used as control representing 100% of bacterial viability. Results are shown as mean ± standard error of the mean (SEM) of three independent assays performed in triplicate. SC, solvent control (DMSO). **, *p*-value < 0.01. *, *p*-value < 0.05. Statistical analysis was done using a Student’s *t*-test.
Table 4. Antibacterial activities of the most active CyC analogs against *M. abscessus* S infected macrophages

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>MIC&lt;sub&gt;50Raw&lt;/sub&gt; (µM)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP</td>
<td>ND</td>
<td>28.3 ±4.7</td>
<td>-</td>
</tr>
<tr>
<td>CyC&lt;sub&gt;7α&lt;/sub&gt;</td>
<td>&gt;100</td>
<td>29.3 ±2.9</td>
<td>&gt;3.4</td>
</tr>
<tr>
<td>CyC&lt;sub&gt;7β&lt;/sub&gt;</td>
<td>&gt;70</td>
<td>65.3 ±6.2</td>
<td>&gt;1.1</td>
</tr>
<tr>
<td>CyC&lt;sub&gt;8α&lt;/sub&gt;</td>
<td>&gt;100</td>
<td>7.9 ±0.23</td>
<td>&gt;12.7</td>
</tr>
<tr>
<td>CyC&lt;sub&gt;8β&lt;/sub&gt;</td>
<td>&gt;70</td>
<td>2.0 ±0.91</td>
<td>&gt;35</td>
</tr>
<tr>
<td>CyC&lt;sub&gt;17&lt;/sub&gt;</td>
<td>&gt;100</td>
<td><em>no effect</em></td>
<td>-</td>
</tr>
<tr>
<td>CyC&lt;sub&gt;18β&lt;/sub&gt;</td>
<td>&gt;100</td>
<td><em>no effect</em></td>
<td>-</td>
</tr>
<tr>
<td>CyC&lt;sub&gt;25&lt;/sub&gt;</td>
<td>&gt;100</td>
<td><em>no effect</em></td>
<td>-</td>
</tr>
<tr>
<td>CyC&lt;sub&gt;26&lt;/sub&gt;</td>
<td>&gt;50</td>
<td><em>no effect</em></td>
<td>-</td>
</tr>
</tbody>
</table>

* Experiments were performed as described in Experimental Section. CC<sub>50</sub>: compound concentration leading to 50% Raw264.7 macrophages toxicity. MIC<sub>50Raw</sub>: minimal compound concentration leading to a 50% decrease in CFU count as compared to untreated cells. Raw264.7 macrophages were infected by *M. abscessus* S at a MOI of 10, and further treated with each CyC or IMP for 24 h. The viable mycobacteria were quantified using the agar plating method. Untreated infected macrophages were used as control representing 100% of bacterial viability. MIC<sub>50Raw</sub> were calculated from curve fitting of CFU% as a function of the inhibitor concentration. Data are expressed as mean values of three independent assays performed in triplicate. IMP, imipenem. SI, selectivity index: SI = CC<sub>50</sub>/MIC<sub>50Raw</sub>. 
CONCLUSION

