Antimycobacterial Agents. Novel Diarylpyrrole Derivatives of BM212 Endowed with High Activity toward Mycobacterium tuberculosis and Low Cytotoxicity

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On the basis of suggestions derived either from a pharmacophoric model for antitubercular agents or from a structure−activity relationship analysis of many pyrroles previously described by us, we report here the design and synthesis of new analogues of 1,5-(4-chlorophenyl)-2-methyl-3-(4-methylpiperazin-1-yl)methyl-1*H*-pyrrole (BM212). Various substituents with different substitution patterns were added to both positions 1 and 5 of the pyrrole nucleus to evaluate their influence on the activity toward *Mycobacterium tuberculosis* (MTB) and atypical mycobacteria. Biological data showed that, although some nontuberculosis mycobacterial strains were found to be sensitive, MIC values were higher than those found toward MTB. The best compound (1-(4-fluorophenyl)-2-methyl-3-(thiomorpholin-4-yl)methyl-5-(4-methylphenyl)-1*H*-pyrrole, **5**) possessed a MIC of 0.4 μg/mL (better than BM212 and streptomycin) and a very high protection index (160), better than BM212, isoniazid, and streptomycin (6, 128, and 128, respectively). Finally, molecular modeling studies were performed to rationalize the activity of the new compounds in terms of both superposition onto a pharmacophoric model for antitubercular compounds and their hydrophobic character.

Introduction

Humankind's battle with tuberculosis (TB) dates back to antiquity. TB, which is caused by *Mycobacterium tuberculosis* (MTB), was a much more prevalent disease in the past than it is today, and it was responsible for the deaths of about 1 billion people during the last two centuries.[1] Improved sanitation and living conditions significantly reduced the incidence of the disease even before the advent of chemotherapy. However, despite the widespread use of the bovine Calmette−Guerin vaccine, which had a great impact on further reduction in TB incidence, TB still remains a leading infectious disease worldwide, especially in third world countries. In fact, MTB is the cause of death of almost 3 million people each year, and it is positioned as the leading bacterial infectious agent.[2−4]

The increase of TB during recent years was largely due to HIV-1 infection, immigration, increased trade, and globalization.[5] Moreover, also the increasing emergence of a drug-resistant TB, especially multidrug-resistant TB (MDR-TB) is particularly alarming. MDR-TB has already caused several fatal outbreaks[6,7] and poses a significant threat to the treatment and control of the disease in some parts of the world, where the incidence of MDR-TB can be as high as 14%.[5] In addition, the TB situation may become even worse with the spread of HIV-1 worldwide, a virus that weakens the host immune system, allows latent TB to reactivate, and makes the person more susceptible to reinfection with either drug-susceptible or drug-resistant strains. The lethal combination of drug-resistant TB and HIV-1 infection is a growing problem that presents serious challenges for effective TB control. In view of this situation, in 1993, the WHO declared TB a global emergency.[8]

One of the major obstacles to global control of TB is represented by the reactivation of the disease in patients who carry a latent infection, in which the bacteria are thought to be in a slow growing or nongrowing state[9,10] and are recalcitrant to treatment by conventional anti-TB drugs.[11,12]

An additional difficulty derives from atypical mycobacteria, which include the *Mycobacterium avium* complex (MAC), an opportunistic pathogen in AIDS patients.[13] Clinical management of MAC infections, especially among AIDS patients, is difficult, partly because of the severely depressed state of the host defense mechanisms in such patients[14] and partly because of the intrinsically low susceptibility of MAC isolates to many antimycobacterial drugs.[14,15]

The current recommended standard TB chemotherapy, called DOTS (directly observed treatment, short-course) has a cure rate of up to 95% and is recommended by the WHO for treating every TB patient. Although DOTS can cure TB, the length of the therapy makes patient compliance difficult, and noncompliance is a frequent source of drug-resistant strains.[7,16] In fact, MDR strains, resistant to many first-line agents, as well as some of the second-line drugs, are increasingly being found.[7,17]

As a consequence, without more effective treatments, the number of infections caused by MDR-TB will probably 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 refractory tuberculosis and MAC infections, are urgently needed.[18]

However, in the last 40 years, only a few drugs have been approved by the Food and Drug Administration (FDA) to treat TB, reflecting the inherent difficulties in discovery and clinical testing of new agents and the lack of pharmaceutical industry research in the area.[19] In particular, in addition to the current

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drugs approved by the FDA for the treatment of TB and to the drugs that commonly are recommended for the treatment of TB but are not FDA approved,[20] a variety of other compounds or classes of compounds is under investigation as potential antimycobacterial drugs. Among them, pyrrole derivatives were first reported by us in 1998[21] as antimycobacterial agents. The most potent derivative (BM212) showed minimum inhibitory concentration (MIC) values ranging from 0.7 to 1.5 μg/mL toward several strains of MTB. Activity toward drug-resistant strains of MTB was similar to that found toward sensitive strains. Finally, it was also active toward nontuberculosis mycobacteria, with MICs higher that that against MTB.

