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Alkylidene Oxapenem β-Lactamase Inhibitors Revisited: Potent Broad Spectrum Activity but New Stability Challenges

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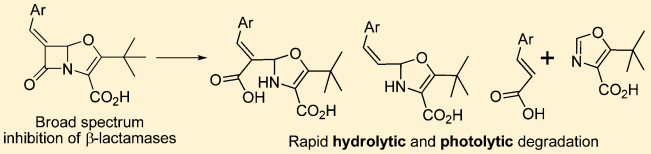
Supporting Information

ABSTRACT: We present a comprehensive study of C6-alkylidene containing oxapenems. We show that this class of β-lactamase inhibitors possesses an unprecedented spectrum with activity against class A, C, and D enzymes. Surprisingly, this class of compounds displayed significant photolytic instability in addition to the known hydrolytic instability. Quantum mechanical calculations were used to develop models to predict the stability of new analogues.



KEYWORDS: *Oxapenems, β-lactamase inhibitors, Gram-negative infections, quantum mechanical calculations, stability*

The β-lactam antibiotics constitute one of the oldest and most widely used classes of antibacterial agents. Unfortunately, resistance to the class is widespread. The primary resistance mechanism is through expression of a collection of enzymes known as β-lactamases that proficiently hydrolyze the β-lactam to an inactive metabolite. One successful strategy to circumvent this resistance combines the β-lactam antibiotic with an agent capable of inhibiting β-lactamases.[1−3] There are currently only three marketed β-lactamase inhibitors, which only inhibit Ambler class A β-lactamases.[4,5] The clinical utility of these established agents is limited by the spread of class C and D enzymes. Hence, there is a significant unmet medical need for broad-spectrum β-lactamase inhibitors with activity against all three classes of serine proteases (class A, C, and D).

β-Lactamase inhibitors based on the oxapenem scaffold have been known for several decades, beginning with a paper describing the synthesis in the 1970s.[6] Later research showed greater potency of C6-alkoxy substituted oxapenems (Figure 1) against several enzymes relative to the marketed drug clavulanic acid.[7−9] These reports mention the susceptibility of the oxapenems to hydrolysis. Pfaendler[10] and Wild[11] established some limited structure−activity relationships (SAR) to enhance stability of the compounds. Wild also described the synthesis of C6-alkylidene oxapenems, containing a double bond attached to the nonbridge carbon on the lactam ring, and noted significant effects of the alkylidene substituent on the stability of the oxapenem core.[12] However, no additional SAR around the alkylidene oxapenems have been reported.

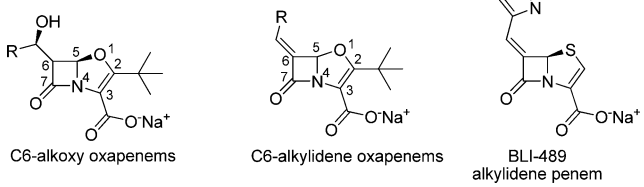


Figure 1. Structures of C6-alkoxy and C6-alkylidene oxapenems, and penem BLI-489.

The structural similarity of the C6-alkylidene oxapenems to the alkylidene penems, exemplified by the clinical candidate BLI-489[13] (Figure 1), caught our attention. Given AstraZeneca's long-standing interest in developing new β-lactamase inhibitors,[14,15] we decided to revisit this scaffold and explore its spectrum against clinically relevant β-lactamases, particularly from class C and D. Herein, we report the extraordinary β-lactamase inhibitory activity of oxapenems with a C6-alkylidene substituent. In addition, we describe predictive models based on quantum mechanical calculations to predict the stability of oxapenem compounds.

