Synthesis and Mechanistic Evaluation of 7-Vinylidenecephem Sulfones as β -Lactamase Inhibitors

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Abstract: Representative 7-vinylidenecephalosporins 1 were synthesized from 7-aminocephalosporanic acid and were biologically evaluated as β -lactamase inhibitors. These chiral allenes were prepared stereospecifically from a cephalosporin-derived propargylic triflate using organocopper reagents. The sodium salts of a few such unsaturated cephalosporanates were evaluated as β -lactamase inhibitors of the type C enzyme derived from Enterobacter cloacae strain P99. One compound, sodium 7-($2'\alpha$ -tert-butylvinylidene)cephalosporanate sulfone (16), was found to be an excellent progressive inhibitor of this enzyme, exhibiting a second-order rate constant of inactivation of $k_{3}' = 1.7 \times$ 10^{6} 1/(mol-min) and a turnover number of 12. A potential mechanism of inhibition was investigated. The corresponding terminally deuterated allene sodium $7-(2'\alpha$ -tert-butyl-2'- β -deuteriovinylidene)cephalosporanate sulfone (21) was prepared and biologically evaluated. The deuterated compound inhibited the enzyme with a rate constant of $k_3' = 2.7 \times 10^5$ 1/(mol·min), representing an isotope effect of 6.3. The deuterated compound had an IC₅₀ value which was approximately twice that of the protio compound, and had a turnover number of 25. A mechanism of inhibition which is consistent with this data was proposed. The mechanism of inhibition involves an acyl enzyme which becomes stabilized toward hydrolysis through its conversion to a vinylogous urethane (β -aminoacrylate). This intermediate is formed by an elimination reaction which transforms the allene into an enyne. The inhibition disappears extremely slowly, presumedly due to hydrolysis of the stabilized acyl enzyme. This pattern of reactivity is further confirmed by a ¹H NMR study of the nonenzymatic hydrolysis of the inhibitor under basic conditions. A second type of enzymatic inhibition, which does not disappear with time, was also observed. This second (irreversible) type of inhibition required longer incubation times and higher ratios of inhibitor to enzyme and showed no isotope effect.

Introduction

Bacteria possess an astounding ability to develop and transfer resistance to antimicrobial agents. "There are countless antibiotics: more than 50 penicillins, 70 cephalosporins, 12 tetracyclines, 8 aminoglycosides, 1 monobactam, 3 carbapenems, 9 macrolides, 2 new streptogramins, and 3 dihydrofolate reductase inhibitors.¹ Despite all these antibiotics, a person could die in a hosptial in New York, San Francisco, Paris, Barcelona, Tokyo, or Singapore as a result of a resistant bacterial infection."² In 1941, penicillin G could kill almost all strains of *Staphylococcus aureus*. By 1944, however, *S. aureus* had developed a penicillinase capable of destroying penicillin, and by 1946, it was estimated that 60% of hospital isolates in the United Kingdom were penicillin resistant.³ Today more than 95% of these strains worldwide are penicillin and ampicillin resistant.

The most important mechanism of microbial resistance to β -lactam antibiotics is the bacterial production of β -lactamases, enzymes which hydrolytically destroy β -lactam antibiotics. This type of resistance can be transferred horizontally by plasmids that are capable of rapidly spreading the resistance—not only to other members of the same strain but *even to other species*. Due to such rapid gene transfer, a patient can become infected with different organisms, each possessing the same β -lactamase.

 β -Lactamase enzymes have been organized into four molecular classes (A, B,⁴ and D⁶) based on amino acid sequence. Alternative organizational schemes emphasize biological activity associated with the enzymes and divide the enzymes into groups (types 1-47 or I-IV⁸). Class A, which includes RTEM and the β -lactamase of S. aureus, class C, which includes the lactamase derived from P99 Enterobacter cloacae, and class D are serine hydrolases. Class A enzymes have a molecular weight of about 29 000 and preferentially hydrolyze penicillins. The class B lactamases are metalloenzymes and have a broader substrate profile than the proteins in the other classes. Class C enzymes include the chromosomal cephalosporinases of Gramnegative bacteria and have molecular weights of approximately 39 000. The recently recognized class D enzymes exhibit a unique substrate profile which differs significantly from those of both class A and class C.

One strategy for overcoming this rapidly evolving bacterial resistance is the synthesis and administration of β -lactamase inhibitors. Reviews of β -lactamase inhibition are available.⁹ Frequently, β -lactamase inhibitors do not possess antibiotic activity themselves and are thus administered together with an

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⁽⁵⁾ Jaurin, B.; Grundstrom, T. Proc. Natl. Acad. Sci. U.S.A. 1981, 78, 4897 (defines class C).

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⁽⁸⁾ Skyes, R. B. J. Infect. Dis. 1982, 145, 762.

antibiotic. One example of such a synergistic mixture is "augmentin", which contains the antibiotic amoxicillin and the β -lactamase inhibitor clavulanic acid.

The class C cephalosporinases, in particular, are responsible for the resistance of Gram-negative bacteria to a variety of both traditional and newly designed antibiotics.¹⁰ The *E. cloacae* species, which possesses a class C enzyme, is now the third greatest cause of nosocomial infections in the United States. This class of enzymes often have poor affinities for inhibitors of the class A enzymes, such as clavulanic acid, a commonly prescribed inhibitor, and common *in vitro* inactivators, such as 6β -iodopenicillanate.¹¹

Several years ago, we prepared a β -lactam with an exocyclic allene fused to the α -position (i.e., an α -vinylidene- β -lactam).¹² Intrigued by the possibility that this previously unknown substructure might have medicinal value, we proceeded to incorporate such an exocyclic allene into the 6-position of the penicillin nucleus.¹³ Recently, we described the preparation of the sodium 6-(2' α -tert-butylvinylidene)penicillinate sulfone and briefly described its biological activity as a potent inhibitor of the type C β -lactamase derived from *E. cloacae* strain P99.¹⁴ We would now like to report the synthesis of representative 7-vinylidenecephems **1** as well as the biological evaluation and



detailed mechanistic examination of sodium 7-(2' α -tert-butylvinylidene)cephalosporanate sulfone, a potent inhibitor of this enzyme. We will demonstrate that 7-(2' α -tert-butylvinylidene)cephalosporanate sulfone has a large second-order rate constant of inhibition of this type C β -lactamase. In a deuterium labeling experiment and by a ¹H NMR analysis, we will investigate a potential mechanistic pathway for this inhibition.

Results

Synthesis. The 7-vinylidenecephems were prepared by modifying the synthetic procedures we had developed in the penicillin series. As shown in Scheme 1, chiral 7-aminocephalosporanic acid (2) was esterified with dipehnyldiazomethane.¹⁵ Treatment with excess triethylamine and trifluoromethane-sulfonic anhydride, followed by hydrolysis of the resultant trifluoromethanesulfonyl imine, produced benzhydryl 7-oxocephalosporanate¹⁶ (4). Due to its instability, this ketone was used without further purification. The reaction between 4 and ethynylmagnesium bromide stereospecifically produced the corresponding propargylic alcohol 5. Conversion to the triflate 6 with trifluoromethanesulfonic anhydride, followed by careful treatment with either $(t-C_4H_9)_2$ CuCNLi₂ or copper(I) bromide,

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yielded either benzhydryl 7-(2'a-tert-butylvinylidene)cephalosporanate (7) or benzhydryl 7-(2' α -bromovinylidene)cephalosporanate (8), respectively. The synthesis of these chiral allenes proceeded with 100% stereospecificity via an anti S_N2' displacement of the leaving group.¹⁷ Reduction of 8 with a Zn-Cu couple produced the parent terminally unsubstituted benzhydryl 7-vinylidenecephalosporanate (9), as shown in Scheme 2. Deprotection of compounds 7-9 produced the corresponding sodium salts 13-15, respectively. Sulfones 10-12 were synthesized by oxidation of 7-9 with excess mchloroperbenzoic acid (m-CPBA). Sodium salts 16 and 17 could be obtained by the deprotection of sulfones 10 and 12, respectively. However, deprotection of 11 under the same conditions failed to produce the corresponding carboxylate salt 14. The results are summarized in Scheme 3. The terminally deuterated analog of 16, 7-(2' β -deuterio-2' α -tert-butylvinylidene)cephem sulfone 21, was made in a similar fashion from dideuterioacetylene.

IC₅₀ Determination. The aforementioned 7-vinylidenecephems were evaluated as inhibitors of the β -lactamase of *E. cloacae* P99. The IC₅₀ value of each compound was determined as follows. Following a 10 min incubation of a dilute solution of enzyme (2.56 nM) and inhibitor (<0.64 μ M), a 50 μ L aliquot of this incubation mixture was then further diluted into 1 mL of nitrocefin solution, and the rate of hydrolysis was measured during a 1 min period by monitoring the absorbance of nitrocefin as a function of time. The 7-vinylidenecephems were evaluated relative to known inhibitors tazobatam, clavulanic acid, and 6-(2' α -tert-butylvinylidene)penam sulfone. The data are presented in Table 1. All of the synthesized compounds compared

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C.; Isom, H.; Siriwardane, U. J. Org. Chem. 1993, 58, 1325.
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⁽¹⁷⁾ The configurational assignment was made by analogy with the corresponding reaction in the penicillin series, which was established by X-ray crystallography (see ref 14).

| Table 1. | PP99 | β -Lactamase | Inhibitory | / Activity |
|----------|------|--------------------|------------|------------|
|----------|------|--------------------|------------|------------|

| compound | IC ₅₀ (µg/mL) |
|---|--------------------------|
| 7-(2'a-tert-butylvinylidene)cephem 13 | 3.91 |
| 7-(2'a-tert-butylvinylidene)cephem sulfone 16 | 0.05 |
| 7- $(2'\alpha$ - <i>tert</i> -butyl- $2'\beta$ -deuteriovinylidene)cephem sulfone 21 | 0.10 |
| 7-vinylidenecephem 15 | 25.83 |
| 7-vinylidenecephem sulfone 17 | 0.11 |
| $6-(2'\alpha$ -tert-butylvinylidene)penam sulfone | 0.39 |
| 7- $(2'\alpha$ -bromovinylidene)cephem 14 | 6.16 |
| tazobactam | 0.22 |
| clavulanic acid | 17.24 |

Scheme 3



favorably to clavulanic acid. $7-2'\alpha$ -tert-butylvinylidene)cephem sulfone **16** was found to be the most potent inhibitor, approximately 5-fold better than tazobactam and 350-fold better than clavulanic acid. Furthermore, **16** was 8-fold more effective than its penam analog, and 80-fold more potent than the corresponding $7-(2'\alpha$ -tert-butylvinylidene)cephem sulfide **7**. Decomopsition of these inhibitors in the buffer solution may account for some of the trends in biological activity. For example, while the most active inhibitor **16** was unchanged in solution for at least 24 h, the unsubstituted allene sulfone **17** was found to be unstable in solution with a 10% decrease in inhibitory activity after 1 h, and marked decomposition in D₂O buffer by ¹H NMR.

