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SYNTHESIS OF L-IDARO-1,4-LACTONE, AN INHIBITOR OF α-L-IDOSIDURONASE

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ABSTRACT

L-Idaro-1,4-lactone was synthesized by two different, published methods: (*1*) epimerization of monopotassium D-glucarate by refluxing in aqueous barium hydroxide, and (*2*) oxidation of L-iditol by heating in dilute nitric acid. The lactone, formed by heat dehydration from aqueous solution at low pH, was purified by paper chromatography, and quantitated by gas–liquid chromatography using inositol as the internal standard. The monolactone inhibited human, seminal-fluid α-L-idosiduronase activity, with either phenyl or 4-methylumbelliferyl α-L-idosiduronic acid as the substrate, to the same degree as D-glucaro-1,4-lactone inhibits α-D-glucosiduronase.

INTRODUCTION

D-Glucaro-1,4-lactone has been shown[1] to be a fairly specific, competitive inhibitor of β-D-glucosiduronase (EC 3.2.1.31), but has been reported[2] to have weaker inhibitory activity for α-L-idosiduronase (EC 3.2.1.76). A preliminary report of its use as a ligand in the affinity-chromatographic isolation of β-D-glucosiduronase has appeared[3], but to the best of our knowledge, this has not been widely exploited. The synthesis of L-idaro-1,4-lactone was undertaken to determine whether it would inhibit α-L-idosiduronase activity in a manner comparable to the inhibition of β-D-glucosiduronase by D-glucaro-1,4-lactone.

We now report the synthesis of L-idaric acid, based on two published methods using different starting-materials. The monolactone of L-idaric acid was found to be a potent inhibitor of α-L-idosiduronase.

EXPERIMENTAL

Materials. — Monopotassium D-glucarate, D-glucaro-1,4-lactone, and L-sorbose were purchased from Sigma Chemical Co., St. Louis, Missouri. Passage of an aqueous

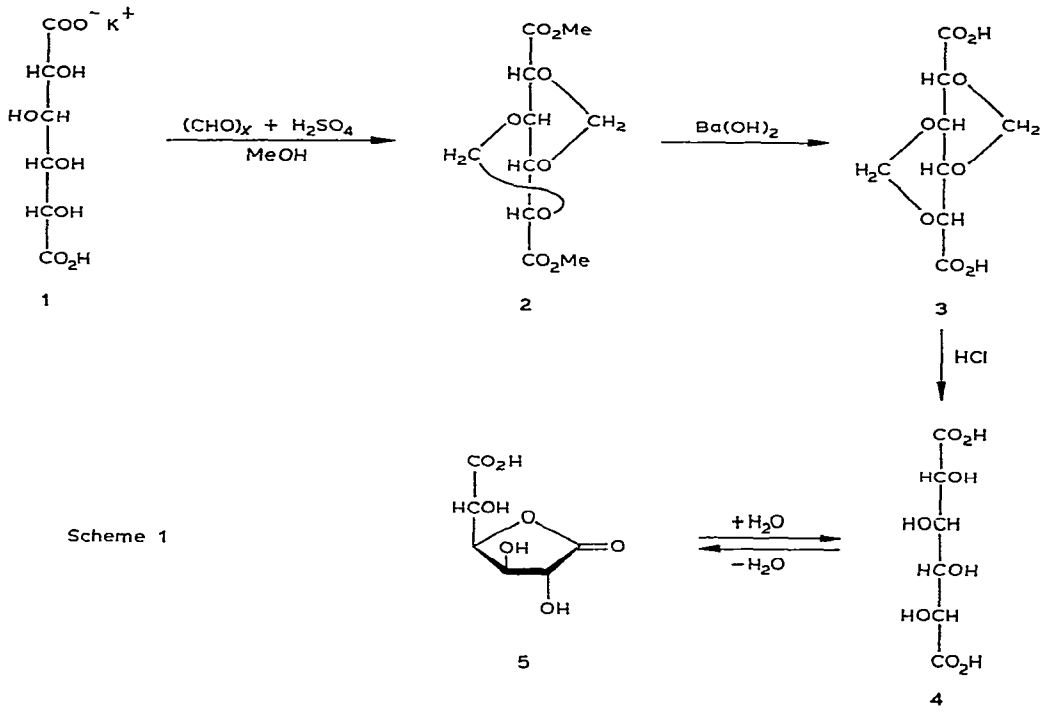
solution of monopotassium D-glucarate (20 g) through a column of AG-50W (H^+) ion-exchange resin (200–400 mesh) yielded D-glucaric acid, D-glucaro-6,3-lactone, and D-glucaro-1,4-lactone in the ratios of ~1:4:3 after drying to crystallization over phosphorus pentaoxide in a vacuum desiccator. The 6,3-lactone was extracted from a portion of this mixture with aqueous acetone, as described[4]. Raney nickel No. 28 was obtained from R. F. Cabirac of the W. R. Grace Co., Baltimore, Maryland. Crystals of authentic L-iditol, $[\alpha]_D$ −3.5°, were kindly supplied by Dr. Gilbert Ashwell, N.I.H., Bethesda, Maryland. Human, seminal fluid was used as the source of α-L-idosiduronase[5]. Phenyl α-L-idosiduronic acid, synthesized by Dr. B. Weissman, was obtained from Dr. E. F. Neufeld, N.I.H., Bethesda, Maryland. 4-Methylumbelliferyl α-L-idosiduronic acid (free from any detectable D-*gluco* isomer), as synthesized by Koch–Light Industries, Colnbrook, England, was purchased from Research Products International, Elk Grove, Illinois.

