Design, Synthesis, and Biological Evaluation of 4-Alkyliden-beta Lactams: New Products with Promising Antibiotic Activity Against Resistant Bacteria

Francesco Broccolo,[§] Gianfranco Cainelli,*[,†] Gianluigi Caltabiano,[‡] Clementina E. A. Cocuzza,[§] Cosimo G. Fortuna,[‡] Paola Galletti,[†] Daria Giacomini,[†] Giuseppe Musumarra,*[,‡] Rosario Musumeci,[§] and Arianna Quintavalla[†]

Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, I-40126 Bologna, Italy, Department of Chemical Sciences, University of Catania, Viale A. Doria 6, I-95125 Catania, Italy, and Department of Clinical Medicine, Prevention and Biotechnology, University of Milano-Bicocca, Via Cadore 48, I-20052 Monza, Italy

Received November 17, 2005

The design, synthesis, and antibacterial activity of 4-alkyliden-azetidin-2-ones as new antimicrobial agents against multidrug-resistant pathogens is reported. 4-Alkyliden-azetidin-2-ones were easily obtained using an original protocol starting from 4-acetoxy-azetidinones and diazoesters. Parent compounds were further elaborated to obtain a small library of 4-alkylidene derivatives. A molecular modeling approach using GRID descriptors based on the concept of VRS identified attractive drug candidates and contributed to the rationalization of functional group effects in QSARs. The in vitro antibacterial activity of the new agents was evaluated against 43 recent clinical isolates of antibiotic-susceptible and -resistant Gram-positive and Gram-negative pathogens by determining their minimum inhibitory concentrations (MICs). The most active compound showed MIC values ranging from 0.25 to 32 mg/L against some of the bacterial species tested. Interestingly, some compounds demonstrated similar activity against methicillin-susceptible and -resistant strains of *Staphylococcus aureus* suggesting possible alternative mechanisms of action of these agents, supported by citotoxicity and preliminary scanning electron microscopy studies.

Introduction

Since their introduction, β-lactam antibiotics proved to be chemotherapeutics of incomparable effectiveness, conjugating a broad spectrum of activity with low toxicity.[1] β-Lactams are a large class of antibiotics characterized by the presence of the azetidin-2-one ring, which is the core of the biological activity, and differentiated by side chains, unsaturations, heteroatoms, and, in many cases, by the presence of another five- or six-membered ring.

The great vivacity of the research in this field led to the development of classical β-lactam substrates such as penicillins and cephalosporins together with nonclassical ones such as carbapenems and monobactams obtained via semi or total syntheses. To complete and increase the importance of β-lactams, nonclassical bicyclic and monocyclic β-lactam substrates with different biological activities have appeared in recent literature: serine-dependent enzyme inhibitors,[2] matrix-metallo protease inhibitors,[3] and even apoptosis inductors.[4]

The problem of microbial resistance, related to the use of these agents over the last 50 years, is nowadays widely recognized, and treatment options in clinical practice are limited by multidrug-resistant bacteria.[5] Despite the need for new antibiotics, large pharmaceutical companies are devoting fewer resources to their development because these agents do not provide as great a return on investment relative to that of compounds for some other therapies.[6] However, in the United States, antimicrobial drugs have had the first or second shortest mean and median clinical development times in every four-year period since 1982.[7] Therefore, the phenomenon of bacterial resistance forces the continuous modification of side chains and structures of known active compounds and the development of new ones.

Herein we report the synthesis and antibacterial activity of a new class of monocyclic beta-lactams substituted in C-4 with an alkyliden carboxy side chain named 4-alkyliden-azetidin-2-one with the aim to contribute to the development of new antimicrobial agents against multidrug-resistant pathogens.

Preliminary results of the antibacterial activity of some of these 4-alkyliden-beta lactams disclosed the opportunity for the application of molecular modeling to relate chemical structures to antibiotic activity and to point out structural modifications that might increase antibiotic potency.

Despite significant advances in the elucidation of the structures of penicillin-binding proteins (PBPs), the overall structural basis for multidrug bacterial resistance has not been clarified. PBPs are a heterogeneous family of enzymes with transpeptidase and transcarboxylase activities involved in the synthesis and cross-linking of the peptidoglycan component of bacterial cell walls, which is fundamental for the manteinance of bacterial cell morphology and integrity. The above considerations suggest that molecular descriptors based on the concept of virtual receptor site (VRS) should be adopted.[8] We selected a molecular interaction field (MIF) approach using grid independent descriptors (GRIND),[8] calculated by using the program ALMOND,[9] which is based on the assumption that the process of ligand−receptor interaction can be represented with the help of the MIF. Accordingly, when a drug binds a receptor of unknown structure, some of the regions defined in its VRS are expected to overlap groups of the real receptor site; therefore, a subset of the VRS regions should be relevant for representation of the binding properties of the ligand.

Results and Discussion

Very few examples of alkyliden-azetidin-2-ones have been reported in the literature. In the 1980s, some C-4 alkyliden

* To whom correspondence should be addressed. Tel: +39-051-20995-10. Fax: +39-051-20994-56. E-mail: gianfranco.cainelli@unibo.it (G.C.). Tel: +39-095-7385012. Fax: +39-095-334175. E-mail: gmusumarra@unict.it (G.M.).

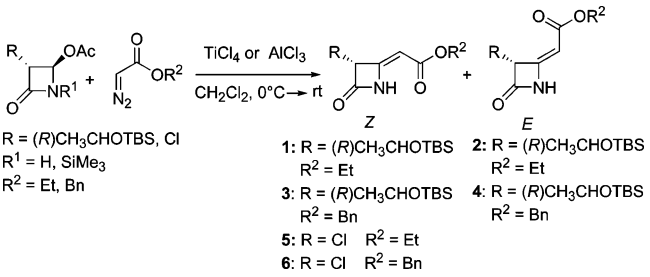
† University of Bologna.

‡ University of Catania.

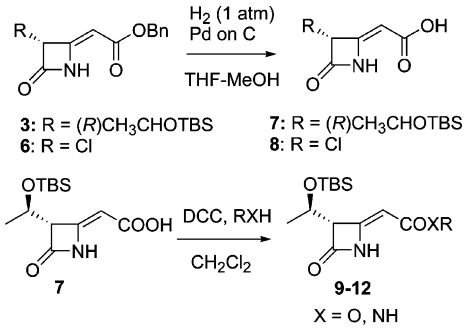
§ University of Milano-Bicocca.

10.1021/jm0580510 CCC: $33.50
Published on Web 04/08/2006

Scheme 1



Scheme 2



derivatives were synthesized and tested for antimicrobial activity with poor results.[10] Recently, we developed a process to obtain 4-alkyliden-azetidin-2-ones[11] (Scheme 1), which could be further modified to obtain a small library of 4-alkyliden compounds.

