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Fructose 1,6-Bisphosphate Aldolase from *Staphylococcus carnosus*: Overexpression, Structure Prediction, Stereoselectivity, and Application in the Synthesis of Bicyclic Sugars

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Abstract: The gene for the fructose 1,6-bisphosphate aldolase from *Staphylococcus carnosus* ($FruA_{sca}$) was subcloned for overexpression in *Escherichia coli* using the expression vector pKK223-3. An efficient, single-step purification by DEAE ion-exchange chromatography furnished the recombinant enzyme ready for synthetic applications. Sequence analysis indicated that $FruA_{sca}$ shares the overall α/β-barrel structure and most of the active site residues with the structurally well-defined FruA catalyst from rabbit muscle which signaled its functional equivalence for synthetic applications. A preparative study with generic aliphatic and hydroxylated aldehydes indeed confirmed a high level of stereoselectivity for both newly created asymmetric centers, and suggested a kinetic enantioselectivity for anionically charged 3-hydroxyaldehydes. In fact, the monomeric $FruA_{sca}$ was found to tolerate even the presence of highly reactive glutardialdehyde derivatives, which otherwise rapidly denature the rabbit muscle enzyme, and to allow their stereoselective conversion to bicyclic sugars.

Keywords: aldol reactions · α/β-barrel protein · asymmetric synthesis · carbohydrates · enzyme catalysis

Introduction

Asymmetric C–C bond formations are the most important and most challenging problems in synthetic organic chemistry. In Nature, such reactions are facilitated by lyases which catalyze the addition of carbonucleophiles to C=O bonds in a manner that is mechanistically classified as an aldol addition.[1] With respect to synthetic applications, the four stereochemically distinct dihydroxyacetone phosphate (DHAP) dependent enzymes[2] are particularly appealing because they control the creation of two new asymmetric centers at the termini of the newly formed C–C bond, thus allowing an effective combinatorial synthesis of stereoisomers.[3–5]

Mechanistically, activation of nucleophilic substrates by aldolases is achieved in two different ways.[6] Class I enzymes bind their substrates covalently by Schiff base formation to an active site lysine residue;[7] these catalysts are typically tetrameric proteins[8] composed of subunits of ~40 kDa and are found usually in higher plants and in mammalian or (as an exception) in specific microbial organisms. Class II aldolases utilize a divalent cation cofactor (usually Zn^{2+}) for substrate coordination;[9] these enzymes are usually dimers[10, 11] of ~39 kDa subunits and are commonly found in fungi and bacteria.

The variant glycolysis aldolases that cleave fructose 1,6-bisphosphate (FBP) have been pivotal to mechanistic advancement, structural insight, and synthetic utility.[12] In vivo, fructose 1,6-bisphosphate aldolases (FruA) catalyze the reversible addition of DHAP to D-glyceraldehyde 3-phosphate (GA3P) to give FBP. These enzymes are highly specific for the DHAP nucleophile and tolerate only a few isosteric replacements in the phosphate moiety,[13–15] but they readily accept a broad range of aldehydes at synthetically useful rates as the electrophile.[3, 17] The class I FruA from *Oryctolagus cuniculus* (rabbit) muscle ($FruA_{rab}$; for the systematic acronym nomenclature see[3]) has to be regarded as *the* classical reference catalyst since it has long been commercially available, has tested positive with literally hundreds of

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aldehydes as substrate analogues in place of the natural substrate D-glyceraldehyde 3-phosphate, and has proved to be eminently useful for the stereoselective synthesis of carbohydrates as well as related natural and nonnatural compounds.[3] However, its main limitations as a practical catalyst—and obstacles to any large-scale, industrial utilization[16]—remain in its relatively low stability under the usual reaction conditions with half-lives of only a few days in buffered solution, and in the narrow restrictions for application of cosolvents.[17]

In comparison, it has been reported that the prokaryotic class I aldolases from *Staphylococcus aureus* and *Pseudomonas aerogenes* display unusually high heat and pH stability,[18] which is most likely due to the fact that these ~33 kDa enzymes are active as monomers. More recently it was found that the related, monomeric class I aldolase from *Staphylococcus carnosus* ($FruA_{sca}$) also has a high pH and temperature stability,[19] and the encoding gene has been cloned and the DNA sequence determined.[20] Furthermore, this catalyst has been shown by a TLC screening to accept a number of aldehydes as substitutes of the natural substrate GA3P.[21] Although the $FruA_{sca}$ enzyme is now offered commercially,[22] a more detailed investigation of its synthetic utility was hampered so far by the high cost of the marketed catalyst and by the efforts required for purification of the wild type enzyme.[19]

Here we report on the construction of a new and highly efficient heterologous overexpression system for $FruA_{sca}$ in *E. coli* from which the enzyme can be obtained by a single-step purification in a state ready for synthetic applications. We also present a sequence comparison with other class I FruA proteins which suggests a common three-dimensional fold and thus a similarly broad synthetic applicability. The extraordinary stability of the enzyme is demonstrated by the preparation of bicyclic sugars from a glutardialdehyde derivative.

Results and Discussion

$FruA_{sca}$ overexpression

The *S. carnosus* gene *fda* encoding the $FruA_{sca}$ had previously been cloned as a 5.2 kb *Pst*I fragment of chromosomal DNA into the pBluescriptII KS + vector.[20] The resulting construct, designated pBluescriptII-fda10, was now used for the construction of a suitable overexpression system. The vector pKK223-3 was chosen because it contains the strong hybrid *tac* promoter and *rrn*B ribosomal termination for controlled protein expression,[23] and because it has proved to be highly successful in the overproduction of other aldolases.[24, 25] The *fda* gene was amplified from pBluescriptII-fda10 by mutating PCR technique to insert two flanking recognition sequences for appropriate restriction enzymes using the primers described in Scheme 1. The sense primer introduced an *Xma*I restriction site immediately before the gene's natural Shine–Dalgarno sequence which is located eight base pairs from the ATG start codon, and the antisense primer incorporated a *Pst*I restriction site after the stop codon. From the PCR amplification only a single band with the expected molecular weight (950 bp) was observed upon DNA agarose gel electrophoresis, and no further purification was required. As summarized by the cloning strategy shown in Figure 1, the *Pst*I and *Xma*I digested insert was unidirectionally ligated into the pKK223-3 vector and the construct, denoted pKK*fda*, was transformed into competent *E. coli* JM105 strain. Out of six colonies selected from LB-ampicillin plates, four carried the desired functional insert as determined by enzymatic assay for FruA activity.[26]

N-terminal primer (5′ → 3′): a a t a c c c g g g AGAGGAGTGTCAATAAATGAACC
Xma I S. D. Start

C-terminal primer (5′ → 3′): t c t a c t g c a g ATTAAGCTTTGTTTACTGAAGC
Pst I Stop

Scheme 1. Oligonucleotide primers used for the subcloning of $FruA_{sca}$. Recognition sequences for *Xma*I and *Pst*I restriction endonucleases are doubly underlined. Sequence stretches with identity to genetic DNA are indicated by capital letters; mutating sequences introduced by PCR extension are given by lowercase letters.

