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Synthesis of Small Organic Molecule, Pa-P-Ester: A Novel Inhibitor Against Pathogenic Gram-Negative Bacteria; *Salmonella typhimurium* and *Escherichia coli*

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ABSTRACT

Gram-negative bacteria are ubiquitous in nature. These bacteria are responsible for causing many noxious diseases such as: neurological abnormalities, gastroenteritis, and life-threatening Typhoid fever in human being and many other animals. Therefore, Salmonella has been a cause to longstanding worldwide health problem and became a reason for significant mortality globally. The toxicity nature of these bacteria is because of their high tendency to survive in the host body rather quickly due to the presence of protective cell wall which defends from invasion of exogenous toxic agent. Therefore, developing new organic small molecule with ideally disposed functional unit which can easily prevent the growth of bacteria is always demanding. We report herein, a synthetically simple and elegant, small organic molecule, PA-P-ester which having highly tunable prerequisite properties with respect to protective cell wall of the bacteria. The molecule, PA-P-ester shows high antibacterial activity against pathogenic gram-negative bacteria such as: *Salmonella typhimurium* and *Escherichia coli*.

KEY WORDS: GRAM-NEGATIVE BACTERIA, SALMONELLA TYPHIMURIUM, ESCHERICHIA COLI, INFECTED DISEASES, ANTIBACTERIAL ACTIVITY, SMALL ORGANIC MOLECULE, PATHOGENIC BACTERIA.

INTRODUCTION

Gram-negative bacteria such as: *Salmonella typhimurium* and *Escherichia coli* are ubiquitous in nature which are considered as highly toxic pathogen among all other bacteria. These bacteria are having a protective cell wall, made up of lipopolysaccharide coat (LPS). This protective cell wall of these gram-negative bacteria acts as defense wall that prevents the foreign invasion into the bacteria body. As a result, these bacteria can easily sustain in the host body even in highly adverse condition and turn out to be toxic pathogen eventually. However, most of the gram-positive bacteria devoid of such protective cell wall and therefore, they have poor resistivity in hostile environment than gram-negative bacteria.

In day today life these bacteria some way or other impact our lives, even if these are pretty small living organism in the earth.[1] Among many gram-negative bacteria, Salmonella and E. Coli are two major pathogens that infect thousands of lives worldwide even today. [1, 2, 16] These bacteria are existed in different shape such as: spheres, spirals and rods in nature and are having wide range of size varies from 0.4 to 3 µM. The varying shape is due to the presence of actin-like bacterial cyto-skeleton.

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[3, 4] Among many of these gram-negative bacteria, *Salmonella* species are found to be highly toxic pathogen. According to the recent findings, *Salmonella* species are responsible for causing wide spectrum of diseases starting from neurological abnormalities to gastroenteritis to life-threatening Typhoid fever in human being.[5, 7, 11-13] Therefore, *Salmonella* has been a cause to longstanding worldwide health problem and became a reason for significant mortality globally.[6, 11-13].

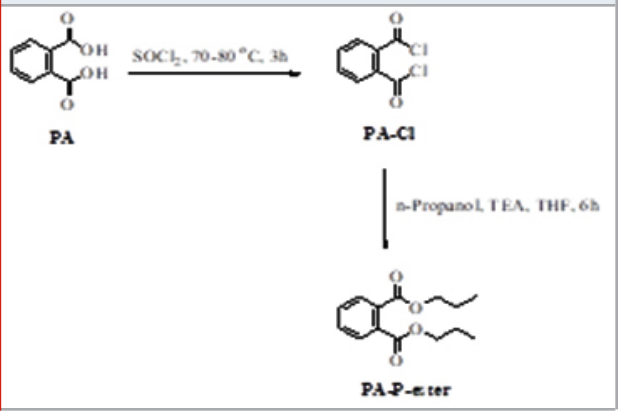
The bacteria S. Typhimurium is a wild in nature which infects a broad range of hosts. However, this specific bacterium belongs to *Salmonella* species. In addition to that, there are few class of Salmonella such as: *S. Typhi*, *S. Pullorum*, and *S. Gallinarum* are exquisitely host-restricted.[6] Furthermore, the bacterium Salmonella enterica serovar *Typhimurium* causes food-borne disease as well.[8] In addition to this, there is a highly diverse group of gram-negative bacteria called *Escherichia coli* which are mostly found in the environment, foods and intestines of people and animal. It has been known in the literature that most of *E. coli* are not harmful, rather helps to keep our digestive track healthy. However, there are few varieties of *E. coli* are highly responsible for causing a broad spectrum of infections such as: diarrhea, food poisoning, pneumonia, urinary tract infections etc. There are different categories of *E. coli* such as: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorragic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) which are responsible for causing diarrhea.[14].

