Mechanism-Based Inhibition of Bacterial γ -Butyrobetaine Hydroxylase

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Abstract: The branched-chain hydrocarbon acids 5,5-dimethylhexanoic acid and 3-(2,2-dimethylcyclopropyl)propanoic acid were examined as substrates and inhibitors of the γ -butyrobetaine hydroxylase from *Pseudomonas* sp. AK1, an α -ketoglutarate-dependent, non-heme iron-containing dioxygenase. The former compound is a very slow alternate substrate of the hydroxylase. It is oxygenated at carbon 3 at ca. 0.1% of the rate of hydroxylation of the natural substrate γ -butyrobetaine, but the reaction is extensively uncoupled: α -ketoglutarate decarboxylation occurs almost 6 times as frequently as carbon hydroxylation. In contrast, 3-(2,2-dimethylcyclopropyl)propanoic acid was observed to be a first-order time-dependent inactivator of the hydroxylase. Inactivation occurred only in the presence of both oxygen and α -ketoglutarate, and the natural substrate provided nearly complete protection from inactivation; thus this cyclopropane-bearing analogue must be a mechanism-based inhibitor of the enzyme.

The final step in the biosynthesis of D-carnitine (2) is the oxygenation of γ -butyrobetaine (1) catalyzed by γ -butyrobetaine hydroxylase (EC 1.14.11.1; henceforth BB hydroxylase). The enzyme, which is typical of the α -ketoglutarate-dependent nonheme iron dioxygenases,¹ has been highly purified from a pseudomonad,² bovine liver,³ and human kidney.⁴ Perhaps because of the relative simplicity of substrate 1 (when compared with the polypeptide substrates of the better known prolyl and lysyl hydroxylases of collagen biosynthesis), BB hydroxylase has been the subject of several mechanistic studies in recent years.



It has been shown that the enzyme is indeed a dioxygenase by means of ¹⁸O₂ labeling,⁵ and the hydroxylation has been found to proceed with retention of configuration at C-3.⁶ α -Ketoglutarate is required for hydroxylation of substrate 1, but decarboxylation of α -ketoglutarate may occur in the absence of 1 if product 2 is present in the reaction mixture.⁷ Such uncoupling of α -ketoglutarate decarboxylation from γ -butyrobetaine hydroxylation usually occurs to only a small extent in the normal reaction, but under some circumstances can increase. In the absence of potassium the ratio of decarboxylation to hydroxylation by the rat liver enzyme may be as high as 3,8 and when $[2,2,3,3,4,4-{}^{2}H_{6}]-\gamma$ -butyrobetaine is used as the substrate for the enzyme from human kidney, the ratio of decarboxylation to hydroxylation rises to 7.5.9 Blanchard and Englard¹⁰ have conducted

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extensive kinetic isotope effect studies with the bovine enzyme using tritium-labeled γ -butyrobetaines, and they conclude, on the basis of an analysis of the α - and β -secondary tritium isotope effects, that carbon 3 of γ -butyrobetaine must be sp²-hybridized in the transition state of the hydroxylation step. These data are consistent with a mechanism (discussed most eloquently by Blanchard and Englard, but based in large part on earlier proposals by Hamilton¹¹ and Siegel¹²) in which the decarboxylation of α -ketoglutarate is coupled to oxygen-oxygen bond cleavage to form succinate, CO₂, and an enzyme-bound ferryl-oxo complex (Fe^{IV}=O). This species then abstracts the pro-R hydrogen at C-3 of γ -butyrobetaine to give a ferric-hydroxyl complex and a carbon radical, and recombination of the carbon and hydroxyl groups follows (with or without an intermediate one-electrontransfer step) to give the product carnitine.

Such a mechanism is reminiscent of recent mechanistic proposals for the hydroxylation reactions catalyzed by the cytochromes P-450 in which the oxygenating species is a ferryl-oxo porphyrin cation radical.¹³ A number of mechanism-based inactivators of cytochromes P-450 have been described that contain cyclopropanes as the key functional group involved in the inactivation process,¹⁴ and indeed a variety of enzymatic reactions that are believed to proceed via radical intermediates may be inhibited by substrate analogues containing cyclopropyl groups,¹⁵ prominent examples are monoamine oxidase¹⁶ and dopamine β -hydroxylase.¹⁷ similar strategy might prove effective for the design of mechanism-based inactivators of γ -butyrobetaine hydroxylase and, by extension, other α -ketoglutarate-dependent dioxygenases.

