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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

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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.

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 Zn²⁺) 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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[‡] Part 13, see ref. [15].

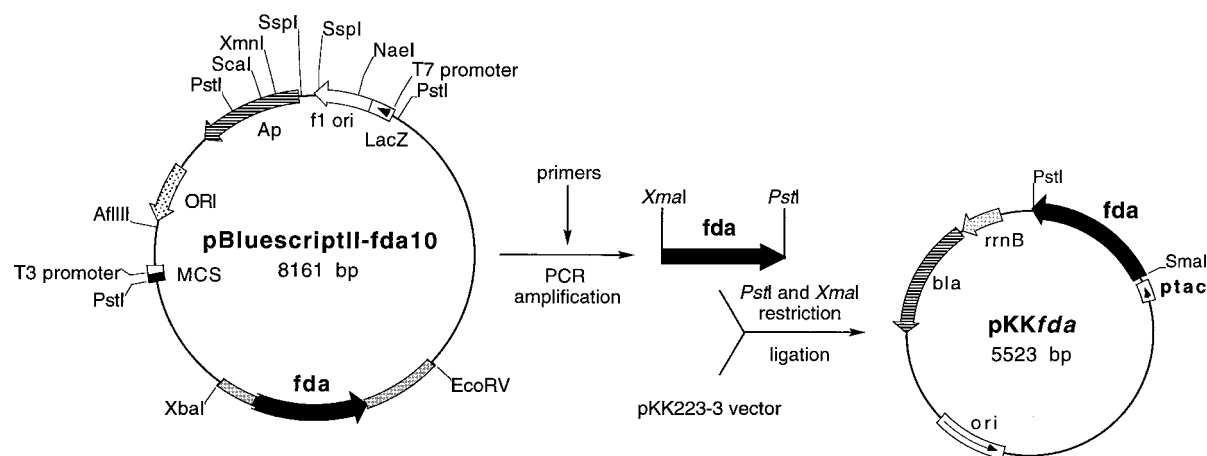


Figure 1. Summary of the subcloning of the FruA_{sca} gene for protein overexpression.

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

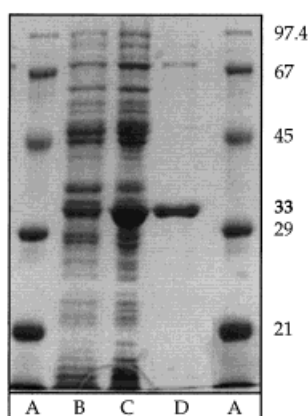


Figure 2. Electrophoretic analysis of the expression of the recombinant FruA_{sca} in *E. coli* by SDS-PAGE separation on a 12% gel. Protein bands were stained by Coomassie blue. A: Molecular weight markers with the size indicated in kilodaltons to the right; expected subunit size of FruA_{sca} is indicated in boldface. B: Total cell lysate of strain JM105/pKKfda without induction. C: Total cell lysate of strain JM105/pKKfda 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 3left). A number of additional ionic and polar residues converge towards the opening of the active site pocket for substrate binding (Figure 3right).

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

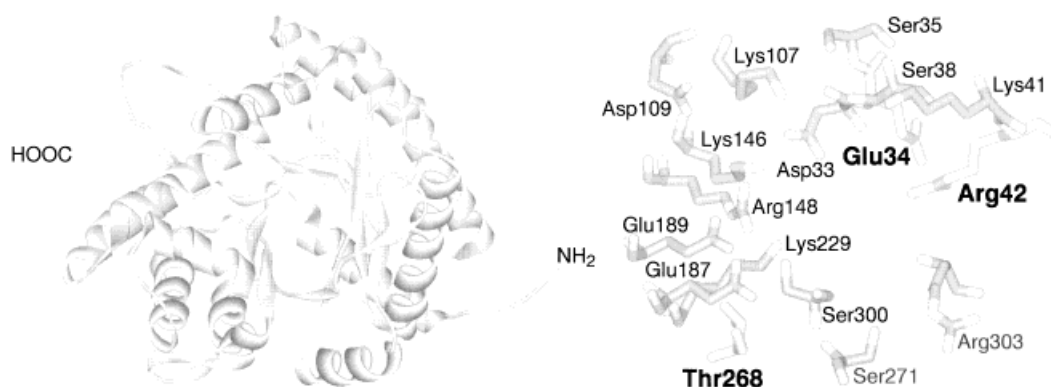


Figure 3. Left: Structural view of the fructose 1,6-bisphosphate aldolase, determined by X-ray structure analysis of FruA_{rab}, showing the α/β -barrel protein architectural motif. The plot was generated from PDB file 1ADO.^[10] β -Strands are shown in cyan and α -helical segments in red. Right: Location 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