Previous reports have shown that a *tert*-butyl substituent at the C2 position led to the highest stability for oxapenems.[11,12] Therefore, we focused our efforts on modifications of the C6 position while keeping the C2 *tert*-butyl substituent in place. The synthesis of the alkylidene oxapenems was carried out according to previously described methods (Scheme 1).[12] Thus, acetoxyazetidinone **1** was converted into methyl thioether **2**, followed by protection of the β-lactam nitrogen with a silyl group. An aldol condensation between racemic **3** and various aldehydes gave access to **4** as a mixture of eight

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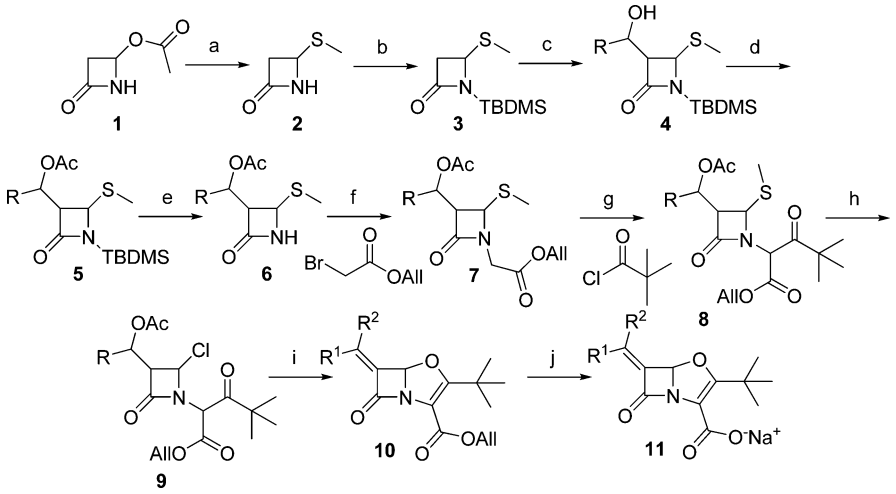
Scheme 1. Synthesis of Oxapenems[a]



[a]Reagents: (a) EtOH, NaSMe, 0−25 °C, 60−70%; (b) DMF, TBDMSCl, DABCO, 80−90%; (c) RCHO, LDA, THF, −78 °C, 40−50%; (d) Ac_2O, TEA, DMAP, 80−90%; (e) TBAF/AcOH, THF, 60−80%; (f) Cs_2CO_3/ACN, 50−70%; (g) LHMDS/THF, 60−70%; (h) NCS, silica, DCM, 30−40%; (i) ^{t}BuOK/^{t}BuOH, THF, 20−30%; (j) TPP, $Pd(PPh_3)_4$/DCM, sodium-2-ethyl hexanoate, 40−50%.

diastereomers. Protection of the hydroxyl group (→ **5**) was followed by desilylation (→ **6**) and alkylation to give **7**. Acylation of the acetate in **7** with pivaloyl chloride gave access to intermediate **8**. Conversion of the methyl thioether into a chloride (**9**) set the stage for a base-mediated cyclization with concomitant elimination of the acetoxy group to form the C6-alkylidene (**10**). It should be noted that the last step removed two chiral centers, resulting in a significant reduction of complexity as the mixture no longer consisted of eight diastereomers but was instead a single racemate. The synthesis was completed by deallylation to give target compound **11** as the sodium salt. The yields for each step were generally moderate to good. A slightly lower yield was observed for the key aldol condensation (**3** → **4**) as well as for the last two steps. The lower yields for the last two steps presumably result from the susceptibility of alkylidene penems to hydrolysis.

We next evaluated the inhibitory activities of the oxapenems against representative serine β-lactamases from class A, C, and D and compared them with those of the marketed β-lactamase inhibitor clavulanic acid (**12**, Table 1). β-Lactamase inhibitory activities were evaluated following 5 min preincubation in an enzymatic turnover assay utilizing the colorimetric substrate nitrocefin. The racemic 2-furan *E*-alkylidene **13**, first reported by Wild et al.,[12] was significantly more potent than enantiomerically pure clavulanic acid, particularly against class C and D enzymes. In fact, **13** showed single digit to double digit nanomolar IC_{50}s against all class A, C, and D β-lactamases tested. The observed inhibitory activity for **13** is among the best reported to date for any β-lactamase inhibitor. To assess the role of the alkylidene substituent, compound **14**, lacking the alkylidene, was synthesized. It was evident by comparing **13** and **14** that the conjugated double bond at the C6 position of the oxapenem scaffold is essential to obtain broad-spectrum activity, particularly against class A enzymes. However, the desalkylidene compound **14** remained surprisingly potent against class C enzymes (and to a slightly lesser extent against class D enzymes).

