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# Design and synthesis of novel antibacterial agents that targets the bacterial fatty acid pathway

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## Abstract

Antibacterial resistance is a global threat that is rapidly overtaking every known drug which has been designed for curbing its spread rendering them ineffective. This emphasizes the urgent need for the development of novel agents with unique targets that would show no resistance when employed against bacterial pathogens, using *Escherichia coli* and *Staphylococcus aureus* as test bacterial strains. The FAS II pathway has now become a prime candidate for developing significant and novel antibacterials as Platensimycin, a newly discovered inhibitor of the pathway further validates it. Three compounds were synthesized from carboxylic acids derivatives known as Acyl chlorides whose –OH group has been replaced by a chlorine atom and whose general formula is represented as RCO-Cl and a primary amine having one alkyl group on the nitrogen atom giving rise to its formula as $RNH_2$ and were subjected to spectroscopic analysis to confirm their chemical structure using NMR, TLC and LC-MS instruments. Biological assays involving the potency test of this synthesized compounds against bacteria confirmed any antibacterial activity as all three novel compounds showed inhibition activity even at the lowest concentration tested. The methyl $CH_3$ groups as well as the various benzene substituted rings of the compounds showed to be contributors to their inhibition activity. Recrystallization of this novel compounds is recommended in the future as a means of purifying it as well as the application of minimal inhibitory concentration (MIC) assay to ensure that this antibacterial agents are selected effectively to improve treatment outcomes.

**Keywords:** Antibacterial; Resistance; Inhibition; Amide coupling; Concentrations

## 1. Introduction

There is urgent need for new antibacterial drugs as a result of the growth of bacterial resistance to all classes of currently available therapies (1). Innovative antibacterial structural classes are especially needed because they are unlikely to demonstrate cross-resistance with the current drug classes that dominate the antibacterial market (such as β-lactams, macrolides, and quinolones) (1). Antibiotic resistance has steadily increased in recent years among numerous prevalent bacterial infections, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*. Methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *pneumococcus*, and vancomycin-resistant *Enterococcus faecalis* (VRE) are three prevalent bacterial infections that are difficult to treat at the moment (2). To combat these resistant strains, physicians frequently have to use their second or even third choice of antibiotic where vancomycin, which is typically considered a last-resort treatment, will lose its efficacy if the trend continues (2).

The pharmaceutical sector has reduced its research on novel antibacterials even though the prevalence of infections that are resistant to antibiotics is still rising. In fact, only two antibiotics with novel modes of action; linezolid and daptomycin have reached the market in the last 40 years (Fig 1) (2,3).

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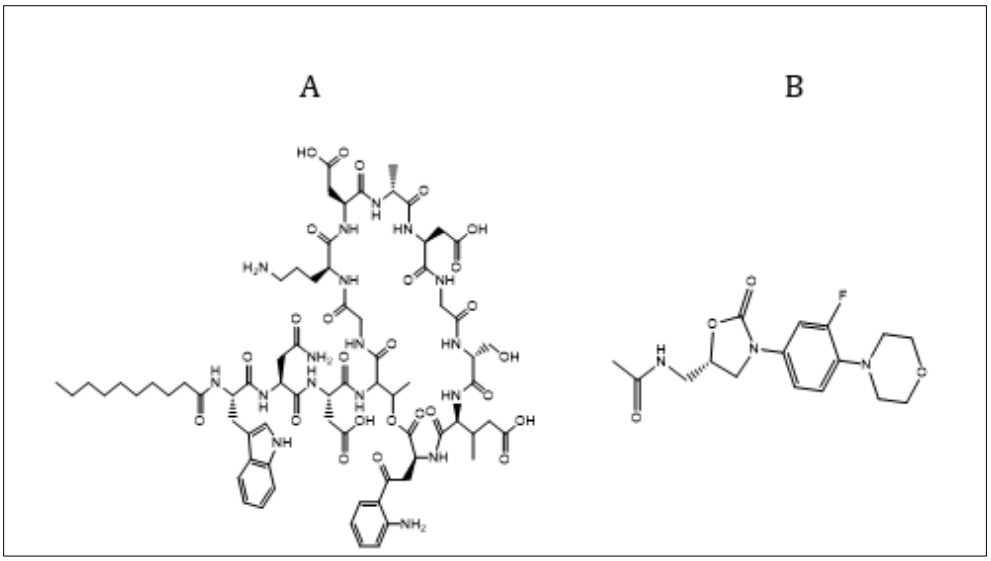


**Figure 1** Chemical structure of Daptomycin (A) and Linezolid (B)

ADHBA – 3-amino-2,4-dihydroxybenzoic acid; BSAC – British Society for Antimicrobial Chemotherapy; CLSI – Clinical and Laboratory Standards Institute; E.coli – Escherichia coli; FabF – (3-oxoacyl –[acyl-carrier protein] synthase 2); FabH – (3-oxoacyl-[acyl-carrier protein] synthase 3); FAS II – Fatty acid synthase type II; GNB – Gram negative bacteria; LC-MS – Liquid chromatography-mass spectrometry; MIC – Minimum inhibitory concentration; MRSA – Methicillin-resistant *Enterococcus Faecalis*; NCIMB – National collection of Industrial, Food and Marine Bacteria; NMR – Nuclear magnetic resonance; PTM – Platencimysin; PTN – Platencin; RNA – Ribonucleic acid; SAR – Structure activity relationship.

