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Efficient enzymatic synthesis of L-rhamnulose and L-fuculose

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ABSTRACT

L-Rhamnulose (6-deoxy-L-*arabino*-2-hexulose) and L-fuculose (6-deoxy-L-*lyxo*-2-hexulose) were prepared from L-rhamnose and L-fucose by a two-step strategy. In the first reaction step, isomerization of L-rhamnose to L-rhamnulose, or L-fucose to L-fuculose was combined with a targeted phosphorylation reaction catalyzed by L-rhamnulose kinase (RhaB). The by-products (ATP and ADP) were selectively removed by silver nitrate precipitation method. In the second step, the phosphate group was hydrolyzed to produce L-rhamnulose or L-fuculose with purity exceeding 99% in more than 80% yield (gram scale).

Among 36 hexoses and pentoses (12 ketoses and 24 corresponding aldoses), only seven monosaccharides are naturally present in substantial quantities (D-xylose, D-ribose, L-arabinose, D-galactose, D-glucose, D-mannose, and D-fructose).[1] With the exception of D-fructose, all other ketoses are defined as 'rare sugars'.[2] Despite their limited accessibility, rare ketoses offer a lot of potential for applications in pharmaceutical, medicinal, food, and synthetic chemistry.[3] For example, D-psicose has about 70% of the sweetness but only 0.3% of the energy calories of sucrose.[4] It can also inhibit hepatic lipogenous enzyme activity, helping reduce abdominal fat accumulation.[5] L-xylulose was reported to be an inhibitor of glycosidase[6], while also serving as an indicator for acute or chronic hepatitis and liver cirrhosis.[7] The investigations of the new properties of rare ketoses and their applications have drawn much attention recently.[8]

L-Rhamnulose and L-fuculose are two crucial rare deoxy ketoses that offer many potential applications. For example, L-rhamnulose is a precursor of furaneol that has been used in the flavor industry for its sweet strawberry aroma.[9] In addition, L-rhamnulose and L-fuculose play important roles in sugar metabolism.[10] In bacteria, L-rhamnose and L-fucose must be converted to their ketose 1-phosphate forms, which are later split into dihydroxyacetone phosphate and L-lactaldehyde by L-rhamnulose 1-phosphate aldolase (RhaD) or L-fuculose 1-phosphate aldolase (FucA) to facilitate further metabolic function.[11] RhaD and FucA are two powerful biocatalysts and have been widely used in synthetic chemistry to produce rare ketoses or their derivatives.[12] Moreover, L-rhamnulose and L-fuculose can also be directly isomerized or epimerized into other rare sugars.[13] Therefore, L-rhamnulose and L-fuculose are not only primary targets for investigating the mechanistic and regulatory aspects of sugar metabolism, but also important starting materials in synthetic chemistry. An efficient system to readily provide both ketoses in considerable amounts is highly attractive in enabling the studies of both deoxy ketoses.

There are two methods that could be used to produce L-rhamnulose and L-fuculose. The most common method is isomerizing L-rhamnose to L-rhamnulose or L-fucose to L-fuculose.[13,14] However, aldose–ketose isomerization mediated by either chemical or enzymatic method (isomerase) is reversible, with reaction equilibrium being very unfavorable for ketose formation.[15] For example, only 11% of L-fucose can be isomerized to L-fuculose by L-fucose isomerase (FucI) in the final reaction equilibrium.[14] Moreover, an extensive isomer separation step is still necessary to obtain a ketose in pure form. Ion-exchange chromatography (Ca^{2+} form) is the main method for sugar isomer separation.[16] Nevertheless, it was reported that L-rhamnulose is hard to be separated from L-rhamnose using ion-exchange chromatography (Ca^{2+} form) column.[13b] They even can't be separated well by HPLC. Commercially available product only has 80% purity (Sigma–Aldrich). Selective degradation of unwanted isomer by bacteria to isolate the desired ketose has also been explored[17],

