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A ^{13}C-N.M.R. STUDY OF INTERMEDIATES IN THE L-TYPE PENTOSE PHOSPHATE CYCLE

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ABSTRACT

The structures in aqueous solution of all major contributing forms of D-*altro*-heptulose 1,7-diphosphate, and D-*glycero*-D-*altro* and D-*glycero*-D-*ido*-octulose 1,8-diphosphates have been established by ^{13}C-n.m.r. spectroscopy. Assignments to individual carbon atoms were made with the aid of isotopically enriched analogues and by comparison with related sugars.

INTRODUCTION

The identification of D-*glycero*-D-*altro*- and D-*glycero*-D-*ido*-octulose 1,8-diphosphates in tissues having active pentose phosphate metabolism has led to the postulation of a new pentose phosphate pathway involving these octulose diphosphates as intermediates[1]. Evidence in favour of the new L-type pathway stems from the ^{14}C-labelling patterns found in the hexose phosphates formed during the *in vitro* dissimilation of 1-^{14}C-labelled ribose phosphate by liver enzymes[2].

The distribution of ^{14}C in the intermediates and products of the pentose pathway is equivocal. Partial redistributions of the ^{14}C label may occur during exchange reactions catalysed by transketolase and transaldolase. Conclusive evidence for the intermediary role of the octulose diphosphates, which occurs at the beginning of the proposed pathway, is being sought by the use of a specifically labelled pentose phosphate precursor in liver enzyme preparations and determination of the resulting labelling pattern in the isolated octulose diphosphates. The ^{14}C methodology used previously is unsuitable for the present study as no specific degradative procedures are available for the octuloses. The use of specifically labelled ^{13}C precursors, combined with ^{13}C-n.m.r. spectroscopy, offers a non-degradative method of monitoring the distribution of the ^{13}C label to the various intermediates of the pathway. Before this experiment could be performed, it was necessary to assign the ^{13}C resonances to the carbon atoms of all of the major contributing forms of both octulose diphosphates as well as to D-*altro*-heptulose 1,7-diphosphate, another intermediate specific to the L-type pathway. The structures of these sugar phosphates and their ^{13}C assignments are reported here.

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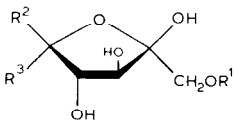
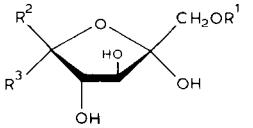
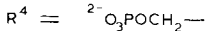
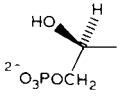
RESULTS AND DISCUSSION

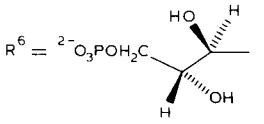
D-altro-*Heptulose 1,7-diphosphate.* — ^{13}C-N.m.r. spectroscopy of D-*altro*-heptulose 1,7-diphosphate in aqueous solution reveals it to be a mixture of three equilibrating structures, in the ratios 74:13:13. These figures were obtained by comparing the integrals of all assignable, corresponding carbon resonances rather than relying on the integrals of anomeric carbon resonances alone, which are weak. The











	R^1	R^2	R^3
1	PO_3^{2-}	R^4	H
2	PO_3^{2-}	R^5	H
3	PO_3^{2-}	R^6	H
7	H	H	R^4
8	PO_3^{2-}	H	R^6

	R^1	R^2	R^3
4	PO_3^{2-}	R^4	H
5	PO_3^{2-}	R^5	H
6	PO_3^{2-}	R^6	H
9	H	H	R^4
10	PO_3^{2-}	H	R^6

preponderant form is the β-furanose **2**, the ^{13}C-n.m.r. shift values of which correlate well with those of the configurationally related β-D-fructofuranose 1,6-diphosphate[3] (**1**, Table I). The n.m.r. characteristics of the second contributing form correspond to those of α-D-fructofuranose 1,6-diphosphate[3] (**4**), indicating the presence of the α-furanose **5**. The assignment of ^{13}C resonances to individual carbon atoms is clear cut, except for C-3 and C-5 in the minor α-furanose **5**. The observation of two-bond POC ($^2J_{CP}$ 4-5 Hz) and three-bond POCC ($^3J_{CP}$ 5–12 Hz) coupling-constants allowed identification of C-1, C-2, C-6, and C-7 in all tautomeric forms.

