Inhibition of Serine β-Lactamases by Vanadate−Catechol Complexes[†]

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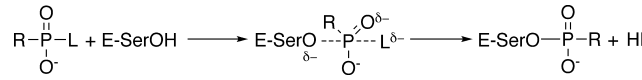
Received June 19, 2008

ABSTRACT: All three classes of serine β-lactamases are inhibited at micromolar levels by 1:1 complexes of catechols with vanadate. Vanadate reacts with catechols at submillimolar concentrations in aqueous buffer at neutral pH in several steps, initially forming 1:1, 1:2, and, possibly, 1:3 complexes. Formation of these complexes is followed by the slower reduction of vanadate (V^{V}) to vanadyl (V^{IV}) and oxidation of the catechol. Vanadyl−catechol complexes, however, do not inhibit the β-lactamases. Rate and equilibrium constants of formation of the 1:1 and 1:2 complexes of vanadate with catechol itself and with 2,3-dihydroxynaphthalene were measured by stopped-flow spectrophotometry. Typical examples of all three classes of serine β-lactamases (the class A TEM-2, class C P99, and class D OXA-1 enzymes) were competitively inhibited by the 1:1 vanadate−catechol complexes. The inhibition was modestly enhanced by hydrophobic substituents on the catechol. The 1:1 vanadate complexes are considerably better inhibitors of the P99 β-lactamase than 1:1 complexes of catechol with boric acid and are likely to contain penta- or hexacoordinated vanadium rather than tetracooordinated. Molecular modeling showed that a pentacoordinated 1:1 vanadate−catechol complex readily fits into the class C β-lactamase active site with coordination to the nucleophilic serine hydroxyl oxygen. Such complexes may resemble the pentacoordinated transition states of phosphyl transfer, a reaction also catalyzed by β-lactamases.

The β-lactam antibiotics continue to be an important component of our antibacterial armament despite much effort to find new alternatives (*1, 2*). Bacterial resistance to the β-lactams continues to rise, however, as it does for all antibiotics in widespread clinical use. A major source of resistance to β-lactams is provided by β-lactamases, enzymes that catalyze the hydrolytic destruction of these antibiotics (*3*). β-Lactamase inhibitors have, therefore, been used for more than 20 years now to synergize the activity of a number of β-lactams. Although this concept of application of β-lactamase inhibitors has been validated, there are still only a small number of inhibitors in general medical practice (*4*). All of these are β-lactams themselves and act as mechanism-based inhibitors, but they are, in general, all susceptible to hydrolysis by mutant β-lactamases (*5*). There is much interest, therefore, in new chemical entities with β-lactamase-inhibitory potential (*6*). There are few generally effective, noncovalent, fast reversible, or non-β-lactam inhibitors, for example.

Well-known non-β-lactam inhibitors of serine β-lactamases include a variety of boronates (*7*−*10*) and phosphonates (*11, 12*). These compounds react with the β-lactamase active site serine to form stable anionic tetrahedral complexes that are thought to structurally and electronically resemble the transition states and/or high-energy tetrahedral intermediates of β-lactamase catalysis (*13*−*15*). Since the phosphonates achieve such structures by means of a phosphoryl transfer reaction, presumably involving a pentacoordinated phosphorus intermediate (Scheme 1), it follows that the serine β-lactamase active site must also strongly bind oxyanions with geometry expanded beyond tetrahedral. This has been shown to be true in one instance. Complexes of vanadate with hydroxamic acids have been shown to inhibit a class C β-lactamase, where the inhibition constants of the active 1:1 complexes are submicromolar (*17*). ^{51}V NMR[1] spectra indicated that the complex formed at the β-lactamase active site contained penta- or hexacoordinated vanadium. A typical class A β-lactamase did not, however, appear to be inhibited by these complexes.

Scheme 1



Beyond the hydroxamic acids, vanadate is known to form tight complexes in aqueous solution with catechols (*18, 19*). A number of catechol-containing complexes of vanadium(V) have been isolated and shown by X-ray crystallography to contain six-coordinated vanadium (*20*−*24*). This paper shows that 1:1 complexes of vanadate with a variety of catechols (**1**−**12**) inhibit typical examples of all three classes (A, C, and D) of serine β-lactamases. It seems likely, therefore, that a broad class of polyoxo species may inhibit these enzymes. Tetrahedral catechol-borates appear to be less effective as inhibitors.

[†] This research was supported by National Institutes of Health Grant AI-17986.

