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SYNTHESIS AND *IN VITRO* ANTIMYCOBACTERIAL ACTIVITY DETERMINATION OF PHTHALIMIDE DERIVATIVES

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Phthalimide derivatives were synthesized using the concept of molecular hybridization or combination of two pharmacophores in one drug. In this paper, the concept was utilized to synthesize new antimycobacterial compounds to combat emerging resistant *Mycobacterium tuberculosis* strains. Four new phthalimides were successfully prepared using a single-step condensation reaction between phthalic anhydride and four different sulfonamides: sulfadimethoxine, sulfathiazole, sulfamethizole, and sulfamethoxypyridazine. IR and ^{1}H NMR spectroscopic and mass spectrometric data verified the identity and structure of the synthesized compounds. The antimycobacterial activity of the synthesized phthalimides and their starting materials was evaluated according to CLSI standard procedures using the agar proportion method. The calculated LogP of both starting materials and phthalimides were also compared.

Sulfathiazole, sulfadimethoxine, and sulfamethizole showed significant activity against *Mycobacterium tuberculosis* H_{37}Rv strain at 0.01 mg/ml, the lowest concentration at which they were tested. One of the four new compounds, 4-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-*N*-(1,3-thiazol-2-yl)benzenesulfonamide, which was synthesized from sulfathiazole also exhibited the same activity. The derivatives from sulfadimethoxine and sulfamethizole, however, showed a decrease in activity. The phthalimides overall gave higher LogP values. The active new compound demonstrates that molecular hybridization may be used to design antimycobacterial compounds with improved activity and pharmacokinetic properties.

Keywords: phthalimide derivative, molecular hybridization, *Mycobacterium tuberculosis*, sulfonamide

INTRODUCTION

Tuberculosis (TB) is a pulmonary illness caused by *Mycobacterium tuberculosis* characterized by symptoms which include coughing with thick mucus, fever, fatigue, and chest pain (Gale et al., 2007). Around one third of the world's population are infected, resulting in some two million deaths per year (Hudson, Imamura, Gutteridge, Tanyok, & Nunn, 2003). Today, first-line drugs used in short-course treatment involve different combinations of isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin. However, due to inappropriate dosage, duration prescribed, and poor patient compliance, drug resistant strains started to emerge (Gale et al., 2007). Multi-Drug Resistant TB is a form of TB that fails to respond to first-line drugs. In 2009, 3.3% of all new TB cases were estimated to be multi-drug resistant (World Health Organization [WHO], 2010). New drugs to treat TB are thus urgently required, specifically those which involve shorter treatment regimen than first-line drugs.

Molecular hybridization in drug design is the combination of the pharmacophores of two bioactive substances to produce a new hybrid compound with improved efficacy compared to the parent drugs (Vegas-Junior, Daniello, da Silva Bolzani, Barreiro, & Fraga, 2007). A pharmacophore summarizes the important binding groups required for activity (Patrick, 2009). In the case of phthalimides, the moieties combined are the phthalimide portion and the sulfonamide group with the amido and amidified sulfonic acid groups *para* to each other in an aromatic ring (Figure 1).

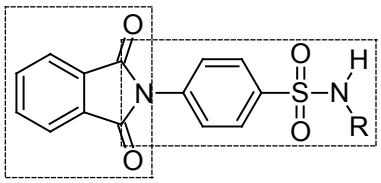


Figure 1. *General structure of a phthalimide derivative (R refers to the heteroaromatic moiety attached to the sulfonamide N).*

Sulfonamides (Figure 2) are bacteriostatic agents whose structure contains amino and amidified sulfonic acid group positions *para* to each other in an aromatic ring. They exert their antibacterial action by acting as a competitive inhibitor of *para*-aminobenzoic acid for dihydropteroate synthase, an enzyme needed to synthesize tetrahydrofolate in bacteria (Lemke, Williams, Roche, & Zito, 2008). Examples of sulfonamides are the starting materials to be used for the synthesis: sulfathiazole **(1)**,sulfamethizole **(2)**, sulfadimethoxine **(3)**, and sulfamethoxypyridazine **(4)**. One sulfonamide, sulfathiazole, has an inherent antimycobacterial activity (Lougheed, Taylor, Osborne, Bryans, & Buxton, 2009) (MIC > 5 μM) although it is not implicated in TB drug therapy.

