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Antimycobacterial compounds. Optimization of the BM 212 structure, the lead compound for a new pyrrole derivative class

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Abstract—Our work on antitubercular agents led to the identification of **BM 212** as a lead compound among a series of pyrrole derivatives with good in vitro activity against *mycobacteria* and *candidae*. Further studies led us to synthesize additional pyrroles bearing the thiomorpholinomethyl moiety and different aryl substituents at N1 and C5. Some of them revealed very active, prompting us to design the new pyrrole derivatives **5**–**20** in the hope of increasing the activity and better understanding the influence of *ortho* halogens on the antimycobacterial activity. Microbiological data showed interesting in vitro activity toward *Mycobacterium tuberculosis* and atypical mycobacteria.

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1. Introduction

Tuberculosis (TB) is still the greatest single infectious cause of mortality worldwide. In particular, *M. avium* complex (MAC) infections are frequently encountered among patients with AIDS in the US, European countries and other nations.[1,2]

The drug-resistant TB has become a serious concern as increasing numbers of TB cases are reported to be caused by strains of *Mycobacterium tuberculosis* resistant to one or more antitubercular drugs.[3–5] In this context, a vigorous search for new drugs for the treatment of the disease revealed necessary.

In our previous work, we have reported the synthesis and both antimycobacterial and antifungal activities of some pyrrole derivatives,[6–9] finding that most of the synthesized compounds showed interesting antimycobacterial properties. Among them, **BM 212** revealed the most active, and it appeared to be endowed with particularly potent and selective both antimycobacterial and antifungal activities.[7]

The successive modifications of **BM 212**, done on the basis of what previously observed by Barbachyn[10] for thiomorpholine and by us about the introduction of halogen atoms, led to the identification of the substituents at C5, N1[8] and C3[9] as very important for activity. Preliminar microbiological results showed the importance of the presence of the thiomorpholinomethyl moiety at the pyrrole C3 and a *p*-chlophenyl substituent at N1 and C5.

More recently, among pyrrole derivatives synthesized by our research group, compound **22** was found more potent, less toxic and more selective with respect to the lead compound **BM 212**.[11]

Moreover, we previously built and optimized a four-feature pharmacophore model for antimycobacterial compounds, consisting in a hydrophobic region, a hydrogen bond acceptor group and two aromatic rings.[12]

Keywords: Pyrrole derivatives; Thiomorpholinomethyl substituent; Antimycobacterial activity; Pharmacophore model.

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In the attempt to increase the fitting to the pharmacophoric model and, consequently, to optimize the bind to the hypothetical receptor, we pursuing our studies synthesizing the pyrrole derivatives **31-44**[13] maintaining the alternative presence of a thiomorpholinomethyl (as in **22**) or a *N*-methylpiperazinomethyl moiety (as in **BM 212**) at the C3 position of the pyrrole ring and replacing one of the phenyl rings at N1 or C5 of **22** with more lipophilic aromatic groups (as an α-naphtyl or *o*-Cl- or *o*-F-phenyl groups) to obtain a better superimposition with the aromatic features of pharmacophoric model. The remaining phenyl ring was left unsubstituted. Moreover, we also synthesized derivative **43**, with unsubstituted phenyl rings at both N1 and C5. Unexpectedly on the basis of the pharmacophoric suggestions, **43** showed an interesting activity against *M. tuberculosis* (MIC = 1 μg/mL).[13]

In this context, to better understand the influence exerted on activity and cytotoxicity by different substitutions at the N1 and C5 phenyl rings, and to refine the structure–activity relationship analysis on such pyrrole derivatives, we have synthesized and tested new compounds bearing *ortho* halogenated phenyl rings at both the N1 and C5 pyrrole positions (namely, compounds **5**–**12**, Table 1).

Following what reported in the literature about their importance, F and Cl were the halogens chosen to be introduced into the phenyl rings. On the other hand, to evaluate the influence of larger and more hydrophobic groups toward the antimycobacterial activity, N1 or C5 naphthyl derivatives have been also synthesized and tested (namely, compounds **13**–**20**, Table 1).

In this paper, we reported the synthesis of the new pyrrole derivatives **5**–**20**, along with their in vitro activity toward *M. tuberculosis* and atypical mycobacteria. Moreover, molecular modeling studies were performed to rationalize their activity in terms of superposition onto a pharmacophoric model for antitubercular compounds and in terms of their lipophilic properties.

2. Chemistry

Compounds **5**–**20** were prepared as illustrated in Scheme 1, from the appropriate arylaldehyde, methyl vinyl ketone and 4-methylthiazolium bromide, as previously reported by us.[13] The pyrroles and the Mannich bases were obtained by a procedure previously described by us.[8]

All the new compounds were identified by elemental analyses and NMR data, that were reported in the experimental part only for compounds **5** and **6**, taken as representative examples of the thiomorpholinomethyl and *N*-methylpiperazinomethyl derivatives, respectively.

Physicochemical data for compounds **5**–**20** were shown in Table 5.

