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Research paper

Azetidiniamines as a novel series of non-covalent broad-spectrum inhibitors of β -lactamases with submicromolar activities against carbapenemases KPC-2 (class A), NDM-1 (class B) and OXA-48 (class D)

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ABSTRACT

The occurrence of resistances in Gram negative bacteria is steadily increasing to reach extremely worrying levels and one of the main causes of resistance is the massive spread of very efficient β -lactamases which render most β -lactam antibiotics useless. Herein, we report the development of a series of imino-analogues of β -lactams (namely azetidiniamines) as efficient non-covalent inhibitors of β -lactamases. Despite the structural and mechanistic differences between serine- β -lactamases KPC-2 and OXA-48 and metallo- β -lactamase NDM-1, all three enzymes can be inhibited at a submicromolar level by compound **7dfm**, which can also repotentiate imipenem against a resistant strain of *Escherichia coli* expressing NDM-1. We show that **7dfm** can efficiently inhibit not only the three main clinically-relevant carbapenemases of Ambler classes A (KPC-2), B (NDM-1) and D (OXA-48) with K_i 's below 0.3 μ M, but also the cephalosporinase CMY-2 (class C, 86% inhibition at 10 μ M). Our results pave the way for the development of a new structurally original family of non-covalent broad-spectrum inhibitors of β -lactamases.

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1. Introduction

The discovery of penicillin was the start of a golden age for antibiotic therapy that is now threatened by exponentially increasing antibiotic resistance phenomena [1]. Because β -lactams (Fig. 1a) have been, and still are, the most prescribed antibiotics worldwide, resistance against them is particularly alarming. In Gram negative bacteria (GNB), the main mechanism of β -lactam resistance is due to the production of β -lactamases, enzymes capable of hydrolyzing β -lactams. Even carbapenems, the most powerful β -lactams, are not spared by metallo- β -lactamases (MBLs) such as NDM-1, IMP-1 or VIM-1 (class B) and/or by some clinically

worrisome serine β -lactamases (SBLs) such as KPC-2 or OXA-48 (classes A and D, respectively) [2,3]. The discovery of novel antibiotics acting on novel targets is difficult to foresee and strategies to overcome β -lactam resistance [4,5] especially *via* β -lactamase inhibition [6–8] may preserve our current therapeutic arsenal. This strategy was implemented more than 30 years ago with the development of β -lactamase inhibitors (BLIs) including clavulanic acid and sulbactam (Fig. 1b), but was not actively pursued until recently. Since 2012 several new broad-spectrum inhibitors of class A and class C β -lactamases have emerged. Of significant interest, avibactam [9,10] and vaborbactam [11] (Fig. 1b) were approved by the FDA for clinical use whilst many of their congeners, in particular diazabicyclooctanes [12–20], and various boron derivatives (see below), including benzosiloxaboroles [21], are currently undergoing preclinical or clinical development. These compounds are mainly able to efficiently inhibit SBLs, including carbapenemases, of class A, C and sometimes D, but generally do not inhibit MBLs (class B). In parallel, continuous efforts for the development of efficient MBL inhibitors [22,23] have recently led to the identification of

promising molecules that can bind to zinc atoms of the active site of MBLs, such as thiaziazole [24] or thiols [25,26] – including the clinically available antihypertensive agent L-captopril [27] –, aspergillomarasmine A [28] and other iminodiacetic acids [29,30], rhodanines [31] and their thienolate derivatives [32] or heteroaryl compounds [33,34] such as ANT2681 [35] or ANT431 [36] (Fig. 1c).

Yet there is still no MBL inhibitor available for clinical use. Because of the emergence of bacterial isolates producing two or even three different carbapenemases of different classes, the development of inhibitors capable of simultaneously inhibiting SBLs and MBLs becomes a relevant, yet challenging, strategy. Apart from some polyphenolic derivatives [37] and tetrazoles [38] with moderate activities, only heteroaryl phosphonates [39] and boronic acid derivatives [40–44] have been reported to efficiently inhibit both SBLs and MBLs (Fig. 1d). Rigid cyclic analogues of vaborbactam such as VNRX-5133 (currently in phase 3 clinical trials) were the first molecules shown to inhibit all classes of β -lactamases (Fig. 1d) [40–43]. VNRX-5133 exhibits submicromolar inhibitory activity against several clinically important MBLs (NDM-1, VIM-1 or VIM-2)

