RESEARCH / INVESTIGACIÓN

Synthesis, *in silico* studies and antibacterial assessment of α-amino phosphonates derivatives

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Abstract: The widespread of multi-resistant strains due to the lack of specific treatment and the propagation of infectious diseases requires all resources to remedy this scourge. This study is therefore aimed to assess the antibacterial activity of four synthetic α-Aminophosphonate 4(a-d). Methods: Firstly, α-Aminophosphonate has been synthesized and characterized, then molecular docking of these compounds 4(a-d) into the active binding site of *Escherichia coli* MurB enzyme (PDB Id: 1MBT) was performed to gain a comprehensive understanding of their biological activity. These compounds have been subjected to in vitro antibacterial screening against three multi-resistant strains *E. coli*, *S. aureus*, and *L. monocytogenes*. These compounds showed crucial antibacterial behavior against all studied strains. Thus, their docking estimation supported the in vitro results and showed that the 4c derivative has considerable binding energy towards the active site of *Escherichia coli* MurB. These findings provide critical information for the exploration of α-amino phosphonates as novel antibacterial agents.

Key words: α-Aminophosphonate, Docking, Antibacterial.

Introduction

The emergence and widespread bacterial resistance to antibiotics dramatically sapped our ability[1] to remedy this infection and precipitate an alarming public health dilemma[2]. Nowadays, the new obsession of researchers is to innovate new therapeutic approaches able to overcome these microbial diseases[3]. Several studies have been focused on the synthesis of eco-friendly substances[4]. Among these molecules, α-amino phosphonates take a leading position[5], especially in biological and environmental applications[6–9].

These compounds attracted the renewed interest of biologists due to their wide-ranging activities; they are recognized as an overwhelming inhibitor of enzymes like serine[10], UDP-galactopyranose mutase[11], and the viral enzyme human immunodeficiency virus protease. Likewise, they limited metastatic progression, activated different apoptosis pathways, and induced anti-tumoral cytotoxicity.

In this regard, the current study pertains to the synthesis and characterization of some α-amino phosphonates derivatives 4(a-c), then we evaluated *in silico* their antimicrobial potential[12] based on a computer-aided simulation[13]. Then, we tested their antibacterial behavior on resistant strains and their synergistic effect with some standard antibiotics.

All this investigation is essential to understand better the mechanism of action of these molecules, which could offer an exceptional framework that may lead us to the discovery of new potent antibiotics.

Methods

The general protocol of α-amino phosphonates synthesis

The multicomponent synthesis of the 4 derivatives of α-amino phosphonates was carried out through the well-established literature protocol[14,15]. We grafted an aromatic aldehyde (1a), aniline (2), and diethyl phosphite (3) in the presence of $Na_2CaP_2O_7$ as a catalyst[16]. A plausible reactional mechanism of the one-pot domino reaction is illustrated in Scheme S1of ESI.

Characterization of th α-aminophosphonates 4(a-d)

Characterization of the Diethyl(phenyl)-N-(phenyl) aminomethylphosphonate 4a

^{1}H-NMR (CDCl3) δ: 1.2 (3H, J_{HH}= 7.2 Hz, t, OCH_2CH_3); 1.4 (3H, J_{HH}= 7.2 Hz, t, OCH_2CH_3); 3.73-4.3 (4H, m, OCH_2CH_3); 4.9 (1H, J_{HP}= 24.6 Hz, d, CHP), 5 (1H, s, NH); 6.6-7.8 (10H, m, HAr).13C-NMR (CDCl3) δ: 16.46 (d, 3JCP= 6.03 Hz, OCH_2CH_3); 16.7 (d, 3JCP= 6.03 Hz, OCH_2CH_3); 56.35 (d, 1JCP = 149 Hz, CHP); 63.5 (d, 2JCP = 6.79Hz, OCH_2CH_3); 63.53 (d, 2JCP = 6.79 Hz, OCH_2CH_3); 114.13 (s); 118.64 (s); 128.1 (s); 128.85 (s); 129.43 (s); 136.2 (s); 146.6 (s). IR (KBr) υ 3304(NH), 2985(CH), 1605 (C=C), 1514 (C=C), 1240 (P=O),1020 (P-O) cm^{-1}.

