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Synthesis of 5-Substituted Isophthalic Acids and Competitive Inhibition Studies with Bovine Liver Glutamate Dehydrogenase (39200)

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Isophthalic acid was competitive (1, 2) with L-glutamate for binding to bovine liver glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating) EC 1.4.1.3). Replacement of a ring atom with quaternary nitrogen increased the hydrophilicity of the molecule and decreased its effectiveness as an inhibitor (1) (cf. ref. 3). From this it was inferred that displacement of bound water between enzyme and inhibitor contributed to the interaction energy.

The present paper reports the effects of placing various substituents on the 5-position of isophthalic acid on inhibitor potency as a function of pH. The following compounds were synthesized or purchased and were examined for effectiveness as competitive inhibitors: trimesic acid (5-carboxyisophthalic acid); 5-hydroxy-, 5-methoxy-, 5-fluoro-, 5-bromo-, 5-cyano-, and 5-methylisophthalic acid.

Materials. Bovine liver glutamate dehydrogenase was obtained as a crystalline homogenous protein from Sigma Chemical Company. The enzyme was collected by centrifugation at 4° and was subsequently dissolved in sodium phosphate buffer (0.15 *M* Na_2HPO_4 solution adjusted to pH 7.8 with 5.0 *M* H_2SO_4). Denatured protein was removed from enzyme solutions by centrifugation at 12,000*g* for 20 min at 4° (4). Stock enzyme solutions (6 mg/ml) were kept refrigerated at 4°. Protein concentrations were estimated by the biuret method (5). Water of 10^6 ohms resistance was used throughout the experiments.

$NADP^+$, NAD^+, and L-glutamic acid were obtained from Sigma Chemical Company. Trimesic acid and 5-methylisophthalic acid were received from Aldrich Chemical Company. The dimethyl ester of 5-aminoisophthalic acid and 5-aminoisophthalic acid were purchased from Baker Chemical Company. 5-Hydroxyisophthalic acid was received from K and K Laboratory, Inc. These compounds were purified until pertinent physical properties agreed with accepted values.

Synthesis of 5-bromoisophthalic acid. The procedures of Gilman and Swayampati (6) and Buck and Ide (7) were used to obtain 5-bromoisophthalic acid from the dimethyl ester of 5-bromoisophthalic acid. A mixture of 120 ml of glacial acetic acid, 16.72 g (80 mmole) of the dimethyl ester of 5-aminoisophthalic acid, and 27.2 ml of aqueous 48% hydrobromic acid was stirred in an ice-salt bath. Sixty milliliters of water that contained 5.52 g (80 mmole) of sodium nitrite was added dropwise to the above solution while maintaining the reaction temperature at 0–5°. The solution was stirred at this temperature for 30 min and then it was filtered. A suspension of cuprous bromide was prepared from 23 g of copper sulfate pentahydrate, 11 g of sodium bromide in 72 ml of water, and 5 g of sodium metabisulfite and 3.28 g sodium hydroxide in 36 ml of water. The two solutions described above were mixed, and a white precipitate of cuprous bromide immediately appeared. The suspension was heated on a steam bath until a temperature of 70° was obtained.

The diazonium solution was added slowly to the hot suspension of cuprous bromide. Next, 24 ml of aqueous 48% hydrobromic acid was added, and the mixture was stirred at room temperature for 1 hr. The mixture was extracted with ethyl acetate. The organic phase was washed serially with water, aqueous 5% sodium hydroxide, water, saturated sodium chloride solution prior to drying over anhydrous sodium sulfate. The

solvent was removed under reduced pressure to give 20.08 g (yield of 92%) of an orange oil that was not purified but was saponified as described below.

A mixture of 6.48 g (24 mmole) of the diester described directly above, 65 ml of methanol, 65 ml of water, and 20 g of 85% potassium hydroxide pellets was heated at reflux on the steam bath for 24 hr. After removal of methanol by reduced pressure, the solution was acidified with cold concentrated hydrochloric acid. The resulting solid was filtered, washed with water, and then air dried to give 4.28 g (yield of 71%) of a white solid with a melting point of 277–280°. Recrystallization from 550 ml of water afforded 3.21 g of 5-bromoisophthalic acid, which was a pale beige solid with a melting point of 282–284° (reported (8) mp 282–283°). Analysis calculated for $C_8H_5BrO_4$: C, 39.2, H, 2.0; found: C, 39.4, H, 2.4.