Results and Discussion

Reaction of BB Hydroxylase with 5,5-Dimethylhexanoate (3). Early studies with substrate analogues have shown that BB hydroxylase exhibits a relatively strict substrate specificity,¹⁸ so that

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Inhibition of γ -Butyrobetaine Hydroxylase

potential alternate substrates and inhibitors should vary minimally from the normal substrate, compound 1. 5,5-Dimethylhexanoate (3) is identical with the normal substrate 1 except that the nitrogen of 1 has been replaced by carbon. This substitution alters the charge of the molecule at physiological pH, but its shape is not significantly different. It is therefore not surprising that compound 3 is a good competitive inhibitor of BB hydroxylase with a K_i of 0.16 ± 0.03 mM,¹⁹ which is roughly an order of magnitude lower than the $K_{\rm M}$ for γ -butyrobetaine (2.4 mM²⁰). However, if 3 is to provide a reasonable starting point for the design of mechanism-based inactivators of the enzyme, it must itself be a substrate.

In preliminary studies to determine if compound 3 was oxidized by BB hydroxylase, only very small amounts of material cochromatographing with an authentic sample of the expected hydroxylation product 6 were detected. Analysis was by HPLC of underivatized material [column A (see Experimental Section); methanol-0.1% aqueous phosphoric acid gradient; detection at 214 nm] or phenacyl ester derivatives [column A; acetonitrilewater gradient; detection at 254 nm]. A tritium wash-out assay was developed to provide a more sensitive and accurate method of detection. A synthesis of 3 that permitted the introduction of hydrogen isotopes specifically at carbon 3 was devised in which the key step was the sodium borohydride reduction of diethyl (3,3-dimethylbutylidene)malonate (5), followed by hydrolysis and decarboxylation of the resulting diester (see below). When this reaction was carried out with sodium borodeuteride as the reducing agent, ¹H NMR analysis of the product [²H]-3 showed that a single deuteron had been introduced and that it was located exclusively at carbon 3. A similar reduction of diethyl(3,3-dimethylbutylidene)malonate employing sodium borotritide gave [³H]-3 of high purity and specific activity.



When [³H]-3 is subjected to oxidation by BB dioxygenase, two radioactive products are obtained: tritium radioactivity is found in the water, and a tritium-labeled compound cochromatographing with authentic $\mathbf{6}$ is observed. For the estimation of the kinetic constants for the oxidation of [3H]-3, assay mixtures contained (at pH 7.0, duplicates for all) N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES, 80 μ mol), catalase (2.2 mg), ferrous sulfate (1.1 μ mol), ascorbate (11 μ mol), α -ketoglutarate (2.3 µmol), BB dioxygenase (0.3 mg, specific activity 2.0 µmol min⁻¹ mg⁻¹), and various concentrations of [³H]-3 in a total volume of 0.75 mL. All components except the labeled substrate were preincubated for 3 min at 37 °C, and the reactions were initiated by addition of [³H]-3. The remaining procedures were the same as in the standard enzyme assay (see Experimental Section), save that columns of Dowex 1X8-400 (OH⁻) were employed (instead of Dowex 50). Enzyme activity was calculated from the amount of tritium washed into the water. The K_M for 3 was found to be 0.4 mM, and V_{max} was calculated to be 0.43 nmol min⁻¹ mg⁻¹only 0.02% of the rate of oxidation of γ -butyrobetaine. When a correction is applied for the observed tritium kinetic isotope effect $[^{T}(V/K) = 4.9$; see below], the V_{max} is still only 0.1% of that for γ -butyrobetaine.²¹



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Tritium Kinetic Isotope Effect and Uncoupling in the Enzymatic Oxidation of 3. In the normal oxidation of 1 by bacterial BB hydroxylase, there is only a small degree of uncoupling of α -ketoglutarate oxidative decarboxylation from carbon hydroxylation; Holme et al.9 reported decarboxylation/hydroxylation ratios of 1.02 and 1.12 for unlabeled γ -butyrobetaine and [2,2,3,3,4,4- ${}^{2}H_{6}$]- γ -butyrobetaine, respectively. Furthermore, the primary tritium isotope effect for C-3 hydroxylation of 1 is small; using [methyl-14C,(3R)-3-3H]-\gamma-butyrobetaine, Blanchard and Englard calculated T(V/K) = 1.5 from the specific activity of the tritium released by hydroxylation and ${}^{T}(V/K) = 1.3$ from the ${}^{3}H/{}^{14}C$ ratio of the unreacted substrate.¹⁰ However, the extremely low rates of oxidation of the alternate substrate 3 observed in our tritium wash-out experiments prompted us to wonder if this reaction exhibits a substantial tritium isotope effect or significant uncoupling of decarboxylation and hydroxylation.

