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SYNTHESIS OF (Z)-2-HYDROXY-5-(1-(2-(PYRAZINE-2-CARBONYL)HYDRAZONO)HEXYL)BENZOIC ACID, A PYRAZINAMIDE ANALOG OF SALICYLIC ACID

Glenn V. Alea, Michelle Pia Anne B. Austria, Alan Christian S. Lim, Faith Marie G. Lagua, and Michael Dominic M. Ajero
Chemistry Department-De La Salle University Manila

Abstract: 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, (Z)-2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid (**4)** was synthesized and characterized. The compound was prepared via molecular hybridization of 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 Craft's acylation of methyl salicylate followed by base hydrolysis of the acylated product. This was coupled with the Pyrazinamide moiety via imine formation. (Z)-2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid **(4)** was obtained as an off-white powder in 62% yield. The addition of a salicylic acid derivative with a six-carbon alkyl chain may increase the lipophilicity of the drug and enhance its permeability to the Mycobacterial cell wall. It may also exhibit activity against resistant strains of *Mycobacterium tuberculosis* towards Pyrazinamide.

Key Words: Tuberculosis; Pyrazinamide; Salicylic acid; Friedel Craft's Acylation; Imine Formation

1. INTRODUCTION

Tuberculosis (TB) is one of the leading causes of death in the world from an infection of acid-fast bacilli *Mycobacterium tuberculosis.*[1] This complex of 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.[2,3]

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 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. [4]

The increase in the development of these multi-drug resistant strains of tuberculosis (MDR-TB) and much worse extensively-drug resistant strains (XDR-TB) which is resistance to the second-line of drugs used to treat the disease has steered 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[5]. This is due to its sterilizing activity, which kills a population of persistent tubercle bacilli that are not affected by other drugs. 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. [6] This stimulated researches that are dedicated to the synthesis of PZA analogues that will show an increased efficacy compared to PZA. Some of these analogues exhibit both bacteriostatic and bactericidal effect on different strains of mycobacterium, *in vitro.*[7]

In another related study, the presence of weak acids such as benzoic acid, fatty acids and salicylic acid could enhance PZA activity against *Mycobacterium tuberculosis*[8] in a nutrient starved incubation condition. It was also shown that the co-administration of PZA with non-steroidal anti-inflammatory drugs, specifically ibuprofen and aspirin, has shown an increase in potency of PZA in a mouse model infected with *Mycobacterium tuberculosis* H37Rv[9].

In view of these results, we envisioned the synthesis of a molecular hybrid of the Pyrazinamide drug and a salicylic acid derivative with a six-carbon alkyl chain. The addition of the alkyl chain may increase the lipophilicity of the compound and aide in its diffusion through the mycobacterial cell wall. This compound may show an increase in the efficacy of the Pyrazinamide drug towards susceptible and resistant strains of *Mycobacterium tuberculosis.*

2. METHODOLOGY

In this study, a pyrazinamide analog of salicylic acid **(4)** was generated via Friedel-Crafts acylation of Methyl salicylate with Hexanoyl chloride using Zinc chloride as catalyst,

followed by base hydrolysis to generate the corresponding salicylic acid derivative **(3)**. Coupling with Pyrazine-2-hydrazide afforded the target compound **(4)** in a relatively high yield.

These compounds were then characterized for their melting point, IR spectra, Mass spectra, and ^{1}H-NMR spectra.

2.1. Materials, Reagents and Apparatus

All reagents used were in analytical grade with ≥99% purity. All chemicals and solvents used were purchased from Sigma-Aldrich Chemicals, Singapore and Merck Chemicals, Philippines except for pyrazine-2-hydrazide which was prepared in a previous study[10].

For the characterization of the target compounds, functionalities were determined using Nicolet-500 FT-IR Spectrometer. The mass spectra of the compounds were obtained using Brűker 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 Ateneo de Manila University. The melting points of the target compounds and all intermediate products were obtained using Fischer-Johns Mel-Temp Apparatus. 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.

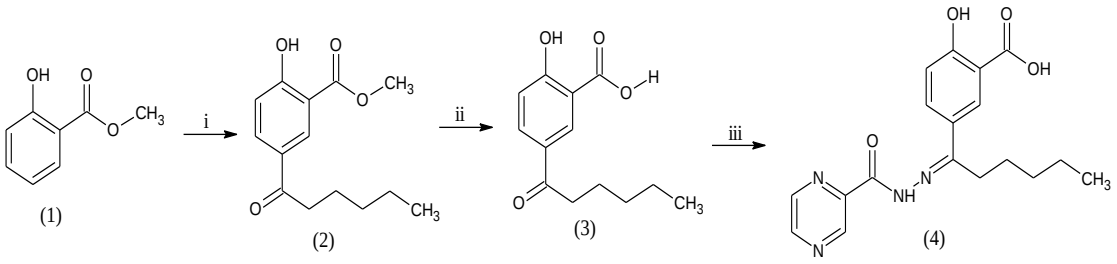

Figure 1. i. Hexanoyl Chloride, Zinc Chloride, DCM; ii. Ethanol, Sodium Hydroxide, Δ60°C; iii. Ethanol, Pyrazine-2-Hydrazide

The precursor compounds Methyl 5-n-hexanoylsalicylate **(2)** and 5-n-hexanoylsalicylic acid **(3)** were prepared via Friedel Crafts acylation using $ZnCl_2$ as catalyst followed by base hydrolysis as shown in Figure 1.

