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Mechanistic Studies of a Protonolytic Organomercurial Cleaving Enzyme: Bacterial Organomercurial Lyase†

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ABSTRACT: Mechanistic studies of the protonolytic carbon–mercury bond cleavage by organomercurial lyase from *Escherichia coli* (R831) suggest that the reaction proceeds via an S_E2 pathway. Studies with stereochemically defined substrates *cis*-2-butenyl-2-mercuric chloride (**1**) and *endo*-norbornyl-2-mercuric bromide (**2**) reveal that a high degree of configurational retention occurs during the bond cleavage, while studies with *exo*-3-acetoxynortricyclyl-5-mercuric bromide (**3**) and *cis-exo*-2-acetoxy-bicyclo[2.2.1]hept-5-enyl-3-mercuric bromide (**4**) show that the protonolysis proceeds without accompanying skeletal rearrangement. Kinetic data for the enzymatic reactions of *cis*-2-butenyl-2-mercuric chloride (**1**) and *trans*-1-propenyl-1-mercuric chloride (**6**) indicate that these substrates show enhanced reaction rates of ca. 10–200-fold over alkylvinylmercurials and unsubstituted vinylmercurials, suggesting that the olefinic methyl substituent may stabilize an intermediate bearing some positive charge. Enzymatic reaction of 2-butenyl-1-mercuric bromide (**5**) yields a 72/23/5 mixture of 1-butene/*trans*-2-butene/*cis*-2-butene, indicative of intervening S_E2' cleavage. The observation of significant solvent deuterium isotope effects at pH 7.4 of $V_{max}(H_2O)/V_{max}(D_2O) = 2.1$ for *cis*-2-butenyl-2-mercuric chloride (**1**) turnover and $V_{max}(H_2O)/V_{max}(D_2O) = 4.9$ for ethylmercuric chloride turnover provides additional support for a kinetically important proton delivery. Finally, the stoichiometric formation of butene and Hg(II) from **1** and methane and Hg(II) from methylmercuric chloride eliminates the possibility of an S_N1 solvolytic mechanism. As the first well-characterized enzymatic reaction of an organometallic substrate and the first example of an enzyme-mediated S_E2 reaction the organomercurial lyase catalyzed carbon–mercury bond cleavage provides an arena for investigating novel enzyme structure–function relationships.

Organomercurial lyase mediates the first of two steps in the microbial detoxification of organomercurial salts (Summers, 1985; Robinson & Tuovinen, 1984; Silver & Kinscherf, 1982; Summers & Silver, 1978). This first step involves protonolysis of the carbon–mercury bond to yield Hg(II) and the corresponding hydrocarbon (eq 1 and 2). In a second step the

$$\text{R-Hg-X} \xrightarrow{\text{organomercurial lyase}} \text{R-H} + \text{Hg(II)} \quad (1)$$

$$\text{Hg(II)} \xrightarrow[\text{NADPH}\quad\text{NADP}^+]{\text{mercuric reductase}} \text{Hg(O)} \quad (2)$$

Hg(II) is reduced to volatile Hg (0) in an NADPH-dependent reaction mediated by mercuric reductase, completing the detoxification (Fox & Walsh, 1982, 1983; Brown, 1985). In the preceding paper we have described the overproduction, purification, and preliminary characterization of cloned bacterial organomercurial lyase (Begley et al., 1986) from *Escherichia coli* (R831) (Schottel, 1978). This enzyme is one of the few known to process an organometallic compound.

The task of organomercurial lyase is by no means trivial given the stability of organomercurial salts toward strong acids (Jensen & Rickborn, 1968) and bases (Makarova & Nesmeyanov, 1967). The mechanism by which the enzyme effects bond cleavage is thus of fundamental interest. In addition, the environmental consequences of heavy-metal pollutants

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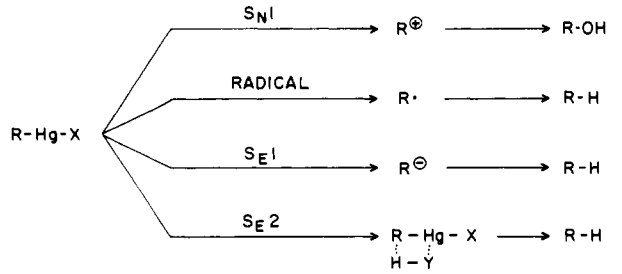


FIGURE 1: Possible mechanistic pathways for protonolysis of organomercurials.

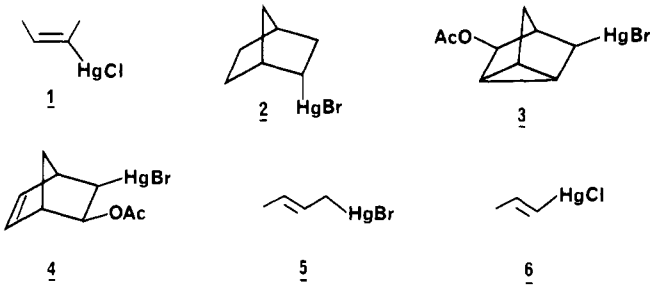


FIGURE 2: Organomercurials used in mechanistic study of organomercurial lyase.

(Thayer, 1984), the microbiological significance of the detoxification pathway (Brown, 1985; Williams & Silver, 1984), and reports of a similar enzymatic process in mammals (Norseth & Clarkson, 1970; Ishihara & Suzuki, 1976) contribute to the significance of this bacterial enzymatic reaction. We report herein our efforts to decipher the mechanistic route of this enzymatic organometallic reaction.

Electrophilic reactions of the carbon–mercury bond can in principle occur via any of four different pathways, depending on the structure of the organomercurial and the reaction conditions (Figure 1) (Jensen & Rickborn, 1968; Bamford & Tipper, 1973). For example, solvolytic S_N1 cleavage generally occurs either when the intermediate carbenium ion is stabilized (Rappoport et al., 1972) or when the mercury atom bears a good leaving group (e.g., acetate) (Jensen & Ouellette, 1961a,b, 1963a,b; Jensen & Rickborn, 1968). The rarer S_E1-type cleavage has been observed only when the resulting carbanion is particularly stabilized (Dodd & Johnson, 1969; Coad & Johnson, 1967; Hughes et al., 1964). Homolytic radical cleavage (S_H2) has been demonstrated in the protonolyses of diorganomercurials, although not conclusively in the case of organomercurial salts (Ingold & Roberts, 1971). Finally, S_E2-type cleavage has been demonstrated by kinetic and stereochemical studies in the protonolyses of vinylmercurial salts and diorganomercurials, while kinetic data have been presented that suggest this pathway for alkylmercurial salts (Jensen & Rickborn, 1968; Kreevoy, 1957; Kreevoy & Hansen, 1961).

