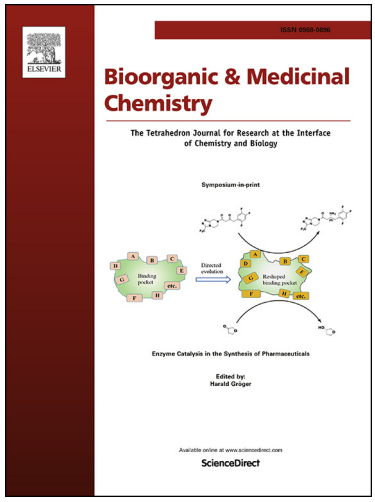
Accepted Manuscript



Specificity of Extended O-Aryloxycarbonyl Hydroxamates as Inhibitors of a Class C β-Lactamase

Alexandra A. Malico, K. Dave, S.A. Adediran, R.F. Pratt

PII: S0968-0896(19)30035-5
DOI: https://doi.org/10.1016/j.bmc.2019.02.023
Reference: BMC 14759

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 6 January 2019
Revised Date: 6 February 2019

Please cite this article as: Malico, A.A., Dave, K., Adediran, S.A., Pratt, R.F., Specificity of Extended O-Aryloxycarbonyl Hydroxamates as Inhibitors of a Class C β-Lactamase, *Bioorganic & Medicinal Chemistry* (2019), doi: https://doi.org/10.1016/j.bmc.2019.02.023

Specificity of Extended O-Aryloxycarbonyl Hydroxamates as Inhibitors of a Class C β-Lactamase

Alexandra A. Malico, K. Dave, S.A. Adediran and R.F. Pratt*

Department of Chemistry, Wesleyan University, Middletown, CT 06459, USA

Keywords

β-Lactamase inhibitor

Irreversible inhibitor

Covalent inhibitor

O-Aryloxycarbonyl hydroxamate

Inhibition kinetics

*Corresponding author

Email address: rpratt@wesleyan.edu

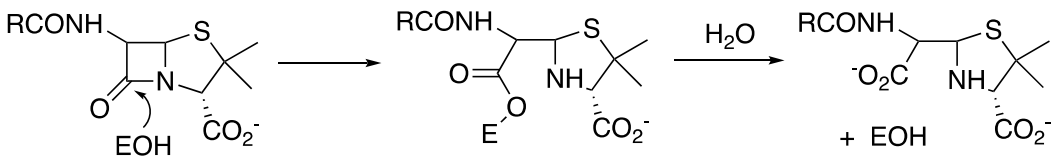
Abstract

Class C β-lactamases have previously been shown to be efficiently inactivated by O-aryloxycarbonyl hydroxamates. O-Phenoxycarbonyl-N-benzyloxycarbonylhydroxylamine (**1**) and O-phenoxycarbonyl-N-(R)-[(4-amino-4-carboxy-1-butyl)oxycarbonyl]hydroxylamine (**2**), for example, were found to be effective inactivators. The present paper describes a structure-activity study of these molecules to better define the important structural elements for high inhibitory activity. The results show that a well-positioned hydrophobic element (which may interact with the Tyr221 residue of the enzyme) and a negatively charged element, e.g. a carboxylate group (which may interact with Arg204), are required for high reactivity with the enzyme. The new compounds were found to inactivate by forming a carbonyl cross-linked enzyme (probably Ser64OCONHLys 315) as for **1** rather than the inert hydroxamoyl derivative observed with **2**.

1. Introduction

Bacteria resist the antibiotic action of β-lactams in several ways, most particularly by the production of β-lactamases.[1] These enzymes catalyze the hydrolysis of β-lactams and readily evolve to do this most efficiently. To overcome the threat posed by β-lactamases to both classical and new β-lactams, there has been much interest for some years now in β-lactamase inhibitors.[2-4] Such molecules can, in principle, inactivate the β-lactamases and thus allow β-lactams to inhibit bacterial cell wall synthesis, incapacitating the bacteria. Mechanistically, there are two groups of β-lactamases, the serine β-lactamases (classes A, C, and D) and the metallo-β-lactamases (class B). In the former group, an active site serine residue provides a nucleophilic hydroxyl group that catalyzes β-lactam hydrolysis by a double displacement mechanism via a covalent acyl-enzyme intermediate (Scheme 1).[5] Most effective serine β-lactamase inhibitors, including those that are currently used in medicine, act by covalent modification of the enzyme, generally of the active site serine hydroxyl group. The recently developed diazabicyclooctanes[6] and cyclic boronic acids[7] are of this type. Although non-covalent inhibitors of serine β-lactamases have been discovered, they have not yet achieved a sufficiently broad spectrum of high activity to allow clinical development.[8-11]

Scheme 1. The β-lactamase reaction.



Some time ago, we reported a new class of covalent inhibitors of serine β-lactamases, the O-aryloxycarbonyl hydroxamates.[12,13] The lead compound **1** (Figure 1) had major activity against class C β-lactamases. The activity of various close analogues of **1** has also been described,[13,14] as well as that of cyclic variants.[15] These compounds have also been shown to be very effective inhibitors of N-terminal hydrolases such as the proteasome[16] and penicillin acylase.[17] Most recently, the synthesis and activity of the peptide analogue **2** against β-lactamases has been reported.[18] This compound was designed to be an inhibitor of bacterial DD-peptidases and thus an antibiotic. It had little DD-peptidase inhibitory activity, however, but was surprisingly active, more so than **1**, against the *Enterobacter cloacae* P99 class C β-lactamase. The latter activity was interpreted in terms of the modeled structure shown in Figure 2, where interactions between the polar terminus of the inhibitor and the protein were proposed to provide the specificity of **2** as an inhibitor.[18] Hydrophobic interaction between the trimethylene chain of the inhibitor and the aromatic ring of Tyr221 may also be important. To determine the relative contributions of the interactions described above, a new series of compounds **3** – **7** (Figure 1) was designed. The synthesis of these compounds and their activity as inhibitors of the class C P99 β-lactamase are described in this paper.