Design of New Pyrrole Derivatives. In an effort to search novel derivatives of BM212 endowed with a better biological profile, we have previously reported the synthesis and both antimycobacterial and antifungal activities of many pyrrole derivatives,[22−24] finding that most of such compounds showed interesting antimycobacterial properties. Moreover, modifications of BM212 (based on the suggestion that a thiomorpholin-4-yl substituent onto a five-membered heterocyclic ring is a crucial key for antitubercular activity)[25] allowed the identification of several very active antitubercular compounds and showed the importance of the (thiomorpholin-4-yl)methyl moiety at the pyrrole C3 and a *p*-halophenyl substituent at both N1 and C5.[26−29]

Moreover, in the absence of any knowledge regarding both the drug target and the biologically active conformation of the previous antitubercular agents, a ligand-based drug design approach has been applied to better understand the relationships between the structure of compounds and their activity and also to aid in the design of more potent inhibitors. Accordingly, the application of a pharmacophoric generation technique led us to build[26] and optimize[30] a pharmacophoric model for antimycobacterial compounds, consisting of four chemical features represented by a hydrophobic region (HY), a hydrogen-bond acceptor group (HBA), and two aromatic rings (RA1 and RA2) (Figure 1).

Analysis of the superposition mode of previously published antitubercular compounds suggested that a phenyl ring bearing a lipophilic group (such as a methyl, ethyl, or isopropyl substituent) at the para position could have profitable interactions with the HY feature of the pharmacophoric model, with a consequent enhancement in activity.[28] On the basis of such a hypothesis, in an attempt to increase the fitting to the pharmacophoric model and, consequently, to optimize the interactions with the hypothetical receptor, we pursued our studies by synthesizing new pyrrole derivatives maintaining the alternative presence of a (thiomorpholin-4-yl)methyl or a (4-methylpiperazin-1-yl)methyl moiety (as in BM212) at the C3 position of the pyrrole ring and introducing a methyl group into one of the phenyl rings at N1 or C5, to allow for a better superposition with the corresponding pharmacophoric feature HY.

In this paper, we report the synthesis of the new derivatives **1**−**28**, characterized by (i) a *p*-fluorophenyl ring at position 1 or 5, found to be important for antitubercular activity; (ii) an additional phenyl group (at position 5 or 1, respectively) bearing F, Cl, and Me substituents with different substitution patterns, to test the hypothesis that a more lipophilic group (such as the methyl one) could improve activity, probably on the basis of the fact that the mycobacterial cell wall structure is very waxy, hydrophobic, and characterized by a high lipid content, and thus, it requires a hydrophobic compound to be penetrated; (iii) a (thiomorpholin-4-yl)methyl or a (4-methylpiperazin-1-yl)methyl moiety at position 3 of the pyrrole nucleus, to support previous findings suggesting that thiomorpholine[31] derivatives were in principle more active than the corresponding *N*-methylpiperazine analogues.

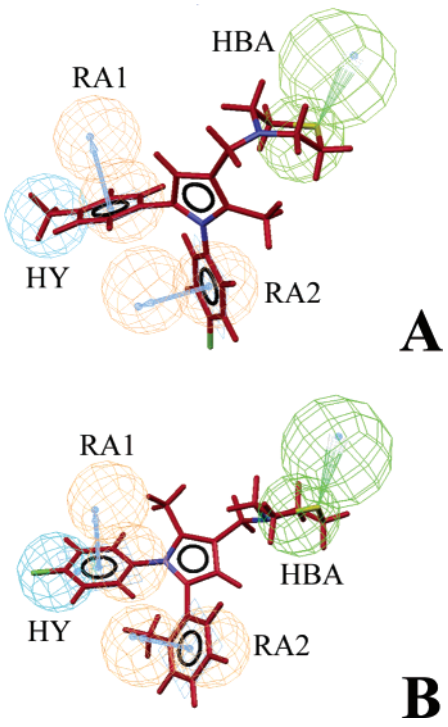
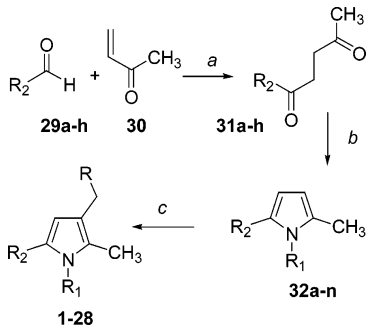


Figure 1. (A) Graphical representation of the superposition pathway of compound **5** onto the pharmacophoric model for antitubercular compounds. The phenyl ring at the nitrogen of the pyrrole ring is accommodated within the aromatic ring feature RA2, the sulfur atom of the thiomorpholine ring at C3 is the hydrogen-bond acceptor group HBA, and the *p*-methylphenyl substituent at position 5 is able to fill both the hydrophobic feature HY (with the methyl group) and the additional aromatic ring RA1 (with its phenyl portion). The methyl substituent at C2 lies in an empty region of space where no pharmacophoric feature is located. (B) Superposition of **9** into the pharmacophoric model showing an alternative binding mode due to the reversed location of substitutents at N1 and C5 of the pyrrole ring. Portions of the pharmacophoric model are color-coded: green for hydrogen-bond acceptor groups (HBA), orange for aromatic rings (RA), and blue for hydrophobics (HY).

