UDC 547-327

NEW p-AMINODIPHENYLAMINE AMIDE COMPOUNDS: DESIGN, SYNTHESIS AND ANTI β-lACTAMASES ACTIVITY EVALUATION

Ahmed A. Saleh, Ahmed A. J. Mahmood*

Department of Pharmaceutical Chemistry, College of Pharmacy,
University of Mosul, Mosul, Iraq
e-mail:ahmedsot@uomosul.edu.iq

Received 03.09.2023
Accepted 11.11.2023

Abstract: *β-Lactams, such as penicillins and cephalosporins, have long been recognized as the most effective antibiotics for the treatment of infectious diseases. However, the major limitation to their effectiveness is the bacterial production of β-lactamase enzymes, which hydrolyze the β-lactam ring in these drugs, rendering them inactive. To overcome this resistance mechanism, β-lactamase inhibitors, such as clavulanic acid, are commonly used in combination with β-lactams. By inhibiting the action of β-lactamase enzymes, these inhibitors restore the efficacy of β-lactam antibiotics. In recent studies, researchers have employed docking techniques to investigate the interaction between β-lactamase enzymes and potential inhibitors. Specifically, the β-lactamases TEM-1 (1pzp) and IMP-1 (1JJE) were used as targets for designing new compounds. A series of novel compounds were generated by synthesizing 6 amides compounds as acid chloride derivatives and reacting them with p-aminodiphenylamine to form amide bonds. These compounds were then characterized by the use of various physical and spectroscopic methods to confirm their structures. Next, the synthesized compounds were subjected to biological testing to evaluate their efficacy against β-lactamase-producing Gram-positive and Gram-negative bacteria. This was accomplished by determining the minimum inhibitory concentration (MIC) of the compounds against three different strains of bacteria. Additionally, the possible anti β-lactamase activities of the compounds were compared to that of clavulanic acid. The results of this study revealed that five of the synthesized products exhibited effect similar to that of clavulanic acid for only one bacterial strain (Staph. aureus). Furthermore, the findings of the docking study suggest that the β-lactamase active pocket has a preference for hydrophobic substituents, as the synthesized products with these groups showed the highest binding score. In conclusion, the use of β-lactamase inhibitors, such as clavulanic acid, in combination with β-lactam antibiotics has proven effective in combating bacterial resistance. The development of novel compounds with anti β-lactamase activity holds promise for improving the treatment of infectious diseases. By understanding the preferences of the β-lactamase active pocket and designing compounds with hydrophobic substituents, researchers can enhance the affinity and efficacy of these inhibitors. This research contributes to the ongoing efforts to combat antibiotic resistance and improve patient outcomes in the field of infectious disease treatment.*

Keywords: *p-aminodiphenylamine, TEM-1 β-lactamase, IMP-1 β-lactamase, amides, docking, β-lactamase inhibitors.*

DOI: 10.32737/2221-8688-2024-1-20-32

Introduction

β-lactam antibiotics, the pioneering warriors in the battle against bacteria, continue to hold their ground as an exceptional class of antibiotics. Their remarkable ability to fight off bacterial infections while selectively targeting harmful microbes sets them apart. The rise of antibacterial resistance has now reached critical levels, posing a menacing threat to public health on a global scale [1]. Infections caused by drug-resistant bacteria have become a pressing concern, transcending borders and affecting populations worldwide. The emergence of genes encoding highly efficient β-lactamases, capable of breaking down β-lactam antibiotics, is on the

rise among both Gram-positive and Gram-negative bacteria. This is a troubling trend that demands urgent attention and action [2].

β-Lactamase production represents the most relevant mechanism of resistance mainly in Gram(-)ve [3]. For that reason, two strategies to overcome β- lactamase-mediated resistance are used: (a) the optimization of β- lactamase-stable antibiotics and (b) the development of selective β-lactamase inhibitors (BLIS) to be co-administered with a β-lactam antibiotics [4]. Even though a lot of efforts had been made for the development of new antibiotics to overcome drug-resistant [3]. Therefore, there is an urgent need to design and develop new antibiotics or anti β-lactamases to handle this situation [3,5].

Serine β-lactamases are enzymes belonging to the molecular classes A, C, and D, and they play a significant role in the resistance of bacteria to β-lactam antibiotics. These enzymes are responsible for the hydrolysis of the β-lactam ring, which is a crucial step in inactivating these antibiotics. The hydrolysis process involves the formation of a covalent acyl-intermediate between the catalytic serine residue and the β-lactam ring within the enzyme's active site. This covalent intermediate is essential for the efficient breakdown of the antibiotic molecule [6]. On the other hand, the metal-β-lactamases of molecular class B have an additional requirement for one or two zinc ions for their catalytic activity. These enzymes have a distinct mechanism, and their hydrolysis process involves a transition state that includes a zinc-stabilized hydroxide ion. The presence of zinc ions is crucial for the stability and function of these metallo-β-lactamases [7].

β-lactamase inhibitors play a crucial role in combating bacterial resistance to β-lactam antibiotics. These inhibitors are designed to target the active site of the β-lactamase enzyme, which is responsible for breaking down β-lactam antibiotics and rendering them ineffective. By co-administering β-lactamase inhibitors with β-lactam antibiotics, their combined action can enhance the effectiveness of the antibiotics by preventing the breakdown of the β-lactam ring, which is essential for their antibacterial activity [8]. However, it is important to note that the currently used β-lactamase inhibitors themselves have limitations. One such limitation is that these inhibitors contain the β-lactam ring, which makes them susceptible to degradation by β-lactamases. This means that they are subject to the same time-limited application as the antibiotics they are meant to enhance. As a result, the effectiveness of these inhibitors can be compromised over time, resulting in reduced efficacy in combating bacterial resistance. Another challenge in using β-lactamase inhibitors is the emergence of inhibitor-resistant β-lactamases (IRTs). Over time, the active site of β-lactamases can undergo mutations that allow them to bypass the binding of β-lactamase inhibitors. These inhibitor-resistant β-lactamases have evolved to confer resistance to all the currently marketed β-lactamase inhibitors, further worsening the problem of bacterial resistance [9].

