Monosaccharides

Deutsche Ausgabe: DOI: 10.1002/ange.201505714
Internationale Ausgabe: DOI: 10.1002/anie.201505714

Facile Enzymatic Synthesis of Ketoses

*Liuqing Wen, Kenneth Huang, Mohui Wei, Jeffrey Meisner, Yunpeng Liu, Kristina Garner, Lanlan Zang, Xuan Wang, Xu Li, Junqiang Fang, Houcheng Zhang, and Peng George Wang**

Abstract: *Studies of rare ketoses have been hampered by a lack of efficient preparation methods. A convenient, efficient, and cost-effective platform for the facile synthesis of ketoses is described. This method enables the preparation of difficult-to-access ketopentoses and ketohexoses from common and inexpensive starting materials with high yield and purity and without the need for a tedious isomer separation step.*

The only seven monosaccharides that occur naturally in substantial quantities are D-xylose, D-ribose, L-arabinose, D-galactose, D-glucose, D-mannose, and D-fructose.[1] Of all possible structures, the twelve ketopentoses and ketohexoses[2] can be divided into two types based on their C-3 configuration: (3*S*)-ketoses and (3*R*)-ketoses (Figure 1). With the exception of D-fructose, all of these ketoses are defined as "rare sugars".[2] Despite their lower accessibility, rare ketoses offer enormous potential for applications in food, pharmaceutical, medicinal, and synthetic chemistry.[3]

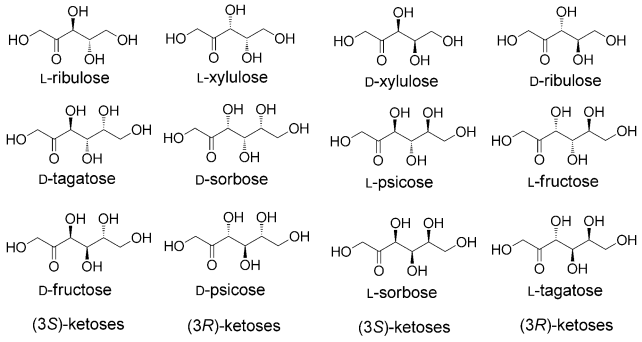


Figure 1. Twelve naturally occurring ketopentoses and ketohexoses including six (3*S*)-ketoses and six corresponding (3*R*)-ketoses.

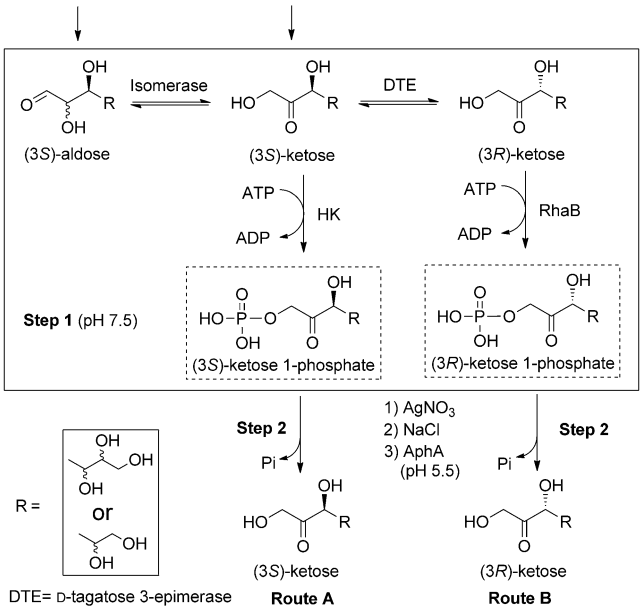
[*] L. Wen, K. Huang, M. Wei, Dr. J. Meisner, Dr. Y. Liu, K. Garner, L. Zang, X. Wang, X. Li, Prof. Dr. P. G. Wang
Department of Chemistry and
Center for Therapeutics and Diagnostics
Georgia State University, Atlanta, GA 30303 (USA)
E-mail: pwang11@gsu.edu
Dr. J. Fang, Dr. H. Zhang, Prof. Dr. P. G. Wang
National Glycoengineering Research Center, Shandong University
Jinan 250100 (China)
Dr. J. Meisner
Current Address: Division of Pulmonary, Allergy and Immunology, Cystic Fibrosis, and Sleep, Department of Pediatrics
Emory University School of Medicine
Atlanta, GA 30322 (USA)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201505714.