Recombinant cells of *E. coli* JM105/pKK*fda* were found to overexpress the $FruA_{sca}$ at about 1 kU g^{-1} wet cell weight (3–4 kU per liter of cell culture) upon induction of the *tac* promoter with 0.5 mM IPTG which corresponds to roughly a factor of 50 relative to wild type *S. carnosus* cells,[19] or to a factor of 20 relative to a previously described autologously cloned *S. carnosus* producer.[20] Analysis by denaturing SDS polyacrylamide gel electrophoresis showed that the fully

Abstract in German: *Das Strukturgen der Fructose-1,6-bisphosphataldolase aus Staphylococcus carnosus ($FruA_{sca}$) wurde für eine Überproduktion in Escherichia coli in den Expressionsvektor pKK223-3 subkloniert. Mittels Einschritt-Reinigung an DEAE-Ionenaustauscher konnte das rekombinante Enzym effizient in für Synthesereaktionen ausreichender Qualität gewonnen werden. Eine Sequenzanalyse deutet darauf hin, daß die $FruA_{sca}$ die α/β-Barrel-Structure und die meisten Aminosäurereste des aktiven Zentrums mit dem stukturell gut charakterisierten FruA-Enzyms aus Kaninchenmuskel teilt, was seine funktionelle Äquivalenz für Syntheseanwendungen nahelegte. Tatsächlich bestätigte eine präparative Studie mit typischen aliphatischen und hydroxylierten Aldehyden ein hohes Maß an Stereoselektivität in der Erzeugung beider neuer Asymmetriezentren und zeigte eine kinetische Enantioselektivität für anionisch geladene 3-Hydroxyaldehyde auf. Die monomere $FruA_{sca}$ erwies sich selbst gegenüber hochreaktiven Glutardialdehydderivativen, welche ansonsten das Kaninchenmuskelenzym rasch denaturieren, als unerwartet stabil, so daß selbst deren stereoselektive Umsetzung zu bicyclischen Zukkern gelang.*

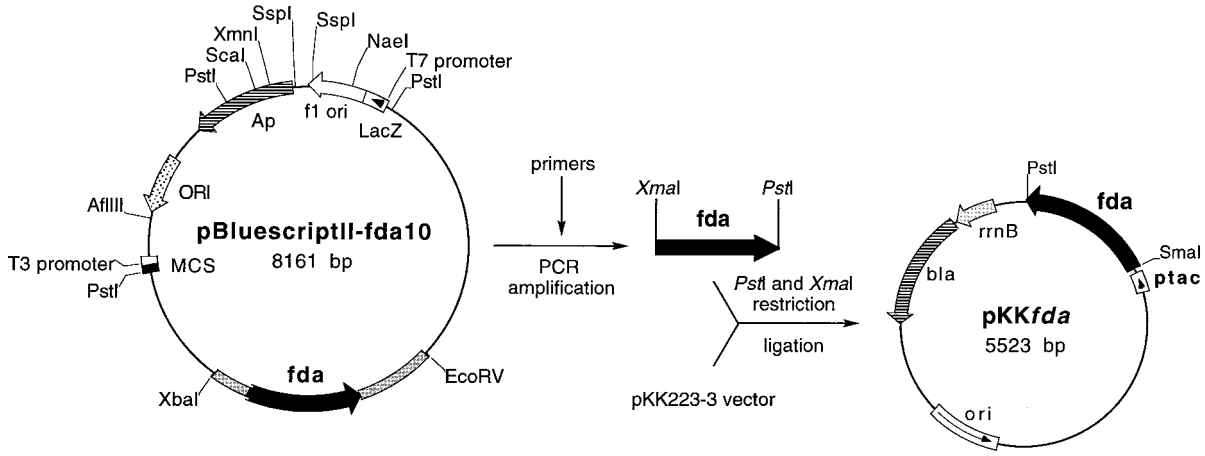

Figure 1. Summary of the subcloning of the $FruA_{sca}$ gene for protein overexpression.

induced recombinant cells contained about 25% of the $FruA_{sca}$ among total soluble cell protein. Despite the high levels of foreign gene production, no formation of inclusion bodies was detected. Because of its strong prevalence, the recombinant $FruA_{sca}$ could be easily obtained in relatively high purity ($\geq$90%) and high yield (87%) by a single anion-exchange chromatography on DEAE sepharose with only one other major band visible at about 70 kDa, as demonstrated by SDS-PAGE analysis (Figure 2). In this state, the enzyme was

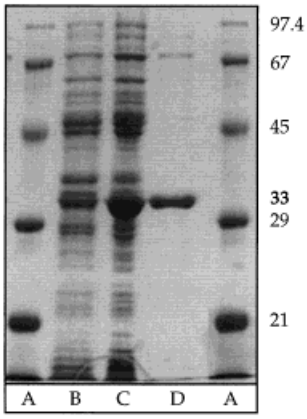

Figure 2. Electrophoretic analysis of the expression of the recombinant $FruA_{sca}$ in *E. coli* by SDS-PAGE separation on a 12% gel. Protein bands were stained by Coomassie blue. A: Molecular weight markers with the size indicated in kilodaltons to the right; expected subunit size of $FruA_{sca}$ is indicated in boldface. B: Total cell lysate of strain JM105/pKK*fda* without induction. C: Total cell lysate of strain JM105/pKK*fda* after induction with 0.5 mM IPTG. D: Purified $FruA_{sca}$ after single-step DEAE chromatography.

found to be quite stable upon storage (95% activity remaining after 12 months at 4 °C) and free from activities interfering in synthetic applications. If desired, practically pure protein (99%) may be obtained by a further gel permeation chromatography (GPC) to remove impurities of higher molecular weight. Also by GPC on Sephadex G150 the molecular weight of the native $FruA_{sca}$ was determined to be about 30 kDa which verified the prior analysis of the aldolase to be active in a monomeric state.[19]

$FruA_{sca}$ protein structure prediction

The application of enzymic catalysts to asymmetric synthesis is intimately tied up to the intricacies of their protein structure. Despite any apparent similarity in primary sequence or primary function, the peculiarities of related catalysts require an empirical scrutiny for their catalyst utility. Thus, when comparing enzymes from different sources but having a common catalytic specificity towards the same natural substrate, each catalyst quality with regard to the breadth of substrate specificity, such as tolerance for non-natural substrate analogues, enantioselectivity, and diastereoselectivity, or physical properties, such as heat, solvent, or pH stability, has to be evaluated individually. In an attempt to investigate the general synthetic features of the $FruA_{sca}$ with respect to the established reference Fru_{rab} enzyme, we first tried to probe the structural relationship of the catalysts in more detail.

Apart from a very narrow sequence comparison of the FruA enzymes from *S. carnosus* with that from human tissues, mays, *Drosophila* and *S. aureus* around the active site lysine (K212) no structural information for the $FruA_{sca}$ was available.[20] Particularly, no data on secondary or tertiary structure elements for the three-dimensional protein folding had been determined. Conversely, the X-ray structures of the highly homologous class I FruA proteins from rabbit (1.9 Å)[7, 8] and human muscle (2.0 Å),[27] and that from *Drosophila melanogaster* (2.5 Å)[28] have been determined at a useful level of accuracy. Their molecular architecture corresponds to that of active tetrameric associates of the respective enzyme subunits, which adopt an eight-stranded α/β- barrel structure motif. The active site cleft is located in the center of the β- barrel with the reactive Lys229 projecting towards the C-terminal opening of the pocket (Figure 3 left). A number of additional ionic and polar residues converge towards the opening of the active site pocket for substrate binding (Figure 3 right).

The deduced amino acid sequence of the *S. carnosus fda* gene (296 aa)[20] was compared to that of the corresponding rabbit gene for the muscle isozyme (364 aa).[29] Sequence alignment of the core structure (280 aa) for the $FruA_{sca}$ and $FruA_{rab}$ revealed an amino acid identity of less than 30% but

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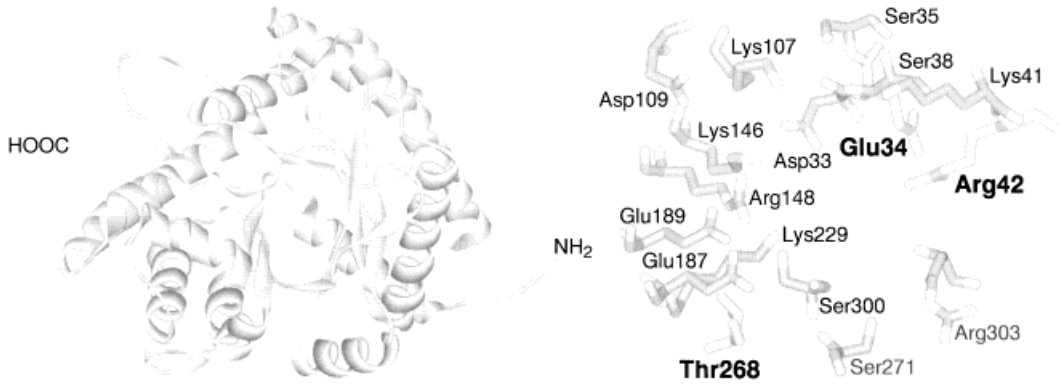