Herein, we have extended our first series of 26 CyC analogs by synthesizing 12 new phosphonate (i.e., CyC_{19-22}) and phosphate (i.e., CyC_{23-30}) compounds in which we varied the R^3 lipophilic chain located on the 7-membered enolphosphorus ring. Evaluation of their antibacterial activity first highlighted CyC_{21} and CyC_{22} as two potential candidates against *M. tuberculosis*. With respect to *M. abscessus*, the MIC determination of this set of 38 CyCs provided CyC_{25} and CyC_{26}, in addition to CyC_{17} and CyC_{18β}, as active inhibitors of R and S variants. We demonstrated that the absence of surface-exposed GPL in the R variant was responsible for the higher susceptibility to the CyCs as compared to the S strain. The absence of GPL may induce some changes in the cell-wall fluidity/permeability, possibly favoring the penetration/action of these inhibitors. Putative enzymes targeted by CyC_{17} or CyC_{26}, the two best extracellular inhibitors, were identified using an ABPP approach. As anticipated, identified proteins were mainly serine or cysteine enzymes involved in mycobacterial lipid metabolism, among them 11 were annotated as essential enzymes in *M. tuberculosis* genome for *in vitro* growth or in infected macrophages. When tested against intracellular bacteria, CyC_{7(α,β)} and CyC_{8(α,β)} appeared as very potent and promising inhibitors with comparable or even lower MIC_{50}\text{Raw} values than that of the standard antibiotics imipenem. Of major interest, such inhibitors were selectively and efficiently acting on major pathogenic mycobacteria inside macrophages, including *M. tuberculosis*^3^ and now also *M. abscessus*. In addition to their low toxicity towards host cells, the CyC inhibitors potentially act as multi-target compounds, confirming their potential against one of the most drug-resistant mycobacterial species. It is very likely that blocking simultaneously enzymes involved in various lipid and/or in cell wall biosynthetic pathways would lead to extracellular and/or intracellular inhibition of *M. abscessus* growth by the selected CyCs. This dual activity of the CyCs is of major importance as it may affect the different stages of the infection process. Moreover, their association with current antibiotics to investigate their potential synergetic activity against resistant *M. abscessus* strains is currently in progress, and could open the way for improving the current treatments against these mycobacterial infections.
EXPERIMENTAL SECTION

Chemistry - synthesis of compounds CyC19 to CyC30

The synthesis of natural Cyclophostin CyC1,4 its phosphonate analogs CyC2α and CyC2β,3 the monocyclic enolphosphonates CyC3-47 and the trans-(α) and cis-(β) diastereoisomers CyC5-10;5 as well as the trans-(α) and cis-(β) Cyclipostin P CyC184 and the corresponding monocyclic phosphonate CyC11-13,5,7 difluorophosphonate CyC14-15 and phosphate CyC16-176,37 analogs have already been reported. The 12 new analogs (CyC19-30) where synthesized in racemic form from various 3-keto esters following already described synthetic routes (See Supplementary Material for detailed procedures and the full chemical characterization of each new compounds),3-6 and have purity of ≥ 95% as determined by HPLC analysis as reported previously.5 The HPLC data were supported by careful analysis of the 1H, 13C and particularly the 31P NMR spectra (see Supplementary Material). Stock solutions (10 mM) in which the CyC compounds were found to be completely soluble in dimethyl sulfoxide (DMSO), were prepared prior to drug susceptibility testing.

Antibacterial evaluation.

Bacterial strains and growth condition. M. marinum ATCC BAA-535/M, M. bovis BCG Pasteur, M. abscessus CIP104536T with either a smooth (S) or rough (R) morphotype, and M. tuberculosis mc²6230 (H37Rv ΔRD1 ΔpanCD65) strains were routinely grown in Middlebrook 7H9 broth (BD Difco, Le Pont de Claix, France) supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma-Aldrich, St. Quentin Fallavier, France) and 10% oleic acid, albumin, dextrose, catalase (OADC enrichment; BD Difco) (7H9-S). In the case of M. tuberculosis mc²6230, 24 µg/mL D-pantothenate (Sigma-Aldrich) was also added in the 7H9-S medium. All cultures were kept at 37 °C without shaking, except M. marinum which was grown at 32 °C. Escherichia coli DH10B and Pseudomonas aeruginosa PA01 were grown at 37 °C in LB Broth Base medium (ThermoFisher Scientific, Illkirch, France). A M. abscessus S mmpL4b KO (M. abscessus S_ΔmmpL4b) mutant displaying an R morphotype40 and its complemented counterpart (M. abscessus S_ΔmmpL4b::C),
which stably expresses MmpL4b under the control of the hsp60 promoter, were also used as previously reported in\textsuperscript{20}.