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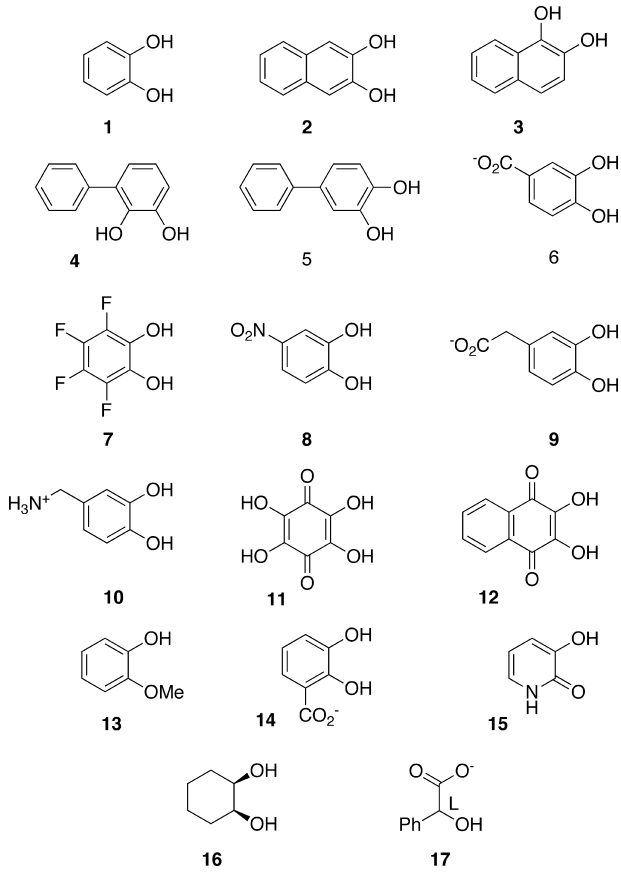
[1] Abbreviations: Centa, 7β-[(thien-2-yl)acetamido]-3-[(4-nitro-3-carboxyphenylthio)methyl]-3-cephem-4-carboxylic acid; MOPS, 3-morpholinopropanesulfonic acid; NMR, nuclear magnetic resonance.

10.1021/bi801153j CCC: $40.75
Published on Web 08/15/2008

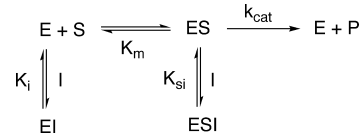


MATERIALS AND METHODS

Materials. The class C β-lactamase of *Enterobacter cloacae* P99 and the class A TEM-2 β-lactamase were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as supplied. The concentrations of stock solutions of these enzymes were obtained spectrophotometrically by the employment of published extinction coefficients of 7.10×10^4 M^{-1} cm^{-1} for the P99 β-lactamase (*25*) and 2.9×10^4 M^{-1} cm^{-1} for the TEM β-lactamase (*26*). The OXA-1 β-lactamase was generously provided by M. Nukaga of Jyosai International University. The following catechols were purchased commercially and used as received: catechol (Acros Organics), 4-nitrocatechol (Aldrich), 2,3-dihydroxypyridine (Acros Organics), *cis*-1,2-cyclohexanediol (Acros Organics), 3,4,5,6-tetrafluorocatechol (Oakwood), 1,2-dihydroxynaphthalene (TCI America), 2,3-dihydroxynaphthalene (TCI America), 3,4-dihydroxyphenylacetic acid (Aldrich), 3,4-dihydroxybenzylamine hydrobromide (Aldrich), 2,3,5,6-tetrahydroxy-1,4-benzoquinone hydrate (Aldrich), 4-phenylcatechol (ChemBridge), 3-phenylcatechol (Fluka), 2,3-dihydroxybenzoic acid (Acros Organics), and 3,4-dihydroxybenzoic acid (Acros Organics). Isonaphthazarin (**12**) was prepared following a literature procedure (*27*). 2-Methoxyphenol was from Sigma, while sodium orthovanadate (99.8%) and vanadyl sulfate trihydrate (>99.99%) were from Aldrich. The β-lactamase substrate Centa was prepared as previously described (*28*), and nitrocefin was purchased from Oxoid.

Steady State Enzyme Kinetics. All kinetics measurements involving the P99 and TEM β-lactamases were carried out in 20 mM MOPS buffer (pH 7.5) at 25 °C, while those involving OXA-1 β-lactamase also contained 50 mM sodium bicarbonate (*29*). β-Lactamase activity was routinely determined spectrophotometrically against 100 μM Centa (P99 β-lactamase), 50−200 μM nitrocefin (TEM β-lactamase), and 50 μM Centa (OXA-1 β-lactamase). Stock solutions (10 mM) of vanadate were prepared by dissolution of sodium orthovanadate in the requisite buffer. Absorption spectra and spectrophotometric reaction rates were measured with a Hewlett-Packard 8452A spectrophotometer.

Scheme 2



The apparent inhibition constant, K_i^{app}, for each of the catechols was determined from experiments in which the vanadate−catechol complex inhibited the P99 β-lactamase-catalyzed turnover of Centa. Initial velocities of Centa hydrolysis, monitored at 410 nm, at a fixed concentration of vanadate (100 μM) and in the presence of several concentrations of catechol (0−200 μM), were determined. These data were fitted to eq 1 by a nonlinear least-squares procedure to obtain the apparent inhibition constant, K_i^{app}. It was assumed that the inhibition of the P99 β-lactamase-catalyzed hydrolysis of Centa (K_m = 6.2 μM) by the vanadate−catechol complexes was largely competitive at the concentrations that were employed (see below).

$$v = v_0(K_m + [S])/[K_m(1 + [I]/K_i^{app}) + [S]] \qquad (1)$$

where v and v_0 represent the initial velocity in the presence and absence of the inhibitor, respectively. The apparent inhibition constant, K_i^{app}, was similarly determined for the TEM and OXA-1 β-lactamases except that a fixed concentration of catechol (500 μM) was used while the vanadate concentration was varied (0−600 μM). The effect of catechol concentration (0−2.0 mM) on the activity of the P99 β-lactamase, in the presence of a fixed boric acid concentration (1.0 mM), was also determined.