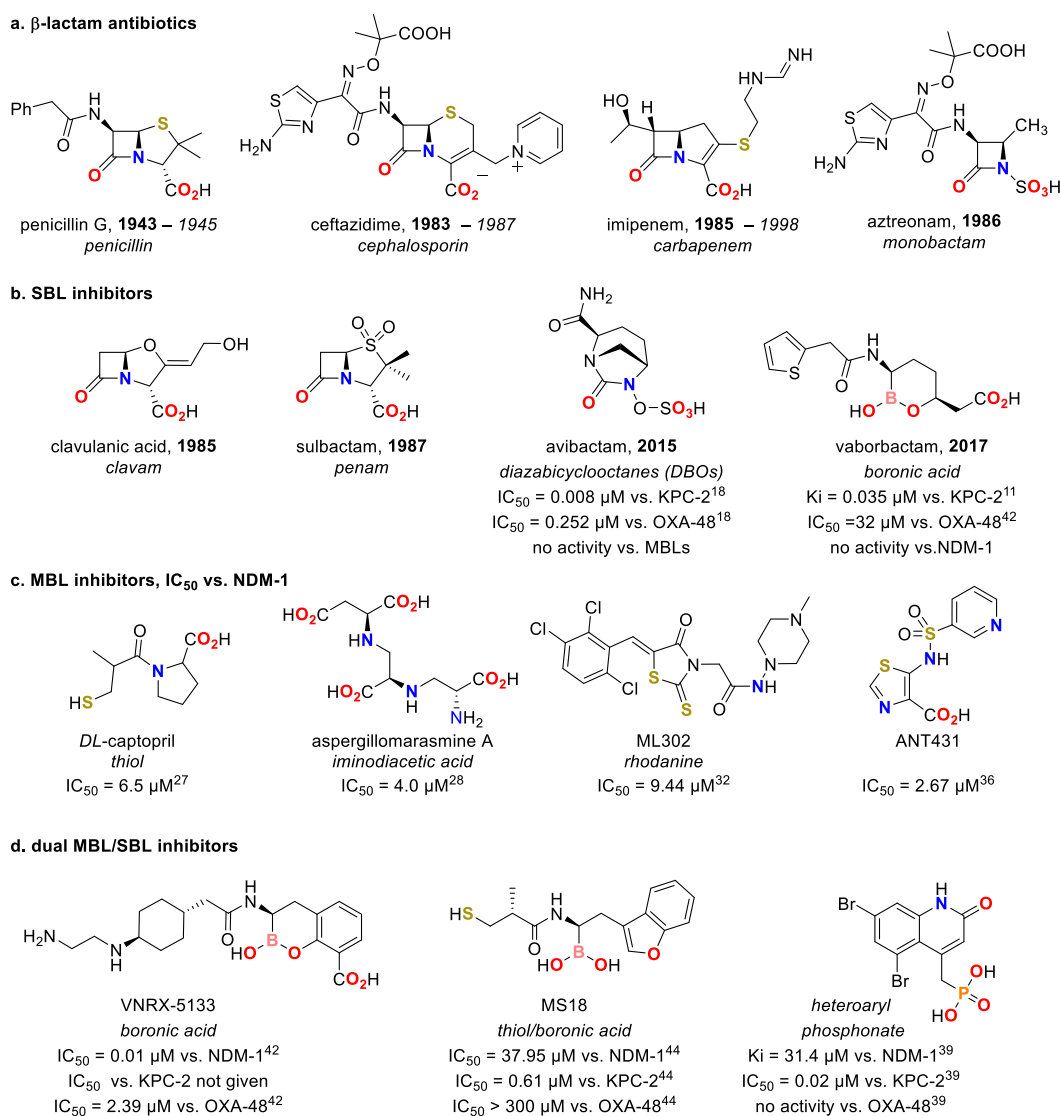


Fig. 1. Representative structures of (a) β -lactam antibiotics (in **bold**: year of entry to the market; in *italics*: year of resistance appearance); (b) serine- β -lactamase inhibitors (in **bold**: year of entry to the market) and inhibitory activities of avibactam and vaborbactam activities vs. carbapenemases; (c) metallo- β -lactamase inhibitors and inhibitory activity vs. NDM-1; (d) dual serine-/metallo- β -lactamase inhibitors and their representative inhibitory activities vs. carbapenemases.

and SBLs (TEM-116, CMY-2 or OXA-10). Very recently, thiol/boronic acid hybrids (such as MS18) were developed and demonstrated interesting dual inhibition properties against SBLs and MBLs [44]. MS18 and its congeners also possess a rather broad scope but show limited effects against NDM-1 (class B) and OXA-48.

In this context, we sought to develop novel inhibitors that could block the activities of both SBLs and MBLs with comparable efficiency, specifically targeting the three clinically-relevant carbapenemases: KPC-2 (class A), NDM-1 (class B) and OXA-48 (class D).

We addressed this challenge by exploring uncharted chemical space around the β -lactam nucleus. Synthetically, the β -lactam ring can be obtained by the Staudinger [45] [2 + 2] cycloaddition between a ketene and an imine (Scheme 1a) [46]. We recently reported that carefully substituted ynamides [47,48] can be used as precursors for the *in situ* generation of ketenimines [49,50] under mild conditions, which can be intercepted by various heterocyclic nucleophiles [51] or can undergo a microwave-assisted [2 + 2] cycloaddition with imines [52]. By replacing the ketene (C=C=O) with the *in situ* generated iminoketene (C=C=NR), the reaction directly led to an azetidininime (Scheme 1b). Such imino- β -lactams have only been rarely studied with respect to their synthesis [53,54] and never with respect to their biological and particularly, antibiotic activities. Taking into account their structural similarities with β -lactams, we thus decided to evaluate their inhibitory activity against carbapenemases.

2. Results and discussion

2.1. Synthesis

Taking into consideration the scope of our previously developed methodology and the fact that only aryl groups can be easily incorporated on the imine (Ar¹) and on positions 1 (Ar³) and 4 (Ar²) of the four-membered-ring [52], we devised a convergent synthetic plan to access as many structural variations as possible on the azetidininime scaffold.

The Ar¹ group originated from aniline **1x**, which was protected by a Boc group to give **2x** (Scheme 2). Coupling of **2x** with brominated triisopropylsilylacetylene provided ynamide **3x** after TBAF-promoted desilylation. Condensation of benzaldehyde **4y** with aniline **5z** afforded imines **6yz**. The key [2 + 2] cycloaddition was performed with microwave heating and gave azetidininimes **7xyz** with concomitant loss of the Boc protecting group. The *para*-phenols **7aam**, **7abm** and **7dfm** were obtained from *para*-alkoxy derivatives **7aaa**, **7aba**, and **7dfc**, respectively, by ether cleavage using BBr₃ (for OMe) or AlCl₃ (for OBn). Overall, more than forty compounds were prepared using this route.

2.2. Initial results

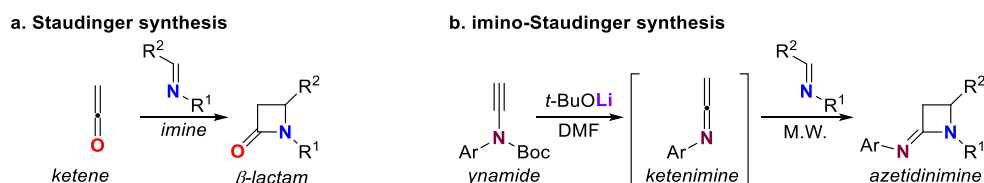
The first compound evaluated was **7aaa**, bearing a *para*-anisyl group on the endocyclic nitrogen. This aryl group was initially chosen for synthetic reasons as it should electronically favor the reaction and could also be easily derivatized. In the presence of

10 μ M of **7aaa**, the hydrolysis of imipenem by NDM-1 was inhibited by 90% with a K_i between 1.0 and 2.4 μ M (Table 1). This very encouraging initial result prompted us to try and gain more insights about this inhibitory activity by performing *in silico* molecular modeling studies (Fig. 2). It appeared that the azetidininime does not seem to behave like a β -lactam during metallo beta-lactamase catalyzed hydrolysis, in which case the carbonyl group is chelated by the zinc ions during the hydrolysis process [22]. Here, the methoxy group of **7aaa**, rather than the imino moiety, would be coordinated by both zinc ions in the active site and the four-membered ring would act as a scaffold to position the two phenyl rings in hydrophobic regions of the enzyme active site.