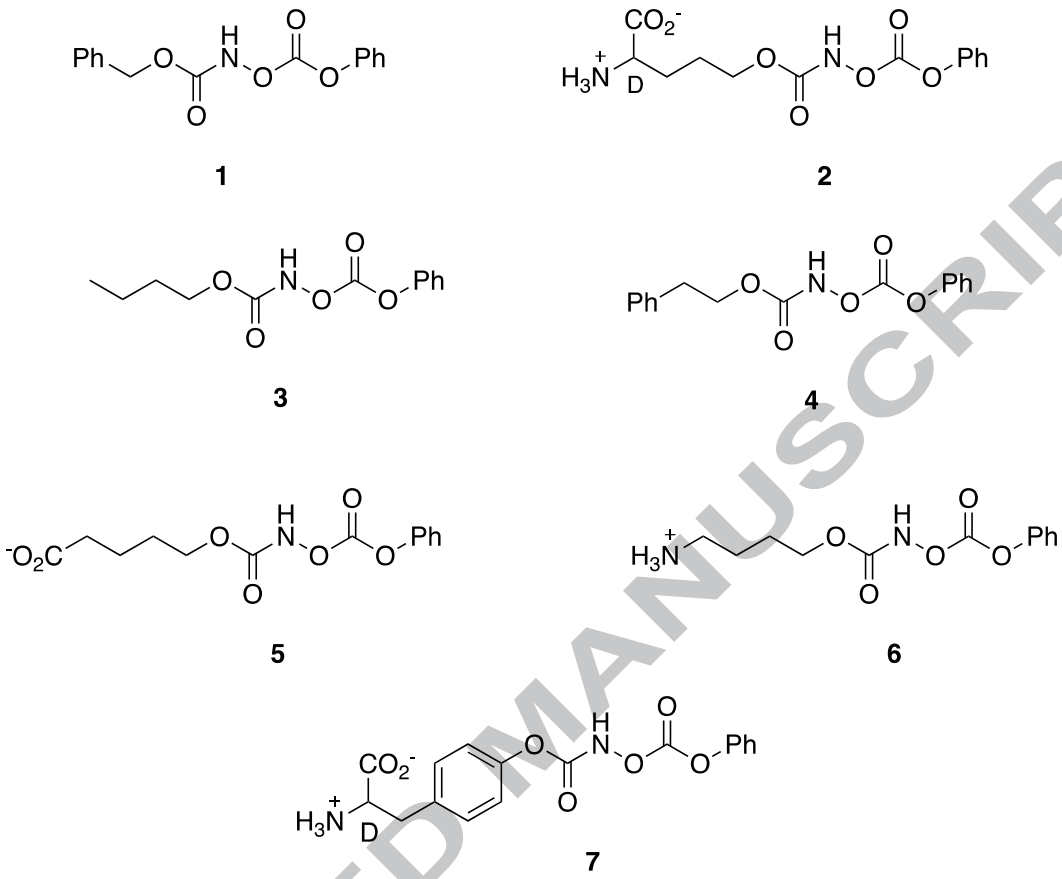


Fig. 1. Aryloxycarbonyl hydroxamate inactivators of Class C lactamases

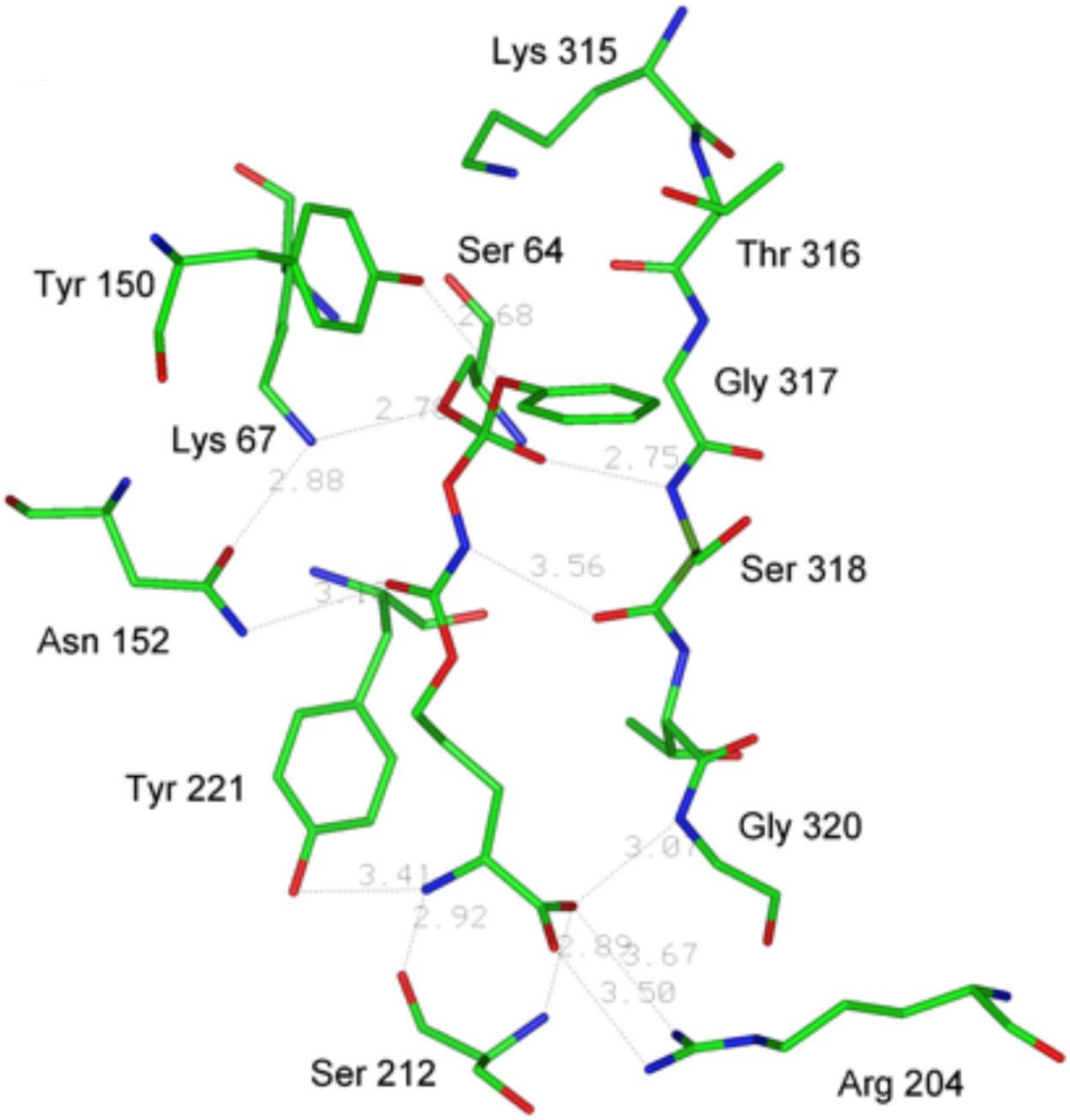


Fig. 2. An energy-minimized model of the tetrahedral intermediate formed on reaction of compound **2** with the P99 β-lactamase. Only heavy atoms are shown. The preparation of this model is described in reference 18.

2. Results and Discussion

2.1 Synthesis

The O-aryloxycarbonyl hydroxamate inhibitors **3** - **7** were prepared essentially as described earlier,[12-14] by acylation of the appropriate hydroxamic acid with phenyl chloroformate in the presence of the base imidazole (Schemes 4-8). Synthesis of the relevant hydroxamic acid for the functionalized inhibitors **5** – **7** (Schemes 6-8), was achieved by activation of an alcohol or phenol by reaction with carbonyl diimidazole in the presence of imidazole. The resulting acyl-imidazole was then treated with a hydroxylamine hydrochloride in the presence of imidazole. Protecting groups were removed in the final step by treatment with trifluoroacetic acid. The final products were characterized by their IR and ^{1}H NMR spectra and by high resolution mass spectrometry; the acyl hydroxamate moiety has a distinctive carbonyl stretching absorption around 1800 cm^{-1} in IR spectra and a highly downfield NH resonance around 12 ppm in ^{1}H NMR spectra.[12-14]

2.2 Kinetics of the inhibition reaction

Aryloxycarbonyl hydroxamates have been shown to be irreversible inactivators of class C β-lactamases.[12-14] The current compounds **3** - **7** also irreversibly inactivated a representative class C β-lactamase, that from *Enterobacter cloacae* P99. In order to determine the kinetics of reaction of these compounds with the β-lactamase, solutions containing a fixed concentration of the enzyme and varied concentrations of the inhibitor were incubated and the activity of the enzyme monitored as a function of time in each case (see Experimental section for details). No return of activity of the enzyme was observed after the activity reached a minimum value, i.e. the inactivation reaction is irreversible, as previously observed.[12-14] Examples of two such experiments with compound **4** are shown in Figure 3A.

A titration of enzyme activity with various concentrations of **4** was also performed (see experimental section for details) with results shown in Figure 3B. The data from these experiments were fitted simultaneously to Scheme 2 as described in the experimental section and yielded the rate parameters presented in Table 1. Similar experiments were carried out with the other compounds giving rate parameters that are also presented in Table 1. Comparable results, previously published,[12,18] for compounds **1** and **2** are also presented in Table 1.

Scheme 2. Kinetic mechanism for reaction of the inhibitors **3** – **7** with the P99 β-lactamase.

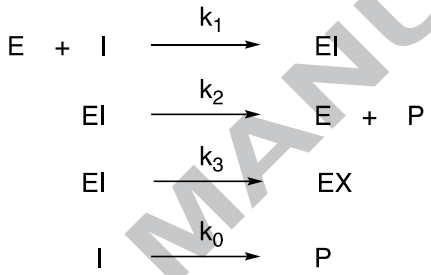
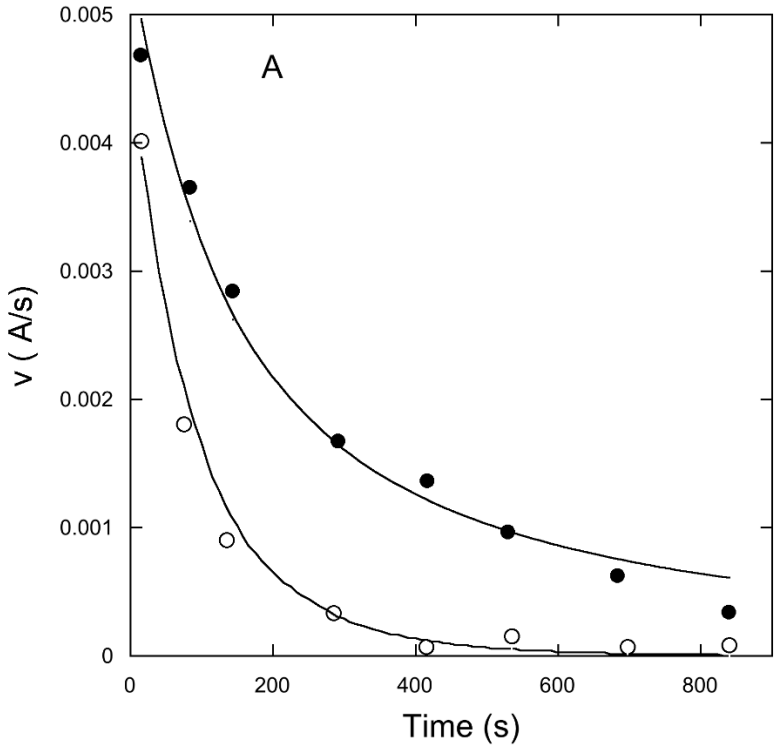


Fig. 3. (A) Activity of the P99 β-lactamase (0.15 μM) as a function of time in the presence of **4** [5.0 μM (●) and 10.0 μM (○)]. (B) Activity of the P99 β-lactamase (0.15 μM) after complete reaction with **4** at various concentrations. In both A and B the points are experimental and the lines represent a least squares fit to all of the data.



