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Antibacterial activity of a new monocarbonyl analog of curcumin MAC 4 is associated with divisome disruption

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ABSTRACT

Curcumin (CUR) is a symmetrical dicarbonyl compound with antibacterial activity. On the other hand, pharmacokinetic and chemical stability limitations hinder its therapeutic application. Monocarbonyl analogs of curcumin (MACs) have been shown to overcome these barriers. We synthesized and investigated the antibacterial activity of a series of unsymmetrical MACs derived from acetone against *Mycobacterium tuberculosis* and Gram-negative and Gram-positive species. Phenolic MACs 4, 6 and 8 showed a broad spectrum and potent activity, mainly against *M. tuberculosis*, *Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus* (MRSA), with MIC (minimum inhibitory concentration) values ranging from 0.9 to 15.6 µg/mL. The investigation regarding toxicity on human lung cells (MRC-5 and A549 lines) revealed MAC 4 was more selective than MACs 6 and 8, with SI (selectivity index) values ranging from 5.4 to 15.6. In addition, MAC 4 did not demonstrate genotoxic effects on A549 cells and it was more stable than CUR in phosphate buffer (pH 7.4) for 24 h at 37 °C. Fluorescence and phase contrast microscopies indicated that MAC 4 has the ability to disrupt the divisome of *Bacillus subtilis* without damaging its cytoplasmic membrane. However, biochemical investigations demonstrated that MAC 4 did not affect the GTPase activity of *B. subtilis* FtsZ, which is the main constituent of the bacterial divisome. These results corroborated that MAC 4 is a promising antitubercular and antibacterial agent.

1. Introduction

Curcumin (CUR) is a β-diketone which is safe for humans [1] and recognized for its antibacterial effects against Gram-positive, Gram-negative and mycobacterial species [2,3]. Studies about its antibacterial activity have shown that the divisome and the bacterial membrane are potential targets of CUR [4,5]. Microscopy analyses demonstrated that treatment of *Bacillus subtilis* with CUR caused disruption of the divisome [5], which is a multiprotein complex operating on the cell division [6]. This effect has been correlated with the ability of CUR to inhibit the assembly of the cytoskeletal protein FtsZ (filamenting temperature-

sensitive protein Z) in protofilaments by stimulating its GTPase activity [4]. *In silico* investigations have elucidated the mode of interaction of CUR with FtsZ from *B. subtilis* and *Escherichia coli* [7], as well as *Mycobacterium tuberculosis* [8].

Despite the *in vitro* and *in vivo* antibacterial effects [9,10], CUR has poor chemical stability, low absorption and fast metabolism [11], requiring repetitive doses, which hinders its therapeutic use. One of the strategies to overcome these barriers is the design of synthetic analogs. Among them, monocarbonyl compounds constitute one of the main classes of CUR analogs which have been investigated, especially for their antitumor and anti-inflammatory properties [12,13]. Monocarbonyl

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analogues are designed by replacing the β -diketone moiety by a monoketone group, since the β -diketone is easily hydrolyzed under basic conditions [14] and it appears to be a substrate of a series of aldo-keto reductases [15]. Several monocarbonyl analogues of curcumin (MACs) have shown improved chemical and metabolic stability [16–19], as well as they exhibited an increase in intestinal permeability [20] and water solubility [21].

In addition to better physical-chemical properties and pharmacokinetic parameters than CUR, MACs have potent activity against bacterial species. Several studies have demonstrated the antimycobacterial activity of symmetrical MACs derived from piperidone against *M. tuberculosis*, *Mycobacterium bovis* and *Mycobacterium marinum* [22–25]. Regarding this class, Subhedar and co-authors described its selectivity against mycobacteria species [24]. On the other hand, the effects of symmetrical MACs derived from cyclopentanone and cyclohexanone have been more frequently reported against Gram-positive and Gram-negative species, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterobacter cloacae* and *E. coli* [26–31].

Tuberculosis is an infectious disease caused by some mycobacteria species, especially by *M. tuberculosis* [32]. The first-line drugs used to treat tuberculosis patients have been the same since the 70's [33]. The treatment consists of two stages: for 2 months, a combination of isoniazid, pyrazinamide, rifampicin and ethambutol, followed by the association of isoniazid and rifampicin for additional 4 months [34]. Nevertheless, the treatment period can be extended to 18–24 months for patients infected with resistant strains, with the inclusion of some second-line drugs, such as moxifloxacin and amikacin [35,36]. The long period of treatment and its adverse effects are factors that contribute to reducing patients' adherence to treatment [37]. Recently, three new anti-tuberculosis drugs were approved, bedaquiline and delamanid [38], ending a period of more than 4 decades without the approval of a new drug against tuberculosis. However, the adaptive capacity of *M. tuberculosis* has already led to the emergence of resistant strains to the two new antimycobacterial drugs [39]. Considering these problems, the development of new agents against *M. tuberculosis*, which can especially combat multidrug-resistant strains, is crucial.

In a previous study, our group investigated the antibacterial mode of action of a simplified curcumin (SCUR), a symmetrical MAC derived from acetone. We reported SCUR lost its ability to perturb *B. subtilis* cell division, but it demonstrated action on the membrane [5], which is a target of CUR [40]. Although some studies have described the antibacterial activity of symmetrical MACs, investigations on unsymmetrical MACs are unexplored. Herein, we synthesized a series of unsymmetrical MACs 1–8 derived from acetone and investigated the effects of methoxyl and hydroxyl substituents on antitubercular and antibacterial activities. The toxicity on human pulmonary cells and the selectivity indexes of most active compounds (MACs 4, 6 and 8) were determined. The most selective compound (MAC 4) was investigated regarding its genotoxic effects and chemical stability. Additionally, we evaluated the ability of MAC 4 to interfere with the assembly of the cell division apparatus and to perturb the GTPase activity of *B. subtilis* FtsZ.

2. Results and discussion

2.1. Chemistry

MACs 1–8 were prepared in two steps, using synthetic route A (Fig. 1). First, benzylideneacetone (1) was prepared by monocondensation of acetone with benzaldehyde using NaOH solution as catalyst, resulting in 89% yield. The intermediate 1 was condensed with methoxylated and hydroxylated benzaldehyde derivatives in H₂SO₄ solution as catalyst, furnishing MACs 1–3 and 5–8 in 23–74% yields.

