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Bromomesaconic and Bromocitraconic Acids. Potential Active Site Labeling Reagents for Dicarboxylic Acid Metabolizing Enzymes[1a]

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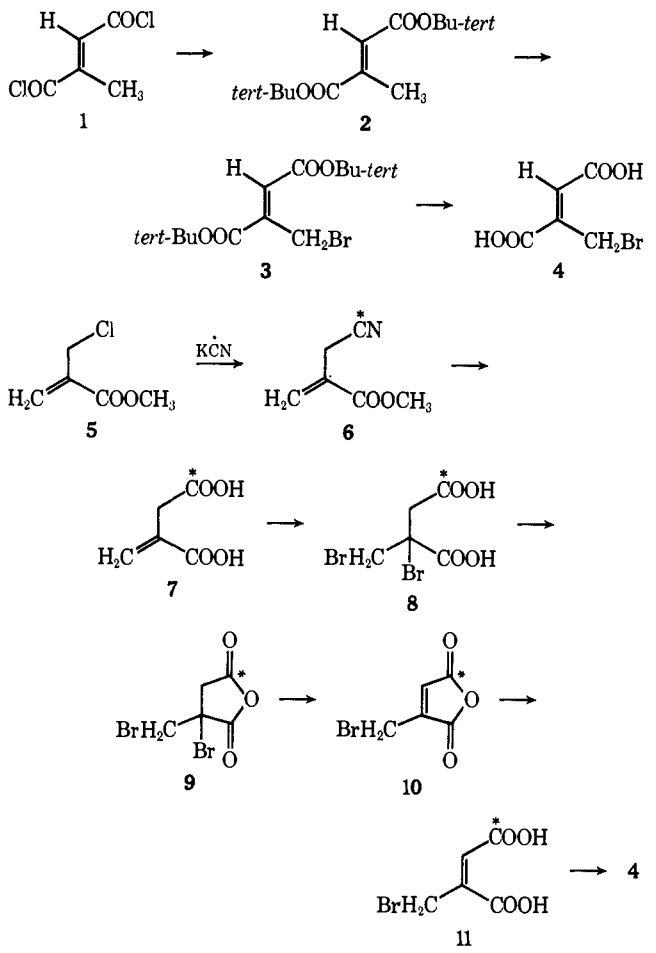
The synthesis of unlabeled and ^{14}C-labeled bromomesaconic acid (**4**) is described. Stable aq solutions of the isomeric compound, bromocitraconic acid (**11**), were prepared by hydrolysis of the anhydride **10**, but all attempts to isolate **11** led to the isomer **4**. Compound **4** is a potent active site specific irreversible inhibitor for yeast and pig heart fumarase; **11** also inactivated fumarase, but at a slower rate, and did not inactivate aspartate transcarbamylase.

In a preliminary communication,[2] we described the inactivation of fumarase by the substrate analog bromomesaconic acid (**4**), the most potent irreversible inhibitor for fumarase yet reported. Details of the synthesis of unlabeled and ^{14}C-labeled **4**, needed for active site labeling studies, and of the previously unobtained isomer, bromocitraconic acid (**11**), are described here. Both **4** and **11** are of interest as potential active site



specific irreversible inhibitors for a variety of enzymes which metabolize dicarboxylic acids.

Chemistry.—Two synthetic pathways to **4** were devised, the first providing an unambiguous synthesis of **4**, and the second a convenient route to the ^{14}C-labeled material as well as the isomer **11**.

Bromination of di-*tert*-butyl mesaconate (**2**), prepared by the action of NaO-*tert*-Bu on the acid chloride **1**,[3] using NBS, produced the bromo ester **3**. Deblocking of **3** in CF_3CO_2H gave bromomesaconic acid **4**.

Because of the unavailability of [^{14}C]mesaconic acid, an alternate route to **4a** was investigated.[4] Treatment of methyl 2-(chloromethyl)acrylate (**5**)[5] with [^{14}C]KCN gave the nitrile **6a**, which was not isolated, but was hydrolyzed in acid[6] to itaconic acid (**7a**). Bromination of **7a** produced the dibromo adduct **8a**,[7] which was dehydrated with $(CF_3CO)_2O$ to give the anhydride **9a**. Dehydrohalogenation of **9a** with Et_3N gave bromocitraconic anhydride (**10a**) in about 27% overall yield from [^{14}C]KCN.

