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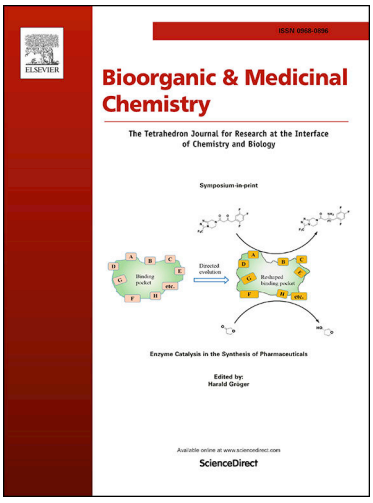

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New compounds for a good old class: synthesis of two B-lactam bearing cephalosporins and their evaluation with a multidisciplinary approach

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Abstract: Antimicrobial resistance is spreading massively in the world and is becoming one of the main health threats of the 21st century. One of the possible strategies to overcome this problem is to modify the known classes of antibiotics in a rational way, with the aim of tuning their efficacy. In this paper, we present the synthesis and the evaluation of the biological activity of a series of two β-lactam bearing cephalosporin derivatives, in which an additional isolated azetidinone ring, bearing different substituents, is joined to the classical cephalosporanic nucleus by a chain of variable length. A computational approach has been also applied in order to predict the molecular interactions between some representative derivatives and selected penicillin-binding proteins, the natural targets of β-lactam antibiotics. All these derivatives are active against Gram-positive bacteria, with MIC_{100} comparable or even better than that of the reference antibiotic ceftriaxone, and show no or very low cytotoxic activity on different cell lines. Overall, these molecules appear to be able to exert their activity in particular against microorganisms belonging to some of the species more involved in the development of multidrug resistance.

Keywords: Cephalosporins; Gram-positive; bifunctional compounds; penicillin-binding proteins; docking.

1. Introduction

In the 21st century, one of the most frightening threats for the human health is the widespread diffusion of antimicrobial resistance, caused by the abuse and misuse of antibiotics in the last decades.[1] This threat is increased by the difficulty in finding new classes of molecules active against microorganisms, since the last class of antibiotics was discovered about 30 years ago.[2] Therefore, while awaiting the discovery of a brand-new antibiotic compound, it is essential to widen the panel of existing drugs, trying to develop innovative molecules from the old available scaffolds.

Nearly 90 years after their discovery, β-lactam antibiotics are still widely used because they couple high specificity and potency with relatively low toxicity and costs of productions.[3] The key of their mechanism of action is their resemblance with the natural substrate of penicillin-binding proteins (PBPs), bacterial enzymes belonging to different classes depending on their molecular mass, structure and different additional activities, involved in the synthesis and maintenance of the peptidoglycan, an essential component of the prokaryotic cell wall.[4] The antibiotics of this class act as suicide inhibitors for PBPs, forming a covalent intermediate that blocks the activity of these enzymes, causing ultimately the lysis of the bacteria. Unfortunately, soon after the introduction of these drugs in clinics, bacteria resistant to their activity were discovered, and to date, multi-drug resistance is common among many microorganisms, especially the so-called ESKAPE pathogens (from the initials of the following bacteria: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species).[5] Therefore, many different classes of β-lactam antibiotics have been created, either to overcome the increasing bacterial resistance or to improve their pharmacological features.[6]

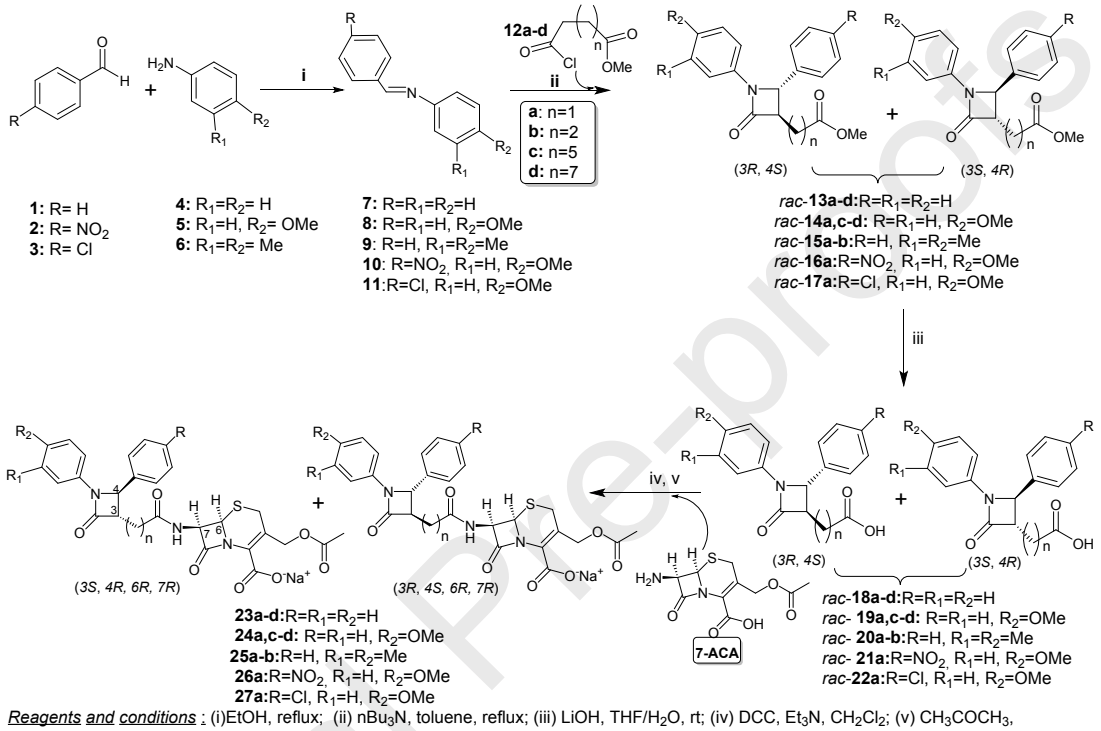
As a part of our long-standing research for new potential antibiotics[7-12], during the last years our efforts have been devoted to the development of an innovative class of bifunctional β-lactam compounds. In particular, recently we have developed the prototype of a class of new 7-aminocephalosporanic acid (7-ACA) derivatives, containing an additional β-lactam ring with different substituents, joined to the amino-nitrogen of 7-ACA scaffold via an amide bond. This new compound shows an antibacterial activity against Gram-positive bacteria and no cytotoxicity towards human cells, even at a concentration higher than the one inhibiting the growth of bacteria.[13] Moreover, a recent computational study predicted an increased resistance of this molecule towards β-lactamases, the enzymes responsible for the most part of the bacterial resistance for these compounds.[14]