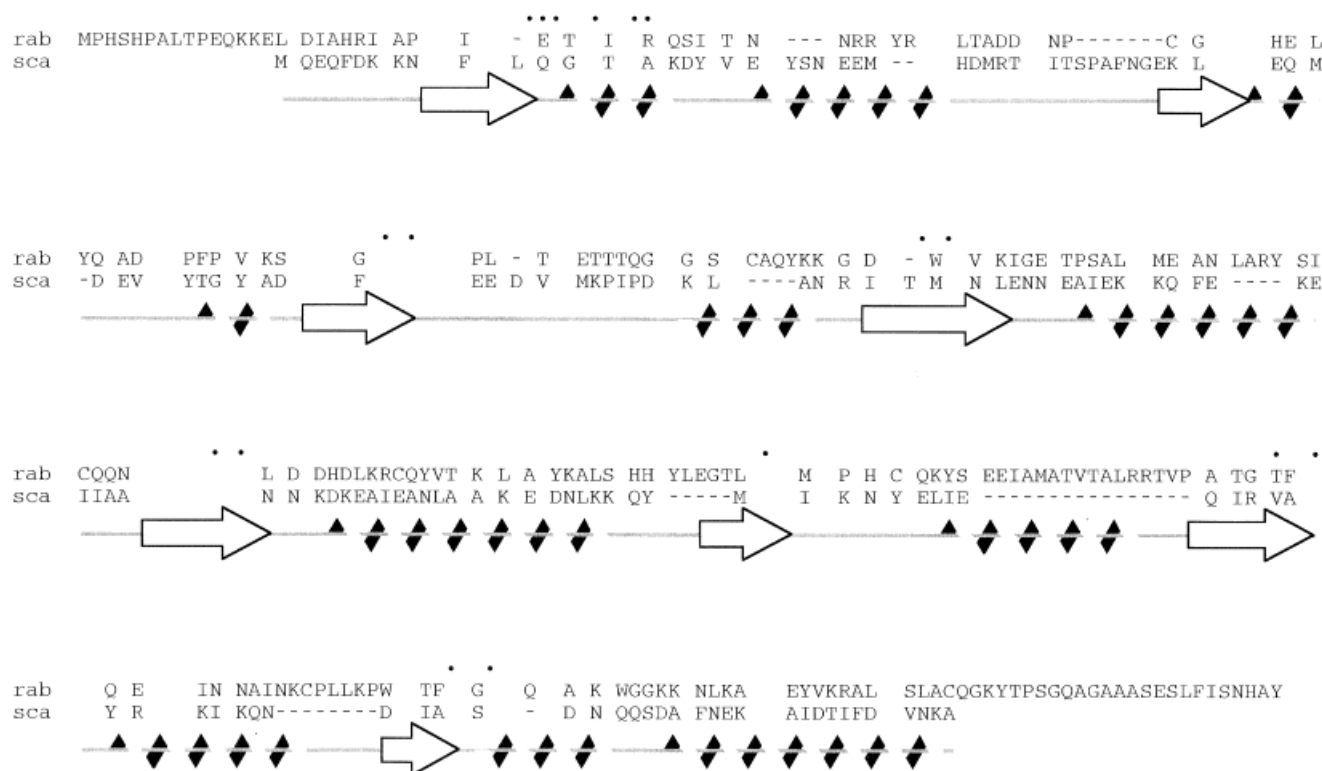


Figure 4. Sequence-based alignment of the amino acid sequences of fructose 1,6-bisphosphate aldolases from rabbit muscle (FruA_{rab}) and *Staphylococcus carnosus* (FruA_{sca}). Red amino acid single-letter codes highlight sequence identity, blue amino acid residues indicate conservative replacements. 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 (A₁–H₂); 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}.