Labelling experiments of the structurally related D-*glycero*-D-*altro*-octulose 1,8-diphosphate (see later) assisted in the assignment of C-3 and C-4 of the major β-furanose **2** and confirmed those assignments reported[3] for **1**. The two primary carbon atoms of **2** may be differentiated by comparing their chemical shifts with those of β-D-*altro*-heptulofuranose 7-phosphate, the preponderant tautomer of D-*altro*-heptulose 7-phosphate in aqueous solution (see Experimental). Its C-1 signal is shifted upfield by 2.9 p.p.m. compared with the diphosphate and is not coupled to phosphorus, whereas C-7 and all other carbon resonances are observed at almost identical frequencies. Similar upfield shifts are experienced by two of the primary

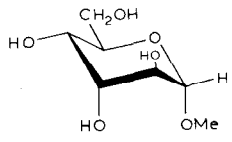
TABLE I

^{13}C CHEMICAL SHIFTS[a] AND ^{31}P–^{13}C COUPLING CONSTANTS (Hz) OF HEXULO-, HEPTULO-, AND OCTULOFURANOSE PHOSPHATES

Structure	*C-1*($^2J_{CP}$)	*C-2*($^3J_{CP}$)	*C-3*	*C-4*	*C-5*($^3J_{CP}$)	*C-6*($^2J_{CP}$ *or* $^3J_{CP}$)	*C-7*($^2J_{CP}$ *or* $^3J_{CP}$)	*C-8*($^2J_{CP}$)
1[b]	66.9(4.0)	102.0[d]	76.9	75.1	80.4[d]	65.4(4.5)		
2	66.4(4.4)	101.9(9.2)	77.3	75.7	81.1	72.5(5.9)	65.6(4.4)	
3	66.5[e](3.4)	102.1(7.3)	77.4	74.9	81.2	71.8	71.9(6.8)	66.2[e](4.9)
4[b]	65.4	105.7[d]	82.4	77.5	82.7[d]	65.4		
5	66.9(4.4)	105.6(5.9)	83.5[e]	76.9	82.3[e]	71.4(7.3)	65.6(4.4)	
6	65.6(4.4)	105.5(7.3)	83.5[e]	76.3	82.4[e]	71.3	69.1(11.7)	65.6(4.4)
7[b]	64.3	102.8	76.6	76.2	77.9(6.6)	63.8(4.4)		
8	67.1(4.0)	101.6(7.3)	76.6	76.2	77.7	69.3	71.1(5.6)	65.8(4.8)
9[b]	[c]	106.4	80.9	76.6	81.2(7.3)	[c]		
10	67.1[e](4.0)	106.1(5.6)	81.3[e]	76.8	81.2[e]	70.4	69.5(6.4)	65.8[e](4.8)

[a]In p.p.m. relative to Me_4Si. [b]Reported by Koerner *et al.*[10]. [c]Obscured by overlapping signals. [d]*J* values not determinable. [e]C-1/C-8 and C-3/C-5 assignments could be interchanged.

carbon atoms of the two minor contributing forms (**5** and **12**) of D-*altro*-heptulose 7-phosphate, allowing differentiation between C-1 and C-7. Because of their similar concentrations, it was not possible to distinguish between individual C-1 or C-7 resonances of these two minor tautomers.



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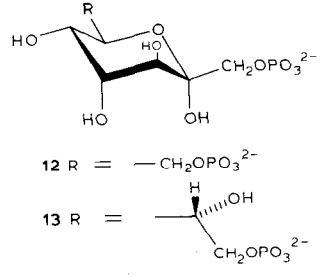


The third component has the pyranose structure **12**. Its anomeric carbon atom absorbs at 98.7 p.p.m., a frequency characteristic of pyranoses. The other carbon atoms (C-3–C-6) also show substantial upfield shifts compared with those of the two furanoses (Table II). Model compounds that would allow assignment of the anomeric configuration of D-*altro*-heptulopyranose are less readily available. Calculations of relative free-energies of both chair conformations of the two anomers were performed according to the method of Angyal[4], but neglecting any interactions due to the phosphate groups. The results predict that the α-pyranose in the $^5C_2$ conformation **12** (E_{conf} = 4.1 kcal.mol^{-1}) is strongly favoured over its β anomer (6.3) and the α (6.8) and β (5.8) anomers in the $^2C_5$ conformation.

