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β-Lactamase Inhibitors Derived from *N*-Tosyloxy-β-Lactams

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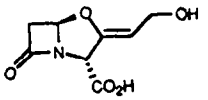
Abstract–Electrophilic *N*-tosyloxy-β-lactams, *N*-tosyloxy-4-phenyl-2-azetidinone (**2b**) and *N*-tosyloxy-3-(*S*)-phthalimido-4-(*S*)-2-azetidinone (**2c**), are described. These agents are novel potent β-lactamase inhibitors.

The development of resistance to β-lactam antibiotics raises concerns about the future ability of this most valuable class of antibiotics to combat infection. The various mechanisms of resistance development include decreased permeability of bacterial outer membranes, bacterial modification of target enzymes and interception and inactivation of the antibiotic by hydrolytic enzymes.[1] The last of these, β-lactamase-induced hydrolysis, is primarily responsible for the development of resistance by many clinically important bacteria.[2] Thus, the continued search for new effective antibacterial agents based on the β-lactam framework is often coupled to a search for effective β-lactamase inhibitors. In fact, coadministration of β-lactam antibiotics with β-lactamase inhibitors has become an effective treatment of bacterial infection caused by resistant strains. One of the most widely used antibacterial prescriptions is Augmentin®, a mixture of amoxicillin and clavulanic acid (**1**), a potent natural β-lactamase inhibitor.[3,4]



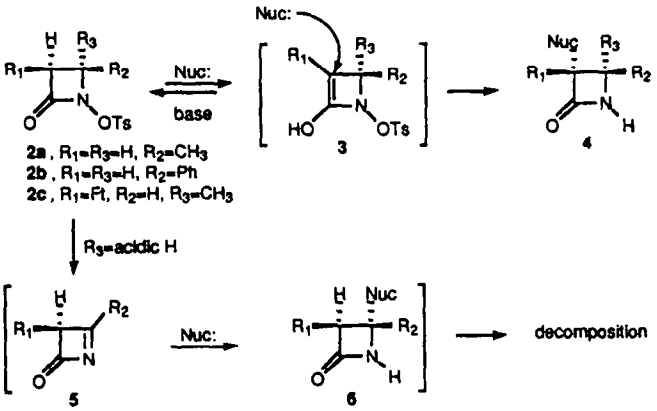
1, clavulanic acid

The most common and effective β-lactamase inhibitors are structurally similar to β-lactam antibiotics in that they retain the β-lactam ring and its reactive carbonyl group for enzymatic recognition and initiation of enzymatic reaction. Effective inhibitors also often contain or generate an additional electrophilic site for simultaneous or subsequent attack by pendant enzymatic nucleophiles. The consequential chemistry and/or blockage of the active site of the β-lactamase minimizes or inhibits substrate turnover and allows the coadministered antibiotic to inactivate its target. Herein we describe a new class of β-lactamase inhibitors based on electrophilic agents derived from *N*-tosyloxy-β-lactams.

Results and Discussion

Chemistry

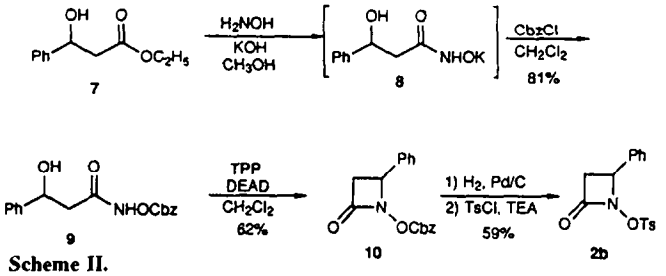
During studies leading to new methods for the peripheral functionalization of β-lactams, we found that activated *N*-hydroxy-β-lactams such as *N*-tosyloxy-β-lactams are susceptible to *nucleophilic* attack at C_3 with concomitant cleavage of the N–O bond (Scheme I).[5] Most interestingly, while the C_3 position of 2-azetidinones (β-lactams) is often used as a nucleophilic carbon in β-lactam chemistry, it serves as an *electrophilic* site in this novel reaction. Further studies revealed that increasing the C_3–H acidity by variation of R_1 from H to Ft (phthalimido) accelerated the reaction, effectively enhancing the electrophilicity at C_3. Apparently, electron withdrawing substituents at C_3 facilitate rate determining enolization (to **3**) and a subsequent S_N2' reaction to give the observed product. Addition of acidifying groups (R_2) at C_4 apparently altered the reaction path to generate imine **5** which was susceptible to nucleophilic attack at C_4 and eventually led to decomposition. While this chemistry has tremendous synthetic potential, the fact that the reactions clearly allowed selective generation of new electrophilic sites on the β-lactam, with the potential of inducing additional enzymatic reactions, encouraged us to study the activity of a variety of *N*-tosyloxy-β-lactams as β-lactamase inhibitors.