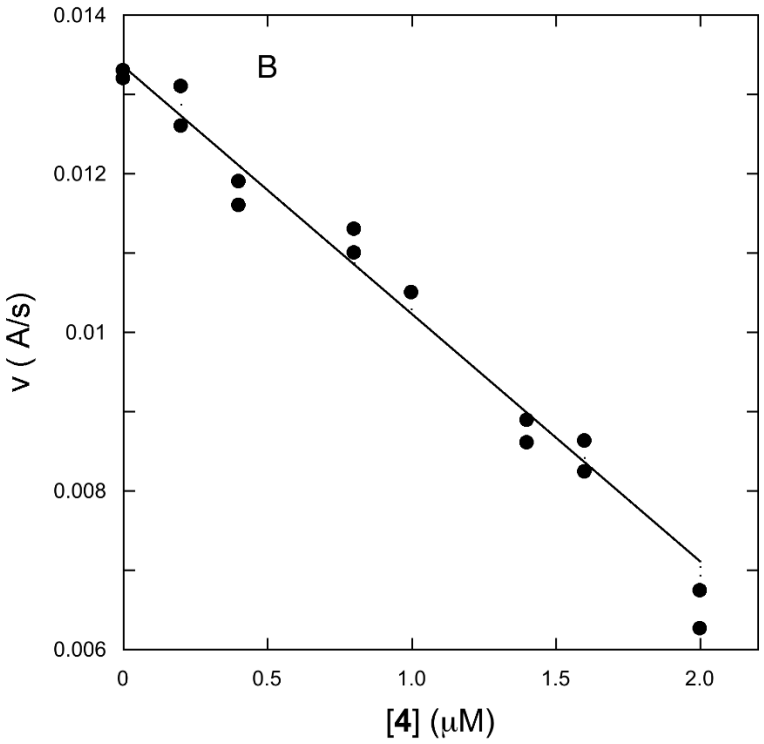


Table 1. Rate constants for reaction of inhibitors **1** – **7** with the P99 β-lactamase.

Compound	k_1 ($M^{-1}s^{-1}$)	k_2/k_3	k_i ($M^{-1}s^{-1}$)[a]
1[b]	$(6.1 \pm 0.2) \times 10^3$	2.0 ± 0.1	$(2.0 \pm 0.1) \times 10^3$
2[c]	$(3.50 \pm 0.04) \times 10^4$	1.25 ± 0.20	$(1.6 \pm 0.1) \times 10^4$
3	$(3.6 \pm 1.2) \times 10^3$	2.4 ± 0.4	$(1.1 \pm 0.4) \times 10^3$
4	$(3.7 \pm 1.2) \times 10^4$	25 ± 1	$(1.4 \pm 0.5) \times 10^3$
5	$(2.8 \pm 0.6) \times 10^4$	2.3 ± 0.1	$(8.5 \pm 1.8) \times 10^3$
6	40 ± 5	≤ 1	20 - 40
7	360 ± 40	≤ 1	180 - 360

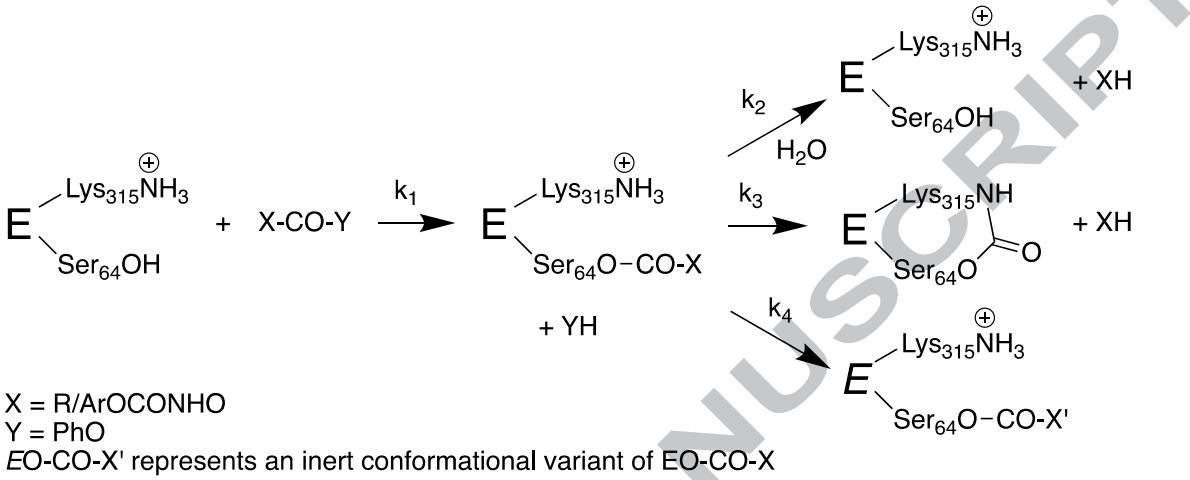
a $k_i = k_1/(1 + k_2/k_3)$
b Data from reference 12.
c Data from reference 18.

2.3 Mass spectra of EI complexes

Electrospray mass spectra of complexes of **3** - **7** with the P99 β-lactamase were obtained as described in the Experimental section. The spectra of all five compounds displayed a dominant peak at M + (26 ± 5) Da, where M is the mass of the parent protein (39,192 Da). This is the same result as obtained with **1**.[12] In that case it was shown by means of a crystal structure that the enzyme inactivation was achieved by insertion of a CO moiety (mass 28 Da), derived from the inhibitor, between the side chain oxygen atom of Ser 64 and the side chain nitrogen atom of Lys 315 (with loss of two protons). Such an insertion probably arose by the mechanism of Scheme 3. In this reaction, both X and Y are displaced by nucleophilic attack, probably first by the active site serine residue that is the nucleophile in normal substrate turnover, and then, in an intramolecular reaction, by Lys 315. In the present cases, the order of displacement of X and Y is not known, but, in the case of **1**, OPh was first displaced.[13] The protons released in the reaction are taken up by X and Y. Hydrolysis of EOCOX, competing with cyclization, leads to the observation of a small amount of turnover

accompanying inactivation (Schemes 2 and 3), as observed for **1** and its analogues previously studied.[12-14,18]

Scheme 3. Reaction of the inhibitors **1** – **7** with the P99 β-lactamase.



Compound **2**, in contrast to **1**, and now to **3** - **7**, has been reported to yield an inert complex that retained the hydroxamate moiety of the inhibitor, i.e M + COX′.[18] It was suggested that this complex arose from a conformational arrangement of the initial acyl-enzyme intermediate to one inert to nucleophilic attack catalyzed by the enzyme and also different from that needed to position it for attack by Lys 315 and cyclization (Scheme 3), the latter of which must occur with **1** and **3** - **7**.[13] It is interesting that the hydroxamate side chains of **5** and **6**, despite their similarity to that of **2**, although lacking the ammonium and carboxylate groups, respectively, cannot achieve an inert M + COX′ conformation. Either the charge or bulk of the polar terminus of **2** must favor the inert conformation rather than the one leading to the cyclization reaction.

It was also noticed that the mass spectrum of the enzyme complex of compound **3** showed a peak at M + 185, i.e. M + CO + COX. Apparently **3** is less specific than the other

compounds, perhaps because of its mobile n-butyl side chain, and is able to acylate a second enzyme functional group, e.g. Lys 67.

2.4 Structure-function issues among O-aryoxycarbonyl hydroxamate β-lactamase inhibitors

A combination of the data of Table 1 and the mass spectrometry results allows several interesting conclusions to be drawn. First, the relative k_1 values (second order rate constants for reaction of E and I – see Scheme 2) will be discussed. The results from previous papers[12,18] show that k_1 for **2** is significantly larger than that for **1**. This result was interpreted in terms of energetically favorable interactions between the polar side chain terminus of **2** and protein residues, as shown in Figure 1, particularly Arg204 and Ser212. These interactions were proposed to restrict the mobility of **2** and thus contribute to its high rate of reaction with the enzyme. The former of these residues was thought to interact with the carboxylate terminus of **2** and the latter with the ammonium terminus. In addition, the aromatic ring of Tyr221 may promote the reaction by hydrophobic reaction with the side chain aromatic ring of **1** and the aliphatic chain of **2**.

That the k_1 value of **3**, with only an aliphatic side chain, is comparable to that of **1** may also reflect its hydrophobic interaction with Tyr221, as described above. Of more interest, however, is the finding that the k_1 value of **4** is greater than those of **1** and **3** and, indeed, comparable to that of **2**. This indicates that specifically placed hydrophobicity in the hydroxamate side chain is of great importance in achieving a rapidly reacting inhibitor of this type. The insertion of an additional methylene group into the side chain of **1** to yield **4** may allow better positioning of the aryl group with respect to Tyr221.