In fact, after enzymatic oxidations of [³H]-3 the amount of tritium radioactivity found in the water (which must originate as a C-3 hydrogen that was removed by hydroxylation) is approximately one-fifth of the amount of radioactivity found in product 6 [which represents the tritium α to the new hydroxy] (tritium on C-3)]. This immediately places a lower limit of about 5 for the value of the primary tritium kinetic isotope effect on the hydroxylation, ${}^{T}(V/K)$.²² The full value of ${}^{T}(V/K)$ cannot be determined unless the stereochemical course of the reaction is known, but the possible outcomes are easily summarized: (1) If hydrogen abstraction is stereospecific, then only intermolecular competition exists between isotopic species,23 and the radioactivity found in product 6 (which comes from the hydrogen not abstracted) may be used to calculate the fractional conversion of

(21) Two reviewers expressed skepticism with regard to the calculation of V_{max} for the unlabeled substrate from tritium wash-out data by using a correction for the observed V/K tritium isotope effect, $^{T}(V/K)$. They noted that the V_{max} isotope effect, ^{T}V , may not be measured with tracer levels of tritium but claimed that for this reason it is improper to apply a correction for $^{\mathsf{T}}(V/K)$ (which may be very different from $^{\mathsf{T}}V$) in the estimation of \mathcal{V}_{\max} for the unlabeled substrate. This conclusion is false; it is indeed $^{\mathsf{T}}(V/K)$ that must be used as the correction factor. In the competing enzymatic oxidations of the protium- and tritium-containing substrates, the observed rate of tritium wash-out, ν_T , is given by eq 1, where V_H , V_T , K_H , K_T , $[A_H]$, and $[A_T]$ are the V_{max} 's, K_M 's, and concentrations of the two substrates. Since tritium is used

$$= \frac{V_{T}[A_{H}]}{K_{T}(1 + [A_{H}]/K_{H}) + [A_{T}]} = \frac{(V_{T}/K_{T})[A_{T}]}{(V_{T}/K_{T})[A_{T}]} = (1)$$

at tracer concentrations, $[A_T]/[A_H]$ is very small, and $[A_T]/K_T \ll [A_H]/K_H$. (Remember that K_H and K_T will not differ by more than an order of magnitude, but $[A_T]$ is very much less than $[A_H]$ —in this particular case, 5 orders of magnitude.) Therefore

ν_T

$$\nu_{\rm T} = \frac{(V_{\rm T}/K_{\rm T})[{\rm A}_{\rm Y}]}{1 + [{\rm A}_{\rm H}]/K_{\rm H}} = \frac{(V_{\rm T}/K_{\rm T})K_{\rm H}[{\rm A}_{\rm T}]}{K_{\rm H} + [{\rm A}_{\rm H}]}$$
(2)

At $[A_T] \rightarrow \infty$ and $[A_H] \rightarrow \infty$ (in constant ratio, tritium is still only a tracer), and remembering that ${}^T(V/K) = (V_H/K_H)(K_T/V_T)$, we have

$$(\nu_{\rm T})_{\rm max} = \frac{(V_{\rm T}/K_{\rm T})K_{\rm H}[A_{\rm T}]}{[A_{\rm H}]} = V_{\rm H}(V_{\rm T}/K_{\rm T})(K_{\rm H}/V_{\rm H})[A_{\rm T}]/[A_{\rm H}] = \{V_{\rm H}/^{\rm T}(V/K)\}[A_{\rm T}]/[A_{\rm H}]$$
(3)

Thus $V_{\rm H}$ is related to the maximum rate of tritium wash-out by factors of $[A_T]/[A_H]$, which is known from the specific activity of the labeled substrate, and ${}^{T}(V,K)$ (not ${}^{T}V$) which is measured in a separate experiment. We thank Prof. W. W. Cleland for the essential details of this derivation. (22) The nomenclature of Northrop is used: Northrop, D. B. In *Isotope Effects on Enzyme Catalyzed Reactions*; Cleland, W. W., O'Leary, M., Narthero D. B. Edd.: University Back Brees, Boltimere 1972; p. 122

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(23) For a full discussion of the types and sensitivities of the measurements that may be made when there exists intermolecular competition between isotopic species, intramolecular competition, or both, see: Melander, L.; Saunders, W. H. Reaction Rates of Isotopic Molecules; John Wiley & Sons: New York, 1980.

⁽¹⁹⁾ In the competitive inhibition experiments, the K_i 's (with standard errors) were determined from least-squares fits of the data to the appropriate rate equation by means of Cleland's program COMP: Cleland, W. W. Methods Enzymol. 1979, 63, 103.

⁽²⁰⁾ Lindstedt, G.; Lindstedt, S.; Tofft, M. Biochemistry 1970, 9, 4336.



