n-hexane/dichloromethane (1:1). The first fraction yields after evaporation of the solvent 180 mg (63%) of colorless **3**. Recrystallization from methanol affords colorless needles, mp 148-149 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.34 and 5.73 (AA'BB', N^{21} = 5.9 Hz, 8 H), 3.64 and 2.05 (AX, ^2J = -12.2 Hz, 4 H), 1.87 (s, 12 H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 143.25 (s, C-1,6,8,13), 138.88 (s, C-7,14), 127.67 (d, C-3,4,10,11), 125.57 (s, C-17,18), 121.98 (d, C-2,5,9,12), 38.74 (t, C-15,16), 21.43 (q, C-19,20,21,22). MS (70 eV): *m/z* 288 (M^+, 98%), 273 (9), 258 (10), 115 (100). IR (KBr): 1607 cm^{-1} (C=C). UV/VIS (dioxane): λ_{max} = 230 nm (ϵ 28 800), 258 (8000), 287 (6300). Anal. Calcd for $C_{22}H_{24}$ (288.4): C, 91.61; H, 8.39. Found: C, 91.82; H, 8.26.

7,14-Diisopropylidene-7,14-dihydro-*anti*-1,6:8,13-bismethano[14]annulene (4). To a solution of 236 mg (1 mmol) of the anti-diketone **7** in 100 mL of dry THF are added 5 mmol of isopropylmagnesium bromide in 3 mL of ether. After being stirred for 2 h at room temperature the mixture is hydrolized with 10 mL of H_2O under ice-cooling. The mixture is poured into dilute H_2SO_4 (ice-cooling) prepared from 10 mL of H_2SO_4 and 20 mL of H_2O, and stirring is continued for 0.5 h. The solution is extracted three times with 50 mL of ether. After washing (water, sodiumbicarbonate solution) the etheral solution is dried ($MgSO_4$). After evaporation of the solvent, the residue is dissolved in *n*-hexane/dichloromethane (1:1) and chromatographed on silica gel (15/3 cm). The first fraction yields 70 mg (24%) of yellow **4**. Recrystallization from methanol yielded yellow crystals, mp 192-193 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.52 and 6.15 (AA'BB', N^{21} = 6.2 Hz, 8 H), 2.75 and 1.92 (AX, ^2J = -12.6 Hz, 4 H), 2.05 (s, 12 H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 139.99 (s, C-7,14), 138.95 (s, C-1,6,8,13), 131.25 (s, C-17,18), 129.31 (d, C-3,4,10,11), 127.43 (d, C-2,5,9,12), 35.32 (t, C-15,16), 24.89 (q, C-19,20,21,22). MS (70 eV): *m/z* 288 (M^+, 18%), 273 (1), 258 (3), 91 (100). IR (KBr): 1599 cm^{-1} (C=C). UV/VIS (dioxane): λ_{max} = 228 nm (ϵ 33 100), 265 (10 100), 325 (12 300). Anal. Calcd for $C_{22}H_{24}$ (288.4): C, 91.61; H, 8.39. Found: C, 91.74; H, 8.36.

Acknowledgment. We are grateful to the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the BASF Aktiengesellschaft in Ludwigshafen for financial support. Thanks are due to Alexander Flatow for recording the PE spectra.

Supplementary Material Available: Tables of atomic coordinates with standard deviations, bond lengths, bond angles, and torsional angles of **6** and **7** (10 pages). Ordering information is given on any current masthead page.

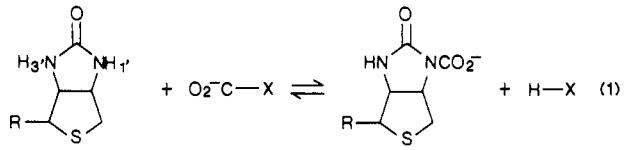
Proton Exchange in Biotin: A Reinvestigation, with Implications for the Mechanism of CO_2 Transfer

Charles L. Perrin* and Tammy J. Dwyer

Contribution from the Department of Chemistry, D-006, University of California, San Diego, La Jolla, California 92093. *Received February 9, 1987*

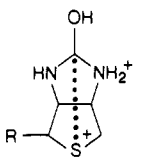
Abstract: Kinetics of proton exchange of biotin and its methyl ester were studied in aqueous buffers. Rate constants were determined by NMR saturation-transfer or line-broadening methods. The reaction was observed to be simply first order in H^+ or OH^-, in disagreement with a previous study (Fry, Fox, Lane, Mildvan *J. Am. Chem. Soc.* **1985**, *107*, 7659). The mechanisms of proton exchange and the implications for the mechanism of biotin-mediated CO_2 transfer are discussed.

The mechanisms of CO_2 metabolism mediated by biotin (**1**) continue to generate interest.[1] The general reaction is of the form of eq 1 or its reverse, where X = $CH_2CO_2^-$ or $CH(CH_3)COSCoA$.



