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Synthesis and characterization of pyruvate–isoniazid analogs and their copper complexes as potential ICL inhibitors

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ABSTRACT

Currently used anti-tubercular drugs target actively growing *Mycobacterium tuberculosis* (*Mtb*) but there are no current therapies targeting persistent mycobacteria. Isocitrate lyase (ICL) is an important enzyme of the glyoxylate shunt pathway used by *Mtb* for sustaining intracellular infection in inflammatory macrophages under conditions of stress such as nutrient depletion and anaerobic metabolism. Since the humans do not possess this enzyme it constitutes an attractive target for selective drug design. Present work describes synthesis and structural characterization of pyruvate–isoniazid conjugates and their copper complexes with potent anti-tubercular activities against *M. tuberculosis* H37Rv.

Tuberculosis (TB) is one of the deadly infectious diseases which has become a global priority due to emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) organisms among the immuno-compromised individuals.[1] World Health Organization (WHO) has estimated 8.5–9.2 million incident cases in 2010 worldwide and 9.6–13.3 million prevalent cases of TB with 0.9–1.2 million deaths due to this infection.[2] Extensive studies on the behavioral pattern of *Mycobacterium tuberculosis (Mtb)*, the most commonly known causative organism of TB, have indicated that the *tubercle bacilli* survive well within the macrophages of their host contrary to the belief that these cells afford a strong initial barrier to bacterial infection.[3]

Isoniazid (INH) is the most common first-line drug used in treating tuberculosis. Serum concentrations of INH are influenced by a number of factors, the most important of being the enzymatic acetylation by *N*-acetyltransferase (NAT) in humans which reduces therapeutic activity of the drug.[4] This phenomenon is under genetic control in humans and can be divided into two categories viz. 'fast acetylators' and 'slow acetylators'. The fast acetylators on long-term treatment of INH lead to significant lowering of drug bioavailability and consequent generation INH resistance.[5] NAT is also present in mycobacterial pathogens. It has been found that recombinant NAT from *M. tuberculosis* acetylates INH in vitro. When corresponding NAT *gene* is over-expressed in a suitable INH-susceptible host such as *Mycobacterium smegmatis*, the resultant organism becomes more resistant to INH.[6] The enzyme deactivates INH by means of a reaction at N2-centre in the hydrazinic chain. Consequently, the logical strategy of avoiding drug resistance dictates that hydrazine unit of INH can be modified with a functional group that blocks acetylation, while maintaining strong antimycobacterial activity.[7,8]

It has been shown that survival of bacteria in activated macrophages and persistent infection of *M. tuberculosis* continues through a carbon-conserving glyoxylate shunt pathway (GSP).[9] The two enzymes of this pathway viz. ICL (isocitrate lyase) and MS (malate synthase), are up-regulated when acetate or fatty acids are the only nutrients available for the bacteria. The gating enzyme for GSP has been found to be ICL that converts isocitrate into succinate and glyoxylate which are further processed into malate by MS, thus bypassing the two decarboxylation steps of tricarboxylic acid cycle (TCA) cycle.[10] Since, ICL is found to play a pivotal role in the persistence of *Mtb* in mice by sustaining intracellular infection in inflammatory macrophages under normal conditions of growth[11] as well as in conditions of stress such as nutrient depletion and anaerobic metabolism related to dormancy,[12] the enzyme is commonly referred as 'persistence factor'.[13] Earlier studies have