The synthetic route A was not able to prepare MAC 4. Its crude product exhibited several compounds after thin layer chromatography analysis, suggesting successive purification steps. The catechol nature of MAC 4 was a barrier to its synthesis by aldol condensation reactions. Thus, the methoxymethyl group (MOM) was required to protect the free hydroxyl groups of respective 3,4-dihydroxybenzaldehyde [41,42]. Benzylideneacetone (1) was used as starting material and three steps were necessary to synthesize MAC 4 using synthetic route B (Fig. 2). First, 3,4-bis(methoxymethoxy)benzaldehyde (2) was synthesized in 73% yield from the protection of 3,4-dihydroxybenzaldehyde with methoxymethyl chloride (MOMCl) in dry acetone and K₂CO₃. In the second step, MOM-protected benzaldehyde (2) was condensed with benzylideneacetone (1) in order to provide MOM-diphenylpentanoid (3) in 42% yield. Lastly, MOM protecting groups were removed by treatment with HCl, providing MAC 4 in a 8% yield.

Structures of compounds were confirmed by their melting point and ¹H and ¹³C NMR spectra data analyses. For all compounds, NMR data, including chemical shifts, integrations multiplicities and coupling constants corresponded to the proposed structures. MACs 1–3 and 5–8 are known compounds and their NMR data are in accordance with previous literature [43–47]. MAC 4 is a new compound and HRMS spectrum data analyses were carried out. Detailed spectral analyses are presented in the Supplementary Information.

2.2. Antitubercular and antibacterial activities

Antitubercular activity of curcumin (CUR), simplified curcumin (SCUR) and unsymmetrical monocarbonyl analogues (MACs 1–8) was evaluated against *M. tuberculosis* H₃₇Rv (ATCC 27294). The antibacterial activity was tested against Gram-negative bacteria, including *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853) and *E. coli* (ATCC 43895), as well as Gram-positive bacteria species, such as methicillin-sensitive *S. aureus* (MSSA) (ATCC 25923), methicillin-resistant *S. aureus* (MRSA) (ATCC 33591), *S. epidermidis* (ATCC 12228) and *Enterococcus faecalis* (ATCC 29212). Antimicrobial potency was expressed as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values in μ g/mL (Table 1). Isoniazid, gentamicin and vancomycin were used as reference antibiotic drugs.

CUR showed activity against MSSA, *S. epidermidis* and *E. faecalis*, with MIC value of 125 μ g/mL. CUR also demonstrated activity against *A. baumannii*, with MIC value of 62.5 μ g/mL. However, it displayed MIC > 125 μ g/mL against *M. tuberculosis*, *P. aeruginosa*, *E. coli* and MRSA. These findings corroborate a previous study carried out by our group

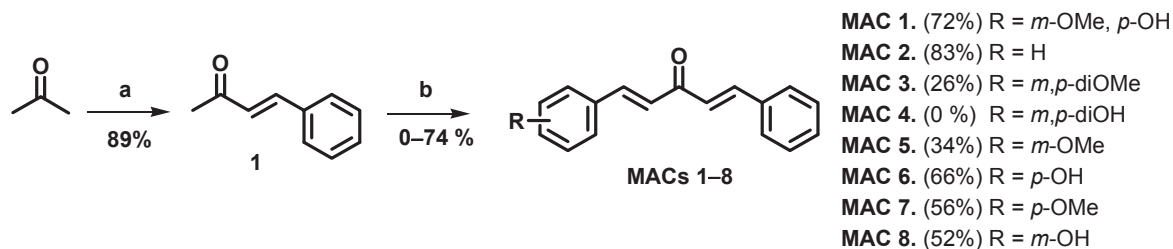


Fig. 1. Synthetic route A of MACs 1–8. Reagents and conditions: (a) benzaldehyde, NaOH, room temperature, 1 h; (b) methoxylated and hydroxylated benzaldehyde derivatives, H₂SO₄, EtOH, room temperature, 24–48 h.

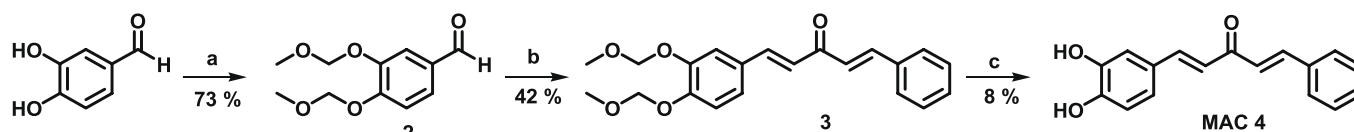


Fig. 2. Synthetic route B of MAC 4. Reagents and conditions: (a) MOMCl, K₂CO₃, dry acetone, N₂ atmosphere, room temperature, 1 h; (b) benzylideneacetone (1), NaOH, EtOH, N₂ atmosphere, room temperature, 8 h; (c) HCl, EtOH, N₂ atmosphere, room temperature, 48 h.

Table 1

Antitubercular and antibacterial activities of curcumin (CUR), simplified curcumin (SCUR) and unsymmetrical monocarbonyl analogs (MACs 1–8) expressed as MIC and MBC values in µg/mL.

| Compounds | R | Mt | | Ab | | Pa | | Ec | | MSSA | | MRSA | | Se | | Ef | |
|------------|-----------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| | | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| CUR | – | >125 | 62.5 | 62.5 | >125 | >125 | >125 | >125 | 125 | >125 | >125 | >125 | 125 | >125 | >125 | >125 | >125 |
| SCUR | – | 7.8 | 15.6 | 31.2 | 62.5 | 125 | >125 | >125 | >125 | 62.5 | 62.5 | 125 | >125 | >125 | >125 | >125 | >125 |
| MAC 1 | <i>m</i> -OMe, <i>p</i> -OH | 3.9 | 7.8 | 7.8 | >125 | >125 | >125 | >125 | >125 | 31.2 | 62.5 | 62.5 | 62.5 | >125 | >125 | >125 | >125 |
| MAC 2 | H | 0.9 | 62.5 | 62.5 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 |
| MAC 3 | <i>m,p</i> -diOMe | 15.6 | 125 | 125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 |
| MAC 4 | <i>m,p</i> -diOH | 0.9 | 3.9 | 3.9 | 31.2 | 62.5 | >125 | >125 | >125 | 125 | 125 | 15.6 | 15.6 | 62.5 | 125 | 62.5 | 125 |
| MAC 5 | <i>m</i> -OMe | 3.9 | 15.6 | 15.6 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 |
| MAC 6 | <i>p</i> -OH | 0.9 | 7.8 | 7.8 | >125 | >125 | >125 | >125 | >125 | 31.2 | 62.5 | 15.6 | 15.6 | 31.2 | 31.2 | >125 | >125 |
| MAC 7 | <i>p</i> -OMe | 7.8 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 | >125 |
| MAC 8 | <i>m</i> -OH | 0.9 | 3.9 | 3.9 | >125 | >125 | >125 | >125 | >125 | 7.8 | 15.6 | 7.8 | 15.6 | 7.8 | 15.6 | >125 | >125 |
| isoniazid | – | 0.04 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| gentamicin | – | – | 1.0 | – | 0.2 | – | – | 0.5 | – | – | – | – | – | – | – | – | – |
| vancomycin | – | – | – | – | – | – | – | – | – | 1.0 | – | 2.0 | – | 2.0 | – | 2.0 | – |

