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Original article

Design, synthesis, biochemical evaluation and antimycobacterial action of phosphonate inhibitors of antigen 85C, a crucial enzyme involved in biosynthesis of the mycobacterial cell wall

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Dedicated to Professor Miha Tišler on the occasion of his 80th birthday.

Abstract

Phosphonate inhibitors of antigen 85C were prepared. The inhibitors, comprising a phosphonate moiety, mycolic acid mimetic and a trehalose surrogate, contain substituted benzyl alcohols, *N*-(ω-hydroxyalky)phthalimide, 2-phenylethanol or 4-(phthalimido)butanol as trehalose mimetics, and an alkyl chain of different lengths mimicking the mycolic acid side chain. The best compounds inhibited the mycolyltransferase activity of antigen 85C with IC_{50} in the low micromolar range and inhibited the growth of *Mycobacterium avium* in culture. The best compounds in the 3-phenoxybenzyl- and ω-(phthalimido)alkoxy series, ethyl 3-phenoxybenzyl butylphosphonate (**4a**) and (1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl) methyl ethyl heptylphosphonate (**5c**) displayed IC_{50} values of 2.0 and 1.3 μM, respectively, in a mycolyltransferase inhibition assay. In a *M. avium* growth inhibition assay MIC of **4a** and (1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl ethyl nonylphosphonate (**5d**) were 248.8 and 84.5 μg/mL, respectively.

Keywords: Antigen 85C; Phosphonate inhibitors; Tuberculosis; Antituberculosis agents

1. Introduction

Tuberculosis (TB), an infection caused by *Mycobacterium tuberculosis* (MTB), has become a major global health concern. Approximately one-third of the world's population harbor latent TB and about eight to ten million new cases occur annually, with almost two million dying from the disease, making it the leading cause of death from a single infectious agent [1]. During the last decades, coinfection with HIV markedly increased the incidence of TB [2]. Additionally, the emergence of drug-resistant and multi-drug-resistant TB has been increasing also in developed countries [1]. The recommended therapy for TB consists of an initial phase of treatment with four drugs – isoniazid, rifampicin, pyrazinamide and ethambutol, taken daily for two months, followed by a continuation phase of treatment with isoniazid and rifampicin for another four months [3]. WHO recommended this so-called DOTS (Directly Observed Therapy, Short course) strategy as the optimal way to treat TB and prevent emergence of resistance. Although DOTS is the best treatment for TB, this complex, lengthy and unpleasant combination of drugs makes patient

Abbreviations: TB, tuberculosis; MTB, *Mycobacterium tuberculosis*; DOTS, Directly Observed Therapy, Short course; TMM, trehalose monomycolate; TDM, trehalose dimycolate; Ag85, antigen 85; mAGP, mycolyl–arabinogalactan–peptidoglycan.

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compliance difficult, which can lead to poor overall cure rates and even to emergence of drug resistance [4]. No truly new TB drugs have been developed for nearly 40 years since the introduction of rifampicin in 1965. It is thus necessary to search for new and more effective antimycobacterial agents with novel mechanisms of action to combat the emergence of drug resistance and to shorten the duration of therapy [5]. Mycobacteria are surrounded by a complex envelope of unusually low permeability, which contributes to the resistance of this bacteria to host defense mechanisms [6,7]. Since several important TB drugs such as isoniazid, ethambutol and ethionamide target mycobacterial cell wall biosynthesis, enzymes involved in this pathway remain the preferred targets in anti-TB drug research [8]. The mycobacterial cell wall consists of three major components forming the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex, among which mycolic acids constitute the outermost layer [9]. Mycolic acids are high molecular weight α-alkyl-β-hydroxy fatty acids unique to *Mycobacterium* and related genera [9–11]. In the mycobacterial cell wall envelope, they are present as free glycolipids, mainly trehalose monomycolate (TMM) and trehalose dimycolate (TDM, cord factor), and as esters of the terminal pentaarabinofuranosyl units of arabinogalactan [12,13].

The antigen 85 (Ag85) complex is a major protein component of the mycobacterial cell wall [14]. It is composed of three proteins (Ag85A, B and C) all of which contribute to cell wall biosynthesis by catalyzing the transfer of mycolic acid from one molecule of TMM to another, resulting in TDM and free trehalose [15] (Fig. 1). In addition, antigen 85 proteins of MTB help maintain the integrity of the cell wall by catalyzing the transfer of mycolic acids to the cell wall arabinogalactan [16,17]. A trehalose analogue, 6-azido-6-deoxytrehalose, inhibits mycolyltransferase activity of all three members of the Ag85 complex *in vitro*, as well as growth of *Mycobacterium aurum*, indicating the importance of TDM for maintaining the integrity of the *Mycobacterium* cell wall [15]. A series of 6,6′-bis(sulfonamido), *N*,*N*′-dialkylamino and related derivatives of 6,6′-dideoxytrehalose were recently designed and synthesized to inhibit the Ag85 complex. The products were active against *M. tuberculosis* and a panel of clinical isolates of *Mycobacterium avium* [18]. Recently, a library of trehalose-based TDM mimics possessing long hydrocarbon chain substituents were prepared, and one of the derivatives displayed strong activity against *Mycobacterium smegmatis* when applied together with isoniazid [19].

The crystal structure of recombinant Ag85C from *M. tuberculosis* reveals an α/β-hydrolase polypeptide fold, with a catalytic triad formed by Ser124, Glu228 and His260 [20]. Near the catalytic triad is a binding site for the carbohydrate moiety with a highly negative electrostatic potential, a hydrophobic tunnel, well suited to accommodate the shorter α-branch of mycolic acids, and a long partially hydrophobic shallow cleft, which can bind mycolic acid's longer β-branch. According to the proposed mechanism of the catalytic mycolyl transfer reaction, Ser124 attacks the carboxyl carbon of TMM to give a mycolyl-enzyme intermediate and free trehalose. In the next step, the 6′-OH group of the second TMM molecule attacks the carboxylate carbon of the acyl-enzyme intermediate, giving TDM. Both, the acylation and deacylation steps proceed via a high-energy tetrahedral transition state [20].

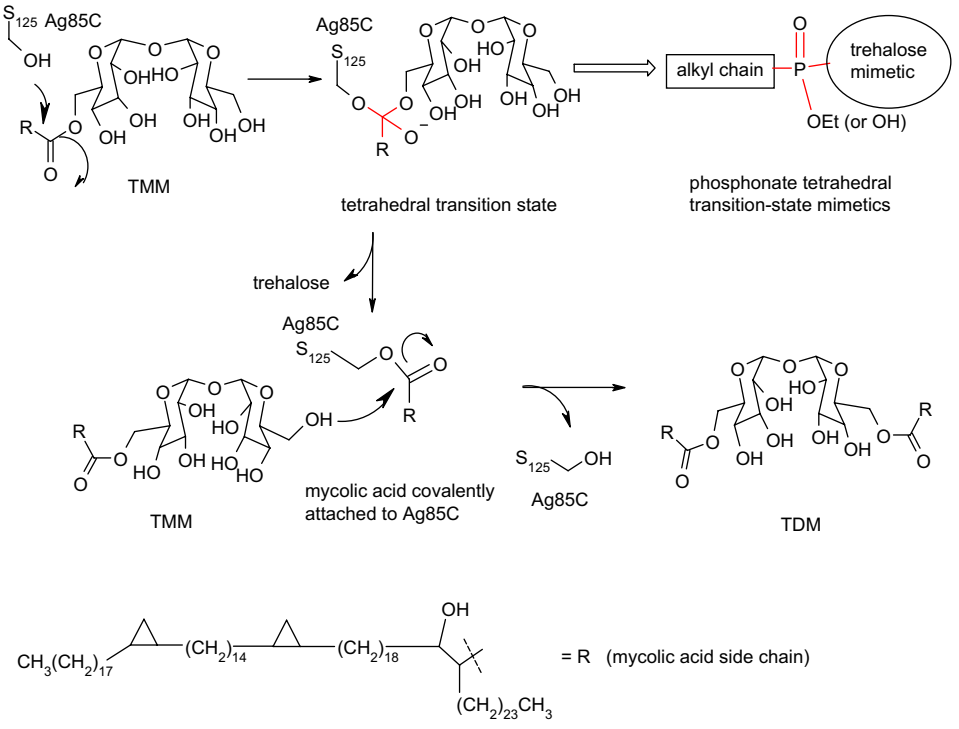


Fig. 1. Proposed mechanism of the mycolyl transfer reaction catalyzed by Ag85C as the basis for designing phosphonate inhibitors.