**Antibiotics.** Amikacin was purchased from Euromedex (Souffelweyersheim, France), and imipenem was provided by Mylan (Saint-Priest, France). Stock solution in water were freshly prepared for each experiment and filtered through a sterilized 0.22-µm-pore-size polycarbonate syringe filter (Millipore, Saint-Quentin-en-Yvelines, France).

**Drug susceptibility testing on solid medium.** This was performed in 24-well suspension culture plates (Greiner bio-one) as described previously\textsuperscript{66}. \textit{E. coli} and \textit{P. aeruginosa} were grown on LB agar medium at 37 °C. All mycobacteria were grown at either 32 °C (\textit{M. marinum}) or 37 °C (\textit{M. abscessus}, \textit{M. bovis} BCG and \textit{M. tuberculosis} mc\textsuperscript{2}6230) on Middlebrook 7H10 agar (BD Difco) supplemented with 10% OADC, and 24 µg/mL D-pantothenate (\textit{M. tuberculosis} mc\textsuperscript{2}6230). The wells were filled with 1 mL of the appropriate medium containing each of the CyC\textsubscript{19}-CyC\textsubscript{30} analogs at a single 30 µM final concentration. Each screening plate contained negative (DMSO) and positive (50 µM antibiotics) controls, as well as one well for sterility control (\textit{i.e.}, medium alone). For the 100% inhibition control we used 50 µM amikacin (Sigma Aldrich) for \textit{M. marinum}, \textit{M. abscessus}, \textit{M. bovis} BCG, \textit{M. tuberculosis} mc\textsuperscript{2}6230 and \textit{E. coli}; and 50 µM carbenicillin (Sigma Aldrich) for \textit{P. aeruginosa}. Each well was spotted with 10 µL of a bacterial culture at 5 × 10\textsuperscript{5} cells/mL. Colonies were counted after 1 day to 2 weeks of incubation at 37 °C, depending on the strain tested, to check bacterial viability. The CyC compounds leading to a minimum of 50% growth inhibition from CFU count were selected for subsequent MIC determination using the REMA assay.

**Resazurin microtiter assay (REMA) for MIC determination.** The concentrations of compound leading to bacterial growth inhibition were first determined using the resazurin microtiter assay (REMA)\textsuperscript{7, 10, 55}. Briefly, log-phase bacteria were diluted to a cell density of 5 × 10\textsuperscript{6} cells/mL and 100 µL of this inoculum was grown in a 96-well plate in the presence of serial dilutions of compounds. After 7-14 days incubation, 20 µL of a 0.025% (w/v) resazurin solution was added to each well (200 µL) and incubation was continued until the appearance of a color change (from blue to pink) in the
control well (bacteria without antibiotics). Fluorescence of the resazurin metabolite resorufin ($\lambda_{\text{excitation}}$, 530 nm; $\lambda_{\text{emission}}$, 590 nm) was then measured, and the concentration leading to 50% growth inhibition was defined as the MIC$_{50}$. See Supplementary Material for detailed protocol.

Determinant of cytotoxic activity for new CyC$_{19}$-CyC$_{30}$ analogs (resazurin assay). The cytotoxicity of compounds against eukaryotic cells was measured based on the reduction of resazurin as a value of cellular viability by metabolic activity. Murine (Raw264.7) macrophages (American Type Culture Collection TIB-71) were cultured from a freezer stock in Dulbecco’s modified Eagle medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum (FBS, Invitrogen) (DMEM$_{\text{FBS}}$). Cells were grown at 37 °C and 5% CO$_2$ to subconfluent concentrations, then 1 $\times$ 10$^5$ cells/well were seeded in 96-well flat-bottom Nunclon Delta Surface microplates with lid (ThermoFisher Scientific, ref. 167008) in a final volume of 200 µL per well and cultured for additional 24 h. The medium was removed by aspiration, and 200 µL of serial two-fold dilution of each compound (CyC$_{19}$ to CyC$_{30}$) in DMEM$_{\text{FBS}}$ were then added to each well. After 24 h incubation, 20 µL of a 0.025% (w/v) resazurin solution was added to each well. Fluorescence was measured following a 4-h incubation at 37 °C and 5% CO$_2$ in the dark, by excitation at 530 nm and emission at 590 nm as described above, leading to relative metabolic activities. Addition of DMSO was used as 100% viability reference and addition of 0.2% Triton X-100 solution served as negative standard (0% viability). All experiments were performed as two independent triplicates.

