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STRUCTURES OF D-*threo*-2,5-HEXODIULOSE 1-PHOSPHATE AND D-*threo*-2,5-HEXODIULOSE 1,6-BISPHOSPHATE (5-KETO-D-FRUCTOSE MONO- AND BIS-PHOSPHATE) IN SOLUTION BY ^{13}C-N.M.R. SPECTROSCOPY*

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ABSTRACT

The mono- (**2**) and bis-phosphate (**3**) derivatives of D-*threo*-2,5-hexodiulose (**1**) (5-keto-D-fructose) were synthesized enzymically and purified by anion-exchange chromatography. The proportions, sizes of ring, and anomeric configurations were determined by F.t. ^{31}P- and ^{13}C-n.m.r. spectroscopy. Compound **2** was found to exist preponderantly (70–78%) in the β-pyranose form with the remainder existing in the 2*R*,5*R*-furanose form. Compound **3** assumes two different furanose forms in solution, one (77–84%) being the 2*R*,5*R*-furanose form and the other the 2*S*,5*R*-furanose form.

INTRODUCTION

Several strains of acetic acid bacteria, such as *Gluconobacter* and *Acetobacter*, have been shown to produce D-*threo*-2,5-hexodiulose (**1**) (5-keto-D-fructose), an unusual dicarbonyl hexose sugar, when grown on D-fructose as the sole carbon source[1,2]. Compound **1** is produced by oxidation of D-fructose at C-5 through the action of D-fructose 5-dehydrogenase[3,4] (EC 1.1.99.11).

Compound **1** is a substrate for yeast hexokinase[5], and both rat and liver fructokinase[6,7] which phosphorylate the sugar in the presence of ATP to give the monophosphate ester, **2**. This ester is phosphorylated by liver phosphofructokinase[8] to yield the bisphosphate ester, **3**. The structure of **1** in solution has been shown[9] by ^{13}C-n.m.r. spectroscopy to be in the β-pyranose form with the 5-keto group hydrated to form a *gem*-diol. In this study, we report the synthesis of the

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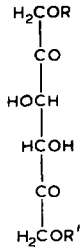
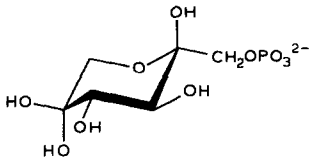




2a

1 R = R′ = H
2 R = PO_3H_2, R′ = H
3 R = R′ = PO_3H_2

mono- (**2**) and bis-phosphate (**3**) esters of **1** and the determination of their structure in aqueous solution by ^{31}P- and ^{13}C-n.m.r. spectroscopy.

RESULTS AND DISCUSSION

D-threo-*2,5-Hexodiulose 1-phosphate* (**2**). — Six major peaks were observed in the proton-noise decoupled, natural abundance ^{13}C-n.m.r. spectrum of **2** (Fig. 1). The ^{31}P–^{13}C coupling pattern indicated that C-1 (δ 67.6) is coupled to P with $^2J_{COP}$ 4.9 Hz and C-2 (δ 98.2) is also coupled to P with $^3J_{CCOP}$ 8.9 Hz (see Table I). No carbonyl resonances were observed in the δ 180–220 spectral region. The ^1H–^{13}C coupling pattern (spectrum not shown) confirmed the presence of two methylene, two methine, and two quaternary carbon atoms. The methylene and quaternary carbon atoms which were not coupled to phosphorus were assigned to C-6 and C-5, respectively. The two methinyl carbon atoms C-3 and C-4 were assigned on the basis of comparison to the chemical shift positions reported previously for **1** which exists in the β-pyranose form in solution[9]. The unphosphorylated sugar **1** showed