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; Mt = *Mycobacterium tuberculosis* H₃₇Rv (ATCC 27294); Ab = *Acinetobacter baumannii* (ATCC 19606); Pa = *Pseudomonas aeruginosa* (ATCC 27853); Ec = *Escherichia coli* (ATCC 43895); MSSA = methicillin-sensitive *Staphylococcus aureus* (ATCC 25923); MRSA = methicillin-resistant *Staphylococcus aureus* (ATCC 33591); Se = *Staphylococcus epidermidis* (ATCC 12228); Ef = *Enterococcus faecalis* (ATCC 29212); – not determined.

which described similar MIC values against *M. tuberculosis*, *P. aeruginosa*, *E. coli*, MSSA and *E. faecalis* [48].

In order to assess the effects of β-diketone by monocarbonyl moiety replacement on antitubercular and antibacterial activities, we compared the CUR and symmetrical simplified curcumin (SCUR). SCUR displayed higher potency than the CUR against *M. tuberculosis*, *A. baumannii*, *P. aeruginosa*, MSSA and MRSA, with MIC values of 7.8, 15.6, 62.5, 62.5 and 125 µg/mL, respectively, indicating that two carbonyls decreased the activity against these species.

We also investigated the effects of methoxyl and hydroxyl substituents removal on the SCUR ring B. Unsymmetrical MAC 1 (unsubstituted ring B) was more active against *M. tuberculosis*, *A. baumannii*, MSSA and MRSA (MIC 3.9, 7.8, 31.2 and 62.5 µg/mL, respectively) than SCUR (MIC 7.8, 15.6, 62.5 and 125 µg/mL, respectively), indicating that the withdrawal of *m*-OMe and *p*-OH groups on the aromatic ring B resulted in an increased activity. In order to evaluate the preliminary relationships between OMe and OH substituents on the aromatic ring A and their antitubercular/antibacterial activities, unsymmetrical MAC 1 was used as a framework.

First, we assayed MAC 2 in order to determine the effects of substituents on the aromatic rings A and B removal. MAC 2 (unsubstituted rings A and B) has MIC values of 62.5, >125 and >125 µg/mL against *A. baumannii*, MSSA and MRSA, respectively. These MIC values were reduced when compared to MAC 1 (MIC 7.8, 31.2 and 62.5 µg/mL, respectively). On the other hand, MAC 2 (MIC 0.9 µg/mL) showed higher antitubercular activity than MAC 1 (MIC 3.9 µg/mL). Fernandes and co-authors reported that lipophilicity is an important physico-chemical property in the development of new antitubercular agents. The lipophilicity increases the permeation of compounds through *M. tuberculosis* mycolic acid membrane [50]. The removal of hydrophilic

substituents on the aromatic ring A may be related to the increase in the antitubercular potency of MAC 2. In our previous study with α,β,γ,δ-unsaturated ketones (cinnamylideneacetophenones), we also found unsubstituted analog was more active against *M. tuberculosis* than its analogs substituted by hydrophilic groups [49].

Second, we evaluated the activities of MAC 3 (*m,p*-diOMe) and MAC 4 (*m,p*-diOH), and compared them to MAC 1 (*m*-OMe, *p*-OH). The comparison between MACs 3 and 1 MIC values revealed the replacement of OH by OMe at *para* position on the aromatic ring A reduced both spectrum and potency. On the other hand, MAC 4 demonstrated potent activity against *M. tuberculosis*, *A. baumannii*, *P. aeruginosa*, MSSA, MRSA, *S. epidermidis* and *E. faecalis* (0.9 µg/mL ≤ MIC ≤ 125 µg/mL), indicating the replacement of OMe by OH at *meta* position increased the spectrum of action and the potency when compared with MAC 1. In addition, the comparison between MIC/MBC values of MAC 4 against MSSA (MIC/MBC = 125 µg/mL) and MRSA (MIC/MBC = 15.6 µg/mL) indicated that the resistant strain was more sensitive to the MAC 4.

Third, the effects of *m*-OMe and *p*-OH substituents on the aromatic ring A were separately assessed, evaluating MACs 5 and 6, respectively. MAC 5 (*m*-OMe) was active only against *M. tuberculosis* and *A. baumannii* (MIC 3.9 and 15.6 µg/mL, respectively). On the other hand, MAC 6 (*p*-OH) demonstrated activity against *M. tuberculosis*, *A. baumannii*, MSSA, MRSA and *S. epidermidis* (0.9 µg/mL ≤ MIC ≤ 31.2 µg/mL). These results suggest that the *p*-OH substituent was more relevant for the activity than the *m*-OMe group. Furthermore, the comparison between the MIC values of MAC 1 (*m*-OMe, *p*-OH) and MAC 6 (*p*-OH) indicated that the presence of *m*-OMe group did not affect the activity against *A. baumannii* and MSSA, but its removal increased the potency against *M. tuberculosis*, MRSA and *S. epidermidis*.

Fourth, we investigated the activities of MAC 7 (*p*-OMe) and MAC 8

(*m*-OH), which are regioisomers of **MAC 5** (*m*-OMe) and **MAC 6** (*p*-OH), respectively. **MAC 7** was inactive (MIC > 125 µg/mL) against Gram-negative and Gram-positive species, and it displayed less activity (MIC 7.8 µg/mL) than its regioisomer **MAC 5** (MIC 3.9 µg/mL) against *M. tuberculosis*. On the other hand, **MAC 8** (MIC 0.9 µg/mL) demonstrated similar antitubercular activity to the regioisomer **MAC 6** (MIC 0.9 µg/mL). Regarding Gram-negative and Gram-positive species, **MAC 8** displayed higher activity than **MAC 6** against *A. baumannii*, MSSA, MRSA and *S. epidermidis*. In general, these results indicated that the position of OMe and OH substituents affects the bioactivity, suggesting that the *meta* position was more relevant for antibacterial and antitubercular activities than *para*.

In order to determine whether the active **MACs** displayed bactericidal or bacteriostatic activity against Gram-negative and Gram-positive species, the ratios between the MBC and MIC values were calculated. Antibacterial agents are generally regarded as bactericidal if MBC/MIC ratio < 4 [51,52]. All active **MACs** exhibited MBC/MIC ratios between 1 and 2, indicating that the compounds have bactericidal activity against these species.