The Ar¹ phenyl substituent is surrounded by Ser251, Asp212, Ala215 and Ser217 for both enantiomers, whereas the Ar² phenyl substituent is positioned near Asn220 in the *R* enantiomer and in the proximity of Val73, Ile35, Met67 and Phe70 in the *S* enantiomer (Fig. 2). The two enantiomers of **7aaa** were separated by chiral chromatography and each tested separately. Both enantiomers showed similar inhibition of NDM-1 (95% inhibition of NDM-1 at 10 μ M, with an IC₅₀ estimated below 5 μ M) that correlates with the racemic mixture activity.

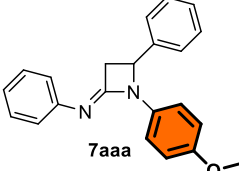
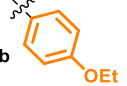
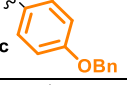
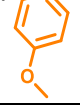
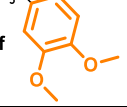


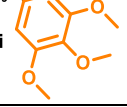


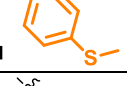
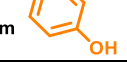
2.3. SAR for enzyme inhibitory activities

A systematic structure-activity relationship study was then undertaken by varying the aromatic groups around the azetidininime nucleus and targeting three carbapenemases: NDM-1, OXA-48 and KPC-2. Preliminary assessment of the compound activities was carried out by first measuring their inhibitory potential at 10 μ M. When the inhibition was lower than 50% at this threshold concentration, the K_i was not determined. In addition to its NDM-1 inhibitory activity, **7aaa** was also found to be moderately active against KPC-2 but did not have any activity against OXA-48 (Table 1). Variations of the Ar³ ring showed that minimal modifications such as replacing the methoxy of **7aaa** by an ethoxy (**7aab**) or a benzyloxy (**7aac**) led to an improvement of the activity against all three enzymes, **7aac** in particular displaying a sub-micromolar activity against NDM-1 ($K_i = 0.2 \mu$ M). An activity profile similar to **7aab** and **7aac** was observed when the methoxy group was in the *meta* position (**7aad**). However, the presence of two methoxy groups (**7aaf**), or a benzo[d][1,3]dioxole moiety (**7aag**), led to a slight decrease of efficiency against NDM-1 and a loss of activity for OXA-48 and KPC-2. This was partially restored by incorporation of a 2,3-dihydrobenzo[b][1,4]dioxane, as **7aah** was found to be quite similar to **7aad** in terms of inhibition profile. While the 3,4,5-trimethoxy derivative **7aai** proved ineffective (which could also be due to its low solubility), aniline-derived compound **7aaj** was found to inhibit all three enzymes with K_i 's of 4.62, 0.8 and 2.41 μ M, for NDM-1, OXA-48 and KPC-2, respectively. The incorporation of an iodine atom (**7aak**) on the *para* position led to solubility issues preventing data from being obtained for OXA-48 and KPC-2 although the anti NDM-1 activity could nevertheless be evaluated to be in the 4.0–5.0 μ M range. The thiomethyl derivative **7aal** was also poorly soluble in water, but a high inhibition of NDM-1 could



Scheme 1. (a) Staudinger synthesis to access β -lactams; (b) imino-Staudinger synthesis to access azetidininimes.

Table 1Influence of Ar³ on NDM-1, OXA-48 and KPC-2 inhibitory activities compared to **7aaa**.N.E.: no effect (at 10 μ M); N. D.: not determined;^a K_i values were determined only for inhibitions >50% at 10 μ M.^b because of some solubility issues (presence of precipitate during the assay) the K_i values were not averaged so a range is given instead.^c the compound was contaminated by residues of heptane.

Compound	K_i (μ M) or % inhibition ^a		
	NDM-1	OXA-48	KPC-2
 7aaa	1.0–2.4 ^b	N.E.	3.6–5–2 ^b
 7aab	1.0–2.4 ^b	45% inhibition at 10 μ M	1.4–3.6 ^b
 7aac	0.20 \pm 0.01	31% inhibition at 10 μ M	1.15 \pm 0.07
 7aad	0.65 \pm 0.07^c	28% inhibition at 10 μ M	3.6–7.2 ^{b,c}
 7aaf	2.4–4.9 ^{b,c}	N.E.	N.E.
 7aag	2.39 \pm 0.29 ^c	N.E.	N.E.
 7aah	0.73 \pm 0.05^c	37% inhibition at 10 μ M ^c	3.6–7.2 ^{b,c}
 7aai	N.E.	N.E.	N.E.
 7aaj	4.62 \pm 0.22	0.8 \pm 0.06	2.41 \pm 0.23
 7aak	1.9–2.4 ^b	N.D.	N.D.
 7aal	<0.5^b	N.E.	50% inhibition at 10 μ M
 7aam	0.36 \pm 0.03	26% inhibition at 10 μ M	45% inhibition at 10 μ M

Color code: **K_i <0.5 μ M** K_i <1.0 μ M K_i <10 μ M IC_{50} >10 μ M

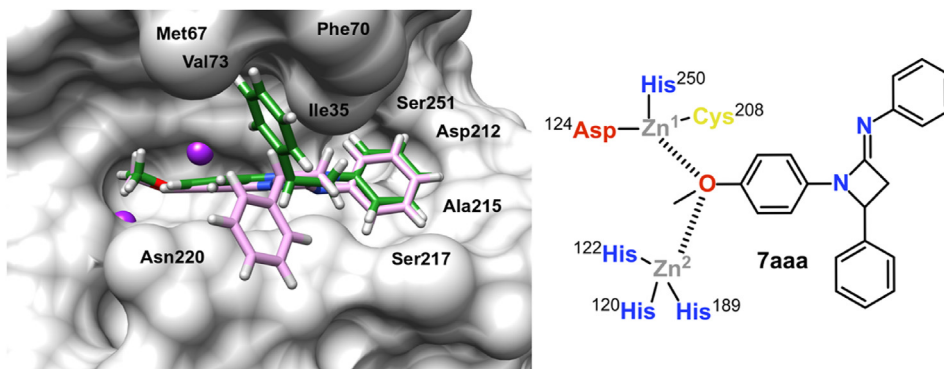
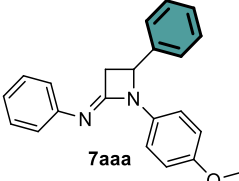









Fig. 2. Docking of compound **7aaa** in the active site of NDM-1 (left) and schematic drawing of its key interactions with the zinc ions (right). The R and S enantiomers of **7aaa** are colored in pink and green, respectively, and the zinc ions are colored in purple.