Compounds **5** and **6** are structural analogues of **2** designed for evaluation of the importance of the terminal carboxylate and ammonium groups of the side chain, respectively; comparison of the reactivity of **5** and **6** is therefore of considerable interest. It is clear from the data of Table 1 that the side chain terminal carboxylate of **5**, presumably because of its interaction with Arg 204, is much more effective in inducing reactivity than the side chain ammonium group of **6** with its putative interaction with Ser212. Whereas **5** has comparable activity to **2**, **6** has little activity at all. This indicates that the ammonium group contributes very little to the reactivity of **2**. It must, however, contribute to the formation of a more inert form of the acyl-enzyme (k_4, Scheme 3), not susceptible to hydrolysis. [18]

Compound **7** was conceived of as an easily prepared combination of **1** and **2**, containing the hydrophobic ring of **1** and the polar side chain terminal elements of **2**. Its lack of significant activity (Table 1), however, indicates a less than optimal placement of both motifs with respect to the respective enzyme functional groups. Indeed, its failure emphasizes the specificity of **2**, **4** and **5**.

The real effectiveness of these compounds as enzyme inactivators, as opposed to enzyme acylators, is provided by the rate parameter k_i, which takes into account not only the acylation rate (k_1) but also the partition of the acyl-enzyme between hydrolytic turnover (k_2) and enzyme inactivation (k_3) (Scheme 2); in terms of Scheme 2, $k_i = k_1/(1 + k_2/k_3)$. Calculated values of k_i from the experimentally determined k_1 and k_2/k_3 values are also shown in Table 1. The k_i parameter thus identifies **2** and **5** as the most effective inactivators. Notably **4**, although the most reactive compound with the enzyme (k_1), is a less effective inactivator because of the partitioning of its derived acyl-enzyme in favor of hydrolysis. This may derive from a relatively large k_2 value, a small k_3 value, or a combination of both.

3. Conclusions

In this paper, we have identified two important side chain determinants for the class C β-lactamase inactivating power of O-aryloxycarbonyl hydroxamates. For high activity, the hydroxamate side chain should incorporate a hydrophobic element, as seen in **4** and a negatively charged element, as seen in **2** and **5**, both of which should have optimal positions with respect to the enzyme surface during the acylation and inactivation reactions. It is likely that a combination of these elements, both optimally placed (unlike in **7**), would produce a more potent β-lactamase inactivator than those discovered to date, both in O-aroyl hydroxamates and also in other platforms. The specificity of the hydroxamate moiety itself and the aryloxide leaving group have been previously examined.[13,14]

4. Experimental section

4.1. General procedures

The solvents ethyl acetate, toluene, and dichloromethane (DCM) were purchased from Pharmco. Hexanes, sodium sulfate and magnesium sulfate were purchased from Macron Fine Chemicals, hydrochloric acid from Pharmco, benzene from EMD chemicals, triethylamine from Fluka Chemica, phenyl chloroformate and imidazole from Sigma Aldrich, pyridine from Spectrum, 1,1'-carbonyldiimidazole (CDI), 4-butyl chloroformate, hydroxylamine hydrochloride, and δ-valerolactone from Acros Organics, 1-amino-4-butanol and D-tyrosine t-butyl ester from Chem-Impex, and O-benzylhydroxylamine hydrochloride from Aesar. Thin layer chromatography plates were purchased from AnalTech. Silica gel for flash chromatography (32 – 63 μm) was obtained from Silicycle.

^{1}H NMR spectra were obtained from a Varian 300 MHz spectrometer and IR spectra from a Perkin Elmer Spectrum BX FT-IR system. NMR chemical shifts are reported as δ [ppm]. High

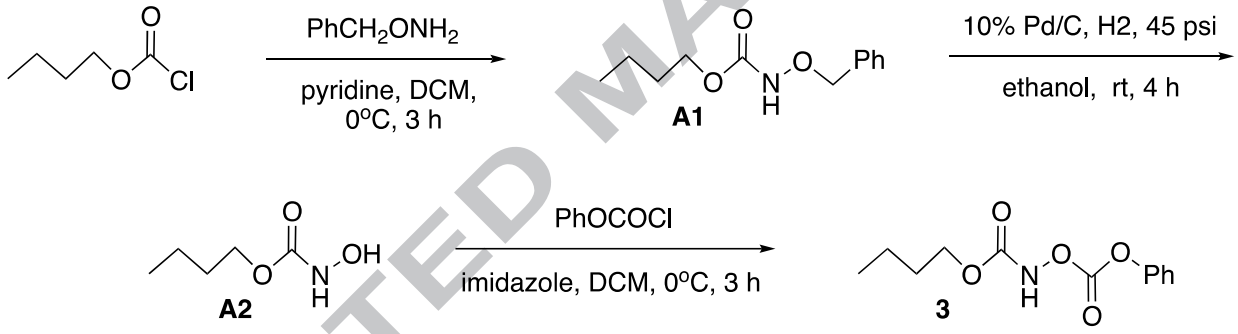
resolution mass spectra were obtained from the Mass Spectrometry Laboratory, University of Illinois at Urbana-Champaign.

The class C β-lactamase from *Enterobacter cloacae* P99 was purchased as a pure protein from the Centre for Applied Microbiology and Research, Porton Down, Wiltshire, U.K. and used as received. Bovine serum albumin (BSA) and MOPS buffer were purchased from Sigma Aldrich. Absorption spectra and spectrophotometric reaction rates were obtained from a Hewlett Packard 8453 UV spectrophotometer.

4.2. Chemical Syntheses

*Synthesis of **3** (Scheme 4)*

Scheme 4



O-Benzyl-N-(4-butyloxycarbonyl) hydroxylamine (**A1**) A solution of O-benzylhydroxylamine hydrochloride (1.48 g, 12.1 mmol) and pyridine (2.22 g, 28.2 mmol) in DCM (60 mL) was added drop-wise under a nitrogen atmosphere at 0°C to a solution of 4-butyl chloroformate (1.65 g, 12.1 mmol) in DCM (120 mL). The reaction mixture was stirred for three hours at 0°C and then slowly brought back to room temperature. The organic layer was evaporated to dryness and the residues were dissolved in 50:50 ethyl acetate:ether. The organic layer was washed with 1 M HCl, dried over magnesium sulfate, and evaporated to dryness to obtain **A1** as a colorless oil. Yield: 1.41 g, 52%. The product thus obtained was used in the next step without further

purification. ^{1}H NMR ($CDCl_3$, 300 MHz): 0.95 (t, 3H, J = 6.0 Hz), 1.41 (quint, 2H), 1.66 (hext, 2H), 4.14 (m, 2H), 4.85 (s, 2H), 7.18 (s, 1H, -NH), 7.38 (s, 5H).

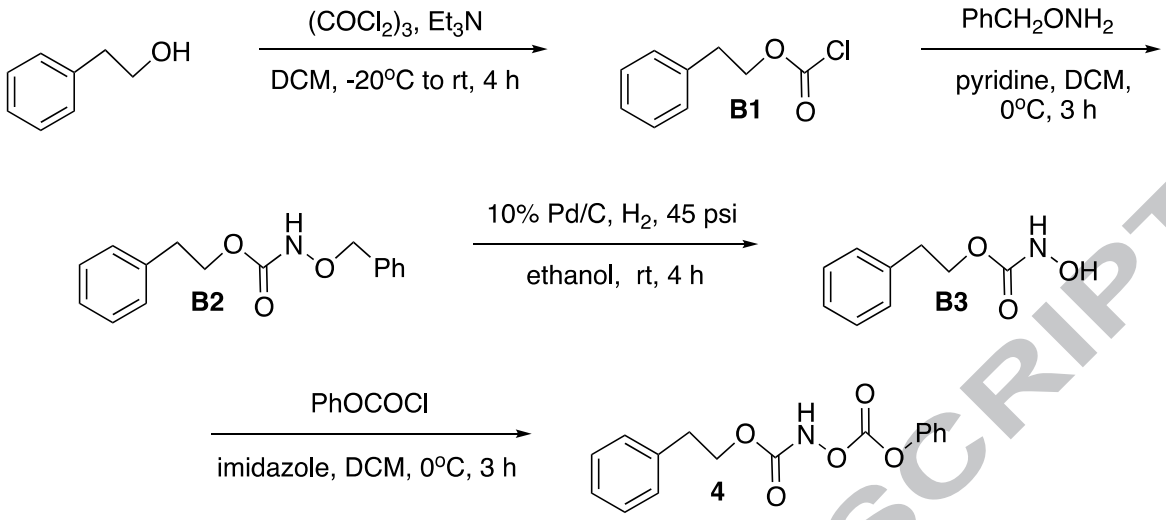
N-(4-butyloxycarbonyl) hydroxylamine **(A2)** Compound **A1** (1.41 g, 6.3 mmol) and 10% Pd/C catalyst (0.2 g) were added to ethanol (20 mL). The reaction was hydrogenated in a Parr apparatus at 45 psi for 4 hours. The catalyst was removed by filtration and the filtrate was concentrated by rotary evaporation to obtain **A2** as a colorless oil. Yield 0.55 g, 64%.

