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Short communication

Synthesis and antimycobacterial activity of some coupling products from 4-aminobenzoic acid hydrazones

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Abstract – Various 2,3,4-pentanetrione-3-[4-[[(5-nitro-2-furyl/pyridyl/substituted phenyl)methylene]hydrazinocarbonyl]phenyl]hydrazones **3a–j** were synthesized by the reactions of acetylacetone with the diazonium salts of 4-aminobenzoic acid-[(5-nitro-2-furyl/pyridyl/substituted phenyl)methylene]hydrazides **2a–j** at 0–5 °C. The structures of these compounds were determined using spectral data. All the synthesized compounds were evaluated for their antimycobacterial activity against *Mycobacterium fortuitum* ATCC 6841 and *Mycobacterium tuberculosis* H37Rv. Of the compounds screened, **2e**, **2i**, **3e** and **3g** were found to be active against *M. fortuitum* at an MIC value of 32 μg/mL. Compound **3a**, which exhibited > 90% inhibition in the primary screen at 12.5 μg/mL against *M. tuberculosis* H37Rv, was the most promising derivative for antituberculosis activity. Results obtained from the level II screening showed that the actual MIC and IC_{50} values of **3a** were 3.13 and 0.32 μg/mL, respectively. The same compound was also tested against *Mycobacterium avium*, which was observed not to be susceptible to **3a**. © 1999 Éditions scientifiques et médicales Elsevier SAS

hydrazide-hydrazones / coupling products / antimycobacterial activity / cytotoxicity

1. Introduction

Antituberculosis and antibacterial effects have been shown with various 4-aminobenzoic acid substituted benzalhydrazones [1]. Furthermore, the coupling products starting from diazonium salts of 4-aminobenzoic acid hydrazide substituted benzalhydrazones with indole have also been reported to possess promising antibacterial and antitubercular activities [2]. These observations prompted us to synthesize some novel coupling products of 4-aminobenzoic acid hydrazide substituted benzalhydrazone derivatives.

Earlier reports indicated that certain compounds bearing aromatic amine functions, such as sulfaguanidine [3], PABA [4], benzocaine [4], sulfanilamide [5], 4-aminobenzoic acid hydrazide [6], oxadiazole [7, 8], thiadiazole [7], quinazolinone [7], 4-aminobenzoic acid hydrazide substituted benzalhydrazones [2], substituted anilines [9, 10], 1,2,4-triazoline-3(2H)-thiones [11, 12], 1,2,4-triazoline-3(2H)-one [12], 1,3,4-oxadiazoline-2(3H)-thione [11, 12], 3,5-dimethyl-1H-pyrazole [11] and 4-aminobenzoic acid-[(4-fluorophenyl)methylene]-hydrazide [12] might be selected to couple their diazonium salts with compounds possessing an active hydrogen.

2. Chemistry

4-Aminobenzoic acid hydrazide **1** was prepared by the reaction of ethyl 4-aminobenzoate with hydrazine hydrate. 4-Aminobenzoic acid-[(5-nitro-2-furyl/pyridyl/substituted phenyl)methylene]hydrazides **2a–j** were synthesized by condensation of **1** with appropriate aldehydes [13] as original compounds, except **2a** [14] and **2c** [15], which were previously reported elsewhere. The diazonium salts of 4-aminobenzoic acid-[(5-nitro-2-furyl/pyridyl/substituted phenyl)methylene]hydrazide were then coupled with acetylacetone in ethanol (50%) con-

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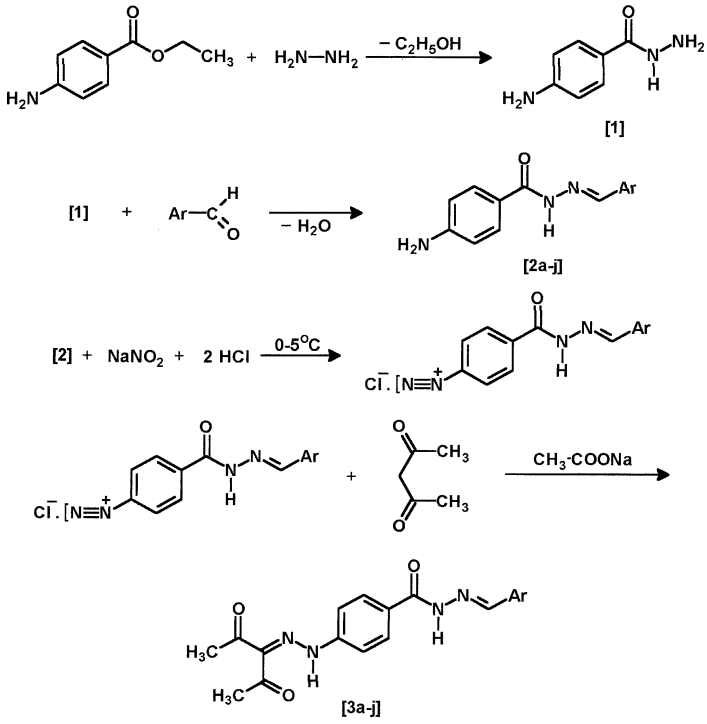