Synthesis of 4-Alkyliden-beta-lactams. 4-Alkyliden-azetidin-2-ones can be easily obtained using an original and well-established protocol starting from 4-acetoxy-azetidinones and diazoesters in the presence of Lewis acids.[11] Compounds **1**−**6** were obtained starting from 3-chloro- or 3-(2-*tert*-butyldimethylsilyloxyethyl)-4-acetoxy-azetidin-2-ones and ethyl- or benzyl-diazoacetate, with $TiCl_4$ or $AlCl_3$ (Scheme 1). The products are obtained in good yields with diastereomeric *Z*/*E* ratios depending on the Lewis acid and reactants: with ethyldiazoacetate and $TiCl_4$, we obtained (**1**/**2**) *Z*/*E* = 80:20 and with $AlCl_3$ *Z*/*E* = 53:47, with benzyldiazoacetate and $TiCl_4$ (**3**/**4**) *Z*/*E* = 90:10, and with $AlCl_3$ *Z*/*E* = 60:40. Starting from 3-chloro 4-acetoxy azetidinone, we obtained only *Z* isomers **5** and **6** with ethyl and benzyl diazoacetate, respectively. In all cases, *Z* and *E* diastereoisomers could be easily separated by flash chromatography.

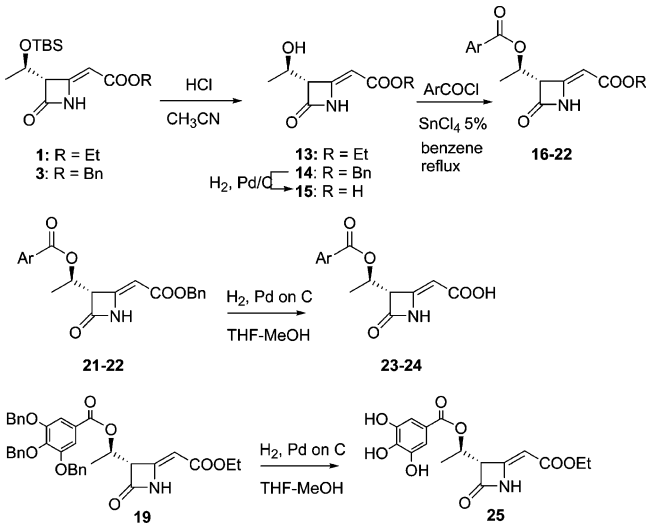
Parent compounds **1**−**6** offer two points for a further elaboration: the C-3 hydroxy side chain and the C-4 ester function. Exploiting modifications on the C-4 side chain, benzyl esters **3** and **6** were quantitatively cleaved using H_2 (1 atm) and Pd on C in THF/MeOH, 1/1 to afford the corresponding acids **7** and **8** as outlined in Scheme 2. Acid **7** can, in turn, be easily transformed in esters or amides **9**−**12** by DCC coupling using benzyl alcohols, phenols, or an amino-ester (Scheme 2).

The C-3 hydroxy-ethyl chain can be further derivatized to several aromatic esters by preliminary deprotection of the silyl group followed by acylation in the presence of $SnCl_4$ (5%) in refluxing benzene (Scheme 3) giving products **16**−**22**.

Compounds **23** and **24** were obtained from corresponding benzyl esters **21** and **22** via hydrogenolysis in THF/MeOH, 1/1, and Pd 10% on C. With the same hydrogenolysis procedure, compound **15** from **14** and **25** from **19** were obtained (Scheme 3).

Microbiological Evaluation. In Table 1 the preliminary results of antibacterial activity toward sensible and antibiotic-resistant bacteria (both Gram-positive and Gram-negative) of

Scheme 3



4-alkyliden beta lactams **1**−**5** and **18** are reported. As reference compounds, we tested two drugs in clinical use, such as amoxicillin **27**, ceftriaxone **28**, and a saturated analogue **26**, which is important for the evaluation of the influence of unsaturation on C-4 in alkyliden-beta-lactams (Chart 1). Some of these 4-alkyliden derivatives demonstrated antibacterial activity.

Table 1 shows that compounds **4** and **5** exhibited some antibacterial activity against the bacterial strains tested. Compound **4** appeared to have some activity (MIC_{50} of 32−64 mg/L) against Gram-positive pathogens, such as *S. pyogenes*, *S. pneumoniae*, and *Enterococcus spp*. Against the latter bacterial strains, compound **4** appeared a possible alternative to inactive-marketed compound **28** (ceftriaxone). Compound **5** showed some antibacterial activity against the Gram-positive strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* as well as against the Gram-negative isolates of *H. influenzae*. In particular, its antibacterial activity against *Staphylococcus epidermidis* was comparable to that of reference compound **28** (ceftriaxone). The other alkyliden-azetidinones shown in Table 1 demonstrated relatively poor antibacterial activity.

Considering that these 4-alkyliden-beta lactams apart from the monocyclic azetidinone framework have a very different structure with respect to monobactam antibiotics (e.g., Aztreonam) their antibacterial activity results are significant. To follow up and assess these microbiological results, we approached a rational design of these new compounds. Difficulties in finding a single receptor target for beta-lactam action against a wide panel of microorganisms prevents the use of specific docking procedures. Therefore, we adopted a VRS approach, where ligands interact with a complex receptor of unknown structure, and QSARs are aimed at identifying pieces of the structure that could be valuable for improving antibacterial activity.

The structures in Chart 1 were selected to derive the first model by a novel methodology adopting GRIND[8] descriptors. The inclusion of totally different structures, such as those of currently marketed drugs amoxicillin **27** and ceftriaxone **28**, is possible because of an important peculiarity in these novel descriptors insensitive to the position and orientation of the molecular structures in the space. Therefore, because the GRIND program does not need the alignment of compounds, 3D-QSAR analysis requires less time compared to that of other 3D-QSAR approaches where the need for the alignment of compounds is

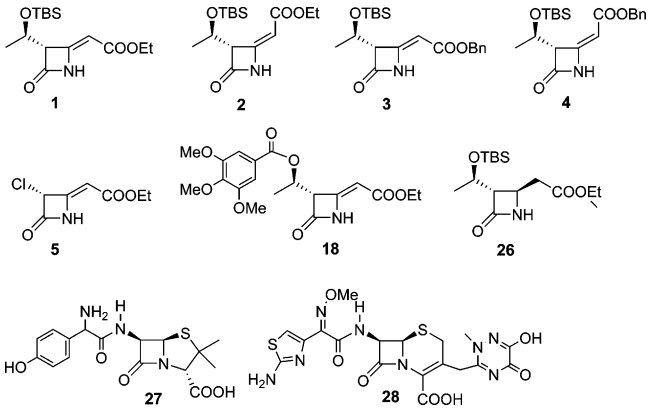
Table 1. MICs (mg/L) for Compounds **1**−**5**, **18**, **26**, and Reference Compounds **27** and **28**[a]

	S. pyogenes (6)			*S. pneumoniae* (9)			*Enterococcus* spp (2)			MRSA (3)		
compd	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
1	128 −>128	128	128	128	128	128	>128	>128	>128	>128	>128	>128
2	128	128	128	128−>128	128	128	>128	>128	>128	>128	>128	>128
3	128 −>128	128	>128	128	128	128	>128	>128	>128	>128	>128	>128
4	32−64	64	64	16−32	32	32	64	64	64	128−>128	>128	>128
5	64−128	128	128	128	128	128	>128	>128	>128	64−128	128	128
18	128 −>128	128	>128	128	128	128	>128	>128	>128	64−>128	>128	>128
26	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
27	<0.06	<0.06	<0.06	<0.06−8	0.12	8	2	2	2	64−128	64	128
28	<0.06	<0.06	<0.06	<0.06−2	0.12	2	>128	>128	>128	>128	>128	>128

