Elucidation of the Order of Oxidations and Identification of an Intermediate in the Multistep Clavaminate Synthase Reaction†

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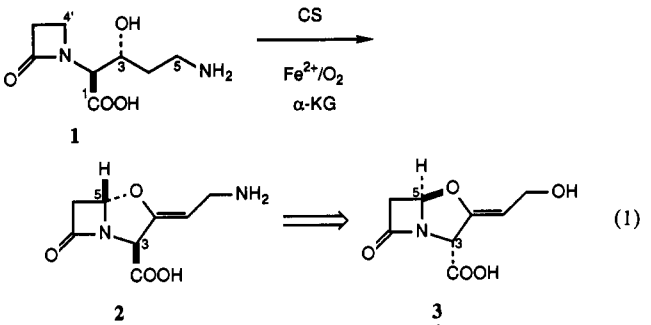
ABSTRACT: The enzyme clavaminate synthase (CS) catalyzes the formation of the first bicyclic intermediate in the biosynthetic pathway to the potent β-lactamase inhibitor clavulanic acid. Our previous work has led to the proposal that the cyclization/desaturation of the substrate proclavaminate proceeds in two oxidative steps, each coupled to a decarboxylation of α-ketoglutarate and a reduction of dioxygen to water [Salowe, S. P., Marsh, E. N., & Townsend, C. A. (1990) *Biochemistry 29*, 6499–6508]. We have now employed kinetic isotope effect studies to determine the order of oxidations for CS purified from *Streptomyces clavuligerus*. By using (4′*RS*)-[4′-^{3}H,1-^{14}C]-*rac*-proclavaminate, a primary $^T(V/K)$ = 8.3 ± 0.2 was measured from [^{3}H]water release data, while an α-secondary $^T(V/K)$ = 1.06 ± 0.01 was determined from the changing ^{3}H/^{14}C ratio of the product clavaminate. Values for the primary and α-secondary effects of 11.9 ± 1.7 and 1.12 ± 0.07, respectively, were obtained from the changing ^{3}H/^{14}C ratio of the residual proclavaminate by using new equations derived for a racemic substrate bearing isotopic label at both primary and α-secondary positions. Since only the first step of consecutive irreversible reactions will exhibit a V/K isotope effect, we conclude that C-4′ is the initial site of oxidation in proclavaminate. As expected, no significant changes in the ^{3}H/^{14}C ratio of residual substrate were observed with [3-^{3}H,1-^{14}C]-*rac*-proclavaminate. However, two new tritiated compounds were produced in this incubation, apparently the result of isotope-induced branching brought about by the presence of tritium at the site of the second oxidation. One of these compounds was identified by comparison to authentic material as dihydroclavaminate, a stable intermediate that normally remains enzyme-bound. On the basis of the body of information available and the similarities to α-ketoglutarate-dependent dioxygenases, a comprehensive mechanistic scheme for CS is proposed to account for this unusual enzymatic transformation.

Bacterial resistance to penicillin antibiotics is largely attributable to the hydrolytic activity of β-lactamase enzymes. One pharmacological approach to counter this phenomenon is the use of the potent inhibitor clavulanic acid (**3**), a natural product whose clinical efficacy derives from the inherent chemical reactivity of its bicyclic core. Lactamase-catalyzed cleavage of the azetidinone amide bond triggers a fragmentation driven by the release of strain energy and an enol → keto tautomerization that ultimately leads to irreversible alkylation and inactivation of the enzyme (Fisher et al., 1978; Charnas et al., 1978; Reading & Farmer, 1981; Frère et al., 1982). The key biosynthetic step in the construction of the fused bicyclic skeleton of **3** is the oxidative cyclization of proclavaminic acid (**1**) to clavaminic acid (**2**) (eq 1) (Elson et al., 1987). This complex reaction is catalyzed by the enzyme clavaminate synthase (CS)[1] and requires Fe^{2+}, α-KG, and O_2. Enzymatic activity has been found in several clavulanate-producing strains of microorganisms (Elson et al., 1987) and has been purified for detailed characterization from *Streptomyces clavuligerus* (Salowe et al., 1990).

As we have noted previously, the requirements for O_2 and α-KG as cosubstrates and Fe^{2+} as essential metal ion are characteristic of a class of dioxygenases that perform hydroxylations at unactivated carbon centers with concomitant formation of succinate and carbon dioxide (Hayaishi et al., 1976). A shared mechanism of oxygen activation, which may involve a ferryl iron–oxo intermediate (Siegel, 1979), is suggested by the extensive similarities between these enzymes and CS. For example, insertion of oxygen at C-4′ of proclavaminate occurs with retention of configuration analogous to the stereochemical course of the hydroxylation reactions catalyzed by the dioxygenases (Basak et al., 1990). It is significant, however, that molecular oxygen is not incorporated into clavaminate by CS (Krol et al., 1989). This fact underscores the redirection of conventional hydroxylation chemistry by the enzyme toward cyclization and desaturation, potentially through substrate heteroatom ligation to iron. The



1 **2** **3** (1)

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[1] Abbreviations: CS, clavaminate synthase; α-KG, α-ketoglutaric acid (2-oxopentanedioic acid); IPNS, isopenicillin N synthase; DAOCS, deacetoxycephalosporin C synthase; BBH, γ-butyrobetaine hydroxylase; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; SOS, sodium 1-octanesulfonate; THF, tetrahydrofuran; DBN, 1,5-diazobicyclo[4.3.0]non-5-ene.

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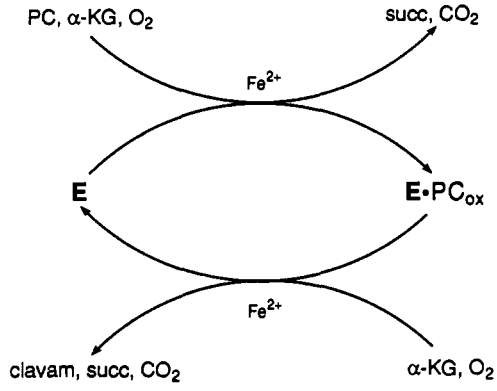
Scheme I[a]



[a] PC = proclavaminate, α-KG = α-ketoglutarate, succ = succinate, clavam = clavaminate, PC_{ox} = enzyme-bound intermediate

overall reaction stoichiometry suggests the existence of two oxidative steps in the conversion of **1** to **2**, each coupled to a decarboxylation of α-KG, and requires in both the reduction of dioxygen to *water*. The proposed reaction cycle depicted in Scheme I is supported by kinetic analysis (Salowe et al., 1990).

The prediction of two stepwise oxidations raises specific mechanistic questions that are addressed in this paper. Kinetic isotope effects for reaction at C-4′ and C-3 in proclavaminate have been determined to unambiguously establish the order of the oxidative steps in the conversion of proclavaminate (**1**) to clavaminate (**2**). In the course of these studies two other reaction products were observed to be released into solution from apparent isotope-induced branching events (Arigoni, 1975; Samuelson & Carpenter, 1981; Korzekwa et al., 1989). The relative concentration of these alternate products was monitored as a function of extent of reaction. On the basis of its appearance and disappearance and its chromatographic behavior, one of these products was proposed to be the intermediate itself of normal reaction, i.e., PC_{ox} in Scheme I. Its identity was confirmed by independent synthesis from clavulanic acid. On the basis of the body of kinetic, stereochemical, and structural information available, a comprehensive mechanistic scheme is proposed to account for this unusual enzymatic transformation.

Materials and Methods

General procedures for chemical synthesis were those previously described (Townsend et al., 1988). Flash chromatography (Still et al., 1978) was performed with EM Science silica gel 60 (230–400 mesh ASTM). Reagents were obtained from Aldrich and used as received unless otherwise noted. NMR spectral data were obtained on a Varian XL/VXR 400 spectrometer. Radial chromatography was carried out on a Chromatotron (Harrison Research).

*Synthesis of [1-^{14}C]-rac-Proclavaminic Acid (**10**)*

Benzyl [1-^{14}C]Glycinate Tosylate Salt (**5**). A 50-mL round-bottomed flask was charged with glycine (75.0 mg, 1.0 mmol) and a solution of [1-^{14}C]glycine (1.0 mCi, ICN) in 0.01 N HCl (10 mL). The solution was neutralized with 1 N NaOH and the water removed by azeotropic distillation with absolute ethanol followed by toluene. A stir bar was added along with toluenesulfonic acid monohydrate (232.6 mg, 1.22 mmol), benzyl alcohol (550 μL, 5.32 mmol) and toluene (15 mL). A Dean–Stark trap and condensor were attached and the reaction was heated to reflux for 20 h. The reaction mixture was cooled, poured into ether (25 mL), and refrigerated. The precipitated crystals were collected, washed thoroughly with ether, and dried: mp 131–132 °C [lit. mp 132–134 °C (Zervas et al., 1957)]. This material was carried on directly to the next step.

Benzyl [1-^{14}C]-N-(3-Bromopropionyl)glycinate (**6**). A 100-mL three-neck round-bottomed flask equipped with a stir bar was charged with a solution of **5** in THF/water (25 mL, 1:1). The solution was cooled in an ice bath, a pH electrode was inserted, and 3-bromopropionyl chloride was added dropwise (140 μL, 1.39 mmol) along with a solution of 1 N NaOH such that the pH of the reaction remained between 5.5 and 6.5. After the addition was complete, the mixture was stirred for 1 h, and then ethyl acetate was added (70 mL). The layers were separated and the organic phase was washed with brine (2 × 70 mL) and dried (anhydrous Na_2SO_4). After concentration in vacuo, the residue was filtered through a small pad of silica gel with ethyl acetate. Evaporation of the solvents and recrystallization from ethyl acetate/petroleum ether gave the product as fine white needles: mp 70–71 °C (184.8 mg, 0.62 mmol, 62% from glycine). Radioinactive material prepared identically gave the following spectral data: IR ($CHCl_3$) 3425, 3002, 1743, 1678, 1514, 1190, 950 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.83 (t, J = 6.6 Hz, 2 H, H-3), 3.63 (t, J = 6.6 Hz, 2 H, H-4), 4.13 (d, J = 5.12, 2 H, NHCH_2), 5.20 (s, 2 H, COOCH_2), 6.10 (br s, 1 H, NH), 7.35–7.38 (m, 5 H, ArH); ^{13}C NMR ($CDCl_3$) δ 26.8, 39.3, 41.5, 67.3, 128.4, 128.5, 128.6, 135.0, 169.6, 169.7; MS m/z 299 (M^+, 0.8%), 165, 135, 109, 91 (100%); accurate mass 299.0161, calcd for $C_{12}H_{14}NO_3{}^{79}Br$ 299.0157.