Figure 1. Synthetic route to compounds **3a–j**.

taining sodium acetate. The resulting coupling products were formulated as 2,3,4-pentanetrione-3-[4-[[(5-nitro-2-furyl/pyridyl/substituted phenyl)methylene]hydrazinocarbonyl]phenyl]hydrazones **3a–j** in view of spectral findings (*figure 1*). Spectral investigations also revealed that the aromatic primary amine function present at **1** was not affected during the synthesis of **2a–j**, although no protective precautions were taken, probably because the -NH_2 moiety of hydrazide function is more nucleophilic than the Ar-NH_2 group.

The novel compounds **3a–j** gave satisfactory elemental analyses. Their structures were established using spectral data (UV, IR, ^{1}H-NMR and EI-MS).

UV spectra of the synthesized compounds showed three absorption maxima at 236–249, 265–303 and 369–402 nm values. The lack of absorption bands between 332–360 nm [16, 17] and above 400 nm [18], which could be attributed to an azo function, together with observation of bands at around 369–402 nm, indicated that compounds **3a–j** were in a hydrazone form [19].

The IR spectra of **3a–j** exhibited the N–H band of hydrazone, the C=O band of ketone and hydrazone arising from stretching vibrations at expected wavenumbers according to the literature [3, 4].

In the ^{1}H-NMR spectra, methyl protons of the ketone moiety gave singlets at 2.52, 2.62 ppm ($CDCl_3$, only for compound **3b**) or 2.42–2.46 ppm (DMSO-d_6, for compounds **3a**, **3c–j**). The azomethine proton (-CH=N-) and NH proton of the hydrazide hydrazone N_1–H (-CONHN=CH-) exhibited the expected singlets at 8.32–8.71 ppm and 11.49–13.52 ppm, respectively. Unlike the others, which were dissolved in DMSO-d_6, the N_1–H proton of compound **3b** resonated at 9.09 ppm, probably due to the use of $CDCl_3$ as solvent [20]. The ^{1}H-NMR spectra of **3b** in $CDCl_3$ and **3c–d** and **3f–j** in DMSO-d_6 displayed the hydrazone N_2–H(-CH=N–NH-) protons at 13.71–14.58 ppm. The hydrazone N–H protons of **3a** and **3e** were observed to exchange with deuterium in DMSO-d_6 [12]. In addition, in the ^{1}H-NMR spectra of compounds **3a–j**, signals arising from the >CH–N=N- structure at 3.00–4.00 ppm [3, 4] were not observed. This finding also supported the idea that the structures of these compounds might be given in hydrazone form. The phenolic proton of compound **3g** displayed a singlet at 9.69 ppm.