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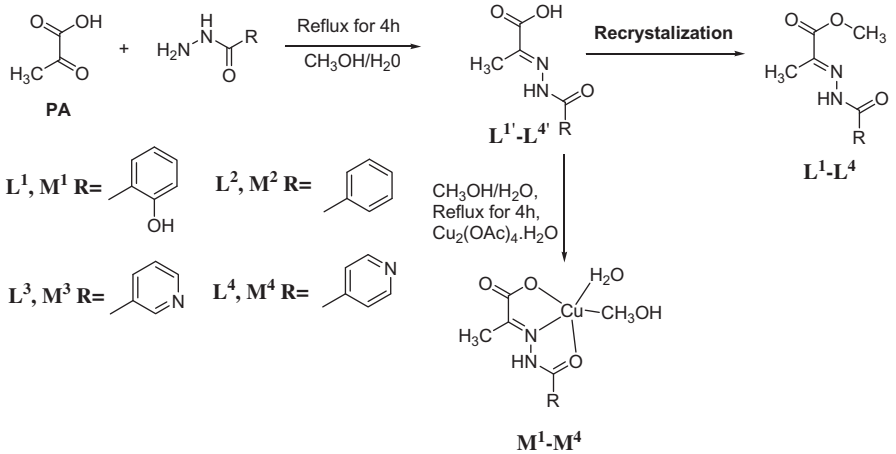
found that 3-nitropropionate (3-NP) and 3-bromopyruvate (3-BP) compounds inhibit ICL of *M. avium* with K_i values of 3 and 120 μM, respectively.[13] Recently, few derivatives such as 3-nitropropionamides,[14] 5-nitro-2-furoic acid hydrazones,[15] 5-nitro-2,6-dioxohexahydro-4-pyrimidinecarboxamides,[16] phthalazin-4-ylacetamides,[17,18] and isatinyl semicarbazone[19] have also been reported as mycobacterial ICL inhibitors. Some known ICL inhibitors are the structural analogs of the reaction products of shunt pathway and include compounds such as succinate, [itaconate, itaconic anhydride, 3-nitropropionate], glyoxylate [3-bromopyruvate] and oxalate, respectively.[20] There have been two reports in recent years describing conjugates of pyruvate with thiophene hydrazone and aminoguanizone but without any investigations on their biological and especially anti-tubercular activity.[21] Clinically employed present TB drugs target actively growing mycobacterium but there are no current therapies for targeting persistent mycobacteria. Additionally humans do not have active glyoxylate shunt operative like mycobacteria and targeting ICL obviously offers some specificity.[22] Hence, targeting ICL with compounds having common structural motif of 3-NP and 3-BP combined with known anti-tubercular hydrazide drugs may lead to a novel class of small molecule inhibitors acting on rapidly multiplying bacteria as well as persistent strains of *Mtb*. Thus, in present study, we have explored NAT-protected Schiff bases of pyruvic acid with INH like hydrazides (**PA**; L^1–L^4) along with their copper complexes (**MPA**; M^1–M^4, respectively) (Scheme 1) to target growing and persistent mycobacterium species.

In order to identify possible binding sites of pyruvic acid conjugates into ICL protein, the acid counterparts of ligands ($L^{1'}$–$L^{4'}$) were docked into pre-defined sites of ICL (PDB ID 1F8M) confirming that hydrazide conjugates of pyruvic acid do not induce any major steric changes in the parent compound pyruvic acid except to allow more hydrogen bonding interactions. The binding energies of the docked compounds were in the range of −7.51 to −8.89 Kcal/mole compared to the parent compound's −8.36 Kcal/mole, respectively. The highest binding energy was exhibited by $L^{4'}$ followed by $L^{2'}$, pyruvic acid (**PA**), $L^{3'}$ and $L^{1'}$ confirming that all molecules bind tightly into the active site of ICL protein. The carbonyl oxygen of the **PA** showed hydrogen bonding interaction with GLY192 (2.158 Å), while $L^{4'}$ exhibited hydrogen bonding interaction involving nitrogen of hydrazide moiety with GLY287 (2.131 Å). Similarly, $L^{3'}$ undergoes two hydrogen bond interactions involving carbonyl oxygen of the carboxylic acid moiety with HIS323 (2.136 Å), ASN319 (1.839 Å), and carbonyl oxygen of the hydrazide moiety with SER317 (1.935 Å). The carbonyl oxygen of the hydrazide moiety of $L^{1'}$ and carbonyl oxygen of carboxylic acid moiety of $L^{2'}$ have shown hydrogen bonding interactions with HIS193 (1.899 Å) and ASN319 (1.911 Å), respectively (Fig. 1 and Table 1). Hence, it was concluded that oxygen atoms either of carboxylic acid moiety or hydrazide moiety favors Van der Waals interactions with amino acid residues in the ICL protein leading to stabilization of ligand into the protein cavity.

The compounds (L^1–L^4, M^1–M^4) were synthesized according to Scheme 1 by condensation reactions of equimolar quantities of pyruvic acid and respective hydrazide, respectively in absolute methanol with continuous stirring at 55–60 °C for 4 h. The resulting precipitates were filtered, dried under vacuum and recrystallized from methanol–water. The purified pyruvic acid hydrazides and their copper complexes were characterized by UV, IR spectroscopy, Cyclic Voltammetry and X-ray crystallography. The compositional and spectral data on all synthesized compounds is summarized in Supplementary data. All synthesized compounds are colorless to pale yellow in color, while their copper conjugates are dark green in color. The characterization data indicated the formation of a methyl ester at carboxylic end of pyruvic acid during recrystalization from methanol–water system.[23]

