New Derivatives of BM212: A Class of Antimycobacterial Compounds Based on the Pyrrole Ring as a Scaffold

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Abstract: During our investigation in the area of antimycobacterial agents, we have identified the 1,5-(4-chlorophenyl)-2-methyl-3-(4-methylpiperazin-1-yl)methyl-1*H*-pyrrole (**BM212**) as the lead compound for a new class of antimycobacterial pyrrole derivatives with potent *in vitro* activity against mycobacteria and with low cytotoxicity. We have also identified the salient structural features of **BM212**, while structure-activity relationships (SAR) and molecular modeling studies on pyrrole compounds allowed us to design and synthesize additional analogues. Among them, seven compounds revealed a very high activity (better than that of **BM212** toward mycobacteria) and a very interesting protection index, comparable to that of reference compounds, such as isoniazid, streptomycin and rifampin.

Key Words: BM212, lead compound, mycobacterium tuberculosis, atypical mycobacteria**,** *in vitro* activity, intramacrophagic activity, pyrroles, pharmacophore, cytotoxicity, protection index.

INTRODUCTION

Mycobacterium tuberculosis (MTB), responsible for tuberculosis (TB) in humans, causes the death of almost 3 million people each year and it is positioned as the leading bacterial infectious agent [1]. According to a recent report compiled by the World Health Organization (WHO), the total number of new cases of TB worldwide in 2002 had risen to approximately 9 million [2] and it is estimated that between 2002 and 2020, approximately 1000 million people will be newly infected, over 150 million people will get sick, and 36 million will die of TB if proper control measures are not instituted [3]. This is despite the undoubted success of widespread implementation of the "DOTS" (directly observed therapy, short-course) strategy, now covering 180 countries and accessible by over 70% of the world's population. A key driver of the TB increase is synergy with the HIV-1 epidemic, which is having a devasting impact in some parts of the world, such as the WHO African Region, where 31% of new TB cases were attributable to HIV-1 co-infection [4]. Furthermore, the emergence of strains of MTB resistant to all the first-line drugs (multidrug resistant TB, MDR-TB) is causing serious concern in some countries [5].

Other important members of the genus *Mycobacterium* are *Mycobacterium leprae*, which infects 12 million patients worldwide, and the so-called "atypical mycobacteria", which include *Mycobacterium avium* complex (MAC), an opportunistic pathogen in AIDS patients [6]. Clinical management of MAC infections, especially among AIDS patients, is difficult, partly because of the severely depressed state of the host defence mechanisms in such patients [7-9], and partly because of the intrinsically low susceptibility of MAC isolates to many antimycobacterial drugs [7, 10]. One of the hallmarks of TB is the persistent phase of infection. During this phase, bacteria are thought to be in a slow-growing or non-growing state [11] and are recalcitrant to treatment by conventional anti-TB drugs [12]. Patients who carry a latent infection are at risk of reactivation of the disease and this is one of the factors which causes a major obstacle to the global control of TB.

The current short-course MTB drug treatment regimen uses an initial 2-month phase of daily therapy with isoniazid (INH), rifampicin (RIF) and pyrazinamide (PZA), in addition to either streptomycin (SM) or ethambutol (EMB)[13]. This regimen is followed by daily therapy with INH and one other primary drug for the next 4 months. Drugs such as ethionamide and fluoroquinolones (FQs) are used as secondary or alternative agents to treat infections caused by strains resistant to commonly used medicines. Long-term therapies, lasting between 6 and 9 months, have frequently led to patient non-compliance and, in turn, contributed to the emergence of MDR-TB [14, 15]. MDR strains, such as the notorious strain W [16], are increasingly being found. They are resistant to many first-line agents including INH, rifampin, ethambutol, streptomycin and pyrazinamide, as well as to some of the second-line drugs, such as ethionamide and the quinolones [17]. Without effective treatments, the fear is that the number of infections caused by MDR-TB will increase out of control. Therefore, the development of new antimicrobial drugs with potent anti-TB and/or anti-MAC activity and new protocols for chemotherapy of the patients with intractable TB and MAC infections, are urgently needed.

It is astonishing that with this background, there have been no new drugs registered to treat TB in the last 40 years. This reflects the inherent difficulties in discovery and clinical testing of new agents and the lack of pharmaceutical industry research in the area [18]. The Global Alliance for TB

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Drug Development (GATB; www.tballiance.org) was established to address this need. Its top priority is the development of a new agent that will shorten the duration of chemotherapy from the current 6-8 months to two months or less, although new drugs with activity against MDR-TB and latent TB are also needed [19]. Fundamental uncertainties in many aspects of the biology of the organism have substantially hampered the ability to identify critical targets whose inhibition would correlate with sterilizing activity. Sterilizing activity refers to the ability of a drug to kill those organisms, known as "persisters", that survive treatment with agents targeting essential processes in dividing bacteria. It is only by discovering new agents with improved sterilizing activity that a shorter treatment regimen can be developed. Furthermore, the new compounds must have a novel mode of action, since such agents are likely more effective against drug resistant strains, and they must be selective for mycobacteria and compatible with HIV drugs.

For clinical control of tuberculosis, including MDR-TB, the new rifamycin derivatives rifabutin (RBT; Mycobutin™, Pharmacia) and rifapentine (RPT; Priftin™, Aventis) [20-22] have been developed. Moreover, a new benzoxazinorifamycin, rifalazil (RLZ: formerly KRM-1648), which has a much more potent *in vitro* and *in vivo* antimycobacterial activity than RBT and RIF [23-27], is now under Phase II clinical study as an antituberculous drug in the United States (US). Fluoroquinolones (FQs), including ciprofloxacin (CPF), levofloxacin (LVFX) and sparfloxacin (SPFX) are also used as the second-line antituberculous drugs for MDR-TB [22, 28-32]. However, there are some flaws for these drugs such as: (i) the therapeutic efficacy of RBT against MDR-TB is somewhat flawed because of the anti-RBT cross-resistance, which is acquired by the majority of RFP-resistant MTB isolates [33]. Similar data are also reported for RPT and RLZ [20, 34]; (ii) all the FQs possess only modest antituberculous activity in treating TB, whereas quinolone-resistant MTB strains are rapidly increasing [30, 32, 35-38]. In addition, although a number of new FQs have been synthesized and developed as chemotherapeutic drugs against common bacterial infections (e.g., sitafloxacin, gatifloxacin, moxifloxacin, grepafloxacin, WQ-3034, HSR-903, T-3811, etc.), only the former three quinolones exhibit the same level of *in vitro* activity against MTB isolates as that of SPFX [22, 39, 40].

With regard to the chemotherapy of MAC infections, ordinary drugs are generally low in efficacy for the following reasons [7-9]: (i) the susceptibility of MAC to the majority of ordinary antimycobacterial drugs, including the above FQs, is generally low due to its impermeability to these agents [7, 22, 41]. Moreover, the range of susceptibilities of MAC isolates to most antimicrobial drugs, excluding macrolides, is very broad; (ii) polyclonal MAC infections in AIDS patients may contribute significantly to the lack of success in treating MAC infections. Although new macrolides, such as clarithromycin (CAM) and azithromycin (AZM), display appreciable levels of anti-MAC activity [22, 42, 43], pulmonary MAC infections are still intractable, even by multidrug therapy involving these macrolides [22, 44]. Therefore, an urgent need now exists for the development of new antimicrobials.