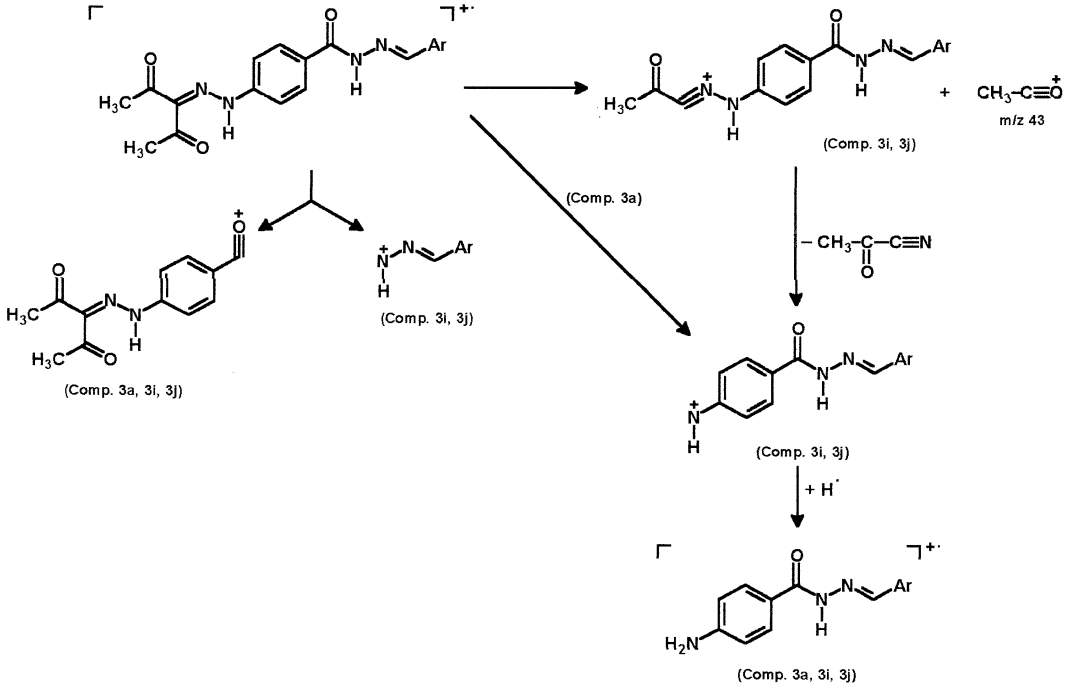


Figure 2. Mass fragmentation pattern of **3a**, **3i** and **3j**.

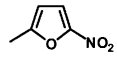
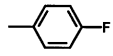
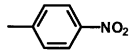
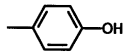
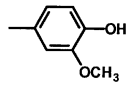
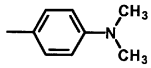
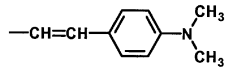
EI-Mass spectra of three selected prototypes, **3a**, **3i** and **3j** confirmed their molecular weights and displayed characteristic fragment ions as shown in *figure 2*. The major fragmentation pathway appeared by the cleavage of -CONH-N= bonds of a hydrazide-hydrazone moiety. Initial loss of an acetyl fragment (m/z 43) followed by expulsion of CH_3–CO–CN from the molecular ion and the subsequent acquirement of a hydrogen radical afforded the fragment ions at m/z 282 and 308 for compounds **3i** and **3j**, respectively. The same type of fragment ion also appeared at m/z 274 in the EI-mass spectrum of compound **3a** by the cleavage of >C=N–NH- bonds of hydrazone followed by protonation. This latter ion exhibited the expected fragmentation pattern of a hydrazide-hydrazone structure [13].

3. Results and discussion

The synthesized compounds **2a–j** and **3a–j** were evaluated for in vitro antimycobacterial activity against *M. fortuitum* ATCC 6841 using the microdilution method [21–24]. Of the screened compounds, **2e** and the related coupling product **3e**, compounds **2i** and **3g** were found as active as tobramycin against the examined strain. The remaining compounds showed less activity (64 μg/mL) or no considerable effect (> 128 μg/mL) as shown in *table I*.

Compounds **2a–j** and **3a–j** were also tested for in vitro antituberculosis activity against *M. tuberculosis* H37Rv using the BACTEC 460 radiometric system [25, 26]. Rifampicin was used as the standard in the tests. Primary antituberculosis activity screening results of these compounds can be seen in *table II*. Compounds **2b**, **2i**, **3e** and **3h** were inactive against *M. tuberculosis* H37Rv, whereas remaining compounds showed varying degrees of inhibition in the primary screen. The compounds which exhibited < 90% inhibition in the primary screen (MIC > 12.5 μg/mL) were not evaluated further. Compound **3a** effecting > 90% inhibition in the primary screen at 12.5 μg/mL was re-tested at lower concentrations against *M. tuberculosis* H37Rv to determine the actual minimum inhibitory concentration in a broth microdilution assay using alamar blue. The MIC was defined as the lowest concentration inhibiting 99% of the inoculum. Compound **3a** was also tested against *M. avium*, a naturally drug-resistant opportunistic pathogen, using the same technique. Clarithromycin was used as the standard in this assay. However, no inhibition was observed with

Table I. Antimycobacterial activity of **2a–j** and **3a–j**.

