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# Formation of 5-Methyl-4-hydroxy-3[2*H*]-furanone in Cytosolic Extracts Obtained from *Zygosaccharomyces rouxii*

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Formation of the flavor compound and precursor 4-hydroxy-5-methyl-3[2*H*]-furanone (HMF, norfuraneol) was demonstrated in cytosolic protein extracts obtained from *Zygosaccharomyces rouxii* after incubation with a number of carbohydrate phosphates. 4-Hydroxy-5-methyl-3[2*H*]-furanone was produced from D-fructose-1,6-diphosphate, D-fructose-6-phosphate, D-glucose-6-phosphate, 6-phosphogluconate, D-ribose-5-phosphate, and D-ribulose-1,5-diphosphate. Enzyme assays revealed D-fructose-1,6-diphosphatase, phosphohexose isomerase, D-glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase activity in the cytosolic extracts. Model studies showed the spontaneous formation of HMF from D-ribulose-5-phosphate. It is assumed that D-ribulose-5-phosphate is generated in cytosolic extracts by the action of the investigated enzymes from the carbohydrate phosphates and is then chemically transformed to HMF. The hypothesis was proven by the production of HMF in solutions containing commercially available enzymes and [6-$^{13}$C]-D-glucose-6-phosphate.

**KEYWORDS: Norfuraneol; *Zygosaccharomyces rouxii*; yeast; D-ribulose-5-phosphate**

## INTRODUCTION

4-Hydroxy-5-methyl-3[2*H*]-furanone (HMF, norfuraneol) was prepared for the first time in low yields by heating arabinose, xylose, ribose, or ribose 5-phosphate in the presence of amine salts (*1*−*4*). The compound has a caramel-like, burnt aroma or roasted chicory root odor, and its isolation from beef broth indicates that it is a component of the flavor associated with cooked beef (*5*). Two tautomeric structures can be considered for this compound, that is, 4-hydroxy-5-methyl-3[2*H*]-furanone and 4-hydroxy-2-methyl-3[2*H*]-furanone, but the 2-methyl tautomer is not stable enough to be separated from the 5-methyl isomer (*6*). Studies on the formation mechanism of HMF showed that it is derived from Amadori products formed during the Maillard reaction (*7*, *8*), but it has also been identified as a natural constituent of raspberry, guava, tomato, and insects (*9*−*12*). A rich source for HMF is shoyu (100 ppm), a fermented soy sauce (*13*, *14*). *Zygosaccharomyces rouxii* was identified as one of the shoyu yeasts, and model fermentations demonstrated the ability of this yeast strain to produce 4-hydroxyfuranones (*15*−*18*). Alternatively, HMF is formed as a major product of the LuxS in vitro reaction in consequence of *S*-adenosylhomocysteine degradation (*19*).

Recently, we detected HMF within *Z. rouxii* cells and observed the formation of 5-methyl-4-hydroxy-3[2*H*]-furanone in cytosolic extracts obtained from *Z. rouxii* cells after incubation with a number of carbohydrate phosphates. The objective of the paper was the demonstration of the biosynthetic pathway of HMF in yeast cells. The production of the furanone was studied in detail by enzyme assays, and a proposed formation pathway was evidenced by model experiments using commercially available enzymes as well as isotopically labeled precursors.

## EXPERIMENTAL PROCEDURES

**General Methods.** High-pressure liquid chromatography (HPLC) analysis with UV and evaporation light scattering detection was performed using an HPLC system equipped with a Spark Holland Basic marathon autosampler (Spark Holland, Emmen, The Netherlands) connected to a Knauer Maxistar pump and a Knauer variable-wavelength monitor (Knauer, Berlin, Germany) (285 nm) as well as an evaporation light scattering detector (Sedere, Alfortville Cedex, France) kept at 40 °C and 2.4 bar of compressed air. Knauer Eurochrom 2000 software was used for data acquisition and evaluation. For HPLC analysis with diode array detection (DAD) a Hewlett-Packard (Waldbronn, Germany) 1100 HPLC gradient pump and a Hewlett-Packard 1100 photodiode array detector were used including Hewlett-Packard Chemstation software for data acquisition and evaluation. For RP analysis a Eurospher 100-C18 column (length = 250 mm × 4 mm i.d., particle size = 5$\mu$m) (Knauer) was employed. A binary gradient starting from 95% A (0.05% formic acid in water) and 5% B (acetonitrile) to 80% A within 10 min and then to 0% A in 30 min was used at a flow rate of 1 mL/min. Injection volume was 20 $\mu$L. HMF yields were quantified using a standard curve of commercial HMF. HMF concentration was calculated as a mean value of duplicate analyses. For anion exchange HPLC a Nucleosil 100-10 SB column (length = 250 mm × 4 mm i.d., particle size = 10 ± 1.5 $\mu$m) (Macherey-Nagel) was used. A binary gradient starting from 92% A (water) and 8% B (1 M ammonium acetate, pH 4) to 10% A within 10