Infra-red spectra of compounds showed a medium intensity band of C=N stretch in the region of 1674–1620 cm^{-1} confirming the formation of Schiff Base.[24] The fingerprint IR region for free ligands exhibited stretching frequencies in the range of 1715–1755 and 1000–1300 cm^{-1} which can be assigned to carbonyl (–C=O, –C–O) ester which were shifted to 1668–1690 cm^{-1} in the corresponding copper complexes indicating involvement of the carbonyl in metal co-ordination.[24] The broad IR band in the range of 3000–3200 cm^{-1} indicates the presence of co-ordinated water molecule in the metal complexes.[25] During metal complexation the ligands were found to act as tridentate donor atom species co-ordinating through azomethine nitrogen, hydrazinic and pyruvate carbonyl atoms, respectively.[26] The electronic spectra of ligands (L^1–L^4) exhibit intra-ligand absorptions ($\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$) in the range 260–280 and 300–400 nm.[27] The characteristic d–d transitions for M^1–M^4 complexes are observed at 650–700 nm typical of five co-ordinate square-pyramidal copper(II) complexes.[28] The room temperature magnetic moments of the synthesized copper complexes are found in the range of 1.79–1.85 BM indicative of monomeric compounds with square-pyramidal geometry.[29] Cyclic voltammetric studies showed irreversible ligand based redox peaks in the range of −1.20 to −0.30 V attributed to the reduction of azomethine linkage,[30] wherein anodic counterpart is not observed. The metal-based redox peaks in the range of +0.35 to +0.82 V (Table 3, Supplementary data are attributed to the $Cu^{2+/1+}$ redox couple.[31] The reversible nature of this redox couple is



Scheme 1. Chemical structures of **PA** (L^1–L^4) and **MPA** (M^1–M^4).

