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Schiff base conjugates of *p*-aminosalicylic acid as antimycobacterial agents

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Abstract—Schiff base conjugates of *p*-aminosalicylic acid have been synthesized and characterized by elemental analysis, IR and ^{1}H NMR spectroscopy and cyclic voltammetry. Compounds containing hydroxyl-rich side chains show enhanced antimycobacterial activity against *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG. Higher Clog*P* values and superior radical scavenging activities are thought to be the contributing factors for their enhanced antimycobacterial activities.

Tuberculosis (TB) is one of the most refractory and deadly infectious killer diseases worldwide caused by the pathogen *Mycobacterium tuberculosis* which is responsible for infecting nine million people globally with three million deaths every year according to World Health Organization (WHO) report.[1] Over the years, the organism has acquired resistance to nearly all first-line antitubercular drugs such as isoniazid, rifamcin, ethionamide, etc., posing serious problems in treating the infection clinically.[2] The problem of clinical treatment has become more acute especially in immuno-compromised AIDS patients where the rise in TB incidence and consequent deaths over the past two decades has escalated by more than 12%. There is thus an urgent need to evolve new antitubercular drugs.

Mycobacteria have a specific need to acquire iron for their metabolism, as described by Ratledge.[3] This task is accomplished by the synthesis of specialized compounds known as siderophores,which, in the mycobacteria, are carboxymycobactin in the pathogens and exochelins in saprophytic bacteria.[3] Mycobactin, which is found exclusively intracellularly in the envelope of mycobacteria, acts as an intracellular storage compound for iron and allows the subsequent slow release of iron into the cytoplasm of the cells at a rate, which can be accommodated by the cells as they up-regulate the synthesis of various iron-containing proteins. Salicylic acid, which can also be found extracellularly in cultures of mycobacteria grown with a deficiency of iron, is a precursor of mycobactin and carboxymycobactin that are essential for growth of *M. tuberculosis* in macrophages.[4] Salicylate, however, is too weak a chelator of iron for it to act as a siderophore.[5] Some time ago Brown & Ratledge[6] suggested that *p*-aminosalicylic acid (PAS), which is a second-line antitubercular drug, acts as an antisalicylate compound by interfering with the metabolism of salicylic acid, possibly by preventing its conversion to carboxymycobactin and mycobactin.[3]

More recent evidence[7] has shown that when *Mycobacterium smegmatis* was treated with PAS, the concentration of salicylate increased by 5- to 6-fold and that of mycobactin decreased by about 70–80% in keeping with these proposals. In addition, a salicylate-requiring auxotroph had a considerably heightened sensitivity to PAS. However, other evidence,[8] which carefully eschewed mention of all contrary evidence, has suggested that PAS may be acting as an anti-folate compound as PAS-resistant mutants of *Mycobacterium bovis* show diminished activity of thymidylate synthase. Whatever is the site of action of PAS, it is easy to synthesize and is an inexpensive drug for the treatment of tuberculosis. As it is now many years since various analogues of PAS have been synthesized[9] it would now seem desirable to revisit this molecule and produce further modifications of it in order to find novel chemotherapeutic agents that may possibly be effective against existing PAS-resistant strains.

Keywords: *para*-Amino salicyclic acid; Mycobacterium bovis; Antimycobacterial agents.
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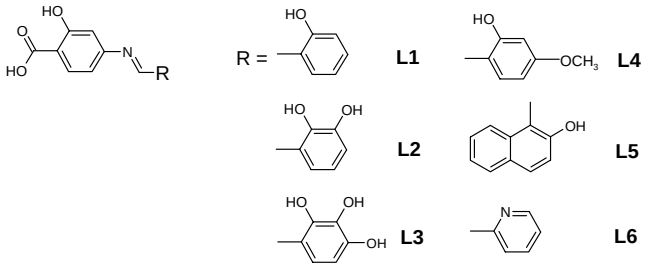

Scheme 1.

In the present work, we describe synthesis and characterization of some new multifunctional conjugates of PAS (Scheme 1) capable of metal chelation along with the results on the evaluation of their antimycobacterial activity against *M. smegmatis* and *M. bovis* (BCG).