Scheme 1[a]



[a] Reagents: (a) 1: 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide, NEt_3, 75−78 °C, 5 h and 2: 2 N HCl. (b) Amine, *p*-toluensulfonic acid, 100 °C, 5 h. (c) 1: Thiomorpholine or *N*-methylpiperazine, CH_3CN, HCHO, CH_3COOH, rt, 8 h and 2: NaOH 20% w/v.

Chemistry. Synthesis of the target compounds is shown in Scheme 1. Briefly, a reaction of a suitable benzaldehyde **29** with methyl vinyl ketone **30**, according to the Stetter conditions,[28] was very versatile in the preparation of the 1,4-diketones **31a**−**h**. Following the usual Paal−Knoor (thermal) condensation, compounds **31a**−**h**, after prolonged reflux in the presence

of the appropriate amine, cyclized to yield the expected 1,5-diarylpyrroles **32a**−**n** in satisfactory yield. Construction of the side chain at C3 was achieved in good yield by reaction of compounds **32a**−**n** with formaldehyde and thiomorpholine or *N*-methylpiperazine, according to the Mannich reaction conditions.

Biology. Compounds were preliminarly assayed in a rapid screening test for their activity toward two clinical strains (i.e., *Mycobacterium fortuitum CA10* and *M. tuberculosis B814)* at the single dose of 100 μg/mL. Those substances that resulted as active in this first screening against at least one of the two mycobacteria were analyzed for their minimum inhibitory concentration (MIC). Only compounds showing MIC values of 16 μg/mL or lower were then studied for their inhibitory activity toward *M. tuberculosis CIP 103471* and a panel of atypical mycobacteria, such as *Mycobacterium marinum* CIP *6423*, *M. avium CIP 103317*, and *Mycobacterium smegmatis* CIP *10359*. MIC values, expressed as μg/mL, were determined for each compound on each of the test mycobacteria.

Cytotoxic activity assays was performed in Vero cells to determine the maximum nontoxic dose ($MNTD_{50}$ expressed as μg/mL), defined as the drug concentration that decreased cell multiplication less than 50% of the control.

In vitro activity of compounds **1**−**28** toward MTB 103471, as well as the cytotoxicity and protection index (PI), were reported in Table 1. Moreover, in vitro activity toward atypical mycobacteria was also reported (Table 2). Isoniazid, streptomycin, rifampicin, and BM212 were used as reference compounds.

Compounds **5** and **17** were also evaluated for their activity toward intracellular MTB (Table 3), as well as toward a panel of 11 resistant and multi-drug-resistant (MDR) clinical isolate strains of MTB (Table 4).

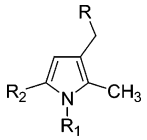
Results and Discussion

Biological data reported in Table 1 showed a general trend that confirmed previous suggestions on the importance of the (thiomorpholin-4-yl)methyl moiety as a substituent of the pyrrole ring.[27−29] In fact, thiomorpholine derivatives showed very good in vitro activity toward MTB and poor toxicity, with a biological profile better than that of the corresponding *N*-methylpiperazine analogues. It is also important to note that compounds **3**, **5**, and **17**, in addition to showing a high in vitro activity toward MTB (MIC of 0.5, 0.4, 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, **5** showed a high protection index (160), better than that of BM212 (6), isoniazid (128), and 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 toward *M. avium* (Table 2). The only exception to this trend was represented by **12**, found 4-fold more active toward *M. avium* (MIC of 2 vs 8 μg/mL), as well as **6** and **26** that showed an identical in vitro activity toward both mycobacteria (4 μg/mL). Moreover, the corresponding thiomorpholine compounds **11** and **25** 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 a chloro substituent was present in both the phenyl rings at N1 and C5) against *M. avium*. All the remaining compounds revealed themselves to be completely inactive against atypical mycobacteria, thus showing a good selectivity versus MTB. The sole compound **18** was found to have a very relevant inhibitory activity toward *M. smegmatis* (0.3 μg/mL).

Compounds **5** and **17** were also evaluated for their activity toward intracellular MTB. It is important to note that while the inhibitory activity toward extracellular MTB accounts for the ability of tested compounds to treat active tuberculosis, assays on intracellular MTB assess the ability of tested compounds to inhibit mycobacteria during the latent phase of tuberculosis, before the latent tuberculosis infection itself progresses to active disease. Biological results reported in Table 3 show that both compounds exert bactericidal activity on intracellular mycobacteria at a 3 μg/mL concentration, comparable to that of rifampicin, used as the reference compound. This result is very important because mycobacteria can reside for years inside lymphoid cells and macrophages (during the latent phase of tuberculosis), and many traditional drugs are unable to get throw it. 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 infection).