3. Results

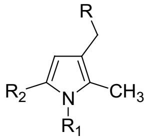
The in vitro activities of compounds **5**–**20** against *M. tuberculosis* 103471, *M. gordonae* 6427, *M. smegmatis* 103599, *M. marinum* 6423 and *M. avium* 103317 were listed in Tables 1 and 2. Cytotoxicity and protection index (PI) were also evaluated for all the synthesized compounds and resulting data were reported in Table 1.

4. Discussion

Antimycobacterial activity: As reported in the experimental section, on the new pyrrole derivatives, we carried out the same biological screening protocol performed on previously published compounds, to allow for a better comparison of all activity data. **BM 212**, **22** and **43** were obviously employed as reference compounds.

Interesting results were obtained for antimycobacterial activity. In fact, as a general trend, a comparison between *N*-methylpiperazinomethyl and thiomorpholinomethyl derivatives confirmed the importance, for in vitro activity, of the thiomorpholinomethyl introduction into the pyrrole structure.[10–13] Accordingly, all the active compounds contained the thiomorpholinomethyl fragment, with the exception of derivatives **16** and **20** that, however, showed high toxicity. Some additional considerations have to be done regarding the introduction of the further *ortho* halogenated phenyl substituent in the molecule. It is important to point out that, on the contrary to what was observed for the correlated compounds **31**–**44** previously synthesized, derivatives **5**, **7** and **9**, were found to be active against *M. tuberculosis*, while compounds **11**, **15**, **16**, and **20** revealed active not only against *M. tuberculosis* but also toward atypical mycobacteria (in particular *M. avium*). Concerning activity against atypical mycobacteria, compound **11**, bearing the thiomorpholinomethyl moiety at C3 and an *o*-chloro substituted phenyl ring at both the N1 and C5 positions, showed a weak activity against *M. avium* but also a moderate toxicity. Among the N1 α-naphthyl derivatives, **16** and **20**, bearing a *N*-methylpiperazinomethyl moiety at C3, and an *o*-F- or *o*-Cl-phenyl ring, respectively, at the position 5, showed a moderate activity, but high toxicity. Compound **15**, C3 thiomorpholinomethyl and C5 *o*-F-phenyl substituted, showed the best activity toward *M. avium*. It is important to underline that the corresponding previously synthesized compound, in which the phenyl group at C5 was unsubstituted, was completely inactive.[13] The activity of **15** toward *M. avium* was comparable to that of the reference compound **22**, N1 *p*-F-phenyl and C5 phenyl substituted, even if it was less active against *M. tuberculosis* and more toxic. These results evidenced the importance of the fluorine substitution toward antimycobacterial activity. Compound **5**, in which both the phenyl rings were *o*-F-substituted, showed a very good activity against *M. tuberculosis*, while it was completely inactive against atypical mycobacteria, thus showing a better selectivity. A very good protection index was observed for compound **5** (the most active compound of the

Table 1. Structure, antimycobacterial activity against *M. tuberculosis* 103471, calculated log *P*, cytotoxicity, and protection index (PI) of the pyrrole derivatives **5**–**44** and **BM 212**, isoniazid, streptomycin, and rifampin



Compd[a]	R[b]	R$_1$	R$_2$	MIC (μg/mL)	log *P*[c]	MTD$_{50}$[d] (μg/mL) VERO cells	Protection index (PI)
5	B	2-F–Ph	2-F–Ph	1	5.58	32	32
6	A	2-F–Ph	2-F–Ph	8	5.10	8	1
7	B	2-Cl–Ph	2-F–Ph	4	6.04	8	2
8	A	2-Cl–Ph	2-F–Ph	>16	5.56	16	
9	B	2-F–Ph	2-Cl–Ph	4	6.04	8	2
10	A	2-F–Ph	2-Cl–Ph	16	5.56	4	
11	B	2-Cl–Ph	2-Cl–Ph	4	6.50	8	2
12	A	2-Cl–Ph	2-Cl–Ph	16	6.02	4	
13	B	2-F–Ph	1-Naphthyl	>16	6.28	16	
14	A	2-F–Ph	1-Naphthyl	>16	5.81	2	
15	B	1-Naphthyl	2-F–Ph	4	6.28	4	1
16	A	1-Naphthyl	2-F–Ph	4	5.81	4	1
17	B	2-Cl–Ph	1-Naphthyl	>16	6.74	128	
18	A	2-Cl–Ph	1-Naphthyl	>16	6.26	16	
19	B	1-Naphthyl	2-Cl–Ph	>16	6.74	64	
20	A	1-Naphthyl	2-Cl–Ph	4	6.26	2	
21	A	4-F–Ph	Ph	16	4.90		
22	B	4-F–Ph	Ph	0.4	5.37	8	20
23	A	Ph	4-F–Ph	16	4.90		
24	B	Ph	4-F–Ph	0.5	5.37		
25	A	4-Cl–Ph	4-F–Ph	16	5.56		
26	B	4-Cl–Ph	4-F–Ph	2	6.04		
27	A	4-F–Ph	4-F–Ph	16	5.10		
28	B	4-F–Ph	4-F–Ph	1	5.58		
29	A	4-F–Ph	4-Cl–Ph	2	5.56		
30	B	4-F–Ph	4-Cl–Ph	1	6.04		
31	B	2-F–Ph	Ph	8	5.37		
32	A	2-F–Ph	Ph	16	4.90		
33	B	Ph	2-F–Ph	4	5.37		
34	A	Ph	2-F–Ph	16	4.90		
35	B	2-Cl–Ph	Ph	16	5.83		
36	A	2-Cl–Ph	Ph	>16	5.36		
37	B	Ph	2-Cl–Ph	16	5.83		
38	A	Ph	2-Cl–Ph	>16	5.36		
39	B	1-Naphthyl	Ph	>16	6.08		
40	A	1-Naphthyl	Ph	>16	5.60		
41	B	Ph	1-Naphthyl	>16	6.08		
42	A	Ph	1-Naphthyl	>16	5.60		
43	B	Ph	Ph	1	5.17	32	32
44	A	Ph	Ph	16	4.69		
BM 212	A	4-Cl–Ph	4-Cl–Ph	0.7	6.02	4	5.6
Isoniazid						32	128
Streptomycin						>64	128
Rifampin						64	213

