Probable Role of Clavaminic Acid as the Terminal Intermediate in the Common Pathway to Clavulanic Acid and the Antipodal Clavam Metabolites

Laura A. Egan, Robert W. Busby, Dirk Iwata-Reuyl,† and Craig A. Townsend*

Contribution from the Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

Received September 4, 1996⊗

Abstract: Emerging chemical and genetic evidence suggests that separate biochemical solutions have evolved to synthesize the four known classes of β-lactam antibiotics. One of these classes contains clavulanic acid (**1**) and a family of structurally related but antipodal clavam metabolites **2**−**5**, **7**, and **8** which lack a carboxylate at C-3, have a different oxidation state, and exhibit stereochemical features at C-2. Previous work has demonstrated the incorporation of ornithine/arginine in the identical regiochemical sense in all of these natural products, and has established the common intermediacy of the monocyclic β-lactam proclavaminic acid (**12**) as well. In this paper the quite advanced bicyclic intermediate clavaminic acid (**14**) has been synthesized in doubly ^{13}C-labeled form by preparative incubation of recombinant clavaminate synthase. The intact and equally efficient incorporation of **14** into valclavam (**7**) and 2-(2-hydroxyethyl)clavam (**8**), together with $^{18}O_2$-incorporation experiments, has been interpreted to define clavaminic acid as the final intermediate shared in the biosynthesis of clavulanic acid and the antipodal clavams. A mechanistic rationale of this interrelationship and the late stages of the respective biosyntheses is proposed.

Introduction

Growing biochemical and genetic evidence suggests that separate biosynthetic strategies have evolved to create the four known classes of β-lactam antibiotics.[1] Among these the potent β-lactamase inactivator clavulanic acid (**1**) is produced by *Streptomyces clavuligerus* together with the antipodal 2-alanylclavam (**2**)[2] and the stereochemically related metabolites **3**−**5** (Scheme 1).[3] The allied strain *Streptomyces antibioticus* does not produce clavulanic acid, but accumulates valclavam (**7**)[4−7] and 2-(2-hydroxethyl)clavam (**8**).[8] Apart from having the 5*S*-configuration at their ring junction, these clavams share a common stereochemistry at C-2 and an oxidation state more reduced at this center than clavulanic acid, and they lack the C-3 carboxyl found in **1**. These differences notwithstanding, experiments described in this paper establish a common biosynthetic pathway among these structures to the probable branch point of clavaminic acid (**14**), a highly advanced intermediate.

Early experiments demonstrated that clavulanic acid (**1**) is derived from the urea cycle amino acids ornithine and arginine,[9] but subsequently, mutant studies clearly showed that arginine is the more direct precursor from primary metabolism.[10] The first cyclic intermediate in the anabolic process is believed to be **10**,[11] which is hydroxylated by the α-ketoglutarate (α-KG)-dependent oxygenase clavaminate synthase (CS)[12,13] to give **11** (Scheme 2). Through the action of a second enzyme, proclavaminate amidino hydrolase (PAH),[11,14,15] the guanidino group of **11** is hydrolyzed in an arginase-like reaction[15] to afford proclavaminic acid (**12**) and urea. Remarkably, while **11** is not a substrate for CS, the terminal amino compound **12** is, and a stepwise[16,17] pair of oxidative reactions ensue mediated again by CS to give clavaminic acid (**14**).[18] A penultimate oxidation and "enantiomerization"[19] gives the unstable aldehyde **15**, which is reduced by the NADPH-dependent clavulanate aldehyde dehydrogenase (CAD) to clavulanic acid (**1**).[20]

It was initially hypothesized that 2-alanylclavam (**2**) might derive from ornithine (**6b**) in the opposite regiochemical sense

* To whom correspondence should be addressed. Phone: (410) 516-7444. Fax: (410) 516-8420. E-mail: Townsend@jhunix.hcf.jhu.edu.

† Current address: Department of Chemistry, Portland State University, Portland, OR 97207.

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

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

(2) Müller, J.-C.; Toome, V.; Pruess, D. L.; Blount, J. F.; Weigele, M. *J. Antibiot.* **1983**, *36,* 217−225.

(3) Brown, D.; Evans, J. R.; Fletton, R. A. *J. Chem. Soc., Chem. Commun.* **1979**, 282−283.

(4) Röhl, F.; Rabenhorst, J.; Zähner, H. *Arch. Microbiol.* **1987**, *147,* 315−320.

(5) Baldwin, J. E.; Claridge, T. D. W.; Goh, K.-C.; Keeping, J. W.; Schofield, C. J. *Tetrahedron Lett.* **1993**, *34,* 5645−5648.

(6) Janc, J. W.; Egan, L. A.; Townsend, C. A. *Bioorg. Med. Chem. Lett.* **1993**, *3,* 2213−2216.

(7) Postels, H.-T.; König, W. A. *Tetrahedron Lett.* **1994**, *35,* 4535−4538.

(8) Wanning, M.; Zähner, H.; Krone, B.; Zeeck, A. *Tetrahedron Lett.* **1981**, *22,* 2539−2540.

(9) Townsend, C. A.; Ho, M.-F. *J. Am. Chem. Soc.* **1985**, *107,* 1065−1066.

(10) Valentine, B. P.; Bailey, C. R.; Doherty, A.; Morris, J.; Elson, S. W.; Baggaley, K. H.; Nicholson, N. H. *J. Chem. Soc., Chem. Commun.* **1993**, 1210−1211.

(11) Elson, S. W.; Baggaley, K. H.; Davidson, M.; Fulston, M.; Nicholson, N. H.; Risbridger, G. D.; Tyler, J. W. *J. Chem. Soc., Chem. Commun.* **1993**, 1212−1214.

(12) Elson, S. W.; Baggaley, K. H.; Fulston, M.; Nicholson, N. H.; Tyler, J. W.; Edwards, J.; Holms, H.; Hamilton, I.; Mousdale, D. M. *J. Chem. Soc., Chem. Commun.* **1993**, 1211−1212.

(13) Baldwin, J. E.; Merritt, K. D.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H. *J. Chem. Soc., Chem. Commun.* **1993**, 1301−1302.

(14) Aidoo, K. A.; Wong, A.; Alexander, D. C.; Rittammer, R. A. R.; Jensen, S. E. *Gene* **1994**, *147,* 41−46.

(15) Wu, T. K.; Busby, R. W.; Houston, T. A.; McIlwaine, D. B.; Egan, L. A.; Townsend, C. A. *J. Bacteriol.* **1995**, *177,* 3714−3720.

(16) Baldwin, J. E.; Adlington, R. M.; Bryans, J. S.; Bringhen, A. O.; Coates, J. B.; Crouch, N. P.; Lloyd, M. D.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H.; Cassells, R.; Nicholson, N. *J. Chem. Soc., Chem. Commun.* **1990**, 617−619.

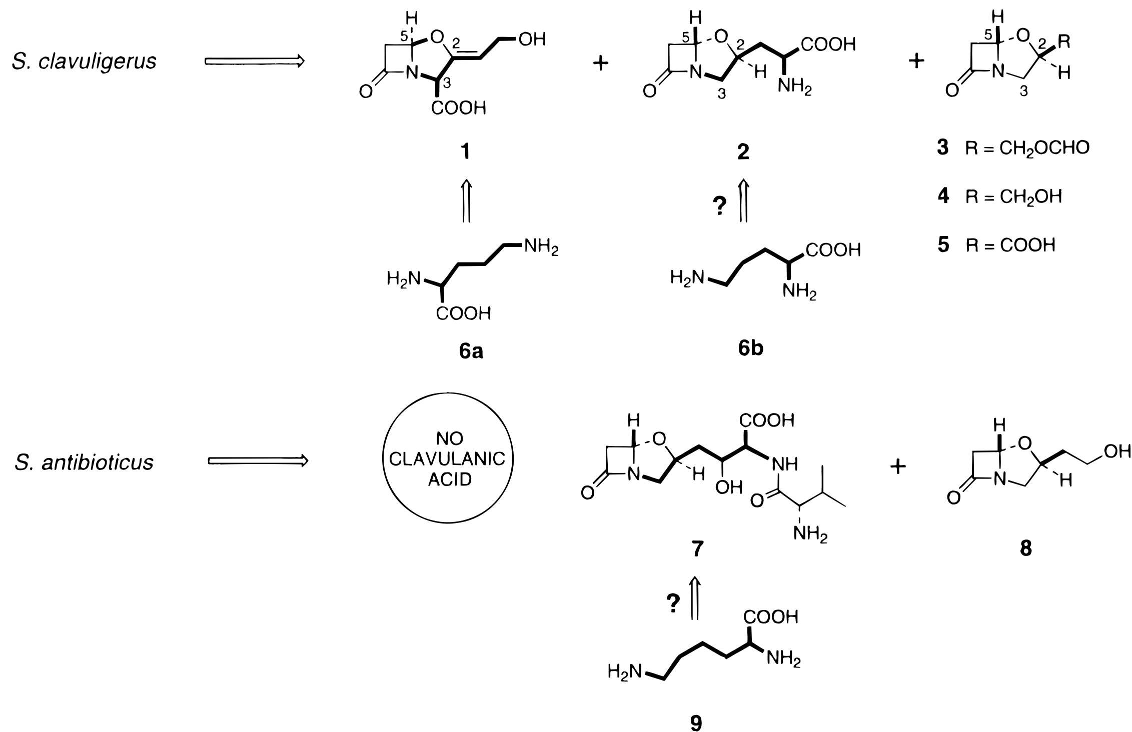
(17) Salowe, S. P.; Krol, W. J.; Iwata-Reuyl, D.; Townsend, C. A. *Biochemistry* **1991**, *30,* 2281−2292.