1: R = $HOCO(CH_2)_4$

Recent results[2] show that X = OPO_3H^- also fits this equation, rather than alternatives involving phosphorylated biotin. Many questions have arisen over the details of the C-X and H-X bond-breaking/making steps: What is the timing of those steps? Might the base that removes the proton of HX be biotin?[3] Might that base be the conjugate base of biotin, formed by removal of the 3' proton?[4] Although recent results[5] show that C-X and H-X bond breaking/making occur stepwise, rather than concerted,[3] similar questions may be raised regarding the breaking of the N-H bond and the formation of the N-C bond. In an attempt to answer these questions, Fry, Fox, Lane, and Mildvan[6] (FFL&M) have studied the kinetics of NH proton exchange in biotin and some of its analogues. All of these show a normal base-catalyzed exchange, first order in OH^-. Most of them also show a normal acid-catalyzed exchange, first order in H^+. However, biotin itself and its methyl ester were found to undergo an exchange of the 1'NH that is second order in H^+ (Figure 1), even though exchange of the 3'NH is normal. They attributed this exchange to a transannular interaction with the sulfur, which stabilizes the diprotonated intermediate (**2**: R = $HOCO(CH_2)_4$ or $CH_3OCO(CH_2)_4$). However, X-ray and NMR studies[7] on complexes of



2

biotin with Lewis acids show no evidence for such interaction. Also, kinetic isotope effects[5c] are inconsistent with transannular C-S bonding. Since the second-order exchange (as well as intermediate **2**, plausible only in superacid[8]) is so unprecedented,

(1) Attwood, P. V.; Keech, D. B. *Curr. Top. Cell. Regul.* **1984**, *23*, 1. Dugas, H.; Penney, C. *Bioorganic Chemistry: A Chemical Approach to Enzyme Action*; Springer: New York, 1981; pp 458-477. Wood, H. G.; Barden, R. E. *Annu. Rev. Biochem.* **1977**, *46*, 385.

(2) Hansen, D. E.; Knowles, J. R. *J. Am. Chem. Soc.* **1985**, *107*, 8304.

(3) Retey, J.; Lynen, F. *Biochem. Z.* **1965**, *342*, 256. Arigoni, D.; Lynen, F.; Retey, J. *Helv. Chim. Acta* **1966**, *49*, 311.

(4) Fung, C. H.; Gupta, R. K.; Mildvan, A. S. *Biochemistry* **1976**, *15*, 85. Goodall, G. J.; Prager, R.; Wallace, J. C.; Keech, D. B. *FEBS Lett.* **1983**, *163*, 6.

(5) (a) Stubbe, J.; Fish, S.; Abeles, R. H. *J. Biol. Chem.* **1980**, *255*, 236. (b) O'Keefe, S. J.; Knowles, J. R. *J. Am. Chem. Soc.* **1986**, *108*, 328. O'Keefe, S. J.; Knowles, J. R. *Biochemistry* **1986**, *25*, 6077. (c) Attwood, P. V.; Tipton, P. A.; Cleland, W. W. *Ibid.* **1986**, *25*, 8197.

(6) Fry, D. C.; Fox, T. L.; Lane, M. D.; Mildvan, A. S. *J. Am. Chem. Soc.* **1985**, *107*, 7659.

(7) Berkessel, A.; Breslow, R. *Bioorg. Chem.* **1986**, *14*, 249.

0002-7863/87/1509-5163$01.50/0 © 1987 American Chemical Society

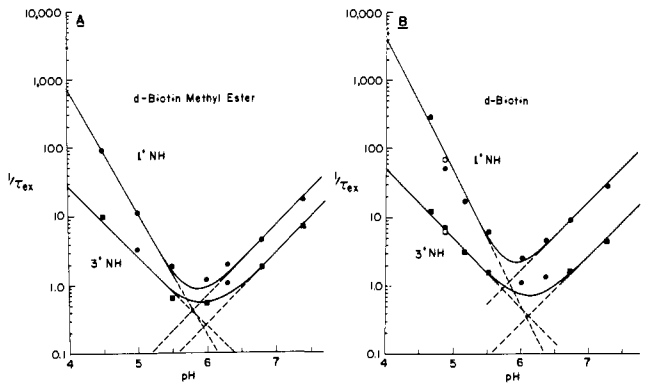


Figure 1. Rate constants for proton exchange in biotin methyl ester and biotin, reprinted from ref 6.

we have reinvestigated the kinetics of proton exchange. We now report that exchange is simply first order in H^+ and that there are no unusual features to proton exchange in biotin.

Experimental Section

Materials. *d*-Biotin and its methyl ester were purchased from Sigma and used without further purification. Imidazole was recrystallized twice from benzene. Otherwise reagent chemicals were used. Samples were prepared to contain 1–10 mM biotin (depending on solubility) plus 0.1 M KCl in 25 mM imidazole buffer (pH >6) or 10, 15, or 20 mM acetate buffer (pH <6). The sample pH was adjusted with HCl or NaOH, and after the NMR measurements the pH stability was verified with a pH microelectrode that fits into the NMR tube.

