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Original article

New series of isoniazid hydrazones linked with electron-withdrawing substituents

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ABSTRACT

A series of new isoniazid hydrazones was synthesized by two procedures. In the first isoniazid was activated with diethoxymethyl acetate and condensed with the appropriate anilines. Alternatively, substituted anilines were activated by diethoxymethyl acetate and subsequently condensed with isoniazid. NMR study confirmed that both synthetic approaches gave the same tautomer. All compounds were screened for *in vitro* antimycobacterial activity. Most of them exhibited the same activity against *Mycobacterium tuberculosis* (MIC 1 μmol L^{-1}) as isoniazid (INH), better activity against *Mycobacterium kansasii* 325/80 (MIC 0.125–0.250 μmol L^{-1}), high value of selectivity index (SI) and IC_{50} between 0.0218 and 0.326 mmol L^{-1}. Compound **2o** with the best SI was used as a model compound for the stability test and was found to be stable at neutral pH, but under acidic conditions it slowly hydrolysed.

1. Introduction

Tuberculosis (TB) remains a major cause of mortality throughout the world. Resistance of *Mycobacterium tuberculosis* to antituberculosis drugs becomes very serious problem [1]. Besides MDR-TB, in 2006 more dangerous form of *M. tuberculosis* resistant against first line antituberculosis drugs, quinolones, and one of the second line anti-TB injectable drugs, called XDR-TB, was observed. The most dangerous bacteria, resistant against both the first and the second line TB drugs were presented in 2009 and are called totally resistant TDR-TB [2]. These facts clearly indicate the need for the development of new antituberculosis agents [3].

Isonicotinic acid hydrazide (isoniazid, INH) belongs to the group of the first line antituberculosis drugs being in clinical practice over 50 years. Chemical modifications of isonicotinic acid hydrazide were performed on all parts of the molecule, but the activity of these derivatives against *M. tuberculosis* has not yet exceeded that of INH [4]. To overcome the resistance, combination of INH molecule with other active molecules is frequently applied [5]. We are interested in isonicotinoyl hydrazones as an effective pharmacophore. Hydrazones have been demonstrated to posses many interesting biological activities including antimicrobial, anticonvulsant, antitubercular, antitumor and others [6]. Several hydrazones of 2-pyrazinecarbohydrazide were recently designed as analogues of pyrazinamide, another first line drug for TB treatment, to reduce the toxicity [7–10]. In our previous study we described the possible synergistic effect of two components [11], thus we expected the same for the newly synthesized series. The linkage between isonicotinoyl hydrazone and an appropriate partner by methine bridge can be gradually hydrolyzed to release active molecules [12]. The combined molecules could serve as prodrugs with an increase of bioavalibility and membrane permeability, targeting to the active site and protecting against multidrug-resistance. An introduction of electron-withdrawing substituents into the molecule of antitubercular active compounds generally increases activity as well as lipophilicity, which is important for the transport through the mycobacterial cell [4].

This work was aimed at enhancing the antimycobacterial activity of INH by conjugation with aniline group having electron-

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withdrawing substituents. Potential increases in biological effect of substituents were calculated from SAR studies [4]. INH was linked with monosubstituted or disubstituted anilines through a CH fragment into *N*-phenyl-*N*′-(pyridin-4-ylcarbonyl)hydrazonoformamide derivatives. All compounds were tested on mycobacterial inhibition against one tuberculous strain *M. tuberculosis* and three non-tuberculous mycobacterial strains—*Mycobacterium avium* and two strains of *Mycobacterium kansasii*; one of them was isolated from the patient.

2. Results and discussion

2.1. Chemistry

Two different approaches were used for the preparation of *N*-phenyl-*N*′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2**). Method A involved the reaction of isoniazid (INH) with diethoxymethyl acetate into *N*′-isonicotinoylethoxymethylene hydrazone (**1**), which was subsequently reacted with the appropriate aniline derivative (Scheme 1).

Some chloro-, bromo- and nitroanilines did not react with the compound **1** by the Method A. In these cases an alternative approach was applied that involved the reaction of the aniline derivatives with diethoxymethyl acetate into **3**, which upon substitution with INH provided the desired compounds **2** (Method B, Scheme 1).

The hydrazonoformamide structure of compounds **2** was confirmed by NMR spectroscopy. In proton NMR spectrum of crude **2a** in DMSO-d_6 solution two sets of resonances were observed with the relative integrals of 1:0.3 (Fig. 1) and were assigned to *E*-**2a** and *Z*-**2a** (Fig. 2). The assignment was based on the distinct correlations in ^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC, ^{1}H–^{13}C HMBC and ^{1}H–^{15}N HMBC spectra. The data are summarized in Tables 1 and 2. The structure of the minor compound that can be seen from Fig. 1 could not be elucidated because in 2D NMR spectra the corresponding low-intensity cross peaks were scrambled in the noise.

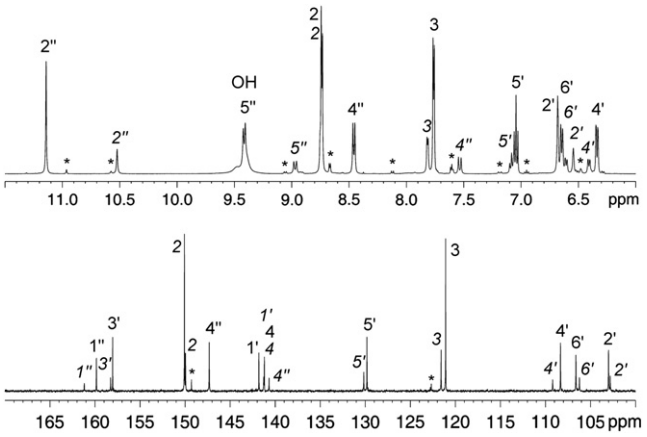
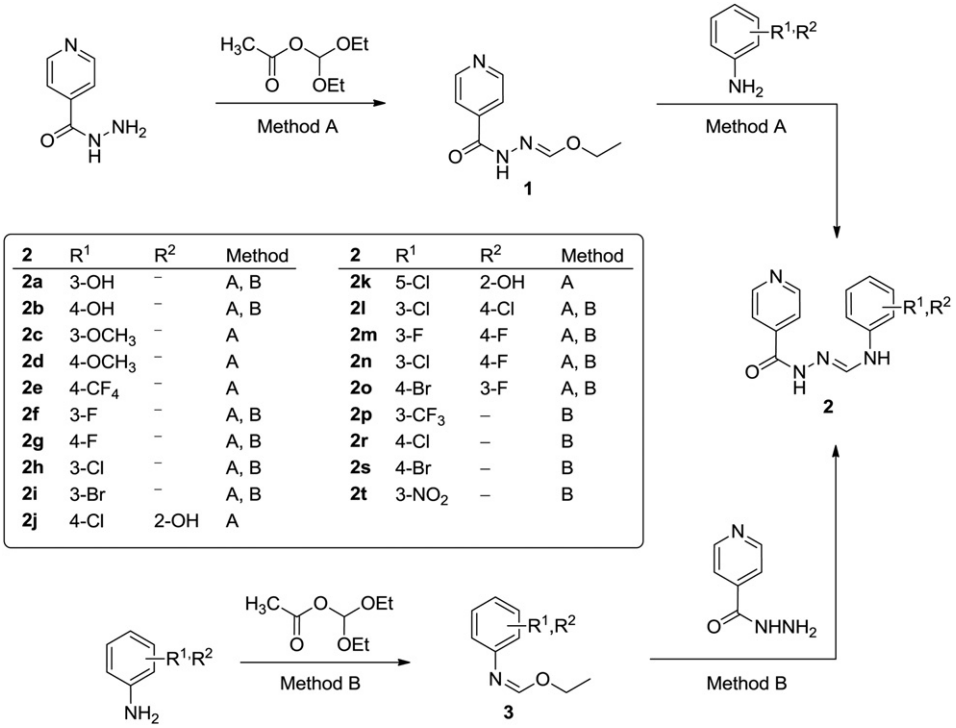


