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Cite this: DOI: 10.1039/c2md00251e

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CONCISE ARTICLE

Unprecedented inhibition of resistant penicillin binding proteins by bis-2-oxoazetidinyl macrocycles†

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Received 4th October 2011, Accepted 25th November 2011
DOI: 10.1039/c2md00251e

Since the discovery of penicillin, bacteria have counteracted the action of antibiotics leading to a worrisome situation about antibiotic efficiency. During our research on non-traditional 1,3-bridged β-lactams embedded into macrocycles as potential inhibitors of Penicillin Binding Proteins (PBPs), we unexpectedly synthesized bis-2-oxoazetidinyl macrocycles arising from a dimerization reaction under ring closing metathesis (RCM) conditions. These molecules were revealed to be good inhibitors of the D,D-peptidase from *Actinomadura* R39, which is commonly used as a model of PBPs. To pursue the research on this type of novel compounds, a complete family of cyclodimers **4** and **5** was synthesized and evaluated against R39, and high molecular weight D,D-peptidases: PBP2a of methicillin-resistant *Staphylococcus aureus* and PBP5 of resistant *Enterococcus faecium*. Some bis-2-oxoazetidinyl macrocycles exhibited very promising activities against PBP2a. In order to explain the biological results, docking experiments of one cyclodimer (**5e**) into the R39 and PBP2a crystallographic structures were performed. The 3D structures of all the dimers were studied by quantum chemistry calculations and the reactivity of one cyclodimer (**5e**) was evaluated using an elaborate model of the R39 active site. Our results highlighted that the activity of the compounds is most probably related to their conformational adaptability, depending on the size of the macrocycles and the geometrical constraints induced by intramolecular H bonds.

Introduction

The introduction of penicillins (*i.e.* the β-lactam antibiotics) into the health care system is one of the most important contributions to medical science in the 20th century. However a consequence of the high use of antibiotics is the emergence of resistant bacteria. The mechanical strength of the bacterial cell wall is conferred by an essential cross-linked biopolymer, the final step of biosynthesis of which is the transpeptidation of peptidoglycan strands catalyzed by D,D-peptidase enzymes. β-Lactam antibiotics, by inhibiting these D,D-peptidases, also called penicillin-binding proteins (PBPs), disable the normal cross-linking of peptidoglycan and leave the bacteria sensitive to cell lysis.[1] Structural modification of the original PBPs[2] is one of the mechanisms of bacterial resistance[1] and a major example of such a phenomenon is the methicillin-resistant *Staphylococcus aureus* (MRSA). In this pathogen, responsible for nosocomial infections in hospitals, the resistance to β-lactam antibiotics is conferred by the expression of a novel PBP with very low β-lactam affinity, named PBP2a.[3] To face the growing resistance towards existing β-lactam antibiotics, the development of new compounds is required.

Recently, in the course of our research on non-traditional β-lactams (*i.e.* 2-azetidinones) as potential inhibitors of PBPs, we attempted to synthesize large ring 1,3-bridged 2-azetidinones **B** *via* ring-closing metathesis (RCM) of precursors **A** as the key-step for macrocyclization.[4] However, we observed that a cyclodimerization giving the bis-2-oxoazetidinyl macrocycles **C** was the preferred outcome; the desired cyclomonomer **B** was obtained in a single case (R = Boc and $n = 2$) as the minor RCM product (Fig. 1). Nevertheless, the inhibition potential of the β-lactam precursors **A** and RCM products **C** was tested against R39, a low molecular weight D,D-peptidase[5] which is commonly used as a model of bacterial serine-enzymes. Compounds from three families, differing in the nature of the N–C(3) substituent (R = Boc, Me, H) have been evaluated. The precursors **A** of the

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† Electronic supplementary information (ESI) available: Experimental procedures, characterization data, copies of ^{13}C NMR spectra, experiments of temperature coefficients for amide protons, protocols of biochemical evaluation, information on computational chemistry, acyl-enzyme modeling, absolute energies and figures of compounds **4** and **5**. See DOI: 10.1039/c2md00251e

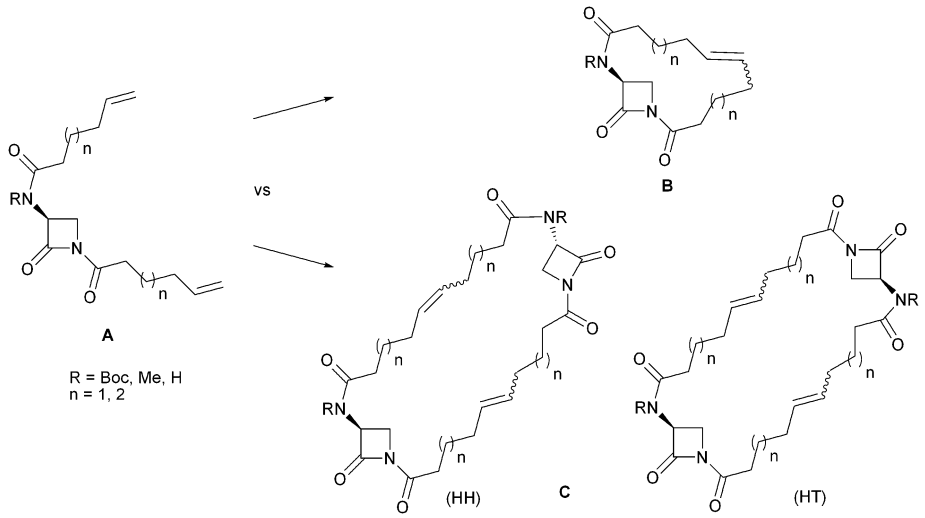


Fig. 1 Cyclic monomer *versus* cyclic dimer.