NMR Methods. Proton NMR spectra were obtained with an Oxford 360-MHz magnet interfaced to a Nicolet 1180E computer and pulse programmer. The probe temperature was 22 °C. Suppression of the water signal was achieved with a 21412-pulse sequence.[9] Suitable signal to noise was obtained with 36 to 360 acquisitions (36 acquisitions × 10 experiments, separately inspected for anomalous line shapes) and a 2500- or 4000-Hz sweep width. The upfield signal at δ 6.40 (δ 6.54 at 0 °C) has been assigned[6,10] as the 1'NH.

Between pH 4.9 and 7.6 rate constants were measured by a saturation-transfer technique. The extent of saturation transfer, $t_{NH}(OH)$, is given by eq 2, where $I_{NH}(OH)$ is the steady-state intensity of either NH peak under conditions of selective saturation of the OH peak, and I_{NH}^0

$$t_{NH}(OH) = [I_{NH}^0 - I_{NH}(OH)]/I_{NH}^0 \quad (2)$$

is the equilibrium intensity of the NH peak without saturation. Experimentally, $t_{NH}(OH)$ approaches 1 under conditions of fast exchange. The rate constant is given in eq 3, where $T_{1,NH}^{app}$ is the apparent spin–lattice

$$k_{obsd} = t_{NH}(OH)/T_{1,NH}^{app} \quad (3)$$

relaxation time of the NH protons. This time was measured by an inversion-recovery method, using 21412 pulses not only for the 90° observation pulse but also for the 180° inversion pulse. This method selectively inverts both NH magnetizations, but not the OH. Then, owing to the 10^4-fold excess of OH over NH, the reverse saturation transfer, from NH to OH, is always negligible, even at fast exchange, so that the OH magnetization remains at equilibrium. Each NH relaxation is consequently a single exponential, with rate constant $1/T_{1,NH}^{app} = k_{obsd} + 1/T_{1,NH}^0$, where $T_{1,NH}^0$ is the inherent spin–lattice relaxation time of an NH proton. Equation 3 then readily follows from the differential equations describing the magnetization.[11]

Below pH 4.9 and above pH 7.6 rate constants were measured by line broadening. The rate constant is given by eq 4,[12] where $\delta\nu_{1/2}$ is an NH peak width at half height (evaluated with a computer program that could

$$k_{obsd} = \pi(\delta\nu_{1/2} - \delta\nu_{1/2}^0) \quad (4)$$

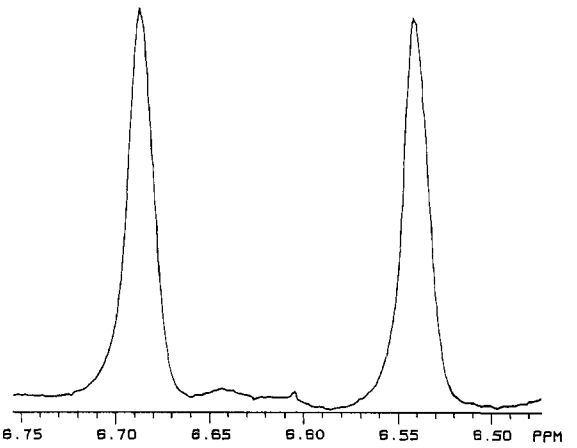


Figure 2. NH singlets of *d*-biotin at 0 °C and pH 6.07. Peak width is 5.4 Hz.

Table I. Rate Constants for Base- and Acid-Catalyzed Proton Exchange in *d*-Biotin and Its Methyl Ester

	$10^{-6}k$(1'NH), M^{-1} s^{-1}	$10^{-6}k$(3'NH), M^{-1} s^{-1}
biotin + OH^-	63 ± 11	11 ± 2
biotin + H^+	1.3 ± 0.2	0.4 ± 0.04
ester + H^+	0.84 ± 0.03	0.36 ± 0.02

fit the overlapping peaks to Lorentzians) and $\delta\nu_{1/2}^0$ is 7.0 Hz, the inherent width of the 3'NH at 22 °C and pH 6.2, where exchange broadening contributes only 0.14 Hz.

Rate constants usually could be determined with a precision of ±5%. However, precision varies, since peaks begin to overlap and broaden. In the two cases (k_{obsd} ca. 15 s^{-1}) where both saturation transfer and line broadening could be used, agreement between the two methods is within the precision (±20%) of the T_1 measurements.

Near pH 6.2, and down to 0 °C, both the 1'NH and the 3'NH are singlets. Figure 2 shows these peaks. This agrees with previous observations on biotin in $CDCl_3$[13] and in superacid,[8] but it is in disagreement with the 8.6-Hz doublet observed for the 1'NH by FFL&M.[6] We are unable to account for this discrepancy, but so large a coupling constant is unprecedented for the HCNH dihedral angle in biotin. Indeed, imidazolone shows a CH_2 triplet with coupling constant 0.5 Hz,[13] which is due to fortuitously equal couplings to both NH.[14]

Results

Dilute solutions of biotin, at pH near neutrality, are not well buffered. To avoid problems of pH instability, kinetic measurements were performed on buffered solutions. The observed rate constants of NH proton exchange do depend slightly on the concentration of acetate buffer, but not on the concentration of imidazole buffer. Such general-acid catalysis, without general-base catalysis, has also been observed[14] for many other amides. It is possible to subtract the general-acid catalysis by extrapolating to zero buffer concentration. This extrapolation is only a small correction. It is also proper to subtract the contribution to k_{obsd} due to general-acid catalysis by biotin itself, since this cannot be relevant to any enzymatic reaction. We assume that the carboxylic acid form of biotin (pK_a taken as 4.82, that of valeric acid[15]) has the same catalytic rate constant as does acetic acid. This is 1.2 × 10^3 M^{-1} s^{-1} for the 1'NH and 1.5 × 10^2 M^{-1} s^{-1} for the 3'NH, obtained as the slope of the plot of k_{obsd} vs. the concentration of acetic acid. This correction too is small.

