

COMPOSITIONS AND METHODS FOR PRODUCING CHEMICALS AND DERIVATIVES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under U.S.C. §119(e) to continuation-in-part of U.S. Application Serial No. 14/222,453, filed March 21, 2014, continuation-in-part of U.S. Application Serial No. 14/033,300, filed September 20, 2013, which claims the benefit of priority under 35 U.S.C. §119(e) of U.S. provisional application serial number 61/704,408, filed September 21, 2012, which are each hereby incorporated by reference in their entireties, including all tables, figures, and claims.

INCORPORATION OF SEQUENCE LISTING

[0002] The material in the accompanying Sequence Listing is hereby incorporated by reference into this application. The accompanying sequence listing text file, name SGI1660_3WO_Sequence_Listing.txt, was created on March 20, 2015 and is 191 KB. The file can be assessed using Microsoft Word on a computer that uses Windows OS.

BACKGROUND OF THE INVENTION

[0003] In recent years, an increasing effort has been devoted to identify new and effective ways to use renewable feedstocks for the production of organic chemicals. Among a plethora of downstream chemical processing technologies, the conversion of biomass-derived sugars to value-added chemicals is considered very important. In particular, six-carboned carbohydrates, i.e. hexoses such as fructose and glucose, are widely recognized the most abundant monosaccharides existing in nature, therefore can be suitably and economically used as the chemical feedstocks.

[0004] The production of furans and furan derivatives from sugars has attracted increasing attention in chemistry and in catalysis studies, and is believed to have the potential to provide one of the major routes to achieving sustainable energy supply and chemicals production. Indeed, dehydration and/or oxidation of the sugars available within biorefineries with integrated biomass conversion processes can lead to a large family of products including a wide range of furans and furan derivatives.

[0005] Among the furans having the most commercial values, furan-2,5-dicarboxylic acid (also known as 2,5-furandicarboxylic acid, hereinafter abbreviated as FDCA) is a valuable intermediate with various uses in several industries including pharmaceuticals, pesticides, antibacterial agents, fragrances, agricultural chemicals, as well as in a wide range of manufacturing applications of polymer materials, *e.g.* bioplastic resins. As such, FDCA is considered a green alternative of terephthalic acid (TPA), a petroleum-based monomer that is

one of the largest-volume petrochemicals produced yearly worldwide. In fact, the US Department of Energy has identified FDCA as one of the top 12 priority compounds made from sugars into a value-added chemical for establishing the “green” chemistry of the future, and as such, it has been named one of the “sleeping giants” of the renewable intermediate chemicals (Werpy and Petersen, *Top Value Added Chemicals from Biomass*. US Department of Energy, Biomass, Vol 1, 2004).

[0006] Although various methods have been proposed for commercial scale production of FDCA (for review, see, *e.g.*, Tong et al., *Appl. Catalysis A: General*, 385, 1-13, 2010), the main industrial synthesis of FDCA currently relies on a chemical dehydration of hexoses, such as glucose or fructose, to the intermediate 5-hydroxymethylfurfural (5-HMF), followed by a chemical oxidation to FDCA. However, it has been reported that current FDCA production processes via dehydration are generally nonselective, unless immediately upon their formation, the unstable intermediate products can be transformed to more stable materials. Thus, the primary technical barrier in the production and use of FDCA is the development of an effective and selective dehydration process from biomass-derived sugars.

[0007] It is therefore desirable to develop methods for production of this highly important compound, as well as many other chemicals and metabolites, by alternative means that not only would substitute renewable for petroleum-based feedstocks, but also use less energy and capital-intensive technologies. In particular, the selective control of sugar dehydration could be a very powerful technology, leading to a wide range of additional, inexpensive building blocks.

Summary of the Invention

[0008] The present invention provides methods for producing a product of one or more enzymatic pathways. The pathways used in the methods of the invention involve one or more conversion steps such as, for example, an enzymatic conversion of guluronic acid into D-glucarate (Step 7); an enzymatic conversion of 5-ketogluconate (5-KGA) into L-Iduronic acid (Step 15); an enzymatic conversion of L-Iduronic acid into Idaric acid Step 7b); and an enzymatic conversion of 5-ketogluconate into 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step 16); an enzymatic conversion of 1,5-gluconolactone to gulurono-lactone (Step 19). In some embodiments the methods of the invention produce 2,5-furandicarboxylic acid (FDCA) as a product. The methods can include both enzymatic and chemical conversions as steps. Various pathways are also provided for converting glucose or fructose or sucrose or galactose into 5-dehydro-4-deoxy-glucarate (DDG), and for converting the same sugars into FDCA. The

methods can also involve the use of engineered enzymes that perform reactions with high specificity and efficiency.

[0009] In a first aspect the invention provides a method for producing a product of an enzymatic or chemical pathway from a starting substrate. The pathway can contain any one or more of the following conversion steps: an enzymatic conversion of guluronic acid into D-glucarate (Step 7); an enzymatic conversion of 5-ketogluconate (5-KGA) into L-Iduronic acid (Step 15); an enzymatic conversion of L-Iduronic acid into Idaric acid (Step 7b); and an enzymatic conversion of 5-ketocluconate into 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step 16); an enzymatic conversion of 1,5-gluconolactone to gulurono-lactone (Step 19).

[0010] In one embodiment the product of the enzymatic pathway is 5-dehydro-4-deoxy-glucarate (DDG). In various embodiments the substrate of the method can be glucose, and the product can 5-dehydro-4-deoxy-glucarate (DDG). The method can involve the steps of the enzymatic conversion of D-glucose to 1,5-gluconolactone (Step 1); the enzymatic conversion of 1,5-gluconolactone to gulurono-lactone (Step 19); the enzymatic conversion of gulurono-lactone to guluronic acid (Step 1B); the enzymatic conversion of guluronic acid to D-glucarate (Step 7); and the enzymatic conversion of D-glucarate to 5-dehydro-4-deoxy-glucarate (DDG) (Step 8).

[0011] In another method of the invention the substrate is glucose and the product is DDG, and the method involves the steps of: the conversion of D-glucose to 1,5-gluconolactone (Step 1); the conversion of 1,5-gluconolactone to gluconic acid (Step 1a); the conversion of gluconic acid to 5-ketogluconate (5-KGA) (Step 14); the conversion of 5-ketogluconate (5-KGA) to L-Iduronic acid (Step 15); the conversion of L-Iduronic acid to Idaric acid (Step 7b); and the conversion of Idaric acid to DDG (Step 8a).

[0012] In another method of the invention the substrate is glucose and the product is DDG and the method involves the steps of the conversion of D-glucose to 1,5-gluconolactone (Step 1); the conversion of 1,5-gluconolactone to gluconic acid (Step 1a); the conversion of gluconic acid to 5-ketogluconate (5-KGA) (Step 14); the conversion of 5- ketogluconate (5-KGA) to 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step 16); the conversion of 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) to 4-deoxy-5-threo-hexosulose uronate (DTHU) (Step 4); and the conversion of 4-deoxy-5-threo-hexosulose uronate (DTHU) to DDG (Step 5).

[0013] In another method of the invention the substrate is glucose and the product is DDG, and the method involves the steps of: the conversion of D-glucose to 1,5-gluconolactone (Step 1); the conversion of 1,5-gluconolactone to gluconic acid (Step 1a); the conversion of gluconic acid to 5-ketogluconate (5-KGA) (Step 14); the conversion of 5-ketogluconate (5-

KGA) to L-Iduronic acid (Step 15); the conversion of L-Iduronic acid to 4-deoxy-5-threo-hexosulose uronate (DTHU) (Step 7B); and the conversion of 4-deoxy-5-threo-hexosulose uronate (DTHU) to DDG (Step 5).

[0014] Any of the methods disclosed herein can further involve the step of converting the DDG to 2,5-furan-dicarboxylic acid (FDCA). Converting the DDG to FDCA in any of the methods can involve contacting DDG with an inorganic acid to convert the DDG to FDCA.

[0015] In another aspect the invention provides a method for synthesizing derivatized (esterified) FDCA. The method involves contacting DDG with an alcohol, an inorganic acid at a temperature in excess of 60 C to form derivatized FDCA. In different embodiments the alcohol is methanol, butanol or ethanol .

[0016] In another aspect the invention provides a method for synthesizing a derivative of FDCA. The method involves contacting DDG with an alcohol, an inorganic acid, and a co-solvent to produce a derivative of DDG; optionally purifying the derivative of DDG; and contacting the derivative of DDG with an inorganic acid to produce a derivative of FDCA. The inorganic acid can be sulfuric acid and the alcohol can be ethanol or butanol. In various embodiments the co-solvent can be any of THF, acetone, acetonitrile, an ether, butyl acetate, an dioxane, chloroform, methylene chloride, 1,2-dichloroethane, a hexane, toluene, and a xylene.

[0017] In one embodiment in the derivative of DDG is di-ethyl DDG and the derivative of FDCA is di-ethyl FDCA, and in another embodiment the derivative of DDG is di-butyl DDG and the derivative of FDCA is di-butyl FDCA.

[0018] In another aspect the invention provides a method for synthesizing FDCA. The method involves contacting DDG with an inorganic acid in a gas phase.

[0019] In another aspect the invention provides a method for synthesizing FDCA. The method involves contacting DDG with an inorganic acid at a temperature in excess of 120 C.

[0020] In another aspect the invention provides a method for synthesizing FDCA. The method involves contacting DDG with an inorganic acid under anhydrous reaction conditions.

Description of the Drawings

[0021] FIG. 1 is a electrophoretic gel of crude lysates and purified enzymes of proteins 474, 475, and 476.

[0022] FIGS. 2A-2H is a schematic illustration of the pathways of Routes 1, 2, 2A, 2C, 2D, 2E, 2F, respectively.

[0023] FIGS. 3A-3c present a schematic illustration of the pathways of Routes 3, 4, and 5, respectively.

[0024] FIG. 4 is an HPCL-MS analysis of the dehydration of gluconate with gluconate dehydratase to produce DHG by pSGI-359.

[0025] FIG. 5 is a graphical illustration of semicarbazide assay plots for measuring the activity of gluconate dehydratases.

[0026] FIGS. 6A-6B provide Lineweaver-Burk plots for the oxidation of glucuronate and iduronate with three enzymes of the invention.

[0027] FIG. 7A shows the results of an HPLC analysis of time points for the isomerization of 5KGA and Iduronate using enzymes DTHU isomerases in the EC 5.3.1.17 family. Controls: dead enzyme is a control with heat inactivated enzyme. Med Bl refers to reactions without isomerase add/n. Time points, x axis 1=0.5 h; 2=1; 3=2 h; 4=16h. Figure 7b shows an HPLC analysis of time points for the isomerization of 5KGA and iduronate using enzymes in the EC 5.3.1.17 family. Controls: dead enzyme is a control with heat inactivated enzyme; Med Bl: refers to reactions without isomerase add/n. Time points, X axis: 1=0 h; 2= 1 h ; 3= 2 h ; 4=17 h.

[0028] FIG. 8 shows product formation for the isomerization of 5KGA and iduronate with enzymes in the EC 5.3.1.n1 family. The data were obtained from enzymatic assays.

[0029] FIG. 9: HPLC analysis of the formation of 2,5-DDH and the reduction of 5KGA concentration over time. Total ion counts for 2,5-DDH are shown.

[0030] FIG. 10 is a HPLC-MS chromatogram showing the production of guluronic acid lactone from 1,5-gluconolactone. An overlay of a trace of authentic guluronic acid is shown.

[0031] FIG. 11 is a schematic illustration of the Scheme 6 reaction pathway.

[0032] FIGS. 12A-12B are LC-MS chromatograms showing 5-KGA and DDG reaction products, respectively.

[0033] FIG. 13 is an LC-MS chromatogram showing FDCA and FDCA dibutyl ester derivative reaction products.

[0034] FIG. 14A is a GC-MS analysis of a crude reaction sample of the diethyl-FDCA synthesis from the reaction of DDG with ethanol. Single peak corresponded to diethyl-FDCA. FIG. 14B is an MS fragmentation of the major product from the reaction of DDG with ethanol.

[0035] FIG. 15A is a GC-MS analysis of a crude reaction sample of the diethyl-FDCA synthesis from the reaction of DDG with ethanol. Single peak corresponded to diethyl-FDCA. FIG. 15B is a MS fragmentation of the major product from the reaction of DDG with ethanol.

[0036] FIG. 16 is a schematic illustration of the synthesis of FDCA and its derivatives from DTHU.

[0037] FIG. 17 is a schematic illustration of Scheme 1. Cell free enzymatic synthesis of DDG from glucose. Enzymes are ST-1: glucose oxidase; ST-1A: hydrolysis-chemical; ST-14: gluconate dehydrogenase (pSGI-504); ST-15: 5-dehydro-4-deoxy-D-glucuronate isomerase (DTHU IS, pSGI-434); ST-7B: Uronate dehydrogenase (UroDH, pSGI-476)); ST-8A Glucarate dehydratase (GlucDH, pSGI-353); ST-A: NAD(P)H oxidase (NADH_OX, pSGI-431); ST-B: Catalase. Figure 17b shows the concentration of reaction intermediates over the first 3h as analyzed by HPLC. Formation of DDG is shown in both reactions.

Detailed Description of the Invention

[0038] The present invention provides methods for producing a product of an enzymatic pathway. The methods can comprise the enzymatic conversion of a substrate into a product. By utilizing the enzymatic and chemical pathways of the invention it is possible to synthesize a wide variety of products in a highly efficient and economical manner. One product that can be produced by the methods and pathways of the invention is 2,5-furanyl dicarboxylic acid (FDCA), which can be produced at commercial scales according to the invention. The methods can comprise one or more enzymatic and/or chemical substrate-to-product conversion steps disclosed herein. In some embodiments the enzymes utilized perform enzymatic conversion steps using activities unknown for the enzymes. These novel activities can therefore be employed in the invention to perform the conversion steps and perform a substrate to product conversion as part of a enzymatic and/or chemical pathway. Any of the products of any of the pathways disclosed herein (e.g., DDG, iduronic acid, idaric acid, glucaric acid, FDCA, etc.) can be produced on a commercial scale. i.e. in quantities of at least 1 gram or at least 10 grams or at least 100 grams or at least 1 kg in a single bioreactor or reaction vessel, as disclosed herein.