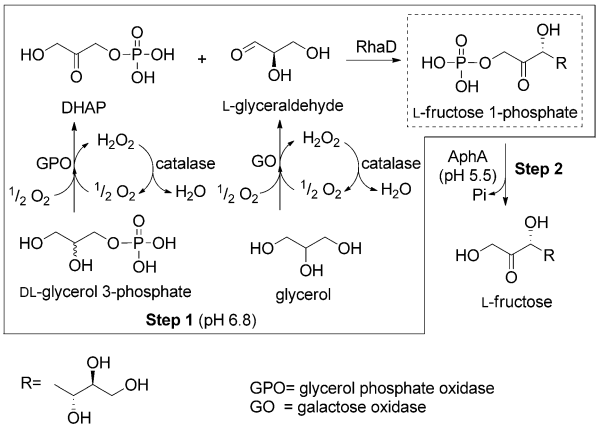
Aldose–ketose isomerization is the most important method for ketose preparation,[4] even though the reaction equilibrium is very unfavorable for ketose formation.[5] Significant progress has been achieved by using both chemical and enzymatic isomerization schemes over the last two decades.[6] Approaches to improve aldose–ketose conversion, including the addition of borate to break the aldose–ketose reaction equilibrium,[7] directed evolution,[8] and the discovery of novel enzymes in nature,[9] have also been suggested. Nevertheless, these methods require a complicated isomer separation step to obtain the ketose in pure form. Furthermore, only four rare ketoses (D-xylulose, D-ribulose, L-ribulose, and D-tagatose) can be directly isomerized from the most common aldoses, and the synthesis of other rare ketoses has thus been more challenging. Chemical methods for the synthesis of these rare ketoses require tedious protection/deprotection manipulations.[10] Alternatively, enzymatic preparation by epimerizing ketoses at the C-3 position,[11] oxidizing polyols,[12] or relying on aldol condensation[13] proceeds regio- and stereoselectively without protection. However, these processes suffer from low yields, expensive starting materials, or complicated isomer separation. Therefore, most of the rare ketoses are still not readily available, which in turn has hindered studies of this fundamental class of carbohydrates.

Herein, a convenient, efficient, and cost-effective platform for ketose syntheses is described. All of the rare ketopentoses and ketohexoses were produced by utilizing this platform, with the exception of L-sorbose. L-sorbose, a starting material for the production of vitamin C, has been produced in industrial settings and is consequently inexpensive.[14]

Our strategies are based on a "phosphorylation→dephosphorylation" cascade reaction. In the first reaction step (Scheme 1), we combined thermodynamically unfavorable bioconversions of common (3*S*)-sugars to the desired (3*S*)-ketoses (route A) or (3*R*)-ketoses (route B) with phosphorylation reactions by substrate-specific kinases (fructokinase (HK) from humans in route A and L-rhamnulose kinase (RhaB) from *Thermotoga maritima* MSB8 in route B). In the second reaction step, adenosine phosphates (ATP and ADP) were selectively removed by a convenient method called silver nitrate precipitation. Then, acid phosphatase (AphA) was added to hydrolyze the phosphate groups and produce the desired ketoses. Notably, both reaction steps can be performed in a one-pot fashion. The preparation of L-fructose from DL-glycerol 3-phosphate and glycerol by relying on aldol condensation using rhamnulose bisphosphate aldolase (RhaD) is illustrated in Scheme 2. L-Psicose was further prepared from L-fructose by using the targeted phosphorylation strategy shown in Scheme 3.



