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Laboratory note

Synthesis, antimycobacterial activities and cytotoxicity on V79 of 3-[4′-Y-(1,1′-biphenyl)-4-yl]-*N*,*N*-dimethyl-3-(4-X-phenyl)-2-propen-1-amine derivatives

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Abstract – The derivatives of 3-(4′-bromo-[1,1′-biphenyl]-4-yl)-3-(4-X-phenyl)-*N*,*N*-dimethyl-2-propen-1-amine (**5a**–**m**) were synthesised through a Friedel–Crafts acylation followed by Wittig reaction. The effects of the compounds on standard strains of *Mycobacterium* sp. (ATCC) and *M. tuberculosis* isolated from clinical specimens were evaluated. Also the toxicity was determined on V79 cells line using neutral red uptake (NRU), nucleic acid content (NAC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to measure the cellular viability. © 2001 Éditions scientifiques et médicales Elsevier SAS

synthesis / cytotoxicity / V79 cells / antimycobacterial activity / 2-propen-1-amine

1. Introduction

Tuberculosis caused by members of the *Mycobacterium tuberculosis* complex is an infectious disease that has damaged humanity since the beginning of the century. An extra complication was detected in the presence of multidrug-resistant or synergism with the human acquired immune deficiency syndrome (AIDS) epidemic. At this moment, there is no new drug generation able to eliminate the bacillus, and the World Health Organization has reported the increase in the number of infections and mortality [1]. In fact, mycobateria are known to easily acquire resistance to streptomycin, isoniazid, ethambuthol, pirazinamide and rifampicin, the most effective agents used in tuberculosis chemotherapy [2].

Moreover, these conventional antitubercular agents are also known to exhibit only restricted activity against nontuberculosis mycobacteria. AIDS is greatly accompanied by the development of opportunistic infections, particularly those involving the *Mycobacterium avium* complex. Also, it is very difficult to treat the infections caused by the *M. avium* complex because of its diversified drug resistance patterns to the majority of the antimycobacterial agents employed in clinics [3].

In a preliminary communication, we showed the antimycobacterial activity of the derivatives **5a** and **5c** in *figure 1* [4]. In this study, new derivatives **5i**–**m** were synthesized and the whole series (**5a**–**m**) (*figure 1*) were evaluated against several strains of mycobacteria. The antimycobacterial activities were evaluated through a microplate Alamar Blue assay [5] and the toxicity of the compounds **5j**–**m** was determined on V79 cells line [6].

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2. Chemistry

2-Propen-1-amine derivatives **5** were prepared by Friedel–Crafts reaction between 4-bromo-biphenyl and the corresponding benzoyl chloride to give the ketones **3a**–**m** and by subsequent Wittig reaction of the latter with β-(*N*,*N*-dimethylamino)ethyltriphenylphosphonium bromide (*figure 1*) [7].

3. Biology

The mycobacteria type strains and the *M. tuberculosis* isolated from clinical specimens were subcultured on Lowenstein–Jensen medium, and subcultured in Middlebrook 7H9 broth medium, until a bacterial density corresponding to a 1.0 McFarland turbidity standard. The suspensions were further diluted 1:25 in Middlebrook 7H9 broth medium before the inoculation (4×10^5 mycobacteria mL^{-1}) [4].

The cytotoxicity effect expressed as cell viability was assayed on a permanent lung fibroblast cell line derived from Chinese hamsters (V79). V79 fibroblasts were grown as monolayers in Dulbecco's modification of Eagle's medium (DMEM), supplemented with heat-inactivated foetal calf serum, penicillin and streptomycin in a humidified incubator with CO_2 in air. At the end of incubation, three independent endpoints for cytotoxicity were evaluated: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nucleic acid content (NAC) and neutral red uptake (NRU) [6].