Figure 3. Left**:** Structural view of the fructose 1,6-bisphosphate aldolase, determined by X-ray structure analysis of $FruA_{rab}$, showing the α/β-barrel protein architectural motif. The plot was generated from PDB file 1ADO.[10] β-Strands are shown in cyan and α-helical segments in red. Right**:** Location of active site residues in the $FruA_{rab}$ enzyme as identified by X-ray crystal structure analysis.[8] Labeling of amino acid residues is indicated according to the primary sequence of $FruA_{rab}$. Residues that are conserved in the *S. carnosus* aldolase ($FruA_{sca}$), as determined by sequence alignment, are shown in standard color code for the elements, and side chains of non-conserved residues are highlighted in yellow. The corresponding substitutions occurring in $FruA_{sca}$ are Glu34Gln, Arg42Ala, and Thr268Val.

with enough indication for a likely similar overall fold. When making an allowance for conservative replacements, the homology rose to a remarkable 45 % (Figure 4). By inspection of this analysis, it becomes evident that there is a strong clustering of similarity in particular stretches of the primary sequence which strongly correlate with all eight β-sheet segments established for the $FruA_{rab}$ enzyme (sheets designated a – h in the crystal structure;[8] almost 80 % homology). In addition, out of the total of 17 amino acid residues contained in the active site, which are situated within the β-strands or at loops proceeding from the latter, 14 are identical and only three are not conserved (Figure 3 right). Since the secondary structure elements are thus responsible for the formation the β-barrel core and also mediating the catalytic activity, it can thus safely be deduced that the $FruA_{sca}$ shares with the $FruA_{rab}$ not only a common α/β-barrel structure but

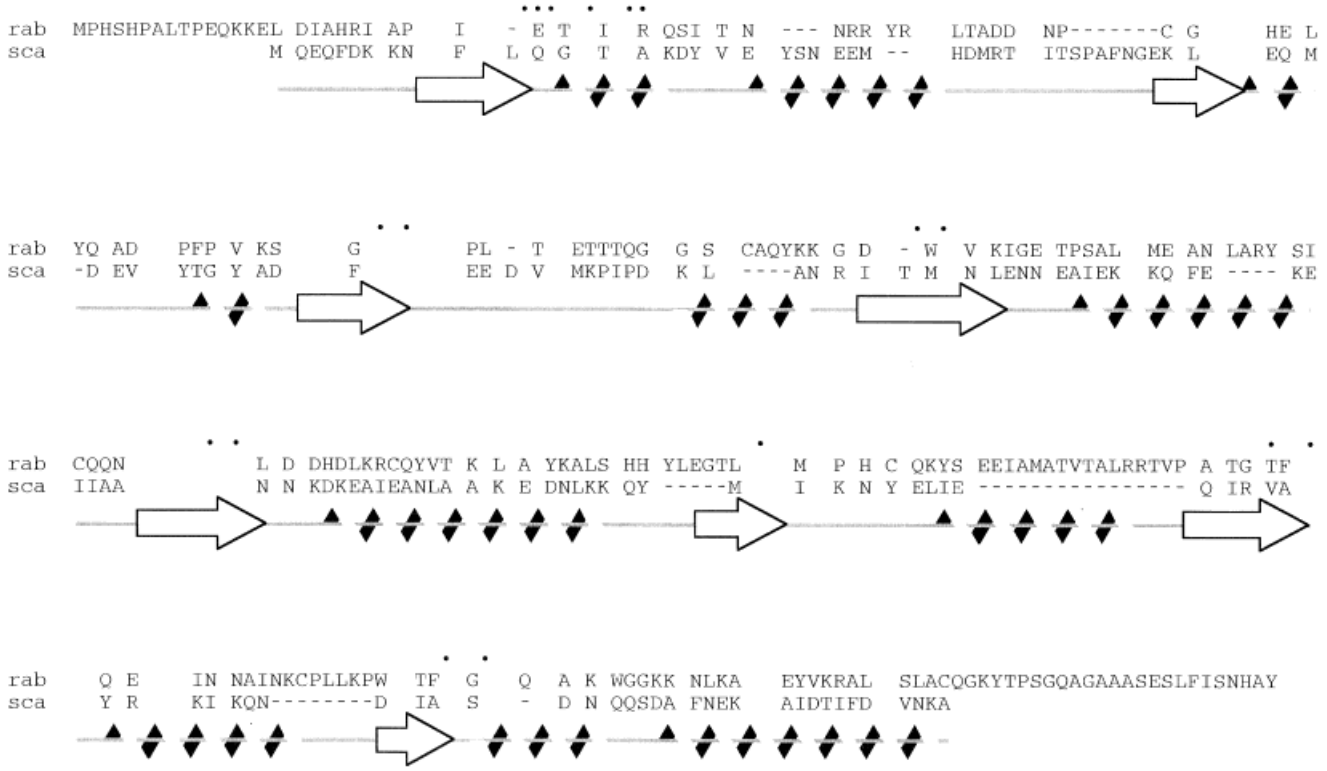

Figure 4. Sequence-based alignment of the amino acid sequences of fructose 1,6-bisphosphate aldolases from rabbit muscle ($FruA_{rab}$) and *Staphylococcus carnosus* ($FruA_{sca}$). Red amino acid single-letter codes highlight sequence identity, blue amino acid residues indicate conservative replacements. Black dots above the sequence of $FruA_{rab}$ indicate polar active site residues. Elements of secondary structure, as determined by X-ray analysis of $FruA_{rab}$,[8] are drawn below the alignment. Arrows represent β-strands (a – h), and coils represent α-helices ($A_1 - H_2$); other secondary structure elements, such as random coils and turns, are represented by a solid line; coloration of the symbols corresponds to that used in Figure 3.

will also very likely have quite similar catalytic properties. A comparable, if less pronounced, prediction was deduced by using a recently developed algorithm for sequence-derived fold recognition.[30]

Interestingly, the regions with higher diversity are located in the α-helical protein segments which make up the periphery of the three-dimensional structure. The highest disagreement in fact is found in helices E and F which flank the β-strand containing the Schiff-base forming lysine (K229 in $FruA_{rab}$, K212 in $FruA_{sca}$). The fact that these helices are mostly responsible for creating the rigid, nonpolar surface contacts between aggregated subunits of the tetrameric aldolases[31] makes it clear that profound alterations towards improved hydrophilicity (Figure 5) are mandatory for these particular entities to maintain a monomeric state of the $FruA_{sca}$.

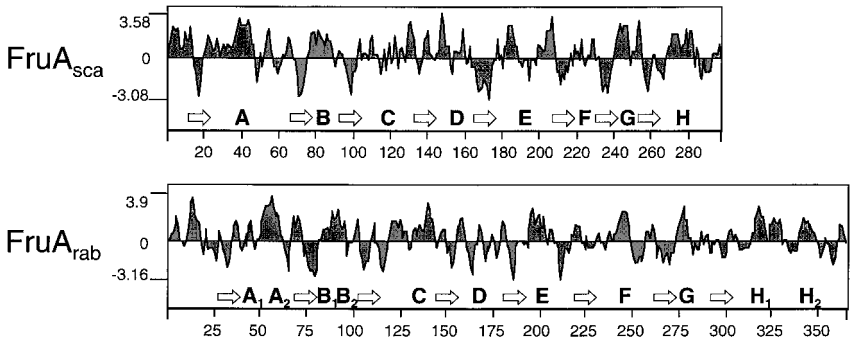

Figure 5. Plots of hydropathy index against residue number for fructose 1,6-bisphosphate aldolases from *Staphylococcus carnosus* ($FruA_{sca}$) and rabbit muscle ($FruA_{rab}$). The peaks and troughs are filled for an easy comparison of the profiles. Both plots were generated with an average segment length of five residues. Secondary structure elements for $FruA_{rab}$ are indicated by arrows for β-strands and by capital letters for α-helices, as deduced from the crystal structure; corresponding structure elements are tentatively assigned for $FruA_{sca}$ as derived from the sequence alignment shown in Figure 4.

$FruA_{sca}$ stereoselectivity

This $FruA_{sca}$ catalyst had been shown earlier to accept a number of aldehydes as substitutes of the natural substrate GA3P.[21] However, the simple TLC screening method used to detect product formation was unsuited to reveal the constitutional identity of the product formed nor its configurational purity. An evaluation of the stereoselectivity of the aldolase, however, was particularly desirable as a crucial precondition for potential applications in asymmetric synthesis.