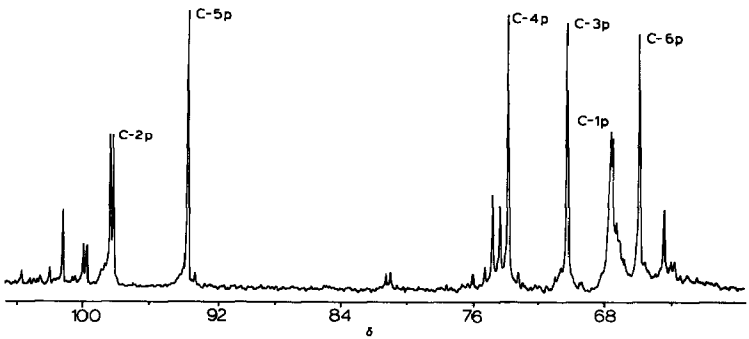


Fig. 1. Proton-decoupled, natural abundance, F.t. ^{13}C-n.m.r. spectrum of compound **2** for a 80mM solution recorded at 50 MHz and 22°. Chemical shifts, ^1H- and ^{31}P-coupling constants for the signals shown are reported in Table I (p = pyranose form).

TABLE I

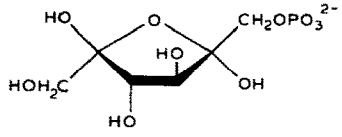
C-N.M.R. DATA[a] (δ) FOR β-D-*threo*-2,5-HEXODIULO-2,6-PYRANOSE (**1**), β-D-*threo*-2,5-HEXODIULO-2,6-PYRANOSE 1-PHOSPHATE (**2a**), (2*R*,5*R*)-β-D-*threo*-2,5-HEXODIULOSE HYDRATE-2,5-FURANOSE 1-PHOSPHATE (**2e**), (2*R*,5*R*)-β-D-*threo*-2,5-HEXODIULOSE HYDRATE-2,5-FURANOSE 1,6-BISPHOSPHATE (**3c**), AND (2*R*,5*S*)-β-D-*threo*-2,5-HEXODIULOSE HYDRATE-2,5-FURANOSE 1,6-BISPHOSPHATE (**3b**)

C-atom	**1**	**2a**	**2e**	**3c**	**3b**
1	64.6	67.6	[b]	67.3	[b]
	($^1J_{CH}$ 145)	($^1J_{CH}$ 146, $^2J_{POC}$ 4.9)		($^1J_{CH}$ 147, $^2J_{POC}$ 4.8)	
		($^3J_{HCOP}$ 5.3)		($^3J_{HCOP}$ 5.4)	
2	98.9	98.2	99.8	100.2	102.8
		($^3J_{POCC}$ 8.9)	($^3J_{POCC}$ 9.9)	($^3J_{POCC}$ 9.8)	($^3J_{POCC}$ 9.5)
3	69.5	70.4	74.6	74.8	81.2
	($^1J_{CH}$ 136)	($^1J_{CH}$ 148)		($^1J_{CH}$ 147)	($^1J_{CH}$ 147)
4	74.2	74.1	75.0	74.8	75.5
	($^1J_{CH}$ 146)	($^1J_{CH}$ 147)		($^1J_{CH}$ 147)	($^1J_{CH}$ 148)
5	92.7	93.7	101.2	100.2	100.9
				($^3J_{POCC}$ 9.8)	($^3J_{POCC}$ 9.4)
6	65.9	66.0	64.5	67.3	[b]
	($^1J_{CH}$ 145)	($^1J_{CH}$ 148)		($^1J_{CH}$ 147, $^2J_{POC}$ 4.8)	
				($^3J_{HCOP}$ 5.4)	

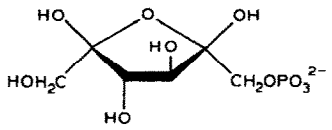
[a]Recorded at 50 MHz. ^{31}P–^{13}C, ^{31}P–^1H, and ^1H–^{13}C coupling constants (in Hz), where resolved, are indicated below the chemical-shift values. [b]Signals were obscured by other resonances.

resonance positions at δ 69.5 and 74.2 for C-3 and -4, respectively, corresponding closely to the values of δ 70.4 and 74.0 obtained for the methinyl carbon atoms of the monophosphate ester reported herein. The analogous chemical shifts for the β-pyranose form of D-fructose 1-phosphate are δ 69.0 (C-3) and 70.4 (C-4), while those for the β-furanose form of D-fructose 1-phosphate are substantially deshielded (δ 77.4 for C-3, and 75.2 for C-4). These chemical shifts, in addition to the unique signal at δ 93.7 for the hydrated ketone group at C-5, are indicative of a pyranose form. An α-pyranose ring structure is unlikely because C-6 of the α-pyranose form of fructose 1-phosphate resonates[10] at δ 61.9, a position far upfield of the C-6 signal of **2**. The most reasonable structure one may propose, for the major form of **2** therefore, is that of a β-pyranose ring (β-D-*threo*-2,5-hexodiulo-2,6-pyranose 1-phosphate, **2a**), in analogy to **1** and D-fructose 1-phosphate.