4. Results and discussion

4.1. Chemistry

4.1.1. Synthesis

The compound **1i** was prepared in a 50 mL flask containing 7.2 mmol of 4-acetoxybenzoic acid; 27 mmol of thionyl chloride was added and the mixture was refluxed under stirring for 6 h. The excess thionyl chloride was removed by distillation under vacuum to recover the 4-acetoxybenzoic acid chloride (**1i**) [8]. The ketone **3i** was synthesized by a classical Friedel–Crafts reaction between the corresponding acid chloride (**1i**) and 4-bromobiphenyl (**2**) (*figure 1*). Briefly, the acid chloride **1a**–**i** (2 mmol) was added dropwise under agitation at room temperature to a solution containing 4-bromobiphenyl (1.72 mmol), $AlCl_3$ (3 mmol) and CS_2 (4 mL). The mixture was refluxed for 7 h and cooled to room temperature by addition of crushed ice. The solvent was distilled and the reaction product was extracted with CH_2Cl_2 (100 mL) and treated with saturated solution of Na_2CO_3 for **3i**. The organic layer was separated, dried over $MgSO_4$, filtered and evaporated under reduced pressure to give the solid crystalline product **3i**. The purification was performed through re-crystallisation with CH_2Cl_2–hexane. Compound **3m** was prepared using 1.36 mmol of **3g** and refluxed with 5.27 mmol of $AlCl_3$ and 7 mL of benzene for 4 h under stirring [9]. The reaction was stopped with water addition and acidified with HCl. The product **3m** was extracted with CH_2Cl_2 and purified by re-crystallisation with CH_2Cl_2–hexane. Compound **5i** was prepared in a flask containing β-dimethylamino ethyl triphenyl phos-

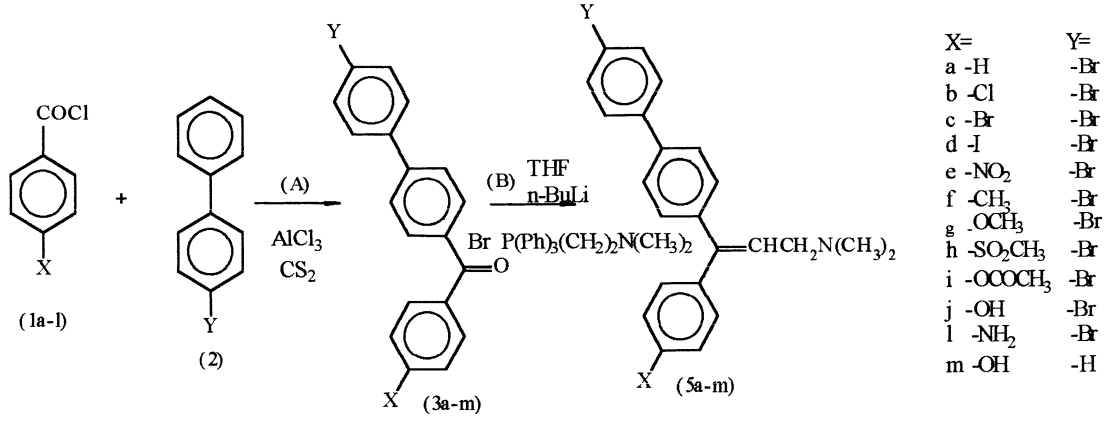

Figure 1. General procedure for the synthesis of compounds **5a**–**m**. (A) Friedel–Crafts reaction with reflux during 7 h; and (B) Wittig reaction overnight.

Table I. Qualitative analysis of bromine from the compound **3m** by EDXRF.

Element	Concentration (%)	Error
Al	1.149	0.0680
P	0.124	0.0103
Cl	0.038	0.0026
Ca	0.016	0.0005
Fe	0.001	0.0001
Cu	0.003	0.0001
Zn	0.001	0.0001
Br	0.641	0.0028
OCHN	98.028 (difference)	–

phonium bromide (2.15 mmol) and THF (25 mL) and a solution of *n*-butyllithium in 2.5 mmol hexane (2.21 mmol) 0 °C with stirring under argon atmosphere. After 30 min, the ketone **3i** was added to the mixture and the reaction left overnight at room temperature with stirring. The products **5i** was extracted with CH_2Cl_2 and purified by percolation in a silica gel column and by TLC using ethyl acetate–CH_3OH 5% as eluent. Compound **5j** was prepared with 0.4 mmol of **5i**, and refluxed for 4–5 h with 5 mL of potassium methoxide (Scheme 3). Compound **5j** was obtained through an extraction with CH_2Cl_2 in acid solution. The solvent was removed by distillation under vacuum and **5j** was purified by TLC using ethyl acetate–CH_3OH 5%–triethylamine 0.4% as eluent. Compound **5l** was prepared by reaction of 0.11 mmol of **5e**, 6 mmol of iron (Fe^0) and 25 mL of an alcoholic solution EtOH–HCl (2 mol L^{-1}), and refluxed for 6 h [10]. The solution was alkalinised to pH 8.3 with an alcoholic solution of KOH 15% and 38 μL of an alcoholic solution of H_2SO_4 (3 mol L^{-1}) was added. After filtration and distillation of the EtOH the organic product **5l** was extracted with CH_2Cl_2. The product was purified first by percolation in a silica gel column and by TLC in silica gel using ethyl acetate–CH_3OH 5% as eluent. Two other tentative approaches to synthesize **5l** were carried out by reduction of **5e** with $NaBH_2S_3$ [11] and by high-pressure hydrogenation in ethanolic solution for 30 min [12] with Pd/C 10%, but in both cases, the reactions were ineffective.

