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Original article

Large ring 1,3-bridged 2-azetidinones: Experimental and theoretical studies

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ABSTRACT

The relationship between angular strain and (re)activity of bicyclic 2-azetidinones is still an open question of major concern in the field of penicillin antibiotics. Our study deals with original 13-membered-ring 1,3-bridged 2-azetidinones related to the carbapenem family, and featuring a "planar amide" instead of the "twisted amide" typical of penam derivatives. The bicycles **11** and **12** were obtained from acetoxy-azetidinone **7**, via the key-intermediate **10**, by using the RCM (ring closing metathesis) strategy. Theoretical predictions and experimental results of hydrolysis showed that the large bicycle **12**, endowed with high conformational flexibility, is more reactive than the bicycle **11**, including a C=C bond of *E* configuration, and the monocyclic 2-azetidinone precursor **10**. The processing of 2-azetidinones **10**–**12** in the active site of serine enzymes has been computed by *ab initio* methods, considering three models. Due to geometrical parameters of the enzymic cavity (nucleophilic attack from the α-face), precursor **10** was predicted more active than **11** and **12** in the acylation step by Ser–OH. Indeed, bicycles **11** and **12** are modest inhibitors of PBP_{2a}, while **10** is a good to excellent inhibitor of PBP_{2a} and R39 bacterial enzymes.

1. Introduction

Antibiotics of the penicillin family are characterized by the presence of a β-lactam ring involved into a quite rigid bicyclic structure (Scheme 1, **1** and **2**). It is widely accepted that the high reactivity of β-lactam antibiotics results from the lack of amide resonance in the 2-azetidinone ring [1–3]. In penams (**1**), penems (**2**, X = S) and carbapenems (**2**, X = CH_2), the π electrons of the N–C=O function cannot be delocalized over the three atoms owing to the structural constraints which prevent the amide substituents to lie in the same plane. The deviation versus coplanarity (i.e. pyramidality of the bridge-head nitrogen atom) is usually expressed by Woodward's [4–6] or Burton's [7] parameters.

The experimental and theoretical interest of the scientific community in the so-called "twisted amides" [8] has been constant since more than 60 years, as exemplified with the recent achievement of the 2-quinuclidone (**3**) synthesis in 2006 [9]. In the field of antibiotics, [2] a lot of works has been devoted to the search of more strained, fused bicyclic β- [10,11] and γ- [12,13] lactam systems. However, there is still no clear relationship between the lactam structural features (*N*-pyramidality, bond angles and distances), chemical reactivity and biological activity.

Our aim is to explore a totally different route for designing reactive bicyclic β-lactams. During the 2-azetidinone ring opening under nucleophilic attack (basic hydrolysis or processing by a serine enzyme), an important conformational reorganisation of the other ring has to occur. The conformational flexibility of this second ring, namely the capacity to adjust itself to new geometrical parameters, should decrease the activation barrier of the β-lactam N1–C2 bond cleavage. To investigate this idea, we decided to prepare large ring 1,3-bridged β-lactam compounds (Scheme 2) and to study their reactivity, theoretically and experimentally. In contrast with fused lactams, bridged lactams as potential antibiotics are scarcely described in the literature; we found only three relevant examples corresponding to skeletons **4** [14], **5** [15] and **6** [16], but these structures display high angular strain and therefore, twisted amide bonds (Scheme 1).

In this article, we illustrate the study of conformationally flexible 1,3-bridged β-lactams with the 13-membered-ring compounds **11** and **12** (Scheme 3) derived from acetoxy-azetidinone **7**, commonly used as precursor of thienamycin derivatives (carbapenem family) [17]. We selected RCM reaction (Ring Closing Metathesis) [18–20] as key-step for forming the large ring (Scheme 2), the required precursor being readily accessible by sequential functionalisation of nitrogen N1 and deprotected hydroxyl group on C5. We chose acylation reactions to introduce the two side-chains for the following reasons: (i) the synthetic easiness providing a rapid access to compounds of interest; (ii) the

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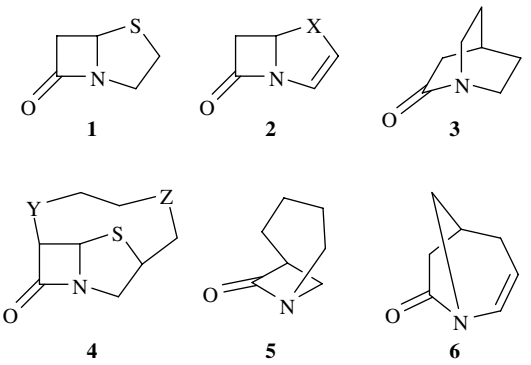


Scheme 1. Bicyclic lactams with "twisted amide" bond (skeletons are drawn with omitting the substituents).

potential biological activities of the novel β-lactams, since N1-acylated compounds have been previously recognized as inhibitors of various proteases [21–24].

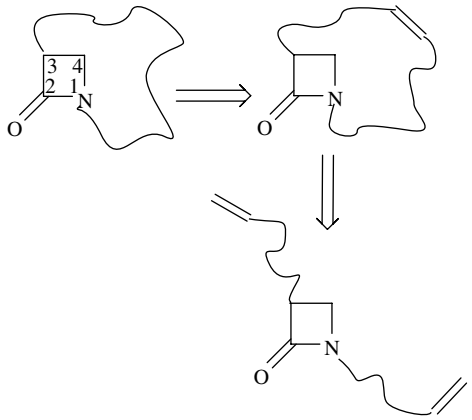
A complete comparative study of the chemical and biochemical reactivity of β-lactams **10**–**12** has been performed. Theoretical predictions could be experimentally confirmed, that pointed out the importance of conformational flexibility. Moreover, interesting activities for the inhibition of serine enzymes were recruited.

2. Results and discussion

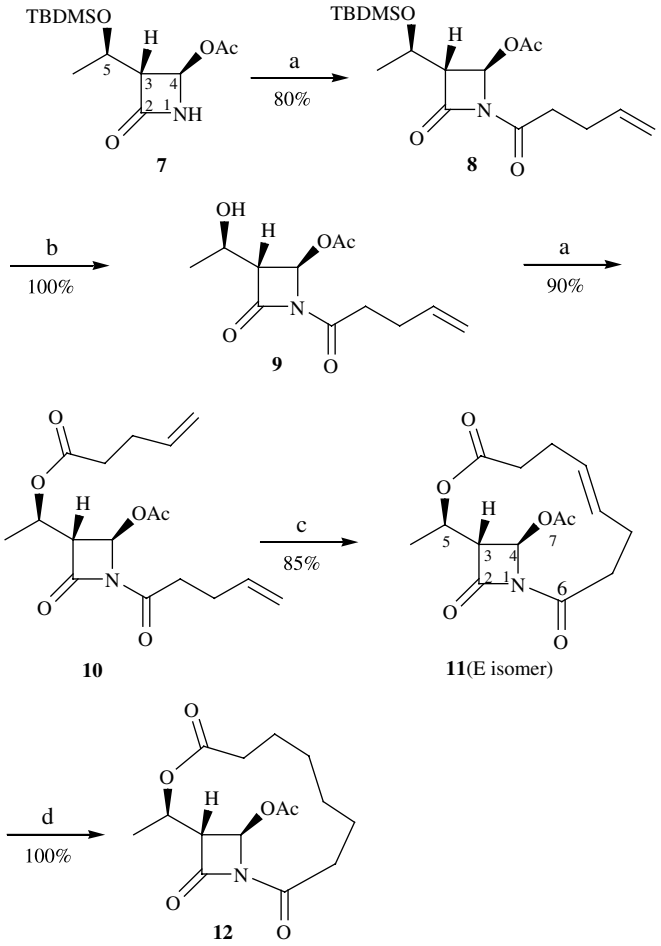
2.1. Synthesis (Scheme 3)

Commercially available (3*R*, 4*R*, 5*R*) chiron **7** was transformed into precursor **10** in three steps with an overall yield of 72%. *N*-Acylation with 4-pentenoyl chloride was conducted in refluxing dichloromethane (DCM), in the presence of pyridine. Silyl ether deprotection of **8** was not effective by using cesium fluoride in methanol, and gave poor yields by using tetrabutylammonium fluoride in aqueous acetic acid and THF solution [25]. The protocol established by Murakami et al. [26] for the synthesis of oxacephems afforded the best results: alcohol **9** was quantitatively obtained by treatment of **8** with HCl–AcOH in acetonitrile at 0 °C. The purity of crude **9**, controlled by ^{1}H NMR, was judged adequate to directly perform the next step. Thus **10** resulted from the reaction of crude alcohol **9** with 4-pentenoyl chloride and pyridine in DCM at room temperature. After chromatographic purification, the product was recovered in 90% yield.