The stereoselectivity of the purified $FruA_{sca}$ enzyme was determined by a series of small-scale preparative reactions in the direction of synthesis, employing two aliphatic, two hydroxylated, and one carboxyl aldehydes as representative substrate analogues (Table 1). In each case, reaction rates were sufficiently fast to maintain the product formation under kinetic control. At about 90% conversion, crude product mixtures were analyzed by high-field ^1H NMR for diastereomeric composition, using authentic reference materials obtained from parallel reactions catalyzed by $FruA_{rab}$ or as available from prior studies.[25]

From nonpolar aliphatic acetaldehyde and propionaldehyde, a very small percentage of *erythro*-configurated stereo-

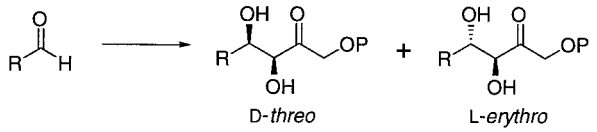

Table 1. Evaluation of $FruA_{sca}$ stereoselectivity.

Aldehyde	R	D-*threo* [%]	L-*erythro* [%]	Yield [%]
acetaldehyde	CH_3	97	3	90
propionaldehyde	C_2H_5	98	2	80
glycolaldehyde	CH_2OH	>95	<5	90
rac-glyceraldehyde	$CHOH{-}CH_2OH$	>95	<5	90
succinic semialdehyde	$(CH_2)_2COOH$	>95	<5	90

isomers was detectable by the distinctive signals of 3-/4-H. Thus, the $FruA_{sca}$ enzyme has a very high capacity to correctly bind the CHO group, even in the most discriminate case of CH_3 versus H differentiation. Within the limits of detection by high-field proton and carbon NMR spectroscopy ($\leq$5%), only single diastereomers were formed from 2- or 3-hydroxylated glycolaldehyde and glyceraldehyde, as well as from succinic acid semialdehyde. In context with similar observations this suggests that polar substrates are more strongly bound by additional hydrogen bonding or Coulomb attraction. Thus, substrate analogues comprising structural features of the natural substrate glyceraldehyde 3-phosphate seem to be excellently suited for complete stereochemical control over the newly generated vicinal diol unit of an absolute (3*S*,4*R*)-*trans* configuration (D-*threo*).

It had been shown previously that the $FruA_{rab}$ displays a pronounced kinetic enantioselectivity for D-configurated 2-hydroxyaldehydes when these bear an anionic group at a distance similar to that in the natural acceptor.[32] In an effort to study the complementary behavior towards 3-hydroxyaldehydes for both of the FruA enzymes, racemic maleic 4-semialdehyde **3** (readily obtainable by a Barbier-type[33] chain extension of glyoxylic acid **1**, followed by ozonolysis) was submitted to enzymic aldolization in the presence of substoichiometric amounts of DHAP (0.3 equivalents; Scheme 2). NMR spectroscopic analysis of the crude material revealed that the initially produced diastereomeric open-chain intermediates **4** and **5** expectedly had cyclized to form pyranoid rings. As evident from the coupling pattern of the methylene group, the major component **6** derives from D-**3** and adopts a β-$^6C_3$-chair conformation. The minor stereoisomer, produced from L-**3**, was determined not to be the anticipated corresponding $^3C_6$-chair but rather the derived intramolecular anomeric lactone **7**, probably due to the acidic conditions maintained upon sample preparation. Both cata-

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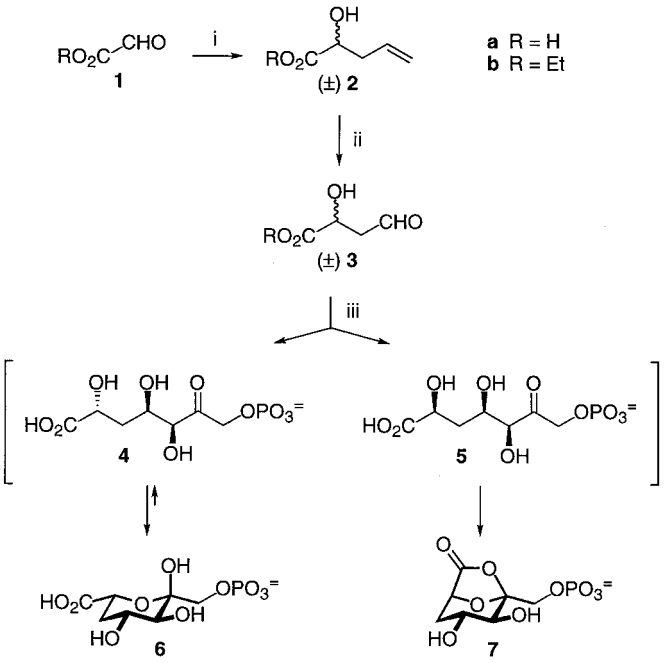


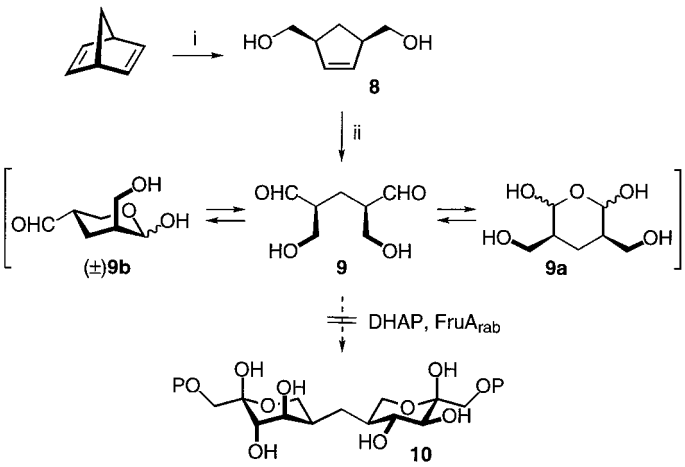
Scheme 2. Kinetic enantioselectivity for 2-hydroxy-4-oxobutanoate. i) Allyl bromide, Sn, EtOH, 45%; ii) O_3, MeOH, $-78\,^{\circ}C$; Me_2S, $-78\,^{\circ}C \rightarrow$room temperature, ~quantitative; iii) FruA, DHAP; ion exchange, **6**:**7** = 85:15 ($FruA_{rab}$) and 90:10 ($FruA_{sca}$).

lysts showed a similar moderate selectivity for the D-configurated aldehyde ($FruA_{rab}$ D:L = 85:15, $FruA_{sca}$ 90:10). Interestingly, the selectivity was significantly lower for the corresponding ethyl ester ($FruA_{rab}$ D:L = 40:60). The latter result not only points to the importance of an anionically charged group but also strongly corroborates the kinetic nature of the observed selectivity since the all-equatorially substituted **6** could alternatively have arisen by its higher thermodynamic stability.[3] The fact that from the latter reaction no ethyl ester remained in the product is in agreement with earlier observations for related substrate types.[34, 35]

Synthetic applications

Although several attempts at enzymatic addition of DHAP to simple linear α,ω-dialdehydes (glyoxal, glutardialdehyde) have been reported,[36, 21] in no case had a product been isolated and characterized. Presumably, this is because the dialdehydes cause crosslinking of the protein[37, 38] and thus irreversibly destroy its enzymatic activity. However, we have recently discovered that α- or β-hydroxylated dialdehydes are good substrates for a twofold, tandem aldol addition of DHAP.[39, 40] The contrasting results may be explained by the formation of stable intramolecular hemiacetals in aqueous solution which mask the reactivity of free dialdehydes.