Figure 1. Time courses of the inactivation of γ -butyrobetaine hydroxylase by 3-(2,2-dimethylcyclopropyl)propanoic acid (4). Reaction conditions are given in the text. (Panel A) Inactivation of BB hydroxylase at various concentrations of 4: (+) 0 mM; (□) 0.2 mM; (■) 0.5 mM; (×) 2.0 mM. (Panel B) Effects of γ -butyrobetaine (1) and oxygen on inactivation of BB hydroxylase by 4: (+) no 4, aerobic; (×) 2.0 mM 4, 2.0 mM 1 aerobic; (I) 2.0 mM 4, aerobic; (\Box) 2.0 mM 4, anaerobic for 30 min, then air admitted. (Panel C) Effect of α -ketoglutarate on inactivation of BB hydroxylase by 4: (+) no 4, plus α -KG; (\square) 1.0 mM 4, minus α -KG; (\blacksquare) 1.0 mM 4, plus α -KG.

3 during the reaction. Under this assumption, the fractional conversions in two separate isotope effect experiments were calculated, and from a formula used by Blanchard and Englard, 10,24 T(V/K) = 4.9 ± 0.6. (2) If hydrogen abstraction is not stereospecific, then both intermolecular and intramolecular competitions exist, fractional conversions may not be calculated from the available data, and the true value of $^{T}(V/K)$ may be greater than or equal to, but not less than 4.9.

In order to estimate the degree of uncoupling, a duplicate set of enzymatic oxidations was performed, similar to those above but on 3 times the scale, containing [3H]-3 at a concentration of 2.0 mM. In these reactions, during the production of 101 ± 5 nmol of 6 (estimated by tritium wash-out corrected²¹ for an isotope effect of 4.9), 580 ± 20 nmol of succinate was produced (estimated by HPLC analysis; column C; 0.05 N sulfuric acid; detection at 214 nm). Thus the ratio of oxidative decarboxylation of α -ketoglutarate to hydroxylation of 3 was a relatively high 5.7. (See Webbie et al.⁸ for a recent discussion of the various factors that may influence the degree of uncoupling in α -keto acid dependent dioxygenases).

In summary, compound 3 is a very poor substrate for bacterial BB hydroxylase. Even with a correction for the observed tritium isotope effect,²¹ the rate of hydroxylation of 3 is no more than 0.1% of the rate for 1. Furthermore, in the turnover of compound 3, less than 20% of the α -ketoglutarate oxidative decarboxylation events lead to hydroxylation, in contrast to the efficient, fully coupled hydroxylation of 1. The difference in the processing of these two substrates is especially dramatic given their nearly identical shape and similar affinity for the active site of BB hydroxylase.

Mechanism-Based Inhibition of BB Hydroxylase by 3-(2,2-Dimethylcyclopropyl)propanoic Acid (4). As discussed in the introduction, it is likely that the hydroxylation event catalyzed by BB dioxygenase begins with abstraction of the 3R-hydrogen atom from γ -butyrobetaine to give a carbon radical. We wished to test this hypothesis by examining the enzymatic processing of substrate analogues that contained a "free radical clock" functional group.²⁵ The obvious choice for this experiment is the organic acid 4; it is very similar in structure to substrate 3 but incorporates a dimethylcyclopropyl group α to the site of hydroxylation. We note that the (2,2-dimethylcyclopropyl)carbinyl radical formed upon hydrogen abstraction from 4 would open to the homoallyl radical much more rapidly-perhaps 10000 times faster²⁶-than

would a simple cyclopropylcarbinyl radical $(1.3 \times 10^8 \text{ s}^{-1})$,² so that any processing of compound 4 by BB hydroxylase should lead to the formation of open-chain products if C-3 radical intermediates are involved.



Compound 4 proved to be a good competitive inhibitor of BB hydroxylase, with a K_i of 0.22 \pm 0.05 m \dot{M} .¹⁹ If 4 does undergo fragmentation upon oxidation by BB hydroxylase, then the product radicals might react with and inactivate the enzyme. In assays of time-dependent inactivation of BB hydroxylase by this analogue, clear time-dependent inactivation of the enzyme was observed.

Figure 1 illustrates these data. Panel A shows the dependence of the rate of the pseudo-first-order inactivation process on inhibitor concentration. The K_i for 4 was estimated to be 0.9 mM, and extrapolation of the observed inactivation rates to infinite inhibitor concentration gave $k_{\text{inact}} = 0.08 \text{ min}^{-1}$. Panel B shows that BB hydroxylase is almost completely protected from inactivation by 4 by addition of the normal substrate 1 and that the inactivation process is oxygen dependent. Panel C shows that the inactivation process is also dependent on α -ketoglutarate, which provides extremely strong evidence that the inactivation requires enzymatic processing of the inhibitor.²⁷ These data satisfy the usual kinetic criteria for mechanism-based inhibition of an enzyme.28,29

We have not chemically characterized a product of this reaction, nor is it known if the inhibition results from covalent modification of the enzyme. However, the finding of time-dependent inactivation of BB hydroxylase by compound 4 is consistent with the

⁽²⁴⁾ The formula used is $^{T}(V/K) = \log (1.0 - f) / [\log (1.0 - fR_{f}/R_{0})]$ where f is the fractional conversion of 3 to 6 and R_f and R_0 are the ratios of tritium in the water to tritium in product 6 at fractional conversion f and complete conversion, respectively.

