β-Secondary Kinetic Isotope Effects in the Clavaminate Synthase-Catalyzed Oxidative Cyclization of Proclavaminic Acid and in Related Azetidinone Model Reactions

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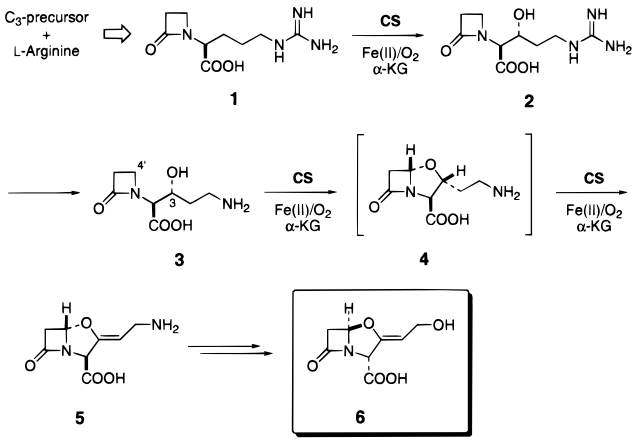
Abstract: Clavaminate synthase is an Fe(II)/α-ketoglutarate-dependent oxygenase that catalyzes three mechanistically distinct reactions in the course of clavulanic acid biosynthesis. Clavulanic acid is of significant chemical importance as a potent inhibitor/inactivator of β-lactamase enzymes, a prominent means of bacterial resistance to, for example, penicillin. Primary and α-secondary $^{T}(V/K)$ kinetic isotope effects have been determined in earlier work for the clavaminate synthase-catalyzed oxidative cyclization of proclavaminic acid, one of the three reactions mediated by this enzyme. In this paper the β-secondary deuterium kinetic isotope effect for this reaction has been determined using remote ^{3}H and ^{14}C labels in an attempt to distinguish between radical or cationic intermediates in the reaction as suggested by the magnitudes of the primary and secondary α-effects. The presence of the adjacent azetidinone nitrogen and the intervention of an azetinone intermediate, formally antiaromatic in the resonance form of the amide, make interpretation of the low β-secondary effect (1.056 ± 0.002 for dideuteriation at C-3′) problematic. To assist interpretation of this result, a 4-chloroazetidinone model system has been constructed dideuteriated at C-3 identically to proclavaminic acid and bearing remote radiolabels. Reaction of this substrate at 25 °C under both radical and solvolysis conditions afforded β-secondary kinetic isotope effect data for direct comparison to the enzymic reaction. The measured effects are similarly small but strongly dependent on the polarity/acidity of the reaction medium. These results are discussed in terms of the commitment to catalysis and the extent to which amide resonance may be favored in the transition state of the oxidative cyclization.

Introduction

The non-heme iron oxygenase clavaminate synthase (CS) catalyzes three distinct oxidative reactions in the course of clavulanic acid (**6**) biosynthesis. These α-ketoglutarate (α-KG)-dependent steps include (1) a conventional hydroxylation of deoxyguanidino proclavaminic acid (**1**, Scheme 1) to guanidino proclavaminic acid (**2**), (2) the oxidative cyclization of proclavaminic acid (**3**) to dihydroclavaminic acid (**4**) and, (3) desaturation of the latter to clavaminic acid (**5**). Remarkably, CS is a monomeric protein having a single iron binding site at which all three transformations take place to achieve the stepwise elevation of chemical potential manifested ultimately in the potent β-lactamase inhibitor **6** itself.[1,2] In each of the CS-catalyzed reactions an equivalent of α-KG is consumed in the activation of molecular oxygen and released as succinate and carbon dioxide. Both the activation of oxygen and the oxidation of substrate may be regarded, therefore, as irreversible processes.

Scheme 1



Mechanistic investigations of clavaminate synthase have taken place at several levels. The behavior of the iron site during substrate binding has been monitored by ligand field CD and MCD measurements to show that the octahedral ferrous resting state of the enzyme remains 6-coordinate but reorganizes upon α-KG binding to accommodate bidentate binding of the cofactor.[3] Upon the further addition of the substrate, deoxyguanidino proclavaminic acid (**1**), however, the site becomes 5-coordinate, square pyramidal. Presumably the open coordination site on iron

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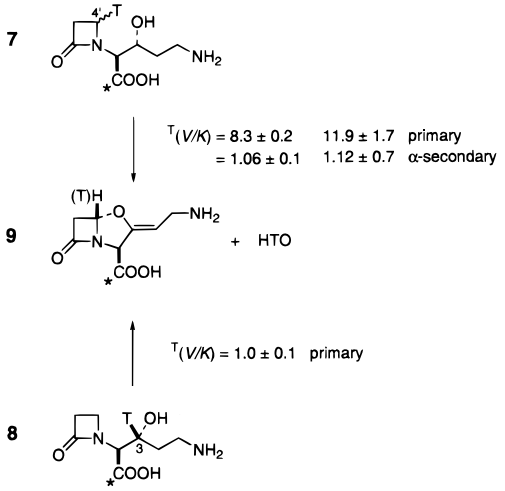
Scheme 2

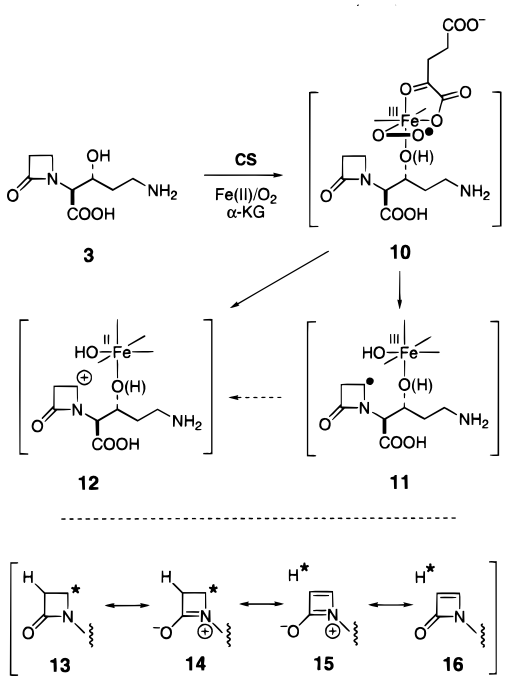


is available to react with dioxygen to initiate the oxidative cycle,[4] as has been proposed analogously for isopenicillin N synthase and supported by NO binding.[5] The dynamics of substrate oxidation were first revealed in kinetic isotope effect experiments to establish the order of the oxidative reactions between proclavaminic acid (**3**) and clavaminic acid (**5**); that is, does oxidative cyclization precede desaturation or vice versa?

The fact that the CS-catalyzed reactions in Scheme 1 are sequential and irreversible[6] was exploited in *V/K* kinetic isotope effect measurements.[7] Proclavaminic acid bearing tritium equally at the two diastereotopic C-4′ positions was prepared (**7**, Scheme 2), and similarly a specimen with tritium at C-3 (**8**). Each was mixed with material radiolabeled with ^{14}C at C-1 (*) to serve as an internal standard and a kinetically unbiased marker of the extent of reaction. For a normal kinetic isotope effect $k_H/k_D > 1$, it would be anticipated that the ^{3}H-labeled substrate relative to the ^{14}C-labeled would be enriched in the unreacted starting material and depleted in the product as the reaction progressed. It was shown by Northrup that the competition between the labeled pairs of substrates is governed by the kinetic parameter $^T[V/K]$, which will reflect events only through the first irreversible step of the reaction.[8] Using this method, discrimination against tritiated molecules should only occur when hydrogen isotope is present at the position oxidized first. For the [4′-^{3}H]-proclavaminic acid (**7**), tritium located on the α-face (*pro-S* locus) would be lost during oxidative cyclization[9] and would appear as HTO in the medium to provide a measure of the primary kinetic isotope effect, while tritium located on the *β*-face (*pro-R*) would provide a measure of the α-secondary kinetic isotope effect in both the product and unreacted substrate. By monitoring the release of HTO from **7** as function of the extent of reaction, a $^T[V/K] = 8.3 \pm 0.2$ was determined for the primary kinetic isotope effect. The corresponding appearance of tritium activity relative to the ^{14}C-internal standard in clavaminic acid (**9**) afforded $^T[V/K] = 1.06 \pm 0.01$ for the α-secondary effect. A more complex analysis of the changing ^{3}H/^{14}C ratio of residual substrate gave independent determinations of both of these parameters with somewhat larger errors: primary $^T[V/K] = 11.9 \pm 1.7$, α-secondary $^T[V/K] = 1.12 \pm 0.07$ (Scheme 2).[7] In contrast, $^T[V/K]$ observed for tritium substitution at C-3 in **8** was 1.0 ± 0.1, clearly establishing that the order of reactions is as shown in Scheme 1 and involving the transient intermediacy of dihydroclavaminic acid (**4**), whose structure was determined from small steady-state concentrations of this species.[7,10,11]

The normal primary and α-secondary kinetic isotope effects determined in the analysis of the oxidative cyclization of proclavaminic acid (**3**) to dihydroclavaminic acid (**4**), although not maximal, were consistent with a substantial change in hybridization at the carbon undergoing oxidation from sp^3 to sp^2 in the transition state of the reaction. In a related study of the α-KG-dependent dioxygenase *γ*-butyrobetaine hydroxylase, a primary $^T[V/K] = 15$ and α-secondary $^T[V/K] = 1.31$ were measured by Blanchard and Englard.[12] Like these investigators, we interpreted our data to discount carbanion or oxenoid C−H insertion processes in favor of radical or carbenium ion intermediates in the oxidative cyclization.[7]

Scheme 3



***β*-Secondary Kinetic Isotope Effect.** To examine more closely the singular CS-mediated cyclization of proclavaminic acid (**3**), and to attempt to distinguish between radical and cationic processes (Scheme 3), we elected to determine the *β*-secondary kinetic isotope effect during oxazolidine ring formation. Alternatively, substrate analogue studies, so revealing in the investigation of the mechanism of isopenicillin N synthase,[13] were completely unsuccessful in the case of cla-

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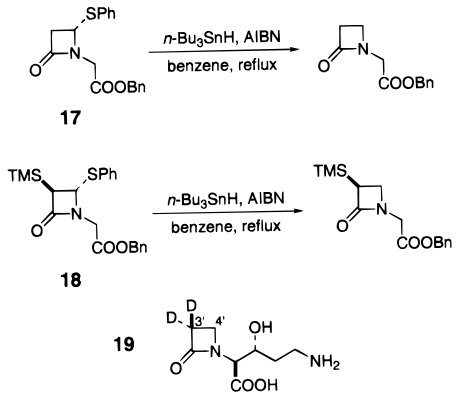
vaminate synthase.[14] The β-secondary effect is well understood to arise principally from the weakening of β-C−H bonds by hyperconjugation to an adjacent empty (cationic) or partially filled (radical) p-orbital.[15,16] It can be argued from first principles that this interaction should be greater for a carbenium ion [2 electrons, 2 (or 3) centers] than for a radical owing to Coulombic repulsion of the unshared electron.[17] This being so, one might expect that a distinction could be made between radical or carbenium ion formation at C-4′ in the transition state of oxidative cyclization. However, a number of elements of the reaction at hand could confound, if not thwart, the envisioned analysis. (1) No β-secondary kinetic isotope effect determinations have been made in chemical models of radical or cationic reaction at C-4 of an azetidinone to serve as benchmarks for the enzymic reaction. We provide these in this paper for comparison to the enzymic reaction.[18] (2) The reaction site lies adjacent to the β-lactam nitrogen. Delocalization of the nitrogen lone pair will suppress the magnitude of all isotope effects measured at the reaction center.[15] However, the countervailing effect of amide resonance, although somewhat reduced in the four-membered ring, will attenuate the extent of this suppression (cf. **13** and **14**, Scheme 3). A qualitative appreciation for the net impact of these effects can be inferred from a comparison of the primary and α-secondary kinetic isotope effects observed for clavaminate synthase and the somewhat higher values reported for γ-butyrobetaine hydroxylase.[12] Therefore, while some reduction of the intrinsic isotope effects at C-4′ in proclavaminic acid is likely to occur through participation by the adjacent β-lactam nitrogen and the step may not be fully rate-determining, the primary effect, especially, remains substantial. (3) That the primary kinetic isotope effect is high is critical to interpretation of the anticipated β-secondary effect.[19] For an enzymic reaction, the commitment to catalysis must be sufficiently low for the observed effect to approximate the intrinsic isotope effect before a meaningful comparison can be made to chemical model reactions. (4) Finally, the envisioned β-secondary kinetic isotope effect determination relies on a hyperconjugative interaction of the C-3 hydrogen of the β-lactam ring. Formally, this participation with the reactive intermediate (*, Scheme 3) would result in azetinone **16** formation. These species are known to be extremely unstable owing, presumably, to the antiaromatic resonance form **15**.[20]

At the outset, it was this last factor that posed the greatest unknown to analysis of the experimental results. Encouragement was taken, however, from an earlier observation that the destabilizing effects imparted by resonance form **15** would not completely override the observation of a β-secondary kinetic isotope effect. Tributyltin hydride reduction of azetidinone **18** was qualitatively 2−3 times faster than that of than **17** (Scheme 4). This rate difference was interpreted to arise from the well-known ability of silyl groups to stabilize radicals and carbenium ions formed β to them through hyperconjugation effects. [21] This

Scheme 4



observation suggested that a β-secondary kinetic isotope effect would, indeed, be detectable in the CS-catalyzed reaction. Moreover, it was hoped that chemical model reactions of the radical and cationic regimes would provide valid comparisons to the biochemical reaction and, in their observed β-secondary kinetic effects, take into account all of these considerations unique to reaction of an azetidinone ring.