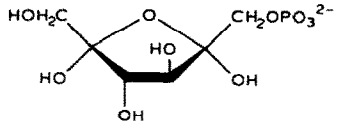
A furanose-ring structure, based on the observation of signals due to C-5 at δ 101.2, and a phosphorus-coupled signal due to C-2 at δ 99.8 ($^3J_{CCOP}$ 9.8 Hz) was assigned to the minor form of **2**. Since **2** has two anomeric centers at C-5 and C-2, a furanose-ring structure may assume four possible anomeric forms. These four forms are structural analogs of α- or β-L-sorbofuranose 6-phosphate (**2d** and **2b**, respectively), and α- or β-D-fructofuranose 6-phosphate (**2c** and **2e**, respectively), (see Scheme 1). The resonances of the furanose form of **2** can be predicted on the basis of the reported ^{13}C-chemical shifts of these structural analogs, except for the presence of OH-5 which would deshield C-5 to a larger extent. Using the ^{13}C-n.m.r. data of Koerner *et al.*[10] on 2,5-anhydro-D-glucitol 6-phosphate and 2,5-anhydro-D-mannitol 6-phosphate, we have predicted the resonance positions of the anomeric



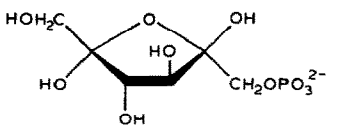
2b (2*S*,5*S*)



2c (2*R*,5*S*)



2d (2*S*,5*R*)



2e (2*R*,5*R*)

Scheme 1. Structures of the four anomeric furanose forms which **2** may assume. Structure **2b** is an analog of β-L-sorbofuranose 6-phosphate and has the 2*S*,5*S* configuration (predicted: C-2, δ 104.7; C-5, δ 106.4). Structure **2c** is an analog of α-D-fructofuranose 6-phosphate and has the 2*R*,5*S* configuration (predicted: C-2, δ 104.9; C-5, δ 105.3). Structure **2d** is an analog of α-L-sorbofuranose 6-phosphate and has the 2*S*,5*R* configuration (predicted: C-2, δ 96.9; C-5, δ 102.8). Structure **2e** is an analog of β-D-fructofuranose 6-phosphate and has the 2*R*,5*R* configuration (predicted: C-2, δ 99.8; C-5, δ 102.4).

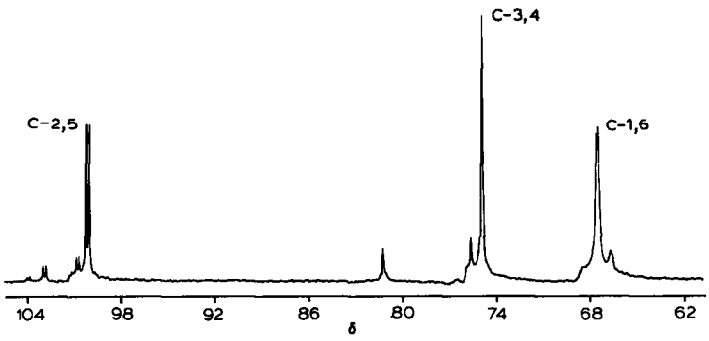
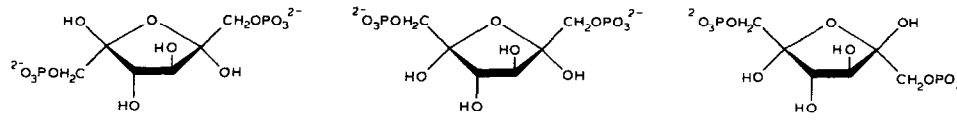