The RCM key step was readily performed under Ahn's conditions [27], i.e. with Grubbs catalyst of second generation (5 mol%), in



Scheme 2. General strategy towards large ring 1,3-bridged β-lactams.



Scheme 3. Synthesis of 13-membered 1,3-bridged 2-azetidinone. a) Cl-CO–$(CH_2)_2$–CH=CH_2, pyridine, CH_2Cl_2, reflux or 20 °C; b) HCl–AcOH (5:7), CH_3CN, 0 °C; c) Grubbs catalyst (2.5 mol%), toluene, 80 °C; d) H_2, Pd–C, EtOAC, 20 °C.

diluted solution of **10** (5 mM in DCM), at room temperature. Only one cyclized compound was formed (TLC and NMR analysis of the crude mixture) corresponding to **11** with the *E* configuration of the C=C double bond ($J_{H,H}$ = 15.5 Hz). Purification by column chromatography on silica gel afforded 85% yield of **11** which structure was confirmed by X-ray diffraction analysis of a single crystal shown in Fig. 1. The main bond lengths, angles, torsions and distances from the plan, characteristic of the bicycle **11**, are summarized in Table 1.

Finally, catalytic hydrogenation led quantitatively to the saturated bicyclic azetidinone **12**.

All the spectroscopic data (Section 4) indicated that the azetidinones **10**–**12** are "non-twisted" amides (Table 2). The IR spectra showed very similar values of carbonyl stretching frequencies for monocyclic (**10**) and bicyclic compounds (**11** and **12**). The β-lactam protons H3 and H4 also displayed the same pattern for the three compounds **10**–**12**, but differences arose for H5 proton of the 1-hydroxyethyl side-chain between monocyclic (**10**) and bicyclic β-lactams (**11**, **12**). However, the two bicyclic derivatives are quite similar considering the dihedral angle H4–C4–C5–H5 (see *J* values). The ^{13}C chemical shifts of the C=O functions were found to be almost identical in compounds **10**–**12**. The X-ray structure of **11** (Fig. 1) confirmed the planarity of the amide β-lactam bond. The typical features of **11** compared to classical penicillins (penams **1** [28] and penems **2** [29]) are summarized in Table 3: the large ring 1,3 bridged 2-azetidinone (**11**) is definitively not a penicillin-like β-lactam compound.

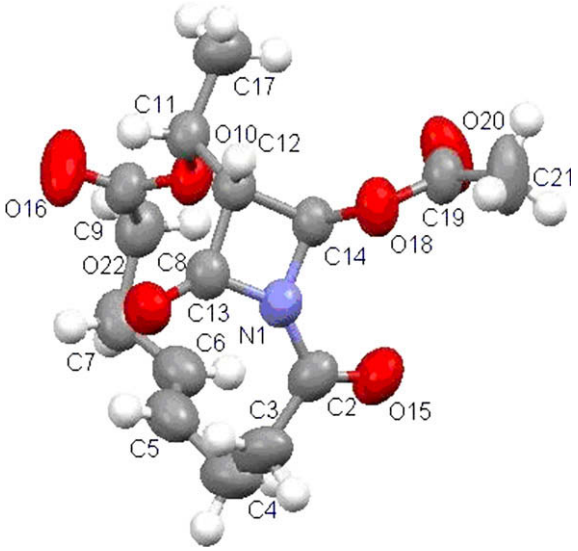


Fig. 1. X-ray structure of **11** (ORTEP view).

2.2. Conformational study – heat of formation

A lot of conformers can exist for the monocyclic molecule **10**. Considering rotations around N1–C6, C4–O7 and C3–C5 bonds (see numbering of Scheme 3), and a minimum of 2 conformers per rotations, at least 8 conformers could be drawn! In the bicyclic compounds **11** and **12**, rotation around C3–C5 being suppressed, a minimum of 4 conformers could be associated with each structure. This has been examined by *ab initio* calculations in vacuum (B3Lyp with the double basis set 6-31G(d)). Several conformers of the precursor **10**, the 1,3-bridged compound **11** with *E* or *Z* configuration of the double bond, and final compound **12** have been localized on the potential energy surfaces. Three representative conformers are pictured in Scheme 4 and shown in Fig. 2 for compound **11**. In conformer I, the two imide carbonyls are aligned and the 13-membered bridge unfolds up to the β-face of the four-membered ring. A complete turning of the C3 thienamycin-like side-chain gives rise to the more stable conformer II with the large cycle covering the azetidinone α-face. The same situation is found in the most stable conformer III, but with an antiparallel arrangement of the C2 and C6 carbonyls. For all conformers, the *E* stereoisomer appears more stable than the *Z* one (see Scheme 4). Conformer III (*E* configuration) is precisely the one rooted in the crystalline solid state of **11** (Figs. 1 and 2).

In solution, the coexistence of conformers for the bicycle **11** (or **12**) was not experimentally visible by NMR spectroscopy. However, conformers were detected in the case of the monocyclic precursor **10**. ^{1}H NMR spectrum recorded in D_2O (25 °C, addition of ≤5% d_6-DMSO for complete dissolution) showed two set of signals in the 3:1 ratio: 6.55 δ and 6.31 δ for H4, 5.33 δ and 5.21 δ for H5, 3.74 δ and 3.38 δ for H3, 2.13 δ and 2.00 δ for $MeCO_2$, 1.35 δ and 1.29 δ for MeCH5 (Fig. 3). Coalescence occurred at 72 °C, giving the same pattern as the spectrum recorded in $CDCl_3$ at 25 °C (see Section 4).

The heat of formation of **11** by RCM reaction was calculated for both stereoisomers, *E* and *Z*, by reference to the open precursor **10** minus ethylene, in the respective conformations I–III (Table 4). The *E* stereoisomer was found to be more stable than the *Z* one, in agreement with the experimental results (NMR and X-ray data). For all considered conformers, cyclisation of **10** into *E*-**11** is favoured by about 5–7 kcal mol^{-1}. The reaction is slightly endothermic in the case of conformer III in which the ester function (C3 side chain) is orientated on the outside of the β-lactam ring and the carbonyl dipoles of the imide function are opposite.

Table 2
Selected spectroscopic data.

Cmpd	IR (C=O)[a] (film, cm^{-1})	^{1}H NMR (δ, mult., *J*)[b] (500 MHz, $CDCl_3$, ppm)	^{13}C NMR (C=O carbons)[c] (125 MHz, $CDCl_3$, ppm)
10	1806, 1742, 1722	6.48 (d, 1.6 Hz), 3.28 (dd, 1.6; 5.8 Hz), 5.29 (dq, 1.8; 6.4 Hz)	162.2, 168.8, 169.2, 171.7
11	1809, 1742, 1716	6.53 (d, 1.6 Hz), 3.19 (dd, 1.6; 1.8 Hz), 5.58 (dq, 1.8; 6.7 Hz)	163.0, 169.1, 170.7, 171.6
12	1807, 1743, 1716	6.50 (d, 1.5 Hz), 3.20 (dd, 1.5; 1.7 Hz), 5.53 (dq, 1.7; 6.9 Hz)	163.5, 169.0, 171.1, 171.9

[a] ν Values are given in the following order: β-lactam, ester and lactone, imide carbonyl functions.
[b] δ Values are given in the following order: H4, H3 and H5 (numbering of Scheme 3).
[c] δ Values are given in the following order: C2, OAc, C6, lactone (numbering of Scheme 3).