Scheme I.

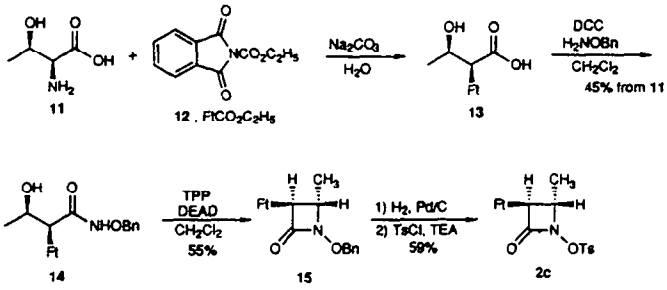
Details of the synthesis and characterization of substrates **2a** were described earlier.[5] The syntheses of the C_4- and C_3-activated derivatives, **2b** and **2c**, respectively, are

summarized in Schemes II and III. The synthesis of **2b** began by hydroxaminolysis[5] of ethyl 3-hydroxy-3-phenyl propionate (**7**) to produce hydroxamate salt **8** which was directly protected with CbzCl to give **9**. Cyclization of **9** under Mitsunobu conditions[6] produced β-lactam **10**. Careful hydrogenolysis with Pd/C gave the corresponding *N*-hydroxy-β-lactam. Since free *N*-hydroxy-β-lactams are very prone to rearrangement,[5,6b] the hydrogenolysis catalyst was removed by filtration and the reaction mixture was treated immediately with *p*-toluenesulfonyl chloride (TsCl) and triethylamine (TEA) to produce the desired 4-phenyl-*N*-tosyloxy-2-azetidinone (**2b**).



Scheme II.

C_3-Activated β-lactam **2c** was derived from L-threonine (**11**). Thus, reaction of **11** with ethoxycarbonyl-phthalimide (**12**) gave *N*-protected-L-threonine **13** cleanly.[7] DCC-mediated coupling of **13** with *O*-benzyl-hydroxylamine gave hydroxamate **14**. Cyclization with the usual Mitsunobu conditions produced β-lactam **15**. As before, hydrogenolysis followed by tosylation gave desired substrate **2c**.



Scheme III.

As previously described,[5] β-lactam **2b** reacts in the presence of base apparently to produce unstable imine **5** which is susceptible to nucleophilic attack at C_4. On the other hand, β-lactam **2c** is prone to nucleophilic attack at C_3. While initiation of similar chemistry in the active site of a β-lactamase enzyme, before, after or instead of protease-like reaction at the β-lactam carbonyl group, was anticipated to deactivate the enzyme, the first concern was that neither **2b** nor **2c** would be recognized by a β-lactamase because the molecules are not very soluble in water and they lack the usual pendant carboxylate with its net negative charge. Still, studies of the β-lactamase inhibitory activity of **2b** and **2c** yielded fascinating results.

Biological studies: β-lactamase inhibition and antimicrobial activity

Compounds **2b** and **2c** were as potent as clavulanate as inhibitors of purified TEM β-lactamase from *Escherichia coli* (Table 1).

Table 1. Inhibition of TEM β-lactamase

Compound	IC_{50}, μM[a]	K_i, μM[b]
1, clavulanic Acid	7	4.7
2b	5	5.9
2c	5	3.4

[a] IC_{50}, Inhibitory concentration of compound required to inhibit enzyme activity by 50%.
[b] K_i, Inhibition constant determined by Lineweaver–Burke plot.

Lineweaver–Burke analysis of **2b** and **2c** (Figure 1) revealed inhibition constants (K_i) comparable to that of clavulanic acid, and kinetics of inhibition compatible with the interpretation of competitive inhibition. Enzyme activity could be partially recovered after prolonged incubation (>6 h), suggesting that inactivation may be reversible.

