Reactions of Enolic Biotin Models

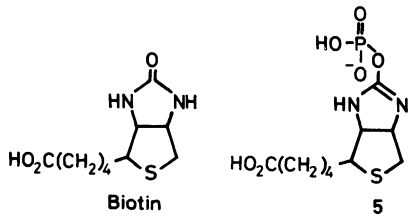
Hiroki KONDO,* Katsuhito MIURA, Eiji SEKI, and Junzo SUNAMOTO
Department of Industrial Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852
(Received April 27, 1985)

In order to delineate the mechanism of biotin-dependent carboxylation reactions, the following model experiments have been carried out. In reactions of carbonate derivatives such as methyl chloroformate with 2-ethoxy-2-imidazoline (**1**), a putative enolic biotin model, or 2-imidazolidinone (**2**), the former showed a much higher reactivity to provide the methoxycarbonylated **1** (**3**). Reactions of **3** with benzenethiolate, cyclohexylamine and carbanions have also been investigated as a model of transcarboxylation from *N*-carboxybiotin to an acceptor substrate. Only carbanions attack the carbonyl carbon of **3**, leading to rupture of the C-O bond, but not of the N-C bond. A net "carboxyl" transfer was effected with 1-methoxycarbonylimidazole (**9**) as a donor substrate. Implications of these results in biotin catalysis are discussed.

Biotin is the coenzyme involved in the synthesis of long-chain fatty acids in biological systems.[1-3] This enzymatic carboxylation takes place in two discrete steps as shown in Eqs. 1a and 1b. Biotin-dependent carboxylation reactions are interesting from a synthetic as well as a biochemical point of view. Despite efforts it has been unsuccessful to duplicate biotin-mediated carboxylation in nonenzymatic systems. One of the major reasons for this lies in the poor reactivity of the ureido nitrogen of biotin upon which carboxylation occurs.[4] Enzymes appear to overcome this problem by utilizing the free energy released upon hydrolysis of ATP to activate one or more of the reactants (Eq. 1a). Several hypotheses have been proposed to account in chemical terms for the role of ATP in biotin carboxylation. One of the attractive hypotheses claims that ATP might phosphorylate the ureido oxygen of biotin to generate an enolic structure (**5**).[5] This mechanism attracted considerable attention in recent years,[6-8] though rigorous structural proof of the thermodynamically unstable **5** has not been provided. In this article we attempted to assess the reactivity of a stable model compound of putative **5** toward several carbonate derivatives. Furthermore, model carboxyl-transfer reactions from a methoxycarbonylated biotin model to an acceptor substrate have also been investigated.

$$\text{E-Biotin} + HCO_3^- + \text{ATP} \rightleftharpoons \text{E-Biotin-}CO_2^- + \text{ADP} + \text{Pi} \quad (1a)$$

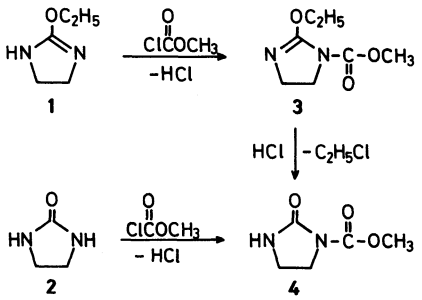
$$\text{E-Biotin-}CO_2^- + \text{RH} \rightleftharpoons \text{E-Biotin} + RCO_2H \quad (1b)$$



Results

Reactions of 1 and 2 with Carbonate Derivatives.

