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Synthesis and Characterization of Pyrazinamide Analogs of Acetylsalicylic Acid and Salicylic Acid

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Pyrazinamide (PZA) is one of the first-line of drugs used to treat tuberculosis. It is an important player in shortening the treatment time of the disease from almost a year to only about six months. The occurrence of resistant strains of the bacteria towards PZA threatens its effectiveness in killing semi-dormant and persistent bacilli in the current and future therapy methods to combat the disease. In this study, PZA analogs of salicylic acid (compounds 5a and 5b) and acetylsalicylic acid (compounds 6a and 6b) were synthesized and characterized. The synthesis involved the preparation of salicylic acid and acetylsalicylic acid derivatives with varying acyl chains via Friedel-Crafts acylation of methyl salicylate, followed by subsequent hydrolysis and acetylation to produce the respective precursor compounds. These were then coupled with 2-pyrazinehydrazide to produce the desired PZA analogs. These analogs may exhibit increased potency against PZA-resistant and susceptible strains of *Mycobacterium tuberculosis*. Characterizations of the compounds were done by IR spectroscopy, high-resolution mass spectrometry, and ^{1}H-NMR spectroscopy.

Key words: aspirin, Friedel-Crafts acylation, imine formation, pyrazinamide, salicylic acid, tuberculosis

INTRODUCTION

Tuberculosis (TB) is now ranked above HIV as a leading cause of death worldwide (WHO 2016). It is caused by *Mycobacterium tuberculosis*, which may be acquired through the ambient air upon interaction with people that expel the bacteria by coughing (Zhang et al. 2003).

In the previous global TB report of 2015 published by the World Health Organization (WHO), the global prevalence, mortality, and incidence rate of the disease has reached half of its 1990 levels. These improvements were seen specifically in nine of the WHO list of high burden countries, which includes the Philippines. These developments show that effective and timely treatment of the disease could reduce its burden (WHO 2015).

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Despite these great accomplishments by the WHO's STOP TB program that started in 2000, there are 10.4 million incident cases of TB that have been reported in 2015, 61% of which stem from Asia. Mortality due to the disease is still considered relatively high, with 1.4 million deaths in the same year among HIV-negative people and an added 0.39 million deaths among those with HIV. A much more alarming statistic is the 580,000 estimated number of incident cases of people with multi-drug resistant TB (MDR-TB). This is a form of TB caused by bacteria that is resistant to at least isoniazid and rifampicin, two of the first-line of drugs (isoniazid, rifampin, pyrazinamide, and ethambutol) used to treat the disease (WHO 2016).

The Philippines is listed among the countries with the highest burden of TB and MDR-TB (WHO 2016). About 15,000 patients among those with pulmonary TB have been tested positive with MDR-TB in 2015. There is also an estimate of about 322 incident cases of TB (with HIV

disease) for every 100,000 of the population in the same year (WHO 2016).

The current goal of WHO under the END TB Campaign for the year 2016-2035 is to reduce the mortality rate and incidence rate by 95% and 90%, respectively, compared to their 2015 levels. In order to accomplish this goal, the number of TB incident cases should have reduced from currently 133 for every 100,000 population to just about less than 10 for every 100,000 by the year 2035 (WHO 2015). This becomes a great challenge due to the added occurrence of extensively drug resistant TB (XDR-TB), which is resistance to the second-line of drugs, and the higher probability of patients with HIV to develop TB at the same time (Servusova et al. 2013).

These targets may only be achieved with the help of new diagnostics, drugs, and vaccines. One very important first-line of drug – which reduces the treatment time of TB from 9-12 months to just about 6 months – is pyrazinamide (PZA) as shown in Figure 1 (Zhang et al. 2003). A special feature of the drug is its capacity to be used on susceptible and multi-drug resistant strains of the bacteria (Shi et al. 2011). The drug has a unique ability to inhibit semi-dormant bacilli which thrive in acidic environments (Njire et al. 2016). Due to this, PZA is being considered to be combined with future anti-TB drugs like moxifloxacin and pretomanid (PA-824), which are already undergoing Phase 3 clinical trials (Njire et al. 2016).

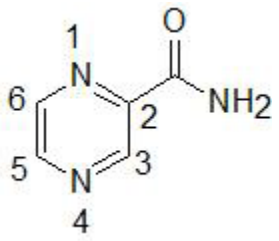


Figure 1. PZA drug (A).

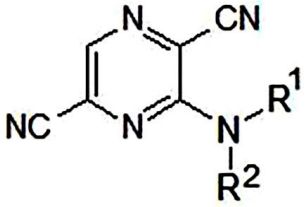
As the mode of action of the PZA drug has not fully been understood, several groups have reported different possible models. One of the initial studies showed that the PZA drug is converted by pyrazinamidase (PZAse) to pyrazinoic acid (POA) inside the bacterial cell. Accumulation of POA in the cytoplasm then reduces the pH inside the cell resulting to a disruption of membrane energetics (Zhang et al. 2003).

In another study, PZA and POA are both taken as competitive inhibitors of NADPH for its binding to *Mycobacterium tuberculosis* fatty acid I synthase (*Mtb* FAS I). The PZA and POA bind at different sites of *Mtb* FAS I, which enables the inhibition of this enzyme via different mechanisms that have not been fully elucidated at the moment (Sayahi et al. 2011). Further investigations on the mechanism of the active metabolite concluded that the formation of POA and the accumulation of its protonated form in the cytoplasm disrupts the proton motive force and eventually decreases the synthesis of respiratory ATP, which directly causes a decrease of cellular ATP levels (Lu et al. 2011). A more specific explanation of the mode of action of PZA was reported by Shi and co-workers (2011), who explain that the effectiveness of the PZA drug is due to the binding of POA to the *Mtb* RpsA ribosomal protein S1. This inhibits the trans-translation process that is an essential mechanism for survival of *Mtb* under stressful conditions (i.e., starvation, presence of other antibiotics, hypoxia, and an acidic environment) (Shi et al. 2011).

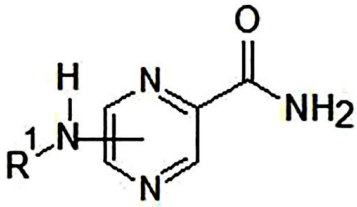
The occurrence of PZA resistance threatens its effectivity as a mainstay in the TB chemotherapy regimen for current treatments and for newly developed anti-TB drugs. One of the main mechanisms of PZA resistance is blamed after the mutations on the *pncA* gene, which codes for the PZAse enzyme that converts PZA to its active POA form (Njire et al. 2016). This has been observed for almost 70-97% of isolated strains of *Mtb*; the other 3-30% of resistant strains do not have mutations on the *pncA* gene, but are due to other variants of the *pncA* gene and other genes like *rpsA* and *panD*, which are related to the other modes of action of PZA (Njire et al. 2016). Findings suggest that the inclusion of PZA to the fluoroquinolone treatment regimens for patients with MDR-TB improved treatment success by 38%. This was also previously seen in a study done for a mouse model, which showed enhanced activities for the second-line of drugs that included PZA than those that did not (Njire et al. 2016). In 2015, a global Phase 3 clinical trial called STAND (Shortening Treatment by Advancing Novel Drugs) was launched by the TB alliance organization and its partners. This aims to test patients with drug-susceptible TB and MDR-TB with PaMZ, a new set of drugs that includes PA-824, moxifloxacin, and PZA. If successful, subsequent development from these trials could eliminate the use of injectable second-line of drugs and also reduce the cost of treatment by 90% (Njire et al. 2016).