New antitubercular/antimycobacterial drugs are now being subjected to further *in vitro* and *in vivo* studies [22, 29, 30]. Among them, the following drugs, reported in Chart **1**, are extremely promising:

1. Oxazolidinones have significant activity against MTB and tubercle bacilli in mice [33]. One derivative, PNU-100480 had activity toward MTB in a murine model [40], and, recently, some PNU-100480 analogues were found to have significant *in vitro* activity against *M. avium* [45].
2. Erythromycin-derived new ketolides (ABT-773 and telithromycin) exibit a potent therapeutic efficacy against MAC infection induced in mice [41].
3. PA-824 is a new nitroimidazole derivative highly active against MTB and non-replicating tubercle bacilli. It is also active toward MDR-TB strains [44].

The strategies [46] followed to generate new TB therapies may involve:

1. Developing new drugs from existing lead molecules used to treat other bacterial infections (e.g. FQs).
2. Modifying an existing drug to improve its antimycobacterial activity and its pharmacokinetic properties to make it less susceptible to the known mechanism of resistance. This is the strategy adopted in developing new rifampin analogues.
3. Discovering new drugs either by random screening or, if a specific target is kown, by a rational design approach.

Two key approaches utilizing interchangeable technologies can be applied to the discovery of novel antibiotics (Strategy 3). The first one is the target-based screening, where comparative genomics and bioinformatic analysis are used to select targets with the appropriate activity spectrum and selectivity. Complete genome sequences of several pathogenic bacteria, including MTB and *M. leprae*, have been determined [47] and many more of such projects are currently under way (e.g. *M. avium*). Even though microbial genomics has little direct impact on antibacterial drug discovery, the possibilities of using genome sequences for target identification are virtually unlimited. Indeed, it is possible to, firstly, compile a list of all potential gene products, secondly, identify the functions that are missing in a particular microorganism, and finally, identify genes common to all (or most) microorganisms in a chosen group or, *vice versa*, unique to a particular pathogen. The second approach is to synthesize and screen compounds that possess antibacterial activity and then to employ genomic tools to identify the molecular target. The recent development of novel biochemical and genomic technologies that facilitate identification and characterization of the mechanism of action of these agents have made this approach as attractive as the genomic target-based screening strategies.

As described above (Strategy 1), one of the strategies followed for searching new drugs, is testing for antitubercular activity compounds already active as antibacterials.

In the past, many pyrroles were synthesized by us as pyrrolnitrin analogues (Chart **1**).

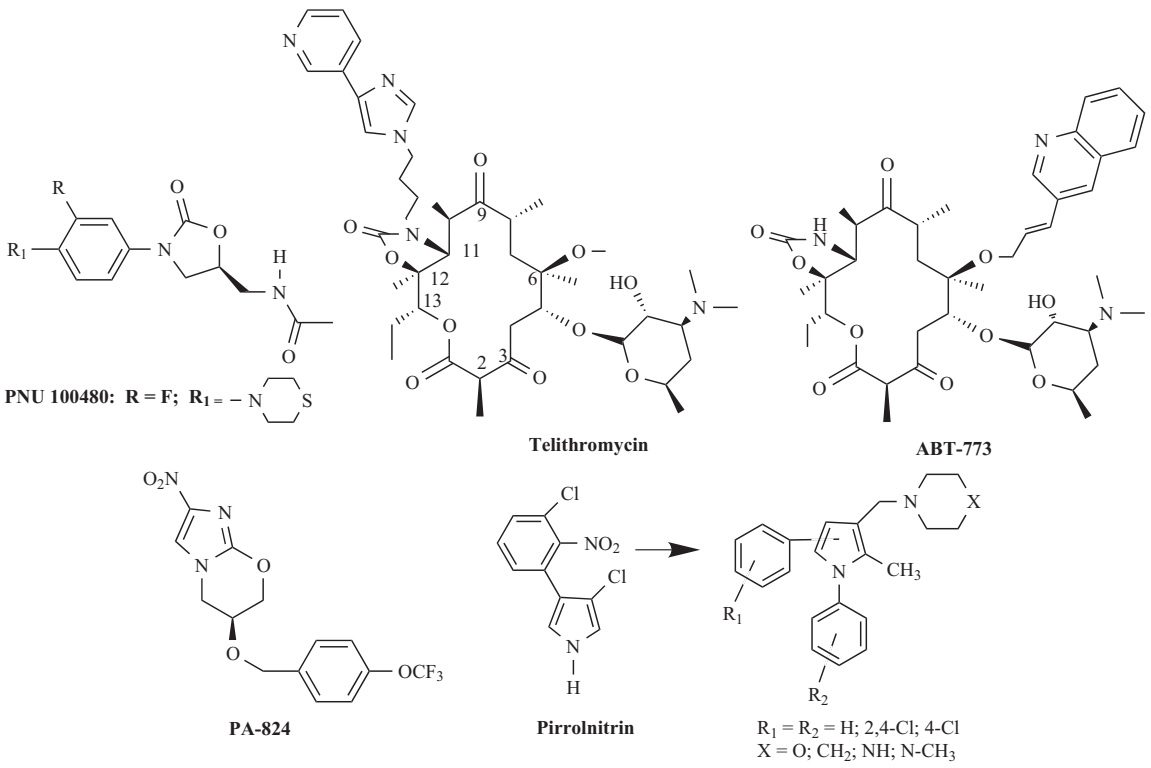
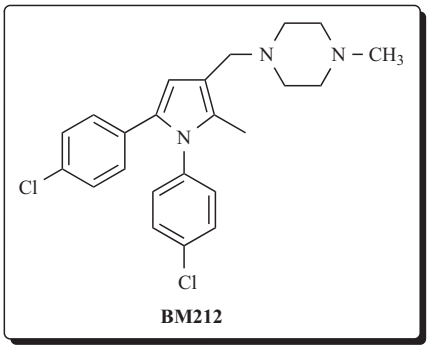


Chart 1.

These compounds, showing both antibacterial and antifungal activities, have been tested against wild-type MTB and drug-resistant clinical isolates, as well as toward *M. avium* and other atypical mycobacteria [48-49].

Among all the synthesized compounds, only some 1,5-diarylpyrrole derivatives showed good antimycobacterial activity. The most active compound was **BM212**, with potent activity against both collection and clinical strains of MTB, non-tuberculosis mycobacteria (such as *M. avium*) and drug-resistant clinical isolates of MTB.



The compound **BM212** exerted its antimycobacterial and bactericidal activities also on intracellular bacilli residing in the U937 human histiocytic lymphoma cell line [37]. It is important to note that, while the inhibitory activity toward extracellular MTB accounts for the ability of test compounds to treat active tuberculosis, assays on intracellular MTB assess the ability of test compounds to inhibit mycobacteria during the latent phase of tuberculosis, before latent tuberculosis infection itself progresses to active disease. Moreover, combating latent tuberculosis infection is one of the major challenges mainly for reducing the high rate of progression to active disease in immunocompromised individuals (in fact, progression is higher in persons with concomitant HIV-1 infection).

On the basis of above results **BM212** was considered the lead compound for this new class of derivatives [48] and one of the most promising future antimycobacterial drug [50]. The only limitation factor was the toxicity. Indeed, it showed a $MNTD_{50}$ = 4 μg/mL, a MIC value of 0.7 μg/mL and a protection index (PI, expressed as $MNTD_{50}$/MIC ratio) of 5.7.

In the attempt to obtain both more active and low toxic compounds, we developed a program to systematically modify **BM212**. Specific goals were:

1. Design and synthesis of new antitubercular compounds starting from the lead molecule **BM212**.
2. Evaluation of new derivatives of **BM212** for their antimicrobial activity against MTB and non-tubercular species of mycobacteria; evaluation of the: (i) activity in macrophages infected with MTB, (ii) combinatorial drug activity, iv) cellular toxicity.
3. Identification and characterization of the cellular target of **BM212**.

