N^{δ}-(5-Hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine, a Novel Methylglyoxal−Arginine Modification in Beer

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N^{δ}-(5-Hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine, or Argpyrimidine, was identified and quantified in beer by high-performance liquid chromatography (HPLC) and coupled gas chromatography−mass spectrometry (HRGC−MS). This novel fluorescent arginine Maillard modification represents the first amino acid modification reported in beer retaining the full backbone of the original amino acid. Two mechanisms of formation could be verified: the major pathway via methylglyoxal and the minor pathway via 5-deoxypentoses. Argpyrimidine concentrations, determined in 35 lager-type beer varieties, reached up to 27 nmol/L and could be positively correlated to beer color and wort content. Within this context, 5-deoxy-D-ribose was identified as a novel intermediate of the Maillard reaction of maltose by HRGC−MS and independent synthesis.

Keywords: *N^{δ}-(5-Hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine; Argpyrimidine; 5-deoxypentose; 5-deoxy-D-ribose; Maillard reaction; beer; amino acid modification*

INTRODUCTION

Methylglyoxal is a known α-dicarbonyl within the Maillard reaction of reducing sugars such as maltose (*1*). In general, this reaction leads initially to the formation of an aminoketose or Amadori product, which is further degraded to yield a very complex reaction mixture having substantial influence on aroma, color, and amine modifications of foods. It is generally accepted that the Maillard reaction proceeds via highly reactive intermediates with α-dicarbonyl moiety like methylglyoxal. Several suggestions have been reported for the synthesis of methylglyoxal within the Maillard reaction (*2*−*5*), but the final mechanism remains unclear. As a consequence of the Maillard reaction, methylglyoxal has been identified and quantified in thermally treated foods such as caramel, coffee, and cocoa, but also in fermented foods such as wine (*6*−*8*). The high reactivity of methylglyoxal leads to modification of functional groups from free or proteinbound amino acids, especially lysine and arginine (*9*−*12*). Recently, we succeeded in identifying N^{δ}-(5-Hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine, or Argpyrimidine, in physiological reaction mixtures of methylglyoxal with proteinbound arginine and in vivo (*13*). Within this context the formation of an imidazolone has been proposed (*14*). However, ongoing studies prove this structure to be a misinterpretation of spectroscopic data and to be identical to Argpyrimidine (*15*; Glomb and Nagaraj, unpublished).

Beer is traditionally manufactured from water, hops, malt, and yeast. During the technology of beer brewing all essential substrates for the Maillard reaction are provided. Proteins are enzymatically broken down to smaller peptides and amino acids, starch is broken down to oligosaccharides, maltose, and glucose. In addition, the reaction is promoted by the high temperatures applied during kilning, mashing, and wort boiling. As proline is the dominating amino acid in beer, research on modifications has been exclusively focused on proline-derived structures. Although countless molecules were identified in model reactions of proline with maltose and glucose, only six could be verified in malt, wort, and beer, representing pyrrolizines, pyridines, azepines, and oxazines (*16*−*21*). Molecules published still incorporate the intact pyrrolidine ring, but lost the carboxylic function of the original amino acid skeleton. The notion that methylglyoxal is formed in beer and second, that beer also contains substantial amounts of arginine, led us to investigate the formation of Argpyrimidine in this beverage. This paper describes the identification and quantitation of this novel arginine-derived structure in beer and verification of an alternative synthesis pathway.

Materials. Chemicals of the highest quality available were obtained from Aldrich (Steinheim, Germany) and Fluka (Neu-Ulm, Germany), unless otherwise indicated. Beer samples were purchased from local retail stores.

Chromatography. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merk, Darmstadt, Germany). Visualization of separated material was achieved with the detection reagent given. Preparative column chromatography was performed on silica gel 60, 63−200 μm (Merk), unless otherwise noted. Solvents were all chromatographic grade. From the individual chromatographic fractions solvents were removed under reduced pressure.

Magnetic Resonance Spectroscopy (NMR). NMR spectra were recorded on a Bruker AC 200 instrument (Rheinstetten, Germany). Chemical shifts are relative to residual nondeuterated solvent as the internal reference.

High-Resolution Gas Chromatography−Mass Spectrometry (HRGC−MS). HRGC−MS was per-

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formed on a HRGC 6160 Mega Series (Fisons Instruments, Mainz, Germany); quartz capillary column (30 m, inner diameter 0.25 mm, DB-5, 0.25 μm, He, 37.3 cm/s, J&W Scientific, Cologne, Germany); injection port, 270 °C; temperature program, after injecting the samples at 100 °C the temperature of the oven was raised at 5 °C·min^{-1} to 200 °C, then raised at 10 °C·min^{-1} to 270 °C, and held for 10 min. For MS analysis the HRGC was connected to a MAT ITD 700 (Finnigan, Bremen, Germany); transfer line, 280 °C; EI at 70 eV.

High-Resolution Mass Spectrometry (HRMS). HRMS was applied on a VG 7070 (VG, Manchester, UK) with heptacosane as the internal standard.

Syntheses. *Argpyrimidine.* Argpyrimidine was prepared mainly according to Shipanova et al. (*13*).