Alongside these developments in clinical trials, research is still relevant to the discovery of PZA analogs that could perform better or in the same way as PZA. Several modifications were already done to study the structure activity relationships (SAR) of introducing different substituents on the pyrazine nucleus (Zitko et al. 2011, 2012; Servusova et al. 2014) and also keeping the PZA group intact and coupling it with other molecules that could improve its activity (Sriram et al. 2006; Imramovsky et al. 2007; Vergara et al. 2009; Alea et al. 2013) as shown in Figure 2 and Figure 3. PZA analogs that made substitutions on the pyrazine ring were

N-substituted 6-amino-5-cyano-pyrazine-2-carboxamides (R^1 = Cl or alkylamino groups) (Zitko et al. 2011) (B)

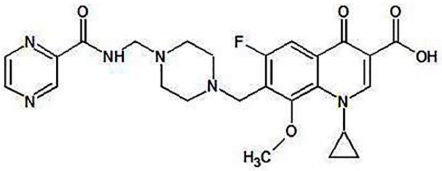


N-substituted 3-aminopyrazine-2,5-dicarbonitriles (R^1 = alkyl group, R^2 = H) (Zitko et al. 2012) (C)

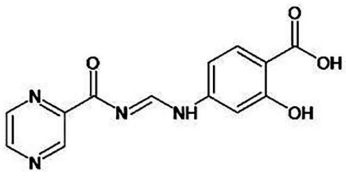


N-substituted 5-amino and 6-aminopyrazine-2-carboxamides (R^1 = octyl group) (Servusova et al. 2014) (D)

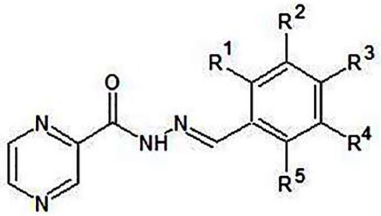
Figure 2. Previously synthesized PZA analogs with PZA ring substitutions.



PZA derivative of Mannich bases (Sriram et al. 2006) (E)



PZA fused with PAS (Para-aminosalicylic acid) (Imramovsky et al. 2007) (F)



N-acylhydrazone derivatives of PZA (Vergara et al. 2009) (G)

Figure 3. Previously synthesized PZA analogs with intact PZA ring.

successful in making compounds that are comparable and more effective than PZA *in vitro* against *Mtb*. In a series of publications, Zitko and co-workers showed that the substitution of chlorine and an alkylamino group in position 6 of the pyrazine ring resulted to derivatives (B) that have minimum inhibitory concentration (MIC) values comparable to PZA towards *Mtb* H37Rv and towards non-tuberculous mycobacterial strains that are not susceptible to PZA (Zitko et al. 2011). In their next work, they placed a cyano group in position 2 instead of a carboxamide and different lengths of alkyl amino groups were introduced at position 3. The results showed that alkyl chains of 5-7 carbons gradually improved the minimum inhibitory concentrations against *Mtb in vitro* (C). These were consistent with their previous work that strengthens the evidence that lipophilic character is an important determinant of the anti-mycobacterial activity of these compounds (Zitko et al. 2012). This has also shown the importance of having a hydrogen atom in the amino group as substituents, wherein a decrease in the activity relative to PZA was seen when a tertiary amino group is used. Servusova and co-workers also studied substitutions of alkyl amino groups on the pyrazine ring at positions 5 and 6 with the retention of the carboxamide group at position 2. They found that the most potent derivative was 6-octylaminopyrazine-2-carboxamide (D), which contained an octyl amino group at position 6. This report also concludes that this derivative might not have the same mode of action as that of the PZA drug. This is due to the presence of long alkyl substitutions at position 6, which would not be able to fit into the catalytic site of the PZase enzyme according to a docking study that was also done by the group (Servusova et al. 2014).

Other groups also investigated on keeping the PZA group intact and coupling it with other molecules in the hopes of improving its activity. A Mannich base derivative of PZA, 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 (E) showed activity against MDR-TB, with MIC value (0.20 µg/mL) that is 125 times greater than the potency of the parent PZA drug (MIC of PZA = 25.0 µg/ml). This has been explained to be due to the increased lipophilicity of the molecule upon coupling and its independence of activity from pH (Sriram et al. 2006). PZA analogs with other anti-TB drugs were also studied. One of the most potent is a derivative (F) that included *p*-aminosalicylic acid, as it gave an MIC value of 3.13 µg/mL (Imramovsky et al. 2007). Another mechanism by which the integrity of the PZA group is kept intact is via the formation of N-acylhydrazones containing the PZA nucleus (G). The synthesis involved the use of monosubstituted benzaldehyde moieties that have compounds with e-withdrawing groups (i.e., Cl, F, CN, NO_2) attached in the ortho (R^1) and meta (R^2) position of the benzaldehyde ring to exhibit better activity (MIC =

50-100 µg/mL) compared with PZA (MIC > 100 µg/mL) (Vergara et al. 2009).

In a study conducted by Chen and co-workers (2007), the co-administration of weak acids (i.e., benzoic acid, fatty acids, and salicylic acid) with PZA showed lower colony forming units (CFU) of *Mtb* in both normal and nutrient starved incubation conditions. The same increased potency of PZA was observed with the addition of non-steroidal anti-inflammatory drugs (e.g., ibuprofen and acetylsalicylic acid) in a mouse model infected with *Mycobacterium tuberculosis* H37Rv (Byrne et al. 2007). In view of these results, the researchers performed the synthesis of PZA analogs of salicylic acid (5a and 5b) and acetylsalicylic acid (6a and 6b) with varying alkyl chains. Expectations were placed on these new analogs to exhibit substantial increase in potency compared to PZA, which can be considered as a potential drug candidate that could treat susceptible and resistant strains of *Mtb* towards PZA. The synthetic strategy involved the integration of the PZA group via imine formation with the ketone group of the acylated salicylic acid and acetylsalicylic acid. A 4-carbon and 8-carbon acyl chain − on the salicylic acid and acetylsalicylic acid derivatives − were also introduced to increase the lipophilicity of these new analogs and allow it to better diffuse into the mycobacterial cell wall. All of the synthesized compounds were characterized with melting point, FT-IR spectroscopy, high-resolution mass spectrometry, and ^{1}H-NMR spectroscopy.