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min and then to 30% A in 30 min was used at a flow rate of 1 mL/min. Injection volume was 20 μL. High-pressure liquid chromatography−tandem mass spectrometry (HPLC-MS/MS) was performed utilizing a TSQ 7000 tandem mass spectrometer system equipped with an electrospray ionization (ESI) interface (Finnigan MAT, Bremen, Germany) and an Applied Biosystems (BAI, Bensheim, Germany) 140b pump. Data acquisition and evaluation were conducted on a DEC 5000/33 (Digital Equipment, Unterföhring, Germany) using Finnigan MAT ICIS 8.1 software. HPLC separation with MS detection was carried out on an XTerra MS C18 column (length = 150 mm × 2.1 mm i.d., particle size = 3.5 μm) (Waters, Milford, MA) using a binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was acetonitrile. HPLC was programmed as described above except for a flow rate of 200 μL/min. The injection volume was 5 μL, and mass spectra were acquired in the positive ion mode. For pneumatic assisted ESI, the spray voltage was set to 3.5 kV, and the temperature of the heated capillary was 210 °C. Nitrogen served both as sheath (70 psi) and auxiliary gas (10 units). Product ion scanning was performed at a collision gas pressure of 2.0 mTorr and a collision energy of 15 eV with a total scan duration of 1.0 s for a single spectrum.

**Reagents.** Chemicals, salts, and solvents of high purity were obtained from Fluka (Deisenhofen, Germany), Sigma (Deisenhofen, Germany), Aldrich (Deisenhofen, Germany), and ICN (Eschwege, Germany). Solvents were redistilled prior to use. Water of HPLC gradient grade was from Merck (Darmstadt, Germany), and acetonitrile of HPLC gradient grade was from Fisher (Loughborough, U.K.). Hexokinase (from baker's yeast), glucose-6-phosphate dehydrogenase (from baker's yeast), 6-phosphogluconic dehydrogenase (from torula yeast), L-glutamic dehydrogenase (from bovine liver), and phosphoriboisomerase (from spinach) were from Sigma. [6-$^{13}$C]-D-Glucose (99% $^{13}$C) was purchased from Deutero GmbH (Kastellaun, Germany).

**Cultivation of *Z. rouxii*.** The yeast strain used in this investigation was *Z. rouxii* ATCC 13356. YPD medium consisting of yeast extract (5 g/L), peptone (5 g/L), $KH_2PO_4$ (5 g/L), $MgSO_4 \cdot 7H_2O$ (5 g/L), glucose (50 g/L), and NaCl (170 g/L) was prepared according to the method of Hecquet and co-workers (*17*). The medium was sterilized by autoclaving.

**Preparation of *Z. rouxii* Cytosolic Protein Extract.** *Z. rouxii* cells were grown at 30 °C in YPD medium. During the mid-logarithm phase the yeast cells were harvested by centrifugation at 5000*g*, washed twice in cooled (4 °C) buffer (50 mM phosphate buffer at pH 7.5 and 150 mM NaCl), and resuspended in 4 mL of cooled (4 °C) lysis buffer (50 mM phosphate buffer at pH 7.5, 150 mM NaCl, 1 mM PMSF, and 30 μg each of leupeptin, pepstatin, and antipain per milliliter). The cell suspension was mixed with an equal volume of cold glass beads (diameter = 0.5 mm; Roth), and cells were broken by vortexing six times for 1 min with 1 min cooling intervals on ice as described previously (*20*, *21*). Unbroken cells and cell wall debris were removed by centrifugation at 1000*g*, and the supernatant was centrifuged at 27000*g* for 1 h at 4 °C. The supernatant (soluble cytosolic proteins) was removed and subjected to dialysis against 20 mM Tris-Cl (pH 7.5) overnight at 4 °C. The protein concentration was determined according to the method of Bradford (*27*) using bovine serum albumin (Sigma) as standard.

