Intramolecular Model for the Reductive Acyl Transfer Catalyzed by α-Keto Acid Dehydrogenases

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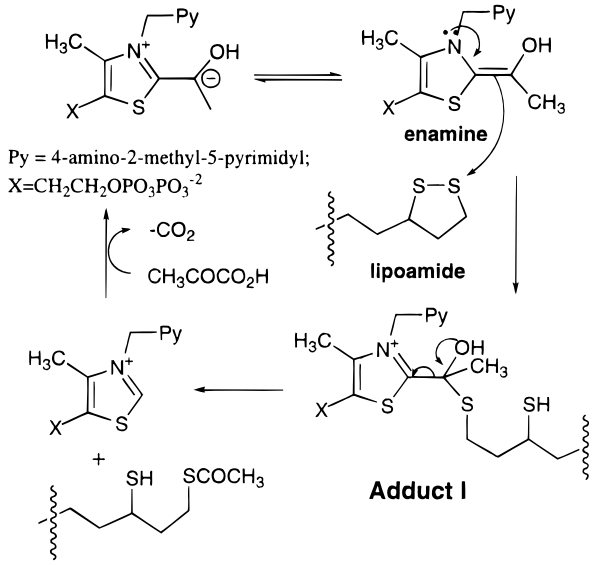
Abstract: An intramolecular model was synthesized for the oxidative acyl transfer between thiamin diphosphate on the E1 and lipoic acid on the E2 subunit of the pyruvate dehydrogenase multienzyme complex. The model incorporates a 2-α-methoxybenzylthiazolium salt as a precursor of the enamine/2-α-carbanion, and lipoic acid. Upon addition of the base, the enamine/2-α-carbanion is generated (detected at 380 nm) and is oxidized by the lipoic acid. The oxidation is very significantly enhanced by the addition of PhHgCl. It is likely that the Hg(II) shifts the equilibrium toward the reductive acylation. This appears to be the first successful model for the reductive acylation for which all intermolecular models (including control experiments in this laboratory) have failed to date. The reaction requires both a high local concentration of reactants and the trapping of the reduced thiolate by an electrophile. It is also evident from the data that oxidation of the enamine/2-α-carbanion intermediate on the 2-oxoacid dehydrogenase multienzyme complexes requires very significant assistance by the protein (at least 10^5-fold rate acceleration as compared to the model here presented), unlike its oxidation by flavin (a model for the enzyme pyruvate oxidase) that requires no significant assistance once the coenzymes are bound to the enzyme (Chiu, C. C.; Pan, K.; Jordan, F. *J. Am. Chem. Soc.* **1995**, *117*, 7027−7028).

The significant progress made during the past decade in delineating the chemistry of structures related to the key α-carbanion/enamine intermediate invoked on all thiamin diphosphate (ThDP)-dependent enzymatic pathways includes generation and spectroscopic characterization,[1] determination of its electrochemical oxidation potentials,[2a] and determination of protolytic equilibria leading to it in nonaqueous[2b] and aqueous[2c] media. Recently, an intermolecular model was reported for oxidation of such an enamine by a flavin analog as a model for the enzyme pyruvate oxidase (POX).[2d] These studies have already provided nonenzymatic rate constants for elementary steps[2c,d] with which the enzymatic turnover numbers could be compared, thereby enabling us to deduce the magnitude of the rate acceleration contributed by the protein environment. We here report synthesis and kinetic studies of the first successful intramolecular model for the chemical step involving the oxidative acyl transfer from the E1 to the E2 component of the pyruvate dehydrogenase multienzyme complex (PDHc), a key player in carbohydrate metabolism.[3a,b] In this reaction, lipoic acid covalently attached to the E2 subunit is reductively acetylated by the ThDP-bound enamine. While the X-ray structures of POX,[4a] pyruvate decarboxylase,[4b,c] and transketolase[4d] have shed better understanding on the function of ThDP, the reductive acyl transfer between enamine-E1 and lipoyl-E2 is least well understood among the series of reactions catalyzed by PDHc. This is, in part, due to the lack of a satisfactory chemical model[5a−c] that would confirm the reduction of lipoic acid by the enamine or the formation of the tetrahedral adduct **I** in Scheme 1 proposed as the key reaction intermediate.[6a,b] Adducts such as **I** have been generated from the enamine and linear sulfide donors[5a] and synthesized[5c] but never observed from the reaction of the enamine and lipoic acid.[7]

Scheme 1. Plausible Mechanism for Reductive Acetylation of Lipoyl-E2 by 2-(1-Hydroxyethylidene)-ThDP at Active Site of Pyruvate Dehydrogenase (E1)



[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1996.