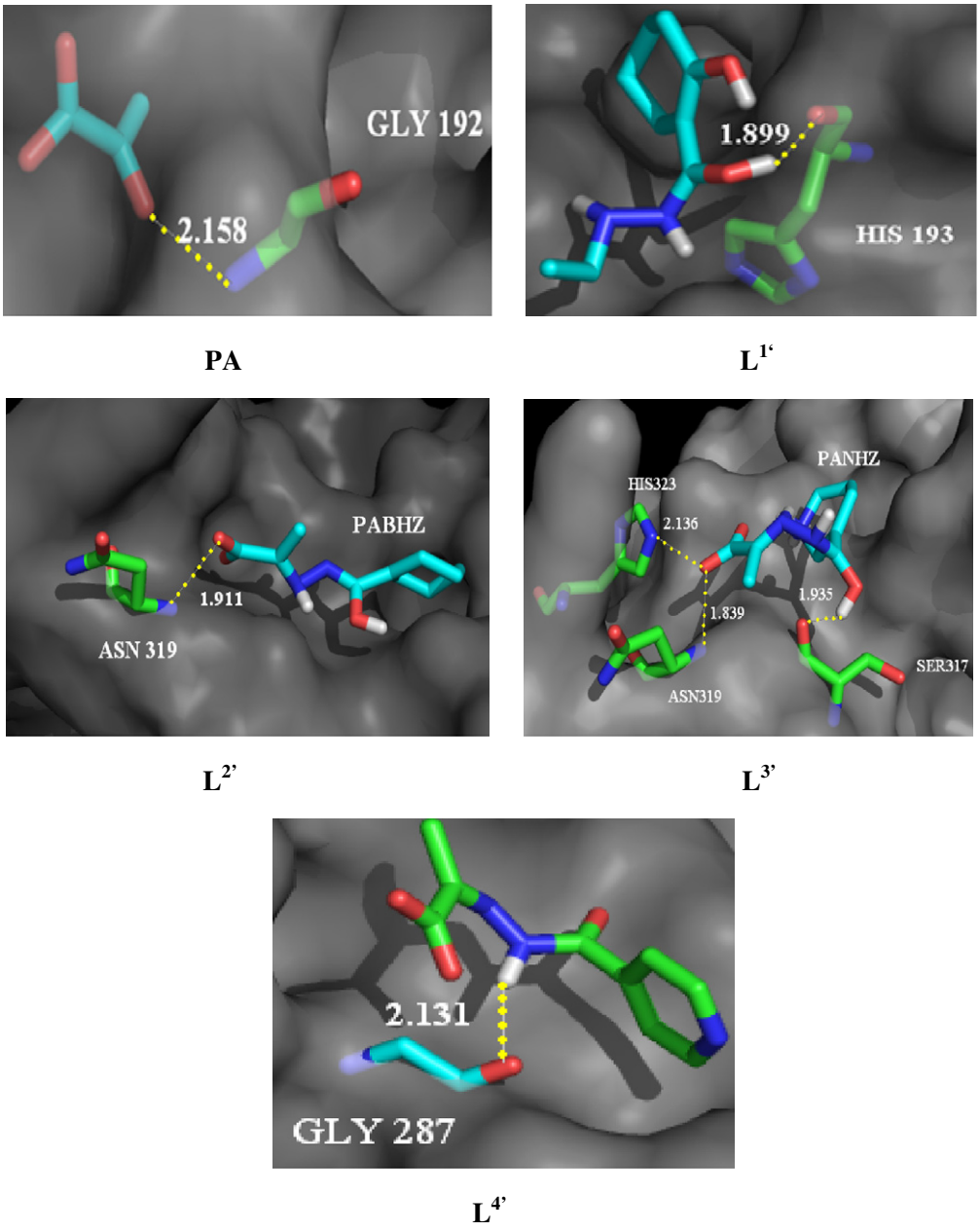


Figure 1. Binding of **PA** (**L**$^{1'}$–**L**$^{4'}$) into the active site of ICL as assessed by molecular docking studies.

Table 1
Docking results and consensus scores of synthesized **PA** (**L**$^{1'}$–**L**$^{4'}$)

Ligand	Binding energy (Kcal/mole)	Docking energy (Kcal/mole)	No. of H bond	H bonding Residue	Distance (Å)
PA	−8.36	−2.34	1	Gly192	2.158
L$^{1'}$	−7.75	−2.86	1	HIS193	1.899
L$^{2'}$	−8.63	−4.12	1	ASN319	1.911
L$^{3'}$	−7.82	−3.67	3	ASN319, HIS323,SER317	1.839, 2.136,1.953
L$^{4'}$	−8.89	−2.78	1	GLY287	2.131

confirmed by scan rate dependence studies. The ratio of anodic to cathodic peak current ip_a/ip_c is nearly equal to unity signifying that these copper complexes are reversibly stable within the timescale of the experiment.[32]

The single crystal X-ray structural characterization of **L**^{1} (Fig. 2) reveals it to be a dimeric, neutral molecule with active involvement of a water molecule connecting two individual molecules of **L**^{1}. The N1–N2 bond length of 1.361(3) Å is consistent with that observed in **L**^{2} as well as in other analogs. The azomethine group (C=N) of **L**^{1} shows partial single bond character with bond length of 1.359(3) Å due perhaps to strong intra-molecular as well as intermolecular H-bonding.[33] The side chain carbonyl [O(2)–C(7)] as well as the ester carbonyl [O(3)–C(10)] in **L**^{1} show a typical double bond character with bond lengths 1.217(3) and

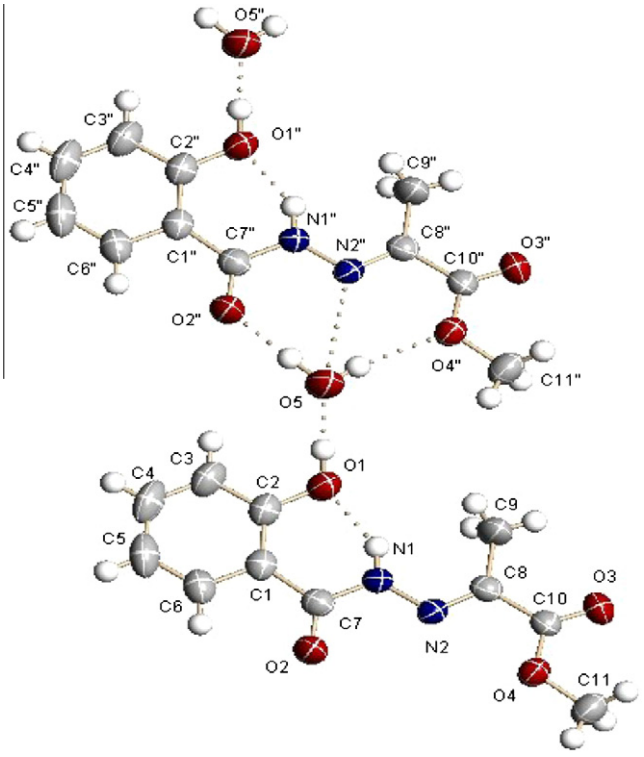


Figure 2. Single X-ray crystal structure of $\mathbf{L^1}$.