MATERIALS AND METHODS

All reagents ($ZnCl_2$, methyl salicylate, pyrazinoic acid, hydrazine hydrate, butanoyl, and octanoyl 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 by the group in a previous study (Alea et al. 2013).

The purity of the product in each reaction was determined by thin layer chromatography. The TLC plates used were 4 cm x 2 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 Ateneo de Manila University's National Chemistry Instrumentation Center (NCIC). The melting points of the target compound and all intermediate products were obtained using the Fischer-Johns Mel-Temp apparatus.

Synthesis of Precursor Compounds to the PZA Analogs of Salicylic Acid (5a and 5b) and Acetylsalicylic Acid (6a and 6b)

Synthesis of Methyl 5-*n*-Alkanoylsalicylate (2a and 2b)

Synthesis of Methyl 5-*n*-Octanoylsalicylate (2a)

A mixture of methyl salicylate (1) (1.7 ml, 13.12 mmol), octanoyl chloride (3.85 ml, 22.55 mmol), and CH_2Cl_2 (5.5 ml) was added drop wise to a mixture of zinc chloride (5.45 g, 39.99 mmol) and CH_2Cl_2 (13.5 ml) under N_2 at ~5-10° C. The mixture was left to stir overnight (~17.5 h) at room temperature.

After overnight stirring, the mixture was poured in a beaker containing 50 ml of ice-water and conc. HCl (3 ml). The resulting solution was extracted with diethyl ether (75 ml). The organic layer was then washed with saturated NaCl solution (75 ml). The clear yellow organic layer was then dried with anhydrous Na_2SO_4 and filtered, while the solvent was evaporated *in vacuo*. The crude product was an oily liquid with a brownish yellow color. The crude product was subjected to column chromatography using 40% Hexane in CH_2Cl_2 as eluent. The product was obtained as white needle-like crystals with the following properties: (0.4643 g, 12.72%); mp: 53-55° C; Rf: 0.55 (20% hexane/ CH_2Cl_2); IR (thin film): 3159 cm^{-1} (Ar-OH), 3005 cm^{-1} (Ar-CH), 1728 cm^{-1} (Ar-Ketone), and 1678 cm^{-1} (Ar-Ester). MS/ESI (m/z) calcd for MF: $C_{16}H_{22}O_4$: 301.141579 [M+Na]; found: 301.1440 [M+Na].

Synthesis of Methyl 5-n-Butanoylsalicylate (2b)

A mixture of methyl salicylate (1) (4.26 ml, 32.87 mmols), butanoyl chloride (3.44 ml, 32.86 mmols), and in CH_2Cl_2 (16 ml) was added drop wise (over 10 min, addition) to a mixture of anh. $ZnCl_2$ (9.9878 g, 73.28 mmols) and CH_2Cl_2 (46 ml). The solution was left to stir overnight at room temperature.

After overnight stirring, distilled water (111.0 ml) was added and the solution was left to stir at room temperature for 1.5 h. Another 50 ml of dis. H_2O was added, followed by CH_2Cl_2 (5 ml). The organic layer was collected, dried with anh. Na_2SO_4, and concentrated *in vacuo*. The crude product was further purified via column chromatograph (40% petroleum ether in CH_2Cl_2). The product was obtained as white small needle-like crystals with the following properties: (0.303 g, 8.295%); mp: 68-70° C; Rf: 0.28, (40% pet ether/CH_2Cl_2); IR (thin film): 3416 cm^{-1} (Ar-OH), 3005 cm^{-1} (Ar-CH), 1674 cm^{-1} (Ar-ketone), and 1674 cm^{-1} (Ar-ester); MS/ESI (m/z) calcd for MF:

$C_{12}H_{14}O_4$: 223.097034 [M+H] and 245.078979 [M+Na]; found: 223.0956 [M+H] and 245.0775 [M+Na].

Synthesis of 5-n-Alkanoylsalicylic Acid Precursors (3a and 3b)

Synthesis of 5-n-Octanoylsalicylic Acid (3a)
A solution of NaOH (0.2378 g, 5.945 mmols) dissolved in distilled water (4.0 mL) was added to a solution of the methyl ester of 5-*n*-octanoylsalicylic acid (2a) (0.2922 g, 1.050 mmols) dissolved in ethanol (3.3 mL). The resulting solution was heated for 1.5 h at a temperature of 60° C. The reaction mixture was cooled to room temperature, after which, conc. HCl (0.5 mL, 6 mmol) was added. The white precipitate formed was suction filtered, washed with cold distilled H_2O, and dried in desiccators. The 5-n-octanoylsalicylic acid was derived as a white flaky solid with the following properties: (0.2432 g, 87.64%); mp: 112-114° C; Rf: 0.20 (8% methanol in ethyl acetate); IR (thin film): 3345 -2359 cm^{-1} (carboxylic acid-OH), 3091 cm^{-1} (Ar-CH), 1674 cm^{-1} (carboxylic acid C=O and ketone C=O), and 1204 cm^{-1} (carboxylic acid C-O); MS/ESI (m/z) calcd for MF: $C_{15}H_{20}O_4$: 265.143984 [M+H] and 287.125929 [M+Na]; found: 265.14619 [M+H] and 287.13090 [M+Na].

Synthesis of 5-n-Butanoylsalicylic Acid (3b)
A solution of NaOH (0.1014 g, 2.535 mmols) dissolved in distilled water (2.0 mL) was added to a solution of the methyl ester of 5-*n*-butanoylsalicylic acid (3a) (0.1036 g, 0.4661 mmols) dissolved in ethanol (2.6 mL). The resulting solution was heated for 1 h at a temperature of 60-65° C. The reaction mixture was cooled to room temperature, after which, conc. HCl (0.2850 mL, 3.42 mmol) was added. The solution did not immediately produce the white precipitate, so an additional 15 drops of conc. HCl was added. The solution was triturated and cooled to allow the crystals to form. The crystals were suction filtered, washed with cold distilled H_2O and dried in a dessicator. The product was derived as a white shiny powdery solid with the following properties: (0.0868 g, 89.43%); mp: 134-138° C; Rf: 0.39, (25 ethyl acetate:1 EtOH: 1 acetic acid); IR (thin film): 3423 cm^{-1} (Ar-OH and carboxylic acid-OH), 1668 cm^{-1} (Ar-ketone), and 1588 cm^{-1} (carboxylic acid-C=O); MS/ESI(m/z) calcd for MF: $C_{11}H_{12}O_4$: 209.081384 [M+H] and 231.063329 [M+Na]; found: 209.0796 [M+H] and 231.0616 [M+Na].