Benzyl [1-^{14}C]-2-(2-Oxoazetidin-1-yl)acetate (**7**). A dry 250-mL three-neck round-bottomed flask was charged with finely powdered potassium hydroxide (69.6 mg, 1.24 mmol), tetra-*n*-butylammonium bromide (61.1 mg, 0.19 mmol) under an argon atmosphere and a solution of dichloromethane/acetonitrile (53 mL, 19:1) was added. After the solution was stirred for a few minutes, a solution of **6** in dichloromethane (7 mL) was added dropwise by syringe over 10 min. After 30 min, thin-layer chromatography showed only a trace of the product. Two drops of water were added and the reaction was stirred an additional 5 h. The reaction mixture was filtered through a small pad of silica gel and concentrated in vacuo, and the product was purified by radial chromatography (Chromatotron, 1 mm of silica; petroleum ether/ethyl acetate, 4:1 to 2:3) to give the product as a clear oil (88.0 mg, 0.40 mmol, 65%). Radioinactive material prepared in a similar manner gave the following spectral data: IR ($CHCl_3$) 3010, 2974, 1774, 1141, 1192 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.02 (t, J = 4.21 Hz, 2 H, H-3), 3.41 (t, J = 4.21 Hz, 2 H, H-4), 5.17 (s, 2 H, CH_2Ph), 7.34–7.37 (m, 5 H, ArH); ^{13}C NMR ($CDCl_3$) δ 37.7, 40.0, 43.2, 67.2, 128.4, 128.6, 128.7, 135.1, 167.9, 168.1; MS m/z 219 (M^+, 5%), 191, 177, 91 (100%); accurate mass 219.0897, calcd for $C_{12}H_{13}NO_3$ 219.0897.

*erythro- and threo-Benzyl [1-^{14}C]-5-(4,5-Diphenyl-2-oxo-4-oxazolin-3-yl)-3-hydroxy-2-(2-oxoazetidin-1-yl)valerate (erythro- and threo-Benzyl [1-^{14}C]Ox-proclavaminates **8** and **9**, Respectively)*. A dry 50-mL round-bottomed flask was charged with a solution of **7** (88.0 mg, 0.40 mmol) in dry ether, and the solution was concentrated in vacuo and dried under high vacuum. A stir bar was added, followed by THF (7 mL) via syringe, and the solution was cooled to −78 °C under an argon atmosphere. Lithium bis(trimethylsilyl)amide (480 μL, 0.48 mmol, 1.0 M in hexanes) was added dropwise by syringe and, after 15 min of stirring, a solution of 4,5-diphenyl-3-(3-oxopropyl)-4-oxazolin-2-one [239.0 mg, 0.815 mmol; prepared by the method of Pansare and Vederas (1987)] in THF (4 mL)

was added dropwise by syringe. After 1 h, the reaction was quenched by the addition of 3 drops of glacial acetic acid and warmed to room temperature. Ethyl acetate (60 mL) was added and the reaction mixture was washed with brine (3 × 60 mL). The organic layer was dried (anhydrous $MgSO_4$) and concentrated in vacuo, and the products were purified by radial chromatography (Chromatotron, 2 mm of silica; petroleum ether/ethyl acetate, 4:1 to 2:3) to give the pure threo diastereomer **9** (22.8 mg, 0.045 mmol, 11%) as well as a mixture of the threo and erythro diastereomers (110.0 mg, 0.21 mmol, 53%) as oils.

A dry 50-mL round-bottomed flask was charged with a solution of the diastereomeric mixture of protected proclavaminic acids above (110.0 mg, 0.21 mmol, 9:1 erythro/threo) in dichloromethane, and the solution was concentrated in vacuo and dried under high vacuum. A stir bar was added along with dry dichloromethane (8 mL) and freshly distilled DBN (28 μL, 0.23 mmol) by syringe. The reaction mixture was stirred at room temperature for 1 h and then filtered through a small pad of silica gel with ethyl acetate, concentrated in vacuo, and purified by radial chromatography (Chromatotron, 2 mm of silica; petroleum ether/ethyl acetate, 4:1 to 2:3), to give 67.8 mg (0.13 mmol, 62%) of pure protected *threo*-proclavaminate **9**. The radioinactive diastereomers of benzyl Ox-proclavaminate prepared similarly gave the following spectral and analytical data. Erythro diastereomer **8**: IR ($CHCl_3$) 3390 (v br), 3014, 1743 (br), 1447, 1377, 1240, 1185 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.86 (m, 2 H, H-4), 2.95 (t, J = 4.0 Hz, H-3′), 3.33 (m, 2 H, H-4′), 3.95 (m, 1 H, H-5), 3.69 (m, 1 H, H-5), 4.07 (m, 1 H, H-3), 4.14 (d, J = 3.6 Hz, 1 H, H-2), 4.54 (br s, 1 H, O*H*), 5.18 (s, 2 H, $COOCH_2$), 7.15–7.29 (m, 5 H, Ar*H*), 7.3–7.4 (m, 6 H, Ar*H*), 7.48–7.55 (m, 4 H, Ar*H*); MS m/z 512 (M^+, 2%), 293, 237, 104, 91 (100%), 84, 77; accurate mass 512.1948, calcd for $C_{30}H_{28}N_2O_6$ 512.1947. Threo diastereomer **9**: IR ($CHCl_3$) 3401 (v br), 3013, 1743 (br), 1449, 1372, 1243, 1186 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.65 (m, 2 H, H-4), 2.98 (t, J = 4.0 Hz, 2 H, H-3′), 3.43 (m, 1 H, H-4′), 3.51 (m, 2 H, H-4′ and H-5), 3.75 (m, 1 H, H-5), 4.18 (d, J = 2.7 Hz, 1 H, H-2), 4.22 (m, 1 H, H-3), 4.42 (br s, 1 H, O*H*), 5.21 (ABq, J = 12.4 Hz, $COOCH_2$), 7.15–7.20 (m, 5 H, Ar*H*), 7.31–7.45 (m, 6 H, Ar*H*), 7.45–7.55 (m, 4 H, Ar*H*). Anal. Calcd for $C_{30}H_{28}N_2O_6$: C, 70.30; H, 5.51; N, 5.47. Found: C, 70.34; H, 5.57; N, 5.39.

[1-^{14}C]-rac-Proclavaminic Acid (**10**). A small Parr hydrogenation vessel was charged with a solution of *threo*-benzyl [1-^{14}C]Ox-proclavaminate (**9**; 90.6 mg, 0.18 mmol) in THF/water (12 mL, 10:1). Palladium on carbon (10%; 109 mg) was added, and the solution was degassed and shaken under a hydrogen atmosphere (40 psi) for 8 h. Analytical thin-layer chromatography showed a substantial amount of starting material still present, so additional catalyst was added, and the solution was degassed and shaken under hydrogen for another 8 h. The reaction mixture was filtered through Celite by washing with small amounts of 1:1 ethanol/water, concentrated in vacuo to approximately 15 mL, and filtered through a 0.22-μm filter to remove the last traces of catalyst. The solution was then further concentrated to 1 mL and purified by reverse-phase HPLC with elution by water. Immediately prior to use, this material was mixed with an appropriately ^3H-labeled proclavaminate and repurified under ion-pairing conditions as described below. Yields were typically 80%, mp 153–154 °C (dec with gas evolution). Radioinactive material prepared similarly gave the following spectral data: IR (1% in KBr) 3373, 2940 (v br), 1703 (br), 1634 (br), 1378, 773 cm^{-1}; ^1H NMR (D_2O) δ 1.89 (m, 2 H, H-4), 2.93 (t, J = 3.97 Hz, H-3′), 3.07 (m, 2 H, CH_2NH_2), 3.43 (m, 1 H, H-4′), 3.50 (m, 1 H, H-4′), 4.00 (d, J = 5.5 Hz, 1 H, H-2), 4.15 (m, 1 H, H-3); ^{13}C NMR (D_2O/dioxane) δ 32.0, 36.2, 38.1, 41.2, 63.5, 70.0, 173.2, 175.3; CI-MS m/z 203 (MH^+, CH_4, 100%), 185, 167, 143, 115; accurate mass (MH^+) 203.1037, calcd for $C_8H_{15}N_2O_4$ 203.1037.

Synthesis of [3-^3H]-rac-Proclavaminic Acid (***15***)

4,5-Diphenyl-3-([3-^3H]-3-hydroxypropyl)-4-oxazolin-2-one (**11**). An oven-dried 25-mL round-bottomed flask equipped with stir bar was charged with 4,5-diphenyl-3-(3-oxopropyl)-4-oxazolin-2-one [147 mg, 0.5 mmol; prepared by the method of Pansare and Vederas (1987)] and absolute ethanol (5 mL) at 0 °C under an atmosphere of argon. Sodium borohydride (1 mg, 0.02 mmol) in absolute ethanol (1 mL) was added, and the resulting mixture was stirred 0.5 h at 0 °C when sodium [^3H]borohydride (ICN, 25.0 mCi, 1.4 Ci/mmol, 0.68 mg) in absolute ethanol (1 mL) was added. The resulting solution was allowed to warm to room temperature over 1 h and stirred a further 18 h. Sodium borohydride (100 mg, 2.6 mmol) was added; the resulting mixture was stirred 0.5 h at room temperature, transferred to a separatory funnel, and partitioned between water (30 mL) and ether (30 mL); and the aqueous phase was acidified to pH = 1 by the addition of 1 N HCl (*CAUTION*: tritium gas is liberated; use a well-ventilated fume hood). After removal of the aqueous phase, the etheral extract was washed with brine (1 × 30 mL), dried over anhydrous $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give the desired tritiated alcohol **11** (167 mg, quant) as a white solid. This material was diluted with 146 mg (0.5 mmol) of unlabeled alcohol to yield, after recrystallization (ethyl acetate/hexanes), 300 mg; mp 102–103 °C [lit. mp 102–103 °C (Pansare & Vederas, 1987)]. Physical and spectral characteristics were identical with those reported by Vederas.

4,5-Diphenyl-3-([3-^3H]-3-oxopropyl)-4-oxazolin-2-one (**12**). Swern oxidation (Mancuso et al., 1978) of **11** (293 mg, 1.0 mmol) according to the procedure of Vederas (Pansare & Vederas, 1987) gave the crystalline aldehyde **12**.

erythro- and threo-Benzyl 5-(4,5-Diphenyl-2-oxo-4-oxazolin-3-yl)-[3-^3H]-3-hydroxy-2-(2-oxoazetidin-1-yl)valerate (**13** *and* **14**, *Respectively*). Benzyl 2-(2-oxoazetidin-1-yl)-acetate (60 mg, 0.27 mmol) was reacted as above with 4,5-diphenyl-3-([3-^3H]-3-oxopropyl)-4-oxazolin-2-one (**12**; 80 mg, 0.27 mmol). After 2 h, acetic acid (17 μL, 0.3 mmol) in water (500 μL) was added at −78 °C, and the mixture was warmed to room temperature and partioned between ethyl acetate (15 mL) and water (15 mL). After workup, the residue was purified by radial chromatography (Chromatotron, 1 mm of silica; 1:1 dichloromethane/ether) to give 17.4 mg (0.034 mmol) of the threo isomer **14** and 27.2 mg (0.053 mmol) of the slower moving erythro isomer **13**, with a combined yield of 33%. The latter was dissolved in dichloromethane (3 mL) and equilibrated with DBN (8 μL, 0.06 mmol) for 1 h at room temperature under an argon atmosphere. Resolution of the diastereomers by radial chromatography as above gave an additional 16 mg (0.03 mmol) of the faster moving threo diastereomer **14** (total 33.0 mg, 0.06 mmol, 24%).