Diethyl(4-methoxyphenyl)-N-(phenyl) aminomethylphosphonate 4b

^{1}H-NMR ($CDCl_3$) δ : 1.25 (3H, J_{HH}= 7.2 Hz, t, OCH_2CH_3); 1.4 (3H, J_{HH}= 7.2 Hz, t, OCH2CH3); 2.45 (3H, s, $C_6H_4CH_3$);3.79-4.31 (4H, m, OCH_2CH_3); 4.87 (1H, JHP= 24.6 Hz, d, CHP), 5 (1H, s, NH); 6.71-7.5 (9H, m, HAr).13C-NMR (CDCl3) δ: 16.5 (d, 3JCP= 5.8 Hz, OCH_2CH_3); 16.71 (d, 3JCP= 5.8 Hz, OCH_2CH_3); 21.4 (s, C6H4CH3); 56 (d, 1JCP = 150 Hz, CHP); 63.48 (d, 2JCP = 6.94 Hz, OCH_2CH_3); 114.14 (s); 118.57 (s); 128 (s); 129.4 (s); 129.55 (s); 129.58 (s); 133 (s); 137.8 (s);146.8 (s). IR (KBr) υ 3325 (NH), 2980 (CH), 1604 (C=C), 1498 (C=C), 1234 (P=O), 1016 (P-O) cm^{-1}.

Characterization of the Diethyl(2-hydroxyphenyl)-N phenyl) Aminomethylphosphonate 4c

^{1}H-NMR ($CDCl_3$) δ : 1.25 (3H, J_{HH}= 7.2 Hz, t, OCH_2CH_3); 1.39 (3H, J_{HH}= 7.2 Hz, t, OCH_2CH_3); 3.86 (3H, s, $C_6H_4OCH_3$);3.8-4.26

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(4H, m, OCH_2CH_3); 4.85 (1H, J_{HP}= 23.1 Hz, d, CHP), 4,95 (1H, s, NH); 6.7-7.53 (9H, m, HAr).^{13}C-NMR ($CDCl_3$) δ: 16.53 (d, $^3J_{CP}$= 5.7 Hz, OCH2CH3); 16.72 (d, $^3J_{CP}$= 5.7 Hz, OCH_2CH_3); 55.44 (s, $C_6H_4OCH_3$); 55.62 (d, $^1J_{CP}$ = 151,2 Hz, CHP); 63.43 (d, $^2J_{CP}$ = 6.9 Hz, OCH_2CH_3); 63.47 (d, $^2J_{CP}$= 6.9 Hz, OCH_2CH_3);114.15 (s); 114.32 (s); 118.57 (s); 128 (s); 129.2 (s); 129.33 (s); 146.67 (s); 159.56 (s). IR (KBr) υ 3300 (NH), 2983 (CH), 1600 (C=C), 1510 (C=C), 1232 (P=O), 1020 (P-O) cm^{-1}.

Characterization of the Diethyl(4-methylphényl)-N(phenyl)aminomethylphosphonate 4d

^{1}H-NMR ($CDCl_3$) δ : 1.25 (3H, J_{HH}= 7.2 Hz, t, OCH_2CH_3); 1.39 (3H, J_{HH}= 7.2 Hz, t, OCH_2CH_3); 3.86 (3H, s, $C_6H_4OCH_3$);3.8-4.26 (4H, m, OCH_2CH_3); 4.85 (1H, J_{HP}= 23.1 Hz, d, CHP), 4,95 (1H, s, NH); 6.7-7.53 (9H, m, H_{Ar}).^{13}C-NMR ($CDCl_3$) δ: 16.53 (d, $^3J_{CP}$= 5.7 Hz, OCH_2CH_3); 16.72 (d, $^3J_{CP}$= 5.7 Hz, OCH_2CH_3); 55.44 (s, $C_6H_4OCH_3$); 55.62 (d, $^1J_{CP}$ = 151,2 Hz, CHP); 63.43 (d, $^2J_{CP}$ = 6.9 Hz, OCH_2CH_3); 63.47 (d, $^2J_{CP}$= 6.9 Hz, OCH_2CH_3);114.15 (s); 114.32 (s); 118.57 (s); 128 (s); 129.2 (s); 129.33 (s); 146.67 (s); 159.56 (s). IR (KBr) υ 3300 (NH), 2983 (CH), 1600 (C=C), 1510 (C=C), 1232 (P=O), 1020 (P-O) cm^{-1}.