Fig. 1. Aromatic part of ^{1}H NMR and ^{13}C NMR spectra of crude **2a** recorded in DMSO-d_6 with the peak assignment of the resonances belonging to *E*-**2a** (plain figures) and *Z*-**2a** (italic figures). For atom numbering scheme, see Fig. 2. Asterisk denotes unidentified minor product (see text).

For the above-mentioned major two species present in the crude sample of **2a** all the expected structural elements could clearly be identified, i.e., the hydrazonoformamide chain, functionalized with the isonicotinoyl and the 3-hydroxyphenyl group at N-2″ and N-5″, respectively. The major differences in proton, carbon and nitrogen resonances belonging to these two molecules were observed for the hydrazonoformamide atoms, whereas the resonances of the remaining atoms appeared at similar values. For both species, however, the ^{1}H–^{13}C HMBC spectrum exhibited cross peak of NH proton, assigned as H-5″, to both C-2′ and C-6′ carbon atoms of the 3-hydroxyphenyl group (Fig. 2, Tables 1 and 2). Along with the other spectral features, collected in Tables 1 and 2, the above observations confirmed the hydrazonoformamide structures of *E*-**2a** and *Z*-**2a**, and ruled out the possibility of the tautomeric form **2a**′ in any of these two molecules. The assignment of *E*-**2a** stereochemistry of the compound was corroborated by NOE cross



2	R^1	R^2	Method	2	R^1	R^2	Method
2a	3-OH	–	A, B	**2k**	5-Cl	2-OH	A
2b	4-OH	–	A, B	**2l**	3-Cl	4-Cl	A, B
2c	3-OCH_3	–	A	**2m**	3-F	4-F	A, B
2d	4-OCH_3	–	A	**2n**	3-Cl	4-F	A, B
2e	4-CF_4	–	A	**2o**	4-Br	3-F	A, B
2f	3-F	–	A, B	**2p**	3-CF_3	–	B
2g	4-F	–	A, B	**2r**	4-Cl	–	B
2h	3-Cl	–	A, B	**2s**	4-Br	–	B
2i	3-Br	–	A, B	**2t**	3-NO_2	–	B
2j	4-Cl	2-OH	A				

Scheme 1. The synthesis of hydrazonoformamides (**2**).

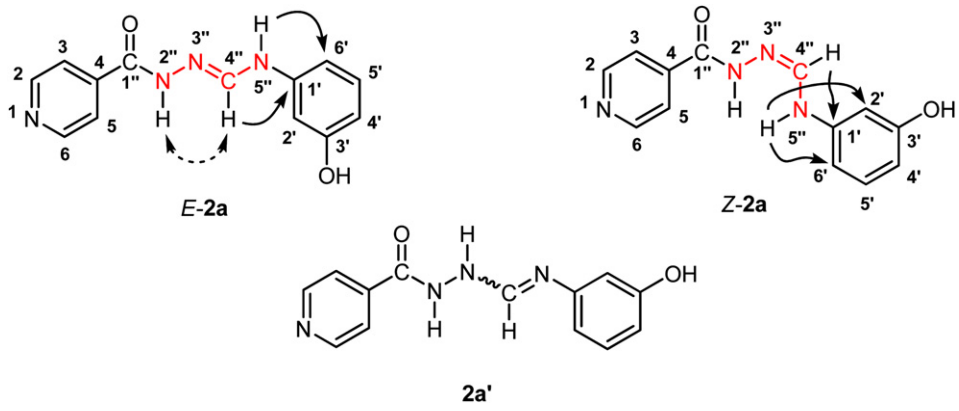


Fig. 2. The structures of isomeric *E*-**2a** and *Z*-**2a** with selected $^{1}H-^{13}C$ HMBC correlations (plain arrow) and NOEs (dotted arrow), and the structure of tautomeric **2a'**.

peak between H-2″ and H-4″ in the $^{1}H-^{1}H$ NOESY spectrum (Fig. 2).

Based on the integration, the 1:0.3 ratio of *E*-**2a** and *Z*-**2a** remained unchanged if the spectra were recorded in DMSO-d_6 at different temperatures in the range of 290–330 K thus indicating that these isomers are most probably not in a dynamic equilibrium but should have been formed during the preparation. Interestingly, the spectra of the samples prepared either by the Approach A or the Approach B shown in Scheme 1 are completely identical.

In contrast to **2a**, the NMR spectra of other hydrazonoformamides **2** generally showed one major set of resonances, presumably belonging to *E*-stereoisomers. This was supported by the distinct ^{15}N NMR chemical shifts of 324.1, 161.1, 252.2 and 106.2 ppm for N-1, N2″, N3″ and N5″ nitrogen atoms, respectively, in the compound *E*-**2f**, which is in good agreement with that of *E*-**2a** (Table 1).

2.2. Antimycobacterial evaluation

In vitro antimycobacterial activity was evaluated against *M. tuberculosis* CNTC My 331/88 (ATCC 27294), *M. avium* CNTC 330/88, *M. kansasii* CNTC My 235/80 and *M. kansasii* 6509/96 (isolated from patient). Minimal inhibitory concentration (MIC) is the lowest concentration of the substance at which the inhibition of the growth of *Mycobacterium* occurs (Table 3). Most of the synthetized compounds **2** showed the same activity as INH, whereas several derivatives had even better activity against *M. kansasii* 235/80. In general, halogenated derivatives exhibited lower MIC values. Compound **2o** was the most active for all the tested strains and was used as a model compound for further evaluation. The best MIC values are emphasized in Table 3.

One may expect an enhancing antibacterial activity of the conjugated aniline molecule having halogens like Cl, Br, F; nitro group, trifluoromethyl group, hydroxy and methoxy group. The contribution of these substituents is evident for many hydrazones [4]. Here, in general, none of them increased the activity in comparison with INH against *M. tuberculosis*. The activity seems to be based predominantly on the isoniazid core. All derivatives exhibited the same or slightly lower effectiveness in comparison with INH. Better results gave hydrazones against *M. avium* and *M. kansasii*. Namely, most of them are more active than INH; the best representatives derived from disubstituted anilines (**2k**, **2l**, **2o**). The most active molecule **2o** having two halogens (4-Br, 3-F) showed MIC 62.5 against *M. kansasii* after 7 days.