Rates of Inactivation. Since the 7- $(2'\alpha$ -*tert*-butylvinylidene)cephem sulfone **16** showed excellent inhibition by this preliminary screening technique, we decided to study it in further detail. A simple kinetic model for this inhibition is shown below and can be analyzed by the method of Kitz and Wilson.¹⁸

$$\mathbf{E} + \mathbf{I} \underbrace{\stackrel{k_1}{\longrightarrow}}_{k_{-1}} \mathbf{E} \cdot \mathbf{I} \stackrel{k_3}{\longrightarrow} \mathbf{E} - \mathbf{I} \stackrel{k_4}{\longrightarrow} \mathbf{E} + \mathbf{I}' \tag{1}$$

$$\ln \frac{[\epsilon]}{[E_{o}]} = \frac{-k_{3}t}{1 + K_{f}/[I]} = -k_{app}t$$
(2)

$$\frac{1}{k_{\rm app}} = \frac{1}{k_3} + \frac{K_{\rm I}}{k_3} \frac{1}{[{\rm I}]}$$
(3)

E represents the β -lactamase, I the inhibitor E-I the reversible Michealis complex, E-I a stabilized intermediate, and I' the hydrolyzed inhibitor. ϵ corresponds to potentially active enzyme

Table 2. Rate Constants of Inhibition

| compound | k_3' (1/(mol·min)) |
|--|--|
| 7-(2'α-tert-butylvinylidene)cephem sulfone 16 7-(2'α-tert-butyl-2'β-deuteriovinylidene)cephem sulfone 21 tazobactam clavulanic acid | 1.7×10^{6} 2.7×10^{5} 7.4×10^{4} 799 |

(all forms of the enzyme except E–I) that are capable of hydrolyzing a substrate upon dilution of the enzyme—inhibitor solution into the substrate solution. k_4 is assumed to be very small. In contrast to the rapdily equilibrating character of a reversible inhibitor, an irreversible (or a very tightly bound) inhibitor is expected to reduce the amount of potentially free enzyme, ϵ , as an easily measurable function of incubation time at each prespecified inhibitor concentration. This type of increasing inhibition has been called "progressive". A rate constant for this change, k_{app} , can then be obtained at each inhibitor concentration by plotting the $\ln([\epsilon]/[E_o])$ vs time.¹⁹ As shown by eq 3, a second plot of the values of $1/k_{app}$ vs 1/[I] is expected to be a straight line with an intercept near the origin (actually the intercept will be $1/k_3$, but this is not an accurate method for the determination of this value).

The enzyme was incubated with five or more different concentrations of inhibitor. At each inhibitor concentration, the activity was regularly assayed and plotted as a function of time (for a 50 min period) by periodically diluting the enzymeinhibitor mixture into nitrocefin (substrate) solution, and continuously monitoring the rate of hydrolysis of this substrate (by measuring the change in the absorbance at 481 nm) for 30 s. The change in absorbance for 30 s was linear, the slope being directly proportional to residual active enzyme, ϵ . At each inhibitor concentration, the amount of inhibition increased with incubation time (during this 50 min period) and a linear relationship between time and $\ln([\epsilon]/[E_o])$ was obtained, indicating progressive inhibition. The (inhibitor) concentrationdependent values of k_{app} were then determined from the slopes of these lines and $1/k_{app}$ plotted vs 1/[I] according to eq 3 to produce a straight line with a slope of K_{I}/k_{3} . Following the convention of Kitz and Wilson, it is assumed that [I] $\ll K_{\rm I}$, in which case eq 2 becomes $k_{app} = (k_3/K_I)[I] = k_3'[I]$. k_3' is a pseudo-second-order rate constant of inhibition and is inversely related to the slope of the line in this second plot. To standardize our data, the second-order rate constant for several known inhibitors were obtained. A graphical analysis of the results with 16 is shown in Figure 1. Comparison with other known inhibitors is shown in Table 2.

In the case of 16, a reasonably straight line was obtained (from the plot of $1/k_{app}$ vs 1/[I]) which intercepted the y-axis near the origin, indicating that k_3 is relatively large.²⁰ This suggests either an irreversible or an extremely tightly bound, very weakly reversible complex. The pseudo-second-order rate constant of inhibition (k_3') for inhibition of this β -lactamase by 16 was determined to be $1.7 \times 10^6 1/(\text{mol-min})$.

Deuterium Isotope Effect. Knowles and Brenner employed 6,6-dideuterio derivatives of penicillinic acid sulfone to investigate the mechanistic pathway of the interaction of the sulfone with RTEM β -lactamase.²¹ Suspecting a somewhat analogous mechanism of action for **16**, we prepared 7-(2' α -tert-butyl-2' β -deuteriovinylidene)cephem sulfone **21**. We were able to

⁽¹⁸⁾ Kitz, R.; Wilson, I. B. J. Biol. Chem. 1962, 237, 3245.

⁽¹⁹⁾ This corresponds to the ratio of their respectives rates of hydrolysis of substrate, since both $[\epsilon]$ and $[E_0]$ are linearly related to the rate of substrate hydrolysis at a constant substrate concentration.

⁽²⁰⁾ This is not, of course, an accurate method for the determination of k_3 .

⁽²¹⁾ Knowles, J. R.; Brenner, D. G. Biochemistry 1981, 20, 3680.



Figure 1. Inhibition of *E. cloacae* P99 β -lactamase by the vinylidenecephem sulfone 16. Panel A shows the progressive inhibition in which enzyme and inhibitor were incubated at 25 °C (at various concentrations) for the times indicated before dilution into nitrocefin solution. The final assay concentration of the enzyme was 2.56 nM. Inhibitor concentrations in the assay were (\blacksquare) 6.4×10^{-7} M, (\blacklozenge) 1.28×10^{-7} M, (\bigstar) 6.4×10^{-8} M, and (\diamondsuit) 3.2×10^{-8} M. Panel B shows the plot of the reciprocals of these concentration-dependent constants vs the reciprocal of inhibitor concentrations for the same compound. k_3' was determined by linear regression to be 1.7×10^6 1/(mol-min).



Figure 2. Double reciprocal plots for both 7-(2' α -tert-butylvinylidene)cephem sulfone 16 and its 2' β -deuterated counterpart 21 showing the marked difference in rates. k_{app} is derived from the slope of the progressive plots as shown in Figure 1. The rate constant of the deuterated compound $k_{3'} = 2.7 \times 10^5$ 1/(mol·min). This corresponds to a deuterium isotope effect of $k_b/k_d = 6.3$.

synthesize the deuterated compound with greater than 95% isotopic purity. To ensure the validity of the subsequent kinetic study, it was important to establish the purity of the carboxylate salt 21. Equimolar solutions of both the protio (16) and deuterio (21) compounds had identical UV spectra (and extinction coefficients) and identical ¹H NMR spectra (and signal to noise ratios after 16 scans), with, of course, the notable absence of the allenic proton.

When a Kitz and Wilson analysis of **21** was performed, a marked deuterium isotope effect was observed (Figure 2). The rate constant for inhibition of **21**, as calculated above, was $k_3' = 2.7 \times 10^5$ 1/(mol·min). This represents a 6-fold decrease in



Figure 3. Reactivation of concentrated I/E ratios. Incubation enzyme concentrations were 43 μ M. Final inhibitor concentrations were adjusted to (\Box) 1/1, (\blacklozenge) 2/1, (\blacktriangle) 3/1, (\blacklozenge) 4/1, and (\blacksquare) 5/1. The final enzyme concentration (in the assay) was 0.01 μ M, representing a 4000-fold dilution.

the rate of inhibition relative to 16. In an IC₅₀ analysis, with a 10 min incubation time, the protio compound 16 was found to be about twice as potent as the deuterated compound 21.

Turnover Ratio. One measure of inhibitory potency that is frequently reported is the turnover number, also called the partition ratio. This ratio corresponds to the number of molecules of inhibitor necessary to deactivate a single molecule of enzyme. For an ideal inhibitor, the value would be unity. In fact, this ratio may not be a constant, but may instead depend on the incubation time of enzyme and inhibitor. Thus, in the case of 16, we studied the time dependence of the inhibition. In contrast to the Kitz and Wilson analysis reported above, this investigation was performed at low molar ratios of inhibitor to enzyme (from 1/1 to 5/1) and in solutions with very high (43 μ M) concentrations of both enzyme and inhibitor.²² Data are obtained by periodically removing a small aliquot of the incubation mixture, extensively diluting it (4000-fold), and treating it with substrate to determine the amount of active enzyme as above. The progress curves at various ratios of inhibitor to enzyme are shown in Figure 3. Notice that, at these low ratios of inhibitor to enzyme, a very slow reactivation can be observed. However, in the case of I/E = 10, less than 25% of activity is recovered even after 28 h. If the incubation is conducted at high dilution, the turnover of inhibitor is even slower. For example at 0.05 μ M and an I/E ratio of 20, less than 4% activity was recovered even after 2.5 days.