[0039] The pathways of the invention are comprised of any one or more of the steps disclosed herein. It is understood that a step of a pathway of the invention can involve the forward reaction or the reverse reaction, i.e., the substrate A being converted into product B, while in the reverse reaction substrate B is converted into product A. In the methods both the forward and the reverse reactions are described as the step unless otherwise noted.

[0040] The methods involve producing a product of a pathway, which can be an enzymatic pathway. The methods involve one or more enzymatic and/or chemical conversion steps, which convert a substrate to a product. Steps that can be included in the methods include, for example, any one or more of: an enzymatic conversion of guluronic acid into D-

glucarate (Step 7); an enzymatic conversion of L-Iduronic acid to 4-deoxy-5-threo-hexosulose uronate (DTHU)(17); an enzymatic conversion of 5-ketogluconate (5-KGA) into L-Iduronic acid (Step 15); an enzymatic conversion of L-Iduronic acid into Idaric acid (Step 7B); and an enzymatic conversion of 5-ketogluconate into 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step 16); an enzymatic conversion of 1,5-gluconolactone to gulurono-lactone (Step 19). Any one or more of the aforementioned steps can be included in a method or pathway of the invention. An enzymatic step or pathway is a step or pathway that requires an enzyme as a catalyst in the reaction to make the step proceed. Chemical steps can be performed without an enzyme as a catalyst in the reaction. Any one or more of the steps recited in the methods can be an enzymatic step. In some embodiments every step of the pathway is an enzymatic step, while in other embodiments one or more steps in the pathway is a chemical step.

[0041] In some embodiments any of the methods can include a step involving the addition of the substrate of the reaction to a reaction mix containing the enzyme that performs the conversion. Thus the method of converting guluronic acid into D-glucarate (step 7) can involve the addition of guluronic acid as starting substrate to the reaction mix; the enzymatic conversion of L-iduronic acid to Idaric acid (7B) can involve the addition of L-Iduronic acid as starting substrate to the reaction mix; the enzymatic conversion of L-Iduronic acid to 4-deoxy-5-threo-hexosulose uronate (DTHU) (17) can involve the addition of L-iduronic acid as starting substrate to the reaction mix. Any of the methods can involve a step of adding glucose, fructose, galactose, sucrose, or mannose or another mono- or di-saccharide to the reaction mixture. Another step that can be included in any of the methods is a step of purifying from the reaction mixture a reaction product. Thus, a step of purifying glucaric acid/D-glucarate or L-Iduronic acid/iduronate, or Idaric acid, or 2,5-diketo hexanedioic/DKHA can be included in any of the methods described herein. Any of the methods disclosed can include a step of isolating or purifying DDG or FDCA from the reaction mixture. And any of the methods can involve a step of adding an enzyme that performs any one or more of the steps described herein to the reaction mixture. A reaction mixture is a mixture of at least one substrate and at least one enzyme and involves the conversion of at least one substrate into at least one enzyme product. Any of the methods can involve a step of adding an isolated enzyme to a reaction mix, the enzyme performing a substrate to product conversion step of a pathway of the invention, and the isolated enzyme being at least 10% purified or at least 20% purified or at least 25% purified or at least 50% purified or at least 70% purified or at least 80% purified or at least 90%, all w/w.

[0042] Since many sugars can be converted into other sugars any of the methods or pathways of the invention can involve the use of glucose, sucrose, fructose or galactose as the starting substrate. Thus, in any pathway or reaction disclosed herein where glucose is the starting substrate it is understood that fructose or sucrose or galactose or mannose or another starting substrate can also be a starting substrate for that pathway or reaction. In some embodiments the sugar is converted into glucose which then enters the pathway but in other embodiments the pathway begins with fructose or sucrose or galactose or mannose or another mono- or di-saccharide.

[0043] The reactions of the invention can occur in a lysate of cells or a cell-free lysate that contains one or more enzymes that perform the enzymatic conversion, but can also occur in a reaction mixture containing components added by the user to form a reaction mixture, or can contain components purified from a cell lysate, or may be contained in a whole cell biocatalyst. The reaction can also occur in a mix made of purified components that have been combined, such as in a mix where the substrate and enzyme were combined to form the reaction mix. The reactions can occur in an in vitro reaction or can occur in a recombinant cell, and therefore the product(s) can be harvested by lysing the cells or by collecting from the culture medium. The reactions can occur in a laboratory container or reaction vessel such as, for example, a centrifuge tube, a test tube, a vial, a beaker, or a glass or metal or plastic container or reactor, a fermenter or fermentation vessel or bioreactor, an algae pond, any of which can be small scale or large scale. Any of the organisms described herein can be utilized as host cells to produce the product of a step or pathway of the invention. The organisms can also be used to produce one or more enzymes of the invention for use in a method of the invention. Various types of organisms can be used. Examples include: bacteria of the family Acetobacteraceae (e.g. bacteria of the genus *Acetobacter*, *Acidiphilium*, *Gluconobacter*, *Gluconoacetobacter*), or bacteria of the family Pseudomonadaceae (e.g., genus *Azotobacter*, *Pseudomonas*), or bacteria of the family Enterobacteriaceae (e.g., of the genus *Escherichia* (e.g., *E. coli*), *Klebsiella*). Yeast can also be used for these purposes such as yeast of the genera *Saccharomyces*, *Ashbya*, *Kluyveromyces*, *Lachancea*, *Zygosaccharomyces*, *Candida*, *Pichia*, *Arxula* or *Trichosporon* or *Blastobotrys*. Cyanobacteria can also be used such as those of the genus *Cyanothece* (e.g. *Cyanothece* strains ATCC 51142, PCC 7424, PCC 7425, PCC 7822, PCC 8801, PCC 8802), or *Microcystis* or *Synechococcus* (e.g., strains *elongatus* PCC 7942, PCC 7002, PCC 6301, CC9311, CC9605, CC9902, JA-2-3B'a(2-13), JA-3-3Ab, RCC307, WH 7803, WH 8102) or *Synechocystis*, or *Thermosynechococcus*. Thus the present invention provides recombinant host cells comprising a recombinant nucleic acid of one or more of SEQ

ID NOs: 4-6, 20-32, 36-38, 47-54, 56, 62-66, 69-70, 72, and 79-84 or a codon-optimized sequence of any of SEQ ID NOs: 1-84. The host cells can also contain a vector of the invention described herein. A “codon optimized” sequence refers to changes in the codons of a sequence to those preferentially used in a particular organism so that the encoded protein is efficiently expressed in the organism carrying the sequence. The recombinant nucleic acid sequence can be comprised on a vector, as disclosed herein.

[0044] In various embodiments the methods of the invention are methods of converting glucose or fructose or sucrose or galactose to DDG, or glucose or fructose or sucrose or galactose to FDCA, or glucose or fructose or sucrose or galactose to DTHU or DEHU, or for converting DDG to FDCA. The methods can involve converting the starting substrate in the method into the product. The starting substrate is the chemical entity considered to begin the method and the product is the chemical entity considered to be the final end product of the method. Intermediates are those chemical entities that are created in the method (whether transiently or permanently) and that are present in the reaction pathway between the starting substrate and the product. In various embodiments the methods and pathways of the invention have about four or about five intermediates or 4-5 intermediates, or about 3 intermediates, or 3-5 intermediates, or less than 6 or less than 7 or less than 8 or less than 9 or less than 10 or less than 15 or less than 20 intermediates, meaning these values not counting the starting substrate or the final end product.

[0045] The invention provides methods of producing FDCA and/or DDG, from glucose or fructose or sucrose or galactose that have high yields. The theoretical yield is the amount of product that would be formed if the reaction went to completion under ideal conditions. In different embodiments the methods of the invention produce DDG from glucose, fructose, or galactose with a theoretical yield of at least 50% molar, or at least 60% molar or at least 70% molar, or at least 80% molar, at least 90% molar or at least 95% molar or at least 97% molar or at least 98% molar or at least 99% molar, or a theoretical yield of 100% molar. The methods of the invention also can provide product with a carbon conservation of at least 80% or at least 90% or at least 95% or at least 97% or at least 98% or at least 99% or 100%, meaning that the particular carbon atoms present in the initial substrate are present in the end product of the method at the recited percentage. In some embodiments the methods produce DDG and/or FDCA from glucose or fructose or sucrose or galactose via dehydration reactions.

Example Synthesis Routes

[0046] The invention also provides specific pathways for synthesizing and producing a desired product. Any of the following described routes or pathways can begin with glucose or

fructose or sucrose or galactose or mannose and flow towards a desired product. In some embodiments D-glucose is the starting substrate and the direction of the pathway towards any intermediate or final product of the pathway is considered to be in the downstream direction, while the opposite direction towards glucose is considered the upstream direction. It will be realized that routes or pathways can flow in either the downstream or upstream direction. While glucose is used as an example starting substrate for pathways described herein, it is also understood that sucrose, fructose, galactose, or mannose or any intermediate in any of the pathways can also be the starting substrate in any method of the invention, and DDG, DTHU, FDCA, or any intermediate in any of the routes or pathways of the invention can be the final end product of a method of the invention. The disclosed methods therefore include any one or more steps disclosed in any of the routes or pathways of the invention for converting any starting substrate or intermediate into any end product or intermediate in the disclosed routes or pathways using one or more of the steps in the disclosed routes or pathways. Thus, for example the methods can be methods for converting glucose or fructose or sucrose or galactose or mannose to DDG, or to guluronic acid, or to galactarate, or to DTHU, or to DEHU, or to guluronic acid, or to iduronic acid, or to idaric acid, or to glucaric acid, or for converting galactarate to DDG, or for converting guluronic acid to D-glucarate, or for converting 5-KGA to L-Iduronic acid, or for converting L-Iduronic acid to Idaric acid, or for converting 5-KGA to 2,5-DDH or DTHU, or for converting DHG to DEHU. In these embodiments the methods utilize the steps disclosed in the methods and pathways of the invention from starting substrate to the relevant end product. One or more of the steps can also be utilized in methods flowing in the “opposite” or upstream direction from the pathways disclosed herein.

[0047] Route 1 is illustrated in Figure 2a. Route 1 converts D-glucose (or any intermediate in the pathway) into 5-dehydro-4-deoxy-glucarate (DDG) via an enzymatic pathway via a series of indicated steps. Route 1 converts D-glucose into DDG via a pathway having 1,5-gluconolactone, gluconic acid, 3-dehydro-gluconic acid (DHG), 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH), and 4-deoxy-L-threo-hexosulose uronate (DTHU) as intermediates and DDG as the final end product. For any of the pathways additional intermediates not shown can also be present. The steps are the enzymatic conversion of D-glucose to 1,5-gluconolactone (Step 1); the enzymatic conversion of 1,5-gluconolactone to gluconic acid (Step 1A); the enzymatic conversion of gluconic acid to 3-dehydro-gluconic acid (DHG) (Step 2); the enzymatic conversion of 3-dehydro-gluconic acid (DHG) to 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step 3); the enzymatic conversion of 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) to 4-deoxy-L-threo-hexosulose uronate (DTHU) (Step 4); and the

enzymatic conversion of 4-deoxy-L-threo-hexosulose uronate (DTHU) to 5-dehydro-4-deoxy glucarate (DDG) (Step 5). Route 1 also comprises sub-routes where the glucose or any intermediate in the pathway as a substrate is converted into any other downstream intermediate as final product, and each substrate to product sub-route is considered disclosed as if each is set forth herein in full.

[0048] Route 2 is illustrated in Figure 2b and converts D-glucose into DDG. The steps in the Route 2 pathway are the enzymatic conversion of D-glucose into 1,5-gluconolactone (Step 1); the enzymatic conversion of 1,5-gluconolactone to gluconic acid (Step 1A); the enzymatic conversion of gluconic acid to guluronic acid (Step 6); the enzymatic conversion of guluronic acid to D-glucarate (Step 7); the enzymatic conversion of D-glucarate to DDG (Step 8). Route 2 also comprises sub-routes where glucose or any intermediate in the pathway as substrate is converted into any other downstream intermediate as final product, and each sub-route is considered disclosed as if each is set forth herein in full. For example in some embodiments the methods comprise steps for the conversion of glucose or gluconic acid as substrate into guluronic acid or D-glucarate as product using one or more of the steps described in Route 2.

[0049] Route 2A is illustrated in Figure 2c. The steps in Route 2A are the enzymatic conversion of D-glucose to 1,5-gluconolactone (Step 1); the enzymatic conversion of 1,5-gluconolactone to guluronic acid lactone (Step 19); the enzymatic conversion of guluronic acid lactone to guluronic acid (Step 1B); the enzymatic conversion of guluronic acid to D-glucarate (Step 7); the enzymatic conversion of D-glucarate to 5-dehydro-4-deoxy-glucarate (DDG) (Step 8). Route 2A also comprises sub-routes where glucose or any intermediate in the pathway as starting substrate is converted into any other downstream intermediate as final end product, and each sub-route is considered disclosed as if each is set forth herein in full. For example in some embodiments the methods comprise steps for the conversion of glucose or guluronic acid lactone as substrate into glucarate or DDG as product using one or more of the steps described in Route 2A.

[0050] Route 2B is illustrated in Figure 2d. The steps in Route 2B are the enzymatic conversion of D-glucose into gluconic acid (Steps 1 and 1A); the enzymatic conversion of gluconic acid into 5-ketogluconate (5-KGA) (Step 14); the enzymatic conversion of 5-KGA into L-Iduronic acid (Step 15); the enzymatic conversion of L-Iduronic acid into Idaric acid (Step 7B); the enzymatic conversion of Idaric acid into DDG (Step 8A). Route 2B also comprises sub-routes where glucose or any intermediate in the pathway as starting substrate is converted into any other downstream intermediate as final end product, and each sub-route is

considered disclosed as if each is set forth herein in full. For example in some embodiments the methods comprise steps for the conversion of glucose or 5-KGA as substrate into iduronic acid or idaric acid as product using one or more of the steps described in Route 2B.