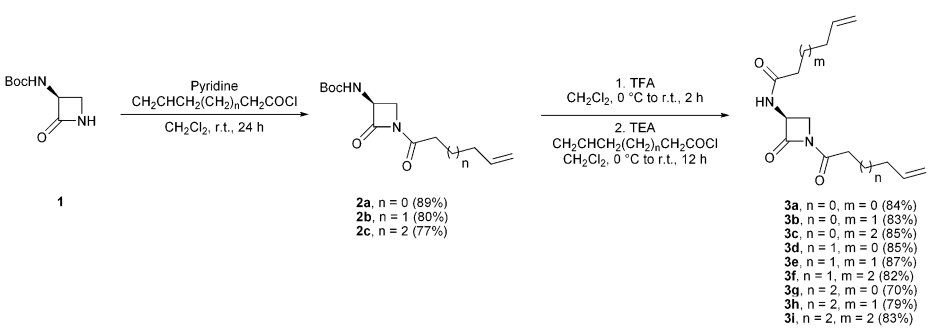
N–H family (R═H) were good inhibitors of R39 and surprisingly, the cyclodimers **C** of the N–H family were even more active than their respective precursors, some of them showing a very good inhibition potential. In order to study more deeply the activity of this series of molecules, non-symmetrical precursors and the corresponding dimers were synthesized, and evaluated against R39, PBP2a and PBP5 (of *Enterococcus faecium*), another example of a high-molecular-weight D,D-peptidase responsible for bacterial resistance.[6] Some bis-2-oxoazetidinyl macrocycles showed promising activities against PBP2a. Non-cyclic dimers were also synthesized to determine if the unique activity of these compounds was due to the geometrical features of the macrocycle or the presence of two β-lactam rings. The 3D structures of all compounds have been studied by quantum chemistry calculations. Molecule **5e** was modelled as an acyl-enzyme docked into the R39 and PBP2a active sites.

Results

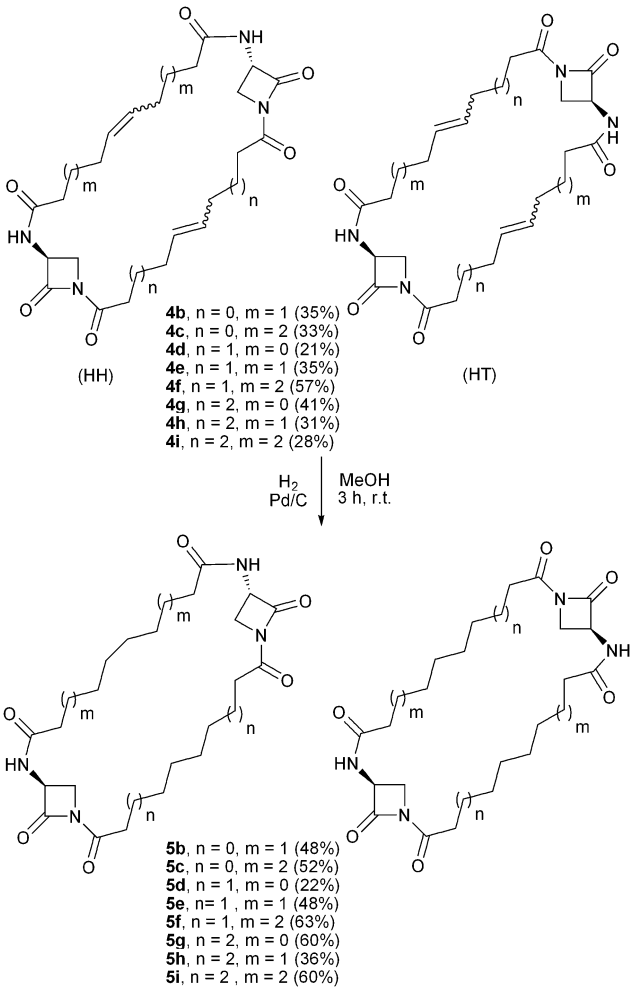
Synthesis

Starting from the chiron **1**,[4] *i.e.* (*S*)-3-(*tert*-butyloxycarbonyl) amino-2-azetidinone, N(1) mono-acylated β-lactams **2a–c** were prepared regioselectively employing pyridine and alkenoyl chlorides. The bis-acylated precursors **3a–i** were then prepared in two steps. The Boc protecting group was removed in the presence of trifluoroacetic acid and the resulting free amine function was acylated with the appropriate alkenoyl chloride using triethylamine as base (Scheme 1). Mono- (**2**) and bis-acylated products (**3**) were isolated in high yields after chromatography.

