Isonicotinoylhydrazothiazoles and Isonicotinoyl-N^4-substituted thiosemicarbazides: Synthesis, Characterization, and Anti-mycobacterial Activity

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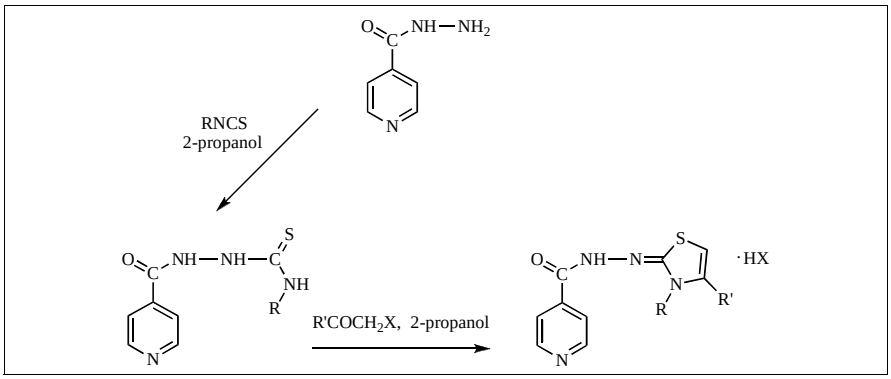
Received September 29, 2005



Differently substituted isonicotinoylhydrazothiazoles and isonicotinoyl-N^4-substituted thiosemicarbazides have been prepared and characterized by means of elemental analysis, ^{1}H-NMR, ^{13}C-NMR, and Mass spectrometry. All the synthesised compounds have been screened in order to evaluate their antimycobacterial activity *in vitro*. Some of the new compounds showed activity towards *M. tuberculosis H37R* and *M. Marinum* in the millimolar range.

J. Heterocyclic Chem., **43**, 1337 (2006).

Introduction.

Since the discovery of Streptomycin, an increasing effort has been devoted to the development of new and more efficient anti-tubercular agents, leading to the introduction of a number of new molecules in clinical practice [1 – 12].

Nevertheless the growth of multi-drug resistant mutant species of mycobacterium (MDR) has become an emerging problem [13], in spite of the fact that *M. tuberculosis* was one of the first infective agents whose genome was sequenced entirely.

A number of biological targets, most of which involved in the synthesis of the mycobacterium cell wall, have been identified for the treatment of mycobacterium sustained infections [14 – 19].

It is known that some thiosemicarbazide derivatives exhibit anti-mycobacterial activity [20]. More recently some isothiosemicarbazide [21] and hydrazo-3,4-disubstituted-thiazole [22] derivatives have been synthesised and tested as anti-mycobacterial agents.

In a previous communication we reported the synthesis and biological evaluation of some differently substituted 9,10-anthracenediones. In particular 1,4-dimethyl-5-methylpiperazino-8-fluoro-9,10-anthracendione exhibits the best activity against *M. avium* NC 08559.06 (1.77 x 10^{-5} *M*). The same compound shows interesting activity towards *M. tuberculosis* INH-R ATCC-35822 and *M. tuberculosis* SM-R ATCC 35820 (7.1 x 10^{-5} M) [23]. Pursuing on our interest in the field of anti-mycobacterial derivatives, we prepared some 1,4-dioxo-3,4-dihydrophtalazine-2(1*H*)-carboxamides and –carbothioamides, with the aim of evaluating their anti-mycobacterial activity [24]. These compounds can be considered aroyl-semicarbazide or -thiosemicarbazide derivatives with a constrained structure.

More recently we have synthesised some aroylisothiosemicarbazides and investigated their activity against *M. tuberculosis* H37RV and *M. avium* ATCC19421 [25]. 1-(4-Nitrobenzoyl)-*S*-allyl-isothiosemicarbazide exhibits the best activity towards *M. tuberculosis* H37RV (8.9 x 10^{-5} M).

In this paper we wish to report on the synthesis and anti-mycobacterial activity of some isonicotinoylhydrazothiazoles and 4-substituted-isonicotinoyl-thiosemicarbazides.