(18) Elson, S. W.; Baggaley, K. H.; Gillett, J.; Holland, S.; Nicholson, N. H.; Sime, J. T.; Woroniecki, S. R. *J. Chem. Soc., Chem. Commun.* **1987**, 1736−1738.

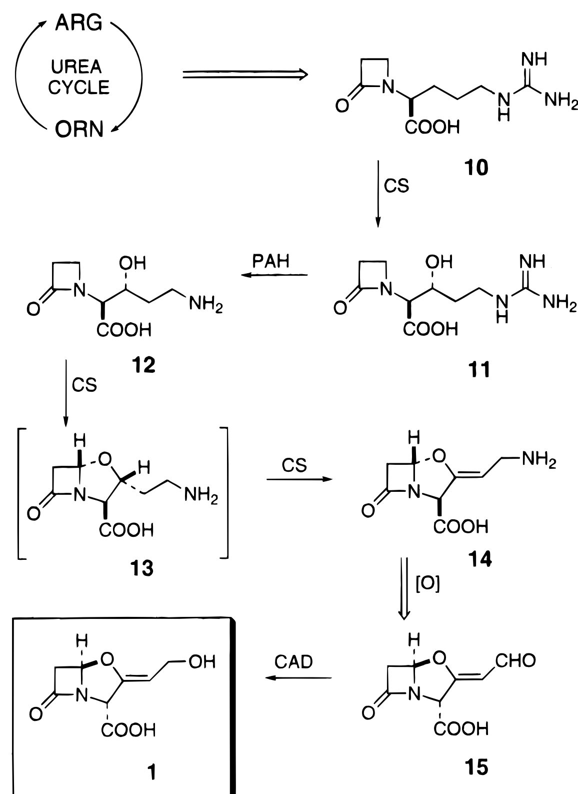
(19) Townsend, C. A.; Krol, W. J. *J. Chem. Soc., Chem. Commun.* **1988**, 1234−1236.

S0002-7863(96)03107-1 CCC: $14.00 © 1997 American Chemical Society

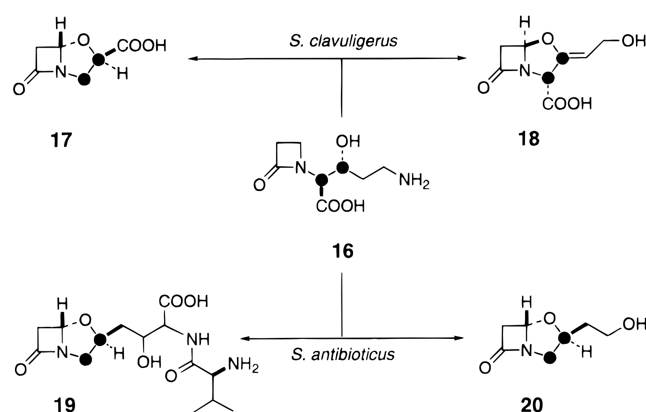
Scheme 1



Scheme 2



Scheme 3



to its utilization in clavulanic acid biosynthesis (**6a**, heavy lines in **2**, Scheme 1).[21] Analogously, valclavam (**7**) could be visualized to be derived from lysine (**9**, heavy lines in **7**)[5] and simultaneously provide a rationale for the absence of clavulanic acid synthesis in this organism (see Scheme 1). Divergence of the biosynthetic pathways to clavulanic acid and to the clavams at the early stage of C_5- or C_6-amino acid attachment to a C_3-precursor[22] was quickly disproved by experiments with [3-^{13}C]-ornithine[21] and [2,3-$^{13}C_2$]proclavaminic acid (**16**, Scheme 3).[6,21] The former was incorporated into clavulanic acid at C-2 as expected, but was found to label C-2 of clavam-2-carboxylic acid (**5**).[21] This location of isotopic enrichment clearly pointed to an orientation of arginine/ornithine *identical* to that occurring in clavulanic acid, implying that a common pathway extended beyond the initial linkage of C_3- and C_5-precursors. Subsequently, administration of [2,3-$^{13}C_2$]proclavaminic acid (**16**) to *S. clavuligerus* gave equally efficient incorporation of paired heavy isotopes into clavam-2-carboxylic acid (**17**) and coproduced clavulanic acid (**18**) as shown in Scheme 3.[21] The common derivation of the highly modified clavam **5**/**17** from an advanced precursor of clavulanic acid (**1**/**18**) was tested further by experiments in *S. antibioticus*. In a similar incorporation study both valclavam (**19**) and 2-(2-hydroxyethyl)-clavam (**20**) were comparably labeled by **16**.[6] Therefore, it

(20) Nicholson, N. H.; Baggaley, K. H.; Cassels, R.; Davison, M.; Elson, S. W.; Fulston, M.; Tyler, J. W.; Woroniecki, S. R. *J. Chem. Soc., Chem. Commun.* **1994**, 1281−1282.

(21) Iwata-Reuyl, D.; Townsend, C. A. *J. Am. Chem. Soc.* **1992**, *114,* 2762−2763.

(22) Townsend, C. A.; Ho, M.-F. *J. Am. Chem. Soc.* **1985**, *107,* 1066−1068.

would appear that all clavams and clavulanic acid have a common biosynthetic origin at least up to and including proclavaminic acid.

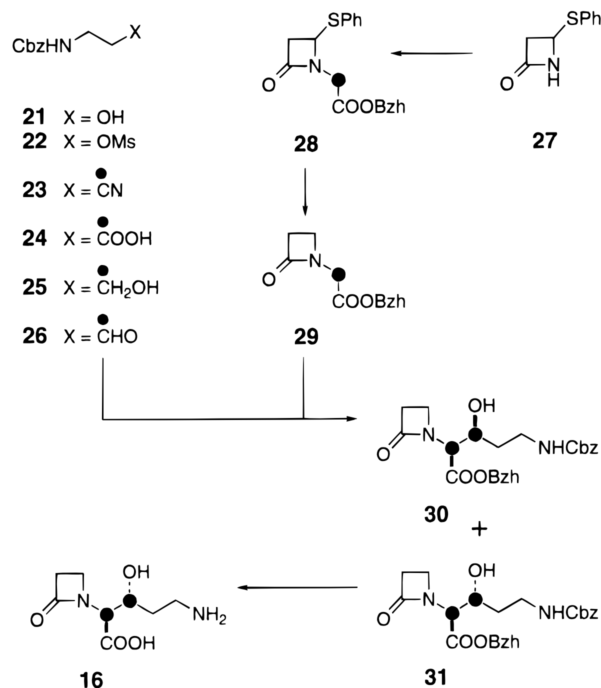
These findings in *S. antibioticus* prompted a search to detect clavaminate synthase (CS) activity in this organism, a strain that does not produce clavulanic acid but presumably must carry out the hydroxylation step necessary for the formation of proclavaminic acid (**12**), that is, the conversion of **10** to **11** in Scheme 2. Two isozymes of this Fe(II)/O_2/α-KG-dependent oxygenase, CS1 and CS2, have been isolated and characterized from *S. clavuligerus*.[23] A third protein, denoted CS3, of virtually identical mass and kinetic properties was indeed purified from *S. antibioticus*.[24] *N*-Terminal sequence information clearly supported the view that all three of these proteins were highly similar.[24] These findings confirmed and extended reports elsewhere[25] in which this CS activity was observed in *S. antibioticus* and established the presence of a functional PAH enzyme as well.