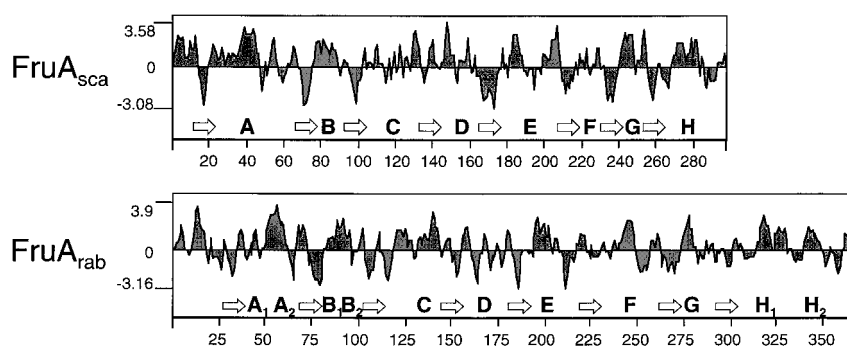


Figure 5. Plots of hydropathy index against residue number for fructose 1,6-bisphosphate aldolases from *Staphylococcus carnosus* (FruA_{sca}) and rabbit muscle (FruA_{rab}). The peaks and troughs are filled for an easy comparison of the profiles. Both plots were generated with an average segment length 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*-configured stereo-

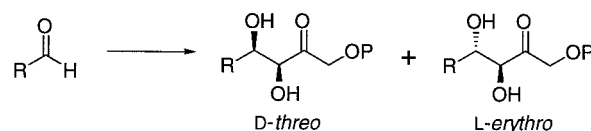


Table 1. Evaluation of FruA_{sca} stereoselectivity.

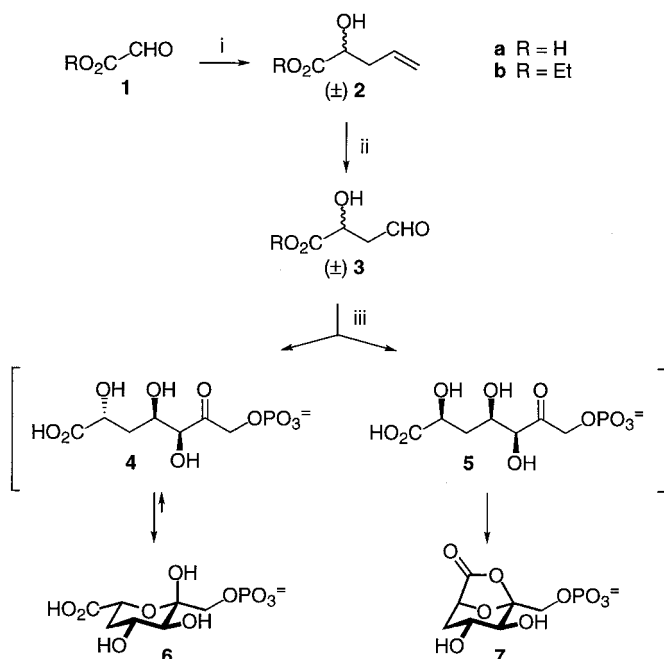
Aldehyde	R	D-threo [%]	L-erythro [%]	Yield [%]
acetaldehyde	CH ₃	97	3	90
propionaldehyde	C ₂ H ₅	98	2	80
glycolaldehyde	CH ₂ OH	> 95	< 5	90
rac-glyceraldehyde	CHOH-CH ₂ OH	> 95	< 5	90
succinic semialdehyde	(CH ₂) ₂ COOH	> 95	< 5	90

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 CH₃ 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 (3S,4R)-*trans* configuration (*D-threo*).

It had been shown previously that the FruA_{rab} displays a pronounced kinetic enantioselectivity for *D*-configured 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 -⁶C₃-chair conformation. The minor stereoisomer, produced from *L*-**3**, was determined not to be the anticipated corresponding ³C₆-chair but rather the derived intramolecular anomeric lactone **7**, probably due to the acidic conditions maintained upon sample preparation. Both cata-



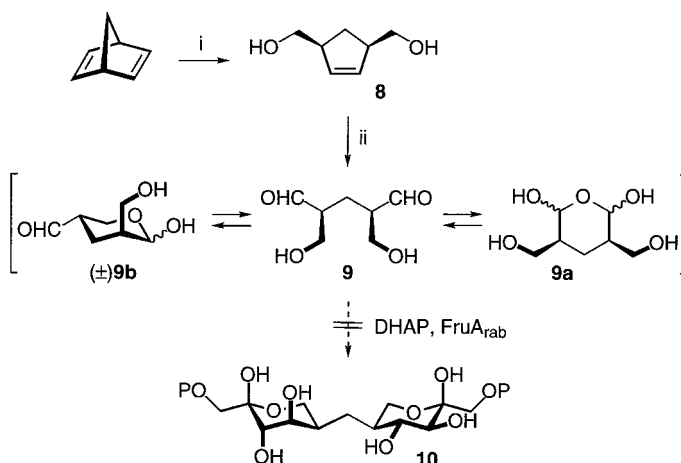
Scheme 2. Kinetic enantioselectivity for 2-hydroxy-4-oxobutanoate. i) Allyl bromide, Sn, EtOH, 45%; ii) O₃, MeOH, –78°C; Me₂S, –78°C → room temperature, ~quantitative; iii) FruA, DHAP; ion exchange, **6:7** = 85:15 (FruA_{rab}) and 90:10 (FruA_{sca}).