[0051] Route 2C is illustrated in Figure 2e. The steps in Route 2C are the enzymatic conversion of D-glucose to gluconic acid (Steps 1 and 1A); the enzymatic conversion of gluconic acid to 5-ketogluconate (5-KGA) (Step 14); the enzymatic conversion of 5-KGA to 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step 16); the enzymatic conversion of 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) to 4-deoxy-5-threo-hexosulose uronate (DTHU) (Step 4); the enzymatic conversion of DTHU to DDG (Step 5). Route 2C also comprises sub-routes where glucose or any intermediate in the pathway as starting substrate is converted into any other downstream intermediate as final end product, and each sub-route is considered disclosed as if each is set forth herein in full. For example in some embodiments the methods comprise steps for the conversion of glucose or gluconic acid as substrate into 2,5-DDH or DTHU using one or more steps described in Route 2C.

[0052] Route 2D is illustrated in Figure 2f. The steps in Route 2D are the enzymatic conversion of D-glucose to gluconic acid (Steps 1 and 1A); the enzymatic conversion of gluconic acid to 5-ketogluconate (5-KGA) (Step 14); the enzymatic conversion of 5-KGA to Iduronic acid (Step 15); the enzymatic conversion of L-Iduronic acid to DTHU (Step 17); the enzymatic conversion of DTHU to DDG (Step 5). Route 2D also comprises sub-routes where glucose or any intermediate in the pathway as starting substrate is converted into any other downstream intermediate as final end product, and each sub-route is considered disclosed as if each is set forth herein in full. For example in some embodiments the methods comprise steps for the conversion of glucose or 5-KGA as substrate into L-iduronic acid or DTHU using one or more of the steps described in Route 2D.

[0053] Route 2E is illustrated in Figure 2g. The steps in Route 2D are the enzymatic conversion of D-glucose to 1,5-gluconolactone (Step 1); the enzymatic conversion of 1,5-gluconolactone to guluronic acid lactone (Step 19); the enzymatic conversion of guluronic acid lactone to guluronic acid (Step 1B); the enzymatic conversion of guluronic acid to 4-deoxy-erythro-hexosulose uronate (DEHU) (Step 17A); the enzymatic conversion of DEHU to 3-deoxy-D-erythro-2-hexulosaric acid (DDH) (Step 7A). Route 2E also comprises sub-routes where glucose or any intermediate in the pathway as starting substrate is converted into any other downstream intermediate as final end product, and each sub-route is considered disclosed as if each is set forth herein in full. For example in some embodiments the methods comprise

steps for the conversion of glucose as substrate into guluronic acid or DEHU using one or more of the steps described in Route 2E.

[0054] Route 2F is illustrated in Figure 2h. The steps in Route 2F are the enzymatic conversion of D-glucose to gluconic acid (Steps 1 and 1A); the enzymatic conversion of gluconic acid to guluronic acid (Step 6); the enzymatic conversion of guluronic acid to 4-deoxy-erythro-hexosulose uronate (DEHU) (Step 17A); the enzymatic conversion of DEHU to 3-deoxy-D-erythro-2-hexulosaric acid (DDH) (Step 7A). Route 2F also comprises sub-routes where glucose or gluconic acid or any intermediate in the pathway as starting substrate is converted into guluronic acid or DDH or any other downstream intermediate as final end product using one or more of the steps of Route 2F, and each sub-route is considered disclosed as if each is set forth herein in full.

[0055] Route 3 is illustrated in Figure 3a. The steps in Route 3 are the enzymatic conversion of D-glucose to gluconic acid (Steps 1 and 1A); the enzymatic conversion of gluconic acid to 3-dehydro-gluconic acid (DHG) (Step 2); the enzymatic conversion of DHG to 4-deoxy-erythro-hexosulose uronate (DEHU) (Step 6A); the enzymatic conversion of DEHU to DDG (Step 7A). Route 3 also comprises sub-routes where glucose or fructose or sucrose or galactose or any intermediate in the pathway as starting substrate is converted into gluconic acid or DDH any other downstream intermediate of Route 3 as final end product using one or more of the steps of Route 3, and each sub-route is considered disclosed as if each is set forth herein in full.

[0056] Route 4 is illustrated in Figure 3b. The steps in Route 4 are the enzymatic conversion of D-glucose to α -D-glucopyranose (Step 9); the enzymatic conversion of α -D-glucopyranose to α -D-glucopyranuronic acid (Step 10); the enzymatic conversion of α -D-glucopyranuronic acid to D-glucaric acid 1,5-lactone (Step 11); the enzymatic conversion of D-glucaric acid 1,5-lactone to D-glucarate (Step 1C); the enzymatic conversion of D-glucarate to DDG (Step 8). Route 4 also comprises sub-routes where glucose or any intermediate in the pathway as starting substrate is converted into glucarate or DDG or any other downstream intermediate as final end product using one or more of the steps of Route 4, and each sub-route is considered disclosed as if each is set forth herein in full.

[0057] Route 5 is illustrated in Figure 3c. The steps in Route 5 are the enzymatic conversion of D-galactose to D-galacto-hexodialdose (Step 9A); the enzymatic conversion of D-galacto-hexodialdose to galacturonate (Step 10A); the enzymatic conversion of galacturonate to galactarate (Step 11A); the enzymatic conversion of galactarate to DDG (Step

13). Route 5 also comprises sub-routes where galactose or any intermediate in the pathway as starting substrate is converted into any other downstream intermediate as final product, and each sub-route is considered disclosed as if each is set forth herein in full. For example in some embodiments the methods comprise steps for the conversion of galactose or another substrate into galacturonate or galactarate using the steps described in Route 5.

[0058] In various other embodiments the invention provides a method of producing a product of an enzymatic and/or chemical pathway from a starting substrate that involves performing Step 1, followed by Step 19, followed by Step 1B to produce a guluronic acid product. Optionally the pathway can continue with Step 7 to produce glucarate. In another embodiment the method involves performing Steps 1 and 1A followed by Step 14, followed by Step 15 to produce Iduronic acid. Optionally the method can continue with Step 7B to produce an Idaric acid product or with Step 17 to produce DTHU. In another embodiment the method involves performing Steps 1 and 1A, followed by Step 14 followed by Step 16 to produce a 2,5-DDH product. In another embodiment the method involves performing Step 1 followed by Step 19 to produce guluronic acid lactone.

The Enzymatic Steps

[0059] There are disclosed a wide variety of enzymes (and nucleic acids that encode the enzymes) that can perform the steps of the methods outlined herein. The enzymes utilized in the enzymatic steps of the invention can be proteins or polypeptides. In addition to the families and classes of enzymes disclosed herein for performing the steps of the invention, homologs having a sequence identity to any enzyme or nucleic acid or to any of SEQ ID NOs 1-84, disclosed herein will also be useful in the invention. Enzymes and nucleic acids that are homologs of SEQ ID NOs: 1-84 have a sequence identity of at least 40% or at least 50% or at least 60% or at least 70% or at least 80% or at least 90% or at least 95% or at least 97% or at least 98% or at least 99% to any nucleic acid or enzyme of SEQ ID NO: 1-84, or to a member of an enzyme class disclosed herein. Percent sequence identity or homology with respect to amino acid or nucleotide sequences is defined herein as the percentage of amino acid or nucleotide residues in the candidate sequence that are identical with the known polypeptides, after aligning the sequences for maximum percent identity and introducing gaps, if necessary, to achieve the maximum percent identity or homology. Homology or identity at the nucleotide or amino acid sequence level may be determined using methods known in the art, including but not limited to BLAST (Basic Local Alignment Search Tool) analysis using the algorithms employed by the programs blastp, blastn, blastx, tblastn and tblastx (Altschul (1997), *Nucleic Acids Res.* 25, 3389-3402, and Karlin (1990), *Proc. Natl. Acad. Sci. USA* 87, 2264-2268),

which are tailored for sequence similarity searching. Alternatively a functional fragment of any of the enzymes or nucleic acids encoding such enzymes or of any enzyme or nucleic acid of SEQ ID NOs 1-84 disclosed herein may also be used. The term “functional fragment” refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion and/or internal deletion (which can be replaced to form a chimeric protein), where the remaining amino acid sequence has at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the corresponding positions in the reference sequence, and/or that retains about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the activity of the full-length polypeptide. The EC numbers provided use the enzyme nomenclature of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology. In other embodiments the functional fragment retains the requirement of the presence of a co-factor necessary for the activity of a protein or protein encoded by SEQ ID NO:1-84.

[0060] Also disclosed is an expression vector having a sequence of SEQ ID NO: 4-6, 20-32, 36-38, 47-54, 56, 62-66, 69-70, 72, and 79-84. The vector can be a bacterial, yeast, or algal vector. Vectors designed for expression of a gene can also include a promoter active in the organism carrying the vector and operably linked to the sequence of the invention. The vector can contain a promoter or expression control sequence operatively linked to a sequence of SEQ ID NOs: 4-6, 20-32, 36-38, 47-54, 56, 62-66, 69-70, 72, and 79-84 or a codon-optimized sequence of any of them. A “promoter” refers to a nucleic acid sequence capable of binding RNA polymerase to initiate transcription of a gene in a 5' to 3' (“downstream”) direction. A sequence is “operably linked” to a promoter when the binding of RNA polymerase to the promoter is the proximate cause of said gene’s transcription.

[0061] Step 1 – Conversion (oxidation or dehydrogenation) of glucose to 1,5-gluconolactone. This step can be performed with various enzymes, such as those of the family oxygen dependent glucose oxidases (EC 1.1.3.4) or NAD(P)-dependent glucose dehydrogenases (EC 1.1.1.118, EC 1.1.1.119). *Gluconobacter oxydans* has been shown to efficiently oxidize glucose to gluconic acid and 5-ketogluconate (5-KGA) when grown in a fermentor. Enzymes of the family of soluble and membrane-bound PQQ-dependent enzymes (EC 1.1.99.35 and EC 1.1.5.2) found in *Gluconobacter* and other oxidative bacteria can be used. Quinoprotein glucose is another enzyme that is useful in performing this step. The specific enzyme selected will be dependent on the desired reaction conditions and necessary co-factors that will be present in the reaction, which are illustrated in Table 1.

[0062] Step 1A – Conversion (e.g., hydrolysis) of 1,5-gluconolactone to gluconate. This step can be performed chemically in aqueous media and the rate of hydrolysis is dependent on pH (Shimahara, K, Takahashi, T., *Biochim. Biophys. Acta* (1970), 201, 410). Hydrolysis is faster in basic pH (e.g. pH 7.5) and slower in acid pH. Many microorganisms also contain specific 1,5-glucono lactone hydrolases, and a few of them have been cloned and characterized (EC 3.1.1.17; Shinagawa, E *Biosci. Biotechnol. Biochem.* 2009, 73, 241-244).

[0063] Step 1B – Conversion of Guluronic acid lactone to guluronic acid. The chemical hydrolysis of guluronic acid lactone can be done by a spontaneous reaction in aqueous solutions. An enzyme capable of catalyzing this hydrolysis is identified amongst the large number of lactonases (EC 3.1.1. XX and more specifically 3.1.1.17, 3.1.1.25).

[0064] Step 2 – Conversion of gluconic acid to 3-dehydro gluconic acid (DHG): Several enzymes, such as gluconate dehydratases, can be used in the dehydration of gluconic acid to dehydro gluconic acid (DHG). Examples include those belonging to the gluconate dehydratase family (EC 4.2.1.39). A specific example of such a dehydratase has been shown to dehydrate gluconate (Kim, S. Lee, S.B. *Biotechnol. Bioprocess Eng.* (2008), 13, 436). Particular examples of enzymes from this family and their cloning are shown in Example 1.

[0065] Step 3: Conversion of 3-dehydro-gluconic acid (DHG) to 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH). Enzymes, 2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase (or DHG dehydrogenases) (EC 1.1.1.127) for performing this conversion have been described.

[0066] Step 4: Conversion of 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) to 4-deoxy-L-threo-hexosulose uronate (DTHU). Enzymes of the family EC 5.3.1.12 can be used in this step, and Step 15 shows that five such enzymes were cloned and shown to have activity for the dehydration of 5-KGA. These enzymes will also show activity towards 2,5-DDH and DTHU.

[0067] Step 5: Conversion of DTHU to 5-dehydro-4-deoxy-glucarate (DDG). DDG can be produced from the chemical or enzymatic oxidation of DTHU, for example with a mild chemical catalyst capable of oxidizing aldehydes in the presence of alcohols. Aldehyde oxidases can be used to catalyze this oxidation. Oxidative bacteria such as *Acetobacter* and *Gluconobacter* (Hollmann *et al Green Chem.* 2011, 13, 226) will be useful in screening. Enzymes of the following families can perform this reaction: aldehyde oxidase EC1.2.3.1, aldehyde ferredoxin oxidoreductase (EC1.2.7.5), and in all the families of EC1.2.1.-XX. Enzymes of the family of uronate dehydrogenases (EC 1.1.1.203) (e.g. see Step 7) will also have this activity. Other enzymes with both alcohol and aldehyde oxidation activity can be used, including enzymes in the alditol oxidase family (see Steps 19 and 6). Other broad

substrate oxidases include soluble and membrane bound PQQ-dependent alcohol/aldehyde oxidases. More specifically soluble periplasmic PQQ oxidases enzymes and their homologs belonging into Type I (EC 1.1.9.1) and II (EC 1.1.2.8) families as well as membrane bound PQQ oxidases belonging into EC 1.1.5.X families are useful. In other embodiments aldehyde dehydrogenases/oxidases that act on DTHU can be used.

