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Synthesis of 2-Hydroxy-5-(1-(2-(pyrazine-2-carbonyl) hydrazono)hexyl) Benzoic Acid, A Pyrazinamide Analog of Salicylic Acid

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The occurrence of resistant strains of *Mycobacterium tuberculosis* has driven current research on combining current anti-tuberculosis drugs and other bioactive molecules to enhance their efficacy against susceptible and resistant strains of the bacteria. In this study, a salicylic acid derivative of pyrazinamide, 2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid was synthesized and characterized. The compound was prepared by coupling a pyrazinamide moiety, one of the first line drugs used to treat tuberculosis and a salicylic acid derivative with a 6-carbon alkyl chain. The salicylic acid derivative was generated via Friedel-Crafts acylation of methyl salicylate followed by base hydrolysis of the acylated product. This was coupled with the pyrazinamide moiety via imine formation. The product, 2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid was obtained as an off-white powder in 62% yield.

Keywords: *tuberculosis, pyrazinamide, salicylic acid, Friedel-Crafts acylation, imine formation*

INTRODUCTION

Tuberculosis (TB) is one of the leading causes of death in the world from an infection of acid-fast bacilli *Mycobacterium tuberculosis* (Todar, 2012). This bacteria commonly infects the lungs but may also attack other organs such as the kidneys, spine and the brain. In the treatment of the disease, the first line of drugs prescribed includes isoniazid, rifampin, ethambutol and pyrazinamide. A second line of drugs that includes kanamycin, capreomycin and amikacin are administered upon the development of multi-drug resistant strains of tuberculosis (MDR-TB) which is resistance of the bacteria against the first line of drugs (WHO, 2012; CDC, 2012).

According to the WHO Global Tuberculosis Report of 2012, there are 8.7 million new cases of tuberculosis in 2011, around 13% of which are co-infected with the HIV disease. Geographically, about 60% of these TB cases are found in South-East Asia and the Western Pacific Region. It has claimed 1.4 million lives last 2011; 1 million of which are patients that are HIV-negative and around 430, 000 are HIV-positive. The burden of tuberculosis is

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further worsened by the increase in the number of cases which are infected with MDR-TB, many cases of which are also found in Eastern Europe and Central Asia (WHO, 2012).

The increase in the development of multi-drug resistant strains of tuberculosis (MDR-TB) and extensively-drug resistant strains (XDR-TB) which is resistance to the fist line and second-line of drugs used to treat the disease respectively has resulted to a vast amount of research on the modification of the first-line of drugs used to treat TB.

One of the first-line of drugs used to treat the disease, pyrazinamide (PZA) plays a unique role in shortening the treatment period from nine months to six months (Zhang *et al*., 2003). This is due to its sterilizing activity, which kills a population of persistent tubercle bacilli that are not affected by other drugs (Vergara *et al*., 2009). Unlike isoniazid and rifampin, PZA has an activity against multi-drug resistant strains that favors its usage over other drugs. However, several studies have shown that this drug is inactive against *Mycobacterium bovis* and other resistant strains of mycobacterium that has a mutation in their *pncA* gene. This gene codes for the pyrazinamidase enzyme, which converts PZA to pyrazinoic acid (POA) as it enters the cell of the bacteria as shown in Figure 1 (Mitchison and Zhang, 2011).