The structural data to the new compounds **3i** and **5i**–**m** were similar to those of **3a**–**h** and **5a**–**h**, respectively. In the ^{1}H-NMR spectra, there are peaks at the aromatic region associated with the hydrogen of the phenyl and biphenyl rings. The groups –CH and –CH_2 are responsible for the two triplets and two doublets due to the presence of the geometric isomers *E* and *Z* [7]. For the compounds **3i** and **5i,** there is a characteristic peak at δ 2.35 and 2.43 due to the hydrogens of their acetyl group and the compound **5l** showed a broad band at δ 3.81 due to the hydrogens of the amine group. For **3m,** the NMR showed the absence of the hydrogen of the methoxyl group (–OCH_3) (present in **3g**) and in the IR spectra, the band at 1256.4 cm^{-1} attributed to the –OCH_3 group of **3g** was absent and band at 3322.9 cm^{-1} was observed due to hydroxyl group of **3m**. Although the compound **3m** has been characterized by ^{1}H-NMR and MS, a further technique, EDXRF, was used in order to confirm the results. The MS showed the absence of the isotopes of bromine, which was present in **3g** (*table I*).

EDXRF showed that the bromine concentration in the sample of **3m** was insignificant as shown in *table I*. The elements OCHN represented 98.028% (by difference) of the composition of the sample (**3m**–$C_{19}H_{14}O_2$). If bromine is present in the molecule ($C_{19}H_{14}BrO_2$) it would be 22.6% and not 0.641 as detected. Probably, this insignificant percentage is related to a residue present in the sample.

The geometric isomers *E* and *Z* of the compounds **5a** and **5c** were prepared with a good yield as previously reported and were now evaluated on mycobacteria strains [14] (*table III*).

4.2. Biology

The susceptibility patterns of the *M. tuberculosis* isolated from clinical specimens are listed in *table II*. From the 12 tested strains only one resistant strain to INH (ALI 258), one resistant to INH and RFP (ALI 3082) and one resistant to INH, EMB, RFP and SM (3415) were found. The others strains were totally susceptible to the classical drugs used in tuberculosis chemotherapy.

One week after inoculation, the results from visually determined MICs were obtained using Alamar Blue dye which is a general indicator of cellular growth and/or viability; the blue, nonfluorescent, oxidized form, becomes pink and fluorescent upon reduction.

The antimycobacterial activities of the all compounds are summarised in *table III*. Compounds **5a** and **5c** with their respective geometric isomers showed good antimycobacterial activity against all the tested strains, as compared EMB, *table IV*), mainly on the isolates of clinical specimens. For the clinical specimens strains (ALI strains) studied, the compounds **5a**–**e** and **5j**–**m** showed an MIC between 4 and 49 μmol L^{-1}. Since the compound **5f** exhibited very low antimycobacterial activities

(around 68–79 μmol L^{-1}) and **5h** (around 34–68 μmol L^{-1}) on standard mycobacteria strains no measurements with the ALI strain were done. The values for **5g** and **5i**, which are similar to **5f**, are not presented in *table III*. It is interesting to note that the isomers **5aE**, **5aZ**, **5cE** and **5cZ** showed activities similar to that of the **5a** and **5c** showing that there is no advantage in the use of the isolated isomers, at least for the in vitro assays. *M. avium*, *M. malmoense*, *M. celatum* and *M. intracellulare* were less susceptible to the compounds which exhibited higher MIC values than the clinical specimen ones. This result was expected since nontuber-

Table II. Susceptibility patterns of clinical isolates of *M. tuberculosis* ($n = 12$).

Strains	Drugs and tested concentrations (μg mL^{-1})				
	PZA 200	INH 0.2	EMB 2	RFP 40	SM 4
ALI 3415	S	R	R	R	R
ALI 258	S	R	S	S	S
ALI 3082	S	R	S	R	S
ALI 3236	S	S	S	S	S
ALI 282	S	S	S	S	S
ALI 536	S	S	S	S	S
ALI 909	S	S	S	S	S
ALI 544	S	S	S	S	S
ALI 452	S	S	S	S	S
ALI 3342	S	S	S	S	S
ALI 552	S	S	S	S	S
ALI 329	S	S	S	S	S

ALI, Adolfo Lutz Institute; S, fully susceptible, R, resistant; PZA, pirazinamid; SM, streptomycin; EMB, ethambutol; INH, isoniazid; RFP, rifampicin.