The existence of CS3 in *S. antibioticus* with all three of its oxidative activities intact pressed further the question[21] whether clavaminic acid (**14**) might be a common intermediate between clavulanic acid (**1**) and the clavam metabolites represented by structures **2**−**7**, and **8**. While the latter share the ring fusion geometry of clavaminic acid (**14**), they lack the C-3 carboxyl of clavulanic acid and have the *opposite* absolute configuration at C-2 of the intermediate **13** involved in the stepwise conversion of **12** to **14** (Scheme 2).[16,17] Moreover, clavaminic acid itself appears to be of the wrong oxidation state to serve readily as a precursor of the clavams saturated at C-2. To determine the extent of the common pathway, [2,3-$^{13}C_2$]clavaminic acid has been prepared and its intact incorporation has been demonstrated into valclavam (**7**) and 2-(2-hydroxyethyl)clavam (**8**). Detailed mechanistic consideration of these findings suggests that clavaminic acid (**14**) is the last biosynthetic intermediate common among these natural products.

Results

Clavaminic acid is a modestly stable amino acid not readily accessible in ^{13}C-labeled form by total synthesis. As CS2 from *S. clavuligerus* has been successfully overproduced in *Escherichia coli*,[26,27] it was resolved at the outset to convert doubly ^{13}C-labeled proclavaminic acid enzymically to the correspondingly enriched clavaminic acid.[28] Racemic [2,3-$^{13}C_2$]proclavaminic acid (**16**) was prepared as outlined in Scheme 4. Label was introduced from potassium [^{13}C]cyanide and sodium [2-^{13}C]bromoacetate. 2-Aminoethanol was protected to give **21**, which was converted to the previously described ^{13}C-labeled acid **24**.[29] Reduction of the mixed anhydride[30] of **24** gave the alcohol **25**, which was readily oxidized[29] to the aldehyde **26**. Labeled bromoacetic acid was converted to its benzhydryl ester[31] and reacted with 4-(phenylthio)azetidin-2-one (**27**)[32] to give **28**,

Scheme 4



which was reduced with nBu$_3$SnH and AIBN to **29**. Deprotonation of **29** with LiHMDS at −78 °C and aldol reaction[29,33−35] with **26** gave a mixture of the *erythro*-**30** and *threo*-**31** products, favoring the former.[17,34] Flash or radial chromatography gave small amounts of pure **30** and **31** as well as mixed fractions. The latter were combined with **30** and equilibrated with DBN[34] to now significantly favor (>3:1) the desired *threo* product **31**. After further careful chromatographic purification, the pooled **31** was hydrogenolyzed[17,29,35] to afford (±)-[2,3-$^{13}C_2$]proclavaminic acid (**16**, $^2J_{CC}$ = 39.3 Hz).

In the presence of Fe(II), α-KG, and molecular oxygen, clavaminate synthase is known to be rapidly inactivated ($t_{1/2}$ = ca. 5 min).[23,24] In the presence of these cofactors and substrate the half-life increases only to about 20 min, from which it can be calculated that CS undergoes fewer than 1000 turnovers before inactivation.[23] Therefore, the preparative use of clavaminate synthase would require several hundred milligrams of protein to produce 50−100 mg of clavaminic acid.

Overproduction of CS2 was carried out essentially as described previously[27] with one modification. The incubation time following induction of the overexpression vector with nalidixic acid was lengthened from 3 to 7 h and gave increased yields of enzyme. A streamlined purification procedure was developed to obtain protein of sufficient purity (>80%) for the large-scale enzymatic conversion of labeled proclavaminic acid to clavaminic acid. This shortened protocol omitted the final gel filtration step using G-75 described in the isolation of the wild-type enzyme.[23]

Optimization of Enzymic Conversion. Maximizing the efficiency of the CS2 cyclization/desaturation reaction was paramount owing to the value of the doubly ^{13}C-labeled substrate and the amount of recombinant enzyme that would be required.

(23) Salowe, S. P.; Marsh, E. N.; Townsend, C. A. *Biochemistry* **1990**, *29,* 6499−6508.
(24) Janc, J. W.; Egan, L. A.; Townsend, C. A. *J. Biol. Chem.* **1995**, *270,* 5399−5404.
(25) Baldwin, J. E.; Fujishima, Y.; Goh, K. C.; Schofield, C. J. *Tetrahedron Lett.* **1994**, *35,* 2783−2786.
(26) Lawlor, E. J.; Elson, S. W.; Holland, S.; Cassels, R.; Hodgson, J. E.; Lloyd, M. D.; Baldwin, J. E.; Schofield, C. J. *Tetrahedron* **1994**, *50,* 8737−8748.
(27) Busby, R. W.; Chang, M. D.-T.; Busby, R. C.; Wimp, J.; Townsend, C. A. *J. Biol. Chem.* **1995**, *270,* 4262−4269.
(28) Such an enzymatic conversion has been carried out previously, although no experimental details were reported: Elson, S. W.; Baggaley, K. H.; Gillett, J.; Holland, S.; Nicholson, N. H.; Sime, J. T.; Woroniecki, S. R. *J. Chem. Soc., Chem. Commun.* **1987**, 1739−1740.
(29) Townsend, C. A.; Basak, A. *Tetrahedron* **1991**, *47,* 2591−2602.
(30) Ishizumi, K.; Koga, K.; Yamada, S.-I. *Chem. Pharm. Bull.* **1968**, *16,* 492−497.
(31) Stelakatos, G. C.; Paganou, A.; Zervas, L. *J. Chem. Soc. C* **1966**, 1191−1199.
(32) Kobayashi, T.; Iwano, Y.; Hirai, K. *Chem. Pharm. Bull.* **1978**, *26,* 1761−1767.
(33) Pansare, S. V.; Vederas, J. C. *J. Org. Chem.* **1987**, *52,* 4804−4810.
(34) Baggaley, K. H.; Elson, S. W.; Nicholson, N. H.; Sime, J. T. *J. Chem. Soc., Perkin Trans. 1* **1990**, 1513−1520.
(35) Iwata-Reuyl, D.; Basak, A.; Silverman, L. S.; Engle, C. A.; Townsend, C. A. *J. Nat. Prod.* **1993**, *56,* 1373−1396.

Several variables were examined to establish optimal conditions on a small scale that could be applied to a large-scale incubation. The variables examined include temperature, pH, buffer composition, substrate and cofactor concentrations, and the substrate: enzyme ratio.

Knowing the oxidative lability of the enzyme and uncertain of the stability of the product, the conversion was examined at 0 and 25 °C. Previous experiments had shown that higher temperatures had an adverse effect on CS stabililty. However, in a 24 h period, little difference was observed in the net yield and the slower, low-temperature reaction was abandoned. Similarly, small variations of the pH around 7.0 had minimal effect on product stability in solution, so the reaction was conducted at this pH, which is the pH optimum of clavaminate synthase.[23] Tris, MOPS, and phosphate buffer were prepared at pH 7, and the enzyme activity was measured. While the organic amine buffers were less easily removed in the final HPLC purification step, reaction in phosphate was only about 50% as efficient.

Significant effects on the overall enzymic conversion were observed in variations of the substrate:cofactor and substrate: CS2 ratios. The concentration of racemic proclavaminate was arbitrarily set at 1 mM on the basis of earlier experience. Periodic substoichiometric additions of α-KG were found to be effective in prolonging the catalytic lifetime of the enzyme and slightly improved the net yield. In the final optimization 10% of the theoretically required α-KG was added every 20 min of the incubation. Rather than a single addition of CS2, it was determined that portionwise addition of the enzyme benefited the overall yield. While the enzymic conversion of proclavaminate to clavaminate can be carried out functionally to completion, large amounts of enzyme are required. In a typical test reaction of 40 μg of (±)-**12**, turnover efficiency increased with further enzyme up to approximately 70 μg of CS2, after which additional protein improved the product yield only marginally. A practical compromise among these variables was achieved by hourly additions of CS2 for the first 4 h of reaction to a final substrate:enzyme molar ratio of 80:1. The optimized conditions were examined on a scale 200-fold greater than the test reactions, but still on 1/5 the scale envisioned for the labeled synthesis. A 68% yield was attained, in heartening agreement with the small-scale test reactions.

Enzymatic Synthesis of [2,3-$^{13}C_2$]Clavaminic Acid. The amount of racemic [2,3-$^{13}C_2$]proclavaminic acid (**16**) was determined using the ninhydrin assay previously described[23] and found to be 1.53 mmol. This was converted to [2,3-$^{13}C_2$]-clavaminic acid (**32**) in a 1.5 L reaction containing 0.1 mM (0.15 mmol, 10 mol %) α-KG, 25 μM ferrous ammonium sulfate, dithiothreitol, and ascorbic acid. Additional 0.15 mmol amounts of α-KG were added every 20 min over 9 h, and partially purified recombinant CS2 estimated to contain 1.0 g of pure enzyme was supplied in equal portions at 0, 1, 2, and 3 h of incubation. We were gratified to find that a 71% yield of **32** was obtained (Figure 1).