The importance of TDM to mycobacterial cell wall stability provides an excellent basis for the development of novel antitubercular drugs. The crystal structure of antigen 85C and its proposed catalytic mechanism [20] allowed us to design phosphonate inhibitors of the mycolyltransferase activity of antigen 85C (Fig. 1), which was briefly reported in our previous communication [35]. Herein we present a full report on the design, synthesis, biochemical evaluation and antimycobacterial activity of the target compounds.

It is well known that incorporation of phosphorus-based transition-state mimetics like phosphonates, phosphonamidates and phosphinates into substrate or product analogues generally leads to powerful enzyme inhibitors, especially protease inhibitors [21]. Given the architecture of the antigen 85C active site and using the concept of phosphonate transition-state analogues, we decided to link together a trehalose mimetic and a mycolic acid side chain surrogate via an ethyl phosphonate moiety, to give simplified tetrahedral transition-state mimetics which would inhibit the mycolyltransferase activity of antigen 85C. Several aromatic ring containing alcohols, e.g. benzyl alcohols, *N*-(ω-hydroxyalky)phthalimides and *N*-(ω-hydroxyethoxyethyl)phthalimide were used to mimic the trehalose of TMM. Alkyl chains of various lengths (C4 to C14) were introduced as hydrophobic groups to mimic either the mycolate α-chain which binds into a 2.1 nm long channel extending through the core of the Ag85C protein or the mycolic acid β-branch which binds to a shallow cleft on the surface. In order to obtain the initial structure–activity relationships, 2-phenylethyl and 4-(phthalimido)butyl analogues of the alkyl chain were also incorporated in the target molecules. The structures of the envisaged potential inhibitors of mycolyltransferase activity of antigen 85C are presented in Fig. 2.

The envisaged inhibitors were docked into the antigen 85C active site. For compound **4a**, a representative of the 3-phenoxybenzyl series, docking experiments predicted that the 3-phenoxybenzyl substituent would locate in the trehalose binding pocket and that the phosphonate moiety would be oriented in the vicinity of Ser124. The alkyl chain would be accommodated in the mycolate α-chain binding channel extending through the core of the protein. A similar binding mode was predicted for the phthalimido compounds **5** with the phthalimido group located in the trehalose binding pocket (Figs. 3 and 4). A recently published crystal structure of a detergent *O*-octylglucoside complexed with antigen 85C revealed a similar binding mode with the octyl chain nestled in the hydrophobic pocket and the glucose moiety occupying the trehalose binding pocket [22]. In a lower-score binding mode of phthalimido compounds **5** docked in the antigen 85C active site, the phthalimido group was sometimes located at the entry to the hydrophobic channel with carbonyl oxygens located close to the catalytic triad.

2. Chemistry

The synthesis of diphenylether phosphonate inhibitors **4a–e** and phthalimido phosphonate inhibitors **5a–f** is presented in Scheme 1. Alkyl bromides **1a–e** were reacted with triethyl phosphite in a Michaelis–Arbuzov reaction [23] to give diethyl phosphonates **2a–e** [24–27] which were partially deprotected with sodium azide [28] to afford monoethyl phosphonates **3a–e** [29,30]. The target mixed phosphonates **4a–e** were obtained by coupling **3a–e** with 3-phenoxybenzyl alcohol using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) [31] as a coupling agent. A series of phthalimido analogues **5a–f** were prepared similarly by coupling the respective monoethyl phosphonates **3a–e** with *N*-(hydroxyethyl)phthalimide or *N*-(2-hydroxyethyl)phthalimide, using BOP reagent. Using the same strategy, 3,4,5-trimethoxybenzylphosphonate **6** was prepared from the corresponding ethyl hydrogen alkylphosphonate **3b**.

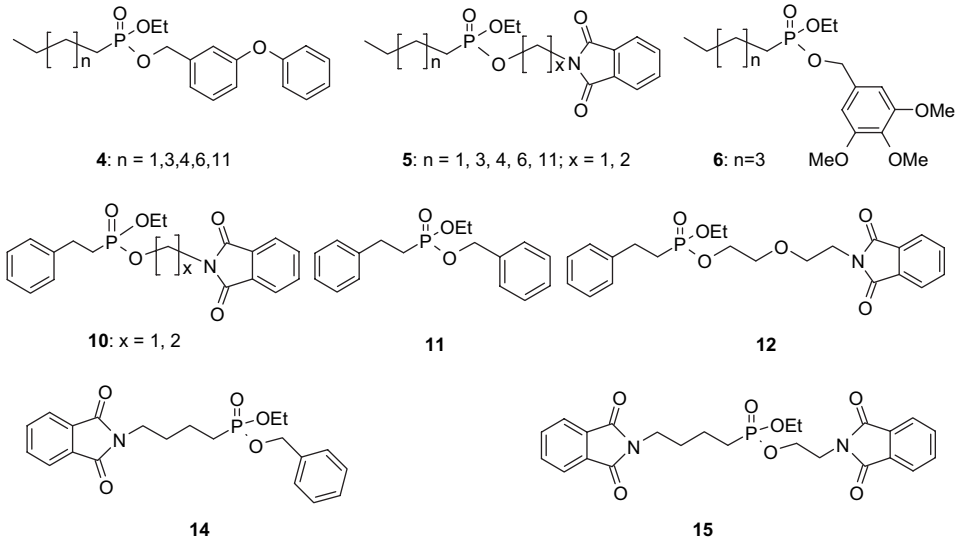


Fig. 2. Target inhibitors of mycolyltransferase activity of antigen 85C.

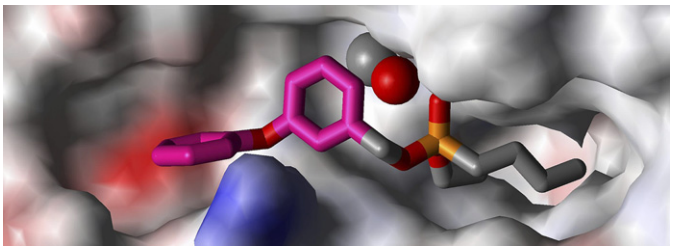
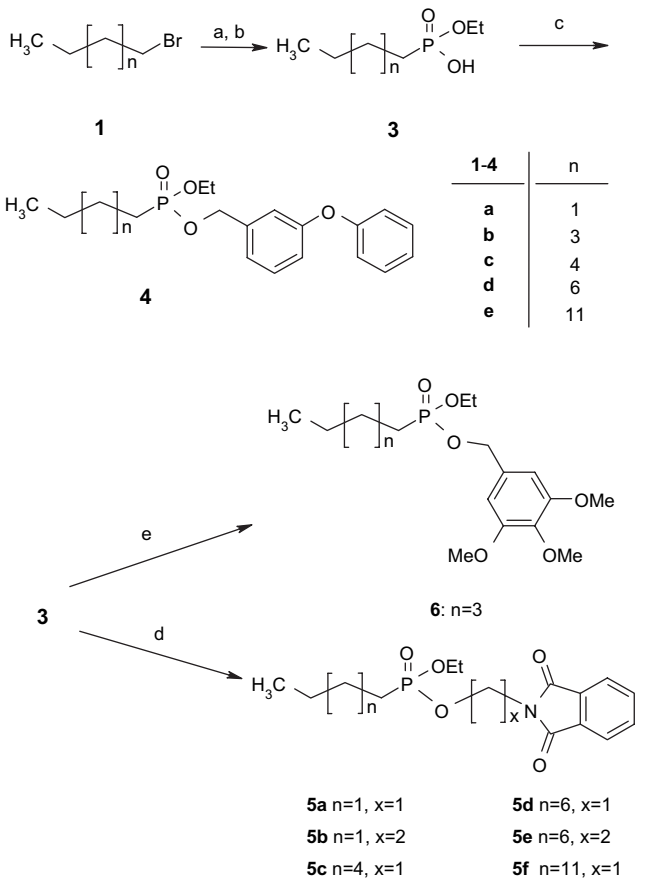


Fig. 3. Hypothetical binding mode of inhibitor **4a** in the antigen 85C active site predicted using AutoDock 3.0. The 3-phenoxybenzyl substituent is located in the trehalose binding pocket and the phosphonate moiety is oriented in the vicinity of Ser124. The alkyl chain is accommodated in the mycolate α-chain binding channel extending through the core of Ag85C.

