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Inhibition of Benzoylformate Decarboxylase by [*p*-(Bromomethyl)benzoyl]formate. Enzyme-Catalyzed Modification of Thiamine Pyrophosphate by Halide Elimination and Tautomerization

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Benzoylformate decarboxylase (benzoylformate carboxy-lyase, BFD; EC 4.1.1.7) from *Pseudomonas putida* catalyzes the decarboxylation of this *a*-keto acid to yield benzaldehyde and CO_2 (Scheme I).[1] The reaction requires thiamine pyrophosphate (TPP) as cofactor suggesting that the mechanism involves the formation of a covalent substrate–cofactor intermediate capable of stabilizing the carbanion generted by decarboxylation. Previous efforts by a number of groups have established this process for the thiaminepyrophosphate-dependent pyruvate decarboxylases.[3] During a study of a number of substituted benzoylformate analogues, we found that [*p*-(bromomethyl)benzoyl]formate (**1**)[4] was a remarkably potent inhibitor of BFD (K_i = 0.3 μM; for benzoylformate, K_m = 0.4 mM). In this report, we establish that the inhibition is due to an unusual enzyme-catalyzed processing of **1** resulting in decarboxylation, bromide ion elimination, and re-aromatization by tautomerization.

Reaction of BFD (1 unit) and **1** (50 μM) afforded quantitative release of bromide ion (Figure 1).[5] In the presence of 1 mM TPP, bromide ion release under these conditions was complete in ~80 min.[6] Addition of 5 mM benzoylformate resulted in a transient inhibition of bromide ion release, clearly indicative of substrate protection of the enzyme. In the absence of excess TPP, however, a slow generation of bromide ion was observed which required >36 h to reach completion. Nonenzymatic, TPP-dependent generation of bromide ion was found to be negligible. The recovery of enzyme activity in the presence of 1 mM TPP as determined by the rate of benzaldehyde formation (Figure 1, inset) exhibited a time course similar to that for bromide ion release. In the absence of excess TPP no recovery of enzyme activity was found during the same time period.

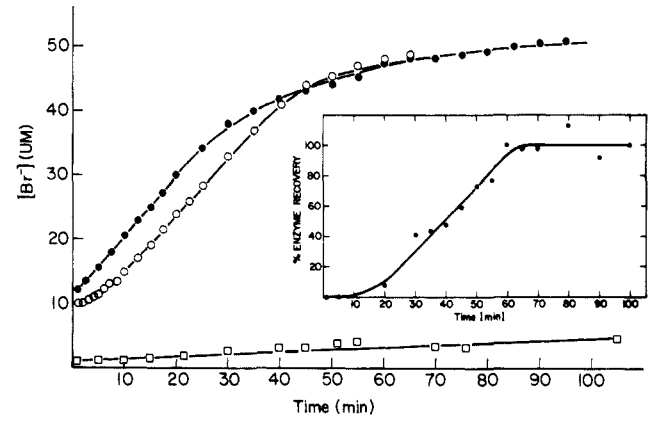
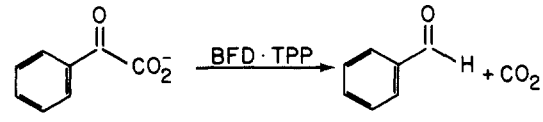
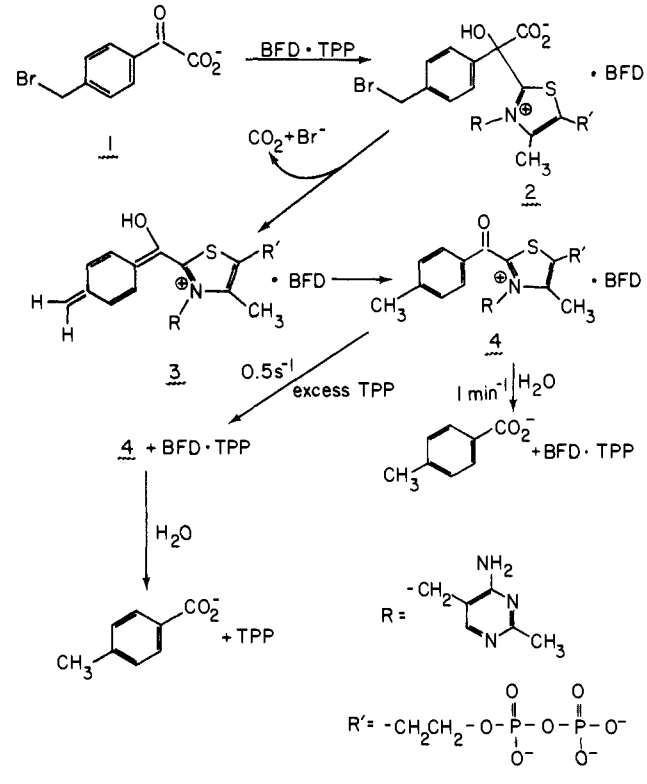