lysts showed a similar moderate selectivity for the D-configured 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 NaBH₄ (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

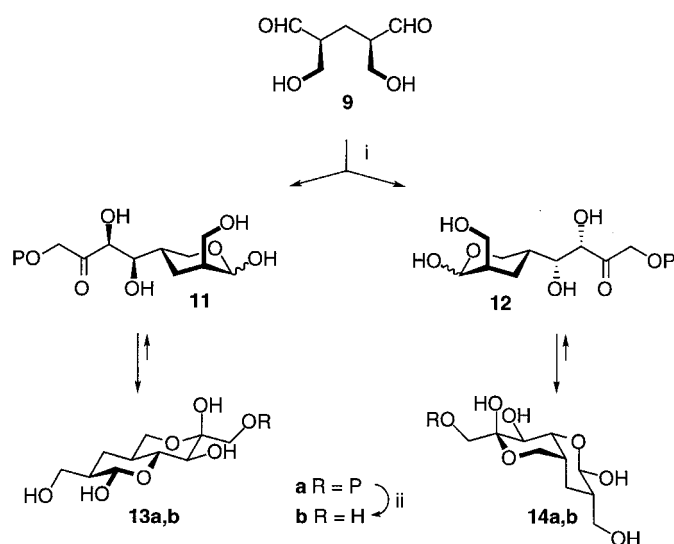


Scheme 3. Two-step synthesis of dialdehyde substrate, and attempted tandem aldol additions using fructose 1,6-bisphosphate aldolase from rabbit muscle (FruA_{rab}). i) O₃, MeOH, –78°C; NaBH₄, room temperature, 55%; ii) O₃, MeOH, –78°C; Me₂S, –78°C → room temperature, ~quantitative. P = PO₃²⁻.

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 δ = 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.

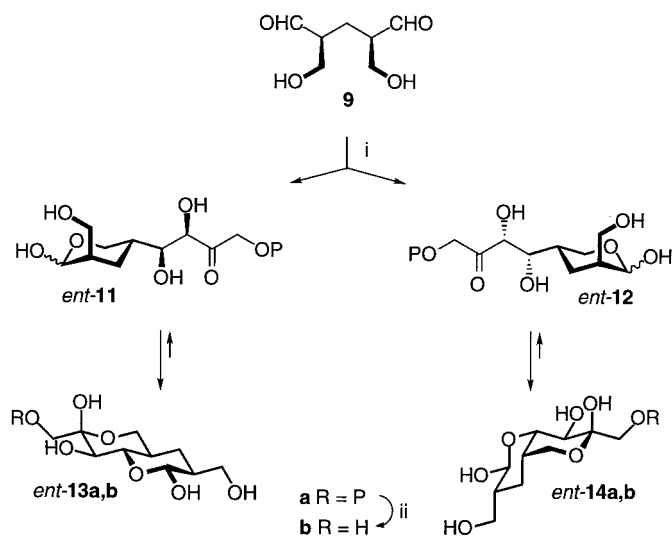
The FruA_{sca} had been reported to show a far superior stability in comparison to the FruA_{rab} under typical reaction conditions.^[21] Since this is quite likely a consequence of its monomeric structure, we anticipated also a higher resistance against crosslinking denaturants. In fact, this supposition 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 C₁₀-dipyranses **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**. $\text{P} = \text{PO}_3^{2-}$.

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**. $\text{P} = \text{PO}_3^{2-}$.

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 GF₂₅₄ 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]

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 μ L reaction mixture containing 1 ng of plasmid pBluescriptII-fda10, 300 nM each of primers, 200 μ M each of deoxynucleoside triphosphates (dNTPs), 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 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 μ L) 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 pKKfda: The PCR amplified *fda* insert was digested by incubation with restriction enzymes *Xma*I and *Pst*I under standard conditions. A 20 μ L reaction mixture containing the DNA insert (1 μ g), 6 mM Tris-HCl (pH 7.5), 50 mM NaCl, 6 mM MgCl₂, 1 mM DTT, and *Xma*I (1 μ L, 5 U) was incubated at 37 °C. After 4.5 h the enzyme was denatured by heating at 65 °C for 10 min. After cooling to ambient temperature and addition of *Pst*I (1 μ L, 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 μ L 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 ZnCl₂, 1 mM MgCl₂, 1 mM spermidine) and treated with calf intestinal alkaline phosphatase at 37 °C for 1 h. The reaction was stopped by addition of 1 μ L 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 μ L of Tris-HCl buffer (10 mM, pH 7.8).

