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Note

Enzymatic synthesis of D-glucosone 6-phosphate (D-*arabino*-hexos-2-ulose 6-(dihydrogen phosphate)) and NMR analysis of its isomeric forms

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Abstract

D-Glucosone 6-phosphate (D-*arabino*-hexos-2-ulose 6-(dihydrogen phosphate)) was prepared from D-glucosone (D-*arabino*-hexos-2-ulose) by enzymatic conversion with hexokinase. The isomeric composition of D-glucosone 6-phosphate in aqueous solution was quantitatively determined by NMR spectroscopy and compared to D-glucosone. The main isomers are the α-anomer (58%) and the β-anomer (28%) of the hydrated pyranose form, and the β-D-fructofuranose form (14%). © 2002 Elsevier Science Ltd. All rights reserved.

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Since D-glucosone (**1**) can be conveniently prepared from D-glucose by enzymatic conversion with pyranose oxidase,[1–3] it has become a useful starting material for various enzymatic and chemical transformations. Catalytic hydrogenation[4,5] of **1** or its enzymatic reduction[3] yields D-fructose in almost quantitative amounts. The enzymatic removal of two molecules of water from **1** results in the formation of cortalcerone, a fungal β-pyrone antibiotic.[6,7] Alkali treatment of **1** leads to multiple products, and in a stereo-controlled benzylic acid type of re-arrangement, D-mannonic acid can be obtained in high yields.[8,9] Prolonged treatment of **1** with pyranose oxidase forms the 3-keto derivative,[10] and it is reported that in the presence of ATP **1** is phosphorylated by yeast hexokinase,[11] but to our knowledge up to now quantitative data on the enzymatic synthesis of D-glucosone 6-phosphate (**2**) and its chemical characterization have not been described in the literature. Since **2** has potential as an intermediate for metabolic engineering, knowledge of its prevalent isomeric form in aqueous solution is of considerable interest. Although for **1** the composition of the isomers in aqueous solution was determined,[12,13] there is to our knowledge no corresponding study on the phosphate **2**. In this communication we report the preparation of D-glucosone 6-phosphate with yeast hexokinase and the evaluation of the isomeric composition of phosphorylated D-glucosone by NMR spectroscopy.

Phosphorylation of D-glucosone (**1**) by hexokinase was first observed in metabolic studies with yeast (for literature see Ref. 11). We used **1** prepared from D-glucose,[2] purchasable yeast hexokinase and ATP for the preparation of 0.5 g (yield 69%) of D-glucosone 6-phosphate (**2**). Because of a higher stability of the hydrated forms of **2**, the purified preparation was typically maintained in aqueous solution.

It is well known that keto aldoses form complex equilibria in solutions due to the second possibility for ring closure. NMR spectroscopy is the method of choice to determine the isomeric forms and the equilibria portions. However, there are only a few reports which describe a detailed examination of the equilibria of keto sugars by NMR (for literature see Ref. 14). The proof of the phosphorylation and the assignment of the structure of D-glucosone 6-phosphate (**2**) was based on

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^1H, ^{13}C and ^{31}P NMR spectra as well as on the comparison with D-glucosone (**1**).

The decoupled ^{31}P NMR spectrum showed only one signal ($\delta = 0.82$ ppm) representing the overlapped shifts of the isomeric forms of **2**. The ^{13}C NMR spectrum (data in Table 1) showed six signals in the anomeric region originating from C-1 and C-2 of the three isomeric forms **2a**, **2b** and **2c**, but no signal at $\delta > 200$ ppm. Therefore, in these forms the carbonyl group not used for ring closure is hydrated which is the usual behavior of 2-keto aldoses.[13,14] From the equilibrium of **1** in aqueous solution,[12,13] a similar distribution and thus similar NMR data can be expected for **2**. In fact, the NMR shifts of the carbon atoms C-1 to C-4 of **2a**, **2b** and **2c** show only small differences to the corresponding forms **1a**, **1b** and **1d** of less than 1 ppm. For C-5 and C-6 the influence of the phosphate group is significant but consistent: The signals for C-5 are upfield shifted by an average 1.5 ppm, and the signals for C-6 are downfield shifted by approximately 4 ppm compared with the corresponding signals of **1a**, **1b** and **1d**. C-5 and C-6 show C,P coupling constants of 5–9 Hz.

By means of a phase-sensitive C,H correlated spectrum the ^1H NMR signals for the isomeric forms **2a**, **2b** and **2c** have been assigned (Table 2). Similarly to the ^{13}C NMR shifts, only the protons neighboring the phosphate group show significant differences in their ^1H NMR shifts compared to the corresponding forms **1a**, **1b** and **1d**. There are consistent shift differences of **2a**, **2b** and **2c** (all downfield shifted): for H-1 and H-3 less than 0.03, H-4 averaged 0.09, H-5 about 0.15, and for the H-6 protons 0.26–0.44 ppm as expected. The H,H coupling constants show sizes comparable to **1**. Due to overlapping of signals only one H,P coupling could be detected.