An incorporation of halogen atoms increases the lipophilicity of the molecule. Whereas mycobacterial cell wall is very lipophilic, the contribution of these lipophilic substituents plays an important role. On the other hand, the hydroxy (**2a**, **2b**) or methoxy (**2c**, **2d**)

Table 1
^{1}H, ^{13}C and ^{15}N NMR chemical shifts, $^{1}H-^{1}H$ COSY, $^{1}H-^{13}C$ HSQC, $^{1}H-^{13}C$ HMBC and $^{1}H-^{15}N$ HMBC for *E*-**2a**. For atom numbering scheme, see Fig. 2.

C	δ_C	H	δ_H (multiplicity, J/Hz[a])	COSY[b]	HSQC[b]	$^{1}H-^{13}C$ HMBC[b]
2(6)	150.08	2 (6)	8.74 (d, 5.8)	H-3(5)	H-2	H-3,6 (2,5)
3(5)	121.07	3 (5)	7.76 (d, 5.5)	H-2(6)	H-3(5)	H-2,5 (3,6)
4	141.20					
1′	141.81					H-2′, 5′, 4″
2′	103.00	2′	6.68 (br s)	H-4′	H-2′	H-6′,4′
3′	158.04					H-2′,5′,4′
4′	108.33	4′	6.34 (d, 8.0)	H-2′,6′,5′	H-4′	H-2′,6′
5′	129.81	5′	7.04 (dd, 8.0, 8.0)	H-2′,4′	H-5′	
6′	106.62	6′	6.65 (d, 8.1)	H-4′,5′	H-6′	H-2′,4′,5″
1″	159.84					H-3,5,2″
4″	147.32	4″	8.46 (d, 8.7)	H-5″	H-4″	H-2″
N	δ_N					$^{1}H-^{15}N$ HMBC[b]
1	323.7					H-2,3,5,6
2″	161.1	2″	11.14 (br s)			H-2″, [c]4″
3″	249.2					H-4″
5″	105.7	5″	9.41 (d, 8.9)	H-4″		H-4″,5″[c]

[a] J values are quoted as directly measured from the spectra.
[b] Unambiguous correlations are listed.
[c] Observed as a doublet due to incompletely suppressed direct NH correlations.

Table 2
^{1}H, ^{13}C and ^{15}N NMR chemical shifts, $^{1}H-^{1}H$ COSY, $^{1}H-^{13}C$ HSQC, $^{1}H-^{13}C$ HMBC and $^{1}H-^{15}N$ HMBC for *Z*-**2a**. For atom numbering scheme, see Fig. 2.

C	δ_C	H	δ_H (multiplicity, J/Hz[a])	COSY[b]	HSQC[b]	$^{1}H-^{13}C$ HMBC[b]
2(6)	149.95	2 (6)	8.74 (d, nd [c])	H-3(5)	H-3(5)	H-3(5)
3(5)	121.58	3 (5)	7.82 (d, 5.1)	H-2(6)		H-2,5 (3,6)
4	141.20					H-2,6
1′	141.26					H-5′,4″
2′	102.81	2′	6.54 (br s)	H-6′,4′	H-2′	H-4′,6′,5″
3′	158.27					H-2′,4′,5′
4′	109.20	4′	6.41 (d, 8.1)	H-2′,5′	H-4′	H-2′,6′
5′	130.16	5′	7.08 (dd, 8.0, 8.0)	H-6′,4′	H-5′	
6′	106.23	6′	6.61 (d, 8.0)	H-2′,5′	H-6′	H-2′,4′,5″
1″	161.19					H-3,5,2″
4″	140.68	4″	7.53 (d, 11.6)	H-2″,5″	H-4″	H-2″
N	δ_N					$^{1}H-^{15}N$ HMBC[b]
1	323.7					H-2,3,5,6
2″	157.2	2″	10.52 (br s)	H-4″		H-2″,[d] 4″
3″	236.2					H-4″
5″	110.8	5″	8.97 (d, 11.6)	H-4″		H-4″

[a] J values are quoted as directly measured from the spectra.
[b] Unambiguous correlations are listed.
[c] Not determined because of overlap with other resonances.
[d] Observed as a doublet due to incompletely suppressed direct NH correlations.

Table 3
Antimycobacterial evaluation on *M. tuberculosis*, *M. avium* and *M. kansasii* cultures.

	R^1	R^2	MIC [μmol L^{-1}]									
			M. tuberculosis 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96		
			14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
2a	3-OH	–	2	2	500	500	250	1000	1000	16	32	32
2b	4-OH	–	4	8	500	500	250	500	500	32	62.5	62.5
2c	3-OCH_3	–	2	2	500	500	250	500	1000	16	16	16
2d	4-OCH_3	–	2	4	500	500	250	500	1000	16	16	32
2e	4-CF_3	–	2	2	500	1000	250	500	1000	32	32	32
2f	3-F	–	1	1	500	1000	125	1000	>1000	16	16	16
2g	4-F	–	1	2	250	500	250	1000	>1000	8	8	8
2h	3-Cl	–	1	1	500	1000	125	500	500	16	16	16
2i	3-Br	–	1	1	250	500	250	1000	1000	8	8	16
2j	4-Cl	2-OH	2	4	250	500	250	500	500	16	16	32
2k	5-Cl	2-OH	2	2	500	500	125	250	250	8	16	32
2l	3-Cl	4-Cl	1	2	250	250	250	500	500	8	16	16
2m	3-F	4-F	2	2	250	250	250	500	1000	8	16	32
2n	3-Cl	4-F	1	2	250	500	250	1000	>1000	16	16	32
2o	4-Br	3-F	1	1	250	250	62.5	250	250	8	8	16
2p	3-CF_3	–	1	1	500	1000	125	250	1000	16	16	32
2r	4-Cl	–	1	1	500	1000	125	250	1000	16	32	32
2s	4-Br	–	2	4	500	500	125	250	500	32	32	32
2t	3-NO_2	–	2	2	250	500	125	250	500	8	16	16
INH	–	–	1	1	>250	>250	>250	>250	>250	2	4	4

substituents seem to be less effective as their contribution to the lipophilicity is lower.

Two compounds (**2k**, **2o**), the most active against *M. kansasii,* were used for the hepatotoxicity evaluation. It is evident that the contribution of halogenated aromatic rings has a positive influence on the reduction of INH hepatotoxicity.

2.3. In vitro cytotoxicity

Cytotoxicity of the most active compounds **2k** and **2o** was determined on human hepatocellular carcinoma cells HepG2 and PBMC (Peripheral Blood Mononuclear Cells) by MTT assay for cellular toxicity. IC_{50} values in mmol L^{-1} are presented in Table 4. Values of selectivity index (SI) indicate rate between IC_{50} of HepG2 cytotoxicity and MIC *M. tuberculosis*. The compounds with SI $\geq$ 10 are considered as promising candidates for further screening [13]. IC_{50} of the tested compounds were in the range of 0.0218–0.326 mmol L^{-1}. Selectivity index calculated for *M. tuberculosis* for compound **2o** exhibits high value of 162.