**General Procedure.** Two milliliter aliquots of the cytosolic protein extract or 20 mM Tris-Cl buffer (pH 7.5) were supplemented with substrate and equal amounts of NAD, NADH, NADP, and NADPH (1 mg each). The incubations were kept with gentle agitation at 30 °C for 30 h.

**Analysis.** The samples were subjected to solid-phase extraction (SPE) using RP18 cartridges (Supelco, 500 mg/3 mL), preconditioned with 6 mL of methanol and 6 mL of water. After application, the cartridges were rinsed with 1 mL of water and eluted with 3 mL of diethyl ether. The water remaining in the diethyl ether extract was removed by freezing (−18 °C). Water (200 μL) was added to the organic phase, and the diethyl ether was removed by a stream of nitrogen. The aqueous phase was analyzed by HPLC with UV detection at 285 nm, HPLC-DAD, and HPLC-ESI-MS/MS.

**Enzyme Assays.** D-Fructose-1,6-diphosphatase, phosphohexose isomerase, d-glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase were determined according to published procedures (*28*−*30*). Enzyme activities were recorded for 10−25 min at room temperature by monitoring NADH formation at 340 nm in intervals ranging from 15 s to 2 min.

**HMF Formation by Phosphoriboisomerase.** A solution (1 mL) containing 20 mM Tris-Cl (pH 7.5), 20 mM D-ribose-5-phosphate, and 10 units of phosphoribose isomerase was incubated at 30 °C with gentle agitation. After 22 h, HMF formation was examined by RP18 HPLC with UV detection at 285 nm.

**Synthesis of [6-$^{13}$C]-D-Glucose-6-phosphate.** To 10 mL of a reaction mixture containing 20 mM $MgCl_2$, 50 mM Tris-Cl buffer (pH 7.5), 1 mM DTT and 15 mM ATP were added [6-$^{13}$C]-D-glucose (20 mM final concentration) and hexokinase (200 units) (*31*). The reaction mixture was incubated at 30 °C with gentle agitation overnight, and phoshorylation was monitored by anion exchange HPLC coupled with an evaporation light scattering detector (ELSD). The solution was diluted with water to 40 mL and loaded onto a DEAE-Sepharose column (2 × 14 cm; Pharmacia) equilibrated with water. The column was eluted with a linear ammonium bicarbonate gradient (0−0.4 M, 300 mL) at a flow rate of 9 mL/h at 4 °C, and fractions of 4.5 mL were collected. Fractions containing [6-$^{13}$C]-D-glucose-6-phosphate were determined by anion exchange HPLC-ELSD, pooled, and lyophilized.

**Model experiment.** The $^{13}$C-labeled D-glucose-6-phosphate (13 mg) was added to a reaction mixture (3 mL) containing 20 mM $MgCl_2$, 50 mM Tris-Cl buffer (pH 7.5), 10 mM ammonium chloride, 1.7 mM NADP, 10 mM α-ketoglutarate, glucose-6-phosphate dehydrogenase (60 units), 6-phosphogluconate dehydrogenase (30 units), and glutamate dehydrogenase (40 units) (*31*). After incubation at 30 °C for 30 h, the sample was subjected to SPE as described above, and the extraxt was analyzed by HPLC-DAD and HPLC-ESI-MS/MS.

## RESULTS

**Transformation of Carbohydrate Phosphates.** *Z. rouxii* cells grown in hyperosmotic medium were isolated by centrifugation and washed three times with 17% NaCl solution. The content of the cells was liberated by grinding the yeast cells with sand in a mortar. Extraction with diethyl ether and subsequent GC-MS analysis revealed HMF as one of the flavor compounds. As enzymatic formation of HMF within the yeast cells was assumed, cytosolic protein extracts were prepared by vortexing *Z. rouxii* cells with glass beads followed by high-speed centrifugation (*20*, *21*). The extracts were dialyzed to remove low molecular weight components (<12 kDa) and were subsequently incubated with carbohydrate phosphates and cofactors (NAD, NADH, NADP, and NADPH) at 30 °C for 30 h. Carbohydrate phosphates have already been described as precursors for HMF (*16*). Samples were subjected to SPE on RP18 cartridges and analyzed by RP18 HPLC with UV detection at 285 nm, GC-MS, or LC-MS. HMF was detected in cytosolic extracts incubated with D-glucose-6-phosphate, D-fructose-6-phosphate, D-fructose-1,6-diphosphate, 6-phosphogluconate, D-ribose-5-phosphate, and D-ribulose-1,5-diphosphate (**Figure 1**). However, HMF concentration was below its odor threshold of 12 mg/L (*11*). HMF was not formed in control experiments without protein extract or with heat-inactivated extract. A pH optimum of pH 8 was determined using D-fructose-1,6-diphosphate as substrate, and stepwise dilution of the cytosolic extract with buffer led to decreasing amounts of HMF. These observations indicated that at least one reaction of HMF formation is enzymatically catalyzed. The formation pathway depicted in **Figure 2** was postulated.