Among the symmetrical precursors ($n = m = 0, 1, 2$), **3a** could not be cyclized (24-membered cyclic dimer not accessible), while ring closing metathesis (RCM) reaction performed on **3e** and **3i** under standard conditions (CH_2Cl_2, 40 °C, 5 mM) in the presence of second generation Grubbs' catalyst (2 × 5 mol%), afforded 28-membered (**4e**) and 32-membered (**4i**) cyclic dimers (probably mixtures of head–head (HH) and head–tail (HT) regioisomers) in modest yields (35% and 28%, respectively). As previously reported,[4] *ab initio* calculations have suggested the presence of intramolecular H bonds in these cyclic dimers, stabilizing particular conformations of the macrocycle that could explain why the cyclic dimers with R ≠ H (*i.e.* R = Boc, Me) were not as good inhibitors of R39 D,D-peptidase as the N–H derivatives. In order to study the possible influence on the biological activity of the length of the two branches of the cyclic dimers, all the non-symmetrical dimers were similarly prepared. Thus the RCM reaction of **3b**, **3c**, **3d**, **3f**, **3g** and **3h** led respectively to the macrocycles **4b** (26-membered), **4c** (28-membered), **4d** (26-membered), **4f** (30-membered), **4g** (28-membered), and **4h** (30-membered), with yields ranging from 31% to 57% (Scheme 2).



Scheme 1 Synthesis of bis-acylated precursors **3**.



Scheme 2 Symmetrical and non-symmetrical cyclic dimers.

Then the dimers were submitted to catalytic hydrogenation in the presence of Pd/C to afford the corresponding saturated macrocycles **5b–i** (Scheme 2).

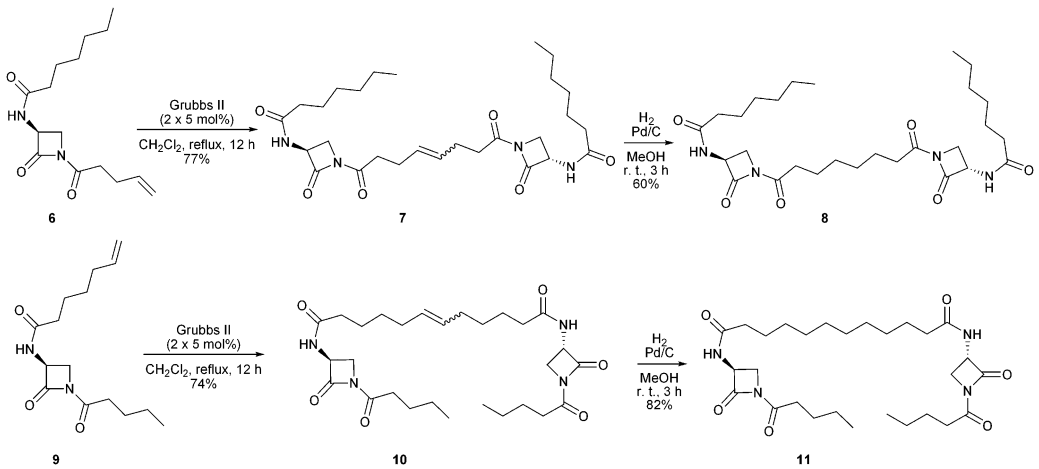
Two non-cyclic dimers were considered as reference compounds for comparison of the biochemical results. Their precursors **6** and **9**, structurally related to the β-lactam **3c** ($n = 0$ and $m = 2$) were prepared by using the same sequence of reactions as depicted before in Scheme 1. Starting from the mono-acylated compound **2a**, the Boc protecting group was removed and the amine function was acylated with heptanoyl chloride and triethylamine giving the bis-acylated β-lactam **6** in 80% yield. Mono-acylation of the chiron **1** in the presence of pyridine and valeroyl chloride (40% yield), followed by Boc deprotection and amine function acylation with hept-6-enoyl chloride and triethylamine afforded **9** in 86% yield. Cross metathesis of precursors **6** and **9** gave the respective non-cyclic dimers **7** and **10** in good yields, and catalytic hydrogenation led to the corresponding saturated products **8** and **11** (Scheme 3).

The accurate structural analysis of the RCM products **4** is a very difficult task due to the presence of non-separable isomers: (i) head–head (HH) and head–tail (HT) regioisomers, (ii) *E* and *Z* stereoisomers because of the uncontrolled configuration of the C=C double bonds. Additionally, the presence of conformers makes the NMR analyses quite complicated (see Experimental section in the ESI†). Using mass spectroscopy, we could observe in some cases contamination products issued from double bond migration (in precursors) leading *in fine* to dimers **4** with the formal extrusion of one CH_2 unit. To avoid this contamination we performed the RCM reactions in the presence of 1,4-benzoquinone,[7] but without achieving the complete disappearance of the lower homologues.