Biological experimental data)

Computational studies

Determination ADMET parameters

The ADMET parameters (absorption, distribution, metabolism, excretion, and toxicity of the synthesized compounds 4(a-d) were calculated using the freely accessible web server Swiss ADME (http://swissadme.ch/index.php#undefined)[17]

Protein-Ligand Docking calculations

After preparing protein/ligands structures, we stimulated the docking interaction using the software AutoDock 1.5.6 (MGL tools- 1.5.6)[18]. Polar hydrogen atoms and Kollman united charges were added to all target proteins, and the resulting file has been saved in pdbqt extension. For the docking calculation, a grid box of 60×60×60 Å in x, y, z directions were created to cover the active site of the target. The default grid points spacing was fixed to 0.375 Å and centred at x = 42.527 y = -46.679, z = 65.559. We used in this work the Lamarckian Genetic Algorithm (LGA) for flexible docking calculations. The LGA parameters including size, energy screening, mutation rate, and crossover rate. After the calculation procedure, we selected the best conformations of the complex from the 10 calculated based on their binding energy scores. Then the complex interaction map was displayed using Discovery Studio Visualizer[17,19].

Microbiology

Bacterial strains

Three bacterial strains *E. coli*, *S. aureus*, and *L. monocytogenes* have been used in this study. The media used for antibacterial screening was the LB and the Brain Heart Infusion (BHI).

Preparation of stock solutions

To prepare a final concentration of 5 µg / ml, we solubilized the selected compounds in DMSO (16.66 µg / ml). Then the stock solutions were stored in sterile containers in obscurity.

Antibiotics

Stocks of antibiotics: ampicillin (100 mg / ml), chloramphenicol (34 mg / ml) and rifampicin (50 mg / ml) were prepared, filtered and stored at -20 ° C until microbiological assays. The chloramphenicol is prepared in ethanol and rifampicin was formulated in methanol.

Antibacterial sensitivity test

The compounds 4(a-d) were tested for their antibacterial activity against *E.Coli*; *S.aureus*, and *L.monocytogene* by using the agar well diffusion method[20]. To explore the antibacterial effect of these compounds, we subculture the different strains by streaking the supercooled media (LB and BHI) with bacterial inoculum adjusted to 10^6 CFU (bacteria/ml) then poured into Petri dishes. After 15 minutes, wells of 6mm were made on each plate by using a sterile cone. Under aseptic conditions, we injected separately 50 µl of the tested molecule; positive control (antibiotic), and negative control (DMSO) in each well. The Petri dishes were incubated at 30 - 37 ° C for 24h. All tests were performed in triplicate.

Test of synergistic effect

We used the diffusion assay described previously to study the synergistic effect between standard antibiotics and the target compounds. Firstly, wells were produced in each plate and filled with 50 µl of the tested samples containing the mixture of molecules and antibiotics. The Petri dishes are maintained at 30 ° - 37 ° C for 24 hours. The use of antibiotics singly serves as a positive control and DMSO is considered a negative control. All tests were obtained in triplicate.

Results

ADMET studies

The ADMET data provides information on the lipophilicity of the ligands, which is predicted by the MIlog P parameter, the hydrogen bonding potential of the compounds, and their molecular flexibility[21]. The different drug-likeness proprieties for all selected compounds were in harmony with Lipinski's rule of five[12] (see table 1).

Binding energy evaluation

Modeling studies are essential to understand the mechanism of action of these designed compounds. Our Docking investigation has been confirmed that the highest energies of binding (ΔG) observed are -6.44 and -6.45 K. cal/mol for the 4c and the 4a (see table 2), which can reflect the good affinity between the protein targeted and ligand in contrast to the ligand of reference (ciprofloxacin).