Table 2

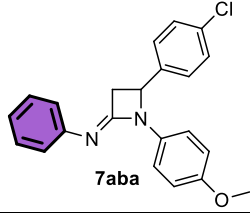
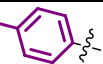
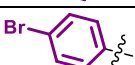
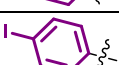
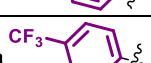
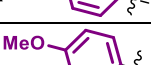
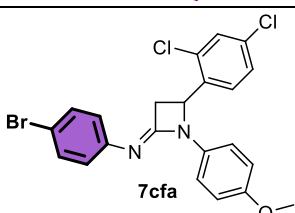
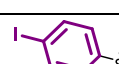
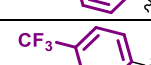
Influence of Ar² on NDM-1, OXA-48 and KPC-2 inhibitory activities compared to **7aaa**.

N.E.: no effect (at 10 μ M); N. D.: not determined;^a K_i values were determined only for inhibitions >50% at 10 μ M.^b because of some solubility issues (presence of precipitate during the assay) the K_i values were not averaged so a range is given instead.^c the compound was contaminated by residues of heptane.

Compound	K_i (μ M) or % inhibition ^a		
	NDM-1	OXA-48	KPC-2
 7aaa	1.0–2.4 ^b	N.E.	3.6–5.2 ^b
 7aba	1.0–2.4 ^b	N.E.	N.E.
 7aca	2.4–4.9 ^{b,c}	N.E.	N.E.
 7ada	N.E.	N.D.	N.E.
 7aea	0.49 ± 0.048	33% inhibition at 10 μ M	0.73 ± 0.05
 7aga	1.0–2.4 ^b	20% inhibition at 10 μ M	1.4–3.6 ^b
 7aha	1.0–2.4 ^b	1.3–2.3 ^b	2.2–5.0 ^b
 7aia	N.E.	N.E.	N.E.

Color code: **$K_i < 0.5 \mu$ M** $K_i < 1.0 \mu$ M $K_i < 10 \mu$ M $IC_{50} > 10 \mu$ M

Table 3Influence of Ar¹ on NDM-1, OXA-48 and KPC-2 inhibitory activities compared to **7aba** and **7cfa**.N.E.: no effect (at 10 μ M);^a K_i values were determined only for inhibitions >50% at 10 μ M.^b because of some solubility issues (presence of precipitate during the assay) the K_i values were not averaged so a range is given instead.

Compound	K _i (μ M) or % inhibition ^a		
	NDM-1	OXA-48	KPC-2
 7aba	1.0–2.4 ^b	N.E	N.E
7bba 	0.24 \pm 0.02	27% inhibition at 10 μ M	1.93 \pm 0.14
7cba 	0.3 \pm 0.06	0.84 \pm 0.04	1.55 \pm 0.18
7dba 	0.33 \pm 0.02	0.83 \pm 0.02	1.81 \pm 0.11
7eba 	0.32 \pm 0.02	32% inhibition at 10 μ M	2.36 \pm 0.21
7fba 	0.86 \pm 0.07	31% inhibition at 10 μ M	N.E.
 7cfa	0.58 \pm 0.04	46% inhibition at 10 μ M	30% inhibition at 10 μ M
7dfa 	0.24 \pm 0.02	0.93 \pm 0.04	1.89 \pm 0.17
7efa 	0.37 \pm 0.02	0.64 \pm 0.04	6.04 \pm 0.27

Color code: K_i<0.5 μ M K_i<1.0 μ M K_i<10 μ M IC₅₀>10 μ M

cephalosporinase CMY-2 (class C) were also inhibited (83% and 86%, at 10 μ M, respectively). These latter results demonstrate that compound **7dfm** can inhibit not only carbapenemases from 3 classes but, more generally, **BLS from all four classes**.

In view of future *in vivo* assays, the phase I metabolic stability of **7dfa**, **7dfc** and **7dfm** was evaluated and the three compounds were found to possess an excellent stability profile with a Cl_{int} between 2.0 and 13.9 μ L/min/mg protein in mouse hepatic cells (Table 7). Their toxicity was measured against both normal cells (MRC-5) and cancer cells (HCT-116) and although **7dfm** was found to have an IC₅₀ in the 20–30 μ M range, neither **7dfa** nor **7dfc** was found to be cytotoxic (IC₅₀ > 100 μ M). Finally, all three compounds were evaluated for the repotentialization of imipenem against the clinical strain of *E. coli* that expresses NDM-1 (amongst other genes of resistance) [56]. In the absence of inhibitor, the MIC was above the resistance threshold at 16 μ g/mL. For **7dfa**, no effect was observed for concentrations up to 100 μ M (although at 200 μ M, the MIC was eventually divided by two). In the presence of 50 μ M (i.e. 30 μ g/mL) of **7dfc**, a notable effect was witnessed as the MIC was divided by 4, bringing it down to the intermediate/resistant limit. The bacteria

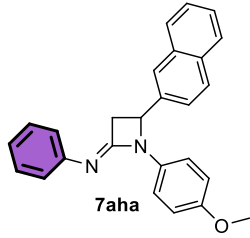
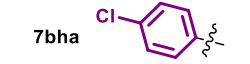
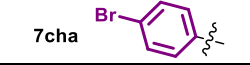
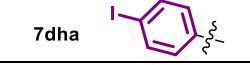
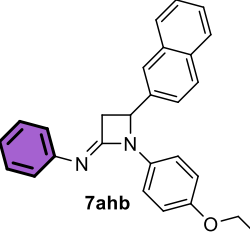
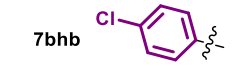
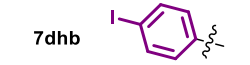
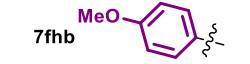
could be made susceptible again (with a MIC of 2 μ g/mL) by using 100 μ M (60 μ g/mL) of the compound. An effect was observed for 20 μ M (i.e. 10 μ g/mL) of **7dfm**, as the MIC was halved. Using 50 μ M (i.e. 25 μ g/mL) of **7dfm**, it could be brought down to 4 μ g/mL, but no improvement could be made using a higher concentration of the inhibitor.

2.5. Molecular modeling

Compound **7dfm** was selected for a more in-depth study of the interaction with NDM-1, KPC-2 and OXA-48. The docking of these enzymes with the two enantiomers of **7dfm** was performed using GOLD [57] and the results for NDM-1 are presented in Fig. 3 and those for KPC-2 and OXA-48 in Figure S1 (see Supplementary Information).