TABLE II

^{13}C CHEMICAL SHIFTS[a] AND ^{31}P–^{13}C COUPLING CONSTANTS (Hz) OF HEPTULO- AND OCTULO-PYRANOSE DIPHOSPHATES AND THEIR CONFIGURATIONALLY RELATED METHYL HEXOSIDES

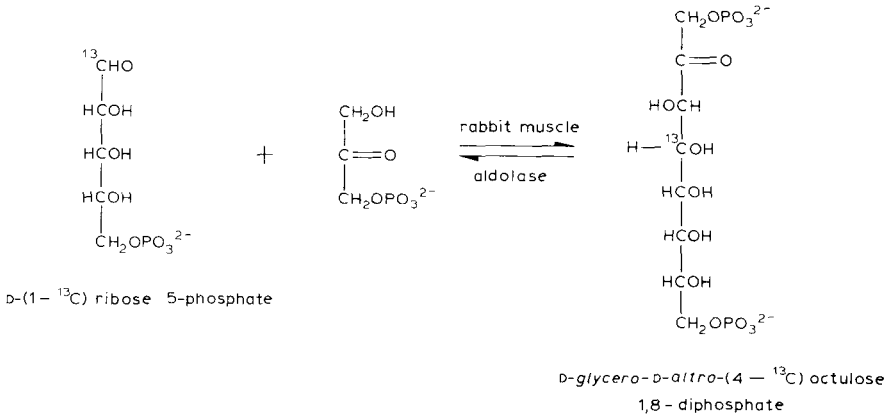
Structure	*C-1*($^2J_{CP}$)	*C-2*($^3J_{CP}$)	*C-3*	*C-4*	*C-5*	*C-6*($^3J_{CP}$)	*C-7*($^2J_{CP}$ *or* $^3J_{CP}$)	*C-8*($^2J_{CP}$)
11[b]	101.1	70.0	70.0	64.8	70.0	61.3		
12	65.6(4.4)	98.8(5.9)	68.9	71.5	64.1	69.0(7.3)	64.5(4.4)	
13	67.0(2.4)	98.6(6.4)	69.4	71.7	65.0	67.4	72.6(6.4)	67.0(2.4)
14[b]	101.5	70.9	71.8	70.3	70.8	60.2		
15	65.8[c](4.0)	99.3(6.4)	69.3[c]	71.0	68.3[c]	68.2[c]	71.7(5.6)	65.5[c](4.0)

[a]In p.p.m. relative to Me_4Si. [b]Reported by Perlin *et al.*[5] and converted to the Me_4Si scale by using $\delta_{Me_4Si} = -\delta_{Cs_2} + 192.8$. [c]C-1/C-8 and C-3/C-5/C-6 assignments could be interchanged.

To ascertain the validity of this calculation, the ^{13}C resonances of this minor isomer of D-*altro*-heptulose 1,7-diphosphate were compared with those of the configurationally and conformationally related methyl α-D-altropyranoside[5] (**11**), and assignments to individual carbon atoms made as shown in Table II. The resultant shifts are largely predictable; replacement of methoxyl by a hydroxyl group causes a significant upfield shift (7–10 p.p.m.) for the carbon atom to which it is attached while not substantially affecting others[6]; replacement of hydrogen by a hydroxymethyl group invariably shifts the resonance of the carbon atom to which it is attached downfield by 4–6 p.p.m.[7], as observed in the corresponding pairs of sugars, α-L-xylopyranose[6]/α-L-sorbopyranose[8], α-D-lyxopyranose[6]/α-D-tagatopyranose[8], and β-D-arabinopyranose[6]/β-D-fructopyranose[8], and thus tends to decrease the upfield shift expected for the replacement of the methoxyl by a hydroxyl group. The effect on other carbon atoms is relatively small and of little diagnostic value, possibly because of conformational changes. The values observed for the third component of D-altroheptulose 1,7-diphosphate (Table II) are well within the range expected for the α-pyranose structure **12**.