Fig. 2. Proton-decoupled, natural abundance, F.t. ^{13}C-n.m.r. spectrum of compound **3** for a 150mM solution recorded at 50 MHz and 22°. Chemical shifts, ^{1}H- and ^{31}P-coupling constants for the signals shown are reported in Table I.

carbon atoms of each of the furanose forms of **2** that might exist in solution. For example, **2b** may be considered as a C-5 hydroxylated analog of β-L-sorbofuranose 6-phosphate. The anomeric C-5 center would, thus, be predicted to have a chemical shift equivalent to the anomeric carbon of β-sorbofuranose 6-phosphate (δ 106.4). The chemical shift of C-2 can be predicted by adding the chemical shift difference observed between equivalent carbon atoms in α-D-fructofuranose 6-phosphate and 2,5-anhydro-D-glucitol 6-phosphate (δ 23.5) to the observed value for C-5 in β-L-sorbofuranose 6-phosphate (δ 81.2) to yield a predicted value of δ 104.7. Similar calculations have been performed on each of the four structures, and are presented in the legend to Scheme 1. Form **2e** emerges as the most likely possibility, owing to the close correspondence in chemical shift positions between the predicted values and the values for the minor form of **2**. Support for form **2e** is enhanced by the observation that, *a priori*, this ring form is that predicted on thermodynamic grounds. Energetically, it is the most favorable conformation, because it avoids *cis*-1,2 interactions of hydroxymethyl (or hydroxylmethyl phosphate) and hydroxyl groups at both anomeric carbons. A *cis*-1,2 orientation of these groups is less stable compared to the *trans*-1,2 orientation of the two groups[11]. Structure **2e** is the only furanose form possible that avoids these unfavorable *cis*-1,2 interactions at both anomeric carbons. Structure **2b** is the most thermodynamically unstable furanose form, having two *cis*-1,2 interactions of this type at both anomeric centers, whereas structures **2c** and **2d** each have one interaction of this type. Therefore, the most likely structure for the minor form of **2** is that of (2*R*,5*R*)-D-*threo*-2,5-hexodiulose hydrate-2,5-furanose 1-phosphate (**2e**), analogous to β-D-fructofuranose 1-phosphate.

D-threo-*2,5-Hexodiulose 1,6-bisphosphate* (**3**). — The natural abundance ^{13}C-n.m.r. spectrum of **3** (Fig. 2) showed three major signals of nearly equal integrated intensity at δ 67.3 ($^{2}J_{COP}$ 4.8 Hz), 74.7, and 100.2 ($^{3}J_{CCOP}$ 9.8 Hz), and no carbonyl or hydrated ketone resonances were observed (see Table I). The 1,6-bisphosphate **3** may only exist in solution as in a furanose form. Since two anomeric centers are present in the molecule at C-2 and C-5, three anomeric configurations are possible



3a (2S,5S) 3b (2R,5S) 3c (2R,5R)

Scheme 2. Structures of the three anomeric forms which **3** may assume. Structure **3a** is an analog of β-L-sorbofuranose and has the 2*S*,5*S* configuration (predicted: C-2, C-5, δ 106.4). Structure **3b** is an analog of both α-L-sorbofuranose and α-D-fructofuranose and has the 2*S*,5*R* or 2*R*,5*S* configuration (predicted: *S*-C, δ 106.4; *R*-C, δ 105.7). Structure **3c** is an analog of β-D-fructofuranose and has the 2*R*.5*R* configuration (predicted: C-2, C-5, δ 102.0).