Table 1. Structure, In Vitro Antimycobacterial Activity against *M. tuberculosis* 103471, Cytotoxicity, and Protection Index (PI) of the New Pyrrole Derivatives **1**−**28** and Compounds Used as References (BM212, Isoniazid, Streptomycin, and Rifampicin)



compd	R[a]	R_1	R_2	MIC (μg/mL)[b]	$MNTD_{50}$ (μg/mL)[c]	PI[d]	log*P*[e]
1[f]	A	4-F-Ph	2-Cl-Ph	4	4	1	6.04
2[f]	B	4-F-Ph	2-Cl-Ph	8	2	0.25	5.56
3[f]	A	4-F-Ph	2-F-Ph	0.5	16	32	5.58
4[f]	B	4-F-Ph	2-F-Ph	>16	4		5.10
5[f]	A	4-F-Ph	4-CH_3-Ph	0.4	64	160	5.86
6[f]	B	4-F-Ph	4-CH3-Ph	4	8	2	5.38
7	A	4-F-Ph	3-CH3-Ph	2	4	2	5.86
8	B	4-F-Ph	3-CH3-Ph	16	8	0.5	5.38
9	A	4-F-Ph	2-CH3-Ph	4	8	2	5.86
10	B	4-F-Ph	2-CH_3-Ph	>16	2		5.38
11[f]	A	4-F-Ph	2,4-Cl_2-Ph	2	64	32	6.70
12[f]	B	4-F-Ph	2,4-Cl_2-Ph	8	16	2	6.23
13[f]	A	4-F-Ph	2,4-F_2-Ph	0.5	16	32	5.78
14[f]	B	4-F-Ph	2,4-F_2-Ph	4	8	2	5.31
15[f]	A	2-Cl-Ph	4-F-Ph	2	8	4	6.04
16[f]	B	2-Cl-Ph	4-F-Ph	4	8	2	5.56
17[f]	A	2-F-Ph	4-F-Ph	0.5	8	16	5.58
18[f]	B	2-F-Ph	4-F-Ph	8	8	1	5.10
19[f]	A	4-CH_3-Ph	4-F-Ph	1	32	32	5.86
20[f]	B	4-CH3-Ph	4-F-Ph	16	8	0.5	5.38
21	A	3-CH3-Ph	4-F-Ph	4	8	2	5.86
22	B	3-CH3-Ph	4-F-Ph	16	16	1	5.38
23	A	2-CH3-Ph	4-F-Ph	8	16	2	5.86
24	B	2-CH_3-Ph	4-F-Ph	>16	2		5.38
25[f]	A	2,4-Cl_2-Ph	4-F-Ph	1	4	4	6.70
26[f]	B	2,4-Cl_2-Ph	4-F-Ph	4	2	0.5	6.23
27[f]	A	2,4-F_2-Ph	4-F-Ph	2	16	8	5.78
28[f]	B	2,4-F2-Ph	4-F-Ph	16	16	1	5.31
BM212	B	4-Cl-Ph	4-Cl-Ph	0.7	4	5.6	6.02
Isoniazid				0.25	32	128	
Streptomycin				0.50	>64	>128	
Rifampicin				0.30	64	213	

[a] A = thiomorpholin-4-yl and B = 4-methylpiperazin-1-yl. [b] MIC = minimum inhibitory concentration toward *M. tuberculosis*. [c] $MNTD_{50}$ = maximal nontoxic dose toward Vero cells. [d] PI = protection index, as the ratio between cytotoxicity and in vitro activity toward *M. tuberculosis*. [e] Calculated by means of the AlogP98 method (see ref 36). [f] Compound submitted to an international patent application (see ref 37).

Table 2. In Vitro Activity (Expressed as MIC Values in μg/mL) of the New Pyrrole Derivatives **1**−**28** and Reference Compounds (BM212, Isoniazid, Streptomycin, and Rifampicin) toward Atypical Mycobacteria (*M. smegmatis*, *M. marinum*, and *M. avium*)

compd	MIC (μg/mL)		
	M. avium	*M. marinum*	*M. smegmatis*
1	16	>16	>16
2	16	>16	>16
3	16	>16	>16
4	>16	>16	>16
5	8	8	16
6	4	>16	8
7	8	>16	>16
8	16	>16	>16
9	8	>16	>16
10	>16	>16	>16
11	4	>16	>16
12	2	>16	>16
13	16	16	>16
14	16	>16	>16
15	>16	>16	>16
16	>16	>16	>16
17	8	>16	8
18	16	>16	0.3
19	8	8	16
20	16	8	>16
21	16	>16	>16
22	>16	>16	>16
23	16	>16	>16
24	>16	>16	>16
25	2	8	16
26	4	8	>16
27	16	16	>16
28	16	16	>16
BM212	0.4	100	25
Isoniazid	32	16	64
Streptomycin	8	32	8
Rifampicin	0.3	0.6	32

Table 3. Activity of Compounds **5** and **17** toward Intracellular (Intramacrophagic) *M. tuberculosis*

compd	inhibition of intramacrophagic mycobacteria (MIC, μg/mL)
5	3
17	3
Rifampicin	3