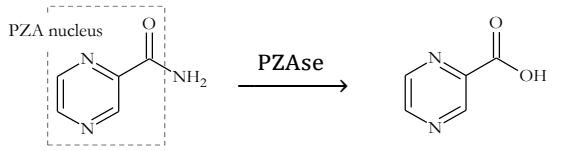


Figure 1. *Conversion of Pyrazinamide to Pyrazinoic acid (POA).*

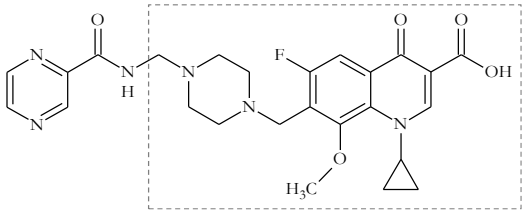
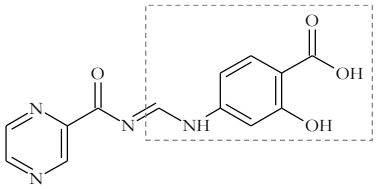
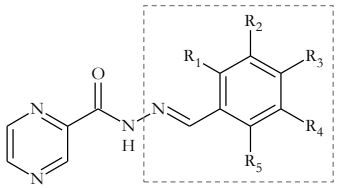
Occurrence of resistance towards the PZA drug, stirred up a huge amount of research work on the modification of the drug to enhance its effectivity against resistant strains of *M. tuberculosis*. Vast arrays of modifications were already done on the PZA nucleus, most of which resulted to an increased potency of the drug compared to the base molecule against resistant strains of *M. tuberculosis* and also towards other non-tuberculous mycobacterial strains (Vergara *et al*., 2009; Sriram *et al*., 2006; Imramovsky *et al*., 2007; Zitko *et al*., 2012).

Most of the modifications done on the PZA drug (Table 1) were made by fusing it with other potential molecules which may improve its activity. These PZA derivatives mostly retained the original structure of the PZA nucleus (Vergara *et al*., 2009; Sriram *et al*., 2006; Imramovsky *et al*., 2007).

The creation of PZA derivatives of Mannich bases has generated analogs with an increased lipophilic character. The synthesis of these Mannich base derivatives of PZA were done via microwave assisted reaction of the PZA molecule with formaldehyde and a secondary amino function of substituted piperazines. This enables the attachment of the PZA core via the methylene group of the Mannich base. One of the synthesized analogs, 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-4-((pyrazine-2-carboxamido)-methyl)piperazin-1-yl)-4-oxoquinoline-3-carboxylic acid (**A**), was the most active compound *in vitro* against MDR-TB with an minimum inhibitory concentration (MIC) of 0.20 μg/ml. This is 125 times greater than the potency of the parent PZA drug against MDR-TB (MIC of PZA = 25.0 μg/ml). The increased anti-TB activity is attributed to the increased lipophilic character of the PZA analog. The calculated logP values of these compounds showed higher values compared to the PZA drug (-1.31). (Sriram *et al*., 2006).

In another related study, PZA was linked with known anti-TB drugs such as isoniazid, p-aminosalicylic acid and ciprofloxacin by the CH fragment. This was done by the reaction of pyrazinamide and N,N-dimethylformamide dimethyl acetal, to create an activated pyrazine derivative, N-(dimethylaminomethylene) pyrazine-2-carboxamide. This allows for the substitution reaction of nucleophilic amino moieties to the activated PZA molecule to generate the derivatives. The higher lipophilic character of 4-(2-Pyrazinecarbonylimino-methyl)aminosalicylic acid (**B**), one of the

Table 1. Modifications on the PZA molecule.

PZA Derivative	Structure
PZA derivatives of Mannich bases (Sriram *et al.*, 2006). *Type of Modification:* Increased Lipophilicity *MIC/logP values:* 0.20 μg/ml vs >25.0 μg/ml (PZA)[a], 0.85 vs -1.31 (PZA)[b]	 **(A)**
PZA fused with PAS (Para-aminosalicylic acid) (Imramovsky *et al.*, 2007). *Type of Modification:* Increased Lipophilicity *MIC/logP values:* 3.13 μg/ml vs 6-60 μg/ml (PZA)[c], 0.11 vs -1.31 (PZA)[d]	 (**B**)
N-acylhydrazone derivatives of PZA (Vergara *et al.*, 2009). *Type of Modification:* Addition of electron withdrawing groups at the ortho and para positions in the fused aromatic ring *MIC/logP values:* 50-100 μg/ml vs >100 μg/ml (PZA)	 **(C)** Compound 6: R_2 = Cl, $R_{1,3,4,5}$ = H Compound 23: R_2 =CN $R_{1,3,4,5}$ = H Compound 27: R_1 = NO_2 $R_{2,3,4,5}$ = H Compound 28: R_2 = NO_2 $R_{1,3,4,5}$ = H

[a] Minimum inhibitory concentration (MIC) required to cause 90% inhibition on MDR-TB
[b] logP value of 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-4-((pyrazine-2-carboxamido)methyl)piperazin-1-yl)-4-oxoquinoline-3-carboxylic acid, (**A**)
[c] Minimum inhibitory concentration agains *M.tb* $H_{37}Rv$
[d] logP value of 4-(2-Pyrazinecarbonyliminomethyl)aminosalicylic acid, (**B**)

synthesized compounds in the study showed higher activity against *M.tuberculosis*, with an MIC value of 3.13 μg/ml. The higher lipophilicities of these compounds enables easy transport through cellular membrane of the mycobacteria, resulting to lower MIC values compared to the parent PZA drug (Imramovsky *et al.*, 2007).