MACs 4, 6 and 8 showed a broad spectrum and potent activity, mainly against *M. tuberculosis* and *A. baumannii*, with MIC values ranging from 0.9 to 7.8 µg/mL. *M. tuberculosis* is the main etiological agent of tuberculosis, a communicable lung disease that is one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent [37]. *A. baumannii* belongs to the ESKAPE pathogens, a bacterial group that also includes *E. faecium*, *S. aureus*, *Klebsiella pneumoniae*, *P. aeruginosa* and *Enterobacter* spp. [53]. These microorganisms are recognized as the leading cause of nosocomial infections worldwide [54]. In addition, the carbapenem resistant *A. baumannii* belongs to the critical category of World Health Organization's priority pathogens list for the development of new antibiotics [55], which reinforces the importance of the search for agents against *A. baumannii*.

2.3. Evaluation of **MACs 4, 6 and 8** toxicity on human cells and determination of selectivity indexes

The evaluation of the effects against human cells is a relevant step to investigate the selectivity and safety of new antibacterial agents. We selected the compounds **MACs 4, 6 and 8**, which demonstrated a broad spectrum and potent activity, in order to evaluate their toxicity on human cells. Since *M. tuberculosis* and *A. baumannii* were the most sensitive species to the selected compounds and cause pulmonary infection [56,57], we investigated the toxicity against human lung cells, including fibroblast (MRC-5) and epithelial (A549) cell lines. **MACs 4, 6 and 8** toxic effects were expressed according to the concentration required in order to reduce cell viability at 50% (IC₅₀) (Table 2). Doxorubicin was used as a reference cytotoxic agent [58].

MAC 4 was less toxic on MRC-5 and A549 cells (IC₅₀ 87.6 and 79.4 µmol/L, respectively) than **MACs 6 and 8** (17.5 µmol/L ≤ IC₅₀ ≤ 52.4 µmol/L). In addition, **MAC 4** was about 120 and 50 times less toxic than doxorubicin on MRC-5 and A549 cells, respectively. In order to compare

Table 2
Toxicity on human lung cells and selectivity indexes of selected compounds.

| Compounds | MRC-5 | | A549 | | SI | |
|--------------|------------------|------|------|------------------|------|-----|
| | IC ₅₀ | Mt | Ab | IC ₅₀ | Mt | Ab |
| MAC 4 | 87.6 ± 2.1 | 15.6 | 6.0 | 79.4 ± 1.3 | 14.2 | 5.4 |
| MAC 6 | 52.4 ± 1.2 | 11.9 | 1.7 | 50.5 ± 2.0 | 11.5 | 1.6 |
| MAC 8 | 17.5 ± 0.9 | 3.8 | 1.1 | 23.3 ± 1.8 | 5.1 | 1.5 |
| CUR | 42.1 ± 0.7 | – | – | 87.6 ± 0.2 | – | – |
| Doxorubicin | 0.7 ± 0.04 | – | – | 1.6 ± 0.2 | – | – |

IC₅₀ (in µmol/L) = concentration required in order to reduce cell viability at 50%; SI (IC₅₀/MIC) = selectivity index; MRC-5 = lung fibroblast cells; A549 = lung epithelial cells; Mt = *Mycobacterium tuberculosis*; Ab = *Acinetobacter baumannii*; – not determined.

the effects on prokaryotic and eukaryotic cells, the selectivity indexes (SI) of selected compounds were determined as the ratio between the IC₅₀ and MIC values against *M. tuberculosis* and *A. baumannii*. **MAC 4** was more selective than **MACs 6 and 8**, with SI values ranging from 5.4 to 15.6. Furthermore, the comparison between the SI values of **MAC 4** suggests it is more promising as an antitubercular agent, since it has higher SI values for this species (SI values of 15.6 and 14.2 for MRC-5 and A549 cells, respectively). According to Orme and collaborators, candidates for new antitubercular agents must have a SI value ≥ 10 [59].

2.4. Genotoxic evaluation of **MAC 4**

In order to further investigate the safety of **MAC 4**, we evaluated its genotoxic effects on human cells by the single cell gel electrophoresis assay (alkaline comet assay). This method is able to measure the damage induced by oxidizing, alkylating or intercalating agents in the DNA [60,61]. A549 cells were exposed to **MAC 4** at ¼IC₅₀ and ½IC₅₀ (19.8 and 39.7 µmol/L, respectively) for 24 h and their nuclei were submitted to electrophoresis. The A549 line was selected because it was the most sensitive to **MAC 4**, and subinhibitory concentrations were used in order to guarantee high percentages of metabolically viable cells. Hydrogen peroxide (1 mmol/L) was used as a reference genotoxic compound.

Fig. 3 presents the genotoxic parameters of **MAC 4**, including the tail moment and the percentage of DNA in tail, which are directly proportional to the DNA break [62]. A549 cells exposed to **MAC 4** at ¼IC₅₀ and ½IC₅₀ did not show significant genotoxic effects compared to the negative control (untreated cells). In addition, the comparison with the genotoxic parameters of cells treated with H₂O₂ (positive control) corroborated the weak genotoxic effect of **MAC 4**. Furthermore, **MAC 4** genotoxic parameters were similar to the **CUR** ones, and **CUR** is considered safe for humans [63].

2.5. Evaluation of **MAC 4** chemical stability

One of the main factors that hinder the clinical use of **CUR** is its poor stability under physiological conditions [11]. In order to assess whether the new monocarbonyl analog has overcome this limitation, we investigated **MAC 4** stability at pH 7.4 and 37 °C by monitoring the decrease in its UV–Vis absorption for 24 h (Fig. 4). The initial concentration of **MAC 4** was reduced at 28% after 24 h, while **CUR** initial concentration reduced at 57%. These data indicate **MAC 4** is more stable than its parent compound. Our findings corroborated other investigations about the increase in the chemical stability of monocarbonyl analogs when compared with **CUR** [17,64], supporting the hypothesis β-diketone moiety of curcumin is correlated with the poor chemical stability [12].

2.6. Effects of **MAC 4** on the divisome of *B. Subtilis*

In order to investigate whether cell division is a target for the new curcumin analog, we evaluated the effects of **MAC 4** on the divisome using *B. subtilis* strain expressing FtsZ-GFP, which labels the bacterial divisome. *B. subtilis* was selected because this species is commonly used in studies on the antibacterial action mode of new agents [65,66] and its division process has been extensively explored [6]. *B. subtilis* was treated with **MAC 4** at ½MIC (73.6 µmol/L) for up to 30 min and analyzed under the microscope. Hexyl gallate was used as a reference divisome disruptor [65].