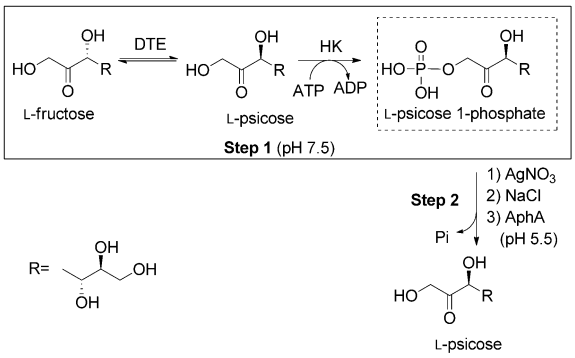
Scheme 1. One-pot two-step enzymatic synthesis of L-ribulose, D-xylulose, and D-tagatose (Route A), and L-xylulose, D-ribulose, D-sorbose, D-psicose, and L-tagatose (Route B).



Scheme 2. One-pot multienzyme reaction for the synthesis of L-fructose.

To achieve the designed outcomes, there are three challenges: 1) the identification of kinases that can phosphorylate the desired ketoses at the C-1 position but not starting sugars or intermediates, 2) the availability of a D-tagatose 3-epimerase (DTE) that specifically epimerizes ketoses at C-3 but not ketose 1-phosphates, and 3) an efficient method to separate sugar phosphates from the adenosine phosphates (ATP and ADP).

The first reaction step for the approaches shown in Schemes 1 and 3 was carried out under slightly basic conditions, in which all of the enzymes were quite active. Nevertheless, monosaccharides are unstable in alkaline media,[15] and the isomerases in the reaction system may also isomerize the intermediate ketose phosphates to a certain extent when the kinase that phosphorylates ketoses at the C-5

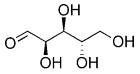
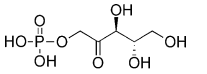
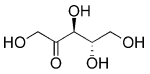
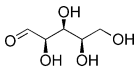
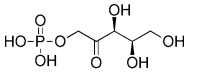
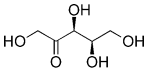
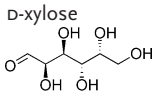
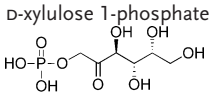
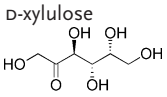
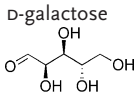
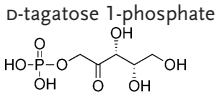
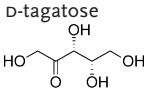
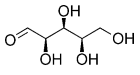
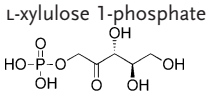
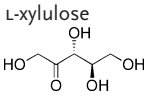
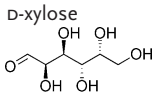
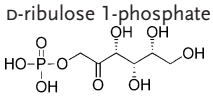
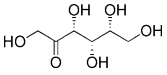
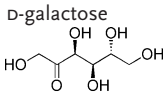
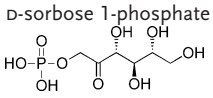
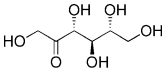
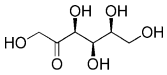
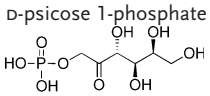
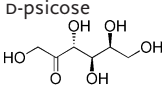
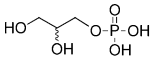
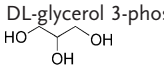
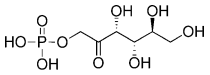
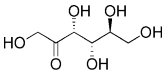
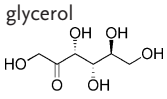
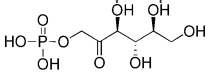
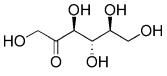


Scheme 3. One-pot multienzyme reaction for the synthesis of L-psicose.