The restricted insert (3 μ L) and restricted and dephosphorylated vector (1 μ L) were incubated overnight at 20 °C with T4 DNA ligase (0.5 U) in the appropriate buffer (66 mM Tris-HCl, 5 mM MgCl₂, 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 pKKfda, 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 OD₅₇₈ 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 OD₅₇₈ 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 Centrifuge 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 μ mol of FBP per minute at 25 °C). Protein purification was performed by ion exchange chromatography on DEAE-Sepharose CL-6B, applying a 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 D₂O 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 ($\pm 2a$): 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 $\times$ 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, CD₃OD): δ = 5.83 (dddd, 1H, 4-H), 5.11 (dd, 1H, 5-H_{eq}), 5.06 (dd, 1H, 5-H_{ax}), 5.01 (br s, 1H, OH), 4.21 (dd, 1H, 2-H), 2.56–2.35 (m, 2H, 3-H); ¹³C NMR (100.6 MHz, CD₃OD): δ = 175.70 (C-1), 134.44 (C-4), 118.29 (C-5), 71.52 (C-2), 39.76 (C-3). Anal: C₅H₈O₃ (C, H).

Ethyl 2-hydroxypent-4-enoate ($\pm 2b$): 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 CHCl₃ (100 mL), neutralized by careful addition of NEt₃, and filtered. The organic layer was dried over MgSO₄, 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, CDCl₃): δ = 5.82 (dddd, 1H, 4-H), 5.15 (dd, 1H, 5-H_{eq}), 5.12 (dd, 5-H_{ax}), 4.31–4.19 (m, 3H, 2-H, CH₂), 3.7 (br s, 1H, OH), 2.57 (m, 1H, 3-H_{ax}), 2.44 (m, 1H, 3-H_{eq}), 1.29 (t, 3H, CH₃); ¹³C NMR (100.6 MHz, CDCl₃): δ = 174.52 (C-1), 132.68 (C-4), 118.57 (C-5), 70.12 (C-2), 61.69 (CH₂), 38.72 (C-3), 14.24 (CH₃). Anal: C₇H₁₂O₃ (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 (Na₃ 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, D₂O): δ = 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, D₂O): δ = 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'-dipyr-anose (13b) and 2,3,4-trideoxy-2,4-di-C-(hydroxymethyl)-D-gulo-octos-7-ulo-1,5:7,4'-dipyr-anose (14b): A solution of diol **8**^[40] (400 mg, 3.1 mmol) in MeOH (20 mL) was cooled to -78°C and purged with a stream of ozone until a blue color persisted. Me₂S (1 mL) was added, and the mixture was stirred at -78°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, HCO₃⁻), 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 (CHCl₃/MeOH 5:1). Compound **13b**: ¹H NMR (500 MHz, D₂O): $\delta = 1.19$ (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' and H-4'), 4.71 (d, 1H, $J_{1,2} = 8.9$, H-1); ¹³C NMR (125 MHz, D₂O): $\delta = 28.75$ (C-3), 41.14 (C-4), 46.07 (C-2), 64.69 and 65.47 (C-2' and C-4'), 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, positive-ion): 273 (5) [$M+\text{Na}$]⁺, 263 (20), 257 (49) [$M+\text{Li}$]⁺, 167 (50), 161 (100). Compound **14b**: ¹H NMR (500 MHz, D₂O): $\delta = 1.65$ –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'), 4.10 (dd, 1H, $^2J = 12.0$, $J_{4,4'eq} = 2.9$, H-4'_{eq}), 3.52 (m, 1H, H-4'_{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); ¹³C NMR (125 MHz, D₂O): $\delta = 27.65$ (C-3), 38.03 (C-4), 46.52 (C-2), 64.86 (C-2'), 65.15 (C-4'), 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) [$M+\text{Na}$]⁺, 257 (100) [$M+\text{Li}$]⁺.

2,3,4-Trideoxy-2,4-di-C-(hydroxymethyl)-L-talo-octos-7-ulo-1,5:7,4'-dipyr-anose (ent-13b) and 2,3,4-trideoxy-2,4-di-C-(hydroxymethyl)-L-gulo-octos-7-ulo-1,5:7,4'-dipyr-anose (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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