Suspecting, therefore, that the β-secondary kinetic isotope effect was likely to be small, a triple-label experiment was undertaken to prepare proclavaminic acid **19** *dideuteriated* (to maximize the β-effect) or unlabeled at C-3′, with ^{3}H or ^{14}C, respectively, at remote, nonreacting positions such that the isotope distribution as a function of the extent of reaction would appear as a changing ^{3}H/^{14}C ratio. That is, ^{3}H would monitor the kinetic fate of dideuteriated proclavaminic acid **19**, while ^{14}C would track its protiated counterpart. [22]

Synthesis of Isotopically Labeled (*2S*,*3R*)-Proclavaminic Acid. A synthesis of enantiomerically pure proclavaminic acid (**3**) starting from β-hydroxyornithine reported previously from this laboratory was not amenable to isotopic substitution at the desired positions.[23] An alternate route was required. The success of Ojima and co-workers in the asymmetric alkylation of enantiomerically pure 4′-phenyl glycyl azetidinyl enolates suggested a similar approach.[24] Although phenyl substitution at C-4′ was not useful for our purpose because of the difficulty of its removal, the availability of 4-thiophenyl azetidinone in both the optically pure *R*- and *S*-forms,[25,26] coupled with its easy reductive removal, encouraged us to investigate the potential of asymmetric aldol condensations from the corresponding glycyl enolates (Scheme 5). Additionally, the introduction of isotopic labels was conceptually straightforward. Thus deuterium could be introduced at C-3 of 4-thiophenyl azetidinone by simple base-promoted exchange, while ^{3}H and ^{14}C could be introduced into the hydroxyornithyl side chain at several sites. We recognized the possibility of decreased selectivity in the coupling reaction owing to both the lower steric profile of thiophenyl

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(18) It is further noted that the hybridization of C-4, while nominally sp^3, will be perturbed to increase p-character to ligands in the strained, four-membered ring, and increase s-character (increase bond strength) to the C−H bonds undergoing change in the course of reaction.

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(22) All substrates were planned to be equally labeled with deuterium at both C-3 methylene positions to amplify the β-isotope effect. While the CS-catalyzed oxidative cyclization is a stereospecific process and the β-secondary kinetic isotope effect may differ depending on which hydrogen (deuterium) at C-3′ is *syn clinal* or gauche to the breaking C−H bond, this stereochemical distinction will escape analysis. However, if a fully developed p-orbital exists at the transition state, the effects of both C-3 deuteria will be the same.

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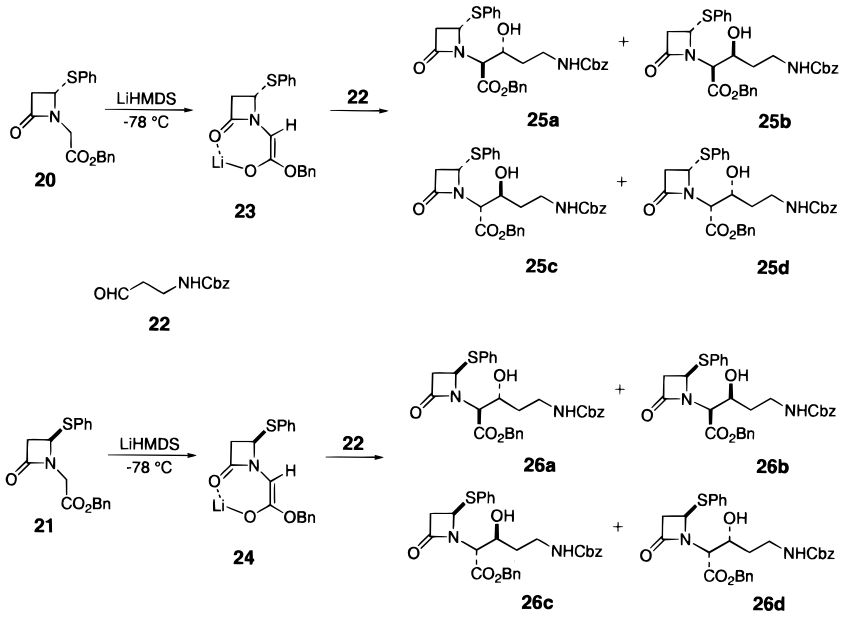
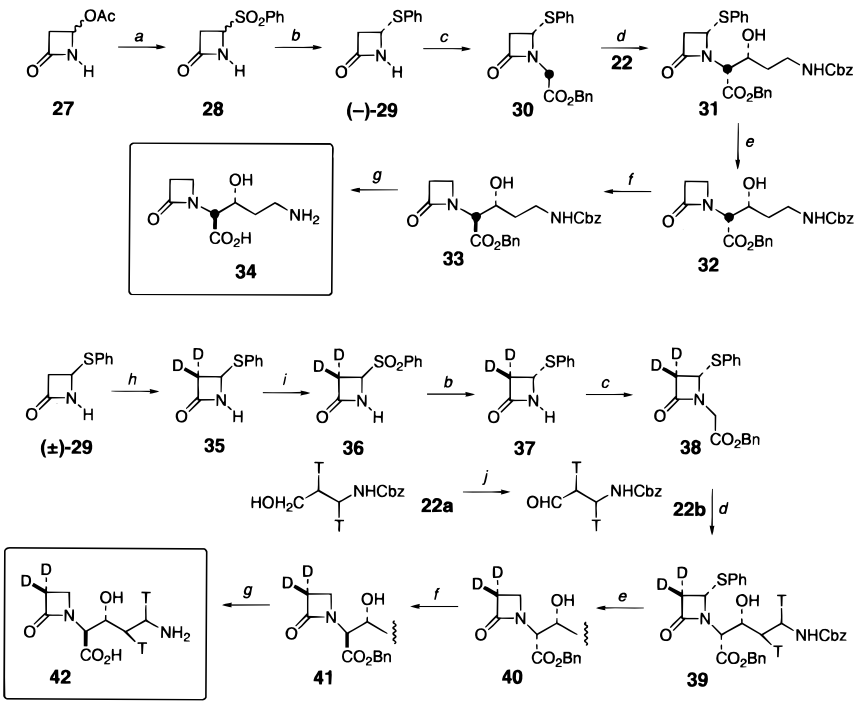
Scheme 5



Scheme 6[a]



[a] Reagents and conditions: (*a*) sodium benzenesulfinate, H_2O, 40 °C; (*b*) PhSH, (+)-cinchonine, PhH, 40 °C; (*c*) LiHMDS, −78 → −20 °C, benzyl [2-^{14}C]bromoacetate or benzyl bromoacetate; (*d*) LiHMDS, −78 °C, 2h, **22** or **22b**; (*e*) $(TMS)_3SiH$, AIBN, PhH, reflux; (*f*) DBN, CH_2Cl_2; (*g*) H_2, 10% Pd/C, EtOH; (*h*) TMSCl, Et_3N, then LDA, −78 °C, TMSCl, then KF, d_4-MeoH; (*i*) $KMnO_4$; (*j*) $(COCl)_2$, DMSO, Et_3N, −78 °C.

compared to phenyl, and the simultaneous creation of two stereogenic centers in the aldol reaction. Generation of the thermodynamic enolates **23** and **24** from the 4*S*- or 4*R*-thiophenyl azetidinones (**20** and **21**, respectively) by treatment with lithium bis(trimethylsilyl)amide at −78 °C followed by addition of *N*-Cbz protected aldehyde **22** provided a mixture of diastereomeric products in the ratios shown in Table 1 in an overall 70% yield. The reaction showed moderate *erythro* selectivity, and the desired L-*threo* isomer **25a**/**26a** was produced in only 5.5−11% as one of the minor isomers depending on which azetidinone was used. Furthermore, the four diastereomers ran in pairs in a variety of chromatographic conditions with the L-*threo* and L-*erythro* constituting one pair, thus making purification of the desired L-isomer difficult. From the remaining pair, however, the major D-*erythro* isomer **25d**/**26d** could be isolated by crystallization from hexane-methylene chloride in high purity. Reductive removal of the thiophenyl group followed by epimerization of the protected D-*erythro* proclavaminate provided the desired L-*threo* diastereomer, which could be

Table 1. Relative Ratios of Aldol Products Derived from the Addition of 20 and 21 to Cbz-protected Aldehyde 22

substrate	L-*threo*	L-*erythro*	D-*threo*	D-*erythro*
20	1.0 (**25a**)	2.0 (**25b**)	0.5 (**25c**)	3.0 (**25d**)
21	0.5 (**26a**)	3.0 (**26b**)	1.0 (**26c**)	2.0 (**26d**)

deprotected to the natural proclavaminate. The yield of the L-*threo* isomer using this approach was 15%, but deemed acceptable in view of the requirements for material of high chemical and enantiomeric purity.

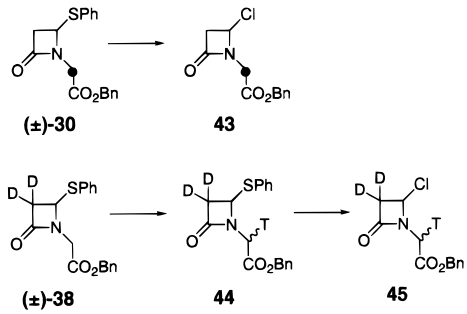
Having established a method for the preparation of enantiomerically pure proclavaminic acid, we proceeded with the synthesis of the isotopically labeled proclavaminates **34** and **42** (Scheme 6). Reaction of 4-acetoxyazetidinone **27** with sodium benzenesulfinate in water at 45 °C gave (±)-4-phenylsulfonyl azetidinone **28** in 81% yield.[26] Displacement of the phenylsulfonyl group with thiophenol in the presence of (+)-cinchonine as the asymmetric catalyst in benzene at 40 °C provided the 4-thiophenyl azetidinone in 97% yield.[25] Fractional crystallization from cyclohexane/benzene gave enantiomerically pure (−)-(4*S*)-thiophenyl azetidinone (**29**) in an optical yield of 29%. The optically impure material could be recycled by oxidizing it back to 4-phenylsulfonyl azetidinone and repeating the displacement with thiophenol and (+)-cinchonine. Replacing (+)-cinchonine with (−)-cinchinidine provided access to (+)−(4*R*)-thiophenyl azetidinone.

For the preparation of ^{14}C-labeled (•) proclavaminate **34**, the (−)-(4*S*)-isomer **29** was coupled with benzyl [2-^{14}C]bromoacetate, prepared from phenyl diazomethane and [2-^{14}C]bromoacetic acid, to give the azetidinone **30** in 47% yield.[27] Aldol condensation with *N*-Cbz-aminopropanal **22** provided the D-*erythro* diastereomer **31** in 33% yield after recrystallization. Because **31** was the last crystalline intermediate in the synthesis, the specific activity was determined at this point giving an average value of 1.22 mCi/mmol. Reduction with tris(trimethylsilyl)silane and AIBN in refluxing benzene[28] gave the protected D-*erythro* proclavaminate **32** in 68% yield. Epimerization with DBN in CH_2Cl_2 provided the L-*threo* isomer **33** in 84% yield, which was deprotected with H_2/Pd−C to give (*2S,3R*)-[2-^{14}C] proclavaminic acid **34** in essentially quantitative yield.