In the present work, we have performed a rational modification of the root bifunctional pharmacophore, on the basis of our previous studies, by introducing substituents on the two phenyl rings bound to the isolated 2-azetidinone moiety and by modulating the length of the chain connecting this ring to the 7-ACA moiety, and we have tested the effects of our modifications on selected strains of Gram-positive and Gram-negative bacteria, as a proof of concept. We have corroborated this approach with the evaluation of the cytotoxicity of these new compounds and the prediction of the interactions of selected compounds, by using a computational biology approach.

2. Results

2.1. Chemistry

The synthetic route to the target compounds **23a-d, 24a, 24c-d, 25a-b, 26a** and **27a** (Figure 1) was designed according to our previous studies with some modifications.[13] The strategy utilized a convergent approach based on the coupling of a variously functionalized β-lactam ring (**13-17**) with commercial (+)-7-ACA. Compounds **7-10** and **13a-16a** (Figure 1) were prepared following the previously described methodology and their spectral data matched with those reported in literature.[15-18]



Reagents and conditions : (i)EtOH, reflux; (ii) nBu_3N, toluene, reflux; (iii) LiOH, THF/H_2O, rt; (iv) DCC, Et_3N, CH_2Cl_2; (v) CH_3COCH_3, $NaHCO_3$, H_2O

Figure 1. Reagents and conditions for the synthesis of target compounds.

The synthesis of the variously functionalized β-lactam rings (**13-17**) included a common origin: a Staudinger [2+2] cycloaddition reaction between a properly substituted imine (**7-11**) and a ketene prepared *in situ* from the appropriate acyl chloride (**12a-d**). The imines (**7-11**) were obtained by condensation of commercially available aldehydes (**1-3**) and anilines (**4-6**) in ethanol at reflux. As regards derivatives **12a-d**, the compounds **12a** and **12b** were prepared starting from the commercially available succinic and glutaric anhydride respectively (see Supporting Information), while **12c** and **12d** were commercially available. Under our selected experimental conditions, racemic *trans*-β-lactams (*rac*-**13-17**) were formed exclusively in the Staudinger reaction as confirmed by ^{1}H-NMR spectra and according to our previous reports.[11] Next, the treatment of these compounds with LiOH solution provided the corresponding acids (*rac*-**18-22**) in quantitative conversion, which were directly subjected to the coupling with commercially available (+)-7-ACA using DCC as a coupling reagent and Et_3N. The subsequent transformation into sodium salt provided target compounds (**23a-d, 24a, 24c-d, 25a-b, 26a, 27a**) as diastereomeric mixture with (*3S, 4R, 6R, 7R*) and (*3R, 4S, 6R, 7R*) absolute configuration at C-3, C-4, C-6 and C-7, respectively, and ensured their better water solubility. Since the separation of diastereomers was disappointing and the computational study

reported in our previous paper[13] evidenced no significant differences among the predicted binding affinities to all PBPs for both diastereoisomers, we decided to perform the biological assays with the diastereomeric mixtures.