We have chosen 2-ethoxy-2-imidazoline (**1**) as a model for the enolic form of biotin and compared its reactivity with that of the keto model compound (**2**) toward several carbonate derivatives, methyl chloroformate, methoxycarbonyl dimethyl phosphate (MDP), and methyl *p*-nitrophenyl carbonate (MPNC). The reactions were routinely followed by ^1H NMR or HPLC, but the products were isolated, where necessary, for identification by a comparison with the authentic specimen. A reaction of **2** with methyl chloroformate gives **4** (Scheme 1), but a large excess of reagent and



Scheme 1.

refluxing (≈60 °C) of the reaction mixture for several hours are usually needed to drive the reaction to completion. On the other hand, a mild condition (one equiv of methyl chloroformate and room temperature) is sufficient to methoxycarbonylate **1** with methyl chloroformate, thus demonstrating that **1** is by far the more reactive than **2** toward this reagent. Conversely, treatment of **1** with methyl chloroformate under the condition adopted for **2** (see above) gave **4** in quantitative yield. It was later found that **4** was produced *via* **3** as stated above and that because of its acid lability **3** is transformed into **4** by the hydrochloric acid generated in the preceeding reaction (Scheme 1).[9]

In addition to methyl chloroformate both MDP and MPNC are able to methoxycarbonylate **1** under mild conditions. Thus treatment of **1** with one equiv of MDP or MPNC at room temperature yielded **3** in excellent yield. MDP possesses potentially two sites for nucleophilic attack,[10] but the reaction with **1** took place solely on the carbon. No conversion of **3** into **4** was observed

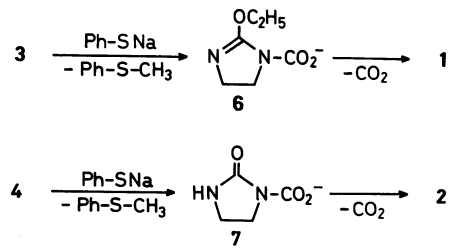
Table 1. Reaction of 1 with carbonate derivatives at 25 °C. The extent of reaction was evaluated by integrating the ^{1}H NMR signal intensities.

Reagent[a]	[Reagent]/[1]	Solvent	Conversion/% 6 min	32 min
$ClCO_2CH_3$	1.0	$CDCl_3$	100	—
MDP	1.1	$CDCl_3$	75±5	100
MPNC	1.0	DMSO-d_6	40±5	80±5

a) MDP, methoxycarbonyl dimethyl phosphate; MNPC, methyl *p*-nitrophenyl carbonate.

when the reaction mixture of **1** and MDP or MPNC was refluxed in chloroform for 30 min, because the acids liberated in these reactions, dimethyl hydrogenphosphate and *p*-nitrophenol, are not acidic enough to bring about such a transformation.[9] The rates of methoxycarbonylation of **1** by these reagents are compared in Table 1. No reaction took place between **2** and MDP or MPNC even after 2 d in refluxing chloroform. This indicates that these two reagents are not electrophilic enough to methoxycarbonylate **2** and that there is a sharp difference between **1** and **2** in the reactivity toward carbonate carbonyls. The difference is too large to allow comparison of the reactivity of both compounds directly on the same scale.

Reactions of 3 and 4 with Nucleophiles. Reactions of **3**, **4**, and related compounds with sulfur and nitrogen bases as well as carbanions have been studied as a model of carboxyl transfer reactions (Eq. 1b). Similar reactions of **3** and **4** with hydroxide ions in water have already been reported.[11] A sample of **3** or **4** was allowed to react with 2 equiv of sodium benzenethiolate in DMSO-d_6 and the progress of reaction was followed by ^{13}C NMR. As has been reported previously,[12] compound **4** reacts with benzenethiolate at room temperature (≈20 °C) to provide methyl phenyl sulfide and **2**. The half time of the reaction was estimated as 30 min. By reference to the similar reaction of hydroxide ions in water,[11] the reaction was presumed to follow the sequence given in Scheme 2.[12] Unlike in the saponification in water, however, compound **7** has never been detected by ^{13}C NMR during the reaction in the present system, indicating that decarboxylation of **7** is fast compared to the demethylation step. The reaction of **3** follows the identical pathway, albeit at a decreased rate. The half time was estimated to be ≈25 min at 66 °C. The nucleophilic attack of benzenethiolate on the methyl carbon of **3** is the rate-determining step and the reaction products obtained were exclusively methyl phenyl sulfide and **1**. The lability of the intermediates (**6** and **7**) hampered comparison of the rates of their decarboxylation.