^{1}H NMR (DMSO, 300 MHz): 0.88 (t, 3H, J = 6.2 Hz), 1.32 (m, 2H), 1.54 (m, 2H), 4.00 (m, 2H), 8.65 (s, 1H, -NH), 9.54 (s, 1H, -OH).

O-(Phenoxycarbonyl)-N-(4-butyloxycarbonyl) hydroxylamine (**3)** Imidazole (0.206 g, 4.1 mmol) in DCM (3.3 mL) was added to **A2** (0.546 g, 4.1 mmol) in DCM (8 mL) under a nitrogen atmosphere at 0°C. The mixture was stirred for 20 minutes. A solution of phenyl chloroformate (0.46 g, 4.1 mmol) in DCM (1.6 mL) was added drop-wise to the reaction mixture over one minute. The resulting mixture was stirred for 45 minutes under nitrogen atmosphere at 0°C and then filtered. Ethyl acetate (10 mL) was added to the filtrate to produce a colorless precipitate. The precipitate was removed by filtration and the filtrate rotary evaporated to dryness and then dried further overnight by means of an oil pump. The crude product was purified by chromatography on silica gel (90:10 hexane:ethyl acetate), yielding **3** as a yellow oil (0.164 g, 14%). ^{1}H NMR (D_6-DMSO, 300 MHz): 1.18 (t, 3H, J = 6.2 Hz), 1.33 (m, 2H), 1.69 (m, 2H), 4.00 (m, 2H), 7.40 (m, 5H), 11.56 (s, 1H, -NH). FTIR (cm^{-1}) 1734, 1799. High resolution ES(+)MS m/z = 276.0840, calcd for $C_{12}H_{15}H_5NO_5$ 276.0848.

*Synthesis of **4** (Scheme 5)*

Scheme 5



2-Phenylethyl chloroformate (**B1**) 2-Phenylethanol (2.06 g, 16.8 mmol) in DCM (62 mL) was added to a pre-cooled solution of triphosgene (5.00 g, 16.8 mmol) in DCM (31 mL) under a nitrogen atmosphere at -20°C and stirred for 10 minutes. Triethylamine (2.04 g, 20.2 mmol) in DCM (9.3 mL) was added to the reaction mixture and stirred to room temperature over 4 hours. The reaction mixture was evaporated to dryness and the resulting residue was stirred in ethyl acetate (60 mL). The resulting suspension was then filtered, the filtrate evaporated to dryness, and further dried overnight by means of an oil pump. The product thus obtained was used for the next step without further purification. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.05 (t, 2H, J = 6.9 Hz), 4.51 (t, 2H, J = 7.2 Hz), 7.30 (m, 5H). FTIR (cm^{-1}) 1778.

O-Benzyl-N-(2-phenylethyloxycarbonyl) hydroxylamine (**B2)** A solution of O-benzylhydroxylamine hydrochloride (2.8 g, 16.8 mmol) and pyridine (3.06 g, 39.1 mmol) in DCM (83 mL) was added drop-wise under a nitrogen atmosphere to a solution of **B1** (3.1 g, 16.8 mmol) in DCM (167 mL) at 0°C. The reaction mixture was stirred for three hours at 0°C and then slowly brought back to room temperature. The reaction mixture was evaporated to dryness and the resulting residue was dissolved in 50:50 ethyl acetate:ether. The organic layer was washed with 1 M HCl, dried over magnesium sulfate, and evaporated to dryness to obtain the

crude product. This material was purified by flash chromatography on silica gel (90:10 hexane:ethyl acetate), yielding the product **B2** as a yellow oil. Yield 1.00 g, 22%. ^{1}H NMR (D_6-DMSO, 300 MHz): 2.89 (t, 2H, J = 6.8 Hz), 4.22 (t, 2H, J= 5.1 Hz), 4.68 (s, 2H), 7.29 (m, 10H), 10.34 (s, 1H).

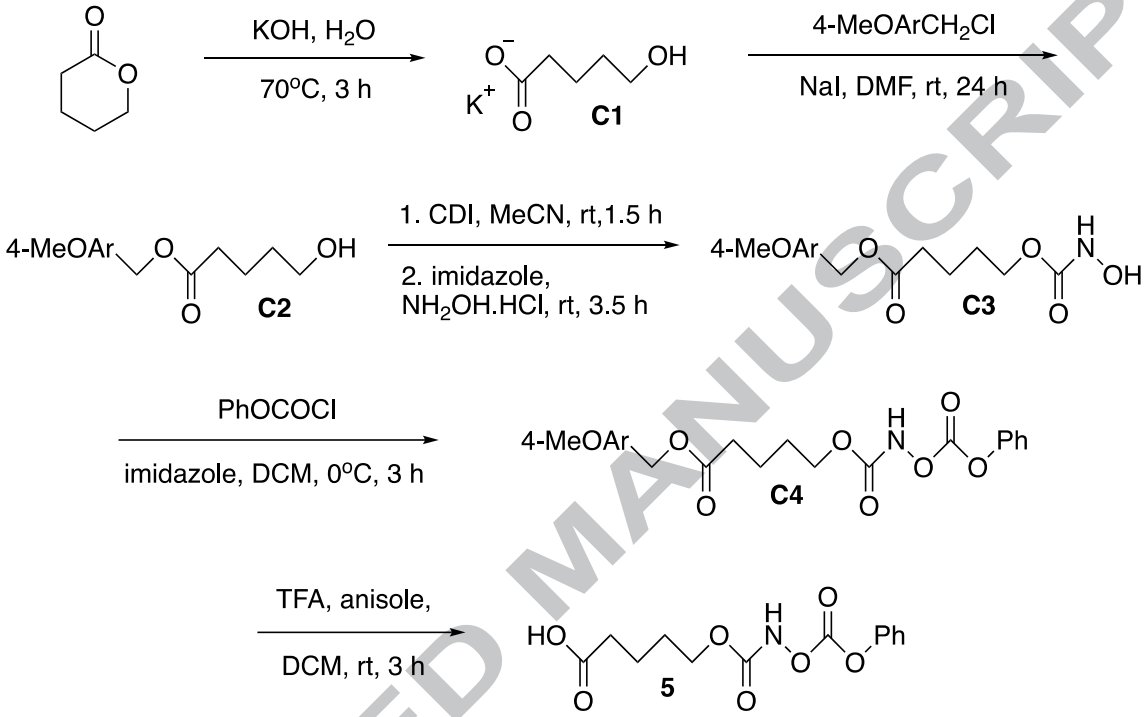
N-(2-phenylethoxycarbonyl) hydroxylamine (**B3)** Compound **B2** (1.00 g, 3.69 mmol) was dissolved in ethanol (20 mL), !0% Pd/C catalyst (0.49 g) was added, and the reaction was hydrogenated at 45 psi for 4 hours. The catalyst was removed by filtration, the filtrate was concentrated using rotary evaporation, and the residue was further dried overnight under an oil pump vacuum. The residue was washed with ethyl acetate and the precipitate was dried under vacuum to obtain **B3** as a colorless solid. Yield 0.50 g, 75%. ^{1}H NMR (D_6-DMSO, 300 MHz): 2.89 (t, 2H, J = 6.6 Hz), 4.16 (t, 2H, J = 7.2 Hz), 7.24 (m, 5H), 8.64 (s, 1H, -NH), 9.62 (s, 1H, -OH).

O-(Phenoxycarbonyl)-N-(2-phenylethoxycarbonyl) hydroxylamine (**4)** Imidazole (0.11 g, 2.2 mmol), dissolved in DCM (3 mL), was added to **B3** (0.40 g, 2.2 mmol) in DCM (7 mL) under a nitrogen atmosphere at 0°C, and the combined solution stirred for 20 minutes. A solution of phenyl chloroformate (0.25 g, 2.2 mmol) in DCM (1.5 mL) was added drop-wise over one minute. The mixture was stirred for a further 45 minutes under nitrogen atmosphere at 0°C and then filtered. Ethyl acetate (10 mL) was added to the filtrate to form a colorless precipitate. The precipitate was removed by filtration and the filtrate was rotary evaporated to dryness and then dried by means of an oil pump for 4 hours. The crude product was purified by recrystallization (98:2 cyclohexane:benzene), yielding the product **4** as a colorless solid. Yield 76 mg, 11%. ^{1}H NMR (D_6-DMSO, 300 MHz): 2.89 (t, 2H, J = 6.7 Hz), 4.31(t, 2H, J = 7.1 Hz), 7.26 (m, 10H),

11.58 (s, 1H). FTIR (cm^{-1}) 1722, 1786. High resolution ES(+)MS m/z = 324.0842, calcd for $C_{16}H_{15}NO_5$ 324.0848.