2.4. Stability measurement

The stability of the most active compound, **2o**, was quantitatively studied by UV–vis spectroscopy at 37 °C on ca. 5×10^{-5} mol L^{-1} solutions. Measurements were carried out in water solutions of HCl, KOH and in appropriate buffer solutions to cover the pH range of 2–13 (Table 5). For each pH the kinetic measurements were carried out where changes in UV–vis spectra were recorded. Wavelength with the highest change of absorbance over the time (λ = 267 nm) was selected to construct a curve of decomposition as the time dependence on absorbance and the observed pseudo-first-order rate constants k_{obs} were calculated (Table 5). Graphical representation of the results, the observed rate constants (k_{obs}) as a function of pH, is shown in Fig. 3.

Fig. 4 shows an illustrative example of k_{obs} determination for compound **2o** in 0.01 M hydrochloric acid solution (pH 2) at 37 °C. This figure shows the time evolution of UV–vis spectra recorded in the range of 200–500 nm. The inset represents time dependence of absorbance extracted from the spectra at λ = 267 nm. The observed pseudo-first-order rate constant k_{obs} of 2.85×10^{-3} s^{-1} was calculated from this absorbance-time dependence.

Table 4
Cytotoxicity evaluation of compounds **2k** and **2o**.

	R^1	R^2	HepG2 IC_{50} [mmol L^{-1}]	PBMC IC_{50}	SI for *M. tuberculosis*. 331/88
2k	5-Cl	2-OH	0.0218	0.075	10.9
2o	3-F	4-Br	0.162	0.326	162
INH	–	–	>1	~4.67	–

3. Experimental

3.1. Synthesis

All chemicals were obtained from Sigma–Aldrich Co. Melting points were determined on the Büchi Melting Point apparatus B-540. Elemental analyses (C, H, N) were performed with a Perkin–Elmer 2400 CHNS/O analyzer. Infrared spectra were recorded

Table 5
Stability of compound **2o** in presented solutions and its rate constant k_{obs}.

Buffer or solution	pH	k_{obs} (s^{-1})
HCl 0.01 M	2.00	0.0028500
HCl 0.007 M	2.16	0.0019200
HCl 0.005 M	2.30	0.0017000
HCl 0.001 M	3.00	0.0005140
HCl 0.0005 M	3.30	0.0003220
Methoxyacetate buffer	3.35	0.0004250
Acetate buffer	4.55	0.0001140
Phosphate buffer	5.81	0.0000710
Imidazole buffer	7.23	0.0000130
N-Methylmorpholine buffer	7.86	0.0000120
TRIS buffer	8.92	0.0000550
Carbonate buffer	9.71	0.0000445
KOH 0.005 M	11.70	0.0001100
KOH 0.1 M	13.00	0.0000691

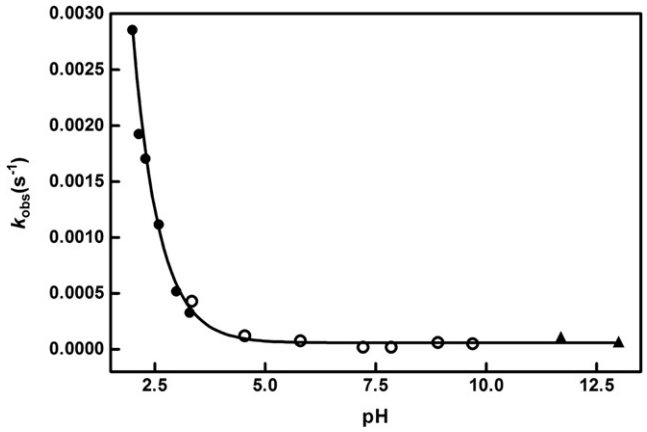


Fig. 3. Dependence of the observed rate constants (k_{obs}, s^{-1}) on pH measured at 37 °C in hydrochloric acid (●), buffers (○) and potassium hydroxide (▲) (Table 5). The ionic strength $I = 1$ mol L^{-1}.

on a Bio-Rad FTS 3000 MX spectrometer in KBr pellets. NMR spectra were measured on a Bruker Avance 300 operating at 300 MHz (^{1}H) and 75 MHz (^{13}C), and Bruker Avance III 500 MHz NMR instrument operating at 500 MHz (^{1}H), 125 MHz (^{13}C) and 50 MHz (^{15}N). The proton and carbon spectra were referenced to TMS as internal standard. The nitrogen chemical shifts were extracted from ^{1}H–^{15}N HMBC spectra. The reported ^{15}N chemical shifts were measured with respect to external 90% CH_3NO_2 in $CDCl_3$ and converted to the δ ^{15}N (liq. NH_3) = 0 ppm scale using the relation: δ ^{15}N (CH_3NO_2) = δ ^{15}N (liq. NH_3) + 380.50 ppm. Chemical shifts are given on the δ scale (ppm). Coupling constants (J) are given in Hz. Multiplicities are indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broadened). Atom numbering scheme for ^{1}H NMR spectra assignment is shown in Fig. 2. Phase sensitive NOESY with gradient pulses in mixing time, of **2a**, was recorded in DMSO-d_6 at 296 K with mixing time of 300 ms and relaxation delay of 2 s. Names of compounds were

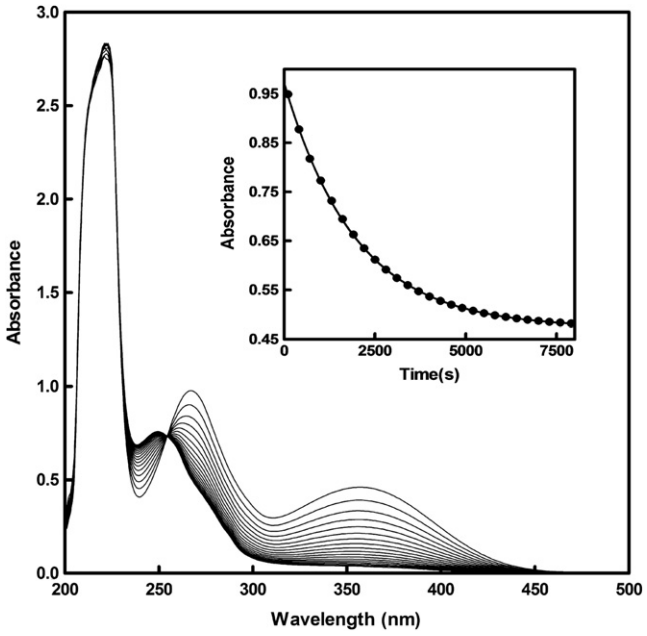


Fig. 4. The changes in UV–vis spectra for the decomposition of compound **2o** in 0.01 M hydrochloric acid solution at 37 °C. The inset represents time dependence of absorbance at 267 nm [$\tau_{1/2}$ = (243 ± 4) s].

generated with ACD/ChemSketch 12.01 and structures were drawn with ChemBioDraw Ultra 11.0 and are formatted as ACS Document 1996.

