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Water-Soluble Class C β-Lactamase Catalytic Residue Mimic: Effect of Proximally Positioned Functional Groups on their pKa Values

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Abstract: Molecules mimicing class C β-lactamase catalytic residues were synthesized to study the effect of proximal positioning of serine, lysine and tyrosine side chains on their pKa values. The three amino acid side chains were mimicked by hydroxymethyl group, alkyl ammonium group and phenol which were linked by a short skeleton to ensure interaction among these functionalities. Comparison of the mimics in water showed a phenolic pKa decrease of up to 3.6 units. The finding supports the possible role of tyrosine as a general acid/base catalyst in acylation of class C β-lactamase.

Discussion of the detailed mechanism of class A and C β-lactamases has been fueled by the recent availability of high- resolution crystal structures, combined with experimental and theoretical studies.[1,2] Yet, besides the notion that they are both serine hydrolases and involve acyl-enzyme intermediates, there is still no consensus on the steps leading to acylation and subsequent hydrolysis. A recent study focusing on the hydrolysis step has suggested that the cyclic ammonia of the acylated substrate acts as a general base activating the hydrolytic water.[1a] On the other hand, crystal structure analyses and mutation studies of class C β-lactamase have suggested that Tyr 150 participates in general acid/base chemistry, particularly in the acylation step.[1d-f] In order for tyrosine to participate in such general acid/base reactions at physiological pH, its pKa must be lowered from its original value of approximately 10 to a near neutral pH. Large pKa alteration has been seen in some lysine pairs and diacids with closely positioned functional groups.[3] Accordingly, small molecules mimicing class C β-lactamase active site were designed, synthesized and analyzed to see whether large alteration in the pKa of a phenol is possible by proximal effects.

The pH profile of penicillin and cephalosporin hydrolysis by class C β-lactamase has indicated two catalytically important ionizing amino acid residues of pKa near 6 and 10,[4] suggesting that the residue with pKa of 6 is that of Tyr 150. Such large alteration of phenolic pKa value has been observed for calix[4]arenes,[5a] *p*-nitrophenol (pKa=7.2) and their derivatives.[5b] The phenomena in calixarene is rationalized by strong intramolecular hydrogen bonding among the phenolic OH groups. The case in nitrophenol derivatives can be explained by through-bond inductive effect. However, the depression of pKa in this enzyme is thought to be accomplished by the presence of two lysine residues adjacent to the phenol.[1d-e,6] In order to study the pKa behavior of Tyr positioned proximal to two Lys and Ser residues, a simple model compound (**1**) was designed and synthesized (Figure 1).