2.3. Reactivity towards hydroxyl anion

Basic hydrolysis of β-lactams has been intensively studied, theoretically [30–34] and experimentally [35,36]. Ring opening is a two-step process involving (i) the formation of a tetrahedral intermediate by nucleophilic addition of OH^- onto the azetidinone carbonyl C2 atom, followed by (ii) the cleavage of the N1–C2 bond, this second step being the rate determining one.

Heat of formation and structure of the tetrahedral intermediates obtained by addition of OH^- onto the β-lactam C=O bond of **10**–**12** (conformer III) have been calculated and compared to a penam reference (Table 5). The N1–C2 bond length is indicative of the intrinsic reactivity of the studied structures: the longer bonds correspond to the more reactive azetidinones. Thus the predicted order of reactivity versus OH^- is as follows: **12** > **11** > **10**. However, our compounds appeared less reactive than the strained penam derivative. The computational results are interesting since the simple chemical "intuition", in this special case, could not be reliable.

Fortunately, the previous order of reactivity could be experimentally confirmed, thus establishing the validity of our computational approach. Basic hydrolysis of azetidinones **10**–**12** was

Table 1
X-ray data for **11** (for atom numbering, see Fig. 1).

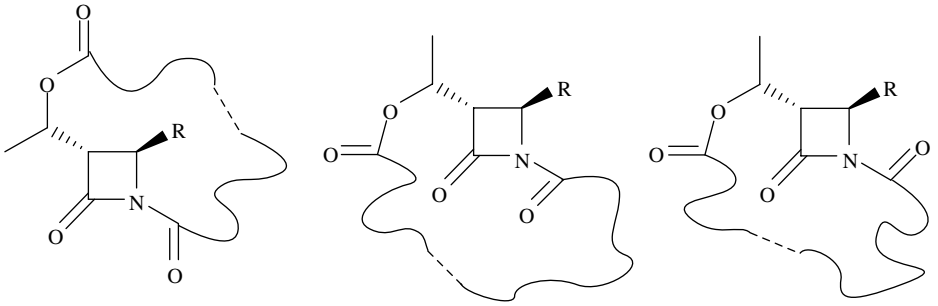
Lengths (Å)		Angles and torsions (°)		Distances[b] (Å)	
N1–C13	1.395 (4)	C13–N1–C14	93.5 (2)	*O22	+0.011
N1–C14	1.465 (4)	C14–N1–C2	127.6 (3)	*C13	+0.034
C14–C12	1.534 (4)	C13–N1–C2	137.4 (3)	*N1	−0.056
C13–C12	1.513 (5)	C13–N1–C14–C12	2.1 (2)	*C2	−0.041
C13–O22	1.200 (4)	C2–N1–C13–O22	9.9 (6)	*O15	+0.052
N1–C2	1.382 (4)	C13–N1–C2–O15	169.1 (3)	C12	+0.225
C2–O15	1.206 (4)	C2–N1–C14–C12	169.9 (3)	C14	+0.172
C6–C5	1.275 (6)	C4–C5–C6–C7	173.0 (4)	h[a]	0.096

[a] N1 distance, in the middle of the calculated plan from atoms linked to N1.
[b] Distances from the best mean plane through the atoms marked by *.

Table 3
Structural features of β-lactams from X-ray data.

Cmpd	*h*[a] (Å)	Bond length[b] (Å) N1–C2; C2=O	Bond angle[b] (Å) C2–N1–C4; C4–N1–C6; C2–N1–C6	θ[c] (°)
11	0.096	1.395; 1.200	93.5; 127.6; 137.4	1.5
Penam [21]	0.38	1.383; 1.206	94.26; 117.24; 125.85	22.65
Penem [22]	0.42	1.383; 1.224	90.55; 113.81; 126.28	29.36

[a] *h*, Woodward's parameter (perpendicular distance of the nitrogen N1 from the plane of its three substituents).
[b] the numbering of compound **11** (see Scheme 3) was used for Penam and Penem representatives.
[c] θ, Burton's parameter (torsion angle measured as 360° minus the sum of the three contiguous angles around N1).



	I	II	III
E-**11**	18.58	8.42	0.00
Z-**11**	24.06	15.15	4.33

Scheme 4. Three localized conformers of **10**–**12**. Energy differences (kcal mol^{-1}) between conformers **11** are given as a function of the C=C double bond configuration.

performed in D_2O at pD = 8 (4 mM solution containing ≤5% d_6-DMSO, 25 °C) and followed by ^{1}H NMR at 500 MHz. Disappearance of the starting materials (% SM) as a function of time (min), clearly showed that **12** reacts faster than **11**, and **11** faster than **10** (Fig. 4). Half-life times ($t_{1/2}$) are respectively about 4 h, 6 h and 16 h. Azetidinone ring opening (N1–C2 cleavage) resulted in a significant shielding of the H3 and H4 protons used as internal probes. No competition with acetate (on C4), or lactone (on C5) hydrolysis could be detected, since chemical shifts of CH_3 and H5 were almost unchanged. N1–C6 cleavage of the imide function was not observed (control of the chemical shift of CH_2 next to C6), in agreement with a previous study of Page devoted to *N*-benzoyl β-lactams [37].

2.4. Reactivity versus serine enzyme models

A concerted mechanism of β-lactam hydrolysis, in neutral medium, could be considered as the simplest model of serine-enzyme machinery [38]. In model A (Scheme 5), the nucleophile is formed by the duplex H_2O–H_2O: the second water molecule acts as a proton carrier of the first water molecule to the nitrogen of the β-lactam function. Two more complex catalytic models have been used in this study, featuring two types of nucleophilic environment. In model B (Scheme 5), the catalytic machinery of the chymotrypsin family is mimicked. The triad formed by Ser-195, His-57 and Asp-102 has been replaced with the triplex 2-formylamino-1-ethanol-H_2O-imidazole. In this model, the hydrogen of the serine mimicry is transferred to the β-lactam nitrogen via a water molecule which interacts with the imidazole ring [39,40]. The mechanism of class-A β-lactamases has been drawn in model C (Scheme 5). In these enzymes, the nucleophilic serine Ser-70 is surrounded by Glu-166, Lys-73 and Ser-130. In order to limit the influence of the global charge on model C, methylamine mimicking Lys-73 is neutral as this aminoacid forms a zwitterion with Glu-166. Ser-70 and Ser-130 have been replaced with 2-formylamino-1-ethanol and methanol respectively [41]. In both models B and C, the formamide moiety mimicks the oxyanion hole stabilization.

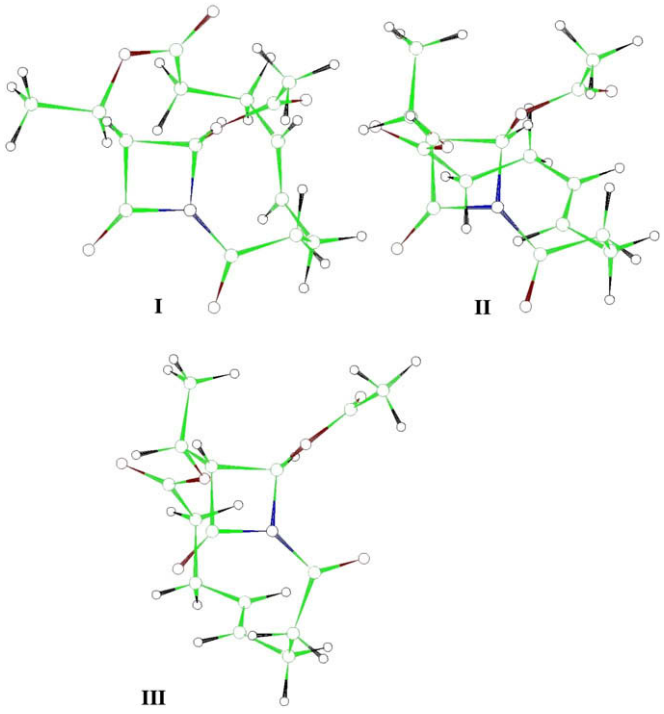


Fig. 2. Structures of calculated conformers of **11** (*E* isomer).