General methods. — Gas–liquid chromatography (g.l.c.) of the trimethylsilyl derivatives of the methyl esters of the aldaric acids and of their lactones, with inositol as the standard for quantitation, was conducted in a capillary column (10 or 50 m) packed with SP-2100, in a Hewlett–Packard 5840A apparatus, under temperature-programmed conditions, using flame-ionization detection, as reported[6]. Also for g.l.c., alditols and their pentaacetates were peracetylated by heating with 1:1 (v/v) pyridine–acetic anhydride, and the products examined in a column (244 cm), packed with 8% of SP-2340 on Chromosorb-W, in the same apparatus. Descending paper-chromatography on untreated Whatman No. 1 and No. 3M paper was performed for 4 h in 3:1:1 (v/v) ethyl acetate–acetic acid–water, with staining by the silver-dip method[7].

Assay of α-L-idosiduronase. — The conditions finally chosen for the assay were[8] (*1*) 1.33mM phenyl α-L-idosiduronic acid, 0.08M sodium formate buffer, pH 3.5, and 0.02% sodium azide (75 μL) for 18 h at 25°, or (*2*) 2.5μM 4-methylumbelliferyl α-L-idosiduronic acid, 0.03M sodium formate buffer, pH 3.7, and 0.03% sodium azide (65 μL) for 1 h at 37°. The 4-methylumbelliferol released (in pmol) was detected with a Turner Model II fluorimeter (Ex λ 265 nm, Em λ 435 nm) after addition of 0.5M glycine–NaOH buffer, pH 10.4 (1.5 mL).

RESULTS AND DISCUSSION

Epimerization of monopotassium D-glucarate. — Monopotassium D-glucarate (**1**; 40 g) was converted into the methyl ester, and thence into the 2,4:3,5-dimethylene acetal with paraformaldehyde and sulfuric acid, as described[9], to yield dimethyl 2,4.3,5-di-*O*-methylene-D-glucarate (**2**); 4.77 g, m.p. 155–157° (lit.[9] yield 7.5 g, m.p. 157.5°) Compound **2** (4.0 g) in an excess of aqueous barium hydroxide solution was boiled under reflux, and extracted as described[9], to give 2,4:3,5-di-*O*-methylene-L-idaric acid (**3**), 1.18 g, recrystallized after passage through a column of AG-50W (H^+) ion-exchange resin, m.p. 297–299°, $[\alpha]_D^{16}$ +73.7° (*c* 3.75, water); lit.[9] yield 1.4 g, m.p. 292° and 299°, $[\alpha]_D^{16}$ +73.7° (*c* 1.817, water). Compound **3** (100 mg) was deformal-



Scheme 1

ized by boiling a solution in 5% hydrochloric acid (10 mL) under reflux for 24 h, or in 10% acid for 6 h, in the presence of 1,3,5-benzenetriol (phloroglucinol) (212 mg) as described[10]. The mineral acid was neutralized with silver carbonate, and the silver salt in solution was decomposed by passing a gentle stream of hydrogen sulfide through the solution, centrifuging off the precipitated sulfide, and decolorizing the supernatant liquor (hot) with activated charcoal (Norit A). The epimerization reactions are shown in Scheme 1.

