Carbohydrate Research, 238 (1993) 287–306
Elsevier Science Publishers B.V., Amsterdam

Facile enzymic *de novo* synthesis and NMR spectroscopic characterization of D-tagatose 1,6-bisphosphate *

Oliver Eyrisch, Gudrun Sinerius and Wolf-Dieter Fessner

Department of Organic Chemistry and Biochemistry, University of Freiburg, Albertstr. 21, D-7800 Freiburg (Germany)

(Received February 20th, 1992; accepted June 1st, 1992)

ABSTRACT

A D-tagatose 1,6-bisphosphate aldolase requiring Zn^{2+} for catalytic activity (class II) was purified from *E. coli* cells grown on galactitol. The aldolase, a homotetramer composed of subunits of mol wt ~28000, had a pH optimum at 7.5 and was highly selective for L-*erythro* as compared to D-*threo* stereochemistry (99:1). This allowed its application in a coupled enzyme system together with glycerol kinase, pyruvate kinase and triose phosphate isomerase for the *de novo*, one-pot synthesis of D-tagatose 1,6-bisphosphate starting from dihydroxyacetone and phosphoenolpyruvate (for the in situ regeneration of adenosine triphosphate), in quantities of 10 mmol. The expeditious process compares very favorably in simplicity and yield (40% overall) with the known multistep chemical preparation even after improvements to the latter accomplished during the present work. The classical sequence, which starts from D-galacturonic acid, was modified at both phosphorylation steps: 1,2:3,4-di-*O*-isopropylidene-D-tagatofuranose was esterified by application of the trivalent phosphitylation agent dibenzyl di-*N*-ethylphosphoramidite followed by hydrogen peroxide oxidation, and a bacterial fructose 6-phosphate kinase was used for enzymic phosphorylation of D-tagatose 6-phosphate. For the latter enzyme, which was the isoenzyme Pfk-2 from a recombinant strain of *E. coli*, kinetic constants were determined. NMR spectroscopic assignments are presented for D-tagatose and its phosphates.

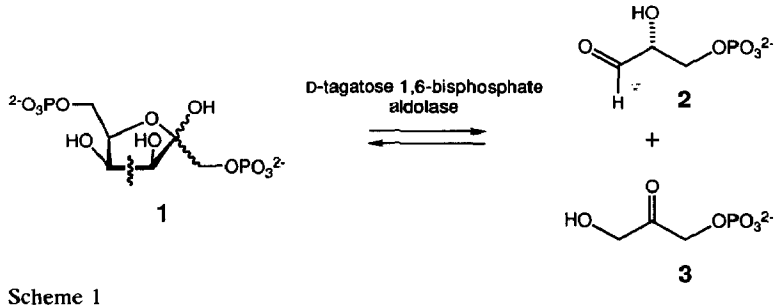
INTRODUCTION

Sugar phosphates play an important physiological role as intermediates and regulators in carbohydrate metabolism[1]. While their chemical synthesis constitutes a considerable challenge[2], enzymic methods provide an appropriate and attractive alternative[3].

An increasing number of aldolases are being introduced as valuable catalysts for asymmetric synthesis[4], with a notable emphasis on applications towards sugars[5]. Particularly, the use of a set of four stereochemically complementary microbial aldolases has been proposed for the directed *de novo* synthesis of monosaccha-

Correspondence to: Dr. W.-D. Fessner, Department of Organic Chemistry and Biochemistry, University of Freiburg, Albertstr. 21, D-7800 Freiburg, Germany.

* Enzymes in Organic Synthesis, Part 4. For Part 3, see ref. 8.



Scheme 1

rides according to a flexible building block scheme[6]. In vivo, the enzymes function to cleave ketose 1(,6)-(bis)phosphates into smaller fragments that can enter the organism's central metabolism. In vitro, three of the aldolases – D-fructose 1,6-bisphosphate (FruA; EC 4.1.2.13)[7,8], L-fuculose 1-phosphate (FucA; EC 4.1.2.17)[6,9] and L-rhamnulose 1-phosphate aldolase (RhuA; EC 4.1.2.19)[6] – have been shown to catalyze the addition of the common aldol donor dihydroxyacetone phosphate to a broad range of aldehydes. C–C bond formation occurs in a highly enantio- and diastereo-selective manner to yield a set of ketose 1-phosphates diastereomeric at C-3 and C-4.

Full flexibility of this synthetic strategy requires the access to the aldolase capable of cleaving (and generating) the fourth diastereomeric configuration, i.e., D-tagatose 1,6-bisphosphate aldolase (TagA). This enzyme, to be classified as another lyase of group [EC 4.1.2] and known to be involved in the metabolism of lactose and D-galactose in *Staphylococcus* and *Streptococcus* species[10,11], was reported to have apparently no stereochemical selectivity, at least with regard to the D-tagatose and D-fructose configurations[10].

Cells of *Escherichia coli* metabolize D-galactose via the Leloir pathway[12], but for the catabolism of galactitol, which can support growth as the only source of carbon and energy, a pathway capitalizing on the aldol cleavage of the intermediate D-tagatose 1,6-bisphosphate was inferred[13], analogous to galactitol dissimilation by *Klebsiella pneumoniae*[14]. While the growth of wild-type cells of *E. coli* on galactitol is restricted to temperatures equal to or below 32°C, resistant mutants have been selected that are able to tolerate temperatures up to 42°C (ref. 13).

Herein we report on the first isolation and characterization of the highly diastereoselective TagA from *E. coli* and detail its application in an expeditious multi-enzymatic synthesis of D-tagatose 1,6-bisphosphate[15] (**1**), its natural substrate. The development was initiated when we required a source of **1** for assaying the TagA, and found that the known route to the compound, consisting of the chemical synthesis and enzymic phosphorylation of D-tagatose 6-phosphate (**6**), was highly unsatisfactory ($\leq 2\%$ overall yield). This original procedure has been modified in an effort to improve its efficiency, and the results are included for comparison.

RESULTS AND DISCUSSION

Purification and characterization of D-*tagatose 1,6-bisphosphate aldolase.*—In crude extracts of galactitol-grown cells of the temperature resistant *E. coli* strain JWL183 (identical to strain L153 referred to previously[13]) both FruA and TagA activities were detected to a comparable extent. A 10-L culture gave 90 g of wet-cell mass containing a total of ~1500 U of TagA and ~1000 U of FruA activity. Complete separation could be achieved by anion-exchange chromatography, proving that the two activities were the property of distinct enzymes. By further application of standard methods of protein purification the TagA was obtained free of extraneous activities that would be incompatible with synthetic applications (e.g., other aldolases, phosphatases, peptidases), in a ~90% pure state, and with 35% overall recovery. This preparation showed a specific activity of 7.5 U/mg, which corresponds to an enrichment factor of ~50.

An attempted isolation of the wild-type protein from strain ECL1, a typical *E. coli* K-12 strain[17], according to the established protocol was accompanied by severe loss of activity during each of the purification steps. Comparative tests for thermal stability over the range 35–60°C indicated an at least 5-fold shorter half-life for the wild-type protein relative to that obtained from the temperature resistant mutant (Fig. 1A). Thus, the selection of a strain for improved growth at elevated temperatures[13] seems to have elicited a structural protein mutation rather than one providing enhanced transcriptional/translational efficiency to compensate for its fast degradation. For obvious reasons, the stable protein of mutant JWL183 was employed in all our subsequent investigations. Denaturing polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) showed a major band for a subunit of mol wt ~28000, followed by faint contaminating bands. Gel permeation studies on the undenatured enzyme gave a mol wt of 110000, indicating that active TagA is a homotetramer similar to the

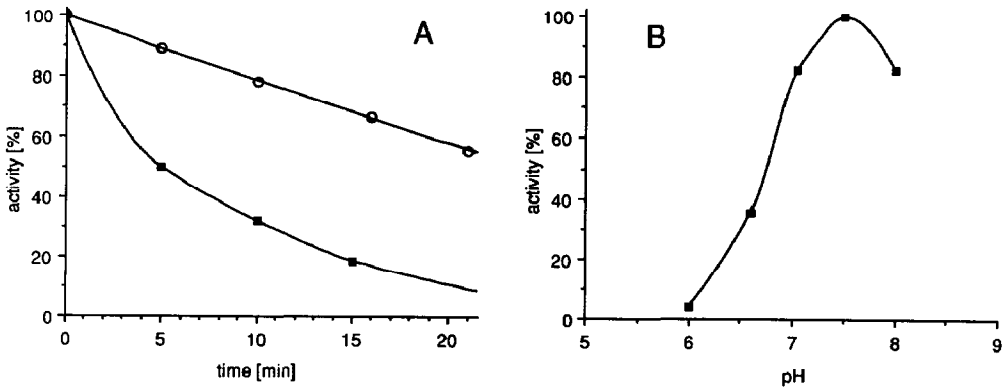


Fig. 1. Characterization of D-tagatose 1,6-bisphosphate aldolase (TagA) from *E. coli*. A: Thermal stability at 45°C; (○) TagA from mutant strain JWL183, (■) TagA from wild-type strain ECL1. B: Effect of pH on enzyme activity. All experiments were conducted in Tris–HCl buffer.