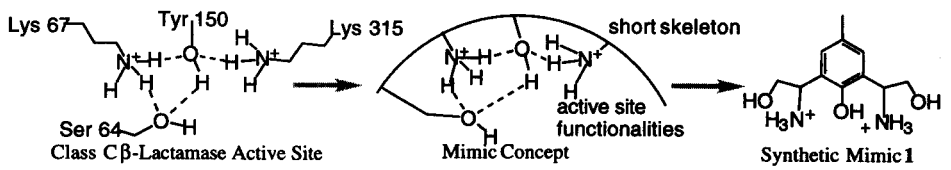

Figure 1

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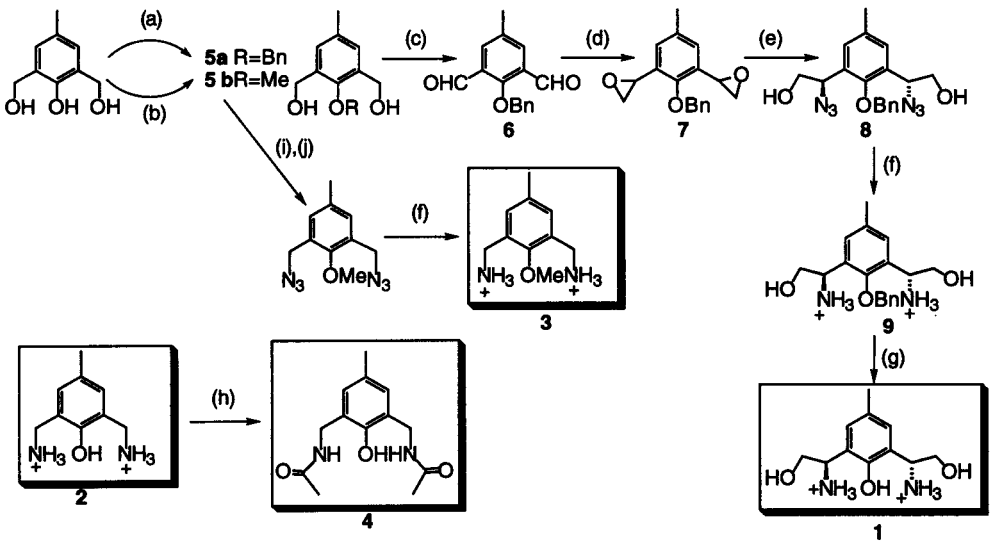
In **1**, Tyr, Lys and Ser are mimicked by phenol, alkyl ammonium and hydroxymethyl groups respectively. These functionalities are linked by a short skeleton to ensure interaction among these functionalities. The symmetric nature of the molecule simplifies its synthesis and analyses (Figure 1). Several synthetic "mutants" of **1** were also prepared and studied for comparison (Scheme 1). Structure **2** lacks the peripheral hydroxyl groups present in **1**, and allows assessment of the contribution of hydroxyl groups to the pKa of the phenol. In molecule **3**, the phenol is protected as a methyl ether, such that the pKa of the amines in a similar system can be isolated. Diamide **4** can still have a hydrogen bonding pattern similar to **2** but allows unambiguous assessment of the phenolic pKa, sandwiched by two uncharged NH's.



Scheme 1. (a) BnBr, K_2CO_3, acetone, reflux 12h (90%); (b) MeI, K_2CO_3, acetone, reflux 12h (65%); (c) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -78 °C to RT (70%); (d) Me_3SI, Bu_4NBr, CH_2Cl_2/50% NaOH, RT 12h; (e) NaN_3, NH_4Cl, EtOH, reflux 12h (39%:24% racemic/meso); (f) Ph_3P, H_2O, THF, RT 48h (90%); (g) H_2 2atm, 10% Pd-C, EtOH, RT 1h (quant); (h) AcCl, pyridine, reflux 3h (35%); (i) $SOCl_2$, 0 °C to RT 2h; (j) NaN_3, DMF, RT 12h (90%).

The syntheses of the active site mimics and their mutants are outlined in Scheme 1.[7] Formation of bis(aminomethyl)-*p*-cresol **2** followed known procedures.[8]

Computational chemistry. Both molecular modeling and semiempirical PM3 molecular orbital (MO) calculations were performed on **A-D** (Figure 2) and their conjugate bases to compare the intrinsic ease of pKa alteration of phenolic hydroxyl and benzyl ammonium due to proximity effects.[9] For **C** and **D**, conjugate bases were formed by either deprotonating the phenol or the ammonium. First, the lowest energy conformations of the acids and their conjugate bases were obtained from molecular modeling (Figure 3).[10a] These conformers were further minimized by MO calculations before finally calculating the heats of formation.[10b] From these heats of formation and that of an isolated proton,[11] the overall deprotonation energies were calculated (Table 1). These results indicate that phenolic deprotonation energy is more easily perturbed ($\Delta E \approx 190$ kcal/mol) than that of ammonium ($\Delta E \approx 60$ kcal/mol) when **A** and **B** are compared to **C** and **D**. However, the ease of deprotonating the ammonium or phenol within **C** and **D** are similar. Thus, from gas phase calculations, the functionality that is likely to deprotonate first in **C** and **D** is unclear.