The intermediate anhydride **10** can be converted to either **4** or **11**. Hydrolysis of **10** in H_2O gives the cis acid **11**, which is identifiable by its nmr spectrum and is stable in dil solution for several days. Removal of H_2O, however, invariably leads to the trans isomer **4**. The ready isomerization of **11** is at present unexplained and is in marked contrast with the isomerization of citraconic acid which occurs only under rather vigorous conditions.[8] Buffered solutions (*ca.* pH 7) of **11** have a half-life of about 5 hr.

Compound **4** is also relatively stable in aq solution; in buffered solutions (pH 7), however, **4** is converted rapidly ($\tau_{1/2}$ *ca.* 15 min)[2] to the lactone, aconic acid.[9]

Enzymology.—Kinetic parameters for the inactivation of fumarase by **4** and **11** were obtained using the method of Schaeffer, *et al.*[10] Both **4** and **11** bind reversibly to fumarase to form complexes, which have dissociation constants K_I (Table I) and which decompose with rate constants k_1 to the inactive forms of the enzyme. When corrections are made for decomposition

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TABLE I

KINETIC PARAMETERS FOR INACTIVATION OF FUMARASE BY BROMOMESACONATE AND BROMOCITRACONATE[a]

	Inhibitor		
	Bromomesaconate	Bromomesaconate	Bromocitraconate
	Species of fumarase		
	Heart	Yeast	Heart
K_I (mM)	0.67	3.6	5.7
k_i (min^{-1})	0.31	0.16	0.16
K_m (fumarate) (mM)	0.90	4.0	0.90
K_I/K_m	0.74	0.90	

[a] Reactions were carried out in 0.1 M sodium phosphate buffer (pH 7.3) at 25°.

of the inhibitors, the observed rates of inactivation (k_{obsd}) follow the rate law,[10] $1/k_{obsd} = (1/k_i)[(K_I)/(I_0)] + 1/k_i$, where ($I_0$) is the initial inhibitor concentration. The fact that both pig heart and baker's yeast fumarase are inactivated by **4** and that the observed K_I's parallel the K_m values for these enzymes shows that the reaction is not species specific. At low (< 1 mM) inhibitor concentrations, the inactivation of fumarase by **11** is 10 to 15 times slower than by **4**, the decrease in rate being reflected primarily in the larger K_I (hence weaker binding) of the cis isomer **11**. The 8.5-fold difference in the K_I's of the cis and trans inhibitors is comparable to the 10-fold difference in binding of maleate and fumarate at pH 7.3.[11]

Aspartate transcarbamylase from *Escherichia coli*, which is strongly inhibited competitively by maleate,[12] was not inactivated by the maleate analog **11** under a variety of conditions. Succinic dehydrogenase was slowly inactivated by **4** and **11**.[13] Neither **4** nor **11** inactivated a mitochondrial malate transport system from rat liver.[14]

Experimental Section

Melting points were taken on a Büchi melting point apparatus in capillary tubes and are uncorrected. Nmr spectra were obtd on a Varian A-60 nmr spectrometer. (Me_4Si in $CHCl_3$). Solvents were removed under vacuum on a Büchi "Rotavapor" rotating evaporator. Elemental analyses were within 0.3% of theory.

Di-*tert*-butyl Mesaconate (2).—A soln of NaO-*tert*-Bu was prepd by adding 9.1 g (0.395 g-atom) of Na to 1800 ml of anhyd *tert*-BuOH and heating at reflux until the Na dissolved. The soln was cooled to room temp, and 30.5 g (0.183 g-atom) of mesaconyl chloride[3] was added with stirring. Stirring was continued for 1.5 hr and 3 g of $NaHCO_3$ was added to destroy excess *tert*-BuO^-. The alcohol was evapd under vacuum and the residue was dissolved in 500 ml of Et_2O. The Et_2O soln was washed twice with 100 ml of 5% $NaHCO_3$, dried (Na_2SO_4), and evapd. The residual oil was distd in the presence of 0.25 g of MgO, and the fraction boiling at 115–118° (10 mm) was collected; yield, 30.5 g (69%). An nmr spectrum (CCl_4) showed the presence of about 80% of **2** [δ 1.31 (s, 18, CH_3C), 1.95 (d, 3, $J \simeq$ 1.6 Hz, $CH_3C{=}C$), 6.2 (d, 1, $J \simeq$ 1.6 Hz, vinyl H) ppm] and 20% of the isomer, di-*tert*-butyl itaconate [δ 1.23 (s, 9, CH_3C), 1.30 (s, 9, CH_3C), 2.87 (s, 2, CH_2), 5.18 (d, 1, $J \simeq$ 1.4 Hz, vinyl H), 5.78 (d, 1, $J \simeq$ 1.4 Hz, vinyl H) ppm].