RhuA ($4 \times 30\,000$)[6] and FucA ($4 \times 25\,000$)[6,9,18], but unlike the FruA ($2 \times 39\,000$)[19] enzymes of *E. coli*. The TagA displays a relatively narrow pH optimum around 7.5 (Fig. 1B) but retains considerable activity ($\geq 40\%$) in the pH range 6.5–7.0 that is the most suitable for synthetic applications. By isoelectric focussing (IEF) a pI value of 4.4 was determined that accords with the easy separability of TagA from FruA (pI 5.1) by ion exchange methods.

The purified TagA catalyzes the reversible cleavage of D-tagatose 1,6-bisphosphate into equal amounts of D-glyceraldehyde 3-phosphate (**2**) and dihydroxyacetone phosphate (**3**) (Scheme 1). The enzyme displays a high specificity and affinity for its natural substrate: The cleavage rate for the 4-epimeric D-fructose 1,6-bisphosphate (**5**) is only 1% of that determined for **1**; however, both bisphosphates are bound competitively with low K_m values of 0.33 mM (**1**) and ~ 0.5 mM (**5**). By this measure, **5** constitutes an effective inhibitor for the cleavage of **1**. D-Fructose 1-phosphate (**8**), on the other hand, at concentrations of up to 5 mM is not converted at any detectable rate ($\leq 0.1\%$). While the affinity of the *E. coli* TagA for its natural substrate is within the range characteristic of other class II aldolases, e.g., the FruA from yeast (0.3 mM)[20] or *E. coli* (~ 0.2 mM)[21], it is 10-fold lower than that of class I aldolases, typified by the FruA from rabbit muscle[22] with a K_m of 0.03 mM. Of the TagA enzymes presently known, it is the one with the highest ratio of TagA to FruA activity (**1** : **5** = 100), exceeding those of *Staphylococcus* and *Streptococcus*[10] (1) or *Klebsiella*[23] (25) aldolases.

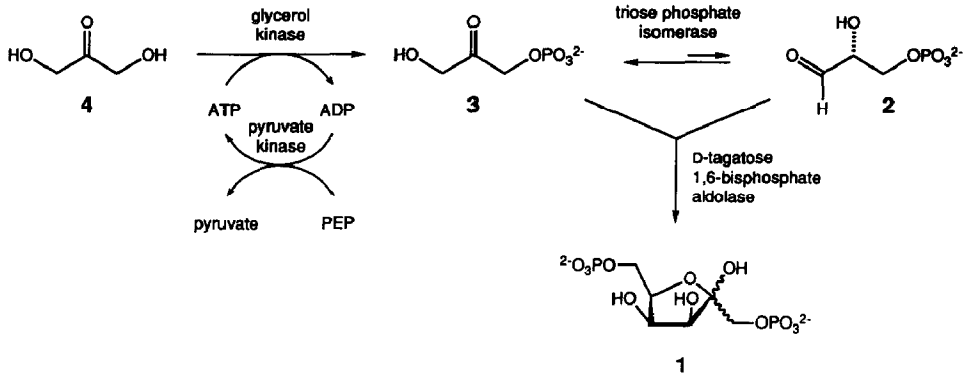
The TagA of *E. coli* indeed belongs to the aldolases of class II, i.e., it requires a divalent metal cation as a cofactor for catalytic function[24]. In particular, TagA activity is completely inhibited (Table I) upon addition of chelating agents like ethylenediaminetetraacetic acid (EDTA) and can be restored by addition of Zn^{2+} ions. Reactivation also occurs upon addition of Co^{2+} (> 100%), Mn^{2+} ($\geq$ 100%), and Ca^{2+} ions (60%), an observation that is in accord with detailed investigations of the cofactor requirements of other aldolases of this group[25]. Conversely, no inactivation occurs upon treatment of the enzyme with sodium borohydride in the

TABLE I

Activity of D-tagatose 1,6-bisphosphate aldolase in the presence of various reagents

Enzyme	Additive(s)	Concentration (mM)	Relative Activity (%)
Native	None		100
Native	DHAP + $NaBH_4$	1 + 10	98
Native	EDTA	5	15
Native	EDTA	15	2
Apoenzyme [a]	Zn^{2+}	20	75
Apoenzyme [a]	Co^{2+}	20	125
Apoenzyme [a]	Mn^{2+}	20	100
Apoenzyme [a]	Ca^{2+}	20	60
Apoenzyme [a]	Cu^{2+}	20	0
Apoenzyme [a]	Mg^{2+}	20	0

[a] Native enzyme inactivated with EDTA, which was left in the sample (see text).



Scheme 2

presence of dihydroxyacetone phosphate, which is evidence against the covalent binding of the substrate by formation of a Schiff base characteristic for class I aldolases[26]. This result establishes that all four dihydroxyacetone phosphate lyases from *E. coli* are mechanistically of the same type, and thereby suggests that these aldolases may have developed from a common ancestor.

Enzymic one-pot synthesis of D-*tagatose 1,6-bisphosphate.*—A preparative exploitation of the TagA in the anabolic direction required that the thermodynamic equilibrium among the components **1**, **2**, and **3** is in favor of C–C bond formation. Whereas in the analogous FruA catalyzed equilibrium the 3,4-*trans* configured adduct **5** strongly predominates ($K_{eq} = [\mathbf{2}][\mathbf{3}]/[\mathbf{5}] = 1.2 \cdot 10^{-4}$ M)[27], the all-*cis* substitution pattern in furanoid D-tagatose 1,6-bisphosphate (**1**, both anomeric forms) must be expected to diminish the relative stability of the product, and hence to decrease the proportion of **1** at equilibrium. Starting from either side (**2** + **3** or **1**), the value of K_{eq} ([**2**][**3**]/[**1**]) was determined and found to be 10^{-3} M. Accordingly, even under optimal conditions, the yield of tagatose 1,6-bisphosphate cannot appreciably exceed 80%.

Given the compatibility of all of the enzymes involved, a multi-enzymic one-pot synthesis could be designed (Scheme 2) that permits the use of commercial dihydroxyacetone (**4**) as the starting material. Phosphorylation by ATP, catalyzed by glycerol kinase and using efficient cofactor recycling based on phosphoenolpyruvate (PEP), readily provides dihydroxyacetone phosphate[28] (**3**), and this can be interconverted in situ with D-glyceraldehyde 3-phosphate (**2**) ($K_{eq} = [\mathbf{3}]/[\mathbf{2}] = 22$)[29] by triose phosphate isomerase. Although the effective concentration of **2** becomes the limiting factor for the subsequent conversion into **1**, the low stationary concentration of **2**, which is the least stable component[30], restricts potential degradation. PEP was given preference over other phosphate donors[31], first because it is quite stable in aqueous solution, which minimizes product contamination by inorganic phosphate, and second because it can be readily prepared on a large scale from pyruvic acid[32]. Substrate conversion and formation of **1** can be

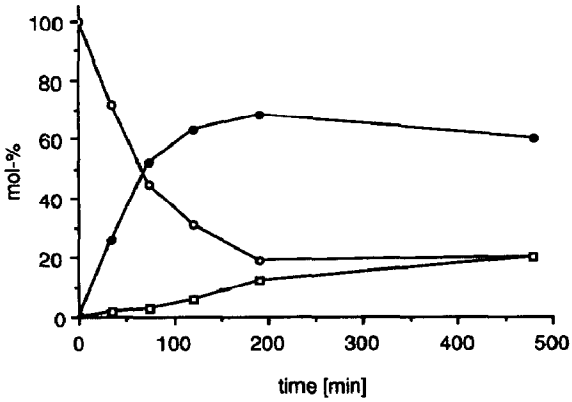


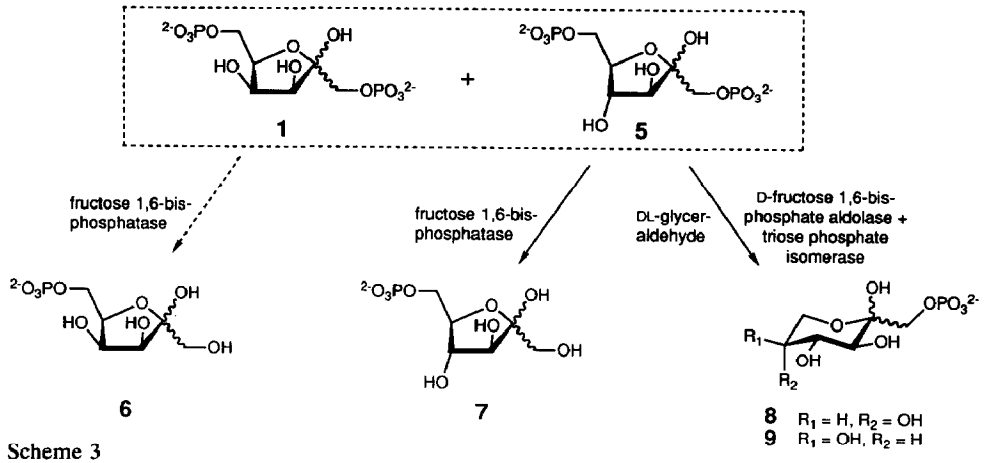
Fig. 2. Product composition during enzymic synthesis of D-tagatose 1,6-bisphosphate starting from dihydroxyacetone phosphate (**3**, 200 mM) in the presence of 50 U/mL triose phosphate isomerase and 8 U/mL D-tagatose 1,6-bisphosphate aldolase, pH 7.0, 25°C . Concentrations of triose phosphates (○, sum of **2** and **3**), D-tagatose 1,6-bisphosphate (●), and D-fructose 1,6-bisphosphate (□) are presented as mol% of total phosphate equivalents.

easily monitored by enzymatic assays for **1** and for PEP/pyruvate, and by TLC using 1:1 saturated ammonia–ethanol as irrigant. Typically, **1** is the slowest moving component, indicative of its high charge and polarity.