In 2017, the WHO released a list of 12 groups of bacteria which pose an increasing threat to human health and are already resistant to the majority of available therapies as "priority pathogens." In a report issued in 2020 which described the clinical pipeline for new antibiotic medicines from 2019, 53 compounds were listed as undergoing research. Out of these, 27 were synthetic and 14 were naturally sourced. However, compared to current antibiotics, only 32 of the 53 target priority diseases have any real advantages. The most virulent and drug-resistant bacteria are Gram negative bacteria (GNB), and only two of the compounds can effectively combat them (4).

Antibiotic resistance is a result of the widespread use of antibiotics, which places a significant selection pressure on its emergence. Antibiotic effectiveness decreases as antibiotic use increases because resistance develops faster as a result of increased frequency. After months or years, clinically significant resistance develops whenever a new antibiotic is brought into modified use. With the exception of vancomycin, which developed resistance roughly 30 years after its initial release, penicillin resistance was found in 1942, and streptomycin resistance in 1944. The indiscriminate use of antibiotics resulted in methicillin-resistant *staphylococcus aureus* (MRSA), which exhibits multiantibiotic resistance against multiple structurally unrelated drugs. In turn, widespread usage of vancomycin, which was regarded as a last-resort antibiotic, resulted in the development of vancomycin-resistant *Enterococci* (VRE) in 1986 (2,5).

The type II fatty acid synthesis system (FAS) in bacteria has emerged as a leading candidate for designing potent and novel antibacterials because it is not only essential for cell survival but also exhibits notable differences between bacterial and human fatty acid synthesis, including the organisation, structure of enzymes, and their specific roles. Because of it's noted variability, this system is a desirable target for the development of antibacterial drugs (6). This route has been validated as a target for antibacterial development by the prolonged usage of the antituberculosis medicine isoniazid and the antiseptic triclosan, both of which are fatty acid biosynthesis inhibitors (7,8).

Platensimycin (Fig. 2) is a promising natural compound for the development of clinically effective antibacterial medicines (FabF inhibitor). Platensimycin was discovered as a result of a focused, differential sensitivity experiment using antisense RNA (9). This natural product from Streptomyces platensis represents a new pharmacologic family of antibiotic with potential inhibitor activity against Gram-positive bacteria, with a MIC of 1 µg/ml against *S. aureus*, *Enterococcus faecalis*, and *Staphylococcus pneumoniae*. It has been established that FabF is the target, and that platensimycin sensitivity to S. aureus is inversely related to FabF expression levels.

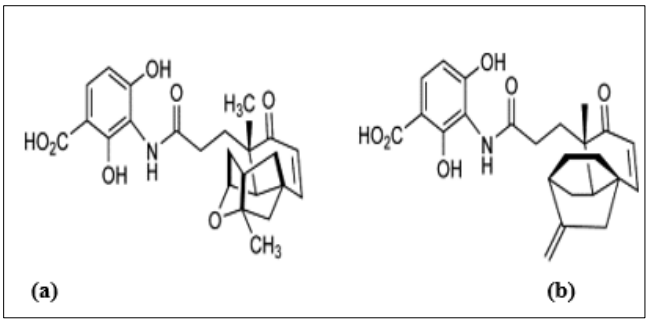


**Figure 2** Chemical structure of Platesimycin (a) and Platencin (b) (9)

Platencin (Fig 2.), a platensimycin analogue, was discovered as a result of ongoing natural product screening. With a variety of Gram-positive bacteria, platencin's *in vitro* activity is equal to platensimycin's, although it performs better against vancomycin-resistant *Enterococcus faecium* and efflux-negative *E. coli*. Platensimycin has poor oral bioavailability and inferior pharmacokinetic features, which are the main drawbacks of most natural products. Platensimycin's complicated tetracyclic moiety has proven difficult for medicinal chemists to completely synthesize. Difficulty arises from the inability to modify some of the reported natural products (cerulenin, thiolactomycin, and platencin) for improved activity towards targeting the FAS II. The need to find new chemically tractable scaffolds as a starting point for the development of fatty acid synthesis inhibitors is highlighted by these unsatisfactory results (9,10).

In this research, we designed and synthesized three novel antibacterial agents, with structural similarities to platensimycin/platencin, from the reaction involving an amine (methyl 3-amino-4-methyloxybenzoate) and three different acid chlorides (stearoyl chloride, palmitoyl chloride and benzoyl chloride).

The objective of this study was to design and synthesize novel antibacterial agents that would target the bacterial fatty acid pathway and, ideally, have little or no resistance. As a result, the goal of this work was to: (i) design and synthesize the antibacterial agents following the reaction of the acid chlorides with the primary amine and, (ii) Identify, characterize, and test the novel agents' antimicrobial activity against Gram positive and Gram negative bacteria.

## 2. Materials and Methods

### 2.1. Reagents

Acid chlorides; stearoyl chloride, palmitoyl chloride, benzoyl chloride were procured from Sigma Aldrich (Dorset, UK). The amine; Methyl-3-amino-4-methoxy-benzoate was also procured from Sigma Aldrich (Dorset, UK). Carboxylic acids; Picolinic acid, Isonicotinic acid and Nicotinic acid. The solvents; Dimethylformamide DMF (PHR1553), Methanol (M/4056/17), Dimethyl Sulfoxide DMSO, Dichloromethane DCM (34856), Dichloromethane, Dichloroethane, Diisopropylethylamine (DIPEA), Ethyl acetate, Hydrochloric acid (HCL), Sodium hydroxide (NaOH) were purchased from Fisher Scientific (Waltham, Massachusetts). Nutrient Agar (CM0003), Nutrient Broth (CM0001), Triclosan (5-chloro-2-(2,4-diclorophenoxy) phenol) (PHR1338) were all purchased from Fisher Scientific (Waltham, Massachusetts).