5-Deoxy-D-ribose was prepared in a 5-step synthesis as described below.

2,3-O-Isopropylidene-5-O-toluol-p-sulfonyl-D-ribono-1,4-lactone **4**. To a solution of 1.0 g (5.3 mmol) 2,3-*O*-isopropylidene-D-ribono-1,4-lactone in 20 mL of anhydrous pyridine, 2.03 g (10.6 mmol) *p*-toluenesulfonyl chloride was added at −15 °C, and the solution was stirred for 10 h at −15 °C and then 10 h at ambient temperature. After addition of 1 mL of ice water, the reaction mixture was poured into 600 mL of ice water under vigorous stirring. The resulting precipitate was filtered, dried, and subjected to column chromatography (CH_2Cl_2). Fractions with material having a R_f 0.27 (TLC, 2:1 hexanes/EtAc, $FeCl_3$/hydroxylamine-reagent) were combined, and the solvents were evaporated to yield colorless crystals (1.1 g, 60%).

HRGC−MS: t_R 31.5 min; *m/z* 327 (M-15, 100%), 155 (50), 127 (30), 91 (90), 85 (80), 68 (45).

HRMS (*m/z*): 327.0538, found; 327.0533, calcd for $C_{14}H_{15}O_7S$, M-15.

^{1}H NMR ($CDCl_3$): δ 1.37 (s, 3H), 1.45 (s, 3H), 2.46 (s, 3H), 4.16 (dd, 1H, J = 11.2 Hz, J = 2.4 Hz), 4.33 (dd, 1H, J = 11.1 Hz, J = 1.9 Hz), 4.68 (m, 1H), 4.73 (d, 1H, J = 5.6 Hz), 4.77 (d, 1H, J = 5.6 Hz), 7.37 (d, 2H, J = 8.4 Hz), 7.74 (d, 2H, J = 8.3 Hz).

^{13}C NMR ($CDCl_3$): δ 21.6, 25.4, 26.5, 68.2, 74.9, 77.3, 79.0, 113.7, 127.8, 130.2, 131.5, 145.8, 173.0.

2,3-O-Isopropylidene-5-iodo-D-ribono-1,4-lactone **5**. To a solution of 1.10 g (3.31 mmol) **4** in 10 mL of anhydrous acetone, 2.13 g (12.8 mmol) of potassium iodide was added, and the mixture was refluxed for about 20 h. Completeness of reaction was monitored by TLC. Insoluble material was filtered off, the solvents were evaporated, and the residue was subjected to column chromatography (EtAc). Fractions with material having a R_f 0.61 (TLC, 2:1 hexanes/EtAc, detection as **4**) were combined, the solvents were evaporated, and the resulting yellow crystals again subjected to column chromatography (3:1 hexanes/EtAc). Fractions with **5** were combined, and solvents were evaporated to yield colorless crystals (0.89 g, 93%).

HRGC−MS: t_R 18.0 min; *m/z* 283 (M-15, 100%), 223 (5), 141 (5), 127 (10), 85 (20), 69 (20), 59 (15).

HRMS: *m/z* 282.9470, found; 282.9468, calcd for $C_7H_8IO_4$, M-15.

^{1}H NMR ($CDCl_3$): δ 1.40 (s, 3H), 1.47 (s, 3H), 3.38 (dd, 1H, J = 11.2 Hz, J = 5.2 Hz), 3.45 (dd, 1H, J = 11.2 Hz, J = 3.5 Hz), 4.61 (d, 1H, J = 6 Hz), 4.63 (dd, 1H, J = 5.3 Hz, J = 3.5 Hz), 4.98 (d, 1H, J = 6 Hz).

^{13}C NMR ($CDCl_3$): δ 5.6, 25.4, 26.4, 75.2, 80.2, 80.7, 114.0, 172.9.

2,3-O-Isopropylidene-5-deoxy-D-ribono-1,4-lactone **6**. 2.54 g (2.54 mmol) of calcium carbonate and Raney nickel catalyst were added to a solution of 0.76 g of (2.56 mmol) **5** in 12 mL of anhydrous ethanol. Completeness of reaction was checked by HRGC−MS (after about 1 h). After the reaction mixture was filtered, solvents were evaporated, and the residue was subjected to column chromatography (4:1 hexanes/EtAc). Fractions with **6** (HRGC−MS) were combined, and the solvents were evaporated to yield a colorless oil (0.34 g, 77%).

HRGC−MS: t_R 7.2 min; *m/z* 172 ($M^{+\bullet}$, 2%), 157 (85), 113 (10), 85 (50), 70 (100), 69 (30).

HRMS: *m/z* 157.0501, found; 157.0508, calcd for $C_7H_9O_4$, M-15.

^{1}H NMR ($CDCl_3$): δ 1.38 (s, 3H), 1.47 (s, 3H), 4.33 (d, 3H, J = 6.9 Hz), 4.50 (d, 1H, J = 5.4 Hz), 4.69 (q, 1H, J = 6.9 Hz), 4.79 (d, 1H, J = 5.5 Hz).

^{13}C NMR ($CDCl_3$): δ 19.5, 25.5, 26.6, 74.6, 78.9, 80.3, 113.7, 173.7.

