A Short Enzymatic Synthesis of L-Glucose from Dihydroxyacetone Phosphate and L-Glyceraldehyde

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Unnatural monosaccharides[1] are potentially useful as nonmetabolizable sweeteners[2,3] and as building blocks for the synthesis of interesting natural and biologically active products.[4] L-Glucose **1** and L-fructose **2**, for example, are known to be low-calorie sweeteners.

Compound **2** was previously prepared by enzyme-catalyzed aldol addition reaction,[1e,5] selective cross-acyloin condensation from tetra-*O*-acetyl-L-arabinose and formaldehyde,[6] L-mannose isomerization catalyzed by cell-free extracts of *Aerobacter aerogenes*,[7] bacterial oxidation of L-mannitol,[8] and chemical synthesis from L-sorbose.[9] **1** was previously prepared via a multichemical transformation from D-glucose[10ab] (12.7%, 8 steps, 326 μmol) or L-arabinose,[10c] via asymmetric epoxidation of (*E*)-2-butenediol[1c] (2.6%, 9 steps, 64 μmol), via asymmetric Diels–Alder reaction[11] (4.5%, 9 steps, 44 μmol), and via chemoenzymatic synthesis[12] from commercially available cycloheptatriene (8.9%, 21 steps, 523 μmol). Compound **1** was also prepared by galactose oxidase-catalyzed[13] stereospecific oxidation of L-glucitol (10–15%, 180 μmol) and by transketolase-catalyzed reaction with L-arabinose.[14]

Most of these methods either give a very low yield for **1**, employ long reaction sequences, or require relatively expensive starting materials. We here report on an alternative route to **1** and **2** from dihydroxyacetone phosphate (DHAP) **3** and L-glyceraldehyde **4** catalyzed by a multienzymatic system composed of rhamnulose-1-phosphate aldolase, acid phosphatase, and fucose isomerase.

This enzymatic process is based on stereospecific aldol additions catalyzed by aldolases.[15] As compound **2** contains 3*R*, 4*S*, and 5*S* stereocenters, rhamnulose-1-phosphate aldolase[1e,5] was used to catalyze the condensation between **3** and **4**. After removal of the phosphate group catalyzed by phosphatase, **2** was isomerized to **1** which was catalyzed by fucose isomerase which is specific for aldoses with 2*S* and 3*R* configurations.[16] The aldolase and isomerase have been cloned and overexpressed in *Escherichia coli*[1e,16,17] strain K12. Another new cloning procedure has been reported,[18] and the recombinant *E. coli* is now available in large quantities for preparative synthesis (ATCC no. 86983 for the aldolase and ATCC no. 87024 for the isomerase).

Our first effort was directed toward a one-pot synthesis using cell-free extracts instead of purified enzymes to avoid protein purification and to simplify the scaling up process. However, unsatisfying results with a low aldose to ketose ratio (**1**/**2** ~2:8) were obtained. After silica gel chromatography, the mixture was separated by Dowex 50W-X8 (Ba^{2+} form) chromatography[19] to yield **2** (26.4% from DHAP, 1.35 mmol) and **1** (4.3% from DHAP, 0.22 mmol). Although the spectral data were in agreement with those of authentic samples, the optical rotation was inconsistent due to the contamination of minor components.

We then carried out a step by step synthesis (Scheme 1);[20] **3** and **4** were enzymatically condensed using rhamnulose-1-phosphate aldolase[1e] to yield L-fructose 1-phosphate **5** which was isolated as barium salt. Crude **5** was converted to sodium salt by treatment with Dowex 50W-X8 (H^+ form) followed by neutralization with NaOH solution. Subsequent phosphate ester hydrolysis with acid phosphatase yielded **2** as a single product, which was isomerized with fucose isomerase to give a 40:60 mixture of **1** and **2** which after separation by Dowex 50W-X8 (Ba^{2+} form) chromatography with water/ethanol (1:1) yielded pure **1** (10.8% from DHAP, 0.78 mmol) and **2** (17.5% from DHAP, 1.27 mmol). NMR spectra and optical rotations of **1** and **2** were in complete agreement with those from authentic samples.