In the course of these investigations we found that besides the known equilibrium components an increasing fraction of D-fructose 1,6-bisphosphate (**5**) was unexpectedly formed as the reaction progressed (Fig. 2). Obviously, this must be a result of the incomplete disastereoselectivity of the TagA enzyme, as manifested in the cleavage direction by the 1% rate for **5**. Accumulation of this undesired 4-epimer benefits from several factors, including its superior thermodynamic stability, its low cleavage rate, and the full reversibility of D-tagatose 1,6-bisphosphate synthesis. Timely termination of the reaction, after the formation of ~ 60–65% of **1**, guarantees a low percentage ($\leq$ 10%) of **5** in the product mixture.

In order to facilitate the isolation of **1** from the reaction mixture it seemed appropriate to convert the contaminating bisphosphate **5** into less charged products. Selective hydrolysis of the 1-phosphate group of **5** is catalyzed by fructose 1,6-bisphosphatase with formation of D-fructose 6-phosphate (**8**, Scheme 3). Of the two commercial phosphatases tested, only the yeast enzyme was found to be specific for **5**, while the one from rabbit muscle also slowly (~ 5% of V_{max}) acted upon the 4-epimeric **1** with formation of D-tagatose 6-phosphate (**6**). This reaction can easily be combined with the above one-pot scheme for convenience to furnish a product of high purity after isolation by ion-exchange chromatography using triethylammonium hydrogencarbonate buffer. Nevertheless, potential contamination of the product by inorganic phosphate, the high pH optimum at 9.5, and the relatively high cost of the phosphatases make this option somewhat inefficient.

Alternatively, **5** can be specifically cleaved by FruA in the presence of **1**, and the dihydroxyacetone phosphate liberated can be recombined quantitatively with an



Scheme 3

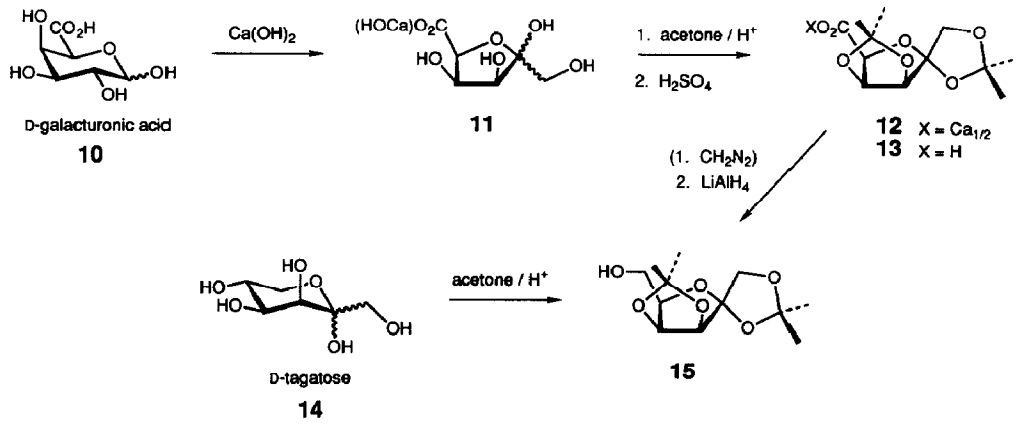
added uncharged aldehyde to produce a ketose 1-phosphate[7]. For obvious reasons, this process requires the previous separation of TagA which, for example, can be achieved by ultrafiltration for the possible recycling of the enzyme. In our study we used DL-glyceraldehyde, which provides favorable reaction rates and equilibria, and product stability, but other aldehydes may be used. Auxiliary triose phosphate isomerase assures the fast and complete conversion of **5** into D-fructose and L-sorbose 1-phosphates (**8** and **9**) as the sole byproducts. This second alternative, although less convenient experimentally, is preferable because of the ease of product purification by ion-exchange chromatography, and because of the lower costs involved.

Both procedures furnish **1** after crystallization as the tetrakis(cyclohexylammonium) salt in a 40% overall yield based on the limiting reactant, the phosphorylating agent PEP. While this result is quite satisfactory for the synthesis of 10 mmol amounts, further optimization may still be possible by the application of larger amounts of TagA. The resulting shorter reaction periods would diminish the degradation of **2** and **3**.

Product identity was confirmed by complete enzymic dephosphorylation of **1** using acid phosphatase, which yielded D-tagatose (**14**) indistinguishable by its spectroscopic data[33] and optical rotation from the product obtained by the chemical synthesis described below, and from an authentic sample.

In case a less costly supply of rabbit muscle fructose 1,6-bisphosphatase would become available, a preparation of D-tagatose 6-phosphate (**6**) by regioselective enzymic hydrolysis of the 1,6-bisphosphate **1** seems to be feasible, and might favorably compare with chemical synthesis[16] in overall yield and convenience.

Chemoenzymic synthesis of D-*tagatose 1,6-bisphosphate* (**1**).—Due to the biological importance of **1** as a crucial metabolite in the galactose or galactitol catabolism of several microorganisms[10,11,13,14], a chemical synthesis had been developed earlier (Scheme 4)[16]. This synthesis exploits the stereochemical relationship with D-galac-

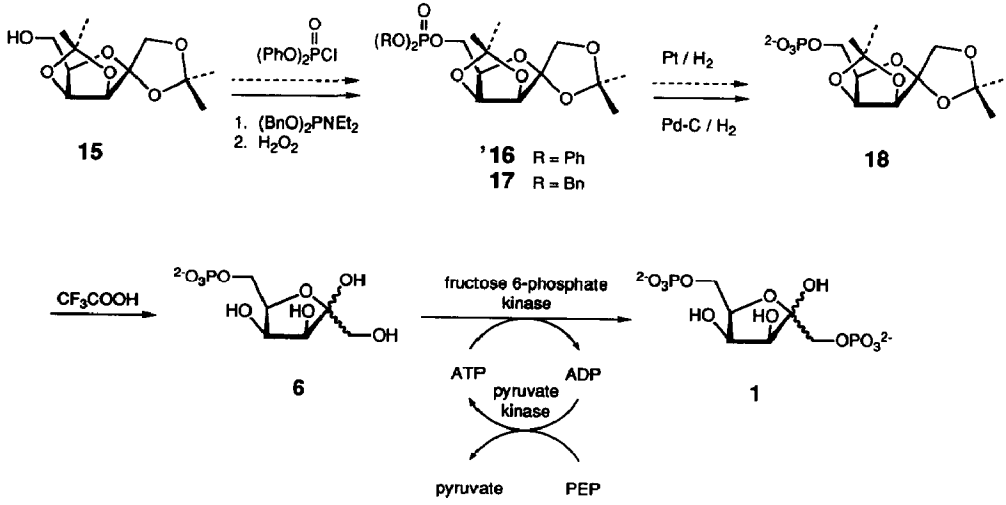


Scheme 4

turonic acid (**10**), a compound that is readily available from the chiral pool, and profits from the observation[34] that the isomerization of **10** to the ketose form D-*arabino*-hex-5-ulosonic acid ("D-tagaturonic acid") is highly favored by the formation of almost insoluble basic lead or calcium (**11**) salts of the product. This low solubility is probably due to stable internal complexation of the counterions. Protection as the diisopropylidene acetal (**12**) followed by reduction of the acid group via its methyl ester produced 1,2:3,4-di-*O*-isopropylidene-D-tagatofuranose (**15**, 12% from **10**). A shorter, more effective version[35] employs commercial D-tagatose (**14**), which can be directly converted into the acetonide **15** (40% yield)[36]. The high cost of **14** as the starting material, however, makes this route impractical for the preparation of **1** in amounts larger than ~ 1 mmol.

The unprotected primary hydroxyl group in **15** had been esterified to give the diphenyl phosphate derivative **16** (Scheme 5)[37], which was then fully deprotected by hydrogenolysis over platinum and treatment of the resulting **18** with sulfuric acid. The D-tagatose 6-phosphate (**6**) thus produced was isolated by precipitation as its barium salt. The second phosphorylation[38] was conducted by enzymic transfer from ATP catalyzed by fructose 6-phosphate kinase (phosphofructokinase, PFK), which finally delivered **1** as a barium precipitate in unspecified yield and purity.