Synthesis of 5-n-Butanoylacetylsalicylic Acid (4b)
A mixture of anh. sodium acetate (0.0082 g) and acetic anhydride (4 ml, 42.31 mmols) was heated to boiling to dissolve the sodium acetate. After which, 5-*n*-butanoylsalicylic acid (3b) (0.1092 g, 0.5244 mmol) was added and the mixture was heated for 15 min at a temp of about 100-120° C. This solution was then cooled to room temperature and poured over crushed ice to allow precipitation to take place. This was left in the refrigerator overnight. The following day, some precipitate was observed. There were still some oily liquid at the bottom, so the solution was triturated and additional ice was added to allow further precipitation of the target compound. More of the precipitate came out of the solution and this was filtered as well. The remaining oily liquid was left in the refrigerator in ice to allow precipitation. This eventually precipitated to a light yellow solid. The white precipitate which came out first in the solution showed only two spots in the TLC. The more polar spot was larger than the other (somewhat negligible; very faint under UV). The light yellow precipitate had more impurities based on its TLC. It has two more non-polar impurities. The larger spot was assumed to be the product. None of the impurities is the starting material (based on TLC using ethyl acetate alone). The products were all combined together and used for the synthesis of 6b.

Synthesis of 2-Pyrazinehydrazide

Synthesis of the Butyl Ester of Pyrazinoic Acid
Triethylamine (5.44 ml, 53.77 mmols) was added slowly to a mixture of pyrazinoic acid (5.0076 g, 40.35 mmols) and acetonitrile (100 ml). The resulting solution was refluxed at 82° C for 30 min. To this solution, bromobutane (6.64 ml, 48.36 mmols) was added drop wise (for 20 min) and it was again refluxed at 82° C for 2.5 h. The mixture was cooled and was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 (150 ml) and washed with 5% $NaHCO_3$ solution (100 ml) followed by water (2x). Saturated brine solution (50 ml) was added upon the presence of an emulsion. The organic layer was dried with anh. Na_2SO_4 and concentrated *in vacuo*. The product was obtained as a very dark brown oily liquid with the following properties: (5.957 g, 81.92%); IR (thin film): 1745 cm^{-1} (ester-C=O), 1304 cm^{-1} (amine-C-N), and 1136 cm^{-1} (ester-C-O); MS/ESI (m/z) calcd for MF: $C_9H_{12}N_2O_2$: 203.079647 [M+Na]; found: 203.08027 [M+Na].

Synthesis of 2-Pyrazinehydrazide
To a solution of butyl pyrazinoate (5.678 g, 31.51 mmols) dissolved in absolute ethanol (150 ml), 25% $N_2H_4 \cdot H_2O$ (33 ml) was slowly added. This solution was refluxed for 2.5 h at 80° C. The solvent was evaporated *in vacuo* and then the crude product was freeze dried to remove the remaining moisture. The crystals were washed with cold ether several times until no more brown coloration was observed. The product was then dried under vacuum. The product was obtained as fine brown crystals with

the following properties: (3.3876 g, 77.82%); mp: 160-162° C; IR (thin film): 3440 cm^{-1} (amine-N-H); 3246 cm^{-1} (amide-N-H), 1648 cm^{-1} (amide-C=O), and 1385 cm^{-1} (amine-C-N); MS/ESI (m/z) calcd for MF: $C_5H_6ON_4$: 139.061986 [M+H] and 161.043931 [M+Na]; found:139.06135 [M+H] and 161.04329 [M+Na].

Synthesis of PZA Analogs of Salicylic Acid (5a and 5b)

Synthesis of the PZA Analog of 5-n-Octanoylsalicylic Acid (5a)

A solution of 5-*n*-octanoylsalicylic acid (3a) (0.1025 g, 0.3877 mmol) dissolved in ethanol (2.0 mL) was added to a solution of 2-pyrazinehydrazide (0.0532 g, 0.385 mmol) dissolved in distilled H_2O (2.0 mL). The solution was left to stir overnight at room temperature. The solid residue that formed was suction filtered and washed with cold anh. diethyl ether. The product was collected as a light yellow powder with the following properties: (0.0792 g, 53.48%); mp: 207-209° C; Rf: 0.47 (25:1:1 ethyl acetate:EtOH:acetic acid); IR (thin film): 2358-3401 cm^{-1} (carboxylic acid-OH), 3342 cm^{-1} (2° amide N-H), 1709 cm^{-1} (carboxylic acid C=O), 1673 cm^{-1} (imine-C=N and, amide-C=O), and 1357 cm^{-1} (Ar-C-N); MS/ESI (m/z) calcd for MF: $C_{20}H_{24}O_4N_4$: 385.187580 [M+H]; found: 385.18410 [M+H]; ^{1}H-NMR (400MHz, DMSO-d_6); δ: 11.03 (1H, s, -N<u>H</u>); 10.57 (1H, s, carboxylic Acid -O<u>H</u>); 9.27(1H; d; J=1.2 Hz; H3); 8.95 (1H; d; J = 2.5 Hz; H6); 8.79 (1H; dd; J = 1.2 and 2.5 Hz; H5); 8.29(1H;d; J = 2 Hz; H20); 8.03(1H; dd; J = 2 and 9 Hz; H24); 7.05 (1H; d; J = 9 Hz; H23); 3.38 (1H; s; -O<u>H</u>); 2.86 (2H; t; J= 7.7 Hz; H12); 1.22-1.53 (10H; m; H17-13); 0.81(3H; t; J = 6.7 Hz; H18).

Synthesis of the PZA Analog of 5-n-Butanoylsalicylic Acid (5b)

A solution of 5-*n*-butanoylsalicylic acid (3b) (0.0442 g, 0.2123 mmol) dissolved in ethanol (6.0 mL) was added to a solution of 2-pyrazinehydrazide (0.0353 g, 0.2555 mmol) dissolved in distilled H_2O (6.0 mL). The solution was allowed to stir for 25.5 h at room temperature or until a precipitate was observed. The solid residue formed was suction filtered and was washed with cold anh. diethyl ether (1 mL, 2x). The product was collected as a light brown powder with the following properties: (0.03000 g, 36.80%); mp: 243-245° C; Rf: 0.32 (25:1:1 ethyl acetate:EtOH:acetic acid); IR (KBr disk): 2517-3433 cm^{-1} (carboxylic acid-OH), 3345 cm^{-1} (2° amide N-H), 1702 cm^{-1} (carboxylic acid C=O), 1665 cm^{-1} (imine-C=N and amide-C=O), and 1399 cm^{-1} (Ar-C-N); MS/ESI (m/z) calcd for MF: $C_{16}H_{16}O_4N_4$: 329.124980 [M+H]; found: 329.1228 [M+H]; ^{1}H-NMR (400MHz, DMSO-d_6) δ: 11.02 (1H, br. S, -N<u>H</u>); 10.57 (1H, br. S, carboxylic acid –O<u>H</u>); 9.26 (1H; s; H3); 8.89 (1H; s;H6); 8.81 (1H; s; H5); 8.29 (1H; s; H16); 8.02 (1H; d; J = 8 Hz; H20); 7.04 (1H; d; J = 8.5 Hz; H19); 3.69 (1H; br. S; -O<u>H</u>); 2.85 (2H; t; 7 Hz; H12); 1.58 (2H; m; H13); 0.96 (3H; t; J = 7 Hz; H14).

