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Antimycobacterial activity of rhodamine 3,4-HPO iron chelators against *Mycobacterium avium*: analysis of the contribution of functional groups and of chelator´s combination with ethambutol

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Rhodamine-labelled 3-hydroxy-4-pyridinone (3,4-HPO) chelators exhibit antimycobacterial activity, related but not limited to their iron binding capacity. We previously found that bacterial growth inhibition observed for chelators with ethyl substituents on the amino groups of the xanthene ring of rhodamine and a thiourea linkage between rhodamine and the chelating unit (**MRH7** and **MRB7**) was different from that of compounds with methyl substituents and an amide linkage (**MRH8** and **MRB8**). In this work we evaluated the antimycobacterial activity of two new chelators (**MRH10** and **MRB9**), expressly designed to allow: (a) the direct comparison of the influence of the functional groups per se and (b) identification of the finest combination to achieve a higher biological activity. The activity of the chelators was assessed, as previously, by measuring their effect against *M. avium*. In this study we also report the antimycobacterial effect of **MRH7**, which proved to be the best performer of all four chelators, in combination with ethambutol, which is one of the antibiotics currently in use to treat mycobacterial infections. The results are indicative that a combination of 3,4-HPO iron chelators with an antibiotic is a promising strategy to fight *M. avium* infections. The current results are relevant for the choice of the best chelator in our set of compounds and also for the design of novel molecular architectures to target cellular membranes.

Introduction

Bacterial resistance to currently available antibiotics is a serious health problem [1, 2] and therefore efforts to develop new molecules that target unconventional mechanisms and pathways involved in infectious processes are urgently required. Iron is an essential element for the growth of all bacteria and also for fungi and protozoa [3] and it has been demonstrated that limiting the iron available for the growth of pathogenic microorganisms can be considered as a strategy to control infections and numerous examples of the use of iron chelation therapies and the consequently improvement in infection susceptibility of several microorganisms have been reported [3-12].

Our group has been particularly interested in mycobacterial infections, namely those caused by the opportunistic infectious pathogen *Mycobacterium avium* that mostly affects patients with compromised immunity [13]. The use of iron chelators to restrict the iron available for mycobacterial growth have been reported [14], namely for *Mycobacterium tuberculosis* [15, 16] and *M. avium* [17-20].

Mycobateria, as many microorganisms, produce various types of siderophores to acquire iron with different characteristics in terms of lipophilicity: the lipophilic mycobactins, which remain cell-wall-associated and hydrophilic carboxymycobactins or exochelins which are released to the extracellular medium [14, 21]. These siderophore types are hexadentate ligands that possess similar hydroxamate binding sites and vary only in lateral chain characteristics, conferring lipophilic character on mycobactins or hydrophilic on carboxymycobactins [14, 22]. The importance of these molecules for bacterial subsistence in their host has been demonstrated by reduced growth of mutant *M. tuberculosis* strains, in which siderophore production is inhibited [23].

3-Hydroxy-4-pyridinones (3,4-HPOs) are bidentate oxygen ligands, which are synthetically versatile allowing the synthesis

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of chelators of variable denticity. These hard ligands show a very high capacity to trap Fe(III) providing an O_6 coordination sphere through the binding of the appropriate number of ligands according to its denticity. Chelation of iron by 3,4-HPO ligands confers an iron-binding moiety different from that provided by natural siderophores (catechol or hydroxamate moiety). Various therapeutic applications have been described for 3,4-HPO ligands [24] and there are also several reports on the effect of these ligands on the inhibition of Gram-positive and Gram-negative bacteria growth [7, 11, 12] and *M. avium* infection [18-20].

In our previous studies regarding the antimycobacterial activity of fluorescent 3,4-HPO chelators we found that the presence of the fluorophores is determinant for the inhibition of *M.avium* intramacrophagic growth since the 3,4-HPO chelating units alone do not have an effect [19, 20]. For the particular case of rhodamine labelled chelators it was demonstrated that two molecular fragments seem to be determinant for a higher activity: (a) a thiourea linkage and (b) ethyl substituents on the amino groups of the xanthene ring. Moreover, it was confirmed that the rhodamine fluorophores *per se* are not active in inhibiting *M.avium* intramacrophagic growth [20].

In order to substantiate the previous results and to identify the finest combination to achieve a higher biological activity we designed the chelators **MRB9** and **MRH10** (Figure 1) that contain these two functional groups in different positions of the molecular framework (in different colour in Figure 1) and measured their antimycobacterial activity in comparison with that of chelator **MRH7** previously studied.

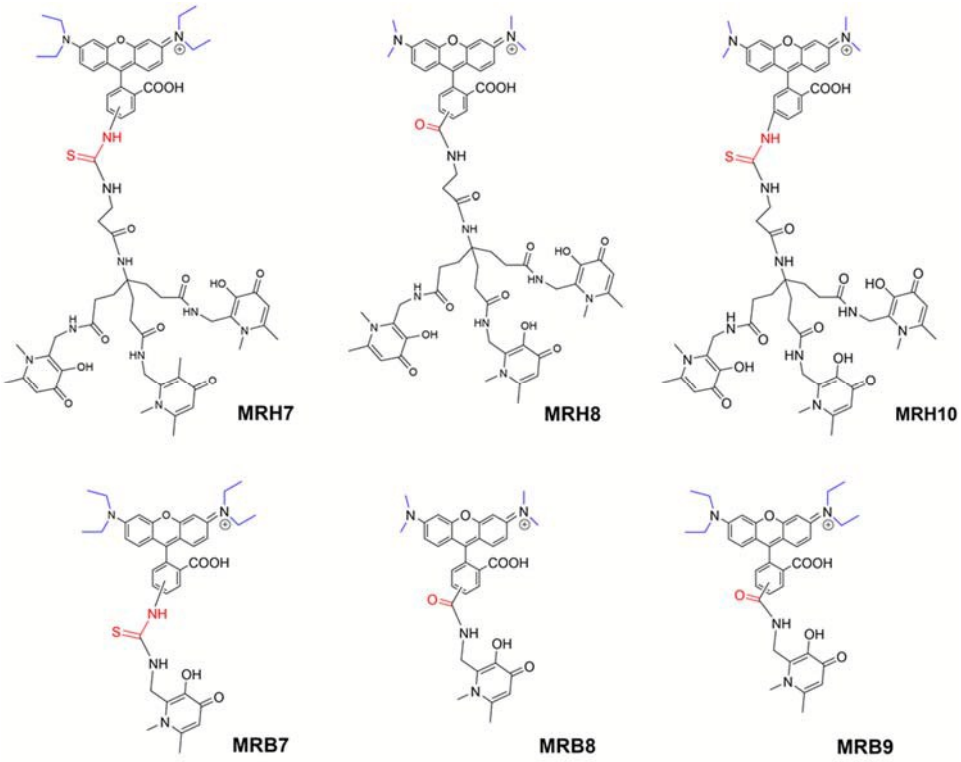

Figure 1. Formulae of rhodamine derived 3,4-HPO iron(III) chelators.