For the synthesis of mixed phosphonates **10a**, **10b**, **11** and **12** (Scheme 2), the key intermediate ethyl hydrogen 2-phenylethyl phosphonate (**9**) [32] was prepared by Michaelis–Arbuzov reaction of (2-bromoethyl)benzene (**7**) with triethyl phosphite to give diethyl phosphonate **8** [33], which was further transformed with sodium azide to phosphonic acid **9**. In the next reaction step this was esterified with *N*-(hydroxymethyl)phthalimide, *N*-(2-hydroxyethyl)phthalimide, benzyl alcohol, or *N*-[2-(2-hydroxyethoxy)ethyl]phthalimide, using BOP coupling strategy. Similarly, mixed phosphonate esters **14** and **15** were obtained from ethyl hydrogen 4-(phthalimido)butylphosphonate (**13**) [34] and benzyl alcohol or *N*-(2-hydroxyethyl)phthalimide (Scheme 3).



Scheme 1. Synthesis of phosphonate inhibitors **4**, **5** and **6**. Reagents and conditions: (a) $P(OEt)_3$, 180 °C, 16 h; (b) NaN_3, DMF, 100 °C, 16 h; (c) 3-phenoxybenzyl alcohol, BOP, DIEA, DMF, rt, 12 h; (d) *N*-(hydroxymethyl)phthalimide or *N*-(2-hydroxyethyl)phthalimide, BOP, DIEA, DMF, rt, 12 h; (e) 3,4,5-trimethoxybenzyl alcohol, BOP, DIEA, DMF, rt, 12 h.

3. Pharmacology

All target compounds were tested for their inhibition of recombinant *M. tuberculosis* antigen 85C mycolyltransferase activity [15]. In this assay the coupling of radioactively labeled trehalose to TMM was monitored in the presence and absence of inhibitors. The target compounds were also tested in an *in vitro M. avium* growth inhibition assay with clarithromycin as positive control [36], in which *M. avium* 2447 was grown in 7H9 medium at 37 °C in the presence and absence of the tested compounds, and the growth of bacteria monitored by measurement of optical density. In our assay, this strain of *M. avium* exhibited susceptibility to clarithromycin with a minimal inhibitory concentration (MIC) of 0.2 μg/mL. The results of both tests are given in Tables 1–3.

4. Results and discussion

Of the ethyl 3-phenoxybenzyl alkylphosphonates **4**, ethyl 3-phenoxybenzyl *n*-butylphosphonate (**4a**) was the most potent in the mycolyltransferase inhibition assay ($IC_{50} = 2.0$ μM) and in the *in vitro M. avium* growth inhibition assay it had a minimal inhibitory concentration of 248.8 ± 129.8 μg/mL. The analogues **4b–d**, containing *n*-hexyl, *n*-heptyl and *n*-nonyl chains were 7–22 times less potent in the mycolyltransferase inhibition assay. In the *in vitro* assay with *M. avium* they displayed MICs of 259.7 ± 126.7 μg/mL (**4b**), 319.0 ±

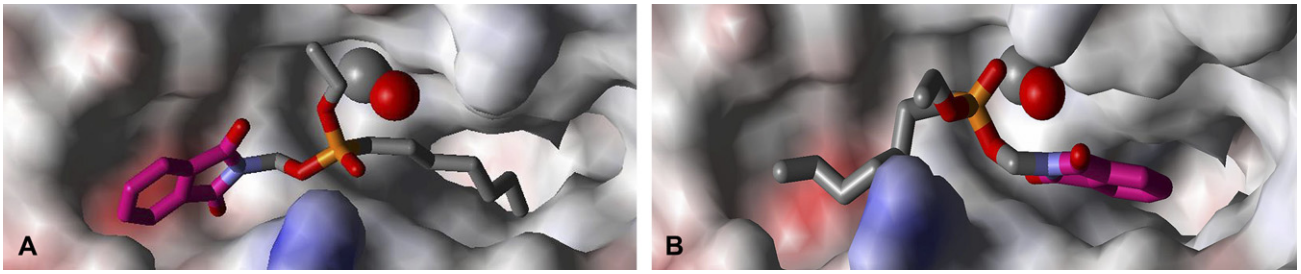
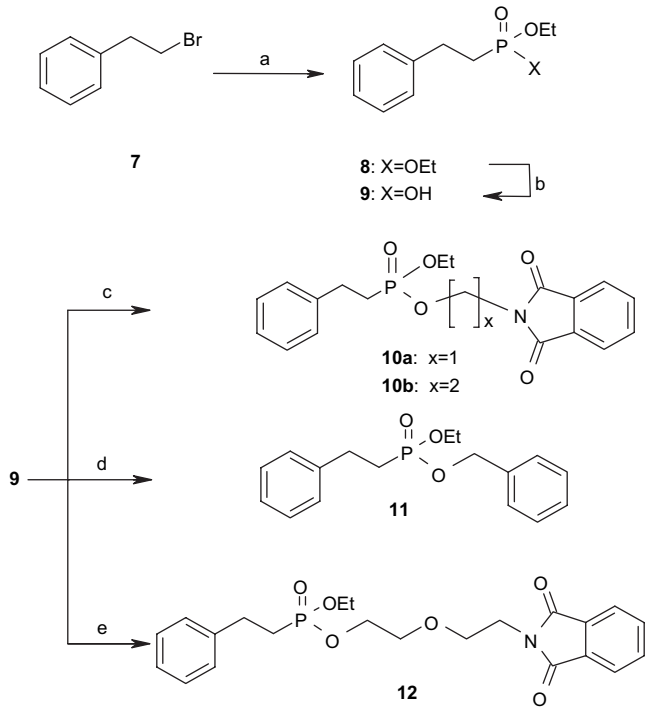
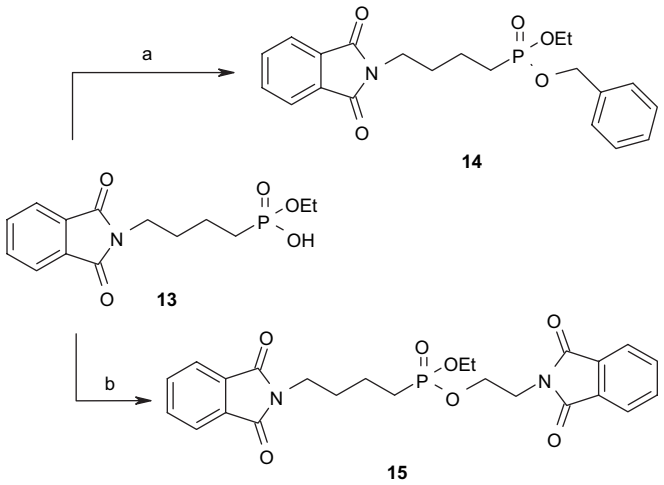


Fig. 4. (A) Highest-score hypothetical binding mode of inhibitor **5c** in the antigen 85C active site as predicted by AutoDock 3.0. The phthalimidomethyl substituent is located in the trehalose binding pocket and phosphonate moiety is oriented in the vicinity of Ser124. The alkyl chain is accommodated in the mycolate α-chain binding channel extending through the core of Ag85C. (B) Lower-score reverse binding mode of **5c** as predicted by AutoDock 3.0.