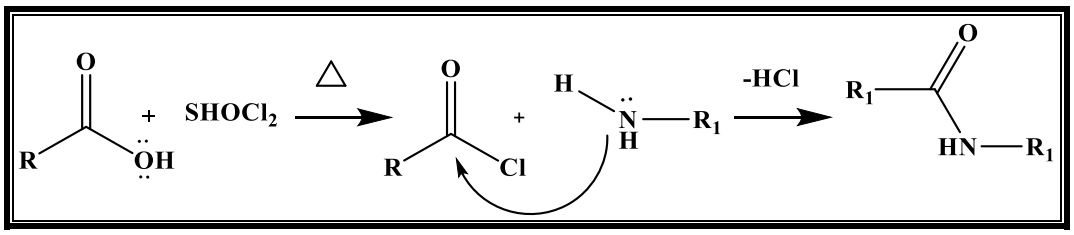
Promising approaches in combating β-lactamases, which are enzymes responsible for antibiotic resistance, involve the development of non-β-lactam inhibitors. These inhibitors have the potential to be effective against a wide range of different β-lactamases, providing a much-needed solution to fight against antibiotic-resistant bacteria [10]. One such group of inhibitors is the diazabicyclooctanes, including avibactam and MK-7655, which have shown activity against class A, C, and D β-lactamases. Additionally, derivatives of boronic acid have also demonstrated efficacy against class A, C, and D β-lactamases [11]. Another notable inhibitor, FTA (3-(4-Phenylamino-phenylamino)-2-(1H-tetrazol-5-yl)-acrylonitrile) was identified and found to bind to the allosteric site, which is distinct from the active site of the enzyme. This unique binding mechanism offers a potential advantage in preventing resistance. Furthermore, a new acylated phenoxyaniline compound has recently been described, exhibiting competitive and reversible inhibition of TEM-1 β-lactamase. This compound hypothetically binds in the vicinity of the enzyme's active site, further reaffirming its potential as an effective inhibitor. These various non-β-lactam inhibitors hold great promise in the battle against antibiotic resistance, offering new strategies to combat the spread of resistant bacteria and improve patient outcomes [12.13].

The aim of this study was to design (by docking) and synthesize a new non-β-lactam inhibitors derived from p-aminodiphenylamine nucleus then testing their anti β-lactamase inhibitory activity.

Materials and Methods

Docking Study: The docking was achieved using the online platform *Mcule* (https://mcule.com/ apps/1-click-docking/) [14]. The docking was made with two β-Lactamase, which are TEM-1 β- lactamase (1PZP) and the IMP-1 β-lactamase (1JJE) enzymes, in order to design an inhibitor for both families. The selection of the best compounds is dependent on the docking scores of the binding energies in both enzymes based on geometric shape complementarity.

Synthesis: In this study, all the used substances were acquired from reputable commercial markets ensuring their quality and reliability. The providers of these substances include "Fluka" from Switzerland, "Merck" from Germany, "Alpha" from India, and "Scharlau" from Spain. To determine the melting points of the substances, open capillaries were utilized. This method allows for accurate measurement of the temperature at which a solid substance transitions from solid to liquid state. By using open capillaries, any potential pressure build-up is mitigated, ensuring precise results. Furthermore, the FTIR spectra recorded using a PerkinElmer infrared spectrophotometer. In addition to FTIR, 1H NMR and 13C NMR spectra were recorded in DMSO-d6 using a Bruker Avance DPX 400 MHz spectrometer. The use of DMSO-d6 as the solvent makes it possible to dissolve the substances and facilitates their analysis. The Bruker Avance DPX 400 MHz spectrometer, with its high frequency and sensitivity, ensures precise and reliable NMR measurements. TMS, or Tetramethylsilane, is used as an internal reference in these NMR experiments to calibrate and normalize the spectra.



Preparation of acid chloride derivatives [15]: In the given experimental procedure, a specified amount of carboxylic acids, measuring 2-3 mmol, were dissolved in a solution containing 10-15 ml of thionyl chloride. The purpose of this step was to facilitate the reaction between the carboxylic acids and the thionyl chloride, which could result in the formation of a new compound. To ensure safety and prevent the release of harmful gases, the reaction mixture was heated under reflux for duration of 30 minutes in a properly ventilated hood. Refluxing involves heating the reaction mixture in a closed system, allowing the vapors to condense and return to the reaction vessel. This method helps to maintain a controlled temperature and prevents the loss of volatile components. Following the reflux step, the excess thionyl chloride was removed by evaporation. Thionyl chloride is a volatile and reactive compound, commonly used as a reagent for various organic transformations. Its removal ensured that only the desired products would be obtained for the subsequent step of the synthesis. The evaporation was achieved by heating the reaction mixture gently, allowing the thionyl chloride to vaporize and escape. This step required caution, as thionyl chloride is toxic and should be handled with care.

Preparation of amides products (Ph An 1-10) [16]: In a carefully controlled experiment, an appropriate acid chloride with a mole ratio of 2.5 X 10^{-3} was added dropwise to a mixture containing p-aminodiphenylamine and pyridine, both with mole ratios of 2.5 X 10^{-3} in 5 ml dichloromethane. This addition process was

carried out at a temperature of 0˚C to ensure optimal conditions for the reaction. The resulting mixture was then left undisturbed at room temperature overnight, allowing sufficient time for the reaction to proceed and the desired product to form. To purify the product, the solvent was evaporated, leaving behind the desired compound. To further eliminate any impurities or byproducts, the product was washed 2-3 times with cold ethanol, a process that effectively removes any unwanted substances. This meticulous purification step ensures that the final product is of high quality and free from any contaminants.

Biological study

Bacterial selection (*β*-lactamases detection): Acidimetric method was used to detect the β-lactamase in one Gram-positive (*Staphylococcus aureus*) and two Gram-negative (*Escherichia coli and Klebsiella pneumonia*,) pathogenic bacteria isolates [17]. Changing color from pink to yellow within 5 min indicates β-lactamase presence. At that (+)ve controls were run in parallel [18]. Furthermore these isolates were chosen to be sensitive to Augmentin and resistant to amoxicillin at one specific concentration, this is to ensure that the clavulanic acid in the Augmentin will inhibit the β-lactamases in those isolates and without it there will be no antibacterial activities for amoxicillin.

Minimum inhibitory concentration (MIC) determination [19]: In order to evaluate the minimum inhibitory concentration (MIC), a broth microdilution method was used. This method involved a sequential process of 10 doubling dilutions of the amides and a standard antibacterial agent, Amoxicillin. These dilutions were prepared in test tubes, with the initial concentration starting at 2000 μg/ml. To initiate the evaluation, bacterial isolates were added to the test tubes at a concentration of 5×10^5 CFU/ml, following the addition of 1 ml of Mueller-Hinton agar [20,21]. The test tubes were then incubated at a temperature of 37°C for duration of 18 hours.