L-Fucose isomerase has been shown to catalyze the isomerization of δ-arabinose (K_m = 35–280 mM)[21] to δ-ribulose (K_m = 10 mM)[21c] and L-fucose (K_m = 17–51 mM)[21a,c,d] to L-fuculose (K_m = 17 mM).[21c] L-Xylose has been found to be a weak substrate for L-fucose isomerase.[22]

(1) (a) Roush, W. R.; Brown, R. J. *J. Org. Chem.* **1982**, *47*, 1371. (b) Minami, N.; Ko, S. S.; Kishi, Y. *J. Am. Chem. Soc.* **1982**, *104*, 1109. (c) Ko, S. Y.; Lee, A. W. M.; Masamune, S.; Reed, L. A., III; Sharpless, K. B.; Walker, F. J. *Tetrahedron* **1990**, *46*, 245. (d) Korobi, Y.; Myles, D. C.; Whitesides, G. M. *J. Org. Chem.* **1992**, *57*, 5899. (e) Fessner, W.-D.; Sinerius, G.; Schneider, A.; Dreyer, M.; Schulz, G. E.; Badia, J.; Aguilar, J. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 555.

(2) (a) Rudney, H. *Science* **1940**, *92*, 112. (b) Brunton, J.; Horner, W. H.; Russ, G. A. *J. Biol. Chem.* **1967**, *242*, 813.

(3) (a) Shallenberger, R. S.; Acree, T. E.; Lee, C. Y. *Nature (London)* **1969**, *221*, 555. (b) Biospherics, Inc.; JP Patent 5712967 A2, August 11, 1982; *Chem. Abstr.* **1982**, *97*, 197120.

(4) (a) Hadfield, A. F.; Sartorelli, A. C. *Carbohydr. Res.* **1979**, *72*, 235. (b) Ishida, H.; Mizuta, A.; Yamamoto, N.; Kiso, M.; Hasegawa, A.; Takeda, K.; Azuma, I. *Agric. Biol. Chem.* **1989**, *53*, 701. (c) Fiandor, J.; García-López, M. T.; De las Heras, F. G.; Méndez-Castrillón, P. P.; Gil-Fernández, C.; Pérez, S.; Vilas, P.; Pérez, C.; García Gancedo, A. *Nucleosides Nucleotides* **1989**, *8*, 257. (d) Broka, C. A.; Ehrler, J. *Tetrahedron Lett.* **1991**, *32*, 5907. (e) Tatsuta, K.; Niwata, Y.; Umezawa, K.; Toshima, K.; Nakata, M. *Tetrahedron Lett.* **1990**, *31*, 1171.

(5) Henderson, I.; Sharpless, K. B.; Wong, C.-H. *J. Am. Chem. Soc.* **1994**, *116*, 558.

(6) Matsumoto, T.; Enomoto, T.; Kurosaki, T. *J. Chem. Soc., Chem. Commun.* **1992**, 610.

(7) Mayo, J. W.; Anderson, R. L. *Carbohydr. Res.* **1968**, *8*, 344.

(8) Dhawale, M. R.; Szarek, W. A.; Hay, G. W.; Kropinski, A. M. B. *Carbohydr. Res.* **1986**, *155*, 262.

(9) (a) Chen, C.-C.; Whistler, R. L. *Carbohydr. Res.* **1988**, *175*, 265. (b) Gizaw, Y.; BeMiller, J. N. *Carbohydr. Res.* **1995**, *266*, 81.

(10) (a) Szarek, W. A.; Hay, G. W.; Vyas, D. M.; Ison, E. R.; Hronowski, L. J. J. *Can. J. Chem.* **1984**, *62*, 671. (b) Sowa, W. *Can. J. Chem.* **1969**, *47*, 3931. (c) Smid, P.; Noort, D.; Broxterman, H. J. G.; van Straten, N. C. R.; van der Marel, G. A.; van Boom, J. H. *Recl. Trav. Chim. Pays-Bas* **1992**, *111*, 524.

(11) Bednarski, M.; Danishefsky, S. *J. Am. Chem. Soc.* **1986**, *108*, 7060.

(12) Johnson, C. R.; Golebiowski, A.; Steensma, D. H. *J. Am. Chem. Soc.* **1992**, *114*, 9414.