Fig. 5 depicts phase contrast and fluorescence microscopy representative images of the negative and positive controls, and treatments with **MAC 4** after 15 and 30 min. Untreated *B. subtilis* cells (negative control) showed normal pattern of septation in which the divisome (marked by the fluorescence of FtsZ-GFP) was located in the middle of the cells as bars perpendicular to the long axis of the rods (Fig. 5A). The same pattern was observed for *B. subtilis* exposed to 1% DMSO (vehicle control) (data not shown). When cells were treated with hexyl gallate (positive control), there was a complete divisome disruption, and the

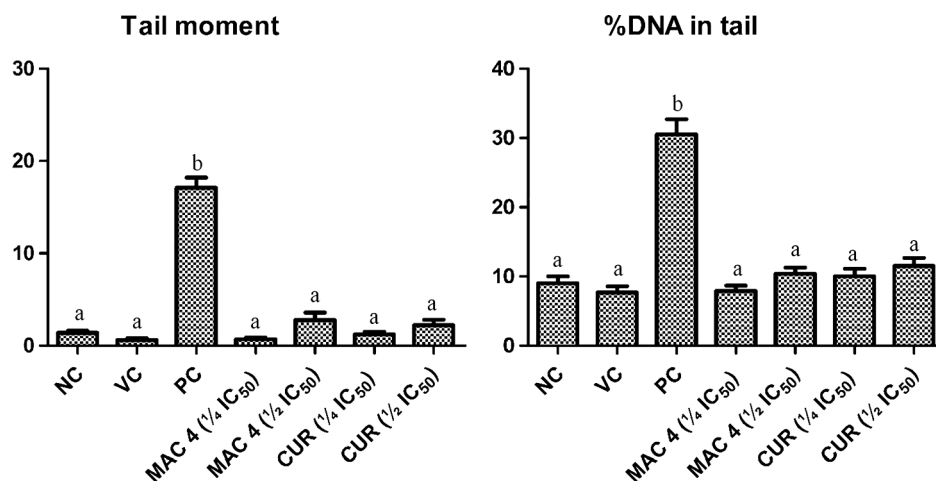


Fig. 3. Genotoxic parameters of **MAC 4** against A549 cell line measured by tail moment and percentage of DNA in tail using the alkaline comet assay. **NC** = negative control (untreated cells); **VC** = vehicle control (cells treated with 1% DMSO); **PC** = positive control (cells treated with H₂O₂ at 1 mmol/L). $\frac{1}{4}$ IC₅₀ values of **MAC 4** and **CUR** = 19.8 and 21.9 μ mol/L, respectively; $\frac{1}{2}$ IC₅₀ values of **MAC 4** and **CUR** = 39.7 and 43.8 μ mol/L, respectively. ANOVA with Tukey's post-test NC versus treatment. The same letters do not differ significantly at 5% level in comparison to all groups (a), different letters significantly differ at 5% level in comparison to all groups (b).

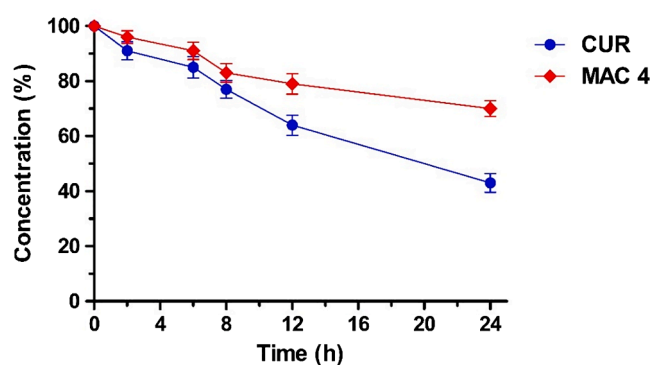


Fig. 4. Chemical stability of **MAC 4** and **CUR** at 35 μ mol/L (pH 7.4 and 37 °C) by monitoring the decrease at their maximum absorbance for 24 h. Data were represented as means \pm SEMs and expressed as percentage.

FtsZ-GFP fluorescence became dispersed in the cytoplasm (Fig. 5B). When *B. subtilis* was exposed to **MAC 4** at $\frac{1}{2}$ MIC for 15 min it was still possible to detect divisome formation, however with some stability interference (Fig. 5C). Although fluorescent septa could be detected in some dividing rods, other cells showed asymmetric division and/or filamentous morphology. After 30 min of treatment with **MAC 4** at $\frac{1}{2}$ MIC, the divisomes were completely unstructured (Fig. 5D). Previous research demonstrated that **CUR** completely disrupted the *B. subtilis* divisome after 15 min [4,5].

The cell division machinery is a complex cellular process, and it makes the localization of each protein involved in the process crucial for the performance of their functions. FtsZ protein constitutes the scaffold at the center of the dividing cells, and it recruits and coordinates several other proteins to accomplish the tasks of proper chromosome segregation, membrane invagination and remodeling, and cell wall synthesis [6]. Since the cell division apparatus is associated with the membrane, membrane perturbations may alter the spatial organization of proteins involved in bacterial division [67]. Thus, we investigated the ability of **MAC 4** at $\frac{1}{2}$ MIC to perturb the membrane of *B. subtilis* strain 168. Nisin was used as a reference compound for membrane disruption [68].

Fig. 6 shows phase contrast and fluorescence microscopy representative images of *B. subtilis* stained with the nucleic acid dyes SYTO9 and propidium iodide (IP). SYTO9 stains the nucleoid of all the cells (here artificially colored in blue), while IP can penetrate only the cells with corrupted membranes (artificially colored in red). Most untreated *B. subtilis* cells were stained in blue (Fig. 6A). The same pattern was observed when *B. subtilis* was exposed to 1% DMSO (data not shown). However, *B. subtilis* cells treated with nisin were stained in red (Fig. 6B).

Exposure to **MAC 4** at $\frac{1}{2}$ MIC, in both periods of 15 and 30 min (Fig. 6C and 6D, respectively), led to a prevalence of blue and pink stained cells, indicating membrane integrity, which corroborated the effect of **MAC 4** on the *B. subtilis* divisome.