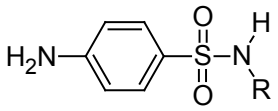
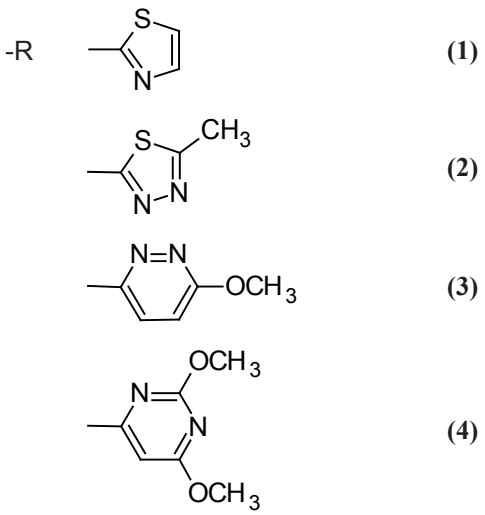


Figure 2. *General structure of a sulfonamide (R refers to the heteroaromatic moiety attached to the sulfonamide N).*



(1)

(2)

(3)

(4)

Several synthetic phthalimides were found to possess toxicity against several bacterial, fungal, and mammalian cells: a series of phthalimides

linked to *N*-acyl substituted phenothiazines were shown to possess antimicrobial activity against several bacteria and fungi (Bansode, Shelke, & Dongre, 2009). A pyrazole ring-containing phthalimide derivative was found to be active against five human cancer cell lines (A549, Bel7402, BGC-823, HCT-8, and A2780) *in vitro* (Yang et al., 2010) thus indicating its potential for anticancer therapy applications. A series of *N*-aryl- and *N*-(1,2,4-triazol-yl)-phthalimides with heteroaromatic moieties were also shown to have hypolipidemic activity, significantly reducing plasma cholesterol and triglyceride levels in Swiss white mice (Sena, Srivastava, Silva, & Lima, 2003).

This paper focused on the synthesis of phthalimide derivatives for potential use as antimycobacterial agents. The synthesis was carried out by condensation of sulfonamides with phthalic anhydride. The phthalimides were characterized by physico-chemical and spectroscopic methods. *In vitro* antimycobacterial activity was then evaluated against *Mycobacterium tuberculosis* H_{37}Rv standard strain.

MATERIALS AND METHODS

Materials, Reagents, and Apparatus

Phthalic anhydride (JT Baker), sulfadimethoxine, sulfamethizole, sulfamethoxypyridazine, and sulfathiazole, *p*-aminosalicylicacid (TCI), isoniazid (Fluka), and solvents used were of AR grade. Plastic-backed Silica Gel 60 GF_{254} TLC plates (Merck) was also used for purity analysis.

IR spectra were obtained from a Nicolet 6700 FT-IR spectrophotometer on KBr disk. Mass spectra were recorded using a Bruker microTOF-Q II LC-MS/MS using ESI-positive ion mode. ^{1}H NMR measurements were carried out at the National Chemistry Instrumentation Center, Ateneo de Manila University on a 400 MHz JEOL lambda NMR spectrometer using DMSO-d_6 as solvent and TMS as internal standard. Melting points were taken on a Fisher Scientific melting point apparatus and a Fluke 51 II Thermometer.

ChemSketch® v. 12.01 was used to draw structures and calculate for LogP values.

Synthesis of 4-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-N-(1,3-thiazol-2-yl) benzenesulfonamide (5)

Sulfathiazole **(1)** (1053 mg, 4.12 mmol), phthalic anhydride (740 mg, 5.00 mmol), 30.0 ml THF, and 50.0 ml glacial acetic acid were mixed, forming a slightly turbid solution. The synthesis required stirring but the mixture eventually clarifies and turns yellowish once it began to boil. Light beige to white-colored amorphous solid was produced (0.9578 g; 60.26%). MP: 216-244°C (with decomposition). R_f: 0.51 (1:1 ether-THF).

IR ν_{max} (cm^{-1}): 1712.06 and 1743.60 (C=O imide); 1145.64 and 1386.64 (S=O).

NMR ^{1}H (500 MHz) δ: 6.87 (1*H*, d,*J* = 4.6 Hz, H_5); 7.30 (1*H*, s, *J* = 4.6 Hz, H_4); 7.35 (2*H*, d, *J* = 8.7 Hz, H_{12}, H_{14}); 7.92 (2*H*, m, H_{22}, H_{23}); 7.98 (2*H*, m, H_{21}, H_{24}); 8.12 (2*H*, d, *J* = 8.7 Hz, H_{11}, H_{15}) ppm.