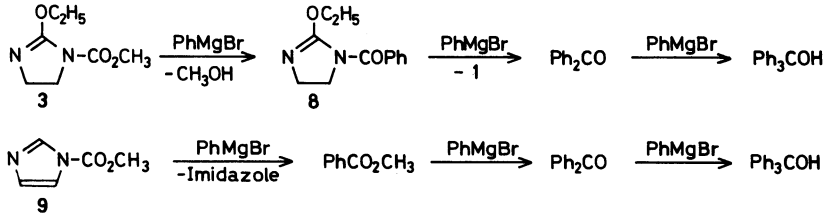


Scheme 2.

A reaction of **3** with cyclohexylamine afforded 1-methoxycarbonyl-2-cyclohexylaminoimidazolidine quantitatively (isolated yield 75%). In contrast, the attack of secondary or tertiary amines on the compound carrying an extra methyl group on 3-N of **3** takes place on the ethyl carbon, not on the 2-position.[7] The presence of a positive charge on the 3-N in the latter substrate appears to be responsible for this difference. Because of the charge the molecule is rendered electron-deficient, thus making the ethyl migration to a nucleophile possible. Compound **4** followed a similar pathway to that for **3** in the reaction with primary amines; initial nucleophilic attack on the ureido carbonyl provided a ring-opened product of substrate.[12]

A reaction of **3** with 3 molar equiv of phenylmagnesium bromide was carried out in THF at 0 °C for 10 h. The reaction mixture was worked up as usual. Inspection of the products by ^{1}H NMR in CCl_4 with CH_2Cl_2 as the internal standard revealed that following compounds had been obtained: 1-benzoyl-2-ethoxy-2-imidazoline (**8**) 52%, benzophenone 6%, triphenylmethanol 11%, and unreacted **3** 14%. This mode of product distribution is consistent with a sequence of reactions shown in Scheme 3. Initial attack of the Grignard reagent on the carbonyl carbon of **3** is followed by scission of the C–O bond, but not of the N–C bond. The product (**8**) undergoes further reactions with phenylmagnesium bromide to give benzophenone and triphenylmethanol. The possibility of an alternative pathway by way of methyl benzoate is ruled out, because the ester, if produced, could have survived under the present reaction conditions (see below). Analogous results were obtained for the reaction of **4** with phenylmagnesium bromide,[13] indicating that the electronic structure of the electrophilic center is not different significantly between the two compounds.

The above results suggest that net carboxyl transfer might be achieved if the basicity of the pertinent nitrogen is lowered. Hence 1-methoxycarbonylimidazole (**9**) was chosen as a donor substrate. Reaction of **9** with phenylmagnesium bromide, indeed, provided methyl benzoate (Scheme 3). For example, a reaction of **9** with 2 molar equiv of phenylmagnesium bromide yielded the following products: methyl benzoate 51%, benzophenone 1%, triphenylmethanol 5%, and unreacted **9** 8%. This result clearly indicates that the N–C bond cleavage or "the carboxyl" transfer has taken place. An alternative pathway that produces 1-benzoylimidazole as the initial product may also be envisioned, because the latter two products can be generated from either methyl benzoate or 1-benzoylimidazole. Furthermore, only 65% of the substrate initially present were identi-



Scheme 3.

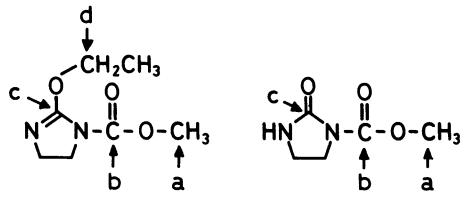
fied as above and the rest might be, at least in part, accounted for by 1-benzoylimidazole, which may be lost during work-up due to its water lability. Nevertheless, the methyl benzoate pathway is the main one, since the ester was obtained as the major product at least in one instance (above).