[a] Compounds **21**–**30** have been reported in Ref. 11, while compounds **31**–**44** have been reported in Ref. 13.

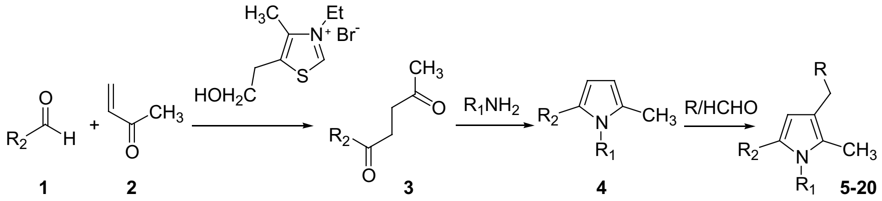
[b] A = N-methylpiperazinyl, B = thiomorpholinyl.

[c] Calculated by means of the AlogP98 method (see Ref. 21).

[d] MTD$_{50}$ = median toxic dose.

series) comparable to that of derivative **43**. Derivatives **7**, **9**, **11**, **15**, **16** and **20** revealed moderately active against *M. tuberculosis*; a moderate toxicity was observed for **7**, **9**, **11**, and **15**. All of them were as toxic as **BM 212** and more toxic than **5**, **43**, and **22**. Finally compounds **13**, **14**, and **17**–**19**, C5 α-naphtyl substituted were found to be completely inactive.

From these microbiological data it is possible to draw the following considerations. (1) *N*-methylpiperazinomethyl derivatives were in general more toxic and less active than corresponding thiomorpholinomethyl compounds. (2) Regarding the thiomorpholinomethyl derivatives, by introducing a second *o*-halophenyl substituent in the molecule, the antimycobacterial activity



Scheme 1.

Table 2. In vitro activity against *M. smegmatis*, *M. marinum*, *M. gordonae* and *M. avium* of compounds **BM 212**, **22**, **43**, **5**–**20**, isoniazid, streptomycin and rifampin

Compound	MIC (μg/mL)			
	M. smegmatis 103599	*M. marinum* 423	*M. gordonae* 6427	*M. avium* 103317
BM 212	25	100	>100	0.4
22	>16	>16	>16	2
43	>16	>16	>16	>16
5	>16	>16	>16	>16
6	>16	>16	>16	>16
7	>16	>16	16	16
8	>16	>16	>16	>16
9	>16	>16	>16	>16
10	>16	8	>16	16
11	>16	16	>16	8
12	>16	>16	>16	16
13	>16	>16	>16	>16
14	>16	>16	>16	16
15	16	16	>16	2
16	>16	8	>16	4
17	>16	8	16	>16
18	>16	>16	>16	>16
19	>16	>16	>16	>16
20	>16	8	>16	4
Isoniazid	64	16	32	32
Streptomycin	8	32	16	8
Rifampin	32	0.6	0.6	0.3

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 into the *ortho* position of the 5-phenyl ring of **31** or the N1-phenyl ring of **33** to give compound **5** with an activity 8- and 4-fold better (MIC = 1 μg/mL) than the corresponding parent compounds (MIC of 8 and 4, respectively). Moreover, a 4-fold enhancement of activity was found by introducing an *o*-F into the unsubstituted phenyl ring of **35** (MIC of 16) and **37** (MIC of 16) to give compounds **7** (MIC of 4) and **9** (MIC of 4), respectively. While an additional *o*-Cl substituent produced compounds with comparable or improved activity with respect to the mono halo derivatives (compare **7** vs **33**, **9** vs **31** and **11** vs **35**), they were also characterized by higher toxicity with respect to the corresponding fluoro derivatives. (3) Naphthyl derivatives **15**, **16**, and **20** bearing an *o*-F- or an *o*-Cl-phenyl ring at C5 showed better activity with respect to the parent compounds with the unsubstituted 5-phenyl ring (**39** and **40**). All the remaining members of this subclass were inactive (namely, **13**, **14**, and **17**–**19**).