Synthesis of 5-cyanoisophthalic acid. A mixture of 60 ml glacial acetic acid, 7.24 g (0.04 mole) 5-aminoisophthalic acid, 10 ml concentrated hydrochloric acid, and 10 ml of water was stirred in an ice-salt bath. Sodium nitrite (2.76 g, 0.04 mole) in 30 ml of water was added dropwise, maintaining the temperature at 0–5°. The solution was stirred at 0–5° for 30 min after the addition was completed.

A mixture of 4.7 g (0.096 mole) of sodium cyanide, 4.3 g cuprous cyanide (0.048 mole), and 15 ml of water was heated on the steam bath until a temperature of 70° was reached.

The diazonium solution prepared directly above was added slowly to the cyanide solution. Periodic heating was necessary to maintain the solution at 60–70°. After the addition was completed the mixture was heated on the steam bath for 45 min. The white solid was removed by filtration, and the filtrate was extracted with ethyl acetate. The organic phase was washed thoroughly with water and then with a 5% sodium bicarbonate solution. The basic aqueous extract was acidified to pH 2 with hydrochloric acid. The resulting mixture was cooled, then filtered. The solid was air dried to give 2.66 g (35%) of an orange solid, mp 249–252°. The crude 5-cyanoisophthalic acid was added to 200 ml of hot ethyl acetate. The mixture was filtered, then 200 ml of petroleum ether (bp 60–75°) was added to the filtrate. The mixture was heated to boiling, then filtered. The solvent was removed under reduced pressure to afford 1.7 g of colorless prisms, mp 253–254° (reported (8) mp 251–251.5°).

Synthesis of 5-methoxyisophthalic acid. To a cold solution of 3.4 g (18 mmole) of 5-hydroxyisophthalic acid, 4.0 g (60 mmole) of 85% potassium hydroxide pellets and 75 ml of water, was added, dropwise, over a 5-min interval, 5.6 ml (7.6 g = 60 mmole) of dimethyl sulfate. The mixture was stirred at room temperature for 1 h and then refluxed for 4 h. Potassium hydroxide, 15 g, was added, and the mixture was again refluxed for 4 hr. The mixture was cooled, aqueous 5% hydrochloric acid was added until a pH of 2 was obtained, and then the resulting precipitate was removed by filtration. Recrystallization from ethyl acetate afforded 0.7 g (yield of 20%) of 5-methoxyisophthalic acid with a melting point of 267–270° (reported (9) melting point was 267–268°).

Synthesis of dimethyl 5-diazoniumisophthalate tetrafluoroborate. A solution of 8.36 g (0.04 mole) of dimethyl 5-aminoisophthalate (Eastman) in 120 ml of glacial acetic acid, 20 ml of water, and 10 ml of concentrated hydrochloric acid was cooled in an ice-salt bath to 0–5°. A solution of 2.76 g (0.04 mole) of sodium nitrite in 30 ml of water was added dropwise while maintaining the temperature between 0–5°. The orange solution was stirred at 0–5° for an additional 30 min. A cold solution of 6.16 g (0.056 mole) of sodium tetrafluoroborate (Ventron) in 20 ml of water was added slowly with stirring. The mixture was allowed to warm up to room temperature over a 30-min period, then 250 ml of ether was added. A white precipitate formed. The mixture was cooled, filtered, washed thoroughly with ether, and then air dried for 18 hr at room temperature. This afforded 9.5 g (77%) of dimethyl 5-diazoniumisophthalate tetrafluoroborate as a white solid.