2.7. Effects of **MAC 4** on the GTPase activity of *B. Subtilis* FtsZ

In order to verify whether the divisome disruption after treatment with **MAC 4** is related to a direct action on FtsZ protein, we evaluated its ability to affect the GTPase activity of FtsZ from *B. subtilis*. GTP binding and hydrolysis are necessary for the polymerization of FtsZ and the assembly of FtsZ protofilaments. Therefore, compounds that affect the GTPase activity of FtsZ are promising inhibitors of the bacterial cell division process [69,70]. We incubated FtsZ with **MAC 4** at MIC and $2 \times$ MIC (147 and 294 μ mol/L, respectively), and its GTPase activity was measured. The protein incubated with 1% DMSO was used as a negative control. The GTPase activity of FtsZ was weakly increased by exposure to **MAC 4** at MIC and $2 \times$ MIC (15% and 23%, respectively) when compared to the negative control. This increase was not significant, and it indicates that the ability of **MAC 4** to disrupt the divisome is not related to a direct action on the GTPase activity of *B. subtilis* FtsZ. Besides FtsZ, there are other proteins involved in divisome dynamics, which can be the molecular target of **MAC 4**. The lack of FtsZ GTPase inhibition of **MAC 4** can be related to β -diketone moiety removal. Kaur and collaborators described *in silico* FtsZ inhibition studies of **CUR** demonstrating that the β -diketone established relevant interactions with glycine residues 21 and 22 of *B. subtilis* FtsZ [7]. In a previous study with monocarbonyl **SCUR**, we also did not identify the ability of this compound to alter the FtsZ GTPase activity [5], corroborating the essentiality of the β -diketone for an effective interaction with FtsZ.

3. Conclusion

In summary, we synthesized a series of unsymmetrical monocarbonyl compounds derived from acetone as part of our ongoing search for antibacterial agents based on the **CUR** structure. **MACs 4, 6 and 8** showed potent activity against Gram-negative and Gram-positive species, and *M. tuberculosis*. The assessment of toxicity on human cells revealed **MAC 4** as the most selective analog. In addition, preliminary investigations of genotoxicity and chemical stability indicated that **MAC 4** is potentially safe and stable under physiological conditions, respectively. **MAC 4** action mode involved divisome disruption, which is an innovative target for the development of new antibacterial agents. Altogether, our results corroborate the **CUR** potential as a privileged framework for the discovery of compounds for bacterial infection treatments, including tuberculosis.

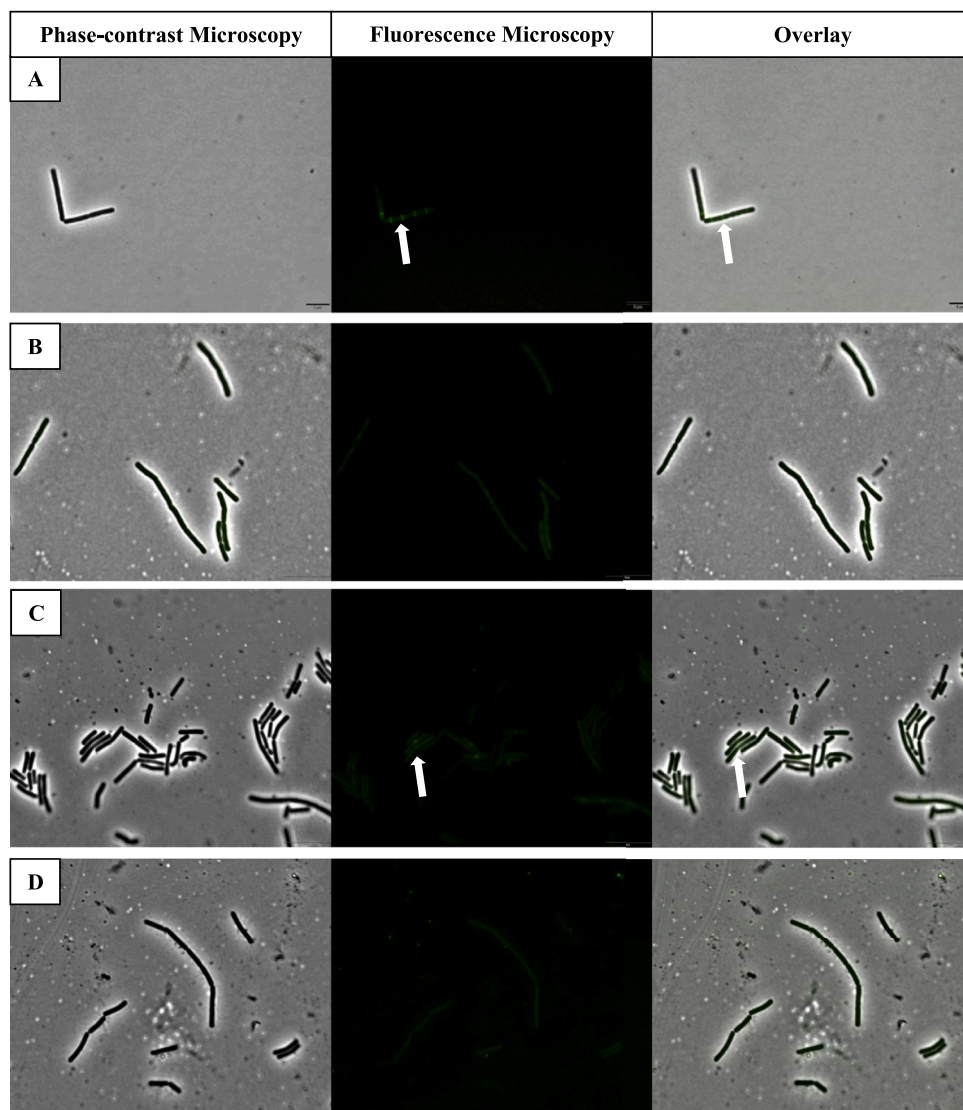


Fig. 5. Effects of MAC 4 on the divisome of *B. subtilis* expressing FtsZ-GFP. (A) Untreated *B. subtilis* (negative control); (B) *B. subtilis* treated with hexyl gallate at 255 $\mu\text{mol/L}$ after 30 min (positive control); (C) *B. subtilis* treated with MAC 4 at $1/2$ MIC (73.6 $\mu\text{mol/L}$) after 15 min; (D) *B. subtilis* treated with MAC 4 at $1/2$ MIC after 30 min. Magnification: 100 \times ; bar 5 μm . The arrows indicate the intact divisome.

4. Experimental

4.1. Chemistry

4.1.1. Material and instruments

CUR and all reagents were purchased from Merck (Kenilworth, USA), and solvents and catalysts from Synth (Diadema, Brazil). Reactions were monitored using thin-layer chromatography (TLC) (Merck, Kenilworth, USA). Silica-gel (200–300 mesh) and LH-20 (Sephadex) (Merck, Kenilworth, USA) were used for column chromatography separations. Melting points (MP) were determined in Tecnonon PFM-IIIV apparatus (MS Tecnonon Instrumentação, Piracicaba, Brazil) and were uncorrected. Nuclear magnetic resonance spectra (^1H and ^{13}C NMR) were carried out on Bruker Avance III 14.1 T (600 MHz) and Bruker Avance III 9.4 T (400 MHz) spectrometers (Bruker, Billerica, USA), using CDCl_3 or $\text{DMSO}-d_6$ as solvents and TMS (tetramethylsilane) as internal reference (Merck, Kenilworth, USA). Chemical shifts (δ) and coupling constants (J) were expressed in ppm and Hz, respectively. Multiplicities were reported as singlet (s), doublet (d) and doublet of doublet (dd). High resolution mass spectrum of MAC 4 was performed on ESI-QqTOF-MS spectrometer (Xevo G2 Xs, Waters, Massachusetts, USA).