All the docked molecules were subjected to 2D protein-ligand interaction analysis and the obtained conformations compared to the Ciprafloxin have been reported in Figures 3. The selected compounds complex to the target enzyme (MurB) represented at least two H-bonds. Accordingly, the docking studies of α-amino phosphonates gathered previously in table 2 revealed their free energy of binding to *E. coli* UDP-N-acetylenolpyruvoylglucosamine reductase (MurB) was higher for all compounds than the score obtained for standard antibiotic (Ciprofloxacin). Hence, we can conclude that *E. coli* MurB is the putative target responsible for the antibacterial activity of these compounds[22]. Their interaction with the target was stabilized by forming hydrogen bonds, hydrophobic interactions, Van der walls Pi-sulfur and Pi-alkyl interactions (Figure 1).

Additionally, the binding mode of the most active compound 4c (binding energy: -6.45 kcal/mol) showed two hydro-

Compounds	MW [a]	MLogP[b]	nHBA [c]	nHBD [d]	nRB [e]	TPSA [f]	Lipinski
Rules	<500	≤4.15	<≤10	≤5	≤10	<160 Å	
4a	319.34 g/mol	4	3	1	8	57.37	Yes; 0 violation
4b	333.36 g/mol	3.65	3	1	8	57.37	Yes; 0 violation
4c	349.36 g/mol	3.28	4	1	9	66.60	Yes; 0 violation
4d	336.34 g/mol	2.42	5	1	6	82.64	Yes; 0 violation

[a]Molecular Weight; [b] Calculated Lipophilicity (MLog Po/w); [c] Number of Hydrogen Bond Acceptor; [d] Number of Hydrogen Bond Donor; [e] Number of Rotatable Bond; [f] Topological Polar Surface Area.

Table 1. Determination of pharmacokinetic parameters for good oral bioavailability of synthesized compounds 4(a-d).

Ligand	Energie (KJ/mol)	Residue involved	RSM(Å)
4a	**-6.44**	**Gly 219 and Ser 64**	**1.52**
4b	-5.59	Arg 207 Ile 59 Val 181 Gly 61	1.54
4c	**-6.45**	**Val 181 and Ile 59**	**1.16**
4d	-6.29	Arg 207 Pro 123 Cyst 218	1.84
Ciprafloxin	**-5.4**	**Arg 207 Pro 123 Val 181 Ile 59 Ser 64**	**0.9**

Table 2. The binding score of the tested compounds docked into the active site of 1MBT.

gen bonds formed between the oxygen atom and the amino acid Ile 59. We also detected the formation of close interaction with Val 181 via a hydrogen bond. Moreover, Pi-alkyl and Pi-sulfur interactions are formed with the residues Leu 80Lys 292 and Met 132. These amino acids reside at the enzyme's active site and are crucial in the biosynthesis of peptidoglycan; any modifications in these amino acids could down-regulate the enzyme activity, which induces bacterial cell death23.

In vitro studies

The antibacterial effect of the four synthesized compounds against the three strains was investigated by using the diffusion method, and the results are reported in Table 3. The obtained data showed clearly that the 4c compound possesses significant activity against the whole strains compared to the other derivatives. We also observed that the 4a and 4b were more actives against *E. coli* and *S. aureus*, while the 4d compound showed a substantial activity only against *L. monocytogenes*. On the other side, no inhibition zone was detected with vehicle control (DMSO).

Synergistic Test

The effect of the combination between the 4 synthesized products and antibiotics viz ampicillin, chloramphenicol, and rifampicin on the viability of the strain are summarized in figures 2 and 3.

By comparing the above results, it's exciting to underline that the synergistic effect of the synthesized products varies as a function of the studied strain and the antibiotic used. The potent inhibition was detected against *S. aureus* and *L. monocytogenes* after rifampicin to the 4 b and 4c compounds. Nevertheless, when we combine ampicillin/4b, chloramphenicol/4b, and chloramphenicol/4c we obtain a sizable activity only against *E. coli* (G- bacteria).