Discussion

From the results presented above, two main conclusions have been drawn. First, the stable enolic biotin model (**1**) possesses a much higher nucleophilicity than the corresponding keto model (**2**) toward a carbonyl carbon of the carbonate derivatives (a hydrogencarbonate surrogate). A similar behavior of both models was reported briefly for the hydrolytic reaction of carboxylic esters in water.[14] Also consistent with these is the observation that whereas 3-methyl-2-imidazolidinone was unable to add to the carbonyl carbon of a carbamate, the corresponding enol could.[15] All these phenomena may be rationalized in terms of a difference in the basicity of the pertinent nitrogen of the two compounds: The pK_a for the conjugate acid of **1** is $\approx$9[14] while the corresponding value for **2** ≈ -1.[16] Taken together, it is suggested that the ultimate function of ATP in the first partial reaction of carboxylation (Eq. 1a) is to enhance basicity and hence nucleophilicity of the nitrogen of biotin in question through phosphorylation of the ureido carbonyl.

It is, however, noted that despite suggestions for the participation of *O*-phosphono enolic structure (**5**) in biotin catalysis, its existence has not been proven in an explicit manner. The enolic structure of biotin in its unphosphorylated form is predicted theoretically to be unstable.[17] It is also pointed out that an alternative explanation may be invoked for the role of ATP, *e.g.*, phosphorylation of hydrogencarbonate, another substrate in the carboxylation of biotin (Eq. 1a).[18] The resulting mixed anhydride is expected to exhibit a higher reactivity than hydrogencarbonate. In fact, this mode of activation of a carboxyl group by ATP is rather general in synthase reactions in which an amide or ester bond is formed concomitant with ATP hydrolysis.[19]

Secondly, it was found that compound **3** undergoes reaction with various nucleophiles in a way quite analogous to that for compound **4**,[12] demonstrating that the electronic structure around the reaction center is not different significantly between the two. Compounds **3** and **4** have several potential sites for nucleophilic attack (Scheme 4), of which sites b and c are present in the *N*-carboxybiotin intermediate as well.



Scheme 4.

As noted above and previously,[7,11,12] oxygen and sulfur nucleophiles attack site a and nitrogen nucleophiles attack mostly site c. Only carbanions attack site b, an observation reminiscent of the fact that the enzymatic carboxyl transfer takes place on the ("active") carbon in most cases.[1-3] The initial addition of a carbanion to the ester carbonyl (site b) of **3** and **4** is followed by scission of the C-O bond. A "carboxyl" transfer was effected by employing 1-methoxycarbonylimidazole (**9**) as the donor substrate. The lower basicity of the imidazole nitrogen to which the methoxycarbonyl group is linked appears to be responsible for this difference; the pK_a of imidazole being $\approx$14,[20] while that of urea 25—33.[21] This implies that the N-C bond cleavage becomes feasible where the basicity of the pertinent nitrogen is low enough to make it a good leaving group. This result might be of value in relation to the mechanism of the enzymatic carboxyl transfer. As shown above, the carboxyl carbon bonded to the ureido nitrogen is a poor electrophile. To achieve transfer of the carboxyl group, one might have to enhance the electrophilicity of the carboxyl carbon and/or the nucleophilicity of the carboxyl-acceptor substrate. One way of doing this would be the electrophilic catalysis. For example, protonation or metal coordination to the ureido ring should bring about an electronic shift from the carboxyl group,[22,23] thereby facilitating nucleophilic attack of the incoming nucleophile on that site (Scheme 4). To corraborate this hypothesis further work is being continued in this laboratory.