[3-^3H]Proclavaminic Acid (**15**). A Parr hydrogenation bottle was charged with benzyl 5-(4,5-diphenyl-2-oxazolin-3-yl)-[3-^3H]-3-hydroxy-2-(2-oxoazetidin-1-yl)valerate (**14**; 33 mg, 0.06 mmol), water (0.5 mL), THF (5 mL), and 10% palladium on carbon (50 mg) under an atmosphere of argon. After the vessel was evacuated, an atmosphere of hydrogen was introduced (50 psi initial pressure) and the mixture was shaken mechanically for 18 h. The catalyst was removed and

the product was isolated as before.

Synthesis of [4'-^{3}H]-rac-Proclavaminic Acid (21)

[3-^{3}H]-3-Hydroxypropionitrile (**17**). A 25-mL round-bottomed flask equipped with a stir bar was charged with ethyl cyanoacetate (**16**; 213 μL, 2.0 mmol), absolute ethanol (5 mL), and sodium borohydride (4.2 mg, 0.1 mmol). The mixture was stirred at room temperature for 0.5 h, at which time a solution of sodium [^{3}H]borohydride (ICN, 50 mCi, 1.4 Ci/mmol, 1.36 mg) in absolute ethanol (3 mL) was added. The resulting mixture was stirred for 18 h, and sodium borohydride (80 mg, 2.11 mmol) was added. After being stirred for 15 min, the reaction mixture was filtered through a short column of silica gel (5 g) with ethyl acetate (200 mL) and the filtrate was concentrated in vacuo to afford a colorless oil (149 mg, 2.0 mmol, 100%). This material was used without further purification in the next reaction. Spectral data gathered in a radioinactive trial experiment were as follows: IR ($CHCl_3$) 3616, 3455 (br), 3021, 2959, 2897, 2255, 1413, 1228, 1186, 1062 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.62 (t, J = 6.6 Hz, 2 H, H-3), 5.88 (t, J = 6.6 Hz, 2 H, H-2).

Sodium [3-^{3}H]-3-Hydroxypropionate (**18**). A 50-mL round-bottomed flask equipped with a stir bar and fitted with a condenser was charged with [3-^{3}H]-3-hydroxypropionitrile (149 mg, 2 mmol), 3-hydroxypropionitrile (137 μL, 2 mmol), water (5 mL), and 1 N sodium hydroxide (4.2 mL, 4.2 mmol). The mixture was stirred at room temperature for 30 min and then heated at reflux for 48 h. The colorless solution was cooled and concentrated nearly to dryness. Absolute ethanol (40 mL) was added to precipitate the salt **18** as a white solid (336 mg, 75%), mp 142 °C [lit. mp 143 °C (Read, 1941)]. Unlabeled material prepared similarly gave the following data: ^{1}H NMR (D_2O) δ 2.42 (t, J = 6.8 Hz, 2 H, H-2), 3.78 (t, J = 6.8 Hz, 2 H, H-3).

Benzyl N-([3-^{3}H]-3-Hydroxypropionyl)glycinate (**19**). A 50-mL round-bottomed flask equipped with a stir bar was charged with sodium [3-^{3}H]-3-hydroxypropionate (**18**; 336 mg, 3.0 mmol), benzyl glycinate tosylate salt (2.0 g, 6 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (1.2 g, 6 mmol), water (10 mL), acetonitrile (10 mL), and ethyl acetate (10 mL). The mixture was stirred at room temperature for 18 h and diluted with ethyl acetate (100 mL), and the organic phase was washed successively with 5% aqueous sodium bicarbonate (3 × 75 mL), 1 N HCl (3 × 75 mL), brine (1 × 75 mL) and dried over anhydrous $MgSO_4$. After filtration, the solvents were removed in vacuo to afford **19**, after recrystallization from dichloromethane/petroleum ether, as white plates (326 mg, 46%), mp 76–77 °C. Radioinactive material prepared similarly gave the following spectral data: IR ($CHCl_3$) 3436, 3012, 2955, 1743, 1666, 1519, 1390, 1049, 955, 908, 867 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.49 (t, J = 5.3 Hz, 2 H, H-3), 3.08 (br s, 1 H, O*H*), 3.89 (t, J = 5.3 Hz, 2 H, H-4), 4.09 (d, J = 5.4 Hz, 2 H, NHCH_2), 5.19 (s, 2 H, COOCH_2), 6.42 (br s, 1 H, N*H*), 7.34–7.39 (m, 5 H, Ar*H*); ^{13}C NMR ($CDCl_3$) δ 38.1, 41.4, 58.9, 67.4, 128.4, 128.6, 128.7, 135.0, 169.8, 172.5; MS m/z 237 (M^+, 5%), 130, 103, 91 (100%); accurate mass 237.1003, calcd for $C_{12}H_{15}NO_4$ 237.1001.

Benzyl 2-(2-Oxo-[4-^{3}H]azetidin-1-yl)acetate (**20**). To a 100-mL round-bottomed flask equipped with a stir bar was added benzyl *N*-([3-^{3}H]-3-hydroxypropionyl)glycinate (**19**; 326 mg, 1.38 mmol) in dichloromethane (20 mL). The solution was cooled to 0 °C under an atmosphere of argon. Carbon tetrabromide (1.00 g, 3.03 mmol) and triphenylphosphine (795 mg, 3.03 mmol) were added, and the resulting solution was stirred 1 h at 0 °C. The mixture was filtered through a short column of silica gel (7.5 g) with ethyl acetate (200 mL), the filtrate was concentrated in vacuo, and the residue was purified by radial chromatography (Chromatotron, 2 mm of silica; 3:1 hexanes/acetone) to give benzyl *N*-([3-^{3}H]-3-bromopropionyl)glycinate (158 mg, 40%), mp 71–72 °C (cf. **6** prepared above).

The bromide so prepared (156 mg, 0.53 mmol) was cyclized as above for **6** to give azetidinone **20** (52.0 mg, 45%), identical in all respects with unlabeled material characterized previously.

[4'-^{3}H]-rac-Proclavaminic Acid (**21**). Benzyl 2-(2-oxo-[4-^{3}H]-azetidin-1-yl)acetate (**20**) was processed as above to prepare the title compound.

Synthesis of Dihydroclavaminic Acid (25)

4-Nitrobenzyl (3R,5R,Z)-2-(2-Azidoethylidene)clavam-3-carboxylate (**23**). An oven-dried 50-mL round-bottomed flask equipped with a stir bar was charged with 4-nitrobenzyl (3*R*,5*R*,*Z*)-2-(2-hydroxyethylidene)clavam-3-carboxylate [**22**; 501 mg, 1.5 mmol; see Brown et al. (1984)], triphenylphosphine (419 mg, 1.6 mmol), THF (13 mL), and a solution of hydrazoic acid (Braun, 1931) in benzene (2.08 mL, 1.6 mmol). The mixture was cooled to −45 °C at which time diethyl azodicarboxylate (252 μL, 1.7 mmol) in THF (1 mL) was added dropwise over 15 min. The solution was stirred for 15 min, and additional hydrazoic acid (750 μL, 0.5 mmol) and diethyl azodicarboxylate (47 μL, 0.3 mmol) were added with stirring. After 30 min, analytical thin-layer chromatography (3:2 petroleum ether/ethyl acetate) indicated nearly quantitative conversion. The mixture was warmed to room temperature and purged with a stream of argon for 15 min to remove the excess hydrazoic acid in a well-ventilated fume hood (*CAUTION*: hydrazoic acid is extremely toxic). Removal of the solvents in vacuo and purification by flash chromatography (60 g of silica gel; 1:1 petroleum ether/ethyl acetate) gave a yellow oil (438 mg), which was triturated with ether (2 mL) to afford, after standing 2 h at 0 °C, a white powder (428 mg, 80%), mp 68–70 °C; $[\alpha]_D$ = +39.5° (*c* 1.0, EtOAc). IR ($CHCl_3$) 3028, 2111, 1805, 1756, 1695, 1609, 1526, 1350, 1309, 1224, 1183, 1173, 1137, 1120, 1042, 1016, 890 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.13 (dd, J = 0.8, 16.8 Hz, 1 H, H-6β), 3.53 (dd, J = 2.8, 16.8 Hz, 1 H, H-6α), 3.85 (dt, J = 0.8, 7.5 Hz, 2 H, CH_2N_3), 4.82 (dt, J = 0.8, 7.5 Hz, 1 H, =C*H*), 5.29 (ABq, J = 16.7 Hz, 2 H, COOCH_2), 5.73 (dd, J = 0.8, 2.8 Hz, 1 H, H-5), 7.52 (d, J = 8.5, 2 H, ArH), 8.35 (d, J = 8.5 Hz, 2 H, ArH); ^{13}C NMR ($CDCl_3$) δ 45.9, 46.5, 60.5, 66.4, 88.5, 94.4, 123.9, 128.6, 128.7, 141.6, 154.7, 166.4, 174.0; MS m/z 359 (M^+, 0.1%), 331, 317 (M^+ − CH_2CO, 32.3%), 275, 234, 136 (100%), 106, 90; accurate mass 359.0868, calcd for $C_{15}H_{13}N_5O_6$ 359.0866.

(2RS,3R,5R)-2-(2-Aminoethyl)clavam-3-carboxylic Acid (**24** *and* **25**). A 250-mL Parr hydrogenation bottle was charged with 4-nitrobenzyl (3*R*,5*R*,*Z*)-2-(2-azidoethylidene)clavam-3-carboxylate (**23**; 50 mg, 0.14 mmol), absolute ethanol (5 mL), and water (1 mL) under an argon atmosphere. Palladium on carbon (10%; 150 mg) was added, and the mixture was degassed and, after introducing an atmosphere of hydrogen (initial pressure 50 psi), was shaken mechanically for 18 h. The catalyst was removed by filtration through Celite with water (20 mL). The filtrate was concentrated and purified by HPLC (Whatman ODS-3 C-18 reverse phase, 9.4 × 250 mm semipreparative column; detection at 220 nm; mobile phase H_2O; flow 3.0 mL/min; retention time 4.5 min) to give 10 mg (36%) of the desired clavams as a hygroscopic white powder as a 3:2 mixture of the (2*R*,3*R*,5*R*) to (2*S*,3*R*,5*R*) diastereomers, **24** to **25**. Major diastereomer **24**: ^{1}H NMR (D_2O) δ 1.82 (m, 1 H, H-8), 1.94

(m, 1 H, H-8), 2.85 (dd, J = 1.0, 16.9 Hz, 1 H, H-6β), 3.06 (t, J = 7.2 Hz, 2 H, H-9), 3.29 (dd, J = 3.0, 16.9 Hz, 1 H, H-6α), 4.43 (d, H = 6.7 Hz, 1 H, H-3), 4.60 (m, 1 H, H-2), 5.58 (dd, J = 1.0, 3.0 Hz, 1 H, H-5). Minor diastereomer **25**: ^{1}H NMR (D_2O) δ 2.04 (m, 1 H, H-8), 2.22 (m, 1 H, H-8), 2.83 (d, J = 16.5 Hz, 1 H, H-6β), 3.06 (t, J = 7.2 Hz, 2 H, H-9), 3.29 (dd, J = 2.6, 16.5, 1 H, H-6α), 3.98 (d, J = 6.7 Hz, 1 H, H-3), 4.50 (m, 1 H, H-2), 5.39 (d, J = 2.6 Hz, H-5).