### 2.2. Instrumentation

Zone of inhibition measurement was done using ProtoCOL3 by Synbiosis (Cambridge, UK). Reactions were done on a magnetic stirring plate (SciQuip, England, UK) with a stirring bar. Vacuum oven (Thermo scientific, Langenselhold, Germany) was used in ensuring total dryness of the crude product. TLC analysis was determined on aluminium silica precoated gel plates (POLYGRAM®, Germany), UV absorbance was monitored using a UV lamp (Mineralight®, US). LC-MS was conducted using a Zorbax SB-C18 guard column on an Agilent 1200 HPLC linked to a 6130 single quadrupole mass spectrometer. Bruker Topspin 1.3 and Icon NMR automation software were used to determine 1H and 13C NMR spectra on a Bruker 400MHz Ultrashied, and chemical shifts were reported in ppm. On an XT4MP device, melting points (uncorrected) were determined (Taike Corp., Beijing, China).

## 2.3. Chemistry Synthesis Procedure

### *2.3.1. General procedure for One Pot synthesis of amides from Carboxylic Acids activated using thionyl chloride*

With a few minor modifications to the chemical solvents utilized, protocols for this synthesis were followed from Leggio et. al (2016) as described (13). In 10ml of dichloromethane, 1 mmol of the provided amine, 3 mmol of DIPEA, and 1 mmol of (thionyl chloride) $SOCl_2$ were added after adding 1 mmol of the carboxylic acids. At room temperature, the mixture was stirred for 20 minutes. The solvent was evaporated at reduced pressure in order to obtain the reaction product. The resultant residue was dissolved in dichloromethane, followed by 1N HCl and 1N NaOH washings.

### *2.3.2. General procedure for the synthesis of methyl-3-methoxy-4-palmitamido benzoate*

The procedure as described by *Montalbetti et. al* in amide coupling via acyl chloride formation was adopted for this procedure but slightly modified as already prepared acyl chlorides was used (12). Using a 1:1 ratio, (0.003moles) 0.8ml of the palmitoyl chloride was pipetted into a 50ml round bottom flask under nitrogen in a fume hood. In the round bottom flask, 0.5g of the amine (methyl 3-amino-4-methoxy benzoate) was added to the liquid. 10ml of DMF was added and the mixture was stirred for 7hours. To crash out the product, 20ml of distilled water was slowly and cautiously added to the reaction, and white precipitates were seen forming. The reaction was left for another 45minutes while a separating apparatus was set up.

The solid was filtered and then dried using a vacuum oven giving a yield of 0.8217g (71%). TLC (eluent: Dichloromethane : Methanol. 13:1): $R_f$= 0.74 Chemical shifts were reported in ppm (d). $^1$H NMR (d-DMSO, 400MHz) δ ppm: 0.85 (3H, *t*, $CH_3$), 1.23 (28H, br *s*, $[CH_2]_{14}$), 1.47 (2H, *m*, $CH_2$-$CH_2$-CO), 2.18 (2H, *m*, $CH_2$-CO), 3.81 (3H, *s*, $OCH_3$), 3.91 (3H, *s*, $CO_2CH_3$), 7.17 (1H, *dd*, Ar-H), 7.71 (1H, *dd*, Ar-H), 8.60 (1H, *s*, Ar-H), 9.18 (1H, *m*, NH). MS (ESI): 420.3 ($C_{25}H_{41}NO_4$, $[M + H]^+$).

### *2.3.3. General procedure for the synthesis of methyl 4-benzamido-3-methoxybenzoate*

The same procedure was repeated as described above with 0.35ml (0.01moles) of the benzoyl chloride, TLC (eluent: Dichloromethane : Methanol. 13:1): $R_f$= 0.76 White product with a yield of 0.3122g (39%), mp: 139-146ºC. $^1$H NMR ($CD_3OD$, 400MHz) δ ppm: 3.92 (3H, *s*, $OCH_3$), 4.02 (3H, *s*, $OCH_3$), 7.18 (2H, *s*, Ar-H), 7.21 (2H, *s*, Ar-H), 7.56 (2H, *t*, Ar-H), 7.62 (2H, *t*, Ar-H), 7.97 (3H, *m*, Ar-H), 8.72 (1H, *s*, NH). MS (ESI): 286.1 ($C_6H_{15}NO_4$, $[M + H]^+$).

### *2.3.4. General procedure for the synthesis of methyl 3-methoxy-4-stearamido-benzoate*

The same procedures was repeated as described above with 0.183g (0.001moles) of stearoyl chloride. White crystals with a yield of 0.8732g (70%). TLC (eluent: Dichloromethane : Methanol, 13:1); $R_f$= 0.82, mp: 98-102ºC. $^1$H NMR ($CDCL_3$, 400MHz) δ ppm: 0.92 (3H, *t*, $CH_3$), 1.30 (32H, br *s*, $[CH_2]_{16}$), 1.72 (2H, *m*, $CH_2$), 2.45 (2H, *m*, $CH_2$-CO), 3.92 (3H, *s*, $OCH_3$), 3.99 (3H, *s*, $CO_2CH_3$), 6.96 (1H, *dd*, Ar-H), 7.31 (1H, *dd*, Ar-H), 7.86 (1H, *dd*, Ar-H), 9.12 (1H, *s*, NH). MS (ESI): 447.66 ($C_{27}H_{45}NO_4$, $[M + H]^+$).

## 2.4. Antibacterial Activity

*Escherichia coli* (4174) and *Staphylococcus aureus* (6571) acquired from NCIMB (Aberdeen) were used to investigate the synthetic compounds' antibacterial activity. One colony was selected from a master plate kept at -80°C and grown into approximately 200 mL of nutrient broth overnight at 37°C and 180 rpm in a shaker. A streak plate per colony was created, which was then incubated for 24 hours at 37°C. These streak plates, which were kept at 4ºC throughout the operation, served as the master plates.