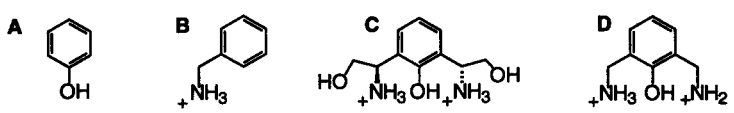


Figure 2

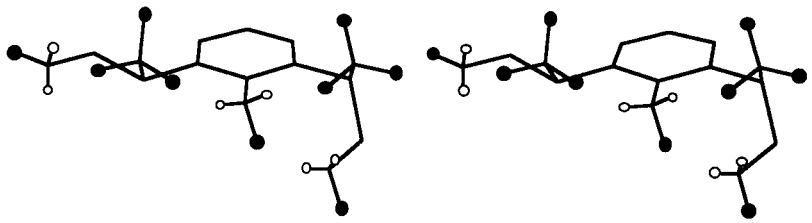


Figure 3. Stereoview of **C** after minimization by MacroModel. ● indicates a hydrogen atom and ○ indicates a lone pair

Experimental pKa determination. Simple aqueous acid-base titrations of **1-4** were performed at 297K and the results are summarized in Table 1.[12] Model compound **1** equipped with both functionalities, has pKa values of 6.3 and 9.0. Clearly, the acidity of one of the functionalities was greatly altered from a basic pKa to a slightly acidic pKa by the presence of other functionalities in close proximity. Removing the hydroxymethyl groups as in **2**, the two pKa values increase to 7.0 and 9.7, thus the hydroxymethyl groups appear to contribute to pKa depression. Furthermore, since the pKa values of **1** and **2** are similar, hydroxyl deprotonation in **1** is unlikely. The pKa values for **3** are different from that of **2**, and suggests that the two deprotonations in **2** are from one phenol and one ammonium, whereas the deprotonations in **3** are from the two ammoniums. In structure **4**, where the amide NH's are still within interacting distance of the phenol, but are neutral and can no longer deprotonate in the pH range studied, the phenolic pKa stayed basic at 9.0. The presence of positively charged ammoniums seem essential for pKa reduction of the phenol.

Table 1. Computational and Experimental Deprotonation Behavior of **A-D** and **1-4**

Computational		Experimental					
			Acidity		UV λ_{max} (nm)		
Molecule	Deprotonation Energy (kcal/mol)	Molecule	pKa_1	pKa_2	pH2-6	pH7-9	pH10-13
A	345	**A**	9.89	n.a.	270	270	287
B	214 - 216	**B**	9.33	n.a.	n.d.	n.d.	n.d.
C	156 (phenol); 154 - 184 ($-NH_3^+$)	**1**	6.3	9.0	282	306	302
D	153 (phenol); 147 - 153 ($-NH_3^+$)	**2**	7.0	9.7	282	306	302
		3	8.5	9.2	n.d.	n.d.	n.d.
		4	9.0	n.a.	281	281	302

Notes: not applicable data (n.a.); not determined data (n.d.)

Spectroscopic study. UV absorption spectra (298K) of **1**, **2** and **4** at pH's corresponding to the various protonation states identified pKa values corresponding to phenol deprotonation (Table 1).[13] In **1**, the red shift from 282 nm to 306 nm upon raising the pH from acidic to near-neutral (corresponding to pKa_1) is indicative of phenol deprotonation. Further raising the pH causes a second deprotonation, and the absorption returns slightly towards the blue to 302 nm. This slight absorption shift may be a counter-ion effect.[5b] For the three different protonation states of **2**, exactly the same λ_{max} values as **1** were observed. The λ_{max} values of **4** at 281nm and 302nm at pH7 and pH11 respectively confirms that this shift is due to the phenol.