Calculated for $[C_{17}H_{12}N_3O_4S_2]^+$ =386.026372 Da; found = 386.02594*m/z*.

4-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-N-(5-methyl-1,3,4-thiadiazol-2-yl) benzenesulfonamide (6)

Sulfamethizole **(2)** (1085 mg, 4.01mmol), phthalic anhydride (828 mg, 5.59mmol), 30.0 ml THF, and 50.0 ml glacial acetic acid were combined, forming a clear yellow solution. A 0.6091 g (37.90%) of white amorphous solid was produced. MP: 256-260°C. R_f: 0.47 (1:1 ether-ethyl acetate).

IR ν_{max} (cm^{-1}): 1714.98 and 1740.78 (C=O

imide); 1152.77and 1386.64 (S=O).

NMR ^{1}H (500 MHz) δ: 2.50 (3*H*, s, H_9); 7.70 (2*H*, d, *J* = 8.70 Hz,H_{13}, H_{15});7.93 (2*H*, m,H_{22}, H_{25}); 7.97 (2*H*, m,H_{23}, H_{24}); 8.00 (2*H*, d, *J* = 8.70 Hz,H_{12}, H_{16}) ppm.

Calculated for $[C_{17}H_{13}N_4O_4S_2]^+$ = 401.037271 Da; found = 401.03687 *m/z*.

Synthesis of 4-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-N-(6-methoxypyridazin-3-yl) benzenesulfonamide (7)

Sulfamethoxypyridazine **(3)** (585 mg, 2.09 mmol), phthalic anhydride (397 mg, 2.68 mmol), 15.0 ml THF, and 20.0 ml glacial acetic acid were combined, forming a clear yellow solution. A 0.8565 g (75.30%) of white fine solid was produced. MP: 260-265°C. R_f: 0.50 (1:6 hexane-ethyl acetate).

IR ν_{max} (cm^{-1}): 1717.22 and 1744.73 (C=O imide); 1135.57 and 1385.58 (S=O).

NMR ^{1}H (500 MHz) δ: 3.39 (3*H*, s, H_{29})7.44 (1*H*, d,*J* = 9.90 Hz,H_{26});7.67 (2*H*, d, *J* = 8.60 Hz,H_{11}, H_{15});7.89 (1*H*, d, *J* = 9.90 Hz,H_{27}); 7.93 (2*H*, m,H_6, H_9);8.00 (2*H*, m,H_7, H_8); 8.02 (2*H*, d, *J* = 8.60 Hz,H_{26}) ppm.

Calculated for $[C_{19}H_{15}N_4O_5S]^+$ =411.075766 Da; found = 411.07506*m/z*.

Synthesis of N-(2,6-dimethoxypyrimidin-4-yl)-4-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl) benzenesulfonamide (8)

Sulfadimethoxine **(4)** (1261 mg, 4.06 mmol), phthalic anhydride (778 mg, 5.25 mmol), 20.0 ml THF, and 50.0 ml glacial acetic acid were mixed together, forming a clear solution. The synthesis afforded 0.5373 g (30.02%) of white amorphous solid.

IR ν_{max} (cm^{-1}): 1741.72 and 1719.88 (C=O imide); 1375.54 and 1157.55 (S=O)

NMR ^{1}H (500 MHz) δ: 3.76 ppm (3*H*, s, H_{31}) and 3.82 (3*H*, s, H_{29}); 6.01 (1*H*, s, H_{27}); 7.94 (2*H*, m, H_6, H_9) and 8.00 (2*H*, m, H_7, H_8);7.74 (2*H*, d, *J* = 8.8 Hz, H_{12}, H_{14}) and 8.12 (2*H*, d, *J* = 8.8 Hz, H_{11}, H_{15}) ppm.

$[M+H]^+$ = 441.08608 *m/z* (Calculated for $[C_{20}H_{17}N_4O_6S]^+$: 441.086331 Da).

Antimycobacterial Assay

Susceptibility testing of *Mycobacterium tuberculosis* standard strain H_{37}Rv against the starting materials and synthesized compounds was performed externally by Connie Ang and Marc Cajucom at the Philippine General Hospital - Medical Research Laboratory using the Agar Proportion Method. The procedure was executed according to Clinical and Laboratory Standards Institute (CLSI) Guidelines. The procedure used was adapted from Schwalbe, Steele-Moore, and Goodwin (2007).