	MSSA (3)			*S. epidermidis* (6)			*H. influenzae* (4)			*other Gram* − (10)		
compd	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
1	8−>128	>128	>128	128 >128	>128	>128	128	128	128	128−>128	>128	>128
2	32−>128	>128	>128	128 >128	128	>128	128	128	128	128−>128	>128	>128
3	>128	>128	>128	128 >128	>128	>128	128−>128	128	>128	128−>128	>128	>128
4	128−>128	128	>128	128 >128	128	>128	128	128	128	128−>128	>128	>128
5	32−64	32	64	16−32	16	32	16−32	32	32	64−128	128	128
18	16−>128	>128	>128	32−>128	128	>128	128−>128	128	>128	128−>128	>128	>128
26	16−>128	>128	>128	>128	>128	>128	128−>128	128	>128	128−>128	>128	>128
27	0.5−8	1	8	0.25−4	1	4	0.5−64	1	64	1−>128	128	>128
28	2	2	2	1−32	8	32	<0.06 0.12	<0.06	0.12	<0.06 >128	<0.06	>128

[a] The number of clinical isolates for each species is indicated in parentheses.

Chart 1. Structures of New 4-Alkyliden Beta Lactams **1**−**5**, **18**, and Reference Compounds **26**−**28** Tested for the Antibacterial Activity Reported in Table 1.



an extremely difficult and time-consuming step. Moreover, the use of these descriptors is not limited to 3D-QSAR and allows the extention of their application in 3D searching, pharmacophore identification, and structure−metabolism relations.

The 3D structures of the compounds in Chart 1were imported in Mol-file and coded as ALMOND descriptors following the procedure described in the Computational Methods section.

To obtain relevant VRSs, it is necessary to adopt probes resembling potentially important groups of the binding site. For compounds interacting with proteins, it seems reasonable to use the DRY probe to represent hydrophobic interactions, the O probe (carbonyl oxygen) to represent hydrogen bond acceptor groups, and the N1 probe (amide nitrogen) to represent hydrogen bond donor groups.

Principal components analysis (PCA) of ALMOND descriptors relative to compounds tested afforded a five-principal-components (PC) model explaining 93.4% of variance, 68.3% 1st PC, 5.2% 2nd PC, 6.2% 3rd PC, 6.0% 4th PC, and 7.7% 5th PC. Figure 1, the scores plot for the above model, depicting the data structure elucidated by 3 PC (already explaining more than 79% of variance), appears to be a simplified graphical representation for an immediate evaluation of 3D structural MIF

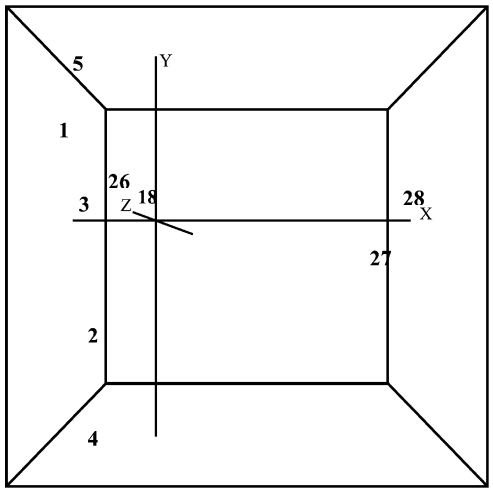


Figure 1. Three-component PCA scores plot for compounds **1**−**5**, **18**, and **26**−**28**.

results for the compounds when interacting with the three above-mentioned probes.

Figure 1 shows that commercially available drugs, amoxicillin **27** and ceftriaxone **28**, exhibit a high value in the *x* axis, the main direction representative of interactions with the VRS expected to be related to overall antibacterial activities. Accordingly, all 4-alkyliden-azetidin-2-ones, exhibiting low activity, lie in the left part of the plot with negative *x* values. It is worth mentioning that **4** and **5**, moderate antibacterials with negative *x* values in Table 1, are characterized by the lowest and the highest *y* values, respectively in Figure 1. The latter features suggest that their activity could be ascribed to peculiar interactions with the selected probes evidenced by the 2nd PC.

Relevant information on the changes in molecular structures in relation to their interactions with the selected probes (Dry, O, and N1) can be obtained by an inspection of the correlograms (Computational methods). In the correlograms for the compounds in Chart 1with the hydrophobic probe, the O probe, and the N1 probe (from left to right) shown in Figure 2, striking differences can be envisaged in their interactions with the O

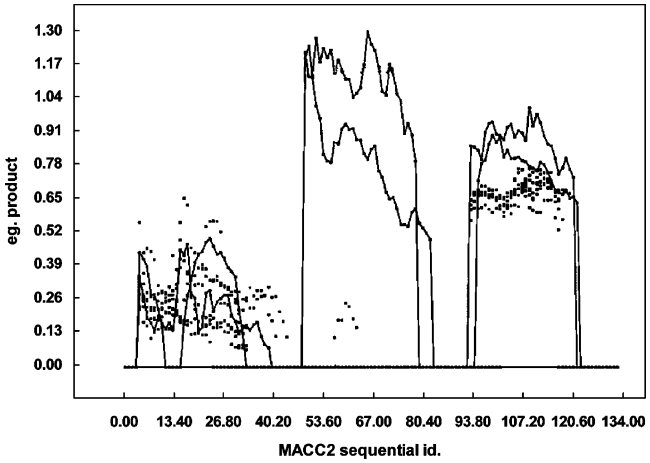


Figure 2. Correlograms for compounds **1**−**5**, **18**, and **26**−**28** with the hydrophobic probe, the O probe, and the N1 probe (from left to right). The two curves indicate profiles for **27** and **28**.

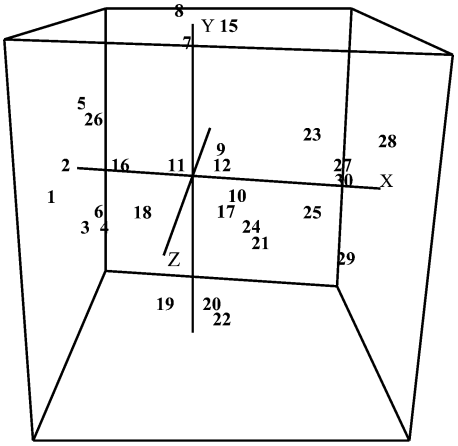


Figure 3. Three-component PCA scores plot for compounds **1**−**30**.

probe (center). Taking into account that every peak in the correlogram indicates the VRS at a distance corresponding to the abscissa of the peak it is evident that apart from **18** showing some interactions in the range 6−10 Å alkyliden-azetidinones do not interact at all with the O probe at distances in the range 2−21 Å. The insertion of hydroxyl groups at appropriate distances in alkyliden-azetidinones is expected to provide better interactions that might result in increased antibacterial activities.