The isomer mixt was used directly in the subsequent bromination reaction, since it was determined in trial experiments that both isomers were converted to the same product.

Di-*tert*-butyl Bromomesaconate (3).—A 6.2-g (0.026 mole) portion of di-*tert*-butyl mesaconate–itaconate isomer mixt, 6.1 g (0.034 mole) of NBS, 1.0 g (0.0042 mole) of Bz_2O_2, and 0.05 g of MgO in 40 ml of $CHCl_3$ was heated at reflux for 1 hr with stirring. The $CHCl_3$ was removed on a rotary evaporator. The residue was triturated with CCl_4, and succinimide was filtered off and washed with a little CCl_4. Traces of succinimide were removed by passing the CCl_4 washings (*ca.* 50 ml) through a column containing 75 g of silica gel. The column was washed with 1000 ml of CCl_4, followed by 500 ml of $CHCl_3$. The washings were collected and evapd. Petr ether was added to the residue giving a ppt of Bz_2O_2. The peroxide was filtered off and the filtrate was evapd, leaving 7.8 g of an oil. An nmr spectrum (CCl_4) showed the presence of about 85% of **3** [δ 1.35 (s, 18, CH_3C), 4.44 (s, 2, CH_2Br), 6.33 (s, 1, vinyl H) ppm] and impurities consisting of benzoyl peroxide and small amounts of starting material and unknown compds. Because of the peroxide content, further purification by distn was not attempted.

Bromomesaconic Acid (4).—A 13-g sample of crude **3** (contg about 0.032 mole) was heated at reflux in a mixt of 20 ml of CF_3COOH and 60 ml of C_6H_6 for 5 hr. The solvent was evapd on a rotary evaporator, the residue was triturated with C_6H_6, and the resulting solid was filtered off. A single crystn from Et_2O–petr ether gave 3.25 g (*ca.* 50%) of a product melting at 176–177°. An nmr spectrum in D_2O showed only two peaks: δ 4.6 (s, 2, CH_2Br) and 6.9 (s, 1, vinyl H) ppm. A sample recrystd from Et_2O–petr ether and dried under vacuum melted at 184–186°. *Anal.* ($C_5H_5BrO_4$) C, H, Br.

2-Bromo-2-(bromomethyl)succinic Acid (8b).—A stirred mixt of 39 g (0.30 mole) of itaconic acid and 50 ml of AcOH was heated on a steam bath, and to it was added, over a period of 2 hr, a soln of 50 g (0.31 mole) of Br_2 in 50 ml of AcOH. Stirring and heating were continued for 1.5 hr longer, and AcOH and Br_2 were removed on a rotary evaporator. CCl_4 (100 ml) was added and evapd. Addnl CCl_4 (50 ml) was added, the mixt was kept in a cold room overnight, and the resulting solid was filtered and washed with 20 ml of CCl_4: yield, 66 g (76%); mp 164–167°. A sample recrystd from $CHCl_3$–petr ether melted at 167–168° (lit.[7] 166–168°).

2-Bromo-2-(bromomethyl)succinic Anhydride (9b).—A mixt of 58 g (0.20 mole) of **8b** and 60 ml of $(CF_3CO)_2O$ was heated at reflux with exclusion of moisture. After 30 min the solvent was removed on a rotary evaporator. Removal of traces of CF_3COOH and $(CF_3CO)_2O$ under high vacuum resulted in 54 g (99%) of a product melting at 54–56°. Recrystn from hexane–$CHCl_3$ gave white needles: mp 58–60° (lit.[15] 50–52°); nmr ($CDCl_3$), δ 3.53 (s, 1), 3.75 (s, 1), 3.91 (s, 1), 4.11 (s, 1) ppm. The lower melting material can be used directly in the next reaction.

Bromocitraconic Anhydride (10b).—A soln of 20 g (0.20 mole) of dry Et_3N in 60 ml of dry Et_2O was added dropwise, over a period of 45 min, to a stirred soln of 48 g (0.18 mole) of **9b** in 100 ml of dry Et_2O at room temp. Stirring was continued for an addnl 1.5 hr, and the resulting black mixt was filtered to remove $Et_3N{\cdot}HBr$. Evapn of Et_2O gave 24 g of a brown oil, which on distn yielded 15.7 g (45%) of pure product: bp 116–117° (1.2 mm); ir (neat), 1835, 1760 cm^{-1} (anhydride C=O); nmr ($CDCl_3$), δ 4.23 (d, 2, $J \simeq$ 1.5 Hz, CH_2Br), δ 6.90 (t, 1, $J \simeq$ 1.5 Hz, vinyl H) ppm. *Anal.* ($C_5H_3BrO_3$) C, H, Br.