The nature of the inhibition of the P99 β-lactamase by the vanadate−catechol complex was studied in more detail at three concentrations of Centa (30, 50, and 100 μM), with the concentration of vanadate at 100 μM and the catechol concentration varied from 0 to 100 μM. The data were fitted with Dynafit (*30*) to Scheme 2 where K_i and K_{si} are the inhibition constants representing the competitive and uncompetitive contributions, respectively, to the observed inhibition.

Stopped-Flow Kinetics. Vanadate (0.5 mM) and catechol (0.5−5.0 mM) solutions were mixed rapidly in a Durrum D-110 stopped-flow spectrophotometer and the ensuing reactions monitored at 430 nm. These data were fitted to Scheme 3 with Dynafit (*30*) where K_{V2} and K_{V4} were fixed at values of 310 M^{-1} and 3.0×10^8 M^{-3} (*31*), respectively. This experiment was repeated with 2,3-dihydroxynaphthalene. The values of K_1 and K_2 obtained were used to fit the inhibition data below.

Job Kinetics Plots. The activity of the P99 β-lactamase was determined as the concentrations of vanadate (0−0.2 mM) and catechol or 2,3-dihydroxynaphthalene (0.2−0 mM) were continuously varied. The resulting data and those for the fixed vanadate concentration experiments described above

Scheme 3

$$V + C \underset{K_1}{\rightleftharpoons} VC \qquad K_1 = k_{-1}/k_1$$

$$VC + C \underset{K_2}{\rightleftharpoons} VC_2 \qquad K_2 = k_{-2}/k_2$$

$$V + V \underset{K_{v2}}{\rightleftharpoons} V_2$$

$$4V \underset{K_{v4}}{\rightleftharpoons} V_4$$

were fitted to Scheme 4 with Dynafit (*30*) to obtain the inhibition constant, K_i, of a 1:1 complex.

Job Absorbance Plot. The effect of continuous variation of vanadate and catechol concentrations, both from 0 to 1 mM, on the absorbance at 420 nm was determined. The absorbance was determined immediately (5−10 s) after manual mixing. A Job plot of the data was fitted to Scheme 3. In this fitting procedure, K_1 and K_2 values were fixed at those obtained from the stopped-flow experiments.

Molecular Modeling. The computations were set up essentially as previously described (*17, 32*) and run on an SGI Octane 2 computer with Insight II 2005 (Accelrys Software, San Diego, CA). The starting point for the simulations was the crystal structure of a covalent complex of the P99 β-lactamase with a phosphonate inhibitor [PDB entry 1bls (*14*)]. The pentacoordinated vanadium anion was constructed using the V−O bond distances and angles calculated by Krauss and Basch (*33*), and the positions of the vanadium atom and the five oxygen ligands were fixed in the subsequent calculations; this procedure was necessary since molecular mechanics parameters for this type of structure were not available. The crystal structure of a pentacoordinated vanadium(V)−catechol complex, however, has structural features similar to those of the model (*34*). Short molecular dynamics runs (40 ps) to locally relax the structure, followed by molecular mechanics energy minimizations on typical snapshots, were then performed.

RESULTS AND DISCUSSION

The class C β-lactamase of *E. cloacae* P99 was inhibited by mixtures of vanadate and catechol (Figure 1). The figure shows the change in initial rates with a varying catechol concentration at a fixed vanadate concentration, but quantitatively identical results, within experimental uncertainty, were obtained from experiments at fixed catechol and varying vanadate concentrations (not shown). Vanadate alone (to 1.0 mM) and catechol alone (to 2.0 mM) did not inhibit. The inhibition appeared to be fast and reversible and was fully expressed 5 s after addition of enzyme to a vanadate−catechol mixture. Incubation of the enzyme with vanadate and catechol for an extended period of time led to irreversible enzyme inhibition. For example, in a mixture of 0.1 mM vanadate and 0.116 mM catechol, the apparent first-order rate constant for inactivation was 1.76×10^{-4} s^{-1}. This slow inactivation may arise from the complex chemistry of the vanadate−catechol reaction (see below) and was not further investigated. The fast, reversible inhibition, presumably by a vanadate−catechol complex, was found to be largely competitive ($K_i^{app} = 2.7$ μM from eq 1) but with a small noncompetitive component (Figure 2). A fit of the latter data to Scheme 2 led to K_i and K_{si} values of 3.7 ± 0.4 and 230 ± 120 μM, respectively. Inhibition appeared to be less efficient at pH 6.5 and 8.5 (K_i^{app} values of 5.1 ± 0.3 and 7.4 ± 0.8 μM, respectively), probably due to dissociations at the enzyme active site (*35*) and of dihydrogen vanadate (*36*).