Considering the life-threatening pathogenic effect of gram-negative bacteria, it has been a continuous endeavour from wide spectrum of scientific community to develop novel antibacterial agent. Till today, Most commonly used antibacterial agents are the derivative of quinolones and fluoroquinolones compounds.[9] It is always been a huge challenge for synthetic chemist to develop molecule with well-defined architecture that can strictly inhibit pathogenic gram-negative bacteria. The poor rate of progress is attributed to the protective cell wall of the gram-negative bacteria which demands a precise molecular structure. Therefore, to overcome this difficulty, herein we have developed a new scaffold with stringent architectural component inbuild into the benzene core. We have demonstrated potential antibacterial activity of this molecule against gram-negative bacteria.

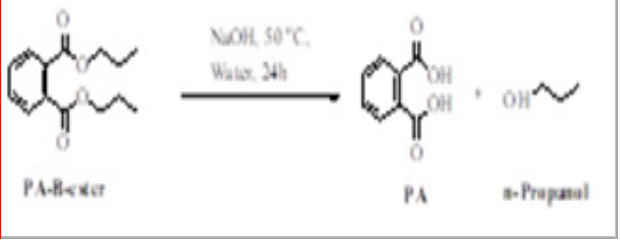
RESULTS AND DISCUSSION

There are several coupling reagents (DCC, HATU, HBTU, EDC, $SOCl_2$ etc.) available now a days to convert acid to corresponding amide or ester. Most of these coupling agents such as: 1) DCC, 2) HATU, 3) HBTU, 3) EDC are highly expensive and therefore, difficult to afford them for academic research. But on the other hand, thionyl chloride ($SOCl_2$) is another robust alternative for the synthesis of an amide or ester from acid. Furthermore, it is comparatively much cheaper than other coupling reagents. This reagent is pretty simple to handle and moreover, it affords high yield. Therefore, this reagent became central to many of the organic transformation for industrial uses. In light of these potential advantages, we have utilized the thionyl chloride (SOCl2) as a coupling agent to generate a new variety of molecular scaffolds which might inhibit toxic pathogen like Gram-negative bacteria such as: *Salmonella* and *E. coli*. (Scheme 1).

Scheme 1: Synthetic scheme for the synthesis of PA-P-ester



Scheme 2: Base hydrolysis of PA-P-ester



In search of an ideal molecular scaffold for the precise installation of n-propanol unit in order to have ester-based molecules, we intended to start with semi-rigid backbone like phthalic acid (PA). We envisioned that benzene ring in PA will provide rigidity along with pi-pi stacking interaction with hydrophobic region of the toxic pathogen which in turn could provide good binding efficacy. In addition to that the ester functionality may help in making hydrogen bonding interaction with hydrophilic part of the pathogen. The remaining butane tail may provide flexibility to the core structure and along with that it may enhance the possibility of cell permeability nature of the molecule. Consequently, synthesizing this kind of molecule may inhibit pathogens. Having these anticipations in mind, we wanted to install the ester moiety into a rigid system, Phthalic acid (PA). Therefore, we persuaded to synthesize compound, PA-P-ester (Scheme 1) with an intention that this compound may show better inhibition property towards pathogen.

