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EXPERIMENTS AND SPECULATIONS ON THE ROLE OF OXIDATIVE CYCLIZATION CHEMISTRY IN NATURAL PRODUCT BIOSYNTHESIS

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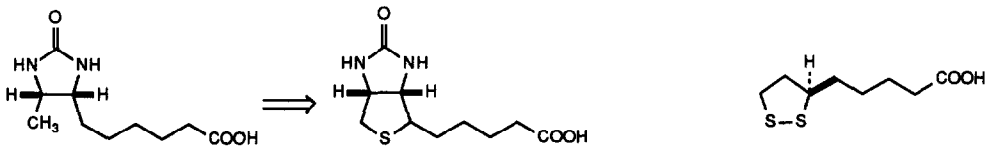
Abstract—Syntheses of [2-^{2}H,3-^{13}C]- and [2-^{2}H,3'-^{2}H$_2$]proclavaminic acid, (**22**) and (**29**) respectively, are described. These labeled substrates were incubated with clavaminate synthase, a key enzyme acting in the biosynthesis of the lactamase inhibitor clavulanic acid, in the presence of ferrous ion, α-ketoglutaric acid and molecular oxygen. The apparent oxidative cyclization/desaturation chemistry evident in the conversion of proclavaminic acid (**8**) to clavaminic acid (**9**) takes place without loss or exchange of label at C-2 and C-3' in the substrate. These observations point to notable similarities to sulfur insertion reactions in natural product biosynthesis and lead to the proposition of a generalized mechanism for oxidative cyclization invoking substrate heteroatom participation. By extension of this mechanistic hypothesis, a new biogenetic speculation is advanced to account for polyether formation, for example, in monensin (**52**) and brevetoxin A (**55**).

Often the late stages of a natural product biosynthetic pathway involve transformations that are fundamentally oxidative. In the main these reactions are direct hydroxylations where subsequent elevations to the ketone or carboxyl oxidation states are possible. However, a developing body of experimental work suggests that an important subset of these reactions can be identified where it may be argued that the biochemical machinery of typical *hydroxylation* chemistry is diverted to carry out *oxidative cyclization* processes.

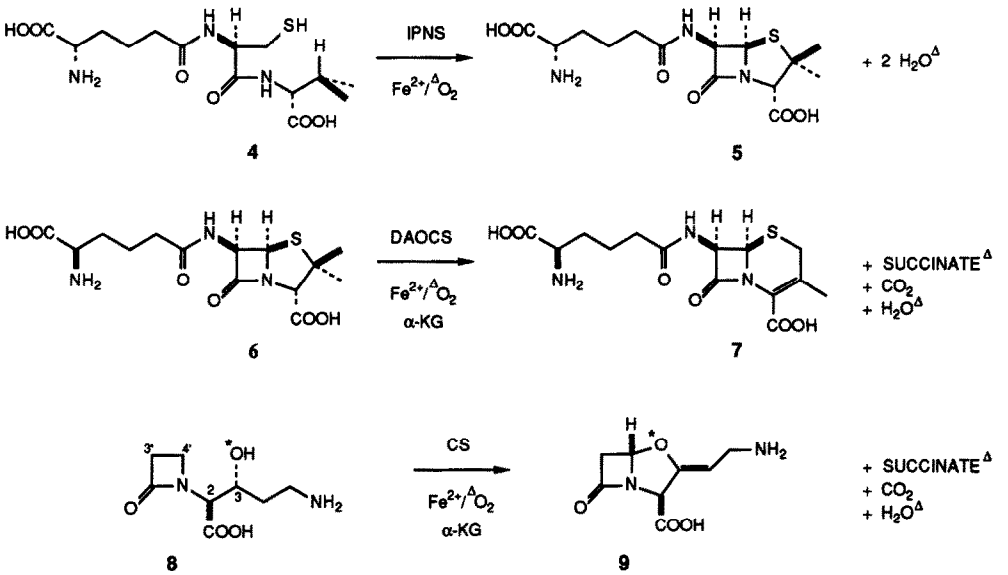


1 **2** **3**

Early studies of this kind include the interesting sulfur insertion reactions evident, for example, in the conversion of dethiobiotin (**1**) to biotin (**2**) or of octanoic acid to lipoic acid (**3**).[1] However, nowhere have these processes been examined more closely than in the oxidative transformations that characterize the formation of important members of the β-lactam antibiotic family. Isopenicillin N synthase (IPNS) carries out the double oxidative cyclization of tripeptide **4** to isopenicillin N (**5**) with concommitant reduction of a single molecule of dioxygen (Δ)[2] to two equivalents of water. Similarly, deacetoxycephalosporin C synthase (DAOCS), another Fe(II)-dependent oxygenase, carries out the ring expansion of penicillin N (**6**) to deacetoxycephalosporin C (**7**) in the presence of α-ketoglutaric acid (α-KG) and molecular oxygen to give carbon dioxide and succinic acid bearing one of the atoms of dioxygen and a mole of water accounting for the other atom from dioxygen (Δ).[3] All of these examples involve *sulfur* as the participating heteroatom. An important analogy to these processes has been demonstrated recently for *oxygen* as the participating heteroatom in the conversion of proclavaminic acid (**8**) to clavaminic acid (**9**), a key intermediate in the biosynthesis of the β-lactamase inhibitor clavulanic acid.[4] The

conversion of **8** to **9** is carried out by clavaminate synthase (CS) which, like DAOCS, requires Fe(II), α-KG and molecular oxygen as co-reactants.[5,6] It should be noted that in each of these three transformations involved in β-lactam antibiotic biosynthesis, molecular oxygen, which in conventional hydroxylation chemistry would appear in an oxidized product, for the case of oxidative cyclization is instead reduced to water. Therefore, the thermodynamically unfavorable creation of these strained bicyclic systems—ring strain which is fundamental to their ultimate expression of antibiotic activity as selective enzyme inhibitors—is mechanistically coupled to the exergonic reduction of dioxygen to water.[6] In this paper we review experiments with clavaminate synthase that have led to recognition of these common mechanistic features and provide experimental details of the syntheses of labeled substrates essential to these studies. A generalized mechanism of oxidative cyclization involving heteroatom participation in iron ligation is proposed and some speculative extensions of this concept to other classes of natural products containing oxacycles is considered.

