JOC*Note*

One-Pot Synthesis of L-Fructose Using Coupled Multienzyme Systems Based on Rhamnulose-1-phosphate Aldolase

Dirk Franke, Timothy Machajewski,[1]
Che-Chang Hsu, and Chi-Huey Wong*

Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

wong@scripps.edu

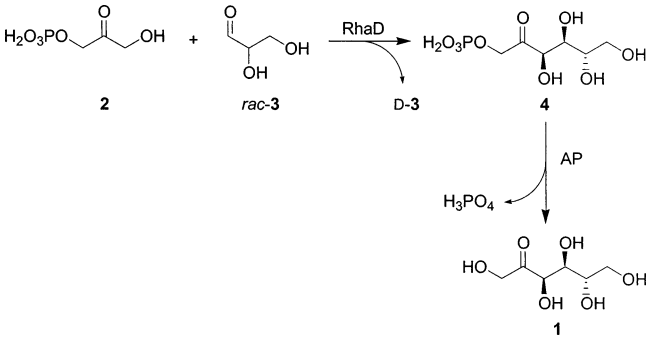
Received January 14, 2003

Abstract: Two methods have been developed for the highly efficient enzymatic synthesis of L-fructose: one is based on rhamnulose-1-phosphate aldolase and acid phosphatase using racemic glyceraldehyde and dihydroxyacetone phosphate as substrates; the other is to generate enantiomerically pure L-glyceraldehyde in situ from glycerol for the aldol reaction, using galactose oxidase catalyzed oxidation of glycerol in the presence of catalase. Using this four-enzyme system, enantiomerically pure L-fructose was obtained. Using the more expensive dihydroxyacetone phosphate, the yield was 55% after purification.

Unnatural monosaccharides have many useful applications. L-Fructose, for example, is known as a nonnutritive sweetener,[2] an inhibitor of various glycosidase,[3] and an insecticide for ants and house flies.[4] In addition, unnatural monosaccharides are potentially useful as chiral building blocks for the synthesis of biologically active compounds.[5] Given the importance of these monosaccharides, the development of their synthesis has been the subject of considerable recent interest.

L-Fructose (**1**) has previously been prepared via chemical[6−8] and chemoenzymatic[9−11] synthesis. Whereas the enzyme-based methods afford very low yields and often require relatively expensive starting materials, attempts to chemically synthesize **1** have been characterized by tedious multistep sequences and low selectivity. Thus, the overall low yields and difficulty in scaling up L-fructose production have prevented further evaluation of its applications.

SCHEME 1. Preparation of l-Fructose (1) Starting from *rac*-Glyceraldehyde (*rac*-3) and DHAP (2)



We reported earlier the enzymatic synthesis of L-fructose (**1**) from dihydroxyacetone phosphate (**2**, DHAP) and L-glyceraldehyde (L-**3**),[12,13] which is carried out by a multienzyme system comprising rhamnulose-1-phosphate aldolase (RhaD) and acid phosphatase (AP) using stereospecific aldol addition reaction by this aldolase.[14] Although **1** is produced rapidly in moderate yield, it suffers from two limitations. First, L-**3** is not commercially available. Although it can be synthesized with osmium-catalyzed asymmetric dihydroxylation of protected acrolein[13] or glycol cleavage of ascorbic acid derivatives,[15] preparation of L-**3** on gram scales is inefficient and the use of toxic heavy metals places a limit on its applications. Additionally, L-**3** is thermodynamically metastable and decomposes easily.[16] Second, the intermediate L-fructose-1-phosphate (**4**) was isolated as a barium salt, and additional steps were required to remove and recycle the toxic metal salts. We report herein two improved methods for the synthesis of **1**, each using a coupled enzyme system, that overcome these limitations.

Our initial strategy was to use racemic glyceraldehyde (*rac*-**3**) and utilize the stereoselectivity of the aldolase to preferentially make L-fructose (**1**) (Scheme 1).