Compound **5**, structurally related to the previously described pyrrole derivatives **21**–**44** and **BM 212**, found to be the most active compound among the new pyrrole derivatives, was also tested against intracellular and resistant mycobacteria. Biological results, reported in Tables 3 and 4, showed that all of the tested strains were inhibited by compound **5**.

Moreover, it exerted bactericidal activity also on intracellular mycobacteria (Table 3).

MIC value was the same found for **BM 212** and lower than that of rifampin and compound **22**. This result was very important because mycobacteria can reside for years inside lymphoid cells and macrophage, and

Table 3. Inhibition of intramacrophagic *Mycobacterium tuberculosis* by compound **5**, **22**, **43**, **BM 212** and rifampin

Compound	MIC (μg/mL)
	Inhibition of intramacrophagic mycobacteria
BM 212	1
22	3
43	1
5	1
Rifampin	3

Table 4. Sensitivity of different strains of *Mycobacterium tuberculosis* resistant to different inhibitors

Strains resistant N.	Strept.	Isoniazid	Rifamp.	Ethamb.	BM 212	22	43	5
M. tuberculosis 15	s[a]	s	r	s	s	s	s	s
M. tuberculosis 150	s	s	r	s	s	s	s	s
M. tuberculosis 585	s	r	r	s	s	s	s	s
M. tuberculosis 535	s	s	s	r	s	s	s	s
M. tuberculosis 541	r[b]	r	s	s	s	s	s	s

[a] s = sensitive.

[b] r = resistant.

Table 5. Physicochemical data of compounds **5**–**20**

Compound	mp (°C)	Yield (%)	Formula (MW)
5	Oil	55	$C_{22}H_{22}N_2SF_2$ (384.49)
6	Oil	45	$C_{23}H_{25}N_3F_2$ (381.47)
7	Oil	45	$C_{22}H_{22}N_2SFCl$ (400.95)
8	Oil	20	$C_{23}H_{25}N_3FCl$ (397.93)
9	Oil	30	$C_{22}H_{22}N_2SFCl$ (400.95)
10	Oil	20	$C_{23}H_{25}N_3FCl$ (397.93)
11	Oil	75	$C_{22}H_{22}N_2SCl_2$ (417.40)
12	Oil	30	$C_{23}H_{25}N_3Cl_2$ (414.38)
13	147–148	30	$C_{26}H_{25}N_2SF$ (416.56)
14	Oil	30	$C_{27}H_{28}N_3F$ (413.54)
15	Oil	70	$C_{26}H_{25}N_2SF$ (416.56)
16	Oil	65	$C_{27}H_{28}N_3F$ (413.54)
17	170–171	80	$C_{26}H_{25}N_2SCl$ (433.02)
18	Oil	45	$C_{27}H_{28}N_3Cl$ (430.00)
19	95–96	75	$C_{26}H_{25}N_2SCl$ (433.02)
20	Oil	65	$C_{27}H_{28}N_3Cl$ (430.00)

many traditional drugs were not able to get throw it. Moreover, the high selectivity of derivative **5** against mycobacteria was worthy of further consideration. In fact, this compound was very active only against *M. tuberculosis*, while it was completely inactive against atypical mycobacteria (see Table 2).

5. Computational investigations

The new pyrrole derivatives have been computationally analyzed by means of the Catalyst software[14] for their fit properties to a five-feature pharmacophoric model (Fig. 1) previously built from us for antitubercular compounds, constituted by a hydrophobic region, two aromatic rings and a hydrogen bond acceptor group.

Moreover, lipophilicity of the new compounds was investigated, suggesting that it was correlated with the antitubercular activity, and provided all the compounds with a high probability to penetrate hydrophobic membranes, such as the mycobacterial cell wall.

Pharmacophore model calculations: Previous results published from our research group on the field of antitubercular agents, combined with biological data reported in this paper suggested a summary of the relationships between the structure of such compounds and their antimycobacterial activity. All the compounds were characterized by a 2-methylpyrrole nucleus bearing various substituents at the positions 1, 3, and 5. In further detail, while a thiomorpholinomethyl or a *N*-methylpiperazinomethyl groups were the substituents at position 3, positions 1 and 5 have been substituted with *ortho* or *para* substituted halophenyl ring or 1-naphthyl ring.