In this context, the branched chain glutardialdehyde **9** was of interest as a potential precursor to hydrolytically stable, one-carbon linked disaccharide mimetics (e.g., **10**). The *meso*-cyclopentene-3,5-dimethanol **8** was prepared by controlled ozonolysis of norbornadiene, followed by reduction with $NaBH_4$ (Scheme 3).[41] Ozonolytic cleavage of the remaining double bond cleanly furnished the corresponding dialdehyde **9**. In contrast to glutardialdehyde itself, which is reported to

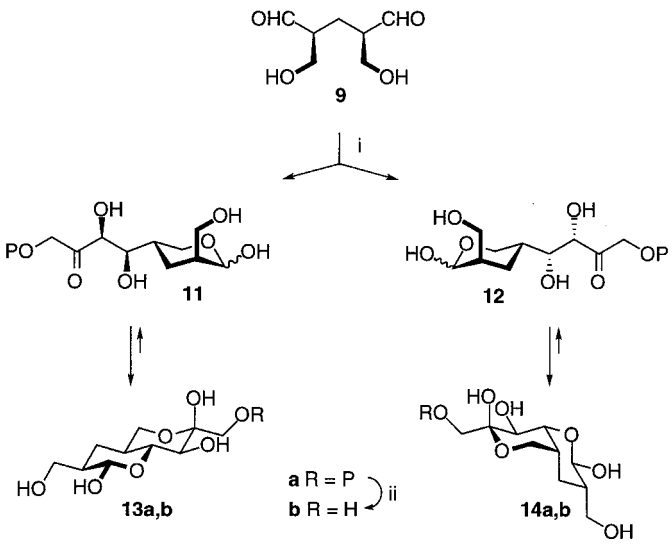


Scheme 3. Two-step synthesis of dialdehyde substrate, and attempted tandem aldol additions using fructose 1,6-bisphosphate aldolase from rabbit muscle ($FruA_{rab}$). i) O_3, MeOH, $-78\,^{\circ}C$; $NaBH_4$, room temperature, 55%; ii) O_3, MeOH, $-78\,^{\circ}C$; Me_2S, $-78\,^{\circ}C \rightarrow$room temperature, ~ quantitative. $P = PO_3^{2-}$.

crosslink FruA even at very low concentration,[42] the derivative **9** was expected to display a moderate reactivity because of its ability for intramolecular cyclization to form stable six-membered hemiacetals. In fact, NMR analysis showed a very complex mixture comprising free aldehydes (broad singlets at $\delta = 9.72$ and 9.67), hydrates, and isomeric structures (e.g. **9a**,**b**) to exist in aqueous solution, corresponding to the different possible modes of cyclization. Owing to the presence of a relatively high portion of free aldehyde, however, to our dismay a copious precipitate formed within few minutes when a solution of dialdehyde **9** and DHAP was incubated with $FruA_{rab}$. No enzymatic activity remained in solution, no trace of aldolization product was detectable from this attempt.

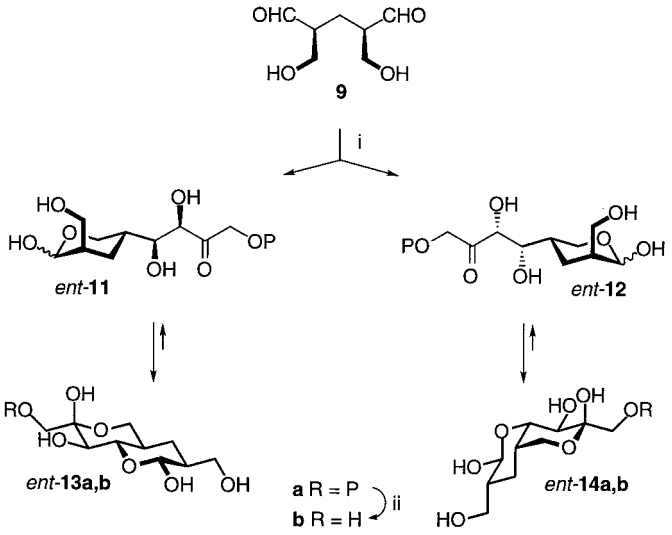
The $FruA_{sca}$ had been reported to show a far superior stability in comparison to the $FruA_{rab}$ under typical reaction conditions.[21] Since this is quite likely a consequence of its monomeric structure, we anticipated also a higher resistance against crosslinking denaturants. In fact, this supposition proved correct: when $FruA_{sca}$ was treated with **9** in the presence of DHAP no precipitate formed but a rapid consumption of DHAP was monitored by enzymatic assay (Scheme 4). Analysis by TLC indicated the formation of monophosphate(s), however, without traces of bisphosphate. Further conversion of monophosphate intermediates to bisphosphates could also not be detected upon prolonged incubation. After enzymatic dephosphorylation, the annulated C_{10}-dipyranoses **13b** and **14b** were obtained as a 3:1 diastereomeric mixture in 26% overall yield from **8**. Apparently, the two primary monophosphates **11** and **12** form rather stable intramolecular acetals **13a** and **14a**, respectively, thus effectively precluding a second addition.

To verify the structure as well as to gain insight into the origin of enantiotopos selectivity, it was desirable to submit substrate **9** to aldolization with the rhamnulose 1-phosphate aldolase (RhuA) having a *threo* stereoselectivity which is configurationally opposite to that of FruA enzymes.[25] Our experience showed that this class II aldolase from *E. coli*,



Scheme 4. Aldol reaction using fructose 1,6-bisphosphate aldolase from *S. carnosus* ($FruA_{sca}$). i) DHAP, $FruA_{sca}$, pH 7.0; ii) alkaline phosphatase, pH 8.0; ratio **13**:**14** = 3:1, 26% from **8**. $P = PO_3^{2-}$.

available commercially or by recombinant overproduction,[25] also is a rather robust catalyst having a very broad substrate tolerance.[3] Gratifyingly, when a solution of **9** and DHAP was treated with RhuA, no protein precipitation occurred and analysis by TLC indicated monophosphate formation (Scheme 5). Again, no bisphosphates were detectable at



Scheme 5. Aldol reaction using rhamnulose 1-phosphate aldolase from *E. coli* (RhuA). i) DHAP, RhuA, pH 7.0; ii) alkaline phosphatase, pH 8.0; ratio *ent*-**13** : *ent*-**14** = 1:3, 32% from **8**. $P = PO_3^{2-}$.

longer incubations. After dephosphorylation, a mixture of two diastereomers was isolated which were identified by NMR analysis to correspond to products *ent*-**13b** and *ent*-**14b** in a ratio of 1:3 (32% overall yield from **8**). Interestingly, the stereoisomer ratio (major component *ent*-**14b**) was opposite to that registered for the $FruA_{sca}$ experiment (major component **13b**), which infers that both catalysts had preferentially attacked the carbonyl group of identical enantiotopicity. Clearly, because of the contrasting identity of the major products, the enantiotopos selectivity in the terminus differentiation is (at least partially) of kinetic, and not of thermodynamic, origin.

Conclusions

We have constructed a new heterologous overexpression system for the $FruA_{sca}$ enzyme which by far excels the autologous production system reported previously. The high productivity and the simplicity of an effective one-step purification scheme make this a suitable source for large quantities of the biocatalyst. We have shown that its reported superior stability is paralleled by a high level of stereoselectivity for aldol addition reactions with generic aliphatic and hydroxylated aldehydes, an experimental outcome which had been suggested, in fact, by the discovery of high sequence and structural similarity around the known active site of the $FruA_{rab}$ and the postulated active site environment of the $FruA_{sca}$.

The synthetic utility of the enzyme in asymmetric synthesis was demonstrated by the formation of a new set of unusual bicyclic sugars from bifunctional precursors of a type that proved to be impossible to convert by using conventional $FruA_{rab}$ catalysis. Kinetic enantioselectivity could be demonstrated for an anionically charged 3-hydroxyaldehyde. Thus, when compared to the $FruA_{rab}$ enzyme as the current standard, the $FruA_{sca}$ is more than functionally equivalent, and promises to become a cost-efficient and overall effective replacement, particularly with regard to potential large-scale applications.[4]

Experimental Section

Materials and methods: Plasmid pBluescriptII-fda10 was provided by Professor Götz, Tübingen. Oligonucleotide primers, vector pKK223-3, *Escherichia coli* strain JM105, and media for protein chromatography were purchased from Pharmacia Biotech. PCR Core Kit, restriction enzymes, and T4 DNA ligase were obtained from Boehringer Mannheim and used according to the manufacturer's instructions. Other routine cloning operations were performed by the standard procedures.[43] The nuclease (Benzonase, grade I) was purchased from Merck, Darmstadt.