The strategy for synthesizing new compounds was based on structure-activity relationship (SAR) analysis done previ-

ously, molecular modeling and literature reports [37,49,51, 52]. Indeed, in the absence of the knowledge of the receptor target for pyrrole derivatives, we performed molecular modeling studies starting from all the compounds synthesized by us at that moment. The structural analogy among the studied compounds, combined with their small activity range of only two orders of magnitude, led to the conclusion that the common feature hypothesis generation [51], using the software Catalyst [53], was the most suitable computational method to be applied to this class of compounds. In fact, the activity range was too narrow to generate a statistically significant quantitative model able to correlate the structural features of these compounds with their biological data.

The common feature hypothesis generation is a computational approach based on the ligand-based drug design (pharmacophore development) method [51]. Such an approach is also based on the assumption that only common chemical groups responsible for high antitubercular activity should be identified by the program and used to build a pharmacophoric model. The goal of this approach was the identification of the spatial location (three-dimensional disposition) of a collection of chemical features (hydrogen bond acceptors, hydrogen bond donors, hydrophobics, aromatic rings, etc, all of them constituting the pharmacophoric model) representing the portions of the ligands interacting with the receptor site.

From these studies, we first identified a preliminary pharmacophoric model, characterized by five-features (two aromatic groups RA, two hydrophobics HY, and a hydrogen bond acceptor HBA) [51], that was then optimized in a new model with four features (two RA, one HY, and one HBA) [54].

DESIGN AND SYNTHESIS OF BM212 DERIVATIVES: COMPOUNDS 1

Previous SAR and molecular modeling studies allowed to identify chemical groups to be added at positions N1, C5 and C3 of the pyrrole ring with the aim of improving antimycobacterial activity. Following what reported by Barbachyn et. al about the thiomorpholine introduction in oxazolidinones already active as antitubercular agents [55] and what reported in the literature about the importance for the activity exherted by F and Cl substituents, the first modifications of **BM212** led to compounds **1a-j** (Table **1**) [52,56] in which we alternatively introduced a *N*-methylpiperazinomethyl or thiomorpholinomethyl moiety at C3 of the pyrrole and a chlorine or a fluorine atom at the para position of one or both the phenyl rings at N1 and/or C5 positions.

It is important to point out that among compounds **1a-j**, derivatives **1b** and **1h** showed a very good Protection Index (PI) ($MNTD_{50}$/MIC ratio), and were more active than the lead compound **BM212**.

In particular **1b** revealed the most active compound and its activity was comparable to that of reference compounds even though its PI was lower than it. These findings confirm our hypothesis and literature reports [52,56], suggesting that the introduction of a fluorine atom in a structure led to the improvement of activity and lowering of toxicity. On the other hand, the presence of two chlorine atoms in the structure seemed to be an important parameter for the activity against atypical mycobacteria. Fig. (**1**) shows the superposition pathway of compound **1b** to the pharmacophoric model.

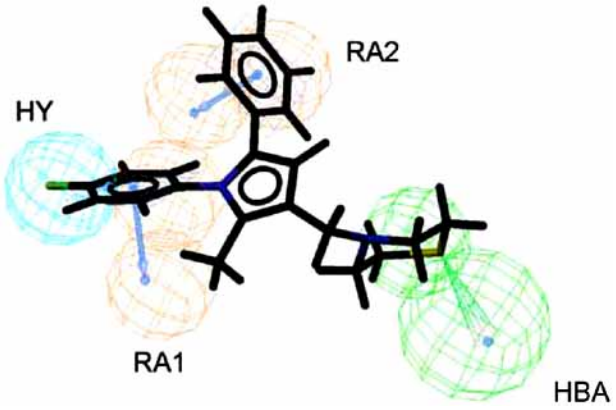
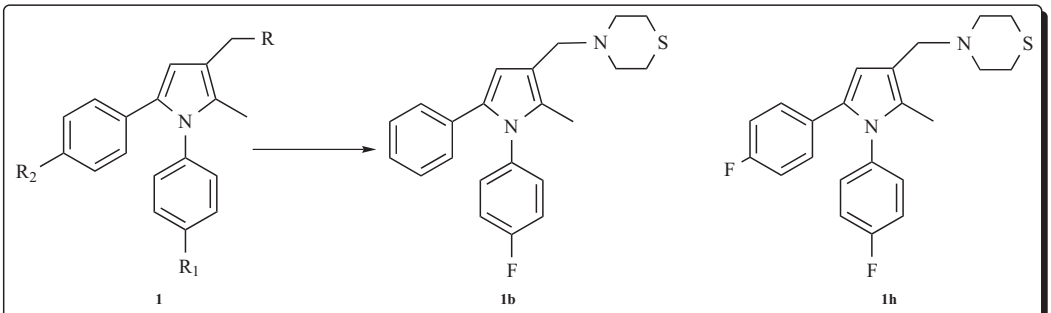


Fig. (1). Compound **1b** superposed to the improved pharmacophoric model.

DESIGN AND SYNTHESIS OF BM212 DERIVATIVES: COMPOUNDS 2

These results, combined with the aim of improving the inhibitory activity and further reducing the cytotoxicity of this class of compounds, led to plan and synthesize the new

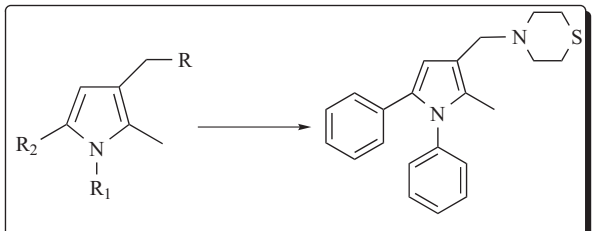


1 1b 1h

R = *N*-methylpiperazinyl, thiomorpholinyl.
R_1 = phenyl; R_2 = F; Cl.
R_2 = phenyl; R_1 = F; Cl.
R_1 = R_2 = F; Cl.

compounds **2a-n** [57]. They were obtained by alternatively introducing a thiomorpholinomethyl or a *N*-methylpiperazinomethyl moiety (as in **1b** and **BM212**, respectively) at C3 of the pyrrole ring and by replacing one of the phenyl rings at N1 or C5 with both more lipophilic aromatic groups (i.e., α-naphthyl) or phenyl rings substituted with the same previous substituents but in different positions (i.e., *o*-Cl-phenyl, *o*-F-phenyl). This choice was supported by the fact that an α-naphthyl or an *o*-Cl-phenyl or an *o*-F-phenyl group could improve the superposition to the aromatic regions of the model and, consequently, led to a better bind to the hypothetical receptor.

The remaining phenyl ring was left unsubstituted. Moreover, we also synthesized derivative **2m**, in which both the phenyl rings at N1 and C5 were unsubstituted.



2 **2m**

R = thiomorpholinyl, *N*-methylpiperazinyl
R_1 = α-naphthyl, R_2 = phenyl, 2-Cl-phenyl, 2-F-phenyl
R_1 = phenyl, 2-Cl-phenyl, 2-F-phenyl, R_2 = α-naphthyl
$R_1 = R_2$ = phenyl, 2-Cl-phenyl, 2-F-phenyl

Interesting results were obtained from the data regarding antimycobacterial activity. Indeed, in general, by a comparison between *N*-methylpiperazinomethyl and thiomorpholinomethyl derivatives, we could confirm the importance of the thiomorpholine introduction in the pyrrole structure regarding the *in vitro* antimycobacterial activity. About substituents at N1 and C5, it is important to point out that, contrary to our expectations, *o*-Fluoro or *o*-Chloro derivatives were less active and more toxic than the corresponding *p*-Fluoro or *p*-Chloro derivatives [56]. These results underlined that a *p*-Fluoro-phenyl moiety at N1 and/or C5 of the pyrrole ring is fundamental for the activity. In fact, *o*-Fluoro compounds are 20 to 8-fold less active than the corresponding *p*-Fluoro derivatives **1a-j**, suggesting that the *ortho* substitution does not increase the superposition with the aromatic portions of the pharmacophore. Activity underwent a further decrease in compounds bearing the α-naphthyl substituent, that revealed completely inactive. On the contrary, a very interesting biological profile was found for compound **2m** in which both the phenyl rings at N1 and C5 are unsubstituted. In addition to be less toxic than **1b** and **BM212**, it shows a very good PI (Table **1**). In particular, even if it is not the most active compound (MIC = 1 μg/mL), its activity is comparable to that of reference compounds and PI is the best among all the compounds 1 and 2. These findings were in contrast with the hypothesis that the introduction of a fluorine atom in a structure could lead to a better activity and a lower toxicity.