(pentose) or C-6 (hexose) position is used. To avoid such unwanted side reactions, kinases that phosphorylate ketoses at the C-1 position were utilized.[16] In route A (Scheme 1), a kinase that can phosphorylate (3*S*)-ketoses (D-xylulose, L-ribulose, and D-tagatose) but not (3*S*)-aldoses (D-xylose, L-arabinose and D-galactose) is necessary. In route B, a kinase that specifically recognizes (3*R*)-ketoses but not (3*S*)-ketoses and (3*S*)-aldoses is required. By screening the substrate specificity of many kinases (data not shown), we discovered that fructokinase (HK) from humans[17] accords well with the requirements of route A, and L-rhamnulose kinase (RhaB) from *Thermotoga maritima* MSB8, a novel enzyme that absolutely requires ketoses with (3*R*)-configuration, accords well with the requirements of route B (see the Supporting Information). HK can also phosphorylate L-psicose efficiently, while no activity towards its corresponding (3*R*)-ketose (L-fructose) was detected, thus making it possible to establish the reaction for the preparation of L-psicose from L-fructose (Scheme 3). Another requirement for the establishment of the transformations shown in (Schemes 1, route B and Scheme 3) is that DTE, which catalyzes the interconversion of (3*S*)- and (3*R*)-ketoses,[11] does not epimerize ketose 1-phosphates, otherwise the products obtained will be a mixture containing both (3*S*)-ketoses and (3*R*)-ketoses. DTE does not recognize D-fructose 6-phosphate or D-ribulose 5-phosphate.[11] We therefore hypothesized that it would not recognize other ketose phosphates such as ketose 1-phosphates. This hypothesis was supported by purity analysis of the final products (Table 1).

In the second reaction step, acid phosphatase was used to hydrolyze the phosphate group of ketose 1-phosphates to produce ketoses under acidic conditions (pH 5.5) in which monosaccharides are stable. However, the presence of adenosine phosphates (ATP and ADP) inhibits the hydrolytic activity of the acid phosphatase.[18] In this work, a convenient method referred to as silver nitrate precipitation was used to selectively remove the adenosine phosphates. Silver phosphate is insoluble and silver ions can thus precipitate ADP and ATP.[19] Interestingly, we noticed that silver ions cannot precipitate monophosphate sugars when the sugars are composed of four or more carbons (Table S2 in the Supporting Information). It appears that binding of the sugar group to phosphate prevents silver phosphate precipitation. By apply-

Table 1: Synthesis of rare ketoses using the two-step strategy shown in Schemes 1 to 3.

Entry	Starting material	Enzymes	Scheme	Intermediate	Ketose	Yield [%][a]	Purity [%][b]
1	 L-arabinose	AraA HK	Route A Scheme 1	 L-ribulose 1-phosphate	 L-ribulose	93	>99
2	 D-xylose	XylA HK	Route A Scheme 1	 D-xylulose 1-phosphate	 D-xylulose	92	>99
3	 D-galactose	AraA HK	Route A Scheme 1	 D-tagatose 1-phosphate	 D-tagatose	92	>99
4	 L-arabinose	AraA DTE RhaB	Route B Scheme 1	 L-xylulose 1-phosphate	 L-xylulose	91	>99
5	 D-xylose	XylA DTE RhaB	Route B Scheme 1	 D-ribulose 1-phosphate	 D-ribulose	91	98.2
6	 D-galactose	AraA DTE RhaB	Route B Scheme 1	 D-sorbose 1-phosphate	 D-sorbose	94	>99
7	 D-fructose	DTE RhaB	Route B Scheme 1	 D-psicose 1-phosphate	 D-psicose	93	>99
8	 L-sorbose	DTE RhaB	Route B Scheme 1	 L-tagatose 1-phosphate	 L-tagatose	95	>99
9	 DL-glycerol 3-phosphate  glycerol	GPO GO catalase RhaD	Scheme 2	 L-fructose 1-phosphate	 L-fructose	70	99
10	 L-fructose	DTE HK	Scheme 3	 L-psicose 1-phosphate	 L-psicose	90	>99

[a] All products were prepared on a preparative (408 mg–628 mg) scale. [b] Defined as the percentage of desired ketose out of the sum of all possible sugars.