The equilibrium state of sedoheptulose (≡ D-*altro*-heptulose) has been determined as 66:18:16 for the β-furanose:α-furanose:α-pyranose, by comparing the integrals of the anomeric carbon signals in the ^{13}C-n.m.r. spectrum. No attempt was made to assign the complete spectrum, and so these figures are probably less reliable[9].

D-glycero-D-altro-*Octulose 1,8-diphosphate.* — ^{13}C-N.m.r. signals of this sugar, when compared with those of D-*altro*-heptulose 1,7-diphosphate (Tables I and II) indicate a mixture of β-furanose **3**, α-furanose **6**, and α-pyranose **13** forms in relative amounts of 74, 19, and 7% in aqueous solution. The close resemblance of the equilibrium distribution to that of D-*altro*-heptulose 1,7-diphosphate demonstrates that stabilities are determined mainly by substitution in the ring and to only a minor extent by interactions in the side chain. Comparison of ^{13}C shifts of the predominant β-furanose **3** with those β-D-fructofuranose 1,6-diphosphate

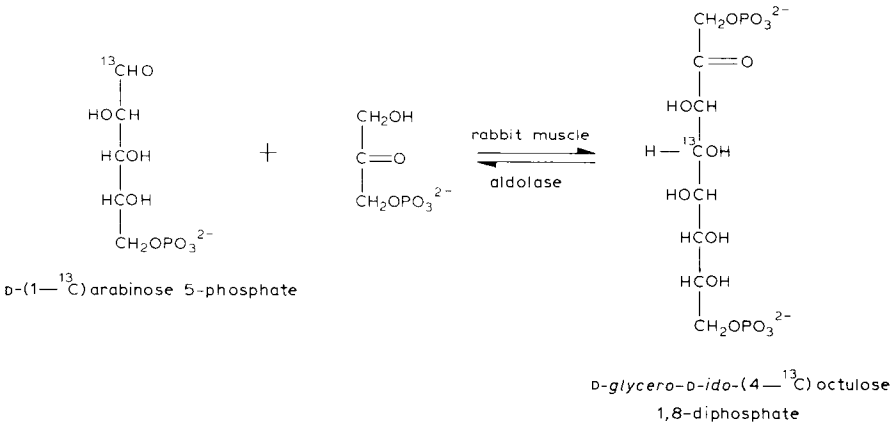


Scheme 1. Enzymic synthesis of D-*glycero*-D-*altro*-(4-^{13}C)octulose 1,8-diphosphate.

(**1**) permitted assignment of most carbon resonances, but the signals at 77.4 and 74.9 p.p.m. could not be identified unambiguously in this way, as the earlier assignment of C-3 and C-4 (76.9 and 75.1 p.p.m.) in **1** did not appear to be unequivocal. The question of operation of the L-type pentose phosphate pathway depends critically on the observation of ^{13}C incorporation at C-4 in both octulose 1,8-diphosphates from D-(1-^{13}C)ribose phosphate as precursor, and unequivocal identification of this particular carbon atom is therefore essential. Hence we prepared D-*glycero*-D-*altro*-(4-^{13}C)octulose 1,8-diphosphate, by rabbit muscle aldolase-catalysed condensation of D-(1-^{13}C)ribose 5-phosphate (90% ^{13}C) with 1,3-dihydroxy-2-propanone phosphate[11] according to Scheme 1.

The ^{13}C-n.m.r. spectrum of the labelled octulose diphosphate shows signals at 74.9, 76.4, and 71.7 p.p.m. only, thus identifying the C-4 atoms of all three contributing forms. The ratio of their integrals reflected closely the distribution as determined from the natural-abundance ^{13}C-n.m.r. spectrum. The spectrum of D-*glycero*-D-*altro*-octulose 1,8-diphosphate admixed with ~1% of the 4-^{13}C-labelled compound exhibited the normal pattern, with the signals at 74.9, 76.4, and 71.7 p.p.m. of approximately doubled intensity.