NMR spectra were recorded on Varian VXR 300 or Unity 500 spectrometers; chemical shifts are referenced to internal TMS or TSP ($\delta = 0.00$). Mass spectra were recorded on a Finnigan MAT 212 system and elemental analyses were performed on a Heraeus CHN-O-Rapid system. A Fischer 502 ozone generator was used for ozonolyses; ultrasound-accelerated reactions were carried out with the aid of a Bandelin Sonorex TK 52 H cleaning bath. Column chromatography was performed on Merck 60 silica gel (0.063 – 0.200 mesh), and analytical thin-layer chromatography (TLC) was performed on Merck silica gel plates 60 GF_{254} using a 1:1 mixture of saturated ammonia/ethanol for development, and anisaldehyde stain for detection. Analytical grade ion exchange media (100 – 200 mesh) were purchased from Bio-Rad. Fructose 1,6-bisphosphate aldolase from rabbit muscle (FruA; [EC 4.1.2.13]; type IV) and triose phosphate isomerase from rabbit muscle ([EC 5.3.1.1]; type I) were purchased from Sigma, and recombinant rhamnulose 1-phosphate aldolase from *E. coli* (RhuA; [EC 4.1.2.19]) was obtained from Boehringer Mannheim. Alkaline phosphatase from bovine intestinal mucosa ([EC 3.1.3.1], synthetic purity grade) was purchased from Fluka. Dihydroxyacetone phosphate (DHAP) was prepared from glycerol phosphate according to the published procedure.[13]

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PCR amplification: The gene *fda* contained in the plasmid pBluescriptII-fda10[20] was amplified by using the standard PCR technique as described in the literature[44] but with mutating oligonucleotide primers as shown in Scheme 1. Amplification was performed in a 100 μL reaction mixture containing 1 ng of plasmid pBluescriptII-fda10, 300 nM each of primers, 200 μM each of deoxynucleoside triphosphates (dNTPs), 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 50 mM KCl. The reaction mixture was heated for 2 min at 95 °C, then *Taq* DNA polymerase (2.5 U) was added, the solution was layered with mineral oil (100 μL) and subjected to 25 cycles of amplification (Eppendorf Mastercycler 5330). Cycle conditions were set for denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min, and elongation at 72 °C for 3 min. The mineral oil was removed and DNA was collected by phenol/chloroform extraction and ethanol precipitation. Analysis by 1% agarose gel electrophoresis showed the desired DNA insert (950 bp) to be the only detectable polymer chain reaction (PCR) product which was used for the subsequent steps without further purification.

Construction of the expression vector pKK*fda*: The PCR amplified *fda* insert was digested by incubation with restriction enzymes *Xma*I and *Pst*I under standard conditions. A 20 μL reaction mixture containing the DNA insert (1 μg), 6 mM Tris-HCl (pH 7.5), 50 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT, and *Xma*I (1 μL, 5 U) was incubated at 37 °C. After 4.5 h the enzyme was denaturated by heating at 65 °C for 10 min. After cooling to ambient temperature and addition of *Pst*I (1 μL, 10 U), the mixture was incubated at 37 °C for another 4.5 h. After denaturation by heating at 65 °C for 10 min, the restricted insert was recovered by phenol/chloroform extraction and precipitation with ethanol (2 vol) and 3 M NaOAc (1/10 vol, pH 5.2). The DNA pellets were taken up with 20 μL Tris-HCl (10 mM, pH 7.8).

The vector pKK223-3 was digested with *Xma*I and *Pst*I under the same conditions. The crude digested vector was taken up in dephosphorylation buffer (50 mM Tris-HCl, pH 9.0, 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$, 1 mM spermidine) and treated with calf intestinal alkaline phosphatase at 37 °C for 1 h. The reaction was stopped by addition of 1 μL of 0.5 M EDTA solution (final concentration 10 mM) and heating at 65 °C for 60 min. Plasmid DNA was recovered by phenol/chloroform extraction followed by ethanol precipitation, and the DNA pellet was taken up in 20 μL of Tris-HCl buffer (10 mM, pH 7.8).

The restricted insert (3 μL) and restricted and dephosphorylated vector (1 μL) were incubated overnight at 20 °C with T4 DNA ligase (0.5 U) in the appropriate buffer (66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM ATP, pH 7.5). After ligation, competent cells of *E. coli* JM105 strain were transformed with the new recombinant plasmid, denominated pKK*fda*, and plated onto LB agar plates supplemented with ampicillin (200 mg L^{-1}). Out of six colonies randomly selected, four carried the desired insert as determined by plasmid isolation and restriction analysis, as well as by enzymatic assay for FruA activity[26] after isopropyl-β-D-thiogalactopyranoside (IPTG) induction.

Expression and purification of $FruA_{sca}$: A positive clone was grown aerobically in 200 mL of LB medium supplemented by ampicillin (200 mg L^{-1}) at 37 °C in a shake flask at 300 rpm. When the turbidity reached an OD_{578} of 1.0, this was used as a starter culture to inoculate a 10-L-fermentor (B.Braun Biostat B) charged with 9.8 L of the same medium. Cells were grown aerobically with stirring at 37 °C to an OD_{578} of about 0.7 when IPTG was added to a concentration of 0.5 mM. After a further 5–6 h, cells were harvested by continuous centrifugation (Heraeus Contifuge 28RS; 15000 rpm, 4 °C) to give a wet weight of 35 g. The pellet was resuspended in Tris-HCl buffer (4 mL g^{-1} cells) and 5000 U of benzonase was added. Cells were disrupted by glass bead milling (IMA cell desintegrator C), and the extracts were clarified by filtration followed by centrifugation at 24000 g for 15 min at 4 °C. Total crude FruA activity, determined spectrophotometrically,[26] amounted to 30000 U (0.86 U mg^{-1} cells; 1 U is defined to catalyze the cleavage of 1 μmol of FBP per minute at 25 °C). Protein purification was performed by ion exchange chromatography on DEAE-Sepharose CL-6B, applying an NaCl gradient for protein elution (100–350 mM). FruA activity eluted at about 150 mM, and active fractions were pooled and concentrated by ultrafiltration (Amicon YM10 membrane) followed by ammonium sulfate (80%) precipitation (total recovered activity 26000 U, 87%).

Protein analysis: Protein concentration was determined by the Bradford method[45] with assay reagents supplied by Bio-Rad, using bovine serum albumin for calibration. Protein purity was analyzed by SDS-PAGE performed according to the method of Laemmli[46] using a 12% gel.

Sequence analysis: The protein sequence of the *S. carnosus* fructose 1,6-bisphosphate aldolase (296 aa) and the corresponding rabbit sequence of the muscle isozyme (364 aa), as derived from published gene sequences (GenBank accession numbers X71729 and K02300, respectively), were analyzed by using the program MegAlign (DNASTAR Inc.). Clustal method with default parameters was applied for the analysis.

Analysis of hydropathy index: The hydropathy index of the fructose 1,6-bisphosphate aldolases from *S. carnosus* and rabbit muscle was determined and plotted according to the method of Kyte and Doolittle,[47] as implemented in the program Protean (DNASTAR Inc.).

Evaluation of stereoselectivity: An aqueous solution (20 mL) containing fructose 1,6-bisphosphate (25 mM) and the respective aldehyde (50 mM) was adjusted to pH 6.8. After addition of triosephosphate isomerase (10 U) and FruA (from *S. carnosus* or rabbit muscle; 20 U) the mixture was allowed to stand at ambient temperature with intermittent analysis for conversion by TLC. At about 90% conversion, enzymes were removed by short heating to 90 °C and filtration through a pad of charcoal. After removal of the volatiles by roto-evaporation at ≤20 °C under vacuum, the residue was taken up in D_2O for NMR analysis. Products were identified by comparison with reference spectra of authentic compounds,[25] and their proportions were determined by ^1H NMR integration of key signals.