(13) Root, R. L.; Durrwachter, J. R.; Wong, C.-H. *J. Am. Chem. Soc.* **1985**, *107*, 2997.

(14) Bolte, J.; Demuynck, C.; Constant, O.; Hecquet, L. *In Microbial Reagents in Organic Synthesis*; Servi, S., Ed.; Nato ASI Series; Kluwer Academic Publishers: Dordrecht, 1992; p 57.

(15) Wong, C.-H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 412.

(16) (a) Fessner, W.-D.; Schneider, A.; Eyrisch, O.; Sinerius, G.; Badía, J. *Tetrahedron: Asymmetry* **1993**, *4*, 1183. (b) Fessner, W.-D.; Badía, J.; Eyrisch, O.; Schneider, A.; Sinerius, G. *Tetrahedron Lett.* **1992**, *33*, 5231.

(17) Badía, J.; Balomá, L.; Aguilar, J.; Boronat, A. *FEMS Microbiol. Lett.* **1989**, *65*, 253.

(18) (a) Chou, W.-C.; Chen, L.; Fang, J.-M.; Wong, C.-H. *J. Am. Chem. Soc.* **1994**, *116*, 6191. (b) García-Junceda, E.; Shen, G.-J.; Sugai, G.-J.; Wong, C.-H. *Bioorg. Med. Chem.*, in press.

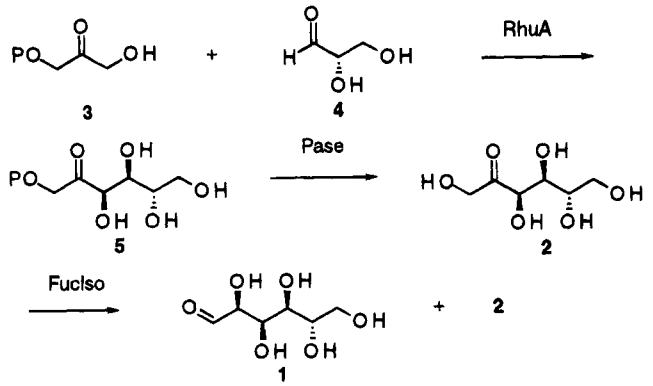
(19) Liu, K. K.-C.; Pederson, R. L.; Wong, C.-H. *J. Chem. Soc., Perkin Trans. 1* **1991**, 2669.

(20) We decided to show the open-chain structures for a better understanding of the stereochemistry of the processes.

(21) (a) Izumori, K.; Yamanaka, K. *Agric. Biol. Chem.* **1974**, *38*, 267. (b) Mortlock, R. P. *Methods Enzymol.* **1966**, *9*, 583. (c) Green, M.; Cohen, S. S. *J. Biol. Chem.* **1956**, *219*, 557. (d) Boulter, J. R.; Gielow W. O. *J. Bacteriol.* **1973**, *113*, 687.

0022-3263/95/1960-4294$09.00/0

Scheme 1



1 (150 mM) was reported not to be a substrate when incubated with isomerase (100 units/mmol) for 6 min.[21d] However, we have found that **1** is a weak substrate for fucose isomerase and the reaction can become useful in organic synthesis when an adequate concentration of **1** (180 mM), long reaction times (3 days), and large amounts of enzyme (9900 units/mmol) are employed. However, we could not reach the 55:45 equilibrium ratio[23] for **1** and **2** using more enzyme and/or a longer reaction time. These results seem to show that compounds with a δ-arabino configuration are good substrates and that those with an L-xylo configuration are weak substrates for L-fucose isomerase.

The process reported here shows some advantages compared to other methods previously reported. It is shorter and gives higher yields for **1** and **2**. It uses relatively inexpensive cell-free extracts, which can be easily prepared from commercially available recombinant *E. coli* strains.