The corrected rate constants k are plotted vs. pH in Figure 3. They are well fitted to eq 5 by a nonlinear least-squares procedure. Values of k_{H^+} and k_{OH^-} are listed in Table I. If a term $k_{H^+}'[H^+]^2$

$$\log k = \log (k_{H^+}[H^+] + 0.8 \times 10^{-14}k_{OH^-}/[H^+]) \quad (5)$$

is included in eq 5, the coefficient k_{H^+}' is not significantly different

(8) Olah, G. A.; White, A. M. *J. Am. Chem. Soc.* **1968**, *90*, 6087.
(9) Redfield, A. G.; Kunz, S. D.; Ralph, E. K. *J. Magn. Reson.* **1975**, *19*, 114. Wright, J. M.; Feigon, J.; Denny, W.; Leupin, W.; Kearns, D. R. *Ibid.* **1981**, *45*, 514.
(10) Abe, A.; Nee, M.; Roberts, J. D.; Wittenberg, W. L.; Leonard, N. J. *J. Am. Chem. Soc.* **1981**, *103*, 4954.
(11) Krishna, N. R.; Huang, D. H.; Glickson, J. D.; Rowan, R., III; Walter, R. *Biophys. J.* **1979**, *26*, 345. Lambert, J. B.; Nienhuis, R. J.; Keepers, J. W. *Angew. Chem., Intl. Ed. Engl.* **1981**, *20*, 487.
(12) Johnson, C. S., Jr. *Adv. Magn. Reson.* **1965**, *1*, 33.
(13) Glasel, J. A. *Biochemistry* **1966**, *5*, 1851.
(14) Wang, W. Ph.D. Thesis, University of California, San Diego, 1984.
(15) Jencks, W. P.; Regenstein, J. *Handbook of Biochemistry*, 2nd ed.; CRC Press: Cleveland, 1970.

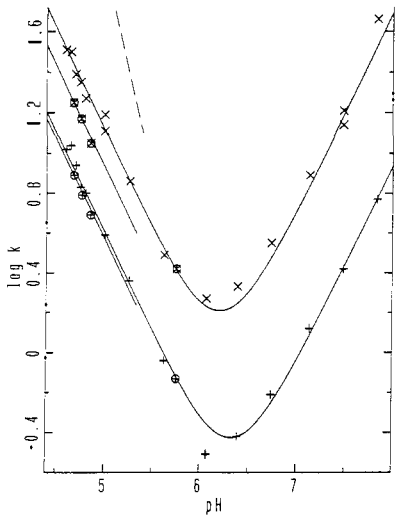


Figure 3. Rate constants for proton exchange in biotin and its methyl ester. Biotin 1'NH (×), 3'(NH) (+), ester 1'NH (⊗), 3'NH (⊕). The solid lines show the computer fit to eq 5 with the rate constants in Table I. The dashed line is of slope −2.

Table II. Salt Effect on Base-Catalyzed Proton Exchange in *d*-Biotin[a]

[KCl], M	k_{obsd}(1'NH), s^{-1}	k_{obsd}(3'NH), s^{-1}
0.00	5.21	1.04
0.05	4.54	0.94
0.10	4.79	0.87

[a] pH 6.96, 10 mM imidazole buffer.

from zero. For ease of comparison, a line of slope −2 is also drawn on Figure 3, and it can be seen that there is no need for the term proportional to $[H^+]^2$ which was observed by FFL&M.[6] This is reassuring, since Herschlag[16] has noted that the reported k_{H^+}' requires that the protonation of the conjugate acid of biotin be faster than diffusion-controlled.

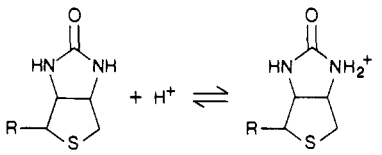
Table II presents the dependence of the rate constants at pH 6.96 on the concentration of KCl. At this pH the reaction is predominantly OH^- catalyzed. It can be seen that the rate is essentially independent of [KCl], in contrast to the 5-to-14-fold increases observed by FFL&M[6] on increasing [KCl] from 0 to 0.1 M. The absence of a salt effect is in agreement with many previous studies on simple amides,[17] and it may be that the apparent salt effect[6] was due to inadequate buffering.