Table 4. In Vitro Activity of Compounds **5** and **17** toward a Panel of Clinical Isolates of *M. tuberculosis* Resistant to Different Antitubercular Agents

clinical isolate	sensitivity or resistance[a]				MIC (μg/mL)	
	streptomycin	isoniazid	rifampicin	ethambutol	**17**	5
149/03	S	r	r	s	2	0.5
421/96	S	s	r	s	2	0.5
586/98	S	s	r	s	2	0.5
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	2	0.5
520/98	R	s	r	s	2	0.5
326/04	R	r	s	r	>32	32
296/04	S	s	r	r	2	0.5
482/98	S	s	r	s	2	0.5
275/05	R	s	s	s	2	0.5

[a] s: sensitive and r: resistant. Streptomycin, isoniazid, rifampicin, and ethambutol were tested at single doses of 1.0, 0.1, 1.0, and 5.0 μg/mL, respectively.

Finally, compounds **5** and **17** were also evaluated for their activity toward a panel of 11 resistant and multi-drug-resistant clinical isolate strains (MDR-TB) of MTB. Table 4 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, **5** 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, **17** 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. Also, compound **17** 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.

Computational Investigations. With the aim of further investigating the relationships between the structure of the new compounds and their in vitro activity toward MTB, we resorted to a ligand-based drug design approach based on a pharmacophoric model previously reported by us for antitubercular agents.[30] It is important to note that the pharmacophore should be considered as a qualitative model mainly intended to identify common chemical features shared by our antimycobacterial compounds. On this basis, the model proved to rationalize the major structure−activity relationships of the studied compounds. In fact, it is able to account for the influence that substituents and substitution patterns at both positions 1 and 5 exert in defining the activity of 1,5-diarylpyrrole derivatives toward MTB. However, the pharmacophoric model is not fully able to explain differences in activity (among the two classes of compounds) when they probably derive from a different substituent at position 3 (namely, the *N*-methylpiperazinomethyl and thiomorpholinomethyl moiety).

Analysis of the orientation of the most active compound (**5**) into the pharmacophoric model showed that such a compound was able to perfectly fulfill all the features (Figure 1A). In detail, the side chain at position 5 was embedded into the HY−RA1 system, the methyl group corresponding to the hydrophobic substituent HY and the phenyl ring to one of the aromatic ring features (RA1) of the model. Moreover, the remaining aromatic ring feature (RA2) was matched by the phenyl ring at position 1, while, as expected, the sulfur atom of the thiomorpholine ring was the hydrogen-bond acceptor group. Changing the substitution pattern of the *p*-methyl group as in the *m*-methyl and *o*-methyl derivatives (**7** and **9**, respectively), the binding mode onto the pharmacophore was found to be reversed (Figure 1B). In fact, while the side chain at position 3 remained in the region of the HBA feature, the substituent at position 1 partially filled HY and RA1 with its *p*-fluoro and phenyl group, respectively. Consequently, RA2 was matched by the phenyl ring at position 5. However, the *m*-methyl and, particularly, the *o*-methyl substituents exerted a conformational constraint toward the phenyl ring at C5, leading to a reorientation of its plan. As a consequence, both the *m*-methylphenyl and the *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). This result, in addition to the partial fit of the *p*-fluorophenyl group onto the HY−RA1 system, could account for the decrease in the activity of these derivatives. In a similar way, the *o*-chloro derivative **1**, showing the same orientation of both **7** and **9**, possessed an activity lower than that of the corresponding fluoro analogue **3**. This was accounted by the pharmacophoric model on the basis of the fact that the conformational rearrangement found for **7** and **9** with respect to **5** was also shown by **1** in comparison to **3**. As a consequence, concerning the latter compound, the fluoro being smaller than the chloro substituent, the phenyl ring at position 5 was able to fulfill RA2. Moreover, the remaining pharma-

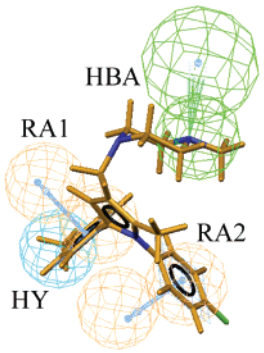


Figure 2. Superposition of compound **6** into the pharmacophoric model. Both the substituted phenyl rings follow a superposition pathway similar to that found for compound **5**. Moreover, the *N*-methylpiperazine ring adopts a conformation with its methylene moiety at position 1 in an axial conformation. However, such a disfavored conformer is the solely able to allow for the match of N4 into the HBA feature of the pharmacophore.

cophoric features were well-satisfied by the N1 *p*-fluorophenyl group (HY and RA1) and by the side chain at position 3, similar to that found for **5**, accounting for the comparable MIC values determined for **3** and **5** (0.5 and 0.4 μg/mL, respectively). As expected, the activity of **13** (0.5 μg/mL) was higher than that of the corresponding dichloro derivative **11** (2 μg/mL) and comparable to that of the monofluoro derivative **3** (0.5 μg/mL). Finally, the dichloro compound **11** showed an activity value slightly lower than that of the corresponding monochloro analogue **1** (4 μg/mL).