2,3-O-Isopropylidene-5-deoxy-D-ribose **7**. To a solution of 178 mg (1.04 mmol) of **6** in 12 mL of anhydrous THF, 4.2 mL (4.2 mmol) of a 1 M diisobutylaluminumhydride solution in toluene was added slowly. After 30 min, the reaction was quenched by 15 mL of a saturated aqueous solution of Rochele's salt, and the temperature was allowed to rise until two layers became visible. The reaction mixture was extracted with diethyl ether, the organic layer was dried over calcium sulfate, and the solvents were evaporated after filtration. The residue was subjected to column chromatography (5:1 hexanes/EtAc). Fractions with material having a R_f 0.39 (TLC, 2:1 hexanes/EtAc, alkaline methanolic triphenyltetrazolium chloride solution) were combined, and the solvents were evaporated to yield a colorless oil (125 mg, 69%).

HRGC−MS: t_R 5.8; *m/z* 159 (M-15, 30%), 157 (20), 113 (10), 99 (15), 85 (10), 71 (30), 59 (100).

HRMS: *m/z* 159.0657, found; 157.0660, calcd for $C_7H_{11}O_4$, M-15.

^{1}H NMR ($CDCl_3$): δ 1.32 (s, 3H), 1.35 (d, 3H), 1.47 (s, 3H), 2.80 (d, 1H, J = 2.1 Hz), 4.36 (q, 1H, J = 7.1 Hz), 4.55 (d, 1H, J 5= .9 Hz), 4.67 (d, 1H, J = 5.9 Hz), 5.43 (d, 1H, J = 2.4 Hz).

^{13}C NMR ($CDCl_3$): δ 21.6, 24.8, 26.4, 83.2, 85.4, 86.3, 103.2.

The NMR data showed also small amounts of the α-anomer.

5-Deoxy-D-ribose. A solution of 25 mg (0.14 mmol)of **7** in 1 mL of 10% aqueous HAc was refluxed for about 2 h. Completeness of the reaction was monitored with TLC (EtAc, R_f 0.55, detection as **7**). Solvents were evaporated, and the residue was dried under high vacuum to yield a colorless oil (18 mg, 93%).

HRGC−MS: t_R (after oximation with hydroxylammonium chloride and trimethylsilylation) 16.0 min (*m/z* 437 ($M^{+\bullet}$, 2%), 320 (25), 219 (45), 191 (10), 147 (20), 131 (20), 129 (30), 117 (20), 100 (5), 73 (100). 16.2 min (*m/z* 422 (M-15, 5%), 320 (20), 219 (40), 191 (5), 147 (20), 131 (20), 129 (30), 117 (15), 101 (5), 73 (100). Peak ratio t_R 16.0:16.2 = 1:3.

HRMS: *m/z* 422.2034, found; 422.2030, calcd for $C_{16}H_{40}NO_4Si_4$, M-15.

^{1}H NMR ($CDCl_3$): δ 1.15 (d, 3H, J = 6.4 Hz, H_5-α), 1.24 (d, 3H, J = 5.9 Hz, H_5-β), 3.72 (t, 1H, J = 5.6 Hz, H_3-α), 3.90 (m, 3H, $H_{2,3,4}$-β), 4.05 (m, 2H, $H_{2,4}$-α), 5.10 (d, 1H, J = 1.2 Hz, H_1-β), 5.26 (d, 1H, J = 4.2 Hz, H_1-α).

^{13}C NMR ($CDCl_3$): δ 19.2 (C_5-α), 20.4 (C_5-β), 71.8 (C_3-α), 76.2 (C_2-α), 76.5 (C_3-β), 76.8 (C_2-β), 79.4 (C_4-α), 79.6 (C_4-β), 97.1 (C_1-α), 102.2 (C_1-β).

^{1}H- and ^{13}C NMR identified the final material as a 1:2.2 mixture of the α- and β-anomer.

High-Performance Liquid Chromatography (HPLC). *Analytical Systems*. A Jasco (Groβ-Umstadt, Germany) ternary gradient unit 980-PU-ND, with degasser, autosampler 851-AS, column oven CO-200 set at 25 °C, and fluorescence detector 920-FP was used. The effluent was monitored at 320 nm for the excitation and 398 nm for the emission. Chromatographic separations were performed in stainless steel columns filled with RP-18 silica gel (VYDAC 218TP54, 250 × 4.6 mm, 5 μm, Hesperia, CA; and Knauer Eurospher 100, 250 × 4.6 mm, 5μm, Berlin, Germany) using a flow rate of 1.0 mL·min^{-1}. The mobile phase used with the VYDAC column was water (solvent A) and MeOH/water (7:3, v/v; solvent B). To both solvents (A and B), 1.2 mL/L of heptafluorobutyric acid was added. Samples were injected at 20% B, the gradient changed to 35% B in 35 min, then changed to 70% B in 5 min, and was held at 70% B for 10 min. The mobile phase used with the Knauer column was water with 1.13 g NaH_2PO_4 x 2 H_2O/L (adjusted to pH 6.5; solvent A) and propanol/water (6:4, v/v) with 1.0 g NaH_2PO_4 x 2 H_2O/L (adjusted to pH 7.0; solvent B). To both solvents A and B, 3 g sodium dodecyl sulfate/L was added. Samples were injected at 2% B, the solvent ratio held at 2% B for 15 min, the gradient then changed to 100% B in 5 min, and held at 100% B for 10 min.