These compounds are structurally related to both isoniazid and to previously reported active compounds [20, 22].

Results and Discussion.

The synthetic pathway to compounds **2a-d** and **3a-h** is outlined in Scheme 1.

Isonicotinoylhydrazide is reacted with the appropriate isothiocyanate to achieve the N^4-substituted isonicotinoylthiosemicarbazides **2a-d**. Compounds **2b**, **2c**, and **2d** have been synthesised slightly modifying literature procedures [25,26].

The formation of neither triazolinethiones, nor oxadiazoles was observed by refluxing compounds **1** with isothiocyanates in 2-propanol.

The formation of triazolinethiones is only observed by refluxing 1-isonicotinoyl-4-substitutedthiosemicarbazides in 2 *N* sodium hydroxyde, as described by Gülerman and coworkers [27].

On the contrary, by refluxing 1-isonicotinoyl-4-phenyl-thiosemicarbazides (**2b**) in 2-propanol with a little excess of pyridine, the formation of 5-(4-pyridinyl)-2-phenyl-amino-1,3,4-oxadiazole (**4**) was obtained.

The structure of compound **4** was confirmed by using analytical and spectroscopic methods, and by comparison with an authentic sample prepared by cyclodesulfurization with I_2/NaOH/ethanol, as described in literature [28].

Products **2a-d** are then reacted under mild conditions with either bromoacetophenone or chloroacetone, to give the target isonicotinoylhydrazo-3,4-disubstituted thiazoles **3a-h**. The reaction has been carried in the absence of a proton acceptor, in order to achieve the salts of compounds **3a-h**, more suitable for biological testing. By using these conditions only the formation of the thiazole derivatives **3a-h** was observed, due to the high regio-selectivity of the reaction.

The possible formation of the mercaptoimidazole derivatives was previously observed by some of us only when isothiosemicarbazones are refluxed in the presence of a proton acceptor and acetonitrile as a solvent [29].

The analytical data of compounds **2a-d** and **3a-h** are reported in Tables 1 and 2 respectively.

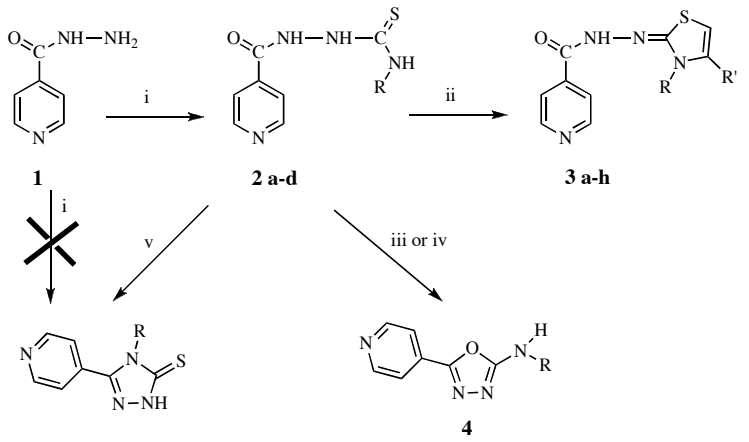
All the synthesized compounds have been characterized by means of both analytical and spectroscopic methods. The main fragmentation pathways exhibited by compounds **3a-h**, in electron ionization conditions, are illustrated in Scheme 2.

Once characterized, all compounds were tested to evaluate their anti-mycobacterial activity against *M. avium, M. tuberculosis H37Rv, M. intracellulare,* and *M. marinum.* MIC values of the synthesised compounds are reported in Table 3.

The most active compounds, **2a**, **2c**, **3a**, **3b**, and **3e** were also tested to evaluate their cytotoxicity. The obtained results, expressed as maximum non-toxic dose (MNTD), are the following: **2a** < 2.97 x 10^{-4} mmol/ml; **2c** = 4.49 x 10^{-4} mmol/ml; **3a** <1.6 x 10^{-4} mmol /ml; **3b** = 2.19 x 10^{-4} mmol/ml; **3e** <1.36 x 10^{-4} mmol /ml.