Synthesis of the PZA Analogs of Acetylsalicylic acid (6a and 6b)

Synthesis of the PZA Analog of 5-n-Octanoylacetylsalicylic Acid (6a)

The PZA analog of 5-*n*-octanoylsalicylic acid (5a) (0.0204 g, 0.05306 mmols) was dissolved in acetic anhydride (14.5 µl, 0.1534 mmols). Anhydrous sodium acetate (0.0012 g) was added to serve as a catalyst. The mixture was heated for about 5 min in a water bath with a temp of ~90° C. The solution changed from a solid mixture in acetic anhydride to a brown liquid upon heating in the water bath. When the solution was removed from heat, the mixture solidified. Distilled H_2O (89 µl) was added and the solution was allowed to cool. Afterwards, an additional 2 ml of water was added. The solution had a brown precipitate. After further cooling, the solid light brown precipitate was filtered and allowed to dry. The solid was further washed with cold diethyl ether (~1 ml, 3x) and allowed to dry. The product was obtained as a cream white solid with the following properties: (0.0093 g, 41.10%); mp: 140-143° C; Rf: 0.50 (25:1:1 ethyl acetate:EtOH:acetic acid); MS/ESI (m/z) calcd for MF: $C_{22}H_{26}O_5N_4$: 449.180090 [M+Na]; found: 449.1777 [M+Na]; ^{1}H-NMR (400MHz, DMSO-d_6) δ: 11.12 (1H, s, -N<u>H</u>); 10.60 (1H, s, carboxylic acid -O<u>H</u>); 9.28 (1H; br. s; H3); 8.96 (1H; br. s; H6); 8.80 (1H; br. s; H5); 8.41 (1H; d; J = 2; H20); 8.09 (1H; dd; J = 2 and 8 Hz; H24); 7.31 (1H; d; J = 8 Hz; H23); 3.38 (3H; br. s; OC<u>H</u>$_3$); 2.92 (2H; t; J= 8 Hz; H12); 1.22-1.38 (10H; m; H17-13); 0.81 (3H; t; J = 7 Hz; H18).

Synthesis of the PZA Analog of 5-*n*-Butanoylacetylsalicylic Acid (6b).

A solution of the 2-pyrazinehydrazide (0.0321 g, 0.2324 mmol) dissolved in ethanol (6 ml) was added to a solution of 5-*n*-butanoylacetylsalicylic acid (4b) (0.0568 g, 0.2270 mmols) dissolved in ethanol (7 ml). The reaction was left to stir for 45 h. Not much precipitate was observed. TLC showed that there was a new spot aside from the starting materials. The 5-*n*-butanoylacetylsalicylic acid (4b) was still seen in the TLC and only a very little amount of 2-pyrazinehydrazide left. Thus, more 2-pyrazinehydrazide was added (0.1 equiv.) to allow further reaction of 4b. The reaction was stirred at room temperature for about 3 h. The reaction was left to stand without stirring for 27.5 h. The reaction was again left to stir for 24 h at room temperature. The TLC of the reaction was again checked and it did not show any improvement since there was still a lot of the starting material present. Thus, another 0.1 equiv. of the 2-pyrazinehydrazide (dissolved in 1

ml dis. H_2O) was added. Another 5 ml of dis. H_2O was added for the next 5 h and the solution was again left to stir overnight. After overnight stirring, not much change was seen in the amount of reactants but some precipitate has formed. The reaction was again left to stir overnight, without covering the reaction vessel to allow the ethanol to evaporate, concentrating the solution. The following day, the solution was concentrated *in vacuo*, as this was done, some brown precipitate formed. This was done until the volume of the reaction mixture was reduced to half. Again another 0.1 equiv of 2-pyrazinehydrazide was added (dissolved in 1.5 ml of dis. H_2O) to further react the remaining starting material. The reaction was left to stir overnight. The following day, a lot more of the brown precipitate formed but the TLC profile did not change. This brown precipitate was filtered and washed with cold diethyl ether (2x). The filtrate was allowed to stir to further react the solution. This was again left overnight. The solution was checked the next day and still not much precipitate has formed. The solution was allowed to stir the whole day and the reaction was stopped for ~27.5 h. The reaction was worked-up by concentrating it *in vacuo* and collecting any precipitate formed. The precipitate collected was immediately washed with cold diethyl ether and left to dry.

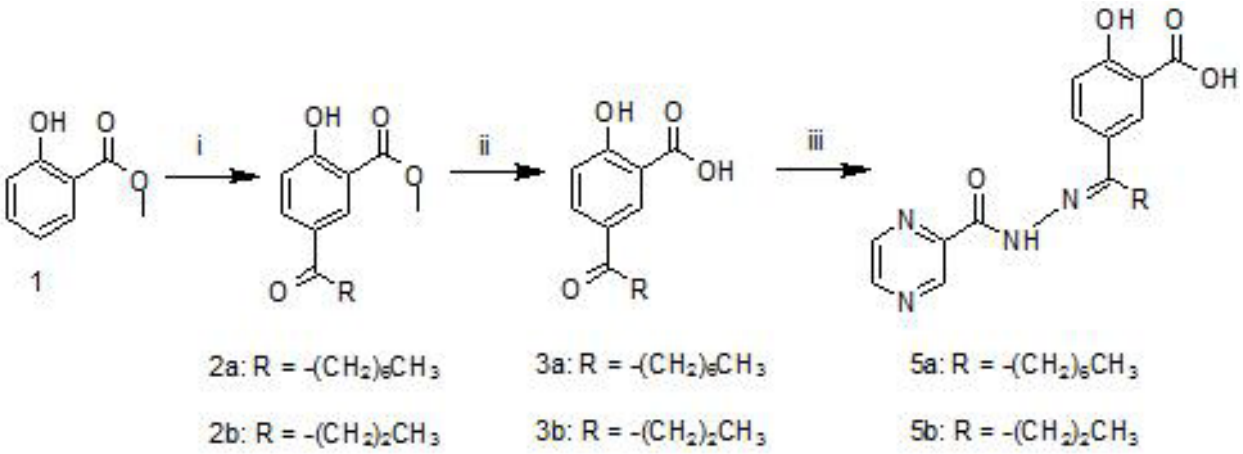
Since the reaction was started, some solids were deposited on the mouth of the round bottom flask; these were also collected and washed with cold diethyl ether as well. The product was obtained as a brown solid (0.0092 g, 8.207%); mp: 198-205° C; Rf: 0.19, (25:1:1 ethyl acetate:EtOH:acetic acid); MS/ESI (m/z) calcd for MF: $C_{18}H_{18}O_5N_4$: 393.117489 [M+Na]; found: 393.1138 [M+Na]; ^{1}H-NMR(400MHz, DMSO-d_6) δ: 10.60 (1H, s, -NH); 10.02 (1H, s, carboxylic acid -OH); 9.17 (1H; d; J = 1.5 Hz; H3); 8.91 (1H; d; J = 2.7 Hz; H6); 8.76 (1H; d; J = 1.5 and 2.4 Hz; H5); 8.37 (1H;d; J = 2; H20); 8.05 (1H; dd; J = 2 and 8 Hz; H24); 6.99 (1H; d; J = 8 Hz; H23); 3.38 (3H; s; OCH_3); 2.92 (2H; t; J= 7 Hz; H12); 1.61 (2H; sxt; H13); 0.91 (3H; t; J = 7 Hz; H14).