Hydrolysis of Bromocitraconic Anhydride in H_2O. Method A.—A mixt of 2.65 g (0.139 mole) of bromocitraconic anhydride **10b** and 0.25 ml (0.14 mole) of H_2O was stirred at room temp for 2 hr, at which time a solid ppt had formed. An nmr spectrum of the reaction mixt in D_2O indicated the presence of about 30% of bromomesaconic acid **4b** [δ 4.6 (s, 2, CH_2Br), 6.9 (s, 1, vinyl H) ppm] and 70% of bromocitraconic acid **11b** [δ 4.15 (s, 2, CH_2Br), 6.25 (s, 1, vinyl H) ppm]. On longer standing the mixture solidified, forming a product which was detd to be almost entirely **4b** by nmr analysis.

Method B.—Bromocitraconic anhydride (**10b**, 0.18 g, 0.94 mmole) was dissolved in 0.5 ml (25 mmoles) of D_2O. Nmr spectra of the soln showed that hydrolysis of the anhydride to bromocitraconic acid **11b** was complete in 45 min at room temp, and that **11b** while in dil soln was stable toward isomerization and hydrolysis of the bromo group for at least 6 days. All attempts to isolate solid **11b** by evapn of dil aq solns gave only isomerized product, however.

Methyl 1-(Chloromethyl)acrylate (5).—Methyl methacrylate 468 g, 4.68 mmoles) was stirred and cooled in an ice–salt bath. Cl_2 gas and dry air were passed through the liquid, the flow of Cl_2

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being adjusted so that the temp of the liquid remained below 3°. After 7.5 hr, when nmr showed that nearly all of the starting material had reacted, the flow of Cl_2 was stopped and air was allowed to bubble through the soln for 30 min more to remove Cl_2 and HCl. Nmr analysis of the product showed about 20% of the desired material as well as about 80% of methyl 2,3-dichloro-2-methylpropionate and other unidentified materials. The mixt was distd using a 1.5-m fractionating column packed with glass helices. An 80-ml fraction, boiling at 62.5–64° (20 mm) and contg about 85% of **5**, was collected over a 5-hr period. Redistn of this fraction at 62–63° (20 mm) [lit.[5] 56–57° (10 mm)] gave 20 ml of pure **5** as well as 30 ml of product contaminated with 5% of methyl 2,3-dichloro-2-methylpropionate: nmr of **5** (neat), δ 3.55 (s, 3, OCH_3), 4.07 (d, 2, $J \simeq 1$ Hz, CH_2Cl), 5.72 (t, 1, $J \simeq 1$ Hz, vinyl H), 6.02 (s, 1, vinyl H) ppm.

[4-^{14}C]Itaconic Acid (7a). Unlabeled KCN (0.65 g) was dissolved in 2.5 ml of H_2O and 0.5 ml of this soln was added slowly to a stirred soln of 2.0 g of methyl 2-(chloromethyl)acrylate. A second 0.5-ml portion of the KCN soln was used to dissolve 6 mg (0.5 mCi; New England Nuclear) of [^{14}C]KCN. The resulting soln, followed by the remainder of the unlabeled KCN soln, was added to the reaction mixt, and stirring was continued at room temp. Alcohol was removed from the reaction mixt on the rotary evaporator, leaving a mixt of H_2O, an oil, and KCl, which was then added to 50 ml of Et_2O. The soln was dried (Na_2SO_4), filtered, and evapd yielding 1.6 g of crude **6a**, an oil; ir (neat), 2250 cm^{-1} (nitrile). Concd HCl (10 ml was added to the crude nitrile, and the stirred mixt was heated on the steam bath. After 1.5 hr, all of the oil had dissolved, and the soln was evapd nearly to dryness on the rotary evaporator. The residue was dissolved in 15 ml of water and the soln was passed through a column contg 20 g of Dowex 50-X8 (H^+ form) to remove ammonium ions. The column was washed with 150 ml of H_2O, and washings were combined and evapd on the rotary evaporator. The residue was dried overnight in a vacuum desiccator and then was triturated with 3 ml of Et_2O. The mixt was kept in the cold room for a few hours, and the insol crude itaconic acid was filtered off and washed with 1 ml of Et_2O; yield, 0.55 g (4.6×10^8 dpm). Unlabeled itaconic acid (0.1 g) was added to the Et_2O filtrate and the Et_2O was evapd. The solid residue was washed with 2 ml of Et_2O and was filtered off, yielding an addnl 78 mg (3.0×10^7 dpm) of **7a**. The 2 crops of crude crystals were combined: yield, 0.63 g; mp 147–157° (lit.[16] 162–165°); 4.9×10^8 dpm (44% from [^{14}C]KCN.