Combination of new molecules with classic antibiotics is an emerging and promising strategy to overcome bacterial resistance mechanisms and to restore antibiotic effectiveness [25]. The clinical potential of the combination of iron chelators with other antibiotics has been demonstrated to fight bacterial [14, 26-30], fungal [31] and protozoal [32] infections, although, no reports were found of the use of combinations therapies involving iron chelators and classic anti-mycobacterial antibiotics used in clinics.

In the present work we report studies in which **MRH7** was tested in combination with ethambutol, a drug, [(S,S)-2,20-(ethylenediimino)-di-1-butanol], that was first reported in the early 1960s [33] and is one of the few well-known and reliable antimycobacterial used in clinics [32, 34]. Ethambutol acts by inhibiting mycobacterial arabinofuranosyl transferases which are responsible for glycosylation steps in the biosynthesis of lipoarabinomannan (LAM) and arabinogalactan (AG), both constituents of the mycobacterial cell wall [35, 36]. Though ethambutol is active against *M. avium* pathogens high doses or combinations therapies are required and resistance phenomena have been described [37-41]. Moreover, there is some evidence that combined therapies using ethambutol have advantages due to the ability of this drug to increase cell wall permeability in *M. avium* and consequently favour the influx of other drugs tested in combination [42, 43]. In addition, there are reports of the ability of ethambutol to chelate trace metals, namely iron and copper, therefore contributing to a

better control of the infection additionally to their usual described mechanism of action [44-46].

In this study we report the *in vitro* inhibitory effect of the new chelators and the *in vitro* inhibitory effect of MRH7, which proved to be the best performer of all four chelators studied by our group, when used in combination with ethambutol against *M. avium.* Taken together, our results suggest that the conjugation of hexadentate HPO iron chelators to a second bioactive molecule is a promising strategy against *M. avium* microorganism and this work is the first report of the used of iron chelators in combination with other antibiotics for the treatment of *M. avium* infection

Experimental

Materials and Methods

Chemistry

General information. Chemicals were obtained from Sigma–Aldrich (grade puriss, p.a.) or Fluka (p.a.) and were used as received unless otherwise specified.

NMR spectra were recorded on a Bruker Avance III 400, operating at 400.15 MHz for ^{1}H and 100.62 MHz for ^{13}C atoms, equipped with pulse gradient units, capable of producing magnetic field pulsed gradients in the z-direction of 50.0 G/cm or on a Bruker Avance III Two-dimensional $^{1}H/^{1}H$ correlation spectra (COSY), gradient selected $^{1}H/^{13}C$ heteronuclear single quantum coherence (HSQC) and $^{1}H/^{13}C$ heteronuclear multiple bond coherence (HMBC) spectra were acquired using the standard Bruker software. NMR and Mass Spectrometry analyses were performed at Laboratory of Structural Analysis, Centro de Materiais da Universidade do Porto (CEMUP) (Portugal). Elemental analyses were performed at the analytical services of University of Santiago (Spain).

Synthesis of 5(6)-carboxytetraethylrhodamine (F9). To a solution of 3-diethylaminophenol (3.037 g; 1.78×10^{-2} mol) in propionic acid (7 mL), 1,2,4-benzenetricarboxylic anhydride (1.755 g; 8.86×10^{-3} mol) and *p*-toluenesulfonic acid (359 mg; 1.86×10^{-3} mol) were added and the mixture was heated to 100º C for 6 days. Subsequently the reaction was worked-up by adding a mixture of ice/water (300 mL), extracting with methanol/dichloromethane 85:15 (3×200 mL) and drying the organic phase with anhydrous sodium sulphate. The product was purified by gradient flash column chromatography, eluting with methanol/dichloromethane (3:7) to afford 5(6)-carboxytetraethyl-rhodamine (**F9**) (168.6 mg; 4%) as a purple solid with a mixture with a 1:1.1 ratio of 5'- 4'- isomers (being the 5'-isomer the least polar one) as determined through NMR spectra analysis. The spectral data are accordingly with the literature [47, 48].

^{1}H NMR (400.15 MHz, MeOD-d_4, ppm): δ 1.30 (t, *J* 7.1 Hz, 24H, 8×CH3-rhodamine, 4'-isomer, 5'-isomer); 3.66 (q, *J* 7.1 Hz, 16H, 8×CH2-rhodamine, 4'-isomer, 5'-isomer), 6.93 (s, 2H, H4, H5), 6.98-7.08 (m, 2H, H2, H7), 7.19-7.25 (m, 5H, H1, H8+ H5' 4'-isomer), 7.87 (s, 1H, H6' 5'-isomer), 8.11 (d, *J* 7.8 Hz, 1H, H4' 5'-isomer), 8.19 (d, *J* 8.1 Hz, 1H, H5' 4'-isomer), 8.27 (d, *J* 8.0 Hz 1H, H3' 5'-isomer), 8.80 (s, 1H, H3' 4'-isomer).

Synthesis of 3-hydroxy-4-pyridinone ligands. The fluorescent bidentate and hexadentate ligands were prepared by the coupling of fluorophore (rhodamine derivatives) to a protected bidentate or hexadentate 3-hydroxy-4-pyridinone (3,4-HPO) unit [20]. The bidentate or hexadentate unit were synthesized in our laboratory following the procedures described in the literature [18, 49]. A diagram of the 3,4-HPO units and fluorophores used to obtain the final fluorescent ligands synthesized in this work and the synthetic procedures are outlined in Figure 2.

3,4-HPO hexadentate unit was coupled tetramethylrhodamine isothiocyanate (**F10**) in the presence of anhydrous *N,N*-dimethylformamide (DMF) and triethylamine to produce **1**, followed by deprotection with BCl_3 to yield the **MRH10**.

Compound **2** was obtained by reaction of 3,4-HPO bidentate unit with 5(6)-carboxytetraethylrhodamine (**F9**) in the presence of anhydrous DMF, dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) in order to generate the activated ester form of the rhodamine derivate and it was subsequently deprotected with BCl_3 to yield the rhodamine **MRB9**.