Thus, further structural modifications on the set of 4-alkyliden beta lactams were aimed at verifying the effects of the carboxylic function in relation to the corresponding ethyl or benzyl esters and the mono or polyphenolic substituents at appropriate positions. A principal components analysis (PCA) of ALMOND descriptors relative to compounds **1**−**30** afforded a second five-principal-components (PC) model explaining 65.3% of variance, 32.5% 1st PC, 15.8% 2nd PC, 7.3% 3rd PC, 5.5% 4th PC, and 4.2% 5th PC. Figure 3, the scores plot depicting the data structure in the above model elucidated by 3 PC (explaining 55.6% of variance), shows clearly that compounds **1**−**30** are equally distributed in the eight octants of the 3D plot. The four commercially available active compounds (**27**−**30**) exhibit highly positive *x* values. Consequently, in the present model, compounds with positive *x* values, whose structures are characterized by phenolic (**9**, **10**, **12**, **17**, **20**, **22**, and **25**) and carboxylic functions (**23** and **24**), are shown to interact better with the selected probes of the VRS and are expected to exhibit antimicrobial activity. Interestingly, Figure 4 points out that several designed compounds exhibit significant interactions with

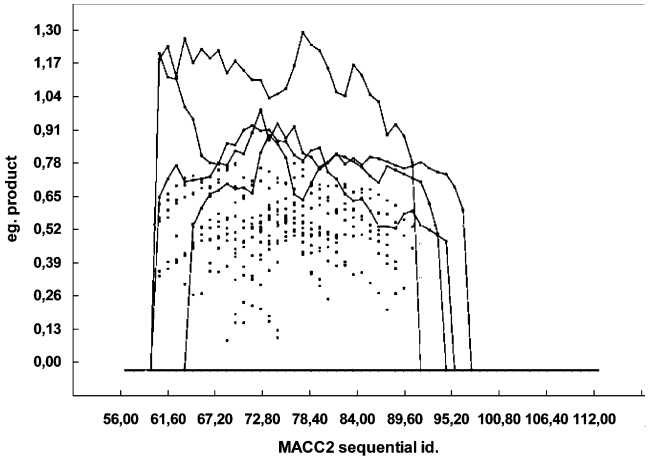


Figure 4. Correlograms for compounds **1**−**30** with the O probe. The four curves indicate profiles for **27**−**30**.

the O probe, which are comparable with the correlogram profiles of marketed drugs **27**−**30**.

Table 2 shows that compound **6** was the most active of the new antibacterial compounds tested (MIC_{50} ranging from 1 to 32 mg/L) with the broadest spectrum of antibacterial activity (*S. pyogenes*, *Enterococcus* spp., MRSA, MSSA, *S. epidermidis*, and *H. influenzae*). Compound **8** was less active but with a similar spectrum of activity as that of compound **6**. The antibacterial activity of chloro derivatives **5**, **6**, and **8**, exhibiting negative *x* values in Figure 3, could be explained by complex processes involving nonspecific toxic effects[12] rather than specific interactions with PBPs, as evidenced by the citotoxicity studies described below.

Compounds **21** and **22** were shown to have a relatively good antibacterial activity, specifically, against methicillin-susceptible staphylococci (MIC_{50} ranging from 2 to 16 mg/L). In particular, the lowest MIC values were shown by compound **22** against *Staphylococcus epidermidis* (0.25 mg/L), value comparable to clinically used antibiotics.

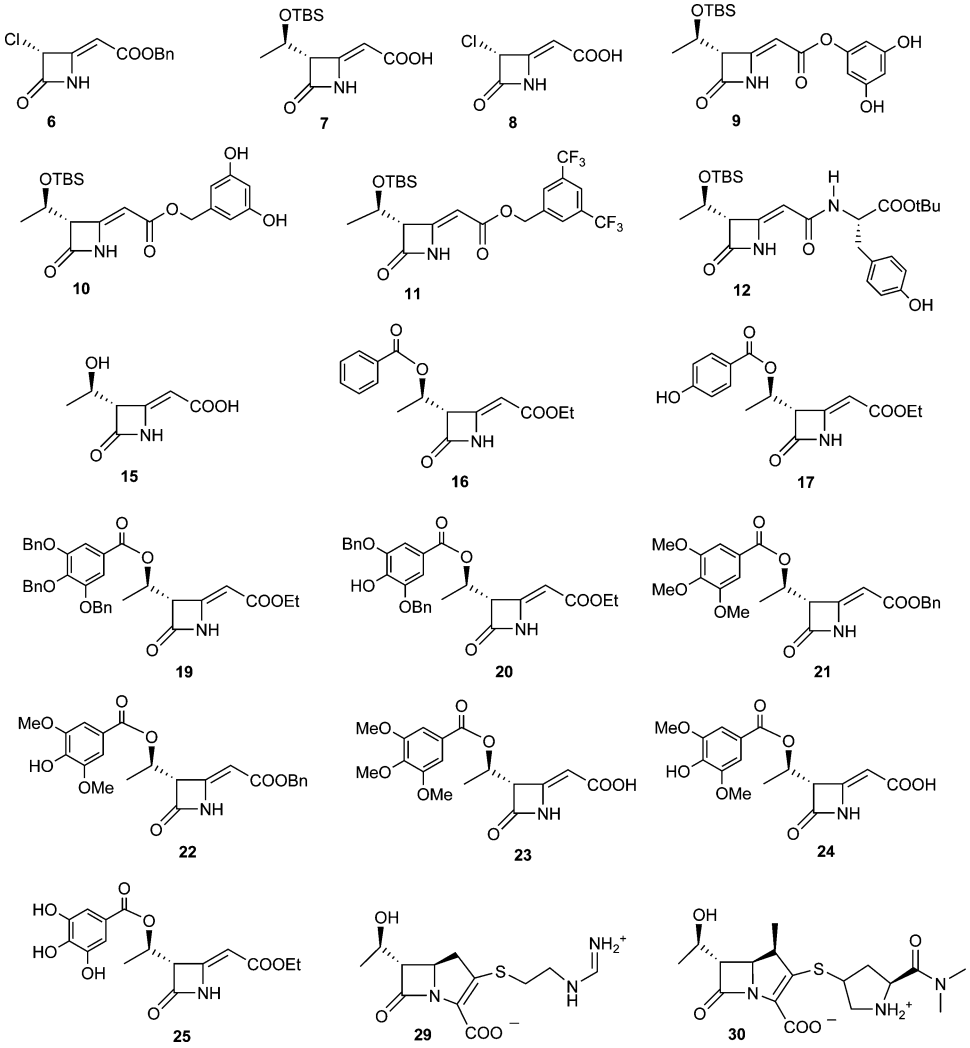
Compounds **9** and **10** were found to have moderate antibacterial activity against some Gram-positive bacteria, such as *S. pyogenes*, *S. pneumoniae*, and *Staphylococci* spp. More interesting, however, was the finding that compounds **6**, **9**, **10**, and **22** showed very similar antibacterial activity against both MRSA and MSSA. Methicillin resistance in strains of *Staphylococcus* spp. is brought about by the acquisition of a new PBP, PBP 2A with low affinity for all beta-lactam agents. Therefore, the methicillin-resistant phenotype usually brings about cross resistance of the isolate to all other beta-lactam agents. The antibacterial activity of compounds **6**, **9**, **10**, and **22** may, therefore, be indicative of an alternative target of action.