DHAP (**2**) and *rac*-**3** were condensed using RhaD to yield L-fructose-1-phosphate (**4**). Conducting phosphate ester hydrolysis with acid phosphatase (AP) without isolation allowed us to avoid the use of barium salts. No diastereomers of **1** were found as byproducts of the condensation and hydrolysis steps (^{1}H NMR). Chromatographic purification was used to remove salts and remaining D-glyceraldehyde (D-**3**) to give pure **1** in 64% yield based on **2**.[17]

As a refinement of this method we sought to produce L-glyceraldehyde (L-**3**) in situ from an inexpensive starting material and couple this reaction to the aldolase-

* Corresponding author.

(1) Current address: Chiron Corporation, Emeryville, CA 94608-2916.

(2) Levin, G. V.; Zehner, L. R.; Saunders: J. P.; Beadle, J. R. *Am. J. Clin. Nutr.* **1995**, *62*, 1161.

(3) Muniruzzaman, S.; Pan, Y. T.; Zeng, Z.; Atkins, B.; Izumori, K.; Elbein, A. D. *Glycobiology* **1996**, *6*, 795.

(4) Levin, G. V.; Zehner, L. R. Eur. Pat. EP 397,027 (Cl. A01N43/08), 14. Nov 1990; US Patent 6,166,193, 24. Nov 1992; *Chem. Abstr.* **1991**, *114*, 242827q.

(5) Györgydeák, Z.; Pelyvás, I. *Monosaccharide Sugars−Chemical Synthesis by Chain Elongation, Degradation, and Epimerization*; Academic Press: San Diego, 1998.

(6) Matsumoto, T.; Enomoto, T.; Kurosaki, T. *J. Chem. Soc., Chem. Commun.* **1992**, *8*, 610.

(7) Chen, C. C.; Whistler, R. L. *Carbohydr. Res.* **1988**, *175*, 265.

(8) Gizaw, Y.; BeMiller, J. N. *Carbohydr. Res.* **1995**, *266*, 81.

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

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

(11) Itoh, H.; Izumori, K. *J. Ferment. Bioeng.* **1996**, *81*, 354.

(12) Alajarin, R.; Garcia-Junceda, E.; Wong, C.-H. *J. Org. Chem.* **1995**, *60*, 4294.

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

(14) Fessner, W. D.; Badia, J.; Eyrisch, O.; Schneider, A.; Sinerius, G. *Tetrahedron Lett.* **1992**, *33*, 5231.

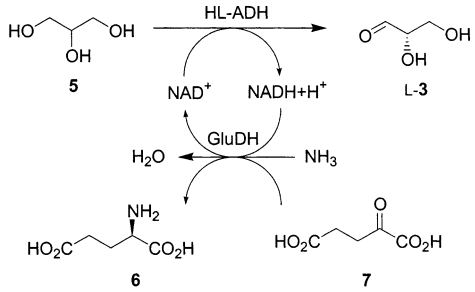
(15) Hubschwerlen, C. *Synthesis* **1986**, 962−964.

(16) Baer, E.; Fischer, H. O. L. *J. Am. Chem. Soc.* **1939**, *61*, 761.

(17) Given the possibility of recovering D-**3** and using the racemization reaction[16] to regenerate *rac*-**3**, it is in theory possible to completely transform *rac*-**3** into **1**.

10.1021/jo030021m CCC: $25.00
Published on Web 07/26/2003

SCHEME 2. Enzymatic Oxidation of Glycerol (5) to Effort L-Glyceraldehyde (L-3) Using HL-ADH and a NAD^+ Regeneration System with α-Keto Glutaric Acid (7) and GluDH



catalyzed condensation with DHAP (**2**). The enzymatic oxidation of glycerol (**5**) to L-**3** has been reported on analytical scales by two approaches: with horse liver alcohol dehydrogenase (HL-ADH)[18] and galactose oxidase (GOase) from *Dactylium dendroides*.[19]

The HL-ADH reaction requires NAD^+ for activity, and because NAD^+ is too expensive for stoichiometric use,[20] a cofactor regeneration system would be required for preparative synthesis of L-**3**. This can be accomplished by the L-glutamic dehydrogenase (GluDH) catalyzed reduction of α-keto glutaric acid (**7**) to give glutamic acid (**6**) (Scheme 2).