Paper chromatography of the deformalized products showed two widely separated spots, L-idaric acid (4) and a single monolactone (5), having mobilities different from those of D-glucaric acid and its 6,3- and 1,4-lactone, as shown in Fig. 1. The products were separated in quantity by preparative chromatography on Whatman No. 3M paper, located by staining segregated, marginal strips, eluted with water, the eluates evaporated to dryness, the residues dissolved in known volumes of water, and the solutions rechecked for purity by paper chromatography, quantitated by g.l.c., and stored frozen. Each product was initially free from any other, but, with prolonged storage and multiple thawings, some of the lactone was converted into free L-idaric acid, whereas the acid afforded only barely detectable amounts of the lactone. The lactone was readily converted into the free acid by titration with M sodium hydroxide, as for uronic acids[11].

For g.l.c., the method of methyl esterification (boiling with M hydrogen chloride in methanol, and evaporating to dryness) prior to forming the Me_3Si derivatives is

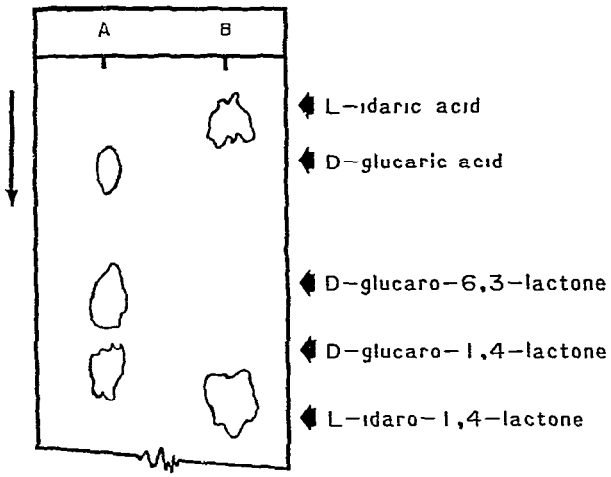


Fig. 1. Paper chromatogram of the aldaric acids and their 1,4-lactones. (A, D-Glucaric acid and lactones, B, L-idaric acid and the lactone from the epimerization reaction.)

such that either L-idaric acid or its monolactone yields two major products in the same proportions. D-Glucaric acid, or its 6,3- or 1,4-lactone, always formed three major compounds in the same proportions when derivatized for g.l.c., all having retention times differing from those of the two major L-idaric acid derivatives. In quantitating the products by g.l.c., with inositol (1 μmol) as the internal standard, the molar ratios were calculated by using either the sum of the area under both major peaks for L-idaric acid and its lactone, or the sum of the area under all three major peaks for D-glucaric acid and its lactones. When the methyl esters were derivatized with acetyl, instead of trimethylsilyl, groups only a single, major product was formed in each case, and that from the L-idarates had a retention time greater than that from the D-glucarates.