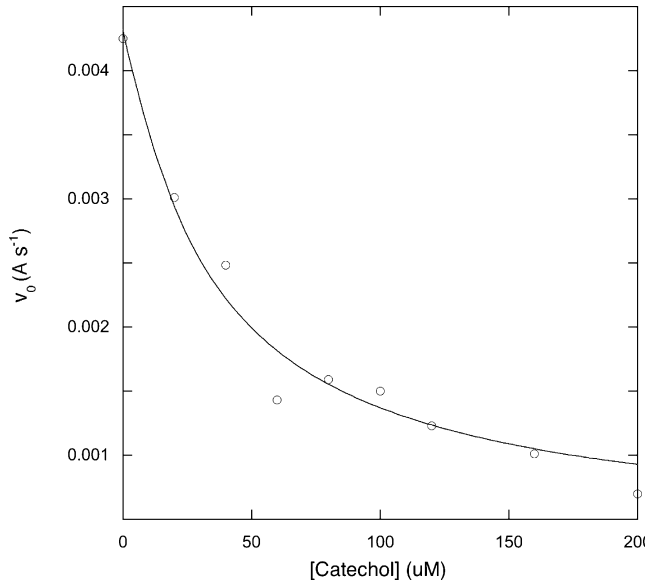


FIGURE 1: Inhibition of the P99 β-lactamase (2.0 nM) by mixtures of vanadate (0.10 mM) and catechol. The substrate was Centa (100 μM). The points are experimental, and the solid line was calculated as described in the text.

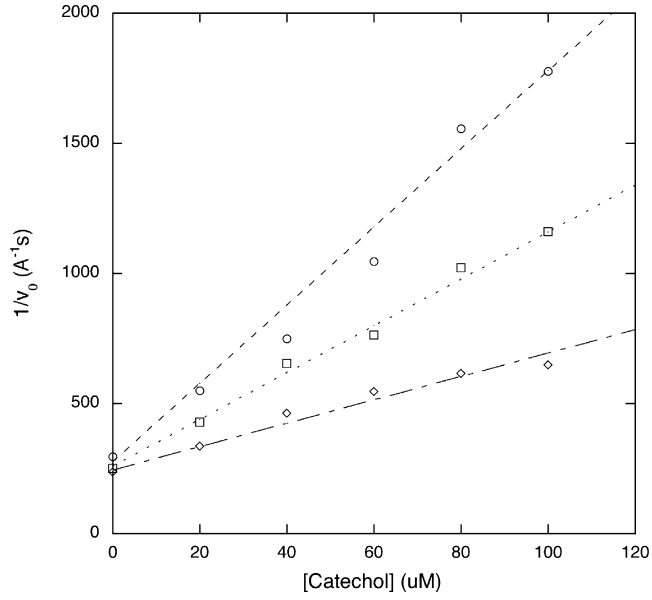


FIGURE 2: Dixon plot of the inhibition of the P99 β-lactamase (2.0 nM) by mixtures of vanadate (0.1 mM) and catechol. The substrate was Centa [(◇) 30, (□) 50, and (○) 100 μM]. The points are experimental, and the lines were calculated as described in the text.

Combinations of vanadate with other catechols (**2**−**12**) yielded the K_i^{app} values listed in Table 1. Most catechols tested formed an inhibitor in the presence of vanadate, with 3-phenylcatechol apparently the most effective of those tested; 4-nitrocatechol, however, was essentially just as effective. Phenol and 2-methoxyphenol were considerably less effective. An interesting group of compounds that afforded no inhibition under the conditions employed consisted of 2,3-dihydroxybenzoic acid **14**, 2,3-dihydroxypyridine (3-hydroxypyrid-2-one) **15**, *cis*-1,2-dihydroxycyclohexane **16**, and L-mandelic acid **17**.

Table 1: Inhibition of β-Lactamases by Catechol−Vanadate Complexes

	K_i^{app} (μM)		
	P99[a]	TEM-2[b]	OXA-1[c]
1	2.7 ± 0.3 (0.53 ± 0.06)[d]	62 ± 4 (27 ± 4)[d]	1.5 ± 0.1 (0.82 ± 0.05)[d]
2	0.58 ± 0.04 (0.16 ± 0.01)[d]	77 ± 13 (46 ± 5)[d]	16 ± 3 (10 ± 2)[d,e]
3	0.86 ± 0.08	nd[g]	nd[g]
4	0.43 ± 0.03	23 ± 4	3.0 ± 0.7[f]
5	0.9 ± 0.2	30 ± 5	1.1 ± 0.3
6	5.7 ± 1.2	200 ± 20	nd[g]
7	1.9 ± 0.5	nd[g]	nd[g]
8	0.51 ± 0.08	160 ± 20	nd[g]
9	4.2 ± 0.9	140 ± 10	nd[g]
10	10.9 ± 0.5	45 ± 11	nd[g]
11	10 ± 1	nd[g]	nd[g]
12	0.65 ± 0.13	nd[g]	nd[g]
13	50 ± 20	nd[g]	nd[g]
phenol	220 ± 40	nd[g]	nd[g]

[a] The class C β-lactamase of *E. cloacae* P99, 0.1 mM vanadate, varied catechol. [b] The class A TEM-2 β-lactamase from *Escherichia coli*, 0.5 mM catechol, varied vanadate. [c] The class D OXA-1 β-lactamase from *Es. coli*, 0.5 mM catechol, varied vanadate. [d] True K_i values of the 1:1 complex; see the text. [e] With 1.0 mM **2**, varied vanadate. [f] With 0.1 mM **4**, varied vanadate. [g] Not determined.