Discussion

The discrepancy between these kinetic results and those of FFL&M[6] may be due to difficulties with NMR techniques when the reaction is too fast. With our instrumentation we were unable to use FFL&M's saturation-recovery method for determining $T_{1,NH}^{app}$. This method requires a homospoil pulse[18] to eliminate transverse OH magnetization, which would create a dynamic-range problem. However, our homospoil pulse takes 5–10 ms to decay sufficiently for the OH resonance to resharpen and be suppressed by the 21412-pulse sequence. This limits the method to rate constants $\ll 10^2$ s^{-1}. Accordingly we used an inversion-recovery method for determining $T_{1,NH}^{app}$. This is applicable to rate constants <20 s^{-1}. For faster reactions this method was found to be unreliable, since the 21412 pulse sequence requires ca. 2 ms to suppress the OH resonance 500 Hz away from the NH resonances. Narutis and Kopple[19] seem to have recognized such limitations, since they too used a combination of methods. We too used line broadening to determine the faster rates. Even this method is limited to rate constants <100 s^{-1}, since otherwise the NH peaks become too broad and overlapping. It is not clear how FFL&M[6] could determine rate constants approaching 300 s^{-1}, since that requires a T_1 measurement on an NH peak that is 100 Hz wide.

The good fit (Figure 3) to eq 5 shows that acid-catalyzed proton exchange is simply first order in H^+, just as the base-catalyzed exchange is first order in OH^-. We certainly find no evidence for an exchange mechanism second order in H^+. Also, within experimental error, there is no acceleration near pH 4.8 and biotin is not substantially accelerated relative to its methyl ester, so that there is no significant intramolecular general-acid catalysis by the COOH group. We can only conclude that the second-order dependence observed by FFL&M[6] is due to a systematic error that gives erroneously high rate constants when the exchange is fast. Indeed, Mildvan[20] has found that the order in H^+ decreases at 10 °C, where the rates are not so high.

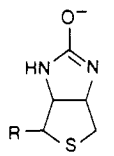
The mechanism of acid-catalyzed exchange is simply N-protonation (eq 6), via the conjugate acid (**3**) as has long been



(6)

3

accepted[21] for ureas. The rate constants are somewhat lower than those of other ureas,[22] since the N-protonation mechanism is expected[23] to be quite sensitive to polar effects. The electron-withdrawing effect of the sulfur on the intermediate, **3**, is closely modeled by its effect on the pK_a of $CH_3SCH_2CH_2NH_3^+$, which is 9.18, 1.4 pK units lower than that of $CH_3CH_2CH_2CH_2NH_3^+$.[15] In contrast, the base-catalyzed exchange rate is nearly the same as in other ureas. This may be attributed to a weaker polar effect of the sulfur on the more distant negative charge in the intermediate **4**.

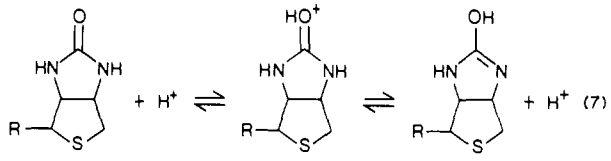


4

In both the acid- and base-catalyzed exchanges, the 1'NH is ca. 4 times as reactive as the 3'NH. This is a consequence of steric hindrance by the side chain, as suggested by Glasel.[13] The similarity of the retardation in both the acid- and base-catalyzed exchanges is a consequence of the similarity of the accessibility requirements of the two mechanisms.[24] This is in contrast to exchange in bovine pancreatic trypsin inhibitor,[25] where the base-catalyzed exchange and the imidic acid mechanism have different accessibility requirements.[24]

Proton exchange in biotin cannot proceed via the imidic acid mechanism (eq 7), involving the isourea ("enol") tautomer **5**, which has often been proposed[1,3] as an intermediate. Rate constants for the imidic acid mechanism for proton exchange in *N*-methylamides $XCONHCH_3$ have been fit[23] to $\log k = 0.43pK_a + 0.46$, where pK_a refers to the corresponding XCOOH. Although the pK_a of

(16) Herschlag, D., through personal communication from W. P. Jencks.
(17) Klotz, I. M.; Frank, B. H. *J. Am. Chem. Soc.* **1965**, *87*, 2721. Schleich, T.; Rollefson, B.; von Hippel, P. H. *Ibid.* **1971**, *93*, 7070. Kim, P. S.; Baldwin, R. L. *Biochemistry* **1982**, *21*, 1.
(18) Waelder, S.; Lee, L.; Redfield, A. G. *J. Am. Chem. Soc.* **1975**, *97*, 2927. Bednar, R. A.; Jencks, W. P. *Ibid.* **1985**, *107*, 7117.
(19) Narutis, V. P.; Kopple, K. D. *Biochemistry* **1983**, *22*, 6233.
(20) Mildvan, A. S., personal communication. Fox, T. L.; Tipton, P. A.; Cleland, W. W.; Mildvan, A. S., in press.
(21) Martin, R. B. *J. Chem. Soc., Chem. Commun.* **1972**, 793.
(22) Vold, R. L.; Daniel, E. S.; Chan, S. O. *J. Am. Chem. Soc.* **1970**, *92*, 6771. Hunston, D. L.; Klotz, I. M. *J. Phys. Chem.* **1971**, *75*, 2123. Martinelli, L. C.; Blanton, C. D.; Whidby, J. F. *J. Am. Chem. Soc.* **1971**, *93*, 5111. Whidby, J. F.; Morgan, W. R. *J. Phys. Chem.* **1973**, *77*, 2999. Yavari, I.; Roberts, J. *Org. Magn. Reson.* **1980**, *13*, 68.
(23) Perrin, C. L.; Arrhenius, G. M. L. *J. Am. Chem. Soc.* **1982**, *104*, 6693.
(24) Perrin, C. L.; Lollo, C. P. *J. Am. Chem. Soc.* **1984**, *106*, 2754.
(25) Tüchsen, E.; Woodward, C. *J. Mol. Biol.* **1985**, *185*, 421.