Compound	MIC values (μg/mL) *M. fortuitum* ATCC 6841	Ar
2a	64	
3a	64	
2b	64	
3b	64	
2c	64	
3c	64	
2d	64	
3d	> 128	
2e	32	
3e	32	
2f	> 128	
3f	> 128	
2g	64	
3g	32	
2h	64	
3h	> 128	
2i	32	
3i	> 128	
2j	64	
3j	> 128	
Tobramycin	32	

compound **3a** at 12.5 μg/mL, whereas clarithromycin exhibited 98% inhibition at 2 μg/mL. Level II assay results of **3a** are given in *table III*. Compound **3a** was found to be active against *M. tuberculosis* H37Rv at 3.13 μg/mL. The same compound was also tested for cytotoxicity (IC_{50}) in VERO cells at concentrations equal to and greater than the MIC for *M. tuberculosis* H37Rv. The IC_{50} value was found at a concentration level of 0.32 μg/mL for compound **3a**. The selectivity index (SI = IC_{50}/MIC) was calculated as 0.1, showing that this compound not only displayed a considerable antituberculosis activity, but also had a remarkable cytotoxicity.

Compounds **2a–j**, whose synthesis was previously reported [13], was the starting point of our new work in order to obtain new coupling products in which aromatic primary amine functions were masked. The aim of this study was not only to screen the antimycobacterial activity of **2a–j** and the resulting **3a–j**, but also to observe the influence of coupling **2a–j** with ethyl acetoacetate on this activity. As a matter of fact, series **3a–j** was found to be more active against *M. tuberculosis* H37Rv than the corresponding **2a–j** in most cases, as shown in *table II*. Even in the case of **3a**, in which activity possibly arises from the presence of the 5-nitro-2-furanyl moiety, the coupling product **3a** was found to be more active, with 95% inhibition (MIC = 3.13 μg/mL), than the corresponding amine **2a** which inhibited the growth of *M. tuberculosis* H37Rv by only 70%. From the above data it can be concluded that **3a** could be a leading compound for further development. Structural modifications, in which ketone functions present in **3a–j** are replaced with different moieties in order to minimize toxicological effects, will be the subject of our continuing studies.

4. Experimental protocols

4.1. Chemistry

Benzocaine, 4-dimethylamino benzaldehyde and acetylacetone were purchased from Merck. All other chemicals were purchased from Sigma.

Melting points were determined using a Büchi-530 melting point apparatus, uncorrected. IR spectra: (KBr, ν, cm^{-1}) Perkin-Elmer FTIR 1600 spectrophotometer. UV Spectra: (ethanol) λ_{max} (log ε) Shimadzu UV 2100S spectrophotometer. ^{1}H-NMR: (DMSO-d_6, $CDCl_3$, TMS as internal standard, chemical shifts, δ, in ppm) Brüker AC 200 L spectrometer. EI-MS: Kratos MS-9/50 spectrometer (for compounds **3i** and **3j**) and ZabSpec EI^+ Magnet (for compound **3a**) at 70 eV. Elemental analyses: Carlo-Erba 1106 instrument.

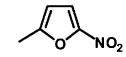
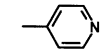
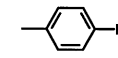
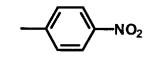
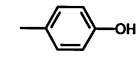
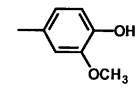
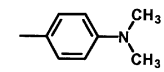
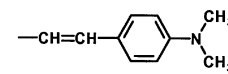
4.1.1. Preparation of aromatic primary amines **2a–j**

Compounds **2a–j** were prepared as described previously [13–15]. M.p.'s: for compound **2a** (271–274 °C, lit. [14] 273–274 °C); for compound **2c** (218 °C, lit. [15] 218–219 °C).

4.1.2. Synthesis of the coupling products **3a–j**

To a cooled solution of compounds **2a–j** (0.01 mol), in 2 mL of hydrochloric acid (37%), an ice-cold solution of 10 mL of sodium nitrite (10%) was added. The reaction mixture was then poured into a mixture of 1 mL of acetylacetone and 50 g of sodium acetate in ethanol (50%) by vigorous stirring. This mixture was allowed to stand in a refrigerator for 24 h. Precipitated solid was collected, washed with water, dried and washed with an appropriate solvent to give **3a–j**.

Table II. Primary antituberculosis activity screen results of **2a–j** and **3a–j**.