Mechanistic studies on the electrophilic reactions of various organomercurials have relied heavily on the use of stereochemically defined substrates (Figure 2) such as **1** and **2** (Casey et al., 1973; Whitesides & SanFilippo, 1970) wherein subsequent retention, inversion, or scrambling of stereochemistry at the reacting carbon center is monitored. Substrates such as **3** and **4** have likewise been used as radical probes wherein skeletal rearrangement is monitored (Whitesides & SanFilippo, 1970). Common in these studies as well as those of others (Jensen & Rickborn, 1968) is the finding that the reaction products (and thus mechanistic pathway) can be dependent on solvent and reaction conditions, as well as substrate and reagent structure. Nevertheless, the use of stereochemically defined substrates has proved useful, and we have utilized this approach as one aspect of our enzymatic studies. In addition to the stereochemical results we report here various kinetic data that further our understanding of the organomercurial lyase catalyzed carbon–mercury bond cleavage.

Experimental Procedures

Materials.[1] The bacterial enzymes organomercurial lyase and mercuric reductase were purified to homogeneity as described by Begley et al. (1986) and Fox and Walsh (1982), respectively. NADPH stock solutions (20 mM, H_2O) and organomercurial stock solutions (100 mM, Me_2SO) were freshly prepared before use. L-Cysteine, NADPH, CAPS, and bis-tris-propane were from Sigma Chemical Co. D_2O (99.8% d), 1-butyne, norbornene, and 1-bromo-2-butene were from Aldrich Chemical Co. 2-Mercaptoethanol was from Baker Co. For experiments utilizing gas chromatography, column A refers to an 80% Porapak N/20% Porapak Q column (0.25 in. × 8 ft) in a Carle-Hach Model AGC-100 instrument operating at 130 °C; column B refers to a 4.1% Carbowax on Chromosorb G column (0.25 in. × 10 ft) in a Perkin-Elmer Sigma-3 instrument operating at 140 °C. Analyses of gaseous products, both for analytical and for kinetic experiments, were carried out by injecting 50-μL aliquots of the reaction headspace (4-mL total volume; injection size $^1/_{80}$th of headspace). nongaseous products were analyzed by extracting the assay mixture with pentane and injecting aliquots into the GC. Calibration of the GC indicated that generally a minimum of 0.1–1.0 pmol of compound could be detected, depending on the compound analyzed. Fourier transform infrared spectra were measured by James Bentsen on a Nicolet 60-SX instrument. Spectra were obtained at room temperature with a 1-mm path length cell, from 240 transient accumulations. Spectral analysis using the calibration curve of SanFilippo (1970) indicated a detection limit of 10% for the *exo*-2-d_1 isomer.

Buffer 1 consisted of 100 mM sodium phosphate and 10 mM L-cysteine (pH 7.4). This buffer oxidizes readily and was prepared immediately before use. Buffer 2 consisted of 50 mM bis-tris-propane, 50 mM CAPS, and 10 mM 2-mercaptoethanol, titrated to the required pH. The corresponding deuterated buffers were prepared by dissolving the components in D_2O, removing the solvent, redissolving in D_2O, and titrating with $NaOD/D_2O$ to the indicated pD. The pD value of these solutions refers to the value measured on a standard pH meter calibrated with H_2O buffers corrected to pD units. *cis*-2-Butenyl-2-mercuric chloride (**1**) was prepared from 2-butyne following the general procedure described by Larock et al. (1972). *trans*-1-Propenyl-1-mercuric chloride (**6**) was prepared as described by Foster and Tobler (1962). 2-Butenyl-1-mercuric bromide (**5**) [mp 90.5–91.0 °C; lit. (Sleezer et al., 1963) 90.8–91.2 °C] was prepared as described by Sleezer et al. (1963). High-field ^1H NMR analysis (250 MHz, $CDCl_3$) of this material indicated a mixture of two compounds (3–3.5/1) [chemical shifts (methyl protons) major, 1.68 ppm, minor, 1.71 ppm; (allylic protons) major, 2.76 ppm, minor, 2.73 ppm], with the major isomer tentatively assigned the *trans*-2-butenyl structure and the minor compound the *cis*-2-butenyl structure based on the literature precedent [see Sleezer et al. (1963)]. No evidence of the 1-butenyl isomer was found. *exo*-3-Acetoxynortricyclyl-5-mercuric bromide (**3**) and *endo*-

[1] Abbreviations: bis-tris-propane, 1,3-bis[[tris(hydroxymethyl)-methyl]amino]propane; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Me_2SO, dimethyl sulfoxide; GC, gas chromatograph; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form.

norbornyl-2-mercuric bromide (**2**) were prepared as described by Whitesides and SanFilippo (1970). *cis-exo*-2-Acetoxybicyclo[2.2.1]hept-5-enyl-3-mercuric bromide (**4**) was prepared as described by Whitesides and SanFilippo (1970) and purified by successive recrystallizations from ethyl acetate, ethanol, 2-butanol, and hexane. 2-Acetoxynortricyclylene and 2-acetoxynorbornane standards were prepared as described by Cristol et al. (1962) and purified by chromatography (silica gel/$AgNO_3$; hexane/ether, 99/1). A standard mixture of all three possible rearrangement products was prepared by sodium borohydride reduction of **3** as described by Whitesides and SanFilippo (1970).

Methods. Protonolysis of cis-2-Butenyl-2-mercuric Chloride (**1**). To a 5-mL sealable Wheaton vial was added buffer 1 (500 μL), H_2O (410 μL), organomercurial solution (10 μL), NADPH (20 μL), mercuric reductase (20 μL of 1.2 mg/mL), and organomercurial lyase (40 μL of 1.1 mg/mL, 2 nmol). After sealing and incubating at 37 °C (30 min), the reaction headspace was analyzed by GC using column 1. The product was identified by coinjection with authentic *cis*- and *trans*-2-butene, with a detection limit of 1%. Less than 1% butene was formed in the absence of organomercurial lyase. Kinetic parameters were determined by measuring the rate of 2-butene formation by GC at various concentrations of **1** (1.5, 1.0, 0.8, 0.6, 0.4 mM). The assay mixture for the kinetic studies was prepared as described above, but using 30 μL of organomercurial lyase (0.9 mg/mL, 1.2 nmol).