It is important to note that Mueller-Hinton agar and bacterial isolates were solely used as the positive control, denoted as A (+)ve control. On the other hand, the negative control, denoted as (-)ve control, consisted solely of Mueller-Hinton agar. By applying this method, researchers were able to determine the MIC, which represents the lowest concentration of a substance required to inhibit the growth of bacteria. The MIC step was done to test the antibacterial activity for both the standard and the synthesized compounds. The MIC for the standard compound will specify the concentration at which the Augmentin will be active (sensitive) and certainly the amoxicillin will be resistance as we mention in the previous step. The MIC evaluation for the synthesized compounds assessed their antibacterial activities (if there is any), and to specify the concentration of these compounds that will be used in the next step. This concentration must be below their MIC and above the clavulanic acid MIC in the Augmentin. This is to ensure that the antibacterial activities of the amoxicillin that appeared in the next step will be caused by the inhibition of the β-lactamase by either the clavulanic acid or the synthesized compound after inoculation of each one with the amoxicillin.

Anti β-lactamase activities evaluation: In the study, the disk diffusion method was employed to assess the anti β-lactamase activity of the synthesized amides [22]. To conduct the experiment, each compound under investigation was utilized as a co-inhibitor along with 1000 μg of amoxicillin (equal to that of Augmentin) that was prepared as disks, with 5μl per disk. These disks containing the amoxicillin and the synthesized compound (in their sub MIC concentration) were then placed on Mueller-Hinton agar medium in a Petri dish, which had been previously inoculated with the bacterial strains to be tested using sterile cotton swabs. The entire setup was then incubated at a temperature of 37°C for duration of 24 hours. The zones of microbial growth that appeared around the disk were subsequently measured and recorded as the diameters of inhibition [23]. As a control, disks containing 1000 μg of amoxicillin alone (5μl/disk) were also prepared and included in the experiment. In order to dissolve the synthesized compounds, DMSO (dimethyl sulfoxide) was utilized as a solvent, ensuring a final concentration of less than 2% to ensure that it did not adversely impact bacterial growth. This precaution was taken to maintain

the integrity and accuracy of the experiment [22].

Results and discussion

Molecular docking study:

The docking for clavulanic acid, avibactam, tazobactam, sulbactam and for the designed amides was carried out on both β-lactamases TEM-1 (1pzp) and the IMP-1 (1JJE). The chemical structures of the designated amide substituents are listed in Table (1), while the result of docking is listed in Table (2).

Table 1. Designed structures of amide substituents

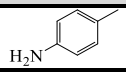
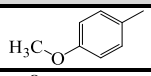
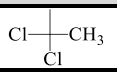
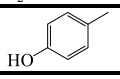
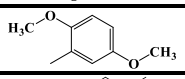
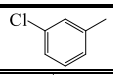
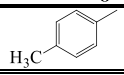
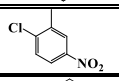
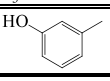
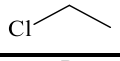
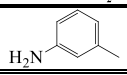
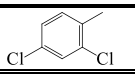
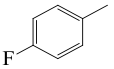
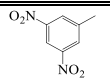
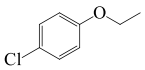
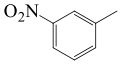
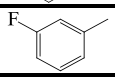
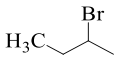
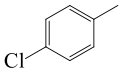
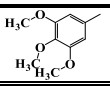
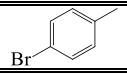
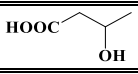
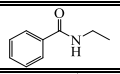
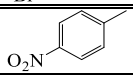
Item	Y	Item	Y	Item	Y
Y1		Y12		Y23	
Y2		Y13		Y24	
Y3		Y14		Y25	
Y4		Y15		Y26	
Y5		Y16		Y27	
Y6		Y17		Y28	
Y7		Y18		Y29	
Y8		Y19		Y30	
Y9		Y20		Y31	
Y10		Y21		Y32	
Y11		Y22		Y32	

Table 2. Docking study results for standard inhibitors and p-aminodiphenylamine amide compounds with TEM-1 β- lactamase (1PZP) and the IMP-1 β-lactamase (1JJE) enzymes (kcal/mol)

No.	Docking Score		No.	Docking Score		No.	Docking Score	
	1PZP	1JJE		1PZP	1JJE		1PZP	1JJE
Y1	-7.2	-8.0	Y12	-7.4	-7.6	Y23	-7.4	-7.6
Y2	-7.0	-7.7	Y13	-7.8	-8.6	Y24	-7.8	-8.6
Y3	-9.0	-8.1	Y14	-9.1	-8.3	Y25	-7.2	-8.0
Y4	-7.6	-9.4	Y15	-7.4	-8.0	Y26	-7.0	-7.7
Y5	-8.5	-7.0	Y16	-7.4	-8.4	Y27	-7.4	-7.6
Y6	-8.5	-9.1	Y17	-7.8	-7.0	Y28	-7.8	-8.6
Y7	-8.5	-7.0	Y18	-7.4	-8.0	Y29	-7.2	-8.0
Y8	-7.0	-7.7	Y19	-7.4	-8.4	Y30	-7.0	-7.7
Y9	-7.4	-8.0	Y20	-7.3	-7.9	Y31	-7.4	-7.6
Y10	-8.4	-9.4	Y21	-7.4	-8.0	Y32	-7.2	-8.0
Y11	-7.0	-10.0	Y22	-9.6	-8.1	Y33	-9.3	-10.7
sulbactam	-5.5	-5.3	Avibactam	-5.4	-7.6	tazobactam	-4.3	-6.4

Clavulanic acid	**-6.1**	**-6.1**						

The docking of the standard inhibitor was carried out not only to consider their score as reference control but also their binding poses used to define the active pocket site amino acids for both enzymes. The amino acids (LEU 196, ALA 199, IEU 200, GLY211, ALA253 and GLY 256) were bound to the most of standard inhibitors in the β-lactamases TEM-1 (1pzp) [24]. While the amino acids (VAL 25, TRP 28, PHE 51, HIS 79, SER 80, ASP 81 and ASN 167) were bound to the most of standard inhibitors in the β-lactamases IMP-1 (1JJE) [25]. The above 6 and 7 amino acids will define the active binding site for the attachment with this both enzymes respectively.

Concerning the interaction of the tested compounds with enzymes (Table 2), we found that 6 compounds with substituents (Y3, Y6, Y11, Y14, Y22 and Y33) are bound to 4-5 out of the 6 amino acids and also 4-6 out of 7 amino acids that defined the binding site of both β-lactamases TEM-1 (1pzp) and the IMP-1 (1JJE) respectively. In addition to 3-5 additional amino acids for each compound, those used to potentiate the interaction with both enzymes (Fig.1). The findings of the docking study suggest that the β-lactamase active pocket has a preference for hydrophobic substituents, as the compounds with these groups showed the highest binding score.