[0068] Step 5 can also be performed using a dehydrogenase from acetic acid bacteria such Gluconobacter and Acetobacter and Gluconoacetobacter, and others. Whole cell activity is identified by screening microorganisms for the oxidation of DTHU. The activity is identified and one or more of the enzymes is cloned. Enzymes with uronate dehydrogenase activity described in Step-7 and 7B are also screened and found to have this activity. A library of soluble periplasmic and membrane bound PQQ-dependent enzymes is also cloned and several enzymes are found having this activity. Some of the enzymes found to have the activity are NAD(P)- or PQQ-dependent dehydrogenases, but others are FAD-dependent aldehyde dehydrogenases. SEQ ID NO: 71-72 are examples of NADP-dependent dehydrogenases, and any one or a combination of them can be used to perform Step 5. SEQ ID NOs: 73-84 are examples of suitable PQQ-dependent dehydrogenases and any one or any combination of them can be used to perform Step 5.

[0069] Steps 6 and 6A: Conversion of gluconic acid to guluronic acid (6) and conversion of 3-dehydro-gluconic acid (DHG) to 4-deoxy-5-erythro-hexosulose uronate (DEHU)(6A). The enzymes described in Step 5 are useful for these conversions. Other useful enzymes include NAD(P)-dependent dehydrogenases in the EC 1.1.1.XX families and more specifically glucuronate dehydrogenase (EC 1.1.1.19), glucuronolactone reductase (EC 1.1.1.20). In addition, a large number O₂-dependent alcohol oxidases with broad substrate range including sugars will be useful (EC 1.1.3.XX), including sorbitol/mannitol oxidases (EC 1.1.3.40), hexose oxidases (EC 1.1.3.5), alcohol oxidases (EC 1.1.3.13) and vanillin oxidase (EC 1.1.3.38). PQQ-dependent enzymes and enzymes present in oxidative bacteria can also be used for these conversions.

[0070] Steps 7 and 7B: Conversion of guluronic acid to D-glucaric acid (7) and conversion of L-Iduronic acid to Idaric acid (7B). These steps can be accomplished with enzymes of the family of uronate dehydrogenases (EC 1.1.1.203) or the oxidases, as described herein. Examples of uronate dehydrogenases include SEQ ID NO: 1-6, and any one or any combination of them can be used to perform Steps 7 and 7B.

[0071] Step 7A: Conversion of 4-deoxy-5-erythro-hexosulose uronate (DEHU) to 3-deoxy-D-erythro-2-hexulosaric acid (DDH). The same enzymes described in Step 5 will be

useful for performing this conversion. Similar to Step 5, for steps 7 and 7B enzymes are identified having the stated activity, which are NAD(P)- or PQQ-dependent dehydrogenases, but others are FAD-dependent aldehyde dehydrogenases. Examples of NADP-dependent gluconate-5-dehydrogenases include SEQ NO: 71-72 and examples of PQQ-dependent dehydrogenases include SEQ ID NO: 73-84, and any one or any combination of them can be used to perform steps 7 and 7B.

[0072] Steps 8 and 8A: Conversion of D-glucaric acid to 5-dehydro-4-deoxy-glucarate (DDG) (Step 8) and conversion of Idaric acid to DDG (Step 8A). Enzymes of the family of glucarate dehydratases (EC 4.2.1.40) can be used to perform these steps. Enzymes of this family have been cloned and have been shown to efficiently convert glucarate to DDG. Two D-glucarate dehydratases (EC 4.2.1.40) were cloned as shown in the Table of cloned glucarate dehydratases below. Both enzymes showed very high activity for the dehydration of Glucarate to DDG using the semicarbazide assay, as described in Step 2.

Cloned glucarate dehydratases

Organism	pSGI (Vector)	Gene ID	WT/SY N
E. coli	353 (pET28)	P0AES2	WT
Pseudomonas (SGI)	244	#8114	WT

[0073] Step 9 and 9A: Conversion of D-glucose to α -D-glucopyranose (9) and conversion of D-galactose to D-galactopyranose (9A). Oxidases such as those of the galactose oxidase family (EC 1.1.3.9) can be used in this step. Mutant galactose oxidases are also engineered to have activity on glucose and have been described (Arnold, F.H. et al *ChemBioChem*, 2002, 3(2), 781). Step 9A can be performed with enzymes of the class EC 1.1.3.9.

[0074] Step 10: Conversion of α -D-glucopyranose to α -D-glucopyranuronic acid (step 10) and D-galactopyranose to galacturonic acid (10A). This step can be performed using an enzyme of the family of aldehyde dehydrogenases. Also an enzyme identified from those of Step 5 will be useful for both of these conversions.

[0075] Step 11 and 11A: Conversion of α -D-glucopyranuronic acid to glucuronic acid 1,5-lactone. Aldehyde dehydrogenases and oxidases as described in Step 5 will be useful in performing this step. Uronate dehydrogenases described in Steps 7 and 7B can also be useful in performing this step. Step-11A is the conversion of galacturonic acid to galactaric acid. The

uronate dehydrogenase (EC 1.1.1.203), for example those described in Steps 7 and 7B, will be useful in performing this step.

[0076] Step 12: Conversion of fructose to glucose. Glucose and fructose isomerases (EC 5.3.1.5) will be useful in performing this step.

[0077] Step 13: Conversion of galactarate to 5-dehydro-4-deoxy-D-glucarate (DDG). Enzymes of the family of galactarate dehydrogenases (EC 4.2.1.42) can be used to perform this step, and additional enzymes can be engineered for performing this step.

[0078] Step 14: Conversion of gluconate to 5-ketogluconate (5-KGA). A number of enzymes of the family of NAD(P)-dependent dehydrogenases (EC1.1.1.69) have been cloned and shown to have activity for the oxidation of gluconate or the reduction of 5KGA. For example, the NADPH-dependent gluconate 5-dehydrogenase from *Gluconobacter* (Expasy P50199) was synthesized for optimal expression in *E. coli* as shown herein and was cloned in pET24 (pSGI-383). The enzyme was expressed and shown to have the required activities. Additional enzymes useful for performing this step include those of the family of PQQ-dependent enzymes present in *Gluconobacter* (Peters, B. et al. *Appl. Microbiol Biotechnol.*, (2013), 97, 6397), as well as the enzymes described in Step 6. Enzymes from these families can also be used to synthesize 5KGA from gluconate.

[0079] Step 15: Conversion of 5-KGA to L-Iduronic acid. This step can be performed with various enzymes from different isomerase families, as further described in Example 4. Examples include isomerases of SEQ ID NOs: 7-19 or a homolog having at least 70% sequence identity to an isomerase of SEQ ID NOs: 7-19; or by an isomerase encoded by a nucleic acid of SEQ ID NOs: 20-32 or a homolog of any of them.

[0080] Step 16: Conversion of 5-KGA to (4S)-4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH). This dehydration can be performed with enzymes in the gluconate dehydratase family (EC 4.2.3.39), such as those described in Example 5 or Step 17. Examples of gluconate dehydratases that can be used for Step 16 include SEQ ID NOs 33-35 (encoded by SEQ ID NOs: 36-38, and any one or any combination of them can be used to perform Step 16, or homologs thereof.

[0081] Step 17 and 17A: L-Iduronate to 4-deoxy-5-threo-hexosulose uronate (DTHU) and Guluronate to 4-deoxy-erythro-5-hexosulose uronate (DEHU).

[0082] Enzymes of the family of dehydratases are identified that can be used in the performance of this step. Enzymes from the families of gluconate or glucarate dehydratases will have the desired activity for performing these steps. Furthermore, many dehydratases of the family (EC 4.2.1.X) will be useful in the performance of these steps. In particular,

enzymes that dehydrate 1,2-dihydroxy acids to selectively produce 2-keto-acids will be useful, such as enzymes of the families: EC 4.2.1.6 (galactonate dehydratase), EC 4.2.1.8 (mannonate dehydratase), EC 4.2.1.25 (arabonate dehydratase), EC 4.2.1.39 (gluconate dehydratase), EC 4.2.1.40 (glucarate dehydratase), EC 4.2.1.67 (fuconate dehydratase), EC 4.2.1.82 (xylonate dehydratase), EC 4.2.1.90 (rhamnonate dehydratase) and dihydroxy acid dehydratases (4.2.1.9). Since known enzyme selectivity is the production of an alpha-keto acid the identified enzymes will produce DEHU and DTHU, respectively, as the reaction products.

Step 19: Conversion of 1,5-gluconolactone to guluronic acid lactone. This step can be performed by enzymes of the family of alditol oxidases (EC 1.1.3.41) or the enzymes described in Step 6. Examples of alditol oxidases that can be used for Step 19 include SEQ ID NOs 39-54 or a homolog of any of them, or by an alditol oxidase encoded by a nucleic acid of SEQ ID NOs: 47-54 or a homolog of any of them; and any one or any combination of them can be used to perform Step 19.

Methods of Converting DDG to FDCA and of making esterified DDG and FDCA

The present invention also provides novel methods of converting DDG to FDCA and FDCA esters. Esters of FDCA include diethyl esters, dibutyl esters, and other esters. The methods involve converting DDG into a DDG ester by contacting DDG with an alcohol, an inorganic acid, and optionally a co-solvent to produce a derivative of DDG. The alcohol can be methanol, ethanol, propanol, isopropanol, butanol, isobutanol, pentanol, hexanol, heptanol, octanol, nonanol, decanol, undecanol, dodecanol, tridecanol, tetradecanol, pentadecanol, hexadecanol, heptadecanol, octadecanol, nonadecanol, eicosanol, dimethyl sulfoxide, dimethylformamide, polyethylene glycol, methyl isobutyl ketone, or any C1-C20 alcohol. The inorganic acid can be sulfuric acid, phosphoric acid, perchloric acid, nitric acid, hydrochloric acid, hydrofluoric acid, hydroboromic acid and hydriodic acid. The co-solvent can be any of or any mixture of THF, acetone, acetonitrile, an ether, butyl acetate, an dioxane, chloroform, methylene chloride, 1,2-dichloroethane, a hexane, toluene, and a xylene. Any combination of the alcohols, inorganic acids, and co-solvents can be utilized in the reactions. The esterified DDG can then be converted into esterified FDCA, for example by contacting it with an acid catalyst.

DDG Purification

[0083] DDG purification for dehydration or esterification was performed by acidifying the DDG, e.g., by lowering the pH of the reaction with the addition of conc HCl to pH ~2.5. At this pH proteins and any residual glucarate precipitate are removed by filtration and the mixture is lyophilized to give a white powder consisting of DDG and the reaction salts. The mixture can be lyophilized at neutral pH after the enzymes have been removed by

filtration. Without further purification the DDG can then be dehydrated to give 2,5-FDCA, or be esterified to dibutyl-DDG (or di-ethyl DDG) prior to dehydration. One or more steps of purifying or esterifying DDG can be added to any of the methods and pathways disclosed herein that produce DDG. Other methods for purifying DDG from the aqueous mixture can also be used. These include separations using membranes or ion exchange resins that capture salts or DDG etc.

[0084] The invention therefore provides a method of purifying DDG that involves acidifying DDG in a solution, filtering the solution through a filter membrane, and removing water from the solution (e.g., by lyophilization or spray drying). The solution with the DDG can be acidified to a pH of 2.5-3.5 or pH of 3.0-4.0 or pH of 3.5-4.5 or pH of 4.0-5.0 or pH of 4.5-5.5 or pH of 5.0-6.0 or pH of 5.5-6.5 or pH of 6.0-7.0 or pH of 6.5-7.5 or pH of 7.0-8.0 or pH of 7.5-8.5 or pH of about 8. The amount of water removed can be greater than 80% or greater than 85% or greater than 87% of the water or greater than 90% of the water or greater than 95% of the water or greater than 97% or greater than 98% or greater than 99% of the water from the solvent comprising the DDG. Yields of greater than 25% or 30% or 35% or 40% or 45% molar can be obtained. In one embodiment the method does not involve a step of ion exchange chromatography.

Methods for synthesizing FDCA and FDCA Derivatives

[0085] The invention also provides various methods of synthesizing FDCA. One method for synthesizing FDCA involves contacting DDG with an alcohol, an inorganic acid at a high temperature to form FDCA. The alcohol can be any alcohol (e.g., any of those described above), and examples include (but are not limited to) methanol, ethanol, propanol, and butanol. Diols can also be used. The high temperature can be a temperature greater than 70 °C or greater than 80 °C or greater than 90 °C or greater than 100 °C or greater than 110 °C or greater than 120 °C or greater than 130 °C or greater than 140 °C or greater than 150 °C to form FDCA. Reaction yields of greater than 20% or greater than 30% or greater than 35% or greater than 40% can be achieved.

[0086] The invention also provides methods for synthesizing derivatives of FDCA. The methods involve contacting a derivative of DDG with an inorganic acid to produce a derivative of FDCA. The inorganic acid can be, for example, sulfuric acid, or any inorganic acid such as those described above. Optionally, the derivative of DDG can be purified prior to contacting it with the second inorganic acid. Non-limiting examples of derivatives of DDG or FDCA include, but are not limited to, methyl DDG, ethyl DDG, propyl DDG, butyl DDG, isobutyl DDG, di-methyl DDG, di-ethyl DDG, di-propyl DDG, di-butyl DDG. The derivative

of FDCA produced can be, but is not limited to, methyl FDCA, ethyl FDCA, propyl FDCA, butyl FDCA, di-methyl FDCA, di-ethyl FDCA, di-propyl FDCA, di-butyl FDCA, and isobutyl FDCA. The derivate of FDCA produced corresponds to the derivative of DDG used in the method. The derivative of FDCA can then be de-esterified to produce FDCA. The method can also be conducted in the gas phase, e.g., using the parameters described below.