The reaction mixture was immediately passed through a DEAE Sephadex A25 column to remove most of the protein. Unlike the smaller scale test incubations, a second protein removal step was required prior to HPLC separation. This was successfully achieved by membrane ultrafiltration. Remaining [2,3-$^{13}C_2$]-(2*S*)-proclavaminic acid (**16**) and its unreactive 2*R*-enantiomer were cleanly resolved from the 3*S*,5*S*-product **32** by preparative reversed-phase HPLC. [2,3-$^{13}C_2$]Clavaminic acid (**32**) was obtained as an off-white solid contaminated by small amounts of MOPS buffer, but no labeled proclavaminate **16**.

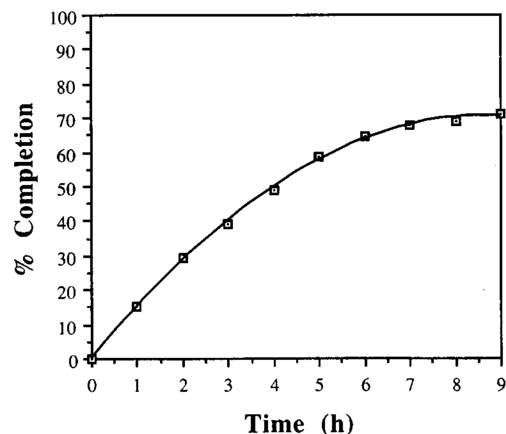
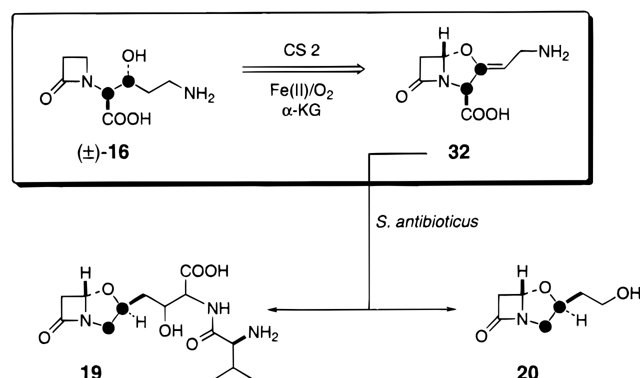


Figure 1. Conversion of (±)-[2,3-$^{13}C_2$]proclavaminic acid (**16**) to (−)-[2,3-$^{13}C_2$]clavaminic acid (**32**) using recombinant CS2 monitored as a function of time.

Scheme 5



Control experiments showed that the observed concentrations of MOPS buffer had no adverse effects on cell growth or clavam production.

Incorporation of [2,3-$^{13}C_2$]Clavaminic Acid. *S. antibioticus* was grown in a modified soy bean medium, and the doubly ^{13}C-labeled clavaminic acid **32** was administered (0.2 mM) at the onset of clavam production as determined by the imidazole assay.[23,36] After 120 h, valclavam (**19**) and 2-(2-hydroxyethyl)-clavam (**20**) were isolated and purified (see the Experimental Section). ^{13}C{^{1}H}NMR analysis revealed that the labeled precursor had been incorporated intact and with equal efficiency (*ca.* 0.2%) into both **19** and **20** (Scheme 5). The coupling constant observed between the enriched C-2 and C-3 sites was $^1J_{CC}$ = 32.6 Hz, in accord with the earlier successful incorporation of [2,3-$^{13}C_2$]proclavaminic acid (**16**).[6] To ensure that the observed incorporation of paired ^{13}C-labels from [2,3-$^{13}C_2$]-clavaminic acid (**32**) was not due to trivial incorporation of unreacted [2,3-$^{13}C_2$]proclavaminic acid (**16**) carried along from the enzyme incubation, the integrity of the purified cyclization product was confirmed by analytical HPLC and ^{1}H- and ^{13}C-spectroscopy. The relatively low but specific incorporation of clavaminic acid was similarly observed in experiments to investigate the biosynthesis of clavulanic acid (**1**).[28]

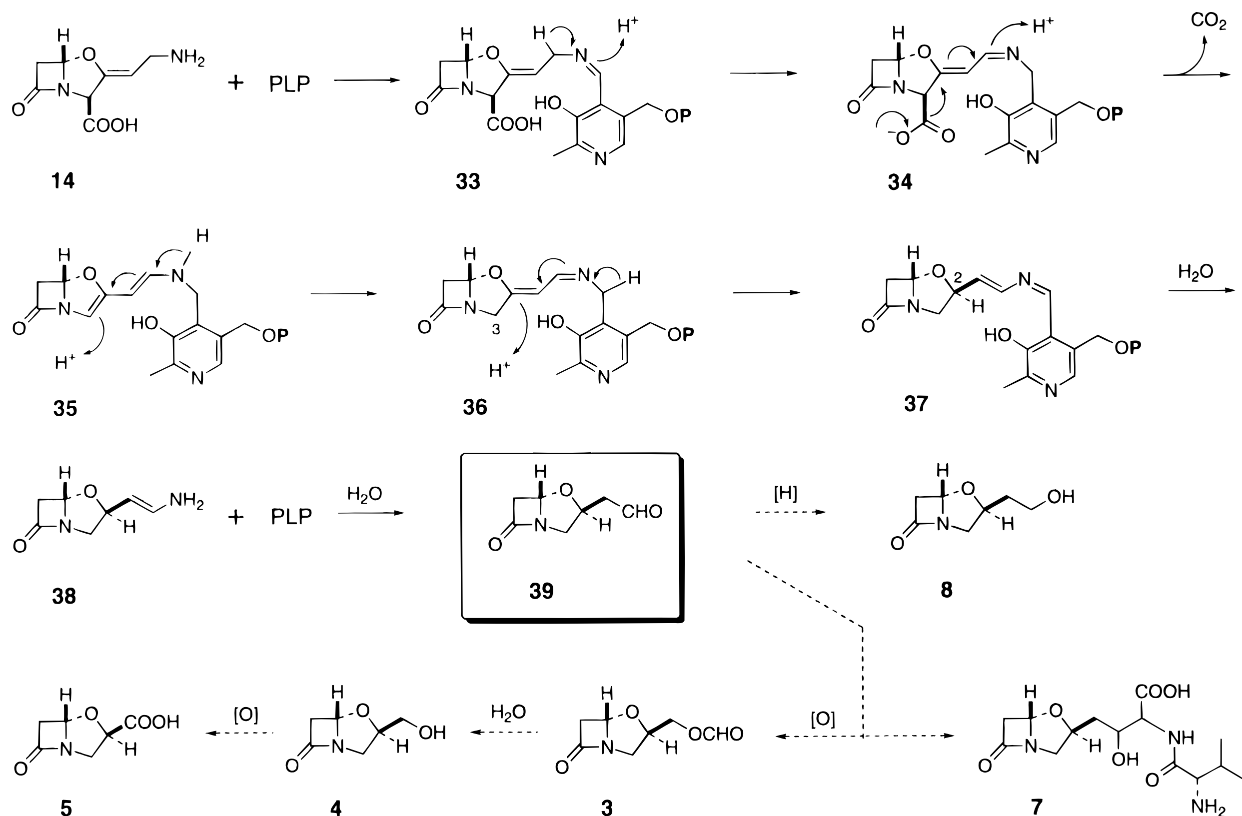
Discussion

The absolute configurations at C-2 and C-5 are identical among all the known clavams[2−4,8] and the more complex clavamycins.[37] Each lacks the C-3 carboxyl seen in clavulanic

(36) Bird, A. E.; Bellis, J. M.; Gasson, B. C. *Analyst* **1982**, *107*, 1241−1245.

(37) Naegeli, H. U.; Loosli, H.-R.; Nussbaumer, A. *J. Antibiot.* **1986**, *39*, 516−524.