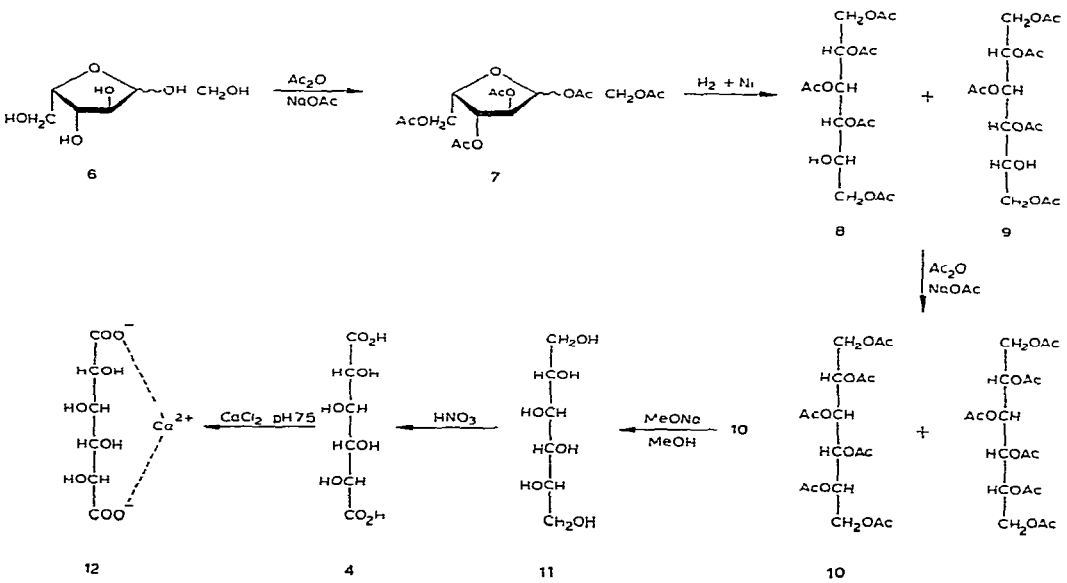
Reduction of L-*sorbose to* L-*iditol* (*and* D-*glucitol*). — L-Sorbose (**6**) was acetylated in lots (8 g) with acetic anhydride and fused zinc chloride at 50°, the excess of acetic anhydride was decomposed with water, the acid was neutralized with sodium hydrogencarbonate, the solution was diluted with water (4 vol.), and the penta-*O*-acetyl-L-sorbose (**7**) was extracted into chloroform (100 mL × 4) as published[12]. The extract was dried (calcium chloride), filtered, and the filtrate flash-evaporated to a dry, crystalline residue of L-sorbose pentaacetate, which was recrystallized from methanol (1.5 vol.); m.p. 96–99° (lit.[12] m.p. 99°). L-Sorbose pentaacetate was reduced with hydrogen at 1–2 atmospheres in the presence of Raney-nickel catalyst as published[12]. Although the authors claimed a 9:1 steric preference for the L-iditol epimer in the penta-*O*-acetylated product, our best efforts yielded only 76% of L-iditol pentaacetate (**8**), 18% of D-glucitol pentaacetate (**9**), plus 6% of an unidentified, fast-running impurity (g.l.c.). (Direct hydrogenation of L-sorbose with hydrogen in the presence of Raney nickel, or direct reduction with sodium borohydride, gave a 1:1 mixture of D-glucitol and L-iditol.)

The mixed pentaacetates obtained from the hydrogenation of L-sorbose pentaacetate (15.7 g) in absolute ethanol were evaporated to a syrup, and peracetylated with fused sodium acetate in acetic anhydride as published[13]. On recrystallization from absolute ethanol, 6.495 g (49.3% of the theoretical) of L-iditol hexaacetate (**10**) was obtained; this was >96% pure (by g.l.c.), and had the same retention time as the hexaacetate prepared directly from authentic L-iditol. Three recrystallizations from ethanol yielded L-iditol hexaacetate containing less than 0.01% of impurity (g l.c.).

L-Iditol hexaacetate (10 g) was deacetylated with sodium in methanol as described[13], mixed with AG-50W (H^+) cation-exchange resin (200–400 mesh) (5 mL) suspended in methanol, the suspension filtered, the filtrate flash-evaporated to a clear syrup, and the syrup stored under vacuum over phosphorus pentaoxide. Alone, L-iditol slowly crystallized in 6 days; seeded with authentic L-iditol, crystallization started within 6 h, and was complete within 48 h. Three such batches yielded 8.059 g (62.3% of the theoretical) of L-iditol (**11**), m.p. 74.5–75°, $[\alpha]_D^{26}$ −3.44° (*c* 10.0, water); authentic L-iditol has[13] m.p. 74–75.5°, $[\alpha]_D$ −3.5° (water).

Anal. Calc. for $C_6H_{14}O_6$: C, 39.56; H, 7.75; O, 52.69. Found*: C, 39.70; H, 7.78; O, 52.65.

Oxidation of L-*iditol.* — Oxidation of finely ground, crystalline L-iditol (5 g) was performed as described[13] with 4.06M nitric acid (sp. gr. 1.15), to yield calcium L-idarate (**12**), 2.65 g (39% of the theoretical). Paper chromatography, and g.l.c., of the product after passage through AG-50W (H^+) ion-exchange resin, showed its identity with L-idaric acid obtained in the epimerization reaction described earlier.



Scheme 2

*Galbraith Laboratories, Inc., Knoxville, Tennessee.