Conclusion. The study of class C β-lactamase model compounds **1-4** showed that adjacent positioning of ammoniums can indeed reduce the pKa of a phenol to 6.3 through proximity effect, even in an aqueous solvent exposed system where polarized interactions are expected to be weakened. This finding lends an experimental and physicochemical support for the possible role of Tyr 150 as a general acid/base catalyst in the acylation of class C β-lactamase. Investigation of β-lactam hydrolysis in the presence of these models, as well as development of elaborated structures of **1** capable of accelerating β-lactam hydrolysis are now underway.

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7. Spectral data of all new compounds are shown below: **1.** ^{1}H-NMR (CD_3OD) δ 7.222 (s, 2 H), 4.679 (dd, 2 H, J = 4.4, 7.6 Hz), 3.918 (dd, 2 H, J = 4.4, 11.6 Hz), 3.846 (dd, 2 H, J = 7.6, 11.6 Hz), 2.327 (s, 3 H); HRMS (FAB in 3-nitrobenzyl alcohol and glycerol) calcd for $C_{11}H_{19}N_2O_3$ (M + H$^+$), 227.1396; found 227.1388; **3.** ^{1}H-NMR (CD_3OD) δ 7.333 (s, 2 H), 4.171 (s, 4 H), 3.857 (s, 3 H), 2.374 (s, 3H); HRMS (FAB in 3-nitrobenzyl alcohol and glycerol) calcd for $C_{10}H_{17}ON_2$ (M+H$^+$), 181.1341; found, 181.1329; **4.** ^{1}H-NMR ($CDCl_3$) δ 9.689 (s, 1 H), 6.911 (s, 2 H), 4.322 (d, 4 H, J = 6.4 Hz), 2.196 (s, 3 H), 1.986 (s, 6 H); HRMS (FAB in 3-nitrobenzyl alcohol and glycerol) calcd for $C_{13}H_{19}O_3N_2$ (M + H$^+$), 251.1396; found 251.1390; **6.** ^{1}H-NMR ($CDCl_3$) δ 10.233 (s, 2 H), 7.891 (s, 2 H), 7.40-7.32 (m, 5 H), 5.133 (s, 2 H), 2.414 (s, 3H); MS (EI) calcd for $C_{16}H_{14}O_3$ (M$^+$), 254.09; found 254; **8.** ^{1}H-NMR ($CDCl_3$) δ 7.45-7.41 (m, 5 H), 7.176 (s, 2 H), 4.99-4.92 (m, 4 H), 3.79-3.73 (m, 4 H), 2.368 (s, 3 H), 1.930 (br s, 2 H); MS (FAB in dithiodiethanol) calcd for $C_{18}H_{20}N_6O_3Na$ (M + Na$^+$), 391.15; found 391.2.
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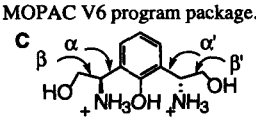
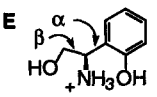
10. (a) Energy minimized structures were obtained by using MM2 forcefield as implemented in MacroModel V3. Systematic minimization was employed, where the torsion angle of the exocyclic C-C bonds in **B**, **C** and **D** were varied by 10° increments prior to minimization. In the case of **C** where there are two sets of two different exocyclic C-C bonds, tortion α and β in compound **E** were minimized initially. After obtaining several α and β values giving rise to several low energy conformations, combinations of these values were used as starting values for α,α',β and β' to find the lowest energy conformation of **C**. (b) Minimizations and calculations of heats of formation were carried out using PM3 semiempirical methods as implemented in MOPAC V6 program package.





11. An experimental value of 367.2 kcal/mol was used for the energy of formation of an H$^+$. Stull, D. R.; Prophet, J. *JANAF Thermochemical Tables*; NSRDS-NBS37.
12. Using a glass electrode pH meter, a 50 to 90 point pH titration curve was obtained by titrating a solution, 10mM in **1**, **2**, **3** or **4**, and 10 mM in HCl (500 μL to 1000 μL) with 50mM NaOH solution in increments of 5-20 μL at 297K.
13. UV measurements were made on a Shimadzu UV-2400PC UV-VIS Recording Spectrophotometer.