Ideally, one should be able to graph the function of enzyme activity remaining vs the molar ratio of inhibitor to enzyme. For an irreversible inhibitor, the plot is usually linear, although reasons for deviations from linearity at extremely low values of I/E have been discussed.²³ One should be able to read the turnover number (the number of equivalents of inhibitor necessary for zero activity) from the x-axis intercept of such a plot. In the case of 16, this quantity clearly depends on whether the incubation solutions were assayed at 90 min or 15 h. As shown in Figure 4, two different values of approximately 6 and 12 (molecules of inhibitor per molecules of enzyme) were generated at these two times, respectively. The 15 h value of 12 was found to be independent of incubation concentration. A corresponding study of the deuterated compound 21 was performed (Figure 5). Table 3 compares the partition coefficients of 16, 21, tazobactam, and clavulanic acid with this lactamase. Note that there is a substantial difference in the turnover ratio of the deuterated and protonated compounds.

⁽²²⁾ In contrast to the Kitz and Wilson study discussed earlier which was performed with relatively large values of I/E.

⁽²³⁾ Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. J. Biol. Chem. 1983, 258, 15046.



Figure 4. Partition coefficient studies at two incubation times carried out as detailed in Figure 3. Set A shows the remaining activity at various inhibitor/enzyme ratios after 90 min of incubation. Set B shows results after 15 h of incubation. The partition coefficient is extrapolated from the linear section of these plots. At 90 min, the partition coefficient is approximately 6, and at 15 h, thepartition coefficient is approximately 12.



Figure 5. Partition coefficient studies of the deuterated (21) (\blacksquare) and protonated (16) (\bullet) compounds after a 15 h incubation. Set A shows the remaining activity at various inhibitor/enzyme concentrations for compound 16, and set B shows the results for the corresponding deuterated compound 21. The partition coefficient of the deuterated compound was found to be approximately 25. Incubation enzyme concentrations were 43 μ M. The final enzyme concentration was 0.01 μ M.

| compound | partition coefficient |
|---|--------------------------|
| 7-($2'\alpha$ - <i>tert</i> -butylvinylidene)cephem sulfone 16 | 12 |
| 7-($2'\alpha$ - <i>tert</i> -butyl- $2'\beta$ -deuteriovinylidene)cephem sulfone 21 | 25 |
| tazobactam | 50° |
| clavulanic Acid | >500 000° |

^a Reference 30.

Gel Filtration. The most demanding test of irreversibility consists of separating the inhibited enzyme from excess inhibitor by gel filtration and monitoring for reactivation. In the gel filtration experiments, enzyme was incubated with specific amounts of inhibitor (I/E = 20/1 to 1000/1) for varying amounts of time (1-1800 min). To remove excess inhibitor, the enzyme-inhibitor solutions were then placed on a Sephadex G-25 column at room temperature. Incubation enzyme concentrations were on the order of 43 μ M. After gel filtration, fractions containing enzyme, as determined by UV analysis, were combined. As a control, an identical solution of enzyme itself (without inhibitor) was subjected to gel filtration, and the same fractions were combined. In the case of the inhibited enzyme, good separation of inhibited enzyme, E-I, from inhibitor was demonstrated by recording an ultraviolet spectrum of each fraction.

Initial experiments were performed with a 2 h (enzyme and inhibitor) incubation time. As shown in Figure 6, these experiments were performed at high concentration and at four



Figure 6. Remaining activity of fully deactivated enzyme after gel filtration as a function of time. The enzyme was incubated with varying amounts of inhibitor **16** for 2 h, after which the mixture was subject to gel filtration, and the recovery of activity as a function of time was monitored. Incubation enzyme concentrations were 43 μ M. Inhibitor concentrations were adjusted to I/E = (\blacksquare) 20/1, (\blacklozenge) 100/1, (\blacktriangle) 500/1, and (\diamondsuit) 1000/1.



Figure 7. Remaining activity (log scale) as a function of incubation time for compound 16, set A (\blacksquare) , and its deuterated analog 21, set B (\bullet) . A solution of enzyme (43 μ M) in excess inhibitor (500/1 as detailed in Figure 6) was incubated for various amounts of time, after which the mixture was subject to gel filtration chromatography (and consequent removal of excess inhibitor) and allowed to recover any remaining activity for 24 h prior to the assay. The slopes are very similar, indicating the absence of a primary deuterium isotope effect for irreversible inhibition.

different ratios of inhibitor to enzyme. After gel filtration, the inhibited enzyme was capable of partially recovering activity at a very slow rate. Twenty-four hours after gel filtration, it was found that the solution incubated at I/E = 100/1 had recovered 80% of its activity and the solution with I/E = 500/1 had recovered 45%. At I/E = 20/1, after gel filtration, the ln- $([\epsilon]/[E_o])$ is plotted vs time. The data obtained are approximately linear for the first hour,²⁴ producing a first-order rate constant of *reactivation* $k_{act} = 4.8 \times 10^{-4} \text{ s}^{-1}$.

An incubation study at i/e = 500:1 was carried out on both 7-(2' α -tert-butylvinylidene)cephem sulfone 16 and its deuterated analog 21 to determine the first-order rate constant of irreverible inactivation. During incubation of the inhibitor-enzyme solution, aliquots of the incubation mixture were removed at specified intervals. For each aliquot, the inhibited enzyme was separated from excess inhibitor by gel filtration through identical Sephadex columns and the combined enzyme fractions allowed to stand for >24 h to allow for possible turnover of bound inhibitor. As shown in Figure 7, the large number of manipulations necessary for each data point (including individual gel filtration and recombination of fractions) introduces a relatively large error. Despite this, it was found that irreversible inhibition increases progressively with incubation time at approximately the same rate for the deuterated and nondeuterated compounds. (Despite the large deviation from linearity, an isotope effect as small as 2 would have been easily visible from this data.) After

⁽²⁴⁾ Reactivation slowed considerably after the first hour.

9 h of incubation, and 24 h after gel filtration, both compounds 16 and 21 had regained 35% of the control activity. By plotting the $\ln(\epsilon)/[E_0]$ vs time, the first-order rate constant for irreversible inactivation was determined to be $k_{\text{inact}} = 1.7 \times 10^{-3} \text{ min}^{-1}$ for both 16 and 21.

Spectrophotometric Studies. Attempts to observe new chromophores associated with the inhibited species were hampered by the large and broad UV absorptions of the inhibitor itself (maxima at 229, 264, and 293 nm) which extend to nearly 350 nm. A related problem in detecting hydrolysis products of the inhibitor (i.e., turnover of inhibitor) by UV was the high efficiency and low turnover number of this inhibitor.

Careful (nonenzymatic) hydrolysis of the inhibitor with NaOD in D₂O produced a new chromophore at $\lambda_{max} = 325$ nm. ¹H NMR analysis of this hydrolysis reaction revealed a reasonably clean spectrum, with new absorptions at $\delta = 7.60$ (s, <1H), 5.84 (br s, <1H), 5.69 (br s, <1H), 1.73 (s, 3H), and 1.14 (s, 9H). Integrations of less than one proton may be attributed to partial exchange with solvent. A corresponding reaction with hydrazine produced a similar spectrum.

Removal of excess inhibitor by gel filtration allowed us to obtain a difference ultraviolet spectrum [i.e., (enzyme + inhibitor) - enzyme]. A spectrum of the combined fractions (after gel filtration) of the enzyme-inhibitor complex, minus the combined fractions of native enzyme (control, also after gel filtration), produced new maxima at 240 and 310 nm.

Micellaneous Studies. Frequently, enzymes which have been inhibited by virtue of their conversion to a stabilized acyl enzyme may be reactivated by treatment with a potent external nucleophile. In the case of inhibition by 16, however, it was found that addition of excess hydrazine did not accelerate the recovery of activity of inhibited mixtures. However, this mechanistic data cannot be used to exclude acyl enzyme intermediates since the active site of P99 β -lactamase has been found to be resistant to external nucleophiles.²⁵

Discussion

The β -lactamase derived from E. cloacae strain P99 is a serine hydrolase. The crystal structure of this enzyme has recently been resolved to 2.0 Å.²⁶ The class C β -lactamase of Citrobacter freundii, which exhibits 75% sequence homology with P99, has also been resolved.²⁷ Serine-64 has been found to be the active site amino acid of importance. The crystal structures indicate a significant empty space near the cephalosporins's 7-position, potentially explaining the tolerance of the enzyme for the rigid and bulky tert-butylvinylidene substituent of 16.

A significant difference also exists in the kinetics of β -lactam hydrolysis between class A and class C β -lactamases. For the class C enzymes, hydrolysis of the acyl enzyme (deacylation) appears to be rate determining, while for the class A enzymes, acylation itself is rate determining.

Pratt has recently reported a stoichiometric inhibition of the P99 enzyme with phosphonate monoesters.²⁸ Bush et al. analyzed extremely efficient inhibition of cephalosporinases with the monophosphams.²⁹ A similar analysis of the inhibition of this enzyme and enzymes from other classes with tazobactam has recently appeared.30

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Scheme 4



Several previous mechanistic studies on β -lactamase inhibition and studies on the biological chemistry of cephalosporins are relevant to the work presented in this paper. These include Knowles' study of the mechanism of action of penicillanic acid sulfone (sulbactam.)³¹ Pratt's study of the hydrolysis of cephalosporins by RTEM-2 and E. cloacae P99,³² and Doherty's inhibition of human leukocyte elastase by esters of C-7substituted cephalosporin sulfones.33

Brenner and Knowles' proposed mechanism for the inhibition of RTEM β -lactamase with penicillanic acid sulfone is shown in Scheme 4. The key intermediate is the imine which is created (after formation of the acyl enzyme) through cleavage of the carbon-sulfur bond. This intermediate is then partitioned in three directions: irreversible inactivation through transimination with the enzyme, turnover through deacylation, and reversible inhibition through tautomerization to a stabilized β -aminoacrylate (vinylogous urethane). Deuteration of the 6-position slows the rate of tautomerization of the imine to the enamine and thereby increases both the amount of irreversible inhibition and the amount of hydrolysis.