**Enzyme Assays.** Enzymatic activity of D-fructose-1,6-diphosphatase, phosphohexose isomerase, D-glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase was determined in cytosolic extracts obtained from *Z. rouxii* to confirm the pathway shown in **Figure 2**. All investigated enzymes were detected in the extracts, and enzyme activites were calculated (**Table 1**). Because different extracts were used for the deter-

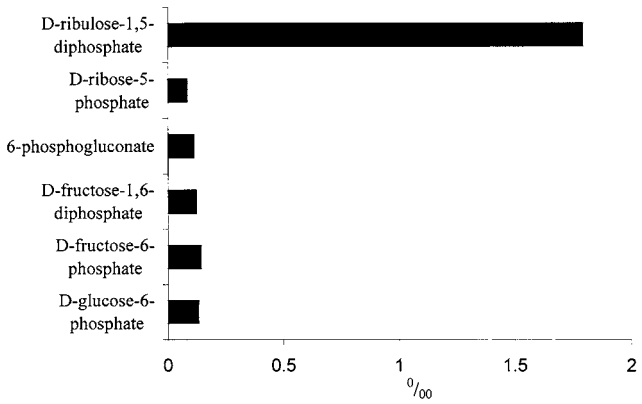


**Figure 1.** HMF formation expressed as conversion rates (‰) obtained after the incubation of *Z. rouxii* cytosolic protein extract with different carbohydrate phosphates. Twenty milligrams of each substrate (except D-ribulose-1,5-diphosphate, 10 mg) and 4 mg of cofactor (NAD, NADH, NADP, and NADPH) were incubated with 2 mL of *Z. rouxii* cytosolic extract in Tris-Cl buffer (pH 7.5) for 30 h. HMF was not formed in the absence of sugar phosphates or the *Z. rouxii* cytosolic extract.

**Table 1.** Enzymatic Activities of D-Fructose-1,6-diphosphatase (**1**), Phosphohexose Isomerase (**2**), D-Glucose-6-phosphate Dehydrogenase (**3**), and 6-Phosphogluconate Dehydrogenase (**4**) in Cytosolic Protein Extracts Obtained from *Z. rouxii*

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| volume activity (units $mL^{-1}$ of extract) | 0.012 | 17.9 | 0.039 | 0.25 |
| specific activity (units $mg^{-1}$ of protein) | 0.0023 | 5.71 | 0.013 | 0.081 |

mination of the enzymatic activities, the factor between volume activity and specific activity varies. The individual enzymatic activities depend on the growth stage of the yeast culture, but the growth cannot be easily synchronized in different vessels. We concluded that D-ribulose-5-phosphate is the ultimate precursor of HMF. The pentulose-phosphate is formed in the cytosolic yeast extracts from the added carbohydrate phosphates because every enzyme of the postulated pathway displayed in **Figure 2** is present in the extract and during storage experiments with D-ribulose-5-phosphate dissolved in buffer (pH 7.5) up to 1% of the pentulose-phosphate was chemically converted to HMF within 30 h at room temperature

**Alternative Formation of D-Ribulose-5-phosphate.** To highlight the importance of D-ribulose-5-phosphate as precursor of HMF, alternative pathways leading to the phosphorylated carbohydrate were investigated. D-Ribulose-5-phosphate was synthesized by commercially available D-ribose-5-phosphate isomerase and kept at pH 7.5 at 30 °C for 22 h. HPLC analysis confirmed the formation of HMF during the enzymatic catalysis (conversion rate = 0.33%). In a second series of experiments D-ribose-5-phosphate and D-ribulose-1,5-diphosphate were incubated under standard conditions with *Z. rouxii* cytosolic extract. HMF was formed from D-ribose-5-phosphate, but a higher concentration of HMF (conversion rate = 1.79‰) was obtained in the case of D-ribulose-1,5-diphosphate (**Figure 1**). HMF was not formed in experiments without cytosolic extract, with heat-inactivated extract, or with D-ribulose. Thus, the presence of D-ribose-5-phosphate isomerase and phosphatase was anticipated in the cytosolic extract because D-ribulose-5-phosphate seemed to be the most efficient precursor for HMF.