Inhibition of R39, PBP2a and PBP5

All bis-acylated β-lactams, precursors (*i.e.* **3**, **6** and **9**) and dimers (*i.e.* **4**, **5**, **7**, **8**, **10** and **11**), were evaluated for their potential inhibition effect on bacterial serine enzymes.

The D,D-peptidase from *Actinomadura* R39 (ref. 5) is usually considered for a preliminary screening of penicillin-like compounds. R39 and the tested azetidinone (100 μM) were incubated together (1 h, 25 °C). After preincubation the residual activity (RA) of the enzyme was determined by measuring the hydrolysis rate of the thiolester substrate S2d (*i.e.* *N*-benzoyl-D-alanyl-thioglycolate),[8] in the presence of Ellman's reagent (for labeling the formed thiol), by monitoring the increase of absorbance at 412 nm. The results are given in Table 1 as percentages (%) of residual activity. The activity in the absence of an inhibitor



Scheme 3 Non-cyclic dimers.

Table 1 Results of the inhibition experiments with R39, PBP2a and PBP5

		R39	PBP5	PBP2a	
Entry	Cmpd	RA (%)	RA (%)	RA (%)	Cycle size
1	**3a**	>100	79	72	
2	**3b**	14 ± 3	93	98	
3	**3c**	14 ± 11	89	91	
4	**3d**	39 ± 12	100	99	
5	**3e**	17 ± 9	100	90	
6	**3f**	21 ± 27	85	74	
7	**3g**	50 ± 3	77	72	
8	**3h**	15 ± 3	82	86	
9	**3i**	23 ± 0	81	80	
10	**4b**	2 ± 1	92	91	26
11	**4c**	3 ± 1	95	73	28
12	**4d**	9 ± 1	86	84	26
13	**4e**	4 ± 8	90	94	28
14	**4f**	1 ± 2	98	78	30
15	**4g**	2 ± 2	96	100	28
16	**4h**	3 ± 1	100	100	30
17	**4i**	0	89	84	32
18	**5b**	2 ± 1	70	70	26
19	**5c**	0	100	29	28
20	**5d**	0	100	55	26
21	**5e**	2	90	70	28
22	**5f**	1 ± 1	100	22	30
23	**5g**	0 ± 3	100	33	28
24	**5h**	2 ± 1	72	63	30
25	**5i**	0	95	61	32
26	**6**	6 ± 0	100	92	
27	**7**	0	100	100	
28	**8**	10 ± 4	99	74	
29	**9**	5 ± 2	100	97	
30	**10**	5 ± 2	89	90	
31	**11**	3 ± 4	90	89	

is set at 100% and therefore low values indicate very active compounds, since the enzyme has been inhibited by the tested compound and consequently cannot hydrolyze its substrate. A tested compound is considered as a "hit" (*i.e.* potential inhibitor of interest) for a RA value below 80%.

Except one product amongst the precursors (entry 1, *i.e.* compound **3a**), all the other precursors and dimers inhibited R39, and the dimers were generally more active than their precursors.

Then all the compounds were evaluated against two high-molecular-weight D,D-peptidases responsible for bacterial resistance to β-lactam antibiotics: PBP2a from *S. aureus* and PBP5 from *E. faecium*.

PBPs and the tested azetidinone (1 mM) were incubated together (4 h, 30 °C). Then fluorescein-labelled ampicillin[9] was added to detect the residual activity (RA). In this protocol, the tested compounds were supposed to be capable of acylating the PBPs (to give stable acyl-enzyme intermediates), and the residual activity of PBPs is determined by the amount of covalent PBP–ampicillin complexes formed, as measured by fluorescence spectroscopy after denaturation and SDS-PAGE separation of the acylated enzymes from the reagent band.

Only a small activity against PBP5 was detected for two precursors (entries 1 and 7) and two saturated cyclic dimers (entries 18 and 24). Several products exhibited a small to moderate activity against PBP2a (entries 1, 6, 7, 9, 11, 14, 18, 20, 21, 24, 25 and 28) but amongst the hydrogenated non-

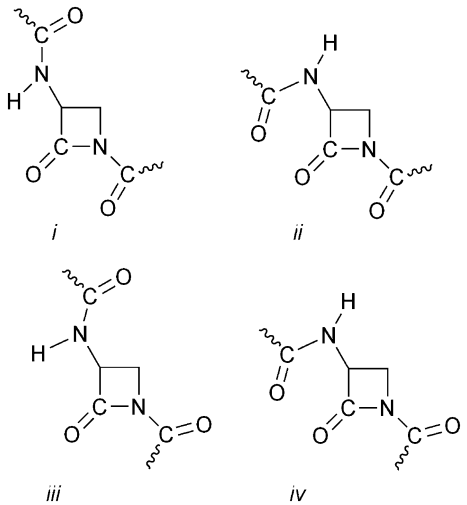


Fig. 2 Conformations (*i*), (*ii*), (*iii*) and (*iv*) of the precursors.

symmetrical cyclic dimers, three compounds, namely **5c**, **5f** and **5g** (entries 19, 22 and 23), showed a very good inhibition potential.

None of the non-cyclic dimers had a significant activity on PBP2a, therefore the activity could not be correlated simply to the presence of two β-lactam rings. We hypothesized that geometrical factors imposed by the macrocycles should be the determining factors.