Another way of attaching the PZA nucleus to other molecules to improve its effectivity is via imine bond formation. N-acylhydrazones containing the PZA nucleus were synthesized by reactions involving monosubstituted benzaldehyde moieties and a derivatized PZA core. This generated compounds with e-withdrawing groups (e.g. Cl, F, CN and NO_2) attached in the ortho and meta position of the benzaldehyde ring to exhibit better activity (MIC = 50-100μg/mL) compared with PZA (MIC > 100 μg/mL) (Vergara *et al.*, 2009).

Some bioactive molecules have been studied upon its co-administration with the PZA drug. Weak acids such as benzoic acid, fatty acids and salicylic acid were co-administered

alongside PZA and showed lower colony forming units (CFU) of *M. tuberculosis* in both normal and nutrient starved incubation conditions (Chen *et al.*, 2007). Non-steroidal anti-inflammatory drugs, specifically ibuprofen and aspirin, upon co-administration with PZA have also shown an increase in potency of the drug in a mouse model infected with *Mycobacterium tuberculosis* H37Rv (Byrne *et al.*, 2007).

In view of these results, we envisioned the synthesis of a pyrazinamide analog with a salicylic acid derivative. The attachment of the PZA nucleus via imine formation afforded an easy pathway of fusing the PZA moiety with an acylated salicylic acid derivative. This allows for the retention of the original PZA nucleus. The addition of the alkyl chain may increase the lipophilicity of the compound and aide in its diffusion through the mycobacterial cell wall (Sriram *et al.*, 2006; Imramovsky *et al.*, 2007). The evidence on the enhanced effectivity of the PZA drug in the presence of weak acids may be further investigated with this lead compound. This compound may show an increase in the efficacy of the pyrazinamide drug towards susceptible and resistant strains of *Mycobacterium tuberculosis.*

EXPERIMENTAL

General Analytical Procedure. All reagents ($ZnCl_2$, methyl salicylate, pyrazinoic acid, hydrazine hydrate, and hexanoyl chloride) used were analytical grade with ≥99% purity. All chemicals and solvents used were purchased from Sigma-Aldrich Chemicals, Singapore and Merck Chemicals, Philippines except for the pyrazine-2-hydrazide which was prepared in a previous study (Lagua, 2011).

The purity of the product in each reaction was determined by thin layer chromatography. The TLC plates used were 4x2 cm in dimension and pre-coated with silica gel (Fluka). Visualizing agents for TLC were ultraviolet light and iodine (I_2) powder chamber.

For the characterization of the target compounds, functional groups were determined using Nicolet-500 FT-IR Spectrometer. The mass spectra of the compounds were obtained using Bruker Mass Spectrometer either in the positive or negative ion mode. The ^{1}H–NMR spectra were obtained using Jeol 400 MHz Nuclear Magnetic Resonance Spectrometer at the National Chemistry Instrumentation Center (NCIC) Ateneo de Manila University. The melting points of the target compound and all intermediate products were obtained using the Fischer-Johns Mel-Temp Apparatus.