Final purification of the labeled proclavaminates was carried out on a Whatman Partisil ODS-3 10-μm reverse phase column (4.6 × 250 mm) in a Waters 600 HPLC system equipped with a Rheodyne injector. The column was equilibrated for several hours with an aqueous ion-pairing solution containing 0.01% TFA/0.01% SOS. The compounds (<500 nmol/injection) were eluted isocratically at 1.5 mL/min. The major peak of radioactivity was lyophilized to remove TFA and redissolved in water. Reinjection on an analytical scale indicated ≥95% radiochemical purity. A control experiment established that SOS did not significantly inhibit CS at concentrations up to 0.5%.

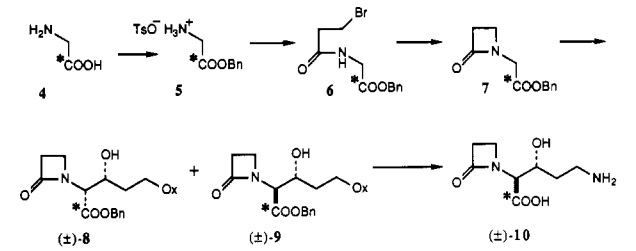
Clavaminate synthase was purified through the Sephadex G-75 step as previously described (Salowe et al., 1990). Incubations contained, in a volume of 130 μL, 50 mM sodium 3-(N-morpholino)propanesulfonate buffer (pH 7.0), 0.5 mM dithiothreitol, 0.1 mM sodium ascorbate, 10 μM ferrous ammonium sulfate, 3 mM sodium α-KG, and 1 mM *rac*-proclavaminate with a ^{3}H/^{14}C dpm ratio of approximately 5. A 20-μL aliquot was removed for the time zero point and added to 5 μL of 1 mM EDTA. The reaction was initiated by the addition of 0.1 mg of enzyme. Five aliquots of 20 μL were removed at various times up to 1 h and terminated with EDTA as above. The samples were frozen at −70 °C until immediately prior to HPLC analysis.

Analysis of the incubation mixture at various time points was carried out on the same HPLC system used for final purification of the proclavaminates, except that the elution buffer contained 0.1% TFA/0.01% SOS. Samples were eluted isocratically at 1.5 mL/min while collecting 0.5-min fractions directly into 7-mL glass miniscintillation vials. Optifluor liquid scintillation cocktail from Packard (6.25 mL) was added promptly for counting. The injector and sample loop (50 μL) were flushed with a minimum of 40 volumes of water and methanol between injections. The vials were counted in a Beckman LS 5801 liquid scintillation counter operating in automatic quench correction mode with a dual label program to directly calculate dpm of ^{3}H and ^{14}C. After subtracting appropriate blank values for each channel, the counts for each peak were added for calculations. The extent of reaction was determined from the ^{14}C distribution as the total dpm in the product divided by half of the total dpm recovered in the injection to account for the nonreacting enantiomer of the racemic substrate. The fractional extent of release of tritiated products (e.g., [^{3}H]water) was determined from the tritium distribution as the total ^{3}H in the product divided by half (for [3-^{3}H] substrate) or one-fourth (for [4′-^{3}H] substrate) of the total ^{3}H recovered in the injection. Data on ^{3}H/^{14}C ratios as a function of extent of reaction were fit to equations given under Results, by assuming constant variance and using a modified nonlinear least-squares regression program (Duggleby, 1984). Values are reported with their associated standard errors.

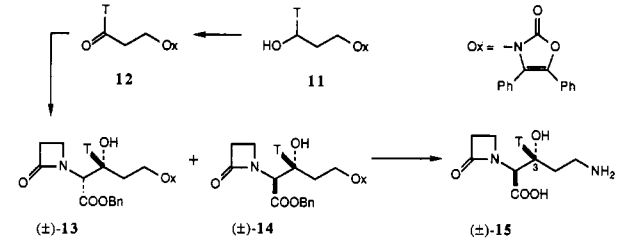
Results

Preparation of Labeled Substrates. To examine the kinetic isotope effects in the double oxidative cyclization catalyzed by clavaminate synthase, [4′-^{3}H]- and [3-^{3}H]proclavaminates were prepared. Moreover, [1-^{14}C]proclavaminate was synthesized to serve as an internal standard to accurately determine relative tritium activities and to provide a precise measure of the extent of reaction. These syntheses were patterned generally on the route to proclavaminic acid developed by workers at Beecham (Baggaley et al., 1987).

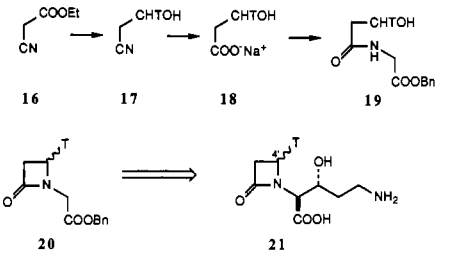
Scheme II



Scheme III



Scheme IV



[1-^{14}C]-*rac*-Proclavaminic acid (**10**) was prepared from [1-^{14}C]glycine (**4**) as shown in Scheme II. Fisher esterification of **4** gave the benzyl ester **5** as its crystalline tosylate salt (Zervas et al., 1957), which was condensed with 2-bromopropionyl chloride to afford the amide **6**. Closure to the glycyl β-lactam **7** was achieved with potassium hydroxide under phase-transfer conditions (Takahata et al., 1981). Treatment of **7** with lithium bis(trimethylsilyl)amide at low temperature and reaction with Ox-protected 3-aminopropanal [Ox = 4,5-diphenyl-4-oxazolidin-2-one, see Sheehan and Guziec (1972)] gave a kinetic mixture of erythro and threo products **8** and **9**, respectively (each as racemates), in a 3:1 ratio. These were equilibrated with DBN to favor the threo isomer (**8**/**9** 1:4), which was separated by flash chromatography (Still et al., 1978) and hydrogenolyzed to give (±)-[1-^{14}C]proclavaminate (**10**).

[3-^{3}H]-*rac*-Proclavaminic acid (**15**) was prepared by reduction of the known, unlabeled N-protected 3-aminopropionaldehyde **12** (Pansare & Vederas, 1987) with sodium [^{3}H]borohydride to give **11**. This was reoxidized (Mancuso et al., 1987) to the tritiated aldehyde **12** and condensed and further processed as above to give racemic [3-^{3}H]proclavaminate (**15**, Scheme III).

[4′-^{3}H]-*rac*-Proclavaminic acid (**21**) was synthesized as shown in Scheme IV. Ethyl cyanoacetate (**16**) was reduced to [3-^{3}H]-3-hydroxypropionitrile (**17**) and hydrolyzed to sodium [3-^{3}H]-3-hydroxypropionate (**18**). Condensation with

benzyl glycinate in the presence of water-soluble carbodiimide gave the hydroxyamide **19**. Application of the Hooz–Gilani procedure (Hooz & Gilani, 1968) afforded the corresponding bromoamide (cf. **6**), which was converted as in Scheme II to the [4′-^{3}H]azetidinone **20** and finally to [4′-^{3}H]proclavaminate (**21**).

Theory. The CS catalytic cycle in Scheme I posits two consecutive irreversible reactions for the transformation of proclavaminate to clavaminate. The experimental approach taken to determine the order of oxidations exploited the consequences of irreversible reactions upon expression of V/K kinetic isotope effects. In the case of a normal primary isotope effect, i.e., $k_H/k_T > 1$, the different reaction rates of isotopically substituted and unsubstituted substrate molecules present in the same reaction mixture results in the enrichment of the isotope in the substrate and its depletion in the product. The competition between different substrates is governed by the kinetic parameter V/K, which only reflects events through the first irreversible step of the reaction (Northrup, 1977). Thus, for the stepwise catalytic sequence proposed for clavaminate synthase, *isotopic discrimination in remaining substrate should only be observed when the heavier hydrogen isotope is at the position oxidized first.* Since the enzyme cannot convert the intermediate back to the original substrate, no substrate V/K effect should be observed with isotopic label at the second position oxidized.

We chose to measure the primary ^{3}H kinetic isotope effects, $^T(V/K)$, using the common double-label procedure in which ^{14}C is present at a nonreactive position and isotopic discrimination appears as a changing ^{3}H/^{14}C ratio. At the time this work was initiated, a stereospecific synthesis for the active (2*S*,3*R*) enantiomer of proclavaminate (**1**) that would allow convenient introduction of radiolabel at the positions of interest was not available. Hence, the tritiated and ^{14}C-labeled substrates were prepared in racemic form. This has important and useful consequences for data analysis. Consider first an experiment using [3-^{3}H,1-^{14}C]-*rac*-proclavaminate (**10**/**15**). The general case of a substrate in an irreversible reaction that can undergo complete turnover is described by

$$S_x/S_0 = (1 - x)^{(1/E1 - 1)} \quad (2)$$

where $E1$ is the primary isotope effect $^T(V/K)$, x is the fractional extent of reaction, and S is the substrate specific activity (^{3}H/^{14}C ratio) at the subscripted extent of reaction (Melander & Saunders, 1980). A plot of S_x/S_0 vs x gives a curve that rises steeply toward infinity as x approaches 1. With a racemate, however, the increasing specific activity of the residual active enantiomer is diluted by the increasing proportion of inactive enantiomer that undergoes no change in specific activity. Consequently, the ^{3}H/^{14}C ratio does not rise to infinity at 100% conversion, but instead returns to its initial value. It can be shown (see Appendix) that the applicable equation is

$$S_x/S_0 = [1 + (1 - x)^{1/E1}]/(2 - x) \quad (3)$$

where the symbols have the same definitions as above. The behavior of eq 3 is shown in Figure 1 for several values of $E1$. The overall effect is that much smaller changes in specific activity occur, particularly at higher extents of reaction, than in the case of a single enantiomer. While this suppression lowers the sensitivity of the experiment, that apparent disadvantage is significantly offset for large to moderate isotope effects by the improved ability to accurately measure specific activity at high extents of reaction by scintillation counting methods.

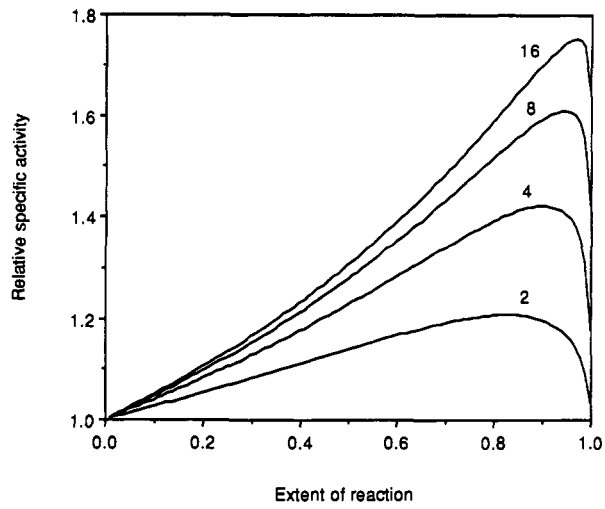


FIGURE 1: Relative specific activity of a racemic substrate as a function of extent of reaction at the indicated isotope effect $E1$ described by eq 3.