Having confirmed the essentiality of the alkylidene substituent for broad spectrum coverage, we examined the role of the alkylidene geometry. As shown in Table 1, compounds **15*E*** and **16*E*** were significantly more potent inhibitors than their corresponding *Z* isomers. Table 1 also showed that replacement of the 2-furan (**13**) with a phenyl group (**15**) resulted in increased potency against class C enzymes, but reduced activity against the class A carbapenemase KPC-2. While compound **16*E***, containing a 3-furan group, was more potent against KPC-2 than the compound with the phenyl substituent (**15*E***), its activity was less than that of the 2-furan (**13**), particularly against class C enzymes.

Finally, we examined the effect of introduction of substituents on the aromatic ring by making the *o*-, *m*-, and *p*-methoxyphenyl alkylidenes (**17**−**19**). As can be seen from Table 1, substitution at each of these 3 positions was tolerated, with the resulting compounds displaying similar or slightly improved potency compared to unsubstituted phenyl **15*E***.

During the course of this preliminary SAR evaluation, we realized that further optimization of activity would be a fruitless endeavor if we did not first address the severe stability issues observed for this initial set of compounds. The stability of the compounds in Table 1 was too low to allow evaluation in more advanced models, such as cellular activity assays (inhibition of bacterial growth) or determination of physicochemical properties. Surprisingly, we found that even under strictly anhydrous conditions, the compounds in Table 1 suffered from rapid degradation. Analysis of the reaction mixtures revealed that the degradation pathways for anhydrous and aqueous degradation differed, raising the interesting possibility that this class of compounds suffered from photolytic instability in addition to hydrolytic instability.

Indeed, under carefully controlled conditions, we were able to separate the two distinct degradation pathways (Scheme 2). Thus, HPLC−MS−MS analysis of the degradation mixture in aqueous buffer with exclusion of light suggested that hydrolytic degradation proceeded through opening of the β-lactam with concomitant decarboxylation (D1). An additional minor peak with an identical mass and mass-spec fragmentation pattern but shorter retention time was also detected, suggesting formation of an isomer of D1 (D2). However, analysis of an anhydrous DMSO solution of **13** subjected to ambient lighting suggested formation of three major degradants from two separate

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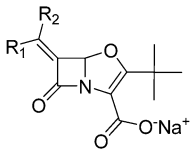
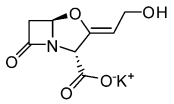
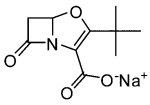
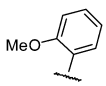
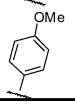
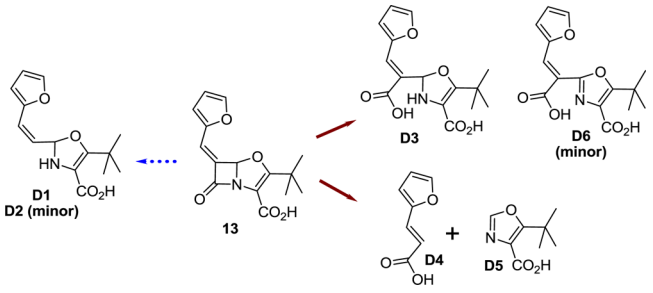
Table 1. Inhibitory Activity of Oxapenems against Representative β-Lactamases