However, two reasons render HL-ADH unattractive for our purposes. HL-ADH is known to operate at a pH optimum of 8.0, which precludes the possibility of coupling the oxidation to the aldolase-catalyzed condensation that would require more acidic conditions. In addition, stoichiometric quantities of **7** would be necessary for NAD^+ recycling and **1** would have to be separated from **6**.

In contrast, GOase does not require the addition of a cofactor and also operates at a similar pH as RhaD, having a pH-optimum of around 6.7,[21] but being also active over a wide pH range from 5.5 to 8.0 (see supplemental information). This would allow for the simultaneous production and condensation of L-glyceraldehyde (L-**3**). This reaction, however, has never been illustrated on preparative scales. Fungal GOase has recently been produced with *Pichia pastoris*[22] as an active fusion protein and with *Escherichia coli* as a mutant generated by directed evolution,[23] making it possible to economically produce large quantities of the enzyme.

To combine the GOase-catalyzed oxidation of glycerol (**5**) with RhaD condensation, we established an analytical method for determining the kinetic parameters of GOase reaction with different substrates. The released hydrogen peroxide in the GOase reaction was used to quantify the enzymatic activity through horseradish peroxidase (HRP) catalyzed oxidation of 2,2′-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) with H_2O_2. The pH range of HRP (5.5−7.5)[24] was compatible with GOase for the working coupled assay.

TABLE 1. Kinetic Parameters for the Oxidation of L-Fructose (1), DHAP (2), L-Glyceraldehyde (L-3), D-Glyceraldehyde (D-3), L-Fructose-1-phosphate (4), Glycerol (5), and Galactose (gal) with GOase in 50 mM Potassium Phosphate Buffer at pH 6.7 and 23 °C

substrate	K_m[a] [mm]	k_{cat}[a] [min^{-1}]	k_{cat}/K_m[b] [mm^{-1} min^{-1}]
5	1150 ± 200	7.7 ± 0.5	0.0067 ± 0.0010
2	4.2 ± 0.8	4.3 ± 0.2	1.0 ± 0.2
1, L-**3**, D-**3**, **4**	nq[c]	≤0.09	nq[c]
gal	55 ± 10	11 ± 1	0.20 ± 0.04

[a] The error intervals resulted from nonlinear regression of the Lineweaver−Burk plot. [b] The error interval was calculated according to Gauss' error propagation law:

$$\Delta\frac{k_{cat}}{K_m} = \sqrt{\left(\frac{1}{K_m}\Delta k_{cat}\right)^2 + \left(\frac{k_{cat}}{K_m{}^2}\Delta K_m\right)^2}$$

[c] Not quantifiable.

Determination of the kinetic parameters of the oxidation of glycerol (**5**) and galactose indicated that the k_{cat} values for both substrates are quite similar (Table 1),[25] but the K_m value for **5** was 20 times higher than for the natural substrate galactose. Quantification of product formation proved that **5** was oxidized with only 1 equiv of oxygen and neither L-glyceraldehyde (L-**3**) nor D-glyceraldehyde (D-**3**) was accepted as a substrate for GOase. L-Fructose (**1**) or L-fructose-1-phosphate (**4**) was not accepted as a substrate at concentrations up to 5 m. We have found that in addition to dihydroxyacetone,[26] its phosphorylated analogue DHAP (**2**) is accepted as a substrate for GOase, resulting in the formation of an unidentified product.[27]

For establishing a preparative route toward the production of L-fructose (**1**) from glycerol (**5**) with GOase and RhaD, two different strategies were investigated.