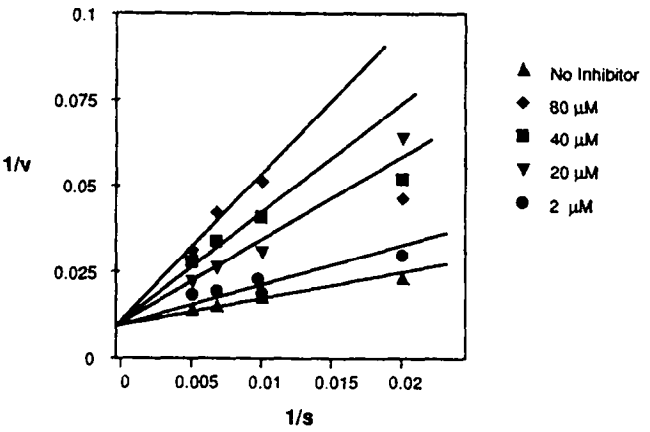


Figure 1. Kinetics of inhibition of TEM β-lactamase by compound **2c**

Like clavulanic acid, compounds **2b** and **2c** had only weak inhibitory activity against a type I enzyme from *Enterobacter cloacae*, with IC_{50}'s for all three compounds >500 μM. Compounds **2b** and **2c** at concentrations of ≤100 μg/mL were unable to reduce the minimal inhibitory concentration of ampicillin against β-lactamase-producing strains of *Escherichia coli*, suggesting that while the compounds may exhibit inhibition comparable to that of clavulanic acid on purified enzyme, they are not able to penetrate the outer membrane efficiently enough to inhibit the enzyme in intact bacterial cells. No antimicrobial activity (minimal inhibitory concentrations >100 μg/mL) against *Escherichia coli* or *Staphylococcus aureus* was detected for **2b** or **2c**.

Conclusion

N-Tosyloxy-β-lactams may act as potent competitive inhibitors of β-lactamase despite their relatively poor solubility and the absence of the negatively charged carboxylate. The utility of these agents is however limited by apparent lack of penetration of the bacterial outer membrane, as would be anticipated by their low

hydrophilicity relative to clavulanic acid and β-lactam antibiotics.

Experimental Section

General methods

Melting points were taken on a Thomas-Hoover Capillary Melting Point Apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were obtained on a General Electric GN-300 spectrometer and were performed in chloroform-*d*. ^{1}H NMR chemical shifts are reported in ppm relative to tetramethylsilane. For ^{13}C NMR, reference was the center peak of chloroform-*d* (77.0 ppm). Infrared spectra were recorded on a Perkin-Elmer 1420 spectrophotometer. TF refers to thin-film and KBr refers to potassium bromide disk. Electron impact mass spectra, chemical ionization mass spectra and fast atom bombardment were recorded on an AEI Scientific Apparatus MS 902 and Finnigan MAT model 8430 spectrometers. Analytical TLC was carried out using commercially available aluminum-backed 0.2 mm silica gel 60 F-254 plates. Flash silica gel column chromatography was conducted using Merck silica gel 60 (230–400 mesh).

All reactions were periodically monitored by TLC and worked up after the complete consumption of starting materials unless specified otherwise. Solvents for flash column chromatography were distilled. Anhydrous methylene chloride, acetonitrile and triethylamine were freshly distilled from CaH_2 and stored under nitrogen. All purchased reagents were of reagent grade quality and were used without further purification.