Preparative Systems. A Waters Chromatography gradient system (Waters, Milford, MA) with two pumps (model 6000A) and a solvent programmer (model 660) was used at a flow rate of 7 mL·min^{-1}. Chromatographic separations were performed on glass columns (Lobar Merk Lichroprep, 310 × 25 mm, RP18, 40−63 μm) and stainless steel columns (VYDAC 218TP1022, 250 × 25 mm, RP18, 10 μm). The mobile phase used with both systems was identical to the analytical VYDAC/MeOH system. On the Lobar system samples were injected at 5% B. This ratio was held for 30 min, then changed to 60% B in 40 min, and held for 50 min. Finally the gradient was changed to 100% B in 5 min, and held for 20 min. On the preparative VYDAC system samples were injected at 10% B, the gradient then changed to 40% B, this ratio held for 30 min, and the gradient then changed to 70% B, and held for 25 min. The gradient was then changed to 100% B in 20 min and held for 30 min.

Quantitation of Argpyrimidine. *In Beer*. Decarbonated beer (1 mL) was applied to ion exchange chromatography (Dowex 50WX4−400, H^+-form, 0.5 mL in a pasteur pipet). After rinsing with 5 mL of H_2O, elution was started with 1N NH_4OH solution. The second mL of NH_4OH solution was collected, solvents were removed in a Savant Speed Vac Plus SC 110A (Life Science International, Frankfurt, Germany), and the residue was reconstituted in 200 μL of H_2O. The reconstitution (100 μL) was injected on the HPLC−VYDAC/MeOH system and the eluate was collected between t_R of 24 and 30 min. For determination of the exact time window, an authentic standard was injected first. Solvents were evaporated (Speed Vac), and the residue was reconstituted in 200 μL of H_2O, and 50 μL was injected on the HPLC−Knauer/propanol system for quantification. For recovery studies, 19.9 pmol Argpyrimidine was added to 1 mL of beer prior to workup. Values plotted are means of two independent analyses for each sample.

In Model Reactions. After sterile filtration, a solution of 50 mM N^{α}-t-BOC-arginine hydrochloride and 50 mM sugar (methylglyoxal, 5-deoxy-D-ribose, D-ribose, D-glucose, D-maltose) in 0.2 M phosphate buffer (pH 7.4) was incubated at 55 °C. Aliquots of 50 μL were drawn at 24, 48, 72, 96, and 168 h and 200 μL of 3 N HCl was added. After 30 min, the solvents were evaporated (Speed Vac) and, after reconstitution with water, the residue worked up as described for beer samples. Analysis was performed only on the HPLC−VYDAC/MeOH system. Values given were obtained by analysis of duplicate incubations.

Identification of Argpyrimidine in Beer by HRGC−MS. Decarbonated beer (1 L) was subjected to ion exchange chromatography (70 g Dowex 50WX4−400, H^+-form). After rinsing with 500 mL of H_2O, elution was started with 250 mL of 1N NH_4OH solution. The first 50 mL of NH_4OH solution was discarded, the rest was combined and the solvents were evaporated. The residue was subjected first to column chromatography (RP18, 40−63 μm, Merk, Darmstadt, Germany, 8:2 methanol/H_2O), then to preparative Lobar−HPLC, and finally to preparative VYDAC−HPLC. Fractions having material eluting at the same t_R as Argpyrimidine (analytical HPLC−VYDAC/MeOH system) were combined, solvents were evaporated, and the residue was subjected to the next chromatographic step. The final resulting residue was dissolved in 500 μL of trifluoroacetic anhydride; after 1 h the solvents were evaporated, and 500 μL of a solution of diazomethane in diethyl ether (Aldrich, Milwaukee, WI) was added. After 10 min the solvents were removed under nitrogen, and the residue was subjected to column chromatography (EtAc). Fractions were monitored by HRGC−MS. For comparison, 1 mg of authentic Argpyrimidine standard was derivatized the same way using 100 μL of trifluoroacetic anhydride and 100 μL of diazomethane solution, but no column chromatography.

Identification of 5-Deoxy-D-ribose. *In Incubations of Maltose and Lysine*. Maltose monohydrate (211 mg, 0.58 mmol) and 53 mg (0.29 mmol) of lysine hydrochloride in 2 mL of phosphate buffer (0.2 M, pH 8.5) were heated at 80 °C for 3 h. The mixture was passed through protonated Dowex 50WX4−400, washed with 20 mL of water, and the solvents were evaporated. The resulting residue was subjected to column chromatography (9:1, CH_2Cl_2/MeOH). Eluate from 90 to 180 mL was combined, solvents were evaporated, and the residue was dissolved in 100 μL of anhydrous pyridine. After addition of 2 mg of hydroxylamine hydrochloride, the solution was stirred for 20 h at ambient temperature. N,O-bis(trimethylsilyl)acetamide (100 μL) was added to the reaction mixture and, after 1 h, analysis was performed on HRGC−MS.