Figure 1. Analysis of BFD-catalyzed bromide ion release from (*p*-(bromomethyl)benzoyl]formate (**1**). Experimental procedure is described in ref 5. In addition to **1** (50 μM) and BFD (1 unit), reaction mixtures contained no added TPP (□), 1 mM TPP (●), and 1 mM TPP plus 5 mM benzoylformate (○). Background at time zero for TPP-containing reactions is due to residual chloride ion. (Inset) Time course of recovery of BFD activity in the presence of **1** (50 μM) and TPP (1 mM). Recovery was measured as the percent of maximal benzaldehyde formation from benzoylformate.[2]

Scheme I



Scheme II



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[⊥] American Cancer Society Faculty Research Awardee (1983–1988).

(1) Benzoylformate decarboxylase was isolated according to: Hegeman, G. D. *Methods Enzymol.* **1970**, *17A*, 674. The enzyme had a final specific activity of 34 units/mg and is stored in the presence of 1 mg/mL bovine serum albumin.[2] A coupled assay[2] with equine liver alcohol dehydrogenase was used. Pyruvate is neither a substrate nor an inhibitor of BFD.[2]

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(4) (*p*-Methylbenzoyl)formic acid (Barnish, I. T.; Cross, P. E.; Danilewicz, J. C.; Dickinson, R. P.; Stopher, D. A. *J. Med. Chem.* **1981**, *24*, 399) was treated with 1.1 equiv of *N*-bromosuccinimide in refluxing CCl_4 for 90 min in the presence of a trace of benzoyl peroxide and Pyrex-filtered UV light (275 W). The organic layer was washed with H_2O, driev over anhydrous Na_2SO_4, and concentrated in vacuo. Recrystallization from hot toluene/hexane afforded pale yellow crystals (mp 68–70 °C; yield 54%). UV (H_2O λ_{max} 265 nm; ϵ 22500 M^{-1} cm^{-1}. 1H NMR (acetone-d_6) δ 7.5 (2 H, d), 7.15 (2 H, d), 4.2 (2 H, s). Elemental analysis for $C_9H_7O_9Br \cdot H_2O$. Theoretical: C, 41.41; H, 3.47. Found: C, 41.14; H, 3.20.

(5) Bromide ion release was measured on an Orion Model 811 pH meter equipped with an Orion Model 94-35 bromide electrode and a Model 90-01 single-junction reference electrode. Experiments were performed on 2 mL of assay solution (see Figure 1) containing 0.1 M potassium phosphate buffer (pH 7.0) with 0.1 M sodium nitrate to stabilize electrode performance. The enzyme was dialyzed against 0.05 M potassium phosphate (pH 6.0) to remove excess thiamine pyrophosphate and chloride, the latter of which affects the electrode. The electrode was not disturbed during the course of each experiment.

(6) The rate of consumption of **1** is ~1% that of benzoylformate (turnover number ~70 s^{-1})[2] under the same reaction conditions.

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We have established that the aromatic product formed quantitatively from the enzymatic processing of **1** is *p*-methylbenzoate,[7] indicating a complete diversion from the normal reaction pathway leading to aldehyde. Moreover, no irreversible inactivation of BFD has been detected.

We believe that these results constitute a novel example of enzyme-catalyzed halide elimination and inhibition of a TPP-dependent enzyme. Decarboxylation and bromide ion elimination from the enzyme-bound adduct formed from TPP and **1** (**2**; Scheme II) would generate the quinone-like **3**. This intermediate may undergo either enzyme-catalyzed or chemical tautomerization to yield (*p*-methylbenzoyl)-TPP (**4**). In the presence of excess TPP dissociation of **4** from BFD would permit an unmodified TPP to "rescue" the enzyme, resulting in a remarkably rapid turnover determined by the dissociation constant (k_{off}) for **4**.[8] Without excess TPP turnover of **1** is evidently dependent upon the hydrolysis of **4**. Since BFD does not normally catalyze hydrolysis of an acyl-TPP, turnover without excess TPP is ultimately determined by this process, which must be relatively slow.[9]

Leung and Frey[10] have reported that 3-fluoropyruvate undergoes decarboxylation, fluoride elimination, and tautomerization by pyruvate dehydrogenase; on the other hand, 3-chloro- and 3-bromopyruvate appear to be much more complex in their reactions with this enzyme.[11] Also, Kuo and Jordan[12] have found that (*E*)-4-(4-chlorophenyl)-2-keto-3-butenoic acid functions as an irreversible (Michael-type) inhibitor of pyruvate decarboxylase. Our findings are somewhat reminiscent of the inactivation of γ-aminobutyric acid transaminase by gabaculine.[13] In that case, covalent modification of pyridoxal was achieved by aromatization of the gabaculine–pyridoxal adduct via an enzyme-catalyzed tautomerization.

