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Synthesis, antimycobacterial activity and docking study of 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives and related hydrazide-hydrazones

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ABSTRACT

A new convenient method for preparation of 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives **5b–g** and coumarin containing hydrazide-hydrazone analogues **4a–e** was presented. The antimycobacterial activity against reference strain *Mycobacterium tuberculosis* H37Rv and cytotoxicity against the human embryonic kidney cell line HEK-293 were tested *in vitro*. All compounds demonstrated significant minimum inhibitory concentrations (MIC) ranging 0.28–1.69 μM, which were comparable to those of isoniazid. The cytotoxicity (IC_{50} > 200 μM) to the "normal cell" model HEK-293T exhibited by 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives **5b–e**, was noticeably milder compared to that of their hydrazone analogues **4a–e** (IC_{50} 33–403 μM). Molecular docking studies on compounds **4a–e** and **5b–g** were also carried out to investigate their binding to the 2-*trans*-enoyl-ACP reductase (InhA) enzyme involved in *M. tuberculosis* cell wall biogenesis. The binding model suggested one or more hydrogen bonding and/or arene-H or arene-arene interactions between hydrazones or pyrazole-fused coumarin derivatives and InhA enzyme for all synthesized compounds.

A reemergence of tuberculosis accompanied by an increasing number of drug resistant *Mycobacterium tuberculosis* strains highlights the urgent need of searching and developing of new antitubercular drugs, capable of bypassing the resistance mechanisms. In the last decades, major advances in molecular biology have increased the knowledge of the mechanisms of resistance to the main anti-TB drugs, with the identification of specific gene mutations that are associated with drug resistance[1,2]

Isoniazid (INH), an essential antitubercular agent recommended by the WHO, is a prodrug that penetrates the tubercle bacilli by passive diffusion and is bio-activated by the bacterial anti-oxidant enzyme (KatG).[3,4] It exerts its anti-tubercular activity *via* interference with the synthesis of mycolic acids, which comprise crucial elements of the mycobacterial cell wall**.** Even with the clinical success of isoniazid, severe adverse effects, especially peripheral neuropathy and hepatotoxicity, are associated with INH-based treatment protocols; moreover its usefulness is further limited by the occurrence of resistance.[5] To overcome the resistance,[6] the drug design strategies frequently employ a combination of the INH molecule with other pharmacophores, rendering antitubercular activity. The novel INH hydrazide derivatives appeared to be promising anti-tubercular agents - more effective and less hepatotoxic than isoniazid.[7–16] In the meantime Ellis *at al.*,[17] have described the mechanism of action of the pyridoxal isonicotinoyl hydrazones (PIC) and suggested that hydrazones act as a lipophilic vehicle for the transport of its intact INH moiety into the mammalian cell and the mycobacterium (Fig. 1). The mechanism of antimycobacterial activity of INH[18] and isonicotinoyl hydrazone derivatives passes through formation of electrophilic intermediate species (i.e. a hydrazyl radical or ion) (Fig. 1). The acyl radical being coupled to NADH or NAD + seems to be crucial in yielding adduct responsible for the inhibition of 2-*trans*-enoyl-ACP reductase (InhA), and in restraining the mycobacterial cell wall synthesis. InhA catalyze the final step in the elongation cycle of the bacterial