ing this selective precipitation ability, sugar phosphates can be easily and cleanly separated from adenosine phosphates (ATP and ADP). The method was tested in the synthesis of L-rhamnulose 1-phosphate from L-rhamnulose by using RhaB (see the Supporting Information). Once no L-rhamnulose could be detected in the reaction by HPLC, ATP and ADP were removed by adding a small excess of silver nitrate, which led to the removal of more than 99% of the ATP and ADP (Figure S2). Then, excess sodium chloride was added to remove the redundant silver ions. The total separation process can be completed in less than 15 min. After desalting by using Bio-Gel P-2 column, L-rhamnulose 1-phosphate sodium was isolated in 94% yield, which is comparable to the yield when using ion-exchange purification methods but it is more convenient. The precipitate can be redissolved in ammonium hydroxide, and the adenosine phosphates (ATP and ADP) or silver ions can be then recycled. Compared to barium precipitation, another sugar phosphate purification method in which sugar phosphate was isolated as a barium salt by using barium,[20] no additional steps to remove toxic ions and no accurate pH control are necessary. Moreover, silver is safer than barium and other metal ions that can be used to precipitate adenosine phosphates, such as mercury.[21] These advantages make this method highly attractive for use in rapidly purifying sugar phosphate.

Having overcome these problems, other conversion-related enzymes including D-xylose isomerase (XylA),[22] RhaD,[23] and AphA[18] from *Escherichia coli*, L-arabinose and D-galactose isomerase (AraA) from *Thermotoga maritima* MSB8,[24] and DTE from *Pseudomonas* Sp. St-24[11] were prepared as described in Supporting Information.

In route A (Scheme 1), L-arabinose, D-xylose, and D-galactose were incubated with their corresponding isomerases and HK to prepare L-ribulose, D-xylulose and D-tagatose,

respectively (Table 1, entries 1–3). The reactions were allowed to proceed until no detectable starting aldoses were found by HPLC (conversion ratios exceeding 99%), thus making isomer separation unnecessary. Silver nitrate precipitation was used to remove ATP and ADP. Proteins were also removed during this process (as confirmed by Bradford assay). Consequently, two steps of the method could be carried out in one pot. After the hydrolysis of phosphate groups in step 2, the solution from each reaction was desalted by using a Bio-Gel P-2 column. L-ribulose, D-xylulose, and D-tagatose were finally obtained in more than 90% yield (Table 1). The products were confirmed by NMR, HPLC and MS analysis (see the Supporting Information). HPLC and NMR analysis indicated product purity exceeding 99%.

In route B (Scheme 1), five (3*R*)-ketoses (L-xylulose, D-ribulose, D-sorbose, D-psicose, and L-tagatose) were prepared from five common (3S)-sugars (L-arabinose, D-xylose, D-galactose, D-fructose, and L-sorbose, respectively). The discovery of DTE made it possible to achieve the interconversion between (3*S*)-ketoses and (3*R*)-ketoses, which is especially important for the preparation of (3*R*)-ketoses.[11] Nevertheless, with the exceptions of two (3*S*)-ketoses (D-fructose and L-sorbose), ketoses are not readily available, and the conversions catalyzed by DTE are an equilibrium reaction. For example, the conversion ratio is only 20% for D-fructose to D-psicose and 27% for L-sorbose to L-tagatose.[25] The separation of (3*S*)-ketoses and (3*R*)-ketoses is difficult owing to their similar properties. In this work, we combined DTE-catalyzed epimerization with targeted phosphorylation of (3*R*)-ketose by RhaB (entries 7 and 8). To avoid using ketoses that are not readily available, we included enzymatic isomerization when starting with (3*S*)-aldose (entries 4–6). All reactions were allowed to proceed until no detectable starting sugars were found by HPLC (conversion ratios exceeding 99%). After the hydrolysis of phosphate groups in step 2, all five (3*R*)-ketoses were obtained in more than 90% yield (Table 1). Given the high substrate specificity of RhaB, the products L-xylulose, D-sorbose, D-psicose, and L-tagatose were obtained in more than 99% purity. D-ribulose was obtained in 98.2% purity while 0.6% of D-xylulose and 1.2% of D-xylose were observed because D-xylose and D-xylulose could be phosphorylated by RhaB to a certain extent (Table S1 in the Supporting Information).