To investigate the mechanisms of action of compounds **6** and **22**, which were found to be most active against MRSA, further studies were carried out to determine the possibility of non-specific toxicity of these agents as well as their effect on bacterial cell wall formation.

Cytotoxicity studies using a human cell lines were carried out evaluating the effect of the new compounds on cell viability at concentrations above and below those shown to be antimicrobial.

The highest cytotoxic effect was observed with compound **6**. The cells showed a decreased viability to 48% at 128 μM following a 3 h exposure. After 21 h, a viability of 81% was observed at a concentration of 8 μM, with a progressive decrease at higher concentrations, reaching 23% at 128 μM. Compound **22** showed little or no cytotoxicity on the cell line after 3 h.

Chart 2. Structures of New 4-Alkyliden Beta Lactams **6**−**12**, **15**−**17**, and **19**−**25** and Reference Compounds **29**−**30** Tested for the Antibacterial Activity Reported in Table 2.



Upon exposure of the cell culture for 21 hours, a slight progressive cytotoxic effect was seen starting at a concentration of 32 μM with a viability of 81%, reaching 64% at 128 μM, compared to that of the control.

Preliminary in vitro cell morphology studies by scanning electron microscopy (SEM) based on the exposure of a MSSA and a MRSA strain to a 8 × MIC concentration of compound **6**, **22**, and penicillin at several time-points pointed out that these new 4-alkyliden-beta lactams bring about similar cellular morphological changes with both MSSA and MRSA. This finding is in contrast to the behavior of penicillin, which, as expected, produced cell lysis in the MSSA strain but not with the MRSA isolate. Compound **6** showed a massive reduction of the bacterial cellular pellet during bacterial preparation for microscopy, which was also associated with large amounts of cell debris on microscopic visualization. This finding together with the results of cytotoxic studies would point to a nonspecific cellular toxicity of **6**, particularly, at high concentrations. Exposure to compound **22** brought about the formation of large abnormally shaped cells. This dysfunction in bacterial cell wall formation may arise from the interaction of this compound with another PBP involved in the synthesis of peptidoglycan and present in both MRSA and MSSA.

The role of PBP2A in the resistance for methicillin-resistant *Staphylococcus aureus* (MRSA)[13] or PBP2X for S*treptococcus pneumoniae*[14] has been pointed out and elegant evidences for the cooperative functioning of PBP2, PBP4, and PBP2A in the antibiotic susceptibility of *Staphylococcus aureus* provided.[15] However, the role of other PBPs remains to be explored, and specific drug interactions with different bacterial strains have not been fully elucidated. In this context, the modeling VRS approach demonstrates its utility in suggesting structural modifications for the design of more active compounds. However, it is worth mentioning that an increased in silico drug−receptor interaction does not necessarily imply an increased in vitro antimicrobial activity because it is well known that the penetration of cell membranes is related to the lipid solubility of the drug, an important property governing the passage through membrane barriers. Therefore, the drug partition coefficient plays a fundamental role in its absorption and consequently, in its activity.

Table 2 shows clearly that the increased activity of compounds **9**, **10**, **17**, **20**, **21**, **22**, and **25**, paralleled by positive *x* values in Figure 3, due to the presence of phenolic OH and/or OMe groups,[16] results in higher affinities of the designed structures for the VRS. Partition coefficients (clogP), calculated by HINT![17] for the above drugs (3.66 for **9**, 2.98 for **10**, 1.99 for **17**, 5.76 for **20**, 2.34 for **21**, 3.67 for **22**, and 1.33 for **25**) point out hydrophobicity values consistent with an acceptable permeability and with the observed in vitro activities.

However, the presence of carboxylic functionalities results in the lack of activity for compounds **7**, **8**, **15**, **23**, and **24.** The

Table 2. MIC (mg/L) for Compounds **6**−**12**, **15**−**17**, **19**−**25** and Reference Compounds **29**−**30**[a]

	S. pyogenes (6)			*S. pneumoniae* (9)			*Enterococcus* spp (2)			MRSA (3)		
compd	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
6	16−128	32	128	64−128	128	128	16	16	16	16−32	16	32
7	128−>128	128	>128	128	128	128	128	128	128	>128	>128	>128
8	4−128	16	128	64−128	128	128	128−>128	128	>128	16−128	128	128
9	8−16	16	16	8−16	16	16	64	64	64	32−64	32	64
10	32	32	32	32	32	32	128	128	128	64−128	64	128
11	128−>128	128	>128	128−>128	128	>128	>128	>128	>128	>128	>128	>128
12	128−>128	>128	>128	64−>128	>128	>128	>128	>128	>128	>128	>128	>128
15	128	128	128	128	128	128	>128	>128	>128	>128	>128	>128
16	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
17	128−>128	128	>128	64−128	128	128	>128	>128	>128	8−>128	>128	>128
19	>128	>128	>128	128−>128	>128	>128	>128	>128	>128	>128	>128	>128
20	32−>128	>128	>128	16−>128	>128	>128	>128	>128	>128	>128	>128	>128
21	128−>128	128	>128	>128	>128	>128	128−>128	128	>128	2−128	128	128
22	128	128	128	128	128	128	128−>128	128	>128	1−64	32	64
23	128−>128	>128	>128	128	128	128	>128	>128	>128	>128	>128	>128
24	128−>128	>128	>128	128	128	128	>128	>128	>128	>128	>128	>128
25	128−>128	>128	>128	128−>128	128	>128	>128	>128	>128	8−128	128	128
29	<0.06	<0.06	<0.06	<0.06−1	0.12	1	0.5−>128	0.5	>128	32−128	64	128
30	<0.06	<0.06	<0.06	<0.06−2	<0.06	2	2−>128	2	>128	32	32	32

	MSSA(3)			*S. epidermidis* (6)			*H. influenzae* (4)			*other Gram* − (10)		
compd	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
6	8−16	8	16	8−32	8	32	1−2	1	2	128−>128	128	>128
7	>128	>128	>128	>128	>128	>128	>128	>128	>128	128−>128	>128	>128
8	32−64	32	64	32−64	64	64	8−64	64	64	16−128	32	128
9	32	32	32	16−64	32	64				128−>128	>128	>128
10	64	64	64	64−>128	128	>128				>128	>128	>128
11	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
12	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
15	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
16	>128	>128	>128	64−>128	>128	>128	>128	>128	>128	>128	>128	>128
17	16−>128	>128	>128	8−>128	8	>128	–	–	–	>128	>128	>128
19	>128	>128	>128	>128	>128	>128	>128	>128	>128	128−>128	>128	>128
20	>128	>128	>128	>128	>128	>128	>128	>128	>128	128−>128	>128	>128
21	8−16	8	16	2−16	4	16	128−>128	>128	>128	128−>128	>128	>128
22	8−128	16	128	0.25−64	2	64	32−128	64	128	128−>128	>128	>128
23	>128	>128	>128	64−>128	>128	>128	128	128	128	128−>128	>128	>128
24	>128	>128	>128	64−>128	>128	>128	128	128	128	128−>128	>128	>128
25	64−128	128	128	8−64	16	64	128	128	128	128−>128	>128	>128
29	<0.06	<0.06	<0.06	<0.06−1	<0.06	1	<0.06−0.12	<0.06	0.12	<0.06−0.12	<0.06	0.12
30	<0.06	<0.06	<0.06	<0.06−64	0.12	64	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06

[a] The number of clinical isolates for each species is indicated in parentheses.

above structure−activity relationships are more evident when comparing active ester precursors **21** (clogP 2.34) and **22** (clogP 3.67) with the corresponding inactive carboxylic acids **23** (clogP −1.48) and **24** (clogP −0.16). The lack of activity of the latter acids (exhibiting positive *x* values in Figure 3 and, therefore, significant interactions with the VRS) as compared to that of the corresponding esters could be related to the permeability difficulties indicated by their higher hydrophilicities.