Deuterolysis of endo-Norbornyl-2-mercuric Bromide (**2**). A solution of organomercurial lyase in deuterated buffer 1 was prepared by concentrating 2 mL of enzyme solution (0.56 mg/mL) to 0.2 mL in a collodion bag concentrator (10000-Da cutoff), adding 10 mL of deuterated buffer 1, reconcentrating to 0.2 mL, and finally diluting with 0.8 mL of deuterated buffer 1. The deuterolysis assay mixture was prepared by combining the above organomercurial lyase solution (1.0 mL), NADPH (50 μL, 20 nM in D_2O), mercuric reductase (50 μL, 1.2 mg/mL), mercurial **3** (30 μL, 0.1 M in Me_2SO-d_6), and D_2O (1.0 mL). After incubation (37 °C, 3 h) the reaction mixture was extracted with CCl_4 (1.0 mL) and the extracts were dried ($MgSO_4$) and finally analyzed by FTIR (detection limit for *exo*-**2**-*d* isomer 10%). The absorption intensities, in combination with the extinction coefficient of the exo isomer (ϵ = 21 L mol^{-1} cm^{-1}; Begley, unpublished data), indicated that the solution analyzed contained ca. 0.4 mg/mL of *endo*-norbornane-2-*d*, indicating 100% turnover of the mercurial to deuteriohydrocarbon.

Protonolysis of exo-3-Acetoxynortricyclyl-5-mercuric Bromide (**3**). To a 5-mL sealable Wheaton vial was added buffer 1 (500 μL), H_2O (250 μL), organomercurial solution (10 μL), NADPH (20 μL), mercuric reductase (20 μL of 1.2 mg/mL), and organomercurial lyase (200 μL of 1.1 mg/mL, 10 nmol). After sealing and incubating at 37 °C (4 h), the reaction mixture was extracted with pentane (500 μL), concentrated to 50 μL, and analyzed directly by GC using column 2. The product was identified by coinjection with authentic standards, with a 1% detection limit for rearranged products. No product was formed in the absence of organomercurial lyase.

Protonolysis of cis-exo-2-Acetoxybicyclo[2.2.1]hept-5-enyl-3-mercuric Bromide (**4**). The protonolysis of **4** was carried out as described for **3** and the product identified by GC (column 2). The detection limit for rearranged products was again 1%.

Protonolysis of 2-Butenyl-1-mercuric bromide (**5**). Enzymatic turnover of **5** was carried out as described for **1**, except with 30 μL of organomercurial lyase (0.9 mg/mL, 1.2 nmol). Products were identified by GC (column 1) via coinjection with pure *cis*-2-butene, *trans*-2-butene, and 1-butene. Kinetic parameters were determined for **5** by measuring total butene formation at six concentrations of **5** (1.5, 1.0, 0.8, 0.6, 0.4, 0.2 mM). The assay mixtures were prepared as described for the kinetic analysis of **1**, except with 20 μL of organomercurial lyase (0.9 mg/mL, 1.2 nmol). Control experiments indicated that <5% protonolysis occurred in the absence of organomercurial lyase.

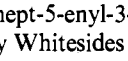
Entry	Substrate	Hydrocarbon	Hg (II)
1	 1000 nmol	 790 nmol	Hg(II) 590 nmol
2	CH_3-Hg-Cl	CH_4 0.47 nmol/min	Hg (II) 0.43 nmol/min

FIGURE 3: Stoichiometry of organomercurial turnover by organomercurial lyase. In entry 1 the total final quantities of both hydrocarbon and Hg(II) were measured. In entry 2 the rates of hydrocarbon and Hg(II) formation were measured.

Protonolysis of trans-1-Propenyl-1-mercuric Chloride (**6**). Kinetic parameters were determined for **6** by measuring the rate of propylene formation by GC (column 1) at various concentrations of **6** (1.5, 1.0, 0.8, 0.6, 0.4 mM). The assay mixtures were prepared as described for the kinetic analysis of **1**.

Determination of Stoichiometry of Hydrocarbon and Mercuric Ion Formation. (A) cis-2-Butenyl-2-mercuric Chloride (**1**) *as Substrate.* To a 5-mL sealable Wheaton vial was added 500 μL of assay buffer 1, 10 μL of mercurial (0.1 M, 1 μmol), 40 μL of mercuric reductase (1.2 mg/mL), 60 μL of NADPH (20 mM, 1.2 μmol), 50 μL of organomercurial lyase (0.9 mg/mL, 2 nmol), and 340 μL of H_2O. After sealing, the mixture was degassed with argon and then incubated at 37 °C for 2 h. A control experiment was run using all components except organomercurial lyase. Butene formation was quantitated by GC, and NADPH consumption was quantitated by measuring the change in absorbance at 340 nm of the initial and final assay mixtures (Fox & Walsh, 1982, 1983). Background NADPH consumption was determined from the absorbance change of the control sample.

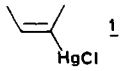
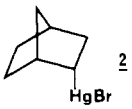
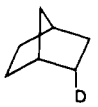
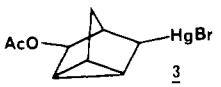
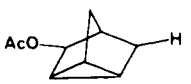
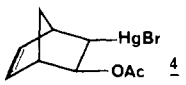
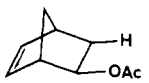
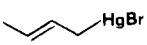
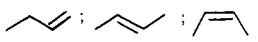
(B) Methylmercuric Chloride as Substrate. The assay mixture was prepared by mixing buffer 1 (2.50 mL), NADPH (100 μL), organomercurial lyase (500 μL), CH_3HgCl (5 μL), and H_2O (1.65 mL). Aliquots (0.95 mL) were added to 5-mL sealable Wheaton vials and incubated at 37 °C for time intervals of 15, 20, 45, 60, and 75 min. At each time, methane production was quantitated by GC (column 1) and Hg(II) was quantitated by adding mercuric reductase (50 μL, 1.2 mg/mL) and measuring the amount of NADPH consumed by the decrease in absorbance at 340 nm (Fox & Walsh, 1982, 1983).

Deuterium Isotope Effect Studies. Kinetic parameters (K_m and V_{max}) were measured in deuterated buffer 2 for ethylmercuric chloride at pD 10.1 as described for the pH–rate studies (Begley et al., 1986) and at pD 7.4 in deuterated buffer 1. Kinetic parameters for *cis*-2-butenyl-2-mercuric chloride (**1**) were likewise measured in deuterated buffer 1 (pD 7.4), as described for the protonolysis of **1**.