			Class A IC_{50} (μM)			Class C IC_{50} (μM)		Class D IC_{50} (μM)		
Cmpd	**R1**	**R2**	**TEM-1**	**CTX-M-15**	**KPC-2**	**P99[a]**	**AmpC[b]**	**OXA-10**	**OXA-24/40**	**OXA-48**
12			0.13	0.003	>200	>200	>200	0.59	>39	15
13	H		0.002	0.002	0.057	0.022	0.058	0.003	0.01	0.007
14			7.9	0.31	>200	0.025	0.16	0.036	1.7	0.075
15*E*	H		0.073	0.061	29	0.006	0.007	0.01	0.26	0.26
15*Z*		H	2.9	2.2	2	0.25	0.29	0.036	8.6	1.9
16*E*	H		0.003	0.002	0.31	4.1	1.8	0.016	0.003	0.026
16*Z*		H	0.61	0.45	0.11	1.6	1.2	0.007	0.79	1.0
17	H		0.005	0.016	7.7	0.004	0.007	0.007	0.19	0.16
18	H		0.056	0.022	44	0.005	0.008	0.022	0.39	0.23
19	H		0.015	0.007	8.9	0.002	0.007	0.003	0.055	0.078

[a]*E. cloacae* ARC3525. [b]*P. aeuruginosa* PAO1, PDC-1.

Scheme 2. Putative Decomposition Pathways for Compound 13 during Photolytic (Red Arrow) and Hydrolytic (Blue Dotted Arrow) Degradation



pathways: ring opening of the β-lactam to give D3 along with cleavage of the β-lactam ring to give a furan acrylic acid (D4) and an oxazole (D5). The detection of the latter two products suggested that this degradation proceeded through a light-induced retro 2 + 2 addition. A small peak for oxazole D6 was detected as well during the photolysis experiment, presumably the result of oxidation of D3. Small peaks for D1 and D2 were also detected during the photolysis experiment, but at significantly lower levels than during aqueous hydrolysis experiments. Comparison of the hydrolytic and photolytic experiments revealed that the typical β-lactam hydrolysis product D3 was not detected when degradation proceeded in the dark. This counterintuitive observation may indicate rapid decarboxylation of the initial hydrolysis product D3. It is conceivable that detection of D3 during photolysis reflects the formation of a reactive intermediate under anhydrous conditions, which is converted into D3 in the aqueous mobile phase during analysis, thereby limiting decarboxylation prior to detection (and explaining the detection of only minor quantities of D1 and D2 in the photolysis mixture). Finally, it should be noted that all structural assignments are tentative, based on HPLC−MS−MS only, and that formation of isomers with identical molecular weight cannot be ruled out.

Having established the two different degradation mechanisms that plagued the C6-alkylidene oxapenems, we set out to develop robust methods to measure the photolytic and hydrolytic stability. In order to measure the photolytic stability of the oxapenems, data was collected in triplicate on anhydrous

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solutions in DMSO using an autosampler rack that had been modified to allow controlled exposure of the samples to light. Since traditional sun-chamber conditions were found to degrade the compounds very rapidly, a fluorescent light was used to slightly accelerate photodegradation over general laboratory lighting for optimal read-out. Samples were tested at 6 min intervals in triplicate with an autosampler using UPLC-MS. Decomposition was determined based on the quantitative peak area remaining for each measurement. Quantitation was done using detection of the DAD-UV signal between 200 and 400 nm.The standard deviation between the triplicate measurement was reasonable for each of the compounds tested (<10% of the reported value). The results of the photolytic stability assays are shown in Table 2 (data sorted by measured half-life).

Table 2. Experimental Photolytic Stability Data and Calculated Absorbance Wavelengths for C6 Alkylidene Oxapenems; Data Are Sorted Based on Longest Measured $t_{1/2}$

compound (nm)	photolytic $t_{1/2}$ (h)	$\lambda_{calc\text{-}long}$
14	115	286
16*Z*	0.38	421
16*E*	0.32	420
18	0.21	443
15*E*	0.21	445
19	0.20	435
17	0.20	446
15*Z*	0.17	444
13	0.22	456

The data in Table 2 showed that every compound with an extended conjugated system suffered from photolytic instability. The one compound that was stable (**14**) lacked the extended conjugated system and was not expected to absorb visible light. For comparison, BLI-489 (which also possesses an extended conjugated system) had a half-life of 0.5 h in this assay, whereas clavulanic acid (lacking the conjugated system) was stable in this assay (half-life > 100 h). We used the time-dependent density functional theory (TD-DFT) method to calculate the wavelengths of the absorption maxima. These data are also recorded in Table 2. Confirming our hypothesis, the calculated absorption maxima for all photolytically labile compounds were in the visible range, whereas the absorbance maximum for the most stable compound (**14**) was outside the visible range. Moreover, the two alkylidene oxapenems with the shortest calculated absorbance wavelengths (**16*Z*** and **16*E***) were somewhat more stable than the alkylidene oxapenems with longer calculated absorbance wavelengths. Within this series, we felt confident that we could use the calculated absorbance maxima to design compounds with improved photolytic stability.