As already evidenced in our previous work, derivatives bearing a (4-methylpiperazin-1-yl)methyl moiety at position 3 of the pyrrole ring showed an activity toward *M. tuberculosis* lower than that of the corresponding (thiomorpholin-4-yl)methyl analogues. In particular, the *o*-chloro derivatives **1** and **15** showed better activity (a 2-fold difference) than **2** and **16**, respectively. A 16−32-fold difference was also found for the corresponding *o*-fluoro derivatives (compare **17** vs **18** and **3** vs **4**, respectively). In an attempt to rationalize these results, an analysis of the fitting mode of the (thiomorpholin-4-yl)methyl and (4-methylpiperazin-1-yl)methyl moieties allowed us to find the N4 of the piperazine as the hydrogen-bond acceptor feature, similar to the sulfur atom of the thiomorpholine ring. However, such a conformer was disfavored because it suffered from the fact that the methylene substituent at N1 was in an axial conformation (Figure 2). This result could, at least in part, account for the better activity usually found for thiomorpholine derivatives in comparison to the corresponding *N*-methylpiperazine analogues. However, as a consequence of the inability of the pharmacophoric model to fully explain the differences in activity usually found between the two classes of compounds, we are aware of the fact that a further refinement of the model is required to better account for the relationships between the structure of compounds and their antimycobacterial activity. Moreover, it is important to note that, by definition, the Catalyst software is unable to codify into a pharmacophoric model all the molecular determinants contributing to activity. As an example, the software does not take into account electronic effects possibly involved in defining the activity. On this basis, we cannot exclude that the pharmacophoric model is unable to fully explain the difference in activity of the two classes of compounds and that different variables, in addition to the fit to the pharmacophoric model, could account for the lower activity of *N*-methylpiperazine derivatives with respect to thiomorpholine compounds.

Reversing the substitution pattern at positions 1 and 5 of **1**−**14** led to compounds **15**−**28**. Among them, similar to that found for compounds **1**−**14**, thiomorpholine derivatives were more active than the corresponding *N*-methylpiperazine analogues. Moreover, an activity of **17** (0.5 μg/mL), identical to that found for **3**, was rationalized through the analysis of its superposition mode onto the pharmacophoric model. In fact, **17** showed an orientation reversed in comparison to that of **3**, with the substituent at position 5 (a *p*-fluorophenyl moiety) matching the HY−RA1 pharmacophoric portion, while the N1 phenyl ring was embedded into the RA2 feature. Despite the 180° rotation around the pyrrole ring, the substituent at position 3 was located in such a way that the thiomorpholine sulfur atom corresponded to the hydrogen-bond acceptor group. A similar orientation was found for **15**, characterized by an activity 4-fold lower than that of **17** (2 vs 0.5 μg/mL), in agreement with what was previously reported for **3** and **1**, respectively. Compound **19**, able to fulfill the whole pharmacophoric model as **5** did, showed an activity of 1 μg/mL, comparable to that of **5** (0.4 μg/mL). Moreover, compound **25**, bearing a 2,4-dichloro phenyl group at N1, was characterized by an activity very similar to that of the corresponding monochloro analogue **15** (1 vs 2 μg/mL), following the same trend found for **11** and **1**.

However, the activity of **27**, expected to be similar to that of **17**, was found to be 4-fold lower (2 vs 0.5 μg/mL), and it was impossible to be rationalized on the basis of suggestions only derived from the superposition to the pharmacophoric features.

Finally, the methyl group at position 2 of the pyrrole ring, located in empty regions of space with no contact involving the features of the pharmacophore, could be considered as a nonpharmacophoric portion of the antitubercular compounds reported, but it possibly contributed to define the mutual orientation (conformations) of substituents at both positions 1 and 3.

Additional calculations were performed in an attempt to rationalize the lower MIC values usually found for thiomorpholine derivatives in comparison to the corresponding *N*-methylpiperazine compounds. For this purpose, log *P* values of the new pyrrole derivatives were calculated with Cerius2 software[32] (Table 1) with the aim of evaluating if any correlation between antitubercular activity and lipophilicity of such compounds occurred. Results showed that log *P* values of the thiomorpholine derivatives were in all cases higher than those found for the corresponding *N*-methylpiperazine counterparts, supporting the hypothesis that a more hydrophobic character is preferred for the antitubercular potency of a compound (at least in vitro).[33] Accordingly, the analysis of log *P* values showed that compounds with MIC values in the range between 0.4 and 1 μg/mL showed high hydrophobicity (5.58−6.70). On the contrary, compounds with the lowest activity (namely, **4**, **8**, **10**, **20**, **22**, **24**, and **28**) were characterized by the lowest hydrophobicity values, their log *P* values ranging from 5.10 to 5.38.