Computational chemistry

The geometry of all the molecules has been fully optimized at the B3LYP level[10] using the 6-31G(d) basis set.[11] A great number of local minima could be trapped for precursors **3**. Four conformations of the precursors referred to as (*i*), (*ii*), (*iii*), and (*iv*) have been located (Fig. 2). The C(3) amide function exhibits the *trans* geometry in all cases;[4] the conformers result from rotations around the C(3)–N and N(1)–CO bonds. The four conformations lie in the same range of stability, the relative energies being less than 7 kcal mol^{-1}; the (*iii*) conformation is always the most stable one (Table 2) (all absolute energies of the precursors in the selected conformations are given in the ESI†).

A lot of conformers can exist for the dimers **4** (only the *trans* C=C configuration has been considered). Two conformations of the β-lactam can be located giving rise to a head–head (HH) or

Table 2 Relative energies of the precursors in the selected conformations

	Relative energy of open precursors/kcal mol^{-1}			
Compound	(*i*)	(*ii*)	(*iii*)	(*iv*)
3a	4.73	5.50	0.00	0.33
3b	4.61	6.84	0.00	1.52
3c	4.71	5.64	0.00	0.40
3d	5.11	5.60	0.00	0.10
3e	4.82	6.27	0.00	0.91
3f	4.62	5.64	0.00	0.13
3g	4.80	5.51	0.00	0.07
3h	4.57	5.53	0.00	0.13
3i	4.88	5.71	0.00	0.37

Table 3 Relative energies and heats of formation of HH and HT unsaturated dimers and relative energies of HH and HT saturated dimers

Unsaturated dimer	Relative energy of HH unsaturated dimer/kcal mol^{-1}	Heat of formation of HH unsaturated dimer/kcal mol^{-1}	Relative energy of HT unsaturated dimer/kcal mol^{-1}	Heat of formation of HT unsaturated dimer/kcal mol^{-1}	Saturated dimer	Relative energy of HH saturated dimer/kcal mol^{-1}	Relative energy of HT saturated dimer/kcal mol^{-1}
4b	0.00	1.13	5.30	6.43	**5b**	0.00	6.93
4c	14.00	14.24	0.00	0.24	**5c**	8.61	0.00
4d	19.06	17.65	0.00	−1.40	**5d**	12.39	0.00
4e	9.59	3.93	0.00	−5.66	**5e**	8.70	0.00
4f	0.00	2.32	7.36	9.68	**5f**	0.00	8.26
4g	14.21	17.22	0.00	3.01	**5g**	14.62	0.00
4h	0.00	11.08	1.85	12.93	**5h**	0.00	0.43
4i	0.00	−1.81	2.43	0.62	**5i**	0.00	1.52

head–tail (HT) arrangement in the macrocycle. There are as many stable HT conformers (*i.e.* **4c**, **4d**, **4e**, **4g**) as stable HH conformers (*i.e.* **4b**, **4f**, **4h**, **4i**).

The heats of formation of the dimers have been calculated with respect to the most stable conformation of their respective open precursor which is the (*iii*) conformation (Table 3) (all absolute energies of the HH and HT conformers are given in the ESI†). The heat of formation of the most stable conformers is negative or slightly positive.

For saturated compounds **5** (Table 3), the conformations often remain in the same local minima as for the unsaturated ones and their relative energies are slightly modified.

Docking

All the cyclodimers display high activities against R39, while some saturated cyclodimers have good activities against PBP2a. So we focused on the saturated cyclodimers **5** to understand their activities.

The molecule **5e** has been docked as an acyl-enzyme complex in the R39 (Fig. 3a) and PBP2a (Fig. 3b) cavities. This symmetrical cyclodimer, featuring a medium activity against PBP2a, has been selected as a representative compound of the whole series **5**.

Using the crystal structures of complexes of R39 and PBP2a[3c] with β-lactam antibiotics as templates, **5e** was docked into their respective active sites, conserving the main interactions found in the X-ray structures. The HH conformer has been considered, according to the reactivity model (see below). As expected, **5e** adapted perfectly to the geometrical constraints of the R39 cavity (RA of **5e** *versus* R39 = 2%). The acyl-enzyme model showed that the alkyl chain of the macrocycle can run into a mainly hydrophobic cavity that normally receives the side chain of the D-α-aminopimelic acid, one of the amino acids constituting the pentapeptide substrate. Consistent with the measured activity (RA = 70% *versus* PBP2a), **5e** fitted quite well into the PBP2a cavity although the cavity is narrower than in R39 (Fig. 3). The residues of PBP2a surrounding the alkyl chain of the macrocycle are not hydrophobic. The flexibility of the macrocycle **5f**, the most active non-symmetrical dimer *versus* PBP2a, allowed the docking of both HH and HT isomers in the active site as acyl-enzymes. The docking in the PBP2a active site highlighted the possibility for the whole macrocycle to completely fill the cavity with the closed lactam cycle sandwiched between Tyr446 and Met641. (The figures of macrocycle **5f** of both HH and HT isomers docked as an acyl-enzyme complex in the PBP2a cavity are given in the ESI†.)