Scheme 6



acid (**1**), as well as having the opposite ring fusion geometry. As shown schematically in Scheme 6, we propose[21] the intermediacy of aldehyde **39**, whose simple reduction in *S. antibioticus* could be visualized to afford 2-(2-hydroxyethyl)-clavam (**8**), or by Baeyer–Villiger-like oxidation in *S. clavuligerus* could give the formate ester **3**, which in turn could be hydrolyzed to the alcohol **4** and further oxidized to clavam-2-carboxylate **5**. Aldol-like reactions can be invoked to generate the carbinol center at C-9 in valclavam (**7**)[6,38] and similarly the various clavamycins. The implied relationship between clavaminic acid (**14**) and the aldehyde **39** poses an interesting question for which several mechanistic rationales can be advanced. Preservation of the stereochemistry at C-5 is required as is a means to promote decarboxylation. Ideally, the resulting pair of electrons should be stored in the substrate to, in effect, reduce the clavaminate double bond and generate the stereocenter at C-2. Among these mechanistic possibilities, pyridoxal can be envisioned to play such a role as outlined in Scheme 6. Initial imine formation between **14** and pyridoxal phosphate (PLP) and isomerization to **34** are proposed to provide a ready path for decarboxylation. Protonation at C-3 of the resulting vinylogous enamine **35** and isomerization and a second protonation are suggested to set the stereochemistry at C-2 and result in formation of the labile azadiene **37**. Hydrolysis of the latter would restore the PLP cofactor and release the new enamine **38**, which upon hydration would give the desired aldehyde **39**.

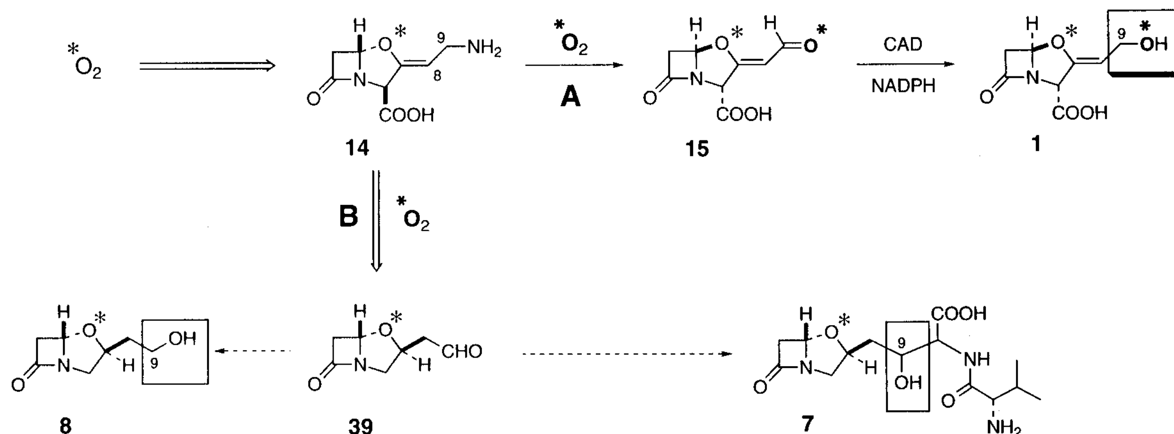
While the transformations proposed in Scheme 6 must be regarded as speculation at present, experimental observations have been made, particularly in clavulanic acid biosynthesis, that lend indirect support to this scheme and further define the extent of the common pathway to the β-lactamase inactivator **1** and the antipodal clavams. It was shown a decade ago that growth of *S. clavuligerus* in an $^{18}O_2$-containing atmosphere gave rise to the incorporation of heavy isotope (*) at the oxazolidine oxygen and, unexpectedly, into the allylic hydroxyl of clavulanic acid (**1**; see Scheme 7).[19] The latter finding suggested enzymic hydroxylation at C-9 to give a transient allylic carbinolamine bearing label from molecular oxygen. This unstable intermediate was proposed to decompose to the aldehyde enantiomeric to **15**. This intermediate was thought to be stereochemically unstable and to “racemize”, from which the mirror image stereoisomer could be selected by a dehydrogenase and reduced to the enantiomeric, now biologically active, clavulanic acid (**1**).[19] This interpretation is not fully correct as Elson and coworkers have demonstrated.[20] In admirably careful experiments it was shown that the aldehyde **15** is unstable but isolable as a single stereoisomer in small quantities from fermentations of *S. clavuligerus*. Moreover, the Beecham group has characterized an NADPH-dependent dehydrogenase responsible for carrying out the final reduction of **15** to **1**.[20] Two facts should be noted at this point. First, the striking “enantiomerization” process that occurs during the oxidative deamination of **14** to **15** must take place prior to, or concomitant with, product release. Second, the oxygen (*) introduced in this process is not lost and appears with undetectable exchange in the product **1**. The intermediate aldehyde **15** is evidently stereochemically stable and, as a vinylogous ester, is slow to undergo exchange of the labeled aldehyde oxygen relative to the rate of enzymic reduction to **1**.

An instructive comparison can be made to fermentation of *S. antibioticus* in an $^{18}O_2$-containing atmosphere[39]—an organism, it is recalled, that does not produce clavulanic acid. This experiment was carried out as previously described[19] and gave efficient incorporation of isotope into the oxazolidine ring sites of valclavam (**7**) and 2-(2-hydroxyethyl)clavam (**8**), as expected on the basis of the intermediacy of clavaminic acid (**14**). In neither of these metabolites, however, could ^{18}O be detected at

(38) Baldwin, J. E.; Goh, K.-C.; Schofield, C. J. *Tetrahedron Lett.* **1994**, *35*, 2779–2782.

(39) Egan, L. A.; Townsend, C. A., Unpublished results.

Scheme 7



C-9 by NMR spectroscopy[40] or mass spectrometry. The complete absence of labeled oxygen (*) at these carbinol sites is somewhat less compelling than their presence in path A where structural features of the intermediate **15** can be seen to retard or prevent loss of heavy isotope. On the other hand, presuming the intermediacy of the unconjugated aldehyde **39** in path B, such exchange would be more greatly favored. However, the absolute configurations of **15** and **39** are opposite, and one might suppose that, if **39** were initially formed by oxidative deamination, a portion of the ^{18}O-label would remain at C-9 in **7** and **8** in the time regime of biosynthesis at neutral pH and a temperature of *ca.* 28 °C. The absence of detectable isotope from molecular oxygen at the carbinol centers is consistent with these oxygens appearing in the biosynthesis from the aqueous medium. Whether mediated by PLP, as suggested in Scheme 6, or by some other means, together these findings point to clavaminic acid (**14**) being the final common intermediate to clavulanic acid (**1**, path A) and all of the remaining clavam natural products by a distinct route (path B).

S. antibioticus, an organism that produces valclavam (**7**) and 2-(2-hydroxyethyl)clavam (**8**), but no clavulanic acid (**1**), contains a single form of clavaminate synthase (CS3).[24] In contrast *S. clavuligerus* produces four stereochemically related clavams **2**−**5** as well as clavulanic acid and harbors two isozymes of this protein (CS1 and CS2).[23] It has been proposed that this difference might imply separate genetic loci for the biosynthetic machinery responsible for clavulanic acid and clavam synthesis.[41,42] The experiments described in this paper demonstrate a common biosynthetic pathway of considerable length in the formation of these natural product groups. The extent to which the corresponding genes encoding these common enzymes are duplicated in *S. clavuligerus*, beyond *cs1* and *cs2*, will be of interest.

Experimental Section

General Methods. Air- and moisture-sensitive reactions were run under an inert atmosphere (Ar or N_2) in oven-dried (150 °C) glassware. Tetrahydrofuran (THF) was freshly distilled from Na/benzophenone ketyl, and dichloromethane (CH_2Cl_2) was freshly distilled from CaH_2. All other solvents for air- or moisture-sensitive reactions were used as received or dried by standard procedures.[43] Nalidixic acid, Brij-58, Q-Sepharose resin, and G-75-50 superfine Sephadex resin were purchased from Sigma Chemical Co. (St. Louis, MO). Glass microbeads (0.5 mm) were purchased from VWR. Collodian apparatus and membranes were purchased from Schleicher & Schuell (Keene, NH). All other chemicals (Aldrich Chemical Co., Milwaukee, WI) were of reagent grade and used without further purification. Fermentation media were obtained from Difco Laboratories (Detroit, MI).

