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Bioproduction of a novel sugar 1-deoxy-L-fructose by *Enterobacter aerogenes* IK7; isomerization of a 6-deoxyhexose to a 1-deoxyhexose

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Abstract—1-Deoxy-L-fructose, a very rare monosaccharide, was produced by hydrogenation of 6-deoxy-L-mannose (L-rhamnose)—the only cheaply available deoxy sugar—to 1-deoxy-L-mannitol (L-rhamnitol) followed by oxidation with *Enterobacter aerogenes* IK7. The entire procedure was conducted in water and shows the power of green environmentally friendly chemistry combined with biotechnology in the preparation of new monosaccharides with potential for novel bioactive properties or alternative foodstuffs; the reactions here are reported on a multigram scale but would be reproducible on a very large scale.

1. Introduction

Carbohydrates are displayed on the surface of almost all cells in all domains of life and thus have a multitude of biological activities; this recognition of the chemotherapeutic potential of rare sugars has resulted a large amount of ingenious effort in the de novo synthesis of small amounts of uncommon sugars.[1] Synergistically, large scale hexose isomerization by chemical methods [for example of D-tagatose as a new sugar substitute[2]] and by biotechnological procedures[3] has been developed under environmentally friendly conditions, allowing the production of hexoses in significant quantities. In contrast there has been relatively little investigation of the isomerization of deoxy sugars, although 6-deoxy-L-mannose (rhamnose) **2**, the only cheaply available deoxy sugar,[4] may be equilibrated (Scheme 1) by chemical[5] or enzymatic[6] techniques to 6-deoxy-L-fructose (rhamnulose) **1**. Herein the conversion of 6-deoxy hexose **2** to 1-deoxy-L-fructose **4** by hydrogenation to give rhamnitol **3** followed by oxidation with the newly isolated microbe *Enterobacter aerogenes* IK7 is reported.

Deoxy sugars are one of the important classes of carbohydrates. These carbohydrates are found in liposaccharides, glycoproteins, glycolipids on bacterial cell surfaces and as part of many secondary metabolites;[7] they play important roles in addition to central metabolism, involved in cell signaling, immunological recognition and in host-pathogen interactions and long been used as therapeutic agents. For example, 2-deoxy-L-ribose suppress tumor growth.[8] Increasing interest in the sugar metabolism of bacteria recognize their monosaccharides—which are not found in mammals—allow for the design of new chemotherapeutic agents, which target enzymes, involved in the synthesis of deoxy sugars. Alteration in these sugar levels may influence the host-pathogen interaction or even viability of bacterium; L-rhamnose is found as a common component of bacterial cell wall, and deletion of genes in L-rhamnose pathway in *vibrio cholerae* have a severe colonization defect.[9] In the uropathogenic strain *Escherichia coli* 075:K5, lack of a functional enzyme dTDP-6-deoxy-L-lyxo-4-hexulose-4-reductase (RmlD) leads to loss of serum resistance.[10] Hence, the biosynthetic pathways of bacterial nucleotide sugars could be considered as potential targets for novel antimicrobial agents; 1-deoxy-D-fructose and its analogues proven to act as potential metabolic inhibitor and antimetabolites.[11]

Both chemical and biotechnological syntheses of rare deoxy sugars should allow studies to clarify their roles; such studies are hampered by the lack of availability of deoxy sugars, which occur in very small amounts, and

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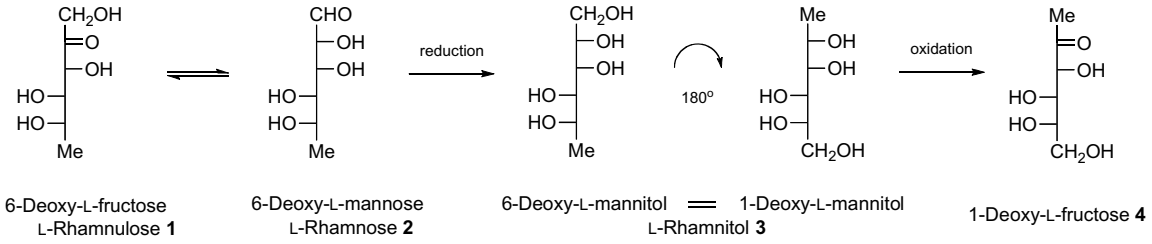