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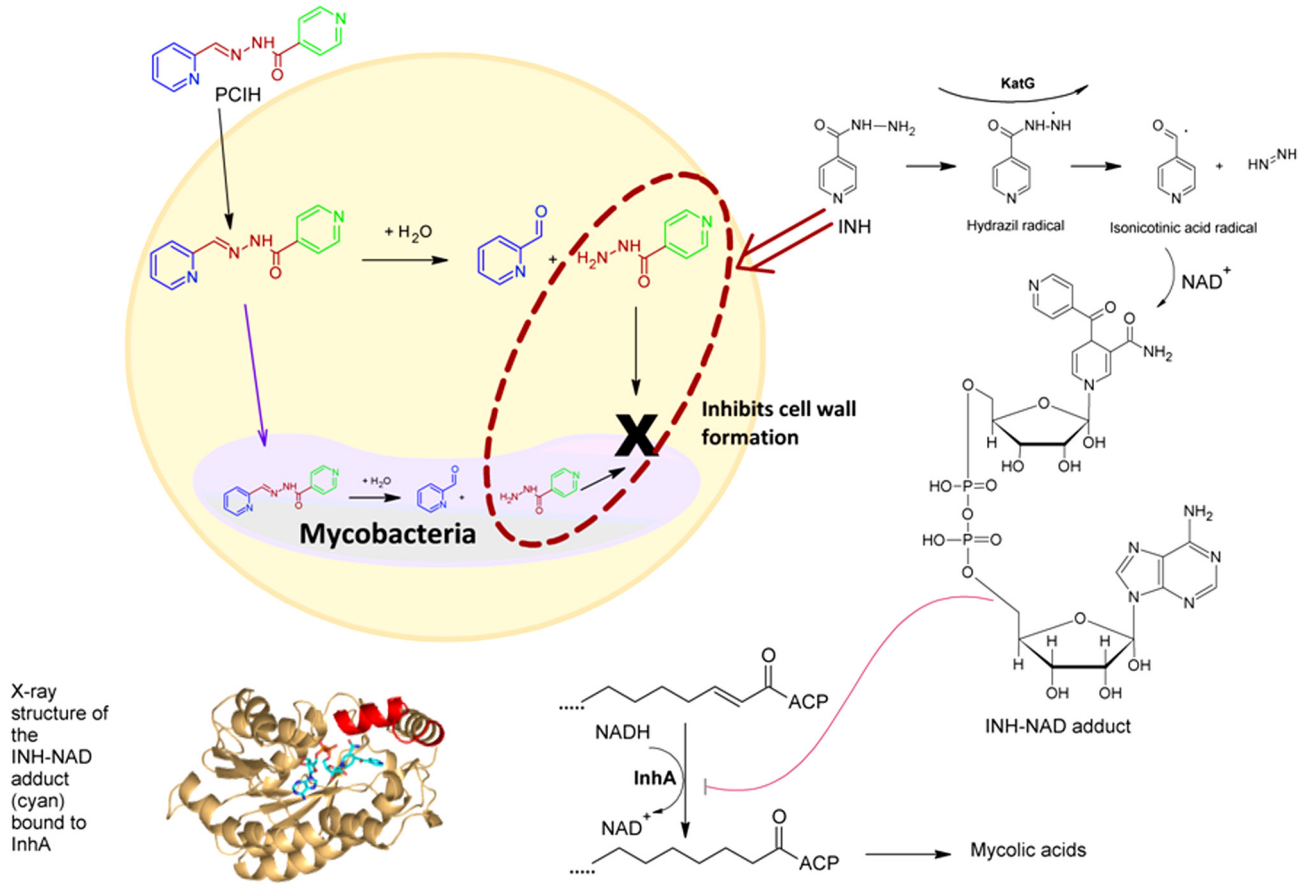


Fig. 1. Schematic representation of the proposed mechanism of action of isoniazid (INH)[18] and hydrazone derivative (2-pyridylcarboxaldehydeisonicotinoylhydrazone – PCIH).[17] X-ray structure of the INH-NAD adduct (cyan) bound to InhA (1zid.pdb).[27] The substrate binding loop is colored red. This portion of the protein (res 196–211) is disordered in the structure of triclosan bound to InhA (2b35.pdb).[24].

fatty acid biosynthesis pathway (type-II fatty acid elongation system- FAS-II) has been recognized as a primary molecular target for developing new anti-TB drugs[19,20] (Fig. 1). There is also evidence that INH can inhibit non-NAD(P) binding proteins such as KasA, the β-ketoacyl-ACP synthase in the FASII pathway.[21] Importantly, since mutations of KatG are the most common mechanisms of resistance to INH,[22,23] inhibitors that bind to the final drug target (s) should be active against the majority of INH-resistant clinicaly isolated strains.[24] Molecular investigation on the genetic basis of the INH-resistance in *M. tuberculosis* strains in Bulgaria targeted two the most frequently reported mutations related to INH resistance, katG 315AGC > ACC and inhA −15C > T[25,26] The global prevalence of the katG S315T substitution in INH-resistant strains highlights the selective advantage conferred by this mutation, which appears to provide the optimal balance between decreased catalase activity and a sufficiently high level of peroxidase activity in KatG. Mutations in the inhA promoter region are thought to increase the InhA protein expression, thereby elevating the drug target levels and producing INH resistance by a drug titration mechanism.