Compound 1. To a solution of tetramethylrhodamine isothiocyanate (**F10**) (0.0319 g, 7.19×10^{-5} mol) in anhydrous DMF (0.5 mL) and triethylamine (0.01 mL) was added the 3,4-HPO hexadentate unit (0.0622g, 5.99×10^{-5} mol) and the mixture was stirred at room temperature in the dark and under argon atmosphere, for 48 hours. The product was purified by gradient flash column chromatography, eluting with chloroform/methanol (7:3) and increasing polarity until chloroform/methanol (4:6) and ammonia. The sample was purified by TLC, eluting with acetonitrile/water/ammonia (3:2:0.05) to afford **1** (0.0430 g; 48%) as a purple solid. Only 5' isomer was obtained as determined through NMR spectra analysis. ^{1}H NMR (400.15 MHz, MeOD-d_4, ppm): δ 1.87 (m, 6H, $CC\underline{H}_2CH_2$); 2.08 (m, 6H, $CCH_2C\underline{H}_2$); 2.33 (s, 9H, 6''-CH_3); 2.45 (t, 6,2 Hz, 2H, $NCH_2C\underline{H}_2CO$); 3.19 (s, 12H, $C\underline{H}_3$-rhodamine); 3.49 (s, 9H, NCH_3); 3.76 (bs, 2H, $NC\underline{H}_2CH_2CO$); 4.45 (s, 6H, $C\underline{H}_2NH$); 5.07 (s, 6H, $C\underline{H}_2C_6H_5$); 6.40 (s, 3H, H-5''); 6.77 (bs, 2H, H-4, H5); 6.8[illegible] (d, *J* 8.9 Hz, 2H, H2, H7); 7.23-7.44 (m, 15H, - $CH_2C_6\underline{H}_5$ and 2H H1, H8); 7.71 (bs, 2H, H4', H6'); 8.07 (d, *J* 9.5 Hz,1H, H3'). ^{13}C NMR (100.62 MHz, MeOD-d_4, ppm): δ 20.9 (6''-CH_3); 30.9 ($CCH_2\underline{C}H_2$); 31.3 ($C\underline{C}H_2CH_2$); 36.3 ($\underline{C}H_2NH$); 37.4 (NCH_3); 40.9 (CH_3-rhodamine); 74.8 ($\underline{C}H_2C_6H_5$); 97.2 (C4, C5); 114.7 (C1, C8); 115.0 (C2, C7); 119.4 (C5''); 124.0 (C4', 6'); 129.4 – 130.1 (C $CH_2\underline{C}_6H_5$); 132.5 (C3'); 138.4 ($\underline{C}q$-C_6H_5); 143.3 (C2''); 147.4 (C3''); 150.9 (C6''); 158.4 (C4''); 158.8 (C4a, C5a); 160.8 (C1', C8a); 175.3 ($\underline{C}ONHCH_2$).

MRH10. Compound **1** (0.0400g, 2.67×10^{-5} mol) was dissolved in anhydrous dichloromethane (20 mL), under argon and cooled to 0ºC. BCl_3 (1 mL) was added dropwise and the

reaction mixture was kept overnight with stirring at room temperature. Methanol (50 mL) was added and the mixture was stirred for 1 h. The solid product that formed was removed by filtration and the solvent was removed under reduced pressure to afford the crude product. Recrystallization of the crude product from methanol/acetone (1:9) afforded the hydrochloride salt **MRH10** (0,0224g, 61%) as a purple solid (5'-isomer). Elemental analysis for ($C_{62}H_{77}N_{11}O_{13}S^{4+}$.4Cl$^-$ .11HCl.6H_2O): Calculated (found): C 39.88 (39.71); H 5.40 (5.10); N 8.25 (8.21). MS: calculated for $C_{62}H_{74}N_{11}O_{13}S^+$.$K^+$: 1251.48 [$M^+$.$K^+$]; found: matrix-assisted laser desorption/ionization time of flight MS: 1251.40 [M^+.K^+]. ^{1}H NMR (400.15 MHz, MeOD-d_4, ppm): δ 1.95 (m, 6H, CC$\underline{H_2}CH_2$); 2.22 (m, 6H, $CCH_2C\underline{H_2}$); 2.63 (s, 9H, 6''-CH_3); 3.02 (m, 2H, $NCH_2C\underline{H_2}CO$); 3.31 (s, 12H, CH_3-rhodamine); 3.97 (s, 9H, NCH_3); 4.52 m, 2H, $NC\underline{H_2}CH_2CO$); 4.65 (s, 6H, $C\underline{H_2}NH$); 7.01 (s, 3H, H5''); 6.86-7.38 (m, 6H, H1 – H8); 7.64-8.49 (m, 3H, H3'- H6'). ^{13}C NMR (100.62 MHz, MeOD-d_4, ppm): δ 21.3 (2''-CH_3); 30.6 ($CCH_2\underline{C}H_2$); 31.0 ($C\underline{C}H_2CH_2$); 35.7 ($NCH_2\underline{C}H_2CO$); 36.3 ($\underline{C}H_2NH$); 40.0 ($N\underline{C}H_3$); 41.1 (CH_3-rhodamine); 44.5 ($N\underline{C}H_2CH_2CO$); 97.5 (C4, C5); 114.2 (C5"); 117.9, 123.8, 124.2, 129.2, 132.0 (C1 – C8); 132.5, 133.5, 134.8 (C3'- C6'); 140.8 (C2''); 144.8 (C3'');150.9 (C4''); 159.0 (C4a, C5a); 161.2 (C6"); 176.3 ($CH_2\underline{C}ONHCH_2$). UV-Vis (λ_{max} / nm) 552; ε = 3.2 x 10^4 mol^{-1} dm^3 cm$^{-1;}$ Fluorescence (λ_{max} / nm) 575.