Synthesis of Methyl-5-n-hexanoylsalicylate (2). Zinc chloride (2.1872 g, 16.04 mmol), methyl salicylate (1.70 mL, 13.12 mmol) and dichloromethane (6.00 mL) were mixed in a 100 mL round bottom flask. To this mixture, hexanoyl chloride (2.00 mL, 14.31 mmol) was added drop-wise forming a cloudy white mixture. Stirring of the reaction mixture for 48 hours produced a dark brown colored solution. The reaction mixture was extracted with CH_2Cl_2 (20 mL) followed by washing with distilled water (10 mL x 2). The organic layer was dried with anh. Na_2SO_4, filtered and concentrated *in vacuo* producing a viscous dark brown liquid. Column chromatography separation using 100% n-C_6H_{14} was carried out to remove the starting material. This was followed by using 60% n-C_6H_{14}/CH_2Cl_2 as solvent system to isolate the target compound. The target compound was generated as yellow needle-like crystals (0.656 g, 20.01 %). *Rf: 0.48 (2:3 n-C_6H_{14}-CH_2Cl_2); m.p.: 41-43°C; IR (thin film): 3550.15 – 3005.39cm^{-1} (aromatic –OH group), 1677.43cm^{-1} (ester C=O), 1677.43cm^{-1} (ketone C=O); MS/ESI (m/z):* calculated for *$C_{14}H_{18}O_4$: 250.29032; found [M+Na]: 273.11236 and [M+H]: 251.1299.*

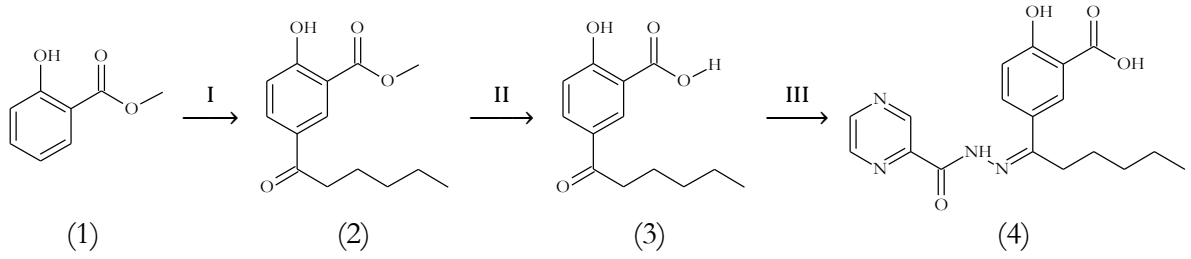
Synthesis of 5-n-Hexanoylsalicylic acid (3). A solution of methyl-5-n-hexanoylsalicylate (**2**) (0.3285 g, 1.312 mmol) dissolved in ethanol (4.00 mL) was mixed with sodium hydroxide (4.058 M) producing a clear orange solution. The reaction was heated to 90°C with continuous stirring for two hours. The solution was cooled to room temperature, and conc. HCl was added drop-wise. The organic components were extracted with CH_2Cl_2 (20.00 mL) and washed with distilled

water (10.00 mL x 2). The organic layer was dried with anh. Na_2SO_4, filtered and concentrated *in vacuo* producing an orange crystal (0.2706 g, 87.29 %). *Rf: 0.52 (25:1:1 ethyl acetate-EtOH-acetic acid); m.p.: 116-118°C; IR (KBr disk): 2508.17cm^{-1}-3465.85cm^{-1}(carboxylic acid –OH), 1672.43cm^{-1}(carboxylic acid C=O), 1672.43cm^{-1} (ketone C=O), 1200.37cm^{-1} (carboxylic C-O); MS/ESI (m/z):* calculated for *$C_{13}H_{16}O_4$: 236.26374; found [M-H]:235.09884.*

Synthesis of 2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid (4). 5-n-Hexanoylsalicylic acid (**3**) (0.2594 g, 1.098 mmol) dissolved in ethanol (4.00 mL) was mixed with a solution of pyrazine-2-hydrazide (0.1529 g, 1.107 mmol) dissolved in distilled water (2.00 mL) in a round bottom flask. Mixing the two solutions immediately formed an off-white precipitate. The reaction mixture was stirred for 48 hours producing an off-white mixture. The organic constituents were extracted with CH_2Cl_2 (20.00 mL), washed with distilled water (10.00 mL x 2) and concentrated *in vacuo* producing an off-white powder (0.3913 g, 62.43 %). *Rf: 0.39 (25:1:1 ethyl acetate-EtOH-acetic acid), m.p.: 239-243°C; IR (KBr disk): 3353.82cm^{-1}: -NH_2, 1674.36cm^{-1}: amide (C=O) and imine (C=N), 1713.23cm^{-1}: carboxylic acid (C=O), 1351.48cm^{-1}: amide (C-N) stretch; MS/ESI (m/z):* calculated for *$C_{18}H_{20}O_4N_4$: 356.37665; found [M-H] 355.13910; ^{1}H-NMR (400MHz, DMSO-d_6) δ: 11.04 (1H, s, carboxylic –OH, H24); 9.28 (1H, d, J value = 1.5, H3); 8.96 (1H, d, J value = 2.4Hz, H6) 8.82-8.81 (1H, dd, J value = 1.5 and 2.5Hz, H5); 8.31 (1H, d, J value =2.3, H18); 8.04-8.02 (1H; dd, J value = 2.4 and 8.8Hz, H22); 7.07 (1H, d, J value = 8.8Hz, H21); 3.92 (1H, s, H24); 1.31 (6H, m, H13-H15); 0.88 (3H, m, J value = 7, H16)*

