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Reaction of Lys-Tyr-Lys Triad Mimics with Benzylpenicillin: Insight into the Role of Tyr150 in Class C β-Lactamase

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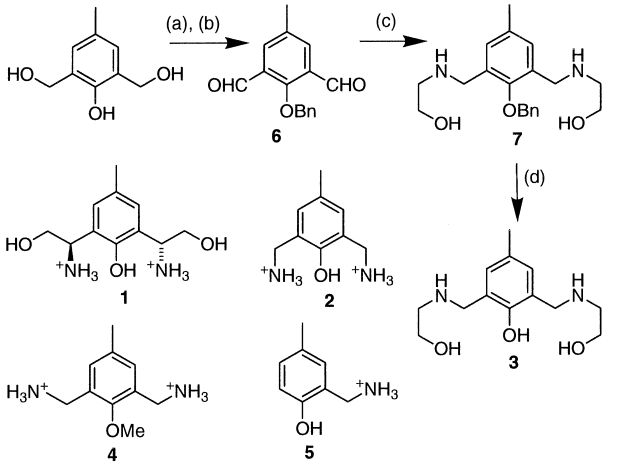
Abstract—Small and simple molecules mimicking a Lys-Tyr-Lys triad and some 'mutant' derivatives were designed and synthesized. These compounds react with benzylpenicillin in water (75 mM phosphate buffer, pH 7), apparently through general base assistance by the phenolic moiety. Class C β-lactamase has a Lys-Tyr-Lys triad in its active site, and our finding gives some insight into the role of this triad in the enzymatic β-lactam hydrolysis mechanism.

Class C β-lactamases efficiently hydrolyze β-lactam antibiotics, including cephalosporines.[1] Studies using transition-state analogue inhibitors,[2] X-ray crystal structures of the native enzyme,[3] inhibitor bound enzymes,[4] and natural mutants[5] have identified the active site. Particularly, the roles of Tyr150, Lys67, and Lys315 in the active site are of interest since they appear to form a hydrogen-bond network with Ser64 at which the acyl-enzyme intermediate forms. Kinetic parameters obtained with Lys67, Lys315, and Tyr150 mutants by Frère et al.[6] and Page et al.[7] suggested that the basic residue activating Ser64 for acylation is Lys67. However, later studies by Frère et al.[8] suggested the combination of Tyr150 and Lys315 as the base. Crystallography[2] and pH dependent kinetic solvent isotope effect studies[9] suggested that the phenol of Tyr150 has a reduced pK_a, and should therefore exist as the phenolate anion capable of functioning as a base. Thus, the base activating Ser64 remains enigmatic, yet these findings at least narrow down the choice of basic residues to Lys67, Lys315, or Tyr150. This means that either lysine or tyrosine is likely to have a largely reduced, near neutral pK_a rather than their typical value close to 10.

We have already reported a model compound which mimics the positioning of Lys67-Tyr150-Lys315 side-chain functional groups of class C β-lactamases, and have used this and its derivatives to measure their functional group acidities.[10] The shallow, solvent-exposed active site of class C β-lactamase is expected to share some similarity to these water-soluble model compounds. The findings with these model compounds agreed well with the proposed negative charge at Tyr150, and positive charges at Lys67 and Lys315 residues under physiological pH. We now describe the use of these compounds to explore their behavior in the presence of a β-lactam.

The study involves the use of compounds **1**–**5** shown in Scheme 1. Compound **3**[11] was synthesized by reductive amination of **6** followed by hydrogenolysis of the benzyl



Scheme 1. (a) BnBr, K_2CO_3, acetone, reflux 12 h (90%); (b) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78 °C to rt (87%); (c) ethanolamine, HOAc, $NaBH_3CN$, MeOH, overnight (54%); (d) H_2 2.5 atm, 10% Pd–C, MeOH, rt 4 h (83%);.

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group (Scheme 1). Compound **5**[12] was prepared according to known procedures. Compounds **1**, **2** and **4** were prepared as described previously.[10]

Compounds **1** and **2** both bring two primary amines close to a phenol. This is similar to the arrangement of the active-site side chains, Lys67, Tyr150, and Lys315 residues (Fig. 1). Compound **3** has functional groups similar to that of **1** but the hydroxyethyl group is attached to the amine, thereby yielding a secondary amine. The methyl ether in 'mutant' **4** precludes deprotonation of the phenol. 'Mutant' **5** addresses the difference between having a phenol between two amines and having a phenol next to a single amine.