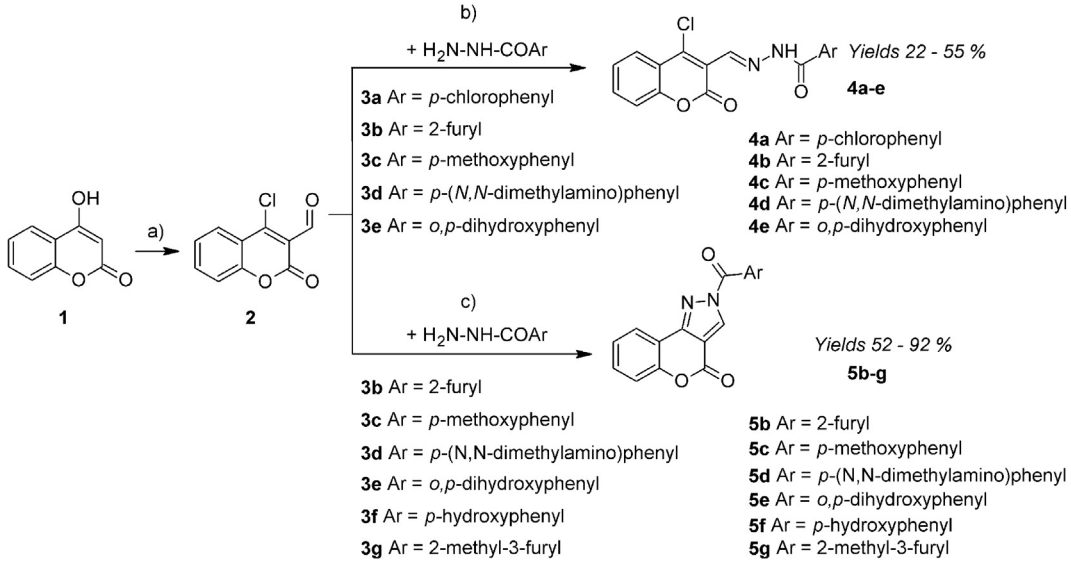
This highlights the crucial need of developing drugs with shorter, simpler regimens as well as with novel mechanisms of action that can be used for treatment of drug resistant forms of the disease. Numerous efforts have been undertaken to develop new INH derivatives and hydrazones[28,29] as anti-TB agents (Saluzide, Ftivazide, Salizide).[30,31] Indeed, the INH derivative LL-3858 is in initial stages of phase II clinical trial for the treatment of TB and may be approved to treat TB in the near future.

Recently we described a series of hydrazide-hydrazones containing coumarin or 2*H*-chromene moieties with potent anti-TB activity and moderate cytotoxicity.[32] Having surveyed comprehensively the literature on pyrazole[33–36] and coumarin[34,37–46] scaffolds as important structural components in the design of anti-tuberculosis agents against *Mycobacterium tuberculosis* we focused on the synthesis of pyrazole-fused coumarin derivatives as less toxic antimycobacterial compounds.

Herein, continuing the research on the biological activity of 2*H*-chromene derivatives, we present the one-pot synthesis, antimycobacterial activity and cytotoxicity of novel 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives **5b–g**. Molecular docking studies were carried out with the InhA enzyme from *M. tuberculosis* in order to explore the structural requirements controlling the observed antimycobacterial activity of **4a–e** and **5b–g**.

The synthesis of hydrazide-hydrazones **4a–e** and *N*-2-substituted coumarin[4,3-*c*]pyrazoles **5b–g** is shown in Scheme 1.

