doi.org/10.36721/PJPS.2023.36.2.REG.409-415.1

Arresting the biosynthesis of Lipid A to hinder *Escherichia coli* and *Pseudomonas aeruginosa* through fatty diglyceride

Hamdullah Khadim Sheikh[1*], Tanzila Arshad[1], Uzma Habib[2], Seyedeh Zahra Mirmohammadi[3], Rafia Usman[4] and Muhammad Mohtasheemul Hassan[1]

[1]Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Pakistan
[2]School of Interdisciplinary Engineering and Sciences (SINES), National University of Science and Technology (NUST), Pakistan
[3]Department of Chemistry, Payame Noor University, Urmia, Iran
[4]Department of Chemistry, NED University of Engineering and Technology Karachi, Karachi, Pakistan

Abstract: Lipid A is a fragment of lipopolysaccharide (LPS) in gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*; hence inhibition of its biosynthesis is one of the plausible ways of preventing such bacteria from growth and thus preventing gastrointestinal diseases caused by *Escherichia coli* and *pseudomonas aeruginosa*. This research revolves around the development of antibiotic glyceride derivatives for the inhibition of the biosynthesis of lipid A. To target the enzymes involved in the biosynthesis of lipid A, four N,N-dimethylaminobenzoate moiety containing fatty diglyceride derivatives were synthesized through a multi-step synthetic scheme starting from glycerol. The molecular structure of the targeted molecules and synthesized intermediates in the synthetic scheme were confirmed by detailed structural analysis through ^{1}N-NMR, mass and IR spectroscopic techniques. Antibacterial activity was evaluated against the gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The derivatives also underwent docking analysis on the pdb's of enzymatic catalysts involved in the biosynthesis of lipid A using AutoDock Vina package. All synthesized fatty esters gave good antibacterial activity and binding energy upto -7 kcal/mol in the docking analysis. A structure-property relationship was established between alkyl chain lengths of diglycerides and their resultant binding energies. These molecules and their resultant activity can assist in further designing and retrosynthesis of molecular derivatives of drug molecules with lipid A biosynthesis as target for its inhibition.

Keywords: Fatty acids, gram-negative bacteria, lipid a, molecular docking analysis, organic synthesis.

INTRODUCTION

Lipid A

Lipid A is a fragment of lipopolysaccharide (LPS) in gram-negative bacteria *Escherichia coli*, *Francisella Novicida*, *Pseudomonas Aeruginosa* etc (Brade *et al*., 2020). It works as a nonpolar anchor for the LPS bonded to the external membrane, hence, its presence is mandatory for the cell growth of gram-negative bacteria ((Sperandeo *et al*., 2017)). The property of immunogenicity in LPS depends upon the polysaccharide components in the structure while its toxicity is existent because of presence of lipid A moiety. Lipid A triggers the TLR4/MD activation and thus induces inflammation in mammalian immune system (Cochet and Peri, 2017). Lipid A itself is an endotoxin as well hence is a drug target.

The biosynthetic scheme or pathway for the development of lipid A is known as the *Raetz* pathway. This collective reaction scheme or pathway can serve as a target for antibiotic drug development as the development of lipid A is necessary for the cell growth of most of the gram-negative bacteria (Raetz *et al*., 2007). The Raetz pathway involves six enzymes i.e. Lpx A, Lpx C, Lpx D, Lpx H, Lpx B and Lpx K. All of these enzymes act as catalysts in the biosynthesis of Lipid A. Inhibitors of Lipid A are supposed to bind with these enzymes to inhibit the formation of intermediates required for the biosynthesis of Lipid A.