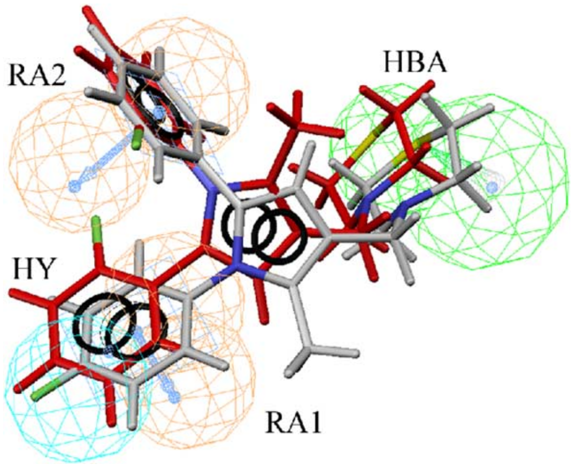


Figure 1. Superposition of compound **5** (red) and **22** (grey) into the pharmacophoric model for antitubercular compounds. Compound **22** matches RA2 with the 5-phenyl ring, HY with the fluorine atom, RA1 with the phenyl ring at N1, and, finally, HBA with the morpholino sulfur atom. Differently, compound **5** undergoes a slight translation to fit both HY and RA2 with the sole 5-phenyl ring. It is important to note the *o*-F substituent directed toward the aromatic sphere corresponding to the receptor counterpart (potentially) interacting with the N1 phenyl ring of **5**. A hydrophobic interaction between the *ortho* halogen and the aromatic portion of the receptor could account for the activity found for *ortho* halogenated compounds and for the decrease in activity found when the size of the halogen increased (i.e., from a fluorine to a chlorine). Pharmacophoric features are color-coded: HY (hydrophobic), blue; RA (aromatic regions), orange; HBA (hydrogen bond acceptor groups), green.

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 **20** was the sole exception of a *N*-methylpiperazinomethyl derivative with an enhanced activity in comparison to the corresponding thiomorpholinomethyl counterpart **19**. 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 sulfur 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 **43** produced variations in activity. In particular, both the N-(*p*-F-phenyl) and the 5-(*p*-F-phenyl) derivatives **22** and **24** showed a slight enhancement in activity (MIC values of 0.4 and 0.5 μg/mL, respectively) with respect to the parent compound. Activity of such two compounds was rationalized by the pharmacophoric model. In fact, superposition of these compounds with the model showed that all the pharmacophoric features were filled by the chemical groups of the molecules. In particular, the fluoro substituent was found to be very important because it matched HY. Moreover, the model evidenced that the optimal substitution was represented by the N-(*p*-F-phenyl) group. However, the 5-(*p*-F-phenyl) moiety was also able to satisfy the HY feature of the model by a reverse orientation of the pyrrole ring that underwent a 180° rotation leading the 5-substituent to fill the RA1-HY system of the model. This finding led to the suggestions that (1) compounds bearing phenyl rings with different halogenation could show activity comparable each other. Accordingly, **30** and **26** showed MIC = 1 and 2 μg/mL, respectively. A comparable MIC value of 1 was found for the difluorinate derivative **28**, also able to interact with the model through alternate orientations differing for a 180° rotation of the pyrrole ring. (2) Moreover, on the basis of the pyrrole rotation, the methyl group at the 2 position was located in two different and empty regions of space, without any contact with pharmacophoric features. As a consequence, the methyl group was not a pharmacophoric portion of the antitubercular compounds, but showed a structural function in defining the mutual orientation of substituents at both the positions 1 and 3.

In disagreement with the suggestions reported above about the *N*-methylpiperazinomethyl derivatives, while the unsubstituted 1,5-diphenyl derivative **44** and the corresponding N-(4-Cl–Ph)-5-(4-F-phenyl) counterpart **25** were inactive, the reverse derivative **29** possessed MIC = 2 μg/mL, and the corresponding dichloro analogue (**BM 212**) was characterized by MIC = 0.7 μg/mL.

The introduction of an *ortho* halo substituent into the structure of compound **43** led to a drop in activity, accounted by the pharmacophoric model on the basis of the inability to fully match the HY feature. In fact, in the series of *ortho* halogenated derivatives, the halo substituents was located in an empty region of space, where any of the pharmacophoric features lie, while the phenyl ring was engaged to partially fill either the HY or the RA1 features of the model. Regarding activity, while *ortho*-fluoro derivatives **31** and **33** retained an appreciable activity, the corresponding *ortho*-Cl compounds **35** and **37** were found inactive, showing that the increase of the size of the halogen or, alternately, the decrease of its electron-withdrawing capabilities was detrimental for activity. Analysis of the fitting mode of *ortho* halogenated compounds into the pharmacophoric model showed that several orientations were characterized by the fluorine substituent pointing toward the RA2 aromatic sphere corresponding to the receptor counterpart (evidenced in Fig. 1). 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). However, on the basis of this hypothesis, we were unable to explain why dichloro derivatives (i.e., compound **11**) retained good activity, that probably was dependent on additional factors such as electronic and lipophilic contributions.

When a system with halogenated phenyl rings at both the positions 1 and 5 was restored, activity increased. In fact, either the chloro-fluoro **7** or fluoro-chloro **9** derivatives or the dichloro compound **11**, showed comparable activity (MIC = 4 μg/mL). As expected, the difluoro compound showed a better activity (MIC = 1 μg/mL).