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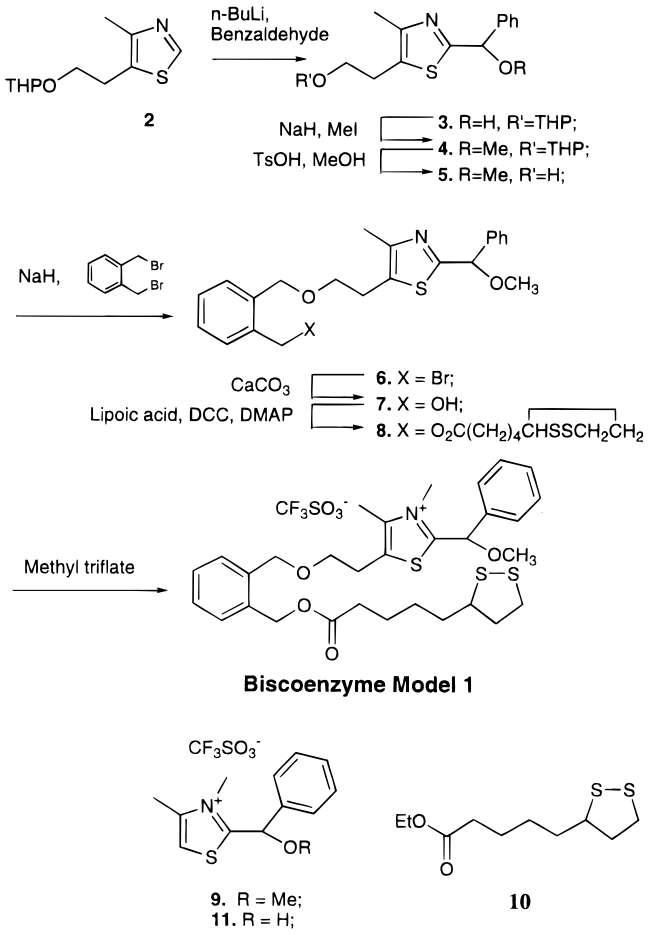
Scheme 2. Synthesis of Bis-coenzyme Model **1**



To make further progress in delineating the mechanism and rates of the reductive acylation of lipoic acid, we have undertaken and completed the synthesis of an intramolecular bis-coenzyme model for PDHc that incorporates two reactive moieties, a precursor of the enamine and the dithiolane ring. The new model offers distinct advantages over the previous unsuccessful ones: (1) an intramolecular system that creates a high local concentration of reactants, especially useful while conducting studies at very low concentrations; (2) a thiazolium derivatized with an aldehyde equivalent that could be converted to the enamine simply by the addition of base;[2] (3) the C2α-OR protected enamine, to avoid dissociation of the aldehyde as a competing step and to enable us to observe the enamine directly by Vis spectroscopy[2c] (phenyl in place of methyl); (4) a thiolate trapping agent to render the reduction irreversible and to prevent side reactions of the newly generated thiolate with the thiazolium ring.

The synthesis of model molecule **1** is outlined in Scheme 2. The THP-protected 4-methyl-5-thiazolethanol **2**[8] was treated with *n*-BuLi and benzaldehyde in THF at −78 °C to give 2-α-hydroxybenzylthiazole **3** in 80% yield. Treatment of the alcohol at 0 °C gave **4**, which upon removal of the THP protecting group provided a high yield of 2-α-methoxybenzylthiazole **5**. Williamson ether synthesis[9] between **5** and α,α′-dibromo-*o*-xylene required THF as solvent and reflux for 36 h (52% yield). The monosubstituted benzylbromide **6** was hydrolyzed in 2:1 dioxane/ H_2O containing $CaCO_3$, and the resulting alcohol **7** was coupled to lipoic acid using standard DCC strategy. Finally, the thiazole was selectively methylated by exposure to methyl triflate in CH_2Cl_2 at 0 °C to provide the bis-coenzyme **1** without damaging the fragile lipoic moiety (31% yield for two steps).