Scheme 1.

the difficulty of their isolation from natural resources. In this context, the use of microorganisms and biocatalysts for the production of these intermediates is becoming increasingly common, because of their ability to catalyze reactions with high stereo, regio, and chemo-selectivity. Biotransformation steps can either complement chemical steps or exist as alternative synthetic routes for a given compound, often with the advantage of reducing the number of reaction steps required.[12] In this study, a novel route for the synthesis of 1-deoxy ketohexose from a 6-deoxyaldose depends on the selective oxidation of a polyol by a newly isolated microbe. The use of microbial cells as biocatalysts is particularly advantageous, since they contain multiple dehydrogenases that are able to accept a broad spectrum of rare polyols to catalyze desired oxidation reactions.

2. Synthesis of 1-deoxy-L-fructose 4 from 6-deoxy-L-mannose (rhamnose) 1

L-Rhamnose **2** in water was reduced to L-rhamnitol **3** by hydrogenation at 1.2 MPa using a nickel catalyst at 50 °C yielded 100% L-rhamnitol when 10% w/v L-rhamnose was used as a substrate. Furthermore, the reduction proceeded under mild conditions at low temperature and with water as solvent; the catalyst could be used for several hydrogenations without loss of activity. Although the synthesis of polyols by hydrogenation processes is well established for common aldoses such as glucose, very few studies on the catalytic reduction of deoxy sugars such as L-rhamnose have been reported (Scheme 2).

Oxidation of L-rhamnitol **3** to 1-deoxy-L-fructose **4** required selective oxidation of the secondary alcohol adjacent to the methyl group. The newly isolated microbe, *E. aerogenes* IK7 was found as the most efficient for the biotransformation; cell production was optimized. Maximum cell growth and activity was observed when the microbe was grown on tryptic soy broth medium, supplemented with D-mannitol as the sole carbon source. After 36 h, well-grown cells were harvested by centrifugation at 12,000*g* for 10 min, washed twice with distilled water and resuspended in 50 mM Tris–HCl buffer (pH 9.0). The resultant cell suspension was used for 1-deoxy-L-fructose production in the bioconversion phase.

The effect of pH on the biotransformation in a number of buffers was studied. The optimum pH at 9.0 was established for 1-deoxy-L-mannitol **3** dehydrogenation in Tris–HCl buffer (Fig. 1). Oxidation of polyols by different microorganisms have been reported at alkaline pH.[13]

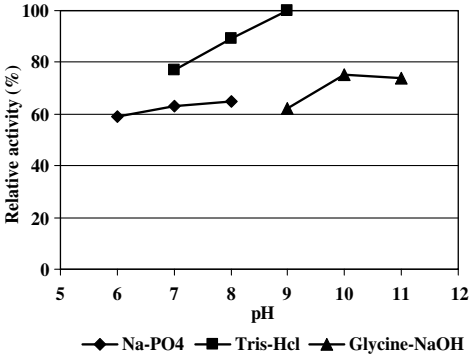
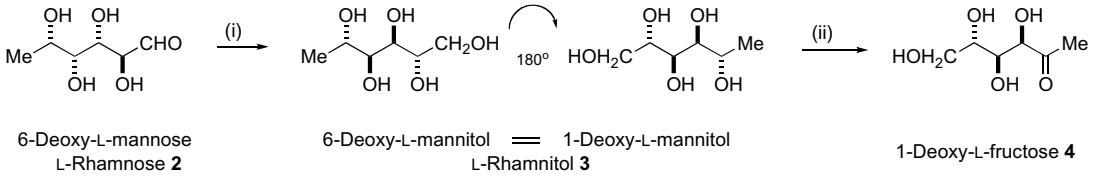


Figure 1. Effect of pH on oxidation.