4-Phenyl O-*Cbz-2-azetidinone (**10**)*

The preparation of this compound started with ethyl 3-hydroxy-3-phenyl propionate (**7**). A solution of $H_2NOH{\cdot}HCl$ (576.4 mg, 8.29 mmol) and KOH (466.9 mg, 8.3 mmol) in methanol (10 mL) was stirred at 0 °C for 30 min. The precipitated KCl was removed by filtration through a plug of celite and the clear solution was transferred to a solution of ethyl 3-hydroxy-3-phenyl propionate (1.472 g, 7.6 mmol) in methanol (20 mL). To this solution was added KOH (466.9 mg, 8.3 mmol) and the reaction mixture was stirred at room temperature overnight. The solvent was evaporated and the residue was dissolved in CH_3CN. To this solution was added CbzCl (0.94 mL) and the reaction mixture was stirred vigorously for 2 h. The precipitate was removed by filtration and the resulting clear solution was concentrated to yield *O*-Cbz 3-hydroxy-3-phenyl propiohydroxamate (**9**) as a white solid (1.94 g, 81%) which was used directly in the next reaction. ^{1}H NMR δ 2.58–2.64 (dd, J_1 = 2.68, J_2 = 15.19, 1H), 2.67–2.75 (dd, J_1 = 9.67, J_2 = 14.87, 1H), 3.31 (b, 1H), 5.13–5.17 (dd, J_1 = 2.89, J_2 = 10.08, 1H), 5.27 (s, 2H), 7.36–7.39 (m, 5H), 9.46 (b, 1H); IR (KBr) 3260, 3140, 2960, 1790, 1675, 1235.

To a solution of **9** (1.9 g, 6.16 mmol) and triphenylphosphine (TPP, 2.37 g, 9.0 mmol) in CH_2Cl_2 (20 mL) was added diethyl azodicarboxylate (DEAD, 1.43 mL, 9.0 mmol). The reaction mixture was stirred overnight and then concentrated. The residue was purified by column chromatography eluting with ethyl acetate and hexanes (1:3) to provide **10** as a colorless oil (1.12 g, 62%). ^{1}H NMR δ 2.75–2.80 (dd, J_1 = 2.90, J_2 = 13.97, 1H), 3.32–3.39 (dd, J_1 = 6.0, J_2 = 13.94, 1H), 5.12–5.14 (dd, J_1 = 2.87, J_2 = 5.98, 1H), 5.22 (s, 2H), 7.35–7.39 (m, 5H); ^{13}C NMR δ 42.5, 60.8, 71.8, 126.4, 128.6, 128.7, 128.9, 129.1, 133.6, 136.1, 153.4, 164.2; IR (TF) 3040, 1805, 1780, 1450, 1375, 1225, 1040, 965 cm^{-1}.

4-Phenyl N-*tosyloxy-2-azetidinone (**2b**)*

Compound **10** (341 mg, 1.15 mmol) in methanol (10 mL) was subjected to hydrogenolysis for 30 min. The catalyst was removed by filtration through a layer of celite and the clear filtrate was concentrated to yield 4-phenyl *N*-hydroxy-2-azetidinone as an oil (174 mg, 92.8%) which was carried onto the next step without further purification.

To a solution of 4-phenyl *N*-hydroxy-2-azetidinone (174 mg, 1.07 mmol) and TsCl (170.28 mg, 0.89 mmol) in CH_3CN was added Et_3N (0.08 mL) at 0 °C. The reaction mixture was stirred for 40 min. After concentration, the residue was purified by column chromatography with ethyl acetate and hexanes (1:3) to provide the desired product (**2b**) as a white solid (203 mg, 59%). m.p. 103–107 °C; ^{1}H NMR δ 2.44 (s, 3H), 2.73–2.79 (dd, J_1 = 3.14, J_2 = 14.4 1H), 3.21–3.28 (dd, J_1 = 6.11, J_2 = 14.43, 1H), 4.93–4.96 (dd, J_1 = 3.14, J_2 = 6.07, 1H), 7.26–7.36 (m, 7H), 7.78–7.81 (d, J = 8.36, 2H); IR (KBr) 3040, 1810, 1595, 1375, 1190, 1175, 1025; MS 317 (M^+), 155, 139, 104, 91; HRMS calcd for $C_{16}H_{15}NO_4S$ 317.07218, found 317.0729.

trans-N-*Tosyloxy-3-phthalimido-4-methyl-2-azetidinone (**2c**)*

L-Threonine (**11**, 2.0 g, 16.8 mmol) together with Na_2CO_3 (1.78 g, 16.8 mmol) was dissolved in H_2O (15 mL). Ethoxycarbonylphthalimide (**12**, 3.708 g, 16.9 mmol) was added and the turbid solution was stirred at room temperature until it became clear (about 30 min). The solution was acidified from pH = 8 to pH = 3 and extracted with CH_2Cl_2 (five 10 mL portions). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to yield protected threonine **13** as a glassy thick oil (3.785 g, 90.5%, crude).