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 **39–42** inactive. Similarly, introduction of an *ortho* chloro or fluoro substituent on the N-phenyl ring led to inactive compounds (**13**, **14**, **17**, and **18**). 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 a halogen was introduced at the *ortho* position of the 5-phenyl ring, an enhancement of activity was found. In particular, while the fluoro derivatives (**15** and **16**) showed an activity of 4, compound **19** bearing a chlorine and a thiomorpholinomethyl moiety was found to be inactive, differently from **20** characterized by an activity of 4. The last finding was in disagreement with the general trend showing that *N*-methylpiperazinomethyl derivatives were characterized by activity lower than the corresponding thiomorpholinomethyl analogues.

To summarize, the pharmacophoric model previously built by our research group for compounds inhibiting *M. tuberculosis* was able to account for the major structure–activity relationships found for the above compounds (Fig. 1). (i) Compounds that better fitted the model was characterized by a thiomorpholinomethyl ring at the position 3 and an *para*-F phenyl ring at the position 1 or 5 (as an example, compounds **22** and **24**). Such compounds were able to fit all the pharmacophoric features. In detail, the F-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. (ii) The sulfur atom of the thiomorpholinomethyl ring represented the hydrogen bond acceptor group, HBA. Piperazine 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. (iii) Introduction of a halogen substituent at the *ortho* position instead of the *para* position, 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*-F-phenyl 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.

Log P calculations: The enhancement in activity usually found for thiomorpholinomethyl derivatives in comparison to the corresponding *N*-methylpiperazinomethyl compounds, was rationalized by means of the Cerius2 software,[14] used to calculate $\log P$ values (reported in Table 1) of the pyrrole derivatives, with the aim of evaluating if any correlation between antitubercular activity and lipophilicity of such compounds, occurred. As a result, we found that all the thiomorpholinomethyl derivatives showed $\log P$ values higher than the corresponding *N*-methylpiperazinomethyl counterparts, in agreement with the suggestion that a more hydrophobic character is better for the antitubercular potency of a compound (at least in vitro). Moreover, compounds with the best activity values, ranging from 0.4 through 1, were characterized by a calculated $\log P$ spanning between 5.37 and 6.04, including **BM 212** (MIC = 0.7 μg/mL). However, higher $\log P$ values associated with the naphthyl derivatives were in disagreement with the previous hypothesis. In fact, with the exception of compounds **15**, **16**, and **20**, activity of such compounds was found to be >16. A recent paper[15] 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 $\log P$ along with a limited enhancement of the molecular volume could lead to more potent derivatives". Regarding compounds with $\log P$ lower that 5.37, they were associated with activity values of 16 or higher, with the exception of compounds **6** and **43** with activity of 8 and 1, respectively.

VolSurf calculations: The pharmacokinetic properties of the pyrrole derivatives were also evaluated with the aim of study their ability to penetrate the lipophilic mycobacterial cell wall. For this purpose, the VolSurf approach has been applied, being this software a chemometric tool able to adequately handle also biological data derived from cell systems, in principle dependent on the kinetic properties of the studied compounds,[16] as in the case of MIC values determined for the pyrrole derivatives presented in this paper. The VolSurf approach consists in several steps of calculations. Briefly, the program calculated the molecular interaction fields (MIF) by contouring the surface of each inhibitor with a probe (namely, the water, dry and carbonyl oxygen probes). In a second step, a series of many molecular descriptors were calculated on the basis of MIFs. Values of such descriptors were subsequently projected into a pharmacokinetic model (namely, the Caco-2 absorption model, provided along with the VolSurf program) to predict the entity of absorption process, that depended heavily on the ability of each compound to diffuse through the lipophilic membranes. Being aware that the mycobacterial cell wall and the membranes used to generated the adsorption models in VolSurf are characterized by phospholipid layers differing in composition and structural organization, results from VolSurf calculations could provide a preliminar rough description of the studied compounds to evaluate their permeation properties, as recently reported in the literature.[17] Projections of the antitubercular pyrrole derivatives into the VolSurf model (Fig. 2) suggested that the compounds could have high probability of being absorbed in such biological systems. An implication deriving from this finding was that our compounds could (potentially) penetrate the hydrophobic barrier of the mycobacteria, thus suggesting that the inactivity of some of them was not the consequence of a poor adsorption, but was probably derived from a poor affinity of compounds for the corresponding receptor counterpart.