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(26) Chatgilialoglu, C.; Ingold, K. U.; Tse-Sheepy, I.; Warkentin, J. Can. J. Chem. 1983, 61, 1077.

⁽²⁷⁾ We note that in Figure 1 the time courses for experimental runs that contain inactivator 4 do not extrapolate to precisely 100% activity at zero time. This is due to the fact that inactivator 4 is also a competitive inhibitor of BB hydroxylase ($K_i = 0.22 \text{ mM}$) and that the dilution of each aliquot of the preincubation mixture into the normal assay mixture was typically only 10-20-fold. Thus the assay of an aliquot of a preincubation mixture that contained 2.0 mM 4 would contain 0.1-0.2 mM 4, which would somehwat slow the normal assay due to ordinary competitive inhibition. (28) Rando, R. R. Science 1974, 185, 320.

⁽²⁹⁾ Walsh, C. Tetrahedron 1982, 38, 871.

concept that a C-3 carbon radical is an intermediate in the hydroxylation reactions catalyzed by this enzyme, and it suggests that cyclopropane-bearing substrate analogues, which have often been used as mechanistic probes and inhibitors in enzymology,¹⁵ may be useful as inhibitors of other α -keto acid dependent dioxygenases.

Experimental Section

General. Melting point determinations, ultraviolet-visible absorption spectroscopy, ¹H and ¹³C nuclear magnetic resonance (NMR) spectrometry, low- and high-resolution mass spectral analyses, and coupled gas chromatography-mass spectrometry (GC-MS) were performed as previously described.³⁰ Simple gas chromatography (GC) was carried out on packed columns as previously described³⁰ or on capillary columns [25 m × 0.53 mm, BP1 stationary phase (Scientific Glass Engineering, Inc.)] on a Hewlett-Packard 5890A chromatograph with a flame ionization detector. High-performance liquid chromatography (HPLC) was performed on a Waters Associates chromatograph using Whatman Partisil ODS 2 (column A) or Whatman Partisil C8 (column B) columns $(25 \times 0.46 \text{ cm})$ for reverse-phase chromatography, or Bio-Rad HPX-87H (columns C) for ion-exchange chromatography. Thin-layer chromatography (TLC) was performed on plates of silica gel GF (2.5 × 10 cm, 0.1-mm thickness). Components were located by observation of the plates under UV light or by treating the plates with a molybdic acid reagent followed by heating. Thin-layer radiochromatography (radio-TLC) was performed similarly, but the plates were cut into 0.5-cm sections which were assayed for radioactivity by liquid scintillation counting. Liquid scintillation counting was performed on a Beckman LS5801 instrument; the scintillation cocktail was a solution of toluene and ethanol (2:1) containing 0.8% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-[2-(5-phenyloxazolyl)]benzene (POPOP). γ -Butyrobetaine (1) was purchased from Aldrich Chemical Co. or synthesized as described previously.³¹ 5,5-Dimethylhexanoic acid (3) [mp 37-38 °C (lit.³² 39 °C)] was prepared by the method of Gutman and Hickinbottom.³²

[2,3-3H]-y-Butyrobetaine ([3H]-1). y-Aminobutyric acid (92.1 mg, 0.894 mmol) and γ -amino[2,3-³H]butyric acid (0.68 µg in 0.25 mL of 0.01 N HCl, 0.25 mCi, New England Nuclear) were added to a suspension of barium hydroxide octahydrate (1.18 g, 7.45 mequiv) in water (3 mL). Methyl iodide (1.06 g, 7.45 mmol) was added and brought into solution with the addition of methanol (10 mL). The reaction flask was tightly stoppered and the solution stirred overnight at room temperature. Sulfuric acid (1.24 mL of a 6 N solution in ethanol, 7.45 mequiv) was added and the barium sulfate removed by centrifugation. The supernatant liquid was neutralized and filtered through a short column of Dowex 1-X8 (OH-) to remove any anionic species. The filtrate was concentrated by rotary evaporation. The residue was dissolved in water (2 mL), the pH was adjusted to neutrality, and the solution was applied to a column of Dowex 50-X8 (H⁺) resin (50-100 mesh, 1.2×25 cm). The column was washed with water (2 volumes) and $[2,3-^{3}H]-\gamma$ -butyrobetaine was eluted with 1 N HCl (3 volumes). The volatiles were removed by thorough rotary evaporation and drying under vacuum over phosphorus pentoxide. The residue (66.3 mg, 41% chemical yield) was dissolved in water (50.0 mL) and divided into 2-mL aliquots for storage at -20 °C. The specific radioactivity of this material was 1.01×10^9 dpm/mmol, and the radiopurity was judged to be >95% by radio-TLC (9:1:1 methanolacetone-concentrated HCl).