Scheme 2. Synthesis of phosphonate inhibitors **10**, **11** and **12**. Reagents and conditions: (a) $P(OEt)_3$, 180 °C, 16 h; (b) NaN_3, DMF, 100 °C, 16 h; (c) *N*-(hydroxymethyl)phthalimide or *N*-(2-hydroxyethyl)phthalimide, BOP, DIEA, DMF, rt, 12 h; (d) $PhCH_2OH$, BOP, DIEA, DMF, rt, 12 h; (e) *N*-[2-(2-hydroxyethoxy)ethyl]phthalimide, BOP, DIEA, DMF, rt, 12 h.

92.7 μg/mL (**4c**), and 188.4 ± 88.3 μg/mL (**4d**), respectively. A drastic drop in mycolyltransferase inhibitory potency was observed with the *n*-tetradecyl analogue **4e** (IC_{50} = 862.3 μM) which, due to its low water solubility, could not be tested in the *M. avium* culture *in vitro*. Thus, in the inhibitors **4**, a short alkyl chain of about 4 carbon atoms is optimal and long alkyl chains are not well tolerated. A lower mycolyltransferase inhibitory potency was also observed in compound **6**



Scheme 3. Synthesis of mixed phosphonates **14** and **15**. Reagents and conditions: (a) $PhCH_2OH$, BOP, DIEA, DMF, rt, 12 h; (b) *N*-(2-hydroxyethyl)phthalimide, BOP, DIEA, DMF, rt, 12 h.

Table 1
Mycolyltransferase inhibitory potencies of compounds **4a–e** and **6** and their inhibition of growth of *Mycobacterium avium*

No.	*n*	Chemical formula	MW	IC_{50} (μM)	MIC (μg/mL)[a]
4a	1	$C_{19}H_{25}O_4P$	348	2.0	248.8 ± 129.8
4b	3	$C_{21}H_{29}O_4P$	376	42.6	259.7 ± 126.7
4c	4	$C_{22}H_{31}O_4P$	390	21.0	319.0 ± 92.7
4d	6	$C_{24}H_{35}O_4P$	418	14.8	188.4 ± 88.3
4e	11	$C_{29}H_{45}O_4P$	488	862.3	n.d.
6	3	$C_{18}H_{31}O_6P$	374	101.6	n.d.

n.d. = Not determined.
[a] MIC of clarithromycin was 0.2 μg/mL.

(IC_{50} = 101.6 μM), which possesses a single phenyl ring instead of a diphenylether moiety (Table 1).

In the second series of inhibitors, *N*-(hydroxymethyl)phthalimide and *N*-(2-hydroxyethyl)phthalimide were used as replacements for trehalose. Encouraging mycolyltransferase inhibitory properties of compounds **5a–f** (IC_{50} values between 1.3 and 87.1 μM), confirmed the favorable choice of both, the trehalose mimicking moiety, and the alkylphosphonate residue predicted to interact with the hydrophobic channel in the antigen 85C core. The observed correlation between mycolyltransferase inhibitory potency and inhibition of *M. avium* growth *in vitro* was weaker than in the diphenylether series of compounds. Whereas compound **5c** was the most potent inhibitor of mycolyltransferase activity (IC_{50} = 1.3 μM), the most potent compound in *M. avium* growth inhibition assay was compound **5d** (MIC of 84.5 ± 56.5 μg/mL) with IC_{50} value of 87.1 μM in the mycolyltransferase inhibition assay. Compound **5b** had an MIC of 406.1 ± 239.9 μg/mL, whereas all other tested compounds of this series showed no detectable activity against *M. avium* in the *in vitro* assay. The best mycolyltransferase inhibitor in this series, compound **5c**, showed no capacity to inhibit the growth of *M. avium* in the *in vitro* assay (Table 2). These results indicate that, besides inhibition of antigen 85C, other targets and/or nonspecific binding are probably involved in the interaction of compounds **5a–f** with mycobacteria.

In order to study the influence of bulkiness of the alkyl groups which are predicted to bind to the hydrophobic channel, phenylethyl side chain containing phosphonates **10a**, **10b**, **11** and **12** were prepared. The length of the phenylethyl group is comparable to the length of the *n*-butyl group which was optimal for mycolyltransferase inhibitory activity. With

Table 2
Mycolyltransferase inhibitory potencies of compounds **5a–f** and their inhibition of growth of *Mycobacterium avium*

No.	*n*	*x*	Chemical formula	MW	IC_{50} (μM)	MIC (μg/mL)[a]
5a	1	1	$C_{15}H_{20}NO_5P$	325	10	Inactive
5b	1	2	$C_{16}H_{22}NO_5P$	339	50.7	406.1 ± 239.9
5c	4	1	$C_{18}H_{26}NO_5P$	367	1.3	Inactive
5d	6	1	$C_{20}H_{30}NO_5P$	395	87.1	84.5 ± 56.5
5e	6	2	$C_{21}H_{32}NO_5P$	409	25.7	Inactive
5f	11	1	$C_{25}H_{40}NO_5P$	466	41.0	n.d.

n.d. = Not determined.
[a] MIC of clarithromycin was 0.2 μg/mL.

Table 3
Mycolyltransferase inhibitory potencies of phosphonic acids **3a–d**, **9** and **13**

No.	*n*	Chemical formula	MW	IC_{50} (μM)
3a	1	$C_6H_{15}O_3P$	166	430.7
3b	3	$C_8H_{19}O_3P$	194	3.56
3c	4	$C_9H_{21}O_3P$	208	1.1
3d	6	$C_{11}H_{25}O_3P$	236	471.3
9	–	$C_{10}H_{15}O_3P$	214	225
13	–	$C_{14}H_{18}NO_5P$	311	110

exception of compound **12** ($IC_{50} = 348$ μM) all these compounds were found to be inactive in the mycolyltransferase inhibition assay, suggesting that the terminal phenyl group precludes binding of the phenylethyl group to the hydrophobic channel of the antigen 85C active site. The same effect could be responsible for the absence of inhibition in the phthalimidobutyl group containing compounds **14** and **15**.

Surprisingly, of the alkylphosphonic acid monoethyl esters, compounds **3b** and **3c** were found to be potent inhibitors of mycolyltransferase activity of antigen 85C (Table 3). This suggests that these compounds act as nonspecific ''promiscuous'' inhibitors. Having a structure that consists of a polar head and a hydrophobic tail, monoethyl phosphonates resemble the charged lipids typically found in micelles and vesicles. It has recently been postulated that such small molecules form aggregates of 50 to over 400 nm in diameter which can absorb or adsorb target enzymes, thereby inhibiting them [37]. Further experiments are needed to confirm this suggestion.

5. Conclusions

Starting from the crystal structure of *M. tuberculosis* antigen 85C, we have designed and synthesized a series of phosphonate inhibitors of mycolyltransferase activity of Ag85C, containing in their structure a phosphonate moiety, a mycolic acid side chain mimetic, and a simple trehalose surrogate. Ethyl 3-phenoxybenzyl butylphosphonate (**4a**) proved to be the most promising compound inhibiting the mycolyltransferase activity of antigen 85C with an IC_{50} value of 2.0 μM and showing substantial inhibition of growth of *M. avium in vitro*. In the phthalimido series of inhibitors **5**, the optimal mycolyltransferase inhibitory activity and the optimal *M. avium* growth inhibition activity do not reside in the same molecule. (1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl ethyl heptylphosphonate (**5c**) displayed the best potency ($IC_{50} = 1.3$ μM) in a mycolyltransferase inhibition assay, whereas in a *M. avium* growth inhibition assay (1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl ethyl nonylphosphonate (**5d**) possessed the highest growth inhibitory potency. Although in this work the new compounds were tested only against *M. avium* 2447 strain, future work will need to consider the screening of several strains to assess variability in susceptibility to our compounds.

Since the antigen 85 proteins have closely similar active site residues it can be expected that the compounds reported in this paper will have similar activities against all three antigen 85 proteins and thus promising antimycobacterial activity. Further insight into the interaction of these inhibitors with the antigen 85C active site is expected from the crystal structures of complexes between antigen 85C and its most potent inhibitors. These studies are in progress and will further aid optimization of the reported inhibitors.