In the beginning, the precursor compound, phthalic acid (PA) converted to corresponding acid chloride, PA-Cl by treating with thionyl chloride under room temperature for 3 hours (Table 1). The mixture of water

and tetrahydrofuran was chosen as the solvent system to carry out the reaction. Minimum amount of water was taken to solubilize the precursor compound, phthalic acid. The resulted acid chloride, PA-Cl was further treated with n-propanol in the presence of triethyl amine as a base to get the desired product, PA-P-ester. Reaction kinetics was completely monitored by thin layer chromatography (TLC) techniques. But surprisingly, TLC result analysis showed no new product formation, rater mostly precursor, PA was observed on TLC (Figure 1). We anticipated that the phthalic acid does not convert to corresponding acid chloride due to high transition state energy barrier which may require high temperature rather than room temperature.

Therefore, to overcome this challenge we further executed the reaction in the reflux condition keeping all other parameters unaltered. TLC result shows very mild product formation (Figure 1) which is negligible. This experiment clearly indicating that, not only temperature but also protic solvent like water and methanol play a role in inhibiting the reaction. In presence of methanol we observed almost the similar results like water. Therefore, we turn our attention to start the reaction using only aprotic solvent like THF. To overcome this problem, we designed two experiment in parallel by using THF as solvent. In the first experiment, the reaction was carried out at room temperature in presence of THF as a solvent and kept all other parameter constant. However, in the second experiment the reaction was carried out in reflux condition in presence of THF as a solvent without altering any other parameters. TLC analysis clearly shows new product formation (Figure 1, TLC-4) in both cases.

The intensity of product spot observed for refluxed reaction is much brighter than room temperature reaction. These results clearly reveal that the reaction must be carry out in aprotic solvent at refluxed condition to get the optimum product formation. With this optimized reaction condition, we observed up to 91 % of product yield (Table-2). The products were further purified by column chromatography in 1% methanol and chloroform system. The desired product, PA-B-ester formation was further confirmed by UV-Visible spectroscopy study (Figure 3) by comparing with commercially available starting material. This data clearly showed the formation of product. Elemental analysis data also support the product formation. Again, to cross verify the product formation we carried out the base hydrolysis reaction (Scheme 2) with sodium hydroxide solution in water as a solvent and isolated the hydrolyzed products using column chromatography techniques. After column purification of crude product, we observed two kinds of material. One material is solid and another one is liquid. The solid material expected to be phthalic acid and liquid material may be n-propanol.

The solid material obtained after hydrolysis was analyzed by comparing the TLC with commercially available starting material. The TLC result clearly shows, the hydrolyzed product is a starting material (Figure 2). It was further confirmed by comparing melting point with the starting material. The melting point of the starting material (207 °C) was found to be same as that of isolated product (207 °C). This indicated that the hydrolyzed product is PA (Scheme 1). The liquid material obtained after hydrolysis was analyzed using TLC with reference to commercially available n-propanol compound. TLC result clearly showed both have the same retention time. This result indicated the second hydrolysis product may be n-propanol. It further confirmed by determining the boiling point of the liquid material which was similar to commercially available n-propanol. This result confirmed that the resulted hydrolysis product is a n-propanol. Therefore, these results strongly support the formation ester derivative, PA-P-ester.

Figure 1: TLC-1-Reaction carried in water+ THF solvent mixture at room temperature; TLC-2-Reaction carried in water as solvent at reflux condition; TLC-3-Reaction carried in methanol as solvent at reflux condition; TLC-4-Reaction carried out in THF at reflux condition

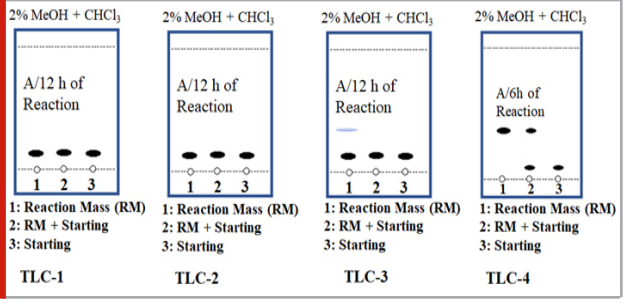


Figure 2: Monitoring the reaction kinetics of PA-P-ester hydrolysis reaction (scheme-2): after 1, 6, and 12 hours of reaction time using TLC.