(Scheme 2), which are structural analogs of β-L-sorbofuranose (**3a**), α-D-fructofuranose (**3b**), and β-D-fructofuranose (**3c**) (the fourth form analogous to α-L-sorbofuranose is identical to form **3b**). Although two of these forms (**3a** and **3c**) have the required symmetry to yield three ^{13}C-resonances for the hexose component, thermodynamic considerations would predict form **3c** to preponderate for **3** as it is the form that lacks a *cis*-1,2 orientation of hydroxymethyl phosphate and hydroxyl groups at both anomeric carbons. This form of **3** has an axis of C_2 symmetry and would be expected to show three ^{13}C-resonances, of equal intensities, at the positions observed in the spectrum. One signal corresponding to C-1 and -6 is observed at δ 67.3, very close to the positions of the signals observed for C-1 and -6 for the minor form of **2** and of C-1 of β-D-fructofuranose 1,6-bisphosphate[10] (δ 66.9). C-3 and -4 resonate at δ 74.8 and C-2 and -5 at δ 100.2, values close to those observed for the minor form of **2**, and for β-D-fructofuranose 1,6-bisphosphate. By comparison, the anomeric carbons of form **3a** would be expected to resonate at δ ~106, a value analogous to the chemical shift reported for the anomeric carbon of β-D-sorbofuranose 6-phosphate[10].

A set of six smaller peaks, of nearly equal intensity, was also observed, and we believe that they correspond to a minor, asymmetric furanose form of **3** (form **3b**, see Table I). The phosphorus-coupled downfield signals appearing at δ 100.9 and 102.8 were assigned to the resonances of C-5 and -2, respectively. Observation of $^1J_{CH}$ coupling through off-resonance decoupling studies (data not shown) revealed that neither signal was coupled to protons, identifying these as anomeric carbons. The signals of the methinyl carbons, C-3 and -4 (δ 81.2 and 75.5, respectively), of this form could be assigned based on a comparison to the C-3 and -4 signals of α-D-fructofuranose 1,6-bisphosphate (δ 82.4 and 77.5, respectively[10]). We propose that the minor furanose form of **3** exists in a 2*R*,5*S* configuration. Relative to the major form of **3**, form **3b** would be produced if either C-2 or -5 underwent mutarotation. When an anomeric carbon undergoes mutarotation, one may expect deshielding of 2–4 p.p.m. associated with a *cis*-C-1–O-3 interaction[11–13]. In the proposed structure **3b**, a single *cis*-1,2 interaction between hydroxymethyl phosphate and hydroxyl groups has been introduced by mutarotation, and there is a 2-p.p.m. downfield shift of one of the anomeric resonances. Finally, a very small phosphorus-coupled resonance appeared at δ 103.8, which may be due to form **3a**.

Anomeric composition and enzyme specificity. — Integration of the signal intensities of the ^{31}P and C-5 resonances gave the equilibrium composition of the anomeric forms of **2** and **3**. Compound **2** has an equilibrium composition of 70–78% as the β-D-pyranose ring (**2a**) and 22–30% as the 2*R*,5*R* form **2e**. There was no evidence of α-D-pyranose or 2-*S*-furanose forms, although each of these forms have been reported to exist as 5% of the total for D-fructose 1-phosphate[10]. Ignoring these forms, the composition of **2** was nearly identical to that reported for D-fructose 1-phosphate (73% β-D-pyranose and 16% 2*R*-furanose). Phosphofructokinase uses as substrate the β-D-furanose form of 2-ketohexose phosphates[14,15], and these results are compatible with the activity of **2** as a substrate for phosphofructokinase with a K_m close to that of D-fructose 6-phosphate and a V ~25% of the value for D-fructose 6-phosphate[8].

Compound **3** has an equilibrium composition of 77–84% of the 2*R*,5*R* form (**3c**) with the remaining 16–23% in a 2*R*,5*S* furanose form (**3b**). Once again, these values are nearly identical to the reported composition of D-fructose 1,6-bisphosphate (77% β-D-furanose; 23% α-D-furanose[10]). No acyclic forms of **3**, either keto or hydrate, were detected in this study, although n.m.r. examination of ^{13}C-enriched D-fructose 1,6-bisphosphate demonstrated that the rapid mutarotation of the sugar bisphosphate causes the ketone resonances to broaden, making quantitation of trace amounts difficult[16]. Considering the similarities in structure and anomeric composition of **3** and D-fructose 1,6-bisphosphate, it is surprising that **3** is such a poor substrate for muscle aldolase (<0.01% of the V for D-fructose 1,6-bisphosphate[8]). The compound is, however, a good competitive inhibitor (K_i 270 μM) and forms a Schiff's base with the active-site lysine residue. The reason for the very poor activity of **3** in the muscle aldolase reaction is unclear, but may be the result of enzyme inactivation by the α-dicarbonyl cleavage product, hydroxypyruvaldehyde phosphate[17].

