Bromomesaconic and Bromocitraconic Acids. Potential Active Site Labeling Reagents for Dicarboxylic Acid Metabolizing Enzymes¹⁸

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Received December 8, 1970

The synthesis of unlabeled and ¹⁴C-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 ¹⁴C-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.

(1) (a) Supported in part by a grant (GM 14729) from the National Institutes of Health; (b) recipient of a Research Career Development Award (GM 17608) from the National Institutes of Health.

(2) R. A. Laursen, J. B. Baumann, K. B. Linsley, and W.-C. Shen, Arch. Biochem. Biophys., 130, 688 (1969). **Chemistry.**—Two synthetic pathways to 4 were devised, the first providing an unambiguous synthesis of 4, and the second a convenient route to the ¹⁴C-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 CF₃CO₂H gave bromomesaconic acid 4.

Because of the unavailability of [¹⁴C]mesaconic acid, an alternate route to **4a** was investigated.⁴ Treatment of methyl 2-(chloromethyl)acrylate (**5**)⁵ with [¹⁴C]KCN gave the nitrile **6a**, which was not isolated, but was hydrolyzed in acid⁶ to itaconic acid (**7a**). Bromination of **7a** produced the dibromo adduct **8a**,⁷ which was dehydrated with (CF₃CO)₂O to give the anhydride **9a**. Dehydrohalogenation of **9a** with Et₃N gave bromocitraconic anhydride (**10a**) in about 27% overall yield from [¹⁴C]KCN.

The intermediate anhydride 10 can be converted to either 4 or 11. Hydrolysis of 10 in H₂O gives the cis acid 11, which is identifiable by its nmr spectrum and is stable in dil solution for several days. Removal of H₂O, 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 $(\tau_{1/2} ca. 15 \text{ min})^2$ 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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(4) In our previous paper (ref 2), we described (without experimental details) a synthesis of labeled 4 starting with [14C]citric acid. Because of the expense of radioactive citric acid and the often unpredictable yields of 4, this route was abandoned.

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(8) R. L. Shriner, S. G. Ford, and L. J. Roll, "Organic Syntheses," Collect. Vol. II, Wiley, New York, N. Y., 1943, p 382.

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TABLE I KINETIC PARAMETERS FOR INACTIVATION OF FUMARASE BY BROMOMESACONATE AND BROMOCITRACONATE^a

	Inhibitor		
	Bromo-	Bromo-	Bromo-
	mesaconate	mesaconate	citraconate
	Species of fumarase		
	Heart	Yeast	Heart
K_1 (m M)	0.67	3.6	5.7
$k_1 (\min^{-1})$	0.31	0.16	0.16
$K_{\rm m}$ (fumarate) (m M)	0.90	4.0	0.90
$K_{ m I}/K_{ m m}$	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 $1/k_{obsd} = (1/k_1)[(K_1)/(I_0)] + 1/k_1$, 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_1 '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_1 (hence weaker binding) of the cis isomer 11. The 8.5-fold difference in the K_1 '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. (Me₄Si in CHCl₃). 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 chloride³ was added with stirring. Stirring was continued for 1.5 hr and 3 g of NaHCO₃ was added to destroy excess *tert*-BuO⁻. The alcohol was evapd under vacuum and the residue was dissolved in 500 ml of Et₂O. The Et₅O soln was washed twice with 100 ml of 5% NaHCO₃, dried (Na₂SO₄), 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 (CCl₄) showed the presence of about 80% of 2 [δ 1.31 (s, 18, CH₃C), 1.95 (d, 3, $J \simeq$ 1.6 Hz, CH₃C=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, CH₃C), 1.30 (s, 9, CH₃C), 2.87 (s, 2, CH₂), 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 Bz₂O₂, and 0.05 g of MgO in 40 ml of CHCl₃ was heated at reflux for 1 hr with stirring. The CHCl₃ was removed on a rotary evaporator. The residue was triturated with CCl₄, and succinimide was filtered off and washed with a little CCl₄. 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 CCl₄, followed by 500 ml of CHCl₅. The washings were collected and evapd. Petr ether was added to the residue giving a ppt of Bz₂O₂. The peroxide was filtered off and the filtrate was evapd, leaving 7.8 g of an oil. An nmr spectrum (CCl₄) showed the presence of about 85% of **3** [δ 1.35 (s, 18, CH₃C), 4.44 (s, 2, CH₂Br), 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 CF₃COOH and 60 ml of C₈H₅ for 5 hr. The solvent was evapd on a rotary evaporator, the residue was triturated with C₆H₆, and the resulting solid was filtered off. A single crystn from Et₂O-petr ether gave 3.25 g (*ca.* 50%) of a product melting at 176-177°. An nmr spectrum in D₂O showed only two peaks: δ 4.6 (s, 2, CH₂Br) and 6.9 (s, 1, vinyl H) ppm. A sample recrystd from Et₂O-petr ether and dried under vacuum melted at 184-186°. Anal. (C₅H₅BrO₄) 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. CCl₄ (100 ml) was added and evapd. Addnl CCl₄ (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 CCl₄: yield, 66 g (76%); mp 164–167°. A sample recrystd from CHCl₃-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_3 -COOH and $(CF_2CO)_2O$ under high vacuum resulted in 54 g (99%) of a product melting at 54–56°. Recrystn from hexane-CHCl₃ gave white needles: mp 58–60° (lit.¹⁵ 50–52°); nmr (CDCl₃), 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 Et₃N in 60 ml of dry Et₂O 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 Et₂O at room temp. Stirring was continued for an addnl 1.5 hr, and the resulting black mixt was filtered to remove Et₃N · HBr. Evapn of Et₂O 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 (CDCl₃), δ 4.23 (d, 2, $J \simeq 1.5$ Hz, CH₂BrO₃) C, H, Br.