Inhibition of Lipid A

There has been interest in synthesis of inhibitors of lipid A biosynthetic enzymes (Austin *et al*., 1990). The designed molecules included -NH-OH (oxime) (Onishi *et al*., 1979) and threonyl hydroxamate derivatives (McClerren *et al*., 2005). Keeping LpxC catalytic enzyme as targets, bulky groups were attached to threonyl hydroxamate to enhance the interaction (Liang *et al*., 2013). Continuing the same structural variation theme, our designed molecules have glyceride part attached to the aniline derivative. In this research work, the performance of a partial fatty glyceride, in combination with an esterified aryl ring, is evaluated as bioactive molecule against gram negative bacteria (*Escherichia Coli* and *Pseudomonas Aeruginosa*). Fatty acids with 12, 14, 16 and 18 carbon atoms (9a-9d) were used to synthesize the glyceride part in the targeted molecules. The rest of the molecule, namely the esterified benzoic acid part, was the same for all molecules [See structures (11a-11d) and (14) in fig. 1]. We verified the synthesized molecules inhibition of Lipid A by antibacterial activity analysis and also by docking analysis of synthetic

*Corresponding author: e-mail: hamdullah.khadim.sheikh@gmail.com

molecule with enzymes. The perceived binding energy of the molecule was plotted on a graph (fig. 2) against the increasing length of the alkyl chain of the fatty acid glyceride part in molecules (11a-11d). This evaluated if the alkyl chain length variation affected the binding energy. Compound (14) was also formed with acetic acids (12) esterified at glycol of (8). This compound provided the evidence that the long alkyl chains of fatty acids are responsible for binding.

MATERIALS AND METHODS

All the reagents involved in the reaction used in the synthesis along with the solvents were procured from Sigma-Aldrich (USA). The solvents used in the synthesis were dried using standard purification techniques. The thin layer (TLC) chromatography technique was performed on Merck coated Si-gel 60 F_{254}, Al sheets (20 x 20 cm) to monitor the progress of reactions and the developed spots were visualized under the ultra-violet (UV) light at wavelengths of 254 and 365 nm. Docking was performed on AutoDock Vina software (version 1.2.0). IR spectra were recorded on Bruker ATR alpha spectrophotometer. The ^{1}H-NMR spectrum were obtained using the solvent $CDCl_3$ while trimethylsilane [$Si(CH_3)_4$] was used as an internal standard. The frequency of the ^{1}H-NMR Bruker spectrophotometer was set at 500 MHz. The high resolution (HR) ESI-MS spectra were run on the QSTAR LC/MS/MS mass spectrometer.

Synthesis

Compound (3), (7) and (8) were synthesized using methods described in the literature (Sheikh and Kazmi, 2017) (Scheme 1). For synthesis of (11a-11d) and (14), carbonyldiimidazole (CDI) (4) (8 mmol) and fatty acid (9a-9d) (8 mmol) were dissolved in tetrahydrofuran (THF) solvent. After 2 hours of stirring the reaction mass at 60^{o}C, compound (8) (2 mmol) was added along with triethylamine base (8 mmol). The reaction mixture was kept at 60-65^{o}C for 4 hours and continued at room temperature for next 20 hours. After completion of the reaction, solvent was evaporated and the product mass was poured into a solution of Na_2CO_3 (0.05 M). The obtained precipitate was filtered and washed multiple times with H_2O. The precipitate was extracted with solvent dichloromethane (DCM) and was further dried by addition of sodium sulfate Na_2SO_4. The subsequent evaporation of the solvent provided the targeted ester group containing product. Further purification of the resulting product (11a) was achieved by flash column chromatography technique (mobile phase was ethyl acetate: hexane). The same procedure was repeated for synthesis of (11b-11d) and (14) with acids (9b-9d) and (12) respectively. Spectroscopic data of the synthesized molecules is given below in table 1 (IR and mass spectroscopy) and table 2 (^{1}H-NMR) while labelled structures of (11a-11d) and (14) for ^{1}H-NMR are given in Scheme 1 in fig. 1.

Antibacterial activity

For antibacterial activity, the agar well diffusion procedure was utilized as described by (Arshad *et al*., 2018) against two gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). A mixture of 100µL of inoculums (106 CFU/mL) of test culture and 20mL of sterile tryptic soya agar was put into petri dishes in a sterile environment and was left for 30-40 minutes at 4^{o}C. After this, 0.1mL (two concentrations; 0.1 and 0.05%) of test solutions (11a-11d) and (14) were poured in made up holes of 6 mm diameter in each plate. Standard antibiotic gentamicin discs (10µg) were used as a positive antibacterial control while DMSO served as the negative control. The plates were then incubated at 37±1^{o}C for 24 hours, after which the inhibition zone around the wall was measured (mm) through a Vernier caliper.