2.2 Synthesis of (Z)-2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid (4)

In a round bottom flask, 5-n-hexanoylsalicylic acid (0.2594g, 1.098mmol) dissolved in ethanol (4.00 mL) was mixed with a solution of pyrazine-2-hydrazide (0.1529g, 1.107mmol) dissolved in distilled water (2.00 mL). Mixing the two solutions immediately formed an off-white precipitate. The reaction mixture was left to stir for 48 hours producing an off-white mixture The organic constituents were extracted with dichloromethane (20.00 mL), washed twice with distilled water (10.00 mL) and concentrated *in vacuo*. (Z)-2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid **(4)** was obtained as an off-white powder in 62.43% yield. *Rf value: 0.39(25 Ethyl acetate: 1 EtOH: 1 Acetic acid), MP: 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): 355.13910 [M-H]; MF: C$_{18}$H$_{20}$O$_4$N$_4$. ^{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)*

All of the synthesized compounds were subjected to melting point determination, IR analysis, and Mass Spectra analysis. Only Compounds (4) was subjected to ^{1}H-NMR analysis for confirmation of the structure.

3. RESULTS AND DISCUSSION

In this research study, a Pyrazinamide analog of salicylic acid, (Z)-2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid **(4)** was synthesized. The compound contains a derivative of salicylic acid which contains a six carbon alkyl chain coupled to a pyrazinamide group. This compound may exhibit better inhibitory effects against susceptible and resistant strains of mycobacterium towards pyrazinamide.

Synthesis of (Z)-2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid (4)

The synthesis of the compound **4** involved the formation of an imine bond between the carbonyl carbon of the acyl chain and the nitrogen atom of the hydrazine group in pyrazine-2-hydrazide. The reaction follows a nucleophilic addition mechanism followed by elimination as shown in Figure 2. In the reaction, the hydrazide group acts as a nucleophile to the carbonyl carbon which drives the delocalization of the electrons to the oxygen resulting in the formation of a resonance-stabilized iminium ion. This was followed by proton transfer generating the unstable carbinolamine. Elimination of water by protonating the hydroxyl group followed by a loss of a proton in the succeeding step generated the imine bond.

Compound **4** was obtained as an off-white powder (0.3913 g, 62.43 %) with a melting point of 239-243°C. The IR spectrum of the compound showed peaks at 3354 cm^{-1}, 1713 cm^{-1},

1674 cm^{-1}, and 1352 cm^{-1} which corresponds to the absorption of an amide N-H bond, a carboxylic acid (C=O), both an amide (C=O) and an imine (C=N) stretch, and an amide (C-N) stretch respectively.

The mass spectrum of compound **4** showed a pseudomolecular ion peak at m/z *355.13910 [M-H]* which was consistent with the expected molecular mass of 356.42 amu corresponding to the molecular formula of the target compound **(**$C_{18}H_{20}N_4O_4$)

The ^{1}H-NMR spectrum of compound (4) was obtained using deuterated DMSO as solvent. The multiplet at 0.87 ppm (J = 7 Hz) corresponds to the protons of the methyl group at the terminal carbon of the alkyl chain. The multiplet at 1.34 corresponds to the methylene protons along the alkyl chain. A triplet at 2.87 (J = 7.8 Hz) corresponds to the methylene protons immediately next to the imine bond. Also, a broad singlet at 3.92 corresponds to the proton of the hydroxyl group in the phenyl ring.