Compound 2. To a solution of 5(6)-carboxytetraethyl-rhodamine (**F9**) (168.6 mg, 3.46×10^{-4} mol) in anhydrous DMF (10 mL), 3,4-HPO bidentate unit (73.3 mg; 2.84×10^{-4} mol), DCC (73.0 mg, 3.50×10^{-4} mol) and *N*-hydroxysuccinimide (47.7 mg, 40.2×10^{-4} mol) were added and the mixture was stirred at room temperature in the dark and under an argon atmosphere, for 2 days. Subsequently, the formed *N*,*N*-dicyclohexylurea (DCU) precipitate was filtered off and the solvent removed under reduced pressure. The product was purified by gradient flash column chromatography, eluting with methanol/chloroform (2:8) to afford **2** (52.5 mg; 25%) as a purple solid. Compound **2** was obtained as a 1.1:1 ratio mixture of 4'- and 5'- isomers as determined through NMR spectra analysis. ^{1}H NMR (400.15 MHz, MeOD-d_4, ppm): δ 1.28 (t, *J* 6.9, 24 H, 8× $CH_2C\underline{H_3}$-rhodamine 5'-isomer, 4'-isomer); 2.39 (s, 3H, 6''-CH_3, 5'-isomer); 2.45 (s, 3H, 6''-CH_3, 4'-isomer); 3.65 (m, 11H, NCH_3, 5' isomer + 8×$C\underline{H_2}CH_3$-rhodamine 5'-isomer, 4'-isomer); 3.72 (s, 3H, NCH_3, 4'-isomer); 4.66 (s, 2 H, $C\underline{H_2}$NH, 5'-isomer); 4.74 (s, 2H, $C\underline{H_2}$NH, 4'-isomer); 5.15 (s, 2H, $C\underline{H_2}C_6H_5$, 5'-isomer); 5.23 (s, 2H, $C\underline{H_2}C_6H_5$, 4'-isomer); 6.45 (s, 1H, H5'', 5'-isomer); 6.51 (s, 1H, H5'', 4'-isomer); 6.90 (d, *J* 2.5 Hz, 4H, H4 + H5 4'-isomer, 5'-isomer); 6.99 (br s, 4H, H2 + H7, 5'-isomer, 4'-isomer); 7.21-7.40 (m, 13H, H1 + H8 + *m-, p-* C_6H_5 5'-isomer, 4'-isomer + H6', 4'-isomer, + *o-* C_6H_5, 5'-isomer); 7.46-7.48 (m, 2H, *o-* C_6H_5, 4'-isomer); 7.64 (d, *J* 1.5, 1H, H6', 5'-isomer); 7.99 (dd, *J* 7.9; 1.8, 1H, H5', 4'-isomer); 8.03 (dd, *J* 8.3; 1.8, 1H, H4', 5'-isomer); 8.10 (br d, *J* 8.0, 1H, H3', 5'-isomer); 8.43 (d, *J* 1.7, 1H, H3', 4'-isomer); ^{13}C NMR (100.62 MHz, MeOD-d_4, ppm): δ 12.8 ($CH_2\underline{C}H_3$-rhodamine 5'-isomer, 4'-isomer); 20.8 (2''-CH_3, 5'-isomer); 20.9 (6''-CH_3, 4'-isomer); 37.1 (CH_2NH, 5'-isomer); 37.2 (CH_2NH, 4'-isomer); 37.4 (NCH_3, 5'-isomer); 37.5 (NCH_3, 4'-isomer); 46.7 ($\underline{C}H_2CH_3$-rhodamine 5'-isomer, 4'-isomer); 74.7 ($\underline{C}H_2C_6H_5$, 5'-isomer); 74.9 ($\underline{C}H_2C_6H_5$, 4'-isomer); 97.1 (C4 + C5, 5'-isomer, 4'-isomer); 114.8 (C1'); 114.9 (C2 + C7, 5'-isomer, 4'-isomer); 119.2 (C5'', 5'-isomer, 4'-isomer); 129.4 (CH); 129.5 (C6', 5'-isomer); 129.6 (C5', 4'-isomer); 129.7 (CH); 129.8 (C4', 5'-isomer); 130.0 (CH *o*-C_6H_5), 130.2 (C3', 4'-isomer); 131.0 (C3', 5'-isomer); 132.9 (C1 + C8 5'-isomer, 4'-isomer); 133.1 (CH); 133.7 (C_q, 5'-isomer); 135.6 (C_q); 136.4 (C_q); 136.8 (C4', 4'-isomer); 138.4 (C_q C_6H_5, 5' isomer); 138.5 (C_q C_6H_5, 4'-isomer); 142.3 (C_q); 143.1 (C2'' 5' isomer); 143.3 (C2'' 4'-isomer); 145.0 (C5', 5'-isomer); 147.6 (C5'', 5'-isomer); 147.7 (C3'', 4'-isomer); 151.0 (C2'', 5' isomer); 151.1 (C6'', 4'-isomer); 156.9 (C9); 159.3 (C4a, 4' isomer); 159.4 (C4a, 5'-isomer); 162.0 (C1', 5'-isomer); 162.2 (C1', 4'-isomer); 168.6 (CONH, 5'-isomer); 169.1 (CONH, isomer); 172.3 (2'-CO_2H, 5'-isomer); 172.5 (2'- CO_2H, 4'-isomer);175.0 (C4'', 5'-isomer); 175.1 (C4'', 4'-isomer).

MRB9. Compound **2** (37.7 mg, 5.50×10^{-4} mol) was dissolved in anhydrous dichloromethane (8 mL), under argon and cooled to 0ºC. BCl_3 (0.8 mL) was added dropwise and the reaction mixture was kept overnight with stirring at room temperature. Methanol (30 mL) was added and the mixture was stirred for 1 h. The solid product that formed was removed by filtration and the solvent was removed under reduced pressure to afford the crude product. Recrystallization of the crude product from methanol/acetone (4:6) afforded the hydrochloride salt **MRB9** (37.1 mg, 82%) as a purple solid. Elemental analysis for ($C_{38}H_{46}N_4O_6^{2+}$.2Cl$^-$.$CHCl_3$): Calculated (found): C 55.43 (55.03); H 5.61 (5.07); N 6.63 (6.52). MS: calculated for $C_{38}H_{45}N_4O_6^+$ 653.33 [M^+]; found: ESI - MS: 653.2952 [M^+]. ^{1}H NMR (400.15 MHz, MeOD-d_4, ppm): δ 1.31 (t, *J* 6.7, 24 H, 8× $CH_2C\underline{H_3}$ -rhodamine 5'-isomer, 4'-isomer); 2.63 (s, 3H, 6'' -CH_3, 5'-isomer); 2.68 (s, 3H, 6'' -CH_3, 4'-isomer); 3.68 (q, *J* 6.7, 16H, 8×$C\underline{H_2}CH_3$-rhodamine 5'-isomer, 4'-isomer); 4.08 (s, 3H, NCH_3, 5'-isomer); 4.15 (s, 3H, NCH_3, 4'-isomer); 4.97 (s, 2H, CH_2NH, 5'-isomer); 5.06 (s, 2H, CH_2NH, 4'-isomer); 7.02-7.11 (m, 9H, H5'', 5'-isomer, 4'-isomer + H1, H2, H4, H5, H7, H8); 7.55 (d, *J* 5.7 Hz, 1H, H6', 4'-isomer); 7.88 (br s, 1H, H6', 5'-isomer); 8.27 (br s, 1H, H4', 5'-isomer); 8.33 (br s, 1H, H5', 4'-isomer); 8.41 (br s, 1H, H3', 5'-isomer); 8.82 (s, 1H, H3', 4'-isomer).^{13}C NMR (100.62 MHz, MeOD-d_4, ppm): δ 12.8 ($CH_2\underline{C}H_3$-rhodamine, 5'-isomer, 4'-isomer); 21.3 (6''-CH_3, 5'-isomer, 4'-isomer); 37.2 (CH_2NH, 5'-isomer); 37.3 (CH_2NH, 4'-isomer); 40.0 (NCH_3, 5' isomer, 4'-isomer); 46.9 ($\underline{C}H_2CH_3$-rhodamine, 5'-isomer, 4' isomer); 97.3 (C5''); 114.2 (C4); 114.6 (C5); 114.7 (C2); 115.5 (C7); 130.4 (C4', 5'-isomer); 130.6 (C6', 5'-isomer); 131.6 (C3', 4'-isomer); 132.1(C1); 132.2 (C6', 4'-isomer); 132.4 (C8); 132.6 (C5', 4'-isomer); 132.9 (C3', 5'-isomer); 135.3 (C2', 5'-isomer); 135.6 (C5', 5'-isomer); 136.4 (C2', 4-isomer); 138.2 (C1', 5' isomer); 138.6 (C1', 4'-isomer); 140.6 (C2'' 5'-isomer); 140.8 (C2'', 4'-isomer); 145.2 (C3''); 150.7 (C6''); 157.2 (C3+C7); 159.3 (C9); 159.6 (C_q); 159.7 (C_q); 161.3 (C4a + C5a); 167.3 (2'-CO_2H); 168.6 (C_q); 168.8 (CONH). UV-Vis (λ_{max} / nm) 559; ε = 5.4 x 10^4 mol^{-1} dm^3 cm$^{-1;}$ Fluorescence (λ_{max} / nm) 583.