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but this method is time-consuming and suffer from low efficiency. The addition of borate into reaction system has been suggested to improve aldose–ketose isomerization because borate can bind ketose stronger than aldose.[18] Such strategy has been applied on the conversion of L-fucose to L-fuculose, in which a 85% conversion ratio was observed.[14] However, the purification steps require the separation of ketose-borate complex and the splitting of the desired ketoses from ketose-borate complex.[19] These tedious manipulations place a limit on the applications of this strategy. The second method for the production of L-rhamnulose and L-fuculose is based on aldol condensation reaction.[20] In this strategy, RhaD or FucA were used to produce L-rhamnulose 1-phosphate or L-fuculose 1-phosphate from dihydroxyacetone phosphate (DHAP) and L-lactaldehyde, and then the phosphate group was hydrolyzed to afford L-rhamnulose or L-fuculose. However, DHAP and L-glyceraldehyde are costly and unstable, reducing the synthetic practicality. Although DL-glycerol 3-phosphate, an inexpensive starting material, has been used to produce DHAP in a one-pot reaction fashion[21], ketose production mediated by aldolase still suffers from low yields and tedious purification manipulations.[20b] Therefore, while L-rhamnulose and L-fuculose are commercially available, they are cost prohibitive (L-rhamnulose, $178/10 mg, Sigma–Aldrich; L-fuculose, $199/10 mg, Carbosynth). The study of L-rhamnulose and L-fuculose has been hindered due to their limit availability. Herein an enzymatic method for the efficient and convenient preparation of rare ketoses L-rhamnulose and L-fuculose from readily available aldoses is reported.

Recently, we developed a convenient, efficient and cost-effective platform for the facile synthesis of ketoses, by which 10 non-readily available ketopentoses (L-ribulose, D-xylulose, D-ribulose, and L-xylulose) and ketohexoses (D-tagatose, D-sorbose, D-psicose, L-tagatose, L-fructose and L-psicose) were prepared from common and inexpensive starting materials with both high yield and purity without having to undergo a tedious isomer separation step.[22] The basic concept of this strategy is based on 'phosphorylation→dephosphorylation' cascade reaction. In this work, this strategy was applied to produce L-rhamnulose from L-rhamnose and L-fuculose from L-fucose, respectively. Thermodynamically unfavorable aldose–ketose conversions were combined with phosphorylation reactions by substrate-specific kinases to increase conversion ratio in step 1. Sugar phosphates were purified by silver nitrate precipitation. The phosphate group was hydrolyzed to produce ketoses in step 2 (Scheme 1).

To apply the described scheme on L-rhamnulose and L-fuculose production in this work, the prerequisite is the availability of a kinase that specifically recognizes L-rhamnulose and L-fuculose but not L-rhamnose or L-fucose. Otherwise, the products obtained finally will be a mixture containing both aldose and ketose. L-rhamnulose kinase is the enzyme that prefers ketoses with (3*R*)-configuration.[23] Recently, we identified an L-rhamnulose kinase from *Thermotoga maritima* MSB8, which show high substrate specificity towards (3*R*)-ketoses as compared to (3*S*)-ketoses or (3*S*)-aldoses.[22] In this work, the substrate specificity of RhaB towards several deoxy sugars was studied (see Supplementary data). RhaB failed to recognize L-rhamnose or L-fucose but had high activity towards L-rhamnulose and L-fuculose (Table 1), indicating its potential for one-pot multienzyme (OPME) reactions[24] to produce L-rhamnulose and L-fuculose.

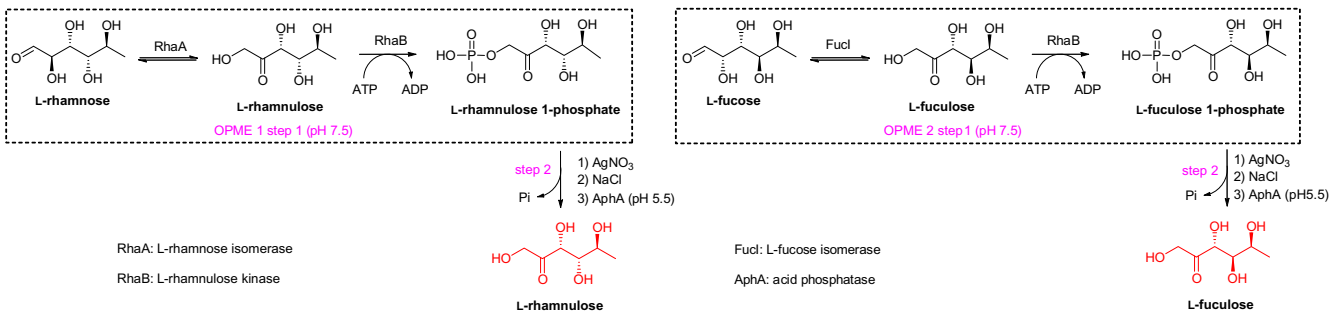
Table 1
Substrate specificity of RhaB towards several deoxy sugars