[0087] Another method for synthesizing FDCA or derivatives of FDCA involves contacting DDG or derivatives of DDG (any described herein) with an inorganic acid in a gas phase, which can be done with a short residence time, e.g., of less than 10 seconds or less than 8 seconds, or less than 6 seconds or less than 5 seconds or less than 4 seconds or less than 3 seconds or less than 2 seconds or less than 1 second. The residence time refers to the time that the sample is present in the reaction zone of the high temperature flow through reactor. The method can also be conducted at high temperatures, for example at temperatures greater than 150 °C, greater than 200 °C, greater than 250 °C, greater than 300 °C or greater than 350 °C. Yields of greater than 25% or greater than 30% or greater than 40% or greater than 45% or greater than 50% molar are obtainable. Another method for synthesizing FDCA involves contacting DDG with an inorganic acid at a temperature in excess of 80 °C or 90 °C or 100 °C or 110 °C or 120 °C. Another method for synthesizing FDCA involves contacting DDG with an inorganic acid under anhydrous reaction conditions. In various embodiments the anhydrous conditions can be established by lyophilizing the DDG in any method of synthesizing FDCA disclosed herein so that the DDG contains less than 10% or less than 9% or less than 8% or less than 7% or less than 6% or less than 5% or less than 4% or less than 3% water or less than 2% water, by weight.

[0088] The methods of the invention for synthesizing FDCA and its derivatives as described herein provide a significantly higher yield than has been available. In different embodiments molar yields of FDCA (v. DDG) can be obtained of greater than 10% or greater than 15% or greater than 20% or greater than 25% or greater than 30% or greater than 35% or greater than 40% or greater than 45% or greater than 50% or greater than 60% or greater than 65% or from about 40% to about 70%, or from about 45% to about 65%, or from about 50% to about 60%.

EXAMPLES

Example 1 – Step 2, Gluconic Acid to 3-dehydro-gluconic acid (DHG)

[0089] Enzymes with natural activity for the dehydration of gluconate are useful in the invention (EC 4.2.1.39). Three enzymes from this family were cloned as shown in Table 1. Enzyme pSGI-365 was cloned and shown to be a dehydratase with broad substrate range

having strong activity for the dehydration of gluconate (Kim, S. Lee, S.B. *Biotechnol. Bioprocess Eng.* **2008**, 13, 436).

Table 1: Enzymes used in this experiment and identity homology. All expressed in *P. fluorescens*

Organism	pSGI (Vector)	Gene ID	WT/SYN	Expression Host
<i>Achromobacter</i>	365 (pRANGER)	E3HJU7	Syn	<i>P. fluorescens</i>
<i>Achromobacter</i>	359 (pRANGER)	#0385	wt	<i>P. fluorescens</i>
<i>Acinetobacter</i>	360 (pRANGER)	#0336	wt	<i>P. fluorescens</i>

	359_Achromob	365_E3HJU7
pSGI-360_Acinetobacter (SGI)	78	79
pSGI-359_Achromobacter (SGI)		95
pSGI-365_Acromobacter		

[0090] Proteins 359, 360, and 365 (SEQ ID NOs 33-35, respectively) showed 2-5 μ mole/min per mg of crude enzyme lysate activity for the synthesis of dehydration of gluconate (gel not shown). pSGI-359 was isolated by precipitation with ammonium sulfate and re-dissolving in buffer and assayed by the semicarbazide assay. Activities of 46.2 U/mL or 5.3 U/mg (1 unit= μ mole/min) for the dehydration of gluconate were calculated from semicarbazide assay plots. Reaction buffer (93 mL) containing Kpi 10 mM pH 8.0 with 2 mM MgCl₂ and 3.5 gr (0.016 mole) of sodium gluconate was mixed with 7 mL of the previous gluconate dehydratase solution. The reaction was incubated at 45 °C for 16 h before one aliquot was analyzed by HPLC-MS (Figure 4). As shown in Figure 4 one new major product with the molecular weight of DHG was produced. The product was also shown to have activity with DHG dehydratases.

[0091] All proteins were cloned on the pRANGER™ (Lucigen, Middleton, WI) expression vector and were expressed in a *Pseudomonas fluorescens* strain. pRANGER™ is a broad host commercially available plasmid vector containing the pBBR1 replicon, Kanamycin resistance and an pBAD promoter for inducible expression of genes. For the enzyme assay a modification of the semicarbazide assay for the quantification of alpha keto acid was used to calculate the activity of each enzyme (Kim, S.; Lee, S.B. *Biochem J.* 2005, 387, 271). SEQ ID NOs: 30-32 and 33-35 show the amino acid and nucleotide sequences, respectively, of the gluconate dehydratases #0385, #0336, and E3HJU7.

Example 2 – Step 3 - 3-dehydro-gluconic acid (DHG) to (4S)-4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH)

[0092] Enzymes of the family (EC 1.1.1.127) can be used to perform this step. Two examples are 2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase and DHG dehydrogenases. Five enzymes from this family were cloned as shown in Table 2 below. pRANGER™ vector was used in every case.

Table 2: Cloned of DHG oxidoreductase (or 2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase)

Organism	pSGI (Vector)	Gene ID	WT/SYN	Expression Host
Agrobacterium sp (SGI)	374	#9041	WT	<i>P. fluorescens</i>
Agrobacterium tumefaciens (SGI)	375	#8939	WT	<i>P. fluorescens</i>
E. coli	376	P37769	WT	<i>P. fluorescens</i>
Sphingomonas (SGI)	395	#5112	WT	<i>P. fluorescens</i>
Hoeflea phototrophica (SGI)	396	#7103	WT	<i>P. fluorescens</i>

[0093] The product prepared from the dehydration of gluconate in Step 2 was used as substrate for assaying the lysates of Table 2. As shown in the following Table 3, enzymes were identified showing activity for the oxidation of DHG in assays measuring NADH formation (absorbance increase at 340 nm).

Table 3: Activity calculations for oxidation of DHG to 2,5-DDH using DHG oxidoreductase.

A unit = $\mu\text{mole/min}$ of NADH

ENZ	U/mg (100 mM DHG)		
	pH=7.5	pH=8.5 (10 mM DHG)	pH=9.5
pSGI_395	0.012	0.070 (0.02)	0.120
pSGI_396	0.033	0.139 (0.018)	0.418
pSGI_374	0.007	0.043 (0.012)	0.091
pSGI_376	0.007	0.121 (0.01)	1.610

[0094] Further verification of the formation of 2,5-DDH by these enzymes was shown in Step 16 where the reduction of 2,5-DDH (made from the dehydration of 5KGA) with pSGI-395 at acidic pH was shown.

Example 3 – Steps 7 and 7B - Conversion of guluronic acid to D-glucaric acid (7) and conversion of L-Iduronic acid to Idaric acid (7B).

[0095] To demonstrate Steps 7 and 7B the following study was performed. Uronate dehydrogenases (EC 1.1.1.203) are enzymes that oxidize glucuronic and galacturonic acid. Three enzymes with sequence similarity to the known uronate dehydrogenase (Expasy: Q7CRQ0; Prather, K.J, et al., *J. Bacteriol.* 2009, 191, 1565) were cloned from bacterial strains as shown in Tables 4 & 5.

Table 4 – Cloned Uronate Dehydrogenases

Organism	pSGI (pET28)	Gene ID	Expression
Agrobacterium	#474	#8807	BL21DE3
Rhizobium	#475	#8958	BL21DE3
Pseudomonas	#476	#1770	BL21DE3

Table 5 – Sequence Identity

	#475	#476	Q7CRQ0
474_Agrobacterium	73	49	90
475_Rhizobium		51	74
476_Pseudomonas			50

[0096] Each protein was expressed with a His tag from pET28 and was purified prior to their screening. Protein gels of the crude lysates and purified enzymes are shown in the gel of Fig. 1. After purification all enzymes were tested for activity against glucuronate, as well as against guluronate and iduronate. Kinetic measurements at different substrate concentrations were performed and the calculated activities and Km values for each enzyme are shown in Table 6. All enzymes showed good activity for glucuronate, and also for L-iduronate and guluronate.

Table 6: Activity and Km value for purified uronate dehydrogenases.

Enzyme	Vmax ($\mu\text{M}/\text{min}/\text{mg}$); and Km (mM)		
	Glucuronate	Iduronate	Guluronate (Vm only)
474	128.2 ; 0.37	0.96 ; 29.8	0.017
475	47.4 ; 0.22	0.59 ; 42.1	0.016
476	90.9 ; 0.34	1.36 ; 29.6	0.014

[0097] Each plasmid shown in Table 4 was transformed in BL21DE3 *E. coli* cells. Clarified lysates were mixed with equal volume of (25 mL) of equilibration buffer and purified on an Ni NTA column. Activity of each purified enzyme was measured in by mixing 0.050 mL of various dilutions of each purified enzyme with 0.95 mL of reaction buffer (100 mM TrisHCl, pH 8.0, 50 mM NaCl, 0.75 mM NAD⁺). The reaction progress was measured by monitoring of the formation of NADH at 340 nm. Figures 6a and 6b provide Lineweaver-Burk

plots for the oxidation of glucuronate and iduronate, with all three enzymes shown in Figure 6. Clear positive slopes were obtained with all enzymes giving the activities shown in the table above. Protein sequences of the uronate dehydrogenases are shown as SEQ ID NOs: 1-3 and the genes as SEQ ID NO: 4-6.

[0098] Pyrroloquinoline (PQQ) dependent aldehyde dehydrogenases also showed good activity for the oxidation of both guluronate and iduronate. These are soluble periplasmic enzymes that were expressed in the *E. coli* cytosol after their periplasmic target sequence was removed. The activities of crude lysates in units ($\mu\text{mole}/\text{min}$) per milligram of total lysate protein are shown in the following Table 6A. The actual activity of each enzyme is at least 2-5x higher if purified (see expression in Figure 3).

Table 6A: Activities of PQQ-dependent dehydrogenases with iduronate and guluronate (Unit= $\mu\text{mole}/\text{min}$)

Enzyme	Iduronate U/mg	Guluronate U/mg
P75804 (SEQ ID NO: 73)	8.7	3.2
9522 (SEQ ID NO: 74)	7.3	6.1
6926 (SEQ ID NO: 75)	9.2	4.1
7510 (SEQ ID NO: 76)	7.3	3.7
7215 (SEQ ID NO: 77)	14.2	8.3
8386 (SEQ ID NO: 78)	4.3	1.5

[0099] The activities shown on Table 6A were measured using an artificial electron acceptor DCPIP (2,6-dichloroindophenol) according to the following protocol: In 0.95 mL of 20 mM Triethanol amine (pH 8.0) containing 0.2 mM DCPIP, 0.2 mM PMS (phnazine ethosulfate) and substrate (10-40 mM), 0.050 mL of enzyme (as crude lysate or 10-100x diluted with buffer) is added and the reaction progress is followed by the change of DCPIP absorbance at 600 nm. Because in their natural state these enzymes are transferring electrons to other proteins or cofactors in the membrane electron transport chain, the *in vitro* activity is measured using artificial electron acceptors with DCPIP being the most common.

[00100] The enzymes on Table 6A were active against a number of other aldehydes including butyraldehyde, butyraldehyde and glycerol (but not glucose). Therefore, these enzymes will oxidize the aldehyde group of iduronate and guluronate to give iduronic and glucaric acid respectively. In order to confirm this selectivity, two of these enzymes, #403 and #412, were expressed in the periplasm of *E. coli* by fusing them with the periplasmic target sequence of #403 (a native *E. coli* enzyme). Both proteins were expressed in the periplasm but in lower levels compared to the cytosol. The previous recombinant cells oxidized

benzaldehyde to benzoic acid in good yields and in lower yields produced glucaric and idaric acid from guluronate and iduronate.

Example 4 - Step-15: Conversion of 5-ketogluconate (5-KGA) to L-Iduronic acid (15) or guluronic acid (15A).

[00101] This example illustrates the identification of an enzyme capable of isomerizing 5-KGA to iduronic acid (Step 15) or guluronic acid (Step 15A). Thirteen enzymes from three different isomerase families were cloned as shown in Table 7, while their % sequence identity is shown in Table 8.

Table 7: Isomerases cloned

EC	Organism	pSGI (pET28)	Gene ID Archetype® or Expasy	WT/SYN
5.3.1.17	Rhizobium	433	#8938	WT
5.3.1.17	E. coli	434	Q46938 (Expasy)	WT
5.3.1.17	Rhizobium	435	#3891	WT
5.3.1.17	Pannonibacter	436	#7102	WT
5.3.1.n1	Lactobacillus	458	A5YBJ4 (Expasy)	SYN
5.3.1.n1	Acidophilum	440	F0J748 (Expasy)	SYN
5.3.1.n1	Bacillus	437	#9209	WT
5.3.1.n1	Ochrobactrum	438	#9732	WT
5.3.1.n1	Halomonas	439	#7403	WT
5.3.1.12	Sphingobacteria	478	#1874	WT
5.3.1.12	Thermotoga	479	Q9WXR9	SYN
5.3.1.12	Bacillus	480	Q9KFI6	SYN
5.3.1.12	Bacillus	481	O34808	SYN

Table 8: % Identities of isomerases

	EC	436	434	435	458	440	437	438	439	481	480	479	478
433	5.3.1.17	<u>65</u>	<u>44</u>	<u>43</u>	16	13	18	11	14	6	11	11	7
436	5.3.1.17		<u>45</u>	<u>46</u>	18	14	15	12	13	5	10	11	7
434	5.3.1.17			<u>46</u>	17	10	15	10	13	6	10	12	7
435	5.3.1.17				18	16	18	14	16	9	11	13	7
458	5.3.1.n1					<u>37</u>	<u>57</u>	<u>41</u>	<u>44</u>	6	7	9	5
440	5.3.1.n1						<u>40</u>	<u>67</u>	<u>50</u>	6	6	6	5
437	5.3.1.n1							<u>46</u>	<u>51</u>	8	7	10	6
438	5.3.1.n1								<u>52</u>	5	5	6	4
439	5.3.1.n1									6	7	8	5
481	5.3.1.12										<u>7</u>	<u>36</u>	<u>54</u>
480	5.3.1.12											<u>7</u>	<u>7</u>
479	5.3.1.12												<u>37</u>
478	5.3.1.12												

[00102] As shown in Table 8, enzymes with medium homology (underlined) within each family were selected for cloning. The data demonstrated that enzymes from all families showed activity for the isomerization of 5-KGA giving L-iduronate as the main product. Two enzymes from the 5.3.1.17 family (433 & 434) were also used in the example showing the formation of DDG from 5-ketogluconate (5KGA).