Hydrogen and carbon-13 NMR spectra were acquired on a Bruker AMX 300 MHz, a Varian Unity*plus* 400 MHz, or a Varian Unity*plus* 500 MHz spectrometer. Chemical shifts of hydrogen resonances are reported as parts per million and referenced to D_2O (4.78 ppm) or $CDCl_3$ (7.26 ppm). Carbon-13 chemical shifts are reported in parts per million and referenced to $CDCl_3$ (77.0 ppm) or internally referenced to 1,4-dioxane (66.5 ppm) for solutions in D_2O. Melting points were determined in open capillary tubes with a Thomas-Hoover Uni-Melt melting point apparatus and are uncorrected. Thin layer chromatography was performed using Analtech (Newark, DE) Uniplate TLC plates stained using $KMnO_4$ (1% in 6.5% Na_2CO_3/0.2% KOH). Low- and high-resolution mass spectral data were obtained on a VG Instruments 70-S GC/MS at 70 eV and are tabulated as *m/z* (intensity expressed as a percent of the base peak). UV−vis spectrophotometry employed a Beckman DU 70 spectrophotometer (Fullerton, CA). 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, Palo Alto, CA), using rotors prepared with silica gel PF-254 with $CaSO_4\cdot{}^1/_2H_2O$ as binder. HPLC chromatography was performed with a Waters 600 multisolvent delivery system equipped with a Rheodyne injector and a Waters 490 programable multiwavelength detector. Protein assays were performed by the method of Bradford using BSA as a standard.[44] SDS−PAGE used the buffer system of Laemmli[45] in a Hoefer SE 400 slab gel electrophoresis unit (San Francisco, CA).

Expression of CS2. Cultures of *E. coli* JM101 cells (100 mL) containing the recombinant pARC2P plasmid[27] were prepared by inoculating the liquid medium (2 × YT[46] containing 300 μg/mL of ampicillin) with single colonies picked from agar plates. The cell cultures were frozen at −86 °C in a 50% glycerol suspension. Overnight cultures were initiated by adding 5 mg of the frozen cell suspension to 2 × YT liquid medium containing 300 μg/mL of ampicillin and then incubated with shaking (300 rpm) at 37 °C. Following overnight incubation, the cultures were diluted into 500 mL of 2 × YT medium (300 μg/mL of ampicillin) to an optical density at 590 nm (A_{590}) of 0.10 and grown at 37 °C with shaking to approximately 1×10^9 cells/mL ($A_{590} = 0.90$). Expression was induced by the addition of nalidixic acid at a concentration of 50 μg/mL, and expression proceeded at 19 °C for 7 h. The cells were harvested by centrifugation at 4000*g* for 10 min, frozen in liquid N_2, and stored at −86 °C.

Abbreviated CS2 Purification Procedure. All steps were conducted at 0−4 °C. General enzyme buffer (GEB) consisted of 50 mM Tris (pH 7.0), 20% glycerol, and 10 μM EDTA with 1 mM benzamidine

(40) Vederas, J. C. *Nat. Prod. Rep.* **1987**, *4,* 277−337.
(41) Marsh, E. N.; Chang, M. D.-T.; Townsend, C. A. *Biochemistry* **1992**, *31,* 12648−12657.
(42) Paradkar, A. S.; Jensen, S. E. *J. Bacteriol.* **1995**, *177,* 1307−1314.
(43) Perrin, D. D.; Armarego, W. L. F. *Purification of Laboratory Chemicals*, 3rd ed.; Pergamon Press: New York, 1988.
(44) Bradford, M. M. *Anal. Biochem.* **1976**, *72,* 248−254.
(45) Laemmli, U. K. *Nature (London)* **1970**, *227,* 680−685.
(46) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning. A Laboratory Manual*; Cold Spring Harbor Laboratory: New York, 1982.

and 1 mM DTT added just prior to use. Partial purification of CS2 was carried out using 50 g of frozen expressed *E. coli* JM101 cell paste thawed in lysis buffer (50 mM Tris (pH 8.5), 20% glycerol, 100 mM KCl, 10 mM DTT, and 10 μM EDTA) in a total volume of 75 mL divided evenly into 3 × 50 mL Falcon tubes. Cells were lysed by the addition of 600 μL/tube (1/50th volume) of 1% lysozyme in 10 mM Tris buffer (pH 8.5). Simultaneously, 300 μL/tube of 1 mg/mL DNase I in 1 × TE (10 mM Tris (pH 8), 10 μM EDTA) was added and the cells were incubated on ice with occasional inverting. After 5 min, 600 μL/tube (1/50th) of a 5% Brij-58 solution was added and incubation at 0 °C continued with inverting for an additional 10 min. The lysis mixture was further divided into six Falcon tubes (15 mL/tube), and nitric acid-washed glass microbeads (0.5 mm, 15 g) were then added to each tube. Cell lysis was completed by vortexing each tube for two 15 s bursts, 1 min apart. The cells were diluted to 700 mL with GEB and stirred for 20 min. A 1% streptomycin sulfate fractionation was used to remove nucleic acids, and the protein was precipitated by the addition of ammonium sulfate to 70% of saturation. Following centrifugation the protein pellet was resuspended in 20 mL of GEB (final volume approximately 30 mL). The solution was dialyzed in a Spectra/Por porous membrane (10 000 molecular weight cut off) against 2 L of GEB for a total of 4 h (the initial GEB was replaced after 2 h). The dialyzed protein was removed, diluted to 150 mL with GEB, and loaded onto a Q-Sepharose column (2.5 × 44 cm, 216 mL) equilibrated in GEB at a rate of 2.0 mL/min. The column was washed with 175 mL of GEB followed by elution with a 1.0 L linear gradient of KCl from 0 to 400 mM in GEB, collecting 10 mL fractions. CS2 eluted at 240 mM KCl, and fractions containing CS activity were pooled and concentrated to approximately 20 mL in an Amicon ultrafiltration cell fitted with a PM 10 membrane. The solution was further concentrated and dialyzed in storage buffer (50 mM MOPS (pH 7.0), 20% glycerol, 10 μM EDTA, and 1 mM DTT) using the Collodian apparatus and stored at −20 °C.

Assay for Clavaminate Synthase Activity. Assays for the conversion of **7** to **8** were conducted as previously reported.[23]

Synthesis of (±)-[2,3-$^{13}C_2$]Proclavaminic Acid (16). [1-^{13}C]-3-[(Benzyloxycarbonyl)amino]propanol (25). The previously described[29] Cbz-protected [1-^{13}C]-β-alanine (**24**; 6.135 g, 27.36 mmol) in dry THF (150 mL) was treated at −10 °C with freshly distilled triethylamine (3.85 mL, 27.6 mmol), and ethyl chloroformate (3.2 mL, 33.5 mmol) was added slowly over 20 min. After stirring for an additional 90 min, the precipitated triethylammonium chloride was removed by filtration through a fritted funnel and the cooled filtrate was added over 40 min to a 5 °C solution of $NaBH_4$ (2.1 g, 55 mmol) in water (80 mL). When the addition was complete, more $NaBH_4$ (1.0 g, 26 mmol) was added and the reaction mixture was stirred for 4.5 h. After acidification with 6 N HCl, the solution was extracted with EtOAc (2 × 350 mL) and the combined organic layers were washed with 5% $NaHCO_3$ (600 mL), and brine (450 mL) and dried over anhydrous $NaSO_4$. Concentration *in vacuo* gave a white solid, which was recrystallized from EtOAc/hexanes to give 4.809 g (22.87 mmol, 84%) of the desired alcohol as white needles: 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, C*H*$_2$Ar), 5.02 (br, 1H, N*H*), 3.68 (dt, J = 5.6, 141.6 Hz, 2H, H-1), 3.37 (m, 2H, H-3), 2.51 (br, 1H, O*H*), 1.70 (m, 2H, H-2); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 157.4, 136.4, 128.6, 128.2, 128.1, 66.9, 59.5 (enriched), 37.7, 32.6 (d, J = 42.3 Hz); MS *m/z* 210 (M$^+$, 1.5), 192, 151, 108, 91 (100), 79; accurate mass 210.1088, calcd for $^{12}C_{10}{}^{13}CH_{15}NO_3$ 210.1085.

[1-^{13}C]-3-[(Benzyloxycarbonyl)amino]propanal (26). A dry 250 mL round-bottomed flask equipped with a stir bar was charged with CH_2Cl_2 (50 mL) and oxalyl chloride (2.1 mL, 24 mmol), and the solution was cooled to −78 °C. DMSO (3.5 mL, 49 mmol) in CH_2Cl_2 (5 mL) and a solution of the labeled alcohol **25** (4.687 g, 22.29 mmol) in CH_2Cl_2 (40 mL) were added. After stirring for an additional 15 min, triethylamine (15.5 mL, 111.4 mmol) was added during 7 min, and stirring was continued for an additional 10 min, whereupon the reaction was allowed to warm to room temperature over 45 min. Water (100 mL) was added, and the layers were separated. The aqueous phase was extracted with CH_2Cl_2 (100 mL), and the combined organic extracts were washed with 1 N HCl (150 mL), 5% $NaHCO_3$ (150 mL), and brine (150 mL). After drying with anhydrous Na_2SO_4, the solution was concentrated *in vacuo* and purified by flash chromatography (80 g of silica gel; petroleum ether:EtOAc, = 8:2 to 3:7) to give 3.528 g (16.94 mmol, 76%) of the desired aldehyde as a fluffy white solid. Recrystallization from EtOAc/petroleum ether provided the aldehyde **26** as white leaves: mp 59−60 °C; ^{1}H NMR ($CDCl_3$) δ 9.80 (d, J_{C-H} = 174.3 Hz, 1H, H-1), 7.35 (m, 5H, Ar*H*), 5.12 (br, 1H, N*H*), 5.10 (s, 2H, C*H*$_2$Ar), 3.50 (m, 2 H, NC*H*$_2$), 2.75 (m, 2 H, COC*H*$_2$); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 201.1 (enriched), 156.2, 136.1, 128.5, 128.1, 128.0, 66.7, 44.0 (d, J = 37.6 Hz), 34.4. Characterization of the unlabeled aldehyde gave the following analytical data: 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.