The synthesis of coumarin based hydrazones **4a–e** was achieved by a classical method reported previously.[33] It involves a reaction of 4-chlorocoumarin-3-carbaldehyde **2** with corresponding hydrazides **3a–e** in abs. EtOH. The detailed investigation of the reaction showed that fused 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one was formed easily when an electron donating group was present at *p*-position in the benzene ring. The reaction of 4-chlorocoumarin-3-carbaldehyde **2** with **3b–g** in EtOH:CH_2Cl_2 (at a 1:3 ratio) resulted in *N*-2-substituted chromeno[4,3-*c*]pyrazol-4-one analogues **5b–g** (Scheme 1) in nearly quantitative yields (85–92%). Compounds **5b–g** were further purified by column



Scheme 1. Synthetic route of coumarin containing hydrazones **4a**–**e** and 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives **5b**–**g**. Reaction conditions: a) $POCl_3$, DMF, 60 °C;[34] b) abs. EtOH, r.t., 15 min; c) CH_2Cl_2/EtOH (1:3), r.t., 15 min–1 h.

chromatography (ethyl acetate/hexane at a 1:10 ratio) before being tested for biological activity. The structure of the newly synthesized coumarin derivatives was confirmed by a combination of ^{1}H NMR, ^{13}C NMR, HR-MS and FTIR (ATR) spectral techniques (see Supplementary data, Fig. S1). In addition an X-ray crystallographic study of compound **5d** was reported (Fig. 2), (Supplementary data, Fig. S2, Table S2, Table S3).

The newly synthesized compounds **4a**–**d** and **5c**–**e** displayed chemical stability under the conditions of antimycobacterial activity determination in DMSO-d_6. No changes were detected in their ^{1}H NMR spectra taken in dimethyl sulfoxide-d_6 after 1, 2, 3 and 12 days at 20 °C of the NMR tubes with the initially prepared solutions (Supplementary data Fig. S2 and S3). No changes also were detected in their ^{1}H NMR spectra in 0.6 ml DMSO-d_6 and 0.05 ml assay media Middlebrook 7H9 (pH 6.6) for 16 h and the compound remained unchanged upon heating to 37 °C the NMR tubes with the initially prepared solutions (Supplementary data Fig. S4 and S5).

The *in vitro* antimycobacterial activity of all compounds against reference strain *Mycobacterium tuberculosis* H37Rv was evaluated using the resazurin microtiter assay (REMA) described by Martin et al.[47] Ethambutol (EMB) and isoniazid (INH) were used as controls. The data for all compounds are shown in Table 1.

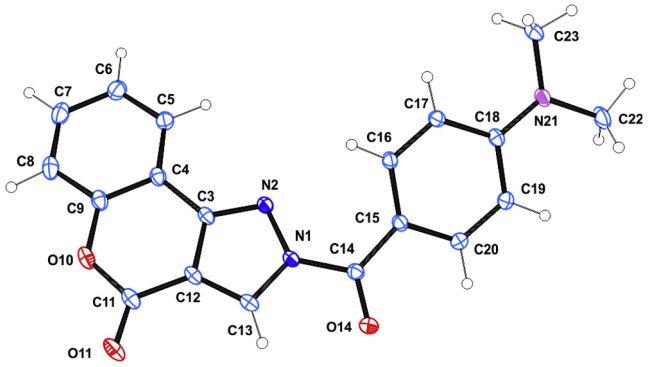
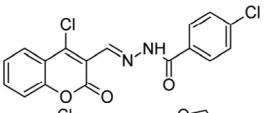
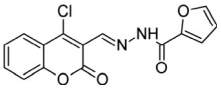
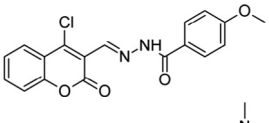
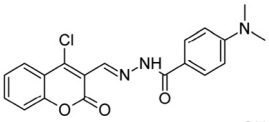
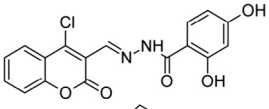
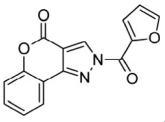
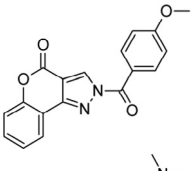
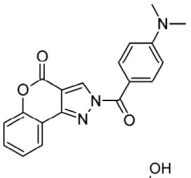
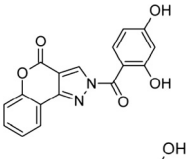
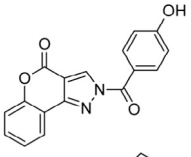
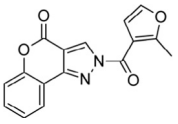


Fig. 2. ORTEP view of the molecular structure of **5d**; atomic displacement parameters are at 50% probability, H atoms are shown as spheres with arbitrary radii.