### *2.4.1. Agar Plate Preparation*

500mL of distilled water was mixed with 14g of agar powder, agitated by hand, autoclaved, and then kept at 55°C. Approximately 25mL of the already prepared agar was poured onto 90mm petri dishes to create agar plates, which were then placed in a laminar flow hood to set for a few hours to solidify. After that, the plates were packaged and kept at 4°C until required.

### *2.4.2. Culture preparation*

Every day, a new culture was grown for use. A single colony was streaked off the previously prepared master plates and added to about 200mL of nutrient broth. This was incubated overnight at 180rpm in a shaker (Fisher Scientific, UK Ltd, Loughborough) at 37 °C.

*2.4.3. Serial dilution preparation*

A series of succeeding dilutions were performed to supply the various concentrations ranges to be tested. 10 mg of the compounds were weighed out into a volumetric flask and 10ml of DMSO was added into it making the stock solution. Serial dilution was now carried out using the ranges of 1000µg/ml, 100µg/ml, 10µg/ml and 1µg/ml and making up with DMSO. The same procedure was done with the Positive control (triclosan).

### 2.5. Toxicity Test

To confirm that only the newly synthesized compounds had an effect on bacterial growth, 10 mL of DMSO was used to test its activity. On the agar plate, 200µL of fresh culture was pipetted, distributed, and given 15 minutes to dry. 20µL of each DMSO concentration were applied to sterile 6 mm filter paper discs, which were then put onto agar plates and incubated at 37 °C overnight. As a negative control, the greatest concentration of DMSO that had no effect on bacterial growth was employed.

*2.5.1. Disc Diffusion Assay*

A disc diffusion experiment was performed to investigate the antibacterial properties of the newly synthesised antibacterial agents. 200 µL of culture was put onto an agar plate and left to dry for 15 minutes. Sterile 6mm discs were put onto the plate with 20µL of the compounds at concentrations of 1mg/mL, 100µg/mL, 10µg/mL, and 1µg/mL, as well as a positive and negative control. Triclosan was employed as a positive control for both *E. coli* and *S. aureus.* Plates were incubated at 37 °C for 24 hours, and the diameter of the inhibition zone was measured.

## 3. Results and discussion

### 3.1. Chemistry

Platencimycin (PTM) and platencin (PTN) are natural compounds that comprises of two distinct moieties linked by an amide bond (Fig. 2). Both compounds include a moiety of 3-amino-2,4-dihydroxybenzoic acid (ADHBA). The structural variations between PTM and PTN are seen in the aliphatic cages that are linked to ADHBA via a flexible propionamide chain. The compound's aliphatic moieties, or ketolides have 17 carbons and a cyclohexenone ring, but differ in the remaining cage moieties. PTM has an unusual tetracyclic ketolide with a fused cyclohexyl-cyclopentyl-furan, whereas PTN contains a tricyclic unit with an exocyclic methylene. (13). Looking at this chemical properties, the acid chlorides were selected with similar conformational properties as benzoyl chloride, stearoyl chloride, palmitoyl chloride all have quite a number of carbon chains and benzene aromatic rings. The amine (methyl 3-amino-4 methoxybenzoate) is the linking bond that would form the amide bond.

Amide bonds are essential for biological macromolecules and polymers structure and function. Because of the importance of this functionality, several techniques to its formation have been developed, ranging from stoichiometric activation of carboxylic acids to more recent developments in catalytic amide bond formation. Some of the available methods for amide coupling are; (i) Peptide coupling method, (ii) Boron based method and, (iii) One pot synthesis method.

In the formation of the amide bond, a common strategy would be the condensation of the carboxylic acid to its activated form which would then react readily with the primary or secondary amine but in this experiment, the activated form of carboxylic acids has a chlorine atom which was easily replaced by an $NH_2$ group to form the corresponding amide.

Of all three methodologies listed, the one pot synthesis method was chosen as it was the easiest and relatively cheap for pharma industries who would adopt this method for designing antibacterial agents with similar activity.

*3.1.1. One pot synthesis of amides from carboxylic acids*

Following the procedure stated by Leggio et. al, 1mmol (0.3742g) of picolinic acid was added to 1 mmol (174µm) of DIPEA (Hunig's base) in 10ml of dichloromethane, then 1 mmol (72.5µm) of thionyl chloride was added into a 25ml round bottom flask with the magnetic stirrer turned on. After 20minutes of stirring at room temperature, it was observed that the reactants did not come to completion as the starting materials were still visibly unchanged. Further analysis into the reaction lead to a TLC analysis of the reaction mixture showing incomplete conversion of the picolinic acid. The reaction was allowed to continue for another hour but still remained the same. DMF was introduced later on in the same reaction flask as a catalyst to enable fast dissolution of the starting materials but it was still unchanged and the reaction turned dark green on its addition with the starting materials still heavily present when TLC analysis was conducted.

Thionyl chloride, $SOCl_2$, which was intended to act as the chlorinating agent in the synthesis of the acyl chloride was not interacting with the amine as expected. Another experiment was carried out employing an extra 4 equivalents of $SOCl_2$ and the reaction was allowed to continue for an hour. Previous research had recommended a variation in the order of reagent addition. The goal of the study was to confirm the one-pot synthesis of amides using thionyl chloride as activating agents, therefore they devised an experiment in which benzoic acid, the carboxylic acid chosen, was treated in the presence of trimethylamine, along with thionyl chloride and the amine. When benzoic acid was added to thionyl chloride first, then $Et_2NH$ and $Et_3N$ were added subsequently, the reaction yield was reduced, and the reaction was still not completed. They repeated the process but adding the thionly chloride first before the benzoic acid and trimethylamine in which the reaction process came to completion (12,14).