PAS (Lancaster, UK) was recrystallized before use, while all aldehydes used in condensation reactions were purchased from Aldrich and were used as received.

Elemental analyses were carried out in the Microanalytical Laboratory at University of Hull, UK. IR spectra were recorded in Nujol in the range 4000–450 cm^{-1} on a Perkin-Elmer 283B FTIR instrument and ^1H NMR was recorded in DMSO-d_6 solvent on the Varian Mercury Vx 300 instruments.

All the Schiff base derivatives were synthesized by stirring a methanolic solution of PAS (typically 1.63 g, 0.01 mol) with corresponding aldehydes (typically 1.22 g, 0.01 mol) in 1:1 stoichiometric ratio at room temperature over 24 h. The precipitates obtained were filtered, washed with methanol, and dried in vacuo over anhydrous $CaCl_2$ and were recrystallized from methanol.

Mycobacterium smegmatis NCIMB 8548 was grown in 250 ml conical flasks, pre-treated to remove trace metals, containing 100 ml medium which consisted of glycerol or glucose, 10 g; KH_2PO_4, 5 g; L-asparagine, 5 g; pH to 7.0, or alternative value, with KOH. Medium was autoclaved with 2% (w/v) aluminium oxide to remove trace metals and dispensed as previously described.[7] When glucose was used, this was sterilized separately. Medium was supplemented with 0.45 μg Zn^{2+} ml^{-1}, 0.1 μg Mn^{2+} ml^{-1} and 40 μg $Mg^{2+}ml^{-1}$ plus for iron-deficient growth 0.04 μg Fe^{2+} ml^{-1} and for iron sufficient growth 2 μg Fe^{2+} ml^{-1}. Cultures were inoculated with 1 ml of 5- to 7-day-old culture grown on iron-deficient medium and were incubated with shaking at 37 °C for 6 to 7 days.[7]

Mycobacterium bovis BCG Glaxo strain was grown as described above. Culture filtrates were acidified, extracted with chloroform and the extracts were analysed by HPLC for the presence of salicylic acid by monitoring the eluate at 237 nm.[7] The reason for using iron-deficient growth conditions for testing the compounds was that it is considered that these are pertinent in vivo conditions that mycobacteria will face when growing in macrophages.[4]

All synthesized Schiff base compounds were characterized by elemental analysis, IR and ^1H NMR spectroscopy (Table 1). The microanalysis data are consistent with the molecular formula assigned to the compounds. The formation of the Schiff base derivatives is confirmed by fingerprinting IR spectra for the appearance of the azomethine stretching frequency (C=N) in the range 1607–1616 cm^{-1} [9] and disappearance of the bands around 3000–3400 cm^{-1} due to the symmetric and asymmetric amino stretches of the *para*-amino groups.[10] The carboxylate carbonyl frequencies in the synthesized derivatives are observed in the region [1629–1657 cm^{-1}],[11] while a broad band located around 3400–3500 cm^{-1} corresponds to the salicylic hydroxyl group.[12] The formation of the Schiff base compounds was also confirmed by ^1H NMR spectrum which shows a proton signal around 8.9 ppm due to –CH=N– chromophore.[13]

The results of the antimycobacterial assays for the compounds **L1**–**L6** against *M. smegmatis* and *M. bovis* BCG are shown in Figures 1 and 2, respectively. *M. smegmatis* is fast-growing mycobacteria with a generation time of 3 h compared to 20 h for that of *M. tuberculosis* and

Table 1. Elemental, IR and ^1H NMR spectral data for the compounds **L1**–**L6**

Compound	Elemental analysis[a]			IR spectroscopy (cm^{-1})			^1H NMR (ppm)	Clog *P*
	% C	% H	% N	*ν*(OH)	*ν*(C=N)	*ν*(C=O)	*δ*(–CH=N)	
L1	65.73 (65.36)	4.19 (4.28)	5.19 (5.44)	3436	1607	1638	8.95	3.42
L2	61.87 (61.53)	3.92 (4.03)	4.81 (5.12)	3420	1616	1630	8.93	2.91
L3	58.73 (58.13)	4.13 (3.80)	4.15 (4.84)	3350	1616	1625	8.72	2.45
L4	63.01 (62.71)	4.42 (4.53)	4.55 (4.87)	3446	1610	1629	8.93	3.40
L5	70.24 (70.35)	4.18 (4.23)	4.23 (4.56)	3435	1608	1657	8.51	4.61
L6	64.07 (64.46)	4.29 (4.13)	10.84 (11.57)	3413	1618	1630	8.60	2.29

Carboxylate group.