2-Hydroxypent-4-enoic acid (±2a): A mixture of glyoxylic acid (2.0 g, 27 mmol), powdered Sn (6.4 g, 54 mmol), and allyl bromide (4.8 mL, 54 mmol) in 50% aqueous ethanol (90 mL) was treated with ultrasound for 6 min under nitrogen (TLC analysis showed complete conversion). The suspension was filtered and the residue washed with ethanol. The combined filtrates were stirred over Zn dust (5 g) for 5 h, then filtered, and concentrated. After extraction of the residue with diethyl ether (4 × 50 mL), the concentrated extract was purified by silica chromatography using chloroform/methanol (5:1) as eluent to furnish a colorless oil (1.6 g, 45%); ^1H NMR (300 MHz, CD_3OD): $\delta = 5.83$ (dddd, 1H, 4-H), 5.11 (dd, 1H, 5-H_c), 5.06 (dd, 1H, 5-H_t), 5.01 (br s, 1H, OH), 4.21 (dd, 1H, 2-H), 2.56-2.35 (m, 2H, 3-H); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta = 175.70$ (C-1), 134.44 (C-4), 118.29 (C-5), 71.52 (C-2), 39.76 (C-3). Anal: $C_5H_8O_3$ (C, H).

Ethyl 2-hydroxypent-4-enoate (±2b): A mixture of ethyl glyoxylate (2.7 g, 26.5 mmol), powdered Sn (6.3 g, 53 mmol), and allyl bromide (4.7 mL, 53 mmol) in 70% aqueous ethanol (75 mL) was treated with ultrasound for 4 min under nitrogen (TLC analysis showed complete conversion). The suspension was diluted with $CHCl_3$ (100 mL), neutralized by careful addition of NEt_3, and filtered. The organic layer was dried over $MgSO_4$, concentrated, and the residue was purified by distillation to yield a colorless liquid (2.4 g, 64%). B.p. 46–50 °C/20 mbar; ^1H NMR (300 MHz, $CDCl_3$): $\delta = 5.82$ (dddd, 1H, 4-H), 5.15 (dd, 1H, 5-H_c), 5.12 (dd, 5-H_t), 4.31-4.19 (m, 3H, 2-H, CH_2), 3.7 (br s, 1H, OH), 2.57 (m, 1H, 3-H_a), 2.44 (m, 1H, 3-H_b), 1.29 (t, 3H, CH_3); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 174.52$ (C-1), 132.68 (C-4), 118.57 (C-5), 70.12 (C-2), 61.69 (CH_2), 38.72 (C-3), 14.24 (CH_3). Anal $C_7H_{12}O_3$ (C, H).

3-Deoxy-D-*xylo*-hept-5-ulopyranosonic acid 7-phosphate (6) and 3-deoxy-D-*lyxo*-5-heptulopyranosono-1,6-lactone 7-phosphate (7): A solution of alkene **2a** (700 mg, 6.0 mmol) in dry methanol (20 mL) was cooled to −78 °C and purged with a stream of ozone until the blue color persisted (10 min). Dimethyl sulfide (2.5 mL) was added, and the mixture was allowed to warm to room temperature with stirring. When a peroxide test proved negative (2 h), water (1 mL) was added, and the solution was concentrated under vacuum to a residual volume of about 1 mL. Water was added to a final concentration of 100 mM. Fructose 1,6-bisphosphate (Na_3 salt; 110 mg, 0.3 mmol) was added to an aliquot of the aldehyde solution (10 mL, 2.0 mmol), and the pH was adjusted to 6.8. After addition of FruA (from *S. carnosus* or rabbit muscle; 10 U) and triose phosphate isomerase (20 U), the mixture was incubated at 25 °C with analysis for conversion by TLC. At about 90% conversion, the solution was worked up as usual to determine the crude product ratio by NMR analysis to be **6**:**7** = 85:15 and 90:10 ($FruA_{rab}$ and $FruA_{sca}$, respectively). A similar reaction sequence was set up but using the ester **2b** instead to give a product ratio of **6**:**7** = 60:40. Compound **6**: ^1H NMR (300 MHz, D_2O): $\delta = 4.36$ (dd, 1H, 2-H), 4.28–3.70 (m, 3H, 4-, 7-H) 3.51 (d, 1H, 5-H), 2.33 (ddd, 1H, 3-H_{eq}), 1.58 (ddd, 1H, 3-H_{ax}), $J_{2,3ax} = 12.4$, $J_{2,3eq} = 2.0$, $J_{3ax,3eq} = J_{3ax,4} = 12.0$, $J_{3eq,4} = 5.0$, $J_{4,5} = 9.4$. Compound **7**: ^1H NMR (300 MHz, D_2O): $\delta = 4.57$ (dd, 1H, 2-H), 4.28–3.70

(m, 3H, 4-, 7-H) 3.58 (d, 1H, 5-H), 2.43 (ddd, 1H, 3-H_{eq}), 1.99 (ddd, 1H, 3-H_{ax}); $J_{2,3ax} = 10.0$, $J_{2,3eq} = 5.0$, $J_{3ax,3eq} = 13.0$, $J_{3eq,4} = 1.5$, $J_{4,5} = 9.4$.

2,3,4-Trideoxy-2,4-di-*C*-(hydroxymethyl)-D-*talo*-octos-7-ulo-1,5:7,4^1-dipyranose (13b) and 2,3,4-trideoxy-2,4-di-*C*-(hydroxymethyl)-D-*gulo*-octos-7-ulo-1,5:7,4^1-dipyranose (14b): A solution of diol **8**[40] (400 mg, 3.1 mmol) in MeOH (20 mL) was cooled to $-78\,^\circ$C and purged with a stream of ozone until a blue color persisted. Me_2S (1 mL) was added, and the mixture was stirred at $-78\,^\circ$C for 1 h. Stirring was continued at room temperature until a peroxide test proved negative (ca. 2 h). The solvent was evaporated and the crude aldehyde **9** was taken up in water (15 mL). To this solution was added DHAP (4.5 mmol), and the pH was adjusted to 6.9 with 1M NaOH. After addition of FruA from *S. carnosus* (200 U), the reaction was monitored by enzymatic assay for DHAP consumption and by TLC analysis. After complete conversion (12 h), the pH was adjusted to 8.0, and alkaline phosphatase (100 U) was added. TLC indicated the reaction to be complete after two days. After desalting (Dowex AG50W-X8, H^+; AG1-X8, HCO_3^-), products were isolated by filtration through a pad of silica to furnish a mixture of **13b**/**14b** in a ratio of 1:3 (200 mg, 26%). Analytical samples were obtained by careful silica gel chromatography ($CHCl_3$/MeOH 5:1). Compound **13b**: ^{1}H NMR (500 MHz, D_2O): $\delta = 1.19$ (q, 1H, $J = 12.2$, H-3$_{ax}$), 1.67 (m, 1H, H-2), 1.74 (m, 1H, H-4), 1.80 (dt, 1H, $^2J = 12.8$, $J_{2,3} = J_{3,4} = 4.0$, H-3eq), 3.53 (t, 1H, $J_{4,5} = J_{5,6} = 10.0$, H-5), 3.69 (d, 1H, $J_{5,6} = 10.0$, H-6), 3.49 and 3.71 (AB, 2H, $^2J = 11.7$, H-8), 3.57–3.71 (m, 4H, H-2^1 and H-4^1), 4.71 (d, 1H, $J_{1,2} = 8.9$, H-1); ^{13}C NMR (125 MHz, D_2O): $\delta =$ 28.75 (C-3), 41.14 (C-4), 46,07 (C-2), 64.69 and 65.47 (C-2^1 and C-4^1), 71.98 (C-5), 66.38 (C-8), 79.74 (C-6), 99.92 (C-1), 100.97 (C-7); MS *m/z* (SIMS, FAB, positve-ion): 273 (5) $[M+Na]^+$, 263 (20), 257 (49) $[M+Li]^+$, 167 (50), 161 (100). Compound **14b**: ^{1}H NMR (500 MHz, D_2O): $\delta = 1.65-1.85$ (m, 3H, H-2,-3), 2.18 (m, 1H, H-4), 3.51 and 3.70 (AB, 2H, $^2J = 11.7$, H-8), 3.61 and 3.72 (ABX, 2H, $^2J = 11.7$, $^3J = 3.4$ and 5.7, H-2^1), 4.10 (dd, 1H, $^2J = 12.0$, $J_{4,4'eq} = 2.9$, H-4$^1_{eq}$), 3.52 (m, 1H, H-4$^1_{ax}$), 4.19 (dd, 1H, $J_{5,6} = 10.5$, $J_{4,5} = 6.0$, H-5), 4.18 (d, 1H, $J_{5,6} = 10.5$, H-6), 4.95 (d, 1H, $J_{1,2} = 8.9$, H-1); ^{13}C NMR (125 MHz, D_2O): $\delta = 27.65$ (C-3), 38.03 (C-4), 46,52 (C-2), 64.86 (C-2^1), 65.15 (C-4^1), 65.49 (C-5), 66.64 (C-8), 77.44 (C-6), 93.88 (C-1), 101.08 (C-7); MS *m/z* (SIMS, FAB, positive-ion): 273 (54) $[M+Na]^+$, 257 (100) $[M+Li]^+$.