6. Experimental

6.1. Synthesis

6.1.1. General

Chemicals were obtained from Acros, Aldrich, Fluka, Merck, Janssen and Sigma and used without further purification. Solvents were used without purification or drying, unless otherwise stated. Reactions were monitored using analytical TLC plates (Merck, silica gel 60 F_{254}) with rhodamine G6 or sulfuric acid staining. Silica gel grade 60 (70–230 mesh, Merck) was used for column chromatography. NMR spectra were obtained on a Bruker Avance DPX 300 instrument. ^{1}H NMR spectra were recorded at 300.13 MHz with tetramethylsilane as internal standard and ^{31}P NMR spectra at 121 MHz using H_3PO_4 as external standard. Mass spectra were obtained with a VG-Analytical Autospec Q mass spectrometer with EI or FAB ionization (MS Centre, Jožef Stefan Institute, Ljubljana). IR spectra were recorded on a Perkin–Elmer FTIR 1600 spectrometer. Elemental analyses were performed by the Department of Organic Chemistry, Faculty of Chemistry and Chemical Technology, Ljubljana, on a Perkin–Elmer elemental analyzer 240 C. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. Melting points were determined using a Reichert hot-stage microscope and are uncorrected. Diethyl phosphonates **2a–e** and **8** and monoethyl phosphonates **3a–e** and **9** were synthesized by modified literature procedures [24–27,29,30,32,33]. Compound **13** was prepared as described [34].

6.1.2. General procedure for the preparation of diethyl alkylphosphonates *2a–e* and *8*

Triethyl phosphite (12.07 g, 72.67 mmol) and 1-bromoalkane (30.28 mmol) were refluxed for 24 h at 160 °C. Excess triethyl phosphite was removed by vacuum distillation, and the residue purified by column chromatography on silica gel to give analytically pure diethyl alkylphosphonate as a colorless liquid.

6.1.3. General procedure for the preparation of ethyl hydrogen alkylphosphonates *3a–e* and *9*

A suspension of diethyl alkylphosphonate (30.28 mmol) and sodium azide (14.76 g, 227.1 mmol) in anhydrous DMF (15 mL) was stirred overnight at 100 °C. The solvent was removed under vacuum. The residue was dissolved in water (100 mL), and the resulting solution washed with ethyl acetate (3 × 30 mL). The aqueous solution was acidified with 4 M HCl to pH 1, and extracted with ethyl acetate (5 × 30 mL). The organic layer was dried over anhydrous Na_2SO_4, and evaporated in vacuum to give ethyl hydrogen alkylphosphonate as a colorless liquid.

*6.1.4. General procedure for the preparation of mixed phosphonates **4a–e**, **5a–f**, **6**, **10a–b**, **11**, **12**, **14** and **15***

N,*N*-Diisopropylethylamine (1.56 g, 12.06 mmol) was added with stirring to a solution of the corresponding monoethyl phosphonate (3.01 mmol), alcohol (4.52 mmol) and BOP (2.00 g, 4.52 mmol) in DMF (6 mL) and stirred overnight at room temperature. The reaction mixture was then poured into ethyl acetate (100 mL), washed with 10% citric acid solution (2 × 25 mL) and saturated $NaHCO_3$ solution (2 × 25 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. The residue was purified by column chromatography on silica gel to give analytically pure product.

*6.1.4.1. Ethyl 3-phenoxybenzyl butylphosphonate (**4a**).* The general procedure described above, using ethyl hydrogen butylphosphonate (**3a**) (0.50 g, 3.01 mmol) and (3-phenoxy)benzyl alcohol (0.91 g, 4.52 mmol), afforded compound **4a** (0.323 g, 30.7%). R_f 0.19 (EtOAc–hexane 1:1). ^{1}H NMR (DMSO-d_6) δ 0.83 (t, 3H, $J = 7.16$ Hz, 3H, $-CH_2-CH_2-CH_3$), 1.18 (t, 3H, $J = 6.97$ Hz, $O-CH_2-CH_3$), 1.25–1.50 (m, 4H, $P-CH_2(-CH_2)_2-CH_3$), 1.65–1.80 (m, 2H, $P-CH_2-(CH_2)_2-$), 3.84–4.05 (m, 2H, $P-O-CH_2-CH_3$), 4.91–5.06 (m, 2H, $O-CH_2-Ph$), 6.95–7.05 (m, 4H, Ar*H*), 7.12–7.20 (m, 2H, Ar*H*), 7.35–7.45 (m, 3H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 36.00. IR (KBr) ν 3466, 2959, 1585, 1488, 1256, 1024, 693 cm^{-1}. MS (FAB) *m*/*z* 349 $(M+H)^+$. Anal. $C_{19}H_{25}O_4P \cdot 0.8H_2O$ (C, H, N).

*6.1.4.2. Ethyl 3-phenoxybenzyl hexylphosphonate (**4b**).* The general procedure described above, using ethyl hydrogen hexylphosphonate (**3b**) (1.32 g, 6.77 mmol) and (3-phenoxy)benzyl alcohol (2.03 g, 10.2 mmol), afforded compound **4b** (0.500 g, 19.6%). R_f 0.31 (EtOAc–hexane 1:1). ^{1}H NMR (DMSO-d_6) δ 0.85 (t, $J = 6.97$ Hz, 3H, $-CH_2-CH_2-CH_3$), 1.14–1.52 (m, 11H, $P-CH_2-(CH_2)_4-CH_3$ and $P-O-CH_2-CH_3$), 1.58–1.79 (m, 2H, $P-CH_2-(CH_2)_4-$), 3.83–4.04 (m, 2H, $P-O-CH_2-CH_3$), 4.90–5.05 (m, 2H, $O-CH_2-Ph$), 6.93–7.05 (m, 4H, Ar*H*), 7.08–7.20 (m, 2H, Ar*H*), 7.33–7.45 (m, 3H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 33.90. IR (KBr) ν 3445, 2931, 1585, 1488, 1448, 1258, 1164, 1024, 847, 693 cm^{-1}. MS (FAB) *m*/*z* 377 $(M+H)^+$. Anal. ($C_{21}H_{29}O_4P \cdot H_2O$) (C, H).

*6.1.4.3. Ethyl 3-phenoxybenzyl heptylphosphonate (**4c**).* The general procedure described above, using ethyl hydrogen heptylphosphonate (**3c**) (0.70 g, 3.37 mmol) and 3-(phenoxy)benzyl alcohol (1.01 g, 5.05 mmol), afforded compound **4c** (0.705 g, 53.7%). R_f 0.29 (EtOAc–hexane 1:1). ^{1}H NMR (DMSO-d_6) δ 0.84 (t, $J = 6.78$ Hz, 3H, $-CH_2-CH_2-CH_3$), 1.14–1.50 (m, 13H, $P-CH_2-(CH_2)_5-CH_3$ and $P-O-CH_2-CH_3$), 1.64–1.79 (m, 2H, $P-CH_2-(CH_2)_5-$), 3.83–4.05 (m, 2H, $P-O-CH_2-CH_3$), 4.91–5.05 (m, 2H, $O-CH_2-Ph$), 6.93–7.05 (m, 4H, Ar*H*), 7.11–7.20 (m, 2H, Ar*H*), 7.35–7.45 (m, 3H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 33.90. IR (NaCl) ν 2930, 2857, 1585, 1488, 1448, 1257, 1216, 1163, 1024, 941, 693 cm^{-1}. MS (FAB) *m*/*z* 391 $(M+H)^+$. Anal. ($C_{22}H_{31}O_4P \cdot 0.3H_2O$) (C, H).