In Incubations of Glycolaldehyde and Hydroxyacetone. Glycolaldehyde dimere (60 mg, 0.50 mmol) and 74 mg (1.00 mmol) of hydroxyacetone in 10 mL of phosphate buffer (0.2 M, pH 8.5) were incubated at 37 °C for 12 h. Solvents were evaporated, and the resulting residue was directly subjected to column chromatography and worked up as for maltose reaction mixtures.

Measurement of Beer Color and Wort. Methods of the European Brewery Convention (EBC) were used: EBC 9.6 and EBC 9.4.

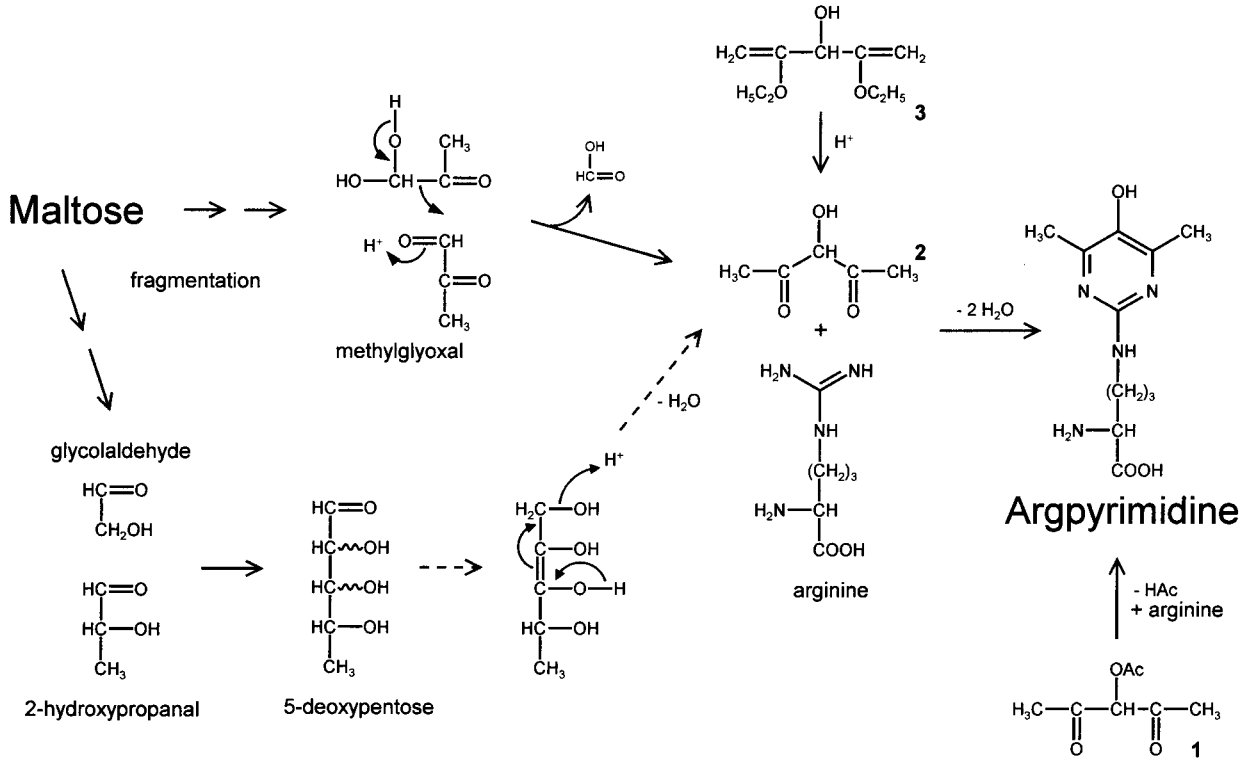

Figure 1. Mechanisms of Argpyrimidine formation from maltose.

RESULTS AND DISCUSSION

Synthesis of Argpyrimdine from Methylglyoxal. We first identified N^{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine or Argpyrimidine as the major fluorescent compound in incubation mixtures of N^{α}-t-BOC-arginine with methylglyoxal and various higher sugars under physiological conditions. The structure was unequivocally confirmed by ^{1}H NMR, ^{13}C NMR, high-resolution FAB−MS, and independent synthesis from 3-*O*-acetyl-2,4-pentanedione **1** (Figure 1). In the reaction scheme shown, a reducing sugar like maltose generates methylglyoxal through interaction with an amine. For the reaction of two molecules of methylglyoxal we proposed an intermolecular disproportionation to form 3-hydroxy-2,4-pentanedione **2**. This step would entail the generation of formic acid, a common Maillard reaction product. Also, this kind of chemistry, basically representing the cleavage of an α-dicarbonyl moiety, can be found as a known reaction pattern within the context of the Maillard reaction, e.g. leading to the formation of imidazolysine/MOLD (*9, 10*). The intermediate reductone **2** can then condense with the guanidino group of arginine to form Argpyrimidine. The formation of **2** could also be explained under participation of the guanidino nitrogens without changing the overall mechanism. As reductones are extremely sensitive to oxidation and fragmentation reactions, we synthesized 3-hydroxy-2,4-diethoxy-1,4-pentanediene **3** as a stable derivative to substantiate our hypothesis. The vinyl ether-protected carbonyl functions can be set free under mild acid conditions. Incubations with the such-generated reductone **2** yielded eight times more Argpyrimidine than when incubations were started from methylglyoxal. Thus, the formation of Argpyrimidine involves methylglyoxal with the generation of a 5-carbon backbone reductone as the crucial intermediate.