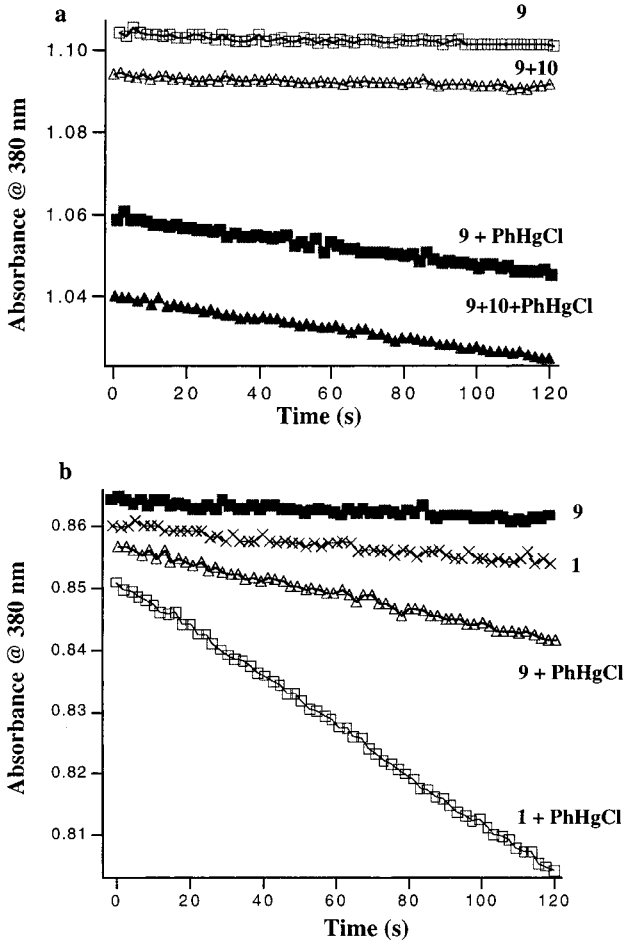


Figure 1. (a) Time dependence of depletion of enamine derived from **9**. The enamine was generated by the addition of Et_3N (0.5 mL) to **9** (0.08 mM in 2 mL of DMSO) in the absence or presence of ethyl lipoate (0.8 mM) and PhHgCl (0.8 mM) at 25 °C. (b) Time dependence of depletion of enamine derived from bis-coenzyme **1** and thiazolium **9**. The enamine was generated by the addition of Et_3N (0.5 mL) to **1** or **9** present at an initial concentration of 0.08 mM in 2 mL of DMSO in the absence or presence of PhHgCl (0.08 mM) at 25 °C.

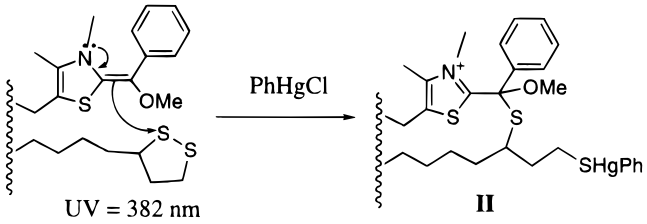
Kinetic studies of model **1** were conducted at 382 nm (ϵ_{382} = 15 000),[2c] monitoring the enamine absorbance. Figure 1a illustrates attempts made with the intermolecular reaction. The enamine was generated from **9** (at 0.08 mM in 2 mL of dry DMSO) by the addition of 0.5 mL of Et_3N, and the addition of even 10-fold molar excess of ethyl lipoate did not change the nearly imperceptibly slow depletion of the enamine. The same experiments were repeated in the presence of 0.8 mM PhHgCl, the zero-order slopes increased by the same factor within experimental error both in the absence and in the presence of ethyl lipoate. Therefore, *the intermolecular reaction with lipoate failed* (as also reported in ref 5a), but apparently PhHgCl could also react with the enamine. Next, a solution of bis-coenzyme **1** (0.08 mM) in 2 mL of DMSO was treated with Et_3N (0.5 mL) to generate the enamine. Equilibrium was reached in less than 30 s. The depletion of the enamine was then monitored for an additional 2 min (Figure 1b). In the absence of PhHgCl there was a very slow rate of depletion of bis-coenzyme **1**; the slope in the presence of intramolecular lipoate is nearly the same as for the depletion of **9**. The presence

(7) The results presented here do not exclude the intervention of an electron transfer mechanism, such as observed under electrochemical oxidation of the enamine,[2a] either in our model or on the enzyme.