The variation of oxidation efficiency with initial substrate concentrations (Fig. 2) shows that 5% w/v of rhamnitol **3** afforded 91% conversion to 1-deoxy-fructose **4** in 12 h, whereas at 10% concentration of **3** a yield of 74% of **4** was obtained. Interestingly, high substrate concentration did not show any significant inhibitory effects. Negligible amounts of by-products were observed, including trace



Scheme 2. Reagents and conditions: (i) Nickel, H_2, H_2O, 1.2 MPa pressure, 50 °C, 100%. (ii) *Enterobacter aerogenes* IK7, 74–91% (the large scale given in the experimental is on 74%).

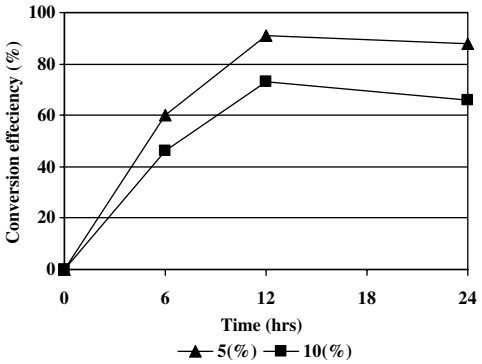


Figure 2. Effect of substrate concentration.

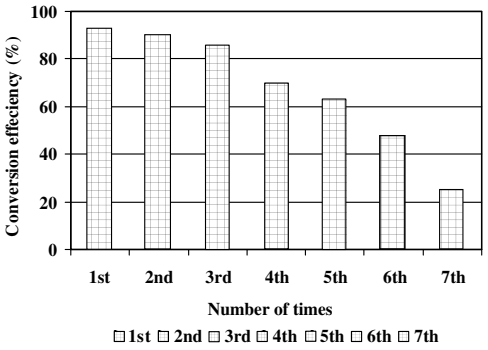


Figure 3. Effect of cell recycling on oxidation.

quantities of 6-deoxy-L-fructose (L-rhamnulose) **1**. Since microbial cells contain multiple enzymes, the formation of **1** may have involved a different dehydrogenase. 1-Deoxy-D-fructose has previously been synthesized by chemical[14,15] and biological method;[16] no previous report on 1-deoxy-L-fructose **1** production has appeared.

The cells were reused several times for the production 1-deoxy-L-fructose (Fig. 3). The conversion rate was very similar in the first three runs; the subsequent decline in activity might be due to loss of cell viability. In the biotransformation, the reduced co-factor is reoxidized and reutilized for oxidation of the substrate. It has been long established that the whole cells contain all the enzymes for regeneration of co-factors and are well protected within their natural cellular environment.[17,18] The crystalline 1-deoxy-L-fructose **4** was analyzed by HPLC (Fig. 4); the retention time of product **4** was indistinguishable from that of its enantiomer 1-deoxy-D-fructose.[20]

Although 1-deoxy-fructose crystallizes in the β-pyranose form,[19] in aqueous solution it exists a mixture of open-chain, furanose and pyranose forms; the ^{13}C NMR spectrum of the isolated ketose **4** is compared (Fig. 5) with that of its enantiomer 1-deoxy-D-fructose, which was prepared by chemical synthesis.[20]

3. Conclusion

In summary, 50 g of L-rhamnose **2** gave 37 g of crystalline 1-deoxy-L-fructose **4** (74% overall yield). Since the strain IK7 requires an inexpensive source of growth carbon D-mannitol, the procedure can be scaled up to provide significant quantities for full evaluation of the deoxysugar, which would be available at sufficiently low cost to stimulate its use in wide variety of fields. To the best of our knowledge, this is the first report on bioproduction of 1-deoxy-L-fructose **4** from 6-deoxy-L-mannitol **3** by *E. aerogenes*. The paper establishes the use of combined chemical and biotechnological pathways for the production of novel deoxy monosaccharides.