For analyis of hydrolytic stability, we diluted stock solutions of the test compounds in DMSO to 200 μM in pH 7.4 buffered aqueous solutions. The solutions were stored in amber vials in racks with a controlled temperature (37 °C). Samples were analyzed by HPLC-UV in triplicate using an autosampler over a period of at least 8 h with a minimum of 10 injections per vial. The quantitative peak area, determined by DAD-UV detection between 210 and 400 nm, remaining for each sample was used to monitor hydrolysis (Figure 2). Wild et al. reported half-lives of oxapenems under different conditions, for **15*E*** (4.3 h) and

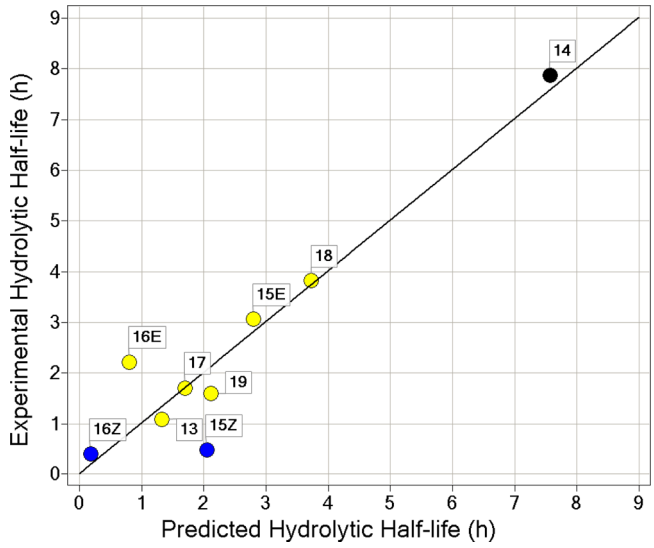

Figure 2. Predictive hydrololytic half-life versus experimental hydrolytic half-life for oxapenem compounds. Data points are colored based on geometry of the alkylidene.

15*Z* (1.4 h),[12] which compared well with our hydrolytic stability data (3.1 and 0.5 h, respectively). Performing the stability experiments multiple times suggested our method was highly reproducible (Supporting Information). Comparison to known inhibitors showed that the oxapenems are significantly less stable: both clavulanic acid and BLI-489 had half-lives of >50 h in this assay.

Having established a reproducible, quantitative method to measure hydrolytic stability, we attempted to provide a predictive model for hydrolytic stability. We anticipated a greater energy barrier from starting material to transition state would translate to greater hydrolytic stability. However, neither the calculated transition state energy (ΔG_{act}) nor endothermicity (ΔG_{rxn}) (calculated using the B3LYP method and the 6-31++G(d,p) basis set with the IEF-PCM solvation model) showed an acceptable correlation with the measured hydrolytic stability. This observation may indicate that different compounds degrade through different pathways or have different rate limiting steps and/or transition states.

At this point, having nine data points for experimental hydrolytic stability of alkylidene oxapenems, we were able to generate a hydrolytic stability QSAR model. The potential descriptors used to generate the model were calculated using the software Semichem CODESSA (Comprehensive Descriptors for Structural and Statistical Analysis; Semichem, Shawnee Mission, KS) following AM1 energy minimization of each structure, and the best model was selected based on the multilinear regression (MLR) method.

Using the MLR method, we generated a QSAR model with a statistically valid correlation between predicted and experimental hydrolytic stability (R^2 = 0.88, *F*-value =53, *t* test = −7.2): predicted hydrolytic half-life = −1571.2 − 112.8·$E_{Res,C-N}$.