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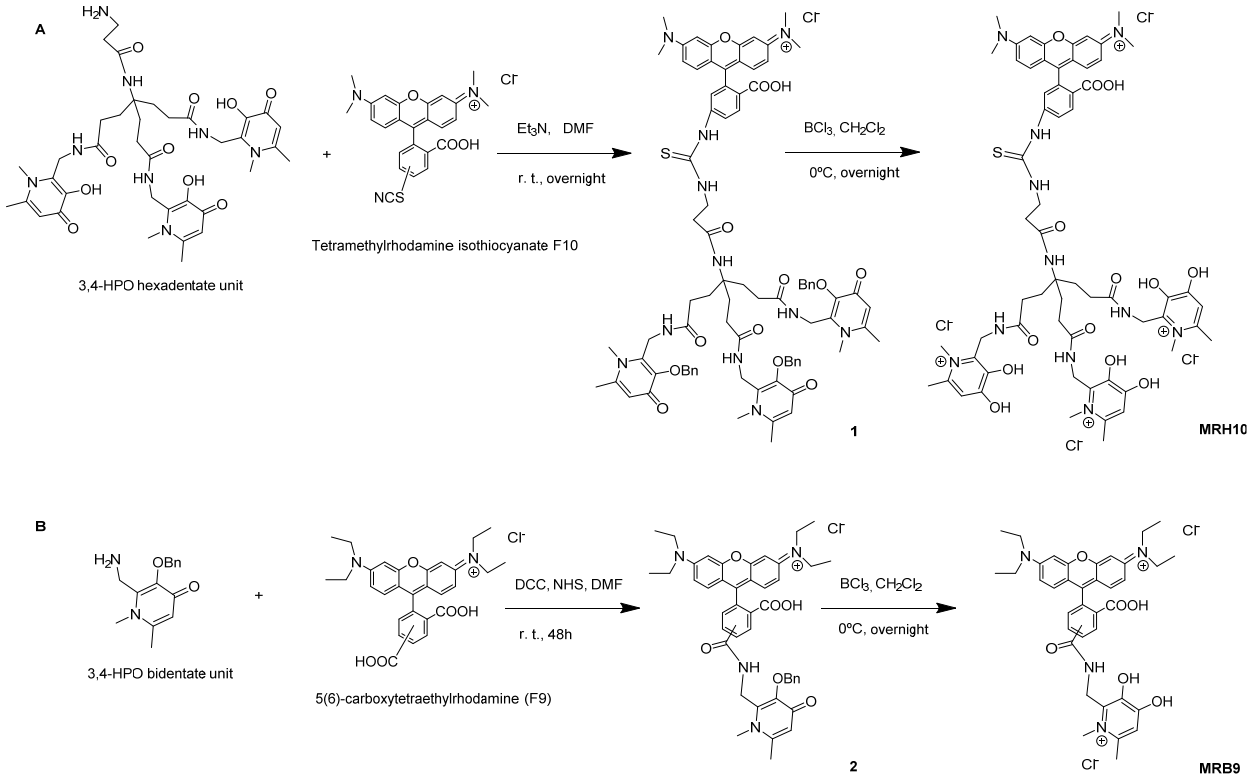

Figure 2. Reaction scheme of the synthetic route for the preparation of the new chelator **MRH10** (A) and **MRB9** (B)

Electronic spectroscopy: absorption and fluorescence measurements. Electronic absorption measurements were performed in a Perkin Elmer Lambda 25 spectrophotometer equipped with a constant-temperature cell holder, using the conditions T= 25ºC, l=1cm cuvettes and wavelength range 225-650 nm.

Fluorescence measurements were performed in a Varian Cary Eclipse spectrofluorometer, equipped with a constant-temperature cell holder, using the conditions T= 25ºC, l=1cm cuvettes. All spectra were recorded with excitation and emission slit widths of 5 nm, 650V, with: i) λ_{exc}=553 nm and λ_{em} from 560 to 700 nm for **MRH10**; ii) λ_{exc}=560 nm and λ_{em} from 561 to 700 nm for **MRB9**.

Stock solutions of the different compounds were obtained by preparing a concentrated solution of the compound in dimethylsulfoxide (DMSO). Samples for absorption and fluorescence measurements were prepared by dilution of a known volume of the DMSO stock solution in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer solution (pH 7.4, I=0.1 M NaCl). The percentage of the DMSO stock solution was always less than 1% in the final volume. The results are summarized in the experimental section.

Biology

Bacteria. *M. avium* strain 2447, smooth transparent variant (SmT) was isolated from an AIDS patient and provided by F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium). Mycobacteria were grown to mid-log phase in Middlebrook 7H9 medium (Difco, Sparks, MD) containing 0.04% Tween 80 (Sigma, St. Louis, MO) at 37ºC.

Bacteria were harvested by centrifugation, suspended in a small volume of saline containing 0.04% of Tween 80 (Sigma) and briefly sonicated in order to disrupt bacterial clumps. The suspension was diluted and stored in aliquots at -80ºC until use.

Bone marrow derived macrophages (BMM). Macrophages were derived from the bone marrow of BALB/c mice. Each femur was flushed with 5 mL of Hanks' balanced salt solution (HBSS, Life Technologies, Paisley, U.K.). The resulting cell suspension was centrifuged and the cells re-suspended in DMEM (Life Technologies, Paisley, U.K.) supplemented with 10 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 10% FBS (Fetal Bovine Serum) (Life Technologies), and 10% of L929 cell conditioned medium (LCCM) as a source of Macrophage Colony Stimulating Factor (M-CSF). According to manufacturer's information, the iron (Fe(III)) content of DMEM is 0.25 mM. The added FBS contributes an additional 3 mM of iron. In order to remove fibroblasts, the cells were cultured overnight, at 37ºC in a 7% CO_2 atmosphere, on a cell culture dish. The non-adherent cells were collected with warm HBSS medium, washed and resuspended in DMEM with 10% LCCM at a cell density of $4x10^5$ cells/ml. One ml of this cell suspension was distributed per well in 24-well plates which were incubated at 37ºC in a 7% CO_2 atmosphere. Three days later, 0.1 mL of LCCM was added. On the 7th day of culture, the medium was renewed.

Infection and treatment of BMM. On the tenth day of culture, the macrophages of each well were incubated with 0.2 mL of DMEM containing 10^6 *M. avium* colony forming units (CFU) for 4 h at 37ºC in a 7% CO_2 atmosphere. The cells were washed with warm HBSS to remove extracellular mycobacteria and reincubated in DMEM with 10% FBS and 10% LCCM with or without the addition of the compounds to be tested. To quantify the number of intracellular mycobacteria at time zero of infection, macrophages from three wells were immediately lysed with 0.1% of saponin.