*6.1.4.4. Ethyl 3-phenoxybenzyl nonylphosphonate (**4d**).* The general procedure described above, using ethyl hydrogen nonylphosphonate (**3d**) (1.00 g, 4.09 mmol) and 3-(phenoxy)benzyl alcohol (1.23 g, 6.14 mmol), afforded compound **4d** (0.253 g, 15.1%). R_f 0.25 (EtOAc–hexane 1:1). ^{1}H NMR (DMSO-d_6) δ 0.85 (t, $J = 6.78$ Hz, 3H, $-CH_2-CH_2-CH_3$), 1.12–1.34 (m, 15H, $P-(CH_2)_2-(CH_2)_6-CH_3$ and $P-O-CH_2-CH_3$), 1.35–1.50 (m, 2H, $P-CH_2-CH_2-(CH_2)_6-$), 1.64–1.78 (m, 2H, $P-CH_2-(CH_2)_7-$), 3.84–4.04 (m, 2H, $P-O-CH_2-CH_3$), 4.91–5.05 (m, 2H, $O-CH_2-Ph$), 6.95–7.05 (m, 4H, Ar*H*), 7.11–7.20 (m, 2H, Ar*H*), 7.34–7.47 (m, 3H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 40.00. IR (KBr) ν 2928, 2855, 1585, 1488, 1257, 1024, 959, 692 cm^{-1}. MS (FAB) *m*/*z* 419 $(M+H)^+$. Anal. ($C_{24}H_{35}O_4P \cdot 1.8H_2O$) (C, H).

*6.1.4.5. Ethyl 3-phenoxybenzyl tetradecylphosphonate (**4e**).* The general procedure described above, using ethyl hydrogen tetradecylphosphonate (**3e**) (0.50 g, 1.63 mmol) and 3-(phenoxy)benzyl alcohol (0.49 g, 2.45 mmol), afforded compound **4e** (0.253 g, 19.8%). R_f 0.38 (EtOAc–hexane 1:2). ^{1}H NMR (DMSO-d_6) δ 0.85 (t, $J = 6.79$ Hz, 3H, $-CH_2-CH_2-CH_3$), 1.14–1.36 (m, 25H, $P-(CH_2)_2-(CH_2)_{11}-CH_3$ and $P-O-CH_2-CH_3$), 1.36–1.50 (m, 2H, $P-CH_2-CH_2-(CH_2)_{11}-$), 1.65–1.78 (m, 2H, $P-CH_2-(CH_2)_{12}-$), 3.84–4.05 (m, 2H, $P-O-CH_2-CH_3$), 4.90–5.05 (m, 2H, $O-CH_2-Ph$), 6.95–7.05 (m, 4H, Ar*H*), 7.11–7.19 (m, 2H, Ar*H*), 7.34–7.46 (m, 3H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 33.90. IR (KBr) ν 2925, 2853, 1586, 1488, 1447, 1257, 1216, 1024, 941, 692 cm^{-1}. MS (FAB) *m*/*z* 489 $(M+H)^+$. Anal. ($C_{29}H_{45}O_4P$) (C, H).

*6.1.4.6. (1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-methyl ethyl butylphosphonate (**5a**).* The general procedure described above, using ethyl hydrogen butylphosphonate (**3a**) (0.50 g, 3.01 mmol) and *N*-(2-hydroxymethyl)phthalimide (0.80 g, 4.52 mmol), afforded compound **5a** (0.210 g, 21.4%). R_f 0.34 (EtOAc–hexane 4:1). ^{1}H NMR (DMSO-d_6) δ 0.81 (t, 3H, $J = 7.16$ Hz, 3H, $-CH_2-CH_2-CH_3$), 1.20 (t, 3H, $J = 7.16$ Hz, $O-CH_2-CH_3$), 1.25–1.49 (m, 4H, $P-CH_2-(CH_2)_2-CH_3$), 1.70–184 (m, 2H, $P-CH_2-(CH_2)_2-$), 3.94–4.08 (m, 2H, $P-O-CH_2-CH_3$), 5.42–5.49 (m, 2H, $O-CH_2-N$), 7.89–8.02 (m, 4H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 33.10. IR (KBr) ν 3499, 2961, 1783, 1731, 1375, 1249, 1004, 726 cm^{-1}. MS (FAB) *m*/*z* 326 $(M+H)^+$. Anal. ($C_{15}H_{20}NO_5P \cdot 0.5\ H_2O$) (C, H, N).

*6.1.4.7. 2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-ethyl ethyl butylphosphonate (**5b**).* The general procedure described above, using ethyl hydrogen butylphosphonate (**3a**) (0.50 g, 3.01 mmol) and *N*-(2-hydroxyethyl)phthalimide (0.86 g, 4.52 mmol), afforded compound **5b** (0.110 g, 9.1%). R_f 0.23 (EtOAc–hexane 4:1). ^{1}H NMR (DMSO-d_6) δ 0.75 (t, 3H, $J = 7.15$ Hz, $-CH_2-CH_2-CH_3$), 1.10 (t, 3H, $J = 6.97$ Hz,

O–CH_2–CH_3), 1.15–1.40 (m, 4H, P–CH_2–(CH_2)$_2$–CH_3), 1.48–1.68 (m, 2H, P–CH_2–(CH_2)$_2$–), 3.77–4.04 (m, 4H, O–(CH_2)$_2$–N), 4.06–4.23 (m, 2H, P–O–CH_2–CH_3), 7.79–7.93 (m, 4H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 33.90. IR (KBr) ν 3473, 2959, 1774, 1715, 1394, 1246, 1035, 960 cm^{-1}. MS (FAB) *m/z* 340 $(M+H)^+$. Anal ($C_{16}H_{22}NO_5P\cdot 0.25H_2O$) (C, H, N).

*6.1.4.8. (1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-methyl ethyl heptylphosphonate (**5c**).* The general procedure described above, using ethyl hydrogen heptylphosphonate (**3c**) (0.50 g, 2.40 mmol) and *N*-(2-hydroxymethyl)phthalimide (0.64 g, 3.61 mmol), afforded compound **5c** (0.88 g, 54.3%). R_f 0.33 (EtOAc–hexane 2:1). ^{1}H NMR ($CDCl_3$) δ 0.87 (t, $J = 6.78$ Hz, 3H, –CH_2–CH_2–CH_3), 1.19–1.65 (m, 15H, P–(CH_2)$_6$–CH_3 and P–O–CH_2–CH_3), 4.11–4.26 (m, 2H, P–O–CH_2–CH_3), 5.58–5.68 (m, 2H, O–CH_2–N), 7.77–7.83 (m, 2H, Ar*H*), 7.92–7.98 (m, 2H, Ar*H*). ^{31}P NMR ($CDCl_3$) δ 33.81. IR (KBr) ν 2930, 2857, 1783, 1731, 1375, 1256, 1048, 1002, 724 cm^{-1}. MS (FAB) *m/z* 368 $(M+H)^+$. Anal. ($C_{18}H_{26}NO_5P\cdot 0.5H_2O$) (C, H); N: calcd, 3.72; found, 4.28.

*6.1.4.9. (1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-methyl ethyl nonylphosphonate (**5d**).* The general procedure described above, using ethyl hydrogen nonylphosphonate (**3d**) (0.50 g, 3.01 mmol) and *N*-(2-hydroxymethyl)phthalimide (0.80 g, 4.52 mmol), afforded compound **5d** (0.188 g, 15.8%). R_f 0.39 (EtOAc–hexane 4:1). mp = 55–59 °C. ^{1}H NMR (DMSO-d_6) δ 0.85 (t, $J = 6.78$ Hz, 3H, –CH_2–CH_2–CH_3), 1.07–1.34 (m, 15H, P–(CH_2)$_2$–(CH_2)$_6$–CH_3 and P–O–CH_2–CH_3), 1.35–1.51 (m, 2H, P–CH_2–CH_2–CH_2–), 1.68–1.83 (m, 2H, P–CH_2–CH_2–CH_2–), 3.94–4.09 (m, 2H, P–O–CH_2–CH_3), 5.39–5.52 (m, 2H, O–CH_2–N), 7.88–8.02 (m, 4H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 33.00. IR (KBr) ν 2920, 2852, 1778, 1720, 1613, 1466, 1381, 1247, 1192, 1044, 847, 722, 616, 531 cm^{-1}. MS (FAB) *m/z*: 396 $(M+H)^+$. Anal. ($C_{20}H_{30}NO_5P$) (C, H, N).