Identification and Quantitation of Argpyrimidine in Beer. In sugar-protein reaction mixtures Argpyrimidine can be detected only after enzymatic hydrolysis, as the molecule is labile to acid- and base-catalyzed protein hydrolysis. Beer contains significant amounts of free amino acids, most importantly proline, but also arginine, in concentrations up to about 500 and 100 ppm, respectively. In addition, methylglyoxal has been quantified in beer within the range of 0.08−0.2 ppm. We therefore developed a method to detect free Argpyrimidine in this beverage. As the molecule was expected to be present only as a minor constituent, a very efficient workup was developed. Beer (1 mL) was first passed through a protonated cation exchanger and washed with water to remove carbohydrates and nonbasic material. Material containing amines was then eluted with diluted ammonia in a very defined small fraction. Removal of solvents resulted in a still voluminous brown residue, which showed that substantial parts of the beer matrix had not been removed. Injected on high-performance liquid chromatography (HPLC), this resulted in a huge background fluorescence present in the upper trace chromatogram of Figure 2 A and omitted direct measurement of Argpyrimidine. The first-step HPLC was performed on a reversed-phase C-18 column with methanol/water eluent and heptafluorobutyric acid as the ion pair reagent. By addition of authentic standard (lower trace, Figure 2A) a six minutes retention time window (CUT) was defined to collect material, which was reinjected on a second HPLC system. The signal in the upper trace, coeluting at about 28 min with the standard, does not correlate with Argpyrimidine. In addition, to follow the elution pattern on the methanol/water HPLC system, the sensitivity of the detector had to be lowered by one magnitude compared to that of chromatogram B. The second HPLC system also consisted of a C-18 column, but with a propanol/water eluent and sodium dodecyl sulfate as the ion pair reagent. Figure B (lower trace) showes a baseline separated signal for Argpyrimidine, which

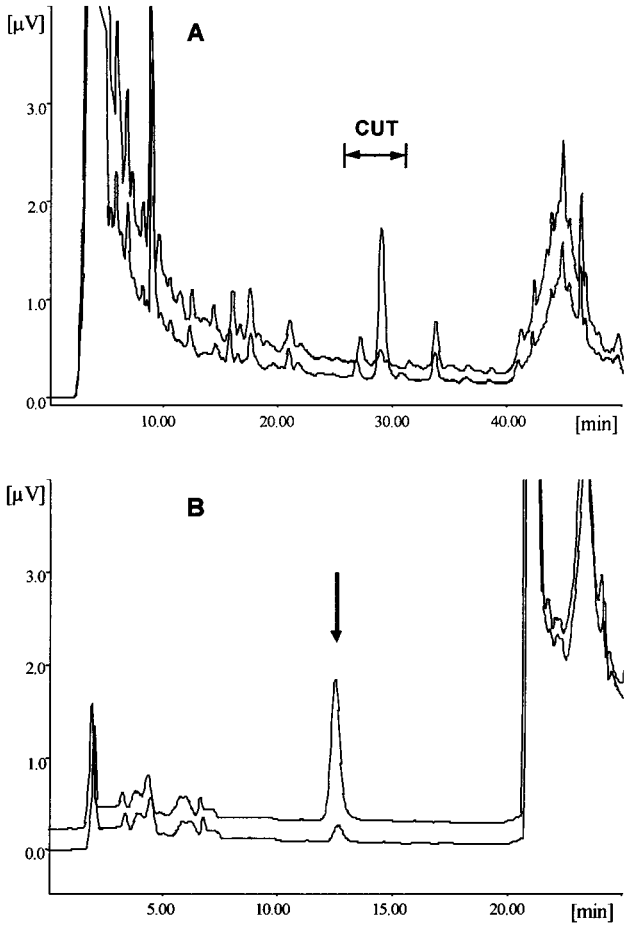

Figure 2. Detection of Argpyrimidine in beer by a 2-step HPLC system. After workup by ion exchange chromatography, samples were injected on HPLC system A, eluate containing Argpyrimidine was collected (CUT) and reinjected on HPLC system B for quantitation. Addition of standard is marked by arrows.

clearly increased after standard addition. The peak in this sample injection represents the absolute amount of 450 fmol Argpyrimidine. By addition of standard, the recovery rate of the total analysis procedure, starting from 1 mL of beer, could be verified to be 67% with a relative standard deviation of 5% ($n = 15$).