Scheme I



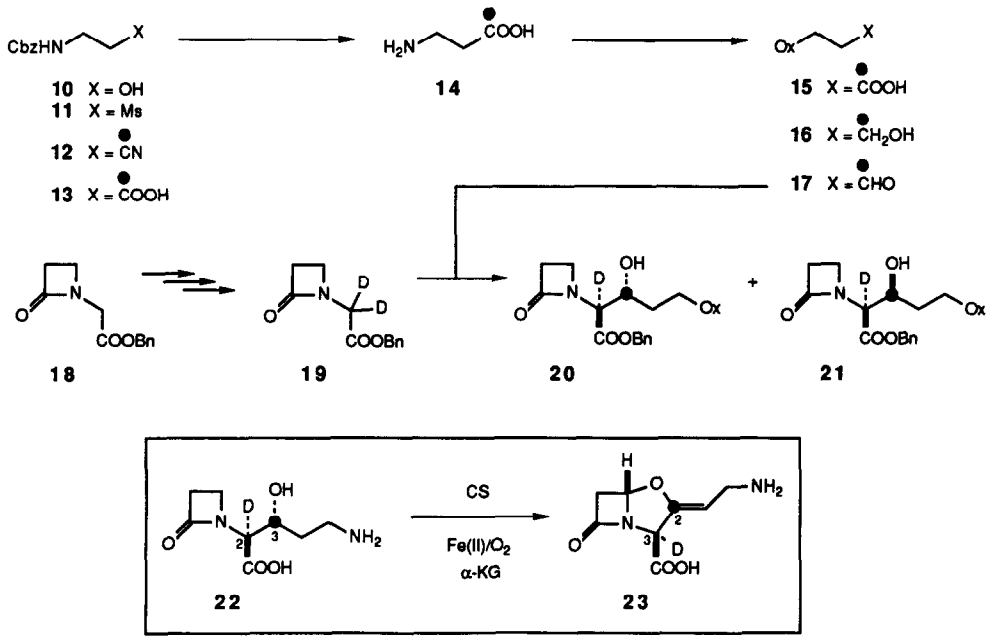
4 **5** **6** **7** **8** **9**

Perhaps the most striking aspect of the *S*-insertion reactions involved in biotin (**2**), lipoate (**3**), isopenicillin N (**5**) and deacetoxycephalosporin C (**7**) biosynthesis is the deceptively simple replacement of a C—H bond by a C—S bond in the absence of any detectable further hydrogen loss or exchange at the same or adjacent carbons. In considering the conversion of proclavaminate (**8**) to clavaminate (**9**) several questions were immediately posed. While obviously hydrogen loss from C-4 in **8** must occur to allow for double bond formation in **9**, does loss or exchange take place at C-2 or C-3'? Similarly, does the C-3 hydroxyl in **8** give rise to the oxazolidine oxygen in **9**, or does it arise from molecular oxygen (Δ) during the oxidative cyclization process itself?

The matter of hydrogen loss was addressed in two experiments. First racemic [2-^{2}H, 3-^{13}C]-proclavaminate (**22**) was prepared as shown in Scheme II. The Ox-protected[7] aldehyde **17** was generated from

[1-^{13}C]β-alanine (**14**)[8] and condensed with the partially deuteriated glycyl β-lactam **19** to give a kinetic aldol product **20/21** favoring the undesired *erytho*-isomer (**20**:**21**, 1:3). The *threo*-isomer **20** was isolated by silica gel chromatography and the remaining **21** containing a small amount of **20** was equilibrated with DBN to give **20** and **21** in a 4:1 ratio. Pure *threo*-isomer **20** was separated by radial chromatography, combined with the material isolated above and deprotected to give [2-^{2}H, 3-^{13}C]proclavaminate (**22**).

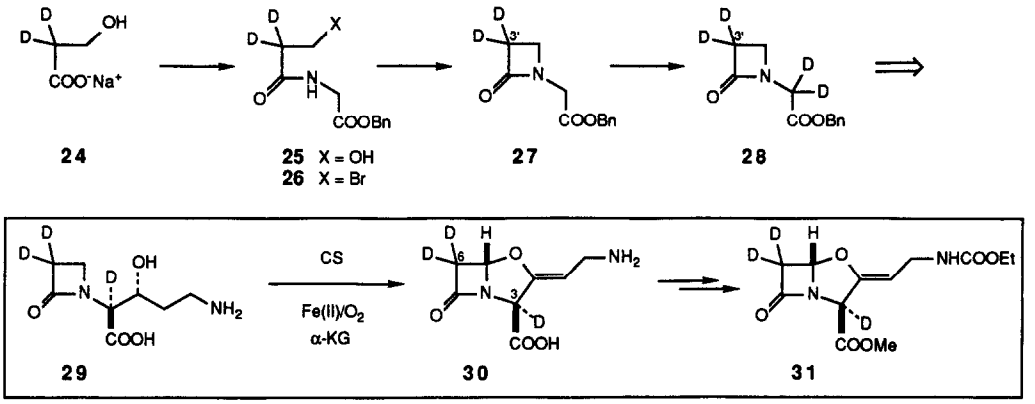
Scheme II



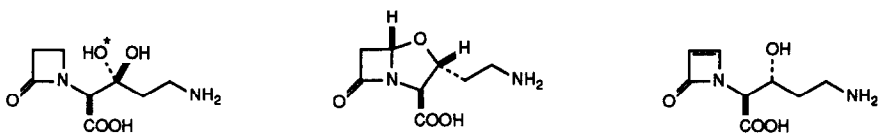
The fate of the C-2 deuterium label was monitored indirectly by its β-isotope shift[9] (Δδ 0.07 ppm upfield for **22** itself) on the position of the C-3 resonance in the ^{13}C-NMR spectrum. The synthesis of the labeled precursor was carried out in such a way that the ratio between C-2-^{1}H and C-2-^{2}H species was roughly equal and reflected in two closely spaced but distinct resonances for C-3. Upon enzymic conversion to clavaminate **23** the ^{1}H/^{2}H-ratio at C-2 was seen to be unchanged as revealed by the appearance of two signals for C-2 in clavaminate in the same ratio as the starting proclavaminate indicating, therefore, *no* exchange of hydrogen/deuterium at C-2 in proclavaminate (**22**) through the oxidative conversion to **23**.[4]

Second, [2-^{2}H, 3'-^{2}H$_2$]proclavaminate (**29**) was prepared to examine the possible loss or exchange of label at C-3' during the oxidative cyclization catalyzed by CS. The deuterium labels destined ultimately for C-3' were introduced at a high level at an early stage of the synthesis during the hydrolysis of 3-hydroxypropionitrile (**24**) in NaOD/D$_2$O (Scheme III). The resulting sodium salt was condensed with benzyl glycinate in the presence of water-soluble carbodiimide to give the crystalline amide **25**. Mass spectral analysis at this point indicated a deuterium content of 97%/site. The remaining steps of the synthesis were quite straightforward (Scheme III) and are detailed in the Experimental Section. As a further check for possible exchange at C-2, the [3'-^{2}H$_2$]azetidinone **27** was labeled as above with deuterium to give **28** containing about 80% d/site in the glycyl side chain.