Conclusions

In conclusion, the reported 4-alkyliden-beta-lactams constitute a family of new compounds with interesting antibacterial properties, possessing, apart from the monocyclic azetidinone framework, a very different structure with respect to monobactam antibiotics.

The antibacterial activity of the 4-alkyliden-beta-lactams tested was, overall, not outstanding; however, some compounds were found to have an inhibitory effect, generally, more marked against Gram-positive pathogens, although the spectrum of activity varied. Interestingly, the generally undifferentiated antibacterial activity of some of the studied compounds against both methicillin-susceptible and -resistant strains of *Staphylococcus aureus* was suggestive of an alternative mechanism of action as compared to that of typical beta-lactams. A molecular modeling approach using GRID descriptors based on the concept of the VRS allowed the identification of interactions through oxygenated functions such as phenolic OH, which are valuable for the antibacterial activity and contributed to the rationalization of functional group effects in QSARs. Although the compounds reported here do not exhibit outstanding antibacterial activities, they could represent lead structures for the development of new 4-alkyliden-beta-lactams specifically designed against resistant bacteria.

Experimental Section

General Procedures. Commercial reagents were used as received without additional purification. Anhydrous solvents were obtained commercially and used without further drying. ^{1}H- and ^{13}C NMR values were recorded on a VARIAN Mercury 400, INOVA 300, or GEMINI 200 instrument using a 5 mm probe. All chemical shifts have been quoted relative to deuterated solvent signals, δ in ppm and *J* in Hz. Data and spectra are reported in the Supporting Information. FT-IR: Nicolet 205 measured as films between NaCl plates and reported in cm^{-1}. TLC: Merck 60 F_{254}. Column chromatography: Merck silica gel 200−300 mesh. HPLC-MS, HPLC: Agilent Technologies HP1100, column ZOBRAX-Eclipse XDB-C8 Agilent Technologies. The compounds were eluted

with CH_3CN/H_2O, gradient from 30 to 80% of CH_3CN. Acid compounds were eluted with a CH_3CN/H_2O mixture containing 0.1% of formic acid. MS: Agilent Technologies MSD1100 single-quadrupole mass spectrometer, full-scan mode from *m/z* 50 to 2600, scan time 0.1 s in positive ion mode, ESI spray voltage 4500 V, nitrogen gas 35 psgi, drying gas flow 11.5 mL/min, fragmentor voltage 20 V. The $[\alpha]^{25}_D$ values were determined with a Perkin-Elmer 343 polarimeter. Melting points were determined using a Büchi apparatus and are uncorrected. The purities of the target compounds were assessed as being >95% using HPLC with two diverse systems (see Supporting Information for details).

Compounds **1**, **2**, **5**, **7**, and **26** have been prepared according to ref 11. Compounds **3**, **4**, and **6** have been prepared according to the same reported procedure[11] using benzyldiazoacetate[18] as the diazocarbonyl compound.

Compounds **8**, **15**, **23**, and **24** were obtained by hydrogenolysis of the corresponding benzylesters. A representative procedure is the following: Compound **21** (0.444 g, 0.976 mmol) was dissolved in an anhydrous mixture THF/MeOH (4 mL/4 mL) and Pd. A 10 wt % of activated carbon, (0.045 g) was added. Finally, the reaction mixture was treated with H_2 (1 atm), and it was stirred at room temperature for 4 h. Then it was filtered and concentrated to give compound **23**.

Compounds **9**, **10**, **11**, and **12** were obtained starting from **7** via DCC coupling with the corresponding amine, amino acid, or alcohol (Scheme 3). A representative procedure is as follows: Compound **7** (0.065 g, 0.228 mmol) was dissolved in anhydrous CH_2Cl_2 (3 mL), and the solution was brought to 0 °C. Then DCC (0.052 g, 0.251 mmol), 1,3,5-trihydroxybenzene (0.045 g, 0.274 mmol), and catalytic DMAP (5.6 mg, 0.0456 mmol) were added at 0 °C. The reaction mixture was stirred at room temperature for 4 h, then concentrated EtOAc was added, and the mixture was filtered. The liquid residue was concentrated and purified by flash chromatography (cyclohexanes−ethyl acetate 60:40) to give compound **9**. Note that in the preparation of **10**, **11**, and **12** catalytic DMAP was not necessary.

Compounds **16**, **17**, **18**, **19**, **20**, **21**, and **22** were obtained by $SnCl_4$ catalyzed O-acylation of the 3-hydroxyethyl chain. Compounds **1** and **3** were deprotected on the 3-hydroxyethyl chain by means of HCl 1N in CH_3CN. A representative procedure for compound **13** is as follows: Compound **1** (0.313 g, 1 mmol) was dissolved in CH_3CN (6 mL), and HCl 1N (1 mL) was added. The reaction was monitored by TLC, and other 1 mL portions of HCl 1N were added till total conversion. After that, saturated NaCl solution was added, and the mixture was extracted with CH_2Cl_2 (5 × 10 mL), dried on Na_2SO_4, concentrated, and the residue purified, when necessary, by flash chromatography (cyclohexane/ethyl acetate 4:6) or by trituration in pentane/Et_2O. Yield 87%.

The obtained desilylated compound **13** (0.045 g, 0.226 mmol) was dissolved in anhydrous benzene (4 mL), and then 3,4,5-trimethoxybenzoyl chloride (0.104 g, 0.452 mmol) and, finally, $SnCl_4$ (0.023 mmol, 23 μL of a solution of 1M in CH_2Cl_2) were added. The solution was heated to reflux and was stirred for 4 h. Then the reaction mixture was quenched in a solution of $NaHCO_3$ (5%), extracted with ethyl acetate (4 × 10 mL), dried on Na_2SO_4, and concentrated. The residue was purified by flash chromatography (cyclohexane/ethyl acetate 8/2) to give compound **18**. Yield 50%.

Compound **25** was obtained in quantitative yield by hydrogenolysis (THF/MeOH 1/1, Pd 10% on C, H_2 1 atm) of the corresponding tribenzyloxyderivative **19**.