Discussion

The *in silico* data have been supported our study *in vitro* performed previously against Multi-resistant strains. According to our docking finding, all the selected compounds had H-bond interactions with the MurB enzyme indicating that the binding affinity increased significatively with the number of the H-bond, which reflects the stability of the complex ligand-protein.

We also observed that the studied compounds fulfill Lipinski's rule requirements and represent a high binding score with an RSM (root square mean) value < 2Å, which confirms their antibacterial inhibiting activity and the reliability of our virtual screening[24]. Based on molecular docking analysis, we can conclude that the putative mechanism of antibacterial activity observed *in vitro* is probably the inhibition of the *E. coli* MurB enzyme. This enzyme catalyzed the second step in forming muramyl sugar[22], which is essential for peptidoglycan biosynthesis[25,26].

On the other side, the sensitivity of the studied strains against these molecules provides their antibacterial potency estimated using an inhibition zone formed around the well. If we should classify these molecules as a function of their increasing antibacterial potential order: the 4c compound takes the place position due to their great effect against the three multi-resistant strains viz *S. aureus*, *E. coli* et *L. monocytogenes*(4c>4b> 4a >4d). At the same time, the three other compounds possessed a variable antibacterial activity as a function of the strain used. For example, the 4a and 4b derivatives possess antibacterial activity against gram-negative strains (*E. coli*); Nevertheless, the 4d derivative is more efficient against gram-positive strains(*L. monocytogenes* and *S.aureus*). It's gratifying to elucidate that this antibacterial sensitivity presu-

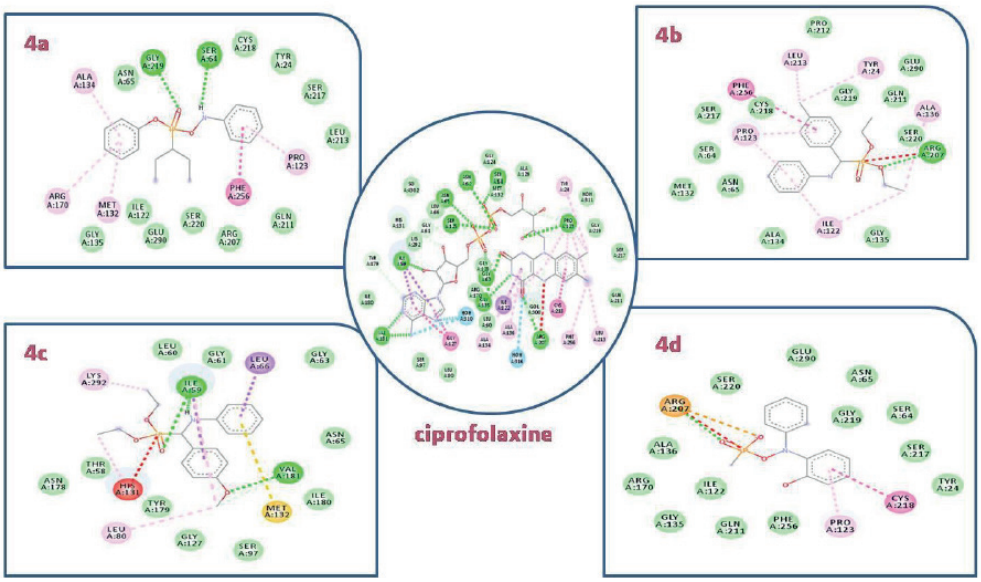

Figure 1. 2D docking diagrams of compounds 4a, 4b, 4c and,4d at the active site of MurB.