Molecular docking analysis

For molecular docking study, AutoDock Vina tool was used (Eberhardt *et al*., 2021). Using the AutoDock tools, the .pdb structures of the derivatives (11a-11d) and (14), after the addition of H atoms and Kollaman charges, were converted into. pdbqt format which is required for the execution of the docking process on AutoDock Vina. For preparation of receptor, structures of enzymes Lpx A (PDB=2QIA), Lpx C (PDB=3P3G), Lpx D (PDB=6P89), Lpx H (PDB=5WLY), Lpx B (PDB=5W8X) and Lpx K (PDB=4EHX) were downloaded from Protein Data Bank (Berman, 2008) in .pdb format. Again, AutoDock tool was used to remove the H_2O molecules from the protein structures while the polar H atoms and Kollaman charges were added. These structures were saved as in. pdbqt format. Every enzymatic structure had specific grid box parameters.

RESULTS

Synthesis

IR and mass spectroscopic data of the synthesized molecules (11a-11d) is given in table 1 while the ^{1}H-NMR data is given in tabular form in table 2.

Antimicrobial activity

All synthesized compounds (11a-11d) and (14) were evaluated for their antibacterial activity against gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*). The standard used was Gentamycin (10 µg) while two concentrations 0.1 and 0.05% were taken for molecules (11a-11d) and (14) (table-3).

Molecular docking analysis (binding energy data)

Molecular docking result as binding energies of (11a-11d) and (14) against all six enzymes, which are responsible for the biosynthesis of lipid A, are listed in table 4. Docking score included H-bonding, the Van der Waals forces and π-stacking (Anslyn and Dougherty, 2004; Li *et al*., 2019). Further details of the interaction of compound

(11b) with the all six enzymes are listed in table 5. The results of the docking analysis are available at http://dx.doi.org/ 10.17632/42dpzcw8y3.1

DISCUSSION

Synthesis

The two OH groups of glycerol were partially protected through ketal formation in such a way that 1,2 diols were protected in form of 1,2-isopropylidene glycerol or solketal while one terminal -OH group remained available for further derivation in the reaction scheme (Hussain *et al*., 2004; Willberg-Keyriläinen and Ropponen, 2019). The second step involved its esterification with of 4-(dimethylamino) benzoic acid. For esterification, the -COOH of 4-(dimethylamino) benzoic acid was reacted with 1,1'-carbonyldiimidazole (CDI) to form (6) (Scheme 1 in fig. 1). Solketal (3) along with the triethyl amine $N(CH_2CH_3)_3$ was then added to the reaction media to obtain the product (7). The ketal ring of (7) was then deprotected in acidic media. 1,2 OH sites on (8) then formed esters with Lauric (9a), myristic (9b), palmitic (9c) and stearic acids (9d) by using carbonyldiimidazole (CDI) in step 2 of Scheme 1(fig. 1). This step activated the carboxylic acid towards acyl addition elimination reaction by incorporating the imidazole ring in place of OH of -COOH as the leaving group. Imidazole is a stable base hence it is a stable leaving group. This makes the C=O more reactive towards acyl addition-elimination by incoming nucleophile. Later esterification of (9a-9d) with (8) was also done via acyl imidazole intermediates (10a-10d) to yield diglyceride esters (11a-11d). To evaluate the impact of long fatty alkyl chains in (10a-10d), molecule (14) was also synthesized with acetic acids esterified at glycol of (8).

Molecular structures of the synthesized molecules (11a-11d) and (14) can be varied to manipulate the resultant properties of targeted molecules. This is possible because glycerides can be of various types with different fatty acids for esterification with (8). Fatty acids can be identical or they can structurally different. The length of alkyl chain of the fatty acid can be medium or long. The alkyl chain of the fatty acid can be saturated or unsaturated, polyunsaturated or conjugated, branched etc. The alkyl chain of the fatty acids may also carry secondary functional groups. There is also the option of causing positional isomerism in the resultant molecule with 1,3 and 1,2 diglycerides (Nitbani *et al*., 2020).