Table III. MIC (μmol L^{-1}) of compounds **5a**–**m** against *M. tuberculosis* H37Ra (H37Ra), *M. tuberculosis* H37Rv (H37Rv), *M. kansasii* (Mk), *M. avium* (Ma), *M. malmoense* (Mm), *M. cellatum* (Mc), *M. intracellulare* (Mi) and strains of clinical specimens of *M. tuberculosis* (identified by numbers).

Strains	Compound													
	5a	**5aE**	**5aZ**	**5b**	**5cE**	**5cZ**	**5c**	**5d**	**5e**	**5f**	**5h**	**5j**	**5l**	**5m**
H37Ra	20.0	10.0	10.0	5.0	34.0	17.0	34.0	9.5	36.5	79.0	68.0	39.0	19.6	49.0
H37Rv	10.0	10.0	10.0	5.0	8.5	4.0	8.5	19.0	36.5	79.0	34.0	5.0	9.8	6.0
Mk	20.0	40.0	20.0	14.0	34.0	34.0	34.0	38.0	73.0	79.0	68.0	39.0	19.6	98.0
Ma	40.0	40.0	40.0	37.5	34.0	34.0	34.0	19.0	73.0	79.0	68.0	39.0	19.6	98.0
Mm	80.0	80.0	40.0	37.5	34.0	34.0	17.0	9.5	73.0	79.0	68.0	39.0	78.0	98.0
Mc	40.0	40.0	20.0	5.0	34.0	34.0	17.0	38.0	36.5	79.0	68.0	5.0	78.0	48.6
Mi	40.0	80.0	40.0	18.7	68.0	68.0	68.0	19.0	73.0	79.0	68.0	78.0	78.0	98.0
ALI 3415	40.0	40.0	40.0	ND	17.0	34.0	34.0	ND	36.5	ND	ND	ND	ND	49.0
ALI 282	≤5.0	≤5.0	≤5.0	≤5.0	≤4.0	≤4.0	≤4.0	≤4.0	18.5	ND	ND	ND	ND	49.0
ALI 536	≤5.0	≤5.0	≤5.0	≤5.0	≤4.0	≤4.0	≤4.0	≤4.0	18.5	ND	ND	ND	≤5.0	ND
ALI 909	10.0	20.0	20.0	ND	17.0	17.0	17.0	ND	ND	ND	ND	ND	39.0	ND
ALI 544	10.0	20.0	20.0	ND	34.0	17.0	17.0	ND	ND	ND	ND	ND	ND	ND
ALI 452	≤5.0	≤5.0	≤5.0	≤5.0	≤4.0	≤4.0	≤4.0	≤4.0	ND	ND	ND	ND	ND	ND
ALI 3342	ND	10.0	10.0	ND	8.5	8.5	8.5	ND	ND	ND	ND	ND	ND	ND
ALI 552	10.0	20.0	20.0	ND	17.0	17.0	17.0	ND	ND	ND	ND	ND	39.0	ND
ALI 258	≤5.0	≤5.0	≤5.0	≤5.0	8.5	8.5	≤4.0	≤4.0	ND	ND	ND	ND	19.5	ND
ALI 3082	20.0	20.0	40.0	ND	17.0	17.0	17.0	ND	ND	ND	ND	ND	ND	ND
ALI 329	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	≤5.0	ND
ALI 3236	20.0	ND	ND	ND	ND	ND	34.0	ND	ND	ND	ND	39.0	19.5	49.0

ND, not determined; ALI, Adolfo Lutz Institute.

Table IV. MIC ($\mu mol\ L^{-1}$) of classical drugs used in the chemotherapy of tuberculosis.

Strains	MIC ($\mu mol\ L^{-1}$)			
	INH	RFP	SM	EMB
M. tuberculosis H37Rv	≤0.22	≤0.036	≤0.086	14.4
M. tuberculosis H37Ra	≤0.22	≤0.036	≤0.086	3.6
M. avium	≥3.65	0.3	0.69	≥14.4
M. kansasii	≥3.65	≥0.6	≤1.37	>14.4
M. malmoense	>3.65	≥0.6	≤1.37	≥14.4
M. cellatum	>3.65	≥0.6	0.69	7.2

INH, isoniazid; RFP, rifampicin; SM, streptomycin; EMB, ethambutol.

Table V. Cytotoxicity of compounds **5a**–**m** on V79 cells.

