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Synthesis of 6-Phosphofructose Aspartic Acid and Some Related Amadori Compounds

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Abstract

We describe the synthesis and characterization of 6-phosphofructose-aspartic acid, an intermediate in the metabolism of fructose-asparagine by *Salmonella*. We also report improved syntheses of fructose-asparagine itself and of fructose-aspartic acid.

Graphical Abstract

6-Phosphofructose-aspartic acid (phosphate monoanion @ low pH)
MM2 Calculation (low dielectric)

Keywords

fructose-asparagine; fructose-aspartic acid; 6-phosphofructose-aspartic acid; *Salmonella*; Amadori rearrangement

1. Introduction

Fructose-asparagine [N-(1-deoxy-D-fructose-1-yl)-L-asparagine, CAS 34393-27-6] is an important nutrient for *Salmonella*. The proposed pathway for its metabolism involves

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deamination followed by phosphorylation to 6-phosphofructose aspartic acid [N-(1-deoxy-6-phospho-D-fructose-1-yl)-L-aspartic acid]¹. These molecules are Amadori rearrangement products resulting from the reaction of a sugar with an amine. Their chemistry and other properties have been ably reviewed². Standard syntheses are conducted by heating the components in an appropriate solvent, most frequently methanol. Attempts to make the title compound failed completely in a variety of solvents because of the insolubility of glucose-6-phosphate. Prof. V. V. Mossine of the University of Missouri suggested to me a procedure that has solved the solubility problem for this molecule and has increased the yield for the synthesis of other Amadori products. One dissolves the components in water, adds glycerol or ethylene glycol, removes the water by rotary evaporation, and finally carries out the reaction by heating. Remarkably, all of the components remain in solution at every stage. Following the reaction, the glycol is separated from the product by the addition of 1- or 2-propanol which are miscible with both glycerol and ethylene glycol but in which the Amadori product is insoluble. Reactions are faster in ethylene glycol than glycerol perhaps due to the viscosity difference^{3,4}. Purification of the crude material presented another problem. Standard procedures for Amadori products use a sulfonic acid ion exchange column to retain the product but not the excess sugar and other non-cationic molecules. Washing with ammonium hydroxide then elutes the product and excess amine. The title compound was so weakly bound to the resin that it was eluted with a water wash but still after the non-ionic components. This results in a better purification than is usual because the product is not contaminated with excess amine that remains bound to the column. Energy minimization calculations using the MM2 force field within ChemDraw reveals a possible reason for this interesting behavior. Shown in Figure 1 are models for both the α - and β -anomers at a pH corresponding to that on the Dowex column. Under these conditions, the secondary amine is protonated and the phosphate group is a mono-anion. The sugar residue is bent in a macrocycle for both anomers so that the phosphate anion is close to the -NH_2^+ group thus weakening its interaction with the resin. The experimental results can be accounted for by a significant contribution of these conformations to the equilibria. This is one possibility; a reviewer has suggested another namely that both 6-PF-Asp and F-Asp are fully protonated on the Dowex column and that their separation is due to a sieving effect, the more extensive hydration of the phosphorylated molecule increasing its effective molecular weight. We favor the first explanation because we think that a molecule with a net charge of +1 will be more strongly bound than one with a net charge of zero. Also, pKa values of molecules within the resin matrix are not well established. Perhaps both effects contribute. 6-Phosphofructose asparagine was made in the same way as 6-phosphofructose aspartic acid. Its behavior on the Dowex column was also the same implying the same protected conformation under acidic conditions, which were confirmed with additional modeling calculations.

The ¹H NMR spectra of 6-PF-Asp and 6-PF-Asn are complex due to about equal concentrations of the α - and β -furanose anomers for both molecules (Figure 1). The ¹³C spectra show the expected 20 resonances for a 1:1 mixture of the alpha and beta anomers. The 5' and 6' carbons show splitting by phosphorus. In addition, we have worked out improved conditions for the synthesis and purification of fructose asparagine and fructose aspartate.