This correlation included a single descriptor, the average two-center core−electron resonance energy for a C−N bond ($E_{Res,C-N}$), which describes the charge transfer ability between all carbon−nitrogen bonds. While a correlation between a property and descriptor does not necessarily indicate a causative relationship, these compounds contain a single nitrogen atom (the atom adjacent to the carbonyl), which is

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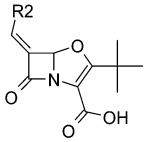
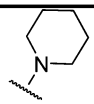
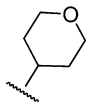
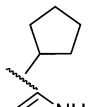
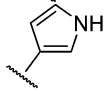
adjacent to the site of attack for hydrolysis. Thus, a mechanistic correlation between the descriptor and the reaction rate seems plausible. The overall trend was quite reasonable (Figure 2) and provided confidence that this model would be able to predict hydrolytic stability of the oxapenems.

With models to predict both hydrolytic and photolytic stability in place, we evaluated a large number of virtual compounds, possessing various aromatic and aliphatic alkylidene substituents, in order to identify candidates for synthesis with acceptable expected stabilities. The criteria we used were predicted hydrolytic half-life > 20 h and predicted $\lambda_{calc\text{-}long}$ < 400 nm. Unfortunately, none of the evaluated compounds met the hydrolytic half-life cutoff. However, several compounds were predicted to have lower $\lambda_{calc\text{-}long}$ (illustrative examples are given in Table 3). Of these, some compounds

Table 3. Predicted Hydrolytic Half-Life and Photolytic $\lambda_{calc\text{-}long}$ for Representative Virtual Alkylidene Oxapenems

Compound		$t_{1/2}$(h)	$\lambda_{calc\text{-}long}$ (nm)
			
20		<0.01	353
21		1.6	382
22		1.2	380
23		<0.01	408

were predicted to be inherently unstable (cf. compounds **20** and **23**). Other compounds (e.g., **21** and **22**) were predicted to be at the lower end of the range of hydrolytic stabilities observed to this point. While we did not expect these compounds to become candidates for further development, we were still interested in preparing these two compounds to test the validity of our predictive models. Unfortunately, attempts to make compounds **21** and **22** failed, apparently due to instability of the final products. The latter was consistent with the low predicted hydrolytic stability (although it should be noted that several compounds in Table 1 were successfully prepared despite similar or lower predicted hydrolytic stabilities).

Since both models showed a reasonable correlation between predicted and measured stabilities, at this point we felt confident that the SAR for hydrolytic and photolytic stability was divergent and that the identification of a compound in the alkylidene oxapenem series combining excellent activity, hydrolytic stability, and photolytic stability would pose a formidable challenge.

While we were ultimately unable to identify a broad spectrum inhibitor for clinical development, this study clearly reveals the unprecedented enzyme inhibitory activity of the oxapenems. Within the constraints of their hydrolytic and photolytic stability, the described oxapenems could be valuable tools to explore inhibition of a broad range of β-lactamases. We have also demonstrated that alkylidene oxapenems suffer from photolytic instability in addition to their known susceptibility to hydrolysis. This new observation may explain why previous attempts to develop C6-alkylidene oxapenems into drug candidates have failed. Finally, the predictive hydrolytic and photolytic stability models presented in this letter may prove valuable for other projects that aim to improve these types of stabilities in β-lactams or other cores.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full experimental details for compounds synthesized and descriptions of biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.M., J.V., and P.S.S. drafted the manuscript. M.M., J.V., S.L., and P.S.S. participated in the design and execution of this study. M.K. and K.R. performed the chemical syntheses. S.T., M.Za., and M.Zh. performed stability studies. T.P. and J.B. performed enzyme inhibition experiments.

Notes

The authors declare the following competing financial interest(s): The authors are current or former employees of AstraZeneca and may possess AstraZeneca stock.

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■ ABBREVIATIONS

SAR, structure−activity relationships; BLI, β-lactamase inhibitor

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