An isotope effect will also be manifested in the changing specific activity of the reaction product. Since the specific activity of [^{3}H]water, the product of interest for a primary isotope effect, cannot be determined from a ^{3}H/^{14}C ratio, an alternative method for measuring isotopic discrimination is employed. The fractional extent of tritium release is determined as a function of extent of reaction (Melander & Saunders, 1980):

$$T_x/T_{1.0} = 1 - (1 - x)^{1/E1} \quad (4)$$

where T_x is the amount of [^{3}H]water found at a particular extent of reaction. $T_{1.0}$, the amount of [^{3}H]water released when the reaction has gone to completion, can be assumed for a racemate to be half of the initial amount of tritium present in the substrate.

Additional considerations arise when more than one isotope effect is present. The synthetic route to [4′-^{3}H]proclavaminate (**21**), in addition to producing racemic substrate, labeled the 4′ *pro-R* and *pro-S* positions equally. Since clavaminate synthase stereospecifically removes the 4′ *pro-S* hydrogen (Basak et al., 1990),[2] there are functionally three kinds of tritium present in the substrate. One-fourth of the tritium resides in (4′*S*)-[4′-^{3}H]-(2*S*,3*R*)-proclavaminate and is subject to a primary isotope effect. Another quarter of the total tritium is located in (4′*R*)-[4′-^{3}H]-(2*S*,3*R*)-proclavaminate and gives rise to an α-secondary isotope effect. The remaining 50% of the tritium resides in either the 4′*R* or 4′*S* position of the enzymatically inactive (2*R*,3*S*)-proclavaminate and undergoes no change in specific activity during the reaction. Equation 5 (derived in the Appendix) describes the change in specific activity of (4′*RS*)-[4′-^{3}H,1-^{14}C]-*rac*-proclavaminate (**10**/**21**) as a function of the extent of reaction:

$$S_x/S_0 = [2 + (1 - x)^{1/E1} + (1 - x)^{1/E2}]/(4 - 2x) \quad (5)$$

where $E1$ is the primary isotope effect and $E2$ is the α-secondary effect.

The primary isotope effect can be determined independently by using eq 4 to analyze [^{3}H]water release data, except that $T_{1.0}$ will be one-fourth of the initial amount of substrate tritium. Since the nonabstracted 4′*R* tritium is retained in the

[2] In earlier work we estimated that the stereoselectivity of the enzyme was conservatively >90% and could well be higher if a V/K isotope effect was present. Since the present work indicates a substantial isotope effect to indeed exist, CS is functionally, if not absolutely, stereospecific.

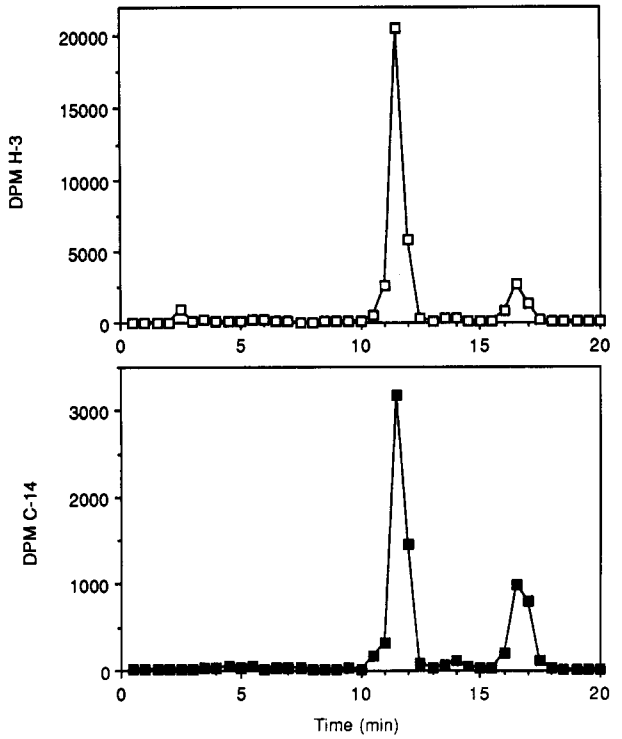


FIGURE 2: Radiochromatogram for the reaction of CS with (4′*RS*)-[4′-^{3}H,1-^{14}C]-*rac*-proclavaminate terminated at extent of reaction = 0.58. The retention times of tritiated reaction components are as follows: [^{3}H]water, 2.5 min; proclavaminate, 11.5 min; clavaminate, 16.5 min.

product, the α-secondary effect can also be determined independently by using the $^3H/^{14}C$ ratio of the clavaminate formed. The applicable equation (Melander & Saunders, 1980) is

$$P_x/P_{1.0} = [1 - (1 - x)^{1/E2}]/x \quad (6)$$

where P_x is the product specific activity ($^3H/^{14}C$ ratio) at extent of reaction x and $P_{1.0}$ will be half of S_0, the initial substrate specific activity.

Determination of the Isotope Effect at C-4′. A representative HPLC chromatogram for the incubation of (4′*RS*)-[4′-^{3}H,1-^{14}C]-*rac*-proclavaminate (**10**/**21**) with CS is presented in Figure 2 and shows that the peaks of interest are well resolved. The data are summarized in Figures 3 and 4. Fitting the [^{3}H]water release data to eq 4 gave a significant primary $^T(V/K) = 8.3 \pm 0.2$ (Figure 3A), while fitting the clavaminate $^3H/^{14}C$ ratio data to eq 6 gave a small normal α-secondary $^T(V/K) = 1.06 \pm 0.01$ (Figure 3B). Inspection of Figure 4 clearly reveals the impact of these combined effects upon the relative specific activity of the residual proclavaminate. Computer fitting to eq 5 gave values for the isotope effects of 11.9 ± 1.7 (primary) and 1.12 ± 0.07 (α-secondary). The agreement upon both primary and secondary $^T(V/K)$s is satisfactory given the difficulties of fitting data to eq 5, which contains the two variable parameters as reciprocal exponents. Simulations show that relatively large variations in the isotope effects, particularly in the case of the dominating primary effect, can have a relatively small visual effect upon the fit.

Determination of the Isotope Effect at C-3 and Discovery of Alternate Reaction Pathways. Since a V/K isotope effect was clearly present at the 4′ position of proclavaminate, no similar isotopic discrimination against substrate labeled at C-3 would be expected if this were the position of the second oxidation in Scheme I. This expectation was borne out experimentally. Examination of Figure 4 reveals no significant

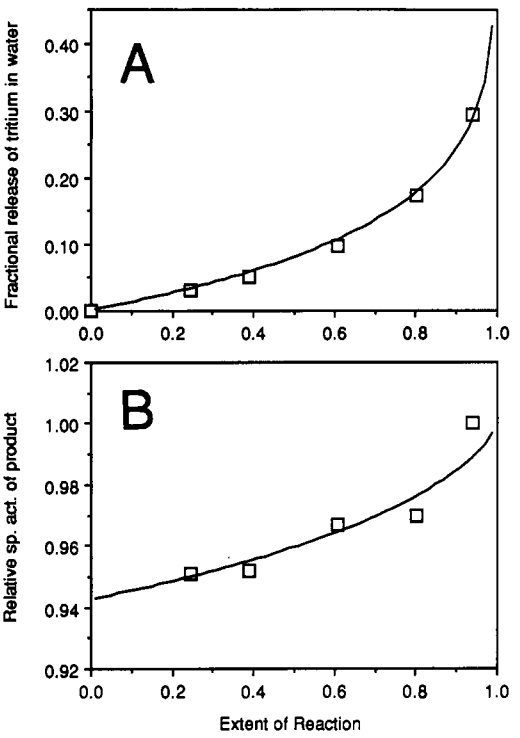


FIGURE 3: Isotope effects for (4′*RS*)-[4′-^{1}H,1-^{14}C]-*rac*-proclavaminate. (A) Fractional extent of tritium release ($T_x/T_{1.0}$) as a function of extent of reaction. The line was drawn by using eq 4 and a primary isotope effect of 8.3. (B) Relative specific activity of clavaminate ($P_x/P_{1.0}$) as a function of extent of reaction. The line was drawn by using eq 6 and an α-secondary isotope effect of 1.06.

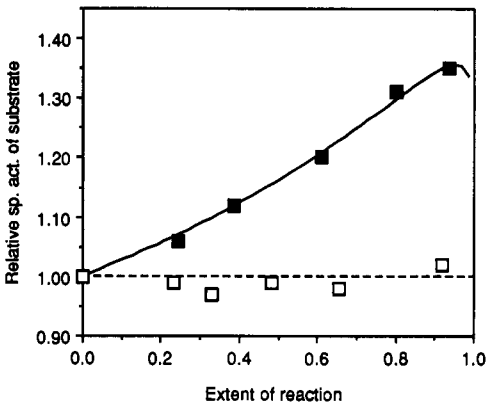


FIGURE 4: Relative specific activity of proclavaminate as a function of extent of reaction for incubations of either (4′*RS*)-[4′-^{3}H,1-^{14}C]-*rac*-proclavaminate (■) or [3-^{3}H,1-^{14}C]-*rac*-proclavaminate (□) with CS. The line for the former was drawn by using eq 5 and isotope effects of 11.9 and 1.12.

increase in the $^3H/^{14}C$ ratio of residual [3-^{3}H,1-^{14}C]-*rac*-proclavaminate (**10**/**15**) over the course of the reaction. Figure 4 is particularly compelling since the experiment with [3-^{3}H]proclavaminate is subject to less suppression of isotopic ratio changes by nonreacting stereoisomers than the experiment with (4′*RS*)-[4′-^{3}H]proclavaminate (**10**/**21**). From this information alone, it would thus appear unequivocal that oxidation occurs first on the β-lactam ring at C-4′.

Inspection of the HPLC chromatogram for the incubation of [3-^{3}H,1-^{14}C]-*rac*-proclavaminate with CS, however, reveals the formation of unexpected additional products. Apart from residual substrate and [^{3}H]water, there are two new peaks (A and B in Figure 5) of radioactivity that contain primarily ^{3}H and little or no ^{14}C. Figure 6 shows the relative distribution of tritium among the products during the course of the reaction. It is particularly notable that the formation of

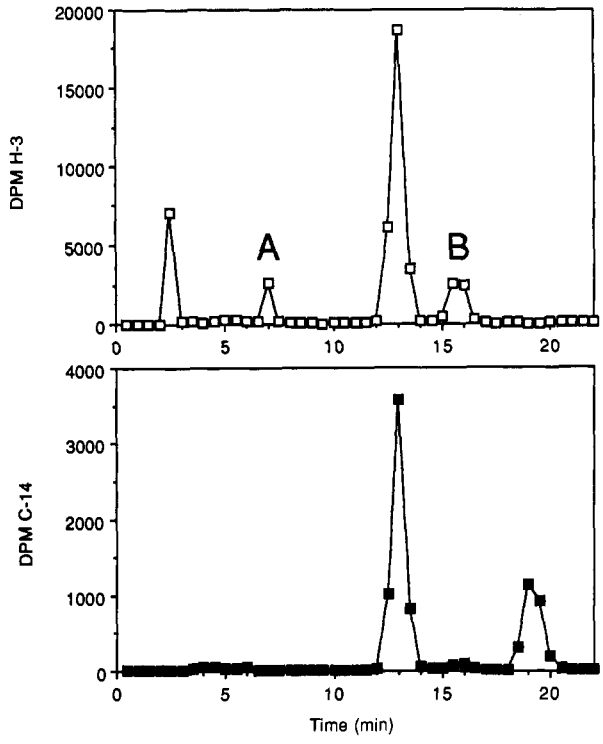


FIGURE 5: Radiochromatogram for the reaction of CS with [3-^{3}H,1-^{14}C]-*rac*-proclavaminate terminated at extent of reaction = 0.64.