Intramacrophage killing assay. Murine (Raw264.7) macrophages (American Type Culture Collection TIB-71) were grown in DMEM$_{\text{FBS}}$ medium at 37 °C and 5% CO$_2$ to subconfluent concentrations, then 5 $\times$ 10$^4$ cells/well were seeded in 96-well flat-bottom Nunclon Delta Surface microplates with lid (ThermoFisher Scientific, ref. 167008) in a final volume of 200 µL per well and cultured for additional 24 h. The cells were infected with M. abscessus S at a multiplicity of infection (MOI) of 1:10 and incubated at 37 °C in the presence of 5% CO$_2$ for 3 h. Cells were then washed three times with DMEM then refed with DMEM$_{\text{FBS}}$ supplemented with 200 µg/mL amikacin for 1 h. at 37 °C and 5% CO$_2$ to kill all extra-cellular bacteria; washed again three times with DMEM prior
to the addition of 2-fold dilutions of CyC compounds or imipenem (IMP) in DMEM\textsubscript{PBS} supplemented with 50 μg/mL \textit{(i.e., 85.4 μM)} amikacin (200 μL final volume). In each plate, negative controls consisting of amikacin (50 μg/mL) and 1% DMSO \textit{(i.e., infected macrophages only)}; as well as positive controls containing amikacin (50 μg/mL) plus 80 μg/mL \textit{(i.e., 267 μM)} IMP were also included. Plates were incubated for 24 h at 37 °C, 5% CO\textsubscript{2}. Cells were washed three times with PBS and lysed by adding 200 μL of 0.1% Triton X-100. Serial dilutions of each culture were then plated at least in duplicate on 7H9 agar medium. Colonies were counted after 4 to 5 days of incubation at 37 °C to check intracellular bacterial viability following treatment with each compound concentration. DMSO-treated infected macrophages corresponded as control representing 100% of bacterial viability. Intracellular MIC\textsubscript{Raw} values were determined by fitting the CFU% sigmoidal dose-response curves in Kaleidagraph 4.2 software (Synergy Software). The lowest compound concentration inhibiting 50% of intracellular bacterial growth was defined as the MIC\textsubscript{50Raw}.

Experiments were done three times independently.

**CyC\textsubscript{17} and CyC\textsubscript{26} target enzymes identification.**

\textit{Activity-based protein profiling (ABPP).} Homogeneous bacterial suspension of \textit{M. abscessus} R in 7H9-S was adjusted at an OD\textsubscript{600} of 40 and then incubated with the selected CyC\textsubscript{17} or CyC\textsubscript{26} inhibitor (400 μM final concentration) or DMSO (control) at 37 °C for 2-3 h. under gentle shaking at 75 rpm. Bacteria were then washed 3 times with PBS containing 0.05% Tween 80, resuspended in PBS buffer at a 1:1 (w/v) ratio and then lysed by mechanical disruption on a BioSpec Beadbeater. Both CyC-treated \textit{M. abscessus} and DMSO-control lysate samples (750 μL – 0.75 mg total proteins) were labeled with 2 μM Desthiobiotin-FP probe for 90 min at room temperature. Samples were enriched for biotinylated proteins using Nanolink streptavidin magnetic beads 0.8 μm (SoluLink), according to the manufacturer’s instructions. The resulting captured biotinylated proteins solution was mixed with 5X Laemmli reducing sample buffer, and heated at 95 °C for 5 min. The released denatured proteins were subjected to tryptic digestion, peptide extraction, and LC-MS/MS analysis as described below. Alternatively, the CyC-treated \textit{M. abscessus} and DMSO-control lysate samples
(100 µL – 100 µg total proteins) were incubated with 2 µM ActivX TAMRA-FP probe (Thermo Fisher Scientific) for 90 min at room temperature and in absence of light. The reaction was stopped by adding 4X Laemmli reducing sample buffer and boiling at 95 °C for 5 min. The labeled proteins were further separated by SDS-PAGE electrophoresis. TAMRA fluorescence (TAMRA: \( \lambda_{\text{ex}} \) 557 nm, \( \lambda_{\text{em}} \) 583 nm) was detected using a ChemiDoc MP Imager (Bio-Rad).