Antibacterial activity

All compounds showed excellent antibacterial activity against both bacteria at both concentrations (0.1 and 0.05 %). Among all of the five synthesized compounds, (11b) showed zone inhibition values close to the standard values (table 3). This shows that the diglyceride of the myristic acid is the most effective in inhibition of the biosynthesis of lipid A compared to the diglycerides of other fatty acids in the same series. This further confirms that reception of the synthesized molecules (11a-11d) at the enzymatic substrates and the resultant bioactivity are sensitive to the alkyl chain length of the esterified fatty acids. These compounds (11a-11d) and (14) showed excellent antibacterial activity as compared to the previously reported triglycerides (Arshad *et al*., 2018) and monoglycerides (Sheikh *et al*., 2018).

Molecular docking analysis

The docked adduct were visualized and analyzed through PyMol software. Since, we targeted enzymatic catalysts involved in the synthesis of Lipid A, interaction with these enzymes can inhibit their catalytic activity and hence Lipid A will be synthesized in lower amount. As all the synthesized compounds have shown excellent *in-vitro* antibacterial activity against gram-negative bacteria (*Escherichia Coli* and *Pseudomonas Aeruginosa*) (table 3), hence docking analysis was performed to analyze the binding energy and mode of interaction's dependence upon the alkyl chain length. The binding results (table 4) showed that, all compounds (11a-11d) and (14) have fair to good level of binding with all six enzymes and thus can inhibit them from taking part in synthesis of lipid A. The highest binding interaction was found to be against the Lpx-K enzyme with a binding energy value of -7 kcal/mol by compound (11b). All compounds (11a-11d) and (14) showed lowest binding energy with the lipid Lpx-A. Compound (14) showed highest interaction which was -4.7 kcal/mol while compound (11c) showed lowest interaction with -3.9 kcal/mol of binding energy with Lpx-A (table 4). Length of the alkyl chain of the fatty acids in the synthesized molecules and binding energy is plotted in fig. 3. Through the study of binding energy values and *in-vitro* antibacterial activity, we found that (11b) was the most active compound against gram-negative bacteria. This can be due to the fact that as in lipid A, the (11b) also have 14 carbon atoms fatty alkyl chain. Details of interactions of (11b) with the reception sites of all the enzymes used in the Raetz synthesis of Lipid A are given in table 5 while the 2D diagrams of binding are given in fig. 3. As can be seen in the details of interaction, the majority of the interactions responsible for binding energy are London dispersion forces which justifies the use of long alkyl chain fatty acid incorporation in the structure of derivatives. The aryl ring of benzoic acid, the $N(CH_3)_2$, C=O group also play their part in establishment of π-σ, π-π and π-alkyl interactions. As expected from more nonpolar character of the molecule, there are just few conventional and unconventional H-bonds formed.

CONCLUSION

We designed and through multistep synthetic scheme, synthesized the diglyceride (11a-11d) and diacetate (14) derivatives to bind with catalytic enzymes in the biosynthesis of Lipid A of *E. coli* and *Pseudomonas aeruginosa*.

Table 4: Spectroscopic data including IR and Mass spectra details of the synthesized molecules (11a-11d).

Compound	C=O (cm^{-1}) conjugated ester	C=C (cm^{-1}) Aromatic	C-O (cm^{-1}) ester	C-N (cm^{-1})	[M+H] (m/z)
11a	1695.7	1617.1	1284.8	1205.9	604.4
11b	1695.3	1616.8	1285.1	1207	660.5
11c	1697.6	1617.1	1292.9	1206.6	717.5
11d	1697.1	1617.6	1286.4	1205.1	772.6
14	1699.2	1623.1	1294.1	1227.8	324.1

Table 4: ^{1}H-NMR details of the synthesized molecules (11a-11d).