Benzhydryl [2-^{13}C]-2-[4′-(Phenylthio)-2′-oxoazetidin-1′-yl]acetate (28). 4-(Phenylthio)azetidin-2-one (**27**)[32] (2.237 g, 12.48 mmol) in THF (25 mL) was cooled to −78 °C, and LiHMDS (13.8 mL, 1.0 M in hexane) was added dropwise. The reaction was stirred for 45 min, and a solution of benzhydryl [2-^{13}C]bromoacetate[31] (4.246 g, 13.87 mmol) in THF (10 mL) was added over 25 min. After stirring for a few minutes at −78 °C, the reaction was allowed to warm to 0 °C over the course of 2 h. The mixture was filtered through a plug of silica gel washing with EtOAc, and the filtrate was concentrated *in vacuo*. Purification by flash chromatography on silica gel gave 2.226 g (5.504 mmol, 44%) of the desired product **28** as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.38−7.18 (m, 15H, Ar*H*), 6.84 (s, 1H, C*H*Ar$_2$), 5.21 (dd, J = 2.3, 5.0 Hz, 1H, H-4′), 4.41 (dd, J = 18.1, 139.4 Hz, 1H, ^{13}C*H*), 3.83 (dd, J = 18.1, 141.1 Hz, 1H, ^{13}C*H*), 3.41 (dd, J = 4.7, 15.4 Hz, 1H, H-3′$_{cis}$), 2.88 (dd, J = 2.6, 15.1 Hz, 1H, H-3′$_{trans}$). Unlabeled material prepared in an analogous manner gave the following spectral data: IR ($CHCl_3$) 3060, 3037, 3013, 1760 (br), 1496, 1454, 1437, 1401, 1365, 1196, 1178, 967, 941, 914, 700 cm^{-1}; ^{13}C{^{1}H} NMR ($CDCl_3$) δ 167.1, 165.4, 139.3, 139.2, 133.7, 130.3, 129.4, 128.7, 128.6, 128.3, 128.2, 127.1, 127.0, 78.3, 59.1, 45.0, 41.3; MS *m/z* 167 (100), 109; accurate mass 403.1246, calcd for $C_{24}H_{21}NO_3S$ 403.1242.

Benzhydryl [2-^{13}C]-2-(2′-Oxoazetidin-1′-yl)acetate (29). The 4-(phenylthio)azetidin-2-one **28** (2.020 g, 5.167 mmol) was dissolved in dry benzene (35 mL), and the solution was thoroughly degassed. AIBN (170.9 mg, 1.041 mmol) and tributyltin hydride (2.8 mL, 10.3 mmol) were added, and the reaction was heated to reflux with stirring under N_2. After 10 h, additional AIBN (102.3 mg, 0.6230 mmol) and tributyltin hydride (1.0 mL, 3.7 mmol) were added, and the reaction was continued for another 9 h. The reaction mixture was concentrated *in vacuo* and the residue taken up in acetonitrile (100 mL) and washed with hexane (2 × 100 mL). The acetonitrile solution was concentrated and the residue purified by radial chromatography (2 mm silica gel; petroleum ether:EtOAc = 9:1 to 1:1) to give 171.9 mg (0.4250 mmol, 8.2%) of unreacted starting material and 880.5 mg (2.971 mmol, 58%) of the reduced azetidinone **29** as a clear oil, which solidified upon standing: mp 69−70.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.32 (m, 10H, Ar*H*), 6.92 (s, 1H, C*H*Ar$_2$), 4.11 (d, J = 139.6 Hz, 2H, ^{13}C*H*$_2$), 3.41 (t, J = 4.2 Hz, 2H, H-4′), 3.03 (t, J = 4.1 Hz, 2H, H-3′); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 43.3 (enriched). Unlabeled material prepared in an analogous manner gave the following spectral data: ^{13}C{^{1}H} NMR ($CDCl_3$) δ 167.9, 167.4, 139.4, 128.6, 128.2, 127.0, 78.1, 43.3, 40.0, 37.7; MS *m/z* 295 (M$^+$, 0.3), 183, 168, 167, 152, 84, 77, 42; accurate mass 295.1212, calcd for $C_{18}H_{17}NO_3$ 295.1208.

Benzhydryl [2,3-$^{13}C_2$]-5-[(Benzyloxycarbonyl)amino]-3-hydroxy-2-(2′-oxoazetidin-1′-yl)pentanoates 30 and 31. Ester **29** (880.5 mg, 2.971 mmol) in THF (50 mL) was cooled to −78 °C, and LiHMDS (3.4 mL, 1.0 M in hexane) was added dropwise. After stirring for 30 min, a solution of the Cbz-protected aldehyde **26** (932.8 mg, 4.480 mmol) in THF (8 mL) was added dropwise, and the reaction was stirred for 2 h. A solution of 5 M acetic acid (600 μL) in THF was added to quench the reaction, and after warming to room temperature, the mixture was transferred to EtOAc (200 mL) and washed with 1 N HCl (200 mL), 5% $NaHCO_3$ (200 mL), and brine (2 × 200 mL). The organic layer was concentrated *in vacuo* and the residue purified by radial chromatography (2 mm silica gel; petroleum ether:ethyl acetate, = 4:1 to 1:1) to give 201.7 mg (0.9687 mmol) of the unreacted aldehyde **26**, 140.0 mg (0.4725 mmol, 16%) of the unreacted azetidinone **29**, 221.6 mg (0.4392 mmol, 15%) of the *threo* diastereomer **31**, and 1.003 g (1.988 mmol, 67%) of a mixture of the *erythro*/*threo* diastereomers.

The latter mixture of diastereomers **30**/**31** (1.003 g, 1.988 mmol) in CH_2Cl_2 (40 mL) was treated with DBN (235 μL, 1.90 mmol), and the solution was stirred for 1 h. After passage through silica gel, the filtrate was concentrated *in vacuo*, and the residue was purified by radial chromatography (2 mm silica gel; petroleum ether:ethyl acetate (4:1 to 1:1) to give 457.3 mg (0.9064 mmol, 46%) of the *threo* diastereomer **31** and 245.0 mg (0.4856 mmol, 24%) of a mixture of the *erythro*/*threo* diastereomers. The *threo* diastereomer **31** gave the following spectral data: ^{1}H NMR ($CDCl_3$) δ 7.33 (m, 15H, Ar*H*), 6.93 (s, 1H, C*H*Ar$_2$), 5.10 (m, 3H, C*H*$_2$Ar and N*H*), 4.59 (m, 0.5H, H-3), 4.41 (m, 1.5H, O*H* and H-2), 4.07 (m, 0.5H, H-3), 3.98 (m, 0.5H, H-2), 3.48 (m, 1H, H-4′), 3.40 (m, 2H, H-5), 3.28 (m, 1H, H-4′), 2.99 (t, J = 4.2 Hz, 2H, H-3′), 1.68 (m, 2H, H-4); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 68.9 (d, J = 37.9 Hz), 61.9 (d, J = 38.0 Hz). Unlabeled material prepared in an analogous manner gave the following spectral data: IR ($CHCl_3$) 3448, 3010, 2955, 1725 (br), 1710, 1514, 1503, 1408, 1231, 1181, 699 cm^{-1}; ^{13}C{^{1}H} NMR ($CDCl_3$) δ 169.3, 167.8, 157.2, 139.3, 136.3, 128.6, 128.5, 128.3, 128.2, 128.1, 127.2, 127.0, 78.6, 68.9, 66.9, 62.1, 40.4, 37.8, 36.4, 34.5; accurate mass (MH$^+$) 503.2179, calcd for $C_{29}H_{31}N_2O_6$ 503.2182.