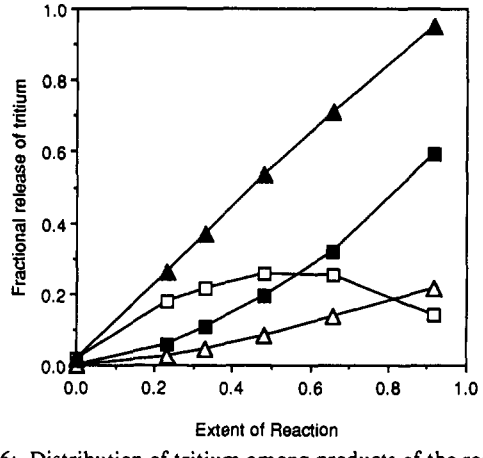
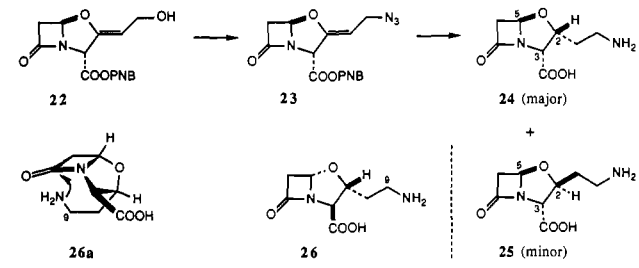


FIGURE 6: Distribution of tritium among products of the reaction of [3-^{3}H,1-^{14}C]-*rac*-proclavaminate with CS as a function of extent of reaction: (■) [^{3}H]water, (△) peak A, (□) peak B, (▲) sum of the above.

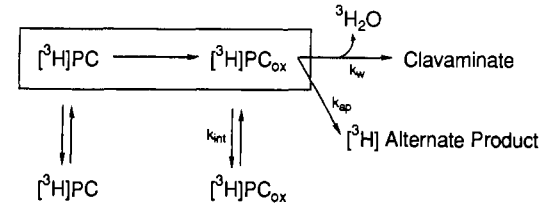
[^{3}H]water is not linear with extent of reaction. While in isolation this would appear to be the result of an isotope effect, when all tritiated products are considered together an essentially linear correlation does exist between tritium released and extent of reaction. This implies that *all* tritiated products are derived from a common intermediate after the initial irreversible oxidation at C-4′. Since the new compounds contain only tritium radiolabel, they must be the result of isotope-sensitive branching from PC_{ox} (Scheme I). That is, the kinetic isotope effect at C-3, which cannot be manifested as a discrimination among substrate molecules, is unmasked by the availability of competing pathways.

One potential alternative fate to normal turnover would be the release of the intermediate PC_{ox} bearing tritium label into solution. As the extent of reaction increases, the intermediate can compete with the diminishing level of proclavaminate for binding in the active site and continue reaction on to clavaminate. The behavior of peak B in Figure 6, whose relative

Scheme V



Scheme VI



concentration rises at partial extents of reaction and then declines, identifies it as a likely candidate for free PC_{ox} that is ultimately consumed. Identification of peak B as corresponding to dihydroclavaminate (**26**) was achieved through comparisons to its authentic enantiomer. *p*-Nitrobenzyl clavulanate (**22**; Scheme V, PNB = *p*-nitrobenzyl) was converted to the allylic azide **23** and exhaustively hydrogenated to a 3:2 mixture of the clavam derivatives **24** and **25**, respectively. Interestingly, addition of hydrogen to the vinyl ether double bond from the concave face of the molecule was favored, as has been observed previously for lithium clavulanate (Cherry & Newall, 1982). The structures of **24** and **25** were readily assigned based on chemical shift correlations of their ^{1}H NMR spectra to other known clavams, in particular H-5 and H-3 (H-5, δ 5.41 vs 5.25; H-3, δ 4.43 vs 3.98 for **24** and **25**, respectively) (Bentley & Hunt, 1980; Wanning et al., 1981; Cherry & Newall, 1982). The diastereomeric clavams **24** and **25** were well separated under the ion-pairing conditions of the HPLC analysis. Only the minor isomer **25** coeluted with the radioactivity in peak B of Figure 5. The structure of PC_{ox}, is therefore, the *enantiomer* of **25**, that is, dihydroclavaminic acid (**26**).

The possibility was entertained that dihydroclavaminate **26**, free in solution, bearing an *endo*-aminoethyl side chain might undergo an intramolecular transamidation reaction (cf. **26a** and analogous precedented reactions; Kobayashi et al., 1978; Bentley & Hunt, 1980) in competition with its return to the synthase and further oxidative conversion to clavaminate (**2**). Such a process could be envisioned to account for the formation of peak A. However, in separate experiments both in buffer alone and under the incubation conditions but lacking enzyme, **24** and **25** failed to show any decomposition in 75 min. In contrast, peak A is detectable within minutes from the start of the incubation of [3-^{3}H,1-^{14}C]proclavaminate. The origin of peak A must, therefore, reside in some other process than exchange into the medium or normal reaction of PC_{ox} to clavaminate.

Another possible fate for the enzyme-bound dihydroclavaminate intermediate is oxidation at a site other than C-2, leading to the formation of an alternate reaction product that, unlike clavaminate, contains tritium. Further consideration of the relative tritium activities present in peak A and in [^{3}H]water as a function of extent of reaction reveals that they are formed in essentially constant ratio (1:2.4, respectively) throughout the reaction. This observation would be consistent with Scheme VI, in which divergent, irreversible reactions

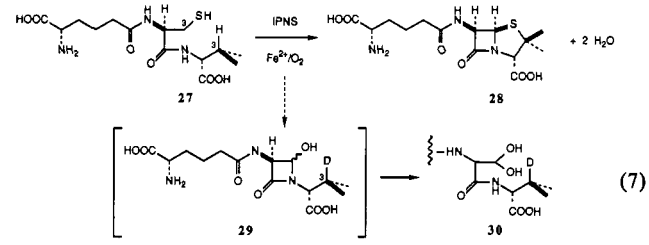
occur on the enzyme (boxed region) either at C-2 to form clavaminate or at another center in dihydroclavaminate (**26**) to give alternate product A. The identity of this product is not known and is currently under investigation. Nonetheless, the ratio of rate constants k_{int}:k_w:k_{ap} in Scheme VI may be estimated from the 23% conversion data point in Figure 6 to be 7.5:2.4:1. Since $k_w \gg k_{int},k_{ap}$ when protium is present at C-3 of proclavaminate, the isotope effect operating in this half of the overall reaction cycle must be quite substantial.

Discussion

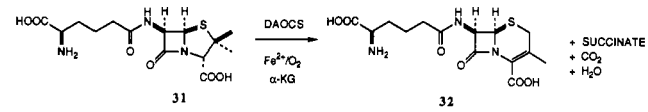
Few isotope effect studies have been carried out on α-ketoglutarate-dependent oxygenases. Among the hydroxylases, only γ-butyrobetaine hydroxylase (BBH) from calf liver has been found to exhibit a large intermolecular tritium selection effect (Blanchard & Englard, 1983). The primary $^T(V/K)$ of 15 and α-secondary $^T(V/K)$ of 1.31 were interpreted as reflecting coupled motions of the primary and α-secondary hydrogens during bond cleavage and rehybridization. In considering possible mechanisms of the hydroxylation reaction itself, Blanchard and Englard viewed their data, in particular the magnitude and direction of the α-secondary effect, as indicating a substantial change in hybridization at the carbon undergoing oxidation from sp^3 to sp^2 in the transition state of the reaction. Such extensive rehybridization was seen to discount carbanion (Holme et al., 1968; Lindblad et al., 1969) or oxenoid C–H insertion (Hamilton, 1971) mechanisms as likely pathways for the enzymic reaction. On the other hand, significant sp^2 character in the transition state is in accord with the intermediacy of radical or carbonium ion intermediates in the oxidation. As discussed quite thoroughly by Blanchard and Englard, homolytic C–H cleavage was favored over hydride abstraction, which implied a central role for ferryl–oxo species (Siegel, 1979) as the active form of oxygen in these reactions.

The magnitudes of the corresponding primary and α-secondary $^T(V/K)$ isotope effects in the CS reaction were determined in two ways. The appearance of radiolabel in the reaction products from (4′*RS*)-[4′-^{3}H,1-^{14}C]proclavaminic acid provided one measure. First, a primary $^T(V/K) = 8.3 \pm 0.2$ was determined from the release of [^{3}H] water, while variation of the $^3H/^{14}C$ ratio in the product clavaminic acid gave an α-secondary $^T(V/K) = 1.06 \pm 0.01$. Second, a modification of the conventional treatment for changes in relative specific activity of the residual substrate was derived (Appendix) to permit the rigorous evaluation of reactions involving a racemic substrate. While less sensitive for low isotope effects than the customary treatment of single enantiomers, it confers the experimental advantage for both large and small isotope effects of allowing accurate determination of $^3H/^{14}C$ ratios at high extents of reaction. Application of eq 5 relevant to C-4′ tritium-labeled substrate yielded primary and α-secondary $^T(V/K) = 11.9 \pm 1.7$ and 1.12 ± 0.07, respectively, in substantial agreement with the values obtained from product release data. Compared to the magnitudes of the primary and α-secondary $^T(V/K)$ isotope effects obtained for calf liver BBH (Blanchard & Englard, 1983), the corresponding effects in the CS reaction, while still substantial, are smaller, notably the α-secondary effect. Whether this difference reflects an altered transition-state geometry for hydrogen abstraction in the CS reaction and/or the effect of different ground state hybridizations of the methylene C–H bonds imposed by the rigid β-lactam ring (greater s-character) is unclear. In any event, caution must be exercised in any comparisons of this kind since in neither case is the extent of suppression of the isotope effect by other partially rate-limiting steps known. We also note that an *intra*molecular tritium isotope effect of 6.5 has been reported for thymine hydroxylase (Holme, 1982). Although not directly comparable to the intermolecular effects discussed above, the intramolecular discrimination among torsionally symmetric hydrogens should closely reflect the intrinsic isotope effect for this case. While a larger data base on α-ketoglutarate-dependent oxygenases and nonenzymatic model systems clearly will be needed before generalizations can be firmly drawn on the oxidative chemistry performed by this class of enzymes, we view our isotope effect data, like Blanchard and Englard for BBH, to support the intervention of radical or cationic intermediates in the oxidative cyclization catalyzed by clavaminate synthase.