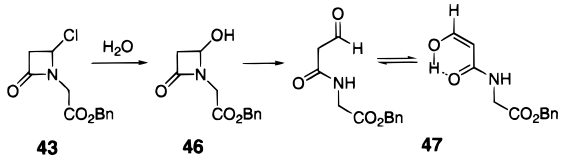
In the preparation of [$^2H_2$,^{3}H]-labeled proclavaminic acid **42,** racemic 4-thiophenyl azetidinone (±)-**29** was first deuteriated by silylating at C-3 followed by desilylation in anhydrous KF/d_4−MeOH. Four cycles of this procedure provided the dideuteriated thiophenyl azetidinone **35**. High resolution ^{1}H NMR spectroscopy revealed an incorporation of 90% deuterium at each methylene position. An attempt to achieve deuteriation at C-3 by anion formation with LDA followed by addition of D_2O was largely unsuccessful. The alkaline medium generated after quenching led to considerable decomposition of the product. Oxidation of **35** with *m*-CPBA gave the phenylsulfonyl azetidinone **36**, which was further elaborated to provide the (−)-(4*S*)-thiophenyl azetidinone **37**. Mass spectrometric analysis gave a deuterium content of 81% $^2H_2$, 18% $^2H_1$, and 1% $^2H_0$ confirming the earlier NMR estimation of ∼90% *d*/site. Coupling of the benzyl azetidinyl acetate **38** with [2,3-^{3}H]-*N*-Cbz propanal **22a** followed by the steps described above provided the [3′-$^2H_2$,2,3-^{3}H]proclavaminic acid **42** with a specific activity of 2.44 mCi/mmol.

Development of Model Systems. For the best kinetic isotope effect comparisons to be made, the model and CS-catalyzed reactions ought to mimic one another as closely as possible. Important criteria for the model were that it ideally should be a single compound, which could react by either a radical or cationic mechanism, and it should be readily synthesized from a dideuteriated intermediate common to the synthesis of the labeled proclavaminic acids. In this way, the deuterium contents of all systems used in the kinetic analysis would be the same. In view of these requirements, we selected 4′-chloroazetidinone **43**. Azetidinones substituted with chlorine at C-4 are known to undergo a variety of ionic reactions,[29] and their reactivities under radical conditions is well precedented.[30,31]

Scheme 7



Scheme 8



Synthesis of the labeled chloroazetidinones is illustrated in Scheme 7. Preparation of the ^{14}C material (•) was accomplished in a simple one-step procedure involving chlorinolysis of the [2-^{14}C]-4′-thiophenyl azetidinone (±)-**30** with Cl_2 in CCl_4 to give the [2-^{14}C]-4′-chloroazetidinone **43** in approximately 60% yield after crystallization (specific activity 0.131 mCi/mmol). The synthesis of [3′-$^2H_2$,2-^{3}H]-4′-chloroazetidinone **45** involved introduction of tritium by treating (±)-**38** with 0.75 equiv of LiHMDS in THF at −78 °C followed by quenching with [^{3}H]-H_2O in THF to produce **44**. Control experiments showed that no exchange occurred in the β-lactam ring under these conditions. The latter on chlorinolysis gave **45**, which had a specific activity of 0.415 mCi/mmol after recrystallization.

To monitor the reactions at different time points, we adopted the same HPLC assay used previously.[7] Initial work revealed that the chloroazetidinone eluted as a single peak with a retention time of approximately 8 min in a solvent system of water−acetonitrile (70:30). We subsequently discovered that the peak was due not to the chloroazetidinone, but to its hydrolysis product **47**. Hydrolysis of **43** to **46** was very rapid, and the ring spontaneously opened to **47**. However, the reaction went to completion with no loss of tritium or ^{14}C. Although deuterium is lost in the hydrolysis, owing to facile exchange during enolization (Scheme 8), the measured ^{3}H/^{14}C ratio in the hydrolyzed product would be identical to that in the labeled chloroazetidinone initially isolated (see Scheme 5). Because of the simplicity of the reactions and the subsequent ease of workup, we chose an alkylsilane-mediated radical reduction of **43**/**45** to the azetidione **48** (Scheme 8) and their acetolysis to the 4′-acetoxyazetidinone **49** as the model radical and cationic reactions, respectively. To avoid extrapolations brought about by the effect of temperature on the magnitude of measured isotope effects,[32] all of the experiments were conducted at 25

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°C. The radical reaction was run in a quartz test tube under argon at constant temperature with irradiation provided by a medium-pressure Hg lamp equipped with a Vycor filter. The acetolysis reaction was carried out by treating **43/45** with anhydrous acetic acid at 25 °C under an argon atmosphere. Termination of the reaction at various time points was achieved by immediately freezing the sample in dry ice. Experiments with a second, less polar solvolytic system employing ethanol−THF (1:1) were carried out in an analogous fashion.

Isotope Effect Determinations in the CS-Catalyzed and Chemical Model Reactions. Theory. For the general case of a substrate in an irreversible reaction substituted with a heavy isotope in *macroscopic* amounts, the isotope effect, $^{D}(V/K)$, is described for residual substrate by

$$^{D}(V/K) = \ln[(1 - x)(1 + S_0)/(1 + S_x)]/\ln[(1 - x)(1 + S_0)/(1 + S_x)S_x/S_0] \quad (1)$$

where x is the total fractional extent of reaction and S is the substrate D/H ratio at the subscripted extent of reaction.[16] When tritium and ^{14}C are used as remote labels for deuterium and protium, respectively, and deuteriation at the site of interest is complete, that is, every molecule containing ^{3}H also contains ^{2}H, then the $^{3}H/^{14}C$ ratio can be used directly as a measure of S in eq 1. However, if deuterium labeling is less than 100%, then there are three substrate species to consider: A_1, which contains deuterium and tritium, A_2, which contains protium and tritium, and A_3, which contains protium and ^{14}C. The D/H ratio is now given by

$$S = A_1/(A_2 + A_3) \quad (2)$$

while the experimentally measured quantity, the $^{3}H/^{14}C$ ratio, is given by

$$S' = (A_1 + A_2)/A_3 \quad (3)$$

To correlate S' with S, one first needs to translate the $^{3}H/^{14}C$ ratio, S', to an apparent D/H mole ratio. The D/H ratio described by eq 2 is a macroscopic mole ratio of the various deuterated and protiated substrate molecules, while eq 3, as defined by $^{3}H/^{14}C$ ratios, is a microscopic quantity and is only a measure of the apparent macroscopic D/H mole ratio when the specific activities of the ^{3}H and ^{14}C labeled substrates are equivalent. The conversion of the microscopic $^{3}H/^{14}C$ ratios, S', to the macroscopic mole ratios, M, is given by

$$M = S'(a_C/a_H) \quad (4)$$

where a_C equals the specific activity of ^{14}C-labeled substrate and a_H equals the specific activity of ^{3}H-labeled substrate. In the case where the two specific activities are equivalent, then M is equal to S'. One can then use the fact that the tritium and ^{14}C-labeled substrates bearing hydrogen at the site of interest (A_2 and A_3), will show no isotope effect throughout the reaction, and their ratio (A_2/A_3), defined as K, will, therefore, remain constant. It follows then that

$$S = A_1/(A_2 + A_3) \quad (5)$$

$$= (A_1/A_3)/(1 + A_2/A_3) \quad (6)$$

$$= (A_1/A_3)/(1 + K) \quad (7)$$

and

$$S' = (A_1 + A_2)/A_3 \quad (8)$$

$$= A_1/A_3 + K \quad (9)$$

or

$$A_1/A_3 = S' - K \quad (10)$$

Therefore

$$S = (S' - K)/(1 + K) \quad (11)$$

$$S' = S(1 + K) + K \quad (12)$$

K can be easily determined from the experimental design as

$$K = (1 - F)M_0 \quad (13)$$

where F is the fraction of deuterium labeling in the substrate and M_0 is the initial apparent D/H mole ratio. Substituting eq 11 for S_0 and S_X in eq 1 then allows for the calculation of the isotope effect.

An analogous derivation can be made for calculating the isotope effect based on product formation, in this case the relevant equation is

$$^{D}(V/K) = \ln[1 - x(1 + S_0)/(1 + R_x)]/\ln[1 - x(1 + S_0)/(1 + R_x)(R_x/S_0)] \quad (14)$$

where R_x is the product D/H ratio at the subscripted extent of reaction and the other symbols are defined as above. It can be seen that for calculations based on residual substrate, measurements made after 50% of reaction are the most sensitive to changes in the $^{3}H/^{14}C$ ratio, while the corresponding calculations based on product formation are most sensitive for measurements made at 0−50% reaction. Furthermore, from a comparison of the maximum measurable change in specific activity, it is clear that eq 1 for residual substrate is inherently more sensitive. For this reason, and in order to make a manageable number of samplings of the reactions, all of our analyses were based on calculations of residual substrate specific activities.

Experiment. Incubations of [3′-$^{2}H_2$,4,5-^{3}H]- and [2-^{14}C]-(*2S,3R*)-proclavaminates **42/34** (Scheme 6) with clavaminate synthase were performed in triplicate and the reaction mixtures analyzed at different time points after separation of the components by HPLC (see Experimental Section). The extent of reaction was determined from *both* the ^{14}C and tritium distributions in the reaction as noted above. A representative HPLC radiochromatogram for an incubation of the labeled clavaminates with CS is shown (Figure 1A). The relevant peaks are well resolved. Fitting the radiochemical data to eq 1 gave a β-secondary isotope effect $^{D}(V/K) = 1.056 \pm 0.002$. Superimposition of the data from three different experiments (Figure 1B) demonstrates that eq 1 is followed well.

A representative radiochromatogram for the radical-mediated reduction of benzyl [3′-$^{2}H_2$,2-^{3}H]- and [2-^{14}C]-4′-chloroazetidinyl acetate **45/43** (Scheme 7) in acetonitrile−THF is presented in Figure 2A and a corresponding radiochromatogram for the acetolysis in Figure 3A. The data are summarized in Figures 2B and 3B, respectively. Fitting the radiochemical data from triplicate experiments to eq 1 gave a β-secondary isotope effect of 1.081 ± 0.002 for the radical reaction. Similar data fitting for the acetolysis reaction gave an unexpectedly low isotope effect of 1.040 ± 0.002. In contrast, the solvolysis reaction carried out in the less polar EtOH−THF mixture (Figure 3C) proceeded with an isotope effect of 1.098 ± 0.005, substantially

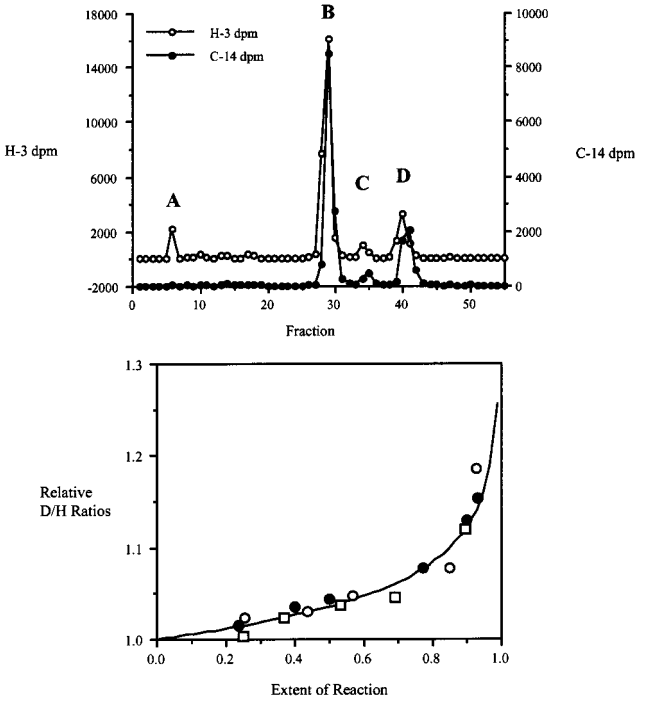


Figure 1. (Top) Radiochromatogram for the reaction of CS with [4,5-^{3}H,3′-^{2}H$_2$,2-^{14}C]-(2*S*,3*R*)-proclavaminate (**42**/**34**) terminated at extent of reaction = 0.306. The retention times of reaction components are as follows: (A) [^{3}H]water, 2.5 min.; (B) proclavaminate, 12 min; (C) dihydroclavaminate, 14 min; (D) clavaminate, 17 min. (Bottom) Relative D/H ratios of [4,5-^{3}H,3′-^{2}H$_2$,2-^{14}C]-(2*S*,3*R*)-proclavaminic acid as a function of extent of reaction upon incubation with CS from triplicate experiments. The curve was drawn using eq 1 with a β-secondary isotope effect = 1.056, K = 0.1334, and R_0 = 1.047.