(7)

5

a carbamic acid (5.25[26]) is uncertain, the extrapolated rate constant for a urea (X = H_2N) is only 5×10^2 M^{-1} s^{-1}. This is so much lower than 1.3×10^6 M^{-1} s^{-1}, the observed value of k_{H^+}, that we may reject this mechanism. Martin[21] reached this same conclusion by deducing that if the observed k_{H^+} were due to the mechanism of eq 7, the pK_b of an isourea would be 10.7, rather than the 4.3 observed for model compounds.[15]

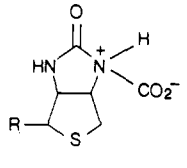
The exchange kinetics provide insight into the mechanism of biotin-mediated CO_2 transfer. We may distinguish 7 possible timings of the proton-transfer steps: (1) N-protonation prior to CO_2 transfer; (2) O-protonation and then N-deprotonation prior to CO_2 transfer; (3) N-deprotonation and then O-protonation prior to CO_2 transfer; (4) N-deprotonation subsequent to CO_2 transfer; (5) N-deprotonation simultaneous with CO_2 transfer; (6) N-deprotonation prior to CO_2 transfer; and (7) any of the previous, along with additional proton-transfer steps. We consider each of these possibilities in turn.

(1) N-Protonation cannot be involved, since the intermediate (**3**) is unreactive as a CO_2 acceptor. Consequently the kinetics of acid-catalyzed exchange, via the dominant mechanism (eq 6), are irrelevant to the mechanism of CO_2 transfer.

(2) The kinetics of acid-catalyzed exchange would have been relevant had exchange proceeded via the imidic acid mechanism (eq 7). This would create the isourea, **5**, whose nitrogen is now more nucleophilic and capable of being a CO_2 acceptor. However, this isourea cannot be involved, since the extrapolation above shows that at pH 7.4 its rate of formation is only 2×10^{-5} s^{-1}, far slower than the 15–74 s^{-1} cited[6] for the carboxylation rates of enzyme-bound biotin.

(3) In principle the isourea might be formed more rapidly by another mechanism, namely O-protonation of the ureide anion (**4**), formed by the base-catalyzed exchange mechanism. A variant is a concerted N-deprotonation and O-protonation. However, the pK_a of the isourea (**5**) may be estimated as 9, from the pK_as of *N*-methylurea (18.3)[27] and of protonated urea (0.18) and *O*-methylurea (9.72).[15] Therefore at physiological pH there is no thermodynamic gain from O-protonation. Besides, O-protonation is counterproductive, since it only reduces the nucleophilicity of the nitrogen and renders the isourea less active than the ureide anion as a CO_2 acceptor.

(4) It has long seemed unlikely[28] that biotin itself accepts CO_2 to form **6** as an intermediate, since the nitrogen of a urea is hardly nucleophilic. Indeed, decomposition of carbamates derived from



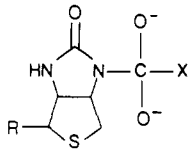
6

weakly basic amines shows general-acid catalysis,[25,29] which is attributed to simultaneous proton transfer and C–N bond cleavage. This concerted mechanism is required because **6** is too strong an acid and too unstable to be an intermediate with a finite lifetime.

(5) Simultaneous N-deprotonation and CO_2 transfer is consistent with general-acid catalysis[29a] of *N*-carboxybiotin decomposition. This decomposition is analogous to enzymatic CO_2 transfer (eq 1) only if the latter occurs by formation of enzyme-bound molecular CO_2 as an intermediate.[30] Such decomposition of O_2^-C–X is more reasonable for X^- = OPO_3H^- but less so for X^- = an enolate, which is a poorer leaving group. If CO_2 transfer is stepwise, via a tetrahedral intermediate, it is then also less likely that N-deprotonation and CO_2 transfer occur simultaneously.