Compound	MIC values (μg/mL) *M. tuberculosis* H37Rv	Inhibition (%)	Ar
2a	> 12.5	70	
3a	< 12.5	95	
2b	> 12.5	–	
3b	> 12.5	16	
2c	> 12.5	16	
3c	> 12.5	35	
2d	> 12.5	6	
3d	> 12.5	20	
2e	> 12.5	11	
3e	> 12.5	–	
2f	> 12.5	23	
3f	> 12.5	13	
2g	> 12.5	21	
3g	> 12.5	52	
2h	> 12.5	26	
3h	> 12.5	–	
2i	> 12.5	–	
3i	> 12.5	25	
2j	> 12.5	56	
3j	> 12.5	76	
Rifampicin	0.25	98	

4.1.2.1. 2,3,4-pentanetrione-3-[4-[[(5-nitro-2-furyl)-methylene]hydrazinocarbonyl]phenyl]hydrazone **3a**

Yield: 65%, m.p. 230 °C (methanol). UV (ethanol): λ_{max} (log ε) = 303 (4.1611), 380 (4.5789); IR (KBr): ν = 3 450–3 420 cm^{-1} (H_2O), 3 331 (NH), 1 672 (C=O, ketone), 1 625 (C=O, hydrazone); EI-MS (70 eV): m/z (%) = 385 (14.15) [M^+], 274 (55.00), 273 (8.41), 262 (22.41), 259 (8.64), 244 (17.14), 231 (4.33), 219 (3.00), 206 (2.50), 120 (33.74), 92 (7.44), 78 (90.77), 65 (3.88), 63 (100); ^{1}H-NMR (DMSO-d_6): δ (ppm) = 2.46 (s, 6H, $COCH_3$), 7.64–7.99 (m, 6H, Ar-H), 8.39 (s, 1H, CH=N), 11.90–12.11 (s, 1H, -CONHN=CH-). Analysis: $C_{17}H_{15}N_5O_6.H_2O$ (% calculated/found) 50.62/50.21 (C); 4.24/3.54 (H); 17.36/18.15 (N).

Table III. Level II antituberculosis activity assay result of **3a**.

Compound	MIC value (μg/mL) *M. tuberculosis* H37Rv	IC_{50} (μg/mL)	SI (IC_{50}/MIC)
3a	3.13	0.32	0.1
Rifampicin	0.125		

4.1.2.2. 2,3,4-pentanetrione-3-[4-[[(4-pyridyl)methylene]hydrazinocarbonyl]phenyl]hydrazone **3b**

Yield: 48%, m.p. 246 °C (ethanol). UV (ethanol): λ_{max} (log ε) = 241 (4.1912), 295 (4.3144), 369 (4.6061); IR (KBr): ν = 3 200 cm^{-1} (NH), 1 690 (C=O, ketone), 1 650 (C=O, hydrazone); ^{1}H-NMR ($CDCl_3$): δ (ppm) = 2.52 (s, 3H, $COCH_3$), 2.62 (s, 3H, $COCH_3$), 7.47–8.01 (m, 8H, Ar-H), 8.67 (s, 1H, CH=N), 9.09 (s, 1H, -CONHN=CH-), 14.58 (s, 1H, -CH=N–NH-). Analysis: $C_{18}H_{17}N_5O_3$ (% calculated/found): 61.53/61.05 (C); 4.84/4.73 (H); 19.93/19.68 (N).

4.1.2.3. 2,3,4-pentanetrione-3-[4-[[(phenyl)methylene]-hydrazinocarbonyl]phenyl]hydrazone **3c**

Yield: 75%, m.p. 199–204 °C (ethanol). UV (ethanol) λ_{max} (log ε) = 290 (4.2762), 369 (4.5297); IR (KBr): ν = 3 260 cm^{-1} (NH), 1 684 (C=O, ketone), 1 642 (C=O, hydrazone); ^{1}H-NMR (DMSO-d_6): δ (ppm) = 2.46 (s, 6H, $COCH_3$), 7.27–8.11 (m, 9H, Ar-H), 8.46 (s, 1H, CH=N), 11.74 (s, 1H, -CONHN=CH-), 13.71 (s, 1H, -CH=N–NH-). Analysis: $C_{19}H_{18}N_4O_3$ (% calculated/found): 65.13/64.30 (C); 5.18/5.20 (H); 15.99/15.41(N).