Pratt and Faraci studied the hydrolysis of cephalosporin sulfides by E. cloacae P99 and TEM-2. They discovered a transient inhibition ($t_{1/2} = 10$ min) of PC1 β -lactamase. The UV absorption associated with the stabilized intermediate was consistent with the formation of the 3-exo-methylenecephem as shown in Scheme 5. This intermediate is hydrolyzed (deacylated) 1000 times slower than its precursor.

Doherty et al. demonstrated that elastase could be inhibited by cephalsporin sulfone esters. The mechanism proposed is shown in Scheme 6.34 Crystallographic evidence has been

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Scheme 5



Scheme 6



obtained for the key bound intermediate involving an attack of a histidine residue.

The preliminary kinetics presented above provides evidence for at least two separate types of inhibition. For example, while 16 and its deuterated analog 21 are clearly different in the IC_{50} analysis, the Kitz and Wilson analysis, and exhibit a different partition ratio, they are similar in the gel filtration study. It is important to note that demonstration of irreversible inhibition (after Sephadex filtration) required relatively concentrated samples and longer incubation times (>2 h) while the comparison of 16 with other known inhibitors (to obtain IC_{50} values) is conducted at higher dilution and shorter incubation times (i.e., 10 min to 1 h). One type of inhibition occurs very rapidly (even in highly dilute inhibitor-enzyme solutions), disappears very slowly with time, and is dramatically affected by deuteration of the allene. A second type of inhibition occurs slowly, requires high concentrations of inhibitor, does not disappear with time, and is not affected by deuteration of the allene. In the more rapid type of inhibition, the observed deuterium isotope effect on the rate of inhibition for 7-(2' α -tert-butyl-2' β deuteriovinylidene)cephem sulfone 21 was $k_h/k_d = 6.3$ (using the Kitz and Wilson plots). This large value obviously represents a primary isotope effect. In the slow type of inhibition, no significant isotope effect was observed.

Any mechanism proposed for the rapid type of inhibition must account for the large isotope effect at the terminal allenyl position as well as for the fact that the sulfone is substantially more active (78-fold) than the sulfide. In addition, it should be noted that the corresponding $6-(2'\alpha$ -tert-butylvinylidene)penam sulfone is also active against this enzyme (approximately 8-fold less active than the cephem). Two potential mechanistic pathways leading to stabilized intermediates are shown in Scheme 7. Note that both pathways demand the elimination of the terminal allenic proton as the rate-determining step (as required by the observation of such a large isotope effect). A good candidate for the stabilized, rapidly formed (but slowly hydrolyzed) intermediate is structure **22**.

In order to establish that the exocyclic allene is necessary for biological activity, we have independently prepared and investigated the biological activity of the corresponding 7-unsubstituted cephalosporanic acid sulfone **25** as well as the 7-(*tert*butylmethylene)cephalosporin sulfone **26**. Both of these materials are at least 1000 times less potent than **16** as inhibitors of this lactamase.³⁵ Thus, all data indicate that the exocyclic allene is a mechanistically important feature of this inhibitor.



The NMR studies provide further corroboration of the proposed mechanism of inhibition. As mentioned earlier, the ¹H NMR of the hydrolyzed inhibitor shows absorptions at $\delta =$ 7.60 (s, <1H), 5.84 (br s, <1H), 5.69 (br s, <1H), 1.73 (s, 3H), and 1.14 (s, 9H), with partial exchange (with the deuterated solvent) under the basic conditions potentially responsible for integrations of less than a full proton. (In fact, in buffered D₂O itself, this cephalosporin sulfone rapidly exchanged 2-position protons with the deuterated solvent, thus accounting for their absence in the NMR spectrum.) The absorptions at 5.84 and 5.69 are assigned to a 3'-exocyclic methylene. This is in full agreement with the absence of absorptions corresponding to the 3-position acetoxymethylene (CH₂OAc) and the upfield shift of the acetoxy methyl absorption $(OC(O)CH_3)$, indicating the production of sodium acetate. The tert-butyl absorption of the hydrolyzed material has been shifted 0.17 ppm downfield from that of the inhibitor itself. This is precisely the direction and magnitude anticipated for a change from a vinylic tert-butyl group to an acetylenic *tert*-butyl group.³⁶ Finally, the absence of the 6-position (ring juncture) proton and appearance of a new signal at $\delta = 7.60$ indicates that the sulfone has indeed departed and the β -aminoacrylate (analogous to 22) has formed.

At present, we have less evidence for the mechanism of irreversible inhibition. By analogy with the work of Doherty, the irreversibly inhibited intermediate could form by addition of an external nucleophile to 22 as shown in Scheme 8. However, the published details of the crystal structure of the P99 β -lactamase indicate no histidine nearby, although there are other aminoa cids in the vicinity which could serve as potential nucleophiles.

Summary

In summary, we have prepared a highly potent, unique inhibitor of the β -lactamase derived from *E. cloacae* P99. Compound **16** progressively inhibited P99 and showed a very high second-order rate constant of inactivation, comparing favorably to other known inhibitors such as 6β -iodopenicillanic acid, the monophosphams, and tazobactam. Two types of inhibition were observed. One type was a highly efficient inhibition which displayed a substantial isotope effect upon deuteration of the terminal allenyl position. At low inhibitor/ enzyme ratios, a portion of this inhibition disappeared at a very slow rate. We have proposed a mechanism of inhibition which is consistent with this data. Secondly, irreversible inhibition of this enzyme could be observed at higher concentrations of inhibitor and longer incubation times. No isotope effect was observed for the irreversible inhibition.

The role of the allene in our proposed mechanism of inhibition is not that of a simple Michael acceptor. Instead, the unsaturation serves to increase the acidity of the terminal allenyl proton, leading (after formation of the acyl enzyme) to an elimination which eventually converts the allene to an enyne (and the acyl enzyme to a vinylogous urethane) which is stabilized toward hydrolysis. As expected, this step shows a substantial isotope effect upon replacement of the terminal

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Scheme 8



allenic proton with deuterium. The elimination and conversion to the enyne are possible only after the opening of the β -lactam through the formation of the acyl enzyme.

Experimental Section

All assays of β -lactamase activity were performed on a Beckman DU-70 spectrophotometer, and hydrolysis rates of β -lactamase substrate, nitrocefin, were monitored at 482 nm. Nitrocefin was purchased from Becton Dickinson Microbiology Systems (Cockeysville, MD). Melting points were uncorrected and determined on a MEL-TEMP capillary melting point apparatus. Infrared spectra were recorded on a Perkin-Elmer Model 710B diffraction grating spectrophotometer or a Perkin-Elmer 1600 Series Fourier transform infrared spectrophotometer. ¹H and ¹³C NMR spectra were recorded on a Bruker WP200SY spectrometer. Proton chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane (0.0). Carbon chemical shifts are reported in parts per million (δ) by using chloroform-d (77.0) as the reference. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Mass spectral data were obtained by FAB techniques from the Midwest Center for Mass Spectrometry at the University of Nebraska-Lincoln, Lincoln, NE. Thin layer chromatography (TLC) was performed on Merck's 0.2 mm Kieselgel 60 F254 silica-coated aluminum plates. The compounds were identified in one or more of the following manners: UV (254 nm), iodine chamber, and/or phosphomolybdic acid spray reagent. The positions of the compounds on the TLC plate are listed as R_f values in the given solvent(s). Flash chromatography was performed by using thick-walled glass columns and Merck's 0.040-0.063 mm Kieselgel 60 silica gel. Reversed-phase chromatography was performed using preparative layer plates purchased from Analtech (RPS-F, 1000 µm). The chromatography solvents were distilled from calcium hydride before use. All additional solvents were obtained from Aldrich in Sure-Seal bottles.

E. cloacae P99 β -lactamase was pruchased from the Center for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.). Tazobactam and clavulanic acid were obtained from American Cyanamid Co. and SmithKline Beecham Pharmaceuticals, respectively. All other reagents were used as received from Aldrich unless otherwise noted. Unless otherwise specified, all yields refer to the isolation of purified material (after chromatography).

IC₅₀ Determination. Phosphate buffer (50 mM, pH 7.2) was prepared by dissolving NaH₂PO₄ (0.840 g) and Na₂HPO₄ (2.56 g) in

500 mL of deionized (Millipore) water. A solution of the β -lactamase derived from *E. cloacae* P99 (1.00 mg of enzyme was dissolved in 100 mL of 50 mM, pH 7.2 phosphate buffer) was prepared. A standard solution of a β -lactamase substrate, nitrocefin, was prepared by dissolving 2.00 mg of nitrocefin in 50 mL of phosphate buffer. A solution of inhibited was prepared by dissolving a specified amount (in the range of 0.5–10 mg) of inhibitor in 10 mL of phosphate buffer. These solutions were allowed to equilibrate to 25 °C in a water bath for at least 15 min.

To determine the rate of enzymatic hydrolysis of nitrocefin in the absence of inhibitor, 500 μ L of the standard nitrocefin solution was further diluted with 450 μ L of buffer and the new solution was allowed to equilibrate to 25 °C in a water bath for 10 min. A 100 μ L sample of enzyme solution was diluted with 400 μ L of phosphate buffer, then 50 μ L of the diluted enzyme solution was added to the second nitrocefin solution (to bring the total volume to 1.00 mL), and the hydrolysis rate of nitrocefin was determined spectrophotometrically by monitoring the change in the absorption at 482 nm.