The iron chelator solutions were obtained by preparing concentrated solutions of the different compounds in DMSO followed by dilution of a known volume of the DMSO stock solution in DMEM. The percentage of the DMSO stock solution was always less than 1% in the final volume.

Chelators were added to the culture medium at the concentrations indicated in each figure (ranging from 5 to 120 μM). Chelators were tested alone and in combination with ethambutol (ETH) at 2 μg/ mL (7.2 μM). Ethambutol was also tested alone at the same concentration used in combination with chelators. Each concentration was tested in triplicates. All the results were confirmed in at least three independent assays. The formulae of the bidentate and hexadentate ligands used in the present study are shown in Figure 1.

Quantification of M. avium inside BMM. After 5 days of infection, the growth of *M. avium* 2447 SmT was evaluated by counting the colony forming units. Macrophages were lysed using 0.1% saponin and the resulting bacterial suspension was diluted in water containing 0.04% Tween 80. The dilutions were plated in Middlebrook 7H10 agar (Difco) and the number of colonies was counted after 8 days at 37ºC. The difference, in terms of $\log_{10}$ CFU/well, between time zero of infection and the last day in culture (day 5), is abbreviated as ''$\log_{10}$ increase''.

Statistical analysis. Data obtained in this work were analysed using the software Graph Pad Prism version 6.0. Data are expressed as mean ± SD for the number of experiments indicated in the legends of the figures. Multiple comparisons were performed using one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison posthoc test. Significance was accepted when p value < 0.05 was obtained.

Results and discussion

Synthesis and characterization of fluorescent 3,4-HPO chelators

The new fluorescent ligands **MRH10** and **MRB9** were prepared using straightforward synthetic protocols [18, 20]. For compound **1**, the commercially available fluorophore tetramethylrhodamine isothiocyanate (**F10**) was coupled to the protected 3,4-HPO hexadentate unit. To obtain compound **2**, the fluorophore 5(6)-carboxytetraethyl-rhodamine (**F9**) was synthetized and coupled to the protected 3,4-HPO bidentate unit. The last step is the deprotection reaction for the removal of the protecting benzyl group with under BCl_3, as it is depicted in Figure 2.

The comparison of the results obtained for the synthesis of **1** and **2** demonstrates that the reaction of tetramethylrhodamine isothiocyanate with the hexadentate 3,4-HPO lead us to obtain ligand **1** in a 48% yield and in the reaction of 5(6)-carboxytetraethyl-rhodamine with the bidentate unit we obtained ligand **2** in 76 % yield. Although both synthetic procedures are very similar, the yield of the reactions are different and this fact could be related with the functional group of the fluorophore that reacts with the amine group pf the 3,4-HPO unit. In the deprotection reactions, no major differences are observed and ligands **MRH10** and **MRB9** was successfully synthetized and the yield of reactions are respectively 61 and 96%.

The structures of the ligands **1**, **MRH10**, **2** and **MRB9** in solution were established by NMR analysis (^{1}H and ^{13}C, 1D and 2D experiments, including COSY, HSQC and HMBC spectra for unequivocal assignment of the most characteristic proton and carbon chemical shifts). The assignment of the resonance signals in ^{13}C NMR spectra of the protected and de-protected compounds was achieved by analysis of 1H/13C HSQC and ^{1}H/^{13}C HMBC spectra, which provide one and multiple bond ^{1}H-^{13}C connectivity.

The ^{1}H NMR spectra of ligand **1** revealed that the resonance signals of the methyl protons of the rhodamine residue appear at 3.19 ppm and those of H1-H8 protons in the spectral are between 6.77 and 7.44 ppm. The two multiplets at high field (1.87-2.08 ppm) were assigned to -$C(CH_2CH_2)_3$ protons. The resonance signals of NCH_2CH_2CO protons appear as triplets or broad singlets at 2.45 and 3.76 ppm. The protons of the methyl group of the pyridinone ring appear at 2.33 ppm and the protons of the methyl linked to the nitrogen of the ring appear at 3.49 ppm.

The resonance signals, at 6.40 ppm, were assigned to the aromatic CH protons (H5") of the pyridinone residue and show HMBC correlation with a carbon at 20.9 ppm assigned as 6''-$\underline{C}H_3$. The set observed in the low field spectral area (7.7-8.1 ppm) and assigned to the protons of di-substituted aromatic ring of rhodamine residues. Due to the characteristic chemical shift of carbon C3', the reaction was isomeric selective and only isomer 2'/5' was obtained.

The signals related with the protecting groups are the singlet at 5.07 ppm that corresponds to the protons of the methyl group and the protons of the benzyl ring appear between 7.23-7.44 ppm. The carbon associated to this methyl group is at 74.8 ppm, the quaternary carbon appear at 138.4 ppm and the last 5 carbons of the benzyl ring appear between 129.4 and 130.1 ppm. The resonance signal at 175.3 ppm was found to give long range coupling to the resonance signal of -$C(CH_2CH_2)_3$ and - $C\underline{H}_2NH$ protons and was attributed to carbonyl carbons of the three amide groups.

After the deprotection (**MRH10**), significant differences in the ^1H and ^{13}C spectra are detected as for example the shift of the protons of methyl group linked to the nitrogen of the pyridinone from 3.49 to 3.97 ppm as also the shift in the protons of the methylene group of the linkage ($C\underline{H}_2NH$) from 4.45 to 4.65 ppm. Their respective carbons are also dislocated to low field region, namely $N\underline{C}H_3$ from 37.4 in the protected ligand and 40.0 in the deprotected form and the shift from 119.4 to 114.2 ppm for the carbon C5''.

The carbon at 140.8 show HMBC correlation with H5'', $C\underline{H}_2NH$ and 6''-$C\underline{H}_3$ protons and was attributed to C2''. The carbon at 144.8 show HMBC correlation with H5'and $C\underline{H}_2NH$ protons and was attributed to C3''. Signals at 150.9 and 161.2 were attributed as carbons C4'' and C6'' due to their HMBC correlations with H5'', $NC\underline{H}_3$ and 6''$C\underline{H}_3$ or H5'', $NC\underline{H}_3$, respectively. The resonance signal at 176.3 ppm was found to give long range coupling to the resonance signal of -$C(CH_2CH_2)_3$ and - $C\underline{H}_2NH$ protons and was attributed to carbonyl carbons of the three amide groups.

The ^1H NMR spectra of compound **2** indicated that the resonance signals of the ethyl protons of the rhodamine residue appear at 1.28 (CH_3) and 3.65 (CH_2) ppm respectively. The peaks of H4 and H5 appear as a doublet at 6.90 ppm while H2 and H7 appear as a broad singlet at 6.99 ppm, being these signals useful of diagnostic of the presence of the rhodamine moiety. The protons of the methyl group of the 3,4-HPO group appear at 2.39 ppm for the 5'-isomer and at 2.45 ppm for 4'-isomer. The proportion between the intensities of these was used to determine the ratio of both isomers in the mixture (1:1.1 of the 5'- and 4'- isomers), being this result coherent with the one found for other analogous peaks of the 2 isomers. The protons of CH_2NH attached to the 3,4-HPO groups appear at 3.49 ppm.