The best activity against *M. tuberculosis* was observed for compounds **2a**, **2c**, **3a**, **3b**, and **3e**, indicating that the

Scheme 1



Reagents and conditions: i) RNCS, 2-propanol, reflux; ii) R'$COCH_2X$, 2-propanol; iii) 2-propanol, pyridine, reflux; iv)I_2 , NaOH, ethanol; v) NaOH 2N reflux

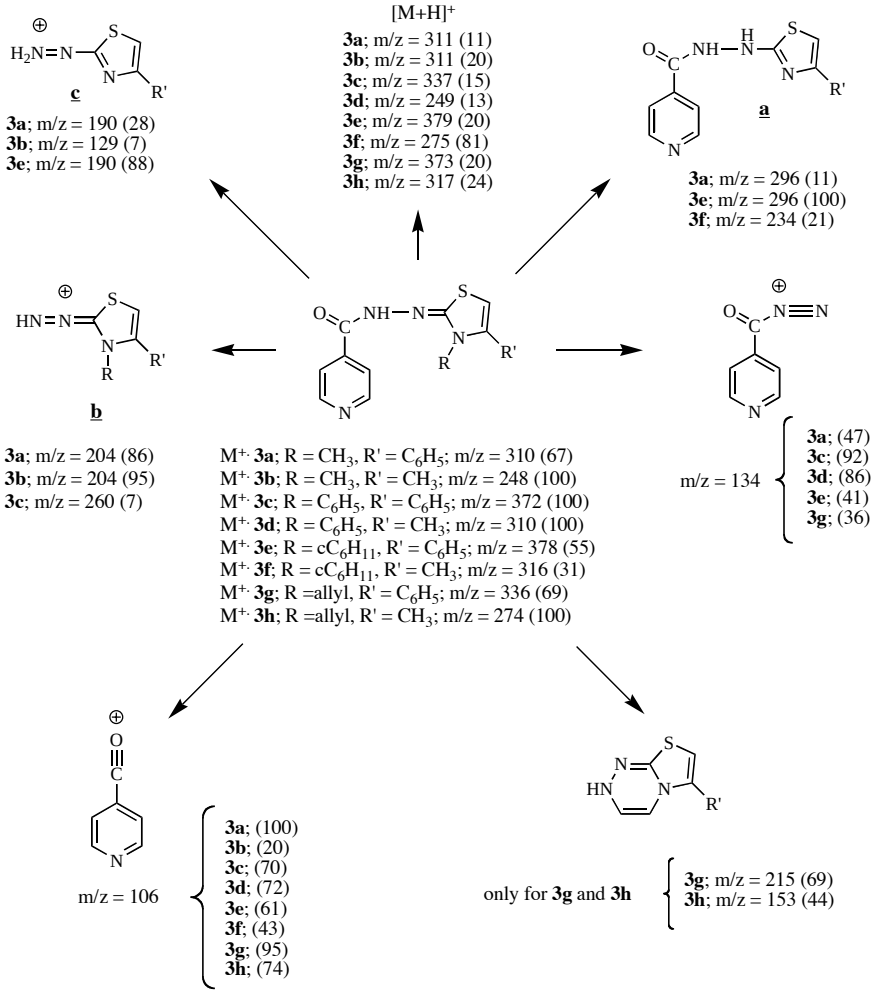
Synthetic pathway to compounds 2**a-d,** 3**a-h** and **4**

Table 1

Analytical Data of Compounds **2a**-**d**

Compound	Formula	R	m/z	Yield%	Mp°C	C%	H%	N%
2 a	$C_8H_{10}N_4OS$	CH_3	210	93	219-20	45.70 (45.91)	4.79 (4.77)	26.65 (26.56)
2 b	$C_{13}H_{12}N_4OS$	C_6H_5	272	98	187-89	57.34 (57.56)	4.44 (4.41)	20.57 (20.49)
2 c	$C_{13}H_{18}N_4OS$	c-C_6H_{11}	278	90	212-4	56.09 (56.35)	6.52 (6.49)	20.13 (20.05)
2 d	$C_{10}H_{12}N_4OS$	CH_2-CH=CH_2	236	87	218-20	50.83 (50.95)	5.12 (5.15)	23.71 (23.70)