From our reaction, the mixture changed color from green to dark purple after the addition of the thionyl chloride to the carboxylic acid and stirring in the presence of heat in an oil bath. Given that the reagents were still in their undissolved state, TLC examination revealed very little separation of the starting components. This one-pot synthesis process was terminated at this point since it used a lot of time and starting materials without producing any results, as investigations have shown that the bulky groups on the carboxylic acid molecule sterically hinder the reaction's progress (14).

Leggio et al. (2016) employed a developed approach on two N-protected α-amino acids, N-nosyl-Lphenylalanine and N-nosyl-L-alanine, to study the steric hindrance present on the carboxylic acid. In 20 minutes and with significantly lower yields, N-nosyl-L-phenylalanine and N-nosyl-L-alanine were transformed into the equivalent N,N-diethylamides. Based on these findings, it was concluded that the steric hindrance provided by the groups on the nitrogen atom of the amine has no effect on reaction progression, despite the fact that reaction times are longer and reaction yields are slightly lower when sterically hindered carboxylic acids are used. (14).

*3.1.2. Chemical synthesis of methyl 3-methoxy-4-palmitamido benzoate*

The synthesis involves the reaction between palmitoyl chloride and methyl 3-amino-4-methoxy benzoate in the presence of DMF. The reaction progressed rapidly with the crashing out of the crude product on addition of distilled water after 3 hours. The disappearance of the two starting materials on TLC occurred after 3 hours of the reaction process and the reaction was deemed to have progressed to completion.

Solid precipitates were recovered during the isolation procedure of the chemical synthesis. Based on TLC and NMR research, it was eventually determined that the solid was methyl 3-methoxy-4-palmitamido benzoate. Vacuum drying of the moist substance produced a white solid.

For the $^1H$ NMR spectra, methyl 3-methoxy-4-palmitamido benzoate displayed four peaks in the aromatic region between 9.3-7.2 ppm, two of which had double protons and the other two having a single proton. In the aliphatic region, six peaks were observed between 0.8-3.7 ppm with two overlapping methylene peaks at 3.9ppm.

The long aliphatic chains of the compound appeared at 1.2ppm which was obviously expected upfield (shielded) and the amide $NH_3$ bond was seen downfield (deshielded) at 9.3ppm due to reduced electron density. The benzene aromatic rings were observed between 8.6-7.17ppm. Impurities were seen in the spectra as water peaks were observed at 3.35 and the solvent residual peak was spotted at 2.5ppm.

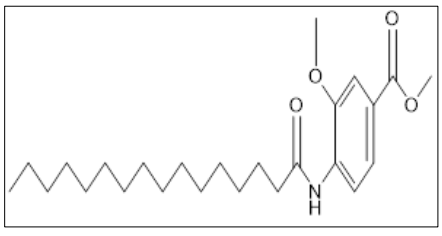


**Figure 3** Chemical structure of methyl 3-methoxy-4-palmitamido benzoate

*3.1.3. Chemical synthesis of methyl 3-methoxy-4-stearamido benzoate*

Except for the number of aliphatic peaks, the $^1H$ spectra is identical with the first product mentioned above. Slight difference would appear in the deuterated solvent used but generally, all peaks would be found almost at the same position.

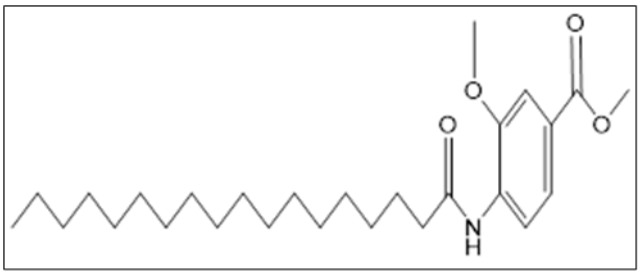

**Figure 4** Chemical structure of methyl 3-methoxy-4-stearamido benzoate

*3.1.4. Chemical synthesis of methyl 4-benzamido 3- methoxy benzoate*

Slightly different from the first two compound structures with two benzene shapes and an amide connecting chain seen. The peaks are found at almost similar positions with no long aliphatic chain peak. Two peaks were displayed in the aliphatic region while 6 peaks were seen in the aromatic region but altogether the NMR analysis confirms the structure of the compound.

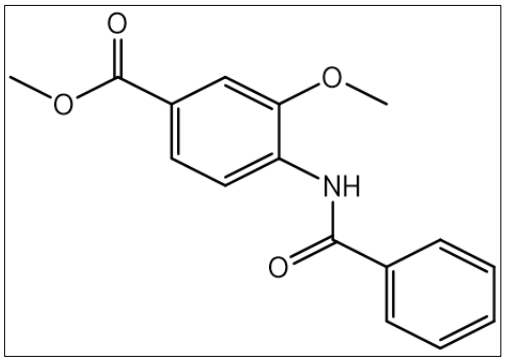

**Figure 5** Chemical structure of methyl 4-benzamido 3-methoxy benzoate

### 3.2. Antimicrobial Activity

*3.2.1. Toxicity Test*

The purpose of this test was to confirm that the DMSO used to dilute the compounds in the serial dilution step was not in any way affecting the bacteria. The agar plate assay was used to further validate that the test, which was conducted on a range of (1-100%), had no impact on the bacterial growth for *S. aureus* and *E. coli*. Furthermore, it was consistent with studies by Verheijen et al. (2019), who stated that "DMSO is commonly considered non-toxic even below 10% (v/v), and in ordinary application, the effects of DMSO are assumed to be minor." (15).