Compound	IC_{50} ($\mu mol\ L^{-1}$)		
	NAC	NRU	MTT
5a	8.6 [a]	4.98 [a]	10.0 [a]
5b	10.0 [a]	6.32	12.0
5c	10.8 [a]	11.0 [a]	5.7 [a]
5d	24.39 [a]	35.45	>40
5e	9.2 [a]/8.2 [b]	5.8 [a]	7.5 [a]
5f	>25	>20	>25
5g	7.0 [a]	5.02	8.30
5h	48.0 [a]	20.0 [a]	40.0 [a]
5j	6.66	7.55	7.19
5l	25.51	28.97	28.03
5m	9.57	16.84	18.02
RFP [c]	200.0	470.0	360.0

[a] De Conti et al. (1998) [6].
[b] De Conti et al. (1996b) [13].
[c] SM, INH and EMB did not exhibit any cytotoxicity up to 1000 $\mu mol\ L^{-1}$ concentration.

culosis mycobacteria are less susceptible to the chemotherapy of tuberculosis.

In the case of H37Ra and H37Rv strains, the halogen at the *p*-position in the phenyl moiety of the compounds **5b**–**d** exerts a significant effect on their activity at lower IC_{50} values than the MIC values. Presumably, in this strain the amino group (**5l**) also exerts an efficient volume effect due to its molar refractivity (MR) value similar to the halogens [20]. Similar explanation for the *p*-OH group (**5j**) is proposed, since the MR value is slightly lower than the others mentioned before. In the cases of ALI-282, ALI-536, ALI-452 and 258 INH in which are involved the compounds **5a**–**d**, it is hard to explain using the same argument as before. Also, it is interesting to notice that **5c** (*p*-Br) and **5d** (*p*-I) are the most effective on the different mycobacteria strains and acted efficiently in almost all the tested strains, standard or from clinical specimens (H37Ra,, Ma, Mm, Mi, ALI-282, ALI-536l, ALI-452, ALI-3342 and ALI-258). Other comparison can be made between compounds **5j** and **5m**. Compound **5j** seems to be more effective with lower MIC than **5m**, which does not contain a bromine atom at the 4′-position of the biphenyl ring. Probably, the bromine gives a lipophilic character to the molecule increasing the ability of the drug to pass through the membrane and consequently there is an increase of the antimycobacterial activity.

In our study the reference drugs INH, RFP, SM and EMB were used on standard strains and the respective MIC are given in *table IV*. The MIC of **5a**–**m** are indicative of a moderated antimycobacterial activity if the data are compared with that of reference drugs, since the MIC doses are higher than those of INH, RFP, SM and EMB with some exceptions as discussed before.

The toxicity to mammalian cells V79 was determined for **5b**, **5d**, **5f**, **5g**, and **5j**–**m**. The results are expressed as MTT, NRU and NAC assays. In *table V* it is possible to observe the IC_{50} values of all the compounds (except compound **5i** which was not studied since it exhibited a low antimycobacterial activity) of the series obtained by extrapolation of the data. Compound **5j** was the most toxic of the series with lower IC_{50} values. However, compounds **5d**, **5h** and **5l** are less toxic to V79 cells with higher IC_{50} values than the others. Probably in this case, a combination of inductive (σI) and MR effects [19] are involved in the toxicity. In the reference drugs INH, SM and BEM did not show any cytotoxicity in V79 cells up to 1000 $\mu mol\ L^{-1}$, but RFP exhibited IC_{50} values of 200, 470 and 360 $\mu mol\ L^{-1}$ for NAC, NRU and MTT, respectively (*table V*).

5. Conclusions

The derivatives of 3-(4′-bromo-[1,1′-biphenyl]-4-yl)-3-(4-X-phenyl)-*N*,*N*-dimethyl-2-propen-1-amine (**5a**–**m**) were synthesised and showed a moderated antimycobacterial activity. Due to the wide range of activity in all the mycobacteria strains, compound **5c** was the best choice for the preparations of new formulations (free form, inclusion complex with β-cyclodextrin and liposomes) in order to improved its

effectiveness on intracellular mycobacteria (macrophage) or in infected animal. Also, improvements are required to obtain new derivatives of this compounds which able to achieve more effective antimycobacterial activity with lower toxicity to the mammalian cells.

6. Experimental protocols

6.1. Materials and methods

The chemicals used in this study were purchased from Aldrich–Sigma or Merck without further purification, except the solvent THF, which was treated with anhydrous calcium hydride, refluxed with metallic sodium and distilled.

The drugs pyrazinoic acid amide (PZA), isonicotinic acid hydrazide (INH), ethambuthol dihydrochloride (EMB), streptomycin sulfate (SM) were obtained from Sigma–Aldrich and Rifampicin (RFP) from Merck.