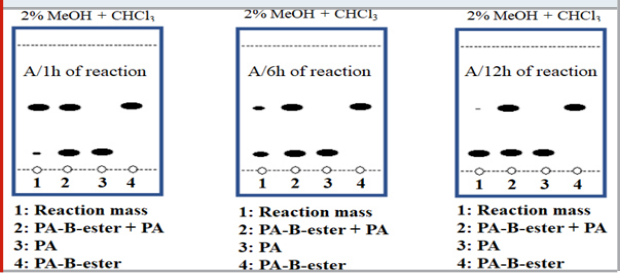
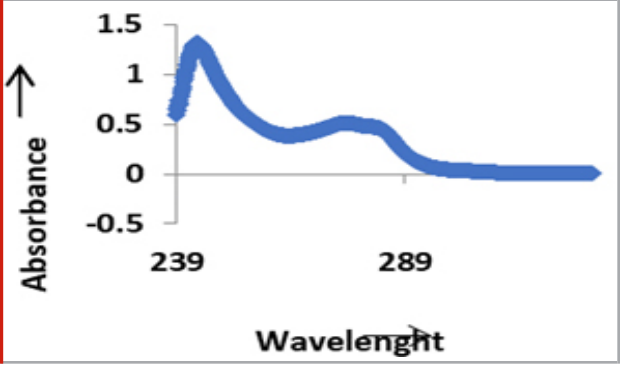


Figure 3: UV-VIS Spectra of the compound, PA-B-ester



The antibacterial activities of PA-P-ester against pathogenic bacteria i.e. *Salmonella typhimurium* and *Escherichia coli* were checked. A standardized concentration of both the inoculums were evenly spread on the surface of two different agar plates. A 500 µM concentration of PA-P-ester was loaded into both the bore wells and incubated for 24h at 30±2oC temperature. The results clearly showed a very strong inhibition zone of diameter 30.2 mm size against *Salmonella typhimurium* after incubation with PA-P-ester for 24 hours (Figure 4a). This result clearly reveals that, PA-P-ester has potential antibacterial activity against *Salmonella typhimurium*. However, the antibacterial activities of PA-P-ester was found to be relatively lesser for *Escherichia coli* than *Salmonella typhimurium* which is evident from the inhibition or clearance zones of both the pathogenic strains (Figure 4 a & b).

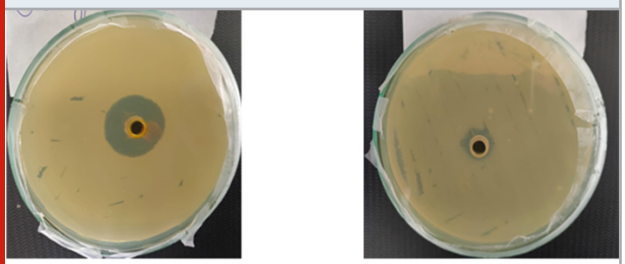
Table 1. Chemicals used for the reaction

Chemicals	Molecular Weight in g/mol	Quantity	Moles
Phthalic acid $\{C_6H_4(COOH)_2\}$	166.13	5 gm	0.03
Thionyl Chloride ($SOCl_2$)	118.97	6.6 ml.	0.075
THF (C_4H_8O)	72.11	100 ml.	0.09
n-Butanol ($C_4H_{10}O$)	74.121	6.04 ml.	0.81

Table 2. Reaction analysis in different solvent system

Solvents	Temperature	TLC Analysis	Yield	Remark
Water + THF	Room Temp. (RT)	No new spot observed	0%	The reaction was not progress in aqueous medium.
	Reflux Condition	No new spot observed	1%	
Methanol +THF	RT	No new spot observed	2-3%	The acid was not converted into acid chloride in methanol solvent.
	Reflux Condition	No new spot observed	12%	
Tetrahydrofuran	RT	New spot observed (not clear)	43%	In RT the rection progress very slowly
	Reflux Condition (70-80° C)	New clear spot observed	91%	and is taking long time. Yield is less. But in refluxed condition the rection progress very fast and observed quantitative yield.