The tested series exhibited prominent antimycobacterial activity against the chosen strain, whereby the MIC values were comparable to those of INH and lower than those of EMB. The cytotoxicity of the selected compounds was evaluated against the human embryonal kidney cell line 293 T, after 72 h of continuous exposure, using the MTT-dye reduction assay.[48]The tested agents caused a 50% cell growth inhibition within the experimental concentration range (12.5–100 μM). In addition, the selectivity index values (SI) were determined as the IC_{50}/MIC ratio. All the compounds were found highly potent against the *M. tuberculosis* H37Rv demonstrating a nanomolar-to-low-micromolar activity (MICs ranging 0.32 to 1.69 μM). The most active compounds **4c** (MIC = 0.28 μM) and **5f** (MIC = 0.32 μM) with *p*-methoxyphenyl and *p*-hydroxyphenyl substituents, respectively, demonstrated a remarkable minimal associated cytotoxicity (IC_{50} = 112.9 μM and IC_{50} > 200.00 μM, respectively). The activity of compounds **4a**–**e** was comparable to that of the hydrazone derivatives presented in our previous study.[22] However, the cytotoxicity of 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives **5b**–**g**, to the "normal cell" model HEK-293 T (IC_{50} > 200 μM) was noticeably milder compared to the one of their open chain hydrazone analogues **4a**–**e** (33–403 μM). The difference in the cytotoxic potential resulted in much better SI values exhibited by derivatives **5b**–**g**. Hence, the 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives **5b**–**g** possess potent antimycobacterial activities combined with low cytotoxicity resulting in SI values higher than those of their open chain analogues **4a**–**e** (Table 1). It was observed that the presence of -OH, OMe and -N(Me)$_2$ functional groups at 4th position in the phenyl ring enhanced the antimycobacterial activity while any alteration in this substitution pattern caused a substantial decrease of the anti-tubercular potency. The addition of a second hydroxyl group in compound **5e** gave a derivative slightly less potent (MIC 0.62 μM) than monohydroxy derivative **5f**. All tested hydrazones, except **4b**, were more lipophilic than INH (Table 1) with log P values higher than those of the controls. It is well known that increasing the lipophilicity is important for the transport through the mycobacterial cell.[49] We observed no correlation between the antimycobacterial potency and log P values within the tested series.

To explain the activity order of **4a**–**e** and **5b**–**g** against *M. tuberculosis* the compounds were docked into the binding site of

Table 1
Antimycobacterial activity and *in vitro* cytotoxicity.

Compd.	Structure	MIC[a](μM)	IC_{50}[b](μM)	SI[c]	Log P[d]
4a		0.55	21.00	33	2.18
4b		0.63	63.62	236	0.61
4c		0.28	112.9	403	1.41
4d		0.54	18.70	20	2.75
4e		0.56	–	–	2.69
5b		0.72	>200.00	>282	1.29
5c		0.62	>200.00	>645	2.09
5d		0.59	>200.00	>408	2.61
5e		0.62	>200.00	>308	1.78
5f		0.32	>200.00	>625	2.06
5g		1.69	>200.00	>119	1.54
EMB.2HCl[e]		1.46	–	–	0.77
INH[e]		0.79	–	–	−0.63

[a] Antimycobacterial activity against reference strain of *Mycobacterium tuberculosis* H37Rv.
[b] *In vitro* cytotoxicity to human embryonal kidney cell line 293 T, IC_{50} (μM).
[c] Selectivity index, SI ratio = IC_{50}/MIC.
[d] LogP was calculated by Marvin 16.2.8.0 (http://www.chemaxon.com).
[e] EMB.2HCl (ethambutol dihydrochloride-reference compound) and [e]INH (Isoniazid - reference compound).

mycobacterial enoyl reductase (InhA). Two docking solutions can be considered as "standard" in a typical docking analysis, namely: 1) *the top score* – the pose with the highest rank; 2) *the best pose* – the pose with lowest root mean square deviation (RMSD) to the reference ligand from the experimentally solved structure. ( ). The docking scores (S_value) of ligands **4a–e** and **5b–g** were found satisfactory in the range of -7.72 to -6.74 (See Supplementary data – Table S1 lists the essence of docking results for the synthesized compounds). The results presented in Table S1 showed that the lowest RMSD was obtained for 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one **5g**, followed by one of the most active compounds **5f**, while the highest rank was recorded for *p*-(dimethylamino)phe-

nyl hydrazone derivative **4d**. The docking scores of the most active compounds of the series, **4c** and **5f**, were found to be −7.27 and −6.89, respectively.