Both enantiomers of **7dfm** appear to interact with NDM-1 in a similar manner as **7aaa** (see Fig. 2). The phenol moiety would be coordinated with the two zinc ions and the 4-iodo-phenyl substituent positioned in the same subpocket in both cases, possibly establishing a stabilizing halogen bond with the side-chain of

Table 4Influence of Ar¹ on NDM-1, OXA-48 and KPC-2 inhibitory activities compared to **7aha** and **7ahb**.N. D.: not determined;^a K_i values were determined only for inhibitions >50% at 10 μ M.^b because of some solubility issues (presence of precipitate during the assay) the K_i values were not averaged so a range is given instead.

Compound	K_i (μ M) or % inhibition ^a		
	NDM-1	OXA-48	KPC-2
 7aha	1.0–2.4 ^b	1.2–2.3 ^b	3.6–5.0 ^b
 7bha	0.73 ± 0.08	18% inhibition at 10 μ M	3.94 ± 0.25
 7cha	0.52 ± 0.03	0.39 ± 0.04	5.23 ± 0.25
 7dha	0.23 ± 0.02	0.85 ± 0.04	1.58 ± 0.12
 7ahb	2.4–3.4 ^b	N.D.	N.D.
 7bhb	0.27 ± 0.03	30% inhibition at 10 μ M	1.96 ± 0.08
 7dhb	0.25 ± 0.02	0.70 ± 0.02	1.84 ± 0.08
 7fhb	0.27 ± 0.03	0.84 ± 0.02	1.81 ± 0.16

Color code: K_i<0.5 μ M K_i<1.0 μ M K_i<10 μ M IC₅₀>10 μ M

Asp212. This would position the 2,4-dichloro-phenyl substituent in a hydrophobic environment bordered by residues Val73, Ile35, Met67 and Phe70 for the *S* enantiomer (Fig. 3b) and in the vicinity of Asn220 for the *R* enantiomer (Fig. 3a).

A comparison between this modeling and the crystallographic structures of NDM-1 in complexes with various ligands published recently [38,39] would suggest that our compounds bind more deeply in the NDM-1 active site cavity and explore different subpockets (Figure S2, see Supplementary Information). Particularly interesting is the lack of inhibition observed for VIM-52, in contrast with VIM-1, as VIM-52 is a His224Arg variant of VIM-1 [55] and this mutation occurs in the L3 loop, which is known to play a role in substrate recognition and specificity [58,59]. Although its carbapenemase activity remains the same, the hydrolysis of ceftazidime and cefepime is slower. Superposition of the two VIM variants on the docking conformations of the **7dfm** enantiomers shows that the loss of activity for VIM-52 could indeed be due to the impossibility of the compound to access the active site, as opposed to VIM-1. This difference is mainly due to the formation of a salt bridge between Arg224 and Glu225, which blocks a significant part of the

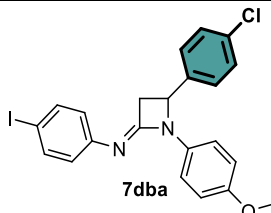
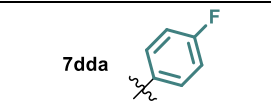
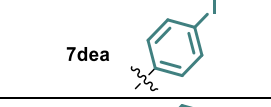
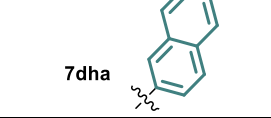
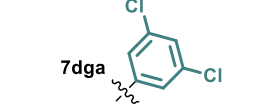
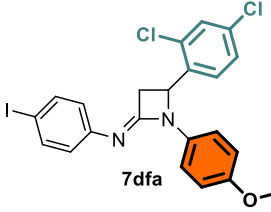
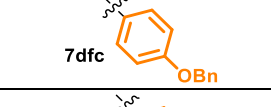
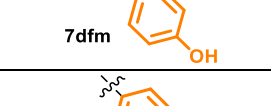
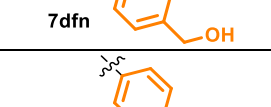
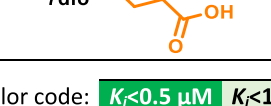
binding pocket in VIM-52. This salt bridge cannot be formed between His224 and Glu225 in VIM-1. Under these conditions, only minor steric hindrance with the 4-iodo substituent of Ar¹ is observed in VIM-1, whereas important clashes are present between Ar¹ and the residues Arg224 and Glu225 in VIM-52 (Figure S3, see Supplementary Information). It is worth nothing that this different behavior of **7dfm** towards VIM-1 and VIM-52 provides experimental support for the binding mode proposed here, confirming that **7dfm** interacts with the L3 loop of NDM-1 and VIM-1, which is not the case with the metallo- β -lactamase inhibitors reported recently by Renslo and Chen [38,39] (Figure S2).

3. Conclusions

The evaluation of azetidinimines, imino-analogues of β -lactams, as β -lactamase inhibitors led to the development of a new family of non-covalent carbapenemase inhibitors [38,39]. Structural optimization led to the identification of phenol **7dfm** as the lead compound, which can strongly inhibit class B MBL NDM-1, class A SBL KPC-2 and class D SBL OXA-48 with K_i 's of 0.07, 0.28 and

Table 5

Influence of Ar² compared to **7dba** and of Ar³ compared to **7dfa** on NDM-1, OXA-48 and KPC-2 inhibitory activities
 N.E.: no effect (at 10 μM); N. D.: not determined;^a K_i values were determined only for inhibitions >50% at 10 μM.