Experimental

Materials. Common chemicals including 2-imidazolidinone (**2**, ethyleneurea) were obtained from commercial sources. The following compounds were prepared by the

literature methods specified; 2-ethoxy-2-imidazoline (**1**),[24] 1-methoxycarbonyl-2-ethoxy-2-imidazoline (**3**),[25] 1-methoxycarbonyl-2-imidazolidinone (**4**),[4] 1-methoxycarbonylimidazole,[26] methoxycarbonyl dimethyl phosphate.[27] 1-Benzoyl-2-ethoxy-2-imidazoline (**8**) was prepared by reaction of 1-benzoyl-2-imidazolidinone with triethyloxonium tetrafluoroborate. The crude product was purified by column chromatography on silica gel with acetonitrile as an eluant and by recrystallization from hexane, mp 46—48 °C. IR (KBr) 1673 (C=O), 1648 cm^{-1} (C=N). NMR (CCl_4): δ=0.84 (t, 3H, $-CH_3$), 3.52 (t, 2H, $-CH_2-$), 3.93 (t, 2H, $-CH_2-$), 4.01 (q, 2H, $-CH_2-CH_3$), 7.10—7.28 (m, 5H, $-C_6H_5$). Found: C, 66.27; H, 6.45; N, 12.92%. Calcd for $C_{12}H_{14}N_2O_2$: C, 66.03; H, 6.46; N, 12.83%.

Apparatus. Electronic and infrared absorption spectra were determined on a Hitachi 124 and Jasco A-100 spectrophotometer, respectively. ^1H NMR spectra were taken on a Jeol JNM-MH-100 spectrometer. Chemical shifts are expressed in parts per million relative to internal TMS. ^{13}C NMR spectra were determined on a Jeol JNM-FX-90Q spectrometer operating at 22.5 MHz. Typical experimental parameters were the following: spectral width of 5000 Hz with acquisition of 8 K data points, 10-μs pulse, and a recovery time of 3s. Mass spectra were determined with a Jeol JMS-DX 300 mass spectrometer. High-performance liquid chromatography (HPLC) was run on a Toyo Soda HLC 802 UR with Merck LiChrospher Si-100 as the stationary phase (4×300 mm) and chloroform–methanol (98:2 by vol.) as the eluant. When run at a flow rate ≈1.0 ml/min following retention times (min) were obtained MPNC 2.7, methyl chloroformate 3.2, **1** 3.8, **3** 4.7, *p*-nitrophenol 6.2, **4** 8.2.

Reaction of 1 with MPNC. A mixture of 120 mg (1.03 mmol) of **1** and 220 mg (1.12 mmol) of MPNC in 20 ml of $CHCl_3$ was refluxed for 12 h. The solvent was removed *in vacuo* and the residue was purified by column chromatography on silica gel (1.6×22 cm) with acetonitrile as an eluant in 62% yield, mp 67.5—70.0 °C (lit,[25] 67—70 °C).

Reaction of 3 with Cyclohexylamine. A sample of **3** (50 mg, 0.29 mmol) and cyclohexylamine (70 μl, 0.58 mmol) were allowed to react in chloroform at 60 °C for 24 h. Evaporation of the solvent *in vacuo* left an oil, to which was added hydrochloric acid equimolar to **3**. The product obtained as a hydrochloride in 75% yield was recrystallized from ethanol, mp 142—145 °C. R_f 0.59 ($CHCl_3$). IR(KBr) 1728 cm^{-1} (C=O). MS *m/z* 225.14715 (M^+). Calcd for $C_{11}H_{19}N_3O_2$ as free base: 225.14761.

Reaction of 9 with Phenylmagnesium Bromide. To a THF solution (12 ml) of phenylmagnesium bromide (24.6 mmol) was added a THF solution (40 ml) of **9** (1.68 g, 13.3 mmol) at 0 °C. The mixture was stirred for 10 h. The reaction was terminated by the addition of 10 ml of water and the solid formed was removed by filtration. The filtrate was extracted with ether (3×10 ml) and chloroform (3×10 ml). The combined extracts were dried over $MgSO_4$. Evaporation of the solvents left an oil, which was dissolved in carbon tetrachloride together with 846 μl (13.3 mmol) of dichloromethane for submission to NMR measurements.