Experimental Section

Materials and Methods. Acid phosphatase (EC 3.1.3.2) was purchased from Sigma. All chemicals and solvents were purchased from Aldrich. Dowex 50W-X8 (200–400 mesh, H^+ form) was thoroughly washed with purified water prior to use. Aldol condensation was monitored enzymatically by DHAP consumption.[24] Hydrolysis was monitored by TLC (silica gel 60 from Merck). Isomerization was monitored by ^{1}H-NMR analysis of H_α and H_β (1 H, $\delta_{H\alpha}$ = 5.22 ppm, $J_{H\alpha}$ = 3.8 Hz, $\delta_{H\beta}$ = 4.63 ppm, $J_{H\beta}$ = 8.0 Hz) for **1** in the pyranoside form and the protons at 4.1–3.9 ppm for **2** in the β-pyranoside (a) and β-furanoside (b) forms (H_{3b} = 4.08 ppm, H_{4b} = 4.08 ppm, H_{6a} = 4.03 ppm, $J_{H6a,H6'a}$ = 12.6 Hz, $J_{H6a,H5a}$ = 1.3 Hz, H_{5a} = 3.99 ppm, $J_{H5a,H4a}$ = 3.6 Hz).[25] Nuclear magnetic resonance (400 MHz) spectra were obtained using HDO (in D_2O, δ = 4.8 ppm) and CH_3CN (in D_2O, δ = 1.60 ppm) as internal references. Flash chromatography was carried out with silica gel 60 (230–400 mesh).

Enzyme Preparation. The *E. coli* cells (ATCC no. 87024) containing recombinant fucose isomerase were grown aerobically to late logarithmic phase (OD_{600}, 0.9) at 37 °C in four 3 L flasks containing LB medium (1 L each) supplemented with ampicillin (250 μg/mL), followed by induction with IPTG (250 μM). The culture was kept overnight (OD_{600}, 2.1) at 30 °C. Cells were harvested by centrifugation (10000*g*; 30 g of wet cells were obtained), resuspended in Tris·HCl buffer (50 mM, pH 7.5, 80 mL), and disrupted by repeatedly being passed through a French press (~1600 lb/in^2) for five times. Cellular debris was removed by centrifugation (16000*g*), and the clear supernatant (total activity, 66 000 units) was concentrated to one-fifth of the volume by ultrafiltration (MW cut off 10 000) and used for the isomerization reaction. For the preparation of aldolase, the *E. coli* cells (ATCC no. 86983) were grown as previously described[18] and the crude extract (total activity, 1330 units) obtained was directly used without being concentrated for the aldol reaction. The aldolase[26] and isomerase[27] were assayed according to the procedures described previously. One unit of enzyme represents 1 μmol of product formed per minute.

L-Fructose-1-phosphate Barium Salt 5. Freshly prepared aqueous solutions of **3** (7.24 mmol)[28] and **4** (9.05 mmol)[29] were combined, and the resulting solution (100 mL) was adjusted to pH 6.8 with 6 N NaOH (2 mL). Rhamnulose-1-phosphate aldolase (50 units, 20 mL)[18] was added, and the reaction mixture was incubated at 25 °C until the consumption of **3** was higher than 90% (2 days). The solution was adjusted to pH 7.0 with 6 N NaOH, $BaCl_2{\cdot}2H_2O$ (2 equiv) was added, and the mixture was refrigerated at 4 °C for 1 h. The precipitate was filtered off through Celite, acetone (250 mL) was added to the solution, and the mixture was allowed to stand at 4 °C overnight. The precipitate was collected by centrifugation, washed with EtOH/Et_2O (1:1, 40 mL), and dried under high vacuum (2.50 g, 87%). The crude barium salt was used directly without characterization for the next reaction.[30]

L-Fructose 2. Crude **5** (2.50 g, 6.33 mmol) was powdered, suspended in H_2O (55 mL), and treated with Dowex 50W-X8 (H^+ form, 200–400 mesh) for 30 min. The resin was filtered off and washed with water (2 × 15 mL), and the solution was adjusted to pH 4.7 with 6 N NaOH. Acid phosphatase (0.28 mL, 125 units) was added, and the mixture was stirred at 25 °C for 43 h, after which the solution was adjusted to pH 7.0 with $Ba(OH)_2{\cdot}8H_2O$ solution, followed by the addition of methanol (2 volumes). The precipitate was filtered off, and the solution was concentrated under reduced pressure (bath temp < 25 °C) for silica gel chromatography with $CHCl_3$/MeOH (2:1) to give **2** (486 mg, 2.70 mmol, 37% from **3**) as a white solid: mp 91–93 °C (lit.[9b] 90–94 °C, lit.[7] 93–95 °C, lit.[9a] 89–90 °C); $[\alpha]_D$ +87.2 (*c* 2.1, H_2O) (lit.[9a] $[\alpha]_D$ +88 (*c* 1.5, H_2O), lit.[9b] $[\alpha]_D$ +94.4 (*c* 1.8, H_2O), lit.[5] $[\alpha]_D$ + 92 (*c* 2, H_2O), lit.[8] $[\alpha]_D$ +86 (*c* 1, H_2O)). NMR (^{1}H and ^{13}C) data were identical to those of D-fructose.