4.1.2. Synthesis of SCUR

SCUR was synthesized by reaction between acetone and vanillin as previously described by Morão and co-authors [5].

4.1.3. Synthesis of MACs 1–3 and 5–8

MACs 1–3 and 5–8 were synthesized in two steps [71].

4.1.3.1. Synthesis of benzylideneacetone (1). A solution of benzaldehyde (8 mmol) in acetone (15 mL) was cooled in an ice bath for 10 min. Acetonic suspension of NaOH (1 mL, 0.5 mol/L) was added dropwise and reaction medium was stirred at room temperature for 1 h. Reaction medium was poured onto crushed ice from deionized water. The crude product was partitioned with ethyl acetate (3×25 mL), and the combined organic phase was concentrated under reduced pressure. Compound 1 was recrystallized in cold EtOH.

4.1.3.2. Synthesis of MACs 1–3 and 5–8. To a solution of benzylideneacetone (1) (5.2 mmol) in EtOH (10 mL) was added the respective benzaldehyde derivative (5.0 mmol). Catalytic amounts of H_2SO_4 were added to the reaction mixture, and the solution was stirred at room temperature for 24–48 h. Reaction medium was poured onto crushed

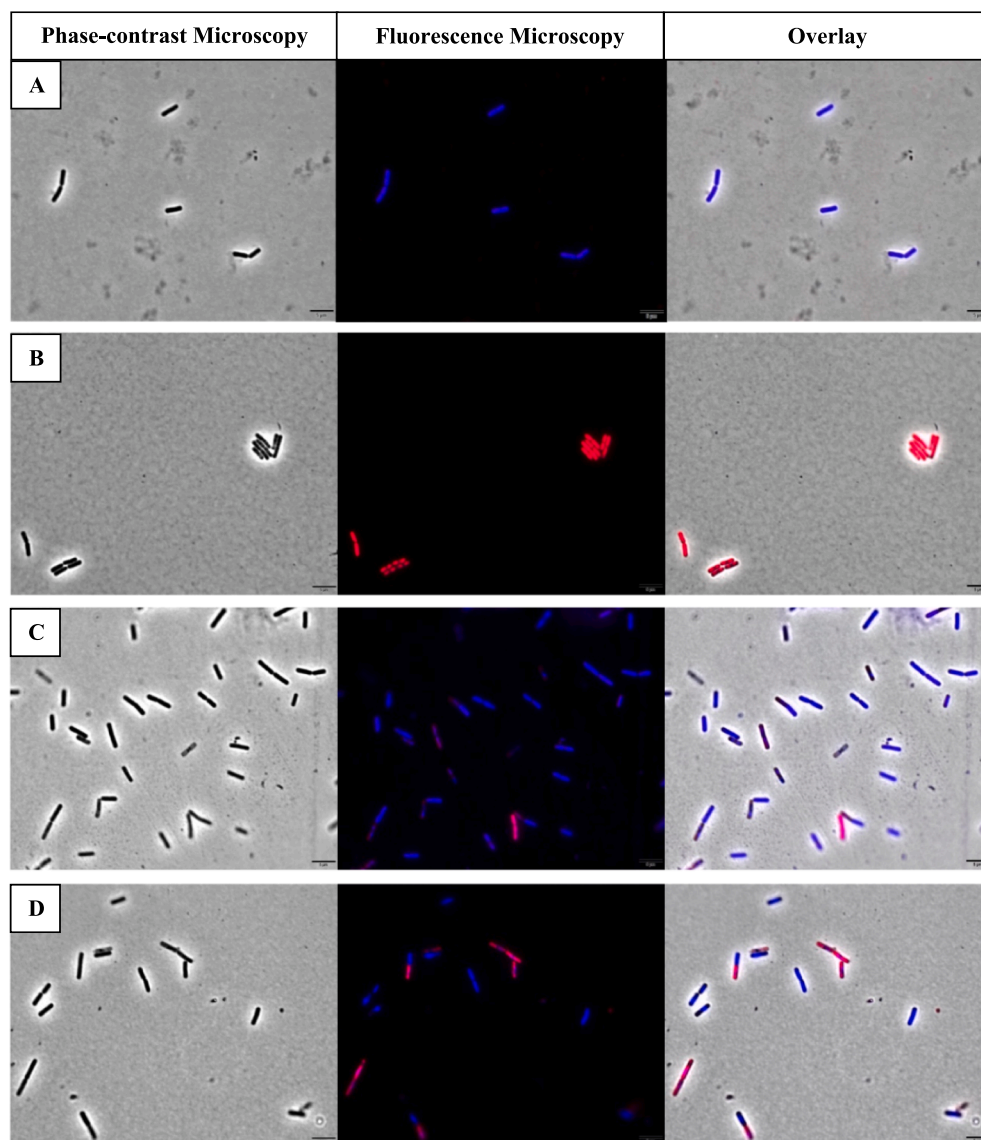


Fig. 6. Effects of MAC 4 on the membrane of *B. subtilis* strain 168 stained with Live/Dead BacLight kit. (A) Untreated *B. subtilis* (negative control); (B) *B. subtilis* treated with nisin at 1.5 $\mu\text{mol/L}$ after 30 min (positive control); (C) *B. subtilis* treated with MAC 4 at $\frac{1}{2}$ MIC (73.6 $\mu\text{mol/L}$) after 15 min; (D) *B. subtilis* treated with MAC 4 at $\frac{1}{2}$ MIC after 30 min. Magnification: 100 \times ; bar 5 μm . Blue cells: intact membrane. Pink-red cells: damaged membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ice. The crude product was partitioned with ethyl acetate (3×25 mL), and the combined organic phase was concentrated under reduced pressure. **MACs 1, 6 and 8** were purified by gel filtration column chromatography (LH-20) using EtOH as eluent. **MAC 2** was recrystallized in EtOH. **MACs 3, 5 and 7** were purified by column chromatography over silica gel using mixtures of hexane and ethyl acetate (4:1), (9:1) and (9:1) as mobile phases, respectively.

4.1.4. Synthesis of MAC 4

MAC 4 was synthesized in three steps [41].