Synthesis of dimethyl 5-fluoroisophthalate. Four identical thermal decompositions were carried out on a total of 18.5 g (0.06 mole) of the dimethyl 5-diazoniumiso-

phthalate tetrafluoroborate described above, and the final reaction mixtures were combined and purified as described below. Into a 100-ml three-necked round-bottomed flask equipped with a magnetic stirrer, thermometer, air condenser leading to a water trap, and heating mantle were added 4.6 g (0.015 mole) of the tetrafluoroborate salt and 50 ml of xylene (Fisher), dried over 3A molecular sieves. The stirred suspension was heated slowly between 45–60 V until a temperature of 139° was reached (approximately 1.5 hr) and even gas evolution (which began around 50°) was maintained by observation of nitrogen being released through the water trap. After gas evolution had ceased for 5 min, the orange solution was allowed to cool to room temperature, then cooled in an ice-water bath and 15 ml of water was added slowly. The mixture was stirred for 5 min. Ether and additional water were added. The organic phase was washed with water, 2% sodium hydroxide solution, water, saturated sodium chloride solution, and then dried over anhydrous sodium sulfate. The ether was removed at reduced pressure (water aspirator), then the xylene was removed under high vacuum. This afforded an orange oil which was dissolved at room temperature in a minimum amount of petroleum ether (bp 60–68°) and cooled in a Dry Ice–isopropyl alcohol bath. An orange oil separated and soon thereafter cream colored crystals began to form. As soon as the crystals began to form, the solution was decanted away from the orange oil and the mixture cooled again in the Dry Ice–isopropyl alcohol bath until crystallization ceased. The solid was removed by filtration, washed with petroleum ether (cooled as above), and air dried to give 6.9 g (55%) of dimethyl 5-fluoroisophthalate as a cream colored solid, mp 49–53° (reported (10) mp 56–57°).

Synthesis of 5-fluoroisophthalic acid. A mixture of 6.9 g (0.033 mole) of dimethyl 5-fluoroisophthalate, described directly above, 190 ml of water, 190 ml of methanol, and 27.1 g (0.41 mole) of 85% potassium hydroxide pellets was heated at reflux for 22 hours. The volume was reduced to 200 ml (water aspirator) and the resulting basic aqueous phase was extracted with ether. The basic aqueous phase was acidified with ice-concentrated hydrochloric acid to pH 2, then extracted with ethyl acetate. The organic phase was washed thoroughly with water, a saturated sodium chloride solution, and then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure (water aspirator) to give 5.27 g of a yellow solid, mp 291–294°. This solid was slurried with an ethyl acetate–petroleum ether (bp 60–68°) mixture and allowed to stand at 5° for 1 hr. The solid was filtered, rinsed with cold petroleum ether and air dried to give 4.84 g (82%) of 5-fluoroisophthalic acid as a cream-colored solid, mp 292–294° (reported (10) mp 295–297°).

Purification of 5-hydroxyisophthalic acid. A 10-g sample of 5-hydroxyisophthalic acid (K&K), mp 269–280°, was treated with various mixtures of methanol, ether, and petroleum ether (bp 60–68°) until 6 g of the pure acid, mp 302–304°, was obtained as a white solid (reported (11) mp 296°). Analysis calculated for $C_8H_6O_5$: C, 52.7, H, 3.3; found: C, 52.6, H, 3.3.

Methods. Enzyme assays were performed at 30 ± 0.1° using a Gilford 300 spectrophotometer connected to a Honeywell Electronik 19 recorder. Velocity was determined as the change in absorbance per minute at 340 nm for the reduction of $NADP^+$ or NAD^+. pH was measured prior to and directly after enzyme assay with use of a Beckman research pH meter. Apparent inhibition constants (K_i) were determined from double reciprocal plots of velocity versus glutamate concentrations and from Dixon plots of reciprocal velocities versus inhibitor concentrations (12). In the latter plot K_i values were determined from the intersection point of lines extrapolated in the fourth quadrant. Varying the glutamate concentrations by four-fold provided a large angle for accurate interpolation of K_i. Experimental errors in the determination of K_i were ± 10%.