RESULTS AND DISCUSSION

The relevance of the PZA drug in the treatment of TB is threatened by the constant occurrence of resistant strains of *Mtb* towards the drug. One particular solution to this is to derivatize the PZA drug by coupling it with other molecules that could enhance its efficacy towards resistant and susceptible strains of the bacteria. Several studies made on PZA analogs have shown that the attachment of lipophilic groups (e.g., long alkyl chains) have improved the inhibitory effect of the resulting derivative compared to PZA (Zitko et al. 2011, 2012; Servusova et al. 2014). In another study, co-administration of PZA with weak acids (Chen et al. 2007) and some NSAIDs (Byrne et al. 2007) has also shown enhanced activity of the drug. This lead the researchers to synthesize a set of PZA analogs fused with salicylic acid and acetylsalicylic acid derivatives that have varying chains of alkyl groups.

PZA Analogs of Salicylic Acid (5a and 5b)

The formation of PZA analogs of salicylic acid was done by the generation of an acylated salicylic acid derivative, followed by its attachment to the PZA nucleus. This attachment was easily done via the imine formation of 2-pyrazinehydrazide with an acyl group of the salicylic acid derivatives as shown in Scheme 1.



Scheme 1. Synthesis of PZA analogs of salicylic acid 5a and 5b. i) $RCOCl/ZnCl_2$; ii) $NaOH/H_2O$; iii) 2-pyrazinehydrazide**.**

To carry out this strategy, Friedel-Crafts acylation was first employed. Methyl salicylate was reacted with the corresponding acid chloride to attach a ketone group. The methyl 5-*n*-octanoylsalicylate (2a) and methyl 5-*n*-butanoylsalicylate (2b) were obtained as white needle like crystals in 13% and 8% yield, respectively. These acylated derivatives of methyl salicylate − with 4-carbon and 8-carbon acyl chains, respectively − were generated in relatively low yields due to the weaker catalytic power of zinc chloride. This left a lot of the methyl salicylate starting material unreacted. The acylated methyl salicylate precursors were then hydrolyzed using sodium hydroxide to generate the salicylic acid moiety. This produced 5-*n*-octanoylsalicylic acid (3a) and 5-*n*-butanoylsalicylic acid (3b) both as a white solid in 88% and 89% yield, respectively. These were then reacted with 2-pyrazinehydrazide to introduce the PZA moiety.

The PZA analog of 5-*n*-octanoylsalicylic acid (5a) was generated as a light yellow powder in 53% yield. The structure of the compound was confirmed by ^{1}H-NMR spectroscopy. The spectrum accounted for the 24 hydrogen atoms in the molecule as shown in Figure 4. The presence of the alkyl chain was signified by the peaks at 0.81 ppm (H18, t, J = 7 Hz, 3H), 1.22-1.53 ppm (H17-H13, m, 10H) and at 2.86 ppm (H12, t, J = 8 Hz, 2H). These signals correspond to the methyl group at the free end of the chain, followed by the methylene protons which are more deshielded being closer to the imine functionality.

The presence of the salicylic acid moiety was confirmed by the signal at 3.38 ppm (H27, s, 1H) and 10.57 ppm (H26, s, 1H), which corresponds to the proton of the phenol group and the carboxylic acid –OH, respectively. The appropriate positions of the substituents in the aromatic ring of the salicylic acid group was confirmed by the signal at 7.05 ppm (d, J = 9 Hz; 1H) corresponding to the Ar-CH proton (H23**)** near the hydroxyl group, which showed ortho coupling with the signal at 8.03 ppm (dd, J = 2 Hz, 8.8 Hz; 1H) corresponding to the Ar-CH proton (H24**)** near the imine group. The presence of a meta coupling of this H24 proton with the more deshielded signal at 8.29 ppm (d, J = 2 Hz; 1H), which corresponds to the Ar-CH proton (**H20**) near the carboxylic acid group, confirms the attachment of the acyl chain at the para position relative to the hydroxy group of the salicylic acid moiety.

The hydrogens at the PZA ring are situated at a more deshielded region due to the electron withdrawing effect of the nitrogen atoms in the ring. The signal at 8.79 ppm (dd, J = 1.2 Hz and 2.5 Hz, 1H), 8.95 ppm (d, J = 2.5 Hz, 1H), and at 9.27 ppm (d, J = 1.2 Hz, 1H) are associated with the H5, H6, and H3 protons, respectively. These assignments were made due to the evidence of an ortho and meta coupling of the signal at 8.79 ppm, which came out as a doublet of doublet. The coupling constant of the signal at 8.95 ppm (equivalent to 2.5 Hz) and that of the signal at 9.27 ppm (equal to 1.2 Hz) supports the location of the H5 proton being ortho to the H6 and meta to the H3 proton. The chemical shifts of these protons also confirm their chemical environment, with H3 being the most deshielded due to its ortho position relative to the amide group and to the nitrogen of the pyrazine ring. The 8.95 ppm signal is assigned to the H6 proton, since it is placed meta relative to the amide group and also close to one of the nitrogens of the pyrazine ring. The singlet at 11.03 ppm (H9a, 1H) corresponds to the NH proton of the amide group; this may indicate the formation of the imine product since it did not give a triplet signal. These values for the PZA protons were consistent with the ones found in literature: δ: 12.52 (1H; s; NH); 9.29 (1H; d; J=1.3 Hz, H3); 8.94 (1H; d; J = 2.4 Hz; H6) and 8.81 (1H; dd; J=2.4 and 1.3 Hz, H5) (Vergara et al. 2009).