RESULTS

Reaction Stoichiometry. The results of the stoichiometry experiments are presented in Figure 3, where it is seen that hydrocarbon and Hg(II) are formed in essentially equal

Table I: Results of Enzymatic Protonolysis of Organomercurials **1–5**[a]

entry	substrate	product	product compn [detection limits (%)]
1	 **1**		>99% (1)
2[b]	 **2**		>90% (10)
3	 **3**		>99% (<1)
4	 **4**		>99% (<1)
5	 **5** (3-3.5:1 trans-cis)		73/23/5

[a] Substrates prepared and enzymatic reactions run as described under Materials and Methods. [b] This reaction run in deuterated buffer 1.

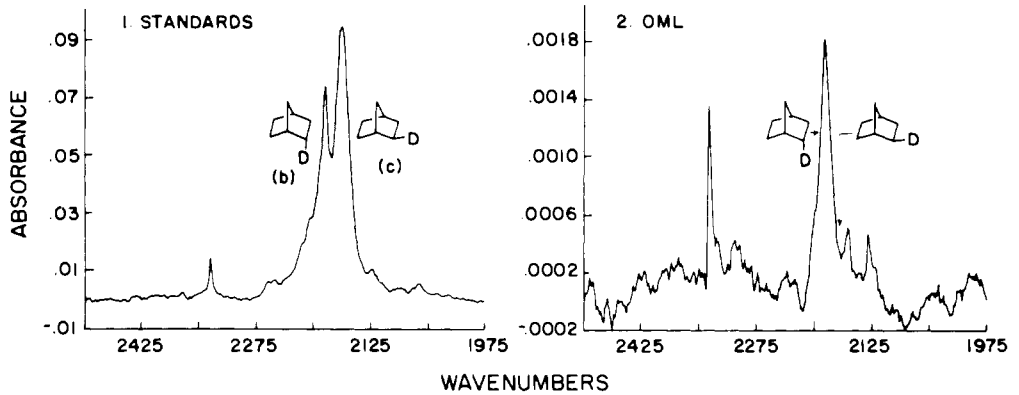


FIGURE 4: FTIR analysis of the enzymatic product from deuterolysis of mercurial **2**.

amounts. In the case of *cis*-2-butenyl-2-mercuric chloride (**1**) the total amounts of butene (790 nmol) and Hg(II) (590 nmol; assayed by coupling Hg(II) to Hg(0) reduction with the NADPH-utilizing mercuric reductase) formed are similar, and in the case of methylmercuric chloride, methane and Hg(II) are formed at essentially equal rates (0.47 and 0.43 nmol/min).

Studies with Organomercurials **1–6**. Results of the enzymatic protonolyses of substrates **1–5** are summarized in Table I.

(*a*) *Stereochemistry*. It is evident that retention of sterochemistry is observed in the enzymatic C–Hg bond cleavages of **1** and **2** (entries 1 and 2). The enzymatic processing of **1** proceeds to give exclusively *cis*-2-butene, with no *trans*-2-butene to the limits of detection by GC (1%). Likewise, deuterolysis of *endo*-mercurial **2** yields the endo deutero product, again to the limits of detection (10% based on isomer C–D peak resolution) by FTIR (Figure 4). In this case product analysis by FTIR was necessitated by the low turnover of **2** and thus the small quantity of norbornane-2-*d* that was obtained (see Methods). As seen in Figure 4, analysis of the C–D stretching region (2100–2200 cm^{-1}) provides a method for detecting the respective *endo*-**2**-d_1 and *exo*-**2**-d_1 products, although the signals are not completely distinct for each isomer. It is clear, however, that the endo deuterio isomer is the major enzymatic reaction product.

Crotylmercurial **5** provides a probe for intervening allylic rearrangement during the bond cleavage, whereby production of isomeric 1- and 2-butenes can be monitored (Figure 7). Interestingly, enzymatic protonolysis of crotylmercurial **5** proceeded to give primarily 1-butene (75%), along with some *trans*- and *cis*-2-butene (23 and 5%, respectively; entry 5).

(*b*) *Radical Probes*. Further studies with tri- and bicyclic mercurials **3** and **4** indicated that enzymatic protonolysis proceeds with *no* concomitant skeletal rearrangement, again to the limits of detection by GC (1%) (entries 3 and 4). Mercurials **3** and **4** serve as probes for radical intermediates since their corresponding carbon radicals undergo rapid skeletal rearrangement (10^8 s^{-1}; Carlsson & Ingold, 1968) as shown in Figure 5 to a diagnostic mixture of the nortricyclene and the bicyclic norbornenes (Whitesides & SanFilippo, 1970). As seen in Figure 6 the three norbornyl rearrangement products are clearly separable by GC (column 2) and would have been readily detected in the enzymatic product mixture starting from nortricyclylmercurial **3**. To further test the absence of this radical manifold in enzymic turnover we also tested the norbornenylmercurial **4** since in model studies it gives exactly the same three hydrocarbons of Figure 5 by the radical route. Again, **4** gave only the single norbornene noted in Table I. These data argue very strongly against radical intermediates in organomercurial lyase catalysis.

Butenylmercurial **1** also serves as a radical probe since the 2-butenyl radical is known to undergo rapid inversion to a mixture of *cis*- and *trans*-2-butene (10^8 s^{-1}); Fessenden & Schuler, 1963). As described in Section a, the protonolysis of **1** proceeded to give exclusively *cis*-2-butene.

(*c*) *Kinetic Parameters*. Due to difficulties in quantitating the products from **2–4** it was not feasible to measure kinetic parameters for these substrates. However, the mercurials **1**, **5**, and **6** yield readily quantitated products at high V_{max} rates and thus are poised for kinetic analysis. Kinetic parameters

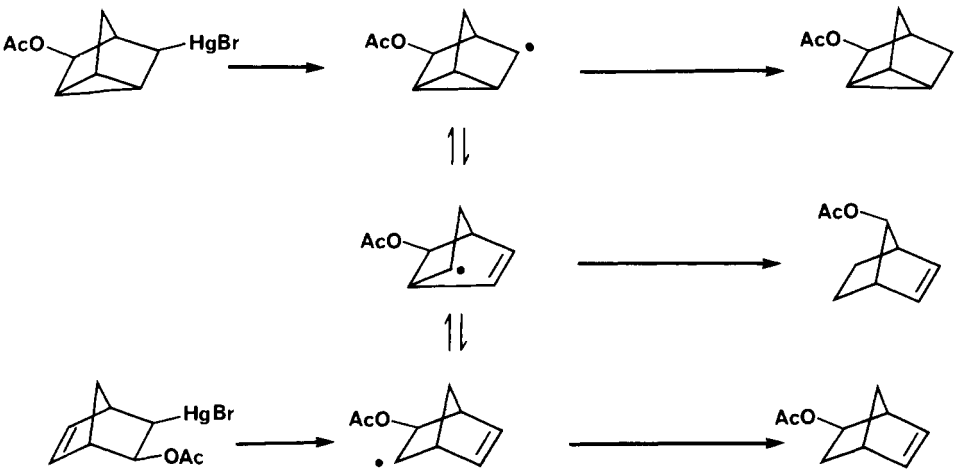


FIGURE 5: Expected products of radical cleavage of organomercurials **3** and **4** [from Whitesides and SanFilippo (1970)].