*Synthesis of **5** (Scheme 6)*

Scheme 6



Potassium 5-hydroxypentanoate (**C1)** δ-Valerolactone (18.6 g, 189 mmol) and potassium hydroxide (10.6 g, 189 mmol) were combined in water (30 mL). This mixture was heated under reflux for three hours. Sufficient water was then to dissolve the salt and the resulting mixture filtered. The resulting filtrate was rotary evaporated to dryness. The resulting salt was suspended in toluene (40 mL) and evaporated to dryness. The crude product was recrystallized (95% ethanol) and dried overnight under an oil pump vacuum to yield **C1**. Yield 27.6 g, 94%. ^{1}H NMR (D_2O, 300 MHz): 1.41 (br, 4H), 2.07 (t, 2H, J = 7.2 Hz), 3.45 (t, 2H, J = 5.7 Hz).

4 Methoxybenzyl 5-hydroxypentanoate (**C2) C1** (18.1 g, 0.116 mol), 4-methoxybenzyl chloride (15.8 g, 0.116 mol), and sodium iodide (333 mg, 2.22 mmol) were dissolved in dimethylformamide (400 mL) and stirred at room temperature for 24 hours. The reaction mixture

was rotary evaporated to dryness and dried further overnight by means of an oil pump. The resulting residue was dissolved in dichloromethane (125 mL) and washed with a saturated solution of sodium bicarbonate and water. The organic layer was separated and dried over magnesium sulfate. The solution was filtered and the filtrate rotary evaporated to obtain **C2** as a yellow oil. Yield 27.6 g, 23%.^{1}H NMR (D_6-DMSO, 300 MHz): 1.41 (m, 2H), 1.56 (m, 2H), 2.31 (t, 2H, J = 4.9 Hz), 3.37 (m, 2H), 3.77 (s, 3H), 4.41 (t, 1H, -OH, J = 3.6 Hz), 5.04 (s, 2H), 7.00 (d, 2H, J = 7.2 Hz), 7.36 (d, 2H, J = 9.1 Hz).

N-[4-(benzyloxycarbonyl)butoxycarbonyl] hydroxylamine (**C3)** To a solution of **C2** (1.00 g, 4.2 mmol) in acetonitrile (20 mL), carbonyl diimidazole (1.05 g, 6.3 mmol) was added and the resulting solution stirred at room temperature for 90 minutes. Imidazole (1.14 g, 16.8 mmol) and hydroxylamine hydrochloride (1.45 g, 21.0 mmol) were then added and the mixture stirred for 4 hours. The imidazole hydrochloride precipitate was removed by filtration and the filtrate partitioned between ethyl acetate and 0.2 M aqueous citric acid. The organic layer was washed with a brine solution, dried over sodium sulfate, and evaporated to dryness to obtain **C3** as a colorless oil. Yield 0.74 g, 60%. ^{1}H NMR (D_6-DMSO, 300 MHz): 1.54 (m, 4H), 2.31 (t, 2H, J = 4.8 Hz), 3.73 (s, 3H), 3.95 (t, 2H, J = 3.6 Hz), 4.38 (q, 2H, J = 5.1 Hz), 4.98 (s, 2H), 6.92 (d, 2H, J = 6.3 Hz), 7.28 (d, 2H, J = 9.0 Hz), 8.62 (s, 1H, -NH), 9.51 (s, 1H, -OH).

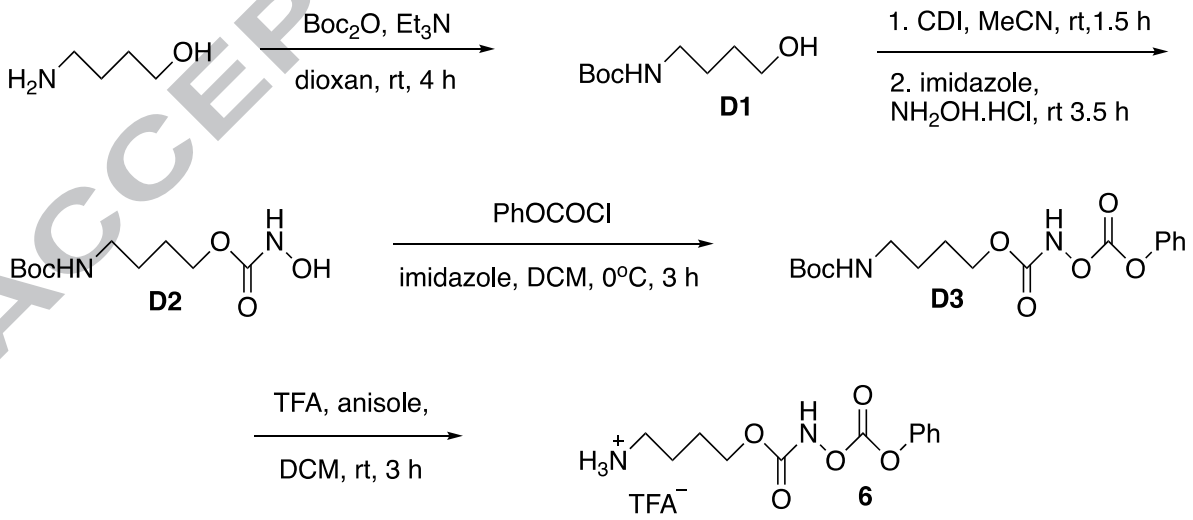
O-Phenoxycarbonyl-N-[(4-benzyloxycarbonyl)butoxycarbonyl] hydroxylamine (**C4)** Imidazole (0.13 g, 2.50 mmol) was added to **C3** (0.74 g, 2.5 mmol) in DCM (10 mL) under a nitrogen atmosphere at 0°C and the mixture stirred for 20 minutes. A solution of phenyl chloroformate (0.31 g, 2.5 mmol) in DCM (1.5 mL) was then added drop-wise over one minute and the ensuing mixture was stirred for 45 minutes under the nitrogen atmosphere at 0°C. The resulting mixture was filtered. Ethyl acetate (10 mL) was added to the filtrate which led to the formation of a

colorless precipitate. The precipitate was removed by filtration and the filtrate was rotary evaporated to dryness and further dried overnight by means of an oil pump. This procedure yielded the crude product as a yellow oil that was purified by flash chromatography on silica gel (90:10 hexane:ethyl acetate). The required product **C4** was thus obtained as a colorless oil (57.6 mg, 59% yield). ^{1}H NMR (D_6-DMSO, 300 MHz): 1.58 (m, 4H), 2.34 (t, 2H, J = 6.7 Hz), 3.74 (s, 3H), 4.11 (t, 2H, J = 5.3 Hz), 4.99 (s, 2H), 7.35 (m, 9H), 11.54 (s, 1H).

O-Phenoxycarbonyl-N-(4-carboxybutyloxycarbonyl) hydroxylamine (**5**) TFA (1 mL) was added drop-wise with stirring under a nitrogen atmosphere at room temperature to a solution of **C4** (100 mg) in DCM (1 mL). The subsequent reaction was monitored by TLC until it reached completion. Excess solvent was removed via rotary evaporation, yielding the required product **5** as a colorless solid (72 mg, 100%). ^{1}H NMR (D_6-DMSO, 300 MHz): 1.57 (m, 4H), 2.23 (t, 2H, J = 6.9 Hz), 4.12 (t, 2H, J = 5.3 Hz), 7.35 (m, 5H), 11.55 (s, 1H). FTIR (cm^{-1}) 1757, 1794. High resolution ES(+)MS 320.0739, calcd for $C_{13}H_{15}NO_7$ 320.0746.

*Synthesis of **6** (Scheme 7)*

Scheme 7



4- t-Butoxycarbonylaminobutanol (**D1**) To a pre-cooled solution of 4-aminobutanol (5.00 g, 56.1 mmol) and triethylamine (8.50 g, 84 mmol) in 66% aqueous dioxane (225 mL), a solution of di-t-butyl dicarbonate (14.0 mL, 61.6 mmol) in dioxane (75 mL) was added and the mixture was stirred at room temperature for 4 hours. The solvent was removed by rotary evaporation and the resulting residue was partitioned between ethyl acetate and 10% aqueous citric acid. The product was extracted from the aqueous layer with ethyl acetate three times and the combined organic layer was washed with a 5% sodium bicarbonate solution and dried over magnesium sulfate. The crude product was purified by fractional distillation from a Kugelrohr apparatus at 130 ºC and 0.1 torr, yielding **D1** as a yellow oil (8.20 g, 77% yield). ^{1}H NMR (D_6-DMSO, 300 MHz): 1.35 (s, 9H), 2.87(q, 4H), 3.35 (q, 4H), 4.34 (t, 1H, -OH, J = 5.3 Hz), 6.75 (t, 1H, J = 3.4 Hz).