	ppm (Integral, Multiplicity, Coupling constant)									
S.No.	$C_{17}H_3$, $C_{17'}H_3$	$(CH_2)_{16}$, $(CH_2)_{16'}$	$C_{15}H_2$, $C_{15'}H_2$	$C_{14}H_2$, $C_{14'}H_2$	$C_{11}H_3$, $C_{12}H_3$	C_1H_2-O-	C_2H_2-O-	C_3H_2-O-	C_7H, C_9H	C_6H, $C_{10}H$
11a	0.91 (6H, t, 6.5 Hz)	1.86 (32H, m)	2.25 (4H, m)	2.56 (4H, m)	3.02 (6H, s)	3.99 (1H, m), 4.23(1H, m)	4.76 (1H, m)	4.41 (1H, m), 4.76 (1H, m)	6.82 (2H, d, 9 Hz)	7.67 (2H, d, 9 Hz)
11b	0.91 (6H, t, 6.5 Hz)	1.85 (40H, m)	2.15 (4H, m)	2.55 (4H, m)	3.02 (6H, s)	3.96 (1H, m), 4.39 (1H, m),	5.0 (1H, m)	4.26 (1H, m), 4.63 (1H)	6.77 (2H, d, 7.5 Hz)	7.58 (2H, d, 7.5Hz)
11c	0.91 (6H, t, 8 Hz)	1.81 (48H, m)	2.09(2H, m), 2.34 (2H, m)	2.51 (2H, m), 2.73(2H, m)	3.02 (6H, s)	4.03 (1H, m),m), 4.12 (1H, m),	4.98 (1H, m)	4.43(1H, m), 4.71 (1H)	6.79 (2H, d, 7.5 Hz)	7.62 (2H, d, 7.8Hz)
11d	0.91 (6H, t, 8 Hz)	1.96 (56H, m)	2.21(4H, m),	2.46 (2H, m)	3.02 (6H, s)	3.93 (1H, m),m), 4.20 (1H, m),	4.98 (1H, m)	4.57 (2H, ///m)	6.8 (2H, d, 7.5 Hz)	7.62 (2H, d, 7.8Hz)
14	2.05(3H, s) 2.11(3H, s)	-	-	-	3.02 (6H, s)	3.98(1H, m), 4.36 (1H, m)	4.95 (1H, m)	4.79 (2H, m),	6.72 (2H, d, 8.2 Hz)	7.74 (2H, d, 7.8Hz)

Table 4: Antimicrobial activity of compounds (11a-11d)

S. No	*Zone inhibition*					
	Escherichia Coli			*Pseudomonas Aeruginosa*		
	Mm			Mm		
Conc. %	0.1	0.05	Std.	0.1	0.05	Std.
11a	21	17.5	22	16.3	15	20
11b	22	22.7	23.5	18	18.6	19
11c	21	20.3	24	17.6	16.7	20
11d	17.3	11	22	14	9	20
14	21	19	22	18	17.3	20

Table 4: Binding energy evaluation data by docking of synthesized molecules with enzymes of synthesis of Lipid A

Enzyme	Lpx A	Lpx C	Lpx D	Lpx H	Lpx B	Lpx K
PDB code	2qia	3p3g	6p89	5wly	5w8x	4ehx
(resolution)	(1.7A)	(1.65A)	(1.48A)	(2.0a)	(1.98A)	(1.9A)
	Binding energy					
11a	-4	-5.7	-6	-5.2	-6	-6.6
11b	-4.1	-5.9	-6.2	-4.9	-6.3	-7
11c	-3.9	-5.2	-5.8	-4.4	-5.2	-6.8
11d	-4.2	-5.3	-6	-4.2	-5.7	-6.1
14	-4.7	-5.6	-5.5	-5.6	-5.5	-6.3

Table 5: Binding interactions of 11b with the reception sites of the enzymes Lpx A, B, C, D, H and K.