The resonance signal at 6.45 ppm was assigned to the aromatic proton (H5") of the 5'-isomer 3,4-HPO residue and shows a HMBC correlation with a carbon at 20.8 ppm of the carbon 6''-CH_3 of the 5'-isomer), while the signal at 6.51 was attributed to the H5" of the 4'-isomer correlated to the carbon 6''-CH_3 of the 4'-isomer at 20.9 ppm.

The aromatic peaks (6.9-7.4 ppm) were assigned to the protons of di-substituted aromatic ring of rhodamine residues. The signals related to the benzyl group are the singlet at 5.17 ppm (5'-isomer) and at 5.23 ppm (4'isomer) corresponding to the methylene protons of benzyl group while the aromatic protons appear as two sets of multiplets comprised between 7.21-7.40 ppm and 7.46-7.48 ppm. The carbons associated to the methylene of benzyl group are found at 74.7 ppm for the 5'-isomer and at 74.9 ppm for the 4'-isomer.

The quaternary carbons in ^{13}C NMR were assigned resorting to HMBC correlation, namely the signals assigned to 2'-CO_2H at 172.3 and 172.5 ppm for 5'- and 4'-isomers respectively due to correlation with the respective H3'proton signals 8.10 (5'-isomer) and 8.43 (4'-isomer). The peak at 168.6 ppm was identified as belonging to the carbon of CONH of 5'- isomer due to correlation with the corresponding peaks at 4.66 ppm ($C\underline{H}_2NH_2$) and with 7.64 ppm (H6') both associated to 5-isomer, similarly the peak at 169.1 ppm was assigned to carbon of CONH of 4'- isomer because of the observed correlation with the peaks at 4.74 ppm ($C\underline{H}_2NH_2$) and 8.43 ppm (H3') associated to 4'- isomer. The resonance signals at 175.0 and 175.1 were assigned to C4'' of 5'- and 4'-isomers respectively, due to correlation with respective H5'' proton signals of 6.45 and 6.51 ppm of both isomers. The carbons at 151.0 and 151.1 ppm were assigned to C6'' of 5'and 4'- -isomers respectively, based on correlations with the respective H3'', $C\underline{H}_2NH$ and 6''-$C\underline{H}_3$ signals. C3'' signals were assigned to 147.6 ppm (5'-isomer) and 147.7 ppm (4'-isomer) based on correlation with the corresponding H3'and $C\underline{H}_2NH$ protons. 143.1 (5'-isomer) and 143.3 (4'-isomer) were attributed to C2'' based on the correlations with corresponding H5'', NCH_3 and $C\underline{H}_2NH$ protons.

In the spectra of **MRB9** the peaks for the 4'- and 5'-isomers present the same chemical shifts for analogous atoms while in their precursor (compound **2**) these appear often fully resolved with distinct chemical shifts, being the peaks of 5'-isomer at higher field than the corresponding ones for 4' isomer. **MRB9** also shows observable increase for chemical shifts of the protons and carbons in the ^1H and ^{13}C spectra respectively, to higher field in comparison to compound **2**. Among others, it is quite noticeable the change observed for the methylamino group of the pyridinone (NCH_3) from 3.65 ppm in compound **2** to 4.08 ppm in **MRB9** (5'-isomer) and from 3.72 to 4.15 ppm (4'-isomer). A similar observation can be made for the methylene group of the linkage ($C\underline{H}_2NH$) varying from 4.66 in 2 to 4.97 (5'-isomer), and from 4.74 to 5.06 (4'-isomer). Their respective carbon signals are also dislocated to lower field region, namely $N\underline{C}H_3$ 37.4 (5'-isomer) and 37.5 (4'-isomer) to 40.0 for both isomers.

The observed difference in the chemical shifts of ^1H and ^{13}C nucleus in the spectra of the protected and deprotected compounds is primarily due to the deprotection of the hydroxyl group and the acidic pH of the deprotection reaction that lead to isolate the final ligands in the enolic form.

Absorption and fluorescence measurements

The absorption electronic spectra of **MRH10** and **MRB9** show two sets of bands in different regions of the spectra, which correspond to π → π* transitions of the π systems of 3,4-HPO and the fluorophores that constitute the chelators structure. The spectral parameters of the new fluorescent chelators are summarized in experimental section. Transitions in the range 281-290 nm are associated with ethylene and benzene bonds of the aromatic ring of the 3,4-HPO. The other set of transitions in the range 552 and 559 nm are signed to tetramethylrhodamine isothiocyanate (**F10**) or 5(6)-carboxy-tetraethyl-rhodamine (**F9**) π systems. The data obtained is in agreement with the results reported in the literature [18, 20]. The molar absorptivity values obtained are in the magnitude (10^4), close to those reported in previous results for similar rhodamine derived ligands [18, 20].

Evaluation of antimycobacterial activity

Effect of MRH10 and MRB9 chelators on the intramacrophagic growth of M. avium 2447 SmT in comparison with MRH7. Intramacrophagic growth of the bacteria was measured, as CFU (Colony Forming Units), after five days in culture in the absence or in the presence of the chelators. The effect of each compound in the intramacrophagic growth of *M. avium* was calculated as the difference in $\log_{10}$ CFU/well between day 0 and day 5. The antimycobacterial effect of the new chelators **MRH10** vs **MRB9** was tested and compared with the effect of the **MRH7**, used herein as a second control, for the same comparable concentrations: (a), (b), (c) and (d). Since chelators **MRH7** and **MRH10** are hexadentate and **MRB9** is bidentate the concentration of the latter is always three times the concentration used for the hexadentate ligands. All the compounds were tested in at least three independent assays and the results are represented in Figure 3.

The results show that all the fluorescent chelators exhibit antimycobacterial effect and this activity seems to be dose-dependent. In comparison with the control, **MRH7** has a significant effect at lower concentrations such as 10 μM while the new chelator **MRH10** only induces a reproducible inhibitory effect leading to a significant decrease on *M. avium* growth at concentrations up to 20 μM. Moreover, **MRB9** is only effective at the highest concentration tested (120 μM).

Comparison of the effect of the three chelators shows that:
(a) at the lowest concentration, no significant differences are observed for the activity of **MHR10** and **MRH7** and the effect of the latter is significantly higher than the obtained for **MRB9**.
(b and c) at intermediate concentrations **MRH7** exhibits the highest activity and its effect is significantly greater than that obtained for **MRB9**. No significant differences on the inhibition of bacterial growth are apparent between **MHR10** and **MRB9.**
(d) at the highest concentration tested, all ligands induce a significant decrease on the intramacrophagic growth of *M. avium* and the effect is not significantly different between them.