Detailed Material and Methods regarding ABPP experiments is given in *Supplementary Material.*

**Protein identification and quantification.** Protein extract were loaded and stacked on a NuPAGE gel (Life Technologies). Stained bands were submitted to an in-gel trypsin digestion. Peptides extracts were reconstituted with 0.1% trifluoroacetic acid in 4% acetonitrile and analyzed by liquid chromatography (LC)-tandem mass spectrometry (MS/MS) using a LTQ-Orbitrap Velos Mass Spectrometer (Thermo Electron, Bremen, Germany) online with a nanoLC Ultimate 3000 chromatography system (Dionex, Sunnyvale, CA). Protein identification and quantification were processed using the MaxQuant computational proteomics platform, version 1.5.3.8 using a UniProt *M. abscessus* ATCC 19977 (Taxon 561007) database (date 2018.01; 4940 entries). The statistical analysis was done with Perseus program (version 1.5.6.0). Differential proteins were detected using a two-sample \( t \)-test at 0.01 and 0.05 permutation based FDR. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (www.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD014255.

Detailed Material and Methods is given in *Supplementary Materials.*
ANCILLARY INFORMATION.

Supporting Information: The Supporting Information is available free of charge on the ACS Publications website.

Detailed protocols, full chemical characterization, NMR spectra and HPLC chromatograms of all new CyC compounds; detailed protocols regarding the MIC determination and targets identification; Figure S1 (Activity based protein profiling workflow for the identification of the proteins covalently bound to each CyC inhibitor) and Figure S2 (Inhibition of the Ag85C \textit{Mabs} by CyC17); Table S1 (antimicrobial susceptibility testing of the new series of 12 CyCs by the agar plate method) and Table S2 (Extracellular antibacterial activities of all CyC analogs against both R & S variants of \textit{M. abscessus}). (PDF)

Tables S3-S8: CyC17 and CyC26 target proteins identified in \textit{M. abscessus} R total lysate and in culture cell by LC-ESI-MS/MS analysis, and full dataset from the proteomics analysis. (XLSX)

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§ AM and JNR contributed equally to this work.

CDS, SC and JFC conceived and designed the experiments. JNR, BPM, RRP, AAB and CDS provided chemical compounds. AM, VLM and SA performed the experiments. AM, SA, LC, CDS, SC and JFC contributed to data analysis and interpretation. AM, JNR, JLH, LK, CDS, SC and JFC wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.
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ABBREVIATIONS USED

ABPP, activity-based protein profiling; AMK, amikacin; CC₅₀, compound concentration leading to 50% of cell cytotoxicity; CF, cystic fibrosis; CFU, colony-forming units; CyC, Cyclipostins & Cyclophostin analogs; GPL, glycopeptidolipids; IMP, imipenem; INH, isoniazid; MIC₅₀, minimal inhibitory concentration leading to 50% of growth inhibition; NTM, non-tuberculous mycobacterial; pFDR, permutation false discovery rate; REMA, resazurin microtiter assay; RFU%, relative fluorescence units.
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