To further verify the identity of the material detected by HPLC, coupled gas chromatography−mass spectrometry (HRGC−MS) was performed. As the sensitivity of this method is much lower than that of fluorescence detection, 1 L of beer had to be worked up. First, material was subjected to ion-exchange chromatography to remove carbohydrates, and then it was fractionated by repeated chromatography on reversed phase columns with consecutively smaller particle size. Finally, the resulting residue was derivatized in two steps with trifluoroacetic anhydride and diazomethane. The fragmentation patterns of mass spectra obtained with electron impact ionization (EI) were virtually identical to the one of the derivatized standard (Figure 3 A and B). With chemical ionization, a pseudo molecular ion of *m*/*z* 379 was obtained, i.e., Argpyrimidine was derivatized to the corresponding N^{α}-trifluoroacetyl-N^{δ}-(5-methoxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine methylester (data not shown). EI in Figure 3B led to a base peak of *m*/*z* 347 representing the expected loss of an $O-CH_3$ fragment. As Argpyrimidine was only a minor constituent even in the fractionated beer sample,

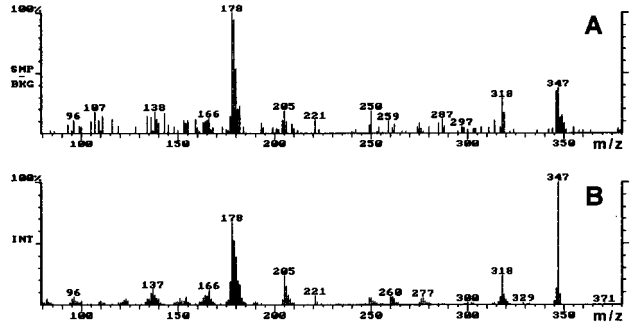

Figure 3. Identification of Argpyrimidine in beer by mass spectrometry after derivatization: (A) beer workup, (B) authentic standard.

HPLC with selective fluorescence detection proved to be the superior method for quantitation. Taken together, the results of the HPLC and HRGC−MS data unequivocally establish the presence of Argpyrimidine in beer.

35 lager-type beer varieties were analyzed for Argpyrimidine following the procedure described above. According to their declaration, they all complied with the German Purity Law, which means they were brewed traditionally using only water, hops, barley malt, and yeast, but no extra ingredients. Also, beer varieties are to be manufactured and classified within certain ranges of wort content (Schankbier 7−8%, Vollbier 11−14%, Starkbier ≥16%). As depicted in Figure 4 A, Argpyrimidine concentration correlated positively with wort content. Obviously, there is considerable scattering because of many variables in the individual brewery recipes such as temperature control and malt selection. The results suggest that higher concentrations of fermentable carbohydrates led to more methylglyoxal formation during mashing and wort boiling, and thus, to more amino acid modifications. Darker beers (closed circles) showed higher amounts of Argpyrimidine levels than comparable lighter varieties within the same wort range. Therefore, considerable modification must also occur during malting, leading probably to both Argpyrimidine itself and methylglyoxal or some methylglyoxal precursor like the Amadori product. This can also been seen in Figure 4B, plotting Argpyrimidine concentration against beer color measured as EBC units. On the other hand, among darker beer varieties, Figure 4B elucidates that darker color does not result in more Argpyrimidine. One explanation might be the use of small quantities of dark specialty malt to achieve the desired final tone of an otherwise regularly brewed product. These malt varieties are often manufactured under extreme conditions that do not lead to amino acids released to the final beverage. Finally, considerable amounts of methylglyoxal should also be released by yeast metabolism during fermentation, but, because of the relatively low temperatures, impact on Argpyrimidine formation must be considered to be small.

Taken together, the detection of Argpyrimidine proves that Maillard modifications of intact amino acids are present in beer. Qualitative and quantitative studies are now needed to identify the impact of the various stages of brewing technology on the formation of such structures. The results could be used to understand and correlate macroscopic changes on a molecular basis and, thus, to define the final quality of beer.

Alternative Synthesis Pathway for Argpyrimidine from Glycolaldehyde and 2-Hydroxypropanal. Glycolaldehyde and 2-hydroxypropanal are, like

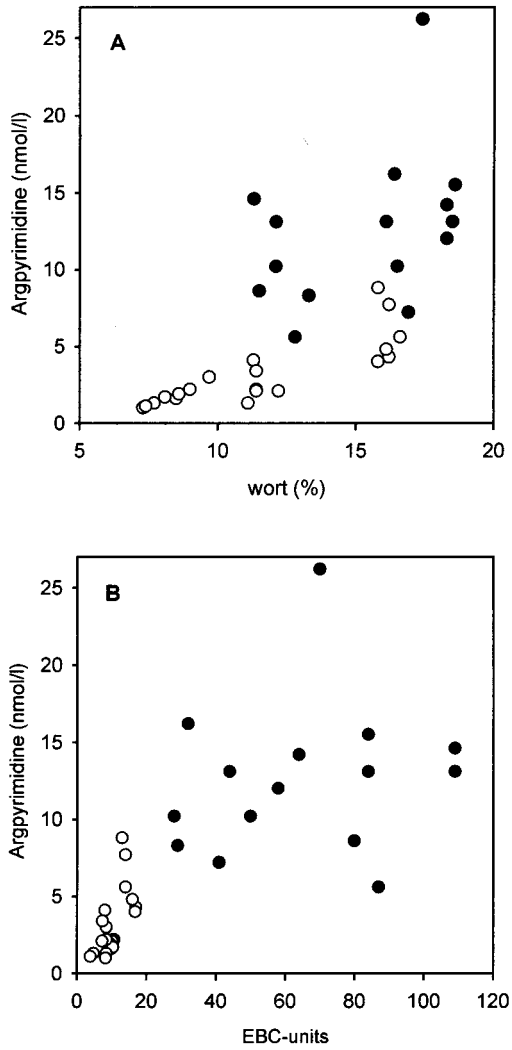

Figure 4. Correlation of Argpyrimidine concentration in beer to wort content (A) and beer color expressed as EBC units (B). Closed circles indicate samples with EBC units of more than 28.