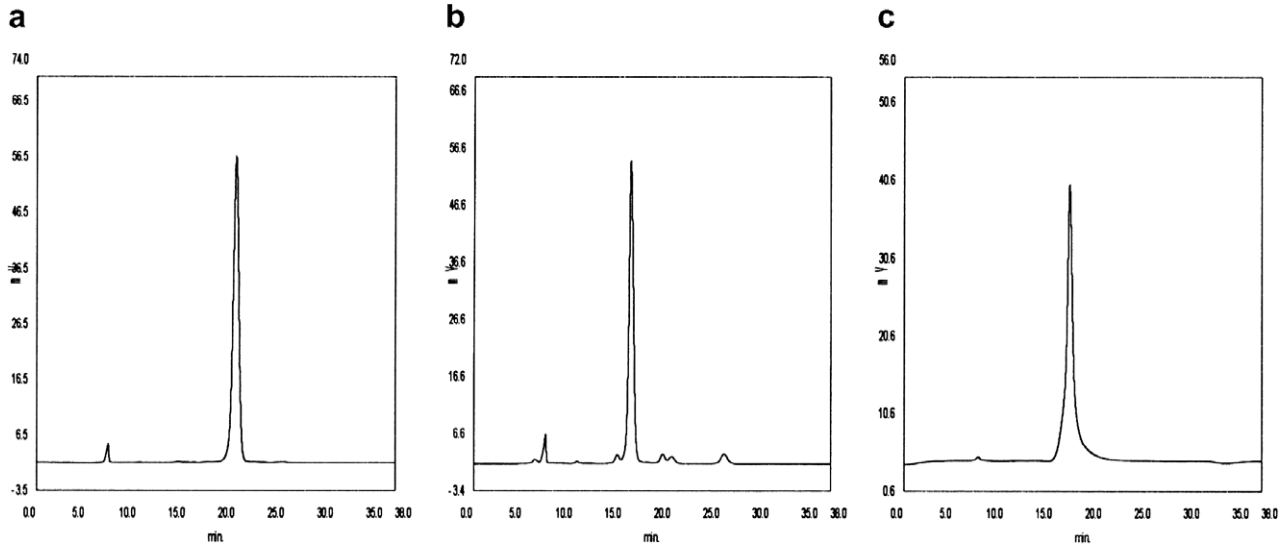


Figure 4. HPLC profiles of 6-deoxy-L-mannitol **3** transformation to 1-deoxy-L-fructose by *Enterobacter aerogenes* IK7. (a) 0 h reaction; (b) 12 h reaction; (c) separated product.