2. Experimental

All specific rotations were at 25°C in water. Exact masses were measured using a Bruker Solarix, 15 tesla, FT-ICR instrument. IR spectra were taken as Nujol mulls on a Shimadzu Affinity-1S instrument. NMR experiments were performed on a Bruker Avance IIIHD 800 MHz spectrometer equipped with a QCI-P cryoprobe at 25°C. ¹³C NMR spectra were recorded on a Bruker Avance IIIHD 700 MHz instrument equipped with a TXO cryoprobe at 25°C. Accurate chemical shifts and scalar couplings were determined using CLIP/CLAP-HSQC experiments with a 1 second acquisition time, with or without ³¹P decoupling⁵. Data were processed using TopSpin 3.5 and the nmrPipe suite of programs⁶. Proton and carbon chemical shifts are referenced indirectly to DSS based on the temperature dependence of the water resonance. Phosphorus chemical shifts were indirectly referenced to 85% phosphoric acid. Stereospecific assignment of the diastereotopic methylene protons of the fructose residues were not performed and therefore the assignments in the Tables may be reversed. Errors in the measurements are approximately 1 ppb for proton, 10 ppb for carbon and phosphorus, and 0.2 Hz for scalar couplings.

2.1 Fructose Asparagine (F-Asn) ^{7,8}

Method A: D-glucose (4.1 g, 22 mmol), L-asparagine (0.5 g, 3.7 mmol), sodium bisulfite (0.2 g, 2 mmol), and malonic acid (0.2 g, 2 mmol) were dissolved with gentle heating in 5 mL water. Ethylene glycol (15 mL) was then added to form a homogeneous solution. About 80% of the water was removed by rotary evaporation at about 50 deg. The solution was placed in an incubator, uncovered, at 70° for about 17 hr to yield a pale yellow still homogeneous solution. Longer heating results in the formation of brown degradation products which make the purification difficult. Ethylene glycol was removed by precipitation of the crude product by the addition of 1-propanol. The precipitated material was dissolved in a small amount of water and applied to 15 g of a Dowex 50 (200–400 mesh, hydrogen ion form) column (2.5 X 8 cm). The column was washed with about 500 mL of water and then the product together with some asparagine was eluted with 0.4 M ammonium hydroxide. The relevant fractions were evaporated first at RT to remove excess ammonia. This minimizes the formation of diglucosyl amine. Then the temperature was increased to 50° to remove water with a final drying by azeotroping with ethanol. A relatively large flask previously treated with dichlorodimethylsilane facilitates removal of the product. The yield is about 60–70% of an off-white free-flowing powder. It has the approximate composition F-Asn.0.25 NH₄⁺ and contains about 10% asparagine as measured by proton NMR using the ratio of the intensity of the 3'-doublet at δ4.1 to the integration of the resonances from δ2.7–2.9 due to the beta protons of asparagine and the asparagine residue of F-Asn.

Method B: Glucose (2 g, 11.1 mmol), L-asparagine (1 g, 7.6 mmol), sodium bisulfite (1 g, 9.3 mmol), and malonic acid (1 g, 9.6 mmol) were suspended in 20 mL methanol and refluxed for 19 h, treated with a little Norite, cooled and the excess asparagine was filtered off. Five volumes of acetone were added to this solution with good stirring. The precipitate was filtered by suction and washed with acetone. Alternatively, the methanol can be

removed by rotary evaporation. The crude material was dissolved in small volume of water and applied to a 2.5×8 cm column of about 10 g of Dowex 50 in the hydrogen ion form and washed thoroughly with water. This removes the excess glucose. Fructose asparagine and a small amount of asparagine were then eluted with dilute ammonia (4 mL conc. ammonium hydroxide in 100 mL water, ca. 0.7 M). F-Asn elutes just as the effluent from the column begins to turn alkaline and it is accompanied by some yellow impurities. The work-up is the same as that described for Method A. The yield is about 400 mg (18–22%) of a yellow free-flowing powder.

2.1.1 Purification of F-Asn—We found that a mixture of asparagine and fructose asparagine (F-Asn) could be well separated by TLC on silica using 1:1 1-propanol:water, Rf Asn, 0.7, Rf F-Asn, 0.1. We were not successful in scaling up this separation on a column. A clean sample of fructose asparagine was obtained by extracting the crude material at RT with methanol. Proton nmr showed no detectable asparagine as judged by the pattern of the β -protons of the asparagine residue in the region δ 2.9–2.7. $[\alpha]_D = -42$ or -46.7 after correction for the presence of a half mole of ethylene glycol ($c=0.25$, pH 6.5). At pH 2 the value is -29.5 after correction. [The ethylene glycol can be removed either by more thorough washing with water on the Dowex column or by extraction of the crude material with acetonitrile in a Soxhlet apparatus]. Compare the literature values of -40^7 and -60^8 , pH values unknown. Exact mass: calcd. 295.11359, found 295.11358. IR(Nujol) : 3331, 3158, 1682, 1620, 1306, 1150, 1078, 1022, 775 cm^{-1} .