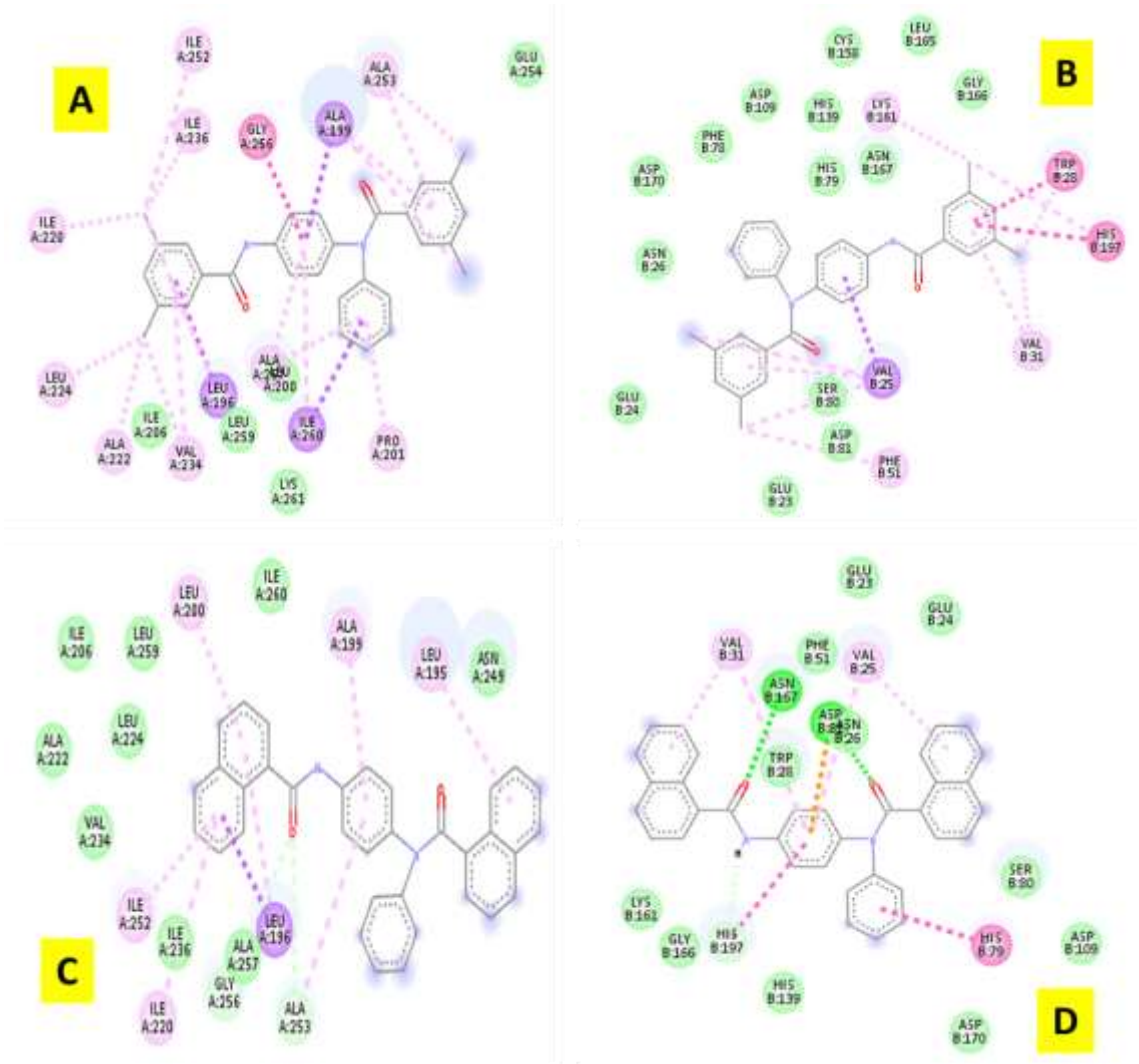


Fig. 1. 2D chemical structure for selected Y22 (A and B) Y33 (C and D) with the β-lactamases TEM-1 (1pzp) and the IMP-1 (1JJE) respectively.

Chemical study results:

The physical properties of the synthesized amides derivatives are listed in the Table (3).

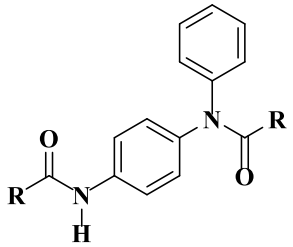
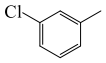
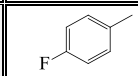
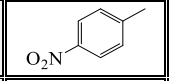
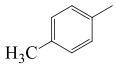


Table 3. Physical properties and the most characteristic peaks (ν_{cm-1}) of the FT-IR spectrum for the p-aminodiphenylamine amide compounds (Pa Am 1-6).

Compd. No.	R	M wt.	m.p(°C)	Color	Yield%	N-H amide	C-H	C=O amide	other
Pa Am 1		461.34	185-187	black	85	w 3322	w 3026	S 1654	C-Cl m 737
Pa Am 2		428.44	184-187	green	87	w 3367	w 3060	S 1643	C-F m 835
Pa Am 3		482.45	218-221	green	83	w 3260	w 3046	S 1645	N-O m 1418
Pa Am 4		420.51	189-202	gray	80	w 3352	w 3032	S 1650	C-H W 2943
Pa Am 5		448.57	196-198	gray	84	w 3282	w 3042	S 1667	C-H W 2914
Pa Am 6		492.58	133-136	Dark green	88	w 3400	w 3044	S 1651	----------

The FTIR indicate the formation of the amide bonds, the disappearance of the N-H peaks at 3456 cm^{-1} and 3375 cm^{-1} of the 1st and 2nd amine of the p-aminodiphenylamine respectively and replaced by the amide N-H at 3260-3400 cm^{-1} and amide C=O at 1643-1667 cm^{-1} conforming the amides formations. In addition to other peaks for each compound that belongs to the substitutions of the acid chlorides used (Table 3).