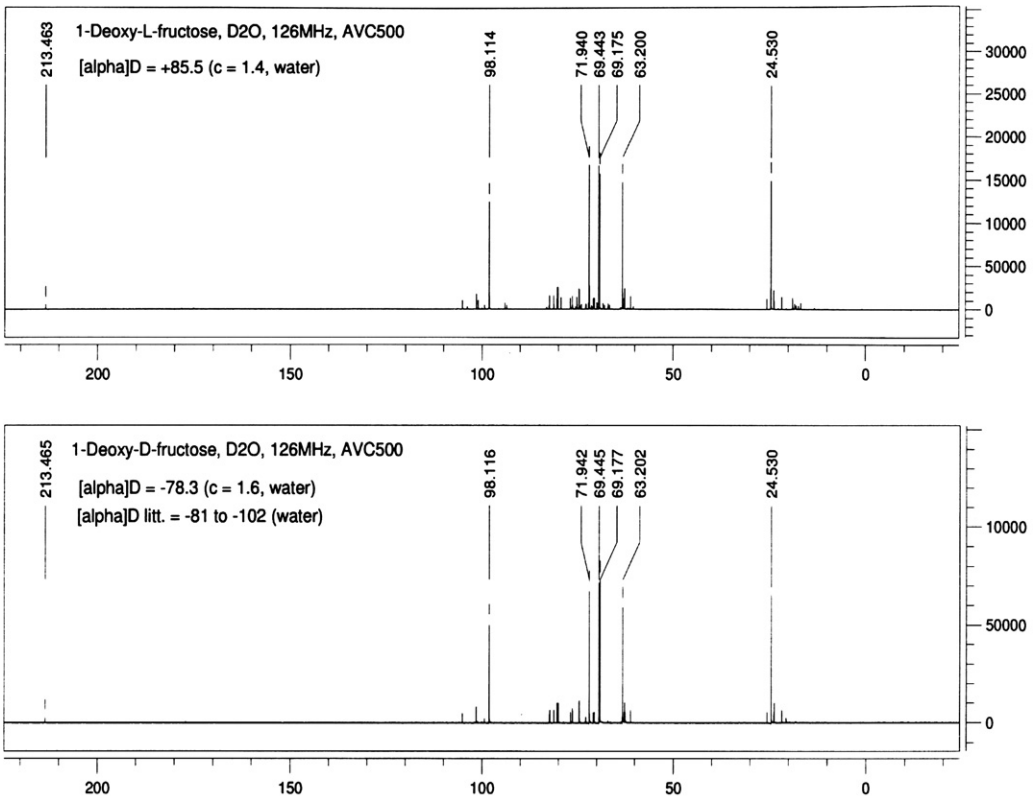


Figure 5. ^{13}C NMR spectrum of 1-deoxy-L-fructose **4** (upper); authentic 1-deoxy-D-fructose (lower).

4. Experimental

4.1. General experimental

L-Rhamnose and all other biochemicals were purchased from Sigma Chemical Co. (MO, USA) and Wako Pure Chemicals (Osaka, Japan), all are certified as reagent grade (RG). 50 mM Tris–HCl buffer, used for L-rhamnitol oxidation, was prepared from tris(hydroxymethyl)aminoethane $H_2NC(CH_2OH)_3$ by adjusting the pH to 9.0 with 1.0 N HCl. L-Rhamnose hydrogenation was performed in TEM-1000M hydrogenation apparatus (Taiatsu Techno Co. Ltd, Japan). Microbe growth and oxidation reactions were carried in bioreactors (TS-M-15L fermentor and TS-M-5L fermentor) from TAKASUGI SEISAKUSHO Co. Ltd. Polyol oxidation and ketose accumulation in the reaction mixture was determined by Nelson–Somogyi method[21] with the help of UV–visible spectrophotometer (UV-1700 pharmaspec, Shimadzu, Kyoto). ^{13}C NMR spectra (Burker AMX 500, 126 MHz) were recorded in D_2O using acetone as internal standard. Optical rotations were recorded on a Jasco R1030 polarimeter, Na^+ lamp, (Jasco, Tokyo, Japan) at 20 °C in deionized H_2O polarimeter with a path length of 1 dm. Concentrations are quoted in g 100 mL^{-1}. The product was analyzed by high-performance liquid chromatography (Hitachi GL-611 column, Tokyo, Japan and Shimadzu RID-6A refractive index detector, Kyoto, Japan) at 60 °C, eluted with 10^{-4} M NaOH at a flow rate of 1.0 mL/min. Melting points of L-rhamnitol and 1-deoxy-L-fructose were measured by differential scanning calorimetry (DSC 8240D, Tokyo, Japan.)

4.2. L-Rhamnitol 3

4.2.1. Preparation of catalyst. Raney nickel (30 g) was dissolved in 300 ml of 20% aqueous sodium hydroxide (300 mL). The reaction mixture was boiled at 80 °C for 6 h and the catalyst was washed several times with water to obtain a pH 9.2. The resultant precipitate was used for hydrogenation.

4.2.2. Hydrogenation reaction. The prepared catalyst was added to a solution of rhamnose **2** (50 g) in water (300 mL); the reaction was made up to a total volume of 500 mL, which was introduced into the reactor at a pressure of 1.2 MPa hydrogen. The reactor was then heated to a temperature of 50 °C for 8 h. The liquid product from the reaction mixture was analyzed by HPLC to afford rhamnitol **3** (50 g, 100 yield) mp 117.3 °C, $[\alpha]_D^{20} = +1.4$ (*c* 1.4, H_2O).