(8) Chiu, C. C. Ph.D. Dissertation, Rutgers University Graduate Faculty at Newark, 1995.

(9) Feuer, H.; Hooz, I. *The chemistry of the ether linkage*; Interscience: New York, 1967; pp 446−468.

Scheme 3. Proposed Mechanism for Reductive Acylation Using a Hg(II) Trapping Agent



of PhHgCl at 0.08 mM increased the rate of enamine depletion for both experiments, much more so for **1**.

The approximately 10-fold increase in rate[10] in the presence of Hg(II) can be attributed to the redox reaction that is assisted by the ability of Hg(II) to shift the unfavorable equilibrium to the adduct **II** (Scheme 3) by irreversible trapping of the free thiolate generated during the reaction of the enamine with the lipoic acid. Efforts to identify **II** have proven futile to date. The ionic character and metal chelation of the product made it difficult to analyze it by GC−MS. Attempts to produce larger amounts of product also failed because higher concentrations of the reaction mixture tended to promote polymerization. A different thiolate trapping agent iodoacetamide exhibited high reactivity with the enamine, even higher than did PhHgCl, hence kinetic studies with this reagent were not pursued.

Treatment of **11** (with an unprotected 2-α-alcohol version of **9**) with Et_3N resulted in the prompt reduction of Hg(II) into elemental Hg by the enamine, probably due to its high redox potential demonstrated previously.[2d] Therefore, derivatives of **1** bearing the free 2-α-hydroxyl group were not synthesized.

Based on these results, we draw the following conclusions: (1) The intramolecular bis-coenzyme **1** can indeed undergo a model reaction for the reductive acylation by providing a high local concentration of reactants. To the best of our knowledge, no other models have demonstrated directly oxidation of the enamine/2-α-carbanion (rather than of the 2- α-hydroxyalkyl-ThDP, as sometimes written) with lipoic acid; (2) Formation of the tetrahedral adduct **I** is a slow process even in the presence of a thiolate trapping agent, suggesting that this may indeed be the rate-limiting step in the enzyme system. Therefore, effective activation of the deceptively unreactive dithiolane ring to reduction remains an important challenge for future research and is most likely one of the important roles of the 2-oxoacid dehydrogenase multienzyme complexes; (3) Since enamines derived from both the 2-α-methoxybenzyl-3,4-dimethylthiazolium and 2-α-hydroxybenzyl-3,4-dimethylthiazolium salts showed inactivity toward lipoic acid (in contrast to the POX model that had a clear preference for the free 2-α-hydroxybenzyl analogs[2d]), the reducing power of the enamine is not a crucial issue for the acyl transfer reaction in PDHc;[11] (4) A comparison of these results with those on POX,[2d] indicates clearly that the redox step needs little or no assistance in POX but needs significant assistance by the protein in PDHc. Dividing the zero-order rate in Figure 1b (difference in slopes for **1** in the absence and the presence of PhHgCl) by the molarity of **1** yields an apparent (i.e., in the presence of 0.08 mM PhHgCl) first-order rate constant of ca. 3.2×10^{-4} s^{-1} for this model reaction, more than 10^5 times slower than the turnover numbers for POX and PDC (both ca. 60 s^{-1}/subunit).[12]

(10) This number is derived from the slopes of lines (4−3)/(2−1) (from the top) in Figure 1b.

(11) Frey, P. A.; Flournoy, D. S.; Gruys, K.; Yang, Y.-S. *Ann. N.Y. Acad. Sci.* **1989**, 21−35.

(12) PDHc has many subunits and sufficient uncertainty about its precise stoichiometry, that comparison with these other ThDP-dependent decarboxylases may be more useful.