Chemical names and spectral characterization of compounds Pa Am1- Pa Am6

Pa Am1 [3-chloro-N-(4-(3-chlorobenzamido)phenyl)-N-phenylbenzamide]. **The ^{1}H NMR of Pa Am1** (δ, ppm) (DMSO-d6): 9.93 (s, 1H, N 14), 8.00 (m, 3H, C 21,30), 7.90 (m, 1H, C 25,26), 7.64-7.73 (m, 4H, C 9,10,12,13), 7.54-7.60 (m, 4H, C 23,24,27,28), 7.38 (d, 2H, C 2,6), 5.11 (d, 2H, C 3,5), 7.03 (d, 1H, C 4). **The ^{13}C NMR of Pa Am1** (δ, ppm) (DMSO-d6): 168.16 (C18), 166.34 (C15), 142.43 (C1), 137.94 (C8), 135.42 (C11), 133.76 (C17,20), 133.19 (C22,29), 131.14 (C23,24,28), 130.97 (C27), 129.30 (C3,5), 128.39 (C21,30), 127.89 (C25), 127.79 (C2,4,6), 126.86 (C9,13,26), 121.71 (C10,12).

Pa Am2 [4-fluoro-N-(4-(4-fluorobenzamido)phenyl)-N-phenylbenzamide]. **The ^{1}H NMR of Pa Am2** (δ, ppm) (DMSO-d6): 10.21 (s, 1H, N 14), 8.07 (m, 2H, C 26,30), 7.89 (m, 2H, C 21,25), 7.84 (dt, 2H, C 10,12), 7.58 (dt, 2H, C 9,13), 7.36-7.43 (m, 5H, C 2,3,4,5,6), 7.28 (m, 4H, C 22,24,27,29). **The ^{13}C NMR of Pa Am2** (δ, ppm) (DMSO-d6): 168.73 (C18), 166.42 (C15), 165.66 (C28), 163.59 (C23), 142.59 (C1), 137.91 (C8), 135.41 (C11), 131.66 (C21,25), 131.29 (C20), 131.15 (C17), 130.15 (C26,30), 129.30 (C3,5), 127.38 (C2,6), 127.18 (C4), 126.55 (C9,13), 121.92 (10,12), 116.17 (C22,24), 115.79 (C27,29).

Pa Am3 [4-nitro-N-(4-(4-nitrobenzamido)phenyl)-N-phenylbenzamide].

The ^{1}H NMR of Pa Am3 (δ, ppm) (DMSO-d6): 10.19 (s, 1H, N 14), 7.86 (d, 2H, C 21,25), 7.73 (d, 2H, C 26,30), 7.34 (m, 6H, C 2,6,10,12,29), 7.17-7.25 (m, 6H, C 3,5,9,13,27), 7.07 (d, 2H, C 22,24), 2.54 (s, 1H, C 4), 2.40 (s, 3H, C 32), 2.24 (s, 3H, C 31). **The ^{13}C NMR of Pa Am3** (δ, ppm) (DMSO-d6): 170.17 (C18), 165.82 (C15), 144.43 (C1), 142.15 (C23), 140.16 (C28), 139.63 (C8), 137.92 (C11), 133.99 (C20), 132.37 (C17), 129.52 (C22,24), 129.39 (C27,29), 129.21 (C26,30), 128.88 (C3,5), 128.49 (C21,25), 128.15 (C2,6), 127.88 (4), 126.68 (C9,13), 121.28 (C10,12), 21.49 (C31), 21.36 (C32).

Pa Am4 [4-methyl-N-(4-(4-methylbenzamido)phenyl)-N-phenylbenzamide]. **The ^{1}H NMR of Pa Am4** (δ, ppm) (DMSO-d6): 10.70 (s, 1H, N 14), 8.14 (m, 2H, C 27,29), 8.04 (m, 2H, C 22,24), 7.85 (m, 2H, C 26,30), 7.75 (m, 2H, C 21,25), 7.53 (m, 2H, C 10,12), 7.46 (m, 2H, C 9,13), 7.22-7.33 (m, 4H, C 2,3,5,6), 2.50 (t, 1H, C 4). **The ^{13}C NMR of Pa Am4** (δ, ppm) (DMSO-d6): 166.40 (C18), 164.62 (C15), 146.90 (C28), 145.72 (C23), 142.60 (C1), 138.30 (C20), 137.86 (C17), 134.71 (C8), 133.16 (C11), 132.80 (C21,25), 130.70 (C26,30), 129.79 (C3,5), 129.55 (C2,6), 128.60 (C4), 127.40 (C9,13), 126.91 (22,24), 124.80 (C27,29), 120.61 (C10,12).

Pa Am5 [N-(4-(3,5-dimethylbenzamido)phenyl)-3,5- -N- dimethyl phenylbenzamide]. **The ^{1}H NMR of Pa Am5** (δ, ppm) (DMSO-d6): 10.27 (s, 1H, N 14), 7.85 (dt, 2H, C 10,12), 7.67 (m, 4H, C 21,25,26,30), 7.57 (dt, 2H, C 9,13), 7.36-7.43 (m, 5H, C 2,3,4,5,6), 7.09 (dt, 2H, C 23,28), 2.29 (s, 4H, C 31,32,33,34). **The ^{13}C NMR of Pa Am5** (δ, ppm) (DMSO-d6): 168.45 (C18), 166.39 (C15), 142.59 (C1), 137.91 (C8), 137.06 (C22,24,27,29), 135.41 (C11), 134.19 (C20), 133.65 (C17), 133.46 (C23,28), 129.30 (C3,5), 128.47 (C21,25), 127.53 (C26,30), 127.38 (C2,6), 127.18 (C4), 126.55 (C9,13), 121.92 (10,12), 20.91 (C31,32,33,34).

Pa Am6 [N-(4-(1-naphthamido)phenyl)-N-phenyl-1-naphthamide]. **The ^{1}H NMR of Pa Am6** (δ, ppm) (DMSO-d6): 10.35 (s, 1H, N 14), 8.97 (d, 1H, C 26), 8.657 (d, 4H, C 25,29,35,38), 8.40 (d, 3H, C 10,12,34), 8.16 (d, 3H, C 23,24,32), 8.05 (m, 1H, C 33), 7.79 (t, 3H, C 13,27,36), 7.73 (dt, 5H, C 2,6,9,28,37), 7.69 (m, 3H, C 3,4,5). **The ^{13}C NMR of Pa Am6** (δ, ppm) (DMSO-d6): 169.11 (C15,18), 133.95 (C1,8,11), 133.42 (C20,30,31), 131.16 (C17,21,22), 130.34 (C23,25), 129.09 (C3,32,34,38), 128.17 (C5,27,29,36), 128.04 (C2,4,6,37), 126.67 (C9,13,24,28), 125.97 (C26,33,35), 125.36 (C10,12).