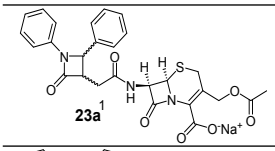
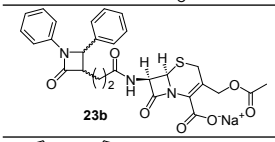
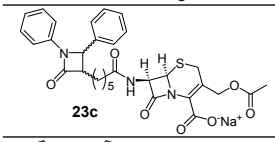
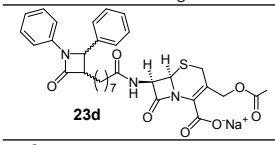
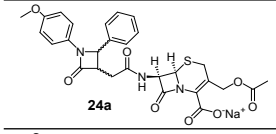
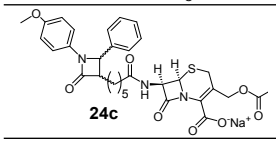
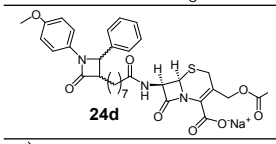
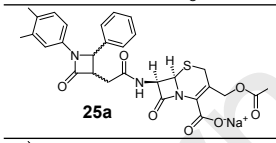
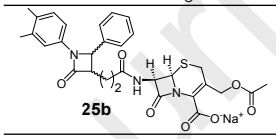
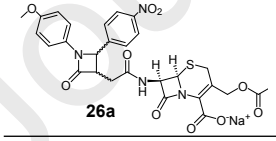
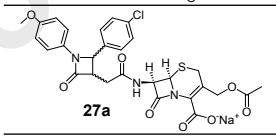
2.2 Antimicrobial Activity

The antimicrobial activity of compounds **23a-d, 24a, 24c-d, 25a-b, 26a** and **27a** was evaluated by comparative analysis of both the MIC and the MLD determined for non-pathogenic Gram negative strains *Escherichia coli* JM107 and *Salmonella enterica* LT2 ATCC 700720, and Gram positive strains *Staphylococcus aureus* (a pathogenic hospital isolate) and *Bacillus sp.* (a non-pathogenic environmental strain). These strains are the same bacteria used in our previous work[13], to have a direct comparison with our previous results. The analysis was also extended to ceftriaxone, a third-generation cephalosporin with a broad-spectrum activity (Table 1).

All compounds were very effective against Gram-positive microorganisms, with MIC_{100} ranging from values ≤0.25 to 32 μg/ml, but not against Gram-negative (up to 256 μg/ml). The most active non-elongated antibiotics were **24a** and **23a**, whose MIC and MLD for *S. aureus* were significantly lower than those of ceftriaxone are (MIC_{100} about 3.5 and 2 times lower for **24a** and **23a**, respectively).

The elongation of the carbon chain did not change the action spectrum of compounds, however, it significantly increased both the activity towards Gram-positive and the bacterial sensitivity to the antibiotics compared to ceftriaxone. As indicated in Table 1 for *S. aureus* the MIC_{100} and MLD of **23d** were 2.5 and 3 times lower than those of **23a**, respectively, and 5.5 and 7 times lower than those of ceftriaxone. On the *Bacillus* sp. the bacteriostatic activity was proportional to the chain length, reaching the lower values for **23c**, whose MIC_{100} value was 5 times lower than that of **23a** and 1.5 lower than that of ceftriaxone. In contrast, compounds elongation significantly decreased the bactericidal activity, also in comparison to ceftriaxone (see MLD). Analysis of compounds **24** evidenced that **24d** was the more efficient against both *S. aureus* and *Bacillus* sp. with MIC_{100} values more than 6 and 3 times lower than **24a**, respectively. Compound **24d** was much more active than ceftriaxone, even compared to compounds **23c-d**. In fact, the MIC_{100} for *S. aureus* and *Bacillus* sp. were 22 times and 3 times lower than those of ceftriaxone are, respectively. This antibiotic also presented the higher bactericidal activity against *S. aureus*, compared to all tested compound and ceftriaxone, while for *Bacillus* sp. the more lethal was the **24c**.