Experimental Section

General. ^{13}C and ^1H NMR studies were carried out on a Bruker WP-200SY or Varian VXR-400S spectrometer. GC−MS spectra were obtained on a Finnigan MAT INCOS 50 mass spectrometer equipped with an HP 5890A GC. UV−Vis spectra were recorded on a Varian DMS-300 spectrometer. Microanalyses were performed by Robertson Microlit Laboratories, Inc. Flash chromatography refers to the method described by Still[13] using Fisher 200−400 mesh silica gel.

Materials. All chemicals were reagent grade and were used without further purification unless otherwise stated. 4-Methylthiazole and 4-methyl-5-thiazolethanol were purchased from Pyrazine Specialties, Inc. All solvents were purchased from Fisher unless otherwise indicated. THF and diethyl ether were dried by distilling from sodium and benzophenone, respectively. Methylene chloride was distilled from CaH_2 and stored over activated 3-Å molecular sieves. DMSO and DMF, purchased from Aldrich as anhydrous grade, were stored over activated 3-Å molecular sieves overnight prior to use. The 3-Å molecular sieves purchased from Aldrich were activated by flame drying followed by purging with Ar.

2-(1′-Hydroxybenzyl)-4-methyl-5-(tetrahydropyranoxyethane)thiazole (3). To the solution of THP-protected 4-methyl-5-thiazolethanol **2** (3.00 g, 13.2 mmol) in dry THF (35 mL) was added *n*-butyllithium (1.51 M, 8.8 mL) dropwise at −78 °C under nitrogen. After 30 min, a solution of benzaldehyde (2.10 g, 19.8 mmol, 1.5 equiv) in THF (5 mL) was added dropwise, and the mixture was stirred at −78 °C for 1 h. The cooling bath was removed, and the mixture was warmed to room temperature. After being quenched with water (5 mL), the reaction mixture was partitioned between ether (100 mL) and saturated NH_4Cl (50 mL). The organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to give the crude product that was purified by flash chromatography (70% EtOAc/petroleum ether). The product was then recrystallized from EtOAc/petroleum ether to yield 3.80 g (86%) of **3** as white cubic crystals: mp 95−98 °C; ^1H NMR ($CDCl_3$/TMS) δ 1.23−1.88 (m, 6 H), 2.27 (s, 3 H), 2.92 (t, 2 H, $J = 6$ Hz), 3.28−3.93 (m, 4 H), 4.62 (m, 1 H), 5.50 (s, 2 H), 7.15−7.46 (m, 5 H). Anal. Calcd for $C_{18}H_{23}NOS{\cdot}H_2O$: C, 64.84; H, 6.95; N, 4.2. Found: C, 63.87; H, 6.99; N, 4.03

2-(1′-Methoxybenzyl)-4-methyl-5-(tetrahydropyranoxyethane)thiazole (4). To the suspension of NaH (1.1 equiv 60%, 270 mg, 11.25 mmol) pre-washed with hexane in dry THF (10 mL) was added thiazole alcohol **3** (3.40 g, 10.2 mmol) in THF (20 mL) at 0 °C under nitrogen. The reaction mixture was stirred at room temperature for 30 min, then MeI (3.0 equiv, 4.34 g, 30.6 mmol) was added in one portion. After 2 h, the reaction was quenched by adding saturated NH_4Cl (10 mL). To the reaction mixture was added ether (100 mL), then it was washed with water and brine and dried over $MgSO_4$. The solvent was removed in vacuo to give the crude product that was purified by flash chromatography (30% EtOAc/petroleum ether) to afford 2.84 g (81%) of **4**. An analytical sample was obtained by vacuum distillation as a colorless oil: bp 108−110 °C (0.05 mmHg); ^1H NMR ($CDCl_3$/TMS) δ 1.25−1.87(m, 6 H), 2.26 (s, 3 H), 2.93 (t, 2 H, $J = 6$ Hz), 3.40 (s, 3 H), 3.28−3.93 (m, 4 H), 4.64 (m, 1 H), 5.51(s, 2 H), 7.17−7.45 (m, 5 H).