Scheme 2



Most relevant fragment ions of compounds **3a**-**h**

Table 2

Analytical Data of compounds **3a-h** (HX)

Compound	Formula	R	R_1	m/z	Yield%	Mp°C	C%	H%	N%
3a	$C_{16}H_{15}BrN_4OS$	CH_3	C_6H_5	310	70	235-38	49.11 (49.31)	3.86 (3.88)	14.32 (14.26)
3b	$C_{11}H_{13}ClN_4OS$	CH_3	CH_3	248	46	218-20	46.40 (46.57)	4.60 (4.58)	19.67 (19.71)
3c	$C_{21}H_{17}BrN_4OS$	C_6H_5	C_6H_5	372	38	258-60	55.64 (55.83)	3.78 (3.76)	12.36 (12.31)
3d	$C_{16}H_{15}ClN_4OS$	C_6H_5	CH_3	310	79	248-50	55.41 (55.27)	4.36 (4.40)	16.15 (16.18)
3e	$C_{21}H_{23}BrN_4OS$	c-C_6H_{11}	C_6H_5	378	38	280 dec	54.90 (55.13)	5.05 (5.04)	12.20 (12.24)
3f	$C_{16}H_{21}ClN_4OS$	c-C_6H_{11}	CH_3	316	31	273-76	54.46 (54.28)	6.00 (5.98)	15.88 (15.93)
3g	$C_{18}H_{17}BrN_4OS$	CH_2-CH=CH_2	C_6H_5	336	56	242-45	51.80 (52.03)	4.11 (4.09)	13.43 (13.38)
3h	$C_{13}H_{15}ClN_4OS$	CH_2-CH=CH_2	CH_3	274	17	195-97	50.24 (49.99)	4.86 (4.88)	18.03 (17.97)

presence of an alkyl moiety either on the N^4 or in position three of the dihydrothiazole ring, leads to the most active compounds.

Despite the fact that the most active compounds exhibit citotoxicity, in the case of compound **2c**, was a satisfactory selectivity index (MNTD/MIC = 20) observed.

Nevertheless, further information on the structure-activity relationships of these compounds can be achieved. By comparing the activities of compounds **3a**, **3c**, **3e**, and **3g** with **3b**, **3d**, **3f**, and **3h**, it seems that on introducing a phenyl moiety in the position 4 of the dihydrothiazole ring the activity towards *M. tuberculosis* increases.

Although none of the tested molecules exhibit an anti-mycobacterial activity, comparable with the reference compounds, in particular with Rifampicin, these results give more than one hint for the development of future work.

As an example, the introduction of differently substituted aryl moieties, in the position four of the dihydrothiazole ring, as well as the replacement of the isonicotinoyl group, will be investigated.

Table 3

Anti-mycobacterial activity
MIC [mmol/ml]