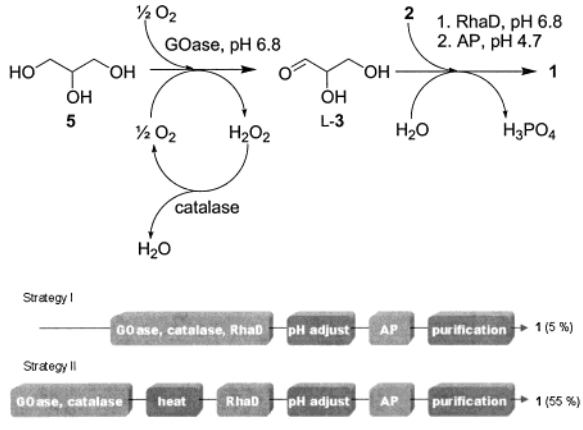
In the first strategy the formation of L-glyceraldehyde (L-**3**) and condensation with DHAP (**2**) was conducted simultaneously (Scheme 3, Strategy I).

Although the K_m value for GOase using **5** as a substrate was 1.15 M, for enhanced diffusion rates, we chose a concentration of 183 mM for **5** to produce L-**3** in situ, which corresponds to an activity of only 14% v_{max}. To compensate for the low turnover rate, a GOase concentration of 5 U mL^{-1} was used, which was significantly higher than the concentration of RhaD (0.8 U mL^{-1}). To prevent inactivation of the enzyme, we supplemented the system with catalase in excess in order to destroy hydrogen peroxide, which was found to inactivate the system.

Combining glycerol (**5**), DHAP (**2**), GOase, catalase, and RhaD resulted in the formation of L-fructose-1-phosphate (**4**) (^{1}H NMR, no other products identified).[28] Simultaneously, the disappearance rate for DHAP (**2**) was 17.5 mM h^{-1} (enzymatic quantification of **2**). Here

(18) Wong, C.-H.; Matos, J. R. *J. Org. Chem.* **1985**, *50*, 1992.
(19) Klibanov, A. M.;. Alberti, B. N.; Marletta, M. A. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 804.
(20) Wong, C.-H.; Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry*; Pergamon Press: Oxford, 1994.
(21) Driscoll, J. J.; Kosman, D. J. *Biochemistry* **1987**, *26*, 3429.
(22) Whittaker, M. M.; Whittaker, J. W. *Protein Expression Purif.* **2000**, *20*, 105.
(23) Sun, L.; Petrounia, I. P.; Yagasaki, M.; Bandara, G.; Arnold, F. H. *Protein Eng.* **2001**, *14*, 699.
(24) Bolscher, B. G. J. M.; Zoutberg, G. R.; Cuperus, R. A.; Wever, R. *Biochim. Biophys. Acta* **1984**, *784*, 189.
(25) Kinetic parameters calculated with *GraFit*, Version 4.0; Erithacus Software Limited: U.K., 1998.
(26) Mendonca, M. H.; Zancan, G. T. *Arch. Biochem. Biophys.* **1987**, *252*, 507.
(27) The possible product phosphoric acid mono(2,3-dioxo-propyl) ester could not be identified using ^{1}H NMR.

SCHEME 3. One-Pot Synthesis of L-Fructose (1) Starting from Glycerol (5) and DHAP (2) Using Coupled Enzymatic Systems with GOase, Catalase, RhaD, and AP



again, the dephosphorylation of **4** could be done with AP in the presence of the other enzymes without prior isolation. Unfortunately, after silica gel purification, only a 5% yield of L-fructose (**1**) could be obtained from DHAP (**2**). This can most likely be attributed to the reaction of DHAP (**2**) with GOase. Increasing the concentration of RhaD to 1.7 U mL^{-1} (factor 2) or glycerol (**5**) to concentrations of 0.4, 0.8, or 1.2 M (factor 2, 4, or 6) did not result in any significant additional formation of L-fructose-1-phosphate (**4**). Comparing the high k_{cat}/K_m value of GOase with DHAP (**2**) as a substrate to that with glycerol (**5**), we concluded that spatiotemporal separation of GOase reaction and RhaD condensation is essential to prevent consumption of **2.**