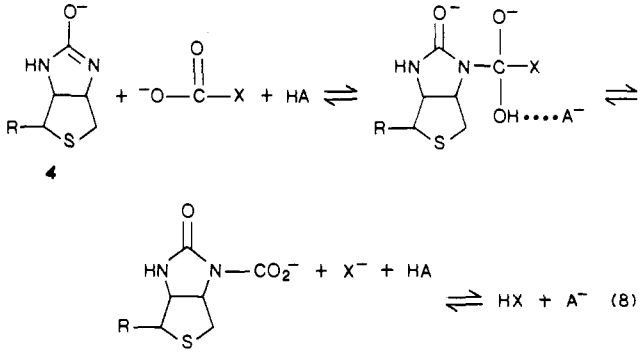
(6) Stepwise N-deprotonation prior to CO_2 transfer requires the ureide anion (**4**) as intermediate. According to our kinetics, and those of FFL&M,[6] the rate of base-catalyzed N-deprotonation, at pH 7.4, is ≥ 12 s^{-1}. As pointed out by FFL&M, this is so close to the rates of carboxylation of enzyme-bound biotin that the steady-state concentration of ureide anion (**4**) cannot be rejected as an intermediate. Even though this is a high-energy species, it is kinetically competent. Also, its nitrogen is more nucleophilic than its oxygen, in contrast to the urea itself.[31] Moreover, it is not necessarily OH^- that acts as the basic catalyst, but any appropriate basic group on the enzyme can deprotonate the biotin. This corresponds to a general-base-catalyzed reaction with Brønsted $\beta = 1$, so that the reduced basicity of the general base, relative to OH^-, is compensated by its increased concentration.

(7) We propose that additional proton transfers are necessary to promote CO_2 transfer and avoid the high-energy[32] intermediate **7**. A CO_2^- group itself, as in *N*-carboxybiotin or oxaloacetate,



7

is not readily transferred. It is quite resistant to nucleophilic attack, except by the most powerful of nucleophiles.[33] Unless CO_2 transfer proceeds via molecular CO_2[30] it is necessary to use a proton donor, HA, to activate the CO_2^- group for nucleophilic attack (eq 8, X = $CH_2COCO_2^-$ or $CH(CH_3)COSCoA$ or perhaps OPO_3H^-). Such a role has been proposed for a cationic site or



(8)

4

hydrogen bond donor,[34] although the emphasis was on rotating the CO_2^- group. The advantage of N-deprotonation prior to, rather than concerted with, CO_2 transfer is that N-deprotonation creates the needed proton donor in the active site. It is for this reason that we prefer the stepwise mechanism (possibility 6) over the concerted one (possibility 5).

Moreover, the proton exchange between this proton-donor/-acceptor site and the one that (de)protonates pyruvate or propionyl CoA may explain the transfer of tritium label[35] from pyruvate

(26) Johnson, S. L.; Morrison, D. L. *J. Am. Chem. Soc.* **1972**, *94*, 1323.
(27) Molday, R. S.; Kallen, R. G. *J. Am. Chem. Soc.* **1972**, *94*, 6739.
(28) Caplow, M. *J. Am. Chem. Soc.* **1965**, *87*, 5774.
(29) (a) Caplow, M.; Yager, M. *J. Am. Chem. Soc.* **1967**, *89*, 4513. Caplow, M. *Ibid.* **1968**, *90*, 6795. (b) Ewing, S. P.; Lockshon, D.; Jencks, W. P. *Ibid.* **1980**, *102*, 3072.
(30) Sauers, C. K.; Jencks, W. P.; Groh, S. *J. Am. Chem. Soc.* **1975**, *97*, 5546.
(31) Hegarty, A. F.; Bruice, T. C. *J. Am. Chem. Soc.* **1970**, *92*, 6575.
(32) Blagoeva, I. B.; Pojarlieff, I. G.; Kirby, A. J. *J. Chem. Soc., Perkin Trans. 2* **1984**, 745.
(33) March, J. *Advanced Organic Chemistry*, 3rd ed.; Wiley-Interscience: New York, 1985; pp 824, 1099.
(34) Thatcher, G. R. J.; Poirier, R.; Kluger, R. *J. Am. Chem. Soc.* **1986**, *108*, 2699.

to propionyl CoA. The proton carrier can then be biotin itself, rather than its isourea tautomer. It is not necessary to derive an estimated pK_a of 6.4 for that tautomer, rather than the 9 estimated above.

Unfortunately these results do not clarify the importance of the sulfur in biotin. Biotin does not show a proton-exchange mechanism second order in H^+. Therefore there is no need to invoke a transannular interaction as in **2**. The electron-withdrawing effect of the sulfur does affect reactivity, but oxygen would show nearly the same effect. The default rationalization is that only biotin itself has the optimum geometry to fit into the enzyme site, but this is not very informative.

Acknowledgment. This research was supported by National Science Foundation Grant CHE85-09312.

Registry No. Carbon dioxide, 124-38-9; biotin, 58-85-5; biotin methyl ester, 608-16-2.

(35) Rose, I. A.; O'Connell, E. L.; Solomon, F. *J. Biol. Chem.* **1976**, *251*, 902.