Receptor	H-Bond	C-H bond	π- σ	π-Alkyl	π- π T- Shaped	Alkyl
Lpx A	HIS A:144; SER A:146; THR A:128	ASP A:126		ARG A:255; ILE A:164; PHE A:162		
Lpx B		LEU A:306; ALA A:127; ASP A:310				LEU A:302; LEU A:328; TRP A:128; ALA A:303; ALA A:152; LYS A:156; PHE A:153; PHE A:149
Lpx C	LYS A:239; PHE A:194					ILE A:159; LYS A: 262; PHE A: 161; LEU A: 62; PYS A:63 LEU A: 18; ILE A: 198; CYS A: 207
Lpx D	GLN A: 32; MET A: 31; SER C: 258; ASP C: 221; ASN C: 240; LEU C: 220		ALA C:219			LYS C: 260; VAL C: 242; ILE C: 224; HIS C: 276; HIS C: 239; TYR A: 47; PHE A: 41; VAL A: 43
Lpx H	ARG A: 157	ARG A: 151; HIS A:195		CYS A:119		ALA A:158; ALA A:154; ALA A:178; ALA A: 182; LYS A:150
Lpx K	ARG A:206			VAL A:244	PHE A:296	PRO A: 279; LEU A:294; TYR A: 187; LYS A:156; LYS A:158 VAL A: 190; VAL A: 244