Briefly, solutions (0.5 mg/ml) of the test compounds in DMSO were prepared. Aliquots (1.0, 0.5, and 0.1 ml) of the solution were then incorporated into enough liquefied Middlebrook 7H10 agar to make 5 ml agar solutions of the compounds with final concentrations of 0.10, 0.50, and 0.01 mg/ml. The solutions were then poured into three out of four quadrants of the petri dish and left to solidify. The fourth quadrant contained the compound-free medium. *Mycobacterium tuberculosis* H_{37}Rv ATCC 27294 was then inoculated in each quadrant and then incubated. Growth appearance was checked after three weeks.

The solvent, DMSO, was used as negative control for the assay, while the known antitubercular drugs *p*-aminosalicylic acid **(9)** and isoniazid **(10)** were used as positive controls.

RESULTS AND DISCUSSION

Phthalimides can be synthesized in a variety of methods. In a study by Li (2010), (1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl) acetic acid, a phthalimide derivative, was synthesized by reflux

heating phthalic anhydride and aminoacetic acid in acetone. A series of phthalimide intermediates were prepared by Pessoa et al. (2010) as follows: Phthalic anhydride and an amine starting material were reflux-heated for three hours with DMF as solvent and DMAP as base catalyst. The synthesis gave good yields in crystalline form. Santos et al. (2009) synthesized phthalimide intermediates with potential activity against *Mycobacterium tuberculosis*. Different sulfonamides were combined with phthalic anhydride in glacial acetic acid and stirred under nitrogen at 130º C for two hours (Figure 3). The product was crystallized upon addition of cold water and recrystallized in ethanol to give variable yields. This procedure was adapted with modifications on the solvent systems used. THF was added to account for the insolubility of the starting materials in glacial acetic acid.

Based on the amount of the limiting reagents used, the amines, the calculated yields varied from 30.02% to 75.30%. The mechanism is thought to follow the usual acid-catalyzed amide formation amidification of phthalic anhydride, which passes through a phthalamic acid intermediate (Sena et al., 2003) and is driven by the formation of the stable phthalimide ring moiety. The compounds gave very high melting points, all exceeding 200 ºC, as expected from literature (Santos et al., 2009). Some showed decomposition, with one compound exhibiting it at above 300 ºC.

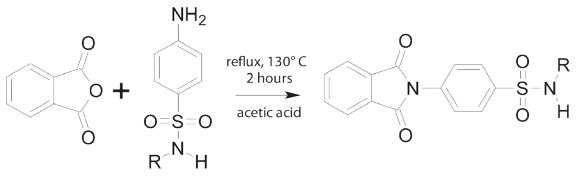


Figure 3. *Synthesis of antimycobacterialphthalimide derivatives (R refers to the heteroaromatic moiety attached to the sulfonamide N in both starting material and product).*

For those synthesized from sulfonamides, strong and sharp IR absorption bands corresponding to the stretching of the C-O bond of the phthalimide carbonyl group appeared at around 1740 cm^{-1} and 1690 cm^{-1} while those absorptions corresponding to the sulfonyl group stretches appeared at around 1360 cm^{-1} and 1150 cm^{-1}.

Mass spectrometric data showed the pseudo-molecular ion peaks $[M+H]^+$ for all compounds, the analysis being run on positive ion mode, with the compounds being converted to cations through formic acid prior to injection into the instrument. A blank determination was performed, by recording the mass spectrum of 0.01% formic acid in DMSO to rule out peaks due to impurities.

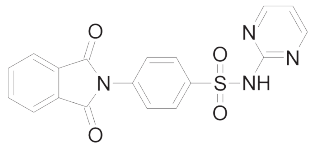
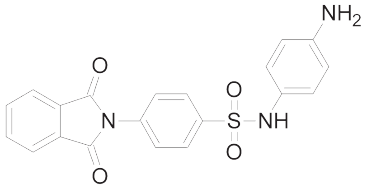
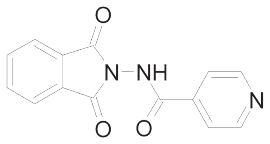
In general, the synthesized compounds showed phthalimide proton resonances at δ 7.6-8.1 ppm and δ 7.7-7.9 ppm for the protons of the phenyl ring associated with the sulfonamide starting material.

The solvent peak of DMSO-d_6 was present in all spectra at 2.49 ppm, as well as traces of impurities corresponding to solvents used in the synthesis and purification like acetic acid, water, and ethanol. The characteristic peaks were identified through literature (Silverstein, Webster, & Kiemle, 2005; Santos et al., 2009).