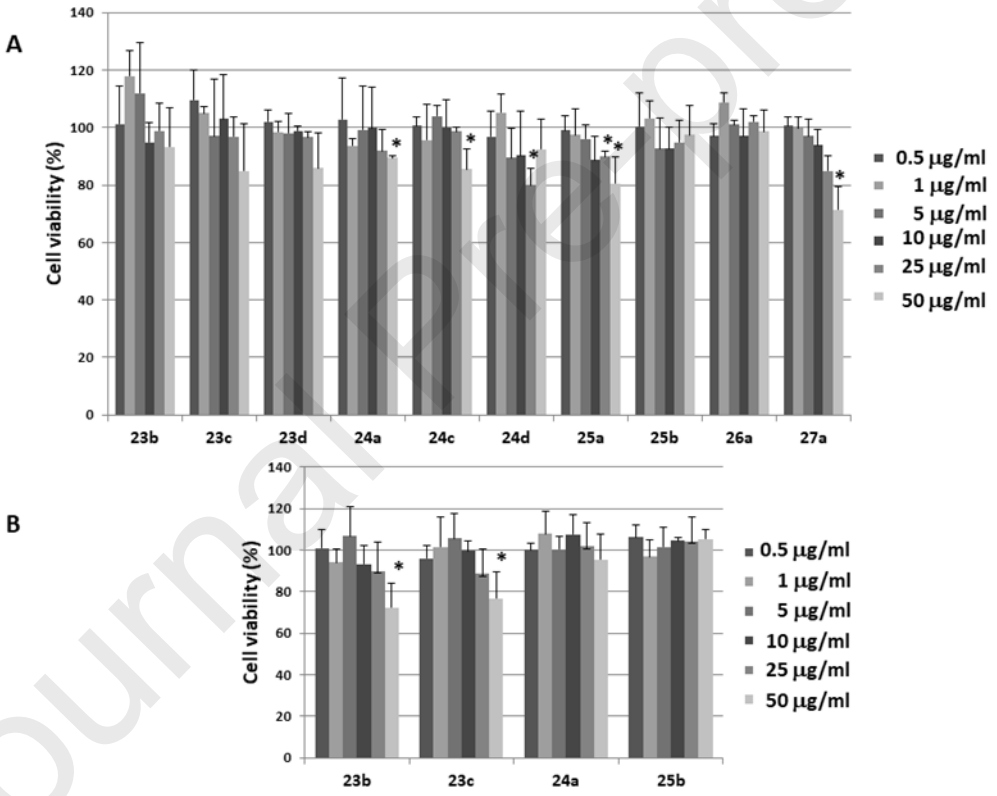
Table 1. Antimicrobial activity of elongated and non-elongated compounds and comparison with ceftriaxone (MICs and MLDs are in μg/ml). The reported values were determined from 0 to 256 μg/ml for each antibiotic, by at least three independent experiments, each in triplicate. In some cases, only the ranges of values at which the toxic doses fall have been determined. Except for >256 μg/ml, the symbols "<" or ">" without the indication of the interval of concentrations mean that MIC and/or MLD are very close to the values to which they refer, and in at least one of the independent experiments it was found to be the same. [1]: data already published in our previous work.[13]

Compound	*S. aureus*			*Bacillus sp.*			*E. coli*	*S. enterica*
	MIC_{50}	MIC_{100}	MLD	MIC_{50}	MIC_{100}	MLD	$MIC_{50}/_{100}$ MLD	$MIC_{50}/_{100}$ MLD
 **23a**[1]	0.75	2.5	>3	<0.25	2.5	>2.5	>256	>256
 **23b**	>0.25, <0.5	2.5	≥3	<0.25	1	>32	>256	>256
 **23c**	>1.5, <2	>3.5, <4	>4	<0.25	0.50	≥32	>256	>256
 **23d**	<0.25	1	≥1	<0.25	0.75	>16	>256	>256
 **24a**	<0.25	1.5	>1.5	<0.25	0.75	≥128	>256	>256
 **24c**	0.75	1.75	>1.75, <2	<0.12	>0.25, <0.35	≥0.75	>256	>256
 **24d**	>0.12	<0.25	0.25	<0.12	0.25	>2, <4	>256	>256
 **25a**	>1	>4, <8	>16	<0.25	3.5	≥3.5	>256	>256
 **25b**	1	>4, <6	>16	≤1	2	64	>256	>256
 **26a**	>4, <8	32	>32	8	32	>256	>256	>256
 **27a**	>4, <8	32	>32	<4	32	>256	>256	>256
Ceftriaxone	>2, <4	5.5	>7	<0.25	0.75	≥1	<0.25/0.25 >1	<0.25/0.25 ≥2

2.3. Cell cytotoxicity

We tested compounds on MRC5, one of the two human cell lines previously used[13], at different concentrations ranging from 0.5 to 50 μg/ml. We observed that no compounds were cytotoxic in the range 0.5-25 μg/ml except in the case of **25a** and **24d** that both significantly reduced cell viability of about 20% at 25 μg/ml. A slight (about 20%) significant reduction of cell viability was observed at the highest dose (50 μg/ml) for several compounds (Figure 2A). We also tested compounds **23b-c**, **24a** and **25b** on a further human cell line, Calu-1, and only observed a reduction of cell viability at the highest concentration of compounds **23b** and **23c** (Figure 2B).

Figure 2. Cytotoxicity of cephalosporin derivatives. (A) Effect on cell viability of MRC5 cells after 24 h exposure to compounds **23b-c**, **24a**, **24c-d**, **25a-b**, **26a**, **27a** (dose-range 0.5–50 μg/ml). Data for **23a** were previously published.[13] (B) Effect on cell viability of Calu-1 cells after 24 h exposure to compounds **23b-c, 24a, 25b** (dose-range 0.5–50 μg/ml). Data are reported as mean ± SD from two or three independent experiments, each in triplicate. Vehicle (DMSO), at the highest concentrations used (0.4% v/v), induced a reduction of cell viability of about 15% (not shown). *p<0.05 vs. the respective vehicle.