2.2 Fructose-Aspartate (F-Asp) ^{9–11}

Glucose (1 g, 5.5 mmol), potassium L-aspartate (0.3g, 1.8 mmol), and 0.1 g NaHSO_3 were dissolved in 3 mL water. Ethylene glycol (3 g) was added and the mixture rotary evaporated at 50° to remove water. The homogeneous solution was heated at 70° in an unstoppered flask for 17 hr. Ethylene glycol was removed by adding the reaction mixture, dropwise, to 200 mL of well-stirred 2-propanol. The precipitated product was dissolved in water and applied to a Dowex 50 column in the hydrogen ion form as reported for the synthesis of fructose asparagine using 0.3 M ammonium hydroxide for elution. Remarkably, under these conditions, and in contrast to the reaction with asparagine, there is only a trace of free amino acid in the Dowex 50 ammonia eluate. Yield: 35–45%.

Exact mass: calcd. 296.09761, found 296.09760. $[\alpha]_D = -72.2$ ($c=0.2$, pH 6.5) (lit.¹⁰ -51.7 , pH unknown).

2.3 6-Phosphofructose Aspartic Acid (6-PF-Asp)

Glucose-6-phosphate disodium salt (hydrate), 0.91 g (3 mmol), 0.2 g aspartic acid (1.5 mmol), and 0.2 g sodium bisulfite (0.21 mmol) were dissolved in 5 mL of water. Ethylene glycol (10 mL) was added. A homogeneous solution was formed. Water was removed on a rotary evaporator. The mixture remains homogeneous. The reaction was carried out at 60° in an unstoppered flask for about 20 hr. The products were precipitated by adding the solution dropwise to 2-propanol, 200 mL, with good stirring. The precipitate was filtered, washed with 2-propanol, and then acetone. The product is a free-flowing yellow powder. About 100 mg of this is dissolved in 0.25 mL of water and applied to an 8 X 1 cm column of Dowex 50

H⁺ X8, 200–400 mesh. It is washed on to the column with a little water and then, using only water as the eluent, 1 mL fractions are collected as soon as the eluate is acid. Most of the colored impurities and most of the remaining ethylene glycol emerge in the first 4 fractions, then the product appears in the next few fractions. It is detected by reaction with ninhydrin. Any excess aspartic acid remains bound to the column. The yield is about 50% of theoretical as a syrup. Trituration with 2-propanol followed by acetonitrile gave a solid.

Exact mass: calcd. 376.06394, found 376.06396

2.3.1 The cyclohexylamine salt of 6-phosphofructose aspartate—

Cyclohexylamine has long been used in sugar phosphate chemistry. The syrupy 6-phosphofructose aspartate (0.2 g) was dissolved in 1 mL water and then treated with cyclohexylamine until the pH was neutral. Then, 2 mL of ethanol was added and the mixture added dropwise to 25 mL of well-stirred acetonitrile. A precipitate formed which was rapidly filtered and then dried over phosphorus pentoxide.

6-phosphofructose aspartate tris(cyclohexylamine) salt. 2H₂O. $[\alpha]_D = -8.15$ or, after correction for the cyclohexylamine, -15.49 ($c=0.9$, pH 6.5). The dextrorotatory shift for the phosphorylated compound is consistent with the values for fructose (-92) and for fructose-6-phosphate ($+2.5$).

Anal.: calc. for C₂₈H₆₁O₁₄N₄P: C, 47.45; H, 8.67; N, 7.90. Found: C, 47.74; H, 8.73; N, 8.27.

2.4 6-Phosphofructose Asparagine

This material was made by reaction of glucose-6-phosphate and asparagine in a manner identical to that described for 6-phosphofructose aspartic acid.