In attempts to improve upon this situation we found that diazomethane esterification of the hexulosonic acid acetal **13** prior to its reduction was not requisite, as the acid could be directly reduced to the primary alcohol **15** by using lithium aluminum hydride. With a stoichiometric alkaline work-up[39] instead of the standard acidic procedure, the isolation of **15** proved not only to be more convenient, particularly on a larger scale, but also much more efficient than in the older method[16] (76% as compared to 41%; 33% overall yield from **10**). In addition, when the previously employed phosphorylating reagent diphenyl phosphorochloridate was replaced by the more recently developed dibenzyl di-*N*-ethyl-phosphoramidite[40] followed by hydrogen peroxide oxidation, the corresponding dibenzyl phos-



Scheme 5

phate ester **17** was obtained in near quantitative yield. Hydrogenation of the latter over Pd–C proceeded under mild conditions to provide the D-tagatose 6-phosphate acetal **18**, with an excellent result overall for the phosphorylation sequence. D-Tagatose 6-phosphate (**6**) was isolated as its sodium salt for characterization and to assure its purity, but for the preparation of **1** it can be used directly in solution without prior isolation. Enzymic phosphorylation by rabbit muscle PFK was conducted using a catalytic quantity of ATP coupled with a cofactor recycling system based on PEP and pyruvate kinase. This option was chosen over the use of stoichiometric amounts of ATP to obviate the pronounced inhibitory effect of the latter[41] on the PFK. Bisphosphate **1** produced by this means was isolated by anion-exchange chromatography using triethylammonium hydrogencarbonate as a volatile buffer and was crystallized as the tetrakis(cyclohexylammonium) salt. The overall yield from D-galacturonic acid **10** was 18%. Although the chemical synthesis thereby could be markedly improved, it still falls short of the one-pot synthesis using TagA as detailed above.

Evaluation of a microbial fructose 6-phosphate kinase.—Because of the strong regulatory effects involved in the use of the mammalian PFK (inhibition by several metabolites including the reaction product[8,37]) and to reduce enzyme costs, the "non-allosteric" fructose 6-phosphate kinase (Pfk-2) of *E. coli* was investigated as a potential substitute. Unlike the major Pfk-1 isozyme this microbial enzyme is known to convert D-tagatose 6-phosphate to **1**, and therefore had been thought to have a function in galactitol metabolism[42]. Its kinetic constants for **6**, however, had been reported to be very unfavorable as compared to those for D-fructose 6-phosphate, i.e., a considerably higher K_m value of 6 mM and a relative V_{max} of less than 20%.

The Pfk-2 isozyme was obtained from recombinant *E. coli* strain[43] DF1020/pFD110 from which the structural genes responsible for the Pfk-1 and Pfk-2 isoenzymes have been deleted[42], and a plasmid coding for Pfk-2 is overexpressed. Since cell extracts from this strain are devoid of competing Pfk-1 activity, isolation of the enzyme was straightforward, and it was successfully applied in the preparative phosphorylation of **6** to **1**.

At variance with literature data, the kinetic constants of Pfk-2 for D-tagatose 6-phosphate were determined to be $K_m = 0.8$ mM, *rel* $V_{max} = 39\%$ (V_{max} for **5** = 100%). The enzyme thus has capabilities within the same range as the rabbit muscle enzyme (for **6**, $K_m = 0.2$ mM, *rel* $V_{max} = 69\%$; for **5**, $K_m = 0.15$ mM, *rel* $V_{max} = 100\%$). Because the overexpressed microbial Pfk-2 is so readily obtainable at low cost, and far less subject to inhibition, e.g., by PEP even at high concentrations (~ 30% activity at > 300 mM)[8], it can be recommended as an effective replacement for the mammalian enzyme.

Chemical shift assignments and tautomeric composition of D-*tagatose 1,6-bisphosphate in aqueous solution.*—Although phosphorylation at C-6 restricts the composition of ketoses in solution to furanoid or open-chain isomers, their individual affinity and reactivity at enzymatic binding sites will depend on the distribution, and the rate of interconversion, of their α- and β-anomeric forms. The unusual all-*cis* substitution pattern of furanoid tagatose derivatives merited a more detailed investigation. Thus, the ^1H and ^{13}C NMR spectra of D-tagatose 1,6-bisphosphate and, for comparison, those of D-tagatose and D-tagatose 6-phosphate were determined at ambient temperature in D_2O at pH 7.0. Chemical shift assignments for the proton signals followed from the vicinal coupling patterns, and from the fact that the C-6(1) methylene AB systems are split upon phosphorylation by additional ^1H–^{31}P coupling. For the carbon spectra, the degree of protonation was determined through DEPT experiments. Signal assignments for phosphate esters were facilitated by the presence of characteristic 2-bond and 3-bond ^{13}C–^{31}P couplings to C-6(1) and C-5(2) for which typically[44] the vicinal couplings $^3J_{POCC}$

TABLE II

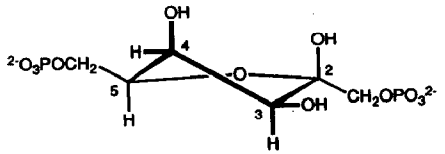
^{13}C NMR chemical shifts and ^{31}P–^{13}C coupling constants for D-tagatose 1,6-bisphosphate (**1**), D-tagatose 6-phosphate (**6**), and D-tagatose (**14**)

Structure	Chemical shifts and J values [a]					
	C-1 ($^2J_{C,P}$)	C-2 ($^3J_{C,P}$)	C-3	C-4	C-5 ($^3J_{C,P}$)	C-6 ($^2J_{C,P}$)
α-1	65.68 (br) [b]	105.09 (6.0)	77.18	71.41	79.01 (6.2)	63.14 (3.0)
β-1	66.31 (br) [b]	102.59 (8.2)	71.11 [c]	71.79 [c]	79.47 (7.1)	62.91 (br) [b]
α-6	62.90	105.61	77.73	71.69	78.65 (8.0)	64.53 (4.0)
β-6	63.15	103.11	70.97 [c]	71.27 [c]	79.43 (7.8)	64.96 (4.3)
α-14	62.87	105.90	77.23	71.69	79.63	62.37 [c]
β-14	63.06	102.97	71.11	71.56	80.46	61.41

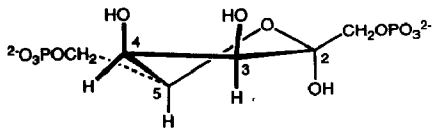
[a] Determined at 100.6 MHz in D_2O at pH 7.0; chemical shifts in ppm relative to tetramethylsilane, measured from internal acetonitrile at 1.30 ppm; J in Hz. [b] br, Broad signals, coupling unresolved. [c] Assignments tentative.

were found to be larger than the geminal values $^2J_{POC}$. The NMR results for incrementally phosphorylated tagatose compiled in Table II parallel those of previously, albeit incompletely, determined data for the furanoid isomers of **14** (ref. 33) and **6** (ref. 44).

According to integrations of the ^{13}C signals (average of the respective sets) and the similarly well-separated H-4 proton signals, **1** at equilibrium consists of β and α anomers in a ratio of 80:20, very close to the values calculated (82:18) and measured (83:17) for **6**. For the β and α anomers of **1** the signal for H-4 appears as a triplet, with coupling constants of 4.8 and 5.5 Hz, respectively, For each individual case, the equivalence of $J_{3,4}$ and $J_{4,5}$ is indicative of a population of conformers of similar energy interconverting by pseudorotation[45], particularly plausible for all-*cis* substitution. The magnitude of the respective couplings is consistent with a predominant contribution of the $^4T_3$ conformation for the β and the $^OT_5$ conformation for the α anomer.



$^4T_3$ (β)



$^OT_5$ (α)

Although the contrathermodynamic arrangement of ring substituents in phosphates **1** and **6** should strongly destabilize cyclic forms, no signals for the linear keto forms or their hydrates could be detected within the sensitivity limits of the measurement ($\sim$ 2%). The nonobservance of the relevant C-2 signals is indeed not surprising, since these resonances would likely be obscured by line-broadening due to relatively rapid interconversion between the keto and cyclic forms. The detection of open-chain or hydrated keto forms would thus require the higher sensitivity attainable with appropriately ^{13}C-labeled compounds, which are as yet unavailable.

In conclusion, we have isolated for the first time and characterized the D-tagatose 1,6-bisphosphate aldolase from *E. coli* as a useful catalyst for asymmetric C–C bond formation with comparatively high diastereoselectivity. The enzyme thus allows a novel, very simple preparative synthesis of the valuable metabolic intermediate D-tagatose 1,6-bisphosphate (**1**), and consequently of the rare keto sugar D-tagatose (**14**), from readily available achiral sources without the need for tedious or costly manipulation of protecting groups.