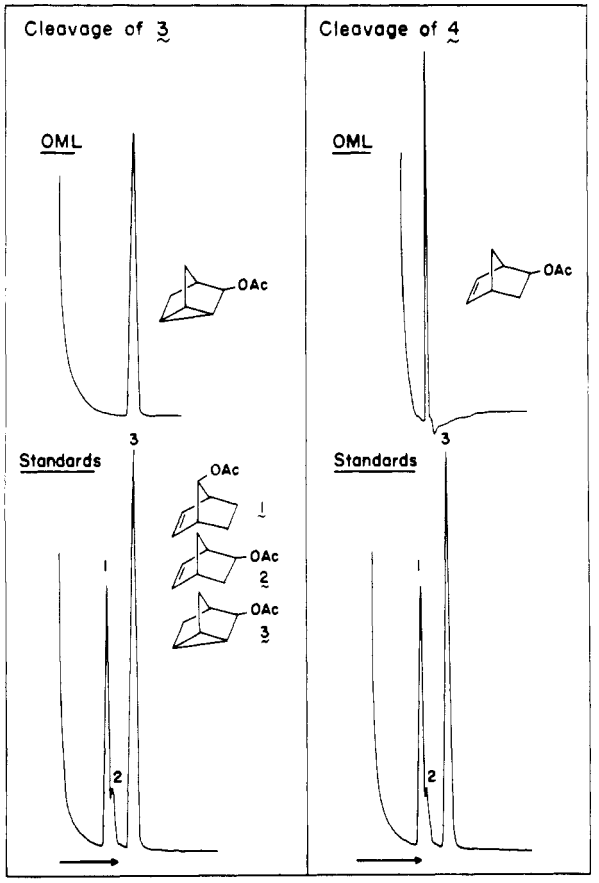


FIGURE 6: GC analyses of organomercurial lyase cleavage of **3** and **4**, with standards comparison.

are presented in Table II, along with data for other pertinent mercurials (Begley et al., 1986). These data indicate that these mercurials (in particular 1) are exceptionally good substrates (entries 8–10), with K_m = 3.3 mM and V_{max} = 10.7 μmol mg^{-1} min^{-1} (turnover number 240 min^{-1}) for butenylmercurial **1** and K_m = 2.4 mM and V_{max} = 3.5 μmol mg^{-1} min^{-1} (turnover number 78 min^{-1}) for propenylmercurial **2**. Crotylmercurial **5** is also rapidly turned over by the lyase, with K_m = 1.3 mM and V_{max} = 2.6 μmol mg^{-1} min^{-1} (turnover number 58 min^{-1}). Turnover numbers are defined here as moles of product per minute per mole of enzyme. Thus, for organomercurial lyase (M_r 22 400) a turnover number of 240 min^{-1}, or 4 s^{-1}, is still relatively modest for an enzymic rate, but is in fact more than 2 orders of magnitude faster than the turnover number for methylmercuric chloride.

Table II: Kinetic Parameters for Selected Organomercurial Substrates[a]

entry	substrate	V_{max} (nmol mg^{-1} min^{-1})	K_m	turnover no.[b] (min^{-1})
1	CH_3HgCl	31	0.5	0.7
2	HgCl	245	0.5	5.5
3	HgCl	880	0.9	20
4	HgBr	110	0.8	2.5
5	HgCl	40	0.8	0.9
6	HgOAc	670	0.9	15
7	HgBr	520	1.0	12
8	HgBr	2600	1.3	58
9	HgCl	3500	2.4	78
10	HgCl	10700	3.3	240

[a] Parameters determined for entries 2 and 8–10 as described under Methods. Data for other entries are from Begley et al. (1986). [b] Turnover number is defined as moles of product per minute per mole of enzyme.

Table III: Solvent Deuterium Isotope Effect Data[a]

entry	substrate	V_H/V_D	K_H/K_D	pH
1	HgCl	2.1	1.0	7.4
2	HgCl	4.9	0.8	7.4
3	HgCl	1.6	1.2	10.1

[a] Kinetic parameters measured as described under Methods.

Solvent Deuterium Isotope Effect Studies. Kinetic data obtained for *cis*-2-butenyl-2-mercuric chloride (**1**) and ethylmercuric chloride turnover in H_2O and in D_2O are summarized in Table III. In both cases solvent isotope effects on V_{max} were observed. In particular, at pH (pD) 7.4 analysis of butenylmercurial **1** indicated $V_{max}(H_2O)/V_{max}(D_2O)$ = 2.1 and $K_m(H_2O)/K_m(D_2O)$ = 1.0, while for ethylmercuric chloride $V_{max}(H_2O)/V_{max}(D_2O)$ = 4.9 and $K_m(H_2O)/K_m(D_2O)$ = 0.8. These values determined at pH 7.4 are off the max-

imum of pH 10.1 determined for organomercurial lyase (Begley et al., 1986). Analysis of ethylmercuric chloride at pH (pD) 10.1 indicated a decreased isotope effect of $V_{max}(H_2O)/V_{max}(D_2O) = 1.6$ and $K_m(H_2O)/K_m(D_2O) = 1.2$.