Scheme III



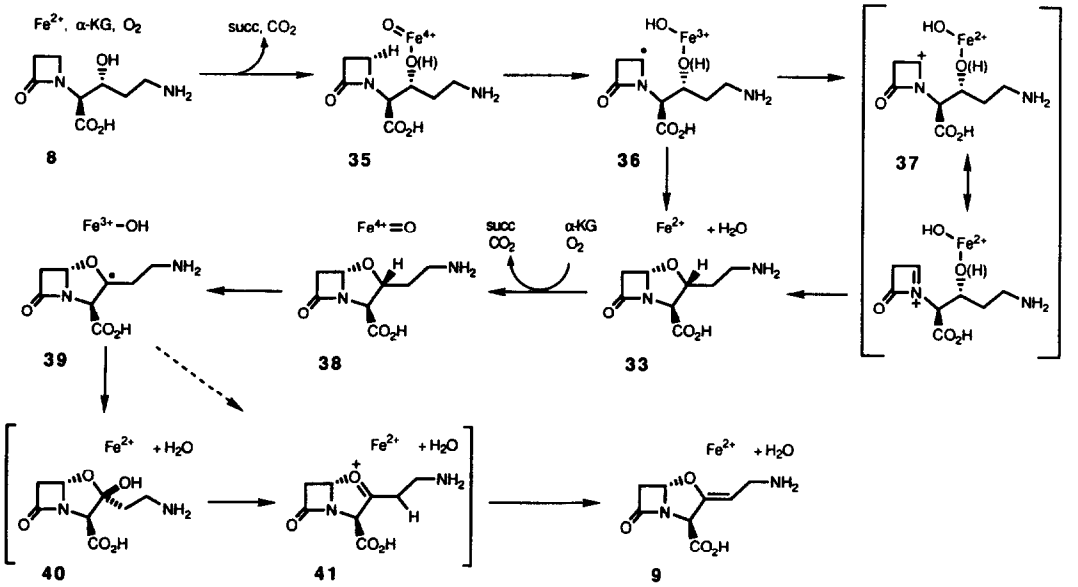
The [2-^{2}H,3'-^{2}H$_2$]proclavaminate (**29**) so obtained was converted in the presence of CS to clavaminic acid (**30**).[4] To allow accurate analysis of the deuterium content by mass spectrometry, **30** was converted to the *N*-ethoxycarbonyl methyl ester **31**. The ^{1}H-NMR spectrum showed no detectable loss of deuterium from the β-lactam (*i.e.* at C-6) in **31**. As described in greater detail elsewhere,[4] CIMS analysis of **31** confirmed that, within rather narrow confines of experimental error, no loss of deuterium label occurs from C-2 or C-3' in proclavaminic acid through conversion to clavaminic acid.



In a parallel experiment,[4] it was further shown that the C-3 hydroxyl (*) of proclavaminate (**8**) gives rise exclusively to the oxazolidine oxygen in clavaminate (**9**). Therefore, although the oxidation state of C-2 in clavaminate is that of a ketone, no exchange of this oxygen or of the adjacent hydrogen at C-3 is evident through the course of this net four-electron process. This finding suggested that either oxidative oxazolidine formation occured prior to desaturation, or oxidation at C-3 in **8** took place in such a way that the substrate oxygen remained differentiated or isolated from exchange by the enzyme prior to oxidative cyclization to **9** (for example, *gem*-diol formation **32**). Implicit to this interpretation is the assumption of stepwise two-electron oxidations. Extensive steady-state kinetic analyses have demonstrated that, indeed, the catalytic cycle is stepwise.[6] Moreover, recent kinetic isotope effect measurements have established the order of these oxidations in which it was unambiguously shown that reaction at C-4' precedes that at C-3 in proclavaminic acid.[10] These findings point clearly to the intermediacy of dihydoclavaminate (**33**). The latter has been detected at intermediate extents of reaction as the result of isotope-induced branching events[11] with substrate labeled at C-3 with deuterium[12] or tritium.[10] The initial oxidative C—O bond formation at C-4' cannot involve the intermediacy of an (antiaromatic[13]) azetinone **34** as no hydrogen loss is observed to take place at C-3'. The only β-lactam hydrogen lost from proclavaminate is

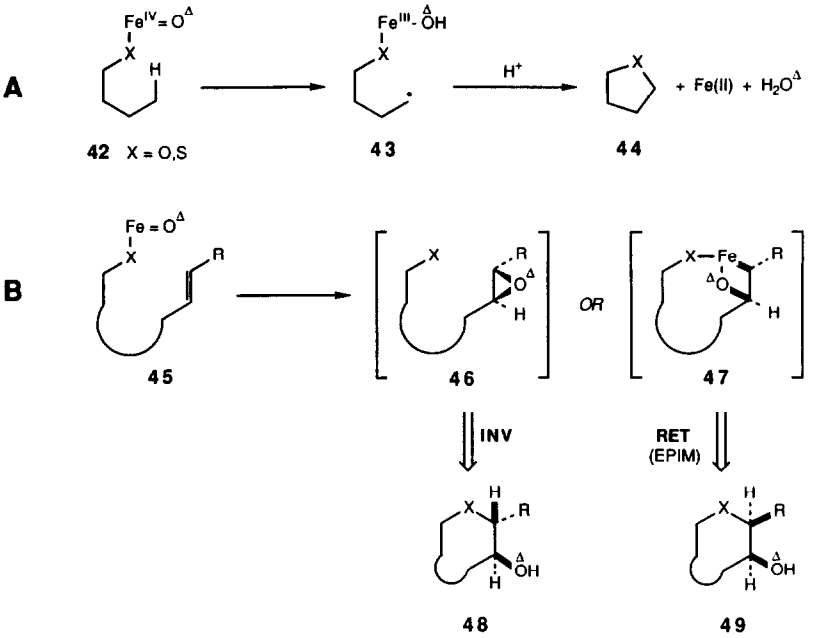
the (4'*S*) to give the bicylic framework of dihydroclavaminate (**33**) with retention of configuration at the newly-formed bridgehead.[14] It is notable that retention is the stereochemical course observed for hydroxylation by the apparently closely related α-KG-dependent dioxygenases.[15] To accomodate all of these observations we propose the mechanistic rationale shown in Scheme IV.

Scheme IV



As noted previously,[6] it is quite probable that the mechanism of oxygen activation by the α-KG-dependent dioxygenases (hydroxylation chemistry) and CS (oxidative cyclization/desaturation chemistry) is closely related. In keeping with recent work with the former class of hydroxylases,[16,17] we invoke as the first chemical step, following binding of iron and co-substrates, the generation of a ferryl ion $[Fe^{IV}=O]^{2+}$ by decarboxylation of α-KG coupled to cleavage of the O—O bond.[10] As the oxidation state of the metal changes during this part of the catalytic cycle, it becomes more electrophilic and the C-3 hydroxyl group of proclavaminate may provide a ligand to the metal, *e.g.* **35**. Such an interaction could play a role in diverting the catalytic machinery of this protein from hydroxylation chemistry to oxidative cyclization chemistry. By analogy to proposed mechanisms of P-450 action, homolytic C—H abstraction of the (4'*S*)-hydrogen by the ferryl species is proposed to form carbon-centered radical **36**. Intramolecular capture of the radical or, after electron transfer, of the corresponding cation (acyliminium ion) **37** gives the strained bicyclic framework of dihydroclavaminate **33** coupled to the release of one mole of water. The alternative collapse of intermediate **36** to the 4'-hydroxyazetidinone is a presumably less favorable overall process thermodynamically and would afford an extremely unstable hemiaminal intermediate prone to ring opening. The second half of the catalytic cycle proceeds in the absence of heteroatom ligation, again through a radical intermediate **39**, and either oxygen "rebound" to **40** followed by loss of water, or directly by electron transfer from **39** to give the oxonium ion **41**. Tautomerization of the latter then would give the product clavaminic acid (**9**).[10]