The use of kinetic isotope effects to deduce the order of chemical steps in a reaction has precedence in the mechanistic studies of two other enzymes involved in the biosynthesis of bicyclic β-lactam natural products. Both isopenicillin N synthase [IPNS, tripeptide **27** to isopenicillin N (**28**)] and deacetoxycephalosporin C synthase [DAOCS, penicillin N (**31**) to deacetoxycephalosporin C (**32**)] perform oxidative cyclization chemistry (eqs 7 and 8) with potential similarity to the CS reaction. Competition experiments with IPNS have established that isotopic discrimination occurs against ^{2}H at C-3 of the substrate cysteinyl residue[3] but that there is no V/K effect with ^{2}H at C-3 of the valinyl moiety in tripeptide **27** (Baldwin et al., 1984). If one assumes that the initial oxidation is irreversible, the results may be interpreted to support a stepwise reaction in which the β-lactam ring is formed initially. This conclusion was later strengthened by the discovery of isotope-induced branching. When substrate **27** deuteriated at C-3 of valine was used, a small amount of compound **30** was produced, presumably because the isotope effect aborted the second cyclization in some fraction of turnovers and an unstable decomposition product of initial oxidation, possibly **29**, was released (Baldwin et al., 1988). Isotope effect experiments have also been used with DAOCS to determine that a methyl hydrogen is lost first in a stepwise ring-expansion process (Baldwin et al., 1987).[4]



(7)



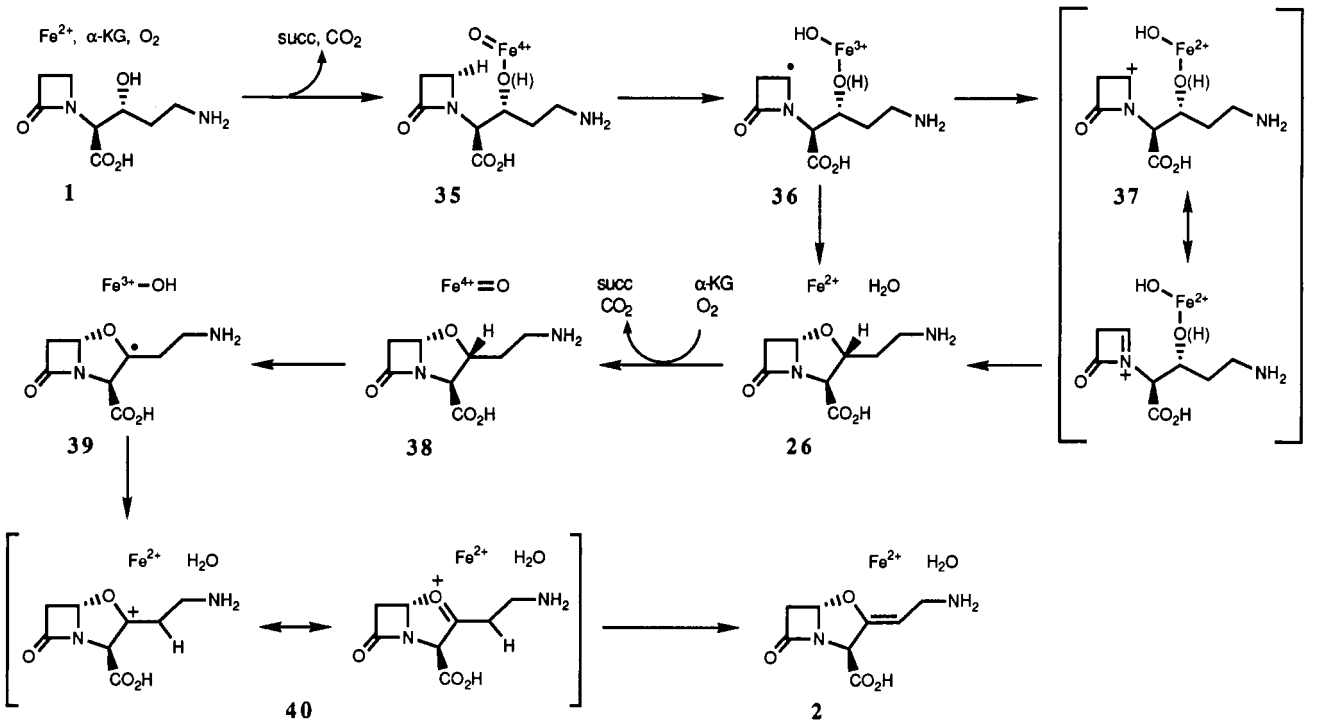
(8)

The enzymatic conversion of proclavaminate to clavaminate requires the loss of four hydrogen atoms in two oxidative events, which can be viewed as formally elevating C-4′ from the alcohol (amine) to the aldehyde (hemiaminal) and C-3 from the alcohol to the ketone (enol ether) oxidation states. While mechanisms can be envisioned that remove any of these

[3] Although not reported by the authors, a range of 1.3–1.8 for $^D(V/K)$ can be calculated from their data on the changing isotope ratio of the substrate and product.

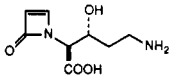
[4] Although not reported by the authors, a range of 2.6–7.4 for $^D(V/K)$ can be calculated from their limited data on the changing isotope ratio of the substrate.

Scheme VII

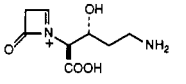


four hydrogens as the first step, the expected bond energies of the hydrogens at carbons 3 and 4′ identify them as the most reasonable candidates for abstraction. We have postulated, on the basis of the overall reaction stoichiometry and kinetic evidence, that the oxidations occur in a stepwise fashion, each step consuming O_2 and α-KG and producing succinate, CO_2, and H_2O (Scheme I; Salowe et al., 1990). Each of these reactions would be expected to be irreversible given the exergonic nature of the coupled oxidative decarboxylation of α-KG and reduction of dioxygen.

The isotopic discrimination observed against proclavaminate labeled with tritium at C-4′ but not at C-3 strengthens our earlier proposal of stepwise reactions and reveals unambiguously that C-4′ is the site of the initial oxidation. The subsequent isotope effect at C-3 slows the second oxidation sufficiently to allow dissociation of an intermediate to become detectable. Three possibilities may be considered for the structure of the initial oxidation product. We have previously argued against **33** because of the lack of isotope exchange at C-3′ during the reaction (Krol et al., 1989) and the intrinsic instability of the antiaromatic azetinone (Olofson et al., 1984). Acyl iminium ion **34**, if released from the enzyme, would be expected to rapidly hydrate to a hydroxyazetidinone, which is known to be unstable toward ring opening (Kamata et al., 1979). Dihydroclavaminate (**26**), on the other hand, should have comparable hydrolytic stability to the structurally similar clavaminate (**2**) and thus was viewed as the probable candidate for the intermediate. The endo-2*R*,3*S*,5*S* configuration postulated for dihydroclavaminate was defined by the absolute stereostructure of proclavaminate (**1**) and the following facts: first, the proclavaminate C-3 tritium label is retained; second, no exchange is observed adjacent to the C-1 carboxyl in the conversion of **1** to **2** (Krol et al., 1989); and third, the oxidative cyclization at C-4′ of **1** occurs with overall retention of configuration (Basak et al., 1990). Coelution during HPLC of the authentic enantiomer of dihydroclavaminate with the intermediate further supports this structural assignment. While this paper was in preparation, an independent report on the reaction intermediate appeared (Baldwin et al., 1990). Incubation of [3-^{2}H]proclavaminate with CS led to the accumulation of a compound that was spectroscopically identical with the enantiomer of dihydroclavaminate, i.e., **25**, prepared synthetically from clavulanate by an alternative route to the one used in the present studies. The compound isolated from the enzymatic reaction could apparently undergo conversion to clavaminate, although insufficient experimental detail was given to evaluate the efficiency of this process. These findings are entirely consistent with our own assignment of the structure of dihydroclavaminate as **26**, and, further, they are in accord with the V/K isotope effects and the dynamic behavior of this intermediate through the course of the reaction determined in the present studies.



33



34

Having established the site of initial oxidation and identified the structure of the intermediate, we are now in a position to propose a fairly detailed working hypothesis for the enzyme's catalytic mechanism. The activation of molecular oxygen can be considered in the context of proposals for α-KG-dependent dioxygenases. Like several of these enzymes, CS catalyzes a slow "uncoupled" reaction. In the absence of proclavaminate, α-KG is decarboxylated to succinate and carbon dioxide with the apparent partial reduction of dioxygen to peroxide (Salowe et al., 1990). It has been suggested that the uncoupled reactions of dioxygenases form a ferryl ion $[Fe{=}O]^{2+}$ that is a normal intermediate in hydroxylation when all substrates are present (Siegel, 1979; Hanauske-Abel & Gunzler, 1982; Blanchard & Englard, 1983). Thymine hydroxylase can perform an olefin epoxidation, consistent with the expected reactivity of this putative oxidant (Thornburg & Stubbe, 1989). Once oxygen activation has been achieved, hydrogen abstraction (either homolytic or heterolytic) and oxygen transfer can be envisioned to occur by analogy to the heme-dependent cytochrome P-450s. In support of a homolytic pathway for dioxygenases, a cyclopropyl ring containing

substrate analogue has been found to inactivate γ-butyrobetaine hydroxylase, presumably through a rapid radical rearrangement, although the actual product of the reaction has not yet been identified (Ziering & Pascal, 1990).

We propose that the first chemical step to occur following binding of substrates and iron at the active site of CS is the generation of the ferryl oxidant by decarboxylation of α-KG coupled to cleavage of the O–O bond (Scheme VII). The hydroxyl group at C-3 of proclavaminate may supply a ligand to the metal during or after this process (Krol et al., 1989). Abstraction of the *pro-S* hydrogen atom at C-4′ then generates the carbon-centered radical **36**, or possibly an equivalent organoiron species. Bond formation between the oxidized carbon and the C-3 hydroxyl, either directly (**36** → **26**) or after a second electron transfer step (**36** → **37** → **26**), produces dihydroclavaminate. The functional divergence of CS from hydroxylases arises because the capture of the radical (or cation) occurs *intramolecularly* with a proclavaminate-derived oxygen instead of *intermolecularly* with the dioxygen-derived hydrogen abstractor. Dihydroclavaminate remains in the active site as an enzyme-bound intermediate for the second oxidative reaction in which a new ferryl species is generated from a second molecule of α-KG and O_2 (**26** → **38**). Hydrogen abstraction in this cycle, however, occurs at C-3 to form carbon-centered radical **39**. Electron transfer then leads to oxonium ion **40**, which tautomerizes to the final product clavaminate (**2**). Alternatively, oxygen "rebound" to the radical **39** would give a hemiketal functionally equivalent through ionic chemistry with oxonium ion **40**.