For the determination of inhibitory activity, a specified volume $(2.5-400 \ \mu L)$ of inhibitor solution was added to a solution prepared from 100 μL of enzyme standard solution and enough phosphate buffer to make the total volume 0.50 mL (total volume = volume of inhibitor solution + 100 μL of enzyme solution + volume of phosphate buffer solution), and the obtained solution was incubated at 25 °C for 10 min. A 50 μL sample of the incubated solution was removed and added to a solution prepared from 500 μL of nitrocefin standard solution and 450 μL of phosphate buffer The hydrolysis rate of nitrocefin by the partially inhibited enzyme was determined spectrophotometrically.

Inhibition Constant Determination. For the determination of inhibitory rate, a specified volume (2.5, 5.0, 120.0, or $50.0 \,\mu$ L) of 0.05 mg/mL inhibitor solution was added to a solution prepared from 100 μ L of enzyme standard solution and enough phosphate buffer to make the total volume 0.5 mL (total volume = volume of inhibitor solution), and the obtained solution + volume of phosphate buffer solution), and the obtained solution was incubated at 25 °C. At various time points, 50 μ L of the incubated solution was removed and added to a solution prepared from 500 μ L of nitrocefin standard solution and 450 μ L of phosphate buffer, and then the hydrolysis rate of nitrocefin was determined spectrophotometrically by monitoring the absorbance at 482 nm.

Dilution Reactivation Studies. For the dilution reactivation studies, 2 mg of enzyme was dissolved in 0.3 mL of buffer and 3.34 mg of 16 was dissolved in 10 mL of buffer. A 50 μ L sample of enzyme solution was incubated with specified amounts of 16 and balanced with buffer to yield a total volume of 200 μ L. At appropriate time intervals, 5 μ L of the incubation mixture was diluted into 500 μ L of buffer solution. Then 25 μ L of this mixture was diluted into 1 mL (2 mg of nitrocefin in 100 μ L of buffer) of solution and assayed over 30 s, and the rate of change compared to that of a control enzyme solution.

Gel Filtration Studies. In the gel filtration (reactivation) experiments, 50 μ L of the above concentrated enzyme-inhibitor mixture that had incubated for 2 h was placed on a Pharmacia PD-10 column, 9 mL, containing G-25M Sephadex packing (preequilibrated with 25 mL of phosphate buffer). The column was eluted with phosphate buffer, and 0.5 mL fractions were collected. The UV spectra of each fraction were obtained, and those containing enzyme (fractions 5-8), as indicated by UV absorption at 280 nm, were combined. The activity of these combined fractions was monitored by diluting 2 μ L of this solution into 1 mL of nitrocefin solution. A plot of the natural logarithm of the remaining activity versus time yielded a straight line over the first 50 min, the slope of which gave the first-order rate constant of activation, k_{act} . Excess inhibitor came off the column in fraction 11 or higher, indicating a clean separation.

For the gel filtration (deactivation) studies, 5.01 mg of 16 (or 21) was dissolved in 0.450 mL of buffer, 1 mg of enzyme was dissolved in 0.15 mL of buffer, and these two solutions were combined. At time intervals of 1, 2, 3, 4, 6, and 9 h) 50 μ L of this enzyme—inhibitor mixture was separated on a column as described above. After the relevant fractions were combined, they were allowed to stand for >24 h to overcome residual reversible inhibition. They were then assayed against a control (enzyme with no inhibitor) which had also passed through a column. A plot of the natural logarithm of the remaining activity vs time line for 16 and 21 produced roughly linear data for each compound, the slope of which corresponded to the irreversible rate of inactivation k_{inact} .

Benzhydryl 7 β -Aminocephalosporanate (3). The title compound was prepared according to the procedure of Sheehan and Commons.³⁷ To a suspension of 7-aminocephalosporanic acid (2) (130.4 g, 0.48 mol) in methanol (480 mL) was added a solution of diphenyldiazomethane¹⁵ (93.0 g, 0.48 mol) in CH₂Cl₂, and the mixture was further stirred (mechanically) at room temperature (rt) for 44 h. The solid was removed by filtration, and the organic layer was concentrated in vacuo and purified by column chromatography (10% CH₃OH in CH₂-Cl₂) to afford the desired ester as a pale yellow solid (86.1 g, 41% yield): $R_f = 0.44$ in 1/9 CH₃OH/CH₂Cl₂, mp 45-46 °C; IR (CHCl₃) 2980, 1780, 1730 cm⁻¹; ¹H NMR (CDCl₃) δ 8.41 (2H, br s), 7.22 (10H, m), 6.91 (1H, s), 5.27 (1H, d, J = 2.8 Hz), 5.15 (1H, d, A or ABq, J = 14.0 Hz), 4.94 (1H, s), 4.84 (1H, d, B of ABq, J = 14.0 Hz), 3.73 (1H, d, A of ABq, J = 16.7 Hz), 3.33 (1H, d, B of ABq, J = 16.7 Hz),1.92 (3H, s); ¹³C NMR (CDCl₃) δ 169.8, 168.8, 160.6, 138.9, 138.7, 129.5, 129.3, 129.1, 128.7, 128.5, 127.97, 127.61, 127.52, 127.18, 126.52, 126.06, 125.4, 79.0, 63.3, 62.6, 58.5, 25.7, 20.1.

Benzhydryl 7-Oxocephalosporanate (4). The title compound was prepared by modifying the procedure of Hagiwara et al.³⁸ To a solution of benzhydryl 7 β -aminocephalosporanate (3) (5.9 g, 13.5 mmol) in anhydrous CH₂Cl₂ (70 mL) at -78 °C was added triethylamine (5.6 mL, 40.4 mmol) dropwise with stirring. After 5 min, trifluoromethanesulfonic anhydride (6.8 mL, 40.4 mmol) was added dropwise to this solution over a 5 min period. The reaction mixture was allowed to warm slow; y to 0 °C over a 1 h period. It was then recooled to -78°C, and triethylamine (5.6 mL, 40.4 mmol) was added over approximately 5 min. The reaction mixture was stirred at -78 °C for an additional 30 min and poured into cold 0.5 N HCl (200 mL). The resultant solution was further stirred for 30 min. The layers were separated, and the aqueous layer was extracted with CH₂Cl₂ (50 mL). The combined organic layers were washed with cold 0.5 N HCl (3 \times 100 mL), dried (Na₂SO₄), and concentrated to produce the title compound (5.8 g, 98% yield) as a pale yellow solid which was used without further purification: IR (CHCl₃) 3005, 1830, 1790, 1740 cm⁻¹; ¹H NMR (CDCl₃) δ 7.39 (10H, m), 7.05 (1H, s), 5.07 (1H, d, A of ABq, J = 13.9 Hz), 4.85 (1H, d, B of ABq, J = 14.0 Hz), 3.64 (1H, d, A of ABq, J = 18.5 Hz), 3.44 (1H, d, B of ABq, J = 18.6 Hz), 2.05 (3H, s); ¹³C NMR (CDCl₃) δ 188.4 (s), 170.3 (s), 160.1 (s), 158.7 (s), 138.8 (s), 138.6 (s), 128.4, 128.2, 128.1, 127.7, 126.9, 126.2, 80.1 (d), 65.8 (d), 62.6 (t), 27.7 (t), 20.4 (q).

Benzhydryl 7 α -**Ethynyl-7** β -hydroxycephalosporanate (5). Ethynylmagnesium bromide (45.2 mL, 22.6 mmol) was slowly added to the cold (-78 °C) solution of 7-oxocephalosporanate 4 (5.5 g, 12.6 mmol) in anhydrous THF (85 mL). It was then further stirred at -78 °C for 1 h and at -40 °C for 1.5 h. The reaction mixture was then quenched with acetic acid (2.9 mL, 50.4 mmol), and ether (500 mL) was then added. The combined organic layers were washed with water

(1 × 100 mL) and brine (1 × 100 mL), dried (Na₂SO₄), and concentrated *in vacuo*. The product was then immediately purified by column chromatography (1/4 EtOAc/CH₂Cl₂) to give the title compound (2.9 g, 50% yield) as a pale yellow fluffy solid: $R_f = 0.56$ in 1/4 EtOAc/CH₂CL₂; mp 50–52 °C; IR (CHCl₃) 3670, 3565, 3300, 3010, 2120, 1790, 1730 cm⁻¹; ¹H NMR (CDCl₃) δ 7.37 (10H, m), 6.95 (1H, s), 5.14 (1H, d, A of ABq, J = 13.9 Hz), 5.08 (1H, s), 4.89 (1H, d, B of ABq, J = 17.8 Hz), 3.53 (1H, d, A of ABq, J = 17.8 Hz), 3.35 (1H, d, B of ABq, J = 17.8 Hz), 2.88 (1H, s), 2.05 (3H, s); ¹³C NMR (CDCl₃) δ 170.7 (s), 162.7 (s), 160.3 (s), 139.3 (s), 139.1 (s), 132.1 (s), 128.42, 128.0, 127.3, 126.9, 125.6, 79.6 (d), 78.3 (s), 77.9 (s), 77.3 (d), 65.4 (d), 62.6 (t), 26.3 (t), 20.5 (q). Anal. Calcd for C₂₅H₂₁NO₆S: C, 64.79; H, 4.54; N, 3.02. Found: C, 64.20; H, 4.39; N, 3.25. Two minor products were isolated from this reaction.

(1) Benzhydryl 7 β -Ethynyl-7 α -hydroxycephalosporanate: yellow solid (0.58 g, 10% yield); $R_f = 0/41$ in 1/4 EtOAc/CH₂Cl₂; IR (CHCl₃) 3550, 3300, 3010, 2105, 1780, 1735 cm⁻¹; ¹H NMR (CDCl₃) δ 7.34 (10H, m), 6.92 (1H, s), 6.45 (1H, s), 2.05 (OH, s), 1.99 (3H, s); ¹³C NMR (CDCl₃) δ 170.7 (s), 165.6 (s), 162.8 (s), 138.8 (s), 128.5, 128.2, 127.6, 127.4, 126.8, 126.6, 122.1 (d), 119.2 (s), 78.9 (s), 78.8 (d), 77.6 (d), 76.9 (s), 65.5 (t), 61.0 (d), 50.2 (d), 20.4 (q).