Since **1** and **2** mimic the active site, we were curious whether these and related synthetic compounds enhance β-lactam ring opening. Thus, we reacted compounds **1**–**5** with benzylpenicillin. Reactions on compounds **1**–**5** with benzylpenicillin were carried out at room temperature, with 5 mM of both reactants, in a 75 mM phosphate buffer solution at pH 7.0. The decrease in concentration of benzylpenicillin was monitored by HPLC. Similarly, the decrease in benzylpenicillin (5 mM) due to hydrolysis was monitored in the absence of compounds **1**–**5**. The time taken for 5 mM benzylpenicillin to decrease to 4.5 mM (10% decrease) is shown in Table 1.

Compounds **1**–**5** were mono-acylated by benzylpenicillin at the amine (Fig. 2). MS and IR spectra characterized the β-lactam acylated products, **1′**–**5′**.[13] MS of the product revealed a molecular ion peak with mass corresponding to the synthetic compound plus benzylpenicillin. The carbonyl stretches in the IR spectra correspond to amides. The absence of a stretch around 1740 cm^{-1} suggested that the reaction yielded an amide, and not an ester.

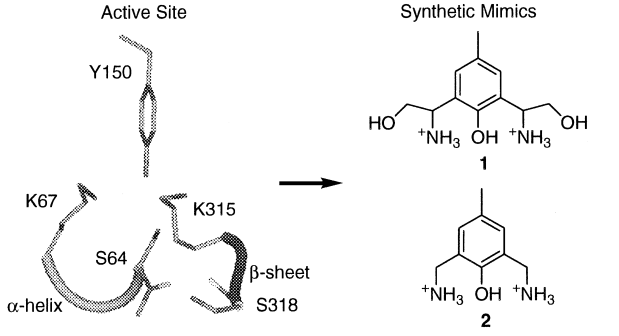


Figure 1. Class C β-lactamase active site and synthetic mimics **1** and **2**.

Table 1. Time taken for 10% decrease in benzylpenicillin concentration and pK_a of **1**–**5**

Compound reacted with benzylpenicillin	Time taken for 10% decrease in benzylpenicillin concentration[a] (h)	pK_{a_1}	pK_{a_2}
2	1.38	7.0	9.7
3	2.45	6.4	9.7
1	3.60	6.3	9.0
5	8.25	8.7	n.d.[b]
4	44.9	8.5	10.2
none	50.8	n.a.[c]	n.a.

[a]All determinations were carried out at least twice. The values obtained from separate runs were within 22% of each other.
[b]n.d., not determined.
[c]n.a., not applicable.

Considering the reaction mechanism, there are two ways in which the phenolic compounds, **1**–**3** and **5**, can form mono-amides. The reaction may proceed (1) via a phenol ester intermediate, followed by subsequent acyl-transfer, or (2) via activation of the amino group by the adjacent phenolate resulting in a direct nucleophilic attack by the amino group on the β-lactam carbonyl carbon.

If the first mechanism is operating, the phenolic ester formation step should be rate limiting because the second step would be a facile intramolecular (6-*exo*-trig) reaction. Keeping in mind the pK_{a_1} values of **1**–**3** (Table 1), if the first mechanism holds, the time taken for a 10% decrease in benzylpenicillin in the presence of **1** or **3** should be similar and should also be shorter compared to that for **2**. This is because **1** and **3**, which have slightly acidic pK_{a_1} values (6.3 and 6.4, respectively), should have larger populations of deprotonated species at pH 7 compared to **2** which has a pK_{a_1} of 7.0. Also, steric hindrance around the phenol appears similar in all of these compounds. Nevertheless, the fastest decrease in benzylpenicillin concentration was observed with **2** (the most basic) followed by **3**, followed by **1** (the most acidic). Thus, these findings did not strongly support the phenol ester mechanism.