**Confirmation of the Enzymatically Catalyzed Reactions and Spontaneous HMF Formation.** [6-$^{13}$C]-D-Glucose-6-phosphate was synthesized and converted with an excess of commercially available D-glucose-6-phosphate dehydrogenase

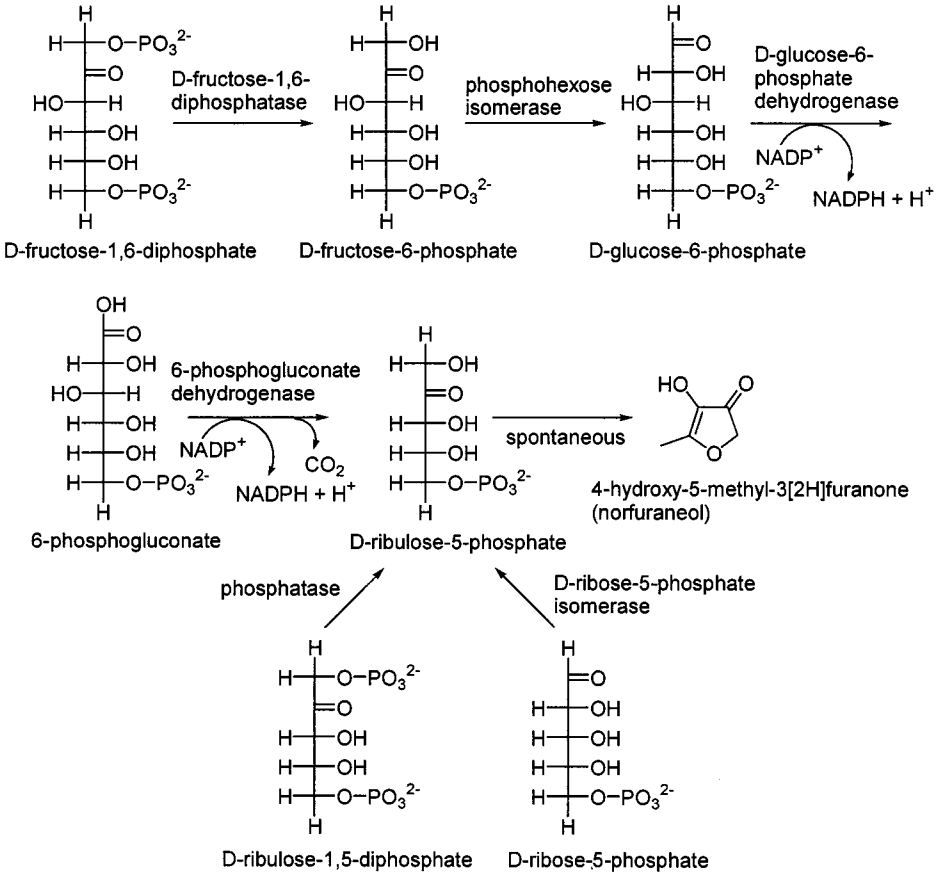


**Figure 2.** Formation pathway of HMF with involved substrates and enzymes in cytosolic extracts obtained from *Z. rouxii*.