Compound	*M. avium*	*M. tuberculosis H37Rv*	*M. intracellulare*	*M. marinum*
2a	>4.76 x 10^{-4}	2.38 x 10^{-4}	>4.76 x 10^{-4}	>4.76 x 10^{-4}
2b	>3.67 x 10^{-4}	>3.67 x 10^{-4}	>3.67 x 10^{-4}	>3.67 x 10^{-4}
2c	>3.59 x 10^{-4}	2.24 x 10^{-5}	>3.59 x 10^{-4}	>3.59 x 10^{-4}
2d	>4.23 x 10^{-4}	>4.23 x 10^{-4}	>4.23 x 10^{-4}	>4.23 x 10^{-4}
3a	>2.55 x 10^{-4}	1.28 x 10^{-4}	>2.55 x 10^{-4}	>2.55 x 10^{-4}
3b	>3.51 x 10^{-4}	1.75 x 10^{-4}	>3.51 x 10^{-4}	>3.51 x 10^{-4}
3c	>2.21 x 10^{-4}	>2.21 x 10^{-4}	>2.21 x 10^{-4}	>2.21 x 10^{-4}
3d	>2.88 x 10^{-4}	2.88 x 10^{-4}	>2.88 x 10^{-4}	>2.88 x 10^{-4}
3e	>2.18 x 10^{-4}	1.09 x 10^{-4}	>2.18 x 10^{-4}	1.09 x 10^{-4}
3f	>2.83 x 10^{-4}	>2.83 x 10^{-4}	>2.83 x 10^{-4}	>2.83 x 10^{-4}
3g	>2.39 x 10^{-4}	>2.39 x 10^{-4}	>2.39 x 10^{-4}	>2.39 x 10^{-4}
3h	>3.21 x 10^{-4}	>3.21 x 10^{-4}	>3.21 x 10^{-4}	>3.21 x 10^{-4}
INH*	1.82 x 10^{-4}	1.39 x 10^{-6}	3.6 x 10^{-4}	3.6 x 10^{-4}
RMP**	1.52 x 10^{-5}	2.31 x 10^{-7}	4.7 x 10^{-7}	3.8 x 10^{-6}

* Isoniazid, ** Rifampicin

EXPERIMENTAL

Melting points are uncorrected and were determined on an Electrothermal 9100 apparatus. Infrared (ir) spectra were recorded on a Perkin-Elmer 1640 FT spectrophotometer (KBr discs or nujol, in cm^{-1}). ^{1}H nmr spectra were recorded on a Bruker AMX (300 MHz) using tetramethylsilane (TMS) as internal standard (chemical shifts in δ values), and dimethyl-sulfoxide-d_6 and deuterochloroform as solvents. Electron ionisation (EI) mass spectra were obtained on a Fisons QMD 1000 mass spectrometer (70 eV, 200 μA, ion source temperature 200°C). The samples were introduced directly into the ion source. Elemental analyses were obtained on a Perkin-Elmer 240 B microanalyser. Tlc chromatography was performed using silica gel plates (Merck F 254).

The structures of all compounds were assigned on the basis of ir, nmr, mass spectra, and elemental analysis.

Synthetic Procedures.

General Procedure for the Synthesis of Isonicotinoyl-N^4-substituted thiosemicarbazides (**2a-d**).

A suspension of isoniazid (0.03 mol) and the appropriate isothiocyanate (0.03 mol) in 100 mL of isopropanol is refluxed under vigorous stirring until complete dissolution of the reagents and for a further 3 hours. After cooling to room temperature a solid is obtained, which is filtered off, washed several times with isopropyl ether, and dried. The following listed compounds were obtained with this procedure.

1-Isonicotinoyl-4-methylthiosemicarbazide (**2a**).

^{1}H-NMR (DMSO-d_6): δ 2.99 (d, 3H, *J* = 4.2, NHCH_3); 7.93 (dd, 2H, *J* = 4.2, 1.5, C_3H and C_5H-pyr); 8.25 (d, 1H, NH, *J* = 4.2, D-exch.); 8.86 (dd, 2H, *J* = 4.6, 1.5, C_2H and C_6H-pyr); 9.95 (s, 1H, NH, D-exch.); 10.76 (s, 1H, NH, D-exch.). ^{13}C NMR (DMSO -d_6): 30.5, 120.18, 120.70, 136.17, 148.25, 164.29, 180.33.

1-Isonicotinoyl-4-phenylthiosemicarbazide (**2b**).

^{1}H-NMR (DMSO-d_6) : δ 7.27 (t, 1H, *J* = 7.1, Ar); 7.44 (t, 2H, *J* = 7.1, Ar); 7.54 (d, 2H, *J* = 6.5, Ar); 7.97 (d, 2H, *J* = 4.2, C_3H and C_5H-pyr); 8.88 (d, 2H, *J* = 4.2, C_2H and C_6H-pyr); 9.92 (s, 1H, NH, D-exch.); 9.99 (s, 1H, NH, D-exch.); 10.97 (s, 1H, NH, D-exch.). ^{13}C NMR (DMSO -d_6): 120.37, 122.03, 122.28, 127.24, 128.13, 129.36, 129.59, 129.86 129.98, 166.38, 182.11.