The protein-ligand interactions (PLI) diagram of the co-crystallized ligand of *M. tuberculosis* InhA inhibited by PT70 in the ligand-binding domain is presented in Fig. 2. It was obtained using "Ligand Interactions" tool of Molecular Operating Environment of Chemical Computing Group (MOE, version 2016.08, https://www.chemcomp.com/MOE-Molecular_Operating_Environment.htm). Docking simulations were performed using the only published X-ray crystallographic structure of InhA from species of *Mycobacterium tuberculosis* (ENR) complexed with 5-hexyl-2-(2-methylphenoxy)phenol (PT70) and co-factor nicotinamide adenine dinucleotide (NAD^+). The crystal structure of *M. tuberculosis* InhA inhibited by PT70 was extracted from Protein Data Bank (http://www.rcsb.org/, PDB ID 2X22). During the docking process, water molecules and all the ligands in the crystal structures were removed, except the co-factor NAD^+. Protein-ligand interactions illustrated in Figures 3 and 4 were in the maximum distance of 4.5 Å÷ between heavy atoms of the ligand and receptor. As seen from Fig. 3, three residues were involved in interactions, namely Phe149, Tyr158 and Met161. Residues Ala198, Met199, Ile202 and Val203 should be explicitly mentioned as very important, since they are involved in the substrate-binding loop.[50] Fig. 3 demonstrated all of them at ligand exposure. Fig. 4a presents the PLI diagrams of the most active compounds **4c** and **5f** and Fig. 4b – the docking conformations and corresponding Connolly surface.

As seen from Figure 4a (left), compound **4c** demonstrated two newly appeared interactions with Met199 (HB) and Leu218 (arene-H interaction with coumarin ring), that was at ligand exposure, but not exhibiting any strong interaction in Fig. 3. All the rest mentioned as important residues were still very close. Compound **5f** (Fig. 4a, right) repeated the interaction with Phe149 and demonstrated one newly appeared interaction with Leu218 (arene-H interaction with coumarin ring). As in the case of compound **4c,** all the rest mentioned as important residues were still very close. It should be noted that both most active TB compounds **4c** and **5f** demonstrated one of the interactions of compound **4d**, ranked by the docking as *the top score* – the pose with the highest rank (See Suppl. Table S1).

All synthesized compounds **4a–e** and **5b–g** as docked in the ligand-binding domain of *M. tuberculosis* InhA inhibited by PT70 with Connolly surface are presented on Fig. 5. As seen from the figure, all synthesized compounds occupied the same binding site as that of PT70 and NAD+, formed a cluster, fitted well in the ligand-binding domain of *M. tuberculosis* InhA and always showed one or more specific protein-ligand interactions as listed in Supplementary data Table S1. This result indicated that the coumarin structure or pyrazole-fused coumarin scaffold was the key fragment for binding of the compound in the InhA pocket and is crucial to attaining favorable MIC values, whereas the substituents in the benzene ring were a key group to enhance MIC values.

The docking study analysis of the tested compounds with respect to the reward for hydrogen bonding revealed compounds **5b–g** to have similar docking scores but protein-ligand interactions different from those of compounds **4a–e**. The results of enzyme inhibition activity of the substituents at *p*-position in hydrazone derivatives showed the following trend: (*p*-$N(Me)_2$ > (*p*-MeO phenyl); *p*-chloro phenyl, *o,p*-diOH phenyl) > (2-furyl moiety). In the case of substitution at aroyl moiety in the coumarin fused pyrazoles, the substitution with electrondonating groups at the benzene or furyl ring was favored. In addition, any discrepancy