[00103] Activity for the isomerization of 5KGA and iduronate using enzymes from Table 7 was measured using an enzymatic method that detected the formation of products by their activity against two different enzymes. For example, isomerization of 5KGA was detected by measuring the activity of the product iduronate using uronate dehydrogenase (pSGI-476). Isomerization of iduronate was detected by measuring the activity 5KGA reductase (pSGI-383, EC 1.1.1.69) of the product 5KGA. Presence of the products was also detected by GC-MS.

[00104] Enzymes from all families showed varying activity for the isomerization of 5KGA and iduronate. Two enzymes from EC 5.3.1.12 were used in a cell free reaction to isomerize 5KGA and ultimately produce DDG as described in the example. The enzymes were purified and showed a single band by gel electrophoresis. The purified isomerases were used in reactions using lysate and buffer containing 5KGA or Iduronate. Product formation was demonstrating using both HPLC and the previously described enzymatic methods. Results for 17h of incubation using both HPLC and enzyme assays are shown in Figure 7a. All enzymes showed good activity for the isomerization of both 5KGA and iduronate. Yields for iduronate isomerization by pSGI433, pSGI 434, pSGI 435, and p SGI 436 were 56%, 48%, 42%, (436 not measured), respectively when measured enzymatically and 78.8%, 78.5%, 73.3% and 76.6%, respectively when measured by HPLC assay. Yields after 16h for 5KGA isomerization by the same enzymes were 18%, 17%, and 19% respectively (436 not measured) when measured by enzymatic assay, and 16.6%, 17.8%, 16.3%, and 16.9%, respectively, when measured by HPLC assay.

EC 5.3.1.12 enzymes

[00105] Enzymes from the EC 5.3.1.12 family (glucuronate isomerases) were also purified by gel electrophoresis, isolated, and used to prepare reactions by mixing with buffer (50 mM HEPES, 1 mM ZnCl₂, pH 8.0) that contained 5 mM of 5KGA or Iduronate. The reactions were incubated at 30 °C and analyzed for product formation using both HPLC and enzymatic methods. Results are shown in Figure 7b.

5.3.1.17 Enzymes

[00106] Enzymes pSGI-478 and pSGI-479 (5-dehydro-4-deoxy-D-glucuronate isomerases) showed isomerization activity for both 5KGA and iduronate. This activity was also confirmed with the enzymatic assays as above. Yields for isomerization of iduronate by pSGI-478 and -479 were 50% and 37%, respectively, when measured enzymatically, and 20% and 18% when measured by HPLC. Yields for 5KGA isomerization were 23% and 26%, respectively, when measured enzymatically, and 24% and 16%, respectively when measured by HPLC. Results are shown in Figure 7a.

5.3.1.n1 Enzymes

[00107] Enzymes in this family were purified by gel electrophoresis. Product formation was measured using enzymatic assays as described above and the results are shown in Figure 8. All enzymes cloned in this family were shown to have activity for the isomerization of 5KGA and iduronate.

[00108] In each case plasmids were transformed in BL21DE3 and proteins purified on a Ni NTA column.

Example 5: Step 16 – 5-keto-gluconate (5KGA) to (4S)-4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH)

[00109] The three gluconate dehydratases described in Step 2 (Example 1) were expressed as described in Example 1, along with a purified glucarate dehydratase from Step 8. Enzymatic reactions for activity were performed and HPLC-MS analysis showed the formation of 2,5-DDH (Figure 9), which was also confirmed by the fact that formation of the new product was accompanied by the reduction of 5-KGA only in the samples containing gluconate dehydratases, as well as by enzymatic assays with DHG dehydratase (pSGI-395). Good slopes at 340 nm indicating large enzyme activity were obtained when NADH, pSGI-395 lysate and aliquots of the previous reactions were mixed (data not shown). This result in combination with the HPLC analysis prove that the gluconate dehydratases examined dehydrate 5KGA to 2,5-DDH.

Example 6: Step 19 – Conversion of 1,5-gluconolactone to guluronic acid δ -lactone.

[00110] 1,5-gluconolactone oxidation is a side activity of enzymes from the alditol oxidases (EC 1.1.3.41) family. These enzymes oxidize various alditols such as sorbitol, xylitol, glycerol and others. Enzymes were identified having activity for the oxidation of 1,5-gluconolactone, as shown in Table 6 below.

Table 6. Alditol oxidases with activity on 1,5-gluconolactone.

Enzyme	Enzyme Source	Sorbitol U/mg	1,5- Gluconolactone			
			U/mg	Reaction Setup		
				Enzyme mg	Substrate mg/mM	Yield
AO#13	<i>Terriglobuds roseus</i>	0.23	0.02	5.3	15 / 85	7%
AO#22	<i>Granulicella mallensis</i>	0.27	0.015	7.6	15 / 85	9%
AO#28	<i>Streptomyces acidiscabies</i>	1.30	0.010	15	15 / 85	8%
AO#36	<i>Actinomycetales (SGI)</i>	1.83	0.102	25	90 / 35	46%
AO#51	<i>Frankia sp</i>	0.59	0.019	NT	NT	NT
AO#57	<i>Propionibacteriaceae (SGI)</i>	1.47	0.051	40	70 / 57	6%
AO#76	<i>Streptomyces sp.</i>	1.45	0.045	8.2	15 / 85	23%
AO#251*	<i>Paenibacillus sp.</i>	0.47	0.003	24	15 8.5	~2%

*crude lysate

[00111] Reactions were prepared using lysates of all the purified enzymes shown on Table 6. Reactions were prepared in 50 mM K-phosphate buffer, pH 7.0 with 0.5 mg/mL catalase and incubated at 30 °C. A new product was observed by HPLC-MS analysis showing the same retention time as guluronate after comparison with authentic standards (Figure 10). This was confirmed by GC-MS, where the product also had the same MS fingerprint as guluronate. It is therefore clear that all the alditol oxidases described in the Table oxidize the 6-OH of 1,5-gluconolactone to produce the guluronic acid lactone. All alditol oxidases were cloned in pET28a with a HisTag and were expressed in BL21DE3 and purified on a Ni NTA column.

Example 7 – Synthesis of FDCA and Other Intermediates

[00112] Purified DDG mono potassium salt was used for the dehydration to 2,5-FDCA. Sulfuric acid was added to DDG and the reaction stirred at 60°C. The in situ yield was calculated (by HPLC-MS) to be ~24% and ~27%.

[00113] The reaction solutions were combined and then diluted by pouring into ice (to neutralize the heat). Approximately equivalent volume of THF was added, and the solution transferred to a separation funnel. Sodium chloride salt was added until separation was achieved. The solution was agitated between additions for best possible dissolution. The aqueous layer was removed, and the THF layer washed 3x more with sat. NaCL solution. Sodium sulfate was added and the solution left sitting overnight. Two layers formed again overnight. The aqueous layer was discarded and then silica gel was added to the solution. It was then concentrated down to solids via rotovap. The solids were loaded into a silica flash column and then separated via chromatographically. The fraction was concentrated and dried.

The isolated yield was 173.9mg. Corrected yield: 24.9%. ^1H and ^{13}C NMR and HPLC-MS analysis confirmed the product

Dehydration of DDG Dibutyl-2,5-FDCA in BuOH/H₂SO₄

[00114] Dehydration of un-derivitized lyophilized DDG containing the dehydration salts in BuOH was done using a Dean-Stark apparatus. Under these conditions, DDG was added to BuOH, and then H₂SO₄ was added and the reaction heated at 140 °C. After stirring for 4 h HPLC-MS analysis shows the disappearance of DDG and the formation of dibutyl-2,5-FDCA. The in situ yield was calculated (by HPLC-MS) to be 36.5%.

[00115] The mixture was extracted with water, 1% NaOH, and again with water. Then the organic layer was concentrated to a final mass of 37.21g. A portion of this mass (3.4423g) was removed and 0.34 g of dibutyl-2,5-FDCA was purified using HPLC. Extrapolating the yield of the isolated product to the total amount of compound isolated from the reaction (37.21g) and taking into account the amount of salts present in the original DDG (~60% pure by weight) the reaction yield was calculated to be 42%. ^1H and ^{13}C NMR and HPLC-MS analysis confirmed the product

Synthesis of dibutyl DDG

[00116] In another aspect the invention provides a method for synthesizing a derivative of DDG. The method involves contacting DDG with an alcohol, an inorganic acid, and optionally a co-solvent to produce a derivative of DDG. Optionally the derivative of DDG can be purified. The reaction can have a yield of the derivative of DDG of at least 10% molar yield or at least 15% molar yield or at least 20% molar yield or at least 25% or at least 30% or at least 35% molar yield or at least 40% molar yield. The inorganic acid can be sulfuric acid and the alcohol can be methanol, ethanol, propanol, butanol, isobutanol, or any C1-C20 alcohol. In various embodiments the co-solvent can be any of THF, acetone, acetonitrile, an ether, butyl acetate, an dioxane, chloroform, methylene chloride, 1,2-dichloroethane, a hexane, toluene, and a xylene. When the alcohol is ethanol the DDG derivative will be DDG mono-ethyl ester and/or DDG diethyl ester. When the alcohol is butanol the DDG derivative will be DDG mono-butyl ester and/or DDG dibutyl ester.

[00117] DDG mono-potassium salt was used for derivatization according to the following protocol. In a 1L Morton type indented reaction vessel equipped with a mechanical stirrer and heating mantle was charged with 60:40 DDG:KCl (31.2 mmol), BuOH, and heptane. In a separate vial, sulfuric acid was added to water, and allowed to cool after dissolution. The solution was then added to the flask. The solution was kept at 30°C.

[00118] The precipitate was filtered off concentrated. The remaining gel was dissolved in EtOAc, and then TLC plates were spotted with the solutions and the plates were sprayed with a phosphomolybdic acid mixture, and then heated to at least 150°C on a hot plate to identify the DDG-DBE fraction. Isolated yield: 4.62 g (15.2 mmol, 47% yield), > 98% purity. ¹H and ¹³C NMR and HPLC-MS analysis confirmed the product.

[00119] Different solvents can be used in the synthesis of DDG esters, such as mixtures of BuOH (5%-95% v/v) with co-solvents such as THF, acetone, acetonitrile, ethers (dibutyl, ditheyl etc), esters such as Butyl-acetate, 1,6-dioxane, chloroform, methylene chloride, 1,2-dichloroethane, hexanes, toluene, and xylenes may be used as cosolvents. Reaction catalysts such as acids (sulfuric, hydrochloric, polyphosphoric or immobilized acids such as DOWEX) or bases (pyridine, ethyl-amine, diethyl-amine, boron trifluoride) or other catalysts commonly used for the esterification of carboxylic acids.

Dehydration of dibutyl-DDG to dibutyl-FDCA in n-BuOH/H₂SO₄

[00120] A stock solution of DDG-DBE (di-butyl ester) was made in butanol and transferred to a clean, dry 100mL round-bottomed flask equipped with a stir bar. To the flask, 25mL of conc. sulfuric acid was added. The flask was sealed and then stirred at 60°C for 2hrs. The in situ yield was calculated to be ~56%. The reaction solution was concentrated and the residue was dissolved in MTBE and transferred to a separation funnel, and then washed with water. The recovered organic layer was concentrated and then separated via HPLC for an isolated yield: 250.7 mg (~90% purity) and 35% isolated yield (corrected for purity). ¹C and ¹³C NMR and HPLC-MS analysis confirmed the product.

Example 8 – Cell free synthesis of DDG and FDCA and derivatives from 5-KGA (Route 2A)

[00121] This example illustrates the enzymatic conversion of 5KGA to DDG using purified enzymes according to Scheme 6 (a sub-Scheme of 2B), and also illustrates the DDG produced being dehydrated to FDCA using chemical steps. The Scheme involves the steps of isomerization of 5KGA (Step 15) and the subsequent oxidation to idaric acid (Step 7B). DDG was also dehydrated under differing chemical conditions to FDCA. The last step (Step-8A) was performed using glucarate dehydratase from *E. coli*.

[00122] Scheme 6 is illustrated in Figure 11. The scheme was performed using a cell free enzymatic synthesis of DDG from 5-KGA. The Scheme involves the performance of steps 15, 7B and 8A (see Fig. 2d). Two additional proteins were used to complete the reaction path, the first being NADH-oxidase (Step A) that is recycling the NAD⁺ cofactor in the presence of oxygen, and catalase (Step B) that decomposes the peroxide produced from the

action of NADH oxidase. The enzymes are shown in the following Table 7. All enzymes contained a HisTag and were purified using an Ni-NTA column. Yields for this synthesis of [00123] DDG were calculated to be at least 88-97%.

Table 7:

STEP	Enzyme	EC	Organism
15	pSGI-433 (DTHU _{IS})	5.3.1.17	<i>Rhizobium</i> (SGI)
15	pSGI-434 (DTHU _{IS})	5.3.1.17	<i>E. coli</i>
7B	pSGI-476 (UroDH)	1.1.1.203	<i>Pseudomonas</i> (SGI)
8A	pSGI-353 (GlucDH)	4.2.1.40	<i>E. coli</i>
A	pSGI-431 (NADH _{OX})	1.6.3.1	<i>Thermus thermophilus</i>
B	Catalase	1.11.1.6	<i>Corynebacterium</i>

[00124] 500 mL of liquid culture was purified for each isomerase for the reaction. Besides the enzymes shown on Table 7, each reaction contained 50 mM TrisHCl (pH 8.0), 50 mM NaCl, 1 mM ZnCl₂ and 2 mM MgCl₂, 1 mM MnCl₂ and 1 mM NAD⁺. Reactions were analyzed by HPLC after 16 h of incubation and Figure 12 presents the chromatograms.

[00125] For dehydration to FDCA, the reaction mixtures of both samples were combined and lyophilized into a white powder, which was split into two samples and each dissolved in AcOH with 0.25M H₂SO₄ or in 4.5 mL BuOH with 0.25M H₂SO₄. Both reactions were heated in sealed vials for 2-4 h at 120 °C. Reaction products are shown in Figure 13.