The experimental results lend stronger support for a mechanism where the amino group directly reacts with the β-lactam, that is the ammonium activation mechanism (Scheme 2). As Scheme 2 shows, compounds **1**–**3** are all in an equilibrium at pH 7 between the overall +2 charge state and the overall +1 state. In the overall +1 state, one of the amines is capable of reacting directly with the β-lactam at pH 7. The difference in **1** and **3** compared to **2** is in the hydroxyalkyl groups. These groups appear to reduce the reactivity of **1** and **3** by steric crowding around the amine, thereby making **2** the most reactive. Between **1** and **3**, although the hydroxyl

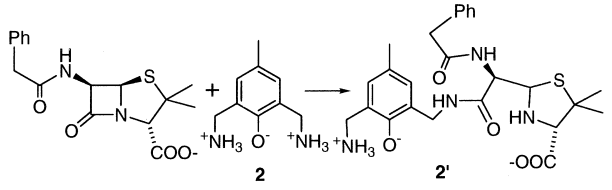
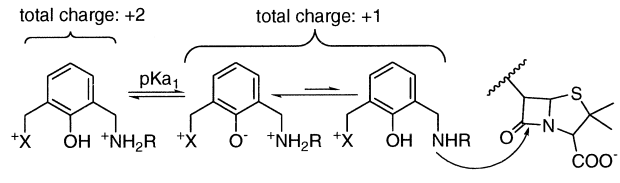
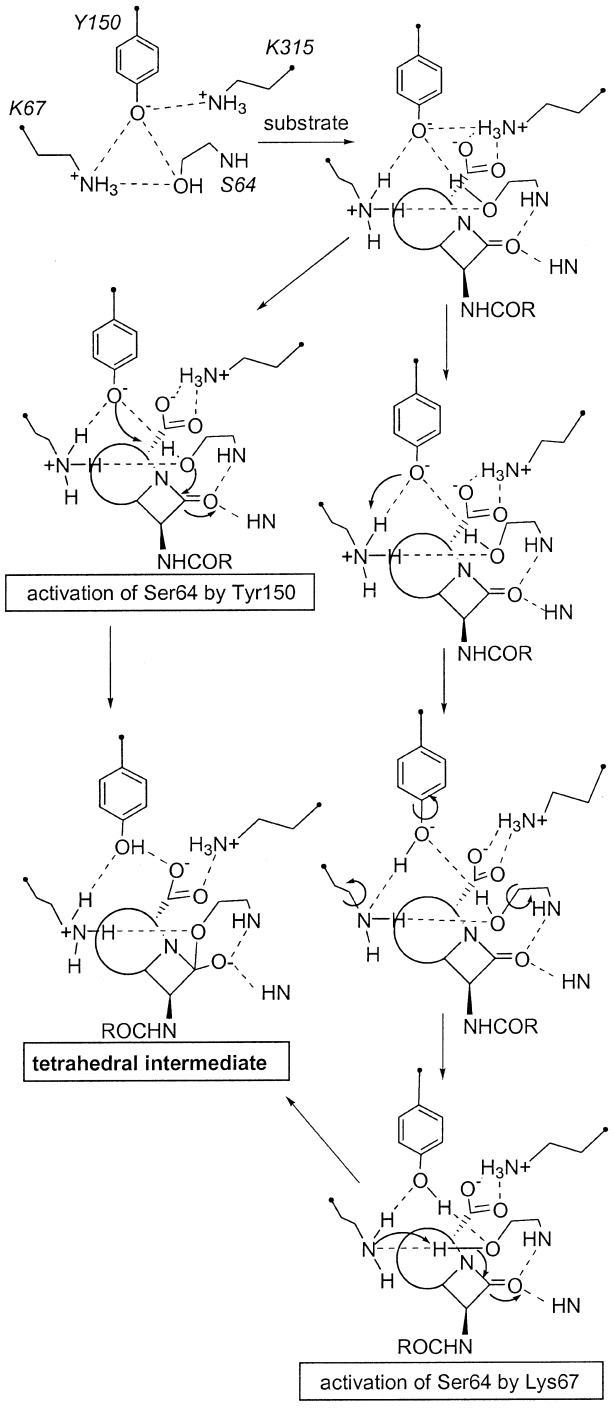


Figure 2. Reaction between benzylpenicillin and **2**. Analogous reactions were carried out with **1** and **3**–**5**.