L-fructose was synthesized by using RhaD because there is a lack of common corresponding sugars. RhaD exclusively produces L-fructose from dihydroxyacetone phosphate (DHAP) and L-glyceraldehyde.[23] However, DHAP and L-glyceraldehyde are costly and unstable. To increase the practicality of the process, two previously reported strategies[23,26] were combined to allow the use of the inexpensive materials glycerol and DL-glycerol 3-phosphate to produce L-fructose 1-phosphate in a one-pot reaction (Scheme 2) in which L-glyceraldehyde is produced from glycerol by galactose oxidase (GO) and DHAP is produced from DL-glycerol 3-phosphate by glycerol phosphate oxidase (GPO). After the hydrolysis of the phosphate group in step 2, L-fructose was finally obtained in 70% yield with a purity of 99%. L-psicose was then prepared from L-fructose by using DTE. However, the conversion of L-fructose into L-psicose by DTE is unfavorable for L-psicose formation, for which the conversion ratio is only 24%.[25] Applying the discovery that HK from humans could efficiently phosphorylate L-psicose but not L-fructose, L-psicose was prepared from L-fructose by using the described targeted phosphorylation strategy (Scheme 3) in 90% yield with a product purity exceeding 99%.

In summary, a novel method for the facile synthesis of ketoses was established. This method relies on substrate-specific kinases and the improved aldol condensation reaction, and makes it possible to use one-pot multienzyme (OPME)[27] reactions to prepare difficult-to-access ketopentoses and ketohexoses from common and inexpensive materials. The described two-step strategy not only provides unprecedentedly high yields but also avoids the need for a complicated isomer separation step. ATP is commercially cheap owing to increased industrial production over the past decade and an ATP-regeneration system has also been suggested,[28] thus making the transformation reaction described herein of particular interest for large-scale production.

This study represents a highly convenient and efficient strategy for ketose synthesis. We anticipate that this method will accelerate progress in understanding the biological roles and synthetic applications of rare ketoses, as well as advancing the synthesis of rare aldoses since the aldose–ketose isomerization reaction is very favorable for aldose formation. Future studies will enable the identification of new kinases for use in sugar syntheses, thereby providing a powerful set of tools for carbohydrate research.

Acknowledgements

Prof. Dr. P. Wang thanks National Institute of Health (R01GM085267), National Basic Research(973) Program of China (2012CB822102), and the Key Grant Project of Chinese Ministry of Education (313033) for financial support.

Keywords: enzymatic synthesis · ketoses · one-pot reactions · phosphorylation · synthetic methods

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 12654–12658
Angew. Chem. **2015**, *127*, 12845–12849

[1] K. Izumori, *J. Biotechnol.* **2005**, *118*, S89–S90.
[2] T. B. Granström, G. Takata, M. Tokuda, K. Izumori, *J. Biosci. Bioeng.* **2004**, *97*, 89–94.
[3] a) Z. Ahmed, *Electron. J. Biotechnol.* **2001**, *4*, 13–14; b) B. Li, S. Varanasi, P. Relue, *Green Chem.* **2013**, *15*, 2149–2157; c) Y. Ishida, K. Kakibuchi, R. Kudo, K. Izumori, S. Tajima, K. Akimitsu, K. Tanaka, *Acta Hortic.* **2012**, *927*, 929–934.
[4] Z. Li, Y. Gao, H. Nakanishi, X. Gao, L. Cai, *Beilstein J. Org. Chem.* **2013**, *9*, 2434–2445.
[5] D. Ekeberg, S. Morgenlie, Y. Stenstrom, *Carbohydr. Res.* **2007**, *342*, 1992–1997.
[6] a) Y. Román-Leshkov, M. Moliner, J. A. Labinger, M. E. Davis, *Angew. Chem. Int. Ed.* **2010**, *49*, 8954–8957; *Angew. Chem.* **2010**, *122*, 9138–9141; b) K. Beerens, T. Desmet, W. Soetaert, *J Ind Microbiol. Biotechnol.* **2012**, *39*, 823–834; c) D. Ekeberg, S. Morgenlie, Y. Stenstrom, *Carbohydr. Res.* **2005**, *340*, 373–377;