EXPERIMENTAL

Materials. — Hexokinase (yeast, Type F-300; EC 2.7.1.1), phosphofructokinase (rabbit muscle), pyruvate kinase (rabbit muscle, Type III; EC 2.7.1.40), and D-fructose-1,6-bisphosphatase (rabbit liver; EC 3.1.3.11) were purchased from Sigma. D-*threo*-2,5-Hexodiulose (5-keto-D-fructose) was prepared as previously described[18]. All chemicals were of the highest purity commercially available.

D-threo-*2,5-Hexodiulose 1-phosphate* (**2**). — The enzymic synthesis of **2** was carried out with slight modifications of the previously reported procedure[18]. The pH of a mixture (50 mL) containing 50mM **1**, 30mM triethanolamine, 48mM potassium phosphoenolpyruvate, 5mM adenosine triphosphate, and 5mM magnesium acetate was adjusted to 7.8 with 50% KOH, and hexokinase (80 units) and pyruvate kinase (180 units) were added. After 7 h, the reaction was shown to be 96% complete by determination of the pyruvic acid formed with lactate dehydrogenase and NADH. Charcoal (2 g; Norit) was added to remove adenine nucleotides, and the

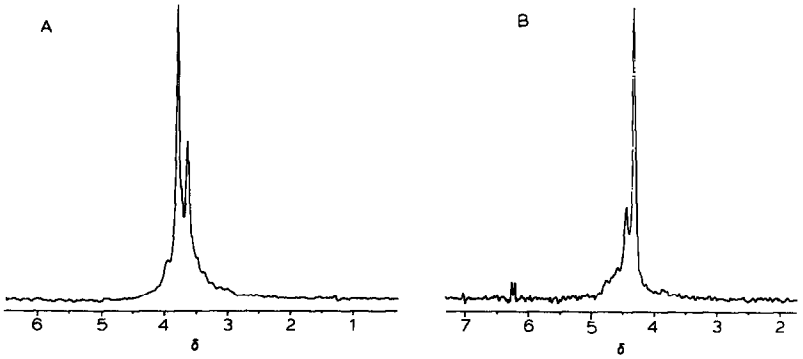


Fig. 3. Proton-decoupled, ^{31}P-n.m.r. spectrum of **2** (A) and **3** (B). Spectra were recorded at 81 MHz and 22° and chemical shifts were relative to external 85% H_3PO_4.

solution was stirred for 1 h. The suspension was filtered and the filtrate applied to a DEAE–Sephacel column (1.5 × 15 cm), equilibrated with 10mM triethanolamine · HCl (pH 7.8) and eluted with a linear KCl gradient (0–150mM, 1 L) in equilibration buffer at a flow rate of 1 mL/min. Fractions (5 mL) that gave a positive phosphate ester[19] reaction were assayed for **2** by use of a coupled-enzyme assay consisting of phosphofructokinase, ATP, pyruvate kinase, potassium phosphoenolpyruvate, lactic dehydrogenase, and NADH, and measuring the disappearance of absorbance at 340 nm. Fractions containing **2** were pooled and barium acetate (1 equiv.) was added followed by ice-cold ethanol (2 vol.). The flocculent, overnight precipitate was centrifuged, washed with absolute ethanol, and dried in a desiccator. The Ba precipitate was redissolved in water with beads of Dowex 50W-X8 (H^+) cation-exchange resin, and enzymic assays of this purified material showed a content of **2** >80% (72% yield from **1**); ^{1}H-decoupled ^{31}P-n.m.r. (Fig. 3A): two closely spaced signals at δ 3.80 (downfield from external 85% H_3PO_4 signal; ~70% by integration), and at δ 3.65 (~30% by integration), and no ^{31}P signal due to inorganic phosphate.