(2) Benzhydryl 7 β -Hydroxycephalosporanate:³⁹ pale yellow solid (0.59 g, 10% yield); $R_f = 0.26$ in 1/4 EtOAc/CH₂Cl₂; IR (CHCl₃) 3680, 3350, 3010, 1795, 1735 cm⁻¹; ¹H NMR (CDCl₃) δ 7.38 (10H, m), 6.93 (1H, s), 5.39 (1H, d, J = 4.9 Hz), 5.15 (1H, d, A of ABq, J =13.8 Hz), 4.97 (1H, d, J = 4.8 Hz), 4.81 (1H, d, B of ABq, J = 13.8 Hz), 3.57 (1H, d, A of ABq, J = 18.6 Hz), 3.39 (1H, d, B of ABq, J =18.7 Hz), 2.16 (OH, s), 2.04 (3H, s); ¹³C NMR (CDCl₃) δ 170.7 (s), 162.5 (s), 160.3 (s), 139.0 (s), 138.7 (s), 129.4, 128.5, 128.4, 128.3, 128.1, 127.7, 127.0, 80.0 (d), 62.4 (d), 57.2 (d), 26.3 (t), 20.4 (q).

Benzhydryl 7 α -Ethynyl-7 β -[[(trifluoromethyl)sulfonyl]oxy]cephalosporanate (6). Trifluoromethanesulfonic anhydride (3.3 mL, 19.1 mmol) was added dropwise (4 s intervals) to a cold (0 °C) solution of pyridine (2.6 mL, 31.8 mmol) and benzhydryl 7 α -ethynyl-7 β -hydroxvcephalosporanate (5) (5.9 g, 12.7 mmol) in anhydrous CH₂Cl₂ (60 mL). The reaction mixture was allowed to warm to rt and monitored by TLC (reaction time 30 min). After concentration the residue was purified by column chromatography (CH2Cl2) to yield the title compound as a white solid (4.67 g, 62% yield): $R_f = 0.63$ in 15% EtOAc in CH₂Cl₂; mp 42-43 °C; IR (CHCl₃) 3300, 3020, 2120, 1810, 1780, 1750 cm⁻¹; ¹H NMR (CDCl₃) δ 7.39 (10H, m), 6.94 (1H, s), 5.29 (1H, d, A of ABq, J = 13.9 Hz), 5.26 (1H, s), 5.09 (1H, d, B of ABq, J = 14.8 Hz), 3.52 (1H, d, A of ABq, J = 16.5 Hz), 3.34 (1H, d, B of ABq, J = 18.3 Hz), 3.29 (1H, s), 2.09 (3H, s); ¹³C NMR (CDCl₃) δ 170.1 (s), 159.4 (s), 155.1 (s), 140.8 (s), 139.2 (s), 139.1 (s), 128.5, 128.1, 126.9, 126.8, 125.3, 118.0 (q, J = 321.11 Hz), 87.3 (s), 84.0 (d), 79.6 (d), 71.9 (s), 66.5 (d), 61.7 (t), 26.5 (t), 20.4 (q). Anal. Calcd for C₂₆H₂₀NO₈S₂F₃: C, 52.44; H, 3.36; N, 2.35; F, 9.58. Found: C, 52.66; H, 3.37; N, 2.33; F, 9.26.

Benzhydryl 7-(2'a-Bromovinylidene)cephalosporanate (8). Method A. Copper(I) bromide (CuBr; 133 mg, 0.93 mmol) was added in one portion to a solution of benzhydryl 7α -ethynyl- 7β -[[(trifluoromethyl)sulfonyl]oxy]cephalosporanate (6) (500 mg, 0.84 mmol) in anhydrous DMF (5.0 mL) at rt and stirred in the dark for 30 min. The DMF was removed in vacuo at rt. The residue was dissolved in ether (50 mL), washed with water $(2 \times 15 \text{ mL})$, dried (Na₂SO₄), and concentrated to produce a yellow solid. This material was purified by column chromatography (CH₂Cl₂) to yield the title compound as a pale yellow solid (140 mg, 32% yield): $R_f = 0.75$ in 15% EtOAc in CH₂Cl₂; mp 63-65 °C; IR (CHCl₃) 3010, 1950, 1780, 1730 cm⁻¹; ¹H NMR (CDCl₃) δ 7.42 (10H, m), 7.01 (1H, s), 6.74 (1H, d, J = 1.17 Hz), 5.38 (1H, d, J = 1.12 Hz), 5.02 (1H, d, A of ABq, J = 13.5 Hz), 4.78 (1H, d, B of ABq, J = 13.4 Hz), 3.60 (1H, d, A of ABq, J = 18.31 Hz), 3.41 (1H, d, B of ABq, J = 18.11 Hz), 2.04 (3H, s); ¹³C NMR (CDCl₃) δ 194.6 (s), 170.3 (s), 160.6 (s), 156.1 (s), 139.1 (s), 138.9 (s), 128.4, 128.1, 128.0, 127.7, 127.1, 124.6, 111.7 (s), 81.8 (d), 79.9 (d), 62.9 (t), 56.2 (d), 27.8 (t), 20.5 (q); HRMS for $[C_{25}H_{20}NO_5SBrNa]^+$, m/z calcd 548.0143, found 548.0146.

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Method B. Lithium bromide (LiBr; 285 mg, 3.3 mmol) and copper(I) bromide (CuBr; 470 mg, 3.3 mmol) were added in one portion to a solution of benzhydryl 7 α -ethynyl-7 β -[[(trifluoromethyl)sulfonyl]-oxy]cephalosporanate (6) (1.5 g, 2.5 mmol) in anhydrous THF (15 mL). The mixture was allowed to stir at rt for 5 min. The THF was removed in vacuo. The residue was dissolved in ether (20 mL), washed with water (1 × 10 mL), dried (Na₂SO₄), and concentrated in vacuo to give a yellow solid (1.30 g, 98% yield). This reaction produced a single isomer, 7-(2' α -bromovinylidene)cephalosporanate. The material was judged to be 95% pure by ¹H NMR and used for the next step without purification.

Benzhydryl 7-Vinylidenecephalosporanate (9). To a solution of benzhydryl 7-(2'a-bromovinylidene)cephalosporanate (8) (2.4 g, 4.6 mmol) in a 1/5 mixture of anhydrous THF/MeOH (60 mL) were added NH₄Cl (0.98 g, 18.4 mmol) and a Zn-Cu couple (0.6 g, 9.2 mmol). After stirring at rt for 30 min, the reaction mixture was concentrated in vacuo. The residue was dissolved in ether (100 mL), washed with water (20 mL), dried (Na₂SO₄), concentrated, and chromatographed (1/1 hexane/CH2Cl2 followed by 1/3 hexane/CH2Cl2) to give a white fluffy solid (1.45 g, 71% yield): $R_f = 0.3$ in CH₂Cl₂; IR (CHCl₃) 3010, 1985, 1790, 1730 cm⁻¹; ¹H NMR (CDCl₃) δ 7.40 (10H, m), 7.0 (1H, s), 5.58 (2H, d, J = 13.4 Hz), 5.29 (1H, t, J = 1.88 Hz), 4.99 (1H, d, A of ABq, J = 13.35 Hz), 4.74 (1H, d, B of ABq, J = 13.3 Hz), 3.57 (1H, d, A of ABq, J = 18.2 Hz), 3.37 (1H, d, B of ABq, J = 18.3 Hz),2.03 (3H, s); ¹³C NMR (CDCl₃) δ 200.0 (s), 170.3 (s), 160.8 (s), 158.7 (s), 139.2 (s), 139.0 (s), 128.4, 128.0, 127.9, 127.7, 127.4, 127.0, 123.0, 105.7 (s), 75.1 (t), 79.7 (d), 63.0 (t), 56.6 (d), 27.8 (t), 20.5 (q); HRMS for $[C_{24}H_{21}NO_5SNa]^+$, i.e., $[M + Na]^+$, m/z calcd 470.1038, found 470.1042.

Benzhvdrvl 7-(2'a-tert-Butvlvinvlidene)cephalosporanate (7). To a suspension of CuCN (0.376 g, 4.2 mmol) in anhydrous THF (30 mL) was added t-BuLi (4.0 mL, 1.7 M in pentane, 6.8 mmol) at -100 °C. The cooling bath was removed until all the solid had gone into the solution (approximately 3 min). This solution was again cooled to -100 °C and was cannulated into a cold (-100 °C) solution of benzhydryl 7 α -ethynyl-7 β -[[(trifluoromethyl)sulfonyl]oxy]cephalosporanate (6) (2.0 g, 3.4 mmol) in anhydrous THF (5 mL) at -100 °C. The solution was further stirred at -100 °C for 1 min before pouring the cold reaction mixture into cold (0 °C) saturated NH4Cl solution (100 mL). The reaction mixture was extracted with ether (2 \times 50 mL). dried (Na₂SO₄), concentrated, and chromatographed (5% EtOAc in CH₂-Cl₂) to give a white fluffy solid (0.913 g, 54% yield): $R_f = 0.80$ in 5% EtOAc in CH2Cl2; mp 113-114 °C; IR (CHCl3) 3000, 2960, 1970, 1770, 1730 cm⁻¹; ¹H NMR (CDCl₃) δ 7.42 (10H, m), 7.05 (1H, s), 5.98 (1H, d, J = 1.63 Hz), 5.25 (1H, d, J = 1.69 Hz), 4.97 (1H, d, A of ABq, J = 13.30 Hz), 4.72 (1H, d, B of ABq, J = 13.23 Hz), 3.55 (1H, d, A of ABq, J = 18.14 Hz), 3.35 (1H, d, B of ABq, J = 18.23Hz), 2.01 (3H, s), 1.18 (9H, s); ¹³C NMR (CDCl₃) δ 194.6 (s), 170.2 (s), 161.1 (s), 159.5 (s), 139.2 (s), 139.0 (s), 128.3, 128.0, 127.9, 127.7, 127.1, 121.9, 113.2 (d), 107.2 (s), 79.6 (d), 63.0 (t), 57.0 (d), 33.6 (s), 29.7 (q), 27.8 (t), 20.5 (q). Anal. Calcd for $C_{29}H_{29}NO_5S$: C, 69.18; H, 5.77; N, 2.78. Found: C, 69.08; H, 6.00; N, 2.73.