3.1.1. General procedures for the synthesis of hydrazonoformamides **2**

3.1.1.1. Method A. Compound **1**: Diethoxymethyl acetate (15 mmol) was added to a stirred solution of isoniazid (10 mmol) in acetonitrile (100 mL) at 55 °C. The reaction mixture was stirred for 30 min at the same temperature. The solvent was evaporated in vacuo and the crude product was washed with diethyl ether (2 × 20 mL). The product, ethyl isonicotinoylhydrazonoformate **1**, was dried in the air and recrystallized from acetonitrile [11].

Compound **2**: A solution of substituted aniline (1 mmol) in ethanol (1 mL) was added to a stirred solution of ethyl isonicotinoylhydrazonoformate **1** (1 mmol) in ethanol (10 mL) at 55 °C. The mixture was stirred for 6 h at the same temperature and for 20 h at room temperature. The product **2** was collected by filtration and recrystallized from the appropriate solvent.

3.1.1.2. Method B. Compound **3**: Diethoxymethyl acetate (411 mg, 3 mmol) was added to a stirred solution of substituted aniline (2 mmol) in acetonitrile (20 mL) at 40 °C. The reaction mixture was stirred for 30 min at the same temperature. The crude product **3** was filtered off and used without further purification for the following reactions.

Compound **2**: A solution of the intermediate **3** (1 mmol) in acetonitrile (1 mL) was added to a stirred solution of isoniazid (137 mg, 1 mmol) in acetonitrile (10 mL) at 55 °C. The mixture was stirred for 6 h at the same temperature and for 20 h at room temperature. The precipitate was collected by filtration and recrystallized from the appropriate solvent.

3.1.2. Data of compounds **2**

*3.1.2.1. N-(3-Hydroxyphenyl)-N′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2a**).* Yield 54% (Method A), 57% (Method B); mp 190–192 °C (ethanol). IR (KBr): 3226, 3085, 3030, 2871, 2601, 2488, 1669, 1631, 1599, 1544, 1497, 1445, 1414, 1366, 1306, 1247, 1164, 1062, 1003, 964, 909, 849, 764, 697, 688 cm^{-1}. Anal. Calcd for $C_{13}H_{12}N_4O_2$ (256.26): C, 60.93; H, 4.72; N, 22.86. Found: C, 61.08; H, 5.02; N, 21.98.

*3.1.2.2. N-(4-Hydroxyphenyl)-N′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2b**).* Yield 54% (Method A), 59% (Method B); mp 190–192 °C (ethanol). IR (KBr): 3212, 3150, 3069, 2610, 1668, 1634, 1601, 1676, 1549, 1519, 1458, 1412, 1366, 1314, 1247, 1170, 1105, 1059, 1005, 1067, 909, 826, 755, 690 cm^{-1}. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 11.06 (s, 1H, NH), 9.12 (s, 1H, NH), 8.71 (d, J = 4.9 Hz, 2H, H2, H6), 8.26 (s, 1H, CH), 7.79 (d, J = 5.1 Hz, 2H, H3, H5), 7.45 (s, 1H, OH), 7.19 (d, J = 7.7 Hz, 2H, H2′,H6′), 6.70 (d, J = 8.1 Hz, 2H, H3′, H5′). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.2, 152.3, 150.3 (2C), 148.4, 141.7, 132.9, 121.4 (2C), 118.4 (2C), 115.8 (2C). Anal. Calcd for $C_{13}H_{12}N_4O_2$ (256.26): C, 60.93; H, 4.72; N, 21.86. Found: C, 60.81; H, 5.15; N, 21.41.

*3.1.2.3. N-(3-Methoxyphenyl)-N′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2c**).* Yield 55% (Method A); mp 164–166 °C (ethanol). IR (KBr): 3041, 2360, 1671, 1645, 1634, 1621, 1600, 1549, 1500, 1474, 1464, 1410, 1360, 1317, 1288, 1230, 1159, 1047, 968, 907, 840, 776, 755, 689 cm^{-1}. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 11.16 (s, 1H, NH), 9.47 (s, 1H, NH), 8.73 (d, J = 5.8 Hz, 2H, H2, H6), 8.47 (d, J = 4.9 Hz, 1H, CH), 7.8 (d, J = 5.6 Hz, 2H, H3, H5), 7.16 (t, J = 8.1 Hz, 1H), 6.93 (s, 1H), 6.78 (d, J = 7.9 Hz, 1H), 6.49 (d, J = 8.1 Hz, 1H), 3.36 (s, CH_3). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.3, 150.4 (2C), 147.5, 142.2, 141.5, 130.2, 121.9, 121.4 (2C), 108.7, 106.7, 102.3, 55.2. Anal. Calcd for

$C_{14}H_{14}N_4O_2$ (270.36): C, 62.21; H, 5.22; N, 20.73. Found: C, 62.14; H, 5.46; N, 20.92.

*3.1.2.4. N-(4-Methoxyphenyl)-N′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2d**).* Yield 29% (Method A); mp 170–172 °C (ethanol). IR (KBr): 3421, 3208, 2836, 1667, 1620, 1549, 1535, 1515, 1466, 1365, 1319, 1308, 1290, 1247, 1179, 1035, 828, 685, 670 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.13 (s, 1H, NH), 9.27 (s, 1H, NH), 8.71 (d, $J = 5.7$ Hz, 2H, H2, H6), 8.35 (s, 1H, CH), 7.78 (d, $J = 5.5$ Hz, 2H, H3, H5), 7.29 (d, $J = 7.7$ Hz, 2H, H2′,H6′), 6.85 (d, $J = 8.0$ Hz, 2H, H3′, H5′), 3.70 (s, CH_3). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.1, 154.2, 150.4 (2C), 148.1, 141.6, 134.3, 121.4 (2C), 118.1 (2C), 114.6 (2C). Anal. Calcd for $C_{14}H_{14}N_4O_2$ (270.36): C, 62.21; H, 5.22; N, 20.73. Found: C, 62.40; H, 5.51; N, 20.82.

*3.1.2.5. N′-(Pyridin-4-ylcarbonyl)-N-[4-(trifluoromethyl)phenyl] hydrazonoformamide (**2e**).* Yield 42% (Method A); mp 191–193 °C (acetonitrile). IR (KBr): 3424, 3234, 3051, 2360, 1651, 1635, 1615, 1549, 1530, 1491, 1458, 1412, 1328, 1270, 1194, 1164, 1115, 1068, 1014, 834, 753, 669 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.09 (s, 1H, NH), 8.71 (d, $J = 4.5$ Hz, 3H, NH, H2, H6), 8.32 (s, 1H, CH), 7.78 (m, 2H, H3, H5), 7.30 (d, $J = 8.0$ Hz, 2H, H2′, H6′), 6.87 (d, $J = 8.2$ Hz, 2H, H3′, H5′). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.1, 154.2 (2C), 150.3, 148.0, 141.6, 134.3, 121.9, 121.4 (2C), 118.1 (2C), 114.6 (2C). Anal. Calcd for $C_{14}H_{11}F_3N_4O$ (308.27): C, 54.55; H, 3.60; N, 18.18. Found: C, 54.81; H, 3.93; N, 18.44.