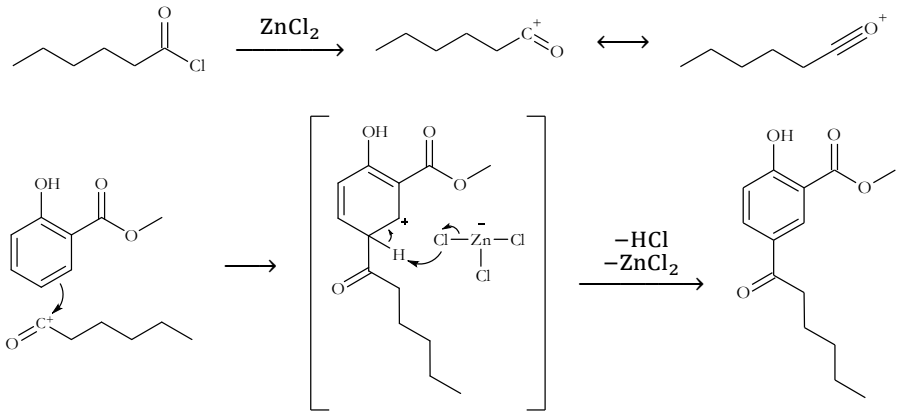
RESULTS AND DISCUSSION

The relevance of the pyrazinamide drug in the treatment of tuberculosis engages current research in the derivatization of the drug to be effective against resistant strains of mycobacteria. The retention of the pyrazinamide nucleus and its coupling with a vast set of compounds which may enhance its effectivity has been the study of a significant amount of research (Vergara *et al.*, 2009; Sriram *et al.*, 2006; Imramovsky *et al.*, 2007). Specifically the attachment of the pyrazinamide core through the formation of an N-acylhydrazone group has proven to be a simple method used in a previous study to effectively fuse the PZA core with potential compounds that may improve its efficacy against *M. tuberculosis* (Vergara *et al.*, 2009). This method has been adopted by the group to create a pyrazinamide analog with a salicylic acid moiety, which is done by creating a salicylic acid derivative with an acyl chain, to which the amine group of the pyrazinamide molecule may be fused via imine formation (Scheme 1). Previous study shows that the co-administration of weak acids lowered the number of CFU in normal and nutrient-starved media of mycobacteria (Chen *et al.*, 2007). This has prompted the group to fuse the pyrazinamide core to a salicylic acid molecule by the attachment of an acyl chain which contains a ketone group. This may be used as the point of attachment to the derivatized pyrazinamide nucleus in the hope that the resulting compound would increase the effectivity of the PZA drug against susceptible and resistant strains of mycobacteria.



Scheme 1. *I) hexanoyl chloride, zinc chloride, DCM, 20.01%; II) ethanol, sodium hydroxide, 60°C, 87.29%; III) ethanol, pyrazine-2-hydrazide, 62.43%.*

The attachment of an alkyl chain to the salicylic acid moiety was done to increase the lipophilicity of the salicylic acid derivative to be attached to the PZA ring. This was done via Friedel-Crafts acylation of methyl salicylate with hexanoyl chloride in the presence of zinc chloride as catalyst. Friedel-Crafts acylation reactions are useful alternative routes to direct alkylation reactions since it provides a less reactive aromatic compound which limits the generation of over alkylated products. This was then deemed to be the best pathway to attach an alkyl chain to the methyl salicylate precursor. The reaction proceeds via the generation of an acylium ion, followed by the bond formation using two pi-electrons from the methyl salicylate ring generating a carbocation intermediate. The removal of a proton adjacent to the carbocation regenerates the aromatic ring (Scheme 2).