Microbiological Assays. (i) Bacterial Strains. A total of 43 clinical isolates were used for the determination of the in vitro antibacterial activity of the studied compounds. The bacterial strains included the following Gram-positive (29) and Gram-negative (14) species: *S. pyogenes* (6), *S. pneumoniae* (9), *Enterococcus* spp. (2), methicillin-resistant *S. aureus* (MRSA) (3), methicillin-susceptible *S. aureus* (MSSA) (3), *S. epidermidis* (6), *H. influenzae* (4), *M. catarrhalis* (1), *E. coli* (3), *P. mirabilis* (2), *S. marcescens* (2), and *K. pneumoniae* (2).

(ii) Determination of Minimum Inhibitory Concentrations (MICs). The in vitro antibacterial activity of the new agents was studied by determining their minimum inhibitory concentrations (MICs) by means of the broth microdilution method according to the CLSI (Clinical and Laboratory Standards Institute, formerly the NCCLS) guidelines. Briefly, serial 2-fold dilutions of each antibiotic were made using the Mueller-Hinton broth in microtiter plates with 96 wells. An equal volume of the bacterial inoculum (5×10^5 CFU/mL) was added to each well on the microtiter plate containing 0.05 mL of the serial antibiotic dilutions. The microtiter plate was then incubated at 35 °C for 16−20 h after which each well was analyzed for the presence of bacterial growth. The MIC was defined as the lowest concentration of antimicrobial agent able to cause inhibition of bacterial growth as shown by the lack of turbidity of the culture medium. The results have been reported as MIC ranges, MIC_{50} and MIC_{90} (the minimum concentrations able to inhibit 50 and 90% of the tested isolates, respectively), for the different bacterial species, despite the small number of strains tested to show the different spectrum of activity of the new compounds.

The in vitro antibacterial activity of 23 new 4-alkyliden-azetidin-2-ones was tested and compared to that of reference beta-lactam agents in clinical use: amoxicillin (Sigma-Aldrich), ceftriaxone (Sigma-Aldrich), imipenem (Merk Sharp Dohme), and Meropenem (AstraZeneca).

Cell Viability Assay. Cells from a human cell line (TPC1) were seeded into 96-well plates (Nunc, Roskilde, Denmark) at 40 000 cells/well in 200 μL of supplemented DMEM medium and cultured overnight at 37 °C and 5% CO_2. On the following day, the medium was changed to a fresh medium and compounds **6** and **22** at concentrations of 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 μM in 200 μL of supplemented medium was added to the wells. Each concentration and controls was tested using six parallel wells. The cells were cultured at 37 °C and 5% CO_2 for 3, 6, and 21 h, and the viability of the cells was determined using an MTT-assay (van de Loosdrecht AA).

The MTT-solution (5 mg/mL) was prepared by dissolving 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and thiazolyl blue (MTT) (Sigma, St. Louis, MO) in sterile D-PBS (Gibco or BioWhittaker, Verviers, Belgium) and mixing with the medium at a ratio of 1:15. The medium was removed, and the cells were washed twice with the medium. After the addition of 100 μL of MTT-solution to each well, the plates were incubated at 37 °C and 5% CO_2 for 3 h. After removing the MTT-solution, the cells were washed with D-PBS, and 100 μL of acidified 2-propanol solvent was added to dissolve the intracellular crystals that may have formed. The plates were then incubated at 37 °C for 15 min with occasional shaking, and the optical density of each well was measured at 600 nm using a micro-ELISA reader.

Computational Methods. MIFs were obtained using the program GRID, version 20.[19] GRIND were generated, analyzed, and interpreted using the program ALMOND version 3.0.0 (www.moldiscovery.com). Computations and graphical displays were performed on SGI O2 workstations (MIPS R12000 processor). The process of ligand−receptor interactions, represented with the help of the MIF, is described using molecular descriptors based on the concept of VRS. Basically, GRIND are a small set of variables representing the geometrical relationships between relevant regions of the VRS and, as such, are independent of the coordinate frame of the space where the MIF is computed. The procedure for obtaining GRIND involves three steps: (i) computing a set of MIFs, (ii) filtering the MIFs to extract the most relevant regions that define the VRS, and (iii) encoding the VRSs into the GRIND variables.

(i) Computing the MIF. To obtain relevant VRSs, it seems reasonable to use the DRY probe to represent the hydrophobic interactions, the O probe (carbonyl oxygen) to represent hydrogen bond acceptor groups, and the N1 probe (amide nitrogen) to represent hydrogen bond donor groups. In default mode, a grid-spacing of 0.5 Å is used with the grid extending 5 Å beyond a molecule.

(ii) Filtering the MIF. A previously decribed procedure,[8] defining the most interesting regions as those characterized by intense favorable (negative) energies of interaction by means of an algorithm to select from each MIF a fixed number of nodes

optimizing a scoring function, was adopted. This function, including the intensity of the field at a node and the mutual distances between the chosen nodes as optimality criteria, extracts from each field a number of nodes (in the order of 150−200) that represent independent, favorable, probe-ligand interaction regions.

(iii) Encoding the VRS into GRIND. GRIND encodes the geometrical relationships between the VRS regions so that they are independent of their positions in the 3D space by using an auto- and cross-correlation transform.[8] For each probe, the procedure computes the product of the interaction energy for each pair of filtered nodes, storing only the highest product, a peculiarity responsible for the reversibility properties of GRIND. This method is called maximum auto- and cross-correlation (MACC) or, more specifically, MACC-2.[9] The values obtained from the analysis can be represented directly in correlogram plots, where the products of the node−node energies are reported versus the distance separating the nodes.

Molecular Description: GRID Force Field. The GRID program[20] was used to describe the molecular structures. GRID is a computational procedure calculating, at each point, the interaction energy between the probe and the target molecule as the sum of Lennard-Jones (E_{LJ}), hydrogen bond (E_{HB}) electrostatic interactions (E_{EL}) and, for specific probes, the entropic contribution (E_{ENT})

$$E_{x,y,z} = \sum_{i=1}^{N} E_{LJ} + \sum_{i=1}^{N} E_{HB} + \sum_{i=1}^{N} E_{EL} + \sum_{i=1}^{N} E_{ENT}$$

GRID contains parameters describing each type of atom in each ligand molecule. These parameters define the strength of the Lennard-Jones, hydrogen bond, and electrostatic interactions used to evaluate the energy functions. GRID give precise spatial information, and this specificity and sensitivity are an advantage because they may be representative of the important chemical groups present in the active site.

Calculation of clogP. The partition coefficients calculations (clogP) were performed by HINT! on the basis of the hydrophobic fragment constant approach of Hansch and Leo[21] in which fragment constants (and some atom constants) for a variety of organic species of biological importance are tabulated.

Acknowledgment. We thank Dr. L. Giurato (Department of Pharmaceutical Sciences, University of Catania) for the calculation of clogP, Mr. Andrea Garelli for HPLC/MS analysis, Mrs. Diana Rambaldi for experimental assistance, and Dr. Antonello Villa (Consorzio MIA, University of Milano-Bicocca) for the expertise on experiments using SEM. This work was supported by MURST (ex-60% and COFIN 2004).