Exact mass: calcd. 375.07992, found 375.07986

2.5 IR Spectra

The IR spectra of these molecules show characteristic absorptions in the carbonyl region. F-Asn gives a pair of broad doublets at 1681, 1672 and 1633, 1614 cm⁻¹, whereas these absorptions are shifted for F-Asp to 1622, 1614 and 1573, 1568 cm⁻¹. Both F-Asn and F-Asp show the C-O stretch at 1080, shifted to 1060 for ¹³C substitution. 6-PF-Asp gave 1730, 1454, 1415, 1203, 1080, 1040, 950, 880, 815 cm⁻¹. The absorptions at 1730, 1040, and 950 cm⁻¹ are attributable to the phosphate group^{12–14}.

2.6 NMR Assignments

2.6.1 6-PF-Asp—As is seen in Fig. 2, 1-dimensional NMR is not sufficient to characterize the spectrum of 6-PF-Asp unambiguously. 6-PF-Asp is a mixture of two very similar anomeric furanose conformers. To identify the connected spin systems in these samples, HSQC-TOCSY were recorded as well as an HP-TOCSY to correlate the phosphate group of the 6-PF-Asp with the fructose ring (Fig 3).

2.6.2 F-Asp—Only one major anomer was observed in the spectra of F-Asp consistent with the preference of molecules of this sort for the β -pyranose form (ref. 2, Table II, p. 318) although these solutions are an equilibrium mixture of five isomers: the α - and β -furanose and pyranose anomers as well as the open-chain form. We have made assignments for F-Asp and F-Asn only for the predominant form which we assume to be the β -pyranose. Partial assignments for the proton and carbon spectra have been made previously^{15,16}. In agreement with Altena et al.¹⁵, the C1' position exhibits clear deuterium isotope shifts consistent with partial exchange with the D₂O solvent.

2.6.3 F-Asn—As with F-Asp, only one major anomer was observed.

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Highlights

A critical solubility problem was overcome to enable the synthesis of 6-phosphofructose-aspartic acid.

A detailed nmr analysis (proton, carbon, and phosphorus) is given

6-Phosphofructose-aspartic acid

Figure 1.
Low pH conformations of 6-phosphofructose-aspartic acid.

Figure 2.
1D ^1H spectrum of 6PF-Aspartate recorded with (blue) or without (red) ^{31}P decoupling.

Figure 3. HSQC-TOCSY (A) and HP-TOCSY (B) experiments to identify the major component spin systems in 6PF-Aspartate.

Table 1

Two major conformers were observed in the spectra for 6-PF-Asp corresponding to its α - and β -furanose anomers. While it was possible to identify the fructose spin systems of each conformer, it was not possible to bridge the assignments to the amino acid moiety. Thus, the conformers of each are tabulated separately. The value for C2' was identified through 2-bond correlations to H1' and H1'' that appear in the HSQC experiments.

6PF-Aspartate					Conformer #1	Conformer #2
Fructose Ring	Conformer #1	Conformer #2			Conformer #1	Conformer #2
Chemical Shifts (ppm)						
H1'	3.458	3.501	¹ J _{C1'H1'}	145.6	146.5	146.1
H1''		3.428	¹ J _{C1'H1''}			
H3'	4.054	4.234	¹ J _{C3'H3'}	145.0	149.0	
H4'	4.213	4.128	¹ J _{C4'H4'}	146.8	149.1	
H5'	3.973	4.216	¹ J _{C5'H5'}	149.6	148.9	
H6'	4.084	4.106	¹ J _{C6'H6'}	147.2	147.6	
H6''	3.985	4.016	¹ J _{C6'H6''}	147.3	146.8	
C1'	45.73	52.98	² J _{H1'H1''}	12.8	13.1	
C2'	98.83	95.91	³ J _{H3'H4'}	8.0	5.1	
C3'	80.82	85.12	³ J _{H4'H5'}	7.7	7.0	
C4'	77.16	78.28	³ J _{H5'H6'}	2.8	2.9	
C5'	81.50	83.99	³ J _{H5'H6''}	5.7	5.0	
C6'	67.61	66.89	² J _{H6'H6''}	11.5	11.7	
³¹ P	0.29	0.34	³ J _{PH6'}	6.3	5.8	
			³ J _{PH6''}	6.8	6.7	
Amino Acid	Conformer #1	Conformer #2		Conformer #1	Conformer #2	
Chemical Shifts (ppm)						
H α	4.364	4.396	¹ J _{CaHa}	147.1	147.3	
H β ₁	3.205	3.201	¹ J _{CβHβ1}	132.8	132.6	
H β ₂	3.142	3.148	¹ J _{CβHβ2}	131.6	131.6	