The authors are grateful to prof. A. Marquet for informing them of her unpublished results and for helpful discussions. They also thank Drs. K. Sakata and N. Nishino for determining mass spectra. This work was supported by a Grant-in-Aid for Special Project Research 57102007 from the Ministry of Education, Science and Culture.

References

1) J. Moss and M. D. Lane, *Adv. Enzymol.*, **35**, 321 (1971).
2) H. G. Wood and R. E. Barden, *Ann. Rev. Biochem.*, **46**, 385 (1977).
3) S. Numa and T. Tanabe, "Fatty Acid Metabolism and its Regulation," ed by S. Numa, Elsevier, Amsterdam (1984), Chap. 1.
4) M. Caplow, *J. Am. Chem. Soc.*, **87**, 5774 (1965).
5) J. Retey and F. Lynen, *Biochem. Z.*, **342**, 256 (1965).
6) R. Kluger and P. D. Adawadkar, *J. Am. Chem. Soc.*, **98**, 3741 (1976).
7) M. J. Cravey and H. Kohn, *J. Am. Chem. Soc.*, **102**, 3928 (1980).
8) C. Blonski, M-B. Gasc, A. Klaebe, J-J. Perie, R. Roques, J. P. Declercq, and G. Germain, *J. Chem. Soc., Perkin Trans. 2*, **1982**, 7.
9) H. Kondo, K. Miura, and J. Sunamoto, *Bull. Chem. Soc. Jpn.*, **55**, 3347 (1982).
10) A. J. Lambie, *Tetrahedron Lett.*, **1966**, 3709.
11) H. Kondo, K. Miura, and J. Sunamoto, *Tetrahedron Lett.*, **23**, 659 (1982).
12) N. Matsumura, Y. Yagyu, H. Kawai, Y. Otsuji, and E. Imoto, *Nippon Kagaku Kaishi*, **1977**, 362.
13) N. Matsumura, K. Matsukawa, Y. Sakaguchi, and H. Inoue, *Bull. Chem. Soc. Jpn.*, **55**, 1667 (1982).
14) A. F. Hegarty, T. C. Bruice, and S. J. Benkovic, *J. Chem. Soc., Chem. Commun.*, **1969**, 1173.
15) C. M. Visser and R. M. Kellogg, *Bioorg. Chem.*, **6**, 79 (1977).
16) M. Caplow, *Biochemistry*, **8**, 2656 (1969).
17) G. M. Maggiora, *J. Theor. Biol.*, **43**, 51 (1974).
18) Y. Kaziro, L. F. Hass, P. D. Boyer, and S. Ochoa, *J. Biol. Chem.*, **237**, 1460 (1962).
19) C. Walsh, "Enzymatic Reaction Mechanisms," Freeman, San Francisco (1979), Chap. 5.
20) G. Yagil, *Tetrahedron*, **23**, 2855 (1967).
21) A. H. Corwin and J. D. Reinheimer, *J. Am. Chem. Soc.*, **73**, 1184 (1951).
22) H. Kondo, D. Horiguchi, S. Ikeda, J. Sunamoto, and K. Tsujii, *J. Org. Chem.*, **44**, 4430 (1979).
23) H. Kondo, K. Miura, S. Uno, and J. Sunamoto, *J. Inorg. Biochem.*, **21**, 93 (1984).
24) G. I. Poos, J. Kleis, and C. K. Cain, *J. Org. Chem.*, **24**, 645 (1959).
25) H. Kohn, M. J. Cravey, J. H. Arceneaux, R. L. Cravey, and M. R. Willcott, III, *J. Org. Chem.*, **42**, 941 (1977).
26) S. Iwasaki, *Helv. Chim. Acta*, **59**, 2738 (1976).
27) A. A. Shamshurin, O. E. Krivoshchekova, and M. Z. Krimer, *Zh. Obsch. Khim.*, **35**, 1877 (1965).