2-(1′-Methoxybenzyl)-4-methyl-5-thiazolethanol (5). Thiazole **4** (2.00 g, 5.99 mmol) and TsOH (114 mg, 0.6 mmol) in methanol (50 mL) were heated to reflux for 1 h. After the solution was cooled to room temperature, the product was isolated by removing the methanol in vacuo followed by workup with ether (100 mL) and saturated aqueous $NaHCO_3$ (50 mL), dried over $MgSO_4$, and concentrated to give the crude product, which was purified by flash chromatography (methanol/EtOAc/petroleum ether, 0.1:70:30). The product was recrystallized from EtOAc/petroleum ether to yield 1.25 g (75%) of **5** as white cubic crystals: mp 139−141 °C; ^1H NMR ($CDCl_3$/TMS) δ 2.35 (s, 3 H, C4-Me), 2.91 (t, 2 H, C5-CH_2, $J = 6.4$ Hz), 3.42 (s, 3 H, OCH_3), 3.75 (t, 2 H, CH_2CH_2OH, $J = 6.4$ Hz), 5.46 (s, 1 H, CHC$_6$H$_5$), 7.30−7.43 (m, 5 H, Ar-H). Anal. Calcd for $C_{14}H_{17}NO_2S$: C, 63.85; H, 6.51; N, 5.32. Found: C, 63.69; H, 6.43; N, 5.14.

α-Bromo-*O*-xylenyl 2-(1′-methoxybenzyl)-4-methyl-5-thiazolethanoxyl Ether (6). To a suspension of oil-free NaH (1.2 equiv, 60%,

(13) Still, W. S.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923−2925.

180 mg) in 10 mL of dry THF was added thiazole **5** (1.02 g, 3.70 mmol) in THF (2 mL) at room temperature under nitrogen. The mixture was heated to reflux for 2 h, cooled to room temperature, and α,α′-dibromoxylene (2.0 equiv, 1.95 g, 7.4 mmol) was added. The mixture was refluxed for 24 h and cooled to room temperature, and 100 mL of ether was added. The mixture was washed with saturated NH_4Cl, water, and brine. The organic layer was dried over $MgSO_4$ and concentrated in vacuo to give the crude product, which was purified by flash chromatography (30% EtOAc/petroleum ether) to provide 0.870 g (53%) of **6** as a light yellow oil: ^{1}H NMR ($CDCl_3$/TMS) δ 2.24 (s, 3 H, C4-Me), 2.93 (t, 2 H, C5-C*H*$_2$, J = 6.3 Hz), 3.38 (s, 3 H, OC*H*$_3$), 3.58 (t, 2 H, CH_2CH_2O, J = 6.3 Hz), 4.41 (s, 2 H, C*H*$_2$Br), 4.57 (s, 2 H, OC*H*$_2$C$_6$H$_5$), 5.43 (s, 1 H, C*H*C$_6$H$_5$), 7.21−7.42 (m, 9 H, Ar-H, Ar-H). Anal. Calcd for $C_{22}H_{24}NO_2SBr$: C, 59.19; H, 5.42; N,3.14. Found: C, 58.27; H, 5.42; N, 2.99.

α-Hydroxy-*O*-xylenyl 2-(1′-methoxybenzyl)-4-methyl-5-thiazolethanoxyl Ether (7). Thiazole **6** (840 mg, 1.88 mmol) and $CaCO_3$ (1.88 g, 18.8 mmol) in water (10 mL) and dioxane (10 mL) were heated to reflux for 12 h under nitrogen. The mixture was filtered and concentrated to remove the dioxane. The water layer was extracted with ether (3 × 30 mL). The combined organic layer was dried over $MgSO_4$ and concentrated in vacuo to give a crude product that was purified by flash chromatography (70% EtOAc/petroleum ether). The product was recrystallized from EtOAc/petroleum ether to yield 3.80 g (86%) of **7** as white cubic crystals: mp 167−171 °C; ^{1}H NMR ($CDCl_3$/TMS) δ 2.22 (s, 3 H, C4-Me), 2.89 (t, 2 H, C5-C*H*$_2$, J = 6.7 Hz), 3.37 (s, 3 H, OC*H*$_3$), 3.57 (t, 2 H, CH_2CH_2O, J = 6.7 Hz), 4.53 (s, 2 H, C*H*$_2$OH), 4.54 (s, 2 H, OC*H*$_2$C$_6$H$_5$), 5.42 (s, 1 H, C*H*C$_6$H$_5$), 7.19−7.38 (m, 9 H, Ar-H, Ar-H). Anal. Calcd for $C_{22}H_{25}NO_3S$: C, 68.90; H, 6.57; N, 3.65. Found: C, 68.63; H, 6.54; N, 3.54