6.2. Chemistry

Reactions were monitored and compound purities were determined by TLC on UV-254 plates. Mass spectra (MS) were taken on a VG Autospec spectrometer under electron impact at an ionisation irradiation energy of 70 eV. The ratios m/z and the relative intensities corresponded to the calculated molecular weights of the compounds prepared. Nuclear magnetic resonance spectra of hydrogen and carbon (^{1}H-NMR and ^{13}C-NMR), spectra were recorded in a Gemini (Varian, USA) or on a Bruker apparatus at a working frequency of 300 or 500 MHz for ^{1}H and 75 or 125 MHz for ^{13}C in $CDCl_3$ using tetramethylsilane (TMS) as the internal standard (chemical shifts in δ, ppm). Analytical TLC was carried out on pre-coated plates (silica gel 60 F_{254}) and spots were visualised with UV light and in an I_2 chamber. IR spectra (KBr) were recorded on a Win-Bomem spectrophotometer (ν_{max} in cm^{-1}) and melting point in a Quimis apparatus. All the compounds were characterised by the above-mentioned techniques and the data for only the new compounds are listed since the data of **3a**–**h** and **5a**–**h** have been reported before [13]. Qualitative analysis of bromine was performed for **3m** by energy dispersive X-ray fluorescence (EDXRF) in a Tracor X-ray Model Spectrace 5000 (*table I*).

6.2.1. Compound *3i*

IR (KBr, cm^{-1}): 1744.1 (ν(C=O), $OCOCH_3$), 1642.5 (ν(C=O), ArCOAr). ^{1}H-NMR (300 MHz, $CDCl_3$/TMS, δ): 8.0–7.1 (m, 12H, Ar), 2.35 (s, 3H, $ArOCOCH_3$). ^{13}C-NMR (75 MHz, $CDCl_3$/TMS, δ): 195.07 (C=O), 168.77 (C=O), 144.02 (C), 138.80 (C), 136.45 (C), 135.21 (C), 132.12 (CH), 132.00 (CH), 131.92 (CH), 131.71 (CH), 130.74 (CH), 128.85 (CH), 128.77 (CH), 127.92 (CH), 126.84 (CH), 126.68 (C), 126.41 (C), 125.40 (CH), 122.64 (C), 122.14 (CH), 122.03 (CH), 121.75 (CH), 21.17 (CH_3). MS (m/z): 396/394 ([$M^{+\bullet}$], 22), 354/352 (73), 261/259 (20), 152 (53), 121 (100), 93 (8), 65 (8). M.p. 185–195 °C; UV (nm, CH_2Cl_2) $\lambda = 254$. Yield 80%.

6.2.2. Compound *3m*

IR (KBr, cm^{-1}): 3322.9 (ν(–OH)), 1644.1 (ν(C=O) ArCOAr). ^{1}H-NMR (300 MHz, $CDCl_3$/TMS, δ): 8.35–7.31 (m, 13 H, Ar). MS (m/z) 274 ([$M^{+\bullet}$], 100), 181 (79), 152 (44), 121 (94). M.p. 161 °C. Yield ~100%.

6.2.3. Compound *5i*

IR (film, cm^{-1}) 1752 (ν(C=O), $ArOCOCH_3$). ^{1}H-NMR (300 MHz, $CDCl_3$/TMS, δ): 7.8–6.8 (m, 24H Ar, E/Z), 6.33 (1t, 1H, –CH, E/Z, $J = 6.96$ Hz), 6.30 (1t, 1H, –CH, E/Z, $J = 6.96$ Hz), 6.28 (1t, 1H, –CH, E/Z, $J = 6.96$ Hz), 3.45 (1d, 2H, $–CH_2$, E/Z, $J = 7.32$ Hz), 3.32 (1d, 2H, $–CH_2$, E/Z, $J = 6.96$ Hz), 2.43 (s, 6H, CH_3 $–OCOCH_3$, E/Z), 2.30 (s, 12H, $–N(CH_3)_2$, E/Z). MS (m/z): 451/449 ([$M^{+\bullet}$], 27), 436 (8), 406 (5), 363 (13), 363 (13), 329 (41), 314 (92), 58 (100). Yield 40%.