The reactivity of β-lactams **10**–**12** has been computed at the RHF level with the minimal basis set MINI-1′; this is imposed by the size of the analyzed systems which involve about 70 atoms [42]. For model A, both approaches of the azetidinone ring have been considered, on the α-face and the β-face of the cycle. For models B and C, only the nucleophilic attack on the α-face has been computed because this approach is the relevant one [43] in the active site of elastases (model B) and class A beta-lactamases (model C). Results are collected in Table 6.

The order of reactivity found for nucleophilic attack by $(H_2O)_2$ from the less hindered β-face (model A, first column) was almost the same as for basic hydrolysis, i.e. **12** > **11** > **10**, independently of the conformer considered, with Δ*E* values ranging from 26.7 to 19.6 kcal mol^{-1} (conformer II) and 29.8 to 28.7 (conformer III). The 13-membered ring 1,3-bridged compounds **11** and **12** showed reactivities of similar order of magnitude as the penam compound attacked from the α-face (Δ*E* = 26.6 kcal mol^{-1}). However, this way of nucleophilic attack appeared to be highly unfavourable for the bicyclic azetidinones **11** and **12** in conformation III (model A, second column), with Δ*E* values of 49.1 kcal mol^{-1} and 51.3 kcal mol^{-1}, respectively. Considering the chymotrypsin model (model B, third column), the penam reference was found highly reactive (Δ*E* = 14.6 kcal mol^{-1}), but not the bridged compounds **11** and **12** in the most stable conformation III (Δ*E* = 39.2 and 43.1 kcal mol^{-1}). Due to the size of the nucleophilic triplex in the β-lactamase model (model C, fourth column), conformation III of the bicyclic azetidinones **11** and **12** was no more accessible; at the transition state, the imide carbonyl turned to give the conformation II. In this model, the penam compound appeared again more

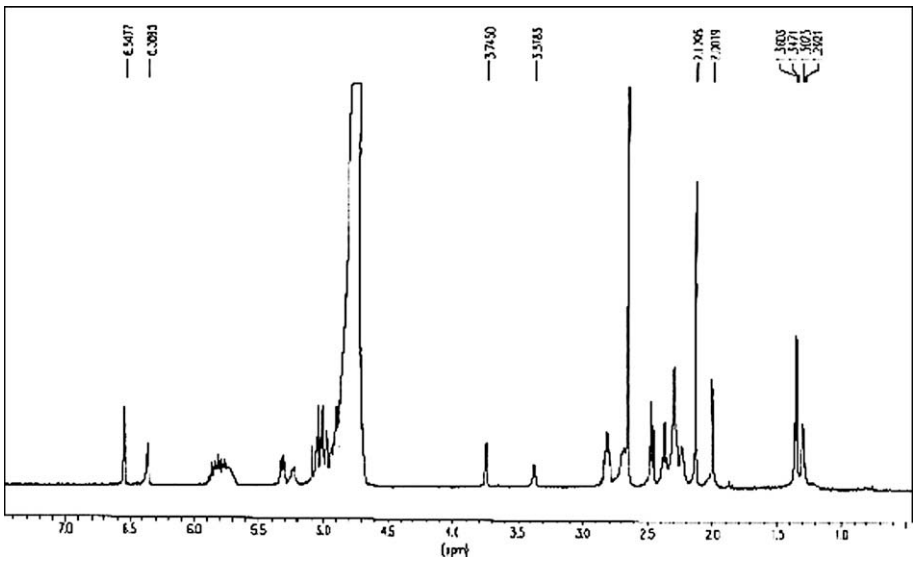


Fig. 3. ^{1}H NMR spectrum of **10**.

reactive ($\Delta E = 17.6$ kcal mol^{-1}) than our large ring bridged compounds ($\Delta E = 35.2$ and 50.9 kcal mol^{-1}). In fact, the unfolding of this ring, in the most stable conformers, hides the α-face of the reactive four-membered ring, thus handicapping the nucleophilic attack. This is not the case for the monocyclic precursor **10** which is predicted to be more reactive versus serine enzymes (models B and C) than both **11** and **12**. Moreover, the conformation of the acetate substituent (on C4) could play an indirect, but probably decisive role in the transition state stabilisation. Indeed, perturbations of the H-bonding network in the enzymic cavity were found depending on the orientation of this group. This is illustrated on Fig. 5 showing two conformers III of **10**, different at the level of the OAc substituent, processed in model C. When the acetate carbonyl was oriented outside versus the β-lactam ring (structure **b**), the methanol molecule of the nucleophilic triplex moved in order to create H-bonding interaction with this function, at the transition state. Such a phenomenon was not detected when the acetate carbonyl pointed inside versus the β-lactam ring (structure **a**).

The theoretical study of **10**–**12** reactivity towards models of serine enzymes emphasized the huge complexity of our systems, precisely due to their conformational flexibility. It is also important to note that only the acylation step has been computed, although the complete catalytic process involves three successive steps: the recognition step (formation of a Michaelis complex), the acylation step (formation of an acyl-enzyme intermediate), and the deacylation step (hydrolysis of the acyl-enzyme intermediate) [44,45].

Taking into account the limitations of our models, it seems nevertheless that the bridged β-lactams **11** and **12** could interact with serine enzymes, under selected conformations (some ΔE values are around 30 kcal mol^{-1}), but that the monocyclic precursor **10** should be more active. This has been tested experimentally.

2.5. Biochemical evaluation

As representative enzyme of the chymotrypsin family (model B), we have considered PPE (porcine pancreatic elastase). The capacity of several β-lactam antibiotics [46,47] and monocyclic β-lactams [48–52] to inhibit elastase has been previously reported.

Compounds **10**–**12** were evaluated for their inhibitory effect on PPE, in a competition experiment with *N*-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide as substrate. The rates of PPE-catalyzed hydrolysis of the substrate were determined, in the presence of different concentrations of tested compounds (from 0.1 μM to 100 μM), thanks to the measurement of UV-absorbance (A) at 410 nm (formation of *p*-nitroaniline) recorded as a function of time. The monocyclic β-lactam **10** was active at 1 μM (Fig. 6), but the bicyclic β-lactams **11** and **12** were still inactive at 100 μM. The active

Table 4
Heat of formation of **11** in kcal mol^{-1} (B3Lyp/6-31G(d)).

ΔE of *E*-isomer/*Z*-isomer		
Conformer I	6.01	11.49
Conformer II	7.29	14.03
Conformer III	2.76	7.09

Table 5
Tetrahedral intermediates formed by addition of OH^- onto the β-lactam C=O bond of **10**, **11** and **12** in conformer III and Penam (B3Lyp/6-31G(d)).

Cmpd	N1–C2 length (Å)	ΔE (kcal mol^{-1})
10	1.589	55.06
11	1.619	53.03
12	1.627	54.98
Penam[a]	1.676	38.09

[a] Penicillin structure without the acylamino side-chain.

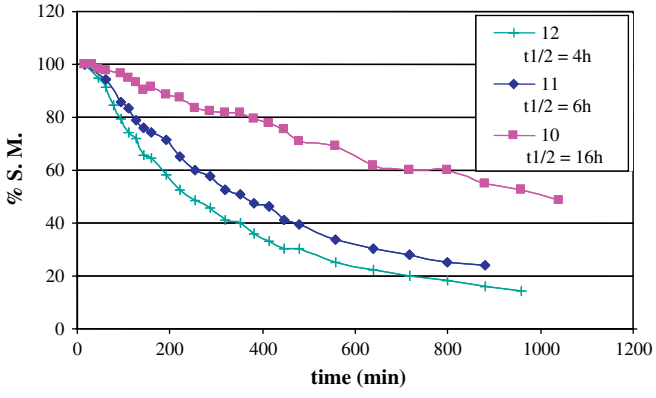
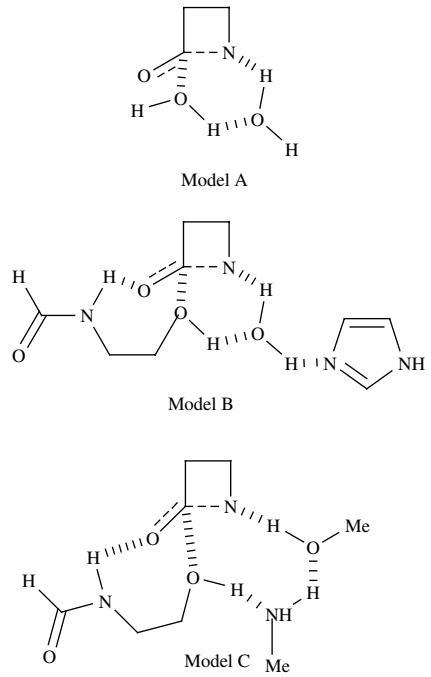


Fig. 4. Rate of hydrolysis of **10**–**12**.