*3.1.2.6. N-(3-Fluorophenyl)-N′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2f**).* Yield 62% (Method A), 80% (Method B); mp 180–182 °C (ethanol). IR (KBr): 3208, 1693, 1651, 1614, 1596, 1548, 1549, 1496, 1463, 1408, 1366, 1320, 1267, 1218, 1152, 1060, 1001, 970, 908, 845, 776, 682 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.22 (s, 1H, NH), 9.62 (s, 1H, NH), 8.79 (d, $J = 4.2$ Hz, 2H, H2, H6), 8.42 (s, 1H, CH), 7.73 (d, $J = 5.1$ Hz, 2H, H3, H5), 7.30 (m, 2H), 7.03 (d, $J = 6.7$ Hz, 1H), 6.71 (t, $J = 5.9$ Hz, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.5, 160.4, 150.4 (2C), 147.1, 142.9, 141.4, 130.9, 121.4 (2C), 112.5, 107.5 (q), 103.4 (q). Anal. Calcd for $C_{13}H_{11}FN_4O$ (258.25): C, 60.46; H, 4.29; N, 21.69. Found: C, 60.29; H, 4.06; N, 21.31.

*3.1.2.7. N-(4-Fluorophenyl)-N′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2g**).* Yield 39% (Method A), 61% (Method B); mp 191–193 °C (ethanol). IR (KBr): 3213, 3128, 3068, 2830, 1667, 1621, 1549, 1539, 1515, 1410, 1358, 1296, 1222, 1155, 1005, 965, 830, 754, 671 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.15 (s, 1H, NH), 9.03 (s, 1H, NH), 8.69 (d, $J = 4.6$ Hz, 2H, H2, H6), 8.38 (s, 1H, CH), 7.76 (d, $J = 5.0$ Hz, 2H, H3, H5), 7.38 (d, $J = 6.4$ Hz, 2H, H2′, H6′), 7.12 (d, $J = 6.9$ Hz, 2H, H3′, H5′). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.3, 158.77, 150.3 (2C), 147.7, 141.5, 137.4, 121.4 (2C), 118.2 (2C), 115.7 (q, 2C). Anal. Calcd for $C_{13}H_{11}FN_4O$ (258.25): C, 60.46; H, 4.29; N, 21.69. Found: C, 60.76; H, 4.05; N, 21.39.

*3.1.2.8. N-(3-Chlorophenyl)-N′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2h**).* Yield 54% (Method A), 77% (Method B); mp 176–178 °C (ethanol). IR (KBr): 3208, 1692, 1650, 1616, 1593, 1548, 1549, 1502, 1463, 1403, 1366, 1320, 1265, 1218, 1154, 1060, 1006, 970, 908, 845, 759, 682 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.21 (s, 1H, NH), 9.56 (s, 1H, NH), 8.77 (d, $J = 5.1$ Hz, 2H, H2, H6), 8.46 (s, 1H, CH), 7.71 (d, $J = 5.6$ Hz, 2H, H3, H5), 7.36 (m, 2H), 7.03 (d, $J = 7.1$ Hz, 1H), 6.92 (m, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.1, 150.2 (2C), 147.1, 142.8, 141.4, 130.9, 123.7, 122.3, 121.4 (2C), 118.2, 115.4. Anal. Calcd for $C_{13}H_{11}ClN_4O$ (274.71): C, 56.84; H, 4.04; N, 20.40. Found: C, 56.62; H, 4.36; N, 20.26.

*3.1.2.9. N-(3-Bromophenyl)-N′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2i**).* Yield 60% (Method A), 80% (Method B); mp 178–180 °C (ethanol). IR (KBr): 3201, 3050, 2892, 1673, 1632, 1593, 1548, 1481, 1408, 1378, 1314, 1283, 1219, 1070, 992, 908, 839, 763, 701, 681 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.21 (s, 1H, NH), 9.60 (s, 1H, NH), 8.73 (d, $J = 5.6$ Hz, 2H, H2, H6), 8.43 (d, $J = 7.0$ Hz, 1H, CH), 7.79 (d, $J = 5.8$ Hz, 2H, H3, H5), 7.60 (s, 1H), 7.42 (m, 1H), 7.21 (m, 1H), 7.07 (m, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.3, 150.4 (2C), 147.1, 142.6, 141.4, 131.2, 123.8, 122.4, 121.4 (2C), 118.7, 115.3. Anal. Calcd for $C_{13}H_{11}BrN_4O$ (319.16): C, 48.92; H, 3.47; N, 17.55. Found: C, 49.24; H, 3.86; N, 17.91.

*3.1.2.10. N-(4-Cchloro-2-hydroxyphenyl)-N′-(pyridin-4-ylcarbonyl) hydrazonoformamide (**2j**).* Yield 30% (Method A); mp 191 °C (ethanol). IR (KBr): 3193, 1693, 1633, 1549, 1488, 1411, 1360, 1317, 1261, 1222, 1061, 1023, 1002, 848, 682 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.02 (s, 1H, NH), 9.04 (s, 1H, NH), 8.73 (d, $J = 2.8$ Hz, 2H, H2, H6), 8.36 (s, 1H, CH), 8.14 (s, 1H), 7.94 (s, 1H, OH), 7.76 (d, $J = 3.9$ Hz, 2H, H3, H5), 7.54 (m, 1H), 6.84 (m, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.9, 151.6 (2C), 147.8, 143.9, 141.2, 130.2, 122.7, 121.0 (2C), 119.2, 115.4, 107.3. Anal. Calcd for $C_{13}H_{11}N_4O_2Cl$ (291.72): C, 53.71; H, 3.81; N, 19.27. Found: C, 54.02; H, 4.06; N, 18.83.

*3.1.2.11. N-(5-Chloro-2-hydroxyphenyl)-N′-(pyridin-4-ylcarbonyl) hydrazonoformamide (**2k**).* Yield 43% (Method A); mp 207–208 °C (ethanol). IR (KBr): 3219, 1695, 1635, 1587, 1542, 1515, 1413, 1389, 1324, 1285, 1060, 1010, 848, 684 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.27 (s, 1H, NH), 9.03 (s, 1H, NH), 8.72 (d, $J = 4.7$ Hz, 2H, H2, H6), 8.31 (d, $J = 17.9$ Hz, 1H, CH), 8.15 (s, 1H), 7.77 (d, $J = 4.9$ Hz, 2H, H3, H5), 7.59 (d, $J = 5.5$ Hz, 1H), 7.54 (s, 1H, OH), 6.81 (m, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.6, 150.6 (2C), 147.8, 144.9, 141.5, 130.2, 122.9, 121.4 (2C), 118.2, 115.4, 108.3. Anal. Calcd for $C_{13}H_{11}N_4O_2Cl$ (291.72): C, 53.71; H, 3.81; N, 19.27. Found: C, 53.39; H, 3.61; N, 19.55.