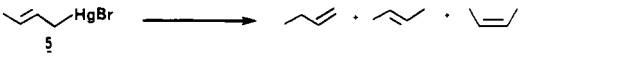
Discussion

Consideration of our results allows us to distinguish among the range of possible mechanisms (Figure 1) for the organomercurial lyase mediated bond cleavage. Solvolysis via an S_N1 pathway appears highly unlikely on the basis of our stoichiometry experiments with **1** and with methylmercuric chloride, where stoichiometric formation of hydrocarbon and Hg(II) was observed. Since the expected solvolysis products are Hg(0) and ROH, our results are clearly incompatible with a solvolytic mechanism. The range of mechanistic possibilities can be narrowed further by eliminating an S_E1 pathway on the grounds of chemical plausibility. Since none of the substrates investigated possess functional groups capable of stabilizing an intermediate carbanion and this pathway has been observed only rarely with suitable substrates in nonenzymatic reactions (Dodd & Johnson, 1969; Coad & Johnson, 1967; Hughes et al., 1964), it appears highly improbable here. The primary task then is to distinguish between a radical and S_E2 pathway. Substrates **1**–**5** provide specific probes for radical intermediates and for determination of the stereochemical course of the bond cleavage at the carbon center undergoing reaction, thus providing important insights into the mechanistic route of the enzymatic bond cleavage.

(*A*) *Stereochemical Reaction Course.* Our results with stereochemically defined substrates **1** and **2** indicate that the enzymatic bond cleavage proceeds with a high degree of configurational retention, consistent with an S_E2 mechanism. Nonenzymic protonolysis of diorganomercurials proceeds primarily with retention of stereochemistry, and this fact is commonly presented as evidence in support of an S_E2 pathway (Gale et al., 1960; Jensen & Rickborn, 1968) although isomerization of the substrates under the chemical protonolysis conditions complicates analysis of the results. Reactions of organomercurials with other electrophiles also proceed with retention of configuration, again presumably via an S_E2 pathway. In particular, reactions of optically active organomercurials with mercuric salts have been found to occur with a high degree of retention of configuration (Jensen, 1960; Charman et al., 1959; Reutov & Uglova, 1959). Likewise, electrophilic brominolysis of **1** proceeds with retention of geometry in pyridine (Casey et al., 1973) as does brominolysis of optically active 2-bromomercuributane (Jensen et al., 1959, 1960). Loss of configurational integrity often results from the intervention of radical intermediates in these reactions under certain conditions (e.g., CS_2 as solvent in the brominolysis reactions); however, observation of configurational retention is generally interpreted as evidence for an S_E2 mechanism.

We note that the S_E2 mechanism does not, however, dictate retention of configuration at the carbon center undergoing reaction, as evidenced in the electrophilic reactions of organotin and boron compounds that proceed with inversion of stereochemistry (Fukuto & Jensen, 1983; Brown et al., 1976). Thus, while inversion of stereochemistry does not automatically preclude an S_E2 mechanism, retention of stereochemistry is suggestive of this pathway, and the enzymatic stereochemical data are therefore of particular interest.

(*B*) *Radical Probes.* Our results with substrates **1**, **3**, and **4** suggest that radical intermediates are not important in the enzymatic reaction (see Figure 5). Since complete retention of geometry is observed with **1** and no rearrangement products are observed with either **3** or **4** during the enzymatic reaction,



5

Entry	Reagent	Ratio of Butenes (1-:trans-2-:cis-2-butene)	Rate 5/Rate n-BuHgCl
1	HCl/EtOAc (X = Br)	99.3:0.2: 0.5	ca. 10^7
2	HCl/EtOAc (X = OAc)	99.2:0.3: 0.5	ca. 10^7
3	organomercurial lyase	72:23:5	ca. 3

FIGURE 7: Protonolysis of **5** with HCl and with organomercurial lyase. The data in entries 1 and 2 were from Sleezer et al. (1963). In entry 3, the enzymatic reaction was carried out at pH 7.4 as described under Methods. The mercurial **5** consised of a ca. 3–3.5/1 mixture of trans-2/cis-2 olefin isomers (see Materials).

the presence of long-lived radical intermediates appears unlikely. We cannot, however, rule out the possibility of rapid, regiospecific radical trapping at the active site. Given the broad substrate specificity of the enzymic reaction, it appears unlikely, though, that a high degree of substrate immobility would exist in the active site leading to regiospecific trapping. Further argument in contradiction of a radical pathway is provided by considering the relative V_{max} rates of the methyl and *tert*-butylmercuric chlorides. The *tert*-butyl radical is known to be some 13 kcal/mol more stable than the methyl radical (Kerr, 1966), and yet both substrates proceed at virtually the same rates (0.7 vs. 0.9 min^{-1}). One would have predicted that some fraction of the calculated 10^9-fold rate difference expected from this 13-kcal energy difference would be manifested in the enzymic reaction if radical intermediates were significant.

(*C*) *Allylic Substrate* **5**. The regiochemical results for the enzymatic protonolysis of crotylmercurial **5** (72% 1-butene; 23% *trans*-2-butene; 5% *cis*-2-butene; Table I, entry 5) are in contrast to those reported for its protonolysis with HCl, which proceeds with exclusive allylic rearrangement to 1-butene (>99%) (Sleezer et al., 1963) (Figure 7). As described under Methods the crotylmercurial **5** used in this study consisted of an inseparable mixture of two olefinic isomers (ca. 3–3.5/1), with the major compound tentatively assigned the *trans*-2-butenyl configuration. No evidence of any 1-butenyl species was found by high-field ^1H NMR analysis. Thus, while our results are not directly comparable with those of Sleezer et al. (1963) who reported using pure *trans*-**5**, it is possible that some minor cis isomer was also present in the earlier study.

Further contrasts between the chemical and enzymatic protonolyses are seen in comparing the relative reaction rates for **5** with *n*-butylmercuric chloride. In particular, while the rate of protonolysis of **5** with HCl is reported to be some 10^7 times faster than for the corresponding *n*-butylmercurial, **5** is cleaved enzymically only ca. 3-fold more rapidly the *n*-butylmercurial (58 vs. 20 min^{-1}).