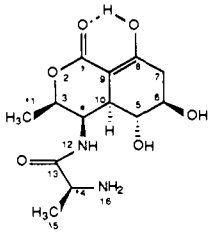
π-Facial Selection in Intermolecular Diels–Alder Reactions: Total Syntheses of (+)-Actinobolin and (+)-5,6,10-*triepi*-Actinobolin

Alan P. Kozikowski,*† Thaddeus R. Nieduzak,† Toshiro Konoike,† and James P. Springer‡

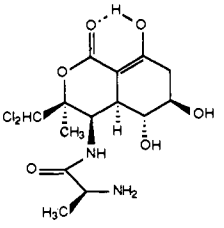
Contribution from the Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, and the Merck Institute, Rahway, New Jersey 07065.
Received February 19, 1987

Abstract: Syntheses of both 5,6,10-*triepi*-actinobolin and the antibiotic actinobolin are described in which a homochiral diene prepared from L-threonine is employed as a key component in a Diels–Alder reaction with an acetylenic dienophile. While the Diels–Alder reaction of this diene with methyl propiolate furnished the cycloadduct required for the synthesis of (+)-actinobolin as the minor diastereomer, the completion of the synthesis required but seven additional steps. The steric and stereoelectronic features responsible for the π-facial course of this cycloaddition reaction are discussed along with the various steps required to complete the syntheses of the title compounds.

Actinobolin (**1**) is a broad spectrum antibiotic first obtained from submerged aerated broth cultures of *Streptomyces griseoviridus* var. *atrofaciens* by Haskell and Bartz.[1a] The substance



Actinobolin (1)

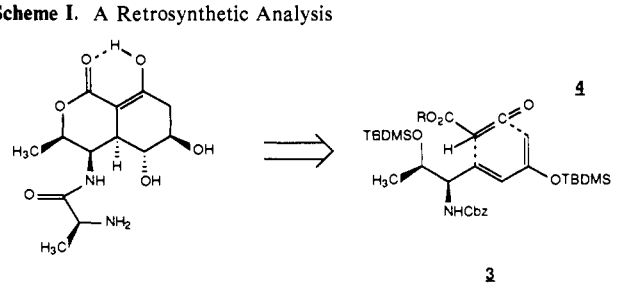


Bactobolin (2)

is an amphoteric, water-soluble lactone that readily forms crystalline salts with acids. It chelates iron, aluminum and other metal ions. Actinobolin was found to be a potent inhibitor of various Gram-positive and Gram-negative bacteria, and it was found to possess some antileukemic activity as well.[1b,c] The structure of actinobolin was determined through a combination of chemical degradations,[1d] derivatizations, and spectral analyses which were additionally aided by a computer program designed to evaluate the structural implications of the experimental data.[1e-g] Closely related to actinobolin structurally is the chlorine-containing antibiotic bactobolin (**2**), a compound isolated from a culture broth of *Pseudomonas* BMG-13-147.[2] Bactobolin exhibits both stronger antibacterial activity and more pronounced antileukemic activity than does actinobolin.

In this article we describe our efforts to synthesize actinobolin in the laboratory through an intermolecular Diels–Alder strategy.

Scheme I. A Retrosynthetic Analysis



As shown below, (Scheme I), we envisioned the assembly of actinobolin through reaction of the silyloxydiene **3** with some carbalkoxyketene equivalent **4**. The construction of the diene component from L-threonine, the π-facial course of the reaction of this diene with methyl propiolate, and the conversion of the Diels–Alder products to *triepi*-actinobolin and actinobolin are detailed in the following sections.[3,4]

† University of Pittsburgh.
‡ Merck Institute.

(1) (a) Haskell, T. H.; Bartz, Q. R. *Antibiot. Ann.* **1959**, 1958/1959, 505. (b) Pittillo, R. F.; Fisher, M. W.; McAlpine, R. J.; Thompson, P. E.; Ehrlich, J.; Anderson, L. F.; Fiskin, R. A.; Galbraith, M.; Kohberger, D. L.; Manning, M. C.; Reutner, T. F.; Roll, D. R.; Weston, K. *Antibiot. Ann.* **1959**, 1958/1959, 497. (c) Burchenal, J. H.; Holmberg, E. A. D.; Reilly, H. C.; Hemphill, S.; Reppert, J. A. *Antibiot. Ann.* **1959**, 1958/1959, 528. (d) Munk, M. E.; Sodano, C. S.; McLean, R. L.; Haskell, T. H. *J. Am. Chem. Soc.* **1967**, *89*, 4158. (e) Nelson, D. B.; Munk, M. E.; Gash, K. B.; Herald, D. L. *J. Org. Chem.* **1969**, *34*, 3800. (f) Antosz, F. J.; Nelson, D. B.; Herald, D. L.; Munk, M. E. *J. Am. Chem. Soc.* **1970**, *92*, 4933. (g) Nelson, D. B.; Munk, M. E. *J. Org. Chem.* **1970**, *35*, 3832.

(2) (a) Kondo, S.; Horiuchi, Y.; Hamada, M.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1979**, *32*, 1069. (b) Ishizuka, M.; Fukasawa, S.; Masuda, T.; Sato, J.; Kanbayashi, N.; Takeuchi, T.; Umezawa, H. *Ibid.* **1980**, *33*, 1054. Hori, M.; Suzukake, K.; Ishikawa, C.; Asakura, H.; Umezawa, H. *Ibid.* **1981**, *34*, 465. Veda, I.; Munakata, T.; Sakai, J. *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem.* **1980**, *B36*, 3128.

0002-7863/87/1509-5167$01.50/0 © 1987 American Chemical Society