Benzhydryl 7-(2'a-Bromovinylidene)cephalosporanate Sulfone (11). To the solution of sulfide 8 (0.65 g, 1.23 mmol) in CH_2Cl_2 (10 mL) and pH 6.4 phosphate buffer solution (10 mL) was added in one portion m-CPBA (85%, 0.853 g, 4.94 mmol). The mixture was stirred rapidly as possible overnight at room temperature. After separating the layers, the aqueous layer was extracted with ether $(1 \times 10 \text{ mL})$. The combined organic layers were washed with 5% NaHSO₃ (1 \times 5 mL) and saturated NaHCO₃ (1 × 5 mL), dried (Na₂SO₄), concentrated, and chromatographed (5% EtOAc in CH2Cl2). A yellow solid was obtained (0.32 g, 46% yield): $R_f = 0.35$ in 5% EtOAc in CH₂Cl₂; IR (CHCl₃) 3020, 1950, 1800, 1740, 1350, 1130 cm⁻¹; ¹H NMR (CDCl₃) δ 7.39 (10H, m), 7.00 (1H, s), 6.94 (1H, d, J = 1.38 Hz), 5.39 (1H, d, J = 1.13 Hz), 5.09 (1H, d, A of ABq, J = 14.25 Hz), 4.75 (1H, d, B of ABq, J = 14.33 Hz), 4.06 (1H, d, A of ABq, J = 18.25 Hz), 3.82 (1H, d, B of ABq, J = 18.17 Hz), 2.06 (3H, s); ¹³C NMR (CDCl₃) δ 196.8, 170.1, 159.6, 155.3, 138.7, 138.6, 128.6, 128.4, 128.3, 127.6, 127.1, 125.8, 125.4, 104.4, 83.8, 80.6, 69.0, 61.9, 51.3, 20.4.

Benzhydryl 7-Vinylidenecephalosporanate Sulfone (12). This compound was prepared from the sulfide 9 as described for the benzhydryl 7-(2' α -bromovinylidene)cephalosporanate sulfone (11)

(yield 55%, 0.590 g): $R_f = 0.35$ in 5% EtOAc in CH₂Cl₂; mp 155– 156 °C; IR (CHCl₃) 3010, 1985, 1790, 1730, 1340, 1125 cm⁻¹; ¹H NMR (CDCl₃) δ 7.43 (10H, m), 6.99 (1H, s), 5.70 (2H, dd, J = 1.65Hz, J = 5.31 Hz), 5.33 (1H, s), 5.03 (1H, d, A of ABq, J = 14.02 Hz), 4.70 (1H, d, B of ABq, J = 14.01 Hz), 4.04 (1H, d, A of ABq, J = 18.12 Hz), 3.79 (1H, d, B of ABq, J = 18.40 Hz), 2.03 (3H, s); ¹³C NMR (CDCl₃) δ 201.7 (s), 170.1 (s), 159.8 (s), 157.6 (s), 138.8 (s), 138.6 (s), 128.4, 128.2, 128.1, 127.5, 127.0, 124.0, 98.8 (s), 86.4 (t), 80.3 (d), 69.5 (d), 61.9 (t), 51.0 (t), 20.3 (q); HRMS for [C₂₅H₂₁NO₇-SNa]⁺, i.e., [M + Na]⁺, *m*/z calcd 502.0936, found 502.0931.

Benzhydryl 7-(2'α-*tert*-Butylvinylidene)cephalosporanate Sulfone (10). This compound was prepared from the sulfide 7 as described above in benzhydryl 7-(2'α-bromovinylidene)cephalosporanate sulfone (11) (yield 65%, 0.692 g): $R_f = 0.42$ in 2% EtOAc in CH₂Cl₂; mp 163-164 °C; IR (CHCl₃) 3010, 2960, 1970, 1790, 1740, 1340, 1125 cm⁻¹; ¹H NMR (CDCl₃) δ 7.40 (10H, m), 7.01 (1H, s), 6.18 (1H, d, J = 1.66 Hz), 5.30 (1H, s), 5.02 (1H, d, A of ABq, J = 13.93 Hz), 4.68 (1H, d, B of ABq, J = 13.93 Hz), 4.02 (1H, d, A of ABq, J = 18.27 Hz), 3.76 (1H, d, B of ABq, J = 18.20 Hz), 2.03 (3H, s), 1.19 (9H, s); ¹³C NMR (CDCl₃) δ 197.1 (s), 170.1 (s), 160.0 (s), 158.6 (s), 138.8 (s), 138.7 (s), 128.5, 128.3, 128.2, 127.6, 127.1, 126.5, 123.1, 114.6 (d), 100.1 (s), 80.4 (d), 70.1 (d), 62.0 (t), 51.2 (t), 34.0 (s), 29.7 (q), 20.4 (q); HRMS for [C₂₉H₂₉NO₇SNa]⁺, i.e., [M + Na]⁺, m/z calcd 558.1562, found 558.1567.

Sodium Salt of 7-(2'a-Bromovinylidene)cephalosporanic Acid (14). To a solution of benzhydryl 7-($2'\alpha$ -bromovinylidene)cephalosporanate (11) (300 mg, 0.57 mmol) in anhydrous CH₂Cl₂ (6 mL) was added anisole (0.62 mL, 5.7 mmol) at -78 °C followed by addition of AlCl₃ solution (1.43 mL, 1.0 M in nitrobenzene, 1.43 mmol) in one portion. The mixture was stirred for 15 min at -78 °C and poured into rapidly stirred cold water (60 mL) containing NaHCO₃ (0.48 g, 5.7 mmol) followed by addition of EtOAc (50 mL). It was further stirred for 5 min and filtered using Celite 545. The aqueous layer was separated and concentrated in vacuum to 2 mL and further purified by reversed-phase preparative layer chromatography ($R_r = 0.62$ in 5%) EtOH in water). Lyopholization produced a pale yellow fluffy solid (105 mg, 48% yield): IR (Nujol) 1975, 1750, 1610, 1410 cm⁻¹; ¹H NMR (DMSO- d_6) δ 7.56 (1H, d, J = 0.7 Hz), 5.62 (1H, s), 5.00 (1H, d, A of ABq, J = 13.5 Hz), 4.76 (1H, d, B of ABq, J = 13.28 Hz), 3.56 (1H, d, A of ABq, J = 17.91 Hz), 3.17 (1H, d, B of ABq, J =17.84 Hz), 2.05 (3H, s); HRMS for [C₁₂H₉NO₅SBrNa₂]⁺, i.e., [M + Na]⁺, m/z calcd 403.9180, found 403.9168.

Sodium Salt of 7-Vinylidenecephalosporanic Acid (15). This compound was prepared from the corresponding ester 9 (500 mg, 1.12 mmol) as described for the sodium salt of 7-(2' α -bromovinylidene)-cephalosporanic acid (14) above to give the title compound as a white fluffy solid (220 mg, 65% yield): $R_f = 0.65$ in 5% EtOH in water; IR (Nujol) 1980, 1760, 1735, 1590 cm⁻¹; ¹H NMR (DMSO- d_6) δ 5.81 (2H, d, J = 1.5 Hz), 5.41 (1H, s), 4.95 (1H, d, A of ABq, J = 12.11 Hz), 4.71 (1H, d, B of ABq, J = 12.05 Hz), 3.50 (1H, d, A of ABq, J = 17.20 Hz), 3.24 (1H, d, B of ABq, J = 17.40 Hz), 1.99 (3H, s); HRMS for [C₁₂H₁₁NO₅SNa]⁺, i.e., [M + H]⁺, m/z calcd 304.0255, found 304.0250.

Sodium Salt of 7-(2' α -tert-Butylvinylidene)cephalosporanic Acid (13). This compound was prepared from the corresponding ester 7 (420 mg, 0.83 mmol) as described for the sodium salt of 7-(2' α bromovinylidene)cephalosporanic acid (14) above to give the title compound as a white fluffy solid (118 mg, 39% yield): $R_f = 0.80$ in 15% EtOH in water; IR (Nujol) 1975, 1760, 1720, 1610 cm⁻¹; ¹H NMR (DMSO- d_6) δ 6.20 (1H, s), 5.33 (1H, s), 4.93 (1H, d, A of ABq, J =12.02 Hz), 4.69 (1H, d, B of ABq, J = 11.97 Hz), 3.48 (1H, d, A of ABq, J = 17.66 Hz), 3.20 (1H, d, B of ABq, J = 18.09 Hz), 1.99 (3H, s), 1.10 (9H, s); HRMS for [C₁₆H₁₉NO₅SNa]⁺, i.e., [M + H]⁺, m/z calcd 360.0881, found 360.0882.