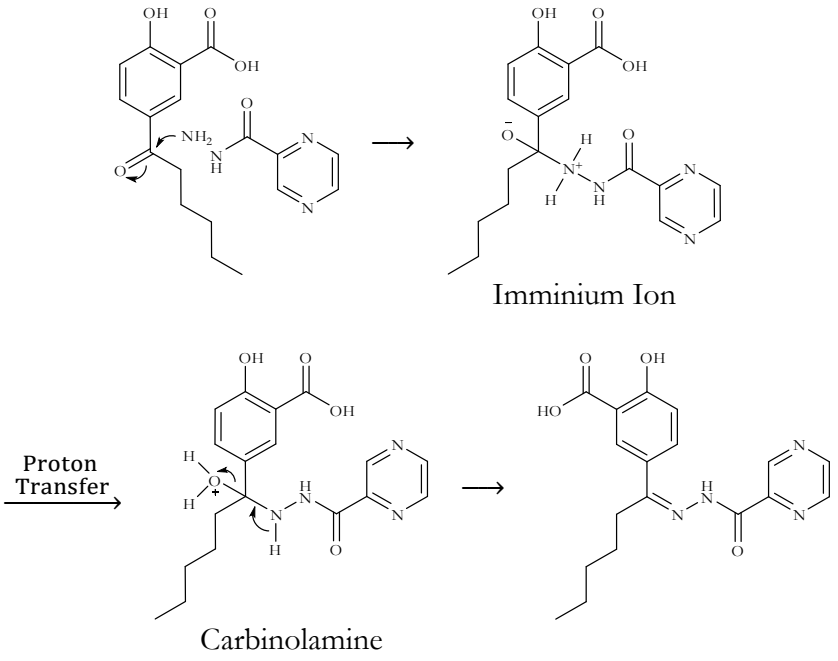
Scheme 2. *Mechanism of Friedel-Crafts Acylation for the synthesis of compound (2).*

This afforded methyl-5-n-hexanoylsalicylate (**2**) in 20.01% yield. The low yield may be attributed to the substantial amount of unreacted starting material and the presence of some by-products generated after the reaction. The low reactivity of the zinc chloride catalyst may have contributed to the relatively low yield (Heany, 1991).

Methyl-5-n-hexanoylsalicylate (**2**) was generated as yellow needle like crystals with a sharp melting point (41-43 °C). The TLC pure product (Rf = 0.48; 2:3 Hexane-CH_2Cl_2) gave an Rf value that is lower than that of methyl salicylate (Rf value = 0.61; 2:3 Hexane-CH_2Cl_2) which confirms that the addition of an acyl chain has increased the polarity of the molecule. The IR spectrum of the compound showed a signal at 1677 cm^{-1} which signifies the attachment of the ketone group. The mass spectrum of the compound showed a pseudomolecular ion peak at 273.11236 [M+Na] and 251.12995 [M+H] which are consistent with the expected mass of compound corresponding to a molecular formula of $C_{14}H_{18}O_4$ (m/z = 250.32).

To generate the salicylic acid group, the methyl salicylate derivative (**2**) was reacted with NaOH to allow hydrolysis of the ester group to carboxylic acid functionality. This generated 5-n-hexanoylsalicylic acid (**3**) as orange crystals in high yield (87.29 %). This structural change was confirmed by the broad signal at 3466 – 2508 cm^{-1} in the IR spectrum of the compound which corresponds to the carboxylic acid OH. The one-spot TLC profile with an Rf value of 0.52 (25:1:1 ethyl acetate-EtOH-acetic acid) and sharp melting point of the salicylic acid derivative further confirms the purity of the compound. The expected mass of m/z = 236.29 corresponding to the molecular formula of the compound ($C_{13}H_{16}O_4$) was confirmed by the presence of a pseudomolecular ion peak at 235.09884[M-H].