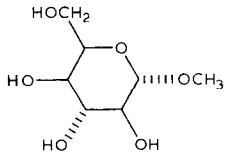
D-glycero-D-ido-*Octulose 1,8-diphosphate.* — The presence of two furanoses and one pyranose, in the ratio 67:14:19, is indicated by the ^{13}C-n.m.r. spectrum of this compound. The carbon chemical shifts of the major form correspond well with those of α-L-sorbofuranose 6-phosphate[10] (**7**), showing that β-D-*glycero*-D-*ido*-octulofuranose 1,8-diphosphate (**8**) is the major component of the equilibrating mixture (Table I). The minor furanose is identified as α-D-*glycero*-D-*ido*-octulofuranose 1,8-diphosphate (**10**) by the similarity of its ^{13}C-resonances with those of β-L-sorbofuranose 6-phosphate[10] (**9**). The small difference of chemical shifts between C-3 and C-4 of the major contributing β-furanose (76.2 and 76.6 p.p.m.), together with the ambiguity encountered in the literature in the assignment of C-3 and C-4 of the model compounds α- and β-L-sorbofuranose 6-phosphates[10], was re-



Scheme 2. Enzymic synthesis of D-*glycero*-D-*ido*-(4-^{13}C)octulose 1,8-diphosphate.

solved by the synthesis of D-*glycero*-D-*ido*-(4-^{13}C)octulose 1,8-diphosphate, from D-(1-^{13}C)arabinose 5-phosphate and 1,3-dihydroxy-2-propanone phosphate, catalysed by rabbit muscle aldolase, according to Scheme 2.

The ^{13}C-n.m.r. spectrum of the labelled compound identified the C-4 resonances of the three contributing structures (Tables I and II) and confirmed their relative abundances in the mixture as determined from the natural-abundance, ^{13}C-n.m.r. spectrum. The comparatively large difference in chemical shifts between C-1 and C-8 of β-D-*glycero*-D-*ido*-octulofuranose 1,8-diphosphate and the availability of D-*glycero*-D-*ido*-octulose 8-phosphate prepared enzymically[12] enabled assignment of C-1 and C-8 in **8**. Removal of the phosphate group shifts the C-1 resonance upfield by 2.7 p.p.m. and eliminates the carbon–phosphorus coupling, while the C-8 resonance remains in the same position.



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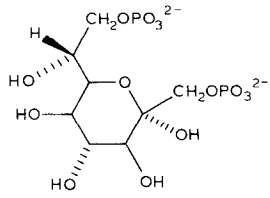

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The ^{13}C-n.m.r. shifts of the third component of D-*glycero*-D-*ido*-octulose 1,8-diphosphate (19%) (Table II) suggest the α-pyranose structure **15**. However, the expected correlation between the chemical-shift values of **15** and the configurationally related methyl α-D-idoside **14** is not good, even after taking into account the effects of the different substituents on the anomeric carbon atom. This suggests that **15** may have a different conformation from that of **14**, which is known by ^{1}H-n.m.r. to exist as a 3:2 mixture of the $^4C_1$ and the $^1C_4$ forms[13]. The β-pyranose ($^2C_5$) struc-

ture can be ruled out, as there is no correlation between the ^{13}C chemical shifts of this third tautomer and those of α-L-sorbopyranose[8]. Calculations of conformational free-energies for the α- and β-pyranose forms strongly support the $^5C_2$ conformation of the α-pyranose structure (4.8 kcal.mol^{-1}) for **15** over its $^2C_5$ conformer (6.8) and the two β-pyranose forms (5.8 and 7.0).

EXPERIMENTAL

General methods. — Unless stated otherwise, all ^{13}C-n.m.r. spectra were recorded at 25° with a Bruker CXP-200 n.m.r. spectrometer operated at 50.3 MHz. Samples were dissolved in D_2O and adjusted to pH 7.0 by the addition of 0.1M NaOH or 0.1M HCl as required. Methanol was added as an internal standard. Its chemical-shift value of 49.7 p.p.m. with respect to Me_4Si was calculated by measuring the chemical shift of methanol relative to 1,4-dioxane in D_2O solution (17.7 p.p.m.) and using the literature value of 67.4 p.p.m. for the chemical shift of 1,4-dioxane relative to Me_4Si. 1,3-Dihydroxy-2-propanone phosphate, β-hydroxypyruvate, D-arabinose 5-phosphate, D-ribose 5-phosphate, D-glucose 6-phosphate, D-*altro*-heptulose 1,7-diphosphate, and D-*altro*-heptulose 7-phosphate were of commercial origin (Sigma) and used without purification. D-Erythrose 4-phosphate was prepared from D-glucose 6-phosphate[14]. Rabbit muscle aldolase was obtained from Boehringer–Mannheim. Transketolase was prepared from spinach[15]. K^{13}CN as supplied by Merck, Sharp and Dohme, Canada, was of 90% isotopic purity.