Compound	K _i (μM) or % inhibition ^a		
	NDM-1	OXA-48	KPC-2
 7dba	0.38 ± 0.03	0.85 ± 0.08	1.77 ± 0.18
 7dda	0.50 ± 0.04	0.45 ± 0.04	6.49 ± 0.29
 7dea	0.37 ± 0.03	0.51 ± 0.05	5.66 ± 0.47
 7dha	0.26 ± 0.03	0.86 ± 0.06	1.60 ± 0.15
 7dga	2.04 ± 0.15	0.53 ± 0.06	42% inhibition at 10 μM
 7dfa	0.22 ± 0.02	0.91 ± 0.09	1.97 ± 0.12
 7dfc	0.36 ± 0.03	41% inhibition at 10 μM	3.69 ± 0.23
 7dfm	0.07 ± 0.02	0.07 ± 0.04	0.28 ± 0.01
 7dfn	1.30 ± 0.12	N.E.	3.89 ± 0.37
 7dfo	0.035 ± 0.003	N.E.	4.15 ± 0.38

Color code: K_i<0.5 μM K_i<1.0 μM K_i<10 μM IC₅₀>10 μM

0.07 μM, respectively, which compared quite favorably with the most recently developed MBL-specific and *poly*-carbapenemase inhibitors (see Fig. 1). This molecule can also inhibit other MBLs (NDM-4, NDM-7, NDM-9 and VIM-1), as well as a class C cephalosporinase (CMY-2). Molecular modeling with NDM-1 showed that this compound does not mimic a β-lactam within the enzyme

active sites and that both enantiomers can interact with the enzymes. While this gives hints towards the mode of action of these derivatives against NDM-1 as the prototypical MBL, there are chances that they act in a very different fashion against SBLs. Studies are underway to ascertain the mode of actions of this family of compounds against specific SBLs. The lead compound **7dfm** and

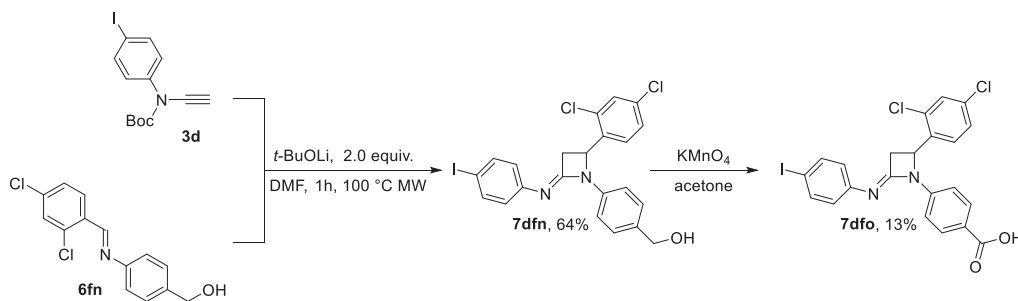
Scheme 3. Synthesis of compounds **7dfn** and **7dfo**.

Table 6

Additional enzymatic inhibitory activities (% at 10 μ M) for **7dfm**.

Compound	NDM-4	NDM-7	NDM-9	VIM-1	VIM-52	CTX-M-15	CMY-2
7dfm	100%	100%	100%	100%	0%	83%	86%

two submicromolar inhibitors of NDM-1 **7dfa** and **7dfc** exhibited excellent metabolic stability and moderate to very low cytotoxicity. Significant repotentialization of imipenem against an NDM-1 expressing resistant *E. coli* strain was achieved with both **7dfm** and **7dfc**. Further refinement of the structure of these molecules will be pursued to improve the efficiency and the drug-like properties of these β -lactamase *pan* inhibitors, before initiating *in vivo* studies.

4. Materials and methods

4.1. General procedure for azetidinimine synthesis

The imine (1.0 equiv.), the ynamide (2.0 equiv.) and the additive – silica gel (1.0 equiv.) or ZnOTf_2 (10 mol%) – were successively added in a microwave sealable tube and placed under argon before the addition of *t*-BuOLi – solid or 2.2 M in solution in THF – (2.0 equiv.) followed by extra dry DMF (0.3 M). The sealed tube was placed in a microwave apparatus for 1 h at 100 $^\circ\text{C}$. The crude material was purified by flash chromatography on silica gel or by preparative TLC. See Supporting Information for detailed synthetic procedures and characterizations of the compounds.

4.2. Cloning of the NDM-1, OXA-48 and KPC-2 genes

The sequences without the peptide signal (predicted by SignalIP 4.1 Server) of *bla*_{NDM-1}, *bla*_{OXA-48} and *bla*_{KPC-2} genes, encoding for

Table 7

Metabolic stability, cytotoxicity and imipenem repotentialization for **7dfa**, **7dfc** and **7dfm**.

	Stability		IC ₅₀ (μM) ^c		MIC ($\mu\text{g}/\text{mL}$) ^d			
	Non-NADPH ^a	CL _{int} ^b	MRC-5	HCT-116	0 μM	20 μM	50 μM	100 μM
7dfa	83%	2.0	>100	>100	16	16	16	16
7dfc	100%	7.6	>100	>100	16	16	4	2
7dfm	96%	13.9	19.9	32.3	16	8	4	4

^a Stability after 45 min in the presence of mouse hepatic microsome in the absence of NADPH.

^b CL_{int} was determined using mouse hepatic microsomes and is given in $\mu\text{L}/\text{min}/\text{mg}$ protein.

^c Inhibition of cell proliferation.

^d Minimum inhibitory concentration for imipenem using *E. coli* GUE-NDM1 [56] clinical strain. No antibiotic effect was observed when the bacteria were incubated with **7dfa**, **7dfc** or **7dfm** at 20 μM , 50 μM or 100 μM .

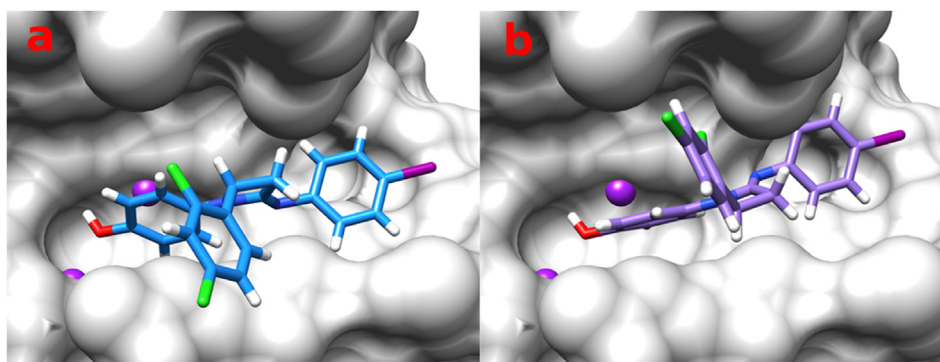


Fig. 3. Docking conformations of the R (a) and S (b) enantiomers of compound **7dfm**, in the active site of NDM-1 (class B) represented as gray surface; the zinc ions are colored in purple.