Sodium Salt of 7-(2' α -tert-Butylvinylidene)cephalosporanic Acid Sulfone (16). This compound was prepared from the corresponding ester 10 (250 mg, 0.47 mmol) as described for the sodium salt of 7-(2' α bromovinylidene)cephalosporanic acid (14) above to give the title compound as a white fluffy solid (110 mg, 60% yield): $R_f = 0.50$ in 20% EtOH in water; IR (Nujol) 1980, 1765, 1730, 1615, 1330, 1130 cm⁻¹; ¹H NMR (DMSO- d_6) δ 6.39 (1H, s), 5.84 (1H, s), 4.92 (1H, d, A of ABq, J = 12.07 Hz), 4.64 (1H, d, B of ABq, J = 12.08 Hz), 4.05 (1H, d, A of ABq, J = 17.71 Hz), 3.78 (1H, d, B of ABq, J = 17.63 Hz), 1.99 (3H, s), 1.10 (9H, s); HRMS for $[C_{16}H_{19}NO_7SNa]^+$, i.e., $[M + H]^+$, m/z calcd 392.0779, found 392.0780.

Benzhydryl 7 α -(2'-Deuterioethynyl)-7 β -hydroxycephalosporanate. A solution of ethylmagnesium bromide (1 M in THF, 57.2 mL, 57.2 mmol) was added to a solution of ethynylmagnesium bromide (0.5 M in THF, 57.2 mL, 28.6 mmol) under argon at room temperature. The reaction was heated to 55-60 °C for 1 h. After cooling the reaction to 50 °C, D₂O (2.29 g, 114 mmol) was added dropwise to produce a very slow stream of gas. This gas (C2D2 and residual ethane) was bubbled through a cold trap (chilled to -78 °C) and into a flask containing another solution of ethylmagnesium bromide (1 M in THF, 2.29 mL, 2.29 mmol) and THF (20 mL) at 15-20 °C with vigorous stirring. After addition of D₂O to the first solution was complete, 1 mL of DOAc was added slowly to the initial reaction to ensure completion. After no more gas was produced, this (deuterioethynyl)magnesium bromide solution was cooled to -78 °C and a solution of benzhydryl 7-oxocephalosporanate (4) (0.5 g, 1.14 mmol) in anhydrous THF (10 mL) was added slowly. The reaction was then further stirred at -78 °C for 1 h and at -40 °C for 1 h and 30 min. The reaction mixture was then quenched with DOAc (2.9 mL, 50.4 mmol), and 500 mL of ether was added. The ethereal solution was washed with water $(3 \times 500 \text{ mL})$ and brine $(1 \times 400 \text{ mL})$, dried (Na₂SO₄), and concentrated in vacuo. It was then immediately purified by column chromatography (1/4 EtOAc/CH₂Cl₂) to give the title compound: R_f = 0.56 in 1/4 EtOAc/CH₂Cl₂; mp 53-55 °C; IR (CHCl₃) 3018, 1793, 1732 cm⁻¹; ¹H NMR (CDCl₃) δ 7.32 (10H, m), 6.92 (1H, s), 5.12 (1H, d, A of ABq, J = 13.9 Hz), 5.09 (1H, d, B of ABq, J = 13.9 Hz), 5.02 (1H, s), 3.47 (1H, d, A of ABq, J = 17.8 Hz), 3.30 (1H, d, B of ABq, J = 17.8 Hz), 3.30 $(1H, d, B \text{$ J = 17.8 Hz), 2.04 (3H, s); ¹³C NMR (CDCl₃) δ 170.7 (s), 162.7 (s), 160.3 (s), 139.4 (s), 139.2 (s), 132.2 (s), 128.5, 128.1, 128.03, 127.4, 126.9, 125.6, 79.6 (d), 78.3 (s), 77.7 (s), 77.5 (d), 77.1, 76.5, 65.4 (d), 62.7 (t), 26.4 (t), 20.6 (q).

Benzhydryl 7 α -(2'-Deuterioethynyl)-7 β -[[(trifluoromethyl)sulfonyl]oxy]cephalosporanate (18). Trifluoromethanesulfonic anhydride (0.55 mL, 3.23 mmol) was added dropwise (4 s intervals) to a cold (0 °C) solution of pyridine (0.435 mL, 5.39 mmol) and benzhydryl 7α-(2'-deuterioethynyl)-7 β -hydroxycephalosporanate (17) (1.0 g, 2.16 mmol) in anhydrous CH2Cl2 (10 mL). The reaction mixture was allowed to warm to rt and monitored by TLC (reaction time 30 min). After concentration the residue was purified by column chromatography (CH₂Cl₂) to yield the title compound as a white solid (1.1 g, 85% yield): $R_f = 0.63$ in 15% EtOAc in CH₂Cl₂; mp 42-44 °C; IR (CHCl₃) 2941, 2581, 1982, 1806, 1735 cm⁻¹; ¹H NMR (CDCl₃) δ 7.36 (10H, m), 6.93 (1H, s), 5.28 (1H, d, A of ABq, J = 14.48 Hz), 5.08 (1H, d, B of ABq, J = 14.48 Hz), 3.51 (1H, d, A of ABq, J = 16.7 Hz), 3.33 (1H, d, B of ABq, J = 16.7 Hz), 3.29 (1H, s), 2.08 (3H, s); ¹³C NMR $(CDCl_3) \delta 170.14, 159.5, 155.2, 140.9, 139.3, 128.77, 128.19, 127.18,$ 127.02, 87.36, 79.68, 71.57, 66.56, 61.83, 26.59, 20.46.

Benzhydryl 7-(2'\alpha-tert-Butyl-2'\beta-deuteriovinylidene)cephalosporanate (19). To a suspension of CuCN (0.094 g, 1.05 mmol) in anhydrous THF (30 mL) was added *t*-BuLi (0.987 mL, 1.68 mmol) at -100 °C. The cooling bath was removed until all the solid had gone into the solution (approximately 3 min). This solution was again cooled to -100 °C and was cannulated into a cold solution of benzhydryl 7 α -(2'-deuterioethynyl)-7 β -[[(trifluoromethyl)sulfonyl]oxy]cephalosporanate (18) (0.500 g, 0.839 mmol) in anhydrous THF (5 mL) at -100 °C. The solution was further stirred at -100 °C for 1 min beofre pouring the cold reaction mixture into cold (0 °C) saturated NH₄Cl solution (50 mL). The reaction mixture was extracted with ether (2 × 50 mL), dried (Na₂SO₄), concentrated, and chromatographed (5% EtOAc in CH₂Cl₂) to give a white fluffy solid (0.172 g, 41% yield): $R_f = 0.8$ in 5% EtOAc in CH₂Cl₂; mp 110–113 °C; IR (CHCl₃) 2962, 1966, 1773, 1739, cm⁻¹; ¹H NMR (CDCl₃) δ 7.45 (10H, m), 7.00 (1H, s), 5.22 (1H, s), 4.93 (1H, d, A of ABq, J = 13.19 Hz), 4.68 (1H, d, B of ABq, J = 13.18 Hz), 3.53 (1H, d, A of ABq, J = 18.31 Hz), 3.33 (1H, d, B of ABq, J = 18.61 Hz), 2.00 (3H, s), 1.18 (9H, s); ¹³C NMR (CDCl₃) δ 194.8, 170.4, 161.2, 159.7, 139.3, 139.1, 128.5, 128.2, 128.01, 127.6, 127.84, 127.2, 122.04, 107.4, 77.8, 63.2, 57.2, 33.7, 29.9, 28.0, 20.7.

Benzhydryl 7-(2'α-tert-Butyl-2'β-deuteriovinylidene)cephalosporanate Sulfone (20). This compound was prepared from 0.100 g of the sulfide 19 as described fro benzhydryl 7-(2'α-bromovinylidene)cephalosporanate sulfone (11) (yield 75%, 0.08 g): $R_f = 0.42$ in 2% EtOAc in CH₂Cl₂; mp 165–168 °C; IR (CHCl₃) 3032, 2960, 1790, 1740, cm⁻¹; ¹H NMR (CDCl₃) δ 7.35 (10H, m), 6.97 (1H, s), 5.02 (1H, d, A of ABq, J = 13.93 Hz), 4.66 (1H, d, B of ABq, J = 13.93Hz), 4.02 (1H, d, A of ABq, J = 18.27 Hz), 3.76 (1H, d, B of ABq, J = 18.20 Hz), 2.03 (3H, s), 1.19 (9H, s); ¹³C NMR (CDCl₃) δ 197.1, 170.1, 160.1, 158.6, 138.9, 138.7, 128.5, 128.3, 127.84, 127.7, 127.1, 126.5, 123.1, 100.2, 80.5, 70.1, 62.1, 51.3, 34.0, 29.8, 20.5.

Sodium Salt of 7-(2' α -tert-Butyl-2' β -deuteriovinylidene)cephalosporanic Acid Sulfone (21). This compound was prepared from the corresponding ester 20 (0.08 mg, 0.147 mmol) as described for the sodium salt of 7-(2' α -bromovinylidene)cephalosporanic acid (14) above to give the title compound as a white fluffy solid (35 mg, 61% yield): $R_f = 0.50$ in 20% EtOH in water; ¹H NMR (DMSO- d_6) δ 5.85 (1H, s), 4.92 (1H, d, A of ABq, J = 11.9 Hz), 4.65 (1H, d, B of ABq, J = 11.9Hz), 4.06 (1H, d, A of ABq, J = 17.28 Hz), 3.79 (1H, d, B of ABq, J = 17.58 Hz), 1.99 (3H, s), 1.10 (9H, s).

NMR Hydrolysis Study. An 8 mg sample of 7-(2' α -tert-butyl-2' β -deuteriovinylidene)cephalosporanate sulfone **16** was dissolved in 0.5 mL of D₂O, 1.5 μ L of NaOD solution (40% w/w in D₂O) was added, and the reaction was followed by ¹H NMR for 15 min. This resulted in what appeared to be a 1/1 mixture of two compounds. Then an additional 1.5 μ L of NaOD solution was added, and the reaction was followed to completion (within another 15 min): ¹H NMR (D₂O) δ 7.60 (s, <1H); 5.84 (br s, <1H), 5.69 (br s, <1H), 1.73 (s, 3H), 1.14 (s, 9H).

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Supplementary Material Available: Figures showing ¹H NMR spectra of basic hydrolysis of 16 in D_2O (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.