methylglyoxal, known Maillard reaction intermediates of higher sugars (*22* and *23*). Both carbonyl structures could undergo aldol condensation to form 5-deoxypentoses, which, after enolization and elimination of water, would result in 3-hydroxy-2,4-pentanedione **2** (Figure 1). As in the case of methylglyoxal, reaction of this precursor with the guanidino group of arginine should give Argpyrimidine. However, up to now there are no reports on the formation of 5-deoxypentoses within the context of the Maillard reaction. To establish these structures as novel sugar degradation intermediates and to test for the proposed alternative reaction pathway leading to Argpyrimidine, we synthesized 5-deoxy-D-ribose as an authentic reference. Two procedures have been published using methyl-2,3-*O*-isopropylidene-β-D-ribofuranoside (*24*) and 2,3-*O*-isopropylidene-D-ribono-1,4-lactone (*25*) as the educts, respectively. As 2,3-*O*-isopropylidene-D-ribono-1,4-lactone is commercially readily available, we followed the latter strategy with major modifications.

The formation of 5-deoxy-D-ribose was established in reaction mixtures of glycolaldehyde and hydroxyacetone, thus verifying the proposed aldol condensation reaction. Because of the expected reactivity of 5-deoxypentoses, only minor quantities were detected after column chromatography and derivatization for HRGC−MS by first

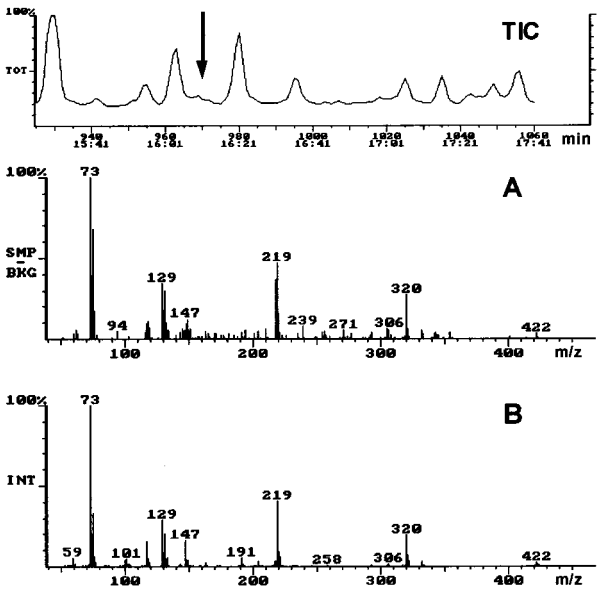

Figure 5. Identification of 5-deoxy-D-ribose in maltose reaction mixtures by HRGC−MS after derivatization: (TIC) total ion chromatogram, (A) mass spectra of sample workup, and (B) authentic standard.

Table 1. Argpyrimidine in Incubations of N^{α}-t-BOC-Arginine with Various Sugars[a]

incubation time (d)	5-deoxy-D-ribose	D-ribose	D-glucose	D-maltose	methylglyoxal
	Argpyrimidine (μmol/mol arginine)				
1	26	9	n.d.[b]	n.d.	33100
2	44	29	n.d.	n.d.	42800
3	116	52	n.d.	n.d.	47500
4	116	73	n.d.	n.d.	54200
7	214	94	17	12	49800

[a] Both reactants 50 mM at 55 °C in 0.2 M phosphate buffer, pH 7.4. [b] n.d., not detectable.

oximation with hydroxylamine and finally trimethylsilylation. Figure 5 shows the detection of 5-deoxy-D-ribose in incubations of maltose and lysine at 80 °C. The mass spectrum obtained at about 16.1 min of the total ion chromatogram (TIC) is virtually identical to the one from authentic 5-deoxyribose. The fragment at *m*/*z* 422 represents the characteristic loss of a methyl group from the molecular ion. Although only trace amounts were detectable in the TIC, these data unequivocally establish the formation of 5-deoxypentoses as novel Maillard intermediates of maltose.

Argpyrimidine formation was compared in incubations of N^{α}-t-BOC-arginine with methylglyoxal, 5-deoxy-D-ribose, and various other sugars at 55 °C (Table 1). The small quantities derived from 5-deoxy-D-ribose indeed verify the proposed alternative synthesis pathway, but, contrary to expectations, prove it to be of only minor importance. Obviously, methylglyoxal is by far the better precursor of Argpyrimidine within the reaction schemes shown in Figure 1.

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