EXPERIMENTAL

General.—^1H and ^{13}C NMR spectra were recorded on a Bruker WM-400 instrument operating at 400 and 100.6 MHz, respectively, with sodium (2,2,3,3-$^2H_4$)-3-(trimethylsilyl)-propionate and acetonitrile (δ_H = 0.00 and δ_c = 1.3, respectively, in D_2O), or tetramethylsilane (δ 0.00 and 0.00, $CDCl_3$) as internal stan-

dards. Optical rotations were determined with a Perkin–Elmer 241 polarimeter. Melting points were obtained with a Büchi 510 apparatus and are uncorrected. Reactions were monitored by TLC on Silica Gel 60 F_{254} (Merck), with detection by UV light or by staining with anisaldehyde[46]. Commercial enzymes were purchased from Sigma: acid phosphatase (sweet potato, type XA), fructose 6-phosphate kinase (rabbit muscle, type III), fructose 1,6-bisphosphatase (rabbit muscle and *Torula* yeast), fructose 1,6-bisphosphate aldolase (rabbit muscle, type IV), glycerol kinase (*E. coli*), glycerol phosphate dehydrogenase (rabbit muscle, type I), pyruvate kinase (rabbit muscle, type III), and triose phosphate isomerase (rabbit muscle, type III-S). Photometric measurements were made with an Eppendorf photometer 1101M or a Perkin–Elmer spectrophotometer Lambda 15. Protein concentration was determined by the Bradford method[47] with assay reagents supplied by Bio-Rad, using bovine serum albumin for calibration. Media for column chromatography (DEAE-Sepharose CL-6B and Sephadex G-150) and precast gels for isoelectric focussing (Ampholine PAGplate 3.5-9.5) were obtained from Pharmacia and used according to the manufacturer's instructions. Membranes YM-10 for ultrafiltration were purchased from Amicon. PEP monopotassium salt was prepared by the procedure of Hirschbein et al.[32].

Enzyme assays.—The D-tagatose 1,6-bisphosphate aldolase (TagA) assay solution (500 μL) contained D-tagatose 1,6-bisphosphate (2.4 mM), reduced nicotinamide adenine dinucleotide (NADH, 0.2 mM) and potassium chloride (60 mM) in Tris buffer (60 mM, pH 7.5), to which was added glycerol phosphate dehydrogenase (3 μL, 1U). Control runs also included triose phosphate isomerase (3 μL, 20 U). After addition of an aliquot of the TagA containing solution (10–25 μL), NADH oxidation was determined photometrically at 366 nm. One unit of TagA is defined as the activity catalyzing the cleavage of 1 μmol of D-tagatose 1,6-bisphosphate per minute at 25°C and pH 7.5.

The activity of fructose 1,6-bisphosphatase was estimated by analysis for remaining substrate at appropriate time intervals in reaction mixtures containing D-fructose 1,6-bisphosphate (**5**) or D-tagatose 1,6-bisphosphate (**1**) at 10 mM concentration. Activities of the enzymes glycerol kinase, pyruvate kinase, D-fructose 6-phosphate kinase, D-fructose 1,6-bisphosphate aldolase (FruA), and triose phosphate isomerase, and concentrations of the substrates dihydroxyacetone phosphate, D-glyceraldehyde 3-phosphate, D-fructose 1,6-bisphosphate, phosphoenolpyruvate, and pyruvate were determined by photometric assays performed according to standard methods[48].

Purification of D-tagatose 1,6-bisphosphate aldolase (TagA).—E. coli strain JWL183 (ref. 13) was grown aerobically to late logarithmic phase at 37 °C in a 1-L shake flask in 200 mL of a mineral salt medium[49] supplemented with 1% casamino acids and 0.5% galactitol. This culture was used as the inoculum for a 10-L fermentor (New Brunswick) containing the same medium, and growth was continued with aeration at 37°C. Bacteria were harvested at the end of the log phase by continuous centrifugation (Sharples centrifuge) to yield ~ 90 g of wet cell mass.

Cells were resuspended in four times their wet weight of cold preparation buffer (20 mM Tris, 10 mM mercaptoethanol, 1 mM $ZnCl_2$, pH 7.2) and disrupted by three-fold passage through a French press. Extracts were clarified by centrifugation at 24000*g* (total activity 1400 U) and applied to a DEAE-Sepharose column (5 × 32 cm) equilibrated with preparation buffer. The column was washed with preparation buffer containing 150 mM NaCl (500 mL) and protein was eluted with a linear gradient of NaCl (150–400 mM, 2.4 L). Active fractions were pooled and concentrated to a volume of 30 mL by ultrafiltration (total activity 650 U). The concentrate was applied to a Sephadex G-150 column (5 × 78 cm) equilibrated with preparation buffer containing 100 mM NaCl and active fractions were pooled and concentrated to a volume of 10 mL by ultrafiltration (total activity 500 U).

Molecular weight determination.—SDS gel electrophoresis was performed according to the method of Laemmli[49] using 12% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue. The following proteins were used for calibration: bovine serum albumin (66000), chicken ovalbumin (45000), yeast alcohol dehydrogenase (39800), bovine carbonic anhydrase (29000), chymotrypsinogen (25000), and myoglobin (17000). From a semilogarithmic plot of the relative electrophoretic mobility against the mol wt a linear calibration curve was obtained with a correlation coefficient of 0.99. The subunit mol wt of the TagA was calculated as ~ 28000. In a similar manner, the mol wt was determined by gel chromatography using a Sephadex G-150 column (2 × 95 cm) that had been calibrated with blue dextran (2000000), rabbit muscle aldolase (160000), bovine serum albumin (68000), and cytochrome C (12300). The mol wt of the native enzyme was calculated as ~ 110000.

pH Optimum.—For determination of the pH profile the standard assay for TagA was modified by adjusting the pH of the assay buffer in 0.5 unit increments in the range 6.0–9.0.

Temperature stability.—Aliquots of a solution of purified TagA (28 U/mL) in Tris buffer (20 mM, pH 7.2) containing 10 mM mercaptoethanol and mM $ZnCl_2$ were heated at 35, 45, and 60°C in a water bath. At 5 min intervals samples were withdrawn, rapidly cooled in an ice bath, and the remaining activity was determined.

Kinetic measurements.—The kinetic parameters of TagA for D-tagatose 1,6-bisphosphate and D-fructose 1,6-bisphosphate were derived by measuring the reaction rate at various substrate concentrations ranging from 0.1 to 5.0 mM in the presence of ~ 0.1 U of TagA per assay. Studies of the two PFK enzymes with D-fructose 6-phosphate and D-tagatose 6-phosphate employed substrate concentrations ranging from 0.1 to 5.0 mM and ~ 0.1 U of the respective enzyme per assay. Data processing was performed according to standard linearization methods (Lineweaver–Burk, Eadie–Hofstee and Hanes–Woolf)[51] as implemented in the program *EnzymeKinetics* (Trinity Software).

Inactivation and metal replacement studies.—For the test of class I activity[26], purified TagA in 200 mM phosphate buffer at pH 6.0 was treated with mM

dihydroxyacetone phosphate, then a solution of $NaBH_4$ (M in 10 mM NaOH) was slowly introduced during 10 min to produce a final concentration of 10 mM. The remaining activity was determined in aliquots removed from the mixture and neutralized with acetic acid prior to the measurements. For verification, a sample of rabbit muscle FruA was subjected to an analogous treatment, which caused complete loss of activity.

For the test of class II activity, a sample of the TagA solution was treated with 100 mM EDTA in small aliquots up to a final concentration of 15 mM, at which point the remaining activity had dropped below ~2% of the original value (dialysis against 200 mM EDTA for 20 h proved less efficient). Samples of this mixture were then assayed in buffer modified by supplementation with one of various divalent metal salts at 20 mM.

Equilibrium constant.—Solutions of D-tagatose 1,6-bisphosphate (**1**) at various concentrations (20, 40, and 150 mM) in Tris buffer (20 mM, pH 7.2) containing 10 mM mercaptoethanol and mM $ZnCl_2$ were incubated with TagA (5 U/mL) at 25°C. Conversion was monitored by enzymic assay in aliquots (50 μL) that were removed at 1 h intervals. The reaction was stopped by the addition of 7% perchloric acid (30 μL), and the precipitate was removed by centrifugation. The supernatant was neutralized by the addition of M NaOH (20 μL). The resulting solution was assayed for dihydroxyacetone phosphate under standard conditions, and the sum of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate equivalents was determined separately in the presence of added triose phosphate isomerase. Bisphosphates **1** and **5** were assayed independently as described above.

Purification of the Pfk-2 from E. coli.—A 200-mL starter culture of *E. coli* strain DF1020/pFD110 (ref. 43) was grown aerobically to late log phase at 37°C on LB medium supplemented with tetracycline (5 μg/mL). A 10-L fermentor containing the same medium was inoculated with this culture. After aerobic cultivation at 37°C, bacteria were harvested by centrifugation to provide 40 g of wet cell mass. A portion of the latter (11 g) was resuspended in preparation buffer (10 mM Tris, 10 mM mercaptoethanol, 0.1 mM EDTA, 5 mM $MgCl_2$, pH 7.5), cells were disrupted mechanically, and the suspension was centrifuged as described above. The crude extract (total activity 31 000 U) was subjected to DEAE-Sepharose chromatography, and the kinase was eluted using a linear gradient of NaCl (0–500 mM, 500 mL). Active fractions were pooled and concentrated to 9 mL by ultrafiltration (total activity 14 500 U).