Scheme V



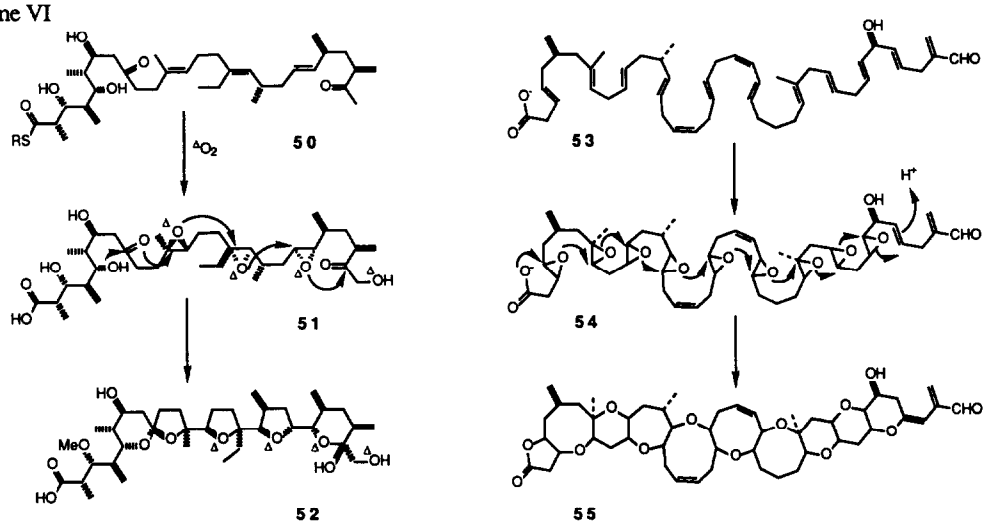
42 X = O,S 43 44 45 46 47 48 49

Important parallels can be seen between this mechanistic proposal for CS and that of Baldwin for IPNS.[18] For the latter enzyme recent spectroscopic measurements support the interaction of the substrate thiol with the active site iron in the anoxic resting enzyme.[19] The central feature of both of these proposals is the formation of a heteroatom-ferryl complex **42** (Scheme V-A) where X=O for CS and X=S for IPNS. Hydrogen abstraction 1,5 to X gives **43**, which collapses through a radical or ionic pathway to generate a new 5-membered ring, a mole of water and the ferrous resting state of the enzyme. The comparatively lax structural specificity shown by IPNS has allowed a very interesting series of experiments to be carried out by the Oxford group where various unsaturated amino acids have replaced valine in analog tripeptides of the normal ACV substrate **4** (Scheme I).[18] Some of these, in addition to giving products of oxidative cyclization expected for IPNS, yield *oxygenated* cyclization products labeled by $^{18}O_2$ and having stereochemical features that can be accounted for by two mechanistic rationales. These are shown in their essential details in Scheme V-B for a generic unsaturated substrate **45**. Oxygen transfer to form an epoxide **46** is precedented in other systems[20] and would lead to C—X bond formation with *inversion* of configuration at the electrophilic epoxide carbon. In contrast, [2+2] cycloaddition of the ferryl species to the olefin would give an oxametallocyclobutane as **47** in which C—X bond formation to **49** would be expected to proceed with *retention* of configuration observed for the analogous C—H insertion process depicted in Scheme V-A. For the case of X=S (at least) some examples of epimerization during C—X bond formation at saturated and unsaturated centers are known.[18,20]

The possibility that oxygen, a heteroatom much more widely distributed in natural products than sulfur, could give analogous oxidative cyclization chemistry at unsaturated centers (Scheme V-B, X=O) in parallel with its demonstrated ability in CS to undergo C—H insertion reactions (Scheme V-A, X=O), leads to the formulation of a new biogenetic hypothesis for polyether formation. It must be stated unequivocally at the outset that this is

speculation, but in the spirit of a symposium presentation some interesting predictions can be made that are subject, in principle, to experimental test.

Scheme VI

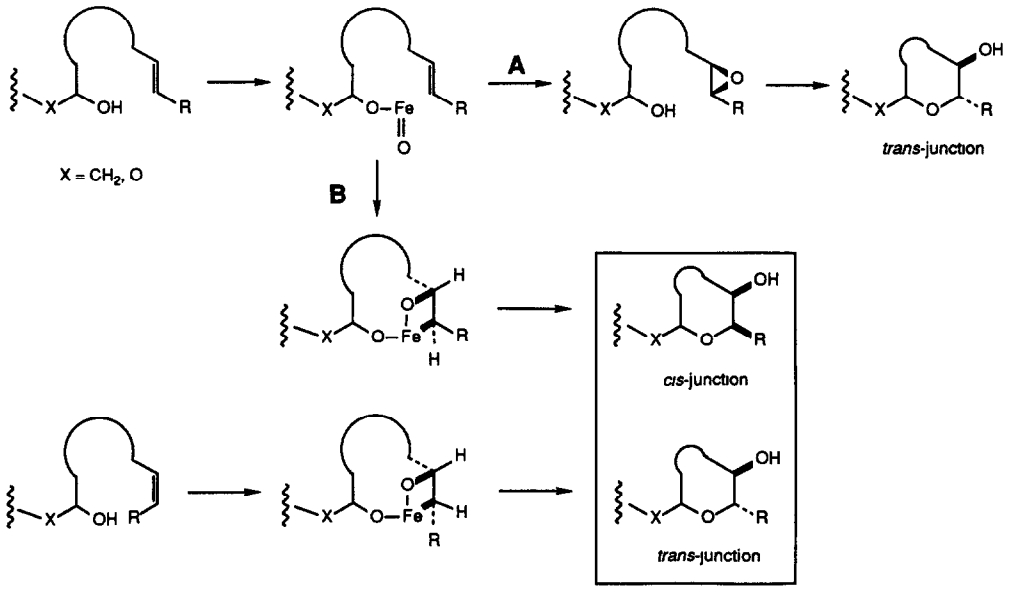


Two natural product groups become relevant to the present discussion: the polyether antibiotics, *e.g.* monensin (**52**), and the group of marine toxins typified by brevetoxin A (**55**, BTX-A, see Scheme VI). A compelling proposal, which to date remains unproved, was advanced originally by Westley[21] to account for the biogenesis of lasalocid and has since been modified in important ways to encompass monensin and most microbial polyether antibiotics.[22] The central contention of this hypothesis is the formation of an all-(*E*) triene **50** ("premonensin"), which was suggested to undergo epoxidation at each of the double bonds to give triepoxide **51**. Cyclization of the latter as indicated in Scheme VI would afford monensin (**52**) correct in all of its stereochemical details. The location and (*E*)-configuration of the double bonds in **50** is in keeping with the extant body of experimental data on fatty acid and polyketide biosynthesis and tacitly posits their formation in a dehydrative step during processive chain elongation.[23] The putative biogenetic role of polyepoxide intermediates has been extended to account for the formation of the brevetoxins, as shown similarly for BTX-A (**55**) in Scheme VI.[24] A significant structural feature of these scimitar-shaped molecules is that all ring junctions are *trans*. Therefore, proceeding, as for the polyether antibiotics, from the all-(*E*) configured double bonds in **53** to the polyepoxide **54**, a cascade of ring closures can be proposed to give BTX-A (Scheme VI).