Results and discussion. Figures 1 and 2 illustrated the interactions of 5-substituted isophthalic acids with bovine liver glutamate dehydrogenase and demonstrated that substitution in the 5 position did not alter the mechanism of enzyme inhibition. It remained competitive with L-glutamate. All

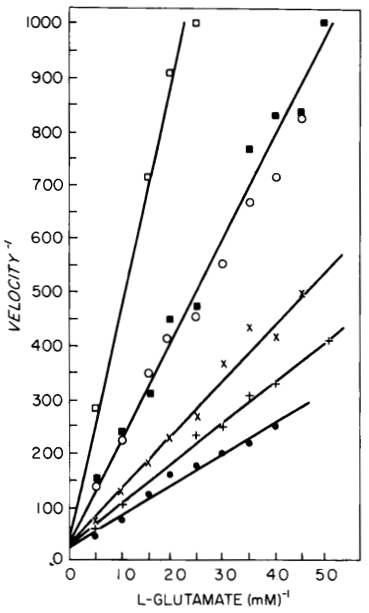

FIG. 1. Double reciprocal plots of velocity against glutamate concentrations in the presence of trimesic acid (5-carboxyisophthalic acid), 5-hydroxyisophthalic acid, 5-bromoisophthalic acid, 5-methoxyisophthalic acid, or 5-methylisophthalic acid at a fixed level of $NADP^+$. Assays were conducted at 30° in 3.0 ml of 0.1 *M* Na_2HPO_4–0.1 *M* $Na_4P_2O_7$ buffer adjusted to pH 7.1 with 5 *M* H_2SO_4. The assay system contained 0.2 m*M* $NADP^+$, enzyme (10 μg/ml), inhibitor, and varying amounts of L-glutamate. The reaction was started by addition of enzyme in buffer. Concentrations of inhibitors were 5 m*M* trimesic acid (□), 5 m*M* 5-hydroxyisophthalic acid (■), 5 m*M* 5-bromoisophthalic acid (○), 5 m*M* 5-methoxyisophthalic acid (X), and 5 m*M* 5-methylisophthalic acid (+). Note that the inhibitors gave slopes that intersected with control (●) on the ordinate.

the compounds (some of which, for brevity, are not depicted here) 5-carboxy-, 5-hydroxy-, 5-methoxy-, 5-fluoro-, 5-bromo-, 5-cyano-, and 5-methylisophthalic acid were competitive inhibitors of bovine liver glutamate dehydrogenase. Replacement of NAD^+ with $NADP^+$ did not alter the mechanism of interaction, inhibition still remained competitive with respect to glutamate. Replacement of one substituent (other than hydrogen) with another one at the 5 position did not greatly alter the inhibition potency of the isophthalate. Although these substituent changes were generally sufficient to change biological responses by at least 3 orders of magnitude in other systems via hydrophobic bonding (π effects (13)) or electronic charge (σ effects (13)), such free energy dependencies were not found here, Table I. In general less than 1-

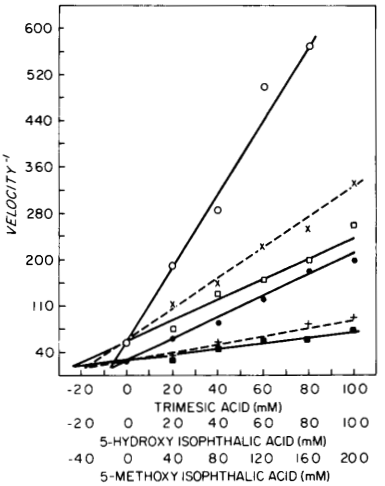

FIG. 2. Dixon plot of reciprocal velocity against inhibitor concentration at fixed levels of L-glutamate and $NADP^+$. Assays were conducted at 30° in 3.0 ml of 0.1 *M* Na_2HPO_4–0.1 *M* $Na_4P_2O_7$ buffer, pH 7.10. The assay system contained 0.6 m*M* $NADP^+$, enzyme (10 μg/ml), varying amounts of inhibitor, and L-glutamate. The reaction was started by addition of enzyme in buffer. The competitive inhibitors were trimesic acid (5-carboxyisophthalic acid) in the presence of 0.5 m*M* L-glutamate (○) and 2.0 m*M* L-glutamate (●), 5-hydroxyisophthalic acid in the presence of 0.5 m*M* L-glutamate (+) and 2.0 m*M* L-glutamate (X), and 5-methoxyisophthalic acid in the presence of 0.5 m*M* L-glutamate (□) and 2.0 m*M* L-glutamate (■). Values above the named inhibitor represent its concentration on the abscissa. Extrapolation of slopes for two different substrate concentrations gave an intersection point in the fourth quadrant that represented the apparent K_i value for the inhibitor.