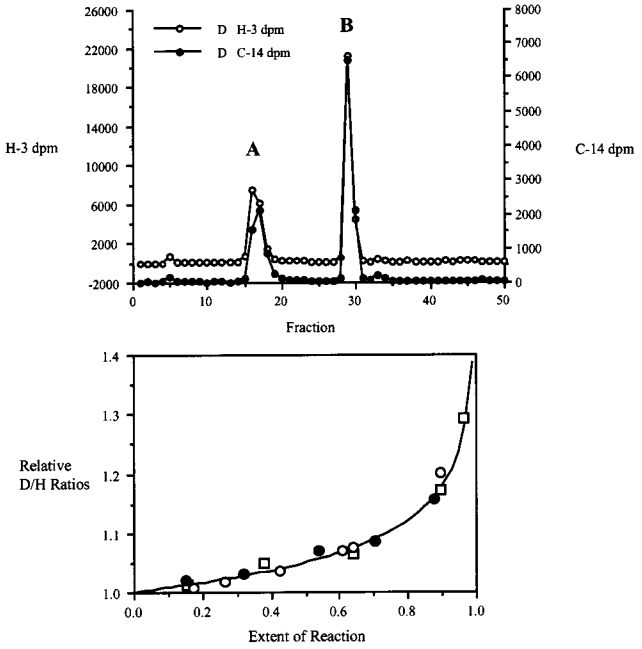


Figure 2. (Top) Radiochromatogram for the radical mediated reduction of benzyl [3′-^{2}H$_2$,2-^{3}H,2-^{14}C]-4′-chloroazetidinyl acetate (**45**/**43**) terminated at extent of reaction = 0.639. The retention times of the reaction components are as follows: (A) (**45**/**43**), 9 min; (B) benzyl [3′-^{2}H$_2$,2-^{3}H,2-^{14}C]-azetidinyl acetate, 15 min. (Bottom) Relative D/H ratios of benzyl [3′-^{2}H$_2$,2-^{3}H,2-^{14}C]-4′-chloroazetidyl acetate (**45**/**43**) upon the radical mediated reduction to azetidinone (**48**) from triplicate experiments. The curve was drawn using eq 1 with a β-secondary isotope effect = 1.081, K = 0.0975, and R_0 = 0.7997.

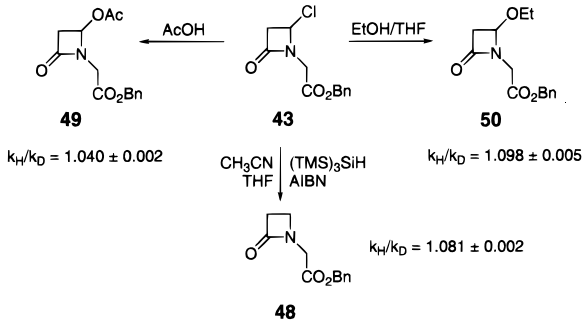
higher than that for the acetolysis reaction. These findings are summarized in Scheme 9.

Analysis. The experimentally determined β-secondary kinetic isotope effects for the CS-catalyzed and chemical model reactions were relatively small and represent the effect of *two* deuteria at the remote site. This outcome was anticipated by the presence of the azetidinone nitrogen adjacent to the site of reaction, and the antiaromatic character imparted to the β-lactam ring by the hyperconjugative interaction directly responsible for expression of the isotope effect itself (cf. **15** and **16**, Scheme 3). As all reactions were run at 25 °C and based on a common synthetic intermediate having the same deuterium content, we believe the reactions can be justly compared. Particularly surprising was the observation that solvolysis in acetic acid should yield a β-secondary isotope effect one-half the magnitude of a corresponding radical reaction (Scheme 9).

Silanes are known to function as hydride donors under some conditions.[33,34] Although neither the reaction conditions of the radical model, nor the geometric constraints against chloride displacement in a four-membered ring seemed to favor such a process, a control reaction was carried out. Thus, [2-^{14}C]-chloroazedinone **43** was exposed to tris(trimethylsilyl)silane in acetonitrile−THF (1:1) and AIBN at 25 °C under an inert atmosphere, but in the absence of UV irradiation. After 6 h, only starting material was present, demonstrating that reduction proceeded, as expected, by a radical mechanism only.

For a given molecular structure, a compelling argument can be made that the β-secondary kinetic isotope effect should be

Scheme 9



larger for a carbenium ion than the corresponding radical. This case can be made on the basis of the energetics of the interacting orbitals[17] and rotational barriers at radical and cationic centers.[35] Thus, while a special circumstance might apply in the case of the β-lactam ring, we take the view that the polarity or proton-donating ability of the medium is unusually important in this particular instance. That is, hyperconjugation affords the azetinone structure **16** (Scheme 3). In a polar environment the contribution of structure **14** will be enhanced and hyperconjugation will lead to the unstable antiaromatic azacyclobutadiene configuration **15**. That is, the β-secondary kinetic isotope effect will be maximally suppressed as amide resonance is maximally stabilized. The overriding importance of this effect is apparent when the solvolysis is run in a less acidic medium, ethanol−THF (1:1), and a significantly higher β-secondary isotope effect is observed. The principal driver of the experimentally determined kinetic isotope effect, therefore, would appear to be solvent stabilization of amide resonance, behavior favored by

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(34) Kursanov, D. N.; Loim, N. M.; Baranova, V. A.; Moiseeva, L. V.; Zalukaev, L. P.; Parnes, Z. N. *Synthesis* **1973**, 420−422.
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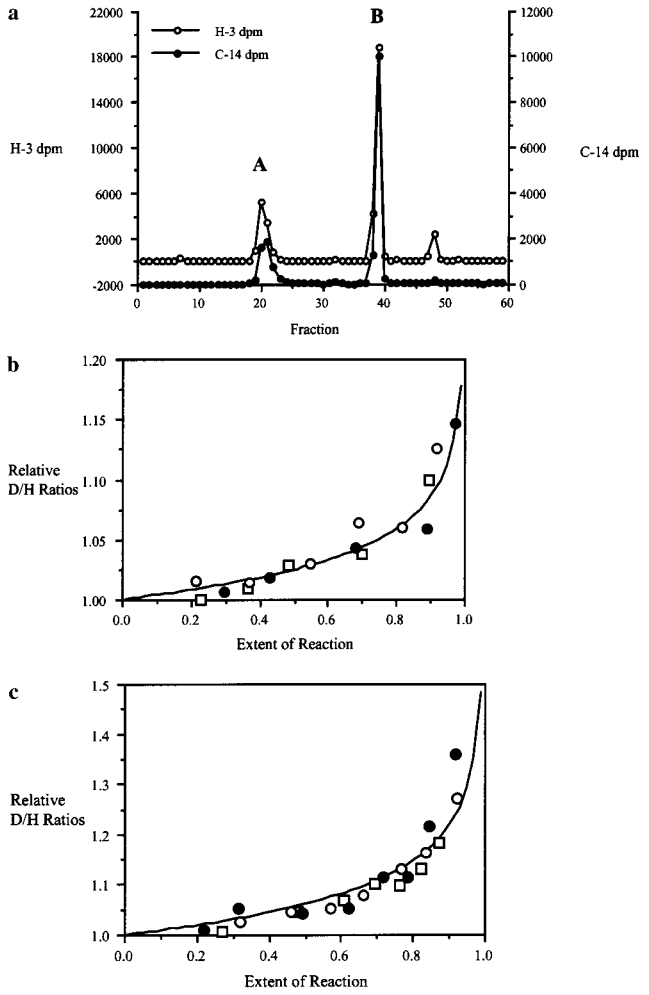


Figure 3. (Top) Radiochromatogram for the acetolysis of benzyl [3′-$^2H_2$,2-^{3}H,2-^{14}C]-4′-chloroazetidinyl acetate (**45**/**32**) terminated at extent of reaction = 0.701. The retention times of the reaction components are as follows: (A) (**45**/**43**), 10 min; (B) benzyl [3′-$^2H_2$,2-^{3}H,2-^{14}C]-4′-acetoxyazetidinyl acetate, 18 min. (Bottom left) Relative D/H ratios of benzyl [3′-$^2H_2$,2-^{3}H,2-^{14}C]-4′-chloroazetidinyl acetate (**45**/**43**) upon the acetolysis to acetoxyazetidinone (**49**) from triplicate experiments. The curve was drawn using eq 1 with a β-secondary isotope effect = 1.040, K = 0.0733, and R_0 = 0.6131. (Bottom right) Relative D/H ratios of benzyl [3′-$^2H_2$,2-^{3}H,2-^{14}C]-4′-chloroazetidinyl acetate **45**/**43** upon the ethanolysis to ethoxyazetidinone **50** from triplicate experiments. The curve was drawn using eq 1 with a β-secondary isotope effect = 1.098, K = 0.0733, and R_0 = 0.6131.

acetic acid and, to a lesser extent, by ethanol−THF. Superimposed on the relatively large variation in β-secondary kinetic isotope effect brought about by medium effects is the question of mechanism. Whatever kinetic effect is measured for the radical, it will likely be larger for carbenium ion formation at C-4′.

This being so, it is noteworthy that the β-secondary effect observed for the enzymic reaction $^T(V/K) = 1.056 \pm 0.002$ is smaller than that observed for the radical reaction in acetonitrile−THF (1:1; ϵ = 36.6 and 7.5, respectively). At least two factors can account for this difference, although their combined significance cannot be firmly established. First, the commitment to catalysis, while probably low, is not accurately known as the intrinsic primary effect will surely be reduced by the adjacent azetidinone nitrogen and may be only partially rate-determining. Even if this were calculable, however, the effective dielectric of the enzyme active site is not known.[36] The apparent suppression of the β-secondary kinetic isotope effect raises the interesting question about the extent of polarization of the lactam bond during CS-catalyzed oxazolidine ring formation. The low value of the β-secondary effect suggests that it might be quite highly polarized. Advantage to catalysis could result from this phenomenon by favoring the formation of a radical over a carbenium ion at C-4′ and, thereby, lowering the activation energy for the difficult catalytic task of removing hydrogen from an otherwise comparatively unactivated C−H bond of increased s-character.

Conclusion

While the experimental data make distinction between a radical and cationic mechanism of oxidative cyclization uncertain, on purely chemical grounds we favor formation of an iron-oxo intermediate as **52** followed by a homolytic reaction path in which activated molecular oxygen abstracts the α-hydrogen atom (4′-*proS*) from proclavaminic acid (**3**) in the rate-determining step to give a radical as **53**. The unexpected observation of an especially small β-secondary deuterium kinetic isotope effect, however, could point to a high degree of amide resonance in the transition state of C-4′−H bond cleavage. Such polarization may favor a radical intermediate as it would be intrinsically less stabilized than a carbenium ion by hyperconjugative interaction leading to the antiaromatic species **15**. Whether this radical species undergoes direct insertion of the substrate oxygen (path A, Scheme 10),[7,37] or by electron transfer gives the cation/acyliminium **54** followed by ionic cyclization (path B) cannot be distinguished at present. Such a process may, or may not, involve coordination of proclavaminate to iron as shown in Scheme 10.[7] Altermatively, transient generation of an iron−carbon bond in **55** can be visualized. The existence of an open coordination site on iron in both IPNS[5] and CS[4] (e.g., **51**) prior to oxygen binding opens the possibility of an agostic mechanism[38] in which the metal inserts into the C-4′−H bond.[39] We regard such a reaction path by Fe(II) as highly unlikely. Finally, a simultaneous process could be advanced in which C-4′ hydrogen abstraction is synchronous with iron−carbon bond formation (**56**, path D). An analogous mechanism has been proposed for the second oxidative cyclization catalyzed by IPNS.[13,40,41] To the extent that C−H bond breaking and C−Fe bond forming are synchronous, it would seem unlikely that reactive intermediates would be stabilized by hyperconjugation, or that the metal center would be sufficiently close to have an agostic interaction with a C-3′ hydrogen. Little, if any, β-secondary kinetic isotope effect would be expected, therefore, for reaction by this mechanism, and the primary kinetic isotope effect would be predicted to be small as well. We favor an explanation for the kinetic behavior of clavaminate synthase based on the electronic constraints imposed by an azetidinone

(36) This is likely to be quite variable and not accurately represented by a macroscopic value (Warshel, A. *J. Biol. Chem.* **1998**, *273*, 27035−27038). Nonetheless, macroscopic dielectrics for active sites have been variously estimated to range from 4 to 40 with 10 taken as an average value (Warshel, A. *Annu. Rev. Biophys. Biophys. Chem.* **1991**, *20*, 267−298). Jordan has experimentally determined an ϵ = 13−15 in a very recent example (Jordan, F.; Li, H.; Brown, A. *Biochemistry* **1999**, *38*, 6369−6373).