the mature proteins: NDM-1 (from amino 29 to 293), OXA-48 (from amino 23 to 261), KPC-2 (from amino 30 to 293) and deleted of the stop codon, were amplified by PCR using primers including NdeI and XhoI restriction sites. Then, PCR product was cloned into pET41b vector (Invitrogen®, Life Technologies, Cergy-Pontoise, France), using NdeI and XhoI restriction enzymes, to obtain a C-Term His8-tag. The accuracy of the recombinant plasmid was verified by sequencing, using a T7 promoter and T7 terminator with an ABI Prism 3100 automated sequencer (Applied Biosystems, Thermo Fisher Scientific, Les Ulis, France). The nucleotide sequences were analyzed by using software available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

4.3. Protein expression and purification for NDM-1, OXA-48 and KPC-2

The recombinant plasmids harboring β -lactamase genes were transformed into *E. coli* BL21 (DE3), and an overnight culture was used to inoculate 2 L of Luria Bertani medium broth containing 50 μ g/mL of kanamycin. Bacteria were cultured at 37 °C, until an OD of 0.6 at 600 nm was reached. The expression of the β -lactamase genes was carried out at 37 °C for 3 h, with 1 mM of IPTG as an inducer. Cells were pelleted by centrifugation, at 6000 g for 15 min, then resuspended with 25 mM of phosphate sodium pH 7.4, 300 mM of K_2SO_4 (for OXA-48) or NaCl (for NDM-1 and KPC-2), and 10 mM of imidazole. Bacterial cells were disrupted by sonication, and the bacteria debris was removed by 2 centrifugations: first at 10,000 g for 1 h at 4 °C, and then the supernatants obtained were centrifuged at 96,000 g for 1 h at 4 °C. The soluble fraction was filtered and then passed through a HisTrapTMHP column (GE Healthcare®, Velizy-Villacoublay, France). The recombinant proteins were eluted, using elution buffer (25 mM of phosphate sodium pH 7.4, 300 mM of K_2SO_4 or NaCl, and 500 mM of imidazole). The eluted proteins were concentrated by using Vivaspın 20 (10000 MWCOPEs Sartorius®, Aubagne, France), dialyzed against 0.1 M of HEPES (pH 7.5) buffer and 150 mM K_2SO_4 or NaCl and then loaded onto a HiLoad 16/600 Superdex 75 pg (GE Healthcare). The protein purity, estimated by SDS-PAGE (see [Figures S4–S6](#) in the Supplementary Information), was more than 99%, and the pooled fractions were dialyzed against 100 mM sodium phosphate buffer pH 7 (and 50 μ M $ZnSO_4$ for NDM-1) and concentrated, using Vivaspın columns. Protein concentration was determined by Bradford Protein assay (Bio-Rad, Marnes-La-Coquette, France).

4.4. In vitro β -lactamase inhibition assay

Spectrophotometric assays were performed using ULTROSPEC 2000 UV spectrophotometer and the SWIFT II software (GE Healthcare, Velizy-Villacoublay, France). Assay conditions were as follows: 100 mM phosphate buffer, pH 7 (supplemented with 50 μ M $ZnSO_4$ when testing NDM-1, and with 50 mM $NaHCO_3$ [60,61] when testing OXA-48), 100 μ M imipenem (Sigma-Aldrich, Saint-Quentin Fallavier, France). The reaction was monitored at 297 nm, time course 600 s at 25 °C with 3 min of incubation (compound/carbapenemase). Enzyme concentrations were: 1 nM for NDM-1, 1.7 nM for KPC-2 and 5.8 nM for OXA-48. Initial steady-state velocities from imipenem hydrolysis were measured at a wavelength of 297 nm. IC_{50} values for each inhibitor compound were assayed at seven different concentrations, and performed in triplicate. The K_i of each inhibitor was determined according to the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + [S]/K_m)$, where [S] is the imipenem concentration and K_m is the Michaelis constant of the enzymes [62]. Percentage of inhibition was determined when the IC_{50} of the compound was lower than 10 μ M.

4.5. Minimal inhibition concentrations

MIC values were determined by broth microdilution, in triplicate, in cation-adjusted Mueller Hinton broth according to the Clinical Laboratory Standards Institute (CLSI, <https://clsi.org/>) guidelines. The enterobacterial clinical strain *E. coli* NDM-1 GUE expressing the carbapenemase NDM-1 was used [56]. Experiments were performed in microtiter plates containing the medium with imipenem and inhibitors (dissolved in DMSO). Three inhibitor concentrations were tested: 50, 100 and 200 μ M. Plates were incubated overnight at 37 °C for 18–24 h. In all MIC assays, a negative control (bacteria incubated with different concentrations of compound) was performed. No antibiotic effect was observed, for all the compounds, even with the highest concentration tested (200 μ M).

4.6. Incubations in hepatic microsomes

Compounds (5 μ M) were incubated in 0.5 mg/mL of pooled male mouse liver microsomes (from Biopredic, France), in 0.1 M phosphate buffer at pH 7.4 at 37 °C. After prewarming the mixture for 5 min, reactions were initiated by the addition of NADPH (1 mM). Incubations (400 μ L) were performed at 37 °C for 0, 5, 15, 30 and 45 min in duplicate and the reaction immediately terminated by adding 200 μ L of cold acetonitrile. Samples (including the control to evaluate non NADPH-dependent stability) were centrifuged and the supernatant fractions analyzed by UPLC-MS/MS with multiple reaction monitoring (MRM). Diphenhydramine was used as positive control in the mouse liver microsomes tests. The MRM area response of the analyte was set to 100% with the T0 incubation, the relative decrease in MRM area ratio intensity over time against that of the control (percent parent decrease) was used to determine the half-life ($t_{1/2}$) of compounds in the incubation. Half-life values were calculated from the relationship:

$T_{1/2}$ (min) = 0.693/k, where k is the slope of the Ln concentration vs time curve. The intrinsic clearance (CL_{int}) was calculated as: $CL_{int} = (0.693 \times \text{incubation volume } (\mu\text{L})) / (t \text{ (min)} \times \text{mg of microsomal protein})$.

4.7. Cell culture and proliferation assay

Assays were carried out at the Institut de Chimie des Substances Naturelles by the CIBI screening platform. Cell lines were obtained from the American Type Culture Collection (Rockville, USA) and were cultured according to the supplier's instructions. Briefly, human MRC-5 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS) and 1% glutamine and HCT116 colorectal carcinoma cells were grown in RPMI 1640 containing 10% FCS and 1% glutamine. All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 . Cell growth inhibition was determined by an MTS assay according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, the cells were seeded in 96-well plates (2.5×10^3 cells/well) containing 200 μ L of growth medium. After 24 h of culture, the cells were treated with the test compounds at different final concentrations. After 72 h of incubation, 40 μ L of resazurin was added for 2 h before recording absorbance at 490 nm with a spectrophotometric plate reader. The IC_{50} value corresponded to the concentration of compound inducing a decrease of 50% in absorbance of drug-treated cells compared with untreated cells. Experiments were performed in triplicate. Paclitaxel was used as the reference compound.