1-Isonicotinoyl-4-cyclohexylthiosemicarbazide (**2c**).

^{1}H-NMR (DMSO-d_6): δ 1.15-1.40 (m, 6H, CH_2); 1.67-1.91 (m, 4H, CH_2); 2.62-2.68 (m, 1H, CH); 7.94 (dd, 2H, *J* = 4.2, 1.5, C_3H and C_5H-pyr); 8.23 (s, 1H, NH, D-exch.); 8.87 (dd, 2H, *J* = 4.2, 1.5, C_2H and C_6H-pyr); 9.41 (s, 1H, NH, D-exch.); 10.70 (s, 1H, NH, D-exch.). ^{13}C NMR (DMSO –d_6): δ 25.05, 25.27, 31.94, 53.21, 121.39, 121.83, 139.88, 150.12, 164.35, 180.65.

1-Isonicotinoyl-4-allylthiosemicarbazide (**2d**).

^{1}H-NMR (DMSO-d_6): δ 4.21 (t, 2H, J = 5.4, NHCH_2); 5.22 (dd, 2H, J = 15.74, 10.7, 1.5, =CH_2); 5.90-5.99 (m, 1H, CH=); 7.93 (dd, 2H, J = 4.6, 1.5, C_3H and C_5H-pyr); 8.50 (s, 1H, NH, D-exch.); 8.87 (dd, J = 4.6, 1.5, 2H, C_2H and C_6H-pyr); 9.59 (s, 1H, NH, D-exch.); 10.78 (s, 1H, NH, D-exch.). ^{13}C-NMR (DMSO –d_6): 44.15, 70.32, 104.06, 122.83, 144.13, 145.96, 165.34, 181.77.

General Procedure for the Synthesis of *N*'-(3,4-Disubstitutedthiazole-2(3*H*)-ylidene)isonicotinohydrazides•HBr **3a**, **3c**, **3e**, and **3g**.

The opportune 1-isonicotinoyl-4-substituted-thiosemicarbazide (4.8 mmol) is allowed to react with 2-bromoacetophenone (4.8 mmol) suspended in 40 mL of isopropanol. The reaction mixture is stirred for 2 hours at room temperature (monitored by TLC). The solvent is then removed under reduced pressure and the resulting precipitate is purified by crystallisation with ethanol and dried.

The following listed compounds were synthesized using this procedure.

N'-(3-Methyl-4-phenylthiazole-2(3*H*)-ylidene)isonicotinohydrazide hydrobromide (**3a**).

^{1}H-NMR (DMSO-d_6): δ 3.60 (s, 3H, N-CH_3); 7.01 (s, 1H, C_5H-thiaz); 7.68-7.79 (m, 5H, Ar); 8.18 (d, 2H, J = 4.9, C_3H and C_5H-pyr); 9.05 (d, 2H, J = 4.6, C_2H and C_6H-pyr); 12.00 (s, 1H, NH, D-exch.).^{13}C-NMR (DMSO-d_6): δ 34.7, 98.2, 122.4, 126.4, 128.0, 129.3, 129.9, 134.3, 140.9, 144.8, 149.8, 159.6, 163.0.

N'-(3,4-Diphenylthiazole-2(3*H*)-ylidene)isonicotinohydrazide hydrobromide (**3c**).

^{1}H-NMR (DMSO): δ 6.20 (s, 1H, C_5H-thiaz); 7.19-7.40 (m, 10H, Ar); 7.77 (d, 2H, J = 4.2, C_3H and C_5H-pyr); 8.68 (d, 2H, J = 4.2, C_2H and C_6H-pyr); 11.67 (s, 1H, NH, D-exch.). ^{13}C-NMR (DMSO-d_6): δ 108.2, 122.2, 122.8, 124.2, 126.4, 128, 128.7, 129.6, 134.3, 140.9, 141.3, 147.5, 149.8, 154, 168.2.