2.4. Computational data

The results of the computational analysis of the interactions of two representative compounds, **23d** and **24a**, with some representative PBPs from both Gram-positive and Gram-negative species (Supplementary Table S1)[19-24] are reported in Tables 2-5. In agreement with our previous work[13], there are no significant differences neither among the binding energies predicted for the enzymes belonging to the Gram-positive bacteria and those predicted for Gram-negative bacteria, nor between the binding energies predicted for the two diastereoisomers obtained from the synthesis of the compounds. Indeed, the error made by AutoDock in the prediction of the binding energies is about 1-1.5 kcal/mol, i.e. the

same order of magnitude of these differences. Moreover, the β-lactam ring belonging to the 7-ACA moiety is still predicted to be more reactive towards acylation with respect to the additional, isolated β-lactam ring, as the binding energies predicted for this moiety are consistently lower. In agreement with the microbial assays, the energies predicted for the interaction of **23d** with the enzymes are better than ceftriaxone and those of its precursor **23a** (the compound **8** reported in our previous publication[13]). This is evident in particular for the reaction of the 7-ACA moiety. On the contrary, the binding energies predicted for the interaction of compound **24a** are similar or even slightly worse than those of compound **23a**, and in line with those obtained for ceftriaxone[13].

The detailed interactions of **23d** and **24a** with all the enzymes tested are shown in Supplementary Tables S2-S5. As it was already shown for the precursor **23a**[13], H-bonds prevail among other interactions for both compounds and involve mainly the polar groups linked to the 7-ACA nucleus, the amide group linking 7-ACA to the chain with the isolated azetidinone ring, and the carbonyl groups of the azetidinone rings, especially in their open form, when they interact with the catalytic Ser residue. A comparison of these interactions among all the active sites of the enzymes tested shows that these H-bonds are made with polar residues, in particular Ser, Thr and Asn, and often also with positively charged residues (Lys and Arg). Moreover, in the majority of the active sites, the two phenyl rings on the isolated azetidinone moiety are involved in π interactions of different nature with aromatic residues (Phe, Tyr) and sometimes with charged or polar residues (Arg, Lys, Glu) (Supplementary Tables S6-S9). Interestingly, in the complexes between **24a** and the enzymes from Gram-positive bacteria, the methoxy group bound to one of the two phenyl rings is rarely involved in interactions (Supplementary Tables S2 and S3) whereas, in the complexes with the enzymes from Gram-negative bacteria, that methoxy group is more frequently involved in H-bonds with polar residues such as Gln, Ser and Asn, or even charged residues (Supplementary Tables S4 and S5). On the other hand, the elongated chain of compound **23d** shows an enhanced conformational mobility and often points outside the active site of the enzymes, making contacts with other residues far from it, on the protein surface.

Table 2. Results of covalent docking between compound 23d and selected PBPs of Gram-positive bacteria. [1]:when the result with the best energy is not associated to the most populated cluster, the energy and the number of poses of the most populated cluster is additionally reported. [2]: the additional β-lactam ring has been considered reactive towards the acylation. [3]: the β-lactam ring of the 7-ACA moiety has been considered reactive towards the acylation. [4]: the results obtained for ceftriaxone and previously published[13] are reported here for sake of comparison.

	3VSL (PBP3 from *S. aureus*)		**1TVF (PBP4 from *S. aureus*)**		**2J9P (PBP4a from *B. subtilis*)**	
Compound 23d	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**
(3R,4S), ring A reactive[2]	-12.75 (2) -11.61 (14)	34	-12.06 (12) -10.90 (21)	22	-12.95 (32)	24
(3R,4S), ring B reactive[3]	-15.41 (1) -15.36 (23)	24	-14.54 (2) -14.33 (16)	28	-12.81 (5) -12.11 (21)	20
(3S,4R), ring A reactive[2]	-12.28 (3) -12.27 (15)	30	-12.55 (27)	22	-12.25 (11) -11.81 (20)	29
(3S,4R), ring B reactive[3]	-15.07 (8) -13.25 (12)	30	-13.88 (8) -12.85 (22)	21	-13.37 (13) -11.70 (15)	30
***Ceftriaxone*[4]**	***-14.29 (36)***	***27***	***-12.22 (17)*** ***-12.00 (20)***	***22***	***-11.75 (4)*** ***-10.65 (12)***	***28***

Table 3. Results of covalent docking between compound 23d and selected PBPs of Gram-negative bacteria. [1]: when the result with the best energy is not associated to the most populated cluster, the energy and the number of poses of the most populated cluster is additionally reported. [2]: the additional β-lactam ring has been considered reactive towards the acylation. [3]: the β-lactam ring of the 7-ACA moiety has been considered reactive towards the acylation. [4]: the results obtained for ceftriaxone and previously published[13] are reported here for sake of comparison.

	5FGZ (PBP1b from *E. coli*)		**2EX8 (PBP4 from *E. coli*)**		**3OCL (PBP3 from *P. aeruginosa*)**	
Compound 23d	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**
(3R,4S), ring A reactive[2]	-12.61 (8) -12.39 (24)	32	-11.28 (4) -10.51 (9)	36	-13.60 (18) -13.48 (19)	29
(3R,4S), ring B reactive[3]	-14.45 (4) -13.48 (19)	36	-13.35 (5) -13.30 (11)	34	-15.11 (1) -13.85 (11)	27
(3S,4R), ring A reactive[2]	-13.19 (12) -11.70 (12)	30	-12.47 (20)	32	-14.33 (10) -12.86 (19)	32
(3S,4R), ring B reactive[3]	-13.83 (8) -13.76 (25)	35	-13.65 (20) -13.56 (26)	27	-15.01 (4) -14.51 (15)	30
Ceftriaxone[4]	***-11.87 (4) -11.39 (20)***	***30***	***-12.76 (11) -11.77 (22)***	***14***	***-13.99 (5) -11.98 (18)***	***25***

Table 4. Results of covalent docking between compound 24a and selected PBPs of Gram-positive bacteria. [1]: when the result with the best energy is not associated to the most populated cluster, the energy and the number of poses of the most populated cluster is additionally reported. [2]: the additional β-lactam ring has been considered reactive towards the acylation. [3]: the β-lactam ring of the 7-ACA moiety has been considered reactive towards the acylation. [4]: the results obtained for ceftriaxone and previously published[13] are reported here for sake of comparison.