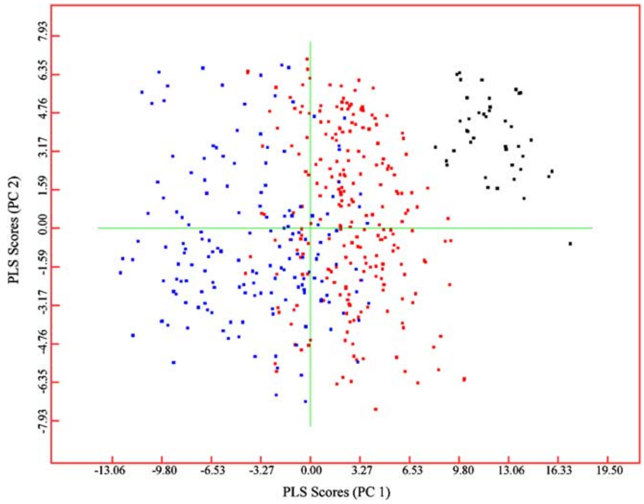


Figure 2. Projection of the 45 pyrrole derivatives (black) into the PLS score plot of the Caco-2 cells adsorption model provided along with the VolSurf software. Compounds used to generate the model are color-coded: blue for low-adsorbed compounds (on the left), red for high-adsorbed compounds (on the right). It is important to note that all the pyrrole derivatives lie in the upper right corner of the graphic, suggesting a high probability to diffuse through the lipophilic membranes and penetrate the hydrophobic barrier of the mycobacteria.

6. Experimental

Melting points were uncorrected and taken on a Fischer–Jones apparatus. NMR spectra were recorded

Table 6. Microanalyses of compounds **5**–**20**

Compd	%C	%H	%N	%S	F	Cl	
5	68.73	5.77	7.29	8.34	9.88		Calc
	68.70	6.00	7.31	8.36	9.87		Found
6	72.41	6.61	11.02		9.96		Calc
	72.39	6.63	11.05		9.99		Found
7	65.90	5.53	6.99	8.00	4.74	8.84	Calc
	65.91	5.55	7.00	8.01	4.74	8.82	Found
8	69.42	6.33	10.56		4.77	8.91	Calc
	69.41	6.30	10.55		4.76	8.90	Found
9	65.90	5.53	6.99	8.00	4.74	8.84	Calc
	66.00	5.52	6.98	8.01	4.76	8.85	Found
10	69.42	6.33	10.56		4.77	8.91	Calc
	69.44	6.34	10.57		4.80	8.90	Found
11	63.31	5.31	6.71	7.68		19.99	Calc
	63.30	5.28	6.72	7.70		20.00	Found
12	66.66	6.08	10.14			17.11	Calc
	66.65	6.06	10.11			17.11	Found
13	74.97	6.05	6.72	7.70	4.56		Calc
	75.00	6.03	6.73	7.71	4.57		Found
14	78.41	6.82	10.16		4.59		Calc
	78.40	6.81	10.14		4.60		Found
15	74.97	6.05	6.72	7.70	4.56		Calc
	74.98	6.04	6.71	7.73	4.58		Found
16	78.41	6.82	10.16		4.59		Calc
	78.40	6.85	10.14		4.58		Found
17	72.12	5.82	6.47	7.40		8.19	Calc
	72.13	5.84	6.50	7.38		8.21	Found
18	75.41	6.56	9.77			8.24	Calc
	75.39	6.56	9.80			8.21	Found
19	72.12	5.82	6.47	7.40		8.19	Calc
	72.13	5.84	6.49	7.41		8.21	Found
20	75.41	6.56	9.77			8.24	Calc
	75.40	6.55	9.75			8.22	Found

for all the synthesized compounds on a 200 Brucker spectrometer using deuterochloroform as solvent and TMS as internal standard. Microanalyses (Table 6) of compounds **5**–**20** were performed by the Servizio di Microanalisi dell' Area di Ricerca di Roma del CNR. Fluka aluminum oxide (activity II–III, according to Brockmann) was used for chromatographic purifications. Fluka Stratocrom aluminum oxide plates with fluorescent indicator were used for thin-layer chromatography (TLC) to check the purity of the compounds.

7. Chemistry

Diketones **3**: The title compounds were prepared according to the general procedure previously described.[11]

Pyrroles **4**: The title compounds were prepared according to the general procedure previously described.[8]

Mannich bases **5**–**20**: To a stirred solution of the appropriate pyrrole **4** (5.6 mmol) in 20 mL of acetonitrile, a mixture of *N*-methylpiperazine or thiomorpholine (5.6 mmol), formaldehyde (5.6 mmol) (40% in water) and 5 mL of acetic acid was added dropwise. After the addition was complete the mixture was stirred at room temperature for 3 h. The mixture was then treated with a solution of sodium hydroxide (20%, v/v) (100 mL) and extracted with ethyl acetate (200 mL). The organic extracts were combined, washed with water (200 mL) and dried. After removal of solvent, the residue was purified by column chromatography using benzene for thiomorpholinomethyl and chloroform for *N*-methylpiperazinomethyl derivatives as eluants, respectively. The eluates were combined after TLC control and the solvent was removed to give the pure product.

Physicochemical data are reported in Table 5.

5: ^{1}H NMR (CDCl$_3$) δ: 1.97 (s, 3H, CH$_3$), 2.56–2.69 (m, thiomorpholine 8H), 3.39 (s, 2H, CH$_2$), 6.24 (s, 1H, H-4), 6.83–7.29 (m, 9H, aromatic protons).