N'-(3-Cyclohexyl-4-phenylthiazole-2(3*H*)-ylidene)isonicotinohydrazide hydrobromide (**3e**).

^{1}H-NMR ($CDCl_3$): δ 1.11-1.38 (m, 6H, CH_2); 1.70-2.17 (m, 4H, CH_2); 3.69-3.76 (m, 1H, CH); 5.90 (s, 1H, C_5H-thiaz); 7.25-7.46 (m, 5H, Ar); 7.66 (d, 2H, J = 4.6, C_3H and C_5H-pyr); 8.70 (d, 2H, J = 4.6, C_2H and C_6H-pyr); 11.65 (s, 1H, NH, D-exch.). ^{13}C-NMR ($CDCl_3$): δ 24.9, 25.9, 28.5, 59.5, 98.2, 120.7, 128.7, 128.96, 129.3, 132.0, 142.1, 150.0, 160.3, 168.3.

N'-(3-Allyl-4-phenylthiazole-2(3*H*)-ylidene)isonicotinohydrazide hydrobromide (**3g**).

^{1}H-NMR ($CDCl_3$): δ 4.48 (t, 2H, J = 5.4, N-CH_2); 5.19 (dd, 2H, J = 15.74, 10.7, 1.5, =CH_2); 5.85-5.90 (m, 1H, CH=); 6.96 (s, 1H, C_5H-thiaz); 7.43-7.61 (m, 5H, Ar); 8.13 (dd, 2H, J = 4.6, 1.5, C_3H and C_5H-pyr); 9.07 (dd, 2H, J = 4.6, 1.5, C_2H and C_6H-pyr); 11.78 (s, 1H, NH, D-exch.). ^{13}C-NMR ($CDCl_3$): δ 48.7, 66.4, 102.0, 118.0, 122.9, 128.8, 129.2, 129.9, 130.6, 134.98, 141.6, 145.9, 146.7, 170.2, 190.2.

General Procedure for the Synthesis of *N*'-(3,4-disubstitutedthiazole-2(3*H*)-ylidene)isonicotinohydrazides•HCl **3b**, **3d**, **3f**, and **3h**.

A mixture of the appropriate 1-isonicotinoyl-4-substituted-thiosemicarbazide (4.8 mmol) and 2-chloroacetone (4.8 mmol) in 50 mL of isopropanol is stirred and heated under reflux until complete dissolution of the reagents and for a further 1.5 hours. The solution is then allowed to cool to room temperature, thus obtaining a solid that is purified by crystallisation with alcohol and dried.

The following listed compounds were synthesized using this procedure.

N'-(3,4-Dimethylthiazole-2(3*H*)-ylidene)isonicotinohydrazide hydrochloride (**3b**).

^{1}H-NMR (DMSO): δ 2.20 (s, 3H, thiaz-CH_3); 3.60 (s, 3H, N-CH_3); 6.62 (s, 1H, C_5H-thiaz); 8.27 (dd, 2H, J = 4.9, 1.1, C_3H and C_5H-pyr); 8.92 (dd, 2H, J = 4.6, 1.1, C_2H and C_6H-pyr); 11.68 (s, 1H, NH, D-exch.). ^{13}C-NMR (DMSO-d_6): δ 13.7, 31.0, 98.0, 121.5, 121.8, 140.0, 149.8, 164.4, 167.1

N'-(3-Phenyl-4-methylthiazole-2(3*H*)-ylidene)isonicotinohydrazide hydrochloride (**3d**).

^{1}H-NMR ($CDCl_3$): δ 2.15 (s, 3H, thiaz-CH_3); 5.77 (s, 1H, C_5H-thiaz); 6.96 (d, 2H, J = 7.6, Ar); 7.09 (t, 1H, J = 7.6, Ar); 7.28 (t, 2H, J = 7.1, Ar); 7.62 (d, 2H, J = 3.8, C_3H and C_5H-pyr); 8.54 (d, 2H, J = 3.8, C_2H and C_6H-pyr); 11.54 (s, 1H, NH, D-exch.). ^{13}C-NMR ($CDCl_3$): δ 14.9, 97.0, 120.9, 122.0, 122.3, 127.2, 128.2, 129.1, 129.8, 129.9, 150.3, 165.0.