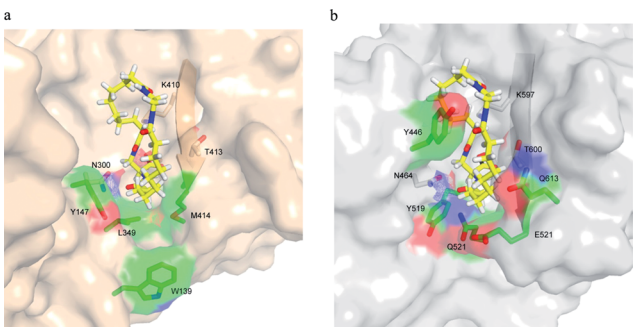


Fig. 3 (a) Acyl-enzyme complex between **5e** and R39. (b) Acyl-enzyme complex between **5e** and PBP2a. Legend: residues surrounding the alkyl chain of the macrocycle in the bottom of the active site are highlighted with carbon atoms coloured green, nitrogen blue and oxygen red. The carbon atoms of **5e** are coloured yellow and hydrogen white. The active serine is hidden by the ligand.

The explanation of the difference between non-resistant and penicillin-resistant PBP activity remains an open question. The main feature could be related to the access to the nucleophilic serine of resistant PBPs which is more ploughed in than the R39 one. So we hypothesized that the conformational flexibility of the bis-2-oxoazetidinyl macrocycles could favor their insertion into the "closed" conformation of PBP2a.

The significant differences in the biological results between unsaturated (**4**) and the corresponding saturated macrocycles (**5**) could also be due to conformational features.

Reactivity

To go a step further, the potential acylating power of the bis-2-oxoazetidinyl macrocycle **5e** (HH dimer) has been estimated by computational chemistry. The reaction considered is the concerted β-lactam ring opening by nucleophilic attack of the active serine residue. The location of the transition state (TS) structure has been performed with an elaborated model of the R39 active site representing the three conserved motifs of the PBP family, as reported from the X-ray data.[5] The first motif is composed of the nucleophilic serine Ser49 and the following amino acids Asn50, Met51 and Lys52. The second and third motifs are formed by Ser298, Asn299 and Asn300, and Lys410, Thr411, Gly412 and Thr413, respectively.[12] The TS structure has

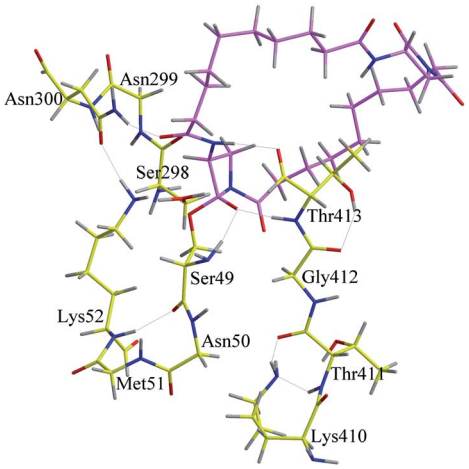


Fig. 4 Compound **5e** at the transition state in the model of the R39 active site. Legend: C of **5e** are in purple, others are in yellow, H are in grey, O are in red, N are in blue, and hydrogen bonds are shown as thin sticks.

been fully optimized in all the directions given by the degrees of freedom at the RHF/MINI-1′ level[13] (216 atoms, 632 basis functions) (Fig. 4). (The stereo view corresponding to Fig. 4 is given in the ESI†.) At this equilibrium structure, Ser49, Lys52 and Ser298 side-chains form a pseudo-8-membered ring with the C(2)–N(1) bond of one β-lactam ring in order to transfer the Oγ hydrogen to the β-lactam N(1) *via* the Lys amino group.

Similarly to the docking, to be well orientated into the model (and hence into the active site), the carbonyl of the amide on position C(3)–N has to rise below the 4-membered ring and the acyl chain of the macrocycle has to expand to the right upper corner C(4) of the processed β-lactam. This precise type of conformation of one β-lactam ring has been chosen for the representation of compounds **5c**, **5f** and **5g** depicted in Fig. 5 (all figures of compounds **4b–i** and **5b**, **5d–i** are given in the ESI†). This type of arrangement was the one used for the docking of compound **5e**.

Depending on the size of the cycle growing from 26 to 32 bonds, the "pitch of screw" has a great influence on the conformation either in the HH or the HT arrangement. For some molecules, a conformation where the acyl chain of the macrocycle is more orientated towards the carbonyl C(2) of one β-lactam ring, is localized for **4/5c**, **4/5d** and **4/5e** HT geometries. This could be related to the inadequacy of this conformation to accommodate the active site. The molecules **4/5c**, **4/5e** and **4/5g** containing the same number of methylene units (28-membered ring) are interesting as they can adopt an active-site compatible geometry in their HH arrangement (Fig. 5 and 6). In the case of **4/5i** (32-membered ring), the conformation looks the same in HH and HT arrangements. All the optimized geometries are local minima. What is remarkable is the ability of some molecules to adapt their conformation to the active site. An example is detailed with compound **5e**. The HT geometry is the most stable with regard to the HH(1) one which is 8.70 kcal less stable. A modification of the geometry has been applied to dock this molecule into R39 as well for the localisation of the TS structure in the theoretical model. Starting from these two different