D-threo-*2,5-Hexodiulose 1,6-bisphosphate*. — The pH of a mixture (40 mL), essentially as previously described[18], containing 45mM **1**, mM ATP, 5mM triethanolamine · HCl buffer (pH 7.8), 44mM potassium phosphoenolpyruvate, and 5mM magnesium acetate was adjusted to 7.4 with 50% KOH (w/v). Hexokinase and pyruvate kinase (1000 units each) were added. When the formation of **2** was shown to be complete by enzymic assay, solid D,L-dithiothreitol, ATP, magnesium acetate, and M triethanolamine (pH 7.4) were added to give the respective final concentrations (in 150 mL) 1mM , 1mM, 5mM, and 10mM. The pH was adjusted to 7.1 with 50% KOH (w/v), and phosphofructokinase (900 units), extensively dialyzed against 10mM triethanolamine · HCl (pH 7.1), was added. Potassium phosphoenolpyruvate was added in aliquots (20 × 100 μmol), and the pH of the mixture was maintained at pH 7 by the addition of 2M KOH. The reaction was terminated after all **2** had been converted into **3**. Charcoal (1 g) was then added to remove adenine nuc-

leotides and the solution was stirred overnight. The charcoal was removed by filtration, and the solution applied to a DEAE–Sephacryl column (1.5 × 15 cm) equilibrated with 10mM triethanolamine · HCl (pH 7.8) and eluted with a linear gradient of KCl (1 L; 0–360mM) in equilibration buffer at a flow rate of 1 mL/min. Fractions containing phosphate esters were identified by incubating aliquots with potato acid phosphatase (2 mg/mL in 100mM sodium acetate, pH 5.0), and measuring inorganic phosphate released[20]. These fractions were assayed for **2** (as discussed earlier), which was shown to be absent. In addition, the presence of the sugar bisphosphate was ascertained qualitatively by the addition of aliquots of these fractions to assay mixtures containing D-fructose-1,6-bisphosphatase, phosphofructokinase, pyruvate kinase, lactate dehydrogenase, MgATP, PEP, and NADH. The presence of **3**, which is a substrate for D-fructose-1,6-bisphosphatase, creates a cycle that results in the disappearance of NADH. Fractions that contained **3** were precipitated by the addition of barium acetate (2 equiv.) and absolute ethanol to a 20% (v/v) concentration. The flocculent precipitate was centrifuged and washed with 50% (v/v) aqueous ethanol. An efficiently stirred suspension of the precipitate was carefully titrated to pH 2.1 with 10% H_2SO_4 (v/v) and, after 1 min, titrated to pH 5.1 with 20% KOH. $BaSO_4$ was removed by centrifugation, and the solution of **3** was concentrated by rotary evaporation at reduced pressure (60% yield from **1**); ^{1}H-decoupled ^{31}P-n.m.r. (Fig. 3B): two closely spaced signals at δ 4.34 (downfield from the signal of external 85% H_3PO_4; 77% by integration) and at δ 4.45 (23% by integration), and no ^{31}P-signal due to inorganic phosphate.

^{31}P- and ^{13}C-n.m.r. spectrometry. — Natural-abundance ^{31}P- and ^{13}C-n.m.r. data were recorded at 81 and 50 MHz, respectively, with a Varian XL-200 spectrometer operating in a pulsed F.t. mode. Broad-band, proton-noise decoupling was employed for ^{1}H-decoupled spectra, and a sweep width of 10 000 Hz with 8000 data points for ^{13}C-n.m.r. spectra, and 5000 Hz with 8000 data points for ^{31}P-n.m.r. spectra. Repetition times were 2–4 s with a determined pulse-angle of 90°. Samples were prepared in 10-mm tubes (1.5 mL) and contained mM EDTA and 20% D_2O (v/v) as an internal lock. The concentration of **2** was 80mM, and that of **3** 150mM. Final pH of the samples was 4.6 for **2** and 5.1 for **3**. Spectra were recorded at 22°, with 1,4-dioxane as an internal ^{13}C-n.m.r. standard (δ 67.4), or 85% H_3PO_4 as an external ^{31}P-n.m.r. standard. The spectra shown in Figs. 1 and 2 were recorded in the absence of 1,4-dioxane, and then 1,4-dioxane was added and the spectrum recorded again.

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