(37) Townsend, C. A. *Biochem. Soc. Trans.* **1993**, *21*, 208−213.

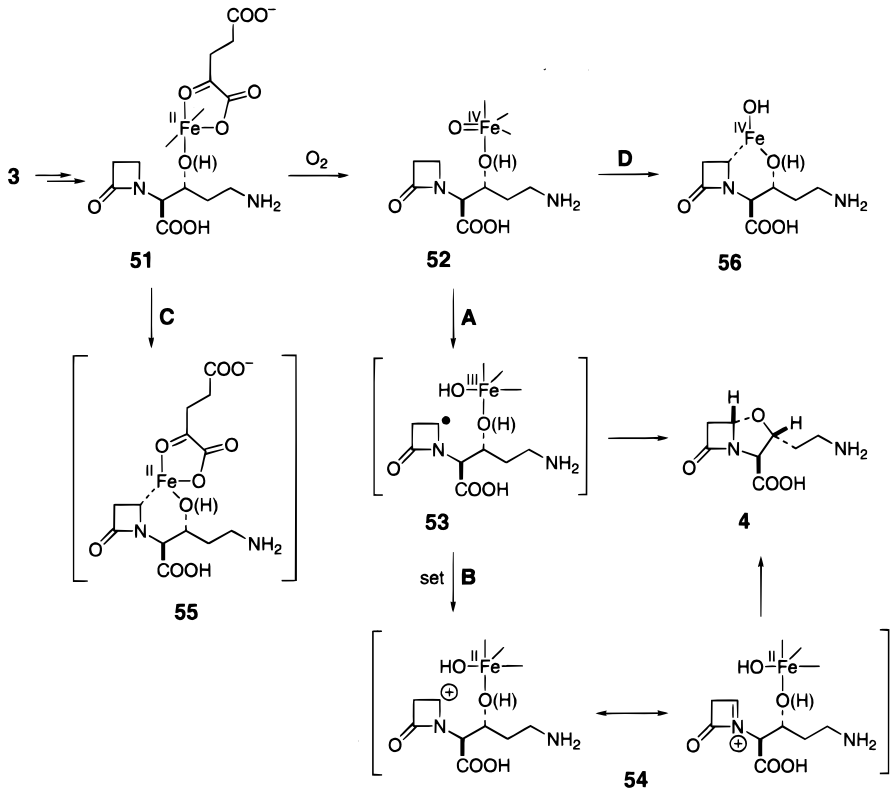
(38) Brookhart, M.; Green, M. L. H. *J. Organomet. Chem.* **1983**, *250*, 395−408.

(39) The microscopic reverse of this process would be protodemetalation. For the case of protodemercurations, β-secondary kinetic isotope effects have been observed to be very low or nonexistent, in contrast to the CS-catalyzed reaction (Bencivengo, D. J.; Brownawell, M. L.; Li, M.-Y.; San Filippo, Jr., J. *J. Am. Chem. Soc*. **1984**, *106*, 3703−3704).

(40) Baldwin, J. E.; Adlington, R. M.; Marquess, D. G.; Pitt, A. R.; Porter, M. J.; Russell, A. T. *Tetrahedron* **1996**, *52*, 2537−2556.

(41) Baldwin, J. E.; Adlington, R. M.; Marquess, D. G.; Pitt, A. R.; Russell, A. T. *J. Chem. Soc., Chem. Commun.* **1991**, 856−858.

Scheme 10



ring and, to a lesser extent, to commitment effects. The alternative rationale implied by synchronous C−H bond breaking and C−Fe bond forming would bypass the formation of discrete radical or ionic intermediates. As a consequence, the attendant rehybridization at C-4′ would be avoided, and, hence, expression of kinetic isotope effects would be markedly decreased. While this view cannot be strictly excluded, we regard it as less likely based on the magnitude of the primary kinetic isotope effect observed, the three model reactions presented and the effect that amide resonance may play in the transition state of the CS-catalyzed reaction.

Experimental Section

All air- or moisture-sensitive reactions were run under an inert atmosphere (Ar or N_2) in flame- or oven-dried glassware with magnetic stirring unless otherwise noted. Moisture-sensitive reagents were added to reaction vessels by dry syringes equipped with oven-dried needles through rubber septa. Reaction temperatures refer to bath temperatures.

Tetrahydrofuran (THF) and diethyl ether (Et_2O) were freshly distilled from Na/benzophenone ketyl; dichloromethane (CH_2Cl_2) was freshly distilled from CaH_2. All other solvents and reagents for air- or moisture-sensitive reactions were used as received or dried by standard procedures (Perrin, D. D.; Armarego, W. L. F.; Perrin, D. R. *Purification of Laboratory Chemicals*; Pergamon: Oxford, 1980.)

^{1}H and ^{13}C NMR spectra were obtained on a Varian XL/VXR-400 or a Bruker AMX 300 NMR spectrometer. Chemical shifts of hydrogen resonances are reported on the δ-scale and referenced to tetramethylsilane (0.0 ppm), deuteriochloroform (7.26 ppm), or acetone-d_6 (2.23 ppm). Coupling constants are reported in Hertz. Carbon-13 chemical shifts are also reported on the δ-scale and referenced to deuteriochloroform (77.0 ppm), or *p*-dioxane-d_8 (66.5 ppm). Low- and high-resolution mass spectrometric data were obtained on a VG Instruments 70-S GC/MS at 70 eV and are tabulated as *m*/*z* (intensity relative to the base peak). IR spectra were obtained on a Perkin-Elmer 1600 Series FT-spectrophotometer ($CHCl_3$ solution or a KBr disk). Ultraviolet−visible spectra were obtained on Beckman DU 70 spectrophotometer. Optical rotations were obtained with a Perkin-Elmer 141 polarimeter using a 1 dm cell at 25 °C, with concentrations expressed in g/100 mL. Melting points were determined in open capillary tubes with a Thomas-Hoover Uni-Melt melting point apparatus and are uncorrected. Microanalysis were performed by Atlantic Microlab, Inc. of Norcross, GA. A Cahn 25 Automatic Electrobalance was used for the accurate weighing of micro-samples (50 μg − 4 mg). Radioactive samples were quantitated by liquid scintillation counting using a Beckman LS 5801 Liquid Scintillation Counter with Opti-Fluor (Packard) scintillation fluid. All MPLC (flash) chromatography was carried out using Merck Kieselgel 60 (230−400 mesh) silica gel. Radial chromatography was performed on a Chromatotron (Harrison Research), using rotors prepared with silica gel PF-254 with $CaSO_4 \cdot 0.5H_2O$ as binder. TLC analysis was performed using Analtech Uniplate TLC plates (cat # 21521) with basic $KMnO_4$ (1% in 6.5% Na_2CO_3/0.2% KOH for visualization. HPLC chromatography was performed with a Waters 600 multisolvent delivery system equipped with a Rheodyne injector and a Waters 490 programable multiwavelength detector. Fractions were collected with an Isco Cygnet automatic fraction collector. Reaction temperatures for kinetic isotope effect experiments were maintained with a Lauda RM-6 circulating bath. Photochemically initiated reductions were performed in a quartz test tube with illumination from a Hanovia medium-pressure mercury lamp (no. 679A36) equipped with a Vycor sleeve. Micro-samples were concentrated under high vacuum in a Savant SVC 100D circulating speed vacuum.

Synthesis of (2*S*,3*R*)-[2-^{14}C]-Proclavaminic Acid (34). 4-Phenylsulfonyl-azetidinone (28). 4-Acetoxyazetidinone (**27**, 13.15 g, 101.8 mmol), H_2O (120 mL), and sodium benzenesulfinate (20.05 g, 122.2 mmol were stirred in an oil bath (40 °C) for 17 h. The product was partioned between ethyl acetate (200 mL) and H_2O (80 mL additional) and the aqueous layer back-extracted with ethyl acetate (3 × 200 mL). The combined organic layers were washed with saturated brine (2 × 600 mL), dried (anhydrous Na_2SO_4), and concentrated in vacuo to give a white solid. Recrystallization from ethyl acetate/petroleum ether gave the desired product as white prisms (15.13 g, 71.61 mmol, 70%): mp 155.5−157 °C [lit.[26] mp 156−157 °C].

(4*S*)-4-Thiophenyl-azetidinone (29). To a solution of the azetidinone **28** (15.13 g, 71. 61 mmol) in benzene (1.5 L) were added (+)-cinchonine (25.30 g, 85.94 mmol and thiophenol (25 mL, 24 mmol),

and the mixture was stirred at 40 °C under argon for 41 h. The mixture was filtered through a pad of silica gel washing with benzene to remove the cinchonine, the filtrate concentrated in vacuo, and the residue applied to a column of silica gel (60 g, benzene). The column was eluted with benzene until thiophenol was no longer detected in the eluent, and elution was continued with ethyl acetate to recover the desired product. Concentration in vacuo gave a white solid, which when recrystallized from benzene/cyclohexane and methylene chloride/petroleum ether, gave optically pure **29** (3.724 g, 20.77 mmol, 29%) as white needles and optically impure product (8.342 g, 46.54 mmol, 65%) as white plates. The optically pure material gave the following physical and spectral data: mp 68−69 °C [lit.[25] mp 58−60 °C]; $[\alpha]_D = -137°$ (*c* = 1.0, $CHCl_3$) [lit.[25] $[\alpha]_D = -105°$]; ^{1}H NMR ($CDCl_3$) δ 7.49−7.37 (m, 5H, Ar*H*), 6.27 (b, 1H, N*H*), 5.03 (dd, *J* = 2.4, 4.9 Hz, 1H, H-4), 3.40 (ddd, *J* = 1.9, 5.0, 15.3 Hz, 1H, H-3), 2.96 (dd, *J* = 2.4, 15.3 Hz, 1H, H-3); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 166.1, 133.4, 131.3, 129.3, 128.6, 54.2, 45.4; MS *m*/*z* 179 (M^+, 7%), 119, 110, 77, 70 (100%); accurate mass 179.0406, calcd for C_9H_9NOS 179.0405.

Benzyl [2-^{14}C]-(4′*S*)-(4′-thiophenyl-2′-oxoazetidin-1′-yl) acetate (30). A solution of azetidinone (−)-**29** (161.8 mg, 0.9027 mmol) in THF (13 mL) was cooled to −78 °C, and a solution of LiHMDS (950 μL, 1.0 M in hexane) was added dropwise via syringe in 5 min. After 50 min, [2-^{14}C]-methyl bromoacetate (196.1 mg, 0.8561 mmol) in THF (5 mL) was added dropwise via syringe over 10 min. The reaction was warmed to −20 °C over 45 min and stirred for 1 h at −20 °C. After the reaction was quenched with a solution of acetic acid in THF, ethyl acetate (100 mL) was added, and the mixture was washed with 1 N HCl (100 mL), 5% $NaHCO_3$ (100 mL) and saturated brine (2 × 100 mL). The organic layer was dried (anhydrous Na_2SO_4) and concentrated in vacuo, and the residue was purified by radial chromatography (1 mm silica gel; ethyl acetate:hexane, 1:19 to 1:1) to give the desired product (131.3 mg, 0.4011 mmol, 47%) as a colorless oil. Radioinactive material prepared similarly gave the following spectral data: $[\alpha]_D = +98.5°$ (*c* 0.72, $CHCl_3$) IR ($CHCl_3$) 3016, 1767, 1405, 1387, 1345, 1260, 1230, 1188, 941 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.41−7.28 (m, 10H, Ar*H*), 5.22 (dd, *J* = 2.3, 5.1 Hz, 1H, H-4′), 5.11 (ABq, *J* = 12.1 Hz, 2H, C*H*$_2$Ph), 4.32 (*A*Bq, *J* = 18.2 Hz, 1H, H-2), 3.78 (A*B*q, *J* = 18.2 Hz, 1H, H-2), 3.18 (dd, *J* = 5.1, 15.2 Hz, 1H, H-3′β), 2.88 (dd, *J* = 2.3, 15.2 Hz, 1H, H-3′α); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 167.7, 165.3, 134.8, 133.8, 130.1, 129.3, 128.7, 128.6, 128.5, 67.3, 58.9, 36.6, 41.1; MS *m*/*z* 327(M^+, 0.3%), 218 (M^+ − SPh, 9%), 181, 176, 135, 109, 91 (100%); accurate mass 327.0931, calcd for $C_{18}H_{17}NO_3S$, 327.0929.