*3.1.2.12. N-(3,4-Dichlorophenyl)-N′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2l**).* Yield 33% (Method A), 32% (Method B); mp 192 °C (acetonitrile). IR (KBr): 3443, 2988, 1693, 1633, 1549, 1487, 1411, 1360, 1316, 1262, 1061, 1023, 849, 684 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.02 (s, 1H, NH), 9.78 (s, 1H, NH), 8.73 (d, $J = 4.9$ Hz, 2H, H2, H6), 8.54 (s, 1H, CH), 8.25 (s, 1H), 7.76 (s, 1H), 7.54 (d, $J = 4.9$ Hz, 2H, H3, H5), 7.02 (m, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.5, 150.7 (2C), 147.2, 142.3, 140.1, 131.8, 130.2, 122.9, 121.2 (2C), 116.9, 110.0. Anal. Calcd for $C_{13}H_{10}N_4OCl_2$ (309.16): C, 50.51; H, 3.26; N, 18.12. Found: C, 50.12; H, 3.66; N, 18.47.

*3.1.2.13. N-(3,4-Difluorophenyl)-N′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2m**).* Yield 24% (Method A), 30% (Method B); mp 193 °C (ethanol). IR (KBr): 3189, 2915, 1694, 1633, 1557, 1487, 1411, 1361, 1261, 1183, 1151, 1061, 1023, 1003, 920, 848, 752, 682 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.09 (s, 1H, NH), 9.80 (s, 1H, NH), 8.73 (d, $J = 5.4$ Hz, 2H, H2, H6), 8.54 (s, 1H, CH), 8.36 (m, 1H), 7.76 (s, 1H), 7.53 (d, $J = 5.8$ Hz, 2H, H3, H5), 7.10 (m, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 161.7, 151.2 (2C), 148.1, 141.9, 140.5, 132.2, 131.4, 122.6, 121.2 (2C), 117.9, 112.1. Anal. Calcd for $C_{13}H_{10}N_4OF_2$ (277.26): C, 56.52; H, 3.65; N, 20.28. Found: C, 56.12; H, 3.91; N, 19.89.

*3.1.2.14. N-(3-Chloro-4-fluorophenyl)-N′-(pyridin-4-ylcarbonyl) hydrazonoformamide (**2n**).* Yield 22% (Method A), 45% (Method B); mp 193–195 °C (ethanol). IR (KBr): 3200, 2915, 1686, 1632, 1602, 1549, 1489, 1411, 1360, 1261, 1222, 1062, 905, 848, 682 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.03 (s, 1H, NH), 9.78 (s, 1H, NH), 8.74 (d, $J = 4.9$ Hz, 2H, H2, H6), 8.38 (s, 1H, CH), 8.14 (m, 1H), 7.76 (s, 1H), 7.53 (d, $J = 4.9$ Hz, 2H, H3, H5), 6.99 (m, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.8, 158.1, 150.2 (2C), 147.0, 145.7, 140.4, 133.4, 126.8, 121.5 (2C), 115.8, 110.9. Anal. Calcd for $C_{13}H_{10}N_4OClF$ (293.71): C, 53.35; H, 3.44; N, 19.14. Found: C, 53.74; H, 3.09; N, 19.46.

*3.1.2.15. N-(4-Bromo-3-fluorophenyl)-N′-(pyridin-4-ylcarbonyl) hydrazonoformamide (**2o**).* Yield 12% (Method A), 17% (Method B); mp 193–195 °C (ethanol). IR (KBr): 3201, 2989, 1686, 1631, 1602, 1548, 1489, 1411, 1359, 1261, 1222, 1062, 905, 848, 682 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.03 (s, 1H, NH), 9.80 (s, 1H, NH), 8.74 (d, $J = 4.5$ Hz, 2H, H2, H6), 8.40 (d, $J = 13.2$ Hz, 1H, CH), 8.15 (s, 1H), 7.77 (d, $J = 5.5$ Hz, 2H, H3, H5), 7.54 (m, 1H), 7.00 (m, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.4, 157.4, 150.4 (2C), 146.7, 142.3, 141.1, 133.8, 121.4 (2C), 114.1, 105.0, 98.3. Anal. Calcd for $C_{13}H_{10}N_4OBrF$ (338.17): C, 46.31; H, 2.99; N, 16.62. Found: C, 46.01; H, 3.39; N, 16.41.

*3.1.2.16. N′-(Pyridin-4-ylcarbonyl)-N-[3-(trifluoromethyl)phenyl] hydrazonoformamide (**2p**).* Yield 71% (Method B); mp 189–191 °C (acetonitrile). IR (KBr): 3240, 3100, 3041, 1657, 1633, 1600, 1559, 1545, 1497, 1478, 1340, 1324, 1263, 1206, 1188, 1172, 1132, 1105, 1072, 999, 901, 881, 833, 791, 710, 697, 670, 639 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.25 (s, 1H, NH), 9.78 (d, $J = 6.0$ Hz, 1H, NH), 8.74 (d, $J = 5.8$ Hz, 2H, H2, H6), 8.52 (d, $J = 5.6$ Hz, 1H, CH), 7.84 (d, $J = 4.2$ Hz, 1H), 7.76 (d, $J = 5.7$ Hz, 2H, H3, H5), 7.52 (m, 2H), 7.24 (d, $J = 6.9$ Hz, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.3, 150.4 (2C), 147.1, 141.7, 141.3, 130.4, 130.0, 124.2 (q, $J = 259.6$ Hz, CF_3), 121.4 (2C), 120.1, 117.5, 112.5. Anal. Calcd for $C_{14}H_{11}F_3N_4O$ (308.27): C, 54.55; H, 3.60; N, 18.18. Found: C, 54.54; H, 3.25; N, 18.51.

*3.1.2.17. N-(4-Chlorophenyl)-N′-(pyridin-4-ylcarbonyl)hydrazono-formamide (**2r**).* Yield 34% (Method B); mp 178–180 °C (ethanol). IR (KBr): 3234, 1634, 1586, 1494, 1407, 1314, 1289, 1262, 1093, 1011, 820, 675 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.20 (s, 1H, NH), 9.57 (d, $J = 6.4$ Hz, 1H, NH), 8.73 (d, $J = 4.4$ Hz, 2H, H2, H6), 8.42 (d, $J = 6.0$ Hz, 1H, CH), 7.75 (d, $J = 4.5$ Hz, 2H, H3, H5), 7.34 (d, $J = 4.4$ Hz, 2H, H2′, H6′), 7.20 (d, $J = 8.7$ Hz, 2H, H3′, H5′). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.3, 150.4 (2C), 147.2, 141.4, 139.9, 129.1 (2C), 124.8, 121.4 (2C), 118.1 (2C). Anal. Calcd for $C_{13}H_{11}N_4OCl$ (274.71): C, 56.84; H, 4.04; N, 20.40. Found: C, 56.90; H, 4.31; N, 20.49.