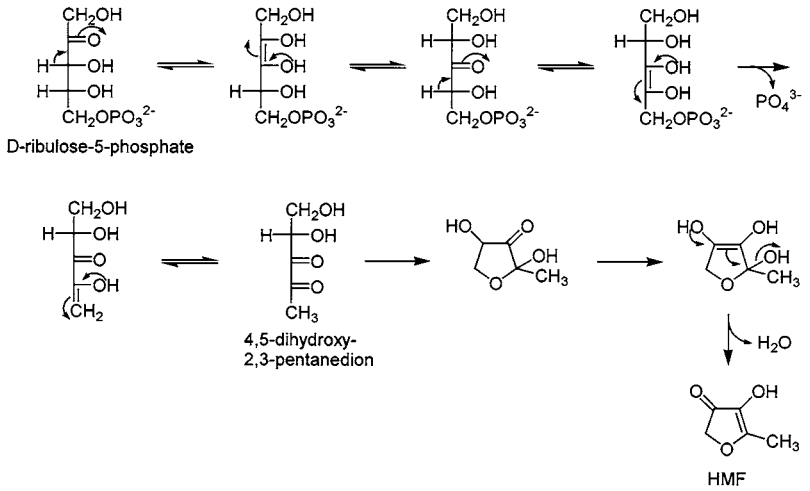


**Figure 3.** Proposed formation pathway of HMF from D-ribulose-5-phosphate.

and 6-phosphogluconate dehydrogenase in Tris-Cl buffer (pH 7.5). The reaction mixture was incubated at 30 °C for 30 h. HMF was detected by RP18 HPLC analysis after SPE. HPLC-ESI-MS analysis confirmed the postulated pathway depicted in **Figure 2** as a single labeled hydroxyfuranone showing an *m*/*z* 157 due to the acetonitrile adduct of the protonated molecular ion was detected. This result implies that in situ synthesized [5-$^{13}$C]-D-ribulose-5-phosphate was transformed into HMF under the assay conditions.

## DISCUSSION

Recently, the formation of HMF and its homologues 4-hydroxy-2-ethyl-5-methyl-3[2*H*]furanone (HEMF) and 4-hydroxy-2,5-dimethyl-3[2*H*]furanone (HDMF) by *Z. rouxii* was studied (*16*, *17*). The authors concluded that D-ribulose-5-phosphate/D-xylulose-5-phosphate and D-fructose-1,6-diphosphate are the precursors of HEMF and HDMF, respectively. In media containing D-ribulose-5-phosphate a significant amount of HMF was detected, but in the absence of the yeast the same amount was formed (*16*). Thus, HMF can be formed nonenzymatically from D-ribulose-5-phosphate as we confirmed with our results. We observed always small amounts of HMF in *Z. rouxii* extracts independent of the culture conditions. Thus, cytosolic protein extracts were prepared, and the production of HMF was studied in detail. On the basis of our results on *Z. rouxii* extracts we postulate the formation pathway in **Figure 2** involving enzymatically catalyzed reactions and a spontaneous chemical transformation. All of the postulated enzymes were detected in the extracts, and model experiments with commercially available enzymes and $^{13}$C-labeled D-glucose-6-phosphate demonstrated the production of HMF.

HMF has already been reported as a side product of the phosphoribose isomerase catalysis with the substrate D-ribose-5-phosphate (*22*). However, HMF was detected only after the enzymatic transformation of D-ribose-5-phosphate with phosphoribose isomerase isolated from spinach but not with the enzyme from rabbit muscle. The authors interpreted their observation as an anomaly of the spinach enzyme. As HMF is nonenzymatically formed from D-ribulose-5-phosphate in a buffer at pH 6−9, we assume that the enzyme preparation from rabbit muscle was either not active or that an intermediate of the transformation was removed by side activities.

Furthermore, HMF was identified as a major product in the LuxS in vitro reaction in consequence of *S*-ribosylhomocysteine degradation leading to the formation of 4,5-dihydroxy-2,3-pentanedione, which can easily be transformed to HMF by cyclization and dehydration (*19*). A similar process was described for the HMF formation in the Maillard reaction from pentoses (*23*). Therefore, we assume a 3,4-enediol formation due to the keto−enol tautomerization of the pentose phosphate followed by phosphate elimination resulting in the release of 4,5-dihydroxy-2,3-pentanedione (**Figure 3**). $^{13}$C NMR spectroscopy of D-ribulose-5-phosphate in aqueous solution showed that the pentulose derivative exists only as the free keto sugar, as evidenced by the chemical shift of its C-2 atom (*24*). This open chain form explains the high reactivity of the sugar phosphate. Keto−enol tautomerization is pronounced, facilitating the elimination of the phosphate leaving group. Similar nonenzymatic reactions are described for D-ribulose-1,5-diphosphate (*25*), dihydroxyacetone-phosphate, and glyceraldehyde-3-phosphate (*26*).

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