If we reconsider the possible biogeneses of these two natural product groups from the perspective of the oxidative cyclization model dicussed above and summarized in Scheme V, an alcohol, hemiacetal or carboxyl can be visualized among the various members of these classes to provide an oxygen ligand to "prime" a succession of oxidative ring closure processes as illustrated in Scheme VII. In each ring-forming reaction a new hydroxyl ligand is produced to propagate the cascade. In path A the hypothetical heteroatom-ferryl species is proposed to transfer oxygen to form an epoxide in a simple, but stepwise extension of the Westley-Cane mechanism. Alternatively, the [2+2] cyclization pathway B would lead to the incorrect *cis*-ring junction in the case of the brevetoxins or the epimeric inter-ring stereochemistry for the polyether antibiotics. However, proceeding from the (Z)-olefin, the *trans*-junction can be achieved. Were the latter process to be acting, for example, in brevetoxin

biosynthesis, it would be predicted that *all* of the double bonds would be of the (*Z*)-configuration rather than a mixture of (*E*) and (*Z*) as shown in **53** (Scheme VII). Biochemically, the all-(*Z*) configuration would be consistent with oxidative desaturation processes to give the unconjugated (*Z*)-double bond from a largely hydrocarbon precursor. Such a proposal is somewhat less compelling for polyethers as monensin (**52**) whose clear polyketide origins would tend to favor (*E*)-double bond formation as a natural course of processive chain elongation. However, premonensin (**50**) has been synthesized but, unfortunately, all attempts to observe either its production in fermentation or its cyclization to monensin have failed.[25] While unexpected, the all-(*Z*) triene corresponding to **50** may serve as the elusive precursor of this important antibiotic.

Scheme VII



Acknowledgment. We are grateful to Dr. S. P. Salowe for conducting the incubations of **22** and **29** with clavaminate synthase and for many helpful discussions together with W. J. Krol and D. Reuyl.

EXPERIMENTAL SECTION

Melting Points were determined in open capillaries using a Thomas-Hoover apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were obtained using Varian XL-400 instrument. Mass spectra were recorded on a VG 70-S mass spectrometer. IR spectra were obtained using Perkin-Elmer 1600 series FT instrument.

Synthesis of DL-[2-^{2}H,3-^{13}C]Proclavaminic Acid (22).

2-Benzyloxycarbonylaminoethanol (10). A 250 mL round-bottomed flask equipped with a stir bar was charged with ethanolamine (3.6 mL, 60 mmol), sodium bicarbonate (5.55 g, 66 mmol), dioxane-H_2O (1:1, 100 mL), and the solution was cooled to 0 °C. Benzyl chloroformate (9.4 mL, 66 mmol) in dioxane-H_2O (1:1, 20 mL) was added over 20 min. The mixture was allowed to warm to room temperature over 1 h and was stirred an additonal 4 h. After transferring to a separatory funnel and partioning between EtOAc (100 mL) and H_2O (100 mL), the organic phase was washed with 1N HCl (3 x 50 mL), 5% $NaHCO_3$ (3 x 50 mL), brine (1 x 100 mL) dried over anhydrous $MgSO_4$, filtered, and the filtrate concentrated *in vacuo* to give a yellow oil, which subsequently solidified. Recrystallization from CH_2Cl_2-pentane afforded the title compound 10.5 g (90%) as fine white needles; mp 62-63 °C, lit[26] mp 54 °C; IR ($CHCl_3$): 3628, 3453, 3015, 2949, 2886, 1716, 1516, 1456, 1233, 1144, 1077, 1028 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7 35 (s, 5H, Ar*H*), 5.26 (bs, 1H, N*H*), 5.11 (s, 2H, PhCH_2), 3.72 (t, J = 5.0 Hz, 2H, CH_2OH), 3.36 (q, J = 5.1 Hz, 2H, NCH_2), 2 31 (bs, 1H, O*H*) Anal. Calcd for $C_{10}H_{13}NO_3$: C, 61.52; H, 6.71; N, 7.18. Found: C, 61.29, H, 6.63; N 7.12.

2-Benzyloxycarbonylaminoethyl-1-methanesulphonate (11). A flame-dried 250 mL round-bottomed flask equipped with a stir bar was charged with 2-benzyloxycarbonylaminoethanol (7 g, 35.75 mmol), CH_2Cl_2 (75 mL), and the solution was cooled to -10 °C. Triethylamine (5.4 mL, 38.75 mmol) and methanesulfonylchloride (3.0 mL, 38.75 mmol) were added with vigorous stirring. The mixture was stirred at -10 °C for 0.5 h, allowed to warm to room temperature, and stirred an additional 18 h. After removal of the solvent *in vacuo*, the residue was partioned between EtOAc (100 mL) and H_2O (100 mL), the organic phase was washed successively with 1N HCl (3 x 100 mL), 5% $NaHCO_3$ (3 x 100 mL) and brine (1 x 100 mL), dried over anhydrous $MgSO_4$, filtered, and the filtrate concentrated *in vacuo*. The yellow oil thus obtained was purified by flash chromatography (CH_2Cl_2-EtOAc, 2:1) to give 9 g (92%) of the desired product as white needles; mp 61-62 °C; IR ($CHCl_3$): 3454, 3030, 2959, 1721, 1516, 1456, 1365, 1342, 1224, 1176, 1015, 971, 917, 796, 698 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.4 (m, 5H, Ph), 5.1 (bs, 3H, PhC*H*$_2$ & N*H*), 4.3 (t, *J* = 5.3 Hz, 2H, OC*H*$_2$), 3.5 (dd, *J* = 5.3 & 5.3 Hz, 2H, NC*H*$_2$), 2.9 (s, 3H, SC*H*$_3$). Anal. Calcd for $C_{11}H_{15}NO_5S$: C, 47.52; H, 7.70; N, 3.96. Found: C, 47.53; H, 7.81; N, 3.92.

[1-^{13}C]-3-Benzyloxycarbonylaminopropionitrile (12). A flame-dried 100 mL round-bottomed flask equipped with a stir bar was charged with 2-benzyloxycarbonylaminoethane-1-methanesulfonate (30.17 g, 105 mmol), K^{13}CN, (99.5 atom % ^{13}C, 2.5 g, 37.8 mmol), and DMSO (30 mL) under an atmosphere of argon. After stirring for 7 days at room temperature, the mixture was poured into H_2O (100 mL), extracted with ether (3 x 100 mL), the organic phase was washed with H_2O (3 x 50 mL), 5% $NaHCO_3$ (3 x 50 mL), brine (1 x 100 mL), dried over anhydrous $MgSO_4$, filtered, and the filtrate concentrated *in vacuo*. The residue was purified by flash chromatography (7:1 hexane-acetone) to give 5.6 g (72%) of the desired nitrile as white leaves; mp 69 °C; IR ($CHCl_3$): 3452, 3020, 2955, 1718, 1516, 1455, 1263, 1239, 1145, 1070, 1000, 742, cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.36 (m, 5H, Ar*H*); 5.12 (s, 3H, N*H* & PhC*H*$_2$), 3.48 (m, 2H, NHC*H*$_2$), 2.62 (dt, *J* = 9.4 & 6.3 Hz, 2H, C*H*$_2$^{13}CN); MS: m/z 205 (M^+, 9.8%), 144, 131, 108 (100%), 91, 79; accurate mass: Calcd for $^{12}C_{10}{}^{13}C_1H_{12}N_2O_2$ 205.0932, found 205.0934.