	3VSL (PBP3 from *S. aureus*)		**1TVF (PBP4 from *S. aureus*)**		**2J9P (PBP4a from *B. subtilis*)**	
Compound 24a	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**
(3R,4S), ring A reactive[2]	-10.91 (17) -10.01 (19)	18	-9.94 (47)	8	-10.76 (20) -9.52 (21)	14
(3R,4S), ring B reactive[3]	-14.16 (24)	19	-12.04 (1) -11.47 (32)	12	-11.75 (40)	18
(3S,4R), ring A reactive[2]	-11.43 (10) -10.95 (24)	19	-9.59 (28)	11	-10.47 (12) -10.31 (44)	10
(3S,4R), ring B reactive[3]	-13.91 (21)	26	-11.70 (53)	10	-11.19 (18) -10.64 (25)	10
***Ceftriaxone*[4]**	***-14.29 (36)***	***27***	***-12.22 (17)*** ***-12.00 (20)***	***22***	***-11.75 (4)*** ***-10.65 (12)***	***28***

Table 5. Results of covalent docking between compound 24a and selected PBPs of Gram-negative bacteria. [1]: when the result with the best energy is not associated to the most populated cluster, the energy and the number of poses of the most populated cluster is additionally reported. [2]: the additional β-lactam ring has been considered reactive towards the acylation. [3]: the β-lactam ring of the 7-ACA moiety has been considered reactive towards the acylation. [4]: the results obtained for ceftriaxone and previously published[13] are reported here for sake of comparison.

	5FGZ (PBP1b from *E. coli*)		**2EX8 (PBP4 from *E. coli*)**		**3OCL (PBP3 from *P. aeruginosa*)**	
Compound 24a	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**	**Predicted ΔG (kcal/mol) and number of poses in the selected cluster[1]**	**Total number of clusters**
(3R,4S), ring A reactive[2]	-11.32 (22)	17	-10.99 (19) -10.00 (34)	10	-12.49 (11) -12.05 (41)	12
(3R,4S), ring B reactive[3]	-13.36 (10) -11.86 (15)	21	-12.65 (49)	13	-13.79 (30)	16
(3S,4R), ring A reactive[2]	-11.66 (25) -10.64 (31)	12	-11.31 (8) -10.26 (25)	12	-12.72 (9) -12.26 (26)	19
(3S,4R), ring B reactive[3]	-13.00 (14) -12.57 (15)	20	-11.99 (21) -11.78 (33)	16	-13.79 (32)	13
***Ceftriaxone*[4]**	***-11.87 (4)* *-11.39 (20)***	***30***	***-12.76 (11)* *-11.77 (22)***	***14***	***-13.99 (5)* *-11.98 (18)***	***25***

3. Discussion

Starting from a literature survey of the basic structure-activity relationships in N-aryl-C4-aryl-2-azetidinones[25-28] and from our previous studies[11,13], we hypothesized that the nature of the substituent groups on the aromatic rings of the additional β-lactam moiety in our compounds could affect the interactions with the active sites and afford a stronger and more efficient biological activity. Therefore, we designed and synthesized a series of novel bifunctional cephalosporin analogues containing electron-withdrawing (NO_2, Cl) and/or electron-donating groups (OMe) on the two phenyl rings at the 1- and 4-positions of the additional β-lactam nucleus, in order to assess the substituent influence on the activity compared to progenitor compound **23a**. In the second instance, we hypothesized that the distance between 7-ACA and the additional substituted β-lactam might also enhance the antibacterial activity, possibly due to the increased conformational flexibility such as to fulfill the

geometric requirements of better interactions. Therefore, we introduced non-functionalized polymethylene chains from one to seven carbons as flexible spacers.

The comparison of the microbiological results shows that all new synthesized compounds are very effective against the Gram-positive selected bacteria and confirmed that carbon chain elongation significantly increases their antimicrobial properties compared to ceftriaxone. Particularly, the introduction of electron-donating OMe group on the phenyl ring at C-4 position of the additional β-lactam ring displays increased activity (bacteriostatic or both bacteriostatic and bactericidal) compared to reference cephalosporin and **23a**. However, the simultaneous presence of OMe group at C-4 position and an electron-withdrawing group on the phenyl ring at the 1-position completely cancels the positive effect of the first and results in a significant reduction of the antimicrobial activity with respect to the basic compound **23a**. The results display also the beneficial influence of lengthening the alkyl chain spacer in the series **23a-d** and **24a-d**.