N'-(3-Cyclohexyl-4-methylthiazole-2(3*H*)-ylidene)isonicotinohydrazide hydrochloride (**3f**).

^{1}H-NMR ($CDCl_3$): δ 1.14-1.37 (m, 6H, CH_2); 1.63-2.09 (m, 4H, CH_2); 2.22 (s, 3H, CH_3); 3.22-3.26 (m, 1H, CH); 6.34 (s, 1H, C_5H-thiaz); 8.18 (dd, 2H, J = 4.6, 1.3, C_3H and C_5H-pyr); 8.85 (dd, 2H, J = 4.6, 1.3, C_2H and C_6H-pyr); 10.43 (s, 1H, NH, D-exch.). ^{13}C-NMR ($CDCl_3$): δ 13.25, 24.6, 29.6, 31.3, 59.6, 97.2, 122.7, 137.1, 140.1, 150.0, 164.5, 166.9.

N'-(3-Allyl-4-methylthiazole-2(3*H*)-ylidene)isonicotinohydrazide hydrochloride (**3h**).

^{1}H-NMR ($CDCl_3$): δ 2.13 (s, 3H, CH_3); 4.45 (d, 2H, J = 2.5, N-CH_2); 5.03 (dd, 2H, J = 17.2, 10.5, =CH_2); 5.66 (s, 1H, C_5H-thiaz); 5.83-5.92 (m, 1H, CH=); 7.67 (d, 2H, J = 3.8, C_3H and C_5H-pyr); 8.63 (d, 2H, J = 3.6, C_2H and C_6H-pyr); 10.56 (s, 1H, NH, D-exch.). ^{13}C-NMR ($CDCl_3$): δ 16.7, 44.1, 65.3, 100.1, 103.2, 122.7, 135.4, 143.9, 145.89, 165.2, 178.5.

Microbiological Assay.

The determination of MIC against Mycobacteria was carried out by the two-fold agar dilution method [30] in 24-multiwell plates (Nunc, Naperville, H, USA) using 7H11 agar (Difco Laboratories) containing the compounds under investigation at concentrations that ranged between 100 and 0.19 μg/ml, on which 100 μl of the test bacterial suspension were spotted.

Suspensions to be used for drug susceptibility testing were prepared from 7H9 broth cultures containing 0.05% Tween 80, washed, suspended in 0.1% Tween 80-saline to yield a turbidity no 1 McFarland, and then diluted in saline to obtain inocula of $3x10^5$- $1.5x10^4$ cells/100μl of bacterial suspension. After a 21-day (slow growers) or 7-day (rapid growers) cultivation in a CO_2 (5% CO_2-95% humidified air) incubator at 37 °C, the growth of organisms was scored. The MIC was defined as the minimum concentration causing complete growth inhibition of organisms or allowing no more than five colonies to growth.

Cytotoxicity Assay.

Cell toxicity of compounds **2a**, **2c**, **3a**, **3b**, and **3e** was tested *in vitro* by a cell viability assay as described by Denizot and Lang [31]. Monolayers of Vero cells in 96-multiwell plates were incubated with the testing compounds at concentrations of 1000 - 62.5 μg/ml in RPMI 1640 medium (Gibco) for 48 h. The medium was then replaced with 50 μl of 1 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) in RPMI without phenol red (Sigma). The cells were incubated at 37°C for 3 h, the untransformed MTT was removed, and 50 μl of isopropanolic HCl 0.04 *N* were added to each well. After few minutes at room temperature to ensure that all crystals were dissolved, the plates were read at a 560 nm test wavelength and a 690 nm reference wavelength with a 96-well plate reader (Sunrise Tecan, Grödig/Salzburg, Austria).

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