***O*-Xylenyl 2-(1′-methoxybenzyl)-4-methyl-5-thiazolethanoxyl Ether Lipoate (8).** To the solution of thiazole **7** (220 mg, 0.52 mmol) in CH_2Cl_2 (2 mL) was added DMAP (0.2 equiv, 14 mg, 0.11 mmol), lipoic acid (0.68 mmol, 140 mg), and DCC (0.52 mmol, 129 mg) at 0 °C under N_2. The solution was stirred overnight at room temperature. After dilution with ether (10 mL), the mixture was filtered and washed with ether (5 mL). The filtrate was concentrated to give the crude product that was purified by flash chromatography (40% EtOAc/petroleum ether) to provide 260 mg of **8** (79%), which solidified after standing at −20 °C: mp 87−89 °C; ^{1}H NMR (400 MHz, $CDCl_3$/TMS) δ 1.30−1.48 (m, 2 H), 1.58 (m, 4 H), 1.80 (m, 1 H), 2.24 (s, 3 H, C4-Me), 2.28 (t, 2 H, J = 7 Hz), 2.38 (m, 1 H), 2.91 (t, 2 H, C5-C*H*$_2$, J = 6.7 Hz), 2.98−3.12 (m, 2 H), 3.37 (s, 3 H, OC*H*$_3$), 3.43 (m, 1 H), 3.57 (t, 2 H, CH_2CH_2O, J = 6.7 Hz), 4.51 (s, 2 H, C*H*$_2$OCO), 5.08 (s, 2 H, OC*H*$_2$C$_6$H$_5$), 5.43 (s, 1 H, C*H*C$_6$H$_5$), 7.19−7.41 (m, 9 H, Ar-H, Ar-H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 15.1, 24.8, 27.3, 26.8, 34.2, 34.7, 38.6, 40.3, 56.4, 57.7, 63.7, 70.5, 83.2, 127.1, 128.3, 128.5, 128.6, 128.8, 129.1, 129.6, 134.5, 136.6, 139.9, 148.4, 168.8, 173.5.

Bis-coenzyme Model (1). To the solution of thiazole **8** (100 mg, 0.175 mmol) in CH_2Cl_2 (2 mL) at −15 °C under N_2 was added methyl triflate (28.7 mg, 0.175 mmol) in one portion. After 30 min, the dry ice/ethylene glycol bath was removed, and the mixture was stirred at room temperature until the side product (R_f = 0.20, CH_2Cl_2/MeOH, 15:1) started to accumulate. The reaction mixture was directly applied to preparative TLC (CH_2Cl_2/MeOH, 15:1) to give 55 mg (43%) of **1** as a brown oil: ^{1}H NMR (400 MHz, $CDCl_3$/TMS) δ 1.30−1.48 (m, 2 H), 1.59 (m, 4 H), 1.80 (m, 1 H), 2.28 (t, 2 H, J = 7 Hz), 2.35 (s, 3 H, C4-Me), 2.38 (m, 1 H), 2.96 (t, 2 H, C5-C*H*$_2$, J = 6.7 Hz), 2.98−3.12 (m, 2 H), 3.39 (s, 3 H, OC*H*$_3$), 3.43 (m, 1 H), 3.63 (t, 2 H, CH_2CH_2O, J = 6.7 Hz), 3.82 (s, 3 H), 4.51 (s, 2 H, C*H*$_2$OCO), 5.08 (s, 2 H, OC*H*$_2$C$_6$H$_5$), 5.93 (s, 1 H, C*H*C$_6$H$_5$), 7.15−7.36 (m, 9 H, Ar-H, Ar-H); ^{13}C NMR (400 MHz, $CDCl_3$/TMS) δ 12.8, 24.5, 27.6, 29.4, 34.1, 34.7, 38.2, 38.4, 40.3, 56.7, 57.9, 63.5, 68.2, 71.4, 79.1, 128.6 (2C), 128.7, 129.5, 129.6, 129.8, 130.6, 133.1, 134.2, 134.3, 135.5, 143.8, 173.4, 173.5.

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