In a second attempt (Scheme 3, Strategy II) L-glyceraldehyde (L-**3**) was synthesized with GOase using catalase and free oxygen prior to condensation with DHAP (**2**). The formation of L-**3** could be quantified in an end-point assay using the NADH-dependent reaction of the aldehyde with alcohol dehydrogenase. Alternatively, Schiff's reagent was used in a photometrically end-point assay for quantification, which was more accurate but less sensitive. Using 75 U mL^{-1} GOase and 93 U mL^{-1} catalase, 50 mM L-**3** could be produced from 0.87 M glycerol (**5**) within a 48 h process time. With regard to a theoretical yield of 96% (not including the oxygen recycling) this corresponds to a relative yield of 6%. For the elimination of the remaining GOase activity, the proteins were heated. We found that L-**3** is stable in aqueous reaction mixture at pH 6.7, when heated to 100 °C over a time period of 10 min (alcohol dehydrogenase test, Schiff's test). Condensation of L-**3** thus produced with DHAP (**2**), dephosphorylation, and purification gave L-fructose (**1**) in a highly pure form and 55% yield. We concluded that the production of **1** using GOase is favorable as a result of the low cost of the starting material and the possibility of integrating the four-enzyme system into a continuous process with only one separation step. It is also remarkable that from achiral starting materials the product is formed with three new chiral centers in only one synthetic operation.

In summary, we have described two methods for the preparation of L-fructose (**1**). The condensation of racemic glyceraldehyde (*rac*-**3**) proceeds in high yield and eliminates the necessity to use toxic osmium[13] or palladium catalysts[15] to introduce the required oxygenation. In addition, the use of toxic barium for precipitation was eliminated, thereby providing an efficient and environmentally friendly process for the production of enantiomerically pure **1**. Additionally, the stereoselective oxidation of glycerol (**5**) by galactose oxidase, coupled with the aldolase reaction in situ, provides **1** in a relatively high yield.

Experimental Section

Enzyme Sources. Acid phosphatase type XA (EC 3.1.3.2, from sweet potato), galactose oxidase (EC 1.1.3.9, from *Dactylium dendroides*, partially purified), horseradish peroxidase type VI (EC 1.11.1.7, from horseradish), glycerol-3-phosphate dehydrogenase type I (EC 1.1.1.8, from rabbit muscle), alcohol dehydrogenase (EC 1.1.1.1, from baker's yeast), and catalase (EC 1.11.1.6, from bovine liver) were purchased commercially. All enzymes have been used as received and have not been desalted. Rhamnulose-1-phosphate aldolase (EC 4.1.2.19) was isolated from *E. coli* as described previously.[29]

Assay for Monitoring Aldol Condensation with RhaD. Aldol condensation was monitored indirectly by the decrease in DHAP concentration. This was done by reduction with NADH-dependent glycerol-3-phosphate dehydrogenase in 50 mM potassium phosphate buffer (pH 7.5) at 23 °C, while monitoring the decrease of absorbance at 340 nm.

Assay for GOase activity by Coupling with HRP/ABTS Assay. The determination of substrate acceptance for GOase and Michaelis−Menten parameters was done by quantification of hydrogen peroxide with a coupled HRP/ABTS assay at 23 °C. Reaction mixture contained (final volume 500 μL): 500 μg mL^{-1} (0.91 mM) ABTS, 4.3 mg L^{-1} (0.75 U mL^{-1}) HRP, 840 mg L^{-1} (5.0 U mL^{-1}) GOase, 50 mM potassium phosphate buffer (pH 6.7). For the determination of kinetic parameters for D-galactose as a substrate, the GOase concentration was 250 mg L^{-1} (1.5 U mL^{-1}). The reaction was initiated by addition of the substrate. The absorption was monitored over a time period of 10 min at a wavelength of 410 nm, which is the absorption maximum for oxidized $ABTS^+$. The pH operating experiments for oxidation of glycerol (**5**) and D-galactose were done in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, 5.5 ≤ pH ≤ 6.5) and 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS, 6.5 ≤ pH ≤ 8.0). The pH was adjusted with 10 N NaOH.

Assay for Quantification of L-Glyceraldehyde (L-3) with Alcohol Dehydrogenase. l-Glyceraldehyde (L-**3**) was quantified using an enzymatic assay based on the aldehyde reduction with NADH-dependent alcohol dehydrogenase from baker's yeast. The assay was performed in 50 mM potassium phosphate buffer (pH 7.0) using 3.4 mg mL^{-1} (990 units mL^{-1}) alcohol dehydrogenase. The rate of decrease of absorption at 340 nm was measured 2 min after addition of the enzyme. Linear correlation of the rate and concentration of substrate was observed at up to 5 mM L-**3** for the final concentration.