Being **2m** analog of **1b** and **BM212**, it was also tested toward intracellular and resistant mycobacteria. Microbiological data showed that all the tested strains were inhibited by **2m** and that it exerted bactericidal activity also on intracellular mycobacteria. The MIC is the same of **BM212** and lower than Rifampin and **1b**. [57] This result is very important because mycobacteria can reside for years inside lymphoid cells and macrophages and traditional drugs are not able to get throw it. Another important aspect was the high selectivity of **2m** against mycobacteria. In fact, this compound is very active only toward MTB, while it is completely inactive against atypical mycobacteria, with the exception of *M. avium* (see Table **1**).

DESIGN AND SYNTHESIS OF BM212 DERIVATIVES: COMPOUNDS 3

To better refine the structure-activity relationships of antitubercular pyrroles, compounds **3a-h** were also synthesized, bearing ortho halogenated phenyl rings at both the N1 and C5 positions of pyrrole [58]. Moreover, compounds **3i-p,** in which a naphthyl substituent replaced one of the *o*-halophenyl groups at N1 or C5 of the pyrrole ring, Table **1**), were also synthesized. As previously reported, F and Cl were chosen as substituents of the phenyl rings.

From the microbiological data, it was possible to draw the following considerations: (i) in principle, *N*-methylpiperazinomethyl derivatives were more toxic and less active than the corresponding thiomorpholinomethyl compounds; (ii) regarding the thiomorpholinomethyl derivatives, by introducing a second *o*-halophenyl substituent in the molecule, the antimycobacterial activity increased. Moreover, the nature of the halogen played an important role in influencing activity. In fact, the best improvement in activity was found when a fluorine atom was introduced at the ortho position of the C5-phenyl ring of **2a** or the N1-phenyl ring of **2c** to give compound **3a** with an activity 8- and 4-fold better (MIC = 1 μg/mL) than the corresponding parent compounds (8 and 4 μg/mL, respectively). Moreover, a 4-fold enhancement of activity was found by introducing an *o*-Fluoro into the unsubstituted phenyl ring of **2e** (16 μg/mL) and **2g** (16 μg/mL) to give compounds **3c** (4 μg/mL) and **3e** (4 μg/mL), respectively. While the introduction of an additional *o*-Chloro substituent produced compounds with comparable or improved activity with respect to the mono halo derivatives (compare **3c** *vs* **2c**, **3e** *vs* **2a** and **3g** *vs* **2e**), they were also characterized by higher toxicity with respect to the corresponding fluoro derivatives; (iii) naphthyl derivatives **3k**, **3l** and **3p**, bearing an *o*-Fluoro- or an *o*- Chloro-phenyl ring at C5, showed better activity with respect to the parent compounds with the unsubstituted 5-phenyl ring (**2i** and **2j**). All the remaining members of this subclass, **3i**, **3j**, and **3m, 3n,** were inactive.

Compound **3a**, structurally related to the previously described pyrrole derivatives **1, 2** and **BM212**, was found to be the most active derivative. It was also tested against resistant mycobacteria, showing that all of the tested strains were inhibited by **3a**. Moreover, it exerted bactericidal activity also on intracellular mycobacteria. MIC value was the same found for **BM212** and lower than that of rifampin and **1b**. The high selectivity of **3a** toward mycobacteria, as previously observed for **2m,** was worthy of further consideration. In fact, this compound was very active only against MTB, while it was completely inactive toward atypical mycobacteria. Pharmacophore mapping of compound **3a** in comparison to **1b** is reported in Fig. (**2**).

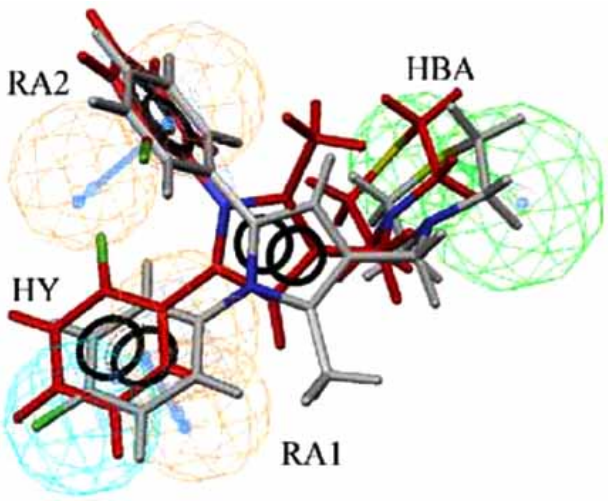


Fig. (2). Superposition of compound **3a** and **1b** into the pharmacophoric model for antitubercular compounds. Pharmacophoric features: HY (hydrophobic); RA (aromatic regions); HBA (hydrogen bond acceptor groups).

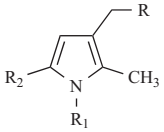
From all the microbiological data derived from these studies, the following considerations could be drawn:

1. compounds with the thiomorpholinomethyl moiety at position C3 of the pyrrole are in general more active and less toxic than the corresponding N-methylpiperazinomethyl derivatives;
2. the presence of the fluorine atom plays an important role in influencing activity;
3. the simultaneous presence of an ortho substituent (in particular, a fluorine), or a para substituent (in particular, a fluorine) at positions N1 and C5, or the unsubstitution at both the phenyl rings, leads to very active compounds;
4. the introduction of a naphthyl substitutent at one of the phenyl rings leads to compounds poor active or completely inactive.

DESIGN AND SYNTHESIS OF BM212 DERIVATIVES. COMPOUNDS 4

Considering all these results, we planned the synthesis of the new derivatives **4a-n** and **5a-n**, designed on the basis of both structure-activity relationships collected from analogue compounds previously reported and suggestions derived from the analysis of a pharmacophoric model for antitubercular compounds [59]. The following structural and lipophilic contributions to the antimicrobial activity have been evaluated: (i) the *para*-Fluoro substitution conjugated with the *ortho*-halo one, toghether with the mutual position of the

Table 1. Structure, Antimycobacterial Activity Against *M. tuberculosis* 103471, *M. avium* 103317, Cytotoxicity, Calculated logP and Protection Index (PI) of the Pyrrole Derivatives 1a-j, 2a-n, 3a-p, BM212, Isoniazid, Streptomycin, and Rifampin



Compd	R[a]	R_1	R_2	*M. tuberculosis* MIC (μg/mL)	*M. avium* MIC (μg/mL)	logP	MTD_{50}[b] (μg/mL) VERO cells	Protection Index (PI)
1a	A	4-F-Ph	Ph	16	>16	4.90	16	1
1b	B	4-F-Ph	Ph	0.4	2	5.37	8	20
1c	A	Ph	4-F-Ph	16	>16	4.90	4	0.25
1d	B	Ph	4-F-Ph	0.5	>16	5.37	4	8
1e	A	4-Cl-Ph	4-F-Ph	16	>16	5.56	8	0.5
1f	B	4-Cl-Ph	4-F-Ph	2	>16	6.04	8	4
1g	A	4-F-Ph	4-F-Ph	16	>16	5.10	16	1
1h	B	4-F-Ph	4-F-Ph	1	>16	5.58	8	8
1i	A	4-F-Ph	4-Cl-Ph	2	>16	5.56	16	8
1j	B	4-F-Ph	4-Cl-Ph	1	>16	6.04	8	8
2a	B	2-F-Ph	Ph	8	>16	5.37	8	1
2b	A	2-F-Ph	Ph	16	>16	4.90	2	0.125
2c	B	Ph	2-F-Ph	4	>16	5.37	4	1
2d	A	Ph	2-F-Ph	16	>16	4.90	2	0.125

(Table 1. Contd….)