Conclusion

We report here a series of novel 1,5-diarylpyrroles 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. Several of the new derivatives were found to have an in vitro activity toward *M. tuberculosis* better than that of the hit compound BM212, and comparable to that of isoniazid, streptomycin, and rifampicin, used as the reference compounds. In addition, they were also characterized by low cytotoxicity with a protection index up to 160, which was better

than that found for both isoniazid and streptomycin and comparable to that of rifampicin. Although the new compounds cannot yet be considered as potential clinical candidates, nevertheless, they are of great interest as hit structures in the search of new antitubercular agents.

Experimental Procedures

Chemistry. All chemicals used were of reagent grade. Yields refer to purified products and are not optimized. Melting points were determined in open capillaries on a Gallenkamp apparatus and are uncorrected. Microanalyses were carried out by means of a Perkin-Elmer 240C or a Perkin-Elmer Series II CHNS/O Analyzer 2400. Merck silica gel 60 (230−400 mesh) was used for column chromatography. Merck TLC plates, silica gel 60 F_{254} were used for TLC. Fluka aluminum oxide (activity II−III, according to Brockmann) was used for chromatographic purifications. Fluka Stratocrom aluminum oxide plates with fluorescent indicators were used for thin-layer chromatography (TLC) to check the purity of the compounds.

^{1}H NMR spectra were recorded with a Bruker AC 400 spectrometer in the indicated solvent (TMS as internal standard): the values of the chemical shifts are expressed in ppm and the coupling constants (*J*) in Hz. Mass spectra were recorded on either a Varian Saturn 3 spectrometer or a ThermoFinnigan LCQ-deca.

General Procedure for the Preparation of Pentane-1,4-diones 31a−h. These compounds were prepared according to the Stetter reaction as shown in Scheme 1, by reaction of suitable benzaldehyde, triethylamine, methyl vinyl ketone, and 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide. Briefly, a mixture of the appropriate benzaldehyde **29a−h** (0.09 mol), triethylamine (19.5 mL, 0.14 mol), methyl vinyl ketone **30** (5.8 mL, 0.07 mol), and 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide (3.53 g, 0.014 mol) was heated at 75−80 °C for 5 h under nitrogen and then cooled. The residue was treated with 2 N HCl (10 mL). After extraction with dichloromethane, the organic layer was washed with aqueous sodium hydrogen carbonate and water. The organic fractions were dried over Na_2SO_4, filtered, and concentrated to give a crude orange liquid. After chromatography on aluminum oxide (activity II−III, according to Brockmann) (hexane/ethyl acetate, 7:3 v/v), the desired compounds **31** were isolated as light yellow solids that, after recrystallization from cyclohexane, gave an analytical sample as white needles.

Examples. 1-(2,4-Difluorophenyl)pentane-1,4-dione (31g). Mp 41 °C (yield 45%), NMR ($CDCl_3$) 7.95 (m, 1H), 6.90 (m, 1H), 6.83 (m, 1H), 3.23 (m, 2H), 2.88 (t, 2H), 2.23 (s, 3H). Anal. ($C_{11}H_{10}F_2O_2$) C, H, F.

1-(2,4-Dichlorophenyl)pentane-1,4-dione (31f). Mp 37 °C (yield 40%), NMR ($CDCl_3$) 7.73 (m, 1H), 7.45 (m, 1H), 7.15 (m, 1H), 3.20 (m, 2H), 2.62 (t, 2H), 2.23 (s, 3H). Anal. ($C_{11}H_{10}Cl_2O_2$) C, H, Cl.

General Procedure for the Preparation of 1,5-Diarylpyrroles 32a−n. These compounds were prepared by means of the Paal−Knorr reaction as shown in Scheme 1, by condensing a 1,4-diketone with the appropriate amine. Briefly, a mixture of the proper diketone **31a−h** (2.28 mmol) and the suitable amine (2.28 mmol) in the presence of *p*-toluensulfonic acid (30 mg, 0.17 mmol) was heated at 100 °C for 5 h. The reaction mixture was cooled, filtered, and concentrated. The crude material was purified by chromatography with cyclohexane as the eluent to give, in satisfactory yield, the expected 1,5-diarylpyrrole as a solid. Recrystallization from cyclohexane gave the required product.

Examples. 1-(4-Fluorophenyl)-2-methyl-5-(2-chlorophenyl)-1*H*-pyrrole (32a). Mp 127 °C (yield 60%); ^{1}H NMR ($CDCl_3$) 7.26 (m, 2H), 7.12−7.05 (m, 4H), 6.98−6.95 (m, 2H), 6.30 (m, 1H), 6.13−6.12 (m, 1H), 2.14 (s, 3H). Anal. ($C_{17}H_{13}ClFN$) C, H, N, Cl, F.

1-(2-Methylphenyl)-2-methyl-5-(4-fluorophenyl)-1*H*-pyrrole (32l). Mp 170 °C (yield 65%); ^{1}H NMR ($CDCl_3$) 7.27−7.24 (m, 4H), 7.04−7.00 (m, 2H), 6.82−6.78 (m, 2H), 6.33 (m, 1H), 6.09−6.08 (m, 1H), 1.99 (m, 3H), 1.82 (s, 3H). Anal. ($C_{18}H_{16}FN$) C, H, N, F.