*3.2.2. Disc Diffusion Assay*

*E. coli* and *S. aureus,* two bacterial strains on the WHO's priority pathogens list classified as "critical" and "high," respectively, were utilized as test subjects for the antibacterial activity of the newly synthesized compounds (Mendelson and Matsoso, 2015). Figure 6 shows an image of a disc diffusion experiment after incubation

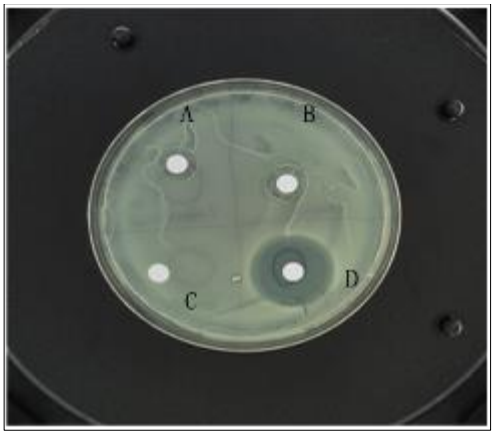


**Figure 6** Sample disc diffusion assay plate with Compounds (A) and (B) of the newly synthesized compounds at 100µg/ml on *E.coli* bacterial strain culture, negative control (C) at 100% DMSO, Positive control (D) Triclosan at 100µg/ml.

To verify the efficacy of the novel antibacterial agents created during the antimicrobial testing, Triclosan, a conventional antibacterial agent, was utilized. It works by preventing the bacterial enoyl-acyl carrier protein reductase (FabI), a vital enzyme in the production of fatty acids in bacteria, from functioning at sublethal quantities. It is proposed that it acts through a variety of non-specific mechanisms, including membrane destruction, at bactericidal doses (11).

The "Kirby-Bauer method," as it is often called, entails seeding a lawn of bacteria on the surface of an agar medium with paper discs saturated with specified doses of antibacterial compounds. The presence or absence of a zone of inhibition around the discs was verified after incubating the plate overnight, as indicated above.

A ruler was used to measure the circle's diameter, and the results were recorded. The plate was then placed in the ProtoCOL3 plate reader by Synbiosis, which read the diameter of each zone. We multiplied this diameter by two and then took the average of the numbers to arrive at the conclusions. The table below displays the results for each compound's average inhibition zones.

**Table 1** Average diameter of inhibition zone of the three novel compounds at 1mg/ml, 100µg/ml, 10µg/ml and 1µg/ml against *E.coli.*

| **Compound** | **1mg/ml** | **100µg/ml** | **10µg/ml** | **1µg/ml** |
|---|---|---|---|---|
| Methyl 3-methoxy-4-palmitamido benzoate | 15.33 | 10.67 | 9.67 | 8.23 |
| Methyl 3-methoxy-4-stearamido benzoate | 12 | 10.5 | 9.5 | 8 |
| Methyl 4-benzamido-3- methoxy benzoate | 10 | 9.17 | 8 | 7.87 |
| Triclosan | 23.56 | 13.45 | 11.11 | 10 |

Zone of inhibition (mm) / concentration (ml)
(Escherichia coli)

**Table 2** Average diameter of inhibition zone of the three novel compounds at 1mg/ml, 100µg/ml, 10µg/ml and 1µg/ml against *Staphylococcus aureus.*

| **Compound** | **1mg/ml** | **100µg/ml** | | **10µg/ml** | **1µg/ml** |
|---|---|---|---|---|---|
| Methyl 3-methoxy-4-palmitamido benzoate | 12 | 10.5 | | 10 | 8.67 |
| Methyl 3-methoxy-4-stearamido benzoate | 10.83 | 10.24 | | 9.87 | 8.7 |
| Methyl 4-benzamido-3- methoxy benzoate | 11 | 9.83 | | 9.17 | 8.33 |
| Triclosan | 22.11 | 11 | | 10.33 | 9.33 |

Zone of inhibition (mm) / concentration (ml); *(Staphylococcus aureus)*

DMSO had no inhibition activity against *E.coli* and *S.Aureus* at 100% concentration used throughout the assay.

The antibacterial assay results indicate that all three recently synthesised compounds were effective against *S. aureus* and *E. coli* at the different doses used. To simplify the explanation of their various actions, the three compounds will now be represented as m1, m2, and m3, in the order in which they occur in the tables above. Gram-negative *E. coli*, which has been known to be highly resistant to antibiotics, was successfully inhibited by all the novel compounds even at the lowest concentration range tested (4).

Because most antibiotics must pass through the outer membrane of Gram negative bacteria to reach their targets, it has been proposed that the outer membrane of Gram negative bacteria is the primary cause of resistance to a range of antibiotics, including β-lactams, colistin, and others. Gram-negative bacteria can develop resistance by changing the hydrophobic characteristics of their outer membranes or by mutating porins and other proteins. Gram-positive bacteria, without a doubt, lack the critical layer, making Gram-negative bacteria more resistant (16).

The effectiveness of each amide derivative's antibacterial properties against various bacterial strains was assessed (*E.coli* 4174 and *S.aureus* 6571). Although none of them came close to the positive control, which had an inhibition value of 23.53mm, they all showed considerable and powerful inhibitory effects against Gram-negative *E. coli*. This is particularly admirable considering that they are all new chemicals that provide an excellent target for bacterial inhibition. These makes the compounds less polar and able to traverse membranes a little easier. The biological tests show that the compounds m1–m3 are effective *E. coli* inhibitors when used as antibacterial agents. The catalytic triad tunnel of Cys-His-Asn, which is conserved in different bacteria, is typically found in the FabH active site. Chain elongation and substrate binding are tightly controlled by this catalytic trio. Since the catalytic triad of FabH's Cys breaks the alkyl chain of CoA, it indicates that interactions between Cys and substrate are crucial for substrate binding.