MIC and MLD also reveal that the compounds have a bactericidal action on *S. aureus*, similarly to ceftriaxone (almost superimposable MIC and MLD values), while for *Bacillus* sp. is more evident a bacteriostatic character (greater separation between MIC and MLD values). This aspect underlines some differences in the antimicrobial mechanism between two genera, which could be based on differences in the cellular PBPs and/or in the affinity with common ones, both resulting in characteristic PBPs profiles of inhibition. The importance of the combination of PBPs that are inhibited on the degree of cellular alteration and lethality has been shown in both Gram-positive and Gram-negative bacteria (*B. subtilis, E. coli, Streptococcus pneumoniae*).[4, 29-31]

We tested the cytotoxic effect of our compounds on MRC5 and Calu-1 cells. We found a significant reduction of cell viability (not over 30%) only at concentrations at least 10 times higher than MIC_{50} both in MRC5 and in Calu-1 cells, even if the two cell lines displayed a slight different sensibility towards the compounds. However, the effect on cell viability of both cell lines was comparable to that of **23a** and of the commercial ceftriaxone on MRC5 cells.[13] Therefore, these compounds show no in vitro cytotoxicity on the tested cell lines at the doses at which they exert a bacteriostatic and/or bactericidal activity.

The computational analysis is intended to provide an interpretation of the molecular phenomena underlying the experimental data obtained in this work, although it is not possible to compare them directly, given the different scale and the complexity of the phenomena that can affect the microbiological assays. Moreover, given the extremely high number of PBPs present in bacteria, this analysis does not claim to be exhaustive, but only to provide suggestions for a better comprehension of the activity of this new class of compounds.

From this analysis, these bifunctional molecules are predicted to bind with good affinity to PBPs belonging both to Gram-positive and Gram-negative microorganisms, despite the microbiological results. This is not unexpected since, as we postulated previously[13-14], probably the lack of activity of these compounds towards this last type of bacteria is due to their external membrane that impairs the penetration of these big compounds in the periplasmic space. The predicted binding energy of the elongated compound **23d** for the two PBPs belonging to *S. aureus* is consistently better than that predicted for the enzyme from *B.*

subtilis, especially for the 7-ACA moiety. This effect is evident also for compound **24a** towards PBP3 belonging to *S. aureus*, whereas in the case of the PBP4 the predicted affinities are similar for *S. aureus* and *B. subtilis*. Overall, these data suggest that these compounds could bind stronger to their target enzymes in *S. aureus*, and this might explain their more pronounced bactericidal activity towards this Gram-positive organism.

Comparing the interactions of compound **24a** with the residues of the active sites of the PBPs with those of progenitor compound **23a**, it is possible to note that the addition of the methoxy group on the phenyl rings bound to the isolated azetidinone moiety seems to increase the number of interactions of this moiety, especially with those enzymes belonging to Gram-negative bacteria. Furthermore, the predicted binding affinity of the elongated compound **23d** for the PBPs is generally improved with respect to the precursor **23a**, especially when the β-lactam ring belonging to 7-ACA is the target for the acylation. We did not perform a direct assay on isolated PBPs to prove it, nevertheless these data are in agreement with the microbiological assays and suggest that the long chain of this compound, by pushing the two moieties far away each other's, allows reducing the steric hindrance of the portion not directly involved in the acylation with the enzyme, thereby favoring the interaction of the other portion with the active site. From these data, it is not surprising that compound **24d**, including both modifications, is the one with the best antimicrobial activity. Additionally, the docking results show that the number of clusters obtained for this compound is very large, in line with the enhanced conformational flexibility of the connecting chain. The position of this chain is quite different in the several results obtained for each simulation (data not shown). In some complexes obtained with docking simulations, this chain is bended and the free moiety can interact with the protein's surface, probably with aspecific interactions, whereas in other cases, the chain is more elongated and protrudes outside the active site (that usually in PBPs is formed by a large cavity at the protein's surface, very accessible to the solvent). This suggests that, once one of the two moieties is covalently bound to the target PBPs, the other moiety might move freely in the medium, being available for the interaction with another target enzyme. The two parts are then potentially able to act independently with fewer constraints than in the precursor **23a**. However, it is important to point out that these compounds are very large and that the long chain is characterized by a high number of torsional degrees, reaching almost the maximum value that can be managed by AutoDock. Further studies by using different approaches, e.g. molecular dynamics simulations in solvent, could be applied to study this effect in more details. For the same reasons, since these complexes violate some of the Lipinski's rules, more studies will be necessary to develop drug-like compounds from this new class of bifunctional cephalosporins.

4. Materials and Methods

4.1 Chemistry

Detailed experimental procedures utilized for the preparation of all new compounds, including the intermediates, and spectral characterization are reported in the Supplementary File S6. All new compounds were characterized by NMR spectroscopy (Bruker Avance-400 and BRUKER Avance-600 spectrometers), ESI-(+)-MS measurements (Waters 4 micro quadrupole mass spectrometer equipped with electrospray ion source), elemental analysis

(FlashEA 1112 Series with Thermal Conductivity Detector) and melting points (DSC 2920 TA INSTRUMENTS) as reported in the Supplementary File S6.