Hydrolysis of Bromocitraconic Anhydride in H₂O. Method A.—A mixt of 2.65 g (0.139 mole) of bromocitraconic anhydride **10b** and 0.25 ml (0.14 mole) of H₂O was stirred at room temp for 2 hr, at which time a solid ppt had formed. An nmr spectrum of the reaction mixt in D₂O indicated the presence of about 30% of bromomesaconic acid **4b** [δ 4.6 (s, 2, CH₂Br), 6.9 (s, 1, vinyl H) ppm] and 70% of bromocitraconic acid **11b** [δ 4.15 (s, 2, CH₂Br), 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 solu showed that hydrolysis of the anhydride to bromocitraconic acid 11b was complete in 45 min at room temp, and that 11b while in dil solu 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 solus 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 Cl₂ was stopped and air was allowed to bubble through the soln for 30 min more to remove Cl₂ 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.⁶ 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, OCH₃), 4.07 (d, 2, $J \simeq 1$ Hz, CH₂Cl), 5.72 (t, 1, $J \simeq 1$ Hz, vinyl H), 6.02 (s, 1, vinyl H) ppm.

 $\simeq 1$ Hz, vinyl H), 6.02 (s, 1, vinyl H) ppm. [4-14C] 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 [14C]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 H₂O, an oil, and KCl, which was then added to 50 ml of Et₂O. The soln was dried (Na₂SO₄), 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 Et₂O. 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 \times 10⁸ dpm). Unlabeled itaconic acid (0.1 g) was added to the Et₂O filtrate and the Et₂O was evapd. The solid residue was washed with 2 ml of Et₂O and was filtered off, yielding an addnl 78 mg $(3.0 \times 10^7 \text{ dpm})$ of 7a. The 2 crops of crude crystals were combined: yield, 0.63 g; mp 147-157° (lit.¹⁶ 162-165°); $4.9 \times 10^8 \text{ dpm} (44\% \text{ from } [^{14}\text{C}]\text{KCN}.$

[4-14C] Bromomesaconic Acid (4a).—Crude [4-14C] itaconic

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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 CCl4 was added and evapd. The alternate addn and evapn of CCl4 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 (CF₃CO)₂O with exclusion of moisture. After 1 hr, the reaction mixt was cooled and evapd on a rotary evaporator. Traces of CF₃COOH 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 Et₂O. A soln of 0.39 g of dry Et₃N in 10 ml of dry Et₂O was added slowly to the anhydride soln with stirring and exclusion of moisture. The black soln was filtered to remove Et₃N · HBr, and the Et₂O 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 H₂O. Excess H₂O 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 [4-14C]bromomesaconic acid, 0.1 g (~42% from bromomesaconic anhydride); mp 184-186°; sp act., 1.10×10^5 dpm/ *u*mole

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 Na₂HPO₄ were dissolved in H₂O, the pH was adjusted to the desired value, and final vol adjustments were made by adding H₂O. 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. Na₂HPO₄ 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 Acetylcholinesterase¹

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Received December 3, 1970

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_t , 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 cor-

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relating 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

(7) F. Hobbiger in "Heffter-Heubners Handbuch der experimentellen Pharmakologie," Vol. 15, G. B. Koelle, Ed., Springer, Berlin, 1963, p 921.