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6PF-Aspartate					Conformer #1	Conformer #2
Fructose Ring		Conformer #1	Conformer #2		Conformer #1	Conformer #2
Chemical Shifts (ppm)			Scalar Couplings (Hz)			
C α	59.81	59.79	$^3J_{\text{H}\alpha\text{H}\beta 1}$	4.9	5.2	
C β	35.64	36.03	$^3J_{\text{H}\alpha\text{H}\beta 2}$	6.7	6.6	
			$^2J_{\text{H}\beta 1\text{H}\beta 2}$	18.3	18.3	

Table 2

Chemical shifts and scalar couplings measured for F-Asp. The value for C2' was identified through a 2-bond correlation to H3' that appears in the HSQC experiments.

F-Aspartate			
<i>Chemical Shifts (ppm)</i>	<i>Scalar Couplings (Hz)</i>		
H1' (H1'')	3.406	$^1J_{C1'H1'}$	144.8
H1'' (H1')	3.231	$^1J_{C1'H1''}$	143.8
H1' (D1'')	3.392	$^1J_{C3'H3'}$	143.4
H1'' (D1')	3.218	$^1J_{C4'H4'}$	142.7
H3'	3.786	$^1J_{C5'H5'}$	147.1
H4'	3.904	$^1J_{C6'H6'}$	143.3
H5'	4.015	$^1J_{C6'H6''}$	151.4
H6'	4.050	$^1J_{C\alpha H\alpha}$	146.2
H6''	3.781	$^1J_{C\beta H\beta 1}$	130.7
H α	3.835	$^1J_{C\beta H\beta 2}$	129.4
H β_1	2.818	$^2J_{H1'H1''}$	12.8
H β_2	2.685	$^3J_{H3'H4'}$	10.1
C1' (H ₂)	55.10	$^3J_{H4'H5'}$	3.1
C1' (HD)	54.84	$^3J_{H5'H6'}$	1.4
C2'	98.21	$^3J_{H5'H6''}$	1.9
C3'	72.81	$^2J_{H6'H6''}$	13.3
C4'	72.14	$^3J_{H\alpha H\beta 1}$	4.2
C5'	71.65	$^3J_{H\alpha H\beta 2}$	9.8
C6'	66.65	$^2J_{H\beta 1 H\beta 2}$	17.4
C α	62.85		
C β	38.10		

Table 3

Chemical shifts and scalar couplings measured for F-Asn. The value for C2' was identified through a 2-bond correlation to H3' that appears in the HSQC experiments. The signals for H1' and H1'' were degenerate and appear only as a singlet, thus $^2J_{H1'H1''}$ was not measureable.

F-Asparagine			
<i>Chemical Shifts (ppm)</i>	<i>Scalar Couplings (Hz)</i>		
H1'/H1'' (singlet)	3.361	$^1J_{C1'H1'/H1''}$	142.3
H3'	3.793	$^1J_{C3'H3'}$	142.5
H4'	3.900	$^1J_{C4'H4'}$	141.3
H5'	4.013	$^1J_{C5'H5'}$	147.1
H6'	4.040	$^1J_{C6'H6'}$	143.4
H6''	3.763	$^1J_{C6'H6''}$	151.3
H α	4.013	$^1J_{C\alpha H\alpha}$	145.1
H β_1	2.962	$^1J_{C\beta H\beta_1}$	130.4
H β_2	2.861	$^1J_{C\beta H\beta_2}$	130.4
C1'	51.60	$^2J_{H1'H1''}$	n.a.
C2'	98.43	$^3J_{H3'H4'}$	10.1
C3'	72.80	$^3J_{H4'H5'}$	3.1
C4'	72.15	$^3J_{H5'H6'}$	1.2
C5'	71.64	$^3J_{H5'H6''}$	1.9
C6'	66.57	$^2J_{H6'H6''}$	13.1
C α	54.02	$^3J_{H\alpha H\beta_1}$	4.2
C β	37.19	$^3J_{H\alpha H\beta_2}$	8.0
		$^2J_{H\beta_1 H\beta_2}$	16.9