This salicylic acid derivative was then attached to the pyrazinamide nucleus via an imine formation reaction. The PZA molecule was first converted to pyrazine-2-hydrazide to allow for the reaction between the amino group of the hydrazide and the ketone group



Scheme 3. *Mechanism of Imine formation for the synthesis of compound (4).*

of the acyl chain in the salicylic acid moiety (Scheme 3) as adopted from the procedure used by Vergara and co-workers in the synthesis of N-acylhydrazones (Vergara *et al.*, 2009).

This route afforded the target compound (Figure 2) as an off-white powder in 62.43 % yield. The crude product generated after the reaction only required washing with water to remove the unreacted pyrazine-2-hydrazide and salicylic acid derivative. The structure of compound (4) was confirmed by ^{1}H-NMR. The singlet at 11.04 ppm confirms the presence of the NH proton of the amide group attached to the pyrazinamide moiety which is similar to the data generated in previous studies (Swamy, *et al.*, 2007). The absence of any doublet in the same region signifies the attachment of the amino group to the ketone functionality.

The relevant signals (Table 2) attributed to the pyrazinamide ring are given by the peaks at 8.81 -8.82 ppm (dd, J = 1.5, 2.5 Hz) due to the H5 proton. This shows ortho and meta coupling with the signals at 9.28 and 8.96 ppm which corresponds to H6 (J = 2.4 Hz) and H3 (J = 1.5 Hz) protons respectively.

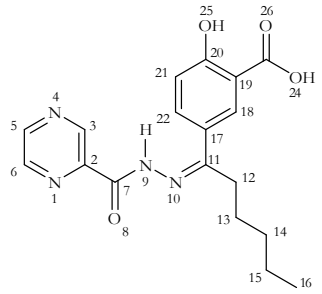


Figure 2. *2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl) hydrazono)hexyl) benzoic acid (4).*

The attachment of the acyl chain at the para position relative to the OH group in the salicylic acid ring was further confirmed by the signal of the H22 proton at 8.04 ppm (dd, J = 2.4, 8.8 Hz). This confirms the ortho coupling of this proton with the with the H21 proton at 7.07 ppm (d, J = 8.8 Hz) and its meta coupling with the H18 proton at 8.30 ppm (d, J = 2.3 Hz).

The protons at the alkyl chain were also confirmed with the multiplet signal at 1.34 ppm corresponding to the methylene protons in the chain. The triplet at 2.87 ppm (J = 7.8 Hz) correspond to the methylene protons nearest the imine bond, as shown in the table below.

Table 2. ^{1}H-NMR Results for 2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono) hexyl)benzoic acid (4).

Proton Chemical Shift, δ (ppm)	Integration	Multiplicity	Coupling Constant, J (Hz)	Assignment
0.87	3H	(m)	7	H16
1.34	6H	(m)		H13-H15
2.87	2H	(t)	7.8	H12
3.92	1H	(s)		H25
7.04-7.07	1H	(d)	8.8	H21
8.04-8.02	1H	(dd)	2.4, 8.8	H22
8.31-8.30	1H	(d)	2.3	H18
8.82-8.81	1H	(dd)	1.5, 2.5	H5
8.96	1H	(d)	2.4	H6
9.28	1H	(d)	1.5	H3
11.04	1H	(s)		H24

The attachment of the PZA nucleus is further confirmed by the signal at 1674 cm^{-1} corresponding to the C=N stretch of the imine functionality. The TLC profile of the compound showed an Rf value of 0.39 (25:1:1 ethyl acetate-EtOH-acetic acid) which proves that the addition of the PZA ring made the compound more polar compared to that of compound 3. The purity of the compound was further confirmed by a melting point range at 239-243°C.

The pseudomolecular ion peak at *355.13910 [M-H]* proved to be consistent with the expected molecular mass of 356.42 amu corresponding to the molecular formula of the target compound ($C_{18}H_{20}N_4O_4$).

CONCLUSION

A new salicylic acid derivative of pyrazinamide, 2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid (**4**) was synthesized and characterized. The preparation involved the Friedel-Crafts acylation of methyl salicylate (**1**), the base hydrolysis of the acylated product followed by its coupling with pyrazine-2-hydrazide. This scheme proved to be a viable method, generating the target compound in *62 %* yield. This compound may exhibit an increased activity of the PZA drug against *M.tuberculosis*. Thus, it is recommended that the compounds be tested for antimycobacterial activity to determine its effectiveness for the treatment of tuberculosis.

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