1.192(3) Å, respectively. $\mathbf{L^1}$ shows extensive hydrogen bonding interactions involving the solvent water molecule, salicylic hydroxyl group (on the adjacent molecule), azomethine nitrogen and the hydrazinic carbonyl as well as ester oxygen stabilizing the whole molecule.

The ligand $\mathbf{L^2}$ crystallizes as a monomeric species (Fig. 3) having an orthorhombic space group Pna21 with crystal parameters a = 8.0735(8) Å, b = 8.4887(8) Å, c = 16.0227(16) Å, respectively. The C=N– azomethine bond length [N(2)–C(8)]–1.287(2) Å in $\mathbf{L^2}$ has a double bond character as against that observed in $\mathbf{L^1}$ [1.359(3) Å], probably due to the absence of intramolecular hydrogen bonds. The bond length of the hydrazonic carbonyl [C(7)–O(1)] is found to be 1.233(2) Å which is slightly longer than the one found in $\mathbf{L^1}$ **[O(2)–C(7)–1.217(3)** Å] but is consistent with other similar compounds.[34]

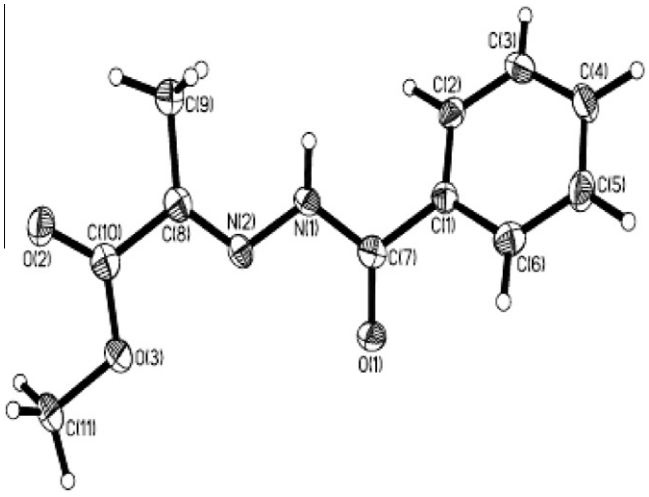


Figure 3. Single X-ray crystal structure of $\mathbf{L^2}$.

The copper complex $\mathbf{M^1}$ (Fig. 4) consists of a neutral [Cu(L)(H_2O)(MeOH)] molecule. The square pyramidal geometry around the central copper atom consists of a NO4 donor-atom set where the tridentate ligand $\mathbf{L^1}$ donates the hydrazonic carbonyl oxygen, azomethinic nitrogen and carbonyl oxygen of the pyruvic moiety, respectively. The remaining coordinating sites around the copper atom are occupied by the oxygen atoms of water and methanol molecule, respectively. The $Cu–O_{water}$ distance is 1.9247(18) Å, which is comparatively shorter than the normal range (2.15–2.25 Å) observed for similar compounds.[35] The Cu–O(3) bond distance in this compound [1.9797(15) Å] is in agreement with values normally observed for the square pyramidal copper complexes. Intramolecular hydrogen bonding is observed between the azomethinic nitrogen and the hydroxyl oxygen of the salicylic unit, where the proton pre-dominantly residing on the oxygen atom and making a hydrogen bond of 1.95 Å with the nitrogen atom.[36]

The screening of the synthesized ligands ($\mathbf{L^1}$–$\mathbf{L^4}$) and their copper complexes ($\mathbf{M^1}$–$\mathbf{M^4}$) for their antibacterial activity against *M. tuberculosis* H37Rv strain (Fig. 5) indicate percent inhibition ranging from 6% to 92%. Compounds containing isonicotinoyl group ($\mathbf{L^4}$ and $\mathbf{M^4}$) are found to be the most active among the group, perhaps owing to its well-known antibacterial activity retained in the present compound. We believe that their close structural resemblances with the known ICL inhibitors like 3-BP and 3-NP make them potential candidates for further explorations. 3-BP has been found to inhibit ICL via dehalogenation to form covalent adduct with the active site nucleophile Cys 191.[13] The pyruvate moiety in this compound occupies the site where the second carboxylate of succinate is located and forms hydrogen bonds with the side chains of His 193 ND1, Asn 313 ND2, Ser 315 OG, Ser 317 OG, Thr 347 OG1 and a solvent molecule. In this inhibitor–enzyme complex, the glyoxylate binding site is occupied by solvent molecules that co-ordinate with Mg^{2+} ion.[13] The divalent cation Mg^{2+} was found to be essential for activation of ICL and in absence of Mg^{2+} the enzyme was found to be inactive.[37] In previous studies, the ability of pyruvate hydrazides to form chelates with magnesium was reported by Yang et al.[38] suggesting that this could be an additional mechanism of inhibition of ICL by these compounds. Similar mechanisms may also be operative in case of present pyruvate conjugates. In present work, we have found the significant enhancement in anti-tubercular activity of present ligand after complexation with copper which may be explained on the basis