[4-^{14}C]Bromomesaconic Acid (4a).—Crude [4-^{14}C]itaconic acid (**7a**, 0.61 g) was dissolved in 5 ml of HOAc, and the soln was heated on a steam bath with stirring. A soln of 0.9 g of Br_2 in AcOH was added dropwise over a period of 30 min, and the mixt was stirred and heated an addnl 30 min. It was evapd to an oil on the rotary evaporator, and 10 ml of CCl_4 was added and evapd. The alternate addn and evapn of CCl_4 was repeated several times until the residue remained solid: yield of **8a**, 1.04 g; mp 164–167°. This material was heated at reflux with 10 ml of $(CF_3CO)_2O$ with exclusion of moisture. After 1 hr, the reaction mixt was cooled and evapd on a rotary evaporator. Traces of CF_3COOH were removed in a vacuum desiccator under high vacuum: yield of crude **9a** (white solid), 0.96 g; mp 51–55°. The crude anhydride **9a** (0.96 g) was dissolved in 20 ml of dry Et_2O. A soln of 0.39 g of dry Et_3N in 10 ml of dry Et_2O was added slowly to the anhydride soln with stirring and exclusion of moisture. The black soln was filtered to remove $Et_3N \cdot HBr$, and the Et_2O was evapd leaving a dark oil; yield of crude **10a**, 0.52 g (~27% from KCN). A portion (0.22 g) of the crude **10a** was dissolved in 2 hr, by stirring with 1.0 ml of H_2O. Excess H_2O was removed under vacuum on the rotary evaporator, and the oily residue was allowed to stand exposed to air overnight, during which time the oil solidified. The solid was washed with 2 ml of C_6H_6 and was filtered; yield, 0.2 g; mp *ca.* 160°. The crude product was dissolved in 0.2 ml of water, the soln was cooled in an ice bath, and then 0.3 ml of concd HCl was added. The white ppt which formed was filtered off, washed with a small amount of cold 6 *N* HCl, and dried under high vacuum: yield of pure [4-^{14}C]bromomesaconic acid, 0.1 g (~42% from bromomesaconic anhydride); mp 184–186°; sp act., 1.10×10^6 dpm/μmole.

Inhibitor Solutions for Enzyme Studies. A. Bromomesaconic Acid (4).—Stock solns of **4** have a half-life of about 15 min above pH 7. Weighed samples of **4** and Na_2HPO_4 were dissolved in H_2O, the pH was adjusted to the desired value, and final vol adjustments were made by adding H_2O. These operations were carried out as quickly as possible, and the stock solns were used immediately for inhibitor runs.

B. **Bromocitraconic Acid** (11).—Stock solns of 11, have a half-life of about 5 hr at pH 7. Calculated amts of bromocitraconic anhydride (**10**) were hydrolyzed by stirring in H_2O for about 1 hr. Na_2HPO_4 was then added and the pH adjusted to the desired value. The addn of anhydride **10** directly to buffered solns leads to unknown side reactions.

Enzymes.—Pig heart fumarase was purchased from Calbiochem. Baker's yeast fumarase was prepared in partially pure form by the method of Cataldi and Stoppani.[17]

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Nucleophilicity of Some Reactivators of Phosphorylated Acetylcholinesterase[1]

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A rational approach is presented toward the establishment of a structure–activity relationship in a series of reactivators of DFP-inhibited AChE. The value of k_r, the first-order reactivation rate constant, at pH 7.4 is a function of both the nucleophilicity of the reactivator molecule and its basicity, reaching an optimum for compounds with a pK_a value in the range 7.6–8.0.

Heterocyclic oximes are recognized antidotes against intoxication with organophosphates.[2-4] In a series of studies[1,5,6] we have attempted to determine the structure–activity relationship of these compounds by correlating a relevant thermodynamic property, such as pK_a, with their nucleophilicity toward a common substrate, such as diisopropyl phosphorofluoridate (DFP). This is an arbitrary approach which dwells on the assumption that nucleophilicity toward this substrate reflects the same property toward phosphorylated acetylcholinesterase. The premise that all good reactivators of phosphorylated AChE are also good nucleophiles stems from a large volume of experimental information,[7]

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