6.2.4. Compound *5j*

IR (film, CH_2Cl_2, cm^{-1}) 3322 (ν(ArOH)). ^{1}H-NMR (300 MHz, $CDCl_3$/TMS, δ): 7.54–6.64 (m, 24H Ar, E/Z), 6.13 (1t, 1H, –CH, E/Z, $J = 6.96$ Hz) 6.00 (1t, 1H, –CH, E/Z, $J = 6.96$ Hz), 3.12 (1d, 2H, $–CH_2$, E/Z, $J = 6.96$ Hz); 3.07 (1d, 2H, $–CH_2$, E/Z, $J = 6.96$ Hz), 2.27 (s, 12H $–N(CH_3)_2$, E/Z). MS (m/z): 409/407 ([$M^{+\bullet}$], 100), 394/392 (34), 365/363 (41), 176 (47), 107 (29), 70 (46), 58 (63). Yield 40%.

6.2.5. Compound *5l*

IR (film, CH_2Cl_2, cm^{-1}): 3054 (ν(N–H)), 1265 (ν(C–N)). ^{1}H-NMR (300 MHz, $CDCl_3$/TMS, δ): 7.60–6.59 (m, 24H Ar, E/Z), 6.28 (1t, 1H, –CH, E/Z, $J =$ 6.96 Hz), 6.22 (1t, 1H, –CH, E/Z, $J = 7.14$ Hz), 3.81 (s, 4H $–NH_2$, E/Z), 3.43 (1d, 2H, $–CH_2$ E/Z, $J = 6.96$ Hz), 3.39 (1d, 2H, $–CH_2$, E/Z, $J = 7.32$ Hz), 2.49 (s, 12H $–N(CH_3)_2$, E/Z). MS (m/z) 408/406 ([$M^{+\bullet}$] 100), 393/391 (50), 130 (34), 106 (55), 70 (30), 58 (55). Yield 30%.

*6.2.6. Compound **5m***

IR (film, CH_2Cl_2, cm^{-1}): 3322 (ν(ArOH)). ^{1}H-NMR (500 MHz, $CDCl_3$/TMS, δ): 7.86 (s, 2H –OH, *E*/*Z*), 7.53–6.7 (m, 26H, Ar, *E*/*Z*), 6.14 (1t, 1H, –CH, *E*/*Z*, $J = 6.96$ Hz), 6.05 (1t, 1H, –CH, *E*/*Z*, $J = 6.96$ Hz), 3.28 (1d, 2H, $-CH_2$, *E*/*Z*, $J = 6.84$ Hz), 3.25 (1d, 2H, $-CH_2$, *E*/*Z*, $J = 6.84$ Hz), 2.34 (s, 12H, $-N(CH_3)_2$, *E*/*Z*). ^{13}C-NMR (500 MHz, $CDCl_3$/TMS, δ): 157.46 (C), 157.82 (C), 146.71 (C), 146.39 (C), 140.36 (C), 140.22 (C), 140.21 (C), 140.14 (C), 140.03 (C), 137.74 (C), 132.00 (CH), 130.64 (CH), 129.79 (CH), 128.56 (CH), 128.55 (CH), 128.49 (CH), 127.73 (CH), 127.20 (CH), 127.06 (CH), 126.74 (CH), 126.71 (CH), 126.66 (CH), 126.54 (CH), 120.08 (C), 118.33 (C), 115.53 (CH), 115.50 (CH), 57.23 (CH_3), 56.95 (CH_3), 43.48 (CH_2), 43.43 (CH_2). MS (*m*/*z*): 329 ([$M^{+\bullet}$], 100), 314 (41), 285 (50), 176 (29), 107 (30), 70 (26), 58 (45). Yield 80%.

*6.2.7. Isolation of the isomers from compounds **5a** and **5c***

The geometric isomers *E* and *Z* of **5a** were isolated with preparative TLC on silica gel 2–25 μm (Aldrich) using ethyl acetate–hexane–NH_4OH (79:20:1) as eluent. For the geometric isomers *E* and *Z* of **5c**, a HPLC separation was achieved by a Waters 600E Millipore model with Hi-Chrom preparative HPLC column, Spherisorb S5W 5μ 25 cm×10 mm using ethyl acetate–C_6H_{14}–TEA (79:20:1) as eluent [14].

6.3. Biological assays: antimicrobial susceptibility testing

The type strains of *M. tuberculosis* H37Rv ATCC 27294, *M. tuberculosis* H37Ra ATCC 25177, *M. avium* ATCC 15769, *M. kansasii* ATCC 12478, *M. intracellulare* ATCC 25169 and *M. malmoense* ATCC 29571 were kindly provided by Dr Robert G. Cooksey of the Center for Disease Control and Prevention—CDC (Atlanta, USA) and the *M. tuberculosis* isolated from clinical specimens were obtained from patients with tuberculosis at Adolfo Lutz Institute and enumerated (ALI, Ribeirão Preto, SP, Brazil). All isolates were grown on Loweinstein–Jensen medium and were examined for growth rate, microscopy colony morphology and pigmentation. For identification, they were submitted to biochemical tests for niacin production, nitrate reduction, catalase, arylsulphatase, pyrazinamidase, urease and lipase (Tween 80 hydrolysis) [15].