Model A: H_2O-H_2O duplex (concerted chemical hydrolysis at pH 7)

Model B: 2-formylamino-1-ethanol + H_2O + imidazole
(model of elastase with Ser-195 and His-57)

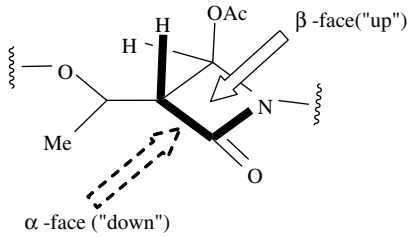
Model C: 2-formylamino-1-ethanol + $MeNH_2$ + MeOH
(model of PBPs with Ser-70, Lys-73 and Ser-130)

Scheme 5. Models of concerted nucleophilic attack on the β-lactam ring.

compound **10** behaved as a "slow substrate" or "reversible inhibitor". Indeed, after pre-incubation of PPE with **10**, at different concentrations (1, 25, 50, 100 μM) and various times (20, 40, 60 min), followed by dilution with the substrate solution, the enzyme recovered slowly its activity (Fig. 7).

Our compounds were also tested against bacterial enzymes (model C), one class-A β-lactamase (TEM-1) and representative PBPs (Penicillin Binding Proteins). Indeed, our reactivity model of

Table 6
Activation energy of concerted nucleophilic attack (ΔE in kcal mol^{-1}).



Cmpd (conformer)	Model A (β-face)[a]	Model A (α-face)[a]	Model B (α-face)[a]	Model C (α-face)[a]
10 (I)	–	–	28.91	37.41
10 (II)	26.72	33.10	–	–
10 (III)	29.69	41.30	30.81	32.75
11 (I)	–	–	26.47	38.29
11 (II)	24.46	37.22	–	35.17
11 (III)	29.83	49.07	39.22	–
12 (I)	–	–	30.78	36.28
12 (II)	19.61	34.84	–	50.89
12 (III)	28.70	51.33	43.16	–
Penam	/	26.56	14.62	17.59

[a] Definition of nucleophilic attack from α-face or β-face.

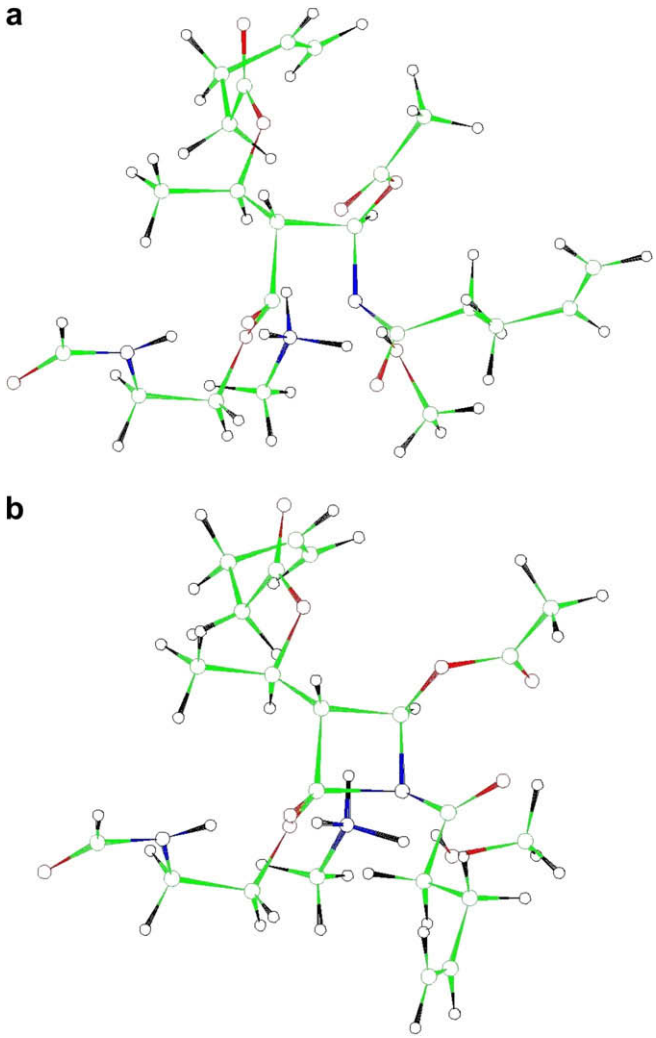


Fig. 5. Processing of **10** in model C; two conformers III, **a** and **b**.

serine β-lactamases mimicks the entities involved during the acylation mechanism, namely Ser-70, Lys-73 and Ser-130. These amino acids are also present in the active site of D,D-peptidases, as proven by crystallographic studies of *Actinomadura* R39 [53] and *Streptomyces* K15 [54].

The potential inhibition of TEM-1 by β-lactams **10**–**12** was evaluated as above, in competition experiments with a chromogenic substrate (nitrocefine), but none of the compounds was found active at 100 μM. R39 is a model enzyme of low molecular weight D,D-peptidases [53]. The other considered PBPs are high molecular weight

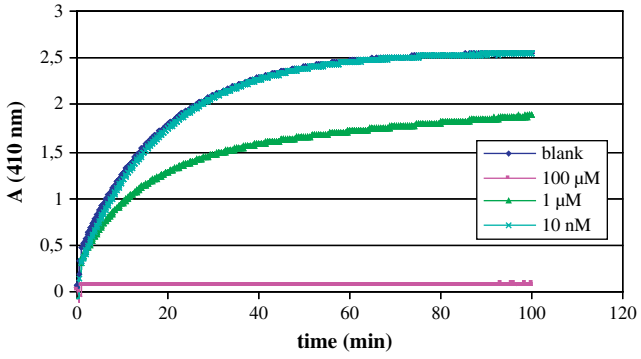


Fig. 6. Inhibition of PPE by azetidinone **10**.

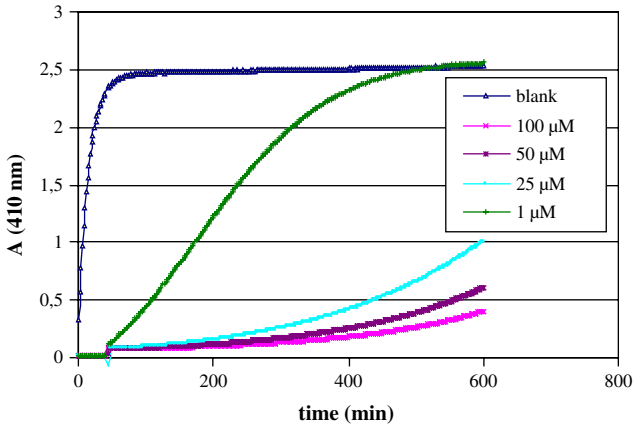


Fig. 7. Reversibility of PPE inhibition by **10** (60 min incubation).

enzymes responsible for bacterial resistance to β-lactam antibiotics: [55–57] PBP_{2a} from *Staphylococcus aureus* ATCC433000 [58,59], PBP_{5fm} from *Enterococcus faecium* [60], and PBP_{2x} from *Streptococcus pneumoniae* 5204 [61–63]. The peptidases (0.8 or 2.5 μM) and the azetidinones **10**–**12** (100 μM) were incubated during 16 h, then fluorescent ampicillin (25 μM) was added. After 45 min, the proteins were denaturated, and the fluorescence intensity (resulting from the covalent protein–ampicillin complexes) was measured. In this protocol, the tested compounds were supposed to be able of acylating the PBPs, and the residual activities of the proteins were thus determined. Results of Table 7 are expressed in percentages of initial activity. Thus low % values indicate very active compounds. Benzylpenicillin (Pen G), used as positive control, showed values of about 5–10%. The monocyclic azetidinone **10** was highly active against R39, moderately active against PBP_{2a}, and weakly active against PBP_{5fm}. The bicyclic azetidinones **11** and **12** were weakly active only against PBP_{2a}. None of the compounds inhibited PBP_{2x}. In opposition to the chemical reactivity, the biochemical tests pointed out azetidinone **10** as the most active compound. Nevertheless promising activities of azetidinones **11** and **12** were recruited versus PBP_{2a}, a clinically relevant target of MRSA strains.