Diethyl (3,3-Diethylbutylidene)malonate (5). A mixture of diethyl malonate (3.04 g, 19.0 mmol), 3,3-dimethylbutanal³³ (2.80 g, 28.0 mmol), acetic anhydride (12.2 g, 12.0 mmol), and zinc chloride (0.410 g, 3.04 mmol) was heated under argon at a gentle reflux for 48 h. The cooled mixture was diluted with benzene (75 mL), washed with water (4×) and brine, and concentrated by rotary evaporation. Purification by column chromatrography (silica gel, 19:1 hexanes-ether) yielded the desired product (3.42 g, 74%) as a colorless oil: ¹H NMR (CDCl₃) δ 0.96 (s, 9 H), 1.29 (t, J = 7 Hz, 3 H), 1.33 (t, J = 7 Hz, 3 H), 2.19 (d, J = 8 Hz, 2 H), 4.20 (q, J = 7 Hz, 2 H), 4.26 (q, J = 7 Hz, 2 H), 7.16 (t, J = 8 Hz, 1 H).

5,5-Dimethyl[3-2H]hexanoic Acid ([2H]-3). Sodium borodeuteride (21.6 mg, 0.516 mmol) was added in one portion to a solution of diethyl (3,3-dimethylbutylidene)malonate (118 mg, 0.488 mmol) in absolute ethanol (2 mL), which was cooled in an ice bath and protected with a drying tube. The ice bath was removed, and stirring was continued for 6 h. Acetone (0.5 mL) was added, and the volatiles were removed by rotary evaporation. The reaction mixture was taken up in ether, washed

with water, and concentrated by rotary evaporation. The residue was heated in a mixture of 1:1 acetic acid-concentrated hydrochloric acid (2 mL) at reflux for 16 h. The cooled reaction mixture was poured into brine and thoroughly extracted with ether. The combined ether extracts were dried (Na₂SO₄) and concentrated to yield the crude product (57 mg). Purification by column chromatography (silica gel, 0.5:10:90 AcOH-ether-hexanes) afforded the desired [²H]-3 as a crystalline solid (27 mg, 38%): mp 37-38 °C [lit.32 39 °C (unlabeled)]; 1H NMR (CD-Cl₃) δ 0.89 (s, 9 H), 1.20 (d, J = 7 Hz, 2 H), 1.59 (m, 1 H), 2.31 (d, J = 7 Hz, 2 H).

5,5-Dimethyl[3-3H]hexanoic Acid ([3H]-3). Diethyl (3,3-dimethylbutylidene)malonate (9.4 mg, 39 μ mol) in absolute ethanol (2 mL) was added via syringe to a vial containing sodium borotritide (2 mg, 44 μ mol, 100 mCi/mmol), and the reaction mixture was stirred for 6 h. Acetone (2 mL) was added, and stirring was continued for 15 min. The volatiles were removed by evaporation at room temperature with the aid of a vacuum pump. To the residue were added acetic acid (0.5 mL) and concentrated hydrochloric acid (0.5 mL), and the mixture was heated at a gentle reflux for 16 h. The cooled reaction mixture was diluted with water, and the organics were extracted into ether. The combined ether extracts were washed with brine and dried (MgSO₄). The ether was removed by vacuum pump at room temperature. Unlabeled 5,5-dimethylhexanoic acid (53.2 mg, 0.369 mmol) was added to the residue, and this material was recrystallized three times from pentane at -60 °C The remaining crystals (48.0 mg, 0.333 mmol) were transferred to a 50-mL volumetric flask and diluted to volume with ethanol for storage. The total radioactivity was 1.84 mCi (specific radioactivity, 5.53 mCi/ mmol). A portion of the labeled acid (2.1 mg) was further purified by preparative TLC (silica gel GF, 200:1 ether-acetic acid). The purified acid (1.73 mg) was stored in ethanol solution. The specific radioactivity of this material was 5.27 mCi/mmol. The purity of this compound was judged to be >99% by GC analysis (BP1 capillary, 60-150 °C, 5 °C/ min) of the methyl ester derivative, and the radiopurity was judged to be >98% by radio-TLC (200:1 ether-acetic acid).