N-[(4-t-butoxycarbonylamino)butoxycarbonyl] hydroxylamine (**D2)** To **D1** (1.00 g, 5.3 mmol) in acetonitrile (20 mL), carbonyl diimidazole (1.28 g, 7.95 mmol) was added and the mixture stirred at room temperature for 90 minutes. Imidazole (1.44 g, 21.2 mmol) and hydroxylamine hydrochloride (1.82 g, 26.5 mmol) were then added and the resulting mixture stirred for 4 hours. The imidazole hydrochloride precipitate was removed by filtration and the filtrate was partitioned between ethyl acetate and 0.2 M citric acid. The organic layer was washed with a brine solution, dried over sodium sulfate, and evaporated to dryness to obtain **D2** as a colorless oil. Yield 1.10 g, 83%. ^{1}H NMR (D_6-DMSO, 300 MHz): 1.35 (s, 9H), 1.37 (m, 2H) 1.46 (q, 2H, J = 8.1 Hz), 2.90 (q, 2H, J = 5.9 Hz), 3.96 (t, 2H, J = 2.9 Hz), 6.76 (t, 1H, -NH), 8.62 (s, 1H, -NH), 9.48 (s, 1H, -OH).

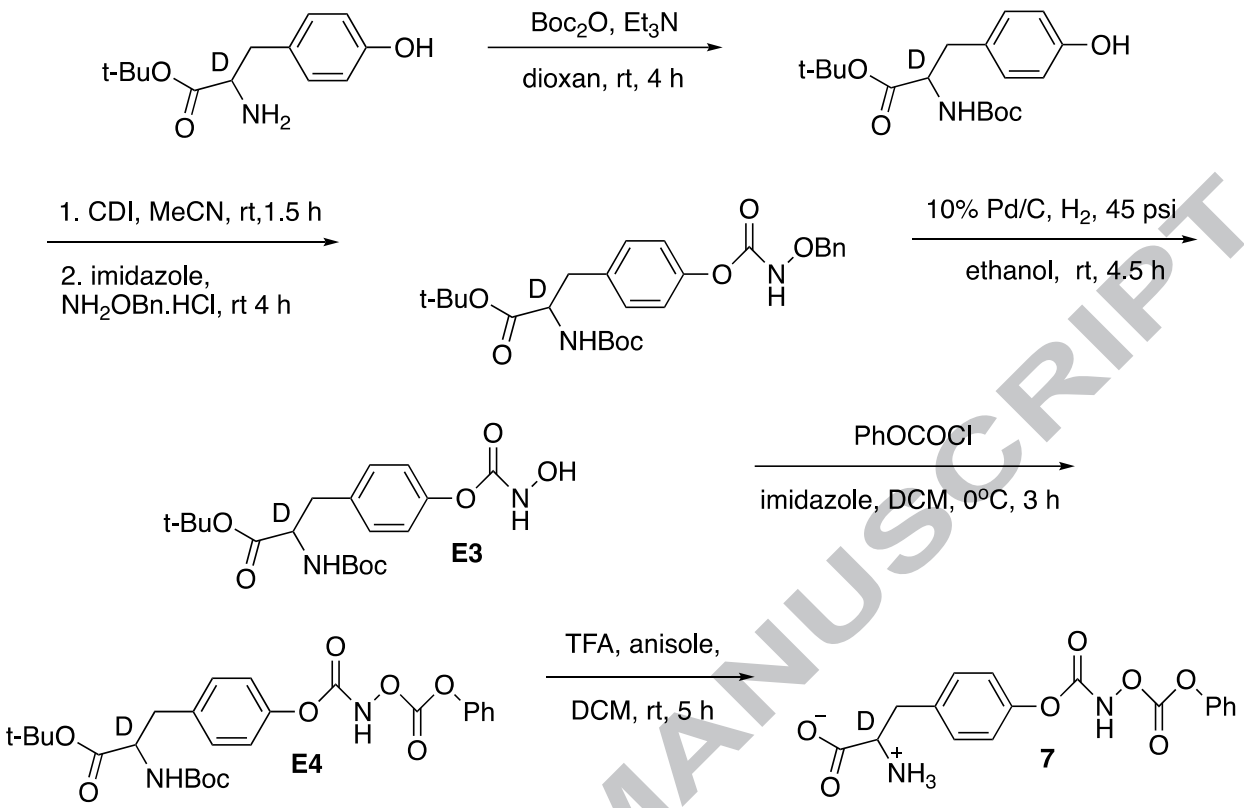
O-Phenoxycarbonyl-N-[4-(t-butoxycarbonyl)butoxycarbonyl] hydroxylamine (**D3)** Imidazole (0.299 g, 4.4 mmol) was added to **D2** (1.10 g, 4.4 mmol) in dichloromethane (10 mL) under a nitrogen atmosphere at 0°C. The mixture was stirred for 20 minutes. To the reaction mixture, a

solution of phenyl chloroformate (0.50 g, 4.4 mmol) in dichloromethane (2 mL) was added drop-wise over one minute. The mixture was stirred for 2 hours under a nitrogen atmosphere at 0°C. The mixture was filtered. To the filtrate, ethyl acetate (10 mL) was added, giving rise to a colorless precipitate. The precipitate was removed by filtration and the filtrate evaporated to dryness. The residue was further dried overnight by means of an oil pump, yielding the crude product as a colorless solid (1.34 g). The crude product was purified by flash chromatography on silica gel (80:20 hexane:ethyl acetate), yielding **D3** as a yellow oil. Yield 427 mg, 25% yield. ^{1}H NMR (D_6-DMSO, 300 MHz): 1.35 (s, 9H), 1.40 (m, 4H), 1.58 (q, 2H, J = 5.4 Hz), 2.91 (q, 2H, J = 5.8 Hz), 4.15 (t, 2H, J = 5.3 Hz), 6.85 (t, 1H, -NH), 7.26 (d, 2H), 7.32 (t, 1H), 7.47 (t, 2H), 11.53 (s, 1H, -NH). FTIR (cm^{-1}) 1757, 1805.

O-Phenoxycarbonyl-N-(4-aminobutoxycarbonyl) hydroxylamine.TFA (**6**) TFA (1 mL) was added drop-wise with stirring under nitrogen at room temperature to a solution of **D3** (100 mg) in dichloromethane (1 mL). The reaction was monitored by TLC to completion over 1 hour. Solvent and excess TFA were removed by rotary evaporation. Further drying under an oil pump vacuum yielded the trifluoroacetate salt of the required product **6** as a yellow oil. Yield 67 mg, 100%. ^{1}H NMR (D_6-DMSO, 300 MHz): 1.64 (m, 4H), 2.49 (q, 2H, J = 1.5 Hz), 4.18 (t, 2H, J = 6.3 Hz),7.26 (d, 2H, J = 6.2 Hz), 7.32 (t, 1H, J = 8.1 Hz), 7.47 (t, 2H, J = 7.8 Hz), 7.66 (br s, 3H, $NH3^{+}$), 11.58 (s, 1H, -NH). FTIR (cm^{-1}) 1744, 1800. High resolution ES(+)MS m/z = 269.1136, calcd for $C_{12}H_{17}NO_5$ 269.1137.

*Synthesis of **7** (Scheme 8)*

Scheme 8



O-(N-Hydroxyaminocarbonyl)-D-tyrosine (**E3**) was prepared as shown in Scheme 7 from N-t-butoxycarbonyl-D-tyrosine t-butyl ester using the same methodology as in the syntheses described above.

O-[(N-Phenoxycarbonyloxy)aminocarbonyl]-N-t-butoxycarbonyl-D-tyrosine t-butyl ester (**E4**) The hydroxamic acid **E3** (100 mg, 0.252 mmol) and imidazole (17.2 mg, 252 mmol) were dissolved in DCM (1.4 mL) and the solution stirred to 0 ^{o}C in an ice bath. Phenyl chloroformate (39.6 mg, 0.252 mmol) dissolved in DCM (0.6 mL) was then added by syringe and the mixture stirred at 0 ^{o}C for 3h. The mixture was filtered and the filtrate rotary evaporated to dryness. The residue was stirred with ethyl acetate (4 mL) at room temperature. The precipitate was removed by filtration and the filtrate evaporated to dryness. Purification of the product was achieved by preparative thin layer chromatography on silica gel (30% ethyl acetate/hexane). Yield 34 mg, 44%. ^{1}H NMR (D_6-DMSO, 300 MHz): 2.96 (m, 2H), 4.00 (m, 1H), 7.08 (d, J = 7.7 Hz, 2H),

7.19 (d, J = 8.7 Hz, NH), 7.25 (d, J = 8.7 Hz, 2H), 7.26 (d, J = 8.7 Hz, 2H), 7.34 (t, J = 7.6 Hz, 1H), 7.48 (t, J = 7.7 Hz, 2H), 12.3 (br s, ONH). FTIR (cm^{-1}) 1700, 1720, 1775, 1805.