Integration of signals which were not overlapped yielded the equilibrium portions of the isomeric forms. In agreement with **1** (Scheme 1) the α-1,5-pyranose **2a** prevailed (58%) followed by its β-anomer **2b** (28%) and the β-2,5-furanose **2c** (14%). A 2,6-pyranose like **1c** can not be formed since a ring closure using C-6 is not possible. As yeast hexokinase acts non-specifically on several hexo-aldoses and on D-fructose,[15] a defined substrate form of **1** is not given in Scheme 1.

1. Experimental

Bioconversion.—D-Glucosone (**1**) was prepared from D-glucose by enzymatic conversion with pyranose oxidase.[2] The bioconversion of **1** to D-glucosone 6-phosphate (**2**) with yeast hexokinase (EC 2.7.1.1; SIGMA, Germany) was performed in 100 mM MOPS buffer (pH 6.5) in a final volume of 60 mL which contained the following components: 2.81 mmol (0.5 g) of D-glucosone, 2.81 mmol (1.55 g) of Na_2ATP, 3.43 mmol (0.7 g) of $MgCl_2 \times 6H_2O$, and 100 units of desalted yeast hexokinase. Following an incubation of 30 h at 25 °C, the hexokinase was removed from the reaction solution by ultrafiltration, using a YM 10 membrane (Millipore,

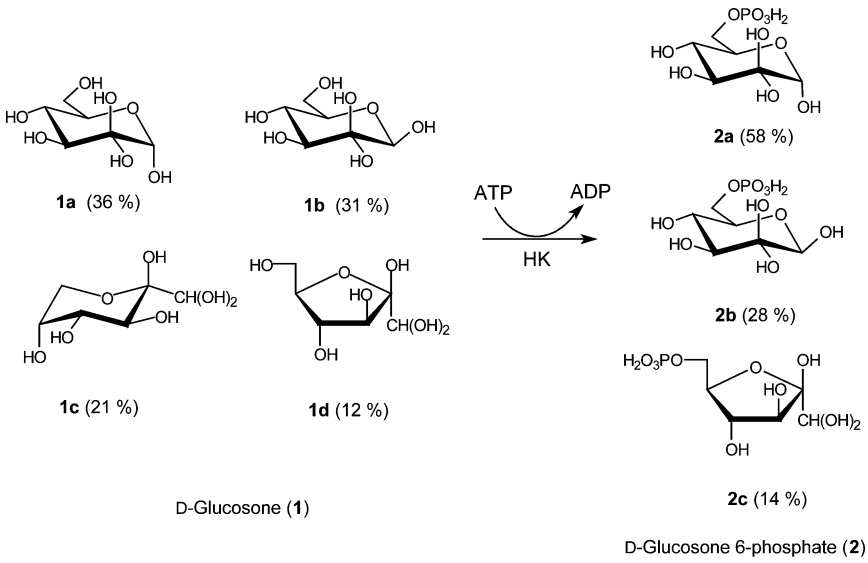
Table 1
^{13}C (125 MHz) NMR data for compounds **1** and **2** in D_2O

Form	C-1	C-2	C-3	C-4	C-5	$J_{5,P}$	C-6	$J_{6,P}$
1a	94.82	93.79	73.78	69.01	72.21	–	61.18	–
2a	94.89	93.72	73.63	68.41	70.90	7.6	65.27	5.1
1b	95.26	93.23	76.57	68.82	76.23	–	61.18	–
2b	95.31	93.17	76.37	68.27	74.71	8.1	65.17	5.1
1c	89.60	97.54	67.96	70.10	69.40	–	63.66	–
1d	90.23	101.00	76.06	74.72	80.73	–	62.22	–
2c	90.15	101.17	75.77	74.48	79.03	8.1	66.11	5.1

Table 2
^1H (500 MHz) NMR data for compounds **1** and **2** in D_2O

Form	H-1	H-3	$J_{3,4}$	H-4	$J_{4,5}$	H-5	$J_{5,6a}$	$J_{5,6b}$	H-6a	$J_{6a,6b}$	H-6b
1a	4.749	3.554	9.1	3.318	10.5	3.681	2.3	n.d.	3.665	12.4	3.541
2a	4.763	3.566	9.4	3.404	9.9	3.823	n.d.	6.1	3.98	n.d.	3.98
1b	4.499	3.333	9.5	3.258	AB	3.270	1.5	5.8	3.702	12.3	3.516
2b	4.528	3.356	AB	3.347	n.d.	3.423	2.0	n.d.	4.038[a]	11.4	n.d.
1c	4.844	3.724	10.0	3.673	1.9	3.790	1.3	3.0	3.821	12.8	3.547
1d	4.742	3.985	8.0	3.875	8.0	3.620	3.1	6.0	3.601	12.5	3.472
2c	4.745	4.009	8.2	3.970	8.7	3.746	n.d.	n.d.	3.86	n.d.	3.84

[a] $J_{6a,P} = 5.6$ Hz.