5,5-Dimethyl-3-hydroxyhexanoic Acid (6). Acetic acid (600 mg, 10.0 mmol) was added dropwise via syringe to a solution of lithium diisopropylamide (20 mmol) in THF (50 mL) at -78 °C. The cooling bath was removed, and the reaction was stirred for 1 h, warmed to 45 °C, and stirred for 30 min. The mixture was cooled to -78 °C and 3,3-dimethylbutanal (1.00 g, 10.0 mmol) was added dropwise via syringe. The cooling bath was removed, and stirring was continued for 1 h. The reaction mixture was cooled in an ice bath, and cold water (50 mL) was added slowly with stirring. The layers were separated and the organic phase was extracted once more with water. The aqueous extracts were combined, washed with ether, cooled in and ice bath, carefully acidified to pH 2 with 3 N HCl, and extracted thoroughly with ether. The combined ether extracts were washed with water and brine, and they were dried over Na2SO4. The volatiles were removed by rotary evaporation to yield 1.3 g of crude compound 6. This material was recrystallized from acetonitrile at -20 °C to yield the desired β -hydroxy acid (1.10 g, 69%): mp 110-111 °C; ¹H NMR (CDCl₃) δ 0.97 (s, 9 H), 1.39 (m, 2 H), 2.41 (d, J = 7 Hz, 2 H), 3.78 (br s, 1 H), 4.18 (m, 1 H); ¹³C[¹H] NMR $(acetone-d_6) \delta 30.5, 42.5, 44.3, 51.0, 66.5, 173.7; MS, m/z 160 (M⁺, 0.2),$ 142 (3), 127 (82), 109 (40), 101 (36), 89 (100). The purity of this compound was judged to be >98% by HPLC (column A, 55:45 to 100:0 acetonitrile-water gradient) and GC (3% OV-17, 195 °C) analyses of the phenacyl ester.

3-(2,2-Dimethylcyclopropyl)propanoic Acid (4). A stirred mixture of iodine (0.635 g, 2.50 mmol), methylene iodide (6.65 g, 2.50 mmol), zinc-copper couple (2.51 g, 39 mmol), and methyl 5-methyl-4-hexenoate (1.75 g, 12.5 mmol) in diethyl ether (1 mL) was heated at reflux under argon for 60 h. The cooled mixture was diluted with ether (20 mL) and filtered through a glass frit. The filtrate was washed successively with cold 1 N HCl, 1 N NaHCO₃, water, and brine, and it was dried over MgSO4. The volatiles were removed by rotary evaporation, and the crude product was purified by column chromatography (silica gel, solvent 17:3 hexane-ether). The fractions containing the desired cyclopropane were combined and concentrated, and the ester was hydrolyzed by gentle reflux in aqueous ethanolic K₂CO₃ (4 g, 35 mmol) for 2 h. The usual aqueous workup yielded 1.48 g (85%) of compound 4 as a colorless oil: ¹H NMR (CDCl₃) δ -0.09 (t, J = 7 Hz, 1 H), 0.38 (m, 1 H), 0.49 (m, 1 H), 1.00 (s, 3 H), 1.02 (s, 3 H), 1.54–1.76 (m, 2 H), 2.40 (t, J = 7 Hz, 2 H), 9.6 (br s, 1 H); MS, m/z 142 (M⁺, 25), 124 (68), 109 (44), 96 (68), 82 (100); exact mass 142.0993, calcd for $C_8H_{14}O_2$ 142.0994. The purity of this material was judged to be 90% by GC analysis (BP1 capillary, 60-150 °C, 5 °C/min) of its methyl ester derivative.

Enzymes and Routine Assay. Catalase (bovine liver) was purchased from Sigma Chemical Co. BB hydroxylase was purified from *Pseudo-monas* sp. AK1 by the method of Lindstedt et al.² Our preparations were typically 90% pure as judged by sodium dodecyl sulfate-polyacrylamide

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gel electrophoresis (SDS-PAGE); the gels showed no major impurities, only a multitude of very minor impurity bands. Routine assays were conducted by monitoring tritium wash-out from $[2,3^{-3}H]-\gamma$ -butyrobetaine by a modification of the method of Englard et al.³⁴ Assay mixtures (pH 7.0) contained an aliquot of enzyme solution, 0.5 μ mol of [2,3-³H]- γ butyrobetaine, 0.75 μ mol of α -ketoglutarate, 4.3 μ mol of ascorbate, 0.5 μ mol of ferrous sulfate, 1 mg of catalase, and 12.5 μ mol of potassium phosphate in a final volume of 0.25 mL. All assay components except the radiolabeled substrate were mixed in open test tubes (13 × 100 mm) and preincubated for 3 min at 37 °C with rotary shaking (160 rpm). The substrate was added, and the incubation was continued for 45 min. The reaction was terminated by addition of 2,2'-bipyridyl (50 µL of a 0.5 M ethanolic solution). The mixture was applied to a small column (1-mL total volume) of Dowex 50X8-400 (H⁺), and the column was eluted with 1.5 mL of water. Aliquots of the effluent were then analyzed for tritium radioactivity. In determination of the enzyme activity, two correction factors must be applied to account for the fact that (a) only one-fourth of the tritium in the labeled substrate is in the 3R position, which is the site of hydroxylation, and (b) there is a small primary tritium kinetic isotope effect for the hydroxylation, ${}^{T}(V/K) = 1.5.{}^{10}$ Under these conditions our most active preparations of BB hydroxylase showed a specific activity of 6.1 µmol min⁻¹ mg⁻¹. More typically, the specific activity was about 2 μ mol min⁻¹ mg⁻¹, but there was no difference in the purity of the various preparations as judged by SDS-PAGE.