[1-^{13}C]-*N*-Benzyloxycarbonyl-β-Alanine (13). A 250 mL round-bottomed flask equipped with a stir bar was charged with methanol (50 mL), 1N NaOH (60 mL), 30% aqueous hydrogen peroxide (38 mL), [1-^{13}C]-3-benzyloxycarbonylamino-propionitrile (4.9 g, 23.8 mmol) at 0 °C, and the mixture allowed to warm to room temperature over 1 h. After stirring for 3 days at room temperature, the methanol was removed *in vacuo* and 1N HCl (80 mL) was added to precipitate the desired product, which was collected by filtration, washed sparingly with cold water and dried to give 2.96 g (55%) of the desired product as white leaves; mp 101-103 °C, lit[27] mp 103-105 °C; ^1H NMR ($CDCl_3$): δ 7.35 (m, 5H, Ph), 5.28 (bs, 1H, N*H*), 5.09 (s, 2H PhC*H*$_2$), 3.47 (t, *J* = 6.0 Hz, 2H, NHC*H*$_2$), 2.60 (t, *J* = 6.0 Hz, 2H, C*H*$_2$COOH); MS: m/z 234 (M^+, 2.6%), 266, 108 (100%), 91, 79; accurate mass: Calcd for $^{12}C_{10}{}^{13}C_1H_{13}NO_4$ 224.0878, found 224.0882.

[1-^{13}C]-3-(4,5-Diphenyl-2-oxo-4-oxazolin-3-yl)propanoic Acid (15). To a 250 mL Parr hydrogenation bottle was added 13 (718 mg, 3.2 mmol), water and absolute ethanol (10 ml each). The solution was degassed and then shaken under hydrogen (room temperature, 50 psi) over 10% Pd-C (40 mg) for 2 h. The catalyst was removed by filtration through Celite and the bed was thoroughly washed with water. The combined filtrate and washings were concentrated to leave a pale yellow oil from which [1-^{13}C]β-alanine (14) was crystallized from water-ethanol as fine white needles (278 mg, 97%); mp 204-205 °C (dec), lit[28] 195-200 °C (dec); ^1H NMR (D_2O): δ 3.18 (m, 2H, NCH_2), 2.54 (m, 2H, CH_2CO); MS (CI, NH_3): m/z 108 (MNH_4^+, 0.21%), 91 (MH^+, 100%); accurate mass: Calcd for $^{12}C_2{}^{13}CH_7NO_2$ 90.0510, found 90.0508. Other spectral properties were identical with authentic material.[29]

[1-^{13}C] β-alanine (278 mg, 3.1 mmol) and tetramethylammonium hydroxide (20% solution in methanol, 1.41 mL, 1 eq) were combined in a 25 mL round-bottomed flask. The methanol was removed *in vacuo*. The residue was redissolved in ethanol and evaporated. The process was repeated twice and the residue was dried under high vacuum at 60 °C overnight and then taken up in dry DMF (5 ml) and 4,5-diphenyl-1,3-dioxol-2-one[7] (762 mg, 3.2 mmol) was added. The mixture was stirred under argon for 2 h. 2N Hydrochloric acid (4 ml) was added and the mixture was diluted with EtOAc (15 ml) and transferred to a separatory funnel. The aqueous phase was extracted with EtOAc (2 x 25 ml). The combined EtOAc layers were washed with water, brine and then dried (Na_2SO_4). Filtration followed by evaporation gave a gummy residue which was thoroughly dried under vacuum. Anhydrous trifluroacetic acid (TFA, 5 mL) was added and the solution was stirred under argon at room temperature overnight. The TFA was removed and the residue was partitioned between CH_2Cl_2 and water (50 mL each), washed with brine and dried (Na_2SO_4). The solution was filtered and evaporated to afford the title compound as a pale yellow solid (900 mg, 94%) which was recrystallized from hexane-EtOAc; mp 165-67 °C; IR ($CHCl_3$): 3170, 3015, 1747, 1708, 1670, 1448, 1371, 1265, 1231, 1121, 1058 cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.40 (br, 1H, COO*H*), 7.56-7.20 (m, 10H, 2 x Ph), 3.77 (m, 2H, NC*H*$_2$), 2.66 (m, 2H, C*H*$_2$CO); ^{13}C NMR ($CDCl_3$): δ 175.44 (^{13}*C*OOH), 174.44, 154.39, 134.87, 130.46, 129.72, 128.80, 127.84, 124.39, 127.55, 126.70, 37.52, 32.43 (d, $J_{C\text{-}C}$ = 55.7 Hz, *C*H$_2$CO); MS: m/z 310 (M^+, 100%), 237, 178, 177, 165, 105, 104; accurate mass: Calcd for $^{12}C_{17}{}^{13}CH_{15}NO_4$ 310.1035, found 310.1037.

4,5-Diphenyl-3-([3-^{13}C]-hydroxypropyl)-4-oxazolin-2-one (16). Ox-protected [1-^{13}C]β-alanine (825 mg, 2.67 mmol) in THF was stirred under argon in a flame-dried 50 mL round-bottomed flask at -5 °C. Triethylamine (410 µL, 1.1 eq) was added and the solution was stirred at -5 °C for 10 min. Then a solution of ethyl chloroformate (308 µL, 1.1 eq) in dry THF (5 mL) was added over a period of 15 min, and the mixture was stirred at 0 °C for 30 min, filtered through a plug of cotton and the filtrate was collected in an addition funnel. The latter was then added dropwise to a solution of $NaBH_4$ (296 mg, 3 eq) in water (1 mL) and THF (5 ml) at 0 °C over a period 30 min. The mixture was then stirred at room temperature for 6 h, adjusted to pH 1 with 1N HCl and then extracted into EtOAc (3 x 50 ml). The combined EtOAc layers were washed with 5% $NaHCO_3$ and brine, then dried over Na_2SO_4, filtered and evaporated. The clear oil obtained was purified by flash chromatography eluting with hexane-EtOAc (1:1) to afford a white

solid. The product was obtained by recrystallization from petroleum ether-CH_2Cl_2 (550 mg, 69%); mp 103-104 °C, lit[30] mp 102-103 °C; ^1H NMR ($CDCl_3$): δ 7.60-7.20 (m, 10H, 2 x Ph), 3.75 (m, 2H, NCH_2), 3.70 (dt, J = 128.0 & 7.0 Hz, 2H, CH_2OH), 2.71 (m, 1H, O*H*). 1.72 (m, 2H, NCH_2CH_2); MS: m/z 296 (M^+, 100%), 237, 178, 165, 163, 105, 104, 77; accurate mass: Calcd for $^{12}C_{17}{}^{13}CH_{17}NO_3$ 296.1242, found 296.1240.