Biological results:

The minimum inhibitory concentration (MIC) results show that the augmentin is a reasonable activity against all bacterial strains at 2000 μg/ml concentration, and amoxicillin at 2000 μg/ml has antibacterial activity against *Staph. aureus* only, while the tested amides show no antibacterial activity by all concentration used in the three bacterial strains. The MIC study was performed for the synthesized amides in order to ensure that when they are co-administered with the amoxicillin as anti β-lactamase they will have no antibacterial activity as we intend to use their sub-MIC, and the appeared activity will be due to the amoxicillin by the aid of the amide compounds that will mask and inhibit the β-lactamase enzyme. From the MIC study, two concentrations of 800 μg and 1600 μg were chosen for the synthesized compounds in order to test their anti β-lactamase activity as compared to 200 μg of the clavulanic acid in the augmentin.

In the next step the anti β-lactamase activity of the synthesized compounds against human pathogenic bacterial isolates was evaluated [19]. Each tested compound was used as co-inhibiter with 1000 μg of amoxicillin prepared as disks (5μl/disk) at 800 μg and 1600 μg concentrations.

Amoxicillin 1000 μg alone and augmentin (1000/200mg) were incubated as well, so the results would be considered as a control for the synthesized compounds (Table 4).

Table 4. Inhibition zones for synthesized compounds as co-inhibitors with amoxicillin against Gram-positive and Gram-negative pathogenic bacteria.

Com. No.	Inhibition zone diameter (mm)						Com. No.	Inhibition zone diameter (mm)					
	Gram(+)ve		Gram (-)ve					Gram(+)ve		Gram (-)ve			
	Staph. aueus		*E. coli*		*K. pneumonia*			*Staph. aueus*		*E. coli*		*K. pneumonia*	
	1:1	1:2	1:1	1:2	1:1	1:2		1:1	1:2	1:1	1:2	1:1	1:2
Augmentin	18	18	21	22	20	22	Pa Am 3	18	19	0	0	0	0
Amoxicillin	9	10	0	0	0	0	Pa Am 4	0	0	0	0	0	0
Pa Am 1	14	14	0	0	0	0	Pa Am 5	10	11	0	0	0	0
Pa Am 2	12	13	0	0	0	0	Pa Am 6	18	17	0	0	0	0

1:1 = 1000 μg/ml Amoxicillin : 800 μg/ml synthesized compound
1:2 = 1000 μg/ml Amoxicillin : 1600 μg/ml synthesized compound

In general, the results represent that all the synthesized compounds had no activity as anti β-lactamase against E. coli and P. aeruginosa indicating that the synthesized compounds did not inhibit the β-lactamases in these strains. This observation may be related to enzymes model in the docking study, which defers from those existed in the tested bacterial strains used, or even they were from different families.

Concerning the *Staph. aureus* bacteria, five of the synthesized compounds showed promising activities, although all of them have no antibacterial activities when used alone. All these compound, in addition to halogen atoms or nitro group, have one or more hydrophobic residue in its structure. This is coming true with the docking results (https://mcule.com/ apps/1-click-docking/) and with other papers [26]. The activity of the synthesized compounds against the Gram (+) ve *Staph. aureus* bacteria could be related to many mechanisms, and we cannot certain that the activity is due to inhibition of the β-lactamases in this strain. More specific studies on bacterial strain bearing β-lactamases enzymes mimic those used in the docking study must be performed in order to confirm such activity. Furthermore the result of this study is not very much encouraging, but still it is a part of ongoing project that utilize many scaffold to test their β-lactamase inhibitory activity hoping to find a universal inhibitor that exceed the clavulanic acids or its group.

Conclusion

The use of β-lactamase inhibitors, such as clavulanic acid, in combination with β-lactam antibiotics, has proven effective in combating bacterial resistance. The development of novel compounds with anti β-lactamase activity holds promise for improving the treatment of infectious diseases. By understanding the preferences of the β-lactamase active pocket and designing compounds with hydrophobic substituents, researchers can enhance the affinity and efficacy of these inhibitors. The current research contributes to the ongoing efforts to combat antibiotic resistance and improve patient outcomes in the field of infectious disease treatment. More specific studies on bacterial strain bearing β-lactamases enzymes must be performed in order to confirm such activity.

References

1. Olsen, O. New promising b-lactamase inhibitors for clinical use. *Eur. J. Clin. Microbiol. Infect Dis.* 2015, vol. 34(7), pp. 1303–1308. doi: 10.1007/s10096-015-2375-0.
2. King, D.T., Sobhanifar, S., and Strynadka, N.C.J. One ring to rule them all: current trends in combating bacterial resistance to the b-lactams. *Protein Sci.* 2016, vol. 25, pp. 787–803. doi: 10.1002/pro.2889.
3. Sakurai Y, Yoshida Y, Saitoh K, Nemoto M, Yamaguchi A, Sawai T. 1990. Characteristics of aztreonam as a substrate, inhibitor and inducer for β-lactamases. *J Antibiot* (Tokyo). 1990, vol. 43, pp. 403–410. doi: 10.7164/antibiotics.
4. Araoka, H., Baba, M., Tateda, K., Ishii, Y., Oguri, T., Okuzumi, K., Oishi, T., Mori, S., Mitsuda, T., Moriya, K., et al. Monobactam and aminoglycoside combination therapy against metallo-b-lactamase-producing multidrug resistant Pseudomonas aeruginosa screened using a 'break-point checkerboard plate'. *Scand J Infect Dis*. 2010, vol. 42(3), pp. 231-3. doi: 10.3109/00365540903443157.
5. Rongfeng Li, Ryan A. O., and Craig A. T. Identification and Characterization of the Sulfazecin Monobactam Biosynthetic Gene Cluster. *Cell Chemical Biology.* 2017 vol. 24(1), pp. 24-34. doi: 10.1016/j.chembiol.2016.11.010
6. Grigorenko V.G., Andreeva I.P., Rubtsova M.Yu., Deygen I.M., Antipin R.L., Majouga A.G., Egorov A.M., Beshnova D.A., Kallio J., Hackenberg C., Lamzin V.S. Novel non-β-lactam inhibitor of β-lactamase TEM-171 based on acylated phenoxyaniline, *Biochimie.* 2017, vol. 132, pp. 45-53, https://doi.org/10.1016/j.biochi.2016.10.011.
7. Adnan A. Zainal, Musab Mohammed Khalaf, Ahmed A. J. Mahmood. Cholinesterase activity in non-alcoholic-fatty liver disease in diabetic patients taking oral antidiabetic drugs. *Current Topics in Pharmacology*, 2021, vol. 25, pp. 43–51.
8. Baquero F., Tedim A.P., Coque T.M. Antibiotic resistance shaping multi-level population biology of bacteria. *Front. Microbiol.*, 2013, vol. 4 (15), pp. 1-15.
9. Tang S., Apisarnthanarak A., Hsu L.Y. Mechanisms of β-lactam antimicrobial resistance and epidemiology of major community-and healthcare-associated multidrug-resistant bacteria. *Adv. Drug Deliv. Rev.*, 2014, vol. 78, pp. 3-13. doi: 101016/jaddr201408003
10. Mahmood, A.A.J., Khalaf, M.M., Zainal, A.A. Cholinesterases activities in diabetic and hyperlipidemic patient. Indian Journal of Public Health Research and Development, 2019, vol. 10(10), pp. 2812–2816. DOI:10.5958/0976-5506.2019.03297.2
11. Pimenta A.C., Fernandes R., Moreira I.S. Evolution of drug resistance: insight on TEM β-lactamases structure and activity and β-lactam antibiotics. *Mini Rev. Med. Chem.*, 2014, vol. 14, pp. 111-122. DOI: 10.2174/1389557514666140123145809
12. Bujor, A.; Hanganu, A.; Tecuceanu, V.; Madalan, A.M.; Tudose, M.; Marutescu, L.; Popa, M.; Chifiriuc, C.M.; Zarafu, I.; Ionita, P. Biological Evaluation and Structural Analysis of Some Aminodiphenylamine Derivatives. *Antioxidants*, 2023, vol. 12, p. 713. https://doi.org/10.3390/antiox12030713
13. Ehmann D.E., Jahic H., Ross P.L., Gu R.F., Hu J., Kern G., Walkup G.K, Fisher S.L. Avibactam is a covalent, reversible, non–β-lactam β-lactamase inhibitor. *PNAS*, 2012, vol. 109(29), pp. 11663-11668. doi: 10.1073/pnas.1205073109.
14. Mahmood, A.A.J., Al-Iraqi, M.A., Abachi, F.T. Design, synthesis and anti-ß-lactamase activity for new monobactam Compounds. *Materials Today: Proceedings*, 2021, vol. 42(3), pp. 1860–1866. DOI:10.1016/j.matpr.2020.12.218
15. Alwan S.M. Computational Calculations of Molecular Properties and Molecular Docking of New and Reference Cephalosporins on Penicillin Binding Proteins and Various β-Lactamases. *Journal of Pharmacy and Pharmacology*. 2016, vol. 4(5), pp. 212-225.