4.3. 1-Deoxy-L-fructose 4

4.3.1. Optimization of conditions. *E. aerogenes* IK7, an isolate of our laboratory, was used for biotransformation of 1-deoxy-L-mannitol to 1-deoxy-L-fructose. The strain was maintained as frozen stock cultures containing 50% (v/v) glycerol at −80 °C for long-term storage and at 4 °C for routine use. The strain was routinely grown on 1% D-mannitol supplemented with 2% TSB medium at

37 °C in Erlenmeyer flasks (120 rpm) for 36 h. Batch fermentations (10 L medium for biomass growth) were performed in a 15 L bioreactor at 37 °C, with agitation rate of 180 rpm and an airflow rate of 1.0/min.

The effect of pH on 1-deoxy-L-fructose production was studied using different buffers (50 mM sodium–phosphate pH 6.0–8.0, 50 mM Tris–HCl pH 7.0–9.0 and 50 mM glycine–NaOH pH 9.0–11.0). Transformation reactions were carried out at 37 °C with shaking at 120 rpm in 30 mL L-shaped tubes, which contains the following reaction mixture: 50 mg (1%) of substrate and 5.0 mL of a cell suspension prepared in 50 mM of different buffers, which had cell density of 20 (A_{600}). Samples were taken at different intervals and relative activity was determined by analytical methods.

After determining the optimal reaction conditions, the conversion rates for various initial concentrations of 6-deoxy-L-mannitol were measured. Transformation reaction was carried out at 37 °C with shaking at 120 rpm in 30 mL L-shaped tubes containing a reaction mixture of the following composition: 250–500 mg (5–10%) of substrate and 5.0 mL of a cell suspension prepared in the same buffer, which had cell density of 50 (A_{600}). Samples were taken at different intervals to determine the conversion rate.

Once, the transformation reaction reached the equilibrium, the reaction is terminated by centrifugation at 12,000*g* for 10 min. The supernatant obtained in this step was allowed to downstream process, the retained cell were washed twice in distilled water and resuspended in 50 mM Tris–HCl buffer (pH 9.0) and reused for further oxidation process. The reaction conditions were same as bioreactor transformation process.

4.3.2. Oxidation of rhamnitol 3 in a bioreactor. The cells were cultivated, harvested, and washed as described above. The transformation reaction was carried out in a 5.0 L bioreactor at 37 °C with an agitation rate of 120 rpm and airflow rate of 0.5 L/min for 12 h. The composition of the reaction mixture was as follows: 50 g (5%) 1-deoxy-L-mannitol **3** (50 g, 5% w/v) with washed cells with a cell density of 50 (A_{660}) were suspended in Tris–HCl buffer (1000 mL, 50 mM, pH 9.0).

After complete consumption of 1-deoxy-L-mannitol **3**, the reaction was terminated by centrifugation at 12,000*g* for 10 min for cell removal. The supernatant obtained in this step was decolorized by treating with activated charcoal and the resultant was filtered to remove the treated charcoal. The filtrate was deionized with a mixture of Diaion SK1B (H^+ form; Mitsubishi Chemical, Tokyo) and Amberlite IRA-411 (CO_3^{2-} form; Muromachi Technos, Tokyo) (IRA-411, CO_3^{2-}) ion-exchange resins. The deionized content was then evaporated and concentrated under vacuum at 40 °C. After concentration, the reaction mixture was separated by one-pass separation system, which consists of eight columns connected with two pumps, each column was filled with 2.5 L of UKB-555 ion-exchange resin (Ca^{2+} form; Mitsubishi Chemical, Tokyo). Separated fractions were concentrated up to 50% and the concentrate was kept in a desiccator for crystallization to give 1-deoxy-L-fructose **4**, (37 g, 74% overall yield) mp 72.2 °C, $[\alpha]_D^{20} = +85.5$ (*c* 1.4, H_2O) {lit.[20] for the enantiomer 1-deoxy-D-fructose $[\alpha]_D^{21} = -80.5$ (*c* 1.0, H_2O)}.

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