The results obtained show that **MRH7** seems to be the most active chelator in the control of the infection, being effective in most concentration values tested. Chelator **MRH10** exhibits a close performance to that of **MRH7** and is significantly better than that of **MRB9.**

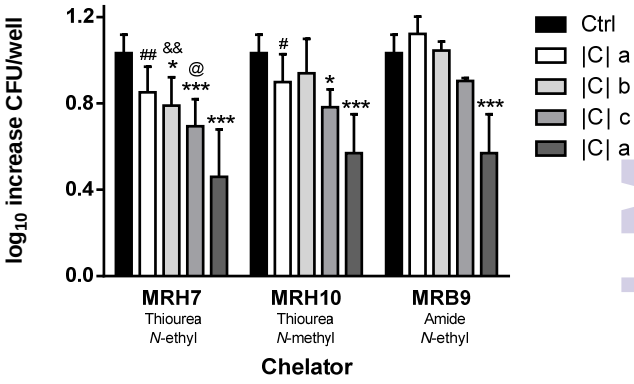

Figure 3. Effect of **MRH7**, **MRH10** and **MRB9** on intramacrophagic growth of *M. avium*. BMM were infected with *M. avium* 2447 (SmT). Each chelator was added, just after infection, at a concentration of 5 (a), 10 (b), 20 (c) and 40 (d) μM for **MRH7** and **MRH10** and 15 (a), 30 (b), 60 (c) and 120 (d) μM for **MRB9**. Infected BMM were incubated for 5 days to measure the intracellular growth of bacteria. The difference, in terms of $\log_{10}$ CFU/well, between days 0 and 5 was designated ''$\log_{10}$ increase''. The graphs show the geometric mean ± SD of the $\log_{10}$ increase per well obtained from three wells for each condition from three of five independent experiments. Statistical analysis was performed using ANOVA test to compare the bacterial growth: i) in the absence or presence of each the chelators and ii) in the presence **MRH7** vs **MRH10** vs **MRB9**, for the same concentrations, (a), (b), (c) and (d). Statistical significance: * p<0.05 and ** p<0.001 when compared with control (no chelator); # p<0.05 for **MRH10** vs **MRB9** or ## p< 0.01 for **MRH7** vs **MRB9**, at concentration (a); && p< 0.01 for **MRH7** vs **MRB9**, at concentration (b); @ p<0.05 for **MRH7** vs **MRB9**, at concentration (c).

Effect of MRH7 in combination with ethambutol (ETH) on the intramacrophagic growth of M. avium 2447 SmT. The effects of the chelator **MRH7** and of the classic antimycobacterial antibiotic, ethambutol (ETH) were evaluated *per se* and in combination. Different concentrations of ethambutol were tested in preliminary studies and the chosen value (7.2 μM) represents the concentration for which the effect of the antibiotic alone is virtually null. The experiments were performed using a fixed concentration of the antibiotic combined with **MRH7** in the concentrations of 5, 10 and 20 μM. The antimycobacterial activity of **MRH7**, ETH and their combination was compared with the control (no treatment) and all the conditions were compared between them. The results obtained are represented in Figure 4.

The results of the combination of **MRH7** with ETH reveal that the effect is significantly different from the control for all the concentration range, even at the lowest concentration (5 μM) of **MRH7**. Also, the effect of the combination of **MRH7** with ETH at 10 and 20 μM is significantly higher than the effect obtained with ETH *per se*.

Considering the combination of **MRH7** with ETH at the highest concentration of chelator (20 μM), the effect is also significantly higher than that obtained for **MRH7** *per se*, at the same concentration.

These data reinforce our hypothesis of the advantageous use of the combination of iron(III) chelators and the clinical used antimycobacterial antibiotic, ETH, as a strategy to fight *M. avium* infections.

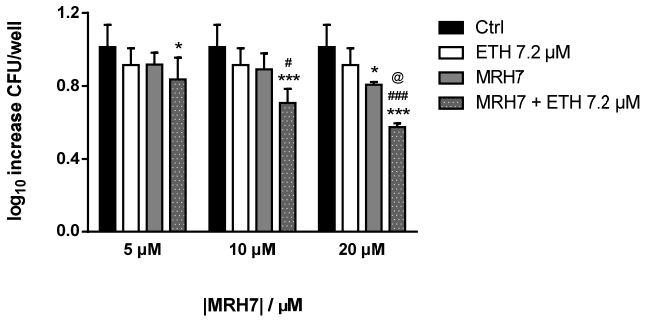

Figure 4. Effect of **MRH7**, ETH and their combination on intramacrophagic growth of *M. avium*. BMM were infected with *M. avium* 2447 (SmT). Each compound was added, just after infection, at a concentration of 5, 10 and 20 μM for **MRH7** and 7.2 μM for ETH. Infected BMM were incubated for 5 days to measure the intracellular growth of bacteria. The difference, in terms of $\log_{10}$ CFU/well, between days 0 and 5 was designated ''$\log_{10}$ increase''. The graphs show the geometric mean ± SD of the $\log_{10}$ increase per well obtained from three wells for each condition from four independent experiments. Statistical analysis was performed using ANOVA test to compare the bacterial growth in the absence or presence of **MRH7**, ETH or their combination and in the presence of **MRH7**, with or without combination of ETH. Statistical significance: * $p<0.05$ and *** $p<0.001$ when compared with control (no compound); # $p<0.05$ or ### $p<0.001$ when compared with ETH 2 μg/ mL; @ $p<0.05$ when compared with **MRH7** (without ETH).

Conclusions

The present results substantiate the premise that the inhibitory effect of rhodamine labelled 3,4-HPO chelators is strongly dependent on the fluorophore and confirm that the importance of the thiourea linkage, the ethyl substituents on the amino groups of the xanthene ring and the advantage of their associated inclusion in the molecular framework.

The relevance of the concomitant presence of the two functional groups for the chelators interaction with membranes has been suggested in an NMR and MD simulation study in which we demonstrate that the presence of N-ethyl groups in the xanthene structure and the thiourea link in the structure of **MRB7**, as opposed to N-methyl groups in the xanthene structure and an amide link in the structure of **MRB8**, seem to facilitate the affinity of MRB7 molecules to the liposome surface and their ability to penetrate deeper into the hydrophobic interior of lipid bilayer [50].

Moreover, the combined administration of chelator **MRH7** with the antibiotic ethambutol proved to be advantageous to achieve a higher antimycobacterial effect when compared with the activity of the chelator and the antibiotic *per se*. The results imply that it is possible to reduce the amount of chelator used to obtain a significant biological effect while simultaneously improving the activity of ETH.

To the best of our knowledge the present work is the first report revealing the advantageous combination of iron chelators and classic antibiotics in the treatment of intracellular infections such as *M. avium* infections.

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Chelator MRH7 (thiourea linkage; ethyl substituents) and its co-administration with ethambutol are the best choices for a higher antimycobacterial effect.