3. Conclusions

We have explored a non-traditional approach for designing reactive β-lactams, and possibly new antibacterial agents. Instead of increasing the angular strain and the N1 pyramidality (“twisted amide”) in the azetidinone ring of 1,4-fused bi- [64,65] or tricyclic systems [66], we considered flexible 1,3-bridged bicyclic systems featuring a “planar amide” and a large ring susceptible to generate a lot of conformers. Such azetidinones, endowed with a thienamycin-like side-chain at C3 and the related stereochemistry at C3–C5, are readily accessible via a convergent RCM strategy, as illustrated by the synthesis of the 13-membered bicycles *E*-**11** and **12** from the commercial chiron **7**, precursor of **10**. The experimental structural data (IR and NMR spectroscopic analyses and X-ray diffraction) confirmed that β-lactams **10**–**12** are well planar amides. Theoretical analysis of our novel systems revealed that their reactivity towards nucleophiles (OH^-, H_2O–H_2O) and serine enzymes (models B and C) indeed depends significantly on the considered conformers.

Table 7
Inhibition of bacterial enzymes. Results are given as percentages of initial activity.

Cmpd[a]	R39 (0.8 μM)	PBP_{2a} (2.5 μM)	PBP_{5fm} (2.5 μM)	PBP_{2x} (0.8 μM)
10	14 ± 2	66 ± 14	84 ± 12	93 ± 6
11	96 ± 4	86 ± 8	104 ± 4	116 ± 5
12	113 ± 18	94 ± 3	113 ± 11	99 ± 4
Pen G	nd[b]	13 ± 3	10 ± 3	<5

[a] Compounds were tested at 100 μM.
[b] Not determined.

The chemical reactivity of compounds **10**–**12** could be correctly predicted by *ab initio* calculations, regarding their half-live times in phosphate buffer. Both bicyclic azetidinones **11** and **12** are more reactive than their monocyclic precursor **10**, probably because some angular strain still operates in the 13-membered bridged series. The enhanced reactivity of **12** (saturated large cycle) versus **11** (C═C double bond with *E*-configuration in the large ring) can result from torsion (or eclipse) strains and transannular interactions which become more important when carbon atoms with sp^2 geometry are transformed into the sp^3 one.

The reactivity of azetidinones **10**–**12** versus serine enzymes appeared hardly predictable by theoretical tools. Nevertheless, the crucial role of geometrical factors (“up” or “down” nucleophilic attack of the β-lactam carbonyl) has been pointed out. Consequently, the monocyclic precursor **10** seems to be more capable of fitting within an enzymic cavity than the bicycles **11** and **12**. Experimentally, we found that **10** behaves as a good inhibitor of a mammalian elastase. The slow (and partial) reversibility of this inhibition is reminiscent of the mechanism of action of clavulanic acid and 6-β-bromopenicillanic acid versus class A β-lactamases (such inhibitors are called “passive covalent inhibitors”) [67,68]. Also, **10** was an excellent inhibitor of the bacterial D,D-peptidase R39. This result abolishes an old dogma in the field of penicillin-type antibiotics, i.e. the need of a carboxylic function on the substrate/inhibitor for promoting a good interaction with PBP enzymes. Yet, the most stimulating biochemical result was the discovery of novel inhibitors of PBP_{2a} featuring non-traditional structures: their activities are moderate (**10**) to modest (**11**) and weak (**12**), but well measurable.

The emergence of penicillin-resistant bacterial strains and the recent dramatic increase of those strains, all over the world, represent a major concern in medicine. Particularly, the prevalence of MRSA (methicillin-resistant *Staphylococcus aureus*) in hospitals has stimulated the development of more potent carbapenems (ME 1036 [64]) and cephalosporins (PPI 0903M [65]), characterized by very bulky substituents on the bicyclic skeletons. The lethal action of such antibiotics is due to their high affinity for PBP_{2a}. The development of large ring 1,3-bridged 2-azetidinones, structurally related to the carbapenem family (2-hydroxyethyl side chain on C3 and thienamycin stereochemistry) and the cephalosporin family (acylamino side chain on C3 and penicillin stereochemistry), is currently continued in our laboratory.

The present preliminary results pave the route of a possible alternative strategy for the discovery of novel ‘hits’ towards resistant infections.

4. Experimental section

4.1. General

Manipulations were performed under an argon atmosphere in flame-dried glassware. Reagents were used as-received and solvents were dried by standard procedures. TLC analyses were performed on aluminium plates coated with silica gel 60 F_{254} (Merck) and visualized with UV (254 nm) and $KMNO_4$ solution. Melting points were measured on Electrothermal apparatus calibrated with benzoic acid (uncorrected m.p.).Column chromatographies were performed on silica gel Merck 60 (40–60 μm). NMR spectra were recorded on a Bruker Avance 500 spectrometer at 500 MHz (^1H) and 175 MHz (^{13}C) in $CDCl_3$ with TMS as an internal standard. IR spectra were recorded on Shimadzu FTIR-8400S spectrometer; compounds were deposited on NaCl plates as a thin film by evaporation from CH_2Cl_2 solution. High Resolution MS were obtained in the Mass Spectrometry Service of the University of Mons-Hainaut, Belgium.

4.2. Synthesis

4.2.1. 1-(Pent-4-enoyl)-(3R,4R)-3-[1(R)-(tert-butyldimethylsilyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (**8**)

To a stirred solution of acetoxy-azetidinone **7** (500 mg, 1.74 mmol) in dry CH_2Cl_2 (15 mL) were added pyridine (0.28 mL, 3.48 mmol) and 4-pentenoyl chloride (0.38 mL, 3.48 mmol). The solution was heated at 35 °C under an argon atmosphere for 65 h. Then the reaction mixture was diluted with CH_2Cl_2 (50 mL) and successively washed with a 3.3 M HCl solution (50 mL), a saturated $NaHCO_3$ solution (50 mL) and brine (50 mL). The organic phase was dried over $MgSO_4$ and concentrated under vacuum. The residue was purified by flash chromatography (cyclohexane/EtOAc 10:1) to furnish imide **8** as a pale yellow oil (515 mg, 80%). $R_f = 0.57$ (cyclohexane/EtOAc 5:2); ^{1}H NMR: $\delta = 6.58$ (d, $J = 1.6$ Hz, 1H; H4), 5.82 (ddt, $J = 6.5$, 10.5, 17.2 Hz, 1H; CH=CH_2), 5.08 (dd, $J = 1.6$, 17.2 Hz, 1H; CH=CH_2), 5.01 (dd, $J = 1.6$, 10.5 Hz, 1H; CH=CH_2), 4.29 (qd, $J = 2.5$, 6.7 Hz, 1H; H5), 3.12 (dd, $J = 1.6$, 2.5 Hz, 1H; H3), 2.81 (td, $J = 7.6$, 16.9 Hz, 1H; CO–CH_2), 2.74 (td, $J = 7.6$, 16.9 Hz, 1H; CO–CH_2), 2.40 (td, $J = 6.5$, 7.6 Hz, 2H; CH_2–C=C), 2.10 (s, 3H; $COCH_3$), 1.31 (d, $J = 6.7$ Hz, 3H; CH_3–CH–O), 0.82 (s, 9H; Si*t*Bu), 0.03 (s, 3H; $SiCH_3$), 0.07 (s, 3H; $SiCH_3$); ^{13}C NMR: $\delta = 169.1$ (C6), 168.9 (OCOMe), 164.4 (C2), 136.2 (CH=C), 115.8 (C=CH_2), 74.1 (C4), 65.0 (C3), 64.1 (C5), 35.6 (COCH_2), 27.4 (CH_2C=), 25.5 (C(CH_3)$_3$), 21.7 (CH_3CH–O), 20.8 (CH_3CO_2), 17.6 (C(CH_3)$_3$), −4.3 ($SiCH_3$), −5.5 ($SiCH_3$); IR: $\nu = 1808$, 1759, 1721, 1642, 1306 [1]; HR-ESI-TOF-Mass (positive-mode): *m/z* calcd for $C_{18}H_{31}NO_5Si$ + Na: 392.1869; found: 392.1863.