General Procedure for the Preparation of Compounds 1−28. These compounds were prepared by means of the Mannich reaction as shown in Scheme 1, starting from a suitable pyrrole and thiomorpholine or *N*-methylpiperazine in acetonitrile and acetic acid, in the presence of formaldehyde. In detail, to a stirred solution of an appropriate pyrrole **32a−n** (5.6 mmol), in acetonitrile (20 mL), a mixture of thiomorpholine (0.57 g, 5.6 mmol) or *N*-methylpiperazine (0.56 g, 5.6 mmol), formaldehyde (0.18 g, 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 8 h. The mixture was then treated with a solution of sodium hydroxide (20%, w/v) and extracted with ethyl acetate. The organic extracts were combined, washed with water, and dried. After the removal of solvent, the residue was purified by column chromatography, using aluminum oxide and ethyl acetate for (thiomorpholin-4-yl)methyl derivatives and chloroform for (4-methylpiperazin-1-yl)methyl derivatives. The eluates were combined after TLC control, and the solvent was removed to give **1−28** as solids in satisfactory yield. Recrystallization from diethyl ether gave the required products.

Examples. 1-(4-Fluorophenyl)-2-methyl-3-(thiomorpholin-4-yl)methyl-5-(2-chlorophenyl)-1*H*-pyrrole (1). Mp 95 °C (yield 83%); ^{1}H NMR ($CDCl_3$) 7.27−7.25 (m, 2 H), 7.10−7.04 (m, 4H), 7.03−6.92 (m, 2H), 6.28 (s, 1H), 3.49 (s, 2H), 2.80 (m, 4H), 2.72 (m, 4H), 2.08 (s, 3H). Anal. ($C_{22}H_{22}ClFN_2S$) C, H, N, S, Cl, F.

1-(2-Methylphenyl)-2-methyl-3-(thiomorpholin-4-yl)methyl-5-(4-fluorophenyl)-1*H*-pyrrole (23). Oil (yield 50%); ^{1}H NMR ($CDCl_3$) 7.26−7.23 (m, 4H), 7.00−6.98 (m, 2H), 6.80−6.75 (m, 2H), 6.31 (s, 1H), 3.51−3.44 (m, 2H), 2.76 (m, 4H), 2.70 (m, 4H), 1.96 (s, 3H), 1.90−1.77 (m, 3H). Anal. ($C_{23}H_{25}FN_2S$) C, H, N, S, F.

Microbiology. Compounds. Compounds **1−28** 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.[34] Growth media were Mueller−Hinton (Difco) containing 10% OADC (oleic acid, albumine, and dextrose complex) for *M. fortuitum* and Middlebrook 7H11 agar (Difco) with 10% ADC (albumine and dextrose complex) for *M. tuberculosis*. The substances were preliminarily screened at a single dose of 100 μg/mL. Compounds that resulted active in the preliminary test were assayed for detecting the minumum inhibitory concentration (MIC). Only compounds with MIC values of 16 μg/mL or less were then further studied for inhibitory activity against a variety of mycobacteria in Middlebrok 7H9 broth using the NCCLS procedure. The mycobacteria used for biological tests were *M. tuberculosis CIP 103471* and atypical mycobacteria, such as *M. marinum* CIP *6423*, *M. avium* CIP 103317, and *M. smegmatis* CIP 103599 (from the Institute Pasteur collection, CIP). Results are reported in Tables 1 and 2. For each compound, MIC values (expressed in μg/mL) were determined on each strain of mycobacteria.

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 compound 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 inoculated with test mycobacteria at a concentration of 10^5 to 10^6 colony forming units (CFU)/mL and incubated at 37 °C for 3−21 days. A control tube without any drug was included in each experiment. Isoniazid, streptomycin, rifampicin, and BM212 were used as controls.

Inhibitory Activity on Clinical Isolates of *M. tuberculosis*. Compounds **5** and **17** were also assayed toward a panel of 11 clinical isolates of *M. tuberculosis* in Middlebrook 7119 broth enriched with 10% ADC (Difco) using the macrodilution broth method.

Cytotoxic Activity Assays. Vero cells were inoculated in 6-well plates each containing 9×10^4 cells and incubated in Dulbecco's Minimum Essential Medium (DMEM) with 5% fetal calf serum

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

Computational Methods. All calculations and graphic manipulations were performed on a SGI Origin 300 server and SGI Octane2 workstations by means of the Catalyst 4.10 software package.[35]

All the compounds used in this study were built using the 2-D−3-D sketcher of Catalyst. A representative family of conformations was 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 a 20 kcal/mol range above the lowest energy conformation found.

The Compare/Fit command within Catalyst was used to align the studied compounds onto the pharmacophoric model. Particularly, the Best Fit option was selected, which manipulated 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 Alog *P*98 values of the studied compounds. Such a descriptor is an implementation of the atom type-based Alog *P* method using the latest published set of parameters.[36]

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Supporting Information Available: Experimental details (details of synthesis and elemental analysis data of compounds). This material is available free of charge via the Internet at http://pubs.acs.org.

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