Substrate	RhaB activity (%)
L-Rhamnulose	100
L-Fuculose	81.3
L-Rhamnose	ND
L-Fucose	ND

ND: no detectable activity was observed.

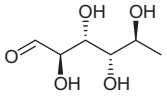
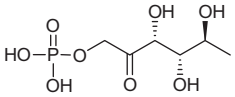
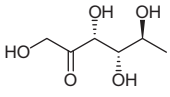
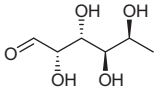
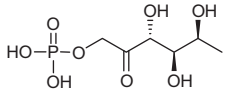
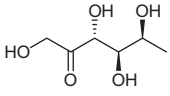
Having met the prerequisite, other conversion-related enzymes including L-rhamnose isomerase (RhaA)[25], L-fucose isomerase (FucI)[26], and acid phosphatase (AphA)[27] from *Escherichia coli* were prepared as described in Supplementary data. To test the potential of RhaB in OPME reactions, analytical scale reactions (Table 2) were performed in one-pot (164 μg scale), and tested by TLC. Reactions without isomerases were done as negative controls. Once the formation of sugar phosphates was observed on TLC while no reactions were observed on the control reactions, preparative reactions (gram scale) were performed.

In the first reaction step, L-rhamnose was incubated with RhaA and RhaB in one-pot in the presence of ATP as the phosphate donor (OPME 1, Scheme 1). No buffer was used in consideration to purification. The reaction pH was held near 7.5, where all enzymes are quite active, using sodium hydroxide as the reaction occurred. In this one-pot two-enzyme system, RhaA isomerized L-rhamnose to L-rhamnulose, which was immediately phosphorylated by RhaB. It seems that L-rhamnulose was taken out of the reaction balance, and thus the reaction was driven towards the formation of L-rhamnulose in its ketose 1-phosphate form (L-rhamnulose 1-phosphate). The reaction was monitored by TLC and HPLC equipped with ELSD detector (HPX-87H column). Once the reaction finished (conversion ratio exceeding 99%), silver nitrate precipitation was used to precipitate ATP and ADP.[22] In detail, silver nitrate was added into reaction system until no new precipitate formed, and the precipitate was removed by centrifugation. Sodium chloride was added to precipitate the remaining silver ions, and the precipitate was removed by centrifugation. After desalting by using P-2 column, L-rhamnulose 1-phosphate was isolated in 91% yield. The product was analyzed by NMR and MS (see Supplementary data). The NMR spectra and MS data are well in accord with previously reported data[20b,28], confirming the isolated product is L-rhamnulose 1-phosphate. In the second



Scheme 1. Two-step strategy for the enzymatic synthesis of L-rhamnulose and L-fuculose from L-rhamnose and L-fucose.

Table 2
Enzymatic synthesis of L-rhamnulose and L-fuculose from L-rhamnose or L-fucose using the strategy shown in Scheme 1

Entry	Starting material	Enzymes	Intermediate	Step 1 yield (%)	Product	Total yield (%)	Scale (mg)	Purity[a] (%)
1	 L-rhamnose	RhaA RhaB AphA	 L-rhamnulose 1-phosphate	91	 L-rhamnulose	82	1080	>99
2	 L-fucose	FucI RhaB AphA	 L-fuculose 1-phosphate	93	 L-fuculose	84	1099	>99

[a] Defined as the percentage of desired ketose out of the sum of starting aldose and product.