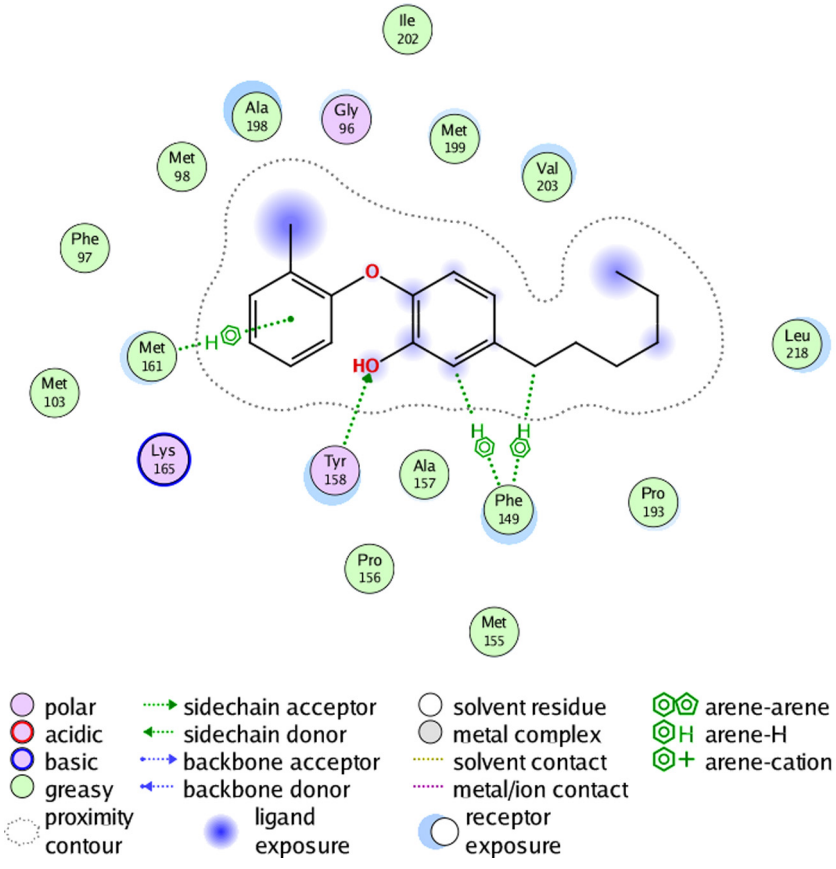


Fig. 3. Interaction diagram of the ligand-binding domain of *M. tuberculosis* InhA inhibited by PT70 with TCU (5-hexyl-2-(2-methylphenoxy)phenol) (PDB ID 2X22).