In other experiments the *erythro* and *threo* diastereomers **30** and **31** were isolated as oils, which solidified on standing under vacuum. Recrystallization from EtOAc/hexanes gave white crystalline solids, *erythro* mp 97−99 °C and *threo* mp 119−121 °C.

(±)-[2,3-^{13}C$_2$]Proclavaminic Acid (16). Protected *threo*-proclavaminate **31** (1.04 g, 2.06 mmol) was dissolved in a solution of ethanol H_2O (40 mL, 7:1) and 10% Pd/C (200 mg) was added. The mixture was degassed, and H_2 was bubbled through for 30 min. The solution was passed through a 0.22 μm filter, washing with water, the ethanol was removed *in vacuo*, and the aqueous solution lyophilized to give 315.0 mg (1.540 mmol, 75%) of the product **16** as a fluffy, white solid: ^{1}H NMR (D_2O/acetone) δ 4.52 (dt, J = 4.5, 9.0 Hz, 0.5H, H-3), 4.46 (t, J = 4.4 Hz, 0.5H, H-2), 4.01 (m, 1H, 0.5 H-2 and 0.5 H-3), 3.64 (m, 1H, H-4′), 3.55 (m, 1H, H-4′), 3.18 (m, 2H, H-5), 3.05 (t, J = 3.9 Hz, 2H, H-3′), 1.88 (m, 2H, H-4); ^{13}C{^{1}H} NMR (D_2O/dioxane) δ 68.6 (d, J = 39.3 Hz), 60.2 (d, J = 39.3 Hz). Physical and spectral data of unlabeled material prepared in an analogous manner were as previously described.[17]

Conversion of (±)-[2,3-^{13}C$_2$]Proclavaminic Acid (16) to (−)-[2,3-^{13}C$_2$]Clavaminic Acid (32). A 2 L Erlenmeyer flask was equipped with a stir bar containing 1.5 L of the following: 50 mM MOPS (pH 7), 0.5 mM DTT, 0.1 mM ascorbic acid, 25 μM ferrous ammonium sulfate, 1.0 mM [2,3-^{13}C$_2$]-D,L-proclavaminic acid (**16**, 1.53 mmol), and 0.1 mM α-KG (0.15 mmol). The reaction was initiated by the addition of 2.5 mL of a partially-pure CS2 solution (equivalent to 100 mg/mL pure CS2) and allowed to proceed at room temperature for 9 h with constant stirring. Throughout the entire reaction, 0.15 mmol of α-KG (306 μL of a 500 mM stock solution) was added every 20 min. Additional 2.5 mL aliquots of partially-pure CS2 (*ca.* 250 mg of pure enzyme) were added at 1, 2, and 3 h. Aliquots of 200 μL were removed hourly and added to Eppendorf microcentrifuge tubes containing 10 μL of a 4 mM solution of EDTA. Samples were frozen in liquid N_2 until the reaction was complete. These samples were then thawed, and derivatized with imidazole, and the A_{312} was read in the usual manner in order to monitor the extent of reaction which was determined to be 71%. The theoretical yield was calculated using the amount of L-enantiomer available.

Purification of [2,3-^{13}C$_2$]-(3*S*,5*S*)-Clavaminic Acid (32). The final reaction mixture was loaded onto a DEAE Sephadex column, pH 6 (2.5 × 41 cm, 200 mL) which was subsequently washed with 2 column volumes of double distilled H_2O. The eluent was frozen and partially lyophilized overnight. The high concentration of MOPS hampered lyophilization and caused the concentrated sample to thaw. The final protein removal step employed an Amicon ultrafiltration cell fitted with a PM 10 membrane. The filtrate was then frozen and lyophilized overnight to approximately 5 mL of a pink, viscous solution, which was then purified by HPLC [Whatman Partisil ODS 3, 10 μm (22 × 250 mm), mobile phase H_2O (10 mL/min), monitored at λ = 220 nm]. The entire sample was purified in this manner (200 μL/injection) with a t_r of 8.5 min for clavaminate and t_r of 6.0 min for proclavaminate. The product contained approximately 5% of the MOPS buffer present in the initial reaction mixture: ^{1}H NMR (D_2O) δ 5.77 (d, J = 2.7 Hz, 1H, H-5), 4.82 (t, 1H, H-8), 4.76 (bs, 1H, H-3), 3.67 (m, 2H, H-9), 3.57 (dd, J = 2.7, 17.1 Hz, 1H, H-6α), 3.15 (d, J = 17.1 Hz, 1H, H-6β); ^{13}C{^{1}H} NMR (D_2O) δ 181.5, 175.6, 161.1, 92.3, 90.8, 74.7, 66.0, 48.2.

Growth of *S. antibioticus* for Feeding Experiments. Seed Medium. D,L-Methionine (0.4 g), D-glucose (4.0 g), yeast extract (4.0 g), and malt extract (10 g) were dissolved in 1 L of tap H_2O (pH 7.3). A 500 mL Erlenmeyer flask containing 100 mL of seed medium was inoculated using sterile techniques with 200 μL of a spore suspension of *S. antibioticus* (Tü 1718) prepared as described by Wanning.[47] Once inoculated, the seed medium was grown for 48 h at 27 °C with shaking (300 rpm).

Modified Soy Fermentation Medium. In 1 L of tap H_2O, 3.75 mmol each of L-alanine, L-arginine, L-aspartic acid, L-glutamate, and L-serine were dissolved along with 30 g of D-mannitol (pH 7.2). Ten 500 mL Erlenmeyer flasks each containing 2 g of freshly ground soy beans and 100 mL of fermentation medium were inoculated with a 1% inoculum of the 48 h seed culture.

Administration of [2,3-^{13}C$_2$]Clavaminic Acid (32) to *S. antibioticus*. [2,3-^{13}C$_2$]Clavaminic acid (**32**; 26 mg) was administered to *S. antibioticus* cultures (7 × 100 mL in 500 mL Erlenmeyer shake flasks) as a filtration-sterilized, aqueous solution (pH 7.0) in pulsed feedings of 0.1 mmol/L aliquots at 56 and 65 h. At 121 h, doubly ^{13}C-labeled valclavam (**19**) and 2-(2-hydroxyethyl)clavam (**20**) were isolated as described below.

Isolation and Purification of Valclavam (7/19) and 2-(2-Hydroxyethyl)clavam (8/20). Clavam isolation was carried out according to the procedure of Wanning with some modifications.[47] The fermentation broth was harvested by centrifugation, and the supernatant was passed through cheesecloth, filtered through a Celite pad, and brought to 2% w/v in NaCl. The solution was loaded onto an Amberlite XAD-4 column (800 mL) and the column rinsed with 1 L of H_2O. Desorption of valclavam (**7**/**19**) was achieved by elution with 1 L of a 20% aqueous methanol solution. The column was then eluted with 1 L of methanol to collect 2-(2-hydroxyethyl)clavam (**8**/**20**).

After concentration of the first of these by rotary evaporation (without heat), the aqueous residue was passed through a Sephadex-DEAE A-25 column (100 mL). The eluent was frozen and lyophilized to provide crude valclavam as an orange powder. Further purification of valclavam (**7**/**19**) was achieved by reversed-phase HPLC (Whatman Partisil ODS-3, 10 μm, 9.4 × 250 mm; mobile phase H_2O). Following concentration of the 100% methanol fraction by rotary evaporation (without heat), the remaining aqueous residue (60 mL) was diluted with an equal volume of water (60 mL) and extracted with EtOAc (3 × 100 mL). Sodium chloride was used to disrupt the emulsion which normally formed between the two layers. The EtOAc extracts were combined, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to provide a brown oil. The oil was further purified by flash chromatography using a stepwise gradient of 50:50 to 25:75 hexane/EtOAc to 100% EtOAc. The product was identified using TLC (50:50 EtOAc/hexane) where the clavam of interest eluted with an R_f of 0.19. After product fractions were pooled and concentrated *in vacuo*, the product was obtained as a clear, pale amber oil.

^{13}C{^{1}H} NMR analysis of valclavam (**19**) and 2-(2-hydroxyethyl)clavam (**20**) revealed intact incorporation of the ^{13}C-labels at C-2 and C-3 in both clavams with an efficiency of 0.2% ($^2J_{CC}$ = 32.6 Hz).

Acknowledgment. We are grateful to the National Institutes of Health for financial support of this research (Grant AI14937) and to the NIH (NMR: Grants RR 01934, RR 04794, and RR 06468; MS: Grant RR 02318) and NSF (NMR: Grant PCM 83-03176) for major funding to acquire the analytical instrumentation used.

JA963107O

(47) Wanning, M. Ph.D. Thesis, University of Tübingen, Tübingen, Germany, 1980.