Benzyl (2*R*,3*R*,4′*S*)-[2-^{14}C]-5-(*N*-Benzyloxycarbonyl)-amino-3-hydroxy-2-(4′-thiophenyl-2′-oxoazetidin-1′-yl)pentanoate (31). Azetidinone 30 (131.3 mg, 0.4011 mmol) in THF (10 mL) was cooled to −78 °C, a solution of LHMDS (480 μL, 1.0 M in hexane) was added dropwise via syringe and the reaction stirred for 2 h. Cbz-aldehyde 22b (202.9 mg, 0.9791 mmol) in THF (3 mL) was then added dropwise and the mixture stirred a further 2 h. After the reaction was quenched by the addition of acetic acid in THF, the mixture was diluted with ethyl acetate (70 mL) and washed with 1 N HCl (50 mL), 5% $NaHCO_3$ (50 mL), and saturated brine (50 mL). The organic layer was dried (anhydrous Na_2SO_4) and concentrated in vacuo, and the residue was purified via radial chromatography (2 mm silica gel; ethyl acetate: hexane, 1:19 to 1:1), to give the starting azetidinone 30 (25.0 mg, 0.076 mmol, 19%), and the desired D-*erythro* 31 (59.1 mg, 0.111 mmol, 28%) as a white solid. Recrystallization from ethyl acetate/cyclohexane provided the product as fine, white needles (48.8 mg, 0.091 mmol, 23%), with a specific activity of 1.22 mCi/mmol. Radioinactive and nondeuteriated material prepared similarly gave the following physical and spectral data: mp 102−103 °C; $[\alpha]_D = +73°$ (*c* 1.4, $CHCl_3$); IR ($CHCl_3$) 3446, 3006, 2950, 1748, 1735, 1509, 1261, 1233, 1216, 1188, 1040 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.36−7.28 (m, 15H, Ar*H*), 5.21 and 5.12 (ABq, *J* = 11.2 Hz, 2H, C*H*$_2$Ph), 5.11 (s, 2H, C*H*$_2$Ph), 5.22 (bs, 1H, N*H*), 4.96 (bs, 1H, O*H*), 4.88 (dd, *J* = 2.4, 5.1 Hz, 1H, H-4′), 4.20 (m, 1H, H-3), 3.99 (bs, 1H, H-2), 3.45−3.31(m, 2H, H-5), 3.34 (dd, *J* = 5.1, 15.4 Hz, 1H, H-3′β), 2.89 (dd, *J* = 2.1, 15.3 Hz, 1H, H-3′α), 2.05−1.88 (m, 2H, H-4); MS *m*/*z* 425 (M^+ − SPh, 2.2), 407 (M^+ − SPh − H_2O), 383 (M^+ − SPh − CH_2CO), 327, 219, 218, 176, 91 (100); accurate mass 425.1717, calcd for $C_{23}H_{25}N_2O_5$ (M^+ − SPh) 425.1713; Anal. Calcd for $C_{29}H_{30}N_2O_5S$: C 65.15, H 5.66, N 5.24, S 6.00; found: C 65.00, H 5.61, N 5.18, S 5.90.

Benzyl (2*R*,3*R*)-[2-^{14}C]-5-(*N*-Benzyloxycarbonyl)-amino-3-hydroxy-2-(2′-oxoazetidin-1′-yl)pentanoate (32). Thiophenyl β-lactam 31 (48.8 mg, 0.091 mmol), tris(trimethylsilyl)silyl hydride (75 μL, 0.24 mmol), and AIBN (∼5) were disolved in dry, distilled benzene (4 mL) and the solutionwas degassed by bubbling Ar for 30 min. The reaction mixture was brought to reflux with stirring. Additions of tris-(trimethylsilyl)silyl hydride (3 × 75 μL) and AIBN (3 × 5 mg) were made over the next 15 h, and the reaction was terminated after 19 h. The benzene was removed in vacuo and the residue purified via radial chromatography (1 mm silica gel; ethyl acetate:hexane, 1:4 to 100% ethyl acetate), giving 26.3 mg (0.062 mmol, 68%) of the desired product as a colorless oil. Radioinactive and nondeuteriated material prepared similarly gave the following physical and spectral data: $[\alpha]_D = -24°$ (*c* 1.7, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.36 (m, 10H, Ar*H*), 5.22 (ABq, *J* = 12.0 Hz, 2H, C*H*$_2$Ph), 5.16 (bs, 1H, N*H*), 5.09 (s, 2H, C*H*$_2$Ph), 4.65 (bs, 1H, O*H*), 4.18 (m, 1H, H-3), 4.08 (br, 1H, H-2), 3.43 (m, 1H, H-5), 3.32 (m, 2H, H-4′), 3.25 (m, 1H, H-5), 2.95 (t, *J* = 4.0 Hz, 2H, H-3′), 1.87 (m, 1H, H-4), 1.78 (m, 1H, H-4); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 168.5, 168.0, 156.8, 136.4, 135.0, 128.5, 128.3, 128.1, 127.9, 127.8, 69.1, 67.1, 66.5, 61.7, 39.6, 37.8, 36.2, 33.4; MS *m*/*s* 219, 148, 128, 108, 91 (100%), 79, 65; accurate mass 426.1799, calcd for $C_{23}H_{26}N_2O_6$ 426.1791.

Benzyl (2*S*,3*R*)-[2-^{14}C]-5-(*N*-Benzyloxycarbonyl)-amino-3-hydroxy-2-(2′-oxoazetidin-1′-yl)pentanoate (33). To D-*erythro* diastereomer **32** (26.3 mg, 0.062 mmol) in CH_2Cl_2 (4 mL) was added DBN (10 μL, 0.08 mmol), and the reaction was stirred for 1 h. Silica gel (∼500 mg) was added to terminate the reaction, and the mixture filtered through a pad of silica gel, washing with ethyl acetate. The filtrate was concentrated in vacuo to give 22.0 mg (0.052 mmol, 84%) of the protected proclavaminic acids in ratio of > 4:1 favoring the desired (2*S*,3*R*) (*threo*) diastereomer. Radioinactive and nondeuteriated material prepared similarly gave the following physical and spectral data: $[\alpha]_D = +22.3°$ (*c* 0.85, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.33 (m, 10H, Ar*H*), 5.22 (ABq, *J* = 12.0 Hz, 2H, C*H*$_2$Ph), 5.13 (br, 1H, N*H*), 5.07 (s, 2H, C*H*$_2$Ph), 4.47 (d, *J* = 8.2 Hz, 1H, O*H*), 4.27 (m, 1H, H-3), 4.14 (d, *J* = 3.1 Hz, 1H, H-2), 3.44 (m, 3H, H-5 and H-4′), 3.28 (m, 1H, H-4′), 3.00 (t, *J* = 4.0 Hz, 2H, H-3′), 1.68 (m, 2H, H-4); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 169.3, 168.5, 157.1, 136.3, 135.1, 128.6, 128.5, 128.3, 128.2, 128.1, 128.0, 68.9, 67.4, 66.8, 61.8, 40.3, 37.7, 36.3, 34.4; MS *m*/*z* 219, 148, 128, 108, 91 (100%), 79, 65; accurate mass 426.1799, calcd for $C_{23}H_{26}N_2O_6$ 426.1791.

(2*S*,3*R*)-[2-^{14}C]-Proclavaminic Acid (34). Hydrogenation of diastereomer **33** (22.0 mg, 0.052 mmol) in ethanol (4 mL) containing 10% Pd/C (∼5 mg) by bubbling hydrogen through the Ar degassed solution for 30 min with stirring. The catalyst was removed by filtration and the filtrate concentrated to approximately 0.5 mL in vacuo. The material was purified twice via reverse phase HPLC, first using a Partisil ODS-3 C-18 column, then using a Spheresorb S5 ODS-2 C-18 column, both with H_2O as eluent. Radioinactive and nondeuteriated material prepared in a similar manner gave the following physical and spectral data: mp 153−154 °C; $[\alpha]_D = +6.85°$ (*c* 0.35, H_2O) [lit.[23] $+7.3°$ (*c* = 1.0, H_2O)]; IR (KBr) 3373, 2940, 1703, 1634, 1378, 773 cm −1; ^{1}H NMR (D_2O/acetone) δ 4.15 (ddd, *J* = 3.9, 5.5, 9.4 Hz, 1H, H-4′), 4.00 (d, *J* = 5.5 Hz, 1H, H-2), 3.50 (m, 1H, H-4′), 3.43 (m, 1H, H-4′), 3.07 (m, 2H, C*H*$_2$NH$_2$), 2.93 (t, *J* = 4.0 Hz, 2H, H-3′), 1.89 (m, 2H, H-4); ^{13}C{^{1}H} NMR (D_2O/dioxane) δ 175.3, 173.2, 70.0, 63.5, 41.2, 38.1, 36.2, 32.0; CI-MS *m*/*z* 203 (MH^+, 100%), 185, 167, 143, 115; accurate mass (MH^+) 203.1037, calcd for $C_8H_{15}N_2O_4$ 203.1037.

Synthesis of (2*S*,3*R* 3′-^{2}H$_2$,4,5-^{3}H]-Proclavaminic Acid (42). [2,3-^{3}H]-*N*-benzyloxycarbonyl-3-aminopropanol (22a). To $NaHCO_3$ (377.1 mg, 4.489 mmol) and β-alanine (181.0 mg, 2.032 mmol) in a 50 mL flask was added a solution of [2,3-^{3}H]-β-alanine (5 mCi) in 5 mL of 0.01 N HCl followed by H_2O washings (5 mL) and dioxane (7.5 mL). The solution was cooled in an ice bath, and benzyl chloroformate (450 μL, 3.15 mmol) dissolved in dioxane (2.5 mL) was added in approximately 15 min. The mixture was stirred for 45 min, warmed to room temperature, and stirred a further 3 h. The dioxane was removed in vacuo, and the resulting solution was transferred to a separatory funnel, diluted with 5% $NaHCO_3$ (30 mL), and washed with ethyl

acetate (50 mL) to remove excess benzyl chloroformate. The aqueous layer was then acidified with 6 N HCl, diluted with an equal volume of saturated brine, and extracted with ethyl acetate (2 × 80 mL). The combined organic layers were dried (anhydrous Na_2SO_4) and concentrated in vacuo to give a white solid. After drying under high vacuum, the residue was dissolved in THF (11 mL) and the solution cooled in an ice bath. Triethylamine (295 μL, 2.11 mmol) was then added dropwise, and after the mixture stirred for 30 min, a solution of ethyl chloroformate (215 μL, 2.25 mmol) in THF (2 mL) was added dropwise. The reaction mixture was stirred for 2 h and filtered through a fritted funnel containing Celite to remove precipatated triethylammonium hydrochloride. The filtrate and THF washings (20 mL) were taken up in a syringe and added dropwise to a 0 °C solution of sodium borohydride (215.0 mg, 5.69 mmol) in H_2O (20 mL). When the addition was complete, additional sodium borohydride (114 mg, 3.02 mmol) was added, and the reaction stirred for 5 h, warming to room temperature. The reaction mixture was transferred to a separatory funnel, diluted with ethyl acetate (120 mL) and then washed with 1 N HCl (100 mL), 5% $NaHCO_3$ (100 mL), and brine (100 mL). The organic layer was then dried (anhydrous Na_2SO_4) and concentrated in vacuo to give a white solid, which was recrystallized from ethyl acetate/petroleum ether to give the product as white needles (236.2 mg, 1.129 mmol, 56% from β-alanine). Radioinactive material prepared in a similar manner gave the following physical and spectral data: mp 44−45 °C; IR ($CHCl_3$) 3630, 3450, 3015, 2950, 1706, 1520, 1250, 1225, 1135, 1072, 1028 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.35 (m, 5H, Ar*H*), 5.11 (s, 2H, CO_2CH_2), 5.02 (bs, 1H, N*H*), 3.68 (t, J = 5.6 Hz, 2H, CH_2-OH), 3.37 (m, 2H, CH_2NH), 2.51 (bs, 1H, O*H*), 1.70 (m, 2H, C-2); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 136.4, 128.5, 128.1, 128.0, 66.7, 59.4, 37.7, 32.4; MS *m*/*z* 209 (M^+, 2%), 108, 91(100%), 79, 65; accurate mass 209.1054, calcd for $C_{11}H_{15}NO_3$ 209.1052.