4.1.2.4. 2,3,4-pentanetrione-3-[4-[[(2-fluorophenyl)-methylene]hydrazinocarbonyl]phenyl]hydrazone **3d**

Yield: 67%, m.p. 214 °C (ethanol). UV (ethanol): λ_{max} (log ε) = 233 (4.1269), 297 (4.3132), 371 (4.6158); IR (KBr): ν = 3 450–3 400 cm^{-1} (H_2O), 3 200 (NH), 1 680 (C=O, ketone), 1 625 (C=O, hydrazone); ^{1}H-NMR (DMSO-d_6): δ (ppm) = 2.46 (s, 6H, $COCH_3$), 7.28–8.00 (m, 8H, Ar-H), 8.71 (s, 1H, CH=N), 11.69 (s, 1H, -CONHN=CH-), 13.76 (s, 1H, -CH=N–NH-). Analysis: $C_{19}H_{17}FN_4O_3{\cdot}H_2O$ (% calculated/found): 59.06/59.15 (C); 4.95/4.82 (H); 14.50/15.02 (N).

4.1.2.5. 2,3,4-pentanetrione-3-[4-[[(4-fluorophenyl)-methylene]hydrazinocarbonyl]phenyl]hydrazone **3e**

Yield: 41%, m.p. 218 °C (ethanol). UV (ethanol): λ_{max} (log ε) = 285 (4.5454), 371 (4.8213); IR (KBr): ν = 3 500–3 450 cm^{-1} (H_2O), 3 284 (NH), 1 684 (C=O, ketone), 1 625 (C=O, hydrazone); ^{1}H-NMR (DMSO-d_6): δ (ppm) = 2.42 (s, 6H, $COCH_3$),7.30–7.94 (m, 8H, Ar-H), 8.47 (s, 1H, CH=N), 11.81 (b, ½H, -CONHN=CH-). Analysis: $C_{19}H_{17}FN_4O_3{\cdot}H_2O$ (% calculated/found): 59.06/58.57 (C); 4.95/4.88 (H), 14.50/14.47(N).

4.1.2.6. 2,3,4-pentanetrione-3-[4-[[(4-nitrophenyl)-methylene]hydrazinocarbonyl]phenyl]hydrazone **3f**

Yield: 14%, m.p. 284 °C (ethanol). UV (ethanol): λ_{max} (log ε) = 236 (4.1306), 265 (4.0161) sh, 372 (4.5728); IR (KBr): ν = 3 500–3 450 cm^{-1} (H_2O), 3 330 (NH), 1 672 (C=O, ketone), 1 637 (C=O, hydrazone); ^{1}H-NMR (DMSO-d_6): δ (ppm) = 2.46 (s, 6H, $COCH_3$), 7.70–8.31 (m, 8H, Ar-H), 8.56 (s, 1H, CH=N), 12.09 (b, 1H, -CONHN=CH-), 13.74 (b, 1H, -CH=N–NH-). Analysis: $C_{19}H_{17}N_5O_5{\cdot}½H_2O$ (% calculated/found): 56.43/56.38 (C); 4.48/4.14 (H);17.32/17.18 (N).

4.1.2.7. 2,3,4-pentanetrione-3-[4-[[(4-hydroxyphenyl)-methylene]hydrazinocarbonyl]phenyl]hydrazone **3g**

Yield: 38%, m.p. 239 °C (ethanol). UV (ethanol): λ_{max} (log ε) = 240 (4.4540), 275 (4.0277) sh, 372 (4.8302); IR (KBr): ν = 3 500 cm^{-1} (OH), 3 295 (NH), 1 672 (C=O, ketone), 1 642 (C=O, hydrazone); ^{1}H-NMR (DMSO-d_6): δ (ppm) = 2.46 (s, 6H, $COCH_3$), 6.83–8.00 (m, 8H, Ar-H), 8.36 (s, 1H, CH=N), 9.69 (s, 1H, Ar-OH), 13.52 (b, ½H, -CONHN=CH-), 14.28 (b, ½H, -CH=N–NH-). Analysis: $C_{19}H_{18}N_4O_4{\cdot}2H_2O$ (% calculated/found): 56.71/56.07 (C); 5.51/4.80 (H); 13.92/14.68 (N).