Enzyme-catalyzed eliminations of halide ion through an intervening phenyl group are intriguing phenomena which have been proposed for the formation of quinone–methide intermediates by bioreductive alkylating agents.[14] The present study constitutes a clear example of this process by a carbanionic elimination.[15]

Acknowledgment. This work was supported by USPHS Grants GM 35066 and GM 35067 (J.W.K.) and AM 17323 and CA 37655 (G.L.K.). L.J.D. gratefully acknowledges an NSF Predoctoral Fellowship (1981–1984) and is a Ph.D. candidate at Yale University. We thank Paul Weiss and Dr. Paul F. Cook for help with preliminary experiments. We also thank Dr. Frank Jordan for a sample of (*E*)-4-(4-chlorophenyl)-2-keto-3-butenoic acid.

(7) An ethereal extract of an acidified, completed reaction mixture was treated with diazomethane. The resulting methyl *p*-toluate was identified and quantitated by comparison with an authentic sample, using gas chromatography–mass spectrometry.

(8) These results suggest that $k_{off} \sim 0.5\ s^{-1}$. The reported Km for TPP is 1 μM.[1]

(9) We estimate that $k_{hydrolysis} \sim 1\ min^{-1}$. While this rate is slow relative to the other enzymatic rate constants, it agrees well with the reported instability of 2-benzoyl-3,4-dimethylthiazolium iodide, an analogue of **4**, to hydrolysis and methanolysis: White, F. G.; Ingraham, L. L. *J. Am. Chem. Soc.* **1962**, *84*, 3109.

(10) Leung, L. S.; Frey, P. A. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 274. In contrast, our preliminary studies show that [*p*-(fluoromethyl)-benzoyl]formate is a normal substrate for and not an inhibitor of BFD. This suggests that the intervening phenyl group imposes an energy barrier sufficient to prevent fluoride but not bromide elimination. The chloro analogue is currently under study.

(11) Apfel, M. A.; Ikeda, B. H.; Speckhard, D. C.; Frey, P. A. *J. Biol. Chem.* **1984**, *259*, 2905. Pyruvate dehydrogenase is a multienzyme complex containing three distinct enzymatic activities: pyruvate decarboxylase, dihydrolipoamide transacetylase, and dihydrolipoamide dehydrogenase. Both the inherent complexity of this system and the potential for affinity labeling by 3-chloro- and 3-bromopyruvates probably account for the multiple sites and modes of inactivation that were observed for these compounds.

(12) Kuo, D. J.; Jordan, F. *Biochemistry* **1983**, *22*, 3735; *J. Biol. Chem.* **1983**, *258*, 13415. We have determined that (*E*)-4-(4-chlorophenyl)-2-keto-3-butenoic acid does not inhibit BFD.

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(14) Lin, A. J.; Sartorelli, A. C. *J. Org. Chem.* **1973**, *38*, 813.

(15) (Halomethyl)coumarin derivatives have been shown to inactivate α-chymotrypsin by oxyanion-induced halide elimination: Bechet, J.-J.; Dupaix, A.; Yon, J.; Wakselman, M.; Robert, J.-C.; Vilkas, M. *Eur. J. Biochem.* **1973**, *35*, 527–539.

Tris(benzocyclobutadieno)benzene, the Triangular [4]Phenylene with a Completely Bond-Fixed Cyclohexatriene Ring: Cobalt-Catalyzed Synthesis from Hexaethynylbenzene and Thermal Ring Opening to 1,2:5,6:9,10-Tribenzo-3,4,7,8,11,12-hexadehydro[12]-annulene

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1,3,5-Cyclohexatriene is commonly introduced in organic textbooks as the hypothetical model for a bond-fixed benzene, yet the search for this structural unit has been elusive.[1] We report two facile approaches to the tris(benzocyclobutadieno)benzene nucleus **1**, including the parent **1b**, in which the central benzene ring exists in this form, and the first thermal retrocycloaddition of a benzene ring to a trialkyne. Our strategy relies on an iterative sequence of palladium-catalyzed alkynylations followed by cobalt-catalyzed cyclobutabenzoannelations.[2]

The simplest approach to **1** is outlined in Scheme I and involves the also theoretically interesting hexaethynylbenzene (**2**)[3] as an intermediate and its 3-fold cyclization in which a record number (for cobalt) of six rings and nine bonds are formed to give what must be an extraordinarily strained molecule.[4] Protodesilylation then furnishes the title compound **1b**.[5] Scheme II depicts a stepwise approach to the title compound, providing chemical structural corroboration, support for a stepwise cyclization path in Scheme I, and another derivative of **1**.[5] It starts with bis(2-bromophenyl)ethyne (**3**)[6] which was converted to the corresponding diiodide **4**[7] (necessary for the success of the subsequent step), subsequently to be subjected to a novel palladium-catalyzed

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(2) Berris, B. C.; Hovakeemian, G. H.; Lai, Y.-H.; Mestdagh, H.; Vollhardt, K. P. C. *J. Am. Chem. Soc.* **1985**, *107*, 5670.