L-Glucose 1. **2** (486 mg, 2.7 mmol, 180 mM) was dissolved in Tris·HCl buffer (7.5 mL, 50 mM, pH 7.5) containing $MnCl_2$ (2 mM) and mercaptoethanol (2 mM). The solution was adjusted to pH 7.5 with 1 N NaOH, and the fucose isomerase-containing cell-free extract (26 600 units) was added. The mixture was stirred at 25 °C for 72 h and then adjusted to pH 7.0. Methanol (2 volumes) was then added, and the precipitate was filtered off through Celite. The solution was concentrated and chromatographed on silica gel with $CHCl_3$/MeOH (2:1). The solution was concentrated, and the residue was dissolved in buffer and treated as above by repeating the whole process and keeping a 180 mM concentration for **2**. The resulting residue was chromatographed on Dowex 50W-X8 (200–400 mesh, barium form) with water/ethanol (1:1) to give **1** (141 mg, 0.78 mmol, 28.9%), $[\alpha]_D$ −50.7 (*c* 1, H_2O) (lit.[12] $[\alpha]_D$ −52 (*c* 0.8, H_2O), lit.[1c] $[\alpha]_D$ −47.2 (*c* 0.83, H_2O), lit.[10a] $[\alpha]_D$ −52.9 (*c* 5.48, H_2O), lit.[10b] $[\alpha]_D$ −53 (*c* 3.0, H_2O)), and **2** (229 mg, 1.27 mmol, 47%). NMR (^{1}H and ^{13}C) data were identical to those of an authentic sample.

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(22) The determined apparent K_m value for L-xylose was 0.4 M, although L-xylose actually became inhibitory at concentrations over 0.25 M. The extrapolated V_{max} for L-xylose only represented 0.83% of the value for L-fucose or D-arabinose: Oliver, E. J.; Mortlock, R. P. *J. Bacteriol.* **1971**, *108*, 293.

(23) Bock, K.; Meldal, M.; Meyer, B.; Wiebe, L. *Acta Chem. Scand.* **1983**, *37B*, 101.

(24) Bergmeyer, H. U. *Methods of Enzymatic Analysis*, 3rd ed.; Verlag Chemie: Deerfield, FL, 1984; Vol. 2, pp 146–7.

(25) The integration of these protons represents 2H from the total integration for the mixture of tautomers. D-fructose exists as a mixture of β-pyranose (~70%), β-furanose (~25%), α-furanose (~5%), and α-pyranose (traces) in D_2O solutions: (a) De Bruyn, A.; Anteunis, M.; Verhegge, G. *Carbohydr. Res.* **1975**, *41*, 295. (b) Jaseja, M.; Dais, P.; Perlin, A. S. *Magn. Reson. Chem.* **1990**, *28*, 283. (c) Pelmore, H.; Eaton, G.; Symons, M. C. R. *J. Chem. Soc., Perkin Trans. 2* **1992**, 149.

(26) Chiu, T.-H.; Feinhold, D. S. *Biochemistry* **1969**, *8*, 98.

(27) Dische, Z.; Borenfreund, E. *J. Biol. Chem.* **1951**, *192*, 583.

(28) Jung, S.-H.; Jeong, J.-H.; Miller, P.; Wong, C.-H. *J. Org. Chem.* **1994**, *59*, 7182.

(29) This compound is commercially available. It can be prepared in large scale using the osmium-catalyzed asymmetric dihydroxylation reaction (ref 5).

(30) Straub, A.; Effenberger, F.; Fischer, P. *J. Org. Chem.* **1990**, *55*, 3926.