4.1.2.8. 2,3,4-pentanetrione-3-[4-[[(4-hydroxy-3-methoxyphenyl)methylene]hydrazinocarbonyl]phenyl]hydrazone **3h**

Yield: 69%, m.p. 178 °C (ethanol). UV (ethanol): λ_{max} (log ε) = 237 (4.1504), 293 (4.1156) sh, 372 (4.4509); IR (KBr): ν = 3 500 cm^{-1} (OH), 3 307 (NH), 1 678 (C=O, ketone), 1 625 (C=O, hydrazone); ^{1}H-NMR (DMSO-d_6): δ (ppm) = 2.46 (s, 6H, $COCH_3$), 3.83 (s, 3H,-OCH_3), 6.84–7.31 (m, 3H, Ar-H), 7.66 (d, 2H, o-NH, *J* = 8.6 Hz), 7.96 (d, 2H, m-NH, *J* = 8.6 Hz), 8.34 (s, 1H, CH=N), 9.48 (s, 1H, Ar-OH), 11.56 (b, 1H, -CONHN=CH-), 13.69 (b, ½H, -CH=N–NH-). Analysis: $C_{20}H_{20}N_4O_5{\cdot}4H_2O$ (% calculated/found): 51.28/51.40 (C); 5.98/4.76 (H); 11.96/11.84 (N).

4.1.2.9. 2,3,4-pentanetrione-3-[4-[[(4-dimethylaminophenyl)methylene]hydrazinocarbonyl]phenyl]hydrazone **3i**

Yield: 22%, m.p. 228 °C (ethanol). UV (ethanol): λ_{max} (log ε) = 240 (4.0491), 384 (4.4508); IR (KBr): ν = 3 450–3 400 cm^{-1} (H_2O), 3 225 (NH), 1 678 (C=O, ketone), 1 625 (C=O, hydrazone); EI-MS (70 eV): m/z (%) = 393 (66.18) [M^+], 351 (1.88), 350 (2.42), 308 (1.04), 282 (14.09), 281 (1.71), 231 (100), 163 (29.73), 162 (15.75), 146 (47.29), 120 (50.04), 92 (10.39), 43 (48.83); ^{1}H-NMR (DMSO-d_6): δ (ppm) = 2.46 (s, 6H, $COCH_3$), 2.98 (s, 6H,-$N(CH_3)_2$), 6.77 (d, 2H, o-$N(CH_3)_2$, *J* = 8 Hz), 7.55 (d, 2H, m-$N(CH_3)_2$, *J* = 8 Hz), 7.68 (d, 2H, o-NH, *J* = 8.3 Hz), 7.97 (d, 2H, m-NH, *J* = 8.3 Hz), 8.32 (s, 1H, CH=N), 11.49 (s, 1H, -CONHN=CH-), 13.80 (s, 1H, -CH=N–NH-). Analysis: $C_{21}H_{23}N_5O_3{\cdot}H_2O$ (% calculated/found): 61.31/61.39 (C); 6.08/6.08 (H);17.03/17.45 (N).

4.1.2.10. 2,3,4-pentanetrione-3-[4-[[3-(4-dimethylaminophenyl)-2-propenylidene]hydrazinocarbonyl]phenyl]hydrazone **3j**

Yield: 47%, m.p. 225 °C (ethanol). UV (ethanol): λ_{max} (log ε) = 249 (4.4045), 402 (4.8812); IR (KBr): ν = 3 177 cm^{-1} (NH), 1 672 (C=O, ketone), 1 649 (C=O, hydrazone); EI-MS (70 eV): m/z (%) = 419 (7.39) [M^+], 376 (3.98), 308 (25.37), 231 (46.63), 188 (9.57), 172 (30.68), 145 (4.34), 136 (7.60), 120 (100), 92 (20.39), 65 (12.30), 43 (32.25); ^{1}H-NMR (DMSO-d_6): δ (ppm) = 2.46 (s, 6H, $COCH_3$), 3.23 (s, 6H, -$N(CH_3)_2$), 6.71 (d, 2H, o-$N(CH_3)_2$, *J* = 8.6 Hz), 6.76–6.96 (m, 2H, -CH=CH-), 7.43 (d, 2H, m-$N(CH_3)_2$, *J* = 8.6 Hz), 7.65 (d, 2H, o-NH, *J* = 8.6 Hz), 7.94 (d, 2H, m-NH, *J* = 8.6 Hz), 8.33 (d, 1H, -CH=N), 11.46 (s,1H, -CONHN=CH-), 13.72 (s, ½H, -CH=N–NH-). Analysis $C_{23}H_{25}N_5O_3 \cdot H_2O$ (% calculated/found): 63.14/63.69 (C); 6.22/5.95 (H); 16.01/15.57(N).