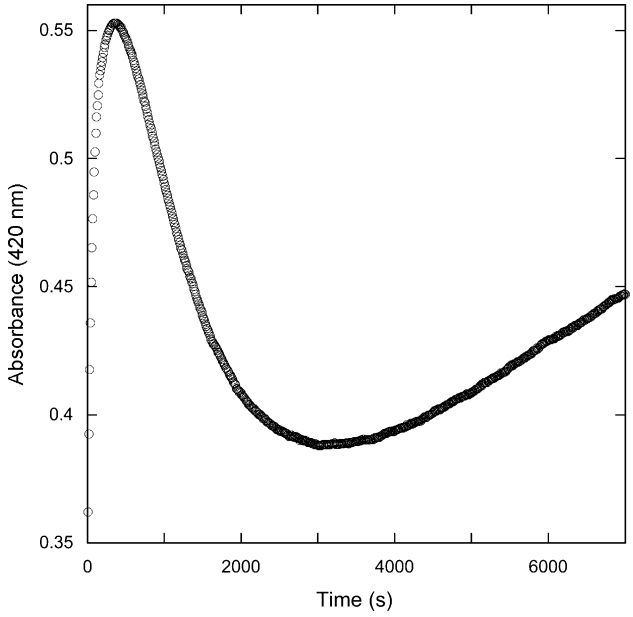


FIGURE 3: Slow absorption changes at 420 nm arising from reaction of vanadate (0.5 mM) and catechol (3.0 mM) at pH 7.5.

Representative class A (TEM-2) and class D (OXA-1) β-lactamases were also inhibited by vanadate−catechol mixtures (Table 1). The degree of inhibition of the OXA-1 enzyme matched that observed for the class C β-lactamase, but that of TEM-2 was considerably lower, at least with the compounds tested to date. 4-Phenylcatechol yielded the best inhibitor of the OXA-1 β-lactamase and 3-phenylcatechol the best for TEM-2. The affinity of the P99 and OXA-1 enzymes for hydrophobic ligands is well-known (*9, 37*−*40*), although in neither case were **4** and **5** significantly better than catechol itself.

Reactions between Vanadate and Catechol. The literature shows, qualitatively and, under some circumstances, quantitatively, that vanadate forms complexes with catechols and then oxidizes them (*41*−*43*). Our qualitative observations confirm a complex reaction pathway. For example, Figure 3 shows the absorption at 420 nm after vanadate (0.5 mM) and catechol (3 mM) are mixed. The figure shows the formation and decay of an intermediate, reaching a maximal

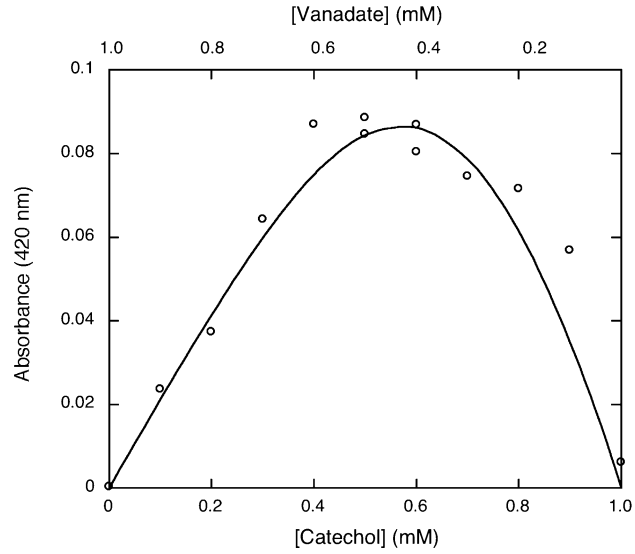


FIGURE 4: Job plot showing the effect of continuous variation of vanadate and catechol concentrations on the absorbance at 420 nm. The points are experimental, and the solid line was calculated as described in the text.

concentration at ~500 s, followed by a slower reaction. Spectra at appropriate times showed that the intermediate was associated with maximal absorption around 420 nm. Decay of this intermediate was correlated with the formation of an absorption peak at 640 nm, indicated by the presence of a blue color, which is known to arise from the formation of vanadium(IV). The final slow reaction was accompanied by the formation of a brown color with associated broad, long wavelength absorption. These observations are qualitatively the same as those reported by Kustin and co-workers, who speculated that the final brown products are quinone-derived polymers (*42, 43*). The reactions leading to these products involved atmospheric oxygen since removal of most of the latter by nitrogen flushing of reaction solutions led to a much slower final phase of reaction. Similar slow reactions were observed with the other catechols, including 4-nitrocatechol and the 2,3-dihydroxyquinones **11** and **12**.