2,3,4-Trideoxy-2,4-di-*C*-(hydroxymethyl)-L-*talo*-octos-7-ulo-1,5:7,4^1-dipyranose (*ent*-13b) and 2,3,4-trideoxy-2,4-di-*C*-(hydroxymethyl)-L-*gulo*-octos-7-ulo-1,5:7,4^1-dipyranose (*ent*-14b): From a similar reaction of diol **8** (430 mg, 3.40 mmol) and DHAP (3.50 mmol) but using RhuA (200 U) instead, a mixture of *ent*-**13b**/*ent*-**14b** was obtained in the ratio of 1:3 (272 mg, 32%).

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[1] B. L. Horecker, O. Tsolas, C. Y. Lai in *The Enzymes*, *Vol. VII*, 3rd ed. (Ed.: P. D. Boyer), Academic Press, New York, **1972**, pp. 213–258.
[2] W.-D. Fessner in *Microbial Reagents in Organic Synthesis* (Ed.: S. Servi), Kluwer Academic, Dordrecht, **1992**, pp. 43–55.
[3] W.-D. Fessner, C. Walter, *Top. Curr. Chem.* **1997**, *184*, 97–194.
[4] W.-D. Fessner, *Curr. Opin. Chem. Biol.* **1998**, *2*, 85–97.
[5] S. Takayama, G. J. McGarvey, C.-H. Wong, *Ann. Rev. Microbiol.* **1997**, *51*, 285–310.
[6] W. J. Rutter, *Fed. Proc.* **1964**, *23*, 1248–1257.
[7] N. Blom, J. Sygusch, *Nat. Struct. Biol.* **1997**, *4*, 36–39.
[8] J. Sygusch, D. Beaudry, M. Allaire, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 7846–7850.
[9] W.-D. Fessner, A. Schneider, H. Held, G. Sinerius, C. Walter, M. Hixon, J. V. Schloss, *Angew. Chem.* **1996**, *108*, 2366–2369; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 2219–2221.
[10] N. S. Blom, S. Tetreault, R. Coulombe, J. Sygusch, *Nat. Struct. Biol.* **1996**, *3*, 856–862.
[11] S. J. Cooper, G. A. Leonard, S. M. McSweeney, A. W. Thompson, J. H. Naismith, S. Qamar, A. Plater, A. Berry, W. N. Hunter, *Structure* **1996**, *4*, 1303–1315.
[12] T. Gefflaut, C. Blonski, J. Perie, M. Willson, *Prog. Biophys. Mol. Biol.* **1995**, *63*, 301–340.
[13] W.-D. Fessner, G. Sinerius, *Angew. Chem.* **1994**, *106*, 217–220; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 209–212.
[14] R. Duncan, D. G. Drueckhammer, *J. Org. Chem.* **1996**, *61*, 438–439.
[15] H.-L. Arth, W.-D. Fessner, *Carbohydr. Res.* **1998**, *305*, 313–321.
[16] B. Bossow, W. Berke, C. Wandrey, *BioEngineering* **1992**, *8*, 12–14.
[17] M. D. Bednarski, E. S. Simon, N. Bischofberger, W.-D. Fessner, M. J. Kim, W. Lees, T. Saito, H. Waldmann, G. M. Whitesides, *J. Am. Chem. Soc.* **1989**, *111*, 627–635.
[18] F. Götz, S. Fischer, K.-H. Schleifer, *Eur. J. Biochem.* **1980**, *108*, 295–301.
[19] H. P. Brockamp, M. R. Kula, *Appl. Microbiol. Biotechnol.* **1990**, *34*, 287–291.
[20] C. Witke, F. Götz, *J. Bacteriol.* **1993**, *175*, 7495–7499.
[21] H. P. Brockamp, M. R. Kula, *Tetrahedron Lett.* **1990**, *31*, 7123–7126.
[22] Boehringer Mannheim, *Biochemicals Catalog*, **1998**.
[23] J. Brosius, A. Ullrich, M. A. Raker, A. Gray, T. J. Dull, R. R. Gutell, H. F. Noller, *Plasmid* **1981**, *6*, 112–118.
[24] A. Ozaki, E. J. Toone, C. H. von der Osten, A. J. Sinskey, G. M. Whitesides, *J. Am. Chem. Soc.* **1990**, *112*, 4970–4971.
[25] W.-D. Fessner, G. Sinerius, A. Schneider, M. Dreyer, G. E. Schulz, J. Badia, J. Aguilar, *Angew. Chem.* **1991**, *103*, 596–599; *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 555–558.
[26] A. U. Bergmeyer, *Methods of Enzymatic Analysis*, *Vol. VI*, 3rd ed., VCH, Weinheim, **1984**, pp. 342–350.
[27] S. J. Gamblin, G. J. Davies, J. M. Grimes, R. M. Jackson, J. A. Littlechild, H. C. Watson, *J. Mol. Biol.* **1991**, *219*, 573–576.
[28] G. Hester, O. Brenner-Holzach, F. A. Rossi, M. Struck-Donatz, K. H. Winterhalter, J. D. G. Smit, K. Piontek, *FEBS Lett.* **1991**, *292*, 237–242.
[29] D. R. Tolan, A. B. Amsden, S. D. Putney, M. S. Urdea, E. E. Penhoet, *J. Biol. Chem.* **1984**, *259*, 1127–1131.
[30] D. Fischer, D. Eisenberg, *Protein Sci.* **1996**, *5*, 947–955.
[31] Z. Q. Zhang, D. L. Smith, *Protein Sci.* **1996**, *5*, 1282–1289.
[32] W. J. Lees, G. M. Whitesides, *J. Org. Chem.* **1993**, *58*, 1887–1894.
[33] C. J. Li, *Tetrahedron* **1996**, *52*, 5643–5668.
[34] N. J. Turner, G. M. Whitesides, *J. Am. Chem. Soc.* **1989**, *111*, 624–627.
[35] W.-D. Fessner, G. Sinerius, *Bioorg. Med. Chem.* **1994**, *2*, 639–645.
[36] F. Effenberger, A. Straub, *Tetrahedron Lett.* **1987**, *28*, 1641–1644.
[37] I. Chibata, *Immobilized Enzymes. Research and Development*, Wiley, New York, **1978**.
[38] M. D. Trevan, *Immobilized Enzymes. An Introduction and Applications in Biotechnology*, Wiley, New York, **1980**.
[39] O. Eyrisch, W.-D. Fessner, *Angew. Chem.* **1995**, *107*, 1738–1740; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1639–1641.
[40] M. Petersen, M. T. Zannetti, W.-D. Fessner, *Top. Curr. Chem.* **1997**, *186*, 87–117.
[41] C. A. Grob, H. R. Pfaendler, *Helv. Chim. Acta* **1970**, 2156–2159.
[42] J. Hajdu, M. Solti, P. Friedrich, *Acta Biochim. Biophys. Acad. Sci. Hung.* **1975**, *10*, 7–16.
[43] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, **1989**.
[44] H. G. Gassen, G. E. Sachse, A. Schulte, *PCR. Grundlagen und Anwendungen der Polymerase-Kettenreaktion*, Fischer, Stuttgart, **1994**.
[45] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.
[46] U. K. Laemmli, *Nature* **1970**, *227*, 680–685.
[47] J. Kyte, R. F. Doolittle, *J. Mol. Biol.* **1982**, *157*, 105–132.

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