All of these properties suggest that these small molecule inhibitors of FabH enzymatic activity could be candidates for the synthesis of selective, nontoxic, and broad-spectrum antibacterials. (17).

Having an average 10mm inhibition zone measurement, the three compounds with benzene rings and 4-methoxy substituted benzene rings showed impressive antibacterial activity against *E. coli*. The results that were obtained will be helpful in the search for stronger antibacterial drugs.

In a study by L. Shi et al (2010) which synthesized twenty (20) new Schiff bases and a primary amine as potent inhibitors of *E.coli* FabH, four (4) of the compounds having a methyl group and long aliphatic chains like that of m1 and m2 were found to be averagely active against all the tested bacterial strains. It was indicated in that study that the long aliphatic chains was responsible for the subtle decline in antibacterial activity (17).

Although the compounds m1-m3 had ≥10mm inhibition values, we can conclude that the longer aliphatic chain and large volume acid substituent group at the amide binding sites played a huge role in the antimicrobial responsiveness. Specifically, compounds m1 and m2 showed similar moderate antibacterial activity against all bacterial strains. According to the results of a broth dilution method used to analyse the activity of long chain fatty alcohols against *Staphylococcus aureus*, the most effective number of carbon atoms in long chain fatty alcohols in terms of growth-inhibitory activity ranged from 13 to 15. The broth dilution method was also used to measure bactericidal activity, which revealed that long-chain fatty alcohols with aliphatic carbon chains of 12 or 13 carbons had the strongest growth-inhibitory effect (18).

When compared to the positive control (Triclosan), which had an inhibition value of 23.56mm across all the compounds, we can see that all the newly synthesised compounds exhibit potent and promising antibacterial activity against the bacteria even though at an average value.

The thick cell wall of *Staphlylococcus aureus* is composed of peptidoglycan, which is itself composed of tetrapeptide-linked N-acetylglucosamine and N-acetylmuramic acid. According to studies, some antibacterial drugs like penicillin function by attaching to proteins and preventing the cell enzymes from forming new cell walls. Recent case studies have shown that *Staphylococcus aureus* produces the enzyme β-lactamase, and that β-lactam antibiotics can bind to this enzyme. It has been established that the mechanisms of antibiotic resistance are located in the cells, which are made up of plasmids, prophages, and transposons all contained within a 2800 bp circular chromosome (Fig.7) (19).

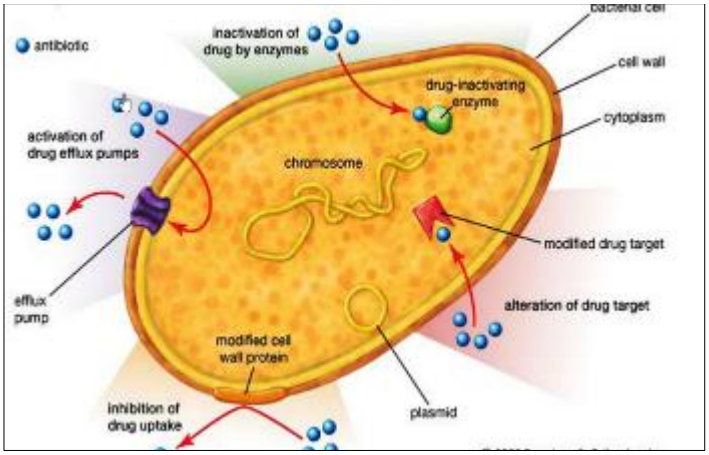


**Figure 7** Mechanism of Antibiotic resistance in biological cells (19)

Given the results found for *E.coli* and in comparison to what has been obtained in *S.aureus,* we can see that Methyl 3-methoxy-4-palmitamido benzoate (m1) sustained the highest inhibition value (12mm) for the bacteria strain proving to be a more potent compound for future analysis and study in its improvement.

When comparing to Triclosan, we notice that the three compounds are somewhat promising as they exhibit greater chances of being powerful antibacterials against this threat of resistance that has cut across all medically known facts. At the lowest concentration of 1µg/ml, the positive control had an average of 9.33mm while the three compounds were between 8.3-8.7mm which shows promise.

Hueck et al. (1966) discovered fungiastatic, bacteriostatic, and algistatic activity in 164 fatty nitrogen compounds, including primary alkyl amines, secondary amines, tertiary amines, diamines, and quaternary ammonium compounds. Both gram-positive and gram-negative organisms were resistant to these amides' antibacterial activities (17). Kabara et al. (1972) attempted to correlate structure with biological activity in a different study in which they revisited the antibacterial activity of fatty acids and other lipids. With gram-positive and gram-negative bacteria, the effects of various chain lengths and the influence of various functional groups were investigated. The findings revealed that compounds with a chain length of 11 to 13 carbon atoms had the strongest antibacterial activity in a series of alkyl amines from C8 to C20. Although amine compounds affected both gram-positive and gram-negative organisms, gram-positive species were more susceptible to amine antibacterial action than gram-negative organisms (20).

The microorganisms that were most resistant were susceptible to chain lengths ranging from 10 to 12 carbons. Chain lengths 2 to 3 carbon atoms longer had an influence on the more susceptible organisms. The most susceptible gram-negative bacteria were generally more resistant than the most resistant gram-positive bacteria. (20).