Compd	R[a]	R_1	R_2	*M. tuberculosis* MIC (μg/mL)	*M. avium* MIC (μg/mL)	logP	MTD_{50}[b] (μg/mL) VERO cells	Protection Index (PI)
2e	B	2-Cl-Ph	Ph	16	>16	5.83	2	0.125
2f	A	2-Cl-Ph	Ph	>16	>16	5.36	4	-
2g	B	Ph	2-Cl-Ph	16	>16	5.83	2	0.125
2h	A	Ph	2-Cl-Ph	>16	>16	5.36	4	-
2i	B	1-naphthyl	Ph	>16	>16	6.08	4	-
2j	A	1-naphthyl	Ph	>16	>16	5.60	2	-
2k	B	Ph	1-naphthyl	>16	>16	6.08	4	-
2l	A	Ph	1-naphthyl	>16	>16	5.60	4	-
2m	B	Ph	Ph	1	16	5.17	32	32
2n	A	Ph	Ph	16	>16	4.69	4	0.25
3a	B	2-F-Ph	2-F-Ph	1	>16	5.58	32	32
3b	A	2-F-Ph	2-F-Ph	8	>16	5.10	8	1
3c	B	2-Cl-Ph	2-F-Ph	4	16	6.04	8	2
3d	A	2-Cl-Ph	2-F-Ph	>16	>16	5.56	16	-
3e	B	2-F-Ph	2-Cl-Ph	4	>16	6.04	8	2
3f	A	2-F-Ph	2-Cl-Ph	16	16	5.56	4	0.25
3g	B	2-Cl-Ph	2-Cl-Ph	4	8	6.50	8	2
3h	A	2-Cl-Ph	2-Cl-Ph	16	16	6.02	4	0.25
3i	B	2-F-Ph	1-naphthyl	>16	>16	6.28	16	-
3j	A	2-F-Ph	1-naphthyl	>16	16	5.81	2	-
3k	B	1-naphthyl	2-F-Ph	4	2	6.28	4	1
3l	A	1-naphthyl	2-F-Ph	4	4	5.81	4	1
3m	B	2-Cl-Ph	1-naphthyl	>16	>16	6.74	128	-
3n	A	2-Cl-Ph	1-naphthyl	>16	>16	6.26	16	-
3o	B	1-naphthyl	2-Cl-Ph	>16	>16	6.74	64	-
3p	A	1-naphthyl	2-Cl-Ph	4	4	6.26	2	0.5
BM212	A	4-Cl-Ph	4-Cl-Ph	0.7	0.4	6.02	4	5.6
Isoniazid				0.25	32	32	32	128
Streptomycin				0.50	8	>64	>64	128
Rifampin				0.30	0.3	64	64	213

[a]A = *N*-methylpiperazinyl, B = thiomorpholinyl.

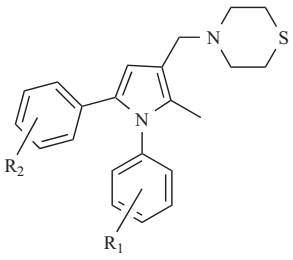
[b]MTD_{50} = median toxic dose.

substituents; (ii) the *para*-Fluoro substitution conjugated with the methyl introduction in the remaining phenyl ring, considering the mycobacterial cell wall structure, very waxy, hydrophobic and characterized by a high lipid content.

In detail, **(**a) the thiomorpholinomethyl and the *N*-methylpiperazinomethyl moieties are alternatively placed at pyrrole C3; **(**b) at least one of the phenyl rings is *p*-Fluoro substituted; **(**c) the remaining phenyl ring is *ortho*-halo or *ortho,para*-dihalo, or *ortho*- or *meta*- or *para*-methyl (more lipophilic) substituted.

Several of the new derivatives were found to have an *in vitro* activity toward MTB better than that of **BM212**, and

comparable to that of **1b**, **2m**, **3a**, isoniazid, streptomycin and rifampicin, used as the reference compounds. In addition, they were also characterized by low cytotoxicity, with protection index up to 160, better than that found for both isoniazid and streptomycin, and comparable to that of rifampicin. Although the new compounds cannot be considered as potential clinical candidates, nevertheless they are of great interest as hit structures in the search of new antitubercular agents.



4, **5**

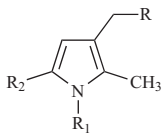
R_1 = 4-F; R_2 = 2-Cl; 2-F; 2,4-Cl_2; 2,4-F_2; 2-CH_3; 3-CH_3; 4-CH_3 (**4a-n**)
R_2 = 4-F; R_1 = 2-Cl; 2-F; 2,4-Cl_2; 2,4-F_2; 2-CH_3; 3-CH_3; 4-CH_3 (**5a-n**)

From microbiological data, reported in Table **2**, it is possible to underline that all the synthesized structures were found to be more active against MTB than *M. avium,* depending on the nature and the corresponding position occupied by the substituents.

It is also important to note that compounds **4a, 4m** and **5m**, in addition to show a high *in vitro* activity toward MTB (MIC of 0.4, 0.5, and 0.5 μg/mL, respectively, comparable to that found for isoniazid, 0.25 μg/mL, streptomycin, 0.5 μg/mL, and rifampicin, 0.3 μg/mL), were also characterized by a very low toxicity. In particular, **4a** showed a high protection index (160), better than that of BM212 (5.6), isoniazid (128), streptomycin (128), and slightly lower than that of rifampicin (213).

Regarding activity toward atypical mycobacteria, the new compounds were in principle found to be more active against MTB than *M. avium* (Table **2**). The only exception to this trend was represented by **4h**, found 4-fold more active toward *M. avium* (MIC of 2 *vs* 8 μg/mL), as well as **4b** and **5h** that showed identical *in vitro* activity toward both mycobacteria (4 μg/mL). Moreover, the corresponding thiomorpholinomethyl compounds **4g** and **5g** also showed an interesting inhibitory activity toward *M. avium* (4 and 2 μg/mL, respectively). These results were in good agreement with the high activity showed by **BM212** (in which chloro and/or N-methylpiperazinomethyl substituents were present at N1, C3

Table 2. Structure, *In Vitro* Antimycobacterial Activity Against *M. tuberculosis* 103471, *M. avium* 103317, Cytotoxicity, Calculated logP and Protection Index (PI) of the New Pyrrole Derivatives 4a-n and 5a-n and Compounds Used as References (BM212, Isoniazid, Streptomycin and Rifampin)



Compd	R[a]	R_1	R_2	*M. tuberculosis* MIC (μg/mL)[b]	*M. avium* MIC (μg/mL)[b]	log*P*	$MNTD_{50}$ (μg/mL)[c] VERO cells	(PI)[d]
4a	B	4-F-Ph	4-CH_3-Ph	0.4	8	5.86	64	160
4b	A	4-F-Ph	4-CH_3-Ph	4	4	5.38	8	2
4c	B	4-F-Ph	3-CH_3-Ph	2	8	5.86	4	2
4d	A	4-F-Ph	3-CH_3-Ph	16	16	5.38	8	0.5
4e	B	4-F-Ph	2-CH_3-Ph	4	8	5.86	8	2
4f	A	4-F-Ph	2-CH_3-Ph	>16	>16	5.38	2	
4g	B	4-F-Ph	2,4-Cl_2-Ph	2	4	6.70	64	32
4h	A	4-F-Ph	2,4-Cl_2-Ph	8	2	6.23	16	2
4i	B	4-F-Ph	2,4-F_2-Ph	0.5	16	5.78	16	32
4j	A	4-F-Ph	2,4-F_2-Ph	4	16	5.31	8	2
4k	B	4-F-Ph	2-Cl-Ph	4	16	6.04	4	1
4l	A	4-F-Ph	2-Cl-Ph	8	16	5.56	2	0.25
4m	B	4-F-Ph	2-F-Ph	0.5	16	5.58	16	32
4n	A	4-F-Ph	2-F-Ph	>16	>16	5.10	4	

(Table 2. Contd….)