4,5-Diphenyl-3-([3-^{13}C]-3-oxopropyl)-4-oxazolin-2-one (17). Swern oxidation of **16** (254 mg, 0.86 mmol) according to the procedure by Vederas[30] gave the crystalline aldehyde (238 mg, 94%); mp 101-103 °C, lit[30] mp 102-103 °C; ^1H NMR ($CDCl_3$): δ 9.66 (d, J = 176.3 Hz, 1H, C*H*O), 7.56-7.19 (m, 10H, 2 x Ph), 3.80 (m, 2H, NCH_2), 2.79 (m, 2H, CH_2CHO), MS: m/z 294 (M^+, 4.8%), 237, 122, 106, 105, 77; accurate mass: Calcd for $^{12}C_{17}{}^{13}CH_{15}NO_3$ 294.1085, found 294.1088.

Benzyl [2-$^2H_2$]-2-(2-Oxoazetidin-1-yl)acetate (19). Lithium hexamethyldisilazide (1M in hexane, 1.34 mL) was added to freshly distilled THF (5 mL) in a flame-dried 25 mL round-bottomed flask under argon and stirred at -78 °C. A solution of unlabelled glycyl β-lactam[10] **18** (292 mg, 1.34 mmol) in dry THF (5 mL) was added in about 10 min and then stirred at -78 °C for 30 min. A solution of deuterioacetic acid (CH_3COOD, 86 μL, 0.74 mmol) in D_2O (600 μL) was added and the mixture was slowly warmed to 0 °C. It was transferred to a separatory funnel using EtOAc (50 mL) and washed with water, 5% $NaHCO_3$ and brine (50 mL each). The organic phase was dried over Na_2SO_4, filtered and evaporated to leave the glycyl β-lactam as an oil (50% enriched with deuterium at C-2). The process was repeated twice and the title compound **19** was finally isolated pure by radial chromatography using a 2 mm plate and hexane-EtOAc (1:1) as eluent (250 mg, 85%). ^1H NMR showed the deuterium content to be about 80%; MS: m/z 221 (M^+, 2.9%), 179, 91, 44 (100%); accurate mass: Calcd for $C_{12}{}^1H_{11}{}^2H_2NO_3$ 221.1021, found 221.1023.

***threo* Benzyl 5-(4,5-Diphenyl-2-oxo-4-oxazolin-3-yl)-[2-^{2}H,3-^{13}C]-3-hydroxy-2-(2-oxoazeitidin-1-yl)pentanoate (20).** Aldol condensation was carried out between **17** (100 mg, 0.34 mmol) and **19** (75 mg, 0.34 mmol) using lithium hexamethyl disilazide as the base.[10] Work up followed by epimerization with DBN gave the title compound **20** (109 mg, 62%); ^1H NMR ($CDCl_3$) δ 7.54-7.21 (m, 15H, 3 x Ph), 5.22, 5.19 (ABq, J = 12.4 Hz, 2H, CH_2Ph), 4.40 (br s, 1H, O*H*), 4.22 (dm, J = 140.0 Hz, 1H, H-3), 4.19 (m, 0.12 H, H-2), 3.75 and 3.49 (m, 2H, H-5), 3.45 and 3.41 (m, 2H, H-4'), 2.98 (t, J = 4.0 Hz, 2H, H_2-3'); MS: m/z 514 (M^+, 25.0%), 295, 294, 238, 237, 105, 91(100%); accurate mass: Calcd for $^{12}C_{29}{}^{13}C^1H_{27}{}^2HN_2O_6$ 514.2044, found 514.2045.

DL-[2-^{2}H,3-^{13}C]Proclavaminic Acid (22). Prepared from **20** (40 mg, 0.078 mmol) by hydrogenolysis using 10% Pd-C (40 mg) in ethanol-water (6 mL, 2:1), the product was purified by reverse phase HPLC (Whatman ODS-3 C-18 R.P., 9.4 x 250 mm semi-prep column; detection at 220 nm; mobile phase H_2O; flow 3.0 mL/min; retention time 4.1 min); ^1H NMR (D_2O) δ 4.10 (dm, J = 145.7 Hz, 1H, H-3), 4.02 (m, 0.2H, H-2), 3.48 and 3.42 (m, 2H, H-4'), 3.06 (m, 2H, H-5), 2.92 (t, J = 4.0 Hz, 2H, H-3'), 1.77 (m, 2H, H-4). Other spectral properties were identical with unlabeled material.[10]

Synthesis of DL-[2-^{2}H,3'-$^2H_2$]Proclavaminic Acid (29).

Benzyl ([2-$^2H_2$]-3-Hydroxypropionyl)glycine (25). A solution of 3-hydroxypropionitrile (1.05 g, 14.4 mmol) and 10N NaOD (1.44 mL, 14.4 mmol) in D_2O (3 mL) was heated to reflux under argon for 72 h. The solution was then concentrated under vacuum and ethanol was added. The precipitated white solid of sodium [2-$^2H_2$]-3-hydroxypropionate **24** was collected (1.54 g, 94%). **24** (600 mg, 5.26 mmol)) and benzyl glycinate tosylate (2.67 g, 7.89 mmol) were dissolved in D_2O (5 mL), acetonitrile (5 mL) and ethyl acetate (5 mL). To this solution 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.51 g, 7.89 mmol) was added and the mixture was stirred at room temperature for 24 h. The mixture was then partitioned between EtOAc and water (50 mL each). The EtOAc layer was washed with 1N HCl, 5% $NaHCO_3$ and brine (50 mL each) and then dried over Na_2SO_4. Filtration followed by evaporation left an oily residue from which the desired amide **25** was isolated by flash chromatography using EtOAc-hexanes (2:1) as eluent. The product was recrystallized from CH_2Cl_2-petroleum ether as white needles (503 mg, 40%), mp 75-76 °C, lit[10] 76-77 °C; ^1H NMR ($CDCl_3$): δ 7.36 (m, 5H, Ph), 6.27 (bs, 1H, N*H*), 5.20 (s, 2H, CH_2Ph), 4.11 (d, J = 5.4 Hz, 2H, NCH_2), 3.90 (s, 2H, CH_2OH), 2.80 (bm, 1H, O*H*); ^{13}C NMR ($CDCl_3$): δ 171.90, 169.92, 135.08, 128.58, 128.55, 128.30, 67.25, 58.64, 41.25; MS: (m/z) 239 (M^+, 3.66), 148, 132, 108, 105; accurate mass: Calcd for $C_{12}{}^1H_{13}{}^2H_2NO_4$ 239.1127, found 239.1128

Benzyl ([2-$^2H_2$]-3-Bromopropionyl)glycine (26). To a solution of hydroxyamide **25** (239mg, 1 mmol) in freshly distilled CH_2Cl_2 (5 mL) at 0 °C, carbon tetrabromide (664 mg, 2 mmol) and triphenylphosphine (524 mg, 2 mmol) were added and stirred under argon at 0 °C for 2 h. Neutral alumina (2 gm) was added and the mixture was evaporated to leave a dry yellow powder. This was applied to a silica gel column and eluted with EtOAc-hexane (1:3) to afford the bromide **26** as a white solid, which was recrystallized from CH_2Cl_2-petroleum ether as needles (171 mg, 77%); mp 72-73 °C, lit[10] mp 71-72 °C; ^1H NMR ($CDCl_3$): δ 7.36 (m, 5H, Ph), 6.06 (bs, 1H, N*H*), 5.20 (s, 2H, CH_2Ph), 4.13 (d, J = 5.1 Hz, 2H, NCH_2), 3.62 (s, 2H, CH_2Br); ^{13}C NMR ($CDCl_3$): δ 169.76, 169.62, 134.95, 128.65, 128.60, 128.40, 67.37, 41.46, 26.73; MS: m/z 303, 301 (M^+, 0.92, 1.0 %), 196, 194, 169, 167, 139, 137, 111, 109, 91 (100%); accurate mass: Calcd for $C_{12}{}^1H_{12}{}^2H_2NO_3{}^{79}Br$ 301.0283, found 301.0280.