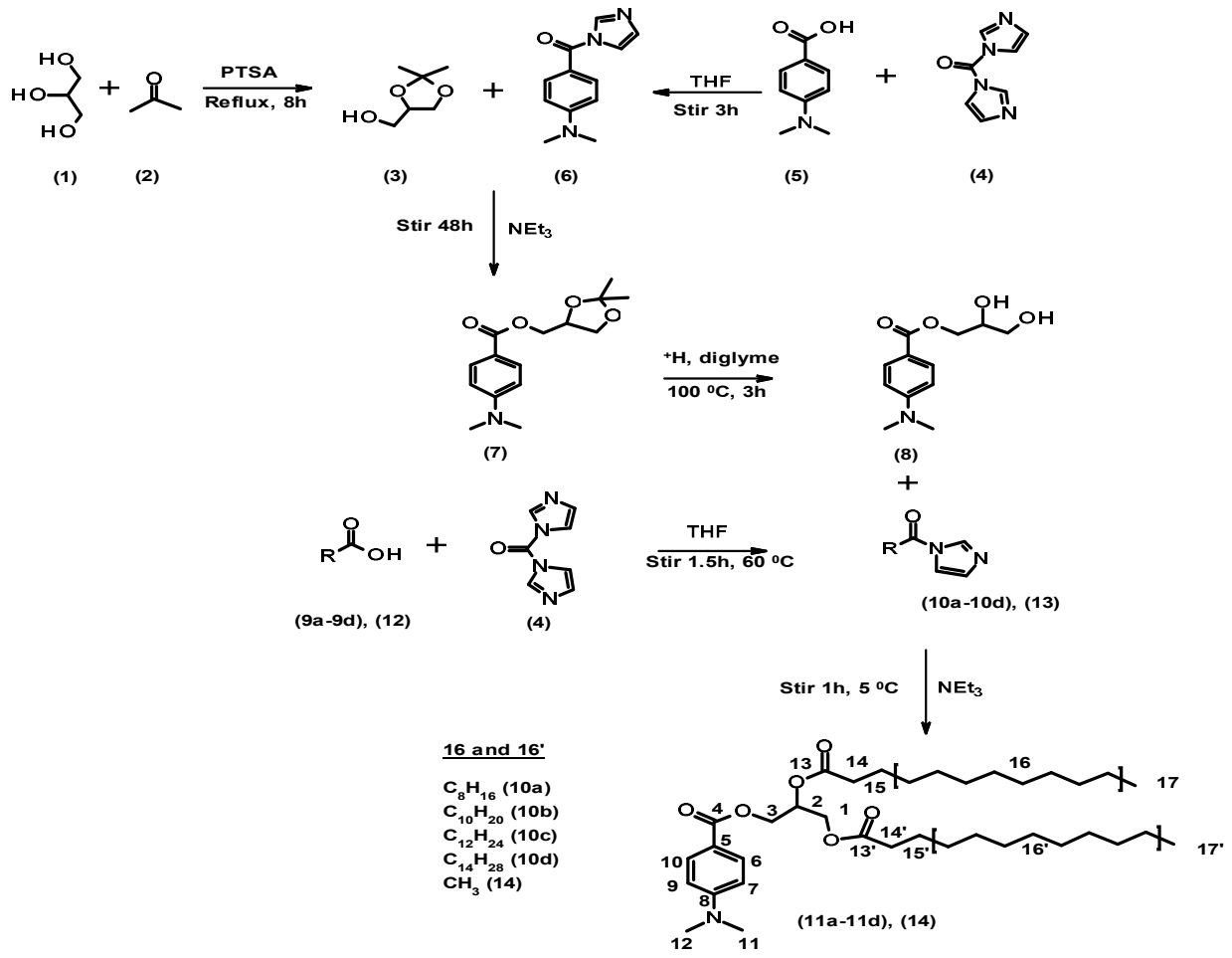

Fig. 2: Scheme 1: Synthesis of (11a-11d) and (14) from glycerol. Structure of (11a-11d) for ^{1}H-NMR is also given.

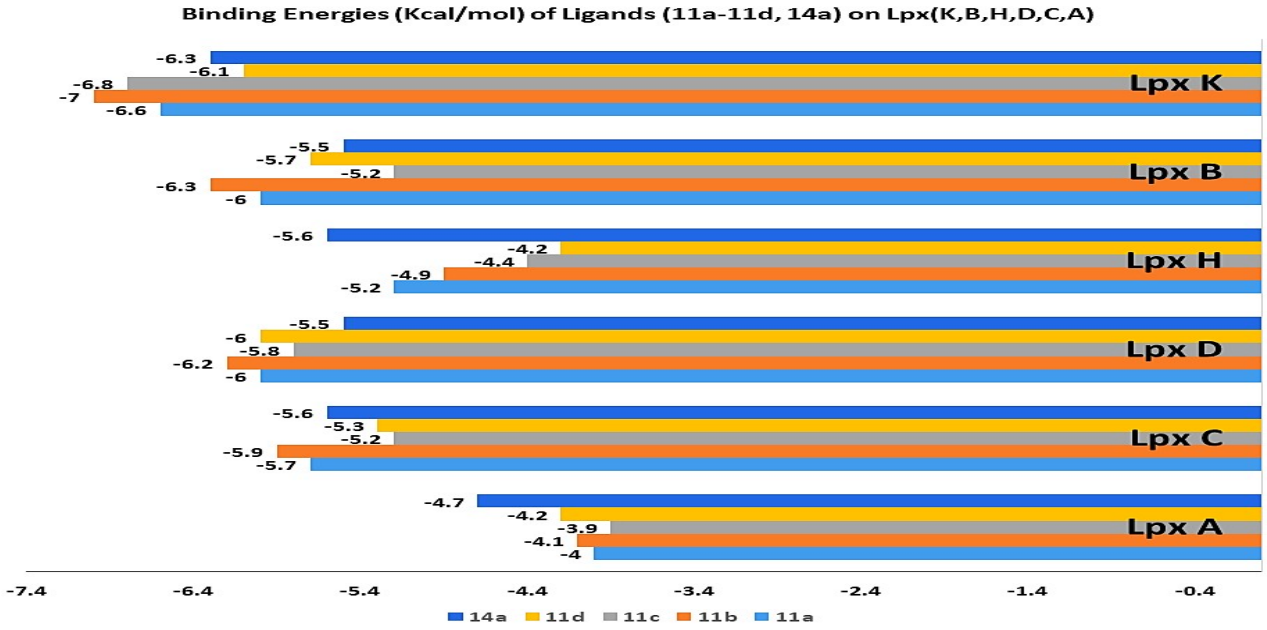

Fig. 2: Plot of binding energies against alkyl chain lengths of synthesized diglycerides (11a-11d) and (14a)