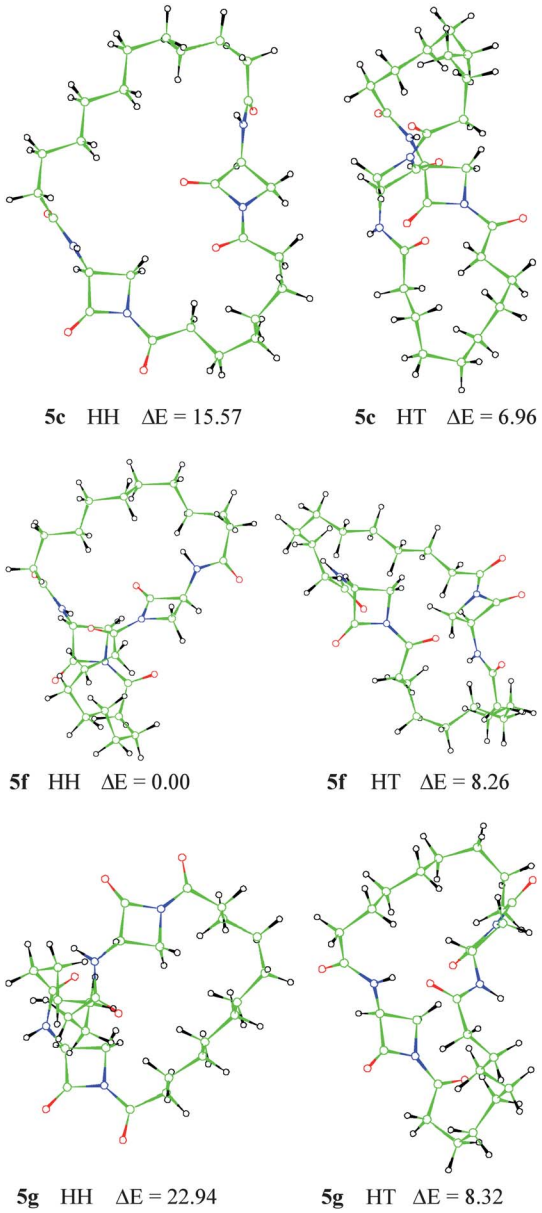


Fig. 5 Representative conformers of **5c**, **5f** and **5g**. Relative energies are given in kcal mol^{-1}.

geometries, a reoptimization at the B3LYP/6-31G(d) level leads to two other local minima HH(2) (from docking) and HH(3) (from the theoretical model) with relative energies of 9.51 kcal and 14.73 kcal (Fig. 6). The biological results could be related to the conformational adaptability of the compounds which is a tenuous equilibrium between the size of the cycle and the geometric constraints imposed by its conformation.

Discussion

All the dimers **4/5** exhibit a high activity against a non-resistant PBP (R39) and solely some saturated cyclodimers **5** show a good activity against a resistant PBP (PBP2a). The fine analysis of the behaviour of the bis-2-oxoazetidinyl macrocycles **4/5** is a problem of huge complexity, for a lot of reasons. The RCM

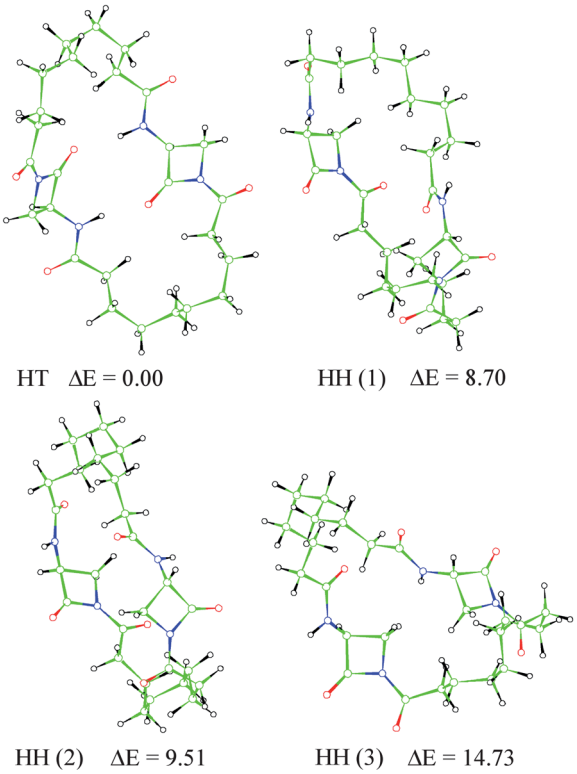


Fig. 6 Conformers of **5e**. Relative energies are given in kcal mol^{-1}.

reaction affords most probably a distribution of HH and HT regioisomers, as well as mixtures of *E*/*Z* olefins. For the hydrogenated compounds **5** the problem of *E*/*Z* geometry has been suppressed, but there are still HH and HT isomers for which the distribution product is not known. For each regioisomer, several conformations exist. The conformers are more or less stabilized and present different geometrical constraints due to the presence in the cyclodimers of intramolecular H bonds. Proton NMR experiments were performed in order to determine if the amide protons of cyclic dimers were involved in intramolecular H-bonding. One amide proton of **5c** exhibited a different behaviour compared to the non-cyclic dimer **11**, which supported the involvement of one intramolecular hydrogen bond in the cyclic dimer (results are given in the ESI†).