Calcium D-arabino-*hex-5-ulosonate hydroxide* (**11**).—D-Galacturonic acid (**10**, 60.0 g, 309 mmol) was dissolved in 15 L of cold 0.15% $Ca(OH)_2$ solution with vigorous stirring. On keeping the solution at room temperature for 7 days crystals formed, and these were filtered off, washed with ice–water, and dried in vacuo to yield **11** (56.4 g, 86%; lit.[16] 83%). For spectral characterization, a sample was mixed with ion-exchange resin (Dowex 50W-X8, H^+ form) prior to the measurements. ^1H NMR data (D_2O): (in this list *R* indicates the anomer that would be designated β if numbering were as in D-tagatose, *S* denotes the corresponding "α"

anomer, and *h* a lactone-hemiacetal form)* δ 3.59 (s, H-6*R*), 3.66 (d, *J* 12.0 Hz, H-6a*S*), 3.72 (d, *J* 12.0 Hz, H-6b*S*), 4.03 (dd, *J* 9.0, 1.5 Hz, H-3*h*), 4.21 (d, *J* 4.5 Hz, H-4*R*), 4.25 (d, *J* 4.8 Hz, H-4*S*), 4.28 (d, *J* 1.5 Hz, H-2*h*), 4.32 (d, *J* 9.0 Hz, H-4*h*), 4.46 (d, *J* 4.5 Hz, H-3*R*), 4.48 (s, H-2*R*), 4.49 (dd, *J* 4.8, 3.8 Hz, H-3*S*), 4.54 (d, *J* 19.5 Hz, H-6a*h*), 4.63 (d, *J* 3.8 Hz, H-2*S*), and 4.65 (d, *J* 19.5 Hz, H-6b*h*); ^{13}C NMR (D_2O): δ 62.86 (C-6*R*), 63.29 (C-6*S*), 70.92 (C-4*R*), 72.41 (C-3*R*), 72.93 (C-3*S*), 78.47 (C-4*S*), 80.50 (C-2*S*), 81.44 (C-2*R*), 103.82 (C-5*R*), 106.05 (C-5*S*), 175.76 (C-1*S*), and 176.19 (C-1*R*). Integration showed the ratio of the *S* and *R* anomers and lactone hemiacetal to be 27:61:12.

*Calcium (5*R*)-3,4:5,6-di-*O*-isopropylidene*-D-arabino-*hex-5-ulo-5,2-furanosonate* (**12**).—To a solution of the calcium hexulosonate hydroxide **11** (49.8 g, 234 mmol) in 1.1 L of anhyd acetone was added concd H_2SO_4 (81.0 g, 830 mmol) and powdered 4A molecular sieves (60 g). The mixture was stirred at 25°C for 5 h when TLC indicated complete conversion. The suspension was filtered and $Ca(OH)_2$ (~ 75 g, 1.0 mol) was added to the solution until it became slightly alkaline. Following filtration, the solvent was removed in vacuo to yield **12** (38.9 g, 57%; lit.[16] 48%) as a colorless solid; ^{1}H NMR ($CDCl_3$): δ 1.29, 1.35, 1.41, and 1.45 (4 s, CH_3), 4.21 (d, *J* 9.8 Hz, H-6a), 4.28 (d, *J* 9.8 Hz, H-6b), 4.51 (d, *J* 3.8 Hz, H-2), 4.56 (d, *J* 6.0 Hz, H-4), and 5.14 (dd, *J* 6.0, 3.8 Hz, H-3); ^{13}C NMR ($CDCl_3$): δ 24.73, 25.60, 25.81, and 26.22 [2 C(*C*H_3)$_2$], 68.55 (C-6), 80.05 and 80.43 (C-3 and C-2), 84.11 (C-4), 111.50, 111.50 and 112.50 [C-5 and 2 *C*(CH$_3$)$_2$], and 173.74 (C-1).

*(5*R*-3,4:5,6-Di-O-isopropylidene*-D-arabino-*hex-5-ulo-5,2-furanosonic acid* (**13**). —Calcium salt **12** (6.3 g, 21.5 mmol) was added with vigorous stirring to an ice-cooled mixture of 10% H_2SO_4 (33 mL), water (100 mL), and ether (100 mL). After 5 min the organic layer was separated, washed with cold water (4 × 100 mL), and dried ($MgSO_4$). Evaporation in vacuo yielded **13** (5.1 g, 87%; lit.[16] 73%); ^{1}H NMR ($CDCl_3$): δ 1.32, 1.40, 1.41, and 1.47 (4 s, CH_3), 4.25 (d, *J* 10.8 Hz, H-6a), 4.34 (d, *J* 10.8 Hz, H-6b), 4.62 (d, *J* 4.5 Hz, H-2), 4.64 (d, *J* 6.0 Hz, H-4), and 5.11 (dd, *J* 6.0, 4.5 Hz, H-3); ^{13}C NMR ($CDCl_3$): δ 25.08, 25.86, 26.12, and 26.32 [2 C(*C*H$_3$)$_2$], 68.95 (C-6), 78.61 and 80.58 (C-2 and C-3), 84.20 (C-4), 112.28, 112.32, and 113.69 [C-5 and 2 *C*(CH$_3$)$_2$], and 171.13 (C-1).

*1,2:3,4-Di-O-isopropylidene-α-*D*-tagatofuranose* (**15**).—A solution of carboxylic acid **13** (5.04 g, 18.4 mmol) in anhyd diethyl ether (250 mL) was cautiously added during 1 h to a suspension of $LiAlH_4$ (4.59 g, 121 mmol) in the same solvent (50 mL) at 25°C. After stirring for 5 h, water (4.6 mL), 15% NaOH (4.6 mL), and water (13.8 mL), in succession, were slowly introduced. After the reaction mixture had become completely colorless, anyhyd $MgSO_4$ (~ 50 g) was added and stirring was continued for 10 min. After filtration, the solvent was evaporated and the residual syrup was purified by chromatography on silica gel by elution with EtOAc.

* The sequence rule designations *R* and *S* are used when the anomeric carbon (C-5) has a higher locant than the configurational reference carbon (C-4).

Evaporation and recrystallization from petroleum ether (30–50°C) afforded colorless needles of **15** (3.64 g, 76%; lit.[16] 41%); ^{1}H NMR ($CDCl_3$): δ 1.32, 1.40, 1.44, and 1.48 (4 s, CH_3), 2.17 (dd, OH), 3.93 (m, H-6a,6b), 4.07 (m, J 3.8 Hz, H-5), 4.08 (d, J 9.8 Hz, H-1a), 4.28 (d, J 9.8 Hz, H-1b), 4.64 (d, J 6.0 Hz, H-3), and 4.85 (dd, J 6.0, 3.8 Hz, H-4); ^{13}C NMR ($CDCl_3$): δ 24.54 and 25.80 [C(CH_3)$_2$], 26.31 [C(CH_3)$_2$], 60.81 (C-6), 69.07 (C-1), 78.79 and 80.38 (C-4 and C-5), 85.26 (C-3), 111.53, 111.65, and 112.73 [C-2 and 2C(CH$_3$)$_2$]. Anal. Calcd for $C_{12}H_{20}O_6$ (260.3): C, 55.37; H, 7.74. Found: C, 55.14; H, 7.78.

*1,2:3,4-Di-*O*-isopropylidene-α-*D*-tagatofuranose 6-(dibenzyl phosphate)* (**17**).—To a solution of alcohol **15** (1.00 g, 3.8 mmol) in anhyd tetrahydrofuran (15 mL) was added dibenzyl di-*N*-ethylphosphoramidite[40] (2.53 g, 7.9 mmol) and 1,2,4-triazole (525 mg, 7.6 mmol), and the mixture was stirred at room temperature for 24 h. It was then cooled to −78°C, 30% H_2O_2 (1.3 mL, 11.5 mmol) was added in a single portion, and the mixture was allowed to warm to 25°C with stirring for 90 min. Following evaporation, the colorless oily residue was dissolved in ether (50 mL) and extracted successively with N sodium hydrogensulfite (10 mL), N HCl (2 × 25 mL), satd $NaHCO_3$ (25 mL), and water (25 mL). The organic layer was dried ($MgSO_4$) and the solvent was removed by evaporation. Column chromatography on silica gel using EtOAc as eluent provided **17** (2.1 g, 98%) as a colorless, chromatographically homogeneous syrup; ^{1}H NMR ($CDCl_3$): δ 1.28, 1.36, 1.38, and 1.43 (4 s, CH_3), 4.01 (d, J 9.4 Hz, H-1a), 4.24 (d, J 9.4 Hz, H-1b), 4.11–4.36 (m, H-5,6a,6b), 4.59 (d, J 6.0 Hz, H-3), 4.74 (dd, J 6.0, 3.4 Hz, H-4), 4.95 and 5.01 (dAB, J 13.2, 7.9 Hz, PhCH_2), 5.08 (d, J 7.5 Hz, PhCH_2); ^{13}C NMR ($CDCl_3$): δ 24.41 and 25.57 (2 CH_3), 26.01 (2 CH_3), 65.17 (d, $J_{C,P}$ 5.1 Hz, C-6), 68.81 (C-1), 68.92 (d, $J_{C,P}$ 2.7 Hz, 2 PhCH_2), 77.29 (d, $J_{C,P}$ 4.7 Hz, C-5), 79.28 (C-4), 84.71 (C-3), 111.42, 111.61, and 112.58 [C-2 and 2 C(CH$_3$)$_2$], 127.55 [Ph-C(p)], 128.19 and 128.23 [Ph-C(o,m)], and 135.40 [Ph-C(*ipso*)]. Anal. Calcd for $C_{26}H_{33}O_9P$ (520.5): C, 60.00; H, 6.39. Found: C, 60.21; H, 6.23.