4.2.2. 1-(Pent-4-enoyl)-(3R,4R)-3-[1(R)-hydroxyethyl]-4-(acetoxy)-azetidin-2-one (**9**)

β-Lactam **8** (850 mg, 2.3 mmol) dissolved in CH_3CN (100 mL) was treated with 17 M AcOH (0.95 mL, 16.1 mmol) and 12 M HCl (0.96 mL, 11.5 mmol) at 0 °C for 3 h. The solvent was removed under vacuum and the residue diluted with EtOAc (50 mL). The organic phase was washed with a 10% Na_2CO_3 solution and brine (2 × 50 mL), then dried over $MgSO_4$ and concentrated. The crude alcohol was purified by flash chromatography (cyclohexane/EtOAc 5:3) to yield **9** as a pale yellow oil (490 mg, 83%). Compound **9** can also be used without purification in the next step. $R_f = 0.40$ (cyclohexane/EtOAc 5:3); ^{1}H NMR: $\delta = 6.33$ (d, $J = 1.4$ Hz, 1H; H4), 5.72 (ddt, $J = 6.8$, 10.3, 17.1 Hz, 1H; CH=CH_2), 4.98 (dd, $J = 1.5$, 17.1 Hz, 1H; CH=CH_2), 4.91 (dd, $J = 1.5$, 10.3 Hz, 1H; CH=CH_2), 4.13 (qd, $J = 5.4$, 6.4 Hz, 1H; H5), 3.30 (br s, 1H; OH), 3.08 (dd, $J = 1.4$, 5.4 Hz, 1H; H3), 2.70 (m, 2H; CO–CH_2), 2.30 (td, $J = 6.8$, 7.4 Hz, 2H; CH_2–C=C), 2.02 (s, 3H; $COCH_3$), 1.24 (d, $J = 6.4$ Hz, 3H; CH_3–CH–O); ^{13}C NMR: $\delta = 169.9$ (C6), 169.5 (O$COCH_3$), 163.3 (C2), 136.0 (CH=C), 115.8 (C=CH_2), 75.2 (C4), 64.9 (C3), 63.8 (C5), 35.6 (COCH_2), 27.4 (CH_2–C=C), 20.9 (CH_3CHO), 20.7 (CH_3CO_2); IR: $\nu = 3501$, 1805, 1755, 1718, 1641, 1311 cm^{-1}; HR-ESI-TOF-Mass (positive-mode): *m/z* calcd for $C_{12}H_{17}NO_5$ + Na: 278.1004; found: 278.0992.

4.2.3. 1-(Pent-4-enoyl)-(3R,4R)-3-[1(R)-(pent-4-enoyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (**10**)

To a stirred solution of β-lactam **9** (800 mg, 3.14 mmol) in dry CH_2Cl_2 (20 mL) were added pyridine (0.38 mL, 6.28 mmol) and 4-pentenoyl chloride (0.69 mL, 6.28 mmol). The reaction was performed at room temperature under an argon atmosphere for 3 h. The mixture was diluted with CH_2Cl_2 (50 mL) and successively washed with a 3.3 M HCl solution (50 mL), a saturated $NaHCO_3$ solution (50 mL) and brine (50 mL). The organic phase was dried over $MgSO_4$ and concentrated. The residue was purified by flash chromatography (cyclohexane/EtOAc 5:2) to provide **10** as a pale yellow oil (950 mg, 90%). $R_f = 0.56$ (cyclohexane/EtOAc 5:2); ^{1}H NMR: $\delta = 6.48$ (d, $J = 1.6$ Hz, 1H; H4), 5.82 (ddt, $J = 6.4$, 10.3, 17.0 Hz, 1H; CH=CH_2), 5.78 (ddt, $J = 6.4$, 10.3, 17.0 Hz, 1H; CH=CH_2), 5.29 (qd, $J = 5.8$, 6.4 Hz, 1H; H5), 5.08 (dd, $J = 1.6$, 17.0 Hz, 1H; CH=CH_2), 5.05 (dd, $J = 1.6$, 17.0 Hz, 1H; CH=CH_2), 5.02 (dd, $J = 1.6$, 10.3 Hz, 1H; CH=CH_2), 5.00 (dd, $J = 1.6$, 10.3 Hz, 1H; CH=CH_2), 3.28 (dd, $J = 1.6$, 5.8 Hz, 1H; H3), 2.80 (m, 2H; NCOCH_2), 2.40 (m, 4H; OCOCH_2 and CH_2C = C), 2.34 (m, 2H; CH_2C = C), 2.11 (s, 3H; OCOCH_3), 1.39 (d, $J = 6.4$ Hz, 3H; CH_3–CHO); ^{13}C NMR: $\delta = 171.7$ (O$COCH_2$), 169.2 (C6), 168.8 (O$COCH_3$), 162.2 (C2), 136.2 (CH=CH_2), 135.9 (CH=CH_2), 115.9 (CH=CH_2), 115.6 (CH=CH_2), 74.5 (C4), 65.6 (C5), 62.5 (C3), 35.7 (NCO–CH_2), 33.3 (OCOCH_2), 28.6 (CH_2C=C), 27.4 (CH_2C=C), 20.6 (OCOCH_3), 18.1 (CH_3CHO); IR: $\nu = 1806$, 1742, 1722, 1642, 1313 cm^{-1}; HR-ESI-TOF-Mass (positive-mode): *m/z* calcd for $C_{17}H_{23}NO_6$ + Na: 360.1423; found: 360.1412.

4.2.4. (E,11R,12R,14R)-14-(Acetoxy)-11-(methyl)-2, 9, 13-trioxo-10-oxa-1-aza-bicyclo-[10.1.1]-tetradec-5-ene (**11**)

Grubbs catalyst of second generation was used (Scheme 6).

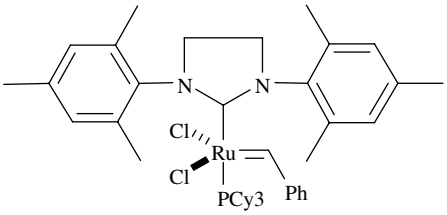
To a stirred solution of β-lactam **10** (355 mg, 1.05 mmol) in dry CH_2Cl_2 (200 mL, [5 mM]) was added Grubbs catalyst (44.7 mg, 0.053 mmol). The reaction was performed at room temperature under an argon atmosphere for 5 h. Then the solvent was removed and the residue purified by flash chromatography (cyclohexane/EtOAc 5:2) to provide **11** as a white solid (268 mg, 83%). $R_f = 0.54$ (cyclohexane/EtOAc 5:3); m.p. = 117.8 °C; ^{1}H NMR: $\delta = 6.53$ (d, $J = 1.6$ Hz, 1H; H4), 5.58 (qd, $J = 1.8$, 6.7 Hz, 1H; H5), 5.46 (m, 1H; CH=CH), 5.40 (m, 1H; CH=CH), 3.27 (m, 1H; NCO–CH), 3.19 (dd, $J = 1.6$, 1.8 Hz, 1H; H3), 2.46 (m, 1H; OCO–CH), 2.40 (m, 3H; NCO–CH + CH_2C=C), 2.30 (m, 3H; OCO–CH + C=C–CH_2), 2.13 (s, 3H; OCOCH_3), 1.40 (d, $J = 6.7$ Hz, 3H; CH_3CHO); ^{13}C NMR: $\delta = 171.6$ (O$COCH_2$), 170.7 (NCOCH_2), 169.1 (O$COCH_3$), 163.0 (C2), 130.4 (CH=C), 128.3 (C=CH), 74.1 (C4), 64.9 (C5), 62.7 (C3), 34.7 (NCOCH_2), 33.7 (OCOCH_2), 30.3 (CH_2C=C), 28.2 (C=C–CH_2), 20.8 (OCOCH_3), 18.4 (CH_3CO); IR: $\nu = 1809$, 1742, 1716 cm^{-1}; HR-ESI-TOF-Mass (positive-mode): *m/z* calcd for $C_{15}H_{19}NO_6$ + Na: 332.1110; found: 332.1101.