(3) Diercks, R.; Armstrong, J. C.; Boese, R.; Vollhardt, K. P. C. *Angew. Chem.* **1986**, *98*, 270.

(4) The ring strain in biphenylene has been estimated to lie somewhere between 64.3 and 75.4 kcal mol^{-1}: Bedford, A. F.; Carey, J. G.; Millar, I. T.; Mortimer, C. T.; Springall, H. D. *J. Chem. Soc.* **1962**, 3895. Good, W. D. *J. Chem. Thermodyn.* **1978**, *10*, 553.

(5) All new compounds gave satisfactory analytical and/or spectral data. For example, **1a**: yellow crystals (from acetone), mp (acetone solvate) >315 °C; MS, *m/e* (relative intensity) 732.3295 (M^+, 49, calcd for $C_{42}H_{60}Si_6$ 732.3311), 119 (15), 73 (100); ^1H NMR ($CDCl_3$, 200 MHz) δ 0.42 (s, 36 H), 2.16 (s, acetone, 6 H), 7.53 (s, 6 H); ^{13}C NMR ($CDCl_3$) δ 2.33, 125.50, 131.23, 147.56, 148.88; IR (KBr), 2960, 2905, 1750 (acetone), 1270, 1253, 1067, 850, 753, 653 cm^{-1}; UV (hexane) λ_{max} 242 (log ϵ = 4.72), 251 (4.79), 291 (4.90), 299 (5.09), 314 (5.33), 347 (4.47), 364 (4.33), 376 sh (4.21), 394 sh (4.06) nm. **1b**: yellow crystals, mp 248 °C; MS/ *m/e* (relative intensity) 300.0937 (M^+, 100, calcd for $C_{24}H_{12}$ 300.0939), 272 (22); ^1H NMR (300 MHz, acetone-d_6) δ 7.24 (AA′ m, 6 H), 7.31 (BB′ m, 6 H); (250 MHz, $CDCl_3$) δ 7.19 (s); ^{13}C NMR ($CDCl_3$) δ 119.79, 128.63, 130.13, 148.44; IR (KBr), 3050, 2920, 1775, 1760, 1451, 1432, 1358, 1167, 1120, 750, 732, 697 cm^{-1}; UV (hexane) λ_{max} 227 sh (log ϵ = 4.22), 234 (4.39), 243 (4.56), 269 (4.38), 282 (4.61), 297 (4.85), 324 sh (3.91), 336 (4.02), 352 (3.90), 363 sh (3.7), 379 sh (3.4) nm. **4**: colorless crystals, mp 103 °C. **5**: colorless crystals, mp 174–175 °C; MS, *m/e* (relative intensity) 418.1571 (M^+, 100, calcd for $C_{28}H_{26}Si_2$ 418.1573), 73 (22); ^1H NMR (200 MHz, $CDCl_3$) δ 0.26 (s, 18 H), 7.34 (m, 4 H), 7.50 (m, 2 H), 7.60 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ −0.39, 75.22, 78.21, 88.02, 91.88, 92.23, 123.99, 127.00, 128.23, 128.86, 132.33, 132.75; IR (KBr) 2970, 2210, 2107, 1490, 1253, 1040, 860, 760 cm^{-1}. **6**: yellow crystals, mp 186 °C; MS, *m/e* (relative intensity) 418.1572 (M^+, 100, calcd for $C_{28}H_{26}Si_2$ 418.1573), 73 (30); ^1H NMR (200 MHz, $CDCl_3$) δ 0.27 (s, 18 H), 7.00 (m, 8 H); ^{13}C NMR ($CDCl_3$) δ 0.07, 99.80, 100.3, 119.2, 119.5, 129.36 (2C), 134.1, 147.8, 149.8, 149.0, 152.5; IR (KBr) 2142 cm^{-1}; UV (cyclohexane) λ_{max} 235 (log ϵ = 4.53), 247 (4.53), 280 (4.70), 293 (4.82), 305 (4.51), 314 (4.37), 320 (4.37), 331 (4.62), 398 (3.53), 422 (3.58), 452 (3.51) nm. **1c**: yellow crystals, mp 239 °C.

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