[00126] Samples 1 and 2 represent authentic standard and the 3h time point from the reaction in AcOH/ H₂SO₄, respectively. Spiking of sample 2 with sample 1 gave a single peak further verifying the FDCA product. Samples 1 and 3 (Figure 13) represent authentic standard and the 4h time point from the reaction in BuOH/ H₂SO₄, respectively. The formation of FDCA from the enzymatic reactions further confirms the presence of DDG in these samples.

Example 9 – Synthesis of DDG from Glucose and Gluconate

[00127] This example shows the enzymatic conversion of glucose and gluconate to DDG. The reaction was conducted with purified enzymes, and crude lysates as a catalyst. Enzymes and substrates were combined in a bio-reactor as shown in the Table below:

	Substrate	ST-1	ST-14 pSGI-504	ST-15 pSGI-434	ST-7B pSGI-476	ST-8A pSGI-353	ST-A pSGI-431	ST-B
Rxn-1	Glucose 600 mg	2 mg	7 mL ¹	50 mL ²	7.5 mL ¹	1 mL ³	4 mL ⁴	2 mg
Rxn-2	Gluconate 700 mg	-	7 mL	50 mL	7.5 mL	1 mL	4 mL	2 mg

1. Lysate from 500 mL liquid culture of recombinant *E. coli* with plasmid
2. Lysate from 2L liquid culture of BL21DE3/pSGI-434
3. Purified enzyme, ~30 Units of activity (or 3 mg of purified GlucD)
4. Lysate from 250 mL of culture

[00128] The reaction was incubated at 35 °C and dissolved oxygen and pH were kept at 20% and 8 respectively. Time points were analyzed by HPLC-MS and the results are shown in Figure 17b. Extracted chromatograms verified the DDG mass (not shown) and corresponding MS fragmentation. The results clearly showed production of DDG during incubation of the enzymes with either glucose or gluconate.

Example 10 - Construction of expression cassettes for recombinant glucarate dehydratases

[00129] The following example describes the creation of recombinant nucleic acid constructs that contained coding sequence of a D-glucarate dehydratase activity (GDH, EC 4.2.1.40) for heterologous expression in *E. coli* cells.

[00130] Genes encoding D-Glucarate dehydratase from *E. coli* (Expasy: P0AES2;), *Acinetobacter ADP1* (Expasy: P0AES2), as well as a proprietary *Pseudomonas* bacterial strain (#8114) were PCR-amplified from genomic DNA.

[00131] Each of the PCR-amplified genes was subsequently cloned into the bacterial transformation vector pET24a(+), in which the expression of each of the GDH genes was placed under control of a T7 promoter. The nucleotide sequences of each of the PCR-amplified inserts were also verified by sequencing confirmation.

Example 11 - *E. coli* strains expressing recombinant glucarate dehydratases.

[00132] Each of the expression vectors constructed as described in Example 9 was introduced into NovaBlue(DE3) *E. coli* by heat shock-mediated transformation. Putative transformants were selected on LB agar supplemented with Kanamycin (50 µg/ml). Appropriate PCR primers were used in colony-PCR assays to confirm positive clones that contained each of the expression vectors.

[00133] For each expression vector, a bacterial colony was picked from transformation plates and allowed to grow at 30°C in liquid LB media supplemented with Kanamycin (50 µg/ml) for two days. The culture was then transferred into vials containing 15% glycerol and stored at -80°C as a frozen pure culture.

Example 12 - Demonstration of *in vitro* synthesis of DDG by using cell lysate of recombinant *E. coli* cells expressing a GDH enzyme

[00134] This Example describes how *in vitro* synthesis of DDG intermediate was achieved using recombinant glucarate dehydratase (GDH) enzymes produced in *E. coli* cells.

[00135] Preparation of cell lysates: Recombinant bacterial strains constructed as described previously in Example 2 were grown individually in 3 mL of liquid LB media supplemented with Kanamycin (50 µg/ml) at 30°C on a rotating shaker with rotation speed pre-set at 250 rpm for 1 day. This preculture was used to inoculate 100 mL of TB media containing Kanamycin (50ug/ml), followed by incubation at 30°C on a rotating shaker pre-set at 250 rpm for 2-3 hour until early log phase ($OD_{600} \sim 0.5-0.6$) before isopropyl D-1 thiogalactopyranoside (IPTG; 0.25 mM final concentration) was added to induce protein expression. Cells were allowed to grow for another 18 hours at 30°C before they were harvested by centrifugation, resuspended in 15 mL of lysis buffer (10 mM phosphate buffer, pH 7.8, 2 mM $MgCl_2$) and were lysed by sonication. The production of recombinant enzymes in *E. coli* cells was quantified using standard pre-cast SDS-PAGE gels system (BioRad), and specific activity was measured according to a procedure described by Gulick *et al.* (*Biochemistry* 39, 4590-4602, 2000). Crude cell lysates or purified enzymes (using the HisTag) were then tested for the ability to convert gram amounts of glucarate to DDG as described in greater detail below.

Enzymatic Dehydration of Glucarate

[00136] A large scale oxidation of glucarate using glucarate dehydratase was prepared. 350 mL of water 25 g of glucaric acid sodium salt (0.1 mole) and 4.5 gr of KOH (0.8 mole) were mixed in an Erlenmyer flask. Residual solid glucarate was dissolved by the slow addition of 5M KOH solution (~3 mL) and the pH was adjusted to 7.4. In this solution 100 mg of purified glucarate dehydratase and 2 mM $MgCl_2$ were added, and the mixture was placed in an orbital shaker at 30 °C for 20 h. The next day the precipitate is removed by filtration. The pH of the reaction was essentially unchanged. Analysis of the reaction revealed the presence of only DDG in the solution, indicating >95% yield.

Purification of DDG product from enzymatic reactions:

[00137] DDG produced via enzymatic dehydration was purified by using either of the two following techniques. The enzymatic dehydration reactions were acidified to pH~3.0 with 6M HCl, filtered to eliminate precipitate, and subsequently lyophilized to produce a white powder consisting of DDG and salts. The same DDG purity (but lower amount of salts) can be obtained if the reaction was filtered through a 10 KDa membrane to remove proteins and then lyophilized. Without any further purification both previous lyophilized powders can be dehydrated to FDCA (or its esters) or can be esterified to dibutyl DDG as shown in other examples of this application.

[00138] Results of HPLC-MS analyses indicated that DDG product constituted at least 95% of the total products in the samples obtained from either of the two purification techniques.

Example 13 - Demonstration of *in vitro* synthesis of FDCA from DDG in one-step chemical reaction

[00139] Applicants have discovered that the synthesis of FDCA (*i.e.* the free acid form) could be achieved by a chemical conversion of DDG to FDCA in the presence of H₂SO₄. The reaction was performed as follows. Approximately 20 mg of DDG acid (crude lyophilized powder with salts previously purified as described in Example 3) and 0.25 M of H₂SO₄ were added into an air tight sealed tube containing 1 mL of water and 1 mL of DMSO. The DDG was found completely dissolved in this solution. The reaction was stirred at 105°C for 18 hours. Results of an HPLC-MS analysis performed on a crude reaction sample indicated the formation of FDCA free acid (FDCA: 2,5-furan dicarboxylic acid) as the major product, as well as insignificant amounts of some other unidentified byproducts. As a control in HPLC-MS analysis, a commercial FDCA was analyzed in the same conditions.

Example 14 - Demonstration of *in vitro* synthesis of FDCA-esters (dimethyl-, diethyl-, dibutyl-, and isopropyl- esters)

Synthesis of diethyl-2,5 FDCA from purified DDG:

[00140] In an air tight sealed tube, 18 mL of EtOH, 0.2 gram (1 mmole) of DDG acid, previously purified as described in Example 11, and 0.25 M of H₂SO₄ were added. The DDG acid was not completely dissolved in this solution. The reaction was gently stirred at 105°C for 18 hours. Results of a GC-MS analysis of a crude reaction sample indicated that the formation of diethyl-FDCA the major product. As a control, an authentic FDCA was chemically synthesized, esterified to diethyl-FDCA and analyzed in the same conditions.

Example 15 - Synthesis of dibutyl-2,5 FDCA from purified DDG

[00141] In an air tight sealed tube, 18 mL of n-BuOH, 0.2 gram (1 mmole) of DDG acid, previously purified as described in Example 11, and 0.25 M of H₂SO₄ were added. The DDG acid was not completely dissolved in this solution. The reaction was gently stirred at 105°C for 18 hours. As shown in FIGURE 15, results of the GC-MS analysis of a reaction sample indicated that diethyl-FDCA (FDCA: 2,5-furan dicarboxylic acid) was formed as the major product. As a control, an authentic FDCA was chemically synthesized, esterified to diethyl-FDCA, and analyzed in the same conditions.

Example 16 - Synthesis of dibutyl-2,5 FDCA from crude DDG (unpurified):

[00142] 0.2 gram (1 mmole) of crude DDG acid, which was an unpurified lyophilized powder obtained directly from the enzymatic dehydration of glucarate as described in Example 11, was added into an air tight sealed tube containing 18 mL of n-BuOH, followed by addition of 0.25 M of H₂SO₄. The crude DDG acid was not completely dissolved in this solution. The reaction was gently stirred at 105°C for 18 hours. Results of a GC-MS analysis of a crude reaction sample indicated that diethyl-FDCA (FDCA: 2,5-furan dicarboxylic acid) was formed as the major product. The GC-MS result indicated that the present of contaminant salts in crude/unpurified lyophilized powder did not significantly affect the reaction outcome. As a control, an authentic FDCA was chemically synthesized, esterified to diethyl-FDCA, and analyzed in the same conditions.

Example 17 - *In vitro* production of FDCA and/or esters using immobilized acids

[00143] In industrial practices, immobilized acids offer many advantages for performing dehydrations since they can typically operate in several types of solvent (aqueous, organic or mixed, *etc.*). In addition, they can be easily recycled and be re-used. Following some examples of the synthesis of esters of FDCA using immobilized AMBERLYST®15 (Rohm and Haas, Philadelphia, PA) and DOWEX®50 WX8 (Dow Chemical Co, Midland, MI).

Synthesis of dibutyl-FDCA from crude DDG by using DOWEX®50 WX8

[00144] In an air tight sealed tube, 2 mL of n-Butanol, 20 mg of crude DDG acid (unpurified lyophilized powder containing salts) and 200 mg of DOWEX®50 WX8 were combined. The DDG was not completely dissolved in this solution. The reaction was gently stirred at 105°C for 18 hours. Results of the GC-MS analysis of a crude reaction sample indicated that diethyl-FDCA (FDCA: 2,5-furan dicarboxylic acid) was formed as the major product. This GC-MS result indicated that the present of contaminant salts (phosphate and NaCl) in crude/unpurified lyophilized powder did not significantly affect the reaction outcome. As a control, an authentic FDCA was chemically synthesized esterified to diethyl-FDCA and analyzed in the same conditions.

Synthesis of dibutyl-FDCA from crude DDG by using AMBERLYST®15

[00145] In an air tight sealed tube, 2 mL of n-Butanol, 20 mg of crude DDG acid (crude lyophilized powder with salts) and 200 mg of AMBERLYST®15 (Rohm and Haas, Philadelphia, PA) were combined. The DDG was not completely dissolved in this solution. The reaction was gently stirred at 105°C for 18 hours. Results of the GC-MS analysis of a crude reaction sample indicated that diethyl-FDCA (FDCA: 2,5-furan dicarboxylic acid) was formed as the major product. This GC-MS result indicated that the present of contaminant

salts (phosphate and NaCl) in crude/unpurified lyophilized powder did not significantly affect the reaction outcome. As a control, an authentic FDCA was chemically synthesized esterified to diethyl-FDCA and analyzed in the same conditions.

Synthesis of ethyl-FDCA from crude DDG by using AMBERLYST®15

[00146] In an air tight sealed tube, 2 mL of ethanol, 20 mg of crude DDG acid (unpurified lyophilized powder containing salts) and 200 mg of AMBERLYST®15 (Rohm and Haas, Philadelphia, PA) were combined. The DDG was not completely dissolved in this solution. The reaction was gently stirred at 105°C for 18 hours. Results of the GC-MS analysis of a crude reaction sample indicated that diethyl-FDCA (FDCA: 2,5-furan dicarboxylic acid) was formed as the major product. This GC-MS result indicated that the present of contaminant salts (phosphate and NaCl) in crude/unpurified lyophilized powder did not significantly affect the reaction outcome. As a control, a commercial FDCA was chemically esterified to diethyl-FDCA and analyzed in the same conditions.

Synthesis of diethyl-FDCA from crude DDG by using DOWEX®50 WX8

[00147] In an air tight sealed tube, 2 mL of ethanol, 20 mg of crude DDG acid (unpurified lyophilized powder containing salts) and 200 mg of DOWEX®50 WX8 were combined. The DDG was not completely dissolved in this solution. The reaction was gently stirred at 105°C for 18 hours. Results of the GC-MS analysis of a crude reaction sample indicated that diethyl-FDCA (FDCA: 2,5-furan dicarboxylic acid) was formed as the major product. This GC-MS result indicated that the present of contaminant salts (phosphate and NaCl) in crude/unpurified lyophilized powder did not significantly affect the reaction outcome. As a control, a commercial FDCA was chemically esterified to diethyl-FDCA and analyzed in the same conditions.

Example 18 - Production of FDCA derivatives

[00148] The synthesis of a number of high-value FDCA derivatives is described in Figure 16 in which dehydration of DTHU produces furfural-5-carboxylic acid, *i.e.* FCA, which is then chemically or enzymatically oxidized to FDCA, be reduced to FCH, or be transaminated (using chemical reductive amination or transaminase) to amino acid-AFC.

Example 19 – Production of di-butyl FDCA in a gas phase reaction

[00149] In this example the inlet of the GC was used as a high temperature reactor to catalyze the dehydration of di-butyl DDG to di-butyl FDCA. The resulting products were chromatographically separated detected by mass spectrometry. A solution of di-butyl DDG (10 mM) and sulfuric acid (100 mM) in butanol was placed in a GC vial. The vial was injected

into a GC and FDCA Dibutyl ester was observed. The reaction occurred in the 300 °C inlet (residence time = 4 seconds). The average yield of 6 injections was 54%.