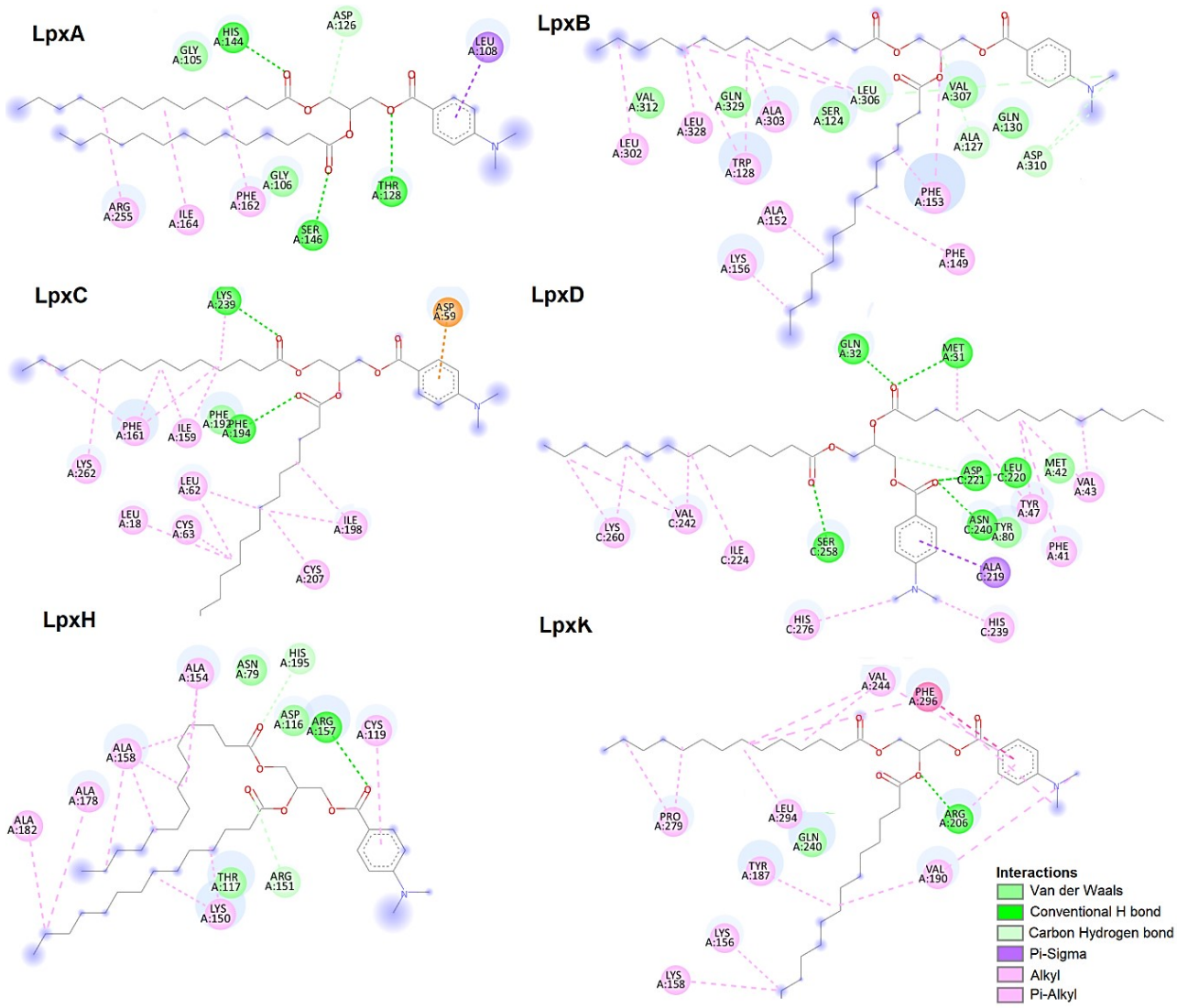

Fig 3: 2D diagrams of the binding interactions of molecule (11b) with substrates LpxA, B, C, D, H and K.

These structures were used for docking analysis with the enzymes involved in the biosynthesis of Lipid A. Synthesized molecules showed enhanced binding to the enzymatic catalysts in the biosynthesis of Lipid A compared to diacetate (14) because of the presence of two esterified fatty acid alkyl chains on edge of the synthesized diglyceride molecules.

This implies that the alkyl chains of the esterified fatty acids are responsible for binding with the substrate through induced dipole-induce dipole interaction. Molecules showed good *in vitro* anti-bacterial activity as well which can lead to further development of these derivatives into potential drug candidates.

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