Reversible Inhibition Studies. For the determination of reversible inhibition of BB hydroxylase by substrate analogues, a series of routine assays were performed that contained various concentrations of [2,3-³H]- γ -butyrobetaine and various concentrations of the potential inhibitor.

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For these assays, all components except the substrate and inhibitor were mixed and preincubated as described above, and then the inhibitor was added, followed immediately by the substrate to initiate the reaction. In this way the inhibitors could have no effect on the activation of the enzyme during the preincubation period. The remainder of the assay procedures were as above, and the inhibition data were analyzed in the form of Lineweaver-Burk plots.¹⁹

Time-Dependent Inactivation Studies. For the determination of time-dependent inactivation of BB dioxygenase by substrate analogues, the following procedure was used. A series of reaction mixtures were prepared, each of which contained (at pH 7.0) 100 µg of BB dioxygenase, 1.2 μ mol of α -ketoglutarate, 4.5 μ mol of ascorbate, 0.6 μ mol of ferrous sulfate, 1 mg of catalase, and 50 µmol of potassium phosphate in a final volume of 0.28 mL. After preincubation for 3 min at 37 °C with rotary shaking (160 rpm), 20-µL aliquots of inhibitor stock solutions of appropriate concentrations were added. The incubations were continued, and at 10-min intervals aliquots were taken from the mixtures for the assay of remaining enzyme activity by using the standard routine assay procedure.

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Registry No. [3H]-1, 124201-55-4; 3, 24499-80-7; [2H]-3, 124201-56-5; [³H]-3, 124201-57-6; 4, 124201-52-1; 5, 124201-53-2; 6, 124201-54-3; D₂, 7782-39-0; T₂, 10028-17-8; γ-amino[2,3-³H]butyric acid, 13048-68-5; diethyl malonate, 105-53-3; 3,3-dimethylbutanal, 2987-16-8; methyl 5-methyl-4-hexenoate, 35901-76-9; BB hydroxylase, 9045-31-2.

Man-Designed Bleomycin with Altered Sequence Specificity in DNA Cleavage[†]

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Abstract: The synthetic approach to the concerted antitumor mechanism of bleomycin is studied by introducing a dynamic change into the O₂-activation moiety and DNA-binding site. A model PYML(6)-bleomycin previously reported, possessing an oxygen-activating methoxypyridine moiety and a DNA-binding bithiazole moiety, exhibits a nucleotide cleavage mode virtually identical with that of bleomycin. Herein reported is a newly designed bleomycin analogue, PYML(6)-(4R-APA)-distamycin, wherein the 4-methoxypyridine moiety and a DNA-binding distamycin component are connected through an (R)-4-aminopentanoic acid linker moiety. Synthesis of PYML(6)-(4R-APA)-distamycin is carried out by condensation of the hydroxyhistidine-pentanoic acid fragment with the methoxypyridine moiety, followed by introduction of the distamycin moiety. PYML(6)-(4R-APA)-distamycin cleaves a G4 phage DNA fragment (100 base pairs) at 1 µM concentration in the presence of Fe(II), oxygen, and dithiothreitol and induces dramatically altered adenine/thymine specificity. It is indicated that the specific recognition of base sequences for the cleavage is mainly controlled by the DNA affinity site and that the (R)-4-aminopentanoic acid linker seems to determine the proper arrangement of the iron-oxygen site and the distamycin moiety on DNA.

Bleomycins (BLMs) are chemotherapeutic agents used for the clinical treatment of Hodgkin's lymphoma, carcinomas of the skin, head, and neck, and tumors of the testis.¹ The drug was isolated from Streptomyces verticillus as a copper chelate by Umezawa

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and his co-workers in 1966, and the structure was shown to be a glycopeptide consisting of an unusual hexapeptide and a disaccharide.² In addition to its clinical usefulness, BLM attracts current interest because of its unique biochemical functions. It has been well documented that BLM cleaves DNA preferentially at G-C (5' \rightarrow 3') and G-T (5' \rightarrow 3') sequences in the presence of oxygen and ferrous ion.³ The biochemical capability of BLM

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