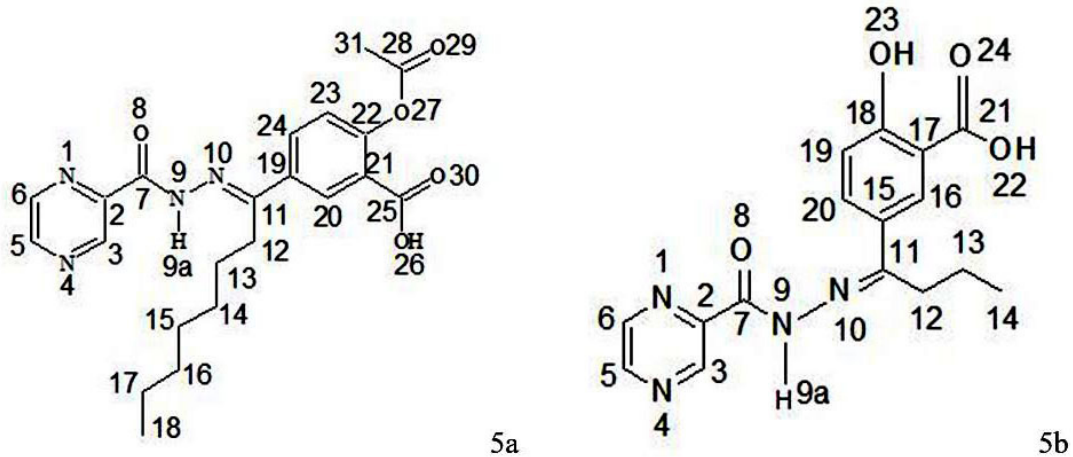


Figure 4. ^{1}H-NMR (400 MHz) labels for compounds 5a and 5b in DMSO-d_6.

The attachment of the PZA ring was also confirmed by the presence of a peak at 1673 cm^{-1} in the IR spectrum of the compound, which corresponds to a C=N stretch due to an imine functionality and the loss of a ketone functionality.

The mass spectrum of the compound that showed a pseudomolecular ion peak at m/z 385.18410 $[M+H]^+$ corresponds to the molecular formula expected of the target compound, which is $C_{20}H_{24}O_4N_4$.

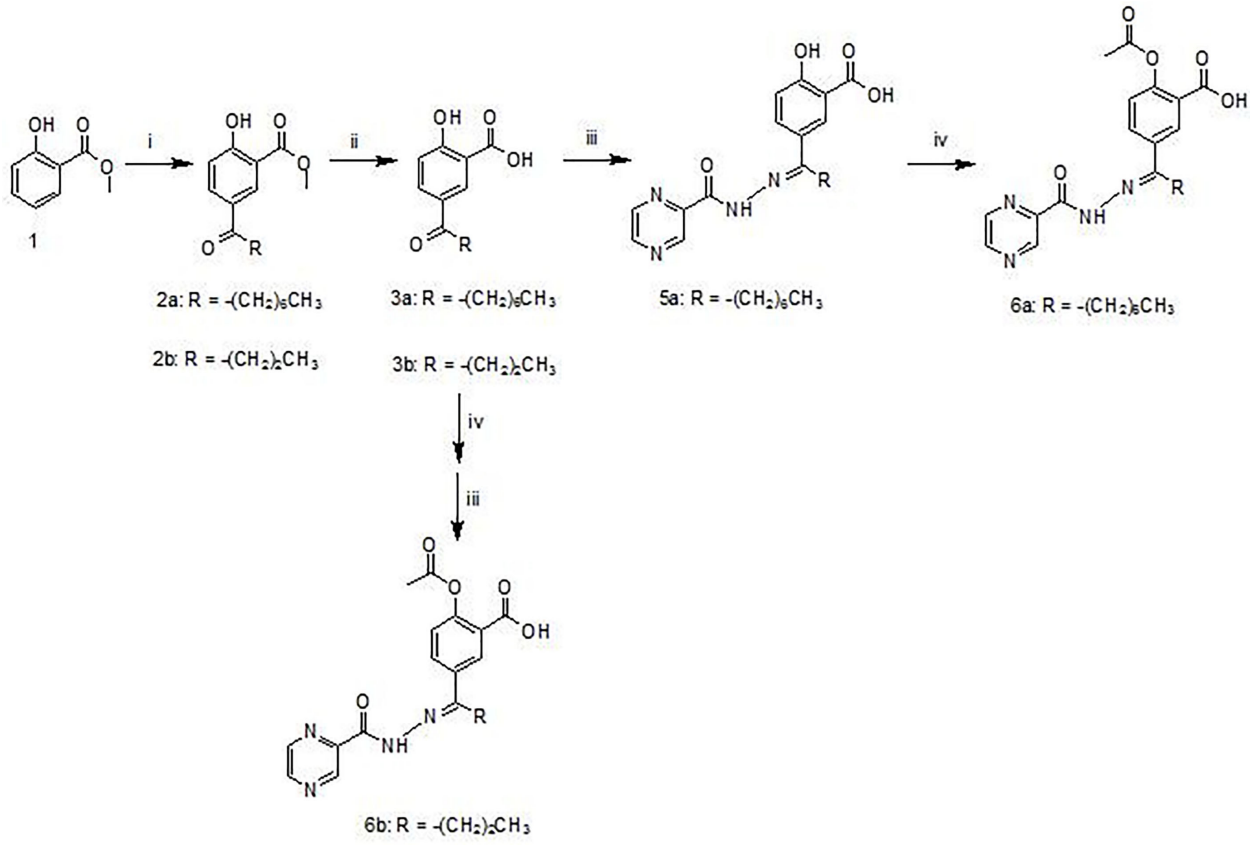
The PZA analog of 5-*n*-butanoylsalicylic acid (5b) was generated as a light brown powder in 37% yield. Its ^{1}H-NMR spectrum, which accounted for 16 hydrogens, showed signals that are consistent with the signals found in 5a as shown in Figure 4**.** The only difference is seen in the occurrence of a multiplet at 1.58 ppm (H13, m, 2H), which integrates for only 2 hydrogens due to the attachment of a shorter alkyl chain with only 3-carbon atoms. Similar range of chemical shifts, multiplicities, and couplings were also found for the salicylic acid group and PZA group of compound 5b.

The expected molar mass of the compound was confirmed by the pseudomolecular ion peak at 329.1228 $[M+H]^+$, which is due to the mass of the compound recorded with a hydrogen. These signals were consistent with the molecular formula of $C_{16}H_{16}O_4N_4$.

PZA Analogs of Acetylsalicylic acid (6a and 6b)

The synthesis of the PZA analog of 5-*n*-octanoylacetylsalicylic acid (6a) and the PZA analog of 5-*n*-butanoylacetylsalicylic acid (6b) followed an almost similar synthetic pathway as the generation of their salicylic acid counterparts (5a and 5b). The only difference is that in the preparation of the PZA analog of 5-*n*-octanoylacetylsalicylic acid (6a), the conversion of the salicylic acid group to the acetylsalicylic acid group via acetylation of the hydroxy group was done after its coupling with the PZA moiety as shown in Scheme 2. On the other hand, 6b was generated via the formation of the acylated salicylic acid precursor 3b, followed by acetylation with acetic anhydride to generate the acetylsalicylic acid moiety. This was then reacted *in situ* with 2-pyrazinehydrazide to generate the target compound 6b as shown in Scheme 2.