O-[(N-Phenoxycarbonyloxy)aminocarbonyl]-D-tyrosine (**7**) The N-protected material **E4** (140 mg) was dissolved in DCM (1 mL) at room temperature. Redistilled anisole (59 μL, 2 eq) was added with stirring, followed by TFA (1.0 mL). This mixture was stirred at room temperature for 5 h, after which benzene (2 mL) was added and the solvents and TFA were removed by rotary evaporation. An additional benzene addition (1 mL) was followed by evaporation. The residue was stirred with diethyl ether (4 mL) and the solid residue isolated by decantation. This procedure was repeated twice more and the residue, an off-white powder, was dried under an oil pump vacuum. Yield 38 mg, 42%. ^{1}H NMR (D_6-DMSO, 5% CF_3CO_2D): 3.09 (m, 2H), 4.12 (m, 1H), 7.12 (d, J = 8.7 Hz, 2H), 7.24 (d, J = 7.3 Hz, 2H), 7.28 (d, J = 8.5 Hz, 2H), 7.30 (t, J = 8.5 Hz, 1H), 7.44 (t, J = 7.5 Hz, 2H). FTIR (cm^{-1}) 1740, 1805. High resolution ES(+)MS m/z = 361.1031, calcd for $C_{17}H_{17}NO_7$ 361.1036.

4.3 Analytical and Kinetics Methods

The concentration of the P99 β-lactamase in stock solutions was measured spectrophotometrically, assuming the extinction coefficient of the enzyme to be 7.1 x 10^4 $M^{-1}cm^{-1}$ at 280 nm.[19] All kinetics experiments were performed at 25 °C in 20 mM MOPS buffer, pH = 7.5. Stock solutions of **3** - **7** (10 mM) were prepared in dry DMF. Spontaneous hydrolyses of **3**, **5**, and **6** (each 5.0 μM) were monitored spectrophotometrically at 270 nm. The full progress curves were then fitted to a first order rate equation and the rate constants k_0 thus obtained. Rate constants for hydrolysis of **4** and **7** could not be obtained in this way since no spectral change was observed over the relevant time period. The rate constants for **4** and **7** were assumed to be the same as that for **1** in fitting of the inactivation kinetics for these compounds.

Inactivation kinetics The P99 β-lactamase (150 nM) was incubated with inhibitor (2.0 μM and, separately, 5.0 μM **3**; 5.0 μM and, separately, 10.0 μM **4**; 0.5 μM and, separately, 2.0 μM **5**; 20.0 μM **6**; and 2.5 μM and, separately, 5.0 μM and 10.0 μM **7**) in MOPS buffer containing 0.1% bovine serum albumin. At suitable times, aliquots of the reaction mixture (20 μL) were then added to cuvettes containing 980 μL of MOPS buffer containing cephalothin substrate (300 μM, K_m = 15.4 μM). Initial rates of hydrolysis of the substrate were monitored at 270 nm. The initial rates, proportional to active enzyme concentrations, were plotted against time of reaction. Titration experiments were also performed. Here, enzyme (150 nM) was incubated with various concentrations of inhibitor (0 - 0.50 μM **3**, 0 - 2.0 μM **4**, 0 - 0.40 μM **5**, 0 - 2.0 μM **6**, and 0 - 2.0 μM **7**) and the inactivation reaction allowed to proceed until no further loss of enzyme activity occurred. Initial rates of hydrolysis of cephalothin were then determined as described above and the initial rates plotted as a function of initial inhibitor concentration. These data, from both types of experiment, taken together, were fitted to Scheme 2 by means of the Dynafit program.[20] To do this, since $(k_2 + k_3) >> k_1[I]$, k_2 was fixed at 100 s^{-1} and the values of k_1 and k_3 allowed to vary. The fit, therefore, allowed determination of k_1 and k_2/k_3 values.

Mass spectra of inactivated enzymes Electrospray mass spectra of the P99 β-lactamase before and after inactivation by **3** – **7** were obtained exactly as previously described.[13,18] This involved acid precipitation of the enzyme by trichloroacetic acid followed by the washing and drying of the precipitate formed.

Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors do thank Wesleyan University, however, for institutional support.

References

1. Frère J-M, Sauvage E, Kerff, F. From ”an enzyme able to destroy penicillin” to carbapenemases: 70 years of β-lactamase misbehavior. *Current Drug Targets* 2016; 17: 974-982.
2. Garber K. A β-lactamase inhibitor revival provides new hope for old antibiotics. *Nature Rev Drug Discov.* 2015; 14:445-447.
3. Bush K. A resurgence of β-lactamase inhibitor combinations effective against multidrug-resistant Gram-negative pathogens. *Int J Antimicrob Agents*. 2015; 46: 483-493.
4. Wright GD. Empowering older antibiotics. *Cell*. 2016; 167: 301.
5. Fink AL, Page, MI. The mechanisms of catalysis by β-lactamases. In: Frère J-M, ed. *Beta-Lactamases*. New York, NY: Nova Science; 2012: 161-198.
6. Lahiri SD, Mangani S, Durand-Reville T, Benvenuti, M, De Luca F, Sanyal G, Docquier J-D. Structural insight into potent broad-spectrum inhibition with reversible recyclization mechanism: avibactam in complex with CTX-M-15 and *Pseudomonas aeruginosa* AmpC β-lactamases. *Antimicrob. Agents Chemother.* 2013; 57: 2496-2505.
7. Hecker SJ, Reddy KR, Totrov M, Hirst, GC, Lomovskaya O, Griffith DC, King P, Tsivkovski R, Sun D, Sabet M, Tarazi Z, Clifton MC, Atkins K, Raymond A, Potts KT, Abendroth J, Boyer SH, Loutit JS, Morgan EE, Durso S, Dudley MN. Discovery of a cyclic boronic acid β-lactamase inhibitor (RPX7009) with utility vs class A serine carbapenemases. *J Med Chem.* 2015; 58: 3682-3692.
8. Powers RA, Morandi F, Shoichet BK. Structure-based discovery of a novel, noncovalent inhibitor of AmpC β-lactamase. *Structure*. 2002; 10: 1013-1023.

9. Babaoglu K, Simeonov A, Irwin JJ, Nelson ME, Feng B, Thomas CJ, Cancian L, Costi MP, Maltby DA, Jahav A, Inglese J, Austin CP, Shoichet BK. *J Med Chem*. 2008; 51: 2502-2511.
10. London N, Miller RM, Krishnan S, Uchida K, Irwin JJ, Eidam O, Gibold L, Cimermancic P, Bonnet R, Shoichet BK, Taunton J. Covalent docking of large libraries for the discovery of chemical probes. *Nature Chem Biol.* 2014; 10: 1066-1072.
11. Pemberton OA, Zhang X, Nichols DA, DeFrees K, Jaishankar P, Bonnet R, Adams J, Shaw LN, Renslo AR, Chen Y. Antibacterial spectrum of a tetrazole-based reversible inhibitor of serine β-lactamases. *Antimicrob Agents Chemother*. 2018; 62: e02563-17.
12. Wyrembak PM, Babaoglu K, Pelto RB, Shoichet BK, Pratt RF. O-Aryloxycarbonyl hydroxamates: New *ß*-lactamase inhibitors that cross-link the active site. *J. Am. Chem. Soc.* 2007; 129: 9548-9549.
13. Pelto RB, Pratt RF. Kinetics and mechanism of inhibition of a serine *ß*-lactamase by O-aryloxycarbonyl hydroxamates. *Biochemistry.* 2008; 47: 12037-12046.
14. Tilvawala R, Pratt RF. Covalent inhibition of serine ß-lactamases by novel hydroxamic and acid derivatives. *Biochemistry.* 2013; 52: 3712-3720.
15. Tilvawala R, Pratt RF. Kinetics of a two-stage pro-inhibitor of serine β-lactamases. *Biochemistry*. 2013; 52: 7060-7070.
16. Adediran SA, Lin G, Pelto RB, Pratt RF. Crossover inhibition as an indicator of convergent evolution of enzyme mechanisms: a β-lactamase and a N-terminal nucleophile hydrolase. *FEBS Lett.* 2012; 586: 4186-4189.

17. Adediran SA, Pratt RF. Penicillin acylase and O-aryloxycarbonyl hydroxamates: Two acyl-enzymes, one leading to hydrolysis, the other to inactivation. *Arch Biochem Biophys*. 2017; 614: 65-71.

18. Tilvawala R, Cammara M, Adediran SA, Brodbelt JS, Pratt RF. A new covalent inhibitor of class C β-lactamases reveals extended active site specificity. *Biochemistry*. 2015; 54: 7375-7384.

19. Joris, B., DeMeester, F., Galleni, M. Reckinger, G., Coyette, J. Frere, J.-M., Van Beeumen, J. The β-lactamase of *Enterobacter cloacae* P99. Chemical properties, N-terminal sequence and interaction with 6-β-halogenopenicillinates. *Biochem. J.* 1985; 228: 241-248.

20. Kuzmic. P. Program DYNAFIT for the analysis of enzyme kinetic data: Application to HIV proteinase. *Anal. Biochem.* 1996; 237: 260-272.

Table of Contents Graphic

Specificity of Extended O-Aryloxycarbonyl Hydroxamates as Inhibitors of a Class C β-Lactamase

Alexandra A. Malico, K. Dave, S.A. Adediran and R.F. Pratt*