DOI:10.17265/2328-2150/2016.05.004
16. Rafiq Z., Saranya S. and Vaidyanathan R. Computational Docking and *In Silico* Analysis Of Potential Efflux Pump Inhibitor Punigratane. *International Journal Of Pharmacy And Pharmaceutical Sciences. 2018*, vol 10, issue 3, p. 27. DOI:10.22159/ijpps.2018v10i3.21629
17. Bidya, S. and Suman, R.S. Comparative Study of Three β Lactamase Test Methods in Staphylococcus aureus Isolated from Two Nepalese Hospitals. *Open Journal of Clinical Diagnostics.* 2014, vol. 4, pp. 47-52. doi.org/10.4236/ojcd.2014.41009
18. Mahmood, A.A.J., Al-Iraqi, M.A., Abachi, F.T. New 4, 4'-Methylenedianiline Monobactame Compounds: Synthesis, Antioxidant and Antimicrobial Activities Evaluation. *AIP Conference Proceedings*, 2022, 2660, 020024
19. Kowalska-Krochmal B, Dudek-Wicher R. The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. *Pathogens.* 2021, vol. 10(2), p.165. doi: 10.3390/pathogens10020165. PMID: 33557078; PMCID: PMC7913839.
20. Sperling D., Karembe H., Zouharova M., Nedbalcova K. Examination of the minimum inhibitory concentration of amoxicillin and marbofloxacin against Streptococcus suis using standardised methods. *Vet Med - Czech*, 2020, vol. 65(9), pp. 377-386. DOI: 10.17221/111/2020-VETMED
21. Mahmood, A.A.J. Synthesis, antioxidant and antimicrobial activities for new 4,4'-methylenedianiline amide compounds. *Egyptian Journal of Chemistry*, 2021, vol. 64(12), pp. 6999–7005. DOI: 10.21608/EJCHEM.2021.80123.3949
22. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal*. 2016, vol. 6(2), pp. 71-79. doi: 10.1016/j.jpha.2015.11.005.
23. Arun K., Lalit Sharma, and Monika Kaura. Synthesis And Comparative Antimicrobial Study Of Beta-Lactam Derivatives. *International Journal Of Pharmaceutical And Chemical Sciences*. 2013, vol. 2 (2), pp. 560-565.
24. Hellemann E, Nallathambi A, Durrant JD. Allosteric inhibition of TEM-1 β lactamase: Microsecond molecular dynamics simulations provide mechanistic insights. *Protein Sci*. 2023, vol. 32(4):e4622. doi: 10.1002/pro.4622.
25. Drawz SM, Bonomo RA. Three decades of beta-lactamase inhibitors. *Clin Microbiol Rev*. 2010, vol. 23(1), pp. 160-201. doi: 10.1128/CMR.00037-09.
26. Avci F.G., Altinisik F.E., Vardar Ulu D., Ozkirimli O. E., Akbulut B. S..(2016). An evolutionarily conserved allosteric site modulates beta-lactamase activity, *J. Enzyme Inhib. Med. Chem.* 2016, vol. 31, pp. 33–40. DOI:10.1080/14756366.2016.1201813.

p-AMİNODİFENİLAMİN AMİDİN YENİ BİRLƏŞMƏLƏRİ: DİZAYNI, SİNTEZİ VƏ ANTİ β –LAKTAMAZ AKTİVLİYİNİN QİYMƏTLƏNDİRİLMƏSİ

Əhməd A. Saleh, Əhməd A. J. Mahmud*

Əczaçılıq Kolleci, Əczaçılıq Kimyası Kafedrası,
Mosul Universiteti, Mosul, İran
e-mail:ahmedsot@uomosul.edu.iq