The regiochemical outcome of the enzymatic reaction of **5** can be explained in two ways. It appears most likely that the active-site acid group is suitably situated to allow both S_E2' and S_E2 cleavage to occur, leading to the three isomeric butenes. Alternatively, the allylic mercurial may undergo rearrangement either in the thiol-containing buffer or in an enzymic binding induced process to a 1-butenyl-3-mercurial species and then undergo an enzymic S_E2 protonolysis. Such rearrangement has been proposed in the protonolyses of the 2-butenyltrimethyltins with various acids, where varying mixtures of 1-butene and *cis*- and *trans*-2-butene are observed depending on the solvent and acid used (Verdone et al., 1975). We cannot absolutely rule out a radical intermediate, although such a species appears unlikely since the 2-butenyl-1-carbinyl radical is known to equilibrate in solution to a rearranged

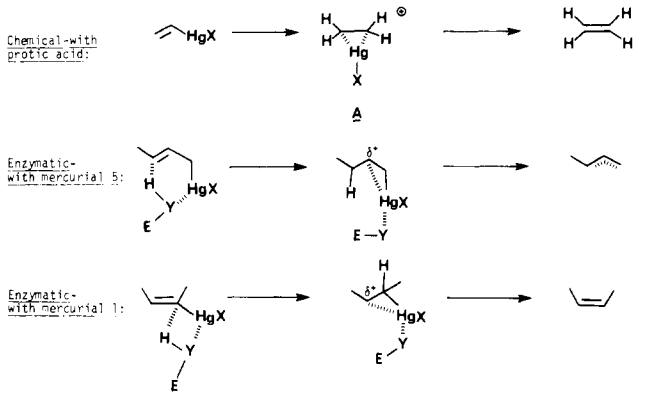


FIGURE 8: Protonolysis of alkenyl- and allylmercurials.

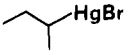
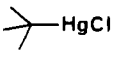
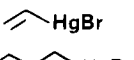
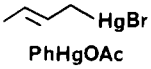
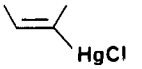
product mixture consisting of ca. 41/23/36 *trans*-2-butene-*cis*-2-butene/1-butene (Denney et al., 1967).

That the large rate difference observed in the chemical protonolysis of the crotyl- and butylmercurials (crotyl 10^7-fold faster) is not reflected in the enzymatic reaction rates may be due to a number of factors. One possibility is that a stereoelectronic requirement for rate acceleration (i.e., overlap of the C–Hg σ bond with the olefin π system) cannot be met by the enzyme. A more likely explanation, however, is shown in Figure 8. Rate acceleration via mercury stabilization of positive charge at C2 in the transition state as proposed for the alkenylmercurials **1** and **6** (vide infra) requires proton delivery at C3 in the case of **5**. It may be that the active-site conjugate acid group in organomercurial lyase is not optimally positioned for proton delivery to C3, thus precluding the full kinetic expression of the transition-state stabilization offered by the mercury atom.

(*D*) *Kinetic Data.* Involvement of a protic enzyme moiety in the lyase-mediated carbon–mercury bond cleavage is supported by the high rates observed in the enzymic reactions of substituted alkenylmercurials **1** and **6** (Table II). Stabilization by metals leading to a carbenium ion intermediate such as **A** (Figure 8) has been proposed to explain the facile acid protonolyses of alkenylmetal species (Kreevoy & Kretchmer, 1964; Negishi, 1980), and it appears reasonable that a similar process is operating in the enzymatic reaction. The rate acceleration observed for both **1** (240 min^{-1}) and **6** (78 min^{-1}) in contrast with the 6- to 20-fold lower V_{max} of processing of vinylmercuric bromide (12 min^{-1}; Table II, entries 7, 9, and 10) suggests that stabilization of intermediate positive charge by the electron-releasing C2 methyl substituent is important in lowering the energy of a kinetically important transition state, thus leading to rapid bond cleavage. It appears unlikely that the rate accelerations seen with **1** and **6** reflect differences in enzyme on or off rates since (a) the structural changes in going from ethyl, vinyl, *n*-butyl, *sec*-butyl, or *tert*-butyl to propenyl or *cis*-2-butenyl are minimal and (b) the product Hg(II) is identical for all substrates.

In an S_E2 mechanism a rate-determining transition state involving partial carbonium ion character at the C2 position will also have partial protonation at the C1 position in that transition state. This hypothesis is supported by the solvent deuterium isotope effects observed for the butenylmercurial **1** in the enzymatic reaction, indicative of a kinetically significant proton transfer. In particular, the $V_{max}(H_2O)/V_{max}(D_2O)$ of 2.1 for *cis*-butenylmercurial **1** suggests a kinetically important proton-transfer step partially limits V_{max} in this case. The observation of a large solvent isotope (k_H/k_D = 2.96 for $HClO_4$) in the chemical protonolysis has similarly been interpreted as evidence of this protonolysis pathway

Table IV: Rate Data for Cleavage of Organomercurials via Protonolysis

substrate	turnover no.[a] (s^{-1})	rate of chemical protonolysis[b] (s^{-1})
CH_3HgCl	1.2×10^{-2}	1.5×10^{-8} [c]
	3.3×10^{-1}	4.8×10^{-9} [c,d]
	4.2×10^{-2}	1.4×10^{-9} [c,e]
	1.4×10^{-2}	
	1.9×10^{-1}	4.9×10^{-4} [f]
	1.0	4.9×10^{-2} [g]
PhHgOAc	2.5×10^{-1}	4.5×10^{-6}
	4.0	

relative ease of cleavage of R–Hg by H^+ (easiest to hardest)[h]			bond strengths
R = aryl	R = alkyl	C–Hg bonds[i]	(kcal/mol)
p-anisyl	methyl	Ph–Hg–Cl	66 ± 3
phenyl	ethyl	CH_3–Hg–Cl	64.3 ± 2
p-chlorophenyl	*n*-propyl	CH_3CH_2–Hg–Cl	60.3 ± 3
	n-butyl		
	isoamyl		
	benzyl		
	tert-butyl		

[a] Data for enzymatic turnover of organomercurials; from this work and Begley et al. (1986). [b] These numbers were calculated from data in the references indicated and are corrected to the enzymatic reaction conditions. We note that Jensen and Rickborn (1968) have questioned the accuracy of some of Kreevoy's rate data. [c] Data from Kreevoy and Hansen (1961). [d] Value for *n*-propylmercurial protonolysis. [e] Value for isopropylmercurial protonolysis. [f] Data from Kreevoy and Kretchmer (1964). [g] Data from Kreevoy et al. (1966). [h] Data from Jensen and Rickborn (1968). [i] Data from Skinner (1964).

(Kreevoy & Kretchmer, 1964). The dependence of the solvent deuterium isotope effect on pH for ethylmercuric chloride (Table III) provides additional support that delivery of a proton is at least partially rate limiting at pH 7.4. A similar effect has been utilized in this laboratory in a study of dihydroorotate oxidase, where changes in pH allowed manipulation of the rate-limiting step (Pascal & Walsh, 1984). It appears that a similar phenomenon is involved here and provides strong evidence for the involvement of an active-site moiety.