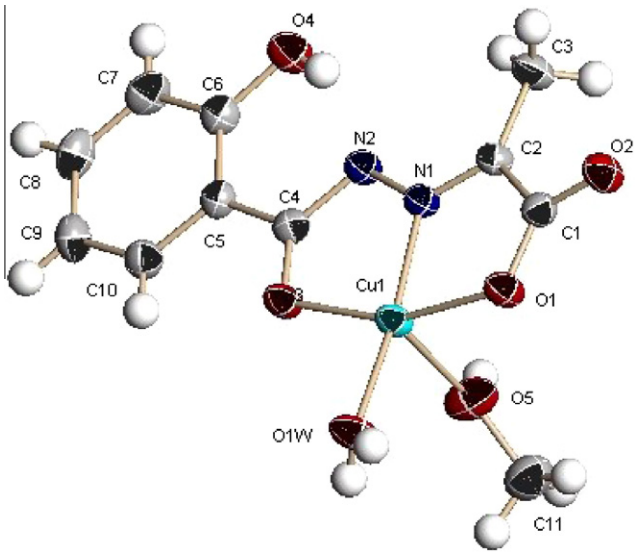


Figure 4. Single X-ray crystal structure of $\mathbf{M^1}$.

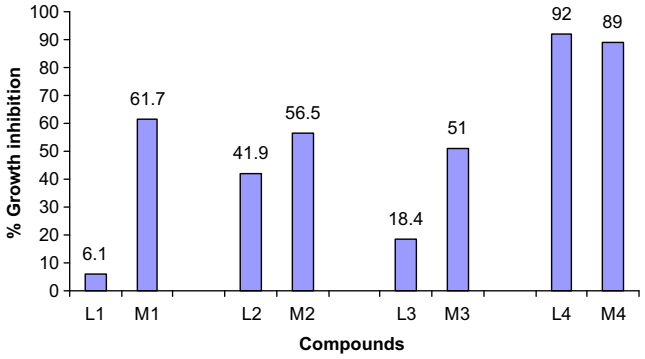


Figure 5. Antibacterial activity against H37Rv of **PA** ($\mathbf{L^1}$–$\mathbf{L^4}$) and **MPA** ($\mathbf{M^1}$–$\mathbf{M^4}$).

of literature reports stating that *Mtb* is much more susceptible to copper than other bacteria and is killed in vitro by copper concentrations lower than those found in phagosomes of macrophages. It has also been suggested that the mammalian host uses copper to control *Mtb* infection and copper resistance mechanisms are crucial for *Mtb* virulence.[39] Ito and group have shown that INH produced DNA damages in the presence of Cu(II) ions due to active oxygen species other than the hydroxyl free radical, presumably by the Cu-(1)–peroxide complex.[40] In many cases metal coordination has been used to confer antibacterial and antiproliferative properties to different INH derivatives due to enhanced lipophilicity conferred by metal complexation.[41,42] It has also been reported that presence of Cu^{2+} improves the uptake of INH and analogous hydrazides into both susceptible and resistant mycobacteria.[43]

In summary, we have been able to synthesize pyruvate–isoniazid conjugates and their copper complexes with potent anti-tubercular activities against *M. tuberculosis* H37Rv. The compounds obviously need to be investigated further for their mechanistic details of their ICL inhibitory activities.

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Supplementary data

Experimental details of synthetic procedures and characterization data of $\mathbf{L^1}$–$\mathbf{L^4}$ and $\mathbf{M^1}$–$\mathbf{M^4}$, crystal data tables of $\mathbf{L^1}$, $\mathbf{L^2}$ and $\mathbf{M^1}$ (Table 2) were given. **CCDC 632417, 679623, 633548** contains the supplementary crystallographic data for $\mathbf{L^1}$, $\mathbf{L^2}$, and $\mathbf{M^1}$, respectively. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223 336 033; or e-mail: deposit@ccdc.cam.ac.uk.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.047.

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