Scheme 2.

group is three bonds away from the amino nitrogen, in both cases, **1** has primary amines and **3** has secondary amines. Secondary amines are generally more basic compared to primary amines, as revealed by their pK_{a_2} values, and its nitrogen is expected to be more nucleophilic. This agrees well with the faster reaction observed with **3** compared to **1**. In the presence of compound **5**, which is a mono-amine, a 10% decrease in benzylpenicillin concentration takes nearly 6 times longer compared to the case with **2**. If the longer time required for the 10% decrease is solely due to the halved effective amine concentration, **5** should take approximately twice as long as **2** to decrease benzylpenicillin by 10%. Also, the pK_a values show that the second amine contributes to reduce pK_{a_1} in **2**. Compound **4** lacking the phenolic hydroxyl group was the least reactive. Its presence hardly affected the reduction of benzylpenicillin concentration.



Scheme 3.

Briefly summarizing these results, the time taken for 10% decrease in benzylpenicillin concentration only loosely corresponds to the pK_{a_1} value, which according to previous UV studies is the pK_a of the phenolic hydroxyl group for compounds **1**–**3** and **5**.[10] This means that the precise amount of phenolate species is probably not so important in determining the time required for a 10% decrease of benzylpenicillin.

Certain aspects of our findings with the model compounds seemed to have some relevance to the actual enzyme mechanism. Combining our findings with previous findings by others on class C β-lactamase, the mechanism of Ser64 activation, and subsequent formation of the tetrahedral intermediate may be like that shown in Scheme 3. In this hypothetical mechanism, the free enzyme has a partially deprotonated Tyr150 at physiological pH, based on the pK_a's of compounds **1**–**3**.[14] The substrate then binds and forms the Michaelis complex. According to molecular modeling, the negatively charged carboxylate (or sulfonate) of the β-lactam antibiotic should be at a hydrogen bonding distance to Lys315 and Thr316.[3,15–17] Crystal structures of enzyme–inhibitor complexes[3] reveal that there is space near Lys315 to accommodate a hydrogen bond acceptor. Next, as a result of the carboxylate–Lys315 salt bridge, the effect of positive charge on Tyr150 and the hydrogen bond between Lys315 and Tyr150 are expected to weaken. This would create a situation similar to that of model compound **5**, and the phenolic pK_a of Tyr150 should rise. At this point, Tyr150 is hydrogen bonded to Ser64 and Lys67, therefore these hydrogen bonds should be strengthened, and this might trigger the nucleophilic attack of Ser64 on the β-lactam carbonyl carbon.[18] This step which could be either stepwise or concerted has some similarity to amine activation seen in compounds **1**–**3**. The circuitous proton abstraction via Lys67 should also be possible, but would involve reorientation of the hydrogen bonds. The two routes to Ser64 proton abstraction have been assessed by β-secondary and solvent deuterium kinetic isotope effects.[19] Although a clear-cut conclusion could not be drawn, the results seem to favor the direct proton acceptance mechanism by Tyr150. Yet, perhaps this possibility of alternative pathways to achieve the same goal brings forth the versatility of these enzymes and makes mechanism elucidation through mutation studies and modified substrates difficult.

In summary, class C β-lactamase active-site model compounds and their 'mutants' demonstrated the variability of the phenolic pK_a and to a lesser extent the amino pK_a. Acylation of these compounds by benzylpenicillin suggested that the phenol activates the

adjacent ammonium for acylation at physiological pH. A hypothetical mechanism of Ser64 activation by Tyr150 in the formation of the tetrahedral intermediate in class C β-lactamase can be proposed by incorporating the findings from compounds **1**–**5** to previous experimental and computational findings by others. The likely course of proton transfer is a direct transfer from Ser64 to Tyr150; however, a relay of proton transfers from Lys67 to Tyr150, and then from Ser64 to Lys67 may also be possible.

Our next goal in understanding the reaction mechanism at the atomic level is to clarify the pK_a values and protonation state of the active-site residues in the native enzyme. Accordingly, construction of class C β-lactamase variants that should allow clarification of the basicity of Lys67 and Tyr150 is now under way.

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