The fast reversible inhibition, however, arises from a species immediately present after manual mixing of vanadate and catechol (5 s), i.e., prior to the reactions described in the previous paragraph. This inhibition, therefore, cannot involve V^{IV}. Indeed, catechol had no effect on the activity of the P99 β-lactamase in the presence of vanadyl sulfate. The absorption spectra mentioned above indicate that the species present immediately after mixing of vanadate and catechol also have absorption at 400−430 nm. Figure 4 shows a Job plot of this absorption (420 nm) versus the continuous variation of vanadate and catechol concentrations. This plot indicates that a simple 1:1 complex cannot alone be responsible for this absorption. The line fitting the data to Scheme 3 suggests that a mixture of 1:1 and 2:1 catechol: vanadate ratios are required. In Scheme 3, V represents vanadate, C catechol, VC a 1:1 complex of V and C, and VC_2 a 1:2 complex.

More quantitative resolution of the kinetics and thermodynamics of the rapid reactions occurring on mixing vanadate and catechol at pH 7.5 was obtained from stopped-flow experiments. Figure 5 shows the results of such experiments in which the absorption increases at 430 nm after rapid

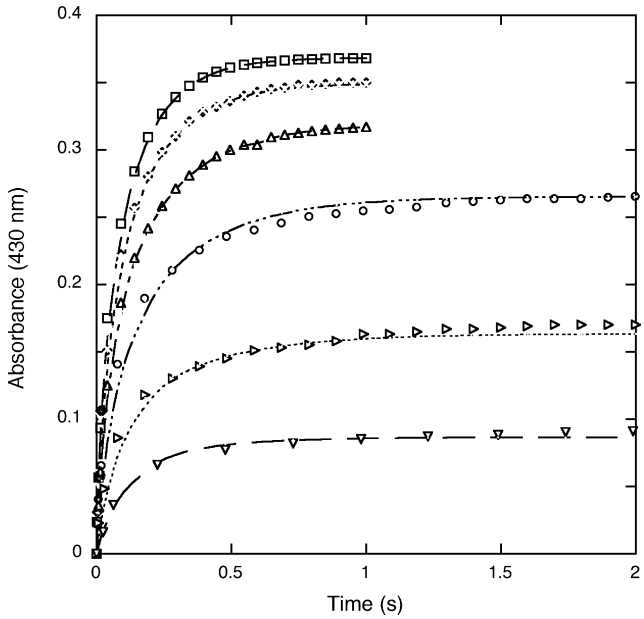


FIGURE 5: Fast absorption changes at 430 nm arising from reactions of vanadate (0.5 mM) and catechol [(▽) 0.5, (right-pointing triangle) 1.0, (○) 2.0, (△) 3.0, (◇) 4.0, and (□) 5.0 mM] at pH 7.5. The points are experimental, and the lines were calculated as described in the text.

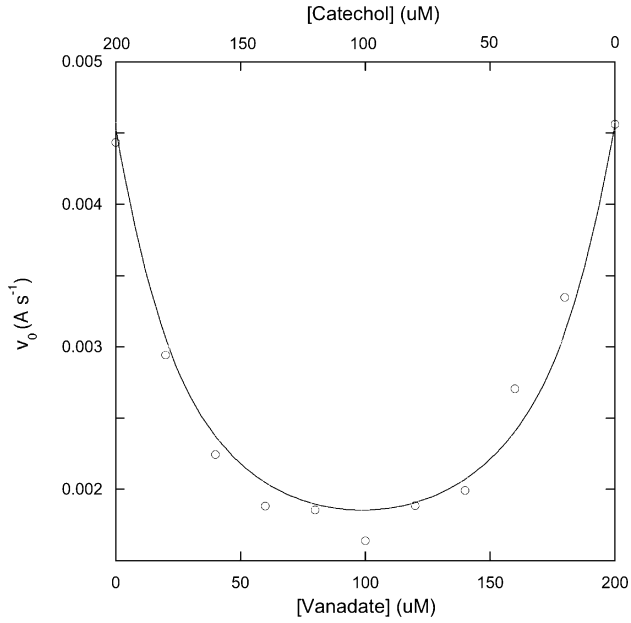


FIGURE 6: Job plot showing the effect of continuous variation of vanadate and catechol concentrations on the initial rates of Centa (100 μM) hydrolysis catalyzed by the P99 β-lactamase. The points are experimental, and the solid line was calculated as described in the text.