Compd	R[a]	R_1	R_2	*M. tuberculosis* MIC (μg/mL)[b]	*M. avium* MIC (μg/mL)[b]	log*P*	$MNTD_{50}$ (μg/mL)[c] VERO cells	(PI)[d]
5a	B	4-CH_3-Ph	4-F-Ph	1	8	5.86	32	32
5b	A	4-CH_3-Ph	4-F-Ph	16	16	5.38	8	0.5
5c	B	3-CH_3-Ph	4-F-Ph	4	16	5.86	8	2
5d	A	3-CH_3-Ph	4-F-Ph	16	>16	5.38	16	1
5e	B	2-CH_3-Ph	4-F-Ph	8	16	5.86	16	2
5f	A	2-CH_3-Ph	4-F-Ph	>16	>16	5.38	2	-
5g	B	2,4-di-Cl-Ph	4-F-Ph	1	2	6.70	4	4
5h	A	2,4-di-Cl-Ph	4-F-Ph	4	4	6.23	2	0.5
5i	B	2,4-di-F-Ph	4-F-Ph	2	16	5.78	16	8
5j	A	2,4-di-F-Ph	4-F-Ph	16	16	5.31	16	1
5k	B	2-Cl-Ph	4-F-Ph	4	>16	5.56	8	2
5l	A	2-Cl-Ph	4-F-Ph	2	>16	6.04	8	4
5m	B	2-F-Ph	4-F-Ph	0.5	8	5.58	8	16
5n	A	2-F-Ph	4-F-Ph	8	16	5.10	8	1
BM212	A	4-Cl-Ph	4-Cl-Ph	0.7	0.4	6.02	4	5.6
Isoniazid				0.25	32		32	128
Streptomycin				0.50	8		>64	>128
Rifampin				0.30	0.3		64	213

[a]A = *N*-methylpiperazinyl B = Thiomorpholinyl

[b]MIC = Minimum inhibitory concentration toward MTB.

[c]$MNTD_{50}$ = Maximal non toxic dose toward Vero cells.

[d]PI = protection index, as the ratio between cytotoxicity and in vitro activity toward MTB.

and C5) against *M. avium*. All the remaining compounds revealed completely inactive against atypical mycobacteria, thus showing a good selectivity *versus* MTB. The sole compound **5n** was found to have a very relevant inhibitory activity toward *M. smegmatis* (0.3 μg/mL).

Compounds **4a** and **5m** were also evaluated for their activity toward intracellular MTB. Biological results showed that both compounds exert bactericidal activity on intracellular mycobacteria at 3 μg/mL concentration, comparable to that of rifampin, used as the reference compound. Finally, compounds **4a** and **5m** were also evaluated for their activity toward a panel of eleven resistant and MDR clinical isolate strains of MTB. Table **3** shows that all of the tested strains were inhibited by such compounds at concentrations ranging from 0.5 to 2 μg/mL. The sole exception was represented by the 326/04 strain, sensitive to such compounds at concentrations higher than 32 μg/mL. In detail, **4a** showed a very good activity (0.5 μg/mL) toward the whole panel of clinical isolates of MTB, with the exception of the 326/04 strain (32 μg/mL). Similarly, **5m** was characterized by an activity of 0.5 μg/mL toward 43/05 and 158/97, while the remaining clinical isolates were inhibited at a 2 μg/mL concentration. Compound **5m** was inactive toward the isolate 326/04, known to be sensitive to rifampicin, while resistant to isoniazid, streptomycin, and ethambutol. These experimental evidences make these compounds extremely interesting when compared to the compounds now used in therapy, which tend to be less active against drug-resistant mycobacteria.

Fig. (**3**) shows compound **4a** mapped to the pharmacophoric model.

From the whole study, that is still continuing in the attempt to find new compounds more active and less toxic and also directed to the identification of the possible mode of action of these compounds, eight hits derived from **BM212**, were found, as reported in Chart **2**.

COMPUTATIONAL INVESTIGATIONS

All the pyrrole derivatives have been computationally analyzed by means of the Catalyst software [51] for their fit properties to a four-feature pharmacophoric model (Fig. (**1**)) previously built from our group for antitubercular compounds.

All the structures were characterized by a 2-methylpyrrole nucleus bearing various substituents at the positions N1, C3, and C5. While a thiomorpholinomethyl or a *N*-

Table 3. Activity Toward MDR-TB Strains

Strain	SM 1 μg/mL	INH 0,1 μg/mL	RIF 1 μg/mL	EMB 5μg/mL	4a MIC (μg/mL)	5m MIC (μg/mL)
149/03	S	R	R	S	0,5	2
421/96	S	S	R	S	0,5	2
586/98	S	S	R	S	0,5	2
43/05	S	S	S	S	0,5	0,5
158/97	S	S	S	R	0,5	0,5
134/02	R	R	S	S	0,5	2
520/98	R	S	R	S	0,5	2
326/04	R	R	S	R	32	> 32
296/04	S	S	R	R	0,5	2
482/98	S	S	R	S	0,5	2
275/05	R	S	S	S	0,5	2
H37Rv	S	S	S	S	0,5	2

methylpiperazinomethyl groups were the substituents at position 3, positions 1 and 5 have been substituted with various ortho-, meta- or para- substituted halophenyl ring or 1-naphthyl ring.

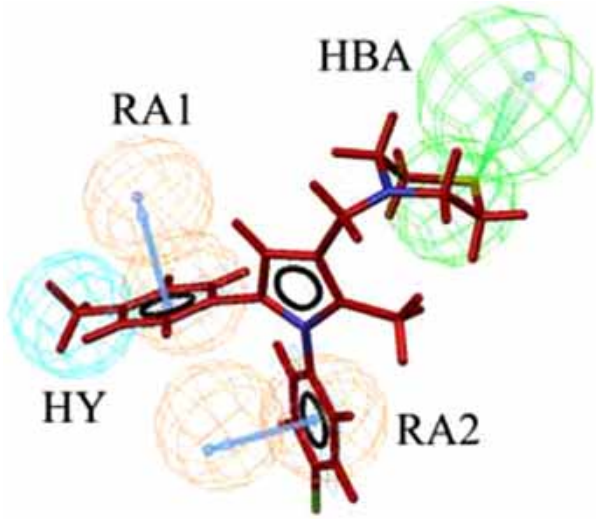


Fig. (3). Compound **4a** mapped to the pharmacophoric model.

In principle, activity data showed that a thiomorpholinomethyl moiety at the position 3 was a better substituent with respect to the *N*-methylpiperazinomethyl one. In fact, compound **3p** was the sole exception of a *N*-methylpiperazinomethyl derivative with an enhanced activity in comparison to the corresponding thiomorpholinomethyl counterpart **3o**. This trend has been accounted by the pharmacophoric model on the basis of the fact that the piperazino N4 nitrogen atom was unable to be the hydrogen bond acceptor, similarly to the sulphur atom of the thiomorpholinomethyl analogues. In the *N*-methylpiperazinomethyl series, a different orientation of the six-membered ring was found, leading the N1 to match the HBA feature of the model.