6: ^{1}H NMR (CDCl$_3$) δ: 1.98 (s, 3H, N–CH$_3$), 2.29 (s, 3H, CH$_3$), 2.45 (m, *N*-methylpiperazine 8H), 3.42 (s, 2H, CH$_2$), 6.30 (s, 1H, H-4), 7.1–7.34 (m, 10H, aromatic protons).

8. Microbiology

Compounds: Compounds **5**–**20** and reference drugs were dissolved in DMSO at a concentration of 10 mg/mL and stored cold until used.

Antimycobacterial activity: Compounds were preliminarily assayed against two freshly isolated clinical strains, *M. fortuitum* CA10 and *M. tuberculosis* B814, according to the dilution method in agar.[18] Growth media were Mueller-Hinton (Difco) containing 10% of OADC (oleic acid, albumine and dextrose complex) for *M. fortuitum* and Middlebrook 7H11 agar (Difco) with 10% of OADC (albumine and dextrose complex) for *M. tuberculosis*. Substances were tested at the single dose of 100 μg/mL. The active compounds were then assayed for inhibitory activity against a variety of mycobacterium strains in Middlebrok 7H9 broth using the NCCLS procedure. Data are reported in Tables 1 and 2. The mycobacterium species used for biological tests were *M. tuberculosis 103471* and, among atypical mycobacteria, *M. smegmatis* 103599, *M. gordonae* 6427, *M. marinum* 6423 and *M. avium 103317* (from the Institute Pasteur collection).

In all cases, minimum inhibitory concentrations (MICs in μg/mL) for each compound were determined. The MIC was defined as the lowest concentration of drug that yielded an absence of visual torbidity. Stock solutions of substances were prepared by dissolving a known weight of agent in DMSO. The stock solutions were sterilized by passage through a 0.2 μm Nylon membrane filter. Serial 2-fold dilutions of the compounds with water were prepared. The tubes were incubated at 37 °C for 3–21 days. A control tube without any drug was included in each experiment. Isoniazid (INH), streptomycin and Rifampin were used as controls.

Inhibitory activity of BM 212, 22, 43 and 5 on multi-drug-resistant and intramacrophagic mycobacteria: The mycobacteria used were *M. tuberculosis* 15, *M. tuberculosis* 150, *M. tuberculosis* 585, *M. tuberculosis* 535 and *M. tuberculosis* 541. Compound **BM 212**, **22**, **43**, and **5** were tested on multiresistant *M. tuberculosis* strains in

Middlebrook 7119 broth enriched with 10% ADC (Difco) using the macrodilution broth method. The bactericidal activity of such compounds on intracellular mycobacteria was studied on U937 cells (INC-FLOW), a human hystiocytic cell line. Cells were differentiated into macrophages with 20 ng/mL of phorbol myristate acetate (PMA, Sigma) and grown in RPMI 1640 medium with 10% fetal calf serum.

9. Cytotoxic activity assays

The Vero cells were inoculated in 6-well plates each containing 9×10^4 cells and incubated in DMEM with 5% FS for 24 h at 37 °C in a 5% CO_2 incubator. After 24 h of culture, the medium was changed and a new medium containing decreasing doses of the substances under study was added.

After 5 days, the cells were trypsinized and counted in a Neubauer chamber under a light microscope. All the tests were done in triplicate. The maximum 50% non-toxic dose ($MNTD_{50}$) was defined as the drug concentration that decreased cell multiplication less than 50% with respect to the control.

10. Computational methods

All calculations and graphic manipulations were performed on a SGI Origin 300 server and SGI Octane workstations by means of the Catalyst (version 4.9),[14] Cerius2 (version 4.8.1)[14] and VolSurf (version 3.0.11)[19,20] software packages.

All the compounds used in this study were built using the 2D–3D sketcher of Catalyst. A representative family of conformations were generated for each molecule using the poling algorithm and the 'best quality conformational analysis' method. Conformational diversity was emphasized by selection of the conformers that fell within 20 kcal/mol range above the lowest energy conformation found.

The Compare/Fit command within Catalyst has been used to predict activity values of the studied compounds. Particularly, the Best Fit option has been selected, which manipulates the conformers of each compound to find, when possible, different mapping modes of the ligand within the model.

The QSAR+ module of Cerius2 was used to calculate AlogP98 values of the studied compounds. AlogP98 was chosen because it was demonstrated to be more reliable in comparison to clogP for compounds having more than 45 atoms, while results from both methods are comparable for compounds with 21–45 atoms. The AlogP98 descriptor is an implementation of the atom-type-based AlogP method using the latest published set of parameters.[21]

VolSurf software has been used as a tool to calculate in silico pharmacokinetic properties of the pyrrole derivatives, by evaluation their absorption from lipophilic membranes.[22] VolSurf descriptors show the advantage of being relatively independent on conformational sampling. For this reason, results from VolSurf calculations are only marginally influenced by conformational averaging and sampling. Further detail on the software can be found at the Molecular Discovery web page http://www.moldiscovery.com/soft_volsurf.php.

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