TABLE I. INHIBITION OF BOVINE LIVER GLUTAMATE DEHYDROGENASE AT PH 7.4.

Compound	K_i (m*M*)[a]
Isophthalic acid	0.8
5-Carboxyisophthalic acid	0.7
5-Hydroxyisophthalic acid	2.0
5-Fluoroisophthalic acid	2.4
5-Methoxyisophthalic acid	3.8
5-Bromoisophthalic acid	3.5
5-Cyanoisophthalic acid	2.3
5-Methylisophthalic acid	11.0

[a] K_i values were calculated from experiments similar to these in Fig. 2.

order change occurred from the substitutions in the 5 position of isophthalic acid at pH 7.4. Free energy correlations were not attempted with these molecules.

Figure 3 presented the effects of pH on the extent of competitive inhibition by some 5-substituted isophthalic acids. A break in the plot (12) of pK_i (negative logarithm of the inhibition constant K_i) *versus* pH indicated the presence of a perturbant group on the enzyme which influenced combination of the substituted isophthalic acid but not the parent compound with the enzyme. This group had an apparent pK_a of 7.4–7.8.

Protonation of the unknown group (pK_a 7.4–7.8) facilitated binding of the 5-substituted isophthalate. This ionized moiety might be the residue lysine which has been previously shown (14, 15) to complex with pyridoxal phosphate, an inhibitor of glutamate dehydrogenase. Alternatively, histidine might have been involved in the combination since it has a pK of 6. Our data did not distinguish between either possibility.

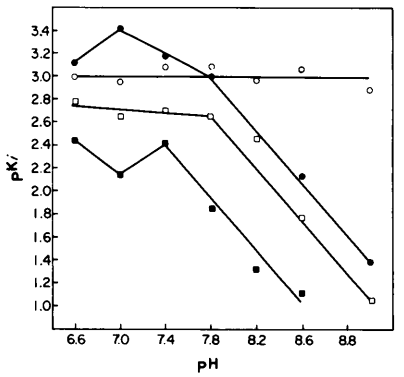

FIG. 3. Profile of inhibitor potency (pK_i) as a function of pH. Assays were conducted at 30° in 3.0 ml of 0.1*M* Na_2HPO_4–0.1 *M* $Na_4P_2O_7$ buffer. The assay system contained 0.6 m*M* $NADP^+$, enzyme (10 μg/ml), 0.5 m*M* or 2.0 m*M* L-glutamate, and varying amounts of inhibitor. The reaction was started by addition of enzyme in buffer. Apparent K_i values were determined graphically from Dixon plots of reciprocal velocity against inhibitor concentration (see typical examples in Fig. 2). Negative common logarithms of apparent K_i values (pK_i) of the competitive inhibitors were plotted as a function of pH for isophthalic acid (○), trimesic acid (●), 5-hydroxyisophthalic acid (□), 5-methoxyisophthalic acid (■).

Examination of the ionizable or hydrogen bond properties of the substituent added to isophthalate showed that no group was capable of pH change at the pK region found.

Summary. Isophthalic acid, 5-carboxy-, 5-hydroxy-, 5-methoxy-, 5-fluoro-, 5-bromo-, 5-cyano-, and 5-methylisophthalic acid were inhibitors competitive with L-glutamate for bovine liver glutamate dehydrogenase. The extent of inhibition by the derived compounds was not much greater than that obtained with the parent compound, isophthalic acid. A plot of pK_i versus pH showed the presence of an ionizable group (pK_a 7.4–7.8) at the enzyme active site which interacted with the substitutent at the 5 position of the substituted isophthalates.

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