Scheme 1. Isomeric forms (equilibria ratios in brackets) of D-glucosone (**1**) and D-glucosone 6-phosphate (**2**) in aqueous solution. As yeast hexokinase (HK) acts non-specifically (see text), a defined substrate form of **1** is not given.

Bedford, MA, USA). The course of the conversion was followed by TLC on Silica plates using ethyl acetate–pyridine–acetic acid–water (36:36:7:21) as the mobile phase. D-Glucosone gave a distinct spot at $R_f = 0.72$ with dinitrophenylhydrazine–sulfuric acid reagent, whereas D-glucosone 6-phosphate yielded a smear with a front at $R_f = 0.24$.

Product purification.—The above ultrafiltrate was concentrated by evaporation to a volume of 30 mL, and then applied to a preparative column (900 × 20 mm) of DOWEX 50W-X8 (loaded with Ca^{2+}) at 71 °C, and then eluted with water at a flow rate of 2.5 mL/min.[2] Fractions of high purity as identified by TLC were collected, concentrated by evaporation and lyophilized. From 0.5 g of D-glucosone 0.5 g of D-glucosone 6-phosphate were obtained as a yellowish–brown solid which corresponded to a yield of 69%.

NMR spectroscopy.—NMR spectra were recorded on a Bruker AMX 500 spectrometer at 500.14 MHz for ^1H, 125.76 MHz for ^{13}C and 202.46 MHz for ^{31}P at 300 K in D_2O. 1D and 2D spectroscopy were performed with standard Bruker software. The chemical shifts refer to TMS and were given for ^1H and ^{13}C in relation to internal acetone ($\delta = 2.030$ and 30.50 ppm) and for ^{31}P to external 85% H_3PO_4. Coupling constants J are given in Hz. H-6 methylene protons were marked as H-6a and H-6b, whereas H-6a is shifted to downfield. Signals not detectable due to overlapping or low intensity were designated as n.d., and AB systems as AB.

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References

1. Liu, T. N. E.; Wolf, B.; Geigert, J.; Neidleman, S. L.; Chin, J. D.; Hirano, D. S. *Carbohydr. Res.* **1983**, *113*, 151–157.
2. Huwig, A.; Danneel, H. J.; Giffhorn, F. *J. Biotechnol.* **1994**, *32*, 309–315.
3. Leitner, C.; Neuhauser, W.; Volc, J.; Kulbe, K. D.; Nidetzky, B.; Haltrich, D. *Biocat. Biotransform.* **1998**, *16*, 365–382.
4. Geigert, J.; Neidleman, S. L.; Hirano, D. S. *Carbohydr. Res.* **1983**, *113*, 159–162.
5. Freimund, S.; Huwig, A.; Giffhorn, F.; Köpper, S. *Chem. Eur. J.* **1998**, *4*, 2442–2455.
6. Koths, K.; Halenbeck, R.; Moreland, M. *Carbohydr. Res.* **1992**, *232*, 59–75.
7. Gabriel, J.; Volc, J.; Kubátová, E.; Palková, Z.; Pospíšek, M. *Carbohydr. Res.* **1994**, *252*, 297–301.
8. Lindberg, B.; Theander, O. *Acta. Chem. Scand.* **1968**, *22*, 1782–1786.
9. Ericsson, B.; Lindgren, B. O.; Theander, O. *Cellul. Chem. Technol.* **1973**, *7*, 581–591.
10. Volc, J.; Sedmera, P.; Havlicek, V.; Prikrylová, V.; Daniel, G. *Carbohydr. Res.* **1995**, *278*, 59–70.
11. Bayne, S.; Fewster, J. A. *Adv. Carbohydr. Chem.* **1956**, *11*, 43–96.
12. Shaked, Z.; Wolfe, S. *Methods Enzymol.* **1988**, *137*, 599–615.
13. Freimund, S. Ph.D. Thesis, University of Oldenburg, 1997.
14. Freimund, S.; Huwig, A. *Carbohydr. Lett.* **2000**, *3*, 419–425.
15. Bergmeyer, H. U.; Grassel, M.; Walter, H. E. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed., 3rd ed.; VCH: Weinheim, 1983; Vol. 2, pp 222–223.