Supporting Information Available: ^{1}HNMR and ^{13}CNMR data and spectra for all new compounds and HPLC for key target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Wright A. J. The penicillins. *Mayo Clin. Proc.* **1999**, *74*, 290−307.
(2) Konaklieva, M. I. Beta-lactams as inhibitors of serine enzymes. *Curr. Med. Chem.:-Anti-Infect. Agents* **2002**, *1*, 215−238. As recent examples of beta-lactam inhibitors of HLE see: (b) Singh, R.; Micetich, R. G. Monobactams as enzyme inhibitors. *IDrugs* **2000**, *3*, 512−517. (c) Clemente, A.; Domingos, A.; Grancho, A. P.; Iley, J.; Moreira, R.; Neres, J.; Palma, N.; Santana, A. B.; Valente, E. Design, synthesis and stability of *N*-acyloxymethyl- and *N*-aminocarbonyloxymethyl-2-azetidinones as human leukocyte elastase inhibitors. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1065−1068. (d) Koteva, K. P.; Cantin, A. M.; Neugebauer, W. A.; Escher, E. Synthesis and evaluation of novel β-lactam inhibitors of human leukocyte elastase. *Can. J. Chem.* **2001**, *79*, 377−38.
(3) Cainelli, G.; Galletti, P.; Garbisa, S.; Giacomini, D.; Sartor, L.; Quintavalla A. 4-Alkylidene-azetidin-2-ones: Novel inhibitors of leukocyte elastase and gelatinase. *Bioorg. Med. Chem.* **2003**, *11*, 5391−5399.
(4) Kazi, A.; Hill, R.; Long, T. E.; Kuhn, D. J.; Turos, E.; Dou, Q. P. Novel *N*-thiolated-beta-lactam antibiotics selectively induce apoptosis in human tumor and transformed, but not normal or nontransformed, cells. *Biochem. Pharmacol.* **2004**, *67*, 365−374.
(5) World Health Organization. *Overcoming antimicrobial resistance;* WHO Report on Infectious Diseases, 2000. www.who.int/infectious-disease-report/2000/index.html.
(6) Power, J. H. Development of drugs for antimicrobial-resistant pathogens. *Curr. Opin. Infect. Dis.* **2003**, *16*, 547−551.
(7) Reichert, J. M. Trends in drug development and approval times for new therapeutics in the United States. *Nat. Rev. Drug Discovery* **2003**, *2*, 695−702.
(8) Pastor, M.; Cruciani, G.; McLay, I.; Pickett, S.; Clementi, S. Grid-independent descriptors (GRIND): A novel class of alignment-independent three-dimensional molecular descriptors. *J. Med. Chem.* **2000**, *43*, 3233−3243.
(9) ALMOND Molecular Discovery, Ltd. www.moldiscovery.com.
(10) Bachi, M. D.; Goldberg, O.; Grass, A.; Vaya, J. Properties and reactions of 4-thioxo-2-azetidinones. *J. Org. Chem.* **1980**, *45*, 1481−1484. (b) Prasad, K.; Kneussel, P.; Schulz, G.; Stutz, P. A new method of carbon extension at C-4 of azetidinones. *Tetrahedron Lett.* **1982**, *23*, 1247−1250; (c) Greengrass, C. W.; Hoople, D. W. Reaction of 4-acetoxy-2-azetidinone with tertiary carbanions: Preparation of 4-alkyl- and 4-alkylidene-2-azetidinones. *Tetrahedron Lett.* **1981**, *22*, 1161−1164. (d) Battaglia, A.; Cainelli, G.; Giacomini, D.; Martelli, G.; Panunzio, M. Cycloaddition reaction of heterocumulenes and ester enolates: A novel synthesis of 4-alkylidene-azetidin-2-ones. *Tetrahedron Lett.* **1987**, *28*, 4347−4350.
(11) Cainelli, G.; Giacomini, D.; Galletti, P.; Quintavalla, A. Synthesis of novel 4-(2-oxoethylidene)azetidin-2-ones by a Lewis acid mediated reaction of acyldiazo compounds. *Eur. J. Org. Chem.* **2003**, *9*, 1765−1774
(12) Bogdanov, P. M.; Dabbene, V. G.; Albesa, I.; de Bertorello, M. M.; Brinòn, M. C. Mechanism of antibacterial and degradation behavior of a chlorinated isoxazolynaphthoquinone. *Biochem. Biophys. Res. Commun.* **1999**, *263*, 301−307
(13) Lim, D.; Strynadka, C. J. Structural basis for the β-lactam resistance of PBP2a from methicillin-resistance *Staphylococcus aureus. Nat. Struct. Biol.* **2002**, *9*, 870−876.
(14) Dessen, A.; Mouz, N.; Gordon, E.; Hopkins, J.; Dideberg, O. Crystal structure of PBP2x from a highly penicillin-resistant *Streptococcus pneumoniae* clinical isolate: a mosaic framwork containing 83 mutations. *J. Biol. Chem.* **2001**, *276*, 45106−45112.
(15) Leski, T. A.; Tomasz, A. Role of Penicillin-Binding Protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of *Staphylococcus aureus*: Evidence for cooperative functioning of PBP2, PBP4, and PBP2A. *J. Bacteriol.* **2005**, *187*, 1815−1824.
(16) Jones, R. N.; Erwin, M. E. In vitro evaluation of Ro 09-1227, a novel catechol-substituted cephalosporin. *Antimicrob. Agents Chemother.* **1992**, *36*, 233−238.
(17) Kellogg, G. E.; Semus, S. F.; Abraham, D. J. HINT − A new method of empirical hydrophobic field calculation for CoMFA. *J. Comput.-Aided Mol. Des.* **1991**, *5*, 545−552.
(18) Benzyldiazoacetate is a known compound: Angle, S. R.; Belanger, D. S. Stereoselective synthesis of 3-hydroxyproline benzyl esters from *N*-protected-beta-aminoaldehydes and benzyl diazoacetate. *J. Org. Chem.* **2004**, *69*, 4361−4368, and references citated herein. We synthesized this compound using the procedure as described for similar compounds in: Danheiser, R. L.; Miller, R. F.; Brisbois, R. G.; Park, S. Z. An improved method for the synthesis of alpha-diazo ketones. *J. Org. Chem.* **1990**, *55*, 1959−1964.
(19) *GRID*, version 20; Molecular Discovery, Ltd. (www.moldiscovery.com).
(20) Goodford, P. J. A computational procedure for determining energetically favourable binding sites on biologically important macromolecules. *J. Med. Chem.* **1985**, *28*, 849−857. (b) Boobbyer, D. N. A.; Goodford, P. J.; Mcwhinnie, P. M.; Wade, R. C. New hydrogen-bond potentials for use in determining energetically favourable binding sites on molecules of known structure. *J. Med. Chem.* **1989**, *32*, 1083−1094. (c) Wade, R.; Clerk, K. J.; Goodford, P. J. Further development of hydrogen bond function for use in determining energetically favourable binding sites on molecules of known structure. Ligand probe groups with the ability to form two hydrogen bonds. *J. Med. Chem.* **1993**, *36*, 140−147.
(21) Hansch, C.; Leo, A. *Substituent Constant for Correlation Analysis in Chemistry and Biology*; Wiley and Sons: NewYork, 1979.

JM0580510