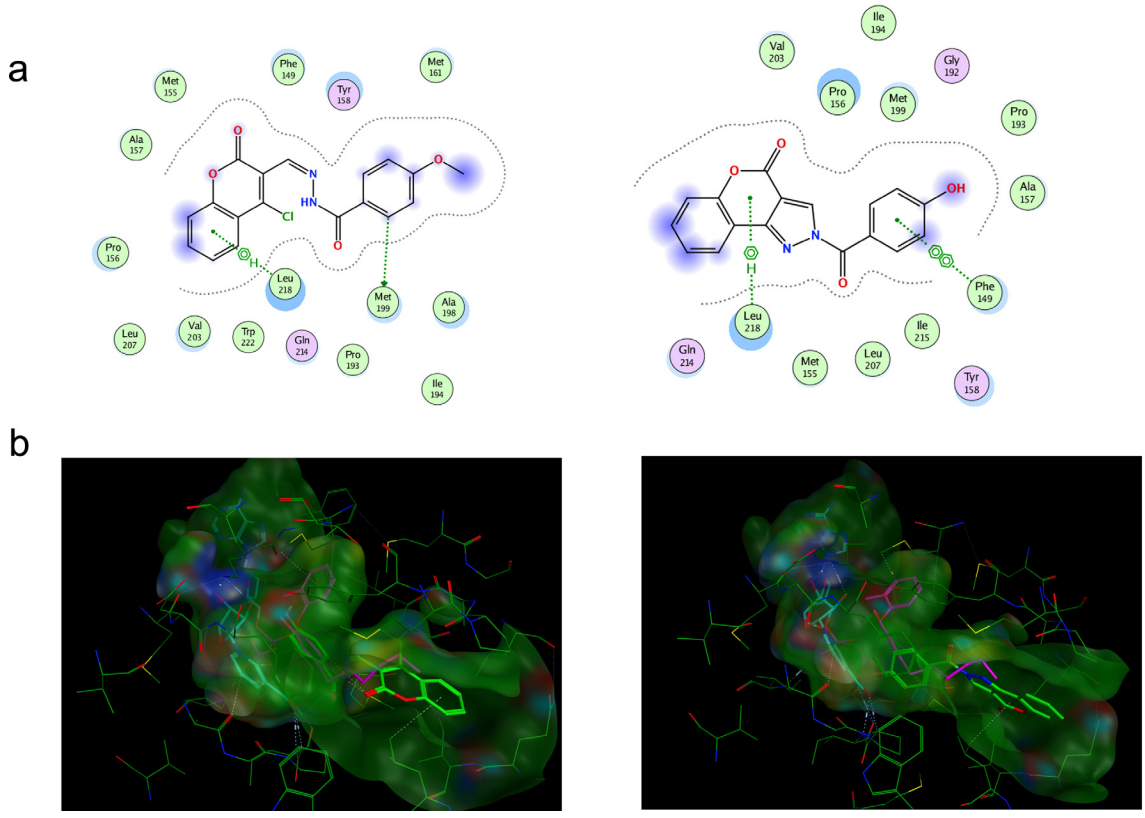


Fig. 4. Docking results for the most active TB compounds **4c** and **5f.**

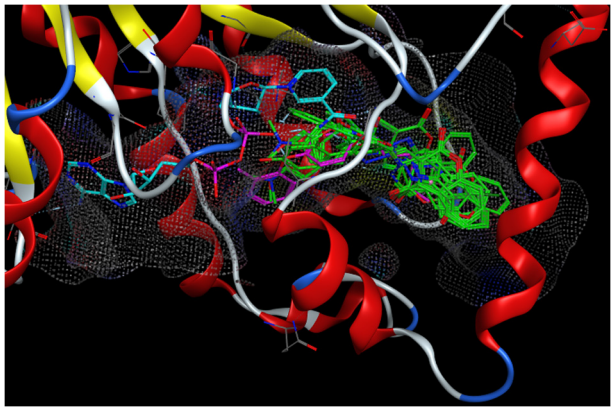


Fig. 5. Docking conformation of all synthesized compounds (in green), PT70 (in magenta), NAD + (in cyan) and corresponding Connolly surface.

between the docking data and the actual activity of the tested compounds could be ascribed to pharmacokinetic and biopharmaceutical properties, that make a given compound better accessible by the receptor site and condition superior activity even when the binding interaction is a less prominent one. To the best of our knowledge, this is the first literature report on the antimycobacterial activity of pyrazole-fused chromene derivatives.

In summary, a series of coumarin-linked hydrazide–hydrazones **4a**–**e** and 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-ones **5b**–**g** were synthesized *via* an efficient one-pot protocol. All compounds demonstrated significant minimum inhibitory concentrations (MIC) ranging from 0.28 to 1.69 μM against reference *M. tuberculosis* H37Rv strain. The cytotoxicity against the human embryonic kidney cell line HEK-293 was also evaluated and the selectivity of the antiproliferative effects was thus assessed. All compounds displayed good SI values ranging from 33 to more than 645. In general, 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives **5b**–**g** possessed potent antimycobacterial activity combined with low cytotoxicity what resulted into SI values higher than that of their open chain analogues **4a**–**e**. The activity of the tested compounds may be attributed to the significant interactions with the inhibitor binding cavity of *M. tuberculosis* Enoyl-ACP reductase and/or related to ability of the tested compounds to penetrate mycobacterial cells. The above observations show that the novel hydrazide–hydrazones **4a**–**e** and 2-aroyl-[1]benzopyrano[4,3-*c*]pyrazol-4(1*H*)-one derivatives **5b**–**g** have potential as antimycobacterial agents. The results of the study can be utilized to further optimize and improve the potency and selectivity toward ENR enzyme by varying the basic skeleton and the substituents.

Acknowledgements

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.05.011.

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