4.2. Microbiological procedures

4.2.1. In vitro evaluation of antimycobacterial activity against M. fortuitum

Preparation of Mycobacterial inoculum required a few modifications due to the difficulty of obtaining a homogenous suspension of *M. fortuitum* in the broth used. Four or five colonies of *M. fortuitum* which were previously grown in tryptic soy agar (TSA) after 72 h of incubation at 30 °C were collected by means of a swab and suspended in 4.5 mL of Mueller-Hinton broth enriched with Tween 80 (0.2%). Following the inclusion of 4–5 glass beads, this mixture was whirled using a vortex-mixer to ensure a good suspension. The density of this culture was then adjusted to a turbidity equivalent to that of a 0.5 McFarland standard and finally, the adjusted culture was diluted with sterile water so that, after inoculation, each microplate well had an inoculum size of 1.5×10^5 cfu/mL.

Antimycobacterial testing of all compounds was carried out in Mueller-Hinton broth enriched with Tween 80 (0.2%) at pH 7.3. Tobramycin, which is an active antibiotic against rapidly growing mycobacteria, was selected as the standard drug. Quality control strains used in the present study were *E. coli* and *S. aureus*. Tobramycin was used at a concentration range of 4–0.03 μg/mL against the quality control strains. The standard drug and test compounds were dissolved in water and DMSO, respectively, and were diluted with the broth used. The concentration intervals were 32–0.5 μg/mL and 128–1 μg/mL for the standard drug and the test compounds, respectively. Microplate wells, containing 100 μl of broth with Tobramycin or the test compounds, were then inoculated with 10 μl of *M. fortuitum* suspension whose preparation is described above. Sheep-blooded agar was used for the purity control. After incubation for 72 h at 30 °C, the last microplate well with no growth of microorganism was recorded to represent the MIC expressed in μg/mL [21–24].

4.2.2. In vitro evaluation of antimycobacterial activity against M. tuberculosis *H37Rv and* M. avium

A primary screen was conducted at 12.5 μg/mL (or molar equivalent of highest molecular weight compound in a series of congeners) against *M. tuberculosis* H37Rv in BACTEC 12B medium using the BACTEC 460 radiometric system. Compounds effecting < 90% inhibition in the primary screen (MIC > 12.5 μg/mL) were not evaluated further. Compounds demonstrating at least 90% inhibition in the primary screen were re-tested at lower concentration (MIC) in a broth microdilution assay with alamar Blue. The MIC was defined as the lowest concentration inhibiting 99% of the inoculum. These compounds were also tested against *M. avium*, a naturally drug-resistant opportunistic pathogen, using the same technique. Concurrent with the determination of MIC's, compounds were tested for cytotoxicity (IC_{50}) in VERO cells at concentrations equal to and greater than the MIC for *M. tuberculosis* H37Rv. After 72 h exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega CellTiter 96 Non-radioactive Cell Proliferation Assay.

4.2.3. BACTEC radiometric method of susceptibility testing

Inocula for susceptibility testing were either from a positive BACTEC isolation vial with a growth index (GI) of 500 or more, or a suspension of organisms isolated earlier on a conventional medium. The culture was well mixed with a syringe and 0.1 mL of a positive BACTEC culture was added to each of the vials containing the test drugs. The drug vials contained rifampicin (0.25 μg/mL). A control vial was inoculated with a 1:100 dilution of the culture. A suspension equivalent to a McFarland No. 1 standard was prepared in the same manner as a BACTEC positive vial, when growth from a solid medium was used. Each vial was tested immediately on a BACTEC instrument to provide CO_2 in the headspace. The vials were incubated at 37 °C and tested daily with a BACTEC instrument. When the GI in the control reads at least 30, the increase in GI (Δ GI) from the previous day in the control was compared with that in the drug vial. The following formula was used to interpret results:

Δ GI control > Δ GI drug = susceptible

Δ GI control < Δ GI drug = resistant

If a clear susceptibility pattern (the difference of Δ GI of control and the drug bottle) was not seen at the time the control GI is 30 the vials were read for 1 or 2 additional days to establish a definite pattern of Δ GI differences.

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