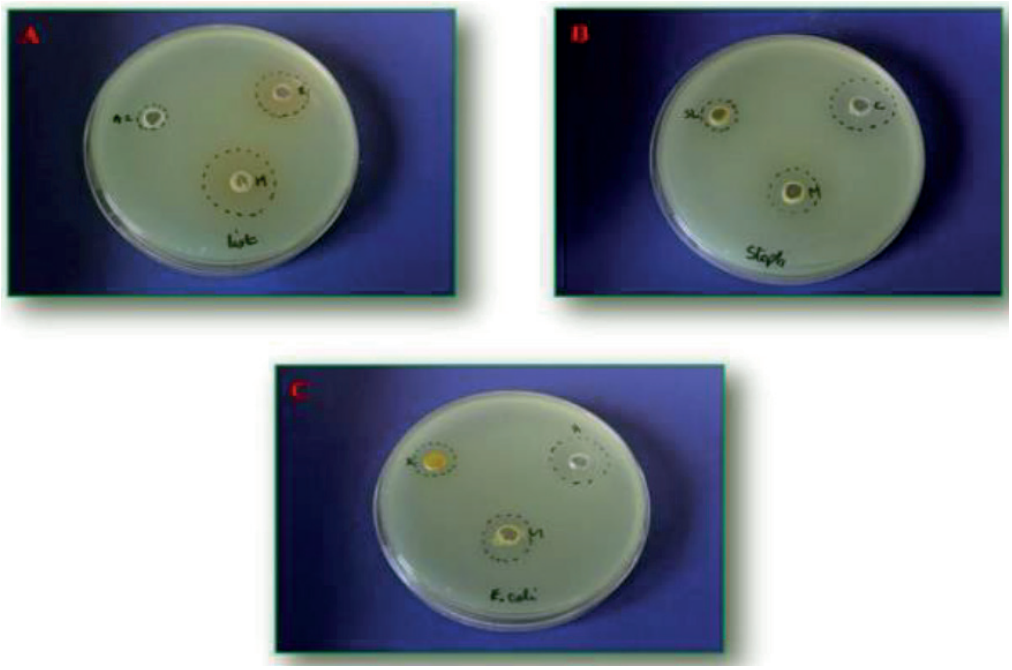

Figure 2. Synergistic effect between α-amino phosphonates and commercial antibiotics. A: a mixture between rifampicin and compound 4b, B: chloramphenicol supplemented to 4c, and C: ampicillin added to the compound 4c. *M: molecule/antibiotic, List: L. *monocytogenes*, Staph: *S. aureus.*

mably related to the structure of the tested compounds, as described the substitution of bioactive motive such methyl previously; methoxy, or metoxybenzaldhyde in the phenyl ring enhance this activity[27]; as results when the aromatic aldehyde is replaced with hydroxyl scaffold, we notice a slight and inconspicuous antibacterial activity[28,29].

Our results are in harmony with some previous studies. It's gratifying to cite the research work published in 2011[30], and they showed the excellent antibacterial and antifungal behavior of these compounds[30]. Another research work achieved in 2020[5] also confirmed their main antibacterial activity against a panel of gram-positive and harmful strains.

Despite their excellent antibacterial effects, in some cases, these pathogens strains can escape from the effect of these substances[31,32]. This new situation compelled the researchers to develop new promising strategies. Our study selected a simple method by combining the synthesized compounds and some antibiotics of references.

The approach adopted herein has given a satisfactory result. On the one hand, we noticed an increase in antibacterial activity. On the other hand, we obtained a powerful effect with a lower concentration of antibiotics.

Conclusions

In this study, four derivatives of α-amino phosphonates were successfully synthesized, then assessed for their antibacterial activity *in vitro* and *silico*.

The obtained data revealed that all studied compounds are biologically active and possess potent antibacterial activities, especially the 4*a* derivative.