This route generated compound 6a as a cream white solid in 41% yield. The structure of the compound was confirmed using ^{1}H-NMR spectroscopy. The spectrum



Scheme 2. Synthesis of the pyrazinamide analog of acetylsalicylic acid 6a and 6b. i) $RCOCl/ZnCl_2$; ii) $NaOH/H_2O$; iii) 2-pyrazinehydrazide; and iv) acetic anhydride/NaOAc.

of the compound accounted for the 26 hydrogen atoms in the molecule as shown in Figure 5. A similar set of signals were observed with those generated with compounds 5a and 5b. The presence of the alkyl chain was again confirmed by the peaks observed at 0.81 ppm (H18, t, J = 7 Hz, 3H), 1.23-1.38 ppm (H17-H13, m, 10H), and 2.92 ppm (H12, t, J = 8 Hz; 2H). These correspond to the methyl group and the methylene protons, respectively, of the hydrocarbon chain nearest to the imine group.

The structure of the acetylsalicylic acid moiety was confirmed by the broad singlet at 3.38 ppm (H31) and the singlet at 10.60 ppm (H26), which corresponds to the three hydrogens of the methyl group in the ester moiety and the carboxylic acid –OH, respectively.

The doublet at 7.31 ppm (J = 8 Hz; 1H) corresponds to the Ar-CH proton (H23) near the ester group, which is shifted to a more deshielded region compared to the signals seen for the same hydrogen in compound 5a and 5b. This is due to the ester functionality present instead of just a hydroxyl group, which has a lesser electron withdrawing effect. The doublet of a doublet at 8.09 ppm (J = 2 Hz and 8 Hz; 1H) is assigned to the Ar-CH proton near the imine group (H24). This is due to the observed J value of 8 Hz for the signal at 7.31 ppm which showed ortho coupling with the signal at 8.09 ppm. On the other hand, meta coupling of the signal given by H24 with the deshielded signal at 8.41 ppm (H20, J = 2 Hz; 1H), which corresponds to the Ar-CH proton near the carboxylic acid group further confirms the appropriate position of H24.

The hydrogen atoms at the PZA ring for compound 6a were consistent with those found in 5a and 5b**.** The signals at 8.80 ppm, 8.96 ppm, and at 9.28 ppm are assigned accordingly to the H5, H6, and H3 protons for it follows the same multiplicities and coupling patterns as those seen in 5a and 5b.

The mass spectrum of the compound that showed a pseudomolecular ion peak at m/z 449.1777 $[M+Na]^+$ corresponds to the molecular formula expected of the target compound, which is $C_{22}H_{26}O_5N_4$.

The PZA analog of 5-*n*-butanoylacetylsalicylic acid (6b) was generated as a brown solid in 8% yield. The compound had a melting point of ~198-205° C, with the large range possibly explainable by the presence of some impurities in the sample.

The structure of the compound was further confirmed by the ^{1}H-NMR, which accounted for the 18 hydrogen atoms in the molecule as shown in Figure 5. Similar set of signals to those of 6a were observed for the alkyl chain in the molecule except for the presence of a sextet at 1.61 ppm (H13, J = 7 Hz), which corresponds to the two hydrogen atoms assigned to the methylene protons attached to the end methyl group in the alkyl chain. This signifies that these protons are in between a methyl and a methylene group to give the observed multiplicity. All the other signals were consistent with that observed in 6a, which confirms the expected structure for 6b.

The mass spectrum of the compound that showed a pseudomolecular ion peak at m/z 393.1138 $[M+Na]^+$ corresponds to the molecular formula expected of the target compound, which is $C_{18}H_{18}O_5N_4$.

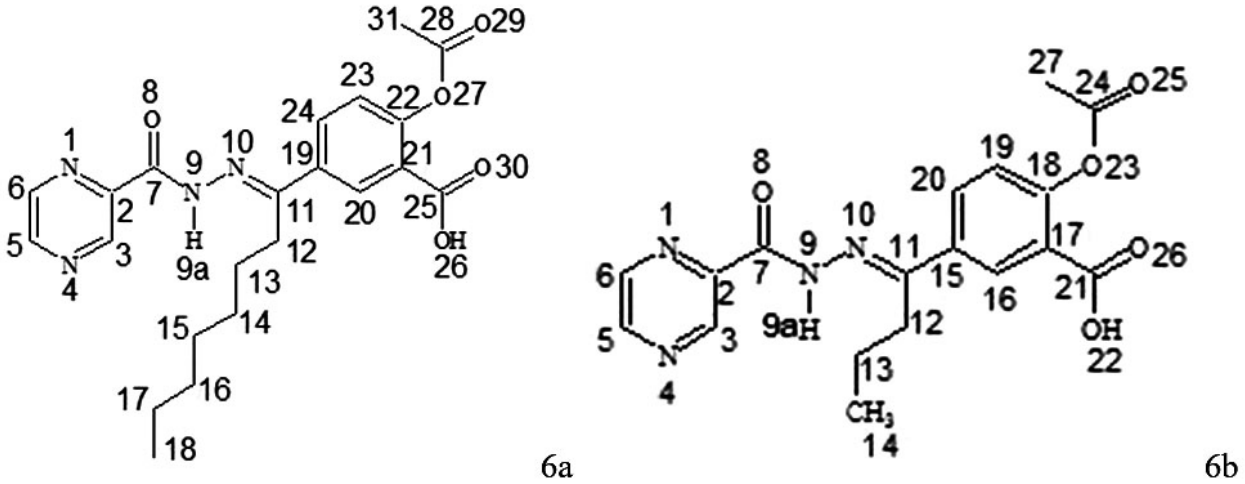


Figure 5. ^{1}H-NMR (400 MHz) labels for compound 6a and 6b in DMSO-d_6.

CONCLUSION

New PZA analogs of salicylic acid (5a and 5b) and acetylsalicylic acid (6a and 6b) were synthesized and characterized. The PZA analog of salicylic acid (5a and 5b) were obtained in 53% and 37% yields from 3a and 3b, respectively. The overall yields are 25% and 34% from 2a and 2b, respectively. The preparation of the acylated salicylic acid followed by base hydrolysis and coupling with 2-pyrazinehydrazide proved to be a viable way to generate the target compounds. In the synthesis of the PZA analog of acetylsalicylic acid (6a), the most viable method involved the base catalyzed acetylation of 5a**.** It was generated in 41% yield from 5a with an overall yield of 10% from 2a. The preparation of 6b, on the other hand, involved the acetylation of acylated salicylic acid followed by its coupling with 2-pyrazinehydrazide. It was obtained in 8% yield from 4b, with an overall yield of 8% from 2b.

These PZA analogs of salicylic acid and acetylsalicylic acid may exhibit increased potency against PZA-resistant strains of *Mycobacterium tuberculosis.* It is therefore recommended that anti-mycobacterial testing be employed to determine the effectivity of the synthesized compounds. It is also recommended that the ^{13}C-NMR spectra for all the derivatives be determined.

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