4.2.5. (11R,12R,14R)-14-(Acetoxy)-11-(methyl)-2, 9, 13-trioxo-10-oxa-1-aza-bicyclo-[10.1.1]-tetradecane (**12**)

Precursor **11** (266 mg, 0.87 mmol) dissolved in EtOAc (15 mL) was placed under H_2 atmosphere (1 atm) at room temperature in the presence of Pd catalyst (20 mg, 10% on C) for 17 h. Filtration and concentration under vacuum furnished **12** as a white solid (270 mg, 100%). $R_f = 0.55$ (cyclohexane/EtOAc 5:3); m.p. = 113.3 °C; ^{1}H NMR: $\delta = 6.50$ (d, $J = 1.3$ Hz, 1H; H4), 5.53 (qd, $J = 1.7$, 6.9 Hz, 1H; H5), 3.22 (dt, $J = 3.9$, 14.1 Hz, 1H; NCOCH), 3.20 (m, 1H; H3), 2.34 (dt, $J = 4.7$, 14.1 Hz, 1H; NCOCH), 2.22–2.31 (m, 2H; OCOCH_2), 2.10 (s, 3H; OCOCH_3), 2.01 (m, 1H), 1.76 (m, 1H), 1.60 (m, 1H), 1.49 (m, 1H), 1.38 (d, $J = 6.9$ Hz, 3H; CH_3CHO), 1.19–1.35 (m, 4H); ^{13}C NMR: $\delta = 171.9$ (O$COCH_2$), 171.1 (NCOCH_2), 169.0 (O$COCH_3$), 163.8 (C2), 74.8 (C4), 65.4 (C5), 62.5 (C3), 32.9 (NCOCH_2), 32.7 (OCOCH_2), 25.6 (CH_2), 25.4 (CH_2), 25.3 (CH_2), 25.1 (CH_2), 20.8 (OCOCH_3), 18.3 (CH_3CHO); IR: $\nu = 1807$, 1743, 1716 cm^{-1}; HR-ESI-TOF-Mass (positive-mode): *m/z* calcd for $C_{15}H_{21}NO_6$ + Na: 334.1267; found: 334.1252.

4.3. Single-crystal X-ray crystallography

Compound **11** was recrystallized from ether/acetone (slow evaporation at room temperature). X-ray intensity data were



Scheme 6. Grubbs catalyst.

collected at 298(2) K with a MAR345 image plate detector using Mo Kα ($\lambda = 0.71069$ Å) monochromatized radiation. The unit cell parameters were refined using all the collected spots after the integration process. Fig. 1 is drawn in the ORTEP style [69]. The structure was solved by direct methods with SHELXS97 and refined by full-matrix least-squares on F^2 using SHELXL97 [70]. All the non-hydrogen atoms were refined with anisotropic temperature factors. The hydrogen atoms were calculated with AFIX and included in the refinement with a common isotropic temperature factor. The structure has been deposited within the Cambridge Crystallographic data Centre, no CCDC 684061.

Crystal system = monoclinic; space group = $P2_1$; unit cell dimensions: $a = 9.396(3)$ Å; $b = 9.161(3)$ Å; $c = 9.815(3)$ Å; volume = 819.0(5) Å^3; $Z = 2$; $Dx = 1.25$ g cm^{-3}.

A total of 10 605 reflections were collected of which 1962 were independent ($R_{int} = 0.049$) 1859 observed reflections ($I > 2.5\sigma(I)$); 2θ max = .44.5°; final $R = 0.046$ (all data); $wR2 = 0.117$; $S = 1.09$; largest peak and hole 0.14, −0.14 e Å^{-3}.

4.4. NMR kinetic study

Phosphate buffer (1 mL, 50 mM, pH 8 adjusted with NaOH solution) was freeze-dried and the residue was dissolved in D_2O (99.9%, 1 mL). A solution of azetidinone (25 μL, 8.10^{-2} M) in DMSO-d_6 was diluted with deuteriated phosphate buffer (475 μL) to give a final concentration of 4 mM. Chemical hydrolysis was followed by ^{1}H NMR at 500 MHz as a function of time.

4.5. Theoretical evaluation

Conformational studies, calculations of heat of formation and reactivity versus hydroxyl anion were carried out using the well-established B3Lyp hybrid density functional [71] using the split valence polarized 6-31G(d) basis set [72].

Reactivity versus serine enzyme models were performed at the *ab initio* RHF level using the minimal basis set MINI-1' [73]. All the calculations have been performed with the suite of programs Gaussian [74].

4.6. Biochemical evaluation

4.6.1. Assay with PPE

To a solution (2 mL) of substrate at 25 °C (*N*-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide; 300 μM in TRIS buffer, 100 mM, pH 7.5) and tested compound (20 μL; 10^{-2}–10^{-4} M in *N*-methyl-pyrrolidone (NMP)), or solvent (20 μL of NMP, for control), was added porcine pancreatic elastase (PPE from Sigma at 0.35 mg/mL; 0.67 μL of 6 μM solution in acetate buffer, 50 mM, pH 5; [E] = 200 nM). Absorbance at 410 nm, corresponding to the appearance of *p*-nitroaniline (substrate hydrolysis product) was measured with a Varian Cary 3 BIO spectrophotometer, as a function of time. Tested concentrations of inhibitors were 100, 50, 25 and 1 μM.

The reversibility of the inhibition was controlled by the incubation/dilution method. Enzyme solution (67 μL) and inhibitor solution (20 μL) were incubated during 20, 40 and 60 min before the addition of substrate solution (2 mL). Absorbance at 410 nm for different concentrations of inhibitor (100, 50, 25 and 1 μM) was measured as a function of time.

4.6.2. Assay with TEM-1

The assay mixture was prepared at 25 °C from the substrate solution (nitrocefine at 1 mM in phosphate buffer, 100 mM, pH 7 containing 5% DMSO; 100 μL) and the tested compound (10^{-2}–10^{-4} M in DMSO; 10 μL), or solvent (10 μL DMSO, for control), diluted in phosphate buffer (890 μL, 50 mM, pH 7). At $t = 0$, the enzyme solution was added (class A β-lactamase TEM-1 at 1.2 μM in phosphate buffer, 50 mM; pH 7; 1 μL, [E] = 1.2 nM). Absorbance at 486 nm, corresponding to the appearance of nitrocefine hydrolysis product, was measured with a Varian Cary 3 BIO spectrophotometer, as a function of time. Tested concentrations of inhibitors were 100, 10 and 1 μM. TEM-1 enzyme was obtained from the Unité de Biochimie (UCL, Louvain-la-Neuve), professors J. Fastrez and P. Soumillon.

4.6.3. Assay with PBPs

The tested enzymes (R39, PBP_{2a}, PBP_{5fm}, PBP_{2x}) were produced and purified at the University of Liège (Centre d'ingénierie des protéines, professors J-M. Frère, B. Joris and M. Galleni). The inhibition studies were performed at 30 °C in sodium phosphate buffer (50 mM, pH 7) containing 5% to 10% DMF to ensure product solubility. In the case of PBP_{2a} and PBP_{5fm}, 0.5 M NaCl was added. Compounds were tested at 100 μM concentration; experiments were performed in triplicate. Inhibitor (100 μM) and protein (0.8 or 2.5 μM) were mixed and incubated for 16 h. The residual free protein was counter labeled with fluorescein-labeled ampicillin (25 μM) for 45 min. Then, the reaction was stopped by addition of SDS-PAGE loading buffer and heating at 100 °C for 4 min. The reaction mixture was submitted to SDS-PAGE. The fluorescent complexes (formed by reaction of residual PBPs and ampicillin) were visualised using a Molecular Imager FX and quantified with the Quantity One Software of BIORAD. In each assay, background fluorescence was subtracted.

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