d) S. Saravanamurugan, M. Paniagua, J. A. Melero, A. Riisager, *J. Am. Chem. Soc.* **2013**, *135*, 5246 – 5249.
[7] J. F. Mendicino, *J. Am. Chem. Soc.* **1960**, *82*, 4975 – 4979.
[8] H. Leemhuis, R. M. Kelly, L. Dijkhuizen, *IUBMB Life* **2009**, *61*, 222 – 228.
[9] J. K. Vester, M. A. Glaring, P. Stougaard, *Microb. Cell Fact.* **2014**, *13*, 72.
[10] a) T. Mukaiyama, Y. Yuki, K. Suzuki, *Chem. Lett.* **1982**, 1169 – 1170; b) T. Matsumoto, T. Enomoto, T. Kurosaki, *J. Chem. Soc. Chem. Commun.* **1992**, 610 – 611.
[11] H. Itoh, H. Okaya, A. R. Khan, S. Tajima, S. Hayakawa, K. Izumori, *Biosci. Biotechnol. Biochem.* **1994**, *58*, 2168 – 2171.
[12] W. Poonperm, G. Takata, K. Morimoto, T. B. Granstrom, K. Izumori, *Enzyme Microb. Technol.* **2007**, *40*, 1206 – 1212.
[13] T. D. Machajewski, C. H. Wong, *Angew. Chem. Int. Ed.* **2000**, *39*, 1352 – 1375; *Angew. Chem.* **2000**, *112*, 1406 – 1430.
[14] R. Giridhar, A. K. Srivastava, *World J. Microbiol. Biotechnol.* **2001**, *17*, 185 – 189.
[15] J. C. Speck, Jr., *Adv. Carbohydr. Chem.* **1958**, *13*, 63 – 103.
[16] R. W. Nagorski, J. P. Richard, *J. Am. Chem. Soc.* **1996**, *118*, 7432 – 7433.
[17] a) A. Asipu, B. E. Hayward, J. O'Reilly, D. T. Bonthron, *Diabetes* **2003**, *52*, 2426 – 2432; b) R. Bais, H. M. James, A. M. Rofe, R. A. Conyers, *Biochem. J.* **1985**, *230*, 53 – 60.
[18] G. M. Rossolini, M. C. Thaller, R. Pezzi, G. Satta, *FEMS Microbiol. Lett.* **1994**, *118*, 167 – 173.
[19] G. M. Whitesides, P. E. Garrett, M. G. Siegel, Patents (US4164444 A), **1979**.
[20] C.-H. Wong, S. D. McCurry, G. M. Whitesides, *J. Am. Chem. Soc.* **1980**, *102*, 7938 – 7939.
[21] K. Tanaka, M. Honjo, Patents (US3079379 A), **1963**.
[22] A. S. Rozanov, S. N. Zagrebelny, A. B. Beklemishev, *Appl. Biochem. Microbiol.* **2009**, *45*, 31 – 37.
[23] D. Franke, T. Machajewski, C. C. Hsu, C. H. Wong, *J. Org. Chem.* **2003**, *68*, 6828 – 6831.
[24] D. W. Lee, H. J. Jang, E. A. Choe, B. C. Kim, S. J. Lee, S. B. Kim, Y. H. Hong, Y. R. Pyun, *Appl. Environ. Microbiol.* **2004**, *70*, 1397 – 1404.
[25] H. Itoh, K. Izumori, *J. Ferment. Bioeng.* **1996**, *81*, 351 – 353.
[26] Z. Li, L. Cai, Q. Qi, P. G. Wang, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 7081 – 7084.
[27] H. Yu, K. Lau, V. Thon, C. A. Autran, E. Jantscher-Krenn, M. Xue, Y. Li, G. Sugiarto, J. Qu, S. Mu, L. Ding, L. Bode, X. Chen, *Angew. Chem. Int. Ed.* **2014**, *53*, 6687 – 6691; *Angew. Chem.* **2014**, *126*, 6805 – 6809.
[28] Z. Liu, J. Zhang, X. Chen, P. G. Wang, *ChemBioChem* **2002**, *3*, 348 – 355.

Received: July 3, 2015
Published online: August 14, 2015