Xülasə: Penisilinlər və sefalosporinlər kimi β-laktamlar da uzun müddət yoluxucu xəstəliklərin müalicəsi üçün ən təsirli antibiotiklər kimi tanınıb. Bununla belə, onların effektivliyinə əsas məhdudiyyət, bu dərmanların tərkibindəki β-laktam halqasını hidroliz edərək onları qeyri-aktiv edən β-laktamaza fermentlərinin bakteriya istehsalıdır. Bu müqavimət mexanizmini aradan qaldırmaq üçün β-laktamaza inhibitorları, məsələn, klavulan turşusu, β-laktamlarla birlikdə istifadə

olunur. β-laktamaza fermentlərinin təsirini əngəlləməklə, bu inhibitorlar β-laktam antibiotiklərinin effektivliyini bərpa edirlər. Son tədqiqatlarda tədqiqatçılar β-laktamaza fermentləri və potensial inhibitorlar arasındakı qarşılıqlı əlaqəni araşdırmaq üçün dokinq üsullarından istifadə etmişlər. Xüsusilə, β-laktamazlar TEM-1 (1pzp) və IMP-1 (1JJE) yeni birləşmələrin dizaynı üçün hədəf kimi istifadə edilmişdir. 6 amid birləşməsini xlor anhidrid törəmələri kimi sintez edərək və amid rabitələri yaratmaq üçün p-aminodifenilaminlə reaksiyaya girərək bir sıra yeni birləşmələr yaradılmışdır. Bu birləşmələr daha sonra strukturlarını təsdiqləmək üçün müxtəlif fiziki və spektroskopik üsullardan istifadə etməklə xarakterizə edilmişdir. Sonra sintez edilmiş birləşmələr β-laktamaza istehsal edən qram-müsbət və qram-mənfi bakteriyalara qarşı effektivliyini qiymətləndirmək üçün bioloji sınaqdan keçirilmişdir. Bu, üç müxtəlif bakteriya ştammına qarşı birləşmələrin minimum inhibitor qatılığını (MİQ) təyin etməklə həyata keçirilmişdir. Bundan əlavə, birləşmələrin mümkün anti β-laktamaz fəaliyyəti klavulan turşusu ilə müqayisə edilmişdir. Bu tədqiqatın nəticələri, sintez edilmiş məhsullardan beşinin yalnız bir bakteriya ştammı (Staph. aureus) üçün klavulan turşusuna bənzər təsir göstərdiyini ortaya qoymuşdur. Bundan əlavə, dokinq tədqiqatının nəticələri göstərir ki, β-laktamaz aktiv hidrofobik əvəzedicilərə üstünlük verir, çünki bu qruplarla sintez edilmiş məhsullar ən yüksək rabitə göstərmişdir. Yekun olaraq, klavulan turşusu kimi β-laktamaza inhibitorlarının β-laktam antibiotikləri ilə birlikdə istifadəsi bakterial müqavimətlə mübarizədə effektivliyini sübut etmişdir. Anti β-laktamaz fəaliyyəti ilə yeni birləşmələrin inkişafı yoluxucu xəstəliklərin müalicəsinin yaxşılaşdırılması üçün vədlər verir. β-laktamaz aktiv cibinin üstünlüklərini başa düşmək və hidrofobik əvəzediciləri olan birləşmələri dizayn etməklə tədqiqatçılar bu inhibitorların oxşarlığını və effektivliyini artıra bilərlər. Bu tədqiqat antibiotik müqaviməti ilə mübarizə və yoluxucu xəstəliklərin müalicəsi sahəsində xəstələrin nəticələrini yaxşılaşdırmaq üçün davam edən səylərə töhfə verir.

Açar sözlər: p-aminodifenilamin, TEM-1 β-laktamaza, IMP-1 β-laktamaza, amidlər, dokinq, β-laktamaza inhibitorları.

НОВЫЕ СОЕДИНЕНИЯ п-АМИНОДИФЕНИЛАМИН АМИДА: ДИЗАЙН, СИНТЕЗ И ОЦЕНКА АНТИ β-ЛАКТАМАЗНОЙ АКТИВНОСТИ

Ахмед А. Салех, Ахмед А. Дж. Махмуд*

Кафедра фармацевтической химии Фармацевтического колледжа,
Университет Мосула, Мосул, Ира
e-mail: ahmedsot@uomosul.edu.iq

Резюме: β-лактамы, такие как пенициллины и цефалоспорины, уже давно признаны наиболее эффективными антибиотиками для лечения инфекционных заболеваний. Однако основным ограничением их эффективности является выработка бактериями ферментов β-лактамаз, которые гидролизуют β-лактамное кольцо этих препаратов, делая их неактивными. Чтобы преодолеть этот механизм резистентности, обычно используются ингибиторы β-лактамаз, такие как клавулановая кислота, в сочетании с β-лактамами. Ингибируя действие ферментов β-лактамаз, эти ингибиторы восстанавливают эффективность β-лактамных антибиотиков. В недавних исследованиях исследователи использовали методы стыковки, чтобы изучить взаимодействие между ферментами β-лактамаз и потенциальными ингибиторами. В частности, β-лактамазы TEM-1 (1pzp) и IMP-1 (1JJE) были использованы в качестве мишеней для создания новых соединений. Ряд новых соединений был получен путем синтеза 6-амидных соединений в виде производных хлорангидридов и взаимодействия их с п-аминодифениламином с образованием амидных связей. Затем эти соединения были охарактеризованы с использованием различных физических и спектроскопических методов для подтверждения их структуры. Далее синтезированные соединения были подвергнуты биологическим испытаниям для оценки их эффективности против грамположительных и грамотрицательных бактерий, продуцирующих β-лактамазу. Это было достигнуто путем определения минимальной ингибирующей концентрации (МИК) соединений против трех различных штаммов бактерий. Кроме

того, возможную анти-β-лактамазную активность соединений сравнивали с активностью клавулановой кислоты. Результаты исследования показали, что пять синтезированных продуктов продемонстрировали эффект, аналогичный эффекту клавулановой кислоты, только на один штамм бактерий (Staph. aureus). Кроме того, результаты исследования докинга позволяют предположить, что активный карман β-лактамаз отдает предпочтение гидрофобным заместителям, поскольку синтезированные продукты с этими группами показали самый высокий показатель связывания. В заключение следует отметить, что использование ингибиторов β-лактамаз, таких как клавулановая кислота, в сочетании с β-лактамными антибиотиками доказало свою эффективность в борьбе с бактериальной резистентностью. Разработка новых соединений с анти β-лактамазной активностью обещает улучшить лечение инфекционных заболеваний. Понимая предпочтения активного кармана β-лактамазы и создавая соединения с гидрофобными заместителями, исследователи могут повысить сродство и эффективность этих ингибиторов. Это исследование способствует постоянным усилиям по борьбе с устойчивостью к антибиотикам и улучшению результатов лечения пациентов в области лечения инфекционных заболеваний.

Ключевые слова: п-аминодифениламин, β-лактамаза TEM-1, β-лактамаза IMP-1, амиды, докинг, ингибиторы β-лактамаз.