Introduction of a halogen substituent at the para position of the 1- or 5-phenyl ring of compound **2m** produced variations in activity. In particular, both the *N*-(*p*-Fluoro-phenyl) and the 5-(*p*-Fluoro-phenyl) derivatives **1b** and **1d** showed a slight enhancement in activity (MIC values of 0.4 μg/mL and 0.5 μg/mL, respectively) with respect to the parent compound. Superposition of these compounds with the model showed that the fluoro substituent matched HY, and that the optimal substitution was represented by the *N*-(*p*-Fluoro-phenyl) group. However, the 5-(*p*-Fluoro-phenyl) moiety was also able to satisfy HY by a reverse orientation of the pyrrole ring (180° rotation) leading the 5-substituent to fill RA1-HY features. This finding led to the suggestions that **(**1) compounds bearing phenyl rings with different halogenation could show activity comparable each other (compare **1k** and **1f** showing MIC = 1 μg/mL and 2 μg/mL, respectively). A comparable MIC value of 1 was found for the difluorinate derivative **1h**, characterized by a 180° rotation of the pyrrole ring, was also able to interact with the model through alternate orientations (2). The methyl group at the 2 position was accommodated within two regions of space, never occupied by pharmacophoric features.

When the structure of **1b** was modified by introduction of an *ortho* halo substituent, a drop in activity was found, probably due to the unability to fully match the HY feature of the pharmacophoric model. In fact, in the series of *ortho* halogenated derivatives, while the phenyl ring partially match HY and RA1, the halogen is far from any feature. *Ortho*-fluoro derivatives **2a** and **2c** retained an appreciable activity, while the corresponding *ortho*-Chloro compounds **2e** and **2g** were found inactive. These data suggested that the increase of the halogen size or, alternately, the decrease of its

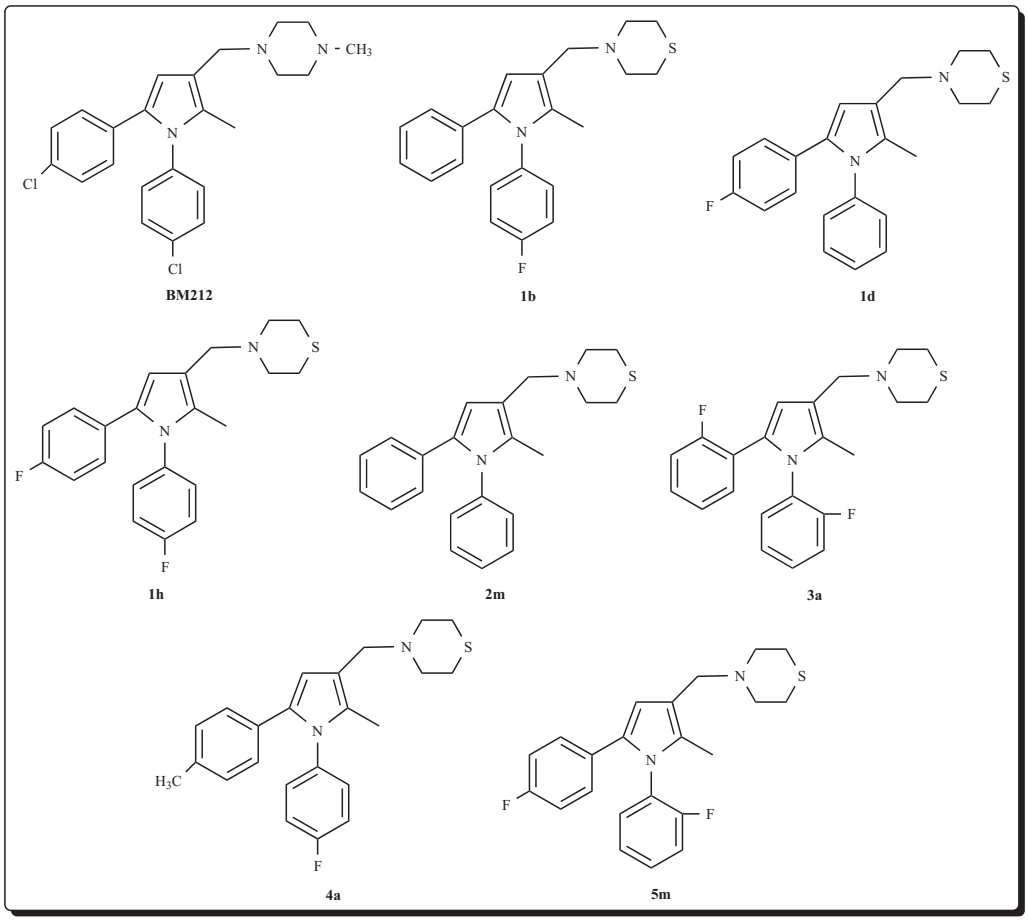


Chart 2.

electron withdrawing capabilities led to lower activity. Several orientations of *ortho* halogenated compounds into the pharmacophoric model were characterized by the fluorine substituent pointing toward the projection sphere of RA2, representing a portion of the receptor counterpart. As a consequence, the halogen atom and the aromatic portion of the receptor could be engaged in a lipophilic interaction accounting for the interesting activity found for *ortho* halogenated compounds. Such an interaction could also rationalize the decrease in activity when the size of the *ortho* halogen atom increased (on the basis of steric bumps, instead of profitable interactions, between the halogen and the receptor).

Regarding the *ortho* halogenated compounds, accordingly with what observed for unsubstituted or *para* halogenated derivatives, the *N*-methylpiperazinomethyl analogues were found to show a lower activity with respect to the corresponding thiomorpholinomethyl compounds.

Finally, when an α-naphthyl and a phenyl ring were alternately introduced as substituents at the positions 1 and 5 of the pyrrole nucleus, activity underwent a deep decrease, being compounds **2i-l** inactive. Similarly, introduction of an *ortho* chloro or fluoro substituent on the *N*-phenyl ring led to inactive compounds (**3i**, **3j**, **3m**, and **3n**). Low activity of all these naphthyl derivatives was not dependent on the substituent at the position 3. In fact, both thiomorpholinomethyl and *N*-methylpiperazinomethyl compounds were characterized by activity values >16. Differently, when the fluoro was introduced at the *ortho* position of the 5-phenyl ring, as in **3k,** an enhancement of activity was found when the thiomorpholinomethyl moiety was present at C3.