According to Scheme VII, discrimination against tritium can occur at both points of the mechanism involving hydrogen abstraction, although only the first oxidation at C-4′ results in a changing specific activity of the substrate, as found experimentally (Figure 4). Since the enzyme has a sequential kinetic mechanism (Salowe et al., 1990) and the isotope-sensitive bond-breaking step lies after the irreversible activation of dioxygen, it might be expected that no discrimination among substrate molecules would be observed. This paradox can be reconciled by proposing that proclavaminate is required in the active site for efficient generation of the oxidant but can dissociate from the activated complex **35**. The more sluggishly reacting tritiated substrate can thereby be exchanged for a swifter protiated one. During or prior to release of the isotopic substrate, the iron–oxo species may decompose nonproductively (e.g., forming peroxide) so that recharging with fresh α-KG and O_2 is required. The net effect then is the promotion of an uncoupled reaction that aborts turnover of the isotopically labeled proclavaminate. Experimental results with γ-butyrobetaine hydroxylase support the feasibility of this proposal. Deuteriation increases the ratio of α-KG decarboxylation to γ-butyrobetaine hydroxylation by the enzyme from 1.2 to 7.5 (Holme et al., 1984). Preliminary experiments with CS have found elevated levels of CO_2 formation when [4′-^{2}H]proclavaminate replaces unlabeled substrate (S. P. Salowe, unpublished results). Rejection of a slow substrate concomitant with an uncoupled decarboxylation probably occurs as well during the second CS oxidative event when the enzyme is confronted with tritium at C-3 of the dihydroclavaminate intermediate. In this case the phenomenon is manifested by the preferential release of dihydroclavaminate bearing tritium at C-2 as compared to protium at this locus (compare levels of ^{3}H and ^{14}C for peak B in Figure 5). Moreover, enzyme-bound dihydroclavaminate bearing tritium at the site of the second oxidation is seen to partition in constant ratio to clavaminate itself with release of [^{3}H]water and to a second product, peak A in Figure 5, that retains the tritium label. This product thus would appear to be derived from oxidation at a reaction site other than C-2 of **26**. The structure of this alternative product is not known at present, but control experiments demonstrated that it is not derived from decomposition of dihydroclavaminate itself. In the event, it is clear that both hydrogen abstraction events at C-4′ and C-3 in proclavaminate present significant kinetic barriers in the overall enzymatic transformation.

Acknowledgments

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Appendix

If A represents the concentration of all substrate isomers in a reaction and F is that fraction of A that is enzymatically active, then at fractional extent of reaction x the concentration of inactive isomer is $(1 - F)A$ while the concentration of the active isomer is $(1 - x)FA$. It follows that the fractions of the inactive and active isomers in the remaining total substrate, K and L respectively, are

$$K = (1 - F)A/[(1 - F) + (1 - x)F]A = (1 - F)/(1 - xF) \quad (9)$$

$$L = (1 - x)FA/[(1 - F) + (1 - x)F]A = (1 - x)F/(1 - xF) \quad (10)$$

The experimentally measured specific radioactivity of the total substrate, S, is a weighted average of the specific activities of the active and inactive isomers, S_a and S_i, respectively:

$$S = KS_i + LS_a \quad (11)$$

Since the specific activity of the inactive isomer remains unchanged during the reaction at S_0, the initial specific activity of the total substrate, eq 11 can be rearranged to

$$S/S_0 = K + L(S_a/S_0) \quad (12)$$

Substituting eq 13 (Melander & Saunders, 1980) and the definitions of K and L into eq 12 leads, after algebraic simplification, to eq 14.

$$S_a/S_0 = (1 - x)^{(1/E - 1)} \quad (13)$$

$$S/S_0 = [1 - F + F(1 - x)^{1/E}]/(1 - xF) \quad (14)$$

In the case of a racemate, $F = 0.5$ and eq 14 further simplifies to eq 3 in the text.

The equations can be modified to deal with substrate containing isotopic label at two positions that give different isotope effects. Let G represent the fraction of active isomer with isotopic label subject to isotope effect $E1$ and $(1 - G)$ be the fraction subject to isotope effect $E2$. Equation 11 then becomes

$$S = KS_i + GLS_1 + (1 - G)\,LS_2 \quad (15)$$

where S_1 and S_2 are the specific activities of the substrates experiencing isotope effects $E1$ and $E2$, respectively. It follows that

$$S/S_0 = K + GLS_1/S_0 + (1 - G)LS_2/S_0 \quad (16)$$

Using eq 13 for both isotope effects, substituting the definitions of K and L, and simplifying gives

$$S/S_0 = [1 - F + FG(1 - x)^{1/E1} + (1 - G)F(1 - x)^{1/E2}]/(1 - xF) \quad (17)$$

for (4′*RS*)-[4′-^{3}H]-*rac*-proclavaminate, $F = G = 0.5$ and eq 17 reduces to eq 5 in the text.

Registry No. **5**, 131216-02-9; **6**, 131216-03-0; **7**, 131216-04-1; **8**, 131235-92-2; **9**, 131216-05-2; **10**, 131216-06-3; **11**, 131216-07-4; **12**,

131216-08-5; **13**, 131216-09-6; **14**, 131216-10-9; **15**, 131216-11-0; **16**, 105-56-6; **17**, 131216-12-1; **18**, 131216-13-2; **19**, 131216-14-3; **20**, 131216-15-4; **21**, 131216-16-5; **22**, 57943-83-6; **23**, 65788-54-7; **24**, 131320-38-2; **25**, 131320-39-3; T, 10028-17-8; clavaminate synthase, 122799-56-8; [1-^{14}C]glycine, 56-39-3; 3-bromopropionyl chloride, 15486-96-1; 4,5-diphenyl-3-(3-oxopropyl)-4-oxazolin-2-one, 110354-14-8; benzyl 2-(2-oxoazetidin-1-yl)acetate, 114342-03-9; benzyl glycinate tosylate salt, 1738-76-7; benzyl *N*-([3-^{3}H]-3-bromopropionyl)glycinate, 130676-99-2.

REFERENCES

Arigoni, D. (1975) *Pure Appl. Chem. 41*, 219–245.

Baggaley, K. H., Sime, J. T., Nicholson, N. H., Elson, S. W., Gillett, J., Holland, S., & Woroniecki, S. R. (1987) *J. Chem. Soc., Chem. Commun.*, 1738–1739.

Baldwin, J. E., Adlington, R. M., Moroney, S., Field, L. D., & Ting, H.-H. (1984) *J. Chem. Soc., Chem. Commun*, 984–986.

Baldwin, J. E., Adlington, R. M., Aplin, R. T., Crouch, N. P., Schofield, C. J., & Ting, H.-H. (1987) *J. Chem. Soc., Chem. Commun.* 1654–1656.

Baldwin, J. E., Adlington, R. M., Bradley, M., Norris, W. J., Turner, N. J., & Yoshida, A. (1988) *J. Chem. Soc., Chem. Commun.*, 1125–1128.

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. (1990) *J. Chem. Soc., Chem. Commun.*, 617–619.

Basak, A., Salowe, S. P., & Townsend, C. A. (1990) *J. Am. Chem. Soc. 112*, 1654–1656.

Bentley, P. H., & Hunt, E. (1980) *J. Chem. Soc., Perkin Trans. 1*, 2222–2227.

Blanchard, J. S., & Englard, S. (1983) *Biochemistry 22*, 5922–5929.

Braun, J. V. (1931) *Annalen 490*, 125.

Brown, A. G., Corbett, D. F., Goodacre, J., Harbridge, J. B., Howarth, T. T., Ponsford, R. J., Stirling, I., & King, T. J. (1984) *J. Chem. Soc., Perkin Trans. 1*, 635–650.

Charnas, R. L., Fisher, J., & Knowles, J. R. (1978) *Biochemistry 17*, 2185–2189.

Cherry, P. C., & Newall, C. E. (1982) in *Chemistry and Biology of β-Lactam Antibiotics* (Morin, R. B., & Gorman, M., Eds.) Vol. 2, pp 361–402, Academic Press, New York.

Duggleby, R. J. (1984) *Comput. Biol. Med. 14*, 447–455.

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

Englard, S., Blanchard, J. S., & Midelfort, C. F. (1985) *Biochemistry 24*, 1110–1116.

Fisher, J., Charnas, R. L., & Knowles, J. R. (1978) *Biochemistry 17*, 2180–2184.

Frère, J.-M., Dormans, C., Lenzini, V. M., & Duyckaerts, C. (1982) *Biochem. J. 207*, 429–436.

Hamilton, G. A. (1971) *Prog. Bioog. Chem. 1*, 83–157.

Hanauske-Abel, H. M., & Gunzler, V. (1982) *J. Theor. Biol. 94*, 421–455.

Hayaishi, O., Nozaki, M., & Abbott, M. (1976) *Enzymes (3rd Ed.) 12*, 119–189.

Holme, E. (1982) *Biochim. Biophys. Acta 707*, 259–266.

Holme, E., Lindstedt, G., Lindstedt, S., & Tofft, M. (1968) *FEBS Lett. 2*, 29–32.

Holme, E., Lindstedt, S., & Nordin, I. (1984) *Biosci. Rep. 4*, 433–440.

Hooz, J., & Gilani, S. S. H. (1968) *Can. J. Chem. 46*, 86–87.

Kamata, S., Yamamoto, S., Haga, N., & Nagata, W. (1979) *J. Chem. Soc., Chem. Commun.*, 1106–1107.

Kobayashi, T., Iwano, Y., & Hirai, K. (1978) *Chem. Pharm. Bull. 26*, 1761–1767.

Korzekwa, K. R., Trager, W. F., & Gillette, J. R. (1989) *Biochemistry 28*, 9012–9018.

Krol, W. J., Basak, A., Salowe, S. P., & Townsend, C. A. (1989) *J. Am. Chem. Soc. 111*, 7625–7627.

Lindblad, B., Lindstedt, G., Tofft, M., & Lindstedt, S. (1969) *J. Am. Chem. Soc. 91*, 4604–4606.

Mancuso, A. J., Huang, S. L., & Swern, D. J. (1987) *J. Org. Chem. 52*, 4804–4810.

Melander, L., & Saunders, W. H. (1980) *Reaction Rates of Isotopic Molecules*, pp 91–128, Wiley, New York.

Northrup, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrup, D. B., Eds.) pp 122–152, University Park Press, Baltimore, MD.

Olofson, R. A., Morrison, D. S., & Banerji, A. (1984) *J. Org. Chem. 49*, 2653–2654.

Pansare, S. V., & Vederas, J. C. (1987) *J. Org. Chem. 52*, 4804–4810.

Read, R. R. (1941) *Organic Synthesis*, Collect. Vol. 1, pp 321–322, Wiley, New York.

Reading, C., & Farmer, T. (1981) *Biochem. J. 199*, 779–787.

Salowe, S. P., Marsh, E. N., & Townsend, C. A. (1990) *Biochemistry 29*, 6499–6508.

Samuelson, A. G., & Carpenter, B. K. (1981) *J. Chem. Soc., Chem. Commun.*, 354–356.

Sheehan, J. C., & Guziec, F. S., Jr. (1972) *J. Am. Chem. Soc. 94*, 6561–6562.

Siegel, B. (1979) *Bioorg. Chem. 8*, 219–226.

Still, W. C., Kahn, M., & Mitra, A. (1978) *J. Org. Chem. 43*, 2923–2925.

Takahata, H., Ohnishi, Y., Takehara, H., Tsuritani, K., & Yamazaki, T. (1981) *Chem. Pharm. Bull. 29*, 1063–1068.

Thornburg, L. D., & Stubbe, J. (1989) *J. Am. Chem. Soc. 111*, 7632–7633.

Townsend, C. A., Christensen, S. B., & Davis, S. G. (1988) *J. Chem. Soc., Perkin Trans. 1*, 839–861.

Zervas, L., Winitz, M., & Greenstein, J. R. (1957) *J. Org. Chem. 22*, 1515–1521.

Ziering, D. L., & Pascal, R. A. (1990) *J. Am. Chem. Soc. 112*, 834–838.