4.8. PCR, cloning, expression, and DNA sequencing for NDM-4, NDM-7, NDM-9, VIM-1, VIM-52, CTX-M-15 and CMY-2

Whole-cell DNA of the enterobacteriales expressing β -lactamases NDM-4, NDM-7, NDM-9, VIM-1, VIM-52, CTX-M-15 and CMY-2 was extracted, using the QIAamp DNA mini kit (Qiagen, Courtaboeuf, France) and used as a template to amplify all the different genes. The sequences without the peptide signal (predicted by SignalP 4.1 Server, <http://www.cbs.dtu.dk/services/SignalP-4.1/>) encoding for the mature protein, were obtained by PCR amplification, using the forward primers, which included an *NdeI* restriction site, and the reverse primer which included an *XhoI* restriction site and a deletion of the stop codon of the gene to allow the expression of an C-Term His tag. Then, PCR product was cloned into pET41b vector (Invitrogen®, Life Technologies, Cergy-Pontoise, France), using *NdeI* and *XhoI* restriction enzymes, to obtain a C-Term His₈-tag. The accuracy of the recombinant plasmid was verified by sequencing, using a T7 promoter and T7 terminator with an ABI Prism 3100 automated sequencer (Applied Biosystems, Thermo Fisher Scientific, Les Ulis, France). The nucleotide sequences were analyzed by using software available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

4.9. Protein purification for NDM-4, NDM-7, NDM-9, VIM-1, VIM-52, CTX-M-15 and CMY-2

An overnight culture of *E. coli* BL21 DE3 harboring recombinant pET41b plasmids was used to inoculate 2 L of LB medium broth containing 50 mg/L kanamycin. Bacteria were cultured at 37 °C until an OD of 0.6 at 600 nm was reached. The expression of the β -lactamase genes was carried out overnight at 22 °C with 0.2 mM IPTG as inducer. Cultures were centrifuged at 6000 g for 15 min and then the pellets were resuspended with the binding buffer (10 mM imidazole, 25 mM sodium phosphate pH 7.4 and 300 mM NaCl). Bacterial cells were disrupted by sonication and the bacterial pellet was removed by two consecutive centrifugation steps at 10000g for 1 h at 4 °C; the supernatant was then centrifuged at 96000g for 1 h at 4 °C. The soluble fractions were filtered and then passed through a HisTrap™ HP column (GE Healthcare) and proteins were eluted with the elution buffer (500 mM imidazole, 25 mM sodium phosphate pH 7.4 and 300 mM NaCl). Finally, a gel filtration step was performed with 100 mM sodium phosphate buffer pH 7 and 150 mM NaCl with a Superdex 75 column (GE Healthcare). The protein purity was estimated by SDS-PAGE. The pooled fractions were dialyzed against 10 mM Tris-HCl pH 7.6, 50 μ M of ZnSO₄ was added for NDM and VIM, then concentrated using Vivaspinn columns (Sartorius, Aubagne, France). The concentrations were determined by measuring the OD at 280 nm and with the extinction coefficients obtained from the ProtParam tool (Swiss Institute of Bioinformatics online resource portal) [63].

4.10. Molecular modeling

The three-dimensional structure of compound **7aaa** [52] was retrieved from the Cambridge Structural Database [64] (CSD refcode KEMJEU) and that of compound **7dfm** was built starting from **7aaa** by manual editing using UCSF Chimera package [65]. The structures of enantiomers were generated using an in-house script. Molecular docking was performed using the GOLD suite [57] (CCDC) and the GoldScore scoring function, with the structures 4HL2 [66], 2OV5 [67] and 4S2P [68] as receptors for NDM-1, KPC-2 and OXA-48, respectively. The binding sites were defined as 15 Å radius spheres centered on the Zn1 ion for NDM-1 and on the OG atom of Ser70 for KPC-2 and OXA-48. In agreement with our

previous studies [69–75] showing that an enhanced conformational search is beneficial, especially for large molecules, a search efficiency of 200% was used to better explore the ligand conformational space. All other parameters were used with the default values. The VIM-52 structure was generated from the crystallographic structure of VIM-1 (PDB code 5N5I) [76] by the *in silico* mutation His224Arg using UCSF Chimera [65], followed by energy minimization of Arg224 and Glu225 to optimize the ionic interaction between these two residues. Images were generated using UCSF Chimera [65].

Supplementary information

The Supplementary data contain detailed synthetic procedures and characterizations of the compounds, the data obtained from the docking of KPC-2 and OXA-48 with **7dfm**, interaction of **7dfm** with VIM-1 and VIM-52, and the copies of the NMR spectra.

Author contributions

This study was conceived by K. Cariou, B. I. Iorga, T. Naas and R. H. Dodd. Synthesis and characterization of the compounds were performed by E. Romero, M. Benchekroun, S. Ventre, A. C. A. D'Hollander, K. Vijayakumar and C. Minard. Purification of the proteins, inhibition assays and MIC assays were performed by S. Oueslati with C. Exilie, L. Tlili and A. Zavala. Metabolic studies were performed by L. A. Nguyen under the supervision of A. Pruvost. Structural analysis and molecular modeling studies were performed by A. Zavala, E. Elisée, E. Selwa, P. Retailleau and B. I. Iorga. K. Cariou and B. I. Iorga drafted the manuscript which was amended and commented on by all authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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List of abbreviations

CTX-M-15	Cefotaximase-Munich 15
CMY-2	Cephamecinase-2
DMSO	dimethyl sulfoxide
GNB	Gram-negative Bacilli
HCT-116	Human Colorectal Carcinoma cells
KPC	Klebsiella Pneumoniae Carbapenemase
MBL	Metallo- β -lactamase
MIC	Minimum Inhibitory Concentrations
MRC-5	Medical Research Council cell strain 5
ND	Not Determined
NDM	New Delhi metallo- β -lactamase
NE	no effect
OXA	Oxacillinase

SAR Structure Activity Relationship
 SBL Serine- β -lactamases
 VIM Verona Integron metallo- β -lactamase

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmech.2021.113418>.

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