[3,4-^{3}H]-*N*-benzyloxycarbonyl-3-aminopropanal (22b). Cbz-alcohol **22a** (236.2 mg, 1.129 mmol), in CH_2Cl_2 (5 mL) was added dropwise to a −78 °C solution oxalyl chloride (110 μL, 1.26 mmol) in CH_2Cl_2 (3 mL) and DMSO in CH_2Cl_2 (2.5 mL). The reaction was stirred a further 15 min, then triethylamine (790 μL, 5.67 mmol) was added dropwise in 1.5 min. The mixture was stirred an additional 5 min at −78 °C, warmed to room temperature over 45 min, diluted with ethyl acetate (60 mL) and washed with 1 N HCl (40 mL), 5% $NaHCO_3$ (40 mL), and brine (2 × 40 mL). The organic layer was dried (anhydrous Na_2SO_4) and concentrated in vacuo to give a pale yellow oil. Crystallization from ethyl acetate/cyclohexane afforded the product (176.3 mg, 0.8508 mmol, 76%) as fine white needles, with a specific activity of 2.45 mCi/mmol. Radioinactive material prepared in a similar manner gave the following physical and spectral data: mp 59−60 °C; ^{1}H NMR ($CDCl_3$) δ 9.80 (s, 1H, C-1), 7.35 (m, 5H, Ar*H*), 5.12 (bs, 1H, N*H*), 5.10 (s, 2H, CO_2CH_2), 3.50 (q, 2H, J = 6.0 Hz, CH_2NH), 2.73 (t, 2H, J = 5.74 Hz, CH_2CO); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 201.1, 157.1, 136.3, 128.4, 128.0, 127.9, 66.6, 43.9, 34.4; MS *m*/*z* 207 (M^+, 3%), 108, 91 (100%), 79, 65; accurate mass 207.0900, calcd for $C_{11}H_{13}NO_3$ 207.0895.

[3′-^{2}H$_2$]-4-Thiophenyl-2-azetidinone (35). To a solution of racemic 4−thiophenyl azetidinone **29** (5.053 g, 28.19 mmol) in THF (55 mL) at 0 °C under Ar, diisopropylamine (4.35 mL, 31.0 mmol) was added dropwise. The reaction was stirred for 1 h and filtered through a fritted funnel with THF (45 mL) to remove the precipitated diisopropylammonium hydrochloride. The clear filtrate was cannulated dropwise to a −78 °C solution of LDA [prepared from disopropylamine (5.9 mL, 49.1 mmol) and *n*−BuLi (28.2 mL, 1.5 M in hexane)]. When the addition was complete, the reaction was stirred for 20 min, and trimethyl silyl chloride (6.5 mL, 51.2 mmol) was added dropwise. After the mixture stirred 2 h, the reaction was stopped by the addition of 5 M acetic acid (12 mL) in THF and warmed to room temperature. The reaction mixture was diluted with EtOAc (400 mL) and washed with 1 N HCl (2 × 300 mL), 5% $NaHCO_3$ (350 mL), and brine (2 × 300 mL). The organic layer was dried (Na_2SO_4) and concentrated in vacuo, and the crude mixture was purified by flash chromatography to give, after recrystallization from CH_2Cl_2/pentane, the *trans* (2.125 g, 30%)- and *cis* (3.442 g, 49%)-isomers as white needles. *trans*-Isomer: mp 90−90.5 °C; IR ($CHCl_3$) 3401, 3008, 1751, 1479, 1334, 1253, 1091, 859, 847 cm^{-1}; ^{1}H NMR ($CDCl_3$) 7.45 (m, 2H, ArH), 7.34 (m, 3H, ArH), 6.01 (b, 1H, NH), 4.77 (d, J = 2.4 Hz, 1H, H−4), 2.72 (dd, J = 0.9, 2.4 Hz, 1H, H−3), 0.14 (s, 9H, TMS); ^{13}C {^{1}H} NMR ($CDCl_3$) δ 168.6, 133.4, 132.1, 129.4, 128.7, 56.5, 50.09, −3.0; MS *m*/*z* 251 (M^+, 0.5%), 236, 208, 193, 182, 151, 142; accurate mass 251.0802, calcd for $C_{12}H_{17}NOSSi$ 251.0800. Anal. Calcd. for $C_{12}H_{17}NOSSi$: C, 57.33; H, 6.82; N, 5.57; S 12.75. Found C, 57.35; H, 6.84; N 5.53; S, 12.66. *Cis* isomer: 7.30 (m, 5H, ArH), 6.02 (b, 1H, NH), 5.22 (d, J = 5.2 Hz, 1H, H−3), 3.23 (1H, dd, J = 1.5, 5.2 Hz, 1H, H−4), 0.31 (s, 9H, TMS); ^{13}C {^{1}H} NMR ($CDCl_3$) δ 168.8, 134..6, 131.4, 129.4, 127.7, 58.7, 49.5, −1.2; MS *m*/*z* 236 (M − CH_3, 0.14%), 179, 167, 151, 142, 109, 70 (100%); accurate mass 236.0565, calcd for $C_{11}H_{14}NOSSi$ (M − CH_3) 236.0565; Anal. Calcd for $C_{12}H_{17}NOSSi$: C, 57.33; H, 6.82; N, 5.57; S, 12.75. Found: C, 57.29; H 6.85; N, 5.55; S, 12.67.

A mixture containing both *cis*- and *trans*-azetidinones (9.5 g, 37.85 mmol) was dissolved in d_4-MeOH (6 mL) under argon. The solution was then transferred to another flask containing dry KF (2.2 g, 1 equiv) under argon at 0 °C. After stirring at 0 °C for 5 min, the solution was warmed to room temperature over a period of 10 min and filtered through silica gel with EtOAc. The combined filtrate and washings were evaporated to leave an oil from which 4-thiophenyl azetidinone was crytallized as a white solid from hexanes and EtOAc having 46% deuteriation at C-3 per site (6.2 g, 92%). The procedure of silylation at C-3 and desilylation in deuterated methanol was repeated 4 times and the level of deuteriationat C−3 was raised to ∼90% per site.

[3-^{2}H$_2$]-4-Phenylsulfonyl-azetidinone (36). To [3-^{2}H$_2$]-4-thiophenyl azetidinone **35** (1.02 g, 5.64 mmol) in acetone at 0 °C was added 2 N H_2SO_4 (4 mL) followed by the slow addition of $KMnO_4$ (1.3 g, 8.2 mmol) in water (25 mL). After 1 h at room temperature, the mixture was filtered through Celite, which was washed with EtOAc. The combined filtrate and washings were concentrated to ∼50 mL and then partitioned between EtOAc and water (50 mL each). The aqueous layer was reextracted with EtOAc (50 mL). The combined EtOAc layers were dried (Na_2SO_4) and evaporated to leave the title compound as a white crystalline solid (1.1 g, 93%).

(4*S*)-[3-^{2}H$_2$]-4-Thiophenyl-azetidinone (37). The title compound was prepared following the same procedure as that described for the preparation of **29**.

Benzyl (4′*S*)-[3-^{2}H$_2$]-(4′-Thiophenyl-2′-oxoazetidin-1′-yl) Acetate (38). The title compound was prepared following the same procedure as that described for the preparation of **30**.

Benzyl (2*R*,3*R*,4′*S*)-[3′-^{2}H$_2$,4,5-^{3}H]-5-(*N*-Benzyloxycarbonyl)-amino-3-hydroxy-2-(4′-thiophenyl-2′-oxoazetidin-1′-yl)pentanoate (39). The dideuteriated azetidinone **38** (206.0 mg, 0.6254 mmol) in dry THF (6 mL) was cooled to −78 °C, and LHMDS (750 μL, 1 M in hexanes) was added dropwise. The reaction was stirred 2 h, and a solution of the tritiated aldehyde **22b** in THF (6 mL) was added dropwise in 10−15 min. After 2 h, the reaction was quenched with excess acetic acid in THF (400 μL, 2.0 mmol). After stirring for a few min at −78 °C, the mixture was warmed to room temperature, transferred to a separatory funnel with ethyl acetate (60 mL), and washed with 1 N HCl (2 × 50 mL), 5% $NaHCO_3$ (50 mL), and brine (50 mL). The organic layer was dried (anhydrous Na_2SO_4) and concentrated in vacuo, and the crude material was purified by radial chromatography (1 mm silica gel; 10% ethyl acetate in hexanes to 100%). Recrystallization from ethyl acetate/cyclohexane gave 88.5 mg (0.165 mmol, 19%) of the desired D-*erythro* diastereomer **39** as fine white needles, with a specific activity of 2.44 mCi/mmol. For physical and spectral data of radioinactive material prepared in an analogous manner, see **31** above.

Benzyl (2*R*,3*R*)-[3′-^{2}H$_2$,4,5-^{3}H]-5-(*N*-benzyloxycarbonyl)-3-hydroxy-2-(2′-oxoazetidin-1′-yl)pentanoate (40). The thiophenyl β-lactam **39** (88.5 mg, 0.165 mmol) and AIBN (∼10 mg) were dissolved in dry benzene (5 mL) and tris(trimethylsilyl)silyl hydride (220 μL, 0.713 mmol) was added. The solution was sparged with Ar for approximately 40 min, and the reaction heated to reflux. Additions of tris(trimethylsilyl) hydride (3 × 220 μL) and AIBN (3 × 10 mg) were made over approximately 10 h, and the reaction terminated after 16.5 h. The benzene was removed in vacuo, and the residue purified by radial chromatography (1 mm silica gel; ethyl acetate:hexane, 1:4 to 100% ethyl acetate) to give 58.1 mg (0.136 mmol, 82%) of the desired product

as a colorless oil. For physical and spectral data of radioinactive material prepared in an analogous manner, see **32** above.

Benzyl (2*S*,3*R*)-[3′-^{2}H$_2$,4,5-^{3}H]-5-(*N*-Benzyloxycarbonyl)-amino-3-hydroxy-2-(2′-oxoazetidin-1′-yl)pentanoate (41). The D-*erythro* diastereomer **40** (58.1 mg, 0.136 mmol) in CH_2Cl_2 (5 mL) was treated with freshly distilled DBN (17 μL, 0.14 mmol. After stirring at room temperature for 2 h, the mixture was filtered through silica gel washing with ethyl acetate. Concentration of the filtrate in vacuo and purification of the residue by radial chromatography (1 mm silica gel; ethyl acetate: hexane, 1:4 to 1:1) gave 39.8 mg (0.093 mmol, 69%) of the desired L-*threo* diastereomer **41** as a colorless oil and 9.2 mg (0.022 mmol, 16%) of the D-*erythro* diastereomer. For physical and spectral data of radioinactive material prepared in an analogous manner, see **33** above.

(2*S*, 3*R*)-[4,5-^{3}H,3′-^{2}H$_2$]-Proclavaminic acid (42). Protected proclavaminate **41** (20.0 mg, 0.047 mmol) in ethanol (4 mL) was hydrogenated over 10% Pd/C (~5 mg). The solution was degassed with bubbling Ar for approximately 30 min followed by The catalyst was removed by filtration through a 0.22 μm filter with H_2O, and the ethanol removed in vacuo. Purification was as described for **34** above. For other physical and spectral data of radioinactive, nondeuteriated material, see **34** above.

Synthesis of Benzyl [2-^{3}H,3′-^{2}H$_2$]-2-(4′-Chloro-2′-oxoazetidin-1′-yl)acetate and Benzyl [2-^{14}C]-2-(4′-Chloro-2′-oxoazetidin-1′-yl)-acetate (43) and (45). Benzyl [2-^{14}C]-2-(4′-Chloro-2′-oxoazetidin-1′-yl)acetate (43). To (±)-[2-^{14}C]azetidinone **30** (200 mg, 0.611 mmol) in CCl_4 (3 mL) was added a freshly prepared solution of Cl_2 in CCl_4 (3 mL, 1% w/v) at 0 °C. After stirring for 20 min, the mixture was concentrated in vacuo, the residue was triturated with hexane (2 × 10 mL), and the hexane was removed by pipet. The residual oil was purified by radial chromatography (1 mm silica gel; ethyl acetate: hexane, 1:4) followed by recrystallization from CH_2Cl_2/petroleum ether to give approximately 15 mg of the product as fine white needles having a specific activity of 0.131 mCi/mmol. Unlabeled material prepared in an analogous manner gave the following physical and spectral data: mp 70−71 °C; IR ($CHCl_3$) 3018, 1776, 1747, 1384, 1262, 1228, 1193, 933 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.37 (m, 5H, Ar*H*), 5.82 (dd, J = 1.3, 4.0 Hz, 1H, H-4′), 5.19 (ABq, J = 12.2 Hz, 2H, C*H*$_2$Ar), 4.35 (*A*Bq, J = 18.2 Hz, 1H, C*H*$_2$CO$_2$), 3.75 (A*B*q, J = 18.2 Hz, 1H, C*H*$_2$CO$_2$), 3.63 (dd, J = 4.0, 15.3 Hz, 1H, H-3′$_{cis}$), 3.24 (d, J = 15.3 Hz, 1H, H-3′$_{trans}$); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 167.5, 163.9, 134.8, 128.8, 128.5, 67.6, 66.6, 50.3, 40.9; MS *m*/*z* 253 (M$^+$, 2.0%), 118, 91 (100%), 76, 65, 55, 43, 39; accurate mass 253.0508, calcd for $C_{12}H_{12}ClNO_3$ 253.0506.