*3.1.2.18. N-(4-Bromophenyl)-N′-(pyridin-4-ylcarbonyl)hydrazono-formamide (**2s**).* Yield 49% (Method B); mp 186–188 °C (ethanol). IR (KBr): 3220, 1699, 1631, 1586, 1548, 1491, 1313, 1258, 1075, 1004, 846, 815, 693, 676 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.21 (s, 1H, NH), 9.58 (d, $J = 7.1$ Hz, 1H, NH), 8.72 (d, $J = 6.1$ Hz, 2H, H2, H6), 8.42 (d, $J = 6.7$ Hz, 1H, CH), 7.74 (d, $J = 6.0$ Hz, 2H, H3, H5), 7.44 (d, $J = 8.9$ Hz, 2H, H2′, H6′), 7.29 (d, $J = 8.9$ Hz, 2H, H3′, H5′). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.4, 150.5 (2C), 147.2, 141.5, 140.3, 132.1 (2C), 121.5 (2C), 118.6 (2C), 112.7. Anal. Calcd for $C_{13}H_{11}N_4OBr$ (319.17): C, 48.92; H, 3.47; N, 17.55. Found: C, 49.20; H, 3.81; N, 17.89.

*3.1.2.19. N-(3-Nitrophenyl)-N′-(pyridin-4-ylcarbonyl)hydrazono-formamide (**2t**).* Yield 39% (Method B); mp 190–191 °C (ethanol). IR (KBr): 3232, 3080, 1641, 1636, 1530, 1350, 1265, 1209, 1149, 1068, 998, 840, 736, 678 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 11.29 (s, 1H, NH), 9.93 (d, $J = 7.3$ Hz, 1H, NH), 8.74 (d, $J = 6.0$ Hz, 2H, H2, H6), 8.56 (d, $J = 7.2$ Hz, 1H, CH), 8.27 (s, 1H), 7.84 (d, $J = 5.3$ Hz, 1H), 7.77 (d, $J = 1.9$ Hz, 2H, H3, H5), 7.69 (d, $J = 7.6$ Hz, 1H), 7.58 (m, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 160.4, 150.4 (2C), 148.7, 146.8, 142.1, 141.3, 130.6, 122.7, 121.4 (2C), 115.8, 110.4. Anal. Calcd for $C_{13}H_{11}N_5O_3$ (285.26): C, 54.74; H, 3.89; N, 24.55. Found: C, 54.49; H, 4.12; N, 24.43.

3.2. In vitro biological assays

3.2.1. In vitro antimycobacterial evaluation on M. tuberculosis 331/88, M. avium 330/88, M. kansasii 235/80 and M. kansasii 6509/96 cultures

In vitro antimycobacterial activity was evaluated against *M. tuberculosis* CNTC My 331/88, *M. avium* CNTC 330/88, *M. kansasii* CNTC My 235/80 and *M. kansasii* 6509/96. All strains were obtained from the Czech National Collection of Type Cultures (CNCTC) with exception of *M. kansasii* 6509/96, which is a clinical isolate. Antimycobacterial activity was measured in Sula semi-synthetic medium (SEVAC, Prague) at 37 °C. Compounds were dissolved in dimethyl sulfoxide solution (max. 5% DMSO in water) and added to the medium within a concentration range 1000, 500, 250, 125, 62, 31, 16, 8, 4, 2 and 1 μmol L^{-1}. Minimal inhibitory concentration (MIC) was determined after incubation at 37 °C for 7, 14 and 21 days.

3.2.2. In vitro cytotoxicity of compounds by MTT assay

HepG2 human hepatoma cells (ATCC HB-8065) and freshly prepared human PBMC (peripheral blood mononuclear cells) [14] were cultured in RPMI-1640 medium without phenol red supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 160 μg mL^{-1} gentamycin [15,16]. Cell cultures were maintained at 37 °C, 5% CO_2 in water-saturated atmosphere.

Cells were plated into 96-well plate with initial cell number of 5×10^3 per well (in the case of PBMC 2.0×10^5 cells/well). After 24 h incubation at 37 °C prior to the experiment, cells were treated with compounds in 100 μL serum free medium overnight. Control cells were treated with serum free medium. Four parallel measurements were performed in all cases.

After overnight incubation at 37 °C, the cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay [17,18]. Then 45 μL MTT-solution (2 mg mL^{-1}) was added to each well. The respiratory chain [14] and other electron transport systems [19] reduce MTT and thereby form non-water-soluble violet formazan crystals within the cell [20]. The amount of these crystals can be determined spectrophotometrically and serves to estimate the number of mitochondria and hence the number of living cells in the well [21]. After 4 h of incubation, cells were centrifuged for 5 min (2000 rpm) and supernatant was removed. The obtained formazan crystals were dissolved in 50 or 100 μL DMSO and optical density (OD) of the samples was measured at $\lambda = 540$ nm and $\lambda = 620$ nm using ELISA Reader (iEMS Reader, Labsystems, Finland). OD_{620} values were subtracted from OD_{540} values. The percent of cytotoxicity was calculated using the following equation:

$$\text{Cytotoxicity (\%)} = [1 - (OD_{treated}/OD_{control})] \times 100;$$

where $OD_{treated}$ and $OD_{control}$ correspond to the optical densities of the treated and the control cells, respectively. In each case, two independent experiments were carried out with 4–8 parallel measurements. The 50% inhibitory concentration (IC_{50}) values were determined from the dose-response curves. The curves were defined using MicrocalTM Origin1 (version 7.5) software.

3.3. Stability testing

All of the kinetic measurements were carried out in a 1 cm closable cell using a Hewlett–Packard 8453 diode array spectrophotometer at 37 ± 0.1 °C. The observed pseudo-first-order rate constants k_{obs} were calculated from absorbance-time dependences at the wavelength of $\lambda = 267$ nm and at the substrate concentrations of ca. 5×10^{-5} mol L^{-1}. Ionic strength $I = 1$ mol L^{-1} was adjusted by KCl. In all kinetic runs, the standard deviation in the fit was always less than 1% of the quoted value and was more usually between 0.2 and 0.4% of the quoted value. The pH of individual buffers was measured using PHM 93 Radiometer Copenhagen apparatus equipped with glass electrode. Redistilled water, commercially available substituted acetic acids, amines, and potassium chloride (p.a.) for adjustment of ionic strength of buffer solutions were used.

4. Conclusion

A new series of isoniazid hydrazones linked by the CH fragment with substituted anilines possessing electron-withdrawing substituents were prepared by two synthetic approaches. Nearly all compounds exhibited the same inhibitory effect as a standard (INH, MIC 1 μmol L^{-1}), they were more active against *M. kansasii* (MIC 62.5–125 μmol L^{-1}). The best activity was observed for *N*-(4-bromo-3-fluorophenyl)-*N*′-(pyridin-4-ylcarbonyl)hydrazonoformamide (**2o**) (*M. tuberculosis* 1 μmol L^{-1}; *M. avium* 250 μmol L^{-1}; *M. kansasii* 62.5 μmol L^{-1}; *M. kansasii* clinical isolate 8 μmol L^{-1}). This compound is stable under neutral pH, slowly hydrolyses under acidic conditions and exhibits promising selectivity index ($SI = 162$) that predetermines it for further screening.

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