The relative insensitivity of organomercurial lyase to substrate structure seen in comparing the protonolysis of mercurial **5** with that of *n*-butylmercuric chloride is further manifested in the relatively constant V_{max} values obtained for a range of structurally dissimilar organomercurial salts. As seen in Table II, K_m and V_{max} values are remarkably constant for a range of compounds, including vinyl, aryl, and primary, secondary, and tertiary alkylmercurials. It is apparent then that only in the case of the alkenylmercurials where an alternate reaction pathway involving olefinic protonation (with stabilization of partial positive charge by the mercury atom) is possible are any significant rate differences observed. The relative rates for the chemical and enzymatic protonolysis of various mercurials are presented in Table IV, where several features are immediately evident. First, while the turnover numbers are quite modest for an enzymatic reaction, they nevertheless represent a 10^6–10^7-fold acceleration over the chemical protonolysis rates. Furthermore, there is no apparent correlation of either the ease of chemical protonolysis or bond strengths with the enzymatic rates. We note that we have not yet

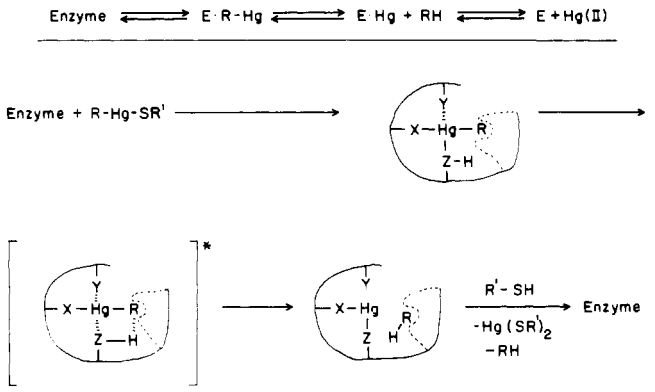


FIGURE 9: Mechanistic hypothesis for the organomercurial lyase catalyzed protonolysis of organomercurials.

determined the rate-limiting step for the enzymatic reaction. However, assuming the reactions all proceed to give a common enzyme–Hg(II) complex [see Begley et al. (1986)], it appears that Hg(II) release cannot be completely rate limiting, given the $>10^2$-fold variation of rate with substrate.

(*E*) *Mechanistic Hypothesis.* Presented in Figure 9 is one possible mechanistic pathway for the enzymatic reaction. While details of this sequence regarding the active-site residues [there are no metals or cofactors (Begley et al., 1986)] remain to be discerned, it is reasonable to propose the mechanistic scheme shown on the basis of our data obtained so far. The organomercurial substrates undergo initial binding to the enzyme via coordination to group X to give an enzyme–substrate complex (saturation kinetics are observed with all substrates in Table II). Coordination of another nucleophilic moiety, Y, to the organomercurial substrate would then activate the carbon–mercury bond toward protonolysis. While evidence for such coordination in chemical protonolyses is meager at best, several X-ray crystallographic structures of organomercurial complexes with amino acids reveals the presence of such secondary bonding interactions (Wong et al., 1973, 1974; Furmanova et al., 1980). In addition, nucleophilic acceleration of electrophilic reactions of the carbon–mercury bond (e.g., with I_2) are well documented (Beletskaya et al., 1965; Charman et al., 1961; Kreevoy et al., 1966; Sayre & Jensen, 1979). Carbon–mercury bond cleavage would then occur via the indicated S_E2 transition state to yield the hydrocarbon and enzyme-coordinated Hg(II). At this point we cannot conclusively say whether the S_E2 path to protonolysis involves a three- or a four-centered transition state, although we propose a four-centered pathway in light of previous suggestions of this route during electrophilic reactions of organomercurial salts (Jensen & Rickborn, 1968). Finally, exchange of Hg(II) with thiols in the solvent leads to free enzyme ready for another catalytic cycle. While organomercurials are known to undergo rapid exchange with oxygen and sulfur ligands (10^6–10^8 s^{-1}) (Rabenstein & Reid, 1984; Rodriquez et al., 1978; Eigen & Wilkins, 1965) Hg(II) release from the enzyme may be governed by factors such as solvent accessibility and conformational constraints in the active site, leading to a partial effect of mercury release on rate. Without excess thiols in the buffer as a reservoir to partition product Hg(II) out of the enzyme active site, the enzyme will accumulate as an inhibited enzyme–Hg(II) complex (Begley et al., 1986).

The overall sequence shown leaves many questions to be addressed. In particular, the structural features that allow the enzyme to bind an organomercurial but yet readily release Hg(II) are of considerable importance, as is the identity of the active-site moiety involved in proton delivery. One mechanistic possibility is that hydrophobic interactions of the organomercurial hydrocarbon moiety with the enzyme active site play an important role in substrate binding, rather than binding of the Hg atom to an active-site moiety. This hypothesis is reasonable given the very low solubility of organomercurials in aqueous systems and high solubility in nonpolar hydrocarbons. The failure of the enzyme to undergo inactivation with a variety of group-specific reagents (Begley, unpublished data) indicates that other methods may be necessary to elucidate the active-site structure. In this vein efforts are currently under way to crystallize the enzyme (in collaboration with E. Pai, University of Heidelberg). Ultimately we hope to decipher the enzymic features that provide the unique activation toward protonolysis of a variety of normally quite inert organomercuric salts.

Summary. On the basis of our results reported herein we suggest that the enzymatic protonolysis of organomercuric salts by organomercurial lyase is best explained by an S_E2 mechanism. As such, organomercurial lyase is the first enzyme known to catalyze an S_E2 reaction as well as the first well-characterized enzyme that processes an organometallic substrate. The elucidation of structural features of the active site as well as additional mechanistic details will require further detailed studies and will be reported in due course.

ACKNOWLEDGMENTS

We thank Dr. James Bentsen for acquiring the FTIR data for deuterolysis of mercurial **2**. We also thank Dr. Spencer Shames for his helpful comments and suggestions during the course of this work.

Registry No. **1**, 104506-39-0; **2**, 16888-31-6; **3**, 31002-56-9; **4**, 31002-55-8; **5**, 18355-68-5; **6**, 1871-04-1; MeHgCl, 115-09-3; EtHgCl, 107-27-7; BuHgCl, 543-63-5; *s*-BuHgCl, 38455-12-8; *t*-BuHgCl, 38442-51-2; PhHgOAc, 62-38-4; CH_2=CHHgBr, 16188-37-7; D_2, 7782-39-0; organomercurial lyase, 72560-99-7.

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