*1,2:3,4-Di-*O*-isopropylidene-α-*D*-tagatofuranose 6-(dihydrogen phosphate)* (**18**).—Dibenzyl ester **17** (260 mg, 0.5 mmol) was dissolved in 15 mL of anhyd EtOH, 5% Pd–C (50 mg) was added, and the suspension was vigorously stirred under an H_2 atmosphere for 20 h. The mixture was filtered and concentrated in a rotary evaporator at temperatures below 20°C (bath temperature) under high vacuum, and the residue was recrystallized from pentane to yield **18** (163 mg, 95%), identical to a sample prepared according to the literature procedure[37]; ^{1}H NMR ($CDCl_3$–CD_3OD 5:1): δ 1.30, 1.39, 1.40, and 1.45 (4 s, CH_3), 4.04 (d, J 9.8 Hz, H-1a), 4.12 (br, 2 OH), 4.10–4.30 (m, H-5,6a,6b), 4.26 (d, J 9.8 Hz, H-1b), 4.63 (d, J 6.0 Hz, H-3), and 4.83 (dd, J 6.0, 9.8 Hz, H-4); ^{13}C NMR ($CDCl_3$–CD_3OD 5:1): δ 23.79 and 24.95 [C(CH_3)$_2$], 25.37 [C(CH_3)$_2$], 63.48 (d, $J_{C,P}$ 4.7 Hz, C-6), 68.34 (C-1), 77.30 (d, $J_{C,P}$ 8.8 Hz, C-5), 78.95 (C-4), 84.28 (C-3), 111.11, 111.15, and 112.15 [C-2 and 2 C(CH$_3$)$_2$].

D-Tagatose 6-phosphate, disodium salt (**6**).—An aqueous solution (5 mL) of acetonide **18** (163 mg, 0.5 mmol) was acidified with trifluoroacetic acid (0.5 mL)

and stirred at room temperature for 24 h. After concentration in a rotary evaporator at 20°C (bath temperature) under high vacuum to a residual volume of 0.5 mL, the solution was rediluted to 10 mL and passed through an ion-exchange column (Dowex 50W-X8, Na^+ form; 0.5 × 2 cm) to provide, after evaporation as described before, the disodium salt of **6** (140 mg, 98%).

Enzymic phosphorylation of **6**.—An aqueous solution (60 mL) containing disodium D-tagatose 6-phosphate (**6**, 4.70 g, 15.6 mmol), monopotassium phosphoenolpyruvate (2.86 g, 13.7 mmol), ATP disodium salt (212 mg, 0.35 mmol), and $MgCl_2$ (71 mg, 0.35 mmol) was neutralized with 2 M NaOH. After vacuum degassing, pyruvate kinase (300 U) and fructose 6-phosphate kinase (300 U) were added, and the solution was allowed to stand at room temperature without stirring. Conversion was followed by TLC using 1:1 satd ammonia–EtOH, and by enzymic assay for PEP and pyruvate. After 40 h, the solution was filtered through charcoal, diluted with water (360 mL), and passed through an anion-exchange column (Dowex AG1-X8, HCO_3^- form, 1.5 × 30 cm). The column was washed with water (150 mL) and triethylammonium hydrogencarbonate buffer (250 mM, 450 mL) to remove pyruvate and impurities, then the product was eluted with 450 mM triethylammonium hydrogencarbonate (900 mL). Repeated lyophilization and redissolution in water (3 × 50 mL), ion exchange to give the free acid (Dowex AG50W-X8, H^+, 1.5 × 21 cm), and neutralization with cyclohexylamine followed by crystallization from 90% aq EtOH provided **1** (6.47 g, 60%), identical to the compound described next.

D-*Tagatose 1,6-bisphosphate, tetrakis(cyclohexylammonium) salt* (**1**) *by one-pot enzymic synthesis*.—An aqueous solution (200 mL) of dihydroxyacetone (6.75 g, 75 mmol), monopotassium phosphoenolpyruvate (10.30 g, 50 mmol), and ATP (1.50 g, 2.5 mmol) was adjusted to pH 7.0 by the addition of M NaOH. Magnesium chloride (0.20 g, 1 mmol) and β-mercaptoethanol (33 μL, 0.5 mmol) were added and the solution was degassed under vacuum. Glycerol kinase (300 U) and pyruvate kinase (350 U) were introduced and the mixture was allowed to stand at 25°C for 24 h, after which the formation of dihydroxyacetone phosphate was complete as determined by enzymic assays for dihydroxyacetone phosphate, phosphoenolpyruvate, and pyruvate. Triose phosphate isomerase (1000 U) and purified D-tagatose 1,6-bisphosphate aldolase (TagA, 200 U) were added, and incubation was continued for a further 40 h, after which enzymic assay indicated the formation of D-tagatose 1,6-bisphosphate (**1**) and D-fructose 1,6-bisphosphate (**5**) in a ~9:1 ratio. After separation of the enzymes by ultrafiltration, DL-glyceraldehyde (0.45 g, 5 mmol), D-fructose 1,6-bisphosphate aldolase (100 U), and triose phosphate isomerase (500 U) were added to the filtrate. After standing overnight, the presence of **5** was no longer detectable by TLC or enzymic assay.

In a separate run, contaminating **5** was removed by adjusting the pH of the filtrate to 8.0 and incubating with fructose 1,6-bisphosphatase (40 U) at 25°C for 3 days. The respective mixtures were filtered through charcoal and applied to an anion-exchange resin (Dowex AG 1-X8, HCO_3^- form, 3 × 20 cm). The column was

washed with deionized water (4 bed volumes), then the product was eluted by applying a 0–500 mM gradient of triethylammonium hydrogencarbonate buffer (600 mL). Fractions containing **1** were combined, passed through a Dowex AG 50W-X8 (H^+) column, and neutralized with cyclohexylamine to provide, after concentration and crystallization from EtOH, **1** (14.74 g, 40%) as the tetrakis(cyclohexylammonium) salt; mp > 160°C (dec.), $[\alpha]_D$ + 1.4° (*c* 1, H_2O); ^1H NMR (D_2O): δ 3.82 (dd, *J* 10.5, 5.3 Hz, H-1aβ), 3.88 (dd, *J* 10.5, 6.0 Hz, H-1bβ), 4.00 (dd, *J* 10.5 Hz, H-6aβ), 4.14 (dd, *J* 10.5 Hz, H-6bβ), 4.24 (m, H-5β), 4.30 (d, *J* 4.8 Hz, H-3β), 4.49 (t, *J* 4.8 Hz, H-4β), 4.44 (m, 5-Hα), and 4.56 (t, *J* 5.5 Hz, H-4α). Integration showed the ratio of α and β anomers to be 20:80. Anal. Calcd for $C_{30}H_{66}N_4O_{12}P_2$ (736.8): C, 48.90; H, 9.03; N 7.60. Found: C, 49.00; H 8.86; N 7.29.

D-*Tagatose* (**14**).—To an aqueous solution (10 mL) of the tetrakis(cyclohexylammonium) salt of **1** (100 mg, 0.14 mmol), adjusted to pH 6.0 by the addition of HCl, was added acid phosphatase (5 U), and the mixture was allowed to stand at room temperature for 6 h. The solution was desalted (Dowex AG 50W-X8, H^+ form, then Dowex AG 1-X8, HCO_3^- form) and evaporated in vacuo to furnish **14** (23 mg, 94%), which proved identical to an authentic sample by comparison of its mp, NMR data, and optical rotation.

ACKNOWLEDGMENTS

Support of this work by the Deutsche Forschungsgemeinschaft (Project Fe 244/2-1), the Wissenschaftliche Gesellschaft Freiburg and the Fonds der Chemischen Industrie is gratefully acknowledged. O.E. is indebted to the Ministerium für Wissenschaft und Kunst, Baden-Württemberg for a scholarship. We thank Professors J. W. Lengeler (University of Osnabrück), D.G. Fraenkel (Harvard Medical School, Boston), and J. Aguilar (University of Barcelona) for the generous gift of bacterial strains, Dr. D. Hunkler for NMR spectra, and M. Petersen for skilful experimental assistance.