4.1.4.1. Synthesis of 3,4-bis(methoxymethoxy)benzaldehyde (2). A suspension of K_2CO_3 (50 mmol) in dry acetone (25 mL) was cooled in an ice bath for 10 min. After this period, 3,4-dihydroxybenzaldehyde (5 mmol) and methoxymethyl chloride (MOMCl) (20 mmol) were added sequentially. The reaction mixture was stirred at room temperature for 1 h under N_2 atmosphere. The suspension was diluted with deionized water (25 mL), and the crude product extracted with ethyl acetate (3×25 mL). The combined organic layer was concentrated under reduced pressure. Compound **2** was purified by column chromatography over silica gel using hexane and ethyl acetate (7:3) as mobile phase.

4.1.4.2. Synthesis of compound 3. A solution of compound **2** (2 mmol) and benzylideneacetone (**1**) (2.2 mmol) in EtOH (7 mL) was cooled in an ice bath for 10 min. Ethanolic solution of NaOH (1 mL, 1 mol/L) was added dropwise to the reaction mixture. The solution was stirred at room temperature for 8 h under N_2 atmosphere. Reaction medium was poured onto crushed ice. The crude product was partitioned with ethyl acetate (3×15 mL), and the combined organic phase was concentrated under reduced pressure. Compound **3** was purified by column chromatography over silica gel using hexane and ethyl acetate (3:2) as mobile phase.

4.1.4.3. Synthesis of MAC 4. A solution of compound **3** (1 mmol) in EtOH (5 mL) and 33% HCl (0.3 mL) was stirred at room temperature for 48 h under N_2 atmosphere. Reaction medium was poured onto crushed ice. Precipitated crude product was filtered, washed with cold water and dried at room temperature. **MAC 4** was purified by gel filtration column chromatography (LH-20) using EtOH as mobile phase.

4.2. Antibacterial assay

Bacteria species used in this study were *A. baumannii* (ATCC 19606), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 43895), methicillin-sensitive

S. aureus (ATCC 25923), methicillin-resistant *S. aureus* (ATCC 33591), *S. epidermidis* (ATCC 12228), *E. faecalis* (ATCC 29212) and *B. subtilis* strain 168. MIC and MBC values were determined by the microdilution method according to the Clinical and Laboratory Standards Institute with minor modifications [72]. CUR, SCUR and MACs 1–8 were diluted in DMSO and assayed in concentrations ranging from 125 to 0.5 µg/mL. Gentamicin and vancomycin were used as reference antibiotic drugs against Gram-negative and Gram-positive bacterial species, respectively. MIC value was defined as the lowest concentration of compound that inhibited visible microbial growth. MBC was determined by subculturing aliquots from the wells with no visual bacterial growth onto trypticase soy agar (TSA) plates, which were incubated at 37 °C for 24 h. MBC value was defined as the lowest concentration of compound that allowed no visible growth on the solid medium. Three independent assays were performed.

4.3. Antitubercular assay

Antitubercular activity was evaluated against *M. tuberculosis* H₃₇Rv (ATCC 27294) according to Resazurin Microtiter Assay [73], with minor modifications described by our previous work [49]. CUR, SCUR and MACs 1–8 were prepared in DMSO and evaluated in concentrations ranging from 125 to 0.5 µg/mL. Isoniazid was used as reference antitubercular drug. Three independent assays were performed. MIC value was defined as the lowest concentration resulting in 90% inhibition of growth of *M. tuberculosis*.

4.4. Cytotoxicity assay

Cell lines used in this study were MRC-5 (normal human lung fibroblast cell line ATCC CCL-171) and A549 (human lung adenocarcinoma epithelial cell line ATCC CCL-185). The IC₅₀ values were determined as described by Silva and co-authors [74]. Cells were treated with CUR and MACs 4, 6 and 8 in concentrations ranging from 100 to 1 µmol/L. Doxorubicin was used as a reference cytotoxic drug. Three independent assays were performed. The IC₅₀ value was calculated from an analytical curve through a regression analysis and it represents the concentration required to reduce the viability of cells at 50%.

4.5. Genotoxicity assay

The cell line used in this investigation was the A549 (human lung adenocarcinoma epithelial cell line ATCC CCL-185). The genotoxicity was assessed using the alkaline version of the comet assay (or single cell gel electrophoresis) [60]. Minor modifications in this protocol and their detailed procedures were reported in previous work by Polaquini and co-authors [48]. Cells were treated with CUR and MAC 4 at respective ½IC₅₀ and ¼IC₅₀ values. Hydrogen peroxide (1 mmol/L) was used as reference genotoxic compound. The level of DNA damage was assessed by image analysis system (TriTekCometScore™ 1.5, 2006) and the percentages of DNA in the tail and tail moment were calculated. The results were expressed as the mean ± standard error of three independent experiments performed in duplicate.

4.6. Chemical stability assay

Chemical stability of MAC 4 (at λ_{max} 375 nm) and CUR (at λ_{max} 426 nm) was analyzed by monitoring their UV–visible absorptions in phosphate buffer (pH 7.4) at 37 °C. Minor modifications in this protocol and their detailed procedures were reported in our previous work [48].

4.7. Phase contrast and fluorescence microscopy assays

The effect of MAC 4 on the divisome of *B. subtilis* was investigated according to Morão and co-authors [5]. *B. subtilis* (*amy::Pspac-ftsZ-gfpmut1*) was cultivated in the presence of 0.02 mmol/L IPTG (isopropyl

β-D-1-thiogalactopyranoside) to induce the expression of FtsZ-GFP (Green Fluorescent Protein) from the *Pspac* promoter. Cells were exposed to MAC 4 at its ½MIC (73.6 µmol/L) for 15 and 30 min at 29 °C. Hexyl gallate (255 µmol/L) was used as a reference divisome disruptor [65].

The membrane disruption assay was performed as described by Polaquini and co-authors [48]. *B. subtilis* strain 168 was treated with MAC 4 at its ½MIC (73.6 µmol/L) for 15 and 30 min at 29 °C. Membrane integrity was assessed using the Live/Dead BacLight Bacterial Viability kit (Thermo-Scientific L7012). Nisin (1.5 µmol/L) was used as reference membrane disruptor. All visualizations were carried out using an Olympus BX61 microscope (Olympus, Melville, USA) equipped with a monochromatic camera OrcaFlash 2.8 (Hamamatsu, Shizuoka, Japan). Image documentation and processing were done using the software CellsSen Dimension (Olympus, Melville, USA).

4.8. Anti-FtsZ assay

FtsZ of *B. subtilis* was expressed and purified using the ammonium sulfate precipitation method as described by Król and Scheffers [75]. FtsZ GTP hydrolysis rate was determined using the malachite green phosphate assay as described by Król and co-authors [65].

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.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2021.104668>.

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