*6.1.4.10. 2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-ethyl ethyl nonylphosphonate (**5e**).* The general procedure described above, using ethyl hydrogen nonylphosphonate (**3d**) (0.41 g, 1.74 mmol) and *N*-(2-hydroxyethyl)phthalimide (0.80 g, 4.52 mmol), afforded compound **5e** (0.147 g, 20.6%). R_f 0.25 (EtOAc–hexane 4:1). ^{1}H NMR (DMSO-d_6) δ 0.86 (t, $J = 6.18$ Hz, 3H, –CH_2–CH_2–CH_3), 1.04–1.40 (m, 17H, P–(CH_2)–(CH_2)$_7$–CH_3 and P–O–CH_2–CH_3), 1.53–1.68 (m, 2H, P–CH_2–CH_2–CH_2–), 3.77–3.94 (m, 4H, N–CH_2–CH_2–O), 4.06–4.23 (m, 2H, CH_3–CH_2–O), 7.81–7.92 (m, 4H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 33.90. IR (KBr) ν 2927, 2854, 1774, 1716, 1393, 1241, 1034, 844, 720 cm^{-1}. MS (FAB) *m/z* 410 $(M+H)^+$. Anal. ($C_{21}H_{32}NO_5P\cdot 1.67H_2O$) (C, H, N).

*6.1.4.11. (1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-methyl ethyl tetradecylphosphonate (**5f**).* The general procedure described above, using ethyl hydrogen tetradecylphosphonate (**3e**) (0.50 g, 1.63 mmol) and *N*-(2-hydroxymethyl)phthalimide (0.43 g, 2.45 mmol), afforded compound **5f** (0.168 g, 22.1%). mp = 65–70 °C. ^{1}H NMR (DMSO-d_6) δ 0.89 (t, $J = 6.60$ Hz, 3H, –CH_2–CH_2–CH_3), 1.15–1.42 (m, 25H, P–(CH_2)$_2$–(CH_2)$_{11}$–CH_3 and P–O–CH_2–CH_3), 1.50–1.67 (m, 2H, P–CH_2–CH_2–(CH_2)$_{11}$–), 1.73–1.87 (m, 2H, P–CH_2–(CH_2)$_{12}$–), 4.06–4.23 (m, 2H, P–O–CH_2–CH_3), 5.58–5.68 (m, 2H, O–CH_2–N), 7.70–7.98 (m, 4H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 33.80. IR (KBr) ν 3491, 2917, 2848, 1778, 1722, 1466, 1378, 1246, 1044, 982, 844, 720, 616, 532 cm^{-1}. MS (FAB) *m/z* 488 $(M+Na)^+$. Anal. ($C_{25}H_{40}NO_5P\cdot 0.5H_2O$) (C, H, N).

*6.1.4.12. Ethyl 3,4,5-trimethoxybenzyl hexylphosphonate (**6**).* The general procedure described above, using monophosphonate **3b** (0.96 g, 4.95 mmol) and (3,4,5-trimethoxy)-phenylmethanol (1.07 mL, 4.52 mmol), afforded compound **6** (0.200 g, 11.0%). R_f 0.12 (EtOAc–hexane 1:1). ^{1}H NMR (DMSO-d_6) δ 0.85 (t, $J = 6.78$ Hz, 3H, –CH_2–CH_2–CH_3), 1.18–1.26 (m, 3H, P–O–CH_2–CH_3), 1.26–1.52 (m, 8H, P–CH_2–(CH_2)$_4$–CH_3), 1.67–1.80 (m, 2H, P–O–CH_2–CH_3), 3.66 (s, 3H, CH_3–O–Ar), 3.78 (s, 6H, 2 × CH_3–O–Ar), 3.90–4.08 (m, 2H, CH_3–CH_2–O–P), 4.86–4.98 (m, 2H, Ph–CH_2–O–), 6.71 (s, 2H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 33.80. IR (KBr) ν 2935, 1594, 1508, 1460, 1424, 1334, 1237, 1129, 1047, 1013, 967 cm^{-1}. MS (EI) *m/z* 374 (M^+). Anal. ($C_{18}H_{31}O_6P\cdot 0.2H_2O$) (C, H).

*6.1.4.13. (1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-methyl ethyl phenethylphosphonate (**10a**).* The general procedure described above, using ethyl hydrogen 2-phenethylphosphonate (0.45 g, 2.1 mmol) and *N*-(hydroxymethyl)phthalimide (1.39 g, 3.15 mmol), afforded compound **10a** as a colorless liquid (0.564 g, 71.90%). R_f 0.36 (EtOAc). ^{1}H NMR (DMSO-d_6) δ 1.20 (t, $J = 6.97$ Hz, 3H, OCH_2CH_3), 2.06–2.21 (m, 2H, CH_2CH_2P), 2.70–2.84 (m, 2H, PhCH_2CH_2), 3.95–4.09 (m, 2H, OCH_2CH_3), 5.47 (d, $J = 8.66$ Hz, 2H, OCH_2N), 7.12–7.30 (m, 5H, *ArH*), 7.88–8.03 (m, 4H, Ar*H*). IR (KBr) ν 3496, 2983, 1783, 1375, 1248, 1049, 1003, 869, 713 cm^{-1}. MS (FAB) *m/z* 374 $(M+H)^+$. Anal. ($C_{19}H_{20}NO_5P$) (C, H, N).

*6.1.4.14. (1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-ethyl ethyl phenethylphosphonate (**10b**).* The general procedure described above, using ethyl hydrogen 2-phenethylphosphonate (0.63 g, 3.28 mmol) and *N*-(2-hydroxyethyl)phtalimide (1.45 g, 3.28 mmol), afforded compound **10b** as a colorless liquid (0.67 g, 79.7%). R_f 0.50 (EtOAc). ^{1}H NMR (DMSO-d_6) δ 1.09 (t, $J = 6.97$ Hz, 3H, OCH_2CH_3), 1.89–2.04 (m, 2H, CH_2CH_2P), 2.61–2.73 (m, 2H, OCH_2CH_2N), 3.81–3.93 (m, 4H, PhCH_2CH_2 and OCH_2CH_2N), 4.13–4.22 (m, 2H, OCH_2CH_3), 7.13–7.30 (m, 5H, Ar*H*), 7.81–7.93 (m, 4H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 32.3. IR (KBr) ν 3478, 2982, 1774, 1714, 1604, 1394, 1245, 1072, 1035, 961, 720 cm^{-1}. MS (FAB) *m/z* 388 $(M+H)^+$. Anal. ($C_{20}H_{22}NO_5$) (C, H, N).

*6.1.4.15. Benzyl ethyl phenethylphosphonate (**11**).* The general procedure described above, using ethyl hydrogen 2-phenethylphosphonate (0.41 g, 1.90 mmol) and benzyl alcohol (1.26 g, 2.84 mmol), afforded compound **11** as a yellowish liquid (0.46 g, 79.8%). R_f 0.67 (EtOAc–hexane 1:1). ^{1}H NMR (DMSO-d_6) δ 1.20 (t, $J = 7.16$ Hz, 3H, OCH$_2$*CH*$_3$), 2.02–2.16 (m, 2H, CH$_2$*CH*$_2$P), 2.72–2.83 (m, 2H, Ph*CH*$_2$CH$_2$), 3.91–4.06 (m, 2H, O*CH*$_2$CH$_3$), 4.95 (s, 2H, O–C*H*$_2$–Ph), 7.16–7.45 (m, 10H, Ar*H*). ^{31}P NMR (DMSO-d_6) δ 32.3. IR (KBr) ν 3431, 2929, 1600, 1451, 1244, 1010, 850 cm^{-1}. MS (FAB) *m/z* 305 $(M + H)^+$. Anal. ($C_{17}H_{21}O_3P \cdot 0.25H_2O$) (C, H).