Phthalimide derivatives previously synthesized (Santos et al., 2009) have been shown to possess antimycobacterial activity when subjected to an *in vitro* assay against *Mycobacterium tuberculosis* H_{37}Rv using Alamar Blue susceptibility assay. In this paper, an agar proportion method used to screen susceptibility of *Mycobacterium tuberculosis* strains were performed as antimycobacterial assay for the compounds (Schwalbe et al., 2007). Results of the biological assay showed whether a particular compound exhibited appreciable antimycobacterial activity at a specified concentration. The negative control for the assay, DMSO, apparently showed toxicity

towards the *Mycobacterium tuberculosis* cells at compound concentrations 0.1 mg/ml and 0.05 mg/ml, since this would mean an equivalent to 20% and 10% DMSO concentration in the media. It showed no toxicity at compound concentration of 0.01 mg/ml. Hence, only results for that compound concentration were considered. At the lowest concentration of 0.01 mg/ml, positive controlsexhibited antimycobacterial activity as expected, while the negative control, DMSO showed no activity.

Table 1.
Antimycobacterial Activity of Some Phthalimide Derivatives

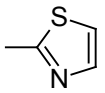
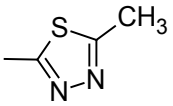
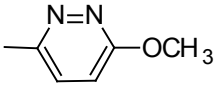
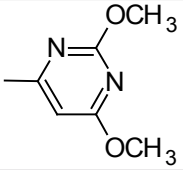
Structure of phthalimide derivative	MIC (µg/ml)
	3.9
	7.8
	5.0

For comparison, the sulfonamide starting materials were also evaluated for their antimycobacterial activity to investigate whether molecular hybridization would actually improve their bioactivity. Of the four sulfonamides used, three exhibited considerable activity at the concentration being investigated: **(1)**, **(2)**, and **(4)**. These compounds are not routinely used in the treatment of tuberculosis. Table 2 summarizes the antimycobacterial activities of the starting materials and synthesized compounds.

The phthalimide derivatives of sulfadimethoxine and sulfamethizole did not show the same activity at 0.01 mg/ml. This could be possibly explained by the steric hindrance brought about by the 1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl moiety attached to the *para* amino group of the sulfonamide. The structural feature could have prevented the compound from binding to the active site of the enzyme dihydropteroate synthetase and thus could not have inhibited its action.

On the other hand, **(5)** demonstrated good antimycobacterial activity comparable to that of its starting material. Given that it is just as active, the compound poses an advantage over its starting material in the sense that it is less polar. The additional phthalimide structural feature confers lipophilicity to the compound, thus enabling it to penetrate cell membranes more easily. LogP values of the compounds were calculated using ChemSketch® to support this. Higher LogP values suggested greater lipophilicity. The bioactivity, physicochemical, and pharmacokinetic properties of this new phthalimide derivative thus warrants further investigation to completely establish its advantage over **(1)**.

Table 2.
Antimycobacterial Activity and LogP Values of Phthalimide Derivatives

	Amine starting material			Synthesized phthalimide derivative		
-R		*Activity at 0.01 mg/ml*	*LogP*		*Activity at 0.01 mg/ml*	*LogP*
	**(1)**	Active	0.05	**(5)**	Active	1.49
	**(2)**	Active	0.51	**(6)**	Not active	1.53
	**(3)**	Not active	0.32	**(7)**	Not active	1.53
	**(4)**	Active	1.48	**(8)**	Not active	2.69

CONCLUSION

Four phthalimides were successfully synthesized using the single-step condensation reaction, four of which are new compounds. IR and ^{1}H NMR spectroscopic data verified their structural features. Pseudomolecular ion peaks of m/z = $[M+H]^+$ strongly corresponding to the calculated values according to MS measurements.

One of the new compounds, **(5)**, showed significant activity against *Mycobacterium tuberculosis* H_{37}Rv strain at 0.01 mg/ml, the lowest concentration at which it was tested. It showed the same activity as its starting material, **(1)**. However, the new compound posed an advantage over the starting material in the sense that it is less polar, as supported by its calculated LogP value. This implies that it is more lipophilic and would be able to penetrate cell membranes more easily.

Further investigation on the antimycobacterial activities of **(5)** may be undertaken by evaluating its bioactivity at lower concentrations. Comparison of its bioactivity with its starting material, **(1)**, should be explored at lower concentrations to verify the effectiveness of molecular hybridization in increasing the efficacy of potential medicinal agents.

ACKNOWLEDGEMENTS

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