Assay for Quantification of L-Glyceraldehyde (L-3) with Schiff's Test. For the quantification of l-glyceraldehyde (L-**3**) using Schiff's test, 10 μL of aqueous sample containing up to 83 mM L-**3** was dissolved in 0.4 mL of Schiff's reagent for aldehydes. Absorption was monitored at a wavelength of 560 nm over a time period of 30 min while incubating at 23 °C. Maximal absorption was measured after approximately 15 min. The concentrations of L-**3** were calculated from the maximal absorption using a reference calibration curve with *rac*-**3** and acetaldehyde as substrates.

(28) We found that crude GOase resulted in the formation of a product mixture, while **4** was produced only when partially purified GOase was used. Therefore, partially purified enzyme was used in all further experiments.

(29) Garcia-Junceda, E.; Shen, G.-J.; Sugai, T.; Wong, C.-H. *Bioorg. Med. Chem.* **1995**, *3*, 945.

Improved Synthesis of L-Fructose (1) from Glycerol (5) in a Three-Stage Enzymatic Process. GOase (3750 units) and catalase (4650 units) were added to a solution of 5 mL (44 mmol) glycerol (**5**) in 45 mL of 50 mM potassium phosphate buffer (pH 6.7). The mixture was carefully shaken at 23 °C for 48 h while vented to the atmosphere. The formation of L-glyceraldehyde (L-**3**) in the reaction mixture was monitored using the Schiff test. After a process time of 48 h, 4.5 g L^{-1} (50 mM) L-**3** were achieved. For inactivation of remaining GOase the solution was heated to 100 °C for 5 min using a microwave oven and immediately cooled to 23 °C with a water bath. All nonsoluble ingredients were removed by filtration and pH 6.8 was adjusted (1 N NaOH); 24 mg (3.4 mmol) DHAP (**2**),[30] 3.8 mg (28 μmol) zinc(II) chloride, and 35 units RhaD were added. The mixture was shaken at 23 °C for 3 days. The pH was adjusted to 4.7 with 1 N HCl, and 100 μL (110 units) AP in aqueous solution was added. For hydrolysis the reaction mixture was shaken at 37 °C for another 16 h. After cooling to 23 °C the pH was adjusted to 7.0 with 1 N NaOH, and the solution was diluted with 150 mL of methanol. The white precipitate was removed by filtration through Celite. All volatiles were removed in vacuo, and the yellow residue was further purified using silica gel chromatography with $CHCl_3$/MeOH (2:1). Fractions containing **1** were pooled and concentrated in vacuo, and 350 mg (1.9 mmol) of **1** was obtained as a white solid. With regard to DHAP (**2**) as the starting material, this correlates with a yield of 55%. ^{1}H NMR, ^{13}C NMR, and optical rotation were in complete agreement with authentic samples.[31]

Acknowledgment. A Feodor-Lynen fellowship of the Alexander-von-Humboldt Foundation to D.F. is gratefully acknowledged. We are grateful to Drs. Tom Tolbert, Lac Lee, and Jungie Liu and Mrs. Sarah Hanson for stimulating discussions.

Supporting Information Available: General experimental information; procedure for the synthesis of **1** from *rac*-**3**; procedure for the synthesis of **1** from **5** with a two-stage enzymatic process; Lineweaver−Burk plots for the determination of kinetic constants for GOase with galactose, glycerol, and DHAP as substrates; pH rate profile for the oxidation of glycerol and galactose with GOase; standard curve for the quantification of glyceraldehyde with Schiff's reagent; calculation of theoretical achievable yield for oxidation of glycerol; and ^{1}H NMR spectra of **1** generated from *rac*-**3** and **5**, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

JO030021M

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

(31) Authentic **1** was prepared from L-**3** following the method described in ref 12.