D-altro-*Heptulose 7-phosphate.* — ^{13}C-N.m.r. data (50 MHz, D_2O): The title compound consists of a mixture of β-furanose (67%), α-furanose (17%), and α-pyranose (16%) forms; β-furanose: δ 63.5 (C-1), 65.6 (d, 4.9 Hz, C-7), 72.7 (d, 6.1 Hz, C-6), 76.3 (C-4), 76.9 (C-3), 81.3 (C-5) and 102.5 (C-2); α-furanose: δ 65.2 (C-1), 65.6 (d, 4.9 Hz, C-7), 71.7 (d, 7.5 Hz, C-6), 77.3 (C-4), 82.6 and 82.8 (C-3 and C-5) and 105.5 (C-2); α-pyranose: δ 63.6 (C-1), 64.1 (C-5), 64.5 (d, 4.9 Hz, C-7), 69.2 (d, 7.3 Hz, C-6), 69.5 (C-3), 72.0 (C-4) and 98.6 (C-2).

D-glycero-D-altro-*Octulose 1,8-diphosphate.* — D-Ribose 5-phosphate disodium salt (685 mg, 2.5 mmol) and 1,3-dihydroxy-2-propanone phosphate (27 mg, 0.16 mmol) were incubated[11] in the presence of rabbit muscle aldolase (5 mg) for 3 h at 25°. Protein was removed by ultrafiltration and the title compound isolated by anion-exchange chromatography. The column was eluted with a linear gradient, starting with water (500 mL) in the mixing bottle and 4M formic acid–M ammonium formate (500 mL) in the reservoir. Fractions containing D-*glycero*-D-*altro*-octulose 1,8-diphosphate were pooled and passed through a column of Dowex 50 (H^+). Formic acid was removed by continuous extraction with ether and the aqueous layer made neutral with M NaOH to give the tetrasodium salt of the title compound, in 80% yield, as determined colorimetrically by using the cysteine–sulfuric acid reaction described by Paoletti *et al.*[11].

D-glycero-D-ido-*Octulose 1,8-diphosphate.* — D-Arabinose 5-phosphate disodium salt (493 mg, 1.8 mmol) and 1,3-dihydroxy-2-propanone dihydrogenphos-

phate (20 mg, 0.12 mmol) were incubated[11] in the presence of rabbit muscle aldolase (4 mg) for 24 h at 25°. The title compound was isolated, as already described, in 60% yield.

D-glycero-D-ido-*Octulose 8-phosphate.* — Because of the small quantity (10 mg) prepared enzymically[12] and the resulting low signal-to-noise ratio, the ^{13}C-n.m.r. data of only the major β-furanose form are reported (15 MHz, D_2O): δ64.3 (C-1), 65.7 (bd, C-8), 69.6 (C-6), 71.3 (d, 5.8 Hz, C-7), 76.4, 76.9 and 77.3 (C-3, C-4 and C-5), and 102.1 (C-2).

D-glycero-D-altro-*(4-^{13}C)Octulose 1,8-diphosphate.* — D-(1-^{13}C)Ribose 5-phosphate (17 mg, 0.06 mmol) obtained from D-erythrose 4-phosphate and K^{13}CN as described by Serianni *et al.*[16] was treated with an excess of 1,3-dihydroxy-2-propanone dihydrogenphosphate (153 mg, 0.9 mmol) in the presence of aldolase (4 mg) using the same conditions already detailed to give, after conventional processing, the title compound in 50% yield. Unlabelled D-*glycero*-D-*altro*-octulose 1,8-diphosphate in approximately equal concentration was added as carrier to facilitate chromatographic separation.

D-glycero-D-ido-*(4-^{13}C)-Octulose 1,8-diphosphate.* — D-(1-^{13}C)-Arabinose 5-phosphate (19 mg, 0.07 mmol) obtained[16] from D-erythrose 4-phosphate and K^{13}CN, was treated with an excess of 1,3-dihydroxy-2-propanone dihydrogenphosphate in the presence of aldolase, as already described for the unlabelled compound, to give the title compound in 30% yield.

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