[a] Values in parentheses are calculated ones.

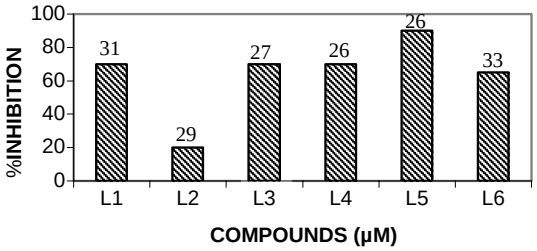

Figure 1. Antimycobacterial activities of the Schiff base conjugates of PAS (**L1**–**L6**) against *Mycobacterium bovis*BCG.

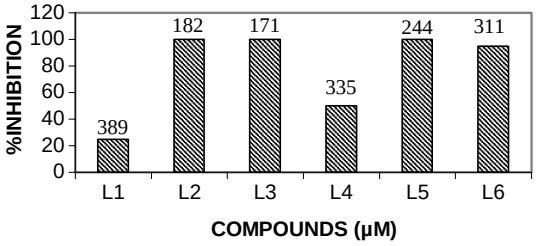

Figure 2. Antimycobacterial activity of the Schiff base conjugates of PAS (**L1**–**L6**) against *Mycobacterium smegmatis*.

hence is normally employed for the rapid screening of antitubercular compounds.[14] It also has similar rates of *homologous recombination to M. tuberculosis*.[15] *M. smegmatis*, unlike *M. tuberculosis* or BCG, has a second route of iron uptake that does not involve salicylic acid[3] and, consequently, is considerably less sensitive to PAS than are the pathogenic mycobacteria.[7] *M. bovis* BCG resembles *M. tuberculosis* being a slow-growing pathogen[16] and has 99.9% similarity of DNA sequences with that of *M. tuberculosis*.[17]

In the present study, all the susceptibility testing do not include the actual MICs, but we have noted the percent inhibition at a single concentration and the detailed studies are presently underway. The inhibitory concentration values for almost all of the new compounds as seen from the preliminary data are considerably lower than PAS in case of *M. smegmatis* (MIC > 650 μM) as well as *M. bovis* BCG (MIC < 52 μM) indicating conjugation of hydroxyl-rich ligands is effective in synergistically enhancing the antimycobacterial activity of these compounds. It is difficult to find reliable data in the literature concerning MICs, because experimental conditions are so different that it could be erroneous to compare published values.

Conjugation of heterocyclic ring gave a slight improvement in the activity of the compounds. This is important, as many of the existing first-line antitubercular drugs possess such structural motif.

Rapid drug resistance developed by *M. tuberculosis* is generally due to decreased drug permeability induced by the bacteria by thickening their cell wall so that only lipophilic derivatives can penetrate such waxy cell wall.[8]

The recent genomic data on *M. tuberculosis* have shown that about 12% of its genes are involved in cell wall and lipid biosynthesis processes.[18] On comparing the Clog P[19] values of PAS (0.71) with **L5** (4.62), the Schiff base side chain in the new derivatives clearly contributes substantially in enhancing the liposolubility of the parent compound thereby facilitating the entry of these molecules through lipid-enriched bacterial cell wall.[20]

Interestingly, the HPLC analysis of the culture filtrates showed no increased accumulation of salicylic acid (results not shown), suggesting the probable mechanism of action of these compounds is not through blocking the conversion of salicylate into mycobactin or carboxymycobactin. In this connection, there have been prior reports indicating that the orthohydroxy Schiff bases of isoniazid had higher antimycobacterial activity than isoniazid where the enhanced activity was attributed to radical scavenging activity of the compound.[21] It is likely a similar mechanism may be operating in case of the compounds described here.

PAS conjugates containing hydroxyl-rich Schiff base ligands have enhanced antimycobacterial activities against *M. smegmatis* and *M. bovis* BCG that may be useful in developing new and potent antimycobacterial agents.

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