GC Settings: Direct liquid inject / MS detector

Inlet: 300° C, total flow 29.51 ml/min, split ratio 10:1, split flow 24.1 ml/min, Septum Purge flow 3 mL/min.

GC liner: 4 mm, glass wool (P/N 5183-4647)

Column Flow: 2.41 ml/min He constant pressure control

Oven Program: At 40 °C hold for 2 min, then ramp 25 °C/min to 275 °C, then ramp 40 °C/min to 325 °C, hold for 2 min.

Column: HP-5MS, Agilent Technologies, 30m x 0.25mm x 0.25um.

Total Runtime: 14.65 minutes

MSD Transfer line: 290 °C

MS Source: 250 °C

MS Quad: 150 °C

Retention times:

2,3-FDCA Dibutyl ester: 9.3 min

2,5-FDCA Dibutyl ester: 9.7 min

[00150] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

[00151] No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinence of the cited documents. It will be clearly understood that although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

[00152] It should also be understood that the foregoing examples are offered to illustrate, but not limit, the invention.

CLAIMS

What is claimed is:

1. A method for producing a product of an enzymatic or chemical pathway from a starting substrate, the pathway comprising one or more conversion steps selected from the group consisting of:
 - an enzymatic conversion of guluronic acid into D-glucarate (Step 7);
 - an enzymatic conversion of 5-ketogluconate (5-KGA) into L-Iduronic acid (Step 15);
 - an enzymatic conversion of L-Iduronic acid into Idaric acid Step 7b); and
 - an enzymatic conversion of 5-ketocluconate into 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step 16);
 - an enzymatic conversion of 1,5-gluconolactone to gulurono-lactone (Step 19).
2. The method of claim 1 wherein the one or more conversion steps is the enzymatic conversion of guluronic acid into D-glucarate (Step 7).
3. The method of claim 1 wherein the one or more conversion steps is the enzymatic conversion of 5-ketogluconate (5-KGA) into L-Iduronic acid (Step 15).
4. The method of claim 1 wherein the one or more conversion steps is the enzymatic conversion of L-Iduronic acid into Idaric acid Step 7b).
5. The method of claim 1 wherein the one or more conversion steps is the enzymatic conversion of 5-ketogluconate into 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step 16).
6. The method of claim 1 wherein the one or more conversion steps is the enzymatic conversion of 1,5-gluconolactone to gulurono-lactone (Step 19).
7. The method of claim 1 wherein the product of the enzymatic pathway is 5-dehydro-4-deoxy-glucarate (DDG).
8. The method of claim 1 wherein the substrate is glucose and the product is 5-dehydro-4-deoxy-glucarate (DDG), comprising the steps of:
 - the enzymatic conversion of D-glucose to 1,5-gluconolactone (Step 1);
 - the enzymatic conversion of 1,5-gluconolactone to gulurono-lactone (Step 19);
 - the enzymatic conversion of gulurono-lactone to guluronic acid (Step 1B);
 - the enzymatic conversion of guluronic acid to D-glucarate (Step 7);
 - the enzymatic conversion of D-glucarate to 5-dehydro-4-deoxy-glucarate (DDG) (Step 8).
9. The method of claim 1 wherein the substrate is glucose and the product is DDG, comprising the steps of:

- the conversion of D-glucose to 1,5-gluconolactone (Step 1);
the conversion of 1,5-gluconolactone to gluconic acid (Step 1a);
the conversion of gluconic acid to 5-ketogluconate (5-KGA) (Step 14);
the conversion of 5-ketogluconate (5-KGA) to L-Iduronic acid (Step 15);
the conversion of L-Iduronic acid to Idaric acid (Step 7b); and
the conversion of Idaric acid to DDG (Step 8a).
10. The method of claim 1 wherein the substrate is glucose and the product is DDG, comprising the steps of:
the conversion of D-glucose to 1,5-gluconolactone (Step 1);
the conversion of 1,5-gluconolactone to gluconic acid (Step 1a);
the conversion of gluconic acid to 5-ketogluconate (5-KGA) (Step 14);
the conversion of 5- ketogluconate (5-KGA) to 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step 16);
the conversion of 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) to 4-deoxy-5-threo-hexosulose uronate (DTHU) (Step 4); and
the conversion of 4-deoxy-5-threo-hexosulose uronate (DTHU) to DDG (Step 5).
11. The method of claim 1 wherein the substrate is glucose and the product is DDG, comprising the steps of:
the conversion of D-glucose to 1,5-gluconolactone (Step 1);
the conversion of 1,5-gluconolactone to gluconic acid (Step 1a);
the conversion of gluconic acid to 5-ketogluconate (5-KGA) (Step 14);
the conversion of 5-ketogluconate (5-KGA) to L-Iduronic acid (Step 15);
the conversion of L-Iduronic acid to 4-deoxy-5-threo-hexosulose uronate (DTHU) (Step 7b); and
the conversion of 4-deoxy-5-threo-hexosulose uronate (DTHU) to DDG (Step 5).
12. The method of claim 8 further comprising the step of converting the DDG to 2,5-furandicarboxylic acid (FDCA).
13. The method of claim 9 further comprising the step of converting the DDG to 2,5- furandicarboxylic acid (FDCA).
14. The method of claim 10 further comprising the step of converting the DDG to 2,5- furandicarboxylic acid (FDCA).
15. The method of claim 11 further comprising the step of converting the DDG to 2,5- furandicarboxylic acid (FDCA).

16. The method of claim 12 wherein converting the DDG to FDCA comprises contacting DDG with acid to convert the DDG to FDCA.
17. The method of claim 13 wherein converting the DDG to FDCA comprises contacting DDG with acid to convert the DDG to FDCA.
18. The method of claim 14 wherein converting the DDG to FDCA comprises contacting DDG with acid to convert the DDG to FDCA.
19. The method of claim 15 wherein converting the DDG to FDCA comprises contacting DDG with acid to convert the DDG to FDCA.
20. A method for synthesizing a derivative of FDCA comprising:
contacting DDG with an alcohol, an inorganic acid at a temperature in excess of 60 C to form FDCA.
21. The method of claim 20 wherein the alcohol is butanol or ethanol.
22. The method of claim 20 having a yield of at least 25% molar.
23. A method of synthesizing a derivative of DDG comprising:
contacting DDG with an alcohol, an inorganic acid, and optionally a co-solvent to produce a derivative of DDG.
24. The method of claim 23 wherein:
 - a) the alcohol is ethanol or butanol;
 - b) the inorganic acid is sulfuric acid; and
 - c) the co-solvent is selected from the group consisting of: THF, acetone, acetonitrile, an ether, ethyl acetate, butyl acetate, an dioxane, chloroform, methylene chloride, 1,2-dichloroethane, a hexane, a heptane, toluene, carbon tetrachloride, petroleum ether, and a xylene.
25. A method for synthesizing derivative of FDCA comprising:
contacting a derivative of DDG with an inorganic acid to produce a derivative of FDCA.
26. The method of claim 25 having a yield of greater than 25% molar.
27. The method of claim 26 wherein the derivative is DDG is selected from the group consisting: methyl-DDG, ethyl-DDG, butyl-DDG, di-methyl DDG, di-ethyl-DDG, and di-butyl DDG.
28. The method of claim 25 further comprising that the derivative of FDCA is de-esterified to yield FDCA.
29. The method of claim 25 further comprising a step of polymerizing the derivative of FDCA.
30. A method for synthesizing FDCA comprising:

contacting DDG with an inorganic acid in a gas phase.

31. A method for synthesizing FDCA comprising:

contacting DDG with an inorganic acid at a temperature in excess of 120 C.

32. A method for synthesizing FDCA comprising:

contacting DDG with an inorganic acid under anhydrous reaction conditions.

33. A method for producing a product of an enzymatic or chemical pathway from a starting substrate, the pathway comprising one or more conversion steps selected from the group consisting of:

the conversion of DTHU to DDG (Step-5);

the conversion of gluconic acid to guluronic acid (Step-6);

the conversion of DEHU to DDH (Step 7A).

the conversion of guluronic acid to DEHU (Step 17A);

34. The method of claim 33 wherein the substrate is glucose and the product is DDG, comprising the steps of:

the conversion of D-glucose to 1,5-gluconolactone (Step 1);

the conversion of 1,5-gluconolactone to gluconic acid (Step 1a);

the conversion of gluconic acid to 3-dehydro-gluconic (DHG) (Step-2)

the conversion of 3-dehydro-gluconic (DHG) to 4,6-Dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step-3)

the conversion of 2,5 DDH to 4-deoxy-5-threo-hexosulose uronate (DTHU) (Step 4)

the conversion of DTHU to DDG (Step-5).

35. The method of claim 33 wherein the substrate is glucose and the product is DDG, comprising the steps of:

the conversion of D-glucose to 1,5-gluconolactone (Step 1);

the conversion of 1,5-gluconolactone to gluconic acid (Step 1a);

the conversion of gluconic acid to guluronic acid (Step-6)

the conversion of guluronic to glucarate (Step-7)

the conversion of glucarate to DDG (Step-8)

36. The method of claim 33 wherein the substrate is glucose and the product is DDG, comprising the steps of:

the conversion of D-glucose to 1,5-gluconolactone (Step 1);

the conversion of 1,5-gluconolactone to gluconic acid (Step 1a);

the conversion of gluconic acid to 5-ketogluconate (5-KGA) (Step 14);

- the conversion of 5- ketogluconate (5-KGA) to 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) (Step 16);
- the conversion of 4,6-dihydroxy 2,5-diketo hexanoate (2,5-DDH) to 4-deoxy-5-threo-hexosulose uronate (DTHU) (Step 4); and
- the conversion of 4-deoxy-5-threo-hexosulose uronate (DTHU) to DDG (Step 5).
- 37.** The method of claim 33 wherein the substrate is glucose and the product is DDG, comprising the steps of:
- the conversion of D-glucose to 1,5-gluconolactone (Step 1);
 - the conversion of 1,5-gluconolactone to gluconic acid (Step 1a);
 - the conversion of gluconic acid to 5-ketogluconate (5-KGA) (Step 14);
 - the conversion of 5-ketogluconate (5-KGA) to L-Iduronic acid (Step 15);
 - the conversion of L-Iduronic acid to 4-deoxy-5-threo-hexosulose uronate (DTHU) (Step 7B);
 - the conversion of 4-deoxy-5-threo-hexosulose uronate (DTHU) to DDG (Step 5).
- 38.** The method of claim 33 wherein the substrate is glucose and the product is DDH, comprising the steps of:
- the conversion of D-glucose to 1,5-gluconolactone (Step 1);
 - the conversion of 1,5-gluconolactone to guluronic acid lactone (Step 19);
 - the conversion of guluronic acid lactone to guluronic acid (Step 1B);
 - the conversion of guluronic acid to DEHU (Step 17A);
 - the conversion of DEHU to DDH (Step 7A).
- 39.** The method of claim 33 wherein the substrate is glucose and the product is DDH, comprising the steps of:
- the conversion of D-glucose to 1,5-gluconolactone (Step 1);
 - the conversion of 1,5-gluconolactone to gluconic acid (Step 1a);
 - the conversion of gluconic acid to guluronic acid (Step 6);
 - the conversion of guluronic acid to DEHU (Step 17A);
 - the conversion of DEHU to DDH (Step 7A).
- 40.** The method of claim 33 wherein the one or more conversion steps is the conversion of DTHU to DDG (Step-5).
- 41.** The method of claim 33 wherein the one or more conversion steps is the conversion of gluconic acid to guluronic acid (Step-6).
- 42.** The method of claim 33 wherein the one or more conversion steps is the conversion of DEHU to DDH (Step 7A).

43. The method of claim 33 wherein the one or more conversion steps is the conversion of guluronic acid to DEHU (Step 17A).
44. The method of claim 2 wherein the conversion of guluronic acid into D-glucarate is performed by a uronate dehydrogenase of SEQ ID NO: 1-3 or a homolog having at least 70% sequence identity to SEQ ID NOs: 1-3; or by a uronate dehydrogenase encoded by a nucleic acid of SEQ ID NOs: 4-6 or a homolog having at least 70% sequence identity to a nucleic acid of SEQ ID NOs: 4-6. The method of claim 4 wherein the conversion of L-iduronic acid into Idaric acid is performed by a uronate dehydrogenase of SEQ ID NO: 1-3 or a homolog having at least 70% sequence identity to SEQ ID NOs: 1-3; or by a uronate dehydrogenase encoded by a nucleic acid of SEQ ID NOs: 4-6 or a homolog having at least 70% sequence identity to a nucleic acid of SEQ ID NOs: 4-6.
45. The method of claim 3 wherein the conversion of 5-KGA into L-iduronic acid is performed by an isomerase of SEQ ID NOs: 7-19 or a homolog having at least 70% sequence identity to an isomerase of SEQ ID NOs: 7-19; or by an isomerase encoded by a nucleic acid of SEQ ID NOs: 20-32 or a homolog having at least 70% sequence identity to a nucleic acid of SEQ ID NOs: 20-32.
46. The method of claim 5 wherein the conversion of 5-KGA into 2,5-DDH is performed by a gluconate dehydratase of SEQ ID NOs: 33-35 or a homolog having at least 70% sequence identity to a gluconate dehydratase of SEQ ID NOs: 33-35; or by a gluconate dehydratase encoded by a nucleic acid of SEQ ID NOs: 36-38 or a homolog having at least 70% sequence identity to a nucleic acid of SEQ ID NOs: 36-38.
47. The method of claim 6 wherein the conversion of 1,5-gluconolactone into guluronolactone is performed by an alditol oxidase of SEQ ID NOs: 39-46 or a homolog having at least 70% sequence identity to an alditol oxidase of SEQ ID NOs: 39-46; or by an alditol oxidase encoded by a nucleic acid of SEQ ID NOs: 47-54 or a homolog having at least 70% sequence identity to a nucleic acid of SEQ ID NOs: 47-54.