Scheme 4

$$V + C \underset{K_1}{\rightleftharpoons} VC$$
$$VC + C \underset{K_2}{\rightleftharpoons} VC_2$$
$$V + V \underset{K_{v2}}{\rightleftharpoons} V_2$$
$$4V \underset{K_{v4}}{\rightleftharpoons} V_4$$
$$E + VC \underset{K_i}{\rightleftharpoons} EVC$$
$$E + S \underset{K_m}{\rightleftharpoons} ES$$
$$ES \xrightarrow{k_{cat}} E + P$$

mixing of various concentrations (0.5−5.0 mM) of catechol with vanadate (0.5 mM) are displayed. These data could not be fitted to a simple scheme involving a single 1:1 complex but could be fitted to Scheme 3. Dissociation constants for VC and VC_2 were 0.32 and 2.29 mM, respectively, with the following rate constants: $k_1 = 9900 \pm 600$ s^{-1} M^{-1}, $k_{-1} = 3.17 \pm 0.15$ s^{-1}, $k_2 = 1050 \pm 35$ s^{-1} M^{-1}, and $k_{-2} = 2.4 \pm 0.1$ s^{-1}.

The corresponding complexes with 2,3-dihydroxynaphthalene were comparably tight ($K_1 = 0.25$ mM, $K_2 = 3.34$ mM), but VC formed more rapidly (Figures S1 and S2): $k_1 = (2.13 \pm 0.02) \times 10^4$ s^{-1} M^{-1}, $k_{-1} = 5.28 \pm 0.05$ s^{-1}, $k_2 = 490 \pm 8$ s^{-1} M^{-1}, and $k_{-2} = 1.64 \pm 0.02$ s^{-1}. In this case, it is very clear that two complexes are formed sequentially because of the greater disparity between k_1 and k_2.

The absorption data of Figure 4 could then be very nicely fitted (solid line) to Scheme 3 with the equilibrium constant values determined above. It appears, therefore, that under the conditions of the β-lactamase inhibition described above, the solutions contain 1:1 and 1:2 complexes of vanadate and catechol as the dominant species.

The amplitude and rate of the third phase of reaction, observed immediately after manual mixing (Figure 3), also increased with catechol concentration (not shown). This suggests that the first intermediate seen in Figure 3 may be a VC_3 complex; such complexes have been prepared and characterized previously (*44*). At least one of the catechol−vanadate complexes must lead to the redox reaction yielding V^{IV}. A complete scheme for the reaction of catechol and vanadate after manual mixing is shown below (Scheme 5); it is possible, however, that VC and/or VC_2 may be the redox active species as well as, or instead of, VC_3.

Nature of the Inhibitor. A kinetics Job plot for the rapid reversible inhibition of the P99 β-lactamase by vanadate−catechol mixtures is shown in Figure 6. The solid line shown as fitting these data derives from Scheme 4, where EVC represents a 1:1:1 inhibitory complex. This result shows that the inhibitor is formally VC, the 1:1 complex of vanadate and catechol. The fits to the data of Figure 6 yielded the true K_i value for the VC complex, 0.53 ± 0.06 μM. Another estimate, 0.51 ± 0.06 μM, could be obtained from the fit of Scheme 4 to the data depicted in Figure 1; these two

Scheme 5

$$V + C \underset{K_1}{\overset{\text{fast}}{\rightleftharpoons}} VC$$
$$VC + C \underset{K_2}{\overset{\text{fast}}{\rightleftharpoons}} VC_2$$
$$VC_2 + C \rightleftharpoons VC_3 \longrightarrow V^{IV}C_n \xrightarrow{O_2} \text{catalytic catechol oxidation}$$

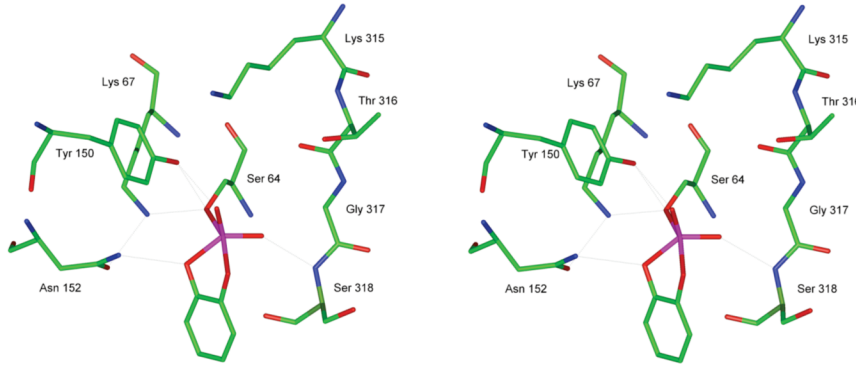
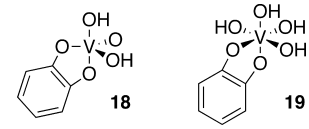


FIGURE 7: Stereoview of an energy-minimized model of the ternary complex of vanadate, catechol, and the active site of the P99 β-lactamase.

estimates are obviously in good agreement. Similar results were obtained for 2,3-dihydroxynaphthalene (Figure S3), and thus estimates of K_i of 0.12 ± 0.01 and 0.15 ± 0.01 μM, respectively, were obtained.

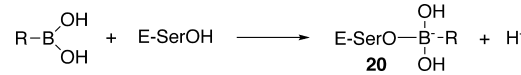
These values are compiled in Table 1 along with similarly derived values for the TEM-2 and OXA-1 β-lactamases. It is clear that these complexes are submicromolar inhibitors of class C and D β-lactamases. Further ligand screening would be needed to obtain an equally good inhibitor of the class A enzyme.