The introduction of a lipophilic group aimed at better matching the hydrophobic region of the pharmacophoric model led to compounds able to perfectly fulfill all the features of the model. As an example (Fig. (**3**)), the most active compound **4a** filled HY and RA1 with its methyl group and phenyl ring, respectively, constituting the side chain at C5. The second phenyl ring at N1 corresponded to the RA2 feature, while the hydrogen bond acceptor was represended by the sulphur atom of the thiomorpholine nucleus. As usual, the methyl group at C2 was accommodated within an empty region of space where no pharmacophoric feature are located. It could be considered as a no-pharmacophoric portion of the antitubercular compounds reported, but it possibly contributed to define the mutual orientation (conformations) of substituents at both positions 1 and 3. When the position of the methyl group was changed from *para* to meta and *ortho* (**4c** and **4e**, respectively), a reorientation was found within the pharmacophore. In particular, the *p*-fluoro and

phenyl group of the side chain at position 1 were accommodated into HY and RA1, respectively, while the substituent at position 3 remained in the region of HBA. As a consequence, the phenyl ring at position 5 matched RA2. However, the *m*-methyl and, particularly, the *o*-methyl substituents induced a conformational rearrangement of the phenyl ring at C5, leading to a reorientation of its plane. Consequently, both the *m*-methylphenyl and *o*-methylphenyl substituents were unable to fully satisfy the directionality property of the RA2 feature (the fit toward this feature decreased, possibly leading to less profitable interactions with the aromatic counterpart of the putative receptor), accounting for the decrease in the activity of such derivatives. In a similar way, the *o*-Chloro derivative **4k**, able to match the model in the same orientation of **4c** and **4e**, showed an activity lower than that of the corresponding fluoro analogue **4m**. This trend was accounted by the pharmacophoric model considering that the conformational rearrangement of **4c** and **4e** in comparison to **4a** was also found for **4k** with respect to **4m**. As a consequence, concerning the latter compound, being the fluoro smaller than the chloro substituent, the phenyl ring at position 5 was able to fulfill RA2. Moreover, the remaining pharmacophoric features were well satisfied by the N1 *p*-Fluoro phenyl group (HY and RA1), and by the side chain at position 3, similarly to that found for **4a**, accounting for comparable MIC values of **4m** and **4a** (0.5 and 0.4 μg/mL, respectively). Not surprisingly, **4i** showed an activity (0.5 μg/mL) higher than that of the corresponding dichloro derivative **4g** (2 μg/mL) and comparable to that of the mono fluoro derivative **4m** (0.5 μg/mL). Finally, the dichloro compound **4g** showed an activity value slightly lower than that of the corresponding mono chloro analogue **4k** (4 μg/mL).

As expected on the basis of previous biological data, derivatives bearing a thiomorpholinomethyl moiety at the position 3 of the pyrrole core showed an antimycobacterial activity toward MTB better than that found for the *N*-methylpiperazinomethyl analogues. In particular, the *o*-chloro derivatives **1** and **15** showed better activity (a 2-fold difference) than **4l** and **5l**, respectively. A 16- to 32-fold difference was also found for the corresponding *o*-fluoro derivatives (compare **5m** versus **5n**, and **4m** versus **4n**, respectively). The preference of a thiomorpholino group for activity was rationalized considering that the nitrogen atoms of the piperazine ring are unable to be the hydrogen bond feature, while the sulphur atom of the thiomorpholino ring was accommodated into the HBA sphere. However, it is also important to note that the *N*-methylpiperazinomethyl derivatives could adopt an alternative orientation leading N1 of the piperazine ring to be the hydrogen bond acceptor group. However, such conformations were disfavoured because they suffered from a marked distortion of the piperazino ring.

Among compounds **5a-n**, obtained by reversing the substitution pattern at positions 1 and 5 of **4a-n**, a thiomorpholinomethyl moiety at position 3 was better for activity in comparison to the corresponding *N*-methylpiperazinomethyl side chain, similarly to that found for compounds **4a-n**. Moreover, activity of **5m** (0.5 μg/mL), identical to that found for **4m**, was rationalized through the analysis of its superposition mode onto the pharmacophoric model. In fact, **5m** showed an orientation reversed in comparison to that of **4m**, with the substituent at position 5 (a *p*-Fluoro phenyl moiety) matching the HY-RA1 pharmacophoric portion, while the N1 phenyl ring was embedded into the RA2 feature. Although the 180^0 rotation around the pyrrole ring, the substituent at position 3 was located in such a way that the thiomorpholino sulphur atom corresponded to the hydrogen bond acceptor group. A similar orientation was found for **5k**, characterized by an activity 4-fold lower than that of **5m** (2 *vs* 0.5 μg/mL), in agreement with what previously reported for **4k** and **4m**, respectively. Compound **5a**, able to fulfill the whole pharmacophoric model as **4a** did, showed an activity of 1 μg/mL, comparable to that of **4a** (0.4 μg/mL). Moreover, compound **5g**, bearing a 2,4-dichloro phenyl group at N1, was characterized by an activity very similar to that of the corresponding mono chloro analogue **5k** (1 *vs* 2 μg/mL), following the same trend found for **4g** and **4k**.

In conclusion, compounds that better fitted the pharmacophoric model were characterized by:

(i) a thiomorpholinomethyl moiety at the position 3; the sulphur atom of the thiomorpholinomethyl portion represented the hydrogen bond acceptor group, HBA. *N*-methylpiperazine derivatives were also able to fill HBA, but with their N1, in a different conformation with respect to thiomorpholinomethyl compounds, accounting for variation in activity between the two classes of compounds;

(ii) an *o*-Fluoro phenyl ring, or a *p*-Fluoro phenyl ring at position 1 and/or 5 (as compounds **1b, 1d, 1h**, **3a** and **4a**). Such compounds were able to fit all the pharmacophoric features. In detail, the Fluoro-phenyl moiety matched the HY-RA1 system, while the unsubstituted phenyl ring was located within the RA2 feature. Moreover, compounds were allowed to undergo a 180° rotation to satisfy the features, when the substituents at N1 and C5 were mutually changed;

(iii) introduction of a halogen substituent at the ortho position instead of the para position, in only one of the phenyl rings, was detrimental for activity. In addition, a chlorine atom produced a decrease in activity higher than a fluoro substitution; (iv) a naphthyl group, that in principle could fill by itself both HY and RA1, was found to be unable to satisfy at the same time such two features, with a consequent decrease in activity with respect to the corresponding *p*-Fluorophenyl derivatives;

(v) the 2-methyl group was not involved in any interaction with pharmacophoric elements, but showed a role in influencing the conformational properties of substituents added at the positions 1 and 3 of the pyrrole ring.

Additional calculations were performed in an attempt to rationalize the lower MIC values usually found for thiomorpholinomethyl derivatives in comparison to the corresponding *N*-methylpiperazinomethyl compounds. For this purpose, logP values of the new pyrrole derivatives were calculated with Cerius2 software [60] (Table **1**) with the aim of evaluating if any correlation between antitubercular activity and lipophilicity of such compounds, occurred. Results showed that logP values of the thiomorpholinomethyl derivatives were in all cases higher than those found for the corresponding *N*-methylpiperazinomethyl counterparts, supporting the hypothesis that a more hydrophobic character is preferred for

the antitubercular potency of a compound (at least *in vitro*) [58].

Compounds with the best activity values, ranging from 0.4 through 1, were characterized by a calculated logP spanning between 5.37 and 6.04, including **BM212** (MIC = 0.7 μg/mL). However, higher logP values associated with the naphthyl derivatives were in disagreement with the previous hypothesis. A recent paper [61] also describing antimicobacterial pyrroles gave a justification for such finding, in terms of overall molecular volume, hypothesizing a limitation in the size of substituents. In fact, the authors reported that "a higher value of logP along with a limited enhancement of the molecular volume could lead to more potent derivatives". Regarding compounds with logP lower that 5.37, they were, in general, associated with activity values of 16 or higher.

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ABBREVIATIONS

AZM	=	Azithromycin
CPF	=	Ciprofloxacin
CAM	=	Clarithromycin
EMB	=	Ethambutol
FQs	=	Fluoroquinolones
INH	=	Isoniazid
LVFX	=	Levofloxacin
MAC	=	*M. avium* complex
MTB	=	*M. tuberculosis*
MDR-TB	=	Multidrug resistant tuberculosis
PI	=	Protection Index
PZA	=	Pyrazinamide
RBT	=	Rifabutin
KRM	=	Rifalazil
RIF	=	Rifampicin
RPT	=	Rifapentin
SPFX	=	Sparfloxacin
SM	=	Streptomycin
SAR	=	Structure-activity Relationships
TB	=	Tuberculosis

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