Hueck et al. discovered that dioctylamine has potent antibacterial activity against both gram-positive and gram-negative bacteria after researching two secondary amines. An aliphatic chain of seven carbons separating the two amino groups was found to be the optimum for *E.coli* inhibition, according to these authors. Longer chain compounds, on the other hand, were not studied. The two primary amino groups are essential for antibacterial action, according to the research; replacing one amino group with an OH radical decreases activity. Longer chain mono-amines were found to be more effective against gram negative bacteria than gram positive bacteria.

In comparison to our results, the longer aliphatic chains of the compounds in combination with the amide gives quite a promising effect that could be said to be behind the effectiveness against the bacterial strains.

The series of N,N-dimethyl-substituted alkyl tertiary amines (chain lengths from C11 to C18) also displayed the primary and secondary amines' pattern of action. Activity against gram-negative bacteria decreased when a chain length of 14 carbon atoms was attained, while activity against gram-positive bacteria rose as chain length grew (20).

Following our results in table 2, apart from the concentrations of 1 mg/ml for both *E.coli* and *S.aureus* in which the gram-negative bacterial strain showed the highest inhibition value from all three compounds, the gram positive bacteria demonstrated very good inhibition at the other three concentrations (100µg/ml, 10µg/ml and 1µg/ml) tested even greater than those of gram-negative. This research finding is generally consistent with some findings in the studies cited

above. However a series of antibacterial assays that would further test their bacterial inhibitory effects needs to be done in order to validate the potentials of this novel compounds as well as elucidate the structural relationship which would look at the relationship between the chemical structures of the compounds and their biological activities towards the bacterial strains.

## 4. Future Work

In the limited time available for this study, three compounds were designed and synthesized however some other biological assays still needs to be carried out in order to validate this novel compounds as bacterial FAS II targets.

### 4.1. Recrystallization of the novel compounds

Going back to the NMR analysis and Melting point determination, we could see that there was presence of impurities which showed up in the spectra and induced the long period it took all three compounds to melt. It is advised in the future to purify this novel compounds after synthesis through recrystallization or column chromatography.

### 4.2. Minimum Inhibitory Concentration (MIC)

In this study, Disc diffusion assay was used to measure zones of inhibition values of the newly synthesized compounds but isn't sufficient as a confirmatory test for the newly designed compounds to be considered antibacterials.

The British Society for Antimicrobial Chemotherapy (BSAC) and the Clinical and Laboratory Standards Institute (CLSI) both propose using MICs as the starting point for wider preclinical studies of new antibacterial medicines. Measuring the minimal inhibitory concentrations is mostly done to make sure that antibiotics are selected effectively to improve treatment outcomes. Each of the novel medications will have a susceptibility interpretation noted next to it in the MIC report (21). The use of incompatible amounts of antibacterials produces the selective pressure that has determined the evolution of bacterial pathogen resistance, as demonstrated by antibiotics at sub-MIC doses. As a result, determining MIC is becoming increasingly critical in order to make the optimal option when administering antibacterials.

One technique to be used for this is a broth dilution assay, which combines a media, antibacterial chemicals, and the test microorganism (in this case, we will still work with *E.coli* and *S.aureus*). Turbidity typically determines the MIC (22).

The majority of the synthesised compounds displayed significant antibacterial activities that could be further investigated in clinical settings, according to Lei Shi et al's (2010) MIC assay for potent inhibitors of β-ketoacyl-acyl carrier protein synthase III (FabH) as potential antibacterial agents (16).

### 4.3. Structure-Activity Relationship (SAR)

In order to find amide-based next generation antibiotics for expanded clinical uses and bacterial resistance readiness, the structure-activity relationship (SAR) of the newly synthesized amide derivatives will be of considerable importance. SAR is regarded as the most crucial idea in drug development because it identifies precisely which positions on novel compounds can be modified to enhance their potency, *in vivo* efficacy, selectivity, and other properties while also distinguishing those positions from others where modifications are not possible (23).

SAR's primary objectives are to determine as precisely as possible the ranges of variation in compound structures that are consistent with the emergence of specific outcomes, such lethal endpoints. Secondly, it would describe the probable ways that modifications to the composition and general traits of the newly synthesized compounds might have an impact on endpoint potency (24).

Payne et al. (2002) used high throughput screening to determine the structure-activity relationship (SAR) of a few compounds from the GlaxoSmithKline compound collection. Following preliminary lead optimization studies, a few of these compounds were found to have increased potency against *S. aureus* and *E. coli* (25). Some of the structural changes we could make to the compounds are looking at the effects of the chain length, an ester versus carboxylic acid as well as ether versus phenol or what a longer ether chain could have as an effect.

## 5. Conclusion

The present study was focused on replicating novel antibacterial agents like that of platensimycin that would exhibit potency and promising inhibition against the bacterial strains; gram-negative and gram-positive bacteria that were used in the experiment. Three novel compounds were successfully synthesized, confirmed for chemical structure and tested

for their antibacterial activities using disc diffusion which showed a promising future in the fight against bacterial resistance to already existing antibacterial drugs. Even at the lowest dose of 1 μg/ml, these novel compounds inhibited Gram-negative bacteria, which has been demonstrated in recent literature to be highly resistant to practically all novel classes of antibacterial drugs. When compared to previous studies, it is clear that there has been a considerable advancement; nonetheless, additional research and testing are urged to further determine the readiness, efficacy, and potency of this novel compounds before it is moved to clinical and pre-clinical trials.

## Compliance with ethical standards

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*Disclosure of conflict of interest*

No conflict of interest to be disclosed.

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