Since the inhibitor is short-lived, it would be difficult at present to characterize it structurally more fully. Crystal structures of various vanadate−catechol complexes invariably show five or six coordination to vanadium with the catechol as a chelating ligand (*20*−*24*). It is likely, therefore, that the major 1:1 complex in solution contains chelated catechol. The geometry at vanadium is also an issue, with coordination numbers between and including 4 and 6 possible. One-to-one complexes of vanadate with hydroxamic acids in neutral aqueous solution contain five- or six-coordinated vanadium (*17, 45, 46*). It is thus quite likely that the 1:1 complexes of vanadate with catechol under these conditions can be represented as **18** or **19**, or anions derived from these. These complexes would be labile, however, so that even if the structure of the dominant complex in solution were known, the inhibitory complex with a β-lactamase may contain a different arrangement of ligands. It is also likely, based on available precedent with boronate (*47, 48*) and vanadate (*46, 49*) inhibitors of hydrolases, that the vanadium is coordinated to the active site serine hydroxyl oxygen atom in the inhibitory complex. A model of such a complex formed from the P99 β-lactamase is shown in Figure 7, where a catechol chelate with five-coordinated vanadium is shown at the active site. This structure includes one vanadyl oxygen in the oxyanion hole, while the other, taking the general position of the leaving group in a tetrahedral transition state, is hydrogen bonded to the side chain hydroxyl group of Tyr150. One catechol oxygen is hydrogen-bonded to the side chain amido nitrogen of Asn152 just as is the side chain amide oxygen of substrates or transition state analogue inhibitors (*14, 16*). The O_γ atom of active site Ser64 is hydrogen-bonded to the Lys67 side chain ammonium group. A 1:1 catechol−vanadate complex can therefore fit well into the active site, although other orientations are possible. A combination of catechol structure−activity studies and further modeling may be able to reduce the number of possibilities.

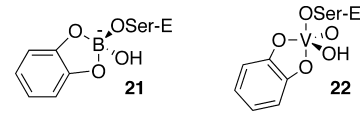


Comparison with Analogous Boric Acid Complexes. Serine β-lactamases are inhibited by boric acid and boronic acids which form rapidly reversible tetrahedral adducts (**20**) at the active site (Scheme 6) (*7*−*10*). These components are

Scheme 6



believed to be effective inhibitors because of the steric and electronic resemblance between **20** and the anionic tetrahedral transition states and intermediates of acyl transfer reactions. Boric acid is known to form chelated complexes with diols, including catechols (*50*−*52*). We were interested in comparing the inhibitory ability of tetrahedral catechol-borate complexes, which may have the structure **21**, with those of vanadates, which are expected to more likely have trigonal bipyramidal **22** or octahedral geometry (see above).



Measurements of inhibition caused by catechol (0−2 mM) in the presence of boric acid (1.0 mM) suggested, assuming formation in solution of an inhibitory 1:1 catechol−borate complex with a dissociation constant of 2.87 mM (*50*), that the K_i value of such a complex would be ~40 μM. This result indicates that a tetrahedral borate complex is considerably weaker as a P99 β-lactamase inhibitor than the 1:1 catechol−vanadate complex (0.53 μM). This discovery is quite striking since tetrahedral adducts, mimicking acyl transfer transition states, are generally very effective inhibitors of acyl transferases. Simple boronates appear to be weaker inhibitors of class A and D β-lactamases than of class C β-lactamases (*53*), so vanadate complexes may well also be stronger inhibitors of these enzymes than would be the corresponding borates.

The results described in this paper show that 1:1 catechol−vanadate complexes, with only minimal structural optimization to date, are quite effective inhibitors of representative examples of all three classes of serine β-lactamases. This discovery extends earlier results with 1:1 hydroxamic acid−vanadate complexes, which appeared to be selective inhibitors of only class C enzymes (*17*). Although the structure of the inhibitory complex in the present case could not be easily studied because of the redox lability of catechol−vanadium(V) mixtures, it is likely, as demonstrated with the hydroxamic acids, that the inhibitor contains penta- or hexacoordinated vanadium derived from addition of the active site serine hydroxyl to vanadium (Figure 7). Indeed, a crystal structure of just such an arrangement with chymotrypsin has been described previously (*44*). The weaker inhibition of a class C β-lactamase by catechol−borate complexes, which would certainly form tetrahedral adducts, than by vanadate−catechol complexes is also evidence of the vanadate inhibitors having higher coordination numbers. Although catechol−vanadate complexes are obviously not practically useful as new β-lactamase inhibitors, they do point toward new arrangements of negatively charged oxygen atoms that can bind tightly to all classes of the serine β-lactamase active site. One such arrangement, already noted, but now extendable, is found in the transition states of covalent phosph(on)ate inhibitors (*12, 17, 46*).

SUPPORTING INFORMATION AVAILABLE

Kinetics of the reaction between vanadate and 2,3-dihydroxynaphthalene at pH 7.5 and inhibition of the P99 β-lactamase by the complex formed. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI801153J