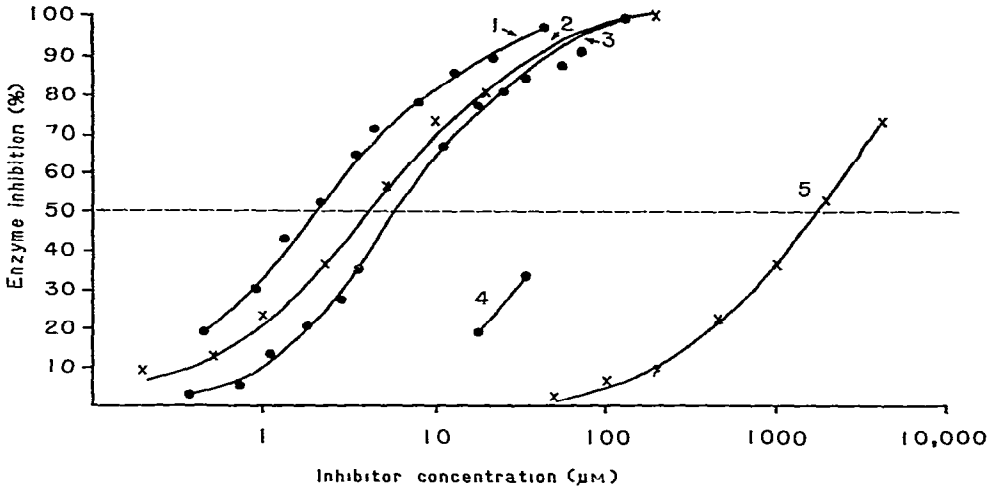


Fig 2 Inhibition of L-idosiduronase. [Curves 1, 3, and 4: inhibition of L-idosiduronase by L-idaro-1,4-lactone (1, with 4-methylumbelliferyl glycoside; 3, with phenyl L-idosiduronic acid); and by L-idaric acid (4, with 4-methylumbelliferyl glycoside). Curves 2 and 5: inhibition of D-glucosiduronase by D-glucaro-1,4-lactone (curve 2) and D-glucaric acid (curve 5), after Levvy[1].]

Steps in the oxidation reaction are shown in Scheme 2. Alternatively, L-idaric acid may be precipitated as the monopotassium salt[14]; a sample of the monohydrate was assayed

Anal. Calc. for $C_6H_{11}KO_9$: C, 27 07; H, 4.16; K, 14.69; O, 54.08; H_2O, 6.77. Found*: C, 27.85; H, 4.15; K, 14.54; O, 53.06; H_2O, 4.67.

Inhibition of L-idosiduronase. — To the enzyme-assay mixtures described were added various amounts of an aqueous solution of L-idaro-1,4-lactone, instead of water, in the final mix. Assays of inhibition by phenyl L-idosiduronic acid were performed in duplicate, and assays of inhibition by the 4-methylumbelliferyl glycoside in triplicate, at each concentration tested. The optimal pH for the latter assay was 3.7 (data not shown). Fig. 2 shows the inhibition of the L-idosiduronase by L-idaro-1,4-lactone using both substrates, or by L-idaric acid using 4-methylumbelliferyl glycoside, and, for comparison, also shows the inhibition of β-D-glucosiduronase by D-glucaro-1,4-lactone and D-glucaric acid (after Levvy[1]). L-Idaro-1,4-lactone achieved 50% inhibition of L-idosiduronase at a concentration of 80 μM with phenyl L-idosiduronic acid as the substrate, and at 40 μM with 4-methylumbelliferyl glycoside as the substrate. This inhibition is comparable to that of D-glucosiduronase by D-glucaro-1,4-lactone. L-Idaric acid inhibited L-idosiduronase at one-tenth the concentration of D-glucaric acid that is required for comparable inhibition of D-glucosiduronase.

Levvy's final concentration[1] of substrate (phenyl D-glucosiduronic acid) was 630μM, which is about twice as dilute as our phenyl L-idosiduronic acid and 300 times as concentrated as our 4-methylumbelliferyl glycoside. Although the concentration

*Galbraith Laboratories, Inc., Knoxville, Tennessee.

of L-idaro-1,4-lactone was determined after purification, some of the lactone may have been hydrated to the free acid, and consequently, the true concentration of lactone shown in these two curves may have been even lower for any given inhibition. The corollary may be true for the large inhibition of L-idosiduronase seen here with L-idaric acid. Although only "barely detectable" amounts of L-idaric acid are spontaneously converted into the lactone, as little as 1 % would be sufficient to cause significant inhibition of the enzyme. To explain inhibition of D-glucosiduronase by his preparations of "purified" D-glucaric acid, Levvy postulated[1] similar contamination with D-glucaro-1,4-lactone. That a given concentration of L-idaro-1,4-lactone more effectively inhibits L-idosiduronase when the concentration of substrate is 0.001 (4-methylumbelliferyl glycoside *vs.* phenyl L-idosiduronic acid) suggests a competitive type of inhibition.

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