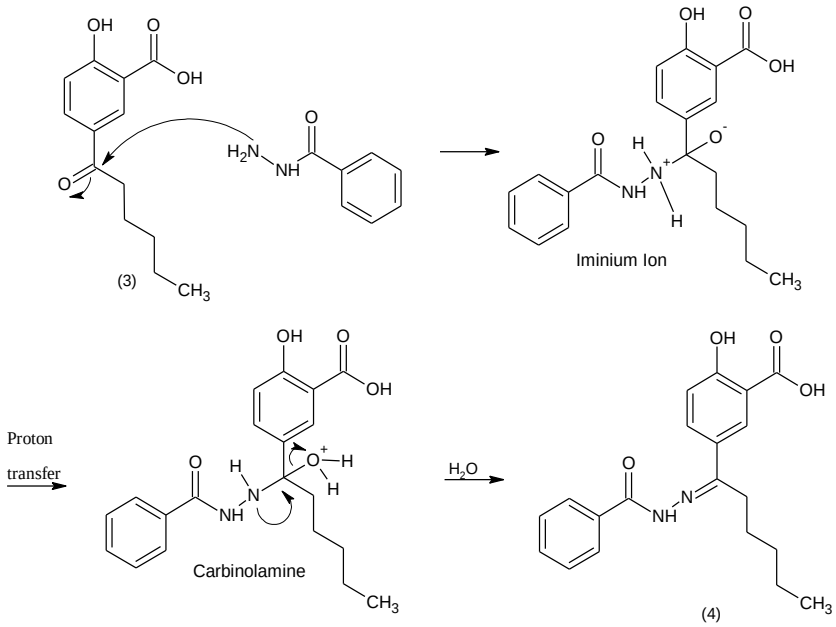

Figure 2. Mechanism of Imine formation for the synthesis of Compound **4**

The signals present at around 7.07 (J = 8.8Hz) and 8.30 ppm (J = 2.3 Hz) corresponds to the hydrogen atoms in the phenyl ring. The ortho-positioning of the alkyl chain was confirmed by the presence of a doublet of a doublet at 8.04 ppm (J value = 2.4, 8.8 Hz) corresponding to the

H22 proton which shows ortho coupling with the H21 proton (J value = 8.8 Hz) and meta coupling with the H18 proton (J value = 2.3 Hz). The doublet of a doublet at 8.82-8.81 ppm (J = 1.5, 2.5 Hz) is attributed to H5 proton in the pyrazinamide ring which showed 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. The singlet at 11.04 ppm corresponds to the proton of the hydroxyl group of the carboxylic acid moiety. All these signals observed from the ^{1}H-NMR is consistent with the structure of compound 4.

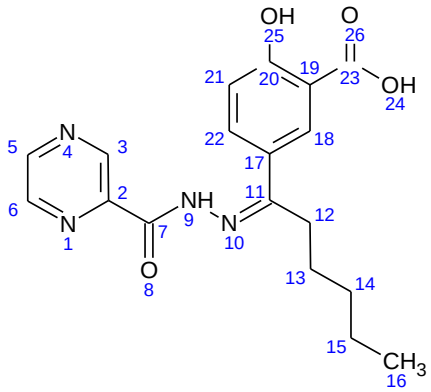

Figure 3. (Z)-2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid **(4)**

4. CONCLUSION

A salicylic acid derivative of Pyrazinamide, (Z)-2-hydroxy-5-(1-(2-(pyrazine-2-carbonyl)hydrazono)hexyl) benzoic acid (4) was synthesized and characterized. The preparation involved the Friedel Craft's acylation of methyl salicylate (1), 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 *62.43%* yield. This compound may exhibit an increased potency against *M.tuberculosis*. Thus, it is recommended that the compounds be tested for antimycobacterial activity to determine the effectiveness of the synthesized compound for the treatment of tuberculosis.

5. ACKNOWLEDGEMENT

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6. REFERENCES

1) Todar, K. (2012), *Todar's Online Textbook of Bacteriology: Tuberculosis (Mycobacterium tuberculosis).* Retrieved from World Wide Web: http://textbookofbacteriology.net/tuberculosis.html
2) World Health Organization, (March 2012). WHO Global TB Control Report 2011. Retrieved from World Wide Web: http://www.who.int/tb/publications/global_report/2011/gtbr11_full.pdf
3) Center for Disease Control and Prevention, (19 July 2012). Division of Tuberculosis Elimination. Retrieved from World Wide Web: http://www.cdc.gov/tb/
4) World Health Organization (October 2012) WHO Global Tuberculosis Report 2012. Retrieved from the World Wide Web:http://www.who.int/tb/publications/global_report/gtbr12_executivesummary.pdf
5.) Zhang, Y.; Wade, M. M.; Scorpio, A.; Zhang, H.; Sun, Z. (2003). Mode of action of pyrazinamide: disruption of *Mycobacterium tuberculosis* membrane transport and energetics by pyrazinoic acid. *Journal of Antimicrobial Chemotherapy*. 52, 790 – 795**.**
6.) Mitchison, D. A.; Zhang, Y. (2011) Recent Developments on the Study of Pyrazinamide: An Update. *Antituberculosis Chemotherapy*, *Prog Respir Res.* Donald, P. R.; van Helden, P. D., Eds.; Karger: Basel,; Vol. 40; pp 32 – 43.
7.) Yamamoto, S.; Toida, I.; Watanabe, N.; Ura, T.(1995). In Vitro Antimycobacterial Activities of Pyrazinamide Analogs. *Antimicrobrial Agents and Chemotherapy.* 39, (9), 2088 – 2091.
8.) Chen, Z. F.; Huang, Q.; Li, Y. Y.; Zhang, Y.; Ren, Y.; Li, K. S.; Fu, Z. J.; Xu, S. Q. (2007). Nutrient starved incubation conditions enhance pyrazinamide activity agains *Mycobacterium tuberculosis. Zhonghua Jie He He Hu Xi Za Zhi*. May, 30, (5) 359 – 362.
9.) Byrne, S. T.; Denkin, S. M.; Zhang, Y. (2007). Aspirin and ibuprofen enhance pyrazinamide treatment of murine tuberculosis. *Journal of Antimicrobial Chemotherapy*, 59, 313 – 316.
10.)Lagua, F.M. Synthesis and Characterization of Novel Pyrazinamide Analogues of Salicylic acid and Aspirin. Unpublished Graduate Thesis, De La Salle University 2011