4.2 Microbiological assays and bacterial strains

The compounds were tested on the same bacteria strains used previously[13], both Gram-negative as *E. coli* JM109 and *Salmonella enterica* LT2 ATCC 700720, and Gram-positive as *S. aureus* and *Bacillus* sp. The non-pathogenic strains *E. coli* JM109 and *Salmonella enterica* subsp. *Enterica* serovar *Typhimurium*, strain LT2 ATCC 700720 were purchased from Promega (http://www.promega.com/products) and Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Culture (http://www.dsmz.de), respectively. *S. aureus* and *Bacillus* sp. were derived from the collection deposited in the microbiology laboratory directed by dr. G. Vigliotta. The first is a pathogenic hospital isolate, while the second one is a non pathogenic environmental strain.

The minimum concentration inhibiting growth (MIC_{100}), the minimum concentration that reduced growth by 50% compared to the control samples grown in the absence of antibiotic (MIC_{50}) and the minimum lethal dose (MLD) of each antibiotic derivative were estimated as described previously[13], according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI).[11,32] The toxic doses were determined from 0 to 256 μg/ml for each antibiotic, by at least three independent experiments, each in triplicate.

Results were compared to that of ceftriaxone (Sigma-Aldrich, Milan, Italy), a reference cephalosporin agent active towards a broad range of Gram-positive and Gram-negative bacteria, in clinical use for the treatment of a variety of infections such as meningitis, gonorrhea and community-acquired pneumonia.[33-34]

4.3 Evaluation of cytotoxicity

Cytotoxicity of compounds was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma-Aldrich) colorimetric test performed on MRC5, a human hembryonic lung cell line, and on Calu-1 cells, a human lung cancer cell line (Interlab Cell Line Collection, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy), as previously described.[13,35] For each compound the MTT assay was performed at least two times in triplicate.

4.4 Computational section

The structures of the PBPs used in this work[19-24] were downloaded from the Protein Data Bank.[36] These representative proteins were selected in order to include enzymes from both Gram-positive and Gram-negative bacteria tested in this work, belonging to different classes according to the Ambler classification.[4] In addition, quality criteria of their structures were taken into account, as it has been done in our previous works.[13-14]

The structures of the representative compounds **23d** and **24a** were designed and saved in 3D .pdb format using ChemDraw and Chem3D Pro 12.0 (Perkin Elmer). Since their chemical synthesis produces a diastereoisomeric mixture (see Results), we designed and analysed both diastereoisomers (3R, 4S) and (3S, 4R). Moreover, for each diastereoisomer, the reaction with both β-lactam rings was simulated. Therefore, for each protein and each compound, four different docking simulations were performed.

To perform covalent docking, the flexible side chain approach[37] implemented in AutoDock v. 4.2[38] was used. Chimera[39] was used to modify the structures and, if necessary, to optimize them, according to the protocol for the covalent docking. The parameters used to setup simulations on compound **24a** were the same used in our previous studies[13-14], whereas for **23d** the maximum number of energy evaluations was increased to 25,000,000 to take into account the high number of torsions in this molecule, and the dimensions of the box was increased to 75x75x80 to widen the space available for the molecule to change its conformation. The values of the interactions between compound **23a** (previously identified as compound **8**[13]) and the PBPs, or between ceftriaxone and PBPs were recalculated using these new parameters, but they resulted not significantly different with respect to those already published (data not shown). The conformations corresponding to the best energetic and to the most populated cluster of poses obtained from covalent docking, were analysed with Discovery Studio (DassaultSystèmes, 2015) to identify the most important interactions between the enzymes and the two compounds.

5. Conclusions

We have presented the synthesis and a multidisciplinary analysis of the biological effects of different series of new cephalosporin derivatives bearing two β-lactam rings, with proven antimicrobial activity on Gram-positive bacteria such as *S. aureus* and *Bacillus* sp.. These compounds show to have bactericidal/bacteriostatic effects at concentrations at which they are well tolerated by human cells. A computational analysis has allowed to dissect the interactions that these compounds can have with their target enzymes, PBPs. All together, these data suggest that these molecules are promising starting points for the development of new antibiotics.

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Legend to figures

Figure 1. **Reagents and conditions for the synthesis of target compounds**.

Figure 2. Cytotoxicity of cephalosporin derivatives. (A) Effect on cell viability of MRC5 cells after 24 h exposure to compounds **23b-c, 24a, 24c-d, 25a-b, 26a, 27a** (dose-range 0.5–50 µg/ml). Data for **23a** were previously published.[13]. (B) Effect on cell viability of Calu-1 cells after 24 h exposure to compounds **23b-c, 24a, 25b** (dose-range 0.5–50 µg/ml). Data are reported as mean ± SD from two or three independent experiments, each in triplicate. Vehicle (DMSO), at the highest concentrations used (0.4% v/v), induced a reduction of cell viability of about 15% (not shown). *$p<0.05$ vs. the respective vehicle.

Graphical abstract