Benzyl ([3-$^2H_2$]-2-Oxoazetidin-1-yl)acetate (27). To powdered KOD [obtained by dissolving KOH (67 mg, 1.185 mmol) in D_2O (0.5 mL) and drying under vacuum overnight] a mixture of CH_2Cl_2 (9.5 mL) and acetonitrile (0.5 mL) was added followed by tetrabutylammonium bromide (64 mg, 0.20 mmol) and stirred under argon. A solution of the bromide **26** (191 mg, 0.632 mmol) in a mixture of CH_2Cl_2 (9.5 mL) and acetonitrile (0.5 mL) was added slowly over a period of 30 min. Two drops of D_2O were added and stirring was continued for a further period of 1 h. The mixture was then filtered through silica gel and the bed was thoroughly washed with EtOAc. The combined filtrate and washings were evaporated to leave an oily residue, which upon

chromatography over silica gel eluting with EtOAc-hexane (1:2) gave the title β-lactam **27** as a clear oil (100 mg, 72%); ^{1}H NMR ($CDCl_3$): δ 7.38 (m, 5H, Ph), 5.16 (s, 2H, CH_2Ph), 4.02 (s, 2H, H-2), 3.40 (s, 2H, H-4'). Other spectral properties were identical with those reported in the literature.[10]

Benzyl [2-$^2H_2$]-([3-$^2H_2$]-2-Oxoazetidin-1-yl)acetate (28). The title compound was prepared from **27** by exchanging the C-2 hydrogens three times according to the procedure described for the preparation of **19.** ^{1}H NMR showed about 80% deuterium content at C-2; MS: m/z 223 (M^+, 3.1%), 179, 91, 44 (100%); accurate mass: Calcd for $C_{12}{}^1H_9{}^2H_4NO_3$ 223.1147, found 223.1148.

***threo* Benzyl 5-(4,5-Diphenyl-2-oxo-4-oxazolin-3-yl)-[2-^{2}H]-3-hydroxy-2-([3-$^2H_2$]-2-oxoazetidin-1-yl)pentanoate.** The title compound was made from **28** (95 mg, 0.43 mmol) and the 3-Ox propanal (138 mg, 0.47 mmol) following the procedure as described for **20.** ^{1}H NMR ($CDCl_3$): δ 7.54-7.21 (m, 15H, 3 x Ph), 5.21 and 5.19 (ABq, 2H, J = 12.4 Hz, CH_2Ph) 4.40 (d, J = 7.9 Hz, 1H, OH), 4.22 (m, 1H, H-3), 3.75 and 3.49 (m, 2H, H -5), 3.45 and 3.41 (ABq, J = 5.7 Hz, 2H, H-4'), 1.70 (m, 2H, H-4); trace signals for H-2 at 4.19 ppm and for H-3' at 2.98 ppm were observed. MS: m/z 515 (M^+, 35.3%), 294, 293, 238, 237, 105, 104, 91 (100%); accurate mass: Calcd for $C_{30}{}^1H_{25}{}^2H_3N_2O_6$ 515.2136, found 515.2140.

DL-[2-^{2}H, 3'-$^2H_2$]Proclavaminic Acid (29). The triply labeled substrate was prepared from its protected form above (30 mg, 0.058 mmol) by hydrogenolysis (10% Pd-C) and purified by HPLC as described before; ^{1}H NMR (D_2O): δ 4.10 (m, 1H, H-3), 3.48, 3.42 (ABq, J = 6.2 Hz, 2H, H-4'), 3.09 (m, 2H, H-5), 1.79 (m, 2H, H-4). Trace signals for H-2 at 4.02 ppm and for H-3' at 2.92 ppm were observed. Other spectral properties were identical with unlabelled material[10].

Isolation and Derivatization of Clavaminic Acid from the Incubation Mixture

The crude incubation mixture (2.5 mL),[4] after protein removal by ultrafiltration, was directly injected into the HPLC column (Whatman ODS-3 C-18 R.P.,9.4 x 250 mm semi-prep; detection at 220 nm; mobile phase H_2O; flow 3.0 mL/min; injected volume 250 μL). The clavaminate had a retention time of 5.6 min. The fractions corresponding to the clavaminate peak were pooled, lyophilized and straightway derivatized by the method described below.

The lyophilite contained about 420-500 μg of clavaminate. To it 140 μL of a solution of $NaHCO_3$ (8.4 mg) in water (1.4 mL) was added and the solution was stirred at 0 °C. 70 μL of a solution of ethyl chloroformate (47 μL) in acetone (7 ml) was added slowly (5 min). The solution was stirred at 0 °C for 15 min and then slowly warmed to room temperature over a period of 1 h. A solution of brine (2 mL) was added and the pH was carefully adjusted to 2.3 with 0.01N HCl and then extracted with EtOAc (2 x 10 mL). The combined EtOAc layers were dried over Na_2SO_4, filtered, evaporated to *ca.* 2 mL and treated with ethereal dizomethane at 0 °C. After stirring for 1 h at room temperature, the excess diazomethane was removed in a stream of argon. The solvents were removed under vacuum and the residue was purified by passing through a short column of silica gel (2 gm) eluting with EtOAc-petroleum ether (gradient 1:3 to 1:1). The methyl *N*-ethoxycarbonyl clavaminate was visualised with 2% TPTA reagent (2,3,5-triphenyl-2H-tetrazolium chloride), which gives a pink spot. The fractions showing positive reaction with TPTA reagent were combined, the solvents removed and the oily residue was dried under vacuum and finally analyzed by ^{1}H NMR spectroscopy and mass spectrometry. An authentic sample of unlabeled enantiomeric material prepared in same manner in 88% yield gave the following spectral data: ^{1}H NMR ($CDCl_3$): δ 5.70 (dd, J = 0.7 & 2.8 Hz, 1H, H-5), 5.03 (d, J = 1.1 Hz, 1H, H-3), 4.77 (m, 2H, H-8 & N*H*), 4.12 (q, J = 6 8 Hz, 2H, CH_2CH_3), 3.87 (m, 2H, H-9), 3.79 (s, 3H, CO_2CH_3), 3.49 (dd, J = 2.8 & 16.7 Hz, 1H, H-6α), 3.08 (dd, J = 0.7 & 16.7 Hz, 1H, H-6β), 1.25 (t, J = 6.9 Hz, 3H, CH_2CH_3); accurate mass (CI, isobutane) Calcd for $C_{12}H_{16}N_2O_6 + H^+$ 285.1086, found 285.1087.

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