REFERENCES

1 B. Axelrod, in D.M. Greenberg (Ed.), *Metabolic Pathways,* Vol. 1, Academic Press, New York, 1967, pp 271–306.
2 C.E. Ballou and D.L. MacDonald, *Methods Carbohydr. Chem.,* 2 (1963) 270–297; S.P. Colowick and N.O. Kaplan (Eds.), *Methods Enzymol.,* 3 (1957) 110–231.
3 M.D. Bednarski, H.J. Waldmann, and G.M. Whitesides, *Tetrahedron Lett.,* 27 (1986) 5807–5810; M. Kapuscinski, F.P. Franke, I. Flanigan, J.K. MacLeod, and J.F. Williams, *Carbohydr. Res.,* 140 (1985) 69–79; A.S. Serianni, E. Cadman, J. Pierce, M.L. Hayes, and R. Barker, *Methods Enzymol.,* 89 (1982) 83–92.
4 W.-D. Fessner, in G. Helmchen, R.W. Hoffmann, J. Mulzer, and E. Schaumann (Eds.), *Houben-Weyl: Methoden der Organischen Chemie,* Vol. E22, *Stereoselective Synthesis of Organic Compounds,* Thieme, Stuttgart, in press.
5 E.J. Toone, E.S. Simon, M.D. Bednarski, and G.M. Whitesides, *Tetrahedron,* 45 (1989) 5365–5422; D.G. Drueckhammer, W.J. Hennen, R.L. Pederson, C.F. Barbas, C.M. Gautheron, T. Krach, and C.-H. Wong, *Synthesis,* (1991) 499–525.

6 W.-D. Fessner, G. Sinerius, A. Schneider, M. Dreyer, G.E. Schulz, J. Badía, and J. Aguilar, *Angew. Chem.*, 103 (1991) 596–599; *Angew. Chem. Int. Ed. Engl.*, 30 (1991) 555–558.
7 M.D. Bednarski, E.S. Simon, N. Bischofberger, W.-D. Fessner, M.-J. Kim, W. Lees, T. Saito, H. Waldmann, and G.M. Whitesides, *J. Am. Chem. Soc.*, 111 (1989) 627–635; J.R. Durrwachter, D.G. Drueckhammer, K. Nozaki, H.M. Sweers, and C.-H. Wong, *ibid.*, 108 (1986) 7812–7818; C.H. von der Osten, A.J. Sinskey, C.F. Barbas, R.L. Pederson, Y.-F. Wang, and C.-H. Wong, *ibid.*, 111 (1989) 3924–3927; A. Straub, F. Effenberger, and P. Fischer, *J. Org. Chem.*, 55 (1990) 3926–3932; M. Schultz, H. Waldmann, H. Kunz, and W. Vogt, *Liebigs Ann. Chem.*, (1990) 1019–1024; H.P. Brockamp and M.-R. Kula, *Tetrahedron Lett.*, 31 (1990) 7123–7126.
8 W.-D. Fessner and C. Walter, *Angew. Chem.*, 104 (1992) 643–645; *Angew. Chem. Int. Ed. Engl.*, 31 (1992) 614–616.
9 A. Ozaki, E.J. Toone, C.H. von der Osten, A.J. Sinskey, and G.M. Whitesides, *J. Am. Chem. Soc.*, 112 (1990) 4970–4971.
10 D.L. Bissett and R.L. Anderson, *J. Biol. Chem.*, 255 (1980) 8750–8755.
11 V.L. Crow and T.D. Thomas, *J. Bacteriol.*, 151 (1982) 600–608.
12 E.S. Maxwell, K. Kurahashi, and H.M. Kalckar, *Methods Enzymol.*, 5 (1962) 174–189.
13 J. Lengeler, *MGG, Mol. Gen. Genet.*, 152 (1977) 83–91.
14 J. Markwell, G.T. Shimamoto, D.L. Bissett, and R.L. Anderson, *Biochem. Biophys. Res. Commun.*, 71 (1976) 221–227.
15 W.-D. Fessner and O. Eyrisch, *Angew. Chem.*, 104 (1992) 76–78; *Angew. Chem. Int. Ed. Engl.*, 31 (1992) 56–58.
16 R.L. Anderson, W.C. Wenger, and D.L. Bissett, *Methods Enzymol.*, 89 (1982) 93–98.
17 E.C.C. Lin, *Annu. Rev. Microbiol.*, 30 (1976) 535–578.
18 Y.-M. Chen, Z. Lu, and E.C.C. Lin, *J. Bacteriol.*, 171 (1989) 6097–6105.
19 P.R. Alefounder, S.A. Baldwin, R.N. Perham, and N.J. Short, *Biochem. J.*, 257 (1989) 529–534.
20 O.C. Richards and W.J. Rutter, *J. Biol. Chem.*, 236 (1961) 3185–3192.
21 S.A. Baldwin, R.N. Perham, and D. Stribling, *Biochem. J.*, 169 (1978) 633–641.
22 E.E. Penhoet, M. Kochman, and W.J. Rutter, *Biochemistry*, 8 (1969) 4396–4402.
23 R.L. Anderson and J.P. Markwell, *Methods Enzymol.*, 90 (1982) 232–234.
24 B.L. Horecker, O. Tsolas, and C.Y. Lai, in P.D. Boyer (Ed.), *The Enzymes*, 3rd ed., Vol. 7, Academic Press, New York, 1972, pp 213–258.
25 J.T. Kadonaga and J.R. Knowles, *Biochemistry*, 22 (1983) 130–136; N.B. Schwartz and D.S. Feingold, *Bioinorg. Chem.*, 1 (1972) 233–243.
26 B.L. Horecker, P.T. Rowley, E. Grazi, T. Cheng, and O. Tchola, *Biochem. Z.*, 338 (1969) 36–51.
27 A.L. Lehninger, J. Sice, and E.V. Jensen, *Biochim. Biophys. Acta*, 17 (1955) 286–287.
28 D.C. Crans and G.M. Whitesides, *J. Am. Chem. Soc.*, 107 (1985) 7019–7027.
29 P. Oesper and O. Meyerhof, *Arch. Biochem. Biophys.*, 27 (1950) 223–225.
30 J.P. Richard, *J. Am. Chem. Soc.*, 106 (1984) 4926–4936.
31 D.C. Crans, R.J. Kazlauskas, B.L. Hirschbein, C.-H. Wong, O. Abril, and G.M. Whitesides, *Methods Enzymol.*, 136 (1987) 263–280.
32 B.L. Hirschbein, F.P. Mazenod, and G.M. Whitesides, *J. Org. Chem.*, 47 (1982) 3765–3766.
33 S.J. Angyal and G.S. Bethell, *Aust. J. Chem.*, 29 (1976) 1249–1265.
34 F. Ehrlich and R. Guttmann, *Chem. Ber.*, 67 (1934) 573–589.
35 E.L. Totton and H.A. Lardy, *Methods Enzymol.*, 3 (1957) 174–176.
36 T. Reichstein and W. Bosshard, *Helv. Chim. Acta*, 17 (1934) 753–761.
37 T.A.W. Koerner, R.J. Voll, A.-L.E. Ashour, and E.S. Younathan, *J. Biol. Chem.*, 251 (1976) 2983–2986.
38 T.-C. Tung, K.-H. Ling, W.L. Byrne, and H.A. Lardy, *Biochem. Biophys. Acta*, 14 (1954) 488–494.
39 V.M. Micovic and M.L. Mihailovic, *J. Org. Chem.*, 18 (1953) 1190–1200.
40 J.W. Perich and R.B. Johns, *Tetrahedron Lett.*, 28 (1987) 101–102; R.L. Pederson, J. Esker, and C.-H. Wong, *Tetrahedron*, 47 (1991) 2643–2648.
41 D.P. Bloxham and H.A. Lardy, in P.D. Boyer (Ed.), *The Enzymes*, 3rd ed., Vol. 8, Academic Press, New York, 1973, pp 239–278.
42 J. Babul, *J. Biol. Chem.*, 253 (1978) 4350–4355; F. Daldal, J. Babul, V. Guixé, and D.F. Fraenkel, *Eur. J. Biochem.*, 126 (1982) 373–379; F. Daldal, *J. Mol. Biol.*, 168 (1983) 285–305.

43 D.F. Fraenkel et al., unpublished results.
44 T.A.W. Koerner, R.J. Voll, L.W. Cary, and E.S. Younathan, *Biochemistry*, 19 (1980) 2795–2801.
45 J.F. Stoddard, *Stereochemistry of Carbohydrates*, Wiley, New York, 1971.
46 E. Stahl and U. Kaltenbach, *J. Chromatog.*, 5 (1961) 351–355.
47 M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
48 A.U. Bergmeyer, *Methods of Enzymatic Analysis*, 3rd ed., VCH, Weinheim, 1984.
49 S. Sridhara, T.T. Wu, T.M. Chused, and E.C.C. Lin, *J. Bacteriol.*, 98 (1969) 87–95.
50 U.K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
51 H. Segel, *Enzyme Kinetics*, Wiley, New York, 1975.