With biological evaluation and docking results in hand, we

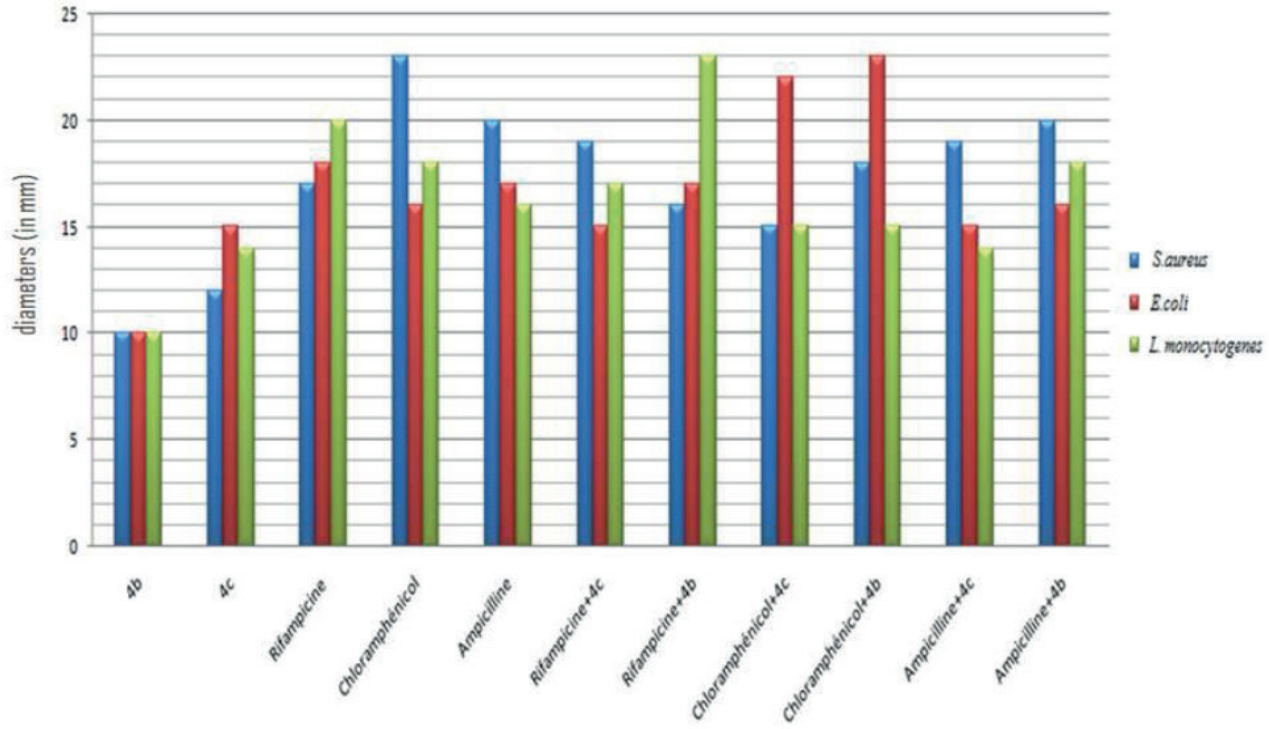

Figure 3. The plot of a synergistic effect of α -amino phosphonates/antibiotic on the growth inhibition of studied strains.

Stains	*E. coli*			*L. monocytogenes*			*S. aureus*		
	IZD* expressed in mm								
	1\2	1\4	1\8	1\2	1\4	1\8	1\2	1\4	1\8
4a	**12**	**10**	**9**	**10**	**9**	**8**	**11**	**9**	**7**
4b	**11**	**12**	**11**	**10**	**8**	**6**	**10**	**8**	**6**
4c [a]	**19**	**17**	**14**	**18**	**17**	**14**	**18**	**17**	**16**
4d	**10**	**8**	**7**	**12**	**12**	**10**	**10**	**8**	**6**

*IZD is the inhibition zone diameter calculated in mm.

Table 3. Antibacterial activity of the synthesized compounds 4(a-d).

can propose a plausible mode of action of these compounds before evaluating their biological activity *in vitro* and *in vivo*.

Limit of studies

Given the above facts, a single bioassay is not able to reflect the full picture of the derivatives' effects on multi-resistant bacteria.

Recommendations for future studies

We recommend using another bacterial species (Gram- and Gram+) to provide broad information about the antibacterial potential of these compounds.

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Finding statement

This research was conducted within the university research plans and benefited from the general funds granted to the physiopathology, molecular genetics, and biotechnology laboratories.

Conflicts of Interest

None

Author contributions

B. Addoum, A. Soukri, B. El Khalfi, and H. Elmakssoudi contributed to the study's design. S. Harrati wrote and reviewed the draft of the manuscript.

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