Using a model of the R39 active site, we have determined the geometrical requirements necessary to form the transition state of the acyl-enzyme complex: (i) the macrocyclic chain has to expand to the right upper corner C(4) of the processed β-lactam; (ii) the carbonyl of the amide on position C(3)–N has to rise below the β-lactam ring to allow the interaction with the NH of the Asn300 amino acid residue.

This model of reactivity could also be applied to the case of PBP2a that shares with R39 the same three conserved motifs in the catalytic cavity. The differences are to be found at the entry and at the bottom of the respective cavities.[3,5] Globally, the active serine of PBP2a is less accessible, because it is more buried into the active site.

Amongst the saturated cyclodimers **5**, because of the intramolecular H bonds, compounds are more or less distorted and some of them look like a screw. This structural feature is more pronounced for the non-symmetrical dimers compared to the symmetrical ones of the same size. As a matter of fact, the best activities were recruited for the "non-symmetrical" compounds (**5c**, **5d**, **5f**, and **5g**).

The poor activity (or the absence of activity) of all the dimers **4/5** against PBP5 could not be rationalized. Ceftobiprole, a cephalosporin derivative with high affinity for PBP2a of MRSA, is not similarly active against PBP5 of *E. faecium*. The role of the antibiotic side-chains (at positions 7 on the β-lactam ring and 3 on the fused six-membered ring) on their specificity is not yet elucidated.[14] These chains are supposed to contribute to the good positioning of the β-lactam carbonyl *versus* the active serine of the target PBP. In particular, the different chains fixed on the cephalosporin fused ring (ceftobiprole and ceftazidime, for example) could behave as a "lever arm" inducing an unpredictable inhibition pattern against the set of PBPs.[15] So, it is not surprising that our compounds show different affinities for PBP2a and PBP5.

Conclusion

Currently, the mode of action of non-resistant and resistant PBPs, at the molecular level, is not fully understood. The comprehension of molecular interactions between proteins and small molecules relies on complementary studies based on experimental (measured activities, X-ray structures) and theoretical data (modeling, docking experiments), making the foundation of structure-based drug design.[16]

Herein we have disclosed a novel family of β-lactam compounds capable of acylating PBPs, namely the bis-2-oxoazetidinyl macrocycles. 26- to 32-Membered bis-2-oxoazetidinyl macrocycles devoid of side-chain (R=H) are synthesized using the RCM reaction as the key-step for cyclodimerization. All the compounds are good inhibitors of R39; some saturated macrocycles reveal very promising activities against PBP2a of MRSA while the corresponding linear dimers are inactive confirming that the activity is not imputable to the presence of two β-lactam cores in the molecules. The structure of our novel inhibitors is totally different from that of penicillins or cephalosporins, and abolishes the traditional dogma linked to the β-lactam antibiotics, such as the requirement of a 5- or 6-membered cycle fused to the N(1)–C(4) atoms of the β-lactam ring, and the presence of a carboxylic function on this fused ring.

Geometrical and conformational factors have been identified to be responsible for the activity of all compounds against R39 D, D-peptidase, and of some compounds against PBP2a from MRSA. The activity of lipophilic β-lactams (such as **4** and **5**) could be related to the specificity of PBP2a which processes only cell-wall precursors bearing a pentaglycine strand attached to a lysine residue of the stem peptide.[17] The bis-2-oxoazetidinyl macrocycles which feature branches of different lengths for connecting the two four-membered rings are the most active ones against PBP2a (*i.e.* compounds **5c**, **5d**, **5f**, **5g**). Due to the occurrence of intramolecular H-bonds inducing geometrical constraints, the so-called non-symmetrical dimers **5** look like screws. Their pitch of screw, depending on the macrocycle size with the optimum for 28-membered cycles, combined with their high conformational adaptability could probably explain why, for instance, the compounds **5c** and **5g** compared to **5e** are able to

slip in, and to adapt to the closed conformation of the PBP2a active site. In the context of the urgent need of new antimicrobial agents with activity against MRSA and other resistant pathogens, our work provides an alternative approach for the rational design of active β-lactams.

Acknowledgements

This work was supported by the Interuniversity Attraction Pole (IAP P6/19 PROFUSA), F.R.S.-FNRS, UCL and ULg (computational facilities). G.D. and J.M.-B. are senior research associates of the F.R.S.-FNRS (Belgium). Ir. Raoul Rozenberg and Dr Cécile Le Duff have contributed to the structural analysis. Chafia Ouali (University Denis Diderot Paris VII) is thanked for her assistance in the synthesis of some compounds.

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