*6.1.4.16. 2-[2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)ethoxy]ethyl ethyl phenethylphosphonate (**12**).* The general procedure described above, using ethyl hydrogen 2-phenethylphosphonate (0.51 g, 2.38 mmol) and 2-[2-(2-hydroxyethoxy)ethyl]-1*H*-isoindole-1,3(2*H*)-dione (1.58 g, 3.57 mmol), afforded compound **12** (0.65 g, 63.4%). R_f 0.63 ($CHCl_3$–MeOH 9:1). ^{1}H NMR (DMSO-d_6) δ 1.6 (t, $J = 6.7$ Hz, 3H, OCH$_2$*CH*$_3$), 1.89–2.05 (m, 2H, CH$_2$*CH*$_2$P), 2.65–2.79 (m, 2H, Ph*CH*$_2$CH$_2$), 3.58 (t, $J = 4.52$ Hz, 2H, CH$_2$*CH*$_2$N), 3.67 (t, $J = 5.56$ Hz, 2H, O*CH*$_2$CH$_2$N), 3.77 (t, $J = 5.56$ Hz, 2H, OCH$_2$*CH*$_2$O), 3.85–4.07 (m, 4H, O*CH*$_2$CH$_3$ and O*CH*$_2$-CH$_2$O), 7.15–7.32 (m, 5H, Ar*H*), 7.78–7.89 (m, 4H, Ar*H*). IR (KBr) ν 3467, 2944, 1774, 1712, 1395, 1244, 1032, 966, 796, 722 cm^{-1}. MS (FAB) *m/z* 432 $(M + H)^+$. Anal. ($C_{22}H_{26}NO_6P$) (C, H, N).

*6.1.4.17. Benzyl ethyl 4-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)butylphosphonate (**14**).* The general procedure described above, using ethyl hydrogen 4-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)butyl-phosphonate (**13**) (0.417 g, 1.34 mmol) and benzyl alcohol (0.890 g, 2.01 mmol), afforded compound **14** (0.386 g, 71.84%) as a yellow oil. R_f 0.22 (EtOAc). ^{1}H NMR (DMSO-d_6) δ 1.18 (t, $J = 6.9$ Hz, 3H, OCH$_2$*CH*$_3$), 1.38–1.55 (m, 2H, CH$_2$*CH*$_2$P), 1.61–1.87 (m, 4H, NCH$_2$C*H*$_2$ and C*H*$_2$CH$_2$P), 3.57 (t, $J = 6.7$ Hz, 2H, CH$_2$*CH*$_2$N), 3.87–4.05 (m, 2H, O*CH*$_2$CH$_3$), 4.96 (s, 2H, OC*H*$_2$Ph), 7.26–7.42 (m, 5H, Ar*H*), 7.80–7.90 (m, 4H, Ar*H*). IR (KBr) ν 3458, 2941, 1770, 1711, 1397, 1244, 1020, 961, 721 cm^{-1}. MS (FAB) *m/z* 402 $(M + H)^+$. Anal. ($C_{21}H_{24}NO_5P \cdot 0.25H_2O$) (C, H, N).

*6.1.4.18. 2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)ethyl ethyl 4-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)butylphosphonate (**15**).* The general procedure described above, using ethyl hydrogen 4-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)butyl-phosphonate (**13**) (0.419 g, 1.35 mmol) and 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethanol (0.386 g, 2.02 mmol), afforded compound **15** (0.44 g, 68.1%). mp = 59–62 °C. R_f 0.47 (EtOAc–hexane 10:1). ^{1}H NMR (DMSO-d_6) δ 1.07 (t, $J = 7.2$ Hz, 3H, OCH$_2$*CH*$_3$), 1.31–1.46 (m, 2H, CH$_2$*CH*$_2$P), 1.54–1.75 (m, 4H, C*H*$_2$CH$_2$CH$_2$P), 3.51 (t, $J = 6.8$ Hz, 2H, CH$_2$*CH*$_2$N), 3.77–3.91 (m, 4H, O*CH*$_2$C*H*$_2$N), 4.08–4.18 (m, 2H, O*CH*$_2$CH$_3$), 7.77–7.91 (m, 8H, Ar*H*). ^{31}P NMR (DMSO-d_6): δ 36.5. IR (KBr) ν 3466, 2942, 1771, 1712, 1613, 1395, 1244, 1034, 957, 720 cm^{-1}. MS (FAB) *m/z* 485 $(M + H)^+$. Anal. ($C_{24}H_{25}N_2O_7P$) (C, H, N).

6.2. Molecular docking

Automated docking was used to determine the orientation of inhibitors bound in the active site of Ag85C. A genetic algorithm method, implemented in the program AutoDock 3.0, was employed [38]. The structures of inhibitors were prepared using HyperChem 7.5 (HyperChem, version 7.5 for Windows; Hypercube, Inc.: Gainesville, FL, 2002). The crystal structure of Ag85C was retrieved from the RCSB protein database (PDB entry 1DQZ) and all water molecules removed. Polar hydrogen atoms were added and Kollman charges [39], atomic solvation parameters and fragmental volumes were assigned to the protein using AutoDock Tools (ADT). For docking calculations, Gasteiger–Marsili partial charges [40] were assigned to the ligands and nonpolar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The grid map, which was centered at Ser124 of the protein, was generated with AutoGrid. The grid dimensions were large enough to cover the inhibitors and the enzyme's active site. Lennard-Jones parameters 12-10 and 12-6, supplied with the program, were used for modeling H-bonds and van der Waals interactions, respectively. The distance-dependent dielectric permittivity of Mehler and Solmajer [41] was used to calculate the electrostatic grid maps. Random starting points, random orientation, and torsions were used for all ligands. The translation, quaternion, and torsion steps were taken from default values in AutoDock. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters. The number of docking runs was 100, the population in the genetic algorithm was 250, the number of energy evaluations was 500,000, and the maximum number of iterations 27,000.

6.3. Biological assays

6.3.1. Mycolyltransferase assay [15]

Mycolyltransferase assays were performed by suspending 62.5 μg of TMM in 50 μL of 0.1 M potassium phosphate (pH 7.5) and 10 mM DTT and sonicating the mixture for 15 min. To this mixture was added 100 μg of enzyme and ^{14}C-trehalose (0.25 μCi, 30.4 mCi/mmol, Amersham) along with the inhibitor in a final volume of 200 μL and the contents incubated at 37 °C for 30 min. The reaction was stopped by adding 3 mL of $CHCl_3$/CH_3OH (2:1) and 300 μL of water. This mixture was vortexed, centrifuged and the upper aqueous phase was discarded. The lower organic layer was washed twice using 0.5 mL of $CHCl_3$/CH_3OH/H_2O (3:47:48) and finally dried under a stream of nitrogen. The resulting organic extractable material containing the products of the mycolyltransferase reaction was resuspended in 100 μL of $CHCl_3$/CH_3OH (2:1). A 50 μL aliquot was dried prior to the addition of 5 mL of scintillation fluid and counted. Thin-layer chromatography (TLC)-autoradiography of the organic extractable products was performed with silica gel TLC plates using the solvent system $CHCl_3$/CH_3OH/NH_4OH (80:20:2). TDM and TMM standards were visualized by spraying with 10% α-naphthol in 5% sulfuric acid in ethanol and heating at 110 °C. The mycolyltransferase assays were performed in

triplicate and 3–5 independent experiments performed for each series of inhibitors.

6.3.2. M. avium growth inhibition assay [36]

M. avium 2447 was grown in Middlebrook 7H9 medium (Difco, St. Louis, MO) at 37 °C in the presence and absence of the test compounds, and the growth of bacteria was monitored by optical density ($OD_{610\ nm}$) measurement. Cultures were performed in 96 flat bottom well plates in a total volume of 200 μL and starting the cultures at a mycobacterial density of 10^4 colony forming units per well. The maximum concentration tested was 20 to 150 μg/mL, depending on the solubility of each compound. Exponential growth of *M. avium* was observed until day 15 of growth. From the growth curves obtained, the specific growth rates (k) were calculated. The k values were plotted against the concentration of the different compounds tested and when there was a linear decrease in k as a function of the drug concentration, the minimal inhibitory concentration (MIC, $k = 0$) was extrapolated by calculating the best fit correlation. When no apparent dose response relation between k and drug concentration was found the compound was considered inactive. Only in the cases of compound **5d** and clarithromycin it was possible to perform the assay using the actual MIC. In all other cases, solubility in the culture medium was inferior to the MIC which was then extrapolated from the values obtained with concentrations of the drug which led to incomplete inhibition of growth of the mycobacteria. Three independent determinations were performed for each compound. Data are shown as the mean of MIC calculated for each experiment ± one standard deviation.

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