Benzyl [2-^{3}H,3′-^{2}H$_2$]-2-(4′-Thiophenyl-2′-oxoazetidin-1′-yl)acetate (44). THF (2 mL) and a solution of LiHMDS (150 μL, 1.0 M in hexane) was cooled to −78 °C, dideuterated azetidinone (±)-**38** (47.9 mg, 0.146 mmol) in THF (3 mL) added dropwise. The reaction was stirred for 45 min, quenched by the addition of a solution of [^{3}H]H_2O (20 μL, 1.0 Ci/mL) in THF (100 μL), and warmed to room temperature. The mixture was filtered through a pad of silica gel and anhydrous Na_2SO_4 with ethyl acetate, and the filtrate was concentrated in vacuo to give a yellow oil. The residue was purified by radial chromatography (1 mm silica gel; ethyl acetate:hexane, 1:3) to give 37.4 mg (0.114 mmol, 78%) of the tritiated product as a colorless oil.

Benzyl [2-^{3}H,3′-^{2}H$_2$]-2-(4′-Chloro-2′-oxoazetidin-1′-yl)acetate (45). The labeled azetidinone **44** (134.4 mg, 0.4080 mmol) in CCl_4 (3 mL) was cooled to 0 °C, and a freshly prepared solution of Cl_2 in CCl_4 (3 mL, 1% w/v) added. After stirring 20 min, the reaction mixture was concentrated in vacuo, the residue triturated with hexane (2 × 10 mL), and the hexane removed by pipet. The residual oil was purified by radial chromatography (1 mm silica gel; ethyl acetate:hexane, 1:4) followed by recrystallization from CH_2Cl_2/petroleum ether to give approximately 10 mg of the product as fine white needles with a specific activity of 0.415 mCi/mmol. For physical and spectral data of radioinactive material prepared in an analogous manner, see **43** above.

Benzyl 2-(4′-Acetoxy-2′-oxoazetidin-1′-yl)acetate (49). Chloroazetidinone **43** (51.0 mg, 0.201 mmol) and glacial acetic acid (3 mL) were treated with triethylamine (30 μL), and after stirring for 2 h, the reaction mixture was diluted with ethyl acetate (60 mL) and washed with 5% $NaHCO_3$ (2 × 50 mL) and brine (2 × 50 mL). The organic layer was dried (anhydrous Na_2SO_4) and concentrated in vacuo, and the residue was purified by radial chromatography (1 mm silica gel; ethyl acetate:hexane, 1:3) to give 45.0 mg (0.162 mmol, 81%) of the desired product as a colorless oil. IR ($CHCl_3$) 3028, 1779, 1749, 1405, 1381, 1351, 1236, 1224, 1194, 1043, 929 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.36 (m, 5H, Ar*H*), 6.06 (dd, J = 1.5, 4.2 Hz, 1H, H-4′), 5.17 (ABq, J = 14.0 Hz, 2H, C*H*$_2$Ar), 4.22 (*A*Bq, J = 18.0 Hz, 1H, C*H*$_2$CO$_2$), 3.94 (A*B*q, J = 18.0 Hz, 1H, C*H*$_2$CO$_2$), 3.33 (dd, J = 4.1, 15.1 Hz, 1H, H-3′$_{cis}$), 3.06 (d, J = 14.7 Hz, 1H, H-3′$_{trans}$), 2.07 (s, 3H, C*H*$_3$); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 171.6, 168.0, 165.7, 135.0, 128.7, 128.6, 128.4, 76.5, 44.5, 42.9, 20.7; MS *m*/*z* 277 (M$^+$, 0.13%), 235, 217, 189, 173, 143, 142, 100, 91 (100%); accurate mass 277.0957, calcd for $C_{14}H_{15}NO_5$ 277.0950.

Determination of the β-2° Kinetic Isotope Effect in the Reaction of (2*S*,3*R*)-[3′-^{2}H$_2$,4,5-^{3}H]- and (2*S*,3*R*)-[2-^{14}C]-Proclavaminic Acid 34 and 42, Respectively with Clavaminate Synthase. Incubations were run in Wheaton glass mini-vials equipped with a spin vane at 25 °C and contained, in a volume of 280 μL or 468 μL, 50 mM MOPS buffer (pH 7.0), 0.5 mM DTT, 0.1 mM sodium ascorbate, 0.01 mM ferrous ammonium sulfate, 3 mM α-KG, 0.3−0.35 mM [3′-^{2}H$_2$; 4,5-^{3}H]-(2*S*, 3*R*)-proclavaminic acid, and 0.15−0.2 mM [2-^{14}C]-(2*S*,3*R*)-proclavaminic acid. The ^{3}H/^{14}C ratios were approximately 2.0−4.0. A 40 μL aliquot was removed for the time zero point and added to 10 μL of 4 mM EDTA. The reaction was initiated with the addition of 0.52 mg of protein. Six aliquots of 40 μL were removed and terminated with 10 μL of 4 mM EDTA at 0.5, 2, 6, 9, 20, and 30 min, with additional enzyme (0.13 mg) added at 7 min. The samples were frozen in dry ice until immediately prior to HPLC analysis.

Analysis of the incubation mixture at various time points was carried out on a Whatman Partisil ODS-3 10 mm C-18 column (4.6 × 250 mm), with isocratic elution of an ion-pairing solution containing 0.1% TFA/0.01% sodium octane sulfonate (OSA). Samples were eluted at 1.5 mL/min while collecting 13 drop (~30 s) fractions directly into 7 mL glass mini-scintillation vials. Optifluor liquid scintillation cocktail (6.25 mL) (Packard) was added for counting. After subtracting appropriate blank valuesfor each channel, the dpm values for the relevant fractions were added. The extent of reaction was determined from both the ^{14}C and ^{3}H distribution as the total dpm in the product divided by the total dpm recovered in the injection. The data on ^{3}H/^{14}C ratios as a function of extent of reaction were computer fit to eq 1 using a modified nonlinear least-squares regression program and by assuming constant variance. Values are reported with their standard errors.

Determination of the β-2° Kinetic Isotope Effect in the Solvolysis Reaction of Benzyl [2-^{14}C]-2-(4′-chloro-2′-oxoazetidin-1′-yl)acetate (43) and Benzyl [2-^{3}H,3′-^{2}H$_2$]-2-(4′-chloro-2′-oxoazetidin-1′-yl)acetate (45) with Acetic Acid. A dry Wheaton glass mini-vial equipped with a dry spin vane and rubber septum was charged with 300−430 μg of benzyl [2-^{14}C]-2-(4′-chloro-2′-oxoazetidin-1′-yl)acetate (**43**) and 275−310 μg of benzyl [2-^{3}H,3′-^{2}H$_2$]-2-(4′-chloro-2′-oxoazetidin-1′-yl)-acetate (**45**). The ^{3}H/^{14}C ratios were approximately 2−3. Dry THF (500 μL) was added, and the solution was transferred by syringe to a second dry mini-vial equipped with a spin vane. A 30 μL aliquot was removed for the time zero point, and the THF in the reaction vial removed under a dry stream of Ar or N_2. Dry, freshly distilled acetic acid (500 μL, distilled from acetic anhydride), equilibrated to 25 °C, was then added by syringe. The reaction was run under a dry atmosphere of N_2 or Ar with stirring. Seven to eight aliquots of 30 μL were removed at various times up to 6 h, and the samples were frozen in dry ice until immediately prior to HPLC analysis. The solvent was then removed in a Speed-Vac, the residue taken up in 30 μL of CH_3CN, and 25 μL analyzed by HPLC.

Analysis of the reaction mixture a various time points was carried out on a Whatman Partisil ODS-3 10 μm C-18 column with elution at 1 mL/min, using the following gradient: 80% H_2O and 20% CH_3CN to 50% H_2O in 15 min, held at 50% for 10 min, then back to 80% H_2O in 15 min. The column eluent was collected in approximately 30 s fractions directly into 7 mL glass mini-scintillation vials, and 6.25 mL of Optifluor liquid scintillation cocKtail (Packard) added for counting. Extent of reaction and data on ^{3}H/^{14}C ratios as a function of reaction were calculated as described above.

Determination of the β-2° Kinetic Isotope Effect in the Solvolysis Reaction of Benzyl [2-^{14}C]-2-(4′-Chloro-2′-oxoazetidin-1′-yl)acetate (43) and Benzyl [2-^{3}H, 3′-$^{2}H_2$]-2-(4′-Chloro-2′-oxoazetidin-1′-yl)-acetate (45) with Ethanol. A dry Wheaton glass mini-vial equipped with a dry spin vane and rubber septum was charged with 320−480 μg of benzyl [2-^{14}C]-2-(4′-chloro-2′-oxoazetidin-1′-yl)acetate **43** and 340−420 μg of benzyl [2-^{3}H, 3′-$^{2}H_2$]-2-(4′-chloro-2′-oxoazetidin-1′-yl)acetate **45**. The ^{3}H/^{14}C ratios were approximately 2−3. Dry THF (300 μL) was added, and, after stirring thoroughly, the solution (250 μL) was transferred by syringe to a second dry mini-vial equipped with a spin vane. A 15 μL aliquot was removed and added to 15 μL of H_2O for the time zero point**.** Dry, denatured ethanol (235 μL, Aldrich), equilibrated to 25 °C, was then added. Seven to eight aliquots of 30 μL were removed and added to 30 μL at various times up to 3 h, and the samples were frozen in dry ice until prior to HPLC analysis, when they were thawed and immediately injected.

Analysis of the reaction mixture was carried out as described immediately above for solvolysis in acetic acid. Data analysis was identical to that described above for the clavaminate synthase reaction.

Determination of the β-2° Kinetic Isotope Effect in the Photochemically Initiated Reduction of Benzyl [2-^{3}H,3′-$^{2}H_2$]-2-(4′-Chloro-2′-oxoazetidin-1′-yl)acetate 45 and Benzyl [2-^{14}C]-2-(4′-Chloro-2′-oxoazetidin-1′-yl)acetate 43. A dry Wheaton glass mini-vial equipped with a dry spin vane and rubber septum was charged with 260−310 μg of benzyl [2-^{14}C]-2-(4′-chloro-2′-oxoazetidin-1′-yl)acetate **43** and 270−360 μg of benzyl [2-^{3}H, 3′-$^{2}H_2$]-2-(4′-chloro-2′-oxoazetidin-1′-yl)acetate **45**. The ^{3}H/^{14}C ratios were approximately 3. A solution of dry, degassed CH_3CN:THF (350 μL, 1:1; CH_3CN dried at reflux over CaH_2, then distilled; degassing achieved with five cycles of freeze/pump/thaw) was added followed by tris(trimethylsilyl)silyl hydride (150 μL) and a solution of AIBN (20 μL, ∼40 mM) in dry, degassed CH_3CN:THF (1:1). After stirring, the solution was transferred to a dry quartz test tube equipped with a dry stir bar and rubber septum. A 30 μL aliquot was removed for the time zero point and the reaction photolyzed at 25 °C with a medium-pressure mercury lamp equipped with a Vycor sleeve. Two to four additions of AIBN (20 μL) were made during the course of the reaction. Six aliquots of 30 μL were removed at various times up to 6 h and the samples frozen in dry ice until immediatly prior to HPLC analysis. The solvent and silyl hydride were then removed in a Speed-Vac, the residue taken up in 30 μL CH_3CN, and 25 μL analyzed by HPLC.

Analysis of the reaction mixture was carried out as described above for solvolysis in acetic acid. Data analysis was identical to that described above for the clavaminate synthase reaction.

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