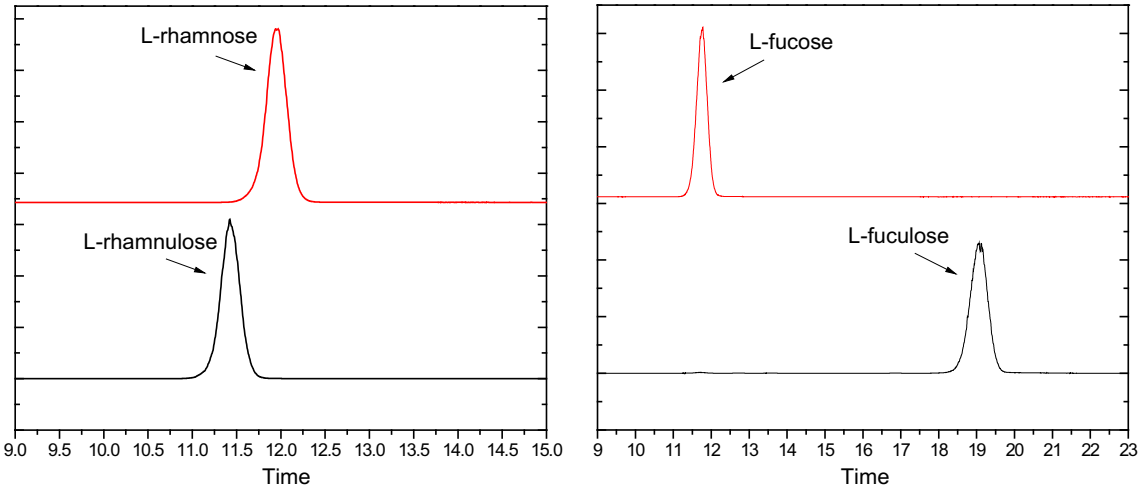


Figure 1. HPLC profiles of L-rhamnulose (HPX-87H column) and L-fuculose (Sugar-Pak 1 column) compared with starting aldoses.

reaction step, the phosphate group of L-rhamnulose 1-phosphate was hydrolyzed by AphA in pH 5.5 to produce L-rhamnulose. Once phosphate sugar was no longer observed on TLC, the solution was concentrated and purified by P-2 column to afford final product in 82% yield with regard to L-rhamnose. The product was analyzed by HPLC, NMR and MS (See Supplementary data). No obvious peak of L-rhamnose can be found by HPLC (Fig. 1) and no characteristic peak of aldose can be found in NMR spectra (See Supplementary data) indicating product purity exceeding 99%.

In our previously reported strategy, phosphorylation and dephosphorylation reactions were carried in a one-pot fashion. Although a total isolated yield exceeding 90% was reached, the hydrolyzing reaction required a long reaction time and a large amount of acid phosphatase due to unfavorable reaction conditions. In this work, the intermediate of phosphate sugar was purified by P-2 column to remove most of impurities (mainly salt) which affect the activity of acid phosphatase. Although a slightly lower isolated yield was obtained, this two-step strategy reduced reaction time and usage of acid phosphatase.

Similarly, preparative scale synthesis of L-fuculose from L-fucose was also carried in gram scale. In the first reaction step, L-fucose was incubated with FucI and RhaB in the presence of ATP as phosphate donor (OPME 2, Scheme 1). Conversion ratio exceeding 99% can be reached. Following the same manipulation as described above, L-fuculose 1-phosphate was isolated in 93% yield. NMR and MS data are well in accord with the previously reported data,[20b,28] confirming the isolated product is L-fuculose 1-phosphate (see Supplementary data). After hydrolyzing the phosphate group of L-fuculose 1-phosphate by AphA in step 2, L-fuculose was obtained in 84% yield with regard to L-fucose. The product was analyzed by HPLC, NMR and MS (see Supplementary data). HPLC and NMR analysis indicates a product purity exceeding 99%.

In summary, based on the 'phosphorylation→de-phosphorylation' cascade reaction and silver nitrate precipitation purification method, L-rhamnulose and L-fuculose were efficiently and conveniently prepared from readily available starting aldoses (L-rhamnose and L-fucose) in more than 80% yield in gram scale. Using a single desalting column (P-2 column), product with high purity was obtained. ATP is also commercially inexpensive owing to increased industrial production over the past decade and an ATP-regeneration system has also been suggested[29], thus making the preparation process described herein of particular interest for large-scale production. We anticipate that this work will accelerate the progress in understanding the biological roles and synthetic applications of L-rhamnulose and L-fuculose.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.12.051.

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