The *M. tuberculosis* strains isolated from clinic specimens were classified as sensitive or resistant to the drugs PZA, INH, EMB, RFP and SM by the proportion method using Lowenstein–Jensen medium [16]. The critical concentration of the drugs were 200 μg mL^{-1} for PZA; 0.2 μg mL^{-1} for INH; 2 μg mL^{-1} for EMB; 40 μg mL^{-1} for RFP and 4 μg mL^{-1} for SM. The stock solutions were prepared in water 10 mg L^{-1} (except for RFP which was prepared in methanol) and diluted in Loweinstein–Jensen medium before solidification. The isolates were considered resistant if there was a growth on the medium containing drug more than 1% for INH, RFP and EMB and 10% for PZA and SM compared with the growth on the drug-free medium (*table II*).

The mycobacteria type strains and the *M. tuberculosis* isolated from clinical specimens were subcultured in Lowenstein–Jensen medium at 37 °C for 3 weeks and subcultured in Middlebrook 7H9 broth medium at 37 °C for 10 days, until a bacterial density corresponding to a 1.0 McFarland turbidity standard. The suspensions were further diluted 1:25 in Middlebrook 7H9 broth medium before the inoculation (4×10^5 mycobacteria mL^{-1}).

6.4. Stock solutions

Stock solutions of compounds **5a**–**m** were prepared in DMSO at 1 g L^{-1} concentration and the dilutions were performed in Middlebrook 7H9 broth medium to obtain 100 μl of dilution in each well. The drugs INH, RFP, SM and EMB were used as references and the tests were performed by the microplate Alamar Blue assay as previous reported [5]. The visual MICs were defined as the lowest drug concentration that prevented a colour change from blue to pink.

6.5. Cytotoxicity to mammalian cells

The toxicities of the compounds were determined on V79 cells (Chinese hamster lung fibroblast generously supplied by Dr R. Meneghini from the Universidade de São Paulo, Brazil) by a multi-endpoints cytotoxicity method [6]. The cytotoxicity effect expressed as cell viability was assayed on a permanent lung fibroblast cell line derived from Chinese hamsters (V79). V79 fibroblasts were grown as monolayers in Dulbecco's modification of Eagle's medium (DMEM), supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. Cells were plated at density of 3×10^4 cell mL^{-1} in 24- or 96-well plates. The medium was removed 48 h after cell seeding and replaced with medium containing the com-

dissolved in DMSO and then diluted in DMEM. Cells were exposed for 24 h to test medium with or without the drugs (control). Each drug concentration was tested in triplicates and the assays repeated three times in separate experiments. At the end of incubation, three independent endpoints for cytotoxicity were evaluated: MTT, NAC and NRU.

6.5.1. NAC

Cell number in control and treated wells was estimated from their total nucleic acid content according to Cingi et al. [17]. Cells were washed twice with cold phosphate buffered saline (PBS) and a soluble nucleotide pool was extracted with cold ethanol. The cell monolayers were then dissolved in 1 or 0.1 mL of NaOH (0.5 mol L^{-1}) and incubated at 37 °C for 1 h and the absorbance read at 260 nm. Results are expressed by a comparison of absorbance between cells treated with drugs and controls (no drugs).

6.5.2. MTT

The tetrazolium reduction assay was performed according to the method of Denizot and Lang [18]. Briefly, cells were washed once with phosphate buffered saline (PBS) and 0.1 mL of serum-free medium containing MTT (1 mg mL^{-1}) was added to each well. After incubation for 4 h, the supernatant was removed and the blue formazan product obtained was dissolved in 0.1 or 1 mL of ethanol with stirring for 15 min on a microtitre plate shaker and the absorbance was read at 570 nm.

6.5.3. NRU

The neutral red uptake assay was performed according to the method of Borenfreund and Puerner [19]. After 4 h of incubation with serum-free medium containing neutral red (50 μg mL^{-1}), the cells were washed quickly with PBS–Ca^{2+} and then 0.1 mL of a solution of 1% (v/v) acetic acid in 50% (v/v) ethanol was added to each well to extract the dye. After agitation on a microtitre plate shaker the absorbance was read at 540 nm.

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