To a solution of **13** (3.785 g, 15.2 mmol) and *O*-benzyl hydroxylamine (2.06 g, 16.7 mmol) in CH_2Cl_2 (20 mL) was added a solution of DCC (3.45 g, 16.7 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred for 2 h and then filtered through celite. The clear solution was poured into water and acidified to pH = 4.5 (from pH = 6). The aqueous layer was extracted with CH_2Cl_2 three times. The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography eluting with ethyl

acetate/hexanes (1:1) to afford hydroxamate **14** as a thick oil (2.65 g, 45% overall from L-threonine).

To a solution of **14** (2.65 g, 7.5 mmol) and TPP (2.556 g, 9.75 mmol) in CH_3CN (5.0 mL) was added DEAD (1.55 mL, 9.75 mmol). The reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography with ethyl acetate/hexanes (1:1) to afford β-lactam **15** (1.39 g, 55%). ^1H NMR δ 1.26–1.28 (d, J = 6.5, 3H), 4.00–4.07 (dq, J_1 = 2.21, J_2 = 6.10, 1H), 4.727–4.734 (d, J = 2.22, 1H), 5.09–5.13 (d, J = 11.19, 1H), 5.14-5.18 (d, J = 11.15, 1H), 7.38–7.54 (m, 5H), 7.74–7.77 (dd, J_1 = 3.05, J_2 = 5.53, 2H), 7.86–7.88 (dd, J_1 = 3.09, J_2 = 5.38, 2H); ^{13}C NMR δ 15.9, 56.5, 59.3, 78.3, 123.4, 128.3, 128.8, 129.2, 131.3, 134.3, 134.9, 159.7, 166.6; IR (KBr) 2980, 1795, 1775, 1720, 1395 cm^{-1}; MS (CI with isobutane) 337 (MH^+), 231, 188, 107.

Compound **15** (317 mg, 0.94 mmol) in methanol (10 mL) was subjected to hydrogenolysis for 3 h. The catalyst was removed by filtration through a layer of celite and the filtrate was concentrated to yield the corresponding *N*-hydroxy-β-lactam as an oil which was carried onto the next step without further purification.

To a solution of the *N*-hydroxy-β-lactam in CH_2Cl_2 (5.0 mL) was added TsCl (179.8 mg, 0.94 mmol) and TEA (0.13 mL, 0.94 mmol). The reaction mixture was stirred at room temperature for 2 h. After concentration under reduced pressure, the residue was purified by column chromatography eluting with ethyl acetate/hexanes (1:2) to afford compound **2c** (332 mg, 88%) as a white solid. ^1H NMR δ 1.53–1.56 (d, J = 6.25, 3H), 2.47 (s, 3H), 4.47–4.55 (dq, J_1 = 2.91, J_2 = 6.24, 1H), 4.83–4.84 (d, J = 2.89, 1H), 7.41–7.44 (d, J = 8.24, 2H), 7.76–7.79 (dd, J_1 = 3.15, J_2 = 5.59, 2H), 7.87–7.90 (dd, J_1 = 3.13, J_2 = 5.38, 2H), 8.05–8.08 (d, J = 8.26, 2H); ^{13}C NMR δ 16.0, 21.6, 56.6, 60.9, 123.6, 129.4, 129.8, 130.0, 131.2, 134.5, 146.5, 161.0, 166.2; IR (KBr) 2980, 1810, 1720, 1400, 1195, 1180, 940 cm^{-1}; FAB (MH^+) 410; Ms 330, 228, 185, 155, 130, 104, 76.

Assays of β-lactamase inhibition and microbiological activity

β-Lactamase inhibition studies used purified enzyme prepared as previously described.[8] For determination of β-lactamase inhibition, activity of enzyme in the presence of inhibitor was measured by following the hydrolysis of the chromogenic substrate nitrocefin spectrophotometrically at 482 nm. Assay conditions were 50 mM phosphate buffer, pH 7.0, at 27 °C. Measurement was initiated by addition of substrate immediately after addition of the inhibitor, with no prior preincubation. Tests for antimicrobial activity and synergy with β-lactam antibiotics were performed by standard microdilution assay.[9]

Acknowledgments

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