Figure 5: Antibacterial activities of PA-B-ester against pathogenic bacterial stains: (a) Staining of PA-B-ester against *Salmonella Typhimurium*, (b) Staining of PA-B-ester against *Escherichia Coli*.



(Salmonella Typhimurium) (a) (Escherichia Coli) (b)

This antibacterial activity could be due to the presence of well-balanced hydrophobic, hydrophilic and long chain hydrocarbon molecules. Owing to these well-balanced properties of PA-P-ester, might be proficient to cross the bacterial cell membrane resulting in the inhibition of the bacterial strain growth.

Experimental section
MATERIAL AND METHODS

The chemicals and solvents were purchased from Spectro chem Ltd and Sigma Aldrich. All the chemicals were directly used without further purification. Normal phase column chromatography purification was carried out by using MERCK silica gel 60 (particle size: 100-200 mesh). Reactions were monitored wherever possible by thin layer chromatography (TLC). Silica gel G (Merck) was used for TLC and column chromatography was undertaken on silica gel (100–200 mesh) in hexane, hexane–ethyl acetate or chloroform. UV and visible peaks of synthesized organic compound was measured in chloroform as a solvent in the range of 200-400 nm. The wavelength (in nm) was taken in X-axis and absorbance in the Y-axis. It shows maximum absorbance at 243.6 nm wavelength. Melting points were recorded in a Fisher-Johns melting point apparatus.

Test organisms: The test bacterial cultures including *Escherichia coli* (MTCC No.- 614), *Salmonella typhimurium* (MTCC No.-3224) were collected from IMTECH Chandigarh. All the bacterial cultures were maintained in

nutrient agar slants. The slants were kept in refrigerator for use during further experiments.

Antimicrobial Assay: A standardized concentration of inoculums with fixed volume was spread evenly or swabbed on the surface of gelled agar plates. A hole which ranges from 6 - 8 mm in diameter was punched with a sterile cork borer aseptically in plates. A fixed volume (50 µl) of the sample solution was then introduced into the bored agar well and incubated at optimum temperature (Bacteria - 30±2°C for 24 hrs) depending upon the test microorganism. [17].

Synthesis of PA-P-ester: Phthalic acid (5g) was taken in a round bottom flask (RBF). To this solid mass, thionyl chloride (6.5 ml) was added dropwise over a period of 10 minutes at 10-15 °C. The temperature of reaction mass was raised to 70-80 °C and stirred it for 3 hours. Slowly cooled the reaction mass temperature to 0-5 °C and added THF (50 ml) into it. To this ice-cold solution, triethylamine was added slowly over a period of 1h. Then n-propanol (6.4 ml) was added into the reaction mass at 0-5 °C. Slowly raised the reaction mass temperature to room temperature and stirred it for 12h. TLC was checked and reaction was found to be completed. The organic layer was dried over anhydrous Na2SO4, filtered and evaporated. Finally, the crude compound was purified using column chromatography.

CONCLUSION

In conclusion, to our knowledge, it is a very unique example where PA-P-ester molecule strongly inhibit the growth of pathogenic gram-negative bacteria like *Salmonella typhimurium* and *Escherichia coli*. This molecule may open up new doorway for the treatment of diseases, causing by these bacteria. To our opinion, this work may provide new insight to design potential molecule of tunable property with respect to protective cell wall of the bacteria which will enhance the cell permeability of the molecule. Thereby, it will inhibit the pathogenic gram-negative bacterial growth.

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